Transdifferentiation Requires iNOS Activation
Role of RING1A S-Nitrosylation

Shu Meng, Gang Zhou, Qilin Gu, Palas K. Chanda, Frank Ospino, John P. Cooke

**Rationale:** We have previously shown that innate immunity is necessary for transdifferentiation of fibroblasts to endothelial cells. A major signaling molecule involved in innate immunity is inducible nitric oxide synthase (iNOS). Accordingly, we hypothesized that iNOS-generated nitric oxide (NO) might enhance transdifferentiation.

**Objective:** To elucidate the role of NO in epigenetic plasticity during transdifferentiation.

**Methods and Results:** We exposed the BJ fibroblasts to transdifferentiation formulation that included endothelial growth factors and innate immune activator polyinosinic:polycytidylic acid to induce endothelial cells. Generation of transdifferentiated endothelial cells was associated with iNOS expression and NO elaboration. In the absence of polyinosinic:polycytidylic acid, or in the presence of antagonists of NFκB (nuclear factor kappa B) or iNOS activity, NO synthesis and induce endothelial cell generation was reduced. Furthermore, genetic knockout (in murine embryonic fibroblasts) or siRNA knockdown (in BJ fibroblasts) of iNOS nearly abolished transdifferentiation, an effect that could be reversed by iNOS overexpression. Notably, polyinosinic:polycytidylic acid induced nuclear localization of iNOS, and its binding to, and nitrosylation of, the epigenetic modifier ring finger protein 1A (RING1A) as assessed by immunostaining, Co-IP, and mass spectrometry. Nitrosylation of RING1A reduced its binding to chromatin and reduced global levels of repressive histone marker H3K27 trimethylation. Overexpression of a mutant form of RING1A (C398A) lacking the nitrosylation site almost abrogated transdifferentiation.

**Conclusions:** Overall, our data indicate that during transdifferentiation, innate immune activation increases iNOS generation of NO to S-nitrosylate RING1A, a key member of the polycomb repressive complex. Nitrosylation of RING1A reduces its binding to chromatin and decreases H3K27 trimethylation level. The release of epigenetic repression by nitrosylation of RING1A is critical for effective transdifferentiation. (Circ Res. 2016;119:e129-e138. DOI: 10.1161/CIRCRESAHA.116.308263.)

**Key Words:** chromatin ■ endothelial cell ■ epigenetic repression ■ mass spectrometry ■ innate immunity

Transdifferentiation is the process by which a somatic cell undergoes a metamorphosis into a different type of somatic cell. This process of cellular transformation plays a role in malignancy, but it may also play a role in the physiological response to injury. For the purpose of regenerative medicine applications, several groups have converted somatic cells, such as fibroblasts into neurons,1 cardiomyocytes,2 or endothelial cells (ECs)3 by overexpressing specific lineage transcription factors. Transdifferentiation from fibroblasts to endothelial cells may be useful in cardiovascular regeneration. For example, an effective transdifferentiation strategy might modify the healing process by preventing the formation of scar tissue and facilitating the generation of vascularized tissues, in response to an injury.

Most investigators who are transdifferentiating cells for regenerative medicine applications have achieved their goal using viral vectors encoding transcriptional factors–mediating cell lineage.4,5,7 By contrast, we have developed a nonviral strategy using small molecules and growth factors. This strategy was made possible by leveraging our observation that cellular transformations (either nuclear reprogramming to pluripotency or transdifferentiation to another cell type) require innate immune activation.6,7 Indeed, the viral vectors used in nuclear reprogramming studies are more than passive vehicles; by activating innate immunity, they facilitate reprogramming.7 We have shown that innate immune activation increases the expression of histone acetyltransferase family members and suppresses the expression of histone deactylase (HDAC) family members and DOT1L (DOT1-like histone lysine methyltransferase).7 These changes in the balance of epigenetic modifiers would be expected to increase epigenetic plasticity.

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Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DAF-FM DA</td>
<td>4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>iECs</td>
<td>induce endothelial cells</td>
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<td>INOS</td>
<td>inducible nitric oxide synthase</td>
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<td>MEF</td>
<td>murine embryonic fibroblast</td>
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<td>MS</td>
<td>mass spectrometry</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>PIC</td>
<td>polynosinic/polycytidylic acid</td>
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<td>PRC</td>
<td>polycomb repressive complex</td>
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<td>RING1A</td>
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It is well known that inducible nitric oxide synthase (iNOS) is a major effector of innate immune signaling. Activation of innate immunity leads to NFκB (nuclear factor kappa B)-mediated expression of iNOS, which S-nitrosylates proteins to affect their activity and cellular location. Accordingly, we hypothesized that innate immune activation of iNOS and S-nitrosylation of epigenetic modifiers might contribute to epigenetic plasticity required for transdifferentiation. Driven by this hypothesis, we now describe the discovery of a novel iNOS-dependent post-translational modification of the repressive epigenetic machinery to facilitate transdifferentiation.

**Methods**

### Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Other reagents included fetal bovine serum, Taqman real-time polymerase chain reaction (RT-PCR) primers, Taqman master mix, 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate), RNAiMAX, and 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA); polyinosinic:polycytidylic acid (PIC) and NFκB inhibitors Bay117082, celastrol, and dexamethasone (Invivogen, San Diego, CA); antibody against mouse CD144 (BD Biosciences, San Jose, CA); antibody against iNOS, HRP-conjugated goat anti-mouse or rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); antibody against β-tubulin, ring finger protein 1A (RING1A), H3K27 trimethylation (H3K27me3) and H3K9 dimethylation (H3K9me2; Abcam, Cambridge, United Kingdom); bone morphogenetic protein-2, vascular endothelial growth factor, and basic fibroblast growth factor (R Peprotech, Rocky Hill, NJ); and Scramble and iNOS siRNA (Integrated DNA Technologies, Coralville, IA).

### Cell Culture and Induce Endothelial Cell Generation

BJ human neonatal foreskin fibroblast cells (BJ fibroblasts; Stengert), wild-type (WT) and iNOS−/− murine embryonic fibroblasts (MEFs; Cell Biologics Inc, Chicago, IL) were cultured and maintained in Dulbecco modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin (5% CO₂, 37°C). We transdifferentiated human and mouse fibroblasts to induce endothelial cells (iECs) using our previously established protocol. Briefly, cells were treated with PIC (30 ng/mL) daily for 7 days, and the medium was gradually transitioned to a 10% knockout serum replacement. Cells were treated with endothelial cell growth medium 2 (2; Lonza) containing 20 ng/mL bone morphogenetic protein-4, 50 ng/mL vascular endothelial growth factor, and 20 ng/mL basic fibroblast growth factor for another 7 days. For maintenance of transdifferentiation, the cells were cultured for another 2 weeks in the presence of basic fibroblast growth factor, vascular endothelial growth factor, and 0.1 mM 8-bromoadenosine-3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP), with the medium changed every 2 days. In some experiments, inhibitors of iNOS or NFκB were present throughout the transdifferentiation protocol; they were readded when the transdifferentiation medium was refreshed.

### RNA Extraction and Real-Time PCR

mRNAs were extracted from cultured cells and reverse transcribed into cDNA, and then specific primers for targeted genes were used for PCR amplification. For real-time PCR, a SYBR-green PCR system or Taqman system was used and ran on QuantStudio real-time PCR system from Life Technologies. Gene expression data using the ΔΔCt method was normalized to the expression of the 18S gene and plotted as relative fold changes.

### Western Blot and Immunoprecipitation

Western blot analysis was performed as previously described. Immunoprecipitation was performed using the Pierce Classic Magnetic IP/Co-IP Kit (Thermo Scientific, Waltham, MA) following manufacturer’s manual.

### Chromatin Immunoprecipitation–qPCR

Chromatin immunoprecipitation was performed using a previously established method. Recovery of genomic DNA was calculated as the ratio of copy numbers in the immunoprecipitate to the input control. Primers of the CD144 promoter were custom designed and synthesized from IDT as follows; primer sequence (5′–3′), (F): CAGCCCTTTGGTTTCTTTGG; (R): GGAGTCAAAGTGACCAGCT.

### Fluorescent Assay for Nitric Oxide

BJ fibroblasts were stained with DAF-FM DA to access intracellular nitric oxide (NO). Cultured cells exposed to different experimental conditions were incubated with DAF-FM DA 10 μmol/L at 37°C for 30 minutes. Fluorescence was quantified by measuring fluorescence intensity at 495 nm excitation and 515 nm emission by a fluorescence plate reader (Tecan M1000 PRO).

### Immunofluorescent Staining

After BJ fibroblasts were cultured in 8-well chamber slides and exposed to experimental conditions, cells were fixed, permeabilized, blocked with 5% normal donkey serum, and stained for RING1A and iNOS. The nuclei were stained with DAPI for 10 minutes.

### Analysis of S-Nitrosylation by Mass Spectrometry

Protein from both control and PIC-treated BJ fibroblasts was immunoprecipitated using RING1A antibody, resolved on sodium dodecyl sulphate (SDS)-PAGE gel and stained with Coomassie Blue. After destaining, the gel was scanned and the RING1A band was cut and sent to the mass spectrometry-proteomics core facility (Baylor College of Medicine) for mass spectrometry (MS) analysis. The gel band was further destained and double digested with trypsin and GluC in situ. Peptides in the gel were extracted, quantified and 200 ng of each sample was analyzed using the Eksigent nanoLC system and then the ABCIEX TripleTOF 5600 mass spectrometer. The Eksigent nanoLC and the ABIEX TripleTOF 5600 mass spectrometer were controlled by Analyst software version 1.6 (ABCEIX Inc). The MS spectra (m/z 100–2000) were acquired with internal mass calibration. Raw data were converted to MGF format and then imported into Peptides software v6.0 for further peak generation and database search with the SPIDER function. The search sequence was E3 ubiquitin-protein ligase RING1A of human (NP_002922.2 GI:51479192).
and ligated into EcoRI/Sall digested pCMV-Tag2C to generate pCMV-Tag2C-RING1A. We further created pCMV-Tag2C-RING1A-C398A using a PCR-based method. Briefly, PCR reaction was performed using primer pair: C398A Mutation-Forward (5′-CGGCCACTGGAGCTGGCCTATGCTCCCACCAGATACAAAG-3′) and C398A Mutation-Reverse (5′-TTGTGACGATAGGCGAGCCCGTCGACGACGAC-3′). The PCR products were treated with DpnI and transformed into Escherichia coli Top10 competent cells by heat shock. Single clones were sequenced to examine the C398A mutation.

**Flow Cytometry**

Cells were trypsinized and filtered through a 40-µm nylon mesh to generate single-cell suspensions. Cells were stained with antibody for cell surface marker detection. Data were analyzed by flowjo software.

**Data Analysis**

Results are expressed as the mean±SEM. Statistical comparisons between 2 groups were performed via Student t test. One-way ANOVA was used to compare the means of multiple groups. *P<0.05 was considered statistically significant.

**Results**

**iNOS Is Induced During Transdifferentiation**

Our laboratory has previously established a nonviral methodology to transdifferentiate fibroblasts to iECs.7 We activate innate immunity with a toll-like receptor 3 agonist PIC to induce epigenetic plasticity. In this state of epigenetic plasticity, fibroblasts are exposed to a media containing endothelial cytokines and small molecules that favor endothelial phenotype. The iECs that we obtain using this protocol are highly similar to human dermal microvascular endothelial cells as assessed by immunohistochemical markers, acetylated low-density lipoprotein uptake, the generation of capillary-like networks in matrigel, the generation of NO, and angiogenic cytokines and by RNAseq analysis.7

We examined NOS gene expression at days 0, 6, 14, 21, and 28; by day 28 of the transdifferentiation protocol, CD31+ iECs can be detected, as quantified by FACS analysis (Figure 1A and 1B). We observed that the expression of iNOS increased throughout transdifferentiation, peaking at day 21 (Figure 1C). By contrast, the expression of eNOS (endothelial NOS) was not detectable until day 28 when there was evidence of transdifferentiation to a mature endothelial cell phenotype (Figure 1D). Throughout the whole process, nNOS (neuronal NOS) was not detectable (data not shown).

**NO Is Generated During Transdifferentiation**

To determine whether the upregulation of iNOS expression increased the generation of NO, we used DAF-FM DA staining. DAF-FM DA is essentially nonfluorescent until it reacts with NO to form a fluorescent benzotriazole.13 On day 3 of the transdifferentiation protocol, we observed a significant increase in DAF-FM DA fluorescence in both image analysis (Figure 2A) and fluorescence readings (Figure 2B) in the group treated with PIC compared with the vehicle-treated control. An inhibitor of all NOS isoforms l-NAME (l-NG-nitroarginine methyl ester) and the more specific iNOS inhibitors (S)-methylisothiourea sulfate and BYK 191023 dihydrochloride (BYK) each reduced NO generation. In addition, the NFκB inhibitors Bay117082, celastrol, or dexamethasone each blocked iNOS upregulation and NO synthesis induced by PIC (Figures 2A, 2B, and 3D).

**Transdifferentiation Requires iNOS Induction and NO Generation**

To determine whether generation of NO is required for transdifferentiation, we performed the transdifferentiation studies in the presence of the NOS inhibitor l-NAME or in the presence of NO scavengers uric acid or ebselen. Each of these agents substantially reduced transdifferentiation, as assessed at day 28 by FACS for CD31+ iECs (Figure 3A and 3C). This indicates that NO generation is required for efficient transdifferentiation.
We hypothesized that by inhibiting NFκB activity, we would suppress the induction of iNOS and thereby reduce the efficiency of transdifferentiation. Indeed an inhibitor of NFκB activity, Bay117082, decreased iNOS gene expression (Figure 3D) and reduced the yield of iECs (Figure 3B and 3C). Two other known antagonists of NFκB-mediated iNOS induction, celestrol, and dexamethasone also reduced the yield of iECs by 90% (Figure 3B and 3C). These studies confirm that NFκB-mediated induction of iNOS is required for effective transdifferentiation.

Genetic Deficiency of iNOS Abolishes Transdifferentiation

To further test the role of iNOS in transdifferentiation, we assessed the transdifferentiation of WT and iNOS−/− MEFs to iECs. We confirmed the increasing expression of iNOS during transdifferentiation in WT MEF at days 0, 3, and 14 (Figure 4C), whereas iNOS gene expression was not detected in iNOS−/− MEF. We observed that transdifferentiation was nearly abolished in the iNOS−/− MEF. To confirm this work in human cells, we knocked down iNOS in BJ fibroblasts by siRNA (Figure 4D). This knockdown of iNOS substantially reduced the yield of iECs (Figure 4E). The effect of siRNA knockdown was reversed by iNOS overexpression using a pcDNA3.1-his-iNOS plasmid14 (Figure 4F). These data suggest that iNOS is required for transdifferentiation.

RING1A Is S-Nitrosylated During Transdifferentiation

Our data indicated that the NFκB–iNOS–NO pathway is required for transdifferentiation. We were interested to know whether this pathway exerted its effect on transdifferentiation by post-translational modification of epigenetic modifiers, so as to enhance epigenetic plasticity. Because NO species may react with cysteine to form SNO proteins, and peroxynitrite can react with l-tyrosine to form 3-nitro-l-tyrosine (3-NT), we quantified SNO and 3-NT levels as an index of iNOS activity. As shown in western analysis, cell lysate protein SNO (Figure 5A) and 3-NT levels are significantly increased as early as day 3 after the induction of transdifferentiation (Online Figure I).

It has been previously reported that HDAC2, RING1A, and CBX1 can be S-nitrosylated.15–18 Accordingly, we turned our attention to detecting S-nitrosylation of epigenetic modifiers focusing first on HDAC2, RING1A, and CBX1. We observed that RING1A was SNO modified at day 3 during transdifferentiation (Figure 5B), but not HDAC2 or CBX1 (Online Figure II). We confirmed the RING1A S-nitrosylation by immunoprecipitation of RING1A, followed by probing with SNO antibody. We found that in PIC-treated BJ fibroblasts RING1A was S-nitrosylated (Figure 5C). Consistent with our hypothesis, NFκB inhibitor Bay117082 could block this S-nitrosylation (Figure 5C), suggesting that NFκB–iNOS pathway is upstream of RING1A S-nitrosylation.

To identify the specific cysteine that is S-nitrosylated, we performed MS analysis of the RING1A immunoprecipitated from cell lysate of fibroblasts treated with vehicle or PIC. The MS studies revealed cysteine at 398 position is heavily S-nitrosylated (Figure 6A) by PIC stimulation. In addition, nitroso-cysteine modifications could also be detected in 279 and 332 position cysteines, although the modification level is low (Online Figure III).

To further examine whether S-nitrosylation of RING1A at 398 cysteine is critical for transdifferentiation, we performed site-directed mutagenesis. We first constructed plasmids containing wild-type (pCMV-Tag2C-RING1A) or mutant RING1A (C398A mutation, pCMV-Tag2C-RING1A-C398A plasmid). The GCC codon encoding the 398 position cysteine was mutated into a TGC codon-encoding alanine, which we confirmed by sequencing (Figure 6B). Then both plasmids were transfected into BJ fibroblasts, which were then subjected to the transdifferentiation protocol with or without PIC. As shown in Figure 6C, C398A-RING1A is minimally S-nitrosylated after PIC, suggesting that the 398 position cysteine is the major site of S-nitrosylation. Subsequently, we assessed the effect of overexpression of the mutant RING1A on transdifferentiation. Fibroblasts-overexpressing...
C398A-RING1A were resistant to transdifferentiation (Figure 6D), by comparison to fibroblasts overexpressing the wild-type form of RING1A. Altogether, these data suggest that S-nitrosylation of RING1A at 398 cysteine is critical for transdifferentiation.

**Discussion**

A Novel Mechanism Underpinning Transdifferentiation

Discussion

The new and salient findings of this article are that (1) during transdifferentiation of fibroblasts to endothelial cells, the expression of iNOS is induced, and NO is generated; (2) the generation of NO is required for efficient transdifferentiation; (3) NO facilitates transdifferentiation, in part, by blocking repressive epigenetic markings; (4) this effect is achieved at least, in part, by iNOS binding to and nitrosylating RING1A, and (5) the S-nitrosylation of RING1A causes it to dissociate from the chromatin, impairing the ability of the PRC to maintain H3K27 trimethylation. Most importantly, we have identified a critical role for NO in transdifferentiation. Our results indicate that iNOS serves as an S-nitrosylase that directly binds to an epigenetic modifier to regulate chromatin remodeling during transdifferentiation. The temporal and spatial characteristics of the S-nitrosylation may provide the specificity of the epigenetic effect. We have previously shown that innate immunity is required for efficient nuclear reprogramming.4 This is the first demonstration that NO generated during innate immune signaling regulates epigenetic plasticity.

These observations significantly extend our previous work, which revealed that innate immune activation and nuclear region. Colocalization of iNOS and RING1A in the nucleus (Figure 7) can also be detected. A physical association of iNOS with the epigenetic modifier RING1A could provide for specificity of the RING1A-SNO modification.

**RING1A Nitrosylation Reduces Its Chromatin Binding and Suppressive Chromatin Marks in Endothelial Promoters**

RING1A is a critical component of polycomb repressive complex (PRC) 1 and is required for stabilizing repressive epigenetic marks, such as H3K27 trimethylation (H3K27me3).19,20 To examine whether the SNO modification of RING1A has any impact on cellular H3K27me3 level, we performed Western blot and found that cellular H3K27me3 levels were decreased in PIC-treated cells. This effect of PIC was abrogated by using an NFxβ inhibitor Bay117082 to block iNOS induction (Figure 8A). This result suggested that the RING1A-SNO modification may destabilize the PRC complex to reduce H3K27 trimethylation.

To confirm that SNO modification of RING1A impairs its binding to chromatin, and thereby reduces H3K27me3, we used chromatin immunoprecipitation assay. Specifically, we examined the promoter region of CD144, an endothelial-specific gene that is fully repressed in fibroblasts. After PIC treatment for 3 days, RING1A binding to CD144 promoter is decreased (Figure 8B) together with decreased levels of H3K27me3 (Figure 8C). In addition, H3K9me2 is also decreased in the CD144 promoter (Figure 8D). These studies are consistent with a functional effect of S-nitrosylation to reduce RING1A association with the chromatin and thereby to decrease the suppressing histone marks at promoters of endothelial genes during transdifferentiation.

**Figure 3. Nitric oxide synthase (NOS) and NFκB (nuclear factor kappa B) inhibitors abrogate transdifferentiation.** Human fibroblasts were treated with the transdifferentiation factors and polyinosinic:polycytidylic acid (PIC) for 3 d in the presence or absence of antagonists to the NOS pathway or to NFκB activation. We used the NOS inhibitor L-NAME ((L-NAME, nitroarginine methyl ester) 100 μmol/L), or the NO scavengers Ebselen (Ebs; 10 μmol/L) or uric acid (UA; 100 μmol/L); or the NFκB inhibitors Bay117082 (Bay; 3 μmol/L), celastrol (Cel; 2.5 μmol/L) or dexamethasone (dexa; 100 nmol/L). A, Representative FACS data of CD31+ cells obtained with the transdifferentiation protocol in the presence of L-NAME, Ebselen, or uric acid. B, Representative FACS data of CD31+ induce endothelial cell (iEC) population with Bay117082, celastrol, or dexamethasone. C, Quantification of percentage of CD31+ cells of (A) and (B). *P<0.05, vs PIC group. D, Relative fold change in gene expression levels of inducible NOS (iNOS) at day 3 during transdifferentiation. Cells were treated with the transdifferentiation cocktail in the presence or absence of PIC or with PIC plus Bay117082. *P<0.05, vs vehicle-treated CT. #P<0.05, vs PIC treatment. Data are shown as the means±SEM and are representative of 3 independent experiments.

**Figure 4. iNOS Translocates to the Nucleus and Binds RING1A**

A recent study indicates that iNOS may bind to its protein target, providing some specificity of S-nitrosylation.14 To determine whether iNOS physically associates with RING1A, we immunoprecipitated RING1A and probed for iNOS (Figure 5D). These experiments indicated that iNOS does bind to RING1A. Furthermore, we examined the cellular localization of iNOS and RING1A. Under basal conditions, iNOS was primarily observed in the cytoplasm. After PIC treatment, we observed iNOS translocated to the perinuclear and nuclear region. Colocalization of iNOS and RING1A in the nucleus (Figure 7) can also be detected. A physical association of iNOS with the epigenetic modifier RING1A could provide for specificity of the RING1A-SNO modification.

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These observations significantly extend our previous work, which revealed that innate immune activation
is required for cellular metamorphoses. Specifically, we showed previously that toll-like receptor 3 stimulation facilitates nuclear reprogramming of human fibroblasts to iPSCs or their transdifferentiation into endothelial cells. We found that toll-like receptor 3 stimulation by PIC increased the expression of histone acetyltransferase family members and reduced the expression of HDAC family members and Dot1L. These changes would be expected to increase epigenetic plasticity, an expectation that was confirmed by chromatin immunoprecipitation–qPCR studies of the relevant promoter regions.

\[iNOS\text{ Is an Effector of Innate Immunity and Epigenetic Plasticity}\]

Innate immune activation of NFκB induces iNOS expression. Subsequently, iNOS generates NO, which can activate guanylate cyclase to produce the second messenger cGMP. In addition, NO may S-nitrosylate cysteine residues of proteins to modify their function. For example, S-nitrosylation of caspase 3 regulates apoptosis. Others have shown that S-nitrosylation of the epigenetic modifier HDAC2 in embryonic cortical neurons regulates dendritic growth and branching. In addition, iNOS has been reported to translocate into the nucleus both in malignant and normal cells, but the biological implications of this translocation are obscure. Some epigenetic modifiers have been shown to be capable of S-nitrosylation in screening assays, including RING1A and CBX1. However, it is not known whether these proteins are post-translationally modified in cells and whether the modifications modulate their function.

Thus, we hypothesized that iNOS-derived NO is a major effector of innate immunity in transdifferentiation. We found that iNOS is indeed upregulated during transdifferentiation (Figure 1) in association with increases in intracellular NO (Figure 2) and with increased generation of SNO-NT (Figure 5A) and 3-NT modifications of cellular proteins (Online Figure 1). Notably, pharmacological antagonists that reduced intracellular NO levels consistently diminished transdifferentiation efficiency (Figure 3A). Furthermore, inhibitors of NFκB blocked the upregulation of iNOS, reduced NO levels, and also impaired transdifferentiation efficiency (Figure 3B and 3D). Together these studies indicated that iNOS-generated NO is critical for transdifferentiation.

To further confirm iNOS is required for effective transdifferentiation, we derived MEFs from iNOS−/− mice. These mice have a knockout of the iNOS calmodulin-binding domain. Figure 4. Transdifferentiation is abrogated by inducible nitric oxide synthase (iNOS) deficiency. A, Representative FACS data of CD144+ iEC after transdifferentiation of wild-type (WT) and iNOS−/− murine embryonic fibroblasts (MEFs). B, Quantification of CD144+ cells. *P<0.05, iNOS−/− MEFs vs WT MEFs. C, Relative fold change in gene expression levels of iNOS during transdifferentiation of WT or iNOS−/− MEFs at day 0, 3, and 14 by real-time polymerase chain reaction. D, Western blot detection of iNOS expression in BJ fibroblasts transfected with scramble-CT siRNA (10 nmol/L), with iNOS siRNA (10 nmol/L) plus pcDNA3.1-his-iNOS plasmid, or with 10 nmol/L of iNOS siRNA only. E, Quantification of CD31+ cells derived from BJ fibroblasts treated with scramble-CT siRNA, with iNOS siRNA or with iNOS siRNA plus pcDNA3.1-his-iNOS plasmid. Data are shown as the means±SEM and are representative of 3 independent experiments.

\[\text{Figure 5. Inducible nitric oxide synthase (iNOS) binds and S-nitrosylates (SNO) ring finger protein 1A (RING1A). BJ fibroblasts were treated with vehicle, polyinosinic:polycytidylic acid (PIC), or with PIC and the NFκB (nuclear factor kappa B) inhibitor Bay117082 for 3 d, and proteins were collected for Western blot or immunoprecipitation application. A, Western blot detection of iNOS and SNO protein expression. B, Proteins from cell lysate were immunoprecipitated with RING1A or control IgG antibody and subject to Western blot detection of SNO protein. C, Proteins from cell lysate were immunoprecipitated with RING1A or control IgG antibody and subject to Western blot detection of iNOS protein. Data shown are representative of 3 independent experiments.}\]
domain encoded by exon 12 to 13 and are not capable of generating NO after lipopolysaccharide challenge. Accordingly, we observed that iNOS−/− MEFs are resistant to transdifferentiation (Figure 4A). We then observed similar results in human fibroblasts by transient knockdown of iNOS. Overexpression of iNOS in the knockdown cells restores the transdifferentiation capacity (Figure 4E). Taken together, the genetic and pharmacological studies suggest that iNOS-generated NO is required for transdifferentiation.

Nitrosylation of RING1A Is Required for Transdifferentiation

Next, we screened several epigenetic modifiers that have been reported to be S-nitrosylated. Of these, only RING1A is heavily...
H2A monoubiquitination.19,20 Knockout of RING1A reduces Maintenance of H3K27 trimethylation requires PRC1-induced of PRC2–PRC1 has been described, 35,36 in that PRC1 recog-

These experimental results indicate a critical role of the physi-

B inducible nitric oxide synthase (iNOS), H3K27me3, RING1A, tran-

A transdifferentiation protocol.

Figure 8. Innate immune activation decreases ring finger protein 1A (RING1A) binding to chromatin and reduces repressive histone markers. BJ fibroblasts treated with or without polyinosinic:polycytidylic acid (PIC) for 3 d during the transdifferentiation protocol. A, Western blot examination of inducible nitric oxide synthase (iNOS), H3K27me3, RING1A, and S-nitrosylated protein expression. B, RING1A, (C) H3K27me3, and (D) H3K9me2 binding to the CD144 promoter were analyzed by chromatin immunoprecipitation (ChIP)-polymerase chain reaction. *P<0.05, vs vehicle-treated CT.

Data are shown as the means±SEM and are representative of 3 independent experiments.

S-nitrosylated at the 398 position cysteine during innate immune activation as assessed by MS (Figure 6A) and Co-IP studies (Figure 5). The S-nitrosylation of a protein can be achieved through several different mechanisms.10,25,31,32 Recently, it has been shown that protein S-nitrosylation can be achieved in a target-selective manner at a sequence motif recognition site by an inducible heterotrimeric S-nitrosylase complex.14 This protein complex consists of iNOS, S100A8, and S100A9 and recognizes a conserved I/L-X-C-X2-D/E motif. Of note, although RING1A lacks this motif, our Co-IP data indicate that iNOS associates with and S-nitrosylates RING1A (Figure 5D). Immunostaining also confirms the colocalization of iNOS and RING1A in the nucleus (Figure 7). This physical association is likely to provide some specificity of the S-nitrosylation. Indeed, we were not able to rescue the transdifferentiation process in iNOS−/− MEFs using the NO donor SIN1 (data not shown). These experimental results indicate a critical role of the physical association of iNOS with RING1A for transdifferentiation.

RING1A is a major component of the PRC1,33 which acts as a transcriptional repressor.14 A sequential recruitment of PRC2–PRC1 has been described,35,56 in that PRC1 recognizes the H3K27me3 marker that is imposed by PRC2.35,40 Maintenance of H3K27 trimethylation requires PRC1-induced H2A monoubiquitination.19,20 Knockout of RING1A reduces H3K27me3 marks.34 In this regard, we observed that PIC reduced binding of RING1A to, and H3K27 trimethylation of, the endothelial-specific gene promoter CD144 (Figure 8B and 8C). These observations suggest that when RING1A is S-nitrosylated, it dissociates from the chromatin, thereby releasing the PRC complex and destabilizing repressive marks, such as H3K27me3.

Physiological and Pathobiological Relevance of Our Findings

iNOS is a potent cellular defense mechanism, generating reactive nitrogen species that also have cytotoxic effects. We now reveal another role for iNOS in innate immunity, as a facilita-

In some instances, the epigenetic plasticity associated with innate immune activation may have a physiological role. For example, in the setting of experimental myocardial infarction, a substantial number of cardiac fibroblasts may transdifferentiate to endothelial cells to enhance the angiogenic response to ischemic injury.41 Epigenetic plasticity induced by innate immunity might also participate in pathological transdifferentiation. For example, the chronic inflammation associated with gastroesophageal reflux disease is accompanied by a premalignant transdif-

Indeed, it has been long recognized by clinicians that cancers may arise in the setting of chronic inflammation or infection.43,44 Furthermore, iNOS is often expressed in prema-

Conclusions

We have identified a novel mechanism by which innate immune signaling facilitates transdifferentiation. The activation of iNOS and the generation of NO are associated with iNOS binding to, and S-nitrosylating RING1A. This modification causes a dissociation of RING1A from the chromatin. This effect is associated with a reduction in H3K27 trimethylation, a repressive chromatin marking. These effects would be predict-

Acknowledgments

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Disclosures

Dr Cook is an inventor on patents owned by Stanford University related to the manipulation of innate immune signaling for nuclear reprogramming so as to induce pluripotency, transdifferentiation, or other therapeutic cellular modifications. The other authors report no conflicts.

References

Novelty and Significance

**What Is Known?**
- Transdifferentiation describes the process by which a somatic cell undergoes a change in phenotype.
- Transdifferentiation may contribute to disease, such as with endothelial-to-mesenchymal transition, which participates in cardiac fibrosis.
- Transdifferentiation may contribute to regeneration, such as with mesenchymal-to-endothelial transition, which contributes to angiogenesis in ischemic myocardium.

**What New Information Does This Article Contribute?**
- Transdifferentiation of fibroblasts to endothelial cells requires expression of inducible nitric oxide synthase.
- Ring finger protein 1A, a component of the polycomb repressive complex, is bound by inducible nitric oxide synthase and nitrosylated.
- The nitrosylation of ring finger protein 1A reduces repressive epigenetic markings in lineage-specific gene promoters, facilitating mesenchymal-to-endothelial transition.

Transdifferentiation of one cell type to another plays a role in disease, as well as in development and regeneration. The process of transdifferentiation from one cell lineage to another requires epigenetic changes that unlock those genes that are needed for expression of the new phenotype (and which suppress those genes that were responsible for the previous phenotype). An understanding of the epigenetic changes that govern these changes in gene expression (and thus cell phenotype) will lead to new therapeutic avenues. We have shown that activation of innate immunity causes global changes in the expression of epigenetic modifiers that can enhance epigenetic plasticity. In this article, we show that inducible nitric oxide synthase–generated nitric oxide, which is one of the major effectors of innate immunity, also plays a critical role in mediating the epigenetic plasticity that is required for the transdifferentiation of human fibroblasts to endothelial cells. The nitrosylation of ring finger protein 1A, a component of the polycomb repressive complex, causes the dissociation of this complex from the chromatin, thereby reducing repressive epigenetic markings, such as H3K27 trimethylation. These insights may lead to new approaches to enhance therapeutic transdifferentiation, for example, mesenchymal-to-endothelial transition to enhance vascularity in ischemic syndromes.
Transdifferentiation Requires iNOS Activation: Role of RING1A S-Nitrosylation
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Transdifferentiation requires iNOS activation: Role of RING1A S-Nitrosylation

Shu Meng, MD PhD; Gang Zhou, MD PhD; Qilin Gu, PhD; Palas K. Chanda, PhD; Frank Ospino, BS; John P. Cooke*, MD PhD

Center for Cardiovascular Regeneration
Department of Cardiovascular Sciences, Houston Methodist Research Institute, Houston, TX 77030

*Correspondence to:
John P. Cooke, M.D., Ph.D.
Department of Cardiovascular Sciences
Houston Methodist Research Institute
6670 Bertner Ave., Mail Stop: R10-South
Houston, Texas, 77030
Phone: 713-441-8322
Fax: 713-441-7196
Email: jpcooke@houstonmethodist.org

Key words: Innate immunity, epigenetics, endothelial cell, nitric oxide
Material and Methods

Chemicals and Reagents
All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Other reagents included FBS, Taqman RT-PCR primers, Taqman master mix, DAF-FM diacetate, RNAimax and DAPI (Life Technologies, Carlsbad, CA); the TLR3 agonist polyinosinic:polycytidilic acid (PIC) and NFκB inhibitors Bay117082, celastrol and dexamethasone (Invivogen, San Diego, CA); antibody against the S-nitroso (SNO) moiety (Sigma-Aldrich); antibody against 3-nitrotyrosine (NT) (Cayman Chemical, Ann Arbor, Michigan); PE- antibody against human CD31 and APC- antibody against mouse CD144 (BD Biosciences, San Jose, CA); antibody against inducible nitric oxide synthase (iNOS), HRP conjugated goat anti mouse or rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); antibody against β-tubulin, Ring1A, H3K27 trimethylation (H3K27me3) and H3K9 dimethylation (H3K9me2; Abcam, Cambridge, UK); bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ); Scramble and iNOS siRNA (Integrated DNA Technologies, Coralville, IA).

Cell culture and iEC generation
BJ human neonatal foreskin fibroblast cells (BJ fibroblasts) (Stemgent), WT and iNOS-/- murine embryonic fibroblasts (MEF; Cell Biologics Inc, Chicago IL) were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin (5% CO2, 37°C). We transdifferentiated human and mouse fibroblasts to endothelial cells (iEC) using our previously established protocol \(^1\). Briefly, cells were treated with PIC (30 ng/mL) daily for 7 days, and the medium was gradually transitioned to a 10% knockout serum replacement. Cells were treated with endothelial cell growth medium 2 (EGM-2; Lonza) containing 20 ng/mL BMP4, 50 ng/mL VEGF, and 20 ng/mL bFGF for another 7 days. For maintenance of transdifferentiation, the cells were cultured for another 2 weeks in the presence of bFGF, VEGF, and 0.1 mmol/L 8-bromoadenosine-3’:5’-cyclic monophosphate sodium salt (8-Br-cAMP), with the medium changed every 2 days. In some experiments, inhibitors of iNOS or NFκB were present during the first three days of the transdifferentiation protocol; they were re-added when the transdifferentiation medium was refreshed.

RNA extraction and Real-Time PCR
mRNAs were extracted from cultured cells using RNeasy Mini Kit (Qiagen). The mRNA was reverse transcribed into cDNA and then specific primers for targeted genes were used for PCR amplification. For real-time PCR, a SYBR-green PCR system or Taqman system was used and ran on QuantStudio real-time PCR system (Life Technologies). Gene expression data using the ΔΔCt method was normalized to the expression of the 18S gene and plotted as relative fold changes.

**Western blot and Immunoprecipitation**

Western blot analysis was performed as previously described. Cultured cells were homogenized in RIPA buffer containing 1× protease and phosphatase inhibitor cocktail. Protein concentration was quantified using BCA assay (Pierce) and resolved on 4-12% gradient SDS-polyacrylamide gels. The gel was transferred into nitrocellulose membrane using an iBlot System (Life Technology). Blots were stained with 1% Ponceau S for loading controls. Then blots were blocked with 5% nonfat milk in PBST (PBS with 0.1% Tween 20) for 1 hr at room temperature and probed with primary antibody overnight or longer at 4°C. Blots were then washed 4 times with PBST for 10 min. Horse radish peroxidase (HRP) -conjugated goat anti-mouse or rabbit antibodies were incubated for 1 hr at room temperature. Blots were washed for 4 times with PBST for 10 mins. Then antigen-antibody complexes were detected by exposure in a Fluorchem M system (Proteinsimple, San Jose, CA).

Immunoprecipitation was performed using the Pierce Classic Magnetic IP/Co-IP Kit (Thermo Scientific, Waltham, MA) following manufacturer’s manual. After exposure to a variety of experimental conditions, BJ fibroblasts were washed and collected in lysis buffer (pH 7.4, 0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP40, 5% glycerol). After 30 min incubation on ice, the lysate was centrifuged at 13,000×g for 10min to collect the supernatant. The cytosolic extract (500μg) was pretreated with protein A/G magnetic beads and incubated with control IgG, anti-SNO or anti-RING1A antibody overnight at 4°C. Protein A/G magnetic beads were incubated with samples for 2 h at 4°C, pelleted and washed three times with lysis buffer. Proteins were eluted in pH2.0 elution buffer and neutralized with pH8.5 buffer for western blot analysis.

**Chromatin Immunoprecipitation (ChIP) -qPCR**

ChIP was performed using a previously established method. Recovery of genomic DNA was calculated as the ratio of copy numbers in the immunoprecipitate to the input control. Primers of the CD144
promoter were custom designed and synthesized from IDT, as follows: primer sequence (5’ to 3’), (F): 
CAGCCTCTTGGTTCTTCTGG; (R): GGAGTCAAGTGACCCAGCTC.

**Fluorescent assay for nitric oxide (NO)**
To assess intracellular NO, we stained BJ fibroblasts using 4-Amino-5-methylamino-2’, 7’-
difluorofluorescein Diacetate (DAF-FM DA). After the fibroblasts had been exposed to experimental 
conditions, they were incubated with DAF-FM DA 10 µM at 37 °C for 30 min. Fluorescence was 
quantified by measuring fluorescence intensity at 495 nm excitation and 515 nm emission by a 
fluorescence plate reader (Tecan M1000 PRO).

**Immunofluorescent staining for iNOS and RING1A**
After BJ fibroblasts were cultured in 8 well chamber slides and exposed to experimental conditions, cells 
were then fixed with 4% paraformaldehyde, permeabilized with phosphate-buffered saline (PBS) with 
0.1% Triton X-100, blocked with 5% normal donkey serum, and stained for anti-human RING1A or iNOS 
antibody overnight at 4°C. After washes with PBS, the cells were further incubated with Alexa Fluor 488 
or 555 conjugated secondary antibodies at RT for 1 hr. The nuclei were stained with DAPI for 10 min.

**Analysis of S-nitrosylation by mass spectrometry (MS)**
Protein from both control and PIC treated BJ fibroblasts was immunoprecipitated using RING1A antibody, 
resolved on 4-12% gradient SDS/PAGE gel and stained with Coomassie Blue. After destaining, the 
RING1A band was cut and sent to the mass spectrometry-proteomics core facility (Baylor College of 
Medicine). The gel band was further destained and double digested with trypsin and GluC (NEB) in situ. 
Peptides in the gel were extracted, quantified and 200 ng of each sample was analyzed using the 
Eksigent nanoLC system and then the ABIEX TripleTOF 5600 mass spectrometer. The Eksigent nanoLC 
and the ABIEX TripleTOF 5600 mass spectrometer were controlled by Analyst software version 1.6 
(ABIEX Inc.). The MS spectra (m/z 100-2000) were acquired with internal mass calibration. Raw data 
were converted to MGF format and then imported into Peaks software v6.0 for further peak generation 
and database search with the SPIDER function. The search sequence was E3 ubiquitin-protein ligase 
RING1A of human (NP_002922.2  GI:51479192).

**Construction of RING1A overexpression plasmid and site directed mutagenesis**
Human RING1A cDNA fragment was amplified with forward primer (5′-CAGGAATTCTGAAGAACACGCCGGAATG-3′) and reverse primer (5′-GAGGTCGACGTACCTTGGGATCCTTGCTGGGAGC-3′) from pCMV6-XL4-RING1A (OriGene, # SC118280) and ligated into EcoRI/SalI digested pCMV-Tag2C to generate pCMV-Tag2C-RING1A. We further created pCMV-Tag2C-RING1A-C398A using a PCR-based method. Briefly, PCR reaction was set by 10 ng of template (pCMV-Tag2C-RING1A), 1 μM primer pair: C398A Mutation-Forward (5′-CGGCCACTGGAGCTGGCCTATGCTCCCACCAAGTCCAAAG-3′) and C398A Mutation-Reverse (5′-TTGGTGGGAGCATAGGCCAGCTCCAGTGGCCGGGACAC-3′), 200 μM dNTPs and 1 unit of Phusion High-Fidelity DNA Polymerase (NEB, # M0530S). The PCR cycles were initiated at 94°C for 5 minutes, followed by 18 amplification cycles. Each amplification cycle consisted of 94°C for 30 seconds, 68°C for 3 minutes. The PCR products were treated with 5 units of DpnI at 37°C for 2 hours and then 5 μl of each PCR reactions was transformed respectively into E. coli Top10 competent cells by heat shock. Single clones were sequenced to examine the C398A mutation.

Flow cytometry

Cells were trypsinized and filtered through a 40 μm nylon mesh to generate single cell suspensions. Cells were stained with antibody for cell surface marker detection. Data were analyzed by flowjo software.

Data analysis

Results are expressed as the mean±SEM. Statistical comparisons between 2 groups were performed via Student t test. One-way ANOVA was used to compare the means of multiple groups. P<0.05 was considered statistically significant.
References


Online Figure I. 3-NT protein increased during transdifferentiation. BJ fibroblasts were treated with PIC for 0, 3 and 7 days and collected for western blot. Lysates from treated cells were subjected to immunoblotting with 3-NT antibody and β-tubulin antibodies.
Online Figure II. CBX1 and HDAC2 are not S-nitrosylated during transdifferentiation. BJ fibroblasts were treated with PIC or vehicle control for 3 days. Cell lysates were subject to immunoprecipitation with SNO antibody and then immunoblotted for (A) CBX1 antibody or (B) HDAC2 antibody.
Online Figure III

A

IB: SNO

IB: SNO

IB: iNOS

IB: β-tubulin

5% Input IgG IP RING1 IP

45 min exposure Ponceau S staining

B

IB: SNO

IB: SNO

60KD

5% Input IgG IP RING1 IP

45 min exposure Ponceau S staining
Online Figure III

**Online Figure III.** *iNOS binds and S-nitrosylates Ring1A.* BJ fibroblasts were treated with vehicle; PIC; or with PIC and the NFκB inhibitor Bay117082 for 3 days and proteins were collected for western blot or immunoprecipitation application. (A) Original blots of Figure 5A. (B) Original blots of Figure 5B. (C) Original blots of Figure 5C. (D) Proteins from cell lysate were immunoprecipitated with SNO or control IgG antibody and subject to western blot detection of Ring1A. Left panel showed the western blot. Middle and right panels are the original blots.
Online Figure IV. MS analysis of S-nitrosylation site of Ring1A. MS/MS fragmentation spectra for (A) the Cys332-containing peptide and (B) the Cys279-containing peptide. MS spectra of the cysteine-containing peptides identified after MS analysis. Peptide sequence is shown at the top left of each spectrum, with the annotation of the identified matched amino terminus-containing ions (b ions) in black and the carboxyl terminus-containing ions (y ions) in red. For clarity, only major identified peaks are labeled.
Online Figure V

**A**

IB: SNO

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**B**

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**Online Figure V. RING1A-C398A mutation ablished S-nitrosylation.**
BJ fibroblasts were transfected with either pCMV-Tag2C-RING1A-C398A (WT-RING1A) or pCMV-Tag2C-RING1A-C398A (C398A-RING1A) plasmid and then subject to transdifferentiation protocol. At day 3, proteins were collected and immunoprecipitated with Flag or control IgG antibody. Products were examined by western blot detection of SNO protein. (A) Western blot. (B) Original blots of A.
Online Figure VI. Transdifferentiation protocol. Direct reprogramming of human fibroblasts to functional endothelial cells via activation of innate immunity and microenvironmental cues. The figure outlines the time course and sequential treatments.
Online Figure VII

PIC ↓
TLR3 activation ↓
NFκB ↓
iNOS

Direct binding \text{NO generation} ↓
Ring1A S-nitrosylation ↓
↓ Ring1A binding to chromatin ↓
↓ PRC1 stability and ↓ H3K27me3 level ↓
Epigenetic plasticity ↓
Environmental cues

Transdifferentiation

Online Figure VII. Mechanism of epigenetic plasticity induced by innate immune activation.