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Differential effects of toll-like receptor stimulation on mRNA-driven myogenic conversion of human and mouse fibroblasts

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ABSTRACT

Transfection with *in vitro* transcribed mRNAs is a safe and effective tool to convert somatic cells to any cell type of interest. One caveat of mRNA transfection is that mRNAs are recognized by multiple RNA-sensing toll like receptors (TLRs). These TLRs can both promote and inhibit cellular reprogramming. We demonstrated that mRNA transfection stimulated TLR3 and TLR7 and induced cytotoxicity and IFN-β expression in human and mouse fibroblasts. Furthermore, mRNA transfection induced paracrine inhibition of repeated mRNA transfection through type I IFNs. Modified mRNAs (mmRNAs) containing pseudouridine and 5-methycytosine reduced TLR stimulation, cytotoxicity and IFN-β expression in fibroblasts. Repeated liposomal transfection with MyoD mmRNAs significantly enhanced myogenic conversion of human and mouse fibroblasts compared with repeated transfection with MyoD mRNAs. Interestingly, electroporation of mRNAs and mmRNAs completely abrogated cytotoxicity and IFN-β expression and also abolished myogenic conversion of fibroblasts. At a low concentration, TLR7/8 agonist R848 enhanced MyoD mmRNA-driven conversion of human fibroblasts into skeletal muscle cells, whereas high concentrations of R848 inhibited myogenic conversion of fibroblasts. Our study suggests that deliberate control of TLR signaling is a key factor in the success of mRNA-driven cellular reprogramming.

1. Introduction

Repeated transfection with *in vitro*-transcribed (IVT) mRNAs encoding defined factors induced direct reprogramming of human somatic cells into induced pluripotent stem cells (iPSCs) as efficiently as viral transduction [1]. Furthermore, repeated transfection with mRNAs encoding cell fate-determining factors could directly convert mouse and human fibroblasts into many different types of cells, including mouse myoblast-like cells [1], mouse neuronal stem cells [2], mouse cardiomyocytes [3] and human hepatocyte-like cells [4].

Viral RNAs and synthetic analogs can stimulate endosomal toll-like receptors (TLRs), e.g., TLR3, TLR7 and TLR8, and cytoplasmic RNA-sensing pattern recognition receptors (PRRs) and induce expression of type I interferon (IFN) (e.g., IFN-α and IFN-β) and cell death in various human and mouse cells [5–7]. The type I IFNs are anti-infectious cytokines that regulate the growth of infected cells by the inhibition of mRNA translation and cell proliferation [8].

Transfection with IVT mRNAs also stimulates RNA-sensing TLRs and PRRs, resulting in the induction of inflammatory response, cytotoxicity and type I IFN expression [9,10]. RNA-driven cytotoxicity and inflammatory response are one of the major obstacles to mRNA-driven reprogramming of cells [1,9,11]. To overcome this problem, modified nucleosides such as pseudouridine (ψU) and 5-methylcytosine (5 mC) were incorporated into IVT mRNAs and these modified IVT mRNAs (called mmRNAs) ablated activation of RNA-sensing TLRs and PRRs [1,12,13]. On the other hand, TLR-mediated inflammatory responses are required for the successful reprogramming of cells [14,15]. Supplementation with TLR3 agonist polyinosinic-polycytidylic acid (polyI:C) enhanced mmRNA-driven reprogramming of human fibroblasts into iPSCs [1]. Thus, TLR and PRR are simultaneously necessary and inhibitory for mRNA-driven reprogramming of cells. Therefore, it is critical to develop an

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optimal method to deliberately control activation of TLRs and PRRs and improve mmRNA-driven reprogramming of cells.

In the current study, we compared mRNA- and mmRNA-induced cytotoxicity and IFN-β expression between human and mouse fibroblasts and determined improved methods to deliver mRNA, modulate inflammatory responses and cytotoxicity and enhance mRNA-driven cellular reprogramming.

2. Materials and methods

2.1. Generation of MyoD, GFP and actin mRNAs and mmRNAs

Mouse and human MyoD1, mouse actin and green fluorescent protein (GFP) cDNAs were cloned into the transcription vector pSP73-Sph/A64. Generation of IVT mRNAs was conducted as described previously [16]. Briefly, plasmids were linearized with SpeI and *in vitro* transcribed using the MEGAscript® kit (Ambion, Austin, TX). The transcription reaction mixture contained 6 mM 3′-0-Me-m^7G(5′)ppp(5′)G ARCA Cap analog (New England Biolabs, Ipswich, MA), 7.5 mM ATP, 7.5 mM CTP, 7.5 mM UTP, 1.5 mM GTP, T7 polymerase, reaction buffer (all supplied by the kit) and 48 μg/ml linear DNAs. To generate mmRNAs, 5 mC triphosphate and ψU triphosphate (TriLink Biotechnologies, San Diego, CA) replaced CTP and UTP in the reaction mixture. Reactions were incubated for 4 h at 37 °C. IVT mRNAs were treated with DNase I for digestion of the template DNAs, followed by polyadenylation of RNA using a Poly(A) tailing kit (Ambion). To avoid the activation of innate immune responses by RIG-I and PKR, the IVT mRNAs were treated with Antarctic Phosphatase (New England Biolabs) to remove residual 5′-triphosphates, and then the mRNAs were purified using the NucleoSpin® RNA Clean-up kit (MACHEREY-NAGEL, Bethlehem, PA).

2.2. Cell culture

Mouse embryonic fibroblasts (MEF) (ATCC, Manassas, VA) and human lung fibroblasts (hLF) (Lonza, Allendale, NJ) were grown in DMEM with 15% heat-inactivated FBS. Human neonatal dermal fibroblasts (hDF) (Invitrogen, Grand Island, NY) were grown in Medium 106 with Low Serum Growth Supplement (Invitrogen). The cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.3. mRNA transfection and myogenic conversion

Human and mouse cells (1.25×10^4 cells/cm^2) were incubated overnight in a multi-well flat bottom plate (Corning, Tewksbury, MA). The cells were transfected with the IVT mRNAs using DharmaFECT® Duo Transfection Reagent (Thermo Scientific) or Electro Square Porator ECM 830 (BTX, San Diego, CA) as described previously [17]. Briefly, DharmaFECT were mixed in OPTI-MEM I (Invitrogen) at a RNA:DharmaFECT:OPTI-MEMα ratio of 100 ng:0.4 μl:10 μl. This mixture was incubated for 15 min at room temperature. 250 ng of the IVT RNA per 1×10^4 cells were directly added to each well of a multi-well plate. After a 5-h incubation, culture supernatant was aspirated and fresh cell culture media was added. For electroporation, 1×10^5 cells suspended in 100 μl Opti-MEM I were mixed with either 2.5 μg of IVT RNAs in 2-mm cuvettes and were electroporated at 340 V for 500 μs Cells were mixed with 2.15 ml of pre-warmed complete medium immediately after electroporation and seeded into various multi-well plates depending on the experiments. To differentiate into skeletal muscle cells, MyoD mRNA- and MyoD mmRNA-transfected cells were incubated for 7 days with muscle differentiation medium composed of DMEM supplemented with 2% horse serum and insulin/selenium/transferrin (Invitrogen). PolyI:C, R848 (both from Invivogen, San Diego, CA) and B18R (eBioscience, San Diego, CA) were used to modify innate immune activation during myogenic conversion.

2.4. Cytotoxicity assay

Fibroblasts (4×10^3 cells/well) in 90 μl of culture medium were incubated overnight in a 96-well plate. These cells were transfected as described above and incubated for 72 h at 37 °C in a humidified incubator with 5% CO_2. Cytotoxicity was determined using Celltiter 96® MTS Cell Proliferation Assay Kit (Promega, Madison, WI), according to the manufacturer's instructions. The percent cytotoxicity was calculated by using the following equation: % cytotoxicity = $([O.D.]_{untreated} - [O.D.]_{treated})/[O.D.]_{untreated}$ x 100.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The production of IFN-β and IL-8 was determined using human and mouse IFN-β ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ) and BD OptEIA™ IL-8 ELISA sets (BD Biosciences, Franklin Lakes, NJ), respectively, by following the manufacturer's instructions.

2.6. Quantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using Superscript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was carried out using the Taqman Gene Expression Assay (Applied BioSystems, Foster City, CA, USA) with a C1000 Touch Thermal Cycler (Bio-Rad). The PCR reactions for all assays were performed at 50 °C for 2 min, 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s, 60 °C for 30 s. The gene expression level was normalized to GAPDH as an internal reference.

2.7. Statistical analysis

The paired two-tailed Student's *t*-test was applied for determination of statistical significance. A probability of less than 0.05 ($P < 0.05$) was used for statistical significance.

3. Results and discussion

3.1. Mouse and human fibroblasts are sensitive to mRNA-induced cytotoxicity

Consistent with a previous study [12], transfection with unmodified IVT mRNAs stimulated RNA-recognizing TLR3 and TLR7, whereas transfection with modified IVT mmRNAs significantly reduced stimulation of these TLRs (Fig. 1A). Furthermore, human and mouse fibroblasts transfected with IVT mRNAs produced IFN-β and underwent cell death, whereas fibroblasts transfected with IVT mmRNAs significantly decreased IFN-β production and cell death compared with fibroblasts transfected with IVT mRNAs (Fig. 1B and C). Although both human and mouse fibroblasts transfected with IVT mRNAs produced similar amounts of IFN-β human fibroblasts were more sensitive to IVT mRNA-induced cytotoxicity than mouse fibroblasts (Fig. 1C).

3.2. IFN-β secreted from mRNA-transfected cells inhibits repeated mRNA transfections

Repeated transfections with mRNAs are often required for mRNA-driven reprogramming of cells. Innate immune and

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