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Induce Differentiation of Embryonic Stem Cells by Co-Culture System

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Additional information is available at the end of the chapter

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1. Introduction

Stem cells, which are found in all multi-cellular organisms, can divide and differentiate into diverse special cell types and can self-renew to produce more stem cells. In mammals, two main broad types are included, such as embryonic stem (ES) cells and adult stem cells. The former are derived from the inner mass of blastocysts, and the latter have been found in various tissues from adult. In a developing embryo, stem cells can differentiate into all kinds of specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body.

ES cells, derived from the inner cell mass of pre-implantation embryos [1], can proliferate in culture and are able to give rise to all derivatives of the three primary germ layers: endoderm, mesoderm and ectoderm. In other words, they can develop into more than 200 cell types of the adult body when given stimulation for a specific cell type. The endoderm is composed of the entire gut tube and the lungs; the ectoderm gives rise to the nervous system and skin; and the mesoderm gives rise to muscle, bone, blood, and so on.

ES cells, being pluripotent cells, make them an excellent candidate as a source of functional differentiated cells for tissue replacement and regenerative medicine and after disease or injury. Using stem cell in regenerative therapy requires specific stimulation or signals for specific differentiation. If implanted directly, ES cells will randomly differentiate into many different types of cells and cause a teratoma eventually. ES cell researchers still face a few of

the hurdles, including differentiating ES cells into specific cells while avoiding transplant immuno-rejection [2].

Till to date, mouse embryonic stem (mES) cells and human embryonic stem (hES) cells have been used in researches. They require very different environments in order to maintain an undifferentiated state. Mouse ES cells are cultured on a layer of gelatin as an extracellular matrix and require the presence of feeder cells (STO or SNL) and leukemia inhibitory factor (LIF) [3]. Human ES cells are grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic fibroblast growth factor (bFGF or FGF-2) [4]. ES cells will rapidly differentiate without optimal culture conditions or genetic manipulation [5].

The multi-lineage differentiation potential of stem cells is not only an opportunity but also a challenge. An undesired cell type may lead to a pathophysiologic state or a non-functional tissue construct once that stem cells differentiate at the wrong time or place. In order to avoid such maladaptive responses, stem cells have evolved elaborate circuitry that triggers them to respond to differentiation cues only in an appropriate biological context. While most of researchers have been focusing on the role of soluble cues (e.g. growth factors and cytokines) in regulating stem cell differentiation, recent evidence demonstrated that the response to these stimuli are strongly modified by adhesive and mechanical cues, and that these microenvironment factors may be used explicitly to control stem cell differentiation in their own right [6]. With these advances in stem cell research, mimicking cellular microenvironment in vitro is becoming increasingly oriented toward to guide stem cell growth and differentiation.

In a living organism, cells are surrounded by peripheral other cells and embedded in an extracellular matrix (ECM) that defines the architecture, signaling, and biomechanics of the cellular microenvironment. As for stem cell, the word "niche" can be in reference to the in vivo and in vitro stem cell microenvironment. In architecture, the word niche refers to a recess, and in ecology it refers to a habitat where an organism can reside and reproduce. So, the grand position of the stem cell in popular concepts of science is appropriately humbled by the cells dwelling in a place where they might awaken with fleas. The concept of a niche as a specialized microenvironment housing stem cells was first proposed by Schofield, although experimental evidence was first provided by invertebrate models. In the gonads of *Drosophila melanogaster* and *Caenorhabditis elegans*, the germ stem cells reside at the distal end of a tapered structure, and have been shown to depend upon interactions with somatic cells so that stem-cell features could be maintained [7].

Stem cells are defined by their ability in complex multi-dimensional environment name as niche. Within the niche, several factors are important to regulate stem cell characteristics: (i) cell-cell interactions among stem cells; (ii) interactions between stem cells and neighboring differentiated cells; (iii) interactions between stem cells and extracellular matrix, adhesion molecules, soluble factors (growth factors, cytokines), oxygen tension, and other nature of the environment. Applying for regenerative medicine, specific differentiation of stem cells must be induced in vitro, and then specific graft with sufficient quantity and pure quality could be transplanted back into the patient. In order to archive this purpose, the researchers are trying to replicate the stem cell niche conditions in vitro. However, obviously, it is difficult to mimic the biological complexity of the native cell context in the laboratory under standard 2D culture

conditions, since much of the complex interplay of mechanical and molecular factors present in vivo is absent in 2D culture status [8]. This is a major limitation to investigate cellular response in vitro. Therefore, we need generate a new culture system that would be “something between a culture dish and the cells”, to represent cellular environment in a living organism and be more predictive of in vivo systems [9,10]. In particular, to stimulate stem cell potential and obtain biologically response in vitro, a new environment that are associated with their proliferation, differentiation, and assembly into tissues is desired. The researches should abide by the following premise: the function of the complicated factors is known to play a role during development or remodeling, and cellular responses to environment factors are predictable.

In our researches, we co-cultured ES cells with special cells to induce the specific differentiation. The co-culture system could supply stem cell for physical attachment (mechanical stretch), regulating signals, as well inducing factors such as cytokines (soluble or diffused). All these combined cues determined the differentiation of specific type of cells. The co-culture system recapitulated the combinations of parameters in the native environment to convert “collections of cells” into specific cell phenotypes. Hence, the design of co-culture system is necessarily inspired by stem cells research.

Co-culture system (ES cells and certain cell) supplied the physical, structural, and molecular factors to induce cellular differentiation. It opens several exciting possibilities: (i) establish functional implant which is suitable for transplantation and replace of degenerated tissues, (ii) investigate developmental processes and understand stem cell behavior in a native environment; (iii) avoid using biomaterials in order to escape from immuno-rejection. A variety of parameters were outlined in Fig.1. It included co-culture system and engineered 2D culture environment that influence stem cell behavior (e.g., self-renewal, migration, and differentiation).

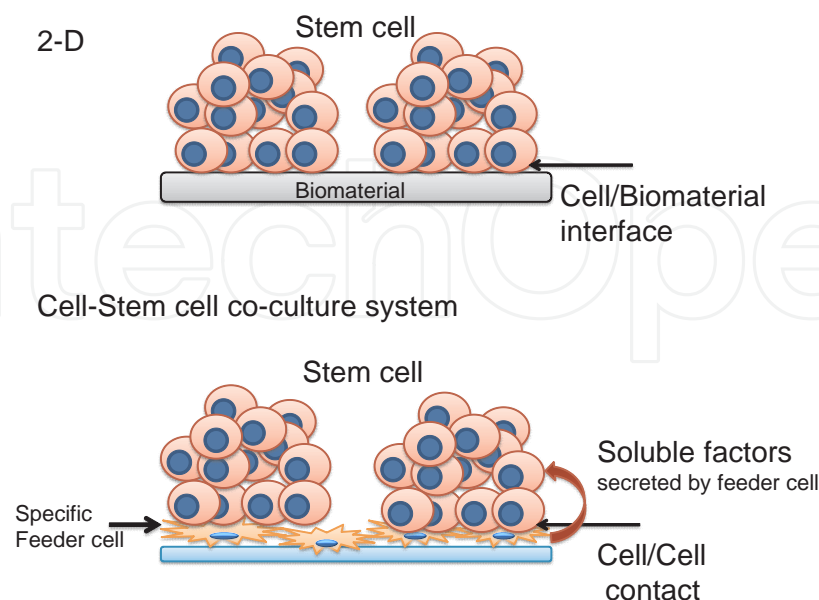


Figure 1. Manipulating the stem cell microenvironment in 2D and cell-stem cell co-culture system. Controllable parameters include matrix properties, cell/cell contact and soluble factors which were secreted by specific feeder cells.

The following section will review several important properties in the design co-culture system to control stem cell differentiation.

2. Interactions with stem cells

2.1. Direct attachment between stem cells and engineered materials

In tissue engineering, either natural or synthetic materials have been investigated for interaction with stem cells and to control their behavior [11]. The benefits to co-culture system include their ability to provide complicated signal to stem cells by physical attachment or chemical excretion. Compared to biological materials, natural or synthetic biomaterials, the former have poor mechanical properties and easily cause immune-response depending on the source of the materials; the later have wide diversity in properties that may be designed according to mechanics, chemistry, and degradation. The toxicity and a limited repertoire of cellular interactions are concerned.

As for the group of natural materials, collagen, matrigel, alginate, and hyaluronic acid (HA) have been used for stem cell researches. Collagen gels have been widely used in stem cell study, including mesenchymal stem cells (MSCs) [12,13] and ES cells [14]. Matrigel consists of a mixture of molecules derived from natural ECM and has been investigated for stem cell culture without feeder cells and inducing differentiation. An improved three-dimension (3D) and serum-free approach was established to differentiate hES cells into functional endothelial cells [16]. Alginate is a seaweed-derived polyanion that forms hydrogels through ionic cross-linking. ES cells have been induced in alginate hydrogels for a variety of applications, typically encapsulated stem cells for transplantation [15]. HA is a polysaccharide found in many tissues and has been modified to form hydrogels. HA hydrogels have been investigated for the culture and growth of undifferentiated human ES cells and MSCs [17, 18].

Synthetic materials were also widely used in stem cell cultures [19]. Materials that degrade through either hydrolytic or enzymatic mechanisms have been synthesized, and the advantage is the tenability and versatility of these physical properties. A hydroxyester has been extensively used in the field of tissue engineering, primarily due to their history of biocompatibility and use in medicine. One composition was seeded with human ES cells for the regeneration of numerous tissues, including vascular and neural structures [20].

2.2. Secreted Soluble factors

Cell and extracellular-matrix components in the stem-cell niche are relatively predictable, although the complexity and integration of these elements is far from known. Soluble mediators of cellular response would also be expected and a number of soluble factors such as growth factors and cytokines are important for stimulate and control the differentiation of stem cells. Adding relative molecules to the culture medium can induce the differentiation. For example, bone marrow stromal cells (BMSCs) have been directed into all kinds of cells [21]. Alternatively, it is advantageous to secrete the molecules directly from

the specific cells. The molecules that can induce differentiation is various, such as basic fibroblast growth factor (FGF), members of the transforming growth factor family (TGF), small molecules such as retinoic acid. Molecule release is typically controlled through diffusion, degradation, or combination of factors. Multiple growth factors have been delivered from the same scaffold based on polymer degradation rates [22]. However, regards to co-culture system, the cells used as basement feeder open up the possibility to control molecule secretion for complex signaling cascades in stem cell differentiation, although the regulating mechanism is difficult to be cleared.

2.3. Mechanical and shape cues to control stem cell differentiation

Using soft lithography techniques, the influence of spatially patterned adhesion molecules on cell differentiation was investigated, such as cell spreading and shape on MSC differentiation, through control the cellular cytoskeleton. MSCs tended to the differentiation of osteoblasts, when they were patterned on larger islands of adhesion ligands, which allowed for cell spreading. On the other hand, MSCs on smaller islands stayed rounded and differentiated into adipocytes [23]. This study indicated that stem cell commitment could be regulated by adhesion molecules and is a consideration in design for inducing the differentiation of stem cells.

During the last decades, much progress has been made in the molecular understanding of early differentiation in stem cells. For example, neural inducer molecules, such as chordin, noggin, and retinoic acid (RA), were identified, and several intracellular mediators of neural differentiation have been characterized. Multiple-step method involving in embryoid body (EB) formation followed by different combination of growth factors was the most common method for inducing differentiation of ES cells [24, 25]. Although the methods can produce a good proportion of different type of specific cells, it has several apparent problems. (i) it is difficult to analyze and control each regulatory step of differentiation in this multiple-step because EBs contains many different kinds of cells, including endoderm, mesoderm, and ectoderm cells; (ii) growth factors have complicated function in vivo. For example, RA, a strong teratogen, is supposed to perturb neural patterning and neuronal identities in EBs as it does in vivo. RA treatment of early embryos causes suppression of forebrain development. It is therefore preferable to avoid RA treatment for therapeutic application unless RA induces the particular type of neurons of one's interest; (iii) in order to avoid infection and rejection, the serum of animal origin should be excluded; (iv) multiple-step method is cost-consuming.

3. Inducing specific differentiation of stem cells using with cell co-culture system

In our research, we introduce an efficient cell co-culture system for in vitro differentiation of specific cell type from ES cells in a serum-free condition that requires neither EBs nor complicated treatment with growth factors.

3.1. Induction of midbrain dopaminergic neurons from primate embryonic stem cells by co-culture with Sertoli cells

In the first study, we have established a new method for generating dopaminergic neurons from primate ES cells by co-culture with Sertoli cells. Neurodegenerative diseases present severe problems due to the limited repair capability of the nervous system [26]. Stem cells have a capacity for unlimited self-renewal, along with the ability to produce multiple different types of terminally differentiated descendants. They are candidate therapeutic tools in neurodegenerative disorders, such as Parkinson's disease, which is characterized by degeneration and death of midbrain neurons that produce dopamine. Transplantation of dopaminergic neurons taken from human fetuses into Parkinson's disease patients shows a remarkable, but inconsistent, ability to replace endogenous degenerated dopaminergic neurons and to ameliorate some of the disease symptoms [27, 28]. However, since treatment of a single Parkinson's disease patient requires dopamine neurons from 6 to 10 human fetuses, replacement therapy is not routinely available. Other sources of dopamine-producing cells, including those from the adrenal medulla or carotid bodies, have been examined for their ability to alleviate Parkinson's symptoms, but these sources are also limited in numbers and/or are not as effective as fetal dopamine neurons [29]. ES cells can proliferate indefinitely and are able to differentiate into cell types of all three germ layers *in vivo* and *in vitro*. These unique properties of ES cells make them an excellent candidate for the treatment of Parkinson's disease [30,31].

Our ES cell-Sertoli co-culture system is technically simple, cost-saving, and the induction is efficient and speedy. This protocol only used both of cells and treated without any inducing factors. After 3 weeks of induction, immuno-staining revealed that $90\% \pm 9\%$ of the colonies contained tyrosine hydroxylase-positive (TH⁺) neurons, and $60\% \pm 7\%$ of the tubulin III-positive (Tuj III⁺) neurons were TH⁺ (Fig2. A-E). At the same time, the possible roles of Sertoli cell in the differentiation of ES cells into Dopaminergic Neurons were investigated. When cultured on the gelatin-coated dish in the same medium but without Sertoli cells, ES cells differentiated into neurons at a low frequency compared with the rate obtained with ES cells cultured on Sertoli cells (Fig2. F, lanes 1 and 2). This suggested that Sertoli cells had an active role in the promotion of neural differentiation of ES cells. We tested whether direct physical contact between ES cells and Sertoli cells was essential for the induction. ES cells cultured on gelatin-coated dishes and separated from co-cultured Sertoli cells by a 0.22- μ m filter membrane were still able to induce significant neural differentiation of ES cells (Fig2. F, lane3; Fig2.H). When Sertoli-conditioned medium was used to culture ES cells without Sertoli cells as a feeder layer, neural differentiation was still induced compared with ES cells cultured alone on gelatin-coated dishes (Fig2. F, lane4; Fig2.I). Together these data suggest that Sertoli cells secrete one or more soluble neural-inducing factors.

Based on the known ability to promote neuronal differentiation [32], GDNF seemed to be a likely candidate for the Sertoli-induced differentiation of primate ES cells. We confirmed the presence of GDNF expression in Sertoli cells by immunohistochemistry (Fig. 2 J, Fig.2 K lane 1) and in the conditioned medium by Western blot analysis (Fig.2 K, lane 2). We therefore tested whether GDNF promoted dopaminergic neuron differentiation of primate ES cells. First, we added GDNF to ES/Sertoli cell co-cultures and compared results with ES cells on a gelatin

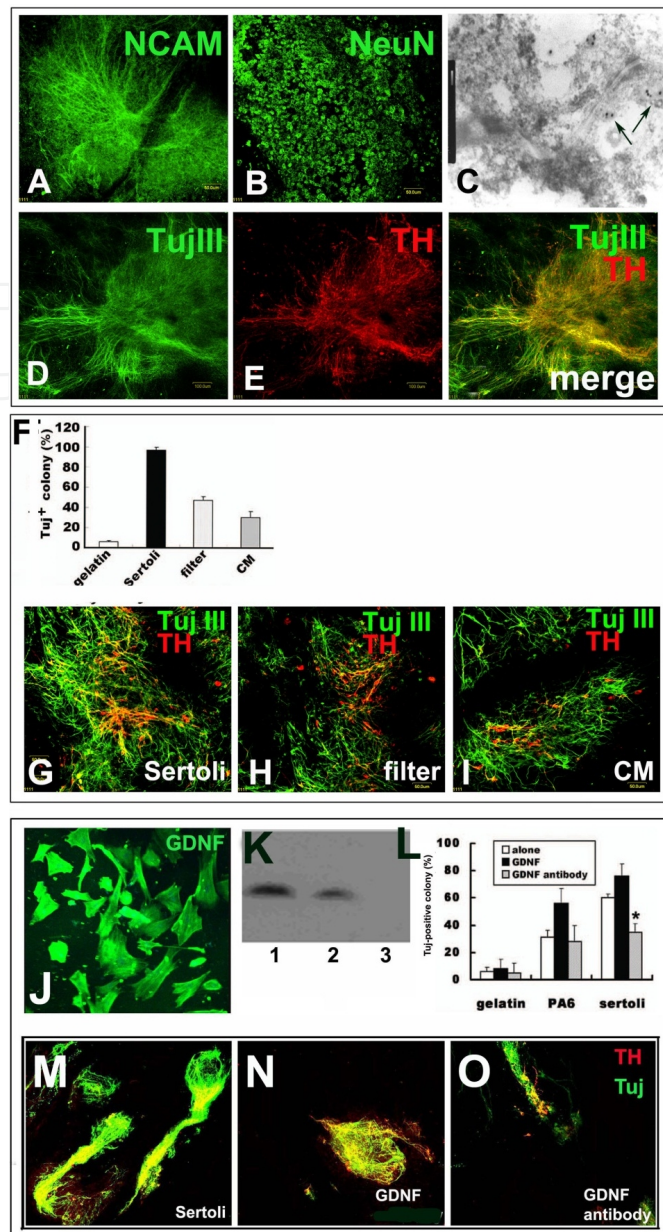


Figure 2. A-E. Sertoli-induced dopaminergic neural differentiation and of monkey embryonic stem cells. Characterization of monkey ES colony induced by Sertoli cells. Expression of NCAM (A), NeuN (B) confirmed the neural identity of cells in ES colony. After 3 weeks, approximately 97% of the ES cell colonies cultured with Sertoli cells were Tuj III⁺ (D). After 3 weeks of culture, immunostaining of Sertoli-induced neurons with anti-TH antibody (E). (C): Electron microscopy showed TH ImmunoGold particles (15nm) were associated with small vesicles presumably containing neurotransmitters located at the presynaptic terminal (arrow). F-I. The physical and chemical role of Sertoli cells in the induction of dopaminergic neurons. (F): Sertoli cells induced the expression of neuronal marker Tuj III in co-cultured monkey embryonic stem (ES) cells even when separated by a filter membrane. CM induced neural differentiation in ES cells cultured on gelatin-coated dish. However the differentiation rate was lower than with direct coculture with Sertoli cells. Tuj III (green) and TH (red) double staining of ES cells when separated from Sertoli cells by a filter (H) and cultured in conditioned medium alone (I). Scale bar = 50um. J-O. The role of GDNF in Sertoli-induced dopaminergic neuron differentiation. (J): Immunostaining of GDNF in Sertoli cells. (K): GDNF protein was detected by Western blot of lysed Sertoli cells (lane 1) and in conditioned medium (lane 2). However, it was not detected in conditioned medium blocked by anti-GDNF antibody (lane 3). (L): In embryonic stem (ES) cells cocultured with conditioned medium derived from Sertoli cells, the TH⁺ cell percentage was reduced significantly when GDNF was blocked by antibody. TH staining of ES cells cultured on Sertoli cells (M), on Sertoli cells treated with extraneous GDNF (N), and on blocked conditioned medium (O). Scale bars = 50um.

coated substrate after 3 weeks in culture. We found that GDNF increased the number of TH⁺ cells in co-culture with Sertoli (Fig. 2 L, N) At the same time, we incubated the conditioned medium with GDNF-blocking antibodies and effectively removed GDNF as determined by Western blot (Fig. 2K, lane3). The number of TH⁺ cells supported by anti-GDNF-treated conditioned medium was reduced to 35% ±6% ($n=6,000$; Fig. 2L), which was significantly less than that induced by Sertoli cells ($*p<.05$; Fig. 2L), but still more than in colonies grown on gelatin alone. GDNF antibody inhibited the TH⁺ neural induction activity of Sertoli cells, as shown by TH staining of ES/Sertoli cell co-cultures and ES cells cultured in conditioned medium blocked by GDNF antibody (Fig. 2 M-O).

Possible roles of Sertoli cells in the differentiation of ES cells into dopaminergic neurons were discussed. Sertoli cells have an active role in the promotion of neural differentiation of ES cells. The mechanism of dopaminergic neuron induction in co-cultures of ES cells with Sertoli cells remains to be understood. First, whether direct physical contact between ES cells and Sertoli cells was essential for the induction was tested through filter membrane to separate ES cells with Sertoli cells. The result showed that Sertoli cells were still able to induce significant neural differentiation of ES cells, indicating that Sertoli cells produce soluble inducing factors. However, Sertoli-conditioned medium could not elicit significant induction. It suggested two possibilities as to the molecular nature of neuron-inducing activity by Sertoli cells. One is that Sertoli cells secrete two different neuron inducing factors, a cell surface-anchored factor and a labile soluble factor. Another might be that the neuron-inducing activity is mediated by secreted factors. At present, we cannot exclude either possibility.

Some factors have been implicated in the regulation of dopaminergic differentiation [33], such as FGF8, Shh, interleukin (IL) 1, IL11, GDNF, and neutralizing antibodies of FGF8 and Shh. Among them, GDNF has the most potent neuroprotective and trophic effects on dopamine neurons in many model systems [34,35,36]. However, it is a large protein and has to be delivered directly to the brain rather than given peripherally. When successfully delivered, GDNF supports the survival and outgrowth of dopamine neurons following transplantation [37]. In addition, GDNF added to cell suspensions of embryonic ventral mesencephalic tissue improves the survival of dopamine neurons following grafting into the degenerative striatum [38]. Other studies have shown that intermittent injections of GDNF in the vicinity of intrastriatal nigral cell suspension grafts have similar effects on improving the survival and/or fiber outgrowth of transplanted dopamine neurons [39,40]. In a previous study [41], neurospheres modified to produce GDNF increased the survival of transplanted dopamine neurons in 6-OHDA-lesioned animals. GDNF is also capable of promoting differentiation of mesencephalic neurospheres towards the neuronal lineage, and more importantly, towards the dopaminergic development indicated by expression of Nurr1 and Ptx3. Buytaert-Hoefen et al. [42] proved that significant differentiation of dopaminergic neurons were not induced when cultured on PA6 stromal cells alone except for the presence of GDNF or striatal astrocytes. Sertoli cells secrete GDNF and promote the survival of transplanted dopaminergic neurons. In the present study, we showed that GDNF plays a role in dopaminergic neuron differentiation when primate ES cells were co-cultured with Sertoli cells. In our study, extraneous GDNF induced the differentiation of dopaminergic neuron. Sertoli-induced neural differentiation of ES cells

was partly suppressed by a low-dose of anti-GDNF antibody. However, blocking GDNF did not completely inhibit the neural differentiation. We cannot be certain that GDNF activity was completely blocked, although it was not found by Western blot. Therefore, the diminished neuronal differentiation that occurred could have been in response to the remaining, unblocked GDNF. Alternatively, if all of GDNF was blocked, then one or more other factors were present in the conditioned medium and promoted differentiation at a reduced rate. If these factors exist, they are more effective in the presence of GDNF. This confirmed the conclusion of Buytaert-Hoefen et al. [42] that GDNF is required but not necessary for the induction of dopaminergic neurons.

From these results, we can conclude that Sertoli cells may stimulate dopaminergic differentiation by a complex combination of growth factors or other factors, including other unidentified components. Among these, GDNF plays some role, but not a decisive role. It is also possible that the supporting environment provided by Sertoli cells, or an interaction between Sertoli cells and primate ES cells, plays a role in their neuron inducing activity.

In addition, isolated Sertoli cells enable survival and function of co-grafted foreign dopaminergic neurons in rodent models of Parkinson's disease. They also promote regeneration of damaged striatal dopaminergic circuitry in those same Parkinson's disease models [43]. In our study, significant TH⁺ cells were found in the degenerative striatum when differentiated primate ES cells were co-transplanted with Sertoli cells. Moreover, 2-month survival of TH⁺ neurons derived from ES cells was observed. It is likely that the nutritive support of the Sertoli cells is responsible for this enhanced TH cell survival.

In common, dopaminergic neurons were generated from neural precursor cells amplified from EBs. One group [44] used a lengthy four-step method to produce TH⁺ neurons at an efficiency of approximately 7% of the Tuj III⁺ neurons. Kawasaki et al. [45] used the term stromal cell-derived inducing activity to describe the dopaminergic neuronal-inducing defect of PA6 cells. The stromal feeder-mediated neural induction had been demonstrated for mouse, primate, and human ES cells [46-48]. In contrast to the previous protocols, our methods does not require growth serum, the formation of EBs, selection of neural precursor cells, retinoic acid, growth factors, or other special treatment. Thus, our method is more suited to detailed analyses of differentiation and transplantation therapy.

3.2. Differentiation of primate ES cells into retinal cells induced by ES cell-derived pigmented cells

In the second study, retinal cells were induced from primate ES cells by co-culturing with ES-derived pigmented cells.

Photoreceptors are the primary sensory neurons residing in the outer nuclear layer (ONL) of the vertebrate retina. Photoreceptor degeneration is a common cause of human visual impairments resulting from light damage, genetic changes and aging. The unfortunate nonrenewable nature of photoreceptors has inspired that these degenerative diseases maybe treatable by transplantation of healthy fetal cells. Previous work has shown that freshly harvested retinal pigment epithelium (RPE) can be effective in rescuing photoreceptors in the

Royal College of Surgeons (RCS) rat, an animal model of indirect photoreceptor degeneration [49]. However, obtaining a sufficient number of suitable donor cells remains a problem.

The isolation of embryonic stem (ES) cells has drawn much attention, given their potential to generate all adult cell types. As ES cells continue to proliferate in an undifferentiated state *in vitro*, an unlimited stem cell source or its derivatives may be secured. It is also a potential benefit that ES cells may be genetically manipulated to permit the selective differentiation and/or isolation of a specific cell type.

Recently, several laboratories have devised hetero chronic co-culture experiments to characterize the nature of the interactions necessary for the differentiation of retinal cells [50,51]. Early embryonic mouse cells (E12) were shown to differentiate into rod photoreceptor cells with a higher frequency when co-cultured with either postnatal rat retinal cells or with later staged embryonic retinal cells that were competent to generate rod photoreceptors. The results suggested that the differentiation of retinal progenitor cells as rod photoreceptors is influenced by locally diffusible signals in the extra cellular environment that are developmentally regulated during the period of retinal histogenesis.

Although the specific factors that promote differentiation of cells as rod photoreceptors are not known, several classes of molecules have been shown to play a role in the developing retina, for example, small peptide growth factors, Shh, taurine, epidermal growth factor (EGF) and fibroblast growth factor (FGF) [52-54]. The vitamin A derivative retinoic acid has been shown to influence cell fate in the developing nervous system. In particular, all-trans retinoic acid has been shown to alter cell fate decisions in the developing limb bud, hindbrain and inner ear [55,56]. In addition, several studies have demonstrated that all-trans retinoic acid and at least one of the nuclear retinoic acid receptors (RAR α) are present in the developing retina [56]. RA caused a dose-dependent, specific increase in the number of cells that developed as photoreceptors in culture throughout the period of retinal neurogenesis [52,53].

Retinal pigmented epithelium (RPE) is a neuroectodermal derivative essential for the survival of photoreceptors. It supplies nutrition and provides several trophic factors that help maintain the normal physiology within the neuro sensory retina and photoreceptors [57,58].

In most of studies, differentiation of the retinal progenitors into photoreceptors was infrequent in the absence of co-culture with embryonic retinal tissues [59,60]. Use of the fetal retina involves some ethical and practical consideration, and the cell supply is a problem. Osakada and colleagues [52,53] induced the generation of putative rod and cone photoreceptors from ES cells by stepwise treatments under defined culture conditions. However, the practical steps were complicated and time consuming. In this study, the monolayer of RPE derived from ES cells was used as an inducing feeder layer which could replicate the microenvironment of retina or sub-retinal to successfully induce photoreceptor cells. This is the first time that ES-derived cells are used to induce the differentiation of ES cells. Furthermore, retinoic acid was used to induce the differentiation of photoreceptors. To our knowledge, a few reports have produced photoreceptor cells in uncontaminated culture conditions except for Takahashi's group [52] Osakada [53] and Lamba's [60,61,62,] research. The present study demonstrated the generation of photoreceptors from primate ES cells under conditions free of animal-derived substances.

During the study of ESC-Sertoli co-culture system, one unexpected finding was the appearance of epithelial cells with massive pigmentation from the second week and they grew at a constant rate. After monkey ES cells were cultured on Sertoli cells for 3 weeks, a patch of pigmented cells was mechanically isolated by using a tip after being loosened with trypsin and plated on a gelatin-coated dish without feeder cells in primate ES cell culture medium. The polygonal morphology with a compact cell-cell arrangement was reminiscent of the pigmented epithelium of the eye, and clearly distinct from pigmented melanocytes derived from neural crest (Fig3 A-I). In recent, transplantation of retinal pigment epithelium (RPE) has become a possible therapeutic approach for retinal degeneration. Meanwhile, RPE is a neural ectodermal derivative essential for the survival of photoreceptors. It serves as nutritional cells and provides several trophic factors that help maintain the normal physiology within the neural sensory retina and photoreceptors. Therefore, we investigated whether retinal cells could be induced by co-culturing ES cells together with RPE. At the same time, RA was treated in order to improve the differentiation efficiency. The results showed that after 10-day co-culture of ES cells and these RPE, some ES derivatives became immuno-positive for rhodopsin. RT-PCR analysis demonstrated expression of retina-related gene markers such as Pax6, CRX, IRBP, Rhodopsin, Rhodopsin kinase and Muschx10A. When RA was added, the distinct increase of photoreceptor specific proteins markers was found. Besides, the differentiation of bipolar, horizontal cells was demonstrated by protein and gene expression. RA treatment also altered more retinal cell differentiation (Fig3. J-M).

We demonstrated that pigment epithelial cells can be generated, enriched, and expanded from primate ES cells when ES cells were co-cultured with Sertoli cells. These ES-converted pigment epithelial cells showed development of several of the characteristics of RPE cells and were able to be used to induce the differentiation of retinal cells. For the clinical application, methods for purifying large numbers of lineage specific cells should be developed. In the present study, RPE cells could be easily identified under a dissecting microscope and selectively expanded into a uniform single cell layer.

The RPE cells contain melanin granules, have a characteristic polygonal morphology, and play some critical roles including (i) forming a barrier separating the retina from the blood vessels of the choroid coat; (ii) regulating nutritive substance transport required for retinal progenitors to differentiate into retinal neurons and the maintenance of retinal cells; (iii) regenerating visual pigments; (iv) digesting the shed parts of photoreceptor cells after having phagocytized them [63]. Therefore, we used ES cell- derived RPE cells to provide epigenetic retinal neurons, not only the efficient induction of photoreceptors, but also other retinal cell lineages, such as bipolar, and horizontal cells. It indicated that the secreted/diffusible factors from RPE or direct cell-cell contact were sufficient to induce retinal cells from ES cells.

A monolayer of ESC-derived RPE cells, an inducing feeder layer that replicates the retinal microenvironment and RA were used to successfully induce photoreceptor differentiation. To our knowledge, this is the first time that ESC-derived cells have been used to induce ESC differentiation. ES/RPE co-culture system can serve as a promising method for therapeutic application and basic research on retinal degeneration disease, although we are still far from an established in vitro or in vivo source of retinal cells.

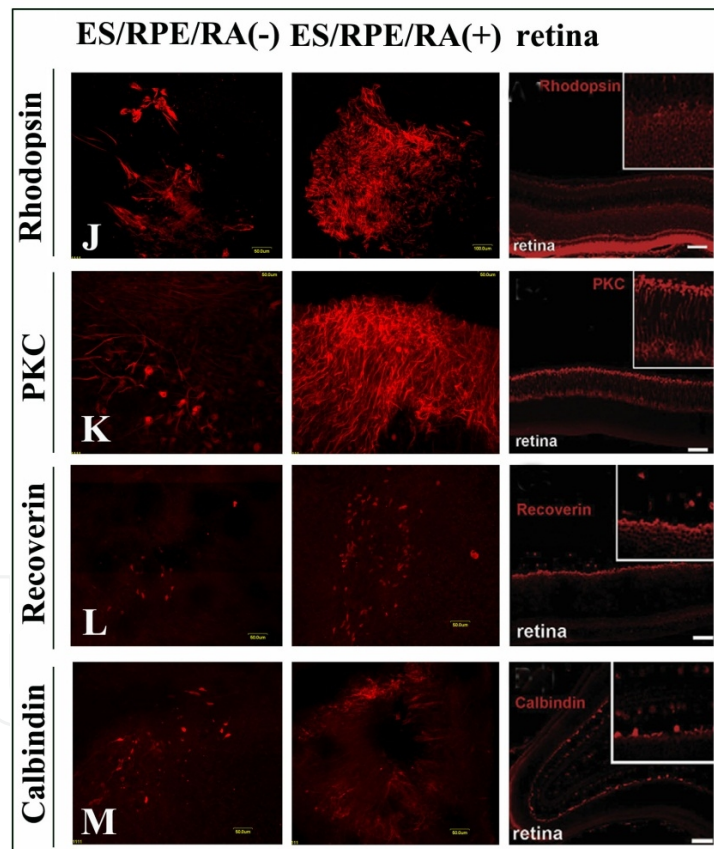
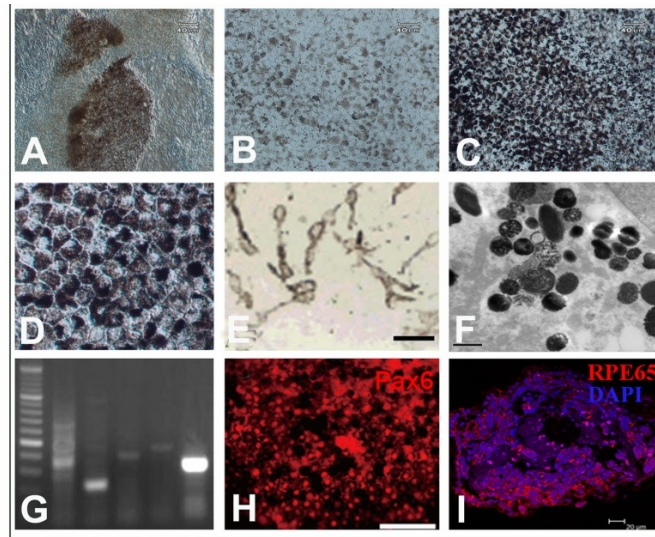


Figure 3. A-I. Analysis of pigmented epithelial cells derived from primate ES cells using LM (A-E), TEM (F), RT-PCR (G), immunofluorescence staining (H-I). (A) LM images showing pigmented cells present in the primate ESC colonies grown at a constant rate on Sertoli feeder cells for 2, 4, and 6 weeks (A,B and C, respectively). The polygonal morphology with a compact cell-cell arrangement (D) is similar to the pigmented epithelium of the eye and clearly distinct from pigmented melanocytes derived from the neural crest (E). (F) TEM images of primate ESC-derived pigmented cells displaying melanin granules. (G) Shown are the RT-PCR-amplified products of RPE cell-specific markers (C1): tyrosinase-related protein-2(Trp-2), RPE65, CRALBP, MertK. (H-I) Immunostaining of RPE cells derived from ES cells with

anti-Pax6 and RPE65 antibody is also shown. J-M. Analysis of differentiated retinal cells induced by ESC-derived RPE cells by immunofluorescence staining. Immunopositive ESC derivatives for retinal cell-specific markers are shown as follows: (J) ES cells were immunopositive for rhodopsin after a 21-day co-culture with RPE cells, and an increased frequency of rhodopsin-positive cells was estimated after RA treatment, and some of them developed into rosettes. The eyes of a db/db mouse (2 weeks) were used as a positive control to identify the positive immunofluorescence staining results and the specificity of retinal antibodies. (K) PKC-positive cells appeared as early as day 3 after starting co-cultures. A drastic increase was observed after RA treatment. Positive control with mouse eyes and PKC antibody is also shown. (L) Recoverin-positive cells appeared after a 10-day o-culture with RPE cells. Expression increased at a constant rate and after RA treatment. Positive control with mouse eyes and recoverin antibody is also shown. (D) After a 10-day co-culture with RPE cells, ES cells became immunopositive for calbindin. Immunopositive ESC derivatives for calbindin are shown at day 2. The number of calbindin-positive cells increased after RA treatment (D2). Positive control with mouse eyes and calbindin antibody is also shown. Scale bars = 50 μ m.

3.3. Bone marrow stromal cells as an inducer for cardiomyocyte differentiation from mouse embryonic stem cells

In the third study, bone marrow stromal cells (BMSCs) were used as an inducer to induce cardiomyocyte differentiation from mouse ES cells.

Several studies reported that different feeder layers induced cardiomyocytes from ES cells [64-67]. It proved that the visceral-endoderm-like cell line, END-2 induced mouse P19EC, mouse and human ES cells to aggregate in co-culture and give rise to cultures containing beating areas. For mouse P19 EC cells, it has been demonstrated that a diffusible factor secreted by the END-2 cells is responsible for the induction of cardiomyocyte formation.

Like END-2 cells, various cell types of stem cells remain in a mature body. Among them, bone marrow stem cells (BMSCs) are unique because of rich functional products. A wide array of cytokines including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin growth factor-1 (IGF-1) were detected in the BMSC cultured medium by ELISA [68]. Furthermore, they secreted hepatocyte growth factor (HGF), and transforming growth factors (TGF-beta) [69], which are all potent cardiomyocyte growth and survival factors, or play an important role in proliferation and differentiation of stem cells [70]. IGF-1 can promote angiogenesis in infarcted myocardium, reduce the degree of myocardial necrosis, maintain the myocardial structure, stimulate proliferation of cardiac fibroblast, and inhibit matrix degradation; thus preventing ventricular dilatation and reducing load capacity of the heart [71]. After binding to its receptor on ES cells, IGF-1 induces expression of a number of cardiac-specific transcription factors such as the zinc finger GATA proteins and Nkx-2.5, a co-activator of GATA-4. GATA-4 and Nkx-2.5 are essential for heart development [72]. HGF is a multifunctional factor promoting cell mitosis. It can promote cell survival and regeneration, inhibit the apoptosis of stem cells, and increase the survival rate of transplanted cells. More importantly, HGF enhances growth and favors cell extracellular matrix interactions; the critical steps during myocardial regeneration after infarction. In the heart, high concentration and wide distribution of FGF has been identified from early embryonic stages. Basic FGF plays a vital role in the growth and differentiation of cardiac myocytes. FGF was found to induce DNA synthesis in adult rat myocytes, cells which were considered capable of regeneration [73]. Members of the

TGF- β superfamily play important roles in cardiac development during embryogenesis [74] as well as in various cardiac pathologies [75]. TGF- β 1 has been shown to induce cardiac differentiation in vitro in embryonic explants and stem cells [76,77] as well as in adult bone marrow-derived cells [70]. Previous studies of non-conditional global VEGF or VEGF isoform gene deletion have demonstrated embryonic and early postnatal mortality associated with severe cardiac abnormalities and abnormal vascularization; thus establishing that systemic VEGF expression is essential normal myocardial development [78]. From these reports BMSCs are expected to function as inducer for cardiac differentiation from ES cells.

To confirm whether BMSCs are a real cardiac inducer to differentiate ES cells into cardiomyocytes, mouse embryoid bodies (EBs) were co-cultured with rat BMSCs. After about 10 days, areas of rhythmically contracting cells in more solid aggregates became evident with bundle-like structures formed along borders between EB outgrowth and BMSC layer. ESC-derived cardiomyocytes exhibited sarcomeric striations when stained with troponin I (Trop I), organized in separated bundles. Besides, the staining for connexin 43 was detected in cell-cell junctions, which demonstrated that ESC-derived cardiomyocytes were coupled by gap junction in culture (Fig4 A-F). In addition, an improved efficiency of cardiomyocyte differentiation from ESC-BMSC co-culture was found in the serum-free medium: 5-fold up-regulation in the number of beating area compared with the serum medium. Effective cardiac differentiation was also recognized in transfer filter assay and in condition medium obtained from BMSC culture. A clear increase in the expression of cardiac genes and TropI protein confirmed further cardiac differentiation by BMP4 and Retinoic Acid (RA) treatment (Fig4 G,H). These results demonstrate that BMSCs can induce cardiomyocyte differentiation from ES cells through soluble factors and enhance it with BMP4 or RA treatment. Serum-free ESC-BMSC co-culture represents a defined in vitro model for identifying the cardiomyocyte-inducing activity from BMSCs and, in addition, a straightforward experimental system for assessing clinical applications.

In our study, two experiments of separation from BMSCs by the filter and using CM without contact of BMSCs were performed. These results showed that both conditions were still able to induce significant cardiac differentiation (Fig4 I-M). As expected previously, these data have shown that it is due to varied soluble factors secreted by BMSCs, though effective soluble factors have to be determined in future. On the other hand, cardiomyocyte bundles were formed long the edge of EB outgrowth, which was a borderline with BMSC layer. These regular arrangements seemed to be associated with BMSC layer structures. Moreover, in fact, direct cell-to-cell interaction between BMSC and EBs was more effective to induce cardiac differentiation. It may be possible that the supporting environment provided by BMSCs or an interaction between BMSCs and EBs may play a role in their cardiac-inducing activity.

Compared with the inducing effects of low dose cytokines alone or with BMSCs alone, our co-culture with BMSCs supplemented with cytokines could increase the differentiation of cardiomyocytes, which indicated that BMSCs had the ability to promote the induction and proliferation of ESC-derived cardiomyocyte and the addition of low dose cytokines had a synergistic effect on this ability.

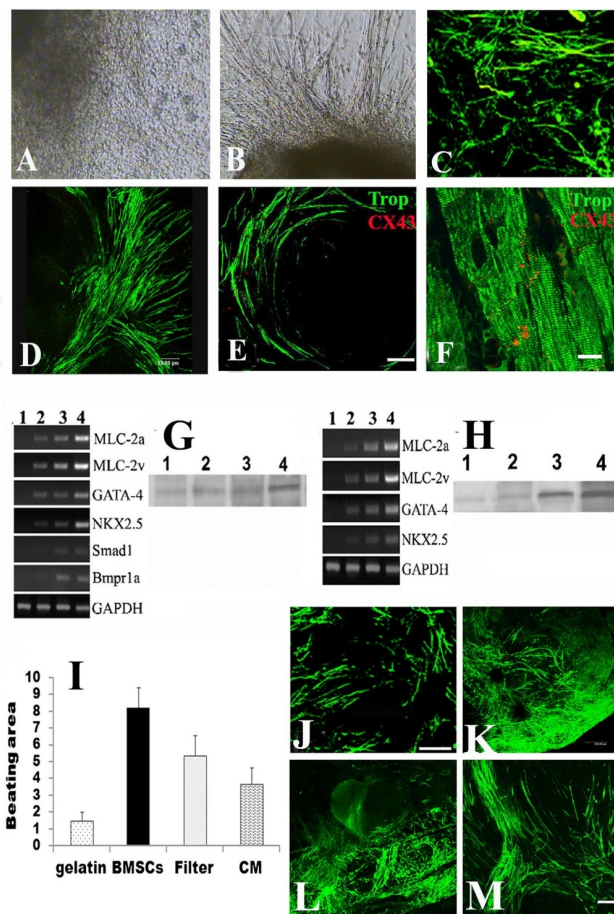


Figure 4. Characterization of cardiomyocyte induced by BMSC co-culture. (A,B) Morphology of serum or non-serum treated EB outgrowth on BMSC layer on day 14. (A) 20% FBS: Multi-angular and flattened cells spread out from EBs. (B) Absence of FBS: fiber-like cells extended radially and formed bundle-like structures each other. Scale Bars = 25um. (C-F) Beating areas stained for Tropl (green) and CX43 (Red). (C) In EBs formed in the presence of FBS, cardiomyocyte fibers are distributed disorderly. (D) Cardiomyocyte fibers are organized regular in EBs formed in the absent of FBS. Radial-morphological-like fibers surrounded or spread from EBs. Beating cells showed spindle, round and tri or multi-angular morphologies with characteristic organized in separated bundles. (E) CX43 staining among Tropl-positive cardiomyocytes showed the presence of gap junctions. (F) The heart of C57BL/6 mice was used as a positive control to identify the positive immunofluorescence staining results and the specificity of retinal antibodies. Bars = 100um. **G-H** Examination of expression changes of cardiomyocytes from EBs after BMP4 and RA treatment. (G) Induction of cardiac-specific genes and BMP signaling molecules in EBs exposed to BMP4, as shown by RT-PCR. Lane 1: undifferentiated ES cells; lane2: EB cultured on gelatin-coated dish, FBS(-); lane3: EB co-cultured with BMSCs, FBS(-); lane4: EB co-cultured with BMSCs, FBS(-), and treated with BMP4. The expression of cardiac markers and BMP signals were increased markedly by BMP4 treatment. Tropl protein was also detected by Western of lysed EB on gelatin-coated dish (lane1), EB on gelatin-coated dish and treated with BMP4 (lane2); EB co-cultured with BMSCs (lane3); EB co-cultured with BMSCs and treated with BMP4 (lane4). Combined using BMP4 and BMSCs induced significant cardiac differentiation. (H) The effect of RA on the differentiation of cardiomyocytes. (D) RT-PCR analysis revealed that both early cardiac genes, MLC-2a and MLC-2v, were increased in RA-treated EBs. Lane 1: undifferentiated ES cells; lane2: EB cultured on gelatin-coated dish, FBS(-); lane3: EB co-cultured with BMSCs, FBS(-); lane4: EB co-cultured with BMSCs, FBS(-), and treated with RA. Tropl protein was detected by Western of lysed EB on gelatin-coated dish (lane1), EB on gelatin-coated dish and treated with RA (lane2), EB co-cultured with BMSCs (lane3); EB co-cultured with BMSCs and treated with RA (lane4). Combined using RA and BMSCs significantly induced cardiac differentiation. **I-M.** The physical and chemical role of BMSCs in cardiomyocyte induction. (I) BMSCs induced beating cardiomyocytes in co-cultured ES cells even when separated by a filter membrane. CM induced cardiac differentiation in ES cells cultured on gelatin-coated dish. However the differentiation rate was lower than with direct co-culture with BMSCs. (J-M) EB on gelatin-coated dish (J); EB co-cultured with BMSCs (K); Tropl staining of ES cells when separated from BMSCs by a filter (L) and cultured in CM alone (M). Scale Bars = 50um.

4. Conclusion

Cell-to-cell interaction is important to differentiate varied cells or tissues from stem cells and/or in embryogenesis. An important component to the stem cell microenvironment is the surrounding matrix, which includes numerous chemical and biophysical cues. The changing local molecular conditions through selecting specific co-culture system might thereby provide promising method to modulate stem cell differentiation. In our researches, we selected the different kinds of cells as a feeder cell to induce the specific differentiation of stem cells. The molecular basis of induction in co-culture system remains to be understood. We tested some factors that have been implicated in the regulation of differentiation, such as GDNF, bFGF, BMP4, or neutralizing antibodies of GDNF. So far, no significant effect factors on the induction were observed. Interestingly, as showed above, the differentiation efficiency of ES cells in co-culture system is as high as the efficiency in the multiple-step method with lots of growth factors treatment, or even higher than that. The possible explain for this is that supporting environment provided by co-culture basement cells, such as Sertoli, RPE or BMSCs, or an interaction between co-culture basement cells and ES cells, plays a role in their inducing activity.

In conclusion, we established a simple and effective system for the differentiation of specific cells from ES cells. Further study is warranted to establish selection methods, analyze cell functions, and transplanted for degeneration diseases. However, the co-culture system can serve as a promising method for therapeutic applications and basic research on degeneration diseases.

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