



## The cytotoxic and immunogenic hurdles associated with non-viral mRNA-mediated reprogramming of human fibroblasts

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### ABSTRACT

Delivery of reprogramming factor-encoding mRNAs by means of lipofection in somatic cells is a desirable method for deriving integration-free iPSCs. However, the lack of reproducibility implies there are major hurdles to overcome before this protocol becomes universally accepted. This study demonstrates the functionality of our in-house synthesized mRNAs expressing the reprogramming factors (OCT4, SOX2, KLF4, c-MYC) within the nucleus of human fibroblasts. However, upon repeated transfections, the mRNAs induced severe loss of cell viability as demonstrated by MTT cytotoxicity assays. Microarray-derived transcriptome data revealed that the poor cell survival was mainly due to the innate immune response triggered by the exogenous mRNAs. We validated the influence of mRNA transfection on key immune response-associated transcript levels, including *IFNB1*, *RIG-I*, *PKR*, *IL12A*, *IRF7* and *CCL5*, by quantitative real-time PCR and directly compared these with the levels induced by other methods previously published to mediate reprogramming in somatic cells. Finally, we evaluated chemical compounds (B18R, chloroquine, TSA, Pepinh-TRIF, Pepinh-MYD), known for their ability to suppress cellular innate immune responses. However, none of these had the desired effect. The data presented here should provide the basis for further investigations into other immunosuppressing strategies that might facilitate efficient mRNA-mediated cellular reprogramming in human cells.

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### 1. Introduction

Reprogramming of somatic cells toward pluripotency brought a promise in the field of regenerative medicine. The first induced pluripotent stem cells (iPSCs) were generated by introduction of transcription factors into human fibroblasts by means of retroviruses. It was achieved by the group of Yamanaka, who transduced fibroblasts with four transcription factors, namely OCT4, SOX2, KLF4 and c-MYC [1,2]. Other research groups refined the protocol by using less or other factors [3–5] or by implementing small molecules to increase the efficiency [4,6–8]. iPSCs produced this way, successfully serve as a tool in disease modeling or drug screening. Nonetheless, they are not suitable to generate patient-specific transplantable cells in a clinical setting because of the abundant

insertional mutagenesis. Therefore, one of the main focuses in the field has been to develop methods, which avoid genome insertions when delivering the transcription factors to somatic cells. Accordingly, the plasmids encoding the reprogramming factors have been delivered to mouse and/or human cells by means of repeated non-viral transfections [9], non-integrating, episomal viral vectors [7,10], or using excisable vectors [11,12]. Even though they succeeded in reducing genomic insertions markedly, the risk of modifying the host genome has never been eliminated completely. Zhou et al. generated iPSCs completely devoid of genomic insertions by introducing recombinant transcription factor proteins [13]. This method, however, was very inefficient. Recently, two alternative methods have been described to generate iPSCs, both inherently incapable of causing insertional mutagenesis. To induce pluripotency Warren et al. delivered the transcription factors as mRNAs complexed with commercially available cationic lipids and claimed high reprogramming efficiency [14]. This, however, required 17 daily mRNA transfections, which has been shown to severely compromise cell viability [15]. Miyoshi et al. produced

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iPSCs by transfection of mature human embryonic stem cell-associated miRNAs [16]. Despite these achievements, the lack of follow-up publications implies there are major hurdles to overcome before these approaches become reproducible and routinely applicable for successful cellular reprogramming [17].

We are experienced in both non-viral mRNA delivery into cells [18,19] and the derivation of human iPSCs [6,20–22]. We have previously applied a slightly adjusted mRNA reprogramming protocol, which enabled us to reduce the number of transfections, to successfully induce the onset of reprogramming in mouse embryonic fibroblasts [23]. In this study we assessed the early response in terms of expression of pluripotency-associated genes upon transfecting human neonatal fibroblasts with mRNAs encoding the Yamanka factors and studied in detail the impact of repeated mRNA transfections on the viability and transcriptome of human neonatal fibroblasts.

## 2. Materials and methods

### 2.1. Cell culture

Human foreskin fibroblasts (HFF1 and BJ, SCRC-1041 and SCRC-2522 from ATCC, respectively) were cultured in DMEM (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany) at 37 °C, 5% CO<sub>2</sub>.

### 2.2. *In vitro* mRNA synthesis

Previously described plasmids (containing a T7 RNA polymerase promoter) encoding a single reprogramming factor (OCT4, SOX2, KLF4 or c-MYC) were purchased from Addgene (Cambridge, USA) [14]. The plasmid encoding green fluorescent protein (GFP) (pGEM4Z-EGFP-A64, kindly provided by Prof. Dr. E. Gilboa, Duke University Medical Center, Durham, NC, USA) was previously described by Nair et al. [24]. To produce mRNAs, plasmids were first purified using a QIAquick PCR purification kit (Qiagen, Venlo, Netherlands) and linearized using restriction enzymes (XbaI for plasmids encoding reprogramming factors and SpeI for the GFP-coding plasmid). Linearized plasmids were used as templates for the *in vitro* transcription reaction using the T7 mMessage mMachine kit according to the manufacturer's instructions (Ambion, Lennik, Belgium). This resulted in the production of m(GFP) that was both capped and polyadenylated. All the other mRNAs (m(OCT4), m(SOX2), m(KLF4) and m(c-MYC)) were capped and subsequently polyadenylated with a poly(A) tailing kit supplied by Ambion. mRNAs were purified by DNase I digestion followed by LiCl precipitation and a 70% ethanol wash. The mRNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and then mRNA was stored in small aliquots at –80 °C at a concentration of 1 µg/µl.

mRNAs encoding the four Yamanaka factors including both pseudouridine and 5-methylcytidine-modified nucleotides were purchased from Stemgent (Miltenyi, Bergisch Gladbach, Germany).

### 2.3. mRNA transfection

HFF1 cells were seeded one day prior to the experiment (200,000 cells/well in 6-well-plates for qRT-PCR experiments, 100,000 cells/well in 12-well-plates for cytotoxicity and immunostaining experiments). Either 4 µg of m(GFP) or a 4 µg mixture of the four separate mRNAs (equal amounts of m(OCT4), m(SOX2), m(KLF4), m(c-MYC)), either produced as described earlier or commercially bought, were pre-diluted in 46 µl Opti-MEM (Invitrogen, Merelbeke, Belgium). These mRNA dilutions were complexed with 4 µl of Lipofectamine RNAiMAX (LF) (1 mg/ml, Invitrogen, Merelbeke Belgium), also pre-diluted in 46 µl Opti-MEM. After 10 min of incubation at RT, 900 µl of Opti-MEM were added and the complete solution was added to one well (6-well-plate) or divided over two wells (12-well-plate) (after removal of growth medium). After 2 h of incubation with the cells, the complexes were removed and regular growth medium was added.

### 2.4. Analysis of GFP expression

To assess the number of GFP-positive cells, culture medium was removed from the wells and the cells were washed with PBS. After detaching the cells with trypsin (0.05%, Life Technologies) and centrifugation, the cells were re-suspended in flow buffer (PBS containing 1% BSA and 0.1% azide). The samples were kept on ice until GFP expression was evaluated by a Beckman Coulter Flowcytometer FC500, equipped with a 488 nm laser. Images showing GFP expression in cells transfected with Lipofectamine/m(GFP) complexes were taken with a confocal microscope LSM 510 (Zeiss, Oberkochen, Germany).

### 2.5. Immunostaining and microscopy analysis

To visualize the nuclear expression of the reprogramming factors, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 12 min at RT, washed two times with 0.05% Tween 20 (Sigma, Munich, Germany) in PBS (PBST) and permeabilized with 1% Triton X-100 (Sigma) in PBS. After two more washes with PBST, cells were blocked with 5% FBS and 1% Bovine Serum Albumin (BSA, Sigma) in PBST for 1 h at RT. Primary antibodies to detect the reprogramming factor proteins were purchased from Santa Cruz Biotechnologies (Heidelberg, Germany): OCT4 (Cat. No. sc-5279), SOX2 (Cat. No. sc-17320), KLF4 (Cat. No. sc-20691) and c-MYC (Cat. No. sc-764). Secondary antibodies were bought from Life Technologies: Alexa Fluor 594 chicken anti-goat IgG (Cat. No. A21468), Alexa Fluor 488 goat anti-mouse IgG (Cat. No. A11001) and Alexa Fluor 488 donkey anti-rabbit IgG (Cat. No. A21206). Nuclei were counterstained with DAPI (100 ng/ml, Vector Laboratories, Burlingame, CA, USA) diluted with PBST (12 min, RT). Cells were visualized using a confocal microscope LSM 510.

### 2.6. Toxicity assays

HFF1 cells were plated in 12-well-plates (90,000 cells/well) and incubated with mRNA complexes prepared as described above for 2 h. Transfections were performed every day. Cell viability was evaluated 24 h post-transfection by an MTT assay (Roche, Vilvoorde, Belgium) performed according to the manufacturer's instructions.

### 2.7. RNA isolation

Total RNA was isolated from cell lysates using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The concentrations of the isolated RNAs were determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer and purity was assessed by agarose gel electrophoresis.

### 2.8. Illumina bead chip hybridization and data analysis

The hybridization protocol has been described earlier by Mah et al. [25]. Briefly, 500 ng total RNA were used as input for amplification and labeling reactions (Illumina TotalPrep RNA Amplification Kit, Ambion) prior to hybridization onto Illumina HumanRef-8 v3.0 Expression BeadChips on the Illumina BeadStation 500 platform (Illumina, San Diego, CA, USA, [www.illumina.com](http://www.illumina.com)). Raw data were converted to background-subtracted, normalized ("rank invariant" algorithm) data with the help of the Gene Expression Module version 1.8.0 provided with the GenomeStudio software (Illumina). Genes were considered "present" if the "Detection P-Value"  $p_{det} < 0.01$ . Significant differential gene expression with respect to Lipofectamine-treated control cells ("mock") required  $p_{det} < 0.01$  in at least one of the samples under investigation and was defined by 1.5 fold up- or down-regulation in combination with an FDR-adjusted (Benjamini and Hochberg algorithm) "Diff P-Value"  $p_{adj} < 0.05$  as output by GenomeStudio. The heatmap was created using the gplots package in R [26,27]. Functional annotation and analyses for KEGG pathway enrichment were conducted using official gene symbols and default settings of the DAVID platform version 6.7 (<http://david.abcc.ncifcrf.gov/home.jsp>) [28,29]. Identification of interferon-regulated genes was achieved with the help of the Interferome database [30]. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [31] and are accessible through GEO Series accession number GSE34943 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34943>).

### 2.9. qRT-PCR

For M-MLV-driven reverse transcription (Affymetrix/USB Corporation, Cleveland, OH, USA), 1 µg total RNA was diluted to 9.5 µl with RNase/DNase-free distilled water (dH<sub>2</sub>O, Life Technologies) and 0.5 µl oligo-dT (1 µg/µl, 15mer) were added. The mixture was incubated at 72 °C for 5 min and cooled down on ice. 15 µl of a master mix were added, containing the following components (per reaction): 9.4 µl RNase/DNase-free dH<sub>2</sub>O, 5.0 µl 5 X M-MLV reaction buffer (USB), 0.5 µl dATP, dCTP, dGTP, dTTP mix (25 mM each, USB), 0.1 µl M-MLV (USB) and the mixture was incubated at 42 °C for 1 h. The enzyme was inactivated at 65 °C for 10 min. The resulting cDNA was kept at 4 °C for direct use or stored at –20 °C. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in 384-well-plates (Applied Biosystems, Foster City, CA) using the SYBRGreen PCR Master Mix (Applied Biosystems). Reactions were carried out on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as previously described [32]. Triplicate amplifications were carried out per gene with three wells without template serving as negative controls. ACTB was amplified along with the target genes as endogenous control for normalization. All primer sequences are provided in Supplementary Table S1.

Data analysis was carried out using the ABI PRISM SDS 2.2.1 software (Applied Biosystems) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Housekeeping gene-normalized, relative mRNA levels of each gene (biological sample versus reference) were calculated based on the 2<sup>–ΔΔCT</sup> method [33]. Data are

presented as mean LOG2 ratios with respect to biological controls and standard deviation.

### 2.10. Analysis of the immune response to different nuclear reprogramming approaches

HFF1 cells were plated in 6-well-plates (200,000 cells/well) one day prior to the experiments. Per well the cells were transduced or transfected with (1) a 1:1:1:1 cocktail of retroviruses encoding four reprogramming factors (OSKM) or a GFP-encoding retrovirus equivalent to the amount of a single reprogramming factor (production of retroviruses and transduction procedure were described earlier [6,20,22]) or (2) 100 pmol of a 1:1:1:1:1 mix composed of the miRNAs miR302a, miR302b, miR302c, miR302d and miR367 or a non-target (scrambled) miRNA (Ambion) or (3) 4  $\mu$ g of in-house synthesized unmodified or purchased modified mRNAs encoding the four reprogramming factors or mRNA encoding GFP. The cells were harvested 24 h after transfection and RNA was isolated for microarray-based global gene expression analysis and the analysis of immune response-associated gene regulation by qRT-PCR (as described above). To determine the immunomodulatory effect of different substances upon mRNA transfection, cells were pre-incubated with these substances 1 h prior to transfection, during the incubation of the complexed RNA with the cells and 24 h post-transfection at the following concentrations: 200 ng/ml Vaccinia B18R (eBioscience/biocompare), 5, 50, 100  $\mu$ M Chloroquine (Sigma), 50, 100, 500 nM Trichostatin A (Sigma), 20  $\mu$ M Pepinh-TRIF (InvivoGen, San Diego, CA, USA), 20  $\mu$ M Pepinh-MYD (InvivoGen).

## 3. Results

### 3.1. Transfection of HFF1 cells with mRNA encoding green fluorescent protein or reprogramming factors

Human foreskin fibroblasts (HFF1) were transfected with 4  $\mu$ g of mRNAs encoding green fluorescent protein (m(GFP)) complexed with 4  $\mu$ l Lipofectamine RNAiMAX (LF) as described in Materials and methods. The flow cytometry and microscopic analysis presented in Fig. 1 shows that approximately 85% of cells expressed GFP with a very high intensity per cell. Next, we transfected HFF1 cells with a mixture of equal amounts of mRNAs encoding the Yamanaka factors (m(OCT4), m(SOX2), m(KLF4), m(c-MYC)). After 24 h, the cells were fixed, stained and analyzed by microscopy. Protein expression was clearly detectable (OCT4, SOX2) or increased above endogenous background levels (KLF4, c-MYC) when compared to mock-transfected control cells (Fig. 2). Importantly, the images confirmed the nuclear localization of the expressed factors.

### 3.2. Viability of HFF1 cells transfected daily with mRNA lipoplexes

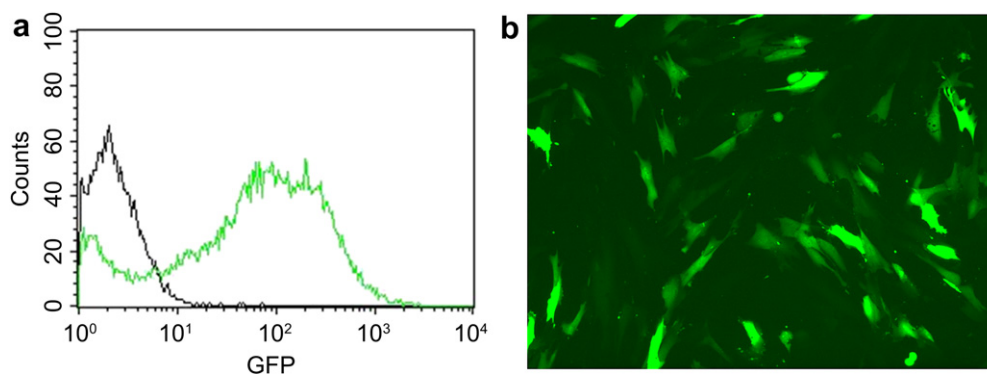
Transfection data can be properly valued only if the potential toxic effects of the system are evaluated. To determine the effect of the transfection protocol on cell viability, HFF1 cells were transfected daily with Lipofectamine complexes carrying mRNAs

encoding the Yamanaka factors. Cell viability was assessed 24 h after each transfection. The data presented in Fig. 3 demonstrate that there was no significant change in the viability of HFF1 cells 24 h after the first transfection. From day 2, however, the number of cells in culture diminished progressively. No cells survived seven consecutive transfections.

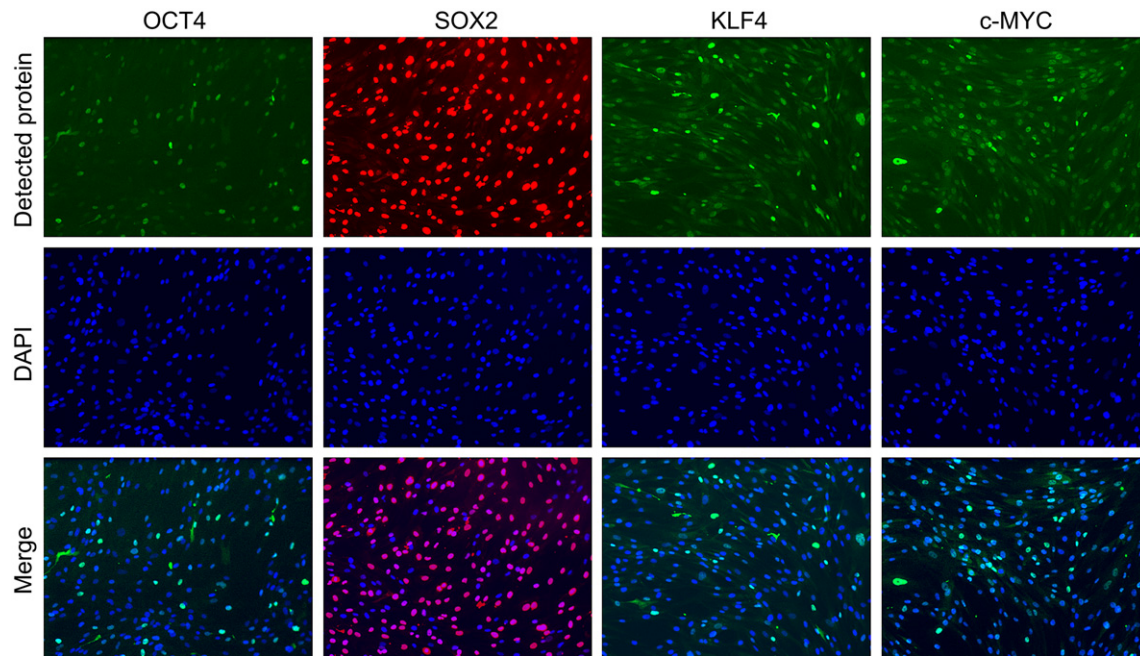
### 3.3. Comparative global gene expression analysis of reprogramming factor mRNA-transfected and mock-transfected human fibroblasts

Several attempts to induce pluripotency in HFF1 and BJ cells by repeated lipofection of the mRNAs encoding the reprogramming factors failed due to progressive loss of cell viability. We decided to investigate the reason for this massive cell death. It is known that transfection of poly(A)-tailed mRNA into human fibroblast cells induces interferon response [15,34]. To get a more detailed insight into which pathways are involved in the immediate response, we analyzed the transcriptomes of two human fibroblast cell lines (HFF1 and BJ) transfected with mRNAs encoding the reprogramming factors and mock-transfected, LF-treated controls. The analysis was performed 24 h post-transfection. The results were compared with the transcriptomes of untreated wild-type human neonatal fibroblasts (HFF1 and BJ cells), human embryonic stem cells (ESCs, lines H1 and H9) and human induced pluripotent stem cells derived from HFF1 and BJ cells (FiPSCs) generated and maintained in our laboratory [20,21]. Hierarchical clustering based on the global gene expression of all samples demonstrated a clear separation of fibroblasts transfected with mRNAs encoding the reprogramming factors from wild-type and mock-transfected fibroblasts as well as from human FiPSCs and ESCs (Fig. 4a). Accordingly, linear correlation coefficient analysis revealed that mRNA-transfected fibroblasts shared decreasing numbers of expressed genes with mock-transfected fibroblasts ( $R^2 = 0.84$ ), wild-type fibroblasts ( $R^2 = 0.73$ ), FiPSCs ( $R^2 = 0.60$ ) and ESCs ( $R^2 = 0.56$ ). Interestingly, mock-transfected and wild-type fibroblasts are closer to FiPSCs and ESCs ( $R^2 \approx 0.71$ ) than are reprogramming factor-transfected fibroblasts from FiPSCs and ESCs ( $R^2 \approx 0.58$ ) (Fig. 4b). This emphasizes that the active transcriptome of reprogramming factor mRNA-transfected fibroblasts is indeed different from both the original donor cells as well as the pluripotent cell lines and that an overall shift toward a pluripotent cell type has not yet occurred.

To investigate if, nevertheless, potential pluripotency-associated genes are up-regulated as early as 24 h after transfection of the reprogramming factors, we generated a Venn diagram based on “detected” genes in mock control cells, reprogramming factor-transfected fibroblasts and the union of FiPSCs and ESCs (the



**Fig. 1.** GFP expression in HFF1 cells. a) 4  $\mu$ g of m(GFP) and 4  $\mu$ l of LF, both pre-diluted in 46  $\mu$ l of Opti-MEM, were mixed and incubated for 10 min at RT. Then, 900  $\mu$ l of Opti-MEM were added and this solution was added to HFF1 cells for 2 h, after which the complexes were removed and regular growth medium added. 10,000 cells were analyzed for GFP expression 24 h post-transfection (black line: untreated control; green line: GFP-positive cells). b) Microscopic image of HFF1 cells lipofected with m(GFP) (magnification 40 $\times$ ).



**Fig. 2.** Nuclear expression of reprogramming factors in HFF1 cells. Cells were transfected with 4  $\mu$ g of mRNA encoding equal amounts of OCT4, SOX2, KLF4 and c-MYC by complexing with 4  $\mu$ l LF as described in Materials and methods. After 24 h, cells were immunostained for the transfected factors. The nuclei were stained with DAPI (magnification 40 $\times$ ).

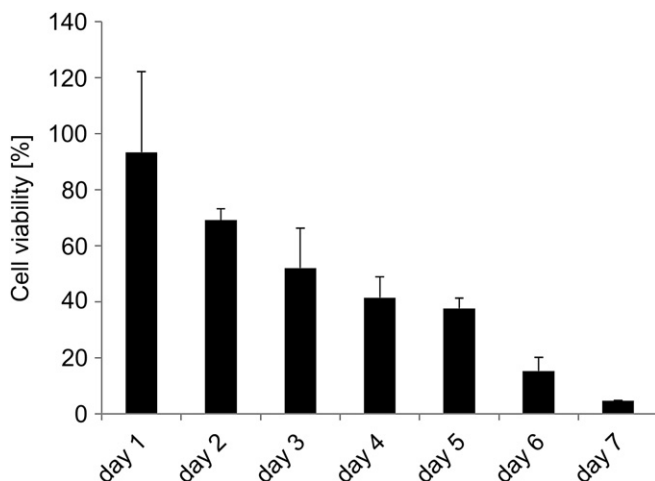
overlapping genes in these two pluripotent cell types), which is presented in Fig. 4c. The list of 148 putative pluripotency-associated genes, which overlap in reprogramming factor-transfected fibroblasts and the union of FiPSCs and ESCs, is given in Supplementary Table S2. It is enriched for cellular membrane and transmembrane protein-encoding genes and genes associated with cell–cell signaling such as *SYT1*, *CXCR7*, *CEACAM1*, *BST2* and *CXCL6*.

To analyze the changes induced by transfection with mRNAs encoding reprogramming factors we filtered the global gene expression data for genes, which were significantly up- or down-regulated in reprogramming factor-transfected fibroblasts with respect to mock-transfected control fibroblasts. As shown in Fig. 4d, a total of 993 genes were significantly differentially regulated

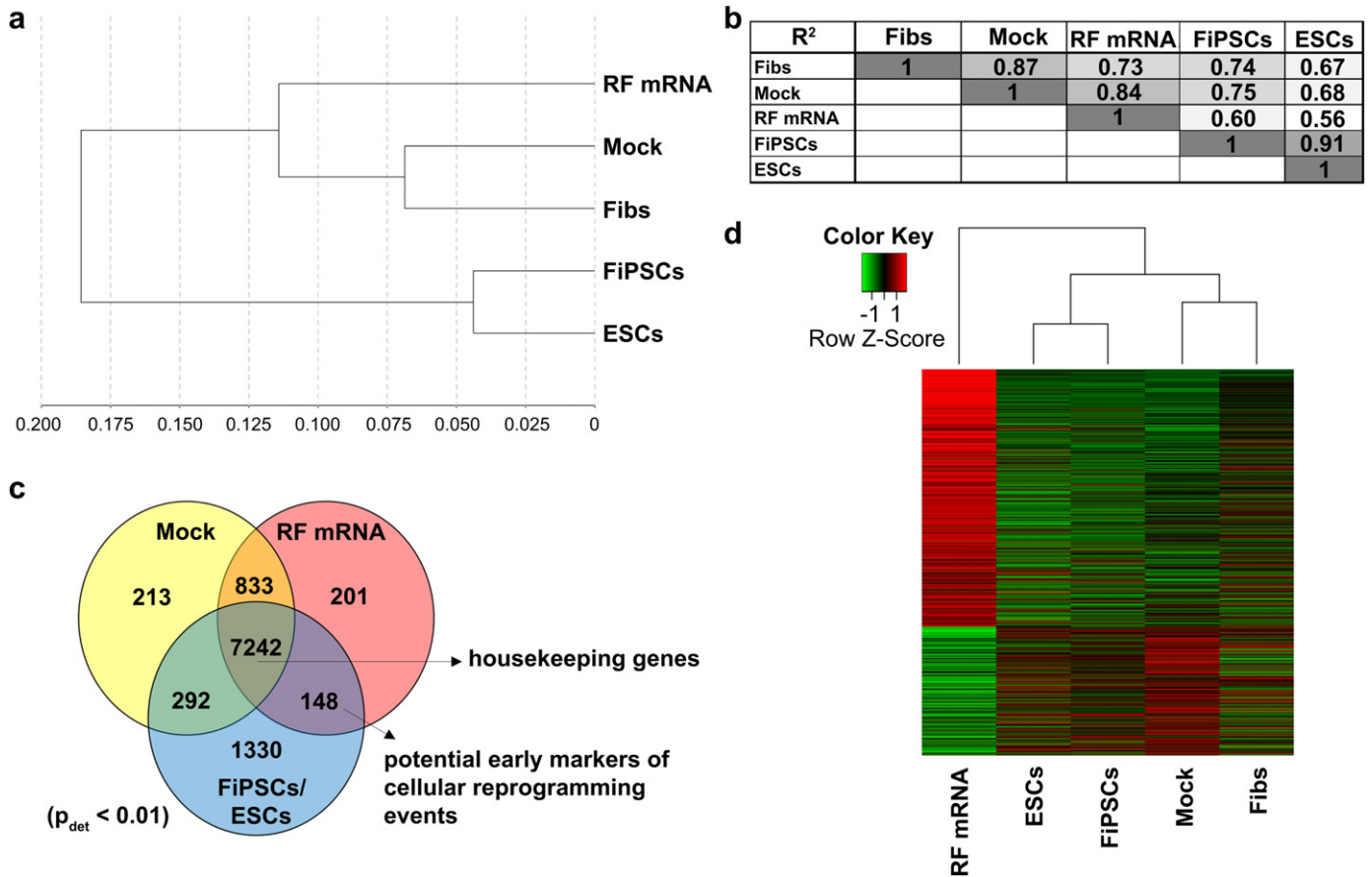
between the two groups. Of these, 662 genes were significantly up-regulated (e.g. *IFNB1*, *CCL5*, *ISG20*, *OAS1*, *MDA5* (*IFIH1*), *RIG-I* (*DDX58*), *IRF7*, *MYD88*, *ADAR*, *TRIF* (*TICAM1*)) and 331 genes were significantly down-regulated (e.g. *CCNB1*, *CCNB2*, *CCNF*, *CDC20*, *BAX*) in mRNA-transfected fibroblast cells when compared to the mock-transfected fibroblasts (Supplementary Table S3 and S4). The DAVID database was used to functionally annotate these gene lists [28,29]. As a result, the three most highly enriched clusters of the 662 up-regulated genes represented (1) the response to bacteria or bacterial structures or drugs, (2) innate immune response and (3) regulation of apoptosis (Supplementary Tables S5). The list of up-regulated genes was further enriched for KEGG pathways such as the Jak-STAT signaling pathway, RIG-I-like receptor signaling pathway, antigen processing and presentation, the cytosolic DNA-sensing pathway, the Toll-like receptor signaling pathway and apoptosis (Supplementary Fig. S1a–c, Supplementary Table S5). The initiation of apoptosis upon mRNA transfection is further supported by the fact that the list of 331 down-regulated genes is highly enriched for genes associated with cell cycle, cytoskeleton and chromosome condensation, which might suggest that proliferation is compromised in mRNA-transfected cells (Supplementary Table S6). Accordingly, the down-regulated genes are significantly involved in cell cycle and p53 signaling pathways (Supplementary Fig. S1d–e and Supplementary Table S6). According to the Interferome database [30], 249 of the 662 up-regulated genes and 40 of the 331 down-regulated genes are so-called interferon-regulated genes (IRGs) (Supplementary Tables S7 and S8).

#### 3.4. Innate immune response in HFF1 cells induced by reprogramming experiments using different delivery techniques

The increased expression levels of immune-related genes observed for the microarray-derived transcriptome data were verified by qRT-PCR. We chose to assess the expression of a set of innate immune response-related genes, known to be regulated during retroviral-based reprogramming [25] and relevant at distinct levels of the antiviral innate immune response. They are



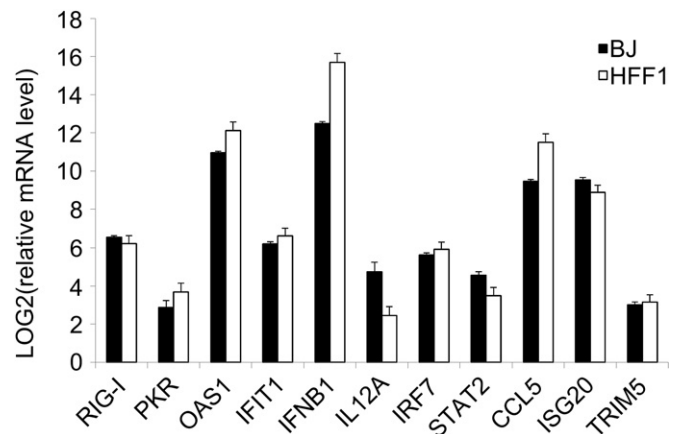
**Fig. 3.** Cytotoxicity of reprogramming factor-encoding mRNA transfections in HFF1 cells. Cells were transfected with 4  $\mu$ g of mRNA encoding equal amounts of OCT4, SOX2, KLF4 and c-MYC by complexing with 4  $\mu$ l LF as described in Materials and methods. The transfection solution was divided over two 12-wells containing HFF1 cells. Transfections were done every day; seven consecutive transfections were performed. MTT assay was done 24 h post every transfection. Graphs represent means  $\pm$  SD.  $n \geq 2$ .



**Fig. 4.** Microarray-based transcriptome analysis of untreated, mock-transfected and reprogramming factor-transfected fibroblasts, fibroblast-derived iPSCs and human ESCs. a) Hierarchical clustering (Pearson's correlation) on the basis of the global gene expression data. (RF mRNA: reprogramming factor mRNA-transfected human neonatal fibroblasts, HFF1, BJ; Mock: mock-transfected fibroblasts, HFF1, BJ; Fibs: wild-type fibroblasts, HFF1, BJ; FiPSCs: fibroblast-derived induced pluripotent stem cells, two HFF1-, two BJ-derived lines; ESCs: human embryonic stem cells, lines H1 and H9). b) Linear correlation coefficients ( $R^2$ ) between the different groups of samples on the basis of the global gene expression data. c) Venn diagram based on “detected” genes in mock-transfected fibroblasts, reprogramming factor-transfected fibroblasts and the union of FiPSCs and ESCs depicting distinct and overlapping transcriptional signatures between the different groups of samples. d) Heatmap portraying the total of 993 significantly differentially expressed genes in reprogramming factor-transfected fibroblasts compared to mock-transfected fibroblasts (662 genes up-regulated, 331 genes down-regulated, cut off: fold change  $\geq 1.5$  up- or down-regulation and  $p_{\text{adj}} < 0.05$ ). For comparison, the corresponding gene expression data for wild-type fibroblasts, FiPSCs and ESCs were included. The heatmap is colored by row-scaled LOG2 average expression signals according to the color key on the top. Samples were clustered by column means.

either directly involved in the recognition and binding of exogenous, putatively pathogenic, nucleic acids (*RIG-I* (*DDX58*) [35,36], *PKR* (*EIF2AK2*) [37,38], *OAS1* [37,39] and *IFIT1* [40]), key regulators of transcription during the innate immune response (*IFNB1* [41]), intra- or extracellular transducers of the stimulus (*IL12A* (*CLMF*, *NKSF1*) [42,43], *IRF7* [41,44], *STAT2* [45], *CCL5* (*RANTES*) [46,47]) or viral restriction factors (*ISG20* [48,49], *TRIM5* [50,51]). Interestingly, all of these genes are regulated by interferons (Supplementary Table S7). As shown in Fig. 5, qRT-PCR demonstrated the up-regulation of all 11 genes under investigation in both BJ and HFF1 fibroblasts upon transfection of the mixture of unmodified reprogramming factor-encoding mRNAs when compared to mock-transfected control cells.

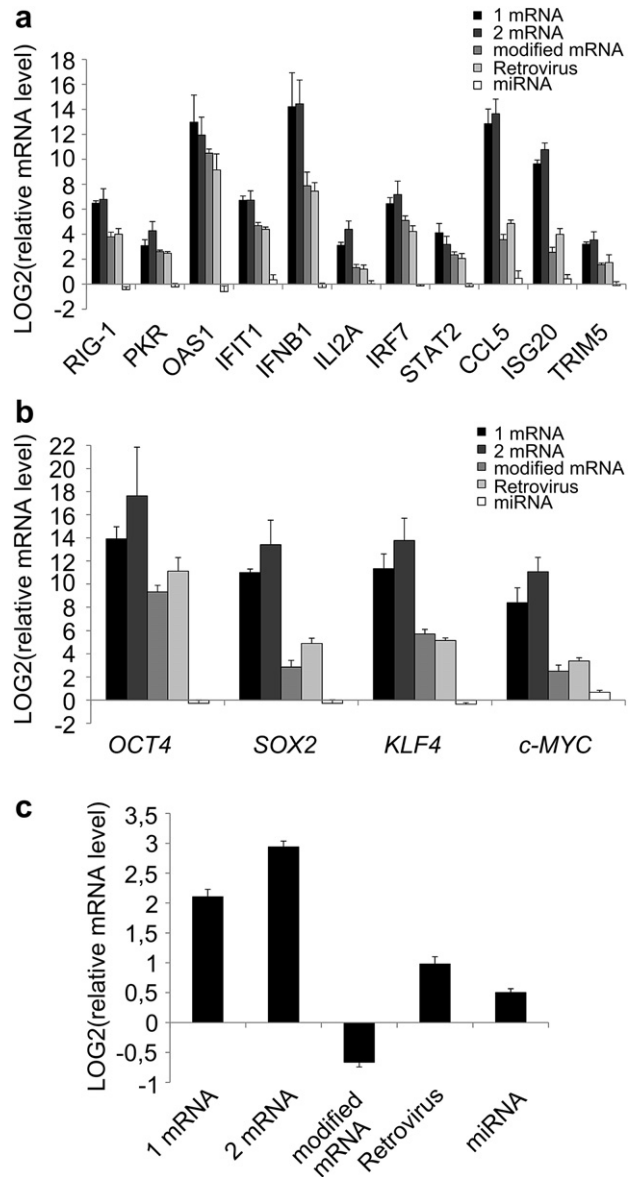
In a next step, we compared the magnitude of this innate immune response during reprogramming experiments utilizing different delivery techniques. To this end, gene expression levels of the same set of innate immune response-associated genes were assessed after (1) retroviral transduction of a 1:1:1:1 cocktail of the reprogramming factors OCT4, SOX2, KLF4 and c-MYC, (2) one single transfection of the reprogramming factors using equal ratios of the in-house synthesized, unmodified mRNAs by means of lipofection, (3) two subsequent of the aforementioned mRNA lipofections (day1, day2), (4) one transfection of a 1:1:1:1 mixture of the Yamanaka



**Fig. 5.** qRT-PCR of innate immune response-associated transcripts in human fibroblast cells upon mRNA transfection. BJ and HFF1 cells were lipofected with a mixture of mRNAs encoding the reprogramming factors in equal ratios (4  $\mu\text{g}$  per 6-well) and harvested 24 h later for RNA isolation. Bars and error bars represent the average of LOG2 ratios of transfected fibroblasts over mock-transfected controls and SD of technical triplicates.  $n = 1$  per cell line.

factors using commercially available, modified mRNAs, (5) one transfection of a mix of pluripotency-promoting miRNAs (miR302s/367). Concerning the series of retroviral and miRNA tests, the experimental set-ups were based on either our own or published reprogramming experiments [16,22,52]. Fig. 6a shows that our unmodified reprogramming factor mRNAs, although capped and poly(A)-tailed, induced the strongest up-regulation of immune-associated genes. Interestingly, the second transfection with the same mRNA cocktail 24 h after the first one, only slightly increased the expression levels of a few genes, namely, *PKR*, *IL12A*, *ISG20*, yet, does not have an overall augmenting immunogenic effect suggesting that the cellular immune response is already nearly maximally up-regulated after the first transfection. The transcript levels of the delivered reprogramming factors itself, however, were only slightly increased after the second transfection when compared to the levels determined after the first transfection (Fig. 6b). In contrast, immunostaining of the introduced factors after two subsequent transfections revealed that the expression of the translated proteins was very weak (data not shown). Transfection of commercially available modified reprogramming mRNAs, which, in contrast to the mRNAs produced ourselves, contained pseudouridine and 5-methylcytidine-modified nucleotides, resulted in markedly reduced expression levels of almost all immune response-associated genes compared to transfections with our unmodified mRNAs (Fig. 6a). Nonetheless, the incorporation of modified nucleotides did not prevent the up-regulation of the innate interferon response and expression levels of the immune response-associated genes were still elevated when compared to mock-transfected controls. Surprisingly, the number of reprogramming factor transcripts in the cell were notably less when compared to the transfections with the same amount of unmodified mRNAs (Fig. 6b). When cells were transduced with a retroviral reprogramming factor cocktail, expression levels of both immune response-associated and reprogramming factor genes of cells were similar to those detected upon transfection of modified mRNAs. Yet, it must be emphasized that retroviral transcripts are usually not yet fully expressed 24 h post-transduction as demonstrated by the increase in immunofluorescent staining intensity of reprogramming factors 24, 48 and 72 h post-transduction in our recent publication [25]. Finally, as anticipated, an up-regulation of endogenous reprogramming factors by miRNAs could not be observed as early as 24 h post-transfection (Fig. 6b). Interestingly however, the transfected miRNAs did not trigger any immune response (Fig. 6a). In contrast to the transfection or transduction of the reprogramming factors, the internalization and functionality of the transfected miRNAs could not be verified by immunostaining of the four factors. Therefore, we chose to analyze *PODXL* transcript levels instead. *PODXL* is a surface marker expressed in human ESCs, iPSCs and embryonal carcinoma cells [53] and is activated as early as 24 h post-retroviral transduction as demonstrated by our previous findings on cellular reprogramming-initiation events [25]. In this respect, the one and two transfections of unmodified RNAs successively up-regulated *PODXL* as did the cocktail of retroviruses and, to a lesser extent, the combination of miRNAs. Surprisingly, the modified mRNAs did not up-regulate *PODXL* 24 h post-transfection (Fig. 6c).

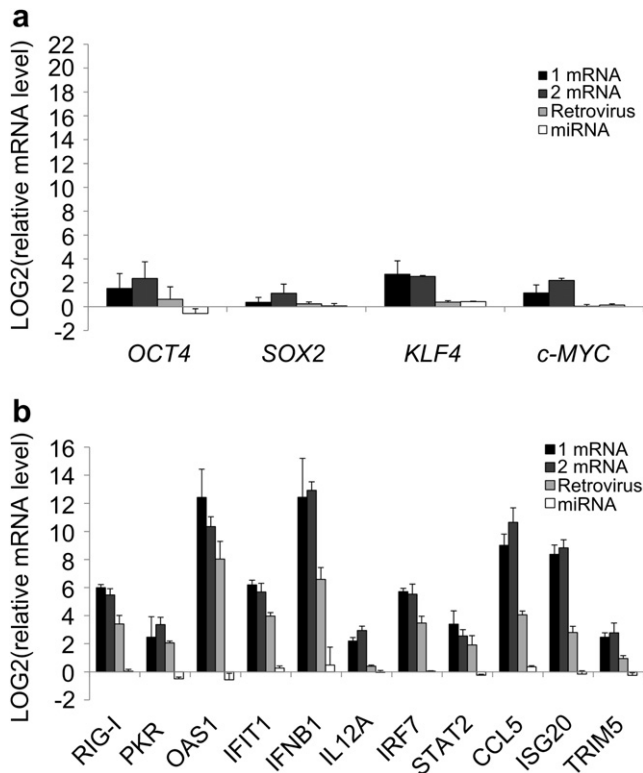
We then investigated whether the immune response is specific for the introduced factors by substituting the reprogramming factors with GFP-encoding mRNAs or retroviruses, or scrambled control miRNAs. Logically, as shown in Fig. 7a, apart from normal variation, we did not detect significant alteration in gene expression levels of reprogramming factors. Yet, with all delivery techniques, the expression levels of immune response genes followed the exact pattern and the absolute transcript quantities were similar when compared to those observed for the respective delivery of reprogramming factor cocktails (Fig. 7b).



**Fig. 6.** Expression levels of innate immune response-associated genes, introduced reprogramming factor genes and *PODXL* in HFF1 cells upon delivery of reprogramming factors by diverse methods. HFF1 cells were transfected with a 1:1:1:1 cocktail of mRNAs encoding OCT4, SOX2, KLF4 and c-MYC. Transfections were carried out once or twice ("1 mRNA", "2 mRNA", respectively; 4  $\mu$ g total per 6-well per transfection). The mRNAs were either synthesized in our laboratory ("mRNA") or commercially bought ("modified mRNA"). Alternatively, HFF1 cells were transduced once with a 1:1:1:1 combination of retroviruses encoding OCT4, SOX2, KLF4 and c-MYC or transfected once with a 1:1:1:1 mix composed of the miRNAs miR302a, miR302b, miR302c, miR302d and miR367 (100 pmol total per 6-well). All samples were harvested 24 h after the last transfection/transduction for RNA isolation. a) Levels of innate immune response-associated transcripts. b) Expression levels of the reprogramming factor genes. c) *PODXL* transcript levels. Bars and error bars represent the average of LOG2 ratios of transfected/transduced fibroblasts over mock-transfected/-transduced controls and SD.  $n = 6$  for "1 mRNA";  $n = 4$  for "2 mRNA";  $n = 3$  for "Retrovirus", "modified mRNA" and "miRNA".

### 3.5. Effect of chemical treatments on innate immune response in HFF1 cells upon transfection of mRNAs encoding reprogramming factors

As demonstrated above, the main roadblock in our mRNA reprogramming experiments was the activation of innate immune response resulting in decreased proliferation and increased loss of cell viability. This might have been a result of the up-regulation



**Fig. 7.** Expression levels of reprogramming factor genes and innate immune response-associated genes in HFF1 cells upon delivery of control genes by diverse methods. Repetition of the HFF1 transfection/transduction experiments as described in Fig. 6. Yet, this time, the total amount of mRNAs and miRNAs used for reprogramming were substituted with the same amount of GFP-encoding mRNA (“1 mRNA”, “2 mRNA”; 4  $\mu$ g total per 6-well per transfection) or a scrambled miRNA (100 pmol total per 6-well). Similarly, cells were transduced with a GFP-encoding retrovirus equivalent to the amount of retrovirus encoding one of the reprogramming factors in the reprogramming OSKM cocktail. a) Expression levels of the reprogramming factor genes. b) Levels of innate immune response-associated transcripts. Bars and error bars represent the average of LOG2 ratios of transfected/transduced fibroblasts over mock-transfected/-transduced controls and SD.  $n = 6$  for “1 mRNA”;  $n = 4$  for “2 mRNA”;  $n = 3$  for “Retrovirus” and “miRNA”.

of one of the key innate immune response regulators, type I interferon *IFNB1*. Interestingly, Yang et al., adopting an integrated genomics approach, identified Nabumetone, a nonsteroidal anti-inflammatory drug, significantly enhanced the induction of pluripotency in mouse cells [54]. With this evidence, we next searched for substances that might suppress the innate immune response we observed from our microarray-based transcriptome data, thereby, increasing mRNA-based reprogramming efficiency. We evaluated the ability of five substances to prevent up-regulation of the different innate immune response-associated genes by means of qRT-PCR when they were incubated before, during and after delivery of the mRNA complexes.

The first molecule to suppress the innate interferon response we tested, was B18R – a virus-encoded decoy receptor specific for type I interferons of various species, which neutralizes signaling via type I interferon receptors. B18R was described to prevent transmembrane signaling and transcriptional regulation of the interferon-regulated genes [55,56] and has been used in the only mRNA-based reprogramming protocol published so far [14]. As presented in Fig. 8a, in our hands this treatment did not show any measurable decrease of innate immune response even though all of these genes are known to be IRGs as determined by the Interferome database. Because this result was in line with our previous finding that B18R did not have any effect on the efficiency of inducing pluripotency in

fibroblast cells [25], we did not pursue investigations using B18R further.

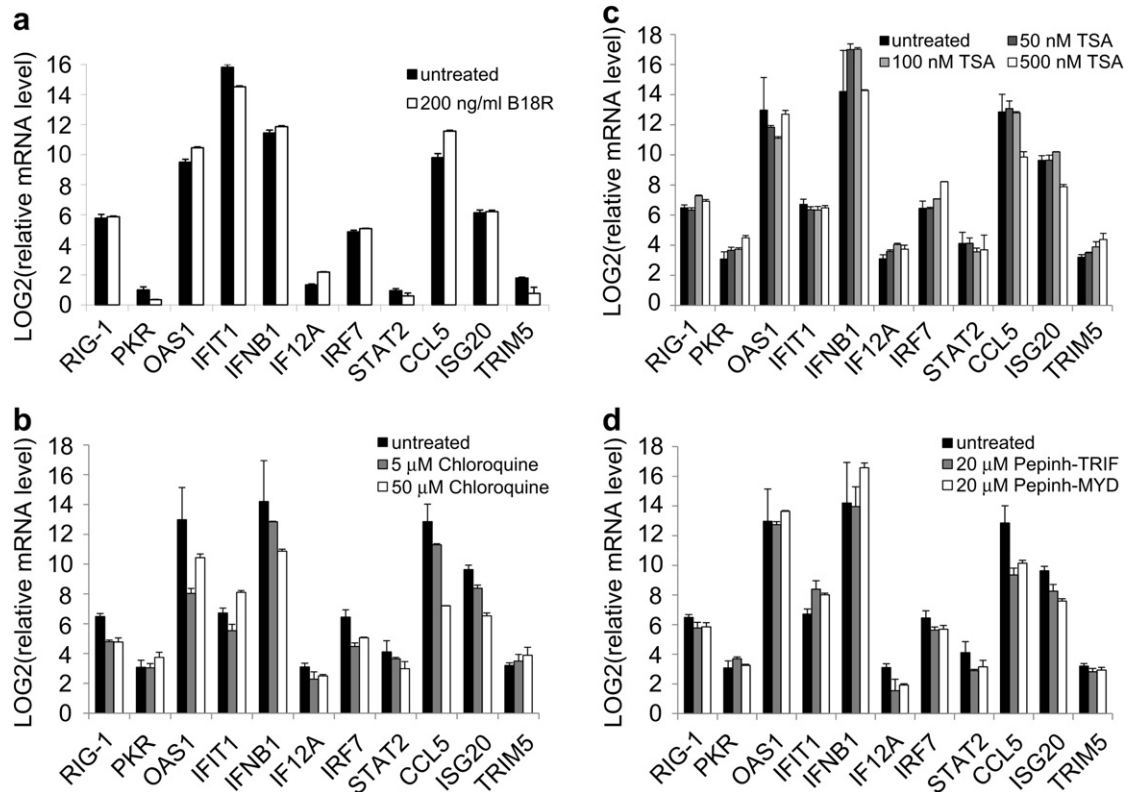
Secondly, chloroquine, originally synthesized as an anti-malaria drug, intrigued us to examine its ability to suppress the immune response initiated by mRNA delivery. This lysosomotropic substance has been reported to exert pleiotropic functions such as enhancing transfection efficiency by facilitating endosomal escape and/or diminishing lysosomal degradation [57,58]. Moreover, chloroquine has been shown to have several immunomodulatory, anti-inflammatory effects such as the ability to inhibit TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production in lipopolysaccharide-stimulated human monocytes/macrophages [59], to reduce type I interferon (IFN- $\alpha$ ) production in CpG-DNA- or viral ssRNA-stimulated plasmacytoid-derived dendritic cells or IL-12 production in ssRNA poly(U)-stimulated monocyte-derived dendritic cells [43,60]. We tested three different concentrations of chloroquine and noticed a strong concentration-dependent cytotoxic effect. Even though the expression levels of a few innate interferon response-associated genes (*RIG-I*, *OAS1*, *CCL5*, *ISG20*) were slightly reduced upon chloroquine treatment when compared to mock-transfected controls (Fig. 8b), these reductions were not sufficient to balance out the cytotoxic effects of this molecule.

Trichostatin A (TSA), a streptomycetes metabolite, was first discovered as an antifungal antibiotic and later as an inhibitor of mammalian histone deacetylases [61,62]. Moreover, it was reported to block nuclear translocation of the transcription factor IRF7 and as a consequence numerous interferons and other pro-inflammatory cytokines [63]. When applied at the suggested concentrations, TSA showed no significant effects on the expression levels of genes tested (Fig. 8c). When used at the highest concentration (500 nM), expression levels of *CCL5* and *ISG20* were reduced when compared to untreated, mRNA-transfected samples, however, the absolute gene expression values were still much higher than those determined for mock-transfected controls.

Finally, we tried Pepinh-TRIF and Pepinh-MYD, two peptide inhibitors designed to contain domains of the signaling adapter molecules TRIF and MyD88, thus facilitating interaction with the respective pattern-recognition receptors TLR3 or TLR7/8 [64,65]. Competing with endogenous TRIF and MyD88 for association with those TLRs, these two peptide inhibitors could attenuate signaling to the nucleus, thereby, suppressing the up-regulation of innate immune response genes. However, treatment of HFF1 cells with each of these peptides at the suggested concentration of 20  $\mu$ M, did not lead to a marked reduction in expression of any of the immune response-associated genes (Fig. 8d).

#### 4. Discussion

The ability of our method to transfect human fibroblast cells with mRNA successfully was demonstrated by the high levels of expression of GFP after m(GFP) lipofection. Moreover, when mRNAs encoding the Yamanaka factors (OSKM) were introduced into HFF1 cells (human neonatal fibroblasts), the proteins were expressed within the cell nucleus as one would expect. Daily transfections with factor-encoding mRNAs caused tremendous cytotoxicity with no viable cells after seven consecutive transfections. Despite our expertise and experience in producing induced pluripotent stem cells (iPSCs) [6,20–22], several attempts to apply our mRNA-mediated technique to fully reprogram HFF1 cells into induced pluripotent cells failed because of this progressive cell death. Accordingly, also Angel and Yanik noticed severe cell damage upon repeated transfection with mRNA [15]. These observations spurred us to investigate the barriers preventing the successful induction of pluripotency in fibroblast cells.



**Fig. 8.** Effect of chemical treatments on the expression of innate immune response-associated transcripts in HFF1 cells upon mRNA transfection. HFF1 cells were transfected once with a 1:1:1:1 mixture of mRNAs encoding OCT4, SOX2, KLF4, c-MYC (4  $\mu$ g total per 6-well). One hour prior to transfection, during transfection and directly following transfection cells were treated with 200 ng/ml B18R (a), given concentrations of chloroquine (b), TSA (c), or pepinh-TRIF or pepinh-MYD (d). All samples were harvested 24 h after transfection for RNA isolation. Bars and error bars represent the average of LOG2 ratios of (treated) transfected fibroblasts over (treated) mock-transfected controls and SD.  $n = 1$  for B18R;  $n = 2$  for Pepinh-TRIF;  $n = 3$  for Pepinh-MYD;  $n = 2$  for each concentration of TSA and chloroquine.

Despite the high expression levels of the exogenously delivered reprogramming factors by means of mRNA lipofection, a general switch in the global gene expression pattern from the fibroblast-transcriptome toward the transcriptome of a pluripotent cell (down-regulation of fibroblast genes, up-regulation of pluripotency-associated genes) was not apparent at 24 h post-transfection of the reprogramming factor-encoding mRNAs. However, we did observe an overlap of the transcriptomes of reprogramming factor-transfected fibroblasts and the union of human FiPSCs and ESCs and the relevance for cellular reprogramming of these highlighted genes warrants further investigation. This is supported by Plews et al., who showed an activation of pluripotency-associated genes upon delivery of mRNAs encoding the reprogramming factors into human fibroblasts, however, complete reprogramming to fully characterizable iPSCs was not attained [66]. The transcriptome data obtained by microarray analysis allowed us to conclude that the key roadblock during reprogramming by means of frequent mRNA transfections is a strong innate immune reaction initiated by cellular mechanisms recognizing foreign nucleic acids, as is the case during microbial or viral infections. This is in line with recent reports [15]. Based on the findings reported in the review of Yoneyama and Fujita [41], the cascade of events provoked in our mRNA-transfected cells can be conceived as follows: first, the transfected, purified, *in vitro* synthesized reprogramming factor-encoding mRNAs are recognized by pattern-recognition receptors (PRRs). Because the secondary structure of the transfected mRNAs is elusive, it is difficult to predict which PRRs are responsible for the detection of the exogenous mRNAs. In principle, given that lipofected nucleic acids are primarily taken up via endocytotic routes [67], TLR3 and TLR7/8 in the endosomes should be the primarily involved PRRs [41]. Yet, it is known

that TLR3 and TLR7/8 are preferentially expressed in immune cells [41]. Accordingly, the microarray data suggest that TLR3 is only expressed in fibroblasts upon mRNA transfection and that TLR7/8 receptors are not expressed in any of the samples under investigation. It is tempting to speculate that cytoplasmic receptors such as RIG-I and MDA5 are essential for the recognition of exogenous mRNA when accessible in the cytoplasm, e.g. after endosomal escape of the transfected mRNA [67]. Concerning these, our microarray data revealed low level expression of both of these receptors in mock-transfected control fibroblasts and strong up-regulation upon mRNA transfection (*RIG-I* (*DDX58*): 47-fold up-regulation, *MDA5* (*IFIH1*): 72-fold up-regulation). Indeed, it has been shown by Kato et al. that induction of type I interferons upon infection of mouse embryonic fibroblasts with several single-stranded RNA viruses was RIG-I- but not TLR-dependent [68]. Although it is not completely clear, which of the suggested receptor(s) is/are eventually responsible for the detection of the foreign mRNAs, our results emphasize that the up-regulation of the receptors involved, triggered an increased translation of type I interferons (such as *IFNB1*) and pro-inflammatory cytokines (such as *CCL2*, *CCL5*, *IL10* and *IL12A*). In turn, type I interferons provoked positive feedback regulation, which enhanced different defense mechanisms against the putative pathogen attack at different levels. Correspondingly, our data suggested that within the mRNA-transfected cells, PRRs like *RIG-I*, *MDA5*, including those, which elicit antiviral/antimicrobial activities, such as *OAS1*, *PKR* and *ADAR*, were significantly up-regulated together with a number of signaling molecules (*JUN* (as part of the transcription factor AP-1), *IRF7*, *MYD88*, *IRAK4* etc.). As a result, protein translation was potentially blocked, the foreign nucleic acid degraded and apoptosis induced. In support of this effect, we



detected down-regulation of interferon-responsive cell cycle regulating genes (*CDC20*, *CCNB1*, *CCNF*). In line with our results, Johnston and co-workers reported induction of *IFNB1*, *PKR*, *OAS1* as well as induction of cell death in human dermal fibroblasts stimulated with dsRNA [69]. It is worth mentioning that interferons as well as the up-regulated pro-inflammatory cytokines act in an autocrine and paracrine fashion, i.e. they transduce their signal into neighboring cells activating the same innate immune mechanisms in those cells and sensitizing them toward foreign nucleic acids [15,41]. Taken together, these findings are in line with our previous observation with respect to retrovirally transduced fibroblasts, i.e. one of the main obstacles to overcome during retroviral-based reprogramming experiments is the innate immune response, which is triggered in the target cells [25].

Despite the lack of TLR7/8 expression in HFF1 and BJ fibroblasts, the TLR-associated adapter molecules *MYD88* and *TRIF* as well as the signaling molecule *IRF7* were highly up-regulated upon transfection of mRNAs encoding the reprogramming factors. Therefore, we were still convinced that both Pepinh-TRIF and Pepinh-MYD as well as TSA would modulate the innate immune response in mRNA-transfected fibroblasts. Unfortunately, none of the chemicals tested showed significant down-regulation of the assessed immune response-associated genes in our hands, although their inhibitory function on distinct levels of the innate immune response activated upon non-viral mRNA transfection was promising.

Beside the quest for alternative ways of suppressing the immune response in mRNA-mediated induction of pluripotency in human somatic cells, it is of importance to investigate the potential of small molecules to increase the efficiency of this reprogramming approach. Several chemical compounds such as the histone deacetylase inhibitor VPA and the cyclic AMP analog 8-Br-cAMP, which transiently down-regulate p53 [4,6] were shown to enhance the induction of pluripotency in somatic cells. Furthermore, treatment of cells with sodium butyrate, a histone deacetylase inhibitor, and other molecules, which modulate distinct signaling pathways, including PS48, an activator of 3'-phosphoinositide-dependent kinase-1 (PDK1) [8], MEK inhibitor PD0325901, GSK3 $\beta$  inhibitor CHIR99021, TGF- $\beta$ /Activin/Nodal receptor inhibitor A-83-01, ROCK inhibitor HA-100 and human leukemia inhibitory factor (LIF) [7], also resulted in increased reprogramming efficiencies. We, therefore, propose that these small molecule cocktails should be tested and also incorporated into mRNA-based cellular reprogramming protocols in the future.

## 5. Conclusions

We have clearly demonstrated the functionality of our mRNA transfection protocol in terms of expression of the translated proteins in the nucleus. Full reprogramming of human neonatal fibroblasts into iPSCs by means of repeated transfection of mRNAs encoding the Yamanaka factors was impeded by activated innate immune response. We intensively investigated this activation by means of comparative global transcriptome analysis and qRT-PCR of selected genes within the innate immune response pathway. The comparison of the immune responses triggered by different delivery methods revealed that the immune response induced by unmodified mRNA was the highest, followed by retroviral transduction and modified mRNA, which induced similar expression levels of immune-associated genes. In contrast, miRNA transfections did not induce a significant response. Supplementation with various anti-inflammatory compounds (B18R, chloroquine, TSA, Pepinh-TRIF, Pepinh-MYD) did not induce a down-regulation of immune response-associated genes in our assays. In summary, our data provide an important basis for developing new approaches for suppressing immune reactions, be it the use of other chemical

compounds and/or siRNA(s), thus, allowing multiple mRNA transfections to facilitate efficient mRNA-mediated induction of pluripotency in human somatic cells.

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## Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.02.025.

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