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E-cadherin gene-engineered feeder systems for supporting undifferentiated growth of mouse embryonic stem cells

Running title: E-cadherin gene-engineered feeders for ES cell culture

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(**ABSTRACT)**

Conventionally, embryonic stem (ES) cells are cultured on a cell layer of mouse embryonic fibroblasts (MEFs) as feeder cells to support undifferentiated growth of ES cells. In this study, cell-cell interactions between mouse ES and feeder cells were artificially engineered via an epithelial cell adhesion molecule, E-cadherin, whose expression is considerable in ES cells. Mouse mesenchymal STO and NIH3T3 cells that were genetically engineered to express E-cadherin were used in ES cell cultures as feeder cells. ES cells cultured on the E-cadherin-expressing feeder cells maintained the expression of stem cell markers, alkaline phosphatase (AP), Oct3/4, Nanog and Sox2, and the efficiency of AP-positive colony formation was comparable to MEFs, and much better than parental STO and NIH3T3 cells. Furthermore, ES cells maintained on the E-cadherin-expressing feeder cells possessed the ability to differentiate into the three germ layers both *in vitro* and *in vivo*. The results indicated that E-cadherin expression in feeder cells could improve the performance of feeder cells, which may be further applicable to create new artificial feeder cell lines.

(INTRODUCTION)

Embryonic stem (ES) cells possessing the inherent capability of infinite proliferation and differentiation into many cell types have been demonstrated to have great potential for cell-based therapies in regenerative medicine (1, 2). The undifferentiated state of ES cells is usually maintained by culture on a feeder cell layer in the presence of anti-differentiation factors such as leukemia inhibitory factor (LIF). Feeder cells produce many growth factors important for self-renewal of ES cells, including activin A, transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), Wnts and bone morphogenetic protein 4 (BMP4). These proteins upregulate the expression of transcription factors such as Oct3/4, Nanog and Sox2 to support undifferentiated growth of ES cells (3-7). Mouse embryonic fibroblasts (MEFs) isolated from mouse fetuses are often used as feeder cells for ES cell cultures, therefore MEFs are primary cells and can only be cultured for several passages without a loss of function. Although the mouse stromal cell line, STO, has been used as a substitute for MEFs, their performance as feeders in ES cell cultures is inferior to the MEFs.

In tissues and organs, cell-cell interactions play crucial roles in maintaining normal physiology. The cell-cell interactions can be mediated in three ways: soluble factors including cytokines; extracellular matrices (ECMs); and cell-cell adhesions. Cell-cell adhesion mediated by various molecules is an important factor in regulating differentiation and proliferation of cells. E-cadherin is a member of the classic cadherin family and expressed mainly in epithelial cells (8). The

extracellular domain on E-cadherin interacts with E-cadherin molecules on neighboring cells in a homotypic calcium-dependent manner, thereby facilitating cell-cell contact such as epithelial islands formed by epithelial cells. In our previous study, the E-cadherin gene was transferred to NIH3T3 fibroblasts (designated 3T3/E-cad cells), and cell-cell interactions between keratinocytes inherently expressing E-cadherin and 3T3/E-cad cells were artificially engineered (9). Similarly, we have succeeded in forming the cell-cell interactions between rat hepatocytes and 3T3/E-cad cells with high frequency, which resulted in enhanced albumin secretion by hepatocytes (9).

Additionally, E-cadherin has a central role in establishing cell-cell adhesive structures during embryogenesis (10), and it is indispensable to colony formation by ES cells (11). Thus, ES cells are known to express E-cadherin, whereas MEFs, STO and NIH3T3 cells do not express E-cadherin. We thought that E-cadherin expression in feeder cells may be a possible approach to enhance the interaction between ES and feeder cells. Therefore, STO and NIH3T3 cells were engineered to express E-cadherin and used for the experiment. Moreover, Nagaoka *et al.* reported that E-cadherin-coated plates could maintain the pluripotency of ES cells without colony formation (12). In the present study, we investigated whether E-cadherin-expressing STO and NIH3T3 cells could improve and support undifferentiated growth of mouse ES cells.

MATERIALS AND METHODS

Cell culture Mouse ES cell lines, H-1 (Riken BioResource Center, Tsukuba, Japan) and 129sv

(Chemicon, Pittsburgh, PA, USA) were cultured on mitotically inactivated feeder cells which were treated with mitomycin C for 2 h. The cells were cultured on 0.1% gelatin- (Nacalai Tesque, Kyoto, Japan) coated tissue culture dishes (Greiner Bio-one, Frickenhausen, Germany) in growth medium composed of Knockout-DMEM™ (Invitrogen, Carlsbad, CA, USA) with 4 mM L-glutamine (Wako Pure Chemical Industries, Osaka, Japan), non-essential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Millipore, Billerica, MA, USA), 100 U/ml penicillin G potassium (Wako), 50 μg/ml streptomycin sulfate (Wako), 15% Knock-out-serum-replacement (Invitrogen) and 10^3 U/l LIF (ESGRO; Millipore). The medium was changed every day and ES cells were passaged every 2–3 days. The MEFs were isolated from fetuses of 14 day pregnant BALB/c mice and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, FL, USA) and 4 mM L-glutamine. STO cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G potassium, 50 μg/ml streptomycin sulfate and non-essential amino acids. NIH3T3 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G potassium and 50 μg/ml streptomycin sulfate. These cells were cultured at 37°C in a 5% CO_2 incubator.

Establishment of E-cadherin-expressing cells An expression plasmid vector for E-cadherin, pcDNA4/E-cad-IRES-EGFP (9) was transfected into STO cells using a lipofection reagent (Lipofectamine2000; Invitrogen). Cells were selected in medium containing 1 mg/ml zeocin

(Invitrogen), and stable E-cadherin-expressing clones (designated as STO/E-cad cells) were established by the limiting dilution method.

Western blot analysis The cell lysates (40 μg total protein) of HaCaT, STO and STO/E-cad cells were subjected to SDS-PAGE on a 7.5% polyacrylamide gel, and the proteins were transferred onto a PVDF membrane (GE Healthcare, Buckinghamshire, UK). After blocking with 5% skimmed milk, the membrane was incubated with a rabbit anti-E-cadherin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, and probed with peroxidase-labeled anti-rabbit IgGs (Santa Cruz Biotechnology) for 1 h. The labeled antibodies were detected with an ECL detection system (GE Healthcare).

Alkaline phosphatase (AP) staining Cells were fixed with 4% paraformaldehyde for 5 min at room temperature and exposed to a solution containing naphthol AS-MX phosphate (Sigma) as a substrate and Fast Violet B Salt (Sigma) as a coupler for 20 min at 37°C. Cells showing AP activity stained dark brown and were observed with a microscope (Olympus, Tokyo, Japan).

RT-PCR analysis After plating of ES cells on gelatin-coated dishes to remove feeder cells, total RNA was extracted from ES cells using RNAiso Plus reagent (Takara, Otsu, Japan). The RNA was reverse-transcribed into cDNA from 1 μg total RNA using a ReverTra Ace First Strand cDNA

synthesis kit (Takara). The specific gene sequences were amplified by PCR using the primers shown in Table 1.

Hanging drop assay Embryoid bodies (EBs) were induced using the hanging-drop method (13). After harvesting of ES cells, they were resuspended in the ES medium without LIF at a concentration of 7,000 cells/ml. Droplets of the cell suspension (15 μl) were placed on the lid of a bacterial grade 100 mm plastic dish (AsOne, Osaka, Japan). The lid was turned upside down and placed on the bottom half of a dish filled with phosphate buffered saline (PBS) and then incubated at 37°C in a 5% CO_2 incubator. After 2 d, the cells were transferred into gelatin-coated dishes and cultured in DMEM supplemented with 15% FBS, non-essential amino acids, 100 U/ml penicillin G potassium and 50 μg/ml streptomycin sulfate.

Teratoma formation assay ES cells (1×10^6 cells) cultured on a feeder layer of STO/E-cad or 3T3/E-cad cells for more than 30 d were transplanted into the femurs of 5-week-old SCID mice (C.B-17/Icr Scid Jcl; Kyudo, Fukuoka, Japan) to form teratomas. At approximately 8 weeks after the injection of ES cells, teratomas were resected from the injection site. The animal experiment was performed according to the guideline of the Ethics Committee for Animal Experiments, Kyushu University.

For histological evaluation, teratomas were washed three times with PBS, fixed with 4%

paraformaldehyde in PBS and embedded in paraffin. Thin sections (4 μm) were prepared and stained with hematoxylin and eosin (H&E). The stained sections were observed using a microscope (Olympus).

Colony forming efficiency assay ES cells were cultured for 7 d on feeder cells and then 1×10^4 ES cells were replated onto MEFs. After 3 d in culture, AP staining was performed as described above. The number of AP-positive cells was counted using microscope images from five fields of view in three separate wells per sample. Colony forming efficiency was determined by the following equation:

Colony forming efficiency (-) = (the number of AP^+ colonies)/(the number of ES cells initially seeded (1×10^4 cells))

ES cell culture using conditioned medium (CM) The feeder cells treated with mitomycin C for 2 h were seeded at 5×10^5 cells/well into 6-well culture plates. On the next day, the medium was replaced with ES cell medium and the culture was further continued for 1 d. Then, the culture broths were collected and filtered using a 0.45 μm cellulose acetate filter (Advantec, Tokyo, Japan). CM was prepared by mixing the respective culture broths with fresh ES cell medium at a ratio of 1:1. ES cells were cultured for 7 d on each feeder cell layer in CM and then 1×10^4 cells were replated onto MEFs. After 3 d of culture, colony forming efficiency was measured.

Magnetic force-based co-culture Co-culture of ES cells on a feeder cell layer applying magnetic force for enhancing the physical contact between the cells was carried out. Magnetite cationic liposomes (MCLs) were prepared from colloidal magnetite nanoparticles (Fe_3O_4, average particle size 10 nm; Toda Kogyo, Hiroshima, Japan) and a lipid mixture consisting of *N*-(α -trimethylammonioacetyl)-didodecyl-D-glutamate chloride, dilauroylphosphatidylcholine and dioleoylphosphatidyl-ethanolamine at a molar ratio of 1:2:2 as described previously (14). For magnetic labeling of ES cells, MCLs were added to ES cells at the net magnetite concentration of 100 pg/cell. After a 2 h incubation, 1×10^5 ES cells were seeded onto a feeder cell layer in 24-well plates. A cylindrical neodymium magnet (30 mm diameter; magnetic induction, 4000 Gauss) was then placed under the 24-well plates to apply a magnetic force vertical to the plate. The cells were cultured for 7 d, including two passages using a magnet. Then 1×10^4 cells were replated onto MEFs. After 3 d of culture, colony forming efficiency was measured.

Statistical analysis All data are expressed as means ± standard deviation (SD). Statistical comparisons were evaluated using a one-way analysis of variance (ANOVA), and values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Establishment of E-cadherin-expressing STO cells Previously, we established stable NIH3T3 transformants expressing E-cadherin (3T3/E-cad) using a plasmid vector with an expression cassette for E-cadherin (9). In the present study, STO cells were transfected using the same plasmid, and E-cadherin-expressing clones were established (STO/E-cad). As shown in Figure 1A, STO/E-cad cells stably expressed E-cadherin (Fig. 1A). Similar to 3T3/E-cad cells (9), STO/E-cad cells grew to form cell islands obviously different from parental STO cells that showed a more scattered and fibroblastic morphology (Fig. 1B). When EGTA was added to the medium as a calcium-chelator in STO/E-cad cell culture to confirm calcium-dependent homotypic interactions between the cells (15), the disruption of cell-cell adhesion was observed (data not shown), suggesting that calcium-dependent interactions were formed between STO/E-cad cells.

Growth of ES cells on E-cadherin-expressing feeder cells We found that both STO/E-cad and 3T3/E-cad cells could effectively support the expansion and self-renewal of mouse ES cells (both H-1 and 129sv cell lines). The AP^+ ES cells cultured on feeder layers of both STO/E-cad (Fig. 2B) and 3T3/E-cad (Fig. 2C) cells exhibited a typical ES cell morphology, formed tightly packed cell colonies with smooth borders, as well as the ability to be cultured on a MEF feeder layer (Fig. 2A), while ES cells cultured on NIH3T3 (Fig. 2D) or STO (Fig. 2E) cells rarely formed AP^+ colonies. Nagaoka *et al.* reported that ES cells cultured on an E-cadherin-coated culture surface using a protein from the extracellular domain of E-cadherin fused with the Fc region of immunoglobulin G as an

adhesion matrix proliferated without colony formation (12). As a mechanism for scattering of ES cells, it was assumed that cells formed a different cytoskeletal organization and E-cadherin-rich protrusions regulated by Rac1 (16). In the present study, ES cells cultured on the feeder layers of E-cadherin-expressing cells formed dense colonies and exhibited similar morphology to culture on MEF feeder layers. The differences may be attributable to localization of E-cadherin molecules, since E-cadherin expressed on cells is mobile on the cell surface and organized to form an adhesion complex at the contact points. The localization of adhesion complex can be a limiting factor for cell migration. On the other hand, the cells can uniformly attach to the E-cadherin-coated surface, and cell migration might be promoted on the E-cadherin-coated surface.

Stem cell marker analysis of ES cells grown on E-cadherin-expressing feeder cells To test whether ES cells cultured on E-cadherin-expressing feeder cells for 20 d (10 passages) can maintain an undifferentiated state, various stem cell markers that characterize the undifferentiated state of ES cells were examined by RT-PCR (Fig. 2F). ES cells (H-1) cultured using both STO/E-cad and 3T3/E-cad cells as the feeder layer, as well as a MEF feeder layer, expressed Oct3/4, Nanog and Zfp42/Rex1 genes, while only low expression of those genes were detected in ES cells cultured on NIH3T3 and STO cells. The same trend was observed for the other ES cell line (129sv; data not shown). Taken together, E-cadherin-expressing cells used as the feeder layer successfully supported the pluripotent state of ES cells.

Pluripotency of ES cells grown on E-cadherin-expressing feeder cells The pluripotency of ES cells (H-1, 129sv) cultured on E-cadherin-expressing feeder cells was investigated by analyzing the capability of EB formation *in vitro* and teratoma formation *in vivo*. ES cells cultured on MEF and E-cadherin-expressing feeder cells were applied to suspension culture using the differentiation medium to form EBs. The expression of marker genes related to the formation of the three germ layers was analyzed by RT-PCR (Fig. 3A). The cells in EBs expressed the marker genes, including Neurod3/ngn1 for ectoderm, Actc1 for mesoderm, and Gata4, α-fetoprotein and BMP2 for endoderm, indicating that *in vitro* pluripotency of ES cells cultured on E-cadherin-expressing feeder cells was maintained.

Next, to evaluate *in vivo* pluripotency, ES cells cultured for more than 30 d on STO/E-cad cells as feeders were transplanted into the femurs of SCID mice, allowing teratoma development at the injection site (Fig. 3Bi). Histological examination of the teratomas revealed that they contained tissue types associated with all three germ layers, such as glands (endoderm, Fig. 3Bii), cartilage (mesoderm, Fig. 3Biii), striated muscles (mesoderm, data not shown), squamous tissues (ectoderm, Fig. 3Biv), and neural epithelium (ectoderm, data not shown). Similar results were obtained by using ES cells on 3T3/E-cad feeder cells (data not shown). These results indicated that ES cells cultured on the E-cadherin-expressing feeder cells maintained pluripotency *in vivo*.

Colony forming efficiency of ES cells cultured on E-cadherin-expressing feeder cells For the quantitative evaluation of feeder ability in E-cadherin-expressing cells, the AP^+ colony forming efficiency of ES cells was measured. As shown in Figure 4A, ES cells cultured on both STO/E-cad and 3T3/E-cad cells as feeders showed significantly higher colony forming efficiencies as compared with those cultured on parental STO and NIH3T3 cells. Furthermore, these colony forming efficiencies were comparable to that cultured on MEFs ($p = 0.45$ *vs.* STO/E-cad feeder, $p = 0.21$ *vs.* 3T3/E-cad feeder).

From the growth curves of ES cells cultured on each feeder layer, specific growth rate of ES cells cultured on each feeder layer was calculated as 1.30±0.31, 0.99±0.14 and 0.65±0.15 d^{-1} for MEF, STO/E-cad and 3T3/E-cad, respectively, and doubling time was calculated as 0.56±0.15, 0.64±0.10 and 1.11±0.24 d for MEF, STO/E-cad and 3T3/E-cad, respectively. ES cells showed comparable growth on MEF and STO/E-cad feeder cells, but slightly inferior on 3T3/E-cad feeder cells. These results were consistent with the colony forming efficiency (Fig. 4A).

To evaluate the effect of soluble factors produced by E-cadherin-expressing STO and NIH3T3 cells, CM derived from the E-cadherin-expressing cell cultures were used for ES cell cultures when the parental cells were used as feeders. As shown in Figure 4B, colony forming efficiencies of ES cells cultured on STO or NIH3T3 cells were not enhanced by the cultures using CM derived from STO/E-cad or 3T3/E-cad cells, suggesting that direct interaction between ES cells and the E-cadherin-expressing cells was important for the undifferentiated growth of ES cells. To further

elucidate the importance of direct contact between the cells, magnetically labeled ES cells were physically forced to attach onto the feeder cells by magnetic force. As shown in Figure 4C, colony forming efficiencies of ES cells cultured on the parental cells (STO and NIH3T3 cells) were significantly improved.

The precise mechanism for the reason why E-cadherin-expressing feeder cells can support the undifferentiated state of ES cells is still unknown. In the case of hepatocytes, Bhandari *et al.* (17) reported that the liver functions of hepatocytes were maintained by co-culture with 3T3 cells, but CM derived from 3T3 cell culture could not substitute for viable 3T3 cells co-cultured with hepatocytes in preserving hepatocyte functions, suggesting that cell-cell interaction is essential for modulating hepatocyte functions. Previously, we also showed that co-culture of hepatoma cells or primary hepatocytes with 3T3 or 3T3/E-cad cells induced improved albumin secretion of hepatic cells (8, 9). From these results, we assume that the imposed signals of soluble and/or insoluble factors from STO and NIH3T3 cells through direct interaction between the cells are responsible for supporting undifferentiated growth of ES cells. The soluble and insoluble factors may include critical cytokines, which are active with a relatively high local concentration, and extracellular matrix components, which bind cytokines onto ES cells making their functions sustainable during ES cell culture.

A major advantage of the system using E-cadherin expressing feeder is that the feeder cells can be infinitely amplified by culture, while growth ability of MEFs is limited. Moreover, signal

transduction between ES and feeder cells may be enhanced leading to reduction of cytokines such as LIF. Although the addition of LIF in culture medium was still necessary to maintain undifferentiated growth of ES cells on E-cadherin expressing feeders, the LIF dependency of ES cells weakened on STO/E-cad feeder compared with MEF feeder (data not shown). Thus, introduction of LIF gene into STO/E-cad cells may be effective for constructing a further attractive feeder system.

It has been known that many signals are involved in self-renewal of ES cells, and the LIF-dependent JAK/STAT signaling pathway is linked to cell adhesion especially through E-cadherin (19). Recently, Soncin *et al.* reported that abrogation of E-cadherin-mediated cell-cell contact in mouse ES cells resulted in reversible LIF-independent self-renewal (20). These results suggested that ES cells were regulated by cell signaling through E-cadherin. To investigate the effect of co-culture with the E-cadherin-expressing cells on E-cadherin signaling of ES cells, semi-quantitative RT-PCR analysis was performed to determine the expression levels of E-cadherin and β-catenin in ES cells cultured on the E-cadherin-expressing cells. However, no significant increase was observed (data not shown). Further studies examining E-cadherin signaling are necessary to elucidate the mechanisms for maintaining the undifferentiated state of ES cells.

In conclusion, we demonstrated that E-cadherin gene transfer to STO and NIH3T3 cells enhanced the ability of feeder cell layers in ES cell cultures. The isolation of MEFs is a laborious and time consuming process requiring the sacrifice of many mice. Although feeder-free culture methods of ES cells have been reported, they can cause chromosomal instability of ES cells (20, 21). Thus,

E-cadherin gene transfer is a possible approach in improving the performance of feeder cells, which may be further applicable to create new artificial feeder cell lines.

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Figure legends

FIG. 1. Establishment of E-cadherin-expressing cells. (A) Western blot analysis of E-cadherin expression in STO/E-cad, STO and HaCaT (as a positive control) cells using anti-E-cadherin antibody. (B, C) Cell morphology of STO/E-cad (B) and STO (C) cells.

FIG. 2. Proliferation of ES cells (H-1) on various feeder layers. (A–E) Alkaline phosphatase staining of ES cells cultured on a feeder cell layer of MEFs (A), STO/E-cad (B), 3T3/E-cad (C), STO (D) or NIH3T3 (E) cells. (F) RT-PCR analysis of genetic markers for the undifferentiated state. ES cells were cultured on MEF, STO/E-cad, 3T3/E-cad, STO or NIH3T3 feeder layers.

FIG. 3. Pluripotency of ES cells grown on E-cadherin-expressing feeder cell layers. (A) RT-PCR analysis of marker genes related to the formation of the three germ layers. ES cells were maintained on a feeder cell layer of E-cadherin-expressing cells or MEFs for 30 d, and then the ES cells were applied to suspension cultures using the differentiation medium to form EBs. After 11 d, the expression of marker genes was analyzed by RT-PCR. (B) Teratoma formation of ES cells cultured on STO/E-cad cells as feeders. At approximately 8 weeks after the injection of ES cells, teratomas were resected from the injection site (i). H&E staining of teratomas demonstrated differentiation into glands (ii), cartilage (iii) and squamous epithelium (iv).

FIG. 4. Colony forming efficiency of ES cells cultured on each feeder layer. (A) ES cells cultured on the respective feeder cells for 7 d and the ES cells were replated onto a MEF feeder layer. Then, AP-positive ES cell colonies were counted 3 d after reseeding. The experiments were performed in triplicate, and the data presented as mean ± SD. *$P < 0.05$. (B) Effect of CM from E-cadherin-expressing cells on colony forming efficiency of ES cells cultured on the respective feeder cells. Normal ES cell medium (open columns) or CM (closed columns) was used for ES cell cultures on feeder cells. When STO and STO/E-cad cells were used as feeder cells, ES cells were cultured using CM derived from cultures of STO/E-cad and STO cells, respectively. Similarly, when NIH3T3 and 3T3/E-cad cells were used as feeder cells, ES cells were cultured using CM derived from cultures of 3T3/E-cad and NIH3T3 cells, respectively. The experiments were performed in triplicate, and the data presented as mean ± SD. *$P < 0.05$. (C) Effect of co-culture of ES cells with feeder cell layers using forced cell-cell interaction *via* magnetic force. MCL-labeled ES cells were cultured on the respective feeder cell layers with (closed columns) or without (open columns) an applied magnetic force for 7 d. The ES cells were replated onto MEF feeder layers. Then, AP-positive ES cell colonies were counted 3 d after reseeding. The experiments were performed in triplicate and the data presented as mean ± SD. *$P < 0.05$.

TABLE 1. Primer sequences for RT-PCR analysis.

Target gene (product size)	Primer sequence	
Oct3/4 (459 bp)	FW: 5’-CTG AGG GCC AGG CAG GAG GAG CAC GAG-3’,	RV: 5’-CTG TAG GGA GGG CTT CGG GCA CTT-3’
Nanog (163 bp)	FW: 5’-GCG GCT CAC TTC CTT CTG ACT T-3’,	RV: 5’-GAC CAG GAA GAC CCA CAC TCA T-3’
Zfp42/Rex1 (287 bp)	FW: 5’-ACG AGT GGC AGT TTC TTC TTG GGA-3’,	RV: 5’-TAT GAC TCA CTT CCA GGG GGC ACT-3’
Neurod3/ngn1 (405 bp)	FW: 5’-CAT CTC TGA TCT CGA CTG C-3’,	RV: 5’-CCA GAT GTA GTT GTA GGC G-3’
Actc1 (124 bp)	FW: 5’-CCA GAT CAT GTT TGA GAC CTT CAA-3’,	RV: 5’-GAA CAT TAT GAG TTA CAC CAT CGC-3’
Gata4 (207 bp)	FW: 5’-CTG GAG GCG AGA TGG GAC GGG ACA CTA C-3’,	RV: 5’-CCG CAG GCA TTA CAT ACA GGC TCA CC-3’
α-Fetoprotein (173 bp)	FW: 5’-TCG TAT TCC AAC AGG AGG-3’,	RV: 5’-AGG CTT TTG CTT CAC CAG-3’
BMP2 (249 bp)	FW: 5’-GGG ACC CGC TGT CTT CTA GTG TTG C-3’,	RV: 5’-TGA GTG CCT GCG GTA CAG ATC TAG CA-3’
GAPDH (150 bp)	FW: 5’-CTA CCC CCA ATG TGT CCG TC-3’,	RV: 5’-GCT GTT GAA GTC GCA GGA GAC-3’

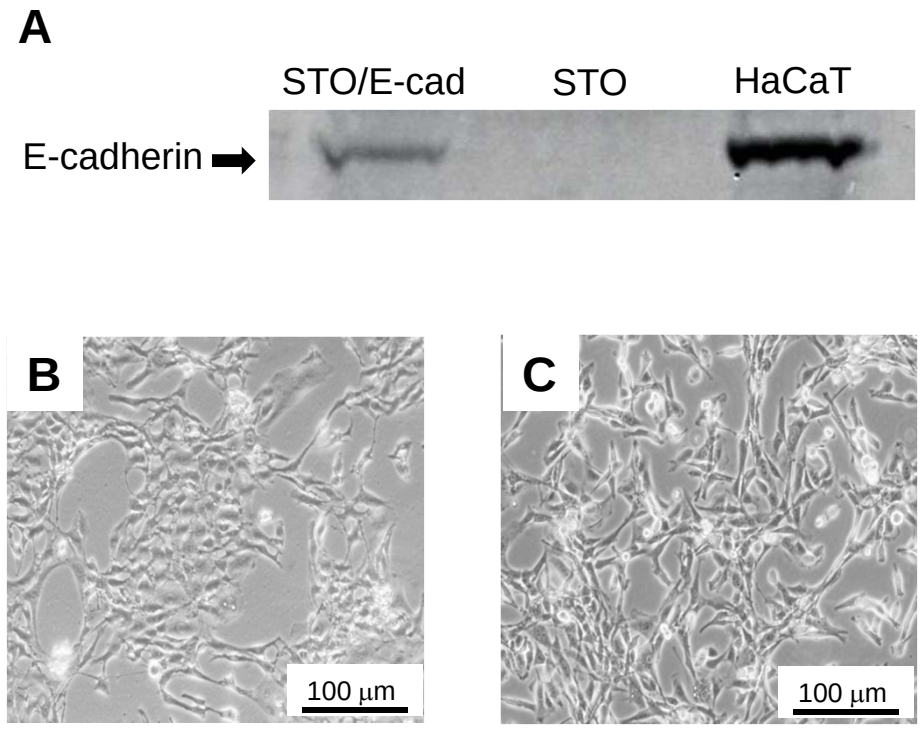
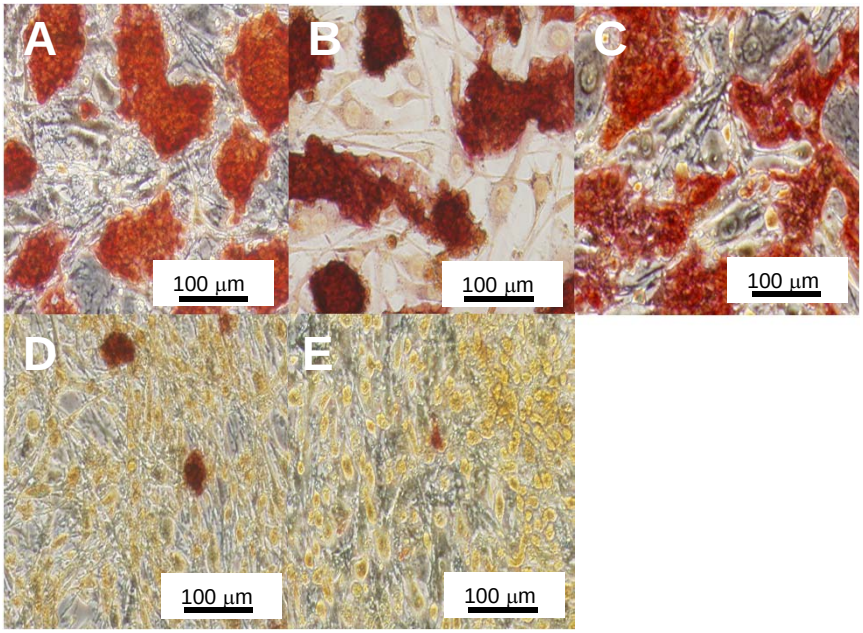


Fig. 1 Horie *et al.*



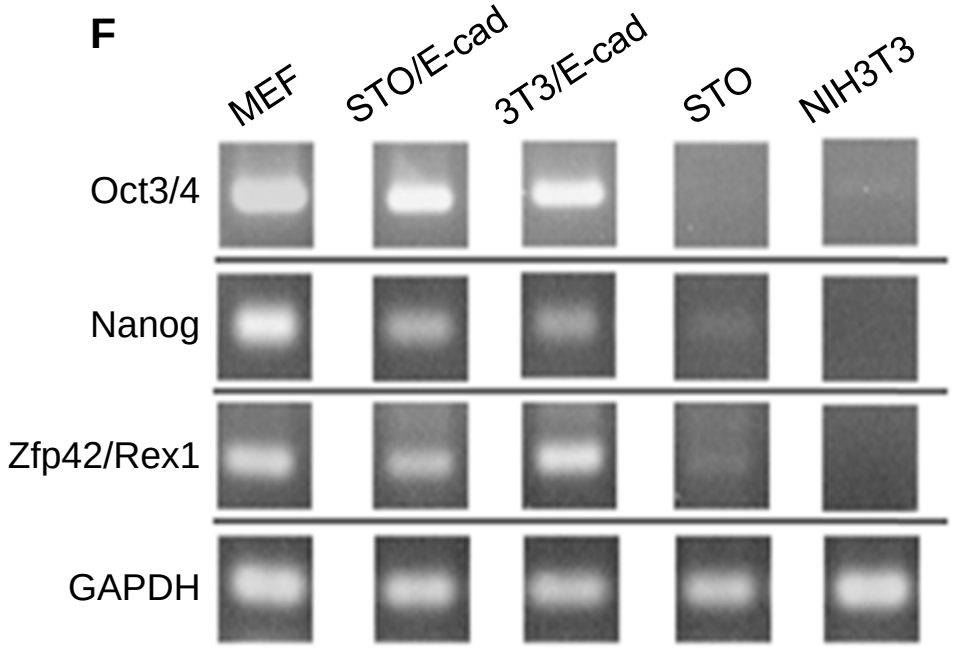


Fig. 2 Horie *et al.*

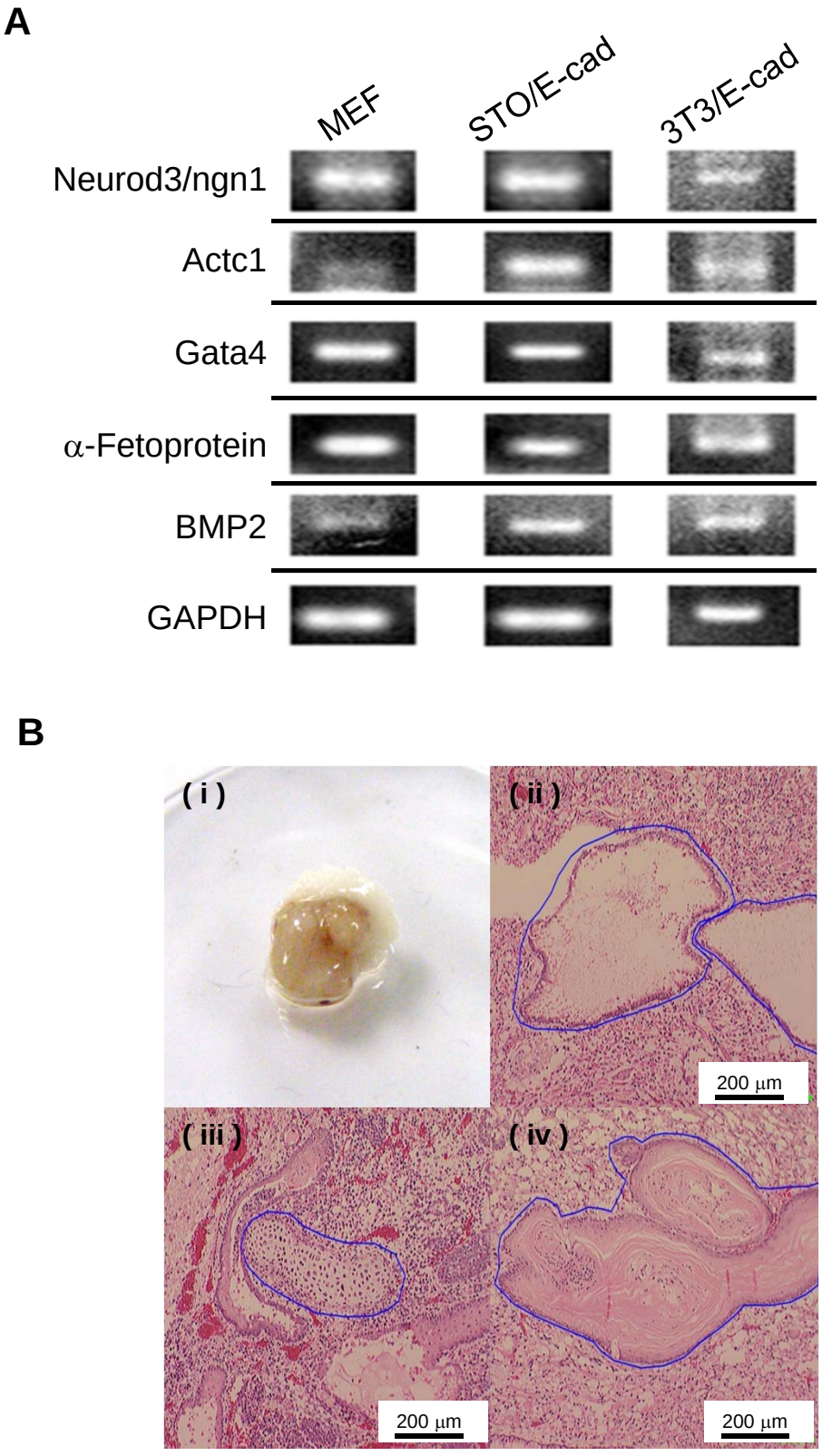


Fig. 3 Horie *et al.*

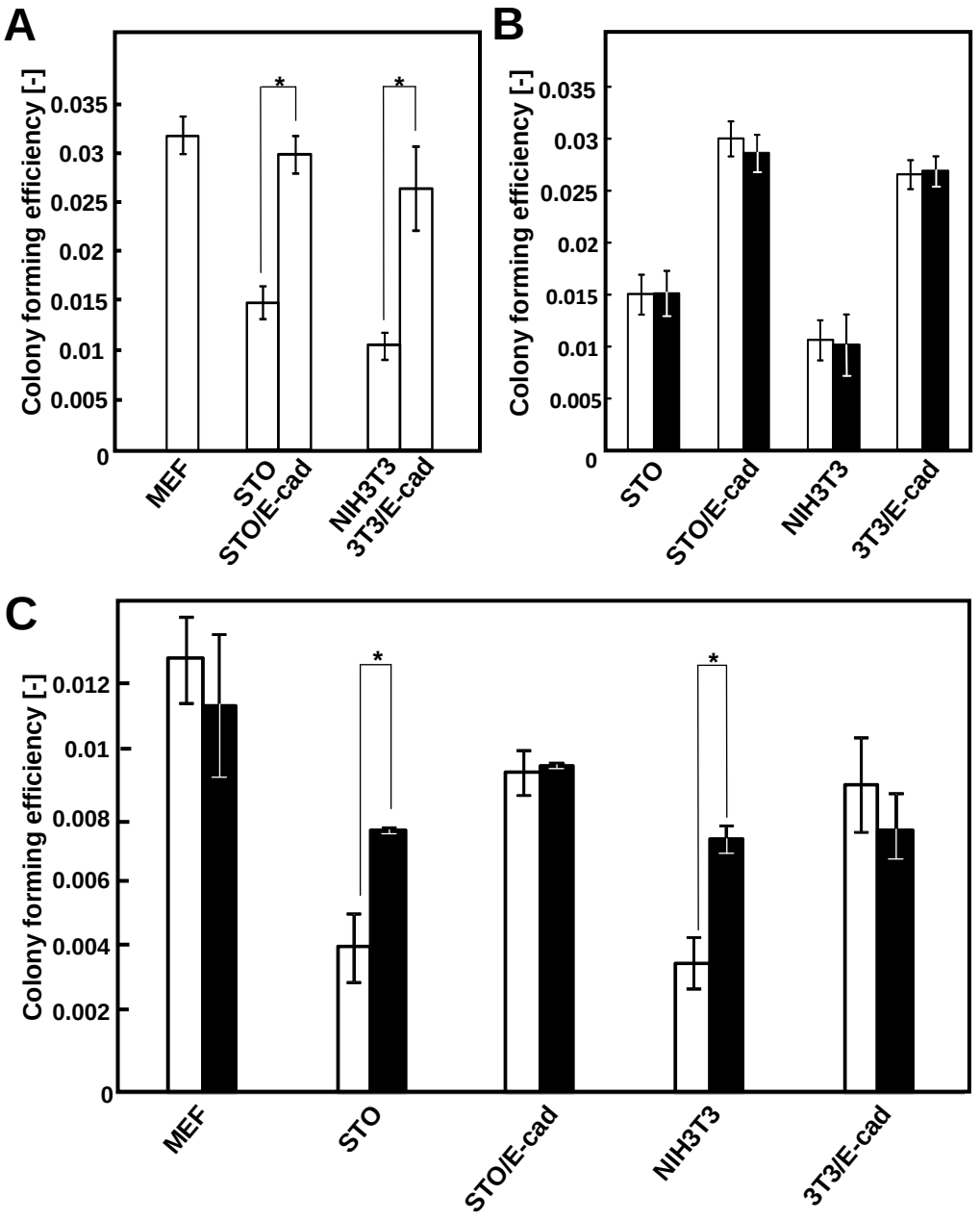


Fig. 4 Horie *et al.*