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A novel stepwise differentiation of functional pancreatic exocrine cells from embryonic stem cells

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10 **Title: A novel stepwise differentiation of functional pancreatic exocrine cells from**
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13 **embryonic stem cells**
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19 Keyword: Pancreatic exocrine cells, mouse embryonic stem cells (ESCs), stepwise
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22 differentiation, Amylase activity, FGF7
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Abstract

Cultivation of functional pancreatic cells isolated from adult mammalian pancreas remains difficult. We developed a differentiation protocol that gradually induced the formation of mouse pancreatic exocrine cells from embryonic stem cells (ESCs). This process mimicked *in vivo* pancreatic development by directing cells through definitive endoderm (DE), gut tube endoderm (GTE), and pancreatic progenitor cells to differentiated cells that expressed pancreatic exocrine enzymes. Mouse ESCs were cultured in hanging drops to form embryoid bodies (EBs). Treatment of EBs with activin A induced the formation of DE cells that expressed marker mRNAs *Goosecoid* and *Mixl1* and which were double-positive with *Foxa2* and *Sox17* proteins. Subsequent treatment of the DE cells by retinoic acid induced the formation of GTE cells that expressed the specific marker *Hnf1b*. Expression of *Goosecoid* and *Mixl1*, was downregulated during this period. Fibroblast growth factor 7 (FGF7) promoted differentiation of PDX1-expressing pancreatic progenitor cells that also expressed *Foxa2* mRNA, an endodermal marker, suggesting derivation from the DE cells. Exocrine cell differentiation was induced with FGF7, glucagon-like peptide-1, and nicotinamide. The differentiated cells expressed mature pancreatic exocrine cell mRNAs, such as *Amylase*, *Elastase*, and *Carboxypeptidase A*. Additionally, they produced pancreatic

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7 elastase, amylase, carboxypeptidase A, and chymotrypsin proteins that were identified
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10 in cytoplasmic granules by immunocytochemistry. Active amylase was released into the
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12 medium. Moreover, FGF7 was associated with differentiation of pancreatic exocrine
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16 cells. The findings reported here offer a novel and effective process to develop
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19 pancreatic exocrine cells from ESCs.
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INTRODUCTION

Pancreatic acinar cells have only minimal proliferative potential *in vitro* and are difficult to culture [1]. Furthermore, there are very few acinar tumor cell lines, and they lack normal physiological functions. In contrast, embryonic stem cells (ESCs) have a virtually endless replicative capacity and have the potential to differentiate into various cells types *in vitro*. Thus they represent an unlimited starting material to generate differentiated cells for preclinical studies and food safety tests. Although some protocols can induce differentiation of pancreatic exocrine cells from ESCs, they produce few cells that achieve any degree of functional maturity [2,3]. Recently, directed differentiation was achieved by incubation of ESCs overexpressing PTF1a and Mist1 with conditioned media of cultured fetal pancreatic rudiments [4].

The molecular mechanism(s) that promote the commitment of stem cells into pancreatic acinar cells remain largely unknown [4]. Activin A, a disulfide-stabilized protein that belongs to the transforming growth factor β (TGF- β) superfamily, is important for early definitive endoderm development and can induce ESCs to differentiate into them [5,6]. *All-trans* retinoic acid (RA) is a well-characterized signaling molecule that acts in anteroposterior patterning of neuroectoderm and mesoderm in

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7 vertebrates [7]. RA is also necessary for formation of the pancreas in *Xenopus laevis*,
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10 zebrafish, and mice [8-10]. A recent study demonstrated that sequential treatment of
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12 mouse ESCs with activin A and RA could induce pancreatic progenitor cells [11]. Finally,
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14 fibroblast growth factor 7 (FGF7) is expressed in the pancreatic mesenchyme and
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16 promotes the growth, morphogenesis, and cytodifferentiation of pancreatic exocrine
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22 cells [12,13].
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25 In this study, we developed a stepwise protocol to promote differentiation of mouse
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28 ESCs into functional pancreatic exocrine cells by mimicking signals that occur during
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30 embryonic development. This protocol used molecular signaling by activin A, RA, and
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33 FGF7 to direct cells through progressive stages resembling the definitive endoderm
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36 (DE) and the gut tube endoderm (GTE). It also induced the development of cells
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39 expressing Pdx1, a typical marker of pancreatic progenitor cells. Ultimately,
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42 Pdx1-positive cells differentiated into cells that expressed exocrine enzymes.
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50 ***Materials and Methods***

51 52 53 **Cell culture and differentiation conditions**

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56 Undifferentiated mouse ESCs (129/sv strain, Cell and Molecular Technologies,
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59 Philipsburg, NJ, USA) were maintained on mitomycin C-inactivated SNL feeder cell
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7 layers in 0.1% gelatin-coated tissue culture dishes. The cells were incubated in
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10 Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Rockville, MD, USA) with
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12 15% knockout-serum replacement (Gibco), 100 μ M nonessential amino acids (Gibco), 1
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14 mM sodium pyruvate (Gibco), 10^3 U/ml of leukemia inhibitory factor (LIF; Chemicon,
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16 Temecula, CA, USA), 100 μ M 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 50 U/ml
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18 penicillin, and 50 μ g/ml streptomycin (Gibco).
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25 To induce differentiation, the ESCs were cultured in feeder-free gelatin-coated
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27 tissue culture dishes using the "hanging drop" method to form embryoid bodies (EBs,
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29 Fig. 1). After 5 days, EBs were plated on gelatin-coated tissue culture dishes in EB
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31 medium composed of Iscove's modified Dulbecco's medium (IMDM, Gibco)
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33 supplemented with 20% fetal bovine serum (FBS), 100 μ M nonessential amino acids, 1
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35 mM sodium pyruvate, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 100 μ M
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37 2-mercaptoethanol. The following day, the EBs were incubated in EB medium with 100
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39 ng/ml activin A (Sigma). After 24 h, activin A was removed, and the EBs were incubated
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41 6 h in EB medium alone, followed by treatment with 1 μ M RA (Sigma). After 24 h, the
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43 medium was switched to serum free medium: DMEM/F12 (1:1) supplemented with N2
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45 supplement (Invitrogen, Carlsbad, CA, USA), B27 supplement (Invitrogen), and 15
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47 ng/ml FGF7 (R&D systems, Inc., Minneapolis, MN, USA). After 3 days, the EBs were
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7 re-plated on gelatin-coated 24-well plates and incubated in DMEM/F12 supplemented
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10 with N2 supplement, B27 supplement, 15 ng/ml FGF7, 10 mM nicotinamide (NA,
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12 Sigma), and 100 ng/ml glucagon-like peptide-1 (GLP-1, 7-36 amide; Bachem AG,
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14 Torrance, CA, USA) to induce differentiation of pancreatic exocrine cells (Fig. 1). In
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19 control cultures, the EBs were treated similarly but without inducing factors.
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25 **Reverse transcriptase polymerase chain reaction (RT-PCR) analysis**

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28 Total cellular RNA was isolated using TRIzol reagent (Invitrogen). Complementary
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31 DNA (cDNA) was synthesized from total RNA using a PrimeScript RT reagent Kit
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34 (Perfect Real Time, TaKaRa Bio Inc., Yokkaichi, Japan) for RT-PCR. The cDNA was
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37 amplified by PCR using specific primers (Table 1) for *Foxa2*, *Goosecoid*, *Hnf1b*, *Hlx9*,
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40 *Nkx6.1*, *Hnf6*, *Pdx1*, *Amylase*, *Elastase 1*, *Elastase 2*, *Carboxypeptidase A*,
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43 *Chymotrypsinogen B*, *P48*, *Mist-1* and β -*actin*.
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47 The PCR was initiated by a 10 min denaturation at 94°C. This was followed by 35
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50 cycles of heating at 94°C (30 sec), 53-65°C (30 sec), and 72°C (30 sec). The final
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53 extension was for 7 min at 72°C. β -*actin* was used as the invariant control. Water was
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56 used as a negative control in place of the cDNA template.
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Real-time PCR

Real-time PCR was carried out with primers (Table 2) for *Foxa2*, *Gooseoid*, *Brachyury*, *Mixl1*, *Hnf1b*, *Pdx1*, *P48*, *Amylase*, *Carboxypeptidase A*, *Chymotrypsinogen B*, *Elastase 1*, *Ins2 (Insulin 2)*, *Glucagon*, and β -*actin* using a SYBR Premix Ex Taq (Perfect Real Time, TaKaRa Bio) in a Thermal Cycler Dice Real Time System (TaKaRa Bio). The cDNA was amplified by a 10 min initial denaturation at 95°C, followed by 40 cycles of heating at 95°C (5 sec) and 60°C (30 sec). The total RNA levels were normalized to levels of β -*actin* mRNA.

Immunofluorescence

For immunofluorescence staining, cultured cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 min, permeabilized with 0.1% Triton X-100 in PBS, and treated with 1.5% normal donkey or goat serum. Then the cells were incubated with primary antibodies at 4°C overnight. Localization of antigens was visualized with IgG secondary antibodies conjugated with fluorescein or TRITC (Alexa 488 and 568, Molecular Probes, Leiden, The Netherlands). Afterwards, the cellular nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes). The following antibodies were used for immunostaining: rabbit

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6 anti-Foxa2 (1:800, Cell Signaling Technology, Inc., Beverly, MA, USA), mouse
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9 anti-Sox17 (1:100, R&D systems), rabbit anti-HNF1b (1:100, Santa Cruz Biotechnology,
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11 Inc., CA, USA), goat anti-PDX-1 (1:200, R&D systems), rabbit anti- α -amylase (1:500,
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13 Sigma), rabbit anti-pancreatic elastase (1:200, Elastin Products Co., Owensville, MO,
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18 USA), goat anti-carboxypeptidase A1 (CPA1, 1:100, Santa Cruz Biotechnology), and
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22 mouse anti-chymotrypsin (1:500, Abcam, Cambridge, UK). Non-immune serum was
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25 used as a negative control in place of the primary antibodies. Mouse fetal foregut and
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28 adult pancreas were used as positive controls to test the specificity of these antibodies.
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32 To estimate the efficiency of differentiation at the cell level, clusters were randomly
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34 selected, and the numbers of positive cells were counted at different stages of
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36 differentiation. The total cell number was determined by DAPI nuclear staining. When
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38 appropriate, data are given as mean \pm standard deviation obtained from at least five
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41 independent experiments.
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50 **Amylase activity assay**

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53 To test for the presence of secreted amylase activity, the induction medium was
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55 changed with fresh induction medium. After incubation for 24 hours, the conditioned
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58 medium was collected and centrifuged. Secreted amylase activity in the supernatant
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7 was measured with Liquid Amylase (CNP3) Reagent Set (Pointe Scientific, Canton,
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10 MI, USA) using α -amylase activity as the standard.
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19 **Results**

20 **Characterization of ESC-derived cells by early differentiation (Stages 1-3)**

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22 We developed a four step protocol for differentiation of ESCs to pancreatic exocrine
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25 enzyme-expressing cells. We focused first on generating DE, followed by
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28 PDX1-expressing cells, and finally, pancreatic exocrine cells. In Stage 1, mouse ESCs
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31 were transitioned through mesendoderm to DE by treatment of EBs with high
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34 concentrations of activin A (100 ng/ml). The mouse ESC-derived DE expressed *Foxa2*
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37 and *Gooseoid*, and the expression levels of these markers were increased by
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40 treatment with activin A compared with non-treated cells (Fig. 2A, B). In addition,
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43 real-time PCR analysis indicated that *Brachyury*, a mesendodermal marker, was highly
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46 expressed in Stage 1 and then sharply decreased afterwards. DE cell markers Sox17
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49 and *Foxa2* were also demonstrated by immunofluorescence (Fig. 2C).
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56 In Stage 2, activin A was removed, and 6 hours later the cells were treated with RA.
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59 The expression of *Gooseoid* and *Mixl1* mRNAs was downregulated (Fig. 2A, B). In
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7 contrast, the expression of *Hnf1b* mRNA, a GTE marker, was up-regulated (Fig. 2A, B).

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10 Consistent with the presence of *Hnf1b* mRNA, HNF1b-positive GTE-like cells were
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12 detected by immunostaining (Fig. 2D). Furthermore, the pancreatic endoderm and
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14 progenitor cell marker *Pdx1* mRNA was also up-regulated in this step (Fig. 2A, B) along
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16 with other pancreatic endodermal markers *Hlxb9* and *Hnf6* mRNA and the pancreatic
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18 progenitor marker *Nkx6.1* mRNA.
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25 In Stage 3, the cells were switched to a serum-free medium containing FGF7 for 3
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27 days. At the end of this period, the expression levels of the pancreatic progenitor cell
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29 markers *Pdx1*, *Nkx6.1* and *P48* were increased (Fig. 2A, B), and there was a increase in
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31 the level of Pdx1 (Fig. 2A, B). Moreover, immunostaining showed that PDX1 proteins
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33 were localized in the nuclei of FGF7-treated cells (Fig. 2E). Additionally, the
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35 PDX1-positive cells existed only in the *Foxa2*-positive cell population, suggesting the
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37 possibility that the PDX1-positive cells were derived exclusively from DE (Fig. 2E).
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39 Conversely, in the absence of FGF7, few cells expressed PDX1 (Fig. 2E), which in our
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41 cultures was essential to the transition of DE cells into pancreatic progenitor cells. This
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43 is consistent with studies that FGF7 is important for pancreatic development [12,13].
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59 **Induction of pancreatic enzyme-expressing cells from progenitor cells (Stage 4)**
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7 During Stage 4, FGF7, GLP-1, and NA were used to recruit PDX1-expressing
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10 progenitor cells to the pancreatic exocrine lineage. The expression of *Amylase*,
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12 *Elastase 1*, *Carboxypeptidase A*, and *Chymotrypsinogen B* mRNAs were continued
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14 through Stage 4, and *Elastase 2* mRNA was expressed later in Stage 4 (Fig. 3A).
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17 *Amylase mRNA* expression, used as a marker for pancreatic exocrine cells, was
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19 confirmed by real-time PCR on Day 4, and it increased until Day 25 (Fig. 3B). The
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21 pancreatic exocrine progenitor cell markers *P48* and *Mist-1* were also present on Day 4
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23 after induction (Fig. 3A), but the expression levels either held steady or decreased.
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26 These data suggested that the pancreatic progenitor cells differentiated into the mature
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28 pancreatic cells during the culture period. Expression of *Amylase* or *Elastase 2* was not
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30 detected in control cells (Fig. 3A).
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41 The presence of mRNAs associated with exocrine progenitor and differentiated
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43 cells does not necessarily mean that the proteins themselves were expressed.
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45 Therefore we used immunocytochemistry to determine if they were. PDX1 continued to
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47 be expressed in the peripheral nucleoplasm of the induced cells at Days 4, 7, and 15
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49 (Fig. 3C). In the mouse fetal foregut at E11, PDX1 was localized to the nuclei (Fig. 3C).
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7 during the culture period. The granular staining pattern, especially for amylase and
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10 elastase, was consistent with storage of digestive enzymes in the zymogen granules
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12 typically present in pancreatic acinar cells (Fig. 3D, E). The amylase and elastase
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14 positive cells frequently formed clusters that were similar to structures found in the
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16 mature pancreas (Fig. 3D, E). Taken together, these findings suggested the
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18 differentiation of mature pancreatic exocrine cells from mouse ESCs.
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25 To determine if the amylase-positive cells were actually pancreatic exocrine cells,
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27 we used double immunostaining for amylase and with CPA1 and with chymotrypsin. At
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29 the Day 22 in Stage 4, most of the amylase-expressing cells were also positive for CPA1
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31 or chymotrypsin (Fig. 3F). We then assessed physiological properties of the mouse
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33 ESC-derived exocrine cells by monitoring the activity of secreted amylase in the culture
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35 medium. Activity levels in the conditioned medium increased until Day 17, but afterward
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37 decreased (Fig. 3G). These data showed that functional pancreatic cells were
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39 generated with our protocol.
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53 **Efficiency of stepwise differentiation and effect of FGF7 on the differentiation of** 54 55 56 **pancreatic exocrine and endocrine cells** 57 58

59 We estimated the efficiency of differentiated cell production at different periods by
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7 counting immunofluorescent positive cells. Typically, for Foxa2-positive DE cells, an
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10 efficiency of $21.9 \pm 4.5\%$ was obtained by activin A treatment compared with non-treated
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13 cells, $4.3 \pm 2.1\%$ ($P < 0.05$, Fig. 4A). For pancreatic progenitor cells, PDX1-positive cells
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16 were present at a frequency of $12.0 \pm 4.7\%$ in FGF7 treated cells, which was a
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19 significantly greater than for non-treated cells, $1.7 \pm 1.7\%$ ($P < 0.05$, Fig. 4B). At Day 15 in
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22 Stage 4, $10.7 \pm 7.4\%$ of the cells stimulated with FGF7, GLP-1, and NA expressed
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25 amylase while only $1.3 \pm 1.8\%$ did in the absence of FGF7 but with GLP-1 and NA
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28 ($P < 0.05$, Fig. 4C). Thus these growth factors play important roles for pancreatic
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31 differentiation, and as a result, improved the differentiation efficiency at various periods.
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34 We next tested the differentiation of ESC-derived pancreatic progenitor cells with
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37 and without FGF7 to confirm the effect of FGF7 on pancreatic exocrine differentiation in
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40 the final stage. Expression of *Amylase*, *Carboxypeptidase A*, *Chymotrypsinogen*, and
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43 *Elastase* mRNAs tended to be greater in cells treated with FGF7 than those without it,
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46 but the differences were not statistically significant (Fig. 4D). On the other hand, the
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49 pancreatic endocrine markers *Ins2* and *Glucagon* remained unchanged under both
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52 conditions (Fig. 4D).
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59 **Discussion**

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7 Although there are many reports of differentiation of pancreatic endocrine cells from
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10 ESCs, only a few reports demonstrate the presence of differentiated exocrine cells
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12 [2,14,15]. Both endocrine and exocrine pancreatic cells are derived from common
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14 progenitor cells that express PDX1 [16,17]. The molecular mechanism for the
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16 differentiation of exocrine cells is poorly understood, so the effective factor(s) that
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18 induce exocrine differentiation from cultured ESCs is not known. We assumed that the
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20 growth factors effective for endocrine cell differentiation also had the potential to
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22 produce exocrine cells. In the present study, we developed a novel stepwise
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24 differentiation protocol using these growth factors. Moreover, we demonstrated the
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26 necessity of FGF7 for producing PDX1-positive pancreatic progenitor cells. The protocol
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28 described here consistently generated pancreatic exocrine enzyme-expressing cells
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30 from mouse ESCs, and these exocrine cells secreted active α -amylase.
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44 A previous report suggested that transforming growth factor (TGF)- β is necessary
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46 for formation of endoderm in vertebrates [18]. Activin A, a member of the TGF- β
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48 superfamily, induces the expression of many endodermal genes, including *Mixl1* and
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50 *Gooseoid*, that are important for early endoderm development [19,20]. Indeed in Stage
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52 1 of the present study, the cells expressed endodermal markers *Foxa2*, *Gooseoid*, and
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54 *Mixl1*, and both *Foxa2* and *Sox17* proteins were present. Furthermore, the expression
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7 level of mRNA for *Brachyury* was very high in this stage, suggesting that the cells had
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10 transited through the mesendoderm stage.

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12 In Stage 2, RA induced differentiation of GTE from DE as demonstrated by
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14 down-regulation of *Gooseoid* and *Mixl1* and up-regulation of *Hnf1b* and *Pdx1*. In
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17 addition, the expression of pancreatic endodermal markers *Hlxb9* and *Hnf6* were initially
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20 detected. RA plays a key role in specification of the foregut endoderm that becomes
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23 pancreas [10], and it regulates the commitment of ESCs to form PDX1-positive
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26 pancreatic endoderm *in vitro* [21]. However, in long term cultures, RA suppresses the
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29 differentiation of exocrine cells from embryonic pancreas [22]. We improved this
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32 procedure by shortening the RA treatment to 24 h.
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37 PDX1 is essential for determination and differentiation of the posterior foregut, in
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40 particular regarding the proliferation and differentiation of the pancreatic progenitor cells
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43 [23,24]. Thus the induction of PDX1-positive cells must be a necessary step for
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46 differentiation of pancreatic exocrine cells. In Stage 3, the treatment of FGF7 resulted in
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49 a large increase in expression of PDX1. Some reports showed that several FGFs are
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52 expressed in pancreatic rudiments where they participate in epithelial-mesenchymal
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55 interactions [12,13]. In particular, FGF7 stimulates both the proliferation of embryonic
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58 pancreatic epithelial cells and the development into acinar exocrine cells expressing
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7 amylase [13]. Furthermore, FGF7 upregulates expression of pancreatic markers,
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10 including *Pdx1* in the developing EB [3]. Our results similarly showed FGF7 promotion
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12 of pancreatic progenitor cell differentiation. In addition to increased expression of *Pdx1*,
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14 pancreatic progenitor markers *Hnf6*, *Nkx6.1*, and *P48* were also notably up-regulated by
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16 FGF7. Additionally, these PDX1-positive cells were also positive for *Foxa2*, suggesting
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18 that they were derived from DE. It is indicated that FGF7 has been demonstrated to play
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20 a key role in the differentiation of GTE into pancreatic progenitor cells and then into
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22 pancreatic exocrine cells *in vitro*.
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31 In Stage 4, ESC-derived PDX1-positive cells treated with FGF7, GLP-1, and NA
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33 became differentiated into functional pancreatic exocrine cells. The molecular
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35 mechanisms by which pancreatic exocrine cell differentiation occurs are largely
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37 unknown. In the absence of FGF7 *in vitro*, most expanded progenitor cells differentiate
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39 into endocrine cells [25]; therefore, FGF7 may play a crucial role for formation of
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41 exocrine cells by suppressing endocrine cell differentiation. Our results are consistent
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43 with the importance of FGF7 in the induction of exocrine differentiation. However, the
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45 effect of FGF7 was limited because ESC-derived non-pancreatic cells also continued to
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47 exist in this culture. GLP-1 and NA are usually used as growth factors for pancreatic
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49 endocrine differentiation [26, 27]. GLP-1 is associated with increased cell proliferation of
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7 both the endocrine and the exocrine component of the pancreas [28]. Additionally, a
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10 previous study showed that the combination with GLP-1 and NA induced both exocrine
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13 and endocrine gene expression [29]. Our data is consistent with the idea that the
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16 combination of FGF7, GLP-1, and NA induce pancreatic exocrine cell differentiation
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19 from mouse ESCs.

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22 In the final stage of differentiation, the transcription factor PDX1 was localized in the
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25 peripheral nucleoplasm although that was localized in nucleoplasm in earlier stages.
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28 The translocation of PDX1 from the nuclear periphery into the nucleoplasm is
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31 associated with the activation of pancreatic cells [30]. As for the adult pancreas, PDX1 is
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34 expressed in endocrine but not in exocrine tissues [31]. Thus, the movement of PDX1
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37 into the peripheral nucleoplasm might promote the differentiation of exocrine cells in this
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40 system.

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43 The differentiated cells secreted increasing amounts of amylase into the medium
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46 through Day 17 of culture. After that, the secretion diminished, which is consistent with
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49 the known difficulty of maintaining differentiated exocrine pancreas in long term culture.
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52 At the same time, the expression of *P48* mRNA, a marker of pancreatic exocrine
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55 progenitor cells, decreased in Stage 4. The role of FGF7 in promoting differentiation was
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58 subtle. When present, it tended to increase the number of amylase-positive cells, but
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7 the results were not statistically significant. These findings suggest that further
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10 pancreatic exocrine differentiation requires other signals in addition to FGF7.
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13 A major contribution of this works is that we generated pancreatic exocrine cells
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16 from mouse ESCs. The combination of growth factors that we used promoted the
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19 recapitulation of embryonic developmental processes by the ESCs. This system offers a
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21
22 new induction model for studying the mechanism of pancreatic exocrine cell formation
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25 and differentiation *in vitro*. In our future works, we will improve the differentiation
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28 efficiency via an inductive or a selective mechanism, and then these differentiated cells
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31 will be available for use in digestion assays and cytotoxicity evaluation. There is even
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34 the possibility that this methodology will have applications in regenerative medicine. In
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37 future works, we will clarify the individual roles of FGF7, GLP-1, NA, and other factors in
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40 pancreatic exocrine cell differentiation.
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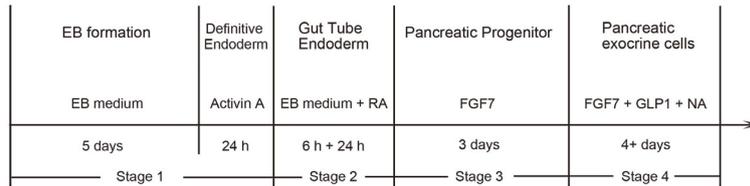
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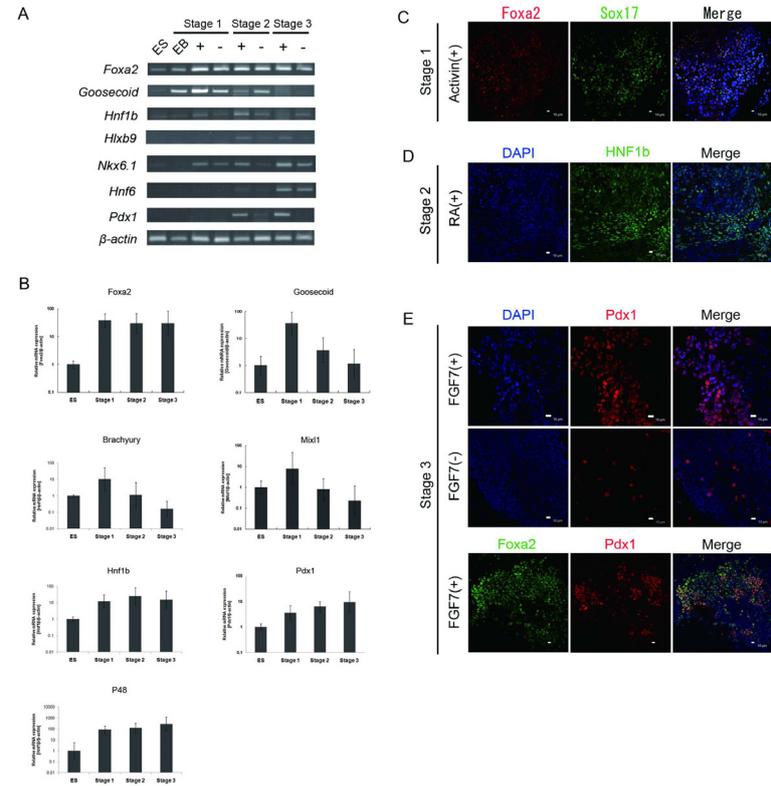
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Fig. 1



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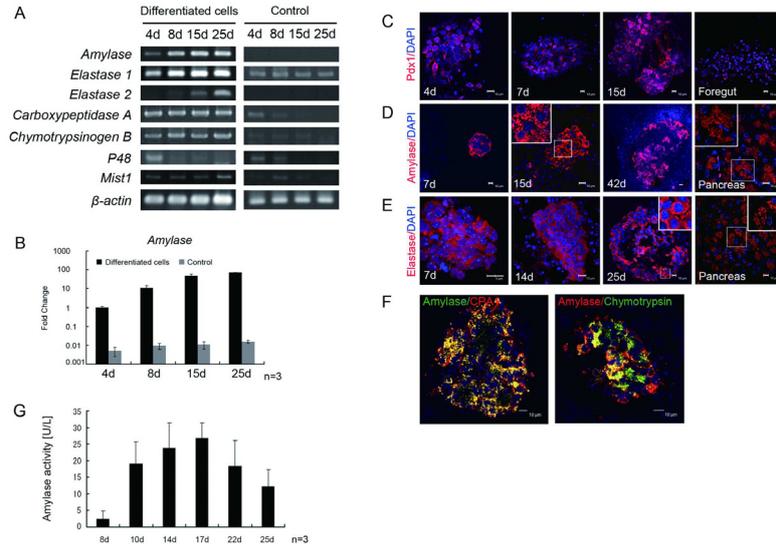
Fig. 2



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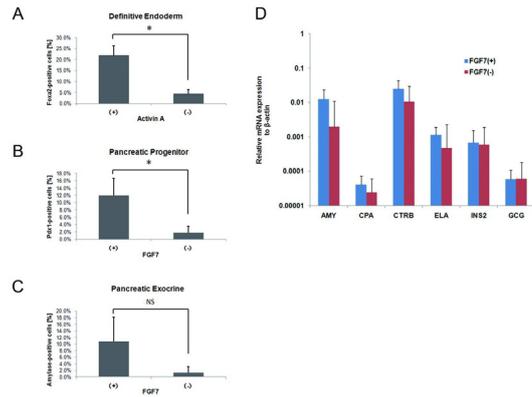
Fig. 3



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Fig. 4



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7 **Figure Legends**
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13 **Figure 1.**
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16 Experimental protocol for the differentiation of embryonic stem cells (ESCs) into
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18 pancreatic exocrine cells. The protocol was divided into four stages including application
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20 of different growth factors at different periods. Undifferentiated mouse ESCs were
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22 cultured using the hanging drop method to induce formation of embryoid bodies (EBs).
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25 The EBs were incubated in EB medium supplemented with activin A, followed by
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28 treatment with *all-trans* retinoic acid (RA). Then EBs were cultured with fibroblast growth
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31 factor 7 (FGF7) for 3 days, and then re-plated and treated with FGF7, glucagon-like
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34 peptide-1 (GLP-1), and nicotinamide (NA).
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44 **Figure 2.**
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47 Derivation and characterization of mouse ESC-derived cells during early differentiation
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49 (Stages 1-3). (A) Reverse transcriptase-PCR analysis of endodermal marker gene
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52 expression of Stage 1 – 3 cells treated with (+) or without (-) inducing growth factors
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55 shown in Figure 1 in EB medium (Stages 1 and 2) or in serum free medium (Stage 3). (B)
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59 Quantitative gene expression levels of mesendodermal marker *Brachyury*, endodermal
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7 markers *Foxa2*, *Goosecoid*, *Mixl1*, *Hnf1b*, and pancreatic progenitor markers *Pdx1*, *P48*
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10 were examined by real-time PCR. The total RNA levels were normalized to *β-actin*
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12 mRNA levels. (C) Immunofluorescent staining of ESC-derived definitive endoderm at
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14 Stage 1. ESCs were positive for both endoderm specific markers *Foxa2* and *Sox17*. (D)
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16 Immunofluorescent staining of ESC-derived GTE at Stage 2 showed the presence of
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18 HNF1b. (E) Immunofluorescent staining of ESC-derived pancreatic progenitor cells at
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20 Stage 3. An increased frequency of *Pdx1*-positive cells was evident after FGF7
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22 treatment. *Pdx1*-positive cells also expressed *Foxa2*. Scale bars: 10 μm.
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34 **Figure 3.**

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37 Characterization of mouse ESC-derived pancreatic exocrine cells by stepwise
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39 differentiation in Stage 4. (A) Expression of pancreatic exocrine cell markers was
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41 examined by RT-PCR in the differentiated cells. Differentiation was promoted by
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43 treatment with FGF7, GLP-1, and NA, which were absent in control cell cultures. The
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45 expression of *Amylase*, *Elastase 1*, *Carboxypeptidase A*, and *Chymotrypsinogen B*
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47 mRNAs were detected from Day 4 after the induction of differentiation. Expression of
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49 *Elastase 2* was detected from Day 8. In the control cells, the expression of these
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51 pancreatic markers was slight or undetectable. (B) Expression of *Amylase* was
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7 examined by real-time PCR in the differentiated cells and untreated controls. The total
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10 RNA levels were normalized to *β-actin* mRNA levels. The mRNA levels of Amylase were
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12 up-regulated from Day 4 through Day 25. (C-E) Immunofluorescent staining for PDX1,
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14 amylase, and elastase at different periods. Mouse fetal foregut and adult pancreas were
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16 used as positive controls. (C) PDX1 was present at the peripheral nucleoplasm as soon
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18 as the fourth day in Stage 4. Amylase (D) and elastase (E) were present in cytoplasm
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20 from one to several weeks. Scale bars: 10 μm. (F) Double immunostaining for amylase
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22 with CPA1 and with chymotrypsin at Day 22. The presence of CPA1 and chymotrypsin
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24 in the same cells as amylase demonstrated that these were differentiated pancreatic
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26 exocrine cells. CPA1, carboxypeptidase A1. Scale bars: 10 μm. (G) Amylase activity in
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28 the conditioned medium of the differentiated cells. Amylase activity levels in conditioned
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30 medium increased from Day 8 until Day 17 and then declined through Day 25.
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Figure 4.

Efficiency of stepwise differentiation and effect of FGF7 on the differentiation of pancreatic exocrine and endocrine cells. (A) The efficiency of conversion to DE cells, shown by the presence Foxa2, was significantly greater for activin A-treated cells than controls ($P<0.05$). (B) For conversion to pancreatic progenitor cells, shown by the presence of PDX1-positive cells, the efficiency was significantly greater for FGF7-treated cells than controls ($P<0.05$). (C) For conversion to pancreatic exocrine cells, shown by the presence of amylase-positive cells, the efficiency was greater for cells treated with FGF7, GLP-1 and NA than with GLP-1 and NA ($P<0.05$). (D) Relative quantitative gene expression for pancreatic exocrine and endocrine markers in pancreatic progenitor cells treated GLP-1 and NA, but either with or without FGF7 at Day 8 in Stage 4. Expression of *Amylase*, *Carboxypeptidase A*, *Chymotrypsinogen*, and *Elastase* mRNAs tended to be greater in cells treated with FGF7 than those without it. AMY, Amylase; CPA, Carboxypeptidase A; CTRB, Chymotrypsinogen B; ELA, Elastase; INS2, Ins 2; GCG, Glucagon. n=3

Table 1

Primers for PCR analysis

Gene	Sense primer	Antisense primer	Annealing T _m (°C)	Product size (bp)
<i>Foxa2</i>	TGGTCACTGGGGACAAGGGAA	GCAACAACAGCAATAGAGAAC	60	289
<i>Goosecoid</i>	ATGCTGCCCTACATGAACGT	CAGTCCTGGGCCTGTACATT	55	517
<i>Hnf1b</i>	GAAAGCAACGGGAGATCCTC	CCTCCACTAAGGCCTCCCTC	60	279
<i>Hlx9</i>	AATGGAAACGCAGCAAAAAG	CCATTGCTGTACGGGAAGTT	60	235
<i>Nkx6.1</i>	TCTTCTGGCCCGGGGTGATG	CCTGCTTCTTCTGGCGGTG	60	311
<i>Hnf6</i>	CAGCACCTCACGCCACCTC	CAGCCACTTCCACATCCTCCG	67	369
<i>Pdx1</i>	CCAAAACCGTCGCATGAAGTG	CTCTCGTGCCCTCAAGAATTTTC	65	392
<i>Amylase</i>	TATTGCCAAGGAATGTGAG	TCAAGTCTGAACCCTGCTAC	58	513
<i>Elastase 1</i>	GTGGCTGCAGGCTATGACAT	GGTGAAGACTGTGGGCTTC	55	410
<i>Elastase 2</i>	TATCAGACCTACCGAGTGCT	ACACCATGCTGGACTTCACA	58	373
<i>Carboxypeptidase A</i>	AATCTCTGCCACCGACGAAG	CAGCACATTGATGGGACGA	60	400
<i>Chymotrypsinogen B</i>	CTGCCCCAGGACACAATGCC	CATCCAGGTCTGAAGATCG	60	413
<i>P48</i>	ATCGAGGCACCCGTTTAC	CGATGTGAGCTGTCTCAGGA	55	226
<i>Mist-1</i>	CGCTGGCCAAGAACTATATC	CCATGAGAGGGCTTATGTC	55	400
<i>β-actin</i>	TTCTTCTTGGGTATGGAAT	GAGCAATGATCTTGATCTTC	53	200

Table 2

Primers for real-time PCR analysis

Gene	Sense primer	Antisense primer	Product size (bp)
<i>Foxa2</i>	GTCGTCCGAGCAGCAACATC	GGGTAGTGCATGACCTGTTCGTAG	90
<i>Mixl1</i>	CTACCCGAGTCCAGGATCCA	CTACCCGAGTCCAGGATCCA	102
<i>Gooseoid</i>	GGAGACGAAGTACCCAGACGTG	ACTTGGCTCGGCGTTCTT	104
<i>Brachyury</i>	CTCCACCTATGCGGACAAT	CCATTGCTCACAGACCAGAG	164
<i>Hnf1b</i>	CATCTGCAATGGTGGTCACAG	GGCTTGCAGTGGACACTGTTT	83
<i>Pdx1</i>	GAAATCCACCAAAGCTCACG	GAATTCCTTCTCCAGCTCCA	130
<i>P48</i>	ATGCAGTCCATCAACGACGCCTTC	GGCTTGCACCAGCTCGCTGAG	140
<i>Amylase</i>	GCACAGTTATCCGCAAGTGGAA	GCACAGTTATCCGCAAGTGGAA	134
<i>Carboxypeptidase A1</i>	GGGACCCTCGTCAGTGTTTA	GCAGAGATTCCGAGAACCTG	155
<i>ChymotrypsinogenB1</i>	GAACTGGCTTCCATTTCTGC	ATGTCATTACGCACGGTGAA	202
<i>Elastase1</i>	AGCACAACCTGAGCCAGAAT	TTGTTAGCCAGGATGGTTCC	196
<i>Ins2</i>	AGAAGCGTGGCATTGTAGATCAGT	CAGAGGGGTAGGCTGGGTAGTG	102
<i>Glucagon</i>	GCACATTACCAGCGACTACAG	GGGAAAGGTCCCTTCAGCATGTCT	154
<i>β-actin</i>	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA	171