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Differentiation of Megakaryocytes/Platelets and Neurons from Human Endometrial Stromal Progenitor Cells

Jinju Wang
Wright State University

Differentiation of Megakaryocytes/Platelets and Neurons from Human Endometrial Stromal Progenitor Cells

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By

Jinju Wang
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jinju Wang ENTITLED Differentiation of Megakaryocytes/Platelets and Neurons from Human Endometrial Stromal Progenitor Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Yanfang Chen, M.D., Ph.D.
Thesis Director

Mariana Morris, Ph.D., Chair
Department of Pharmacology & Toxicology

Committee on
Final Examination

Mariana Morris, Ph.D.

Lawrence S. Amesse, Ph.D.

James E. Olson, Ph.D.

Andrew Hsu, Ph.D.
Dean, Graduate School

ABSTRACT

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Differentiation of Megakaryocytes/Platelets and Neurons from Human Endometrial Stromal Progenitor Cells.[1]

[1] human endometrium is a high dynamic tissue that contains stem/progenitor cells. These endometrial stromal progenitor cells (hESCs) have been differentiated into a number of mesodermal lineages. There is limited information on differentiating hESCs into neurons, and no information on differentiating hESCs into megakaryocytes (MKs) and platelets (PLTs). The aim of this work was to investigate the possibility of differentiating hESCs into two distinct lineages: MKs, with subsequent PLT formation, and neurons.

We isolated hESCs from human endometrial tissue and cultured the cells for 4-6 passages. Before each differentiation experiment, the purity of hESCs was confirmed by flow cytometry analysis which revealed that the cells were highly positive (>95%) for CD90 and CD29 (stromal cell markers), and were negative (<1.3%) for CD45 and CD34 (hematopoietic cell markers).

For MKs differentiation, hESCs (1.8×10^5 cells/well) were cultured in serum-free medium either with (differentiation) or without (control) thrombopoietin (TPO, 50 ng/ml) for 18 days. MKs differentiation was analyzed by flow cytometry and confocal microscopy. PLTs were collected from culture medium supernatant from day10-18, and were defined by flow cytometry and functional study. Our results

show: 1) MKs were successfully generated as evidenced by expression of MK membrane markers (CD41a: 39 ± 3.0% and 1 ± 0.09%; CD42b: 28 ± 2.0% and 1.2 ± 0.06%, differentiation *vs.* control, n=3). 2) Immunocytochemistry analysis showed the differentiation rate of CD41a (38 ± 3.0%) and CD42b (27 ± 2.5%). 3) Generated PLTs were positively labeled with CD41a (90 ± 2%). 4) Functional study of generated PLTs revealed that thrombin (5 U/ml) stimulation up-regulated CD62P expression (26.0 ± 4% and 2.5 ± 1%, thrombin *vs.* control, n=3), and fibrinogen binding (32 ± 3.0% and 1 ± 0.4%, thrombin *vs.* control, n=3). 5) Electronic microscopic examination showed that generated PLTs had similar ultrastructure (storage granules) as normal human peripheral blood PLTs.

For neuron differentiation, hESCs (1.6×10^5 cells/well) were cultured in medium either with (differentiation) or without (control) cytokines (FGF, EGF, NGF and BDNF) for 14 days. Differentiated neurons were analyzed by confocal microscopy and western blot analysis. Our results show: 1) neurons were successfully generated as evidenced by phenotypic expression of neuron specific markers nestin (40 ± 4%), β-tublin III (37 ± 3%), NeuN (15 ± 2%), TH (14 ± 1%), and astrocyte marker GFAP (10 ± 3%). 2) Western blot analysis showed that the expression of transcription genes (Oct4: 0.11 ± 0.01and 0.32 ± 0.01; Sox2: 0.25 ± 0.02 and 0.52 ± 0.03, differentiated *vs.* control, n=4) was down-regulated in differentiated neurons.

Taken together, these data indicate that it’s feasible to generate MKs, functional PLTs and neurons from hESCs. The hESCs could be a potential source for cell-based therapy in regenerative medicine.

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LIST OF ABBREVIATIONS

hESCs	Human endometrial stromal progenitor cells
MK	Megakaryocyte
PLT	Platelet
DMEM/F12	Dulbecco's modified eagle medium nutrient mixture F-12 (ham)
IMDM	Iscove's modified dulbecco's medium
NPBM	Neural progenitor basal medium
TPO	Recombinant human thrombopoietin
BSA	Bovine albumin serum
FBS	Fetal bovine serum
BDNF	Brain derived neurotrophic factor
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
NGF	Nerve growth factor
GFAP	Glial fibrillary acidic protein
TH	Tyrosine hydroxylase
PI	Propidium iodide
DAPI	4, 6-diamidino-2-phenylindole dihydrochloride
TEM	Transmission electron microscopy

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I. INTRODUCTION AND PURPOSE

Structure of Human Endometrium

The endometrium, or uterine mucosa, presents the most inner layer of the uterine wall. It is comprised of an epithelial lining and underlying stroma. The surface (simple columnar) epithelial cells that line the uterine cavity are situated on a basement membrane. Deep to the surface epithelium are glands that extend the full thickness of the mucosa. The glands are separated from each other by a highly cellular connective tissue. Deep to the stroma is the muscular myometrium, which forms the middle and outer uterine wall.

The endometrium is structurally and functionally divided into two major zones, the upper functionalis layer and the lower basalis layer (Gargett, 2007) (**Fig1**). The functionalis layer is shed at menstruation and is subsequently regenerated each month from putative progenitor cells located in the remaining basalis zone (Padykula, 1991).

Endometrium of reproductive-age females is highly dynamic. It has a remarkable capacity for self-renewal, undergoing more than 400 complete cycles of regeneration, differentiation and shedding during the reproductive years (Gargett *et al*, 2008). After the endometrium is shed as part of the physiologic, normal 28-day menstrual cycle, it regenerates to a thickness of 4-7 mm within 4-10 days (McLennan & Rydell, 1965). The regenerative capacity of human endometrium is believed to be at least equivalent to many highly regenerative organs, such as epidermis, intestinal epithelium and bone marrow, where adult stem cells

replenish lost cells to maintain tissue homeostasis (Fuchs & Segre, 2000;Li & Xie, 2005).

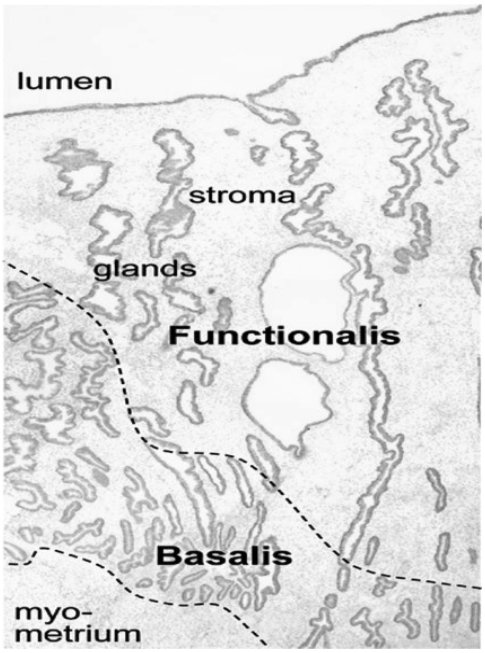

Fig 1. Diagram demonstrating the histologic structure of human endometrium. The endometrium is divided into two major zones: upper functionalis and lower basalis layers (Adapted from Gargett *et al*., 2007).

Presence of Stromal Progenitor Cells in Human Endometrium

The hypothesis that the regenerative ability of endometrium is mediated by adult stem cells or a progenitor cell population was proposed more than two decades ago. It was based on the evidence from histological studies of human and primate endometrium (Padykula *et al*, 1984; Padykula, 1991; Prianishnikov, 1978). However, initial evidence for stem/progenitor cells activity in human

endometrium was not reported until 2004. In that year, Chan and coworkers isolated stromal and epithelial stem cells exhibiting a high proliferative potential (Chan *et al*, 2004). Later, the same group identified the clonogenic epithelial and stromal cells in active, cycling and inactive endometrium (Schwab *et al*, 2005). Further evidence included that endometrial stromal cells are able to differentiate into multiple cell lineages. Wolff et al found that only the endometrial stromal cells can be differentiated into chondrocytes in a defined medium, as compared with other monolayer cells generated from myometrial, fibroid, fallopiantube, and uterosacral ligament tissue (Wolff *et al*, 2007). Most recently, The Dimitrov group reported that human endometrial stromal progenitor cells (hESCs) can be cultured for more than 15 passages and showed high clonogenic efficiency (15%) (Dimitrov *et al*, 2008). Gargett et al described that single endometrial stromal cell can form cellular colonies that can be further serially cloned. Further, it was shown that cloned stromal cells can be differentiated into mesodermal lineages including adipogenic, osteogenic, myogenic and chondrogenic cells in the presence of corresponding inducing factors (Gargett *et al*, 2009). Taken together, these findings strongly indicate that multipotent stromal stem/progenitor cells exist in human endometrium. The putative location of these cells is near blood vessels in the basalis layer of endometrium (Gargett, 2007;Schwab *et al*, 2005) (**Fig 2**).

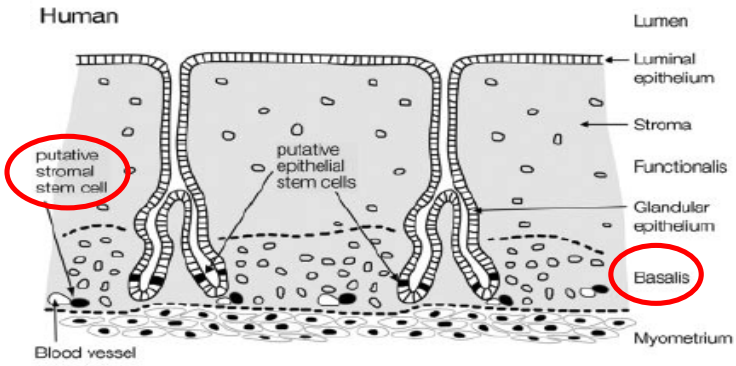

Fig 2. Diagram illustrating putative location of endometrial stem/progenitor cells. Dotted line designates separation between the functionalis and basal layers. Progenitor cells are located in the basal layer, near blood vessels (Adapted from Gargett *et al.,* 2007).

Menorrhagia: a Common Bleeding Disorder in Reproductive-age Females

Menorrhagia is defined as excessively heavy uterine bleeding during normal menstrual cycle intervals and corresponds in adult females to more than 80mL of blood loss per menstrual period. It is common among women of reproductive ages. Women with menorrhagia may experience diminished quality of life parameters, psychosocial issues in addition to developing anemia, diminished reproductive capacity and untoward pregnancy complications. It also places women with this disorder at an increased risk of having unnecessary hysterectomies and other gynecological procedures (Gould, 1995;Hickey & Fraser, 1995). Indeed, one-third women in the United States have hysterectomies before the age of 60 years and menorrhagia is the main

presenting problem in at least one-half of these women. Approximately one-half of women who have a hysterectomy for menorrhagia are found to have an anatomically normal uterus indicating that other causes are responsible for the heavy uterine bleeding (Duckitt & Collins, 2008).

Menorrhagia is a common symptom of von Willebrand disease (vWD) in adult presenting with menorrhagia (Lusher, 1996). Recent studies have demonstrated that menorrhagia also is prevalent in adolescent girls with inherited bleeding disorders, particularly in those with PLT function disorders (PFDs) (Chi *et al*, 2010;El-Hemaidi *et al*, 2007;Hossain *et al*, 2010). PFDs are inherited heterogeneous disorders. The most common PFD subtype, dense granule storage pool deficiency, is a heterogeneous disorder and is frequently found among patients with mild to moderate bleeding diatheses that do not have vWD. Because PLT dense granules contain proaggregation factors for the formation of a haemostatic plug, the overall consequence of poor PLT plug formation result in clinical bleeding disorders, of which menorrhagia is a common problem. Patients with the PFD subtype dense granule storage pool deficiency that experience menorrhagia refractory to medical therapy are in some cases treated with PLT transfusions. One objective of the thesis is to use *ex vivo* methods to generate MKs and PLTs from human endometrial stem/progenitor cells. The overall aim is to then evaluate them for use as a novel therapeutic modality in the treatment of menorrhagia in both adult and adolescent females, from all ethnic-ancestral backgrounds, with menorrhagia and documented platelet storage pool (dense granule) defects. If PLTs with normal dense granules can be generated from

patients in an *in vitro* culture system, it will represent an important first step in using the patients' own stem cells as a source of PLTs for autotransplantation or allotransplantation to treat PLT disorders.

MKs and PLTs

MKs are myeloid cells (constituting less than 1% of these cells) that reside primarily in the bone marrow (BM), and undergo a series of dynamic changes to become mature MKs (Nurden *et al*, 1997). Importantly, MKs are PLT precursor cells. Mature MKs are characterized morphologically by their large cell size, abundant cytoplasm, the presence of numerous intracellular granules and a distinctive demarcation membrane system (DMS). The DMS was originally thought to divide the MK cytoplasm into small fields where individual PLT would assemble to subsequently be released. PLTs are terminally differentiated cells that represent non-nucleated fragments of the MKs. PLTs have multifactorial roles. They are known to play a key role in haemostatic plug formation (Varga-Szabo *et al*, 2008). They also participate in arterial thrombosis (Davi & Patrono, 2007), the innate immune response and metastatic tumor cell biology (Kaushansky, 2009;van der Meer & Pietersz, 2005). PLTs circulate in the blood for a short life span of 7-10 days and need to be replaced in order to maintain normal levels in the blood (Hartwig & Italiano, Jr., 2003). The circulating concentration of PLTs is around 1.5 - 4.5 x 10^{11} per liter of blood in a healthy individual. The circulating PLT level is regulated by both PLT production and destruction. Megakaryocytopoiesis and thrombopoiesis are two essential

processes involved in PLT production (Kuter *et al*, 2001). Megakaryocytopoiesis starts with hematopoietic stem cells (HSCs) that undergo lineage commitment, proliferation and differentiation, all of which are regulated by various cytokines and growth factors. Thrombopoiesis is involved in the process of releasing functional PLTs into the circulation. Abnormalities in these processes can result in clinical bleeding disorders. For example, menorrhagia due to PLT disorders can be caused by either PLT deficiency (thrombocytopenia) or PLT dysfunction. PLT transfusion is widely used for treatment of various PLT-related diseases, such as thrombocytopenia and in cases of menorrhagia that are refactory to medical therapy (Blumberg *et al*, 2008;Kanbur *et al*, 2003;Nevo *et al*, 2007;Stephan *et al*, 1999). In the United States, the total number of PLT transfusions is over 10 million units per year and is increasing annually. This steady increase in demand challenges the US blood transfusion community. Moreover, the short storage life of PLT concentrates, the risk of pathogenic contamination, and the variability of quality and quantity of the donor-derived PLTs compromise the supply of PLTs from traditional donor-dependent method of harvesting PLTs (Matsunaga *et al*, 2006;Stevens *et al*, 2006). Thus, the search for developing an alternative approach to produce large numbers of PLTs is intensely appealing. To this end, producing PLTs from stem cells holds great promise as an alternative approach.

Generation of MKs and PLTs from Stem Cells *in vitro*

Thrombopoietin (TPO), also known as c-Mpl ligand, is a growth factor and a key physiological regulatory factor that induces MK proliferation and maturation (Alexander *et al*, 1996;Gurney *et al*, 1994;Kaushansky *et al*, 1994;Kaushansky, 1995). The *in vitro* use of TPO renders possible the generation of highly enriched MKs with normal structural features and function. Production of PLTs *in vitro* also has been attempted with the goal of using them for transfusions in patients with PLT disorders. Choi et al first demonstrated the feasibility of *in vitro* generation of functional PLTs by stimulating the release of PLTs from cultured MKs differentiated from human peripheral blood (Choi *et al*, 1995). After that, other investigators have successfully produced MKs and PLTs *in vitro* from HSCs and embryonic stem cells (Eto *et al*, 2002;Fujimoto *et al*, 2003;Gaur *et al*, 2006;Matsunaga *et al*, 2006;Takayama *et al*, 2008), as well as from progenitors of subcutaneous adipose tissue (Matsubara *et al*, 2009). The culture-derived PLTs exhibit similar features to PLTs from peripheral blood with respect to morphology and function. Chen et al reported that transplantation of generated MKs can accelerate the recovery of MKs and PLTs and reduce thrombocytopenia in mice model (Chen *et al*, 2009). Furthermore, some clinical trials have shown transplantation of *in vitro* expanded MK progenitors into patients can boost PLTs recovery (Bertolini *et al*, 1997; Matsunaga *et al*, 2006; Paquette *et al*, 2000; Scheding *et al*, 2004). Groups led by Eto and Fujimoto developed approaches to generate a large amount of PLTs *in vitro* from murine embryonic stem cells (Eto *et al*, 2002; Fujimoto *et al*, 2003). Currently, no one

has reported on MK and PLT production from human endometrial-derived stem cells.

Ischemic Stroke and Neuron Stem Cells

Stroke can cause permanent neurological damage and are associated with many complications. It is one of the leading causes of death and disability worldwide and has an incidence of approximately 150-200 in 100,000 (Modan & Wagener, 1992). There are 2 types of stroke: ischemic stroke and hemorrhagic stroke. Ischemic stroke is caused by occlusion of a blood vessel in the brain and constitutes the majority of stroke cases (85-90%) (Qureshi *et al*, 2001). Neurons are the core components of the nervous system. They are electrically excitable cells that process and transmit information to the central nervous systems via various electrical and chemical mechanisms. The nerve system has a limited capacity for self repair owing to the lack of mature neurons to undergo cell division. In most cases, mature neurons are regenerated by adult neural stem cells, which are found mainly in 2 regions in brain: subventricular zone (SVZ) of the lateral ventricles (LV) and subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Lois & varez-Buylla, 1994). Even though adult neural stem cells have the capacity to generate new functional neurons, their regeneration is limited. This is in part due to newly generated neuron stem cells having to migrate long distances from the site of origin to the site of damage and subsequent repair. It has been reported that more than 80% of these immature neurons die before they reach the damaged region during the first week after

stroke, and only a small fraction of damaged neurons can be replaced (Arvidsson *et al*, 2002). Therefore, transplantation of *in vitro* generated neurons is a potential avenue for promoting cerebral repair after neuron damage.

Generation of Neurons from Stem Cells *In vitro*

Stem cells can differentiate into neural lineages that can in turn replace lost neurons. Preclinical experimental studies have included the application of human stem cells from various sources including BM stromal cells, embryonic stem cells, and adipose tissue progenitor cells (Deng *et al*, 2001; Lenka *et al*, 2002; Ning *et al*, 2006; Sanchez-Ramos *et al*, 2000; Woodbury *et al*, 2000). Transplanted embryonic neurons can survive and ameliorate neurological deficits in Parkinson's and stroke models (Hicks *et al*, 2009; Kim *et al*, 2002; Rodriguez-Gomez *et al*, 2007; Takagi *et al*, 2005), respectively. A recent study indicated that transplantation of dopaminergic neurons generated from endometrium could rescue dopamine concentrations in a Parkinson's animal model (Wolff *et al*, 2011). Furthermore, in a clinical trial, transplanted embryonic dopamine neurons have been shown to survive *in vivo*, leading to promising clinical success (Freed *et al*, 2001).

Taken together, recent studies on generating MKs, PLTs and neurons from stem cells is of increasing interest, because of the promising role of MKs and PLTs in transfusion medicine and neurons in transplantation therapy. Stem cells sources include embryonic stem cells (Fujimoto *et al*, 2003; Gaur *et al*, 2006; Nishikii *et al*,

2008; Takayama *et al*, 2008) and HSCs from BM, peripheral blood (PB) and cord blood (CB) (Mattia *et al*, 2002; Proulx *et al*, 2003; Shim *et al*, 2004). However, there are several strengths and limitations of these sources. Human embryonic stem cells provide the feasibility of obtaining abundant of MKs, PLTs and neurons. However, ethical and political issues, potential for tumor formation and the sophisticated experiment technique inherent in their production are formidable obstacles. The limited proliferative ability of HSCs also has been pointed out as a limitation. All of these issues hamper the widespread use of these sources.

Recently endometrial tissue has been identified as a source of multipotent cells that are capable of differentiating into smooth muscle cells, adipocytes, chondrocytes, and osteoblasts (Gargett *et al*, 2009). hESCs can be relatively simply isolated and maintained in cell culture. Further, hESCs can be used as an autologous stem cell source, thereby obviating concerns regarding graft rejection in humans. Currently, there are no reports on generating MKs and PLTs from hESCs. And only one recent publication has even described the possibility of generating neurons from hESCs with combinational inducers (chemical compound and cytokines) (Wolff *et al*, 2011). In the present study, we explored whether hESCs could be differentiated into two distinct cell lines, MKs/PLTs and neurons, in *in vitro* culture systems.

II. HYPOTHESIS AND SPECIFIC AIMS

Hypothesis:

It is hypothesized that human endometrial stromal progenitor cells(hESCs) could be differentiated into MKs/PLTs or neurons in *in vitro* culture systems.

Specific Aims:

Aim-1: To set up a method to culture stromal progenitor cells from human endometrial tissue.

Aim-2: To investigate the feasibility of differentiating hESCs into MKs in an *in vitro* culture system and to evaluate the function and morphology of PLTs released from the generated MKs.

Aim-3: To explore the possibility of neuron differentiation from hESCs in an *in vitro* culture system.

III. EXPERIMENT DESIGN

Aim-1:

Endometrial tissues were collected from selected patients and transferred to the laboratory. The endometrium layer was then dissected, minced, and enzymatically digested into a single-cell suspension. The hESCs were cultured for 4-6 passages and identified by immunocytochemistry and flow cytometry analyses.

Aim-2:

Passages 4-6th of hESCs were cultured in serum-free medium with TPO (50 ng/ml) (differentiation group) or without (control group) for to investigate their ability to differentiate into MKs and subsequently generate PLTs. Generated MKs were identified by flow cytometry and immunocytochemistry methods. Produced PLTs were collected from culture medium and analyzed by flow cytometry. The produced PLTs were characterized functionally by thrombin stimulation study coupled with flow cytometry analysis and electronic microscopic examination.

Aim-3:

hESCs were cultured in medium with (differentiation group) or without (control group) cytokines (10 ng/ml EGF, 10 ng/ml FGF, 10 ng/ml NGF, 15 ng/ml BDNF) for investigating their ability for differentiating into neurons. Generated neurons were determined by immunocytochemistry and western blot analysis of the expressions of transcriptional genes (Oct4 and Sox2).

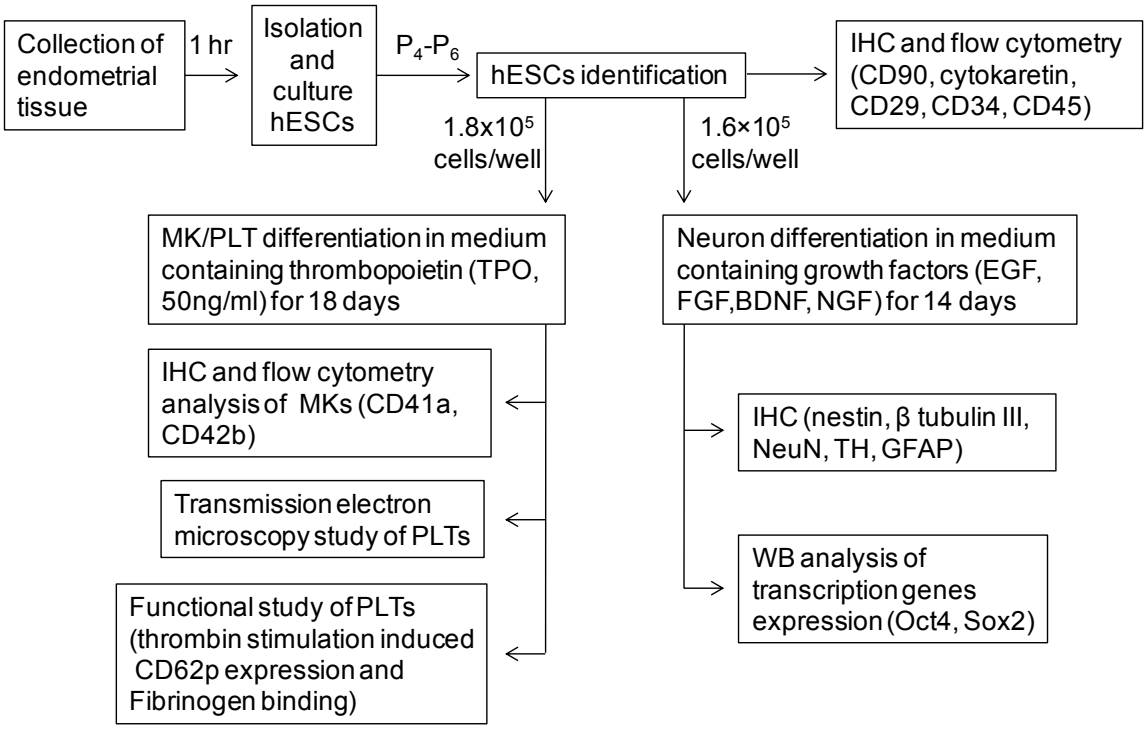

Fig 3. The flow chart shows our experiment protocol. IHC: immunocytochemistry; WB: western blotting.

MATERIALS AND METHODS

Materials

Cytokines

TPO and BDNF were purchased from BD Biosciences (San Jose, CA). FGF, EGF and NGF were purchased from Lonza (Walkersville, MD).

Antibodies

PE-conjugated mouse anti-human CD90, PE-conjugated mouse anti-human CD29, FITC-conjugated mouse anti-human CD45, FITC-conjugated mouse anti-human CD34, PE-conjugated mouse IgG1, FITC-conjugated mouse IgG1, FITC-conjugated mouse anti-CD62P, purified mouse anti-human CD41a, and purified mouse anti-human CD42b were purchased from eBioscience (San Diego, CA). FITC-conjugated mouse anti-human CD41a, FITC-conjugated mouse anti-human CD42b, FITC mouse IgG1 isotype control, and mouse anti-CD90 antibody were purchased from BD Biosciences (San Jose, CA). Mouse anti-Sox2 was purchased from R&D systems (Minneapolis, MN). Rabbit anti-Oct4 was purchased from Abcam Inc (Cambridge, MA). Cy3-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-rabbit, FITC-conjugated goat anti-rabbit, FITC-conjugated goat anti-mouse, and HRP-conjugated antibodies were purchased from Jackson ImmunoResearch Lab (West Grove, PA). Mouse anti-cytokeratin antibody was purchased from Dako (Carpinteria, CA). Nestin, anti-tubulin, NeuN, and TH were purchased from Millipore (Temecula, CA). GFAP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-labeled human fibrinogen, Alexa Fluor 488-conjugated mouse IgG isotype control, and flow cytometry size calibration kit were purchased from Invitrogen (Carlsbad, CA). β-actin was purchased from Sigma (Fairfax, VA).

Reagents

DMEM/F12, IMDM, iron saturated transferrin, L-glutamine, dNTP set, FBS, antibiotic-antimycotic solution, trypsin, and HEPEs were purchased from Invitrogen (Carlsbad, CA). NPBM was purchased from Lonza (Walkersville, MD). 70 μM wire sieve was purchased from BD Biosciences (San Jose, CA). DNAse1, Human insulin, BSA, 2-β-mercaptoethanol, nucleotide kit, PI, RNAase, thrombin, glutaraldehyde, propylene oxide, and spurr resin 812 were purchased from Sigma (Fairfax, VA). Collagenase was purchased from Roche Applied Science (Indianapolis, IN). Uranyl acetate and lead citrate were purchased from Fisher Scientific (Pittsburgh, PA). Osmium tetroxide was purchased from Electron Microscopy Science (Hatfield, PA).

Methods

Harvesting Human Endometrium Tissue Sample

Premenopausal women (30-45 years old) who were undergoing hysterectomy for benign disease indications, leiomyoma, were selected. Informed consent was obtained by each patient and patient confidentiality was protected according to HIPPA guidelines. Ethics approval was obtained from the Institutional Review Boards and Research Committees at the Miami Valley Hospital and Wright State University Research Committee for this study.

Endometrium tissues were obtained for isolation and culture of hESCs. In brief, after the uterus was removed from the patient, a small tissue sample with full thickness endometrium attached to myometrium was dissected and collected immediately. The tissue sample was placed into 20 ml pre-chilled phosphate buffer saline (PBS) buffer containing 10% FBS and 2% antibiotic-antimycotic solution (final concentration: 200 μg/ml penicillin G sodium and 200 μg/ml streptomycin sulfate) and was processed within 30 - 60 min.

Isolation and Culture of hESCs

The endometrium tissue was mechanically and enzymatically digested into a single-cell suspension as previously reported with slight modifications (Chan *et al*, 2004;Dimitrov *et al*, 2008). Briefly, the tissue sample was washed twice with washing buffer (PBS with 25 μM HEPEs and 1% antibiotic-antimycotic solution) to remove excess blood. Then the endometrium layer was dissected from the underlying myometrium, minced into 1-2 mm^3 pieces with a sharp surgical scissor, digested in enzyme digestion buffer (PBS with 25 μM HEPEs, 1mg/ml collagenase, 0.1mg/ml DNAse1 and 1% antibiotic-antimycotic solution) in a humidified 5% CO_2 / 95% air atmosphere at 37°C for 45 min with a brief shaking once every 5 min. Then cell suspension was filtered through a 70 μm wire sieve to remove mucus and undigested tissue. The filtrates were centrifuged at 400 × g for 10 min to collect cells. 0.9 ml ammonium chloride solution was added to lyse excess erythrocytes for 10 min on ice, followed by centrifugation at 400 × g for 10 min, the cell pellets were resuspended with hESCs culture medium (DMEM/F12

supplemented with 10% FBS and 1% antibiotic-antimycotic solution). Cells were plated in 6-well tissue culture plates, allowed to adhere overnight in a humidified 5% CO2/95% air atmosphere at 37°C and cultured as monolayer cells.

For passaging, when cells reach 90% confluence, they were treated with 1.5 ml/well 0.025% trypsin/0.01% EDTA at 37°C for 7 min, then an equal volume of complete DMEM/F12 with 10% FBS medium was added to deactivate the enzyme reaction. Cells were then pelleted. Supernatant was discarded. Pelleted cells were resuspended with hESCs culture medium, split in a 1:3 ratio and cultured until the 4^{th} passage. The culture medium was replaced with fresh warm medium every 2-3 days.

Identification Study of hESCs

Cultured cells at the 4^{th} passage were analyzed by indirect immunocytochemistry and flow cytometry as previous described (Dimitrov *et al*, 2008;Gargett *et al*, 2009).

In brief, for immunocytochemistry staining, a proportion of cells were seeded on coverslips and incubated overnight. After 2 washes with PBS, the cells were fixed with 2% paraformaldehyde (PFA) for 20 min, treated with blocking buffer (PBS with 3% BSA and 3% normal donkey serum) for 1hr at room temperature (RT). Subsequently the cells were incubated with primary monoclonal antibodies mouse anti-human CD90 (Thy-1) (1:25) or mouse anti-human cytokeratin (1:50) overnight at 4ºC. Following by 3 washes with PBS/0.1% BSA, the cells were incubated with secondary antibody, Cy3-conjugated donkey anti-mouse IgG

(1:250), for 1 hr at RT. Washed with PBS/0.1% BSA 3 times (3 min each), mounted with fluorescence mounting medium and imaged under a fluorescence microscope (Leica, Germany).

For flow cytometry analysis, cells were trypsinized at 90% confluence as above described, centrifuged at 400 × g for 10 min. Cell pellets were resuspended and incubated with either PE-conjugated anti-human CD29 and FITC-conjugated anti-human CD45, or PE-conjugated anti-human CD90 and FITC-conjugated anti-human CD34 for 30 min at 4ºC in the dark, individually. Isotype matched (IgG) non-specific antibodies served as negative controls. Final volume per tube was brought to 100 µl for flow cytometric analysis using Accuri C6 flow cytometer and CFlow Plus Analysis software (Ann Arbor, MI). Cells were selected for analysis on the basis of their forward versus side scatter profile to exclude dead cells and debris. All antibody application concentrations followed manufacture instructions and guidelines.

Differentiation of MKs and PLTs from hESCs

In order to differentiate hESCs into the MK lineage, the 4^{th} passage of hESCs were trypsinized with 0.025% trypsin/0.01% EDTA, centrifuged at 400 × g for 10 min, counted using a hemacytometer, and seeded as1.8×10^5 cells/well on 6-well tissue culture plates or coverslipes with hESCs culture medium overnight. Then culture medium was removed and replaced with fresh induction IMDM medium supplemented with 50 ng/ml TPO, 0.5% BSA, 200 µg/ml iron saturated transferring, 10 µg/ml insulin, 2 mM L-glutamine, 4 µg/ml LDL cholesterol, 50 µM

2-â-mercaptoethanol, 20 μM each nucleotide, 20 μM dNTP and 1% antibiotic-antimycotic solution. Cells were cultured for 18 days. One half of the culture medium was replaced in the first 10 days, then all of the culture medium was changed from day 10-18 to collect PLTs from the medium supernatant. Control cells were cultured in complete culture medium (IMDM with 10% FBS and 1% antibiotic-antimycotic solution) without TPO for 18 days. Cells on coverslips were used for MK immunocytochemistry studies.

Characterization Study of MKs Differentiated from hESCs

MKs generated from hESCs were determined by immunocytochemistry and flow cytometry analyses.

For immunocytochemistry, cells on coverslips were fixed with 2% PFA for 20 min at RT, washed with PBS/0.1% BSA 3 times, permeabilized with goat serum blocking buffer (PBS/0.1% BSA and 0.3% triton-100) for 30 min at RT, then incubated with prediluted purified mouse anti-human CD41a (glycoprotein αIIb) and purified mouse anti-human CD42b (glycoprotein αIb) primary antibodies overnight at 4°C. The cells were washed 3 times (5 min each), then incubated with secondary antibody FITC-conjugated goat anti-mouse (1:100), individually, followed by another 3 washes, the cells were incubated with PI/PBS solution in the presence of RNAse (PI/RNAse final concentration: 20 μg/ml) for 15 min at RT in the dark, then mounted with fluorescence mounting solution and imaged under a confocal fluorescence microscope (Leica TCS SP2, Leica, Germany). To

assess the differentiation rate, we counted the number of cells staining positive with a given antibody in 5 individual fields. Control cells were stained concurrently.

For flow cytometry, the cells were detached with 0.025% trypsin/0.01% EDTA, centrifuged at 800 × g for 10 min to pellet the MKs. MKs were then resuspended and fixed with 2% PFA for 20 min at RT, washed with PBS/0.1% BSA 3 times, centrifuged at 800 × g for 10 min, permeabilized with blocking buffer (PBS/0.1% BSA and 0.3% triton X-100) for 30 min at RT. This was followed by incubation with fluorescence conjugated antibodies, FITC-conjugated anti-CD41a, FITC-conjugated anti-CD42b and FITC-conjugated mouse IgG1 isotype control, for 30 min at 4°C in the dark, respectively. The cells were then washed with PBS/0.1% BSA, centrifuged and incubated with 100 µl PBS/0.1% BSA buffer with PI solution (20 ug/ml) containing RNAase (20 ug/ml) for 30 min at RT in the dark. Samples were analyzed by an Accuri C6 flow cytometer and CFlow Plus Analysis software. $CD41a^{+}$cells were defined as MKs and were gated for MK ploidy analysis. All antibody application concentrations followed manufacture instructions and guidelines. Control cells were analyzed simultaneously.

Characterization Study of PLTs Generated from hESCs

PLTs released from generated MKs were defined by flow cytometry analysis. Briefly, culture medium was gently collected from day 10-18 with a 15 ml centrifuge tube and centrifuged at 150 × g for 20 min to eliminate any large cells. The supernatant was fixed with 2% PFA for 20 min at RT, centrifuged at 1700 × g

for 30 min to sediment a PLT pellet. When storing PLTs, the pellet was resuspended with PBS/1% FBS, and stored at 4°C for further analysis. Then fixed and stored PLT pellet were combined, resuspended and incubated with FITC-conjugated anti-CD41a antibody and FITC-conjugated mouse IgG1 isotype control for 30 min at 4°C in the dark, individually. The total volume per tube was brought to 100 µl with PBS/0.1% BSA. 2 µm and 4 µm flow cytometry calibration bead solution was added into each tube. All samples were analyzed using the Accuri C6 flow cytometer. The application concentrations of calibration beads and antibodies followed manufacture instructions and guidelines.

Functional Study of Generated PLTs with Thrombin Stimulation

The functional capabilities of generated PLTs were analyzed for both fibrinogen binding and surface antigen CD62P expression in the presence or absence of agonist thrombin.

To prepare cell samples, PLTs were collected as described above. The PLT pellets were resuspended in a modified Tyrode-HEPEs buffer (138 mM NaCl, 0.36 mM NaH_2PO4, 2.9 mM KCl, 12 mM $NaHCO_3$, 10 mM HEPEs, 5 mM glucose, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4).

The ability of fibrinogen to bind surface receptors on activated PLTs is a prerequisite for PLT aggregation. To determine fibrinogen binding, PLTs were stimulated by thrombin (5U/ml) for 10 min at 37°C without stirring in the presence of Alexa Fluor 488-labeled human fibrinogen (100 µg/ml) or isotype control mouse IgG, respectively. A control group of PLTs underwent concurrent testing.

The control PLTs were stained with Alexa Fluor 488-labeled fibrinogen or isotype control antibodies without thrombin stimulation. All sample mixtures were then diluted with HEPEs buffer. The final volume each sample was brought to 100 µl. All samples were analyzed by Accuri C6 flow cytometer.

The agonist thrombin is responsible for PLT activation and is measured as CD62P expression. To determine the surface expression of P-selectin (CD62P), PLTs were stimulated with 5 U/ml thrombin for 10 min at 37°C, then fixed with 2% PFA for 30 min at RT, washed twice with HEPEs buffer, incubated with either FITC-conjugated anti-CD62P or FITC-conjugated mouse IgG isotype control for 30 min at 4°C in the dark. A control group of PLTs underwent concurrent testing. The control PLTs were fixed without thrombin stimulation and stained. The final volume each sample was brought to 100 µl. All samples were analyzed by Accuri C6 flow cytometer. All antibody application concentrations followed manufacture instructions and guidelines.

Morphologic Study of PLTs by TEM

Culture medium containing PLTs released from generated MKs was gently collected and centrifuged at 150 × g for 20 min to remove nucleated cells. . Control samples of PLTs were obtained from human peripheral blood samples. Peripheral blood was collected from normal patient using standard phlebotomy techniques into a collection tube containing acid-citrated-dextrose (ACD) solution. The control blood sample was centrifuged at 150 × g for 20 min in order to obtain platelet-rich plasma (PRP). The supernatant and PRP were added with equal

volume of 0.5% glutaraldehyde in 0.1 M phosphate buffer, then centrifuged at 1000 × g for 10 min to pellet PLTs. The pellets were then fixed with 500 µl 2% glutaraldehyde in 0.1 M phosphate buffer for 1 hr at 4°C and centrifuged again at 1000 × g for 10 min. The samples were washed with 0.1 M phosphate buffer 3 times (1 min each), fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 2 hrs at 4°C and washed another 3 times (1 min each), then dehydrated with a graded ethanol series (50%, 75%, 95%, 100%) for 5 min each. Subsequently, the samples were treated with 100% propylene oxide 2 times (5 min each), and then treated with 1:2 ratio of spurr resin 812 and propylene oxide, followed by a 2:1 ratio of spurr resin 812 and propylene oxide for 2 hrs. Finally, the samples were embedded in beam capsules with spurr resin 812 according to the manufacture's instruction, baked at 60°C in a hot air oven overnight. Ultrathin sections (60-80 nm) were prepared with MT7000, mounted on 300-mesh copper grids, stained with 2% uranyl acetate for 5 min, rinsed with double distilled water for 5 times, and allowed to dry. The specimens were then stained with lead citrate for 10 min (REYNOLDS, 1963), rinsed with double distilled water another 5 times and allowed to air dry. Finally, all specimens were examined with an EM 208 (Philips) transmission electron microscope at an accelerating voltage of 70 KV.

Differentiation of Neurons from hESCs

To differentiate hESCs into the neuron lineage, the 4^{th} passage of hESCs were trypsinized as noted in the previous sections and cultured at a density of 1.6 × 10^5 cells/well on fibronectin coated 6-well tissue culture plates or coverslips with

hESCs culture medium overnight. The culture medium was then removed and replaced with fresh NPBM medium supplemented with growth factors including 10 ng/ml FGF, 10 ng/ml EGF, 10 ng/ml NGF,15 ng/ml BDNF, and 0.2 mM L-glutamine and 1% antibiotic-antimycotic solution. Cells were cultured for 14 days. Control cells were cultured simultaneously in control culture medium (DMEM/F12 with 10%FBS and 1% antibiotic-antimycotic solution) without cytokines for the same time period as the study cells.

Immunofluorescence Staining of Generated Neurons

The cells on coverslips were fixed with 2% PFA, washed with PBS, permeabilized with blocking buffer (goat serum or donkey serum with PBS/0.1% BSA and 0.3% triton X-100) for 30 min at RT, then incubated with rabbit anti-nestin (1:200), mouse anti-β tubulin III (1:50), mouse anti-NeuN (1:50), rabbit anti-TH (1:1000), rabbit anti-GFAP (1:25) monoclonal antibodies overnight at 4°C in the dark, respectively. After 3 times washes, cells were incubated with corresponding secondary antibody Cy3-conjugated donkey anti-rabbit/mouse (1:250) or FITC-conjugated goat anti-mouse/rabbit (1:100) for 1 hr at RT, individually. Subsequently, cells were washed 3 times, incubated with 1 ml DAPI solution (final concentration: 0.4 μg/ml) for 5 min, mounted with fluorescence mounting solution and imaged under a confocal fluorescence microscope. To assess the differentiation rate, we counted the number of cells staining positive with a given antibody in 5 individual fields. Controls were imaged simultaneously.

Western Blot Analysis of Transcription Gene Expression in Differentiated Neuronal Cells

Protein samples were obtained from undifferentiated hESCs and differentiated neuronal cells following treatment with lysis buffer. Briefly, culture medium was removed from both groups. Cells were washed with PBS 3 times (1 min each). The cells were then scraped off the culture plates in 1 ml PBS, transferred to1.5 ml tubes, and centrifuged at 400 × g for 10 min. 50 µl ice-cold lysis buffer containing protease inhibitor was added to each cell pellet. Samples were centrifuged at 10,000 × g for 5 min. The supernatants were collected as protein. Protein concentrations were determined by the Bradford method using Bio Rad reagent (Bio Rad Laboratories, Hercules, CA). For gel loading, the final sample volume was corrected to 30 µg of total protein. To each of the samples, 10 µl of loading buffer (40% glycerol, 50% Tris-HCL, 10% SDS, bromophenol blue, 0.4% β-mercaptoethanol) was added to samples and boiled for 5 min. Then samples were loaded onto 10% Tris-Glycine SDS-Page gel (Bio Rad Laboratories, Hercules, CA). The gel was electrophoresed at 100 V, 50 mA till the dye came close to the bottom of the gel. Proteins were transferred from the gel to a PVDF membrane overnight at 10 V, 50 mA. Then the PVDF membrane was blocked with 8 ml 5% non-fat milk in 1 × TBS-T buffer for 1 hr at RT. The primary monoclonal antibody rabbit anti-Oct4 (1:500) was probed and incubated overnight at 4°C. The membrane was washed with1× TBS-T buffer for 3 times (5 min each), then probed with secondary antibody horse raddish peroxidase (HRP) conjugated goat anti rabbit (1:4000) for 1 hr at RT, washed with 1× TBS-T for 3

times more, and viewed via the chemiluminescence produced by the ECL substrate (Pierce) and captured by the Fuji LAS3000 imager (Japan). Subsequently, the membrane was washed with 1× TBS-T buffer another 3 times (5 min each), then stripped with stripping buffer for 15 min at RT, blocked with non-fat milk, incubated with primary monoclonal antibody mouse anti-Sox2 (1:2000) overnight at 4°C, probed with secondary antibody HRP-conjugated donkey anti mouse (1:4000), washed and imaged as described above. β-actin was used to determine relative amounts of protein of interest (POI) as follows: normalized value: ($POI_{density}$ - $Background_{density}$) / ($Actin_{density}$ - $Background_{density}$). To do this, monoclonal antibody mouse anti β-actin (1:4000) and secondary antibody HRP-conjugated donkey anti mouse (1:40000) were used. Values were not excluded from the final data calculations unless one of the bands was missing. This method of simultaneous probing is well-established and has been described previously (Mouihate *et al*, 2002).

Statistic Analysis

Results were described as mean ± SEM from at least three independently conducted experiments, unless otherwise specified. The p value < 0.05 was considered to be statistically significant in the paired t-test. All experiments were repeated in triplicate and were all reproducible.

IV. RESULTS

1. Morphology of hESCs

Three days after plating, the cultured cells grew and formed small colonies (**Fig 4A**). Then small colonies gradually formed large colonies (**Fig 4B**) and eventually formed a monolayer at approximately days 8-10 of culture (**Fig 4C**).

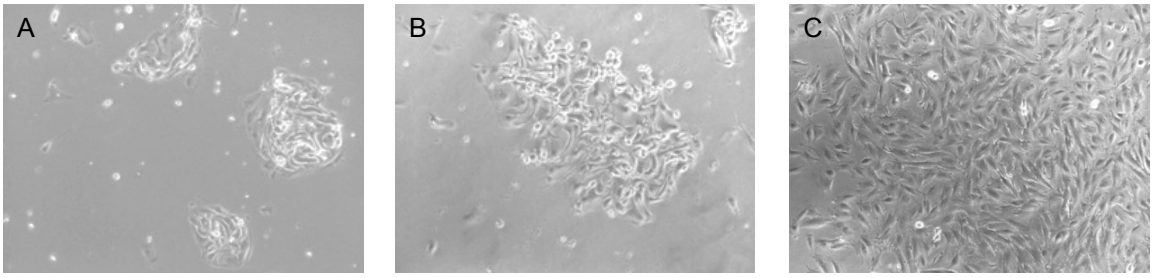

Fig 4. Morphological study of hESCs under light microscope. Representative images of cultured cells: small colonies (A), large colonies (B), and confluence hESCs (C). All magnification: 10 ×.

2. Characterization of hESCs

After the 4th passage culture, the hESCs were identified by immunocytochemistry and flow cytometry. Using flow cytometry, the cultured cells highly positively stained with stromal cell specific markers CD90 (**Fig 5A)** and CD29 (**Fig 5B**), however, negatively stained with hematopoietic lineage markers CD45 (**Fig 5C**) and CD34 (**Fig 5D**). Summarized data (**Fig 5E**) demonstrated the percentages of

phenotypic expression of CD90 (95.2 ± 2%, n=3), CD29 (96.4 ± 3%, n=3), CD45 (1.2 ± 0.05%, n=3), and CD34 (1.15 ± 0.08%, n=3).

Immunocytochemistry revealed the cultured cells exclusively positively stained CD90 (stromal marker) (**Fig 5F**), but negatively stained cytokeratin (epithelial marker) (**Fig 5G**).

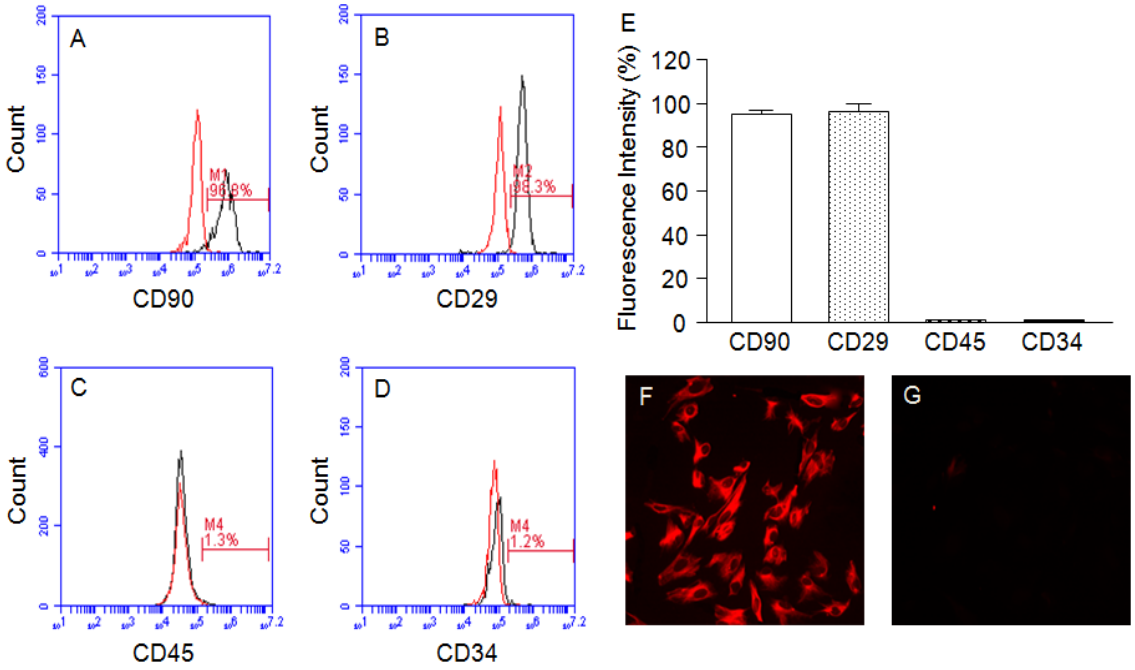

Fig 5. hESCs identification. Flow cytometric representative plots: A, CD90 expression; B, CD29 expression; C, CD45 expression; D, CD34 expression (Black curve: antibodies; Red curve: isotype control); E, summarized data. Data was analyzed as mean ± SEM, n=3, respectively. Immunocytochemistry data: F, CD90 staining; G, cytokeratin staining (Magnification: 20 x).

3. Characterization of hESCs-derived MKs by Immunocytochemistry Analysis

After 7-10 days of differentiation, generated MKs could be visualized under the light microscope (**Fig 6A**). Upon immunofluorescence staining, the generated MKs positively expressed CD41a (**Fig 6B1- 6B3)** and CD42b (**Fig 6C1- 6C3**). Summarized data (**Fig 6D**) demonstrated the percentages of phenotypic expression of CD41a (38 ± 3% and 1 ± 0.5%, differentiation group *vs.* control group, $P<0.01$, n=3), and CD42b (27 ± 2.5% and 0.9 ± 0.4%, differentiation group *vs.* control group, $P<0.01$, n=3) in generated MKs.

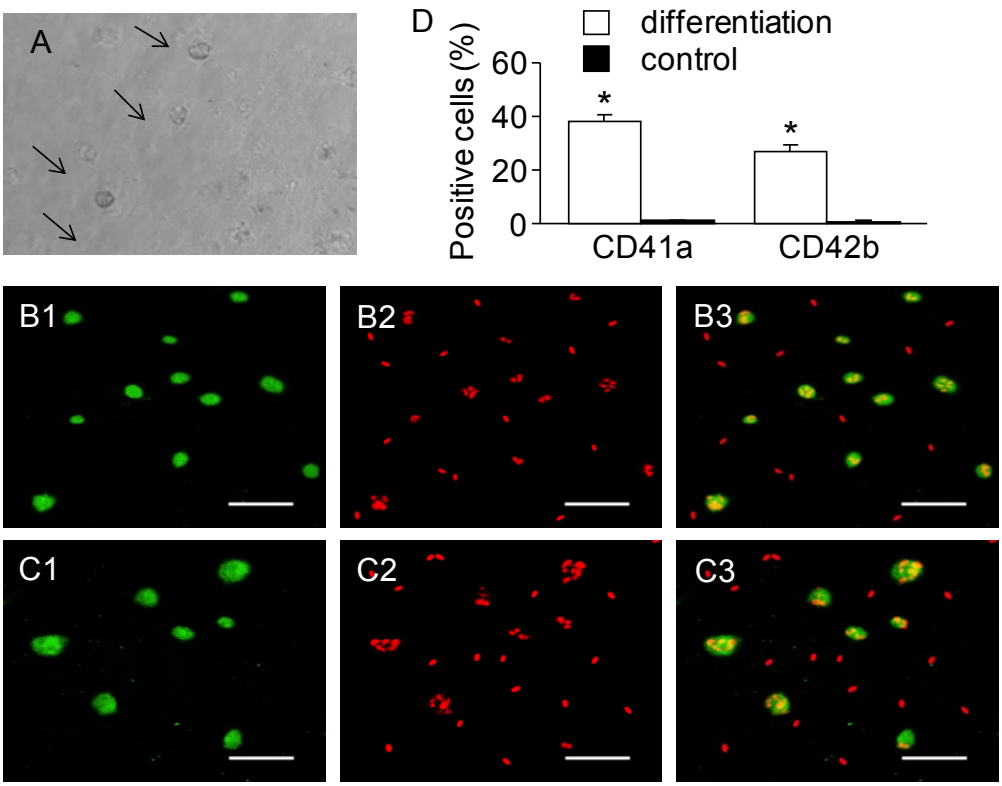

Fig 6. Characterization of hESCs - derived MKs by immunocytochemistry. A, generated MKs can be observed under light microscope after 7-10 days differentiation. B1, CD41a staining, C1,CD42b staining; B2-C2, PI staining; B3-C3, merged; scale bar: 75 μm. D, summarized data shows MKs marker expression on differentiation and control cells. Data was analyzed as mean ± SEM, *$P < 0.01$, n=3.

4. Expression of Surface Protein Markers for hESCs-derived MKs by Flow Cytometric Analysis

The flow cytometric data showed the positive expression of CD41a (**Fig 7A**), and CD42b (**Fig 7B**). Summarized data (**Fig 7C**) demonstrated the positive expression percentages of CD41a (39 ± 3% and 1 ± 0.09%, differentiation group *vs.* control group, $P<0.01$, n=3), and CD42b (28 ± 2% and 1.2 ± 0.06%, differentiation group *vs.* control group, $P<0.01$, n=3). This MK percentage values closely paralleled to those obtained from immunocytochemistry analysis. They were also consistent with the developmental scheme, because CD41a is expressed throughout megakaryocytopoiesis, while CD42b is expressed during the later stages of MK lineage developmental (Tomer, 2004). The number of MKs produced was approximately 5.5×10^4 MKs ($CD41a^+$) versus 1.8×10^5 hESCs.

Undifferentiated hESCs (control) were diploidy and the DNA ploidy was 2N (**Fig 7D**). Generated MKs were polyploidy and the DNA ploidy ranged from 2N to16N (**Fig 7E**).

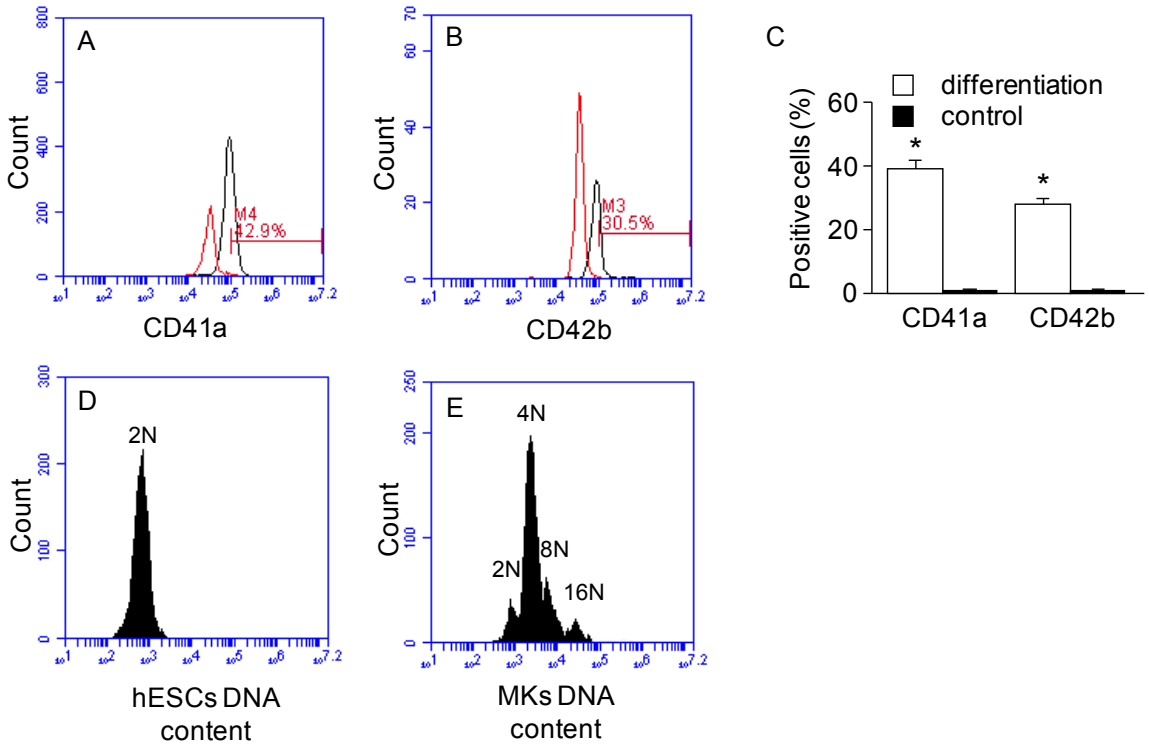

Fig 7. hESCs-derived MKs surface protein expression analysis by flow cytometry. Representative plot of CD41a expression (A), and CD42b expression (B) (Black curve: antibodies; Red curve: isotype control); Summarized data (C) shows MK markers expression on differentiation and control cells. Data was analyzed as mean ± SEM, *P<0.01, n=3. Plot D shows DNA content of undifferentiated hESCs; Plot E shows the polyploidy of generated MKs.

5. Expression of Surface Protein Markers for PLTs by Flow Cytometric Analysis

Culture-derived PLTs were collected from the culture supernatant and incubated with fluorescence-conjugated antibody CD41a as described in the Materials and Methods section. The diameter of the PLTs is approximately 2-3 μm. A gate was fixed in the forward scatter and side scatter histogram by using of 2 μm and 4 μm calibration beads (**Fig 8A**). Collected PLTs were expressed with CD41a (90 ± 2%, n=3) within the fixed gate P1 (**Fig 8B**). The number of PLTs produced was approximately 3 x 10^5 PLTs versus 1.8 x 10^5 hESCs. PLT numbers were reported as a cumulative number up to the point of analysis. Summarized data (**Fig 8C**) showed the MK yield per input of hESC was approximately 0.31 ± 0.01(n=3), the MK yield per input of hESC was approximately 1.7 ± 0.25 (n=3).

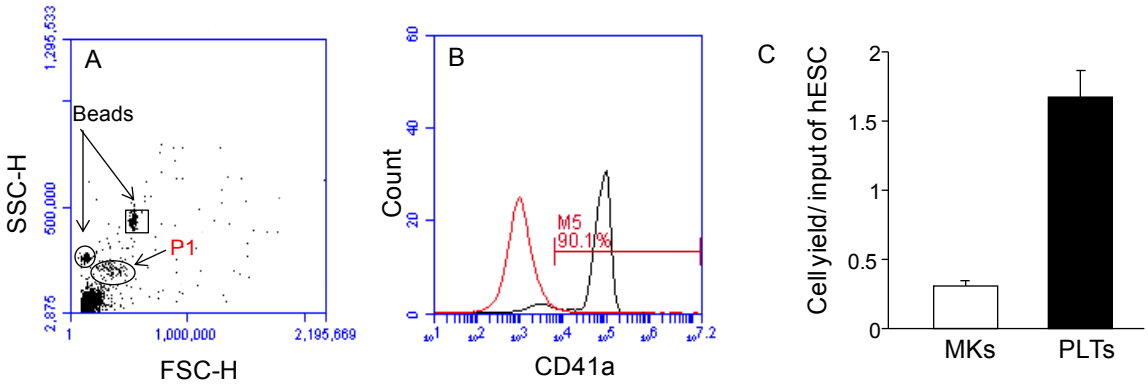

Fig 8. Generated PLTs surface protein expression analysis by flow cytometry. A, representative plot shows the population of PLTs (oval); 2 μm (circle) and 4 μm (square) calibration beads were used as internal size standards; B, CD41a expression in the cells gated on P1 (black curve, red curve: isotype control). C, summarized data of MK and PLT yield per input of hESC. Data was expressed as mean ± SEM, n=3.

6. Expression of Activation-dependent Antigens on Generated PLTs

Normal functional PLTs, express certain cell surface antigens like CD62P and fibrinogen receptor (αII_bIII_a) when activated. P-selectin, presents on the surface of the α-granules of resting PLTs, and is mobilized to the plasma membrane after activation. The PLTs surface protein complex fibrinogen receptor αII_bIII_a changes confirmation after PLTs activation in its conversion to a functional fibrinogen receptor.

Our flow cytometric data showed that the generated PLTs expressed CD62P (**Fig 9A**) and exhibited fibrinogen binding capability (**Fig 9C**). Summarized data (**Fig 9B**) demonstrated the up-regulation of CD62P expression (26 ± 4 % and 2.5 ± 1%, thrombin stimulation group *vs.* control group, $P<0.01$, n=3) after thrombin stimulation. Summarized data (**Fig 9D**) showed the raise of fibrinogen binding (32 ± 3 % and 1 ± 0.4 %, thrombin stimulation group *vs.* control group, $P<0.01$, n=3) after thrombin stimulation. These results indicated that generated PLTs have the ability to become active PLTs.

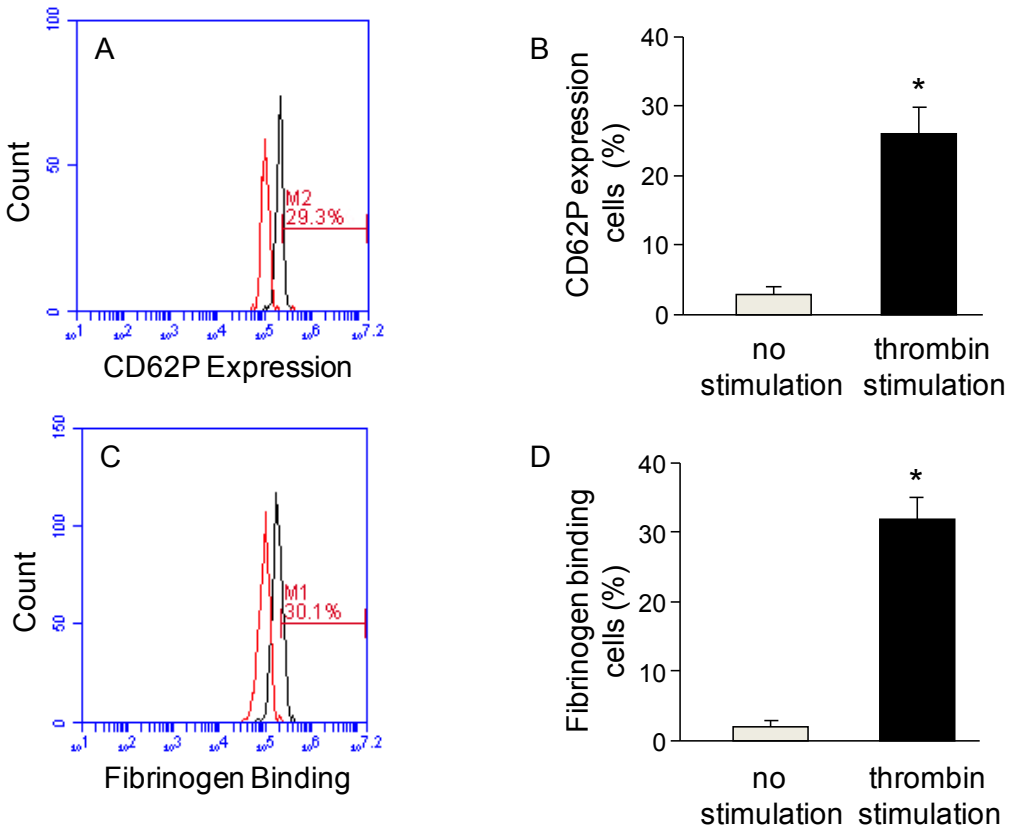

Fig 9. Functional assay of the generated PLTs. Thrombin stimulation enhanced CD62P expression (A) and fibrinogen binding (C) in PLTs released from generated MKs (Black curve, thrombin stimulation; Red curve, isotype control). Summarized data show the significant up-regulation of CD62P expression (B) and fibrinogen binding (D) after thrombin activation. Data was analyzed as mean ± SEM, *P<0.01, n=3.

7. Ultrastructural Similarities between Generated PLTs and Peripheral Blood Derived PLTs

Generated PLTs were examined by TEM. The size and ultrastructural composition of the generated PLTs (**Fig 10A and C**) were similar to those of normal PLTs obtained from human peripheral blood (**Fig 10B and D**). A representative image of generated PLT displayed a discoid shape shown in **Fig 10C**, similar in shape to the peripheral blood PLTs shown in **Fig 10D**. Generated PLTs also showed typical normal PLT features, such as storage granules and mitochondria.

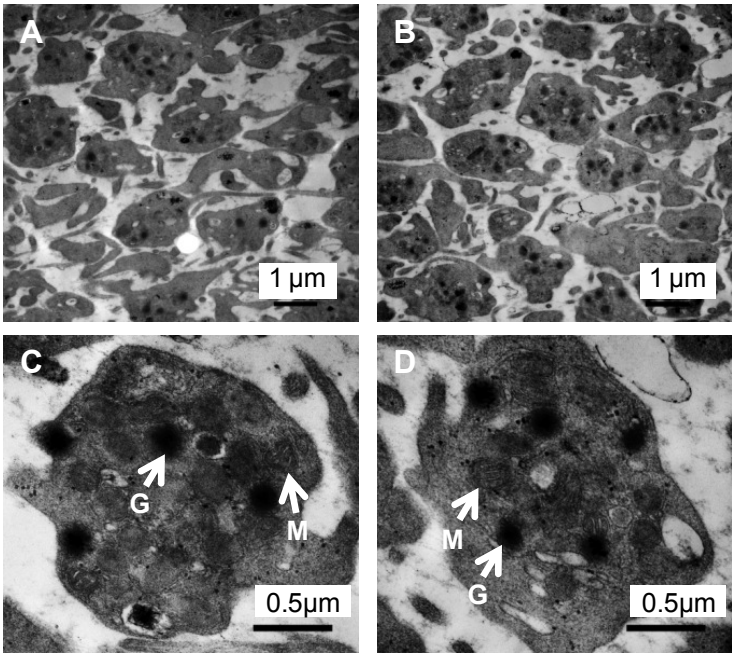

Fig 10. Electron micrographs of PLTs. Generated PLTs (A, C) show typical ultrastructural morphology and cellular organelles as PLTs obtained from peripheral blood (B,D). G: storage granules; M: mitochondria.

8. Expression of Surface Protein Markers for hESCs-derived Neurons by Immunocytochemistry Analysis

After 14 days differentiation, generated neurons exhibited typical neuronal morphology including pyramidal cell bodies and dendritic projections (**Fig 11A**) upon light microscopic examination. Immunocytochemical staining revealed that generated neurons were positively stained for neuron specific markers including nestin (**Fig 11B1-B3**), β-tublin III (**Fig 11C1-C3**), NeuN (**Fig 11D1-D3**), TH (**Fig 11E1-E3**), and astrocyte marker GFAP (**Fig 11F1-F3**). Summarized data (**Fig 11G**) showed the percentages of phenotypic expression of nestin (40 ± 4%, n=3), β-tublin III (37 ± 3%, n=3), NeuN (15 ± 2%, n=3), TH (14 ± 1%, n=3), and astrocyte marker GFAP (10 ± 3%, n=3). The control cells weakly expressed neuron and astrocyte markers (less than 1%).

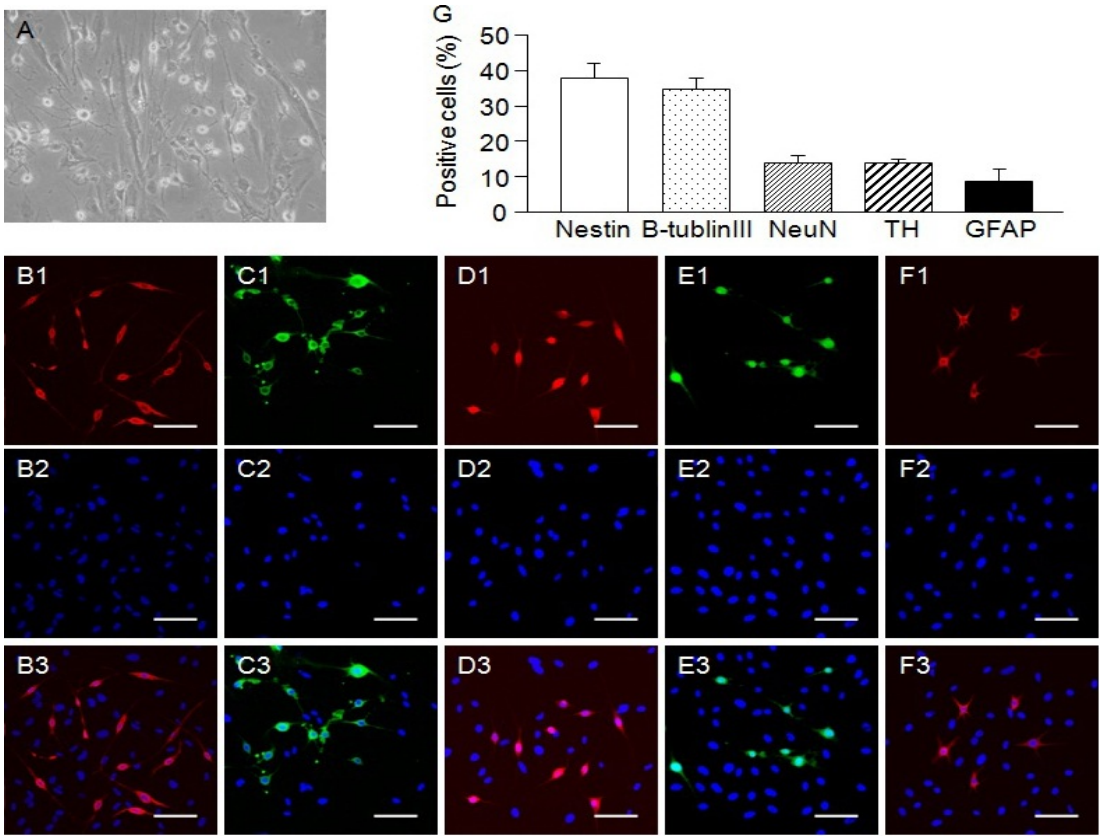

Fig 11. Characterization of generated neurons. A, hESCs treated with neuron differentiation medium for 14 days (Magnification 20 x); B1, nestin staining; C1, β-tublin III staining; D1, NeuN staining; E1, TH staining; F1, GFAP staining; B2-F2, DAPI staining; B3-F3, merged; scale bar, 75 μM. G, summarized data. Data was analyzed as mean ± SEM, n=3.

9. Expression of Transcription Genes Oct4 and Sox2 in the Cells after Neuronal Differentiation

Western blotting data showed the expressions of Oct4 (**Fig 12A**) and Sox2 (**Fig 12B**) on undifferentiated hESCs (control) and differentiated neuronal cells.

Summarized data (**Fig 12C**) demonstrated the significantly down-regulated expression of transcription genes (Oct4: 0.11 ± 0.01and 0.32 ± 0.01, and Sox2: 0.25 ± 0.02 and 0.52 ± 0.03, differentiated group *vs.* control group, *P*<0.01, n=4) in generated neurons.

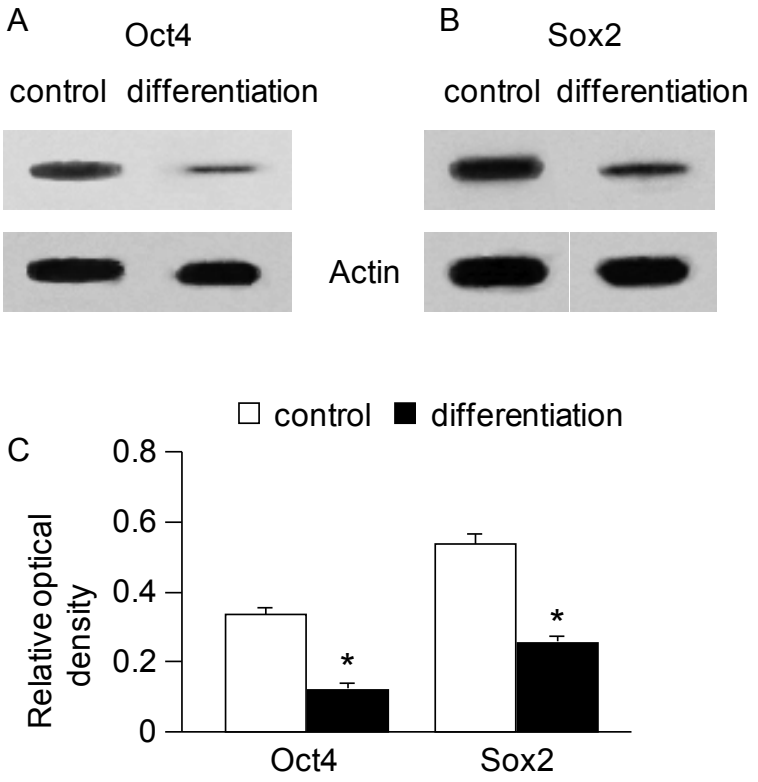

Fig 12. Comparison of the transcription genes expression in neuron differentiation and control cells. Representative bands show the decreased expression of Oct4 (A) and Sox2 (B) in neuronal differentiation cells as compared with control cells. Summarized data (C). Data was analyzed as mean ± SEM, *p<0.01, n=4.

V. DISCUSSION

The major findings of this study are as follows. 1) MKs and functional PLTs can be generated *in vitro* from hESCs. The generated PLTs have similar ultrastructural morphology and functional features with normal PLTs from human peripheral blood. 2) Neuons can also be differentiated from hESCs in an *in vitro* culture system.

Human endometrium is characterized by its self-renewal and cyclic regeneration as part of the normal physiologic course of menstrual cycle in reproductive age females. The upper functionalis layer is shed at menstruation and is thought to regenerate, to a thickness of 0.5 - 1 mm to 5 - 7 mm, from progenitor cells located in the remaining basalis layer (Padykula, 1991). Recent studies have provided evidence for the existence of stromal progenitor cells in endometrium (Chan *et al*, 2004;Dimitrov *et al*, 2008;Schwab *et al*, 2005;Wolff *et al*, 2011).

Based on these reports, we cultured hESCs from human endometrial tissue in this study. The primary cultured cells were composed of a heterogeneous population of stromal cells, epithelial cells and erythrocytes. As Dimitrov et al reported that the frequency of the cells other than hESCs diminishes quickly through 2 - 3 passages in the culture system (Dimitrov *et al*, 2008), we used passages 4 - 6th of hESCs for the differentiation in our experiments. We found that the cultured hESCs of passages 4-6th exclusively expressed stromal cell marker CD90 with none cells expressing epithelial cell marker cytokeratin by

immunocytochemistry analysis. Further analysis by flow cytometry convincingly demonstrated that more than 95% hESCs (P_4-P_6) were positive for stromal cell markers (CD29 and CD90) and negative for hematopoietic cell markers (CD34 and CD45). These data are in agreement with previous findings that showed similar phenotypic expression profiles for their respective hESCs (Chan *et al*, 2004; Dimitrov *et al*, 2008; Schwab *et al*, 2005), demonstrating the stromal progenitor cells exist in the endometrial tissue.

Recent studies have reported on hESCs as a source of multipotent cells that are capable of differentiating into multiple cell lineages including smooth muscle cells, adipocytes, chondrocytes, and osteoblasts (Gargett *et al*, 2009). Additionally, one recent publication described neuron differentiation potentiality from hESCs (Wolff *et al*, 2011). But there are no reports on generating MKs and PLTs from hESCs. The process of MK differentiation and subsequent PLT release by mature MKs are influenced by a variety of cytokines and growth factors. TPO is the primary cytokine to support the proliferation and maturation of MKs (Alexander *et al*, 1996; Gurney *et al*, 1994; Kaushansky *et al*, 1994; Kaushansky, 1995; Schipper *et al*, 1998). It also plays an important role in the formation and function of PLTs *in vitro* (Choi *et al*, 1995; Choi *et al*, 1996; Kaushansky, 1998). Hence, TPO has been widely used in research and in a number of studies on MK and PLT differentiation (Chen *et al*, 2009; Fujimoto *et al*, 2003). Serum is an important supportive component in cell cultures, but it is also a potential source of bacterial, mycoplasmal, and viral contamination and could induce immune-response.

Herein, we firstly described a culture system in which MKs and PLTs can be differentiated from hESCs in serum-free medium supplemented with TPO. The hESCs-derived MKs and PLTs expressed typical MK and PLT specific plasma membrane glycoprotein markers (eg, CD41a, CD42b). Stimulation of generated PLTs through soluble agonist thrombin resulted in activation of the fibrinogen receptor (αII_bIII_a) and enhanced expression of surface antigen P-selectin (CD62P). Electron microscopy studies showed that our generated PLTs have similar morphological and ultrastructural features as PLTs obtained from human peripheral blood including a discoid shape, the presence of storage granules and other organelles like mitochondria. These data are consistent with previous studies about *in vitro* generated MKs and PLTs from other tissue of origin sources (Choi *et al*, 1995;Fujimoto *et al*, 2003;Matsubara *et al*, 2009;Ungerer *et al*, 2004).

Matsubara and colleagues have generated approximately 2×10^6 MKs and 1.5×10^5 PLTs from 10^7 adipocyte precursor cells (Matsubara *et al*, 2009). The Takayama group reported that about $2 - 5 \times 10^5$ MKs and 10^5 PLTs were produced from 10^5 human embryonic stem cells (Takayama *et al*, 2008). In the present study, we obtained approximately 5.5×10^4 MKs and 3×10^5 PLTs from 1.8×10^5 hESCs. The differentiation efficiency of MKs to PLTs among various stem cell sources renders precise comparisons difficult. However, the number of PLTs released from our hESCs-derived MKs was below the theoretical range proposed in other study in which approximately 1000 to 5000 PLTs per MK has been reported to be released *in vivo* from mature MKs (Patel *et al*, 2005).

Release of PLTs from mature MKs is influenced by multiple factors within the BM microenvironment (Larson & Watson, 2006;Patel *et al*, 2005). The low efficiency of PLT release from hESCs-derived MKs may be related to the absence of some *in vivo* stimulus of thrombopoiesis, such as shear flow (Junt *et al*, 2007), in the *in vitro* culture system. Despite these findings, the present study is the first to report that MKs can be feasibly generated from hESCs and the generated MKs released PLTs conformed morphologically and functionally with PLTs obtained from human peripheral blood samples. The capacity to generate functional PLTs from hESCs *in vitro* provides a promising source for PLT in transfusion medicine. Menorrhagia is well recognized as a bleeding disorder in women with inherited bleeding disorders (James, 2005;Kadir *et al*, 1999). PFDs are inherited bleeding disorders frequently found among patients with bleeding diatheses. PFDs occur in 50% of women with unexplained menorrhagia and are prevalent in adolescent with menorrhagia as well (Chi *et al*, 2010; El-Hemaidi *et al*, 2007; Hossain *et al*, 2010; Philipp *et al*, 2003). However, the management of menorrhagia is suboptimal in some patients with PFDs. Some patients are refractory to medical therapy and require repeated PLT transfusions. One possible consequence of this study is *in vitro* generating PLTs with normal function from hESCs provides a potential therapeutic approach to menorrhagia.

In the present study we also reported that neurons can be generated from hESCs in a neuron differentiation medium with growth factors EGF, FGF, NGF and BDNF. Neurotrophic factors (EGF, FGF, NGF, and BDNF) are survival

and/or differentiation factors for neuron progenitor cells. EGF plays an important role in the regulation of cell growth, proliferation and differentiation (Carpenter & Cohen, 1990). FGF has long been known as a key regulator of neuron stem cell proliferation and differentiation (Palmer *et al*, 1999). The importance of FGF *in vivo* for neurogenesis has been confirmed by the neural defects observed in FGF-deficient mice (Vaccarino *et al*, 1999). NGF is the first of a series of neurotrophins shown to mediate the survival of neurons (Freeman *et al*, 2004). BDNF is found to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses (Huang & Reichardt, 2001).

In this study, hESCs following neural differentiation revealed immunocytochemical characteristics of neural lineages. Generated neurons exhibited neuron morphology including pyramidal cell bodies and dendritic projections, and positively stained with neuron specific markers nestin (a filamentous protein expressed by neuronal precursor stem cells) (Lendahl *et al*, 1990), β tubulin III (a marker expressed in immature neurons) (Lee *et al*, 1990), NeuN (a neuron-specific marker expressed in postmitotic cells) (Sarnat *et al*, 1998), and astrocyte marker GFAP (an intermediate filament protein expressed by astrocytes in the central nervous system). These results are consistent with published data of BM stromal cells, which express both neuron and glial cell markers after neuron induction (Lu *et al*, 2006;Sanchez-Ramos *et al*, 2000). Interestingly, the catecholaminergic marker TH, which is the rate-limiting enzyme for dopamine synthesis, is also expressed.

Oct4, a member of the POU family of transcription factors, is characteristically expressed in embryonic cells and necessary for their pluripotency (Pesce & Scholer, 2000). Sox2 is one of the SoxB1family of transcription factors, which are also crucial for stem cell pluripoteny. In our study, we observed that Oct4 and Sox2 were expressed in the initial hESCs population, suggesting that these hESCs contain pluripotent progenitors. After neuronal differentiation, the expression levels of Oct4 and Sox2 were found to be significantly reduced. This finding is in agreement with previous study that reported Oct4 is expressed by some individual stromal cells in human endometrium (Matthai *et al*, 2006) and supports the neuronal differentiation scheme.

Stroke remains a leading cause of death and disability worldwide. Neural tissue has limited potential for intrinsic repair following injury. Stem cell therapy is promising for neural regeneration and thus becomes one of the current trends. An increasing number of studies have shown that stem cell treatment of stroke acts to increase cell survival and angiogenesis, improve synapse formation, and reduce inflammatory responses (Bacigaluppi *et al*, 2009; Lindvall & Kokaia, 2004; Martino & Pluchino, 2006). The capacity to generate neurons from hESCs *in vitro* provides a promising source of transplantation therapy for stroke and neurodegenerative diseases.

One shortcoming of the present study was that we relied only on the morphological changes and protein level of transcriptional genes expression to define the events during neuroectodermal conversion of hESCs. A previous

study attempted to perform electrophysiological experiments on putative neurons derived from hESCs without showing the action potential (Wolff *et al*, 2011). In future studies, we will investigate the membrane action current of generated neurons using standard whole-cell patch-clamp technique and perform the *in vivo* transplantation study.

We will also investigate the differentiation efficiency of PLTs generated from induced pluripotent stem (iPS) cells. iPSCs are a type of pluripotent stem cells that have been reprogrammed from fibroblast cells obtained from either fibroblast cells obtained from either human adult dermal cells, fetuses, or newborn foreskin with defined transcriptional factors (Oct4, Sox2, Klf, C-myc) (Takahashi *et al*, 2007;Yu *et al*, 2007). PLTs are terminally differentiated, anucleate cells. PLTs are unable to retain the pluripotent or tumorigenic properties of iPSCs. A previous finding has already indicated that MKs and PLTs can be generated from hiPSCs, and produced PLTs showed normal function *in vivo* (Takayama *et al*, 2010). Based on these studies, a feasible approach to overcome the relative low efficiency issue is to reprogram iPSCs from hESCs and then differentiate the iPSCs into MKs and PLTs. Such an approach to generating PLTs from hESCs could potentially lead to a constant and safe source of PLTs.

VI. CONCLUSION

In conclusion, 1) our results reported on the feasibility of differentiating hESCs into MKs that subsequently release functional PLTs. This process is achieved using serum-free medium in the presence of TPO in an *in vitro* culture system. The generated PLTs have the potential to serve as an alternative source of PLT concentrates for future use in transfusion medicine to treat PLT-related bleeding disorders like menorrhagia.

2) Our data demonstrated that hESCs can be differentiated into neurons accompany with reduced expression of multipotent transcription genes (Oct4 and Sox2). Correspondingly, these data indicate that hESCs could serve as an alternative source for producing neurons in an *in vitro* culture system. The generated neurons could be a potential source of cell-based therapy to treat stroke, and other neurodegenerative diseases.

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