

—Mini Review—

Induction of Differentiation into Endoderm, Insulin-Secreting Cells from Mouse Embryonic Stem Cells Using Activin A and Retinoic Acid

Kahei Sato* and Mizuki Yamamoto

Department of Applied Biological Science, Nihon University College of Bioresource Sciences, Fujisawa 252-0831, Japan

Abstract: Embryonic stem (ES) cells have been known to differentiate into various progenitor cells. In this study, we investigated the differentiation capacity of mouse ES cells into pancreatic hormone-secreting cells, and insulin-secreting cells. ES cells were cultured in Dulbecco's modified Eagle medium (DMEM) after removal of leukemia inhibitory factor (LIF) for 3 days and then transferred in DMEM supplemented with retinoic acid or activin A. When the culture of embryoid bodies (EBs) derived from ES cells in DMEM added with activin A (2×10^{-9} M or 2×10^{-10} M) for 5 days was performed, endoderm marker genes, GATA4, Sox17 and Foxa2 were expressed in the EBs. However, at 6 days of culture, expression of Sox17 was not observed. When EBs were cultured with activin A (2×10^{-9} M) for 6 days, and followed by 6 days of culture with retinoic acid (10^{-6} M), expression of the pancreatic cell marker genes, GATA4 and Fxa2, were continued, and pancreatic islet genes, insulins 1 and 2, glucagons and somatostatin, were expressed from 5 days of culture. Immunohistochemical analysis gave results similar to RT-PCR. We consider this differentiation method for definitive endoderm production and pancreatic hormone-secreting cells, by using activin A and, retinoic acid is considered to be effective.

Key words : Activin A, Insulin-secretion cell, Mouse embryonic stem cell, Retinoic acid

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of mouse blastocysts and can be maintained

indefinitely in culture [1]. Thomson *et al.* [2] isolated human ES cells from early human embryos and grew them in culture. It is known that these ES cells are indeed capable of becoming any specialized cells.

Embryonic stem (ES) cells are pluripotent cells with the ability to differentiate *in vitro* and *in vivo* into all cell types of the embryo proper. These cells represent a potentially unlimited source of differentiation cells or tissue for transplantation for common diseases such as type 1 diabetes mellitus. During embryogenesis *in vivo*, the liver and pancreas are originated from the definitive endoderm. Studies have been reported the production of glucose responsive insulin producing cells from ES cells [3, 4]. One study showed that with the use of activin A, exendin, cyclopamine and retinoic acid, human ES cells could be induced to endocrine cells capable of synthesizing pancreatic hormones [5]. Activin A is a member of the TGF- β superfamily, and has been shown to mimicking nodal [6]. Using the embryoid body (EB) differentiation model of mES cells, it has been showed that endoderm cells can be induced by treatment with activin A [7]. Also, activin A can induce the formation of neural extension [8]. The potential of activin A to induce ES cells to differentiate into both pancreatic and neural lineages has been demonstrated.

We have been studied the differentiation of mouse ES cells into various types of cells of three germ lineages, endoderm, ectoderm and mesoderm [9–11]. In the present study, we examined the differentiation of mouse ES cells into pancreatic insulin-cells by using activin A, and a novel protocol.

Materials and Methods

Cell culture and differentiation of mouse ES cells

ES cells (TT2 strain) were cultured on gelatinized tis-

sue culture dishes and feeder layer cells in a Dulbecco's modified Eagle medium (DMEM, Nissui pharmaceutical Co.) supplemented with 20% fetal bovine serum (HRJ, Lot no. 9k-4013), 10^{-4} M β -mercaptoethanol and 1000 unit/ml leukemia inhibitory factor (LIF, Esgro Chem.) at 37 °C under 5% CO₂ in air. The feeder layers used were produced by treating the embryonic fibroblasts, from 14.5 days mouse fetuses, with 10 μ g/ml mitomycin-C (Sigma) for 3 h. Feeder layer cells plated at approximately 10⁵ cells/ml in DMEM, supplemented with 20% FBS, were used as the control.

EB cultures were performed by culture using the hanging drop methods. ES cells were resuspended in differentiation medium and spotted as 30 μ l drops on the inverted lid of a 100 mm Petri dish at a concentration of 10,000 cells/ml. The dishes were incubated at 37 °C under 5% CO₂ in air. After 2 days of incubation, EBs were recovered from the feeder cells and subsequently cultured in suspension for 2 days. Then, the EBs were transferred to a 12 well plate. The schedule of treatment with activin A and retinoic acid (RA) for differentiation of mouse embryonic stem cells is shown in Fig. 1.

To induce differentiation of EBs, activin A was added into the medium without LIF. At 0, 4, 5 and 6 days after the start of culture, the culture medium was supplemented with activin A (2×10^{-9} M or 2×10^{-10} M) or RA (10^{-6} M) was supplemented to the culture media, respectively. One group of EBs was cultured for 6 days in medium containing activin A (2×10^{-9} M) and then, the EBs were cultured for 6 days in culture medium containing RA (10^{-6} M).

RT-PCR analysis

Total RNA was isolated from differentiated ES cells, using FastPure RNA Kit. Reverse transcription was performed with 100 ng of total RNA using SuperScript II reverse transcriptase, and 1 μ l aliquots of the reaction mixture were used for PCR with 1.25 unit of Ex Taq polymerase, 25 pmol of specific primers, and 0.2 mM dNTP mixture. The PCR reaction procedure consisted of denaturation at 94 °C for 30 sec, annealing for 30 sec, and extension at 72 °C for 1 min. PCR analysis was performed for each gene with following sets of primers: Oct3/4, 5'-CTCGAACCACATCCTTCTCT, 5'-GGCGTTCTCTTTG-GAAAGGTGTTTC; for Nanog, 5'-TGCCAGGAAGCAGAA-GATG, 5'-GGATACTCCACTGGTGCTGAG; for GATA4, 5'-CGAGATGGGACGGGACACT, 5'-CTCACCCCTCGGC-CATTACGA; for sox17, 5'-GATACGCCAGTGACGAC-CAGAGC, 5'-GAGGTTCACTCCGCGAGTCGTGTC; for Foxa2, 5'-GTAATGCGGAAGTTGGTGGG, 5'-TTGG-TAGTAGGAAGTGTCTGC; for Ins1, 5'-CCTGTTGGTG-

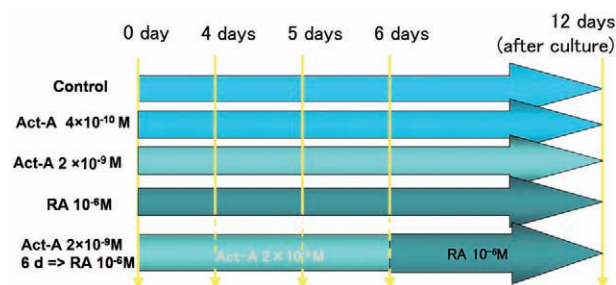


Fig. 1. Schedule of treatment with activin A and retinoic acid for differentiation of mouse embryonic stem cells. Act-A: activin A. RA: retinoic acid.

CACTTCCTAC, 5'-GTTGCAGTAGTCTCCAGCT; for Ins2, 5'-C-TGTGGATGCGCTTCTCCAGCT, 5'-AGTAGTCTC-CAGCTGGTAGAG; for Gcg, 5'-CTTCAAGACACAGAG-GAGAACC, 5'-CACCAGCCAAGCAATGAATTCC; for Sst, 5'-CTGGCTGCGCTCTGCATCGTC, 5'-CAGGAT-GTGAATGTCTTCCAGAAG; for Pdx1, 5'-CAGTGAG-GAGCAGTACTACGC, 5'-GATGATGTGTCTCTCGGT-CAAG; for and GAPDH, 5'-TCATTGACCTCAACTACATGG, 5'-GCTGTAGCCGATTTCATTGTC. PCR products were analyzed using 1.8% agarose gel electrophoresis, and the size of the products was determined by comparison with molecular weight standards after ethidium bromide staining.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline without Ca and Mg (PBS) for 15 min at room temperature. The cells were blocked with PBS containing 5% skim milk for 40 min followed by incubation for 24 h at 4 °C with primary antibodies specific to islet cell markers. Primary antibodies were applied in PBS as follows: rabbit anti-insulin polyclonal antibody (1:100 dilution), rabbit anti-glucagon polyclonal antibody (1:100 dilution), and rabbit anti-somatostatin polyclonal antibody (1:50 dilution) in PBS. The cells were washed with PBS containing 0.05% Tween20 followed by incubation with the secondary antibody, goat anti-rabbit IgG-FITC (1:200 dilution) in PBS, conjugated to fluorescent markers. Nuclei were stained with 10 μ g/ml of propidium iodide (PI).

Results and Discussion

At 4 days of the culture, endoderm marker genes, Sox17, GATA 4, and Fox 2 in EBs treated with activin A were (Fig. 2) expressed. However, at 6 days of culture

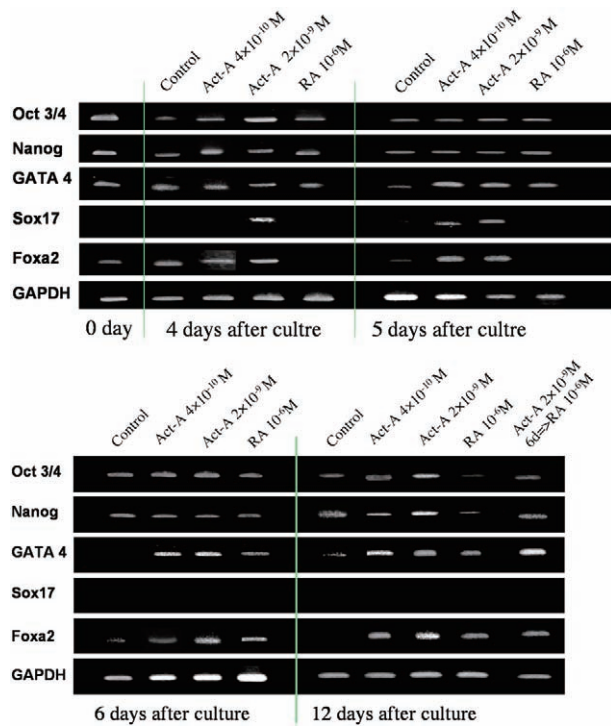


Fig. 2. RT-PCR analysis of endoderm gene expression after culture in the presence of activin A and/or retinoic acid.

the expression of Sox17 was not observed. When EBs were cultured with activin A for 6 days followed by RA for 6 days, the expression of endoderm markers was maintained (Fig. 2). When EBs were cultured for 5 days, pancreatic cell makers, Pdx1, insulin 1, insulin 2, glucagon and somatostatin were expressed clearly (Fig. 3). However, at 6 days of culture glucagon and somatostatin were not seen. When EBs were cultured with activin A (4×10^{-10} M) for 6 days, followed by RA (1×10^{-6} M) for 6 days, they showed the expression of insulin 1 and insulin 2, and somatostatin, but the expression of glucagon was only slight. In the controls the expression of Pdx1, insulin1, insulin 2, glucagon and somatostatin was entirely absent.

Immunohistochemical analysis of the insulin-secreting cells using anti-insulin antibodies revealed that when the cells were cultured with activin A or retinoic acid alone, at 5 or 6 days after culture, staining for insulin was clearly seen in ES cells (Fig. 4). High expression of insulin was seen when cells were cultured with activin A followed by retinoic acid (Fig. 5). The expression of glucagon and somatostatin from ES cells in EBs was similar results, when EBs were cultured with a combination of activin and retinoic acid. In contrast to these results, at 12 days

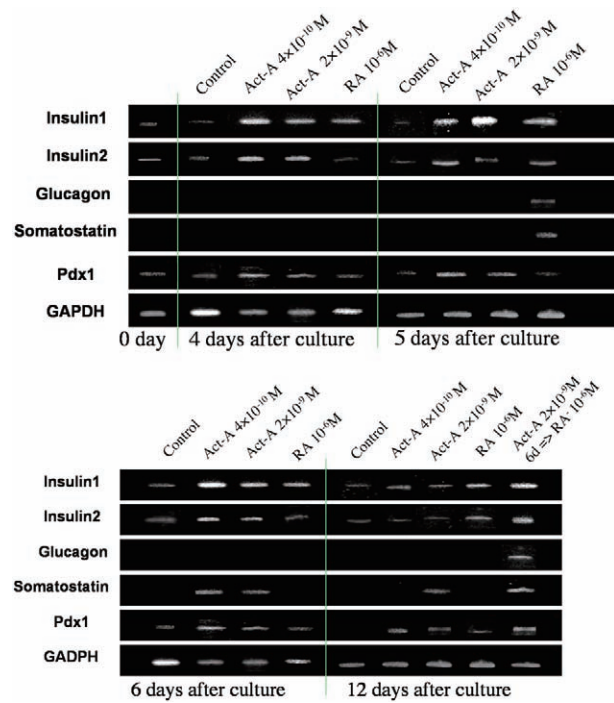


Fig. 3. RT-PCR analysis of pancreatic hormone gene expression. RNA samples were extracted from EBs after culture with activin A and retinoic acid.

of culture with activin A or retinoic acid, insulin expression decreased markedly in ES cells (Fig. 6). These results indicate the maintenance of insulin secretion in ES cells requires culture with retinoic acid after culture of ES cells with activin A, followed by culture with RA.

It has been reported that only the insulin 1 gene is expressed from pancreatic β cells in the rodent [12]. Since in the present study, expression of Pdx1, insulin1, insulin 2, glucagon and somatostatin from cultured cells was confirmed, and we consider the cultured cells were had to differentiated into pancreatic hormone secreting cells. A protocol using activin A and retinoic acid is thought to be useful for studying differentiation of definitive endoderm, pancreatic hormone-secreting cells from murine ES cells. Activin A, a member of the TGF- β superfamily, has been shown to mimicking nodal [6]. Activins interact with two types of cell surface transmembrane receptors, which have intrinsic serine/threonine kinase activities in their cytoplasmic domains. Activin A binds to the type II receptor and initiates a cascade reaction that leads to the recruitment, phosphorylation, and activation of the type I activin receptor. Using the EB differentiation model of mouse ES cells, it has been shown that endoderm cells can be induced to differentiate by treatment with activin A

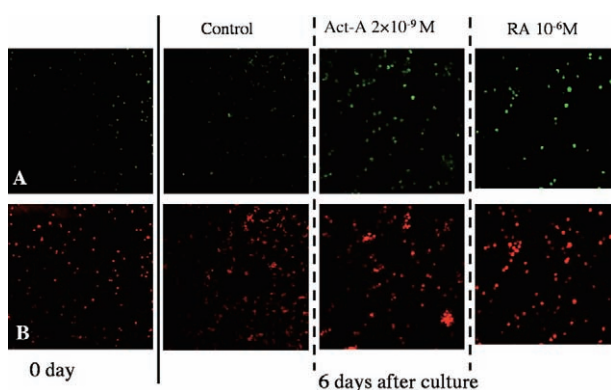


Fig. 4. Insulin expression from mouse ES cells induced by activin A or retinoic acid. A: Immunostaining of cultured cells in response to anti-insulin antibody. B: Propidium iodide (PI) stain. Act-A: activin A. RA: retinoic acid. ES cells showed insulin expression after culture with activin A (2×10^{-9} M) or retinoic acid (10^{-6} M) for 6 days. However, in the control group, expression of insulin was not seen.

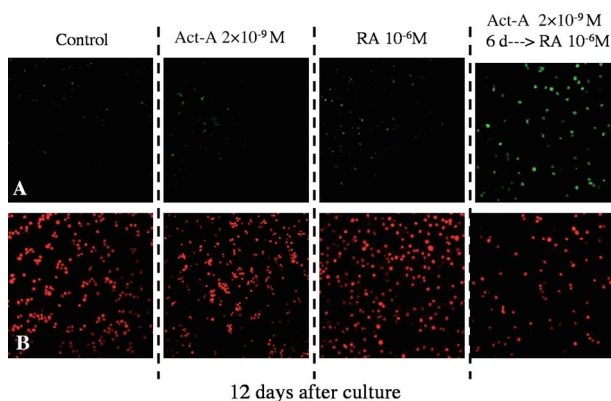


Fig. 5. Expression of insulin from mouse ES cells induced by activin A and retinoic acid. A: Immunostaining of cultured cells in response to anti-insulin antibody. B: Propidium iodide (PI) stain. Act-A: activin A. RA: retinoic acid. When ES cells were cultured with activin A or retinoic acid for 12 days, fluorescence of insulin decreased. However, the expression of insulin in ES cells was maintained when cells were first cultured with the combination of activin A (2×10^{-9} M) for 6 days, followed by retinoic acid (10^{-6} M) for 6 days.

[7, 13]. Using retinoic acid, basic fibroblast growth factor and cAMP, differentiation of mouse ES cells into definitive endoderm has been reported without EB formation [14]. The results of the present study demonstrate that a combination of activin A and retinoic acid in the culture of EBs is an effective and simple method of inducing differ-

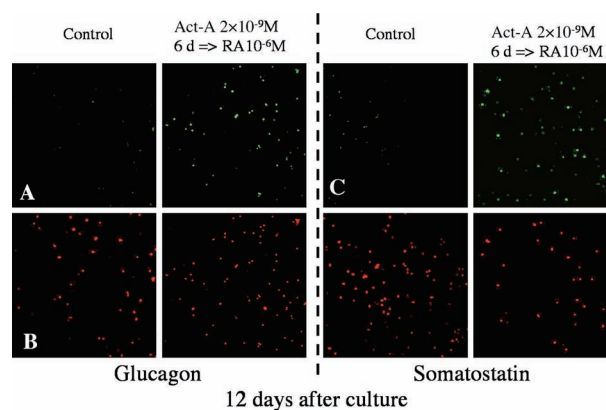


Fig. 6. Expression of glucagon and somatostatin from mouse ES cells induced by using activin A and retinoic acid. A: Immunohistochemical staining of cultured cells in response to anti-glucagon antibody. B: Propidium iodide (PI) stain. C: Immunohistochemical staining of cultured cells in response to anti-somatostatin antibody. Act-A: activin A. RA: retinoic acid. Fluorescence of glucagons was sustained when ES cells were cultured with activin A for 6 days followed by culture with retinoic acid for 6 days.

entiation into pancreatic hormone-secreting cells. Future studies of the detailed mechanisms of activin A and retinoic acid in the differentiation of ES cells into pancreatic endocrine cells from ES cells are desirable.

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