MULTIPOTENT MESOTHELIAL PROGENITORS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS FUNCTION IN DEVELOPMENT AND TISSUE REPAIR

by

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(Under the Direction of Stephen Dalton)

ABSTRACT

The mesothelium, which constitutes the outermost layer of the coelomic organs including the heart, lung, liver and gut, plays a critical role in the development, homeostasis and potentially in repair of the internal organs following injury or disease. During organogenesis, the mesothelium contributes to the vasculature and stroma of the developing organs in addition to acting as an important mitogenic signal. While relatively quiescent in the adult, in response to injury, the mesothelial layer has been shown to reactivate its embryonic developmental program, invade the injured tissue and release cytokines that modulate the injury response. Here, we describe highly efficient methods for the differentiation of human pluripotent stem cells (hPSCs) into mesothelial progenitor cells (MPCs) and define their developmental potential in both in vitro and in vivo models. Differential gene expression analysis of freshly isolated murine embryonic mesothelium was used to validate the characterization of our hPSC-derived MPCs as authentic mesothelium. Clonogenic assays were used to determine the in vitro differentiation potential of hPSC-derived MPCs into fibroblast, smooth muscle and
endothelial lineages and the multipotency of hPSC-derived MPCs was evaluated in vivo by assessing integration of hPSC-derived MPCs into embryonic chick hearts and mechanically-damaged neonatal mouse hearts. At the molecular level, hPSC-derived MPCs are indistinguishable from their in vivo counterparts and respond to signaling molecules that are known to impact mesothelial cell fate decisions during development as shown by their in vitro differentiation into fibroblasts, smooth muscle cells and endothelium in response to PDGF-alpha, PDGF-beta and Vegf signaling, respectively. When transplanted onto developing chick hearts, MPCs incorporate into the host mesothelium and invade the underlying myocardium. MPCs transplanted into mechanically-damaged neonatal mouse hearts migrate into damaged tissue along with endogenous epicardium-derived cells and assemble into coronary vessels in the repair zone. In addition to the utility of these cells for modeling mesothelial development and disease, this study opens up new avenues for tissue engineering and regeneration of the coelomic organs.

INDEX WORDS: Human embryonic stem cells, human induced pluripotent stem cells, mesothelium, pluripotency, differentiation, regeneration, mesenchymal stem cells
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DEDICATION

To my better half, Gregory Joseph Spillers. I will never be able to repay you for your continued patience and support over the last 4 years.
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Human Pluripotent Stem Cells

Human pluripotent stem cells (hPSCs) including human embryonic and human induced pluripotent stem cells are an invaluable resource to the study of human development and disease. Both cell types can be maintained in culture indefinitely under self-renewing conditions and have the ability to differentiate into cells of all three embryonic germ layers (endoderm, ectoderm and mesoderm) with the potential to become any cell type in the human body i.e. they are pluripotent (1, 2). This is in contrast to adult stem cells, which only have the potential to become a select subset of cell types in the human body i.e. they are multipotent. While human embryonic stem cells are isolated from the inner cell mass of pre-implantation blastocysts (1), human induced pluripotent stem cells are derived from adult somatic cells that have been reprogrammed into a pluripotent state (2). Since the first derivation of human embryonic stem cells in 1998, cell culture methods for human pluripotent stem cells have advanced tremendously. Initially cultured in undefined serum based methods, human pluripotent stem cells are now being maintained in completely chemically defined media utilizing recombinant factors that stimulate Fgf, PI3 Kinase and Activin/Nodal signaling (3, 4). The robust cultivation of human pluripotent stem cells in completely chemically defined
media enables researchers to dissect the precise cellular and molecular mechanisms underlying their self-renewal and differentiation into specialized cell types.

While human embryonic stem cells and induced pluripotent stem cells share many fundamental properties, the behavior of these cells has been found to differ slightly, especially when examining epigenetic patterning, gene expression and differentiation efficiency in these cells (5). For this reason, human embryonic stem cells are considered the gold standard for investigating the cellular and molecular events underlying early human embryonic development. On the other hand, human induced pluripotent stem cells are more advantageous for human disease modeling and the generation of cell based therapies. For instance, hIPSCs can be generated from somatic cells, such as skin fibroblasts, of a patient with a specific hereditary disease and then differentiated in culture into the specialized cell type known to produce the particular disease phenotype. This allows for the in vitro study of a patient’s specific disease in the cell type of interest as well as for the evaluation of the efficacy of candidate therapeutic drugs and their corresponding toxicities in a personalized manner. In addition, in patients with diseases where the defect is due to a loss of cells or tissue such as after myocardial infarction or spinal cord injury, hIPSCs can potentially be used to generate sufficient numbers of the depleted cells for transplantation back into the patient as a means of therapy. The benefit of using IPSC technology in this approach is that a patient’s own cells are being used for transplantation, thereby reducing the risk of immune rejection associated with allogeneic or hESC derived transplants.
Embryology of the Mesothelial Cell Layer

The mesothelium is a single layer of cells that line the serous cavities of the body (pericardial, pleural and peritoneal) as well as the outer surface of the coelomic organs located within these cavities including the heart, lung, liver and gut (6). Mesothelial cells lining the body cavities are referred to as parietal mesothelium and form a continuous lining with mesothelium lining the body organs, referred to as visceral mesothelium (see Figure 1) (7). Developmental studies of parietal and visceral mesothelium have shown that both types of mesothelium are derived from lateral plate mesoderm. Early in vertebrate development (end of 3rd week of human development), the lateral plate mesoderm, located on each side of the neural tube is divided by fusion of small coelomic spaces to form the intraembryonic coelom (8).

As shown by the chick embryo cross-section in Figure 2, this division splits the lateral plate mesoderm into two layers: the somatic or parietal mesoderm layer and the splanchnic or visceral mesoderm layer. The dorsally located somatic mesoderm layer associates with the overlying ectoderm to form the somatopleure, while the ventrally located splanchnic mesoderm associates with embryonic endoderm to form the splanchnopleure. The somatopleure and splanchnopleure give rise to the body wall and the organs of the coelomic cavity, respectively. Following lateral folding of the embryo (week 4 of human development), the somatopleure and splanchnopleure on each side of the embryo merge ventrally, creating a continuous lining of the intraembryonic coelom, which is later divided by a process of septation into the pericardial, pleural and peritoneal
cavities, dividing the mesodermal membranes into the serosal membranes of the pericardium, pleura and peritoneum, respectively (weeks 5 and 6 of human development) (8).

While the coelomic space is completely bound by mesoderm at the end of lateral folding, the mesothelium is not yet present on all the visceral organs and serosal surfaces at this stage of development (9-13). In fact, the heart and gut are only composed of two layers at this stage: endocardium/splanchnic mesoderm in the heart and endoderm/splanchnic mesoderm in the gut.

The genesis of the mesothelium from splanchnic mesoderm is not completely understood and recent evidence suggests that it may occur by different mechanisms in the various coelomic organs and their serosal membranes. Most research in this area has focused on development of the mesothelium of the heart known as the epicardium. The epicardium originates from an extra-cardiac structure known as the pro-epicardial organ, which is a protrusion of cells derived from splanchnic mesoderm covering the vitelline veins at the inflow of the developing heart (14). This protrusion of cells consists of an outer epithelial lining and an inner mesenchymal cell core. During cardiac looping, the proepicardium crosses the pericardial cavity, contacts the surface of the myocardium at the atrioventricular canal and spreads over the surface of the heart to form the epicardial layer. Subsequently, some of the cells in the epicardial layer undergo an epithelial to mesenchymal transition (15) and differentiate into the majority of the non-myocardial
cells of the heart including cardiac fibroblasts, coronary smooth muscle cells and some coronary endothelial cells (16-18).

Lineage tracing of splanchnic mesoderm markers, Islet-1 (Isl1) (19, 20) and Nkx2.5 (21-23), using conditional reporter genes have also confirmed that the mesothelium of the heart is formed from splanchnic mesoderm as reporter gene expression was detected in both pro-epicardium and myocardium. Since neither pro-epicardium or sinus horn myocardium express Isl1 or Nkx2.5 based on in situ hybridization and immunostaining, this suggests that these cell types are derived from progenitors that expressed Isl1 and Nkx2.5 at some point during development and subsequently down regulated these factors upon differentiation. The transition through a Nkx2.5 and Isl1+ intermediate during pro-epicardial development is further validated by Cre lineage tracing using Tbx18 (24) and the mesothelial cell marker, Wilms tumor 1 (Wt1) (23), where the pro-epicardium shows co-localization of Nkx2.5/Isl1 Cre lineage reporter expression with Wt1/Tbx18 Cre reporter expression even though by immunostaining, Wt1 has not been found to be present in Isl1 or Nkx2.5 expressing cells.

While Wilm et al 2005 showed that the mesothelial marker, Wilms tumor 1 first materializes in the dorsal mesentery of the gut before spreading over the intestine in a dorsal-ventral fashion similar to the mechanism of epicardial development (25), closer examination of intestinal mesothelial development by Winters et al 2013 suggests otherwise (10). First, by labeling splanchnic mesoderm HH14 chick embryos with GFP, they were able to confirm that the mesothelial layer of the gut is indeed derived from
splanchnic mesoderm similar to the mesothelium of the heart. However, in a quail chick chimera where they segmented quail splanchnopleure along the anterior-posterior axis and transplanted these segments into the coelomic cavity of chick embryos, they found that all transplants were capable of generating a trilaminar intestine, suggesting that mesothelial progenitors are located throughout the splanchnic mesoderm and are not localized to a specific region, contrary to localization of mesothelial progenitors in the pro-epicardium in the heart. Since the liver, lungs and pancreas are all gut-derived structures that are derived from endoderm and splanchnic mesoderm unlike that of the heart, which is completely derived from splanchnic mesoderm, the mesothelium of these organs may follow a pattern more closely resembling that of the gut. Further examination on the development of the mesothelium in these organs is clearly warranted.

**Structure and Function of the Mesothelium**

The mesothelial cell layer consists of a monolayer of flattened, squamous-like cells (approx. 25 um in diameter) covered with microvilli and intermittent cilia on the luminal surface (26). Mesothelial cells have well-developed cell junctions including tight junctions, adherens junctions, gap junctions and desmosomes, helping to establish a semi-permeable diffusion barrier (27). Morphologically, mesothelial cells display characteristics of both epithelial and mesenchymal cells, expressing cytokeratin like epithelial cells as well as the mesenchymal intermediate filaments, vimentin and desmin (28, 29). In normal homeostasis, the mesothelium functions as a protective barrier against physical damage and invading organisms. Through the secretion of
glycosaminoglycans such as hyaluronan and surfactant lubricants, it acts as a frictionless surface allowing unrestricted movement between adjacent organs and surfaces (30). The luminal surface of the mesothelium is also in direct contact with serosal fluid, which is an ultrafiltrate of plasma (31). The mesothelium constantly samples the state of the serosal fluid and the reacts accordingly. In the presence of pathogens, the mesothelium can secrete both pro- and anti-inflammatory cytokines to modulate the immune response as well as upregulate expression of cell adhesion molecules such as ICAM-1 and VCAM-1 to facilitate trafficking of leukocytes (32, 33).

Upon injury, the mesothelium can regulate hemostasis and fibrin clearance through the secretion of both pro-coagulants and fibrinolytic mediators (34) as well as mediate the injury response through the release of growth factors such as transforming growth factor beta (TGFβ), platelet-derived growth factor (PDGF), fibroblast growth factor (Fgf), hepatocyte growth factor (HGF) and members of the epidermal growth factor (EGF) family to stimulate proliferation, cell migration and differentiation of cells in the mesothelium and submesothelium (35). When the balance between these processes is impaired, pathologic conditions such as formation of adhesion bands and tissue fibrosis may occur (36).

Interestingly, the mesothelium has also been found to play a role in regulating tumor growth and adhesion. Following traumatic injury to the mesothelium, upregulation of adhesive molecules and secretion of growth factors such as TGFβ and Fgf into the serosal fluid have been shown to increase tumor adhesion and proliferation at sites distal
to the initial site of injury (37). In addition, the mesothelium normally secretes hyaluronan into the serosal cavity, which can bind CD44 on tumor cells and prevent their attachment to hyaluronan on the surface of the mesothelium (38). Removal of serosal fluid, which may occur following tumor resection can perturb this process and facilitate further tumor attachment.

In addition to regulating tissue homeostasis in the adult, the mesothelium plays an essential role in the development of coelomic organs through both cellular contributions to the developing organs as well as by acting as an important signaling source to underlying tissue. This will be discussed in more detail in the next few sections.

**Mesothelial-derived Cell Lineages**

Mesothelial lineage tracing studies have revealed that during organogenesis, a general trend exists in which a subset of mesothelial cells undergo an epithelial to mesenchymal transition and invade the underlying submesothelial layer. Following this invasion, these submesothelial mesenchymal cells downregulate mesothelial cell markers and differentiate into several lineages within their respective organs, suggesting that the mesothelium is a source of mesenchymal progenitors. Using lineage tracing for mesothelin, a general mesothelial surface marker, Rinkevich et al 2012 identified a mesothelial precursor population capable of clonally generating smooth muscle and fibroblasts in the coelomic organs of the body including the heart, lung, liver, gut and spleen (39) (44). While the mesothelium may give rise to similar mesenchymal lineages,
the signaling events that direct the epithelial to mesenchymal transition as well as the differentiation potential of the different types of mesothelium have been found to differ.

Organ specific differences in the regulation of mesothelial cell invasion are particularly evident when examining the role of the transcription factor Wt1 and hedgehog signaling in heart and lung mesothelial migration, respectively. Von Gise et al 2011 showed that while Wt1 is a marker of mesothelium in the coelomic organs, there are differences in the requirement of Wt1 for a mesothelial epithelial to mesenchymal transition in the heart and gut (40). In particular, in a Wt1 knockout mouse, which perturbed Wnt/beta-catenin and retinoic signaling in the epicardial layer, Wt1 derived cells failed to undergo an epithelial-mesenchymal transition (EMT) and adversely affected heart development through reduced proliferation of compact myocardium and impaired coronary plexus formation. In contrast, gut mesothelial migration was normal in the Wt1 knockout mouse. When examining the requirement of hedgehog signaling, differences between the epithelial to mesenchymal transition of the heart and lungs are also observed. In vitro and in vivo assays showed that inhibition of the hedgehog signaling pathway in mouse lung tissue impaired pleural mesothelial migration into the submesothelial layer, while epicardial derived cells were unaffected (41).

Using mesothelial lineage tracing, while there are several common lineages derived from the mesothelium of the internal organs including the fibroblast and vascular smooth muscle lineages, there are also clear differences. In particular, Msx2 lineage tracing in the liver has shown that mesothelial derived cells contribute to formation of
specialized hepatic stellate cells in addition to smooth muscle actin positive perivascular cells (42). In the lung, Wt1 lineage tracing has shown contribution of mesothelial cells to tracheal and bronchial cartilage, bronchial musculature and a population of endothelial cells in addition to smooth muscle cells and CD34+ interstitial cells (43), whereas the gut has only been shown to give rise to smooth muscle of the blood vessels and mesentery in addition to interstitial fibroblasts (44). The sole source of endothelium in the gut comes from the endothelial plexus that forms during development between the endoderm and splanchnic mesoderm layers (7). In the heart, the cellular contribution of the epicardium to various cardiac lineages has been an area of considerable debate based on conflicting findings in chick and mouse models. Quail-chick chimeras and retroviral labeling in chick models have shown that the mesenchymal cells in the subepicardial space differentiate into smooth muscle and endothelial cells of the coronary vasculature as well as perivascular and interstitial fibroblasts (16, 45). On the other hand, Cre-lox lineage tracing of the epicardial markers, Wilms tumor 1 (Wt1) and Tbx18 have shown that epicardial derived cells are only capable of differentiating into coronary smooth muscle and fibroblasts (24, 46-48). These findings, along with additional studies on a subpopulation of -3d/Scx positive progenitors present within the pro-epicardium that give rise to coronary vascular endothelium in vivo and in vitro, supports the theory that the pro-epicardium is composed of a heterogenous population of cells with various differentiation potentials (49). In addition, a small number of cardiomyocytes have been found to be labeled by Wt1 and Tbx18 lineage tracing (24, 48). However, these findings are highly controversial and may reflect an artifact of nonspecific Cre recombination or labeling of a non-epicardial Wt1/Tbx18+ cell population.
Mesothelial Cell Signaling in Embryonic Development

In addition to contributing to most of the non-parenchymal cells of the coelomic organs, the mesothelium and its derivatives are also potentially an important source of signaling during embryonic development. For example, the epicardium has been found to secrete growth factors in a paracrine manner that are essential for expansion of the myocardial layer of the heart. Evidence for the role of the epicardium in myocardial expansion comes from chick experiments in which blocking pro-epicardial outgrowth in vivo resulted in development of a thin ventricular wall and consequent embryonic lethality (50-52). Consistent with these findings, Stuckmann et al 2003 showed that in explant chick heart slices, removal of the epicardial layer results in reduced cardiomyocyte proliferation (53). Epicardial retinoic acid signaling has been implicated in this interaction between the epicardium and the myocardium.

Retinoic acid synthesis in the heart is predominantly catalyzed by the enzyme, retinaldehyde dehydrogenase 2, Raldh2 (54). Since expression of Raldh2 is temporally and spatially regulated, retinoic acid synthesis is the major control point for regulation of retinoic acid signaling. Retinoic acid functions by binding to one of three nuclear retinoic acid receptors, RARα, RARβ or RARγ, which forms a heterodimer with one of the retinoid X receptors, RXRα, RXRβ or RXRγ and consequently regulates the transcription of specific target genes. Of the six nuclear retinoic acid receptors, only mutations in the RXRα receptor show an embryonic lethal phenotype in mice. While Raldh2-/- mouse embryos show an early heart defect in cardiac looping and chamber
morphogenesis, RXRα-/- mice exhibit ventricular myocardial thinning similar to the phenotype present when development of the epicardium is perturbed (55). When RXRα is conditionally deleted in the epicardium, a phenotype indicative of myocardial proliferation and differentiation defects is apparent including the presence of a thinned compact myocardium and persistence of atrial myosin light chain (mlc2a), which is normally downregulated during myocardial maturation. However, when RXRα is conditionally deleted in the myocardium, those defects are not present (56). These findings suggest that while retinoic acid is not the mitogenic factor secreted by the epicardium to induce myocardial proliferation, retinoic acid signaling induces the epicardium to secrete a factor that is essential to myocardial expansion. Li et al 2011 identified Igf2 as the epicardial mitogen responsible for directly inducing myocardial proliferation (57). Recent studies have shown that retinoic acid induces myocardial expansion through a RA-EPO-Igf2 (retinoic acid-erythropoietin-insulin growth factor 2) signaling axis in which the production of retinoic acid stimulates hepatic erythropoietin (EPO) secretion. EPO then induces the epicardium to secrete Igf2, which binds to its cognate receptor in the ventricular myocardium and activates ERK-mediated proliferation in cardiomyocytes (58).

Epicardial fibroblast growth factor signaling may also play an important role in regulating myocardial expansion. The fibroblast growth factor family consists of 18 signaling ligands and four receptors. Of the four receptors, Fgfr1, 2 and 3 display two splice variants, b and c which are differentially expressed in various tissues. The general trend is that the b variant is expressed in epithelial-like tissues including the epicardium,
whereas the c variant is expressed in mesenchymal-like tissues including the myocardium. The epicardium is known to express Fgfs 1, 2, 4, 9, 16 and 20, while the myocardium expresses the receptors Fgfr1c/2c. Lavine et al 2005 found that Fgf9/- and Fgfr1/2/- knockout mice show a phenotype similar to RXRα/- mice in which there is evidence of severe hypoplasia in the myocardial layer of the heart marked by reduced cardiomyocyte proliferation and early death, suggesting that Fgf9 secreted by the epicardium binds to redundant Fgf receptors 1 and 2c in the myocardium to stimulate proliferation (59). Retinoic acid has been identified as the potential signal that induces Fgf9 expression in the epicardium based on organ culture experiments (55). In addition to direct signaling between the epicardium and the myocardium to induce myocardial expansion, the production of epicardial derived fibroblasts may also play a role in regulating myocardial proliferation. For example, when the Fgf10/Fgfr1/2b signaling axis between the myocardium and epicardium is perturbed, epicardial derived fibroblast do not penetrate the compact myocardium (60). This results in reduced myocardial proliferation. Further evidence for signaling between epicardial derived fibroblasts and myocardium also comes from in vitro experiments, where embryonic and not adult fibroblast stimulate proliferation of myocardium in co-culture through secretion of fibronectin, collagen, and heparin-binding EGF-like growth factor (61).

In the lung, Fgf9 is also expressed in the mesothelial layer during embryonic development and it appears to play an essential role in epithelial growth and branching. For example, when Fgf9 is conditionally deleted in the mesothelium, embryos show hypoplastic lungs with reduced mesenchyme and epithelial branching (62). Further
examination into the mechanism underlying this defect suggests that Fgf9 secreted from the mesothelium binds to Fgf1/2 receptors in the mesenchyme, which stimulates mesenchyme proliferation as well as upregulating expression of Wnt2a (63). Wnt/beta catenin signaling upregulates expression of Fgf1/2c receptors in a feed forward loop. Together, Fgfr1/2 and Wnt/beta catenin signaling in the mesenchyme act to inhibit the production of Noggin. Since Bmp4 in the lung epithelium is responsible for stimulating epithelial proliferation in an autocrine fashion, suppression of noggin, thereby releases inhibition of Bmp4 and consequently stimulates epithelial proliferation and secondarily, epithelial branching.

Anecdotal evidence suggests that the mesothelium of the other coelomic organs such as the liver plays a role in parenchymal expansion, however, the signaling mechanisms underlying these interactions are less clear. For example, in the liver, it has been shown that proliferating hepatocytes are localized to the periphery of the hepatic lobes in close proximity to the mesothelium and that the fetal mesothelium produces mitogenic growth factors, including Ptn (pleiotrophin), Mdk (midkine) and Hgf (hepatocyte growth factor) (64). When the mesothelium is co-cultured with hepatocytes, hepatocyte proliferation increases and in Wt1 knockout mice, where the normal development of the mesothelial layer of the liver is impaired, there is reduced production of the mitogenic growth factors, Ptn and Mdk by the mesothelium and consequently reduced proliferation of hepatocytes.
Mesothelial Activation in Injury and Disease

While in postnatal and adult life the mesothelium is relatively quiescent, in injury and disease the mesothelial is capable of reactivating some of its embryonic developmental programs. For example, using tamoxifen inducible Wt1 Cre lineage tracing in an uninjured adult mouse heart, it was found that the epicardium remains in the outer layer and very few if any cells invade the myocardium in stark contrast to the dynamic role of the epicardium during embryonic development (65). However, after myocardial infarction induced by ligation of the left anterior descending artery, the epicardial layer undergoes organ wide reactivation of early embryonic epicardial transcription factors including Wt1, Tbx18, Raldh2 and Tcf21 and epicardial-derived cells undergo an epithelial to mesenchymal transition and differentiate into fibroblasts and smooth muscle cells, but not cardiomyocytes or endothelial cells. The caveat is that the activation of the epicardium, incites release of pro-inflammatory cytokines and these stromal cell types give rise to the formation of scar tissue instead of functional tissue, often resulting in reduced contractility of the heart, heart failure and ultimately death.

Unlike in the mammalian adult heart, adult zebrafish (66) and neonatal mice in the first week of life (67) show potential for the regeneration of functional tissue with minimal scarring following injury. Similar to the adult mammalian heart, epicardial cells re-enter the cell cycle upon injury and migrate into the injury site. Instead of inducing fibroblast proliferation, signaling from the epicardium induces myocardial cells to divide similar to signaling in embryonic development. Studies on mammalian primary epicardial cultures show that while fetal epicardium has the ability to induce myocardial proliferation,
postnatal epicardium loses this ability. Also, postnatal cardiomyocytes show a reduced responsiveness to embryonic epicardial signaling compared to embryonic cardiomyocytes, reflecting a defect in epicardial-myocardial signaling beginning in postnatal life (68).

Similar to ischemia in the adult heart, a fibrotic response to injury and disease is also evident in the liver, lung and gut and the reactivation of the developmental potential of the mesothelium has been implicated as the culprit. As an example, in the liver, Wt1-Cre lineage tracing in an experimental model of CCl4 induced liver fibrosis revealed that the mesothelial layer contributes to formation of hepatic stellate cells, which are the major fibrogenic cell type in the liver as well as myofibroblasts (69). In the lung, the pleural mesothelium has been implicated as the source of myofibroblasts causing scarring and destruction of the lung architecture in idiopathic pulmonary fibrosis, a devastating progressive lung disease of unknown origin with no known cure (70). Also, in peritoneal sclerosis, a condition that often results from injury to the peritoneal serosa following peritoneal dialysis and in the formation of serosal adhesions following surgery or recurrent effusions, the mesothelium has been recognized as the source of fibrotic cells (71).

Understanding the signaling pathways involved in the mesothelial to mesenchymal transition is also important to the development of therapies for malignant mesothelioma. Malignant mesothelioma is typically caused by asbestos exposure and primarily affects the pleural mesothelium, but can also more rarely affect the peritoneal
and pericardial mesothelium. Asbestos exposure has been found to induce chromosomal aberrations that affect tumor suppressor genes as well as signaling pathways such as Activin A and Erk signaling that affect the proliferation and invasiveness of mesothelial cells (72).

From these examples, it is clear that several disease processes reflect the developmental potential of mesothelial cells and a further examination of the signaling cues directing their differentiation is important to our understanding of these diseases.

**Tissue Engineering Potential of Mesothelial Cells**

Tissue engineering is a growing field that uses cells, engineered scaffolds and biochemical factors to improve, repair or replace damaged or diseased tissue. As the demand for organ transplants increases, research into forming artificial organs has increased tremendously. Recently, Lu et al 2013 showed that human pluripotent stem cells can be used to repopulate a decellularized mouse heart and form contractile heart tissue (73). However, bioengineering a solid organ for longterm transplantation requires incorporation of a number of cell types, especially those that can vascularize the tissue. As a multipotent vascular progenitor and important signaling source during development, the mesothelium shows great potential for use in these types of tissue engineering approaches.
Previously, autologous mesothelial cell transplantation has been shown to be beneficial in several contexts. For example, autologous mesothelial transplantation has been shown to reduce serosal adhesion formation following serosal injury through its fibrinolytic activity (74, 75). Mesothelial cells seeded on tubular constructs have also been utilized in generating vascular (76) and nerve grafts (77, 78). In cardiac transplantation studies, adult mesothelial cells from peritoneal and epicardial origins have been shown to improve heart function after myocardial infarction (79). In these studies, however, the mesothelial cells acted primarily through paracrine signaling mechanisms. Addition of cells of an earlier developmental origin, such as the human IPS-cell derived mesothelial-like cells described here could potentially play a more direct role in tissue remodeling through cellular contribution to vascularization of the injured tissue. Further, obtaining autologous adult mesothelial cells in sufficient numbers for transplantation can in itself cause adverse side effects including fibrosis, adhesions and malignant transformation, making it a poor option for clinical applications in comparison with the use of human IPS-cell derived cells.
Figure 1.1 – Schematic of the different types of mesothelia in the human body. (Adapted from Winters and Bader, 2013) (7).
Figure 1.2 – Transverse sections of a chick embryo during heart and gut tube formation. (a) Posterior region of a HH14 chick embryo prior to lateral folding. (b) Anterior region of the HH14 embryo after lateral folding. (c) Posterior to the heart tube in a HH18 chick embryo. CC, coelomic cavity; DA, dorsal aorta; EC, endocardium; HT, heart tube; I, intermediate mesoderm; N, notochord; NT, neural tube; S, somite; So, somatic mesoderm; Sp, splanchnic mesoderm (Adapted from Winters and Bade) (7).
Figure 1.3 – Functions of the adult mesothelial cell layer. (Adapted from Mutsaers, 2004) (80).
Figure 1.4 – Signaling between the epicardium and myocardium during embryonic development. (Adapted from Brade et al Development, 2011) (58).
Figure 1.5 – The role of the mesothelium in regulating lung epithelial growth and branching during embryonic development. (Adapted from Yin et al Development 2011) (81).
CHAPTER 2

EXPERIMENTAL PROCEDURES

Culture and differentiation of hESCs and hiPS cells.

WA09 human embryonic stem cells (http://stemcells.nih.gov/research/registry/) and Fib-iPS4 human induced pluripotent stem cells (82) were adapted from mouse embryo fibroblast feeder-based culture conditions to feeder-free growth and maintained on a layer of Geltrex (Invitrogen) in chemically defined media (CDM) (3). CDM is composed of DMEM/F12 (Mediatech), 2% probumin (Bovine Serum Albumin fraction V) biotech grade (Millipore), 1 x nonessential amino acids, 50 U/mL penicillin, 50 µg/mL streptomycin, 1 x trace elements A,B,C (all from Mediatech), 10 µg/mL transferrin (Athens Research & Technology), 0.1 mM ß-mercaptoethanol (Gibco), 50 µg/mL ascorbic acid (Sigma), 8 ng/mL bFGF (Invitrogen), 200 ng/mL LR-IGF (Sigma), 10 ng/mL Activin A (R and D Systems) and 10 ng/mL Heregulin β (Peprotech). Cells were passaged every 3-4 days using Accutase (Innovative Cell Technologies) and replated as single cell suspensions on plates coated with 1:200 Geltrex (Invitrogen) at a cell density of 5 x 10⁴ cells/cm² at 37 °C in 5% CO₂. Media was changed every 24 h. To differentiate hPSCs into splanchnic mesoderm (Stage 1) (Figure 2.1), adapted WA09 and Fib-iPS4 cells were plated at a density of 5x 10⁴ cells/cm² on Geltrex-coated plates containing CDM supplemented with 25 ng/ml Wnt3a and 100 ng/mL rhBMP4 (R&D
Cells were cultured at 37° C in 5% CO₂ and media changed every 24 h for a total of 4-6 days.

In order to differentiate Stage 1 cells into mesothelial-like mesenchymal cells (MMCs), Stage 1 cells at day 4 of differentiation were cultured in mesenchymal mesothelial induction media (MMIM) consisting of CDM supplemented with 50 ng/mL rhBMP4 and 4 uM all-trans retinoic acid (Sigma) (Figure 2.1). Media was changed every 48 h for 14-18 d. To convert MMCs into mesothelial-like epithelial cells (MECs), Stage 2 cells were passaged at day 16 into epithelial induction media (EMIM) consisting of CDM plus 4 uM all-trans retinoic acid (Sigma), SB431542 (20 uM), LDN193189 (500 nM), U0126 (20 uM) (all from Tocris) without Fgf2 or Activin A at a density of 1.5 x 10⁵/cm² on Geltrex-coated plates. Media was changed every 48 hours for 12 days.

Alternatively, to convert MECs into MMCs cells, day 12 mesothelial-like epithelial cells were plated at a density of 1.5 x 10⁵/cm² on Marigel-coated. Wt1+ mesothelial-like mesenchymal cells were differentiated into mesenchymal stem cells, by passaging day 16 Stage 2 MMCs into MSC induction media consisting of 20% fetal bovine serum (Sigma), Minimal Essential Media, Alpha (Life Technologies), 50 U/mL penicillin, 50 µg/mL streptomycin (Mediatech), and Glutamax (Life Technologies) at a density of 1.0 x 10⁴/cm² on plastic for 12 days, changing media every 48 h. For Stage 3 differentiations, Stage 2 cells were passaged at day 16 into CDM for 12 days at a density of 1.5 x 10⁵/cm² supplemented with the following: endothelial cells, rhVEGF-A₁₆₅ (R&D Systems, 10ng/ml), SB 431542 (Tocris, 20 mM) with no Activin A; smooth muscle (mural) cells, PDGFB (R&D Systems, 50 ng/ml), TGFβ (R&D Systems; 2 ng/ml); fibroblast (stromal) cells, PDGFA (R&D Systems, 50 ng/ml), TGFβ (R&D Systems; 2 ng/ml) (Figure 2.1).
Flow Cytometry, qRT-PCR analysis, Western Analysis and Immuno-fluorescence

Cells were collected as single cell suspensions following removal from the culture vessel with Accutase (InVitrogen) for hESC-derived splanchnic mesoderm cells or Collagenase Type IV (800 units per ml) (Invitrogen)/0.25% Trypsin EDTA (Sigma) and then analyzed using a Cyan flow cytometer (Dako). Cells were blocked with species-specific serum for 30 min followed by blocking with CD16/32 (eBiosciences) for additional 15 min and stained with specific antibodies shown in Table 2.1. Immunofluorescence was performed on 4% paraformaldehyde-fixed cells in the presence of 10% donkey serum and 0.25% Triton X100 followed by visualization using 2.5% donkey serum in PBS with fluorescent conjugated secondary antibodies. Cells were visualized on a DM6000 B fluorescent microscope (Leica) and confocal images were performed on a LSM710 (Zeiss). mRNA was isolated using RNeasy kit (Qiagen), cDNA was made using iSCRIPT cDNA kit (BioRad) and RT-PCR was performed using TaqMan universal PCR master mix and assays on demand (Applied Biosystems) on a MyIQ (BioRad). TaqMan probe mixes are listed in Table 2.2. Analysis of qRT-PCR data was performed using a iQ5 instrument and Genex software (BioRad). hESC lysates were prepared using RIPA buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.5% SDS), then resolved on a 8-10% polyacrylamide gels. Following transfer to nitrocellulose membranes (0.45 mm; BioRad), blots were probed with primary (Table 2.1) followed by HRP conjugated secondary antibodies (Dako) and developed using Amersham ECL reagents (Amersham Biosciences).
Isolation of embryonic mouse mesothelium

\( Wt1^{\text{creERT2/}}; \) Rosa26\(^{Tdt/+} \) embryonic mice were generated by crossing \( Wt1^{\text{creERT2/}} \) mice with a knock-in of tamoxifen inducible cre-recombinase in the Wt1 locus (Jackson Laboratory Stock #010912) and Rosa26\(^{Tdt/Tdt} \) mice with a \( loxP \)-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant, tdTomato (Jackson Laboratory Stock #007914). At E12.5, pregnant dams were injected with 3 mg of tamoxifen (300 ul of 10 mg/ml tamoxifen/corn oil solution) intraperitoneally at 24 hour intervals for three consecutive days. Following, embryos were harvested at E15.5 and the heart, lung, liver and gut were carefully isolated using sterile technique. The outer mesothelial layer was digested by exposing intact organs to a dissociation buffer containing 1mg/ml Collagenase IV and 0.05% Trypsin as described by Zhou and Pu 2011 (83). Briefly, the intact organs were repeatedly digested (7-8 times) in the Collagnase IV/Trypsin dissociation buffer at 6-7 minute intervals on a 37°C shaker and the supernatants from each digest will be neutralized with horse serum, pooled and filtered through a 70uM nylon mesh (93).

After resuspending heart, lung, liver and gut digestion samples from the \( Wt1^{\text{creERT2/}}; \) Rosa26\(^{Tdt/+} \) E15.5 mice in FACS buffer (0.2% BSA: PBS), TdTomato positive (cells expressing Wt1) and tdTomato negative (cells not expressing Wt1) fractions were sorted using a Moflo (Beckman Coulter) and RNA from TdTomato positive fractions were used for differential gene expression analysis.
Differential gene expression analysis of mouse and human mesothelial samples

RNA from TdTomato positive fractions along with RNA from hESC-derived mesothelial-like epithelial cells (MECs) were extracted with Trizol (Invitrogen) and purified with RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. RNA yield was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and purified RNA (1ug) from each sample was submitted to Hudson Alpha for polyA RNA sequencing (HiSeq v4 50 PE, 25 million reads per sample). Additional raw data for human adult and fetal tissues were downloaded from the Sequence Read Archive (ERP003613, SRP001371) while additional mouse adult and fetal tissues were downloaded from ENCODE and the Sequence Read Archive (SRP049248, SRP018511). Annotation and analysis of all RNAseq data was performed by Kit Nazor, The Scripps Research Institute (TSRI). Briefly, raw mouse and human read count data were read into R and combined into species specific data frames for differential expression analyses using linear modeling strategies. The mouse sample set was comprised of 4 Wt1+ FACS-sorted mesothelium samples and replicated samples from 19 mouse tissues. The human sample set was comprised of 2 replicates of hPSC-derived epithelial mesothelium, 4 replicates of hPSC-derived mesenchymal mesothelium, and replicated samples from 26 human tissues. The RNA-seq expression sets were filtered to include genes having at least 5 reads in 2 or more samples (n_{mouse}= 18839, n_{human}=21762). Raw read counts were normalized using the trimmed mean of M-values (TMM) method (84) and precision weights were calculated using voom (85) prior to differential analysis using the limma empirical Bayes analysis pipeline (86).
human mesothelium gene expression signatures were identified via a series of comparisons between sample groups specified by contrast matrices. More specifically, mesothelium samples (independently for epithelial and mesenchymal human forms) were first compared to all tissue sample groups together as a single group (“vs_Rest”; \(n_{\text{mouse}}=19, n_{\text{human}}=26\)), to the adult tissue sample groups together as a single group (“vs_Adult”; \(n_{\text{mouse}}=17, n_{\text{human}}=18\)), to the fetal tissue sample groups together as a single group (“vs_Fetal”; \(n_{\text{mouse}}=2, n_{\text{human}}=8\)), and to each of the tissue sample groups, independently (\(n_{\text{mouse}}=19, n_{\text{human}}=26\)). Two criteria had to be met in order to be classified as a mesothelium signature gene. First, the gene had to be consistently expressed at either higher or lower levels in each of the “vs_Rest”, “vs_Adult”, and “vs_Fetal” comparisons at FDR <= 0.05. Secondly, the gene had to also be consistently enriched or depleted in at least 70% of the targeted tissue comparisons (\(n_{\text{mouse}}>=13, n_{\text{human}}>=18\)) at FDR <= 0.05.

Gene ranks were determined by sorting the genes according to the number of significant targeted tissue comparisons in descending order and, secondarily, the “vs_Rest” FDR value in ascending order. Rank percentiles were determined in the context of all detected genes in that species (\(n_{\text{mouse}}= 18839, n_{\text{human}}=21762\)).

**Clonal differentiation of human mesothelial-like progenitor cells**

PSA (CD56) (BD Pharmingen cat#556325)/ Flt4 (R&D Systems cat#FAB3492A) double positive stage 2 cells were single cell sorted into sterile 96 well plates (VWR microplate, uclear black with lid, cat#82050-748) containing one part 24 hour conditioned media: one part fresh stage 2 cell media. Media was changed every 24
hours for 4 days to allow for clonal expansion. Following, cells were subjected to one of three conditions: CDM without Activin A + PDGFα (50 ng/ml), CDM without Activin A + PDGFβ (50 ng/ml) + TGFβ1 (2ng/ml), CDM without ActivinA + Vegf165 (50 ng/ml) + SB431542 (20uM) for 12 days, changing media every 48 hours. Cells from all conditions were fixed with 4%PFA:PBS for 10 minutes and stained for either ddr2 (Santa Cruz cat#sc7555), smooth muscle actin (Abcam cat#ab7817) or (Santa Cruz cat#sc8782) von-willebrand factor (Dako cat#A0082). For each condition, 36 wells were stained and analyzed. Staining was visualized on an eVOS fl digital inverted fluorescent microscope and the number of cells positive for each marker as well as the total number of cells in each well was recorded.

**Tube forming assay**

Stage 2 cells were passaged at day 16 and cultured for an additional 12 days on Matrigel-coated dishes in CDM (- Activin A) supplemented with 50 ng/ml rhVEGF-A165 (R&D Systems) at a density of 1.5 x 10^5 cells per cm^2. Media was changed every 48 hours. Tube formation was observed from day 10 post-plating. Tubes were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and probed with antibodies for SMA, vWF and counterstained with DAPI.

**In vivo vascular forming assays**

Collagen plugs were generated by rapidly mixing cold HEPES (25 mM), 18 mM sodium bicarbonate, EBM-2 (Lonza, # cc-3156), 10% fetal bovine serum, human plasma
Chick Implantation and detection of human mesothelium-like cells

Aggregates of hPSC-derived epicardium were implanted into HH stage 14-16 chick embryos in ovo. Windows were cut into egg shells using a Dremel tool and a small slit was made in the vitelline membrane directly over the heart region using an electrolytically sharpened tungsten needle. One to four GFP⁺ cell aggregates, each containing approximately 30-200 cells, were positioned between the heart and the ventral body wall under a fluorescence stereomicroscope, leaving the endogenous pro-epicardium intact. In some cases, aggregates were treated for 2-3 min with Accutase immediately prior to implantation to weaken intercellular contacts. Egg windows were sealed with transparent tape and embryos incubated for 3-6 d, after which embryos were examined under a fluorescence stereomicroscope for the localization of GFP⁺ cells. Embryos in which aggregates had remained in contact with the heart were analyzed further. The heart, adjacent body wall and organs were dissected away from the embryos in 123 mM NaCl and fixed overnight in freshly prepared 4% paraformaldehyde in PBS at 4°C. Tissue fragments were washed in PBS, dehydrated in a graded methanol series and
then processed for immunodetection of GFP in intact tissue fragments or following paraffin sectioning. For whole mount analysis, endogenous peroxidases were quenched and embryos processed for visual immunodetection of GFP as described above. For sectioning, 10-12 mm sections were cut and processed for immunocytochemistry and immunohistochemistry according to standard procedures. Immunocytochemistry: following dewaxing and rehydration, antigen retrieval was performed using 0.1M glycine in PBS for 15 min. Sections were blocked in 5% normal goat serum in PBT for 30-60 min, then incubated for 2 h in a 1:500 dilution of rabbit anti-GFP (Invitrogen). Following washing in PBT, sections were incubated for 1 h with Alexa-flour conjugated Donkey anti-rabbit (Jackson ImmunoResearch Laboratories). Sections were washed in PBS and cover-slipped. Immunohistochemistry: following deparaffinization and rehydration, antigen retrieval was performed using antigen retrieval buffer, high pH (Dako) at 120°C for 20 min and cooled overnight. Sections were blocked with methanol/H2O2, avidin/biotin block (Dako) and protein block serum free (Dako). Sections were then incubated with the primary antibody at 37°C for 2 h in a humid chamber, washed in PBS 3 times (5 min each), then incubated with the biotinylated secondary antibody (1:100 dilution, ABC Elite Kit, Vector Laboratories) in a humid chamber for 30 min at 37°C and washed in PBS 3 times (5 min each). Finally, sections were incubated with horseradish peroxidase-streptavidin (ABC Elite kit, Vector Labs) for 15 min at RT in a humid chamber and washed in PBS 3 times (5 min each). As the last staining step, 3,3'-iaminobenzidine (DAB; Vector Labs) was added to the sections and incubated at RT until a macroscopically appreciable light brown color developed in the sections (generally 30 s
to 5 min) and washed in water. Sections were then counterstained with Gill’s hematoxylin, dehydrated and mounted in Permount (Fisher).

**Mouse embryo organ culture**

Embryonic heart, gut, lung and liver were isolated from E13.5 Wt1\textsuperscript{CreERT2/+}; Rosa\textsuperscript{26Tdr/+} mouse embryos and each organ was incubated in a single well of a 96 well plate (Fisher Scientific: cat#12-565-331) for 60 minutes in 100 ul/organ of a single cell suspension of 1x10\textsuperscript{6} Wt1+ mesothelial cells (Figure 2.2). Following, organs were washed in Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free PBS to remove unattached cells and transferred onto 15 mm Netwell inserts in a 12 well plate (Corning: cat#3477; 74um polyester mesh bottoms) containing CDM without Activin A + 1uM 4-hydroxytamoxifen (Sigma-Aldrich) in ethanol (1 ml added below insert; 0.3 ml added on top of insert). Organs were cultured at 37°C, 5% CO\textsubscript{2} for 72 hours. Media was changed every 24 hours. Organs were fixed for 30 minutes in ice cold 4% PFA in PBS, washed in PBS and then cryo-protected in 30% sucrose in PBS overnight at 4°C. Tissue was then embedded in optimum cutting temperature compound (OCT; Tissue-Tek) and snap frozen.

**Transplantation of Wt1+ mesothelial-like cells in a neonatal mechanical injury model**

$Wt1^{\text{creERT2/+}}$; Rosa\textsuperscript{26Tdr/+} and $Wt1^{+/+}$; Rosa\textsuperscript{26Tdr/+} neonatal mice generated by crossing $Wt1^{\text{creERT2/+}}$ mice (Jackson Laboratory Stock #010912) and Rosa\textsuperscript{26Tdr/Tdr} mice
(Jackson Laboratory Stock #007914) were given an intragastric injection of 1 ug of tamoxifen (Sigma) dissolved in corn oil at noon of the day of birth (P0.5). Six hours after injection, these neonatal mice were separated from their mothers and placed on ice for 5 minutes to induce hypothermia-based anesthesia. The hearts of these neonatal mice were mechanically injured as previously described by Porrello et al 2012. Briefly, a lateral thoracotomy in the 4th intercostal space was performed to expose the apex of the heart. In the sham operated mice, after the heart was exposed, the heart was gently placed back into the chest cavity and the rib cage and chest wall were surgically sewn up using 6-0 non-absorbable Prolene sutures. In mechanically injured mice, 15% of the heart was resected using iridectomy scissors. In mice receiving treatment with hESC-derived Wt1+ mesothelial-like cells, 1 million cells labeled with DiO cell labeling solution were resuspended in 2 ul of Ca^{2+} and Mg^{2+}-free PBS and injected into the pericardial cavity before sewing up the rib cage and chest wall. Once surgery was complete, the neonatal pups were allowed to recover via rapid warming and returned to their mother. The hearts from each of the treatment conditions were harvested at 5, 10, 20 and 30 days post injury. Following removal, collected hearts were washed in PBS, fixed in 4% PFA for 2 hours at 4°C, cryo-protected overnight in 30% sucrose solution and embedded in OCT. To assess the human cellular contribution to the injury site, cryosections of 10μM thickness were probed with antibodies for human-specific golgi (TGN46) or human nuclear antigen (MAB1281) and cell lineage markers including Vimentin (abcam) for fibroblasts, CD31 (Dako) for endothelial cells and Smooth muscle actin (Abcam) for smooth muscle cell identification.
Figure 2.1- Summary of differentiation pathways.
Figure 2.2 – Schematic of organ culture methods.
Table 2.1 – Details of antibodies used in this study.

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All fluorescent secondary antibodies used herein were Donkey a-species Alexa Fluors from Invitrogen or HRP conjugates from Dako
Table 2.2 - qRT-PCR TaqMan assays used in this study from Applied Biosystems.

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</tr>
<tr>
<td>Tbx20</td>
<td>Hs00396596_m1</td>
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<tr>
<td>THBD</td>
<td>Hsoo264920_s1</td>
</tr>
<tr>
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<td>Hs01675818_s1</td>
</tr>
<tr>
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<td>Hs01103754_m1</td>
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<tr>
<td>Zic1</td>
<td>Hs00602749_m1</td>
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CHAPTER 3

RESULTS

Efficient Differentiation of Human Pluripotent Cells into Splanchnic Mesoderm

Given the potential utility of embryonic mesothelial cells in cell based therapies and tissue engineering as multipotent progenitors, we set out to direct the differentiation of human embryonic stem cells into mesothelial-like cells using chemically defined media. Developmental studies have shown that the mesothelium is derived from progenitors with a lateral plate mesoderm followed by a splanchnic mesoderm origin (87, 88). Accordingly, we sought to recapitulate these developmental steps in culture by first defining the culture conditions necessary to differentiate human pluripotent stem cells into splanchnic mesoderm with high efficiency. After subjecting human pluripotent stem cells to recombinant growth factors with known mesoderm inducing activity, it was found that the most effective cocktail of factors tested was a combination of Wnt3a and Bmp4, both of which have well-established roles in early cardiac mesoderm specification (89, 90).

Following Wnt/Bmp treatment for 24 hours, Q-PCR analysis showed upregulation of brachury (T) transcripts, which suggests differentiation through a mesendoderm intermediate (Figure 3.1C). Continued exposure to Wnt3a and Bmp4 resulted in
upregulation of transcripts characteristic of lateral plate mesoderm (Isl1, FoxF1) and splanchnic mesoderm (Nkx2.5, Fgf10, Gata4 and Pitx2). Tbx18 transcripts also increased but with slightly delayed kinetics to that of Isl1 and Nkx2.5. This finding is consistent with vital dye labeling experiments in chick embryos that revealed that a group of unspecified splanchnic mesodermal cells at the venous pole of the heart express the Tbx18 and can give rise to both inflow myocardium and pro-epicardium (86). Western blot analysis further confirmed the transition of hESCs through lateral plate mesoderm and then splanchnic mesoderm states (Figure 3.1D). Brachyury protein increased by day 1 following Wnt/BMP treatment, shortly followed by increased levels of Isl1 and loss of pluripotency markers, Nanog and Oct4. Immunostaining showed that ~95% of Wnt/BMP-treated hESCs were positive for Isl1, Nkx2.5 and Tbx20 within 6 days (Figure 3.1E) and flow cytometry (Figure 3.1F) reiterated the high efficiency of this differentiation.

None of the gene expression changes associated with Wnt/Bmp treatment were observed when pluripotent cells were maintained in normal self-renewal media over the same time period and transcripts associated with somatic (Irx3), paraxial (Focc2, Tbx6), intermediate (Focc2, Tbx6, Pax2) and axial mesoderm (Pax3, 6) were not upregulated in Wnt/Bmp treated cultures (Table 3.1). Furthermore, Q-PCR analysis showed no consistent up-regulation of markers for ectoderm (Pax6, Sox1, Zic1) or primitive/definitive endoderm (AFP, HHex, Sox17, THBD) markers (Figure 3.2). We conclude that under the conditions used, Wnt/BMP treatment of hESCs results in the efficient formation of Spl-m through a LPM intermediate, at the exclusion of other
lineages. Experiments were reproduced in several iPSC lines suggesting this differentiation is generally applicable to a wide range of human pluripotent cell lines (Figure 3.3).

**Efficient Generation of Mesothelial-like Progenitor Cells from Human Pluripotent Cells**

Mesothelium lining the heart, liver and lungs are derived from splanchnic mesoderm progenitors that arise shortly following gastrulation in the developing embryo. In cardiac development, Nkx2.5$^+$ Isl1$^+$ splanchnic mesoderm give rise to progeny that colonize the pro-epicardium and then the epicardium (20, 23, 48, 88, 91). Although the exact details are yet to be resolved, Fgf, BMP and Wnt ligands have been implicated in this process (21, 88, 92). We serendipitously found that addition of all trans-retinoic acid (RA) efficiently converted SplM to a population of cells expressing markers indicative of a mesothelial identity over a 16 day period (Figure 3.4C, D, E). These markers include transcription factors (i.e. Wt1, Tbx18, Tbx5 and epicardin/Tcf21) and metabolic enzymes (i.e. Raldh2) (Figure 3.4C, D, E). Splanchnic mesoderm markers, Isl1 and Nkx2.5, were down-regulated following RA treatment (Figure 3.4C, D). The formation of these cells was recapitulated in hiPSC-derived Isl1$^+$ Nkx2.5$^+$ splanchnic mesoderm (Figure 3.5). Flow cytometry confirmed the highly efficient differentiation of these cells into mesothelial-like cells in these conditions as more than 90% of cells expressed Wt1, Raldh2 and Tbx20 (Figure 3.4F) and the surface markers, CD56 (polysialic acid) and Flt4.
(vascular endothelial growth factor receptor 3) were found to be useful for isolating pure populations of these cells prior to fixation (Figure 3.4F).

Lineage tracing studies have shown that prior to terminal differentiation into stromal and vascular lineages in the developing organs, Wt1+ cells in the mesothelial layer undergo an epithelial to mesenchymal transition and invade the underlying submesothelial layer (40). While both the epithelial and mesenchymal form express mesothelial markers including Wt1, Tbx18 and Raldh2, the mesenchymal form downregulates classical epithelial markers including E-cadherin and Zo-1. Cultured mesothelium including both transformed and primary cultures also exhibits significant morphologic plasticity (36, 93) and alternate between epithelial and mesenchymal forms following passage (94). Factors critical for this transition include TGFb/BMP (95) and Fgf2 (96) signaling. The Wt1+ Tbx18+ Raldh2+ mesothelial-like cells that we have generated from hPSCs using Bmp4, Activin A, Fgf2 and retinoic acid supplementation are mesenchymal in character and accordingly, resemble the submesothelial form in vivo (Figure 3.6A). When TGFb/BMP and Fgf2 signaling is suppressed by factor withdrawal and/or small molecule inhibition however, these cells transition to 'cobblestone'-like, E-cadherin+ ZO1+ epithelia that retain Wt1 expression (Figure 3.6B, 3.8, 3.9). Transition from a mesenchymal to epithelial state under these conditions is therefore consistent with the known behavior of cultured mesothelium. When RA was removed under conditions promoting formation of an epithelial morphology (Smad2,3/1,5,8, MEK/ERK inhibition), Wt1+ expression was lost indicating that retinoic acid signaling and TGFb/BMP/Fgf signaling control separate aspects of the mesothelial state (Figure 3.8). Smad/Fgf2
signaling therefore appears to be critical for the mesenchymal/epithelial characteristics of Wt1+ cells while RA seems critical for maintenance of a mesothelial signature. The mesenchymal to epithelial conversion of these hESC-derived mesenchymal mesothelial-like cells (MMCs) was also found to be reversible (Figure 3.7, 3.9) (i.e. the epithelial form of these cells can undergo an epithelial to mesenchymal transition).

Interestingly, when the mesenchymal form of Wt1+ mesothelial-like cells (MMCs) were plated in mesenchymal-stem cell media for 12 days, they began to resemble mesenchymal stem cells. In particular, by flow cytometry, these hESC-derived MSC-like cells express typical MSC markers including CD90, CD105, CD29, CD13 and CD73 and are negative for hematopoietic markers including CD45 and CD34 (Figure 3.10A). Further, when cultured in adipogenic, chondrogenic or osteogenic differentiation media, these Wt1-derived MSCs are capable of generating adipocytes, cartilage and bone, respectively (Figure 3.10B). Consistent with these findings, pro-epicardial MSC-like stem cells have been previously identified in mice (97).

Differential gene expression analysis of RNAseq data from freshly isolated murine heart, lung, liver and gut embryonic mesothelium was also used to further validate the characterization of our hPSC-derived Wt1+ cells as authentic mesothelium (Figure 3.11). In particular, mesothelial cells were isolated from the heart, lung, liver and gut of embryonic mice and RNAseq was used to identify a unique set of genes specifically enriched in mouse mesothelial subtypes compared to other mouse tissues. The expression profile of these genes were then used for hierarchial clustering of
RNAseq data from a collection of hESC derived cells and human tissues. Based on this analysis, we identified a mesothelial-specific gene expression signature from mouse embryonic mesothelial sub-types and found that hPSC-derived Wt1+ mesothelial progenitor cells show enrichment for mesothelial-specific gene sets.

**Wt1+ mesothelium cells are multipotent and have vascular potential**

The mesothelium is a dynamic cell type capable of giving rise to cells of a variety of different lineages. In vitro and in vivo studies have elucidated several signaling molecules that direct the differentiation of mesothelium into fibroblast, smooth muscle and endothelium during embryonic development. Platelet derived growth factor alpha (PDGFα) and beta (PDGFβ) have been implicated in the differentiation of epicardium into cardiac fibroblasts and smooth muscle, respectively (98, 99). Alternatively, vascular endothelial growth factor (Vegf) has been identified as a pro-angiogenic factor capable of driving the differentiation of epicardium to endothelial cell types (100), which together with SMCs, contribute to formation of the coronary vasculature. To validate our hESC-derived Wt1+ cells as authentic mesothelium, we examined whether these cells respond to signaling cues that are known to direct the differentiation of endogenous mesothelial cells. In these experiments, we used hESC-derived mesothelial-like mesenchymal cells (MMCs) since it has been shown in vivo that differentiation occurs following an epithelial to mesenchymal transition.
hESC-derived Wt1+ MMCs were able to differentiate into fibroblast, smooth muscle and endothelial lineages (Figure 3.12, 3.13). When plated in media containing PDGFα, cells efficiently transitioned into fibroblasts (Ddr2+, pro-collagen+, FM+) (Figure 3.12C). On the other hand, when hESC-derived Wt1+ MMCs were plated in media with PDGFβ and TGFβ1, cells efficiently transitioned to SMCs (smooth muscle actin [SMA]+, calponin+) (Figure 3.12D). Since it has previously been shown that inhibition of TGFβ1 signaling in the presence of VEGF signaling promotes endothelial cell identity in culture (101), we treated hESC-derived Wt1+ MMCs with VEGF and the ALK4 inhibitor, SB431542 to direct endothelial differentiation. In these conditions, hESC-derived Wt1+ MMCs efficiently transitioned to CD31+ vWF+ VE-Cad+ endothelial cells within 12 days (Figure 3.13).

To evaluate if hESC-derived Wt1+ MMCs are homogeneous in their multipotency, we examined their ability to differentiate toward fibroblast, smooth muscle and endothelial fates following amplification at clonal density (Figure 3.14). More specifically, single cell sorts of CD56/Flt4 double positive hESC-derived Wt1+ MMCs were amplified over several days and cultured in the differentiation conditions described above. These clonal assays resulted in highly efficient differentiations of hESC-derived MMCs into fibroblasts, smooth muscle and endothelial cells based on specificity of DDR2, SMA and vWF staining, respectively (Figure 3.14B). These results established that hESC-derived Wt1+ MMCs are uniformly multipotent and are hereon referred to as mesothelium progenitor cells (MPCs).
To establish if multipotent MPCs could form invested vessels in vitro, cells were plated on Matrigel under conditions that we identified as being capable of supporting smooth muscle and endothelial differentiation. MPCs were plated in chemically defined media containing VEGF but in the absence of SB431521 for up to 12 days, resulting in formation of a mixture of smooth muscle and endothelial cells. During this time, MPCs assemble into vessel structures (Figure 3.15A). In contrast to vessels formed by endothelial progenitors however, these vessels were invested with SMA+ SMCs and von Willebrand Factor+ (vWF+) endothelial cells (Figure 3.15A, B, C). The ability of MPCs to generate invested vessels was corroborated by subcutaneously transplanting cells within collagen plugs into flanks of SCID-beige mice (102). Host cells do not infiltrate under these condition (103) allowing for the vascular potential of transplanted cells to be directly assayed. Three weeks after transplantation, plugs were removed from mice, sectioned and evaluated for the formation of vessels. Immunocytochemistry showed the presence of smooth muscle (SMA+) and endothelial (vWF+) cells in vessels from 8/8 plugs (Figure 3.15D, E). These vessels were connected to the host vasculature, as indicated by presence of erythrocytes. Around 75% of all vessels were surrounded by a pericytic layer of cells that stained positive for smooth muscle actin (Figure 3.15E). These observations demonstrate the formation of not only capillaries (vessels lacking smooth muscle cells), but also fully invested vessels (containing smooth muscle and endothelial cells). Vessels were completely absent in 7/7 plugs transplanted without mesothelial cells (data not shown) consistent with previous findings (103). Together, these data indicate that MPCs are multipotent, vasculogenic progenitors capable of invested vessel formation.
Transplanted MPCs assemble into the mesothelial layer of multiple coelomic organs

Pro-epicardium *in vivo* spreads over the heart tube forming an outer epicardial layer and in conjunction with this a subset of these cells undergo an EMT, resulting in the invasion of underlying myocardium (104). To evaluate the invasive properties of hESC-derived Wt1+ MPCs and their ability to integrate into the epicardial layer, GFP-marked MPC aggregates were transplanted into HH stage 14-16 chick embryos, immediately adjacent to the heart tube and in the vicinity of the endogenous PE (Figure 3.16A(I)). This transplantation stage corresponds to when the heart begins to loop and when the epicardium forms in the chick. Embryos containing implanted Wt1+ MPCs were incubated for 3-6 days additional days. Visual inspection showed that approximately half (N=53) of the transplanted embryos had engrafted GFP+ cells that remained in contact with the heart. In most of these embryos, several patches of GFP+ cells were observed on the heart surface, indicative of integration into the host epicardium (Figure 3.16A(II), B). Invasion of the underlying myocardium by human cells was also observed (Figure 3.16A(III)) including their incorporation into nascent coronary vessels (Figure 3.16 B, C). hPSC-derived MPCs therefore function in a developmental context by assembling into the epicardium and nascent coronary vessels. Similarly, human MPCs integrated into the mesothelial layers of liver, lung and gut following transplantation into chick embryos, consistent with these cells having mesothelial characteristics (Figure 3.17). The chick was used as model of choice for developmental *in vivo* studies because the mouse embryo is not a feasible model due to technical barriers relating to embryo accessibility.
To evaluate the ability of hESC-derived MPCs to incorporate into mouse embryonic organs in a developmental context, *ex vivo* mouse organ cultures were used. Embryonic mouse heart, lung, liver and gut were isolated from E13.5 *Wt1*<sup>creERT2/+</sup>; Rosa26<sup>Tdt/+</sup> mice and co-cultured with hESC-derived MPCs for a period of 72 hours. Previously it has been shown that in this context, the endogenous mesothelial layer migrates into the underlying tissue, similar to the epithelial to mesenchymal transition that occurs in embryonic development *in vivo*. Using an inducible Wt1 lineage tracer, we were able to compare the behavior of recently formed endogenous Wt1+ mesothelial cells with hESC-derived MPCs. In this study, both the mesenchymal and epithelial form of hESC-derived mesothelial-like cells were used and it was found that while both forms incorporated into the outer mesothelial layer of the embryonic organs, only the mesenchymal form migrated into the underlying tissue similar to endogenous Wt1+ mesothelial cells (Figure 3.18). This suggests that the mesenchymal form is more migratory.

**MPCs function in tissue repair in vivo**

Following resection of the ventricular apex, the neonatal mouse heart undergoes significant repair including re-muscularization, neo-vascularization and some fibrosis (67). Work in zebrafish suggests that the epicardium may be part of such a response (105). To confirm a role for the epicardium in mouse neonatal repair, we used a Wt1-Cre-GFP mouse reporter line (48) to track endogenous epicardium following mechanical injury (Figure 3.19A). In unperturbed tissue GFP<sup>+</sup> cells marked the outer layer of the
heart, consistent with the mesothelial layer being labeled (Figure 3.19B). Following mechanical injury (5 days post-injury), damaged regions of the heart were re-populated by GFP+ cells, indicative that cells of epicardial origin had migrated into the injury zone (Figure 3.19B). To evaluate if hPSC-derived MPCs had any capacity for repair in this context, the ventricular apex of exposed hearts were resected and DiO-labeled MPCs were injected into the pericardial space. After surgery, pups were returned to their mothers and after 5-30 days, tissue was isolated for analysis. Whole mount immunofluorescence of injured hearts injected with DiO-labeled MPCs demonstrated that following injury, human MPCs had incorporated into the repair zone (Figure 3.20C). Sectioning of tissue from Wt1-Cre lineage traced hearts and hearts transplanted with human MPCs revealed that endogenous epicardium and transplanted human MPCs similarly integrated into the repair zone (Figure 3.21). Closer examination of the repaired tissue showed that human MPCs integrated into both the regenerated mesothelial layer and into the underlying repaired tissue. Human MPCs that integrated in the mouse epicardium maintained Tbx18 expression (Figure 3.23). In the repair zone, human MPCs contributed extensively to both stromal cells (Figure 3.24) and vessel-like structures (Figure 3.25).
Figure 3.1 - Efficient differentiation of human pluripotent cells into Isl1+ Nkx2.5+ splanchnic mesoderm. (a) General approach to specify Isl1+ splanchnic mesoderm (Spl) cells from hESCs (Stage 1 differentiation). (b) Brightfield images of untreated hESCs and hESC-derived splanchnic mesoderm. (c) Q-PCR analysis of Wnt3a and BMP4 treated WA09 hESCs over 4 d. Assays for each transcript were performed in triplicate and fold-changes shown relative to untreated hESCs after normalization with Gapdh. (d) Immunoblot analysis of T, Isl1 and Oct4 over 4 days following treatment of WA09 hESCs with Wnt3a and BMP4. Cdk2 was used as a loading control. (e) Immunostaining of untreated WA09 hESCs or, hESCs treated with Wnt3a (25 ng/ml) and BMP4 (100 ng/ml) for 6 days. Micron bar, 25 um. (f) Flow cytometry analysis following treatment of WA09 hESCs with Wnt3a and BMP4 for 6 days. Data originally generated by David Reynolds and Michael Kulik, Dalton Lab, UGA.
Figure 3.2 - Isl1+ splanchnic mesoderm forms at the exclusion of other lineages following Wnt3a/BMP4 treatment of hESCs. Q-PCR analysis of WA09 hESCs treated with Wnt3a (25 ng/ml) and BMP4 (100 ng/ml) for 0, 4, 6 d. Marker transcripts for endoderm (AFP, HHex, Sox17, THBD) and ectoderm (Pax6, Sox1, Zic1) are shown. Assays were performed in triplicate and shown relative to untreated hESC transcript levels following normalization to Gapdh. Data originally generated by David Reynolds, Dalton Lab, UGA.
Figure 3.3 - Isl1+ splanchnic mesoderm derived from hiPSCs. Fib-iPS4 hiPSCs were cultured for 4 d in hESC media (-) or differentiation media (+) containing Wnt3a (25 ng/ml) and BMP4 (100 ng/ml). (a) Q-PCR analysis of indicated mRNAs was performed in triplicate. Transcript changes are represented as the fold-increase over cells maintained in hESC media (-) following normalization to Gapdh. (b) Immunofluorescence analysis of 4% paraformaldehyde fixed cells probed with the indicated antibodies. Fib-iPS4; iPSCs maintained in self-renewal media for 4 d. + Wnt3a + BMP4; represents iPSCs cultured in media containing Wnt3a and BMP4 for 4 d. DAPI was used to stain nuclei. Micron bar, 100 um. Data originally generated by David Reynolds, Dalton Lab, UGA.
**Figure 3.4 - Efficient differentiation of hESC-derived splanchnic mesoderm to Wt1⁺ mesothelial progenitor cells.**

(a) Summary of the differentiation strategy used to derive mesothelial progenitor cells (MPC) from hESCs (ESC); Stage 2 differentiation. (b) Brightfield Images of hESC derived splanchnic mesoderm and hESC derived mesothelial progenitor cells. (c) Q-PCR analysis as hESC-derived splanchnic mesoderm transitions towards Wt1⁺ mesothelial progenitor cells following RA (4 mM) treatment. Assays were performed in triplicate on samples. Fold-changes are relative to transcript levels in hESCs after normalization to Gapdh. (d) Immunoblot analysis of cell lysates from WA09 hESCs (ESC), Isl1⁺ splanchnic mesoderm (Spl) and Wt1⁺ mesothelial progenitor cells (MPC). Cdk2 was used as a load control. (e) Immunofluorescence staining of hESC-derived mesothelial progenitor cells after 20 d differentiation in the presence of BMP4 and RA. Micron bar, 50 mm. (f) Flow cytometry analysis of hESC-derived Wt1⁺ cells.

Data originally generated by David Reynolds and Michael Kulik, Dalton Lab, UGA.
Figure 3.5 - hiPSC-derived splanchnic mesoderm differentiate to mesothelial progenitor cells in response to RA treatment. qRT-PCR analysis of Fib-iPS4 splanchnic mesoderm (Stage 1; t=0) differentiated for a further 4 d or 14 d (Stage 2) in the presence of 4 mM RA. Assays were performed in triplicate and expressed as the fold-change in transcript levels relative to splanchnic mesoderm after normalization to Gapdh. Data originally generated by David Reynolds, Dalton Lab, UGA.
Figure 3.6 – Generation of Wt1+ mesothelial-like epithelial cells from mesothelial-like mesenchymal cells. (a,b) Immunofluorescence staining of Wt1+ mesothelial-like mesenchymal cells cultured for 12 days in the presence or absence of the indicated growth factors and inhibitors. Retinoic acid (4μM), Bmp4 (50 ng/ml), ActA (10 ng/ml), Fgf2 (8ng/ml), LDN193189 (500 nM), SB431542 (20 uM) and U0126 (20 uM). Micron bar, 50 μM.
Figure 3.7 – Generation of Wt1+ mesothelial-like mesenchymal cells from mesothelial-like epithelial cells. (a,b) Immunofluorescence staining of Wt1+ mesothelial-like epithelial cells cultured for 12 days in the presence or absence of the indicated growth factors and inhibitors. Retinoic acid, (4uM), Bmp4 (50 ng/ml), ActA (10 ng/ml), Fgf2 (8ng/ml), LDN193189 (500 nM), SB431542 (20 uM) and U0126 (20 uM). Micron bar, 50 uM.
Figure 3.8 – Efficiency of the mesenchymal to epithelial conversion of hESC-derived mesothelial progenitor cells. Percentage of cells that are Wt1 positive (a) and human E-cadherin/Zo-1 double positive (b) following 12 days of culture in the presence or absence of retinoic acid, (4uM), Bmp4 (50 ng/ml), ActA (10 ng/ml), Fgf2 (8ng/ml), LDN193189 (500 nM), SB431542 (20 uM) and U0126 (20 uM). Mean +/- 95% CI for each condition displayed. Three fields at 200x magnification used to calculate mean and 95% CI for each condition.
Figure 3.9 – Gene expression analysis of the mesenchymal and epithelial forms of hESC-derived mesothelial progenitor cells. QPCR analysis of mesothelial-like mesenchymal cells (MMC), mesothelial-like epithelial cells derived from mesothelial-like mesenchymal cells (MMC>MEC) and mesothelial-like mesenchymal cells derived from mesothelial-like epithelial cells (MEC>MMC) following culture for 12 days in mesenchymal or epithelial mesothelial induction media. Assays were performed in triplicate on samples. Fold-changes are relative to transcript levels in hESCs after normalization to Gapdh.
Figure 3.10 – hESC-derived mesothelial progenitor cells differentiate into mesenchymal stem cell-like cells. (a) Flow cytometry analysis of Wt1+ mesothelial progenitor cells before (top row) and after (bottom row) being cultured for 12 days in MSC media, probed with indicated antibodies. Red, isotype control. The percentage of positive cells for each antigen is shown in each quadrant of the graph. (b) Bright field images of Oil Red O, Alizarin Red and Alcian Blue staining for undifferentiated MSCs (bottom row) and MSCs differentiated in adipogenic (top left), osteogenic (top middle) and chondrogenic (top right) media for 21 days.
Figure 3.11 - Identifying the molecular signature of mesothelium. Heatmap of the most highly conserved mesothelium signature genes (average rank percentile $\geq 0.95$ for mouse and human mesothelial-like epithelial cell signature genes) (a) and genes that are commonly used to define mesothelium (b), but not conserved according to the defined criteria. Gene names in red text were among the most highly conserved and commonly used to define mesothelium. Data analysis by Kit Nazor, The Scripps Research Institute.
Figure 3.12 - hESC-derived mesothelial progenitor cells efficiently differentiate into smooth muscle and fibroblasts in vitro. (a) Summary of the approach used to generate smooth muscle (SM) and fibroblasts (Fib) from mesothelial-like mesenchymal cells (b) Bright field images showing Epi Wt1⁺ cell aggregates plated on Matrigel for 0 and 24 h (top panels). Micron bar, 200 um. Aggregates were plated in media containing Pdgfa (50 ng/ml) or Pdgfb (50 ng/ml) for 6 days. (c) Pdgfa treated cells were fixed and probed with antibodies for DDR2, FM (fibroblast marker) antigen and pro-collagen. Cells were also stained with DAPI (DNA). Micron bar, 100 um. (d) Pdgfb treated cells were fixed and probed with antibodies for calponin and smooth muscle actin (SMA). DAPI, DNA. Top panels, micron bar, 100 um. Bottom panels, 50 um. Data generated in collaboration with Laura Menendez, Dalton Lab, UGA.
Figure 3.13 – hESC-derived mesothelial progenitor cells efficiently differentiate into endothelium in vitro. (a) Summary of the approach used to generate endothelium from mesothelial-like cells. (b) Immunostaining of Vegf (50 ng/ml) + SB (10uM) treated cells for 12 days with antibodies for vWF and CD31 (endothelial markers). (c) Flow cytometry analysis of Wt1+ cells treated with Vegf (50 ng/ml) + SB (10uM) treated cells for 12 days.
Figure 3.14 – Clonal differentiation of hESC-derived mesothelial progenitor cells into fibroblasts, smooth muscle and endothelium. (a) Schematic illustrating FACS-based strategy used for clonal analysis of Wt1+ mesothelium. (b) Percentage of cells positive for specified lineage markers based on immunostaining following culture for 12 days in fibroblast (PDGFα 50 ng/ml), smooth muscle, (TGFβ 2 ng/ml, Pdgfb 50 ng/ml) and endothelial (rhVEGF-A165 10 ng/ml, SB431542 20 mM) differentiation media. Mean +/- 95% CI for 36 wells per differentiation condition displayed.
Figure 3.15 – hESC-derived mesothelial progenitor cells form fully invested vessels *in vitro* and *in vivo*. Wt1+ mesothelial progenitor cells were plated on Matrigel coated dishes in CDM supplemented rhVEGF-A_{165} (50 ng/ml) but without SB431542. After 12 days tubes were visualized by bright field microscopy (a) or after being probed with antibodies for SMA, vWF and counterstaining with DAPI, as indicated (b,c). Wt1+ mesothelium-collagen constructs were implanted into SCID-beige mice and after 3 weeks resected, fixed, sectioned and probed with vWF (d) or SMA antibodies (e). Invested vessels were seen in 8/8 plugs. *In vivo* collagen plug experiments done by Ian White, Dalton Lab, UGA
Figure 3.16 – Integration of hESC-derived mesothelial progenitor cells during chick epicardial development. (a[i]) Immunofluorescence image of a HH stage 15 chicken embryo immediately following implantation of GFP⁺ Wt1⁺ aggregates adjacent to the developing heart (arrow). (a[ii]) Immunocytochemical visualization of hESC-derived mesothelial progenitor cells on a typical heart four days following implantation, probing with anti-GFP antibody. Three aggregates of GFP⁺ cell are shown to be integrated in the chick epicardium (arrows). (a[iii]) Immunofluorescence localization of human-derived GFP⁺ cells (lavender) in a sectioned chick embryo heart five days following implantation. Arrowheads point to representative GFP⁺ cells. A higher magnification view of the boxed area is shown at upper left. (b) Section of chick heart (as in [ii]) probed with anti-human nuclear antigen antibody (green) and vimentin (red) showing integration of transplanted human Wt1⁺ cells into the host epicardium. Tissue was prepared 4 days after transplantation. Micron bar, 25 um. (c) Section of chick heart probed with anti-human nuclear antigen antibody (blue), vimentin (green) and smooth muscle actin (red) showing incorporation of transplanted human Wt1⁺ cells into blood vessels. Tissue was prepared 4 days after transplantation. Data generated in collaboration with Tatiana Yatskievych and Parker Antin, The University of Arizona.
Figure 3.17 – Integration of hESC-derived mesothelial progenitor cells into other coelomic organs during embryonic chick development. Sections of embryonic chick lung (a), gut (b) and liver (c) probed with human nuclear antigen (green) and vimentin (red). The image shows incorporation of human Wt1⁺ cells into the mesothelium 4 days after transplantation. Data generated in collaboration with Tatiana Yatskievych and Parker Antin, The University of Arizona.
Figure 3.18 - Co-culture of hESC-derived mesothelial progenitor cells and E13.5 mouse heart, lung, liver and gut. Immunofluorescent staining following 72 hours in culture. Wt1 Cre lineage tracing, red. Human Golgi, Green. Cells were also stained with DAPI. MMC, mesothelial-like mesenchymal cells. MEC, mesothelial-like epithelial cells. Micron bar, 50 um.
Figure 3.19 – *Endogenous murine epicardial cells migrate into the injury site following mechanical injury.* (a) Schematic of Wt1 Cre lineage tracing strategy used in b. (b) Heart tissue sections demonstrating Wt1 Cre lineage tracing of epicardial derived cells in uninjured heart tissue (left panel) and a neonatal mouse mechanical injury model (right panel). Green, Wt1-derived. Red, non Wt1-derived. Data generated in collaboration with Ian White, Dalton Lab, UGA.
Figure 3.20 – Whole mount images of Wt1 Cre lineage tracing and hESC-derived human mesothelial progenitor cell incorporation in a neonatal mouse heart mechanical injury model. (a) Schematic of the inducible Wt1-Cre lineage tracing used in b. (b) Whole mount image of Wt1 Cre lineage tracing (red) in injured heart 5 days following injury. Injury was created at P0.5. (c) Whole mount image of hESC-derived mesothelial progenitor cell incorporation 5 days following injury. Human cells labeled with DiO cell labeling solution. Injury was created at P0.5.
Figure 3.21 – Endogenous murine epicardial cells and hESC-derived mesothelial progenitor cells participate in neonatal mouse heart injury repair following mechanical injury. Heart tissue sections from Wt1 Cre lineage traced neonatal mouse hearts injected with DiO labeled hESC-derived mesothelial progenitor cells 30 days following injury. Red, Wt1-derived. Cyan, DiO labeled human cells. DIC, bottom left panel. Micron bar, 50 um.
Figure 3.22 – Human golgi staining can be used to track the integration of human cells in neonatal mouse heart injury repair. Immunofluorescence staining of tissue sections from a neonatal mouse heart injected with human cells 5 days post injury. (a) Human Golgi (red) staining co-localizes with human nuclear antigen (cyan) staining (arrows). Micron bar, 20 um. (b) Human Golgi (red) co-localizes with a human-specific Vimentin staining. Left panel, micron bar, 100 um. Right panel, micron bar, 20 um.
Figure 3.23 – Integration of hESC-derived mesothelial progenitor cells into mouse epicardium following injury. Immunofluorescence staining of tissue sections 7 days post-injury with indicated antibodies. Micron bar, 30 um.
Figure 3.24 - Integration of hESC-derived mesothelial progenitor cells into the injury site following neonatal mouse heart mechanical injury. Immunofluorescence staining of tissue sections 30 days following injury with indicated antibodies. Arrows, human cell incorporation in epicardial layer. Arrowheads, human stromal cells in injury site. Micron bar, 20 um.
Figure 3.25 – Incorporation of hESC-derived mesothelial progenitor cells into blood vessels in the injury site. Immunofluorescence staining of tissue sections 30 days following injury with indicated antibodies. Micron bar, 20 μm.
Table 3.1 - Microarray analysis of hESC-derived Isl1⁺ splanchnic mesoderm. WA09 and BG02 cells were differentiated for 4 d in media containing Wnt3a (25 ng/ml) and BMP4 (100 ng/ml) and mRNA collected along with untreated WA09 and BG02 hESCs cultured for the same time in hESC media. Microarray analysis was performed using Affymetrix Human Genome U133 Plus 2.0 gene chips. Fold-changes (log 2) are compared to parent hESCs. Represented are selected genes that show no significant increase or, a decrease during Wnt/BMP treatment. Data generated by David Reynolds.
CHAPTER 4

FINAL DISCUSSION AND CONCLUSIONS

In this report, we describe highly efficient methods for the conversion of human pluripotent cells into splanchnic mesoderm and then along a pathway reminiscent of development towards a mesothelial-like fate. This is supported by molecular characterization of Wt1+ cells compared to mouse mesothelium subtypes as well as by their differentiation potential in response to known inducers of mesothelial cell fate specification. Wnt3a and Bmp4, factors implicated previously in early mesoderm induction, were used to specify pluripotent cells into Isl1+ Spl-m (89), while Bmp4, ActA, Fgf2 and retinoic acid directed differentiation of these Isl1+ splanchnic mesoderm cells into mesothelial progenitor cells with a mesenchymal morphology.

While it is currently unknown what specific growth factors induce the specification of mesothelium in vivo, hepatic endoderm has been implicated as the source of signaling directing expression of Tbx18 and Wt1 in the heart (106). For example, in the chick embryo, xenotransplantation of quail liver buds and not lung or stomach buds induces expression of proepicardial marker genes, Wt1, Tbx18 and Tcf21 in vivo and in vitro. While Bmp and Fgf secreted from lung buds do not induce expression of pro-epicardial markers, Bmp and Fgf signaling has been implicated in the separation of Tbx18 positive cells into cardiomyocytes and pro-epicardial cells. In particular, exposing
chick explants of Tbx18 positive pro-epicardial precursors to smad-mediated Bmp2 signaling directs specification to the myocardial lineage at the expense of the pro-epicardial lineage, while treatment with Erk-mediated Fgf signaling directs the reverse pathway by inhibiting the Smad receptor and preventing nuclear accumulation of p-Smad1/5/8 (88). This suggests an important role for Erk-mediated Fgf signaling in the differentiation of splanchnic mesoderm into the mesothelium of the heart.

Retinoic acid has also been previously implicated in regulating mesothelial specification, particularly through induction of Wt1 expression. In mouse and chick hearts, retinoic acid has been shown to maintain Wt1 expression as well as inhibit differentiation of epicardium into smooth muscle cells through regulation of the transcription factor, Tcf21 (107). Consistent with these findings, we found retinoic acid to be essential to maintenance of Wt1 expression when converting the mesenchymal form of these hESC-derived MPCs into epithelial MPCs and vice versa.

In early heart development, retinoic acid has also been found to regulate the boundaries of the posterior second heart field by downregulating Isl1 and Fgf8 expression, which is a necessary step towards differentiation to epicardium (108, 109). Similarly, we find that treatment with retinoic acid results in the down-regulation of Isl1 and Nkx2.5 expression. Other signaling pathways are likely to be required for mesothelial specification but currently these await detailed characterization. We anticipate that the hESC model we have developed will be a useful tool to address this question.
Understanding the morphologic plasticity of mesothelial cells and the signals that drive the epithelial to mesenchymal transition are also important to our understanding of coelomic organ development as well as the pathologies that occur when the regulation of the epithelial to mesenchymal transition is perturbed. Here, we have developed a model for studying this transition through manipulation of TGFβ/Bmp and Fgf signaling. It has previously been shown that when the epithelial to mesenchymal transition is blocked, such as that which occurs when Wt1 is knocked out in the epicardium, differentiation into nonmyocardial lineages including the coronary vasculature in the heart is also perturbed (40). This suggests that the epithelial to mesenchymal transition of mesothelial cells is required for its multipotency during development and that identification of a mesenchymal subtype of hESC-derived Wt1 cells may be useful for cell-based therapies, as the mesenchymal subtype may represent a primed form of mesothelium that could differentiate more readily than the epithelial form.

To address the question of whether the mesenchymal human mesothelial progenitor cells that we have generated are indeed multipotent, we clonally expanded hESC-derived MPCs and subjected them to fibroblast, smooth muscle and endothelial differentiation conditions. Since all clones differentiated with high efficiency into each of the indicated lineages, it suggests that these cells are homogeneous in terms of their potential. Further, in the epicardium, it has been suggested that a pro-epicardium is a heterogeneous population of cells and that only a subset of pro-epicardial cells are capable of generating endothelium (49). Accordingly, since our hESC-derived MPCs are able to efficiently differentiate into endothelial cells, it suggests that we have generated a
unique population of cells with greater vascular potential than other forms of mesothelium.

The ability of hESC-derived Wt1+ PE cells to generate fully invested vessels, comprising smooth muscle and endothelial cells, makes them an exciting option for repair and revascularization of damaged tissue, including the heart. The invasive nature of these cells as shown in both developmental and mechanical heart injury models opens up many options in terms of how these cells could be used to re-vascularize damaged tissue. Further work is now required to establish the utility of these cells in animal disease and adult repair models.
CHAPTER 5

REFERENCES


30. S. E. Mutsaers, D. Whitaker, J. M. Papadimitriou, Stimulation of mesothelial cell


52. D. J. Pennisi, V. L. T. Ballard, T. Mikawa, Epicardium is required for the full rate of myocyte proliferation and levels of expression of myocyte mitogenic factors FGF2 and its receptor, FGFR-1, but not for transmural myocardial


75. N. Di Paolo et al., Autologous peritoneal mesothelial cell implant in rabbits and


77. G. Lundborg et al., Nerve regeneration across an extended gap: A 
neurobiological view of nerve repair and the possible involvement of 

78. F. Casta eda, R. K. H. Kinne, Omental graft improves functional recovery of 

79. I. Elmadbouh et al., Mesothelial cell transplantation in the infarct scar induces 
(2005).


81. Y. Yin, F. Wang, D. M. Ornitz, Mesothelial- and epithelial-derived FGF9 have 
distinct functions in the regulation of lung development. *Development*. 138, 

82. I.-H. Park et al., Disease-specific induced pluripotent stem cells. *Cell*. 134, 877– 
886 (2008).

83. B. Zhou, W. T. Pu, in *Cardiovascular Development* (Humana Press, Totowa, NJ, 


