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Reprogramming Fibroblasts to Endothelial Cells: Converted or Born-Again?

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"*These results show that a nucleus can promote the formation of a differentiated intestine cell and at the same time contain the genetic information necessary for the formation of all other types of differentiated somatic cell in a normal feeding tadpole. It is concluded that the differentiation of a cell cannot be dependent upon the incapacity of its nucleus to give rise to other types of differentiated cell.*" This is the concluding paragraph of Sir John Gurdon's seminal study published in 1962, in which he demonstrated that nuclei from differentiated intestinal epithelial cells could give rise to normal tadpoles following transfer into an enucleated recipient egg [1]. Half a century later, Gurdon would receive the Nobel Prize together with Shinya Yamanaka, whose equally seminal work had shown that adult mouse fibroblasts could be reprogrammed to a fully pluripotent stem cell state [2], for their contributions to reprogramming differentiated cells. Unlike Gurdon's nuclear transfer approach, Yamanaka's method relied on the expression of the transcription factors Oct4, Sox2, c-Myc and Klf4 (also known as the "Yamanaka factors") in fibroblasts, which activated the cells' dormant embryonic pluripotency gene network and generated induced pluripotent stem cells (iPSCs). The iPSCs, in turn, could then be differentiated into a variety of desired cell types such as neurons, hepatocytes or cardiomyocytes.

The prescient formulation in Gurdon's 1962 paper emphasizes the reversibility of cell fate decisions without necessarily invoking pluripotency. A differentiated somatic cell such as an intestinal epithelial cell or an adult fibroblast harbors the potential of becoming any other differentiated cell. This idea fueled the search for approaches which would enable the direct conversion of adult differentiated cells into other lineages without generating an intermediate iPSC. Such a direct conversion of fibroblasts or other adult somatic cells into desired cell types would have the pragmatic advantage of expediency. It takes multiple

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weeks to first generate iPSCs from adult cells, and during a second phase, iPSCs have to then be differentiated into, for example, neurons, cardiomyocytes or hematopoietic cells. In addition to this practical advantage, direct lineage conversion may also allay concerns regarding the formation of teratomas or spontaneous differentiation into undesired cell types if one wants to use iPSC-derived cells for therapeutic, regenerative purposes in patients. Undifferentiated pluripotent stem cells can form teratomas, and even though therapeutic applications would most likely use differentiated iPSC-derived cells, there is always a small risk that the obtained iPSC-derived cell populations might be contaminated by some undifferentiated or only partially differentiated iPSC which could form teratomas or give rise to other undesirable cell types following transplantation into the recipient.

At least two distinct approaches have been used to successfully convert adult fibroblasts into functional cardiomyocytes: The first approach involves expressing cocktails of selected transcription factors involved in the specification of the cardiomyocyte lineage during embryonic development[3]. These cardiogenic transcription factor cocktails can also be applied in vivo, where they can directly convert cardiac fibroblasts into functional cardiomyocytes[4, 5]. An alternate means of converting fibroblasts into cardiomyocytes relies on briefly expressing the "Yamanaka factors" in fibroblasts but preventing the formation of pluripotent iPSCs. Instead, the fibroblasts are guided towards the cardiomyocyte lineage by providing the cells with appropriate cardiogenic growth factors and small molecules[6]. The transient activation of pluripotency genes appears to thrust the fibroblasts towards a partially de-differentiated or more pliable state, which then enables the reprogramming using exogenous cardiogenic growth factors and molecules. This latter approach of combining brief activation of pluripotency genes with exogenous differentiation cues (and thus circumventing the formation of pluripotent iPSCs) has also been successfully used to convert fibroblasts into vascular endothelial cells[7–9], whereas the approach using lineage-specific transcription factors to generate endothelial cells was only successful in converting amniotic cells to endothelial cells[10]. The successful amniotic-to-endothelial cell conversion underscores that lineage-specific transcription factors can indeed be used to generate endothelial cells, but its practical use for generating patient-specific endothelial cells was rather limited. Fibroblasts can be easily obtained in the clinical setting through a simple skin biopsy, whereas amniotic cells are quite difficult to come by.

In this issue of *Circulation*, Kim and colleagues now show that adult mouse fibroblasts can be converted into endothelial cells using a lineage-specific transcription factor approach which over-expresses a cocktail of five transcription factors: Foxo1, Er71, Klf2, Tal1, and Lmo2[11]. They initially screened combinations of eleven transcription factors which they deemed important for endothelial cell development and narrowed down the transcription factor combination to the above-mentioned five factors. The screening was performed using mouse fibroblasts with a fluorescent Tie-2 reporter, but flow cytometry confirmed that the generated endothelial cells indeed expressed endothelial-specific surface proteins. More importantly, the newly generated iECs (induced endothelial cells) also functionally behaved like endothelial cells in that they released nitric oxide upon stimulation with acetylcholine or VEGF and enhanced angiogenesis in vivo. According to Kim and colleagues, this specific combination of transcription factors was only able to generate endothelial cells from

fibroblasts, but not from bone marrow mononuclear cells, thus indicating that the starting cell type may dictate what factors are required for lineage conversion to endothelial cells. The fraction of fibroblasts which generated iECs was roughly 4%, as defined by either the Tie-2 reporter or by using the expression of the endothelial-specific surface protein VE-cadherin as an indicator. This percentage may seem low, but it broadly corresponds to range of reprogramming success that has been reported for other forms of lineage conversion. This new method of directly generating endothelial cells from fibroblasts has significant implications for generating patient-specific endothelial cells, especially for regenerative purposes. By providing a lineage-specific direct reprogramming approach, this work now opens up the possibility of future studies which may attempt the in vivo generation of endothelial cells, similar to what has been demonstrated for in vivo fibroblast-to-cardiomyocyte reprogramming[4, 5]. If new cardiac muscle can be generated by locally reprogramming resident cardiac fibroblasts to cardiomyocytes, then surely such a newly generated heart muscle would benefit from the concomitant generation of vascular endothelial cells to ensure oxygenation of the neo-myocardium. Since previous fibroblast-to-endothelial conversion methods relied on transiently expressing pluripotency genes[7–9], they were not well-suited for direct in vivo reprogramming of resident fibroblasts.

The reported success of direct reprogramming to cardiomyocytes varies widely between various research laboratories even when they use the same transcription factors [3, 12] which is why it will be important to await the results of reprogramming success when other research laboratories use the new method proposed by Kim and colleagues to generate endothelial cells. The current study by Kim and colleagues did not investigate whether human fibroblasts can also be reprogrammed to endothelial cells and it still remains to be seen how the results will translate to the human setting. In the case of fibroblast-to-cardiomyocyte conversion, human fibroblasts appear to generate functionally immature cardiomyocytes, as evidenced by low-amplitude calcium transients and rare spontaneous contractility[13], indicating that human fibroblast-to-endothelial reprogramming using endothelial lineage-specific transcription factors may be more challenging.

One limitation of the present study is that it provides little information about the long-term identity and functionality of the generated iECs. Even mature primary endothelial cells in culture can experience a phenotypic drift, and this may be an even bigger concern for reprogrammed cells since it is possible that they may have retained some of their fibroblast identity. In fact, the recent CellNet study analyzed the gene expression patterns of various directly reprogrammed cell types, comparing them to the gene expression patterns of cell types derived from pluripotent stem cells and found that directly reprogrammed cells appear to inadequately silence their source cell program[14]. In practical terms, this suggests that fibroblasts which are first converted to a pluripotent iPSC state and only then differentiated into cardiomyocytes or endothelial cells may be more likely to erase their fibroblast identity than fibroblasts which are directly converted to cardiomyocytes or endothelial cells. The iPSC reprogramming process could provide the cells with a clean slate, allowing them to be "reborn" when they are re-differentiated into a new lineage.

With the variety of methods now available to generate endothelial cells from fibroblasts-iPSC-derived, converted using partial de-differentiation and converted using the endothelial

transcription factor method proposed by Kim and colleagues - how should one choose the optimal method? The answer is that the method of choice is probably context-specific. For example, partial de-differentiation may be well-suited for generating more than one cell type but still avoiding the fully pluripotent state. Kurian and colleagues used a partial dedifferentiation approach and were able to generate "angioblasts", which could give rise to both endothelial cells as well as smooth muscle cells [8] and therefore used for vascular engineering which requires both vascular cell types. On the other hand, the method of Kim and colleagues may be better suited for direct in vivo reprogramming of fibroblasts.

The field of reprogramming fibroblasts to endothelial cells is at its beginning but the road ahead looks quite promising and exciting. We are only gradually realizing how important it is to study cell fate memory and identity during the dynamic process of reprogramming. As our knowledge of the intricate processes which occur during reprogramming increases, we will not only have greater confidence in the scientific validity of the results obtained with the newly generated endothelial cells, but also in the safety of bringing cellular reprogramming to the bedside of cardiovascular patients.

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