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Retinoic Acid Inducible Gene 1 Protein (RIG1)-like Receptor Pathway is Required for Efficient Nuclear Reprogramming

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Abstract

We have revealed a critical role for innate immune signaling in nuclear reprogramming to pluripotency, and in the nuclear reprogramming required for somatic cell transdifferentiation. Activation of innate immune signaling causes global changes in the expression and activity of epigenetic modifiers to promote epigenetic plasticity. In our previous papers, we focused on the role of toll-like receptor 3 (TLR3) in this signaling pathway. Here we define the role of another innate immunity pathway known to participate in the response to viral RNA, the retinoic acid-inducible gene 1 receptor (RIG-1)-like receptor (RLR) pathway. This pathway is represented by the sensors of viral RNA, RIG-1, LGP2 and MDA5. We first found that TLR3 deficiency only causes a partial inhibition of nuclear reprogramming to pluripotency in mouse tail-tip fibroblasts, which motivated us to determine the contribution of RLR. We found that knockdown of iPS-1, the common adaptor protein for the RLR family, substantially reduced nuclear reprogramming induced by retroviral or by mRNA expression of Oct 4, Sox2, KLF4 and cMYC (OSKM). Importantly a double knockdown of both RLR and TLR3 pathway led to a further decrease in iPSC colonies suggesting an additive effect of both these pathways on nuclear reprogramming. Furthermore, in murine embryonic fibroblasts expressing a dox-inducible cassette of the genes encoding OSKM, an RLR agonist increased the yield of iPSCs. Similarly, the RLR agonist enhanced nuclear reprogramming by cell permeant peptides of the Yamanaka factors. Finally, in the dox-inducible system, RLR activation promotes activating histone marks in the promoter

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Disclosures: Drs. Cooke, Sayed and Lee are inventors of the intellectual property, assigned to Stanford University, which was generated by this research.

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Introduction

The forced expression of a small set of reprogramming factors (Oct4, Sox2, Klf4 and c-myc, i.e. “OSKM”) can induce nuclear reprogramming of somatic cells to induced pluripotent stem cells (iPSCs). This landmark development has major implications in regenerative medicine such as disease modeling, drug discovery and patient-specific cell therapy. Since the initial discovery, a variety of alternative strategies have emerged to either increase the yield or safety of the reprogramming process. These include the use of various small molecules that act on epigenetic modifiers to facilitate epigenetic changes required for iPSC generation. Nevertheless, the reprogramming efficiency remains low and is a lengthy process. Thus, a greater understanding of the reprogramming process is likely to yield insights to improve the efficiency and fidelity of nuclear reprogramming. In this regard, we have described a critical signaling pathway required for effective nuclear reprogramming of somatic cells to induced pluripotent stem cells (iPSCs). This discovery occurred as we struggled to generate iPSCs using cell permeant peptide (CPP) versions of the “Yamanaka factors” (OSKM). Our OSKM CPPs could bind in vitro to their cognate consensus sequence; could enter the cell nucleus; and could even rescue pluripotency in iPSCs in which a Yamanaka gene had been knocked down by shRNA. Yet the OSKM CPPs were extraordinarily inefficient at generating iPSCs.

However, when an irrelevant retroviral vector was added with the CPPs, nuclear reprogramming was achieved. Ultimately, we determined that when retroviral vectors were used to deliver the genes encoding the Yamanaka factors, the retroviral dsRNA stimulated Toll-like Receptor 3 (TLR3) to activate NF-κB and IRF3. These transcriptional factors caused global changes in epigenetic modifiers [e.g. downregulation of the histone deacetylase (HDAC) family; upregulation of histone acetyltransferases (HATs)], increasing the probability for the chromatin to assume an ‘open configuration’ state. This increase in epigenetic plasticity permitted the CPPs to activate the network of core pluripotency genes.

In addition to activating TLR3, viral RNA can also stimulate the retinoic acid-inducible gene 1-like receptors (RLR) to mediate host antiviral responses. In human cells, the RLR family comprise RIG-1, MDA5 and LGP2. We were interested to know if the RLR family might also play a role in iPSC generation.

Methods

Cells

BJ human fibroblast cells (Stemgent), and mouse tail-tip fibroblasts (TTFs) isolated from 6-week old TLR3 knockout animals, were cultured and maintained in DMEM with 10% FBS and 1% penicillin/streptomycin (5% CO₂, 37°C). Secondary dox-inducible MEFs were isolated as described previously, from embryos obtained from transgenic
R26iTA:Cdkn1a1lox-4F2A mice expressing the loxP-flanked, dox-inducible polycistronic 4F2A cassette (Oct4, Sox2, Klf4, c-Myc; Jackson Laboratory). These MEFs were expanded for at least two passages after isolation and then frozen in liquid nitrogen.

**Retrovirus preparation**

**Human Fibroblasts**—Retrovirus encoding the reprogramming factors (OSKM) was prepared in HEK293T cells. Briefly, cells were transfected with plasmids encoding VSV-G (envelope protein; 10μg), pUMVC (packaging plasmid; 15μg) and the reprogramming genes (OSKM; 10μg) with lipofectamine for 48hrs. Following transfections, the supernatant was collected, filtered (0.45 um filter) and purified by ultracentrifuge (17,100 rpm for 2.5 hr) to obtain a viral pellet. The viral pellet was resuspended in DMEM to make a 100× stock. For infections in human BJ fibroblasts, (5 × 10^4 cells/well) were seeded a night before and then infected with the resuspended virus and polybrene (8 μg/ml) and incubated for 24hrs.

**Mouse Fibroblasts**—Retrovirus preparations used for reprogramming in MEFs or TLR3KO TTFs were obtained from Stemgent. MEFs and TTFs were infected with the retrovirus using the manufacturer’s protocol.

**Short Hairpin RNA for knockdown studies**

Short hairpin RNA for stable knockdown of IPS1, and scrambled shRNA, were obtained from Invivogen. The target sequence is available on the manufacturer’s website. Briefly, 2 × 10^5 human BJ fibroblasts or mouse fibroblasts (WT or TLR3KO) were seeded in a T75 flask overnight to reach 60–80% confluence at the time of transfection. Cells were transfected with 10ug of plasmid DNA per flask and medium was changed at 24hr post-transfection. Antibiotic selection was started 3 days post-transfection by adding Zeocin (200 μg/ml) to the cells and medium changed every 2–3 days. Following 2–3 weeks of antibiotic selection, transfected cells containing the GFP marker were sorted and expanded.

**Nuclear Reprogramming**

**Retroviral reprogramming**—WT, TLR3KO TTFs or double knockdown (TLR3KO-IPS1KD) TTFs were subjected to retroviral reprogramming (pMX-Oct4, pMX-Sox2, pMX-Klf4, and pMX-cMyc, following Stemgent kit instructions). Following transduction, cells were transferred to dishes containing mitomycin-treated MEF feeder cells with iPSC medium followed by medium changes every 2 days. Cells were monitored for colony formation (generally occurring by day 13 after transduction) and collected for qPCR analysis at regular intervals. Similarly, scramble-shRNA and IPS1-shRNA knockdown human fibroblasts were subjected to this nuclear reprogramming protocol.

**Doxycycline-induced reprogramming**—Secondary MEFs (4 × 10^4 cells/well) isolated from transgenic mice expressing the loxP-flanked, dox-inducible polycistronic 4F2A cassette were plated and treated with 2μg/ml doxycycline with or without 5′ppp-dsRNA (300 ng/ml). Colonies were counted at Day 14 and Day 21.

**mmRNA-based reprogramming**—Scramble-shRNA and IPS1-shRNA knockdown human fibroblasts were transfected with modified mRNA (1ug/ml) encoding the
reprogramming factors on inactivated MEFs (mitomycin-treated) using Lipofectamine RNAiMAX (Life Technologies, NY) prepared in Opti-MEM Reduced Serum Media. Transfection was repeated daily with medium changes containing B18R protein. Colonies were scored at Day 11 and colonies were collected for RNA extraction and qPCR analysis for pluripotent gene expression.

**RNA extraction and quantitative PCR**

Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and first-strand cDNA primed with oligo(dT) primers. qPCR was performed using Taqman gene expression assays (Applied Biosystems). Pluripotent gene expression was analyzed after normalizing to β-actin housekeeping genes and expressed as relative fold change.

**SSEA-1 staining**

For live staining of reprogrammed cells (TTFs or MEFs), primary antibody (anti-mouse SSEA-1, Stemgent) was diluted in cell culture medium (2.5 µg/ml) and added to cells for 30 mins. After several washes with PBS, cells were examined and imaged under a fluorescent microscope.

**Treatment with Cell Permeant Peptides (CPPs)**

Human BJ fibroblasts (8 x 10^4) were serum starved overnight (to induce G1 cell cycle arrest), and then subjected to either retroviral infection (single infection) or CPPs treatment (daily treatments of CPP-Oct4 or CPP-Sox2 at doses of 200nM; Peprotech). 5′ppp-dsRNA (300 ng/ml) was added to BJ fibroblasts at the same time as CPPs or added to secondary MEFs with doxycycline (2µg/ml).

**Western Blotting**

Proteins were extracted from TLR3KO TTFs by solubilizing the cells in RIPA buffer containing 1× protease inhibitor cocktail. Total protein (10 µg) was loaded and resolved on SDS-polyacrylamide gels and then transferred to PVDF membranes. The membranes were then probed with antibodies to TLR3 (1:200, Cell Signaling), and β-actin (Sigma, A5441). Immunoblots were developed with enhanced chemiluminescence reagents (Amersham).

**Chromatin Immunoprecipitation and Chromatin Immunoprecipitation-qPCR**

Quantitative chromatin immunoprecipitation (qChIP) was performed as described previously. Briefly, crosslinked protein-DNA complexes were sonicated to shear chromatin and then incubated with antibodies against H3K4me3 and H3K27me3 overnight at 4C. Following several washes, isolated DNA was concentrated using DNA purification kit and then subsequently used in PCR reactions. Primers for Oct4 promoters were purchased from Cell signaling. For qChIP and qRT-PCR, error estimates are standard deviations. Recovery of genomic DNA as the percentage input was calculated as the ratio of copy numbers in the immunoprecipitate to the input control.
**Statistical Analysis**—Data shown are reported as mean ± standard error of mean (s.e.m.). Differences between groups were calculated by independent t-test. Statistical significance was accepted at P<0.05.

**Results**

1. Partial inhibition of pluripotent gene expression and reprogramming in TLR3 KO Tail-tip fibroblasts

We previously observed that a partial knockdown of TLR3 in human fibroblasts reduced (but did not abolish) the effect of the Yamanaka factors to induce pluripotent gene expression, and reduced the formation of iPSC colonies. To determine if a complete deficiency of TLR3 would fully abolish reprogramming, we used tail-tip fibroblasts (TTFs) isolated from TLR3 KO mice. The TLR3 deficiency was confirmed at the level of gene (Figure S1A) and protein expression (Figure S1B). We began by assessing the response of these cells to the retroviral construct (pMX-Oct4). We focused on core pluripotency genes downstream of Oct4 including Nanog and Sox2 as well as Oct4-activated genes such as Tcf4 and GAP43.

TTFs from TLR3KO or WT were synchronized by serum starvation and then subjected to a single infection with retroviral construct pMX-Oct4 and downstream gene expression was assayed. As expected, as early as day 1 after infection with pMX-Oct4, TTFs from WT mice showed increased expression of the pluripotency (e.g., Nanog) and target genes (Figures 1A–B). In the TTFs from TLR3KO mice the increase in target gene expression in the first 48 hr was attenuated, but not abolished (Figure 1A–B). This observation indicated that there might be other innate immunity pathways involved in nuclear reprogramming using retroviral delivery. Next, we subjected these TTFs to the retroviral reprogramming protocol as described before. Following transduction with all four of the Yamanaka factors, the medium was changed to iPSC medium with medium changes every 2 days. Around day 13, colonies were observed in the WT as well as the TLR3KO TTFs. However, the number of colonies in TLR3KO TTFs was reduced (Figure 1D and 1E). Pluripotent gene expression in TLR3KO iPSCs was not significantly different that WT iPSCs (Figure 1F and Figure S1C–D).

2. RLR pathway knockdown inhibits induction of pluripotent gene expression

Because the complete deficiency of TLR3 did not fully abrogate reprogramming, we investigated the possible role of the family of Retinoic Acid Inducible Gene 1 like receptors (RLR), as this receptor family is known to respond to dsRNA and polyIC. In human cells the family is represented by RIG-1, MDA5 and LGP2, which each exert their effects by signaling through the adaptor protein interferon-beta promoter stimulator 1 (IPS-1). Activation of RLRs induce type 1 interferon and interferon-inducible genes via activation of IRF3, IRF7 and NFkB. To investigate the role of the RLR pathway in the action of retroviral vector encoding Oct4, we generated a stable shRNA knockdown of IPS1 (Figure S2A). We decided to KD IPS1 as it is the common adaptor molecule for RIG-1, MDA-5 and LGP2. As expected shRNA knockdown of IPS1 reduced its expression (Figure S2C). In the stable IPS1 KD fibroblasts, the effect of pMX-Oct4 to induce NFkB (Figure S2B) and pluripotency gene expression (Figure 2A and B) was attenuated.
3. RLR pathway knockdown inhibits nuclear reprogramming

To determine whether RLR signaling was required for generation of human iPSCs using retroviral vectors encoding the Yamanaka factors, we subjected the scrambled shRNA or IPS1 shRNA knockdown cells to retroviral reprogramming. Following two infections with retroviral Yamanaka factors, the transduced BJ fibroblasts were seeded on to mitomycin C-treated MEFs and medium changed as per protocol (Figure 3A). As expected for retroviral generation of human iPSCs, we observed colonies at Day 25 and onwards in the scramble treated fibroblasts. However no colonies were noted in the IPS1 KD cells until day 34. Furthermore, colonies derived from IPS1 KD fibroblasts were significantly smaller and fewer (Figures 3B, 3C). Furthermore, Oct4 expression was reduced in the iPSCs derived from the KD cells (Figure 3D). We further evaluated the pluripotency markers of the IPS1 KD iPSCs at the mRNA level (Fig. S2D). We observed a reduction in the mRNA expression of pluripotency factors, although the typical markers of pluripotency were present by immunohistochemistry (Fig. S2E). Although the iPSCs derived from IPS-1 KD fibroblasts were fewer in number and the colonies were smaller, those iPSCs that were generated could form embryoid bodies, with gene expression for all three germlines in the embryonic bodies (Fig. S2F). These data indicate that the iPSCs derived from the IPS1 KD cells were fewer in number and smaller in size, but they did appear to be pluripotent. Altogether, these results suggest that the RLR pathway plays an important role in the efficient generation of human iPSCs.

To determine if the role of RLR was specific to retroviral reprogramming, or if it might be involved in other reprogramming methods, we subjected the scramble- and IPS1-shRNA fibroblasts to modified messenger RNA (mmRNA)-based reprogramming. The mmRNAs encode the four reprogramming factors OSKM and have been shown previously to yield iPSCs more efficiently. Following the transfection protocol using OSKM mmRNA (Figure 3E), we observed significantly fewer iPSC colonies derived from the IPS1-shRNA fibroblasts by comparison to the scramble control (Figure 3F and G). Similarly, as observed for retroviral reprogramming, the Oct4 expression in iPSC colonies derived from IPS1-shRNA fibroblasts was lower than that in iPSC colonies derived from scramble-shRNA fibroblasts (Figure 3H). These observations suggest that the RLR pathway plays an important role in the results achieved with other nuclear reprogramming methods.

Even though knockdown of RLR pathway inhibited nuclear reprogramming, the colonies derived from both Scramble and IPS1-shRNA fibroblasts exhibited typical ESC-like characteristics. They showed transcriptional expression of pluripotent genes (Figure S2D), immunoreactivity for SSEA3 and OCT4 (Figure S2E), and differentiated into all three embryonic germ layers in vitro (Figure S2F). This suggested that RLR pathway plays a role in iPSC generation and may be less important in iPSC maintenance as IPS-1 knockdown iPSCs were readily maintained in iPSC medium.

4. TLR and RLR pathways have synergistic effects on nuclear reprogramming

Our previous data showed that both TLR3 and RIG1 pathways influence nuclear reprogramming independently. Thus, to further dissect the synergistic effects of both these pathways on nuclear reprogramming, we generated a double-knockdown mouse fibroblast...
line by introducing the shRNA for IPS1 in TLR3KO fibroblasts (Figure 4A). When subjected to retroviral reprogramming using our standard protocol (Figure 4B), the double-knockdown fibroblasts showed a significant decrease in the number of SSEA1 positive colonies when compared to controls i.e. Scramble shRNA in TLR3KO or WT fibroblasts (Figure 4C and 4D). These results suggested that combined deficiency of both the TLR3 and RIG1 pathway had a synergistic effect on nuclear reprogramming. Phenotypic characterization of the few iPSC colonies derived from the double-knockdown fibroblasts showed expression of pluripotency markers at the mRNA (Figure 4E) and protein level (Figure 4F).

5. RLR ligand enhance CPP-induced pluripotent gene expression

Based on the above results that RLR pathway is required for efficient nuclear reprogramming, we hypothesized that addition of an RLR agonist could enhance less efficient reprogramming methods, in particular, cell permeant peptide (CPP)-based nuclear reprogramming. In this approach the Yamanaka factors are introduced in the cell as fusion proteins with a domain that facilitates cell entry. To enhance CPP-based reprogramming, we used 5′ppp-dsRNA, a synthetic RLR agonist. The addition of 5′ppp-dsRNA increased the expression of RLR signaling molecules such as RIG1 (DDX58), MDA5 (IFIH1) and IPS1 (MAVS) (Figure S3E-G). Notably, we observed that 5′ppp-dsRNA (300 ng/ml) enhanced the effect of CPP-Oct4 to induce the expression of pluripotent genes (Figure S3A) and other Oct-4 inducible genes (Figure S3B). Similarly, the RLR ligand also enhanced the effects of CPP-Sox2 on the expression of pluripotent and downstream genes (Figure S3C and S3D).

6. RLR ligand enhances nuclear reprogramming

To validate our hypothesis that the RLR pathway plays an essential role in nuclear reprogramming, we tested the effect of the RLR ligand on another form of reprogramming. For this purpose, we isolated MEFs from embryos expressing a doxycycline (dox)-inducible polycistronic transgene construct encoding the four reprogramming factors. Addition of dox to these MEFs induces the expression of the Yamanaka factors and the generation of iPSCs within about 2 weeks. These MEFs were subjected to dox treatment for 6 days with or without the RLR ligand 5′ppp-dsRNA. The RLR ligand enhanced the expression of the pluripotency genes in doxycycline-treated MEFs (Figure 5A). Furthermore, the generation of iPSCs in doxycycline-treated MEFs was increased by 5′ppp-dsRNA (Figure 5B and C). To determine whether PolyIC (TLR3 agonist) and 5′ppp-dsRNA (RLR agonist) could have synergistic effects on nuclear reprogramming, we added both these agonist to our dox-induced MEFs. Consistent with our previous work, addition of both PolyIC and 5′ppp-dsRNA caused a significant decrease in pluripotent gene expression (Figure 5A) and the number of iPSC colonies (Figure 5B and C), indicating that over-activation of innate immunity could impair reprogramming.

To further determine whether 5′ppp-dsRNA can rescue the reprogramming defect of TLR3KO MEFs (Figure 1D and E), we conducted viral reprogramming of WT and TLR3KO fibroblasts using Yamanaka factors, in the presence or absence of 5′ppp-dsRNA. Consistent with our previous data, we observed a significant decrease in nuclear
reprogramming in TLR3KO fibroblasts when compared to WT fibroblasts. This deficiency could not be rescued by 5′ppp-dsRNA when added to the retroviral Yamanaka factors; indeed we observed a significant decrease in the number of SSEA1+ colonies in both WT and TLR3KO fibroblasts with the combination of retroviral factors and 5′ppp-dsRNA (Figure 5D and 5E). A cell viability assay conducted on WT and TLR3KO fibroblasts showed that addition of 5′ppp-dsRNA to the four-retroviral factors led to significant cell death when compared to retroviral factors alone (Figure 5F). Furthermore, both IFNβ and Casp3 expression levels were significantly elevated in both WT and TLR3KO TTFs when 5′ppp-dsRNA was added to the retroviral transcription factors (Figure 5G).

7. RLR ligand accelerates epigenetic changes during reprogramming

We also determined the effects of the RLR pathway on epigenetic events during nuclear reprogramming in the dox-inducible MEFs. We performed chromatin immunoprecipitation followed by PCR analysis (ChIP-PCR) of the Oct4 and Sox2 promoter to detect trimethylation of histone H3 at lysine 4 (H3K4me3), an epigenetic modification that represents transcriptionally active genes. The RLR ligand 5′ppp-dsRNA accelerated H3K4 trimethylation at both the Oct4 (Figure 6A) and Sox2 promoter (Figure 6C), by comparison to vehicle at day 2 of doxycycline stimulation. In contrast, the dox alone or dox-control MEFs showed no H3K4 trimethylation at these promoters. By day 4 and 6, H3K4 trimethylation was observed in all dox-stimulated cells, although there was a tendency for increased H3K4 trimethylation in the cells that were additionally treated with 5′ppp-dsRNA (Figure S4A and S4C). We also interrogated the Oct4 and Sox2 promoter for the repressive histone marking, H3K27me3. We observed that 5′ppp-dsRNA treatment accelerated the removal of H3K27me3 at both the promoters, by comparison to vehicle at day 2 of doxycycline stimulation (Figure 6B and 6D). At later time points (day 4 and day 6) H3K27 trimethylation was reduced to a similar degree in all dox-stimulated cells (Figure S4B and S4D).

Discussion

Role of the RIG-1 like (RLR) pathway in nuclear reprogramming

In this paper we provide evidence that the RLR pathway mediates nuclear reprogramming induced by retroviral vectors or by mmRNA encoding the Yamanaka factors. Knockdown of the RLR intermediary protein IPS-1 attenuated the expression of downstream pluripotency factors and reduced the generation of iPSC colonies. By contrast, in MEFs containing a doxycycline-inducible cassette of the Yamanaka factors, the RLR agonist 5′ppp-dsRNA accelerated epigenetic changes favoring the expression of Oct4, and increased the expression of pluripotency genes and the generation of iPSC colonies. Similarly, 5′ppp-dsRNA enhanced the effect of CPPs encoding the Yamanaka factors to activate downstream pluripotency genes. These studies indicate that the RLR pathway contributes to these four different methods of nuclear reprogramming.

Role of TLR3 in nuclear reprogramming

Previously, we have shown a role for TLR 3 in mediating viral and mmRNA–based reprogramming. We found that efficient reprogramming with either of these methods require
TLR3-induced activation of NFKB and IRF3. The activation of these transcriptional factors was associated with global changes in the expression of epigenetic modifiers that favor reprogramming. Specifically, members of the histone deacetylase (HDAC) family and Dot1L were downregulated, whereas members of the histone acetyltransferase (HAT) family were upregulated. The altered expression profile of these epigenetic modifiers would be expected to favor the probability of an open chromatin state. Knockdown of TLR 3, TRIF (the intermediary adaptor protein of TLR3), IKKB or IRF-3, each reduced the generation of iPSCs, indicating the importance of the TLR3-mediated pathway in nuclear reprogramming.

We have expanded these findings in the current paper. Whereas in the previous work we relied upon shRNA to induce partial knockdowns of elements in the TLR3 pathway, in the current investigation, we used fibroblasts from TLR3 deficient mice. This cell line permitted us to determine how the complete absence of TLR3 activation would affect reprogramming. In the TLR3 KO fibroblasts, the effect of the Yamanaka factors to induce the downstream pluripotency factors, and to generate iPSCs, was reduced, but it was not abolished. Accordingly, we reasoned that another pattern recognition receptor might be involved in the response to the reprogramming factors.

The importance of innate immune signaling in epigenetic plasticity

RIG-1, LGP2 and MDA5 are members of the RIG-1 like receptor (RLR) family of pattern recognition receptors. The RLR mediate the innate immune response to double stranded or single stranded viral RNA, which is typically short (< 4000nt) with an uncapped 5’ triphosphate. The RLR and TLR family members initiate signaling pathways that differ in their initial steps but converge in the activation of NFKB and IRF3. IPS-1 (also known as mitochondrial antiviral-signaling protein) is an intermediary protein that is required for RLR-dependent IFN production in response to virus infection. As IPS-1 is downstream to all RLRs (RIG-1, LGP2 and MDA5), we proposed to knockdown the entire RLR pathway by knocking down the common adaptor, IPS-1.

We observed that knockdown of IPS1 impaired the effect of retroviral vectors encoding the Yamanaka factors to activate downstream pluripotency genes and to generate colonies of iPSCs. These studies indicate that whereas the Yamanaka transcriptional factors provide directionality for nuclear reprogramming, the viral vector itself plays an important role in the reprogramming by altering the balance of epigenetic modifiers so as to favor an open chromatin state. By generating viral RNA that activates the TLR3 and RLR signaling pathways, the retroviral vectors induce NFKB- and IRF3-mediated changes in the expression of epigenetic modifiers that favor reprogramming. Indeed, by generating a double-knockdown fibroblasts line (by eliminating the RLR pathway in TLR3KO fibroblasts) we further proved that activation of both TLR3 and RLR signaling pathways are crucial for nuclear reprogramming.

The RLR pathway is also involved in mmRNA-based nuclear reprogramming, as IPS1 KD also impaired the upregulation of core pluripotency genes and the generation of iPSCs. However, even in a situation where the RLR pathway is not directly activated by the reprogramming method, as in the case of CPP-based reprogramming, an RLR agonist enhanced the downstream activation of pluripotency genes. Furthermore, in the dox-
inducible MEFs, the RLR agonist accelerated epigenetic alterations, enhanced the expression of pluripotency genes, and increased the generation of iPSCs. These effects are likely due to RLR-induced activation of NFKB and IRF3, and the effects of those transcriptional factors on epigenetic plasticity as we have already demonstrated5.

Of note, an excessive activation of innate immunity may impair nuclear reprogramming. We found that when 5′ppp-dsRNA when added to the retroviral Yamanaka factors, we observed a significant decrease in the number of SSEA1+ colonies. This is likely due to over-activation of innate immunity, excessive generation of inflammatory cytokines, increased caspase activity and apoptosis. Thus, there appears to be an optimal zone of innate immune activation for nuclear reprogramming.

**Role of innate immune signaling in other forms of nuclear reprogramming**

Dr. Yamanaka’s work stimulated a search for sets of transcriptional factors that may induce transdifferentiation of a differentiated cell to another somatic cell type, also known as direct reprogramming. Indeed, by using viral vectors to force the expression of lineage-specific transcriptional factors, fibroblasts have been converted to neurons, cardiomyocytes or ECs23-25. We speculate that these methods to induce transdifferentiation might also rely upon the epigenetic plasticity induced by activation of innate immune signaling. Recently, we have provided evidence for this speculation26. We hypothesized that the viral vectors might be replaced by activators of innate immunity, and that the lineage specific factors might be replaced by inductive growth cues that promote endothelial lineage. We used the TLR3 agonist poly I:C, which has potential for therapeutic use as a immune adjuvant27, 28. The inductive growth cues included physiologically relevant concentrations of VEGF, bFGF, and BMP4, together with 8Br-cAMP and the TGFβ inhibitor SB431542. These growth factors and small molecules are powerful inducers of endothelial development and proliferation, and/or inhibitors of endothelial-to-mesenchyme transformation29-31. Using this non-viral methodology, we generated induced endothelial cells (iECs) from human fibroblasts. These iECs were comparable to authentic endothelial cells as assessed by immunohistochemical, genetic and functional assays in vitro and in vivo. Of most interest to the current manuscript, we found that effective transdifferentiation of human fibroblasts to endothelial cells required innate immune activation26. In human fibroblasts with a partial KD of TLR3, the yield of iECs was reduced by about 40%. More importantly, the iECs obtained seemed to be incompletely reprogrammed as assessed by functional studies (eg. impaired ability to form tubular networks in matrigel). These findings would be consistent with an incomplete activation of epigenetic plasticity and impaired reprogramming with suppression of innate immune activation. We confirmed the importance of innate immune activation in the generation of iECs using a decoy oligonucleotide against p65, which also suppressed the generation of iECs.

**Transflammation and phenotypic fluidity**

Taken together with our published work, our data supports the notion that stimulation of TLR3 or RLR leads to changes in the expression of epigenetic modifiers that favor an open chromatin state. In unpublished work, we now have observed that post-translational changes in subunits of the polycomb complex PRC1 that reduce its ability to maintain repressive...
histone markings is also involved in this phenomenon. Accordingly, we speculate that any activation of innate immunity, triggered by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), alters the expression or activity of epigenetic modifiers so as to facilitate phenotypic fluidity for cellular response to pathogens or injury. We have termed this process “transflammation”\(^3\). This process appears to be required for efficient nuclear reprogramming to pluripotency; and for transdifferentiation of on somatic cell to another. This process may be involved in malignant transformation, such as that which occurs in states of chronic inflammation. Further understanding of this process may provide insights of clinical relevance for therapeutic transdifferentiation or in generation of novel oncotherapeutics.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

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References


Figure 1. Partial inhibition of pluripotent gene expression and reprogramming in TLR3 KO Tail-tip fibroblasts

Changes in pluripotent gene expression levels after exposure of WT and TLR3KO TTFs to retroviral vector encoding Oct4. (A) Relative fold change in gene expression levels of Nanog following exposure of WT TTFs to vehicle (black line) or pMX-Oct4 (green line) and TLR3KO TTFs to vehicle (blue line) or pMX-Oct4 (red line). This relative fold change was determined after a single pMX-Oct4 infection on Day 0. All data represented as mean ± s.d., n=3, *P <0.005. (B) Summary figure showing the average fold-change in the selected genes (i.e. Tcf4, Gap43, Nanog, Sox2 and Oct4) over time for each condition. All data represented as mean ± s.d., n=3, *P <0.05. Changes in reprogramming levels following retroviral OSKM transduction of WT and TLR3KO TTFs. (C) Protocol for mouse iPSC generation using the reprogramming factors, delivered as retroviral vectors. (D) Representative images of mouse iPSCs on day 19 after initiation of retroviral nuclear reprogramming in WT TTFs (left panel) or TLR3KO TTFs (right panel). (E) Total number of SSEA-1+ miPSCs on day 19 in WT and TLR3KO TTFs. All data represented as mean ± s.d., *P<0.05; WT compared to TRL3KO TTFs. (F) Fold change in Oct4 gene expression in WT and TLR3KO TTFs at day 19.
Figure 2. RLR pathway knockdown inhibits pluripotent gene expression following infection with retroviral vector encoding Oct4

(A) Gene expression levels of Nanog following pMX-Oct4 infection in scramble and IPS1 shRNA-knockdown human fibroblasts. (B) Summary figure showing the average fold-change in the selected genes (i.e. Tcf4, Gap43, Nanog, Sox2 and Oct4) over time for each condition. All data represented as mean ± s.d., n=3, *P <0.05.
Figure 3. RLR pathway knockdown inhibits nuclear reprogramming

(A) Protocol for human iPSC generation using retroviral-reprogramming factors encoding OSKM. (B) Representative images of hiPSCs on day 34 following retroviral nuclear reprogramming for scramble and IPS1 shRNA knockdown fibroblasts. (C) Number of iPSC colonies on day 34 in scramble- and IPS1-shRNA knockdown fibroblasts. All data represented as mean ± s.d., n=3, *P <0.05. (D) Oct4 gene expression in scramble- and IPS1-shRNA knockdown fibroblasts at day 34. All data represented as mean ± s.d., n=3, *P <0.05. (E) Protocol for mmRNA-based nuclear reprogramming. (F) Representative images of hiPSCs on day 11 following mmRNA transduction of the reprogramming factors in scramble- and IPS1-shRNA knockdown fibroblasts. (G) Number of mmRNA-transduced hiPSC colonies on day 11 in scramble- and IPS1-shRNA knockdown fibroblasts. All data represented as mean ± s.d., n=3, *P <0.05. (H) Oct4 gene expression in scramble- and IPS1-shRNA knockdown fibroblasts at day 11 following mmRNA-based nuclear reprogramming. All data represented as mean ± s.d., n=3, *P <0.05.
Figure 4. Elimination of both TLR3 and RLR pathway inhibits nuclear reprogramming

(A) Relative gene expression of IPS1 in WT and TLR3KO TTFs following scramble or shRNA-knockdown of IPS1. (B) Protocol for mouse iPSC generation using the reprogramming factors, delivered as retroviral vectors. (C) Representative images of mouse iPSCs on day 19 after initiation of retroviral nuclear reprogramming in Scramble and IPS1-shRNA knockdown WT TTFs (left panel) and Scramble and IPS1-shRNA knockdown TLR3KO TTFs (right panel). (D) Total number of SSEA-1+ miPSCs on day 19 in Scramble and IPS1-shRNA knockdown WT TTFs or Scramble and IPS1-shRNA knockdown TLR3KO TTFs. All data represented as mean ± s.d., *P<0.05; Scramble WT TTFs compared to IPS1-shRNA WT TTFs. *P<0.05; Scramble TLR3KO TTFs compared to IPS1-shRNA TLR3KO TTFs. #P<0.05; IPS1-shRNA WT TTFs compared to IPS1-shRNA TLR3KO TTFs. (E) Pluripotent genes (Oct4, Sox2, and Nanog) expression in iPSCs derived from Scramble and IPS1-shRNA knockdown WT TTFs and Scramble and IPS1-shRNA knockdown TLR3KO TTFs. *P<0.05; Scramble WT TTFs compared to IPS1-shRNA WT TTFs. *P<0.05; Scramble TLR3KO TTFs compared to IPS1-shRNA TLR3KO TTFs. (F) Immunostaining for Oct4 in iPSCs derived from Scramble and IPS1-shRNA knockdown WT TTFs and Scramble and IPS1-shRNA knockdown TLR3KO TTFs. Scale bars, 100 μm.
Figure 5. RLR activation accelerates doxycycline-inducible nuclear reprogramming

MEFs containing the Dox-inducible polycistronic transgene construct encoding OSKM were stimulated by doxycycline (2mg/L), in the absence or presence of 5′ppp-dsRNA. (A) Relative fold change in Oct4 and Sox2 gene expression following treatments with dox alone (black line), dox + control (blue line), dox + 5′ppp-dsRNA (green line) and dox + 5′ppp-dsRNA + PolyIC (red line). All data represented as mean ± s.d., n=3, *P <0.05. (B) Bar graph showing SSEA-1+ colonies at day 14 and day 21 following treatments with dox alone, dox + control, dox + 5′ppp-dsRNA and dox + 5′ppp-dsRNA + PolyIC. All data represented as mean ± s.d., n=3, *P <0.05. (C) Representative images of SSEA-1 live staining of iPSC colonies derived from dox-inducible MEFs following treatments with dox alone, dox + control, dox + 5′ppp-dsRNA and dox + 5′ppp-dsRNA + PolyIC. (D) Total number of SSEA-1+ colonies derived from retroviral reprogramming of WT and TLR3KO TTFs with or without treatment of 5′ppp-dsRNA. All data represented as mean ± s.d., n=3, *P <0.05. (E) Pluripotent genes (Oct4, Sox2, and Nanog) expression in iPSC derived from WT and TLR3KO TTFs with or without treatment of 5′ppp-dsRNA. All data represented as mean ± s.d., n=3, *P <0.05. (F) Bar graph showing relative viability at Day 2 of retroviral reprogramming of WT and TLR3KO TTFs with or without treatment of 5′ppp-dsRNA. All data represented as mean ± s.d., n=3, *P <0.05. (G) Relative fold change in IFNb and Casp3 gene expression in WT and TLR3KO TTFs with or without treatment of 5′ppp-dsRNA.
Figure 6. 5′ppp-dsRNA enhance nuclear reprogramming via epigenetic modifications

(A) ChIP assay to assess H3K4me3 of the Oct4 promoters in secondary MEFs on day 2 following treatments with dox alone, dox + control and dox + 5′ppp-dsRNA. Data represented as mean ± S.D, n = 2, *P < 0.05

(B) ChIP assay to assess H3K27me3 of the Oct4 promoters in secondary MEFs on day 2 following treatments with dox alone, dox + control and dox + 5′ppp-dsRNA. Data represented as mean ± S.D, n = 2, *P < 0.05

(C) ChIP assay to assess H3K4me3 of the Sox2 promoters in secondary MEFs on day 2 following treatments with dox alone, dox + control and dox + 5′ppp-dsRNA. Data represented as mean ± S.D, n = 2, *P < 0.05

(D) ChIP assay to assess H3K27me3 of the Sox2 promoters in secondary MEFs on day 2 following treatments with dox alone, dox + control and dox + 5′ppp-dsRNA. Data represented as mean ± S.D, n = 2, *P < 0.05.