15

Directed Differentiation of Mesendoderm Derivatives from Embryonic Stem Cells

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1. Introduction

In amniotes, mesoderm and endoderm arise during gastrulation, the process that derives the three primary germ layers and establishes the basic body plan of the embryo. However, in recent years there has been a new appreciation for a very early stage of development, when some blastomeres are bipotential and may still contribute to either mesoderm or endoderm (but not ectoderm). This tissue has been termed "mesendoderm" (or sometimes "endomesoderm", but we will use the more common term). Specifically, experiments in nematodes, sea urchins, frogs, or zebrafish showed that when certain single cells were marked at the mid-blastula stage, the labeled cell can contribute to both mesoderm (e.g. blood, heart, muscle) and endoderm (e.g. gut, liver, pancreas) derivatives. Remarkably, the signaling molecules and genetic programs appear to be well conserved across these species (reviewed in Rodaway and Patient, 2001; Wardle and Smith, 2006). Most prominently this involves the nodal signaling pathway (Schier, 2003) and several families of regulatory proteins, including those encoding T-box and GATA transcription factors (Fig. 1). Since zebrafish and frogs are vertebrates, it seems likely that the same developmental programs should function in other vertebrates, including mouse and man.

In the mouse, the three germ layers are derived from the epiblast through gastrulation beginning at approximately day 6.5 of gestation. After implantation, the blastocyst, comprising the inner cell mass inside the trophectoderm, develops into an elongated structure composed of the ectoplacental cone, the extraembryonic ectoderm, the visceral endoderm and the epiblast. Gastrulation begins with the formation of a transient structure known as the primitive streak (PS) in the presumptive posterior end of the embryo through which uncommitted epiblast cells mobilize and egress to form the mesoderm and the endoderm (Tam et al., 2007). On the basis of developmental potential and gene expression patterns, the PS can be divided into anterior, mid and posterior regions, with mesoderm developing from the posterior region and the endoderm developing from the most anterior domain. While the close developmental association between endoderm and mesoderm supports the notion that mesendoderm also generates these two germ layers in mammals, the concept is most strongly supported by studies in the embryonic stem cell system (Tada et al., 2005).

Mouse embryonic stem (ES) cells generated from the blastocyst inner cell mass can be maintained and expanded as a pure undifferentiated population of cells when grown on mouse feeder cells in media containing leukemia inhibitory factor (LIF) and serum (Evans

and Kaufman, 1981). More importantly, ES cells are pluripotent and can be differentiated to a broad spectrum of lineages in vitro, providing a putative source of replacement cells for regenerative therapies. The isolation of human ES cells (hES) increased the interest in the possibility of cell therapies using embryonic stem cells (Thomson et al., 1998). In vitro differentiation of ES cells is induced by removing the ES cells from the feeder layer, or by removing LIF from the culture medium. The cells can be differentiated in aggregates of cells called embryoid bodies (EBs) that are grown in suspension (Fig. 2), in monolayer on extracellular matrix proteins, or in layers cultured on supportive stromal cells (Murry and Keller, 2008). Alternatively, methods have been developed for ES cell differentiation using directed differentiation, involving the addition of factors or small molecules that promote the development, differentiation, and maturation of the ES cells toward specific lineages.

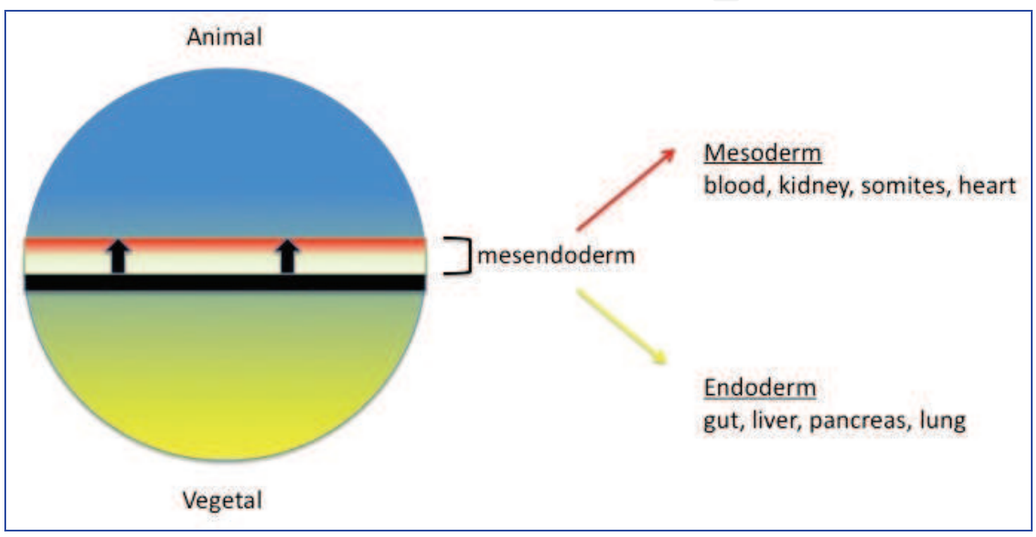

Fig. 1. Shown is a schematized mid-blastula stage zebrafish embryo with the cellularized epiblast toward the animal pole at the top (blue), and the non-cellular yolk cell (yellow) toward the vegetal pole. Unknown signals from the yolk cell induce nodal signaling (black arrows) from the margin (black) to induce bipotential mesendoderm blastomeres. These will generate mesoderm (red) and endoderm (yellow) and associated tissues as indicated by the examples listed (lung, of course, by analogy for mammals)

Directed differentiation provides great promise for generating clinically relevant cell types, including neurons, cardiomyocytes, hepatocytes, insulin-secreting pancreatic beta cells, etc. However, it is clearly not efficient to derive these specific cell types directly from ES cells. Rather, the best strategy is to exploit our knowledge of how the embryo normally generates the cell types during embryonic development, and then applying this knowledge to educate and transition the ES cells toward the desired fate. The fact of a mesendodermal transition stage presents special challenges for harvesting abundant and pure populations of specific progenitors or differentiated cell types from mesoderm or endoderm. During this stage, the same signals are essential for progenitor specification and commitment for multiple different cell types. In this review, we consider progress in directed differentiation for three mesendodermal derivatives: cardiomyocytes (from mesoderm), lung, and liver (both from closely related endoderm). For each tissue, we review what is known about the normal developmental program (signaling pathways and transcriptional regulators), and then

discuss progress in using mouse and human ES cells (and induced pluripotent, or iPS cells) to recapitulate these programs in vitro. Finally, we briefly describe the strategy we are taking to better understand the role of GATA factors as key components of specificity for derivation of mesendodermal cell types.

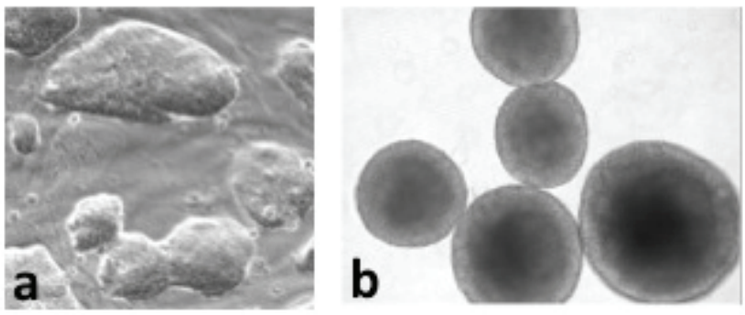

Fig. 2. a) Representative colonies of pluripotent murine ES cells. b) When ES cells are harvested by dissociation and plated back into culture in the absence of LIF, they form round aggregates called embryoid bodies, as shown here. Cells commit to fates from all three germ layers, and differentiate in EBs with a time frame roughly equivalent to normal mouse development

2. Directed differentiation of cardiac cells

Directed differentiation of stem cells toward cardiac tissue forms the basis for a diverse field of research focused on cellular-based therapeutics for myocardial infarction, heart failure, cardiomyopathies, and congenital cardiac defects. Relatively efficient protocols are documented that derive cardiovascular progenitor cells (CPCs) from murine and human ES and iPS cells. Under the appropriate conditions, the pluripotent cells generate CPCs and differentiated cardiomyocytes (CMs) (Boheler et al., 2002; Irion et al., 2008; Mauritz et al., 2008; Wei et al., 2005). Differentiation within the CPC model systems mirrors that of embryonic development, thus providing an accurate and accessible *in vitro* system that facilitates translational research. Major challenges remain to generate in a rationale manner the diversity of cardiac cells, to generate mature and functioning cardiac cells, and to integrate these cells in a productive manner into cardiac tissue.

2.1 Normal cardiogenesis

Cardiac development in mammalian embryos is defined by the expression of a set of conserved temporal and spatial markers. CPCs are derived from mesoderm, marked by the transcription factor brachyury and arising from the PS during gastrulation under the influence of the Wnt, BMP, and Nodal pathways (Tam and Behringer, 1997). Two waves of epiblast cells that are double-positive for both brachyury and fetal liver kinase-1 (Flk1) (also called kinase insert domain receptor, KDR) exit the PS. The first group is c-Kit positive and represents the hematopoietic mesoderm, and the second is the c-Kit negative cardiac mesoderm (Irion et al., 2008). In both hES and mES, early activation of the WNT, BMP, and Nodal pathways induces formation of cardiac mesoderm (Gadue et al., 2006; Laflamme et al., 2007; Lindsley et al., 2006). However, additional studies demonstrated that subsequent WNT pathway inhibition is required to specify cardiac mesoderm, likely at a time-point

after exit from the PS (Naito et al., 2006; Ueno et al., 2007). The CPCs within cardiac mesoderm are identified by their expression of Flk1/KDR and the transcription factor Nkx2.5 (Moretti et al., 2006; Wu et al., 2006). They proceed to the anterior region of the embryo and form a structure known as the cardiac crescent, which fuses to form the primitive heart tube. In addition to Nkx2.5, the GATA and T-box families of transcription factors are key regulators of cardiac development. Their precise roles are yet to be defined; however, the GATA4/5/6 and the TBX1-5, 18, and 20 genes are required for cardiogenesis in all vertebrate models (Peterkin et al., 2005; Plageman and Yutzey, 2005). Combinations of GATA, TBX and NKX2 genes are likely essential to specify cardiac fate from precardiac mesoderm. This has been demonstrated through loss-of-function experiments in both zebrafish (Holtzinger and Evans, 2007) and mouse (Zhao et al., 2008). Loss of single genes leads to morphological defects, because sister genes can functionally compensate for an earlier function in cell specification. Recently, it was shown that expression of Gata4, Tbx5, and Mef2c is sufficient to reprogram fibroblasts to a cardiac fate (Ieda et al., 2010), supporting key functions for these genes in cardiac cell specification. There are two subpopulations of CPCs within the developing heart – the first heart field, which gives rise to the left ventricle and atria, and the second heart field, which expresses Isl1 and Fgf10 and contributes to the right ventricle, outflow tract, and atria (Buckingham et al., 2005).

2.2 Directed differentiation techniques and cardiac subtype generation

Directed differentiation techniques based on recapitulating normal developmental pathways have been employed to enhance CPC generation and CM differentiation in both ES & iPS systems. Spontaneously beating CMs form within murine and human embryoid bodies in suspension cultures at a relatively low efficiency (Doetschman et al., 1985). Co-culture of hES with END-2 cells, which is thought to provide paracrine signals specifying cardiac fate, results in a relatively heterogeneous population of differentiated cells (Mummery et al., 2003). Fetal calf serum formed the basis of cell culture and CM differentiation media in many early studies; however, this is wrought with the inherent inter-batch variability of cytokines. To avoid this potential pitfall, serum-free cultures were developed and enhanced with ActivinA (a Nodal pathway activator), BMP, and Wnt inhibitors, to preferentially form CMs in both mES and hES. This strategy has been further adapted to a high-density monolayer, feeder cell-free system for hES differentiation (Laflamme et al., 2007).

Mouse ES cells display a temporal response to BMP, Wnt, and Activin, wherein there are defined time points of responsiveness and non-responsiveness to these inducing factors (Jackson et al., 2010). The presence of BMP and Wnt appears to be critical for CM specification between days 1.5 through 3 of differentiation. Nodal signaling is required from differentiation day 2 through 5. Thus, the addition of inducing factors can be optimized for CM development. Furthermore, directed differentiation would ideally permit the efficient production of individual subtypes of cardiac cells and their progenitors for therapeutic application. Nkx2.5-expressing mES-derived CMs have been shown to form ventricular, atrial, and pacemaker type cells (Hidaka et al., 2003). The precise details of individual lineage development remain to be elucidated. Lineage specification seems to occur at a relatively early time point, as several groups have shown differential induction consistent with particular CM subtypes (He et al., 2003; Kolossov et al., 2005; Mummery et al., 2003; Satin et al., 2004). Cell structure (as observed by electron microscopy) and gene expression profiles in CPC model systems progress in parallel to known developmental,

electrophysiological, and contractile maturation profiles, and several groups have been able to generate and select for subtype-specific CMs using fluorescent protein tagging techniques. For example, ventricular-like CMs were isolated by selection using eGFP expression under the control of the myosin light chain (MLC) – 2v promoter (Muller et al., 2000). Likewise, Nkx2.5 and Isl1 positive progenitors differentiate into ventricular CMs (Domian et al., 2009), while connexin 40 and 45 positive pacemaker-like CMs are generated under the influence of endothelin (Gassanov et al., 2004).

2.3 Comparison of model systems

Cardiac development is highly conserved between mouse and human models. Findings in mES models have been generally reproducible in hES models and vice versa. Since their initial description in 2006, iPS cells (Takahashi and Yamanaka, 2006) appear to be a comparable model to ES systems for generating CMs, as shown by electrophysiological and CM-specific protein expression profiles (Mauritz et al., 2008). Nonetheless, there are differences among these *in vitro* model systems that represent potential confounders for generalizing results to *in vivo* systems and considering clinical applications. CMs derived from hES proliferate to a much higher degree than those derived from mES, suggesting that there are additional poorly understood growth signaling pathways involved (McDevitt et al., 2005; Snir et al., 2003; Xu et al., 2002). However, hES differentiate at a slower pace and with a lower efficiency. The miPS are similarly slow to differentiate, form smaller CMs, and may have a predilection for a ventricular phenotype (Kuzmenkin et al., 2009; Mauritz et al., 2008).

2.4 Clinical applications

ES cell-based therapeutics for cardiac pathology rely on the ability to develop non-immunogenic, non-neoplastic, functioning CMs or CM progenitors with high fidelity and high efficiency that can be localized to a specific target region. The mES and hES cell derived CMs form stable myocardial grafts in a variety of immuno-compromised animal hosts (Dai et al., 2007; Kehat et al., 2004; Klug et al., 1996; Laflamme et al., 2005) Moreover, hES derived CMs have been shown to restore function in cardiac damaged models. They improve contractility for the infarcted murine heart and provide pacing activity in pig hearts that have undergone atrioventricular node ablation (Cai et al., 2007a; Kehat et al., 2004). Regardless of their direct therapeutic potential, both hES and hiPS already have great value as *in vitro* models for pharmaceutical testing, providing a method of noninvasive assessment of potential cardiotoxicity and arrhythmogenicity (Vidarsson et al., 2010).

3. Directed differentiation of hepatocytes

The liver is the largest internal organ with a mass that accounts for 2% to 5% of body weight. The liver is also the main detoxifying organ of the body and performs numerous essential metabolic, exocrine and endocrine functions. Metabolic functions include synthesis, storage, metabolism and redistribution of nutrients, carbohydrates, fats and vitamins and secretion of plasma proteins including albumin, clotting factors, and apolipoproteins. Endocrine functions include the secretion of insulin-like growth factors, angiotensinogen, and thrombopoietin. Exocrine secretion is mainly in the form of bile. In humans, the liver is composed of two lobes each subdivided into lobules, the basic architectural unit of the liver. Lobules are roughly hexagonal or pentagonal cylinders about 2mm high and 1mm in

diameter formed by single cell sheets of hepatocytes lined by sinusoidal capillaries that radiate toward a small branch of the hepatic vein that extends to the center of each lobule (Si-Tayeb et al., 2010a). At the periphery of each lobule, the interlobular bile ducts, the portal vein, and the hepatic artery run in parallel, forming the portal triad. The basal surface of hepatocytes absorbs metabolites and toxins from blood flowing from the portal vein and the hepatic artery through the sinusoidal capillaries, while bile is secreted from the apical surface of adjoining hepatocytes into the bile canaliculi, and then flows through the interlobular bile ducts to the extrahepatic bile ducts and into the gall bladder.

The primary functional cell types of the liver are the hepatocytes and the cholangiocytes (biliary epithelial cells). Hepatocytes are polarized epithelial cells that account for near 80% of the liver volume (Blouin et al., 1977), and carry out most of the liver functions. Cholangiocytes account for almost 3% of the liver cell population and line the bile ducts contributing to bile transportation. Other liver cell types include endothelial cells from the sinusoids and other liver vasculature, Pit cells (liver natural killer cells), Kupffer cells (resident liver macrophages), and stellate cells.

3.1 Embryonic endoderm development

Hepatocytes and cholangiocytes are derived from the embryonic definitive endoderm (DE), while the remaining liver cell types are derived from mesoderm. In vertebrates, the different populations of the PS are dependent on different levels of Nodal signaling, with the anterior region that contains the DE requiring a higher and more sustained period of Nodal signaling for its specification (Lowe et al., 2001). A conserved network of transcription factors acting downstream of Nodal signaling drives DE specification. With variations in some species, this network includes FoxA2, Gata4 and Gata6, the T-box protein Eomesodermin (Eomes), Mix-like proteins, and Sox17. FoxA2, a member of the Forkhead family of transcription factors, is essential for endoderm differentiation. FoxA2 null embryos can form hindgut but not foregut or midgut endoderm (Dufort et al., 1998), and conditional inactivation has shown that this factor is required for the development of various endoderm-derived organs (Gao et al., 2008; Lee et al., 2005; Wan et al., 2004). Gata4, Gata5 and Gata6 are involved in the specification and differentiation of the endoderm throughout evolution (Woodland and Zorn, 2008). In zebrafish and Xenopus, GATA factors are involved in endoderm patterning downstream of nodal, while in mouse Gata4 and Gata6 have an additional role regulating extraembryonic endoderm development (Zorn and Wells, 2009). However, a triple knockout mouse of all three GATA genes in epiblast-derived tissues has not been reported, leaving open the possibility that these genes are redundantly required for early endoderm development. There is precedence, since they are functionally redundant in a similar way at later stages (Holtzinger and Evans, 2007; Zhao et al., 2008). Eomes is also required for proper endoderm formation in the mouse (Arnold et al., 2008), whereas mouse Mixl1 is essential for definitive endoderm possibly through the control of mesendoderm development (Hart et al., 2002). Sox17 is also a key gene in mouse endoderm development that appears to be essential only for posterior endoderm. In the knockout mutant, anterior endoderm is generated, but posterior and lateral endoderm from midgut and hindgut are reduced and fail to expand (Kanai-Azuma et al., 2002).

Initial endoderm patterning is coincident with the formation of DE during gastrulation. The cells emerging earlier from the PS will form the foregut and those emerging later will give rise to the embryonic midgut and hindgut during morphogenesis. Morphogenetic movements of the primitive gut begin when the sheet of epithelial DE surrounding the

ventral side of the mouse embryo folds over to form two gut pockets (Lewis and Tam, 2006). Anterior axial and lateral endoderm folds ventrally to form the foregut pocket, whose opening moves posterior as the foregut develops, while the hindgut pocket opening moves anterior. Concurrently, the lateral endoderm folds ventrally to meet with the anterior and posterior folds at the yolk stalk, completing the formation of the gut tube (Zorn and Wells, 2009). Thus, morphogenetic movements during gut tube formation allow the convergence of the lateral and medial endoderm progenitors from three spatially separated embryonic domains. The primitive gut tube is patterned along the anterior-posterior axis in a manner that presages the subsequent budding of gut-derived organs along the dorsal and ventral aspect of the foregut (Fig. 3). In addition, morphogenesis results in the close apposition of the foregut progenitors with liver potential and mesoderm from the lateral plate. This relationship is essential for liver specification by inductive signals from this nearby mesoderm.

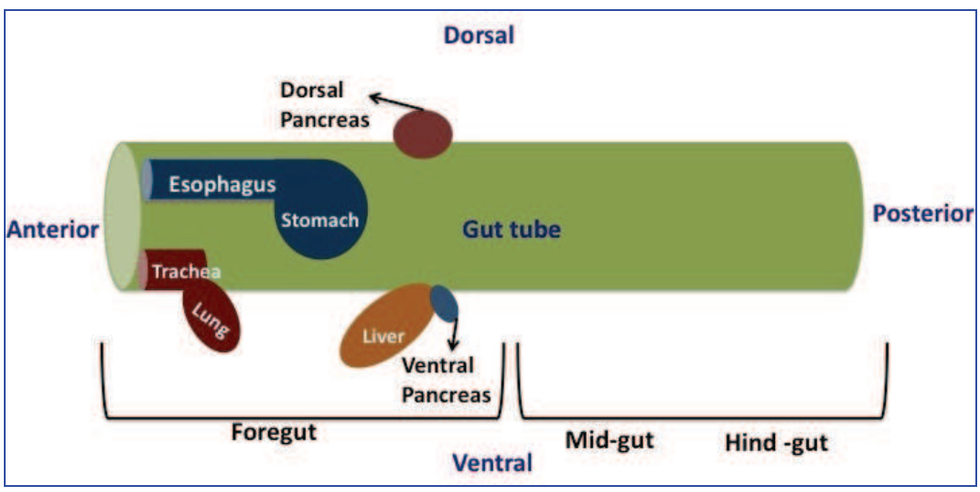

Fig. 3. Schematic illustrating the positional relationship for the various organ domains that emerge by budding from the foregut of the endoderm-derived gut tube at E 9.5 of mouse development

3.2. Liver development

Interaction between foregut endoderm and adjacent mesoderm appears to control two different steps at the onset of liver organogenesis. Initially, signals from the mesoderm regulate regional identity of the endoderm and establish foregut progenitors that are competent to develop into liver (Zorn and Wells, 2009). Genetic and chromatin occupancy studies indicate that FoxA2 and Gata4 factors either mark or help maintain the competence of the endoderm to activate liver genes such as albumin in response to inductive signals (Zaret et al., 2008). Competent cells then respond to several extracellular signals including members of the FGF and BMP families that induce specification to a hepatic fate. FGFs secreted by the cardiogenic mesoderm specifically induce mitogen-activated protein kinase (MAPK) signaling, but not phosphatidylinositol 3-kinase (PI3K) signaling during hepatic gene induction (Calmont et al., 2006). The role of FGF at the onset of liver development is evolutionarily conserved, as FGF also shows hepatogenic properties in frogs and fish. FGF signaling is not sufficient at this stage and BMP signaling from the septum transversum mesenchyme is also required for hepatic induction. BMP signaling may be mediated at least

in part by maintaining the expression of Gata4 that is essential for expression of albumin and other hepatic genes (Rossi et al., 2001). WNT signaling has also been implicated during hepatic induction but its role is complex and may not be evolutionarily conserved (Si-Tayeb et al., 2010a).

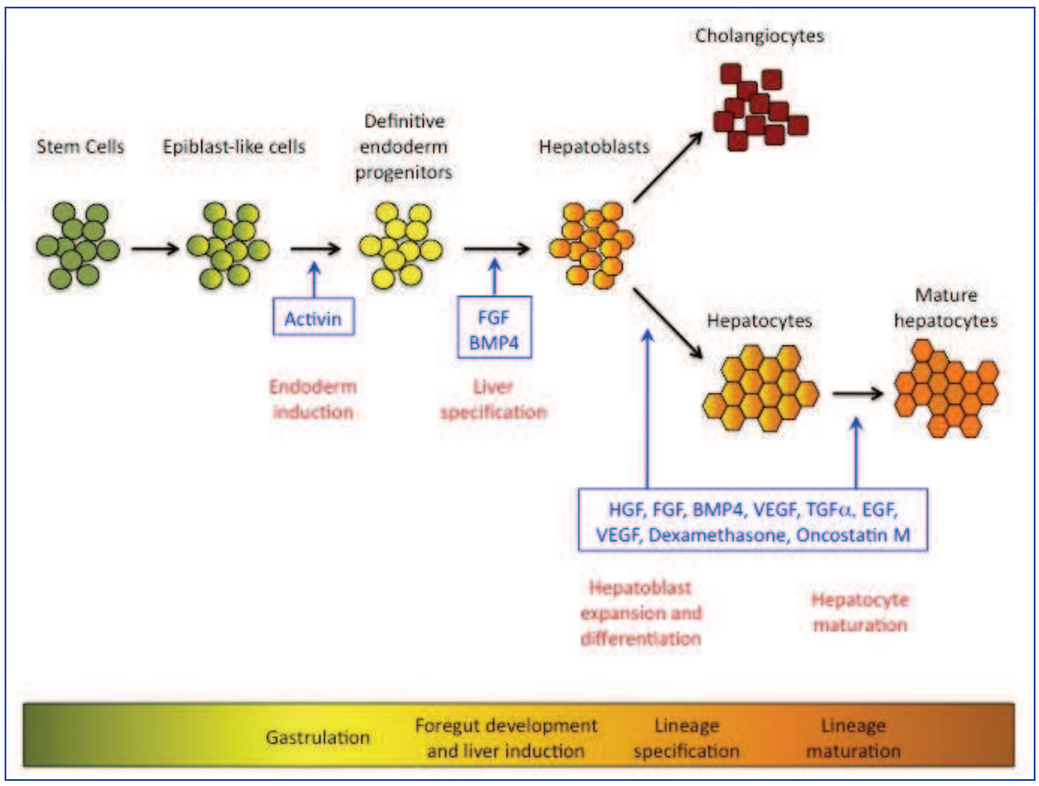

Fig. 4. The schematic illustrates the transition stages that occur in ES cell-based hepatic differentiation cultures (top) relative to normal mouse development (bottom). Also shown are key signaling factors that have been found to strongly promote the transition *in vitro* (blue), based on the known developmental pathways that are required *in vivo*. This strategy therefore demonstrates an effective directed differentiation protocol [as developed by Gouon-Evans, et al. (2006)]

Inductive signals from the cardiac mesoderm and septum transversum mesenchyme regulate the expression of a network of transcription factors, including Hex, Hnf1B, FoxA1, FoxA2 and Gata4, that is critical for the onset of hepatogenesis. At this stage the endoderm in the ventral floor of the foregut becomes thicker and expands to form an outgrowing bud of polarized proliferating cells that is separated from the surrounding mesenchyme by a basement membrane (Lemaigre, 2009). When the hepatic endoderm is specified and the liver bud begins to grow, the cells are referred to as hepatoblasts (Fig. 4). Soon after the liver bud is apparent, the basement membrane is lost and cells delaminate from the foregut and migrate as cords of hepatoblasts into the septum transversum mesenchyme. This process requires the transcription factor Prox1 (Sosa-Pineda et al., 2000). Generation of the liver bud is also accompanied by development of the hepatic vasculature. Interactions with endothelial cells derived from mesoderm are crucial for this budding phase (Matsumoto et al., 2001). Further proliferation enlarges the liver bud. FGF and BMP are also required at this

stage, together with HGF. Genetic studies have revealed that mutations suppressing proliferation and promoting apoptosis result in liver hypoplasia. Signal transduction molecules that are needed for continued fetal liver growth and to prevent apoptosis include members of the anti-apoptotic NFkB complex, integrins and the SAPK/JNK, Ras/Raf-1 and WNT/beta-catenin pathways (Tanimizu and Miyajima, 2007). Transcription factors expressed in the septum transversum mesenchyme (Hlx, N-myc and Jumonji) and hepatoblasts (Foxm1b and Xbp1) are also necessary for growth and proliferation of hepatoblasts at this stage.

Although hepatoblasts already express some genes specific for fully differentiated hepatocytes such as serum albumin, hepatoblasts will give rise to both definitive hepatocytes and cholangiocytes. Differentiation toward cholangiocytes is promoted by Notch and antagonized by HGF, that instead promotes hepatocyte differentiation (Lemaigre and Zaret, 2004). The role of Notch signaling in cholangiocyte development is conserved in fish and in humans. Alagille syndrome, characterized by reduction in intrahepatic bile ducts, is caused by mutations in the Notch ligand Jagged 1 (Li et al., 1997; Oda et al., 1997). In addition, TGF-beta and WNT signaling promotes cholangiocyte development. In response to these and other unknown mesenchymal signals, the expression of cholangiocyte transcription factors OC1, OC2, and HNF1b is increased, while expression levels of the pro-hepatic factors Hnf4a, Tbx3 and C/EBPa are down-regulated. Continued Notch, EGF and HGF signaling is essential for ductal plate remodeling. During mid-gestation, the hematopoietic cells in the liver secrete the cytokine oncostatin M, which in combination with glucocorticoid hormones, HGF and WNT promotes hepatocyte maturation. These factors act in part by regulating liver enriched transcription factors such as C/EBPa, Hnf1a, FoxA factors and Hnf4a (Zorn and Wells, 2009). The polarization of the hepatic epithelium allows the liver to develop its final architecture. After the hepatic epithelium polarizes, canaliculi are created and the basal layer becomes juxtaposed to the fenestrated endothelium that lines the sinusoids.

3.3 Hepatocyte differentiation from embryonic stem cells

The first report of spontaneous differentiation of ES cells into endodermal cells showed expression in EBs of alpha-fetoprotein (Afp), transthyretin and albumin after more than 8–10 days in culture (Abe et al., 1996). After this, reports of spontaneous differentiation were mainly focused on confirming that these markers were expressed by hepatocyte-like cells derived from definitive endoderm and not by cells in yolk sack-like structures derived from visceral endoderm that is also found in EBs (Kumashiro et al., 2005). There is a substantial overlap in markers and regulatory pathways controlling both primitive and definitive endoderm. In addition to identification and analysis of liver-specific markers, these studies characterized hepatocyte-like cells using electron microscopy, and by functional assays such as indocyanine green uptake, urea synthesis, and Periodic acid-Schiff staining for glycogen.

Because of the poorly defined nature of serum, variability between lots, and the low efficiency of protocols containing only serum, subsequent studies aimed to mimic the *in vivo* hepatic differentiation process using combinations of various factors and culture conditions. Hamazaki et al. (2001) presented the first report using growth factors, aFGF and HGF, in addition to serum, to direct the differentiation of embryonic stem cells toward a hepatic fate. Subsequently, other combinations of serum, growth factors and adherent matrices have been tested with varying success (Heo et al., 2006; Hu et al., 2004; Imamura et al., 2004; Kania et al., 2004; Shirahashi et al., 2004; Teratani et al., 2005). In general, all these protocols

direct growth of EBs in serum for several days before plating on an adherent matrix, usually collagen since this protein is found in the connective tissue of the septum transversum that harbors and promotes hepatoblast proliferation. Most protocols include FGF to mimic the secretion of this factor by the cardiac mesoderm, HGF to support mid-stage hepatogenesis, oncostatin M that is produced by hematopoietic stem cells in the embryo liver and induces maturation of fetal hepatocytes, and dexamethasone, which is a synthetic glucocorticoid hormone that induces enzymes involved in gluconeogenesis. A matrix of gelatin and HGF, insulin, transferrin and selenious acid have also been used. In addition to these growth factors and supplements, other protocols add a co-culture step with mesodermal derived cells, nonparenchymal hepatic cells or adult hepatocytes (Cho et al., 2008; Ishii et al., 2005; Shiraki et al., 2008; Soto-Gutierrez et al., 2006a).

The creation of reporter ES cells to identify and monitor early developmental stages allowed the reduction or elimination of serum and the implementation of more efficient protocols in serum-free fully defined media. To monitor DE development, genes expressed in the anterior primitive streak during embryo gastrulation, i.e., Brachyury, Goosecoid, FoxA2 and Sox17, were targeted with reporter molecules. Using these cells it was found that ActivinA, a TGF-beta family member that signals through the same receptor as nodal, efficiently induces DE progenitors *in vitro* (Kubo et al., 2004). These reporter cells also allowed the discovery of the surface receptors Cxcr4, c-Kit, E-cadherin and PDGFRa as useful markers to enrich DE progenitors differentiated from ES cells that have not been genetically modified. By translating findings from embryo development to ES cell culture (Fig. 4), FGF and BMP4 were shown to efficiently induce hepatic fate in the ActivinA-induced endoderm (Gouon-Evans et al., 2006). VEGF was also used during the specification period because the endothelial lineage promotes liver bud growth during embryo development. The hepatoblasts obtained were later expanded and matured in hepatic plating media containing EGF, bFGF, HGF, TGFa, VEGF and dexamethasone. Reporter cells have also been created to monitor and enrich differentiated hepatocyte-like cells (see below).

Efficient hepatic differentiation protocols have also been reported for human embryonic stem (hES) cells. Early reports showed that after EB formation hES cells also differentiate spontaneously into derivatives of the three germ layers, among them endodermal cells and hepatocyte-like cells expressing Afp and albumin (Itskovitz-Eldor et al., 2000; Lavon et al., 2004). An early study that aimed to direct hepatocyte differentiation from hES cells used sodium butyrate as the inducing agent (Rambhatla et al., 2003). Around 15% of the differentiated cells presented morphological features similar to that of primary hepatocytes and expressed albumin, a-1-antitrypsin, cytokeratin 8 and 18, accumulated glycogen and had inducible cytochrome P450 activity. Subsequently, differentiation toward hepatic-like cells was pursued using a variety of protocols that involve the use of growth factors, EB formation, co-culturing with hepatic and non-hepatic cell types, culture in collagen scaffold 3D culture systems, genetic manipulation, and epigenetic modifications (Agarwal et al., 2008; Baharvand et al., 2006; Basma et al., 2009; Cai et al., 2007b; Shirahashi et al., 2004; Touboul et al., 2010). Growth factors and other supplements included different combinations of aFGF, bFGF, EGF, HGF, WNT, all-trans-retinoic acid, oncostatin M, dexamethasone, insulin, transferrin and selenium. Protocols including ActivinA and using monolayer growth instead of EB formation have increased the yield of hES cell-derived definitive endoderm (D'Amour et al., 2005). These protocols have led to higher efficiency in the production of cells displaying hepatic functions including ureagenesis, glycogen storage, albumin, fibrinogen and fibronectin secretion, and CYP activity. Recently, a protocol that

initially enriched definitive endoderm precursors to more than 85% purity generated a population with 90% of cells expressing albumin after hepatocyte differentiation (Duan et al., 2010). Importantly, this population appeared to have complete metabolic function and had the capacity to metabolize drugs at levels that were comparable to primary hepatocytes.
Ethical and safety debates about the use of embryonic stem cells for research and therapy have stimulated the search of alternative approaches to generate pluripotent stem cells. Thus, hES cells may soon be outpaced by iPS cells, generated from postnatal cells by viral-mediated transfer of reprogramming genes (Yamanaka and Blau, 2010). Human iPS cells also open the possibility of creating customized pluripotent stem cell lines which may not only be critical in cell therapy but also in assessing human drug toxicity. More recently iPS cells have been used successfully to model inherited metabolic disorders of the liver (Rashid et al., 2010). Hepatocyte-like cells have been generated from human-iPS cells, including cells obtained by reprogramming of human primary hepatocytes (Liu et al., 2010; Si-Tayeb et al., 2010b; Song et al., 2009; Sullivan et al., 2010; Touboul et al., 2010). Most of these protocols used fully defined culture conditions with growth factors and cytokines that recapitulate essential stages of liver development. In fact, in some cases iPS cells were able to generate hepatocytes more robustly than hES cells, suggesting efficacy of these protocols with pluripotent stem cells of diverse origins (Sullivan et al., 2010). Importantly, iPS cells derived from human foreskin fibroblasts were efficiently induced to form hepatocyte-like cells in culture that were able to integrate into the hepatic parenchyma *in vivo* (Si-Tayeb et al., 2010b).

3.4 Clinical applications and challenges for treating liver disease

Due to the critical roles of the liver in many metabolic processes, liver disease leads to high rates of morbidity and mortality. Liver disease is the fourth leading cause of death among middle-aged adults in the United States. Treatment for patients with acute hepatic failure or end-stage liver disease relies on liver transplantation with approximately 6,000 liver transplant operations performed in the United States every year. The strong regenerative capacity of the liver makes it possible for a healthy donor to provide a portion of liver for transplantation, although scarcity of organ donors limits the efficacy. Therefore, transplantation of hepatocytes is an option to replace whole liver transplantation. Unfortunately, the source of hepatocytes is also limited and cell therapy using hepatocytes or hepatocyte-like cells differentiated from embryonic stem cells or the recently developed iPS cells, is therefore an attractive alternative. While the use of hepatocyte-like cells from ES or iPS cells in the clinic may be a long-term goal, their utility in the short term resides in the assessment of drug toxicity and safety during drug development. Due to the central role that the liver plays in drug metabolism, the measurement of liver toxicity in cell-based models is a critical step in studying the safety of new compounds (Gomez-Lechon et al., 2010).
All available *in vitro* differentiation protocols using ES or iPS cells result in a heterogeneous cell population that includes not only cells with the desired phenotype but also cells with undesired phenotypes, including potentially undifferentiated stem cells. Therefore, ES cell-derived hepatocyte-like cells need to be selected from contaminating undifferentiated and possible tumor forming cells. To this end, selection based on fluorescence-activated cell sorting (FACS) of cells expressing enhanced green fluorescent protein (eGFP) under control of the Afp or albumin promoters has been used (Drobinskaya et al., 2008; Heo et al., 2006; Ishii et al., 2007; Soto-Gutierrez et al., 2006b; Teratani et al., 2005; Yin et al., 2002). In some of

these studies, hepatocyte-like cells were purified using albumin promoter–based cell sorting and transplanted into mouse models for liver injury, or implanted subcutaneously as a bioartificial liver (BAL) device, into mice subjected to 90% hepatectomy, reversing liver failure to variable degrees. Other approaches include the generation of a transgenic ES cell line that expresses a fusion of the hygromycin resistance gene and eGFP under the control of an Afp promoter, or ES cells with bicistronic expression of the eGFP and the puromycin resistance, also under the control of an Afp promoter. Antibiotic selection resulted in an enriched population of cells with hepatoblast and more mature hepatocyte phenotypes that were used in cell replacement experiments. In human cells, reporter genes expressed under the control of the albumin or Afp genes have been used to improve differentiation protocols and to purify differentiated populations for determining global transcriptional profiles (Chiao et al., 2008; Lavon et al., 2004). Differentiated human hepatocyte-like cells have also been enriched by transduction with a lentivirus vector containing the eGFP gene driven by the alpha-1-antitrypsin promoter (Duan et al., 2007).

ES cell-derived hepatocyte-like cells have been tested by transplantation in mouse models of liver disease or damage. Initially, *in vivo* engraftment and proliferation after transplantation was inefficient and teratoma formation accompanied engraftment. However, recent reports show improved results (Gouon-Evans et al., 2006; Heo et al., 2006; Ishii et al., 2007). After transplantation, hepatocyte-like cells not only engrafted and proliferated but they also participated significantly in liver repair and survival. The transplanted cells also showed differentiation into mature hepatocytes and were responsive to normal growth regulation, and proliferated at the same rate as the host hepatocytes after additional growth stimulus from recurrent liver injury. Although in one of these studies teratomas appeared after sixty days (Ishii et al., 2007), no teratomas were observed in the other study for up to 82 days after transplantation (Heo et al., 2006). Recently promising results have been obtained using hepatocyte-like cells in BAL devices to remove toxins from the blood and supply physiologically active molecules important for recovery of hepatic function. In these instances, embryonic derived hepatocytes implanted subcutaneously as a BAL reversed liver failure in mice subjected to 90% hepatectomy (Soto-Gutierrez et al., 2006a) or were able to improve survival in rats with liver failure induced by galactosamine (Cho et al., 2008). The use of BAL devices decreases the risk of teratoma formation. Human hepatocyte-like cells obtained by different protocols have also been transplanted in mouse models. The transplanted cells survive, engraft, and present some functional characteristics of hepatocytes including secretion of liver specific proteins; teratoma formation was reported in some cases. Clearly, novel approaches will need to be developed for the selection of pure hepatic cell populations from i*n vitro* culture systems (for example without using transgenic reporter genes) before transplant protocols will be feasible.

4. Directed differentiation of lung epithelium

The lung develops from DE that is closely related to the liver primordium. Again, this raises a special challenge for directed differentiation, since all of the protocols needed for generating lung progenitors will also generate prehepatic cells. Biasing the protocol too much away from liver may promote the generation of more anterior DE-derived tissue, such as thyroid. A recommended strategy is to use our knowledge of normal mesendoderm development to promote DE, and then, while providing lung-dependent signals, inhibit the progression of non-lung DE-derived progenitors. The normal developmental programs that

derive anterior DE were discussed above in relation to liver. These are equally relevant to lung, but will not be reiterated in this section.

4.1 Phases of lung development

The lung is a respiratory organ with the principal function of transporting oxygen from the atmosphere to blood cells, and to release carbon dioxide from the blood into the atmosphere. Mammals have two lungs with great cellular diversity, with multiple resident epithelial and mesenchymal cell lineages. The lung is a complex system of branched epithelial tubules (airways), which connect to a network of gas exchanging units called alveoli. Pulmonary alveoli are spherical outcroppings that consist of an epithelial layer and extracellular matrix surrounded by capillaries (Ten Have-Opbroek, 1991). The respiratory epithelium in the mammalian lung is composed of a variety of cell types with distinct morphologic and biochemical characteristics. Epithelial cell lineages are arranged in a distinct proximo-distal spatial pattern in the airways. The proximal conducting airways are lined with a ciliated pseudostratified epithelium, but in the distal region they are replaced by a very thin squamous epithelial cell lining and simple cuboidal epithelial cells known as type I and type II airway epithelial cells, respectively (Breeze and Wheeldon, 1977). Type I squamous alveolar cells are responsible for gas exchange in the alveoli and cover a majority (>95%) of the alveolar surface area (Williams, 2003). Type II alveolar cells produce pulmonary surfactant proteins (SP) A, B, C and D, a complex mixture of phospholipids and proteins that is critical for reducing surface tension at the air-liquid interface of the distal lung, to prevent alveolar collapse upon expiration (Fehrenbach, 2001). Non-ciliated Clara cells are present in the small airways and trachea. Clara cells are secretory and the source of Clara cell secretory protein (CCSP) (Bishop and Polak, 2006). Clara cells and type I alveolar cells are unable to replicate and are susceptible to toxic insults. In the event of damage, type II cells can proliferate and/or differentiate into type I cells to compensate for the loss (Evans et al., 1975).

The development and differentiation of lung epithelial cells is accomplished through the proper coordination of various transcription factors including Forkhead box A1 (FoxA1), FoxA2, Foxj1, homeodomain Nkx2.1 (Ttf1), homeodomain box (Hox) A5, the zinc finger Gli transcription factors, basic helix loop helix (bHLH) factors, and GATA transcription factors (Cardoso and Lu, 2006; Costa et al., 2001; Warburton et al., 2000). Also essential are various autocrine and paracrine signaling events, initiated by several families of secreted factors including FGF and TGF-beta family members, for both lung development and morphogenesis.

Lung development in the mouse and human (Fig. 5) can be divided into five overlapping stages (Cardoso and Lu, 2006; Costa et al., 2001; Warburton et al., 2000).

i. **Embryonic phase -** Following gastrulation, definitive endoderm undergoes complex morphogenetic changes that ultimately leads to the formation of the primitive gut tube as described above. The most anterior part of the primitive gut tube is the foregut, while the midgut and hindgut are located at progressively more posterior regions**.** The respiratory system arises from the ventral foregut endoderm. The process initiates with the establishment of respiratory cell fate in the ventral foregut. The foregut endoderm differentiates into various epithelial cell types, which line the inner surface of the developing lung and trachea. This is followed by the development of a tree-like system of epithelial tubules and vascular structures (Metzger et al., 2008) that ultimately gives rise to the mature airways and alveoli.

ii. **Pseudoglandular stage -** This stage is characterized by formation of the bronchial and respiratory bronchiole tree, lined with undifferentiated epithelial cells juxtaposed to the splanchnic mesoderm. By day 12 of mouse lung development branching of the bronchial buds gives rise to the left lung lobes and the four lobes of the right lung.
iii. **Canalicular stage –** During this stage there is extensive branching of the distal epithelium and mesenchyme resulting in formation of terminal sacs lined with epithelial cells integrating with the mesoderm-derived vasculature.
iv. **Saccular stage -** There is a coordinated increase in terminal sac formation and vasculogenesis in conjugation with the differentiation of alveolar epithelial type I and type II cells.
v. **Alveolar stage –** Finally, postnatal lung development features maturation of the terminal respiratory sacs into alveolar ducts and sacs.

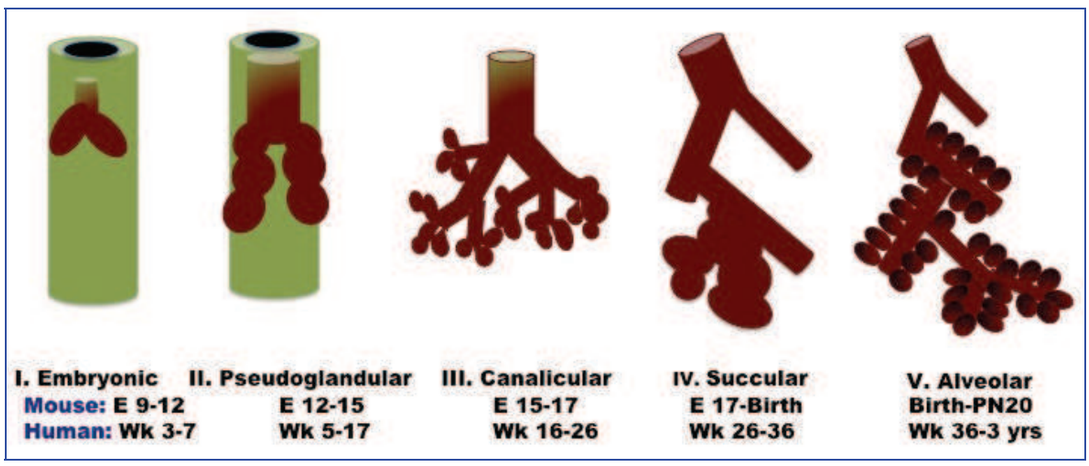

Fig. 5. Shown are the developmental stages of lung morphogenesis for mammals. The schematic primarily depicts airway morphogenesis

4.2 Establishment of endodermal cell fate

As alluded to above, as gastrulation proceeds, the DE cells migrate anteriorly, displacing the extraembryonic visceral endoderm (Lawson and Pedersen, 1987) and this tissue subsequently begins to fold at the anterior and posterior ends, forming the anterior and caudal intestinal portals (Tam et al., 2007). In mouse, by embryonic day 9.5 budding of various domains of endodermally derived organ from the distinct sections of the gut tube begins, and progressively these domains obtain their specific architecture and eventually start differentiating into corresponding organs. The foregut gives rise to lung, thyroid, thymus, esophagus, trachea, stomach, liver and pancreas. Midgut forms the small intestine, and hindgut gives rise to large intestine. Initially, the generation of pre-lung tissue is generally under the control of the same programs responsible for pre-hepatic tissue (see Figs. 3 and 4).

4.3 Establishment of lung epithelial cell fate

Primordial lung bud development originates at E9.5 from the anterior foregut endoderm. As compared to liver and pancreas, the molecular mechanisms responsible for primary lung bud induction are less clear. Precisely when cells fated to lung epithelium originate in the

foregut endoderm is not well defined. Respiratory progenitors are first identified by *in situ* hybridization as a group of Thyroid transcription factor 1 (Titf1, also known as Nkx2.1)-expressing endodermal cells in the prospective lung/tracheal region of the foregut, at E9 (Minoo et al., 1999). Titf1 expression has been reported in respiratory progenitors of the foregut endoderm even before the lung or tracheal primordium can be identified (Serls et al., 2005). However Titf1 is not a specific marker for lung, as endoderm-derived thyroid progenitors also express Titf1 (Lazzaro et al., 1991). The surfactant protein C (SPC) gene is the most specific marker of lung epithelial cells lineage, but its expression is consistently detected only by E10-10.5, after the primary buds form (Wert et al., 1993). FGFs secreted by the cardiac mesoderm influence cell fate decision in the adjacent ventral foregut endoderm in a concentration-dependent manner. Explant cultures of 2-5 somite stage ventral foregut in the presence of high concentrations of FGF2 result in lung lineage specification marked by the expression of Titf1 and Sptfc, whereas a low concentration of FGF2 results in expression of liver markers. The high concentration of FGF2 appears to be instructive for lung specification since dorsal midgut endoderm, which ordinarily does not give rise to lung, also responds to FGF2 induction by expression of Titf1.

4.4 Formation of primary lung bud formation

Once respiratory fate is established in the foregut endoderm, the initial pool of lung progenitors expand giving rise to the primary lung bud. In mammals, signaling by Fgf10 from the surrounding mesoderm and the expression of its receptor FGfr2b on the subset of Titf1-expressing endodermal cells is crucial for lung bud formation. Deletion of either Fgf10 or Fgfr2b results in lung agenesis and multiple abnormalities (Min et al., 1998; Sekine et al., 1999). It has been reported that expression of Fgf10 and formation of lung bud is controlled by retinoic acid signaling (Desai et al., 2006). Disruption of retinoic acid signaling in the foregut affects lung most dramatically resulting is several lung abnormalities including lung agenesis (Mollard et al., 2000). Retinoic acid seems to act as a major regulatory signal integrating the WNT and TGF-beta pathways in the control of Fgf10 induction (Chen et al., 2010).

4.5 Mesenchymal-epithelial interaction

Once the primary lung buds have formed, they extend into the surrounding mesenchyme and begin the process of branching morphogenesis. Branching morphogenesis requires specific interactions between the endodermal epithelium and its mesenchyme (Shannon and Hyatt, 2004). In chick it has been shown that if the lung mesenchyme is removed from the lung rudiments prior to branching, all development is blocked, but if mesenchyme is removed after primary and secondary branching, the subsequent branching morphogenesis is normal. With the optimization of culture techniques, this phenomenon was shown to hold true in mammals as well. Non-lung mesenchyme is capable of inducing formation of lung bud in the gut tube, but this bud does not branch further, again indicating the requirement of lung mesenchyme in branching morphogenesis. Lung mesenchyme is capable of inducing branching morphogenesis in non-lung epithelium such as tracheal epithelium (Wessells, 1970). Since tracheal epithelial cells, just like lung epithelial cells, arise from endoderm, the ability of lung mesenchyme to reprogram tracheal cells may not be surprising. However, lung mesenchyme is also able to induce lung-like patterning in ureteric bud (Lin et al., 2001) and salivary gland (Lawson, 1974).

4.6 Growth factors that regulate lung development

Fibroblast growth factors (FGFs). Although FGF1, FGF2, FGF7, FGF9, FGF10 and FGF18 are all expressed in the developing lung, only FGF10 is essential for lung development. FGF10 is expressed in the mesenchymal cells surrounding distal lung epithelial cells (Bellusci et al., 1997) and its receptor FGFR2IIIb is found throughout the embryonic lung epithelium (Orr-Urtreger et al., 1993). Because FGF10 is produced by the lung mesenchyme and its receptor is on epithelial cells, it is considered a key mediator of the epithelial mesenchymal interaction. FGF10 null mice have no lung development below the trachea (Sekine et al., 1999). *In vitro*, FGF10 causes budding of embryonic lung epithelium in mesenchyme- free cultures, but the ability of FGF10 to sustain expression of SPC was not determined (Bellusci et al., 1997). A high concentration of FGF10 in mesenchymal-free culture conditions caused budding in tracheal epithelium, but again in this study expression of lung specific markers was not studied (Ohtsuka et al., 2001). Rat tracheal epithelium was shown to express lung specific markers (SPC) in the presence of FGF10 and in the absence of lung mesenchyme (Shannon et al., 1999). Other than FGF10, two other members of the FGF family have been shown to influence lung development: FGF9 (Colvin et al., 2001) and FGF7 (Simonet et al., 1995).

Bone morphogenic protein (BMP) 4. In early mouse development, BMP4 is expressed in the splanchnic mesenchyme surrounding the gut tube where the future lung buds will form (Weaver et al., 1999). After lung buds have formed, BMP4 continues to be expressed in the more proximal mesenchyme and also in the epithelium of the distal tips. Over-expression of BMP4 in the distal lung epithelium results in lung hypoplasia with a decrease in the number of SPC-positive cells (Bellusci et al., 1996). Inhibiting BMP4 either by driving noggin expression under the control of the SPC promoter, or by expression of a dominant negative form of the BMP receptor, also resulted in a decrease in the number of SPC positive cells, but with an expansion of epithelial cells expressing proximal markers CC10 and Foxj1 (Weaver et al., 1999). Addition of exogenous BMP4 on either lung (Weaver et al., 2000) or tracheal (Hyatt et al., 2002) epithelium in mesenchyme-free cultures inhibited proliferation. These data suggest that BMP4 is a signal that derives from both epithelial and mesenchymal tissue compartments.

Epidermal growth factor (EGF). EGF receptor (EGFR) signaling has been shown to stimulate murine embryonic branching morphogenesis, epithelial and mesenchymal cell proliferation, and Nkx2.1 and SPC expression (Schuger et al., 1996; Warburton et al., 1993). Similarly, inhibition of EGFR signaling results in decreased branching morphogenesis in culture and a neonatal pulmonary lethal phenotype in the null mutant, associated with decreased branching morphogenesis, and decreased Nkx2.1 and SPC expression levels (Miettinen et al., 1997).

Hepatocyte growth factor (HGF). HGF is expressed in primitive lung mesenchyme, while its receptor, the c-met tyrosine kinase, is expressed in primitive lung epithelium, suggesting the possibility of inductive mesenchymal-epithelial interactions (Sonnenberg et al., 1993).

Platelet derived growth factor (PDGF). PDGF peptides are dimeric ligands formed from two peptide chains, A and B. PDGF-AA and PDGF–BB, and the receptor PDGFR, are expressed in embryonic mouse lung. Abrogation of PDGF-A decreases the size of early embryonic mouse lungs in culture, as well as affecting early branch point formation (Souza et al., 1995). On the other hand, abrogation of PDGF-B reduces the size of the epithelial component of early embryonic mouse lung explants, but does not affect branching.

4.7 Key transcription factors in lung development

Thyroid transcription factor 1 (Ttf1/ Nkx2.1). Ttf1 is a homeodomain protein (also known as thyroid transcription factor-1 (Ttf-1). Although Ttf1 is the earliest known marker for the respiratory system, Ttf1-null mutant mice do have lungs. However, these lungs are highly abnormal, consisting of two main bronchi. In these mice epithelial cells fail to express any of the surfactant proteins or Clara cell CC-10 protein (Minoo et al., 1999), although proximal differentiation is normal. Lack of Ttf1 also affects branching morphogenesis for unknown reasons. Studies have suggested that Ttf1 is essential for the differentiation of distal lung epithelial cells and is required for the expression of several lung markers such as SPC (Kelly et al., 1996). Ttf1 consensus recognition sites are found in the 5′ promoters of several important peripheral lung genes including SPA, B, C, D, CC-10 and Ttf1 itself. It has been demonstrated that Ttf1 promoter activity is stimulated by HNF-3β (Ikeda et al., 1996) and GATA6 (Shaw-White et al., 1999).

Gata6. Unlike in liver, Gata6 is the only GATA factor expressed in the developing lung and this is restricted to distal lung epithelium. Gata6 expression is observed as early as E10.5 (Morrisey et al., 1996). Binding sites for Gata6 are present in several lung-specific promoters including those for the SPA, C and Ttf1 genes. Gata6 is able to trans-activate these promoters in non-lung cells, suggesting a role in the transcriptional regulation of these genes (Bruno et al., 2000; Shaw-White et al., 1999). The key role for Gata6 in the development of lung epithelial cells became clear when Gata6$^{-/-}$ cells were shown to be unable to contribute to the airway epithelium of Gata6$^{-/-}$/C57BL6 chimeric mice (Morrisey et al., 1998). The lungs of these chimeric mice showed defects in lung morphogenesis and down-regulation of lung-specific genes including SPC. The role of Gata6 in distal lung epithelium was studied by expressing a Gata6-Engrailed dominant-negative fusion protein exclusively in distal lung epithelium (Yang et al., 2002). In these mice, airway epithelial cell differentiation was defective, as type I cells were completely absent, while certain aspects of type II cells were also affected. Proximal airway development was also disrupted as shown by decreased levels of CC-10 expression. Lineage tracing of knockout Gata6-/- cells showed that the gene is not required for specification of endoderm, but that it is essential intrinsic to the endoderm for branching morphogenesis and differentiation of epithelium (Keijzer et al., 2001).

Forkhead homologue hepatocyte nuclear family (HNF) proteins. Hnf-3α and Hnf3β are known to have role in regulating transcription of Surfactant protein (SP) and Clara cell secretory protein (CCSP) (Bohinski et al., 1994; Clevidence et al., 1994). The Hnf3β null mutation results in an early embryonic lethal phenotype with complete failure of the primitive foregut to close into a tube (Ang and Rossant, 1994). During lung development HNF3β protein is expressed at higher levels in epithelial cells lining the proximal airways and at lower levels in the distal type II epithelium (Zhou et al., 1996). Consensus HNF binding sites are found in the 5′ promoter regions of several lung specific genes including SPA, B, C, D, and CC-10 in close proximity to Nkx2.1 sites, and it has been reported that HNF-3 activates transcription of Nkx2.1 in respiratory epithelial cells (Ikeda et al., 1996).

Gli transcription factors. Targeted disruption of the Gli2 gene results in diminished lung proliferation and branching. Gli2$^{-/-}$ mice have hypoplastic trachea (Mo et al., 1997). A more severe lung defect is observed with the Gli gene deficiency analyzed in a Gli3 heterozygous background. Gli2$^{-/-}$, Gli3$^{+/-}$ embryonic mouse lungs are more hypoplastic, and the right and left lobes fail to separate (Motoyama et al., 1998). These mice resemble the phenotype observed with Ttf1$^{-/-}$ mice. The most severe phenotype was observed in Gli2$^{-/-}$, Gli3$^{-/-}$ mice,

which display a complete absence of lung, trachea, and esophagus and smaller stomach, liver and pancreas.

4.8 Differentiation of ES cells into lung epithelial cells

Compared to the other derivatives we have discussed, relatively few protocols have been reported that promote the differentiation of ES cells into lung epithelial cells, and even with these, the generation of lung epithelial cells is quite inefficient. To date there are no published protocols capable of generating a homogenous population of functional lung epithelial cells. An early report showed that SPC-positive lung epithelial cells could be derived from mouse ES cells (Ali et al., 2002) by culturing ES cells in a commercially available small airway growth media (SAGM) that supports the growth of lung epithelial cells *in vitro*. However, the ES cells were not responsive to the growth media until they had achieved a relatively advanced state of differentiation, suggesting that the system selected out spontaneously differentiated type II alveolar epithelial cells, rather than actively promoting their differentiation.

Studies from Rippon et al. (2006) further optimized the derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step protocol. In this strategy, definitive endoderm was induced using a high concentration of ActivinA, followed by culturing the floating embryoid bodies in knockout serum replacement (KOSR) media for 10 days. Subsequently, EBs were attached to gelatinized plates and cultured in the presence of KOSR for an additional 10 days. KOSR was replaced with SAGM and cultured for 14 more days. However, the lung epithelial cells derived by this method had a gene expression profile resembling that of the lung committed precursor (SPC^+Ttf1^+) found in foregut endoderm, rather than mature alveolar epithelium. While trying to optimize serum free conditions for lung epithelial cell differentiation, Winkler et al. (2008) observed in the presence of serum relatively high expression levels of surfactant proteins A, B, C and D, CCSP and aquaporin 5, suggesting that epithelial cells expressing the markers for mature type II alveolar epithelium cells could be generated in serum-containing conditions. Surprisingly, a late treatment with ActivinA following serum activation resulted in significantly higher expression levels of SPC compared to an early induction with ActivinA.

Subsequently, other strategies have been used to promote the formation of pulmonary epithelium from ES cells. Co-culture of murine EBs with mesenchyme dissected from the distal tips of mid-gestation murine fetal lungs directed the differentiation of epithelial cells expressing Ttf1 and SPC (Van Vranken et al., 2005). It has also been reported that mouse ES cells can differentiate into type II alveolar epithelial cells when cultured with extracts from murine pneumocytes (Qin et al., 2005). The idea of transitioning lung epithelia through known normal developmental stages, which has been very successful for hepatocytes, may also be applied to lung. For example, initial generation of anterior endoderm using ActivinA, coupled with Wnt3a to suppress primitive endoderm, was successful using a subsequent step of FGF2 induction, to generate SPC-positive cells characteristic of type II alveolar epithelium (Roszell et al., 2009). For the generation of human lung epithelial cells from human ES cells there is to date only a single published protocol (Samadikuchaksaraei et al., 2006). In this case the investigators used commercially available SAGM to support the differentiation. When differentiation of hES was performed in the presence of SAGM, there was up-regulation of SPC levels. Clearly, much progress has been made in understanding the directive differentiation of endoderm derived organs like liver and pancreas, while the

generation of effective protocols for the differentiation of ES cells into lung epithelial cells is still at an early stage, and represents an area of great opportunity. Considerable information is now available concerning the normal programs that regulate lung epithelial development and morphogenesis, and this should continue to be exploited in the near future for additional progress.

5. A strategy to dissect the function of GATA factors for specificity of mesendodermal derivatives

In our own laboratory we are studying organ development and regeneration, and have focused considerable attention toward the role of GATA transcription factors, using zebrafish embryo (Heicklen-Klein et al., 2005) and murine ES cell (Evans, 2008) experimental models. It is particularly intriguing that GATA factors seem to play key roles throughout the various stages of organ development. For example, members of the Gata4/5/6 subfamily may be involved in mesendoderm development (our unpublished results), but also have requirements for subsequent developmental transitions related to both mesoderm and endoderm. This includes, for example, cardiomyocyte specification from precardiac mesoderm (Holtzinger and Evans, 2007), heart tube looping and gut-derived organ budding (Holtzinger and Evans, 2005), and myocardial regeneration in adult zebrafish (Kikuchi et al., 2010). Understanding specificity of function is complicated since the genes are sometimes (but not always) functionally redundant, and they may function cell autonomous, or non-cell-autonomous through activation of secreted signaling factors.

One strategy we are taking is to develop conditional expression systems for specific GATA factors in the context of ES cell directed differentiation protocols, to determine the temporal effect and the ability for the genes to promote specific developmental transition stages. For this purpose we have been using a conditional system established by Kyba, Daley and colleagues (Ting et al., 2005), in which the gene encoding the reverse tetracycline transactivator protein is pre-targeted in the Ainv ES cell line to the constitutive Rosa26 locus, and a tet-operator sequence is pre-targeted just upstream of a loxP site (Fig. 6). This allows the insertion, by homologous recombination, of the coding sequences for any gene, which can then be activated conditionally by the addition of the tetracycline analogue doxycycline into the culture media.

This system provides significant advantages for studying genes like GATA factors that function at multiple stages of embryonic development. For example, forced expression of Gata4 in ES cells efficiently directs the development of primitive endoderm. This is useful for studying primitive endoderm, but abrogates the ability to test functions for subsequent stages, such as cardiomyocyte specification. Using this system we could show that expression of Gata4 during EB development is sufficient to generate DE (although primitive endoderm also forms) and that either type of endoderm is capable of inducing, by a non-cell-autonomous mechanism, the generation of cardiomyocyte progenitors (Holtzinger et al., 2010). Using conditional expression of different GATA factors in this system, we are currently testing their ability to influence mesendoderm development, mesoderm and endoderm patterning, the specification of different anterior DE derivatives including liver and lung, and the maturation of differentiated cell types. A major advantage of the strategy is that the induction is reversible, simply by washing doxycycline out of the media. This turns out to be important, since the transcription factor may have both positive and negative

influences, depending on the time it is expressed (Zafonte et al., 2007). Finally, we have also adapted the system for loss-of-function, by conditional expression of gene-specific shRNAs.

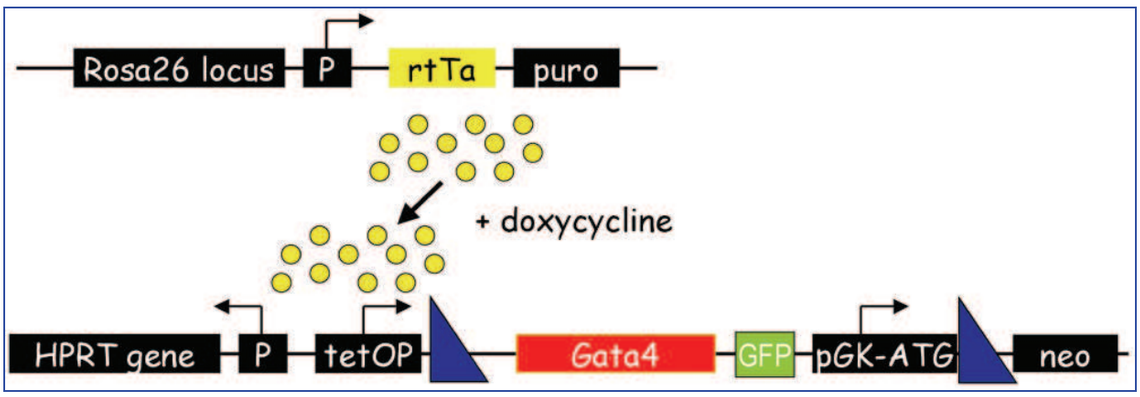

Fig. 6. In the Ainv ES cell line, the reverse tet-transactivator protein (rtTa, yellow balls) is expressed from the Rosa26 locus, and a loxP site (blue-triangle) is located downstream of a tet operator site. We used Cre-mediated recombination to target each flag-tagged Gata4/5/6 cDNA and IRES:GFP cassette into the loxP site, placing e.g. Gata4 (and GFP) under conditional (and reversible) control

6. Conclusion

Recent studies, particularly in zebrafish and frogs, revealed an ancient bipotential germ layer called mesendoderm, regulated by pathways conserved all the way to nematodes. It seems clear that this developmental stage is conserved also in mammals, providing both opportunities and challenges for directing the generation of defined cell types from ES cell cultures. A very successful strategy has been to define the normal developmental pathways that direct progenitors to mesodermal or endodermal fates, and then to recapitulate these pathways in the context of the ES cell *in vitro* system. A major challenge is that the same pathways regulate many diverse programs. However, this challenge can be overcome using conditional strategies (transgenic, or ultimately with small molecules) to fine-tune the timing and dose of the induction for optimization of each desired transitional stage (specification, commitment, proliferation, differentiation, etc.). Perhaps the major challenge moving forward will be to optimize and scale the generation of functional mature cell types, so that these can be applied successfully for therapeutic purposes in regenerative medicine protocols.

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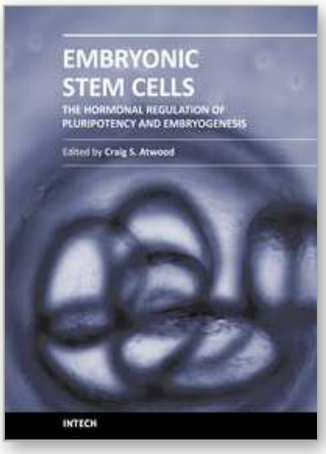

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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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