ARTICLE
Journal of Cellular Biochemistry 112:1022–1034 (2011)

Journal of Cellular Biochemistry

Combined Activin A/LiCl/Noggin Treatment Improves Production of Mouse Embryonic Stem Cell-Derived Definitive Endoderm Cells

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ABSTRACT

Induction of definitive endoderm (DE) cells is a prerequisite for the whole process of embryonic stem (ES) cells differentiating into hepatic or pancreatic progenitor cells. We have established an efficient method to induce mouse ES cell-derived DE cells in suspension embryonic body (EB) culture. Similar to previous studies, mouse ES cell-derived DE cells, which were defined as $Cxcr4^+c\text{-}Kit^+$, $Cxcr4^+E\text{-}cadherin^+$ cells or $Cxcr4^+PDGFRa^-$ cells, could be induced in the serum-free EBs at Day 4 of induction. The activations of Wnt, Nodal, and FGF signaling pathways in differentiating EBs promoted DE cell differentiation, while activation of BMP4 signaling inhibited the process. In the present study, we found that chemical activation of canonical Wnt signaling pathway by LiCl could synergize with Activin A-mediated Nodal signaling pathway to promote induction of DE cells, and inhibition of Bmp4 signaling by Noggin along with Activin A/LiCl further improved the efficiency of DE cell differentiation. The derived DE cells were proved for their capacities to become hepatic progenitor cells or pancreatic progenitor cells. In conclusion, we significantly improved the efficiency of generating mouse ES cell-derived DE cells by combined Activin A/LiCl/Noggin treatment. Our work will be greatly helpful to generate ES cell-derived hepatic cells and ES cell-derived pancreatic cells for future regenerative medicine. J. Cell. Biochem. 112: 1022–1034, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: MOUSE EMBRYONIC STEM CELLS; DEFINITIVE ENDODERM; SUSPENSION CULTURE; EMBRYONIC BODY; DIFFERENTIATION

Embryonic stem (ES) cells can self-renew while maintaining the potential to differentiate into any cell type from three germ layers [Evans and Kaufman, 1981; Murry and Keller, 2008]. There have been substantial progresses in derivation of hepatocyte-like cells and pancreatic cells from mouse and human ES cells [Gouon-Evans et al., 2006; Heo et al., 2006; Soto-Gutierrez et al., 2006; Cai et al., 2007;

The authors disclose no conflicts.

†These authors contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Natural Science Foundation of China; Grant number: 30623003, 30801115, 30901449; Grant sponsor: National Key Basic Research and Development Program of China; Grant number: 2006CB943901, 2006CB943901, 2007CB947102, 2007CB947903, 2009CB941103, and 2010CB945600; Grant sponsor: Chinese National 863 Plan Project; Grant number: 2006AA02Z474; Grant sponsor: N.I.H; Grant number: DK074561 and AI065565, X.W.

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Received 23 April 2010; Accepted 9 November 2010 • DOI 10.1002/jcb.22962 • © 2010 Wiley-Liss, Inc.

Published online 22 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

Duan et al., 2007; 2010; Kroon et al., 2008; Basma et al., 2009; Touboul et al., 2010]. However, the obstacles including low induction efficiency and poor reproducibility for induction method still exist [Snykers et al., 2009]. As the precursors of both liver and pancreas progenitors [Zaret, 2008; Zaret and Grompe, 2008], induction of definitive endoderm (DE) cells is a prerequisite of following hepatic or pancreatic differentiations [Murry and Keller, 2008]. ES cell-derived DE cells can be derived by treatment with recombinant growth factors such as Activin A and/or Wnt 3a [Kubo et al., 2004; D'Amour et al., 2005; Gadue et al., 2006; Nakanishi et al., 2009]. On the other hand, different studies have documented that ES cell-derived DE cell populations in cultures can be isolated either by selection markers under the promoters of genes related to DE, which include Gsc [Tada et al., 2005], Brachyury [Kubo et al., 2004], Foxa2 [Gouon-Evans et al., 2006], Sox17 [Yasunaga et al., 2005], and Hhex [Morrison et al., 2008], or by combined cell surface markers such as Cxcr4, E-cadherin, c-Kit, and PDGFRa (Platelet-Derived Growth Factor Receptor alpha) [Tada et al., 2005; Gouon-Evans et al., 2006; Sherwood et al., 2007]. Generally, the average proportions of derived DE cells in previous reports were less than 40%. Because there were differences in media formulation and in culture processes employed to induce differentiation of ES cells with a low efficiency, it is necessary to optimize the DE cell induction condition and to improve the DE cell induction efficiency in order for following hepatic or pancreatic differentiations.

Here, we report a strategy by modulating multiple signaling pathways to promote differentiation of mouse ES cells into DE cells in suspension embryonic body (EB) culture. We established a 4-day protocol that involves combined Activin A, LiCl, and Noggin (AL2N) treatment in serum-free EBs to derive DE cells at significantly higher proportion (80% of total cells). When cultured under monolayer hepatic or pancreatic induction conditions, the DE cells further differentiated into hepatic progenitor cells or pancreatic progenitor cells with relative morphologies and expressions of hepatic or pancreatic progenitor markers. Our work highlights the combinatorial modulation of multiple signaling pathways to improve the efficiency of DE cell differentiation from ES cells. With the improved induction efficiency in our present protocol, we have moved an important step forward in generating functional DE cells from mouse ES cells, which will be very helpful for improving the methods to derive human DE cells, human hepatic cells, and pancreatic cells for future generative medicine.

MATERIALS AND METHODS

CELL CULTURE AND DIFFERENTIATION

Mouse ES cells (E14; American Type Culture Collection, Manassas, VA) was maintained in 0.1% gelatin-coated T-25 flasks (Corning) in Glasgow minimum essential medium containing 10% knockout serum replacement, 1% defined fetal bovine serum (HyClone, Logan, UT), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoetanol (all from Invitrogen, Carlsbad, CA), 1000 U/ml leukemia inhibitory factor (Chemicon, Temecula, CA). Cells were incubated in a 5% CO_2–air mixture at 37°C.

The serum-containing medium (SCM) contains Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 10% defined fetal bovine serum (Hyclone), 2 mM Glutamine (Invitrogen), 0.5 mM ascorbic acid (Sigma–Aldrich, St. Louis, MO), and 4.5×10^4 M MTG (Sigma–Aldrich). The following modified chemically defined medium (CDM) was used: CDM I consists of 75% Iscove's modified Dulbecco's medium (IMDM), 25% Ham's F12 medium, supplemented with 1× N2 supplements, 0.05% bovine serum albumin (BSA), 2 mM Glutamax-I (all from Invitrogen), 0.5 mM ascorbic acid (Sigma–Aldrich), and 4.5×10^4 M MTG (Sigma–Aldrich). CDM II consists of a 1:1 mixture of Neurobasal medium (Invitrogen) and Dulbecco's Modified Eagle Medium/F12 medium (Invitrogen) supplemented with 0.5× N2 and 0.5× B27 supplements, 0.1% BSA, 2 mM Glutamax-I and 0.1 mM 2-mercaptoethanol.

For suspension EB culture, trypsinized ES cells were seeded (2×10^4 cells/ml) in CDM I in Petri dishes (BD falcon) for 2 days, EBs were collected by brief centrifugation, and resuspended in the same volume of SCM, or in CDM I with or without growth factors and/or chemicals, and further cultured for 2–4 days. For hepatic differentiation, day 4 SF-EBs were trypsinized and replated (5×10^4 cells/cm^2) on gelatin-coated dishes in hepatic progenitor specification medium (CDM II supplemented with 20 ng/ml Activin A, 20 ng/ml BMP4, and 20 ng/ml FGF2) for 2 days, followed in hepatic progenitor expansion medium (CDM II supplemented with 10 ng/ml BMP4, 10 ng/ml FGF2, 20 ng/ml HGF, 20 ng/ml TGFα, 10 ng/ml EGF, and 10^{-7} mol/l dexamethasone) for 6 days. For pancreatic differentiation, day 4 SF-EBs were trypsinized and replated (5×10^4 cells/cm^2) on gelatin-coated dishes for 2 days in CDM II supplemented with 20 ng/ml Activin A, 200 ng/ml Noggin, 2 μM Retinol Acid, 10 ng/ml FGF10, and the cells were then maintained for 4 days in CDM II supplemented with 20 ng/ml Activin A, 200 ng/ml Noggin, 10 ng/ml FGF10, and 10 ng/ml EGF. Medium was changed every other day. Recombinant growth factors were purchased from R&D Systems. Dexamethasone, Ly294002 and LiCl were purchased from Sigma–Aldrich, U0126 was purchased from Merck, and SB431542 was purchased from Tocris.

FACS ANALYSIS AND CELL SORTING

Harvested cells were washed twice in washing buffer (PBS containing 0.2% BSA). For analysis, $1–2 \times 10^5$ cells in 100 μl washing buffer were incubated with chromophore-conjugated antibodies at 4°C for 30 min, followed by washing twice and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Alternatively, primary antibody-bound cells were further incubated with chromophore-conjugated secondary antibodies at 4°C for 30 min, washed twice, and analyzed. Cell sorting was performed as above except that cells were acquired using a FACS Aria I flow cytometer (Becton Dickinson). For each analysis and cell sorting, similar florescence-conjugated isotype control antibodies were used as negative controls to set the quadrants in each graph that were generated automatically by the FlowJo software (Tree Star, Ashland, OR); the proportions of cells in each quadrant were calculated automatically by the FlowJo software (Tree Star), and the values were summarized in the upright quadrant of each graph.

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Cultured cells were fixed with PBS containing 4% paraformaldehyde at room temperature for 20 min, permeabilized and blocked with

blocking buffer (PBS containing 0.1% Triton X-100, 1% BSA, and 10% normal donkey or goat serum) at room temperature for 45 min. Cells were then incubated with primary antibodies at 4°C overnight, followed by appropriate fluorescence-tagged secondary antibodies at room temperature for 1 h. Between each step, cells were washed with PBS containing 0.1% BSA. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich). Stained cells were finally examined under an Olympus IX41 fluorescence microscope Olympus Corporation, Tokyo, Japan and analyzed with Image Pro Plus 5.1 software (Media Cybernetics). For each analysis, additions of appropriate normal IgG antibodies or secondary antibodies were used as negative controls.

ANTIBODIES

The antibodies used in this study were summarized in Supplementary Table I.

REVERSE TRANSCRIPTION PCR (RT-PCR) AND QUANTITATIVE PCR (Q-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Qiagen). 0.5 μg RNA was reverse transcribed into cDNA using First Strand cDNA Synthesis Kit ReverTra Ace -a- (Toyobo, Osaka, Japan). PCR was performed with Taq DNA polymerase (Tiangen, Beijing, China) under the following cycling conditions: 94°C for 5 min, followed by 25–35 cycles of amplification (denaturation at 94°C for 30 s, annealing for 30 s, elongation at 72°C for 1 min), and a final incubation at 72°C for 5 min. Amplified products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Q-PCR analysis was performed with SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) on a Rotor Gene 6500-HRM Real-time PCR cycler (Qiagen). Relative quantification was calculated by normalizing each gene expression against GAPDH or β-Actin following the manufacture's comparative threshold cycle (CT) method (Qiagen). Details of primers for RT-PCR analysis and Q-PCR analysis were summarized in Supplementary Tables II and III.

STATISTIC ANALYSIS

Each experiment was performed at least three times. In Q-PCR analysis and statistical analysis of DE cell population, the statistical significance was calculated by the Student *t*-test, Statistical significance was set at $P < 0.05$ (indicated by "*").

RESULTS

MOUSE ES CELL-DERIVED DE CELLS CAN BE INDUCED ENDOGENOUSLY IN DAY 4 EBS

Previous work showed that when mouse ES cells were differentiated in SCM, DE-specific genes were poorly expressed [Kubo et al., 2004; Tada et al., 2005]. Consistently, when day 2 serum-free EBs (SF-EBs) were resuspended in SCM for 2–4 more days, there were no representative populations of $Cxcr4^+c\text{-}Kit^+$ or $Cxcr4^+PDGFRa^-$ cells from day 3 to day 6 (Supplementary Fig. 1), which indicates DE induction in SCM needs exogenous stimulus, or might be suppressed due to unknown inhibitory factors in the serum.

Subsequently, we focused on optimizing DE inductions in serum-free medium (SFM). When day 2 SF-EBs were directly resuspended in modified SFM (see methods) without exogenous stimulus for 2 days, we consistently detected small populations of $Cxcr4^+c\text{-}kit^+$ (18–20%, n = 3) or $Cxcr4^+PDGFRa^-$ (16–18%, n = 3) cell populations in day 4 SF-EBs (Fig. 1A). Meanwhile, RT-PCR analysis revealed expressions of primitive streak or DE-related genes (Gsc, Cer1, Cxcr4, Hhex, Mixl1, Foxa2, Sox17) in day 4 SF-EBs (Fig. 1B). There were also the expression of Wnt3, Wnt3a, Nodal, and FGF4 (Fig. 1C), which suggest their potential involvement in induction of the small DE cell population. As expected, under conditions that did not significantly affect cell numbers and viabilities (Supplementary Fig. 2A), inhibition of canonical Wnt signaling by DKK1, FGF signaling by U0126 or LY294002, and Nodal signaling by SB431542 respectively led to loss of the representative $Cxcr4^+PDGFRa^-$ cell population in day 4 SF-EBs, while addition of BMP4 (5 ng/ml) reduced $Cxcr4^+PDGFRa^-$ cell population and generated a significant population of $Cxcr4^+PDGFRa^+$ cells (Fig. 1D).

Taken together, these results indicate that DE cells can be induced in day 4 SF-EBs without exogenous stimulus, which is regulated by multiple endogenous signaling pathways. However, the proportion of DE cells under this condition is low (~20%).

ACTIVIN A PROMOTES DE INDUCTION IN A TIME COURSE- AND DOSE-DEPENDENT MANNER

To improve the proportion of DE cells in SF-EBs, the effect of Activin A on DE induction was investigated in our system, as it was demonstrated as an inducer of DE cells in previous protocols [Kubo et al., 2004; D'Amour et al., 2005; Tada et al., 2005]. When day 2 SF-EBs were treated with Activin A (50 ng/ml) for 2–4 more days, we detected higher $Cxcr4^+c\text{-}Kit^+$ cell population (29–36%, n = 3) that peaked on day 4 and decreased thereafter (Fig. 2A). During this process, expression of pluripotent gene Oct4 was down-regulated, and expression of epiblast-specific marker Fgf5 was robust on Day 2; Later, following temporary expressions of primitive streak-specific genes (Mixl1, Gsc, Cxcr4), DE-specific genes (Cer1, Foxa2, Sox17), and mesoderm-specific genes (Flk1 and Gata1) were up-regulated on day 4 and persisted on Day 5, while expression level of Afp was not significant (Fig. 2B). The sequential gene expression dynamics recapitulated those patterns in gastrulation and DE development [McGrath et al., 1999; Tam and Loebel, 2007; Arnold and Robertson, 2009]. Furthermore, when day 2 SF-EBs were treated by different doses of Activin A (0, 1, 10, 25, 50, 100 ng/ml) for following 2 days, expression of both primitive streak and DE specific genes were up-regulated significantly and dose-dependently on Day 4, although expression of extra-embryonic visceral endoderm (VE) specific gene Sox7 was also slightly increased (Fig. 2C). Interestingly, primitive streak-specific and early mesoderm-related gene T was up-regulated by lower dose while down-regulated by higher dose of Activin A treatment (Fig. 2C), an expression pattern similar to in vivo [Latinkic et al., 1997]. Meanwhile, expression of posterior primitive streak-derived hematopoietic marker Gata1 [Kubo et al., 2004] was up-regulated by Activin A at lower dose, but down-regulated by Activin A at higher dose (Fig. 2C). Expressions of

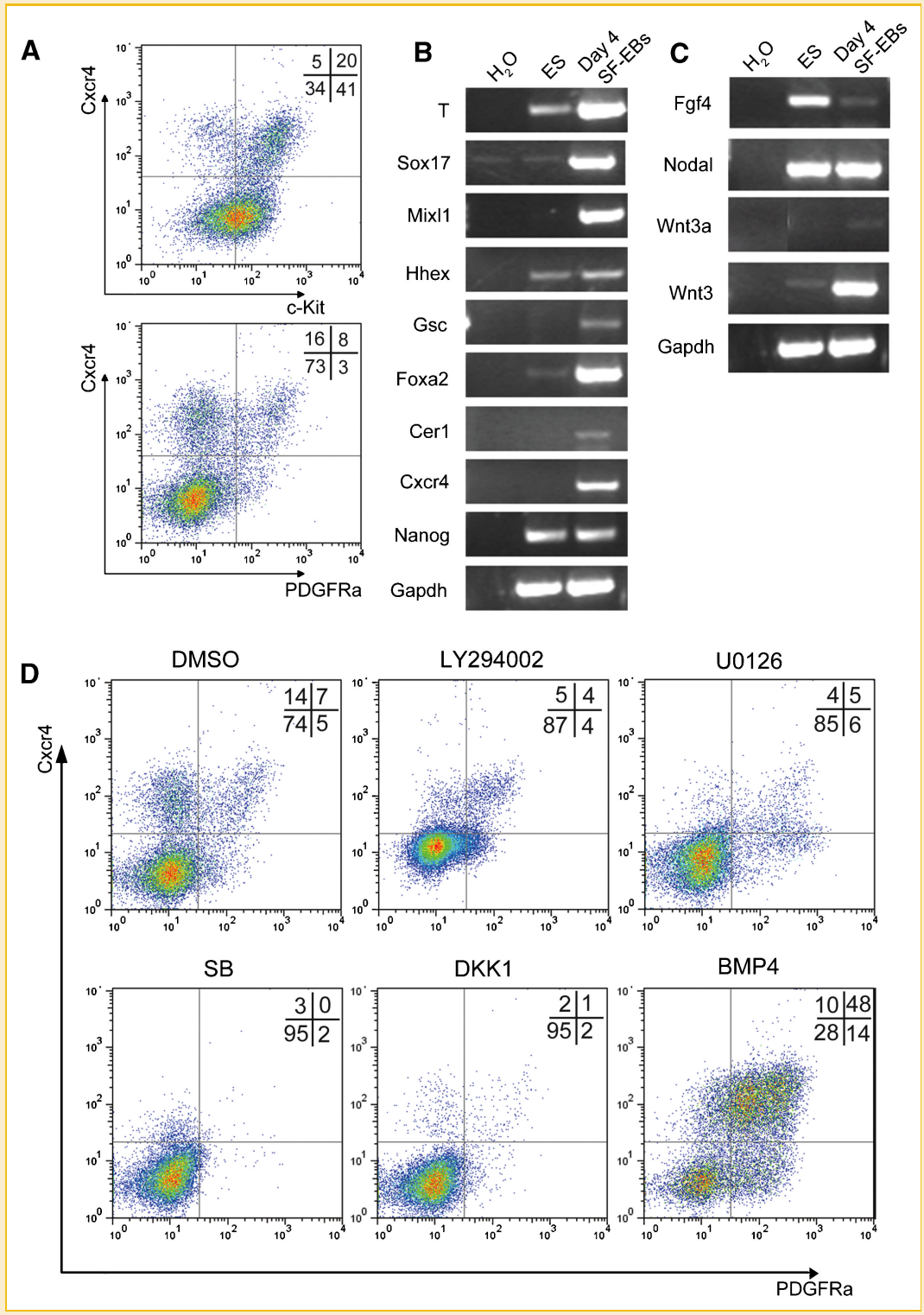

Fig. 1. DE induction in day 4 SF-EBs without exogenous stimulus. (A) FACS analysis revealed small populations of Cxcr4$^+$c-Kit$^+$ (18–20%, n = 3) or Cxcr4$^+$PDGFRa$^-$ (16–18%, n = 3) cells in day 4 SF-EBs without exogenous stimulus. (B, C) RT-PCR analysis revealed expressions of DE-specific genes (B) and signaling molecules (C) in day 4 SF-EBs. (D) Endogenous multiple signaling pathways regulate DE induction in day 4 SF-EBs. Dkk1 (100 ng/ml), BMP4 (5 ng/ml), SB431542 (10 μM), U0126 (10 μM) or LY294002 (20 μM) was added into day 2 SF-EBs, and the proportions of day 4 Cxcr4$^+$PDGFRa$^-$ cell populations were analyzed by FACS and shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

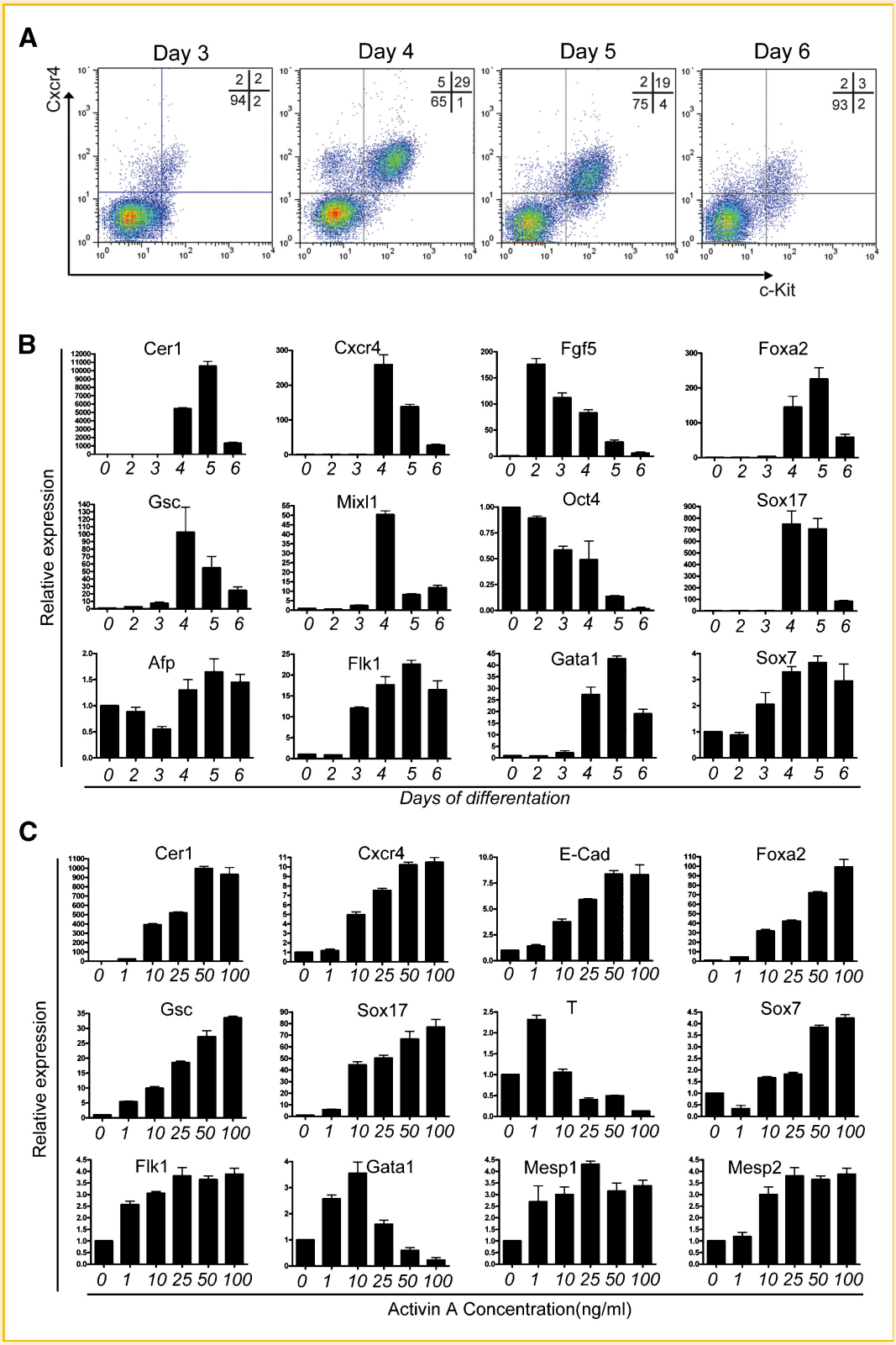

Fig. 2. Activin A treatment promotes DE induction in day 4 SF-EBs. (A, B) day 2 SF-EBs were stimulated with Activin A (50 ng/ml) for 2 days, the proportions of $Cxcr4^{+}c\text{-}Kit^{+}$ cell populations at indicated time were analyzed by FACS (A), and the dynamics of gene expressions from day 0 to day 6 were analyzed by Q-PCR (B). The results were calculated by normalizing to those in the day 0 ES cells, data represent mean ± standard deviation, n = 3. (C) day 2 SF-EBs were stimulated with different doses of Activin A (0, 1, 10, 25, 50, 100 ng/ml) for 2 days, gene expressions in day 4 SF-EBs were analyzed by Q-PCR. The results were calculated by normalizing to those in the day 4 untreated SF-EBs, data represent mean ± standard deviation, n = 3. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cardiovascular progenitor markers Flk1, Mesp1, and Mesp2 [Kattman et al., 2006; Bondue et al., 2008] were slightly up-regulated by Activin A in a dose-dependent manner (Fig. 2C). These gene expression patterns in our results were consistent with previous findings, in which lower dose of Activin A induced a posterior primitive streak fate, while higher dose of Activin A induced an anterior primitive streak fate from ES cells in differentiation [Gadue et al., 2006]. Collectively, these results showed that exogenous Activin A treatment promotes DE induction and generates comparable DE cell populations (~30–40%) in day 4 in SF-EBs as in previous studies [Tada et al., 2005; Gouon-Evans et al., 2006].

CHEMICAL ACTIVATION OF CANONICAL WNT SIGNALING SYNERGIZES WITH ACTIVIN A TO PROMOTE DE INDUCTION AT HIGHER EFFICIENCY

The canonical Wnt signaling has indispensable roles in establishment of primitive streak and specification of mesoderm progenitors [Lickert et al., 2002; Kelly et al., 2004]. It is also crucial for induction of mouse ES cell-derived primitive streak-like cells [Gadue et al., 2006]. In our system, inhibition of canonical Wnt signaling also blocked DE induction in SF-EBs (Fig. 1D), we thus investigated whether activation of Wnt signaling would synergize with Activin A to promote DE induction. To activate canonical Wnt signaling, we used easily available chemical LiCl instead of recombinant Wnt 3a proteins. Although single addition of LiCl (2 mM) on day 2 did not induce higher DE cell population (data not shown), addition of LiCl (2 mM) with Activin A (50 ng/ml, A50L2) did selectively and significantly induce higher proportions of $Cxcr4^+c\text{-}kit^+$ or $Cxcr4^+$E-Cadherin$^+$ cell populations in day 4 SF-EBs (65–71% for "A50L2", n = 3, Fig. 3A and B); Notably, when different doses of LiCl were added, the increase in the proportions displayed a LiCl dose-dependent manner (Fig. 3A and B), although substantial cell death was observed at higher concentrations (5 and 10 mM) (Supplementary Fig. 2B). The percentages of $Cxcr4^+c\text{-}Kit^+$ DE cell population kept on increase, the percentages of $Cxcr4^-$ cells kept on decrease (Fig. 3B). However, the percentages of $Cxcr4^+c\text{-}Kit^-$ populations did not change significantly during the LiCl-induced activation of Wnt pathway (Fig. 3B). Consistently, in response to higher dose LiCl addition, expression of E-Cadherin, Gsc, Cxcr4, and Sox17 were up-regulated; expression of epiblast-specific gene Fgf5 was down-regulated, while the expressions of Sox7, Flk1, and Gata1 were influenced unremarkably (Fig. 3C). These results indicated that $Cxcr4^+c\text{-}Kit^+$ cell population was selectively increased after induction from unsynchronized $Cxcr4^-$ cells, but not from $Cxcr4^+c\text{-}Kit^-$ mesoderm populations without influence of LiCl treatment. Interestingly, addition of LiCl also up-regulated T expression (Fig. 3C), consistent with it being a direct target of canonical Wnt signaling during gastrulation [Yamaguchi et al., 1999; Galceran et al., 2001]. Moreover, when LiCl (2 mM) was added on day 2 with lower dose of Activin A (25 ng/ml), there was still significant increase in the proportions of $Cxcr4^+c\text{-}Kit^+$ or $Cxcr4^+$E-Cadherin$^+$ cell populations in day 4 SF-EBs (Supplementary Fig. 3). Thus, addition of LiCl (2 mM) with Activin A (AL2) could induce higher DE cell population than Activin A (A) in day 4 SF-EBs.

INHIBITION OF ENDOGENOUS BMP4 SIGNALING FURTHER PROMOTES DE INDUCTION IN THE PRESENCE OF ACTIVIN A/LICL

In vitro studies using mouse ES cells revealed that BMP signaling pathway exerts a dominant effect to induce posterior primitive streak cells, and inhibits the induction of Gsc^+ mesendoderm cells by antagonizing Nodal signaling [Tada et al., 2005; Nostro et al., 2008]. In our system, addition of even low dose of BMP4 (5 ng/ml) on day 2 generated a significant population of $Cxcr4^+PDGFRa^+$ cells (Fig. 1D), and reduced Activin A-induced $Cxcr4^+PDGFRa^-$ cell population in day 4 SF-EBs (data not shown), which led us to investigate whether there was residual or endogenous BMP4-mediated inhibitory activity in Activin A (A) or Activin A/LiCl (AL2) -induced SF-EBs. We then added recombinant Noggin protein (200 ng/ml) to inhibit BMP4 signaling together with Activin A (A) or Acivin A/LiCl (AL2) at Day 2, and accordingly analyzed the proportions of DE cell populations and DE-specific gene expressions in day 4 SF-EBs. Although addition of Noggin with Activin A (AN) did not induce higher expressions of DE-specific genes than Acitivin A (A), addition of Noggin with Activin A/LiCl (AL2N) did induce higher expressions of DE-specific genes and lower expression of Sox7 than Activin A/LiCl (AL2) (Fig. 4A). Furthermore, without significantly affecting the cell viabilities (Supplementary Fig. 2B), addition of Noggin slightly but significantly (8–10%, n = 3) increased the proportions of both Activin A (A) -induced and Activin A/LiCl (AL2) -induced $Cxcr4^+PDGFRa^-$ cell populations in day 4 SF-EBs, with the highest proportion approaching 80% (80–85% for "AL2N", n = 3, Fig. 4B and C). These results suggest that inhibition of endogenous BMP4 signaling promotes DE induction in day 4 SF-EBs.

Finally, after Activin A/LiCl/Noggin (AL2N) -induced day 4 SF-EBs were sorted into different cell populations ($Cxcr4^+c\text{-}kit^+$, $Cxcr4^+c\text{-}kit^-$, $Cxcr4^+PDGFRa^-$, $Cxcr4^+PDGFRa^+$, Fig. 5A and B), we analyzed and compared expressions of various linage markers. Results of RT-PCR analysis showed that day 4 $Cxcr4^+c\text{-}Kit^+$ or $Cxcr4^+PDGFRa^-$ populations selectively expressed DE-specific genes (Cer1, Foxa2, Sox17, Gata4, and Hhex), while the $Cxcr4^+c\text{-}Kit^-$ or $Cxcr4^+PDGFRa^+$ populations selectively expressed mesoderm progenitor-specific genes (Gata1, Flk1); However, neither the sorted cell populations nor unsorted EBs expressed neural progenitor markers (Pax6, Sox1) (Fig. 5C). The above results indicated in our EB system that did not favor neural induction, day 4 $Cxcr4^+c\text{-}Kit^+$ or $Cxcr4^+PDGFRa^-$ populations could represent enriched DE cells, and the $Cxcr4^+c\text{-}Kit^-$ or $Cxcr4^+PDGFRa^+$ populations contained mainly mesoderm cells.

Taken together, we have optimized and established an induction cocktail (Activin A + LiCl + Noggin, AL2N) to efficiently derive DE cells in day 4 SF-EBs.

HIGHER DIFFERENTIAL POTENTIAL OF DAY 4 ACTIVIN A/LICL/NOGGIN (AL2N)-INDUCED DE CELLS

To investigate and compare the differential potential of derived DE cells, day 4 SF-EBs were disassociated and replated in culture to undergo hepatic or pancreatic differentiations, respectively. On one hand, under the optimized hepatic induction condition for 4 days (Day 8), replated cells differentiated into mainly epithelial colonies and mesenchymal fibroblast-like cells, and the epithelial colonies from Activin A/LiCl/Noggin (AL2N)-induced SF-EBs were bigger

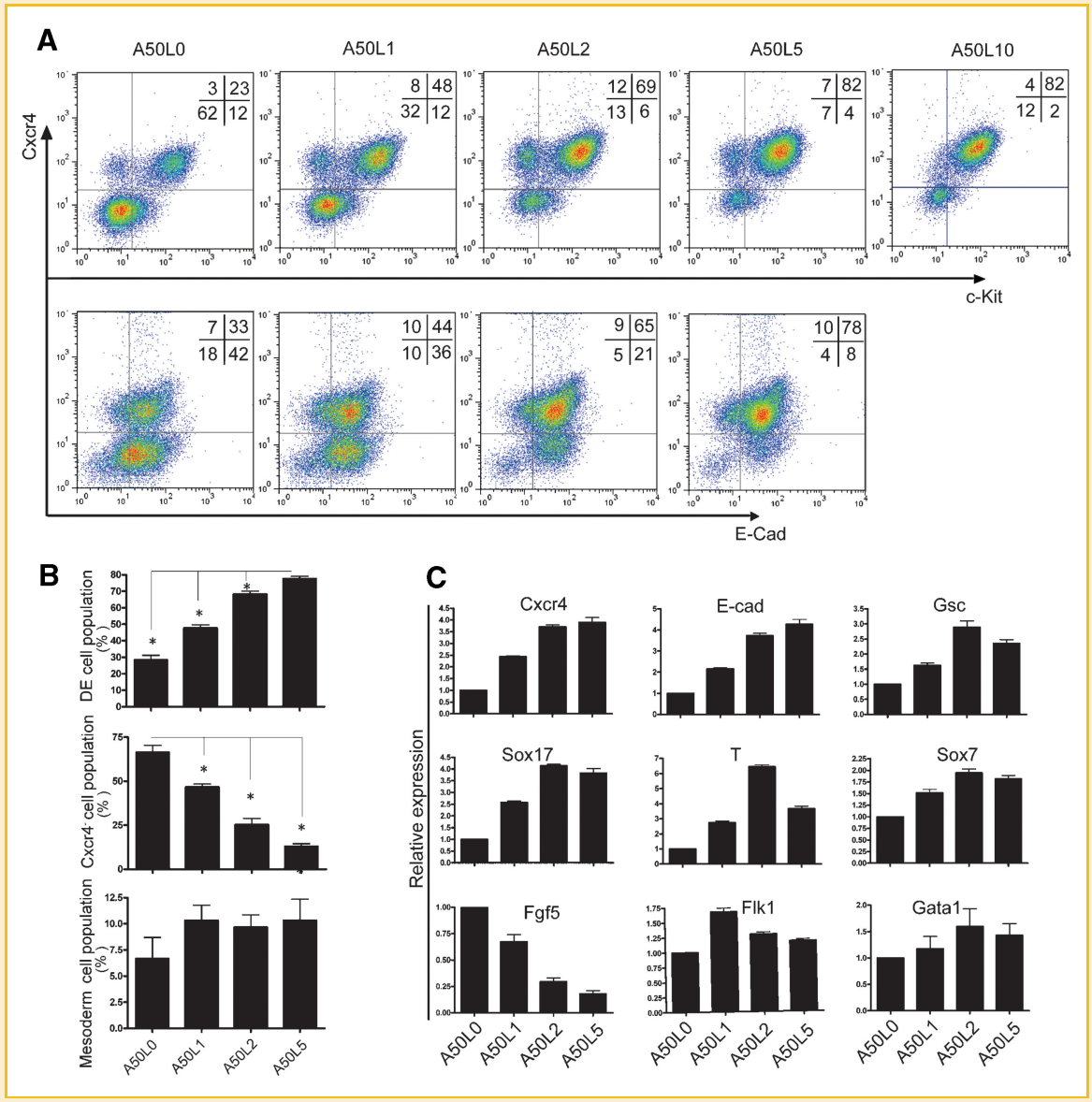

Fig. 3. Chemical activation of canonical Wnt signaling by LiCl promotes DE induction in day 4 SF-EBs with Activin A. day 2 SF-EBs were treated for 2 days with Activin A (50 ng/ml) and different dose of LiCl (0, 1, 2, 5, 10 mM) (A50L0, A50L1, A50L2, A50L5, and A50L10), the proportions of Cxcr4$^+$c-Kit$^+$or Cxcr4$^+$E-Cadherin$^+$ cell populations were analyzed by FACS and shown in (A), a statistic summary on the DE proportions, Cxcr4$^-$ cells, and Cxcr4$^+$c-Kit$^-$ cell populations was shown in (B), and gene expressions analyzed by Q-PCR were shown in (C). The results were calculated by normalizing to those in day 4 Activin A-treated SF-EBs, data represent mean ± standard deviation, n = 3. *, $P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and more homogenous than those from Activin A/Noggin (AN)-induced SF-EBs (Supplementary Fig. 4). The epithelial colonies were identified as hepatic colonies as they expressed Afp, Foxa2, Ck18, and Ck19, genes related to hepatoblasts (Fig. 6A). Following specification, the hepatic colonies continued to proliferate and outgrew to form homogenous epithelial monolayers at day 12 of induction (Fig. 6B), which expressed Aat, Afp, Alb, and Ttr, but not Cps1 or Cyp7a1 (Fig. 6C), indicating they were hepatoblast-like progenitor cells (HPCs). We recently identified and enriched mouse ES cell-derived HPCs in a c-Kit$^-$EpCAM$^+$ cell population with liver repopulation capacity [Li et al., 2010]; using this combination of surface markers, we detected approximately 25% (23–26%, n = 3) c-Kit$^-$EpCAM$^+$ cells in hepatic cultures from Activin A/Noggin (AN)-induced SF-EBs, while the proportion of this population was significantly higher (41–47%, n = 3) in those from Activin A/LiCl/Noggin (AL2N)-induced SF-EBs (Fig. 6D), demonstrating a higher hepatic developmental potential. On the other hand, under a 6-day pancreatic induction condition, we observed cells from both SF-EBs differentiated into epithelial cell clusters expressing Pdx1, one of the early pancreatic genes, and more Pdx1$^+$ cells were detected in the clusters from Activin A/LiCl/Noggin (AL2N)-induced SF-EBs (Fig. 7A); Furthermore, RT-PCR analysis revealed expressions of other pancreatic progenitor markers in day 10 cells, such as NeuroD, Nkx2-2, Nkx6-1, and Isl1 (Fig. 7B), an indication of pancreatic fates.

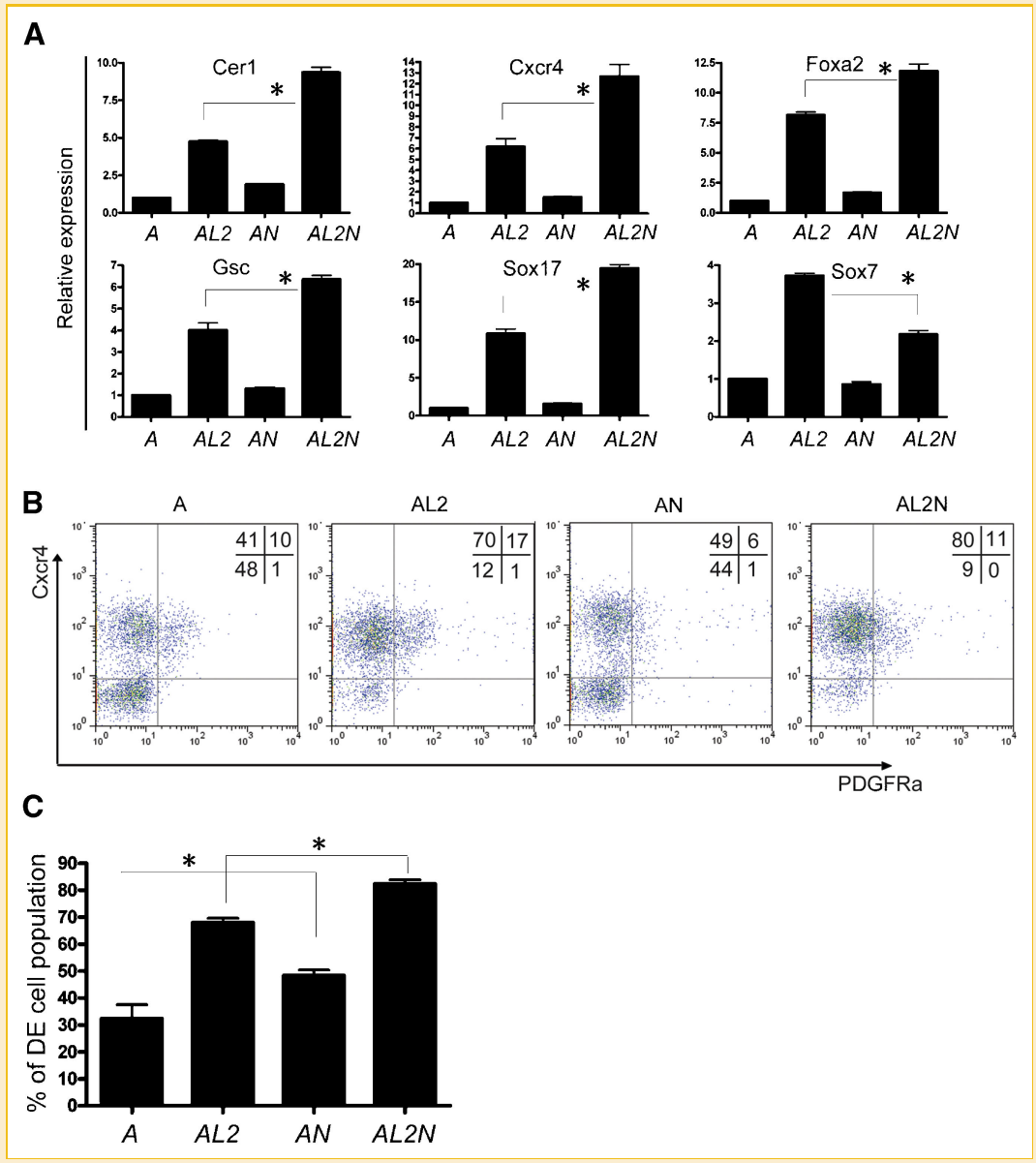

Fig. 4. Combined Activin A/LiCl/Noggin induces enriched DE cell population in day 4 SF-EBs. day 2 SF-EBs were cultured for 2 days under the indicated conditions: A, Activin A (50 ng/ml); AL2, Activin A (50 ng/ml) + LiCl (2 mM); AN, Activin A (50 ng/ml) + Noggin (200 ng/ml); AL2N, Activin A (50 ng/ml) + LiCl (2 mM) + Noggin (200 ng/ml), and gene expressions in day 4 SF-EBs were analyzed by Q-PCR (A), the proportions of $Cxcr4^{+}PDGFRa^{-}$ cell population were analyzed by FACS (B), and statistically summarized in (C). The results in (A) were calculated by normalizing to those in day 4 EBs in condition "A", data represent mean ± standard deviation, n = 3. *, $P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Based on these results, we concluded that DE cells in both Activin A/Noggin (AN)-induced and Activin A/LiCl/Noggin (AL2N)-induced day 4 SF-EBs have the potential to differentiate into hepatic and pancreatic lineages; the higher differential potential of Activin A/LiCl/Noggin (AL2N)-induced DE cells was also consistent with their higher proportions in day 4 SF-EBs (Fig. 4B and C).

DISCUSSION

Pluripotent stem cell-derived functional hepatic cells or pancreatic cells will provide unlimited cell sources for cell replacement therapies in future. Because DE cells are the progenitors for these two types of cells, the protocols to generate DE cells at a higher percentage would benefit to efficiently derive functional hepatic cells or pancreatic cells [Murry and Keller, 2008]. Accumulative genetic and biochemical studies have revealed that Nodal, Wnt, FGF, and BMP signaling pathways are involved in early gastrulation and subsequent development of DE during embryogenesis in vivo [Tam and Loebel, 2007; Arnold and Robertson, 2009]. Similarly, their conserved roles in induction of primitive streak-like or DE-like cells from ES cells, which correlated with the embryogenesis process, have also been independently proved in various induction protocols [Kubo et al., 2004; Tada et al., 2005; Gadue et al., 2006; Gouon-

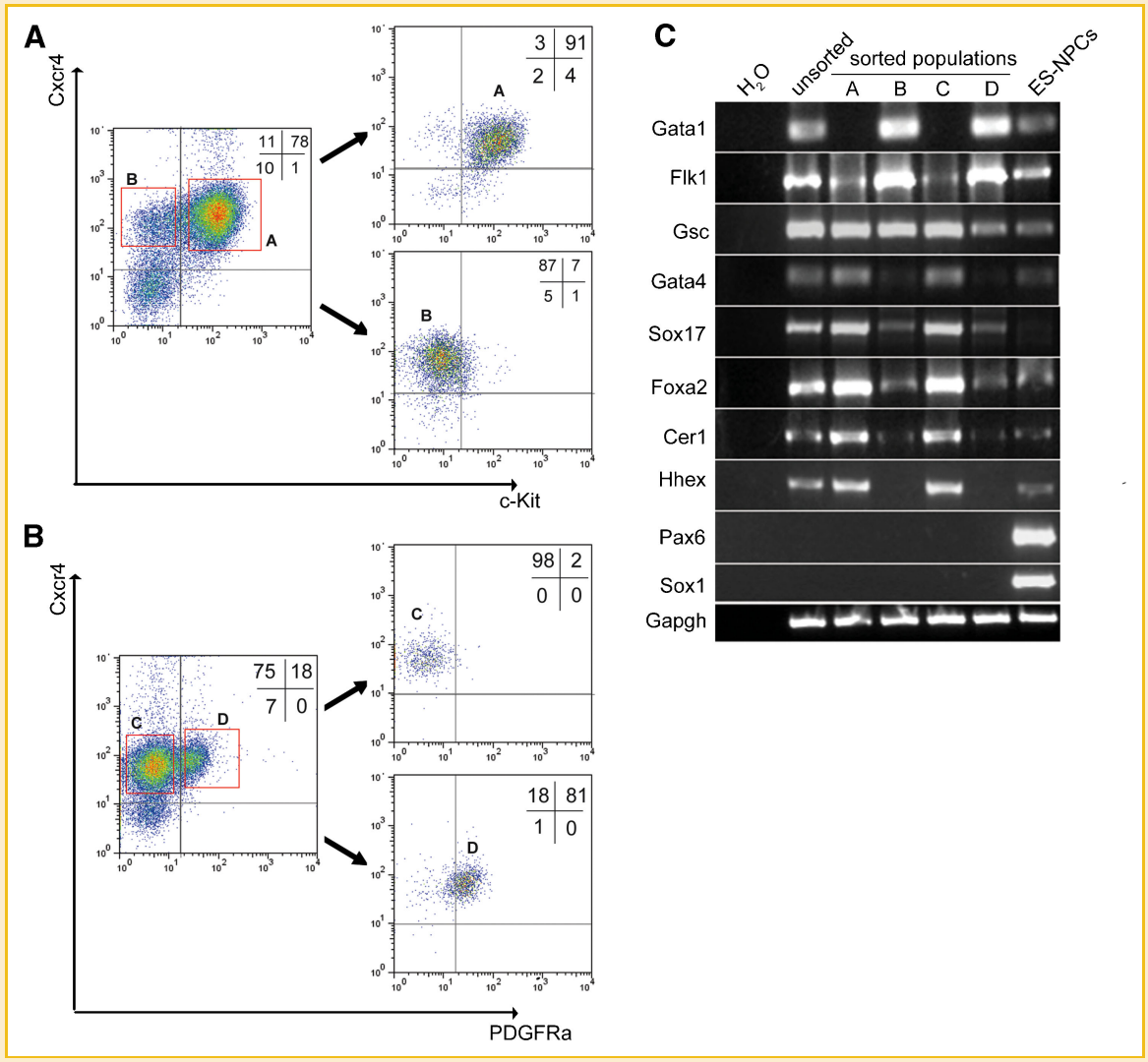

Fig. 5. Analysis of gene expression on FACS-sorted cell populations from day 4 SF-EBs. Activin A/LiCl/Noggin (AL2N)-induced day 4 SF-EBs were sorted into different populations: Cxcr4$^+$c-Kit$^+$ (population "A"), Cxcr4$^+$c-Kit$^-$ (population "B"), Cxcr4$^+$PDGFRa$^-$ (population "C"), and Cxcr4$^+$PDGFRa$^+$ (population "D"). The purities of sorted cell populations were confirmed by FACS analysis (A and B). (C) RT-PCR (30 cycles) analysis revealed differential expressions of various lineage markers in each sorted cell population. Complementary DNA from mouse ES cell-derived neural progenitor cells (ES-NPCs) was used as controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Evans et al., 2006]. Based on these, in the present study we systematically investigated the crosstalk of these pathways and confirmed their actions in induction of DE cells from ES cells in an EB model. Furthermore, we explored how to recapitulate their activations to improve DE cell differentiation from ES cells. We successfully modulated these pathways via a combined Activin A/LiCl/Noggin (AL2N) treatment, which enabled us to obtain higher proportion of DE cells with both hepatic and pancreatic differentiation capacities (Supplementary Fig. 5).

Our work has three aspects of improvement: first, we applied a suspension EB culture system, which is easy to scale up and to manipulate. In contrast, cells cultured adherently can be affected by factors including cell density, coated matrices, which can be avoided by suspension culture. A recent study showed pluripotent human ES cells can be derived, propagated, and differentiated in suspension [Steiner et al., 2010], which represents a significant progress in large-scale production of differentiated cells for clinical applications. Our present protocol is different from previous ones with the steps of disassociation and re-aggregation of cells in DE induction [Gouon-Evans et al., 2006], Instead, day 2 SF-EBs were directly re-suspended and treated by combined Activin A/LiCl/Noggin, which significantly improved the percentage of DE cells. Second, we demonstrated that the combined manipulation of endogenous signaling pathways could lead to induction of DE cells at significantly higher efficiency than previous reports. Although it was indicated in two independent studies that activations of endogenous signaling pathways for either FGF or canonical Wnt were required for generation of DE cells after Activin A induction [Gadue et al., 2006; Morrison et al., 2008], it has been unknown whether exogenous activation of these signaling pathways can

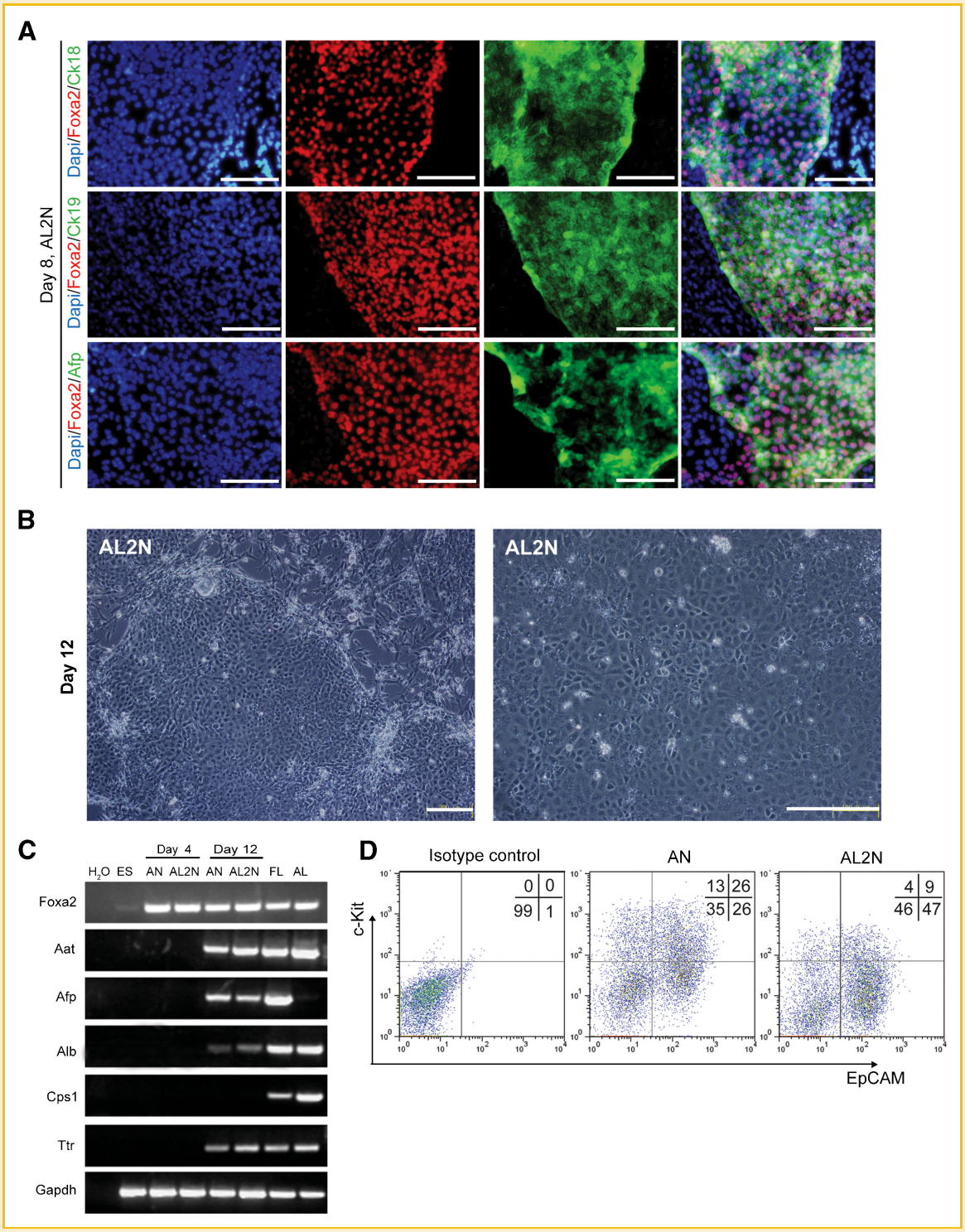

Fig. 6. Hepatic differentiation from day 4 SF-EBs. Activin A/Noggin (AN)- or Activin A/LiCl/Noggin (AL2N)- induced day 4 SF-EBs were disassociated and replated for 4–8 days to undergo hepatic differentiation, immunostainings were carried out on 4 days after replating (Day 8), typical immunostaning views of hepatic cells from Activin A/LiCl/Noggin (AL2N)- induced SF-EBs were shown in (A), and morphologies of these cells at day 12 were shown in (B). (C, D) RT-PCR analysis revealed the expressions of hepatoblast-related genes in day 12 hepatic cells, and the proportions of c-Kit$^-$EpCAM$^+$ hepatic progenitor cells were analyzed by FACS, respectively. FL, fetal liver; AL, adult liver. Scale bars: 100 μm (A), 200 μm (B).

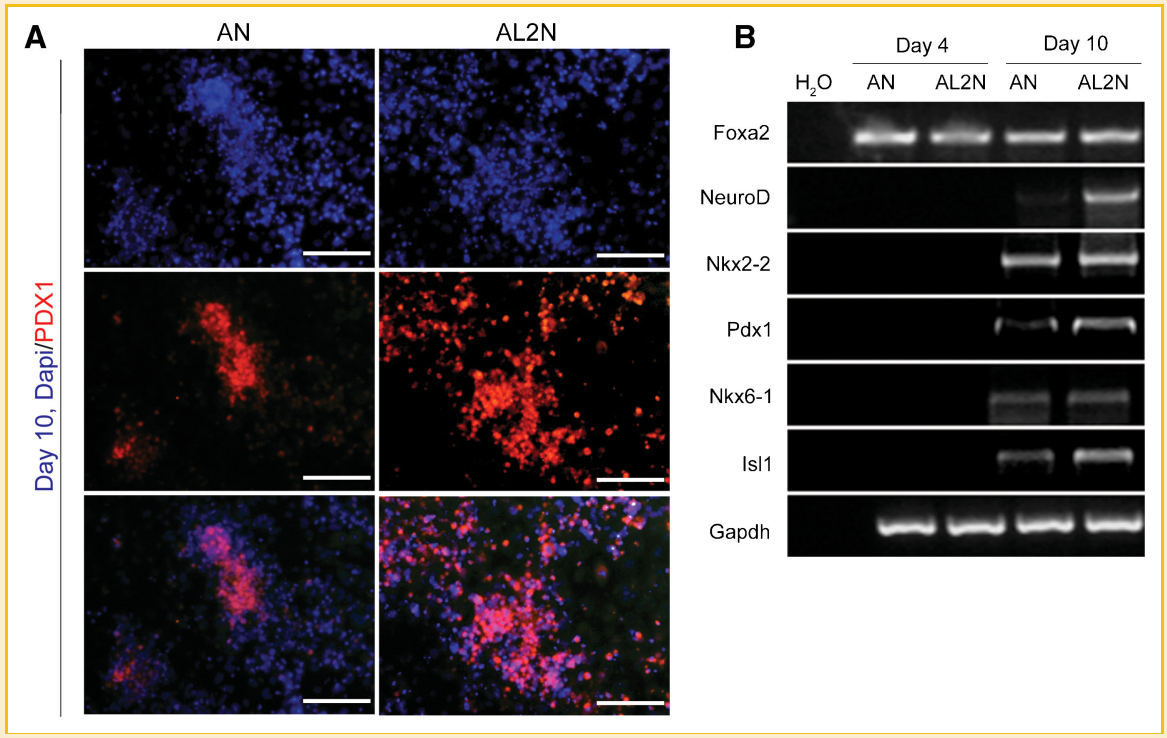

Fig. 7. Pancreatic differentiation from day 4 SF-EBs. Activin A/Noggin (AN)—or Activin A/LiCl/Noggin (AL2N)—induced day 4 SF-EBs were disassociated and replated for 6 days to undergo pancreatic differentiation. (A) Immunostainings were carried out on 6 days after replating (Day 10), representative immunostaning views with anti-Pdx1 antibody were shown. (B) RT-PCR analysis revealed the expressions of pancreatic progenitor genes in day 10 differentiated cells. Scale bars: 100 μm.

synergize with Activin A to induce DE cell population to a higher level. Our data suggest that activation of canonical Wnt signaling has a synergistic effect with Activin A in induction of DE cells from ES cells, and inhibition of BMP4 signaling result an additional improvement of DE cell induction from ES cells. These synergistic effects were further confirmed by the facts that single activation of Wnt, FGF signaling or inhibition of BMP4 signaling was not sufficient to induce DE cells (data not shown), supporting a fundamental role of Nodal signaling in DE induction. Interestingly, although activation of FGF signaling was found in both Activin A-induced and Activin A/LiCl/Noggin (AL2N)-induced day 4 SF-EBs, exogenous addition of either FGF2 or FGF4 had no detectable synergistic effect on DE induction (data not shown), indicating that endogenous FGF signaling is sufficient for DE induction under these conditions. Third, we used LiCl instead of Wnt3a ligand to activate canonical Wnt signaling to induce DE cells with Activin A/Noggin. Currently, recombinant Wnt3a ligand protein with higher purity and activity has not been available yet [Clevers, 2006]. In fact, LiCl as the well-characterized chemical has been widely applied to activate canonical Wnt signaling during studies of animal embryonic development or in vitro pharmacological studies [Klein and Melton, 1996; Clement-Lacroix et al., 2005]. Generally, chemicals are more stable and easier to apply treatment than recombinant proteins, can be synthesized in large quantity and with high purity, and can be conveniently added or removed. Therefore, chemicals especially permeable small molecules hold great potential for therapeutic applications in future [Xu et al., 2008]. Several small molecules have been successfully used in ES cells differentiation processes [Borowiak et al., 2009; Chen et al., 2009; Zhu et al., 2009]. Among them, two molecules named as IDE1 and IDE2 were found to efficiently induce DE cells from ES cells [Borowiak et al., 2009]. Furthermore, it would be more advantageous and economical to use chemicals/small molecules than recombinant proteins in large-scale cell culture for future industrial or clinical applications [Xu et al., 2008]. Recently, Activin A and Wnt3a were applied together under monolayer culture to induce DE cells from human ES cells [Kroon et al., 2008]. In another study, blockade of BMP signaling were shown to abolish mesoderm generation and to induce a cell fate towards the anterior primitive streak progenitors from human ES cells [Sumi et al., 2008]. Thus, it would be valuable to combine activations of these signaling pathways via growth factors and chemicals in order to generate higher human ES cell-derived DE cell populations for further differentiation approaches, either to derived human hepatic cells or to human pancreatic cells.

In summary, we established a method to efficiently derive mouse ES cell-derived functional DE cells by combined Activin A/LiCl/Noggin (AL2N) treatment. The derived DE cells provide a basis for subsequent hepatic or pancreatic differentiation, and our protocol will also be greatly helpful to generate ES cell-derived hepatocytes and ES cell-derived pancreatic cells for future regenerative medicine.

ACKNOWLEDGMENTS

We are grateful to Pingyu Liu for mouse ES cell-derived neural progenitor cell cDNA, and to Xiaowen Lv for sharing the Pdx1 antibody.

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