inefficient. Future methods for generating iPS cells for potential clinical applications will need to increase the speed and efficiency of iPS cell formation while maintaining genome integrity.

SUMMARY ~~OF THE INVENTION~~

The present disclosure~~invention~~ provides compositions and methods for reprogramming the state of differentiation of eukaryotic cells, including human or other animal cells, by contacting the cells with purified RNA preparations comprising or consisting of one or more different single-strand mRNA molecules that each encode a reprogramming factor (e.g., an iPS cell induction factor). The purified single-stranded mRNA molecules preferably comprise at least one modified nucleoside (e.g., selected from the group consisting of a pseudouridine (Ψ), 5-methylcytosine (m^5C), 5-methyluridine (m^5U), 2'-O-methyluridine (Um or $m^{2'\text{-}O}U$), 2-thiouridine (s^2U), and N^6-methyladenosine (m^6A)) in place of at least a portion (e.g., including substantially all) of the corresponding unmodified canonical nucleoside of the corresponding unmodified A, C, G, or T canonical nucleoside. In addition, the single-stranded mRNA molecules are preferably purified to be substantially free of RNA contaminant molecules that would activate an unintended response, decrease expression of the single-stranded mRNA, and/or activate RNA sensors (e.g., double-stranded RNA-dependent enzymes) in the cells. In certain embodiments, the purified RNA preparations are substantially free of RNA contaminant molecules that are: shorter or longer than the full-length single-stranded mRNA molecules, double-stranded, and/or uncapped RNA. In some preferred embodiments, the present disclosure~~invention~~ provides compositions and methods for reprogramming differentiated eukaryotic cells, including human or other animal somatic cells, by contacting the cells with purified RNA preparations comprising or consisting of one or more different single-strand mRNA molecules that each encode an iPS cell induction factor.

In some embodiments, the present disclosure~~invention~~ provides methods for changing the state of differentiation of a somatic cell comprising: introducing an mRNA encoding an iPS cell induction factor into a somatic cell to generate a reprogrammed dedifferentiated cell, wherein the mRNA comprises at least one 5-methylcytidine (or other modified based described herein).

In certain embodiments, the present disclosure~~invention~~ provides methods for reprogramming a cell that exhibits a first differentiated state or phenotype to a cell that exhibits a second differentiated state or phenotype comprising: introducing into the cell that exhibits a first differentiated state a purified RNA preparation comprising modified mRNA molecules that encode at least one

reprogramming factor and culturing the cell under conditions wherein the cell exhibits a second differentiated state. In certain embodiments, the modified mRNA molecules contain at least one modified nucleoside selected from the group consisting of psuedouridine or 5-methylcytidine.

In one aspect, there is provided an in vitro method for reprogramming a human or animal cell that exhibits a first differentiated state or phenotype to a cell that exhibits a second differentiated state or phenotype comprising: introducing into the cell that exhibits a first differentiated state a purified RNA preparation comprising modified mRNA molecules that encode at least one reprogramming factor and culturing the cell under conditions wherein the cell exhibits a second differentiated state; and wherein the modified mRNA molecules contain at least one modified nucleoside selected from the group consisting of: pseudouridine (Ψ), 1-methyl-pseudouridine ($m^1\Psi$), 5-methylcytidine (m^5C), 5-methyluridine (m^5U), 2'-O-methyluridine (Um or $m^{2'\text{-}O}U$), and 2-thiouridine (s^2U), in place of at least a portion of the corresponding unmodified canonical nucleoside. In certain embodiments, the cell is from a human or animal. In further embodiments, the purified RNA preparation: i) comprises first single-stranded mRNAs encoding a first iPS cell induction factor, wherein substantially all of the first single-stranded complete mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and ii) is substantially free of RNA contaminant molecules which are able to activate RNA sensors in said somatic cell. In certain embodiments, the RNA contaminant molecules are selected from the group consisting of: partial mRNAs encoding only a portion of said iPS cell induction factor, RNA molecules that are smaller than the full-length mRNA, RNA molecules that are larger than the full-length mRNA, double-stranded mRNA molecules, and un-capped mRNA molecules.

In some embodiments, the present disclosure~~invention~~ provides methods for reprogramming a somatic cell (e.g., dedifferentiating or transdifferentiating) comprising: contacting a somatic cell with a purified RNA preparation to generate a reprogrammed cell, wherein the purified RNA preparation: i) comprises first single-stranded mRNAs encoding a first iPS cell induction factor, wherein substantially all of the first single-stranded complete mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and ii) is substantially free of contaminant molecules (e.g., RNA contaminant molecules) which are able to activate RNA sensors in the somatic cell. In particular embodiments, the RNA contaminant molecules comprise: partial mRNAs encoding only a portion of the iPS cell induction factor, single-stranded run-on mRNAs encoding the iPS cell induction factor and encoding at least an additional portion of the iPS cell induction factor, double-stranded mRNA molecules, and un-capped mRNA molecules. In certain embodiments, the first single-stranded mRNAs do not also encoding an additional portion of the first iPS cell induction factor.

In some embodiments, the reprogrammed cell is a dedifferentiated cell (e.g., stem cell or stem cell-like cell). In other embodiments, the reprogrammed cell is a transdifferentiated cells (e.g., a skin cells is reprogrammed into a neuronal cell, or other type of change). In further embodiments, the first single-stranded mRNAs encode the complete first iPS induction factor (e.g, the mRNA encodes the entire coding sequence for a particular iPS induction factor). In other embodiments, the contacting further comprises contacting the somatic cell with a growth

factor and/or cytokine (e.g,. after a period of time). In further embodiments, the contact further comprises contacting the somatic cell with an immune response inhibitor.

In certain embodiments, all or nearlly all of the uridine nucleosides in the first single-stranded mRNA are replaced by pseudouridine nucleosides. In other embodiments, all or nearly all of the cytidine nucleosides in the first single-stranded mRNA are replaced by 5-methylcytidine nucleosides or another base recited herein.

In particular embodiments, the present disclosure~~invention~~ provides methods for generating a reprogrammed cell comprising: contacting a somatic cell with a purified RNA preparation to generate a reprogrammed cell that is able to survive in culture for at least 10 days (e.g., at least 10 days ... at least 13 days ... at least 16 days .... at least 20 days ... at least 40 days ... or is able to form a cell-line), wherein the purified RNA preparation comprises first single-stranded mRNAs encoding an iPS cell induction factor, and wherein a majority of the first single-stranded mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue.

In certain embodiments, the purified RNA preparation is free of an amount of RNA contaminant molecules that would activate an immune response in the somatic cell sufficient to prevent the reprogrammed cell from surviving at least 10 days in culture (e.g., at least 10 days ... at least 15 days ... at least 20 days ... at least 40 days, or longer). In other embodiments, the RNA contaminant molecules include: partial mRNAs encoding only a portion of the iPS cell induction factor, single-stranded run-on mRNAs fully encoding the iPS cell induction factor and encoding at least an additional portion of the iPS cell induction factor, double-stranded mRNA molecules, un-capped mRNA molecules, and mixtures thereof. In certain embodiments, the reprogrammed cell that is generated is able to form a reprogrammed cell-line. In other embodiments, the purified RNA preparation is free of an amount of RNA contaminant molecules that would activate an immune response in the somatic cell sufficient to prevent generation of the reprogrammed cell-line.

In particular embodiments, the RNA contaminant molecules are selected from the group consisting of: partial mRNAs encoding only a portion of the iPS cell induction factor, single-stranded run-on mRNAs encoding the iPS cell induction factor and encoding at least an additional portion of the iPS cell induction factor, double-stranded mRNA molecules, un-capped mRNA molecules, and mixtures thereof.

In some embodiments, the present disclosure~~invention~~ provides methods for generating a reprogrammed cell-line comprising: a) contacting a somatic cell with a purified RNA preparation to generate a reprogrammed cell, wherein the purified RNA preparation comprises mRNAs encoding an iPS cell induction factor, and wherein a majority of the mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and b) culturing the dedifferentiated cell to generate a reprogrammed cell-line. In other embodiments, the purified RNA preparation is free of an amount of contaminant molecules that would activate an immune response in the somatic cell sufficient to prevent generation of the reprogrammed cell-line. In certain embodiments, the immune response involves activation of RNA sensors in the somatic cell.

In some embodiments, the present disclosure~~invention~~ provides methods for reprogramming a somatic cell comprising: contacting a somatic cell with a purified RNA preparation to generate a reprogrammed cell, wherein the purified RNA preparation: i) comprises first single-stranded mRNAs encoding a first iPS cell induction factor, wherein substantially all of the first single-stranded mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and ii) is substantially free of: a) partial mRNAs encoding only a portion of the first iPS cell induction factor, and b) double-stranded mRNA molecules. In further embodiments, the first single-stranded mRNA do not also encode an additional portion of the first iPS cell induction factor. In particular embodiments, the first single-stranded mRNA fully encode the first iPS cell induction factor. In other embodiments, the purified RNA preparation is also substantially free (or essentially free or virtually free or free) of single-stranded run-on mRNAs encoding the first iPS cell induction factor and encoding at least an additional portion of the first iPS cell induction factor. In other embodiments, the substantially all of the first single-stranded complete mRNAs are 5' capped. In other embodiments, the purified RNA preparation is also substantially free of un-capped mRNA molecules. In some embodiments, substantially all of the first single-stranded mRNAs comprise at least one psuedouridine residue. In additional embodiments, tsubstantially all of the first single-stranded mRNAs comprise at least one 5-methylcytidine residue. In other embodiments, substantially all of the first single-stranded mRNAs comprise at least one psuedouridine residue and at least one 5-methycytidine residue.

In certain embodiments, the purified RNA preparation comprises a transfection reagent. In other embodiments, the purified RNA preparation is obtained by HPLC purification of an

RNA sample that contains a substantial amount of the partial mRNAs and the double-stranded mRNAs. In further embodiments, the purified RNA preparation is essentially free of the partial mRNAs and the single-stranded run-on mRNAs. In some embodiments, the purified RNA preparation is essentially free or virtually free or free of double-stranded mRNA molecules. In other embodiments, the purified RNA preparation is essentially free or virtually free or free of un-capped mRNA molecules. In some embodiments, substantially all of the first single-stranded mRNAs are polyadenylated. In other embodiments, the first single-stranded complete mRNAs are capped with 7-methylguanosine.

In some embodiments, the first iPS cell induction factor is selected from the group consisting of KLF4, LIN28, c-MYC, NANOG, OCT4, and SOX2. In other embodiments, the purified RNA preparation: i) further comprises second single-stranded mRNAs encoding a second iPS cell induction factor, wherein the second single-stranded mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and ii) is further substantially free of: a) partial mRNAs encoding only a portion of the second iPS cell induction factor, and b) double-stranded mRNAs. In other embodiments, the purified RNA preparation is further substantially free of single-stranded run-on mRNAs encoding a second iPS cell induction factor and encoding at least an additional portion of the second iPS cell induction factor. In some embodiments, the second iPS cell induction factor is selected from the group consisting of KLF4, LIN28, c-MYC, NANOG, OCT4, and SOX2. In certain embodiments, the somatic cell is a fibroblast. In other embodiments, the reprogrammed cell is a pluripotent stem cell. In other embodiments, the dedifferentiated cell expresses NANOG and TRA-1-60. In some embodiments, the cell is in vitro. In further embodiments, the cell resides in culture. In particular embodiments, the cell resides in MEF-conditioned medium.

In an aspect, there is provided a composition comprising a purified RNA preparation, wherein said purified RNA preparation:

i) comprise single-stranded mRNA molecules that encode at least one reprogramming factor, wherein said single-stranded mRNA molecules comprise at least one nucleoside selected from the group consisting of pseudouridine (Ψ), 1-methylpseudouridine, 5-methylcytidine (m^5C), 5-methyluridine (m^5U), 2'-O-methyluridine (Um or $m^{2'\text{-}O}U$), and 2-thiouridine (s^2U), and

ii) is substantially free of RNA contaminant molecules which are able to activate RNA sensors in a human or animal cell.

In some embodiments, the present disclosure~~invention~~ provides compositions comprising a purified RNA preparation, wherein the purified RNA preparation: i) comprises first single-stranded mRNAs encoding a first iPS cell induction factor, wherein the first single-stranded mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and ii) is substantially free of RNA contaminant molecules, which are able to activate RNA sensors in a somatic cell. In certain embodiments, the present disclosure~~invention~~ provides compositions comprising a purified RNA preparation, wherein the purified RNA preparation: i) comprises first single-stranded mRNAs encoding a first iPS cell induction factor, wherein the first single-stranded

complete mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and ii) is substantially free of: a) partial mRNAs encoding only a portion of the first iPS cell induction factor, and b) double-stranded RNA.

In certain embodiments, the purified RNA preparation is also substantially free of single-stranded run-on mRNAs encoding the first iPS cell induction factor and encoding at least an additional portion of the first iPS cell induction factor. In some embodiments, the purified RNA preparation: i) further comprises second single-stranded mRNAs encoding a second iPS cell induction factor, wherein the second single-stranded complete mRNAs comprise at least one pseudouridine residue and/or at least one 5-methylcytidine residue, and ii) is substantially free of: a) partial mRNAs encoding only a portion of the second iPS cell induction factor, and b) single-stranded run-on mRNAs encoding second first iPS cell induction factor and encoding at least an additional portion of the second iPS cell induction factor.

In some embodiments, the present disclosure~~invention~~ provides compositions comprising an in vitro-synthesized mRNA encoding the MYC gene, wherein the in vitro-synthesized mRNA comprises at least one pseudouridine residue and/or at least one 5-methylcytidine residue. In certain embodiments, the compositions are substantially free of RNA contaminant molecules which are able to activate RNA sensors in a somatic cell.

In particular embodiments, the present disclosure~~invention~~ provides methods for inducing a mammalian cell to produce the MYC protein comprising: contacting a mammalian cell with an in vitro-synthesized mRNA encoding the MYC gene, wherein the in vitro-synthesized mRNA comprises at least one pseudouridine residue and/or at least one 5-methylcytidine residue, thereby inducing the mammalian cell to produce the MYC protein.

In an aspect, there is provided an in vitro method for inducing a cell to produce a MYC protein comprising: contacting a cell with an in vitro-synthesized mRNA encoding a MYC gene, wherein the in vitro-synthesized mRNA comprises at least one pseudouridine residue, at least one 1-methyl-pseudouridine, and/or at least one 5-methylcytidine residue, thereby inducing the mammalian cell to produce the MYC protein. In other embodiments, the mammalian cell is a dendritic cell. In other embodiments, the mammalian cell is an alveolar cell, an astrocyte, a microglial cell, or a neuron.

In some embodiments, the present disclosure~~invention~~ provides methods of treating a subject comprising contacting a subject with the MYC protein producing mammalian cell described above and herein.

In additional embodiments, the present disclosure~~invention~~ provides methods of synthesizing an in vitro-transcribed RNA molecule encoding the MYC gene comprising: combining an isolated RNA polymerase, a tempalte nucleic acid sequence encoding the MYG gene, unmodified nucleotides, and pseudouridine or 5-methylcytidine modified nucleotides under conditions such

that an in vitro-transcribed RNA molecule encoding the MYC gene is generated that comprises at least one pseudouridine or 5-methylcytidine residue.

Experiments conducted during the development of embodiments of the present ~~invention~~disclosure demonstrated that mRNA molecules can be administered to cells and induce a dedifferentiation process to generate dedifferentiated cells— including pluripotent stem cells. Thus, the present ~~invention~~disclosure provides compositions and methods for generating iPS cells. Surprisingly, the administration of mRNA can provide highly efficient generation of iPS cells.

The present ~~invention~~disclosure also provides RNA, oligoribonucleotide, and polyribonucleotide molecules comprising pseudouridine or a modified nucleoside, gene therapy vectors comprising same, methods of synthesizing same, and methods for gene replacement, gene therapy, gene transcription silencing, and the delivery of therapeutic proteins to tissue *in vivo*, comprising the molecules. The present ~~invention~~disclosure also provides methods of reducing the immunogenicity of RNA, oligoribonucleotide, and polyribonucleotide molecules.

In some embodiments, the present ~~invention~~disclosure provides methods for dedifferentiating a somatic cell comprising: introducing mRNA encoding one or more iPSC induction factors into a somatic cell to generate a dedifferentiated cell.

In some embodiments, the present ~~invention~~disclosure provides methods for dedifferentiating a somatic cell comprising: introducing mRNA encoding one or more iPSC induction factors into a somatic cell and maintaining the cell under conditions wherein the cell is viable and the mRNA that is introduced into the cell is translated in sufficient amount and for sufficient time to generate a dedifferentiated cell. In some preferred embodiments, the dedifferentiated cell is an induced pluripotent stem cell (iPSC).

In some embodiments, the present ~~invention~~disclosure provides methods for changing the state of differentiation (or differentiated state) of a eukaryotic cell comprising: introducing mRNA encoding one or more reprogramming factors into a cell and maintaining the cell under conditions wherein the cell is viable and the mRNA that is introduced into the cell is translated in sufficient amount and for sufficient time to generate a cell that exhibits a changed state of differentiation compared to the cell into which the mRNA was introduced.

In some embodiments, the present ~~invention~~disclosure provides methods for changing the state of differentiation of a eukaryotic cell comprising: introducing mRNA encoding one or more reprogramming factors into a cell and maintaining the cell under conditions wherein the cell is

viable and the mRNA that is introduced into the cell is translated in sufficient amount and for sufficient time to generate a cell that exhibits a changed state of differentiation compared to the cell into which the mRNA was introduced. In some embodiments, the changed state of differentiation is a dedifferentiated state of differentiation compared to the cell into which the mRNA was introduced. For example, in some embodiments, the cell that exhibits the changed state of differentiation is a pluripotent stem cell that is dedifferentiated compared to a somatic cell into which the mRNA was introduced (e.g., a somatic cell that is differentiated into a fibroblast, a cardiomyocyte, or another differentiated cell type). In some embodiments, the cell into which the mRNA is introduced is a somatic cell of one lineage, phenotype, or function, and the cell that exhibits the changed state of differentiation is a somatic cell that exhibits a lineage, phenotype, or function that is different than that of the cell into which the mRNA was introduced; thus, in these embodiments, the method results in transdifferentiation (Graf and Enver 2009).

The methods of the ~~invention~~present disclosure are not limited with respect to a particular cell into which the mRNA is introduced. In some embodiments of any of the above methods, the cell into which the mRNA is introduced is derived from any multi-cellular eukaryote. In some embodiments of any of the above methods, the cell into which the mRNA is introduced is selected from among a human cell and another animal cell. Although the work presented herein was performed using cells of humans or other animals, the applicants further claim that the methods of the present ~~invention~~disclosure comprising reprogramming human and animal cells by contacting the cells with a purified RNA preparation that consists of one or more purified single-stranded mRNA molecules, each of which encodes a protein reprogramming factor (e.g., a transcription factor) also pertains to reprogramming of other eukaryotic cells (e.g., plant cells and a fungal cells). In some embodiments of any of the above methods, the cell into which the mRNA is introduced is a normal cell that is from an organism that is free of a known disease. In some embodiments of any of the above methods, the cell into which the mRNA is introduced is a cell from an organism that has a known disease. In some embodiments of any of the above methods, the cell into which the mRNA is introduced is a cell that is free of a known pathology. In some embodiments of any of the above methods, the cell into which the mRNA is introduced is a cell that exhibits a disease state or a known pathology (e.g., a cancer cell, or a pancreatic beta cell that exhibits metabolic properties characteristic of a diabetic cell).

The ~~invention~~present disclosure is not limited to the use of a specific cell type (e.g., to a specific somatic cell type) in embodiments of the methods comprising introducing mRNA encoding one or more iPSC cell induction factors in order to generate a dedifferentiated cell (e.g., an iPS cell). Any cell that is subject to dedifferentiation using iPS cell induction factors is contemplated. Such cells include, but are not limited to, fibroblasts, keratinocytes, adipocytes, lymphocytes, T-cells, B-Cells, cells in mononuclear cord blood, buccal mucosa cells, hepatic cells, HeLa, MCF-7 or other cancer cells. In some embodiments, the cells reside *in vitro* (e.g., in culture) or *in vivo*. In some embodiments, when generated in culture, a cell-free conditioned medium (e.g., MEF-conditioned medium) is used. As demonstrated below, such a medium provided enhanced iPS cell generation. The ~~invention~~present disclosure is not limited, however, to the culturing conditions used. Any culturing condition or medium now known or later identified as useful for the methods of the ~~invention~~present disclosure (e.g., to generate iPS cells from somatic cells and maintain said cells) is contemplated for use with the ~~invention~~present disclosure. For example, although not preferred, in some embodiments of the method, a feeder cell layer is used instead of conditioned medium for culturing the cells that are treated using the method.

In some embodiments of any of these methods, the step of introducing mRNA comprises delivering the mRNA into the cell (e.g., a human or other animal somatic cell) with a transfection reagent (e.g., TRANSIT™ mRNA transfection reagent, MirusBio, Madison, WI).

However, the ~~invention~~present disclosure is not limited by the nature of the transfection method utilized. Indeed, any transfection process known, or identified in the future that is able to deliver mRNA molecules into cells *in vitro* or *in vivo*, is contemplated, including methods that deliver the mRNA into cells in culture or in a life-supporting medium, whether said cells comprise isolated cells or cells comprising a eukaryotic tissue or organ, or methods that deliver the mRNA *in vivo* into cells in an organism, such as a human, animal, plant or fungus. In some embodiments, the transfection reagent comprises a lipid (e.g., liposomes, micelles, etc.). In some embodiments, the transfection reagent comprises a nanoparticle or nanotube. In some embodiments, the transfection reagent comprises a cationic compound (e.g., polyethylene imine or PEI). In some embodiments, the transfection method uses an electric current to deliver the mRNA into the cell (e.g., by electroporation). In some embodiments, the transfection method uses a bolistics method to deliver the mRNA into the cell (e.g., a "gene gun" or biolistic particle delivery system.)

The data presented herein shows that, with respect to the mRNA introduced into the cell, certain amounts of the mRNAs used in the EXAMPLES described herein resulted in higher efficiency and more rapid induction of pluripotent stem cells from the particular somatic cells used than other amounts of mRNA. However, the methods of the present ~~invention~~disclosure are not limited to the use of a specific amount of mRNA to introduce into the cell. For example, in some embodiments, a total of three doses, with each dose comprising 18 micrograms of each of six different mRNAs, each encoding a different human reprogramming factor, was used to introduce the mRNA into approximately 3 x 10^5 human fibroblast cells in a 10-cm plate (e.g., delivered using a lipid-containing transfection reagent), although in other embodiments, higher or lower amounts of the mRNAs were used to introduce into the cells.

The ~~invention~~present disclosure is not limited to a particular chemical form of the mRNA used, although certain forms of mRNA may produce more efficient results. However, in some preferred embodiments, the mRNA comprises at least one modified nucleoside (e.g., selected from the group consisting of a pseudouridine (Ψ), 5-methylcytosine (m^5C), 5-methyluridine (m^5U), 2'-O-methyluridine (Um or $m^{2'\text{-}O}U$), 2-thiouridine (s^2U), and N^6-methyladenosine (m^6A)) in place of at least a portion of the corresponding unmodified canonical nucleoside (e.g., in some preferred embodiments, at least one modified nucleoside in place of substantially all of the corresponding unmodified A, C, G, or T canonical nucleoside). In some embodiments, the mRNA is polyadenylated. In some preferred embodiments, the mRNA is prepared by polyadenylation of an in vitro-transcribed (IVT) RNA, the method comprising contacting the IVT RNA using a poly(A) polymerase (e.g., yeast RNA polymerase or *E. coli* poly(A) polymerase). In some embodiments, the mRNA is polyadenylated during IVT by using a DNA template that encodes the poly(A) tail. Regardless of whether the RNA is polyadenylated using a poly(A) polymerase or during IVT of a DNA template, in some preferred embodiments, the mRNA comprises a poly-A tail (e.g., a poly-A tail having 50-200 nucleotides, e.g., preferably 100-200, 150-200 nucleotides, or greater than 150 nucleotides), although in some embodiments, a longer or a shorter poly-A tail is used. In some embodiments, the mRNA used in the methods is capped. To maximize efficiency of expression in the cells, it is preferred that the majority of mRNA molecules contain a cap. In some preferred embodiments, the mRNA molecules used in the methods are synthesized *in vitro* by incubating uncapped primary RNA in the presence a capping enzyme system. In some preferred embodiments, the primary RNA used in the capping enzyme

reaction is synthesized by *in vitro* transcription (IVT) of a DNA molecule that encodes the RNA to be synthesized. The DNA that encodes the RNA to be synthesized contains an RNA polymerase promoter, to which, an RNA polymerase binds and initiates transcription therefrom. It is also known in the art that mRNA molecules often have regions of differing sequence located before the translation start codon and after the translation stop codon that are not translated. These regions, termed the five prime untranslated region (5' UTR) and three prime untranslated region (3' UTR), respectively, can affect mRNA stability, mRNA localization, and translational efficiency of the mRNA to which they are joined. Certain 5' and 3' UTRs, such as those for alpha and beta globins are known to improve mRNA stability and expression of mRNAs. Thus, in some preferred embodiments, the mRNAs that encode reprogramming factors (e.g., iPSC induction factors) exhibit a 5' UTR and/or a 3' UTR that results in greater mRNA stability and higher expression of the mRNA in the cells (e.g., an alpha globin or a beta globin 5' UTR and/or 3' UTR; e.g., a Xenopus or human alpha globin or a beta globin 5' UTR and/or 3' UTR, or, e.g., a tobacco etch virus (TEV) 5' UTR).

The IVT can be performed using any RNA polymerase as long as synthesis of the mRNA from the DNA template that encodes the RNA is specifically and sufficiently initiated from a respective cognate RNA polymerase promoter and full-length mRNA is obtained. In some preferred embodiments, the RNA polymerase is selected from among T7 RNA polymerase, SP6 RNA polymerase and T3 RNA polymerase. In some other embodiments, capped RNA is synthesized co-transcriptionally by using a dinucleotide cap analog in the IVT reaction (e.g., using an AMPLICAP™ T7 Kit or a MESSAGEMAX™ T7 ARCA-CAPPED MESSAGE Transcription Kit; EPICENTRE or CellScript, Madison, WI, USA). If capping is performed co-transcriptionally, preferably the dinucleotide cap analog is an anti-reverse cap analog (ARCA). However, use of a separate IVT reaction, followed by capping with a capping enzyme system, which results in approximately 100% of the RNA being capped, is preferred over co-transcriptional capping, which typically results in only about 80% of the RNA being capped. Thus, in some preferred embodiments, a high percentage of the mRNA molecules used in a method of the present disclosure~~invention~~ are capped (e.g., greater than 80%, greater than 90%, greater than 95%, greater than 98%, greater than 99%, greater than 99.5%, or greater than 99.9% of the population of mRNA molecules are capped). In some preferred embodiments, the mRNA used in the methods of the present ~~invention~~disclosure has a cap with a capl structure, meaning that the 2'

hydroxyl of the ribose in the penultimate nucleotide with respect to the cap nucleotide is methylated. However, in some embodiments, mRNA used in the methods has a cap with a cap0 structure, meaning that the 2' hydroxyl of the ribose in the penultimate nucleotide with respect to the cap nucleotide is not methylated. With some but not all transcripts, transfection of eukaryotic cells with mRNA having a cap with a cap1 structure results in a higher level or longer duration of protein expression in the transfected cells compared to transfection of the same cells with the same mRNA but with a cap having a cap0 structure. In some embodiments, the mRNA used in the methods of the present ~~invention~~disclosure has a modified cap nucleotide. In some experiments performed prior to the experiments presented in the EXAMPLES herein, the present applicants found that, when 1079 or IMR90 human fibroblast cells were transfected with OCT4 mRNA that contained either uridine, or pseudouridine in place of uridine, the pseudouridine-containing mRNA was translated at a higher level or for a longer duration than the mRNA that contained uridine. Therefore, in some preferred embodiments, one or more or all of the uridines contained in the mRNA(s) used in the methods of the present ~~invention~~disclosure is/are replaced by pseudouridine (e.g., by substituting pseudouridine-5'-triphosphate in the IVT reaction to synthesize the RNA in place of uridine-5'-triphosphate). However, in some embodiments, the mRNA used in the methods of the ~~invention~~present disclosure contains uridine and does not contain pseudouridine. In some preferred embodiments, the mRNA comprises at least one modified nucleoside (e.g., selected from the group consisting of a pseudouridine (Ψ), 5-methylcytosine (m^5C), 5-methyluridine (m^5U), 2'-O-methyluridine (Um or $m^{2'\text{-}O}U$), 2-thiouridine (s^2U), and N^6-methyladenosine (m^6A)) in place of at least a portion of the corresponding unmodified canonical nucleoside (e.g., in place of substantially all of the corresponding unmodified A, C, G, or T canonical nucleoside). In some preferred embodiments, the mRNA comprises at least one modified nucleoside selected from the group consisting of a pseudouridine (Ψ) and 5-methylcytosine (m^5C). In some preferred embodiments, the mRNA comprises both pseudouridine (Ψ) and 5-methylcytosine (m^5C). In addition, in order to accomplish specific goals, a nucleic acid base, sugar moiety, or internucleotide linkage in one or more of the nucleotides of the mRNA that is introduced into a eukaryotic cell in any of the methods of the ~~invention~~present disclosure may comprise a modified nucleic acid base, sugar moiety, or internucleotide linkage.

The ~~invention~~present disclosure is also not limited with respect to the source of the mRNA that is delivered into the eukaryotic cell in any of the methods of the ~~invention~~present disclosure. In some embodiments, such as those described in the EXAMPLES, the mRNA is synthesized by in vitro transcription of a DNA template comprising a gene cloned in a linearized plasmid vector or by in vitro transcription of a DNA template that is synthesized by PCR or RT-PCR (i.e., by IVT of a PCR amplification product), capping using a capping enzyme system or by co-transcriptional capping by incorporation of a dinucleotide cap analog (e.g., an ARCA) during the IVT, and polyadenylation using a poly(A) polymerase. In some preferred embodiments, the mRNA is synthesized by IVT of a DNA template comprising a gene cloned in a linearized plasmid vector or a PCR or RT-PCR amplification product, wherein the DNA template encodes a 3'poly(A) tail. In some other embodiments, the mRNA that is delivered into the eukaryotic cell in any of the methods of the ~~invention~~present disclosure is derived directly from a cell or a biological sample. For example, in some embodiments, the mRNA derived from a cell or biological sample is obtained by amplifying the mRNA from the cell or biological sample using an RNA amplification reaction, and capping the amplified mRNA using a capping enzyme system or by co-transcriptional capping by incorporation of a dinucleotide cap analog (e.g., an ARCA) during the IVT, and, if the amplified mRNA does not already contain a template-encoded poly(A) tail from the RNA amplification reaction, polyadenylating the amplified mRNA using a poly(A) polymerase.

With respect to the methods comprising introducing mRNA encoding one or more iPS cell induction factors in order to generate a dedifferentiated cell (e.g., an iPS cell), the ~~invention~~present disclosure is not limited by the nature of the iPS cell induction factors used. Any mRNA encoding one or more protein induction factors now known, or later discovered, that find use in dedifferentiation, are contemplated for use in the present ~~invention~~disclosure. In some embodiments, one or more mRNAs encoding for KLF4, LIN28, c-MYC, NANOG, OCT4, or SOX2 are employed. Oct-3/4 and certain members of the Sox gene family (Sox1, Sox2, Sox3, and Sox15) have been identified as transcriptional regulators involved in the induction process. Additional genes, however, including certain members of the Klf family (Klf1, Klf2, Klf4, and Klf5), the Myc family (C-myc, L-myc, and N-myc), Nanog, and LIN28, have been identified to increase the induction efficiency. Any one or more such factors may be used as desired.

While the compositions and methods of the ~~invention~~present disclosure may be used to generate iPS cells, the ~~invention~~present disclosure is not limited to the generation of such cells. For example, in some embodiments,

mRNA encoding one or more reprogramming factors is introduced into a cell in order to generate a cell with a changed state of differentiation compared to the cell into which the mRNA was introduced. For example, in some embodiments, mRNA encoding one or more iPS cell induction factors is used to generate a dedifferentiated cell that is not an iPS cells. Such cells find use in research, drug screening, and other applications.

In some embodiments, the present ~~invention~~disclosure further provides methods employing the dedifferentiated cells generated by the above methods. For example, such cells find use in research, drug screening, and therapeutic applications in humans or other animals. For example, in some embodiments, the cells generated find use in the identification and characterization of iPS cell induction factors as well as other factors associated with differentiation or dedifferentiation. In some embodiments, the generated dedifferentiated cells are transplanted into an organism or into a tissue residing *in vitro* or *in vivo*. In some embodiments, an organism, tissue, or culture system housing the generated cells is exposed to a test compound and the effect of the test compound on the cells or on the organism, tissue, or culture system is observed or measured.

In some other embodiments, a dedifferentiated cell generated using the above methods (e.g., an iPS cell) is further treated to generate a differentiated cell that has the same state of differentiation or cell type compared to the somatic cell from which the dedifferentiated cell was generated. In some other embodiments, the dedifferentiated cell generated using the above methods (e.g., an iPS cell) is further treated to generate a differentiated cell that has a different state of differentiation or cell type compared to the somatic cell from which the dedifferentiated cell was generated. In some embodiments, the differentiated cell is generated from the generated dedifferentiated cell (e.g., the generated iPS cell) by introducing mRNA encoding one or more reprogramming factors into the generated iPS cell during one or multiple treatments and maintaining the cell into which the mRNA is introduced under conditions wherein the cell is viable and is differentiated into a cell that has a changed state of differentiation or cell type compared to the generated dedifferentiated cell (e.g., the generated iPS cell) into which the mRNA encoding the one or more reprogramming factors is introduced. In some of these embodiments, the generated differentiated cell that has the changed state of differentiation is used for research, drug screening, or therapeutic applications (e.g., in humans or other animals). For example, the generated differentiated cells find use in the identification and characterization

introduced. In some preferred embodiments of this method, the reprogramming efficiency for generating dedifferentiated cells is greater than or equal to 700 dedifferentiated cells (e.g., iPSCs) per 3 x 10^5 input cells into which the mRNA is introduced. In some preferred embodiments of this method, the reprogramming efficiency for generating dedifferentiated cells is greater than or equal to 800 dedifferentiated cells (e.g., iPSCs) per 3 x 10^5 input cells into which the mRNA is introduced. In some preferred embodiments of this method, the reprogramming efficiency for generating dedifferentiated cells is greater than or equal to 900 dedifferentiated cells (e.g., iPSCs) per 3 x 10^5 input cells into which the mRNA is introduced. In some preferred embodiments of this method, the reprogramming efficiency for generating dedifferentiated cells is greater than or equal to 1000 dedifferentiated cells (e.g., iPSCs) per 3 x 10^5 input cells into which the mRNA is introduced. Thus, in some preferred embodiments, this method was greater than 2-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector). In some preferred embodiments, this method was greater than 5-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector). In some preferred embodiments, this method was greater than 10-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector). In some preferred embodiments, this method was greater than 20-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector). In some preferred embodiments, this method was greater than 25-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector). In some preferred embodiments, this method was greater than 30-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector). In some preferred embodiments, this method was greater than 35-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector). In some preferred embodiments, this method was greater than 40-fold more efficient than the published protocol comprising delivery of reprogramming factors with a viral vector (e.g., a lentivirus vector).

The present ~~invention~~disclosure further provides compositions (systems, kits, reaction mixtures, cells, mRNA) used or useful in the methods and/or generated by the methods described herein.

For example, in some embodiments, the present ~~invention~~disclosure provides an mRNA encoding an iPS cell induction factor, the mRNA having pseudouridine in place of uridine.

The present ~~invention~~disclosure further provides compositions comprising a transfection reagent and an mRNA encoding an iPS cell induction factor (e.g., a mixture of transfection reagent and mRNA). In some embodiments, the compositions comprise mRNA encoding a plurality (e.g., 2 or more, 3 or more, 4 or more, 5 or more, or 6) of iPS cell induction factors, including, but not limited to, KLF4, LIN28, c-MYC, NANOG, OCT4, and SOX2.

The compositions may further comprise any other reagent or component sufficient, necessary, or useful for practicing any of the methods described herein. Such reagents or components include, but are not limited to, transfection reagents, culture medium (e.g., MEF-condition medium), cells (e.g., somatic cells, iPS cells), containers, boxes, buffers, inhibitors (e.g., RNase inhibitors), labels (e.g., fluorescent, luminescent, radioactive, etc.), positive and/or negative control molecules, reagents for generating capped mRNA, dry ice or other refrigerants, instructions for use, cell culture equipment, detection/analysis equipment, and the like.

This present disclosure~~invention~~ provides RNA, oligoribonucleotide, and polyribonucleotide molecules comprising pseudouridine or a modified nucleoside, gene therapy vectors comprising same, gene therapy methods and gene transcription silencing methods comprising same, methods of reducing an immunogenicity of same, and methods of synthesizing same.

In one embodiment, the present ~~invention~~disclosure provides a messenger RNA comprising a pseudouridine residue. In another embodiment, the present ~~invention~~disclosure provides an RNA molecule encoding a protein of interest, said RNA molecule comprising a pseudouridine residue. In another embodiment, the present ~~invention~~disclosure provides an *in vitro-transcribed* RNA molecule, comprising a pseudouridine or a modified nucleoside. In another embodiment, the present ~~invention~~disclosure provides an *in vitro-synthesized* oligoribonucleotide, comprising a pseudouridine or a modified nucleoside, wherein the modified nucleoside is m^5C, m^5U, m^6A, s^2U, Ψ, or 2'-O-methyl-U. In another embodiment, the present ~~invention~~disclosure provides a gene-therapy vector, comprising an *in vitro-synthesized* polyribonucleotide molecule, wherein the polyribonucleotide molecule comprises a pseudouridine or a modified nucleoside.

In another embodiment, the present ~~invention~~disclosure provides a double-stranded RNA (dsRNA) molecule containing, as part of its sequence, a pseudouridine or a modified nucleoside and further comprising an siRNA or shRNA. In another embodiment, the dsRNA molecule is greater

than 50 nucleotides in length. Each possibility represents a separate embodiment of the present ~~invention~~disclosure.

In another embodiment, the present ~~invention~~disclosure provides a method for inducing a mammalian cell to produce a recombinant protein, comprising contacting the mammalian cell with an *in vitro*-synthesized RNA molecule encoding the recombinant protein, the *in vitro*-synthesized RNA molecule comprising a pseudouridine or a modified nucleoside, thereby inducing a mammalian cell to produce a recombinant protein.

In another embodiment, the present ~~invention~~disclosure provides a method for treating anemia in a subject, comprising contacting a cell of the subject with an *in vitro-synthesized* RNA molecule, the *in vitro-synthesized* RNA molecule encoding erythropoietin, thereby treating anemia in a subject.

In another embodiment, the present ~~invention~~disclosure provides a method for treating a vasospasm in a subject, comprising contacting a cell of the subject with an *in vitro-synthesized* RNA molecule, the *in vitro-synthesized* RNA molecule encoding inducible nitric oxide synthase (iNOS), thereby treating a vasospasm in a subject.

In another embodiment, the present ~~invention~~disclosure provides a method for improving a survival rate of a cell in a subject, comprising contacting the cell with an *in vitro*-synthesized RNA molecule, the *in vitro-synthesized* RNA molecule encoding a heat shock protein, thereby improving a survival rate of a cell in a subject.

In another embodiment, the present ~~invention~~disclosure provides a method for decreasing an incidence of a restenosis of a blood vessel following a procedure that enlarges the blood vessel, comprising contacting a cell of the blood vessel with an *in vitro*-synthesized RNA molecule, the *in vitro*-synthesized RNA molecule encoding a heat shock protein, thereby decreasing an incidence of a restenosis in a subject.

In another embodiment, the present ~~invention~~disclosure provides a method for increasing a hair growth from a hair follicle is a scalp of a subject, comprising contacting a cell of the scalp with an *in vitro* synthesized RNA molecule, the *in vitro*-synthesized RNA molecule encoding a telomerase or an immunosuppressive protein, thereby increasing a hair growth from a hair follicle.

In another embodiment, the present ~~invention~~disclosure provides a method of inducing expression of an enzyme with antioxidant activity in a cell, comprising contacting the cell with an *in vitro*-

synthesized RNA molecule, the *in vitro-synthesized* RNA molecule encoding the enzyme, thereby inducing expression of an enzyme with antioxidant activity in a cell.

In another embodiment, the present ~~invention~~disclosure provides a method for treating cystic fibrosis in a subject, comprising contacting a cell of the subject with an *in vitro*-synthesized RNA molecule, the *in vitro*-synthesized RNA molecule encoding Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), thereby treating cystic fibrosis in a subject.

In another embodiment, the present ~~invention~~disclosure provides a method for treating an X-linked agammaglobulinemia in a subject, comprising contacting a cell of the subject with an *in vitro* synthesized RNA molecule, the *in vitro*-synthesized RNA molecule encoding a Bruton's tyrosine kinase, thereby treating an X-linked agammaglobulinemia.

In another embodiment, the present ~~invention~~disclosure provides a method for treating an adenosine deaminase severe combined immunodeficiency (ADA SCID) in a subject, comprising contacting a cell of the subject with an *in vitro-synthesized* RNA molecule, the *in vitro*-synthesized RNA molecule encoding an ADA, thereby treating an ADA SCID.

In another embodiment, the present ~~invention~~disclosure provides a method for producing a recombinant protein, comprising contacting an *in vitro* translation apparatus with an *in vitro-synthesized* polyribonucleotide, the *in vitro*-synthesized polyribonucleotide comprising a pseudouridine or a modified nucleoside, thereby producing a recombinant protein.

In another embodiment, the present ~~invention~~disclosure provides a method of synthesizing an *in vitro*-transcribed RNA molecule comprising a modified nucleotide with a pseudouridine modified nucleoside, comprising contacting an isolated polymerase with a mixture of unmodified nucleotides and the modified nucleotide.

In another embodiment, the present ~~invention~~disclosure provides an *in vitro* transcription apparatus, comprising: an unmodified nucleotide, a nucleotide containing a pseudouridine or a modified nucleoside, and a polymerase. In another embodiment, the present ~~invention~~disclosure provides an *in vitro* transcription kit, comprising: an unmodified nucleotide, a nucleotide containing a pseudouridine or a modified nucleoside, and a polymerase. Each possibility represents a separate embodiment of the present ~~invention~~disclosure.