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Embryoid Body Size-Mediated Differential Endodermal and Mesodermal Differentiation Using Polyethylene Glycol (PEG) Microwell Array

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Abstract: Embryoid bodies have a number of similarities with cells in gastrulation, which provides useful biological information about embryonic stem cell differentiation. Extensive research has been done to study the control of embryoid body-mediated embryonic stem cell differentiation in various research fields. Recently, microengineering technology has been used to control the size of embryoid bodies and to direct lineage specific differentiation of embryonic stem cells. However, the underlying biology of developmental events in the embryoid bodies of different sizes has not been well elucidated. In this study, embryoid bodies with different sizes were generated within microfabricated PEG microwell arrays, and a series of gene and molecular expressions related to early developmental events was investigated to further elucidate the size-mediated differentiation. The gene and molecular expression profile suggested preferential visceral endoderm formation in 450 μm embryoid bodies and preferential lateral plate mesoderm formation in 150 μm embryoid bodies. These aggregates resulted in higher cardiac differentiation in 450 μm embryoid bodies and higher endothelial differentiation in 150 μm embryoid bodies, respectively. Our findings may provide further insight for understanding embryoid body size-mediated developmental progress.

Keywords: microwell, embryoid body size, differentiation, lateral plate mesoderm, visceral endoderm.

Introduction

Embryonic stem cells (ESCs) have received extensive attention in the field of cell biology and applied science due to their pluripotency. Hence, extensive research on differentiation of ESCs has been done using various *in vitro* culture techniques. ESCs are known to initiate a range of tissue-specific differentiation *via* the formation of tissue-like spheroids called embryoid bodies (EBs).[19] EB development recapitulates many aspects of early embryogenesis with various gene expressions following the time-dependent sequence of the lineage commitment events to form three germ layers.[1-22]

During EB formation, polarized mesendoderm is known to develop intermediately *via* epithelial-to-mesenchymal transition and generate mesoderm and endoderm.[8] EBs temporally express genes in a manner that recapitulates the sequence of normal development resulting in eventual early cell specification prior to organogenesis.[21] Expression of phenotypic markers of endoderm such as *Forkhead box protein A2 (Foxa2)*, *Sex determining region Y-related HMG box17 (Sox17)*, *GATA binding protein 4 (Gata4)*, and *α-fetoprotein (Afp)*, and mesoderm such as *Brachyury-T*, *Felal liver kinase-1 (Flk1)*, and *Wingless-type MMTV integration site family. member 3A (Wnt3a)* demonstrate the ability of EBs to generate cells for cardio-

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vascular development.[13] However, the typical heterogeneity in the differentiation of EBs hinders production of defined cell types, which can be modulated by culture methods for EB formation. It is reported that *in vitro* cardiac mesoderm formation and subsequent cardiogenic differentiation occurs after 5 days of culture.[15-24] On the other hand, although the culture time of EBs has been considered as an important parameter to induce ESC differentiation *via* three germ layer formation, ESC differentiation was recently reported to be also influenced by EB sizes accompanied with culture time.[5]

In recent years, various microscale technologies have been applied to control stem cell differentiation fate, and microfabrication technique has provided an efficient tool to control EB size homogenously.[9,27,28] In our previous study, a polyethylene glycol (PEG) hydrogel microwell array was developed as an ESC culture platform,[9] enabling control over homogenous EB size. It was demonstrated that microwell-mediated EB size control induced a differential lineage commitment; namely, a higher fraction of larger EBs (450 μm) showed preferential differentiation towards the cardiogenic lineage, whereas a greater number of endothelial cells were generated in smaller EBs (150 μm).[27] The differential expression of *Wnt5a* and *Wnt11*, two members of the noncanonical WNT pathway, was found to be directly involved in the EB size-mediated differentiation into cardiac and endothelial lineage. There is a recent study reporting that endothelial differentiation of ESCs could be optimized by screening size-varying EBs produced by a size-tunable concave microwell array.[54] Another intriguing report has been recently published, which demonstrated that novel size-controllable 3D-configurations of ESCs could direct hepatic differentiation of ESCs.[55] However, upstream developmental events to influence such differential ESC differentiation through the control of the colony sizes have not been elucidated.

Therefore, the central aim of this study is to characterize the differentiation process for elucidating the underlying biology to determine EB size-mediated differential cardiac and endothelial differentiation. To achieve this, different sized EBs (150 and 450 μm) were generated homogenously using a previously established PEG microwell array (150 and 450 μm in diameter) culture platform, and a series of gene and molecular expressions related to early developmental events was investigated. In particular, we tested if the formation of visceral endoderm-like cells in larger EBs (450 μm) may enhance cardiogenic differentiation, whereas the preferential development of lateral plate mesoderm in smaller EBs (150 μm) induced higher endothelial differentiation.

Experimental

Fabrication of Hydrogel Microwell Platforms. Microwell patterns with 150 and 450 μm diameters were generated on a silicon wafer by using an SU-8 photoresist (MicroChem Corp.). Polydimethylsiloxane (PDMS) stamps were used to mold PEG microwells. The PDMS stamps were fabricated by pouring a mixture (10:1) of silicone elastomer base solution and curing agent (Sylgard 184, Essex Chemical) on the patterned silicon master. PEG monomer solution was prepared with PEG dimethacrylate 1,000 (Polysciences, Inc.) mixed with 1% (w/w) of the photoinitiator, Irgacure 2959 (Ciba Specialty Chemicals Corp). Glass slides (75 mm × 25 mm; Fisher) were treated with 3-(trimethoxysilyl) propylmethacrylate (TMSPMA) (Sigma) for 30 min and baked at 70 °C overnight to mount the PEG hydrogel on the surface of the glass substrate. About 200 μL of PEG monomer solution was poured on the TMSPMA-treated glass, and a microfabricated PDMS stamp was placed over the solution. The monomers were cross-linked by exposure to UV light (350-500 nm wavelength, 100 mW/cm^2) for 30 s. After peeling off the PDMS stamp, the remaining PEG hydrogel microwell on the substrate was sterilized with 70% ethanol and washed with Phosphate buffered saline (PBS) overnight before use.

ESC and EB Cultures. Wild type (R1 and E14-Tg2a cell line) or genetically engineered murine ESCs that expressed green fluorescence protein (GFP) upon expression of *Oct4* (R1) or *Gsc* (E14-Tg2a) promoter were cultured on tissue culture flasks coated with 0.1% gelatin (Sigma) using high glucose Dulbecco's Modified Eagles Medium (DMEM; Invitrogen) supplemented with 10% (v/v) ESC qualified-fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 μM β-mercaptoethanol (Sigma), and 1,000 U/mL of leukemia inhibitory factor (LIF; Chemicon). For ESC seeding in microwells, 200 μL of cell suspension (1×10^6 cells per mL) was spread on a glass slide containing microwells. EBs were spontaneously formed and size-controlled within microwells while cultured in alpha Minimal Essential Medium (MEM; Invitrogen) containing 15% heat-inactivated FBS, and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen) with daily exchange of medium.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). The RNA of collected EBs was extracted using RNeasy kit (Qiagen) by following the manufacturer's instructions. RT-PCR was performed using a PTC-100TM thermal cycler (MJ Research Inc.) by following the manufacturer's instructions of SuperScript™ III One-Step RT-PCR System with Platinum Taq kit (Invitrogen). cDNA synthesis was processed at 55 °C for 20 min with 80 ng of total RNA, followed by an initial denaturation step at 94 °C for 2 min. PCR amplification process was then carried under the following conditions: 15 s of denaturing at 94 °C, 30 s of specific primer annealing temperature, and 45 s of extension at 68 °C. Information of the primers used is listed in a supplementary table (Table I). After amplification, PCR products were loaded on 1.2% (w/v) agarose gels containing 0.5 μg/mL of ethidium bromide. Fluorescence was developed under UV light using a gel documentation system (Gel Logic 100 Imaging System, Kodak). Each gene expression was normalized by a correspondent

Table I. Table of Various Genes Examined in this Study, Related to Early Development and Vascular/Cardiac Differentiation

Gene	Gene Product	Expression in Embryo	Primer Sequences	Size	A.T.
Fgf5	Fibroblast growth factor 5	-Expressed in the pluripotent cell pool of epiblast prior to gastrulation[29]	F: AAAGTCAATGGCTCCCACGAA R: CTTCAGTCTGTACTTCACTGG	464	58
Gsc	Goosecoid, a member of the bicoid subfamily of the paired homeobox family of proteins	-First expressed in cells that are undergoing anterior migration during a short period of PS formation, and later during organogenesis of head, limbs and ventrolateral body wall[30]	F: CAGATGCTGCCCTACATGAAC R: TCTGGGTACTTCGTCTCCTGG	158	60
Mixl1	MIX homeobox-like 1	-Expressed during a short period of PS formation prior to the transition towards mesendoderm, extending between posterior ExE tissues and PS[31]	F: ACGCAGTGCTTTCCAAACC R: CCCGCAAGTGGATGTCTGG	196	60
Bra	Brachyury, a founding member of the T-box family of transcription factors	-Expressed during a short period of PS formation prior to the transition towards mesendoderm, extending between posterior ExE tissues and PS[31,32]	F: GCTTCAAGGAGCTAACTAACGAG R: CCAGCAAGAAAGAGTACATGGC	117	60
Wnt3a	Wingless-type MMTV integration site family, member 3a (involved in canonical WNT pathway)	-Expressed through posterior epiblast during gastrulation inducing mesoderm development[31,33]	F: CTCGCTGGCTACCCAATTTG R: CTTCACACCTTCTGCTACGCT	165	60
Dkk1	Dickkopf homologue 1	-Expressed at anterior side of the gastrulating embryo,[3] the extracellular inhibitor of canonical Wnt signaling pathway[34]	F: CTCATCAATTCCAACGCGATCA R: GCCCTCATAGAGAACTCCCG	105	60
Sox17	SRY (sex determining region Y)-box 17	-Expressed in definitive endoderm and its descendants[35]	F: GATGCGGGATACGCCAGTG R: CCACCACCTCGCCTTTCAC	136	60
Foxa2	Forkhead box protein A2	-Expressed in the posterior epiblast and later confined to anterior definitive endoderm[36]	F: CCCTACGCCAACATGAACTCG R: GTTCTGCCGGTAGAAAGGGA	222	60
Bmp4	Bone morphogenic protein	-Extends through ExE ecto-/mesoderm affecting posterior PS[31]	F: TTCCTGGTAACCGAATGCTGA R: CCTGAATCTCGGCGACTTTTT	114	60
Foxf1a	Forkhead box protein F1a	-Expressed in ExE mesoderm (allantois, amnion and yolk sac) as well as lateral plate mesoderm[31,37,38]	F: ACGCCGTTTACTCCAGCTC R: CGTTGTGACTGTTTTGGTGAAG	183	60
Afp	Alpha-fetoprotein	-Expressed in ExE endodermal descendants such as visceral and yolk sac endoderm, and later in endodermal lineage tissues such as fetal liver[39,40]	F: TCCAGAACCTGCCGAGAGTT R: CTGGGGCATACATGAAGGGG	107	60
Ihh	Indian Hedgehog	-Expressed in visceral yolk sac endoderm[39]	F: CTCTTGCCTACAAGCAGTTCA R: CCGTGTTCTCCTCGTCCTT	156	60
Flk1	Fetal Liver Kinase 1 also known as vascular endothelial growth factor receptor 2 (VEGFR-2)	-Expressed in most of the mesodermal lineage cells such as progenies of blood, vascular cells[41]	F: TTTGGCAAATACAACCCTTCAGA R: GCAGAAGATACTGTCACCACC	133	60
Vegf	Vascular endothelial growth factor	-Expressed mainly in embryonic erythroid development[42]	F: CAACATCACCATGCAGATTATGC P: CCACAGGGACGGGATTTCTTG	186	60
Gata1	GATA family of zinc finger transcription factor 1	-Expressed prior to or during the onset of erythroid development[43]	F: TGGGGACCTCAGAACCCTTG R: GGCTGCATTTGGGGAAGTG	134	60
Wnt5a	Wingless-type MMTV integration site family, member 5a (involved in non-canonical WNT pathway)	-Expressed in vasculature of embryonic tissues acting as angiogenic signalling factor on endothelial cells[44]	F: CAACTGGCAGGACTTTCTCAA R: CATCTCCGATGCCGGAACT	128	60
Cd31	Cluster of differentiation 31 also known as platelet endothelial cell adhesion molecule (*Pecam-1*)	-Expressed in the early endothelial precursors during vascular development[45]	F: TGCACCCATCACTTACCACC R: CTTCATCCACCGGGGCTATC	197	60
Tie2	A tyrosine-protein kinase receptor of angiopoietin	-Expressed in the regions where angiogenesis occurs[46]	F: GAGTCAGCTTGCTCCTTTATGG R: AGACACAAGAGGTAGGGAATTGA	77	60
Vecad	Vascular endothelial cadherin also known as *Cd144* (Cluster of Differentiation 144)	-Expressed in vascular development[47]	F: CACTGCTTTGGGAGCCTTC R: GGGGCAGCGATTCATTTTTCT	167	60

Table I. (continued)

Gene	Gene Product	Expression in Embryo	Primer Sequences	Size	A.T.
Nkx2.5	Cardiac NK2 homeobox protein 5	-First expressed in mesodermal cells specified to form heart and throughout the whole heart tissue formation[48]	F: GACAAAGCCGAGACGGATGG R: CTGTCGCTTGCACTTGTAGC	222	60
Gata4	GATA family of zinc finger transcription factor 4	-First expressed in yolk sac endoderm, and later in cardiac tissue, gut epithelium and gonads[49]	F: CCCTACCCAGCCTACATGG R: ACATATCGAGATTGGGGTGTCT	139	60
Wnt11	Wingless-type MMTV integration site family, member 11 (involved in non-canonical WNT pathway)	-Expressed in the developing and postnatal heart[50]	F: GCTGGCACTGTCCAAGACTC R: CTCCCGTGTACCTCTCTCCA	250	60
Tbx5	T-box-containing transcription factor	-Expressed in the developing heart and specification of upper limbs[51]	F: ATGGCCGATACAGATGAGGG R: TTCGTGGAACTTCAGCCACAG	207	60
Nfatc1	Nuclear factor of activated T-cells, cytoplasmic 1	-Exclusively expressed in the initiation of endocardial differentiation during cardiac valve development[52]	F: GACCCGGAGTTCGACTTCG R: TGACACTAGGGGACACATAACTG	97	60
Nrg1	Neuregulin 1, epidermal growth factor family member	-Expressed in developing endocardium during epithelial-to-mesenchymal transition[52]	F: AGTGCCCAAATGAGTTTACTGG R: AGTTCCTCCGCTTCCATAAATTC	97	60
Oct4	Octamer-binding transcription factor 4 also known as a POU-family transcription factor	-Expressed in totipotent embryonic cells and downregulated through developmental progress[53]	F: GGCGTTCTCTTTGGAAAGGTGTTC R: CTCGAACCACATCCTTCTCT	302	55
Hprt	Hypoxanthine-guanine phosphoribosyltransferase	-HPRT RNA levels are known to be very low in a cell, which makes it suitable as an endogenous mRNA control in RT-PCR for highly sensitive quantification of low copy or rare mRNAs.	F: GTTAAGCAGTACAGCCCCAAA R: AGGGCATATCCAACAACAAACTT	131	60

housekeeping gene expression (*Hprt*).

Immunocytochemistry. EBs were collected from microwells by gentle PBS pipetting, and fixed for 20 min at room temperature in 4% (w/v) paraformaldehyde (PFA; BDH Laboratory Supplies). The fixed samples were cryo-sectioned (10 μm) to stain the center area of EBs. For staining 2D-spread EBs, EBs collected from microwells were transferred onto laminin-coated 4-well chamber slides (Vector Laboratories) and then cultured for 7 days, followed by general immunocytochemistry processes. Briefly, the samples were treated with 0.2% (v/v) Triton-X-100 (BDH Laboratory Supplies) for 45 min at room temperature and washed twice with PBS. The samples were then incubated with 3% (v/v) blocking goat, donkey or rabbit serum (Vector Laboratories) in primary diluents composed of 0.05% (w/v) bovine serum albumin (BSA; Sigma-Aldrich), 0.01% (w/v) NaN_3 (Sigma-Aldrich) in PBS for 30 min at room temperature. The serum solution was removed and the samples were incubated with primary antibodies diluted in primary diluents at 4 °C overnight followed by two washes and incubation with secondary antibodies diluted in second-

Table II. Table of Antibodies Used in this Study

	Antigens	Primary Antibody (company, dilution)	Secondary Antibody (company, dilution)
Immuno-cytochemistry	Laminin	Rabbit polyclonal (Abcam, 1/100)	Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen, 1/300)
	Alpha-fetoprotein (*Afp*)	Goat polyclonal (Santa Cruz, 1/50)	Alexa Fluor® 594 donkey anti-goat IgG (Invitrogen, 1/300)
	Cadherin-3 (cdh3)	Mouse monoclonal (Santa Cruz, 1/50)	Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, 1/300)
	Cluster of differentiation 31 (*Cd31*)	Mouse monoclonal (Abcam, 1/100)	Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, 1/300)
	Vascular endothelial cadherin (VE-cad)	Mouse monoclonal (Abcam, 1/100)	Alexa Fluor® 594 rabbit anti-mouse IgG (Invitrogen, 1/300)
	Heavy chain cardiac myosin (MHC)	Mouse monoclonal (Abcam, 1/100)	Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, 1/300)
Flowcytometric analysis	*Cd31*	Anti-mouse *Cd31*-Phycoerythrin (PE) monoclonal antibody (R&D Systems, 1/200)	
	Control IgG	IgG2B Isotype Control-PE (R&D Systems, 1/300)	

ary diluents consisting of 0.05% (w/v) BSA in PBS for 45 min at room temperature (for the whole EB staining, they were incubated overnight at 4 °C). For dual staining, the same steps after the first treatment of primary antibody were repeated for the second reaction. The stained samples were washed three times in PBS and mounted using SlowFade® Gold antifade reagent with DAPI (Invitrogen). An inverted laser scanning confocal microscope (SP5 X MP, Leica) and Leica application suite (LAS) software were used to acquire 3D serial section images of EBs. Sectioned images (2 μm thick) were scanned in 200 MHz and then stacked by Image J (NIH). Antibodies used in this study are listed in Table II.

Flow Cytometric Analysis. Following microwell cultures, the microwell arrays containing EBs were washed once with PBS to remove traces of medium and debris in culture, and then the EBs were retrieved from the microwells by gentle PBS pipetting. EBs were dissociated to single cell suspensions by treatment with 0.15 Wünsch units/mL collagenase (Sigma) solution in DMEM (Invitrogen) for 2 h and Cell stripper TM (Mediatech) for 15 min (37 °C, 5% CO_2) on a shaker. Cells were passed through a 40 μm cell strainer (BD Falcon). The resulting single cells were rinsed and incubated with antibodies in the dark at 4 °C for 40 min (Table II). Dead cells stained by DAPI (Invitrogen) were excluded from the analysis performed using LSRFortessa flow cytometer (BD Biosciences). Positive expression was defined as the level of fluorescence greater than 99% of the one measured using the corresponding isotype-matched control antibodies (R&D systems, Inc).

Statistical Analysis. The error bars on the relative comparisons of gene expressions represent the standard deviation (SD) of the mean obtained from three independent experiments (n=3). Each comparable value from 150 and 450 μm EBs was statistically analyzed with a Student's t-test at a level of significance of $p<0.05$.

Results

PEG Microwell-Mediated Size Control of EBs and Spontaneous Differentiation. In this study, PEG hydrogel microwell array was fabricated to modulate EB size. As shown in

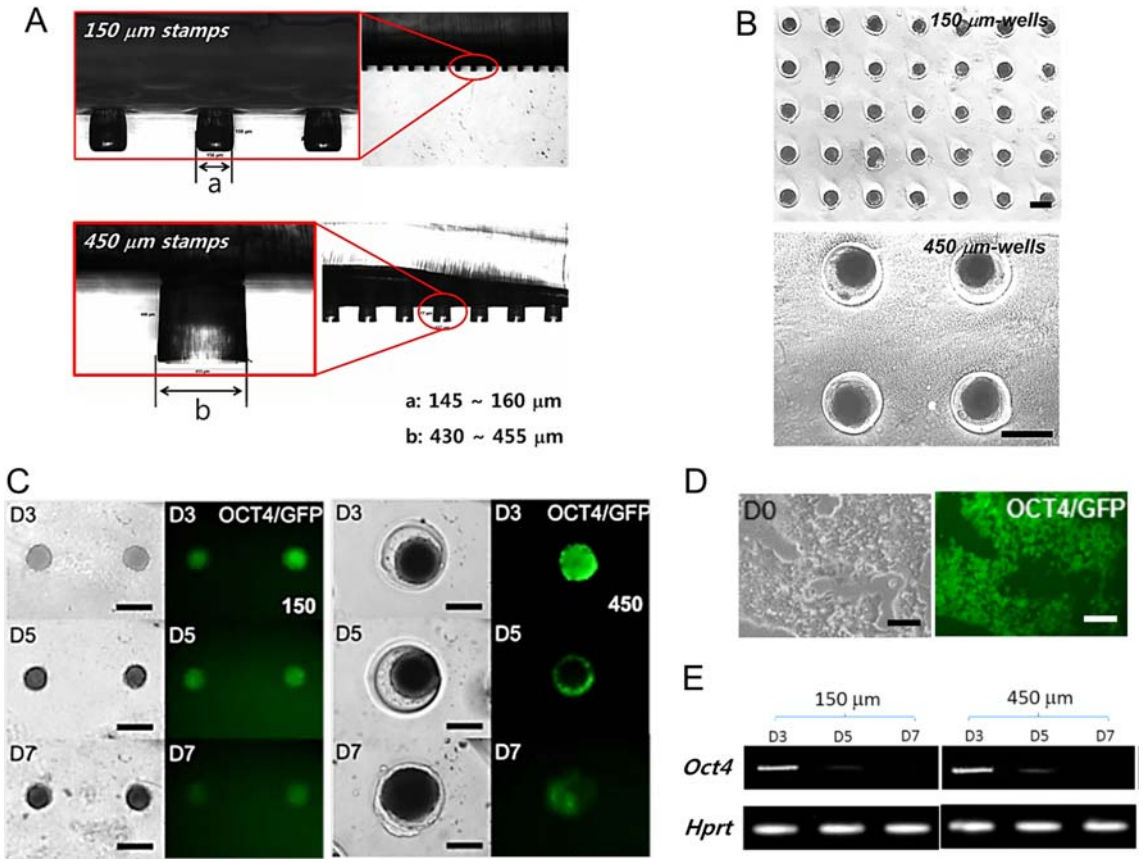

Figure 1. Formation of size-controlled EBs in microwells. Soft lithography technique was used for the fabrication of poly(ethylene glycol) (PEG) hydrogel microwell arrays. (A) Dimensions of poly(dimethylsiloxane) stamps for generation of each microwell array. (B) Size-controlled EBs in culture on day 5. The non-adherent surface property of PEG hydrogel allows for docking of the ESCs within the microwells followed by their growth into EBs confined by the size of the wells. Scale bars, 200 μm for 150 μm wells and 400 μm for 450 μm wells. (C) Phase contrast and fluorescent images of EBs made from *Oct4*/GFP-tagged ESCs within 150 and 450 μm sized microwells. Scale bars, 200 μm. (D) ESCs on D0 prior to seeding in microwells. Scale bars, 100 μm. (E) Gene expressions of *Oct4* expression.

Figure 1(A), different sized PEG microwells were generated on glass plate using PDMS molds with multi-cylinders of 150 and 450 μm in diameters. After PEG microwell fabrication, undifferentiated ESCs suspended as single cells were docked into PEG microwells. After 30 min of incubation, undocked ESCs were removed by gentle washing, and EBs with different sizes could be homogenously formed in each microwells (Figure 1(B)). There was no ESC adhesion on the bottom surface of PEG microwells due to the repellent surface of PEG for cell adhesion. Once EBs formed within microwells, spontaneous differentiation of ESCs within EBs was evaluated by observing *Oct4*-GFP expression. The time-course gradual decrease of *Oct4*-GFP intensity within EBs was observed in both 150 and 450 μm EBs within PEG microwells, which was consistent to the gradual down-regulation of *Oct4* mRNA expression in RT-PCR analysis (Figure 1(C) and (D)).

EB Size-Mediated Mesendodermal Differentiation. Accompanied with spontaneous differentiation of EBs within PEG microwells, gene expressions relevant to mesendoderm formation in the different-sized EBs were investigated (Figure 2). These mesendodermal cells are subsequently segmented to mesoderm, the origin of cardiovascular lineage, and endoderm, a source of inducing signals recruiting mesoderm to the cardiomyocyte lineage.[16-23] Consistent to down-regulation of *Oct4*, the expression of *Fgf5* mRNA were gradually down-regulated over time in both sizes of EBs, indicating spontaneous differentiation of EBs (Figure 2(A)). During spontaneous differentiation, the transient expressions of the mesendoderm-related genes were observed in the 450 μm EBs. The 450 μm EBs displayed temporal distinct increase in mRNA expressions of a mesendoderm marker, goosecoid (*Gsc*),[20] a mesoderm marker, brachyury (*Bra*),[7] and an endoderm maker, *Mixl1*,[3] especially at day 5 of EB culture and rapid down-regulation at day 7. On the other hand, the 150 μm EBs differed from the 450 μm EBs in the expression patterns of mesendodermal genes over time. Although the expression of *Mixl1* showed a

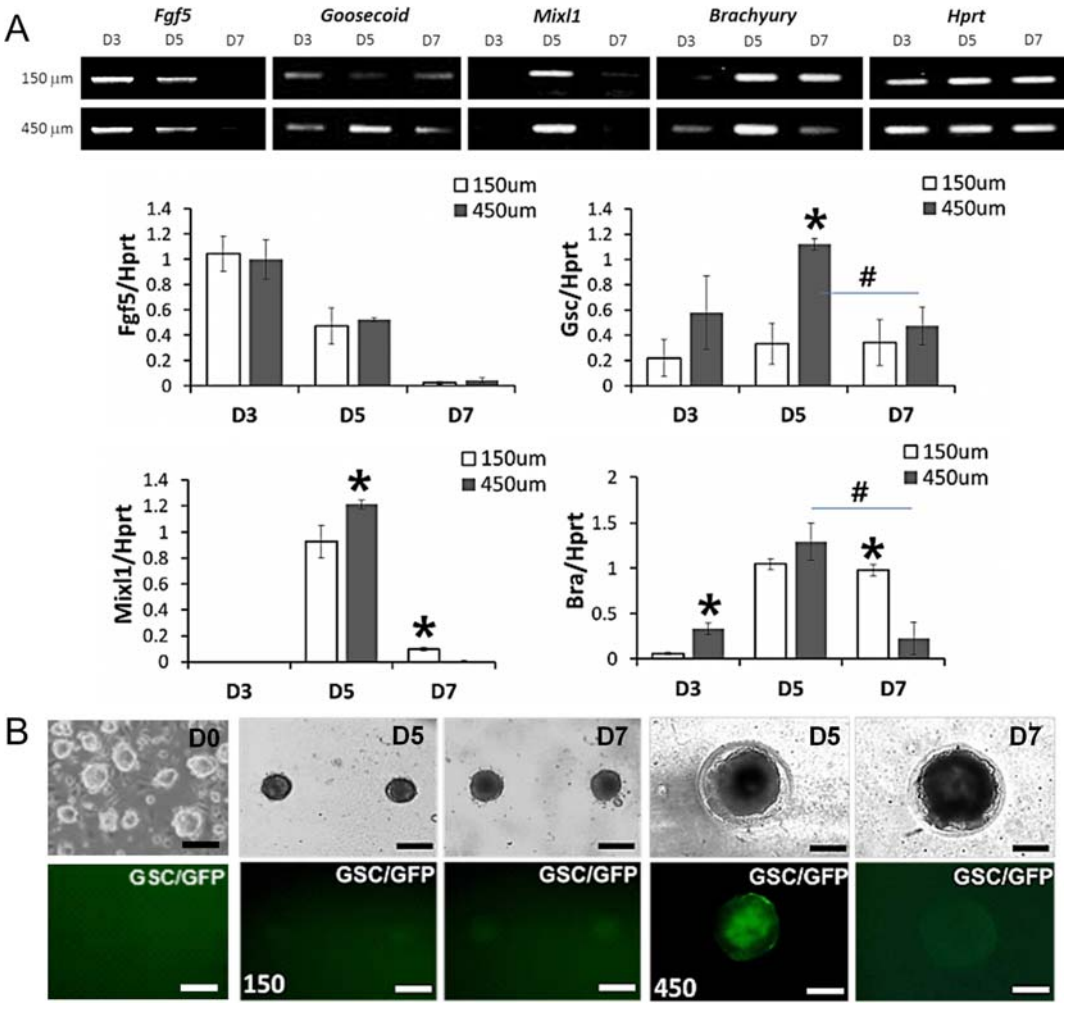

Figure 2. Gene expression profiles related to early developmental processes in the 150 and 450 μm EBs. (A) Gene expressions related to mesendodermal differentiation which is subsequently segmented to mesoderm and endoderm (n=3; *, # $p<0.05$). (B) Phase contrast and fluorescent images of the different-sized EBs made using *Gsc*/GFP-tagged ESCs. D0 show ESCs on a 2D substrate before seeding in microwells. 150 μm EBs and 450 μm EBs on D5 and D7 in microwell culture. Scale bars, 100 μm on D0 and 200 μm in EBs on D5 and D7.

similar trend to that in the 450 μm EBs, the transient upregulation of *Gsc* was not found at day 5 in the 150 μm EBs. Moreover, unlike the 450 μm EBs, the expression of *Bra* upregulated at day 5 was sustained until day 7 in the 150 μm EBs (Figure 2(A)). This pattern of *Gsc* mRNA expression in 150 μm EBs and 450 μm EBs was consistent to the temporal higher intensity of *Gsc*-GFP in 450 μm EBs at day 5 of EB culture within PEG microwells (Figure 2(B)).

EB Size-Mediated Differential Mesodermal and Endodermal Differentiation. Following the characterization of early differentiation such as mesendoderm formation in different-sized EBs, various gene expressions related to meso-

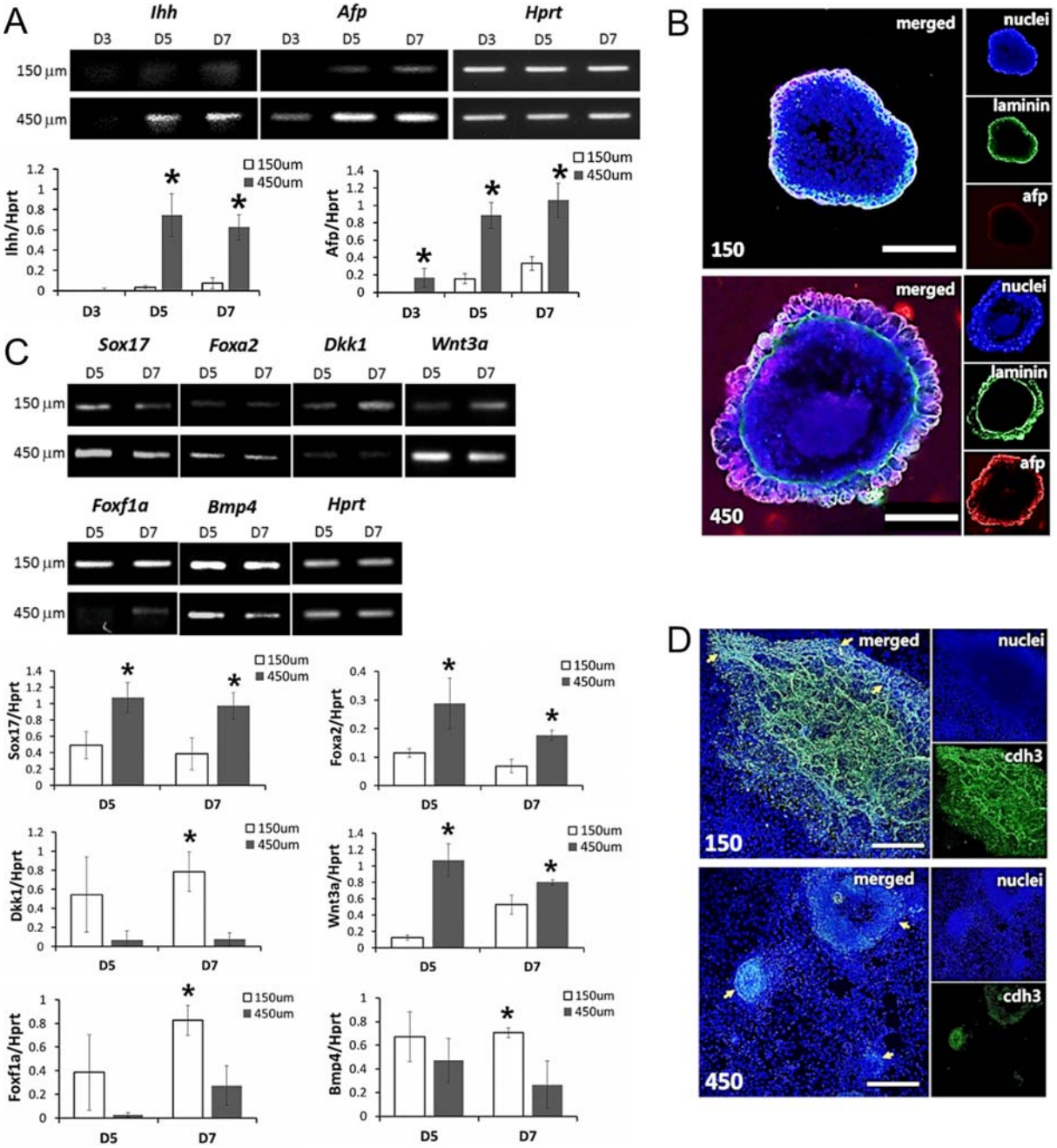

Figure 3. Different tissue development in the 150 and 450 μm EBs. (A) Gene expressions of visceral endoderm lineage differentiation that is to stimulate cardiac differentiation of neighboring cells by paracrine signalling (n=3; *p<0.05). (B) Confocal microscopy images of the different sizes of EBs at day 7 with immunofluorescence for laminin and *Afp*. Scale bars, 100 μm (yop) and 200 μm (bottom). (C) Gene expression profiles related to early gastrulation of size-controlled EBs (n=3; *p<0.05). (D) Cdh3 expressions of different sized EBs cultured for 5 days in microwells, and transferred onto laminin-coated 2D substrates for 7 days of further culturing. Yellow arrows indicate 2D-spread EBs. Scale bars, 200 μm.

derm formation and endoderm formation including visceral endoderm were investigated (Figure 3). Interestingly, the gene expressions related to visceral endoderm formation such as Indian hedgehog (*Ihh*)[17] and alpha-fetoprotein (*Afp*)[14] were up-regulated at significantly higher levels in 450 μm EBs (Figure 3(A)). In addition, immunocytochemical staining showed that a layer of cells showing strong *Afp* molecular expression was found to be at the periphery of 450 μm EB, and the high deposition of laminin, a major component of basement membrane which is layered between ectoderm and visceral endoderm layers,[2] was found to be co-localized with the cells of this layer (Figure 3(B)). In contrast to 450 μm EBs, 150 μm EBs showed relatively weak gene and molecular expressions of *Ihh*, *Afp* and laminin. Such differential endoderm formation in 150 μm EBs and 450 μm EBs was also characterized further by various gene expressions related to endoderm formation. RT-PCR analysis displayed highly up-regulated gene expression profile indicating endoderm formation such as *Foxa2*,[11] *Sox17*,[25] and visceral endoderm formation such as *Wnt3a*[12] in 450 μm EBs in comparison to 150 μm EBs, but showed much lower gene expressions indicating mesoderm formation including lateral plate mesoderm formation such as *Foxf1a* and *Bmp4*,[10-26] and *Dkk1*, an antagonist of *Wnt3a*[18] (Figure 3(C)). In contrast to 450 μm EBs, 150 μm EBs was characterized by relatively

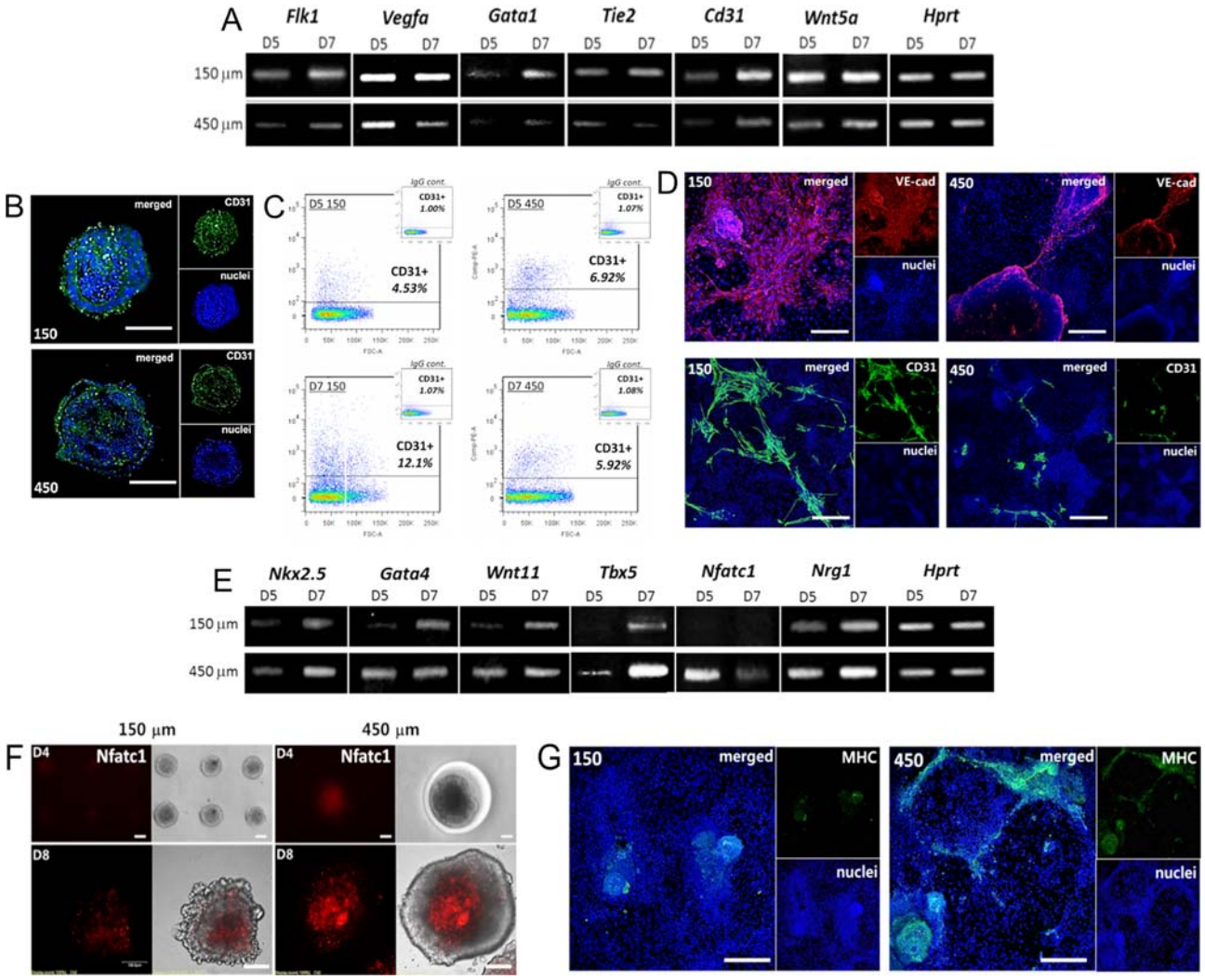

Figure 4. Cardiovascular differentiation in the 150 and 450 μm EBs. (A) Vasculogenic gene expressions of EBs of different sizes. (B) Immunocytochemistry images of the different sizes of EBs on day 7. Cells in both sizes of EBs were positively stained with *Cd31*. Scale bars, 100 μm in 150 μm EB and 200 μm in 450 μm EB. (C) Flow cytometric analysis for *Cd31*+ cell population in EBs of varying sizes. (D) Vascular (Vascular endothelial cadherin (VE-cadherin) and *Cd31*) differentiation proceeded on the 2D substrates. EBs of different sizes were cultured for 5 days in microwells, and then transferred onto laminin-coated 2D substrates for an additional 7 days of culture. Scale bars, 200 μm. (E) Gene expressions of cardiac development including myocardial (*Nkx2.5*, *Gata4*, *Wnt11*, and *Tbx5*) and endocardial (*Nfatc1* and *Nrg1*) differentiation. (F) Fluorescent and phase contrast images of the different sizes of EBs made from *Nfatc1*-nuc-mCherry ESCs. Scale bars, 100 μm. (G) Cardiac (Heavy chain cardiac myosin (MHC)) lineage differentiation proceeded on the 2D substrates. Scale bars, 200 μm.

higher gene expressions of *Bmp4*, *Foxf1a* and *Dkk1*. The differential gene expression profile of ESCs in 150 μm EBs were characterized by strong molecular expression of Cdh3 known to be expressed mostly in lateral plate mesoderm[4] in the replated culture of 150 μm EBs on a laminin-coated tissue culture plate for 7 days (Figure 3(D)).

EB Size-Mediated Differential Cardiogenic and Endothelial Differentiation. Finally, accompanied with early differential endoderm and mesoderm formation in 150 μm EBs and 450 μm EBs, further cardiogenic and endothelial differentiation was characterized by gene expression analysis, flow cytometric analysis, and immunocytochemical staining. As shown in Figure 4(A), gene expressions related to vascular/endothelial differentiation such as *Flk1*, *Vegf*, *Gata1*, *Tie2*, *Cd31*, and *Wnt5a* were highly up-regulated in 150 μm EBs. Immunocytochemical staining obtained from 7 day-cultured EBs showed stronger molecular expression of *Cd31* in 150 μm EBs (Figure 4(B)). Flow cytometric analysis also showed the higher increase of *Cd31*+ population from 4.53% to 12.1% in 150 μm EBs (Figure 4(C)). In addition, the vasculogenic culture of the replated EBs showed well developed VE-cadherin and higher *Cd31* expressions in 150 μm EBs compared to those in 450 μm EBs (Figure 4(D)). These results represented that ESCs in 150 μm EBs tended to differentiate towards endothelial lineage compared to the cells in 450 μm EBs. On the other hand, the expressions of cardiogenic genes such as *Nkx2.5*, *Gata4*, *Wnt11*, *Tbx5*, *Nfatc1*, and *Nrg1* were up-regulated at higher levels in 450 μm EBs in comparison to 150 μm EBs (Figure 4(E)), which was consistent to immunocytochemical results showing the relatively higher molecular expression of *Nfatc1* in 450 μm EBs (Figure 4(F)) and of MHC in the cardiogenic culture of replated EBs (Figure 4(G)). These results represented that ESCs in 450 μm EBs tended to differentiate towards cardiogenic lineage compared to cells in 150 μm EBs.

Discussion

In recent years, microscale biotechnology has opened a new area in the studies of stem cells with the ability to regulate cellular behaviors under controlled microenvironments, and has provided more efficient tools to study cell biology. Microfabrication-mediated control of physical environment around cells in micro-scale could allow the precise modulation of cell to cell interaction. In our previous studies, PEG hydrogel microwell arrays were fabricated to culture ESCs in micro scale, and EB sizes could be modulated in a controlled manner *via* self-aggregation of ESCs within a restricted space with repellent surface against cell adhesion.[6-9] Although many studies reported time-dependent ESC differentiation *via* formation of three germ layers within EBs, our previous study showed that ESC differentiation fate could be also controlled by EB sizes. During suspension culture of EBs, time-dependent increase of EB size approximately from 150 to 450 μm until day 5 was found, and this finding suggested that ESC differentiation might not be influenced only by culture time or EB size independently. Therefore, the EB size-mediated ESC differentiation was evaluated by controlling EB size in microfabricated microwell arrays at the same period of culture, and showed that large EBs generated preferential cardiac differentiation and small EBs generated preferential endothelial differentiation, which was controlled by differential *Wnt5a* and *Wnt11* expressions according to EB size.[27] However, the precise underlying biology to determine both lineage specific differentiations in different sized EBs has not been elucidated.

Hence, in this study, a series of gene and molecular expressions related to early developmental events during EB formation to influence cardiac and endothelial lineage differentiation were investigated. The gradual decrease of *Oct4* (Figure 1(C), (D), and (E)) and *Fgf5* (Figure 2(A)) expressions represented spontaneous differentiation of EBs formed within microwells, which corresponded to the increase in the intensity of goosecoid-GFP of EBs (Figure 2(B)). The upregulated mRNAs of a mesendoderm marker, goosecoid,[20] a mesoderm marker, brachyury,[7] and an endoderm maker, *Mixl1*,[3] indicated spontaneous mesendoderm formation in 150 μm EBs and 450 μm EBs under our culture condition, while mesendoderm formation was found to be higher in 450 μm EBs (Figure 2(A)). It was reported that the goosecoid expression indicating mesendoderm is a divergent point for mesoderm and endoderm.[20] In this study, the development of endoderm and mesoderm during EB formation within microwells was further characterized by assessing gene expression profiles. Relatively higher endodermal differentiation including visceral endoderm formation was found in 450 μm EBs, which was characterized by highly upregulated expression of *Ihh* which is known to be expressed in the visceral endoderm[17] and *Afp*, a visceral endoderm marker[14] (Figure 3(A)). This was consistent to the high deposition of laminin at outer region of EB in immunocytochemical analysis (Figure 3(B)). Laminin is a major component of basement membrane which is layered between ectoderm and visceral endoderm layers, and the basement membrane organization is known to be essential for functioning of visceral endoderm as programmed in the early developmental processes.[2] Such a formation of highly developed endoderm in 450 μm EBs was also proven by various gene expressions displaying the endoderm formation such as the expressions of *Sox17*,[25] *Foxa2*[11] and visceral endoderm formation such as *Wnt3a*.[12] In contrast to ESC differentiation in 450 μm EBs, ESC differentiation in 150 μm EBs was characterized by relatively higher gene expressions of the mesoderm formation such as *Bmp4* and *Foxf1a*. It is well known that *Foxf1a* is downstream target of *Bmp4* in mesodermal signaling,[26] while expressed in lateral plate mesoderm.[10] Such mesoderm formation in 150 μm EBs was also characterized by the molecular expression indicating well developed Cdh3 (also called as P-cadherin), which known to be expressed mostly in lateral plate mesoderm.[4] Furthermore, the relatively higher mRNA expression of *Dkk1*, known as an antagonist of *Wnt3a*,[18] was also found in 150 μm

EBs, which might be related with the lower expression of *Wnt3a*, known to be expressed in visceral endoderm, in comparison with 450 μm EBs. These results of gene and molecular expressions related to the early developmental events represented that ESC differentiation in 450 μm EBs was characterized by well-developed visceral endoderm formation and ESC differentiation in 150 μm EBs was characterized by well-developed lateral plate mesoderm.

The preferential early differentiation of ESCs to visceral endoderm in 450 μm EBs and to lateral plate mesoderm in 150 μm EBs supported further cardiogenic differentiation in 450 μm EBs and endothelial differentiation in 150 μm EBs (Figure 4). Consistent to our previous study,[27] 150 μm EBs showed preferential endothelial differentiation, which was proven by relatively higher gene expressions such as *Flk1*, *Vegf*, *Gata1*, *Tie2*, *Cd31*, and *Wnt5a* in vasculogenic culture of the replated 150 μm EBs in comparison with that of 450 μm EBs. Such enhanced endothelial differentiation was also characterized by VE-cadherin and *Cd31* molecular expressions (Figure 4(D)). In addition, preferential cardiogenic differentiation was developed in cardiogenic culture of the replated 450 μm EBs, which was proven by the upregulated gene expressions such as *Nkx2.5*, *Gata4*, *Wnt11*, *Tbx5*, *Nfatc1*, and *Nrg1*, as well as relatively higher molecular expression of MHC. It has been well proven that visceral endoderm plays a key role of controlling cardiac mesoderm formation and further cardiogenesis,[25] and lateral plate mesoderm supported endothelial differentiation and vasculogenesis.[6]

Conclusions

This study demonstrated that a microfabrication technique provided an efficient culture platform to control and generate homogenous EBs in their size. Further, different preferences of lineage commitments towards cardiac and endothelial lineages in the EBs of different sizes were attributed to the discriminated development of visceral endoderm in 450 μm EBs and lateral plate mesoderm in 150 μm EBs, respectively. Our findings in this study could introduce an interesting insight of EB's developmental progress based on their sizes and a new parameter to control ESC differentiation.

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