Interplay of Oct4 with Sox2 and Sox17: a molecular switch from stem cell pluripotency to specifying a cardiac fate

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Oct4 exerts a dose-dependent dual action, as both a gatekeeper for stem cell pluripotency and in driving cells toward specific lineages. Here, we identify the molecular mechanism underlying this dual function. BMP2- or transgene-induced Oct4 up-regulation drives human embryonic and induced pluripotent stem cells to become cardiac progenitors. When embryonic stem cell pluripotency is achieved, Oct4 switches from the Sox2 to the Sox17 promoter. This switch allows the cells to turn off the pluripotency Oct4-Sox2 loop and to turn on the Sox17 promoter. This powerful process generates a subset of endoderm-expressing Sox17 and Hex, both regulators of paracrine signals for cardiogenesis (i.e., Wnt, BMP2) released into the medium surrounding colonies of embryonic stem cells. Our data thus reveal a novel molecular Oct4- and Sox17-mediated mechanism that disrupts the stem cell microenvironment favoring pluripotency to provide a novel paracrine endodermal environment in which cell lineage is determined and commits the cells to a cardiogenic fate.

Introduction

Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst or induced pluripotent stem cells (iPSCs) derived from reprogrammed somatic cells retain the ability to self-renew in culture and the potential to differentiate into any cell lineage. The ESC-specific protein, Oct4, is one of the most ancient transcription factors (TFs) and is expressed very early during embryonic development (Smith, 1991). In mouse ESCs, together with Sox2 and Nanog, Oct4 cooperatively maintains pluripotency through a tightly regulated transcriptional loop (Loh et al., 2006; Niwa, 2007). However, a new function of this TF has recently emerged in the process of cell lineage determination. In fact, the level of Oct4 in ESCs determines its dual function (Niwa et al., 2000). The mechanism of that process remained unknown. We demonstrated in vitro and in vivo a gene dosage-dependent function of Oct4, driving the ESC or the epiblast toward a mesodermal cardiogenic fate (Zeineddine et al., 2006).

However, the genetic and epigenetic mechanisms underlying the dual function of Oct4, maintaining pluripotency or inducing lineage progression, has remained puzzling. Furthermore, whether this concept applies to human pluripotent cells is also questionable.

Results and discussion

We chose the cardiac lineage, determined as early as within the epiblast (Tam and Schoenwolf, 1999), to analyze the dual function of Oct4. First, we tested whether BMP2-induced cardiac commitment and differentiation of HESC (Tomescot et al., 2007) was associated with modulation of Oct4. Expression of both human Oct4 isoforms, Oct4-iA, maintaining cell pluripotency (Lee et al., 2006), and Oct4-iB of still unknown function, was monitored by real-time quantitative PCR (RT-Q-PCR). Oct4-iA was up-regulated threefold in response to BMP2. Expression of Oct4-iB remained unchanged (Fig. 1 A). We then used a HESC line harboring a reporter gene (HUES-9 pOct4/GFP, Oct4-Sox2 loop and
Oct4p driving EGFP) (Fig. 1 B, inset) to investigate BMP2-mediated regulation of the Oct4p. First, to ensure that GFP was under the tight control of the Oct4p, the HUES-9 pOct4/GFP cell line was nucleofected with COUP-TFI, a repressor of Oct4 (Ben-Shushan et al., 1995). GFP was switched off in cells expressing COUP-TFI, as shown by poor GFP distribution within colonies when compared with a fully green mock colony (Fig. S1 A). Furthermore, overexpression of Oct4-iA in the HUES-9 pOct4/GFP line increased GFP expression as expected from regulation of Oct4p by its own protein (Fig. S1 B). Stimulization of this line with BMP2 up-regulated GFP mRNAs (Fig. 1 B) and FACS revealed a 70% increase in the highly positive GFP population (Fig. 1 C).

To investigate whether a rise in Oct4-iA induces loss in cell pluripotency and promotes cell lineage specification, HESCs were nucleofected with Oct4-iA, or EGFP cDNA (mock cells). Cells were cultured for 4 d with FGF2. Oct4-iA-OEC (35 times), twofold more protein (Fig. 1 E), did not change expression of Oct4-iB (Fig. 1 E). Indeed, only the 48-kD band (Oct4-iA), but not the 35-kD band (Oct4-iB), was increased (Fig. 1 E, inset). SSEA-1 was first used as an early marker of HESC loss of pluripotency (Hoffman and Carpenter, 2005). Less than 1% of mock cells (EGFP nucleofected) but 68% of Oct4-iA nucleofected cells expressed SSEA-1 (Fig. 1 D). This percentage (68%), normalized to the one of cell nucleofection assessed by scoring GFP+ cells (42%) (Fig. 1 D, inset), suggests that Oct4-iA-OEC as well as neighboring cells had entered a differentiation program. To examine which lineage was induced by Oct4, gene expression was monitored by Q-PCR. Oct4-iA-OEC expressed both mesodermal (Brachyury, Tbx6) and cardiac (Mesp1/2, Tbx20, Tbx5, Mef2C, Nkx2.5, cardiac α-actin) markers (Fig. 1 E). Endodermal genes Hex and Sox17 were also switched on by Oct4-iA (Fig. 1 F). Furthermore, the corresponding proteins Sox17, Nkx2.5, and Mef2c were present (Fig. 1 G). High content cell (HCC) imaging assay allowed us to monitor more than 2,500 cells and revealed that 48% of cells expressed Sox17, indicating that all nucleofected cells expressed the protein (when normalized to percentage of Oct4-iA nucleofection 42%, Fig. 1 D). Up to 20% (i.e., 48% of nucleofected cells) expressed Mef2c, and 31% (74% of nucleofected cells) Nkx2.5 (Fig. 1 G). Very low expressions of the hematopoietic CD45, of the endothelial CD31, CD34 markers were detected (Fig. S1 C). Thus, PCR, immunofluorescence, and FACS data demonstrate that up-regulation of Oct4αA forces HESC to exit pluripotency to adopt a cardiovascular fate. Furthermore, the cardiogenic action of Oct4αA was translated into a full cardiac differentiation program. While mock-nucleofected cells gave rise to cardiomyocytes expressing actinin and cardiac troponin T (cTnT), the later proteins were not yet organized in sarcomeric units 4 wk after embryoid body (EB) formation.

In contrast, EBs generated from Oct4-iA-OEC featured 3.1 ± 0.2 (n = 4) as many cTnT+ and actinin+ myocytes as in mock EBs, as assessed by measuring the anti-cTnT and anti-actinin fluorescent areas within whole EBs. Furthermore, the cardiomyocytes were assembled in strands and featured organized and adult-size sarcomeres (2 μm) (Fig. 1 H). Next, we hypothesized that, to trigger specification to a cell lineage, increased Oct4 should disrupt the Sox2/Oct4 pluripotency loop. A potential candidate to replace Sox2 as an Oct4 target, namely Sox17, recently reported as essential for early cardiogenesis (Liu et al., 2007), was expressed in Oct4-iA-OEC (Fig. 1, F and G). We therefore tested the presence of Oct4 on Sox17. ChiP assays designed to pull down Oct4-bound chromatin revealed that BMP2 led to a 10-fold decrease in enrichment of DNA fragments containing the Sox2 distal enhancer (Chew et al., 2005) and a 300-fold increase in the enrichment of DNA fragments containing a region of the Sox17p within the 700-bp upstream of the transcription start site (Fig. 2, A and B). This result obtained in the HUES-24 line was confirmed in two other HESC lines (H9.2 and I4). This was accompanied with induction of Sox17 in BMP2-treated cells (Fig. 2 B, right inset). Oct4-iA-OEC featured a loss in Oct4-iA binding Sox2 DNA including the distal enhancer and a gain in binding Sox17p (Fig. 2, C and D). In line with these data, ChiP from Oct4-iA-OEC using anti-modified-H3 antibodies revealed significant changes in methylation and acetylation of H3 associated with both Sox2 and Sox17 enhancer and promoter, respectively, when compared with mock cells (i.e., GFP nucleofected). Although the ratio of methylations of K4 versus that of K27 of H3, as well as acetylation of H3K9 associated with Sox2 distal enhancer was decreased, these were increased for the Sox17p. This pointed to repression of Sox2 and activation of Sox17p, respectively (Fig. 2 E). Furthermore, the Oct4/Sox2 interaction in control was lost upon up-regulation of Oct4-iA (Fig. 2 F). These transcriptional and epigenetic events led to Sox17 expression in BMP2-stimulated HESC (Fig. 2 G). A similar effect was observed in Oct4-iA-OEC (Fig. 1 G). To gain more insight into the molecular mechanism of the switch of Oct4-iA from the Sox2p to the Sox17p, we manipulated the level of expression of Sox2 in Oct4-iA-OEC, surmising that a stoichiometric competition occurs between Sox2 and Sox17. A displacement of Oct4/Sox2 complex on Sox2 distal enhancer would permit Oct4 binding to Sox17p, turning on the gene and the protein, which would reinforce Sox17 expression by forming an Oct4/Sox17 complex on its promoter. When Sox2 was up-regulated in cells nucleofected with the cDNA (Fig. S1 D), the gain in enrichment of Oct4-iA on the Sox17p was lost. When Sox2 was down-regulated using a shRNA (Chew et al., 2005) (Fig. S1, D and E), Oct4-iA binding to the Sox17p was dramatically increased (Fig. 2 H). We next looked for Oct4–Sox17 interaction in END-2 cells, an endodermal cell line expressing Sox17 (Mummery et al., 1991). Using coimmunoprecipitation after overexpression of Oct4-iA in this cell line, we found that Sox17 binds Oct4 (Fig. 2 I). Altogether, our findings point to Sox17, a novel target of Oct4 also interacting with it, to compete with Sox2 to switch HESC from a pluripotent toward an endodermal/mesodermal fate. To clarify whether Sox17 accounts for the switch of HESC from pluripotency to mesodermal and cardiac commitment of ESC, we used a shRNA approach. Sox17 ShRNA was nucleofected in HESC together with Oct4-iA cDNA. Sox17 mRNA was then reduced in the presence of an increased level of Oct4. The protein was also three times less induced by Oct4 overexpression (Fig. S1 F). The amount of Sox17 upon Oct4-iA up-regulation was thus too limited to significantly trigger cardiogenesis (Fig. 3 A). Similarly, BMP2, which induces Sox17 together with mesodermal and cardiac genes, could no longer induce these genes in the presence of...
Figure 1. Overexpression of Oct4-iA induces cardiogenesis. HUES-24 (A) or HUES-9 pOct4/GFP (B) control or treated for 48 h with BMP2. (A) Oct4 isoforms Q-PCR. (B) Q-PCR of GFP cDNA (n = 2–3). (C) GFP FACS in HUES-9 pOct4/GFP cells (top) nontreated (left) or treated (right) with BMP2. (D–F) Cells nucleofected with Oct4-iA or pCMV-GFP and cultured for 4 d. (D) FACS of GFP (inset = nucleofection efficiency) and SSEA-1 in mock cells (pcDNA backbone, left) and Oct4-iA-OEC (right). (E) Q-PCR of induced genes. (F) Q-PCR of Sox17 and Hex (amplicons on gel) (means ± SEM [n = 3–6] normalized to huGAPDH). *, Statistically significant (P ≤ 0.01). (G) Immunofluorescence of anti-Sox17, -Mef2c, and -Nkx2.5 of Oct4-iA OEC scanned by an arrayscan. Bars, 20 µm. (H) Immunofluorescence of anti-sarcomeric actinin and anti-cTnT of d-30 EBs generated from mock or Oct4-iA OECs.
Sox17 ShRNA (Fig. S1 G). However, Sox17 shRNA did not prevent the loss of Sox2 DNA elements bound to Oct4-iA when overexpressed. Interestingly, under the same condition (i.e., Sox17 silencing), the Sox17p was no longer occupied by Oct4-iA (Fig. 3 B). Thus, collectively, these ChIP experiments (Figs. 2 and 3) showed the occupancy of Sox17p by Oct4. They further revealed
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Oct4-Sox2 ratio, it targets the Oct4–Sox2 enhancer, maintaining Sox proteins and/or DNA elements. At a normal level of Oct4 or complex with Sox17. We can further surmise that the Oct4/Sox2 between both Soxs as shown by the capability of Oct4 to form aing the levels of Sox2 and Sox17 further pointed to a competition methylation, and a decrease in K9 acetylation of H3). Manipulat-

hancer (loss of Oct4 occupancy of the enhancer, increase in K27 expressing an active chromatin state. This leads to gene and protein expression. The opposite scenario occurs on the ing the presence of 150 ng/ml Dkkp (Dkk), 100 ng/ml Noggin, or 10 ng/ml Lefty to prevent Wnt, BMP2, or Nodal signaling, respectively. Fig. 4 C shows that both Dkk and Noggin, but not Lefty, prevented Oct4-iA–induced expression of Brachyury, Mesp, Tbx20, Tbx5, Tbx6, and Nkx2.5. Challenging HESC with BMP2 (10 ng/ml) together with Wnt3a (50 ng/ml) significantly improved the effect of BMP2 on early cardiac gene expression as well as on BMP2 and Wnt3a expression (Fig. S2 C). In line with these data, Oct4-iA-OEC expressed BMP2 and Wnt3a (Fig. 4 D). Thus, BMP2 and Wnt3a are the cardiogenic factors released by Oct4-iA–induced Sox17+ cells. Four other findings confirmed this claim. First, phosphorylated Smad recognized by an anti-

phosphoSmad1,5,8 antibody was barely detectable in mock cells cultured alone, but observed into nuclei of Oct4-iA-OEC in wells in contrast to the membrane staining observed in mock cells (Fig. S3 B). Finally, both anti-BMP2 and anti-Wnt3a antibodies added for 2 d in the medium of Oct4-iA-OEC prevented expression of genes marking the mesoderm (Tbx6), the cardiogenic mesoderm (Mesp1), and cardiac progenitors (Tbx5) (unpublished data). To further investigate whether cells expressing Sox17 exerted their cardiogenic action through an autocrine and/or paracrine mechanism, HUES-9-pOct4/GFP cells were plated in microwells and stimulated for 4 d with BMP2. Cells expressing GFP were stained for Sox17 and Mesp1/2, the earliest cardiac marker. Cells not challenged with BMP2 did not express Sox17 or Mesp1/2 (Fig. 4 E, top). Cells challenged with BMP2 expressed both Sox17 and Mesp1/2. Careful observation of cells revealed a predominance of K4 over K27 methylations, and an increase in K9 acetylation on H3 in the vicinity of the promoter, thus favor-

ing the presence of 150 ng/ml Dkkp (Dkk), 100 ng/ml Noggin, or 10 ng/ml Lefty to prevent Wnt, BMP2, or Nodal signaling, respectively. Fig. 4 C shows that both Dkk and Noggin, but not Lefty, prevented Oct4-iA–induced expression of Brachyury, Mesp, Tbx20, Tbx5, Tbx6, and Nkx2.5. Challenging HESC with BMP2 (10 ng/ml) together with Wnt3a (50 ng/ml) significantly improved the effect of BMP2 on early cardiac gene expression as well as on BMP2 and Wnt3a expression (Fig. S2 C). In line with these data, Oct4-iA-OEC expressed BMP2 and Wnt3a (Fig. 4 D). Thus, BMP2 and Wnt3a are the cardiogenic factors released by Oct4-iA–induced Sox17+ cells. Four other findings confirmed this claim. First, phosphorylated Smad recognized by an anti-

phosphoSmad1,5,8 antibody was barely detectable in mock cells cultured alone, but observed into nuclei of Oct4-iA-OEC in wells including 50% (i.e., nucleofection efficiency) of Oct4-iA-OEC and 50% of non-nucleofected cells (Fig. S3 B). Second, anti-

Smad4 ChIP experiments revealed a binding of Smad to both the Nkx2.5 and Tbx6 promoters, two targets of BMP2 (Fig. S3 A) in Oct4-iA-OEC. Third, Wnt3a signaling pathway was activated in Oct4-iA-OEC, as visualized by nuclear localization of β-catenin in contrast to the membrane staining observed in mock cells (Fig. S3 B). Finally, both anti-BMP2 and anti-Wnt3a antibodies added for 2 d in the medium of Oct4-iA-OEC prevented expression of genes marking the mesoderm (Tbx6), the cardiogenic mesoderm (Mesp1), and cardiac progenitors (Tbx5) (unpublished data). To further investigate whether cells expressing Sox17 exerted their cardiogenic action through an autocrine and/or paracrine mechanism, HUES-9-pOct4/GFP cells were plated in microwells and stimulated for 4 d with BMP2. Cells expressing GFP were stained for Sox17 and Mesp1/2, the earliest cardiac marker. Cells not challenged with BMP2 did not express Sox17 or Mesp1/2 (Fig. 4 E, top). Cells challenged with BMP2 expressed both Sox17 and Mesp1/2. Careful observation of cells revealed

Figure 3. Sox17 mediates Oct4-triggered cardiogenic commitment of HESC. Oct4-iA cDNA alone or together with Sox17 ShRNA was nucleo-

fected in HESC (A) Q-PCR of genes (n = 3). (B) Anti-Oct4ChIP-Q-PCR analy-

sis of Sox2 distal enhancer and Sox17p (n = 3). (B) Anti-Oct4ChIP-Q-PCR analy-

sis of Sox2 distal enhancer and Sox17p (n = 3). Mean ± SEM.

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Figure 4. Overexpression of Oct4-IA induces secretion of paracrine factors. (A) Q-PCR of genes (n = 3–5). (B) Anti-Oct4 ChIP analysis of Sox2 distal enhancer and Sox17p (pSox) in wild-type HESC treated for 4 d with CM of Oct4-IA-OEC. CM from GFP-nucleofected mock cells was used as control (experiment in duplicate). (C) HESC nucleofected with Oct4-IA or GFP (mock cells) and cultured for 4 d in the absence or presence of DKK, Noggin, or Lefty. (D) Q-PCR of BMP2 and Wnt3a in Oct4IA-OEC. (E) anti-Sox17 and -Mesp2 immunofluorescence of Ctrl and BMP2-treated HUES-9 pOct4/GFP cells. (F) Venn diagram for the number of cells positive for GFP, Sox17, and Mesp2 alone or together. Ctrl or BMP2-challenged HUES-9 pOct4/GFP cells in 96-well plates were stained with anti-Sox17 and -Mesp2 antibodies. Numbers in the diagram indicate the total number of cells and the percentage.

* Significantly different from control (P ≤ 0.001).
that a few BMP2-stimulated cells expressed both Sox17 and Mesp1/2, whereas most of them expressed only Sox17 or Mesp1/2. This differential expression of Sox17 and Mesp1/2 was observed in the same or neighboring cell colonies. To further quantify the phenomenon, we set up a high-content imaging assay. 32 wells and 50 fields per well were scanned for expression of GFP, Sox17, and Mesp1/2. Cells expressing GFP, Sox17, Mesp1/2 alone or expressing two or three markers were separately scored. The Venn diagram (Fig. 4 F) first revealed that ~50% of cells responded to BMP2, as 44% did not express Sox17 and/or Mesp1/2. Half of responder cells (33% of total) expressed Sox17 and the other half (26% of total cells) expressed Mesp1/2. The diagram clearly shows that most of the cells expressed only Sox17, with many still highly GFP+ or only Mesp2 having lost GFP. A minority of the cells expressed Sox17 and Mesp1/2; almost no cell expressed the three markers. Although 40–50% cells were nucleoeffected and overexpressed Oct4-iA, 40–60% also expressed several markers including MeF2c and Nkx2.5, which together confer to the cells a cardiac fate (Fig. 1 G), further pointing to a robust paracrine effect of the latter. These findings suggest that the dual function of Oct4 is mediated by a paracrine phenomenon mediated by both BMP2 and Wnt3a. This claim is supported by three series of experiments: first, inhibitors or blocking antibodies, suggesting the presence in the medium of Sox17 expressing cells of both Wnt, known to be a target of Sox17 (Zorn et al., 1999) and BMP2, which cooperates with Wnt pathway (Crease et al., 1998; Kasai et al., 2005; Sumi et al., 2008); second, the BMP2 and Wnt signaling pathways were both activated in wells containing Oct4-iA-overexpressing cells, as revealed by intranuclear localization of both p-Smad1,5,8 and β-catenin in these cells together with the binding of Smad4 to the Nkx2.5p and Tbx6p; and third, expression of BMP2 and Wnt3a mRNAs by Oct4-OEC. Indeed, both Wnt and BMP2 are well-known cardiogenic factors (Ménard et al., 2004; Liu et al., 2007). Wnt exerts a cardiogenic action early on during embryogenesis to establish the primitive streak/mesendoderm and then BMP2 together with Wnt drive the cells toward the posterior primitive streak and mesoderm (Bakre et al., 2007; Sumi et al., 2008). Furthermore, Wnt in the absence of Activin does not promote the hematopoietic lineage (Nostro et al., 2008) as well as Sox17 prevents the development of ectoderm (Séguin et al., 2008).

Then, BMP2 takes over the cardiogenic task. Wnt becoming a cardiogenic inhibitor (Naito et al., 2006). The cardiogenic factors released by Oct4-induced Sox17+ cells may exert their action in an autocrine, and/or in a paracrine manner on neighboring Sox17-negative cells driving them from an undifferentiated to a mesodermal cardiogenic fate. Using HCC imaging to monitor expression of Sox17 and Mesp2 in BMP2-induced Oct4-iA-OEC revealed that one third of Oct4-iA-OEC expressed Sox17 as expected from a direct targeting of Sox17 by the POU factor, whereas many cells expressing Mesp2 did not express any longer Oct4 or Sox17. This observation argues more for a paracrine effect, although we cannot exclude a kinetic effect, cells going through a endo/mesendodermal stage (Sox17+) and then to the cardiogenic mesoderm (Mesp1/2+) turning off Sox17 in line with the inhibitory effect of Mesp1/2 on Sox17 (Bondue et al., 2008).

Our data thus revealed a novel Oct4/Sox17-mediated molecular mechanism that disrupts the microenvironment that favors cell pluripotency and prevents differentiation, to provide a novel paracrine environment more prone to commit the cells toward a cardiac lineage. iPSCs are derived from somatic cells reprogrammed using Oct4, Sox2, Nanog, and Lin28 or Myc (Takahashi et al., 2007). We wondered whether manipulating the Oct4-iA level in such cells could also drive them through the Sox2/Sox17 switch toward a cardiogenic fate. Thus, iPSCs were nucleoeffected with Oct4-iA cDNA and both gene expression and Sox17p occupancy were monitored by Q-PCR. Fig. S3 C shows that 5 d after nucleoeffection, iPSC expressing 10 times more Oct4-iA mRNA than GFP nucleoeffected cells changed of morphology, displaying more flat and irregular colonies in comparison with compact colonies in mock cells expressing GFP. These cells expressed mesodermal and cardiac markers including Brachyury, Mesp1/2, Tbx20, Tbx5, and Nkx2.5, as well as Sox17 (Fig. S3 D). Oct4-iA switched from the Sox2 to the Sox17p (Fig. S3 E). This was associated with up-regulation of Sox17 (Fig. S3 D, inset) and of both Wnt3a and BMP2 mRNAs (Fig. S3 F).

Thus, we have found a novel molecular and cellular circuit in which Oct4 and Sox17 work in combination to drive human pluripotent stem cells toward an endodermal or mesendodermal fate, and in turn toward the fate of cardiac progenitors. This circuit is functional within an ESC colony or "niche", BMP2 acting first in cells surrounding the colony, likely generating a gradient from the outer to the inner part of colonies rather secreting factors such as GDF3 maintaining a basal level of Oct4 and cell pluripotency (Peerroani et al., 2007). Then, mesen/endo/dermal Sox17+ cells amplify the differentiation process by secreting cardiogenic factors (i.e., Wnt3a, BMP2), further acting on the colonies to direct the cells toward a cardiogenic fate (Fig. 5). This molecular and cellular circuitry is likely to occur during the process of somatic cell reprogramming using Oct4 as the main reprogramming inducer, pointing to the importance of an accurate Oct4/Sox2 dosage in these experiments. Oct4 dosage within the nucleus is thus crucial to build specific bridges between different (i.e., Sox2 or Sox17) chromatin domains, resulting in generation of “transcription factories” working either to maintain ES cell pluripotency or to drive them toward a cardiogenic fate.

Materials and methods

Culture and cardiac commitment of human pluripotent stem cells

Two HESC lines and one HiPSC line 11 (Maherali et al., 2008), using human dermal fibroblasts infected by lentivirus harboring the cDNAs encoding Oct4, Sox2, Lin 28, Klf4, and Nanog, were used throughout this study without any difference in results. HUES-24 and HUES-9 pOct4/GFP [a transgenic cell line generated using BAC] and iPS cell were cultured on mouse embryonic fibroblasts [MEFs] prepared from 14 mouse embryos as described previously (Tomescot et al. 2007). HESC were treated for 48 h with 10 ng/ml BMP2 in the presence of 1 μM SU5402, an FGF receptor inhibitor, in KOSSR-DMEM. HESC [both HUES-9 and HUES-24] were used within no more than 10 passages [P28-P38]. Cells are phenotyped every 10 passages using anti- SSEA-3/4, TRA-1-60, and TRA-1-80 antibodies [Millipore]. Less than 5% of cells were positive for SSEA-1 (Millipore). Karyotype was found normal and stable in the course of the experiments. EBs were generated from mock [GFP]-nucleoeffected or Oct4-iA-nucleoeffected in DMEM added with 20% FCS. Cells were let in suspension for 5 d to allow for their aggregation and then EBs were plated on gelatin-coated dishes for the next 3 wks. The blocking anti-BMP2 and anti-Wnt3a antibodies

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(MAB3552 and MAB1324, respectively; R&D Systems), used at a concentration of 7.5 µg/ml medium, were added to mock or Oct4-iA-nucleofected cells for 2 d before RNA extraction and RT-Q-PCR of Tbx6, Mesp1, and Tbx5 genes.

RT-Q-PCR by SYBR green detection
RNA was extracted from ES cells using a QIAGEN or Zymo Research (Proteingene) kit or a Zymo Research RNA MicroPrep (Proteingene) for single colonies. 1 µg of RNA was reverse-transcribed using the SuperScriptII reverse transcription [Invitrogen] and oligo(16)dT. QPCR was performed using a Light-Cycler LC 1.5 or 480 (Roche) according to the manufacturer. Melting curves were used to determine the specificity of PCR products, confirmed using conventional gel electrophoresis and sequencing. Data were analyzed according to Pfaffl (2001). Primers specific for human genes are described in Tomescot et al. (2007) and are available on request.

Primers to amplify enhancer/promoter regions were: Sox17 promoter: forward, 5’-acctctacgctcaggaagag-3’ and reverse 5’-gctttgtcct- gggagaagccgctt-3’ targeting a region within the 700-bp region upstream the transcription start site; Sox2: distal enhancer forward, 5’-ttccgcttgagctcccccttcc-3’ and reverse 5’-gtcgcgttcttgagctctcc-3’. Nkx2.5 promoter: forward 5’-cagtcttgagctcaggtct-3’ and reverse 5’-gctttgtcct- gggagaagccgctt-3’. This was synthesized as a sense and antisense oligonucleotide, annealed and subcloned in pSuper vector (Ambion). The DNA constructs were nucleofected in HESCs, using Amaxa specific nucleo-fector solution I, as recommended by the manufacturer.

Oct4-iA was amplified from RNA extracted from HESC as described in Lee et al. (2006) and subcloned in a pcDNA vector.

Cell immunofluorescence and imaging
Cell immunofluorescence was performed as described previously (Zeineddine et al., 2006). The anti-Sox17 antibody was from R&D Systems (Mab1924); anti-Nkx2.5 antibody from R&D Systems (MAB2444 clone 259416); anti-Mef2c from Aviva; anti-CD31, CD45, and CD34 from Becton Dickinson; anti-phospho-Smad1/5/8 from Cell Signaling Technology; anti–β-catenin from BD Biosciences; anti-sarcomeric actinin from Sigma-Aldrich; and anti–cTnt from Abcam. Slides were mounted using Fluoromount-G (Electron Microscopy Science).

Cardiac TnT and α-actinin fluorescent areas were measured in EBs using a thresholding and setting the cTnT and α-actinin fluorescent areas as regions of interest (ROI) within whole EBs observed at 4x to measure the number of pixels within these ROIs with Image software (National Institutes of Health, Bethesda, MD).

Images were acquired at room temperature with an epifluorescence microscope (AxioImager: Carl Zeiss, Inc.) using an Achroplan 10x objective (NA 0.25). A microscope (Axiostar; Carl Zeiss, Inc.) using an N-Achroplan objective (20x, NA 0.45) (Fig. 1 G), or a laser-scanning microscope (LSM10 Meta; Carl Zeiss, Inc.) using an Achroplan objective 10x (NA 0.25) and Fluor 40x oil (NA 1.3) zoom 2x (Fig. 1 H), and visualized without any processing by LSM browser software (Carl Zeiss, Inc.) or (Fig. 2) a microscope (Multiphoton Radiance 2000MP; Bio-Rad Laboratories) attached to a Nikon Eclipse with a 10x PL-APo (NA 0.45) at room temperature and visualized with Bio-Rad Laboratories software and ImageJ without any processing.

FACS analysis
HESC were trypsinized and washed twice with PBS, filtered through a 70-µm mesh filter before FACS analysis using FACS Calibur (Becton Dickinson) and CELL QUEST software.

Protein immunoprecipitation and Western blots
END-2 cells were cultured in DMEM, supplemented with 10% FCS. Cells were nucleofected with Oct4-iA cDNA using the kit V (Amaxa). Cells were lysed in RIPA buffer. Co-Immunoprecipitation was performed in RIPA buffer and Western blot analysis was conducted as reported previously (Puceat et al., 1998). The anti-Oct4 antibodies used were from Santa Cruz Biotechnology, Inc. Sox2 and Oct4 Western blots were performed using the anti-Sox2 from Santa Cruz Biotechnology, Inc., and anti-Oct4 recognizing both Oct4-iA and -b.
Screening of immunostained cells by high content imaging

Immuno-fluorescence scan of BMP2-treated HUES-9 pOct4/GFP or Oct4iA nucleofected HESC stained with anti-Oct4 and Alexa 546-conjugated anti-mouse IgG or anti-Mesp2 and anti-Alexa 680-conjugated anti-rabbit IgG antibodies or anti-NKx2.5 and anti-Alexa 488-conjugated anti-rabbit IgG antibodies or anti-Mef2c and anti-Alexa 488-conjugated anti-mouse IgG antibodies: HESC were plated in 96-wells plates on MEF and treated or not with 10 ng/ml BMP2 and SU5402 for 4 d. Other cells were nucleofected with Oct4iA cDNA and plated in Labtech. After immunostaining, the HC imaging 96 well-plates (cells in PBS) were scanned with the Arrayscan (Cellomics Thermo Fisher Scientific attached to an inverted microscope [Carl Zeiss, Inc.] and using 20x N-Achromat objective, NA 0.45, at room temperature) using the Cell Health Profiling Bioapplication. The fluorescence threshold was determined from the background obtained in non-BMP2-stimulated cells or mock cells and set as a fixed threshold value to scan the wells of BMP2-treated cells or Oct4iA-nucleofected cells. In BMP2 experiments, 50 fields/well and 32 wells were scanned giving a total of 98,120 cells selected according the morphology, size, and the proximity of their nuclei (i.e., to discriminate HESC colonies from feeder cells). Different types of events were scored using the cell feature parameters and the Boolean operators. The visualization of images and analysis were then performed using the vHCs software.

Online supplemental material

Fig. S1: characterization of HUES-9 pOct4/GFP clone. Non-cardiac potential of differentiation of Oct4-OEC. KO of Sox2/Sox7 and consequences on BMP2-induced gene expression. Fig. S2: paracrine action of Oct4-overexpressing cells. Fig. S3: Oct4-overexpressing cells activate Smad and β-catenin pathways; The Oct4/Sox7 network is conserved in iPSCs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200901040/DC1.


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