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Sonic hedgehog inhibition induces mouse embryonic stem cells to differentiate toward definitive endoderm

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In the experimental group (shh inhibited group), there were significant decreases in the expression of Oct4, Nanog, Shh, GATA4, Brachyury and Goosecoid, while increases were observed for TAT and Pdx1. The expression of Sox17 did not differ between two control and experimental groups. In experimental group, the amount of GSC positive cells was somehow lower but it seems that there was no difference for Sox17. Shh inhibition induces ESCs to differentiate toward definitive endoderm by committing mesendodermal lineages.

Keywords: Definitive endoderm, Dithizone, Hedgehog interacting protein, Mesendoderm, Mouse embryonic stem cell

Pluripotent colonies of embryonic stem cells (ESCs) spontaneously start to differentiate into suspended three-dimensional multicellular formations named embryoid bodies (EBs). This process is regulated by the removal of anti-differentiation factors such as leukaemia inhibitory factor (LIF) and feeder layer cells from their culture condition[1].

EBs recapitulates many aspects of cell differentiation of early mammalian embryogenesis. EBs can differentiate into derivatives of all three germ layers: ectoderm, mesoderm and endoderm[2,3].

Directed differentiation of ESCs could be triggered by the addition of differentiation agents to the media of EBs[4,5], with the goal that many diseases such as Parkinson, diabetes and cardiac failure will be treated by this method[6,7].

However, at the end of the differentiation protocol, there are undifferentiated, immature and unwanted cells that lead to rejection of transplanted tissues or induction of teratoma formation in donors[8,9].

Sonic Hedgehog (Shh) signalling plays an important role in inducing and maintaining the axis of mammalian embryo. Shh expression is normally restricted to embryogenesis and fundamentally affects the patterning of several tissues of the early embryo[10,11,12].

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Many studies have been undertaken to induce endoderm differentiation of ESCs that lead to the generation of many endodermal derivatives such as the pancreas and liver. In most of these reports, endoderm differentiation was triggered by Basic fibroblast growth factor (bFGF) and activins [3,13].

These factors promote endodermal differentiation by blocking Shh in ESCs[10,13]. This was reported by Mfopou *et al.*[14] who showed that inhibition of Shh during EB formation improved the differentiation of stem cells to insulin-secreting cells. Although it is been shown that some endoderm inducers of ESCs inhibit Shh there are not enough data to prove that Shh inhibition could be considered as a pathway for differentiation of definitive endoderm from ESCs. The current study has been conducted to offer if Shh inhibition is a pathway for differentiation of definitive endoderm from ESCs and also to represent the pattern of changes in the markers of three embryonic germ layers. In this regards, relative RT-PCR was done to show the ratio of the expression of markers. Immunocytochemistry and also Dithizone staining were performed to distinguish definitive endoderm cells.

Materials and Methods

In vitro differentiation of embryonic stem cells— Undifferentiated mESCs (MUKF3) were maintained on mitomycin C (10 μg/mL; Sigma; M0503) inactivated

feeder mouse embryonic fibroblast (MEF) cells in DMEM/F12 media (Gibco; 21331-020) supplemented with 15% ESC-qualified fetal calf serum (Gibco; 10439-024), 1000 units/mL LIF (Sigma; L5283), 1% nonessential amino acids (Gibco; 11140-035), 2 mM L-glutamine (Gibco; 25030-024), 0.1 mM β-mercaptoethanol (Sigma; M7522), 100 units/ml penicillin and streptomycin 100 µg/mL (Gibco; 15070-063) at 37 °C and 5% CO_2.

To initiate differentiation, ESCs were removed from feeder cells layer with 0.05% trypsin and 0.5 mM ethylenediaminetetraacetic Acid (EDTA) (Gibco; 25300-054) and counted by haemocytometer. The cells were divided into control and experimental groups in equal numbers (2×10^5 cells/60 mm^2 culture dish). The cells were cultured for 5 days in non-adherent culture dishes to form EBs which were considered as stage 2 in this study.

The media of the control group (group 1) contained DMEM/F12 + 10% knockout serum replacement (KOSR; Gibco; 10829). Experimental group (group 2) received media the same as control group except for the addition of Hedgehog interacting protein (HIP; 10 µg/mL; Sigma-Aldrich). Then, EBs from two groups were harvested and expanded onto separate gelatine-coated (0.1%) culture flasks and allowed to differentiate for additional eight days in the DMEM/F12 media containing 10% FCS.

Reverse transcription–polymerase chain reaction analysis—Total RNA was extracted from differentiated ESCs. Trypsin-EDTA solution was used to dissociate the ESCs from adherent substrata. RNA was prepared using RNAeasy plus mini Kit (Qiagen) that included gDNA eliminator mini-spin columns to avoid DNase digestion and RNeasy mini-spin columns for RNA purification.

Total RNA (≥1 µg) was reverse transcribed using a poly T-tail primer included in the One-Step RT-PCR Kit (Qiagen). cDNA was amplified according to the manufacturer's instructions. Table 1 shows primer pairs, amplicon sizes, and annealing times. Cycle conditions were as follows: 95 °C for 15 min followed by 35 cycles of 94 °C, denaturation for 45 sec, 58 °C to 60 °C annealing for 90 sec, and 72 °C elongation for 1 min, with a final incubation at 72 °C for 10 min. There were 30 cycles for all samples. Experiments were performed in triplicate to ensure reproducibility.

Relative reverse transcription–polymerase chain reaction analysis—The expressions of target genes were quantified against the internal reference gene (GAPDH). Semi-quantitative reverse transcription–polymerase was carried out based on a comparison of CT at constant fluorescence intensity.

Products were electrophoresed on agarose gel (1.5%) and gels were stained with ethidium bromide

Table1—Primers used for RT-PCR analysis of gene expression of differentiated cells from mouse ESCs

Gapdh	ACCTCAACTACATGGTCTAC TTGTCATTGAGAGCAATGCC	58	801 bp
Oct4	GGCGTTCTCTTTGGAAAGGTGTTC CTCGAACCACATCCTTCTCT	58	293 bp
Nanog	AGGGTCTGCTACTGAGATGCTCTG CAACCACTGGTTTTTCTGCCACCG	60	278 bp
Brachyury	CATGTACTCTTTCTTGCTGG GGTCTCGGGAAAGCAGTGGC	60	313 bp
GATA4	CTCCTACTCCAGCCCCTACC GTGGCATTGCTGGAGTTACC	58	591 bp
TAT	CAGAGGACTTGGTGGAGGAG CACGGCTAGACACAGCTCAA	58	551 bp
PDX1	CTTAGCGTGTCGCCACAGCCCTCCA CAGCCGCCTTTCGTTATTCT	58	472 bp
Goosecoid	GCACCATCTTCACCGATGAG AGGAGGATCGCTTCTGTCGT	58	524bp
Sox17	GGCCAGAAGCAGTGTTACACA TTTGATAAAAATCGATGCGAGAGA	58	336 bp
Shh	GGCAGATATGAAGGGAAGAT ACTGCTCGACCCTCATAGTG	59	260 bp

(10 µg/mL) and photographed on an ultraviolet transilluminator (UVIdoc; Uvitec, Cambridge, UK). Gel images were analysed using the UVIband-map program (Uvitec). Semi-quantitative RT–PCR values were presented as the ratio of the specified gene signal divided by the GAPDH signal. RT-PCR was performed as three individual replicates [2].

Immunocytochemistry—Attached EBs were washed with PBS, fixed in 4% paraformaldehyde (20 min), and processed for immunostaining using standard protocols. Briefly, the fixed cells were incubated with anti-Goosecoid, 1:50 (sc-22234); Santa Cruz Biotechnology) or Sox17 (sc-17356; Santa Cruz Biotechnology) as primary antibodies. The non specific binding was blocked before exposure to primary antibodies by incubation in the relevant non-immune serum. For the detection of primary antibody, a fluorescent-labelled secondary antibody, donkey anti-goat IgG-FITC, 1:100 (sc-2024; Santa Cruz Biotechnology) was followed by counterstaining by propidium iodide (P.I) (1 ug/mL) according to the manufacturer's instructions. Gastrulating mice embryos (9.5 days) were used as positive control.

Dithizone (DTZ) staining—A DTZ (194832; Sigma) stock solution was prepared with 50 mg of DTZ in 5 mL of dimethyl sulfoxide (DMSO) and stored at -15 °C. *In vitro* DTZ staining was performed by adding 10 µL of the stock solution to 1 mL of culture medium. The staining solution was filtered (0.2 µm filter) and then used as the DTZ working solution. The culture dishes were incubated at 37 °C for 15 min in the DTZ solution.

The cells were rinsed three times with HBSS, clusters stained crimson red were examined with a stereomicroscope. After examination, the dishes were refilled with DMEM containing 10% FCS. For positive control, the pancreatic islets were isolated from balb/c mice by collagenase digestion and were stained with dithizone (80% purity of islets)[15]. The islets were cultured on gelatine-coated (0.1%) surfaces in DMEM/F12 (Gibco; 21331-020) medium containing 1.1 mmol/L glucose, 100 units/ml penicillin, 100 µg/mL streptomycin, 40 µg/mL gentamicin, and 10% FCS.

Statistical analysis—Data of RT-PCR values are reported as mean± SE. Statistical analysis of these data was done by Student's *t* -test using SPSS 16.0 for windows XP (SPSS, Chicago, IL, USA). *P* value <0.05 was considered significant.

Results

The expression of markers—The relative expression levels and profiles of gene markers are shown in Figs 1 and 2. In the experimental group, there were significant decreases in the expression of Oct4 (*P*=0.000), Nanog (*P*=0.005), Shh (*P*=0.000), GATA4 (*P*=0.04), Brachyury (*P*=0.007) and Goosecoid (*P*=0.04), while increases were observed for TAT (*P*=0.006) and Pdx1 (*P*=0.000). The expression of Sox17 did not differ between two groups.

Immunohistochemistry staining—The Goosecoid (GSC) and Sox17 positive cells are shown in Figs 3 and 4, respectively. In experimental group, the amount of GSC positive cells was somehow lower in comparison to the control group but it seems that there was no difference for Sox17.

Discussion

In this study, Shh inhibition was considered as a probable cellular and molecular pathway for the differentiation of mESCs. At first, the expressions of Oct4 and Nanog as two undifferentiated markers were monitored. The POU family transcription factor Oct4, which is encoded by Pou5f1, is also a pivotal regulator of pluripotency that acts as a gate keeper to prevent ESC differentiation[16].

It has previously been shown that artificial repression of Oct4 in ESCs induces differentiation along the trophectodermal lineage. When over-expressed, ESCs mainly differentiate into primitive endoderm-like cells[17].

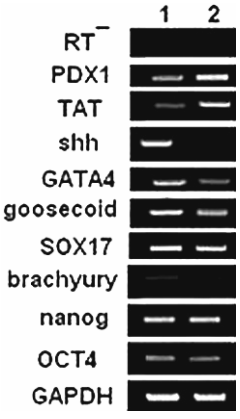

Fig. 1—Temporal expression pattern of genes by reverse transcription polymerase chain reaction (RT-PCR) in two experimental groups. Stages 1 to 3 and the media conditions are explained in material and methods.

Nanog is an NK-2 class homeobox transcription factor that is expressed throughout the pluripotent cells of the inner cell mass (ICM)[18]. Thus, the reduced expression of Oct4 and nanog genes in experimental group has established that Shh inhibition affects differentiation of ESCs.

This study showed that Shh inhibition over expressed pdx1 and tat genes in mESC, while it decreased the expressions of brachyury, gata4 genes. The data of dithizone staining also showed an increase in the number of stained embryonic stem cells following Shh inhibition.

In vivo and *in vitro* experiments showed that Shh inhibition is necessary to induce the precursor of pancreatic tissue, both exocrine and endocrine, which expresses Pdx1[10,13,14,19].

In vivo experiments have shown that during gastrulation of animals, a transient tissue, mesendoderm, is formed. The cells of this tissue can produce both anterior endodermal tissues such as liver and pancreas, and paraxial mesoderm.

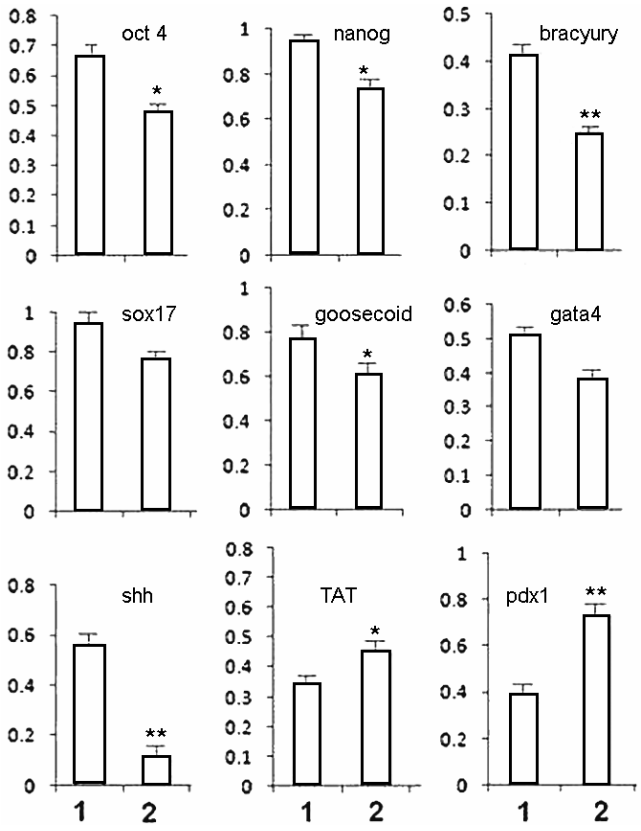

Fig. 2—Relative RT-PCR analysis of the expression of genes of differentiated mouse embryonic stem cells (mESC). The media of forming EBs in group 2 contained Hedgehog interacting protein (HIP; 10 µg/mL). (*P* values:* < 0.05; ** < 0.01)

In preliminary studies, brachyury gene has been considered to be a mesendoderm marker in both *in vivo* and *in vitro* experiments[21-23]. However, Brachyury (T-box transcription factor) is expressed in both primitive node and early mesodermal lineages and could not be suitable for distinguishing mesendoderm from other mesoderm cells. Thus, repression of brachyury suggests that Shh directs the differentiation of mesendoderm to endoderm, in addition to mesoderm.

Yasunaga *et al.*[24] have investigated other mesendoderm markers by addition of activin to a serum-free media of this cell line which selectively induce the *in vitro* emergence of $GSC^{+}Sox17^{+}$ population (definitive endoderm cells) as opposed to $GSC^{-}Sox17^{+}$ population (visceral endoderm cells), and have suggested that GSC is a mesendoderm marker.

Although, the initiation fate of anterior endoderm and paraxial mesoderm are similar because of a common ancestor (mesendoderm), they change at later stages of development.

Many authors performed the differentiation of insulin-producing cells by the selection of nestin-expressing cells[3,25], while other protocols have recommended instead of nestin selection[10,15,26]. In these studies, agents such as bFGF and activins, which inhibit the production of Shh, are considered to be endoderm inducers. However, none of these protocols produce enough number of insulin-producing cells.

It has been shown that EBs differentiate to two layers; an inner layer of columnar primitive ectoderm surrounding a proamniotic-like cavity and an outer layer of primitive or visceral endoderm. This process takes place in 7 days for human ESC and 5 days for mESC[4,27]. The outer layer expresses markers such as GATA4 and nodal which are also mesendodermal makers[28].

The data showed that Shh inhibition during EB formation reduced the expressions of GATA4. Thus, Shh inhibition may reduce the amount of visceral endoderm and only mesendoderm cells are present which express these two markers. Here, it is suggested to separating primitive endoderm as a cell source for inducing endodermal differentiation by Shh inhibition in order to omit visceral endoderm cells at the first stage of stem cell differentiation. However, the idea of separation of primitive endoderm from EBs has been previously introduced by Choi but no additional experiments have been undertaken, and also the effects of Shh inhibition have not been studied in this protocol[29].

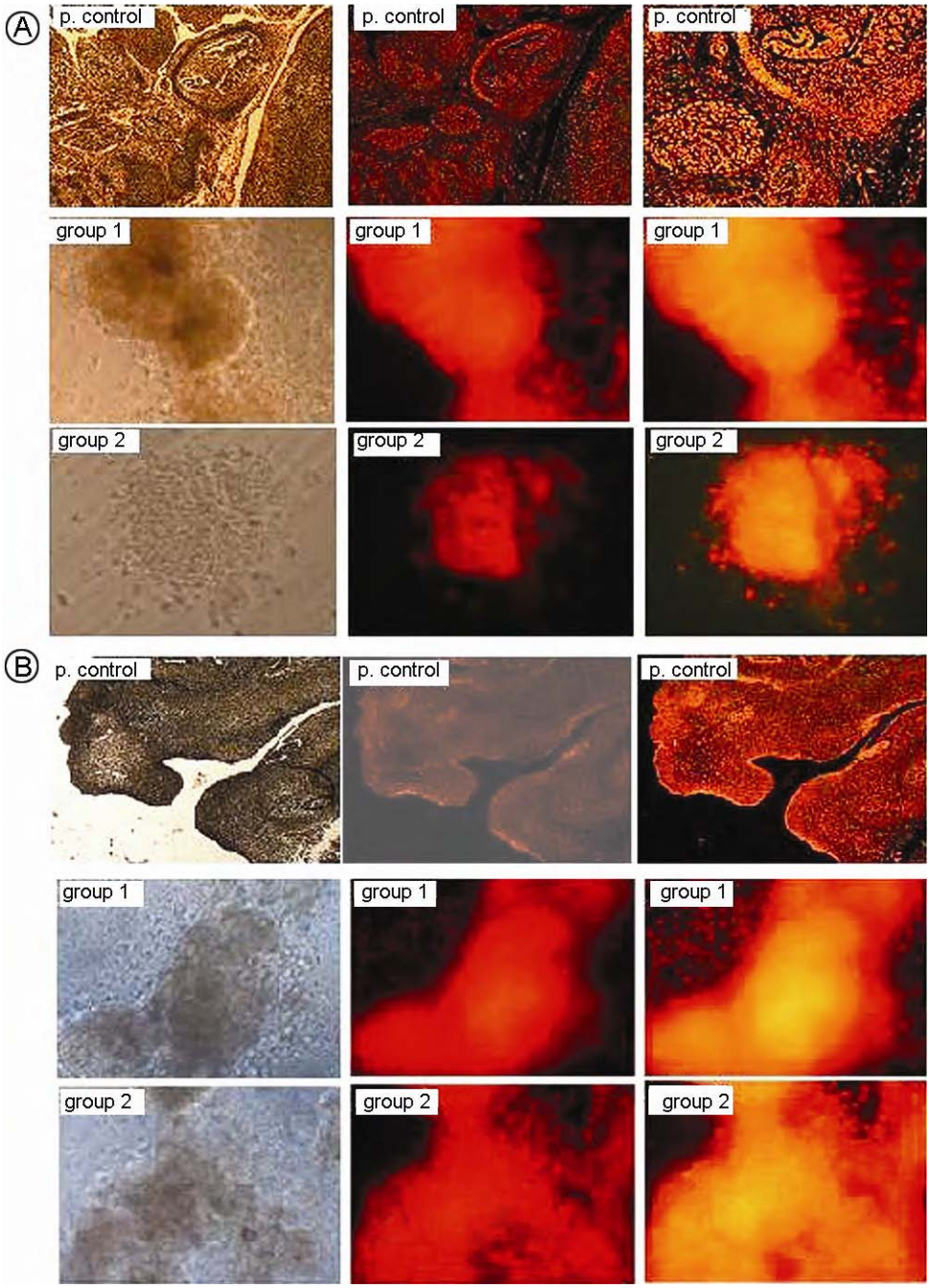

Fig. 3—Immunocytochemistry of mouse embryoid bodies for endoderm markers; A: Goosecoid and B: Sox17. In Group 1 (Control), Group 2 (treated with HIP during forming EB) under fluorescent microscope (X200).

During *in vivo* development of mammalian foregut endoderm, the expression of Shh is inhibited initially and up-regulated in the final stages. Both *in vivo*[30,31] and *in vitro*[32] studies have shown that activin A is an inhibitor of Shh. In a three-step protocol, activin A was added to the media at the first stage of stem cell differentiation during EB formation.

The same results have been produced for TAT expression, a liver precursor marker, thus these issues may be considered for differentiation of the liver from ESC.

These data confirm that certain agents such as activins could be applied for both liver and pancreas formation during ESC differentiation protocols[19,33].

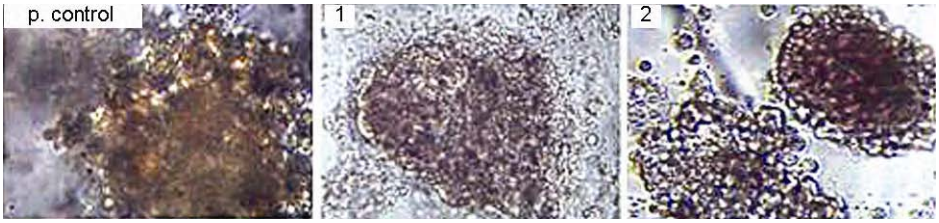

Fig. 4—Dithizone (DTZ) staining of mouse embryoid bodies for showing precursor cells of pancreatic tissue; mouse islets isolated from pancreas (positive control), islet-associated cells in Groups 1 (control) and 2 (treated with HIP during forming EB) under stereomicroscope (X200).

In conclusion, the study presents Shh inhibition as a molecular pathway for induction of definitive endoderm from ESCs by affecting the fate of mesendodermal cells.

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