Generation of Multipotential Mesendodermal Progenitors from Mouse Embryonic Stem Cells via Sustained Wnt Pathway Activation*

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Pluripotent embryonic stem cells (ESCs) are capable of differentiating into cell types belonging to all three germ layers within the body, which makes them an interesting and intense field of research. Inefficient specific differentiation and contamination with unwanted cell types are the major issues in the use of ESCs in regenerative medicine. Lineage-specific progenitors generated from ESCs could be utilized to circumvent the issue. We demonstrate here that sustained activation of the Wnt pathway, using Wnt3A or an inhibitor of glycogen synthase kinase 3β, results in mese/endoderm-specific differentiation. Using monolayer culture conditions, we have generated multipotential “mesendodermal progenitor clones” (MPCs) from mouse ESCs by sustained Wnt pathway activation. MPCs have enhanced potential to differentiate along endothelial, cardiac, vascular smooth muscle, and skeletal lineages than undifferentiated ESCs. In conclusion, we demonstrate that the Wnt pathway activation can be utilized to generate lineage-specific progenitors from ESCs, which can be further differentiated into desired organ-specific cells.

The advantages of lineage-specific progenitor cells over ESCs are that they differentiate into a limited number of cell types of a particular lineage and, therefore, the differentiation will be robust and more efficient. In addition, they can self-renew, and thus, can be maintained as a renewable source of cells. ESCs can aggregate to form embryoid bodies (EBs), which resemble an intact embryo, and thus, many protocols for in vitro differentiation of ESCs utilize formation of EB as the first step. However, there can be a mixture of differentiating cells in using an EB approach, whereas differentiation using monolayer culture can yield more uniform and homogenous results. A successful strategy for the use of ESCs in regenerative medicine could involve formation of lineage-restricted progenitor cells using a monolayer culture system as a first step.

Gene expression analysis and fate maps together indicate that endoderm and mesoderm are derived, at least in part, from bipotent mesendodermal cells that separate during gastrulation. Active canonical Wnt signaling is detected in pregastrulating embryo, through primitive streak (PS) formation, and during gastrulation. The evolutionary conserved Wnt signaling pathway is absolutely essential for mesoderm formation and PS induction. Mice lacking the ligand Wnt3, β-catenin, or the receptors Lrp5/6 do not form PS or mesoderm, thus substantiating the importance of the pathway. In ESCs, the Wnt pathway has been shown to have effects on self-renewal and differentiation; it is specifically required for mesoderm induction via PS formation. The stability of β-catenin, the operative molecule of the canonical Wnt pathway, is controlled by glycogen synthase kinase 3β (GSK-3β) via phosphorylation and subsequent degradation. Upon Wnt pathway activation, GSK-3β is inhibited, and the non-phosphorylated β-catenin is stabilized and enters the nucleus to activate transcription of Wnt-regulated genes. Pharmacological inhibitors of GSK-3β have been used as direct intracellular activators of the canonical Wnt pathway.

The objective of this study was to utilize Wnt pathway activation of ESCs grown under monolayer culture conditions to drive differentiation toward a progenitor population. Using a specific small molecule inhibitor of GSK-3β, we have isolated multipotential “mesendodermal progenitor clones” (MPCs) from mouse ESCs. These MPCs are T+, Flk-1+, Gsc+, and Foxa2+, and they exhibit stable phenotype in culture over a year. More importantly, MPCs have significantly enhanced potential to differentiate along a variety of mesodermal cell types, includ-
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ing skeletal lineage. Thus, our findings can be utilized to differentiate ESCs into lineage-specific progenitor clones, which are multipotential but restricted to meso/endodermal differentiation. These progenitors provide a unique opportunity to better understand mechanisms of early lineage commitment and open new avenues in cell replacement therapy.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse embryonic stem cells, E14TG2a (obtained from ATCC), D3, CS, and V6.4, were cultured feeder-free on 0.1% gelatin-coated dishes in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, 1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM l-glutamine, and leukemia inhibitory factor. Cells were passaged using 0.25% trypsin every 2 days with medium changed every alternate day. Human embryonic stem cells, H1 (National Institutes of Health code: WA01), obtained from WiCell Research Institute, and HuES 6, 7, 8, and 9 (obtained from D. Melton, Harvard University, Boston, MD) were cultured as written in Supplementary A in the supplemental materials (19). 293T- cells were cultured with Dulbecco’s modified Eagle’s medium and 10% fetal calf serum. Wnt3A conditioned medium (CM) and control CM were prepared from L cells overexpressing Wnt3A as described in Ref. 20. All cells were incubated at 37 °C and 5% CO2.

Western Blotting—Total protein was extracted using lysis buffer from Cell Signaling, and 50 μg of protein was loaded per lane and resolved on 12% SDS-PAGE. For details, see Supplementary A in the supplemental materials.

Luciferase Assay—Cells transfected with SuperTOP or SuperFOP plasmids using Lipofectamine 2000 (Invitrogen) were treated as indicated, and luciferase assays were performed using a Promega kit according to the manufacturer’s instructions.

Wnt Pathway Activation and Isolation of MPCs—Mouse ESCs were cultured continuously for 3 weeks in the presence of leukemia inhibitory factor as described above either in growth medium supplemented with 1 μM GSK-3β inhibitor (Eli Lilly) or in 50% growth medium and 50% Wnt3A CM to activate the Wnt pathway. Human ESCs were grown for 2 weeks similarly except that the concentration of GSK-3β inhibitor was 2 μM for H1 and 1 μM for the HuES lines.

Isolation of MPCs—To isolate clones, single cell sorting was performed on E14 cells cultured continuously in the presence of specific inhibitor of GSK-3β (iGSK-3β, 1 μM) and leukemia inhibitory factor using BD fluorescence-activated cell sorter analyzer into 96-well dishes coated with feeders. Each well of 96-well dish had a single colony (or no colony), which was expanded in the presence of iGSK-3β (1 μM) and leukemia inhibitory factor and used for further molecular analysis and differentiation studies.

Immunoﬂuorescence Microscopy—E14 cells, H1 cells, and outgrowth EBs grown on chamber slides were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized using 0.3% Triton-X in phosphate-buffered saline incubated with primary antibodies and secondary antibodies as described in Supplementary A in the supplemental materials.

Molecular Analysis—1 μg of total RNA extracted using TRIzol reagent (Invitrogen) and purified using the spin column (Qiagen) was reverse-transcribed into cDNA using an Archive kit (Applied Biosystems). For details on RT-PCR, see Supplementary A in the supplemental materials. RT-PCR products were confirmed by sequencing. In real-time RT-PCR analysis, untreated mouse/human ESC cDNA was used as control, and data were normalized to β-actin expression and presented as -fold increase over untreated or Ctrl CM-treated ESCs.

Endothelial, Cardiac, and Osteogenic Differentiation Assays—Prior to differentiation assays, MPCs were cultured in the presence of (1 μM) iGSK-3β; however, iGSK-3β was not present during differentiation assays.

Endothelial Sprouting Assay—An endothelial sprouting assay was performed using ES-CULT endothelial kit according to the manufacturer’s instructions (StemCell Technologies). Briefly, 10-day-old EBs formed using E14 cells or MPCs in methylcellulose were harvested, and an equal number of EBs were replated in collagen solution in the presence or absence of cytokines viz vascular endothelial growth factor, basic fibroblast growth factor, interleukin-6, and erythropoietin. EBs were scored on day 3 for sprouts, indicative of endothelial cells. Molecular analysis and immunofluorescence microscopy were performed on day 3 sprouting EBs.

Cardiac Differentiation Assay—The hanging drop method (21) was employed to aggregate 625 cells/25 μl of medium for 2 days. On day 3, EBs were collected and cultured in suspension for 2 days in ES medium. On day 5, EBs were collected, and 20 EBs were plated on 0.1% gelatin-coated 10-cm dishes in ES medium in triplicate. EB was scored as a “beating EB” on day 6 only if each EB was beating in three or more separate areas.

Osteogenic Differentiation Assays—E14 cells and MPCs 23, 31, and 38 were cultured on 0.1% gelatin-coated dishes in ES medium. 24 h later, medium optimized for osteogenic differentiation Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% FBS, 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, and 10 μM β-glycerophosphate was added (22). The medium was changed every 2 days, and cells were passaged as required. Molecular analysis and histochemical stainings were performed at the end of 3 weeks.

Histochemical Staining—For all the stainings, cells were fixed using 4% paraformaldehyde for 15 min and then washed with distilled water. Cells were stained using the alkaline phosphatase kit from Chemicon. Cells were incubated in freshly prepared 2% Alizarin Red S solution for 5 min and then washed with distilled water to remove nonspecific staining. Cells were incubated with 1% Alcian blue for 30 min and washed with 0.1 M HCl, pH 1.0, to remove the excess dye.

RESULTS

Activation of Wnt Pathway Induces Differentiation of ESCs along Meso/Endoderm—Wnt signaling was induced in two ways: by the addition of active Wnt3A CM and a selective inhibitor of GSK-3β. CM made from L cells was used as a control (Ctrl CM). A well-documented luciferase reporter assay was used to assess the ability of Wnt3A and iGSK-3β to activate the Wnt pathway in ESCs. The promoter driving luciferase expression contains either functional (SuperTOP Flash) or non-functional (SuperFOP Flash) β-catenin-responsive T cell factor binding sites. The Wnt3A CM and recombinant Wnt3A (100 ng/ml), when added to mouse ESCs (E14), increased the lucif-
erase reporter activity in the TOP FLASH assays (Fig. 1A), whereas Ctrl CM did not, substantiating the contribution of Wnt3A present in the CM (Fig. 1A).

We used iGSK-3β, a specific inhibitor of GSK-3β, from Eli Lilly as an intracellular activator of the Wnt pathway and compared its activity against BIO, a GSK-3β inhibitor used by others (23). Luciferase reporter assays in E14 cells indicated that both iGSK-3β and BIO at 1 μM increased luciferase activity by several hundredfold. Induction by iGSK-3β was approximately double that of BIO, indicating higher potency of the iGSK-3β over BIO (Fig. 1A). Activation of the Wnt pathway leads to accumulation of dephosphorylated β-catenin in the cytoplasm (20). The addition of Wnt3A CM or iGSK-3β (2 μM) on human ESCs (H1) led to accumulation of active β-catenin, whereas Ctrl CM did not, as determined by Western blotting using an antibody against active/non-phosphorylated β-catenin (Fig. 1B, upper panel). Total β-catenin levels remained the same in all the treatments (Fig. 1B, lower panel). We performed immunostaining to confirm the nuclear localization of β-catenin, an indication of active Wnt signaling. Fig. 1C shows that the iGSK-3β (1 μM)-treated E14 cells (lower panel) showed nuclear accumulation of β-catenin (green), whereas untreated cells did not (upper panel). Thus, both, Wnt3A CM and iGSK-3β activate the Wnt pathway in mouse and human ESCs.

To understand the role of long term activation of the Wnt pathway in ESCs, H1 and E14 cells were treated continuously for 2 and 3 weeks, respectively, with either Wnt3A CM or iGSK-3β. Differentiation of E14 cells was assessed by real-time RT-PCR every 2 days for induction of differentiation (data not shown). The molecular analysis indicated that Wnt pathway activation led to induction of meso/endodermal markers (T-brachury, Gata2, Nkx2.5, Hand1, Foxa2, AFP, Gata4, and Sox17) in E14 cells by 7–10 days, and the induction was stronger at day 21 (Fig. 1, D and E). We observed similar results in H1 cells (supplemental Fig. 1, A and B). Ctrl CM had no effect on up-regulation of markers of differentiation. Both iGSK-3β and Wnt3A gave very similar results of meso/endodermal induction of ESCs, indicating the specific activation of the Wnt pathway and the lack of nonspecific effects of either iGSK-3β or Wnt3A CM (Fig. 1, D and E). Interestingly, neither H1 nor E14 cells expressed markers indicative of ectoderm/neuroectoderm viz Pax6, Sox4, and Nestin in response to Wnt activation (Fig. 1E and supplemental Fig. 1B).

By the end of 3 weeks, robust (up to 100-fold) up-regulations of mesodermal (T-brachury, Nkx2.5, and Hand1) and endodermal (Foxa2, AFP, Gata4, Gata6, and Sox17) markers were observed in E14 cells (Fig. 1E). A 20–100-fold increase in T-brachury and Pitx2, which are direct targets of β-catenin, confirmed activation of the Wnt pathway (Fig. 1E). Immunostaining and Western blotting were performed to confirm the induction of some of the differentiation markers. iGSK-3β-treated H1 cells showed nuclear and perinuclear staining for Foxa2 and T-brachury, respectively (Fig. 1F, right panel). The lack of staining in untreated H1 cells was indicative of the absence of proteins and nonspecific staining (Fig. 1F, left panel). We obtained similar results using H1 cells treated with Wnt3A CM (data not shown). Fig. 1G shows the presence of Foxa2 protein by Western blotting in Wnt3A- and iGSK-3β-treated H1 cells along with HepG2 cells (as a positive control). Untreated H1 cells and Ctrl CM-treated H1 cells lacked the proteins, and β-actin levels showed equal loading (Fig. 1G).

To assess whether this Wnt pathway-induced differentiation is a universal phenomenon or unique to the ESCs tested, we performed the same experiment using multiple mouse and human ESCs. Indeed, we observed that sustained Wnt pathway activation induced meso/endoderm-specific differentiation of multiple human (HuES 6, 7, 8, and 9) and mouse (D3, CS, V6.4) ESCs (supplemental Fig. 2, A–E). Thus, sustained activation of the Wnt pathway induces meso/endodermal differentiation of mouse and human ESCs.

**Active Wnt Signaling Enhances Endothelial Differentiation**—The Wnt pathway is implicated to play a role in differentiation of the vascular and hematopoietic and cardiovascular system (6, 24). Given our results that the Wnt pathway induces meso/endoderm, we hypothesized that activation of the Wnt pathway would impart upon ESCs an enhanced potential to differentiate along these lineages. We compared the potential of untreated and Wnt pathway-activated E14 cells to differentiate along endothelial lineage by performing endothelial sprout assays (see “Experimental Procedures”). About 10% of the EBs formed from untreated E14 cells formed endothelial sprouts (Fig. 1H). In contrast, Wnt pathway-activated cells displayed the significantly increased (50–70%) potential to form endothelial sprouts (Fig. 1H). To assess whether the endothelial sprout formation efficiency can be improved further, we decided to isolate single cell clones from the population of Wnt pathway-activated cells.

**Isolation and Characterization of MPCs from Mouse ESCs**—We isolated single cell clones from long term iGSK-3β-treated E14 cells to assess differences in differentiation potential represented by the individual cells. Single cell clones, hereafter referred to as MPCs, were stably maintained in the presence of iGSK-3β over a year. MPCs have slightly reduced growth rate as compared with undifferentiated ESCs. In contrast to the typical ESC colony morphology where cells are tightly packed, MPCs grow as flat monolayer colonies, and individual cells containing nuclei within a colony were easily discernable (Fig. 2A). Reduced alkaline phosphatase staining intensity indicated possible loss of pluripotency of MPCs as compared with ESCs (Fig. 2A). We also isolated single cell clones from untreated E14 cells, and molecular analysis of these clones indicated no up-regulation of lineage-specific markers confirming that they are undifferentiated ESCs (data not shown). This observation is important as it shows that MPCs are not an artifact of single cell sorting performed to isolate clones.

Molecular analysis on four randomly chosen MPCs indicates that they express a variety of primitive streak markers such as Lefty2, Sprouty2, Wnt3, FGFR8, and Nodal (Fig. 2B), and up to 1200-fold induction of Lhx1 was observed (data not shown). In addition, the MPCs express a variety of mesendodermal markers, such as PDGFRα, chordin, CK18 (Fig. 2B), and goosecoid (Fig. 2C) (25). Real-time RT-PCR on MPCs indicated a striking (up to 300-fold) induction of meso/endodermal markers such as T-brachury, Pitx2, Runx1, Runx2, Sox9, AFP, Foxa2, Gata4, and Sox17 (Fig. 2C), which was higher than that observed in the population of E14 cells treated with iGSK-3β (Fig. 1E). Interest-
ingly, the individual MPCs showed variable up-regulation of mesendodermal markers (e.g. only MPCs 23 and 38 have high levels of goosecoid and T-brachyury, and MPCs 31 and 38 have high AFP), which probably reflects their varied differentiation potential (Fig. 2C). As expected, there was minimal, if any, up-regulation of neuroectoderm markers viz NeuroD, Ngn2, Pax6, and Shh (Fig. 2C). The up-regulation of transcripts for some of the mesendodermal markers such as goosecoid, Foxa2, Sox17, and T-brachyury was confirmed by immunocytochemical analysis (Fig. 2D).
We also assessed the reversible nature of the MPCs by culturing them in the absence of iGSK-3β and performed molecular analysis on markers of differentiation. Culturing MPCs in the absence of the inhibitor led to dramatic reduction in transcript levels for all of the meso/endodermal markers to near ESC levels in 2 weeks (Fig. 2E). This indicated that continuous activation of the Wnt pathway is essential to maintain the MPCs.

In Vitro Differentiation of the Mesendodermal Progenitor Clones—We analyzed the differentiation potential of MPCs along the following mesodermal lineages.

Endothelial Lineage—We performed RT-PCR analysis to assess whether the MPCs 23 and 38 have transcripts for key endothelial markers as an indication of increased propensity to differentiate along endothelial lineage. Transcripts for Flk-1 and Flt-1, the key receptor tyrosine kinases involved in endothelial cell growth and development, along with α-SMA, VE-Cadherin, PECAM1, and podoplanin were present in the MPCs (Fig. 3A) (26–28). Untreated E14 cells had transcripts for PECAM1 and Tie2 only (Fig. 3A), indicating that Wnt pathway activation poises the MPCs to differentiate along endothelial lineage. Molecular analysis further revealed that MPCs 23 and 38 also expressed markers of hematopoietic lineage c-Kit, Scl, β-H1, and β-major, the last two only in MPC 23, and calponin, a vascular smooth muscle (VSM) marker (Fig. 3A) (28, 29). A 20–25-fold induction of Runx1, a transcription factor required for hematopoietic stem cell maintenance, was seen in both the MPCs (Fig. 2C) (30). Collectively, these results suggested that MPCs 23 and 38 are poised to differentiate along endothelial, hematopoietic, and VSM lineages.

Endothelial sprout assays were performed to analyze the endothelial/hematopoietic potential of MPCs. 50–60% EBs formed using MPCs 23 and 38 showed endothelial sprouting in the absence of growth factors, whereas near 100% efficiency of endothelial sprouting was achieved upon the addition of growth factors (Fig. 3B). Results obtained with untreated E14 cells (Fig. 3B) were consistent with the previous experiment (Fig. 1H), i.e. about 10% sprouting in the absence of growth factors. Fig. 3C shows a sprouting EB of MPC 23 as indicated by the long thin outgrowths that branch out from the dense center of the EB. The morphology of the cells in the sprouting EB was somewhat heterogeneous, ranging from large elongated flat cells (of endothelial origin) with fibroblastic morphology (Fig. 3C, black arrows) to relatively small, round cells (of hematopoietic origin) (Fig. 3D, black arrows). To confirm that the elongated flat cells were of endothelial lineage, the sprouting EBs were analyzed by immunofluorescence for cell surface markers indicative of endothelial cells. Fig. 3E shows flat and elongated endothelial-like cells immunostained for Flk-1, Flt-1, PECAM1, CD34, and Tie2 (in red). Molecular analysis on the sprouting EBs showed expression of calponin and vascular SMA (v-SMA), indicative of VSM lineage (Fig. 3F). These observations indicate that MPCs 23 and 38 have increased potential to differentiate into endothelial, hematopoietic, and VSM lineages.

Cardiac Lineage—The myocardial, endothelial, and smooth muscle lineages are believed to develop from a common Flk-1-positive progenitor (31). The presence of high levels of transcripts of T-brachyury and Flk-1 (Fig. 3, A and B) and the early cardiac markers such as Gata4, Hand1, and Tbx5 (Fig. 4A) in the MPCs 23 and 38 indicated that they are poised to differentiate along cardiac lineage (31–33). The potential of MPCs 23, 31, and 38 to differentiate along cardiac lineage was addressed by performing the two-step protocol of hanging drop aggregation followed by plating of equal number of EBs on gelatin-coated dishes (21). Beating cardiomyocytes were scored on day 6 only if beating was observed in three separate areas of an EB. 15% of the EBs formed by untreated E14 cells showed cardiac potential. EBs formed from MPCs 23 and 38 had up to 70%, whereas MPC 31 had about 35% efficiency of forming beating cardiomyocytes (Fig. 4B).

Molecular analysis on the beating EBs was performed to further confirm cardiac differentiation. Hand1, Gata4, Nkx2.5, and Tbx5 markers indicative of primary heart field cells were further up-regulated (Fig. 4C). Up-regulation of Mef2c and myocardin (Fig. 4C), MLC-2a, MLC-2v, and Tbx20 (Fig. 4D), mature cardiomyocytes markers, confirmed cardiac specification (31). Interestingly, beating EBs of MPC 31 showed no expression of MLC-2a, MLC-2v, and myocardin. In addition, neuregulin (Nrg1), a gene expressed in endocardial cells (34), detected only in MPC 38, was suggestive of endocardium (Fig. 4D). The lack of Isl1 and weak expression of FGF10 indicated the absence of secondary heart field cells (31). Beating EBs also expressed transcripts for calponin, v-SMA (Fig. 4D), Flk-1, Flt-1, and VE-cadherin (Fig. 4E). Collectively, the above observations support the conclusion that the MPCs 23 and 38 have enhanced potential to differentiate along cardiac, endothelial, and VSM lineages.

Skeletal Lineage—Canonical Wnt signaling is known to play a role in bone and cartilage development (35, 36). Accordingly, transcripts for Wnt5a and Wnt11 (exclusively in the MPCs)

**FIGURE 1.** Sustained Wnt pathway activation induces meso/endodermal differentiation of undifferentiated ESCs. A, luciferase reporter assays on E14 cells transiently transfected with reporter constructs (SuperTOP Flash (TOP) or SuperFOP Flash (FOP)) and treated with the indicated CM, purified Wnt3A (100 ng/ml), MeBIO, BIO, and iGSK-3β (1 μM each). Note that iGSK-3β is more potent than BIO. B, Western blot analysis on H1 lysates treated with the indicated CM or iGSK-3β (2 μM) using active β-catenin-specific antibody (upper panel) or total β-catenin antibody (lower panel) for equal loading. C, confocal images of immunostaining of E14 cells untreated (upper panel) or treated (lower panel) with Wnt3a CM, iGSK-3β (1 μM), and iGSK-3β (1 μM) using active β-catenin-specific antibody (Anti-ABC Ab). Cells were stained with 4′,6-diamidino-2-phenylindole (blue) for nuclear localization. No primary Ab shows the lack of nonspecific staining. D and E, E14 cells were cultured continuously for 3 weeks in the presence of control CM, Wnt3A CM, or iGSK-3β (1 μM); see “Experimental Procedures” for details). D and E, real-time RT-PCR analysis on the cells after 10 days (D) and 21 days (E), T-T Brachury, F, confocal images of H1 cells, untreated (left panel), and treated with iGSK-3β (2 μM) (right panel) for 2 weeks and immunostained with anti-Foxa2 (upper panel) and anti-T-brachury (lower panel) antibodies. G, Western blot analysis on H1 cells, treated for 2 weeks as indicated, using anti-Foxa2 and anti-β-actin (for equal loading) antibodies. HepG2 cell lysate was loaded as a positive control. H, endothelial sprout assay was performed on E14 cells, control, or treated with Wnt3a CM or iGSK-3β (1 μM) for 3 weeks as described under “Experimental Procedures.” Sprouting EBs were counted on day 3. Data are expressed as the percentage of EBs showing endothelial sprouts. For normalization of real-time RT-PCR, see “Experimental Procedures.” The entire time course of Wnt pathway activation in H1 and E14 cells, analysis of differentiation markers, and endothelial sprout formation assay were repeated three times with highly reproducible results, and the data shown are mean ± S.E. of a representative experiment.
FIGURE 2. Characterization of MPCs. A, alkaline phosphatase staining on E14 cells and MPC. B, RT-PCR analysis on the MPCs for the indicated primitive streak and mesendodermal markers. The no RT control shows the lack of genomic amplification. C, real-time RT-PCR analysis on MPCs to analyze differentiation using the indicated ectoderm and meso/endoderm-specific markers. T, T-brachyury. D, confocal images of immunocytochemical analysis performed on MPC 23 using the antibodies indicated (upper panel). Cells were stained with 4',6-diamidino-2-phenylindole (blue) for nuclear localization, and the lower panel shows the superimposed image. No Primary Ab shows the lack of nonspecific staining. E, real-time RT-PCR analysis on MPCs cultured in the absence of iGSK-3β (Recovery MPC 31 and Recovery MPC 38) for 2 weeks. E14 cells and MPCs cultured in the presence of iGSK-3β (MPC31 and MPC38) are shown for comparison.
Figure 3. MPCs have increased potential to differentiate into endothelial cells. A, RT-PCR analysis on untreated E14 cells and MPCs 23 and 38 using the indicated endothelial and hematopoietic cell-specific markers. B, endothelial Sprout assay performed on EBs formed using untreated E14 cells and MPCs 23 and 38. Data shown are mean ± S.E. of the percentage of EBs with endothelial sprouts. C, bright field picture of a sprouting EB of MPC 23 showing endothelial cells as long, thin outgrowths (black arrows) from the center of the EB. D, sprouting EB under high power; the black arrows indicate the presence of small, round cells. E, confocal images of immunostaining on sprouting EBs of MPC 23 with antibodies against indicated endothelial markers. No Ab control, control without antibody. F, RT-PCR analysis on day 3 sprouting EBs of MPCs 23 and 38 using the indicated endothelial and vascular smooth muscle markers. The no RT control shows the lack of genmic amplification. The entire experiment of forming endothelial sprouts and molecular analysis (B–F) was repeated three times with highly reproducible results, and representative results of one experiment are shown here.

along with Wnt8a and Wnt4 (also present in E14 cells) were detected in MPCs (Fig. 5A). Runx2 and Sox9, key transcription factors in bone and cartilage development, were up-regulated 50–100-fold in MPCs 23 and 38 (Fig. 2C), suggesting that these MPCs could be osteochondro progenitors (37, 38). Osteogenic differentiation potentials of the undifferentiated E14 cells, MPCs 23 and 38, were analyzed in a monolayer culture up to 3 weeks (see “Experimental Procedures”). Undifferentiated E14 cells died. The clones showed an obvious decrease in cell proliferation and survival rate but continued to grow and differentiate. Molecular analysis showed an 8–12-fold increase in bone morphogenic protein receptor 2 (BMPR2) and Msx2 transcripts suggestive of osteogenic differentiation (Fig. 5B) (39). Transcripts for PTHRI and RANKL were present along with osteocalcin and osteopontin, indicating the presence of osteoblasts (Fig. 5C) (40). We were unable to detect osterix (data not shown), indicating the absence of well differentiated osteoblasts. Runx2 and Sox9 transcript levels had increased up to 500-fold (Fig. 5D), confirming differentiation along skeletal lineage. Osteogenic differentiation was marked by high alkaline phosphatase activity in both the clones (Fig. 5, E, panels i and ii). Consistent with the presence of osteogenic markers, the presence of calcium-rich extracellular matrix released by differentiating osteoblasts was confirmed by the appearance of deep red colored Alizarin red-positive regions within the cell monolayer (Fig. 5, E, panels iii and iv).

Three members of Sox family of transcription factors Sox9, Sox5, and Sox6 are required for early chondrogenic differentiation (37, 41). Interestingly, we observed expression of Sox5 and Sox6 (Fig. 5F) and detected up to 400-fold increase in Sox9 transcripts (Fig. 5D), indicating possible differentiation along chondrocytic lineage. MPC 23 expressed Type II collagen and Aggrecan and high levels of Sox9 at the end of week 3 (Fig. 5, B, D, and F). No Alcian blue-positive regions were observed for MPC 23 (data not shown). Very weak Alcian blue staining (Fig. 5G) for MPC38 was suggestive of insufficient chondrogenic differentiation. These observations indicate that MPCs 23 and 38 have enhanced osteogenic potential than chondrogenic.

DISCUSSION

Our current study shows that activation of the Wnt pathway in mouse ESCs can be used to generate multipotential mesendodermal progenitor clones, which have significantly enhanced
potential to differentiate along a variety of mesodermal lineages as compared with undifferentiated ESCs. Activation of the Wnt pathway by using Wnt3A ligand or an intracellular activator gave us reproducible results of differentiation along meso/endoderm in multiple human and mouse ESCs. This substantiates the universal role played by the Wnt pathway in inducing differentiation of ESCs. Of significance is the finding that we did not get any differentiation along neuroectoderm, which agrees with activation of the Wnt pathway inhibits neural differentiation (42).

The lack of 100% efficiency in the endothelial sprout assay with a population of Wnt-activated E14 cells prompted us to isolate MPCs. MPCs have been in continuous culture over a year in the presence of iGSK-3/H9252, indicating a stable phenotype. Strikingly high levels of meso/endodermal markers confirmed beyond doubt that MPCs are differentiated and different from

FIGURE 4. MPCs have increased potential to differentiate into cardiomyocytes and vascular smooth muscle cells. A, real-time RT-PCR analysis on MPCs 23, 31, and 38 for the indicated early cardiac markers. B, analysis of the cardiomyocyte potential of untreated E14 cells and MPCs 23, 31, and 38 by analyzing beating potential of EBs on day 6. EBs were considered beating when beating was observed in three independent areas of an EB. Data shown are mean ± S.E. of the percentage of EBs with three or more beatings. C, real-time RT-PCR analysis on day 7 beating EBs of MPCs 23, 31, and 38 for cardiac-specific markers. D and E, RT-PCR analysis on day 7 beating EBs of MPCs 23, 31, and 38 for mature cardiomyocytes and vascular smooth muscle markers (D) and endothelial markers (E). The no RT control shows the lack of genomic amplification. The entire experiment of forming beating EBs and molecular analysis (B–E) was repeated three times with highly reproducible results, and representative results of one experiment are shown here.
starting ESCs. MPCs are characterized by expression of T-brachyury, Lefty, Nodal, Sprouty 2, and FGF8 typical markers of PS (9, 31). Mesoderm and endoderm are first formed during gastrulation, which involves movement of undifferentiated cells of the epiblast through PS. Although mesoderm is induced in each region, definitive endoderm is induced in only the anterior region of the PS (15, 43). MPCs also express Foxa2, Lhx1, Flk-1, and AFP, markers indicative of anterior PS and anterior definitive endoderm (9, 31). Based on the marker expressions and the lineage-specific differentiation potential, MPCs represent cells of anterior PS. Further evaluations, which are beyond the scope of this study, using early mouse embryos will be needed to explore this further. Interestingly, in the absence of Wnt pathway stimulation, the MPCs reverted back to ESC status. This indicates that the MPCs are not irreversibly committed to the progenitor state they are in and agrees well with the observation that epiblast cells are known to have developmental plasticity (44, 45).

The molecular analysis, immunofluorescence microscopy, and endothelial sprout formations confirmed that MPC 23 had enhanced potential to form endothelial and hematopoietic cells. “Hemangioblast,” the common progenitor for both endothelial and hematopoietic cells, is believed to be present during development (28, 46). Although we do not have conclusive evidence to establish that the MPC 23 is a hemangioblast, it is a possibility, and further analysis can potentially provide a wealth of information on the origins of hemangioblasts (1).

T-brachyury and Flk-1 expression in early mouse embryo mark a broad spectrum of mesodermal progenitors including cardiovascular progenitors (29, 31, 47–49). MPCs 23 and 38 both express T-brachyury and Flk-1, and indeed, both clones have increased potential to differentiate into beating cardiomyocytes, endothelial, and vascular smooth muscle lineages. Interestingly, MPC 31 had the least efficiency of forming beating cardiomyocytes and also had almost no expression of T-brachyury (Fig. 3B) and Flk-1 (data not shown). Thus, our results substantiate further the importance of T-brachyury and Flk-1 in specifying cardiac mesoderm.

MPCs 23 and 38 express Runx2 and Sox9, thus marking them osteochondro progenitors (35, 36). When cultured in osteogenic medium, MPCs, as expected, exhibited osteogenic differentiation but, surprisingly, we also observed chondrogenic differentiation and hypertrophy (specifically in MPC 38) in osteogenic medium. The presence of Wnt5a, Wnt4, and Wnt8a, known to promote chondrocyte differentiation and hypertro-
Wnt Signaling Generates Mesendodermal Progenitors

phy, in MPCs could be contributing to it (50, 51). High β-catenin levels are required for osterix expression, which favors formation of osteoblast over chondrocytes (35, 36). MPC 38 (unlike MPC 23) had elevated levels of Sox9 at the end of 3 weeks and no osterix expression, which could be responsible for the chondrocyte differentiation and hypertrophy. We did not activate the Wnt pathway during the osteogenic differentiation of MPCs; thus, it remains to be investigated whether we would get any change in osteoblast/chondrocyte differentiation by altering the levels of β-catenin. Such studies will also be useful in understanding the underlying molecular mechanism of β-catenin action in skeletal development. We observed that E14 cells cultured as a monolayer in osteogenic medium died, whereas MPCs survived and differentiated. ESCs can be differentiated into osteoblasts via EB formation (40). However, since formation of EBs can reduce yield of desired cell types, we believe ESC-derived MPCs present an attractive option to efficiently produce bone-generating osteoblasts in monolayer culture.

In summary, our findings reported here provide evidence that activation of the Wnt pathway is critical to generate mesendodermal progenitors from mouse ESCs. These progenitors can be a continuous source of cells with remarkable potential to differentiate along endothelial, cardiac, vascular smooth muscle, and skeletal lineages than undifferentiated ESCs. We believe that the mesendodermal progenitors can be of great value in regenerative medicine in addition to being useful as a model system to study early meso/endodermal specifications at the molecular level.

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