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# Therapeutic Transdifferentiation: A novel approach for vascular disease

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## Abstract

Emerging evidence indicates that overexpression of a few “master regulators” can dramatically alter cell type (transdifferentiation), in a process called direct reprogramming. The recent reprogramming of human fibroblasts into cardiomyocytes and endothelial cells augurs a new era in cardiovascular medicine by increasing the feasibility of cardiovascular regenerative therapy.

## Reprogramming fibroblasts to ECs

In a recent seminal paper, Margariti and colleagues described their success in reprogramming human fibroblasts into endothelial cells (ECs) [1]. These induced endothelial cells had all the phenotypic characteristics of genuine endothelial cells. They stained for endothelial markers such as CD31, VE-Cadherin, eNOS and von Willebrand factor. Moreover, they exhibited endothelial behaviors such as network formation in matrigel, and capillary formation in vivo. Furthermore, injection of these cells into the ischemic murine hindlimb improved perfusion, in association with an increase in capillary density. Finally, the cells were capable of contributing to reendothelialization in tissue-engineered vessels.

## Nobel Prize for iPSCs

An important precedent for their accomplishment is the work of Shinya Yamanaka, who won the 2012 Nobel Prize in Physiology or Medicine. Dr. Yamanaka discovered that the forced expression of four “master regulators” (the genes encoding the transcriptional factors Oct4, Sox2, Klf4 and cMyc) could generate “induced pluripotent stem cells” or iPSCs [2, 3]. When fibroblasts are transfected by viral vectors or RNA[4, 5] encoding these genes, a small percentage of the transfected cells will gradually transform (over a period of about 2-4 weeks) into pluripotent stem cells. These iPSCs are capable of becoming endoderm, ectoderm or mesoderm, and any of the somatic cell lineages can potentially be derived. The mechanisms by which the “master regulators” activate the core pluripotency network, and induce the genetic and epigenetic changes required for pluripotency, are incompletely elucidated and the subject of frenetic activity in the stem cell field. As a result, new insights are occurring with regularity, and our understanding of the reprogramming process is deepening. Despite the nascent state of our knowledge, iPSCs appear to have great value as a scientific tool. They have been differentiated into somatic cells for studies of pathobiology, and hold great promise in screening of small molecules for potential therapies[6, 7].

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## Obstacles to iPSC therapies

However, the use of iPSCs (or more likely cells derived from them) for regenerative medicine, is farther off on the horizon. Concerns have been raised about the epigenetic differences between iPSCs and embryonic stem cells [8]. The iPSC-derived cells may retain an epigenetic memory of the parental cells[9] that could influence their function in unanticipated ways. Furthermore, differentiation protocols to therapeutic somatic cells are empirical and inefficient. Finally, methods for purifying the therapeutic cells (and particularly, for excluding parental pluripotent cells) need to be improved[10]. It is theoretically possible that the administration of one pluripotent stem cell could give rise to a teratoma, given the replicative capacity of these cells. Accordingly, a method to generate the desired therapeutic cell directly, ie. from another somatic cell, (direct reprogramming) is appealing because this capability would avoid the risk of administering a stray pluripotent stem cell that could become a teratoma.

## Direct reprogramming

Indeed, several groups have now demonstrated the feasibility of direct reprogramming. Their work owes much to John Gurdon, who also received the 2012 Nobel Prize in Physiology or Medicine. By transferring somatic cell nuclei into an enucleated oocyte, he revealed that cytoplasmic factors in the oocyte were capable of reprogramming the somatic nucleus toward pluripotency[11]. Subsequent nuclear transfer experiments by others, revealed that cytoplasmic factors in one somatic cell could reprogram another, including the studies of Helen Blau which revealed that the mammalian skeletal muscle cell could reprogram the hepatocyte nucleus toward a muscle phenotype[12]. Thus the idea was born that the forced expression of “master regulators ” might permit a form of biological alchemy, where one somatic cell was transformed into another.

This notion was the inspiration for recent work toward directed reprogramming. Typical of these studies were those of Marius Wernig, who manipulated a set of about 20 transcriptional factors known to be important in neuronal development, overexpressing them in fibroblasts[13]. Combinations of these factors were assessed, and a smaller set of four factors were found to be sufficient for generating neurons when transfected into murine fibroblasts. This approach has also been used to generate other somatic cells from fibroblasts, including cardiomyocytes (14, 15).

## Reprogramming fibroblasts to ECs with the Yamanaka factors

Margariti and colleagues took a slightly different approach to reprogramming fibroblasts to endothelial cells. Rather than a set of factors known to be involved in endothelial development, they transfected fibroblasts with the Yamanaka factors, before placing them into endothelial differentiation medium. They reasoned that, after a few days in culture, the overexpression of the Yamanaka factors would generate “partially induced pluripotent cells” (PiPS), ie. cells that were incompletely reprogrammed. Indeed, PiPS cells did not form teratomas in vivo, and did not express pluripotency surface markers. The PiPS were then differentiated toward endothelial cells by prematurely replacing the stem cell media by endothelial differentiation media. Indeed, PiPs displayed the potential to differentiate into endothelial cells (PiPS-EC) when they were exposed to the appropriate culture conditions.

An insight into the nature of this transdifferentiation came from their observation that SET translocation (myeloid leukemia-associated) (SET) similar protein (SETSIP) was induced during the reprogramming process. Notably, when PiPS cells were treated with VEGF, SETSIP translocated to the cell nucleus, directly bound to the VE-cadherin promoter, increasing vascular endothelial cadherin (VE-cadherin) expression levels and EC

differentiation. Thus, one of the key factors in the endothelial culture media was shown to contribute to PiPS differentiation to endothelial lineage.

It can be argued that Margariti and colleagues did not actually induce “direct” reprogramming of fibroblasts to endothelial cells. For direct reprogramming, most others have employed an empirically determined combination of genes encoding transcriptional factors involved in the developmental program of their chosen lineage. By contrast, Margariti and colleagues used the Yamanaka factors to induce an intermediate state of “partial reprogramming”. Complete reprogramming occurs several weeks after exposure to retroviral vectors carrying the Yamanaka factors, under the influence of culture conditions favoring pluripotency. Margariti and colleagues removed the cells from stem cell culture conditions four days after exposure to the Yamanaka factors (well before complete reprogramming), and placed them into culture conditions favoring endothelial lineage. It is possible that the “partially reprogrammed” cells included precursor cells of mesodermal lineage that could respond to the endothelial culture conditions, but the precursor cells were not fully characterized. [Of note, Ding and colleagues have used a similar approach to reprogram fibroblasts to neurons, or to cardiomyocytes (16, 17) suggesting that partially reprogrammed cells are a heterogenous lot, and/or they have sufficient plasticity to differentiate toward different lineages given the right culture conditions]. Single cell transcriptional studies and lineage tracing would have been helpful in characterizing the population of “partially reprogrammed” cells.

## Unanswered questions

That being said, in most of the reported studies of direct reprogramming there has not been a comprehensive analysis of the transitional cells that may be created during the process of transdifferentiation. Furthermore, the epigenetic mechanisms and transcriptional programs underlying the process of transdifferentiation to any cell type are incompletely understood. One of the most perplexing questions is how a handful of transcriptional factors (and in some cases, a single transcription factor [18]!) can induce direct reprogramming of a somatic cell to one of a different germ layer (eg. fibroblast to neuron). This is particularly puzzling when the overexpressed gene does not encode a “pioneering” transcriptional factor, ie. one that does not itself initiate the transcriptional complex that includes other co-factors and epigenetic modifiers.

One potential explanation is that the viral vectors or modified mRNA (mmRNA) that encode the transcriptional factors are having additional effects that are important in reprogramming. Recently, we have discovered that innate immune signaling plays a critical role in reprogramming somatic cells to pluripotency (19). The activation of the toll-like receptor TLR3 by viral vectors or mmRNA triggers a signaling cascade that results in global changes in epigenetic modifiers. We observed that several of the histone de-acetylase (HDAC) family members are dramatically downregulated, whereas some histone acetyl transferase (HAT) genes are upregulated, effects which would favor an “open chromatin” configuration. We found that knockdown of innate immunity signaling in human fibroblasts dramatically reduced their susceptibility to reprogramming by retroviral vectors or mmRNA encoding the Yamanaka factors. By contrast, activation of TLR3 by an irrelevant virus, or by poly I:C, markedly increased the efficiency of reprogramming using the Yamanaka factors in the form of cellpermeant peptides. Thus, activation of innate immunity by viral vectors or mmRNA increases epigenetic plasticity to permit cell transformation, a process we have termed “transflammation”.

## Transflammation and reprogramming

Is it possible that transflammation also plays a role in the “direct reprogramming” using viral vectors or mmRNA? Certainly, the induction of an “open chromatin” configuration would make it easier to explain how a single transcriptional factor might reprogram a somatic cell, together with extrinsic cues from the media and/or extracellular matrix. In this regard, there is insufficient credit attributed to culture conditions that are utilized to favor growth of the desired cell type. It is likely that these culture conditions also influence differentiation to the target somatic cell. Preliminary studies in our laboratory support the notion that activation of innate immunity, and external signals provided by the media and extracellular matrix, may be powerful influences on direct differentiation.

## The promise and peril of direct reprogramming

In any event, direct reprogramming to the desired cell type may have greater potential for regenerative medicine applications. Although human endothelial cells derived from induced pluripotent stem cells (iPSC-EC) are functionally effective in forming capillaries and improving perfusion in vivo (20), generation of pluripotency followed by differentiation to the desired cell type takes months, and there remain concerns about teratoma formation. Direct reprogramming would avoid these concerns (although the fidelity of reprogramming requires confirmation). What is most exciting about direct reprogramming is the possibility that it might be achievable in patients in vivo. Such an approach, if accomplished with small molecules, would avoid the more complex approach of cell delivery. Indeed, recent work reveals that in the ischemic murine myocardium, intramyocardial injection of mmRNA encoding four transcriptional factors is sufficient to induce direct reprogramming of cardiac fibroblasts to cardiac myocytes (14). Although the frequency of the conversion to myocytes was quite low, this exciting result provides compelling support to develop more efficient transdifferentiation techniques for clinical trial.

The clinical applications for direct reprogramming of fibroblasts to endothelial cells are numerous. For example, in ischemic injury such as myocardial infarction, one might convert the cardiac fibroblasts that are migrating and proliferating into the ischemic region into endothelial cells. The intent would be to generate a microvasculature that could provide the nutrition and niche for reparative resident cells that could reconstitute the myocardium. As another example, it is possible that in diseases characterized by fibrosis and loss of the microvasculature (such as scleroderma), reprogramming of fibroblasts to endothelial cells could reverse the scarring process.

However, for the promise of this regenerative therapy to be achieved, greater efficiency of the reprogramming process is required. Furthermore, the fidelity of the reprogramming process must be assured (eg. the induced cells should ideally manifest the same transcriptional and epigenetic profile). Moreover, the integration of the reprogrammed cells within the architecture of the tissue must support organ function (eg. induced cardiomyocytes would need to form a functional synctium with the native cardiomyocytes and not generate an arrhythmic focus; induced endothelial cells would need to form a functional microvasculature with normal arteriovenous communications and lymphatic vessels). Potential adverse effects of direct reprogramming in vivo could include inappropriate differentiation into other cell types (eg. hamartoma) or even tumor cells. In addition, induced cells could be dysfunctional (eg. induced endothelial cells could express more adhesion molecules and promote thrombosis or excessive inflammation). Ideally, the direct reprogramming therapy should utilize small molecules that can target the effected tissue, and avoid gene or cell therapies which raise additional technical and regulatory

obstacles. To conclude, the road to novel regenerative therapy using direct reprogramming will be long and arduous, but the journey has begun.

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