Editorial

Stem Cell INVESTIGATION

SMAD2/3, versatile molecular tools for cellular engineering

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In 2006, the research group directed by Shinya Yamanaka showed for the first time the possibility to reprogram mouse fibroblasts into induced pluripotent stem cells (iPSC) with properties similar to those of embryonic stem cells (ESC) by forced expression of the transcription factors Oct4, Sox2, Klf4 and c-Myc, since then termed Yamanaka factors (1). After this first reprogramming report, these researchers and other groups have successfully used the same factors, or other combinations, to reprogram a wide range of mouse or human somatic cells into iPSC (2-4). Subsequently, a direct conversion of somatic cells with defined characteristics into cell lineages with different functions (also termed direct lineage reprogramming) was achieved using predominantly pioneer/cell-type specific transcription factors playing key roles in the embryonic specification of the cell fate of interest during development (5). During direct conversions, the transition from one functional cell type to another is thought to be executed without passing through an intermediate pluripotent state. Although tumorigenic risks are associated with cells obtained either by reprogramming somatic cells into iPSC or by direct cell conversion, the bypass of the pluripotent state limits the risks of teratoma formation. Since the original iPSC generation report, great advancements and refinements of the experimental procedures have been made to circumvent some issues raised by the reprogramming processes (6). Currently, the reprogramming of virtually any cell type is possible using different combinations of transcription factors, epigenetic regulators, microRNAs and/or small molecules (7).

Reprogrammed cells are now an invaluable tool for *in vitro* disease modelling, high-throughput screens for toxicity tests and drug discovery, and reprogramming raises the possibility for the derivation of patient-specific autologous cells for therapeutic purposes (8). However, the translational potential of these cells is still hampered by the inefficiency of the reprogramming processes, the frequent incomplete cell-fate specification of the reprogrammed cells which may have remnants of the original gene expression pattern and cell function, and *de novo* mutations occurring during the process (6).

Amongst the signalling pathways with essential roles in embryonic development, ESC self-renewal and differentiation, the TGF-β family members ACTIVIN and NODAL are particularly important, acting mostly through the activation of downstream specific transcriptional effectors SMAD2 and SMAD3 (9). ACTIVIN/NODAL instructive roles for embryonic development and complex cell fate decisions are achieved through a precise dosage of their expression levels, an intricate network of interactions with other signalling pathways resulting in the balanced regulation of target genes dependent on SMAD2/3 (9-20). The selective and robust binding of SMAD2/3 to specific loci across the genome and/or their transcriptional activity relies on interactions with other transcriptional factors and epigenetic modulators.

A recent report has established an unprecedented role for SMAD2/3 which may act as general potentiators for human and mouse iPSC reprogramming, and for direct cell lineage conversion, when expressed as constitutively active forms with the adequate reprogramming transcription factors (21). Indeed, constitutively active SMAD2/3 cooperated with Yamanaka factors to accelerate and increase the emerging

number of colonies expressing NANOG. Surprisingly, the authors showed that the endogenous SMAD2/SMAD3 are dispensable for mouse embryonic fibroblasts (MEF) conversion into iPSC by Yamanaka factors (21). Constitutively active SMAD3 only amplified the expression of genes pre-activated by Yamanaka factors, and interacted with OCT4 and SOX2, central pioneer transcription factors of the Yamanaka factors (21). Constitutively active SMAD3 was present at OCT4-targeted genes during reprogramming and promoted the recruitment of histone modifiers and chromatin remodelers to facilitate the switch of gene expression pattern during reprogramming. The ACTIVIN/NODAL signalling pathway, through the activation of SMAD2/3, was previously reported to be critical for self-renewal of human ESC and mouse epiblast-derived stem cells (EpiSC) by a direct regulation of NANOG expression (14,15,18,22,23). Human ESC and mouse EpiSC are primed pluripotent stem cells with distinct properties from those of mouse naïve ESC which are derived from the inner cell mass of the blastocyst. Primed human ESC and mouse EpiSC express the core pluripotency-related transcription factors (such as NANOG, OCT4 and SOX2) concomitantly with genes important to drive differentiation (such as *T/BRACHYURY, SOX17*, *GATA6* and *FGF5*, amongst other genes), and display no expression of genes specifically associated with the metastable naïve pluripotency state (such as *ZFP42/REX1*, *DPPA5*, amongst other genes). Apart from its role in the maintenance of pluripotency, ACTIVIN/NODAL-SMAD2/3 pathway in cooperation with WNT and BMP signalling pathways also actively drives the specification of pluripotent stem cells to mesendoderm cells and prevents the neuroectodermal default differentiation process of these cells (13,14,24). These observations imply that SMAD2/3 activation and function are precisely controlled in pluripotent stem cells, and minor changes to the established balance of active SMAD2/3 may have an impact on cell-fate or cell properties. In the report by Ruetz *et al.* (21), the changes in gene expression promoted by the constitutively active SMAD3 co-expression with Yamanaka factors have indicated a faster reprogramming process, but they do not allow to predict the final cell fate outcome upon completion of the reprogramming process. It would be of interest to evaluate the full potential of constitutively active SMAD2/3 to achieve the complete reprogramming of somatic cells both to naïve and primed iPSC, and test their full differentiation potential to assess unpredictable bias towards specific cell fates.

A significant finding reported by the authors is that constitutively active SMAD2/3 cooperates with a variety of cell-type transcription factors to promote distinct direct cell conversions, suggesting that constitutively active SMAD2/3 may act as general potentiators of transcription factor-triggered cell reprogramming (21). Indeed, constitutively active SMAD2/3 cooperated with CEBPα to convert from mouse B lymphocytes into macrophages, with CEBPα and PRDM16 to convert mouse myoblast cells into adipocytes, and with ASCL1, BRN2A, MYTII and NEUROD1 to generate mature neurons from human fibroblasts (21).

Together, the positive results obtained in cell reprogramming mediated by the forced co-expression of adequate transcription factors and constitutively active SMAD2/3 paves the way for further exploration of the potential of SMAD2/3 for direct conversion of somatic cells into other cell types yet not tested. It would be of interest to assess the potential of proteins interacting with SMAD2/3 and/or modulating their function to improve of the efficiency and specificity of the conversion processes, in combination with activated SMAD2/3 and the appropriate reprogramming factors. In particular, factors known to be critical for the maintenance of pluripotency or the function of the desired cell type to be reprogrammed. For instance, the SMAD2/3 co-repressors SNON (SKIL) and SIP1 which contribute to the maintenance of human ESC pluripotency and mouse EpiSC by inhibiting the transcriptional activity of SMAD2/3 at mesendodermal gene promoters (13,17). Interestingly, the expression of SNON itself in human ESC is under the conjoint control of NANOG, OCT4, SOX2 and SMAD2/3 (13,17). CITED2 is another interesting SMAD2/3 co-activator with potential to synergize with these proteins in reprogramming processes (25). Indeed, CITED2 is important for mouse ESC self-renewal through the regulation of NANOG expression, and for cardiac differentiation (26,27). Moreover, CITED2 co-expression with Yamanaka factors was reported to accelerate and increase the efficiency of iPSC generation (27,28). Additionally, the co-expression of CITED2 with Yamanaka factors reduced the variability of the expression of some core pluripotency-related genes, particularly if reprogramming pre-senescent MEF which are refractory to the process (27,28). Thus, the combined co-expression of constitutively active SMAD2/3 with the Yamanaka factors, and CITED2, SNON or/and SIP1 may even hold a greater potential for a rapid and efficient cell reprogramming than just SMAD2/3 in combination with Yamanaka factors.

The original idea by Shinya Yamanaka to reprogram somatic cells into pluripotent stem cells (1), by forced

expression of factors playing critical roles in the maintenance of ESC identity and functions, has established solid fundaments for cell line reconversion. However, this powerful method still presents associated concerns that need to be addressed to efficiently and reliably originate cell-types of interest. As described recently for constitutively active SMAD2/3 (21), other transcription factors, epigenetic regulators, microRNAs and/or small molecules have been shown to cooperate, complement or substitute Yamanaka factors for reprogramming. This recurrent revisitation of Shinya Yamanaka's pioneer work by researchers worldwide is constantly improving cell engineering processes and bringing reprogrammed cells closer to safe clinical applications.

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Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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