

Polynucleotides comprising 5'UTR - 3' UTR combinations and use thereof.**FIELD OF THE INVENTION**

The present invention relates to the field of artificial polynucleotides and in particular to RNA polynucleotides comprising specific 5'UTR – 3'UTR gene combinations. Provided herein is an RNA polynucleotide comprising at least one 5'UTR element selected from a gene such as ANXA1, E. Coli enolase, RPS14, RPS25, or TCV and at least one 3'UTR selected from a gene such as HBA1, RPS29, Ube2d2a, or UBL5. The invention further relates to a pharmaceutical composition comprising one or more RNA polynucleotides according to the invention and to a method of inducing an enhanced immune response in a subject using said RNA polynucleotides or pharmaceutical composition.

BACKGROUND TO THE INVENTION

Synthetic mRNA has emerged as a powerful tool for the transfer of genetic information, and it is being explored for a variety of therapeutic applications. Many of these applications require prolonged intracellular persistence of mRNA to improve bioavailability of the encoded protein. mRNA molecules or polynucleotides are intrinsically unstable and their intracellular kinetics depend on the 5' untranslated region (5' UTR) and 3' untranslated region (3' UTR) which embrace the coding sequence. Structural features of UTRs such as the sequence, length and secondary structures have a strong impact on translation initiation and peak level expression (mainly 5' UTR) as well as an impact on mRNA stability and duration of expression (mainly 3' UTR).

Accordingly, the combination of UTRs determines the kinetics of expression of mRNA polynucleotides. UTRs used in the synthetic RNA are typically derived from α/β -globin, human heat shock protein or viruses. However, UTR performance may vary between cell types, and customized design of specific UTR for targeted cell type is necessary.

It was therefore an object of the present invention to provide an RNA polynucleotide comprising an optimal 5'UTR/3'UTR combination of specific UTRs resulting in increased stability and/or translation efficiency. Such RNA polynucleotides are particularly useful in mRNA-based therapy, gene therapy and/or genetic vaccination. The purpose of the invention is to provide RNA polynucleotides allowing for increased and prolonged protein expression as well as enhanced biodistribution from said RNA polynucleotide, preferably which exhibit increased translational efficiency. The object underlying the present invention is solved by the claimed subject-matter.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides an RNA polynucleotide comprising:

- 5 i) at least one 5' untranslated region (5'UTR) element comprising of a nucleic acid sequence which is derived from the 5'UTR of a gene selected from ANXA1, E. Coli enolase, RPS14, RPS25, or TCV, or from a fragment or variant thereof;
- ii) at least one open reading frame (ORF) encoding a polypeptide;
- iii) at least one 3' untranslated region (3'UTR) element comprising a nucleic acid sequence which is derived from the 3'UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5, or from
10 a fragment or variant thereof.

It was found that an RNA polynucleotide having at least one of this combination of 5' UTR and 3' UTR resulted in enhanced expression and biodistribution compared to controls.

In a specific embodiment, the 5'UTR of the RNA polynucleotide comprises an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 1 [ANXA1], SEQ ID NO: 2 [eno],
15 SEQ ID NO: 3 [RPS14], SEQ ID NO: 4 [RPS25], or SEQ ID NO: 5 [TCV], or a sequence having at least 95% sequence identity thereto.

In another embodiment, the 3'UTR of the RNA polynucleotide comprises an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 7 [RPS29],
20 SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95% sequence identity thereto.

In another embodiment, the 3'UTR of the RNA polynucleotide comprises an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1],
SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95% sequence identity thereto.

25 In a specific embodiment, the 5'UTR and 3'UTR of the RNA polynucleotide comprise an RNA sequence fragment or variant thereof, corresponding to the DNA sequences selected from any of the following 5' UTR – 3' UTR gene combinations:

- 5'UTR of ANXA1 in combination with 3'UTR of HBA1; or
- 5'UTR of eno in combination with 3'UTR of RPS29; or
- 30 - 5'UTR of eno in combination with 3'UTR of Ube2d2a; or
- 5'UTR of RPS14 in combination with 3'UTR of HBA1; or
- 5'UTR of RPS25 in combination with 3'UTR of Ube2d2a; or

- 5'UTR of TCV in combination with 3'UTR of UBL5.

In a further embodiment, the 5' UTR and 3' UTR of the RNA polynucleotide comprise an RNA sequence or a sequence having at least 95% sequence identity thereto, corresponding to the DNA
5 sequences selected from any of the following combinations:

- SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; or
- SEQ ID NO: 2 [eno] for 5'UTR in combination with SEQ ID NO: 7 [RPS29] for 3'UTR; or
- SEQ ID NO: 2 [eno] for 5'UTR in combination with SEQ ID NO: 8 [Ube2d2a] for 3'UTR; or
- SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; or
- 10 - SEQ ID NO: 3 [RPS25] for 5'UTR in combination with SEQ ID NO: 8 [Ube2d2a] for 3'UTR; or
- SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; or
- SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; or
- SEQ ID NO: 5 [TCV] for 5'UTR in combination with SEQ ID NO: 9[UBL] for 3'UTR.

15 In accordance with a further embodiment of the present invention, the 5'UTR of the RNA polynucleotide further comprises a KOZAK sequence and/or an internal ribosome entry site (IRES). In yet another embodiment, the RNA polynucleotide is a mRNA or non-coding RNA, preferably a mRNA, antisense RNA, siRNA, sgRNA, guide RNA or CRISPR.

20 In another embodiment, the present invention relates to a pharmaceutical composition comprising one or more RNA polynucleotides according to the invention, and at least one pharmaceutically acceptable agent.

In a further embodiment, the present relates to the RNA polynucleotide, or the pharmaceutical composition according to the invention for use in human and/or veterinary medicine. More specific, an RNA polynucleotide or a pharmaceutical composition are provided for use as a vaccine or for use
25 in gene therapy.

In another embodiment, the present invention relates to the use of an RNA polynucleotide, or the pharmaceutical composition according to the invention to enhance the expression and/or translation of a gene product, in particular to enhance initiation of translation. Even more specific, the use of said RNA polynucleotide or said pharmaceutical composition is provided to enhance the stability
30 and/or duration of expression of a gene product, in particular to enhance the biodistribution.

In yet another embodiment, the present invention relates to a method of inducing an enhanced immune response in a subject comprising: administering a therapeutically effective amount of RNA polynucleotide or pharmaceutical composition according to the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

With specific reference now to the figures, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the different embodiments of the present invention only. They are presented in the cause of providing what is believed to be the most useful and readily description of the principles and conceptual aspects of the invention. In this regard no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention. The description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

Fig. 1: Flowchart of the 1st round of mRNA screening. mRNA library consisted of the combination of 21 5' UTRs with 21 3' UTRs and secreted Nanoluc. iMoDCs were transfected in duplicates with 100ng of each mRNA, with subsequent assessment of Transfection efficacy, viability and Nanoluc expression as depicted on the middle panel of the drawing. Then duplicates were pooled and area under the curve was calculated for each mRNA with subsequent selection of top performers in each plate the resulted in the 40 best performing mRNAs. C1, C2, C3, C4 refers to the controls mRNA transfections

Fig. 2: 2nd round of UTR selection. Top 40 best performing mRNAs down selection in A) SkMC, B) in iMoDcs donor 1, and C) in iMoDcs donor 2. The bars annotated with a black dot, indicate selected mRNA species, where the dashed line represents the baseline, where data was normalized to control 3.

Fig. 3: Confirmation of 6 best performers with mRNA encoding luciferase with 8 different cell lines. Top 6 best performing UTR combinations tested in BJ, iMoDC, SkMC, HEK293T, HepG2, K562, CT26 and TC-1 cell lines.

Fig. 4: All six lead candidate 5'UTR - 3'UTR gene combinations result in higher or equal *in vivo* firefly luciferase expression compared to tested control 5'UTR - 3'UTR gene combinations. Quantification of full body bioluminescence signal over time in BALB/c mice injected with TBS, or either of the hit candidate 5'UTR - 3'UTR gene combination firefly luciferase (fLuc) mRNA-LNPs or control 5'UTR - 3'UTR gene combination fLuc mRNA-LNPs.

Fig. 5: Ex Vivo Expression: For all 3 tested mRNA (mulL-7, mulL-21 and mu41BBL) constructs, both UTR V8 and V10 result in significantly higher protein expression compared to the comparative UTR V5 combination.

Fig. 6: In vivo Efficacy: The highest efficacies are obtained using V10 UTRs (Fig. 6D) and V8 UTRs (Fig. 6C). The comparative V5 UTRs (Fig. 6B) clearly perform worse compared to these 2 UTR combination of the invention. TBS is used as negative example (Fig. 6A).

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be further described. In the following passages, different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as
5 being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

As used in the specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. By way of example, "a RNA polynucleotide " means one RNA polynucleotide or more than one RNA polynucleotide. The terms
10 "comprising", "comprises" and "comprised of" as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements, or method steps. The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

15 The term "about" or "approximately" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of +/- 10% or less, preferably +/-5% or less, more preferably +/- 1 % or less, and still more preferably +/- 0.1 % or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier "about" or
20 "approximately" refers is itself also specifically, and preferably, disclosed.

Whereas the term "one or more", such as one or more members of a group of members, is clear per se, by means of further exemplification, the term encompasses inter alia a reference to any one of said members, or to any two or more of said members, such as, e.g., any ≥ 3 , ≥ 4 , ≥ 5 , ≥ 6 or ≥ 7 etc. of said members, and up to all said members.

25 Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a
30 person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the
35 following claims, any of the claimed embodiments can be used in any combination.

All documents cited in the present specification are hereby incorporated by reference in their entirety. Unless otherwise specified, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions may be included to

better appreciate the teaching of the present invention.

In search of RNA polynucleotides exhibiting increased stability and/or translation efficiency, the inventors investigated whether specific 5' UTRs and 3' UTRs could influence the kinetics of expression of said RNA polynucleotides. The inventors unexpectedly found that an RNA polynucleotide comprising a specific customized design of 5'UTR and 3'UTR elements had an advantageous effect on the stability and translation efficiency. It was further established that an RNA polynucleotide comprising a 5' UTR sequence derived from a 5'UTR of a gene selected from ANXA1, E. Coli enolase, RPS14, RPS25, or TCV performs particularly well when combined with a 3' UTR sequence derived from a 3'UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5.

The present invention is particularly suited to be used in a pharmaceutical composition, either in isolation or in the form of a lipid nanoparticle, and therefore also suitable to induce an immune response in a subject. In this context, the RNA polynucleotide according to the invention is also suitable for use as a vaccine or for use in gene therapy. The major advantage of using the RNA polynucleotides according to the invention, is that it enhances the expression and/or translation of a gene product, but more in particular the stability and/or duration of expression of a gene product as well as the biodistribution.

In the context of the present invention, the term "RNA" relates to a nucleic acid molecule which comprises ribonucleotide residues and preferably being entirely or substantially composed of ribonucleotide residues. When reference is made to the term "nucleic acid molecule" it is meant to be the RNA polynucleotide according to the invention, unless otherwise stated. In particular, the RNA polynucleotide is a polymer comprising or consisting of nucleotide monomers which are covalently linked to each other by phosphodiester-bonds of a sugar/phosphate-backbone.

"Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β -D-ribofuranosyl group. In particular, the term refers to double stranded RNA, but may also refer to single stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as modified RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of a RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in RNA molecules can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA. Where in the context of the invention, specific sequences are disclosed, these equally represent corresponding RNA sequences, with the understanding that T is replaced with U.

According to the present invention, the term "RNA polynucleotide" also includes and preferably relates to "mRNA" which means "messenger RNA" and relates to a "transcript" which may be produced using DNA as template and encodes a peptide or protein. mRNA typically comprises a

5' untranslated region (5' -UTR), a protein or peptide coding region and a 3' untranslated region (3'-UTR).

In a particular embodiment, the RNA polynucleotide of the invention is a mRNA or non-coding RNA, preferably a mRNA, antisense RNA, siRNA, sgRNA, guide RNA or CRISPR. Further, the term
5 "RNA polynucleotide" is not restricted to mean "one single molecule" but is, typically, understood to comprise an ensemble of identical molecules. Accordingly, it may relate to a plurality of identical molecules contained in a composition or for example a lipid nanoparticle.

For the sake of clarity, a mRNA encompasses any coding RNA molecule, which may be
10 translated by a eukaryotic host into a protein. Preferably, mRNA is produced by *in vitro* transcription using a DNA template. In one embodiment of the invention, RNA is obtained by *in vitro* transcription. The *in vitro* transcription methodology is known to the skilled person and may comprise a purified linear DNA template containing a promoter, ribonucleotide triphosphates, a buffer system that includes dithiothreitol (DTT) and magnesium ions, spermidine and an appropriate RNA polymerase
15 such as T7 RNA polymerase. The exact conditions used in the transcription reaction depend on the amount of RNA needed for a specific application. There is a variety of *in vitro* transcription kits commercially available.

Accordingly, the invention also provides a nucleic acid molecule which may be a template for
20 the RNA polynucleotide according to the invention which is stabilized and optimized with respect to translation efficiency. In other words, the present invention also relates to an artificial nucleic acid molecule which can be a (modified) RNA or DNA molecule such as a DNA vector which may be used for the production of an mRNA. It may be provided as a double-stranded molecule having a sense strand and an anti-sense strand, for example, as a DNA molecule having a sense strand and
25 an anti-sense strand. The obtainable mRNA, may, in turn, be translated for production of a desired peptide or protein encoded by an open reading frame. If the artificial nucleic acid molecule is a DNA, it may, for example, be used as a double-stranded storage form for continued and repetitive *in vitro* or *in vivo* production of mRNA.

In the context of the present invention, the term "5'-untranslated region (5' UTR)" is to be
30 understood as a particular section of mRNA which is located 5' of the protein coding region (i.e. the open reading frame; ORF) of the mRNA. Typically, the 5' UTR starts with the transcriptional start site and ends one nucleotide before the start codon of the open reading frame. The 5' UTR may comprise elements for controlling gene expression, also called regulatory elements. Such regulatory elements may be, for example but not limited to promoters, enhancers, silencers, insulators, ribosomal binding
35 sites, a 5'-Terminal Oligopyrimidine Tract. The 5' UTR may be post transcriptionally modified, for example by addition of a 5'-cap. In the context of the present invention, a 5' UTR corresponds to the sequence of a mature mRNA which is located between the 5'cap and the start codon. In the context of the present invention, the term "a 5' UTR of a gene", such as "a 5' UTR of ANXA1 gene", is the sequence which corresponds to the 5' UTR of the mature mRNA derived from this gene, i.e. the

mRNA obtained by transcription of the gene and maturation of the pre-mature mRNA. The term "5' UTR of a gene" encompasses the DNA sequence and the RNA sequence of the 5' UTR.

In the context of the present invention, the term "3' untranslated region (3' UTR)" is to be understood as a part of a mRNA which is located between the protein coding region (i.e. the ORF) and the poly(A) sequence of the mRNA. A 3' UTR of the mRNA is not translated into an amino acid sequence. The 3' UTR sequence is generally encoded by the gene which is transcribed into the respective mRNA during the gene expression process. The genomic sequence is first transcribed into pre-mature mRNA, which comprises optional introns. The pre-mature mRNA is then further processed into mature mRNA in a maturation process. In the context of the present invention, a 3' UTR corresponds to the sequence of a mature mRNA which is located 3' to the stop codon of the protein coding region. In the context of the present invention, the term "a 3' UTR of a gene", such as "a 3' UTR of HBA1 gene", is the sequence which corresponds to the 3' UTR of the mature mRNA derived from this gene, i.e. the mRNA obtained by transcription of the gene and maturation of the pre-mature mRNA. The term "3' UTR of a gene" encompasses the DNA sequence and the RNA sequence of the 3' UTR.

As used herein, the term "open reading frame (ORF)" may typically be a sequence of several nucleotide triplets which may be translated into a peptide or protein. An open reading frame preferably contains a start codon, i.e. a combination of three subsequent nucleotides coding usually for the amino acid methionine (ATG or AUG), at its 5'-end and a subsequent region which usually exhibits a length which is a multiple of 3 nucleotides. An ORF is preferably terminated by a stop-codon (e.g., TAA, TAG, TGA). Typically, this is the only stop-codon of the open reading frame. The open reading frame may be isolated or it may be incorporated in a longer nucleic acid sequence, for example in an RNA polynucleotide. An open reading frame may also be termed "protein coding region". Furthermore, the open reading frame may be at least partially codon-optimized. Codon-optimization is based on the finding that the translation efficiency may be determined by a different frequency in the occurrence of transfer RNAs (tRNAs) in cells and is known by skilled artisans in the field of mRNA manufacturing.

The invention thus provides an RNA polynucleotide comprising:

- i) at least one 5' untranslated region (5'UTR) element comprising of a nucleic acid sequence which is derived from the 5'UTR of a gene selected from ANXA1, E. Coli enolase, RPS14, RPS25, or TCV, or from a fragment or variant thereof;
- ii) at least one open reading frame (ORF) encoding a polypeptide;
- iii) at least one 3' untranslated region (3'UTR) element comprising a nucleic acid sequence which is derived from the 3'UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5, or from a fragment or variant thereof.

It was found that an RNA polynucleotide having at least one of these combination of 5' UTR and 3' UTR result in enhanced expression and biodistribution compared to controls.

Preferably, the at least one 5' UTR element and the at least one 3' UTR element are functionally linked to the ORF. This means preferably that the 5' UTR element and the 3' UTR element are associated with the ORF such that they may exert a function, preferably in an additive, more preferably in a synergistic manner, such as a stabilizing function on the expression of the ORF, a protein production increasing function for the protein encoded by the ORF, or a stabilizing function on the RNA polynucleotide. Preferably, the 5' UTR element, the ORF, and the 3' UTR element are associated in 5' to 3' direction. Thus, preferably, the RNA polynucleotide comprises the structure 5'-5' UTR element- (optional)linker-ORF-(optional)linker-3' UTR element-3', wherein the linker may be present or absent. For example, the linker may be one or more nucleotides, such as a stretch of 1 - 50 or 1 -20 nucleotides.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising a nucleic acid sequence which is derived from the 5' UTR of a ANXA1 gene in combination with at least one 3' UTR element comprising a nucleic acid sequence which is derived from the 3' UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5.

In another specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising a nucleic acid sequence which is derived from the 5'UTR of a E. Coli enolase gene in combination with at least one 3' UTR element comprising a nucleic acid sequence which is derived from the 3' UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5.

In yet another embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising a nucleic acid sequence which is derived from the 5'UTR of a RPS14 gene in combination with at least one 3' UTR element comprising a nucleic acid sequence which is derived from the 3' UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising a nucleic acid sequence which is derived from the 5'UTR of a RPS25 gene in combination with at least one 3' UTR element comprising a nucleic acid sequence which is derived from the 3' UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5.

In a further embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising a nucleic acid sequence which is derived from the 5'UTR of a TCV gene in combination with at least one 3' UTR element comprising a nucleic acid sequence which is derived from the 3' UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5.

As described before, the embodiments also encompass fragments and variants of said genes. In the context of the present invention, the term "fragment thereof" encompasses sequences

corresponding to the entire 5' UTR or 3' UTR sequences, i.e. the full length 5' UTR or 3' UTR sequence of said genes, and sequences corresponding to a fragment of the 5' UTR or 3' UTR sequence of said genes. Preferably, a fragment of a 5' UTR or 3' UTR gene comprises a continuous stretch of nucleotides corresponding to a continuous stretch of nucleotides in the full-length 5' UTR or 3' UTR of said gene, which represents at least 20%, preferably at least 30%, more preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, and most preferably at least 90% of the full-length 5' UTR or 3' UTR of said gene. Such a fragment, in the sense of the present invention, is preferably a functional fragment as described herein.

10 In the context of the present invention, the term "variant thereof" refers to a variant of the 5' UTR or 3' UTR of a naturally occurring gene, preferably to a variant of the 5' UTR or 3' UTR of a vertebrate gene, preferably to a variant of the 5' UTR or 3' UTR of a mammalian gene, more preferably to a variant of the 5' UTR or 3' UTR of a human or mouse gene. Such variant may be a modified 5' UTR or 3' UTR of a gene. For example, a variant 5' UTR or 3' UTR may exhibit one or
15 more nucleotide deletions, insertions, additions and/or substitutions compared to the naturally occurring 5' UTR or 3' UTR from which the variant is derived. Preferably, a variant of a 5' UTR or 3' UTR of a gene is at least 40%, preferably at least 50%, more preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90%, most preferably at least 95% identical to the naturally occurring 5' UTR or 3' UTR the variant is derived from.
20 Preferably, the variant is a functional variant as described herein. As used herein, the term "variant" also encompasses the term "homologs" which is to be understood as sequences for the same genes but from other species.

Furthermore, in each of the embodiments, the RNA polynucleotide may comprise more than one 5' UTR element or 3' UTR element as described above. For example, the RNA polynucleotide molecule
25 according to the present invention may comprise one, two, three, four or more 5' UTR elements, and/or one, two, three, four or more 3' UTR elements wherein the individual 5' UTR or 3' UTR elements may be the same or they may be different. For example, the RNA polynucleotide according to the present invention may comprise two essentially identical 5' UTR elements, e.g. two 5' UTR elements comprising or consisting of a nucleic acid sequence which is derived from an E. Coli
30 enolase gene, or fragment or variant thereof, in combination with e.g. two 3' UTR element comprising or consisting of a nucleic acid sequence which is derived from an HBA1 gene, or fragment or variant thereof.

As defined above, the 5' UTR of an ANXA1, E. Coli enolase, RPS14, RPS25, or TCV gene corresponds to the sequence of a 5' UTR of a mature mRNA derived from respectively an ANXA1,
35 E. Coli enolase, RPS14, RPS25, or TCV gene, which preferably extends from the nucleotide located 3' to the 5' cap to the nucleotide located 5' to the start codon. The lengths of the 5' UTRs of said genes may vary between 10 nucleotides up to 500 nucleotides, and are typically less than about 300

nucleotides, preferably less than about 200 nucleotides, more preferably less than about 150 nucleotides. Exemplary 5' UTRs of said genes in the sense of the present invention are the nucleic acid sequences according to SEQ ID NOs. 1 -5 or from a fragment or variant thereof. Preferably, a 5' UTR element in the sense of the present invention functions as a 5' UTR or codes for a nucleotide sequence that fulfils the function of a 5' UTR. The 5' UTR element is preferably suitable for increasing protein production from the artificial RNA polynucleotide.

Analogously, the 3' UTR of a HBA1, RPS29, Ube2d2a, or UBL5 gene corresponds to the sequence of a 3' UTR of a mature mRNA derived from respectively an HBA1, RPS29, Ube2d2a, or UBL5 gene. The lengths of the 3' UTRs of said genes may vary between 10 nucleotides up to 500 nucleotides, and are typically less than about 300 nucleotides, preferably less than about 200 nucleotides, more preferably less than about 150 nucleotides. Exemplary 3' UTRs of said genes in the sense of the present invention are the nucleic acid sequences according to SEQ ID NOs. 6-9 or from a fragment or variant thereof. Preferably, a 3' UTR element in the sense of the present invention functions as a 3' UTR or codes for a nucleotide sequence that fulfils the function of a 3' UTR. The 3' UTR element is preferably suitable for enhancing the stability of an mRNA molecule.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 1 [ANXA1], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto, in combination with at least one 3' UTR element comprising a nucleic acid sequence selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1], SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 2 [E. Coli enolase gene], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto, in combination with at least one 3' UTR element comprising a nucleic acid sequence selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1], SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 3 [RPS14], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto, in combination with at least one 3' UTR element comprising a nucleic acid sequence selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1], SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 4 [RPS25] , or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto, in combination with at least one 3' UTR element comprising a nucleic acid sequence
5 selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1], SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising an RNA sequence corresponding to the DNA sequences selected
10 from SEQ ID NO: 5 [TCV] , or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto, in combination with at least one 3' UTR element comprising a nucleic acid sequence selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1], SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto.

In a specific embodiment, the present invention relates to an RNA polynucleotide comprising at least one 5' UTR element comprising an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 1 [ANXA1], SEQ ID NO: 2 [E. Coli enolase gene], SEQ ID NO: 3 [RPS14] SEQ ID NO: 4 [RPS25] SEQ ID NO: 5 [TCV], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto, in combination with at least one 3' UTR element comprising an RNA
15 sequence corresponding to the DNA sequences selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1], SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95%, 96%, 97%, 98%, 99% sequence identity thereto.
20

Accordingly, the nucleic acid sequence which is derived from the 5' UTR or 3' UTR of a gene is derived from a eukaryotic gene, preferably a plant or animal gene, more preferably a chordate gene,
25 even more preferably a vertebrate gene, most preferably a mammalian gene, such as a human or mouse gene (**e.g. Table 1**). For example, the 5' UTR or 3' UTR element comprises or consists of a nucleic acid sequence which is derived from a nucleic acid sequence, (e.g. an RNA sequence corresponding to the DNA sequences) selected from the group consisting of respectively SEQ ID NOs. 1 - 5 or SEQ ID NOs. 6-9, 18 or fragments or variants thereof, or from the homologs of SEQ
30 ID NOs. 1 -5 or SEQ ID NOs. 6-9, 18. In the context of the present invention, the term "homologs " refers to sequences of other species, e.g. other species than Homo sapiens (human) or Mus musculus (mouse), which are homologous to the sequences according to SEQ ID NOs. 1 -5 or SEQ ID NOs. 6-9, 18. For example, SEQ ID NO. 1 relates to a sequence comprising the 5' UTR of human Annexin A1 (ANXA). A homolog of SEQ ID NO. 1 in the context of the present invention is any such
35 sequence derived from an ANXA gene of another species than human, such as any vertebrate or plant gene, preferably any mammalian ANXA gene other than the human ANXA gene, such as a mouse, rat, rabbit, monkey etc. ANXA gene.

In a particular embodiment, the 5' UTR element and the 3' UTR element are heterologous, e.g. preferably the 5' UTR and the 3' UTR are derived from different genes of the same or of different species. In a preferred embodiment, the 5' UTR element and 3' UTR element is chosen such that it exerts at least an additive, preferably a synergistic function on the protein production from the ORF of the RNA polynucleotide molecule. Preferably, the protein production is increased in at least an additive, preferably a synergistic way by the 5' UTR element and the 3' UTR element. Thus, the protein amount of the protein encoded by the ORF, such as a reporter protein, e.g. luciferase, at a certain time point after initiation of expression of the ORF, e.g. after transfection of a test cell or cell line, is preferably at least the same, preferably higher than what would be expected if the protein production increasing effects of the 5' UTR element and the 3' UTR element were purely additive. The additive, preferably the synergistic effect may, for example, be determined by a luminescence assay.

Preferably, the at least one 5' UTR element and the at least one 3' UTR element act synergistically to stabilize and/or increase protein production from the RNA polynucleotide according to the present invention, e.g. from an mRNA according to the present invention, as described above. In the context of the present invention, the term "stabilizing and/or increase protein production" means that the protein production from the mRNA is stabilized and/or increased compared to the protein production from a reference mRNA, e.g. comprising a reference or control 5' UTR and 3' UTR element (**e.g. Table 2**)

It has now been found that an RNA polynucleotide comprising a 5'UTR and 3'UTR RNA sequence fragment or variant thereof corresponding to the DNA sequences selected from any of the following combinations:

- 5'UTR of ANXA1 in combination with 3'UTR of HBA1; or
- 5'UTR of eno in combination with 3'UTR of RPS29; or
- 5'UTR of eno in combination with 3'UTR of Ube2d2a; or
- 5'UTR of RPS14 in combination with 3'UTR of HBA1; or
- 5'UTR of RPS25 in combination with 3'UTR of Ube2d2a; or
- 5'UTR of TCV in combination with 3'UTR of UBL5.

is particularly suitable to increase expression in different cell types compared to a reference RNA polynucleotide. These RNA polynucleotide molecules are particularly useful in mRNA-based therapy, gene therapy and/or genetic vaccination because they may provide increased and/or prolonged protein expression encoded by the open reading frame from said RNA polynucleotide.

Particularly preferred combinations are 5'UTR of ANXA1 in combination with 3'UTR of HBA1; and - 5'UTR of RPS14 in combination with 3'UTR of HBA1.

More specifically, these advantageous effects were particularly apparent when using RNA polynucleotides comprising a 5' UTR and 3' UTR an RNA sequence or a sequence having at least 95% sequence identity thereof, corresponding to the DNA sequences selected from any of the following combinations:

- 5 - SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; or
- SEQ ID NO: 2 [eno] for 5'UTR in combination with SEQ ID NO: 7 [RPS29] for 3'UTR; or
- SEQ ID NO: 2 [eno] for 5'UTR in combination with SEQ ID NO: 8 [Ube2d2a] for 3'UTR; or
- SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; or
- SEQ ID NO: 3 [RPS25] for 5'UTR in combination with SEQ ID NO: 8 [Ube2d2a] for 3'UTR; or
- 10 - SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; or
- SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; or
- SEQ ID NO: 5 [TCV] for 5'UTR in combination with SEQ ID NO: 9[UBL] for 3'UTR.

Particularly preferred combinations are SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; and SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR.

Alternative preferred combinations are SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; and SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR.

In a very specific embodiment, the present invention provides one or more mRNA molecules encoding IL-7, IL-21 and/or 4-1BBL, comprising a combination of UTRs selected from:

RPS14 5'UTR – HBA1 3'UTR – specifically, SEQ ID NO: 3 [RPS14] - SEQ ID NO: 18 [HBA1]
ANXA1 5'UTR – HBA1 3'UTR- specifically, SEQ ID NO: 1 [ANXA1] - SEQ ID NO: 18 [HBA1]

Particularly suitable RNA polynucleotides are RNA polynucleotides comprising at least one 5' UTR element comprising of a nucleic acid sequence which is derived from the 5'UTR of a E. Coli enolase gene or ANXA gene.

In addition, said combinations of at least one 5' UTR element and the at least one 3' UTR element act synergistically to increase biodistribution from the RNA polynucleotide according to the present invention, e.g. from an mRNA according to the present invention, as described above. In the context of the present invention, the term “biodistribution” means that the transfer of an mRNA or lipid nanoparticle comprising said mRNA within a body, organ or tissue and accumulation thereof is increased compared to the biodistribution from a reference or control mRNA, e.g. comprising a reference 5' UTR and 3' UTR element (see for example RNA polynucleotides comprising control 5' UTR/3' UTR as described in **Table 2**).

More specifically, there is more uniform protein production and biodistribution from the RNA polynucleotide according to the present invention over a predetermined period of time, preferably over 2 hours such as for example over 3 hours, over 4 hours, over 5 hours, over 6 hours, over 7 hours, over 8 hours, preferably over 12 hours, more preferably over 24 hours, even more preferably over 48 hours, most preferably over 72 hours, when compared to a reference RNA polynucleotide molecule. Thus, the level of protein production, e.g. in a mammalian system, from the RNA polynucleotide according to the invention does not drop to the extent observed for a reference RNA polynucleotide, such as a reference mRNA as described above. For example, the amount of a protein (encoded by the ORF) observed 5 hours after initiation of expression, may be comparable to the amount of protein observed 48 hours after initiation of expression, e.g. 48 hours post transfection. In particular, it was found that the biodistribution was enhanced from 5 hours to 24 hours and slightly decreased after 48 and 72 hours but remained overall higher compared to reference RNA polynucleotides.

Said increase in stability of the RNA polynucleotide, said increase in stability of protein production, said prolongation of protein production, said increase in protein production and/or said increase in biodistribution is preferably determined by comparison with a respective reference RNA polynucleotides comprising reference 5' UTR and 3' UTR elements e.g. a 5' UTR element comprising an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 10-13 or a 3' UTR element comprising an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 14-17 (see **Table 2**).

'Increased protein expression' in the context of the present invention may refer to increased protein expression at one time point after initiation of expression compared to a reference molecule or to an increased total protein production within a certain time period after initiation of expression. Thus, the protein level observed at a certain time point after initiation of expression, e.g. after transfection, of the RNA polynucleotide according to the present invention, e.g. after transfection of an mRNA according to the present invention, for example, 2, 3, 4, 5, 7, 8, 12, 24, 48, or 72 hours post transfection, or the total protein produced in a time span of, e.g. 24, 48 or 72 hours, is preferably higher than the protein level observed at the same time point after initiation of expression, e.g. after transfection, or the total protein produced within the same time span, for a reference RNA polynucleotide, such as a reference mRNA comprising a reference 5' and/or a reference 3' UTR. As set forth above, it is a particularly preferred function of the 5' UTR element to affect the increase in protein production from the RNA polynucleotide. Preferably, the increase in protein production effected by the 5' UTR element compared to an RNA polynucleotide comprising a reference 5' UTR and 3' UTR at a given time point post initiation of expression is at least 1.5-fold, more preferably at least 2-fold, more preferably at least 3-fold, even more preferably at least 4-fold, most preferably at least 5-fold of the protein production.

Furthermore, it is preferred that the 3' UTR element and the 5' UTR element have at least an additive,

preferably a synergistic effect on the total protein production from the artificial nucleic acid molecule in a certain time span, such as within 24 hours, 48 hours, or 72 hours post initiation of expression.

Accordingly, the present invention also relates to the use of an RNA polynucleotide, or the pharmaceutical composition according to the invention to enhance the expression and/or translation
5 of a gene product, in particular to enhance initiation of translation. Even more specific, the use of said RNA polynucleotide or said pharmaceutical composition is provided to enhance the stability and/or duration of expression of a gene product, in particular to enhance the biodistribution.

In accordance with a further embodiment of the present invention, the 5' UTR of the RNA polynucleotide further comprises a KOZAK sequence and/or an internal ribosome entry site (IRES).
10 In the context of the present invention, the term "KOZAK sequence" is meant to be a nucleic acid motif that functions as the protein translation initiation site and mediates ribosome assembly ensuring the correct protein sequence is translated. In the context of the present invention, the term "internal ribosome entry site (IRES)" is to be understood as a sequence or motif that separates several open reading frames, for example if the RNA polynucleotide encodes for two or more peptides or proteins.
15 An IRES-sequence may be particularly helpful if the mRNA is a bi- or multicistronic RNA.

Furthermore, the RNA polynucleotide may further comprise additional elements that are known by one skilled in the field of mRNA manufacturing such as a 5'-cap and a poly(A) tail. The optional 5'-cap is preferably attached to the 5'-side of the 5' UTR element. A 5'-cap may be added post-transcriptionally to the 5'-end of an RNA. Preferably, the optional poly(A) sequence is located 3' to
20 the ORF or the at least one 3' UTR element, preferably is connected to the 3'-end of the ORF or the 3'UTR element. The connection may be direct or indirect, for example, via a stretch of 2, 4, 6, 8, 10, 20 etc. nucleotides, such as via a linker of 1 -50, preferably of 1 -20 nucleotides, e.g. comprising or consisting of one or more restriction sites. In one embodiment, the optional polyadenylation signal is located within the 3'UTR element. The length of the poly(A) sequence may vary. For example, the
25 poly(A) sequence may have a length of about 20 adenine nucleotides up to about 300 adenine nucleotides, preferably of about 40 to about 200 adenine nucleotides, more preferably from about 50 to about 100 adenine nucleotides, such as about 60, 70, 80, 90 or 100 adenine nucleotides.

Still further, the RNA polynucleotide according to the invention may comprise one or more typical mRNA modifications, herein termed "modified mRNA nucleosides" which is understood as
30 comprising one or more modified nucleosides, which have useful properties such as the lack of a substantial induction of the innate immune response of a cell into which the mRNA is introduced. These modified nucleic acids enhance the efficiency of protein production, intracellular retention of nucleic acids, and viability of contacted cells, as well as possess reduced immunogenicity.

In some embodiments, modified nucleobases in nucleic acids (e.g. RNA nucleic acids, such as
35 mRNA nucleic acids) comprise 1 -methyl-pseudouridine (m¹y), 1 -ethyl-pseudouridine (e¹y), 5-methoxy-uridine (mo5U), 5-methyl-cytidine (m5C), and/or pseudouridine (y). In some embodiments, modified nucleobases in nucleic acids (e.g., RNA nucleic acids, such as mRNA nucleic acids)

comprise 5-methoxymethyl uridine, 5-methylthio uridine, 1-methoxymethyl pseudouridine, 5-methyl cytidine, and/or 5-methoxy cytidine. In some embodiments, the polyribonucleotide includes a combination of at least two (e.g., 2, 3, 4 or more) of any of the aforementioned modified nucleobases, including but not limited to chemical modifications.

- 5 In another embodiment, the present invention relates to a pharmaceutical composition comprising one or more RNA polynucleotides according to the invention, and at least one pharmaceutically acceptable agent.

In the context of the present invention, by means of the term "pharmaceutical composition" reference is made to a composition having pharmaceutical properties. In other words, reference is made to a composition providing for a pharmacological and/or physiological effect. Pharmaceutical
10 compositions can comprise one or more pharmaceutically acceptable agents such as excipients, carriers, diluents. It can be a solution, a suspension, liquid, or aqueous formulations or any combination thereof. In some embodiments, the pharmaceutically acceptable agent include, but are not limited to, biocompatible vehicles, adjuvants, additives, and diluents to achieve a composition
15 usable as a dosage form. Additional suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences. As used herein and unless otherwise specified, the term "excipient" is to be understood as any substance formulated alongside the active compound included for the purpose of long-term stabilization such as prevention of denaturation or aggregation over the expected shelf life, bulking
20 up liquid or solid formulations that contain potent active compound in small amounts (thus often referred to as "bulking agents", "fillers", or "diluents"), or to confer an enhancement on the active compound in the final dosage form, such as facilitating absorption, reducing viscosity, or enhancing solubility.

In another embodiment, the present invention provides an RNA polypeptide according to the
25 invention which is formulated in liposomes or nanoparticles, such as lipid nanoparticles or polymeric nanoparticles; in particular lipid nanoparticles. The RNA polypeptides defined herein can be formulated in lipid nanoparticles (LNPs) that encapsulate the constructs to protect them from degradation and promote cellular uptake. In the context of the present invention, by means of the term "lipid nanoparticle", or LNP, reference is made to a nanosized particle composed of one or more
30 lipids, e.g. a combination of different lipids. Possible lipids used in the LNP can be for example, but not limited to at least one phospholipids, at least one modified lipids such as PEG lipids, at least one ionizable lipids, at least one sterol. The lipid nanoparticles of the disclosure and the compositions thereof are generally known in the art.

In particular, as defined herein, the term "pharmaceutical composition" is in particular used in the
35 context of said LNP comprising the RNA polynucleotides of the invention in combination with at least one pharmaceutically acceptable carrier or excipient. On the other hand, the term "pharmaceutical formulation" may also refer to naked RNA polynucleotides of the invention which are suspended in a buffer solution and at least one pharmaceutically acceptable carrier or excipient (i.e. without

encapsulation within the LNP). This combination can for example be formulated in a LNP and is in particularly intended for prolonging nucleic acid stability and/or improve delivery.

5 The LNP's may comprise one or more single types of RNA polynucleotides (i.e. RNA polynucleotide with the same 5' UTR, 3' UTR combination), or they may comprise two or more RNA polynucleotides with similar or different types of 5' UTR - 3' UTR combinations. In some embodiments, two or more different RNA polynucleotides are formulated in the same lipid nanoparticle.

10 In a further aspect, the present invention provides the RNA polynucleotide, or the pharmaceutical composition according to the invention for use in human or veterinary medicine. Pharmaceutical compositions are particularly suitable as a vaccine or for use in gene therapy. Vaccines can be prophylactic (example: to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen), or therapeutic (example, to actively treat or reduce the symptoms of an ongoing disease). The administration of vaccines is called vaccination. Finally, the invention provides a method for the prophylaxis and treatment of human and veterinary disorders, by administering a
15 pharmaceutical composition or vaccine to a subject in need thereof.

In the context of the present invention, the term "gene therapy" is to be understood as a treatment of a patient's body or isolated elements of a patient's body, for example isolated tissues/cells, by nucleic acids encoding a peptide or protein, preferably the RNA polynucleotide according to the invention. In the context of the present invention, the term "genetic vaccination" may
20 typically be understood to be vaccination by administration of a nucleic acid molecule encoding an antigen or an immunogen or fragments thereof. Upon transfection of certain cells of the body or upon transfection of the isolated cells, the antigen or immunogen may be expressed by those cells and subsequently presented to the immune system, eliciting an adaptive, i.e. antigen- specific immune response.

25 Accordingly, gene therapy and genetic vaccination may typically comprise at least one of the steps of a) administration of an RNA polynucleotide or pharmaceutical composition as defined herein, directly to a subject - by whatever administration route - or *in vitro* to isolated cells/tissues of a subject, which results in transfection of the subject's cells either *in vivo/ex vivo* or *in vitro*, b) transcription and/or translation of the introduced nucleic acid molecule; and optionally c) re-
30 administration of isolated, transfected cells to the subject, if the nucleic acid has not been administered directly to the subject.

In a further aspect, the present invention relates to a method of inducing an enhanced immune response in a subject comprising: administering a therapeutically effective amount of RNA polynucleotide or pharmaceutical composition according to the invention. The term "immune
35 response" used throughout the description is not intended to be limited to the types of immune responses that may have been exemplified herein such as a particular an immune response against a disease-associated antigen or cells expressing a disease- associated antigen. The term therefore encompasses all infectious agents to which vaccination would be beneficial to the subject.

In the context of the present invention, the term 'therapeutically effective amount' is to be understood as an amount that is sufficient to induce a pharmaceutical effect, such as an immune response, altering a pathological level of an expressed peptide or protein, or substituting a lacking gene product, e.g., in case of a pathological situation.

- 5 The present invention also provides a method for treating or preventing a disease or disorder as described above comprising administering the RNA polynucleotide according to the present invention, or the pharmaceutical composition according to the present invention to a subject in need thereof.

- 10 In the context of the present application, the terms "treatment", "treating", "treat" and the like refer to obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. "Treatment" covers any treatment of a disease in a mammal, in particular a human, and includes: (a) preventing the disease or symptom from occurring in a subject
15 which may be predisposed to the disease or symptoms but has not yet been diagnosed as having it; (b) inhibiting the disease symptoms, i.e. arresting its development; or (c) relieving the disease symptom, i.e. causing regression of the disease or symptom.

- The RNA polynucleotide or the pharmaceutical composition according to the present invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally,
20 vaginally or via an implanted reservoir. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional, intracranial, transdermal, intradermal, intrapulmonary, intraperitoneal, intracardial, intraarterial, and sublingual injection or infusion techniques.

EXAMPLES**Example 1: *In vitro* Screening****5 Material and Methods****iMoDC differentiation and transfection**

Immature human monocytes derived dendritic cells (Mo-iDC) were differentiated from monocytes enriched from buffy coats using positive selection CD14+ beads (StemCell Technologies, EasySep Human CD14 positive Selection kit II, catalogue # 17858) of 2 different donors. Subsequently CD14+ cells were cultured in X-VIVO15 medium (VWR, catalogue # LONZBE02-060F) supplemented with 250 U/ml of IL-4 (Miltenyi Biotec, catalogue # 130-093-922) and 800 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec, catalogue # 130-093-867), and 1% human AB serum (Merck Life Science, catalogue # H4522-100ML) for 7 days, whereby fresh cytokines were added twice over the differentiation period. Cells were harvested at day 7 in phosphate-buffered saline (PBS)/ Ethylenediamine tetraacetic acid (EDTA) (Fisher Scientific, catalogue number # 15433589) and frozen in Cryostor CS10 medium (StemCell Technologies, catalogue # 7930). For the UTRs screening cells of 2 donors were thawed, pooled and transfected using Lipofectamine MessengerMAX Transfection Reagent (Thermo Fisher Scientific, catalogue # LMRNA008) according to manufacturer's instructions (100ng/well, 100ul, ratio 1:3) with 400 UTRs mRNA in duplicates in 96 well plates containing 100*10e3 iMoDCs/well. To ensure equivalent transfection efficacy across all wells the 10% of mCitrine mRNA was co-encapsulated with target Nanoluc mRNAs with different UTRs. Cells viability was monitored using the CellTiter-Fluor Cell Viability Assay (Promega, catalogue # G6081). Quantification of viability and transfection efficacy (Teff) took place after 24h, where Nanoluc secretion levels were followed 6h, 24h, 48h, 72h, 96h. Subsequently, areas under the Nanoluc secretion curve were normalized to transfection efficacy and cell density, and then the top 40 best (top 10%) performing mRNA were down-selected for the next round of screening in another iMoDC donor and human skeletal muscle cells. In the second round of screening 2 other iMoDC donors were assessed for top 40 UTR expression with the readouts similar to 1st round of screening. In confirmational experiments cells were transfected with Fluc and readout was done 24h after transfection.

SkMC culture, differentiation and transfection

Human Skeletal muscle cells (SkMC) were cultured according to manufacturer's instructions (Promo Cell, catalogue # C-12530). Briefly cells were passaged in Skeletal Muscle Cell Growth Medium (Bio-connect, catalogue # C-23060). For differentiation, cells were cultured in Skeletal Muscle Cell Growth Medium until they reached approximately 60 – 80% confluence. Then Growth Medium was replaced with Skeletal Muscle Cell Differentiation Medium (Bio-connect, catalogue # C-23061) . After 5 days of incubation in Skeletal Muscle Cell Differentiation Medium cells were used for transfection with 40

best performing UTRs formulated with Lipofectamine MessengerMAX Transfection Reagent (Thermo Fisher Scientific, catalogue # LMRNA008) according to the manufacturer's instruction (50ng/well, ratio 1:3 in 100 ul). Transfection efficacy and cells viability were followed up as in 1st round of screening, Nanoluc expression was collected over 6h, 24h, 48h, 72h, 96h. In confirmational experiments cells were transfected with Fluc and complete readout was done 24h after.

Cancer Cell lines culture and transfection

4 human cells lines of different origin (HeP2G – liver cancer cell line (ATCC, catalogue # HB-8065), K562 – leukemia cell line (ATCC, catalogue # CCL-243), BJ – fibroblasts (ATCC, catalogue # CRL-2522), HEK293 – human embryonic kidney immortalized cell line (ATCC, catalogue #CRL-1573), and two mouse cell lines CT26 (colorectal carcinoma (ATCC, catalogue # CRL-2638)), TC-1 (immortalized lung epithelial line (kind gift of the University of Leiden, lab of Thorbald Van Hall) were cultured according to manufacturer's instructions. Cells were transfected using Lipofectamine MessengerMAX Transfection Reagent (Thermo Fisher Scientific, catalogue # LMRNA008) with 2 different doses (100 ng vs. 200 ng/well) of mRNA encoding firefly Luciferase (Fluc). Only the 24h time point was assessed in the confirmational experiments.

Data analysis

In all experiments, Nanoluc values were normalized to transfection efficacy, and cell confluency was also taken into account in some experiments. Then Area Under The Curve over all timepoints was assessed using Graph Pad Prism (version 10.0.2(232)). Statistical significance was evaluated using One Way ANOVA given that all assumptions are fulfilled with subsequent post-HOC Tukey test to correct for multiple comparisons. In case of AUC the standard error and degrees of freedom +1 were used for statistical assessment.

Results:

1st round of screening (fig. 1).

In order to select the combination of 5-prime and 3-prime UTRs that enables higher messenger RNA expression and longer intracellular persistence compared to control UTRs (see table 2 further referred as control UTRs) we generated a library of mRNAs where 21 X 5-prime UTRs with 21 X 3-prime UTRs was combined with the coding sequence of secreted NanoLuc. The expression of Nanoluc from 441 mRNA was monitored in human immature Monocyte Derived Dendritic cells because this cell type is frequently targeted in the context of Lipid Nanoparticles Driven therapies and frequently mRNA expression is cell type specific.

We corrected the expression of Nanoluc based on transfection efficacy obtained by co-transfecting cells with 10% of mRNA load consisting of mCitrine mRNA. We also monitored the cells viability to exclude the most toxic mRNA species, that can be observed for example in case of strong innate activation. After transfection efficiency (Teff) normalization, the integrated Nanoluc expression over

6h, 24h, 48h, 72h, 96h was obtained based on Area Under the Curve. Subsequently on every 96-well plates the mRNA with AUC in 60s percentile were selected as best performers that resulted in the total of 40 mRNA selection for the next round of screening and confirmation.

5 **2nd round of selection in primary human cells (Fig 2A, B, C).**

Down selected UTRs were tested in primary human cells namely Skeletal Muscle Cells (skMC). These primary cells are relevant to intramuscular immunization approaches utilizing delivery in Lipid Nanoparticles. Mature SkMC were transfected with top 40 mRNAs using 50ng of mRNA formulated with Messenger Max in ratio 1:3 in duplicates. Nanoluc secretion was evaluated throughout 6h, 24h, 10 48h, 72h, 96h and Transfection efficacy together with viability was assessed after 24h from transfection moment. Kinetic data was corrected using Teff values and also the cell density was taken into account. The Base-line of control 3 mRNA was subtracted from AUC of integrated Nanoluc measurements in GraphPadPrism by division, where all replicates were averaged and mean values were used for the calculation. Subsequently, the 4 best performers were selected: 1) ANXA1-secNluc-HBA1, 2) eno-secNluc-FTH1-201 and eno-secNluc-Ube2d2a, 3) eno-secNluc-TPRKB and 15 eno-secNluc-UBL5, 4) tabA-secNluc-Ube2d2a (Fig 2A).

Consequently the 40 down selected mRNA were transfected in 2 other donors of immature MoDCs to compensate for high variability inherent to different donors, and to repeat original observation. 20 The experiment was performed exactly as in the 1st round of screening with the additional normalization to cells confluency. For donor 1 the best 4 performers were as follows: 1) RPS25-secNluc-Ube2d2a, 2) RPS14-secNluc-HBA1, 3) Eno-secNluc-Ube2d2a, 4) REG1A-secNluc-Ube2d2a. (Fig. 2B). For the donor 2, in turn: 1)TCV-secNluc-HBB-001, 2)HBA1-secNluc-HBA1, 3) TCV-secNluc-UBL5, 4)TCV-secNluc-Med13 and ANXA1-secNluc-HBG2 (Fig. 2C).

25 One combination was overlapping in SkMC and iMoDCs, namely eno-secNluc-Ube2d2a. The HBA1 3-prime UTR demonstrated superior mRNA expression in all donors and cell types in combination with different 5-prime sequences, pointing out that the UTR can boost mRNA properties in different tissues. Thus, the combination of ANXA1 and RPS14 5-prime UTRs with HBA1 3-prime UTRs were 30 selected from SkMC and iMoDC top lists respectively. Similarly, Ube2d2a was also frequently observed over the lists and selected in combination with RPS25. For one of the iMoDCs donor the TCV 5-prime UTR was very frequent, thus we retained this 5' UTR in combination with another popular 3' UTR sequence UBL5 (seen twice). 6th downselected UTRs are summarized in table 1, control UTRs are summarized in table 2.

35

Table 1. Selection of 5' and 3'UTRs

5'UTR	5'UTR sequence – DNA sequence (T = U* in corresponding RNA sequence)	SEQ ID NO:
ANXA1 (human)	AGTGTGAAATCTTCAGAGAAGAATTTCTCTTTAGTTCTTTGCAAGA AGGTAGAGATAAAGACACTTTTTCAAAA	1
Eno (e coli)	TTAACTAGTGACTTGAGGAAAACCTAA	2
RPS14 (human)	GTTAACGCACTGGGTCGTGCTCATTGGTCTGATTGAAGATAGGA ACATTTAACTTCGTACACCCAAGACTTACACTTGAAAA	3
RPS25 (human)	CTTTTTGTCCGACATCTTGACGAGGCTGCGGTGTCTGCTGCTATT CTCCGAGCTTCGCA	4
TCV (turnip crinkle virus)	GGTAATCTGCAAATCCCTGGCACCCGCCTAAAATTGCCCTCATCA ACCTTCTCTCTATTACG	5
3'UTR	3'UTR sequence – DNA sequence (T = U* in corresponding RNA sequence)	
HBA1 (human)	GCTGGAGCCTCGGTGGCCTAGCTTCTTGCCCCTTGGGCCTCCCC CCAGCCCCTCCTCCCCTTCCTGCACCCGTACCCCGTGGTCTTT GAATAAAGTCTGAGTGGGCGGCA	6
RPS29-001 (human)	ATGCTCTTCCTTCAGAGGATTATCCGGGGCATCTACTCAATGAAA AACCATGATAATTCTTTGTATATAAAAATAAACATTTGAAAAAACC	7
Ube2d2a (mouse)	TACTAAAAAAGATATAAAAGTAATTTTATTAACAAGGTAGAGGCA TAACCTTAAAGGCATTTTCATGAA	8
UBL5 (human)	TTGAGAATCCTCATCTTCCTGCCCCGCTTTCCTCTCCCATCCTCAT CCCCCACACTGGGATAGATGCTTGTTTGTA AAAA ACTCACCTTAATA AAGACTTAGATGTTG	9
HBA1 (human) - WT	GCTGGAGCCTCGGTGGCCATGCTTCTTGCCCCTTGGGCCTCCCC CCAGCCCCTCCTCCCCTTCCTGCACCCGTACCCCGTGGTCTTT GAATAAAGTCTGAGTGGGCGGCA	18

* U represents uridine, or a modified version thereof, such as pseudouridine, in particular N1-methyl pseudouridine.

5

Table 2. Control 5' and 3'UTRs

5'UTR	5'UTR sequence – DNA sequence (T = U* in corresponding RNA sequence)	SEQ ID No:
Ctrl 1 ETR (10x gtx 5'UTR element)	CCGGCGGGTTTCTGACATCCGGCGGGTTTCTGACATCCGGCGGGTT TCTGACATCCGGCGGGTTTCTGACATCCGGCGGGTTTCTGACATCC GGCGGGTTTCTGACATCCGGCGGGTTTCTGACATCCGGCGGGTTTC TGACATCCGGCGGGTTTCTGACATCCGGCGGGTTTCTGACATT	10
Ctrl 2 MDRN (Artificial)	AAATAAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGACCCCGGCGC C	11

Ctrl 3 BNT (HBA1)	GAGAATAAACTAGTATTCTTCTGGTCCCCACAGACTCAGAGAGAACC C	12
Ctrl 4 CV (HSD17B4)	GCTCATCTTCCTACCAGAAATCGGCAAGTCACTGACCCTCGTCCCGC CCCCGCCATTCCCCGCCTCCTCCTGTCCCGCAGTCGGCGTCCAGCG GCTCTGCTTGTTCTGTGTGTGTGTCGTTGCAGGCCTTATTC	13
3'UTR	3'UTR sequence – DNA sequence (T = U* in corresponding RNA sequence)	
Ctrl 1 ETR (KSHV-ENE)	CTCGAGTGTTTTGGCTGGGTTTTCTTGTTCGCACCGGACACCTCC AGTGACCAGACGGCAAGGTTTTATCCCAGTGTATATTGTCGAC	14
Ctrl 2 MDRN (HBA1)	GCTGGAGCCTCGGTGGCCTAGCTTCTTGCCCCTTGGGCCTCCCCC AGCCCCCTCCTCCCCTTCCTGCACCCGTACCCCCGTGGTCTTTGAATA AAGTCTGAGTGGGCGGCA	15
Ctrl 3 BNT (AES + 12S rRNA)	CTGGTACTGCATGCACGCAATGCTAGCTGCCCCTTTCCCGTCCTGG GTACCCCGAGTCTCCCCCGACCTCGGGTCCCAGGTATGCTCCCACC TCCACCAGCCCCACTCACCACCTCTGCTAGTTCCAGACACCTCCCAA GCACGCAGCAATGCAGCTCAAAACGCTTAGCCTAGCCACACCCCCA CGGGAACAGCAGTGATTAACCTTTAGCAATAAACGAAAGTTAACT AAGCTATACTAACCCAGGGTTGGTCAATTCGTGCCAGCCACACCC TGGAGCTAGC	16
Ctrl 4 CV (PSMB3+ HSL)	CCCTGTTCCCAGAGCCCACTTTTTTTCTTTTTTTGAAATAAAATAGC CTGTCTTTCACAAAGGCTCTTTTCAGAGCCACCA	17

* U represents uridine, or a modified version thereof, such as pseudouridine, in particular N1-methyl pseudouridine.

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Confirmation for the 6 best performers with other cell lines and mRNA type (Fig 3)

In order to assess the new combinations of UTRs in the context of different coding mRNA sequences, we synthesized mRNA encoding firefly Luciferase (Fluc) ORF with the 6 selected combinations of UTRs. In addition, to check whether cellular environment can affect the mRNA expression because of UTRs we assessed the expression of Fluc in six different human and mouse cell lines. Six human cells lines of different origin HeP2G – liver cancer cell line, K562 – leukemia cell line, BJ – fibroblasts, HEK293 – human embryonic kidney immortalized cell line, Mo-iDCs – immature Monocytes Derived dendritic cells, SkMCs - skeletal muscles cells and two mouse cell lines CT26 colorectal carcinoma and TC-1 immortalized lung epithelial line were transfected with new mRNA at different concentrations 100ng/well and 200ng/well respectively. After 24h the FLuc luminescence, mCitrine Fluorescence and viability were quantified. For viability, fluorescence was converted to percentage viability based on the assumption that untransfected wells were being 100% viable. To take into account transfection efficiency, the expression of Nanoluc (RLU) from transfection with 200ng/well was divided by the mCitrine mRNA expression level (RFU) shown as normalized p/s ratio in figure

3. New UTR combinations show consistently high expression levels in a broad range of cell lines with Fluc mRNA with the top 3 UTR combinations : 1) ANXA-HBA1, 2)eno-RSP29, 3)eno-Ube2d2. Strikingly TCV-UBL5 demonstrated high expression in HEP2G cell lines pointing out that UTRs acting in a tissue specific manner. The best performing UTR combinations after the 2nd round of screening in SkMC and in iMoDCs are listed in table 3.

Table 3. Best performing UTRs combinations

ID	5'-3' UTR
1	ANXA-HBA1
2	eno-RPS29 10
3	eno-Ube2d2
4	RPS14-HBA1
5	RPS25-Ube2d2
6	TCV-UBL5

Example 2: *In vivo* Screening

Materials and methods

Bioluminescence imaging

Bioluminescence imaging was performed 5, 24, 48 and 72 hours after intramuscular administration of 50 µL of TBS or 40 µg/mL firefly luciferase (fLuc) mRNA-LNP with an IVIS Lumina S5 imaging system (PerkinElmer). For non-invasive imaging, female 6-8-week-old BALB/c mice (Charles River) were intraperitoneally injected with 100 µL of 30 mg/mL D-luciferin (Promega, catalogue number # E1605) dissolved in sterile water. Five minutes following administration of D-luciferin, mice were anesthetized in an anesthesia induction chamber using 5% isoflurane and placed on the imaging platform while being maintained under anesthesia using 3% isoflurane. Full body images were taken 10-15 minutes post-D-luciferin administration using the automatic exposure setting. Quantification of the bioluminescence signal was performed using Living Image software (PerkinElmer).

Results

To investigate the effect of the six hit candidate 5'UTR - 3'UTR gene combinations on the RNA polynucleotide stability and/or translation efficiency *in vivo*, female 6-8-week-old BALB/c mice were intramuscularly injected with 50 µL of TBS, one of the six hit candidate 5'UTR - 3'UTR gene combination firefly luciferase (fLuc) mRNA-LNPs or one of the three control 5'UTR - 3'UTR gene combination fLuc mRNA-LNPs. fLuc expression was measured 5, 24, 48 and 72 hours post-administration through quantification of the full body bioluminescence signal following D-luciferin injection using an IVIS platform. Overall, across all time points, all six hit candidate 5'UTR - 3'UTR gene combinations tend to perform better than or equal as the tested internal/benchmark 5'UTR - 3'UTR gene combinations, with the top 3 UTR combinations being 1) eno-RSP29, 2) eno-Ube2d2, and 3) TCV-UBL5. For the earlier time points, ANXA1-HBA1 is the bottom performer in this *in vivo*

expression study, whereas RPS25-Ube2d2a UTR combination is associated with the lowest bioluminescence signal in the later time points (fig. 4).

Example 3: Ex Vivo Expression

5 In this example, we evaluated *ex vivo* expression of novel muLL-7, muLL-21 and mu41BBL UTR's (V8 and V10) compared with the legacy UTR (V5) to check if there is an improved expression in MC-38.

UTR V8 corresponds to RPS14 5'UTR – HBA1 3'UTR

10 UTR V10 corresponds to ANXA1 5'UTR – HBA1 3'UTR

UTR V5 corresponds to the UTRs disclosed in WO2015071295, as comparative example

At 24h, tumors were collected and *ex vivo* expression was assessed by Luminex, procartaplex which enables us to detect muLL-21, muLL-7 and mu41BBL at the same time.

15 Set-up of the experiment:

- Dose: 15 µg / mouse in injection volume 20µl formulated in LNPs
- Read-out type: harvest tumor at 24h and determine protein expression on the Luminex

Inoculate C57BL/6J mice (one-sided; left flank) with 0.5*10e6 tumor cells.

20

When average tumor size reaches 50-100mm³ (day 10 post-inoculation), randomize mice and inject tumors IT with LNPs.

25 As evident in figure 5, for all 3 tested mRNA (muLL-7, muLL-21 and mu41BBL) constructs, both UTR V8 and V10 result in significantly higher protein expression compared to the comparative UTR V5 combination.

Example 4: in vivo Efficacy Study

30 In this example, we evaluated *in vivo* efficacy of novel muLL-7, muLL-21 and mu41BBL UTR's (V8 and V10) compared with the legacy UTR (V5) to check if there is an improved expression in MC-38.

Set-up of the experiment:

- 35
- Dose: 10 µg / mouse in injection volume 20µl formulated in LNPs
 - Read-out type: harvest tumor at 24h and determine survival of the mice up until 35 days after inoculation

Inoculate C57BL/6J mice (one-sided; left flank) with 0.5*10e6 tumor cells.

When average tumor size reaches $> 75 \text{ mm}^3$ (+/- day 11 post-inoculation), randomize mice and inject tumors IT with LNPs. Injections are repeated at 3 days and 7 days post randomization.

- As evidenced in figure 6, the highest efficacies are obtained using V10 UTRs (Fig. 6D) and V8 UTRs (Fig. 6C). The comparative V5 UTRs (Fig. 6B) clearly perform worse compared to these 2 UTR combination of the invention. TBS is used as negative example (Fig. 6A).
- 5

CLAIMS

1. An RNA polynucleotide comprising

5 i) at least one 5' untranslated region (5'UTR) element comprising a nucleic acid sequence which is derived from the 5'UTR of a gene selected from ANXA1, E. Coli enolase, RPS14, RPS25, or TCV, or from a fragment or variant thereof;

ii) at least one open reading frame (ORF) encoding a polypeptide;

10 iii) at least one 3' untranslated region (3'UTR) element comprising a nucleic acid sequence which is derived from the 3'UTR of a gene selected from HBA1, RPS29, Ube2d2a, or UBL5, or from a fragment or variant thereof.

2. The RNA polynucleotide according to claim 1, wherein said 5'UTR of the RNA polynucleotide comprises an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 1 [ANXA1], SEQ ID NO: 2 [eno], SEQ ID NO: 3 [RPS14], SEQ ID NO: 4 [RPS25], or SEQ ID NO: 5 [TCV], or a sequence having at least 95% sequence identity thereto.

15 3. The RNA polynucleotide according to claim 1 or 2, wherein said 3'UTR of the RNA polynucleotide comprises an RNA sequence corresponding to the DNA sequences selected from SEQ ID NO: 6 [HBA1], SEQ ID NO: 18 [HBA1], SEQ ID NO: 7 [RPS29], SEQ ID NO: 8 [Ube2d2a], or SEQ ID NO: 9 [UBL5], or a sequence having at least 95% sequence identity thereto.

20 4. The RNA polynucleotide according to any one of claims 1 to 3, wherein said 5'UTR and said 3'UTR of the RNA polynucleotide comprise an RNA sequence, fragment or variant thereof corresponding to the DNA sequences as selected from any of the following 5' UTR – 3' UTR gene combinations:

- 5'UTR of ANXA1 in combination with 3'UTR of HBA1; or
- 5'UTR of eno in combination with 3'UTR of RPS29; or
- 25 - 5'UTR of eno in combination with 3'UTR of Ube2d2a; or
- 5'UTR of RPS14 in combination with 3'UTR of HBA1; or
- 5'UTR of RPS25 in combination with 3'UTR of Ube2d2a; or
- 5'UTR of TCV in combination with 3'UTR of UBL5.

5. The RNA polynucleotide according to any one of claims 1 to 3, wherein said 5'UTR and said 3'UTR of the RNA polynucleotide comprise an RNA sequence, fragment or variant thereof corresponding to the DNA sequences as selected from any of the following 5' UTR – 3' UTR gene combinations:

- 5 - 5'UTR of ANXA1 in combination with 3'UTR of HBA1; or
 - 5'UTR of RPS14 in combination with 3'UTR of HBA1.

6. The RNA polynucleotide according to any one of claims 1 to 3, wherein said 5' UTR and said 3' UTR of the RNA polynucleotide comprise an RNA sequence, or a sequence having at least 95% sequence identity thereto corresponding to the DNA sequences as selected from any of the following combinations:

- SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; or
 - SEQ ID NO: 2 [eno] for 5'UTR in combination with SEQ ID NO: 7 [RPS29] for 3'UTR; or
 - SEQ ID NO: 2 [eno] for 5'UTR in combination with SEQ ID NO: 8 [Ube2d2a] for 3'UTR; or
15 - SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; or
 - SEQ ID NO: 3 [RPS25] for 5'UTR in combination with SEQ ID NO: 8 [Ube2d2a] for 3'UTR; or
 - SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; or
 - SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; or
 - SEQ ID NO: 5 [TCV] for 5'UTR in combination with SEQ ID NO: 9[UBL] for 3'UTR.

20

7. The RNA polynucleotide according to any one of claims 1 to 3, wherein said 5' UTR and said 3' UTR of the RNA polynucleotide comprise an RNA sequence, or a sequence having at least 95% sequence identity thereto corresponding to the DNA sequences as selected from any of the following combinations:

- 25 - SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR; or
 - SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 6 [HBA1] for 3'UTR.
 - SEQ ID NO: 1 [ANXA1] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR; or
 - SEQ ID NO: 3 [RPS14] for 5'UTR in combination with SEQ ID NO: 18 [HBA1] for 3'UTR.

30 8. The RNA polynucleotide according to any one of claims 1 to 7, wherein said 5'UTR of the RNA polynucleotide further comprises a KOZAK sequence and/or an internal ribosome entry site (IRES).

9. The RNA polynucleotide according to any one of claims 1 to 7, wherein said RNA polynucleotide is a mRNA or non-coding RNA, preferably a mRNA, antisense RNA, siRNA, sgRNA, guide RNA or CRISPR.

10. A pharmaceutical composition comprising one or more RNA polynucleotides according to any of
5 claims 1 – 9, and at least one pharmaceutically acceptable agent.

11. The RNA polynucleotide according to anyone of claims 1-9, or the pharmaceutical composition according to claim 10 for use in human and/or veterinary medicine.

12. The RNA polynucleotide according to anyone of claims 1-9, or the pharmaceutical composition according to claim 10 for use as a vaccine or for use in gene therapy.

10 13. Use of an RNA polynucleotide according to any one of 1-9, or the pharmaceutical composition according to claim 10 to enhance the expression and/or translation of a gene product, in particular to enhance initiation of translation.

14. Use of an RNA polynucleotide according to any one of claims 1-9, or the pharmaceutical composition according to claim 10 to enhance the stability and/or duration of expression of a gene
15 product, in particular to enhance the biodistribution.

15. A method of inducing an enhanced immune response in a subject comprising: administering a therapeutically effective amount of the RNA polynucleotide according to any one of claims 1 to 9 or the pharmaceutical composition according to claim 10 to a subject.