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(71) Applicants: **BIONTECH AG** [DE/DE]; Hölderlinstrasse 8, 55131 Mainz (DE). **TRON - TRANSLATIONALE ONKOLOGIE AN DER UNIVERSITÄTSMEDIZIN DER JOHANNES GUTENBERG-UNIVERSITÄT MAINZ GEMEINNÜTZIGE GMBH** [DE/DE]; Langenbeckstrasse 1, Geb. 708, 55131 Mainz (DE). **UNIVERSITÄTSMEDIZIN DER JOHANNES GUTENBERG-UNIVERSITÄT MAINZ** [DE/DE]; Langenbeckstrasse 1, 55131 Mainz (DE).

(72) Inventors: **SAHIN, Ugur**; Philipp von Zabern Platz 1, 55116 Mainz (DE). **BEISSERT, Tim**; Edith-Stein-Weg 4, 64521 Gross-Gerau (DE). **POLEGANOV, Marco**; Bruchfeldstrasse 32, 60528 Frankfurt (DE). **HERZ, Stephanie**; Weinbergstrasse 7, 54317 Kasel (DE).

(74) Agents: **SCHNAPPAUF, Georg** et al.; ZSP Patentanwälte, Partnerschaftsgesellschaft, Radlkoferstrasse 2, 81373 München (DE).

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(54) Title: METHOD FOR CELLULAR RNA EXPRESSION

(57) Abstract: The present invention relates to expressing RNA in cells and, in particular, enhancing viability of cells in which RNA is to be expressed. Specifically, the present invention provides methods for expressing RNA in cells comprising the steps of preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling in the cells. Thus, preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling in the cells allows repetitive transfer of RNA into the cells.

METHOD FOR CELLULAR RNA EXPRESSION

TECHNICAL FIELD OF THE INVENTION

The present invention relates to expressing RNA in cells and, in particular, enhancing viability of cells in which RNA is to be expressed. The cells are preferably transfected with the RNA such as by repetitive transfection. Specifically, the present invention provides methods for expressing RNA in cells comprising the steps of preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling in the cells. Preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling in the cells allows stable expression of RNA in the cells, in particular, if cells are transfected repetitively with RNA. Alternatively or additionally, preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling enhances survival of the cells, in particular, if cells are transfected repetitively with RNA. Thus, preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling in the cells allows repetitive transfer of RNA into the cells.

BACKGROUND OF THE INVENTION

The advantages of using RNA as a kind of reversible gene therapy include transient expression and a non-transforming character. RNA does not need to enter the nucleus in order to be expressed and moreover cannot integrate into the host genome, thereby eliminating the risk of oncogenesis. Transfection rates attainable with RNA are relatively high, for many cell types even >90%, and therefore, there is no need for selection of transfected cells. Furthermore, the amounts of protein achieved correspond to those in physiological expression.

RNA has been described for as being useful in de-differentiating somatic cells into stem-like cells without generating embryos or fetuses. De-differentiation of somatic cells to cells having stem cell characteristics, in particular pluripotency, can be effected by introducing RNA encoding factors inducing the de-differentiation of somatic cells into the somatic cells (also termed reprogramming transcription factors (rTF)) and culturing the somatic cells allowing the cells to de-differentiate. After being de-differentiated, the cells could be induced to re-differentiate into the same or a different somatic cell type such as neuronal, hematopoietic, muscle, epithelial, and other cell types. Thus, such stem-like cells have medical applications

for treatment of degenerative diseases by "cell therapy" and may be utilized in novel therapeutic strategies in the treatment of cardiac, neurological, endocrinological, vascular, retinal, dermatological, muscular-skeletal disorders, and other diseases.

Furthermore, the use of RNA provides an attractive alternative to circumvent the potential risks of DNA based vaccines. As with DNA, transfer of RNA into cells can also induce both the cellular and humoral immune responses *in vivo*. In particular, two different strategies have been pursued for immunotherapy with *in vitro* transcribed RNA (IVT-RNA), which have both been successfully tested in various animal models. Either the RNA is directly injected into the patient by different immunization routes or cells are transfected with IVT-RNA using conventional transfection methods *in vitro* and then the transfected cells are administered to the patient. RNA may, for example, be translated and the expressed protein presented on the MHC molecules on the surface of the cells to elicit an immune response.

It has been attempted to stabilize IVT-RNA by various modifications in order to achieve higher and prolonged expression of transferred IVT-RNA. However, despite the success of RNA transfection-based strategies to express peptides and proteins in cells, there remain issues related to RNA stability, sustained expression of the encoded peptide or protein and cytotoxicity of the RNA. For example, it is known that exogenous single-stranded RNA activates defense mechanisms in mammalian cells. Furthermore, reprogramming of somatic cells into induced pluripotent stem cells (iPS) requires the continuous expression of reprogramming transcription factors (rTF) and thus, the delivery has to be performed repetitively to assure constant expression of the rTF. However, as demonstrated herein, repetitive RNA-based gene transfer is accompanied with an induction of the IFN-response which hinders the continuous expression of rTF when delivered as mRNA and therefore successful RNA-based reprogramming.

SUMMARY OF THE INVENTION

The invention relates to a method for expressing RNA in a cell comprising the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling.

In one embodiment, the RNA is or has been introduced into the cell such as by electroporation or lipofection. In one embodiment, the RNA is or has been introduced into the cell repetitively.

In one embodiment, the RNA is *in vitro* transcribed RNA.

In one embodiment, preventing engagement of IFN receptor by extracellular IFN inhibits autocrine and/or paracrine IFN functions. In one embodiment, preventing engagement of IFN receptor by extracellular IFN comprises providing a binding agent for extracellular IFN such as a viral binding agent for extracellular IFN. In one embodiment, the viral binding agent for extracellular IFN is a viral interferon receptor. In one embodiment, the viral binding agent for extracellular IFN is vaccinia virus B18R. In one embodiment, the viral binding agent for extracellular IFN is provided to the cell in the form of a nucleic acid encoding the binding agent, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the cell together with the RNA which is to be expressed in the cell.

In one embodiment, the intracellular IFN signalling if not inhibited according to the invention results in inhibition of translation and/or RNA degradation. In one embodiment, inhibiting intracellular IFN signalling comprises inhibiting one or more IFN-inducible antivirally active effector proteins. In one embodiment, the IFN-inducible antivirally active effector protein is selected from the group consisting of RNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (OAS) and RNaseL.

In one embodiment, inhibiting intracellular IFN signalling comprises inhibiting the PKR-dependent pathway and/or the OAS-dependent pathway.

In one embodiment, inhibiting the PKR-dependent pathway comprises inhibiting eIF2-alpha phosphorylation. In one embodiment, inhibiting eIF2-alpha phosphorylation comprises inhibiting PKR and/or providing a pseudosubstrate mimicking eIF2-alpha. In one embodiment, the pseudosubstrate mimicking eIF2-alpha is a viral pseudosubstrate mimicking eIF2-alpha. In one embodiment, the viral pseudosubstrate mimicking eIF2-alpha is vaccinia virus K3. In one embodiment, the viral pseudosubstrate mimicking eIF2-alpha is provided to the cell in the form of a nucleic acid encoding the viral pseudosubstrate, wherein the nucleic

acid is preferably RNA which preferably is or has been introduced into the cell together with the RNA which is to be expressed in the cell.

In one embodiment, inhibiting PKR comprises treating the cell with at least one PKR inhibitor. In one embodiment, the PKR inhibitor inhibits RNA-induced PKR autophosphorylation. In one embodiment, the PKR inhibitor is an ATP-binding site directed inhibitor of PKR. In one embodiment, the PKR inhibitor is an imidazolo-oxindole compound. In one embodiment, the PKR inhibitor is 6,8-dihydro-8-(1H-imidazol-5-ylmethylene)-7H-pyrrolo[2,3-g]benzothiazol-7-one and/or 2-aminopurine. In one embodiment, the PKR inhibitor is a viral inhibitor of PKR. In one embodiment, the viral inhibitor of PKR is vaccinia virus E3. In one embodiment, the viral inhibitor of PKR is provided to the cell in the form of a nucleic acid encoding the inhibitor, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the cell together with the RNA which is to be expressed in the cell. In one embodiment, inhibiting PKR comprises silencing expression of the PKR gene.

In one embodiment, inhibiting the OAS-dependent pathway comprises inhibiting activation of RNaseL. In one embodiment, inhibiting the OAS-dependent pathway comprises inhibiting OAS.

In one embodiment, inhibiting OAS comprises treating the cell with at least one OAS inhibitor. In one embodiment, the OAS inhibitor is a viral inhibitor of OAS. In one embodiment, the viral inhibitor of OAS is vaccinia virus E3. In one embodiment, the viral inhibitor of OAS is provided to the cell in the form of a nucleic acid encoding the inhibitor, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the cell together with the RNA which is to be expressed in the cell.

In one embodiment, the RNA expressed in the cell is not modified by pseudouridine and/or 5-methylcytidine.

In one embodiment, the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling result in an enhancement of stability and/or an enhancement of expression of the RNA in the cell compared to the situation where engagement of IFN receptor by extracellular IFN is not prevented and/or intracellular IFN

signalling is not inhibited. In one embodiment, the enhancement of expression of the RNA in the cell preferably comprises an increase in the level of expression and/or an increase in the duration of expression of the RNA in the cell.

In one embodiment, the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling result in an enhancement of cell viability compared to the situation where engagement of IFN receptor by extracellular IFN is not prevented and/or intracellular IFN signalling is not inhibited.

In one embodiment, the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling comprise treating the cell with (i) vaccinia virus B18R and (ii) vaccinia virus E3 or vaccinia virus K3, or both. In one embodiment, the vaccinia virus B18R, vaccinia virus E3 and/or vaccinia virus K3 are provided to the cell in the form of nucleic acid encoding the vaccinia virus B18R, vaccinia virus E3 and/or vaccinia virus K3, either on the same or on two or more different nucleic acid molecules, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the cell together with the RNA which is to be expressed in the cell.

In one embodiment, the cell is a cell having a barrier function. In one embodiment, the cell is a fibroblast, a keratinocyte, an epithelial cell, or an endothelial cell, wherein the endothelial cell preferably is an endothelial cell of the heart, an endothelial cell of the lung, or an umbilical vein endothelial cell. Preferably, the cell is a human cell.

The invention also relates to the use of (i) means which are suitable for preventing engagement of IFN receptor by extracellular IFN and (ii) means which are suitable for inhibiting intracellular IFN signalling, such as the means described herein, for treating a cell in which RNA is to be expressed. Various embodiments of this method and the means useful therein are described above.

The invention also relates to a method for providing cells having stem cell characteristics comprising the steps of (i) providing a cell population comprising somatic cells, (ii) preventing engagement of IFN receptor of the somatic cells by extracellular IFN, (iii) inhibiting intracellular IFN signalling in the somatic cells, (iv) introducing RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells

having stem cell characteristics into the somatic cells, and (v) allowing the development of cells having stem cell characteristics.

In one embodiment, the method further comprises introducing into the somatic cells miRNA enhancing reprogramming of the somatic cells to cells having stem cell characteristics.

In one embodiment, the one or more factors comprise OCT4 and SOX2. The one or more factors may further comprise KLF4 and/or c-MYC and/or NANOG and/or LIN28. In one embodiment, the one or more factors comprise OCT4, SOX2, KLF4 and c-MYC and may further comprise LIN28 and optionally NANOG. In one embodiment, the one or more factors comprise OCT4, SOX2, NANOG and LIN28.

In one embodiment, the method further comprises the step of culturing the somatic cells in the presence of at least one histone deacetylase inhibitor, wherein the at least one histone deacetylase inhibitor preferably comprises valproic acid, sodium butyrate, trichostatin A and/or scriptaid.

In one embodiment, step (v) comprises culturing the somatic cells under embryonic stem cell culture conditions.

In one embodiment, the stem cell characteristics comprise an embryonic stem cell morphology.

In one embodiment, the cells having stem cell characteristics have normal karyotypes, express telomerase activity, express cell surface markers that are characteristic for embryonic stem cells and/or express genes that are characteristic for embryonic stem cells.

In one embodiment, the cells having stem cell characteristics exhibit a pluripotent state.

In one embodiment, the cells having stem cell characteristics have the developmental potential to differentiate into advanced derivatives of all three primary germ layers.

In one embodiment, the somatic cells are fibroblasts such as lung fibroblasts, foreskin fibroblasts or dermal fibroblasts. Preferably, the somatic cells are human cells.

In one embodiment, the RNA is introduced into the somatic cells by electroporation or lipofection. In one embodiment, the RNA is introduced into the somatic cells repetitively.

In one embodiment, the RNA is *in vitro* transcribed RNA.

In one embodiment, preventing engagement of IFN receptor by extracellular IFN inhibits autocrine and/or paracrine IFN functions. In one embodiment, preventing engagement of IFN receptor by extracellular IFN comprises providing a binding agent for extracellular IFN such as a viral binding agent for extracellular IFN. In one embodiment, the viral binding agent for extracellular IFN is a viral interferon receptor. In one embodiment, the viral binding agent for extracellular IFN is vaccinia virus B18R. In one embodiment, the viral binding agent for extracellular IFN is provided to the somatic cells in the form of a nucleic acid encoding the binding agent, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the somatic cells together with the RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics.

In one embodiment, the intracellular IFN signalling if not inhibited according to the invention results in inhibition of translation and/or RNA degradation. In one embodiment, inhibiting intracellular IFN signalling comprises inhibiting one or more IFN-inducible antivirally active effector proteins. In one embodiment, the IFN-inducible antivirally active effector protein is selected from the group consisting of RNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (OAS) and RNaseL.

In one embodiment, inhibiting intracellular IFN signalling comprises inhibiting the PKR-dependent pathway and/or the OAS-dependent pathway.

In one embodiment, inhibiting the PKR-dependent pathway comprises inhibiting eIF2-alpha phosphorylation. In one embodiment, inhibiting eIF2-alpha phosphorylation comprises inhibiting PKR and/or providing a pseudosubstrate mimicking eIF2-alpha. In one embodiment, the pseudosubstrate mimicking eIF2-alpha is a viral pseudosubstrate mimicking eIF2-alpha. In one embodiment, the viral pseudosubstrate mimicking eIF2-alpha is vaccinia virus K3. In one embodiment, the viral pseudosubstrate mimicking eIF2-alpha is provided to

the somatic cells in the form of a nucleic acid encoding the viral pseudosubstrate, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the somatic cells together with the RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics.

In one embodiment, inhibiting PKR comprises treating the cell with at least one PKR inhibitor. In one embodiment, the PKR inhibitor inhibits RNA-induced PKR autophosphorylation. In one embodiment, the PKR inhibitor is an ATP-binding site directed inhibitor of PKR. In one embodiment, the PKR inhibitor is an imidazolo-oxindole compound. In one embodiment, the PKR inhibitor is 6,8-dihydro-8-(1H-imidazol-5-ylmethylene)-7H-pyrrolo[2,3-g]benzothiazol-7-one and/or 2-aminopurine. In one embodiment, the PKR inhibitor is a viral inhibitor of PKR. In one embodiment, the viral inhibitor of PKR is vaccinia virus E3. In one embodiment, the viral inhibitor of PKR is provided to the somatic cells in the form of a nucleic acid encoding the inhibitor, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the somatic cells together with the RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics. In one embodiment, inhibiting PKR comprises silencing expression of the PKR gene.

In one embodiment, inhibiting the OAS-dependent pathway comprises inhibiting activation of RNaseL. In one embodiment, inhibiting the OAS-dependent pathway comprises inhibiting OAS.

In one embodiment, inhibiting OAS comprises treating the somatic cells with at least one OAS inhibitor. In one embodiment, the OAS inhibitor is a viral inhibitor of OAS. In one embodiment, the viral inhibitor of OAS is vaccinia virus E3. In one embodiment, the viral inhibitor of OAS is provided to the somatic cells in the form of a nucleic acid encoding the inhibitor, wherein the nucleic acid is preferably RNA which preferably is or has been introduced into the somatic cells together with the RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics.

In one embodiment, the RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics is not modified by pseudouridine and/or 5-methylcytidine.

In one embodiment, the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling result in an enhancement of stability and/or an enhancement of expression of the RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics in the somatic cells compared to the situation where engagement of IFN receptor by extracellular IFN is not prevented and/or intracellular IFN signalling is not inhibited. In one embodiment, the enhancement of expression of the RNA in the somatic cells preferably comprises an increase in the level of expression and/or an increase in the duration of expression of the RNA in the somatic cells.

In one embodiment, the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling result in an enhancement of cell viability compared to the situation where engagement of IFN receptor by extracellular IFN is not prevented and/or intracellular IFN signalling is not inhibited.

In one embodiment, the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling comprise treating the somatic cells with (i) vaccinia virus B18R and (ii) vaccinia virus E3 or vaccinia virus K3, or both. In one embodiment, the vaccinia virus B18R, vaccinia virus E3 and/or vaccinia virus K3 are provided to the somatic cells in the form of nucleic acid encoding the vaccinia virus B18R, vaccinia virus E3 and/or vaccinia virus K3, either on the same or on two or more different nucleic acid molecules, wherein the nucleic acid is preferably RNA which preferably is introduced or has been introduced into the somatic cells together with the RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics.

The invention also relates to a method for providing differentiated cell types comprising the steps of (i) providing cells having stem cell characteristics using the method for providing cells having stem cell characteristics according to the invention, and (ii) culturing the cells

having stem cell characteristics under conditions that induce or direct partial or complete differentiation to a differentiated cell type.

The invention also relates to a composition comprising (i) an agent useful for preventing engagement of IFN receptor by extracellular IFN and (ii) an agent useful for inhibiting intracellular IFN signalling. Furthermore, the invention also relates to a kit comprising the composition of the invention. Various embodiments of the composition or kit of the invention are described above for the methods of the invention. In one embodiment, the composition or kit of the invention is useful in the methods of the invention.

In one embodiment, the composition or kit of the invention comprises RNA to be introduced into a cell for expression, e.g. RNA capable of expressing one or more factors allowing the reprogramming of somatic cells to cells having stem cell characteristics as described above.

In one embodiment, an agent useful for preventing engagement of IFN receptor by extracellular IFN inhibits autocrine and/or paracrine IFN functions. In one embodiment, an agent useful for preventing engagement of IFN receptor by extracellular IFN comprises a binding agent for extracellular IFN such as a viral binding agent for extracellular IFN. In one embodiment, the viral binding agent for extracellular IFN is a viral interferon receptor. In one embodiment, the viral binding agent for extracellular IFN is vaccinia virus B18R. In one embodiment, the viral binding agent for extracellular IFN is present in the form of a nucleic acid encoding the binding agent, wherein the nucleic acid is preferably RNA.

In one embodiment, an agent useful for inhibiting intracellular IFN signalling comprises an agent inhibiting one or more IFN-inducible antivirally active effector proteins. In one embodiment, the IFN-inducible antivirally active effector protein is selected from the group consisting of RNA-dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (OAS) and RNaseL.

In one embodiment, an agent useful for inhibiting intracellular IFN signalling comprises an agent useful for inhibiting the PKR-dependent pathway and/or the OAS-dependent pathway.

In one embodiment, an agent useful for inhibiting the PKR-dependent pathway comprises an agent useful for inhibiting eIF2-alpha phosphorylation. In one embodiment, an agent useful

for inhibiting eIF2-alpha phosphorylation comprises an agent useful for inhibiting PKR and/or a pseudosubstrate mimicking eIF2-alpha. In one embodiment, the pseudosubstrate mimicking eIF2-alpha is a viral pseudosubstrate mimicking eIF2-alpha. In one embodiment, the viral pseudosubstrate mimicking eIF2-alpha is vaccinia virus K3. In one embodiment, the viral pseudosubstrate mimicking eIF2-alpha is present in the form of a nucleic acid encoding the viral pseudosubstrate, wherein the nucleic acid is preferably RNA.

In one embodiment, an agent useful for inhibiting PKR comprises at least one PKR inhibitor. In one embodiment, the PKR inhibitor inhibits RNA-induced PKR autophosphorylation. In one embodiment, the PKR inhibitor is an ATP-binding site directed inhibitor of PKR. In one embodiment, the PKR inhibitor is an imidazolo-oxindole compound. In one embodiment, the PKR inhibitor is 6,8-dihydro-8-(1H-imidazol-5-ylmethylene)-7H-pyrrolo[2,3-g]benzothiazol-7-one and/or 2-aminopurine. In one embodiment, the PKR inhibitor is a viral inhibitor of PKR. In one embodiment, the viral inhibitor of PKR is vaccinia virus E3. In one embodiment, the viral inhibitor of PKR is present in the form of a nucleic acid encoding the inhibitor, wherein the nucleic acid is preferably RNA. In one embodiment, an agent useful for inhibiting PKR comprises an agent useful for silencing expression of the PKR gene.

In one embodiment, an agent useful for inhibiting the OAS-dependent pathway comprises an agent useful for inhibiting activation of RNaseL. In one embodiment, an agent useful for inhibiting the OAS-dependent pathway comprises an agent useful for inhibiting OAS.

In one embodiment, an agent useful for inhibiting OAS comprises at least one OAS inhibitor. In one embodiment, the OAS inhibitor is a viral inhibitor of OAS. In one embodiment, the viral inhibitor of OAS is vaccinia virus E3. In one embodiment, the viral inhibitor of OAS is present in the form of a nucleic acid encoding the inhibitor, wherein the nucleic acid is preferably RNA.

In one embodiment, an agent useful for preventing engagement of IFN receptor by extracellular IFN and an agent useful for inhibiting intracellular IFN signalling comprise (i) vaccinia virus B18R and (ii) vaccinia virus E3 or vaccinia virus K3, or both. In one embodiment, the vaccinia virus B18R, vaccinia virus E3 and/or vaccinia virus K3 are present in the form of nucleic acid encoding the vaccinia virus B18R, vaccinia virus E3 and/or

vaccinia virus K3, either on the same or on two or more different nucleic acid molecules, wherein the nucleic acid is preferably RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Viral escape mechanism

Viruses have evolved many escape mechanism that are mediated by viral proteins or viral nucleic acids. RNA that codes for these viral escape proteins can easily be co-transfered with RNA coding for rTF. Antagonistic protein E3 (Vaccinia virus) acts on PKR & OAS, K3 (Vaccinia virus) acts on eIF2a and B18R (Vaccinia virus) acts on IFN. Whereas E3 and K3 are acting intracellular, B18R protein coded by IVT-RNA is secreted from the cell where it binds extracellular type I IFNs and prevents engagement of IFN receptors.

Fig. 2: Repetitive transfer of IVT-RNA (Reprogramming-TF)

(A) CCD1079Sk fibroblasts were electroporated as indicated in the side panel either with 15µg or 5µg of each *in vitro* transcribed (IVT)-RNA encoding the transcription factors OCT4 (O), SOX2 (S), KLF4 (K) and cMYC (M) and cultivated in human embryonic stem (ES) cell medium. Electroporations were performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. At the indicated time points, 10% of the cells were removed from the cultures prior to subsequent electroporation, total RNA was isolated and mRNA-expression of the human ES-marker genes OCT4 (endogenous), TERT, GDF3 and DPPA4 was quantified by qRT-PCR. **(B)** CCD1079Sk fibroblasts were electroporated as indicated in the side panel with 15µg of each IVT-RNA encoding the transcription factors OSKM and cultivated in human ES cell medium. Electroporations were performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. At the indicated time points remaining cells were counted and survival rate in relation to the starting cells was calculated. **(C)** CCD1079Sk fibroblasts were electroporated with 1 µg IVT RNA encoding for firefly luciferase (Luc) and 5 µg IVT RNA encoding for green fluorescent protein (GFP). Electroporations were performed in 2 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. 24h post electroporation, cells were pelleted, total RNA was isolated and mRNA-expression of Interferon (IFN)-a and –b was quantified by qRT-PCR. **(D)** CCD1079Sk fibroblasts were electroporated with 33,4 µg IVT RNA encoding reprogramming mixture (29,5µg rTF (OSKM NANOG (N) LIN28 (L) (1:1:1:1:1:1)), 1,3µg SV40 largeT antigen (lgT), 1,3µg HTLV E6 and 1,25µg GFP). Electroporations were

performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. 48h post electroporation, cells were pelleted, total RNA was isolated and mRNA-expression of the IFN-response genes OAS1, OAS2, MX1, IFITM1 and IRF9 was quantified by qRT-PCR. **(E)** CCD1079Sk fibroblasts were electroporated once with the indicated amounts of IVT-RNA encoding the reporter genes Luc, GFP or the Protein Kinase R (PKR) wild type. Electroporations were performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. Cells were lysed 24h post electroporation and expression and phosphorylation status of the PKR target eukaryotic initiation factor 2a (eIF2a) was monitored by Western Blotting using specific antibodies.

Fig. 3: Use of E3, K3 and B18R in RNA-based gene transfer

(AB) CCD1079Sk fibroblasts were plated into 6 wells (100,000 cells/well) and lipofected the next day using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures was thereby composed of 0.8µg GFP with 0.2µg of each B18R, E3 or K3 (as indicated). IVT-RNA encoding for Luc was used to sum up the mixtures to 1.4µg. Lipofections were performed according to the manufacturers instructions and cells were harvested 48h post transfection. 20% of the cells were used for analysis of GFP expression by FACS **(B)**, whereas the rest of the cells were pelleted, total RNA was isolated and mRNA-expression of IFNb and OAS1 was quantified by qRT-PCR **(A)**. **(C)** CCD1079Sk fibroblasts were plated into 6 wells (100.000 cells/well) and lipofected the next four consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixture was thereby composed of 0.8µg GFP with 0.2µg of each B18R, E3 or K3 (as indicated). IVT-RNA encoding for Luc was used to sum up the mixture to 1.4µg total IVT-RNA. As a control, 1.4µg modified (mod.) IVT-RNA encoding for Luc (0.6µg) and GFP (0.8µg) was used. These RNAs were composed of 100% pseudouridine (psi) and 100% 5-methylcytidine (5mC) instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, cell viability was assayed using the Cell Proliferation Kit II (Roche) and normalized to the mock transfected cells.

Fig. 4: Use of E3, K3 and B18R in RNA-based gene transfer for reprogramming

CCD1079Sk fibroblasts were plated into 6 wells (80,000 cells/well) and lipofected the next four consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures were thereby composed of 0.8µg unmodified GFP or 0.8µg OSKMNL (1:1:1:1:1:1)

either unmodified or modified and either with 0.2µg of each B18R, E3 and K3 unmodified or modified. If necessary IVT-RNA encoding for Luc was used to sum up the mixture to 1.4µg total IVT-RNA. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, cell viability was assayed using the Cell Proliferation Kit II (Roche) with normalization to mock transfected cells (**A**) and by microscopy (**B**). After that, cells were pelleted, total RNA was isolated and mRNA-expression of IFNb and OAS1 was quantified by qRT-PCR (**C**).

Fig. 5: Translation of rTF after repetitive lipofection in the presence of E3, K3 and B18R

CCD1079Sk fibroblasts were plated into 6 wells (100,000 cells/well) and lipofected the next three consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures was thereby composed of 0.2µg GFP with 0.6µg OCT4 or SOX2 or NANOG either unmodified or modified and 0.2µg of each B18R, E3 and K3 (EKB) either unmodified or modified. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, intracellular expression of OSN was monitored by FACS analysis using the human pluripotent stem cell transcription factor analysis kit (BD 560589).

Fig. 6: Reprogramming of HFF using rTF and microRNA in the presence of EKB

HFF fibroblasts (System Bioscience) were plated into 6 wells (100,000 cells/well) and lipofected 5 times a week (Monday to Friday) for two weeks using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT-RNA (**A**). The IVT-RNA mixtures were thereby composed of 0.8µg unmodified GFP or 0.8µg OSKMNL (1:1:1:1:1:1) either unmodified or modified with either 0.2µg of each B18R, E3 and K3 (EKB) either unmodified or modified and 0.4µg of a miRNA mixture composed of miRNAs 302a-d and 367 [0.4µM each]. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections in stem cell media (Nutristem media, Stemgent) were performed according to the manufacturers instructions. On day 6 and day 13, cells were pelleted, total RNA was isolated and mRNA-expression of the human ES-marker TERT, DPPA4, GDF3, LIN28 (endogenous) and REX1 was quantified by qRT-PCR (**B**). Colony growth was observed by microscopy (**C**) and for further analysis, colonies were

stained for the ES surface marker TRA-1-60 using the StainAlive TRA-1-60 antibody (Stemgent) following the manufacturers instructions (**D**).

Fig. 7: Reprogramming of HFF using rTF and microRNA in the presence of EKB (splitting 1:8)

HFF fibroblasts (System Bioscience) were plated into 6 wells (100,000 cells/well) and lipofected 5 times a week (Monday to Friday) for two weeks using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT-RNA (**A**). The IVT-RNA mixtures were thereby composed of 0.8µg OSKMNL (1:1:1:1:1:1) either unmodified or modified with either 0.2µg of each B18R, E3 and K3 (EKB) unmodified or modified and 0.4µg of a miRNA mixture composed of miRNAs 302a-d and 367 [0.4µM each]. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections in stem cell media (Nutristem media, Stemgent) were performed according to the manufacturers instructions. On day 5 and day 12, cells were pelleted, total RNA was isolated and mRNA-expression of the human ES-marker TERT, DPPA4, GDF3, LIN28 (endogenous) and REX1 was quantified by qRT-PCR (**B**). Colony growth was observed by microscopy and for further analysis, colonies were stained for the ES surface marker TRA-1-60 using the StainAlive TRA-1-60 antibody (Stemgent) **(C)** or for the activity of alkaline phosphatase (Vector Red staining kit) following the manufacturers instructions (**D**).

Fig. 8: Titration of EKB

HFF fibroblasts (System Bioscience) were plated into 6 wells (100,000 cells/well) and lipofected the next four consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures were thereby composed of 0.8µg unmodified OSKMNL (1:1:1:1:1:1) with variable amounts of unmodfied B18R, E3 and K3 as indicated. IVT-RNA encoding for Luc was used to sum up the mixture to 1.4µg total IVT-RNA. According to the reprogramming experiments 0.4µg of a miRNA mixture composed of miRNAs 302a-d and 367 [0.4µM each] was added to the samples. As a control, 1.4µg modified (mod.) IVT-RNA encoding for Luc (0.6µg) and OSKMNL (0.8µg; 1:1:1:1:1:1) was used. These RNAs were composed of 100% psi and 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, cell viability was assayed using the

Cell Proliferation Kit II (Roche) (**A**). After that, cells were pelleted, total RNA was isolated and mRNA-expression of IFNb and OAS1 was quantified by qRT-PCR (**B**).

Fig. 9: Effect E3 and K3 alone

CCD1079SK fibroblasts were electroporated with IVT RNA encoding Luc (1 µg), GFP (5 µg) and 3 µg of E3 or K3 or both as indicated. Electroporations were performed in 2 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. (**A**) 10000 cells/well were plated in duplicates into 96-well-plates. Luciferase activity was measured at the indicated time points after electroporation using the Bright Glo Luciferase Assay System (Promega). Mean values of the duplicates are given. (**B**) 300000 cells/well were plated into 6-well-plates and 24h post electroporation, cells were pelleted, total RNA was isolated and mRNA-expression of OAS1 and IFN-b was analyzed by RT-PCR and in case of IFN-b quantified using the Quanti Tect SYBR Green PCR Kit.

DETAILED DESCRIPTION OF THE INVENTION

Although the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodologies, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

In the following, the elements of the present invention will be described. These elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements in this application should be considered disclosed by the description of the present application unless the context indicates otherwise. For example, if in a preferred embodiment RNA comprises a poly(A)-tail consisting of 120 nucleotides and in another preferred embodiment the RNA molecule comprises a 5'-cap analog, then in a preferred embodiment, the RNA comprises the poly(A)-tail consisting of 120 nucleotides and the 5'-cap analog.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", H.G.W. Leuenberger, B. Nagel, and H. Kölbl, Eds., Helvetica Chimica Acta, CH-4010 Basel, Switzerland, (1995).

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, and recombinant DNA techniques which are explained in the literature in the field (cf., e.g., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, J. Sambrook et al. eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be

understood to imply the inclusion of a stated member, integer or step or group of members, integers or steps but not the exclusion of any other member, integer or step or group of members, integers or steps although in some embodiments such other member, integer or step or group of members, integers or steps may be excluded, i.e. the subject-matter consists in the inclusion of a stated member, integer or step or group of members, integers or steps. The terms "a" and "an" and "the" and similar reference used in the context of describing the invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as"), provided herein is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether *supra* or *infra*, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Terms such as "preventing", "reducing" or "inhibiting" relate to the ability to cause an overall decrease, preferably of 5% or greater, 10% or greater, 20% or greater, more preferably of 50% or greater, and most preferably of 75% or greater, in the level. This also includes a complete or essentially complete decrease, i.e. a decrease to zero or essentially to zero.

Terms such as "increasing", "enhancing", or "prolonging" preferably relate to an increase, enhancement, or prolongation by about at least 10%, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably at least 80%, preferably at least 100%, preferably at least 200% and in particular at least 300%. These terms may also

relate to an increase, enhancement, or prolongation from zero or a non-measurable or non-detectable level to a level of more than zero or a level which is measurable or detectable.

The term "recombinant" in the context of the present invention means "made through genetic engineering". Preferably, a "recombinant entity" such as a recombinant protein in the context of the present invention is not occurring naturally, and preferably is a result of a combination of entities such as amino acid or nucleic acid sequences which are not combined in nature. For example, a recombinant protein in the context of the present invention may contain several amino acid sequences derived from different proteins fused together, e.g., by peptide bonds.

The term "naturally occurring" as used herein refers to the fact that an object can be found in nature. For example, a protein or nucleic acid that is present in an organism (including viruses) and can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring.

A nucleic acid is according to the invention preferably deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), more preferably RNA, most preferably *in vitro* transcribed RNA (IVT RNA). Nucleic acids include according to the invention genomic DNA, cDNA, mRNA, recombinantly produced and chemically synthesized molecules. According to the invention, a nucleic acid may be present as a single-stranded or double-stranded and linear or covalently circularly closed molecule. A nucleic acid can, according to the invention, be isolated. The term "isolated nucleic acid" means, according to the invention, that the nucleic acid (i) was amplified *in vitro*, for example via polymerase chain reaction (PCR), (ii) was produced recombinantly by cloning, (iii) was purified, for example, by cleavage and separation by gel electrophoresis, or (iv) was synthesized, for example, by chemical synthesis. A nucleic can be employed for introduction into, i.e. transfection of, cells, in particular, in the form of RNA which can be prepared by *in vitro* transcription from a DNA template. The RNA can moreover be modified before application by stabilizing sequences, capping, and polyadenylation.

As a nucleic acid, in particular RNA, for expression of more than one peptide or protein, either of a nucleic acid type in which the different peptides or proteins are present in different

nucleic acid molecules or a nucleic acid type in which the peptides or proteins are present in the same nucleic acid molecule can be used.

In the context of the present invention, the term "RNA" relates to a molecule which comprises ribonucleotide residues and preferably being entirely or substantially composed of ribonucleotide residues. "Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β-D-ribofuranosyl group. The term "RNA" comprises double-stranded RNA, single-stranded RNA, isolated RNA such as partially or completely purified RNA, essentially pure RNA, synthetic RNA, and recombinantly generated RNA such as modified RNA which differs from naturally occurring RNA by 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.

According to the present invention, the term "RNA" includes and preferably relates to "mRNA". The term "mRNA" means "messenger-RNA" and relates to a "transcript" which is generated by using a DNA template and encodes a peptide or protein. Typically, an mRNA comprises a 5'-UTR, a protein coding region, and a 3'-UTR. mRNA only possesses limited half-life in cells and *in vitro*. In the context of the present invention, mRNA may be generated by *in vitro* transcription from a DNA template. The *in vitro* transcription methodology is known to the skilled person. For example, there is a variety of *in vitro* transcription kits commercially available.

According to the invention, the stability and translation efficiency of RNA may be modified as required. For example, RNA may be stabilized and its translation increased by one or more modifications having a stabilizing effects and/or increasing translation efficiency of RNA. Such modifications are described, for example, in PCT/EP2006/009448 incorporated herein by reference. In order to increase expression of the RNA used according to the present invention, it may be modified within the coding region, i.e. the sequence encoding the expressed peptide or protein, preferably without altering the sequence of the expressed

peptide or protein, so as to increase the GC-content to increase mRNA stability and to perform a codon optimization and, thus, enhance translation in cells.

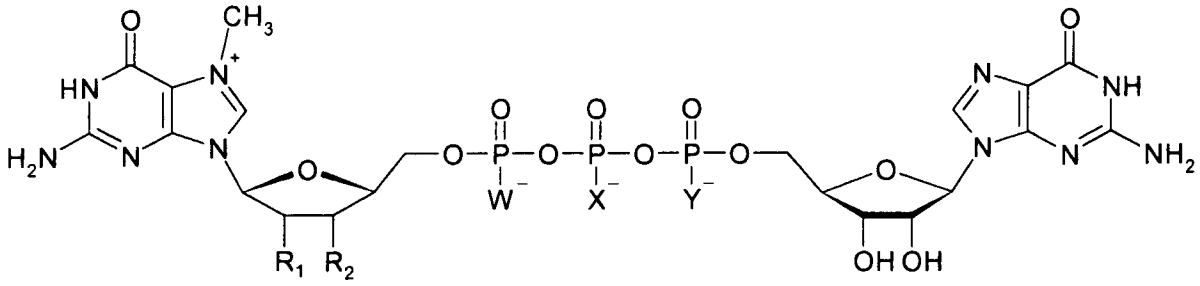
The term "modification" in the context of the RNA used in the present invention includes any modification of an RNA which is not naturally present in said RNA.

In one embodiment of the invention, the RNA used according to the invention does not have uncapped 5'-triphosphates. Removal of such uncapped 5'-triphosphates can be achieved by treating RNA with a phosphatase.

The RNA according to the invention may have modified ribonucleotides in order to increase its stability and/or decrease cytotoxicity. For example, in one embodiment, in the RNA used according to the invention 5-methylcytidine is substituted partially or completely, preferably completely, for cytidine. Alternatively or additionally, in one embodiment, in the RNA used according to the invention pseudouridine is substituted partially or completely, preferably completely, for uridine.

In one embodiment, the term "modification" relates to providing an RNA with a 5'-cap or 5'-cap analog. The term "5'-cap" refers to a cap structure found on the 5'-end of an mRNA molecule and generally consists of a guanosine nucleotide connected to the mRNA via an unusual 5' to 5' triphosphate linkage. In one embodiment, this guanosine is methylated at the 7-position. The term "conventional 5'-cap" refers to a naturally occurring RNA 5'-cap, preferably to the 7-methylguanosine cap (m^7G). In the context of the present invention, the term "5'-cap" includes a 5'-cap analog that resembles the RNA cap structure and is modified to possess the ability to stabilize RNA and/or enhance translation of RNA if attached thereto, preferably *in vivo* and/or in a cell.

Preferably, the 5' end of the RNA includes a Cap structure having the following general formula:



wherein R_1 and R_2 are independently hydroxy or methoxy and W^-, X^- and Y^- are independently oxygen, sulfur, selenium, or BH_3. In a preferred embodiment, R_1 and R_2 are hydroxy and W^-, X^- and Y^- are oxygen. In a further preferred embodiment, one of R_1 and R_2, preferably R_1 is hydroxy and the other is methoxy and W^-, X^- and Y^- are oxygen. In a further preferred embodiment, R_1 and R_2 are hydroxy and one of W^-, X^- and Y^-, preferably X^- is sulfur, selenium, or BH_3, preferably sulfur, while the other are oxygen. In a further preferred embodiment, one of R_1 and R_2, preferably R_2 is hydroxy and the other is methoxy and one of W^-, X^- and Y^-, preferably X^- is sulfur, selenium, or BH_3, preferably sulfur while the other are oxygen.

In the above formula, the nucleotide on the right hand side is connected to the RNA chain through its 3' group.

Those Cap structures wherein at least one of W^-, X^- and Y^- is sulfur, i.e. which have a phosphorothioate moiety, exist in different diastereoisomeric forms all of which are encompassed herein. Furthermore, the present invention encompasses all tautomers and stereoisomers of the above formula.

For example, the Cap structure having the above structure wherein R_1 is methoxy, R_2 is hydroxy, X^- is sulfur and W^- and Y^- are oxygen exists in two diastereoisomeric forms (Rp and Sp). These can be resolved by reverse phase HPLC and are named D1 and D2 according to their elution order from the reverse phase HPLC column. According to the invention, the D1 isomer of $m_2^{7,2'-O}GppSpG$ is particularly preferred.

Providing an RNA with a 5'-cap or 5'-cap analog may be achieved by *in vitro* transcription of a DNA template in presence of said 5'-cap or 5'-cap analog, wherein said 5'-cap is co-transcriptionally incorporated into the generated RNA strand, or the RNA may be generated,

for example, by *in vitro* transcription, and the 5'-cap may be attached to the RNA post-transcriptionally using capping enzymes, for example, capping enzymes of vaccinia virus.

The RNA may comprise further modifications. For example, a further modification of the RNA used in the present invention may be an extension or truncation of the naturally occurring poly(A) tail or an alteration of the 5'- or 3'-untranslated regions (UTR) such as introduction of a UTR which is not related to the coding region of said RNA, for example, the exchange of the existing 3'-UTR with or the insertion of one or more, preferably two copies of a 3'-UTR derived from a globin gene, such as alpha2-globin, alpha1-globin, beta-globin, preferably beta-globin, more preferably human beta-globin.

RNA having an unmasked poly-A sequence is translated more efficiently than RNA having a masked poly-A sequence. The term "poly(A) tail" or "poly-A sequence" relates to a sequence of adenyl (A) residues which typically is located on the 3'-end of a RNA molecule and "unmasked poly-A sequence" means that the poly-A sequence at the 3' end of an RNA molecule ends with an A of the poly-A sequence and is not followed by nucleotides other than A located at the 3' end, i.e. downstream, of the poly-A sequence. Furthermore, a long poly-A sequence of about 120 base pairs results in an optimal transcript stability and translation efficiency of RNA.

Therefore, in order to increase stability and/or expression of the RNA used according to the present invention, it may be modified so as to be present in conjunction with a poly-A sequence, preferably having a length of 10 to 500, more preferably 30 to 300, even more preferably 65 to 200 and especially 100 to 150 adenosine residues. In an especially preferred embodiment the poly-A sequence has a length of approximately 120 adenosine residues. To further increase stability and/or expression of the RNA used according to the invention, the poly-A sequence can be unmasked.

In addition, incorporation of a 3'-non translated region (UTR) into the 3'-non translated region of an RNA molecule can result in an enhancement in translation efficiency. A synergistic effect may be achieved by incorporating two or more of such 3'-non translated regions. The 3'-non translated regions may be autologous or heterologous to the RNA into which they are introduced. In one particular embodiment the 3'-non translated region is derived from the human β-globin gene.

A combination of the above described modifications, i.e. incorporation of a poly-A sequence, unmasking of a poly-A sequence and incorporation of one or more 3'-non translated regions, has a synergistic influence on the stability of RNA and increase in translation efficiency.

The term "stability" of RNA relates to the "half-life" of RNA. "Half-life" relates to the period of time which is needed to eliminate half of the activity, amount, or number of molecules. In the context of the present invention, the half-life of an RNA is indicative for the stability of said RNA. The half-life of RNA may influence the "duration of expression" of the RNA. It can be expected that RNA having a long half-life will be expressed for an extended time period.

Of course, if according to the present invention it is desired to decrease stability and/or translation efficiency of RNA, it is possible to modify RNA so as to interfere with the function of elements as described above increasing the stability and/or translation efficiency of RNA.

The term "expression" is used according to the invention in its most general meaning and comprises the production of RNA and/or peptides or proteins, e.g. by transcription and/or translation. With respect to RNA, the term "expression" or "translation" relates in particular to the production of peptides or proteins. It also comprises partial expression of nucleic acids. Moreover, expression can be transient or stable.

According to the invention, terms such as "RNA expression", "expressing RNA", or "expression of RNA" relate to the production of peptide or protein encoded by the RNA. Preferably, such terms relate to the translation of RNA so as to express, i.e. produce peptide or protein encoded by the RNA.

In the context of the present invention, the term "transcription" relates to a process, wherein the genetic code in a DNA sequence is transcribed into RNA. Subsequently, the RNA may be translated into protein. According to the present invention, the term "transcription" comprises "*in vitro* transcription", wherein the term "*in vitro* transcription" relates to a process wherein RNA, in particular mRNA, is *in vitro* synthesized in a cell-free system, preferably using appropriate cell extracts. Preferably, cloning vectors are applied for the generation of

transcripts. These cloning vectors are generally designated as transcription vectors and are according to the present invention encompassed by the term "vector". According to the present invention, the RNA used in the present invention preferably is *in vitro* transcribed RNA (IVT-RNA) and may be obtained by *in vitro* transcription of an appropriate DNA template. The promoter for controlling transcription can be any promoter for any RNA polymerase. Particular examples of RNA polymerases are the T7, T3, and SP6 RNA polymerases. Preferably, the *in vitro* transcription according to the invention is controlled by a T7 or SP6 promoter. A DNA template for *in vitro* transcription may be obtained by cloning of a nucleic acid, in particular cDNA, and introducing it into an appropriate vector for *in vitro* transcription. The cDNA may be obtained by reverse transcription of RNA.

The cDNA containing vector template may comprise vectors carrying different cDNA inserts which following transcription results in a population of different RNA molecules optionally capable of expressing different peptides or proteins or may comprise vectors carrying only one species of cDNA insert which following transcription only results in a population of one RNA species capable of expressing only one peptide or protein. Thus, it is possible to produce RNA capable of expressing a single peptide or protein only or to produce compositions of different RNAs such as RNA libraries and whole-cell RNA capable of expressing more than one peptide or protein, e.g. a composition of peptides or proteins. The present invention envisions the introduction of all such RNA into cells.

The term "translation" according to the invention relates to the process in the ribosomes of a cell by which a strand of messenger RNA directs the assembly of a sequence of amino acids to make a peptide or protein.

Expression control sequences or regulatory sequences, which according to the invention may be linked functionally with a nucleic acid, can be homologous or heterologous with respect to the nucleic acid. A coding sequence and a regulatory sequence are linked together "functionally" if they are bound together covalently, so that the transcription or translation of the coding sequence is under the control or under the influence of the regulatory sequence. If the coding sequence is to be translated into a functional protein, with functional linkage of a regulatory sequence with the coding sequence, induction of the regulatory sequence leads to a transcription of the coding sequence, without causing a reading frame shift in the coding sequence or inability of the coding sequence to be translated into the desired protein or

peptide.

The term "expression control sequence" or "regulatory sequence" comprises, according to the invention, promoters, ribosome-binding sequences and other control elements, which control the transcription of a nucleic acid or the translation of the derived RNA. In certain embodiments of the invention, the regulatory sequences can be controlled. The precise structure of regulatory sequences can vary depending on the species or depending on the cell type, but generally comprises 5'-untranscribed and 5'- and 3'-untranslated sequences, which are involved in the initiation of transcription or translation, such as TATA-box, capping-sequence, CAAT-sequence and the like. In particular, 5'-untranscribed regulatory sequences comprise a promoter region that includes a promoter sequence for transcriptional control of the functionally bound gene. Regulatory sequences can also comprise enhancer sequences or upstream activator sequences.

Terms such as "enhancement of expression", "enhanced expression" or "increased expression" mean in the context of the present invention that the amount of peptide or protein expressed by a given number of RNA molecules is higher than the amount of peptide or protein expressed by the same number of RNA molecules, wherein expression of the RNA molecules is performed under the same conditions except the condition which results in the enhanced or increased expression of the RNA, such as preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling in a cell versus not preventing engagement of IFN receptor by extracellular IFN and not inhibiting intracellular IFN signalling in a cell. In this context, "same conditions" refer to a situation wherein the same RNA sequences encoding the same peptide or protein are introduced by the same means into the same cells, the cells are cultured under the same conditions (except the condition which results in the enhanced or increased expression) and the amount of peptide or protein is measured by the same means. The amount of peptide or protein may be given in moles, or by weight, e.g. in grams, or by mass or by polypeptide activity, e.g. if the peptide or protein is an enzyme it may be given as catalytic activity or if the peptide or protein is an antibody or antigen or a receptor it may be given as binding affinity. In one embodiment, terms such as "enhancement of expression", "enhanced expression" or "increased expression" mean in the context of the present invention that the amount of peptide or protein expressed by a given number of RNA molecules and within a given period of time is higher than the amount of peptide or protein expressed by the same number of RNA molecules and within the same

period of time. For example, the maximum value of peptide or protein expressed by a given number of RNA molecules at a particular time point may be higher than the maximum value of peptide or protein expressed by the same number of RNA molecules. In other embodiments, the maximum value of peptide or protein expressed by a given number of RNA molecules does not need to be higher than the maximum value of peptide or protein expressed by the same number of RNA molecules, however, the average amount of peptide or protein expressed by the given number of RNA molecules within a given period of time may be higher than the average amount of peptide or protein expressed by the same number of RNA molecules. The latter cases are referred to herein as "higher level of expression" or "increased level of expression" and relate to higher maximum values of expression and/or higher average values of expression. Alternatively or additionally, terms such as "enhancement of expression", "enhanced expression" or "increased expression" mean in the context of the present invention also that the time in which peptide or protein is expressed by RNA molecules may be longer than the time in which the peptide or protein is expressed by the same RNA molecules. Thus, in one embodiment, terms such as "enhancement of expression", "enhanced expression" or "increased expression" mean in the context of the present invention also that the amount of peptide or protein expressed by a given number of RNA molecules is higher than the amount of peptide or protein expressed by the same number of RNA molecules since the period of time in which the RNA is stably present and expressed is longer than the period of time in which the same number of RNA molecules is stably present and expressed. These cases are referred to herein also as "increased duration of expression". Preferably, such longer time periods refer to expression for at least 48 h, preferably for at least 72 h, more preferably for at least 96 h, in particular for at least 120 h or even longer following introduction of RNA or following the first introduction (e.g. in case of repeated transfections) of RNA into a cell.

The level of expression and/or duration of expression of RNA may be determined by measuring the amount, such as the total amount expressed and/or the amount expressed in a given time period, and/or the time of expression of the peptide or protein encoded by the RNA, for example, by using an ELISA procedure, an immunohistochemistry procedure, a quantitative image analysis procedure, a Western Blot, mass spectrometry, a quantitative immunohistochemistry procedure, or an enzymatic assay.

In particular embodiments, the RNA according to the invention comprises a population of

different RNA molecules, e.g. a mixture of different RNA molecules optionally encoding different peptides and/or protein, whole-cell RNA, an RNA library, or a portion of thereof, e.g. a library of RNA molecules expressed in a particular cell type, such as undifferentiated cells, in particular stem cells such as embryonic stem cells, or a fraction of the library of RNA molecules such as RNA with enriched expression in undifferentiated cells, in particular stem cells such as embryonic stem cells relative to differentiated cells. Thus, according to the invention, the term "RNA" may include a mixture of RNA molecules, whole-cell RNA or a fraction thereof, which may be obtained by a process comprising the isolation of RNA from cells and/or by recombinant means, in particular by *in vitro* transcription.

Preferably, according to the invention, the RNA to be expressed in a cell is introduced into said cell, either *in vitro* or *in vivo*, preferably *in vitro*. RNA may be introduced into a cell either prior to, after and/or simultaneously with preventing engagement of IFN receptor by extracellular IFN and/or inhibiting intracellular IFN signalling in the cell. Preferably, engagement of IFN receptor by extracellular IFN is prevented and intracellular IFN signalling is inhibited as long as expression of the RNA in the cell is desired. In one embodiment of the methods according to the invention, the RNA that is to be introduced into a cell is obtained by *in vitro* transcription of an appropriate DNA template.

The RNA used according to the present invention may have a known composition (in this embodiment it is preferably known which peptides or proteins are being expressed by the RNA) or the composition of the RNA may be partially or entirely unknown. Alternatively, the RNA used according to the present invention may have a known function or the function of the RNA may be partially or entirely unknown.

According to the invention, the terms "RNA capable of expressing" and "RNA encoding" are used interchangeably herein and with respect to a particular peptide or protein mean that the RNA, if present in the appropriate environment, preferably within a cell, can be expressed to produce said peptide or protein. Preferably, RNA according to the invention is able to interact with the cellular translation machinery to provide the peptide or protein it is capable of expressing.

According to the invention, RNA may be introduced into cells either *in vitro* or *in vivo*, preferably *in vitro*. The cells into which the RNA has been introduced *in vitro* may, preferably

following expression of the RNA *in vitro* by the methods of the invention, be administered to a patient.

Terms such as "transferring", "introducing" or "transfecting" are used interchangeably herein and relate to the introduction of nucleic acids, in particular exogenous or heterologous nucleic acids, in particular RNA, into a cell. Said terms also include the repetitive introduction of nucleic acids, in particular RNA, into a cell, wherein repetitive mean more than once, e.g. two times or more, three times or more, four times or more, five times or more, six times or more, seven times or more, eight times or more. The time interval between said repetitive introductions of nucleic acids may be 3 days or less, 2 days or less, 24 hours or less or even lower.

According to the present invention, a cell can be an isolated cell or it can form part of an organ, a tissue and/or an organism. According to the present invention, any technique which is suitable to introduce RNA into cells may be used. Preferably, the RNA is introduced into cells by standard techniques. Such techniques comprise transfection of nucleic acid calcium phosphate precipitates, transfection of nucleic acids which are associated with DEAE, the transfection or infection with viruses which carry the nucleic acids of interest, electroporation, lipofection, and microinjection. According to the present invention, the administration of a nucleic acid is either achieved as naked nucleic acid or in combination with an administration reagent. Preferably, administration of nucleic acids is in the form of naked nucleic acids. Preferably, the RNA is administered in combination with stabilizing substances such as RNase inhibitors. The present invention also envisions the repeated introduction of nucleic acids into cells to allow sustained expression for extended time periods.

Cells can be transfected, for example, using commercially available liposome-based transfection kits such as Lipofectamine™ (Invitrogen) and can be transfected with any carriers with which RNA can be associated, e.g. by forming complexes with the RNA or forming vesicles in which the RNA is enclosed or encapsulated, resulting in increased stability of the RNA compared to naked RNA. Carriers useful according to the invention include, for example, lipid-containing carriers such as cationic lipids, liposomes, in particular cationic liposomes, and micelles. Cationic lipids may form complexes with negatively charged nucleic acids. Any cationic lipid may be used according to the invention.

Preferably, the introduction of RNA which encodes a peptide or protein into a cell results in expression of said peptide or protein in the cell. In particular embodiments, the targeting of the nucleic acids to particular cells is preferred. In such embodiments, a carrier which is applied for the administration of the nucleic acid to a cell (for example, a retrovirus or a liposome), exhibits a targeting molecule. For example, a molecule such as an antibody which is specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell may be incorporated into the nucleic acid carrier or may be bound thereto. In case the nucleic acid is administered by liposomes, proteins which bind to a surface membrane protein which is associated with endocytosis may be incorporated into the liposome formulation in order to enable targeting and/or uptake. Such proteins encompass capsid proteins of fragments thereof which are specific for a particular cell type, antibodies against proteins which are internalized, proteins which target an intracellular location etc.

Electroporation or electropermeabilization relates to a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field. It is usually used in molecular biology as a way of introducing some substance into a cell. Electroporation is usually done with electroporators, appliances which create an electro-magnetic field in the cell solution. The cell suspension is pipetted into a glass or plastic cuvette which has two aluminum electrodes on its sides. For electroporation, tyically a cell suspension of around 50 microliters is used. Prior to electroporation it is mixed with the nucleic acid to be transfected. The mixture is pipetted into the cuvette, the voltage and capacitance is set and the cuvette inserted into the electroporator. Preferably, liquid medium is added immediately after electroporation (in the cuvette or in an eppendorf tube), and the tube is incubated at the cells' optimal temperature for an hour or more to allow recovery of the cells and optionally expression of antibiotic resistance.

According to the invention it is preferred that a nucleic acid such as RNA encoding a peptide or protein once taken up by or introduced into a cell which cell may be present *in vitro* or in a subject results in expression of said peptide or protein. The cell may express the encoded peptide or protein intracellularly (e.g. in the cytoplasm and/or in the nucleus), may secrete the encoded peptide or protein, or may express it on the surface. If a peptide or protein (e.g. B18R) is to prevent engagement of IFN receptor by extracellular IFN, secretion of the peptide or protein is preferred. If a peptide or protein (e.g. E3, K3) is to inhibit intracellular IFN signalling, intracellular expression of the peptide or protein is preferred.

If according to the invention RNA capable of expressing certain factors for reprogramming of somatic cells is introduced into somatic cells, it is preferred that this introduction of RNA results in expression of said factors for a time period to complete the reprogramming process and in the development of cells having stem cell characteristics. Preferably, introduction of RNA capable of expression certain factors as disclosed herein into somatic cells results in expression of said factors for an extended period of time, preferably for at least 10 days, preferably for at least 11 days and more preferably for at least 12 days. To achieve such long term expression, RNA is preferably periodically (i.e. repetitively) introduced into the cells more than one time, preferably using electroporation. Preferably, RNA is introduced into the cells at least twice, more preferably at least 3 times, more preferably at least 4 times, even more preferably at least 5 times up to preferably 6 times, more preferably up to 7 times or even up to 8, 9 or 10 times, preferably over a time period of at least 10 days, preferably for at least 11 days and more preferably for at least 12 days to ensure expression of one or more factors for an extended period of time. Preferably, the time periods elapsing between the repeated introductions of the RNA are from 24 hours to 120 hours, preferably 48 hours to 96 hours. In one embodiment, time periods elapsing between the repeated introductions of the RNA are not longer than 72 hours, preferably not longer than 48 hours or 36 hours. In one embodiment, prior to the next electroporation, cells are allowed to recover from the previous electroporation. In this embodiment, the time periods elapsing between the repeated introductions of the RNA are at least 72 hours, preferably at least 96 hours, more preferably at least 120 hours. In any case, the conditions should be selected so that the factors are expressed in the cells in amounts and for periods of time which support the reprogramming process.

Preferably at least 1 μg, preferably at least 1.25 μg, more preferably at least 1.5 μg and preferably up to 20 μg, more preferably up to 15 μg, more preferably up to 10 μg, more preferably up to 5 μg, preferably 1 to 10 μg, even more preferably 1 to 5 μg, or 1 to 2.5 μg of RNA for each peptide, protein or factor is used per electroporation.

Preferably, if a loss of viability of cells occurs by repeated electroporations, previously not electroporated cells are added as carrier cells. Preferably, previously not electroporated cells are added prior to, during or after one or more of the 4^{th} and subsequent, preferably, the 5^{th} and subsequent electroporations such as prior to, during or after the 4^{th} and 6^{th} electroporation. Preferably, previously not electroporated cells are added prior to, during or after the 4^{th} or 5^{th}

and each subsequent electroporation. Preferably, the previously not electroporated cells are the same cells as those into which RNA is introduced.

Terms such as "enhancement of cell viability", "enhanced cell viability" or "increased cell viability" mean in the context of the present invention that the amount of viable or living cells under certain conditions is higher than the amount of viable or living cells under other conditions, wherein cultivation is performed under the same conditions except the condition which results in the enhanced or increased cell viability, such as preventing engagement of IFN receptor by extracellular IFN and inhibiting intracellular IFN signalling in a cell versus not preventing engagement of IFN receptor by extracellular IFN and not inhibiting intracellular IFN signalling in a cell. In this context, "same conditions" refer to a situation wherein the same cells are used, the cells are cultured under the same conditions (except the condition which results in the enhanced or increased cell viability) and the cell viability is measured by the same means. "Same conditions" also encompasses the introduction or repetitive introduction of RNA into cells.

Double-stranded RNA (dsRNA) produced during viral infection activates several cellular antiviral responses. Double-stranded RNA (dsRNA) not only constitutes the genetic material of dsRNA viruses but is also produced in infected cells by positive-strand RNA viruses and some DNA viruses. Among the best characterized cellular antiviral responses is the shutoff of protein synthesis mediated by the RNA-dependent protein kinase (PKR) and the oligoadenylate synthetase (OAS)/RNase L systems. Toll-like receptor 3 (TLR3) and the RNA helicases RIG-I and MDA5 serve as sensors for dsRNA; cf. Fig. 1. Upon activation, they induce signaling cascades culminating in the expression of type I interferons (IFNs). Induction of type I IFNs is controlled predominantly at the transcription level by a family of transcription factors termed the interferon regulatory factors (IRFs).

Interferons are important cytokines characterized by antiviral, antiproliferative and immunomodulatory activities. Interferons are proteins that alter and regulate the transcription of genes within a cell by binding to interferon receptors on the regulated cell's surface, thereby preventing viral replication within the cells. According to the invention, the phrase "engagement of IFN receptor by extracellular IFN" relates to the binding of IFNs, in particular type I IFNs, to interferon receptors on the cell surface.

The interferons can be grouped into two types. IFN-gamma is the sole type II interferon; all others are type I interferons. Type I and type II interferons differ in gene structure (type II interferon genes have three exons; type I, one), chromosome location (in humans, type II is located on chromosome-12; the type I interferon genes are linked and on chromosome-9), and the types of tissues where they are produced (type I interferons are synthesized ubiquitously, type II by lymphocytes). Type I interferons competitively inhibit each others binding to cellular receptors, while type II interferon has a distinct receptor. According to the invention, the term "interferon" or "IFN" preferably relates to type I interferons, in particular IFN-alpha and IFN-beta.

Human IFN-alpha's are encoded by a multigene family consisting of about 20 genes; each gene encodes a single subtype of the human IFN-alpha. Human IFN-alpha polypeptides are produced by a number of human cell lines and human leukocyte cells after exposure to viruses or double-stranded RNA, or in transformed leukocyte cell lines (e.g., lymphoblastoid lines). IFN-alpha's interact with cell-surface receptors and induce the expression, primarily at the transcriptional level, of a broad but specific set of cellular genes.

Human IFN-beta is a regulatory polypeptide with a molecular weight of 22 kDa consisting of 166 amino acid residues. It can be produced by most cells in the body, in particular fibroblasts, in response to viral infection or exposure to other biologics. It binds to a multimeric cell surface receptor, and productive receptor binding results in a cascade of intracellular events leading to the expression of IFN-beta inducible genes which, in turn, produces effects which can be classified as antiviral, antiproliferative, or immunomodulatory.

IFNs induce the expression of a plethora of antiviral genes, which can interfere with the viral replication cycle. According to the invention, the term "antivirally active effector protein" relates to a group of proteins encoded by IFN-stimulated genes (ISGs) the transcription of which is signaled by type I IFNs. These proteins target distinct viral components and distinct stages of the viral life cycle, aiming to eliminate invading viruses. "Antivirally active effector proteins" are involved in different effector pathways individually blocking viral transcription, degrading viral RNA, inhibiting translation, and modifying protein function to control all steps of viral replication. Such proteins include 2',5'-oligoadenylate synthetase (OAS), in particular 2',5'-oligoadenylate synthetase 1 (OAS1), RNA-dependent protein kinase R (PKR), and RNaseL. Both PKR and OAS are directly activated by dsRNA. Hence, dsRNA induces

the expression of these antivirally active effector proteins and is also necessary for their activation.

PKR is constitutively expressed, and induced by type I IFNs. Upon binding to dsRNA, PKR dimerizes and undergoes autophosphorylation to gain full catalytic activity. Once activated, PKR phosphorylates the eukaryotic translation initiation factor eIF2-alpha. In its phosphorylated state, eIF2-alpha forms a stable complex with the nucleotide exchange factor eIF2-beta, which is then no longer recycled for initiation of protein translation by GDP/GTP exchange. Consequently, PKR activation leads to a global block to protein synthesis in the infected cell, which can hamper the production of virus progeny. In this way, PKR in combination with eIF2-alpha constitutes an antiviral pathway (PKR-dependent pathway).

The term "RNA-dependent protein kinase" (protein kinase RNA-activated; PKR) relates to a RNA-binding protein which is an interferon-induced serine/threonine protein kinase initially identified in viral response by virtue of its binding to and activation by the extensive secondary structure formed by viral RNA sequences. Human PKR is 68 kDa with an about 20 kDa N-terminal dsRNA-binding domain and a C-terminal protein kinase domain. *In vitro* PKR is activated by binding to RNA molecules with extensive duplex secondary structure. *In vivo* the enzyme is believed to be activated by viral double-stranded RNA (dsRNA) or viral replicative intermediates comprising dsRNA. Binding to double-stranded RNA to PKR causes a conformational change in the enzyme that alters the ATP-binding site in the kinase domain and leads to autophosphorylation at multiple serine and threonine residues throughout the PKR sequence. RNA-stimulated autophosphorylation increases cellular sensitivity to apoptotic and pro-inflammatory stimuli through a number of putative pathways, including phosphorylation of its known substrate eukaryotic initiation factor 2 (eIF2-alpha).

The term "PKR" preferably relates to human PKR, and, in particular, to a protein comprising the amino acid sequence according to SEQ ID NO: 14 of the sequence listing or a variant of said amino acid sequence. In one embodiment, the term "PKR" relates to a protein comprising an amino acid sequence encoded by the nucleic acid sequence according to SEQ ID NO: 13. The term "PKR" includes any variants, in particular mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present. An allelic variant relates to an alteration in the normal sequence of a gene, the significance of which is often unclear. Complete gene sequencing often identifies

numerous allelic variants for a given gene. A species homolog is a nucleic acid or amino acid sequence with a different species of origin from that of a given nucleic acid or amino acid sequence. One skilled in the art would understand that the cDNA sequence of PKR as described above would be equivalent to PKR mRNA, and can be used for the generation of inhibitory nucleic acids against PKR.

Protein kinase activity including protein kinase autophosphorylation can be measured by a variety of techniques known to the skilled person. One method involves separation of unreacted ATP from the phosphorylated kinase substrate by e.g. precipitating phosphoprotein onto cellulose strips by trichloroacetic acid followed by washing, or adsorption of phosphoprotein onto phosphocellulose strips. For example, dephosphoPKR can be activated by incubation with poly[I:C] and autophosphorylation can be allowed to proceed in the presence of [γ-32P]ATP. The ability of compounds to block this RNA-induced PKR autophosphorylation can be tested. Another method involves detection and quantification of phospho-PKR in relation to the total amount of PKR in the same lysate of cells by Western blotting with antibodies specific for phospho-PKR or full length PKR. Another method involves detection and quantification of the phosphorylated substrate of PKR, e.g. phospho-eIF2-alpha in relation to the total amount of eIF2-alpha in the same lysate of cells by Western blotting with antibodies specific for phospho-eIF2-alpha or full length eIF2-alpha.

The term "eIF2-alpha" preferably relates to human eIF2-alpha, and, in particular, to a protein comprising the amino acid sequence according to SEQ ID NO: 16 of the sequence listing or a variant of said amino acid sequence. In one embodiment, the term "eIF2-alpha" relates to a protein comprising an amino acid sequence encoded by the nucleic acid sequence according to SEQ ID NO: 15. The term "eIF2-alpha" includes any variants, in particular mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present.

OAS is expressed at low constitutive levels and is induced by type I IFNs. The protein accumulates in the cell cytoplasm as inactive monomers. Upon activation by viral dsRNA the enzyme oligomerizes to (in the instance of OAS1) form a tetramer that is able to condense ATP molecules via unusual 2',5'-phosphodiester linkages and synthesizes 2',5'-oligoadenlylates that, in turn, activate the constitutively expressed inactive RNaseL. Binding of 2',5'-oligoadenlylates to RNaseL triggers dimerization of enzyme monomers, via their

kinase-like domains, which then cleaves cellular (and viral) RNAs. As a result, synthesis of viral proteins is inhibited, and viral RNA genomes are directly destroyed. In this way, OAS in combination with RNaseL constitutes an antiviral RNA decay pathway (OAS-RNaseL antiviral pathway or OAS-dependent pathway).

The four OAS genes identified in humans, termed OAS1, OAS2, OAS3 and OASL (OAS-like), have been mapped to chromosome 12 (chromosome 5 in mice). OAS1 has two spliced forms in humans (eight in mice) that produce two, 40 and 46 kDa, proteins that differ at their C-termini by 18 and 54 amino acids, respectively. OAS2 produces four alternatively spliced transcripts that encode two proteins of 69 and 71 kDa. OAS3 encodes a single transcript that produces a 100 kDa protein. These proteins have considerable homology to each other, with OAS1, OAS2 and OAS3 encoding one, two and three, respectively, "OAS" domains. The most distinctive of the OAS proteins is OASL. Two OASL transcripts are expressed producing two proteins of 30 and 59 kDa. The higher molecular weight OASL contains a putative nucleolar localization signal at its C-terminus that, probably, accounts for its unique (from the other OAS isoforms) distribution in the cell. The OASL protein also has an OAS domain, however, mutations at key residues disable the catalytic function of this human protein. Interestingly, one of the two mouse homologues retains its 2',5'-polymerase activity. In addition to the OAS domain, OASL has a unique 160 amino acid C-terminus that encodes a ubiquitin-like domain that is homologous to ISG15. Accordingly, OASL becomes conjugated (ISGylation) to cellular proteins following the treatment of cells with type I IFNs. There appears to be differential expression and induction of each form of the human OAS proteins. Also, each of the three functional OAS proteins has unique biological functions. A tripeptide motif (CFK) within the OAS domains of OAS1 and OAS2 mediate oligomerization, so the catalytically active form of these enzymes is a tetramer and dimer, for OAS1 and OAS2, respectively. This tripeptide motif is not conserved in the OAS domains of OAS3 and OASL and therefore these proteins function as monomers. The polymerization of OAS monomers influences their processivity, with OAS3 synthesizing dimeric molecules of 2',5'-linked oligomers, whereas OAS1 and OAS2 are capable of synthesizing trimeric and tetrameric oligomers. The dimeric 2',5'-linked oligomers are not efficient activators of RNaseL and, consequently, are thought to regulate alternative processes, with one report suggesting a role in gene expression by regulating DNA topoisomerase I. According to the invention the term "2',5'-oligoadenylate synthetase" or "OAS" preferably relates to molecules which are activators of RNaseL and preferably to OAS1 and OAS2.

The term "OAS" preferably relates to human OAS, and, in particular, to a protein comprising the amino acid sequence according to SEQ ID NO: 18 or 20 of the sequence listing or a variant of said amino acid sequence. In one embodiment, the term "OAS" relates to a protein comprising an amino acid sequence encoded by the nucleic acid sequence according to SEQ ID NO: 17 or 19. The term "OAS" includes any variants, in particular mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present.

The 2',5'-dependent RNaseL is expressed as an 80 kDa protein with two kinase-like domains (PUG and STYKc) and eight ankyrin repeats. The enzyme is constitutively expressed as an inactive monomer. Autoinhibition of the enzyme is relieved upon binding of 2',5'-oligomers (generated by OAS proteins) to the ankyrin repeats, and subsequent homodimerization. The active dimeric enzyme then degrades ssRNA.

The term "RNaseL" preferably relates to human RNaseL, and, in particular, to a protein comprising the amino acid sequence according to SEQ ID NO: 22 of the sequence listing or a variant of said amino acid sequence. In one embodiment, the term "RNaseL" relates to a protein comprising an amino acid sequence encoded by the nucleic acid sequence according to SEQ ID NO: 21. The term "RNaseL" includes any variants, in particular mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present.

According to the invention, the term "preventing engagement of IFN receptor by extracellular IFN" relates to an inhibition, i.e. blocking, or reduction, of the interaction of IFNs, in particular type I IFNs, with their specific receptors thus, inhibiting or reducing IFN function. Engagement of IFN receptor by extracellular IFN may be prevented, for example, by the provision of a binding agent for extracellular IFN.

For example, the B18R protein is a vaccinia virus-encoded type I interferon receptor with specificity for mouse, human, rabbit, pig, rat, and cow type I interferons which has potent neutralizing activity. The B18R protein encoded by the B18R gene of the Western Reserve vaccinia virus strain. The 60-65 kD glycoprotein is related to the interleukin-1 receptors and is a member of the immunoglobulin superfamily, unlike other type I IFN-receptors, which

belong to the class II cytokine receptor family. The B18R protein has a high affinity (KD, 174 pM) for human IFN alpha. Among viral host response modifiers, the B18R protein is unique in that it exists as a soluble extracellular, as well as a cell surface protein, enabling blockage of both autocrine and paracrine IFN functions. The B18R protein has been shown to inhibit the antiviral potency of IFN-alpha1, IFN-alpha2, IFN-alpha-8/1/8, and IFN-omega on human cells. The soluble B18R protein is highly potent for neutralizing type I interferons, which include IFN-alpha, beta, delta, kappa.

The term "B18R" preferably relates to a protein comprising the amino acid sequence according to SEQ ID NO: 24 of the sequence listing or a variant of said amino acid sequence. In one embodiment, the term "B18R" relates to a protein comprising an amino acid sequence encoded by the nucleic acid sequence according to SEQ ID NO: 23. The term "B18R" includes any variants, in particular mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present.

According to the invention, the term "intracellular IFN signalling" relates to the intracellular signaling events and effector functions, in particular antiviral functions, activated by IFNs interacting with their specific receptors and includes the functions of proteins that are induced by IFN, in particular antivirally active effector proteins. In particular, the term "intracellular IFN signalling" includes the signal propagation through and effector functions, in particular antiviral functions, exerted by proteins which are part of the PKR-dependent pathway, in particular PKR and eIF2-alpha, and/or the OAS-dependent pathway, in particular OAS and RNaseL.

The term "inhibiting intracellular IFN signalling" relates to an inhibition or reduction of intracellular IFN signalling and may be achieved by inhibiting expression, activity or activation of proteins which are involved in intracellular IFN signaling, in particular proteins which are part of the PKR-dependent pathway and/or the OAS-dependent pathway. For example, many viruses have evolved mechanisms for counteracting the PKR and OAS/RNase L pathways. These mechanisms may be used according to the invention for inhibiting intracellular IFN signaling.

According to the invention, the PKR-dependent pathway may be inhibited by an agent inhibiting or reducing the activity or activation of PKR or by an agent dephosphorylating eIF2-alpha or preventing its phosphorylation, thereby terminating the PKR-induced signal. For example, intracellular IFN signalling may be inhibited according to the invention by utilizing any of the viral defense mechanisms against the PKR signaling cascade. In this respect, the invention may involve the use of decoy dsRNA (e.g. adenovirus VAI RNA; Epstein-Barr virus EBER; HIV TAR), compounds effecting PKR degradation (e.g. poliovirus $2A^{pro}$), compounds inhibiting activation of PKR, e.g. through hiding viral dsRNA (e.g. vaccinia virus E3/E3L; reovirus sigma3; influenza virus NS1, herpes simplex virus type 1 (HSV-1) US11), compounds blocking dimerization (e.g. influenza virus $p58^{IPK}$; Hepatitis C virus NS5A), pseudosubstrates (e.g. vaccinia virus K3/K3L; HIV Tat) or dephosphorylation of substrate (e.g. herpes simplex virus ICP34.5). Vaccinia virus E3 is a 25 kDa dsRNA-binding protein (encoded by gene E3L) that binds and sequesters dsRNA to prevent the activation of PKR and OAS. E3 can bind directly to PKR and inhibits its activity, resulting in reduced phosphorylation of eIF2-alpha. Vaccinia virus gene K3L encodes a 10.5 kDa homolog of the eIF2-alpha subunit that acts as a non-phosphorylable pseudosubstrate of PKR and competitively inhibits phosphorylation of eIF2-alpha. Vaccinia virus C7/C7L inhibits phosphorylation of eIF2-alpha. The ICP34.5 protein from HSV-1 functions as a regulatory subunit of the cellular PP1 phosphatase, directing it to dephosphorylate eIF2-alpha, thereby terminating the PKR-induced signal. The murine cytomegalovirus (MCMV) proteins m142 and m143 have been characterized as dsRNA binding proteins that inhibit PKR activation, phosphorylation of the translation initiation factor eIF2, and a subsequent protein synthesis shutoff.

A decoy RNA is pseudosubstrate RNA that has similar structure to the RNA substrate of an enzyme, in order to make the enzyme bind to the pseudosubstrate rather than to the real substrate, thus blocking the activity of the enzyme.

The term "E3" preferably relates to a protein comprising the amino acid sequence according to SEQ ID NO: 26 of the sequence listing or a variant of said amino acid sequence. In one embodiment, the term "E3" relates to a protein comprising an amino acid sequence encoded by the nucleic acid sequence according to SEQ ID NO: 25. The term "E3" includes any variants, in particular mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present.

The term "K3" preferably relates to a protein comprising the amino acid sequence according to SEQ ID NO: 28 of the sequence listing or a variant of said amino acid sequence. In one embodiment, the term "K3" relates to a protein comprising an amino acid sequence encoded by the nucleic acid sequence according to SEQ ID NO: 27. The term "K3" includes any variants, in particular mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present.

According to the present invention, the term "reducing the activity of RNA-dependent protein kinase (PKR)" relates to measures that result in a lower degree of homodimerization of PKR, in a lower degree of autophosphorylation of PKR and/or in a lower degree of phosphorylation of targets which are kinase substrates of PKR such as eIF2-alpha compared to the normal situation, in particular the normal situation in a cell, wherein the activity of PKR is not reduced/has not been reduced by man. Preferably, said term includes all measures that result in a lower degree of autophosphorylation of PKR and/or in a lower degree of phosphorylation of targets which are kinase substrates of PKR.

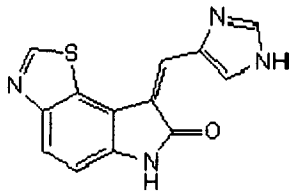
In one embodiment, reducing the activity of RNA-dependent protein kinase (PKR) in a cell comprises treating the cell with an inhibitor of expression and/or activity of PKR. According to the invention, the phrase "inhibit expression and/or activity" includes a complete or essentially complete inhibition of expression and/or activity and a reduction in expression and/or activity.

In one embodiment, said PKR inhibitor is directed at the PKR protein and preferably is specific for PKR. PKR can be inhibited in various ways, e.g. through inhibiting PKR autophosphorylation and/or dimerization, providing a PKR pseudo-activator, or providing a PKR pseudo-substrate. The PKR inhibitor may be an agent which is involved in a viral defense mechanism as discussed above. For example, vaccinia virus E3L encodes a dsRNA binding protein that inhibits PKR in virus-infected cells, presumably by sequestering dsRNA activators. K3, also encoded by vaccinia virus, functions as a pseudosubstrate inhibitor by binding to PKR. Thus, providing vaccinia virus E3L may result in inhibition of PKR. Providing adenovirus VAI RNA, HIV Tat or Epstein-Barr virus EBER1 RNA may result in PKR pseudo-activation. Thus, for example, all viral factors, i.e. virally derived inhibitors,

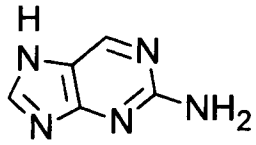
blocking PKR activity such as those described herein may be used for reducing the activity of PKR.

In one embodiment, the PKR inhibitor is a chemical inhibitor. Preferably, the PKR inhibitor is an inhibitor of RNA-induced PKR autophosphorylation. Preferably, the PKR inhibitor is an ATP-binding site directed inhibitor of PKR.

In one embodiment, the PKR inhibitor is 6,8-dihydro-8-(1H-imidazol-5-ylmethylene)-7H-pyrrolo[2,3-g]benzothiazol-7-one. In one embodiment, the PKR inhibitor has the following formula:



In one embodiment, the PKR inhibitor is 2-aminopurine. In one embodiment, the PKR inhibitor has the following formula:



Preferably, an inhibitor as disclosed above is used in a concentration of at least 0.5 μM or higher, at least 1 μM or higher or at least 2 μM or higher and preferably in a concentration up to 5 μM, up to 4 μM, up to 3 μM or up to 2 μM.

In a further embodiment, the inhibitor of activity of PKR is an antibody that specifically binds to PKR. Binding of the antibody to PKR can interfere with the function of PKR, e.g. by inhibiting binding activity or catalytic activity.

In one embodiment, it is envisioned to reduce the activity of PKR in a cell by treating the cell with one or more virally derived inhibitors such as vaccinia virus E3 and/or K3 as well as treating the cell with one or more chemical PKR inhibitors such as 6,8-dihydro-8-(1H-

imidazol-5-ylmethylene)-7H-pyrrolo[2,3-g]benzothiazol-7-one and/or 2-aminopurine.

According to the invention, the OAS-dependent pathway may be inhibited by an agent inhibiting or reducing the activity or activation of OAS and/or RNaseL. For example, vaccinia virus E3 is a 25 kDa dsRNA-binding protein (encoded by gene E3L) that binds and sequesters dsRNA to prevent the activation of OAS.

According to the present invention, the terms "reducing the activity of OAS" preferably relates to measures that result in a lower degree of production of 2',5'-oligoadenlylates and thus, activation of RNaseL.

In one embodiment, reducing the activity of OAS and/or RNaseL in a cell comprises treating the cell with an inhibitor of expression and/or activity of OAS and/or RNaseL. According to the invention, the phrase "inhibit expression and/or activity" includes a complete or essentially complete inhibition of expression and/or activity and a reduction in expression and/or activity.

In one embodiment, inhibition of expression of PKR, OAS or RNaseL, in the following referred to as "target protein", may take place by inhibiting the production of or reducing the level of transcript, i.e. mRNA, coding for the target protein, e.g. by inhibiting transcription or inducing degradation of transcript, and/or by inhibiting the production of the target protein, e.g. by inhibiting translation of transcript coding for the target protein. In one embodiment, said inhibitor is specific for a nucleic acid encoding the target protein. In a particular embodiment, the inhibitor of expression of the target protein is an inhibitory nucleic acid (e.g., antisense molecule, ribozyme, iRNA, siRNA or a DNA encoding the same) selectively hybridizing to and being specific for nucleic acid encoding the target protein, thereby inhibiting (e.g., reducing) transcription and/or translation thereof.

Inhibitory nucleic acids of this invention include oligonucleotides having sequences in the antisense orientation relative to the target nucleic acids. Suitable inhibitory oligonucleotides typically vary in length from five to several hundred nucleotides, more typically about 20-70 nucleotides in length or shorter, even more typically about 10-30 nucleotides in length. These inhibitory oligonucleotides may be applied, either *in vitro* or *in vivo*, as free (naked) nucleic acids or in protected forms, e.g., encapsulated in liposomes. The use of liposomal or other

protected forms may be advantageous as it may enhance *in vivo* stability and thus facilitate delivery to target sites.

Also, the target nucleic acid may be used to design ribozymes that target the cleavage of the corresponding mRNAs in cells. Similarly, these ribozymes may be administered in free (naked) form or by the use of delivery systems that enhance stability and/or targeting, e.g., liposomes.

Also, the target nucleic acid may be used to design siRNAs that can inhibit (e.g., reduce) expression of the nucleic acid. The siRNAs may be administered in free (naked) form or by the use of delivery systems that enhance stability and/or targeting, e.g., liposomes. They may also be administered in the form of their precursors or encoding DNAs.

siRNA preferably comprises a sense RNA strand and an antisense RNA strand, wherein the sense and antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence substantially identical to a target sequence of about 19 to about 25 contiguous nucleotides in a target nucleic acid, preferably mRNA coding for PKR.

It is to be understood that according to the invention instead of the peptides or proteins mentioned above for (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling, nucleic acids encoding the peptides or proteins can be provided. The phrase "provided in the form of a nucleic acid" as used herein is to account for this possibility. For example, cells may be transfected with nucleic acid, in particular RNA, encoding the peptides or proteins and the nucleic acid may be expressed in the cells so as to produce the peptides or proteins.

In one embodiment, cells are treated to (i) prevent engagement of IFN receptor by extracellular IFN and/or (ii) inhibit intracellular IFN signalling prior to, simultaneously with and/or following introduction of RNA encoding the peptide or protein to be expressed, e.g. one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics, or the first introduction (e.g. in case of repeated transfections) of RNA. In one embodiment, cells are treated to (i) prevent engagement of IFN receptor by extracellular IFN and/or (ii) inhibit intracellular IFN signalling following, preferably immediately following introduction of RNA or the first introduction (e.g. in case of repeated transfections) of RNA.

In one embodiment, cells are treated to (i) prevent engagement of IFN receptor by extracellular IFN and/or (ii) inhibit intracellular IFN signalling for at least 24 h, at least 48 h, at least 72 h, at least 96 h, at least 120 h or even longer. Most preferably, cells are treated to (i) prevent engagement of IFN receptor by extracellular IFN and/or (ii) inhibit intracellular IFN signalling for the entire period of time in which expression of RNA is desired, such as permanently, optionally with repeated transfection of RNA.

According to the invention, it is envisioned to (i) prevent engagement of IFN receptor by extracellular IFN and (ii) inhibit intracellular IFN signalling in a cell *in vitro* or *in vivo*, preferably *in vitro*. Thus, according to the present invention, the cell can be an isolated cell or it can form part of an organ, a tissue and/or an organism.

"Antisense molecules" or "antisense nucleic acids" may be used for regulating, in particular reducing, expression of a nucleic acid. The term "antisense molecule" or "antisense nucleic acid" refers according to the invention to an oligonucleotide which is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide or modified oligo-deoxyribonucleotide and which hybridizes under physiological conditions to DNA comprising a particular gene or to mRNA of said gene, thereby inhibiting transcription of said gene and/or translation of said mRNA. According to the invention, an "antisense molecule" also comprises a construct which contains a nucleic acid or a part thereof in reverse orientation with respect to its natural promoter. An antisense transcript of a nucleic acid or of a part thereof may form a duplex with naturally occurring mRNA and thus prevent accumulation of or translation of the mRNA. Another possibility is the use of ribozymes for inactivating a nucleic acid.

In preferred embodiments, the antisense oligonucleotide hybridizes with an N-terminal or 5' upstream site such as a translation initiation site, transcription initiation site or promoter site. In further embodiments, the antisense oligonucleotide hybridizes with a 3'-untranslated region or mRNA splicing site.

By "small interfering RNA" or "siRNA" as used herein is meant an RNA molecule, preferably greater than 10 nucleotides in length, more preferably greater than 15 nucleotides in length, and most preferably 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length

that is used to identify a target gene or mRNA to be degraded. A range of 19-25 nucleotides is the most preferred size for siRNAs.

One or both strands of the siRNA can also comprise a 3'-overhang. As used herein, a "3'-overhang" refers to at least one unpaired nucleotide extending from the 3'-end of an RNA strand. Thus in one embodiment, the siRNA comprises at least one 3'-overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxynucleotides) in length, preferably from 1 to about 5 nucleotides in length, more preferably from 1 to about 4 nucleotides in length, and particularly preferably from about 2 to about 4 nucleotides in length. In the embodiment in which both strands of the siRNA molecule comprise a 3'-overhang, the length of the overhangs can be the same or different for each strand. In a most preferred embodiment, the 3'-overhang is present on both strands of the siRNA, and is 2 nucleotides in length. For example, each strand of the siRNA of the invention can comprise 3'-overhangs of dideoxythymidylic acid ("TT") or diuridylic acid ("uu").

In order to enhance the stability of the siRNA, the 3'-overhangs can be also stabilized against degradation. In one embodiment, the overhangs are stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine nucleotides in the 3'-overhangs with 2'-deoxythymidine, is tolerated and does not affect the efficiency of RNAi degradation. In particular, the absence of a 2'-hydroxyl in the 2'-deoxythymidine significantly enhances the nuclease resistance of the 3'-overhang in tissue culture medium.

The sense and antisense strands of the siRNA can comprise two complementary, single-stranded RNA molecules or can comprise a single molecule in which two complementary portions are base-paired and are covalently linked by a single-stranded "hairpin" area. That is, the sense region and antisense region can be covalently connected via a linker molecule. The linker molecule can be a polynucleotide or non-nucleotide linker. Without wishing to be bound by any theory, it is believed that the hairpin area of the latter type of siRNA molecule is cleaved intracellularly by the "Dicer" protein (or its equivalent) to form a siRNA of two individual base-paired RNA molecules.

As used herein, "target mRNA" refers to an RNA molecule that is a target for downregulation.

siRNA can be expressed from pol III expression vectors without a change in targeting site, as expression of RNAs from pol III promoters is only believed to be efficient when the first transcribed nucleotide is a purine.

siRNA according to the invention can be targeted to any stretch of approximately 19-25 contiguous nucleotides in any of the target mRNA sequences (the "target sequence"). Techniques for selecting target sequences for siRNA are given, for example, in Tuschl T. et al., "The siRNA User Guide", revised Oct. 11, 2002, the entire disclosure of which is herein incorporated by reference. "The siRNA User Guide" is available on the world wide web at a website maintained by Dr. Thomas Tuschl, Laboratory of RNA Molecular Biology, Rockefeller University, New York, USA, and can be found by accessing the website of the Rockefeller University and searching with the keyword "siRNA". Thus, the sense strand of the present siRNA comprises a nucleotide sequence substantially identical to any contiguous stretch of about 19 to about 25 nucleotides in the target mRNA.

Generally, a target sequence on the target mRNA can be selected from a given cDNA sequence corresponding to the target mRNA, preferably beginning 50 to 100 nt downstream (i.e., in the 3'-direction) from the start codon. The target sequence can, however, be located in the 5'- or 3'-untranslated regions, or in the region nearby the start codon.

siRNA can be obtained using a number of techniques known to those of skill in the art. For example, siRNA can be chemically synthesized or recombinantly produced using methods known in the art, such as the Drosophila *in vitro* system described in U.S. published application 2002/0086356 of Tuschl et al., the entire disclosure of which is herein incorporated by reference.

Preferably, siRNA is chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions.

Alternatively, siRNA can also be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing siRNA of the

invention from a plasmid include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter.

Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment.

The siRNA expressed from recombinant plasmids can either be isolated from cultured cell expression systems by standard techniques, or can be expressed intracellularly. The use of recombinant plasmids to deliver siRNA to cells *in vivo* is within the skill in the art. siRNA can be expressed from a recombinant plasmid either as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions.

Selection of plasmids suitable for expressing siRNA, methods for inserting nucleic acid sequences for expressing the siRNA into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art.

The term "cell" or "host cell" preferably relates to an intact cell, i.e. a cell with an intact membrane that has not released its normal intracellular components such as enzymes, organelles, or genetic material. An intact cell preferably is a viable cell, i.e. a living cell capable of carrying out its normal metabolic functions. Preferably said term relates according to the invention to any cell which can be transformed or transfected with an exogenous nucleic acid. The term "cell" includes according to the invention prokaryotic cells (e.g., E. coli) or eukaryotic cells. Mammalian cells are particularly preferred, such as cells from humans, mice, hamsters, pigs, goats, and primates. In one embodiment, the cell is a somatic cell as described herein. In one embodiment, the cell is a cell having a barrier function. Preferably, the cell is a fibroblast such as a fibroblast described herein, a keratinocyte, an epithelial cell, or an endothelial cell such as an endothelial cell of the heart, an endothelial cell of the lung, or an umbilical vein endothelial cell. Preferably, the cell is a human cell.

A fibroblast is a type of cell that synthesizes the extracellular matrix and collagen and plays a critical role in wound healing. The main function of fibroblasts is to maintain the structural integrity of connective tissues by continuously secreting precursors of the extracellular matrix. Fibroblasts are the most common cells of connective tissue in animals. Fibroblasts are

morphologically heterogeneous with diverse appearances depending on their location and activity.

Keratinocytes are the predominant cell type in the epidermis, the outermost layer of the human skin. The primary function of keratinocytes is the formation of the keratin layer that protects the skin and the underlying tissue from environmental damage such as heat, UV radiation and water loss.

Epithelium is a tissue composed of cells that line the cavities and surfaces of structures throughout the body. Many glands are also formed from epithelial tissue. It lies on top of connective tissue, and the two layers are separated by a basement membrane. In humans, epithelium is classified as a primary body tissue, the other ones being connective tissue, muscle tissue and nervous tissue. Functions of epithelial cells include secretion, selective absorption, protection, transcellular transport and detection of sensation.

The endothelium is the thin layer of cells that lines the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. These cells are called endothelial cells. Endothelial cells line the entire circulatory system, from the heart to the smallest capillary. Endothelial tissue is a specialized type of epithelium tissue.

According to the present invention, the term "peptide" comprises oligo- and polypeptides and refers to substances comprising two or more, preferably 3 or more, preferably 4 or more, preferably 6 or more, preferably 8 or more, preferably 10 or more, preferably 13 or more, preferably 16 more, preferably 21 or more and up to preferably 8, 10, 20, 30, 40 or 50, in particular 100 amino acids joined covalently by peptide bonds. The term "protein" refers to large peptides, preferably to peptides with more than 100 amino acid residues, but in general the terms "peptides" and "proteins" are synonyms and are used interchangeably herein.

The present invention also includes "variants" of the peptides, proteins, or amino acid sequences described herein.

For the purposes of the present invention, "variants" of an amino acid sequence comprise amino acid insertion variants, amino acid addition variants, amino acid deletion variants

and/or amino acid substitution variants.

Amino acid insertion variants comprise insertions of single or two or more amino acids in a particular amino acid sequence. In the case of amino acid sequence variants having an insertion, one or more amino acid residues are inserted into a particular site in an amino acid sequence, although random insertion with appropriate screening of the resulting product is also possible.

Amino acid addition variants comprise amino- and/or carboxy-terminal fusions of one or more amino acids, such as 1, 2, 3, 5, 10, 20, 30, 50, or more amino acids.

Amino acid deletion variants are characterized by the removal of one or more amino acids from the sequence, such as by removal of 1, 2, 3, 5, 10, 20, 30, 50, or more amino acids. The deletions may be in any position of the protein. Amino acid deletion variants that comprise the deletion at the N-terminal and/or C-terminal end of the protein are also called N-terminal and/or C-terminal truncation variants.

Amino acid substitution variants are characterized by at least one residue in the sequence being removed and another residue being inserted in its place. Preference is given to the modifications being in positions in the amino acid sequence which are not conserved between homologous proteins or peptides and/or to replacing amino acids with other ones having similar properties. Preferably, amino acid changes in protein variants are conservative amino acid changes, i.e., substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids.

Preferably the degree of similarity, preferably identity between a given amino acid sequence and an amino acid sequence which is a variant of said given amino acid sequence will be at least about 60%, 65%, 70%, 80%, 81%, 82%, 83%, 84%, 84%, 85%, 86%, 87%, 88%, 89%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. The degree of similarity or identity is given preferably for an amino acid region which is at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or about 100% of the entire length of the reference amino acid sequence. For example, if the reference amino acid sequence consists of 200 amino acids, the degree of similarity or identity is given preferably for at least about 20, at least about 40, at least about 60, at least about 80, at least about 100, at least about 120, at least about 140, at least about 160, at least about 180, or about 200 amino acids, preferably continuous amino acids. In preferred embodiments, the degree of similarity or identity is given for the entire length of the reference amino acid sequence. The alignment for determining sequence similarity, preferably sequence identity can be done with art known tools, preferably using the best sequence alignment, for example, using Align, using standard settings, preferably EMBOSS::needle, Matrix: Blosum62, Gap Open 10.0, Gap Extend 0.5.

"Sequence similarity" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions. "Sequence identity" between two amino acid sequences indicates the percentage of amino acids or nucleotides that are identical between the sequences.

The term "percentage identity" is intended to denote a percentage of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Sequence comparisons between two amino acid sequences are conventionally carried out by comparing these sequences after having aligned them optimally, said comparison being carried out by segment or by "window of comparison" in order to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman, 1981, Ads App. Math. 2, 482, by means of the local homology algorithm of Neddleman and Wunsch, 1970, J. Mol. Biol. 48, 443, by means of the similarity search method of Pearson and Lipman, 1988, Proc. Natl Acad. Sci. USA 85, 2444, or by means of computer programs which use these algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

The percentage identity is calculated by determining the number of identical positions between the two sequences being compared, dividing this number by the number of positions compared and multiplying the result obtained by 100 so as to obtain the percentage identity between these two sequences.

Homologous amino acid sequences exhibit according to the invention at least 40%, in particular at least 50%, at least 60%, at least 70%, at least 80%, at least 90% and preferably at least 95%, at least 98 or at least 99% identity of the amino acid residues.

The amino acid sequence variants described herein may readily be prepared by the skilled person, for example, by recombinant DNA manipulation. The manipulation of DNA sequences for preparing peptides or proteins having substitutions, additions, insertions or deletions, is described in detail in Sambrook et al. (1989), for example. Furthermore, the peptides and amino acid variants described herein may be readily prepared with the aid of known peptide synthesis techniques such as, for example, by solid phase synthesis and similar methods.

The invention includes derivatives of the peptides or proteins described herein which are comprised by the terms "peptide" and "protein". According to the invention, "derivatives" of proteins and peptides are modified forms of proteins and peptides. Such modifications include any chemical modification and comprise single or multiple substitutions, deletions and/or additions of any molecules associated with the protein or peptide, such as carbohydrates, lipids and/or proteins or peptides. In one embodiment, "derivatives" of proteins or peptides include those modified analogs resulting from glycosylation, acetylation, phosphorylation, amidation, palmitoylation, myristoylation, isoprenylation, lipidation, alkylation, derivatization, introduction of protective/blocking groups, proteolytic cleavage or binding to an antibody or to another cellular ligand. The term "derivative" also extends to all functional chemical equivalents of said proteins and peptides. Preferably, a modified peptide has increased stability and/or increased immunogenicity.

According to the invention, a variant of a peptide or protein preferably has a functional property of the peptide or protein from which it has been derived. Such functional properties are described herein for OCT4, SOX2, NANOG, LIN28, KLF4 and c-MYC, respectively. Preferably, a variant of a peptide or protein has the same property in reprogramming an

animal differentiated cell as the peptide or protein from which it has been derived. Preferably, the variant induces or enhances reprogramming of an animal differentiated cell.

In one embodiment, the peptide or protein encoded by the RNA is a factor allowing the reprogramming of somatic cells to cells having stem cell characteristics. In one embodiment, the peptide or protein comprises one or more antigens and/or one or more antigen peptides. Preferably, said RNA is capable of expressing said peptide or protein, in particular if introduced into a cell.

A "stem cell" is a cell with the ability to self-renew, to remain undifferentiated, and to become differentiated. A stem cell can divide without limit, for at least the lifetime of the animal in which it naturally resides. A stem cell is not terminally differentiated; it is not at the end stage of a differentiation pathway. When a stem cell divides, each daughter cell can either remain a stem cell or embark on a course that leads toward terminal differentiation.

Totipotent stem cells are cells having totipotential differentiation properties and being capable of developing into a complete organism. This property is possessed by cells up to the 8-cell stage after fertilization of the oocyte by the sperm. When these cells are isolated and transplanted into the uterus, they can develop into a complete organism.

Pluripotent stem cells are cells capable of developing into various cells and tissues derived from the ectodermal, mesodermal and endodermal layers. Pluripotent stem cells which are derived from the inner cell mass located inside of blastocysts, generated 4-5 days after fertilization are called "embryonic stem cells" and can differentiate into various other tissue cells but cannot form new living organisms.

Multipotent stem cells are stem cells differentiating normally into only cell types specific to their tissue and organ of origin. Multipotent stem cells are involved not only in the growth and development of various tissues and organs during the fetal, neonatal and adult periods but also in the maintenance of adult tissue homeostasis and the function of inducing regeneration upon tissue damage. Tissue-specific multipotent cells are collectively called "adult stem cells".

An "embryonic stem cell" or "ESC" is a stem cell that is present in or isolated from an embryo. It can be pluripotent, having the capacity to differentiate into each and every cell present in the organism, or multipotent, with the ability to differentiate into more than one cell type.

As used herein, "embryo" refers to an animal in the early stages of it development. These stages are characterized by implantation and gastrulation, where the three germ layers are defined and established and by differentiation of the germs layers into the respective organs and organ systems. The three germ layers are the endoderm, ectoderm and mesoderm.

A "blastocyst" is an embryo at an early stage of development in which the fertilized ovum has undergone cleavage, and a spherical layer of cells surrounding a fluid-filled cavity is forming, or has formed. This spherical layer of cells is the trophectoderm. Inside the trophectoderm is a cluster of cells termed the inner cell mass (ICM). The trophectoderm is the precursor of the placenta, and the ICM is the precursor of the embryo.

An adult stem cell, also called a somatic stem cell, is a stem cell found in an adult. An adult stem cell is found in a differentiated tissue, can renew itself, and can differentiate, with some limitations, to yield specialized cell types of its tissue of origin. Examples include mesenchymal stem cells, hematopoietic stem cells, and neural stem cells.

A "differentiated cell" is a mature cell that has undergone progressive developmental changes to a more specialized form or function. Cell differentiation is the process a cell undergoes as it matures to an overtly specialized cell type. Differentiated cells have distinct characteristics, perform specific functions, and are less likely to divide than their less differentiated counterparts.

An "undifferentiated" cell, for example, an immature, embryonic, or primitive cell, typically has a nonspecific appearance, may perform multiple, non-specific activities, and may perform poorly, if at all, in functions typically performed by differentiated cells.

"Somatic cell" refers to any and all differentiated cells and does not include stem cells, germ cells, or gametes. Preferably, "somatic cell" as used herein refers to a terminally differentiated cell.

As used herein, "committed" refers to cells which are considered to be permanently committed to a specific function. Committed cells are also referred to as "terminally differentiated cells".

As used herein, "differentiation" refers to the adaptation of cells for a particular form or function. In cells, differentiation leads to a more committed cell.

As used herein, "de-differentiation" refers to loss of specialization in form or function. In cells, de-differentiation leads to a less committed cell.

As used herein "reprogramming" refers to the resetting of the genetic program of a cell. A reprogrammed cell preferably exhibits pluripotency.

The terms "de-differentiated" and "reprogrammed" or similar terms are used interchangeably herein to denote somatic cell-derived cells having stem cell characteristics. However, said terms are not intended to limit the subject-matter disclosed herein by mechanistic or functional considerations.

The term "RNA inducing the development of stem cell characteristics" or "RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics" refers to RNA which when introduced into a somatic cell induces the cell to de-differentiate.

As used herein, "germ cell" refers to a reproductive cell such as a spermatocyte or an oocyte, or a cell that will develop into a reproductive cell.

As used herein, "pluripotent" refers to cells that can give rise to any cell type except the cells of the placenta or other supporting cells of the uterus.

Terms such as "cell having stem cell characteristics", "cell having stem cell properties" or "stem like cell" are used herein to designate cells which, although they are derived from differentiated somatic non-stem cells, exhibit one or more features typical for stem cells, in particular embryonic stem cells. Such features include an embryonic stem cell morphology

such as compact colonies, high nucleus to cytoplasm ratio and prominent nucleoli, normal karyotypes, expression of telomerase activity, expression of cell surface markers that are characteristic for embryonic stem cells, and/or expression of genes that are characteristic for embryonic stem cells. The cell surface markers that are characteristic for embryonic stem cells are, for example, selected from the group consisting of stage-specific embryonic antigen-3 (SSEA-3), SSEA-4, tumor-related antigen-1-60 (TRA-1-60), TRA-1-81, and TRA-2-49/6E. The genes that are characteristic for embryonic stem cells are selected, for example, from the group consisting of endogenous OCT4, endogenous NANOG, growth and differentiation factor 3 (GDF3), reduced expression 1 (REX1), fibroblast growth factor 4 (FGF4), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated 2 (DPPA2), DPPA4, and telomerase reverse transcriptase (TERT). In one embodiment, the one or more features typical for stem cells include pluripotency.

In one embodiment of the method of the invention, the stem cell characteristics comprise an embryonic stem cell morphology, wherein said embryonic stem cell morphology preferably comprises morphological ciriteria selected from the group consisting of compact colonies, high nucleus to cytoplasm ratio and prominent nucleoli. In certain embodiments, the cells having stem cell characteristics have normal karyotypes, express telomerase activity, express cell surface markers that are characteristic for embryonic stem cells and/or express genes that are characteristic for embryonic stem cells. The cell surface markers that are characteristic for embryonic stem cells may be selected from the group consisting of stage-specific embryonic antigen-3 (SSEA-3), SSEA-4, tumor-related antigen-1-60 (TRA-1-60), TRA-1-81, and TRA-2-49/6E and the genes that are characteristic for embryonic stem cells may be selected from the group consisting of endogenous OCT4, endogenous NANOG, growth and differentiation factor 3 (GDF3), reduced expression 1 (REX1), fibroblast growth factor 4 (FGF4), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated 2 (DPPA2), DPPA4, and telomerase reverse transcriptase (TERT).

Preferably, the cells having stem cell characteristics are de-differentiated and/or reprogrammed somatic cells. Preferably, the cells having stem cell characteristics exhibit the essential characteristics of embryonic stem cells such as a pluripotent state. Preferably, the cells having stem cell characteristics have the developmental potential to differentiate into advanced derivatives of all three primary germ layers. In one embodiment, the primary germ layer is endoderm and the advanced derivative is gut-like epithelial tissue. In a further

embodiment, the primary germ layer is mesoderm and the advanced derivative is striated muscle and/or cartilage. In an even further embodiment, the primary germ layer is ectoderm and the advanced derivative is neural tissue and/or epidermal tissue. In one preferred embodiment, the cells having stem cell characteristics have the developmental potential to differentiate into neuronal cells and/or cardiac cells.

In one embodiment, the somatic cells are embryonic stem cell derived somatic cells with a mesenchymal phenotype. In a preferred embodiment, the somatic cells are fibroblasts such as fetal fibroblasts or postnatal fibroblasts or keratinocytes, preferably hair follicle derived keratinocytes. In further embodiments, the fibroblasts are lung fibroblasts, foreskin fibroblasts or dermal fibroblasts. In particular embodiments, the fibroblasts are fibroblasts as deposited at the American Type Culture Collection (ATCC) under Catalog No. CCL-186, as deposited at the American Type Culture Collection (ATCC) under Catalog No. CRL-2097 or as deposited at the American Type Culture Collection (ATCC) under Catalog No. CRL-2522, or as distributed by System Biosciences under the catalog no. PC501A-HFF. In one embodiment, the fibroblasts are adult human dermal fibroblasts. Preferably, the somatic cells are human cells. According to the present invention, the somatic cells may be genetically modified.

The term "factor" according to the invention when used in conjunction with the expression thereof by RNA includes proteins and peptides as well as derivatives and variants thereof. For example, the term "factor" comprises OCT4, SOX2, NANOG, LIN28, KLF4 and c-MYC.

The factors can be of any animal species; e.g., mammals and rodents. Examples of mammals include but are not limited to human and non-human primates. Primates include but are not limited to humans, chimpanzees, baboons, cynomolgus monkeys, and any other New or Old World monkeys. Rodents include but are not limited to mouse, rat, guinea pig, hamster and gerbil.

According to the present invention, one or more factors capable of allowing the reprogramming of somatic cells to cells having stem cell characteristics comprise an assembly of factors selected from the group consisting of (i) OCT4 and SOX2, (ii) OCT4, SOX2, and one or both of NANOG and LIN28, (iii) OCT4, SOX2 and one or both of KLF4 and c-MYC. In one embodiment, said one or more factors capable of being expressed by the RNA comprise OCT4, SOX2, NANOG and LIN28 or OCT4, SOX2, KLF4 and c-MYC. Preferably,

the RNA is introduced into said animal differentiated somatic cell by electroporation or microinjection. Preferably, the method of the invention further comprises allowing the development of cells having stem cell characteristics, e.g. by culturing the somatic cell under embryonic stem cell culture conditions, preferably conditions suitable for maintaining pluripotent stem cells in an undifferentiated state.

OCT4 is a transcription factor of the eukaryotic POU transcription factors and an indicator of pluripotency of embryonic stem cells. It is a maternally expressed Octomer binding protein. It has been observed to be present in oocytes, the inner cell mass of blastocytes and also in the primordial germ cell. The gene *POU5F1* encodes the OCT4 protein. Synonyms to the gene name include *OCT3*, *OCT4*, *OTF3* and *MGC22487*. The presence of OCT4 at specific concentrations is necessary for embryonic stem cells to remain undifferentiated.

Preferably, "OCT4 protein" or simply "OCT4" relates to human OCT4 and preferably comprises an amino acid sequence encoded by the nucleic acid according to SEQ ID NO: 1, preferably the amino acid sequence according to SEQ ID NO: 2. One skilled in the art would understand that the cDNA sequence of OCT4 as described above would be equivalent to OCT4 mRNA, and can be used for the generation of RNA capable of expressing OCT4.

Sox2 is a member of the Sox (SRY-related HMG box) gene family that encode transcription factors with a single HMG DNA-binding domain. SOX2 has been found to control neural progenitor cells by inhibiting their ability to differentiate. The repression of the factor results in delamination from the ventricular zone, which is followed by an exit from the cell cycle. These cells also begin to lose their progenitor character through the loss of progenitor and early neuronal differentiation markers.

Preferably, "SOX2 protein" or simply "SOX2" relates to human SOX2 and preferably comprises an amino acid sequence encoded by the nucleic acid according to SEQ ID NO: 3, preferably the amino acid sequence according to SEQ ID NO: 4. One skilled in the art would understand that the cDNA sequence of SOX2 as described above would be equivalent to SOX2 mRNA, and can be used for the generation of RNA capable of expressing SOX2.

NANOG is a NK-2 type homeodomain gene, and has been proposed to play a key role in maintaining stem cell pluripotency presumably by regulating the expression of genes critical

to embryonic stem cell renewal and differentiation. NANOG behaves as a transcription activator with two unusually strong activation domains embedded in its C terminus. Reduction of NANOG expression induces differentiation of embryonic stem cells.

Preferably, "NANOG protein" or simply "NANOG" relates to human NANOG and preferably comprises an amino acid sequence encoded by the nucleic acid according to SEQ ID NO: 5, preferably the amino acid sequence according to SEQ ID NO: 6. One skilled in the art would understand that the cDNA sequence of NANOG as described above would be equivalent to NANOG mRNA, and can be used for the generation of RNA capable of expressing NANOG.

LIN28 is a conserved cytoplasmic protein with an unusual pairing of RNA-binding motifs: a cold shock domain and a pair of retroviral-type CCHC zinc fingers. In mammals, it is abundant in diverse types of undifferentiated cells. In pluripotent mammalian cells, LIN28 is observed in RNase-sensitive complexes with Poly(A)-Binding Protein, and in polysomal fractions of sucrose gradients, suggesting it is associated with translating mRNAs.

Preferably, "LIN28 protein" or simply "LIN28" relates to human LIN28 and preferably comprises an amino acid sequence encoded by the nucleic acid according to SEQ ID NO: 7, preferably the amino acid sequence according to SEQ ID NO: 8. One skilled in the art would understand that the cDNA sequence of LIN28 as described above would be equivalent to LIN28 mRNA, and can be used for the generation of RNA capable of expressing LIN28.

Krueppel-like factor (KLF4) is a zinc-finger transcription factor, which is strongly expressed in postmitotic epithelial cells of different tissues, e.g. the colon, the stomach and the skin. KLF4 is essential for the terminal differentiation of these cells and involved in the cell cycle regulation.

Preferably, "KLF4 protein" or simply "KLF4" relates to human KLF4 and preferably comprises an amino acid sequence encoded by the nucleic acid according to SEQ ID NO: 9, preferably the amino acid sequence according to SEQ ID NO: 10. One skilled in the art would understand that the cDNA sequence of KLF4 as described above would be equivalent to KLF4 mRNA, and can be used for the generation of RNA capable of expressing KLF4.

MYC (cMYC) is a protooncogene, which is overexpressed in a wide range of human cancers.

When it is specifically-mutated, or overexpressed, it increases cell proliferation and functions as an oncogene. MYC gene encodes for a transcription factor that regulates expression of 15% of all genes through binding on Enhancer Box sequences (E-boxes) and recruiting histone acetyltransferases (HATs). MYC belongs to MYC family of transcription factors, which also includes N-MYC and L-MYC genes. MYC-family transcription factors contain the bHLH/LZ (basic Helix-Loop-Helix Leucine Zipper) domain

Preferably, "cMYC protein" or simply "cMYC" relates to human cMYC and preferably comprises an amino acid sequence encoded by the nucleic acid according to SEQ ID NO: 11, preferably the amino acid sequence according to SEQ ID NO: 12. One skilled in the art would understand that the cDNA sequence of cMYC as described above would be equivalent to cMYC mRNA, and can be used for the generation of RNA capable of expressing cMYC.

A reference herein to specific factors such as OCT4, SOX2, NANOG, LIN28, KLF4 or c-MYC or to specific sequences thereof is to be understood so as to also include all variants of these specific factors or the specific sequences thereof as described herein. In particular, it is to be understood so as to also include all splice variants, posttranslationally modified variants, conformations, isoforms and species homologs of these specific factors/sequences which are naturally expressed by cells.

The term "miRNA" (microRNA) relates to 21–23-nucleotide-long noncoding RNAs found in eukaryotic cells that, by inducing degradation and/or preventing translation of target mRNAs, modulate a plethora of cell functions, including those related to ESC self-renewal/differentiation and cell cycle progression. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression or target degradation and gene silencing. It has been found that miRNAs in the right combination are capable of inducing direct cellular reprogramming of somatic cells to cells having stem cell characteristics in vitro. For example, it has been observed that miRNA cluster 302-367 enhances somatic cell reprogramming.

Preferably, the step of allowing the development of cells having stem cell characteristics used in the methods for providing cells having stem cell characteristics described herein comprises culturing the somatic cells under embryonic stem cell culture conditions, preferbly conditions suitable for maintaining pluripotent stem cells in an undifferentiated state.

Preferably, to allow the development of cells having stem cell characteristics, cells are cultivated in the presence of one or more DNA methyltransferase inhibitors and/or one or more histone deacetylase inhibitors. Preferred compounds are selected from the group consisting of 5'-azacytidine (5'-azaC), suberoylanilide hydroxamic acid (SAHA), dexamethasone, trichostatin A (TSA), sodium butyrate (NaBu), Scriptaid and valproic acid (VPA). Preferably, cells are cultivated in the presence of valproic acid (VPA), preferably in a concentration of between 0.5 and 10 mM, more preferably between 1 and 5 mM, most preferably in a concentration of about 2 mM.

The methods of the present invention can be used to effect de-differentiation of any type of somatic cell. Cells that may be used include cells that can be de-differentiated or reprogrammed by the methods of the present invention, in particular cells that are fully or partially differentiated, more preferably terminally differentiated. Preferably, the somatic cell is a diploid cell derived from pre-embryonic, embryonic, fetal, and post-natal multi-cellular organisms. Examples of cells that may be used include but are not limited to fibroblasts, such as fetal and neonatal fibroblasts or adult fibroblasts, keratinocytes, in particular primary keratinocytes, more preferably keratinocytes derived from hair, adipose cells, epithelial cells, epidermal cells, chondrocytes, cumulus cells, neural cells, glial cells, astrocytes, cardiac cells, esophageal cells, muscle cells, melanocytes, hematopoietic cells, osteocytes, macrophages, monocytes, and mononuclear cells.

The cells with which the methods of the invention can be used can be of any animal species; e.g., mammals and rodents. Examples of mammalian cells that can be de-differentiated and re-differentiated by the present invention include but are not limited to human and non-human primate cells. Primate cells with which the invention may be performed include but are not limited to cells of humans, chimpanzees, baboons, cynomolgus monkeys, and any other New or Old World monkeys. Rodent cells with which the invention may be performed include but are not limited to mouse, rat, guinea pig, hamster and gerbil cells.

De-differentiated cells prepared according to the present invention are expected to display many of the same requirements as pluripotent stem cells and can be expanded and maintained under conditions used for embryonic stem cells, e.g. ES cell medium or any medium that supports growth of the embryonic cells. Embryonic stem cells retain their pluripotency *in*

vitro when maintained on inactivated fetal fibroblasts such as irradiated mouse embryonic fibroblasts or human fibroblasts (e.g., human foreskin fibroblasts, human skin fibroblasts, human endometrial fibroblasts, human oviductal fibroblasts) in culture. In one embodiment, the human feeder cells may be autologous feeder cells derived from the same culture of reprogrammed cells by direct differentiation.

Furthermore, human embryonic stem cells can successfully be propagated on Matrigel in a medium conditioned by mouse fetal fibroblasts. Human stem cells can be grown in culture for extended period of time and remain undifferentiated under specific culture conditions.

In certain embodiments, the cell culture conditions may include contacting the cells with factors that can inhibit differentiation or otherwise potentiate de-differentiation of cells, e.g., prevent the differentiation of cells into non-ES cells, trophectoderm or other cell types.

De-differentiated cells prepared according to the present invention can be evaluated by methods including monitoring changes in the cells' phenotype and characterizing their gene and protein expression. Gene expression can be determined by RT-PCR, and translation products can be determined by immunocytochemistry and Western blotting. In particular, de-differentiated cells can be characterized to determine the pattern of gene expression and whether the reprogrammed cells display a pattern of gene expression similar to the expression pattern expected of undifferentiated, pluripotent control cells such as embryonic stem cells using techniques well known in the art including transcriptomics.

The expression of the following genes of de-differentiated cells can be assessed in this respect: OCT4, NANOG, growth and differentiation factor 3 (GDF3), reduced expression 1 (REX1), fibroblast growth factor 4 (FGF4), embryonic cell-specific gene 1 (ESG1), developmental pluripotency-associated 2 (DPPA2), DPPA4, telomerase reverse transcriptase (TERT), embryonic antigen-3 (SSEA-3), SSEA-4, tumor-related antigen-1-60 (TRA-1-60), TRA-1-81, and TRA-2-49/6E.

The undifferentiated or embryonic stem cells to which the reprogrammed cells may be compared may be from the same species as the differentiated somatic cells. Alternatively, the undifferentiated or embryonic stem cells to which the reprogrammed cells may be compared may be from a different species as the differentiated somatic cells.

In some embodiments, a similarity in gene expression pattern exists between a reprogrammed cell and an undifferentiated cell, e.g., embryonic stem cell, if certain genes specifically expressed in an undifferentiated cell are also expressed in the reprogrammed cell. For example, certain genes, e.g., telomerase, that are typically undetectable in differentiated somatic cells may be used to monitor the extent of reprogramming. Likewise, for certain genes, the absence of expression may be used to assess the extent of reprogramming.

Self-renewing capacity, marked by induction of telomerase activity, is another characteristic of stem cells that can be monitored in de-differentiated cells.

Karyotypic analysis may be performed by means of chromosome spreads from mitotic cells, spectral karyotyping, assays of telomere length, total genomic hybridization, or other techniques well known in the art.

Using the present invention, RNA encoding appropriate factors is incorporated into one or more somatic cells, e.g. by electroporation. After incorporation, cells are preferably cultured using conditions that support maintenance of de-differentiated cells (i.e. stem cell culture conditions). The de-differentiated cells can then be expanded and induced to re-differentiate into different type of somatic cells that are needed for cell therapy. De-differentiated cells obtained according to the present invention can be induced to differentiate into one or more desired somatic cell types *in vitro* or *in vivo*.

Preferably, the de-differentiated cells obtained according to the present invention may give rise to cells from any of three embryonic germ layers, i.e., endoderm, mesoderm, and ectoderm. For example, the de-differentiated cells may differentiate into skeletal muscle, skeleton, dermis of skin, connective tissue, urogenital system, heart, blood (lymph cells), and spleen (mesoderm); stomach, colon, liver, pancreas, urinary bladder; lining of urethra, epithelial parts of trachea, lungs, pharynx, thyroid, parathyroid, intestine (endoderm); or central nervous system, retina and lens, cranial and sensory, ganglia and nerves, pigment cells, head connective tissue, epidermis, hair, mammary glands (ectoderm). The de-differentiated cells obtained according to the present invention can be re-differentiated *in vitro* or *in vivo* using techniques known in the art.

In one embodiment of the present invention, the reprogrammed cells resulting from the methods of this invention are used to produce differentiated progeny. Thus, in one aspect, the present invention provides a method for producing differentiated cells, comprising: (i) obtaining reprogrammed cells using the methods of this invention; and (ii) inducing differentiation of the reprogrammed cells to produce differentiated cells. Step (ii) can be performed *in vivo* or *in vitro*. Furthermore, differentiation can be induced through the presence of appropriate differentiation factors which can either be added or are present in situ, e.g. in a body, organ or tissue into which the reprogrammed cells have been introduced. The differentiated cells can be used to derive cells, tissues and/or organs which are advantageously used in the area of cell, tissue, and/or organ transplantation. If desired, genetic modifications can be introduced, for example, into somatic cells prior to reprogramming. The differentiated cells of the present invention preferably do not possess the pluripotency of an embryonic stem cell, or an embryonic germ cell, and are, in essence, tissue-specific partially or fully differentiated cells.

One advantage of the methods of the present invention is that the reprogrammed cells obtained by the present invention can be differentiated without prior selection or purification or establishment of a cell line. Accordingly in certain embodiments, a heterogeneous population of cells comprising reprogrammed cells are differentiated into a desired cell type. In one embodiment, a mixture of cells obtained from the methods of the present invention is exposed to one or more differentiation factors and cultured *in vitro*.

Methods of differentiating reprogrammed cells obtained by the methods disclosed herein may comprise a step of permeabilization of the reprogrammed cell. For example, cells generated by the reprogramming techniques described herein, or alternatively a heterogeneous mixture of cells comprising reprogrammed cells, may be permeabilized before exposure to one or more differentiation factors or cell extract or other preparation comprising differentiation factors.

For example, differentiated cells may be obtained by culturing undifferentiated reprogrammed cells in the presence of at least one differentiation factor and selecting differentiated cells from the culture. Selection of differentiated cells may be based on phenotype, such as the expression of certain cell markers present on differentiated cells, or by functional assays (e.g., the ability to perform one or more functions of a particular differentiated cell type).

In another embodiment, the cells reprogrammed according to the present invention are genetically modified through the addition, deletion, or modification of their DNA sequence(s).

The reprogrammed or de-differentiated cells prepared according to the present invention or cells derived from the reprogrammed or de-differentiated cells are useful in research and in therapy. Reprogrammed pluripotent cells may be differentiated into any of the cells in the body including, without limitation, skin, cartilage, bone skeletal muscle, cardiac muscle, renal, hepatic, blood and blood forming, vascular precursor and vascular endothelial, pancreatic beta, neurons, glia, retinal, neuronal, intestinal, lung, and liver cells.

The reprogrammed cells are useful for regenerative/reparative therapy and may be transplanted into a patient in need thereof. In one embodiment, the cells are autologous with the patient.

The reprogrammed cells provided in accordance with the present invention may be used, for example, in therapeutic strategies in the treatment of cardiac, neurological, endocrinological, vascular, retinal, dermatological, muscular-skeletal disorders, and other diseases.

For example, and not intended as a limitation, the reprogrammed cells of the present invention can be used to replenish cells in animals whose natural cells have been depleted due to age or ablation therapy such as cancer radiotherapy and chemotherapy. In another non-limiting example, the reprogrammed cells of the present invention are useful in organ regeneration and tissue repair. In one embodiment of the present invention, reprogrammed cells can be used to reinvigorate damaged muscle tissue including dystrophic muscles and muscles damaged by ischemic events such as myocardial infarcts. In another embodiment of the present invention, the reprogrammed cells disclosed herein can be used to ameliorate scarring in animals, including humans, following a traumatic injury or surgery. In this embodiment, the reprogrammed cells of the present invention are administered systemically, such as intravenously, and migrate to the site of the freshly traumatized tissue recruited by circulating cytokines secreted by the damaged cells. In another embodiment of the present invention, the reprogrammed cells can be administered locally to a treatment site in need or repair or regeneration.

In further embodiments, the RNA used in the present invention encodes a peptide or protein which is of therapeutic value. Cells containing the RNA can, for example, be manipulated *in vitro* to express the RNA and thus, the peptide or protein, using the methods of the invention. The cells expressing the peptide or protein can subsequently be introduced into a patient.

In a particularly preferred embodiment, the RNA used in the present invention encodes a peptide or protein comprising an immunogen, antigen or antigen peptide. In one embodiment, the peptide or protein is processed after expression to provide said immunogen, antigen or antigen peptide. In another embodiment, the peptide or protein itself is the immunogen, antigen or antigen peptide. Cells expressing such peptide or protein comprising an immunogen, antigen or antigen peptide can be used, for example, in immunotherapy to elicit an immune response against the immunogen, antigen or antigen peptide in a patient.

An "antigen" according to the invention covers any substance that will elicit an immune response. In particular, an "antigen" relates to any substance that reacts specifically with antibodies or T-lymphocytes (T-cells). According to the present invention, the term "antigen" comprises any molecule which comprises at least one epitope. Preferably, an antigen in the context of the present invention is a molecule which, optionally after processing, induces an immune reaction, which is preferably specific for the antigen. According to the present invention, any suitable antigen may be used, which is a candidate for an immune reaction, wherein the immune reaction may be both a humoral as well as a cellular immune reaction. In the context of the embodiments of the present invention, the antigen is preferably presented by a cell, preferably by an antigen presenting cell, in the context of MHC molecules, which results in an immune reaction against the antigen. An antigen is preferably a product which corresponds to or is derived from a naturally occurring antigen. Such naturally occurring antigens may include or may be derived from allergens, viruses, bacteria, fungi, parasites and other infectious agents and pathogens or an antigen may also be a tumor antigen. According to the present invention, an antigen may correspond to a naturally occurring product, for example, a viral protein, or a part thereof.

In a preferred embodiment, the antigen is a tumor antigen, i.e., a part of a tumor cell which may be derived from the cytoplasm, the cell surface or the cell nucleus, in particular those which primarily occur intracellularly or as surface antigens of tumor cells. For example,

tumor antigens include the carcinoembryonal antigen, α1-fetoprotein, isoferritin, and fetal sulphoglycoprotein, α2-H-ferroprotein and γ-fetoprotein, as well as various virus tumor antigens. According to the present invention, a tumor antigen preferably comprises any antigen which is characteristic for tumors or cancers as well as for tumor or cancer cells with respect to type and/or expression level. In another embodiment, the antigen is a virus antigen such as viral ribonucleoprotein or coat protein. In particular, the antigen should be presented by MHC molecules which results in modulation, in particular activation of cells of the immune system, preferably $CD4^+$ and $CD8^+$ lymphocytes, in particular via the modulation of the activity of a T-cell receptor.

In preferred embodiments, the antigen is a tumor antigen and the present invention involves the stimulation of an anti-tumor CTL response against tumor cells expressing such tumor antigen and preferably presenting such tumor antigen with class I MHC.

The term "immunogenicity" relates to the relative effectivity of an antigen to induce an immune reaction.

The term "pathogen" relates to pathogenic microorganisms and comprises viruses, bacteria, fungi, unicellular organisms, and parasites. Examples for pathogenic viruses are human immunodeficiency virus (HIV), cytomegalovirus (CMV), herpes virus (HSV), hepatitis A-virus (HAV), HBV, HCV, papilloma virus, and human T-lymphotrophic virus (HTLV). Unicellular organisms comprise plasmodia trypanosomes, amoeba, etc.

Examples for antigens that may be used in the present invention are p53, ART-4, BAGE, ss-catenin/m, Bcr-abL CAMEL, CAP-1, CASP-8, CDC27/m, CDK4/m, CEA, CLAUDIN-12, c-MYC, CT, Cyp-B, DAM, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, Gap100, HAGE, HER-2/neu, HPV-E7, HPV-E6, HAST-2, hTERT (or hTRT), LAGE, LDLR/FUT, MAGE-A, preferably MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, or MAGE-A12, MAGE-B, MAGE-C, MART-1/Melan-A, MC1R, Myosin/m, MUC1, MUM-1, -2, -3, NA88-A, NF1, NY-ESO-1, NY-BR-1, p190 minor BCR-abL, Plac-1, Pml/RARa, PRAME, proteinase 3, PSA, PSM, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, SCGB3A2, SCP1, SCP2, SCP3, SSX, SURVIVIN, TEL/AML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2, TPTE and WT, preferably WT-1.

"A portion or fragment of an antigen" or "an antigen peptide" according to the invention preferably is an incomplete representation of an antigen and is capable of eliciting an immune response against the antigen.

In this context, the invention also makes use of peptides comprising amino acid sequences derived from antigens, also termed "antigen peptides" herein. By "antigen peptide", or "antigen peptide derived from an antigen" is meant an oligopeptide or polypeptide comprising an amino acid sequence substantially corresponding to the amino acid sequence of a fragment or peptide of an antigen. An antigen peptide may be of any length.

Preferably, the antigen peptides are capable of stimulating an immune response, preferably a cellular response against the antigen or cells characterized by expression of the antigen and preferably by presentation of the antigen. Preferably, an antigen peptide is capable of stimulating a cellular response against a cell characterized by presentation of an antigen with class I MHC and preferably is capable of stimulating an antigen-responsive CTL. Preferably, the antigen peptides according to the invention are MHC class I and/or class II presented peptides or can be processed to produce MHC class I and/or class II presented peptides. Preferably, the antigen peptides comprise an amino acid sequence substantially corresponding to the amino acid sequence of a fragment of an antigen. Preferably, said fragment of an antigen is an MHC class I and/or class II presented peptide. Preferably, an antigen peptide according to the invention comprises an amino acid sequence substantially corresponding to the amino acid sequence of such fragment and is processed to produce such fragment, i.e., an MHC class I and/or class II presented peptide derived from an antigen.

If an antigen peptide is to be presented directly, i.e., without processing, in particular without cleavage, it has a length which is suitable for binding to an MHC molecule, in particular a class I MHC molecule, and preferably is 7-20 amino acids in length, more preferably 7-12 amino acids in length, more preferably 8-11 amino acids in length, in particular 9 or 10 amino acids in length. Preferably the sequence of an antigen peptide which is to be presented directly is derived from the amino acid sequence of an antigen, i.e., its sequence substantially corresponds and is preferably completely identical to a fragment of an antigen.

If an antigen peptide is to be presented following processing, in particular following cleavage,

the peptide produced by processing has a length which is suitable for binding to an MHC molecule, in particular a class I MHC molecule, and preferably is 7-20 amino acids in length, more preferably 7-12 amino acids in length, more preferably 8-11 amino acids in length, in particular 9 or 10 amino acids in length. Preferably, the sequence of the peptide which is to be presented following processing is derived from the amino acid sequence of an antigen, i.e., its sequence substantially corresponds and is preferably completely identical to a fragment of an antigen. Thus, an antigen peptide according to the invention in one embodiment comprises a sequence of 7-20 amino acids in length, more preferably 7-12 amino acids in length, more preferably 8-11 amino acids in length, in particular 9 or 10 amino acids in length which substantially corresponds and is preferably completely identical to a fragment of an antigen and following processing of the antigen peptide makes up the presented peptide. However, the antigen peptide may also comprise a sequence which substantially corresponds and preferably is completely identical to a fragment of an antigen which is even longer than the above stated sequence. In one embodiment, an antigen peptide may comprise the entire sequence of an antigen.

Peptides having amino acid sequences substantially corresponding to a sequence of a peptide which is presented by the class I MHC may differ at one or more residues that are not essential for TCR recognition of the peptide as presented by the class I MHC, or for peptide binding to MHC. Such substantially corresponding peptides are also capable of stimulating an antigen-responsive CTL. Peptides having amino acid sequences differing from a presented peptide at residues that do not affect TCR recognition but improve the stability of binding to MHC may improve the immunogenicity of the antigen peptide, and may be referred to herein as "optimized peptide". Using existing knowledge about which of these residues may be more likely to affect binding either to the MHC or to the TCR, a rational approach to the design of substantially corresponding peptides may be employed. Resulting peptides that are functional are contemplated as antigen peptides.

"Antigen processing" refers to the degradation of an antigen into fragments (e.g., the degradation of a protein into peptides) and the association of one or more of these fragments (e.g., via binding) with MHC molecules for presentation by "antigen presenting cells" to specific T-cells.

"Antigen presenting cells" (APC) are cells which present peptide fragments of protein antigens in association with MHC molecules on their cell surface. Some APCs may activate antigen-specific T-cells.

The term "immunotherapy" relates to a treatment involving activation of a specific immune reaction.

The term "*in vivo*" relates to the situation in a subject.

The terms "subject" and "individual" are used interchangeably and relate to mammals. For example, mammals in the context of the present invention are humans, non-human primates, domesticated animals such as dogs, cats, sheep, cattle, goats, pigs, horses etc., laboratory animals such as mice, rats, rabbits, guinea pigs, etc. as well as animals in captivity such as animals of zoos. The term "animal" as used herein also includes humans. The term "subject" may also include a patient, i.e., an animal, preferably a human having a disease.

If according to the invention administration to a subject is desired the composition for administration is generally administered in pharmaceutically compatible amounts and in pharmaceutically compatible preparations. The term "pharmaceutically compatible" refers to a nontoxic material which does not interact with the action of the active component of the pharmaceutical composition. Preparations of this kind may usually contain salts, buffer substances, preservatives, excipients and carriers and are administered in a manner known to the skilled person.

The present invention is described in detail by the figures and examples below, which are used only for illustration purposes and are not meant to be limiting. Owing to the description and the examples, further embodiments which are likewise included in the invention are accessible to the skilled worker.

EXAMPLES

Example 1: Repetitive transfer of IVT-RNA (Reprogramming-TF)

Reprogramming of somatic cells into induced pluripotent stem cells (iPS) requires the continuous expression of reprogramming transcription factors (rTF). To avoid the risk of genomic integration which arises when the rTF are delivered virally into the cell, rTF can be efficiently delivered as mRNA by electroporation or lipofection without accompanied modifications of the host genome. Nevertheless the delivery has to be performed repetitively to assure constant expression of the rTF.

CCD1079Sk fibroblasts were electroporated as indicated in the side panel of Fig. 2A either with 15µg or 5µg of each *in vitro* transcribed (IVT)-RNA encoding the transcription factors OCT4 (O), SOX2 (S), KLF4 (K) and cMYC (M) and cultivated in human embryonic stem (ES) cell medium. Electroporations were performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. At the indicated time points, 10% of the cells were removed from the cultures prior to subsequent electroporation, total RNA was isolated and mRNA-expression of the human ES-marker genes OCT4 (endogenous), TERT, GDF3 and DPPA4 was quantified by qRT-PCR. Repetitive electroporation of IVT-RNA coding for 4 rTF (OCT4, SOX2, KLF4 and cMYC) results in rapid induction of some pluripotency markers such as GFD3, DPPA4 and TERT. Other markers such as endogenous OCT4 were not or slightly induced.

CCD1079Sk fibroblasts were electroporated as indicated in the side panel of Fig. 2B with 15µg of each IVT-RNA encoding the transcription factors OSKM and cultivated in human ES cell medium. Electroporations were performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. At the indicated time points remaining cells were counted and survival rate in relation to the starting cells was calculated. Repetitive IVT-RNA transfer is accompanied with massive cell death and successful reprogramming is therefore not achievable.

CCD1079Sk fibroblasts were electroporated with 1 µg IVT RNA encoding for firefly luciferase (Luc) and 5 µg IVT RNA encoding for green fluorescent protein (GFP). Electroporations were performed in 2 mm gap cuvettes using optimized parameters for

CCD1079Sk fibroblasts. 24h post electroporation, cells were pelleted, total RNA was isolated and mRNA-expression of Interferon (IFN)-a and –b was quantified by qRT-PCR. It was observed that electroporation of IVT-RNA is followed by an induction of IFNa and b 24h thereafter; cf. Fig. 2C.

CCD1079Sk fibroblasts were electroporated with 33,4 µg IVT RNA encoding reprogramming mixture (29,5µg rTF (OSKM NANOG (N) LIN28 (L) (1:1:1:1:1:1)), 1,3µg SV40 largeT antigen (lgT), 1,3µg HTLV E6 and 1,25µg GFP). Electroporations were performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. 48h post electroporation, cells were pelleted, total RNA was isolated and mRNA-expression of the IFN-response genes OAS1, OAS2, MX1, IFITM1 and IRF9 was quantified by qRT-PCR. All 5 investigated IFN-response genes are induced 48h post electroporation of IVT-RNA; cf. Fig. 2D. The IFN-response has originally evolved as part of the host innate immune response to viral infections. Viral nucleic acids are efficiently recognized by sensor molecules which leads to antiviral activities including apoptosis, cytoskeletal remodeling, RNA degradation and a halt in protein translation. These mechanisms obviously hinder RNA-based gene transfer.

CCD1079Sk fibroblasts were electroporated once with the amounts of IVT-RNA encoding the reporter genes Luc, GFP or the Protein Kinase R (PKR) wild type indicated in Fig. 2E. Electroporations were performed in 4 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. Cells were lysed 24h post electroporation and expression and phosphorylation status of the PKR target eukaryotic initiation factor 2a (eIF2a) was monitored by Western Blotting using specific antibodies. One of the major player in the IFN response is the PKR which upon activation phosphorylates its target eIF2a leading to an inhibition of translation. We could show that eIF2a is phosphorylated 24h after electroporation of IVT-RNA identifying activation of PKR as one of the prominent obstacles in RNA-based reprogramming.

Repetitive RNA-based gene transfer is accompanied with an induction of the IFN-response which hinders the continuous expression of rTF and therefore successful RNA-based reprogramming.

Example 2: Use of E3, K3 and B18R in RNA-based gene transfer

As a proof of concept unmodified IVT-RNAs coding for the viral escape proteins E3, K3 and B18R (vaccinia virus) were added to a mixture of unmodified IVT-RNA (Luciferase/GFP), lipofected into human foreskin fibroblasts (HFF) and translation of the reporter gene GFP and IFN-response to the RNA was analyzed. Furthermore the survival of the cells after repetitive lipofections was assessed by an Cell Proliferation Kit II (Roche).

CCD1079Sk fibroblasts were plated into 6 wells (100,000 cells/well) and lipofected the next day using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures was thereby composed of 0.8µg GFP with 0.2µg of each B18R, E3 or K3 (as indicated in Fig. 3A,B). IVT-RNA encoding for Luc was used to sum up the mixtures to 1.4µg. Lipofections were performed according to the manufacturers instructions and cells were harvested 48h post transfection. 20% of the cells were used for analysis of GFP expression by FACS (Fig. 3B), whereas the rest of the cells were pelleted, total RNA was isolated and mRNA-expression of IFNb and OAS1 was quantified by qRT-PCR (Fig. 3A). As shown in Fig. 3A, IFNb and the IFN-response gene OAS1 are clearly induced by lipofection of IVT-RNA (Luc/GFP). This induction can be reduced in the case of IFNb by E3/K3 alone, but only the combination of all 3 viral escape proteins is able to reduce significantly both IFNb and OAS1-induction by IVT-RNA 48h post lipofection. As shown in Fig. 3B, expression of the reporter gene GFP is enhanced by addition of E3 and K3. B18R has no effect on the translation of GFP.

CCD1079Sk fibroblasts were plated into 6 wells (100.000 cells/well) and lipofected the next four consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixture was thereby composed of 0.8µg GFP with 0.2µg of each B18R, E3 or K3 (as indicated). IVT-RNA encoding for Luc was used to sum up the mixture to 1.4µg total IVT-RNA. As a control, 1.4µg modified (mod.) IVT-RNA encoding for Luc (0.6µg) and GFP (0.8µg) was used. These RNAs were composed of 100% pseudouridine (psi) and 100% 5-methylcytidine (5mC) instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, cell viability was assayed using the Cell Proliferation Kit II (Roche) and normalized to the mock transfected cells. As shown in Fig. 3C, daily lipofection of unmodified IVT-RNA (Luc/GFP, 4 times) is accompanied with massive loss in cell viability.

The combination of all 3 viral escape proteins is able to overcome this obstacle and enhances survival of the cells even more than the use of modified IVT-RNA (100% psi and 5mC).
It is concluded that repetitive RNA-based gene transfer is possible when the combination of E3, K3 and B18R coded by IVT-RNA is added. Proof of concept was achieved.

Example 3: Use of E3, K3 and B18R in RNA-based gene transfer for reprogramming

After reaching the proof of concept that the addition of IVT-RNA coding for E3, K3 and B18R allows repetitive RNA-based gene transfer, we investigated whether this also holds true for the transfer of a reprogramming mixture. To this aim 6 rTF (OCT4, SOX2, KLF4, cMYC, NANOG, LIN28; short: OSKMNL) were mixed in a molar ratio of 1:1:1:1:1:1 and transferred by lipofection to HFFs. Again, survival of the cells and induction of IFN-response was analyzed after 4 daily repetitive lipofections.

CCD1079Sk fibroblasts were plated into 6 wells (80,000 cells/well) and lipofected the next four consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures were thereby composed of 0.8µg unmodified GFP or 0.8µg OSKMNL (1:1:1:1:1:1) either unmodified or modified and either with 0.2µg of each B18R, E3 and K3 unmodified or modified. If necessary IVT-RNA encoding for Luc was used to sum up the mixture to 1.4µg total IVT-RNA. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, cell viability was assayed using the Cell Proliferation Kit II (Roche) with normalization to mock transfected cells (Fig. 4A) and by microscopy (Fig. 4B). After that, cells were pelleted, total RNA was isolated and mRNA-expression of IFNb and OAS1 was quantified by qRT-PCR (Fig. 4C). As expected viability of the cells transfected with unmodified IVT-RNA (Luc/GFP or OSKMNL) is lost when E3/K3/B18R (EKB) are not present. In this set of experiments the viability is comparable to mock transfected cells when EKB are present. As seen with reporter gene IVT-RNA (see Fig. 3), the survival is even higher in the presence of EKB than with modified IVT-RNA alone. The effects observed in Fig. 4A are visualized in Fig. 4B by representative pictures taken with a microscope. Since the cells transfected with unmodified IVT-RNA did not survive 4 daily repetitive lipofections in this set of experiments, IFN-response could only be analyzed in the remaining samples by qRT-PCR. As can be seen in Fig. 4C, IFN-response measured by the induction of IFNb and OAS1 is nearly diminished in

samples with EKB. The reduction in IFN-response is even lesser than with the use of modified IVT-RNA.

It is concluded that repetitive RNA-based gene transfer with reprogramming rTF is possible when the combination of E3, K3 and B18R coded by IVT-RNA is added. Survival of the cells and reduction of IFN-response is even more pronounced than with modified IVT-RNA.

Example 4: Translation of rTF after repetitive lipofection in the presence of E3, K3 and B18R

Besides the survival of the cells and the reduction of IFN-response, it was also addressed how the rTF are translated after 3 daily lipofections in the presence of E3, K3 and B18R. Expression levels of transferred rTF OCT4, SOX2 and NANOG were analyzed by intracellular FACS-Staining.

CCD1079Sk fibroblasts were plated into 6 wells (100,000 cells/well) and lipofected the next three consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures was thereby composed of 0.2µg GFP with 0.6µg OCT4 or SOX2 or NANOG either unmodified or modified and 0.2µg of each B18R, E3 and K3 (EKB) either unmodified or modified. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, intracellular expression of OSN was monitored by FACS analysis using the human pluripotent stem cell transcription factor analysis kit (BD 560589). Expression levels of NANOG, OCT4 and SOX2 were higher in the presence of EKB when applicated unmodified; cf. Fig. 5.

It is concluded that in the presence of EKB, unmodified IVT-RNA leads to higher expressions of rTF which may result in a more efficient reprogramming.

Example 5: Reprogramming of HFF using rTF and microRNA in the presence of EKB

Repetitive lipofection of rTF-mixture was achieved by addition of EKB to the reprogramming mixture leading to a better survival and higher reduction of IFN-response after daily lipofections. Furthermore higher expression levels of rTF were achieved in the presence of

EKB. In the next experiments these mixture was used for long time lipofections to achieve reprogramming of HFFs. To further enhance reprogramming the microRNAs of cluster 302/367 were added to the reprogramming mixture as well. These miRNAs are thought to be mainly involved in the cellular maintenance of self-renewal and pluripotency, and could lead to reprogramming when expressed by lentiviral vectors (Anokye-Danso et al., 2011).

HFF fibroblasts (System Bioscience) were plated into 6 wells (100,000 cells/well) and lipofected 5 times a week (Monday to Friday) for two weeks using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT-RNA (Fig. 6A). The IVT-RNA mixtures were thereby composed of 0.8µg unmodified GFP or 0.8µg OSKMNL (1:1:1:1:1:1) either unmodified or modified with either 0.2µg of each B18R, E3 and K3 (EKB) either unmodified or modified and 0.4µg of a miRNA mixture composed of miRNAs 302a-d and 367 [0.4µM each]. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections in stem cell media (Nutristem media, Stemgent) were performed according to the manufacturers instructions. On day 6 and day 13, cells were pelleted, total RNA was isolated and mRNA-expression of the human ES-marker TERT, DPPA4, GDF3, LIN28 (endogenous) and REX1 was quantified by qRT-PCR. Colony growth was observed by microscopy and for further analysis, colonies were stained for the ES surface marker TRA-1-60 using the StainAlive TRA-1-60 antibody (Stemgent) following the manufacturers instructions.

As shown in Fig. 6B, analysis of the expression levels of several pluripotency marker revealed that in the sample with unmodified OSKMNL, unmodified EKB and miRNA mixture, all analysed pluripotency marker were highly expressed compared to modified OSMNL, modified EKB and miRNA-mixture or miRNA-mixture with unmodified EKB alone. Unmodified IVT-RNA outstands thereby modification with 5mC and Psi. From d10 on colony formation was observed in the sample with unmodified OSKMNL, unmodified EKB and miRNA mixture; cf. Fig. 6C. In other samples such as miRNA alone no colony formation was observed. In the combination of modified OSKMNL with modified EKB and miRNA 1-3 colonies appeared, which was way behind the combination of unmodified OSKMNL and miRNA-mixture in the presence of EKB. As shown in Fig. 6D, the Ribo-iPS achieved by repetitive transfection with unmodified OSMNL, unmodified EKB and miRNA-mixture could be stained positive for TRA-1-60, a surface marker for human embryonic stem cells.

It is concluded that repetitive RNA-based gene transfer with rTF encoded by unmodified IVT-RNA in combination with unmodified EKB and a miRNA-mixture of mature miRNAs from the cluster 302/367 leads to a highly efficient and robust generation of Ribo-iPS cells characterized by high expression of pluripotency markers and stem cell surface marker. The use of unmodified IVT-RNA thereby outstands the use of modified IVT-RNA.

Example 6: Reprogramming of HFF using rTF and microRNA in the presence of EKB (splitting 1:8)

In the first set of experiments for reprogramming with rTF coded by unmodified IVT-RNA, EKB and miRNA-mixture 302/367 cells were splitted 1:4. In the next experiments cells were splitted 1:8 to avoid a dense growing of the cells.

HFF fibroblasts (System Bioscience) were plated into 6 wells (100,000 cells/well) and lipofected 5 times a week (Monday to Friday) for two weeks using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT-RNA (Fig. 7A). The IVT-RNA mixtures were thereby composed of 0.8µg OSKMNL (1:1:1:1:1:1) either unmodified or modified with either 0.2µg of each B18R, E3 and K3 (EKB) unmodified or modified and 0.4µg of a miRNA mixture composed of miRNAs 302a-d and 367 [0.4µM each]. Modified RNAs were composed of 100% psi and 100% 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections in stem cell media (Nutristem media, Stemgent) were performed according to the manufacturers instructions. On day 5 and day 12, cells were pelleted, total RNA was isolated and mRNA-expression of the human ES-marker TERT, DPPA4, GDF3, LIN28 (endogenous) and REX1 was quantified by qRT-PCR. Colony growth was observed by microscopy and for further analysis, colonies were stained for the ES surface marker TRA-1-60 using the StainAlive TRA-1-60 antibody (Stemgent) or for the activity of alkaline phosphatase (Vector Red staining kit) following the manufacturers instructions.

As shown in Fig. 7B, analysis of the expression levels of several pluripotency marker revealed that in the sample with unmodified OSKMNL, unmodified EKB and miRNA mixture, all analysed pluripotency marker were highly expressed compared to modified OSMNL, modified EKB and miRNA-mixture. Again, unmodified IVT-RNA outstands modification with 5mC and Psi. Also, from d10 on colony formation was observed in both samples (unmodified or modified OSKMNL). These colonies could be stained positive for

TRA-1-60 - a surface marker for human embryonic stem cells; cf. Fig. 7C. As shown in Fig. 7D, the colonies also displayed high activity of Alkaline Phosphatase - another stem cell marker. The obvious higher efficiency of unmodified OSKMNL compared to modified OSKMNL can clearly be seen in the upper panel of Fig. 7D.

It is concluded that repetitive RNA-based gene transfer with reprogramming rTF coded by unmodified IVT-RNA in combination with unmodified EKB and a miRNA-mixture of mature miRNAs from the cluster 302/367 leads to a highly efficient and robust generation of Ribo-iPS cells characterized by high expression of pluripotency markers and stem cell surface marker. The use of unmodified IVT-RNA thereby outstands the use of modified IVT-RNA.

Example 7: Titration of EKB

One possibility to further enhance the efficiency of reprogramming would be the addition of more rTF-IVT-RNA or other enhancing factors encoded by IVT-RNA. Since the lipofection protocol is limited to a certain amount of IVT-RNA, we investigated whether the amount of EKB can be reduced when added to the reprogramming cocktail. HFFs were therefore lipofected with the 6 rTF and miRNA combined with different amounts of EKB reaching from 0.0001µg to 0.2µg (amount used in reprogramming experiments) each. Survival of the cells and induction of IFN-response was analyzed after 4 daily repetitive lipofections.

HFF fibroblasts (System Bioscience) were plated into 6 wells (100,000 cells/well) and lipofected the next four consecutive days using 6µl RNAiMAX (Invitrogen) and 1.4µg IVT. The IVT-RNA mixtures were thereby composed of 0.8µg unmodified OSKMNL (1:1:1:1:1:1) with variable amounts of unmodfied B18R, E3 and K3 as indicated. IVT-RNA encoding for Luc was used to sum up the mixture to 1.4µg total IVT-RNA. According to the reprogramming experiments 0.4µg of a miRNA mixture composed of miRNAs 302a-d and 367 [0.4µM each] was added to the samples. As a control, 1.4µg modified (mod.) IVT-RNA encoding for Luc (0.6µg) and OSKMNL (0.8µg; 1:1:1:1:1:1) was used. These RNAs were composed of 100% psi and 5mC instead of uridine and cytidine which display less immunstimulatory characteristics. Lipofections were performed according to the manufacturers instructions. 24h after the last lipofection, cell viability was assayed using the Cell Proliferation Kit II (Roche). After that, cells were pelleted, total RNA was isolated and mRNA-expression of IFNb and OAS1 was quantified by qRT-PCR.

As shown in Fig. 8A, viability of HFFs after repetitive lipofections was sustained down to a reduction of EKB to 0.025µg IVT-RNA of each factor. Analysis of the IFN-response by measuring the expression levels of IFNb and OAS1 revealed that again the IFN-response to IVT-RNA is clearly reduced down to an amount of 0.025µg of EKB; cf. Fig. 8B. Nevertheless with the residual induction of IFN-response we recommend the use of 0.05µg of EKB.

It is concluded that the amount of EKB can be reduced to 0.025-0.05µg of each IVT-RNA.

Example 8: Effect E3 and K3 alone

So far, only the combination of E3 and K3 was used. In this set of experiments the effect of E3 and K3 alone on translation of the reporter gene Luc and on IFN response was analyzed.

CCD1079SK fibroblasts were electroporated with IVT RNA encoding Luc (1 µg), GFP (5 µg) and 3 µg of E3 or K3 or both as indicated. Electroporations were performed in 2 mm gap cuvettes using optimized parameters for CCD1079Sk fibroblasts. 10000 cells/well were plated in duplicates into 96-well-plates. Luciferase activity was measured at the time points indicated in Fig. 9A after electroporation using the Bright Glo Luciferase Assay System (Promega). Mean values of the duplicates are given. Furthermore, 300000 cells/well were plated into 6-well-plates and 24h post electroporation, cells were pelleted, total RNA was isolated and mRNA-expression of OAS1 and IFN-b was analyzed by RT-PCR and in case of IFN-b quantified using the Quanti Tect SYBR Green PCR Kit.

As shown in Fig. 9A, the translation of Luc was enhanced with E3 or K3 alone as much as with both of them together. As seen for lipofection of IVT-RNA, IFNb and the IFN-response gene OAS1 are clearly induced by electroporation of IVT-RNA (Luc/GFP). These inductions cannot be reduced neither by E3 or K3 nor by the combination of both 24h post electroporation; cf. Fig. 9B.

These experiments indicate that one intracellular viral escape protein (E3 or K3) coded by IVT-RNA may be sufficient to allow repetitive RNA-based gene transfer as seen with the combination EKB.

CLAIMS

1. A method for expressing RNA in a cell comprising the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling.

2. The method of claim 1, wherein the RNA has been introduced into the cell.

3. The method of claim 1 or 2, wherein the RNA has been introduced into the cell repetitively.

4. The method of any one of claims 1 to 3, wherein the RNA has been introduced into the cell by electroporation or lipofection.

5. The method of any one of claims 1 to 4, wherein the RNA is *in vitro* transcribed RNA.

6. The method of any one of claims 1 to 5, wherein preventing engagement of IFN receptor by extracellular IFN inhibits autocrine and/or paracrine IFN functions.

7. The method of any one of claims 1 to 6, wherein preventing engagement of IFN receptor by extracellular IFN comprises providing a binding agent for extracellular IFN.

8. The method of claim 7, wherein the binding agent for extracellular IFN is a viral binding agent for extracellular IFN.

9. The method of claim 8, wherein the viral binding agent for extracellular IFN is a viral interferon receptor.

10. The method of claim 8 or 9, wherein the viral binding agent for extracellular IFN is vaccinia virus B18R.

11. The method of any one of claims 8 to 10, wherein the viral binding agent for extracellular IFN is provided to the cell in the form of a nucleic acid encoding the binding agent.

12. The method of any one of claims 1 to 11, wherein the intracellular IFN signalling results in inhibition of translation and/or RNA degradation.

13. The method of any one of claims 1 to 12, wherein inhibiting intracellular IFN signalling comprises inhibiting one or more IFN-inducible antivirally active effector proteins.

14. The method of claim 13, wherein the IFN-inducible antivirally active effector protein is selected from the group consisting of RNA-dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (OAS) and RNaseL.

15. The method of any one of claims 1 to 14, wherein inhibiting intracellular IFN signalling comprises inhibiting the PKR-dependent pathway and/or the OAS-dependent pathway.

16. The method of claim 15, wherein inhibiting the PKR-dependent pathway comprises inhibiting eIF2-alpha phosphorylation.

17. The method of claim 16, wherein inhibiting eIF2-alpha phosphorylation comprises inhibiting PKR and/or providing a pseudosubstrate mimicking eIF2-alpha.

18. The method of claim 17, wherein the pseudosubstrate mimicking eIF2-alpha is a viral pseudosubstrate mimicking eIF2-alpha.

19. The method of claim 18, wherein the viral pseudosubstrate mimicking eIF2-alpha is vaccinia virus K3.

20. The method of claim 18 or 19, wherein the viral pseudosubstrate mimicking eIF2-alpha is provided to the cell in the form of a nucleic acid encoding the viral pseudosubstrate.

21. The method of claim 14 or 17, wherein inhibiting PKR comprises treating the cell with at least one PKR inhibitor.

22. The method of claim 21, wherein the PKR inhibitor inhibits RNA-induced PKR autophosphorylation.

23. The method of claim 21 or 22, wherein the PKR inhibitor is an ATP-binding site directed inhibitor of PKR.

24. The method of any one of claims 21 to 23, wherein the PKR inhibitor is an imidazolo-oxindole compound.

25. The method of any one of claims 21 to 24, wherein the PKR inhibitor is 6,8-dihydro-8-(1H-imidazol-5-ylmethylene)-7H-pyrrolo[2,3-g]benzothiazol-7-one and/or 2-aminopurine.

26. The method of any one of claims 21 to 23, wherein the PKR inhibitor is a viral inhibitor of PKR.

27. The method of claim 26, wherein the viral inhibitor of PKR is vaccinia virus E3.

28. The method of claim 26 or 27, wherein the viral inhibitor of PKR is provided to the cell in the form of a nucleic acid encoding the inhibitor.

29. The method of claim 14 or 17, wherein inhibiting PKR comprises silencing expression of the PKR gene.

30. The method of any one of claims 15 to 29, wherein inhibiting the OAS-dependent pathway comprises inhibiting activation of RNaseL.

31. The method of any one of claims 15 to 30, wherein inhibiting the OAS-dependent pathway comprises inhibiting OAS.

32. The method of claim 14 or 31, wherein inhibiting OAS comprises treating the cell with at least one OAS inhibitor.

33. The method of claim 32, wherein the OAS inhibitor is a viral inhibitor of OAS.

34. The method of claim 33, wherein the viral inhibitor of OAS is vaccinia virus E3.

35. The method of claim 33 or 34, wherein the viral inhibitor of OAS is provided to the cell in the form of a nucleic acid encoding the inhibitor.

36. The method of any one of claims 1 to 35, wherein the RNA expressed in the cell is not modified by pseudouridine and/or 5-methylcytidine.

37. The method of any one of claims 1 to 36, wherein the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling enhance stability and/or expression of the RNA in the cell.

38. The method of claim 37, wherein the enhancement of expression of the RNA in the cell comprises an increase in the level of expression and/or an increase in the duration of expression of the RNA in the cell.

39. The method of any one of claims 1 to 38, wherein the steps of (i) preventing engagement of IFN receptor by extracellular IFN and (ii) inhibiting intracellular IFN signalling enhance cell viability.

40. The method of any one of claims 1 to 39, which comprises treating the cell with vaccinia virus B18R and one or both of vaccinia virus E3 and vaccinia virus K3.

41. The method of claim 40, wherein the vaccinia virus B18R, vaccinia virus E3 and/or vaccinia virus K3 are provided to the cell in the form of nucleic acid encoding the vaccinia virus B18R, vaccinia virus E3 and/or vaccinia virus K3.

42. The method of any one of claims 1 to 41, wherein the cell is a cell having a barrier function.

43. The method of any one of claims 1 to 42, wherein the cell is a fibroblast, a keratinocyte, an epithelial cell, or an endothelial cell.

44. The method of claim 43, wherein the endothelial cell is an endothelial cell of the heart, an endothelial cell of the lung, or an umbilical vein endothelial cell.

45. The method of any one of claims 1 to 44, wherein the cell is a human cell.

46. A method for providing cells having stem cell characteristics comprising the steps of (i) providing a cell population comprising somatic cells, (ii) preventing engagement of IFN receptor of the somatic cells by extracellular IFN, (iii) inhibiting intracellular IFN signalling in the somatic cells, (iv) introducing RNA capable of expressing one or more factors allowing the reprogramming of the somatic cells to cells having stem cell characteristics into the somatic cells, and (v) allowing the development of cells having stem cell characteristics.

47. The method of claim 46, which further comprises introducing into the somatic cells miRNA enhancing reprogramming of the somatic cells to cells having stem cell characteristics.

48. The method of claim 46 or 47, wherein the one or more factors comprise OCT4 and SOX2.

49. The method of claim 48, wherein the one or more factors further comprise KLF4 and/or c-MYC.

50. The method of claim 48 or 49, wherein the one or more factors further comprise NANOG and/or LIN28.

51. The method of any one of claims 46 to 49, wherein the one or more factors comprise OCT4, SOX2, KLF4 and c-MYC.

52. The method of claim 51, wherein the one or more factors further comprise LIN28 and optionally NANOG.

53. The method of any one of claims 46 to 48, wherein the one or more factors comprise OCT4, SOX2, NANOG and LIN28.

54. The method of any one of claims 46 to 53, further comprising the step of culturing the somatic cells in the presence of at least one histone deacetylase inhibitor.

55. The method of claim 54, wherein the at least one histone deacetylase inhibitor comprises valproic acid.

56. The method of any one of claims 46 to 55, wherein step (v) comprises culturing the somatic cells under embryonic stem cell culture conditions.

57. The method of any one of claims 46 to 56, wherein the stem cell characteristics comprise an embryonic stem cell morphology.

58. The method of any one of claims 46 to 57, wherein the cells having stem cell characteristics have normal karyotypes, express telomerase activity, express cell surface markers that are characteristic for embryonic stem cells and/or express genes that are characteristic for embryonic stem cells.

59. The method of any one of claims 46 to 58, wherein the cells having stem cell characteristics exhibit a pluripotent state.

60. The method of any one of claims 46 to 59, wherein the cells having stem cell characteristics have the developmental potential to differentiate into advanced derivatives of all three primary germ layers.

61. The method of any one of claims 46 to 60, wherein the somatic cells are fibroblasts.

62. The method of claim 61, wherein the fibroblasts are lung fibroblasts, foreskin fibroblasts or dermal fibroblasts.

63. The method of any one of claims 46 to 62, wherein the somatic cells are human cells.

64. A method for providing differentiated cell types comprising the steps of (i) providing cells having stem cell characteristics using the method of any one of claims 46 to 63, and (ii)

culturing the cells having stem cell characteristics under conditions that induce or direct partial or complete differentiation to a differentiated cell type.

65. A composition comprising (i) an agent useful for preventing engagement of IFN receptor by extracellular IFN and (ii) an agent useful for inhibiting intracellular IFN signalling.

66. A kit comprising the composition of claim 65.

Figure 1

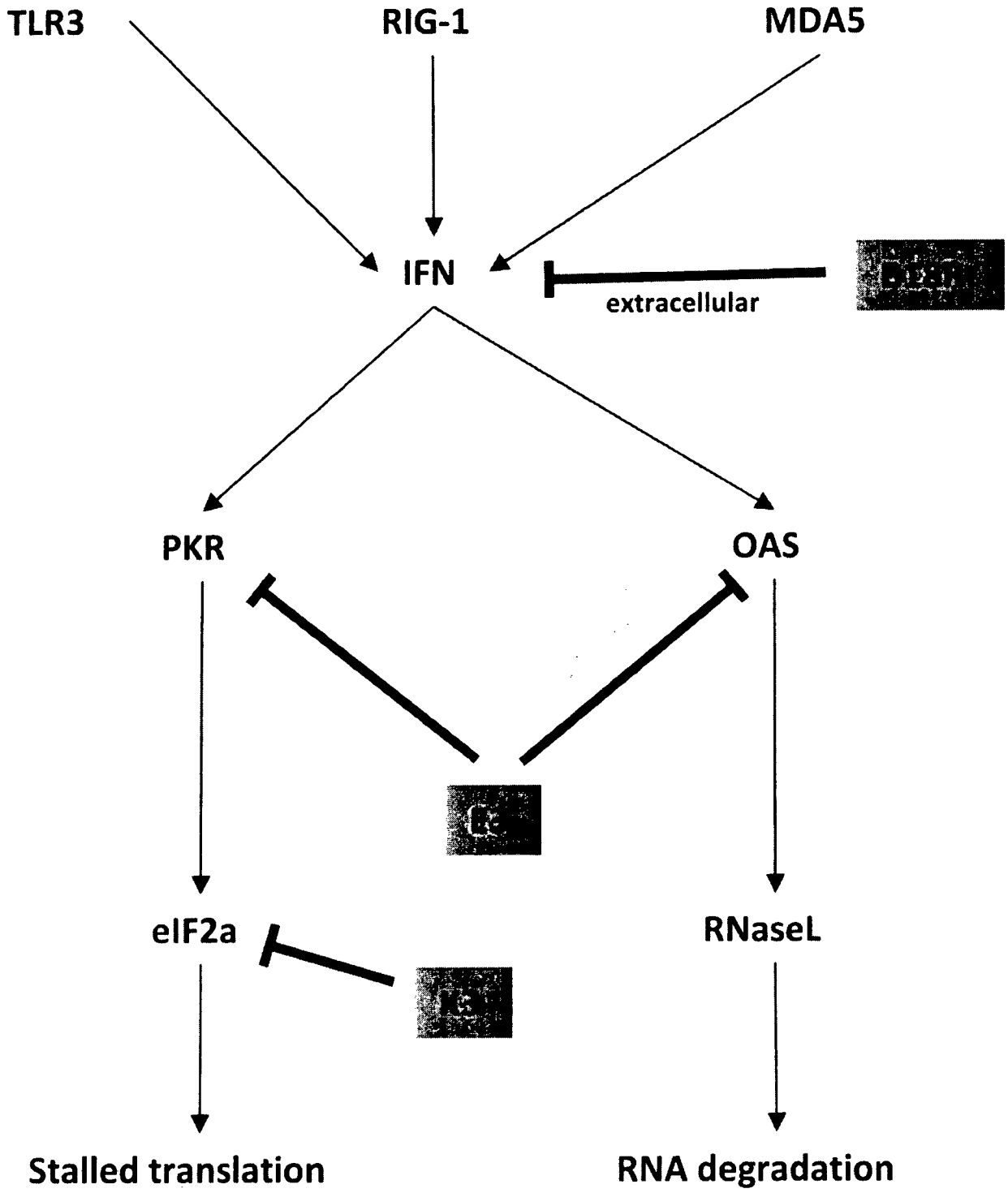

TLR3
RIG-1
MDA5
IFN
extracellular
PKR
OAS
eIF2a
RNaseL
Stalled translation
RNA degradation

Figure 2

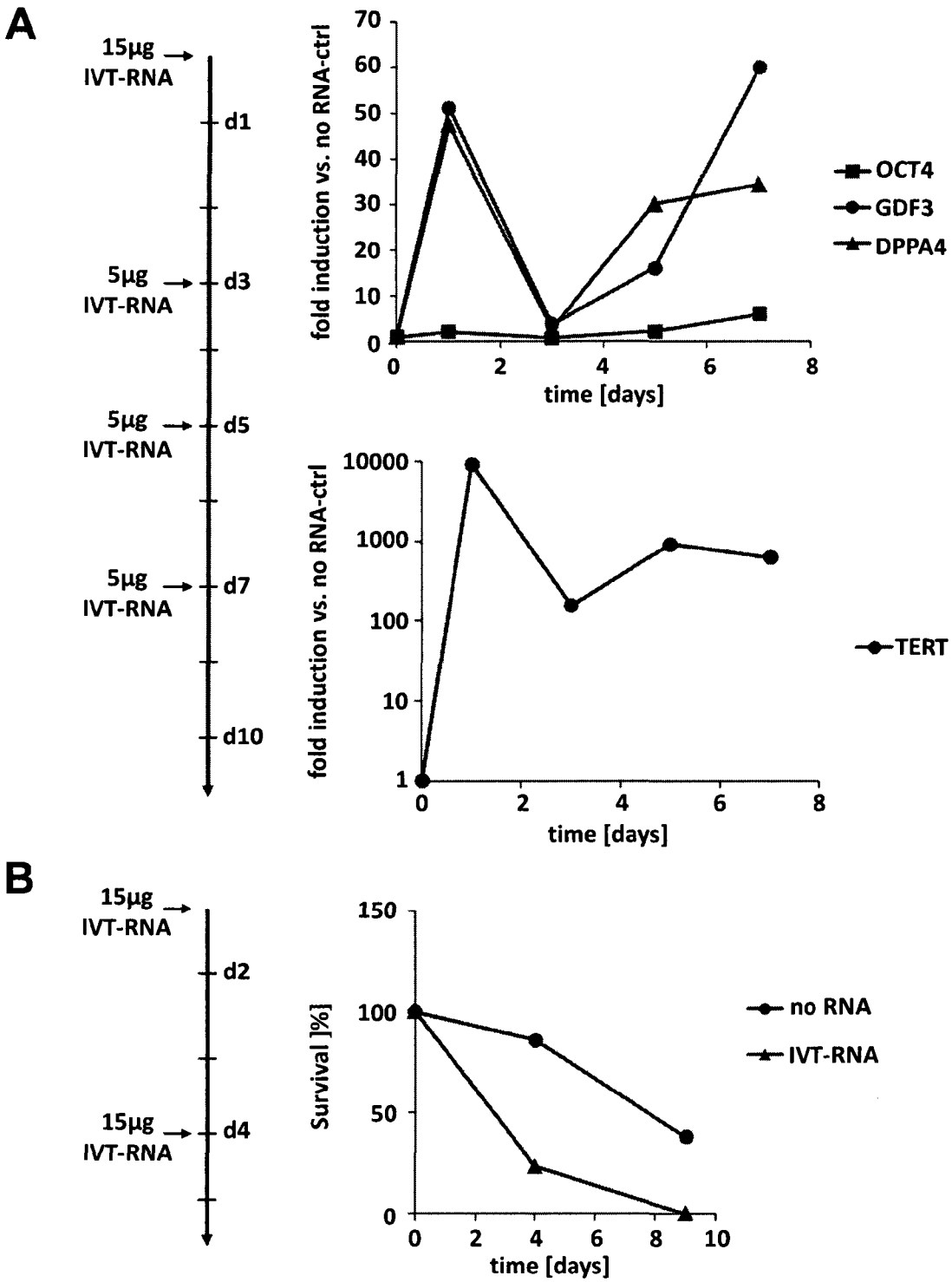
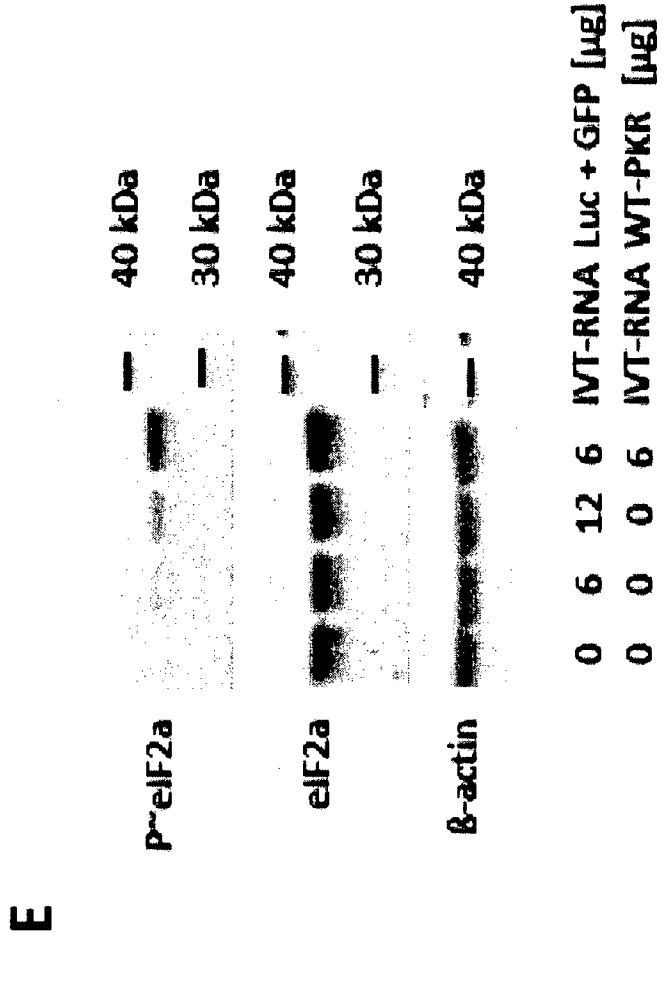

A
15µg IVT-RNA
d1
5µg IVT-RNA
d3
5µg IVT-RNA
d5
5µg IVT-RNA
d7
d10
fold induction vs. no RNA-ctrl
70
60
50
40
30
20
10
0
0
2
4
6
8
time [days]
OCT4
GDF3
DPPA4
fold induction vs. no RNA-ctrl
10000
1000
100
10
1
0
2
4
6
8
time [days]
TERT
B
15µg IVT-RNA
d2
15µg IVT-RNA
d4
Survival [%]
150
100
50
0
0
2
4
6
8
10
time [days]
no RNA
IVT-RNA

Figure 2


fold induction vs. mock
2500
2000
1500
1000
500
0
IFNβ
no RNA
IVT-RNA
fold induction vs. mock
25
20
15
10
5
0
IFNα
no RNA
IVT-RNA

C

Figure 2


E
P-eIF2a
eIF2a
β-actin
40 kDa
30 kDa
40 kDa
30 kDa
40 kDa
0 6 12 6 IVT-RNA Luc + GFP [µg]
0 0 0 6 IVT-RNA WT-PKR [µg]

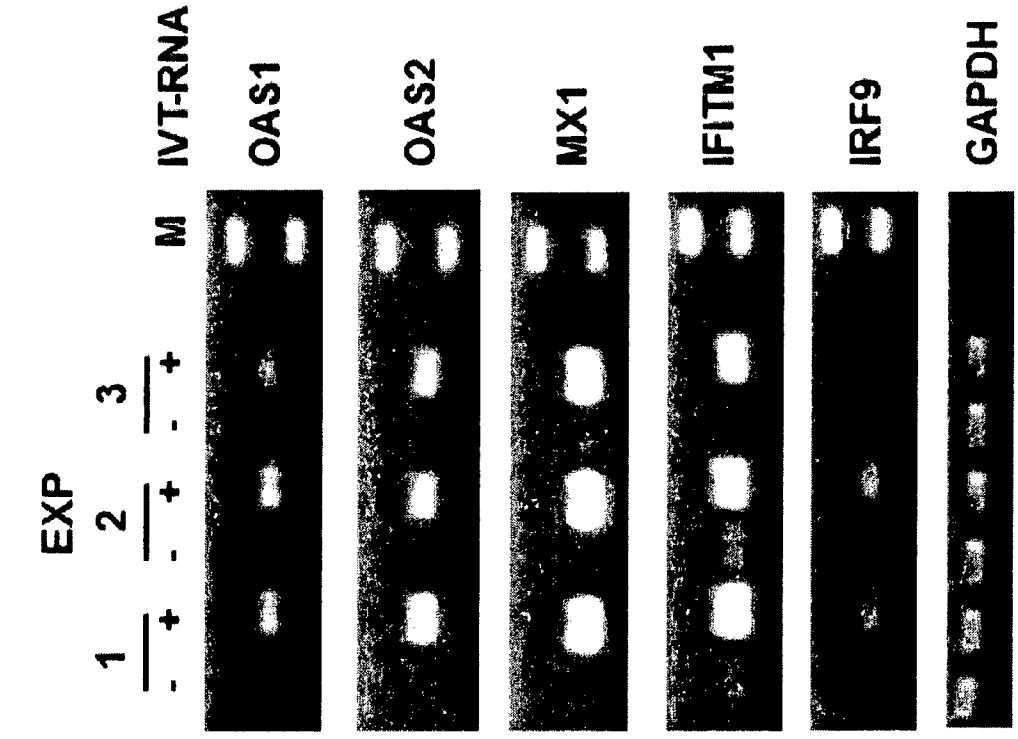

D
EXP
1 2 3
- + - + - +
M
IVT-RNA
OAS1
OAS2
MX1
IFITM1
IRF9
GAPDH

Figure 3

A

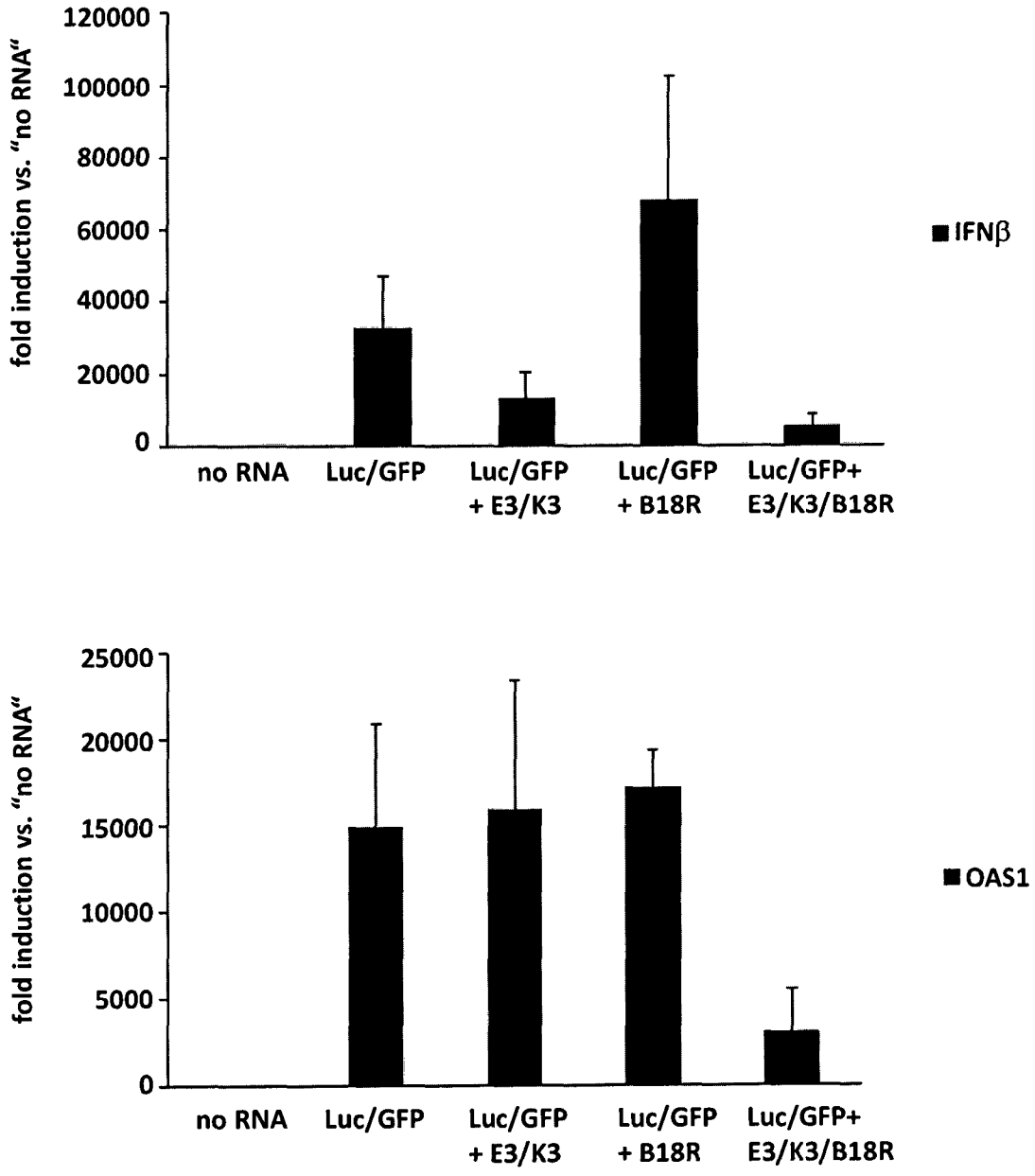
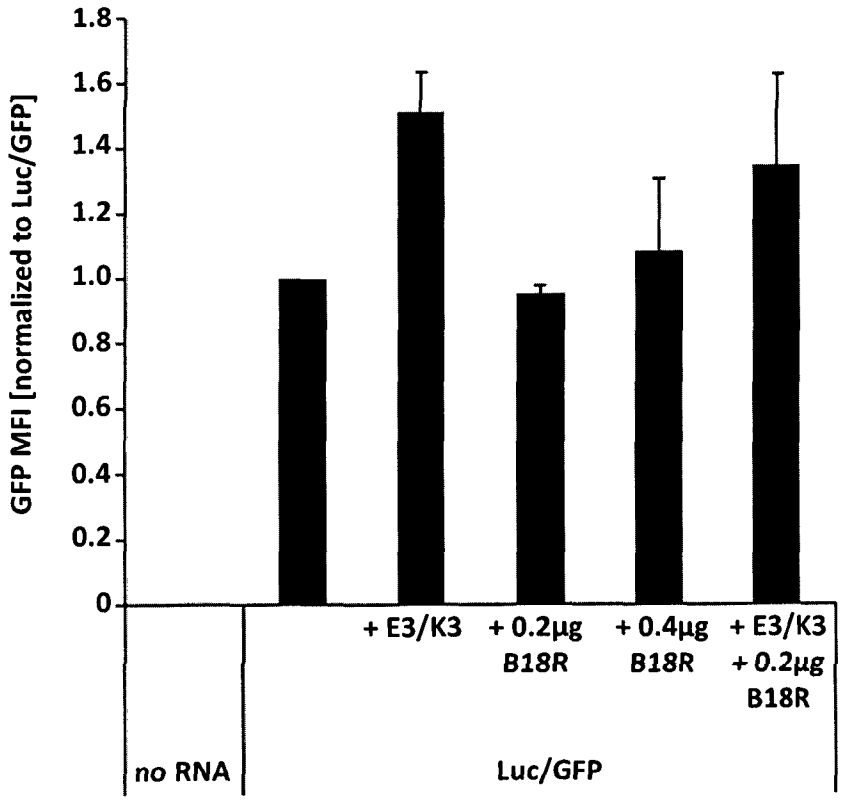

fold induction vs. "no RNA"
120000
100000
80000
60000
40000
20000
0
no RNA
Luc/GFP
Luc/GFP + E3/K3
Luc/GFP + B18R
Luc/GFP+ E3/K3/B18R
IFNβ
fold induction vs. "no RNA"
25000
20000
15000
10000
5000
0
no RNA
Luc/GFP
Luc/GFP + E3/K3
Luc/GFP + B18R
Luc/GFP+ E3/K3/B18R
OAS1

Figure 3

B


GFP MFI [normalized to Luc/GFP]
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
+ E3/K3
+ 0.2µg B18R
+ 0.4µg B18R
+ E3/K3 + 0.2µg B18R
no RNA
Luc/GFP

C

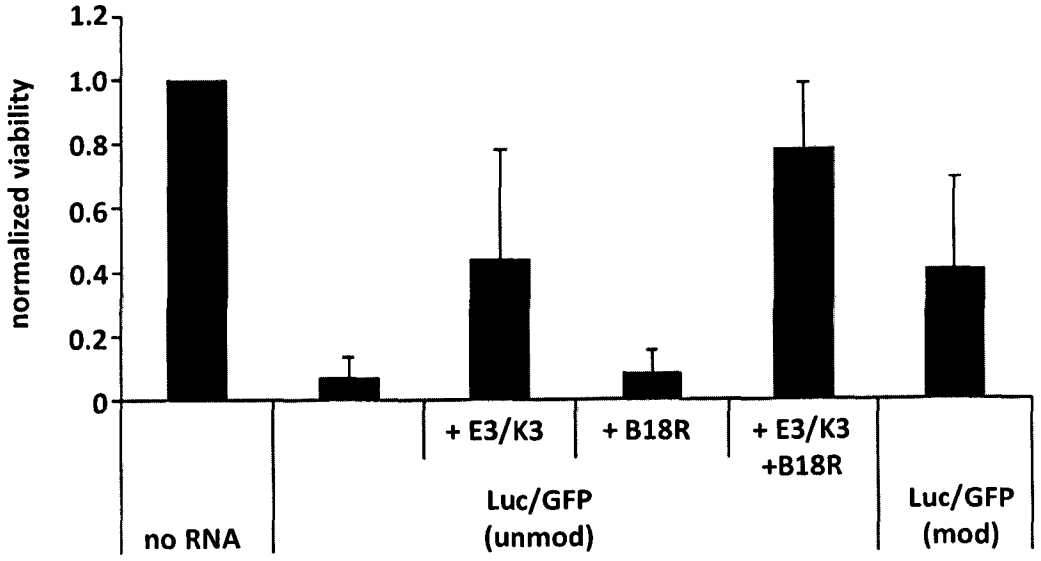

normalized viability
1.2
1.0
0.8
0.6
0.4
0.2
0
+ E3/K3
+ B18R
+ E3/K3 +B18R
no RNA
Luc/GFP (unmod)
Luc/GFP (mod)

Figure 4

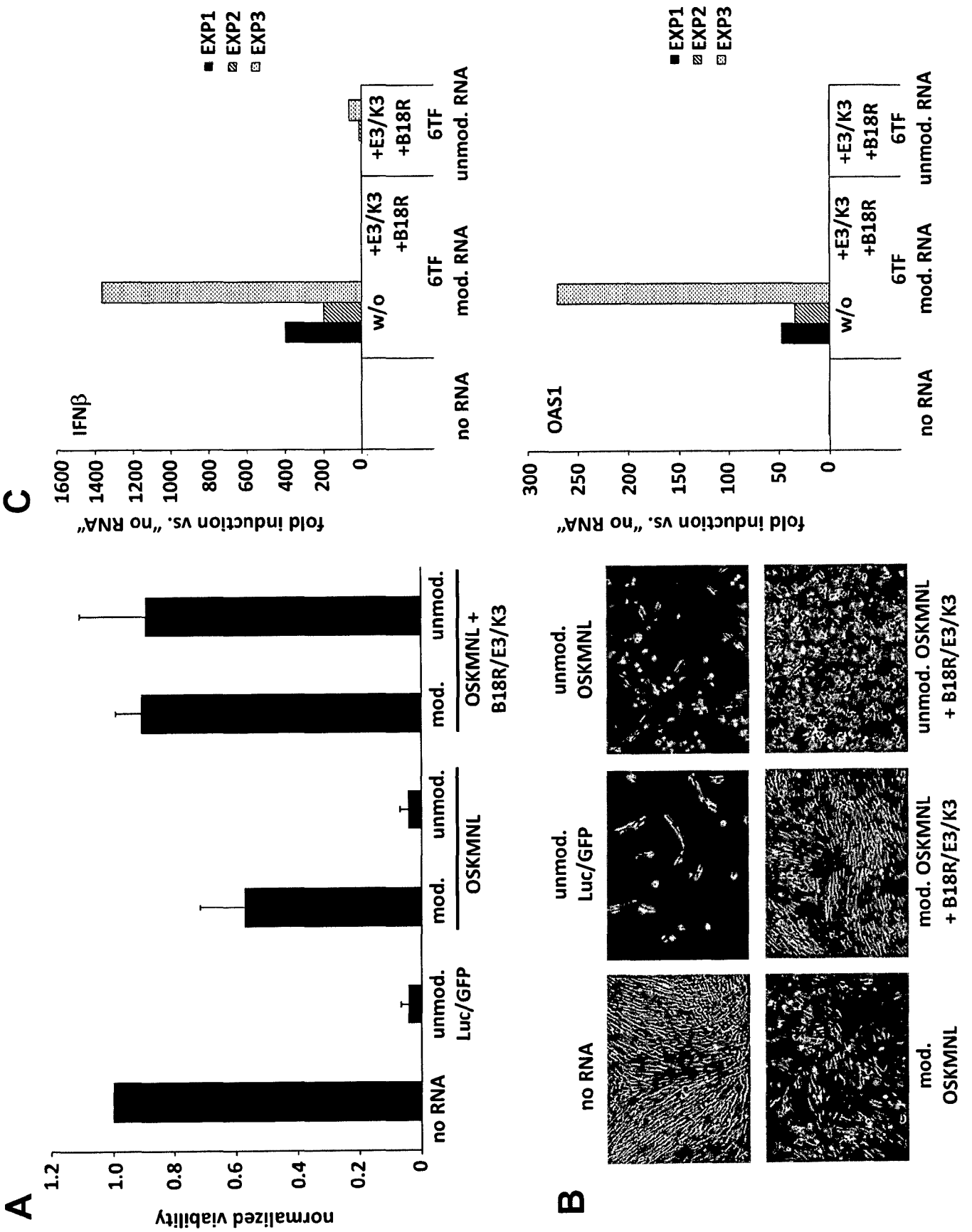

A
normalized viability
1.2
1.0
0.8
0.6
0.4
0.2
0
no RNA
unmod. Luc/GFP
mod.
unmod.
OSKMNL
mod.
unmod.
OSKMNL + B18R/E3/K3
B
no RNA
unmod. Luc/GFP
unmod. OSKMNL
mod. OSKMNL
mod. OSKMNL + B18R/E3/K3
unmod. OSKMNL + B18R/E3/K3
C
IFNβ
fold induction vs. "no RNA"
1600
1400
1200
1000
800
600
400
200
0
EXP1
EXP2
EXP3
no RNA
6TF mod. RNA
w/o
+E3/K3 +B18R
6TF unmod. RNA
+E3/K3 +B18R
OAS1
fold induction vs. "no RNA"
300
250
200
150
100
50
0

Figure 5

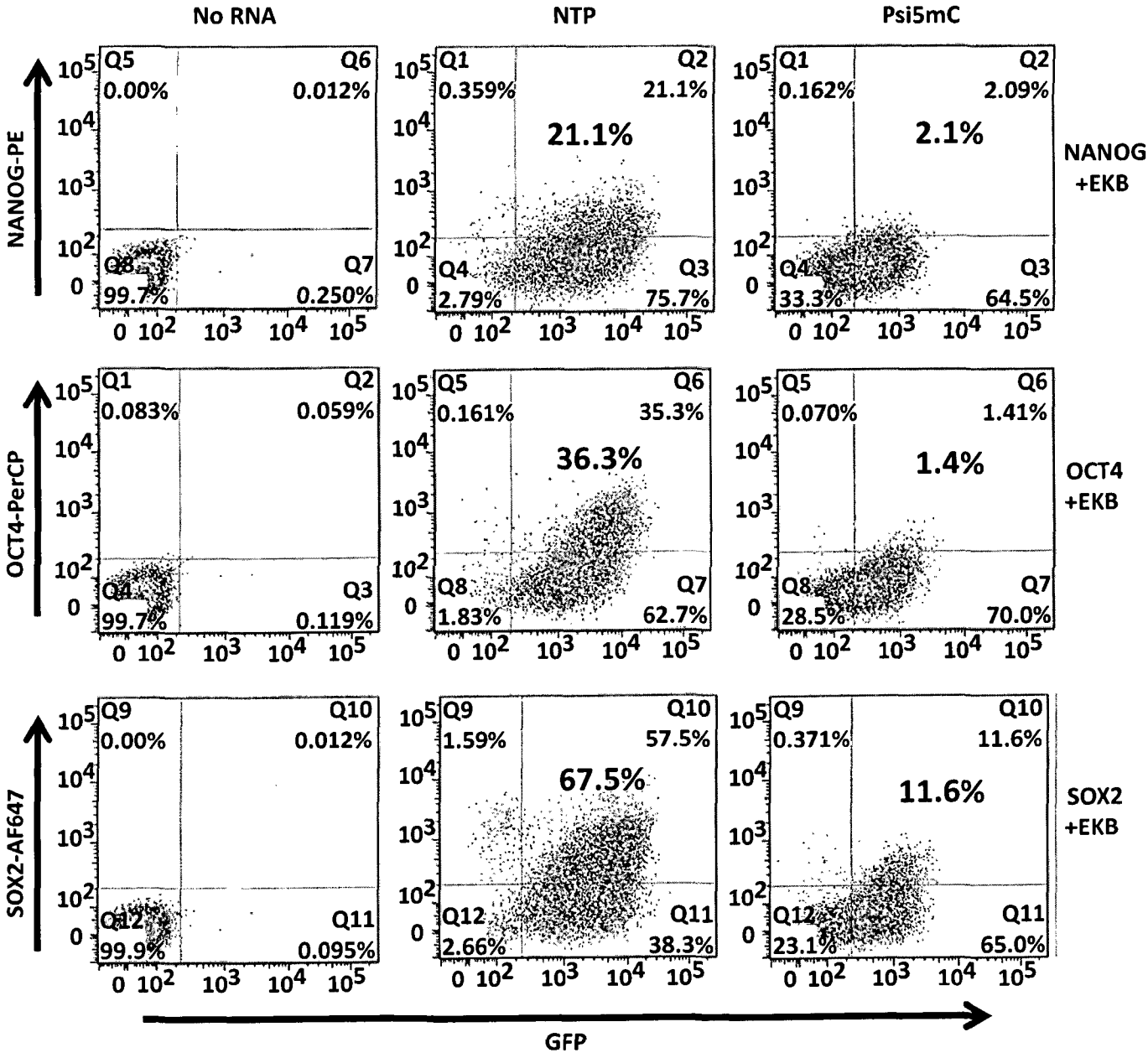
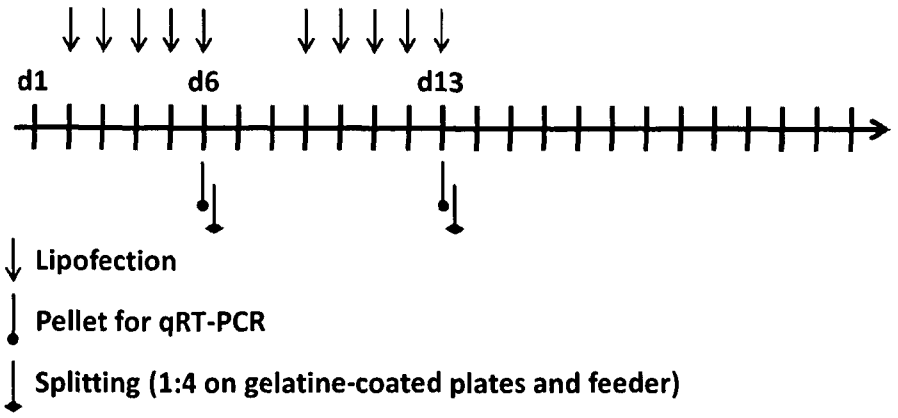
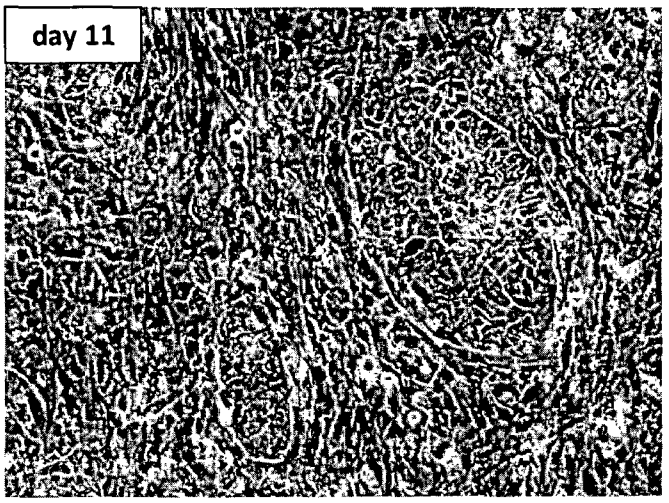

No RNA
NTP
Psi5mC
NANOG-PE
OCT4-PerCP
SOX2-AF647
GFP
NANOG +EKB
OCT4 +EKB
SOX2 +EKB
Q5 0.00%
Q6 0.012%
Q8 99.7%
Q7 0.250%
Q1 0.359%
Q2 21.1%
21.1%
Q4 2.79%
Q3 75.7%
Q1 0.162%
Q2 2.09%
2.1%
Q4 33.3%
Q3 64.5%
Q1 0.083%
Q2 0.059%
Q4 99.7%
Q3 0.119%
Q5 0.161%
Q6 35.3%
36.3%
Q8 1.83%
Q7 62.7%
Q5 0.070%
Q6 1.41%
1.4%
Q8 28.5%
Q7 70.0%
Q9 0.00%
Q10 0.012%
Q12 99.9%
Q11 0.095%
Q9 1.59%
Q10 57.5%
67.5%
Q12 2.66%
Q11 38.3%
Q9 0.371%
Q10 11.6%
11.6%
Q12 23.1%
Q11 65.0%

Figure 6

A


d1
d6
d13
Lipofection
Pellet for qRT-PCR
Splitting (1:4 on gelatine-coated plates and feeder)

C


day 11

D

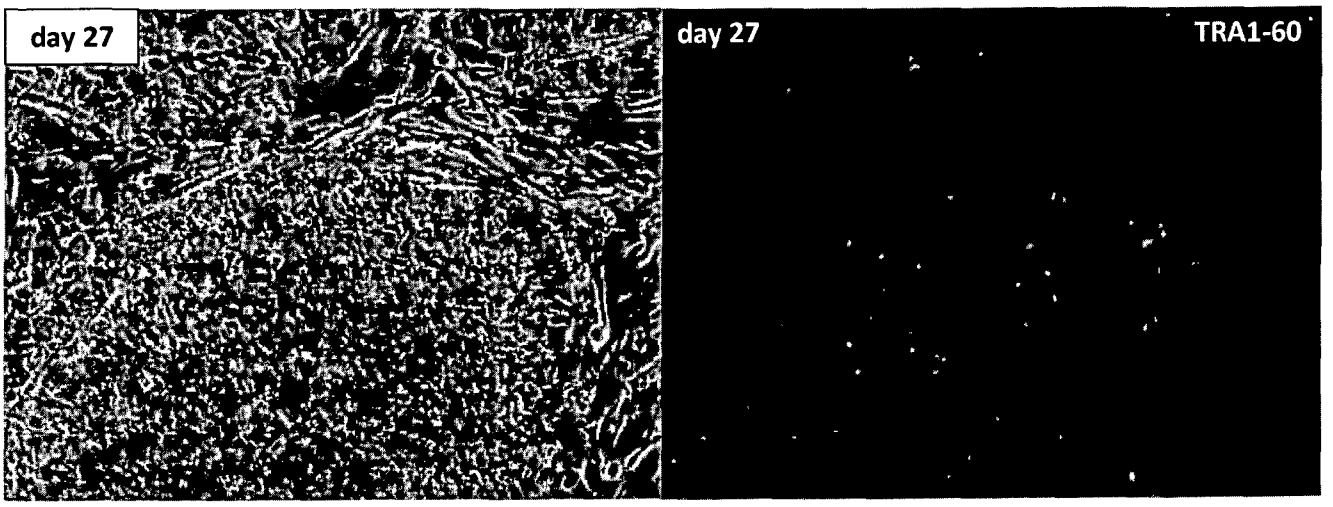

day 27
day 27
TRA1-60

Figure 6

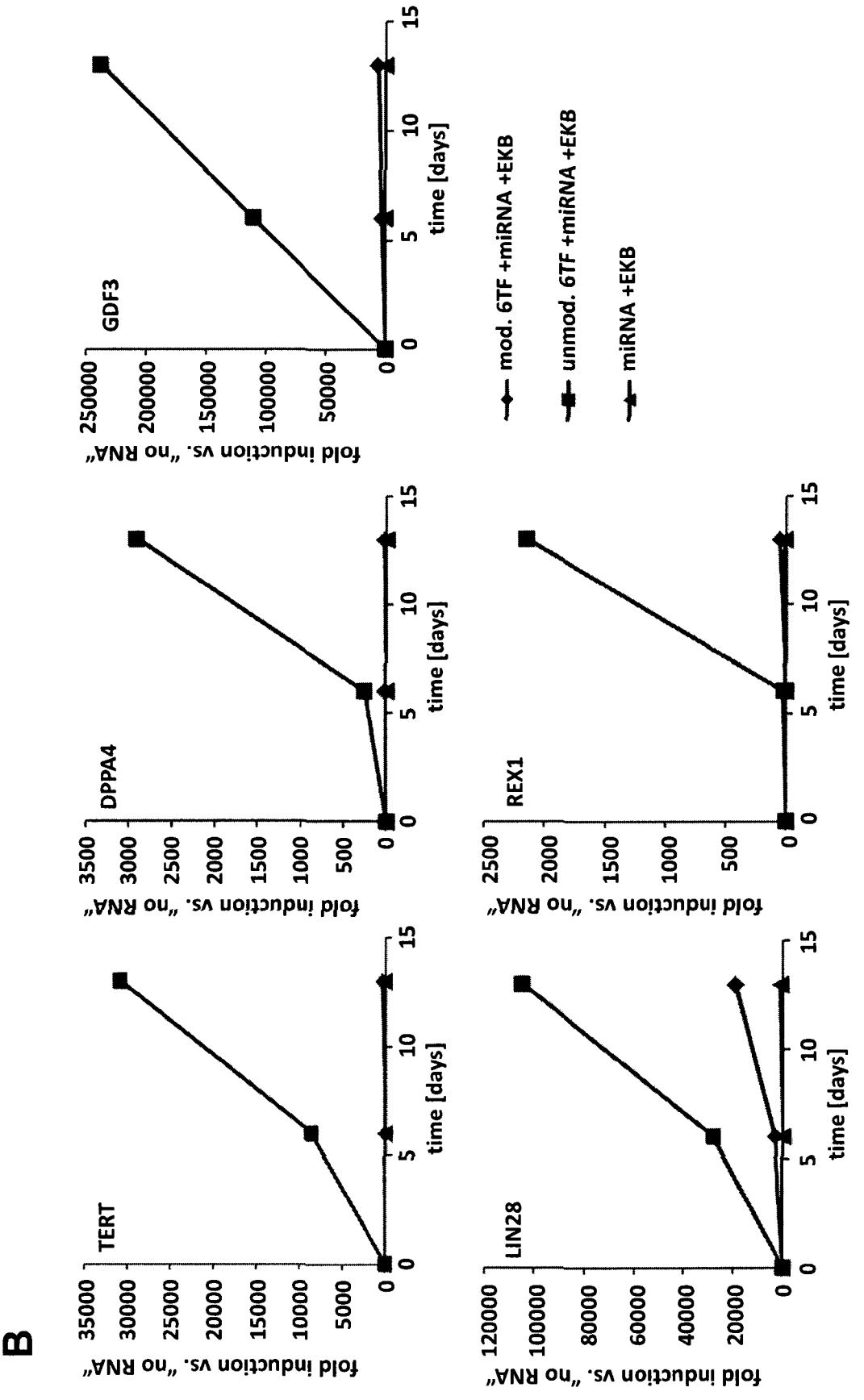

B
TERT
DPPA4
REX1
LIN28
GDF3
fold induction vs. "no RNA"
time [days]
mod. 6TF +miRNA +EKB
unmod. 6TF +miRNA +EKB
miRNA +EKB

Figure 7

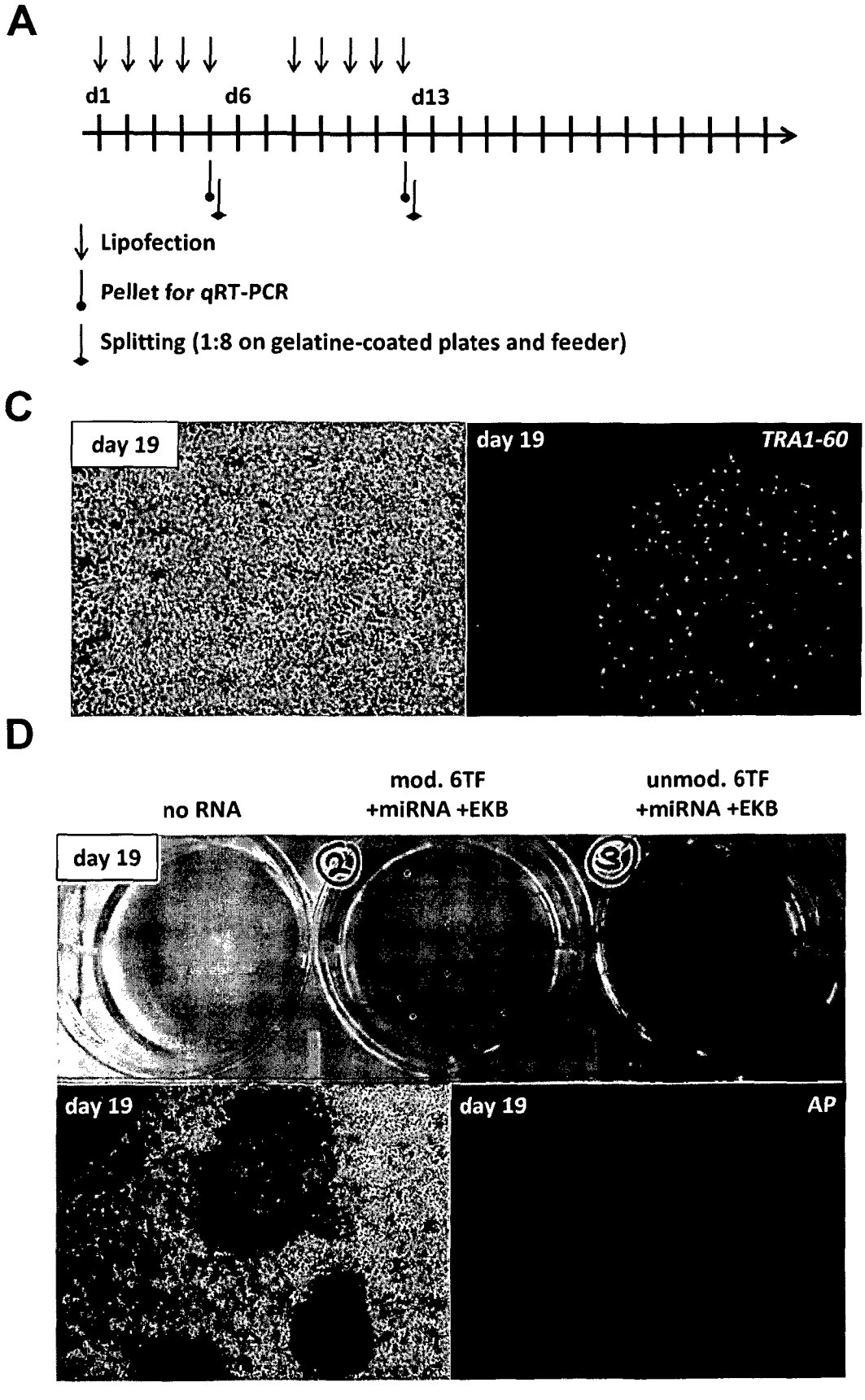

A
d1
d6
d13
Lipofection
Pellet for qRT-PCR
Splitting (1:8 on gelatine-coated plates and feeder)
C
day 19
day 19
TRA1-60
D
no RNA
mod. 6TF
+miRNA +EKB
unmod. 6TF
+miRNA +EKB
day 19
day 19
day 19
AP

Figure 7

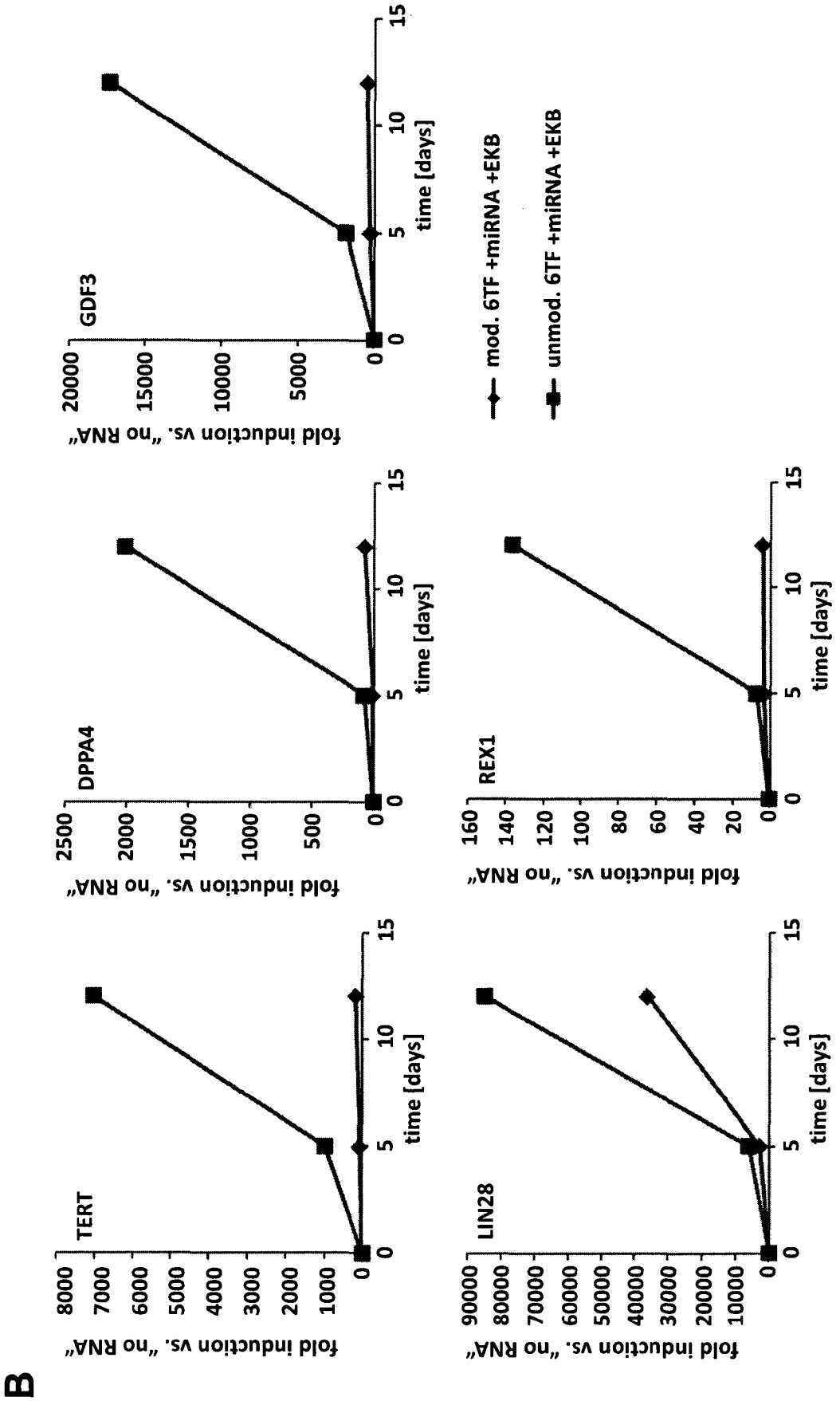

B
GDF3
DPPA4
REX1
TERT
LIN28
fold induction vs. "no RNA"
time [days]
mod. 6TF +miRNA +EKB
unmod. 6TF +miRNA +EKB

Figure 8

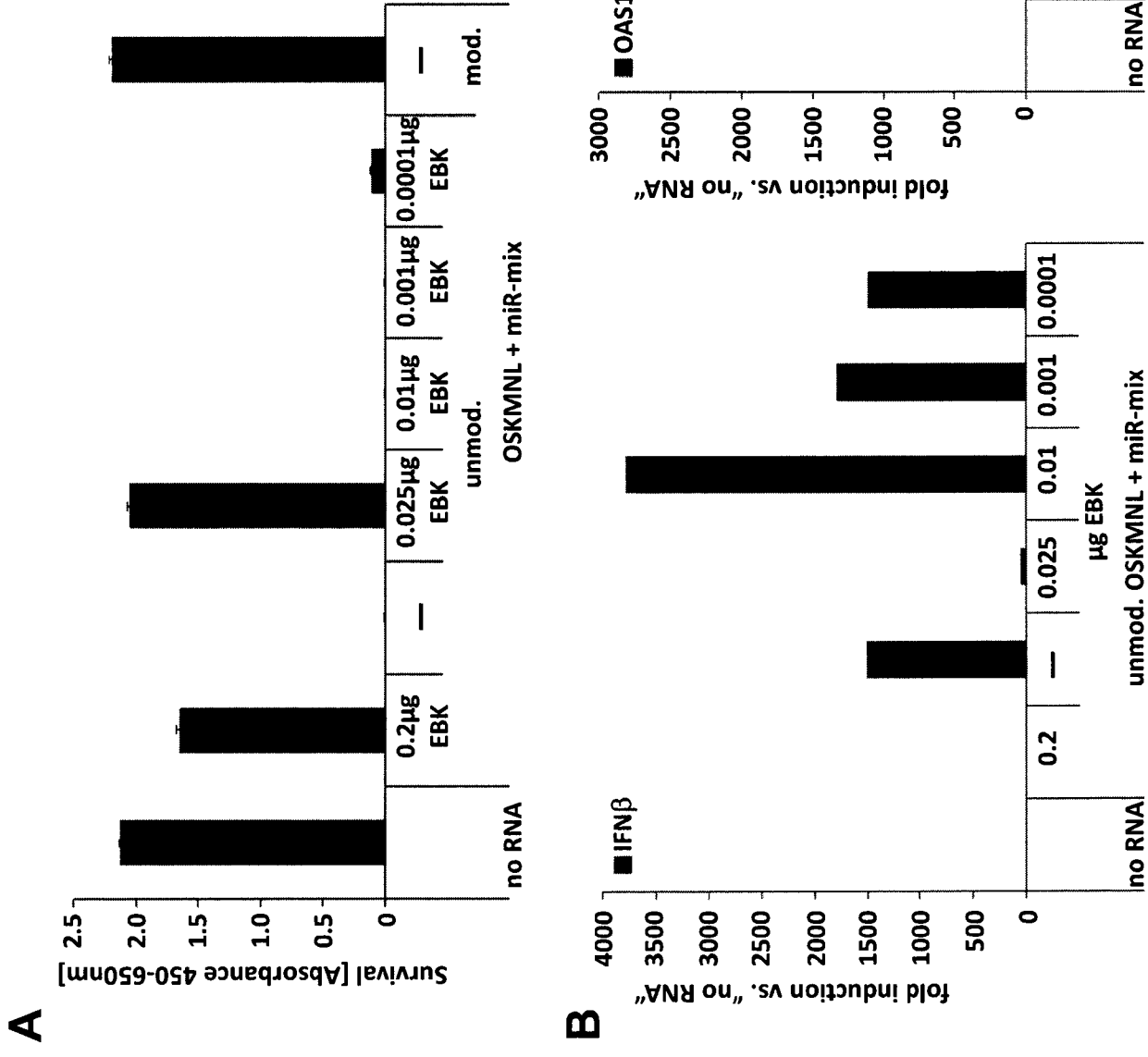
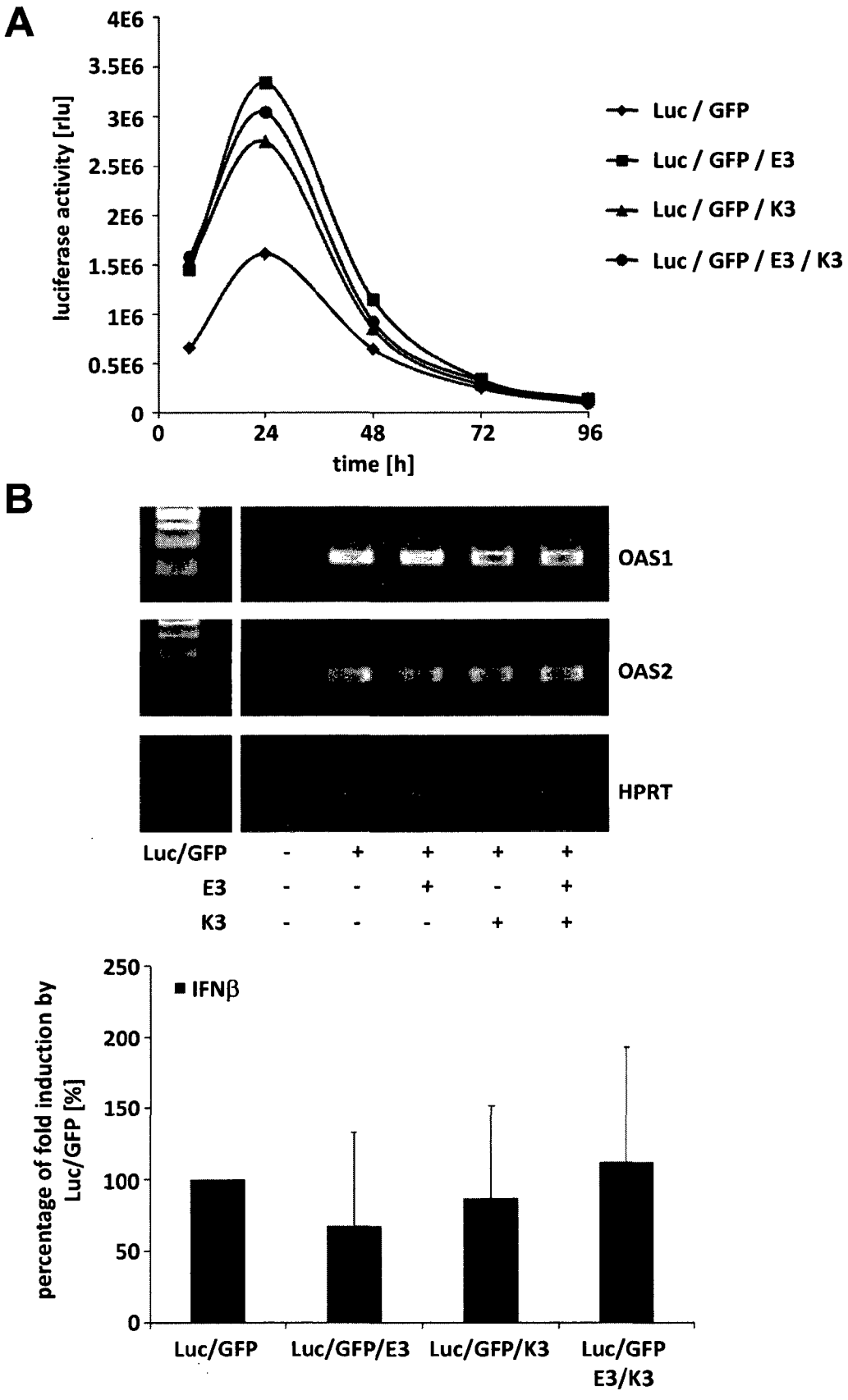

A
Survival [Absorbance 450-650nm]
2.5
2.0
1.5
1.0
0.5
0
no RNA
0.2µg EBK
—
0.025µg EBK
0.01µg EBK
0.001µg EBK
0.0001µg EBK
—
unmod.
mod.
OSKMNL + miR-mix
B
IFNβ
fold induction vs. "no RNA"
4000
3500
3000
2500
2000
1500
1000
500
0
no RNA
0.2
—
0.025
0.01
0.001
0.0001
µg EBK
unmod. OSKMNL + miR-mix
OAS1
fold induction vs. "no RNA"
3000
2500
2000
1500
1000
500
0
no RNA
0.2
—
0.025
0.01
0.001
0.0001
µg EBK
unmod. OSKMNL + miR-mix

Figure 9


A
4E6
3.5E6
3E6
2.5E6
2E6
1.5E6
1E6
0.5E6
0
luciferase activity [rlu]
0
24
48
72
96
time [h]
Luc / GFP
Luc / GFP / E3
Luc / GFP / K3
Luc / GFP / E3 / K3
B
OAS1
OAS2
HPRT
Luc/GFP - + + + +
E3 - - + - +
K3 - - - + +
IFNβ
250
200
150
100
50
0
percentage of fold induction by Luc/GFP [%]
Luc/GFP
Luc/GFP/E3
Luc/GFP/K3
Luc/GFP E3/K3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/004673

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [X] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/004673

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/67 C12N15/68 C12N15/69
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATTHEW ANGEL ET AL: "Innate Immune Suppression Enables Frequent Transfection with RNA Encoding Reprogramming Proteins", PLOS ONE, vol. 5, no. 7, E11756, 23 July 2010 (2010-07-23), pages 1-7, XP055047047, DOI: 10.1371/journal.pone.0011756	1-6, 12-16, 29,36, 39,42, 43, 45-50, 54-66
Y	page 1 - page 6; figures 1-3	7-10, 12-15, 21,46, 51-64

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[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2013	11/02/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schulz, Regine

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN YIN ET AL: "Rhinovirus induces airway epithelial gene expression through double-stranded RNA and IFN-dependent pathways", AMERICAN JOURNAL OF RESPIRATORY CELL AND MOLECULAR BIOLOGY, vol. 34, no. 2, 1 February 2006 (2006-02-01), pages 192-203, XP002487498, AMERICAN LUNG ASSOCIATION, NEW YORK, NY, US ISSN: 1044-1549, DOI: 10.1165/RCMB.2004-0417OC	1,6,7, 12-18, 21-25, 36-39, 42,43, 45,65,66
Y	page 192; figures 1, 3-10; tables 3, 4	12-15, 21-25

Y	LUIGI WARREN ET AL: "Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA", CELL STEM CELL, vol. 7, no. 5, 5 November 2010 (2010-11-05), pages 618-630, XP002640639, CELL PRESS, US ISSN: 1934-5909, DOI: 10.1016/J.STEM.2010.08.012 [retrieved on 2010-09-30] page 619; figures 1, S2, S3, 2, S3, 3, 4, S4, 5, 6; table S1	7-10, 21-25, 46,51-64

X	KUHN ANDREAS N ET AL: "mRNA as a versatile tool for exogenous protein expression.", CURRENT GENE THERAPY, vol. 12, no. 5, 1 October 2012 (2012-10-01), pages 347-361, XP008159357, ISSN: 1875-5631 page 348, left-hand column, paragraph 2nd - page 353, left-hand column, paragraph 3rd page 355, left-hand column, last paragraph - page 357, left-hand column, paragraph 2nd	1,64-66

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GARCÍA-SASTRE ADOLFO ET AL: "Type 1 interferons and the virus-host relationship: a lesson in détente .", SCIENCE, vol. 312, no. 5775, 12 May 2006 (2006-05-12), pages 879-882, XP008159457, (NEW YORK, N.Y.) ISSN: 1095-9203	21
A	page 881, left-hand column, last paragraph - page 882, last paragraph	1,46, 64-66
A	JORDAN R PLEWS ET AL: "Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach", PLOS ONE, vol. 5, no. 12, E14397, 1 December 2010 (2010-12-01), pages 1-10, XP002635012, PUBLIC LIBRARY OF SCIENCE, US ISSN: 1932-6203, DOI: 10.1371/JOURNAL.PONE.0014397 page 1 - page 9; figures 6-7	46-64
A	GEERTRUI TAVERNIER ET AL: "Activation of pluripotency-associated genes in mouse embryonic fibroblasts by non-viral transfection with invitro-derived mRNAs encoding Oct4, Sox2, Klf4 and cMyc", BIOMATERIALS, vol. 33, no. 2, 23 September 2011 (2011-09-23), pages 412-417, XP028101920, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2011.09.062 [retrieved on 2011-09-26] page 412 - page 413	46-64
A	YAKUBOV EDUARD ET AL: "Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors.", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 394, no. 1, 26 March 2010 (2010-03-26), pages 189-193, XP008159426, ISSN: 1090-2104 page 191 - page 193	46-64

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KATHARINA DREWS ET AL: "The cytotoxic and immunogenic hurdles associated with non-viral mRNA-mediated reprogramming of human fibroblasts", BIOMATERIALS, vol. 33, no. 16, 9 February 2012 (2012-02-09), pages 4059-4068, XP028474938, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2012.02.025 [retrieved on 2012-02-14] page 4065, right-hand column, last paragraph - page 4067; figure 8	1,46, 64-66
T	DAISY A. ROBINTON ET AL: "The promise of induced pluripotent stem cells in research and therapy", NATURE, vol. 481, no. 7381, 18 January 2012 (2012-01-18), pages 295-305, XP055050160, ISSN: 0028-0836, DOI: 10.1038/nature10761 page 298, left-hand column, paragraph 3rd - page 303; tables 1, 2	
A	NISHIKAWA SHIN-ICHI ET AL: "The promise of human induced pluripotent stem cells for research and therapy", NATURE REVIEWS MOLECULAR CELL BIOLOGY, vol. 9, no. 9, 1 September 2008 (2008-09-01), pages 725-729, XP002520703, NATURE PUBLISHING, GB ISSN: 1471-0072, DOI: 10.1038/NRM2466 the whole document	64

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