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FIELD OF VISION

Turning scar into muscle

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Abstract

After the demonstration that somatic cells could be reprogrammed to a pluripotent state, exciting new prospects were opened for the cardiac regeneration field. It did not take long for the development of strategies to convert somatic cells directly into cardiomyocytes. Despite the intrinsic difficulties of cell reprogramming, such as low efficiency, the therapeutic possibilities created by the ability to turn scar into muscle are enormous. Here, we discuss some of the major advances and strategies used in direct cardiac reprogramming and examine discrepancies and concerns that still need to be resolved in the field.

Key words: Direct reprogramming; Cardiomyocytes; Induced pluripotent stem cells; Cardiac regeneration; microRNAs

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INVITED COMMENTARY ON HOT ARTICLES

Every cardiologist's dream come true - to transform scar into muscle - seemed closer to becoming real when, in 2010, Ieda *et al*[1] at the Gladstone Institute in California published in the journal *Cell* about the direct conversion of fibroblasts derived from neonatal hearts and tail-tips of mice into cardiomyocyte-like cells using three transcription factors. The group followed the strategy devised by the seminal work of Takahashi *et al*[2], who demonstrated for the first time that somatic cells could be reprogrammed to a pluripotent state through the overexpression of 4 transcription factors. In their original work, Takahashi *et al*[2] used an antibiotic selection gene system to investigate, out of 24 candidates, which transcription factors were critical for the generation of induced pluripotent stem (iPS) cells. Using a similar strategy, Ieda *et al*[1] tested 14 cardiac transcription factors and, by serially withdrawing each factor, they came across a combination of three factors that were necessary and sufficient to induce the reprogramming of fibroblasts into what they called induced-cardiomyocytes (iCM) both *in vitro* and *in vivo*. These three factors were Gata4, Mef2c and Tbx5 (GMT), well known transcription factors activated during cardiogenesis. Major advances that led to this exciting discovery are summarized in Table 1.

The findings of Ieda *et al*[1] were based on the expression of the enhanced green fluorescent protein (eGFP) driven by the α-myosin heavy chain (α-MHC) gene promoter. Therefore, if a fibroblast was converted to an iCM by retroviral transduction with the factors, the cell would become green. Fluorescent activated cell sorting would then allow identification and purification of the iCM. While transducing 14 factors induced only 1.7% of the fibroblasts to become green, GMT transduction

Table 1 Advances in cell reprogramming

Year	Major advances	Ref.
2006	First report to demonstrate that it was possible to reprogram mouse somatic cells to a pluripotent state through the overexpression of 4 transcription factors	[2]
2007	Reprogramming of human cells to a pluripotent state was achieved	[6,7]
2008	First evidence of direct reprogramming of somatic cells from the same organ (pancreatic exocrine cells to beta-cells) without a pluripotency step	[8]
2010	First report of direct reprogramming of somatic cells into an unrelated tissue (tail-tip fibroblasts to neurons)	[9]
2010	Direct reprogramming of somatic cells to cardiomyocytes through the overexpression of 3 cardiac transcription factors was reported	[1]
2012	*In vivo* direct reprogramming of fibroblasts to cardiomyocytes was demonstrated through the injection of viral vectors into infarcted myocardium	[3,4]
2012	First report of direct reprogramming of somatic cells to cardiomyocytes by microRNAs	[5]

resulted in 20% of the cells becoming green. However, expression of eGFP meant that the α-MHC gene promoter was being activated in the transduced cells, not that they had become true cardiomyocytes. Using a second screening round, now examining the expression of cardiac troponin T (cTnT), Ieda *et al*[1] found that only 30% of the green cells were also positive for this cardiac marker. Furthermore, when they compared global gene expression profiles, iCM displayed similar, but not identical, gene expression to neonatal cardiomyocytes. But even though not all reprogrammed cells expressed genes or proteins characteristic of neonatal cardiomyocytes, in those that did, approximately 30% showed intracellular calcium concentration oscillations and fired action potentials after 4-5 wk in culture. In the end, about 1%-2% of the reprogrammed cells were beating iCM. Clearly, the method needed improvement and robustness before one could envisage its application as therapy. In addition, independent confirmation by other laboratories was in order.

The first confirmation that direct reprogramming to a cardiac lineage was possible came from Efe *et al*[10] in 2011. Using the conventional Yamanaka iPS factors (in fact c-Myc was dispensable) plus small molecules that inhibited the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, they were able to show conversion of mouse embryonic fibroblasts into atrial-like cells. This method relied on starting reprogramming into a pluripotent state, just to cut it short by changing culture conditions, and driving a cardiogenic program by using the JAK-STAT inhibitors followed by exposure to BMP4. As mentioned above, all reprogrammed cells were atrial-like, expressing the atrial isoform of myosin light chain and firing action potentials typical of atrial or pacemaker cells. Although clearly distinct from the Ieda strategy[1], Efe *et al*[10] reassured us that turning fibroblasts into cardiac-like cells without passing through the pluripotent state was possible.

In 2012, new data on direct reprogramming into cardiac-like cells appeared in the literature and, as has been frequently the case for stem cells and cell therapies in the heart, reports were controversial. If we direct ourselves firstly towards the transcription factor methodology, Chen *et al*[11], using the lentiviral vectors provided by Srivstava's group, were unable to convert cardiac or tail-tip fibroblasts into iCM. Using three distinct myocardial lineage reporter cells (α-MHC, Nkx2.5 and cTnT promoters coupled to Cre recombinase), they were not able to detect reporter activation in the first two lines (α-MHC and Nkx2.5) in spite of significant overexpression of the GMT factors. In contrast, the cTnT reporter showed robust activation, with 35% of the fibroblasts transduced with GMT expressing the reporter protein (eGFP) and showing over 200-fold higher message levels for cTnT. Nonetheless, expression of other cardiac marker genes was only minimally elevated, indicating a very inefficient reprogramming. In order to discard the absence of cardiac reprogramming due to the lack of an appropriate niche, Chen *et al*[11] transplanted cardiac fibroblasts transduced with GMT into the hearts of infarcted mice. These fibroblasts were obtained from transgenic animals that constitutively expressed luciferase and eGFP, thus allowing the *in vivo* tracking of the cells. Much to their surprise, the authors found that GMT transduced cells survived less than non-transduced ones and, in their majority, expressed fibroblast markers, indicating that conversion to a cardiac-like phenotype was not attained.

In contrast to the results just described, Qian *et al*[3] reported in *Nature* the successful *in vivo* reprogramming of endogenous cardiac fibroblasts to cardiomyocyte-like cells after GMT transduction. It should be noted that the use of a retroviral system to infect the hearts resulted in transduction of the three factors (GMT) to dividing cells only. Therefore, the experiments were performed in mice subject to left anterior descending (LAD) artery ligation, where non-muscle cells, especially fibroblasts, proliferate intensely to form a scar. Using transgenic mice in which the periostin promoter drove either β-galactosidase (β-gal) or enhanced yellow fluorescent protein (eYFP), the authors were able to track the cells that were converted into iCM after GMT. Since periostin is preferentially expressed in fibroblasts and these comprise about 50% of the heart cells, the fibroblasts converted to iCM could be identified by either blue (β-gal) or yellow (eYFP) staining. Performing additional staining for cardiac markers, such as α-actinin, tropomyosin, α-MHC and cTnT, Qian *et al*[3] showed that approximately 35% of the myocytes in the border/infarct zone were double-labeled by cardiac markers and β-gal, indicating their origin as fibroblasts that turned into iCM. The iCM isolated from the border region of infarcted hearts were shown to couple to native cardiomyocytes, to propagate calcium waves, and to fire action potentials and contract after stimulation. Furthermore, in mice that were injected with GMT after acute myocardial infarc-

tion, ejection fraction, stroke volume, cardiac output and scar size were significantly improved when compared to infarcted animals in which only dsRed (a dye) was injected. The authors thus concluded that the improvement in cardiac function was a consequence of the formation of new cardiomyocyte-like cells, the iCM. Of note, the percentage of iCM formed from the total GMT-infected population remained at 12%, even after using adjuvant molecules (in their report, thymosin β4).

In support of Qian *et al*[3] findings, both *in vivo* and *in vitro*, Song *et al*[4] reported in *Nature* the reprogramming of adult tail-tip and cardiac fibroblasts using four transcription factors, namely Gata4, Mef2c, Tbx5 and Hand2 (GMTH). In these experiments, using the three Srivastava's factors (GMT) induced 2.9% iCM and the addition of Hand2 increased that percentage of successful reprogramming to 9.2%. As in the Ieda *et al*[1] paper, the reprogrammed fibroblasts were shown to exhibit different degrees of reprogramming with variable expression of cardiac proteins and distinct levels of sarcomere organization. Using the same approach of targeting dividing cells with retroviral vectors, Song *et al*[4] were able to show that iCM were generated in hearts of mice subject to LAD ligation after intracardiac injection of GMTH. iCM isolated from these hearts and identified by lineage tracing exhibited calcium transients, fired action potentials and contracted (with different degrees of maturity). As described by Qian *et al*[3], the injection of GMTH into infarcted hearts led to significant increases in stroke volume and ejection fraction compared with infarcted animals injected with eGFP retroviruses alone. Scar area was also significantly decreased in the GMTH-treated animals.

A different approach was taken by Jayawardena *et al*[5] using a combinatorial strategy to identify microRNAs (miRs) able to reprogram fibroblasts into cardiac-like cells *in vitro* and *in vivo*. Using reverse transcription polymerase chain reaction for identification of cardiac gene up-regulation, a cardiac promoter (α-MHC) coupled to a reporter gene [cyan fluorescent protein (CFP)], and transgenic mice allowing lineage tracing of cardiac fibroblasts, they transiently transfected distinct combinations of 6 synthetic miRs based on their role in cardiac development into cardiac fibroblasts. The combination of miR-1, -133, -208 and -499 was able to induce reprogramming of the fibroblasts to cardiac-like phenotype. As with the transcription factors described above, the degree of reprogramming seemed to be variable and partial. Addition of an inhibitor of JAK I increased reprogramming efficiency 3-fold, reaching up to 27% of cells expressing the cyan fluorescence in fibroblasts derived from α-MHC-CFP hearts. Injection of a lentivirus with the four miRs into the hearts of double-transgenic mice expressing red tomato under control of the FSP promoter after LAD ligation allowed identification of double-labeled cells, expressing the red tomato protein and cTnT. Quantification of labeled cells in the infarcted hearts indicated up to 1% of reprogramming when using the four miRs, but functional evaluation was not performed in these experiments.

Taken together, these results open exciting new therapeutic possibilities for therapy of cardiac diseases; however, several concerns remain unresolved. First, it seems to be generally accepted that efficiency of *in vitro* direct reprogramming into the cardiac lineage is low. In this context, it needs to be sorted out whether the discrepancies in Ieda's and Chen's work can be explained by differences in cell types, reporter systems, transduction efficiency, levels of GMT expression or in methods for evaluating reprogramming. In addition, as has been demonstrated for iPS cells[12], transcription factor stoichiometry can be critical for the reprogramming process and may be very difficult to control. Nonetheless, it is also important to point out that, even though many strategies could be used to improve reprogramming efficiency *in vitro*, such as the addition of small molecules and the combination of different approaches (transcription factors and miRs), it may be an inherently inefficient process due to the low turnover of cardiomyocytes[13]. In the case of *in vivo* experiments, the consequences of partial reprogramming will have to be assessed from a safety standpoint and experiments in larger animal models will be required. Additionally, the retroviral approach in myocardial infarction models might overcome the *in vitro* efficiency concern, but it raises other issues, especially related to the potential risks of vector integration into DNA. It would be ideal if efficient non-integrating methods of reprogramming could be developed for therapeutic purposes. Indeed, these are thrilling times for the cardiac regeneration field.

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