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Generation of Homogeneous PDX1⁺ Pancreatic Progenitors from Human ES Cell-derived Endoderm Cells

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One key step in producing insulin-secreting cells from human embryonic stem (hES) cells is the generation of pancreatic and duodenal homeobox gene 1 (PDX1)-expressing pancreatic progenitor cells. All-trans retinoic acid (RA) has important roles in pancreas development and is widely used to induce pancreatic differentiation of ES cells. When RA was added directly to the activin A-induced hES cells, <20% cells were positive for the pancreatic marker PDX1, whereas the other cells were mainly hepatic cells. We found that when the activin A-induced hES cells were replated and seeded at low cell densities, the addition of RA induced significant pancreatic differentiation and over 70% of cells in culture expressed PDX1. When the endodermal cells were isolated with the surface marker CXCR4 from the activin A-induced culture and further differentiated with RA, a homogeneous PDX1⁺ cell population (over 95% pure) was generated. The PDX1⁺ cells could further differentiate into cells that expressed pancreatic transcription factors and pancreatic endocrine or exocrine markers. We also found that RA inhibited the hepatic differentiation of endodermal cells that were seeded at low cell densities, and this inhibition may have been through the inhibition of Smad1/5/8 activity. Thus, we present a highly efficient and reproducible protocol for generating PDX1⁺ pancreatic progenitor cells from hES cells.

Keywords: embryonic stem cell, retinoic acid, PDX1, pancreatic progenitor, hepatic, Smad

Introduction

Islet transplantation provides curative effects on type I diabetes (Shapiro et al., 2000, 2006). However, the limited availability of human islet cells constrains this treatment as a clinical therapy. Human embryonic stem (hES) cells can proliferate infinitely and differentiate into many cell types; thus, hES cells are a promising source for islet cells. However, before hES cells can be used to treat diabetes, they should be efficiently and reproducibly induced to differentiate into pancreatic cells (Guo and Hebrok, 2009). Stepwise protocols have been established for generating pancreatic cells from hES cells (D'Amour et al., 2006; Jiang et al., 2007a, b; Shim et al., 2007; Kroon et al., 2008; Johannesson et al., 2009; Zhang et al., 2009). These protocols generally mimic the major events of the pancreas development, which includes several stages such as formation of the primitive gut endoderm, the prospective pancreatic endoderm, the

pancreatic progenitors, the endocrine progenitors, the immature β cells and mature β cells (Oliver-Krasinski and Stoffers, 2008). To date, efficient endoderm differentiation of ES cells has been achieved by activin A treatment (Kubo et al., 2004; D'Amour et al., 2005; Shi et al., 2005). The next step in the route of generating pancreatic β cells is to generate pancreatic and duodenal homeobox gene 1 (PDX1⁺) pancreatic progenitor cells. However, an efficient protocol for this step has not yet been developed.

Multiple extracellular signals and transcription factors are involved in the pancreas development (Jensen, 2004; Jorgensen et al., 2007; Gittes, 2009). PDX1 is one transcriptional factor required for the growth of the pancreatic bud (Offield et al., 1996) and the formation of the mouse and human pancreas (Jonsson et al., 1994; Stoffers et al., 1997). PDX1 expression marks the pancreatic endoderm, the stomach and the duodenal endoderm, but not the liver (Offield et al., 1996; Jensen, 2004). Furthermore, lineage-tracing experiments also showed that all the pancreatic endocrine and exocrine cells are derived from PDX1⁺ cells (Gu et al., 2003). Retinoic acid (RA) signal is required for the development of PDX1⁺ pancreatic cells from the endoderm (Stafford and Prince, 2002; Chen et al., 2004b; Stafford et al., 2004; Martin et al., 2005; Molotkov et al., 2005), and ectopic

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RA signals could alter the fate of the anterior endoderm to adopt a pancreatic fate (Stafford and Prince, 2002). RA promoted the generation of PDX1⁺ pancreatic progenitors from mouse embryonic stem cells (Micallef et al., 2005; Shi et al., 2005), and RA was widely used to induce the pancreatic differentiation of hES cells (D'Amour et al., 2006; Jiang et al., 2007a, b; Shim et al., 2007; Kroon et al., 2008; Johannesson et al., 2009; Zhang et al., 2009). However, the pancreatic differentiation efficiency was generally low. Recently, we found that a large proportion of hepatic cells were generated concomitantly with the small population of PDX1⁺ cells when RA was added to the hES cell-derived endodermal cells (unpublished observation). In mice, the development of PDX1⁺ pancreatic progenitors of the ventral endoderm is in close relation with the liver development (Deutsch et al., 2001). Fibroblast growth factors (FGFs) (Jung et al., 1999) and bone morphogenic proteins (BMPs) (Rossi et al., 2001; Chung et al., 2008) induce the endoderm cells to differentiate into hepatic cells instead of PDX1⁺ pancreatic cells. Also, administration of these signals to hES cell-derived endoderm cells generated >70% hepatic cells (Cai et al., 2007). Because the hepatic differentiation is significant and the pancreatic differentiation efficiency is generally low, we sought for conditions that would drive the hES cell-derived endodermal cells to make the fate choice of the pancreas instead of the liver.

In this study, we showed that after being replated and seeded at low densities, RA was able to efficiently induce hES cell-derived endodermal cells to differentiate into PDX1⁺ pancreatic progenitor cells. We also showed that the presence of RA inhibited hepatic differentiation of replated endodermal cells, possibly through the inhibition of SMAD1/5/8 activity.

Results

RA induced efficient generation of PDX1⁺ cells and inhibited hepatic differentiation when endoderm cells were seeded at low cell densities

To induce the pancreatic differentiation of hES cells, we first induced hES cells to efficiently differentiate into definitive endodermal cells by 3 days of activin A treatment (Cai et al., 2007). Subsequently, RA and keratinocyte growth factor (KGF, also known as FGF7) were added to the endodermal cell culture to test their effects on pancreatic induction (Zhang et al., 2009). After 6 days of RA and KGF treatment, PDX1⁺ cells were located primarily at the edges of some endodermal cell colonies, whereas the other cells present were predominantly α -fetoprotein (AFP)-positive hepatic cells (Figure 1A). The PDX1⁺ cells constituted no more than 20% of the culture population.

We hypothesized that cell–cell interaction might be inhibitory for pancreatic induction. Therefore, activin A-induced cells were dissociated and replated at low density to avoid cell–cell contact. To facilitate viability and growth of the replated cells, the cells were seeded onto 3T3 mouse fibroblast feeder cells. In the absence of RA treatment, 6 days after

replating, most cells expressed hepatic markers AFP and ALB, whereas only a few cells expressed PDX1 (Figure 1B, Supplemental Figure S1A). When RA was included in the replated culture, $72.8 \pm 6.9\%$ of all cells in the culture expressed PDX1⁺, and only a few AFP⁺ or ALB⁺ hepatic cells were detected (Figure 1B, Supplemental Figure S1A). Gene expression analyses of pancreatic (*PDX1*, *NGN3*) and hepatic markers (*AFP*, *ALB*) in the replated cells treated with or without RA also confirmed this result (Figure 1C). The addition of 3T3 feeder cells to the endodermal cells without replating did not clearly increase the PDX1⁺ population (data not shown). Only a few 3T3 feeder cells remained after 6 days of culture, and the residual feeder cells could not be stained by PDX1 (Supplemental Figure S1B).

To further test the hypothesis that cell–cell contact inhibited pancreatic differentiation, the activin A-induced cells were replated at various cell densities and were induced in the presence of RA for 6 days. This treatment efficiently generated PDX1⁺ cells and hepatic differentiation was significantly inhibited when cells were seeded at densities $<5 \times 10^4$ cells/cm² (Figure 1D). When cells were seeded at high density (1×10^5 cells/cm²), AFP⁺ cells were readily detected, while the percentage of PDX1⁺ cells was $<30\%$ (Figure 1D, the lowest panel). These results suggest that cell–cell contact is inhibitory for RA-induced pancreatic differentiation. Thus, RA efficiently induced pancreatic differentiation and inhibited the default hepatic differentiation of replated endodermal cells seeded at low cell densities.

We then applied this protocol to other hES cell lines including H9 and UC06. Although there are differences between cell lines, applying this replating protocol to these additional cell lines generated $63.2 \pm 7.2\%$ and $44.3 \pm 5.2\%$ PDX1⁺ cells, respectively (Supplemental Figure S2).

Sorting of endoderm cells and subsequent differentiation generated homogeneous PDX1⁺ population

To further increase the PDX1⁺ cell purity, we sorted the activin A-induced cells using CXCR4 (Figure 2A), a marker for ES cell-derived endodermal cells (D'Amour et al., 2005; Tada et al., 2005). Sorting with CXCR4 enriched the endodermal cell population because nearly all the cells in the CXCR4⁺ population were positive for the endodermal cell marker SOX17, and >90% of the cells were positive for FOXA2 (Supplemental Figure S3). The sorted cells were seeded onto 3T3 feeder cells at density of 2×10^4 cells/cm² and allowed to further differentiate in the presence of RA. The further differentiation of the CXCR4⁺ endodermal cells generated homogeneous PDX1⁺ cells. In the CXCR4⁺ population, $95.6 \pm 1.4\%$ of cells in culture were PDX1⁺ cells (Figure 2B). In the CXCR4⁻ population, $<10\%$ PDX1⁺ cells were generated (data not shown). These results further proved the effectiveness of RA in inducing PDX1⁺ pancreatic differentiation of the endodermal cells.

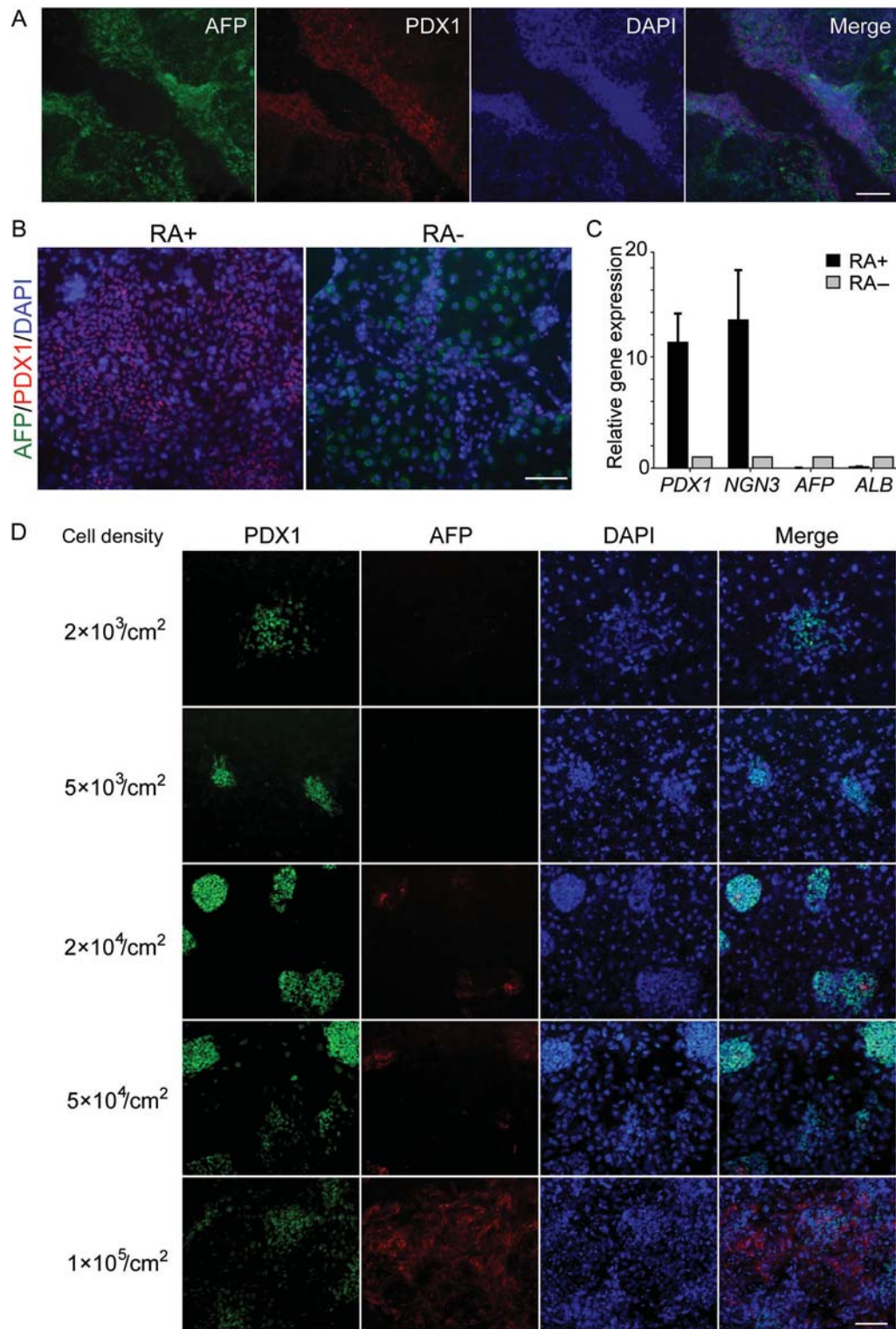


Figure 1 The effects of RA and cell density on pancreatic and hepatic differentiation. **(A)** Location of PDX1-positive cells in the endoderm cell colonies. The endoderm cells without replating were differentiated in the presence of RA and KGF. After 6 days, most of the PDX1 positive cells were found in the edge of some endoderm cell colonies. **(B–D)** The 3-day differentiated cells induced with activin A were replated and further differentiated in the presence or absence of RA for another 6 days. **(B)** The expression of the pancreatic marker PDX1 and hepatic marker AFP in replated endoderm cells cultured in the presence (RA +) or absence (RA –) of RA. **(C)** The gene expression of cells cultured in the presence (RA +) or absence (RA –) of RA. The relative gene expression was normalized to the cells in culture without RA. Three independent experiments were conducted, and the data represent mean \pm SEM. The activin A-induced cells were replated and seeded at indicated densities. After 6 days of differentiation in RA, the expression of AFP and PDX1 in culture was tested using immunofluorescence. RA, all-trans retinoic acid; AFP, α -fetoprotein; Alb, albumin; PDX1, pancreatic and duodenal homeobox gene 1; and NGN3, Neurogenin 3. Scale bars: 100 μ m.

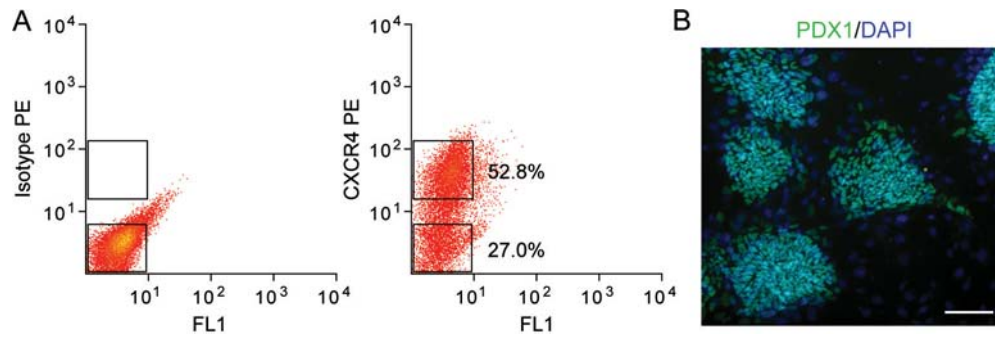


Figure 2 Sorting and further differentiation of activin A-induced cells. **(A)** Sorting the activin A-induced cells with CXCR4. The cells labeled with isotype control were used as the negative control to set the gate. **(B)** After sorting, the cells in the CXCR4⁺ population were further differentiated in the presence of RA for 6 days. More than 95% of cells in culture were PDX1⁺ cells. Scale bars: 100 μ m.

Further differentiation of PDX1-expressing cells

To further clarify the pancreatic identity of the PDX1⁺ cells, we tested the differentiation ability of the generated PDX1⁺ cells. The PDX1⁺ cells were further differentiated in the presence of hepatocyte growth factor (HGF) and Exendin-4 for another 6 days (D'Amour et al., 2006). After 6 days of further differentiation, most PDX1⁺ cells expressed the pancreatic genes FOXA2, HNF6, HNF4 α , HNF1 β and NKX6.1 (Figure 3A). Furthermore, most cells were negative for CDX2 (Figure 3A), which is expressed in the non-pancreatic endodermal cells (Beck et al., 1995). Meanwhile, the further differentiation of the RA-treated cells generated insulin-expressing cells (Figure 3B), whereas no insulin⁺ cells were found in cells that had not been treated with RA (Figure 3C). All the insulin⁺ cells were also positive for C-peptide (Figure 3D), indicating that the insulin was indeed synthesized by the cells themselves and not due to the uptake from the medium (Rajagopal et al., 2003; Hansson et al., 2004). Moreover, amylase⁺ exocrine cells were also detected in the culture (Figure 3E). The gene expression of *INSULIN* and *AMYLASE* was also confirmed by reverse transcription (RT)-PCR (Figure 3F). These results indicate that the generated PDX1⁺ cells have the potential to differentiate into pancreatic hormone-expressing cells.

RA can inhibit hepatic differentiation of cells within 2 days of replating

To study the time window for RA-mediated hepatic fate inhibition during the 6 days after replating, we added RA to the replated activin A-induced cells at different time points after replating. Similar to the RA-treated (6+) replated cultures, if RA was omitted during the first 1 or 2 days (1-5+ or 2-4+), the expression of hepatic markers was minimal, and expression of pancreatic markers was significant, in terms of both mRNA (Figure 4A) and protein expression (Figure 4B, Supplemental Figure S4). When RA was omitted in the first 3 days and was added since the fourth day (3-3+), the hepatic differentiation was substantial and the pancreatic differentiation was inhibited, as detected by RT-qPCR (Figure 4A) and immunostaining

(Figure 4B, Supplemental Figure S4), which was similar to the cultures without RA treatment (6-). These results indicate that RA exerts its hepatic-inhibitory effect on cells that are within 2 days of replating.

RA inhibits Smad1/5/8 phosphorylation in endodermal cells

Because the presence of RA inhibited the hepatic differentiation, we hypothesized that some hepatic induction signals were inhibited by RA. Previous reports showed that BMP is involved in the hepatic specification of ES cell-derived endodermal cells (Gouon-Evans et al., 2006; Cai et al., 2007) as well as the hepatic versus pancreatic fate determination of the endodermal cells (Rossi et al., 2001; Chung et al., 2008). Therefore, we speculated that modulation of BMP signal might be involved in RA-mediated hepatic inhibition. Smads 1, 5 and 8 are the immediate downstream targets of BMP receptors and these factors play a central role in mediating the biological effects of BMPs (Chen et al., 2004a). After the receptor is activated by BMPs, Smad1/5/8 are phosphorylated at the C-terminus and translocated into the nucleus (Canalis et al., 2003). We thus tested Smad1/5/8 activity of the cells at 2 days after replating. The cells at this stage were not yet committed and still possessed the bipotential to differentiate into either hepatic or pancreatic cells (Figure 4). Because the replated cells expressed BMPs (data not shown), the cells clearly exhibited basal Smad1/5/8 activity in the absence of RA (Figure 5A, lane 4). The addition of BMP2 increased the Smad1/5/8 activity (Figure 5A, lane 3). However, adding RA to the culture dramatically decreased the phosphorylation of Smad1/5/8 proteins (Figure 5A, lane 1), as detected by western blot analysis. Similar results were observed when we tested specifically for the phosphorylated Smad1 protein (Figure 5A, lane 2).

The addition of BMP2 did not restore the Smad1/5/8 activity that had been inhibited by RA (Figure 5A, lane 2), suggesting that RA acted downstream of BMP proteins. The addition of RA did not influence the expression of *SMAD1* mRNA (Figure 5B) or protein production (Figure 5A, lane 3), suggesting that RA inhibited Smad1 activity at the post-translational level.

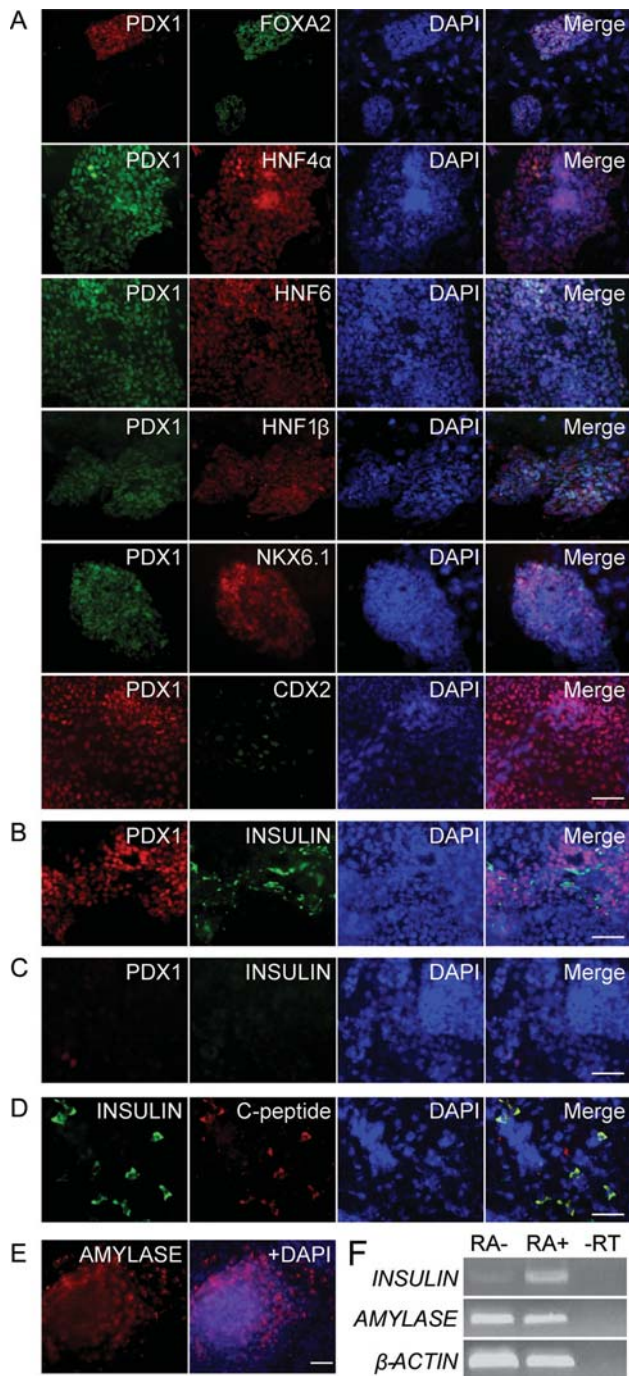


Figure 3 The further differentiation of PDX1⁺ pancreatic progenitor cells. The activin A-induced cells were replated, cultured in the presence or absence of RA for 6 days, and then differentiated in HGF and Exendin 4 for another 6 days. (A) The expression of pancreatic transcriptional factors FOXA2, HNF4 α , HNF1 β , NKX6.1, HNF6 and CDX2 in further differentiated cells with RA treatment. The expression of PDX1 and insulin in the further differentiated cells with (B) or without (C) RA treatment. (D) INSULIN and C-peptide expression in the further differentiated cells with RA treatment. (E) Amylase expression in the further differentiated cells with RA treatment. (F) RT-PCR detection of the gene expression of INSULIN and AMYLASE in the further differentiated cells. The RNA from RA⁺ cells without reverse transcription (RT -) was used as a negative control. HNF, hepatocyte nuclear factor; NKX6.1, NK6 homeobox 1. Scale bars: 50 μ m.

To determine whether the inhibition of Smad1/5/8 activity was responsible for the inhibition of hepatic differentiation, we tested the effects of BMP antagonist Noggin on the hepatic and pancreatic differentiation of the replated cells. Noggin is a relatively specific BMP inhibitor that acts by competitively binding BMP receptors, and it is not known to show any other activity (Canalis et al., 2003). The addition of Noggin inhibited Smad1/5/8 phosphorylation in the replated cells (Figure 5A, lane 5). Furthermore, it inhibited the hepatic differentiation of the endodermal cells (Figure 5C). These results indicate that inhibiting BMP activity could inhibit the hepatic differentiation. Although Noggin inhibited the hepatic differentiation of the endodermal cells, the percentage of PDX1⁺ cells within the population did not increase (Figure 5C), indicating that signals other than Smad1/5/8 inhibition are also needed for efficient pancreatic induction.

The phosphorylated Smad1/5/8 protein was further examined by immunostaining. In the absence of RA treatment, endodermal cells 2 days after replating showed significant amounts of phosphorylated Smad1/5/8 protein, and the majority of phosphorylated Smad1/5/8 proteins were found in the nucleus (Figure 5D). In the presence of RA or Noggin, the proportion of phosphorylated Smad1/5/8 protein was very low (Figure 5D). Six days after replating, no PDX1⁺ cells were positive for the phosphorylated Smad1/5/8 under any differentiation conditions (RA⁺, RA⁻ or Noggin⁺) (Figure 6A). However, some AFP⁺ cells were also positive for phosphorylated Smad1/5/8 (Figure 6B), suggesting that inhibiting the Smad1/5/8 activity may be required for pancreatic induction. These results indicated that RA or Noggin inhibited Smad1/5/8 phosphorylation as well as the hepatic differentiation of endodermal cells.

Discussion

Here we showed for the first time that homogeneous (>95%) PDX1⁺ pancreatic progenitor cells could be generated from hES cells (Figure 2B). These PDX1⁺ progenitor cells expressed pancreatic markers and did not express the hepatic marker AFP, and could differentiate into cells co-expressing NKX6.1 and PDX1, as well as the cells expressing pancreatic exocrine and endocrine markers.

One key factor in our differentiation protocol is the disruption of cell-cell contact by cell dissociation and seeding the cells at low densities. Without cell dissociation, hES cells-derived endoderm cells mainly generated hepatic cells even in the presence of RA (Figure 1A). We also observed that higher seeding density ($1 \times 10^5/\text{cm}^2$) resulted in <30% PDX1⁺ cells (Figure 1D) with significant hepatic differentiation instead. After replating and seeding at low cell density, RA was powerful in inducing pancreatic differentiation and inhibiting hepatic differentiation (Figure 1B, Supplemental Figure S1A), and nearly all the sorted endodermal cells differentiated into PDX1⁺ pancreatic cells (Figure 2B). We speculate that without replating or under high-density conditions, the activation of certain cell-cell contact-dependent signals in endoderm cells may cause resistance to RA signals, and thus yielding high percentage of hepatic versus pancreatic cells. Since notch signaling regulates the pancreas exocrine and

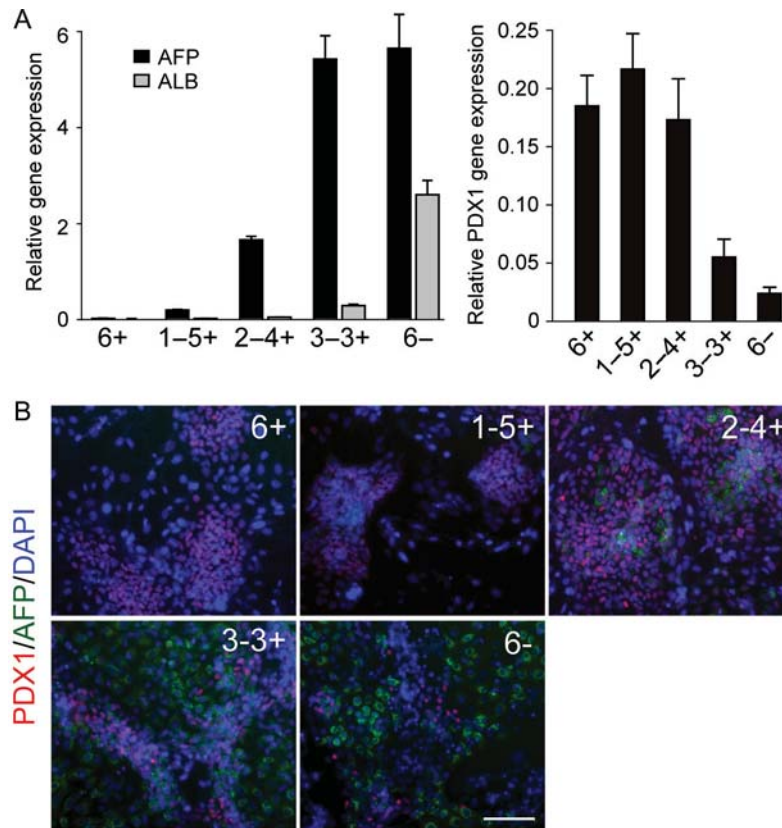


Figure 4 RA can inhibit the hepatic differentiation of the cells that are within 2 days after replating. The activin A-induced cells were replated and cultured for 6 days. RA was included in the culture for 6 days (6 +), or RA was not added in the first day (1-5 +), first 2 days (2-4 +), first 3 days (3-3 +) and the whole 6 days (6 -). **(A)** Relative gene expression of differentiated cells treated with RA at different time. Three independent experiments were conducted and the data represent mean \pm SEM. **(B)** The expression of the hepatic marker AFP and pancreatic marker PDX1 in the differentiated cells with RA added at different time. Scale bars: 100 μ m.

endocrine cell differentiation (Apelqvist et al., 1999; Chen et al., 2004b), and is involved in the effect of RA on the pancreas differentiation (Chen et al., 2004b), we speculate that notch signal may play a role in this lateral inhibition process. Further study is needed to investigate the potential mechanism.

RA induces the pancreatic differentiation of endoderm cells in multiple species (Stafford and Prince, 2002; Chen et al., 2004b; Stafford et al., 2004; Martin et al., 2005; Molotkov et al., 2005). However, the effect of RA on liver development varies in different species. For example, Stafford and Prince (2002) showed that RA is required for both the liver and the pancreas development in *Zebrafish*. However, in *Xenopus*, RA signal is not required for the liver specification (Chen et al., 2004b; Stafford et al., 2004), while increased RA signal inhibits the hepatic gene expression and stimulates dorsal (but not ventral) pancreatic development (Chen et al., 2004b). In mice, RA signal has no effect on the liver development (Martin et al., 2005; Molotkov et al., 2005). In human ES cell differentiation, the role of RA in hepatic differentiation has not been addressed before. Here we showed that RA inhibited the hepatic differentiation of the replated endoderm cells. As BMP is required for hepatic differentiation (Rossi et al., 2001), and RA inhibited the BMP downstream effectors Smad activity, we propose a role for RA in regulating the pancreatic and hepatic cell fate determination of the hES cell-derived

endodermal cells (Figure 7). In replated cultures of endoderm cells not treated with RA, the default differentiation pathway is to become hepatic cells (Figure 1B). The presence of RA inhibits this hepatic differentiation pathway possibly through the inhibition of BMP downstream effectors Smad1/5/8 activity. Noggin inhibits Smad1/5/8 activity as well as the hepatic differentiation, but is not able to induce pancreatic differentiation, and thus RA has additional effects besides inhibiting Smad1/5/8 to induce the pancreatic differentiation of the endodermal cells. A recent report (Chen et al., 2009) suggests that protein kinase C signaling acts downstream of RA in inducing pancreatic differentiation of endoderm cells, thus providing a possibility that protein kinase C signaling may mediate the pancreatic induction effects of RA.

We and others have developed induction protocols to generate pancreatic lineage cells from hESCs (D'Amour et al., 2006; Jiang et al., 2007a, b; Shim et al., 2007; Kroon et al., 2008; Johannesson et al., 2009). In our lab, our differentiation protocols have also been successfully used to generate pancreatic lineage cells from mouse ES cells, mouse nuclear transfer-embryonic stem cells and human-induced pluripotent stem cells (Shi et al., 2005; Jiang et al., 2008; Zhang et al., 2009). All these studies demonstrated that insulin-producing cells could be generated by stepwisely mimicking the major events

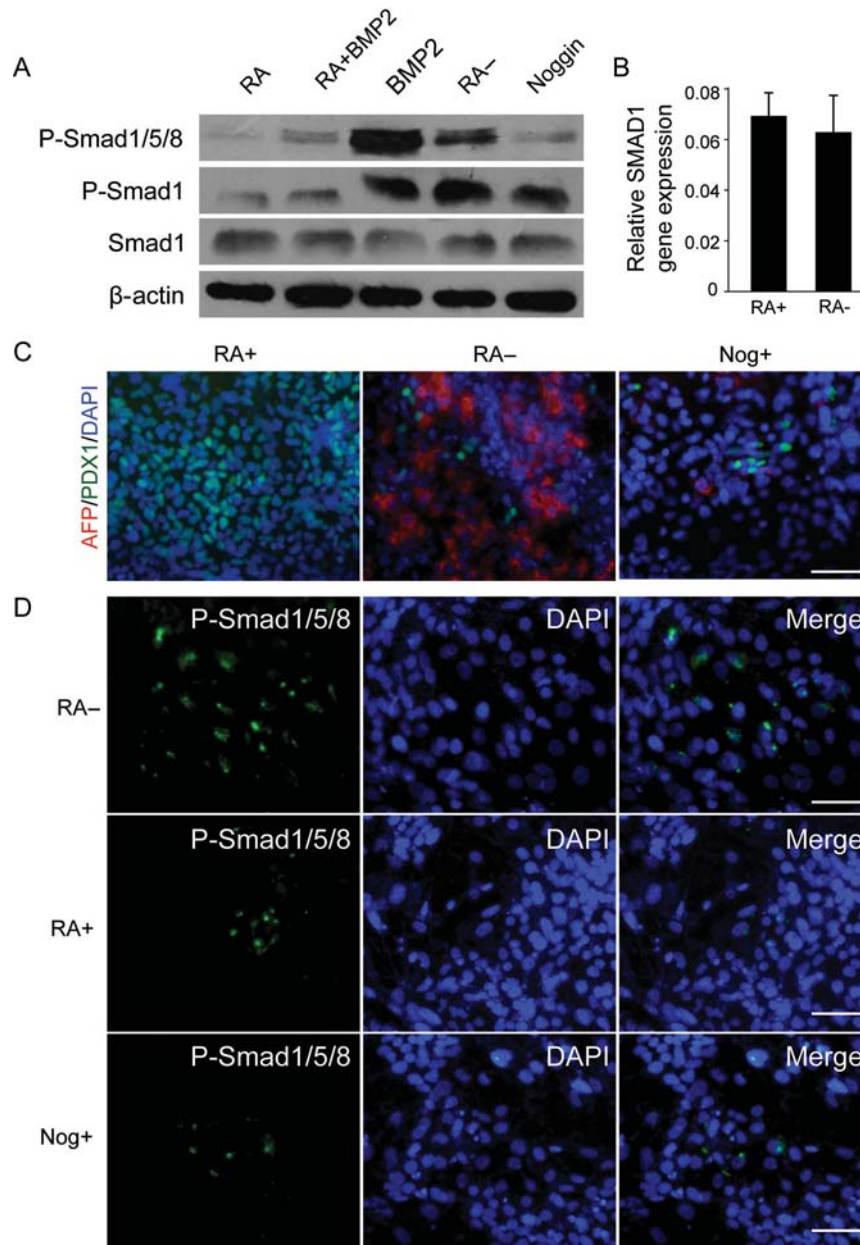


Figure 5 RA inhibits Smad1/5/8 activity. The activin A-induced cells were replated and cultured in media containing different factors. **(A)** Western blot analysis of differentiated cells. Replated cells were cultured in RA, RA + BMP2, BMP2, without RA (RA -) or with Noggin for 2 days. Membranes were probed with antibodies specific to phosphorylated Smad1/5/8, phosphorylated Smad1, Smad1 and β -actin (as a loading control). **(B)** The relative gene expression of SMAD1 in cells treated with or without RA for 2 days. Similar results were produced by two independent experiments with three parallel samples. The data were from one experiment, and they represent mean \pm SEM. **(C)** PDX1 and AFP expression in cells treated without RA (RA -), with RA (RA +) or with Noggin (Nog +) were assessed at Day 6 after replating. The addition of Noggin to the culture inhibited the hepatic marker AFP expression, but did not induce the pancreatic marker PDX1 expression. **(D)** Phosphorylated Smad1/5/8 expression in the culture at Day 2 after replating without RA (RA -), supplemented with RA (RA +), or supplemented with Noggin (Nog +). Scale bars: 100 μ m.

of the pancreas development (Oliver-Krasinski and Stoffers, 2008). However, for each differentiation step, the induction condition has not yet been optimized. In this study, we optimized the PDX1⁺ cells generation step and increased the differentiation efficiency dramatically. Using this new protocol, no obvious cell death was observed during the differentiation processes. We estimated that starting with one hES cell, we could generate one endoderm lineage cell after 3 days of

differentiation, and one endoderm lineage cell could generate ~ 10 PDX1⁺ progenitors in a further 6-day differentiation procedure. Thus, using one hES cell could generate ~ 10 PDX1⁺ progenitors in a 9-day differentiation process. Therefore, by this new protocol, we can obtain large number of nearly homogeneous PDX1⁺ pancreatic progenitors which would provide a defined system to study the mechanism of pancreatic lineage specification from endoderm cells and their further development.

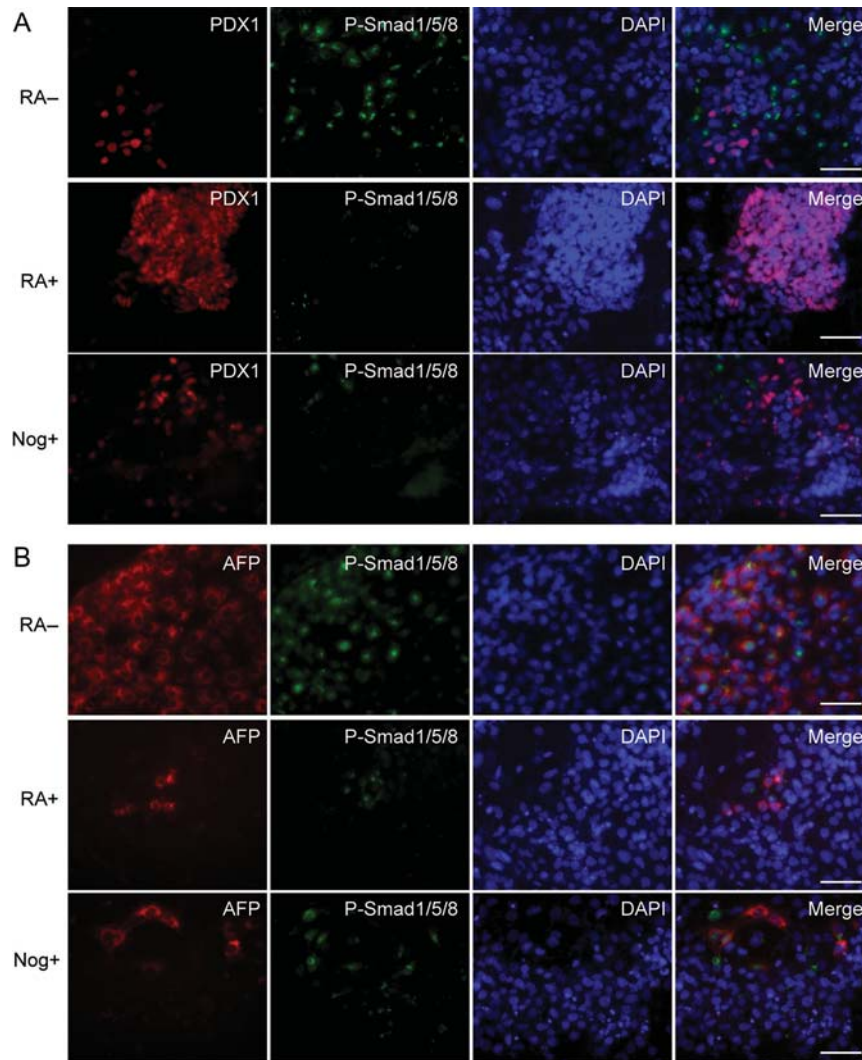


Figure 6 The expression of phosphorylated Smad1/5/8 in the replated cells treated with different factors for 6 days. **(A)** The expression of PDX1 and phosphorylated Smad1/5/8 in the culture without RA (RA -), with RA (RA +) or with Noggin (Nog +). **(B)** The expression of AFP and phosphorylated Smad1/5/8 in the culture supplemented without RA, with RA or with Noggin. Nog, Noggin. Scale bars: 50 μ m.

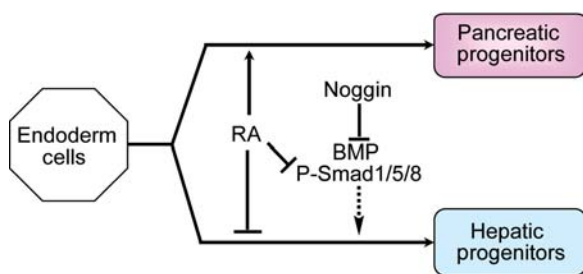


Figure 7 Proposed role of RA in hepatic and pancreatic cell fate determination of hES cell-derived endoderm cells. The endoderm cells could differentiate into hepatic and pancreatic cells. RA initiated the pancreatic program and inhibited hepatic differentiation of endoderm cells. One potential mechanism for the RA-mediated hepatic inhibition is through the inhibition of BMP downstream effector Smad1/5/8 phosphorylation. Noggin inhibited Smad1/5/8 phosphorylation and hepatic differentiation but was not sufficient to induce pancreatic differentiation of endoderm cells.

Finally, the homogeneous PDX1⁺ pancreatic progenitors can be used for gene profiling analysis and for generation of pancreatic specific antibodies.

Materials and Methods

hES cell culture and differentiation

Human ES cell lines H1 and H9 were obtained from Wicell Research Institute (Madison, WI, USA). The UC06 hES cell line was obtained from National Stem Cell Bank (Madison, WI, USA). Unless otherwise stated, all experiments were conducted with H1 cells. All hES cell lines were cultured on mouse embryonic fibroblast (MEF) feeder cells according to previously described protocols (Wang et al., 2005). Briefly, hES cells were cultured on MEF feeder cells in 0.1% gelatin (Sigma, St Louis, MO, USA)-coated plates. The culture medium was Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (DF12)

supplemented with 20% knockout serum replacement, 1% non-essential amino acids, 1% Glutamax, 0.1 mM β -mercaptoethanol (all from Gibco, Grand Island, NY, USA) and 4 ng/ml basic FGF (Peprotech, Rocky Hill, NJ, USA). Cells were split 1:3 every 5 days with 1 mg/ml collagenase IV (Gibco); UC06 cells were split at 1:6 every 4 days with collagenase IV.

For endoderm differentiation, hES cells were seeded onto MEF feeders in growth factor-reduced Matrigel-coated plates (BD Biosciences, Bedford, MA, USA). Two days later, hES cells at ~40% confluency were washed with 1640 medium (Gibco); 100 ng/ml activin A (Peprotech) in 1640 medium was added to the culture, and the medium was replaced daily. On the second day of endoderm differentiation, 0.1% insulin–transferrin–selenium (Gibco) was added to the medium; on the third day, 1% insulin–transferrin–selenium was added to the medium.

At Day 2 of endoderm cell differentiation, 3T3 feeder cells were prepared. The 3T3 cells were treated with 10 μ g/ml mitomycin C (Roche, Mannheim, Germany) for 6 h and then seeded at a density of $4 \times 10^4/\text{cm}^2$ on Matrigel-coated plates. After 3 days of activin A treatments, the endoderm cells were dissociated using trypsin/EDTA (0.25%/0.1 mM) (Gibco) and filtered through a 40 μ m cell strainer (BD Bioscience) to remove clumped cells. Cells were then seeded onto 3T3 feeder cells at a density of $2 \times 10^4/\text{cm}^2$. The basal medium for cell culture was DF12 supplemented with 2% B27 without vitamin A (Gibco), 1% non-essential amino acids, 1% Glutamax, 0.1 mM β -mercaptoethanol and 20 ng/ml KGF. In addition, 2 μ M all-*trans* RA (Sigma), 20 ng/ml BMP 2 or 1 μ g/ml Noggin was added to this basal medium for 2 days to test their effects on Smad phosphorylation, or for 6 days to test their effects on hepatic and pancreatic differentiation. After 6 days, cells were cultured in the basal medium without KGF, and supplemented with 20 ng/ml HGF (Peprotech), 50 ng/ml Exendin-4 and 10 mM nicotinamide (both from Sigma) for another 6 days.

Western blot

Cells treated with different factors were harvested at 2 days after replating and were lysed on ice with RIPA lysis buffer in the presence of PMSF (both from Beyotime, Guangzhou, China). Proteins (20 μ g) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was blocked with 5% skim milk in PBS/0.1% Triton X-100 and incubated with antibodies against phospho-Smad1/5/8 (1:1 000, Cell Signaling Technology), Smad1, phospho-Smad1 (1:500, both from Millipore, Billerica, MA, USA) or β -actin (1:1000, Zhongshan, Beijing, China) overnight at 4°C. Then, the membrane was washed and incubated with secondary antibodies at room temperature for 1 h. After washing, the membrane was visualized with a Super-Signal West Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA).

Immunofluorescence

Cells were washed with PBS, and fixed in 4% PFA (Dingguo, Beijing, China) for 20 min at room temperature. After washed with PBS for three times, the cells were blocked and

permeabilized with PBS containing 10% normal horse serum, 0.1% Triton X-100 and 1% BSA for 45 min at room temperature. After three washes with PBS, diluted primary antibodies were added and incubated overnight at 4°C. The primary antibodies used were: goat anti-human PDX1 (1:200, R&D, Minneapolis, MN, USA), rabbit anti-human PDX1 (for co-staining with HNF1 β) (1:2000, Abcam, Cambridge, UK), mouse anti-human nuclei (1:500, Chemicon, Temecula, CA, USA), goat anti-human FOXA2 (1:200, R&D), goat anti-human SOX17 (1:200, R&D), rabbit anti-human Alb (1:500, DAKO), mouse anti-human AFP (1:500, Sigma), mouse anti-human CDX2 (1:100, Abcam), guinea pig anti-insulin (1:500, DAKO), goat anti-C-peptide (1:200, Millipore), rabbit anti-human HNF4 α (1:100, Santa Cruz), rabbit anti-human HNF6 (1:100, Santa Cruz), goat anti-human HNF1 β (1:100, Santa Cruz), rabbit anti-phospho-Smad1/5/8 (1:200, Cell Signaling Technology), rabbit anti-amylase (1:500, Sigma). The corresponding isotype mouse IgG (BD Pharmingen, Franklin Lakes, NJ, USA) or normal goat and rabbit serum (Zsbio, Beijing, China) were used as negative controls. After five washes with PBS, the cells were incubated with diluted DyLight 549- or DyLight 488-conjugated donkey anti-rabbit, goat, mouse or guinea pig IgG secondary antibodies (1:200, Jackson ImmunoResearch, West Grove, PA, USA) at room temperature in the dark for 1 h. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Roche Molecular Biochemicals, Mannheim, Germany). For calculating the proportion of PDX1⁺ cells in the replated culture, the number of PDX1⁺ cells and DAPI were counted. For each cell line, 20 randomly picked pictures from five independent experiments were used for calculation. Each picture contained at least 500 cells. The percentage of PDX1⁺ cells was determined by dividing the number of PDX1⁺ cells to the number of DAPI. Data were presented as mean \pm SEM.

Cell sorting and culture

The day 3 differentiated cells were dissociated with trypsin/EDTA, filtered through a 40 μ m cell strainer and resuspended in phosphate buffered saline (PBS, Gibco) containing 2% fetal bovine serum (FBS, Gibco). The cells were incubated with PE-conjugated anti-CXCR4 or PE-conjugated isotype control (both from BD Pharmingen) for 30 min at 4°C. After three washings, the cells were sorted with a MoFlo high-performance cell sorter (Cytomation, Fort Collins, CO, USA). The further differentiation of the sorted cells was conducted as the differentiation of the replated cells. For immunostaining analysis of the sorted cells, the cells after sorting were seeded to Matrigel-coated plates in DF12 medium supplemented with 1% ITS. After 8 h, when the cells were attached, they were fixed with 4% PFA and further analyzed with the standard immunostaining protocol.

Reverse transcription PCR and real-time PCR

Total cellular RNA was extracted with TRIzol reagent (Invitrogen) or a Micro Scale RNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and the trace DNA were removed with Turbo DNase which is subsequently inactivated by DNase inactivator (both from Ambion). Then, 2 μ g RNA was used for reverse transcription with a Reverse Transcription

System (Promega, Madison, WI, USA). For PCR, the cycle number was 25, and the primer sequences used were listed in Supplemental Table S1. Real-time PCR analysis was performed on an ABI Prism 7300 Sequence Detection System with the default conditions of the software 1.3. The expression of each gene was normalized to β -*ACTIN* gene expression. The primer sequences for real-time PCR were listed in Supplemental Table S1.

Supplementary Data

Supplementary data for this article are available online at <http://jmc.oxfordjournals.org>.

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