Cell Context-Specific Gene Regulation Using Inhibitory RNAs

The present invention relates to a method of context-specific and cell context-specific gene regulation, and associated nucleic acid constructs, cells and therapeutic uses and methods. In particular, the present invention relates to the context-specific expression of inhibitory RNAs in cells in order to regulate expression of one or more target genes in said cells in a context-specific manner.

Background of the Invention

The following discussion is provided to aid the reader in understanding the disclosure and does not constitute any admission as to the contents or relevance of the prior art.

GEiGS® (or GEiGS) is a gene silencing technology that functions by redirecting endogenous non-coding RNAs (ncRNAs) with RNAi-mediated silencing specificity towards a desired target sequence (e.g. gene transcript). In humans, the redirected ncRNAs are almost exclusively microRNAs (miRNAs). These home in on their targets through sequence complementarity, therefore by changing their sequence it is possible to shift their silencing activity towards a desired target of choice. Precise gene edits are made to the sequence of endogenous miRNAs, in order to provide them with the ability to engage a new desired target through perfect sequence complementarity and silence it via the RNAi pathway.

GEiGS® therefore works by hijacking miRNAs that are already expressed in the cell and redirecting them towards silencing/modulating the expression of a desired target. The GEiGS target is silenced through the RNAi pathway. However, unlike siRNA or shRNA in which silencing is induced through administration of double stranded oligos or transgenes, respectively, GEiGS makes use of endogenous ncRNAs as vectors for silencing the desired targets.

Modifying the sequence of endogenous ncRNAs to redirect their activity is achieved through genome editing technologies, and GEiGS is not dependent on any particular technology and as such can be used with CRISPR, TALENs, zinc finger nucleases and any of their derivatives. While the “engine” of GEiGS (how silencing of the target is achieved) is based on the RNAi mechanism/pathway, the implementation of the technology (how the endogenous ncRNA is hijacked to target the desired gene) is based on genome editing technologies. For these reasons, GEiGS draws strengths from both RNAi and genome editing, enabling stable, tuneable and programmable gene silencing.

Thus, GEiGS (Gene Editing induced Gene Silencing) uses endogenous miRNA loci to drive expression of an interfering RNA (RNAi) specifically designed against new mRNA targets. The present invention is based, at least in part on the realisation that, by redirecting miRNAs or other inhibitory RNAs that have context-specific activity (e.g. developmentally regulated, cell-state-specific or induced in the cell in response to stimulation), GEiGS silencing can be selectively active in a particular context (e.g. cell or tissue type, or in a cell under a certain condition), but not expressed in another context (e.g. type of cell/tissue or under a different condition). Cells can be programmed *via* gene editing to silence pathway genes for example when differentiating from stem cells or following their stimulation by extracellular signals. In this aspect GEiGS is particularly powerful as other RNAi approaches are unable to silence genes in such a context-specific manner.

While the use of a GEiGS approach is preferred in some embodiments of the present invention, other approaches to using inhibitory RNAs to partially or completely silence gene expression are also known in the art, e.g. by inserting a suitable expression construct into the genome of a cell to drive expression of an inhibitory RNA, by providing a suitable expression construct to drive expression of an inhibitory RNA episomally (e.g. on a suitably plasmid or other vector), or by direct delivery of the inhibitory RNA to a cell. Such approaches can also be applied in the present invention, provided that the inhibitory RNAs demonstrate appropriate context-specific activity. For example, suitable inhibitory RNA constructs can be inserted into the genome, e.g. in a locus which encodes one or more endogenous miRNAs. Such an approach, which can be adapted for the current invention, is described in Senis *et al.* ('TALEN/CRISPR-mediated engineering of a promoterless anti-viral RNAi hairpin into an endogenous miRNA locus'. Nucleic Acids Research, 2017, Vol. 45, No. 1 e3 doi: 10.1093/nar/gkw805). Accordingly, in some embodiments of the present invention, a sequence encoding an inhibitory RNA (preferably a promoterless sequence encoding an inhibitory RNA) can be inserted into an endogenous miRNA locus, preferably without disruption or deactivation of any endogenous miRNA.

There remains a need for improved approaches to partially or completely silence gene expression in a more selective or specific manner, e.g. in a context-specific manner. The present invention relates to novel approaches for the use of inhibitory RNAs for context-specific silencing of target gene expression.

Non-limiting examples of potential utility of the present invention include the context-specific silencing of MHC-I on therapeutic cells linked to their stage of functional specification and

preventing their host immune-mediated rejection in appropriate contexts, targeting mechanisms in macrophages preventing their coercion into an immune-suppressive cell in response to the tumour microenvironment and the silencing of immune checkpoints in T-cells thereby minimising their exhaustion and cell death.

These and other advantages and uses of the present invention will be apparent from the following disclosure.

Brief Summary of the Invention

In a first aspect, the present invention provides a method of regulating expression of a target gene in a cell in a context-specific manner, the method comprising:

- providing a cell with a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene;
- wherein the inhibitory RNA is active in the cell in a context-specific manner such that inhibition of the expression of the target gene occurs specifically in a first context, in which the inhibitory RNA is active in said cell.

Suitably, inhibition does not occur or occurs in reduced amount in at least a second context, in which the inhibitory RNA is inactive or less active.

The present invention is thus concerned with the context-specific activity of inhibitory RNAs to provide context-specific inhibition of a target gene. Thus, inhibition of expression of a target gene only occurs, or occurs to a greater extent, in a first context, and the inhibition is absent or reduced in at least a second context. The present invention thus permits the provision of cells (preferably cells for use in therapy) which are modified to inhibit expression of a target gene in a specific context, by virtue of a conditionally active inhibitory RNA.

In some preferred embodiments, the nucleic acid construct is a modified form of an endogenous sequence encoding an inhibitory RNA, preferably an miRNA, which has been modified to target a transcript from the target gene.

In some preferred embodiments the cell is adapted to express at least one further inhibitory RNA (e.g. a second, a third, a fourth, etc. inhibitory RNA) which is/are active in the cell in a context-specific manner such that inhibition of the expression of a second or further target gene occurs specifically in a first context, in which the at least one further inhibitory RNA is active in said cell. Alternatively, the at least one further inhibitory RNA (e.g. a second, a third,

a fourth, etc.) can be active in a second or further context which is different to the first context. Accordingly, the method suitably comprises providing a cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of the target gene, or suitable to inhibit expression of two or more target genes.

In some embodiments the cell is adapted to express 2, 3, 4, 5 or more inhibitory RNAs which are active in the cell in a context-specific manner. Suitably the inhibitory RNAs target more than one target gene. For example, the 2, 3, 4, 5 or more inhibitory RNAs can target 2, 3, 4, 5 or more target genes. In some embodiments two or more inhibitory RNAs can target a single target gene.

In some embodiments, the cell is provided with two or more nucleic acid constructs adapted to express an inhibitory RNA suitable to inhibit expression of one or more target genes in a context-specific manner. In some embodiments a single nucleic acid construct is adapted to express two or more inhibitory RNAs suitable to inhibit expression of one or more target genes in a context-specific manner.

In some embodiments, the cell is provided with a first nucleic acid construct adapted to express a first inhibitory RNA suitable to inhibit expression of a first target gene in a context-specific manner, and at least a second nucleic acid construct adapted to express a second inhibitory RNA suitable to inhibit expression of a second target gene in a context-specific manner. In some embodiments, the cell is provided with a first nucleic acid construct adapted to express a first inhibitory RNA suitable to inhibit expression of a first target gene in a context-specific manner, and at least a second nucleic acid construct adapted to express a second inhibitory RNA suitable to inhibit expression of a second target gene.

In some embodiments, the cell is provided with a single nucleic acid construct adapted to express two or more inhibitory RNAs, wherein one of the two or more inhibitory RNAs is suitable to inhibit expression of a first target gene in a context-specific manner, and another one of the two or more inhibitory RNAs is suitable to inhibit expression of a second target gene in a context-specific manner. In some embodiments, the cell is provided with a single nucleic acid construct adapted to express two or more inhibitory RNAs, wherein one of the two or more inhibitory RNAs is suitable to inhibit expression of a first target gene in a context-specific manner, and another one of the two or more inhibitory RNAs is suitable to inhibit expression of a second target gene.

In some preferred embodiments, the method comprises the step of genetically modifying (editing) an endogenous (typically genomic) sequence encoding an endogenous inhibitory RNA to alter its targeting specificity.

In a further aspect of the invention, there is provided a cell or cell population obtained by the method of the first aspect of the invention.

In a further aspect of the invention, there is provided a genetically modified cell which comprises a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of a target gene;

wherein the inhibitory RNA is active in the cell in a context-specific manner, such that inhibition of the expression of the target gene occurs specifically in a first context, in which the inhibitory RNA is active in said cell.

Optional and preferred features of the first aspect as described above are of course relevant to this second aspect and other aspect described herein.

Suitably, inhibition does not occur or is reduced in at least a second context, in which the inhibitory RNA is inactive or less active.

In some preferred embodiments, the nucleic acid construct is a modified endogenous sequence encoding an inhibitory RNA, preferably an miRNA, which has been modified to target a transcript from the target gene.

In some preferred embodiments the cell is adapted to express at least one further inhibitory RNA (e.g. a second, a third, a fourth, etc. inhibitory RNA) which is/are active in the cell in a context-specific manner such that inhibition of the expression of the gene, or a second or further target gene, occurs specifically in a first context, in which the at least one further inhibitory RNA are active in said cell. Alternatively, the at least one further inhibitory RNA (e.g. a second, a third, a fourth, etc.) can be active in a second or further context which is different to the first context. Accordingly, the method suitably comprises providing a cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of the target gene, or suitable to inhibit expression of two or more target genes.

In some embodiments the cell is adapted to express 2, 3, 4, 5, or more inhibitory RNAs which are active in the cell in a context-specific manner. Suitably the inhibitory RNAs target more than one target gene. For example, the 2, 3, 4, 5, or more inhibitory RNAs can target 2, 3, 4,

5, or more target genes. In some embodiments two or more inhibitory RNAs can target a single target gene. Suitably the inhibitory RNAs can be active in different contexts (e.g. at least a first inhibitory RNA is active in a first context and at least a second inhibitory RNA is active in a second context.

In some embodiments, the cell is provided with two or more nucleic acid constructs adapted to express an inhibitory RNA suitable to inhibit expression of the target gene in a context-specific manner. In some embodiments a single nucleic acid construct is adapted to express two or more inhibitory RNAs suitable to inhibit expression of the target gene in a context-specific manner.

In some embodiments, the cell is provided with a first nucleic acid construct adapted to express a first inhibitory RNA suitable to inhibit expression of a first target gene in a context-specific manner, and at least a second nucleic acid construct adapted to express a second inhibitory RNA suitable to inhibit expression of a second target gene in a context-specific manner. In some embodiments, the cell is provided with a first nucleic acid construct adapted to express a first inhibitory RNA suitable to inhibit expression of a first target gene in a context-specific manner, and at least a second nucleic acid construct adapted to express a second inhibitory RNA suitable to inhibit expression of a second target gene.

In some embodiments, the cell is provided with a single nucleic acid construct adapted to express two or more inhibitory RNAs, wherein one of the two or more inhibitory RNAs is suitable to inhibit expression of a first target gene in a context-specific manner, and another one of the two or more inhibitory RNAs is suitable to inhibit expression of a second target gene in a context-specific manner. In some embodiments, the cell is provided with a single nucleic acid construct adapted to express two or more inhibitory RNAs, wherein one of the two or more inhibitory RNAs is suitable to inhibit expression of a first target gene in a context-specific manner, and another one of the two or more inhibitory RNAs is suitable to inhibit expression of a second target gene.

In a further aspect of the invention there is provided a nucleic acid construct for the context-specific expression of an inhibitory RNA suitable to inhibit expression of the target gene.

In yet another aspect of the invention, there is provided a nucleic acid construct for the context-specific inhibition of expression of a target gene in a cell, the nucleic acid construct encoding an inhibitory RNA suitable to inhibit expression of the target gene, wherein the inhibitory RNA

is active in the cell in a context-specific manner such that inhibition of the expression of the target gene occurs in a first context, in which the inhibitory RNA is active in said cell.

Suitably inhibition does not occur or is reduced in at least a second context, in which the inhibitory RNA is inactive or less active.

In yet another aspect of the invention, there is provided the use of a contextually-active inhibitory RNA, or nucleic acid construct encoding a contextually-active inhibitory RNA, in a method of context-specific inhibition of expression of a target gene in a cell, wherein the inhibitory RNA is active in the cell in a context-specific manner such that inhibition of the expression of the target gene occurs in a first context, in which the inhibitory RNA is active in said cell.

Suitably, inhibition does not occur or is reduced in at least a second context, in which the inhibitory RNA is inactive or less active.

According to a further aspect of the invention, there is provided a composition comprising a cell of the invention.

According to a further aspect of the invention, there is provided a cell or a composition of the invention for use in therapy.

According to a further aspect of the invention, there is provided a method of treating a disease or condition in a subject, wherein the method comprises administering a cell of the invention or composition of the invention in a therapeutic amount to the subject.

According to a further aspect of the invention, there is provided the use of a cell of the invention or a composition of the invention in the manufacture of a medicament for treating a disease in a subject.

In a further aspect, there is provided a reporter nucleic acid comprising or consisting of a constitutive promoter operably linked to a gene encoding a fluorescent protein and a target gene (or a portion of a target gene), wherein the fluorescent protein and the target gene are transcribed as a polycistronic mRNA.

In some preferred embodiments, the target gene is inserted into the 3' untranslated region downstream of the fluorescent protein. In some embodiments, the target gene sequence is

inserted into the 3' untranslated region of the fluorescent protein as a contiguous sequence. In some preferred embodiments, the fluorescent protein and the target gene are transcribed as a single RNA construct.

In some embodiments, the portion of the target gene is one or more exons.

In some preferred embodiments, the target gene is a target gene for one or more inhibitory RNAs. In some preferred embodiments, the target gene is a target gene for one or more inhibitory RNAs according to the present invention. The target gene may be any target gene disclosed herein. In some preferred embodiments, the target gene is PDCD-1, B2M, PPARG, IRF4, KDM6B, FOXP3 or STAT6.

The constitutive promoter may be selected from: simian virus early promoter (SV40), human ubiquitin C promoter (UBC), human elongation factor 1α promoter (EF1A), mouse phosphoglycerate kinase 1 promoter (PGK) and human phosphoglycerate kinase 1 (PGK1). In some preferred embodiments, the constitutive promoter is EF1a.

The fluorescent protein may be selected from: the Green Fluorescent Protein (GFP), the Blue Fluorescent Protein (BFP) and the red fluorescent proteins (e.g. dsRed, mCherry, RFP). In some preferred embodiments, the fluorescent protein is GFP, optionally CopGFP (a fast maturing bright variant).

In some embodiments, the reporter nucleic acid further comprises WPRE (Woodchuck Hepatitis Virus (WHV) Posttranscriptional Regulatory Element). In some preferred embodiments, the reporter nucleic acid is flanked by Long Terminal Repeat (LTR) sequences.

In some embodiments, the reporter nucleic acid further comprises a gene for a cell surface protein operably linked to a constitutive promoter. In some preferred embodiments, the cell surface protein is tNGFR (low affinity NGF receptor) protein. The constitutive promoter operably linked to the gene for a cell surface protein may be selected from: simian virus early promoter (SV40), human ubiquitin C promoter (UBC), human elongation factor 1α promoter (EF1A), mouse phosphoglycerate kinase 1 promoter (PGK) and human phosphoglycerate kinase 1 (PGK1). In some embodiments, the constitutive promoter operably linked to a gene for a cell surface protein is PGK. Suitably, the cell surface expression of tNGFR is used to identify cells which have been successfully transfected using labelled antibodies.

In some embodiments, the reporter nucleic acid is for use in detecting the inhibition or silencing of the target gene by one or more inhibitory RNAs. The reporter nucleic acid is particularly beneficial in detecting the inhibition or silencing of a target gene wherein the target gene is not coding for a cell surface protein in live cells.

In a further aspect, there is provided use of the reporter nucleic acid according to the present invention in detecting the inhibition or silencing of the target gene by one or more inhibitory RNAs.

In a further aspect, there is provided an expression system comprising:
- the reporter nucleic acid according to the invention; and
- an expression nucleic acid comprising a constitutive promoter operably linked to a nucleic acid sequence encoding an inhibitory RNA.

In some embodiments, the expression nucleic acid comprises a constitutive promoter operably linked to a nucleic acid sequence encoding two or more inhibitory RNA. In some embodiments, the expression system comprises two or more expression nucleic acids, each expression nucleic acid comprising a constitutive promoter operably linked to a nucleic acid sequence encoding an inhibitory RNA. Expression systems which have nucleic acid sequence encoding two or more inhibitory RNA or two or more expression nucleic acids are particularly beneficial for screening multiple inhibitory RNAs.

In some embodiments, the inhibitory RNA can be any inhibitory RNA according to any of the embodiments disclosed herein (e.g. in the section “Inhibitory RNAs”). In some embodiments, the inhibitory RNA is an endogenous non-coding RNA encoding sequence, e.g. an endogenous inhibitory RNA encoding sequence, which has been modified to retarget the endogenous inhibitory RNA to silence or partially silence a target gene. In some embodiments, the inhibitory RNA is an endogenous non-coding RNA encoding sequence, e.g. an endogenous inhibitory RNA encoding sequence, which has been modified to retarget the endogenous inhibitory RNA to silence or partially silence a target gene via GEiGS approach. In some embodiments, the inhibitory RNA is a GEiGS mature sRNA sequence (guide), GEiGS Solution sequence (pre-miRNA hairpin) or a GEiGS extended Solution. In some preferred embodiments, the inhibitory RNA is a GEiGS extended Solution.

In some preferred embodiments, the expression nucleic acid is flanked by Long Terminal Repeat (LTR) sequences.

In some embodiments, the reporter nucleic acid and the expression nucleic acid are provided in a single plasmid. In some embodiments, the reporter nucleic acid and the expression nucleic acid are provided in two separate plasmids.

In some embodiments, the reporter nucleic acid and the expression nucleic acid are provided in a single viral vector. In some embodiments, the reporter nucleic acid and the expression nucleic acid are provided in a single lentiviral vector.

In some embodiments, the reporter nucleic acid and the expression nucleic acid are provided in two separate viral vectors. In some embodiments, the reporter nucleic acid and the expression nucleic acid are provided in two separate lentiviral vectors.

In some embodiments wherein the expression system comprises two or more expression nucleic acids, the two or more expression nucleic acids may be provided in two or more separate plasmids or two or more separate viral vectors. In some embodiments wherein the expression system comprises two or more expression nucleic acids, the two or more expression nucleic acids may be provided in two or more separate lentiviral vectors.

In some embodiments, the inhibitory RNA is integrated in a miRNA scaffold or the inhibitory RNA comprises a miRNA scaffold. The miRNA scaffold can be any miRNA scaffold disclosed herein. In some preferred embodiments, the inhibitory RNA is integrated in a miRNA scaffold, the reporter nucleic acid is provided in a lentiviral vector and the expression construct is provided in a lentiviral vector. Embodiments in which the inhibitory RNA is integrated in a miRNA scaffold or the inhibitory RNA comprises a miRNA scaffold are beneficial as the inhibitory RNA integrated in a miRNA scaffold or the inhibitory RNA comprising a miRNA scaffold is shorter than the typical length used for shRNA in a lentiviral construct. This is expected to lead to more efficient processing of the inhibitory RNA integrated in a miRNA scaffold compared to typical shRNA constructs.

In some embodiments, the expression nucleic acid further comprises a gene encoding for a further fluorescent protein operably linked to the constitutive promoter. The further fluorescent protein may be selected from: the Green Fluorescent Protein (GFP), the Blue Fluorescent Protein (BFP) and the red fluorescent proteins (e.g. dsRed, mCherry, RFP).In some embodiments, the further fluorescent protein is dsRed. In some embodiments, the nucleic acid sequence encoding a inhibitory RNA and the gene encoding a further fluorescent protein are transcribed as a single RNA construct. In some embodiments, the nucleic acid sequence

encoding a inhibitory RNA and the gene encoding a fluorescent protein are transcribed as a single RNA construct which is separated via T2A sequence.

In some embodiments, the constitutive promoter in the expression nucleic acid is selected from: simian virus early promoter (SV40), human ubiquitin C promoter (UBC), human elongation factor 1α promoter (EF1A), mouse phosphoglycerate kinase 1 promoter (PGK) and human phosphoglycerate kinase 1 (PGK1). In some embodiments, the constitutive promoter in the expression nucleic acid is EF1a.

In some preferred embodiments, the expression systems has a nucleic acid sequence encoding two or more inhibitory RNA or two or more expression nucleic acids and the reporter nucleic acid and the expression nucleic acid are provided in one or more viral vectors, preferably lentiviral vectors.

In some preferred embodiments, the expression systems comprises two or more expression nucleic acids, the reporter nucleic acid is provided in a lentiviral vector and the two or more expression nucleic acids are provided in separate lentiviral vectors. This embodiment is particularly useful for screening inhibitory RNAs in an efficient and cost effective manner (in a pool) as detailed in Examples 21-22 and Example 10.

In a further aspect, there is provided a method of detecting the inhibitory or silencing activity of one or more inhibitory RNAs on a target gene comprising:
(i) transfecting a cell or cells with the expression system according to the invention;
(ii) binding of the inhibitory RNA to the target gene within the polycistronic mRNA;
(iii) degradation of the polycistronic mRNA; and
(iv) reduction in the fluorescent signal from the fluorescent protein in the cell or cells.

In some embodiments, binding of the inhibitory RNA to the target gene within the polycistronic mRNA results in degradation of the polycistronic mRNA. In some embodiments, degradation of the polycistronic mRNA results in reduction in the fluorescent signal from the fluorescent protein in the cell or cells.

In some embodiments, the method of detecting the inhibitory or silencing activity further comprises incubating the cell or cells under conditions suitable for expression of the reporter nucleic acid and the expression nucleic acid. In some embodiments, the method of detecting the inhibitory or silencing activity further comprises detecting the reduction in the fluorescent signal.

In some embodiments, the reduction in the fluorescent signal from the fluorescent protein in the cell or cells is detected via flow cytometry, fluorescent microscopy or any fluorescent imaging system. In some embodiments, the reduction in the fluorescent signal from the fluorescent protein in the cell or cells is detected via flow cytometry.

The cell or cells may be any cell disclosed herein. In some preferred embodiments, the cell is a macrophage. In some preferred embodiments, the cell is an iPSC. In some preferred embodiments, the cell is a T cell. In some preferred embodiments, the cell is a primary T cell. In some preferred embodiments, the cell is a T cell line, preferably Jurkats.

In embodiments wherein the cell is a T cell, the reporter nucleic acid and the expression nucleic acid are preferably provided in a single lentiviral vectors or in two or more separate lentiviral vectors. Plasmid-based approaches are limited in their ability to transduce T cells and using lentiviral delivery is beneficial in detecting the inhibitory or silencing activity of multiple inhibitory RNAs.

In some preferred embodiments, the expression systems has a nucleic acid sequence encoding two or more inhibitory RNAs or two or more expression nucleic acids and the reporter nucleic acid and the expression nucleic acid are provided in in one or more viral vectors, preferably lentiviral vectors. This embodiment is particularly useful for screening inhibitory RNAs in an efficient and cost effective manner as detailed in Examples 21-22 and Example 10.

In some embodiments, wherein the cell is iPSC, the reporter nucleic acid and the expression nucleic acid are preferably provided in a single lentiviral vectors or in two or more separate lentiviral vectors. Plasmid based screens in iPSC are limited in the number of plasmids which can be transduced and multiple inhibitory RNAs need to be screened in arrays. On the other hand, lentiviral delivery allows a greater number of inhibitory RNAs to be screened at any one time.

In a further aspect, there is provided a method of selecting inhibitory RNA or RNAs which show silencing or inhibition of a target gene from a pool of test inhibitory RNAs, comprising:
(i) transfecting a cell or cells with the expression system according to the invention;
(iii) selecting the inhibitory RNA or RNAs which were transfected into the cell or cells which show reduction in the fluorescent signal from the fluorescent protein.

In some embodiments, the expression system has a nucleic acid sequence encoding each of the pool of test inhibitory RNAs or two or more expression nucleic acids, each encoding one of the pool of test inhibitory RNAs.

In some embodiments, the method of selecting further comprises a step of detecting and selecting the inhibitory RNA which show the highest silencing or inhibition of the target gene within the selected inhibitory RNAs. In some embodiments, the step of detecting and selecting is performed by detecting discreet cell populations which correspond to individual inhibitory RNAs via flow cytometry and selecting the inhibitory RNA is transfected into the cell population with the highest silencing or inhibition of the target gene.

In some embodiments, binding of the inhibitory RNA to the target gene within the polycistronic mRNA results in degradation of the polycistronic mRNA. In some embodiments, degradation of the polycistronic mRNA results in reduction in the fluorescent signal from the fluorescent protein in the cell or cells.

In some embodiments, the method of selecting inhibitory RNAs further comprises incubating the cell or cells under conditions suitable for expression of the reporter nucleic acid and the expression nucleic acid. In some embodiments, the method of selecting inhibitory RNAs further comprises detecting the reduction in the fluorescent signal.

In some embodiments, the reduction in the fluorescent signal from the fluorescent protein in the cell or cells is detected via flow cytometry, fluorescent microscopy or any fluorescent imaging system. In some embodiments, the reduction in the fluorescent signal from the fluorescent protein in the cell or cells is detected via flow cytometry.

The cell or cells may be any cell disclosed herein. In some preferred embodiments, the cell is a macrophage. In some preferred embodiments, the cell is an iPSC. In some preferred embodiments, the cell is a T cell. In some preferred embodiments, the cell is a primary T cell. In some preferred embodiments, the cell is a T cell line, preferably Jurkats.

In embodiments wherein the cell is a T cell, the reporter nucleic acid and the expression nucleic acid are preferably provided in a single lentiviral vectors or in two or more separate lentiviral vectors. Plasmid-based approaches are limited in their ability to transduce T cells and using lentiviral delivery is beneficial in detecting the inhibitory or silencing activity multiple inhibitory RNAs.

In some embodiments, wherein the cell is iPSC, the reporter nucleic acid and the expression nucleic acid are preferably provided in a single lentiviral vectors or in two or more separate lentiviral vectors. Plasmid based screens in iPSC are limited in number and multiple inhibitory RNAs need to be screened in arrays. Lentiviral delivery allows a greater number of inhibitory RNAs to be screened at any one time.

Various aspects, embodiments and examples of the invention are described in further detail below.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

Brief Description of the Figures

Figure 1 shows a schematic representation MHC-I on the surface of cells and the immune surveillance system.

Figure 2 shows a schematic illustration of the interaction of cells comprising non-self MHC-I and those completely lacking MHC-I with cells of the adaptive and innate immune system.

Figure 3 illustrates a strategy to generate hypoimmune cells based on downregulating MHC-I expression to an optimum level that enables evading both T cell- and NK cell-mediated responses.

Figure 4 shows a schematic illustration of the use of the GEiGS workflow to develop, test and implement modified cells according to the present invention.

Figure 5 shows a plasmid map of plasmid VB210602-1567ytv that was used for ectopic (episomal) expression to assess the ability of miRNA sequences determined by GEiGS ("GEiGS solutions") to silence B2M expression.

Figure 6 shows a graph of small RNA real-time PCR (RT-PCR) in iPSCs transfected with plasmids expressing either a negative control-expressing plasmid or B2M-targeting Solution 43. The GEiGS solution is only detected in the corresponding sample indicating the specificity of the assay. Solution 43 is expressed at comparable levels to a panel of endogenous miRNAs.

Figure 7 shows a flow cytometry method and gating to assess the ability of a given GEiGS silencing RNA to reduce B2M expression.

Figure 8 shows a graph illustrating the ability or a range of inhibitory/silencing RNA sequences developed through the GEiGS process ("GEiGS solutions") to reduce B2M expression in cells as determined by flow cytometry. Each GEiGS solution is a combination of miRNA scaffold and specific sequence changes complementary to the B2M mRNA that trigger its degradation. Solutions are developed via the computational workflow to reduce B2M expression Series 1 illustrates the result for all cells expressing DsRed (all successfully transfected cells), while series 2 shows the results for the top 20%, i.e. cells expressing high levels of DsRed and therefore high levels of the GEiGS solution (silencing RNA, sRNA).

Figures 9 and 10 show mean silencing activity of GEiGS in clonal cell lines in which GEiGS Solutions are knocked-in to either one or both alleles corresponding to the genomic location of the original encoded miRNA, using gene editing techniques. Data is expressed as residual B2M expression measured by flow cytometry, averaged over multiple clones of the same genotype.

Figure 11 shows miRNA expression of the endogenous miRNAs of solutions 12 and 30 in iPSCs and pancreatic progenitor cells. It can be seen that expression of the solution 30 miRNA (hsa -mir-21) is specific to pancreatic progenitor cells, whereas the solution 12 miRNA (hsa-mir-302c) is specific to iPSCs.

Figure 12 shows a) results indicating a reduction in surface B2M expression. Of the CD3 positive, mixed T cell population approximately 24% of the cells demonstrated a silencing of B2M, with a reduction in mean fluorescent intensity of 80% , compared with control unedited cells; b) Quantification of B2M silencing in helper (CD4+) and cytotoxic (CD8+) T cells, showing efficient silencing of B2M in both compartments (by ~90%).

Figure 13 shows a schematic illustration of the use of a GEiGS approach to promote activity of T cells in the tumour microenvironment.

Figure 14 shows a schematic illustration of a strategy for the use of GEiGS in macrophage therapy in glioblastoma. GEiGS is used to avoid macrophages adopting a pro-tumour (i.e. tumour associated macrophage, TAM) state, promoting an active, inflammatory phenotype in the tumour microenvironment (TME). Context-specific GEiGS is used to target the IL-4 induced genes STAT6 and IRF4 genes, promoting expression of inflammatory genes.

Figure 15 shows a schematic illustration of how GEiGS-modification can be used to specifically silence further pathways, for example only when T cells reach the tumour. Context -specific GEiGS-mediated inhibition of mitochondrial fission in the TME by redirecting induced miRNAs to conditionally silence DRP1 favours the differentiation and persistence of memory T cells, while allowing mitochondrial dynamics to continue and contribute to mitochondrial homeostasis (such as mitophagy).

Figure 16 shows a scatter plot of the transcriptome of primary T cells, illustrating fold change in counts per million fold change (from naïve to stimulated). This demonstrates that stimulation-induced miRNAs can be detected in primary T cells. Candidate upregulated miRNAs are highlighted by larger circles (data from Amaral et al, 2017, EMBO J, EMBO J. 2017 Feb 1;36(3):346-360)

Figure 17 shows graphs illustrating primary T cell editing efficiency by CRISPR KI (GFP KI at the RAB11A locus) demonstrating that T cells can be edited by CRISPR knock-in (KI) with high efficiency (data from Roth et al, 2018, Nature Nature. 2018 Jul;559(7714):405-409.).

Figure 18a Differential expression analysis of miRNA and mRNA in primary macrophages. Plots show expression level (x-axis, log2 of normalized counts) vs fold-change (y-axis, log2 transformed). Comparison is between pro- (M1) and anti-inflammatory (M2a). Dots represent individual genes and highlighted values are statistically significantly different between conditions – above 0 are high in anti-inflammatory and low in pro-inflammatory, below 0 are low in anti-inflammatory and high in pro-inflammatory

Figure 18b Efficient differentiation and polarisation of THP-1 cells into pro- and anti-inflammatory macrophage-like cells. Plots show flow cytometry detection of known macrophage polarisation markers (CD80 for pro-inflammatory; CD209 for anti-inflammatory);

Figure 18c Correlation analysis of miRNA expression in polarized primary macrophages and THP-1 cells, showing a high degree of correlation between the two experimental models.

Figure 19a. Efficient generation of monocytes from iPSC. The iPSC lines (GEiGS Solution 30 (S30/S30 genotype) and isogenic control) were used to generate enriched monocytes using published protocols, from which greater than 90% of the cells were double positive for the known monocyte markers CD14 and CD45.

Figure 19b. Context specific silencing of B2M in iPSC-derived monocytes. The iPSC lines (GEiGS Solution 30 and isogenic control) were used to generate monocytes which were then stained for residual cell surface B2M expression using flow cytometry (as described for Figs. 9, 10 and 12). When expanded as undifferentiated iPSC, greater than 93% of the cells for both the isogenic control and GEiGS Solution 30 clonal lines expressed B2M, whereas following differentiation into monocytes the GEiGS Solution 30 line showed a marked reduction in the number of cells with the same quantity of cell surface expressed B2M compared with the isogenic control line (16% cells with residual B2M compared with 77%).

Figure 20a shows an illustration of a process for identifying miRNAs for context-specific GEiGS, and then implementation via an ectopic screen to assess target RNA silencing in target cells using a dual reporter. plasmid system.

Figures 20b and c show the plasmid maps of plasmids which are used to perform the ectopic expression screen to identify GEiGS Solutions targeting the genes PPARγ, IRF4, KDM6B and STAT6 in monocyte derived macrophages (see Example 9).

Figure 20d shows the dual reporter construct containing 3' untranslated sequences of target genes in macrophages with which to analyse via the regression of GFP, the specificity of GEiGS Solutions.

Figures 21a and 21b show results of context-specific silencing of B2M in iPSC-derived monocytes, related to the results shown in Fig. 19. Fig. 21a shows expression of hsa-mir-21 in iPSC compared to macrophages; miR-21 is highly expressed in macrophages but not in iPSC (NGS quantification was used). Fig. 21b shows relative B2M expression in GEiGS modified iPSC, monocytes and macrophages (miR-21 redirected to target B2M, i.e. Solution 30) compared to control cells in which the endogenous miR-21 has been knocked out (KO/KO). The GEiGS and control lines were differentiated *in vitro* first to monocytes and subsequently to macrophages. B2M was assayed by flow cytometry showing effective silencing in myeloid cells (84-88% silencing), but not at the iPSC stage.

Figures 21c, 21d, 21e, and 21f show that B2M silencing in myeloid cells results in reduction of cell surface HLA-A/B/C (and therefore MHC-I) in monocytes and macrophages.

Figures 22a, b and c show results of a study indication that GEiGS is very specific, showing no off-target effects. In particular a GEiGS approach as described herein to silence B2M was highly specific, showing no significant off-target effects on expression of other than B2M. The MA plots of the differential gene expression analysis show log2 of mean count per million on the x-axis and log2 of fold-change on the y-axis. Dots represent individual genes tested. Significantly downregulated genes are highlighted in blue, significantly up-regulated genes are highlighter in red). Genes with no significant change in expression level are shown in black. Fig. 22a shows transcriptomic data from cells modified by a CRISPR B2M knock out compared to the control WT/WT parental cell line. The significantly reduced expression level of B2M compared to control is highlighted. No other significant changes in expression levels are for other genes are evident. Fig. 22b shows transcriptomic data in cells in which the miRNA which hsa-mir-20a has been knocked out compared to the control WT/WT parental cell line. Note - hsa-mir-20a is the miRNA which is edited when 'Solution 29' is introduced via GEiGS. No significant changes in expression levels are evident as a result of the knock out of hsa-mir-20a. Fig. 22c shows transcriptomic data in cells in which the miRNA which Solution 29 has been introduced via GEiGS compared to the control WT/WT parental cell line. As can be seen, as with the CRISPR KO, only B2M shows a significant alteration in expression. No other significant changes in expression levels are for other genes are evident.

Figure 23 shows effects of homology arm length on efficiency of homology dependent repair (HDR). Flanking genomic sequences (homology arms) of length 40 base pairs show superior effects for the efficiency of HDR (% HDR) and in a dose dependent manner compared to flanking genomic sequences of length 350 bp or length 150-350 bp which are only marginally better to no donor template alone.

Figure 24 shows that partial knockdown of B2M results in reduced specific lysis by activated NK cells in tissue culture (corresponding to enhanced cell survival following exposure to the innate (NK-mediated) immune system). NK and target cells were cultured under different experimental conditions including exposure to NK-cell activating cytokines IL-2 and/or IL-12. Control iPSC displayed a reduced level of specific lysis compared to a positive control K562 cell line, known to be a potent stimulator of activated NK cell mediated killing. Undifferentiated $B2M^{-/-}$ iPSC showed elevated levels of specific lysis as complete absence of B2M results in complete absence of MHC-I ("missing self" phenotype) and detection and lysis by activated NK cells. Undifferentiated, GEiGS-modified iPSC lines ($iPSC^{S29/29}$) displayed consistently reduced specific lysis by NK cells compared the $B2M^{-/-}$ cell lines across all mixed culture conditions tested

Figure 25 shows Solution 29's ability to buffer the induction of B2M expression (as measured by MHC-I detection) in edited cells following their exposure to the inflammatory stimulus (IFN-γ).

Figure 26 shows the ability of a Solution 30 heterozygous IPSC line to differentiate into CXCR4 expressing definitive endoderm cells. The heterozygous knock-in line (iPSC miR-$21^{S30/+1}$) showed a high proportion of CXCR4 positive cells (approximately 97%), compared with both control (unmodified, 50%) and $B2M^{-/-}$ iPSCs (95%).

Figure 27a shows reduced B2M expression in the proportion of CXCR4 expressing cells generated via in vitro differentiation of the Solution 30 heterozygous IPSC line. The iPSC miR-$21^{S30/+1}$ line showed an approximately 80% reduction in B2M expression compared with control (unmodified) iPSC cell line. As predicted $B2M^{-/-}$ iPSCs do not express the B2M protein.

Figure 27b shows expression of pancreatic and duodenal homeobox-1 (PDX-1) and B2M in pancreatic progenitor cells. Results indicate that a significant proportion of the specified progenitors are positive for PDX-1, a marker gene which is necessary for pancreatic development and Beta-cell maturation. The majority of PDX-1 positive cells also stained for B2M. The iPSC miR-$21^{S30/+1}$ clonal line showed a high proportion of PDX-1 expressing cells which had markedly reduced B2M expression compared with the control (unmodified) iPSCs.

Figure 28 shows the flow cytometry gating strategy used for the determination of 'hits' for GEiGS Solutions against macrophage target genes using the dual reporter system.

Figure 29 shows identification of macrophage target gene (STAT6, IRF4 and KDM6B) GEiGS Solutions from the ectopic screen using the dual reporter system construct.

Figure 30 shows the ectopic screening strategy using dual reporter system to identify 'hits' from designed GEiGS Solutions against T-cell targets of interest.

Figure 31a shows constructs used to validate the use of lentiviral transduction and a pooled screening strategy. **Figure 31b** shows use of lentiviral transduction and a pooled screening strategy to identify 'hits' from designed GEiGS Solutions using primary human T cells.

Figure 32A shows validation of the lentiviral based pooled screening strategy in primary human T cells using verified Solutions for both eGFP and B2M. **Figure 32B** shows DsRed and B2M knockdown corresponding to individual validated Solutions (selected from Example 2) and as combined solutions delivered via lentiviruses in Jurkat cell line.

Figures 33a and b show that multiple Solutions can silence their target genes when ectopically expressed from a single construct with a constitutive promoter in iPSC. The plasmid map for expression of one or more Solutions is shown in Fig. 33a. The plasmid construct is depicted with a miR-30 based Solution targeting eGFP downstream of dsRed reporter in the multiple cloning site (MCS), driven by the constitutive EF1a promoter. The eGFP targeted Solution is replaced with one or more Solutions designed against B2M and eGFP or B2M alone (see Example 23). Figure 33b shows that Solution 101, consisting of miRNA-30 repurposed to target GFP alone, demonstrated an efficient silencing of GFP without affecting the level of expression of B2M in the undifferentiated iPSC and that Solution 179, consisting of two distinct modified miRNA scaffolds (miRNA-20a and miRNA-30), was effective in silencing both B2M and eGFP with apparent equal efficiency.

Figure 34 shows that Solution 177, consisting of modified miRNA-518b (Solution 43) and miRNA-20a (Solution 29), distinct Solutions with diverse sequences targeting the B2M transcript (triggers), appeared more effective in silencing B2M when compared to the Solution 43 alone, indicating a minor additive effect and that the two distinct Solutions do not interfere in each others' ability to silence B2M. Whereas Solution 178 which possesses two copies of Solution 29 and the same trigger sequences, does not have an additive effect in silencing B2M compared with Solution 29 alone.

Detailed Description of Embodiments of the Invention and Examples

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

Definitions

"Context-specific activity", "context-specific inhibition", "context-specific manner" and similar expressions refer to inhibition of the target gene or target RNA by the inhibitory RNA which occurs, preferentially, predominantly or only in a given cellular context. In some embodiments, "context-specific" may be "cell context-specific" inhibition of the target gene or target RNA by the inhibitory RNA which occurs, preferentially, predominantly or only in an individual cell in a given cellular context. In some embodiments, "context-specific" excludes tissue-specific. "Context" in the present context refer to any suitable cell type, cell condition or state of a cell as appropriate. A "first context" is a context in which it is desirable to selectively regulate (e.g. silence) expression of a target gene. In particular, a first context is typically a cellular context in which there is increased activity of a given endogenous inhibitory RNA (e.g. an miRNA) compared to other contexts. Thus, the activity the endogenous RNA can be harnessed to provide context-specific gene silencing. A "second context" may be any context in which it is desirable that the target gene is not silenced or is silenced to a lesser extent. For, example, a first or second context for a cell of the invention may be selected from the following non-exhaustive list: a cell type; a stage of differentiation of a cell; a disease condition; a hypoxic condition; a stimulated condition; an inflammatory condition; a tumour associated condition; etc. In some embodiments the second context is any context other than the first context. In some embodiments the second context is a different stage of differentiation of the cell. In some embodiments the second context is when the cell is not in the intended location (e.g. in the TME). Further contexts are discussed in more detail herein. A target mRNA (i.e., an mRNA that is the target of a an inhibitory RNA in the invention) is typically at least partially silenced in the first context, and is not silenced, or is silenced to a lesser degree, in a context other than the first context. A target mRNA may be an mRNA that is expressed at a high level in the first context, e.g. because it is upregulated in the first context.

The term "RNA interference" and the term "RNAi" are synonymous and refer to the process by which a polynucleotide (e.g. a miRNA or siRNA) comprising at least one polyribonucleotide unit exerts an effect on a biological process. The process includes, but is not limited to, gene silencing by degrading mRNA, attenuating translation, interactions with tRNA, rRNA, hnRNA, cDNA and genomic DNA, as well as methylation of DNA with ancillary proteins. An "inhibitory RNA" is an RNA molecule capable of RNA interference, either as is or after cellular processing. Thus, the term includes, *inter alia*, RNA precursors that are processed to form a miRNA, siRNA, or other RNA that can degrade target mRNA. The term 'sRNA' is also used herein to refer to an inhibitory RNA (i.e. a silencing RNA, abbreviated to sRNA).

The term “silencing” or “gene silencing” refers to a process by which the expression of a specific gene product is lessened or attenuated, in the present case typically by RNA interference. The target gene is typically silenced as a result of the inhibitory RNA targeting (e.g. binding *via* complementarity) a target RNA encoded by the gene. Thus, silencing a gene in the present context is typically achieved through targeting of target RNA transcribed from the gene by the inhibitory RNA as described herein. The silenced gene typically encodes a protein gene product, but it may encode an RNA gene product. The level of gene silencing (also sometimes referred to as the degree of “knockdown” or reduction of expression) can be measured by a variety of means, including, but not limited to, measurement of transcript levels by Northern Blot Analysis, B-DNA techniques, transcription-sensitive reporter constructs, expression profiling (e.g. DNA chips), qRT-PCR and related technologies. Alternatively, the level of silencing can be measured by assessing the level of the protein encoded by a specific gene. This can be accomplished by performing a number of studies including antibody-based detection such as Western Analysis and flow cytometry, measuring the levels of expression of a reporter protein that has e.g. fluorescent properties (e.g., GFP) or enzymatic activity (e.g. alkaline phosphatases), or several other procedures. Typically the level of silencing or reduction is compared to the level of expression of a given protein or mRNA in a control cell, as appropriate. Silencing by at least 30%, 40% 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99%, for example, may be preferred, depending on the situation. Often higher levels of silencing are preferred.

The terms “microRNA”, “miRNA”, or “miR” are synonymous and all refer to non-coding RNAs of, for example, about 19-24 nucleotides in length (and also, as the context will indicate, to DNA sequences that encode such RNAs) that are capable of entering the RNAi pathway and regulating gene expression. “Primary miRNA” or “pri-miRNA” represents the non-coding transcript prior to Drosha processing and includes the stem-loop structure(s) as well as flanking 5′ and 3′ sequences. “Precursor miRNAs” or “pre-miRNA” represents the non-coding transcript after Drosha processing of the pri-miRNA. The term “mature miRNA” can refer to the double stranded product resulting from Dicer processing of pre-miRNA or the single stranded product that is introduced into RISC following Dicer processing. In some cases, only a single strand of an miRNA enters the RNAi pathway. In other cases, both strands of a miRNA are capable of entering the RNAi pathway. miRNAs are found in a wide range of organisms (e.g. insects, mammals, plants, nematodes) and have been shown to play a role in development, homeostasis, and disease aetiology.

The term “silencing RNA form” or “silencing RNAs” or “silencing RNA molecule”, “sRNA” or “trigger sequence/RNA” refers to the mature small RNA being capable of hybridizing with a target RNA (or fragment thereof) and engage the RNAi pathway.

The term “target RNA” refers to a specific RNA that is targeted by the RNAi pathway, resulting in a decrease in the functional activity of the RNA. In some cases, the RNA target is a mRNA (typically B2M) whose functional activity is its ability to be translated. In such cases, the RNAi pathway will decrease the functional activity of the mRNA by translational attenuation or by cleavage. In the instant disclosure, target RNAs are targeted by non-naturally occurring miRNAs. The term “target” can also refer to DNA.

The term “endogenous miRNA” refers to a miRNA produced in an organism through transcription of sequences that naturally are present in the genome of that organism. Endogenous miRNA can be localized in, for example introns, open reading frames (ORFs), 5′ or 3′ untranslated regions (UTRs), or intergenic regions. The organism which produces an endogenous miRNA may be, without limitation, human (and other primates), mouse, rat, fly, worms, fish or other organisms that have an intact RNAi pathway. In some embodiments of the present invention endogenous miRNA loci are altered to retarget the endogenous miRNA to a new target (see, e.g. WO2019/058253, WO2020/183414 and WO2020/183419 for some suitable approaches using the approach referred to as GEiGS). Endogenous miRNAs can be modified *in situ*, i.e. the endogenous miRNA in the genome can be modified directly, or a copy of an endogenous miRNA can be removed from its normal genomic context and modified (e.g. for ectopic expression in the cell, for example either in a different genomic locus or episomally in a suitable vector). A modified copy of an endogenous miRNA can also be inserted in the locus of the original endogenous miRNA so that it is co-transcribed with the original endogenous miRNA; this can be achieved without disrupting or inactivating the original endogenous miRNA (e.g. placing the modified copy of the endogenous miRNA upstream or downstream of the original endogenous miRNA).

The term “nucleic acid construct adapted to express an inhibitory RNA” as used herein means any polynucleotide sequence suitable for expression of the inhibitory RNA as described herein. The nucleic acid construct may be genomic or episomal. In some embodiments, the nucleic acid construct may be a modified endogenous genomic sequence, e.g. which has been genetically modified, e.g. gene edited, so that it is adapted to express the inhibitory DNA. In some embodiments the nucleic acid construct may be a synthetic construct, e.g. an expression cassette in a suitable vector. In other embodiments the nucleic acid construct may be a synthetic construct which has been inserted into the genome. In some preferred

embodiments this construct is a modified endogenous genomic locus in which an endogenous sequence encoding a non-coding RNA has been modified (*in situ*) to provide a sequence encoding an inhibitory RNA which has been retargeted so that it is able to silence a target gene.

The terms "identity" and "identical" and the like refer to the sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, such as between two DNA molecules. Sequence alignments and determination of sequence identity can be done, e.g., using the Basic Local Alignment Search Tool (BLAST) originally described by Altschul et al. 1990 (J Mol Biol 215: 403-10), such as the "Blast 2 sequences" algorithm described by Tatusova and Madden 1999 (FEMS Microbiol Lett 174: 247-250).

Methods for aligning sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in, for example: Smith and Waterman (1981) Adv. Appl. Math. 2:482; Needleman and Wunsch (1970) J. Mol. Biol. 48:443; Pearson and Lipman (1988) Proc. Natl. Acad. Sci. U.S.A. 85:2444; Higgins and Sharp (1988) Gene 73:237-44; Higgins and Sharp (1989) CABIOS 5:151-3; Corpet et al. (1988) Nucleic Acids Res. 16:10881-90; Huang et al. (1992) Comp. Appl. Biosci. 8:155-65; Pearson et al. (1994) Methods Mol. Biol. 24:307-31; Tatiana et al. (1999) FEMS Microbiol. Lett. 174:247-50. A detailed consideration of sequence alignment methods and homology calculations can be found in, e.g., Altschul et al. (1990) J. Mol. Biol. 215:403-10.

The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST™; Altschul et al. (1990)) is available from several sources, including the National Center for Biotechnology Information (Bethesda, MD), and on the internet, for use in connection with several sequence analysis programs. A description of how to determine sequence identity using this program is available on the internet under the "help" section for BLAST™. For comparisons of nucleic acid sequences, the "Blast 2 sequences" function of the BLAST™ (Blastn; Align Sequence Nucleotide BLAST) program may be employed using the default parameters. Nucleic acid sequences with even greater similarity to the reference sequences will show increasing percentage identity when assessed by this method. Typically, the percentage sequence identity is calculated over the entire length of the sequence.

For example, a global optimal alignment is suitably found by the Needleman-Wunsch algorithm with the following scoring parameters: Match score: +2, Mismatch score: -3; Gap penalties: gap open 5, gap extension 2. The percentage identity of the resulting optimal global alignment is suitably calculated by the ratio of the number of aligned bases to the total length

of the alignment, where the alignment length includes both matches and mismatches, multiplied by 100.

The term “complementary” refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands. Complementary polynucleotide strands can base pair in the Watson-Crick manner (e.g., A to T, A to U, C to G), or in any other manner that allows for the formation of duplexes, including the wobble base pair formed between U and G. As persons skilled in the art are aware, when using RNA as opposed to DNA, uracil rather than thymine is the base that is considered to be complementary to adenosine. However, when a U is denoted in the context of the present invention, the ability to substitute a T is implied, unless otherwise stated.

Perfect complementarity or 100% complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand can hydrogen bond with a nucleotide unit of a second polynucleotide strand. Partial complementarity refers to the situation in which some, but not all, nucleotide units of two strands can hydrogen bond with each other. For example, two strands are at least partially complementary when at least 6-7 base pairs can be formed over a stretch of about 19-25 nucleotides. Sequences are said to be “complementary” to one another when each sequence is the (partial or complete) reverse complement (RC) of the other. For example, the sequence 5′ GATC 3′ is perfectly complementary to its reverse complement sequence 3′ CTAG 5′. Sequences can also have wobble base pairing.

The term "expression cassette" as used herein includes a polynucleotide sequence encoding a polypeptide or RNA to be expressed and sequences controlling its expression such as a promoter and optionally an enhancer sequence, including any combination of cis-acting transcriptional control elements.

The term "eukaryotic cell" as used herein refers to any cell of a eukaryotic organism. Eukaryotic organisms include single- and multi-cellular organisms. Single cell eukaryotic organisms include, but are not limited to, yeast, protozoans, slime moulds and algae. Multi cellular eukaryotic organisms include, but are not limited to, animals (e.g. mammals, insects, invertebrates, nematodes, birds, fish, reptiles and crustaceans), plants, fungi and algae (e.g. brown algae, red algae, green algae).

As used herein, the phrase “stem cells” refers to cells which are capable of self-renewing while in an undifferentiated state for extended periods of time in culture, but also retain the capacity

to undergo differentiation into one or more different cell types having a particular, specialized function (e.g., fully differentiated cells). Stem cells can be totipotent, pluripotent, multipotent or unipotent until induced to differentiate into other cell types. Totipotent cells, such as embryonic cells within the first couple of cell divisions after fertilization are the only cells that can differentiate into embryonic and extra-embryonic cells and are able to develop into a viable human being. Preferably, the phrase “pluripotent stem cells” refers to cells which can differentiate into all three definitive embryonic germ layers, i.e., ectoderm, endoderm and mesoderm or remaining in an undifferentiated state. The pluripotent stem cells include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC). The multipotent stem cells include adult stem cells and hematopoietic stem cells.

“Induced pluripotent stem cells” (iPSC; embryonic-like stem cells) refers to cells obtained by de-differentiation of adult somatic cells to endow cells with pluripotent properties, i.e., being capable of differentiating into the three embryonic germ cell layers, i.e., endoderm, ectoderm and mesoderm. Such cells may be obtained from a differentiated tissue (e.g., a somatic tissue such as skin) and undergo de-differentiation by genetic manipulation which reprogram the cell to acquire embryonic stem cells characteristics. The induced pluripotent stem cells may be formed by inducing the expression of Oct-4, Sox2, Kfl4 and c-Myc in a somatic cell. Induced pluripotent stem cells (iPSC) (embryonic-like stem cells) can be generated from somatic cells by genetic manipulation of somatic cells, e.g., by retroviral transduction of somatic cells such as fibroblasts, hepatocytes, gastric epithelial cells with transcription factors such as Oct-3/4, Sox2, c-Myc, and KLF4 such as described in Park et al. Reprogramming of human somatic cells to pluripotency with defined factors. Nature (2008) 451:141-146. iPSCs can be human or non-human.

The phrase "embryonic stem cells" refers to embryonic cells which are capable of differentiating into cells of all three embryonic germ layers (i.e., endoderm, ectoderm and mesoderm), or remaining in an undifferentiated state. The phrase "embryonic stem cells" may comprise cells which are obtained from the embryonic tissue formed after early gestation (e.g., blastocyst) before implantation of the embryo (i.e., a pre-implantation blastocyst), extended blastocyst cells (EBCs) which are obtained from a post-implantation/pre-gastrulation stage blastocyst (see WO2006/040763), embryonic germ (EG) cells which are obtained from the genital tissue of a foetus any time during gestation, preferably before 10 weeks of gestation, and cells originating from an unfertilized ova which are stimulated by parthenogenesis (parthenotes).

The embryonic stem cells of some embodiments of the invention can be obtained using well-known cell-culture methods. For example, human embryonic stem cells can be isolated from human pre-implantation blastocysts. Human blastocysts are typically obtained from human in vivo preimplantation embryos or from in vitro fertilized (IVF) embryos. Alternatively, a single cell human embryo can be expanded to the blastocyst stage.

It will be appreciated that commercially available stem cells can also be used according to some embodiments of the invention. Human ES cells can be purchased from the NTH human embryonic stem cells registry [www.grants.nih.gov/stem_cells/registry/current.htm].

In addition, embryonic stem cells can be obtained from various species, including mouse (Mills and Bradley, 2001), golden hamster [Doetschman et al., 1988, Dev Biol. 127: 224-7], rat [Iannaccone et al., 1994, Dev Biol. 163: 288-92] rabbit [Giles et al. 1993, Mol Reprod Dev. 36: 130-8; Graves & Moreadith, 1993, Mol Reprod Dev. 1993, 36: 424-33], several domestic animal species [Notarianni et al., 1991, J Reprod Fertil Suppl. 43: 255-60; Wheeler 1994, Reprod Fertil Dev. 6: 563-8; Mitalipova et al., 2001, Cloning. 3: 59-67] and non-human primate species (Rhesus monkey and marmoset) [Thomson et al., 1995, Proc Natl Acad Sci U S A. 92: 7844-8; Thomson et al., 1996, Biol Reprod. 55: 254-9].

The phrase "adult stem cells" (also called "tissue stem cells" or a stem cell from a somatic tissue) refers to any stem cell derived from a somatic tissue [of either a postnatal or prenatal animal (especially the human)]. The adult stem cell is generally thought to be a multipotent stem cell, capable of differentiation into multiple cell types. Adult stem cells can be derived from any adult, neonatal or fetal tissue such as adipose tissue, skin, kidney, liver, prostate, pancreas, intestine, bone marrow and placenta.

According to one embodiment, the stem cells utilized by some embodiments of the invention are bone marrow (BM)-derived stem cells including hematopoietic, stromal or mesenchymal stem cells [Dominici, M et al., (2001) J. Biol. Regul. Homeost. Agents. 15: 28-37]. BM-derived stem cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullar spaces.

Hematopoietic stem cells (HSCs), which may also referred to as adult tissue stem cells, include stem cells obtained from blood or bone marrow tissue of an individual at any age or from cord blood of a newborn individual. Preferred stem cells according to this aspect of some embodiments of the invention are embryonic stem cells, preferably of a human or primate (e.g., monkey) origin.

Mesenchymal stem cells (MSCs), the formative pluripotent blast cells, give rise to one or more mesenchymal tissues (e.g., adipose, osseous, cartilaginous, elastic and fibrous connective tissues, myoblasts) as well as to tissues other than those originating in the embryonic mesoderm (e.g., neural cells) depending upon various influences from bioactive factors such as cytokines. Although such cells can be isolated from embryonic yolk sac, placenta, umbilical cord, fetal and adolescent skin, blood and other tissues, their abundance in the BM far exceeds their abundance in other tissues and as such isolation from BM is presently preferred. Adult tissue stem cells can be isolated using various methods known in the art such as those disclosed by Alison, M.R. [J Pathol. (2003) 200(5): 547-50]. Fetal stem cells can be isolated using various methods known in the art such as those disclosed by Eventov-Friedman S, et al. [PLoS Med. (2006) 3: e215].

Hematopoietic stem cells can be isolated using various methods known in the arts such as those disclosed by "Handbook of Stem Cells" edit by Robert Lanze, Elsevier Academic Press, 2004, Chapter 54, pp609-614, isolation and characterization of hematopoietic stem cells, by Gerald J Spangrude and William B Stayton.

Methods of isolating, purifying and expanding mesenchymal stem cells (MSCs) are known in the arts and include, for example, those disclosed by Caplan and Haynesworth in U.S. Pat. No. 5,486,359 and Jones E.A. et al., 2002, Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells, Arthritis Rheum. 46(12): 3349-60.

“Artificial” or “synthetic” when used with reference to polynucleotide sequence means a sequence not found in nature which is, for example, a synthetic modification of a natural sequence, or contains an unnatural sequence.

The terms “polynucleotide”, “nucleotide” or “nucleic acid” are used interchangeably herein and refer to a polymeric macromolecule made from nucleotide monomers particularly deoxyribonucleotide or ribonucleotide monomers chain of nucleotides, regardless of length. Nucleotides include species that comprise purines, e.g., adenine, hypoxanthine, guanine, and their derivatives and analogues, as well as pyrimidines, e.g., cytosine, uracil, thymine, and their derivatives and analogues. The term also encompasses nucleic acids containing known nucleotide analogues or modified backbone residues or linkages, which are naturally occurring and non-naturally occurring, which have similar properties as the reference nucleic acid, and which are intended to be metabolized in a manner similar to the reference nucleotides or are intended to have extended half- life in the system. Examples of such analogues include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs). Suitably the term

"polynucleotide" refers to naturally occurring polymers of deoxyribonucleotide or ribonucleotide monomers. Non-limiting examples of polynucleotides include a gene, a gene fragment, messenger RNA (mRNA), complementary DNA (cDNA), non-coding RNA (ncRNA), microRNA (miRNA), small RNA (sRNA), short interfering RNA (siRNA), short hairpin RNA (shRNA), recombinant polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide of the invention may be provided in isolated or substantially isolated form. By substantially isolated, it is meant that there may be substantial, but not total, isolation of the polypeptide from any surrounding medium. The polynucleotides may be mixed with carriers or diluents which will not interfere with their intended use and still be regarded as substantially isolated. Suitably the polynucleotides of the invention are recombinant. Recombinant means that the polynucleotide is the product of at least one of cloning, restriction or ligation step, or other procedures that result in a nucleic acid molecule that is distinct from a nucleic acid molecule found in nature (e.g. in the case of cDNA).

As used herein the term "operably linked" refer to the arrangement of various nucleic acid elements relative to each other such that the elements are functionally connected and are able to interact with each other in the manner intended. The nucleic acid sequence elements, when operably linked, act together to modulate the activity of one another. By modulate is meant increasing, decreasing, or maintaining the level of activity of a particular element.

As used herein "polycistronic mRNA" is a mRNA which encodes two or more proteins.

As used herein "constitutive promoter" is a promoter which allow for continuous transcription of the gene or genes they are operably linked to. A constitutive promoter may be active in a call under all circumstances.

The term "a fluorescent protein" refers to a polypeptide that emits fluorescence and is typically detectable by flow cytometry, microscopy or any fluorescent imaging system, therefore can be used as a basis for selection of cells expressing such a protein. Examples of fluorescent proteins that can be used as reporters are, without being limited to, the Green Fluorescent Protein (GFP), the Blue Fluorescent Protein (BFP) and the red fluorescent proteins (e.g. dsRed, mCherry, RFP).

The term "reporter nucleic acid" refers to a nucleic acid comprising a constitutive promoter operably linked to a gene encoding a fluorescent protein and a target gene (or a portion of a target gene), wherein the fluorescent protein and the target gene are transcribed as a

polycistronic mRNA. A schematic diagram of a reported nucleic acid according to some embodiments is shown in Fig. 31B.

The term “expression nucleic acid” refers to a nucleic acid comprising a constitutive promoter operably linked to a nucleic acid sequence encoding an inhibitory RNA. A schematic diagram of an expression nucleic acid according to some embodiments is presented in Fig. 31A.

As used herein the term “a pool of test inhibitory RNAs” refers to two or more inhibitory RNAs which are to be experimentally tested for their activity (i.e. their ability to inhibit or silence the target gene).

As used herein the term “transfecting” or “transfection” refers to introducing a nucleic acid into one or more eukaryotic cells.

As used herein, “a method of detecting the inhibitory or silencing activity of one or more inhibitory RNAs on a target gene” refers to a method of assaying or experimentally assessing the extent to which an inhibitory RNA inhibits or silences its target gene.

As used herein, “a method of selecting inhibitory RNAs which show silencing or inhibition of a target gene from a pool of test inhibitory RNAs” refers to a method of screening inhibitory RNAs for their ability to silence or inhibit expression of the target gene and selecting inhibitory RNAs which show silencing or inhibition of a target gene.

Inhibitory RNAs that are active in the cell in a context-specific manner

By “active” in the present disclosure it is meant that the inhibitory RNA is transcribed and processed (if required) such that it is active to inhibit expression of the target gene. Typically inhibition by an inhibitory RNA is via targeting an RNA, typically an mRNA, transcribed from the target gene, but other inhibitory mechanisms are possible.

By “inactive” or “reduced activity” is meant that inhibitory RNA is inactive or less active in the cell such that is unable or less able to inhibit expression of the target gene. Inactivity or reduced activity of the inhibitory RNA can result from an absence or reduced transcription of the inhibitory RNA in the cell, reduced processing of the inhibitory RNA to an active form in the cell, or both.

In some embodiments, the inhibitory RNA is suitable to be transcribed and/or processed in a context-specific manner such that it is active at an inhibitory level in a first context (where inhibition of the target gene is desired), wherein the inhibitory RNA substantially reduces expression of the target gene. In at least a second context (where inhibition of the desired target gene is not desired or wherein a reduction of inhibition is desired) the inhibitory RNA is transcribed and/or processed such that is active at a lower (preferably sub-inhibitory) level or is substantively inactive.

The present invention thus, in some embodiments, harnesses context-specific or cell context-specific activity of inhibitory RNAs (*via*, e.g., context-specific transcription and/or post-transcriptional processing of the inhibitory RNAs) to permit the context-specific regulation of target gene expression. Various inhibitory RNAs that can be used in the present invention are discussed herein.

In some preferred embodiments, regulation of expression of a target gene is cell context-specific, wherein context-specific regulation of target gene expression occurs in a single cell between a first context (e.g. differentiated state) and a second context (e.g. undifferentiated state). Such cell context-specific target gene expression regulation may be distinguished from differential regulation of target gene expression between different cells, for instance between a first cell in a first tissue and a second cell in a second tissue. Suitably, an inhibitory RNA suitable to inhibit expression of a target gene is active in a cell in a cell context-specific manner such that inhibition of the expression of the target gene in said cell occurs specifically in a first context, in which the inhibitory RNA is active in said cell. Optionally the inhibition does not occur, or occurs in reduced amount, in the said cell in at least a second context, in which the inhibitory RNA is inactive or less active.

Activity of endogenous inhibitory RNAs in cells varies depending on cell type and on the cell context (e.g. cell type, tissue type, developmental stage, state of differentiation, stress, microenvironment, activation, etc.). The present invention, in some embodiments, utilises modified forms of one or more endogenous inhibitory RNA(s) which are active in a context-specific or cell context-specific manner to regulate gene expression, e.g. to silence or partially silence one or more target genes in the context in which the one or more endogenous inhibitory RNA(s) is/are active. In particular, in some embodiments, the present invention employs modified forms of endogenous miRNAs that exhibit context-specific activity (typically *via* context specific transcription and/or processing) to regulate, typically partially or completely silence, a target gene.

In some embodiments of the present invention, the expression of the target gene in the first context is reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 95%, at least 97%, at least 98%, at least 99% or 100% compared to a control cell (e.g. cell without the construct for expressing the inhibitory RNA).

In some embodiments of the present invention, the expression of the target gene in the first context is reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or 100% compared to at least a second context.

In some embodiments of the present invention, a GEiGS approach is used to modify an endogenous non-coding RNA encoding sequence, e.g. an endogenous inhibitory RNA encoding sequence, wherein the inhibitory RNA exhibits a context-specific activity, in order to retarget the endogenous inhibitory RNA to silence or partially silence a target gene in the context in which the endogenous inhibitory RNA is active. In some embodiments, a GEiGS approach is used to modify two or more endogenous non-coding RNA encoding sequences, e.g. two or more endogenous inhibitory RNA encoding sequences, wherein the inhibitory RNAs exhibit a context-specific activity, in order to retarget the endogenous inhibitory RNAs to silence or partially silence one or more target genes in the context in which the endogenous inhibitory RNAs are active.

In some embodiments of the present invention a nucleic acid sequence encoding a modified version of an endogenous inhibitory RNA is introduced into the cell, preferably into the genome of cell (but it may be, for example, episomal), wherein the inhibitory RNA exhibits a context-specific activity, in order to silence or partially silence a target gene in the context in which the endogenous inhibitory RNA is active. In some embodiments, therefore, rather than retarget an endogenous inhibitory RNA with context-specific activity, a nucleic acid sequence encoding a modified version of the endogenous inhibitory RNA with context-specific activity can be inserted into the cell, e.g. into the genome of the cell. Thus, in such embodiments, the original ("in situ") endogenous inhibitory RNA can be unmodified. The modified version of the endogenous inhibitory RNA can be inserted into the genome of the cell at any suitable locus for expression. In some embodiments the modified version of an endogenous inhibitory RNA is provided in a synthetic expression cassette.

In some embodiments, two or more nucleic acid sequences encoding modified versions of two or more endogenous inhibitory RNAs are introduced into the cell, preferably into the genome of the cell (but it may be, for example, episomal), wherein the inhibitory RNAs exhibit context-

specific activity, in order to silence or partially silence one or more target genes in the context in which at least one of the two or more endogenous inhibitory RNAs are active. In some embodiments, rather than retarget endogenous inhibitory RNAs with context-specific activity, two or more nucleic acid sequences encoding modified versions of the endogenous inhibitory RNAs with context-specific activity can be inserted into the cell, e.g. into the genome of the cell. Thus, in such embodiments, the original ("*in situ*") endogenous inhibitory RNAs can be unmodified. The modified versions of the endogenous inhibitory RNAs can be inserted into the genome of the cell at any suitable locus for expression. In some embodiments the modified versions of two or more endogenous inhibitory RNAs are provided in two or more synthetic expression cassettes.

In some embodiments a nucleic acid sequence encoding a modified version of the endogenous inhibitory RNA with context-specific activity can be inserted into an endogenous miRNA locus. An approach which can be adapted for the current invention is described, for example, in Senis et al. ('TALEN/CRISPR-mediated engineering of a promoterless anti-viral RNAi hairpin into an endogenous miRNA locus'. Nucleic Acids Research, 2017, Vol. 45, No. 1 e3 doi: 10.1093/nar/gkw805). Accordingly, in some embodiments of the present invention, a promoterless sequence encoding an inhibitory RNA can be inserted into an endogenous miRNA locus, preferably without the disruption or deactivation of any endogenous miRNA.

Inhibitory RNAs

Non-coding RNA molecules can be modified to provide inhibitory RNAs as described herein. According to one embodiment, non-limiting examples of non-coding RNA molecules include, but are not limited to, microRNA (miRNA), piwi-interacting RNA (piRNA), short interfering RNA (siRNA), short-hairpin RNA (shRNA), trans-acting siRNA (tasiRNA), small nuclear RNA (snRNA or URNA), small nucleolar RNA (snoRNA), Small Cajal body RNA (scaRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), extracellular RNA (exRNA), repeat-derived RNA, transposable element RNA and long non-coding RNA (lncRNA). Non-coding RNAs can be modified to become inhibitory RNAs of use in the present invention.

The term "inhibitory RNA" refers to an RNA molecule having a degrading or silencing, e.g. partial or full silencing, effect on its corresponding mRNA having a complementary sequence thereto. In some embodiments of the invention, the inhibitory RNA include siRNAs, shRNAs and microRNAs, preferably microRNAs. The term silencing RNA (sRNA) is also used herein to refer to an inhibitory RNA.

According to one embodiment, non-limiting examples of inhibitory RNA molecules include, but are not limited to, small interfering RNA (siRNA), short hairpin RNA (shRNA), microRNA (miRNA), Piwi-interacting RNA (piRNA) and trans-acting siRNA (tasiRNA).

According to some embodiments, the inhibitory RNA molecule is a capable of inducing RNA interference (RNAi), optionally after cellular processing.

According to some embodiments, the inhibitory RNA molecule is processed from a precursor.

According to some embodiments, the non-coding RNA molecule or RNA silencing molecule is processed from a single stranded RNA (ssRNA) precursor.

According to some embodiments, the non-coding RNA molecule or the RNA silencing molecule is processed from a duplex-structured single-stranded RNA precursor.

According to some embodiments, the non-coding RNA molecule or RNA silencing molecule is processed from a dsRNA precursor (e.g. comprising perfect and imperfect base pairing).

According to some embodiments, the non-coding RNA molecule or the RNA silencing molecule is processed from a non-structured RNA precursor.

According to some embodiments, the non-coding RNA molecule or the RNA silencing molecule is processed from a protein-coding RNA precursor.

According to some embodiments, the non-coding RNA molecule or the RNA silencing molecule is processed from a non-coding RNA precursor.

According to some embodiments, the dsRNA can be derived from two different complementary RNAs, or from a single RNA that folds on itself to form dsRNA.

In some embodiments, the inhibitory RNA or a nucleic acid sequence encoding the inhibitory RNA comprises flanking sequences on both sides (flanking 5′ and 3′ sequences). In some preferred embodiments, the flanking sequences have a length of approximately 40 bp. In some preferred embodiments, the flanking sequences have a length of less than 100 bp, less than 90 bp, less than 80 bp, less than 70 bp, preferably less than 60 bp, less than 50 bp, more preferably less than 49 bp, less than 48 bp, less than 47 bp, less than 46 bp, less than 45 bp, less than 44 bp, less than 43 bp, less than 42 bp, less than 41 bp. Flanking genomic

sequences (homology arms) of length 40 base pairs show superior silencing potency compared to flanking genomic sequences of length 350 bp or length 150-350 bp, as shown in Fig. 23.

Perfect and imperfect based paired RNA (i.e. double stranded RNA, dsRNA), siRNA and shRNA – The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer, also known as endoribonuclease Dicer or helicase with RNase motif, is an enzyme that in humans is encoded by the DICER 1 gene. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). siRNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes with two 3' nucleotides overhangs.

Accordingly, some embodiments of the invention contemplate modifying a genomic sequence encoding a dsRNA to redirect a silencing specificity (including silencing activity) towards a target RNA to silence or partially silence a target gene.

According to one embodiment, dsRNA precursors longer than 21 bp are used. Various studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects - see for example [Strat et al., Nucleic Acids Research, 2006, Vol. 34, No. 13 3803-3810; Bhargava A et al. Brain Res. Protoc. 2004;13: 115- 125; Diallo M., et al., Oligonucleotides. 2003;13:381-392; Paddison P.J., et al., Proc. Natl Acad. Sci. USA. 2002;99: 1443-1448; Tran N., et al., FEBS Lett. 2004;573: 127-134].

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 base pairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is suggested to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position, but not the composition, of the 3'-overhang influences potency of a siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005).

The strands of a double-stranded interfering RNA (e.g., a siRNA) may be connected to form a hairpin or stem-loop structure (e.g., a shRNA). Thus, as mentioned, the inhibitory RNA of some embodiments of the invention may also be a short hairpin RNA (shRNA).

The term short hairpin RNA, "shRNA", as used herein, refers to a RNA molecule having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-CAAGAGA-3' and 5'-UUACAA-3' (International Patent Application Nos. WO2013126963 and WO2014107763). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

The inhibitory RNA molecule of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

Various types of siRNAs are contemplated by the present invention, including trans-acting siRNAs (Ta-siRNAs), repeat-associated siRNAs (Ra-siRNAs) and natural-antisense transcript- derived siRNAs (Nat- siRNAs).

According to one embodiment, inhibitory RNA includes "piRNA" which is a class of Piwi-interacting RNAs of about 26 and 31 nucleotides in length. piRNAs typically form RNA -protein complexes through interactions with Piwi proteins, i.e. antisense piRNAs are typically loaded into Piwi proteins (e.g. Piwi, Ago3 and Aubergine (Aub)).

miRNA - According to another, typically preferred, embodiment the inhibitory RNA molecule may be a miRNA. The term "microRNA", "miRNA", and "miR" are synonymous and refer to a collection of non-coding single- stranded RNA molecules of about 19-28 nucleotides in length, which regulate gene expression. miRNAs are found in a wide range of organisms, including viruses, and have been shown to play a role in development, homeostasis, and disease aetiology.

Initially the pre-miRNA is present as a long non-perfect double-stranded stem loop RNA that is further processed by Dicer into a siRNA-like duplex, comprising the mature guide strand (miRNA) and a similar-sized fragment known as the passenger strand (miRNA*). The miRNA and miRNA* may be derived from opposing arms of the pri-miRNA and pre-miRNA. miRNA* sequences may be found in libraries of cloned miRNAs but typically at lower frequency than the miRNAs.

Although initially present as a double-stranded species with miRNA*, the miRNA eventually becomes incorporated as a single-stranded RNA into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). Various proteins can form the RISC, which can lead to variability in specificity for miRNA/miRNA* duplexes, binding site of the target gene, activity of miRNA (repress or activate), and which strand of the miRNA/miRNA* duplex is loaded in to the RISC. When the miRNA strand of the miRNA:miRNA* duplex is loaded into the RISC, the miRNA* is removed and degraded. The strand of the miRNA:miRNA* duplex that is loaded into the RISC is the strand whose 5' end is less tightly paired. In cases where both ends of the miRNA:miRNA* have roughly equivalent 5' pairing, both miRNA and miRNA* may have gene silencing activity.

The RISC identifies target nucleic acids based on high levels of complementarity between the miRNA and the mRNA, especially by nucleotides 2-8 of the miRNA (referred as "seed sequence").

A number of studies have looked at the base-pairing requirement between miRNA and its mRNA target for achieving efficient inhibition of translation (reviewed by Bartel 2004, Cell 116-281). Computational studies, analysing miRNA binding on whole genomes have suggested a specific role for bases 2-8 at the 5' of the miRNA (also referred to as "seed sequence") in target binding but the role of the first nucleotide, found usually to be "A" was also recognized (Lewis et al 2005 Cell 120-15). Similarly, nucleotides 1-7 or 2-8 were used to identify and validate targets by Krek et al. (2005, Nat Genet 37-495). The target sites in the mRNA may be in the 5' UTR, the 3' UTR or in the coding region. Interestingly, multiple miRNAs may regulate the same mRNA target by recognizing the same or multiple sites. The presence of multiple miRNA binding sites in most genetically identified targets may indicate that the cooperative action of multiple RISCs provides the most efficient translational inhibition.

miRNAs may direct the RISC to downregulate gene expression by either of two mechanisms: mRNA cleavage or translational repression. The miRNA may specify cleavage of the mRNA

if the mRNA has a certain degree of complementarity to the miRNA. When a miRNA guides cleavage, the cut is typically between the nucleotides pairing to residues 10 and 11 of the miRNA. Alternatively, the miRNA may repress translation if the miRNA does not have the requisite degree of complementarity to the miRNA. Translational repression may be more prevalent in animals since animals may have a lower degree of complementarity between the miRNA and binding site.

It should be noted that there may be variability in the 5' and 3' ends of any pair of miRNA and miRNA*. This variability may be due to variability in the enzymatic processing of Drosha and Dicer with respect to the site of cleavage. Variability at the 5' and 3' ends of miRNA and miRNA* may also be due to mismatches in the stem structures of the pri-miRNA and pre-miRNA. The mismatches of the stem strands may lead to a population of different hairpin structures. Variability in the stem structures may also lead to variability in the products of cleavage by Drosha and Dicer. It will be appreciated that the pre-miRNA sequence may comprise from 45-90, 60-80 or 60- 70 nucleotides while the pri-miRNA sequence may comprise from 45-30,000, 50-25,000, 100-20,000, 1,000-1,500 or 80-100 nucleotides

As mentioned above, the methods of some embodiments of the invention are utilized to redirect a silencing activity and/or specificity of a inhibitory RNA molecule (or to generate a silencing activity and/or specificity if a non-coding RNA molecule does not have an intrinsic capability to silence a RNA molecule) towards a target RNA of interest.

According to one embodiment, target RNA is distinct from the original target RNA of the endogenous inhibitory RNA. According to various embodiments, methods of the present invention comprise introducing into a eukaryotic cell a DNA editing agent which redirects a silencing activity and/or specificity of an inhibitory RNA towards a desired target RNA.

As used herein, the term "redirects a silencing specificity" refers to reprogramming the original specificity of a non-coding RNA (typically an inhibitory RNA) towards a non-natural target of the non-coding RNA. Accordingly, the original specificity of the inhibitory RNA is destroyed (i.e. loss of function) and the new specificity is towards a RNA target distinct of the natural target (i.e. RNA of interest), i.e., gain of function. It will be appreciated that only gain of function occurs in cases that the original non-coding RNA has no silencing activity or a construct for expression of an inhibitory RNA is introduced into a cell.

As used herein, the term "target RNA" refers to an RNA sequence bound by an inhibitory RNA molecule. Thus, the target RNA is considered by the skilled artisan as a substrate for the inhibitory RNA.

The consequences of silencing can be confirmed by examination of the outward properties of a eukaryotic cell or organism, or by biochemical techniques. It will be appreciated that the inhibitory RNA molecule of some embodiments of the invention can have some off-target specificity effect/s provided that it does not unduly affect the intended growth, differentiation or function of the cell or organism.

Previous silencing methods utilising shRNA or siRNA are known to have significant off-target effects (Rao DD, Senzer N, Cleary MA, Nemunaitis J. Cancer Gene Ther. 2009 Nov;16(11):807-9. Doi: 10.1038/cgt.2009.53. Epub 2009 Aug 28. PMID: 19713999). Advantageously, in some preferred embodiments, the inhibitory RNA, or the nucleic acid sequence encoding the inhibitory RNA according to the present invention does not have off-target effects. In some preferred embodiments, the inhibitory RNA, or the nucleic acid sequence encoding the inhibitory RNA does not silence unintended targets (any target other than the target it is designed to target, such as B2M).

In some preferred embodiments, the inhibitory RNA may be a miRNA which does not have off-target effects. In some preferred embodiments, the inhibitory RNA may be a miRNA which does not silence unintended targets (any target other than the target it is designed to target, such as B2M).

In some preferred embodiments, the inhibitory RNA, or the nucleic acid sequence encoding the inhibitory RNA does not show loss-of-function effects following redirection of miRNA genes used as scaffolds.

According to one embodiment, the target RNA is endogenous to the cell, e.g. from an endogenous gene. Various genes which express suitable target RNAs are discussed herein.

According to one embodiment, the target RNA or target RNA of interest is exogenous to the cell (also referred to herein as heterologous). In such a case, the target RNA is a product of a gene that is not naturally part of the eukaryotic cell genome (i.e. which expresses the non-coding RNA). Exemplary exogenous target RNAs include, but are not limited to, products of a gene associated with an infectious disease, such as a gene of a pathogen (e.g. an insect, a virus, a bacteria, a fungi, a nematode. An exogenous target RNA (coding or non-coding) may comprise a nucleic acid sequence which shares sequence identity with an endogenous RNA

sequence (e.g. may be partially homologous to an endogenous nucleic acid sequence) of the cell or organism.

The specific binding of an endogenous non-coding RNA molecule with a target RNA can be determined by computational algorithms (such as BLAST) and verified by methods including e.g. Northern blot, In Situ hybridization, QuantiGene Plex Assay etc.

By use of the term "complementarity" or "complementary" is meant that the inhibitory RNA molecule (or at least a portion of it that is present in the processed form, or at least one strand of a double- stranded polynucleotide or portion thereof, or a portion of a single strand polynucleotide) hybridizes under physiological conditions to the target RNA, or a fragment thereof, to effect regulation or function or suppression of the target gene. For example, in some embodiments, a inhibitory RNA molecule has 100 percent sequence identity or at least about 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent sequence identity when compared to a sequence of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 70, 80, 90, 100, 150, 200, 300, 400, 500 or more contiguous nucleotides in the target RNA (or family members of a given target gene).

As used herein, a inhibitory RNA molecules, or their processed small RNA forms, are said to exhibit "complete complementarity" when every nucleotide of one of the sequences read 5' to 3' is complementary to every nucleotide of the other sequence when read 3' to 5'. A nucleotide sequence that is completely complementary to a reference nucleotide sequence will exhibit a sequence identical to the reverse complement sequence of the reference nucleotide sequence.

Methods for determining sequence complementarity are well known in the art and include, but not limited to, bioinformatics tools which are well known in the art (e.g. BLAST, multiple sequence alignment).

According to one embodiment, if the inhibitory RNA molecule is or processed into a siRNA, the complementarity is in the range of 90-100 % (e.g. 100 %) to its target sequence.

According to one embodiment, if the inhibitory RNA molecule is or processed into a miRNA or piRNA the complementarity is in the range of 33-100 % to its target sequence.

According to one embodiment, if the inhibitory RNA molecule is a miRNA, the seed sequence complementarity (i.e. nucleotides 2-8 from the 5') is in the range of 85-100 % (e.g. 100 %) to its target sequence.

According to one embodiment, the inhibitory RNA can be further processed into a small RNA form (e.g. pre-miRNA is processed into a mature miRNA). In such a case, homology is measured based on the processed small RNA form (e.g. the mature miRNA sequence).

As used herein, the term "small RNA form" refers to the mature small RNA being capable of hybridizing with a target RNA (or fragment thereof). According to one embodiment, the small RNA form has a silencing activity.

According to one embodiment, the complementarity to the target sequence is at least about 33 % of the processed small RNA form (e.g. 33 % of the 21-24 nt). Thus, for example, if the inhibitory RNA molecule is a miRNA, 33 % of the mature miRNA sequence (e.g. of the 21 nt) comprises seed complementation (e.g. 7 nt out of the 21 nt).

According to one embodiment, the complementarity to the target sequence is at least about 45 % of the processed small RNA form (e.g. 45 % of the 21-28 nt). Thus, for example, if the inhibitory RNA molecule is a miRNA, 45 % of the mature miRNA sequence (e.g. 21 nt) comprises seed complementation (e.g. 9-10 nt out of the 21 nt).

According to one embodiment, the endogenous inhibitory RNA (i.e. prior to modification) is typically selected as one having about 10%, 20%, 30%, 33%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or up to 99% complementarity towards the sequence of the desired target RNA of interest.

According to a specific embodiment, the inhibitory RNA molecule (i.e. prior to modification) is typically selected as one having no more than 99 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the of interest RNA molecule (i.e. prior to modification) is typically selected as one having no more than 98 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the inhibitory RNA molecule (i.e. prior to modification) is typically selected as one having no more than 97 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the inhibitory RNA molecule (i.e. prior to modification) is typically selected as one having no more than 96 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the inhibitory

RNA molecule (i.e. prior to modification) is typically selected as one having no more than 95 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the inhibitory RNA molecule (i.e. prior to modification) is typically selected as one having no more than 90 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the inhibitory RNA molecule (i.e. prior to modification) is typically selected as one having no more than 85 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the inhibitory RNA molecule (i.e. prior to modification) is typically selected as one having no more than 50 % complementarity towards the sequence of the target RNA of interest. According to a specific embodiment, the RNA molecule (i.e. prior to modification) is typically selected as one having no more than 33 % complementarity towards the sequence of the target RNA of interest.

According to one embodiment, the inhibitory RNA molecule is designed so as to comprise at least about 33 %, 40 %, 45 %, 50 %, 60 %, 70 %, 80 %, 85 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 % or even 100 % complementarity towards the sequence of the target RNA of interest.

According to a specific embodiment, the inhibitory RNA molecule is designed so as to comprise a minimum of 33 % complementarity towards the target RNA of interest (e.g. 85-100 % seed match).

According to a specific embodiment, the inhibitory RNA molecule (e.g. RNA silencing molecule) is designed so as to comprise a minimum of 40 %, 45%, 50 %, 60%, 70%, 80 %, 85%, 90 %, 95 %, 96 %, 97 %, 98 %, 99 % or 100% complementarity towards the target RNA of interest. Higher levels of complementarity are typically preferred when higher levels of inhibition/silencing are preferred.

In order to generate silencing activity and/or specificity of an inhibitory RNA molecule or redirect a silencing activity and/or specificity of an inhibitory RNA molecule towards a target RNA, a sequence of an endogenous non-coding RNA molecule (e.g. inhibitory RNA) is preferably modified using a DNA editing agent.

According to one embodiment, the inhibitory RNA comprises a modified form of an miRNA selected from the group consisting of: hsa-mir-191, hsa-mir-302a, hsa-mir-302c, hsa-mir-93, hsa-mir-106a, hsa-mir-106b, hsa-mir-20a, hsa-mir-363, hsa-mir-518b, hsa-mir-744, hsa-mir-99b, hsa-mir-320a, hsa-mir-520f, hsa-mir-652, hsa-mir-1180, hsa-mir-15b, hsa-mir-23a, hsa-

mir-26b, hsa-mir-335, hsa-mir-361, hsa-mir-1307, hsa-mir-205, hsa-mir-221, hsa-mir-222, hsa-mir-30e, hsa-mir-423, hsa-mir-519c, hsa-mir-92b, hsa-mir-30, hsa-mir-302b, hsa-let-7b, hsa-mir-98, hsa-let-7g, hsa-mir-126, hsa-mir-17, hsa-mir-378a, hsa-mir-671, hsa-mir-155, hsa-mir-20b, hsa-mir-34c, hsa-mir-146a, hsa-mir-29a, hsa-mir-342, hsa-miR-375, hsa-miR-143, hsa-miR-21, hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, hsa-miR-200c, hsa-miR-143, hsa-miR-10a, hsa-miR-122, hsa-miR-22, hsa-miR-192, hsa-miR-146b, hsa-miR-26a, hsa-miR-125a, hsa-miR-150, hsa-miR-10b, hsa-miR-181a, hsa-miR-26a, hsa-miR-92a, hsa-miR-143, hsa-miR-10a, hsa-miR-21, MIR146A (hsa-miR-146a-5p), MIR20A (hsa-miR-20a-5p), LET7C (hsa-miR-let-7c-5p), LET7i (hsa-let-7i-5p), miR-375, miR-340-5p, miR-29a-3p, miR-222-3p, miR-940, miR-203, miR-145, miR-103a, miR-21-3p, miR-181-5p, miR-125b-5p, miR-301a-3p, miR-132, miR-29b-1, miR-27a, miR-146a, miR-222, miR-let7a, miR-320a, miR-146a, miR-let7c, miR-1246, miR-let7d-5p, miR-451, miR-21, miR-23a, miR-27a, miR-24-2, miR-155, miR125b-2, and miR-16.

According to one embodiment, the inhibitory RNA comprises a modified form of an miRNA selected from the group consisting of: hsa-miR-375, hsa-miR-143, hsa-miR-21, hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, hsa-miR-200c, hsa-miR-143, hsa-miR-10a, hsa-miR-122, hsa-miR-22, hsa-miR-192, hsa-miR-146b, hsa-miR-26a, hsa-miR-125a, hsa-miR-150, hsa-miR-10b, hsa-miR-181a, hsa-miR-26a, hsa-miR-92a, hsa-miR-143, hsa-miR-10a, hsa-miR-21, MIR146A (hsa-miR-146a-5p), MIR20A (hsa-miR-20a-5p), LET7C (hsa-miR-let-7c-5p), LET7i (hsa-let-7i-5p), miR-375, miR-340-5p, miR-29a-3p, miR-222-3p, miR-940, miR-203, miR-145, miR-103a, miR-21-3p, miR-181-5p, miR-125b-5p, miR-301a-3p, miR-132, miR-29b-1, miR-27a, miR-146a, miR-222, miR-let7a, miR-320a, miR-146a, miR-let7c, miR-1246, miR-let7d-5p, miR-451, miR-21, miR-23a, miR-27a, miR-24-2, miR-155, miR125b-2, and miR-16.

According to one embodiment, the inhibitory RNA comprises a modified form of an miRNA selected from the group consisting of: hsa-mir-191; hsa-mir-302a; hsa-mir-302c; hsa-mir-93; hsa-mir-106a; hsa-mir-106b; hsa-mir-200c; hsa-mir-20a; hsa-mir-21; hsa-mir-363; hsa-mir-518b; hsa-mir-744; hsa-mir-99b; hsa-mir-320a; hsa-mir-520f; hsa-mir-652; hsa-mir-1180; hsa-mir-15b; hsa-mir-182; hsa-mir-23a; hsa-mir-26b; hsa-mir-335; hsa-mir-361; hsa-mir-1307; hsa-mir-205; hsa-mir-22; hsa-mir-221; hsa-mir-222; hsa-mir-30e; hsa-mir-423; hsa-mir-519c; hsa-mir-92b; hsa-mir-30; hsa-mir-302b; hsa-mir-375; hsa-let-7b; and hsa-mir-98, According to one embodiment, the inhibitory RNA comprises a modified form of an miRNA selected from the group consisting of: hsa-let-7g, hsa-mir-126, hsa-mir-150, hsa-mir-17, hsa-mir-191, hsa-mir-30d, hsa-mir-30e, hsa-mir-363, hsa-mir-93, hsa-mir-378a, hsa-mir-671, hsa-mir-155, hsa-mir-20b and hsa-mir-34c. According to one embodiment, the inhibitory RNA

comprises a modified form of an miRNA selected from the group consisting of: hsa-mir-146a, hsa-mir-29a, hsa-mir-342, hsa-mir-363, hsa-mir-126, hsa-mir-146b, hsa-mir-30d, hsa-mir-20b, hsa-mir-378a, hsa-let-7g, hsa-mir-30e, hsa-mir-671, hsa-mir-17, hsa-mir-20a, hsa-mir-93, hsa-mir-34c, hsa-let-7b, and hsa-mir-155.

Additional miRNAs of interest and preferred contexts for the use of these miRNAs are discussed hereinbelow. Sequences of these miRNAs can be found at miRbase (https://www.mirbase.org/).

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 1 to SEQ ID NO: 17 or selected from SEQ ID NO: 18 to SEQ ID NO: 34. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target B2M. In some preferred embodiments the inhibitory RNA comprises SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30). In some embodiments the inhibitory RNA does not comprises a sequence selected from SEQ ID NO: 1 to SEQ ID NO: 17 or selected from SEQ ID NO: 18 to SEQ ID NO: 34.

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target PPARG.

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target IRF4. Solution 219 (SEQ ID NO: 127 and SEQ ID NO: 151) is a particularly preferred inhibitory RNA targeting IRF4.

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target KDM6B. Solution 245 (SEQ ID NO: 177 and SEQ ID NO: 201) is a particularly preferred inhibitory RNA targeting KDM6B.

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target STAT6.

Solution 260 (SEQ ID NO: 216 and SEQ ID NO: 240) and Solution 266 (SEQ ID NO: 222 and SEQ ID NO: 246) are particularly preferred inhibitory RNA targeting STAT6.

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target B2M. In some embodiments the inhibitory RNA does not comprises a sequence selected from SEQ ID NO: 348 to SEQ ID NO:411 or selected from SEQ ID NO: 412 to SEQ ID NO: 475.

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target FOXP3.

According to one embodiment, the inhibitory RNA comprises a sequence selected from SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673. These sequences correspond to miRNAs (mature sRNA and pre-miRNA hairpin forms) which target PDCD-1.

In some embodiments, the inhibitory RNA is encoded by a nucleic acid sequence encoding the inhibitory RNA. In some embodiments, the nucleic acid sequence encoding the inhibitory RNA comprises a sequence selected from SEQ ID NO: 254-347.

In some embodiments, the nucleic acid sequence encoding the inhibitory RNA comprises a HDR template sequence. In some embodiments, the HDR template sequence is selected from SEQ ID NO: 674-679, 59, 61, 63 and 65.

Two or more inhibitory RNAs suitable to inhibit expression of one or more target genes

The method according to the first aspect of the present invention may comprise providing a cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of the target gene, or suitable to inhibit expression of two or more target genes. The cell according to the second or third embodiments of the present invention may be provided with or comprise a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of the target gene, or suitable to inhibit expression of two or more target genes.

In some embodiments according to the first, second or third aspects of the present invention, the cell is provided with two or more nucleic acid constructs adapted to express an inhibitory

RNA suitable to inhibit expression of one or more target genes. In some embodiments according to first, second or third aspects of the present invention, a single nucleic acid construct is adapted to express two or more inhibitory RNAs suitable to inhibit expression of one or more target genes. Suitably, the inhibition of expression is in a cell context-specific or context-specific manner.

In some embodiments, the method suitably comprises providing a cell with one or two or more nucleic acid constructs adapted to express two or more inhibitory RNAs suitable to inhibit expression of one target gene (i.e. two or more inhibitory RNAs with the same target). In some embodiments, the cell is provided with or the cell comprises one or two or more nucleic acid constructs adapted to express two or more inhibitory RNAs suitable to inhibit expression of one target gene (i.e. two or more inhibitory RNAs with the same target).

In some embodiments, the method suitably comprises providing a cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of one target gene (i.e. two or more inhibitory RNAs with the same target). In some embodiments, the cell is provided with or the cell comprises a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of one target gene (i.e. two or more inhibitory RNAs with the same target). In some preferred embodiments, the cell is a pancreatic cell. In some preferred embodiments, the cell is a T cell. In some preferred embodiments, the cell is a macrophage. In some embodiments the target gene is selected from: B2M, PPARG, IRF4, KDM6B, FOXP3, PDCD-1 and STAT6. In some preferred embodiments the target gene is B2M.

In some embodiments, the two or more inhibitory RNAs are selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M). In some embodiments, the two or more inhibitory RNAs are selected from SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG). In some embodiments, the two or more inhibitory RNAs are selected from SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4). In some embodiments, the two or more inhibitory RNAs are selected from SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B). In some embodiments, the two or more inhibitory RNAs are selected from SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3). In some embodiments, the two or more inhibitory RNAs are selected from SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1). In some embodiments, the two or

more inhibitory RNAs are selected from SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, the method suitably comprises providing a cell with one or two or more nucleic acid constructs adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets). In some embodiments, the cell is provided with or the cell comprises one or two or more nucleic acid constructs adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets).

In some embodiments, the method suitably comprises providing a cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets). In some embodiments, the cell is provided with or the cell comprises a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets). In some preferred embodiments, the cell is a pancreatic cell. In some preferred embodiments, the cell is a T cell. In some preferred embodiments, the cell is a macrophage. In some embodiments, at least one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M). In some embodiments, at least one of the two or more inhibitory RNAs is selected from SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG). In some embodiments, at least one of the two or more inhibitory RNAs is selected from SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4). In some embodiments, at least one of the two or more inhibitory RNAs is selected from SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B). In some embodiments, at least one of the two or more inhibitory RNAs is selected from SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3). In some embodiments, at least one of the two or more inhibitory RNAs is selected from SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1). In some embodiments, at least one of the two or more inhibitory RNAs is selected from SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, the method suitably comprises providing a cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets). In some

embodiments, the cell is provided or the cell comprises a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets).

In some preferred embodiments, the cell is a pancreatic cell. In some preferred embodiments, the cell is a T cell. In some preferred embodiments, the cell is a macrophage. In some embodiments, the two or more target genes are selected from: B2M, PPARG, IRF4, KDM6B, FOXP3, PDCD-1 and STAT6. In some embodiments, one of the target genes is B2M and another one of the target genes is selected from: STAT6, IRF4, DRP1, PPARG, KDM6B, Clever-1 (also known as STAB1 or FEEL-1), LAIR1/2, P-selection glycoprotein ligand (PSGL-1), signal regulatory protein alpha (SIRPa), triggering receptor expressed on myeloid cells 2 (TREM2), proton sensing GPCR (GPR65), inhibitory leukocyte immunoglobulin-like receptor subfamily B4 (LILRB4), RASA-2, CTLA4 and PD-1 (PDCD-1).

In some embodiments, one of the target genes is B2M and another one of the target genes is selected from: PPARG, IRF4, KDM6B, FOXP3, PDCD-1 and STAT6. In some embodiments, the two or more inhibitory RNAs are selected from:

- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, one of the two or more inhibitory RNAs is selected from:
SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M).

In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of a target gene selected from PPARG, IRF4, KDM6B, FOXP3, PDCD-1 and STAT6.

In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is selected from:

- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene B2M and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene PPARG. In some embodiments, one of the two or more inhibitory RNAs inhibits the expression of the target gene B2M and another one of the two or more inhibitory RNAs inhibits the expression of the target gene PPARG. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene PPARG. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22

(Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs inhibits the expression of the target gene PPARG. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M) and another one of the two or more inhibitory RNAs is selected from SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG).

In some embodiments, one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene B2M and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene IRF4. In some embodiments, one of the two or more inhibitory RNAs inhibits the expression of the target gene B2M and another one of the two or more inhibitory RNAs inhibits the expression of the target gene IRF4. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene IRF4. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs inhibits the expression of the target gene IRF4. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M) and another one of the two or more inhibitory RNAs is selected from SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4).

In some embodiments, one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene B2M and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene KDM6B. In some embodiments, one of the two or more inhibitory RNAs inhibits the expression of the target gene B2M and another one of the two or more inhibitory RNAs inhibits the expression of the target gene KDM6B. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18

(Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene KDM6B. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs inhibits the expression of the target gene KDM6B. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M) and another one of the two or more inhibitory RNAs is selected from SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B).

In some embodiments, one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene B2M and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene FOXP3. In some embodiments, one of the two or more inhibitory RNAs inhibits the expression of the target gene B2M and another one of the two or more inhibitory RNAs inhibits the expression of the target gene FOXP3. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene FOXP3. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs inhibits the expression of the target gene FOXP3. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M) and another one of the two or more inhibitory RNAs is selected from SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3).

In some embodiments, one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene B2M and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene PDCD-1. In some embodiments, one of the two or more inhibitory RNAs inhibits the expression of the target gene B2M and another one of the two or more inhibitory RNAs inhibits the expression of the target gene PDCD-1. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene PDCD-1. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs inhibits the expression of the target gene PDCD-1. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M) and another one of the two or more inhibitory RNAs is selected from SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1).

In some embodiments, one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene B2M and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene STAT6. In some embodiments, one of the two or more inhibitory RNAs inhibits the expression of the target gene B2M and another one of the two or more inhibitory RNAs inhibits the expression of the target gene STAT6. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is suitable to inhibit the expression of the target gene STAT6. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs inhibits the expression of the target gene STAT6. In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID

NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M) and another one of the two or more inhibitory RNAs is selected from SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is selected from SEQ ID NO: 216 and/or SEQ ID NO: 240 (targeting STAT6), SEQ ID NO: 222 and/or SEQ ID NO: 246 (targeting STAT6); SEQ ID NO: 127 and/or SEQ ID NO: 151 (targeting IRF4) and SEQ ID NO: 177 and/or SEQ ID NO: 201 (targeting KDM6B).

In some embodiments, one of the target genes is PPARG and another one of the target genes is selected from: IRF4, KDM6B and STAT6. In some embodiments, one of the target genes is PPARG and another one of the target genes is IRF4. In some embodiments, one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG) and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4). In some embodiments, one of the target genes is PPARG and another one of the target genes is KDM6B. In some embodiments, one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG) and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B). In some embodiments, one of the target genes is PPARG and another one of the target genes is STAT6. In some embodiments, one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG) and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, one of the target genes is IRF4 and another one of the target genes is selected from: PPARG, KDM6B and STAT6. In some embodiments, one of the target genes is IRF4 and another one of the target genes is KDM6B. In some embodiments, one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4) and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B). In some embodiments, one of the target genes is IRF4 and another one

of the target genes is STAT6. In some embodiments, one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4) and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, one of the target genes is KDM6B and another one of the target genes is selected from: PPARG, IRF4, and STAT6. In some embodiments, one of the target genes is KDM6B and another one of the target genes is STAT6. In some embodiments, one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B) and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

In some embodiments, one of the target genes is FOXP3 and another one of the target genes is PDCD-1. In some embodiments, one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3) and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1). In some embodiments, one of the target genes is STAT6 and another one of the target genes is selected from: PPARG, IRF4 and KDM6B.

In some embodiments, the method suitably comprises providing a pancreatic cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets).

In some embodiments, the method suitably comprises providing a T cell with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets).

In some embodiments, the method suitably comprises providing a macrophage with a nucleic acid construct adapted to express two or more inhibitory RNAs suitable to inhibit expression of two or more target genes (i.e. two or more inhibitory RNAs with different targets).

Cells and Cellular Contexts

As will be apparent from the above, the present invention relates to inhibitory RNAs that are active in cells in a particular (first) context. In particular embodiments the inhibitory RNAs are

modified forms of endogenous non-coding RNAs, e.g. inhibitory RNAs, with modified target specificity so as to target a desired target gene. It will be apparent that the target gene is different from the (original) gene which is normally targeted by the endogenous non-coding RNA.

In some embodiments the cell which is modified may be in a given context, in which the inhibitory RNA is not active, and wherein the inhibitory RNA becomes active when the cell is in a different context. By way of non-limiting example, a stem cell may be modified such that it comprises a sequence encoding an inhibitory RNA which becomes active when the cell is differentiated into a desired cell type. Alternatively, an immune cell, or a progenitor thereof, may be modified such that it comprises a sequence encoding an inhibitory RNA which becomes active when the immune cell is activated or polarised in a particular way or which enters a particular tissue. In all such cases, the inhibitory RNA is active in a desired (first) context, but is substantially inactive in another context.

In some embodiments of the invention, the inhibitory RNA can be selectively expressed or be selectively processed in a particular cell or tissue type, or in a cell under a certain condition or in a certain state. By way of non-limiting example, the inhibitory RNA can suitably be an inhibitory RNA that is expressed in the cell type of interest (e.g. a pancreatic endocrine hormone expressing cell or immune cell, such as a T-cell or tumour associated macrophage) but which is not expressed in an alternative state of a pancreatic endocrine hormone expressing cell or immune cell (e.g. an inactivated, unresponsive, dysfunctional, or degenerative state), or not expressed in another type of cell (e.g. a precursor/progenitor cell of the pancreatic cell or immune cell).

In some embodiments of the invention, the method comprises modifying a cell to comprise a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene, and wherein the inhibitory RNA becomes active in the cell following a change in the context of the cell. Thus, the inhibitory RNA is initially inactive in the cell, but becomes active once the cell is in a different context (e.g. following activation, polarisation, when the cells present in a particular tissue, etc.).

In some embodiments of the invention, the method comprises modifying a progenitor cell, to comprise a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene, and wherein the inhibitory RNA becomes active in the cell following differentiation, preferably wherein the inhibitory RNA is active in the cell the cell is

fully differentiated. Thus, the inhibitory RNA is initially inactive in the progenitor cell, but becomes active at a later stage in differentiation.

In some embodiments of the invention, the method comprises modifying a progenitor cell (e.g. a stem cell, such as an iPSC) so that when the progenitor cell differentiates to a desired cell type (e.g. fully differentiated) the inhibitory RNA is active in the cell.

In some embodiments of the invention, the method comprises modifying a progenitor cell (e.g. a stem cell, such as an iPSC) so that when the cell differentiates to a desired state (e.g. fully differentiated) and the cell is in a specific context (e.g. following activation, polarisation, when the cells present in a particular tissue, etc.) the inhibitory RNA is active in the cell.

In some embodiments, the inhibitory RNA may be selectively, specifically or preferentially active in a particular cell type. Suitably the inhibitory RNA is inactive or only active at a low level in other cell types.

In some embodiments, the first context is the cell type in which the inhibitory RNA is active, and the second context is another cell type in which inhibition of the target gene is not desired. In some embodiments, the first context is the cell type in which the inhibitory RNA is active, and the second context is any other cell type.

In some embodiments, the inhibitory RNA is selectively active (e.g. selectively expressed or selectively processed) in a particular cell type or tissue type, or in a particular cell type in a certain context. By way of example, the inhibitory RNA can suitably be an inhibitory RNA that is expressed in the cell type of interest (e.g. an enteroendocrine cell, such as a pancreatic beta cell or an immune cell, such as a macrophage or T-cell) but which is not expressed in another type of cell (e.g. a precursor/progenitor cell of the pancreatic beta cell or immune cell).

In some embodiments, the inhibitory RNA may be selectively, specifically or preferentially active in a cell that is in particular stage of differentiation. Suitably the inhibitory RNA is inactive or only active at a low level in cells of the same lineage but at a different stage of differentiation. In such embodiments, the first context is cell at the stage of differentiation in which the inhibitory RNA is active, and the second context can be any other stage of differentiation. In some embodiments the first context is when the cell when it is fully differentiated, e.g. into a therapeutic cell, and the second context is when the cell is undifferentiated or partially differentiated. In some embodiments, the first context is an IPSC-derived pancreatic progenitor, a IPSC-derived monocyte or a IPSC-derived macrophage and the second context

is an undifferentiated iPSC. In some embodiments, the first context is an IPSC-derived pancreatic progenitor and the second context is an undifferentiated iPSC. In some embodiments, the first context is a IPSC-derived monocyte or a IPSC-derived macrophage and the second context is an undifferentiated iPSC. The ability to silence genes conditionally in iPSC-derived differentiated (progenitor) cells but not in the undifferentiated iPSC is beneficial in cell therapy. Conventional constitutive knockout of a gene aiming to modulate the recipient's immune response to a therapeutic cell (e.g. MHC-I, see above) in iPSC and differentiated progenitors used for cell therapy is associated with risks. Trace amounts of contaminating undifferentiated iPSC with the constitutive knockout (e.g. MHC-I) in the therapeutic formulation may go undetected by the recipient's immune system following implantation, begin to proliferate and form a teratoma. On the other hand, silencing such gene only in iPSC-derived differentiated (progenitor) cells means that any contaminating undifferentiated iPSC would be recognised as 'non-self' (e.g. no inhibition of MHC-I) and eliminated by the recipient's immune system following implantation.

In some embodiments, the inhibitory RNA may be active in the cell only when the cell is in a particular state. For example, the inhibitory RNA may be selectively, specifically or preferentially active in a cell which is activated in a particular way, which is subject to stress, which is present in a particular tissue, which is in a tumour microenvironment, which has been infected with a virus, or suchlike. In such embodiments, the first context is the cell state in which the inhibitory RNA is active, and the second context can be any other cell status. In some embodiments, the first context is Treg cell state and the second context is a T cell effector state. In some embodiments, the first context is a cell being in the tumour microenvironment (TME).

In preferred embodiments of the present invention the cell is a eukaryotic cell. According to some embodiments, the eukaryotic cell is from a eukaryotic organism selected from the group consisting of a mammal, an insect, a nematode, a bird, a reptile, a fish, a crustacean, a fungi and an algae. According to preferred embodiments, the eukaryotic cell is a mammalian cell. According to yet further preferred embodiments, the mammalian cell is a human cell.

In certain particular embodiments, the cell is a differentiated cell including without limitation a dendritic cell, a lymphocyte, a red blood cell, platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell and an adipocyte. In particular preferred embodiments, the cell is a therapeutic form of any of said cells. In some preferred

embodiments the inhibitory RNA is active in the relevant differentiated cell, but is inactive or is less active in a precursor of said cell.

In certain particular embodiments, the cell is a stem cell, e.g. a therapeutic stem cell. In some embodiments the stem cell is suitable to differentiate into a differentiated cell including without limitation a dendritic cell, a lymphocyte, a red blood cell, platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell or an adipocyte. In some preferred embodiments the inhibitory RNA is active in the relevant stem cell, but is inactive or is less active in when said stem cell differentiates.

In some preferred embodiments the cell is a pluripotent cell, such as an embryonic stem cell or an iPSC.

In some preferred embodiments the cell is an iPSC or a cell derived from an iPSC.

In some preferred embodiments the cell is a haematopoietic stem cell (HSC).

According to some particularly preferred embodiments, the cell is a therapeutic cell. A therapeutic cell is a cell that is suitable to be administered to a subject with the intention or expectation of providing some therapeutic benefit to the subject. In some embodiments the cell is a therapeutic endocrine cell. In some embodiments the cell is a therapeutic immune cell.

In some preferred embodiments of the invention, the cell is a context-specific hypoimmunogenic cell. More preferably, in some embodiments the cell is a context-specific hypoimmunogenic therapeutic cell. Yet more preferably the cell is a hypoimmunogenic allogeneic therapeutic cell. In such embodiments, the target gene is suitably a gene associated with the MHC-I system.

In some embodiments the inhibitory RNA is specifically active in a cell which is in a context selected from the group consisting of:

- a cell at a particular state of differentiation, for example a fully-differentiated cell, a cell at an intermediate stage of differentiation or an undifferentiated cell;
- a totipotent cell, a pluripotent cell or a multipotent cell;
- a cell present in a particular tissue;

- a cancerous or pre-cancerous cell;
- a tumour associated cell, e.g. a tumour associated immune cell or an immune cell in the tumour microenvironment;
- an activated cell, such as a macrophage polarised into a particular functional state;
- an immune cell which is in a memory or quiescent stem cell state rather than an effector state;
- a cell which is hypoxic; or
- a cell subject to the unfolded protein response.

In some preferred embodiments, the cell is a cell of the endocrine system, preferably a therapeutic cell of the endocrine system. In some embodiments the cell is an enteroendocrine cell, preferably a therapeutic enteroendocrine cell. Endocrine cells are found in a range of endocrine glands in the human and animal body. In some embodiments the cell is from: the pancreas, the thyroid gland, the parathyroid gland, the pituitary gland, the pineal gland, the testis, the ovary, the adrenal gland, the thymus the hypothalamus. Accordingly, the inhibitory RNA may be selectively, specifically or preferentially active in an endocrine cell from one of said endocrine glands.

In some preferred embodiments the cell is a pancreatic cell, more preferably in a pancreatic endocrine hormone expressing cell (for example beta, alpha, delta, or epsilon cells, most preferably a beta cell). In such cases, it is typically preferred that the inhibitory RNA is selectively, specifically or preferentially active such a pancreatic cell. In some preferred embodiments the inhibitory RNA is expressed but at reduced levels in precursor/progenitor cells of said endocrine hormone expressing pancreatic cells, and not expressed in a stem cell, e.g. an iPSC.

In some preferred embodiments, the cell is a pancreatic beta cell. Accordingly, the inhibitory RNA is selectively, specifically or preferentially active in pancreatic beta cells. In some preferred embodiments the inhibitory RNA is not active or is active at a significantly lower level in a precursor/progenitor cell of a pancreatic beta cell such as a stem cell, e.g. an iPSC.

In some embodiments of the invention the inhibitory RNA is selectively active (e.g. selectively expressed or selectively processed) in pancreatic endocrine hormone expressing cells. Suitable inhibitory RNA is selectively active where these cells are adequately vascularised and responding within the expected values for normal fasting blood glucose concentration (between 70 mg/dL (3.9 mmol/L) and 100 mg/dL (5.6 mmol/L). Suitably the inhibitory RNA is inactive or less active when the cells are in an alternative state as a result of exposure to

hypoxia, undergoing oxidative stress, inactivated, glucose unresponsive, glucose responsive but electrically silent, hormone unresponsive, dysfunctional (do not secrete insulin, glucagon, somatostatin, etc), degenerate or hyperproliferative.

In another preferred embodiment of the invention, the cell is an immune cell. Immune cells of interest include neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, dendritic cells, natural killer cells, and lymphocytes (B cells and T cells). Cells of particular interest include T-cells, preferably a cytotoxic T-cells, e.g. T-cells adapted for T-cell therapy such as CAR-T cell therapy, and macrophages. Suitably the inhibitory RNA is selectively active in such immune cells.

In some embodiments, the immune cell is a macrophage, for example an M1 macrophage, an M2 macrophage (e.g. an M2a, M2b, M2c, and M2d macrophage), a Treg macrophage, or a tumour-associated macrophage (TAM). In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in a macrophage, suitably in an M1 macrophage, an M2 macrophage, a Treg macrophage, or a tumour-associated macrophage (TAM). In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in a macrophage with no designated function, preferably a tumour-associated macrophage, e.g. a macrophage adapted for a macrophage therapy such as CAR-M cell therapy or a macrophage adapted with a CAR targeting tumour antigens.

In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in monocytes and macrophages. Suitably the inhibitory RNA is inactive or less active in cells other than monocytes and macrophages. Suitably the inhibitory RNA is inactive in progenitors of monocytes and macrophages such as stem cells (e.g. hematopoietic stem cells, monoblasts or iPSCs). By way of non-limiting example, in such embodiments the inhibitory RNA can be used to drive the monocyte or macrophage towards a pro-inflammatory or anti-inflammatory phenotype, e.g. by silencing one or more anti-inflammatory or pro-inflammatory genes, respectively.

In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in an M2 macrophage (also known as an alternatively activated macrophage). Suitably the inhibitory RNA is inactive or less active in an M1 macrophage (also known as a classically activated macrophage) and/or an unpolarized macrophage. By way of non-limiting example, in such embodiments the inhibitory RNA can be used to drive the M2 macrophage towards a pro-inflammatory phenotype, e.g. by silencing one or more anti-inflammatory genes in the M1 macrophage. Suitably the M2 macrophage can be an M2a, M2b, M2c, or M2d macrophage.

In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in an M1 macrophage. Suitably the inhibitory RNA is inactive or less active in an M2 macrophage and/or an unpolarized macrophage. By way of non-limiting example, in such embodiments the inhibitory RNA can be used to drive the M1 macrophage towards an anti-inflammatory phenotype, e.g. by silencing one or more pro-inflammatory genes in the M1 macrophage.

In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in an unpolarized macrophage. Suitably the inhibitory RNA is inactive or less active in a polarized macrophage, e.g. an M1 or M2 macrophage. By way of non-limiting example, in such embodiments the inhibitory RNA can be used to drive the monocyte or macrophage towards a pro-inflammatory or anti-inflammatory phenotype, e.g. by silencing one or more anti-inflammatory or pro-inflammatory genes, respectively.

In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in a tumour associated macrophage (TAM) or in a macrophage which is exposed to the tumour microenvironment (TME). Suitably the inhibitory RNA is inactive or less active in an M1 macrophage (also known as a classically activated macrophage) and/or an unpolarized macrophage or when the macrophage is not exposed to the TME. By way of non-limiting example, in such embodiments the inhibitory RNA can be used to drive the TAM or macrophage exposed to the TME towards a pro-inflammatory (anti-tumour) phenotype, e.g. by silencing one or more anti-inflammatory genes in the M1 macrophage.

Accordingly, in some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in a T-cell. In some preferred embodiments the inhibitory RNA is selectively, specifically or preferentially active in a tumour infiltrating cytotoxic T-cell, e.g. a T-cell adapted for T-cell therapy such as CAR-T cell therapy.

In some preferred embodiments the inhibitory RNA is not expressed or is expressed at a significantly lower level in a progenitor/precursor cell of these immune cells, such as a stem cell, e.g. an iPSC or a partially differentiated immune cell progenitor.

In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in an immune cell when in a particular state of activation. In some embodiments the inhibitory RNA is not expressed or is expressed at a significantly lower level in the immune cell when it is not in said state of activation. In some preferred embodiments the inhibitory

RNA is inactive or is active at a significantly lower level in a progenitor/precursor cell of the immune cell such as a stem cell, e.g. an iPSC. By way of non-limiting example, an inhibitory RNA can be used to promote activity of T cells in the tumour microenvironment (see Fig. 13), e.g. by targeting genes associated with an immunosuppressive state (PD-1 (PDCD-1), CTLA4, etc.) in a context-specific manner.

In some embodiments of the invention, the inhibitory RNA can be selectively active in immune cells in the tumour microenvironment (TME). For example, the inhibitory RNA can be selectively active when these cells are responding to tumour-derived chemokines (e.g. CXCL9/10/11/12, CCL2/5, CCL17/22), hypoxia (ppO2 8-10mmHg or lower), tumour related immune suppressive cytokines (e.g. IL10, TGF-beta, IL4, IL13), immune cell check point ligands (e.g. for receptors PD-1 (PDCD-1), LAG3, TIM3, CTLA-4, Clever-1), cancer associated fibroblast (CAF) signals (e.g. IL-11, CXCL12, GDF), tumour matrix (e.g. fibronectin, versican, collagen-11A1, SFRP2) or metabolites (e.g. lactate). Suitably the inhibitory RNA are inactive or are active at lower levels when the cells are in an alternative state or migrate away from the tumour microenvironment or remain inactivated, inactivatable, exhausted, rendered anergic or polarised into a state that is immune-suppressive.

In some embodiments of the invention the inhibitory RNA is inactive or is less active in the immune cells when they migrate away from the tumour microenvironment, or are altered as mentioned and as a result are no longer able to silence the expression of receptor genes, their signal transduction components, transcription factors controlling immune checkpoints (e.g. PD-1 (PDCD-1), LAG3, TIM3, CTLA-4, Clever-1), tumour-derived chemokines, immune-suppressive cytokines, CAF ligands, tumour matrix molecules fibronectin, versican, collagen-11A1, SFRP2, metabolite transporters (e.g. lactate, monocarboxylate transporter-1 (SLC16A1), L-Kynurenine receptor (aryl hydrocarbon receptor (RP85; bHLHe76), or the mitochondrial fission regulator DRP1 (dynamin related protein 1) and as a result the immune cells become inactivated, exhausted, immune-suppressive, or anti-inflammatory.

In some preferred embodiments, the inhibitory RNA is selectively, specifically or preferentially active in:

- M2 macrophages compared to M0 macrophages, or vice versa;
- M2 macrophages compared to M1 macrophages, or vice versa;
- M1 macrophages compared to M0 macrophages, or vice versa;
- M2a macrophages compared to M0 macrophages, or vice versa;
- M2a macrophages compared to M1 macrophages, or vice versa;
- M2c macrophages compared to M0 macrophages, or vice versa;

- M2c macrophages compared to M1 macrophages, or vice versa;
- M2c macrophages compared to M2a macrophages, or vice versa;
- M0, M1 or M2 macrophages compared to monocytes, or vice versa;
- an immune cell in the TME (e.g. T cell or TAM) compared with and immune not in the TME; or
- stimulated T-cells compared to naïve T cells, or vice versa.

In some embodiments of the invention, the inhibitory RNA is expressed in the cell as a modified form of an endogenous non-coding, typically inhibitory, RNA that is selectively, specifically or preferentially expressed in the first context, e.g. cell type or tissue type of interest, when the cell is in a particular state of activation or differentiation, etc. This can be achieved through modification of the endogenous inhibitory RNA in the cell, or through introduction of a modified form of the endogenous inhibitory RNA. Preferably it is achieved through modification of the endogenous inhibitory RNA *in situ*, e.g. through GEiGS.

Various databases providing details of tissue and cell expression profiles of inhibitory RNAs are well known. The skilled person can readily use these to identify suitable endogenous inhibitory RNAs for modification to provide context-specific activity of an inhibitory RNA as contemplated in the present invention. See, for example, the miRmine Human miRNA Expression Database, Panwar et al. Bioinformatics. 2017 May 15; 33(10): 1554–1560 at https://guanfiles.dcmb.med.umich.edu/mirmine/; the DIANA-miTED microRNA tissue expression database - see Kavakiotis et al., “DIANA-miTED: a microRNA tissue expression database”, Nucleic Acids Research, Volume 50, Issue D1, 7 January 2022, Pages D1055–D1061 http://www.microrna.gr/mited)

Furthermore, techniques to experimentally analyse the expression profile of inhibitory RNAs are well known in the art. Thus, for any given context (e.g. cell type or condition) the skilled person can readily determine which endogenous RNAs are expressed at high or low levels and thus select one or more suitable endogenous RNAs for modification to target a desired RNA, and thereby silence a desired target gene.

Suitable methods for analysing miRNA expression in a given cell are known in the art, and one approach suitable for use in the present invention is as follows: *Small RNA and miRNA isolation* - Small RNAs including miRNAs are isolated using the miRvana RNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer' s protocol. RNA is quantified using Qubit or Nanodrop spectrophotometer (Thermo Fisher, Wilmington, DE, USA) and quality is determined by Agilent 6000 nanochip (Agilent Technologies, Palo Alto, CA, USA). *miRNA*

measurement: Quantitative Real-Time PCR Analysis is carried out by as follows: RNAs are reverse transcribed and PCR amplified with miScript reverse transcription kit and miScript SYBR PCR kit (Qiagen, Valencia, CA, USA) using ABI 7500 real-time PCR system following the manufacturer's protocols. Values from duplicate reactions are averaged and normalized to the level of U6 SnoRNA. Relative expression levels are calculated following comparative Ct method as previously described (Schmittgen and Livak. Nat Protoc (2008) 3: 1101-1108). Alternatively, miRNAs are detected and relatively quantified using small RNA sequence analysis (as described in www.illumina.com/techniques/sequencing/rna-sequencing/small-rna-seq.html or Wake et al., BMC Genomics (2016) 17(1):1).

In some embodiments, the inhibitory RNA is expressed at a level of at least 1000 RPM (reads per million mapped reads) in the cell in the first context, at least 2000 RPM in the cell in the first context, at least 3000 RPM in the cell in the first context, at least 4000 RPM in the cell in the first context, at least 5000 RPM in the cell in the first context, at least 10000 RPM in the cell in the first context, at least 20000 RPM in the cell in the first context, or at least 50000 RPM in the cell, or at least 100000 RPM in the cell in the first context.

In some embodiments, in a second context, where it is desired that the inhibitory RNA is inactive or less active, the inhibitory RNA is expressed at a level of less than 25%, less than 20%, less than 15%, less than 10%, less than 5% or less than 1% compared to the cell in first context. In some embodiments, the inhibitory RNA is expressed at a level of less than 5000 RPM in the cell in the second context, less than 4000 RPM in the cell in the second context, less than 3000 RPM in the cell in the second context, less than 2000 RPM in the cell in the second context, less than 1000 RPM in the cell in the second context, less than 500 RPM in the cell in the second context, less than 300 RPM in the cell in the second context, less than 200 RPM in the cell in the second context, less than 100 RPM in the cell in the second context, or less than 50 RPM in the cell in the second context.

In some embodiments an endogenous inhibitory RNA that is modified to provide the inhibitory RNA of the present invention is expressed at a level of at least 1000 RPM (reads per million mapped reads) in the cell in the first context, at least 2000 RPM in the cell in the first context, at least 3000 RPM in the cell in the first context, at least 4000 RPM in the cell in the first context, at least 5000 RPM in the cell in the first context, at least 10000 RPM in the cell in the first context, at least 20000 RPM in the cell in the first context, or at least 50000 RPM in the cell, or at least 100000 RPM in the cell in the first context.

In some embodiments, in the second context the endogenous inhibitory RNA that is modified to provide the inhibitory RNA of the present invention is expressed at a level of less than 25%, 20%, 15%, 10%, 5% or 1% compared to the cell in first context.

In some embodiments the endogenous inhibitory RNA that is modified to provide the inhibitory RNA of the present invention is expressed at a level of less than 5000 RPM in the cell in the second context, less than 4000 RPM in the cell in the second context, less than 3000 RPM in the cell in the second context, less than 2000 RPM in the cell in the second context, less than 1000 RPM in the cell in the second context, less than 500 RPM in the cell in the second context, less than 300 RPM in the cell in the second context, less than 200 RPM in the cell in the second context, less than 100 RPM in the cell in the second context, or less than 50 RPM in the cell in the second context.

For pancreatic tissue, for example, hsa-miR-375, hsa-miR-143, and hsa-miR-21 are expressed at particularly high levels (189993.6, 186579.2, and 65231.3 RPM, respectively according to the miRmine database) with a range of other miRNAs also expressed at substantial but more moderate levels (e.g. hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, hsa-miR-200c, etc.).

For liver tissue, for example, hsa-miR-143, hsa-miR-10a, and hsa-miR-122 are expressed at particularly high levels (93940.6, 92139.6, and 80063.1) RPM, respectively according to the miRmine database) with a range of other miRNAs also expressed at substantial but more moderate levels (e.g. hsa-miR-22, hsa-miR-192, hsa-miR-146b, hsa-miR-26a, hsa-miR-125a, hsa-miR-150, etc.).

For brain tissue, for example, hsa-miR-10b, hsa-miR-181a, are expressed at particularly high levels (198835.2, 51936.2) RPM, respectively according to the miRmine database) with a range of other miRNAs also expressed at substantial but moderate levels (e.g. hsa-miR-26a, hsa-miR-92a, hsa-miR-143, hsa-miR-10a, hsa-miR-21, etc.).

For T cells, for example miRNAs are known to be critical regulators of tumour-associated T lymphocytes (Xing Y, et al. Tumor Immune Microenvironment and Its Related miRNAs in Tumor Progression. Front Immunol. 2021 May 18;12:624725. doi: 10.3389/fimmu.2021.624725. PMID: 34084160; PMCID: PMC8167795).

For macrophages and monocytes, for example, in some embodiments the inhibitory RNA is a modified version of MIR146A (e.g. hsa-miR-146a-5p), MIR20A (e.g. hsa-miR-20a-5p), LET7C

(hsa-miR-let-7c-5p), or LET7i (hsa-let-7i-5p). Such miRNAs can be used, for example, to induce silencing when monocytes differentiate into macrophages, and silencing will be maintained to block the initiation of M2-specific programmes.

NGS analysis shows MIR146A (hsa-miR-146a-5p) is highly expressed in M0, M1 and M2 macrophages; qPCR also shows strong induction from proliferative THP-1 monocytes to M0 like state. Thus, MIR146A (hsa-miR-146a-5p) is of particular interest as an inhibitory RNA for differentiated macrophages (e.g. M0, M1 and M2 macrophages) as opposed to monocyte precursors.

For MIR20A (hsa-miR-20a-5p), a combination of NGS and qPCR indicates high expression in proliferative THP-1 monocytes as well as M0, M1 and M2-like cells, thus MIR20A (hsa-miR-20a-5p)is of particular interest as an inhibitory RNA for monocytes and differentiated macrophages.

For LET7C (hsa-miR-let-7c-5p), NGS shows M2a-specific induction (to be confirmed by qPCR). Thus, LET7C (hsa-miR-let-7c-5p) is of particular interest as an inhibitory RNA for differentiated macrophages, especially M2 and/or M2a macrophages.

For LET7i (hsa-let-7i-5p) NGS shows very strong expression in M0, M1, M2a, M2c macrophages, with a moderate level of induction relative to proliferative THP-1 monocytes. Thus, LET7i (hsa-let-7i-5p) is of particular interest as an inhibitory RNA for differentiated macrophages, and may show a reduced level of expression in monocytes. LET7i (hsa-let-7i-5p) may therefore be of interest as an inhibitory RNA for differentiated macrophages for macrophages and monocytes, but with reduced inhibition (i.e. less silencing) of a target gene in monocytes compared to macrophages.

Further examples of differentially expressed miRNAs in macrophages are set out in Tables 15-20. Tables 15-20 below provide a summary of results for differentially expressed miRNAs identified in different macrophage cell states, as follows:

- Table 15 - M1 macrophages compared to M0 macrophages.
- Table 16 - M2a macrophages compared to M0 macrophages.
- Table 17 - M2a macrophages compared to M1 macrophages.
- Table 18 - M2c macrophages compared to M0 macrophages.
- Table 19 - M2c macrophages compared to M1 macrophages.
- Table 20 - M2c macrophages compared to M2a macrophages.

Suitable miRNAs for use in the present invention can be selected from these tables. Note, a positive fold change represents an increase in the first named call type compared to the second named cell type, and a negative fold change represents an increase in the second named cell type compared to the first. Thus, in Table 15 a positive fold change represents increased expression in M1 compared to M0, and a negative fold change represents increased expression in M0 compared to M1. Generally miRNAs with higher levels of differential expression are preferred (i.e. high log FC values) to provide more specific silencing in a given cell state. miRNAs with higher expression levels may also be preferred in some cases.

Further examples of differentially expressed miRNAs in stimulated vs naïve T cells are set out in Table 21. Table 21 summarises the results of NGS differential analysis, equivalent to the analysis performed to determine the results shown in Tables 15-20. Again, generally miRNAs with higher levels of differential expression are preferred (i.e. high log FC values) to provide more specific silencing in a given cell state. miRNAs with higher expression levels may also be preferred in some cases.

More generally, miRNAs are known to be critical regulators of tumour-associated macrophages (Chatterjee, et al. MicroRNAs: As Critical Regulators of Tumor- Associated Macrophages. Int J Mol Sci. 2020 Sep 27;21(19):7117. doi: 10.3390/ijms21197117. PMID: 32992449; PMCID: PMC7582892). This suggests further miRNAs which can be adapted for context-specific gene silencing in TAMs, e.g. to silence genes which promote an M2-like immunosuppressive or quiescent phenotype.

Additional examples from the literature for miRNAs relevant in M2-like macrophages include (Table 1):

miRNA	**Function in Macrophage**	**Reference**
miR-375	M2-like & promotes tumour progression	Frank, A.-C.; Ebersberger, S.; Fink, A.F.; Lampe, S.; Weigert, A.; Schmid, T.; Ebersberger, I.; Syed, S.N.; Brüne, B. Apoptotic tumor cell-derived microRNA-375 uses CD36 to alter the tumor-associated macrophage phenotype. Nat. Commun. 2019, 10, 1–18
miR 340-5p	M2 polarisation in glioblastoma	Liu, Y.; Li, X.; Zhang, Y.; Wang, H.; Rong, X.; Peng, J.; He, L.; Peng, Y. An miR-340-5p-macrophage feedback loop modulates the progression and tumor microenvironment of glioblastoma multiforme. Oncogene 2019, 38, 7399–741
miR 29a-3p	M2 polarisation in oral squamous cell carcinoma	Cai, J.; Qiao, B.; Gao, N.; Lin, N.; He, W. Oral squamous cell carcinoma-derived exosomes promote M2 subtype macrophage polarization mediated by exosome-enclosed miR-29a-3p. Am. J. Physiol. Cell Physiol. 2019, 316, C731–C74
miR 222-3p	M2 like in ovarian cancer	Ying, X.; Wu, Q.; Wu, X.; Zhu, Q.; Wang, X.; Jiang, L.; Chen, X.; Wang, X. Epithelial ovarian cancer-secreted

		exosomal miR-222-3p induces polarization of tumor-associated macrophages. Oncotarget 2016, 7, 43076.
miR 940	Promotes M2-like ovarian cell carcinoma	Chen, X.; Ying, X.; Wang, X.; Wu, X.; Zhu, Q.; Wang, X. Exosomes derived from hypoxic epithelial ovarian cancer deliver microRNA-940 to induce macrophage M2 polarization. Oncol. Rep. 2017, 38, 522–528
miR 203	Promotes M2 in colorectal Carcinoma	Takano, Y.; Masuda, T.; Iinuma, H.; Yamaguchi, R.; Sato, K.; Tobo, T.; Hirata, H.; Kuroda, Y.; Nambara, S.; Hayashi, N. Circulating exosomal microRNA-203 is associated with metastasis possibly via inducing tumor-associated macrophages in colorectal cancer. Oncotarget 2017, 8, 78598
miR-145	Promotes M2 in colorectal Carcinoma	Shinohara, H.; Kuranaga, Y.; Kumazaki, M.; Sugito, N.; Yoshikawa, Y.; Takai, T.; Taniguchi, K.; Ito, Y.; Akao, Y. Regulated polarization of tumor-associated macrophages by mir-145 via colorectal cancer–derived extracellular vesicles. J. Immunol. 2017, 199, 1505–1515
miR-103a	M2 polarisation in lung cancer	Hsu, Y.-L.; Hung, J.-Y.; Chang, W.-A.; Jian, S.-F.; Lin, Y.-S.; Pan, Y.-C.; Wu, C.-Y.; Kuo, P.-L. Hypoxic lung-cancer-derived extracellular vesicle microRNA-103a increases the oncogenic effects of macrophages by targeting PTEN. Mol. Ther. 2018, 26, 568–581
miR 21-3p, miR-181-5p, miR-125b-5p	M2 polarisation in epithelial ovarian cancer	Chen, X.; Zhou, J.; Li, X.; Wang, X.; Lin, Y.; Wang, X. Exosomes derived from hypoxic epithelial ovarian cancer cells deliver microRNAs to macrophages and elicit a tumor-promoted phenotype. Cancer Lett. 2018, 435, 80–9
miR-301a-3p	M2-like in pancreatic cancer	Wang, X.; Luo, G.; Zhang, K.; Cao, J.; Huang, C.; Jiang, T.; Liu, B.; Su, L.; Qiu, Z. Hypoxic tumor-derived exosomal miR-301a mediates M2 macrophage polarization via PTEN/PI3Kγ to promote pancreatic cancer metastasis. Cancer Res. 2018, 78, 4586–4598
miR-132, miR-29b-1, -27a, miR-146a, miR-222	Promotes M2b-like	Graff, J.W.; Dickson, A.M.; Clay, G.; McCaffrey, A.P.; Wilson, M.E. Identifying functional microRNAs in macrophages with polarized phenotypes. J. Biol. Chem. 2012, 287, 21816–21825
miR-let7a, miR-320a, miR-146a	M2-like polarisation	Park, J.E.; Dutta, B.; Tse, S.W.; Gupta, N.; Tan, C.F.; Low, J.K.; Yeoh, K.W.; Kon, O.L.; Tam, J.P.; Sze, S.K. Hypoxia-induced tumor exosomes promote M2-like macrophage polarization of infiltrating myeloid cells and microRNA-mediated metabolic shift. Oncogene 2019, 38, 5158–5173 Fortunato, O.; Borzi, C.; Milione, M.; Centonze, G.; Conte, D.; Boeri, M.; Verri, C.; Moro, M.; Facchinetti, F.; Andriani, F. Circulating mir-320a promotes immunosuppressive macrophages M2 phenotype associated with lung cancer risk. Int. J. Cancer 2019, 144, 2746–2761 Li, Y.; Zhao, L.; Shi, B.; Ma, S.; Xu, Z.; Ge, Y.; Liu, Y.; Zheng, D.; Shi, J. Functions of miR-146a and miR-222 in tumor-associated macrophages in breast cancer. Sci. Rep. 2015, 5, 18648
miR-let7c	Inhibits M1, promotes M2-like	Banerjee, S.; Xie, N.; Cui, H.; Tan, Z.; Yang, S.; Icyuz, M.; Abraham, E.; Liu, G. MicroRNA let-7c regulates macrophage polarization. J. Immunol. 2013, 190, 6542–6549.
miR-1246	Promote M2-like	Qian, M.; Wang, S.; Guo, X.; Wang, J.; Zhang, Z.; Qiu, W.; Gao, X.; Chen, Z.; Xu, J.; Zhao, R. Hypoxic glioma-derived exosomes deliver microRNA-1246 to induce M2

		macrophage polarization by targeting TERF2IP via the STAT3 and NF-κB pathways. Oncogene 2020, 39, 428–442
miR-let7d-5p	Promote M2-like	Baer, C.; Squadrito, M.L.; Laoui, D.; Thompson, D.; Hansen, S.K.; Kiialainen, A.; Hoves, S.; Ries, C.H.; Ooi, C.-H.; De Palma, M. Suppression of microRNA activity amplifies IFN-γ-induced macrophage activation and promotes anti-tumour immunity. Nat. Cell Biol. 2016, 18, 790–802
miR-451, miR-21	Macrophage polarisation in glioblastoma	van der Vos, K.E.; Abels, E.R.; Zhang, X.; Lai, C.; Carrizosa, E.; Oakley, D.; Prabhakar, S.; Mardini, O.; Crommentuijn, M.H.; Skog, J. Directly visualized glioblastoma-derived extracellular vesicles transfer RNA to microglia/macrophages in the brain. Neuro-Oncol. 2015, 18, 58–69

Thus, variants these miRNAs may be of interest for context-specific inhibition in M2 macrophages. For example, in some embodiments, variants of these miRNAs may be used to silence suitable target genes to shift the macrophage to a pro-inflammatory state (e.g. an M1-like state). miRNAs selectively expressed at high levels in M2 macrophages may be of particular interest in context-specific silencing of target genes in therapeutic macrophages for the treatment of cancer.

Additional examples from the literature for miRNAs relevant in M1-like macrophages include (Table 2):

miRNA	**Function in Macrophage**	**Reference**
miR-23a/ miR-27a/ miR24-2 cluster	M1-like, inhibits M2-like in breast cancer	Ma, S.; Liu, M.; Xu, Z.; Li, Y.; Guo, H.; Ge, Y.; Liu, Y.; Zheng, D.; Shi, J. A double feedback loop mediated by microRNA-23a/27a/24-2 regulates M1 versus M2 macrophage polarization and thus regulates cancer progression. Oncotarget 2016, 7, 1350
miR155 / miR125b-2	M1 like phenotype in pancreatic cancer	Su, M.-J.; Aldawsari, H.; Amiji, M. Pancreatic cancer cell exosome-mediated macrophage reprogramming and the role of microRNAs 155 and 125b2 transfection using nanoparticle delivery systems. Sci. Rep. 2016, 6, 30110
miR-16	M1-like in breast cancer	Chen, X.; Zhou, J.; Li, X.; Wang, X.; Lin, Y.; Wang, X. Exosomes derived from hypoxic epithelial ovarian cancer cells deliver microRNAs to macrophages and elicit a tumor-promoted phenotype. Cancer Lett. 2018, 435, 80–9

Thus, these miRNA variants may be of interest for context-specific inhibition in M1 macrophages. For example, in some embodiments, variants of these miRNAs may be used to silence suitable target genes and to shift the macrophage to an anti-inflammatory or pro-regenerative (tissue repair) state (e.g. an M2-like state). miRNAs selectively expressed at high levels in M1 macrophages may be of particular interest in context-specific silencing of target genes in therapeutic macrophages for the treatment of chronic inflammatory and fibrotic conditions.

By way of non-limiting example, the present invention in some preferred embodiments relates to cell-type conditional mRNA target silencing, such as:

(1) the generation of hypoimmunogenic cells for therapies via the partial silencing of either B2M or classical HLA-(A/B/C) molecules;

(2) the specification of therapeutic macrophages targeting the solid tumour microenvironment via the silencing in these cells of mRNA targets (and mechanisms) defining an immune-suppressive state; or

(3) enhancing the persistence of therapeutic T cells via the silencing of immune checkpoints.

Table 3 gives further non-limiting exemplary cell contexts in which context-specific gene silencing may be provided in accordance with the present invention.

Table 3 - Exemplary context-specific (cell state) target gene silencing

Disease	**Cell type**	**First State[a]**	**Function to Inhibit**	**Target Gene(s)**	**Second State[b]**	**Desired Therapeutic Function**
Ovarian/breast cancer (1)	Macrophage	M2-like	Pro-vascular, matrix remodelling, immune suppression	Clever-1 (also known as STAB1 or FEEL-1), LAIR1, DRP1	M1-like	Pro-inflammatory, anti-TME TAM
Macula dystrophy (2)	Retinal pigment epithelium	Proliferating Mesenchyme	Epithelial to mesenchyme transition and cell migration	B2M/HLA-A/B/C, SNAIL, SLUG, a/b crystallin	Polarised retinal pigmented epithelium	RPE which is conditionally hypoimmune providing photoreceptor support
Glioblastoma (high CLEC2D lectin expressing)(3)	CD8 T cells (CD161+)	Inactive; CD7, CD28, PD-1 (PDCD-1), CTLA-4	Exhausted (anergic) TILS unable to kill tumour cells	CD161 (KLRB1)	Activated in response to immunosuppressive TME signals (IL-10, TGF-b, IL-13, IL-4)	Anti-tumour cytotoxicity, not inhibited by other checkpoints (PD-1/PD-L1)
Lysosome storage disorder – MPS-III (4)	Monocyte (macrophage)	CD11b microglia secreting enzyme	Cells active but no enzyme secretion	Repressor. Prevents enzyme expression	CD11b microglia with no enzyme secretion	Local sustained therapeutic enzyme production (sulphamidase)
Lysosome storage disorder –	Glia progenitor cells (Migratory, responsive to IL-6,	mGluR5+, astrocytes	HSPG secretion	EXTL2/EXTL3 (HSPG anabolic genes)	mGluR5+, astrocytes	Substrate reduction therapy; Reduction in HSPG synthesis in astrocytes

MPS-III (4)	RAGE, TNF-a)					
ALS (MND) (5)	Monocyte (macrophage)	M1-like macrophage (CSFR1+, IL34 responsive)	Pro-inflammatory		Polarisation to M1-like M1-like macrophage (CSFR1+, IL34 responsive)	anti-inflammatory, neuroprotective
Drug refractory epilepsy(6)	CNS Progenitors (MGE)	Astrocytes, microglia, VSMC, endothelium	Continued seizures	Mylk, Myl9, ESAM, Aldh11, Enkur, C1qb, Fcrls	GABA Inter-neuron (parvalbumin+, somatostatin+) Dlx1, Lhx6, NKx2.1	Integrating, inhibitory inter-neurone, hypoimmune
Neuropathic pain(7)	Microglia progenitor	Microglia (CD11b)	Pro-inflammatory	B2M/HLA-A/B/C, receptors for inflammatory mediators	$CD11c^{High}$, P2RY12+	Anti-inflammatory, conditionally hypoimmune

miRNAs selectively expressed at high levels in M2 macrophages may be of particular interest in context-specific silencing of target genes in therapeutic macrophages for the treatment of cancer.

References from Table 3:

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Roth P et al.. Malignant glioma cells counteract antitumor immune responses through expression of lectin- like transcript-1. Cancer Res. 2007 Apr 15;67(8):3540-4. doi: 10.1158/0008-5472.CAN-06-4783. PMID: 17440061.

Di W, et al.. Clinical characterization and immunosuppressive regulation of CD161 (KLRB1) in glioma through 916 samples. Cancer Sci. 2022 Feb;113(2):756-769. doi: 10.1111/cas.15236. Epub 2021 Dec 24. PMID: 34881489; PMCID: PMC8819299.

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(7) Kohno K, et al.. A spinal microglia population involved in remitting and relapsing neuropathic pain. Science. 2022 Apr;376(6588):86-90. doi: 10.1126/science.abf6805. Epub 2022 Mar 31. PMID: 35357926.

Nucleic Acid Constructs Adapted to Express an Inhibitory RNA

In some embodiments, the nucleic acid construct adapted to express an inhibitory RNA is part of the genome of the cell (i.e. it is a genomic sequence).

In some embodiments of the present invention the nucleic acid construct adapted to express an inhibitory RNA is episomal.

In preferred embodiments the nucleic acid construct is adapted to express a miRNA.

In preferred embodiments the nucleic acid construct expresses a modified form of an endogenous inhibitory RNA which is expressed in a context-specific manner, and wherein the inhibitory RNA has been modified compared to the endogenous RNA to inhibit expression of the target gene. Accordingly, the context-specific expression of the endogenous inhibitory RNA is maintained, but the targeting specificity of the inhibitory RNA is redirected to inhibit expression of a target gene (which is different from the gene targeted by the endogenous inhibitory RNA).

In some embodiments, the nucleic acid construct adapted to express an inhibitory RNA is part of the genome of the cell and is present in an endogenous locus in the genome which encodes an endogenous non-coding RNA, e.g. an endogenous inhibitory RNA.

In some embodiments, the nucleic acid construct adapted to express an inhibitory RNA is a modified endogenous nucleic acid sequence which encodes an endogenous non-coding RNA, e.g. an retargeted endogenous inhibitory RNA that has been modified *in situ*.

In some embodiments of the present invention, a sequence encoding an inhibitory RNA (preferably a promoterless sequence encoding an inhibitory RNA) is inserted into an endogenous miRNA locus, preferably without disruption or deactivation of any endogenous miRNA. The endogenous miRNA locus may encode the endogenous miRNA which has been modified to target a new target mRNA. Thus, in some embodiments a modified version of an endogenous miRNA is inserted into the genomic locus that contains the original (unmodified) endogenous miRNA, and wherein the original miRNA is not disrupted or deactivated. Thus, the expression or activity of the original (unmodified) endogenous miRNA can be unaltered.

In some embodiments, the nucleic acid construct adapted to express an inhibitory RNA is a modified endogenous nucleic acid sequence which encodes an endogenous non-coding RNA in which the endogenous non-coding RNA (e.g. an endogenous inhibitory RNA) has been modified so that it inhibits expression of the target gene (e.g. targeting a transcript from the target gene). In some embodiments, the nucleic acid construct adapted to express an inhibitory RNA is comprised in a synthetic expression cassette. In some embodiments, the nucleic acid construct adapted to express an inhibitory RNA is comprised in an expression vector.

In some embodiments of the invention the nucleic acid construct adapted to express an inhibitory RNA is part of the genome of the cell and is present in a locus which does not encode an endogenous non-coding RNA.

The nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene may be introduced into the cell via any method known to the skilled person. In some embodiments, the nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene is introduced via lentiviral delivery.

Target Genes

It will be apparent that the present invention provides for the targeting of a wide range of target genes in any given context. Of particular interest in the present invention are target

genes which can be partially or completely silenced in order to induce a particular phenotype, property or behaviour of the cells in a given context. For example, one might seek to alter the immunogenicity of a cell, make a cell active or quiescent, or make a cell pro-inflammatory or anti-inflammatory.

In some embodiments of the invention it is desirable to target genes which alter, preferably reduce, the immunogenicity of cells. This is of particular interest for therapeutic cells, especially allogeneic therapeutic cells, for example allogeneic stem cell-derived islet cells. As discussed herein, genes of the MHC-I complex are of particular interest for generating hypoimmunogenic cells.

Accordingly, in some embodiments the target gene is a gene of the MHC-I complex. In some preferred embodiments the target gene is β_2-microglobulin (B2M). In other embodiments the target gene is an HLA-A, HLA-B and/or HLA-C gene. In some embodiments the target gene is a combination of HLA-A and B or C, or HLA-B and C, or HLA-A/B/C genes.

In some embodiments target genes for inflammatory pathways in allogeneic stem cell-derived therapeutic cells (e.g. islet cells) to alter, preferably reduce, the immunogenicity of cells that are not related to MHC-I complex are selected from: TAP1/2, CD74, PSMB9, the chemokine ligands 10 (CXCL10), CXCL5, CXCL9, signalling molecules STAT1, JAK1/2, HNRNPA1P48, GBP2, and TRIM.

In some embodiments of the invention it is desirable to target genes which alter the activity of immune cells. For example, it can be desirable to target genes which, when inhibited, lead to a pro-inflammatory phenotype or which lead to an immunosuppressive (anti-inflammatory) phenotype.

It will be apparent that there are some conditions in which it is desirable to render immune cells pro-inflammatory, e.g. in tumours. In other conditions it is desirable to render immune cells immunosuppressive or anti-inflammatory, e.g. in autoimmune or other inflammatory diseases. Macrophages and T cells are of particular interest given their key roles in immune activity and signalling in many contexts.

By way of non-limiting example, in macrophages it may be desirable to target one or more genes to render the cells pro-inflammatory or at least avoid an immunosuppressive state. This may be of particular interest for context-specific expression in the TME where

macrophages typically become tumour associated macrophages (TAMs), key cells that create an immunosuppressive microenvironment by producing cytokines, chemokines, growth factors, and triggering the inhibitory immune checkpoint proteins release in T cells. Thus, it would be desirable to modify macrophages such one or more inhibitory RNAs are active in the TME which target one or more genes where inhibition of said one or more genes promotes a pro-inflammatory or non-immunosuppressive state.

Accordingly, in some embodiments, the target gene is preferably one or more gene associated with promoting an immunosuppressive state in an immune cell, particularly a macrophage (thus, promoting a pro-inflammatory phenotype). For example the target gene can be one or more selected from the group consisting of: STAT6, IRF4, DRP1, PPARG, KDM6B, Clever-1 (also known as STAB1 or FEEL-1), LAIR1/2, P-selection glycoprotein ligand (PSGL-1), signal regulatory protein alpha (SIRPa), triggering receptor expressed on myeloid cells 2 (TREM2), proton sensing GPCR (GPR65), inhibitory leukocyte immunoglobulin-like receptor subfamily B4 (LILRB4), and PD-1 (PDCD-1).

In some embodiments, the target gene is preferably selected from one or more genes expressed in a tumour associated macrophage (TAM). For example the target gene can be one or more selected from the group consisting of: TREM2, Clever-1 (also known as FEEL-1 and STAB-1), LAIR1/2, inhibitory leukocyte immunoglobulin-like receptor subfamily B4 (LILRB4).

TREM2 plays an immunosuppressive role in the development of cancer as shown in Katzenelenbogen, et al. which is incorporated herein by reference [Katzenelenbogen, Y., Sheban, F., Yalin, A., Yofe, I., Svetlichnyy, D., Jaitin, D. A., Bornstein, C., Moshe, A., Keren-Shaul, H., Cohen, M., Wang, S. Y., Li, B., David, E., Salame, T. M., Weiner, A., & Amit, I. (2020). Coupled scRNA-Seq and Intracellular Protein Activity Reveal an Immunosuppressive Role of TREM2 in Cancer. Cell, 182(4), 872-885.e19. https://doi.org/10.1016/J.CELL.2020.06.032]. Systemic blockade of Clever-1 (also known as FEEL-1 and STAB-1) with antibodies can elicit lymphocyte activation in patients with solid tumours, yet there exists many tissues expressing the molecule which can act as a ‘sink’ to limit ‘on tumour effect’ as shown in Virtakoivu, et al. which is incorporated herein by reference [Virtakoivu, R., Rannikko, J. H., Viitala, M., Vaura, F., Takeda, A., Lonnberg, T., Koivunen, J., Jaakkola, P., Pasanen, A., Shetty, S., de Jonge, M. J. A., Robbrecht, D., Ma, Y. T., Skytta, T., Minchom, A., Jalkanen, S., Karvonen, M. K., Mandelin, J., Bono, P., & Hollmen, M. (2021). Systemic Blockade of Clever-1 Elicits Lymphocyte Activation Alongside Checkpoint Molecule Downregulation in Patients with Solid Tumors: Results from a Phase I/II Clinical Trial. Clinical

Cancer Research : An Official Journal of the American Association for Cancer Research, 27(15), 4205–4220. https://doi.org/10.1158/1078-0432.CCR-20-4862]. LAIR-1 activation by tumour specific isoforms of collagen inhibits a pro-inflammatory macrophage phenotype in vitro as shown in Keerthivasan, et al. which is incorporated herein by reference [Keerthivasan, S., Şenbabaoğlu, Y., Martinez-Martin, N., Husain, B., Verschueren, E., Wong, A., Yang, Y. A., Sun, Y., Pham, V., Hinkle, T., Oei, Y., Madireddi, S., Corpuz, R., Tam, L., Carlisle, S., Roose-Girma, M., Modrusan, Z., Ye, Z., Koerber, J. T., & Turley, S. J. (2021). Homeostatic functions of monocytes and interstitial lung macrophages are regulated via collagen domain-binding receptor LAIR1. Immunity, 54(7), 1511-1526.e8. https://doi.org/10.1016/J.IMMUNI.2021.06.012].Inhibitory leukocyte immunoglobulin-like receptor subfamily B4 (LILRB4)[Sharma, N., Atolagbe, O. T., Ge, Z., & Allison, J. P. (2021). LILRB4 suppresses immunity in solid tumors and is a potential target for immunotherapy. The Journal of Experimental Medicine, 218(7). https://doi.org/10.1084/JEM.20201811] acts as a myeloid cell checkpoint also suppressing the anti-tumour, pro-inflammatory state of macrophages as shown in Sharma, et al. which is incorporated herein by reference.

T cells also have a key role in the TME. Tumour infiltrating lymphocytes (TILs) and CAR-T cells are typically tolerised through the activity of immune checkpoint pathway, such as PD-1 (PDCD-1), CTLA4, LAG3, and TIM3. Accordingly, in some embodiments, the target gene is preferably one or more gene associated with an tolerised state in an T cell. For example the target gene can be one or more selected from the group consisting of: PD-1 (PDCD-1), CTLA4, LAG3, TIGIT (T-cell-immunoreceptor with Ig and ITIM domains), and TIM3. The target gene can also preferably be one or more genes upregulated in an exhausted state in an T cell. For example, the target gene can be one or more selected from the group consisting of*TOX* subfamily (thymocyte selection-associated high mobility group box protein), *NR4A1* (nuclear receptor 4A1; Nur77, TR3, NGFI-B) and *CBL-B* (E3 ubiquitin-protein ligase).

Additional example of target gene in T cells is RASA-2.

RASA-2 (the RAS GTPase activating protein) ablation in T cells boosts antigen sensitivity and long term function as shown in Carnevale, et al. which is incorporated herein by reference [Carnevale J, Shifrut E, Kale N, Nyberg WA, Blaeschke F, Chen YY, Li Z, Bapat SP, Diolaiti ME, O'Leary P, Vedova S, Belk J, Daniel B, Roth TL, Bachl S, Anido AA, Prinzing B, Ibañez-Vega J, Lange S, Haydar D, Luetke-Eversloh M, Born-Bony M, Hegde B, Kogan S, Feuchtinger T, Okada H, Satpathy AT, Shannon K, Gottschalk S, Eyquem J, Krenciute G, Ashworth A, Marson A. RASA2 ablation in T cells boosts antigen sensitivity and long-term function. Nature. 2022 Sep;609(7925):174-182. doi: 10.1038/s41586-022-05126-w. Epub 2022 Aug 24. PMID: 36002574; PMCID: PMC9433322].

In some embodiments of the invention it is desirable to target genes which are expressed on the cell surface. For example, it can be desirable to silence genes encoding antigens, to prevent cells being recognised by antibodies which recognise the antigens. Accordingly, in some embodiments the target gene is preferably one or more genes expressed on the surface of cells, such as T cells or progenitors thereof (e.g. haematopoietic stem cells (HSCs) or other T cell progenitors, such as common lymphoid progenitors or small lymphocytes). For example, the target gene may be a T cell specific surface protein, such as CD52. Genes encoding other cell surface proteins may also be of interest, and thus the invention contemplates context-specific silencing of genes encoding cell surface proteins other than CD52. Suitably the target gene encode any marker protein specific for a given cell type, e.g. an immune cell type. In some preferred embodiments, the target gene encodes a cell surface protein which is a target for an antibody used in the depletion of immune cells or progenitors thereof (e.g. lymphodepletion), such that the cell in which the target gene is silenced is protected from the antibody. By way of non-limiting example, this approach can be used to target one or more genes selected from genes encoding CD117, CD27, CD45, CD90, CD110 and CD184. See, e.g., Czechowicz *et al.* 'Selective hematopoietic stem cell ablation using CD117-antibody-drug-conjugates enables safe and effective transplantation with immunity preservation Nat Commun.' 2019 Feb 6;10(1):617.

In some preferred embodiments, the target gene encodes CD52. In some preferred embodiments of the invention, CD52 is silenced in a context-specific manner in activated T cells. In some embodiments, genes encoding both B2M and CD52 are target genes for context-specific silencing. In some embodiments target genes encoding both B2M and CD52 are downregulated in the same context, for instance in T cells, in activated T cells and/or in T cell progenitors. Context-specific silencing of CD52 and/or B2M in activated T cells is a particularly preferred embodiment.

In some instances it may be desirable to context-specifically silence a target gene in cells in which other genes are constitutively silenced, e.g. knocked-out or knocked-down (e.g., via CRISPR). Constitutive silencing can be also achieved using GEiGS, e.g. using a solution based on a miRNA scaffold with broad expression pattern (e.g. in T cells, an miRNA which is expressed in activated as well as resting T cells). For example, methods of the invention can be used to context-specifically silence B2M in modified T cells or T cell progenitors, e.g. in activated T cells, in which CD52 has been constitutively silenced, e.g. knocked out (i.e., CD52-CAR-T cells).

Silencing of CD52 can be particularly desirable for use in cell therapy, e.g. cell-based cancer therapy, where anti-CD52 antibodies are used to 'condition' patients prior to administration of the therapeutic cells. One particularly significant example is the use of anti-CD52 antibodies prior to allogeneic T cell therapy, but anti-CD52 antibodies are also useful in allogeneic hematopoietic stem cell transplantation (HSCT). To elaborate, anti-CD52 antibodies (e.g. Alemtuzumab) are administered to patients that are about to receive an allogeneic CAR-T cell infusion or allogeneic HSCT. This treatment kills the patients' own T cells creating a niche for the transplanted cells to expand post-infusion and/or target the cancer. Silencing of CD52 in a patient administered therapeutic CAR-T or HSCs cells prevents their elimination by circulating anti-CD52 antibodies, allowing a window in which the therapeutic cells survive and can kill the cancer cells. In some cases, context-specific silencing of CD52 may be advantageous, e.g. to reduce risk of undesirable T-cell phenotypes, as knocking out CD52 may lead to generates dysfunctional T cells (see Kinsella, et al. 'CD52/GPI− T-Cells Are Enriched for Alloreactive Specificity and Predict Acute Graft-Versus-Host-Disease After Stem Cell Transplantation', Transplantation and Cellular Therapy, Volume 27, Issue 6, 2021, Pages 475.e1-475.e9). Accordingly, as mentioned above, the silencing of CD52 in activated T cells is one particularly preferred embodiment of the invention. In other cases, however, constitutive silencing of CD52 may be desirable, e.g. via CD52 KO, or via GEiGS using a solution based on a miRNA scaffold with broad expression pattern in T cells (i.e., expressed in activated as well as resting T cells). See also Zhao et al. 'The Immunological Function of CD52 and its Targeting in Organ Transplantation'. Inflamm. Res. (2017) 66(7):571–8 and Kamali, et al. 'CRISPR/Cas9-mediated knockout of clinically relevant alloantigenes in human primary T cells' BMC Biotechnology (2021) 21:9 for further information on CD52 and silencing thereof. It will be appreciated that other immune cell markers relevant for cell depletion can be targeted and silenced to protect cells from other cell depletion approaches.

In other embodiments, one or more of CD117, CD27, CD45, CD90, CD110 and CD184 may be silenced in cells, either constitutively or in a context-specific manner. In some embodiments B2M is silenced in a context specific manner, and one or more of CD117, CD27, CD45, CD90, CD110 and/or CD184 is silenced in a context specific manner. In some embodiments B2M is silenced in a context specific manner, and one or more of CD117, CD27, CD45, CD90, CD110 and/or CD184 is silenced in a context specific manner in the same context as B2M. In some embodiments B2M is silenced in a context specific manner, and one or more of CD117, CD27, CD45, CD90, CD110 and/or CD184 is constitutively silenced. CD117, CD27, CD45, CD90, CD110 and/or CD184 represent other targets for immune cell depletion.

In some approaches, such as allogeneic adoptive T cell therapy, it may also be desirable to silence the endogenous T cell receptor (TCR) in order to reduce off-tumour, graft-versus-host-disease (GvHD). Silencing of the endogenous TCR is typically permanent, e.g. via knock-out of the *TRAC* gene. Accordingly, in some embodiments, the endogenous TCR is silenced in the cell, suitably by knocking out or otherwise silencing the TRAC gene, e.g. via GEiGS using a solution based on a miRNA scaffold with broad expression pattern in T cells. However, the silencing of the endogenous TCR could also be context-specific, e.g. where the TRAC gene is a target gene according to the present invention. Thus, in some embodiment the TRAC gene is a target gene for context-specific silencing, e.g., for specific silencing in T cells or in activated T cells.

In some embodiments CD52 and TCR are both silenced in a T cell. In some embodiments CD52, TCR and B2M are silenced in a T cell. In some preferred embodiments TCR is constitutively silenced, suitable by knocking out TCR in the T cell (e.g. by knocking out the TRAC gene) or via GEiGS, e.g. using a solution based on a miRNA scaffold with broad expression pattern in T cells (i.e., expressed in activated as well as resting T cells). In some embodiments TCR is knocked out in the T cell, and CD52 and B2M are silenced in a context-specific manner, suitably wherein CD52 and B2M are silenced in activated T cells. In some embodiments TCR and CD52 are knocked out in the T cell, and B2M is silenced in a context-specific manner, suitably wherein B2M is silenced in activated T cells. Suitable approaches for context-specific silencing target genes in T-cells in general or in activated T-cells are discussed herein.

Methods of Modifying a Cell

In some embodiments the method comprises the step of modifying the cell to provide the nucleic acid construct adapted to express an inhibitory RNA.

In some embodiments, the method uses a gene editing technology to provide inhibitory RNA molecules designed to target and interfere with an RNA molecule of interest (endogenous or exogenous to the eukaryotic cell, preferably endogenous to the eukaryotic cell).

The gene editing technology of some embodiments of the invention comprises genome editing of an inhibitory RNA molecule (e.g. endogenous) yet it is stable and heritable. The present inventors use a Genome Editing induced Gene Silencing (GEiGS) platform capable of utilising a eukaryotic cell's endogenous non-coding RNA molecules including e.g. RNA silencing molecules (e.g. siRNA, miRNA, piRNA, tasiRNA, tRNA, rRNA, antisense RNA, etc.) and

modifying them to target any RNA target of interest. Using GEiGS, the skilled person is able to screen potential non-coding RNA molecules, editing a few nucleotides in these endogenous RNA molecules, and thereby redirecting their activity and/or specificity to effectively and specifically target any RNA of interest including, for instance, mRNA encoding B2M protein.

Accordingly, there is provided a method of inhibiting the translation of an RNA transcript derived from a target protein coding gene (e.g., B2M), in a cell comprising introducing into the cell an RNA editing agent conferring a silencing specificity of a non-coding RNA molecule towards a target RNA of interest, wherein the non-coding RNA molecule is modified such that the sequence of the non-coding RNA molecule is altered to target the RNA transcript of a target gene of interest, thereby modifying the translation of the RNA transcript into a protein molecule.

Various non-limiting examples of genome editing methods and RNA editing agents used to introduce nucleic acid alterations in inhibitory RNA molecules can be used according to specific embodiments of the invention.

Genome Editing using engineered endonucleases - This approach refers to a reverse genetics method using artificially engineered nucleases to typically cut and create specific double-stranded breaks (DSBs) at a desired location(s) in the genome, which are then repaired by cellular endogenous processes such as, homologous recombination (HR) or non-homologous end-joining (NHEJ). NHEJ directly joins the DNA ends in a double-stranded break (DSB) with or without minimal ends trimming, while HR utilizes a homologous donor sequence as a template (i.e. the sister chromatid formed during S-phase) for regenerating/copying the missing DNA sequence at the break site. In order to introduce specific nucleotide modifications to the genomic DNA, a donor DNA repair template containing the desired sequence must be present during HR (exogenously provided single stranded or double stranded DNA).

Genome editing cannot be performed using traditional restriction endonucleases since most restriction enzymes recognize a few base pairs on the DNA as their target and these sequences often will be found in many locations across the genome resulting in multiple cuts which are not limited to a desired location. To overcome this challenge and create site-specific single- or double-stranded breaks (DSBs), several distinct classes of nucleases have been discovered and bioengineered to date. These include the meganucleases, Zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALENs) and CRISPR/Cas9 system.

Meganucleases - Meganucleases are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cys box family and the HNH family. These families are characterized by structural motifs, which affect catalytic activity and recognition sequence. For instance, members of the LAGLIDADG family are characterized by having either one or two copies of the conserved LAGLIDADG motif. The four families of meganucleases are widely separated from one another with respect to conserved structural elements and, consequently, DNA recognition sequence specificity and catalytic activity. Meganucleases are found commonly in microbial species and have the unique property of having very long recognition sequences (>14bp) thus making them naturally very specific for cutting at a desired location.

This can be exploited to make site-specific double-stranded breaks (DSBs) in genome editing. One of skill in the art can use these naturally occurring meganucleases, however the number of such naturally occurring meganucleases is limited. To overcome this challenge, mutagenesis and high throughput screening methods have been used to create meganuclease variants that recognize unique sequences. For example, various meganucleases have been fused to create hybrid enzymes that recognize a new sequence.

Alternatively, DNA interacting amino acids of the meganuclease can be altered to design sequence specific meganucleases. Alternatively, meganucleases with site specific cutting characteristics can be obtained using commercially available technologies e.g., Precision Biosciences' Directed Nuclease Editor™ genome editing technology.

ZFNs and TALENs - Two distinct classes of engineered nucleases, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have both proven to be effective at producing targeted double- stranded breaks (DSBs).

ZFNs and TALENs restriction endonuclease technology utilizes a non-specific DNA cutting enzyme which is linked to a specific DNA binding domain (either a series of zinc finger domains or TALE repeats, respectively). Typically a restriction enzyme whose DNA recognition site and cleaving site are separate from each other is selected. The cleaving portion is separated and then linked to a DNA binding domain, thereby yielding an endonuclease with very high specificity for a desired sequence. An exemplary restriction enzyme with such properties is FokI. Additionally FokI has the advantage of requiring dimerization to have nuclease activity and this means the specificity increases dramatically as each nuclease partner recognizes a unique DNA sequence. To enhance this effect, FokI nucleases have been engineered that can only function as heterodimers and have increased catalytic activity. The heterodimer functioning nucleases avoid the possibility of unwanted homodimer activity and thus increase specificity of the double- stranded break (DSB).

Thus, for example to target a specific site, ZFNs and TALENs are constructed as nuclease pairs, with each member of the pair designed to bind adjacent sequences at the targeted site. Upon transient expression in cells, the nucleases bind to their target sites and the FokI domains heterodimerize to create a double- stranded break (DSB). Repair of these double-stranded breaks (DSBs) through the non-homologous end-joining (NHEJ) pathway often results in small deletions or small sequence insertions (Indels). Since each repair made by NHEJ is unique, the use of a single nuclease pair can produce an allelic series with a range of different insertions or deletions at the target site.

In general, NHEJ is relatively accurate (about 75-85% of DSBs in human cells are repaired by NHEJ within about 30 min from detection) in gene editing erroneous NHEJ is relied upon as when the repair is accurate the nuclease will keep cutting until the repair product is mutagenic and the recognition/cut site/P AM motif is gone/mutated or that the transiently introduced nuclease is no longer present. The deletions typically range anywhere from a few base pairs to a few hundred base pairs in length, but larger deletions have been successfully generated in cell culture by using two pairs of nucleases simultaneously. In addition, when a fragment of DNA with homology to the targeted region is introduced in conjunction with the nuclease pair, the double-stranded break (DSB) can be repaired via homologous recombination (HR) (e.g. in the presence of a donor template) to generate specific modifications.

Although the nuclease portions of both ZFNs and TALENs have similar properties, the difference between these engineered nucleases is in their DNA recognition peptide. ZFNs rely on Cys2- His2 zinc fingers and TALENs on TALEs. Both of these DNA recognizing peptide domains have the characteristic that they are naturally found in combinations in their proteins. Cys2-His2 Zinc fingers are typically found in repeats that are 3 bp apart and are found in diverse combinations in a variety of nucleic acid interacting proteins. TALEs on the other hand are found in repeats with a one-to-one recognition ratio between the amino acids and the recognized nucleotide pairs. Because both zinc fingers and TALEs happen in repeated patterns, different combinations can be tried to create a wide variety of sequence specificities. Approaches for making site-specific zinc finger endonucleases include, e.g., modular assembly (where Zinc fingers correlated with a triplet sequence are attached in a row to cover the required sequence), OPEN (low- stringency selection of peptide domains vs. triplet nucleotides followed by high- stringency selections of peptide combination vs. the final target in bacterial systems), and bacterial one-hybrid screening of zinc finger libraries, among others. ZFNs can also be designed and obtained commercially from e.g., Sangamo Biosciences™ (Richmond, CA).

T-GEE system (TargetGene's Genome Editing Engine) - A programmable nucleoprotein molecular complex containing a polypeptide moiety and a specificity conferring nucleic acid (SCNA) which assembles in-vivo, in a target cell, and is capable of interacting with the predetermined target nucleic acid sequence is provided. The programmable nucleoprotein molecular complex is capable of specifically modifying and/or editing a target site within the target nucleic acid sequence and/or modifying the function of the target nucleic acid sequence. Nucleoprotein composition comprises (a) polynucleotide molecule encoding a chimeric polypeptide and comprising (i) a functional domain capable of modifying the target site, and (ii) a linking domain that is capable of interacting with a specificity conferring nucleic acid, and (b) specificity conferring nucleic acid (SCNA) comprising (i) a nucleotide sequence complementary to a region of the target nucleic acid flanking the target site, and (ii) a recognition region capable of specifically attaching to the linking domain of the polypeptide. The composition enables modifying a predetermined nucleic acid sequence target precisely, reliably and cost-effectively with high specificity and binding capabilities of molecular complex to the target nucleic acid through base-pairing of specificity-conferring nucleic acid and a target nucleic acid. The composition is less genotoxic, modular in their assembly, utilize single platform without customization, practical for independent use outside of specialized core-facilities, and has shorter development time frame and reduced costs.

CRISPR-Cas system and all its variants (also referred to herein as "CRISPR") - Many bacteria and archea contain endogenous RNA-based adaptive immune systems that can degrade nucleic acids of invading phages and plasmids. These systems consist of clustered regularly interspaced short palindromic repeat (CRISPR) nucleotide sequences that produce RNA components and CRISPR associated (Cas) genes that encode protein components. The CRISPR RNAs (crRNAs) contain short stretches of homology to the DNA of specific viruses and plasmids and act as guides to direct Cas nucleases to degrade the complementary nucleic acids of the corresponding pathogen. Studies of the type II CRISPR/Cas system of Streptococcus pyogenes have shown that three components form a RNA/protein complex and together are sufficient for sequence- specific nuclease activity: the Cas9 nuclease, a crRNA containing 20 base pairs of homology to the target sequence, and a trans-activating crRNA (tracrRNA) (Jinek et al. Science (2012) 337: 816-821).

It was further demonstrated that a synthetic chimeric guide RNA (sgRNA) composed of a fusion between crRNA and tracrRNA could direct Cas9 to cleave DNA targets that are complementary to the crRNA in vitro. It was also demonstrated that transient expression of Cas9 in conjunction with synthetic sgRNAs can be used to produce targeted double- stranded breaks (DSBs) in a variety of different species (Cho et al, 2013 (Targeted genome engineering

in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol.* 2013;31(3):230-232.); Cong et al, 2013 (Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-823.; DiCarlo et al, 2013 (Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res. 2013;41(7):4336-4343); Hwang et al., 2013a, b (Nature Biotechnology volume 31, pages227–229 (2013); Heritable and precise zebrafish genome editing using a CRISPR-Cas system. *PLoS One*. 2013;8(7):e68708 Jinek et al., 2013(RNA-programmed genome editing in human cells eLife 2:e00471); Mali et al, 2013 (RNA-guided human genome engineering via Cas9. Science. 2013 Feb 15;339(6121):823-6)).

The CRISPR/Cas system for genome editing contains two distinct components: a sgRNA and an endonuclease e.g. Cas9. The sgRNA (also referred to herein as short guide RNA (sgRNA)) is typically a 20- nucleotide sequence encoding a combination of the target homologous sequence (crRNA) and the endogenous bacterial RNA that links the crRNA to the Cas9 nuclease (tracrRNA) in a single chimeric transcript. The gRNA/Cas9 complex is recruited to the target sequence by the base pairing between the sgRNA sequence and the complement genomic DNA/RNA. For successful binding of Cas9, the genomic target sequence must also contain the correct Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence. The binding of the gRNA/Cas9 complex localizes the Cas9 to the genomic target sequence so that the Cas9 can cut both strands of the DNA causing a double-strand break (DSB). The double-stranded breaks (DSBs) produced by CRISPR/Cas can undergo homologous recombination or NHEJ and are susceptible to specific sequence modification during DNA repair.

The Cas9 nuclease has two functional domains: RuvC and HNH, each cutting a different DNA strand. When both of these domains are active, the Cas9 causes double strand breaks (DSBs) in the genomic DNA or RNA.

A significant advantage of CRISPR/Cas is that the high efficiency of this system is coupled with the ability to easily create synthetic sgRNAs. This creates a system that can be readily modified to target modifications at different genomic sites and/or to target different modifications at the same site. Additionally, protocols have been established which enable simultaneous targeting of multiple genes. The majority of cells carrying the mutation present biallelic mutations in the targeted genes.

However, apparent flexibility in the base-pairing interactions between the sgRNA sequence and the genomic DNA target sequence allows imperfect matches to the target sequence to be cut by Cas9.

Modified versions of the Cas9 enzyme containing a single inactive catalytic domain, either RuvC- or HNH-, are called ‘nickases’. With only one active nuclease domain, the Cas9 nickase cuts only one strand of the target DNA, creating a single-strand break or 'nick'. A single strand break, or nick, is mostly repaired by single strand break repair mechanism involving proteins such as but not only, PARP (sensor) and XRCC1/LIG III complex (ligation). If a single strand break (SSB) is generated by topoisomerase I poisons or by drugs that trap PARP1 on naturally occurring SSBs then these could persist and when the cell enters into S-phase and the replication fork encounter such SSBs they will become single ended DSBs which can only be repaired by HR. However, two proximal, opposite strand nicks introduced by a Cas9 nickase are treated as a double-strand break, in what is often referred to as a 'double nick' CRISPR system. A double-nick, which is basically non-parallel DSB, can be repaired like other DSBs by HR or NHEJ depending on the desired effect on the gene target and the presence of a donor sequence and the cell cycle stage (HR is of much lower abundance and can only occur in S and G2 stages of the cell cycle). Thus, if specificity and reduced off-target effects are crucial, using the Cas9 nickase to create a double-nick by designing two sgRNAs with target sequences in close proximity and on opposite strands of the genomic DNA would decrease off-target effect as either sgRNA alone will result in nicks that are not likely to change the genomic DNA, even though these events are not impossible.

Modified versions of the Cas9 enzyme containing two inactive catalytic domains (dead Cas9, or dCas9) have no nuclease activity while still able to bind to DNA based on sgRNA specificity. The dCas9 can be utilized as a platform for DNA transcriptional regulators to activate or repress gene expression by fusing the inactive enzyme to known regulatory domains. For example, the binding of dCas9 alone to a target sequence in genomic DNA can interfere with gene transcription.

Additional variants of Cas9 which may be used by some embodiments of the invention include, but are not limited to, CasX and Cpfl (also known as Cas12a). CasX enzymes comprise a distinct family of RNA-guided genome editors which are smaller in size compared to Cas9 and are found in bacteria (which is typically not found in humans), hence, are less likely to provoke the immune system/response in a human. Also, CasX utilizes a different PAM motif compared to Cas9 and therefore can be used to target sequences in which Cas9 PAM motifs are not found.

The CRISPR system may be fused with various effector domains, such as DNA cleavage domains. The DNA cleavage domain can be obtained from any endonuclease or exonuclease. Non-limiting examples of endonucleases from which a DNA cleavage domain can be derived

include, but are not limited to, restriction endonucleases and homing endonucleases (see, for example, New England Biolabs Catalog or Belfort et al. (1997) Nucleic Acids Res.). In exemplary embodiments, the cleavage domain of the CRISPR system is a FokI endonuclease domain or a modified FokI endonuclease domain. In addition, the use of Homing Endonucleases (HE) is another alternative. HEs are small proteins (< 300 amino acids) found in bacteria, archaea, and in unicellular eukaryotes. A distinguishing characteristic of HEs is that they recognize relatively long sequences (14-40 bp) compared to other site-specific endonucleases such as restriction enzymes (4-8 bp). HEs have been historically categorized by small conserved amino acid motifs. At least five such families have been identified: LAGLIDADG; GIY-YIG; HNH; His-Cys Box and PD-(D/E)xK, which are related to EDxHD enzymes and are considered by some as a separate family. At a structural level, the HNH and His-Cys Box share a common fold (designated bba-metal) as do the PD-(D/E)xK and EDxHD enzymes. The catalytic and DNA recognition strategies for each of the families vary and lend themselves to different degrees to engineering for a variety of applications. Exemplary Homing Endonucleases which may be used according to some embodiments of the invention include, without being limited to, I-CreI, I-TevI, I-HmuI, I- PpoI and I-Ssp68031.

Modified versions of CRISPR, e.g. dead CRISPR (dCRISPR-endonuclease), may also be utilized for CRISPR transcription inhibition (CRISPRi) or CRISPR transcription activation (CRISPRa).

Other versions of CRISPR which may be used according to some embodiments of the invention include genome editing using components from CRISPR systems together with other enzymes to directly install point mutations into cellular DNA or RNA.

Thus, according to one embodiment, the editing agent is an DNA editing agent. Preferably, the DNA editing agent comprises a DNA editing system. More preferably, the DNA editing system comprises a meganuclease, a zinc finger nucleases (ZFN), a transcription-activator like effector nuclease (TALEN), homology directed repair (HDR), CRISPR-endonuclease, dCRISPR-endonuclease, or a homing endonuclease.

According to one embodiment, the DNA editing agent does not comprise an endonuclease. According to another embodiment, the DNA editing agent comprises an endonuclease. Suitably, in some embodiments the endonuclease is any one of Cas9, Cas12a, CasX, CasY, CasPhi, MAD7 or Cas13 or functional variants thereof. According to a preferred embodiment, the endonuclease is Cas9. According to another preferred embodiment, the endonuclease comprises a catalytically inactive endonuclease.

According to yet a further embodiment of the invention, the DNA editing agent is linked to a reporter for monitoring expression in a cell, e.g. a eukaryotic cell. The reporter may be a fluorescent reporter protein. The term "a fluorescent protein" refers to a polypeptide that emits fluorescence and is typically detectable by flow cytometry, microscopy or any fluorescent imaging system, therefore can be used as a basis for selection of cells expressing such a protein. Examples of fluorescent proteins that can be used as reporters are, without being limited to, the Green Fluorescent Protein (GFP), the Blue Fluorescent Protein (BFP) and the red fluorescent proteins (e.g. dsRed, mCherry, RFP). A non-limiting list of fluorescent or other reporters includes proteins detectable by luminescence (e.g. lucif erase) or colorimetric assay (e.g. GUS). According to a specific embodiment, the fluorescent reporter is a red fluorescent protein (e.g. dsRed, mCherry, RFP) or GFP.

Cell Therapies

The present invention is applicable to a wide range of therapeutic cells. In some preferred embodiments the cells have reduced immunogenicity, e.g. through context-specific silencing of the MHC-I system. However, silencing of other genes may be relevant in many other contexts to modify the phenotypes of cells (e.g. to promote a pro- or anti-inflammatory phenotype).

Therapeutic cells may suitably be derived from pluripotent stem cell lines, such as an embryonic stem cell line or an induced pluripotent stem cell line. Other therapeutic cells are suitably derived from tissue resident, multipotent stem cells or progenitor cells with distinct developmental origins such as haematopoietic stem cells (HSC, hemangioblast), bone, cartilage and adipose stem cells (mesenchymal, lateral plate), striated muscle stem cells (skeletal muscle (satellite), cardiac muscle, epicardium), smooth muscle stem cells (blood vessel pericyte), hemangioblast endothelial stem cells, neural stem cells, glial progenitors, Müller glia cells, non-myelinating Schwaan cells, hair follicular stem (bulge) cells, liver stem cells, cholangiocytes, basal epithelial stem cells in epidermis (skin), the proximal trachea/bronchioles and gastro-intestinal (GI) tract, alveolar type II cells in the lung, kidney mesenchyme.

These stem/progenitor cells are suitable to differentiate into differentiated cells including, without limitation, a dendritic cell, a lymphocyte, a myeloid cell, a red blood cell, platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta, alpha cell), a liver cell (hepatocytes, cholangiocyte), a skeletal muscle cell (multinucleate myotubes), a keratinocyte, a cardiomyocyte (atrial, ventricular), conduction cardiac cells, a vascular smooth muscle cell, a

neuronal cell, glial cells, a choroid cell of the pia, an ocular (corneal) cell, a photoreceptor cell, a retinal interneurone, retinal ganglion cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell or an adipocyte.

A therapeutic cell is a cell that is suitable to be administered to a subject with the intention or expectation of providing some therapeutic benefit to the subject.

Non-limiting examples of specific cell and tissue therapies of interest in the present invention include, replacement (regeneration, restoration) cornea, retinal cells (retinal ganglion, amacrine, photoreceptors), retinal pigmented epithelium, anti-inflammatory cell therapy for acute and chronic CNS injury and degeneration, neuronal replacement such as inhibitory interneurons, glial replacement for white matter degeneration and multiple sclerosis, neuropathic pain, therapeutic endocrine cells for regenerating function of the pancreas, thyroid, or adrenal glands, therapeutic liver cells for detoxification, replacement (regeneration, restoration) of skeletal and cardiac muscle, and immune cells such as macrophages, (CAR) T cells, regulatory T cells, neutrophils, granulocytes, NK cells, NK/T cells and eosinophils.

Therapeutic cells of the endocrine system, preferably include an enteroendocrine cell, preferably a therapeutic enteroendocrine cell such as those of the pancreas, the thyroid gland, the parathyroid gland, the pituitary gland, the pineal gland, the adrenal gland, the thymus the hypothalamus.

Preferred therapeutic cells for the pancreas are endocrine hormone expressing cell (beta, alpha, delta, epsilon cells, most preferably beta cells.

Preferred therapeutic macrophage cells are those which are anti-tumour and pro-inflammatory as a cancer treatment.
Other preferred therapeutic macrophage cells are those which are anti-inflammatory, pro-regenerative, pro-vascularization and pro-tissue remodelling to treat chronic fibrotic diseases such as idiopathic pulmonary fibrosis, chronic kidney disease, non-alcoholic steatohepatitis (NASH), scleroderma, rheumatoid arthritis, ulcerative colitis and myelofibrosis.

Preferred therapeutic T cells are those which are anti-tumour effector cells for hard-to-treat cancers or memory T-cells, or alternative regulatory T cells as a treatment for auto-immune diseases such as psoriasis, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis and pernicious anaemia.

Compositions

The cells or cell populations of the present invention may be formulated for delivery in a composition. Compositions of the invention suitably comprise an cells or cell populations of the invention. The composition may be provided in the form of a kit.

Thus, in an embodiment of the invention, there is provided a composition comprising a cell or cell population of the invention. Compositions of the invention may also contain other compounds, which may be biologically active or inactive. Suitably, the composition of the invention is a sterile composition suitable for parenteral administration.

While many acceptable carriers known to those of ordinary skill in the art may be employed in the compositions of the invention, the optimal type of carrier used will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, parenteral, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration, preferably parenteral e.g., intramuscular, subcutaneous or intravenous administration. For parenteral administration, the carrier preferably comprises water and may contain buffers for pH control, stabilising agents e.g., surfactants and amino acids and tonicity modifying agents e.g., salts and sugars. If the composition is intended to be provided in lyophilised form for dilution at the point of use, the formulation may contain a lyoprotectant e.g., sugars such as trehalose. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed.

Thus, compositions of the invention may comprise buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the invention may be formulated as a lyophilizate.

The effective amount of the isolated cells of the invention for transplantation or for treating a disease condition depends on a number of factors, such as the type of tissue, the severity of the disease condition, the transplantation reaction, the reason for transplantation, and the age and general health of the patient. The effective amount can be determined by a skilled

researcher or clinician by routine practice. Due to the reduced immunogenicity of the transplanted cells, relative large amount of cells can be tolerated by a patient to achieve the desired therapeutic effects. Alternatively, the cells can be repeatedly transplanted at intervals until a desired therapeutic effect is achieved.

The route for administration of the cells of the invention is not limited in any particular way. Exemplary delivery routes include without limitation intravenous, intramuscular, subdermal, intraperitoneal, transcutaneous, intracutaneous, and subcutaneous route. The cells of the present invention can also be administered topically by injection. For example, the cells can be injected into an injured joint, a fractured bone, an infarct site, an ischemic site or their periphery.

In certain particular embodiments, the cells are administered via a delivery device including without limitation a syringe. For example, the cells can be suspended in a solution or a pharmaceutical composition contained in such a delivery device. The "solution" or "pharmaceutical composition" comprises a physiological compatible buffer and optionally a pharmaceutically acceptable carrier or diluent in which the cells of the invention remain viable. The use of such carriers and diluents is well known in the art. The solution includes without limitation physiologically compatible buffers such as Hank's solution, Ringer' solution, or physiologically buffered saline. The cells can be kept in the solution or pharmaceutical composition for short term storage without losing viability. In certain particular embodiments, the cells are frozen for long term storage without losing viability according to cryopreservation methods well-known in the art.

Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran, but still fluid to the extent that can be easily delivered by syringe injection. The solution is preferably sterile, stable under the conditions of manufacture and storage and is free of microorganism contamination through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. The cells contained in the solution can be stem cells or differentiated cells as described herein, in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients indicated above.

The cells may be administered systemically (e.g., intravenously) or locally (e.g., directly into a myocardial defect under the guidance of echocardiogram, or by direct application to damaged tissues or organs accessible during open surgery). For injections, the cells may be in an injectable liquid suspension preparation or in a biocompatible medium which is injectable in liquid form and becomes semi-solid at the site of damaged tissue. A syringe, a controllable

endoscopic delivery device or other similar devises can be used so long as the needle lumen is of sufficient diameter (e.g. at least 30 gauge or larger) to avoid physical damages to the cells during delivery.

In certain other embodiments, the cells can be transplanted via a solid support, e.g., a planar surface or three-dimensional matrix. The matrix or planar surface is surgically implanted into the appropriate site in a patient. For example, a patient needing a pancreatic graft can have differentiated cells on a solid support surgically implanted in the peritoneum or the portal vein of the liver. Exemplary solid support includes without limitation a patch, a gel matrix (such as GELFOAM® from Pharmacia-Upjohn), polyvinyl alcohol sponge (PVA) collagen gel implants (such as IVALON, Unipoint Industries, High Point, NC) and other similar or equivalent devices. A variety of other encapsulation technologies can be used with the cells of the invention, for example, WO 91/10470; WO 91/10425; U.S. Pat. No. 5,837,234; U.S. Pat. No. 5,01 1 ,472; U.S. Pat. No. 4,892,538).

Aspects of the invention are further demonstrated by the following non-limiting examples.

Examples

Introduction

Advantages of GEiGS

Since its discovery in the 1990s RNAi has been leveraged in various forms to silence desired target genes, mostly using oligos (siRNA) or ectopically expressed hairpins (shRNA). miRNA-based vectors for shRNA have been used extensively – these are mimics of endogenous miRNAs in which the stem sequence has been swapped for the shRNA sequence of choice. Several scaffolds are used, the most popular being miR-30 (Fellmann *et al.*, 2013). miR30-based vectors are more efficient than standard hairpin constructs, being more effective at silencing the target and having a superior off-target profile, and for this reason they are now used in shRNA clinical applications (Esrick *et al.,* 2021).

In its current form miR-based shRNA is still implemented by ectopic expression, usually as a lentiviral transgene integrated in the genome at random or within safe harbour loci such as AAVS1, where they are being transcribed from exogenous regulatory elements. GEiGS on the other hand uses modification of endogenous loci encoding silencing molecules to redirect the silencing specificity of the silencing RNAs encoded in these loci, leveraging the program of

expression of the endogenous loci, which brings very distinct advantages, namely the ability to induce stable, tuneable, programmable and specific gene silencing.

1. **Stability**. siRNA oligos are transient in nature, while shRNA suffers from variegated expression due to transgenes being inactivated through epigenetic mechanisms. By contrast, endogenous loci are never unpredictably silenced, so the GEiGS silencing RNA (sRNA) is stably expressed.

2. **Tunability**. GEiGS allows the redirection of miRNA scaffolds with different levels of expression, therefore the abundance of the GEiGS sRNA can be tuned according to the cell engineering requirements. This is notoriously difficult to achieve through current RNAi approaches and impossible through CRISPR KO which is binary in nature.

3. **Programmability**. Importantly, GEiGS can provide programmable gene silencing. By redirecting miRNAs that are developmentally regulated or are cell state-specific, GEiGS silencing can be programmed to be deployed only when therapeutic cells reach a specific stage in differentiation.

4. **Specificity**. Importantly in ensuring that the expression level of the redirected miRNA scaffold and therefore the abundance of the GEiGS® sRNA are retained at physiological levels in the cells, the normal biogenesis pathway for sRNA generation is not super-saturated with the consequential benefit for far lower occurrences of off-target gene silencing effects. This is starkly contrasted with shRNA and siRNA approaches which notoriously generate artefactual off target gene effects.

The GEiGS technology is deployed through the following workflow (see Fig. 4):

1. Computational design of GEiGS® solutions.
2. Experimental validation of the solutions silencing activity through ectopic expression.
3. Gene editing of the endogenous miRNA scaffold in the desired cell type.

GEiGS solutions validated experimentally can be used in therapeutic applications, for example, either as lentiviral transgenes or edited into the genome to be expressed from an endogenous miRNA locus, to silence for example, B2M in primary human cells. Further information of GEiGS can be found in WO2019/058253, WO2020/183414 and WO2020/183419.

As an alternative to GEiGS, suitable inhibitory RNA constructs can be inserted into the genome, e.g. in a locus which encodes one or more endogenous miRNAs. Such an approach

which can be adapted for the current invention is described, for example, in Senis et al. ('TALEN/CRISPR-mediated engineering of a promoterless anti-viral RNAi hairpin into an endogenous miRNA locus'. Nucleic Acids Research, 2017, Vol. 45, No. 1 e3 doi: 10.1093/nar/gkw805). Accordingly, in some embodiments of the present invention, a promoterless sequence encoding an inhibitory RNA can be inserted into an endogenous miRNA locus, preferably without the disruption or deactivation of any endogenous miRNA.

Conditionally hypoimmunogenic therapeutic cells:

Of particular interest in the present invention are conditionally hypoimmunogenic therapeutic cells. Cellular therapies are a class of advanced medical therapies in which patients are treated through the transplantation of cells that may or may not have undergone prior engineering to enhance their functionality. Cellular therapies such as bone marrow transplantation or blood transfusions are examples of well-known cellular therapy that have existed in medicine for many decades. However, more recently, through the use of genetic engineering techniques, novel cell therapies have been developed and used successfully in many different disease indications such as diabetes, cancer and blindness.

Cell therapies can be autologous, that being therapeutic cells that originate from the patient that is being treated or allogeneic where the cells originate from an unrelated donor. Autologous approaches have the benefit of maximising the likelihood of engraftment post-transplantation but can be expensive to manufacture and may not be applicable to very sick patients that are not capable of donating their cells for preparation or who suffer from terminal illnesses.

In contrast, allogeneic cell therapies can in principle be manufactured more efficiently from healthy donors, reducing cost, and in theory can be administered off-the-shelf and be used to treat acutely ill, recently diagnosed patients. However, allogeneic therapies currently require aggressive immunosuppression to reduce the risk of graft rejection post-transplantation. Even then, administration of immunosuppression does not guarantee the rejection of the graft over a longer period. To become mainstream, allogeneic cell therapies will have to overcome the graft rejection barrier.

Graft rejection is primarily driven by the host adaptive immune system reacting to unmatched Major Histocompatibility Complex I (MHC-I complex) expressed on the surface of transplanted cells. The MHC proteins, also known as human leukocyte antigens, are encoded by a cluster of gene complexes known as the human leukocyte antigen (HLA) system. HLA genes are co-dominantly expressed and are highly polymorphic. The expression of many different alleles is advantageous for the adaptive immune system to defend against cancerous cells or

pathogens such as viruses or bacteria. However, it is this high degree of polymorphism that presents a major barrier to allogenic transplants such as allogenic cell therapies.

MHC-I is present on the surface of virtually all human cells and, as discussed, plays important roles in immunosurveillance (Fig. 1) and in recognition of self from non-self. MHC-I is a dimer of β2-microglobulin and an HLA chain. The HLA chain is highly polymorphic, i.e., individuals express very diverse combinations of the dimer in the population. Consequently, the immune system is trained to selectively tolerate only those MHC-I variants expressed by the individual. In the context of a transplant, unless the donor and host express exactly the same HLA variants, which is highly unlikely unless close blood relatives are involved, the host immune system will recognise the non-self MHC-I variants on the surface of the donor cells and will mount a response (also known as alloimmunity). MHC-I is therefore the main source of immunogenicity in allogeneic transplants.

Numerous approaches have been taken to overcome the graft rejection barrier. Traditionally, graft recipients receive life-long immunosuppression, which is not only not always effective but also leaves the recipient vulnerable to serious illness such as infectious disease or cancer. Genetic engineering approaches have also been considered. One such approach is to generate hypoimmunogenic cells by completely knocking out (KO) MHC-I expression (see, e.g., WO2012/145384). While this strategy successfully evades reaction from the host adaptive immunity, it exposes the graft to rejection by cells of the innate immune system through the activity of Natural Killer (NK) cells. NK cells play an important role in detection of aberrant cells and react strongly against cells that lack MHC-I expression, as this is sometimes a hallmark of malignant cells. The complete absence of MHC-I is also referred to as the “missing self” phenotype, and these cells are effectively detected and lysed by NK cells. At the mechanistic level, this is triggered because MHC-I molecules function as inhibitory ligands for NK cells. MHC-I KO cells need additional engineering to stably express tolerising ligands to avoid killing by innate immune cells (Fig. 2).

The present invention aims to address this problem by creating conditionally hypoimmunogenic cells through context-specific partial silencing of MHC-I. As discussed, the MHC-I complex is a heterodimer made up of a hypervariable HLA protein and beta-2 microglobulin (B2M). By partially knocking down B2M expression, there is partial silencing of MHC-I which can allow transplanted cells to evade both the innate (NK-mediated) and adaptive (T-cell mediated) immune system driven rejection.

One approach to generate conditionally hypoimmunogenic cells is to use inhibitory RNAs, e.g. *via* the Gene Editing induced Gene Silencing (GEiGS) system, to modulate the expression of

MHC-I down to a level that prevents a significant adaptive immune response, while still being sufficient to inhibit the NK cell mediated response.

The potential of partial MHC-I silencing was exemplified in an *in vivo* study in which hypoimmunogenic islet cells were generated by siRNA-mediated MHC-I knock-down and subsequently transplanted in NOD mice, showing improved graft survival (Wang et al., 2012, Diabetes. 2012 Dec;61(12):3247-54). While this study shows the efficiency of RNAi-mediated gene silencing to partially silence MHC-I, the transient nature of siRNA means the approach would not be feasible for a clinical application. On the other hand, GEiGS for example, would be perfectly suitable for this approach because it has the potential to provide stable and tuneable silencing.

However, a risk of hypoimmunogenic therapeutic cells is that, because they are well-adapted to evade the immune system, should they, for example, migrate from their desired location and/or de-differentiate they would not be targeted by the immune system. This is a particular concern where therapeutic cells have the potential to de-differentiate, e.g. to revert to an earlier stage of differentiation where they may be more likely to proliferate and or migrate away from their intended site.

Accordingly, it would be advantageous if hypoimmunogenicity were made conditional upon the cells being in their desired context, i.e. in their desired level of differentiation and/or present in their intended location.

Accordingly, in some embodiments the cell is a conditionally hypoimmunogenic cell, typically a hypoimmunogenic therapeutic cell.

In some embodiments the inhibitory RNA which targets B2M (e.g. comprising a sequence according to any one of SEQ ID NOs: 1-17) and is selectively active in a therapeutic cell in the desired context.

For example, the inhibitory RNA which targets B2M is expressed at a level of at least 1000 RPM in the cell, at least 2000 RPM (reads per million mapped reads) in the cell, at least 3000 RPM in the cell, at least 4000 RPM in the cell, at least 5000 RPM in the cell, at least 10000 RPM in the cell, at least 20000 RPM in the cell, or at least 50000 RPM in the cell, or at least 100000 RPM in the cell in the desired context. Preferably the inhibitory RNA is expressed at a level of at least 50000 RPM or at least 100000 RPM in the cell in the desired context.

For pancreatic tissues, for example, hsa-miR-375, hsa-miR-143, and hsa-miR-21 are expressed at particularly high levels (189993.6, 186579.2, and 65231.3 RPM, respectively

according to the miRmine database) with a range of other miRNAs also expressed at substantial but more moderate levels (e.g. hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, hsa-miR-200c, etc.).

In some embodiments the inhibitory RNA is a hsa-miR-375, hsa-miR-143 or hsa-miR-21 which has been modified to target B2M. In some embodiments the inhibitory RNA is a hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, hsa-miR-200c which has been modified to target B2M.

In one exemplary embodiment of the invention, the target gene is B2M. In some embodiments of the invention, the expression of B2M in the cell in the first context is modified in a range of 1%-90%, optionally 5%-90%, optionally 10%-90%, when compared to a control cell.

The expression of B2M may be modified, degraded, reduced or silenced according to the present invention. In one embodiment of any aspect of the invention, the expression of the B2M is modified within a range of 1%-90%, optionally 5%-90%, optionally 10%-90%. Suitably, for example, the expression of the protein is within a range of 1%-80%, 1%-70%, 1%-60%, 1%-50%, 1%-40%, 1%-30%, 1%-20%, 1%-10%, 1%-5%, 5%-80%, 5%-70%, 5%-60%, 5%-50%, 5%-40%, 5%-30%, 5%-20%, 5%-10%, 10%-80%, 10%-70%, 10%-60%, 10%-50%, 10%-40%, 10%-30%, 10%-20%, 20%-90%, 30%-90%, 40%-90%, 50%-90%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% compared to a control cell. It is an advantage of the present invention that the skilled person can modulate, typically reduce, expression of a protein by a desired amount, e.g. to a suitable level compared to a control cell. In some embodiments the expression level of B2M or any other cell surface protein in a cell is determined by the amount of B2M or other protein expressed on the surface of cell. The amount of B2M present on the surface of cell can be determined by various conventional techniques, e.g. by flow cytometry.

In some embodiments of the invention, the inhibitory RNA is inactive or is less active when the pancreatic endocrine hormone expressing cells are altered as mentioned and as a result are no longer able to silence the expression of B2M or HLA-A/B/C and become immunogenic.

Context-specific gene silencing in immune cells:

As discussed above, in some embodiments of the invention the inhibitory RNA is specifically active in immune cells, e.g. in specific types of immune cells or in immune cells in a particular state of activation or polarisation.

By way of example, the solid tumour microenvironment (TME) poses particular challenges to overcome for developers of CAR-T and other engineered immune effector cell therapies. Solid tumours are made of a variety of other cells in addition to cancer cells themselves, such as stroma (cancer associated fibroblasts (CAFs)), and immune cells such as macrophages, T cells (helper, cytotoxic, regulatory). Solid tumours create a microenvironment allowing them to recruit, control and subdue immune effector cells into maintaining immune tolerance and promoting tumour growth. Evidence indicates that the TME can subvert the activity of tumour infiltrating T cells, even CAR-T cells engineered to be highly active against tumours expressing specific antigens (Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. Blood Cancer J. 2021 Apr 6;11(4):69. doi: 10.1038/s41408-021-00459-7. PMID: 33824268; PMCID: PMC8024391). Tumour cells use cytokines, immune checkpoint signals and metabolic intermediates as signals to maintain tolerance. Metabolic intermediates are potent signals for cellular differentiation in immune cells, for example tumour cell generated lactate (Colegio OR, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, Cyrus N, Brokowski CE, Eisenbarth SC, Phillips GM, Cline GW, Phillips AJ, Medzhitov R. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. Nature. 2014 Sep 25;513(7519):559-63. doi: 10.1038/nature13490. Epub 2014 Jul 13. PMID: 25043024; PMCID: PMC4301845) has been demonstrated to polarise macrophages to an immunosuppressive state – tumour associated macrophages (TAM) - through a mechanism dependent on HIF1a and this can play an important role in tumour growth. CAFs and the extracellular matrix produced in the TME of high-grade cancers also contribute to the generation of immunoregulatory phenotypes supportive of tumour growth (Puttock et al,. 2022. bioRxiv. doi.org/10.1101/2022.08.11.503568). Extracellular matrix (ECM) remodelling accompanies invasive carcinomas, the establishment of an immunosuppressive environment and poor clinical response to immunotherapy (Chakravarthy et al 2018. Nature Comms. 9, 4692, doi:10.1038/s41467-018-06654-8). These ECM compositions positively correlate with tumour associated macrophage (TAM) infiltration.

TAMs are a key cell type in the TME and solid tumours have a high content. This is an alternatively activated (M2-like) state, and is correlated with poor prognosis in several malignancies, including ovarian cancer (Zhang et al. 2014. J Ovarian Res 7, 19, doi:10.1186/1757-2215-7-19). TAMs are widely considered the key cell type in the establishment and maintenance of an immune tolerant, TME. They are currently the target of several anti-cancer therapies (PI3K-gamma inhibitor Eganelisib, Infinity Pharm. https://www.infi.com/home/our-development-program/ipi-549/). TAMs are key in creating the immunosuppressive TME and they do this via the production of cytokines, chemokines, growth

factors, and trigger the inhibitory immune checkpoint proteins in T cells. There are multiple pathways in the TME contributing to macrophage polarization into TAMs.

Therapeutic CAR-T cells are subject of inhibition in the TME via similar pathways. TILs and CAR-Ts are tolerised through the activity of immune checkpoint pathway, such as PD-1 (PDCD-1), CTLA4, LAG3, TIM3.

Different states of immune cells are associated with different metabolic programs, because of their distinct energetic needs and it is possible to influence the fate of immune cells by altering their cellular metabolism (Chang CH, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, Huang SC, van der Windt GJ, Blagih J, Qiu J, Weber JD, Pearce EJ, Jones RG, Pearce EL. Cell. 2013 Jun 6;153(6):1239-51. doi: 10.1016/j.cell.2013.05.016.). Controlling the cell metabolism through mitochondrial architecture was shown to influence cell fate (Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q, Huang SC, O'Neill CM, Edelson BT, Pearce EJ, Sesaki H, Huber TB, Rambold AS, Pearce EL. Cell. 2016 Jun 30;166(1):63-76. doi: 10.1016/j.cell.2016.05.035. Epub 2016 Jun 9).

Promoting mitochondrial fusion increases oxidative phosphorylation and fatty-acid oxidation in activated anti-tumour T cells, favouring memory T cell formation, which improves persistence of anti-tumour activity. Inhibition of DRP1 gene favours mitochondrial fusion (blocking their fission) and leads to an increase in oxidative phosphorylation and fatty-acid oxidation.

Other metabolic targets regulating T cell fate include the mammalian target of rapamycin (mTORC1). Pharmacological inhibition of mTORC1 increased fatty-acid oxidation and memory T cell formation, whereas a complete knock-out of this gene is not desirable as it decreases both memory and effector T cell formation.

It is unlikely antagonism of a single pathway, or combinatorial inhibition of disease pathways in solid cancer will prevent TAM formation. Macrophage polarisation to TAMs is dependent on miRNAs, as macrophage-specific deletion of DICER blocks this process completely (Baer C Squadrito ML, Laoui D, Thompson D, Hansen SK, Kiialainen A, Hoves S, Ries CH, Ooi CH, De Palma M. Suppression of microRNA activity amplifies IFN-γ- induced macrophage activation and promotes anti-tumour immunity. Nat Cell Biol. 2016 Jul;18(7):790-802. doi: 10.1038/ncb3371) and T cell activation induces expression of specific miRNAs. The inducible and conditional type of target silencing in GEiGS modified cells makes possible combinatorial cell immunotherapies.

Modified (e.g. GEiGS-modified) macrophages or T/CAR-T cells can leverage changes in miRNA expression that are induced in response to the TME. TME-responsive (e.g. GEiGS-modified) macrophages expressing suitable inhibitory miRNAs can promote a pro-inflammatory programme inside the TME, facilitating activation of tumour infiltrating lymphocytes (TILs) and other T cells (CAR-Ts). miRNA loci normally induced or upregulated in or by the TME are redirected to silence targets thereby preventing or reversing the TAM state and promoting the pro-inflammatory phenotype. Maintenance of the pro-inflammatory polarized state would be conditional upon exposure to TME cues. Should the cells escape the solid TME the miRNAs driving the pro-inflammatory phenotype would be downregulated and system-wide, immune-related off-tumour toxicities (e.g. cytokine release syndrome) would be avoided. Other approaches use constitutively induced pro-inflammatory macrophage polarization but lack control to prevent inadvertent off tumour effects.

Therapeutically it would be beneficial to also generate T/CAR-T cells that are also TME-responsive. Modified (e.g. GEiGS-modified) T-cells would detect the presence of the TME signals and silence, for example, PD-1 (PDCD-1), CTLA4, LAG3 or TIM3, blocking the activity of these immune checkpoint pathways. Other approaches are based on constitutive silencing of these checkpoints but this could lead to severe immune-related adverse effects in patients (cytokine release syndrome). Futhermore, conditional (e.g. GEiGS-mediated) block of mitochondrial fission in the TME by redirecting induced miRNAs to conditionally silence DRP1 favours the differentiation and persistence of memory T cells, while allowing mitochondrial dynamics to continue and contribute to mitochondrial homeostasis (such as mitophagy).

Modification of such cells as described herein (e.g. GEiGS-modification) would allow to specifically silence these pathways only when immune cells (e.g. T cells or macrophages) reach the tumour.

These are merely some of the potential applications of the invention described herein, and the skilled person could apply this approach in many other cell types and contexts.

Example 1 – Computational design of GEiGS solutions

B2M solutions were generated using a computational pipeline. Pipeline inputs for GEiGS targeting B2M are: the mRNA sequence of B2M (GenBank Accession: NM_004048.4), the human genome reference sequence (GRCh38), miRNA annotations (miRbase v22) and cell type specific miRNA quantifications.

Expression of miRNAs in human iPSCs and ESCs was quantified using publicly available next-generation sequencing (NGS) datasets: ENCODE accession ENCSR958UOC, ENCSR430YFL.

The computational pipeline run generated a short-list of 50 GEiGS® solutions expected to be able to silence B2M. The GEiGS® solution designs were comprised of a modified sequence of an endogenous pre-miRNA (hairpin) - also referred to as the endogenous miRNA scaffold - modified to encode a novel silencing RNAi trigger instead of its native guide strand. The novel GEiGS® silencing RNA matched the B2M mRNA by sequence complementarity and was predicted to lead to the silencing of B2M through the RNA interference pathway.

The solution design process also generated corresponding negative control constructs - also referred to as 'dummy' solutions - in which the same endogenous miRNA scaffold was modified as in the corresponding GEiGS® solution but to express a scrambled sequence of the GEiGS® silencing RNA.

Example 2 – Validation of GEiGS solution silencing activity through plasmid-based ectopic expression

19 designs (17 B2M GEiGS solutions and 2 'dummy' controls) were used to generate extended GEiGS solutions. Extended solutions were comprised of a GEiGS solution as designed using the computational pipeline, plus 50-150 bases of genomic sequence flanking on both sides of the endogenous pre-miRNA that the respective GEiGS solution was based on. This was included to avoid omitting important sequences that act as a signal for the enzyme Drosha.

The solutions tested were as follows:

Table 4. Sequences of computationally-designed B2M-targeting RNAi trigger sequences and GEiGS solutions

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
Sol-8	hsa-mir-191	TTCAGTGTAGTACAAGAGATAGA [1]	CGGCTGGACAGCGGGttcagtgtagtacaagagatagaTTGTCTCCAGAGCATTCTCTATCTGCTGACTACACTGCCCCTGCTCTCCTGCCT [18]
Sol-11	hsa-mir-302a	TTTCAATTCTCTCTCCATTCTTC [2]	CCACAAGATTGGAGAGAGTATTGAAACTTTGAAACTAAAGAAGtttcaattctctctccattcttcTGG [19]

Sol-12	hsa-mir-302c	TTCAGTAAGTCAACTTCAATGT [3]	CCTACATTTTAGTTGACTTACTGCAGCTGTGTGAAACAAAAGTttcagtaagtcaacttcaatgtAGG [20]
Sol-19	hsa-mir-93	TTCAGTGTAGTACAAGAGATAGA [4]	CTGGGGGCTCttcagtgtagtacaagagatagaTGTGATTACCCAACTCTTCTCTGTACTTACACTGCCCGAGCCCCCGG [21]
Sol-20	hsa-mir-106a	TAAATTTTCCCCCAAATTCTAAG [5]	CCTTGGCCATGTtaaattttcccccaaattctaagCTTTTTGAGATCTTGAATTAGGGGGAAAATTCTAACATTACCATGG [22]
Sol-21	hsa-mir-106b	TATATGACAAAATGTTTCATTC [6]	CCTGCCGGGGCtatatgacaaaatgtttcattcGTGGTCCTCTCCGTGCGACCAAACATTGTGTCATAGCTGCTCCAGCAGG [23]
Sol-26	hsa-mir-191	TACTTTATCAAATGTATAAGAAG [7]	CGGCTGGACAGCGGGtactttatcaaatgtataagaagTTGTCTCCAGAGCATTCCTTCTTAGCCATTGATAAAGCCCCTGCTCTCCTGCCT [24]
Sol-27	hsa-mir-200c	TAGAATTATAAAGAAGATCATGT [8]	CCCACTGACTTCTTAATAATTCTTGGGTGCGGTTGGGAGTCTCtagaattataaagaagatcatgtGG [25]
Sol-29	hsa-mir-20a	TAACAAATTTCCAATAATCCTGT [9]	GTAGCACtaacaaatttccaataatcctgtTGTTTAGTTATACAGATTATTGGAAATTTGTAAAGTACTGC [26]
Sol-30	hsa-mir-21	TGTTAACATTATTATAACCCTAC [10]	TGTCGGGtgttaacattattataaccctacTGTTGAATCTCATGGAGGGTCATAATATGTTAACATCTGACA [27]
Sol-35	hsa-mir-302a	TTATATTTCTAAATTTTCCCCCA [11]	CCACGGGGTAAAATTTAGTAATATAACTTTGAAACTAAAGAAGttatatttctaaattttcccccaTGG [28]
Sol-36	hsa-mir-302c	TAACATTATTATAACCCTACAT [12]	CCTATGTATTGTTATAATAATGTCAGCTGTGTGAAACAAAAGTtaacattattataaccctacatAGG [29]
Sol-41	hsa-mir-363	TATTATAACCCTACATTTTGTG [13]	TGTTGTCAAAATGTACAGTATATAATATTGATGAGTATCATAGGAGAAAtattataacccctacattttgtgAACC [30]
Sol-43	hsa-mir-518b	TTAACTTATGCACGCTTAACTA [14]	TCATGCTGTGGCAGTTCAGCGTGAATAAGTTTCAATTGTCTGAAAGAAAAttaacttatgcacgcttaactaTTACGGTTTGA [31]
Sol-47	hsa-mir-744	TAACTATCTTAACAAGCTTTGA [15]	TTGGGCAAGGtaactatcttaacaagctttgaGTCTTACTGAAGGTTTCCTGGAAACCACGCACATCAAAGCTGTATAAAATAGAACCTTACTCGGTC [32]
Sol-49	hsa-mir-93	TTACTTTATCAAATGTATAAGAA [16]	CTGGGGGCTCttactttatcaaatgtataagaaTGTGATTACCCAACTTCTATAATTTGTATAAAGTCCCGAGCCCCCGG [33]
Sol-50	hsa-mir-99b	TTTATCAAATGTATAAGAAGTA [17]	GGCACCtttatcaaatgtataagaagtaGGGCCTTCGCTACTCACATCCTATTGATTTGATAACCGTGTC [34]

Neg Ctrl 1	hsa-mir-302a	TTAACTAAACAACAACAAACAG [52]	CCAACCCTttaactaaacaacaacaaacagGAAACAAAATTGTTTGTTGTGGTTTAGTTGTGGGGATGG [53]
Neg Ctrl 2	hsa-mir-200c	TTTACGTCGATAAATCAGGACAC [54]	CCCGTTCCGATTTAACGACGTAATGGGTGCGGTTGGGAGTCTCtttacgtcgataaatcaggacacGG [55]

The sequences of the extended solutions were as follows [SEQ ID NO: #]:

>hsa-mir-302a_11_B2M-exon2-2

GCCAGCACACCTTTTGTTACAAAATTTTTTTGTTATTGTGTTTTAAGGTTACTAAGCTTGT
TACAGGTTAAAGGATTCTAACTTTTTCCAAGACTGGGCTCCCCACAAGATTGGAGAGAG
TATTGAAACTTTGAAACTAAAGAAGTTTCAATTCTCTCTCCATTCTTCTGGTAAGTCTTCC
TTTTACATTTTTATTATTTTTTTTAGAAAATAACTTTATTGTATTGACCGCAGCTCATATATT
TAAGCTTTATTTTGTATTTTTACATCTGTTA [35]

>hsa-mir-302a_35_B2M-exon4

GCCAGCACACCTTTTGTTACAAAATTTTTTTGTTATTGTGTTTTAAGGTTACTAAGCTTGT
TACAGGTTAAAGGATTCTAACTTTTTCCAAGACTGGGCTCCCCACGGGGTAAAATTTAG
TAATATAACTTTGAAACTAAAGAAGTTATATTTCTAAATTTTCCCCCATGGTAAGTCTTCC
TTTTACATTTTTATTATTTTTTTTAGAAAATAACTTTATTGTATTGACCGCAGCTCATATATT
TAAGCTTTATTTTGTATTTTTACATCTGTTA [36]

>hsa-mir-302c_12_B2M-exon2-2

TTTACTTCTCCAAAATAGAACACGCTAACCTCATTTGAAGGGATCCCCTACATTTTAGTT
GACTTACTGCAGCTGTGTGAAACAAAAGTTTCAGTAAGTCAACTTCAATGTAGGTGTCT
CCAAGCCAGCACACCTTTTGTTACAAAATTTTTTTGTTATTGTGTTTTAAGGTTACTAAGC
TTGTTACAGGTTAAAGGATTCTAACTTTTTCCAAGA [37]

>hsa-mir-302c_36_B2M-exon4

TTTACTTCTCCAAAATAGAACACGCTAACCTCATTTGAAGGGATCCCCTATGTATTGTTA
TAATAATGTCAGCTGTGTGAAACAAAAGTTAACATTATTATAACCCTACATAGGTGTCTC
CAAGCCAGCACACCTTTTGTTACAAAATTTTTTTGTTATTGTGTTTTAAGGTTACTAAGCT
TGTTACAGGTTAAAGGATTCTAACTTTTTCCAAGA [38]

>hsa-mir-20a_29_B2M-exon4

GAATGATTTTTACTAATTTTGTGTACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTAT
CTGATGTGACAGCTTCTGTAGCACTAACAAATTTCCAATAATCCTGTTGTTTAGTTATAC
AGATTATTGGAAATTTGTAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCCTGTCGC
CCAATCAAACTGTCCTGTTA [39]

>hsa-mir-200c_27_B2M-exon4

AGCAGGGCTCACCAGGAAGTGTCCCCAGGGACTCGGGTGGTGGGGGGATGGGAGCC
AGGGATCTGCAGCTTTTCCGCAGGGATCCTGGGCCTGAAGCTGCCTGACCCAAGGTG
GGCGGGCTGGGCGGGGGCCCACTGACTTCTTAATAATTCTTGGGTGCGGTTGGGAGT
CTCTAGAATTATAAAGAAGATCATGTGGCCCCTGTCCCTGTGTCAGCAACATCCATCGC
CTCAGGTCCCCAGCCCTTAGCTGGCTGCAGCCCCCTCCCCACTTCCCACGCACCCCG
GAAGCCCCTCGTCTTGAGCTGAGAGCGTTGCACAAGGGGTGG [40]

>hsa-mir-21_30_B2M-exon4

GTTCGATCTTAACAGGCCAGAAATGCCTGGGTTTTTTTGGTTTGTTTTTGTTTTTGTTTTT
TTATCAAATCCTGCCTGACTGTCTGCTTGTTTTGCCTACCATCGTGACATCTCCATGGCT
GTACCACCTTGTCGGGTGTTAACATTATTATAACCCTACTGTTGAATCTCATGGAGGGT
CATAATATGTTAACATCTGACATTTTGGTATCTTTCATCTGACCATCCATATCCAATGTTC
TCATTTAAACATTACCCAGCATCATTGTTTATAATCAGAAACTCTGGTCCTTCTGTCTGG
TGGCACTTAGAGTCTTTTGTGCCATAATGCA [41]

>hsa-mir-363_41_B2M-exon4

TATCGTCTTATTTTAACTTTTAAAAGCCGTAAGTTCTGATATTTAGTCATTGTAAAATGAT
CTGTTTTGCTGTTGTCAAAATGTACAGTATATAATATTGATGAGTATCATAGGAGAAATAT
TATAACCCTACATTTTGTGAACCGCAGGACCTTTGTTGGCGACATTCCTGATCAGCGCT
ACAGTAAGCTAGATGGTAAAAAATCCTTTCTCTAGTTGCTGCTATTAAAAAATAATTTGAA
ATATATTTGGATTTCTAAAAACTATGATAGCTA [42]

>hsa-mir-93_19_B2M-exon2-2

AGGCCTTTTCCCCACTTCTTAACCTTCACTGAGAGGGTGGTTGGGGTCTGTTTCACTCC
ATGTGTCCTAGATCCTGTGCTACAGACCTTCCTTTCTGTCCTCCCGTCTTGGACCTCAG
TCCTGGGGGCTCTTCAGTGTAGTACAAGAGATAGATGTGATTACCCAACTCTTCTCTGT
ACTTACACTGCCCGAGCCCCCGGGACACGTTCTCTCTGCCAATTGTCTTCTTGGCTGA
GCTCCCCAAGCTCCATCTGTCATGCTGGGGAGCCCAGTGGCGTTCAAAAGGGTCTGGT
[43]

>hsa-mir-93_49_B2M-exon4

AGGCCTTTTCCCCACTTCTTAACCTTCACTGAGAGGGTGGTTGGGGTCTGTTTCACTCC
ATGTGTCCTAGATCCTGTGCTACAGACCTTCCTTTCTGTCCTCCCGTCTTGGACCTCAG
TCCTGGGGGCTCTTACTTTATCAAATGTATAAGAATGTGATTACCCAACTTCTATAATTT
GTATAAAGTCCCGAGCCCCCGGGACACGTTCTCTCTGCCAATTGTCTTCTTGGCTGAG
CTCCCCAAGCTCCATCTGTCATGCTGGGGAGCCCAGTGGCGTTCAAAAGGGTCTGGT
[44]

>hsa-mir-106a_20_B2M-exon4

TTTACTTGTTTAGTTTCTAATATGTGTGTTTGTTTTGTTGTTTTAACCAGGTGAGTCTG
CATGGATCTGTGAGGACGGAAAAGAAGAGCTCCTGGAAGACTTAAAATTTTGCTACAGG
AATAGGCCTTGGCCATGTTAAATTTTCCCCCAAATTCTAAGCTTTTTGAGATCTTGAATT
AGGGGGAAAATTCTAACATTACCATGGTGATTTAGTCAATGGCTACTGAGAACTGTAGT
TTGTGCATAATTAAGTAGTTGATGCTTTTGAGCTGCTTCTTATAATGT [45]

>hsa-mir-106b_21_B2M-exon4

TGCTGGCTATCCTGCGCCTTTCCACTGCTCTGGTAAGTGCCCAAATTGCTGGAGGGCC
ATCTGTTTTGACCCTTAAAGGGGTAGCTCCTTACCGTGCTCTCATTGCCGCCTCCCCAC
CTCCCGCTCCAGCCCTGCCGGGGCTATATGACAAAATGTTTCATTCGTGGTCCTCTCC
GTGCGACCAAACATTGTGTCATAGCTGCTCCAGCAGGGCACGCACAGCGTCCGTGGA
GGGAAAGGCCTTTTCCCCACTTCTTAACCTTCACTGAGAGGGTGGTTGGGGTCTGTTTC
ACTCCATGTGTCCTAGATCCTGTGCTACAGACCTTCCTTTCTGTCCTCCCG [46]

>hsa-mir-191_8_B2M-exon2-2

GTCTTGTTCCCTCTAGACTCCGTTTCACAACCTACTCCCGGGTCTTCCTCCTGGGACTG
GGGATGGGCGGGTTCTGATGTGGCCCCAGGGCGAGTGACCTGGGGGCAGGAGCTCC
CCCGCCCCCCGCCAACGGCTGGACAGCGGGTTCAGTGTAGTACAAGAGATAGATTGTC
TCCAGAGCATTCTCTATCTGCTGACTACACTGCCCCTGCTCTCCTGCCTGAGCAGCGC
CCTGGCCCAGATGGGGTGCCCCTGACCCCCAGACATACTTTACTGAGCTGCTTGGGTC
TCAGTTCCTCTCAGTTGCGCCCTCAGGCTGGAGGTGATGGGTGTAGACGTGGGAGAG
CCGAGG [47]

>hsa-mir-191_26_B2M-exon4

GTCTTGTTCCCTCTAGACTCCGTTTCACAACCTACTCCCGGGTCTTCCTCCTGGGACTG
GGGATGGGCGGGTTCTGATGTGGCCCCAGGGCGAGTGACCTGGGGGCAGGAGCTCC
CCCGCCCCCCGCCAACGGCTGGACAGCGGGTACTTTATCAAATGTATAAGAAGTTGTC
TCCAGAGCATTCCTTCTTAGCCATTGATAAAGCCCCTGCTCTCCTGCCTGAGCAGCGCC

CTGGCCCAGATGGGGTGCCCCTGACCCCCAGACATACTTTACTGAGCTGCTTGGGTCT
CAGTTCCTCTCAGTTGCGCCCTCAGGCTGGAGGTGATGGGTGTAGACGTGGGAGAGC
CGAGG [48]

>hsa-mir-744_47_B2M-exon4

CATTAACATGTTTTAAACTTCAGGCCCTTCTACTGCCAAGGTGAGTTCAGGCTGGGCGG
CTGCACCCCTGGGAGCAGGGCAGTGCTGCACTGAGCCAGGCGGGAGCTGGAAGAAG
ACGCAGCACACTGGGTTGGGCAAGGTAACTATCTTAACAAGCTTTGAGTCTTACTGAAG
GTTTCCTGGAAACCACGCACATCAAAGCTGTATAAAATAGAACCTTACTCGGTCCTGAC
CGGCTCGGCTTCTGTTTGTTTATTTCATCTCTACTCAGTACTGCCCTGTTCCCTGGTTTT
AAAGTTGTACTGAAATGCATACCTTGTGATAATGTTGTCACATTTTGTCCTTATTGTGTC
GTGCC [49]

>hsa-mir-518b_43_B2M-exon4

TTTATGTTCTGGATTCCAGAAAACATGCAAACAGGGCAAATAAATGCATCTTTATTTTGT
GTCCATTTTAACCTGGTCAAGGAAAATTCCAACAGCAACATCAAAAAACCAGTGTTGGA
GCAAGAATATGTCATGCTGTGGCAGTTCAGCGTGAATAAGTTTCAATTGTCTGAAAGAA
AATTAACTTATGCACGCTTAACTATTACGGTTTGAGTAAAGCAGCGTTGAAGTTGATGCT
GATCTTGGTAATACATTTGCAGAGCGTGCTTATCATCAGACGTGGACGATGGTGGGGTT
CTGTTTTGGTTTTGTTTTTTTCTAAGACAGGGTCTCCGTTGCCCAG [50]

>hsa-mir-99b_50_B2M-exon4

CTTCTGGGTTCTTTAGGGAGGAGGGGGATGAGAGCCTTGACTCCAGGGTCCCTGATGA
GGAAGGGGCTGAGGGCCTGGACTCCTGGGTTCCTTGGGGAGGAGGGGCCGGGGGCC
CGGACTCCTGGGTCCTGGCACCTTTATCAAATGTATAAGAAGTAGGGCCTTCGCTACTC
ACATCCTATTGATTTGATAACCGTGTCGGGGGCTCACCATCGCGGCTGGGGCCTCCCC
GGCCCTCCCCCTCATCCCTGGTCCTCCTGGTCCCTGTCTGTCTGTCTGTCGGGTCTGT
CCACC [51]

>hsa-mir-302a_23_None (negative control)

GCCAGCACACCTTTTGTTACAAAATTTTTTTGTTATTGTGTTTTAAGGTTACTAAGCTTGT
TACAGGTTAAAGGATTCTAACTTTTTCCAAGACTGGGCTCCCCAACCCTTTAACTAAACA
ACAACAAACAGGAAACAAAATTGTTTGTTGTGGTTTAGTTGTGGGGATGGTAAGTCTTC
CTTTTACATTTTTATTATTTTTTTTAGAAAATAACTTTATTGTATTGACCGCAGCTCATATAT
TTAAGCTTTATTTTGTATTTTTACATCTGTTA [56]

>hsa-mir-200c_13_None (negative control)

AGCAGGGCTCACCAGGAAGTGTCCCCAGGGACTCGGGTGGTGGGGGGATGGGAGCC
AGGGATCTGCAGCTTTTCCGCAGGGATCCTGGGCCTGAAGCTGCCTGACCCAAGGTG
GGCGGGCTGGGCGGGGGCCCGTTCCGATTTAACGACGTAATGGGTGCGGTTGGGAGT
CTCTTTACGTCGATAAATCAGGACACGGCCCCTGTCCCTGTGTCAGCAACATCCATCGC
CTCAGGTCCCCAGCCCTTAGCTGGCTGCAGCCCCCTCCCCACTTCCCACGCACCCCG
GAAGCCCCTCGTCTTGAGCTGAGAGCGTTGCACAAGGGGTGG [57]

In addition to the negative control constructs listed in Table 4 above, an additional miR30-based construct was purchased from Vector Builder (Neg Ctrl 0).

The extended solutions were synthesised and cloned into a bespoke expression plasmid (plasmid VB210602-1567ytv, see Figure 5). The plasmid is a mammalian expression system in which the EF1 alpha (EF1a) promoter drives transcription of the extended GEiGS solution. On the same plasmid DsRed Express 2 (DsRed) cDNA is expressed from the human PGK (hPGK) promoter. Upon transfection into mammalian/human cells, both the GEiGS solution and DsRed cDNA were transcribed. DsRed can be used as a transfection marker, indicating the successfully transfected cells. The level of DsRed expression in the cell (fluorescence intensity) can be assayed by flow cytometry and can be used as a proxy for the expression level/abundance of the GEiGS solution.

The cloned solutions were individually transfected into the human iPSCs line WTC-11 (Allen Institute of Cell Science) by nucleofection (Lonza). The expression of the GEiGS sRNA within the transfected cells from the vector was measured by qPCR and compared to the expression of the corresponding endogenous miRNA scaffold. This showed that the GEiGS sRNA was expressed at comparable levels to the endogenous miRNA scaffold (Figure 6), indicating that expression from the plasmid is not supra-physiological, therefore unlikely to cause significant off-target effects or interfere with the normal functions of the RNAi pathway in the cell.

To assess the effectiveness of solutions in silencing B2M, transfected cells were collected 72 hours post-transfection, stained for surface B2M expression with an APC-coupled antibody, and analysed by flow cytometry. If the GEiGS solution design is effective, transcription of the extended GEiGS solution should lead to its successful sequential post-transcriptional processing by DROSHA and DICER, expression of the mature GEiGS silencing RNA, incorporation into RISC and ultimately B2M mRNA degradation. Validation of the efficiency of post-transcriptional processing of the extended GEiGS solution, the efficiency of incorporation into RISC, and the potential of the GEiGS sRNA to trigger B2M mRNA degradation was assessed.

By using flow cytometry to concomitantly measure the expression of DsRed (transfection and solution expression reporter) and B2M (GEiGS solution target) we were able to generate dose-dependent curves showing B2M residual expression vs solution abundance in the cell (Figure 7). As expected, for negative control constructs the level of B2M did not change irrespective of the level of expression of the GEiGS solution. For effective solutions, B2M levels decreased in direct correlation with the levels of the solution expression in the cell. In other words, cells in which transfection led to higher levels of DsRed/solution expression showed reduced level of B2M.

The efficiency of the GEiGS solutions was quantified through the flow cytometry gating shown in Figure 7. Debris were gated out using the FSC-A and SSC-A flow cytometry channels, and subsequent doublets were gated out using the FSC-A and FSC-H channels. Filtered data was subsequently plotted as B2M vs DsRed. The quantification was performed in two different ways:

Placing a gate on all the cells expressing DsRed (when compared to an untransfected control), and then extracting a mean value for the B2M fluorescence intensity in this subpopulation.

Placing a more selective gate solely on the cells expressing DsRed at high level (top 20%), and subsequently calculating the mean fluorescence intensity for B2M in this subpopulation.

Both approaches were in agreement and allowed comparison of the effectiveness of the different B2M-targeting GEiGS solutions. Between the 17 solutions tested experimentally, a broad spectrum of B2M silencing was achieved, ranging from 10%-90% (Figure 8). The most effective solutions showed strong silencing activity. When expressed at moderate levels, 2 of the 17 solutions tested knocked down endogenous B2M by >80%, and 4 of the 17 knocked down endogenous B2M by >65%. When expressed at high levels, 3 of the 17 solutions tested knocked down endogenous B2M by >80%, and 7 of the 17 solutions tested knocked down endogenous B2M by >65%. Solution activities were reproducible between experimental replicates (mean CV = 14%, median CV = 11%) indicating this assay was robust.

This set of solutions can therefore be used to achieve distinct levels of MHC-I silencing over a broad range of MHC-I expression in engineered cells, allowing the identification of MHC-I levels that can evade both adaptive and innate immune responses post-transplantation in the host.

Example 3 – Gene editing of the endogenous miRNA scaffold in the desired cell type.

Solutions 8, 20, 29 and 30 (see Table 2) were implemented as full GEiGS, i.e. by editing the endogenous loci of the respective miRNA scaffolds. Gene editing was performed by CRISPR knock-in using the following gRNA and HDR template sequences:

Table 5:

Solution (miRNA scaffold)	**gRNA sequence [SEQ ID NO: #]**	**HDR template sequence [SEQ ID NO: #]**	**Type of HDR donor**
Sol-8 (hsa-mir-191)	GGATTCCGTTGCCCGCTGTCC [58]	CCTCAGTCAAACTGAGGACTTCAGATACTCTCATCCACGAAGACGGTTGGGGTGGTTTTTCCCCAGGAAGTAAGAGGGCTATCTTTAGCGAGATGCGGGCTGAGGCAGCCCTGGGGGAGTCACTACCATTGCAGCCCTACCCAGAGGTCCTGGGTCCACCCTCCCTCGCCAATCCTCCCCAGGGTTCCAGAGATGGCCACCAGCCTCGGCTCTCCCACGTCTACACCCATCACCTCCAGCCTGAGGGCGCAACTGAGAGGAACTGAGACCCAAGCAGCTCAGTAAAGTATGTCTGGGGGTCAGGGGCACCCCATCTGGGCCAGGGCGCTGCTCAGGCAGGAGAGCAGGGGCAGTGTAGTCAGCAGATAGAGAATGCTCTGGAGACAATCTATCTCTTGTACTACACTGAACCCGCTGTCCAGCCGTTGGCGGGGGGCGGGGGAGCTCCTGCCCCCAGGTCACTCGCCCTGGGGCCACATCAGAACCCGCCCATCCCCAGTCCCAGGAGGAAGACCCGGGAGTAGGTTGTGAAACGGAGTCTAGAGGGAACAAGACGCTCACCTGGGCCACAGAACAATAGAAGGGGAGGTGGGGGCCTCCCCTGGGACCGGGTCCCGATCCGGATTAGATCCGTGGGGCAGGATTAGCCCTGTTGGTCCCACGAAGCCTGGGCCACTGGTGCGGTGACGGTGGTGGGGCGGGCTTCGGAATCCGTCAGGAGGGAAGCGCGCCCGTGGGGCCGGAGAGGAAGTAAACCCGC [59]	ssDNA
Sol-20 (hsa-mir-106a)	GCACTGTAAGCACTTTTACA [60]	TATGAGCACTTTGGTACTACTAGGACCCAATCTTATCCTCTGTACACAACTGTACACTGCACAGTCCCCACCATCTGTTCCCAACAAACAATAAAAAGGCAAAAAACAAACCACTGAAATGTGTCCTATTCAAATTACTACTCAAGGCATACTTTTAAAATGCTGTATATTAGGCAGCCTGTGCCAGAAGGGGCATTTAGGGCAGTAGATTCTAAGCTGCTTCACTAACTGCACTAGATGCACCTTAACACAAGAGACACATTATAAGAAGCAGCTCAAAAGCATCAACTACTTAATTATGCACAAACTACAGTTCTCAGTAGCCATTGACTAAATCACCATGGTAATGTTAGAATTTTCCCCCTAATTCAAGATCTCAAAAAGCTTAGAATTTGGGGGAAAATTTAACATGGCCAAGGCCTATTCCTGTAGCAAAATTTTAAGTCTTCCAGGAGCTCTTCTTTTCCGTCCTCACAGATCCATGCAGACTCACCTGGTTAAAACAACAAAACAAACACACACATATTAGAAACTAAACAAGTAAACTACTAGTTAATATTCCTGAAACAGGCTTTCCTTTGGCTTACAGAGTCTAAGCTAGGTTTTGTGGTTTCAACCAAATCCTGAGAAAT [61]	ssDNA
Sol-29 (hsa-mir-20a)	CACTAAAGTGCTTATAGTGC [62]	AGGGCCTGCTGATGTTGAGTGCTTTTTGTTCTAAGGTGCATCTAGTGCAGATAGTGAAGTAGATTAGCATCTACTGCCCTAAGTGCTCCTTCTGGCATAAGAAGTTATGTATTCATCCAATAATTCAAGCCAAGCAAGTATATAGGTGTTTTAATAGTTTTTGTTTGCAGTCCTCTGTTAGTTTTGCATAGTTGCACTACAAGAAGAATGTAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGTACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTAACAAATTTCCAATAATCCTGTTGTTTAGTTATACAGATTATTGGAAATTTGTAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCCTGTCGCCCAATCAAACTGTCC	ssDNA

		TGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCA TCCAGCTGTGTGATATTCTGCTGTGCAAATCCATGCAAAAC TGACTGTGGTAGTGAAAAGTCTGTAGAAAAGTAAGGGAAAC TCAAACCCCTTTCTACACAGGTTGGGATCGGTTGCAATGCT GTGTTTCTGTATGGTATTGCACTTGTCCCGGCCTGTTGAGT TTGGTGGGGATTGTGACCAGAAGATTTTGAAAATTAAATATT ACTGAAGATTTCGACTTCCACTGTTAAATGTACAAGATACAT GAA [63]	
Sol-30 (hsa-mir-21)	ATGTTG ACTGTT GAATCT CA [64]	AGCATGTACTCTGGTTTCAACAGACACAAATTTATATGTTAA CCCAGTTTTCTTGCCGTTCTGTAAGTGTTTTATTCTTAGTGT GATTTTTTTCCATTGGGATGTTTTTGATTGAACTTGTTCATTT TGTTTTGCTTGGGAGGAAAATAAACAATTTTACTTTTTTCCTT TAGGAGCATTATGAGCATTATGTCAGAATAGAATAGAATTG GGGTTCGATCTTAACAGGCCAGAAATGCCTGGGTTTTTTTG GTTTGTTTTTGTTTTTGTTTTTTTATCAAATCCTGCCTGACTG TCTGCTTGTTTTGCCTACCATCGTGACATCTCCATGGCTGTA CCACCTTGTCGGGTGTTAACATTATTATAACCCTACTGTTGA ATCTCATGGAGGGTCATAATATGTTAACATCTGACATTTTGG TATCTTTCATCTGACCATCCATATCCAATGTTCTCATTTAAAC ATTACCCAGCATCATTGTTTATAATCAGAAACTCTGGTCCTT CTGTCTGGTGGCACTTAGAGTCTTTTGTGCCATAATGCAGC AGTATGGAGGGAGGATTTTATGGAGAAATGGGGATAGTCTT CATGACCACAAATAAATAAAGGAAAACTAAGCTGCATTGTG GGTTTTGAAAAGGTTATTATACTTCTTAACAATTCTTTTTTTC AGGGACTTTTCTAGCTGTATGACTGTTACTTGACCTTCTTTG AAAAGCATTCCCAAAATGCTCTATTTTAGATAGATTAACATT AACCAACAT [65]	dsDNA

The gRNAs were obtained from Integrated DNA Technologies (IDT) as synthetic sgRNAs. Cas9 protein was also obtained from IDT. sgRNA/Cas9 ribo-nucleoproteins (RNP) were transfected into iPSCs using nucleofection. Five days post-transfection CRISPR pools were cloned in 96-well plates using the F.SIGHT platform (Cytena), and single cell deposition was confirmed using the NYONE imaging system (Synentec). Cell colonies were expanded for 7-10 days and subsequently were genotyped by PCR, sanger sequencing and NGS amplicon sequencing. Confirmed GEiGS lines were analysed by flow cytometry to assess the level of B2M expression.

For Solution 29 both heterozygous (WT/KI) and homozygous (KI/KI) clones were isolated, thus expressing the sRNA mono- and bi-allelically, respectively. Because MIR106A is X-linked (hemizygous), the GEiGS clones isolated for solution 20 were only expressing the sRNA mono-allelically. For solution 30 homozygous (KI/KI) clones were generated more efficiently with Cas12a (Cpf1) protein (also from IDT) instead of Cas9.

GEiGS lines showed homogenous silencing of B2M, with very similar variance of B2M expression between the control parental line (WT/WT) and the GEiGS lines (Figure 9). Quantification of B2M silencing in multiple clones, showed consistent B2M silencing between different clones of the same genotype, reflecting the reproducible silencing activity of GEiGS (Figure 10). On average, heterozygous WT/KI clones for solution 29 showed silencing of B2M

by 89% ± SD 2% (n=4), while homozygous KI/KI clones for the same solution showed silencing by 96% ± SD 0.5% (n=2) (Figure 10). For solution 20, hemizygous KI clones showed B2M silencing by 65% ± SD 1.2% (n=4) (Figures 9 and 10) and for solution 30 a homozygous KI clone displayed approximately 31% level of B2M silencing (n=1).

Example 4 – Gene editing of the endogenous miRNA scaffold in primary human T lymphocytes.

Solution 30 (see Example 2 and Table 4 and 5) was implemented as full GEiGS i.e. by editing the endogenous loci of the respective miRNA scaffolds. A number of stimulation-induced miRNAs can be detected in primary T cells (see Amarel *et al.* 2017 - EMBO J. 2017 Feb 1;36(3):346-360. doi: 10.15252/embj.201694335).

Gene editing was performed by CRISPR knock-in using a methodology described in the paper by Roth et al. 2018 (Nature. 2018 Jul;559(7714):405-409. doi: 10.1038/s41586-018-0326-5).

The method used in Roth et al. (2018) preferably recommends fresh cells, bulk T cells or sub-populations sorted by fluorescence activated cell sorting (FACS), and with cells from whole blood or leukapheresis, though it also is used with cells that have been recovered from cryo-preservation.

Table 6:

Solution (miRNA scaffold)	**gRNA sequence**	**HDR template sequence**	**Type of HDR donor**
Sol-30 (hsa-mir-21)	ATGTTGACT GTTGAATCT CA [64]	AGCATGTACTCTGGTTTCAACAGACACAAATTTATATGTTA ACCCAGTTTTCTTGCCGTTCTGTAAGTGTTTTATTCTTAGT GTGATTTTTTTCCATTGGGATGTTTTTGATTGAACTTGTTC ATTTTGTTTTGCTTGGGAGGAAAATAAACAATTTTACTTTTT TCCTTTAGGAGCATTATGAGCATTATGTCAGAATAGAATAG AATTGGGGTTCGATCTTAACAGGCCAGAAATGCCTGGGTT TTTTTGGTTTGTTTTTGTTTTTGTTTTTTTATCAAATCCTGC CTGACTGTCTGCTTGTTTTGCCTACCATCGTGACATCTCC ATGGCTGTACCACCTTGTCGGGTGTTAACATTATTATAAC CCTACTGTTGAATCTCATGGAGGGTCATAATATGTTAACAT CTGACATTTTGGTATCTTTCATCTGACCATCCATATCCAAT GTTCTCATTTAAACATTACCCAGCATCATTGTTTATAATCA GAAACTCTGGTCCTTCTGTCTGGTGGCACTTAGAGTCTTT TGTGCCATAATGCAGCAGTATGGAGGGAGGATTTTATGGA GAAATGGGGATAGTCTTCATGACCACAAATAAATAAAGGA AAACTAAGCTGCATTGTGGGTTTTGAAAAGGTTATTATACT TCTTAACAATTCTTTTTTTCAGGGACTTTTCTAGCTGTATG ACTGTTACTTGACCTTCTTTGAAAAGCATTCCCAAAATGCT CTATTTTAGATAGATTAACATTAACCAACAT [65]	dsDNA

The gRNAs for Sol-30 were obtained from Integrated DNA Technologies (IDT) as synthetic sgRNAs. Cas9 protein was obtained from IDT. Immediately after isolation from donor

peripheral blood mononuclear cell isolates (PBMC), T cells were stimulated for 2 days with anti-human CD3/CD28 magnetic dynabeads (ThermoFisher) at a beads to cells concentration of 1:1, along with a cytokine cocktail of IL-2 at 200 U ml^{-1}, IL-7 at 5 ng ml^{-1}, and IL-15 at 5 ng ml^{-1}. Beads were removed prior to electroporation in accordance with standard methods (see, e.g., Roth *et al.*). The sgRNA/Cas9 ribo-nucleoproteins (RNP) were transfected into primary human T cells using electroporation. After electroporation, T cells were cultured in media with IL-2 at 500 U ml^{-1}. Throughout the culture period T cells were maintained at an approximate density of 1 million cells per ml of media. Three days after electroporation cells were isolated for analysis of surface B2M expression using flow cytometry and gDNA and RNA extracted for PCR, Sanger sequencing and NGS amplicon sequencing to assess the efficiency of gene editing.

Results indicate a reduction in surface B2M expression (Fig. 4). Of the CD3 positive, mixed T cell population approximately 24% of the cells demonstrated a silencing of B2M (Fig. 12a) with a reduction in mean fluorescent intensity of 80% (n=1), compared with control unedited cells. Analysis confirms that both CD4 and CD8 edited T cells demonstrated similar reduction in B2M surface expression (Fig. 12b).

Example 5 – Demonstrating evasion of both T cell and NK-cell mediated immune responses in culture to clonal GEiGS-modified cell lines

To determine whether partial silencing of B2M enables cells to evade both adaptive and innate immune cell responses, published methods are in use to establish the reactivity in cell culture of donor-derived cytotoxic T-lymphocytes (CTLs) or NK (Natural Killer or LAK, lymphokine activated killer) cells to the GEiGS-modified cell lines (Haga K *et al.* 2006, Hacke K *et al.* 2009, Han X *et al.* 2019). Donor CTLs and NK cells are conveniently isolated from commercial suppliers of peripheral blood mononuclear cells (PBMC).

T lymphocytes are initially preactivated in tissue culture to make them allo-reactive. This is performed via stimulation with cells, cell extracts or stimulated cells presenting HLA-antigens which are recognised as foreign (as they are un-matched) by the donor T lymphocytes. Once activated the donor cells are then mixed in culture with a GEiGS-modified cell line at a defined ratio (typically 10 donor cells : 1 GEiGS-modified cell), though other ratios are tested. After a defined period in mixed cell culture (48hrs), effector donor T lymphocyte activation by the GEiGS-modified cell line is analysed by measuring proliferation, or inflammatory cytokine secretion (measured by ELISA), or the extent of GEiGS-modified cell cytotoxicity mediated by the T lymphocytes is assessed by quantifying GEiGS-modified cell line death by measuring release of the cytoplasmic enzyme lactate dehydrogenase into the culture medium, and/or

release of pre-loaded Calcein AM, and/or staining with a cell death marker (e.g. propidium iodide), and/or time-course visualisation of GEiGS-modified cells preloaded with a fluorescent marker (e.g., CFSC). Undifferentiated iPSC lines (knock-in for Solution 20 or 29), iPSC lines differentiated into pancreatic progenitor cells (knock-in for Solution 30) do not express quantitatively the same level of B2M as the parental iPSC line.

NK cells are non-HLA-restricted and potent effectors which recognise and kill cells that are deficient in surface expression of MHC-I ('missing self'), such as malignant cells. In a related co-culture assay using NK cells from the same PBMC donor, these are mixed in culture with a GEiGS-modified cell line at a defined ratio (typically 5:1 or 1:1). After a defined period in mixed cell culture (4-48hrs), the extent of GEiGS-modified cell cytotoxicity mediated by the NK cells is assessed by quantifying GEiGS-modified cell line death (as above). Undifferentiated iPSC lines (knock-in for Solution 20 or 29), iPSC lines differentiated into pancreatic progenitor cells (knock-in for Solution 30) do not express quantitatively the same level of B2M as the parental iPSC line.

Hence, we demonstrate that efficient and dose-dependent reduction in surface expression of HLA in human cells, yields enhanced resistance to allo-reactive T lymphocyte mediated cytotoxicity, while avoiding non-MHC restricted killing.

Example 6 – Selection of GEiGS solutions for partial silencing of B2M using miRNA scaffolds that are expressed in a context-specific manner for validation via plasmid-based ectopic expression and GEiGS.

Table 7. Sequences of computationally-designed B2M-targeting RNAi trigger sequences and GEiGS solutions based on miRNAs expressed more highly in differentiated cells (pancreatic and primary human T cells):

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
Sol-8	hsa-mir-191	TTCAGTGTAGTACAAGAGATAGA [1]	CGGCTGGACAGCGGGttcagtgtagtacaagagatagaTTGTCTCCAGAGCATTCTCTATCTGCTGACTACACTGCCCCTGCTCTCCTGCCT [18]
Sol-30	hsa-mir-21	TGTTAACATTATTATAACCCTAC [10]	TGTCGGGtgttaacattattataaccctacTGTTGAATCTCATGGAGGGTCATAATATGTTAACATCTGACA [27]
Neg Ctrl 1	hsa-mir-302a	TTAACTAAACAACAACAAACAG [52]	CCAACCCTttaactaaacaacaacaaacagGAAACAAAATTGTTTGTTGTGGTTTAGTTGTGGGGATGG [53]

Neg Ctrl 2	hsa-mir-200c	TTTACGTCGATAAATCAGGACAC [54]	CCCGTTCCGATTTAACGACGTAATGGGTGCGGTTGGGAGTCTCtttacgtcgataaatcaggacacGG [55]

A number of the extended designs tested were based on endogenous pre-miRNAs expressed more highly in undifferentiated iPSC rather than differentiated progeny from the stem cells. These solutions are listed as follows:

Table 8. Sequences of computationally-designed B2M-targeting RNAi trigger sequences and GEiGS solutions based on miRNAs expressed highly in undifferentiated iPSC:

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
Sol-12	hsa-mir-302c	TTCAGTAAGTCAACTTCAATGT [3]	CCTACATTTTAGTTGACTTACTGCAGCTGTGTGAAACAAAAGTttcagtaagtcaacttcaatgtAGG [20]
Sol-20	hsa-mir-106a	TAAATTTTCCCCCAAATTCTAAG [5]	CCTTGGCCATGTtaaattttcccccaaattctaagCTTTTTGAGATCTTGAATTAGGGGGAAAATTCTAACATTACCATGG [22]
Sol-29	hsa-mir-20a	TAACAAATTTCCAATAATCCTGT [9]	GTAGCACtaacaaatttccaataatcctgtTGTTTAGTTATACAGATTATTGGAAATTTGTAAAGTACTGC [26]
Sol-43	hsa-mir-518b	TTAACTTATGCACGCTTAACTA [14]	TCATGCTGTGGCAGTTCAGCGTGAATAAGTTTCAATTGTCTGAAAGAAAAttaacttatgcacgcttaactaTTACGGTTTGA [31]

>hsa-mir-191_8_B2M-exon2-2

GTCTTGTTCCCTCTAGACTCCGTTTCACAACCTACTCCCGGGTCTTCCTCCTGGGACTG
GGGATGGGCGGGTTCTGATGTGGCCCCAGGGCGAGTGACCTGGGGGCAGGAGCTCC
CCCGCCCCCCGCCAACGGCTGGACAGCGGGTTCAGTGTAGTACAAGAGATAGATTGTC
TCCAGAGCATTCTCTATCTGCTGACTACACTGCCCCTGCTCTCCTGCCTGAGCAGCGC
CCTGGCCCAGATGGGGTGCCCCTGACCCCCAGACATACTTTACTGAGCTGCTTGGGTC
TCAGTTCCTCTCAGTTGCGCCCTCAGGCTGGAGGTGATGGGTGTAGACGTGGGAGAG
CCGAGG [47]

>hsa-mir-302c_12_B2M-exon2-2

TTTACTTCTCCAAAATAGAACACGCTAACCTCATTTGAAGGGATCCCCTACATTTTAGTT
GACTTACTGCAGCTGTGTGAAACAAAAGTTTCAGTAAGTCAACTTCAATGTAGGTGTCT

CCAAGCCAGCACACCTTTTGTTACAAAATTTTTTTGTTATTGTGTTTTAAGGTTACTAAGC
TTGTTACAGGTTAAAGGATTCTAACTTTTTCCAAGA [37]

>hsa-mir-106a_20_B2M-exon4

TTTACTTGTTTAGTTTCTAATATGTGTGTTTGTTTTGTTGTTTTAACCAGGTGAGTCTG
CATGGATCTGTGAGGACGGAAAAGAAGAGCTCCTGGAAGACTTAAAATTTTGCTACAGG
AATAGGCCTTGGCCATGTTAAATTTTCCCCCAAATTCTAAGCTTTTTGAGATCTTGAATT
AGGGGGAAAATTCTAACATTACCATGGTGATTTAGTCAATGGCTACTGAGAACTGTAGT
TTGTGCATAATTAAGTAGTTGATGCTTTTGAGCTGCTTCTTATAATGT [45]

>hsa-mir-20a_29_B2M-exon4

GAATGATTTTTACTAATTTTGTGTACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTAT
CTGATGTGACAGCTTCTGTAGCACTAACAAATTTCCAATAATCCTGTTGTTTAGTTATAC
AGATTATTGGAAATTTGTAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCCTGTCGC
CCAATCAAACTGTCCTGTTA [39]

>hsa-mir-21_30_B2M-exon4

GTTCGATCTTAACAGGCCAGAAATGCCTGGGTTTTTTTGGTTTGTTTTTGTTTTTGTTTTT
TTATCAAATCCTGCCTGACTGTCTGCTTGTTTTGCCTACCATCGTGACATCTCCATGGCT
GTACCACCTTGTCGGGTGTTAACATTATTATAACCCTACTGTTGAATCTCATGGAGGGT
CATAATATGTTAACATCTGACATTTTGGTATCTTTCATCTGACCATCCATATCCAATGTTC
TCATTTAAACATTACCCAGCATCATTGTTTATAATCAGAAACTCTGGTCCTTCTGTCTGG
TGGCACTTAGAGTCTTTTGTGCCATAATGCA [41]

>hsa-mir-518b_43_B2M-exon4

TTTATGTTCTGGATTCCAGAAAACATGCAAACAGGGCAAATAAATGCATCTTTATTTTGT
GTCCATTTTAACCTGGTCAAGGAAAATTCCAACAGCAACATCAAAAAACCAGTGTTGGA
GCAAGAATATGTCATGCTGTGGCAGTTCAGCGTGAATAAGTTTCAATTGTCTGAAAGAA
AATTAACTTATGCACGCTTAACTATTACGGTTTGAGTAAAGCAGCGTTGAAGTTGATGCT
GATCTTGGTAATACATTTGCAGAGCGTGCTTATCATCAGACGTGGACGATGGTGGGGTT
CTGTTTTGGTTTTGTTTTTTTCTAAGACAGGGTCTCCGTTGCCCAG [50]

Example 7 – Demonstrating B2M silencing in pancreatic progenitors specified in culture from clonal GEiGS-modified iPSC

Solution 30 (see Table 7) is implement as full GEiGS to demonstrate that B2M silencing can be programmed to be deployed only when therapeutic cells reach a specific stage of

differentiation, as in the case of iPSC-derived pancreatic cells. The endogenous loci of the respective miRNA (hsa-mir-21) is modified (as described in Example 3), as this miRNA scaffold is known to be expressed in a developmentally regulated and cell state specific manner in the pancreatic cells (Jin W et al 2019, see also Fig. 11).

Pancreatic progenitor cells are generated from the control parental line (WT/WT) using a commercially available set of reagents and a validated methodology (Stem Cell Technologies; STEMDiff™ Pancreatic Progenitor Kit) that reproducibly generates progenitors via a four stage process of definitive endoderm, primitive gut tube, posterior foregut endoderm and pancreatic progenitors. Specified progenitors produced after fourteen days in continuous culture are isolated and assessed by flow cytometry for the level of staining for pancreatic progenitor markers (PDX-1, NKX6.1, co-stained for B2M). Results indicate a significant proportion of the specified progenitors are positive for both pancreatic cell markers and the majority of PDX-1 positive cells also staining for B2M.

For solution 30, both heterozygous (WT/KI) and homozygous (KI/KI) clones were isolated, thus expressing the sRNA mono- and bi-allelically, respectively. GEiGS clones are assessed for the level of homogenous, quantitative and reproducible B2M silencing at the pancreatic progenitor stage of specification as the cells are differentiated from iPSC using the current method, with B2M expression being compared with the control parental line (WT/WT). The level of B2M silencing is conditional upon the progenitors remaining in the differentiated state, with this being lost when the progenitors de-differentiate, proliferate or become degenerative.

Example 8 – Demonstrating B2M silencing in monocytes specified in culture from clonal GEiGS-modified iPSC

Solution 30 (see Table 7) implemented as full GEiGS demonstrates that B2M silencing can be programmed to be deployed in other types of therapeutic cells including iPSC-derived monocytes. As in Example 3, the endogenous loci of the respective miRNA (hsa-mir-21) is modified. The homozygous (KI/KI) clones for Solution 30 were isolated, thus expressing the sRNA bi-allelically. GEiGS Solution 30 clones were assessed for the level of homogenous, quantitative and reproducible B2M silencing as the cells were differentiated into monocytes from iPSC. The has-mir-21 scaffold is expressed in a developmentally regulated and cell state specific manner in monocytes (Sheedy FJ. Turning 21: Induction of miR-21 as a Key Switch in the Inflammatory Response. Front Immunol. 2015 Jan 29;6:19. doi: 10.3389/fimmu.2015.00019. PMID: 25688245; PMCID: PMC4310327).

There are many published protocols for the generation of monocytes and macrophages from human pluripotent stem cells (for example Happle, C., Lachmann, N., Ackermann, M.,

Mirenska, A., Gohring, G., Thomay, K., Mucci, A., Hetzel, M., Glomb, T., Suzuki, T., et al. (2018). Pulmonary transplantation of human induced pluripotent stem cell-derived macrophages ameliorates pulmonary alveolar proteinosis. Am. J. Respir. Crit. Care Med. 198, 350–360; Lachmann, N., Ackermann, M., Frenzel, E., Liebhaber, S., Brennig, S., Happle, C., Hoffmann, D., Klimenkova, O., Luttge, D., Buchegger, T., et al. (2015). Large-scale hematopoietic differentiation of human induced pluripotent stem cells provides granulocytes or macrophages for cell replacement therapies. Stem Cell Reports 4, 282–296 and Zhang, H., Xue, C., Shah, R., Bermingham, K., Hinkle, C.C., Li,W., Rodrigues, A., Tabita-Martinez, J., Millar, J.S., Cuchel, M., et al. (2015). Functional analysis and transcriptomic profiling of iPSC derived macrophages and their application in modelling Mendelian disease. Circ. Res. 117, 17–28.

Monocytes were generated from the isogenic control parental line (parental control WT/WT line) and the homozygous (KI/KI) clone for Solution 30 using a validated embryoid body-based protocol over a 3-4 week period. Undifferentiated iPSC were first differentiated into myeloid precursors and then monocytes generated using X-VIVO™ 15 Serum-free Hematopoietic Cell Medium (Lonza) supplemented with the cytokines IL-3 and Macrophage Colony-Stimulating Factor (M-CSF). All reagents were commercially available.

Monocytes were efficiently produced after approximately 3 weeks in continuous culture and were isolated and assessed by flow cytometry for the level of staining for the monocyte markers CD14 and CD45 markers (see Fig. 19a). Greater than 90% of the cells were double positive for the known monocyte markers CD14 and CD45. The iPSC lines (GEiGS Solution 30 (S30/S30 genotype) and isogenic control) were also used to generate monocytes which were then stained for residual cell surface B2M expression using flow cytometry. When expanded as undifferentiated iPSC, greater than 93% of the cells for both the isogenic control and GEiGS Solution 30 clonal lines expressed B2M, whereas following differentiation into monocytes the GEiGS Solution 30 line showed a marked reduction in the number of cells with the same quantity of cell surface expressed B2M compared with the isogenic control line (16% cells with residual B2M compared with 77%) (see Fig. 19b).

A number of the Solutions identified for silencing B2M (e.g. Solution 30) were implemented as full GEiGS, i.e. by editing the endogenous loci of the respective miRNA scaffolds in iPSC and differentiating the stem cells into myeloid cells.

GEiGS was implemented using gRNAs for individual Solution 30, as obtained from Integrated DNA Technologies (IDT) as synthetic sgRNAs. Cas9 protein is obtained from IDT. Isolated

iPSC were electroporated using the Lonza 4D-Nucleofector device (4D-25 Nucleofector Core Unit: Lonza, AAF-1002B; 4D-Nucleofector X Unit: AAF-1002X) (Buffer P3, device setting CM-137) and then contacted with the sgRNA/Cas9 ribo-nucleoprotein complexes (RNP) and the appropriate dsDNA HDR template either produced internally or made commercially (IDT or Genewiz).

After electroporation, clone selection and characterisation, clonal iPSC cell lines with either homozygous knock-out of the miRNA-21 gene or homozygous knock-in of the Solution 30 design into the miR-21 locus, were differentiated into monocytes and then monocyte-derived macrophages in macrophage medium (DMEM high glucose supplemented with 10% FBS [Gibco], 2mMGlutaMAX, 100 U/ml penicillin, 100 µg/ml streptomycin, and M-CSF 100 ng/ml [Peprotech]) and the medium changed every 2–3 days by adding half the volume of medium with 1× cytokines into each well.

After several days differentiation in culture, cells were isolated for analysis of target MHC-I protein expression (B2M or HLA-A/B/C) and the relative B2M and HLA-A/B/C expression in GEiGS modified iPSC, monocytes and macrophages (miR-21 redirected to target B2M, i.e. Solution 30) compared to control cells in which the endogenous miR-21 has been knocked out (KO/KO). The GEiGS and control lines were differentiated in vitro first to monocytes and subsequently to macrophages. B2M and HLA-A/B/C were assayed by flow cytometry showing effective silencing in myeloid cells (84-88% silencing), but not at the iPSC stage (Figures 21c-f).

The relative B2M protein expression levels between the S30 undifferentiated iPSC and its undifferentiated homozygous KO control iPSC line were about the same, indicating that disrupting the endogenous miR-21 locus does not produce an unspecific B2M silencing effect in the iPSC stage. Subsequently, when the iPSC were differentiated, the myeloid progenitors, monocytes and macrophages from the (wild type) control (unedited), homozygous miR-21 KO and the homozygous S30 (KI/KI) cell lines were able to differentiate effectively and with the same efficiency, however the homozygous S30 (KI/KI) cell line showed a specific reduction of B2M protein expression only at the stage of myeloid progenitors, monocytes and macrophages (Fig. 21a). This demonstrates that modifying the miR-21 locus to incorporate Solution 30 does not perturb the ability of the stem cells to differentiate into myeloid lineage cells and that B2M cell surface expression is specifically reduced only the myeloid lineage stage cells.

An antibody to cell surface expressed classical HLA-A/B/C indicated that, as predicted for B2M silencing via Solution 30, there was a reduced cell surface expression of HLA-A/B/C as B2M is required for the correct processing and cell surface distribution of MHC-I complex proteins (Figures 21e and 21f).

This example indicates that silencing of B2M results in reduced cell surface expression of HLA-A/B/C and therefore MHC-I complex in a context-specific manner (only in myeloid lineage cells but not in undifferentiated iPSC.

Example 9 – Computational designs of GEiGS solutions silencing genes in macrophages

GEiGS Solutions targeting genes in macrophages were generated using the computational pipeline. Pipeline inputs are: the mRNA sequence of the major transcripts for the gene PPARG, STAT6, IRF4 and KDM6B (from literature), the human genome reference sequence (GRCh38), miRNA annotations (miRbase v22) and cell type specific miRNA quantifications from analysis of human primary donor macrophages polarised into classical (M1) or non-classical states (M2) using cytokine stimulation.

From RNAseq analysis of polarised macrophages, four individual miRNA scaffolds (LET7i, MIR146A, MIR20A and LET7C) were selected based on differential expression in distinct cell states. These miRNA scaffolds were selected based on their high expression level in monocytes, M1 or M2 macrophages. Expression levels were determined experimentally using two approaches, namely next generation sequencing (NGS) using monocytes and macrophages activated to M1 or M2 states, as illustrated in Fig. 18a, and qPCR performed on macrophages that were produced when proliferative THP-1 cells were induced to differentiate to M0-like cells (See Figs 18a and 18c). Fig 18c confirms that these two approaches provided correlating results.

Tables 15-20 below provide a summary of results for differentially expressed miRNAs identified in different macrophage cell states using NGS, as follows:

- Table 15 - M1 macrophages compared to M0 macrophages.
- Table 16 - M2a macrophages compared to M0 macrophages.
- Table 17 - M2a macrophages compared to M1 macrophages.
- Table 18 - M2c macrophages compared to M0 macrophages.
- Table 19 - M2c macrophages compared to M1 macrophages.
- Table 20 - M2c macrophages compared to M2a macrophages.

An additional scaffold based on an optimised miRNA 30 sequence was also included.

The computational pipeline identified specific designs suitable for targeting the genes of interest based on modified forms of the selected context-specific miRNAs. The miRNA designs from the computational model was broadly in line with published literature describing conventional siRNA designs to target these genes giving further reassurance in the solutions proposed. The solutions were ranked based on their on-target scores and predicted low off-target scores. Both published and novel sequences were used to assist with design of the trigger sequences. Computational analysis was used to select the top ranked sequences for each target gene, ensuring retention of diversity based on the original data source. Further computational analysis was used to shortlist between 22 and 25 individual GEiGS solutions designed per macrophage target gene. This was based on assessment of predictions to form a mature miRNA and the quantification of the differential expression of each miRNA scaffold in polarised cell states following experimental induction.

The GEiGS solution designs were comprised of a modified sequence of an endogenous pre-miRNA (hairpin) - also referred to as the endogenous miRNA scaffold - modified to encode a novel silencing RNAi trigger instead of its native guide strand. The novel GEiGS silencing RNA matched the mRNA targets by sequence complementarity and each was predicted to lead to the silencing of each target mRNA via the RNA interference pathway.

The solution design process also generated corresponding negative control constructs - also referred to as ‘scrambled’ solutions - in which the same endogenous miRNA scaffold was modified as in the corresponding GEiGS solution but to express a scrambled sequence of the GEiGS silencing RNA.

Example 10 – Validation of GEiGS solution silencing activity in macrophages via plasmid-based ectopic expression

Approximately 102 solutions were designed in total and according to the following distribution across the target genes:

Target gene PPARG: 22 solutions distributed across the different miRNA scaffolds, plus an additional, 4 based on miRNA 30;

Target gene IRF4: 24 solutions distributed across the different miRNA scaffolds, plus an additional 4 based on miRNA 30;

Target gene KDM6B: 24 solutions distributed across the different miRNA scaffolds, plus an additional 4 based on miRNA 30; and

Target gene STAT6: 24 solutions distributed across the different miRNA scaffolds, plus an additional 4 based on miRNA 30.

These Solutions were designed to avoid macrophages adopting a pro-tumour (i.e. tumour associated macrophage, TAM) state, promoting an active, inflammatory phenotype in the tumour microenvironment (TME).

An additional 8 'scrambled' solutions were designed in which the same endogenous miRNA scaffolds were modified to express a scrambled sequence of selected GEiGS silencing RNAs.

Extended solutions were comprised of a GEiGS solution as designed using the computational pipeline, plus approximately 130 bases of genomic sequence flanking on both sides of the endogenous pre-miRNA that the respective GEiGS solution was based on. This was included to avoid omitting important sequences that act as a signal for the enzyme Drosha.

Solutions to test are as follows:

Tables 9-12. Sequences of computationally -designed gene-targeting RNAi trigger sequences and GEiGS solutions, on a gene by gene basis:

Table 9 - Gene target: **PPARG**

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
Sol-180	hsa-mir-let7c	TGTCATAGATAAGCTTCAATCT [66]	GCATCCGGGTTGTCATAGATAAGCTTCAATCTTAGAGTTACACCCTGGGAGTTAGATTGAAGCTTAACTAGGACTCCTTGGAGC [88]

Sol-181	hsa-mir-let7c	TAGATAAGCTTCAATCTGATTG [67]	GCATCCGGGTTAGATAAGCTTCAATCTGATTGTAGAGTTACACCCTGGGAGTTCAATCAGATTGAATCTTCTCTTCCTTGGAGC [89]
182	hsa-mir-let7c	TACTTTGATTGCACTTTGGTAC [68]	GCATCCGGGTTACTTTGATTGCACTTTGGTACTAGAGTTACACCCTGGGAGTTGTACCAAAGTGCATTCATAGTTCCTTGGAGC [90]
183	hsa-mir-let7c	TTGTGTAAATGATCTCGTGGAC [69]	GCATCCGGGTTTGTGTAAATGATCTCGTGGACTAGAGTTACACCCTGGGAGTTGTCCACGAGATCACTTAGACATCCTTGGAGC [91]
184	hsa-mir-let7c	TTGAAGTTTCAGGTCATACTTG [70]	GCATCCGGGTTTGAAGTTTCAGGTCATACTTGTAGAGTTACACCCTGGGAGTTCAAGTATGACCTGTAACGTCATCCTTGGAGC [92]
185	hsa-let-7i	TAGATAAGCTTCAATCTGATTG [71]	CTGGCTAGATAAGCTTCAATCTGATTGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCAACTAGATTGAAGCTTATCTGCTA [93]
186	hsa-let-7i	TGTCATAGATAAGCTTCAATCT [72]	CTGGCTGTCATAGATAAGCTTCAATCTGGTCGGGTTGTGACATTGCCCGCTGTGGACGTAGACTGAAGCTTATCTATGACGCTA [94]
187	hsa-let-7i	TATCACTGGAGATCTCCGCCAA [73]	CTGGCTATCACTGGAGATCTCCGCCAAGGTCGGGTTGTGACATTGCCCGCTGTGGAGACTTGTCGGAGATCTCCAGTGATGCTA [95]
188	hsa-let-7i	TACTTTGATTGCACTTTGGTAC [74]	CTGGCTACTTTGATTGCACTTTGGTACGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGTGGCAAAGTGCAATCAAAGTGCTA [96]
189	hsa-mir-146a	TTGAAGTTTCAGGTCATACTTG [75]	CCGATGTGTATCCTCAGCTTTTGAAGTTTCAGGTCATACTTGGTGTCAGTGTCAGAAGTCTGCTCTGAAACTTCACAGCTGGGATATCTCTGTCATCGT [97]
190	hsa-mir-146a	TTGTGTAAATGATCTCGTGGAC [76]	CCGATGTGTATCCTCAGCTTTTGTGTAAATGATCTCGTGGACGTGTCAGTGTCAATCTAGGAAATCATTTACACACAGCTGGGATATCTCTGTCATCGT [98]
191	hsa-mir-146a	TATCACTGGAGATCTCCGCCAA [77]	CCGATGTGTATCCTCAGCTTTATCACTGGAGATCTCCGCCAAGTGTCAGTGTCAGTGGCCGAAATCTCCAGTGATCAGCTGGGATATCTCTGTCATCGT [99]
192	hsa-mir-146a	TAGATAAGCTTCAATCTGATTG [78]	CCGATGTGTATCCTCAGCTTTAGATAAGCTTCAATCTGATTGGTGTCAGTGTCAAGATCCGAATGAAGCTTATCTCAGCTGGGATATCTCTGTCATCGT [100]
193	hsa-mir-146a	TGTCATAGATAAGCTTCAATCT [79]	CCGATGTGTATCCTCAGCTTTGTCATAGATAAGCTTCAATCTATGTCAGTGTCACGATTCAAACTTATCTATGACCAGCTGGGATATCTCTGTCATCGT [101]
194	hsa-mir-20a	TAGATAAGCTTCAATCTGATTGT [80]	GTAGCACTAAAGTGCTTATAGTGCAGGTAGTGTTTAGTTATCTACTGCATTATGAGCACTTAAAGTACTGC [102]
195	hsa-mir-20a	TTGTGTAAATGATCTCGTGGACT [81]	GTAGCACTTGTGTAAATGATCTCGTGGACTTGTTTAGTGTTAGTTACGAGATCATTTACATGTAGTACTGC [103]
196	hsa-mir-20a	TTGAAGTTTCAGGTCATACTTGT [82]	GTAGCACTTGAAGTTTCAGGTCATACTTGTTGTTTAGTTATACAATATGACCTGAAACTTTATAGTACTGC [104]
197	hsa-mir-20a	TACTTTGATTGCACTTTGGTACT [83]	GTAGCACTACTTTGATTGCACTTTGGTACTTGTTTAGTTATAGTACAAAGTGCAATCAAAGAAAGTACTGC [105]
198	hsa-mir-30opt	TTGTGTAAATGATCTCGTGGAC [84]	TTCCACGAGATCATTTACACAATAGTGAAGCCACAGATGTATTGTGTAAATGATCTCGTGGAC [106]

199	hsa-mir-30opt	TATCACTGGAGATCTCCGCCAA [85]	GTGGCGGAGATCTCCAGTGATATAGTGAAGCCACAGATGTATATCACTGGAGATCTCCGCCAA [107]
200	hsa-mir-30opt	TGTGGAGATGCAGGCTCCACTT [86]	CAGTGGAGCCTGCATCTCCACATAGTGAAGCCACAGATGTATGTGGAGATGCAGGCTCCACTT [108]
201	hsa-mir-30opt	TGTCATAGATAAGCTTCAATCT [87]	CGATTGAAGCTTATCTATGACATAGTGAAGCCACAGATGTATGTCATAGATAAGCTTCAATCT [109]

Table 10 - Gene target: **IRF4**

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
Sol-202	hsa-mir-let7c	TAGATCTGTGGTAATCTTCTGG [110]	GCATCCGGGTTAGATCTGTGGTAATCTTCTGGTAGAGTTACACCCTGGGAGTTCCAGAAGATTACCTCAGCTCTTCCTTGGAGC [134]
Sol-203	hsa-mir-let7c	TAAGATGTATACATCACTGCAG [111]	GCATCCGGGTTAAGATGTATACATCACTGCAGTAGAGTTACACCCTGGGAGTTCTGCAGTGATGTAAACAGCTTTCCTTGGAGC [135]
204	hsa-mir-let7c	TtTCTTGAATAGAGGAATGGCG [112]	GCATCCGGGTTTTCTTGAATAGAGGAATGGCGTAGAGTTACACCCTGGGAGTTCGCCATTCCTCTACTTACGAATCCTTGGAGC [136]
205	hsa-mir-let7c	TTCATAGCTAGTTCATTCCCTA [113]	GCATCCGGGTTTCATAGCTAGTTCATTCCCTATAGAGTTACACCCTGGGAGTTTAGGGAATGAACTTGCTCTGATCCTTGGAGC [137]
206	hsa-mir-let7c	TGTCGATGCCTTCTCGGAACTT [114]	GCATCCGGGTTGTCGATGCCTTCTCGGAACTTTAGAGTTACACCCTGGGAGTTAAGTTCCGAGAAGTCATGGACTCCTTGGAGC [138]
207	hsa-let-7i	TAAGATGTATACATCACTGCAG [115]	CTGGCTAAGATGTATACATCACTGCAGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCTGAAGTGATGTATACATCTTGCTA [139]
208	hsa-let-7i	TGTCGATGCCTTCTCGGAACTT [116]	CTGGCTGTCGATGCCTTCTCGGAACTTGGTCGGGTTGTGACATTGCCCGCTGTGGAGATAAGCTCCGAGAAGGCATCGACGCTA [140]
209	hsa-let-7i	TAAATATTCTTCAGCAAAGAGG [117]	CTGGCTAAATATTCTTCAGCAAAGAGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCTGTTTGCTGAAGAATATTTGCTA [141]
210	hsa-let-7i	TAGATCTGTGGTAATCTTCTGG [118]	CTGGCTAGATCTGTGGTAATCTTCTGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCATAAGATTACCACAGATCTGCTA [142]
211	hsa-let-7i	TCTTACAAAATGGTGTGTAAGG [119]	CTGGCTCTTACAAAATGGTGTGTAAGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCTCGCACACCATTTTGTAAGGCTA [143]
212	hsa-mir-146a	TAAGATGTATACATCACTGCAG [120]	CCGATGTGTATCCTCAGCTTTAAGATGTATACATCACTGCAGGTGTCAGTGTCAATGCACTGCTGTATACATCTTCAGCTGGGATATCTCTGTCATCGT [144]
213	hsa-mir-146a	TCAAACACTAATACCTACCTGC [121]	CCGATGTGTATCCTCAGCTTTCAAACACTAATACCTACCTGCGTGTCAGTGTCAACAGGCAGATATTAGTGTTTGCAGCTGGGATATCTCTGTCATCGT [145]
214	hsa-mir-146a	TCTTACAAAATGGTGTGTAAGG [122]	CCGATGTGTATCCTCAGCTTTCTTACAAAATGGTGTGTAAGGGTGTCAGTGTCAGCTTA

			GATTCCATTTTGTAAGCAGCTGGGATATCT CTGTCATCGT [146]
215	hsa-mir-146a	TAAATATTCTTCAGC AAAGAGG [123]	CCGATGTGTATCCTCAGCTTTAAATATTCT TCAGCAAAGAGGATGTCAGTGTCAGCTCT CTGATGAAGAATATTTCAGCTGGGATATCT CTGTCATCGT [147]
216	hsa-mir-146a	TtTCTTGAATAGAGG AATGGCG [124]	CCGATGTGTATCCTCAGCTTTTTCTTGAAT AGAGGAATGGCGGTGTCAGTGTCAGGCC ACTCATCTATTCAAGAACAGCTGGGATATC TCTGTCATCGT [148]
217	hsa-mir-20a	TAAGATGTATACATC ACTGCAGT [125]	GTAGCACTAAGATGTATACATCACTGCAGT TGTTTAGTTATACTGAGTGATGTATACATC TAAAGTACTGC [149]
218	hsa-mir-20a	TtTCTTGAATAGAGG AATGGCGG [126]	GTAGCACTTTCTTGAATAGAGGAATGGCG GTGTTTAGTTATCCGCATTCCTCTATTCAA GACCAGTACTGC [150]
219	hsa-mir-20a	TTCATAGCTAGTTCA TTCCCTAG [127]	GTAGCACTTCATAGCTAGTTCATTCCCTAG TGTTTAGTTATCTAGGAATGAACTAGCTAT GCCAGTACTGC [151]
220	hsa-mir-20a	TCTTACAAAATGGTG TGTAAGGT [128]	GTAGCACTCTTACAAAATGGTGTGTAAGGT TGTTTAGTTATACCTACACACCATTTTGTAA AAAGTACTGC [152]
221	hsa-mir-20a	TAGATCTGTGGTAAT CTTCTGGA [129]	GTAGCACTAGATCTGTGGTAATCTTCTGGA TGTTTAGTTATTCCAAAGATTACCACAGAT CAAAGTACTGC [153]
222	hsa-mir-30opt	TtTCTTGAATAGAGG AATGGCG [130]	AGCCATTCCTCTATTCAAGAAATAGTGAAG CCACAGATGTATTTCTTGAATAGAGGAATG GCG [154]
223	hsa-mir-30opt	TAGATCTGTGGTAAT CTTCTGG [131]	ACAGAAGATTACCACAGATCTATAGTGAAG CCACAGATGTATAGATCTGTGGTAATCTTC TGG [155]
224	hsa-mir-30opt	TCAAACACTAATACC TACCTGC [132]	TCAGGTAGGTATTAGTGTTTGATAGTGAAG CCACAGATGTATCAAACACTAATACCTACC TGC [156]
225	hsa-mir-30opt	TTCATAGCTAGTTCA TTCCCTA [133]	GAGGGAATGAACTAGCTATGAATAGTGAA GCCACAGATGTATTCATAGCTAGTTCATTC CCTA [157]

Table 11 - Gene target: **KDM6B**

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
Sol-226	hsa-mir-let7c	TAGAGAACTGAGAT GACGAGGA [158]	GCATCCGGGTTAGAGAACTGAGATGACGA GGATAGAGTTACACCCTGGGAGTTTCCTC GTCATCTCTGTTGTCTTCCTTGGAGC [182]
Sol-227	hsa-mir-let7c	TAATATTGGATGCAT AGAGATC [159]	GCATCCGGGTTAATATTGGATGCATAGAG ATCTAGAGTTACACCCTGGGAGTTGATCT CTATGCATGCAGGATTTCCTTGGAGC [183]
228	hsa-mir-let7c	TGTTGATCTTCGGTT TCACAGA [160]	GCATCCGGGTTGTTGATCTTCGGTTTCAC AGATAGAGTTACACCCTGGGAGTTTCTGT GAAACCGATGATGAACTCCTTGGAGC [184]
229	hsa-mir-let7c	TAGGACTCTCGAAA CTTCCCTT [161]	GCATCCGGGTTAGGACTCTCGAAACTTCC CTTTAGAGTTACACCCTGGGAGTTAAGGG AAGTTTCGTGAGGCCTTCCTTGGAGC [185]

230	hsa-mir-let7c	TATTAATCCACACGAGGTCTCC [162]	GCATCCGGGTTATTAATCCACACGAGGTCTCCTAGAGTTACACCCTGGGAGTTGGAGACCTCGTGTTGATGGATTCCTTGGAGC [186]
231	hsa-let-7i	TAACGTCTATGTACATACACGA [163]	CTGGCTAACGTCTATGTACATACACGAGGTCGGGTTGTGACATTGCCCGCTGTGGAGATTCGAGTATGTACATAGACGTTGCTA [187]
232	hsa-let-7i	TAGAGAACTGAGATGACGAGGA [164]	CTGGCTAGAGAACTGAGATGACGAGGAGGTCGGGTTGTGACATTGCCCGCTGTGGAGATTCTGCGTCATCTCAGTTCTCTGCTA [188]
233	hsa-let-7i	TAGGATCTTGAATTCGGCTGGC [165]	CTGGCTAGGATCTTGAATTCGGCTGGCGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGCCTGCCGAATTCAAGATCCTGCTA [189]
234	hsa-let-7i	TAATATTGGATGCATAGAGATC [166]	CTGGCTAATATTGGATGCATAGAGATCGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGATGTCTATGCATCCAATATTGCTA [190]
235	hsa-let-7i	TAGGACTCTCGAAACTTCCCTT [167]	CTGGCTAGGACTCTCGAAACTTCCCTTGGTCGGGTTGTGACATTGCCCGCTGTGGAGATAAGTGAAGTTTCGAGAGTCCTGCTA [191]
236	hsa-mir-146a	TAGAGAACTGAGATGACGAGGA [168]	CCGATGTGTATCCTCAGCTTTAGAGAACTGAGATGACGAGGAGTGTCAGTGTCAGCCTCATCCTCTCAGTTCTCTCAGCTGGGATATCTCTGTCATCGT [192]
237	hsa-mir-146a	TAATATTGGATGCATAGAGATC [169]	CCGATGTGTATCCTCAGCTTTAATATTGGATGCATAGAGATCGTGTCAGTGTCAAATCTGTAAGCATCCAATATTCAGCTGGGATATCTCTGTCATCGT [193]
238	hsa-mir-146a	TGTTGATCTTCGGTTTCACAGA [170]	CCGATGTGTATCCTCAGCTTTGTTGATCTTCGGTTTCACAGAGTGTCAGTGTCAGCTGTAAATTCGAAGATCAACCAGCTGGGATATCTCTGTCATCGT [194]
239	hsa-mir-146a	TAGGATCTTGAATTCGGCTGGC [171]	CCGATGTGTATCCTCAGCTTTAGGATCTTGAATTCGGCTGGCGTGTCAGTGTCAACCAGACGTATTCAAGATCCTCAGCTGGGATATCTCTGTCATCGT [195]
240	hsa-mir-146a	TATTAATCCACACGAGGTCTCC [172]	CCGATGTGTATCCTCAGCTTTATTAATCCACACGAGGTCTCCGTGTCAGTGTCAAGAGAGCTAGTGTGGATTAATCAGCTGGGATATCTCTGTCATCGT [196]
241	hsa-mir-20a	TAACGTCTATGTACATACACGAG [173]	GTAGCACTAACGTCTATGTACATACACGAGTGTTTAGTTATCTCGGTATGTACATAGACGTAAAGTACTGC [197]
242	hsa-mir-20a	TAATATTGGATGCATAGAGATCA [174]	GTAGCACTAATATTGGATGCATAGAGATCATGTTTAGTTATTGATTCTATGCATCCAATATAAAGTACTGC [198]
243	hsa-mir-20a	TATTAATCCACACGAGGTCTCCG [175]	GTAGCACTATTAATCCACACGAGGTCTCCGTGTTTAGTTATCGGAACCTCGTGTGGATTAAAAAGTACTGC [199]
244	hsa-mir-20a	TAGGATCTTGAATTCGGCTGGCT [176]	GTAGCACTAGGATCTTGAATTCGGCTGGCTTGTTTAGTTATAGCCGCCGAATTCAAGATCCAAAGTACTGC [200]
245	hsa-mir-20a	TATACTTCTGGCCAGATAGGGT [177]	GTAGCACTATACTTCTGGCCAGATAGGGTGTGTTTAGTGATCATCTATCTGGCCAGAAGTAAAAGTACTGC [201]
246	hsa-mir-30opt	TAGAGAACTGAGATGACGAGGA [178]	TGTGAGCAACTCCTGTACTGTATAGTGAAGCCACAGATGTATACAGTACAGGAGTTGCTCACC [202]

247	hsa-mir-30opt	TATTAATCCACACGAGGTCTCC [179]	TAGATTCTTTCTATGGGCTTTATAGTGAAGCCACAGATGTATAAAGCCCATAGAAAGAATCTC [203]
248	hsa-mir-30opt	TTGATCATCTTGAACAAGTCGG [180]	ACAGCATCTATCTGGAGAGCAATAGTGAAGCCACAGATGTATTGCTCTCCAGATAGATGCTGG [204]
249	hsa-mir-30opt	TAGGACTCTCGAAACTTCCCTT [181]	ACGACTTGTTCAAGATGATCAATAGTGAAGCCACAGATGTATTGATCATCTTGAACAAGTCGG [205]

Table 12 - Gene target: **STAT6**

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
Sol-250	hsa-mir-let7c	TTGCATAGTCTCATACACGTAT [206]	GCATCCGGGTTTGCATAGTCTCATACACGTATTAGAGTTACACCCTGGGAGTTATACGTGTATGAGTCTAGGCATCCTTGGAGC [230]
Sol-251	hsa-mir-let7c	TACATTTAACATATCCTAGGTA..[207]	GCATCCGGGTTACATTTAACATATCCTAGGTATAGAGTTACACCCTGGGAGTTTACCTAGGATATGCTAGCTGTTCCTTGGAGC [231]
Sol-252	hsa-mir-let7c	TGAAGTATTTGTCTGAAAGTGG [208]	GCATCCGGGTTGAAGTATTTGTCTGAAAGTGGTAGAGTTACACCCTGGGAGTTCCACTTTCAGACATATAGTTCTCCTTGGAGC [232]
Sol-253	hsa-mir-let7c	TGTATGTTCCTGCCTATCCGTC [209]	GCATCCGGGTTGTATGTTCCTGCCTATCCGTCTAGAGTTACACCCTGGGAGTTGACGGATAGGCAGTAACCTACTCCTTGGAGC [233]
Sol-254	hsa-mir-let7c	TTGTCATTGAAGATCTTCTGGG [210]	GCATCCGGGTTTGTCATTGAAGATCTTCTGGGTAGAGTTACACCCTGGGAGTTCCCAGAAGATCTTGAGTTACATCCTTGGAGC [234]
Sol-255	hsa-let-7i	TACACGAAGAATCTCAGCCCTT [211]	CTGGCTACACGAAGAATCTCAGCCCTTGGTCGGGTTGTGACATTGCCCGCTGTGGAGATAAGTGCTGAGATTCTTCGTGTGCTA [235]
Sol-256	hsa-let-7i	TTGCATAGTCTCATACACGTAT [212]	CTGGCTTGCATAGTCTCATACACGTATGGTCGGGTTGTGACATTGCCCGCTGTGGAGATATAGGTGTATGAGACTATGCAGCTA [236]
Sol-257	hsa-let-7i	TACATTTAACATATCCTAGGTA [213]	CTGGCTACATTTAACATATCCTAGGTAGGTCGGGTTGTGACATTGCCCGCTGTGGAGATTACATAGGATATGTTAAATGTGCTA [237]
Sol-258	hsa-let-7i	TGAAGTATTTGTCTGAAAGTGG [214]	CTGGCTGAAGTATTTGTCTGAAAGTGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCAATTTCAGACAAATACTTCGCTA [238]
Sol-259	hsa-let-7i	TGTATGTTCCTGCCTATCCGTC [215]	CTGGCTGTATGTTCCTGCCTATCCGTCGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGACTGATAGGCAGGAACATACGCTA [239]
Sol-260	hsa-mir-146a	TACATTTAACATATCCTAGGTA [216]	CCGATGTGTATCCTCAGCTTTACATTTAACATATCCTAGGTAGTGTCAGTGTCAGAC

			CTCGGTTATGTTAAATGTCAGCTGGGATA TCTCTGTCATCGT [240]
Sol-261	hsa-mir-146a	TGAAGTATTTGTCTGA AAGTGG [217]	CCGATGTGTATCCTCAGCTTTGAAGTATT TGTCTGAAAGTGGGTGTCAGTGTCAACG CTCTCCGACAAATACTTCCAGCTGGGAT ATCTCTGTCATCGT [241]
Sol-262	hsa-mir-146a	TTGCATAGTCTCATAC ACGTAT [218]	CCGATGTGTATCCTCAGCTTTTGCATAGT CTCATACACGTATGTGTCAGTGTCGTTAC GCGTCTGAGACTATGCACAGCTGGGATA TCTCTGTCATCGT [242]
Sol-263	hsa-mir-146a	TACACGAAGAATCTCA GCCCTT [219]	CCGATGTGTATCCTCAGCTTTACACGAA GAATCTCAGCCCTTTGGTCAGTGTCCCA GGGATGCGATTCTTCGTGTCAGCTGGGA TATCTCTGTCATCGT [243]
Sol-264	hsa-mir-146a	TGTATGTTCCTGCCTA TCCGTC [220]	CCGATGTGTATCCTCAGCTTTGTATGTTC CTGCCTATCCGTCGTGTCAGTGTCAAAC GGCTAAGCAGGAACATACCAGCTGGGAT ATCTCTGTCATCGT [244]
Sol-265	hsa-mir-20a	TTTGCATAGTCTCATA CACGTAT [221]	GTAGCACTTTGCATAGTCTCATACACGTA TTGTTTAGTTATATACTGTATGAGACTAT GCACTAGTACTGC [245]
Sol-266	hsa-mir-20a	TACATTTAACATATCC TAGGTAC [222]	GTAGCACTACATTTAACATATCCTAGGTA CTGTTTAGTTATGTACTAGGATATGTTAA ATGAAAGTACTGC [246]
Sol-267	hsa-mir-20a	TGAAGTATTTGTCTGA AAGTGGC [223]	GTAGCACTGAAGTATTTGTCTGAAAGTG GCTGTTTAGTTATGCCGTTTCAGACAAAT ACTTAAAGTACTGC [247]
Sol-268	hsa-mir-20a	TGTATGTTCCTGCCTA TCCGTCC [224]	GTAGCACTGTATGTTCCTGCCTATCCGT CCTGTTTAGTTATGGATGATAGGCAGGA ACATAAAAGTACTGC [248]
Sol-269	hsa-mir-20a	TTGCATAGTCTCATAC ACGTATC [225]	GTAGCACTTGCATAGTCTCATACACGTAT CTGTTTAGTTATGATAGTGTATGAGACTA TGTTAGGTACTGC [249]
Sol-270	hsa-mir-30opt	TGACAACACGTTGAC TGATTCT [226]	CGAATCAGTCAACGTGTTGTCATAGTGA AGCCACAGATGTATGACAACACGTTGAC TGATTCT [250]
Sol-271	hsa-mir-30opt	TGTATGTTCCTGCCTA TCCGTC [227]	TACGGATAGGCAGGAACATACATAGTGA AGCCACAGATGTATGTATGTTCCTGCCTA TCCGTC [251]
Sol-272	hsa-mir-30opt	TTGCATAGTCTCATAC ACGTAT [228]	CTACGTGTATGAGACTATGCAATAGTGAA GCCACAGATGTATTGCATAGTCTCATACA CGTAT [252]
Sol-273	hsa-mir-30opt	TTGTCATTGAAGATCT TCTGGG [229]	ACCAGAAGATCTTCAATGACAATAGTGAA GCCACAGATGTATTGTCATTGAAGATCTT CTGGG [253]

The sequences of the extended solutions were as follows in Table 13 below:

Sol. ID	Extended Solution Sequence (cloned fragment) [SEQ ID NO: #]
180	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTGTCATA GATAAGCTTCAATCTTAGAGTTACACCCTGGGAGTTAGATTGAAGCTTAACTAGGACTCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [254]
181	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTAGATAA

	GCTTCAATCTGATTGTAGAGTTACACCCTGGGAGTTCAATCAGATTGAATCTTCTCTTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [255]
182	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTACTTTGA TTGCACTTTGGTACTAGAGTTACACCCTGGGAGTTGTACCAAAGTGCATTCATAGTTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [256]
183	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTTGTGTA AATGATCTCGTGGACTAGAGTTACACCCTGGGAGTTGTCCACGAGATCACTTAGACATCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [257]
184	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTTGAAGT TTCAGGTCATACTTGTAGAGTTACACCCTGGGAGTTCAAGTATGACCTGTAACGTCATCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [258]
185	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAGATAA GCTTCAATCTGATTGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCAACTAGATTGAAGCTTATCTGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [259]
186	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTGTCATAG ATAAGCTTCAATCTGGTCGGGTTGTGACATTGCCCGCTGTGGACGTAGACTGAAGCTTATCTATGACGCTAGTG CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [260]
187	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTATCACTG GAGATCTCCGCCAAGGTCGGGTTGTGACATTGCCCGCTGTGGAGACTTGTCGGAGATCTCCAGTGATGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [261]
188	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTACTTTGA TTGCACTTTGGTACGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGTGGCAAAGTGCAATCAAAGTGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [262]
189	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTTGAAGTTTCAGGTCATACTTGGTGTCAGTGTCAGAAGTCTGCTCTGAAACTTCACAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [263]
190	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTTGTGTAAATGATCTCGTGGACGTGTCAGTGTCAATCTAGGAAATCATTTACACACAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [264]
191	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTATCACTGGAGATCTCCGCCAAGTGTCAGTGTCAGTGGCCGAAATCTCCAGTGATCAGCTGGGATATCTCTG TCATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGA CTGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [265]
192	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTAGATAAGCTTCAATCTGATTGGTGTCAGTGTCAAGATCCGAATGAAGCTTATCTCAGCTGGGATATCTCTGT

	CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [266]
193	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTGTCATAGATAAGCTTCAATCTATGTCAGTGTCACGATTCAAACTTATCTATGACCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [267]
194	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTAAAGTGCTTATAG TGCAGGTAGTGTTTAGTTATCTACTGCATTATGAGCACTTAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [268]
195	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTTGTGTAAATGATC TCGTGGACTTGTTTAGTGTTAGTTACGAGATCATTTACATGTAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [269]
196	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTTGAAGTTTCAGGT CATACTTGTTGTTTAGTTATACAATATGACCTGAAACTTTATAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [270]
197	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTACTTTGATTGCACT TTGGTACTTGTTTAGTTATAGTACAAAGTGCAATCAAAGAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [271]
198	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGTTCCACGAGATCATTTACACA ATAGTGAAGCCACAGATGTATTGTGTAAATGATCTCGTGGACGCCTACTGCCTCGGACTTCAAGGGGCTAGAA TTCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAA ATGGTATAAATTAAATCACTTT [272]
199	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGGTGGCGGAGATCTCCAGTGA TATAGTGAAGCCACAGATGTATATCACTGGAGATCTCCGCCAAGCCTACTGCCTCGGACTTCAAGGGGCTAGAA TTCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAA ATGGTATAAATTAAATCACTTT [273]
200	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAAAGTATGACCTGAAACTTCA ATAGTGAAGCCACAGATGTATTGAAGTTTCAGGTCATACTTGGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [274]
201	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGATTGAAGCTTATCTATGAC ATAGTGAAGCCACAGATGTATGTCATAGATAAGCTTCAATCTGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [275]
202	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTAGATCT GTGGTAATCTTCTGGTAGAGTTACACCCTGGGAGTTCCAGAAGATTACCTCAGCTCTTCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [276]
203	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTAAGATG TATACATCACTGCAGTAGAGTTACACCCTGGGAGTTCTGCAGTGATGTAAACAGCTTTCCTTGGAGCACACTTG

	AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [277]
204	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTTTCTTGA ATAGAGGAATGGCGTAGAGTTACACCCTGGGAGTTCGCCATTCCTCTACTTACGAATCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [278]
205	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTTCATAGC TAGTTCATTCCCTATAGAGTTACACCCTGGGAGTTTAGGGAATGAACTTGCTCTGATCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [279]
206	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTGTCGAT GCCTTCTCGGAACTTTAGAGTTACACCCTGGGAGTTAAGTTCCGAGAAGTCATGGACTCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [280]
207	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAAGATGT ATACATCACTGCAGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCTGAAGTGATGTATACATCTTGCTAGTG CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [281]
208	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTGTCGATG CCTTCTCGGAACTTGGTCGGGTTGTGACATTGCCCGCTGTGGAGATAAGCTCCGAGAAGGCATCGACGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [282]
209	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAAATATT CTTCAGCAAAGAGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCTGTTTGCTGAAGAATATTTGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [283]
210	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAGATCTG TGGTAATCTTCTGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCATAAGATTACCACAGATCTGCTAGTG CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [284]
211	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTCTTACAA AATGGTGTGTAAGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCTCGCACACCATTTTGTAAGGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [285]
212	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTAAGATGTATACATCACTGCAGGTGTCAGTGTCAATGCACTGCTGTATACATCTTCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [286]
213	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTCAAACACTAATACCTACCTGCGTGTCAGTGTCAACAGGCAGATATTAGTGTTTGCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [287]
214	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTCTTACAAAATGGTGTGTAAGGGTGTCAGTGTCAGCTTAGATTCCATTTTGTAAGCAGCTGGGATATCTCTGT

	CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [288]
215	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTAAATATTCTTCAGCAAAGAGGATGTCAGTGTCAGCTCTCTGATGAAGAATATTTCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [289]
216	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTTTCTTGAATAGAGGAATGGCGGTGTCAGTGTCAGGCCACTCATCTATTCAAGAACAGCTGGGATATCTCTG TCATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGA CTGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [290]
217	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTAAGATGTATACAT CACTGCAGTTGTTTAGTTATACTGAGTGATGTATACATCTAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [291]
218	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTTTCTTGAATAGAG GAATGGCGGTGTTTAGTTATCCGCATTCCTCTATTCAAGACCAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [292]
219	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTTCATAGCTAGTTC ATTCCCTAGTGTTTAGTTATCTAGGAATGAACTAGCTATGCCAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [293]
220	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTCTTACAAAATGGT GTGTAAGGTTGTTTAGTTATACCTACACACCATTTTGTAAAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [294]
221	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTAGATCTGTGGTAA TCTTCTGGATGTTTAGTTATTCCAAAGATTACCACAGATCAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [295]
222	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAGCCATTCCTCTATTCAAGAA ATAGTGAAGCCACAGATGTATTTCTTGAATAGAGGAATGGCGGCCTACTGCCTCGGACTTCAAGGGGCTAGAA TTCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAA ATGGTATAAATTAAATCACTTT [296]
223	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGACAGAAGATTACCACAGATCT ATAGTGAAGCCACAGATGTATAGATCTGTGGTAATCTTCTGGGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [297]
224	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGTCAGGTAGGTATTAGTGTTTG ATAGTGAAGCCACAGATGTATCAAACACTAATACCTACCTGCGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [298]
225	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGGAGGGAATGAACTAGCTATG AATAGTGAAGCCACAGATGTATTCATAGCTAGTTCATTCCCTAGCCTACTGCCTCGGACTTCAAGGGGCTAGAA

	TTCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAA ATGGTATAAATTAAATCACTTT [299]
226	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTAGAGAA CTGAGATGACGAGGATAGAGTTACACCCTGGGAGTTTCCTCGTCATCTCTGTTGTCTTCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [300]
227	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTAATATTG GATGCATAGAGATCTAGAGTTACACCCTGGGAGTTGATCTCTATGCATGCAGGATTTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [301]
228	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTGTTGATC TTCGGTTTCACAGATAGAGTTACACCCTGGGAGTTTCTGTGAAACCGATGATGAACTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [302]
229	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTAGGACT CTCGAAACTTCCCTTTAGAGTTACACCCTGGGAGTTAAGGGAAGTTTCGTGAGGCCTTCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [303]
230	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTATTAATC CACACGAGGTCTCCTAGAGTTACACCCTGGGAGTTGGAGACCTCGTGTTGATGGATTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [304]
231	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAACGTCT ATGTACATACACGAGGTCGGGTTGTGACATTGCCCGCTGTGGAGATTCGAGTATGTACATAGACGTTGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [305]
232	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAGAGAA CTGAGATGACGAGGAGGTCGGGTTGTGACATTGCCCGCTGTGGAGATTCTGCGTCATCTCAGTTCTCTGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [306]
233	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAGGATCT TGAATTCGGCTGGCGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGCCTGCCGAATTCAAGATCCTGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [307]
234	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAATATTG GATGCATAGAGATCGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGATGTCTATGCATCCAATATTGCTAGTG CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [308]
235	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTAGGACTC TCGAAACTTCCCTTGGTCGGGTTGTGACATTGCCCGCTGTGGAGATAAGTGAAGTTTCGAGAGTCCTGCTAGTG CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [309]
236	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTAGAGAACTGAGATGACGAGGAGTGTCAGTGTCAGCCTCATCCTCTCAGTTCTCTCAGCTGGGATATCTCTG

	TCATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGA CTGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [310]
237	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTAATATTGGATGCATAGAGATCGTGTCAGTGTCAAATCTGTAAGCATCCAATATTCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [311]
238	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTGTTGATCTTCGGTTTCACAGAGTGTCAGTGTCAGCTGTAAATTCGAAGATCAACCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [312]
239	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTAGGATCTTGAATTCGGCTGGCGTGTCAGTGTCAACCAGACGTATTCAAGATCCTCAGCTGGGATATCTCTG TCATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGA CTGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [313]
240	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTATTAATCCACACGAGGTCTCCGTGTCAGTGTCAAGAGAGCTAGTGTGGATTAATCAGCTGGGATATCTCTG TCATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGA CTGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [314]
241	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTAACGTCTATGTAC ATACACGAGTGTTTAGTTATCTCGGTATGTACATAGACGTAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGC CTGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGT GATATTCTGCTGTGCAAATCCATGCAAAACT [315]
242	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTAATATTGGATGCA TAGAGATCATGTTTAGTTATTGATTCTATGCATCCAATATAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [316]
243	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTATTAATCCACACG AGGTCTCCGTGTTTAGTTATCGGAACCTCGTGTGGATTAAAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGC CTGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGT GATATTCTGCTGTGCAAATCCATGCAAAACT [317]
244	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTAGGATCTTGAATT CGGCTGGCTTGTTTAGTTATAGCCGCCGAATTCAAGATCCAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGC CTGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGT GATATTCTGCTGTGCAAATCCATGCAAAACT [318]
245	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTATACTTCTGGCCA GATAGGGTGTGTTTAGTGATCATCTATCTGGCCAGAAGTAAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGC CTGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGT GATATTCTGCTGTGCAAATCCATGCAAAACT [319]
246	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGGCCTCGTCATCTCAGTTCTCT ATAGTGAAGCCACAGATGTATAGAGAACTGAGATGACGAGGAGCCTACTGCCTCGGACTTCAAGGGGCTAGA ATTCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAA AATGGTATAAATTAAATCACTTT [320]
247	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGTGAGACCTCGTGTGGATTAAT ATAGTGAAGCCACAGATGTATATTAATCCACACGAGGTCTCCGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT

	TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [321]
248	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGACGACTTGTTCAAGATGATCA ATAGTGAAGCCACAGATGTATTGATCATCTTGAACAAGTCGGGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [322]
249	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCAGGGAAGTTTCGAGAGTCC TATAGTGAAGCCACAGATGTATAGGACTCTCGAAACTTCCCTTGCCTACTGCCTCGGACTTCAAGGGGCTAGAA TTCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAA ATGGTATAAATTAAATCACTTT [323]
250	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTTGCATA GTCTCATACACGTATTAGAGTTACACCCTGGGAGTTATACGTGTATGAGTCTAGGCATCCTTGGAGCACACTTG AGCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGT ATCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [324]
251	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTACATTTA ACATATCCTAGGTATAGAGTTACACCCTGGGAGTTTACCTAGGATATGCTAGCTGTTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [325]
252	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTGAAGTA TTTGTCTGAAAGTGGTAGAGTTACACCCTGGGAGTTCCACTTTCAGACATATAGTTCTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [326]
253	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTGTATGTT CCTGCCTATCCGTCTAGAGTTACACCCTGGGAGTTGACGGATAGGCAGTAACCTACTCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [327]
254	AAAGTTTACTGGTAAAATGTTAGAATATTATATCTATATCCTTGCCAAGCCCTTAGGTGTATGGCTGCCATATTT GGAGGAGCTGACTGAAGATATGATAAGGAGTTTGAAGCAACATTGGAAGCTGTGTGCATCCGGGTTTGTCATT GAAGATCTTCTGGGTAGAGTTACACCCTGGGAGTTCCCAGAAGATCTTGAGTTACATCCTTGGAGCACACTTGA GCCGTCGAGGAATTCTTCATCACTTTAACCTGATTGAGCCAATTTGTGTGCAAGAAGGTAATGTGTCATGAGTA TCTTGGATCATTGATTTGGGCCTTTATTACATTAAAATGATCATTTCA [328]
255	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTACACGA AGAATCTCAGCCCTTGGTCGGGTTGTGACATTGCCCGCTGTGGAGATAAGTGCTGAGATTCTTCGTGTGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [329]
256	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTTGCATAG TCTCATACACGTATGGTCGGGTTGTGACATTGCCCGCTGTGGAGATATAGGTGTATGAGACTATGCAGCTAGTG CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [330]
257	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTACATTTA ACATATCCTAGGTAGGTCGGGTTGTGACATTGCCCGCTGTGGAGATTACATAGGATATGTTAAATGTGCTAGTG CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [331]
258	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTGAAGTAT TTGTCTGAAAGTGGGGTCGGGTTGTGACATTGCCCGCTGTGGAGATCCAATTTCAGACAAATACTTCGCTAGTG

	CTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGACA GCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [332]
259	CGCGGCGCGGGGGGAAGGCAAAGGAAAAGAAGGGGCGGCGGGCCGACGTCGGCGGCGAGGAAGGACGGA GGAGCGCCGGAGCCGCTTTGCTGCCCTCCGCGTGGTCCCGTGCCTCCCCGACACCATGGCCCTGGCTGTATGTT CCTGCCTATCCGTCGGTCGGGTTGTGACATTGCCCGCTGTGGAGATGACTGATAGGCAGGAACATACGCTAGT GCTGGTGATGCTCAGCGCCGCGGAGGACAATGGCTGGGAATCCCCTTTGTTTTCCGGGGCGCTGCCTGGGGAC AGCCCGCGAAGCCTCGGCGCCCGGCCGGCGCGGCCACCACGGAAACCGTTAGTTT [333]
260	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTACATTTAACATATCCTAGGTAGTGTCAGTGTCAGACCTCGGTTATGTTAAATGTCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [334]
261	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTGAAGTATTTGTCTGAAAGTGGGTGTCAGTGTCAACGCTCTCCGACAAATACTTCCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [335]
262	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTTGCATAGTCTCATACACGTATGTGTCAGTGTCGTTACGCGTCTGAGACTATGCACAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [336]
263	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTACACGAAGAATCTCAGCCCTTTGGTCAGTGTCCCAGGGATGCGATTCTTCGTGTCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [337]
264	TGAGTGCCAGGACTAGACCTGGTACTAGGAAGCAGCTGCATTGGATTTACCAGGCTTTTCACTCTTGTATTTTAC AGGGCTGGGACAGGCCTGGACTGCAAGGAGGGGTCTTTGCACCATCTCTGAAAAGCCGATGTGTATCCTCAGC TTTGTATGTTCCTGCCTATCCGTCGTGTCAGTGTCAAACGGCTAAGCAGGAACATACCAGCTGGGATATCTCTGT CATCGTGGGCTTGAGGACCTGGAGAGAGTAGATCCTGAAGAACTTTTTCAGTCTGCTGAAGAGCTTGGAAGAC TGGAGACAGAAGGCAGAGTCTCAGGCTCTGAAGGTATAAGGAGTGTGAGTTCCTGTGAGAAAC [338]
265	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTTTGCATAGTCTCAT ACACGTATTGTTTAGTTATATACTGTATGAGACTATGCACTAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCCT GTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTGA TATTCTGCTGTGCAAATCCATGCAAAACT [339]
266	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTACATTTAACATATC CTAGGTACTGTTTAGTTATGTACTAGGATATGTTAAATGAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCCT GTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTGA TATTCTGCTGTGCAAATCCATGCAAAACT [340]
267	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTGAAGTATTTGTCT GAAAGTGGCTGTTTAGTTATGCCGTTTCAGACAAATACTTAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGC CTGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGT GATATTCTGCTGTGCAAATCCATGCAAAACT [341]
268	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTGTATGTTCCTGCCT ATCCGTCCTGTTTAGTTATGGATGATAGGCAGGAACATAAAAGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [342]
269	TAGTTGTGCAAATCTATGCAAAACTGATGGTGGCCTGCTATTTCCTTCAAATGAATGATTTTTACTAATTTTGTGT ACTTTTATTGTGTCGATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTTGCATAGTCTCAT ACACGTATCTGTTTAGTTATGATAGTGTATGAGACTATGTTAGGTACTGCTAGCTGTAGAACTCCAGCTTCGGCC

	TGTCGCCCAATCAAACTGTCCTGTTACTGAACACTGTTCTATGGTTAGTTTTGCAGGTTTGCATCCAGCTGTGTG ATATTCTGCTGTGCAAATCCATGCAAAACT [343]
270	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGAATCAGTCAACGTGTTGTC ATAGTGAAGCCACAGATGTATGACAACACGTTGACTGATTCTGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [344]
271	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGTACGGATAGGCAGGAACATA CATAGTGAAGCCACAGATGTATGTATGTTCCTGCCTATCCGTCGCCTACTGCCTCGGACTTCAAGGGGCTAGAA TTCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAA ATGGTATAAATTAAATCACTTT [345]
272	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCTACGTGTATGAGACTATGCA ATAGTGAAGCCACAGATGTATTGCATAGTCTCATACACGTATGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [346]
273	TGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCGA CTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGACCAGAAGATCTTCAATGACA ATAGTGAAGCCACAGATGTATTGTCATTGAAGATCTTCTGGGGCCTACTGCCTCGGACTTCAAGGGGCTAGAAT TCGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAA TGGTATAAATTAAATCACTTT [347]

The gene sequences in each individual target transcript for which the Solution trigger sequences were designed against are summarised in the following Table 14, which lists their individual ENSEMBL transcript and Exon IDs. HDR templates are designed on this basis.

Table 14:

Soln ID	Solution Name	Transcript ID	Exon# [intron#]	ENSE ID
180	20_PPARG-hsa-let-7c	ENST00000397010.7	5	ENSE00003693703
181	22_PPARG-hsa-let-7c	ENST00000397010.7	5	ENSE00003693703
182	23_PPARG-hsa-let-7c	ENST00000487973.1	Intron 3	Chr10: 22,448,990-22,454,165
183	12_PPARG-hsa-let-7c	ENST00000397010.7	6	ENSE00003577748
184	16_PPARG-hsa-let-7c	ENST00000397010.7	3	ENSE00003663534
185	22_PPARG-hsa-let-7i	ENST00000397010.7	5	ENSE00003693703
186	20_PPARG-hsa-let-7i	ENST00000397010.7	5	ENSE00003693703
187	14_PPARG-hsa-let-7i	ENST00000397010.7	6	ENSE00003577748
188	23_PPARG-hsa-let-7i	ENST00000487973.1	Intron 3	Chr10: 22,448,990-22,454,165
189	16_PPARG-hsa-mir-146a	ENST00000397010.7	3	ENSE00003663534
190	12_PPARG-hsa-mir-146a	ENST00000397010.7	6	ENSE00003577748
191	14_PPARG-hsa-mir-146a	ENST00000397010.7	6	ENSE00003577748
192	22_PPARG-hsa-mir-146a	ENST00000397010.7	5	ENSE00003693703
193	20_PPARG-hsa-mir-146a	ENST00000397010.7	5	ENSE00003693703
194	22_PPARG-hsa-mir-20a	ENST00000397010.7	5	ENSE00003693703
195	12_PPARG-hsa-mir-20a	ENST00000397010.7	6	ENSE00003577748

196	16_PPARG-hsa-mir-20a	ENST00000397010.7	3	ENSE00003663534
197	23_PPARG-hsa-mir-20a	ENST00000487973.1	Intron 3	Chr10: 22,448,990-22,454,165
198	12_PPARG-hsa-mir-30opt	ENST00000397010.7	6	ENSE00003577748
199	14_PPARG-hsa-mir-30opt	ENST00000397010.7	6	ENSE00003577748
200	15_PPARG-hsa-mir-30opt	ENST00000397010.7	4	ENSE00003791065
201	20_PPARG-hsa-mir-30opt	ENST00000397010.7	5	ENSE00003693703
202	33_IRF4-hsa-let-7c	ENST00000696871.1	9	ENSE00001957207
203	41_IRF4-hsa-let-7c	ENST00000380956.9	3 UTR	ENSE00003968729
204	30_IRF4-hsa-let-7c	ENST00000696871.1	9	ENSE00001957207
205	37_IRF4-hsa-let-7c	ENST00000380956.9	3 UTR	ENSE00003968729
206	47_IRF4-hsa-let-7c	ENST00000696871.1	3	ENSE00003692200
207	41_IRF4-hsa-let-7i	ENST00000380956.9	3 UTR	ENSE00003968729
208	47_IRF4-hsa-let-7i	ENST00000696871.1	3	ENSE00003692200
209	50_IRF4-hsa-let-7i	ENST00000380956.9	3 UTR	ENSE00003968729
210	33_IRF4-hsa-let-7i	ENST00000696871.1	9	ENSE00001957207
211	49_IRF4-hsa-let-7i	ENST00000380956.9	3 UTR	ENSE00003968729
212	41_IRF4-hsa-mir-146a	ENST00000380956.9	3 UTR	ENSE00003968729
213	34_IRF4-hsa-mir-146a	ENST00000380956.9	3 UTR	ENSE00003968729
214	49_IRF4-hsa-mir-146a	ENST00000380956.9	3 UTR	ENSE00003968729
215	50_IRF4-hsa-mir-146a	ENST00000380956.9	3 UTR	ENSE00003968729
216	30_IRF4-hsa-mir-146a	ENST00000696871.1	9	ENSE00001957207
217	41_IRF4-hsa-mir-20a	ENST00000380956.9	3 UTR	ENSE00003968729
218	30_IRF4-hsa-mir-20a	ENST00000696871.1	9	ENSE00001957207
219	37_IRF4-hsa-mir-20a	ENST00000380956.9	3 UTR	ENSE00003968729
220	49_IRF4-hsa-mir-20a	ENST00000380956.9	3 UTR	ENSE00003968729
221	33_IRF4-hsa-mir-20a	ENST00000696871.1	9	ENSE00001957207
222	30_IRF4-hsa-mir30opt	ENST00000696871.1	9	ENSE00001957207
223	33_IRF4-hsa-mir30opt	ENST00000696871.1	9	ENSE00001957207
224	34_IRF4-hsa-mir30opt	ENST00000380956.9	3UTR	ENSE00003968729
225	37_IRF4-hsa-mir30opt	ENST00000380956.9	3 UTR	ENSE00003968729
226	85_KDM6B-hsa-let-7c	ENST00000448097.7	12	ENSE00001295739
227	108_KDM6B-hsa-let-7c	ENST00000448097.7	20	ENSE00001289989
228	109_KDM6B-hsa-let-7c	ENST00000448097.7	13	ENSE00001315243
229	94_KDM6B-hsa-let-7c	ENST00000448097.7	13	ENSE00001315243
230	90_KDM6B-hsa-let-7c	ENST00000448097.7	20	ENSE00001289989
231	110_KDM6B-hsa-let-7i	ENST00000448097.7	3 UTR	ENSE00001696731
232	85_KDM6B-hsa-let-7i	ENST00000448097.7	12	ENSE00001295739
233	96_KDM6B-hsa-let-7i	ENST00000448097.7	12	ENSE00001295739
234	108_KDM6B-hsa-let-7i	ENST00000448097.7	20	ENSE00001289989
235	94_KDM6B-hsa-let-7i	ENST00000448097.7	13	ENSE00001315243
236	85_KDM6B-hsa-mir-146a	ENST00000448097.7	12	ENSE00001295739
237	108_KDM6B-hsa-mir-146a	ENST00000448097.7	20	ENSE00001289989

238	109_KDM6B-hsa-mir-146a	ENST00000448097.7	13	ENSE00001315243
239	96_KDM6B-hsa-mir-146a	ENST00000448097.7	12	ENSE00001295739
240	90_KDM6B-hsa-mir-146a	ENST00000448097.7	20	ENSE00001289989
241	110_KDM6B-hsa-mir-20a	ENST00000448097.7	3 UTR	ENSE00001696731
242	108_KDM6B-hsa-mir-20a	ENST00000448097.7	20	ENSE00001289989
243	90_KDM6B-hsa-mir-20a	ENST00000448097.7	20	ENSE00001289989
244	96_KDM6B-hsa-mir-20a	ENST00000448097.7	12	ENSE00001295739
245	97_KDM6B-hsa-mir-20a	ENST00000448097.7	12	ENSE00001295739
246	85_KDM6B-hsa-mir-30opt	ENST00000448097.7	12	ENSE00001295739
247	90_KDM6B-hsa-mir-30opt	ENST00000448097.7	20	ENSE00001289989
248	93_KDM6B-hsa-mir-30opt	ENST00000448097.7	21	ENSE00001314215
249	94_KDM6B-hsa-mir-30opt	ENST00000448097.7	13	ENSE00001315243
250	61_STAT6-hsa-let-7c	ENST00000300134.8	3 UTR	ENSE00001107465
251	65_STAT6-hsa-let-7c	ENST00000300134.8	3 UTR	ENSE00001107465
252	78_STAT6-hsa-let-7c	ENST00000300134.8	4	ENSE00003484230
253	59_STAT6-hsa-let-7c	ENST00000300134.8	3 UTR	ENSE00001107465
254	62_STAT6-hsa-let-7c	ENST00000300134.8	13	ENSE00003661799
255	66_STAT6-hsa-let-7i	ENST00000300134.8	3 UTR	ENSE00001107465
256	61_STAT6-hsa-let-7i	ENST00000300134.8	3 UTR	ENSE00001107465
257	65_STAT6-hsa-let-7i	ENST00000300134.8	3 UTR	ENSE00001107465
258	78_STAT6-hsa-let-7i	ENST00000300134.8	4	ENSE00003484230
259	59_STAT6-hsa-let-7i	ENST00000300134.8	3 UTR	ENSE00001107465
260	65_STAT6-hsa-mir-146a	ENST00000300134.8	3 UTR	ENSE00001107465
261	78_STAT6-hsa-mir-146a	ENST00000300134.8	4	ENSE00003484230
262	61_STAT6-hsa-mir-146a	ENST00000300134.8	3 UTR	ENSE00001107465
263	66_STAT6-hsa-mir-146a	ENST00000300134.8	3 UTR	ENSE00001107465
264	59_STAT6-hsa-mir-146a	ENST00000300134.8	3 UTR	ENSE00001107465
265	69_STAT6-hsa-mir-20a	ENST00000300134.8	3 UTR	ENSE00001107465
266	65_STAT6-hsa-mir-20a	ENST00000300134.8	3 UTR	ENSE00001107465
267	78_STAT6-hsa-mir-20a	ENST00000300134.8	4	ENSE00003484230
268	59_STAT6-hsa-mir-20a	ENST00000300134.8	3 UTR	ENSE00001107465
269	61_STAT6-hsa-mir-20a	ENST00000300134.8	3 UTR	ENSE00001107465
270	54_STAT6-hsa-mir-30opt	ENST00000300134.8	19	ENSE00003520637
271	59_STAT6-hsa-mir-30opt	ENST00000300134.8	3 UTR	ENSE00001107465
272	61_STAT6-hsa-mir-30opt	ENST00000300134.8	3 UTR	ENSE00001107465
273	62_STAT6-hsa-mir-30opt	ENST00000300134.8	13	ENSE00003661799

In addition to the negative control constructs listed in Tables 9-13 above, an additional miR30-based construct was purchased from Vector Builder.

A dual plasmid expression/reporter system was used to test the specificity of the individual extended solutions via their ability to bind with perfect complementarity to their cognate target

gene sequences. The basis for the system is outlined in Fig. 20a. The combined target gene sequences to be tested were inserted as a contiguous sequence into the 3' untranslated region downstream of the GFP coding region of the transcript (CopGFP fast maturing bright variant; see pCDH_reporter plasmid map. Vector Fig. 20b) and expression was driven by EF1a promoter. On the same vector, a PGK promoter drives expression of the low affinity NGF receptor, which was expressed on the cell surface and is used to identify successfully transfected cells using labelled antibodies in flow cytometry.

Each extended solution was synthesised and cloned into a second commercially available expression plasmid (Vector Builder; GEiGS Validation system_v1. Vector 2, Fig. 20c). This plasmid was also a mammalian expression system with the EF1 alpha (EF1a) promoter driving transcription of open reading frames for a fluorescent reporter (dsRED), antibiotic resistance (*pac*; N-acetyl puromycin transferase) and the sequence of each extended GEiGS solution. The polycistronic transcript contained protein coding sequences separated by short T2A sequences (encoding amino acid sequence EGRGSLLTCGDVEEN**PG**P (SEQ ID NO: 683)). The T2A sequences ensured that the dsRED and N-acetyl puromycin transferase were translated as separate proteins due to ribosomal skipping. The dsRED was used to identify successfully transfected cells by flow cytometry. The level of dsRed expression in the cell (fluorescence intensity) was assayed and used as a proxy for the expression level/abundance of the GEiGS solution.

When plasmids were co-transfected into cells, each solution was individually expressed from Vector 2. Upon binding to the respective target gene sequences downstream of coding region for CopGFP (Vector 1), degradation of the whole transcript occurred and a decrease (regression) in the GFP signal was detected by flow cytometry. A schematic of the reported vector is provided in Fig. 20d.

The Solutions identified by the sequences in Tables 9-13 were screened using this methodology. Additionally, the screen verified the activity of Solution 260 (SEQ ID NO: 216, SEQ ID NO: 240, SEQ ID NO: 334), Solution 266 (SEQ ID NO: 222, SEQ ID NO: 246 and SEQ ID NO: 340), Solution 219 (SEQ ID NO: 127, SEQ ID NO: 151 and SEQ ID NO: 293) and Solution 245 (SEQ ID NO: 177, SEQ ID NO: 201 and SEQ ID NO: 319).

Several different protocols are available to deliver plasmid DNA to the monocytic cell line (THP-1) or iPSC-derived monocytes. For the reported screens, cell types were transfected according to the manufacturer's protocols for use of lipid-based reagents (Mirus TransIT® Transfection reagent) once the optimal titre was established for reagent to DNA mixture required for DNA delivery to the cells. Typical initial test ratios of TransIT reagent to plasmid

DNA were in the range of 2:1 (vol.:vol.) but more typically 4:1 or 5:1. This transfection method is useful for hard to transfect primary cells, such as donor monocytes or T cells.

Briefly, cells to transfect were passaged 20-24 hours before use and plated at a density of between 1 and 5 x10^5 cells/ml of culture medium, ideally between 3 and 5 x10^5 cells/ml, to ensure that cells were actively dividing, and the appropriate density was achieved at the time of transfection (>80% confluence). The plasmid DNA was made highly pure and contamination free. The actual ratio of TransIT reagent to plasmid DNA was determined by the format of cell culture (diameter of tissue culture well). The complex was prepared shortly before and left to complex at room temperature for 15-30 minutes using serum free growth medium, before its addition to the cells in a drop-wise manner. Cells to be transfected were grown in the presence of serum and after addition of the reagent:DNA complexes the cells were left in culture for 72 hours. After culture, cells were resuspended and analysed by flow cytometry. The levels of GFP regression of GFP in cells that stably expressing dsRED was quantified. Cells that have regressed GFP and retained dsRED were compared to those cells expressing 'scrambled' solutions with stable dual expression of GFP and dsRED.

The expression of the GEiGS sRNA (expressed from Vector 1) within the transfected cells was also measured by qPCR and compared to the expression of the corresponding endogenous miRNA scaffold in either THP-1 or monocytes. This showed that the GEiGS sRNA was expressed at comparable levels to the endogenous miRNA scaffold, indicating that expression from the plasmid was not supra-physiological, therefore unlikely to cause significant off-target effects or interfere with the normal functions of the RNAi pathway in the cell.

Example 11 - Gene editing of an endogenous miRNA scaffold in macrophages.

A number of the Solutions identified above (Example 10; Tables 9-14) are being implemented as full GEiGS, i.e. by editing the endogenous loci of the respective miRNA scaffolds in human monocytes and iPSC. Several stimulation-induced miRNAs can be detected in primary human monocyte derived macrophages and the THP-1 cell line (see Fig. 18a, b and c). Gene editing is performed by CRISPR knock-in using a methodology described in the paper by Freund *et al.* 2020 (J. Expal. Med. 2020; 217 No. 7 e20191692; doi: doi.org/10.1084/jem.20191692). This paper describes the delivery of Cas9-RNP complexes into cells and the routine generation of >90% gene edited cells without the need for cell selection.

The methodology uses that of Freund *et al.* preferably recommends fresh bulk myeloid cells or CD14+ monocytes isolated from PBMC by fluorescence activated cell sorting (FACS),

though it also is used with cells that are derived from iPSC by *in vitro* differentiation and are cultured.

PBMCs are isolated using Sepmate tubes (StemCell Technologies) and ACK (ammonium-chloride-potassium) lysing buffer (Thermo Fisher Scientific) following the manufacturer's protocol from buffy coats or leukopaks from healthy donors. Human monocytes are isolated from the PBMCs using the human monocyte isolation kit (Miltenyi) according to the manufacturer's protocol. Monocytes are used freshly enriched or aliquoted and frozen in 10% DMSO and FBS for further use.

The gRNAs for individual Solutions are obtained from Integrated DNA Technologies (IDT) as synthetic sgRNAs. Cas9 protein is obtained from IDT. Immediately after isolation from donor peripheral blood mononuclear cell isolates (PBMC), or isolated from iPSC, or freshly thawed the monocytes ($0.5–5 \times 10^6$) are electroporated using the Lonza 4D-Nucleofector device (4D-Nucleofector Core Unit: Lonza, AAF-1002B; 4D-Nucleofector X Unit: AAF-1002X) (Buffer P3, device setting CM-137) and then contacted with the sgRNA/Cas9 ribo-nucleoprotein complexes (RNP) and the appropriate dsDNA HDR template either produced internally or made commercially (IDT or Genewiz).

After electroporation, monocyte-derived macrophages are cultured in macrophage medium (DMEM high glucose supplemented with 10% FBS [Gibco], 2mMGlutaMAX, 100 U/ml penicillin, 100 µg/ml streptomycin, and M-CSF 100 ng/ml [Peprotech]) and the medium changed every 2–3 days by adding half the volume of medium with 1× cytokines into each well.

Several days after electroporation cells are isolated for analysis of target gene expression using flow cytometry and gDNA and RNA extracted for PCR, Sanger sequencing and NGS amplicon sequencing to assess the efficiency of gene editing.

<u>Example 12 - Analysis of potential off-target effects caused by GEiGS demonstrates that GEiGS is very specific, showing no off-target effects</u>

Systematic unbiased whole transcriptome analysis of Solution 29 (S29) GEiGS lines and corresponding controls was performed to assess whether GEiGS-mediated silencing causes off-target effects.

Isogenic GEiGS modified iPSC cells and control iPSC cell lines were generated from the same parental iPSC line and RNA-seq was used to measure gene expression. A differential gene

expression (DGE) analysis was performed using the parental line as reference to detect transcriptional changes genome-wide in an unbiased way. This approach detects both direct changes in gene expression caused by GEiGS (specific target silencing, potential silencing of unintended targets, and potential upregulation of the cognate target of the redirected miRNA) as well as indirect changes caused by knock-on effects from the direct changes in gene expression. RNA-seq and DEG analysis were performed using standard techniques.

The GEiGS lines analysed were homozygous for solution 29 knock-in (S29 KI/KI), which is a B2M-targeting GEiGS solution based on MIR20A as scaffold (see Example 3 for details of implementation of GEiGS for Solution 29). The following control lines were also generated:

(i) B2M homozygous KO (B2M KO/KO) generated by CRISPR KO, to detect transcriptional changes caused specifically by B2M silencing; and

(ii) hsa-mir-20a homozygous KO (hsa-mir-20a KO/KO), to detect changes in gene expression caused by the loss of activity of the miRNA gene used hijacked as scaffold for solution 29.

Each experimental sample included at least two biological replicates (independent clones with the same genotype) in two technical replicates (independent cultures of the same clone). DGE analysis was performed using the R package edgeR, using false discovery rate (FDR) 0.05 as a statistical significance threshold, with no effect size cutoff.

Results are shown in Fig. 22a-22c. When comparing B2M KO/KO to the parental cell line, it can be seen that the CRISPR KO of B2M led to a very specific and significant reduction of mRNA levels transcribed from the B2M gene (91% down-regulation); B2M is the only differentially expressed gene (Fig. 22a). No other changes in gene expression were detected indicating loss of MHC-I expression in a steady state culture of iPSC has no consequence on the physiology of the cell (Fig. 22a).

In the KO of hsa-mir-20a, no changes in expression were caused by the loss of the edited miRNA, providing experimental evidence for the functional redundancy in the miRNA network that is leveraged by GEiGS (Fig. 22b).

In the GEiGS modified cells in which Solution 29 had been introduced into the endogenous hsa-mir-20a locus, again there was very specific and significant reduction of mRNA levels transcribed from the B2M gene compared to the parental cell line; B2M is again the only differentially expressed gene (Fig. 22c). This shows that GEiGS is very specific, only silencing the intended target and no other unintended targets are silenced.

Notably, off-target effects previously associated with silencing of unintended targets when using siRNA or shRNA are not detected. This further supports the application of GEiGS for the highly specific silencing of target genes, avoiding off-target effects such as silencing of unintended targets as well as potential loss-of-function effects following redirection of miRNA genes used as scaffolds.

Example 13 - Additional GEiGS solutions targeting B2M

Further B2M GEiGS solutions were designed as set out in Example 1 and are detailed in Table 22. As before, pipeline inputs were: the mRNA sequence of B2M (GenBank Accession: NM_004048.4), the human genome reference sequence (GRCh38), miRNA annotations (miRbase v22) and cell type specific miRNA quantifications.

Expression of miRNAs in human iPSCs and ESCs was quantified using publicly available next-generation sequencing (NGS) datasets: ENCODE accession ENCSR958UOC, ENCSR430YFL.

The GEiGS® solution designs were comprised of a modified sequence of an endogenous pre-miRNA (hair–in) – also referred to as the endogenous miRNA scaffold – modified to encode a novel silencing RNAi trigger instead of its native guide strand. The novel GEiGS® silencing RNA matched the B2M mRNA by sequence complementarity and was predicted to lead to the silencing of B2M through the RNA interference pathway.

Extended solutions are comprised of a GEiGS solution as designed computationally, plus 40 bases or 50-150 bases of genomic sequence flanking on both sides of the endogenous pre-miRNA that the respective GEiGS solution was based on. This is included to avoid omitting important sequences that act as a signal for the enzyme Drosha.

The B2M GEiGS solutions detailed in Table 22 are tested for silencing activity via the plasmid-based ectopic expression assay detailed in Example 2. As before, a broad spectrum of B2M silencing is observed, ranging from 5%-99%. The most effective solutions show strong silencing activity.

Table 22 Sequences of computationally-designed B2M-targeting RNAi trigger sequences and GEiGS solutions

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**

Sol-1	hsa-mir-320a	TAAACCTGAATCTTTGGAGTAC [348]	CTCCCCTCTACTCCAAATTTTCAGGTCTACCCGGAGTCGGGtaaacctgaatctttggagtacAAAAGGATG [412]
2	hsa-mir-520f	TAAACCTGAATCTTTGGAGTAC [349]	TCTCAGGCTGTGGTACTCCAAAGATTCAGGTTTTCTGTGGTCAGAAAGAAAAGtaaacctgaatctttggagtacTACCGTTTGGGA [413]
3	hsa-mir-652	TAAACCTGAATCTTTGGAGTAC [350]	ACGAATGGCTATGCACTGTACTCCAAAGAGAGAGTCAGGTTTACACATAGACTATAATTGtaaacctgaatctttggagtacAGTGCACAACCTACAC [414]
4	hsa-mir-106a	TTCAGTAAGTCAACTTCAATGTC [351]	CCTTGGCCATGTttcagtaagtcaacttcaatgtcCTTTTTGAGATGACTTGAAATTGACTTACTGCAAACATTACCATGG [415]
5	hsa-mir-1180	TTTTTCAATTCTCTCTCCATTC [352]	GCTGCTGGGGAGAGCGATTGAAAAATAGTGCTCCTGGTTGtttttcaattctctctccattcCGGCGGC [416]
6	hsa-mir-15b	TTTTTCAATTCTCTCTCCATTC [353]	TTGAGGCCTTAAAGTACTGtttttcaattctctctccattcTGCTACAGTCAAGAGACTGGAGAGTGATTTGAAAACTAGAAATTTAAGGAAATTCAT [417]
7	hsa-mir-182	TTCTTCAGTAAGTCAACTTCAATG [354]	GAGCTGCTTGCCTCCCCCCGTTttcttcagtaagtcaacttcaatgGGTGAGGTAACAGGATCCCATTGTTGACGACCTGAAGACTATGGGGCGAGGACTCAGCCGGCAC [418]
9	hsa-mir-23a	TTCAGTGTAGTACAAGAGATAG [355]	GGCCGGCCTATCCTTGTACGACACGGAATTGCTTCCTGTCACAAttcagtgtagtacaagagatagACCGACC [419]
10	hsa-mir-26b	TTCAGTAAGTCAACTTCAATGT [356]	CCGGGACCCAGttcagtaagtcaacttcaatgtGTGTGCTGTCCACATTGAAGTCCACTTACTGACTCGGGGACCGG [420]
13	hsa-mir-320a	TTTCAATTCTCTCTCCATTCTT [357]	CTCCCCTCAGAATGGAGTTAGAATTGCAACCCGGAGTCGGGtttcaattctctctccattcttAAAAGGATG [421]
14	hsa-mir-335	TTCAGTAAGTCAACTTCAATGTC [358]	TGTTTTGAGCGGGGGttcagtaagtcaacttcaatgtcTTGTCATAAACACATTGAAATTGACTTACCGAACCTCCTCTCATTTGCTATATTCA [422]
15	hsa-mir-361	TTTCAATTCTCTCTCCATTCTT [359]	GGAGCtttcaattctctctccattcttTTTATAATTTCAAAAAAACAATGAAGTGTGAGAATTGATTGCTTC [423]

16	hsa-mir-518b	TTCTTCAGTAAGTCAACTTCAA [360]	TCATGCTGTGGCTGAACTTGACTAACTGAAGTCAATTGTCTGAAAGAAAAttcttcagtaagtcaacttcaaTTACGGTTTGA [424]
17	hsa-mir-520f	TTTCAATTCTCTCTCCATTCTT [361]	TCTCAGGCTGTGAAGAATGGAGAGAGAATTGAATCTGTGGTCAGAAAGAAAAGtttcaattctctctccattcttTACCGTTTGGGA [425]
18	hsa-mir-652	TTCAGTGTAGTACAAGAGATAG [362]	ACGAATGGCTATGCACTCTATCTCTTGTAGAGAGTACACTGAACACATAGACTATAATTGttcagtgtagtacaagagatagAGTGCACAACCTACAC [426]
22	hsa-mir-1180	TTAACTATCTTAACAAGCTTTG [363]	GCTGCTGCGCTTGTCAAATAGTTAATAGTGCTCCTGGTTGttaactatcttaacaagctttgCGGCGGC [427]
23	hsa-mir-1307	TAACAAATTTCCAATAATCCTGT [364]	CATCAAGACCCAGCTGAGTCACTGTCACTGCCTACCAAACTGGATTATAGATATATTGTCACGTCTGTGTTGCCAATCGtaacaaatttccaataatcctgtTAGATAGGCGGTCATGCATACGAATTTTCAGCTCTTGTTCTGGTGAC [428]
24	hsa-mir-15b	TAACATTATTATAACCCTACAT [365]	TTGAGGCCTTAAAGTACTGtaacattattataaccctacatTGCTACAGTCAAGAATCTAGGGTTTTATTAATGTTCTAGAAATTTAAGGAAATTCAT [429]
25	hsa-mir-182	TATCAATATTAAAAAGCAAGCAAG [366]	GAGCTGCTTGCCTCCCCCCGTTtatcaatattaaaaagcaagcaagGGTGAGGTAACAGGATCCCTTGGCTTTTGACTATTGATCTATGGGGCGAGGACTCAGCCGGCAC [430]
28	hsa-mir-205	TTCATTCATTATAACAAATTTC [367]	AAAGATCCTCAGACAATCCATGTGCTTCTCTTGttcattcattataacaaatttcTCTCATACCCAACGAAATTTGATATAATGAATGTTCAGGAGGCATGGAGCTGACA [431]
31	hsa-mir-22	TTAACATTATTATAACCCTACA [368]	GGCTGAGCCGCATGTAGGGTTATATAATGATTAATATGTCCTGACCCAGCTAttaacattattataaccctacaTGCCCTCTGCC [432]
32	hsa-mir-221	TAACATTATTATAACCCTACATT [369]	TGAACATCCAGGTCTGGGGCATAATTAGGGTTTTAATAATGTATTTTAGTGTTCGTTAGGCAACtaacattattataaccctacattAGGCTACCTGGAAACATGTTCTC [433]
33	hsa-mir-222	TGTTAACATTATTATAACCCTACA [370]	GCTGCTGGAAGGTGTAGGTACCCTCAATTAGGGTTATAATAAGTTAAATCCTGTCTTTCGTAATCAGCtgttaacattattataaccctacaTGATGGCATCTTCTAGCT [434]

34	hsa-mir-23a	TATAAAGAAGATCATGTCCATG [371]	GGCCGGCCATGGCATGATCGTCTTGATATTGCTTCCTGTCACAAtataaagaagatcatgtccatgACCGACC [435]
37	hsa-mir-30e	TTTATGATTTATTTAACTTGTGGA [372]	GGGCAGTCTTTGCTACtttatgatttatttaacttgtggaGTAAGGTGTTCAGAGGTCCACAAGTTATAAATCATAAAGCGGCAGGCTGCCA [436]
38	hsa-mir-320a	TTATTATAACCCTACATTTTGT [373]	CTCCCCTCCAAAATGTATTGTTATAACAACCCGGAGTCGGGttattataaccctacattttgtAAAAGGATG [437]
39	hsa-mir-335	TAACATTATTATAACCCTACATT [374]	TGTTTTGAGCGGGGGtaacattattataaccctacattTTGTCATAAACATGTAGGGATATAATAATCTTACCTCCTCTCATTTGCTATATTCA [438]
40	hsa-mir-361	TTATTATAACCCTACATTTTGT [375]	GGAGCttattataaccctacattttgtTTTATAATTTCAAAAAACCAAATATATGTGGTTATAATTTGCTTC [439]
42	hsa-mir-423	TAACTTATGCACGCTTAACTATC [376]	ATAAAGGAAGTTAGGATAGTTAAGAGGCAGAAGTATATTTCTATTTTCCAAAtaacttatgcacgcttaactatcCTTGCTTCCTAACCCGCGC [440]
44	hsa-mir-519c	TAACATTATTATAACCCTACAT [377]	TCTCAGCCTGTGACGTAGGGTTATAAAAATGTTACTGTTGTCTGAAAGAAAAGtaacattattataaccctacatTACAGTTTGAGA [441]
45	hsa-mir-520f	TTTATGATTTATTTAACTTGTG [378]	TCTCAGGCTGTGCACAAGTTAAATAAATCATAATCTGTGGTCAGAAAGAAAAGtttatgatttatttacttgtgTACCGTTTGGGA [442]
46	hsa-mir-652	TTCATTATAACAAATTTCCAAT [379]	ACGAATGGCTATGCACTATTGGAAATTTGGAGAGTATAATGAACACATAGACTATAATTGttcattataacaaatttccaatAGTGCACAACCTACAC [443]
48	hsa-mir-92b	TTTTATGATTTATTTAACTTGT [380]	CGGGCCCCGGGCGGGCGACAAGGATAAATAACATCATAAAATTGTTTTTTCCCCCGCCAAtttttatgatttatttaacttgtGGCCCCCCCGGCCC [444]
152	hsa-mir-302a	TTAACTTATGCACGCTTAACTAT [387]	CCACTAGTAAAGCGTGCGAGAGTTAACTTTGAAACTAAAGAAGTTAACTTATGCACGCTTAACTATTGG [451]
153	hsa-mir-302a	TAACAAATTTCCAATAATCCTGT [388]	CCACCAGGTTTATTGGAATTTTGTTACTTTGAAACTAAAGAAGTAACAAATTTCCAATAATCCTGTTGG [452]
154	hsa-mir-302b	TTAACTTATGCACGCTTAACTAT [389]	GCTCACTTCAAGTATAGCGTGCATAAGTTATCTGTGACTTTAAAAGTTAACTTATGCACGCTTAACTATGAGT [453]

155	hsa-mir-302b	TAACAAATTTCCAATAATCCTGT [390]	GCTCCCTTCAAGGTATATTGGAAATTTGTTTCTGTGACTTTAAAAGTAACAAATTTCCAATAATCCTGTGAGT [454]
156	hsa-mir-302c	TTAACTTATGCACGCTTAACTAT [391]	CCTATAGTCTGGCGTGCATAAGTCAACTGTGTGAAACAAAAGTTAACTTATGCACGCTTAACTATAGG [455]
157	hsa-mir-302c	TAACAAATTTCCAATAATCCTGT [392]	CCTACAGGTCTATTGGAAATTTGCTACTGTGTGAAACAAAAGTAACAAATTTCCAATAATCCTGTAGG [456]
158	hsa-mir-302a	TACAAGAGATAGAAAGACCAGTC [393]	CCACACTGTTCTTTCTATGTCTTGTACTTTGAAACTAAAGAAGTACAAGAGATAGAAAGACCAGTCTGG [457]
159	hsa-mir-302b	TACAAGAGATAGAAAGACCAGTC [394]	GCTCGCTTCCCTGTACTTTCTATCTCTTGTTCTGTGACTTTAAAAGTACAAGAGATAGAAAGACCAGTCGAGT [458]
160	hsa-mir-302c	TACAAGAGATAGAAAGACCAGTC [395]	CCTGACTGTGCTTTCTATCTCTTCTACTGTGTGAAACAAAAGTACAAGAGATAGAAAGACCAGTCAGG [459]
161	hsa-mir-375	TTAACTTATGCACGCTTAACTA [396]	CCCAGTTCAAGCGTCTTTACGTTAACCGGACCTGAGCGTTTAACTTATGCACGCTTAACTAGGC [460]
162	hsa-mir-375	TAACAAATTTCCAATAATCCTG [397]	CCAAGGACTTATTGCCTATCTGTTACCGGACCTGAGCGTTAACAAATTTCCAATAATCCTGGGC [461]
163	hsa-mir-375	TACAAGAGATAGAAAGACCAGT [398]	CCTCTGGATCTTTCCCACTATTGTACCGGACCTGAGCGTTACAAGAGATAGAAAGACCAGTGGC [462]
309	hsa-let-7b	TTGACTTTCCATTCTCTGCTGG [399]	CGGGGTTGACTTTCCATTCTCTGCTGGTCAGGGCAGTGATGTTGCCCCTCGGCAAATCCAGCAGAGAATGGAAAGTCACCCTG [463]
310	hsa-let-7g	TTGACTTTCCATTCTCTGCTGG [400]	AGGCTTGACTTTCCATTCTCTGCTGGTGAGGGTCTAGGATACCACCCGGTACCGGAGATCCAGCAGAGAATCGAAAGTCAGCCA [464]
311	hsa-mir-98	TTGACTTTCCATTCTCTGCTGG [401]	AGGATTCTGCTCATGCCAGGGTTGACTTTCCATTCTCTGCTGGGTGGGGTAGGGATATTAGGCCCCAATTAGAAGATCCATCAGAGAATGGAAAGTCTCCCTGGTGTGTGGCATATTCA [465]
312	hsa-mir-98	GGCGTCTCGGTGGCACGACTTGT [402]	AGGATTCTGCTCATGCCAGGGGGCGTCTCGGTGGCACGACTTGGTGGGGTAGGGATATTAGG

			CCCCAATTAGAAGACCAATTCGTGCCACCGAG ATGACCCTGGTGTGTGGCATATTCA [466]
313	hsa-let-7b	TAACAAATTTCCAATAATC CTGT [403]	CGGGGTAACAAATTTCCAATAATCCTGTCAGG GCAGTGATGTTGCCCCTCGGAAGCTCAGGATT ATTGGAAATTTGTTCCCTG [467]
314	hsa-let-7g	TAACAAATTTCCAATAATC CTGT [404]	AGGCTAACAAATTTCCAATAATCCTGTGAGGG TCTATGATACCACCCGGTACAGGAGATCAGG ATTATTGGCAATTTGTTGCCA [468]
315	hsa-mir-98	TAACAAATTTCCAATAATC CTGT [405]	AGGATTCTGCTCATGCCAGGGTAACAAATTTC CAATAATCCTGGTGGGGTAGGGATATTAGGC CCCAATTAGAAGATCAGTATTATTGGAAATTT GTCCCCTGGTGTGTGGCATATTCA [469]
316	hsa-let-7b	TTAACTTATGCACGCTTAA CTAT [406]	CGGGGTTAACTTATGCACGCTTAACTATCAGG GCAGTGATGTTGCCCCACGGAAGATTAGTTAA GCGTGCATAAGTTACCCTG [470]
317	hsa-let-7g	TTAACTTATGCACGCTTAA CTAT [407]	AGGCTTAACTTATGCACGCTTAACTATGAGGG TCTATGATACCACCCGGTACAGGAGATTAGTT AAGCGTGATTAAGTTAGCCA [471]
318	hsa-mir-98	TTAACTTATGCACGCTTAA CTAT [408]	AGGATTCTGCTCATGCCAGGGTTAACTTATGC ACGCTTAACTAGTGGGGTAGGGATATTAGGC CCCAATTAGAAGCTTAGCTAAGCGTGCATAAG TTTCCCTGGTGTGTGGCATATTCA [472]
319	hsa-let-7b	TACAAGAGATAGAAAGA CCAGTC [409]	CGGGGTACAAGAGATAGAAAGACCAGTTCAG GGCAGTGATGTTGCCCCTCGGAAGATACTGG TCTTTCTATCTCTTGTCCCTG [473]
320	hsa-let-7g	TACAAGAGATAGAAAGA CCAGTC [410]	AGGCTACAAGAGATAGAAAGACCAGTTGAGG GTCTATGATACCACCCGGTACAGGAGATACTG GTCTTTCTCTCTCTTGTGCCA [474]
321	hsa-mir-98	TACAAGAGATAGAAAGA CCAGTC [411]	AGGATTCTGCTCATGCCAGGGTACAAGAGAT AGAAAGACCAGTGTGGGGTAGGGATATTAGG CCCCAATTAGAAGATACTTGTCTTTCTATCTCT TGCCCCTGGTGTGTGGCATATTCA [475]

Example 14 - Assessment of effects of homology arm length on efficiency of homology directed repair

The concentration and type of HDR (homology directed repair) templates with different lengths of homology arms was varied to assess if this impacts the efficiency of editing miRNA loci for the insertion of GEiGS templates via homology directed repair. ssDNA (single stranded DNA) may increase accurate editing efficiency compared to dsDNA (double stranded DNA) templates by promoting duplex formation and therefore HDR, as well as reducing the risk of

repair by end-joining mechanisms that would lead to undesired editing outcomes [Skarnes WC, Pellegrino E, McDonough JA. Improving homology-directed repair efficiency in human stem cells. Methods. 2019 Jul 15;164-165:18-28. doi: 10.1016/j.ymeth.2019.06.016. Epub 2019 Jun 16. PMID: 31216442]. Furthermore, shorter homology may allow a greater amount and concentration of donor to be transfected into cells and therefore promote efficient HDR editing.

iPSCs were transfected with Cas9-guide RNA complexes at 4 μM (micromolar) and HDR templates at varying concentrations (μM). Different forms of HDR templates were tested: short ssDNA with 40 bp-long right and left homology arms with modified ends (137 bp total length, purchased with AltR HDR modfications from Integrated DNA Technologies) were compared to long ssDNA with 350 bp-long right and left homology arms (632 bp total length, purchased from Integrated DNA Technologies) or short HDR blocks (dsDNA purchased with from Integrated DNA Technologies with modified ends) with 150- or 350bp-long right and left homology arms (137 or 632 bp total length).The efficiency of HDR was assessed by performing next generation sequencing on the amplified miRNA locus of interest (the miR-21 locus) and analysed using an allele quantification software (CRISPresso2)[Clement K, Rees H, Canver MC, Gehrke JM, Farouni R, Hsu JY, Cole MA, Liu DR, Joung JK, Bauer DE, Pinello L. CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nat Biotechnol. 2019 Mar; 37(3):224-226. doi: 10.1038/s41587-019-0032-3. PubMed PMID: 30809026] (Fig. 23). Fig. 23 shows that flanking genomic sequences (homology arms) of length 40 base pairs show superior effects for the efficiency of HDR (% HDR) and in a dose dependent manner, compared to flanking genomic sequences of length 350 bp or length 150-350 bp which are only marginally better to no donor template alone.

At similar concentrations (0.357 or 2 μM) short ssDNA resulted in higher editing efficiency (%HDR) compared to long ssDNA or short dsDNA HDR blocks. The greatest editing efficiency was observed after increasing the concentration of short ssDNA to 8 μM. Higher concentrations of long ssDNA or short HDR blocks could not be tested due to physical constraints in synthesising these templates.

Example 15 - Demonstrating evasion of NK-cell mediated immune responses in culture to clonal GEiGS-modified cell lines

Natural Killer (NK) cells recognise and kill cells which are deficient in surface expression of MHC-I ('missing self'). NK cell assays were set up to determine how their killing activity against GEiGS-modified iPSC was modulated by B2M silencing.

Pre-isolated CD56+ NK cells were thawed and stimulated to proliferate in IL-2/IL-12, IL-2 alone or without cytokines. On the day of the assay, NK cells were counted and stained with CellTrace™ CFSE Cell Proliferation Kit. Target cells included K562 cells as a positive control for cell killing. It is known that the tumour cell line K562 serves as a control to assess the extent of activated NK cell mediated killing (Zamai L, AR Mariani, G Zauli, L. Rodella, R. Rezzani, FA Manzoli and M Vitale. Kinetics of in vitro natural killer activity against K562 cells as detected by flow cytometry. 1998. Cytometry 32(4):280-285. DOI: 10.1002/(sici)1097-0320(19980801)32:4<280::aid-cyto4>3.0.co;2-m).

NK cells were activated with the cytokines IL-2 or IL-12, or a mixture of IL-2 and IL-12, prior to mixing the cells at a defined ratio (typically 5 : 1 or 1:1). Control (unmodified) iPSC, $B2M^{-/-}$ iPSC and GEiGS-modified iPSC, were stained with Zombie NIR. The Zombie stain allows for differentiating between cells that died before the start of the killing assay as opposed to cells that died during the course of the assay. Target cells were typically seeded into a 96 well plate at a density of 40 000 cells/well.

NK cells were then added to the wells at varying ratios to the target cells. Typical ratios evaluated were 5(NK):1(target) or 2.5:1 or 1:1. Extra wells of target cells were included from which the maximal and spontaneous release could be calculated at the end of the assay. The plate was then incubated for a defined period (4-48 hours) at 37°C, 5% CO_2.

Following incubation, maximal release was determined by lysing samples with 20% Triton-X. All wells were then stained with Propidium iodide (PI). Once the staining was complete the plate was run on the flow cytometer. CFSE staining allowed us to identify the NK cells, Zombie NIR stained cells that died prior to the killing assay, while PI stained cells that had died during the killing assay. Spontaneous release values were subtracted from the PI values and the remaining amount was the percentage of target cells killed by the NK cells.

Specific lysis was observed against K562 cell line in the presence or absence of cytokine stimulation, as a positive control (Fig. 24). Control iPSC displayed a reduced level of specific lysis (approximately 50% of the maximum level of cell killing observed for K562 cells), whereas as expected, undifferentiated $B2M^{-/-}$ iPSC showed elevated levels of specific lysis (Fig. 24). This is because complete absence of B2M results in complete absence of MHC-I ("missing self" phenotype) and these cells are effectively detected and lysed by NK cells. Undifferentiated, GEiGS-modified iPSC lines ($iPSC^{S29/29}$), did not express quantitatively the same level of B2M as the control iPSC line from which it is derived (i.e. they express lower levels of B2M and thus lower levels of MHC-I cell surface expression) and therefore displayed consistently reduced specific lysis by NK cells compared the $B2M^{-/-}$ cell lines across all mixed culture conditions tested.

This demonstrates that partial knockdown of B2M results in partial reduction of MHC-I cell surface expression which in turn allows the cells to evade the innate (NK-mediated) immune system shown by reduced specific lysis of the GEiGS-modified iPSC lines ($iPSC^{S29/29}$) cells by NK-cells. Therefore, partial knockdown of B2M results in enhancing cell survival following exposure to cells of the innate (NK-mediated) immune system.

This example shows that the inhibitory RNA according to the present invention is effective in inhibiting the expression of the target gene, in this case B2M (which in turn leads to a measurable functional effect, reduced lysis by activated NK cells in this case). Solution 29 (SEQ ID NO: 9 and SEQ ID NO: 26) is effective in inhibiting the expression of the target gene.

Example 16 - Ability of GEiGS Solution 29 to buffer against the induced expression of B2M following exposure of cells to an inflammatory cytokine

GEiGS has the potential to provide stable and tunable knock-down of B2M, and therefore partial silencing of MHC-I expression rather than completely knocking it out (KO). MHC-I KO is useful for effective evasion of the recipients adaptive immunity, but exposes the therapeutic cells to rejection by cells of the innate immune system.

Autoimmune disorders or cancer are typified by chronic inflammation in patients. In Type 1 diabetes and cancer therefore, the cells or cellular compositions transplanted as interventions will be exposed to inflammatory stimuli. Many different factors can affect the basal and inducible expression of MHC-I on the cell surface. These include factors to be found in diseased, infected and inflamed tissues, such as cytokines, growth factors [interferons (IFNs), tumour necrosis factor (TNF), and type II interferon (IFN-y)]. IFNs have a capacity to activate the expression of major and minor HLA genes, components of the MHC-I complex.

Partial silencing of B2M is expected to prevent MHC-I upregulation in therapeutic cells in response to inflammatory cytokines.

Various media conditions were tested to assess their effects on B2M expression in iPSC. Interferon-gamma (IFN-γ or IFN-g) was included at a concentration of 20ng/ml, a supra-physiological concentration used to induce IFN-response genes in experimental tissue culture. Exposure of control (unmodified) iPSC to IFN-y for 72 hours (WT+INFg) led to a more than 6 times increase in the relative expression level of B2M as analyzed by flow cytometry using anti-B2M antibodies, compared with unstimulated iPSC alone (WT).

Unexpectedly, while the homozygous (KI/KI) Solution 29 clonal line expressing the B2M sRNA bi-allelically displayed an elevated expression of cell surface B2M in the presence of INF-γ (S29+INFg), this increase was much reduced (7 fold lower) compared with that for the parental

line exposed to IFN-γ for 72 hours (WT+INFg), suggesting that GEiGS has a profound buffering effect in preventing the upregulation of MHC-I on cells in response to an inflammatory cytokine (Fig. 25)). The relative fold change in B2M cell surface expression (MHC-I cell surface expression) between S29 and S29+INF- γ (approximately 10 times) is approximately the same as the relative fold change between WT and WT+INF- γ. In this experiment, the cell surface level of B2M is indicative of the cell surface level of MHC-I since MHC-I complex is a heterodimer made up of a hypervariable HLA protein and beta-2 microglobulin (B2M).

This demonstrates that GEiGS solutions of the invention targeting B2M can also reduce B2M (and therefore MHC-I) levels in chronic inflammatory conditions. This indicates that such solutions could be of therapeutic benefit to patients, even those suffering from chronic inflammatory diseases. Additionally, this example illustrates that the effects of the inhibitory RNA is not limited by the copy number of target transcripts in the cell.

Any of SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 are expected to produce a similar effect to that observed with Solution 30.

Example 17 - Differentiation of GEiGS Solution 30 knock-in iPSC line into definitive endoderm and progenitors of the pancreatic lineage

Homozygous Solution 30 knock-in iPSC lines were differentiated into definitive endoderm and pancreatic lineage cells. A methodology for specifying pancreatic cells from pluripotent stem cells has been adopted from Balboa et al., Functional, metabolic and transcriptional maturation of human pancreatic islets derived from stem cells. Nat Biotechnol. 2022 Jul;40(7):1042-1055. Doi: 10.1038/s41587-022-01219-z. Epub 2022 Mar 3. PMID: 35241836; PMCID: PMC9287162.

There are many different protocols that have been published describing the specification of pancreatic lineage cells from iPSC and the skilled person is aware of other methods for specifying pancreatic cells from pluripotent stem cells. For example, see the references provided below.1: DAmour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol. 2005 Dec;23(12):1534-41. Doi: 10.1038/nbt1163. Epub 2005 Oct 28. PMID: 16258519.

2: Schulz TC, Young HY, Agulnick AD, Babin MJ, Baetge EE, Bang AG, Bhoumik A, Cepa I, Cesario RM, Haakmeester C, Kadoya K, Kelly JR, Kerr J, Martinson LA, McLean AB, Moorman MA, Payne JK, Richardson M, Ross KG, Sherrer ES, Song X, Wilson AZ, Brandon EP, Green CE, Kroon EJ, Kelly G, DAmour KA, Robins AJ. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PloS One.

2012;7(5):e37004. Doi: 10.1371/journal.pone.0037004. Epub 2012 May 18. PMID: 22623968; PMCID: PMC3356395.

3: Kumar M, Jordan N, Melton D, Grapin-Botton A. Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. Dev Biol. 2003 Jul 1;259(1):109-22. Doi: 10.1016/s0012-1606(03)00183-0. PMID: 12812792.

4: Melton D. The promise of stem cell-derived islet replacement therapy. Diabetologia. 2021 May;64(5):1030-1036. Doi: 10.1007/s00125-020-05367-2. Epub 2021 Jan 16. PMID: 33454830; PMCID: PMC8012315.

5: Peterson QP, Veres A, Chen L, Slama MQ, Kenty JHR, Hassoun S, Brown MR, Dou H, Duffy CD, Zhou Q, Matveyenko AV, Tyrberg B, Sörhede-Winzell M, Rorsman P, Melton DA. A method for the generation of human stem cell-derived alpha cells. Nat Commun. 2020 May 7;11(1):2241. Doi: 10.1038/s41467-020-16049-3. PMID: 32382023; PMCID: PMC7205884.

6: Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, Peterson QP, Greiner D, Melton DA. Generation of functional human pancreatic β cells in vitro. Cell. 2014 Oct 9;159(2):428-39. Doi: 10.1016/j.cell.2014.09.040. PMID: 25303535; PMCID: PMC4617632.

The *in vitro* differentiation of pancreatic cells from iPSCs reproducibly progresses via a four stage process of definitive endoderm, primitive gut tube, posterior foregut endoderm and pancreatic progenitors. Specified progenitors produced after fourteen days in continuous culture, are isolated and assessed by flow cytometry for the level of staining for pancreatic progenitor markers (PDX-1, NKX6.1) as well as co-staining for B2M (MHC-I).

Solution 30 miRNA (hsa-mir-21) was demonstrated to be expressed at a low level in undifferentiated iPSCs and upregulated in differentiated cells, particularly pancreatic cells, compared with undifferentiated iPSC (see Example 7, Fig. 11). This cell does not express quantitatively the same level of B2M as the control (undifferentiated) iPSC line (i.e. it expresses less B2M). For comparison, B2M expression levels in undifferentiated iPSCs comprising a Solution 30 construct (S30 KI/KI) have been shown to be similar to those in control (undifferentiated) iPSCs (miR KO/KO) (Fig. 21b). When expanded as undifferentiated iPSC, greater than 93% of the cells for both the isogenic control and GEiGS Solution 30 clonal lines expressed B2M (Fig, 19b and Fig. 21b), whereas following differentiation into pancreatic progenitors the GEiGS Solution 30 line showed a marked reduction in the cell surface expression of B2M compared pancreatic progenitors derived from control iPSC (Fig. 27).

Heterozygous solution 30 knock-in clones were generated and differentiated into definitive endoderm and subsequent pancreatic progenitors. The iPSC clonal line (S30-DP1-F12) is a compound heterozygous genotyped with Solution 30 knocked into one allele and the other

allele possessing a single base insertion which does not disrupt the transcription of the pre-miRNA or its scaffold secondary structure. The iPSC line (miR-21$^{S30/+1}$) was differentiated for 4 days using established methodology to specify definitive endoderm (detailed in Balboa D, et al 2022) and the proportion of cells expressing an early marker (CXCR4) assessed by flow cytometry.

The heterozygous knock-in line (iPSC miR-21$^{S30/+1}$) showed a high proportion of CXCR4+ve cells, on a par with both control (unmodified) and B2M$^{-/-}$ iPSCs (Fig. 26). To confirm that the differentiating cells expressed reduced amounts of B2M, the population of CXCR4+ve cells were assessed for their B2M expression levels. The iPSC miR-21$^{S30/+1}$ line showed an approximately 80% reduction in B2M expression compared with control (unmodified) iPSC (Fig. 27a).

The iPSC lines were differentiated for a further 7-10 days in culture, to a stage at which definitive endoderm cells express the pancreatic lineage marker, PDX-1. The pancreatic and duodenal homeobox-1 (PDX-1) marker gene is necessary for pancreatic development and Beta-cell maturation.

Results indicates a significant proportion of the specified progenitors at this later stage in development are positive for PDX-1 and the majority of PDX-1 positive cells also staining for B2M (Fig. 27b). The iPSC miR-21$^{S30/+1}$ clonal line showed a high proportion of PDX-1 expressing cells which had markedly reduced B2M expression compared with the control (unmodified) iPSCs. The sRNA from Solution 30 was expressed mono-allelically.

These results demonstrate that B2M expression can be reduced using inhibitory RNA of the invention in differentiated cells . Additionally, heterozygous knock-in of inhibitory RNA of the invention is sufficient to achieve significant and sustained knockdown. Finally, this example shows that inhibitory RNA show context-specific expression, low expression in undifferentiated iPSC and high expression in pancreatic progenitors.

Any of SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 are expected to produce a similar effect to that observed with Solution 30.

Example 18: Computational designs for GEiGS solutions silencing genes in T-cells

GEiGS Solutions targeting genes in T-cells were generated using the computational pipeline. Pipeline inputs are: the mRNA sequence of the major transcripts for the gene targets in T cells: FOXP3, PD-1 (PDCD-1) (from literature), the human genome reference sequence (GRCh38),

miRNA annotations (miRbase v22) and cell type specific miRNA quantifications from analysis of T-cells using qPCR.

T cell target genes were selected from literature and published databases of transcripts defined in RNAseq analysis from activated T -cells.

The computational pipeline identified specific designs suitable for targeting the genes of interest based on modified forms of the selected context-specific miRNAs. The miRNA designs from the computational model were broadly in line with published literature describing conventional siRNA designs to target these genes giving further reassurance in the solutions proposed. The solutions were ranked based on their on-target scores and predicted low off-target scores. Both published and novel sequences were used to assist with design of the trigger sequences. Computational analysis was used to select the top ranked sequences for each target gene, ensuring retention of diversity based on the original data source. Further computational analysis was used to shortlist between 22 and 25 individual GEiGS solutions designed per T-cell target gene. This was based on assessment of predictions to form a mature miRNA and the quantification of the differential expression of each miRNA scaffold in polarised cell states following experimental induction.

The GEiGS solution designs were comprised of a modified sequence of an endogenous pre-miRNA (hairpin) - also referred to as the endogenous miRNA scaffold - modified to encode a novel silencing RNAi trigger instead of its native guide strand. The novel GEiGS silencing RNA matched the mRNA targets by sequence complementarity and each was predicted to lead to the silencing of each target mRNA via the RNA interference pathway.

The solution design process also generated corresponding negative control constructs - also referred to as ‘scrambled’ solutions - in which the same endogenous miRNA scaffold was modified as in the corresponding GEiGS solution but to express a scrambled sequence of the GEiGS silencing RNA.

Sequences of computationally-designed FOXP3-targeting RNAi trigger sequences and GEiGS solutions for T-cells are provided in Table 23.

Regulatory T cells (iTreg) are known to be induced from CD4+ T cells during their activation. During manufacture of CAR-T cells, T cells are activated and it is desirable to minimise or reduce the proportion of iTreg cells. Treg cells are also induced from tumour infiltrating T cells as they are exposed to the immunosuppressive TME. Minimising or reducing the proportion of iTreg cells during manufacture or in the TME are desirable as this would reduce suppression of conventional CAR T-cells, increasing cytokine secretion and their antitumour effects.

However, Treg cells are important for maintaining immune system homeostasis, preventing auto-immunity and the undesirable persistence of cytotoxic, effector T cells when these conventional effector T cells are no longer required.

Tregs express (1) immune checkpoint receptors and suppress effector T cells and antigen presenting cells (2) secrete negative regulators of the immune response (IL-10, IL-35, TGFb), (3) induce apoptosis of immune effector cells, (4) perturb metabolism of effector T cells reducing their function (e.g produce adenosine binding to A2A receptor yielding suppression of both antigen presenting and effector T and NK cells). Patients unresponsive to anti-PD1 (PDCD1) antibody treatment are known and this is due to the antibodies activating all T-cells, making iTregs more immunosuppressive (Kumagai et al 2020).

FOXP3 is considered essential for development and maintenance of the T regulatory compartment. Experimental disruption of FOXP3 gene expression yields reduced suppressive Treg activity and restoration of conventional T cell signalling and cytokine secretion in vitro and in vivo assays. FOXP3 has been successfully targeted using antisense oligonucleotides (AZD8701; monotherapy or in combination with Durvalumab (NCT04504669), demonstrating that RNAi against the gene could modulate the phenotype of T-cells to be conventional effector T cells and convey a therapeutic effect, though no clinical benefit has been reported to date.

It would be difficult to know when and how much RNAi against FOXP3 to give to a patient to optimise the therapeutic benefit of conventional effector CAR-T cells while minimising CAR-activated Treg cells in the TME, while ensuring a beneficial safety profile when CAR-T cells are in circulation and no longer required to be acting against the tumour.

These designs (Table 23) are created to provide context specific silencing of FOXP3 in CAR-T-cells only when they are in the TME (TME state). This should ensure a persistent 'on tumour' therapeutic effect with an optimum 'off tumour' strong safety profile.

Table 23. Sequences of computationally-designed FOXP3-targeting RNAi trigger sequences and GEiGS solutions for T-cells

GEiGS Solution ID	miRNA scaffold	GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]	GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]

Sol-336	hsa-let-7g	TAAGACCTTCTCA CATCCGGGC [476]	AGGCTAAGACCTTCTCACATCCGGGCTGAGG GTCTAAGATACCACCCGGTACTGGAGATGCC CGGATGTGACAAGGTCTTGCCA [526]
337	hsa-mir-126	TAAGACCTTCTCA CATCCGGGC [477]	AGGCTAAGACCTTCTCACATCCGGGCTGAGG GTCTAAGATACCACCCGGTACTGGAGATGCC CGGATGTGACAAGGTCTTGCCA [527]
338	hsa-mir-150	TAAGACCTTCTCA CATCCGGGC [478]	CTCCCCATGGCCCTGTAAGACCTTCTCACAT CCGGGCCTGGGCTCAGAGGCCGGATGTGAC CGGTCTTACAGGGACCTGGGGAC [528]
339	hsa-mir-17	TAAGACCTTCTCA CATCCGGGC [479]	GTCAGAATAATGTTAAGACCTTCTCACATCCG GGCCTGATATGTGCAGCTACGGATATGTGAA GGTCTGACGCATTATGGTGAC [529]
340	hsa-mir-191	TAAGACCTTCTCA CATCCGGGC [480]	CGGCTGGACAGCGGGTAAGACCTTCTCACAT CCGGGCCTTGTCTCCAGAGCATTCGGCCCG GCCGTGGAAGGTCTCCCCTGCTCTCCTGCCT [530]
341`	hsa-mir-30d	TAAGACCTTCTCA CATCCGGGC [481]	GTTGTTAAGACCTTCTCACATCCGGGCCTGT AAGACACAGCTAAGGCCCGGATGCGAAGGT CTTTGCTAC [531]
342	hsa-mir-30e	TAAGACCTTCTCA CATCCGGGC [482]	GGGCAGTCTTTGCTACCCGGATGTGAGTTAA GGTCTTACTGTAAGGTGTTCAGAGGAGTAAG ACCTTCTCACATCCGGGCGGCAGGCTGCCA [532]
343	hsa-mir-363	TAAGACCTTCTCA CATCCGGGC [483]	TGTTAGTCGGATGTGCAAACGGTCTTATTGAT GAGTATCATAGGAGAAATAAGACCTTCTCAC ATCCGGGCAACC [533]
344	hsa-mir-93	TAAGACCTTCTCA CATCCGGGC [484]	CTGGGGGCTCTAAGACCTTCTCACATCCGTG CCTGTGATTACCCAACGGCCGGAGTGAGTAA GGTCTCCCGAGCCCCCGG [534]
345	hsa-let-7g	TTGCAGACACCAT TTGCCAGC [485]	AGGCTTGCAGACACCATTTGCCAGCATGAGG GTCTATGATACCACCCGGTACAGGAGATTGC TGGCAAATGCTGTCTGCAGCCA [535]

346	hsa-mir-126	TTGCAGACACCATTTGCCAGC [486]	CGCTGGCGACGGGACTGGCAAATGGAGTCTGCACGCTGTGACACTTCAAACTTGCAGACACCATTTGCCAGCACCGTCCACGGCA [536]
347	hsa-mir-17	TTGCAGACACCATTTGCCAGC [487]	GTCAGAATAATGTTTGCAGACACCATTTGCCAGCAGTGATATGTGCACCTATGGCATATAGTGTCTGTGTTGCATTATGGTGAC [537]
348	hsa-mir-30d	TTGCAGACACCATTTGCCAGC [488]	GTTGTTTGCAGACACCATTTGCCAGCACTGTAAGACACAGCTAAGTGCTGGCAGCGTGTCTGCATGCTAC [538]
349	hsa-mir-30e	TTGCAGACACCATTTGCCAGC [489]	GGGCAGTCTTTGCTAGCTGGCAAATGGCTTGTCTGCAACTGTAAGGTGTTCAGAGGAGTTGCAGACACCATTTGCCAGCAGGCAGGCTGCCA [539]
350	hsa-mir-363	TTGCAGACACCATTTGCCAGC [490]	TGTTATCTGGCAAATCATGATCTGCAATTGATGAGTATCATAGGAGAAATTGCAGACACCATTTGCCAGCAAACC [540]
351	hsa-mir-30d	TTTCTTGCGGAACTCCAGCTTAT [491]	GTTGTTTTCTTGCGGAACTCCAGCTTACTGTAAGACACAGCTAAGTAAGCTGGAACCGCAAGAATGCTCC [541]
352	hsa-mir-363	TTTCTTGCGGAACTCCAGCTTAT [492]	TGTTGTAGCTGGAGTCACGACAAGAAATTGATGAGTATCATAGGAGAAATTTCTTGCGGAACTCCAGCTTAAACC [542]
353	hsa-mir-150	TTGTGTGACTTTTCCAGCCTTGA [493]	CTCCCCATGGCCCTGTTGTGTGACTTTTCCAGCCTTGCTGGGCTCAGACCAGGCTGGAAACCCACACAACAGGGACCTGGGGAC [543]
354	hsa-mir-17	TTGTGTGACTTTTCCAGCCTTGA [494]	GTCAGAATAATGTTTGTGTGACTTTTCCAGCCTTGATGATATGTGCATATTGGCTGTAACGGTCACATTTCGCATTATGGTGAC [544]
355	hsa-mir-30d	TTGTGTGACTTTTCCAGCCTTGA [495]	GCTGTTTGTGTGACTTTTCCAGCCTTGCTGTAAGACACAGCTAAGCAAGGCTGGCAGTCACACATGCTAC [545]

356	hsa-mir-363	TTGTGTGACTTTTCCAGCCTTGA [496]	TGTTGTAGGCTGGAACCGTACACACAATTGATGAGTATCATAGGAGAAATTGTGTGACTTTTCCAGCCTTGAACC [546]
357	hsa-mir-93	TTGTGTGACTTTTCCAGCCTTGA [497]	CTGGGGGCTCTTGTGTGACTTTTCCAGCCTTGATGTGATTACCCAACTCAGGTTGAAAGTGTCACACCCCGAGCCCCCGG [547]
358	hsa-mir-150	TTTGTAGACACCATTTGCCAGTA [498]	CTCCCCATGGCCCTGTTTGTAGACACCATTTGCCAGTCTGGGCTCAGTAGTGGCAAATGGCCCTACAAACAGGGACCTGGGGAC [548]
359	hsa-mir-17	TTTGTAGACACCATTTGCCAGTA [499]	GTCAGAATAATGTTTTGTAGACACCATTTGCCAGTATGATATGTGCATTTAGGCAGCTGATGTCTACATTTGCATTATGGTGAC [549]
360	hsa-mir-191	TTTGTAGACACCATTTGCCAGTA [500]	CGGCTGGACAGCGGGTTTGTAGACACCATTTGCCAGTATTGTCTCCAGAGCATTCTACTGGCTCATGTGTCTACACCCCTGCTCTCCTGCCT [550]
361	hsa-mir-30d	TTTGTAGACACCATTTGCCAGTA [501]	GCTGTTTTGTAGACACCATTTGCCAGTCTGTAAGACACAGCTAAGACTGGCAAAATGTCTACAATGCTAC [551]
362	hsa-mir-363	TTTGTAGACACCATTTGCCAGTA [502]	TGTTCGTGGCAAATGCAGTACTACAAATTGATGAGTATCATAGGAGAAATTTGTAGACACCATTTGCCAGTAACC [552]
363	hsa-mir-30d	ATGTTGTGGAACTTGAAGTAGT [503]	GTAGTATGTTGTGGAACTTGAAGTAGTCTGAAAGACACAGCGAAGATTGCTTCGCTTCGCAACACGCAAC [553]
364	hsa-mir-378a	ATGTTGTGGAACTTGAAGTAGT [504]	AGGGTAGTTTAGGTTCCGTAGAGTGTGTAACCGAGAAATTGCATGTTGTGGAACTTGAAGTAGTCT [554]
365	hsa-mir-671	ATGTTGTGGAACTTGAAGTAGTCC [505]	GCAGGTGAACTGGCAGGCCAGGAAGAGGATGTTGTGGAACTTGAAGTAGTCCTGATGGATGTTTTGGACTACTTCATTCCACACAACCTCTTTCGGGCCGTAGAGCCAGGGCTGGTGC [555]

366	hsa-mir-30d	ATGTTGTGGAGGAACTCTGGGA [506]	GATGTATGTTGTGGAGGAACTCTGGGACTGTAAGACACAGCGAAGTCTCAGGGTATCCGCGACGCGCTAC [556]
367	hsa-mir-378a	ATGTTGTGGAGGAACTCTGGGA [507]	AGTGCCCGAGTTCCTTTGCGGAGTGTGCTACCAAGAAATAGCATGTTGTGGAGGAACTCTGGGACT [557]
368	hsa-mir-155	ATGTTGTGGAGGAACTCTGGGA [508]	CTGATGTTGTGGAGGAACTCTGGGATTTTTGCCTTCAACTGATCCCAGGTTCTCCACAACATCAG [558]
369	hsa-mir-20b	ATGTTGTGGAGGAACTCTGGGAA [509]	AGTACATGTTGTGGAGGAACTCTGGGAATTGCGGCAGGATTGTACGGAGGTTCTTCACGATCCAGTACT [559]
370	hsa-mir-30d	ATCCAGAAGATGGTCCGCCTGG [510]	GTCGTATCCAGAAGATGGTCCGCCTGGCTGTAAGACACAGCTAAGCCAGGTGGGTTCTTTTGGACGCTTC [560]
371	hsa-mir-30d	TTTGTGTTGAGTGAGGGACAGG [511]	GTTGTTTTGTGTTGAGTGAGGGACAGGCTGTAAGACACAGCTAAGCCTGTCCCTATCAACACAATGCTAC [561]
372	hsa-mir-30d	TTTTTGGCAAGGCAGTGTGTGT [512]	GTTGTTTTTTGGCAAGGCAGTGTGTGTCTGTAAGACACAGCTAAGACACACACTATTGCCAAAATGCTAC [562]
373	hsa-mir-30d	ATTTTTGGCAAGGCAGTGTGTGT [513]	GCCGATTTTTGGCAAGGCAGTGTGTGTCTGAAAGACCCAGCCAAGATGTATACTATTGCCGAGATTCTAC [563]
374	hsa-mir-34c	ATGTTGTGGAGGAACTCTGGGAA [514]	AGTCTAGTTGCAATGTTGTGGAGGAACTCTGGGAAGAAGAGTCCTTCTAGGTTCCCTCACCGCGATGCAAAAAGATC [564]
375	hsa-mir-30d	TTATTGGGGACGGTACTGTGGG [515]	GTTGTTTATTGGGGACGGTACTGTGGGCTGTAAGACACAGCTAAGCCCACAGTAATCCCCAATATGCTAC [565]
376	hsa-mir-30d	ATCCTGAGGGTACTGACGCTGC [516]	GTTGTATCCTGAGGGTACTGACGCTGCCTGAAAGACACAGCAAAGGCAGCGTTATCTCTCAGGGCGCTAC [566]

377	hsa-mir-378a	ATCCTGAGGGTACTGACGCTGC [517]	AGGGGGAGTCAGTGCTTTCGGCCTGTGCTACCAAGAAATAGCATCCTGAGGGTACTGACGCTGCCT [567]
378	hsa-mir-93	TGGTGCATGAAATGTGGCCTGTC [518]	CTGGGGGCTCTGGTGCATGAAATGTGGCCTGTCAGTAAAAACCCAACGACGGTCTGTTTGCGTGCGCGTCGAGCCCCCGG [568]
379	hsa-mir-30d	AGGAACTCTGGGAATGTGCTGT [519]	GGGGTAGGAACTCTGGGAATGTGCTGTCTGTAAGACACAGCTAAGATAGCACGTACAGGGTTTCCGCGAC [569]
380	hsa-mir-93	AGGAACTCTGGGAATGTGCTGTT [520]	CTGGGGGCTCAGGAACTCTGGGAATGTGCTGTTCGTAAGTACCCCACAACGCGCTTCCCGAGGGTTCGACGAGCCCCCGG [570]
381	hsa-mir-93	AACCTCACTTCTTGGTCCCTGTG [521]	CTGGGGGCTCAACCTCACTTCTTGGTCCCTGTGTGTGATTACCCAACCACGGGACAAGATAGTGAGGCCCGAGCCCCCGG [571]
382	hsa-mir-30d	AACCTCACTTCTTGGTCCCTGT [522]	GTAGCAACCTCACTTCTTGGTCCCTGTCTGAAAGACACAGCTAAGATGGGGGTCTGAGTGAGGTAGCGAC [572]
383	hsa-mir-30d	AAACCTCACTTCTTGGTCCCTGT [523]	GTAGAAACCTCACTTCTTGGTCCCTGTCTGTAAGACACAGCCAAGATGGGGGTCTGAGTGAGGTCTCCAC [573]
384	hsa-mir-30d	AAGACCTTCTCACATCCGGGCC [524]	GCGGTAAGACCTTCTCACATCCGGGCCCTGTAAGACACAGCGAAGGGTTCGGGTAGGAGGGTTTAGCAAC [574]
385	hsa-mir-30d	TTGCAGACACCATTTGCCAGCA [525]	GCTGTTTGCAGACACCATTTGCCAGCACTGTAAGACACAGCCAAGTGCTGGTAATGTGTCTGCATGCCAC [575]

Example 19 - Macrophage target Solution screens in iPSC

The efficiency of GEiGS solutions for silencing the target genes of interest for macrophages was analysed using the dual reporter system described in Example 10 above, in iPSC. The effect of individual solutions was quantified through the flow cytometry gating strategy shown

in Fig. 28. Debris were gated out using the FSC-A and SSC-A flow cytometry channels, and subsequent doublets were gated out using the FSC-A and FSC-H channels.

Viable and successfully transduced cells were then identified using antibodies to the tNGFR. This population of viable, tNGFR positive cells were then assessed for the extent of copGFP regression. The level of dsRed expression in the tNGFR positive cells (fluorescence intensity) was assayed, with dsRed being a proxy for the expression level/abundance of the GEiGS solution. Quantification of the proportion of dsRed cells also showing copGFP regression indicated GEiGS Solutions effective against the target sequences of interest.

Some of the solutions identified by these screens were Solution 260 (targeting STAT6), Solution 266 (targeting STAT6), Solutions 219 (targeting IRF4) and Solution 245 (targeting KDM6B) (Fig. 29). As shown in Fig. 29, that expression of the particular target gene (STAT6, IRF4 or KDM6B) was reduced using via the tested solutions.

This example shows that Solution 260 (SEQ ID NO: 216, SEQ ID NO: 240, SEQ ID NO: 334), Solution 266 (SEQ ID NO: 222, SEQ ID NO: 246 and SEQ ID NO: 340), Solution 219 (SEQ ID NO: 127, SEQ ID NO: 151 and SEQ ID NO: 293) and Solution 245 (SEQ ID NO: 177, SEQ ID NO: 201 and SEQ ID NO: 319) reduce expression of their target genes. Additionally, this example shows that the screening methodology described in Example 10 is suitable for screening the efficiency of GEiGS Solutions silencing the target genes of interest.

Example 20 - Gene editing of an endogenous miRNA scaffold in macrophages.

The Solutions identified in Example 19 are being implemented as full GEiGS, i.e. by editing the endogenous loci of the respective miRNA scaffolds in human monocytes and iPSC, as outlined in Example 11.

HDR and gRNA sequences designed for editing miR146A to insert GEiGS Solutions 266, 219 and 245 are provided below.

Solution 266 40-40 HDR F

GATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTACATTTAACATA TCCTAGGTACTGTTTAGTTATGTACTAGGATATGTTAAATGAAAGTACTGCTAGCTGTAG AACTCCAGCTTCGGCCTGTCGCCCAATCAAA [SEQ ID NO: 674]

Solution 266 40-40 HDR R

TTTGATTGGGCGACAGGCCGAAGCTGGAGTTCTACAGCTAGCAGTACTTTCATTTAACA TATCCTAGTACATAACTAAACAGTACCTAGGATATGTTAAATGTAGTGCTACAGAAGCTG TCACATCAGATAGACCAGGCAGATTCTACATC [SEQ ID NO: 675]

Solution 219 40-40 HDR F

GATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTTCATAGCTAGT TCATTCCCTAGTGTTTAGTTATCTAGGAATGAACTAGCTATGCCAGTACTGCTAGCTGTA GAACTCCAGCTTCGGCCTGTCGCCCAATCAAA [SEQ ID NO: 676]

Solution 219 40-40 HDR R

TTTGATTGGGCGACAGGCCGAAGCTGGAGTTCTACAGCTAGCAGTACTGGCATAGCTA GTTCATTCCTAGATAACTAAACACTAGGGAATGAACTAGCTATGAAGTGCTACAGAAGC TGTCACATCAGATAGACCAGGCAGATTCTACATC [SEQ ID NO: 677]

Solution 245 40-40 HDR F

GATGTAGAATCTGCCTGGTCTATCTGATGTGACAGCTTCTGTAGCACTATACTTCTGGC CAGATAGGGTGTGTTTAGTGATCATCTATCTGGCCAGAAGTAAAAGTACTGCTAGCTGT AGAACTCCAGCTTCGGCCTGTCGCCCAATCAAA [SEQ ID NO: 678]

Solution 245 40-40 HDR R

TTTGATTGGGCGACAGGCCGAAGCTGGAGTTCTACAGCTAGCAGTACTTTTACTTCTGG CCAGATAGATGATCACTAAACACACCCTATCTGGCCAGAAGTATAGTGCTACAGAAGCT GTCACATCAGATAGACCAGGCAGATTCTACATC [SEQ ID NO: 679]

MIR146A_gRNA

TCTGACACTGACACAACCCA [SEQ ID NO: 680]

MIR146A_gRNA2

TCTGACACTGACACAACCCA [SEQ ID NO: 681]

MIR146A_gRNA3

GAATTCAGTTCTCAAAGCTG [SEQ ID NO:682]

Example 21 – Validating solutions in T cell lines (Jurkats) and primary T cells

An overview of a protocol for a validation screen in T cell lines is provided in Fig. 30.

Briefly, extended solutions are designed computationally, including plus 50-150 bases of genomic sequence flanking on both sides of the endogenous pre-miRNA that the respective GEiGS solution was based on (as described in Example 2 above).

A lentivirus pooled library is produced in a T cell (Jurkat) cell line using a dual reporter construct system as described in Example 10. Confirmed Solutions were tested in a GFP regression assay in primary T cells.

Preliminary data illustrates that performing pooled screens of Solutions and using lentivirus transduction to express them in primary cells is feasible.

The following T cell target is validated in this screen:

PD-1 (PDCD-1)

- Inhibitory receptor expressed on T cells
- PD-1 ligand (PD-L1) is present on APCs and cancers cells and is used as a mechanism for evading immune-mediated clearance
- CRISPR KO has shown improved T cell killing in trial, but total knockout may impact CAR T cell persistence

These designs (Table 24) were created to show context specific expression of the Solutions against PDCD-1 in the CARTs when the cells are in the TME state only. This should yield 'on tumour' activation and therapeutic effect of CARTs which is more durable. Removing the silencing effect and enabling re-expression of PDCD-1 in T cells that are 'off tumour' is important as this is predicted to generate a memory T cell compartment, of benefit for a more sustained, persisting CAR-T cell following reactivation as needed.

Sequences of computationally-designed PDCD-1-targeting RNAi trigger sequences and GEiGS solutions for T-cells are shown in Table 24 below

Table 24 Sequences of computationally-designed PDCD-1-targeting RNAi trigger sequences and GEiGS solutions for T-cells

GEiGS Solution ID	**miRNA scaffold**	**GEiGS mature sRNA sequence (guide) [SEQ ID NO: #]**	**GEiGS solution sequence (pre-miRNA hairpin) [SEQ ID NO: #]**
PDCD-1-Sol-1	hsa-mir-146a	TTTAATTATAATTATAATATAAT[576]	CCGATGAGTGTCCTCAGCTTTTAATTATAATTATAATATAATGCGTCAGTGTCGGTTG

			TCTTCTAGTTATAATTACAGCTGGGACAT CGCCGACATCGA [625]
PDCD-1-Sol-2	hsa-mir-29a	TTAATTATAATTATAA TATAAT [577]	AATTGTGTTATACTCATGATTGCAGAGTC AATATAATTTTCTTAATTATAATTATAATAT AATT [626]
PDCD-1-Sol-3	hsa-mir-29a	TTTAATTATAATTATA ATATAAT [578]	AATTATGTTATATGAGTGATTACAGATAC AAGATAAGAGTTTTAATTATAATTATAATA TAATT [627]
PDCD-1-Sol-4	hsa-mir-342	TTAATTATAATTATAA TATAATA [579]	GAAACGGGGCACAAGGTGTTTTGTGTGT GTTGTGTCGTTGGAACATGGATAATGGA ATTGTTAATTATAATTATAATATAATACAC CTTGGCCCCCCTA [628]
PDCD-1-Sol-5	hsa-mir-363	TTAATTATAATTATAA TATAAT [580]	CGTTCATGTGTTGTACCTGCTGGTTGATT GATGAGGAACAAAGGAGAAATTAATTATA ATTATAATATAATAACC [629]
PDCD-1-Sol-6	hsa-mir-126	TTAATTATAATTATAA TATAAT [581]	CGCCGGCGACGGTATATGTTATGGTAGT GATTGCGCAGTGACACTTCAAACTTAATT ATAATTATAATATAATCCGTCCACGGCA [630]
PDCD-1-Sol-7	hsa-mir-126	TTTAATTATAATTATA ATATAAT [582]	CGCTGGCGACGGCATATATTATAATCGT GATTGCGCCGCGACACTTCGAATTTAATT ATAATTATAATATAATCCGTCCACGGCA [631]
PDCD-1-Sol-8	hsa-mir-146b	TTAATTATAATTATAA TATAATAG [583]	CCTGGCACTTAATTATAATTATAATATAAT AGGAGCTCTAGCCATTTCATATTATCTTA TAATTAGTGCCCGG [632]
PDCD-1-Sol-9	hsa-mir-30d	TAATTATAATTATAAT ATAAT [584]	ATTGGTTAATTATAATTATAATATAATCTG AAAGACACAGCTAAGATTATATTACTTGT AATTGTTCTAC [633]
PDCD-1-Sol-10	hsa-mir-30d	TTTAATTATAATTATA ATATAAT [585]	GTCGTTTAATTATAATTATAATATAATCTG AAAGACACAGCCAAGGTTGTATTGCTTAT GGTTACGCCAC [634]
PDCD-1-Sol-11	hsa-mir-20b	TTAATTATAATTATAA TATAATA [586]	AGTACTTAATTATAATTATAATATAATATC TCGGCATGATGCTAGTGTTCTAATTGTAG TTCCTGTACT [635]
PDCD-1-Sol-12	hsa-mir-378a	TTAATTATAATTATAA TATAAT [587]	AGGCTACGTTATGATTGTAGTAGGGAGT TACCTAGAAATAGCTTAATTATAATTATAA TATAATCT [636]

PDCD-1-Sol-13	hsa-mir-29a	ATTATAATTATAATATAATAGA [588]	ATCTATTATATTTTTATTATAACTGAGTCAATATAATTTTCATTATAATTATAATATAATAGAT [637]
PDCD-1-Sol-14	hsa-mir-29a	AATTATAATTATAATATAATAGA [589]	ATCTATTATATTCATATTATGGCTTAGTCAAAATAACTCTAATTATAATTATAATATAATAGAT [638]
PDCD-1-Sol-15	hsa-mir-363	ATTATAATTATAATATAATAGA [590]	TGTTGATGTTATGTTCAGGCTTATAGTGTGAAGAGTACCATAGGAGAACATTATAATTATAATATAATAGAAACC [639]
PDCD-1-Sol-16	hsa-let-7g	ATTATAATTATAATATAATAGA [591]	AGGCATTATAATTATAATATAATAGATGAGGGTCTATGATACCACCCGGTACAGGAGATTCTATTATATTACAATTATAAGCCA [640]
PDCD-1-Sol-17	hsa-let-7g	AATTATAATTATAATATAATAGA [592]	AGGAATTATAATTATAATATAATAGATGAGGGTCGATGATACCACCCGGTACAGGTGATTTTGTTATATTACAATTGTGATCCA [641]
PDCD-1-Sol-18	hsa-mir-126	ATTATAATTATAATATAATAGA [593]	CGCTGGCGACGGGATATTATATTATTATTATAACGCTGTGACACTTCAAACATTATAATTATAATATAATAGACCGTCCACGGCA [642]
PDCD-1-Sol-19	hsa-mir-126	AATTATAATTATAATATAATAGA [594]	CGCTGGCGACGGGATATTATATTATTATTATAACGCTGTGACACTTCAAAAATTATAATTATAATATAATAGACCGTCCACGGCA [643]
PDCD-1-Sol-20	hsa-mir-30d	ATTATAATTATAATATAATAGA [595]	GTGGCATTATAATTATAATATAATAGACTGAAAGACACAGCTAAGTTTATTGTGCTAATTGTGAAGCAAC [644]
PDCD-1-Sol-21	hsa-mir-30d	AATTATAATTATAATATAATAGA [596]	GATGAATTATAATTATAATATAATAGACTGTAAGACACAGCAAAGTCTGTTGTGCTAGTTATGGGTCTAC [645]
PDCD-1-Sol-22	hsa-mir-30e	ATTATAATTATAATATAATAGA [597]	GGGCAGCCTTTGCCACATTATAATTATAATATAATAGACTGTAAGGTGCACAGAGGAGTTTATTGTGGTAGTTATAGTGCGGCAGGCTGCCA [646]
PDCD-1-Sol-23	hsa-mir-30e	AATTATAATTATAATATAATAGA [598]	GGGCCGACTATGCCAAATTATAATTATAATATAATAGACTGAAAGGAGAGCAGAGGAGTTTATTGTGGTAGTTATAGTTCGGCAGTCGGCCA [647]

PDCD-1-Sol-24	hsa-mir-20b	ATTATAATTATAATATAATAGAA [599]	GGTACATTATAATTATAATATAATAGAATCCGGGCATGATTATAGTTATTTTATGGTTGTACCAGTACC [648]
PDCD-1-Sol-25	hsa-mir-671	ATTATAATTATAATATAATAGAAC [600]	GCAGGTGAACTGGCAGGCCAGGAAGAGGATTATAATTATAATATAATAGAACTGATGGATGTTTTGTTCTATTATAATAATTTAAACCTCTTTCGGGCCGTAGAGCCAGGGCTGGTGC [649]
PDCD-1-Sol-26	hsa-mir-17	TATAATATAATAGAACCACAGGG [601]	GTCAGAATGAGGTTATAATATAATAGAACCACAGGGAGATAGGAGCTACTCGTGGTGCTTTTGTATTAGACGCCTCATGGTGAC [650]
PDCD-1-Sol-27	hsa-mir-20a	TATAATATAATAGAACCACAGGG [602]	GTAGCACTATAATATAATAGAACCACAGGGGGGTTAGTAAGTCCGTGGTTTTGTTGTATTGAAAGTACTGC [651]
PDCD-1-Sol-28	hsa-mir-29a	TATAATATAATAGAACCACAGG [603]	ATTTGTGGTTTTCGAATGTTGTCAGAGTAAAAAGAAAGTTCTATAATATAATAGAACCACAGGT [652]
PDCD-1-Sol-29	hsa-mir-29a	TTATAATATAATAGAACCACAGG [604]	GTTTGTGGTTCTTACATGTTATCAGACGCAATACAAAGGTTTATAATATAATAGAACCACAGGC [653]
PDCD-1-Sol-30	hsa-mir-363	TATAATATAATAGAACCACAGG [605]	TGTTGGTGTGGTTCTCCTGCTGTTATGTTGACGAGGACCAAAGGAGAAATATAATATAATAGAACCACAGGAACC [654]
PDCD-1-Sol-31	hsa-mir-363	TTATAATATAATAGAACCACAGG [606]	CGATGGTGTGGTTCTCCTGCTGTTATGATGATGAGTACCATAGGAGAATTATAATATAATAGAACCACAGGATCC [655]
PDCD-1-Sol-32	hsa-let-7g	TATAATATAATAGAACCACAGG [607]	AGGCTATAATATAATAGAACCACAGGTGAGGGTCTATGATACCACCCGGTACAGGAGATCCTGTGGTTCTACTATATTATGCCA [656]
PDCD-1-Sol-33	hsa-let-7g	TTATAATATAATAGAACCACAGG [608]	AGGTTATAATATAATAGAACCACAGGTGAGGGTCTATGATACCACCCGGTACAGGAGATCCTGTGGTTCTACTATATTATGCCA [657]
PDCD-1-Sol-34	hsa-mir-126	TATAATATAATAGAACCACAGG [609]	CGCCGGCGACGGGATGTGGTTCTATAGTGTTGTCGCAGTGACACCTCAAACTATAATATAATAGAACCACAGGCCGTCCACGGCA [658]

PDCD-1-Sol-35	hsa-mir-126	TTATAATATAATAGAACCACAGG [610]	CGCCGGCGACGGGATGTGGTTTTGTAATGTTGTCGCTGTGACACTTCAAATTATAATATAATAGAACCACAGGCCGTCCACGGCA [659]
PDCD-1-Sol-35	hsa-mir-93	TATAATATAATAGAACCACAGGG [611]	CTGGGGGCTCTATAATATAATAGAACCACAGGGTCTGAATACCAAAGCTCGTGGTCTGTCTGTATTACCCGAGCCCCCGG [660]
PDCD-1-Sol-36	hsa-mir-20b	TATAATATAATAGAACCACAGGG [612]	AGGACTATAATATAATAGAACCACAGGGTTTTGGCATGACCGGAGTGGTGTTGTTGTATTACCTGTCCT [661]
PDCD-1-Sol-37	hsa-mir-363	ATATAATAGAACCACAGGGAAG [613]	CGTTGCTCCTTGTGGCACTCGTTATATTTGATGAGCACCATAGGAGAAAATATAATAGAACCACAGGGAAGAACC [662]
PDCD-1—Sol-38	hsa-mir-363	AATATAATAGAACCACAGGGAAG [614]	TGATGCTTCCTGTGGCACTCGTTATGTTTGATGAGCACCATAGGAGAAAATATAATAGAACCACAGGGAAGATCC [663]
PDCD-1-Sol-39	hsa-mir-126	ATATAATAGAACCACAGGGAAG [615]	CGCTGGCGACGGGATTTTTGTGGTTGTGTTATGCGCGGTGACACTTCAAACATATAATAGAACCACAGGGAAGCCGTCCACGGCA [664]
PDCD-1-Sol-40	hsa-mir-126	AATATAATAGAACCACAGGGAAG [616]	CGCTGGCGACGGGATTTCTGTGGTTATGTTATGCGCTGTGGCACACCAAAAATATAATAGAACCACAGGGAAGCCGTCCACGGCA [665]
PDCD-1-Sol-41	hsa-mir-30e	ATATAATAGAACCACAGGGAAG [617]	GGGCAGTCTTTGCTACATATAATAGAACCACAGGGAAGCTGTAAGGTGTTCAGAGGAGCTTCCCTGTTTCTATTATATGCGGCAGGCTGCCA [666]
PDCD-1-Sol-42	hsa-mir-30e	AATATAATAGAACCACAGGGAAG [618]	GGGCAGTCTATGCCAAATATAATAGAACCACAGGGAAGCTGAAAGGAGTGCAGAGGAGCTTTCTTGTTTTTGTTGTATTCGGCAGGCTGCCA [667]
PDCD-1-Sol-43	hsa-mir-20b	ATATAATAGAACCACAGGGAAGG [619]	AGTACATATAATAGAACCACAGGGAAGGTTTAGGCATGACTCAACTCTGGGGTTTTGTTATCCAGTACT [668]
PDCD-1-Sol-45	hsa-mir-34c	ATATAATAGAACCACAGGGAAGG [620]	AGTCTAGTTACCATATAATAGAACCACAGGGAAGGTTATAGGAGCTTTCCGTGGTCCTATGGTAAGGTAAAAAGATT [669]

PDCD-1-Sol-46	hsa-mir-29a	AACCCCTTTAAATAATTTCAGG [621]	ACTTGGGATTATACTGGGGGGTCTGAGATAATATAAGGTTCAACCCCTTTAAATAATTTCAGGT [670]
PDCD-1-Sol-47	hsa-mir-363	AACCCCTTTAAATAATTTCAGG [622]	CGTTGGTGGGGTTATCAAGCAGGGGTTTAGAAGAGTACCAGAGGAGATAAACCCCTTTAAATAATTTCAGGAACC [671]
PDCD-1-Sol-48	hsa-let-7b	TAAAGGTGGAGGGGTTTCCTGC	CGGGGTAAAGGTGGAGGGGTTTCCTGCTCAGGGCAGTGATGTTGCCCCTCGGGAGATACAGGAAACCCCTCCACCTTTCCCTG [672]
PDCD-1-Sol-49	hsa-mir-155	ATAATTATAATATAATAGAACC [624]	CTGATAATTATAATATAATAGAACCTTTTTGCCTCCAACTGAGGTTCTTTAATTATAATTATCAG [673]

Example 22 - Feasibility of protocol for validating targets in T-cells

To demonstrate the feasibility of the protocol outlined in Example 21 and Fig. 30, the screen was carried out using validated solutions targeting B2M having different predicted levels of silencing in activated T cells based on their known B2M silencing specificity on other human cell types (e.g. iPSC).

This experiment demonstrated the utility of Solutions delivery via a lentivirus (LV), as opposed to plasmid based delivery, into T cells, a notoriously difficult cell type to ectopically express Solutions in via plasmid based systems. Additionally, it validated the dual reporter system in which 3' UTR sequences of the B2M transcript were placed contiguously with encoding sequences for copGFP. Effective Solutions are those with B2M silencing specificity as a result of binding with perfect complementarity to the cognate B2M target gene sequences.

Constructs for the lentiviral (LV) delivery test and validation of the new reporter system for B2M silencing based on copGFP regression in T cells are described (Fig. 31a/b). Constructs with high (Solution 29), intermediate (Solution 12), low (Solution 50) and no (scrambled) B2M silencing activity are shown in Fig. 31a. The predictions of activity were based on previous effectiveness seen or predicted in Examples 2, 12 and 15 and Figs. 10, 22 and 24 for Solution 29, Example 7 and Fig. 11 for Solution 12 and Example 2, Fig.8 for Solution 50.

Additionally, in order to validate LV-based Solution delivery, this reporter system was transduced into Jurkat cell lines. Jurkat cells were transduced with a pooled mixture of Solution

12, Solution 29, Solution 50 or Scramble (negative control) as individual LV constructs, to generate a B2M Jurkat reporter pool of cells.

Distinct multiplicities of infection (MOI) were evaluated (1, 5, and 10) for each pool of LV constructs. dsRED expression from each Solution construct was used to monitor the appearance of and to set the gate for detection of Solution expressing cell pools. Cell surface detection of the low affinity NGF-receptor protein on cell surface was used to gate for cell pools expressing the copGFP sequence containing the B2M 3'UTR binding sequences for each Solution.

The gating strategy to identify those cells expressing both dsRED (Solution sequence) and copGFP is outlined in Fig 31b. Levels of copGFP expression was monitored on day 3, 6 and 9 post transduction and verification of copGFP regression in B2M Jurkat reporter cells containing an effective Solution was confirmed using residual B2M cell surface expression in flow cytometry.

As shown in Fig. 32A, GFP regression as a result of transcript degradation due to Solution binding to the B2M 3'UTR in the construct displayed the predicted pattern of silencing specificity: Solution 29 > Solution 12 > Solution 50. There was close correlation between the quantitative amount of copGFP regression and detectable amounts of residual B2M protein staining, with the silencing effect demonstrated by these disparate analytical approaches being both dose (MOI) and time dependent (days) following transduction.

When analysed in flow cytometry and gated for Solution expressing cells (high dsRED) and copGFP expression, the B2M reporter Jurkat pools displayed a pattern of B2M silencing Solution effect that closely mimics that predicted for each individual Solution if expressed alone, confirming that LV pool transduction of T-cells will be an effective alternative to plasmid based systems for further Solution design testing and validation (Fig 32b).

This example indicates that inhibitory RNA according to the present invention may successfully be delivered using lentivirus. Additionally, this example indicates that the proposed methodology and reporter system can be successfully used to detect and quantify the silencing activity of inhibitory RNA. Additionally, the proposed method can be used to detect discrete cell populations from a pooled transduction in T cells.

Example 23 - Demonstration of the ability of GEiGS to silence one or more target genes in cells when distinct Solutions were ectopically expressed from a single construct.

Constructs were used to ectopically express Solutions targeting B2M and eGFP (Solution 179) or two distinct B2M silencing Solutions as a concatemer (Solution 29 and Solution 43, or two copies of Solution 29), via transfection into iPSC, as described in Example 2. The Solutions were expressed from a polycistronic construct driven by the constitutive promoter EF1a in a plasmid. The plasmid map for expression of one or more Solutions is shown in Fig. 33a.

Two separate iPSC lines were used; a characterised, clonal cell line in which one copy of the sox-2 gene has been modified to incorporate mEGFP so as to not disrupt the protein coding sequence (Allen Institute , AICS-0074-026:WTC-mEGFP-sox2-cl26) and the parental control iPSC line used to generate this tagged line (WTC, Coriell Institute for Medical Research, cell line biorepository, sample GM25256). Cells were collected three days after transfection and analysed by flow cytometry as previously described (Example 2).

B2M and eGFP

GEiGS Solution 101 consists of a miR-30 scaffold modified to incorporate eGFP trigger sequences (miR-30_GFP_Leb_130) to target GFP alone and with extended homology arms of 130 bp represented of the adjacent scaffold sequences (Fellman et al., 2013). The miR-30 GFP sequences are extracted from the publication Lebbink RJ, Lowe M, Chan T, Khine H, Wang X, McManus MT. Polymerase II promoter strength determines efficacy of microRNA adapted shRNAs. PLoS One. 2011;6(10):e26213. doi: 10.1371/journal.pone.0026213. Epub 2011 Oct 21. PMID: 22031824; PMCID: PMC3198731. Solution 179 consists of a polycistronic sequence consisting of Solution 29 (targeting B2M) and miR-30_eGFP_Leb_130 (targeting GFP).

Compared with a negative control vector transfection, Solution 101, consisting of two distinct modified miRNA scaffolds (miRNA-20a and miRNA-30), demonstrated an efficient silencing of GFP without affecting the level of expression of B2M in the undifferentiated iPSC (Fig. 33). Solution 179 was effective in silencing both B2M and eGFP with apparent equal efficiency (Fig. 33).

This demonstrates that multiplexed GEiGS solutions expressed from the same construct can be effective at silencing multiple targets.

Two separate Solutions targeting B2M

GEiGS Solution 177 was made consisting of a Solution 29 (based on miRNA-20a) combined with Solution 43 (based on modified miR-518b), distinct Solutions with diverse sequences targeting the B2M transcript. Solution 178 was made consisting of two copies of the Solution 29 (targeting B2M, the same trigger sequences).

Compared with a negative control vector transfection, Solution 177 (consisting of modified miRNA-518b (Solution 43) and miRNA-20a (Solution 29), distinct Solutions with diverse sequences targeting the B2M transcript (triggers)) appeared more effective in silencing B2M when compared to the Solution 43 alone, indicating a minor additive effect and that the two distinct Solutions do not interfere in each other's ability to silence B2M (Fig. 34). Whereas Solution 178 which possesses two copies of Solution 29 and the same trigger sequences, does not have an additive effect in silencing B2M compared with Solution 29 alone. (Fig. 34). This shows that the two solutions were still effective when used together, and did not disrupt each other's activity.

This demonstrates that multiplexed GEiGS solutions expressed from the same construct can be effective at silencing the same target.

The reader's attention is directed to all papers and documents which are filed concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Each feature disclosed in this specification (including any accompanying claims, abstract and drawings), may be replaced by alternative features serving the same, equivalent, or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated otherwise, each feature disclosed is one example only of a generic series of equivalent or similar features.

The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

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Table 15 – Differential expression of miRNAs in M1 macrophages compared to M0 macrophages.

miRNA	logFC	PValue	FDR
hsa-miR-141-3p	7.666848	0.003704	0.017851
hsa-miR-7702	5.407818	0.007547	0.032117
hsa-miR-187-3p	3.031303	0.001534	0.00944
hsa-miR-6503-3p	-2.67918	9.64E-08	1.29E-05
hsa-miR-145-5p	-2.79059	0.003584	0.017573
hsa-miR-582-3p	-2.85977	5.35E-06	0.000143
hsa-miR-3679-5p	-3.74853	0.011067	0.043861
hsa-miR-4709-3p	-3.86194	0.001669	0.010115

hsa-miR-1294	-6.63775	0.000836	0.005666
hsa-miR-2682-5p	-6.92959	1.34E-05	0.000246
hsa-miR-940	-7.47698	0.004094	0.019268
hsa-miR-500b-3p	-7.60683	0.000283	0.002636
hsa-miR-3177-3p	-7.78132	0.001715	0.01024
hsa-miR-30c-2-3p	-8.23771	0.000343	0.002982

In Tables 15-20: logFC = log2 fold change in expression of miRNA expression; for FDR a value of 0.05 or lower was considered significant (FDR (the false discovery rate) is the p-value corrected for multiple testing).

Table 16 - Differential expression of miRNAs in M2a macrophages compared to M0 macrophages.

miRNA	logFC	PValue	FDR
hsa-miR-1-3p	9.142547135	0.000480712	0.003433659
hsa-miR-125b-2-3p	7.57778722	2.60166E-05	0.000325208
hsa-miR-125b-5p	6.242469755	0.000607209	0.004048058
hsa-miR-193b-3p	6.073443673	0.000120056	0.001091419
hsa-miR-365a-5p	5.688884132	0.001456091	0.007564109
hsa-miR-99a-5p	4.582802096	1.57808E-10	3.15617E-08
hsa-miR-449c-5p	4.436973282	0.015340226	0.04869913
hsa-miR-504-5p	3.97572707	0.00291562	0.013720566
hsa-miR-342-3p	3.055637639	9.38342E-06	0.000170608
hsa-miR-212-3p	2.794946576	1.19445E-07	6.82544E-06
hsa-miR-212-5p	2.635409754	2.71537E-06	6.3891E-05
hsa-miR-511-3p	2.605235828	2.00458E-08	1.48586E-06
hsa-miR-95-3p	-2.590142373	1.00488E-08	1.33983E-06
hsa-miR-6502-5p	-2.834512489	0.00016272	0.001328329
hsa-miR-30c-2-3p	-3.303394843	0.009966492	0.035594615
hsa-miR-629-3p	-3.479508824	0.005017899	0.02027434
hsa-miR-4425	-3.684218929	0.012059518	0.040198392
hsa-miR-335-3p	-3.925313699	0.000900755	0.00545967
hsa-miR-3614-5p	-4.860124371	0.000546288	0.0037675
hsa-miR-4662a-5p	-6.627666078	0.005708904	0.0221705
hsa-miR-3614-3p	-6.787299435	0.009637813	0.034730857
hsa-miR-152-5p	-7.422938688	0.011132673	0.038527834
hsa-miR-4632-3p	-7.487728561	0.000809181	0.005137655
hsa-miR-130a-3p	-7.492446665	0.00132074	0.007139134
hsa-miR-4684-3p	-8.224393561	0.00148938	0.007577525
hsa-miR-187-3p	-8.269827772	0.000105662	0.001006308

Table 17 - Differential expression of miRNAs in M2a macrophages compared to M1 macrophages.

miRNA	logFC	PValue	FDR
hsa-miR-125b-2-3p	12.1170339	8.8682E-06	7.5474E-05
hsa-miR-211-5p	9.624993754	0.00251707	0.008390232
hsa-miR-1-3p	9.142547135	0.000491995	0.002162614
hsa-miR-500b-3p	8.687592736	9.35603E-05	0.0005271
hsa-miR-4787-3p	8.286478937	0.004524366	0.013814857
hsa-miR-2682-5p	7.545479568	5.84013E-06	5.56203E-05
hsa-miR-940	7.28184577	0.004816985	0.014487174
hsa-miR-1294	6.718423854	0.000773671	0.003190396
hsa-miR-125b-5p	6.322698622	0.000583161	0.002508221
hsa-miR-7848-3p	5.878254731	0.021475469	0.049368894
hsa-miR-3177-3p	5.474361562	0.014941055	0.035786958
hsa-miR-511-3p	5.049264634	9.90509E-12	3.96203E-09
hsa-miR-99a-5p	4.851930028	8.28516E-11	1.10469E-08
hsa-miR-511-5p	4.612927715	1.3629E-09	9.08601E-08
hsa-miR-191-3p	4.133855154	0.014546499	0.035264241
hsa-miR-4709-3p	4.096622796	0.001107328	0.004178597
hsa-miR-3679-5p	4.032285047	0.007525539	0.020339296
hsa-miR-582-3p	3.673732403	3.39244E-07	6.78489E-06
hsa-miR-365a-5p	3.666646183	0.011650858	0.029495843
hsa-miR-193b-3p	3.551994644	0.002726285	0.008938639
hsa-miR-504-5p	3.404671669	0.006928101	0.019379305
hsa-miR-3065-5p	3.010279043	0.00125504	0.00469174
hsa-miR-6503-3p	2.901421407	3.6965E-08	1.4786E-06
hsa-miR-342-3p	2.778096605	2.40938E-05	0.000169079
hsa-let-7c-5p	2.663941442	4.08574E-11	8.17148E-09
hsa-miR-1180-3p	2.600515508	0.003108733	0.010028172
hsa-miR-335-3p	-2.974968238	0.006132495	0.017775347
hsa-miR-200a-5p	-2.977173334	0.021739835	0.04969105
hsa-miR-194-5p	-3.186658132	0.000115684	0.000593249
hsa-miR-629-3p	-3.288150158	0.007035746	0.019408955
hsa-miR-7702	-5.056434136	0.01064888	0.027840209
hsa-miR-4684-3p	-5.700373339	0.014914773	0.035786958
hsa-miR-3614-5p	-5.905946527	0.000111488	0.000593249
hsa-miR-4662a-5p	-5.997528397	0.011114527	0.028752094
hsa-miR-708-5p	-6.856705277	0.000954764	0.003744174
hsa-miR-4632-3p	-7.384344413	0.000900512	0.003566386
hsa-miR-3150b-3p	-7.518570966	0.010041886	0.026426016
hsa-miR-3614-3p	-7.90264502	0.003923573	0.012166116
hsa-miR-130a-3p	-9.494017973	0.000190173	0.000905587
hsa-miR-187-3p	-11.30113124	5.6467E-06	5.50898E-05

Table 18 - Differential expression of miRNAs in M2c macrophages compared to M0 macrophages.

miRNA	logFC	PValue	FDR
hsa-miR-6852-5p	7.31207716	0.008210526	0.039821617
hsa-miR-5690	3.344774977	1.1165E-06	4.46598E-05
hsa-miR-30c-2-3p	2.862059938	0.00994013	0.045881497
hsa-miR-146b-5p	-2.610028581	7.92793E-09	1.05706E-06
hsa-miR-877-5p	-2.620154346	0.000908076	0.007264607
hsa-miR-146b-3p	-2.760529745	3.60364E-09	1.05706E-06
hsa-miR-760	-4.338196785	0.000620882	0.005398976
hsa-miR-5001-3p	-7.616499089	9.11377E-08	5.24291E-06
hsa-miR-4521	-7.67561929	0.000232009	0.002508203
hsa-miR-187-3p	-8.269827772	7.53554E-05	0.001310528

Table 19 - Differential expression of miRNAs in M2c macrophages compared to M1 macrophages.

miRNA	logFC	PValue	FDR
hsa-miR-30c-2-3p	11.09977099	1.83122E-05	0.000132806
hsa-miR-2682-5p	8.528625389	1.7675E-06	2.35667E-05
hsa-miR-500b-3p	7.922153506	0.000201492	0.001111546
hsa-miR-7848-3p	7.281151778	0.007008297	0.02092029
hsa-miR-1294	7.047950663	0.000534573	0.002486388
hsa-miR-3177-3p	7.041552106	0.003255136	0.010672577
hsa-miR-511-3p	4.747716311	2.0674E-11	4.1348E-09
hsa-miR-30a-5p	4.696239829	5.70281E-07	1.14056E-05
hsa-miR-145-5p	4.584610486	7.70299E-05	0.000489079
hsa-miR-511-5p	4.543092341	1.61573E-09	1.07715E-07
hsa-miR-5690	3.817542733	2.89259E-07	7.23148E-06
hsa-miR-582-3p	3.447148404	6.8584E-07	1.24698E-05
hsa-miR-4709-3p	3.220483802	0.005526047	0.017135028
hsa-miR-10527-5p	3.203911641	0.012955314	0.033218753
hsa-miR-148a-3p	2.599889098	1.29924E-07	3.99767E-06
hsa-miR-181d-5p	-2.629985342	3.78384E-11	5.04512E-09
hsa-miR-29b-3p	-2.831921193	4.18616E-09	2.39209E-07
hsa-miR-365b-5p	-3.082956863	0.000653255	0.002935979
hsa-miR-3614-5p	-3.181631287	0.004352563	0.01381766
hsa-miR-212-5p	-3.242874665	3.52215E-07	8.28742E-06
hsa-miR-155-5p	-3.469745209	1.18242E-10	1.18242E-08
hsa-miR-760	-3.770677152	0.001711491	0.006280701
hsa-miR-147b-3p	-3.815053426	2.38874E-12	9.55495E-10
hsa-miR-708-5p	-4.256269083	0.008648629	0.024710369
hsa-miR-365a-5p	-6.739494977	0.004938795	0.015555259
hsa-miR-193b-3p	-7.047707667	0.000641273	0.002914877
hsa-miR-5001-3p	-8.541992666	2.55951E-08	1.02381E-06
hsa-miR-4521	-9.52278279	3.355E-05	0.000227458

hsa-miR-187-3p	-11.30113124	4.53369E-06	4.53369E-05

Table 20 - Differential expression of miRNAs in M2c macrophages compared to M2a macrophages.

miRNA	logFC	PValue	FDR
hsa-miR-4662a-5p	9.348255725	0.00047084	0.002025117
hsa-miR-4632-3p	7.497839444	0.00079925	0.003134314
hsa-miR-6852-5p	7.31207716	0.010031802	0.027094697
hsa-miR-371b-5p	7.219453668	0.015541552	0.039345702
hsa-miR-130a-3p	6.986768673	0.0023061	0.007514097
hsa-miR-4684-3p	6.622655104	0.006147267	0.017316245
hsa-miR-30c-2-3p	6.165454781	0.000161622	0.000850643
hsa-miR-3605-3p	4.596065976	2.08846E-08	5.83328E-07
hsa-miR-335-3p	4.474597009	0.000326639	0.001519252
hsa-miR-5690	4.46200323	6.25173E-08	1.25035E-06
hsa-miR-6502-5p	4.305243385	2.65568E-06	3.42668E-05
hsa-miR-30a-5p	4.069213387	2.34323E-06	3.23204E-05
hsa-miR-145-5p	3.063386652	0.0014814	0.005290715
hsa-miR-146b-3p	-2.692750753	4.93477E-09	2.81987E-07
hsa-miR-501-5p	-2.714853631	1.78487E-09	1.21087E-07
hsa-miR-212-3p	-3.230910696	2.26172E-08	5.83328E-07
hsa-miR-760	-3.257822982	0.00457889	0.013771099
hsa-miR-877-5p	-3.648728035	5.34795E-05	0.000330311
hsa-miR-342-3p	-3.68742296	1.38557E-06	2.13164E-05
hsa-miR-212-5p	-4.117038851	2.27188E-08	5.83328E-07
hsa-miR-99a-5p	-4.710910062	1.1549E-10	2.3098E-08
hsa-miR-211-5p	-5.056196079	0.017596334	0.043990836
hsa-miR-125b-5p	-5.274535987	0.001506669	0.005328066
hsa-miR-504-5p	-6.194943045	0.000221776	0.00110888
hsa-miR-6516-5p	-7.037234375	0.010092774	0.027094697
hsa-miR-581	-8.055101431	0.004817854	0.01417016
hsa-miR-4521	-8.24202353	0.000123215	0.000694168
hsa-miR-4787-3p	-8.286478937	0.004440363	0.013558362
hsa-miR-5001-3p	-8.62013036	2.33331E-08	5.83328E-07
hsa-miR-1-3p	-9.142547135	0.000484093	0.002059971
hsa-miR-365a-5p	-10.40614116	0.000165153	0.000857936
hsa-miR-193b-3p	-10.59970231	1.38615E-05	0.000106627
hsa-miR-125b-2-3p	-12.1170339	8.68261E-06	7.2355E-05

Table 21 - Differential expression of miRNAs in stimulated vs naïve T cells.

miRNA	logFC	PValue	FDR
hsa-miR-155-5p	13.9985385	1.9992E-05	0.00684813

hsa-miR-4518	12.1028255	1.6948E-06	0.00355576
hsa-miR-1268b	11.6464798	0.00018681	0.01507409
hsa-miR-206	11.1845304	1.8405E-05	0.00684813
hsa-miR-3662	10.5553387	3.8412E-05	0.00684813
hsa-miR-6850-5p	10.4611029	0.00050689	0.03127828
hsa-miR-877-5p	10.4168157	6.8547E-05	0.00684813
hsa-miR-330-3p	10.3900119	6.7658E-05	0.00684813
hsa-miR-7974	10.2118564	5.0739E-05	0.00684813
hsa-miR-183-5p	9.74654448	0.00027493	0.01988953
hsa-miR-1292-5p	9.1763364	0.00092691	0.03904408
hsa-miR-21-3p	8.9764274	0.00078476	0.03658713
hsa-miR-1270	8.88235386	0.001133	0.04244688
hsa-miR-92a-2-5p	8.85994102	0.00104476	0.04135686
hsa-miR-34c-5p	8.78843713	5.8036E-05	0.00684813
hsa-miR-4746-5p	8.68997612	0.001289	0.04662636
hsa-miR-29b-3p	6.83690794	0.00078404	0.03658713
hsa-miR-18b-5p	5.65186734	0.000584	0.03403439
hsa-miR-146b-5p	4.11359996	0.00016045	0.01402578
hsa-miR-7-5p	3.68882468	0.00102448	0.04133371
hsa-miR-18a-3p	3.40970779	0.00022643	0.01759437
hsa-miR-671-5p	3.16051535	0.0006136	0.03442744
hsa-miR-378a-3p	3.09043338	0.00083337	0.03800881
hsa-miR-146a-5p	3.08064818	0.0011087	0.04229193
hsa-miR-18a-5p	3.03535246	0.00085584	0.03820303
hsa-miR-132-3p	3.01931611	0.0011928	0.04390347
hsa-miR-29a-5p	2.95541586	0.0007246	0.03658713
hsa-miR-20b-5p	2.74883001	0.00017397	0.01459916
hsa-miR-27a-5p	2.72893584	0.00073952	0.03658713

Numbered embodiments

1. A method of regulating expression of a target gene in a cell in a context-specific manner, the method comprising:
   - providing a cell with a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene;
   - wherein the inhibitory RNA is active in the cell in a context-specific manner such that inhibition of the expression of the target gene occurs specifically in a first context, in which the inhibitory RNA is active in said cell.

2. The method of embodiment 1 wherein inhibition does not occur, or occurs in reduced amount, in at least at least a second context, in which the inhibitory RNA is inactive or less active.

3. The method of embodiment 1 or 2 wherein the nucleic acid construct is a modified form of an endogenous sequence encoding an inhibitory RNA, preferably an miRNA, which has been modified to target a transcript from the target gene.

4. The method of any preceding embodiment wherein the cell is adapted to express at least one further inhibitory RNA which is/are active in the cell in a context-specific manner.

5. The method of embodiment 4 wherein inhibition of the expression of a second or further target gene occurs specifically in a first context, in which the inhibitory RNAs are active in said cell.

6. The method of embodiment 4 wherein inhibition of the expression of a second or further target gene occurs specifically in a second or further context, in which the at least one further inhibitory RNAs are active in said cell.

7. The method of any one of embodiments 4 to 6 wherein the inhibitory RNAs target more than one target gene, optionally wherein one of the target genes is B2M and another one of the target genes is selected from: PPARG, IRF4, KDM6B, FOXP3, PDCD-1 and STAT6, optionally wherein one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or

more inhibitory RNAs is selected from: SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG); SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4); SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B); SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3); SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1) or SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6); or wherein one of the target genes is PPARG and another one of the target genes is selected from: IRF4, KDM6B and STAT6, or wherein one of the target genes target genes is IRF4 and another one of the target genes is selected from: PPARG, KDM6B and STAT6, or wherein one of the target genes is KDM6B and another one of the target genes is selected from: PPARG, IRF4, and STAT6, or wherein one of the target genes is FOXP3 and another one of the target genes is PDCD-1.

8. The method of any preceding embodiment wherein the cell is provided with two or more nucleic acid constructs adapted to express an inhibitory RNA suitable to inhibit expression of one or more target genes in a context-specific manner, or

wherein a nucleic acid construct is provided which is adapted to express two or more inhibitory RNAs suitable to inhibit expression of the target gene in a context-specific manner.

9. The method of any preceding embodiment wherein the method comprises the step of genetically modifying a sequence encoding an endogenous inhibitory RNA to alter its targeting specificity so that it targets the transcript of a target gene.

10. The method of any preceding embodiment comprising genetically modifying an endogenous genomic sequence in the cell encoding an endogenous inhibitory RNA to retarget the endogenous inhibitory RNA to the transcript of a target gene.

11. The method of embodiment 9 wherein the method comprises the step of inserting into the genome of the cell at a suitable locus a sequence encoding at least one modified endogenous inhibitory RNA with altered targeting specificity so that it targets the transcript of a target gene.

12. The method of any preceding embodiment, wherein the method comprises modifying a cell to comprise a nucleic acid construct adapted to express an inhibitory RNA suitable

to inhibit expression of the target gene, and wherein the inhibitory RNA becomes active in the cell following a change in the context of the cell.

13. The method of any preceding embodiment wherein a progenitor cell (e.g. a stem cell, suitably an iPSC) is modified to comprise the nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene, and wherein the inhibitory RNA is active in the cell following differentiation, preferably wherein the inhibitory RNA is active in the cell the cell is fully differentiated.

14. The method of any preceding embodiment wherein the cell is a human cell.

15. The method of any preceding embodiment wherein the cell is a is differentiated cell selected from the group consisting of: a leukocyte, a dendritic cell, a lymphocyte, a monocyte, a macrophage, a red blood cell, platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell and an adipocyte.

16. The method of any preceding embodiment wherein the cell is a cell is a stem cell, suitably a stem cell which is suitable to differentiate into a differentiated cell selected from the group consisting of: a leukocyte, a dendritic cell, a lymphocyte, a red blood cell, a platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell or an adipocyte.

17. The method of any preceding embodiment wherein the cell is a pluripotent cell, such as an embryonic stem cell or an iPSC.

18. The method of any preceding embodiment wherein the cell is a therapeutic cell.

19. The method of any preceding embodiment wherein the cell is an allogeneic therapeutic cell.

20. The method of any preceding embodiment wherein the cell is a context-specific hypoimmunogenic cell, preferably a context-specific hypoimmunogenic therapeutic cell.

21. The method of any preceding embodiment wherein the cell is an endocrine cell or a progenitor thereof, optionally a pancreatic endocrine cell or a progenitor thereof, preferably a pancreatic beta cell or a progenitor thereof.

22. The method of any preceding embodiment wherein the cell is an immune cell, or a progenitor thereof.

23. The method of any preceding embodiment wherein the cell is a macrophage or a T cell, or a progenitor thereof.

24. The method of any preceding embodiment wherein the inhibitory RNA is specifically active in a cell which is in a context selected from the group consisting of:
- a cell at a particular state of differentiation, for example a fully-differentiated cell, a cell at an intermediate stage of differentiation or an undifferentiated cell;
- a totipotent cell, a pluripotent cell or a multipotent cell;
- a cell present in a particular tissue;
- a cancerous or pre-cancerous cell;
- a tumour associated cell, e.g. a tumour associated immune cell or an immune cell in the tumour microenvironment;
- a polarised cell, e.g. a macrophage;
- an immune cell which is activated or polarised;
- a cell which is hypoxic; or
- a cell subject to the unfolded protein response.

25. The method of any preceding embodiment wherein the cell is an allogeneic therapeutic cell, or a progenitor thereof, and wherein the inhibitory RNA is active in the allogeneic therapeutic cell which is in a fully differentiated state and/or which is present in the correct tissue.

26. The method of embodiment 25 wherein the inhibitory RNA is substantially inactive when the cell is in a less differentiated state and/or wherein the cell is not in the correct tissue.

27. The method of any preceding embodiment wherein the inhibitory RNA targets a target gene associated with the MHC-I complex, optionally wherein the target gene is B2M, HLA-A, HLA-B and/or HLA-C gene such that expression of the MHC-I complex on the cell is reduced when the inhibitory RNA is active.

28. The method of any preceding embodiment wherein the cell is an allogeneic therapeutic cell selected from the group consisting of:
    - a therapeutic endocrine cell or a progenitor thereof, preferably a pancreatic beta cell or a progenitor thereof;
    - a therapeutic CNS cell or a progenitor thereof, suitably a neuron or a glial cell (such as an astrocyte, an oligodendrocytes, an ependymal cell, or a microglial cell) or a progenitor thereof;
    - a therapeutic immune cell or a progenitor thereof;
    - a therapeutic epithelial cell or a progenitor thereof;
    - a therapeutic myocyte or a progenitor thereof, for example a cardiac myocyte, skeletal myocyte, and smooth myocyte;
    - a therapeutic pluripotent stem cell.

29. The method of any preceding embodiment wherein the cell is, or is derived from, tissue resident, multipotent stem cells or progenitor cells with distinct developmental origins such as haematopoietic stem cells (HSC, hemangioblast), bone, cartilage and adipose stem cells (mesenchymal, lateral plate), striated muscle stem cells (skeletal muscle (satellite), cardiac muscle, epicardium), smooth muscle stem cells (blood vessel pericyte), hemangioblast endothelial stem cells, neural stem cells, glial progenitors, Müller glia cells, non-myelinating Schwaan cells, hair follicular stem (bulge) cells, liver stem cells, cholangiocytes, basal epithelial stem cells in epidermis (skin), the proximal trachea/bronchioles and gastro-intestinal (GI) tract, alveolar type II cells in the lung, kidney mesenchyme.

30. The method of embodiment 27 wherein the target gene is involved in the MHC-I complex, optionally wherein the target gene is the B2M gene, an HLA-A, an HLA-B and/or an HLA-C gene, preferably wherein the target gene is B2M.

31. The method of any preceding embodiment wherein the cell is a allogeneic pancreatic beta cell, or a progenitor thereof, wherein the inhibitory RNA is active in beta cells, preferably fully differentiated beta cells when present in the pancreas, and wherein the target gene is the B2M gene and/or an HLA-A, HLA-B and/or HLA-C gene.

32. The method of any preceding embodiment wherein the inhibitory RNA is a modified form of an endogenous miRNA selected from the group consisting of: hsa-miR-375, hsa-miR-143, hsa-miR-21, hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, and hsa-miR-200c.

33. The method of any preceding embodiments wherein the inhibitory RNA comprises one of the following sequences or a functional variant thereof:
- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

34. The method of any preceding embodiment wherein the cell is a macrophage, or a progenitor thereof, and wherein the inhibitory RNA is specifically active in a macrophage in one of the following contexts:
- the macrophage is an unpolarised macrophage;
- the macrophage is classically activated (M1);
- the macrophage is alternatively activated (M2);
- the macrophage is a regulatory macrophages (Mreg); or
- the macrophage is in the TME;

or wherein the miRNA is selected from the group consisting of: MIR146A (e.g. hsa-miR-146a-5p), MIR20A (e.g. hsa-miR-20a-5p), LET7C (hsa-miR-let-7c-5p), and LET7i (hsa-let-7i-5p).

35. The method of any preceding embodiment wherein the target gene is selected from the group consisting of:
- an immunosuppressive gene;
- a pro-inflammatory gene;
- a gene which promotes immunosenescence.

36. The method of any preceding embodiment wherein the cell is a macrophage or a progenitor thereof, the inhibitory RNA is active in the TME and the target gene is an immunosuppressive gene.

37. The method of any preceding embodiment wherein the target gene is an immunosuppressive gene selected from STAT6, IRF4, DRP1, PPARG, KDM6B, Clever-1, LAIR1/2, P-selection glycoprotein ligand (PSGL-1), signal regulatory protein alpha (SIRPa), triggering receptor expressed on myeloid cells 2 (TREM2), proton sensing GPCR (GPR65), inhibitory leukocyte immunoglobulin-like receptor subfamily B4 (LILRB4), and PD-1 or wherein the target gene is RASA-2.

38. The method of any preceding embodiment wherein the inhibitory RNA is a modified form of an endogenous miRNA selected from the group consisting of: miR-375, miR-340-5p, miR-29a-3p, miR-222-3p, miR-940, miR-203, miR-145, miR-103a, miR-21-3p, miR-181d-5p, miR-125b-5p, miR-301a-3p, miR-132, miR-29b-1, miR-27a, miR-146a, miR-222, miR-let7a, miR-320a, miR-let7c, miR-1246, miR-let7d-5p, miR-451 and miR-21 .

39. The method of any preceding embodiment wherein the cell is a T cell, or progenitor thereof, and wherein the inhibitory RNA is specifically active in the T cell in one of the following contexts:
- the T cell is a CD8+ T cell;
- the T cell is a CD4+ T cell;
- the T cell is a CD4+ T helper cell, optionally a Th1, Th2, Th17, Th9, Tfh or Th22 cell;
- the T cell is a memory T cell;
- the T cell is a CD4+ T_{reg} cell, optionally a $FOXP3^+$ T_{reg} cells or a $FOXP3^-$ T_{reg} cell;
- the T cell is exhausted; or
- the T cell is in the TME.

40. The method of any preceding embodiment wherein the target gene is selected from the group consisting of:
- an immunosuppressive gene;
- a pro-inflammatory gene;
- a gene which promotes immunosenescence;
- a gene involved in an immune checkpoint.

41. The method of any preceding embodiment wherein the cell is a T cell or a progenitor thereof, the inhibitory RNA is active in the TME and the target gene is an immunosuppressive gene, a gene which promotes immunosenescence and/or a gene involved in an immune checkpoint.

42. The method of any one of embodiments 39 to 41 wherein the target gene is an immunosuppressive gene selected from PD-1, CTLA4, LAG3, TIGIT (T-cell-immunoreceptor with Ig and ITIM domains), and TIM3.

43. The method of any one of embodiments 39 to 42 wherein the inhibitory RNA is a modified form of miR-21.

44. A cell or cell population obtained by the method of any preceding embodiment.

45. A genetically modified cell which comprises a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of a target gene;
wherein the inhibitory RNA is active in the cell in a context-specific manner, such that inhibition of the expression of the target gene occurs specifically in a first context, in which the inhibitory RNA is active in said cell.

46. The cell of embodiment 45 wherein inhibition does not occur or is reduced in at least a second context, in which the inhibitory RNA is inactive or less active.

47. The cell of any one of embodiments 45 to 46 wherein the nucleic acid construct is a modified endogenous sequence encoding an inhibitory RNA, preferably an miRNA, which has been modified to target a transcript from the target gene.

48. The cell of any one of embodiments 45 to 47 wherein the cell is adapted to express at least one further inhibitory RNA which is/are active in the cell in a context-specific manner.

49. The cell of embodiment 48 wherein inhibition of the expression of a second or further target gene occurs specifically in a first context, in which the inhibitory RNAs are active in said cell.

50. The cell of embodiment 48 wherein inhibition of the expression of a second or further target gene occurs specifically in a second or further context, in which the at least one further inhibitory RNAs are active in said cell.

51. The cell of any one of embodiments 48 to 50 wherein the inhibitory RNAs target more than one target gene, optionally wherein one of the target genes is B2M and another one of the target genes is selected from: PPARG, IRF4, KDM6B, FOXP3, PDCD-1 and STAT6, optionally wherein one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG); SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4); SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B); SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3); SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1) or SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6); or wherein one of the target genes is PPARG and another one of the target genes is selected from: IRF4, KDM6B and STAT6, or wherein one of the target genes target genes is IRF4 and another one of the target genes is selected from: PPARG, KDM6B and STAT6, or wherein one of the target genes is KDM6B and another one of the target genes is selected from: PPARG, IRF4, and STAT6, or wherein one of the target genes is FOXP3 and another one of the target genes is PDCD-1.

52. The cell of any one of embodiments 48 to 51 wherein the cell is provided with two or more nucleic acid constructs, each adapted to express an inhibitory RNA suitable to inhibit expression of one or more target genes in a context-specific manner, or

wherein a nucleic acid construct is provided which is adapted to express two or more inhibitory RNAs suitable to inhibit expression of the target gene in a context-specific manner.

53. The cell of any one of embodiments 45 to 52 wherein the cell comprises a genetically modified a sequence encoding an endogenous inhibitory RNA with altered targeting specificity so that it targets the transcript of a target gene.

54. The cell of any one of embodiments 45 to 53 comprising a genetically modified endogenous genomic sequence encoding an endogenous inhibitory RNA which has been retargeted to the transcript of a target gene.

55. The cell of any one of embodiment 45 to 54 comprising an insertion into the genome at a suitable locus of a sequence encoding at least one modified endogenous inhibitory RNA with altered targeting specificity so that it targets the transcript of a target gene.

56. The cell of any one of embodiments 45 to 55 comprising a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene, and wherein the inhibitory RNA becomes active in the cell following a change in the context of the cell.

57. The cell of any one of embodiments 45 to 56 wherein the cell is a progenitor cells which has been modified to comprise a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene, and wherein the inhibitory RNA is active in the cell following differentiation, preferably wherein the inhibitory RNA is active in the cell when the cell is fully differentiated.

58. The cell of any one of embodiments 45 to 57 wherein the cell is a human cell.

59. The cell of any one of embodiments 45 to 58 wherein the cell is a is differentiated cell selected from the group consisting of: a leukocyte, a dendritic cell, a lymphocyte, a monocyte, a macrophage, a red blood cell, platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell and an adipocyte.

60. The cell of any one of embodiments 45 to 59 wherein the cell is a cell is a stem cell, suitably a stem cell which is suitable to differentiate into a differentiated cell selected from the group consisting of: a leukocyte, a dendritic cell, a lymphocyte, a red blood cell, a platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell or an adipocyte.

61. The cell of any one of embodiments 45 to 60 wherein the cell is a pluripotent or totipotent cell, such as an embryonic stem cell or an iPSC.

62. The cell of any one of embodiments 45 to 61 wherein the cell is a therapeutic cell.

63. The cell of any one of embodiments 45 to 62 wherein the cell is an allogeneic therapeutic cell.

64. The cell of any one of embodiments 45 to 63 wherein the cell is a context-specific hypoimmunogenic cell, preferably a context-specific hypoimmunogenic therapeutic cell.

65. The cell of any one of embodiments 45 to 64 wherein the cell is an endocrine cell or a progenitor thereof, optionally a pancreatic endocrine cell or a progenitor thereof, preferably a pancreatic beta cell or a progenitor thereof.

66. The cell of any one of embodiments 45 to 65 wherein the cell is an immune cell, or a progenitor thereof.

67. The cell of any one of embodiments 45 to 66 wherein the cell is a macrophage or a T cell, or a progenitor thereof.

68. The cell of any one of embodiments 45 to 67 wherein the inhibitory RNA is specifically active in a cell which is in a context selected from the group consisting of:
- a cell at a particular state of differentiation, for example a fully-differentiated cell, a cell at an intermediate stage of differentiation or an undifferentiated cell;
- a totipotent cell, a pluripotent cell or a multipotent cell;
- a cell present in a particular tissue;
- a cancerous or pre-cancerous cell;
- a tumour associated cell, e.g. a tumour associated immune cell or an immune cell in the tumour microenvironment;
- a polarised cell, e.g. a macrophage;
- an immune cell which is activated or polarised;
- a cell which is hypoxic; or
- a cell subject to the unfolded protein response.

69. The cell of any one of embodiments 45 to 68 wherein the cell is an allogeneic therapeutic cell, or a progenitor thereof, and wherein the inhibitory RNA is active in the allogeneic therapeutic cell which is in a fully differentiated state and/or which is present in the correct tissue.

70. The cell of embodiment 68 or 69 wherein the inhibitory RNA is substantially inactive when the cell is in a less differentiated state and/or wherein the cell is not in the correct tissue.

71. The cell of any one of embodiments 45 to 70 wherein the inhibitory RNA targets a target gene associated with the MHC-I complex, optionally wherein the target gene is B2M, HLA-A, HLA-B and/or HLA-C such that expression of the MHC-I complex on the cell is reduced in a context in which the inhibitory RNA is active.

72. The cell of any one of embodiments 45 to 71 wherein the cell is an allogeneic therapeutic cell selected from the group consisting of:
- a therapeutic endocrine cell or a progenitor thereof, preferably a pancreatic beta cell or a progenitor thereof;
- a therapeutic CNS cell or a progenitor thereof, suitably a neuron or a glial cell (such as an astrocyte, an oligodendrocytes, an ependymal cell, or a microglial cell) or a progenitor thereof;
- a therapeutic immune cell or a progenitor thereof;
- a therapeutic epithelial cell or a progenitor thereof;
- a therapeutic myocyte or a progenitor thereof, for example a cardiac myocyte, skeletal myocyte, and smooth myocyte;
- therapeutic pluripotent stem cell.

73. The cell of any one of embodiments 45 to 72 wherein the cell is, or is derived from, tissue resident, multipotent stem cells or progenitor cells with distinct developmental origins such as haematopoietic stem cells (HSC, hemangioblast), bone, cartilage and adipose stem cells (mesenchymal, lateral plate), striated muscle stem cells (skeletal muscle (satellite), cardiac muscle, epicardium), smooth muscle stem cells (blood vessel pericyte), hemangioblast endothelial stem cells, neural stem cells, glial progenitors, Müller glia cells, non-myelinating Schwaan cells, hair follicular stem (bulge) cells, liver stem cells, cholangiocytes, basal epithelial stem cells in epidermis (skin), the proximal trachea/bronchioles and gastro-intestinal (GI) tract, alveolar type II cells in the lung, kidney mesenchyme.

74. The cell of any one of embodiments 45 to 73 wherein the target gene is involved in the MHC-I complex, optionally wherein the target gene is the B2M gene, an HLA-A, an HLA-B and/or an HLA-C gene, preferably wherein the target gene is B2M.

75. The cell of any one of embodiments 45 to 74 wherein the cell is a allogeneic pancreatic beta cell or a progenitor thereof, the inhibitory RNA is active in fully differentiated beta cells, preferably fully differentiated beta cells when present in the pancreas, and wherein the target gene is the B2M gene and/or an HLA-A, HLA-B and/or HLA-C gene.

76. The cell of any one of embodiments 45 to 75 wherein the inhibitory RNA is a modified form of an endogenous miRNA selected from the group consisting of: hsa-miR-375, hsa-miR-143, hsa-miR-21, hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, and hsa-miR-200c.

77. The cell of any one of embodiments 45 to 76 wherein the inhibitory RNA comprises one of the following sequences or a functional variant thereof:
- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

78. The cell of any one of embodiments 45 to 77 wherein the cell is a macrophage, or a progenitor thereof, and wherein the inhibitory RNA is active in a macrophage in one of the following contexts:
- the macrophage is an unpolarised macrophage;
- the macrophage is classically activated (M1);
- the macrophage is alternatively activated (M2);
- the macrophage is a regulatory macrophages (Mreg); or
- the macrophage is in the TME;

or wherein the miRNA is selected from the group consisting of: MIR146A (e.g. hsa-miR-146a-5p), MIR20A (e.g. hsa-miR-20a-5p), LET7C (hsa-miR-let-7c-5p), and LET7i (hsa-let-7i-5p).

79. The cell of any one of embodiments 45 to 78 wherein the target gene is selected from the group consisting of:
- an immunosuppressive gene;
- a pro-inflammatory gene;
- a gene which promotes immunosenescence.

80. The cell of any one of embodiments 45 to 79 wherein the cell is a macrophage or a progenitor thereof, the inhibitory RNA is active in the TME and the target gene is an immunosuppressive gene or a gene which promotes immunosenescence.

81. The cell of any one of embodiments 45 to 80 wherein the target gene is an immunosuppressive gene selected from STAT6, IRF4, DRP1, PPARG, KDM6B, Clever-1, LAIR1/2, P-selection glycoprotein ligand (PSGL-1), signal regulatory protein alpha (SIRPa), triggering receptor expressed on myeloid cells 2 (TREM2), proton sensing GPCR (GPR65), inhibitory leukocyte immunoglobulin-like receptor subfamily B4 (LILRB4), and PD-1 or wherein the target gene is RASA-2.

82. The cell of any one of embodiments 45 to 82 wherein the inhibitory RNA is a modified form of an endogenous miRNA selected from the group consisting of: miR-375, miR-340-5p, miR-29a-3p, miR-222-3p, miR-940, miR-203, miR-145, miR-103a, miR-21-3p, miR-181d-5p, miR-125b-5p, miR-301a-3p, miR-132, miR-29b-1, miR-27a, miR-146a, miR-222, miR-let7a, miR-320a, miR-let7c, miR-1246, miR-let7d-5p, miR-451 and miR-21.

83. The cell of any one of embodiments 45 to 77 wherein the cell is a T cell, or progenitor thereof, and wherein the inhibitory RNA is specifically active in the T cell in one of the following contexts:
- the T cell is a CD8+ T cell;
- the T cell is a CD4+ T cell;
- the T cell is a CD4+ T helper cell, optionally a Th1, Th2, Th17, Th9, Tfh ot Th22 cell;
- the T cell is a memory T cell;
- the T cell is a CD4+ T_{reg} cell, optionally a FOXP3^{+} T_{reg} cells or a FOXP3^{-} T_{reg} cell;
- the T cell is exhausted (e.g. a cell ; or

- the T cell is in the TME.

84. The cell of embodiment 83 wherein the target gene is selected from the group consisting of:
- an immunosuppressive gene;
- a pro-inflammatory gene;
- a gene which promotes immunosenescence;
- a gene involved in an immune checkpoint.

85. The method of any one of embodiments 83 to 84 wherein the cell is a T cell or a progenitor thereof, wherein the inhibitory RNA is active in the TME and wherein the target gene is an immunosuppressive gene, a gene which promotes immunosenescence and/or a gene involved in an immune checkpoint.

86. The cell of any one of embodiments 83 to 85 wherein the target gene is an immunosuppressive gene selected from PD-1, CTLA4, LAG3, TIGIT (T-cell-immunoreceptor with Ig and ITIM domains), and TIM3.

87. A nucleic acid construct for the context-specific inhibition of expression of a target gene in a cell, the nucleic acid construct encoding an inhibitory RNA suitable to inhibit expression of the target gene, wherein the inhibitory RNA is active in the cell in a context-specific manner such that inhibition of the expression of the target gene occurs in a first context, in which the inhibitory RNA is active in said cell.

88. A nucleic acid construct according to embodiment 87 wherein the construct comprises a sequence selected from the following list, or a functional variant thereof:
- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);

- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

89. An inhibitory RNA which comprises or consists of a sequence selected from the following list, or a functional variant thereof:
- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

90. Use of a contextually-active inhibitory RNA, or nucleic acid construct encoding a contextually-active inhibitory RNA, in a method of context-specific inhibition of expression of a target gene in a cell, wherein the inhibitory RNA is active in the cell in a context-specific manner such that inhibition of the expression of the target gene occurs in a first context, in which the inhibitory RNA is active in said cell.

91. The use of embodiment 88 wherein the inhibitory RNA comprises or consists of a sequence selected from the following list, or a functional variant thereof:
- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);

- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

92. A composition comprising a cell according to any one of embodiments 44 to 86.

93. A method of treatment comprising the administering to a subject a therapeutically effective amount of a cell according to any one of embodiments 44 to 86 or a composition according to embodiment 92.

94. A cell according to any one of embodiments 44 to 86 or a composition according to embodiment 92 for use in therapy.

Claims

1. A method of regulating expression of a target gene in a cell in a context-specific manner, the method comprising:
   - providing a cell with a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene;
   - wherein the inhibitory RNA is active in the cell in a context-specific manner such that inhibition of the expression of the target gene occurs specifically in a first context, in which the inhibitory RNA is active in said cell,
   optionally wherein inhibition does not occur, or occurs in reduced amount, in at least a second context, in which the inhibitory RNA is inactive or less active.

2. The method of claim 1 wherein the nucleic acid construct is a modified form of an endogenous sequence encoding an inhibitory RNA, preferably an miRNA, which has been modified to target a transcript from the target gene.

3. The method of claim 1 or 2 wherein the method comprises the step of genetically modifying a sequence encoding an endogenous inhibitory RNA to alter its targeting specificity so that it targets the transcript of a target gene.

4. The method of any preceding claim wherein the cell is a context-specific hypoimmunogenic cell, preferably a context-specific hypoimmunogenic therapeutic cell.

5. The method of any preceding claim wherein the cell is an immune cell, or a progenitor thereof, such as wherein the cell is a macrophage or a T cell, or a progenitor thereof.

6. The method any preceding claim wherein the inhibitory RNA targets a target gene associated with the MHC-I complex, optionally wherein the target gene is B2M, HLA-A, HLA-B and/or HLA-C gene such that expression of the MHC-I complex on the cell is reduced when the inhibitory RNA is active, preferably when the target gene is B2M.

7. The method of any preceding claim wherein the inhibitory RNA is specifically active in a cell which is in a context selected from the group consisting of:
   - a cell at a particular state of differentiation, for example a fully-differentiated cell, a cell at an intermediate stage of differentiation or an undifferentiated cell;
   - a totipotent cell, a pluripotent cell or a multipotent cell;
   - a cell present in a particular tissue;

- a cancerous or pre-cancerous cell;
- a tumour associated cell, e.g. a tumour associated immune cell or an immune cell in the tumour microenvironment;
- a polarised cell, e.g. a macrophage;
- an immune cell which is activated or polarised;
- a cell which is hypoxic; or
- a cell subject to the unfolded protein response.

8. The method of any preceding claim wherein the cell is an allogeneic therapeutic cell selected from the group consisting of:
- a therapeutic immune cell or a progenitor thereof;
- a therapeutic endocrine cell or a progenitor thereof, preferably a pancreatic beta cell or a progenitor thereof;
- a therapeutic CNS cell or a progenitor thereof, suitably a neuron or a glial cell (such as an astrocyte, an oligodendrocytes, an ependymal cell, or a microglial cell) or a progenitor thereof;
- a therapeutic epithelial cell or a progenitor thereof;
- a therapeutic myocyte or a progenitor thereof, for example a cardiac myocyte, skeletal myocyte, and smooth myocyte;
- a therapeutic pluripotent stem cell.

9. A cell or cell population obtained by the method of any preceding claim.

10. A genetically modified cell which comprises a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of a target gene;

wherein the inhibitory RNA is active in the cell in a context-specific manner, such that inhibition of the expression of the target gene occurs specifically in a first context, in which the inhibitory RNA is active in said cell,

optionally wherein inhibition does not occur or is reduced in at least a second context, in which the inhibitory RNA is inactive or less active.

11. The cell of claim 10 wherein the nucleic acid construct is a modified endogenous sequence encoding an inhibitory RNA, preferably an miRNA, which has been modified to target a transcript from the target gene.

12. The method of any one of claims 1 to 8 or the cell of any one of claims 10 or 11 wherein the cell is adapted to express at least one further inhibitory RNA which is/are active in the cell in a context-specific manner,

optionally wherein inhibition of the expression of a second or further target gene occurs specifically in a first context, in which the inhibitory RNAs are active in said cell or

wherein inhibition of the expression of a second or further target gene occurs specifically in a second or further context, in which the at least one further inhibitory RNAs are active in said cell.

13. The cell of any one of claims 10-12 wherein the cell comprises a genetically modified a sequence encoding an endogenous inhibitory RNA with altered targeting specificity so that it targets the transcript of a target gene.

14. The cell of any one of claims 10-13 comprising a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene, and wherein the inhibitory RNA becomes active in the cell following a change in the context of the cell.

15. The cell of any one of claims 10-14 wherein the cell is a context-specific hypoimmunogenic cell, preferably a context-specific hypoimmunogenic therapeutic cell.

16. The method or cell of any preceding claim wherein the inhibitory RNAs target more than one target gene, optionally wherein one of the target genes is B2M and another one of the target genes is selected from: PPARG, IRF4, KDM6B, FOXP3, PDCD-1 and STAT6, optionally wherein one of the two or more inhibitory RNAs is selected from SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), SEQ ID NO: 10 or 27 (Solution 30), SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M), and another one of the two or more inhibitory RNAs is selected from: SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG); SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4); SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B); SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3); SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1) or SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6); or wherein one of the target genes is PPARG and another one of the target genes is

selected from: IRF4, KDM6B and STAT6, or wherein one of the target genes target genes is IRF4 and another one of the target genes is selected from: PPARG, KDM6B and STAT6, or wherein one of the target genes is KDM6B and another one of the target genes is selected from: PPARG, IRF4, and STAT6, or wherein one of the target genes is FOXP3 and another one of the target genes is PDCD-1.

17. The method or the cell of any preceding claim wherein the cell is a progenitor cell which has been modified to comprise a nucleic acid construct adapted to express an inhibitory RNA suitable to inhibit expression of the target gene, and wherein the inhibitory RNA is active in the cell following differentiation, preferably wherein the inhibitory RNA is active in the cell when the cell is fully differentiated.

18. The method or cell of any preceding claim wherein the cell is a differentiated cell selected from the group consisting of: a leukocyte, a dendritic cell, a lymphocyte, a monocyte, a macrophage, a red blood cell, platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell and an adipocyte

or wherein the cell is a cell is a stem cell, suitably a stem cell which is suitable to differentiate into a differentiated cell selected from the group consisting of: a leukocyte, a dendritic cell, a lymphocyte, a red blood cell, a platelet, a hematopoietic cell, a pancreatic islet cell (e.g. a beta cell), a liver cell, a muscle cell, a keratinocyte, a cardiomyocyte, a neuronal cell, a skeletal muscle cell, an ocular cell, a mesenchymal cell, a fibroblast, a lung cell, a GI tract cell, a vascular cell, an endocrine cell or an adipocyte.

19 . The method or cell of any one preceding claim wherein the inhibitory RNA is a modified form of an endogenous miRNA selected from the group consisting of: hsa-miR-375, hsa-miR-143, and hsa-miR-21, hsa-miR-30d, hsa-miR-192, hsa-miR-30a, hsa-miR-182, hsa-miR-148a, hsa-miR-127, and hsa-miR-200c and/or wherein the inhibitory RNA comprises one of the following sequences or a functional variant thereof:

- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);

- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

20. The method or cell of any preceding claim wherein the cell is a macrophage, or a progenitor thereof, and wherein the inhibitory RNA is active in a macrophage in one of the following contexts:
- the macrophage is an unpolarised macrophage;
- the macrophage is classically activated (M1);
- the macrophage is alternatively activated (M2);
- the macrophage is a regulatory macrophages (Mreg); or
- the macrophage is in the TME;

or wherein the miRNA is selected from the group consisting of: MIR146A (e.g. hsa-miR-146a-5p), MIR20A (e.g. hsa-miR-20a-5p), LET7C (hsa-miR-let-7c-5p), and LET7i (hsa-let-7i-5p).

21. The method or cell of any preceding claims wherein the cell is a T cell, or progenitor thereof, and wherein the inhibitory RNA is specifically active in the T cell in one of the following contexts:
- the T cell is a CD8+ T cell;
- the T cell is a CD4+ T cell;
- the T cell is a CD4+ T helper cell, optionally a Th1, Th2, Th17, Th9, Tfh ot Th22 cell;
- the T cell is a memory T cell;
- the T cell is a CD4+ T_{reg} cell, optionally a $FOXP3^+$ T_{reg} cells or a $FOXP3^-$ T_{reg} cell;
- the T cell is exhausted (e.g. a cell ; or
- the T cell is in the TME.

22. A nucleic acid construct for the context-specific inhibition of expression of a target gene in a cell, the nucleic acid construct encoding an inhibitory RNA suitable to inhibit expression of the target gene, wherein the inhibitory RNA is active in the cell in a context-

specific manner such that inhibition of the expression of the target gene occurs in a first context, in which the inhibitory RNA is active in said cell wherein the construct comprises a sequence selected from the following list, or a functional variant thereof:

- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or
- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

23. An inhibitory RNA which comprises or consists of a sequence selected from the following list, or a functional variant thereof:

- SEQ ID NO: 1 or 18 (Solution 8), SEQ ID NO: 5 or 22 (Solution 20), SEQ ID NO: 9 or 26 (Solution 29), or SEQ ID NO: 10 or 27 (Solution 30) (targeting B2M);
- SEQ ID NO: 66 to SEQ ID NO: 87 or SEQ ID NO: 88 to SEQ ID NO: 109 (targeting PPARG);
- SEQ ID NO: 110 to SEQ ID NO: 133 or SEQ ID NO: 134 to SEQ ID NO: 157 (targeting IRF4);
- SEQ ID NO: 158 to SEQ ID NO: 181 or SEQ ID NO: 182 to SEQ ID NO: 205 (targeting KDM6B);
- SEQ ID NO: 348 to SEQ ID NO:411 or SEQ ID NO: 412 to SEQ ID NO: 475 (targeting B2M);
- SEQ ID NO: 476 to SEQ ID NO: 525 or SEQ ID NO: 526 to SEQ ID NO: 575 (targeting FOXP3);
- SEQ ID NO: 576 to SEQ ID NO: 624 or SEQ ID NO: 625 to SEQ ID NO: 673 (targeting PDCD-1); or

- SEQ ID NO: 206 to SEQ ID NO: 229 or SEQ ID NO: 230 to SEQ ID NO: 253 (targeting STAT6).

24. A composition comprising a cell according to any one of claims 9-21.

25. A cell according to any one of claims 9-21 or a composition according to claim 24 for use in therapy.

26. A reporter nucleic acid comprising or consisting of a constitutive promoter operably linked to a gene encoding a fluorescent protein and a target gene (or a portion of a target gene), wherein the fluorescent protein and the target gene are transcribed as a polycistronic mRNA.

27. The reporter nucleic acid according to claim 26, wherein the target gene is inserted into the 3' untranslated region of the fluorescent protein.

28. The reporter nucleic acid according to claims 26 or 27, wherein the target gene is a target gene for an inhibitory RNA and/or wherein the target gene is selected from: PDCD-1, B2M, PPARG, IRF4, KDM6B, FOXP3 or STAT6.

29. The reporter nucleic acid according to claims 26-28, wherein the constitutive promoter is EF1a and/or wherein the fluorescent protein is GFP, optionally CopGFP.

30. The reporter nucleic acid according to claims 26-29 further comprising:
- WPRE;
- Flanking Long Terminal Repeat (LTR) sequences; and/or
- a gene for a cell surface protein operably linked to a constitutive promoter, optionally wherein the constitutive promoter is PGK, optionally wherein the cell surface protein is tNGFR.

31. The reporter nucleic acid according to claims 26-30 for use in detecting the inhibition or silencing of the target gene by one or more inhibitory RNAs.

32. Use of the reporter nucleic acid according to claims 26-30 in detecting the inhibition or silencing of the target gene by one or more inhibitory RNAs.

33. An expression system comprising:

- the reporter nucleic acid according to claims 26-31; and
- an expression nucleic acid comprising a constitutive promoter operably linked to a nucleic acid sequence encoding an inhibitory RNA.

34. The expression system according to claim 33, wherein:
- the expression nucleic acid is flanked by Long Terminal Repeat (LTR) sequences;
- the constitutive promoter in the expression nucleic acid is EF1a;
- the inhibitory RNA is an endogenous non-coding RNA encoding sequence, e.g. an endogenous inhibitory RNA encoding sequence, which has been modified to retarget the endogenous inhibitory RNA to silence or partially silence a target gene via GEiGS approach, or wherein the inhibitory RNA is a GEiGS extended Solution and/or
- the expression nucleic acid further comprises a further fluorescent protein operably linked to the constitutive promoter, optionally wherein the further fluorescent protein is dsRed;

35. The expression system according to claims 33 or 34, wherein the reporter nucleic acid and the expression nucleic acid are provided in two separate lentiviral vectors.

36. The expression system according to claims 33-35, wherein the expression system comprises two or more expression nucleic acids.

37. The expression system according to claim 36, wherein the two or more expression nucleic acids are provided in two or more separate lentiviral vectors.

38. A method of detecting the inhibitory or silencing activity of one or more inhibitory RNAs on a target gene comprising:
- transfecting a cell or cells with the expression system according to any one of claims 33-37
- binding of the inhibitory RNA to the target gene within the polycistronic mRNA;
- degradation of the polycistronic mRNA; and
- reduction in the fluorescent signal from the fluorescent protein in the cell or cells.

39. A method of selecting inhibitory RNAs which show silencing or inhibition of a target gene from a pool of test inhibitory RNAs, comprising:
- transfecting a cell or cells with the expression system according to claims 33-37; and

- selecting inhibitory RNAs which were transfected into the cell or cells which show reduction in the fluorescent signal from the fluorescent protein.

40. The methods of claim 38 or 39 further comprising:

- incubating the cell or cells under conditions suitable for expression of the reporter nucleic acid and the expression nucleic acid; and/or
- detecting the reduction in the fluorescent signal, optionally via flow cytometry.

41. The method of claims 38-40, wherein the cell is a macrophage, an iPSC or a T cell, optionally a primary T cell or a T cell line.