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Application of Embryonic Stem Cells as a Novel Tool in Drug Screening

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1. Introduction

Toxicological screening using animals are necessary for drug development registration. This approach is time-consuming, costly, labour intensive, stressful for the animals and susceptible to inaccuracies due to individual differences between animals. So, the screening of candidate chemicals in early development is often replaced with *in vitro* cell culture systems (Pearson, 1986; Liebsch & Spielmann, 2002). *In vitro* studies using cell lines were capable of providing more rapid, precise, relevant information than some animal studies, and economical approach for the evaluation of the pharmaco-toxicological profiling of target drugs, characterised by a low compound requirement and short duration (Pearson, 1986; Kari et al., 2007). Also, it is possible to include mechanistic studies, and to test for toxicity that is specific to humans: sensitivity differences between humans and rodents can affect animals (Kari et al., 2007). Among the *in vitro* screening systems, primary cell cultures and/or target organ-specific cell lines can be used to measure the general toxicity of a test compound (Zhou et al., 2006).

However, the sensitivity of hepatotoxicity using primary human hepatocytes or the HepG2 cell line cannot predict effects in early development and toxicological differences, which depend on the state of differentiation in hepatocytes (Knasmuller et al., 2004; Xu et al., 2004). In addition to, primary cells such as hepatocytes in particular and many transformed human hepatocyte-derived cell lines (immortalized cultures, i.e Fa2-N4 cells, HepaRG cells) have limitations in their life span and can have donor-dependent variations (Mills et al., 2004). Also, they have disadvantages such as discontinuous phenotypic characteristics, functional properties and genetic instability. Therefore, more promised future is waiting for hepatocyte-like cells as the source of hepatocytes regarding the approach of stem cells use in the high throughput testing (Duret et al., 2007).

Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells that specialized functions in specific tissues and make up the organ (Thomson et al., 1998; Zhang & Wang, 2008; Schnerch et al., 2010). The classification of stem cells divided into embryonic stem cells (ESCs) and adult stem cells (ASCs) according to derivative origins. ESCs, which are derived from the inner cell mass of blastocysts after fertilization, can unlimited self-renewal and have pluripotent could be rise to cells derived from all three germ lineages. Otherwise, ASCs derived from the specialized cell types of the tissue from which are originated have limited self-replicate and mutilpotent could be giving rise to specialized cells into multiple-lineages not all three germ lineages.

Since Thomson and colleagues were firstly reported to potentials for therapeutic and drug discovery using embryonic stem cells derived from inner cell mass of human blastocysts in 1998, stem cell research have been spotlighted more public and professional interest than other subject in biology field (Thomson et al., 1998; Fuh & Brinton, 2009; Astori et al., 2010; Rashid et al., 2010).

Although the field of stem cell research has grown rapidly, there are focused on the cell therapy using stem cells in regenerative medicine because stem cells have a great fascinating potential could be differentiation into the specialized cells to replace damaged cells or tissues. ESCs is an attractive source for cell therapy, however, there are several obstacles including ethical problems to clinical application using ESCs in degenerative medicine. Especially, the optimal guideline for differentiation of ESCs have been shown to be controversy in the condition of differentiation into target cells due to various factors used to induce the differentiation in ESCs (Denham & Dottori, 2009; Zagami et al., 2009; Bourzac et al., 2010).

Due to the reason, many scientists have been found a research field to make the best use of the potential benefits of stem cells. Stem cell-based systems have striking advantages to select of lead candidates and development of new therapeutic drugs because their proliferation ability and plasticity to generate several cell types. In addition, it is possible to analyze effects or toxicities of target drugs according to differentiation steps from immature to mature cells could be function. *In vitro* assaying of embryotoxicity using embryonic stem cells for the early determination of the teratogenic potential of a compound have been attempted (Kim et al., 2006; Kulkarni & Khanna, 2006). Also, in vitro screening system using mouse embryonic stem cells have been suggested by the European Center for Validation of Alternative Methods (ECVAM) committee (Spielmann et al., 2001; Genschow et al., 2002). Therefore, drug screening system using stem cells is a large range of opinion on this issue. The application of ESCs in drug screening is a promising, innovative alternative that appears to have early efficacy, while reducing the adverse outcomes at later stages of development. Furthermore, it is very sensitive in toxicity screening, as compared to other cell lines. For this reason, the application of ESCs could be an important new tool for developing a unique *in vitro* model with the potential to predict genotoxicity in humans. Drug screening using ESCs focuses on reproductive biology and embryo development. The embryonic stem cell test (ESCT) can detect developmental abnormalities such as abnormal embryogenesis and malformations caused by mutagenic or embryotoxic substances in early embryogenesis.

With this intention, this chapter review outlines the limitation of traditional toxicity screening including in vitro and in vivo screening system. This brief review outlines the construction of more efficient mouse ESC-derived embryoid bodies (EBs) for use as a vasculogenesis model and as a tool for screening the cytotoxicity of new compounds, and the cytotoxicity of 5-fluorouracil (5-FU), and to examine its effects on cell viability, proliferation, and differentiation in mouse ESC-derived endothelial differentiation. Also, we also consider the obstacles that need to be overcome to make the embryonic stem cells cost-effective, to enable guided differentiation to target cells, and to establish a reproducible *in vitro* assay to increase its efficacy as a screening system. Finally, we introduce the latest research on the ESCT and the potential of using ESCs for drug screening.

2. Characterization of embryonic stem cells

Stem cells have the capacity to self-renew as well as the ability to generate differentiated cells that specialized functions in specific tissues and make up the organ. The classification

of stem cells divided into embryonic stem cells (ESCs) and adult stem cells (ASCs) according to derivative origins. ESCs, which are derived from the inner cell mass of blastocysts after fertilization, can unlimited self-renewal and have pluripotent could be rise to cells derived from all three germ lineages. Otherwise, ASCs derived from the specialized cell types of the tissue from which are originated have limited self-replicate and mutilpotent could be giving rise to specialized cells into multiple-lineages not all three germ lineages.

Since mouse embryonic stem cells (mESCs) and human embryonic stem cells (ESCs) were established in 1981 and in 1998, ESCs are a hot issue in stem cell research (Martin, 1981; Thomson et al., 1998). Especially, unique characterization of human ESCs shows unlimited proliferation activity through the expression of stemness markers such as Oct-4, alkaline phospatase, Nanog, SSEA-4, TRA-1-60, and TRA—81 in condition of co-cultured with feeder cells (Figure 1).

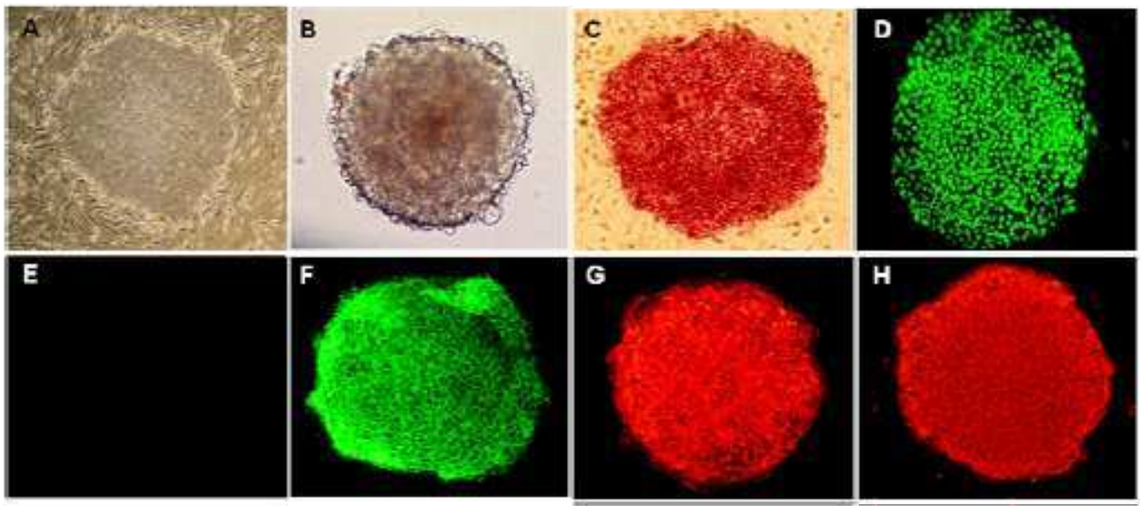

Fig. 1. Characterization of human embryonic stem cells. Morphology of human ES cells cultured with feeder cells (A) and embryonic body (B), Expression of stemness markers, Alkaline phosphatase (C), Oct-4 (D), SSEA-1 (E), SSEA-4 (F), TRA-1-60 (G), and TRA-1-80

Also, human ESCs in vitro can be expanded indefinitely in the undifferentiated state and still retain the capacity for differentiation into endodermal, mesodermal, and ectodemal lineages cells under specific condition in vitro. The potential for differentiation of human ESCs could be confirmed by teratoma formation in SCID mice transplanted human ESCs. Teratoma is an encapsulated benign tumor with tissues or organ components resembling normal derivatives of all three germ layers (Figure 2). The potentials offer a therapeutic intervention in the treatment of degenerative diseases that affect various tissues and their therapeutic effects may be influenced by numerous factors such as cell count, differentiation potential, transplant method, and disease model (Petersen et al., 1999; Huttmann et al., 2003; Kuo et al., 2008).

The characterization of human ESCs derived from early embryos that seem to share many of the properties of mouse ESCs has refocused attention on the in vitro properties of ESCs (Thomson et al., 1998). Therefore, mouse and human ESCs will provide new insights into embryonic development as well as its translation into therapeutic outcomes.

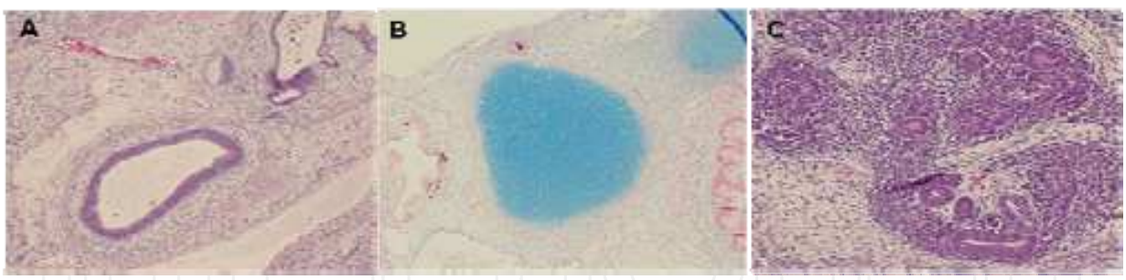

Fig. 2. Differentiation potential of human ESCs by teratoma formation in SCID mice engrafted human ESCs. Teratoma showing tissues from all three germ layers; endoderm (gut epithelium, A), mesoderm (cartilage, B), and ectoderm (neural rosettes, C)

3. Limitation of traditional toxicity screening

Toxicogenomics, which combines toxicology and genomics, is a scientific field that studies how the gene and protein activity within particular cell or tissues of an organism is involved in responses to toxicants. Also, they studies for genetics, mRNA expression, cell and tissue-wide protein expression and metabolomics to understand the role of gene-toxicants interactions in disease. From these processing, new biomarkers for toxicity can discover as well as predictive in toxicology. However, the relationship between dose and its effects on the exposed organism still controversy because there are several factors including age, sex, individual variability, and species differences affected susceptibility and variability in toxicology.

Toxicological screening using animals are necessary for drug development registration. Although there are several animal models from zebra fish to monkey for in vivo toxicity screening as well as drug development, however, there are many kinds of considerations in vivo toxicity screening using animal model. Especially, embryotoxicity tests using animals are a traditional strategy to identify potentially hazardous chemicals. They can also be used to confirm the absence of toxic properties in the development of potentially useful new substances (Spielmann, 2009). Because this approach is time-consuming, costly, labour intensive, stressful for the animals and susceptible to inaccuracies due to individual differences between animals, the screening of candidate chemicals in early development is often replaced with *in vitro* cell culture systems (Pearson, 1986; Liebsch & Spielmann, 2002; Bremer & Hartung, 2004; Knight, 2007). Animal ethics in vivo screening system have been becoming emphasized by Institutional Animal Care and Use Committees (IACUC). In addition, it is difficult to check the correct time point for toxicity testing in screening using animal model. Therefore, there is a need for alternative methods to evaluate the potential reproductive toxicity of chemical substances, by *in vitro* systems. To develop a new alternative screening test, many scientists have tried to use cell lines, primary cell cultures of dissociated cells from mice or rat embryo limb buds, midbrains for micromass tests, or whole embryos from rat (Steele et al., 1983).

In vitro studies using cell lines were capable, or potentially capable, of providing more rapid, precise, relevant information than some animal studies, and economical approach for the evaluation of the pharmaco-toxicological profiling of target drugs, characterised by a low compound requirement and short duration (Pearson, 1986; Kari et al., 2007). Also, it is

possible to include mechanistic studies, and to test for toxicity that is specific to humans: sensitivity differences between humans and rodents can affect animals (Kari et al., 2007). Among the *in vitro* screening systems, primary cell cultures and/or target organ-specific cell lines can be used to measure the general toxicity of a test compound (Zhou et al., 2006). However, the sensitivity of hepatotoxicity using primary human hepatocytes or the HepG2 cell line cannot predict effects in early development and toxicological differences, which depend on the state of differentiation in hepatocytes (Knasmuller et al., 2004; Xu et al., 2004). In addition to, primary cells such as hepatocytes in particular and many transformed human hepatocyte-derived cell lines (immortalized cultures, i.e Fa2-N4 cells, HepaRG cells) have limitations in their life span and can have donor-dependent variations (Mills et al., 2004). However, they have disadvantages such as discontinuous phenotypic characteristics, functional properties and genetic instability.

In toxicological research, the development of alternative *in vitro* toxicity screening systems to replace *in vivo* screening methods using animal experiments and conventional screening systems is important. Although applications using primary cells derived from rodent embryo or tissue-specific cell lines originating from humans have been tried, the validation of cytotoxicity during cellular biological processing must be improved in order to establish new and alternative *in vitro* screening methods, and to increase the efficiency of toxicological analysis through these methods (Tiffany-Castiglioni et al., 1999). Therefore, more promised future is waiting for hepatocyte-like cells as the source of hepatocytes regarding the approach of stem cells use in the high throughput testing (Duret et al., 2007).

4. In vitro toxicity screening using embryonic stem cells

Embryonic stem cells are generally obtained from inner cells mass in blastocysts of either mice or humans. Especially, drug or toxicity screening using human ESCs have many advantages over primary cells and immortalized cell lines for *in vitro* toxicity screening, including unique properties such as unlimited self-renewal, plasticity to generate various cell types and availability of cells of human origin (Davila et al., 2004; Kulkarni & Khanna, 2006; Zhang & Wang, 2008). However, there are some disadvantages as they do not grow as well and are more difficult to cultivation than mouse ESCs. Also, the stem cell technologies for directing them to differentiate are less defined than those for mouse ESCs and it is necessary to provide human ESCs that can be employed in a practical manner for compound screening.

Due to the reason, an Embryonic Stem Cell Test (EST), which mirrors growth and differentiation, is an *in vitro* test system well-suited for the evaluation of the embryotoxic potential of substances (Evans & Kaufman, 1981; Martin, 1981; Smith, 1992; Spielmann et al., 1997; Ramalho-Santos et al., 2002; Wobus & Boheler, 2005). The regulation of the differentiation of mouse ES cells is controlled by critical transcription factors, such as STAT3 (Niwa et al., 1998), Oct4 (Nichols et al., 1998), and Nanog (Chambers et al., 2003): Previous studies have shown that embryonic stem (ES) cells require the expressions of different transcription factors to specify the stem cell state, and that these contribute to the generation of entirely different lineages on changing culture conditions and altering the expression levels of Oct4, a key determinant of the pluripotency for *in vitro* systems (Wobus & Boheler, 2005). In addition, these mES cell systems were reported to be very useful for measuring embryotoxicity (Seiler et al., 2004). Moreover, mouse embryonic stem cells can be routinely employed to screen chemical compounds for teratogenic effects (Scholz et al., 1999; Rohwedel et al., 2001; Vanparys, 2002).

In this chapter, we introduce the possibility of endothelial-like cells derived from mouse embryonic bodies as an in vitro vasculogenesis model and the usefulness of mouse ESCs for in vitro toxicity screening for 5-fluorouracil (5-FU), anti-angiogenesis agents.

5. Endothelial-like cells derived from mouse embryonic bodies as an in vitro vasculogenesis model

The formation of new blood vessels (vasculogenesis) during embryonic development is an important basic step. This process involves the differentiation of angioblasts from mesoderm during the early developmental stages and their organization from a primitive vascular network (Vittet et al., 1996), and is characterized by the expression of endothelial cell specific molecules during the formation of vascular structures in ES-derived embryoid bodies (EBs). A number of markers including vascular endothelial growth factor receptors-2 (Flk-1) (Yamaguchi et al., 1993), platelet endothelial cell adhesion molecule (PECAM) (Redick & Bautch, 1999), and vascular endothelial (VE) cadherin as markers of vasculogenesis have been reported (Dejana et al., 1999). Endothelial, endothelial-like cells, and endothelial precursor cells (EPCs) derived from stem cells have been explored to establish a toxicity screening system for endothelial-specific toxicants (Kim & von Recum, 2008). The feasibility of these screening systems depends on the differentiation processes of the ESCs used; guided differentiation into target cell types and accurate investigation of the mechanisms of endothelial toxicity are necessary. Recently, we reported that endothelial-like and endothelial cells derived from mouse ESCs using EGM medium and optimal protocols are more sensitive to 5-FU toxicity than undifferentiated endothelial cells as well as a mouse endothelial cell line (Kim & von Recum, 2008).

Doetschman et al (1985) were the first to show an *in vitro* mouse model based on the differentiated ES cells. When mES cells are grown in the absence of feeder cells and myeloid leukaemia inhibitory factor (LIF), they are able to differentiate spontaneously (Doetschman et al., 1985). EBs were formed by spheroid aggregation during post-implantation in embryonic tissues and maintained in conditioned medium containing a cocktail of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6) and erythropoietin (EPO) in 1% methylcellulose to induce the formation of EBs (Wang et al., 1992; Vittet et al., 1996). This model system, which forms a primitive vascular plexus, provides an attractive tool for investigating the mechanisms involved in vasculogenesis, i.e., angioblast differentiation, proliferation, migration, endothelial cell-cell adhesion, and vascular morphogenesis (Doetschman et al., 1985; Risau et al., 1988; Wang et al., 1992). In addition, co-cultures with stromal cells, even adherent monolayer cultures in the absence of LIF (Ying et al., 2003), have been used to differentiate mES cells *in vitro*. Moreover, the expression levels of markers of endothelial differentiation at the mRNA and protein levels differ according to endothelial cell differentiation and culture conditions (Doetschman et al., 1985; Risau et al., 1988; Vittet et al., 1996). For these reasons, the mechanism of endothelial cell differentiation involved in the regulation of vasculogenesis remains unclear. However, the exact molecular biological role of 5-FU on cell cycle regulation in the endothelial differentiation of mouse ESCs has not been fully explained yet.

Therefore, we demonstrated that the population of endothelial cells derived from mES cells and to apply this as a tool for the screening of agents with vascular developmental toxicity. Also, we investigated that the specific action of 5-FU on the endothelial differentiation of cells derived from mouse ESCs, finally, we demonstrated that the correlation between cell cycle regulation and endothelial differentiation in mouse ESCs exposed by 5-FU.

5.1 Materials and methods

5.1.1 Cell line and culture conditions

Mouse D_3 ES cells (ATCC Cat. No. CRL-1934, Rockville, MD, USA) were co-cultured with mitomycin C-treated mouse embryonic fibroblast (MEF) cells in high glucose DMEM (Gibco-BRL, Invitrogen, Carlsbad, CA) containing 15% fetal bovine serum (FBS; Hyclone, Ogden, UT), 1000 U/ ml of LIF/ESGRO (Chemicon, Temecula, CA), and basic ES medium components [50 U/ml of penicillin and 50 µg/ml streptomycin (Gibco-BRL, Invitrogen, Carlsbad, CA), 1% non-essential amino acids (Gibco-BRL, Invitrogen, Carlsbad, CA) and 0.1 mM β-mercaptoethanol (Gibco-BRL, Invitrogen, Carlsbad, CA)]. Mouse endothelial cells (C166) (ATCC Cat. No. CRL-2581, Rockville, MD), used as a control for toxicity testing, were cultured in endothelial cell basal medium-2 (EBM-2) containing 5% FBS and cytokine cocktail. The hanging drops method (20 µl per drop; $1x10^5$ cells ml^{-1}) was used to induced differentiation as described by Heuer and colleagues (Heuer et al., 1993) with minor modifications. After incubation for 3 more days, EBs were transferred to gelatin-coated wells of Chamber slides (Nunc, Denmark) or 60mm dishes to allow attachment. To promote endothelial cell differentiation, 3-day-old EBs were placed in DMEM containing 10% FBS and medium consisting of EBM-2, 5% FBS, growth factor cocktail, and ascorbic acid (EGM2-MV Bullet Kit; Clonetics/BioWhittaker, Walkersville, MD).

5.1.2 Cell viability and proliferation analysis

The viability and proliferative activity of ES cells were analyzed using the 3-(4,5-dimethylthiazol-2yl)-2,5,-diphenyl tetrazolium bromide (MTT) assay and by 5-bromo-2'-deoxyuridine (BrdU) incorporation for 10 days, respectively. For the MTT assay, mEBs were hanging drop cultured for 2 days and then grown in standard culture medium (DMEM containing 10% FBS) for 24 hours to allow attachment. 20 µl of MTT (5mg/ml) was then added to 200 µl of the culture medium on days 3, 4, 6, 8, and 10, followed by incubation at 37°C for 4 hr. After incubation, the MTT solution was carefully removed and 150 µl of DMSO (Sigma, St Louis, MO) was added to each well. The plates were then shaken on a plate mixer until the crystals dissolved. The absorbance of the resulting colored solution was measured at 570 nm in a Genios luminometer (TECAN, Austria) at a reference wavelength of 630nm. BrdU detection kits (Roche Molecular Biochemicals, Indianapolis, IN) were used to measure BrdU incorporation. For BrdU assays, mEBs were hanging drop cultured for 2 days and then grown in standard culture medium (DMEM containing 10% FBS) for 24 hours to allow attachment. On days 3, 4, 6, 8, and 10, 10 µl of 100 µM BrdU was added to each well, and the cells were incubated for 4 h. After removing the medium, the cells were fixed and DNA was denatured using FixDenat reagent (Roche Applied Sciences) for 30 min. The reagent was then removed, anti-BrdU-POD solution was added and the plates were incubated for 90 min at room temperature. The cells were then washed three times with washing solution, after which 100 µl of substrate solution was added and the absorbance was measured at 370 and 490 nm using a Genios luminometer (TECAN, Austria).

5.1.3 RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Cells were directly sorted into tubes containing Trizol (Gibco-BRL, Invitrogen, Carlsbad, CA) and mRNA was extracted according to the manufacturer's protocol. The isolated RNAs were quantified using a spectrophotometer (SmartSpec 3000, Bio-Rad). First-strand cDNA was synthesized from 2 µg of total RNA using an oligo (dT) primer and a SuperScript First-

Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. First-strand cDNAs were amplified in a final volume of 25 µl containing 0.5 U Taq DNA polymerase (TaKaRa Biotechnology, Korea) and 10 pmol of each target primer. PCR conditions were as follows: 5 minutes at 94°C, 30 amplification cycles (denaturation at 94°C for 1 minute, annealing at 55°C or 60°C for 1 minute, and extension at 72°C for 1 minute), followed by a final extension at 72°C for 5 minutes. The PCR primers and the size of the amplified products are shown in Table 1. The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. cDNA samples were adjusted to yield equal GAPDH amplifications.

Gene	Sequences	Size (bp)
FLk-1	F: 5′-CAGCTTCCAAGTGGCTAAGG-3′ R: 5′-CAGAGCAACACACCGAAAGA-3′	264
PECAM	F: 5′- GCCTGGAGAGGTTGTCAGAG-3′ R: 5′- GGTGCTGAGACCTGCTTTTC-3′	357
VE-Cadherin	F: 5′- ACCGGATGACCAAGTACAGC-3′ R: 5′- TTCTGGTTTTCTGGCAGCTT-3′	292
GAPDH	F: 5′- TGTTCCTACCCCCAATGTGT-3′ R: 5′- TGTGAGGGAGATGCTCAGTG-3′	396

Table 1. Sequences of oligonucleotide primers used for RT-PCR analysis

5.1.4 Immunocytochemistry

After inducing differentiation, the cells were exposed to 5-FU with/without probucol for 24 hours and fixed with freshly prepared MeOH/DMSO (4:1) overnight at 4°C. Cells were blocked with blocking solution containing 1% BSA and 0.1% Tween 20 for 30 min, and then incubated with rabbit anti-mouse FLK-1 (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rat anti-mouse PECAM (1:100) (MEC 13.3, Santa Cruz), or goat anti-mouse VE-cadherin (1:100) (Santa Cruz), or rabbit anti-mouse PCNA (1:100) (Santa Cruze, Biotechnology, Inc) at 4°C overnight. After washing, cells were incubated with goat anti-rabbit IgG-TRITC (1:100) (Chemicon, Temecula, CA), goat anti-rat IgG-FITC (1:100) (Santa Cruz), or donkey anti-goat IgG-FITC (1:200) (Santa Cruz) as secondary antibodies, respectively. Stained slides were embedded in 30% Mowiol (Calbiochem-Novabiochem, Schwalbach, Germany). Images were obtained and analyzed using a Bio-Rad confocal microscope (Radiance 2000 FCMP, Bio-Rad, USA)

5.1.5 Flow cytometry

Cultured cells were harvested using cell dissociation buffer (Sigma, St Louis, MO). Cells were resuspended at 10^6 cells/100 µl in suspension buffer, and then were incubated with 1 µg/100 µl of PE anti-mouse FLK-1 (Avas 12a1, PharMingen) and FITC anti-mouse PECAM (MEC 13.3, PharMingen), or (FITC) rat anti-mouse VE-cadherin (11D4.1, PharMingen) for 30 minutes at 4°C. Negative controls were incubated for 30 min at 4°C with fluorochrome-labeled irrelevant isotype control antibodies: 1 µg/100 µl PE rat IgG2a′ (PharMingen, San Diego, CA), 1 µg/100µl FITC rat IgG2a′ (PharMingen), or 1 µg/ 100 µl anti-rat FITC-conjugated IgG (Santa Cruz). In order to analyse how Oct-4 expression varies in mouse

ESCs through different stages of endothelial cell differentiation, endothelial differentiation induced cells for 0, 4, 7 and 10 days were harvested using cell dissociation buffer (Sigma, St Louis, MO). To analyse PCNA expression, the cells were exposed to 5-FU with/without probucol for 24 hours at day 9. After 24 hours incubation, control cells and treated cells were harvested using cell dissociation buffer (Sigma, St Louis, MO). Cells were re-suspended at 10^6 cells/100 µl in suspension buffer and then incubated with 1µg/100 µl of rabbit anti-mouse Oct-4 (Santa Cruz, Biotechnology, Inc), or rabbit anti-mouse PCNA (1:100) (Santa Cruze, Biotechnology, Inc) for 1 hour at 4°C. Negative controls were incubated for 1 hour at 4°C with fluorochrome labelled irrelevant isotype control antibodies: 1 µg/ 100 µl goat anti-rabbit FITC-conjugated IgG (Chemicon) or goat anti-rabbit IgG-TRITC (1:100) (Chemicon, Temecula, CA) for primary antibodies, respectively. After staining, cells were analyzed without fixation using a FACS Calibur flow cytometer (Becton Dickinson, MA) using 5 µg/ml of propidium iodide (Sigma) to exclude dead cells. Data was analyzed using CellQuest software (Becton Dickinson, MA).

For cell cycle analysis in differentiation into endothelial cell of mouse ESCs, the treated cells were trypsinised and fixed in cooled 70% ethanol at 4°C. The cells were then incubated in 0.3 ml of DNA staining solution (100 µg/ml PI, 1 mg/ml RNase A (DNase-free) in PBS). The cells were then transferred into D-Hank's solution. The cell suspension was stored on ice in a dark room for a minimum of 30 min and analysed within 2 hours. Data analysis was carried out using CellQuest software (Becton Dickinson, MA).

5.1.6 Capillary tube formation by endothelial cells on matrigel

To induce tube formation on matrigel, mES cells were grown in EGM-2 medium for 10 days and then prepared as a single-cell suspension. $5x10^4$ ES cells were then plated on Matrigel (Becton Dickinson, MA), incubated at 37°C, and analyzed using a phase contrast microscope (Nikon, Eclipse TE 2000-U, Japan).

5.1.7 Cytotoxicity analysis

The cytotoxic effects of 5-Fluorouracil on mES cells and mouse endothelial cell (C166) were analyzed using a validated embryonic stem cell test protocol and MTT assay (Spielmann et al., 1997; Scholz et al., 1999). Briefly, 1000 cells were seeded into each well of a 96-well microtitre plate and grown in the presence of a concentration range of 5-FU and probucol. A negative control containing solvent diluted in medium was also included. At day 9, the cells were exposed to 5-FU (10 µM) with/without probucol (50 µM) in a total volume of 200 µl for 24 hours. The 5-FU and probucol were dissolved in cell culture medium and ethanol, respectively. The final ethanol concentration in the wells was 0.1%. The controls were incubated with equal volumes of drug solvents to avoid changes that could be due to solvent. 20 µl of MTT (5mg/ml) was added to 200 µl culture medium on day 10, followed by incubation at 37°C for 4 hrs. After incubation, the MTT solution was carefully removed and 150 µl of DMSO (Sigma, St Louis, MO) was added to each well. The plates were shaken on a plate mixer until all crystals had dissolved. The absorbance of the resulting coloured solution was measured at 570 nm with a Genios luminometer (TECAN, Austria) at a reference wavelength of 630nm. Cytotoxicity was expressed as a percentage of cells surviving, relative to untreated cultures, and the concentration required to inhibit cell growth by 50% (IC_{50}) was calculated. Each experiment was performed using six replicates for each drug concentration and repeated in triplicate.

5.1.8 Microarray

For cDNA microarray analysis, differentiated mouse ESCs treated with/without 5-FU (10 μM) for 24 hours were collected. Their total RNA was extracted using Trizol (Gibco-BRL, Invitrogen, USA) and mRNA was extracted according to the manufacturer's protocol. The quantity and quality of total RNA and amplified RNA were assessed by using a Bioanalyzer 2100 (Agilent Technologies). The Applied Biosystems Mouse Genome Survey Microarray contains 32,996 60-mer oligonucleotide probes, representing 32,181 individual mouse genes. Digoxigenin-UTP labelled cRNA was generated and linearly amplified from 2 μg of total RNA using Applied Biosystems Chemiluminescent RT-IVT Labelling Kit v.2.0 and manufacturer's protocol (Applied Biosystems). Array hybridization (five arrays per sample), chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit (Applied Biosystems) and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (Applied Biosystems), following the manufacturer's protocol. Images were auto-gridded, then spot and spatially normalised. Chemiluminescent signals were quantified, corrected for background, and the final images and feature data were processed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer software v1.1. Data and images were collected through an automated process for each microarray using the 1700 analyzer. A total of 10 arrays were run for the two groups (five technical replicates for each group). A global median normalisation, which normalises signal intensities across all microarrays to achieve the same median signal intensities for each array, was performed on the Applied Biosystems data sets. For Applied Biosystems arrays, the detection threshold was set as $S/N > 3$ with a quality flag < 100. Correlation and coefficient of variation (CV) analyses were performed using Matlab® software (Mathworks, Natick, MA). Differential expression analysis was done using two different statistical methods: (1) ANOVA analysis was performed using Avadis software. Differentially expressed genes between control and 5-FU (10 μM) treated groups were determined based on the following criteria: (a) $p < 0.001$ in ANOVA analysis; (b) average change between control and 5-FU (10 μM) treated groups > 2 fold; (c) detectable in more than 50% samples. (2) Significance Analysis of Microarray (SAM: http://www-stat.stanford.edu/~tibs/SAM), a supervised learning statistical software that performs a modified *t*-test to identify genes with significant changes in expression, and uses permutations to estimate the false discovery rate (FDR). Hierarchical clustering of log ratios was performed using the software (http://rana.lbl.gov/EisenSoftware.htm) Cluster and Treeview; Euclidean correlation, median centring and complete linkage were applied in all clustering applications.

5.1.9 Western blot analysis

Control cells and treated Cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 50 mM Tris) containing protease inhibitor for 1 h at 4°C. The supernatant was separated by centrifugation, and protein concentration was determined with a Bradford protein assay kit II (Bio-rad). Proteins (25 μg/well) denatured with Laemmli sample buffer (Sigma) were separated by 10% SDS-polyacrylamide gel (Bio-Rad) under a constant current of 50 mV. Proteins were transferred onto nitrocellulose membranes (0.45 mm, Amersham Life Sciences). The membranes were blocked with a 5% BSA solution for 3 hrs, washed with PBS containing 0.2% Tween 20, then incubated with the primary antibody overnight at 4°C. Human specific antibodies against CDK-2, CDK-4, Cyclin D1, Cyclin E, $p21^{WAF1/CIP1}$,

p27 ^{Kip1}, p53, and β-actin from Santa Cruz Biotechnology (Santa Cruz Biotechnology) were used to probe the separate membranes. The immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase conjugated antibody after washing for 2 hours at room temperature. The specific protein bands were detected by enhanced chemiluminescence (Pierce), with X-Omat AR films (Kodak). As a protein loading control, parallel gels were subjected to Western blot analysis using a β-actin antibody (Santa Cruz Biotechnology).

5.1.10 Statistical analysis

All results are expressed as percentages of untreated control values or as the means ± SD of three independent experiments, each with six replicates. Statistical significance was determined using the Student's *t*-test for paired data. A P value of < 0.05 was regarded as significant, and IC_{50} values were calculated using Sigmaplot version 9.0.

5.2 Results

5.2.1 Expressions of endothelial markers at the mRNA and protein levels during the early stages of differentiation

We analyzed the mRNA expression levels of endothelial markers, i.e., FLK-1, PECAM, and VE-Cadherin, during different culture conditions and differentiation stages within 7 days of plating embryoid bodies by RT-PCR (Figure 3A). In case of the expression of the FLK-1 gene, no difference was found between DMEM containing FBS and EGM-2 media.

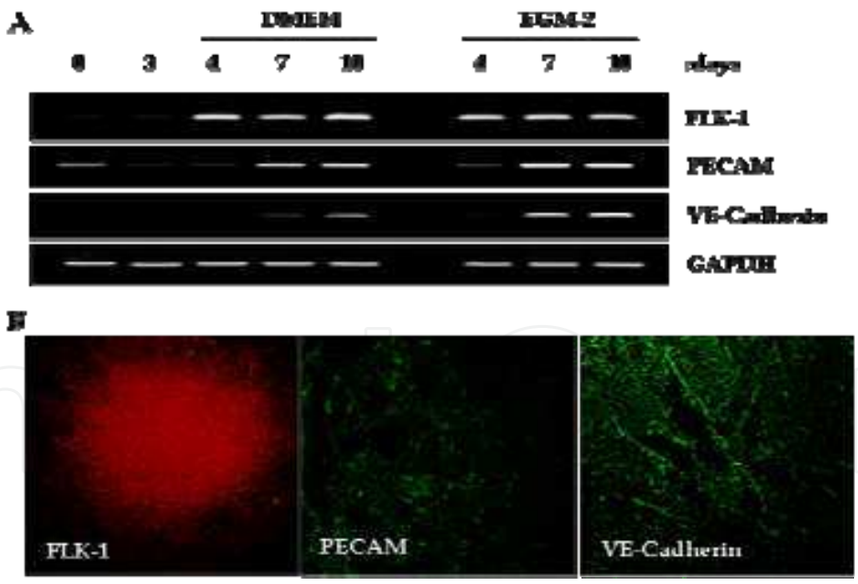

Fig. 3. Expression patterns of endothelial cell-specific markers at the mRNA and protein levels in mES-derived embryoid bodies. Gene expression in ES-derived EBs by RT-PCR (A). Expressions of FLK-1, PECAM, and VE-cadherin in differentiated mES cells at day 10 by immunofluorescence (B) (× 40)

However, the expressions of PECAM and VE-Cadherin mRNA were higher in EGM-2 than in DMEM containing FBS, and their expressions in EGM-2 medium were higher during the early stages. Expression of endothelial cell differentiation markers at the mRNA and protein

levels were similar with respect to time course (data not shown). We carried out immunocytochemistry for endothelial cell markers in mEB cells from EGM-2 medium (Figure 3B), and observed capillary-like structures on day 10. These were stained with specific antibodies for FLK-1, PECAM and VE-Cadherin. The immunoreactivity of each marker was strong and was observed throughout EBs cells.

In addition, we performed flow cytometry analysis for the expressions of FLK-1, PECAM, and VE-Cadherin with time (Figure 4). Cells which are positive for these markers were observed to increase with time. For cells grown in DMEM containing 10% FBS medium and EGM-2 medium for 10 days the percentages expressing these markers were; FLK-1 4.0% and 5.3%, PECAM 10.9% and 44.5%, and VE-Cadherin 5.2% and 6.4%, respectively. These results demonstrate that the expressions of endothelial cell markers at the mRNA and protein levels were greater in cells grown in EGM-2 medium than in DMEM containing 10% FBS medium.

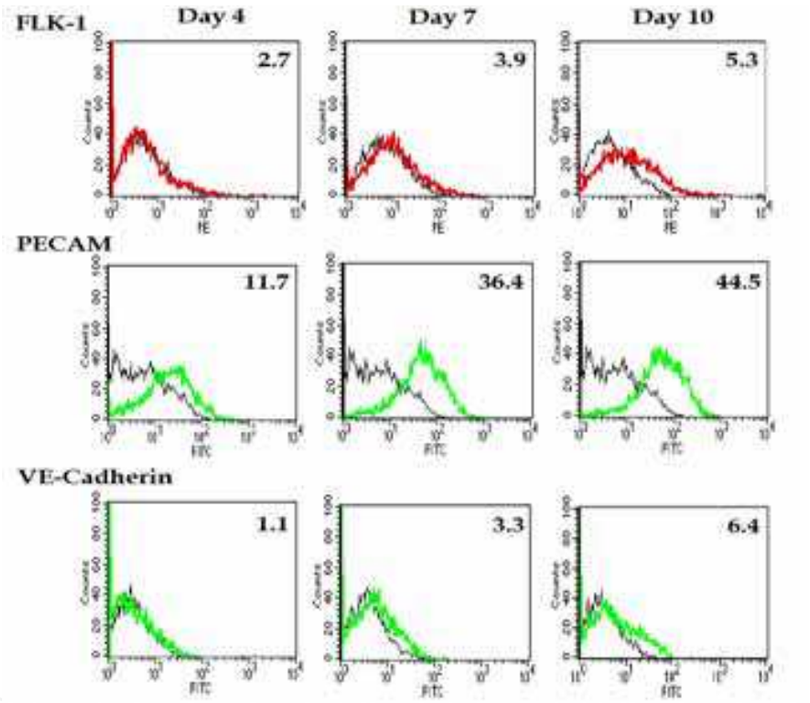

Fig. 4. Kinetics of the expressions of FLK-1, PECAM and VE-cadherin during the differentiation of mES cells according to flow cytometric analysis. Numbers indicate the percentages of target antibody-positive cells

5.2.2 Formation of capillary structures by differentiated mES cells on matrigel

Mouse ES cells cultured in EGM-2 medium for 10 days differentiated into the endothelial cell lineage. Thus, we used gelatin coated dishes and a Matrigel system to confirm whether the mES cells cultured EGM-2 medium effectively construct capillary structures. We cultured mES derived endothelial cells in EGM-2 medium on gelatin coated dishes and Matrigel. After 5 days, we found that mES cells on the gelatin coated dishes could not construct capillary-like structures (Figure 5A), on the other hand, mES cells on Matrigel rapidly formed capillary-like structures, which resembled those formed by HUVEC and other endothelial cell populations (Figure 5B).

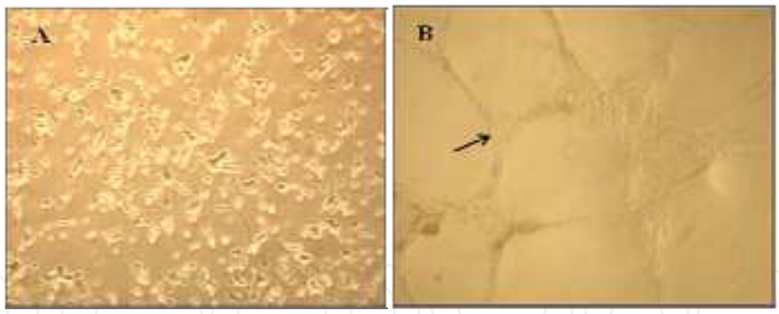

Fig. 5. Formation patterns of vascular-like structures of mES cells. Morphologies of mouse ES cell-derived endothelial cells on a gelatin-coated plate (A) and a matrigel-coated plate (B) (×100). Arrow indicates vascular-like structure derived from mouse ESCs

5.2.3 Differentiated mouse ES cells were more sensitive than mouse endothelial cells (C166) to 5-Fluorouracil

In the sensitivity to expose 5-FU in mouse ES cells, the respective concentrations of 5-FU for 50% reduction (IC_{50}) in early stage (day 3) and late stage (day 9) assays for 24hr were 126 uM and 7.9 uM, respectively (data not shown). This data suggests that the sensitivity of differentiated endothelial cells to toxicity of 5-FU are more sensitive than undifferentiated endothelial cells. In order to confirm the usefulness of the vasculogenesis model produced from differentiated mouse ESCs using EGM-2 medium, we performed vasculogenesis cytotoxicity assays, by treating cells with 5-fluorouracil, a strong inhibitor of vessel

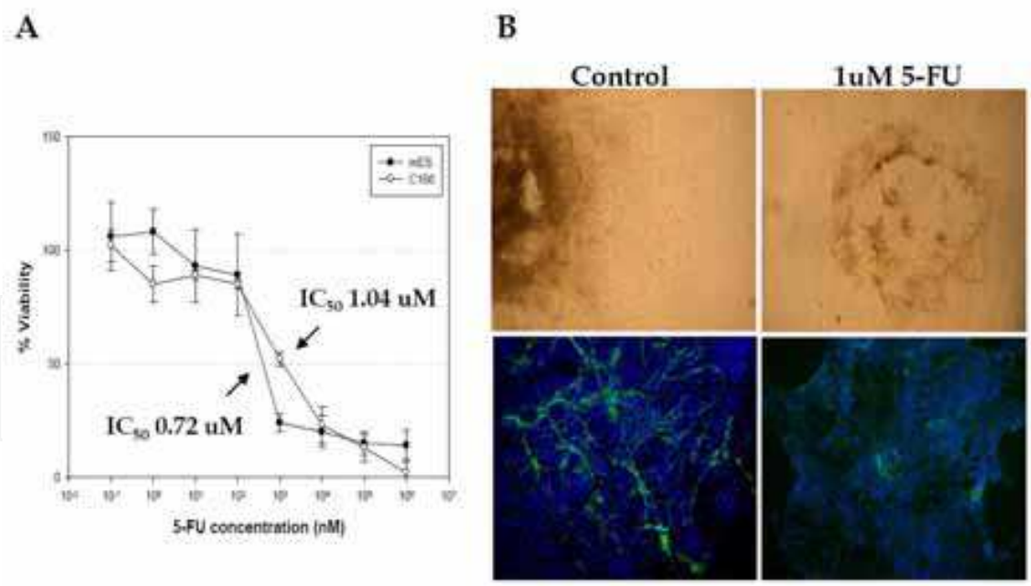

Fig. 6. Illustration of the effects of 5-Fluorouracil on the 72-hour survival of mouse embryonic stem cells and C166 mouse endothelial cells as determined by MTT assays (A). The inhibitory concentrations (IC_{50}) of 5-Fluorouracil in mES cells and C166 cells were 0.72 μM and 1.04 μM, respectively. The morphology and PECAM expression of mouse ES cells exposed to 5- Fluorouracil (1 μM) (B). The expression of PECAM was decreased by 5-fluorouracil treatment in mouse ES cells

formation, to both mouse ES cells and C166 cells. Generally, the growth rates of mouse ES and C166 cells were inhibited in a dose-dependent manner by 5-fluorouracil (Figure 6A). However, the concentrations 5-fluorouracil that caused a 50% reduction in mouse ES and C166 cells were 0.72 and 1.04 uM, respectively. In addition, the expression of PECAM significantly decreased in mouse ES derived endothelial cells which are exposed 5-FU (Figure 6B). From these results, we confirm that differentiated mouse ES cells are sensitive to 5-fluorouracil comparing traditional screening systems (C166) for cell toxicity, so that endothelial cells derived mES can use as an in vitro model for vasculogenesis and toxicity screening.

5.2.4 Effect of 5-FU on the endothelial differentiation of cells derived from mouse ESCs

To study the role of 5-FU in endothelial differentiation, mouse ESCs were differentiated into endothelial precursor cells and treated with 5-FU (10 μM). Oct-4 expression and cell viability were analysed. The expression of Oct-4 gradually decreased, with 100%, 90.6%, 81.3%, 50.5%

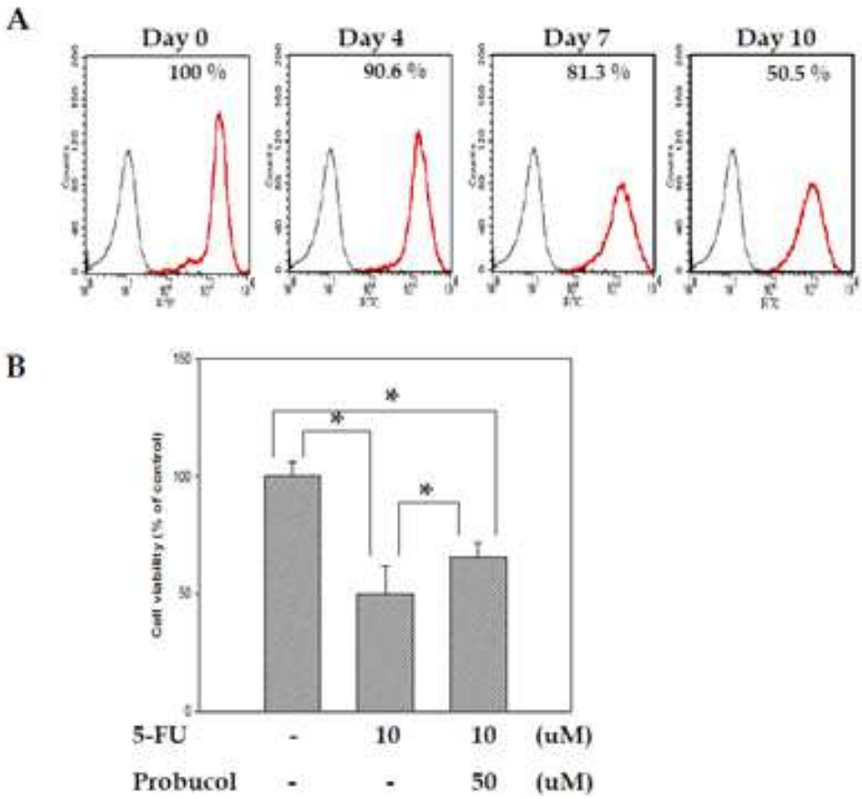

Fig. 7. The expression of Oct4 and the cytotoxicity of 5-Fluorouracil in endothelial precursor cells, derived from mouse embryonic stem cells. The expression of Oct4 gradually decreased during endothelial differentiation (A). After inducing endothelial differentiation for 9 days with EGM-2 medium, the cells were exposed to 5-fluorouracil (10 μM) with/without probucol (50 μM) for 24 hrs. Analysis of the cell viability of the endothelial differentiated cells exposed to 5-fluorouracil were assessed by MTT assays (B). The experiments were performed in 3 independent runs (n = 6). Standard error bars are shown. Significance was tested by the student *t-test* (* $p < 0.01$). Numbers indicate the percentages of target antibody-positive cells

expression on differentiation days 0, 4, 7, and 10, respectively (Figure 7A). In a previous study, we found that the percentage of PECAM expression increased by up to 44.5% in mouse ES-derived endothelial precursor cells, grown in EGM-2 medium for 10 days. There is a strong correlation between the decreased Oct-4 expression and increased PECAM expression during the endothelial differentiation of cells derived from mouse ESCs. Next, we examined the viability of endothelial precursor cells when they were exposed to 5-FU (10 μM), using MTT assays. As shown in the supplemental data, cells exposed to various concentrations of 5-FU and probucol for 24 hours displayed a decrease in cell viability in a concentration dependent manner. The IC_{50} of 5-FU and the concentration of probucol needed to avoid affecting cell viability were 10 μM, 50 μM, respectively. The viability was significantly decreased to 49.8% of the control in the endothelial differentiated cells exposed to 5-FU (10 μM) for 24 hours. The viability was significantly recovered to 65.7% in cells exposed to 5-FU (10 μM) combined with probucol (50 μM) ($P < 0.01$) (Figure 7B).

5.2.5 5-FU inhibits cell proliferation in endothelial differentiated cells derived from mouse ESCs.

The morphology of mouse ES-derived endothelial precursor cells after treatment with the IC_{50} values of 5-FU (10 μM) for 24 hours was changed and detached, compared to the

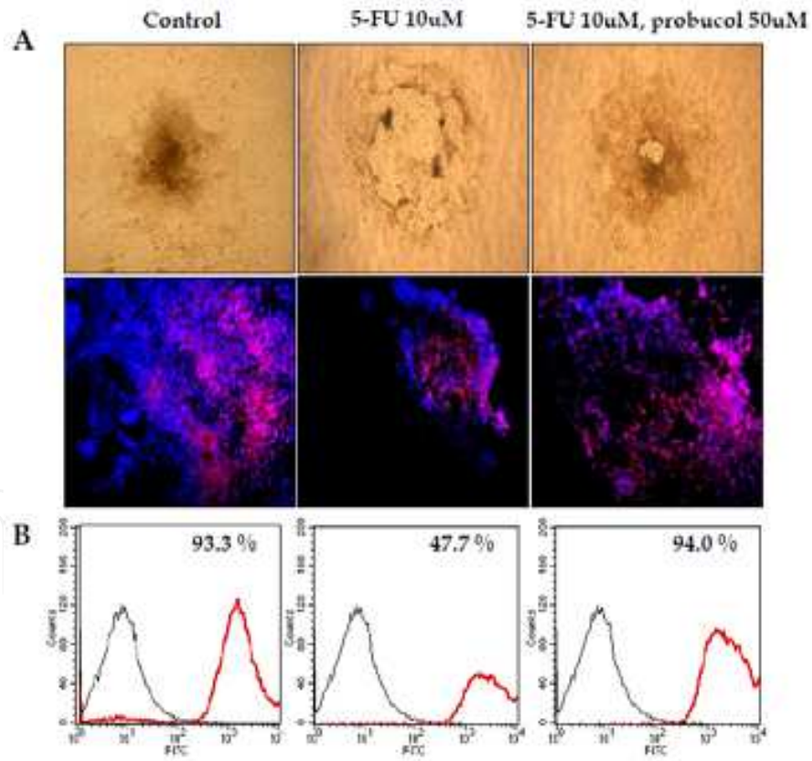

Fig. 8. Anti-proliferative affect of 5-fluorouracil in endothelial precursor cells. Morphology (A, top panel) and PCNA expression (A, bottom panel) of control and cells exposed to 5-fluorouracil (10 μM) with/without probucol (50 μM) for 24hrs. Cell nuclei were stained with DAPI. The percentages of cells with PCNA expression were analysed by FACS (B). Numbers indicate the percentages of target antibody-positive cells. Original Magnification: × 40 for (A)

control cells which grew as confluent aggregates with rounded and polygonal cell morphology. However, probucol (50 μM) treatment to the 5-FU treatment groups for 24 hours induced a morphology similar to that of the control cells (Figure 8A, top panel). In order to investigate the effects of 5-FU on the proliferation of endothelial precursor cells derived from mouse ESCs, PCNA expression was assessed by immunocytochemistry and FACS analysis. After inducing endothelial differentiation for 9 days, we observed that the endothelial precursor cells exposed to 5-FU (10 μM) for 24 hours had decreased PCNA expression, whilst the probucol (50 μM) treated group had recovered its PCNA expression (Figure 8A, bottom panel). These results correlated with the PCNA expression of the endothelial precursor cells exposed to 5-FU (47.7%), as shown by FACS analysis (Figure 8B).

5.2.6 Expression of PECAM in endothelial differentiated cells is down-regulated by 5-FU

To test whether the treatment of mouse ES-derived endothelial precursor cells with 5-FU can influence endothelial differentiation through expression of endothelial specific genes, the expression levels of PECAM were analysed by immunocytochemistry and RT-PCR. The expression of PECAM was dramatically decreased in the 5-FU treatment group, compared to the control group. However, the expression of PECAM was maintained in the probucol treatment group (Figure 9). These findings demonstrate that, in accordance with the morphological differentiation analysis, 5-FU decreases endothelial-specific mRNA levels and so inhibits the expression of genes involved in endothelial differentiation.

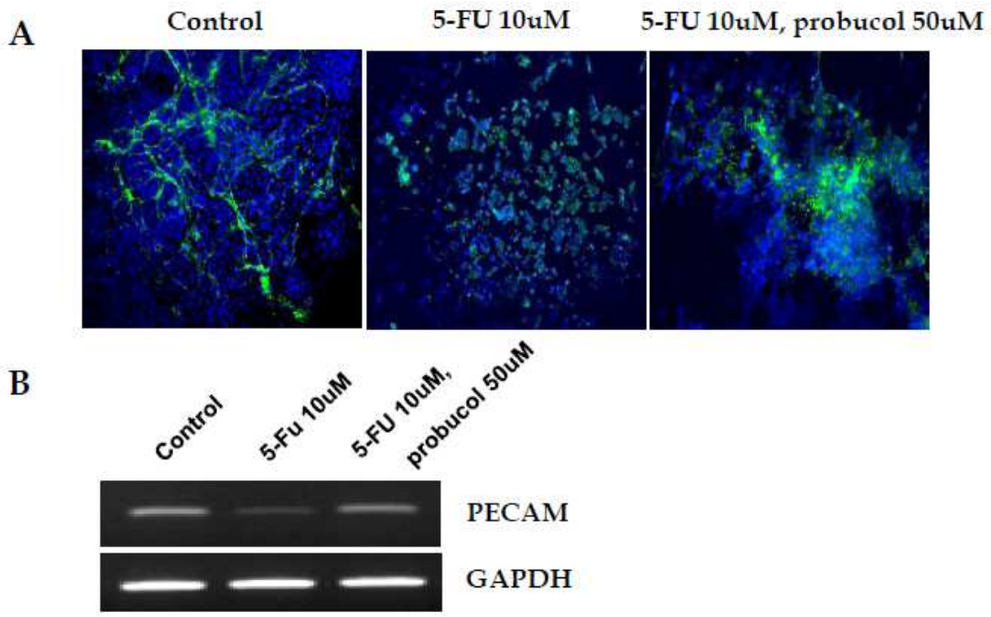

Fig. 9. PECAM expression of endothelial precursor cells, derived from mouse embryonic stem cells. Expression of PECAM in control and cells exposed to 5-fluorouracil (10 μM) with/without probucol (50 μM) for 24hrs were analysed by immunocytochemistry. Cell nuclei were stained with DAPI (A). mRNA levels of PECAM and GAPDH were evaluated by RT-PCR (B). GAPDH used as internal standard. Original Magnification: × 40 for (A)

5.2.7 Gene profiling of mouse embryonic stem cells exposed to 5-FU using Microarray analysis

In order to determine gene expression changes in cells exposed to 5-FU (10 μM), the RNA contained in both the control cells, and the cells exposed to 5-FU treatment for 24 hours at differentiation day 9 was collected. As shown a Fig. 4, a total of 11,668 genes out of 32,996

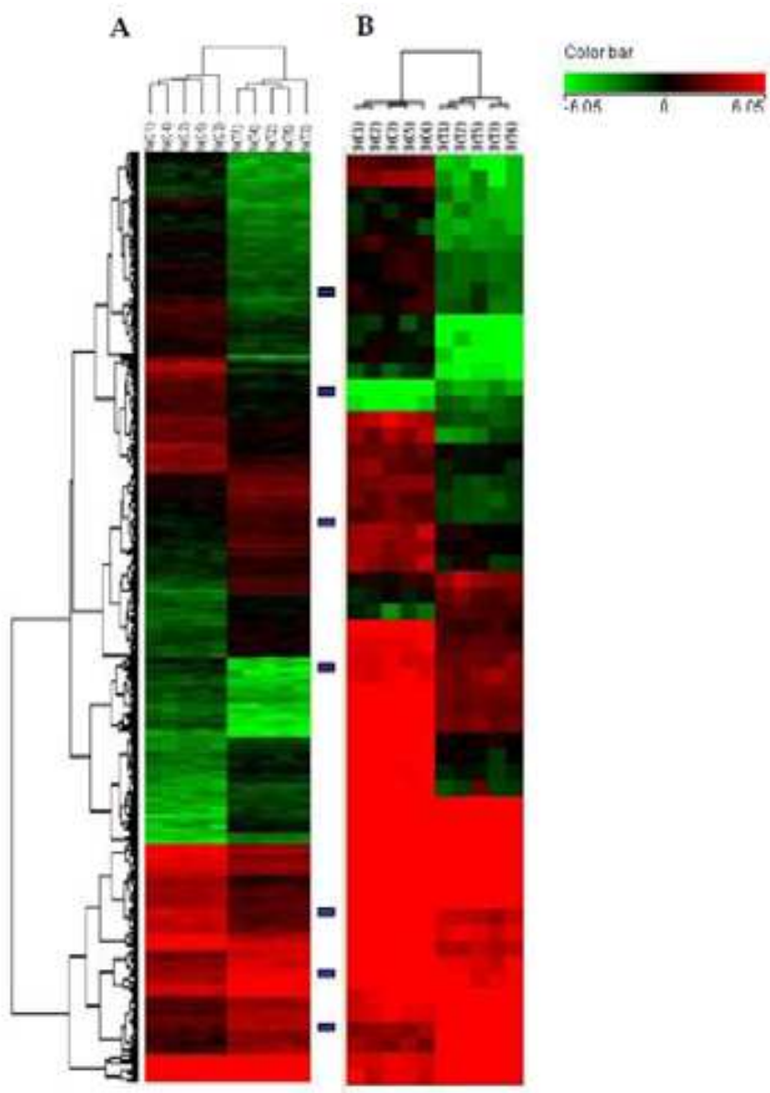

Fig. 10. Gene expression profiling of endothelial precursor control and cells exposed to 5-fluorouracil using microarray analysis. Hierarchical clustering analysis was conducted using control (n=5) and 5-FU treated samples (n=5). The black bars on right side of *A* illustrate the location of clusters shown. The dendrogram in *B* shows the samples identified as being in the cell cycle category, between control and 5-FU treated cells. The intensity of red and green colour is proportional to the relative up-regulation (red) or down-regulation (green) of gene expression in the differentiated samples, compared to that in the undifferentiated reference. Gene names and accession numbers are from Unigene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene)

cDNAs were selected as differentially expressed genes by the ANOVA test ($p < 0.001$). A hierarchical cluster analysis yielded major clusters in the 11,668 expressed genes (Figure 10). These gene expression patterns were classified into functional groups, based on their biological process as defined by the Gene Ontology (GO) annotation system. Most of the observed genes were related to physiological processes, including apoptosis, cell cycle, developmental processes, and signal transduction in all clusters (Table 2). These results suggest that 5-FU treatment affects the expression of numerous genes via the alteration of several processes. Notably, expression of 58 genes out of the 1,439 genes in the cell cycle category was modulated by more than 2-fold, between the control cells and endothelial differentiated cells exposed to 5-fluorouracil (data not shown).

Function (Panther classcification system)	Number
Angiogenesis	4
Apoptosis	30
Cell adhesion	22
Cell cycle	58
Cell proliferation and differentiation	9
Cell structure and motility	36
Developmental processes	80
Homeostasis	7
Immunity and defence	45
Signal transduction	126
Cell adhesion molecule	20
Cell junction protein	8
The others	994/1439

Table 2. Gene content list of the AB 1700 mouse chip

5.2.8 5-FU induces arrest of G1/S phase in endothelial-like cells derived from mouse ESCs

To confirm the effects of 5-fluorouracil (10 μM) with and without probucol (50 μM) in the cell cycle, the cell cycle distribution was analysed by flow cytometry (Figure 11). Generally, the frequencies of G0/G1 phase and S-phase were 62.7% and 33.5% in mouse ESCs, respectively. In contrast, mouse ES-derived endothelial precursor cells exposed to 5-FU (10 μM) for 24 hours showed a decrease to 31.1% in G0/G1 phase and an increase to 60.6% in S phase. Treatment with 50 μM probucol for 24 hours showed 35.0% of cells in G0/G1 phase and 62.8% in S-phase (Figure 11A). These data suggest that 5-FU arrests cells at the G1/S phase boundary in endothelial precursor cells, derived from mouse ESCs. This was followed by decreased proliferation of mouse ES-derived endothelial precursor cells, similar to that reported previously with other cell types (Vittet et al., 1996).To determine whether the expression levels of cell cycle related proteins were changed, we analysed Cyclins, CKDs,

CKDIs, and p53 expression after treatment with 5-FU (10 μM), with and without probucol (50 μM), by Western blot analysis. The expression of Cyclin E, CDK2, $p21^{WAF1/CIP1}$, and p53 was up-regulated in ES-derived endothelial precursor cells exposed to 5-FU, compared to those in the control group and cells treated with 5-FU (10 μM) and probucol (50 μM) (Figure 11B). Although the expression of cyclin D1 remained constant in control cells and mouse ES-derived endothelial precursor cells exposed to 5-FU, they were remarkably decreased by probucol treatment. Otherwise, there were no differences in the expression of CKD4 and $p27^{Kip1}$, the tumour suppressor and inhibitors of Cyclin E / CDK2 in mouse ES-derived endothelial precursor cells, according to 5-FU and probucol treatments (Figure 11B). From these results, we suggest that 5-FU might inhibit G1-related Cyclin/CDK activities through the augmentation of $p21^{WAF1/CIP1}$ expression and by binding to cyclin D/CDK complexes.

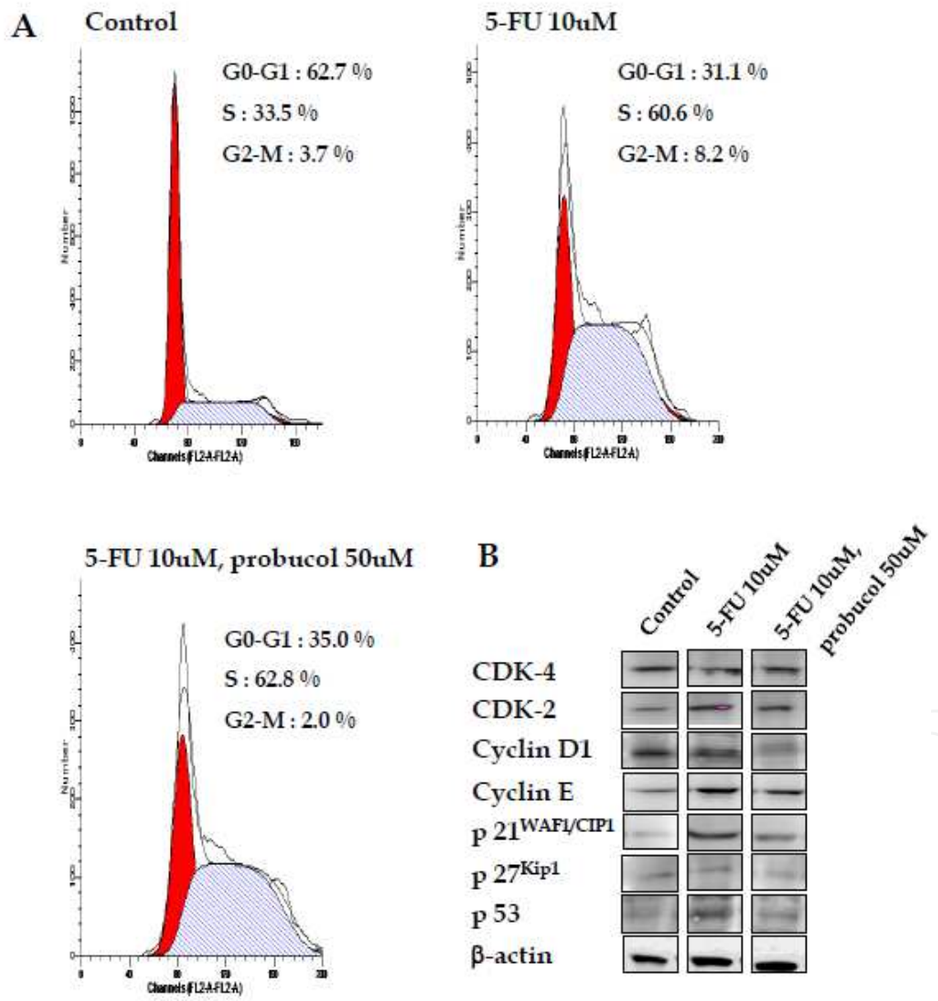

Fig. 11. Cell cycle arrest in endothelial precursor cells after 5-Fluorouracil exposure. DNA distribution histogram, using PI labelling (x-axis) and total number of cells in each channel (y-axis) in control and cells treated with 5-fluorouracil (10μM) with/without probucol (50 μM) (A). Western blot analysis of the expression of G1/S phase-related Cyclins, CDKs, and CDKIs in control and cells exposed to 5-fluorouracil (10μM) with/without probucol (50 μM) (B)

6. Conclusion

In toxicological research, the development of alternative *in vitro* toxicity screening systems to replace *in vivo* screening methods using animal experiments and conventional screening systems is important. Although applications using primary cells derived from rodent embryo or tissue-specific cell lines originating from humans have been tried, the validation of cytotoxicity during cellular biological processing must be improved in order to establish new and alternative *in vitro* screening methods, and to increase the efficiency of toxicological analysis through these methods (Tiffany-Castiglioni et al., 1999). So, toxicity screening using stem cells is highlighted as an alternative cell source for *in vitro* toxicity screening because of several limitations of traditional *in vitro* assays using primary cells or cell lines. For examples, they couldn't demonstrating the biological process involved in a toxic response to xenobiotics comparing to in vivo toxicity testing using animal model. In comparison to *in vivo* studies, the screening system using embryonic stem cell is highly accurate at predicting cellular toxicity, and outperforms classical assays, such as, the fetal limb micromass and post-implantation whole rat embryos culture assays (Scholz et al., 1999).

Embryonic stem cell testing (EST) using the EB system provides a useful tool for analyzing the embryotoxic effects of chemical compounds (Spielmann et al., 1997; Scholz et al., 1999; Huuskonen, 2005). Whole embryo culture showed the best concordance between in vivo classification and in vitro test results with 80% correct classifications versus 78% for EST and 71% for limb bud micromass. Moreover, strong embryotoxicants showed a predictivity of 100% in each of the test systems (Genschow et al., 2002). The powerful advantages of ESCs when they are applied to toxicology come as a result of their unique properties compared to primary cells: self-renewal, plasticity to generate various cells types, and that they are a readily available alternative source to replace primary cells. Therefore, stem cell based screening systems for toxicants offer a very promising technology; it is possible to obtain large numbers of cells for consistent analysis and the study at the different stages of differentiation. In addition, toxicity screenings using ESCs have been validated as a reliable source for *in vitro* developmental toxicology studies (Rohwedel et al., 2001).

In the present study, we developed a vasculogenesis model for the effective differentiation of mES cells into the endothelial lineage using EGM-2 medium. In addition, the availability of differentiated mouse embryonic bodies was confirmed by examining vascular toxicity using 5-Fluorouracil. Since Hirashima and colleagues reported that mES cells are important for studies for murine development, several systems for mES cell differentiation have been reported (Hirashima et al., 1999; Feraud & Vittet, 2003; Davila et al., 2004; Wobus & Boheler, 2005)**.** The majority of reports issued during the past decade have focused on murine ES cells derived embryoid body (EB) formation assays. Differentiation system using embryoid bodies that have the potential to generate various embryonic cell lineages spontaneously induce differentiation (Doetschman et al., 1985; Risau et al., 1988; Wang et al., 1992). These studies show that the differentiation of ES derived EBs is more effective than ES cells in studies of vasculogenesis alone (Risau et al., 1988; Wang et al., 1992; Vittet et al., 1996; Bloch et al., 1997; Wartenberg et al., 1998; Hirashima et al., 1999). Many strategies have been used for endothelial differentiation, i.e., the suspension method (Risau et al., 1988; Wang et al., 1992), a method using semisolid medium (Vittet et al., 1996), and ES cell aggregation by

the hanging drop method (Goumans et al., 1999). Vittet and colleagues confirmed that ES-derived endothelial cells acquire cell-specific markers in a time-dependent manner after LIF removal, which suggests that endothelial markers are expressed in sequential steps, which closely resemble endothelial cell differentiation *in vitro* during embryonic development (Vittet et al., 1996). In the present study, when mEBs formed by the hanging drop method for 3 days were cultured in EGM-2 medium containing 5% FBS and cocktailed cytokines for 10 days, the mRNA expressions of endothelial markers were found to show patterns similar to Vittet's groups. However, the expression levels of endothelial markers of PECAM by FACS analysis were higher in the early stage than reported by Vittet. In addition, the expression levels of these markers were maintained to day 10. Although the proliferation activity of mES cells cultured in EGM-2 medium was lower than that of cells cultured in DMEM containing 10% FBS, the differentiation activity of those grown in EGM-2 medium was higher. These results suggest that serum contained potent stimulators of cell growth, such as, amino acids, growth factors, vitamins, proteins, hormones, lipids, and minerals can affect both proliferation and differentiation. Of these components, albumin protein has been previously reported to stimulate the proliferation of cells and to suppress their differentiation (Ishida & Yamaguchi, 2004). Therefore, we suggested that factors that stimulate differentiation were more abundant in EGM-2 medium than in DMEM medium containing 10% FBS.

A tyrosine kinase receptor for VEGF (FLK-1) is the marker of the lateral plate mesoderm and the earliest differentiation marker of endothelial cells and blood cells (Eichmann et al., 1997). Thus, an early stage defined by FLK-1 and PECAM expression might reflect commitment towards the endothelial lineage. PECAM is a member of the immunoglobulin superfamily expressed by endothelial cells and a subset of hematopoietic cells in the adult organism (Watt et al., 1995; DeLisser et al., 1997). It also was recently reported that undifferentiated ES cells express PECAM (Vittet et al., 1996; Ling & Neben, 1997). Because ES cells resemble the inner cell mass of the blastocyst, this suggested that PECAM may be expressed embryonically earlier than the described post-implantation stages. From these results, we confirmed that undifferentiated ES cells express PECAM, and we showed for the first time that this early expression mirrors PECAM expression in the mouse blastocyst. PECAM is expressed continuously during ES cell differentiation and characterizes a population of cells that are $PECAM^+$ but not part of patent blood vessels. These cells have a possible counterpart *in vivo* in the yolk sac mesodermal cells that express PECAM before blood island formation. These findings are consistent with the model that ES cell differentiation recapitulates yolk sac development. (Redick & Bautch, 1999). These results define PECAM as the first cell adhesion molecule to be ICM-specific and suggest a potential role for this molecule prior to vascular differentiation. (Robson et al., 2001).

Otherwise, expression of VE-cadherin was observed in later maturation (Vittet et al., 1996). VE-cadherin is expressed at the earliest stages (E7.5) of vascular development in the mesodermal cells of the yolk mesenchyme. At later embryonic stages, VE-cadherin expression is restricted to the peripheral layer of blood islands that gives rise to endothelial cells and to the endothelium of most vessel types (Breier et al., 1996). According to our data, the mRNA expressions of Flk-1, PECAM, and VE-cadherin in mES cells cultured in EGM-2 medium were detected on day 4, day 4, and day 7, respectively. These results are similar to those of previous reports (Vittet et al., 1996; Hirashima et al., 1999; Magnusson et al., 2004).

Festag and their colleagues reported that the exposed six test compounds such as all-trans-retinoic acid (RA), 5-FU, diphenylhydantoin (DPH), valproic acid (Val), saccharin (Sacch), and penicillin G (Pen G), with known embryotoxic potential could disturb differentiation potential of murine embryonic stem cell as well as inhibit the differentiation of ES cells into endothelial cells (Festag et al., 2007). Because the sensitivity of differentiated murine embryonic stem cells to 5-FU comparing to endothelial cell line did not mentioned, the guideline as a correct mathematic model would not applied to a different evaluation of the experiments. However, we demonstrate that the differentiation of mES cells into endothelial cells as a measure of 5-FU, which is an anti-angiogenesis chemical toxicity is more sensitive than mouse endothelial cells (C166). In addition, we confirmed that the sensitivity to 5-FU for in the processing of endothelial differentiation from mouse embryonic bodies. These results suggest that EGM-2 medium is suitable to differentiate the endothelial cells from mES cells. In addition, the differentiated endothelial cells derived mES cells were more sensitive to vascular toxicity testing than traditional method. This devised system could be used as a tool for understanding of mechanism of vasculogenesis and for the screening of mesodermal-derived target organs toxicant.

Furthermore, we demonstrate that 5-fluorouracil, an anti-cancer drug, can induce cytotoxicity in endothelial differentiation of mouse ESCs via inhibition of processes involved in cell viability, proliferation, and the cell cycle. During the induction of endothelial differentiation in cells derived from mouse ESCs, the expression of mesodermal lineage-related genes was up-regulated at 7 days after differentiation using EBs, followed by the up-regulation of endoderm lineage related genes at 14 days (Heo et al., 2005). Based on these reports, we induced endothelial differentiation from mouse ESCs for 10 days using EGM-2 to maximise endothelial differentiation, and confirmed the expression patterns of Oct-4 and PECAM. The frequencies of cells undergoing endothelial differentiation gradually increased through subsequent differentiation days; the expression of Oct-4 was 50.5% at endothelial differentiation day 10. These findings show that the characteristics of cells at endothelial differentiation day 10 are similar to endothelial precursor cells and endothelial-like cells. Because of this, these models can be used to evaluate the cytotoxicity of 5-FU at different stages of endothelial differentiation.

A number of anti-cancer agents have been implicated in vascular toxicity and their effects have been attributed to direct toxicity to the endothelium, such as the HUVEC and C166 cell lines. 5-FU gives an increase in the permeability of endothelial monolayers, as well as inducing vascular collapse and tumour necrosis (Watts et al., 1997). 5-FU, as a cytostatic agent with a strong embryotoxic potential, is known to have an effect in rapidly proliferating cells (Parker & Cheng, 1990; Shimizu et al., 2001). These reports are well matched with our results. The cytotoxicity of 5-FU in mouse ESCs was relieved by the addition of probucol (50 μM), an antagonist of 5-FU. This prevented the endothelial injury usually caused by 5-FU, but the rate of recovery for damaged endothelial cells did not completely return to normal (Kita et al., 1987; Kaneko et al., 1996).

In order to understand the biological mechanism of target cell specific toxicants, DNA microarrays have been used to analyse gene profiling, to show the alteration of gene expression (Gunji et al., 2004; Mori et al., 2007; Fumoto et al., 2008). However, studies of gene profiling and the mechanisms within ESCs exposed to special toxicants, including 5-FU, are still rare. Thus, we analysed expression patterns of genes involved in inducing

endothelial differentiation, using a DNA microarray with untreated and 5-FU-treated cells. As shown in the DNA microarray data, a huge number of changes in gene expression were identified by 5-FU treatment. Among these changes, we focused on genes involved in the cell cycle, as our data indicated that 5-FU induces cell proliferation inhibition. In contrast to the somatic cell cycle, ESCs have a unique feature, an abbreviated cell cycle (Becker et al., 2006). This is thought to be controlled through an unusual mechanism of CDK regulation, followed by a very short period of cell cycle. This allows ESCs to preserve their unlimited differentiation potential (Stead et al., 2002). In ESCs, the expressions of cyclin D1 and Cyclin D3 are low, and Cyclin D2 is not expressed, moreover, the expression of CDK-4 and the CDK 4-associated kinase activity is also weak, compared to somatic cells (Burdon et al., 2002). These previous reports lacked a consensus with our data, which showed increased expression of CDK4 and Cyclin D1 during endothelial differentiation. This discrepancy might originate from the differences between cell sources.

Until now, the mechanism that has been reported to explain the cell cycle arrest by 5-FU treatment is a G1/S phase arrest in cancer cell lines, induced by blocking DNA synthesis through the inhibition of thymidylate synthase (Pinedo & Peters, 1988). When damaged by 5-FU treatment, in addition, the expression of the tumour suppressor *p53* is increased *p53* has been identified as a participant in the DNA damage response, resulting in either cell cycle arrest or death. It activates and regulates the transcription of cell cycle arrest or apoptosis related genes (Lane, 1992). The expression of *p21*, which a member of the WAF1/CIP1 family, and activates CKI which works as a linker with the *p53*-dependent pathway is up-regulated (Gartel et al., 1998). Once activated, the *p53* gene product works together with the $p21^{WAF1/CIP1}$ protein, and binds to the cyclin D-CDK4 and cyclin E-CDK2 complexes to inhibit their kinase activities (el-Deiry et al., 1993; Sherr & Roberts, 1999). Finally, increased expression of $p21^{WAF1/CIP1}$ and *p53* is involved in the G1/S-phase arrest of the cell cycle. These reports are in accordance with our data. In addition, the probucol treatment in ESCs, which blocks the cell cycle arrest by 5-FU, appears to aid the repair of damaged cells. Taken together, 5-FU affects endothelial differentiation by decreasing cell viability, proliferation and differentiation, as well as inducing the G1/S phase arrest. These toxicity screening are capable to use the mouse ESC system, therefore, mouse ESCs might be a useful model to use as a tool for screening the cytotoxicity of new compounds.

However, there are still obstacles to overcome in toxicity screening using ESCs. For example, they do not grow as well and are more difficult to maintain and expand than other cell types and the procedures for directing them to differentiate are limited until now. Although stem cells allow for optimal preclinical evaluation of compounds directly on "relevant" human cells prior to clinical testing, it is important because relatively little information is usually obtained during preclinical development procedures on the manner in which novel drugs act on human tissues. Therefore, we should develop new sources of stem cells could be alternative to the use of ESCs in drug screening.

7. References

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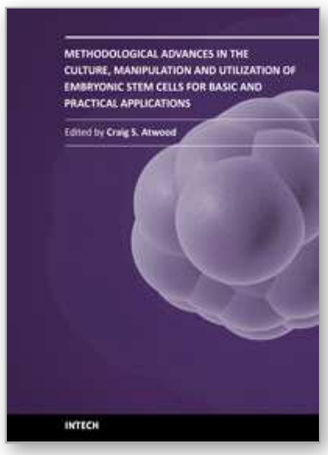

Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications

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