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ORIGINAL ARTICLE

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Pancreatic tissue formation from murine embryonic stem cells *in vitro*

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Abstract The *in vitro* formation of organs and/or tissues is a major goal for regenerative medicine that would also provide a powerful tool for analyzing both the mechanisms of development and disease processes for each target organ. Here, we present a method whereby pancreatic tissues can be formed *in vitro* from mouse embryonic stem (ES) cells. Embryoid body-like spheres (EBSs) induced from ES cell colonies were treated with retinoic acid (RA) and activin, which are candidate regulators of pancreatic development *in vivo*. These induced tissues had decreased expression of the *sonic hedgehog* (*shh*) gene and expressed several pancreatic marker genes. ES cell-derived pancreatic tissue was composed of exocrine cells, endocrine cells, and pancreatic duct-like structures. In addition, the ratio of exocrine to endocrine cells in the induced tissue was found to be sensitive to the concentrations of RA and activin in the present experiment.

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Introduction

Mouse embryonic stem (ES) cells can differentiate into various cell types *in vitro* (Loebel et al., 2003), including the insulin-secreting β cells of the pancreas (Soria et al., 2000; Lumelsky et al., 2001; Hori et al., 2002; Shiroi et al., 2002; Blyszczuk et al., 2003; Kim et al., 2003; Leon-Quinto et al., 2004) but not formed any organs or tissues (Le Douarin, 2000). The promise of transplanting such cells into patients with diabetes has driven extensive research into factors that induce the differentiation of endocrine cells. However, the pancreas is a complex organ composed of exocrine cells, endocrine cells, and pancreatic ducts, which are considered to differentiate from a common precursor cell (Slack, 1995; Percival and Slack, 1999), making the *in vitro* formation of whole pancreas a far more complex undertaking.

Retinoic acid (RA) and transforming growth factor-β (TGF-β) play key roles in the regulation of pancreatic organogenesis (Kim and Hebrok, 2001; Kumar and Melton, 2003). RA signaling is involved in patterning along the anteroposterior axis of the endoderm during the late gastrula stage of zebrafish, African clawed frog (*Xenopus laevis*), and quail. While inhibition of RA signaling in the late stages had little effect on the expression of anterior mesodermic markers, it abrogated pancreatic marker expression. In addition, zebrafish and *Xenopus* embryos treated with exogenous RA showed enlargement of the pancreas, and in zebrafish also of the liver, in the anterior direction (Stafford and Prince, 2002; Chen et al., 2004; Stafford et al., 2004).

Another important mediator of pancreas differentiation is sonic hedgehog (Shh); its down-regulation by signals from the notochord is essential for initiating

differentiation of the dorsal pancreas in mouse and chicken embryos (Kim et al., 1997). In tissue culture experiments using endoderm isolated from chicken embryos, activin, a member of the TGF-β family, inhibited *Shh* expression and induced transcription of pancreatic marker genes, thereby mimicking the effects of the notochord signaling (Kim et al., 1997; Hebrok et al., 1998). Some reports have also suggested that differentiation into pancreatic endocrine cells is regulated by TGF-β signal transduction mediated via activin receptors (Sanvito et al., 1994; Ritvos et al., 1995; Miralles et al., 1998; Yamaoka et al., 1998; Shiozaki et al., 1999; Kim et al., 2000). Culture of the mouse embryonic pancreas *in vitro* in the presence of activin or TGF-β1 led to enhancement of endocrine cell formation, particularly of β cells and pancreatic polypeptide cells (Sanvito et al., 1994), while normal epithelial branching and the development of exocrine cells were disturbed (Ritvos et al., 1995). Furthermore, follistatin, an antagonist for TGF-β signaling (including that via activin), was shown to stimulate the pancreas and differentiate into exocrine cells and reduce the differentiation of endocrine cells (Miralles et al., 1998). In addition, transgenic mice expressing a dominant-negative form of the type II activin receptor showed hypoplasia of the islets (Yamaoka et al., 1998; Shiozaki et al., 1999).

Since both RA and activin have considered to regulate pancreas development, we examined whether treatment with both these factors could induce the differentiation of ES cells into pancreatic cells.

Materials and methods

Maintenance and differentiation of ES cells

Mouse-derived ES cells (E14 and CMTI-1) were seeded onto mouse embryonic fibroblasts pretreated with mitomycin C (10 ng/ml; Sigma, St. Louis, MO) for 2.5 hr. The cells were then incubated in Dulbecco's modified Eagle's medium (DMEM; high glucose with L-glutamine and pyruvate; Gibco 11995-065, GIBCO, Invitrogen, Carlsbad, CA) supplemented with 15% ES cell qualified fetal bovine serum (FBS; Gibco), MEM non-essential amino acid solution (Gibco), 0.001% β-mercaptoethanol (Sigma), and LIF ESGRO (1,500 U/ml, Chemicon, Temecula, CA).

For the differentiation of ES cells, 3-day ES cell colonies were detached from the feeder cells by the treatment of 1 mg/ml of collagenase/dispase (Roche Diagnostic, Indianapolis, IN) (Fig. 1). The clusters of ES cells were transferred into a low cell-binding dish (Nalge Nunc, Rochester, NY). They were cultured at floating condition in DMEM supplemented with 15% KnockOut Serum Replacement (KSR; Gibco) and the culture medium was renewed 2 days later. Four days after embryoid body-like spheres (EBSs) began to form, they were transferred into a low cell-binding 96-well plate (Nunc) and incubated for additional 2 days in DMEM supplemented with 15% KSR containing activin A (0, 10, 25, 50 ng/ml) and all-trans RA (0, 0.001, 0.01, 0.1, or 1 μM; Sigma). The EBSs were then attached to the wells of tissue culture plates or dishes (TPP) that had been coated overnight with 0.1% gelatin (Sigma). The cells were incubated in DMEM supplemented with 10% KSR, and the medium was renewed every third day.

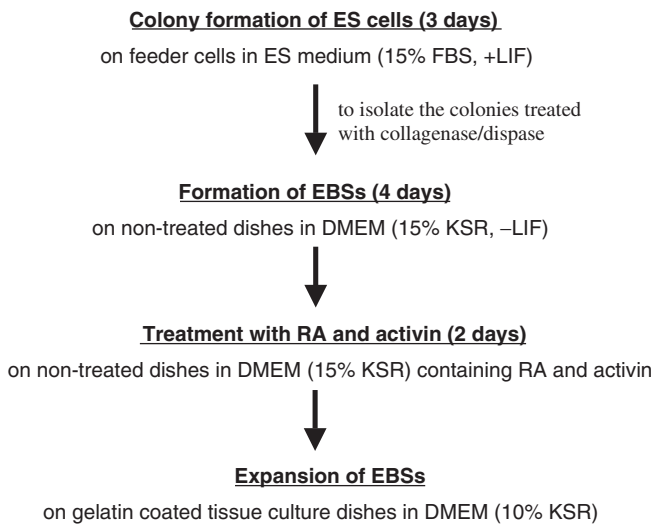

Fig. 1 Experimental protocol for the differentiation of embryonic stem (ES) cells into pancreatic tissue with retinoic acid (RA) and activin. Colonies of ES cells were maintained onto feeder cells and incubated in ES medium containing LIF for 3 days. These ES colonies were detached from the feeder cells by collagenase/dispase treatment. The ES cell clusters were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% KnockOut Serum Replacement (KSR) in floating condition. Four days after embryoid body-like spheres (EBSs) began to form, they were transferred in a low cell-binding multi-well plate and incubated for additional 2 days in DMEM supplemented with 15% KSR, containing various concentrations of activin A and all-trans RA. The EBSs were then attached to the gelatin-coated tissue culture dish and continued to culture in DMEM supplemented with 10% KSR until the examination.

Immunohistochemistry

Thirteen days after the end of treatment (19 days after the beginning of EBS formation), the EBSs were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 40 min at room temperature. The fixed cells were freed from the dish wall and embedded in an LR Gold Resin System (structure probe, Electron Microscopy Sciences, Hatfield, PA), an acrylic resin, and sectioned to yield 600 nm semithin sections. The sections were blocked for 40 min at room temperature in 3% bovine serum albumin (BSA) and phosphate-buffered saline (PBS). They were then exposed to pancreas-specific primary antibodies for 12 hr at 4°C. After washing with PBS, the sections were exposed to labeled secondary antibody for 8 hr at 4°C. The primary antibodies used were rabbit anti-α amylase antibody (1:1,000 dilution, Sigma), anti-insulin monoclonal antibody (1:400 dilution, Sigma), goat anti-C-peptide antibody (1:800 dilution, Linco Research, Millipore, Billerica, MA), rabbit anti-Pdx-1 antibody (1:200 dilution, Chemicon); the secondary antibodies were Alexa-Fluor 488- and Alexa-Fluor 594-conjugated (Molecular Probes, Invitrogen, Carlsbad, CA). The sections were observed under a fluorescence microscope and photographed with AquaCosmos (Hamamatsu Photonics, Hamamatsu, Japan) connected to an ORCA-3CCD camera. For the control, pancreas from 8-week-old mouse was fixed and processed as described above. For smooth muscle detection, EBSs were fixed as described above, blocked with 3% BSA in PBS for 40 min at room temperature, and permeabilized with 0.1% Triton X-100 for 30 min. They were then exposed to fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-α-smooth muscle actin antibody (1:500 dillution; Sigma) for 12 hr at 4°C.

To calculate the C-peptide-positive cells in the induced EBS, 1 μm serial sections were prepared from each specimen, and examined at every 20 μm thickness by immunostained with anti-C-peptide