HIF1α Regulates Early Metabolic Changes due to Activation of Innate Immunity in Nuclear Reprogramming

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SUMMARY

Innate immune signaling has recently been shown to play an important role in nuclear reprogramming, by altering the epigenetic landscape and thereby facilitating transcription. However, the mechanisms that link innate immune activation and metabolic regulation in pluripotent stem cells remain poorly defined, particularly with regard to key molecular components. In this study, we show that hypoxia-inducible factor 1α (HIF1α), a central regulator of adaptation to limiting oxygen tension, is an unexpected but crucial regulator of innate immune-mediated nuclear reprogramming. HIF1α is dramatically upregulated as a consequence of Toll-like receptor 3 (TLR3) signaling and is necessary for efficient induction of pluripotency and transdifferentiation. Bioenergetics studies reveal that HIF1α regulates the reconfiguration of innate immune-mediated reprogramming through its well-established role in throwing a glycolytic switch. We believe that results from these studies can help us better understand the influence of immune signaling in tissue regeneration and lead to new therapeutic strategies.

INTRODUCTION

The discovery that somatic cells can be converted to induced pluripotent stem cells (iPSCs) has opened unprecedented opportunities in regenerative medicine (Takahashi et al., 2007). This has led to seminal work in iPSC-based disease modeling and drug discovery (Sayed et al., 2016, 2019), and more recently iPSC derivatives have been used for cell therapy (Mandai et al., 2017). Disease modeling efforts have been particularly important as it has allowed us to understand the etiologies of human diseases (Sayed and Wu, 2017). Indeed, these efforts have benefited from ever-improving protocols to generate and differentiate these iPSCs to different cell types (Malik and Rao, 2013). Despite advances in basic understanding, the translation of this information to patient care has been limited, mainly due to safety concerns. To eventually move the iPSC technology into the clinic, it will be critical to understand the different signaling pathways involved in achieving pluripotency.

The immune system has been known to play an important role in regeneration and repair (Aurora and Olson, 2014). Indeed, following an injury, immune cells, such as macrophages, act as key regulators in tissue regeneration, and in lower vertebrates, such as amphibians, they can regenerate many organs, including the heart (Godwin et al., 2017). We showed that viral vectors that were used to deliver the reprogramming factors played an independent signaling role in the reprogramming process by activating innate immunity via Toll-like receptor 3 (TLR3) (Lee et al., 2012) or retinoic acid-inducible gene 1 receptor (Sayed et al., 2017). Furthermore, by modulating the same innate immune pathway, we were successful in transdifferentiating human fibroblasts to endothelial cells (iECs) (Sayed et al., 2015).

Mitochondrial metabolism and its bioenergetics have been shown to have a profound influence on nuclear reprogramming (Folmes et al., 2011). To maintain the increased anabolic demands of the rapidly proliferating iPSCs, the somatic cells shift their metabolism from oxidative to glycolytic-based energetics (Zhang et al., 2012) during reprogramming. Indeed, hypoxic conditions promote iPSC yield (Mohyeldin et al., 2010) dependent on hypoxia-inducible factor 1α (HIF1α), a post-translationally regulated transcription factor known as a central regulator of adaptation to limiting oxygen tension (Prigione et al., 2014). In addition to its well-characterized role during hypoxic stress, HIF1α is also activated under normoxic conditions during inflammation (van Uden et al., 2008). Innate immune signaling depends on regulatory activation via the nuclear transcription factor NF-kB, and crosstalk between inflammatory signaling and HIF1α signaling is well...
recognized (D’Ignazio et al., 2016). Moreover, the transcription of HIF1α has been recognized as dependent on NF-κB (Rius et al., 2008).

In this study, we investigated the role of HIF1α in innate immune-activated nuclear reprogramming. We found that human fibroblasts when stimulated with polyinosinic-polycytidylic acid [poly(I:C)], an immunostimulator of TLR3, activated HIF1α early in the reprogramming process. Similarly, loss-of-function studies confirmed that HIF1α was necessary for efficient iPSC generation as well as the transdifferentiation of fibroblasts. Furthermore, bioenergetic studies showed that HIF1α enabled innate immune-mediated reprogramming by promoting a glycolytic shift.

RESULTS

Transcriptional Profiling of Human Fibroblasts Treated with TLR3 Agonist

We have shown that early activation of innate immunity was key to efficient reprogramming by altering the epigenetic landscape allowing somatic cells to either reprogram to iPSCs or transdifferentiate to other somatic cells. To identify the early regulators of this process, we performed RNA sequencing (RNA-seq)-based expression analysis on human fibroblasts treated with poly(I:C). As these changes take place in the initial phase of reprogramming, we focused on the transcriptional genes that were expressed at days 2, 4, and 6 following poly(I:C) treatment. RNA samples were converted to cDNA libraries and sequenced by Ampli-seq, followed by gene expression analysis using the RPKM (reads per kilo base per million mapped reads) expression levels to identify differentially expressed genes. Genome-wide Ampli-seq analysis revealed that human fibroblasts treated with poly(I:C) showed a striking differential change in patterns of gene expression at each of these time points (Figure 1A). Moreover, gene ontology analysis of differentially expressed genes identified by RNA-seq showed the expected activation of genes associated with inflammation and interferon signaling (Sen and Sarkar, 2005) (Figure S1A).

As poly(I:C) enhanced the reprogramming efficiency and transdifferentiation via the inflammatory pathway by activating NF-κB, we focused on identifying those responsive genes that were transcription factors related to nuclear reprogramming and inflammation. For this, we selected genes from three categories: transcription factors, inflammatory genes, and reprogramming genes from online databases for further bioinformatic analysis and found that a set of eight genes overlapped in these three categories (Figure 1B). To validate the expression of these eight genes in our Ampli-seq dataset, we annotated their expression at the different time points and found that, with the exception of HIF1α, all other genes showed a gradual increase in their expression from days 0 to 6 (Figures 1C and S1B). In contrast, HIF1α showed an immediate increase in their expression as early as day 2, suggesting that HIF1α might be an early regulator during innate immune stimulation. Importantly, transcriptional analysis of our RNA-seq data further showed that downstream targets of HIF1α, such as pyruvate dehydrogenase kinase 1 (PDK1) and recombination signal binding protein for immunoglobulin kappa J region (RBPJ), showed an upregulation in their expression levels following poly(I:C) treatment (Figure 1C).

Activation of Innate Immunity Stimulates Early Expression of HIF1α

To validate our RNA-seq data, we stimulated human fibroblasts with poly(I:C) and evaluated the expression of HIF1α. Consistent with our previous data, poly(I:C) treatments increased the expression of TLR3 (Figure S2A) and NF-κB (Figure S2B) in human fibroblasts. Importantly, human fibroblasts treated with poly(I:C) also showed an increase in HIF1α both at the mRNA (Figure 1D) and protein levels (Figure 1E) as early as day 2. Moreover, the protein stability of HIF1α was only detectable at the early time-points (days 2–6) following poly(I:C) treatment, and, with the removal of the innate immune stimulation, HIF1α levels reverted back to basal levels by day 8 (Figure S2C), suggesting that HIF1α activation depends on innate immune stimulation. To further investigate whether this activation was due to innate immune stimulation, we assessed the HIF1α expression in TLR3 knockout (KD) (Figure S2D) human fibroblasts. Indeed, poly(I:C) treatment increased HIF1α expression in scramble-small hairpin RNA (shRNA), but failed to show an increase in HIF1α levels in TLR3KD cells at the mRNA (Figure 1F) and protein levels (Figure 1G). Furthermore, tail-tip fibroblasts isolated from TLR3 knockout mice (Figure S2E) also exhibited decreased HIF1α expression (Figure S2F) and protein stability (Figure S2G) compared with wild-type cells following stimulation with poly(I:C). Taken together, these results show that innate immune signaling drives the mRNA expression and protein stability of HIF1α.

HIF1α Regulates Efficient Innate Immune-Activated Nuclear Reprogramming

As HIF1α can modulate early cell fate during nuclear reprogramming (Prigione et al., 2014), we investigated whether HIF1α can regulate innate immune-activated nuclear reprogramming. For this, we generated an shRNA-based stable cell line of HIF1α-KD human fibroblasts (Figure S3A). As expected, scramble-treated cells showed a significant increase in HIF1α levels at days 2, 4, and 6 of poly(I:C) treatment; however, HIF1α-KD cells failed to show an increase in their HIF1α levels at these
Interestingly, HIF1α reverted back to basal levels in scramble-treated cells following removal of poly(I:C) by day 8, suggesting that HIF1α activation is required only during the early stages of reprogramming. Similarly, HIF1α protein levels remained at basal levels in HIF1α-KD cells at later time points.

Next, we investigated the role of HIF1α in innate immune-mediated nuclear reprogramming. We focused on using cell permissive peptides (CPPs) encoding the reprogramming factors (i.e., CPP-Oct4) as these CPPs do not stimulate innate immunity, thereby allowing us to evaluate the effects of poly(I:C) on HIF1α. As expected, human fibroblasts treated with scramble-shRNA and exposed to CPP-Oct4 and poly(I:C) showed an increase in pluripotency genes. On the contrary, HIF1α-KD cells when treated with CPP-Oct4 and poly(I:C) not only failed to increase the expression of pluripotency genes, including OCT4 and SOX2 (Figure 2A), but also other downstream target genes, including NANOG, GAP43, and TCF4 (Figure S3C). Next, we conducted protein-based nuclear reprogramming on scramble or HIF1α-KD human fibroblasts using our established protocol in which cells were simultaneously treated with CPP-encoding reprogramming factors (CPP-OSKM) and poly(I:C) (30 ng/mL) for 6 days (Figure 2B). As expected, scramble-treated cells exhibited higher yield of iPSCs in the presence of poly(I:C) (Figures 2C and 2D); however, the same protocol failed to enhance the yield of iPSCs in HIF1α-KD cells. Moreover, the iPSCs generated by the HIF1α-KD cells by day 35 expressed lower levels of
OCT4 and SOX2 when compared with scramble fibroblasts (Figures 2E and S3D). Here, these results suggest that HIF1α is a key downstream regulator in innate immune-mediated nuclear reprogramming.

**TLR3 Signaling Is Required for Innate Immune-Activated Transdifferentiation**

Another form of nuclear reprogramming is transdifferentiation or direct differentiation of one somatic cell type to another (Sayed et al., 2013). Indeed, activation of innate immunity supports the direct differentiation of human fibroblasts to endothelial cells (iECs) when placed in an optimal microenvironment (Sayed et al., 2015); however, the TLR signaling pathway that plays a predominant role in transdifferentiation remains to be determined. For this, we generated two stable KD cell lines by directing shRNA toward adaptor proteins that mediate interactions between different TLRs. Specifically, we knocked down myeloid differentiation primary response gene 88 (MyD88) adaptor molecule that is common to all TLRs, except TLR3, which on the other hand is mediated by TIR domain-containing adaptor-inducing interferon-β (TRIF). These KD cells were then subjected to transdifferentiation toward ECs, using a lentiviral-based vector delivering EC lineage-specific transcription factor, ETV2 (Figure S4A), a strategy previously shown to be effective (Lee et al., 2017; Morita et al., 2015). This lentiviral construct was transduced in human fibroblasts previously treated with scrambled shRNA or shRNA directed toward MyD88 or TRIF. Following infection, transduced cells were transitioned to a transdifferentiation protocol and, at day 14, cells were sorted using fluorescence-activated cell sorting to determine the yield of iECs. As seen in Figure S4B, TRIF-KD cells yielded a significantly lower iEC generation when compared with scramble or MyD88-KD cells, suggesting a role for TLR3 signaling in innate immune-mediated transdifferentiation.

**HIF1α Regulates Efficient Innate Immune-Activated Transdifferentiation**

As HIF1α functions as a transcriptional regulator of the adaptive response to hypoxia, and since hypoxia can induce epithelial-to-mesenchymal transformation, we first
sought to determine whether hypoxia alone could initiate transdifferentiation. For this, human fibroblasts were subjected to a 1% O2 atmosphere until day 7 and then cultured in the transdifferentiation medium containing growth factors and small molecules (Figure S4C). As expected, human fibroblasts exposed to poly(I:C) showed approximately ~4% of cells expressing CD31; however, cells only exposed to hypoxia failed to yield any CD31-positive cells (Figure S4D), suggesting that HIF1α induction by hypoxia is not sufficient to drive transdifferentiation in the absence of other factors that are induced downstream of TLR3. This complemented our previous work, which showed that the effects of TLR3 activation to enhance the yield of reprogramming is partly due to its regulation of the expression and distribution of epigenetic modifiers (Lee et al., 2012; Sayed et al., 2017).

Next, to determine whether HIF1α also acts as a downstream regulator of innate immune signaling for transdifferentiation, we subjected our HIF1α-KD fibroblasts to our transdifferentiation protocol (Figure 3A). As shown in Figure 3B, HIF1α-KD cells when treated with poly(I:C) and cultured in transdifferentiation medium showed a significantly lower percentage of iECs when compared with scramble cells. Although morphologically indistinct from scramble iECs (Figure S4E), HIF1α-KD iECs showed decreased expression of EC markers, such as CD31 (Figure 3C), CD144, and eNOS (Figure S4F). Next, we assessed the functional properties of these transdifferentiated iECs, and found that HIF1α-KD iECs showed decreased capacity to form networks of tubular structures (Figures 3D and 3E), decreased capacity to uptake acetylated low-density lipoprotein (Figures 3F and 3G), and decreased capacity to produce nitric oxide when stimulated with acetylcholine (Figure 3H) when compared with scramble iECs.

**Innate Immunity Mediates Metabolic Reprogramming via HIF1α**

It is well established that induction of HIF1α can significantly improve the efficiency of iPSC generation by initiating a glycolytic switch. Similarly, recently it was shown that innate immune signaling promoted a glycolytic switch, thereby allowing transdifferentiation of somatic cells (Lai et al., 2019). To determine whether the innate signaling enhanced nuclear reprogramming via HIF1α-mediated glycolytic switch, we first exposed TLR3-KD cells to poly(I:C) and assessed their bioenergetic markers. As expected, poly(I:C) treatment of scramble cells decreased the rate of cellular oxidative phosphorylation (OXPHOS) as evident by a reduced oxygen consumption rate (Figure 4A) and increased glycolytic metabolism based on an increased extracellular acidification rate (Figure 4B). On the contrary, TLR3-KD cells maintained the rate of cellular OXPHOS and glycolytic metabolism (Figure 4C), suggesting that innate immunity is driving the glycolytic switch.

Next, to determine whether HIF1α acts as a downstream regulator of this glycolytic switch, we tested the effects of poly(I:C) on HIF1α-KD cells. As seen in Figures 4D–4F, HIF1α-KD cells did not decrease the rate of mitochondrial respiration or increase the glycolytic metabolism in the presence of poly(I:C). Taken together, our data suggest that activation of innate immunity results in an early induction of HIF1α, which initiates a glycolytic switch contributing toward enhanced nuclear reprogramming and transdifferentiation.

**DISCUSSION**

Studies have shown that the immune system is critical for tissue regeneration. Lower vertebrates can regenerate their major body organs, including their hearts by recruiting macrophages to the injury sites (Godwin et al., 2017). Moreover, even neonatal mice can regenerate their heart following apical resection (Porrello et al., 2011) or myocardial infarction (Porrello et al., 2013) by recruiting macrophages. However, as adults we have lost this regenerative property either due to the failure of adult cardiomyocytes to proliferate or due to development of fibrosis (Kurose and Mangmool, 2016). We discovered that activation of innate immunity is required for nuclear reprogramming (Lee et al., 2012) and transdifferentiation of somatic cells (Sayed et al., 2015). In these studies, we observed that innate immunity causes global changes in the epigenetic landscape keeping the chromatin in an “open configuration,” thereby allowing the somatic cells to be subjective to reprogramming. Importantly, our work showed that NFκB played a central role in innate immunity-mediated nuclear reprogramming (Liu et al., 2017).

NFκB is a protein complex expressed in all cell types and is known to interact with other transcription factors to regulate gene expression (D’Ignazio et al., 2016). Our RNA-seq data showed upregulation of one such transcription factor, HIF1α, in human fibroblasts when treated with poly(I:C) (Figures 1A–1C). Similarly, poly(I:C) also increased HIF1α protein stability at early time points of innate immune stimulation (Figure S3C). Importantly, knockdown studies of the innate immune pathway further validated that HIF1α was an early regulator in the inflammatory process (Figures 1F, 1G, and S3D–S3G). HIF1α, a member of the hypoxia-inducible factor 1 family is considered a master regulator of the cellular response to low oxygen conditions, where it regulates many biological processes, including glycolysis, proliferation, migration, and apoptosis (Semenza, 2012). However, in addition to hypoxia, HIF1α is also activated in response to inflammation (Schaffer and Taylor, 2015). Similarly, recent work has
Figure 3. HIF1α Regulates Efficient Innate Immune-Activated Transdifferentiation

(A) Protocol for direct differentiation of human fibroblasts to endothelial cells.

(B) MACS plot of iECs generated from scramble and HIF1α-KD human fibroblasts. n = 3 as three independent experiments, *p < 0.05.

(C–H) Characterization of scramble and HIF1α-KD iECs.

(C) mRNA expression of CD31. n = 3 with each done in duplicate, *p < 0.005. (D) Representative images of capillary-like networks. (E) Quantification of the number of tubes. n = 3 as three independent experiments, *p < 0.005. (F) Representative images of acetylated low-density lipoprotein (LDL) uptake. (G) Quantification of Ac-LDL uptake. n = 3 as three independent experiments, *p < 0.05. (H) Quantification of NO production by iECs in response to acetylcholine and Ca²⁺ ionophore A23187. n = 3 with each done in duplicate, *p < 0.05. All data represented as mean ± SEM. Statistical analyses were performed using one-way ANOVA corrected with Bonferroni method.
shown that an extensive crosstalk exists between HIF1α and NF-κB, which includes common stimuli and shared targets. Despite the knowledge that NF-κB can activate HIF1α, little is known about the downstream role of HIF1α, especially in the process of innate immune-mediated nuclear reprogramming.

Our results showed that knockdown of HIF1α impaired poly(I:C)-mediated protein-based nuclear reprogramming (Figures 2C–2E), suggesting that HIF1α is a downstream regulator of innate immune-mediated nuclear reprogramming.

The TLR3-NF-κB pathway activates HIF1α, which initiates a reconfiguration of the somatic cell glucose metabolism causing a glycolytic switch. Indeed, previous work has shown that HIF1α plays a central role in mediating the “Warburg effect,” where somatic cells transform their cellular bioenergetics and shift to glycolysis-based metabolism (Corcoran and O’Neill, 2016). As HIF1α is up-regulated by NF-κB under inflammatory conditions, we speculated that activation of innate immunity induces nuclear reprogramming by initiating the Warburg effect. Our Seahorse and bioenergetics data showed that HIF1α is a key mediator for somatic cells to undergo reprogramming by initiating a glycolytic shift (Figure 4).

In summary, we have identified a key missing link in our discovery that activation of innate immunity is required for...
nuclear reprogramming. Our knockdown and seahorse studies suggest that TLR3-NF-kB innate immune pathway activates HIF1α, a transcription factor that initiates a reconfiguration of the somatic cell glucose metabolism causing a glycolytic switch. Moreover, we have demonstrated that HIF1α is an early regulator in the process of innate immune-mediated nuclear reprogramming. We anticipate that results from our study can help better understand the process of cellular reprogramming and thus lead to new therapeutic strategies.

EXPERIMENTAL PROCEDURES

Full details are provided in Supplemental Experimental Procedures.

Cell Culture

Human foreskin fibroblasts and tail-tip fibroblasts from TLR3 knockout mice were cultured in DMEM (Gibco) with 10% FBS at 5% CO₂, 37°C. ECs were maintained using EC growth medium-2MV (EGM2, Lonza) on gelatin-coated plates. Cells were cultured with medium change every 2 days and passaged using TrypLE (Thermo Fisher Scientific).

RNA-Seq by Ampli-Seq

RNA was extracted using a QIAGEN RNeasy Plus kit as per manufacturer’s instructions and synthesized into cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen). Barcoded libraries were then prepared using IonAmpliSeq Transcriptome Human Gene Expression Kit (Invitrogen) under manufacturer’s instructions; 50 pM per library was loaded on Ion PI v3 chips using an Ion Chef automated platform (Invitrogen) and targeted sequencing of >20,000 RefSeq transcripts using Ion Proton Sequencer following the manufacturer’s instructions. Read counts were aligned and normalized using ampliSeqRNA plugin of Ion Torrent Suite. Hierarchical clustering of RPKM values was performed using Omics Explorer v.3.2 software.

ACCESSION NUMBERS

RNA sequencing data for this article is available on the GEO under accession number GEO: GSE142217.

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

C.L., F.H., M.-T.Z., C.C.C., and M.M. collected and analyzed the data. H.R. carried out bioinformatic analysis. I.Y.C. and K.S. designed the study and wrote the manuscript. D.S. collected and analyzed the data, and wrote the manuscript. N.S. conceived and designed the study, collected and analyzed the data, gave financial support, wrote the manuscript, and approved the final manuscript.

CONFLICTS OF INTEREST

Dr. Sayed is an inventor of the intellectual property, assigned to Stanford University, related to the use of innate immune signaling for nuclear reprogramming and transdifferentiation.

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