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Review article

hPSC-derived lung and intestinal organoids as models of human fetal tissue

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ABSTRACT

In vitro human pluripotent stem cell (hPSC) derived tissues are excellent models to study certain aspects of normal human development. Current research in the field of hPSC derived tissues reveals these models to be inherently fetal-like on both a morphological and gene expression level. In this review we briefly discuss current methods for differentiating lung and intestinal tissue from hPSCs into individual 3-dimensional units called organoids. We discuss how these methods mirror what is known about *in vivo* signaling pathways of the developing embryo. Additionally, we will review how the inherent immaturity of these models lends them to be particularly valuable in the study of immature human tissues in the clinical setting of premature birth. Human lung organoids (HLOs) and human intestinal organoids (HIOs) not only model normal development, but can also be utilized to study several important diseases of prematurity such as respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), and necrotizing enterocolitis (NEC).

1. Background

The gastrointestinal and respiratory systems are intricate, complex, and critical organ structures. Development of both structures begins following the establishment of the primary germ layers during gastrulation. As embryogenesis progresses, the endoderm will undergo complex gut-tube morphogenesis, resulting in epithelium lining the primitive gut tube with the mesoderm contributing to the majority of the supporting structures around it (Sinagoga and Wells, 2015; Wells and Spence, 2014). The gut tube is functionally and geographically divided into three regions along the rostral-caudal axis: the foregut, the midgut and the hindgut (Grapin-Botton, 2005; Zorn and Wells, 2009). Historically, these regions have been distinguished anatomically based on vascularization by different vessels coming off of the descending aorta: the foregut is vascularized by the celiac trunk, the midgut by the superior mesenteric artery, and the hindgut by the inferior mesenteric artery (Sadler, 2012). The foregut will give rise to the oral cavity, pharynx, esophagus, respiratory tract (proximal conducting airways and distal alveolar tissue), stomach, liver, pancreas, and proximal duodenum. The midgut and hindgut give rise to the rest of the small intestine (distal duodenum, jejunum, and ileum) as well as the colon, and the common cloaca (Grapin-Botton, 2005; Zorn and Wells, 2009). The cloaca will be separated by the urorectal septum to form the rectum and anal canal on the dorsal portion of the embryo and the urogenital sinus on the ventral portion (Grapin-Botton, 2005; Seifert et al., 2009a, 2008, 2009b; Zorn and Wells, 2009). The endoderm of the urogenital sinus will give rise to the epithelial lining of the bladder and urethra (Seifert et al., 2008; Zorn and Wells, 2009). With so many critically timed morphological and functional changes, this delicate orchestration leaves the developing embryo vulnerable to a plethora of congenital anomalies and disease processes.

For the past several decades, our understanding surrounding normal and abnormal human development has been limited to the use of rare human samples, and inferences from studies using animal models. Interestingly, there is significant interspecies variation in the context of the respiratory and digestive systems development. For instance, the postnatal mouse intestine continues to develop for two weeks after birth before the crypt domains are properly formed, and before Paneth cells differentiate (Kim et al., 2012). In contrast, human studies have indicated that crypts and Paneth cells are present by the third trimester of gestation (Heida et al., 2016). Similarly, there are intrinsic differences between humans and mice within lung tissue as well. Basal cells, which are

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stem cells that regenerate epithelial lineages, are present throughout the human conducting airways but are confined to the trachea of mice (Boers et al., 1998; Nakajima et al., 1998). Human airways also contain goblet cells, which are less common in rodent airways (Rock et al., 2010). Despite some limitations that come with cell lines and animal models, there is no doubt that our understanding of the cellular and molecular basis of organ development has been substantially advanced by model organism research. Therefore, our recent ability to couple model organism research with complex, organ-like *in vitro* human models (organoids) provides new opportunities to understand diseases that present important challenges in the clinical setting today (St Johnston, 2015).

Organoids are complex multi-cellular structures that exhibit some of the structural and functional features of the native organ. These tissues fall under two broad categories: organoids that are obtained *de novo* through the differentiation of human pluripotent stem cells (hPSCs), and those that are derived from a native tissue source, for example, from a tissue biopsy of an individual (Dedhia et al., 2016). hPSC-derived and tissue-derived culture systems for both lung and intestinal organoids have been have been extensively reviewed recently (Dedhia et al., 2016; Dye et al., 2016; Fatehullah et al., 2016; Nadkarni et al., 2015). Therefore, we will briefly discuss the work that has contributed to our ability to differentiate hPSCs into lung and intestinal organoid tissues, however; this review will be primarily focused on highlighting important clinical challenges that affect the lungs and intestines of neonates for which hPSC-based systems may be used to shed new light on poorly understood disease processes.

2. hPSC derived lung and intestinal organoids: models of immature human tissues

Human pluripotent stem cells are human cells with the ability to differentiate into any cell type in the human body. They include human embryonic stem cells (hESCs) (Thomson et al., 1998) as well as induced pluripotent stem cells (iPSCs), which are somatic cells reverted to a pluripotent state through cellular reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Seminal work in this area has led to the emergence of a whole field focused on understanding how human pluripotent stem cells can be used to better understand human development and disease 'in a dish' (St Johnston, 2015). In general, the field has adopted an approach known as directed differentiation. This approach uses information gained from decades of animal model research to coax cells into different lineages through a step-wise process that attempts to mimic gastrulation, patterning and lineage commitment (Turner et al., 2016). Early work in this area made major contributions to our ability to generate endoderm lineages, as well as pancreatic beta-like cells, and liver hepatocyte-like cells (D'Amour et al., 2006; Kroon et al., 2008; Si-Tayeb et al., 2010).

More recently, many groups have achieved differentiation of complex 3-dimensional organ-like structures from hPSCs, including intestine, stomach, lung, kidney, brain and retina among others (D'Amour et al., 2006; Dye et al., 2015; Kroon et al., 2008; Lancaster et al., 2013; McCracken et al., 2014; Nakano et al., 2012; Si-Tayeb et al., 2010; Takasato et al., 2015; Wells and Spence, 2014). As the epithelial lining of the intestine and lung are both derived from the endodermal germ layer, successful differentiation of intestine and lung organoid tissues *in vitro* begins by directing pluripotent stem cells into the endodermal lineage by stimulating Transforming Growth Factor Beta (TGF-beta) signaling using ActivinA, which mimics the Nodal signaling that is critical for mesendoderm specification in the embryo (D'Amour et al., 2005). Following gastrulation *in vivo* and *in vitro*, the endoderm is

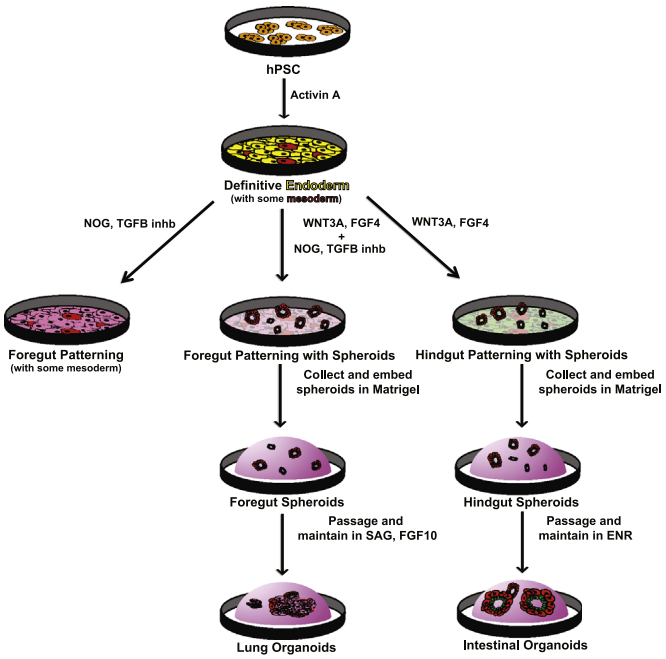

Fig. 1. Directed Differentiation of hPSCs to a Lung and Intestinal Organoid FatehPSC with the use of ActivinA can be differentiated into predominantly endoderm (in yellow) with some proportion of cells adopting a mesoderm fate (in red). When exposed to foregut (Noggin, small molecule TGFB inhibitor) or hindgut (WNT3A, FGF4) specific growth factors the endoderm is patterned accordingly. WNT3A and FGF4 appear to be critical for 3-dimensionality and spheroid formation, so that when added to foregut specific growth factors, foregut spheroids are formed. Spheroids collected and placed in Matrigel droplets are allowed to grow in three dimensions into organoid tissue. These organoids are maintained and passaged in tissue specific growth factors (lung: SAG and FGF10) (Intestinal: EGF, Noggin, R-Spondin [ENR]) for up to a year.

structured as a monolayer of epithelial cells. In the embryo, this sheet of cells is patterned along the rostral-caudal axis into the foregut, midgut and hindgut. *In vivo* signaling pathways thought to be important in rostral/foregut endoderm patterning include Nodal inhibitors (Lefty1 and Cerberus-like) and Bone Morphogenic Protein (BMP) inhibitors (Chordin and Noggin), which act to inhibit posterior patterning (Perea-Gomez et al., 2002; Tiso et al., 2002; Yamamoto et al., 2004). Signaling pathways important in caudal/hindgut endoderm patterning *in vivo* include Fibroblast Growth Factors (FGFs), Retinoic Acid (RA), WNT, and BMP (Wang et al., 2006). *In vitro*, addition of WNT3A and FGF4 to the definitive endoderm (DE) monolayer causes cells to adopt a caudal/hindgut fate and to form small aggregates called spheroids that detach and float above the adherent monolayer (Fig. 1). These spheroids can be placed in an extracellular matrix such as Matrigel where they continue to expand and grow in three dimensions, giving rise to larger organ-like structures called intestinal organoids (Spence et al., 2011; Wells and Spence, 2014). Expansion and growth of intestinal organoids required several growth factors that were shown to be critical for long term *ex vivo* growth of tissue-derived intestinal crypts (Ootani et al., 2009; Sato et al., 2009). For rostral/foregut specification *in vitro*, hPSC derived DE can be patterned into foregut by inhibiting BMP/TGFβ signaling using Noggin (NOG) and/or small molecule inhibitors (Fig. 1) (Green et al., 2011; Hannan et al., 2013; 2015; McCracken et al., 2014).

Concomitant stimulation of DE cultures with spheroid-inducing factors (WNT3A/FGF4) and foregut pattering factors (NOG/TGFβ-inhibitors) led to the formation of foregut spheroids, which expand into larger lung-organoid structures. As with intestinal organoids, expansion of hPSC-derived lung organoids was guided by information from animal development and from work

demonstrating how to culture primary lung tissue *in vitro* (Lee et al., 2014; Rock et al., 2009). The ability of hPSC-derived tissues to self assemble *in vitro* is a remarkable, but poorly understood process, especially given that growth factor gradients thought to be critical for tissue organization in the developing embryo are often lacking from *in vitro* systems (Turner et al., 2016). While more work is needed to understand the self-assembly of tissues *in vitro*, it has been suggested that spheroid/organoid formation is not due to an intrinsic ability of tissues to "self-organize", rather *de novo* formation of these structures is the result of initiating genetic programs that lead to self-assembly of structures *in vitro* that resemble *in vivo* structures following morphogenesis (Turner et al., 2016). The notion that organoids form by initiating a complex genetic program that ultimately leads to self-assembling structures is an intriguing idea given the observation that directed differentiation of hPSCs into endoderm is an inefficient process, leading to the differentiation of both endoderm and non-endodermal derivatives, including mesoderm (Fig. 1) (Spence et al., 2011). While not yet experimentally tested, it is possible that cell-cell interactions and cell-cell signaling resulting from 'inefficient' differentiation may be important for recapitulating aspects of the developmental programs required for formation 3-dimensional structures. Indeed, intestine, lung and gastric organoids derived from hPSCs all contain epithelium and supporting mesenchyme in a three-dimensional structure reminiscent of the developing human fetus (Dye et al., 2015; Finkbeiner et al., 2015b; McCracken et al., 2014; Spence et al., 2011; Watson et al., 2014).

In many cases, lung and intestinal organoids share several properties: they can be maintained in culture for several months and they possess a complex and diverse epithelium as well as supporting mesenchymal tissue, although it should be noted that mesenchyme free systems also exist (Fordham et al., 2013; Gotoh et al., 2014; Hannan et al., 2013; Konishi et al., 2016). Human lung organoid (HLO) epithelium includes basal cells and ciliated cells, as well as diverse mesenchymal cells (myofibroblasts, fibroblasts, and smooth muscle cells) that can be found in both proximal conducting airway-like structures as well as areas that possess alveolar cell types (Fig. 2) (Dye et al., 2015). Human intestinal organoids (HIOs) also include many functionally distinct cell types including epithelial cells (enterocytes, enteroendocrine cells, goblet cells, and Paneth cells) and mesenchymal cells (myofibroblasts, fibroblasts, and smooth muscle cells) which are organized into cyst-like structures with a columnar epithelium-lined lumen with supporting mesenchyme found adjacent to the basal surface of the epithelium (Spence et al., 2011; Wells and Spence, 2014).

Interestingly, a common feature of many hPSC-derived cells, tissues and organoids in many cases, is that they remain in a transcriptional and functional state that is similar to fetal human tissue (Dye et al., 2015; Finkbeiner et al., 2015b; Fordham et al., 2013; Hannan et al., 2013; Hrvatin et al., 2014; Si-Tayeb et al., 2010; Takasato et al., 2015). It is currently unclear why organoid models fail to 'mature' *in vitro*, and further work is required to better understand this phenomenon. A common method in the field to further mature hPSC-derived tissues, including organoids, is to transplant *in vitro* derived tissues into immunocompromised mice (Finkbeiner et al., 2015b, 2015a; Watson et al., 2014). In many cases, this has led to remarkable morphological and functional maturation into an adult-like state. For example, when intestinal organoids are placed under the renal capsule, they become vascularized by the host, can flourish for many months, and are remodeled such that the simple epithelium seen *in vitro* gives rise to crypt and villus-like structures that closely resemble the sophisticated organization of the mature intestine. This includes deep crypts that possess Paneth cells and cells expressing the intestinal stem cell markers LGR5 and OLFM4 as well as longer and more mature villi that express brush border enzymes and other markers of mature small intestine such as dipeptidyl peptidase 4, glucose transporter type 2, and villin (Finkbeiner et al., 2015b, 2015a; Watson et al., 2014). Thus, hPSC-derived tissues that are fetal/immature *in vitro* appear to become morphologically and functionally similar to the mature adult epithelium when transplanted *in vivo.* Consistent with the concept of *in vivo* maturation, intestinal tissue generated by transplanting undifferentiated hPSCs into mice in order to form teratomas proved to be similar to the mature adult intestine. Intestinal organoids generated from teratoma-derived tissue, having come from an *in vivo* environment, yielded more mature adult like gene expression and behavior (Forster et al., 2014). It is important to note, however; that despite the strong similarities between organoids and fetal tissue, and transplanted organoids and adult tissue, many comparisons

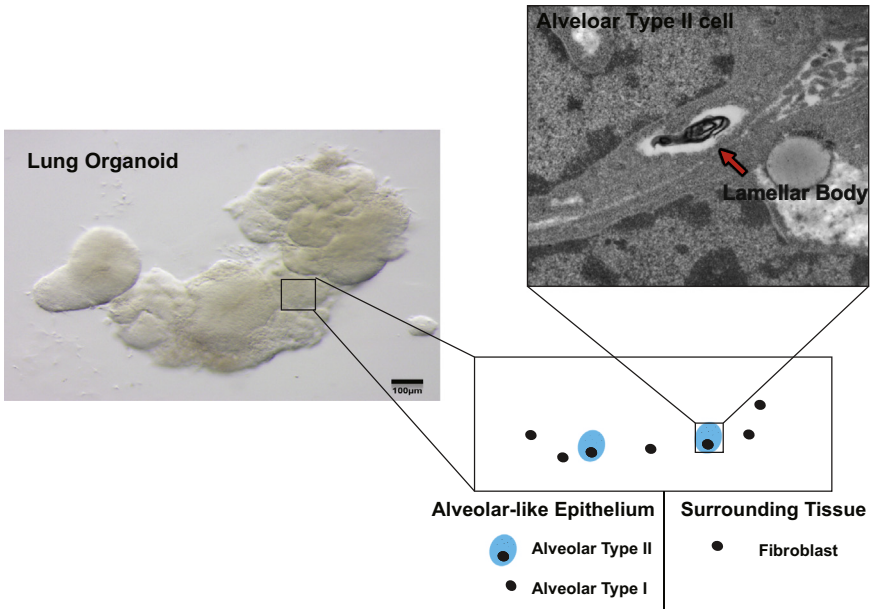

Fig. 2. Alveolar like Tissue in Lung Organoids contains Alveoli type 1 and 2 Pneumocytes and Lamellar Bodies. Lung organoids contain cell types associated with alveoli such as alveolar type 1 and 2 pneumocytes (AECI and AECII cells) surrounded by fibroblasts. AECII cells are more rounded in shape and found located between elongated AECI cells. These AECII cells contain lamellar bodies, which are responsible for manufacturing surfactant proteins both in the lung organoids and *in vivo* human lung tissue.

conducted to date have been carried out on mixed populations of cells. For example, intestinal organoids have been compared to whole thickness fetal intestine (Finkbeiner et al., 2015b). Thus, while similarities between tissues exist, how similar specific tissue compartments are (*i.e.* epithelium *vs.* epithelium) or how similar individual cell types are between *in vitro*-derived and organ-derived tissues is currently unknown. In this light, systems where hPSCs have been differentiated into epithelium-only organoids (Fordham et al., 2013; Hannan et al., 2013) can be leveraged in order to more fairly compare differences with the human fetal epithelium (Fordham et al., 2013).

The immature/fetal nature of these hPSC-derived *in vitro* models makes them the only non-primary human fetal model system to suitable for exploring the immature lung or intestine. Therefore, these organoid models are uniquely poised to model diseases of the immature human neonate. In the same vein, due to similarities with the adult intestine, transplanted hPSC-derived organoids are more appropriate for modeling mature/adult tissue. Importantly, our understanding of human development to aid premature infants born with immature intestine and lungs is particularly limited. This is unfortunate for patients in the neonatal intensive care unit, who experience a delay, derailment, or arrest in development at these stages. The remainder of this review will focus on clinical challenges for which immature/fetal organoids present important new avenues for furthering our understanding of human development and disease.

3. The premature lung: potential applications for HLOs

Prematurity (infants born at less than 37 weeks gestation) is a significant problem in both the developed and developing world. Preterm births in the United States accounted for 11.39% of all births in 2013 (Martin et al., 2015). We are fortunate that care of the premature infant has made incredible strides in the past several decades. It is well-established that the current margin of viability is 23–24 weeks of gestation, though there is ongoing debate prompting consideration of the viability of 22 week fetuses as well (Rysavy et al., 2015). Compare this to 1963 when President John F. Kennedy's premature son died of immature lung development at the gestational age of 34 weeks.

Major advances in neonatal care can be largely attributed to two major breakthroughs, both involving the immature respiratory system of the preterm. First, since alveolar type II cells (AECII) in the immature lung does not generate sufficient surfactant protein, the commercial availability of surfactant in the 1990s allowed for the delivery of this surface tension relieving choline based phospholipid directly to the lungs via an endotracheal tube (Suresh and Soll, 2005). With surface tension relieved, compliance of the immature lung was significantly improved so that mechanical ventilation could be more effective and less likely to cause detrimental damage to alveoli via high pressure and air leaks. Pulmonary surfactant was first discovered in the 1950s and was later described as the key player in “hyaline membrane disease” now known as “respiratory distress syndrome of the premature infant” or RDS (Notter and Shapiro, 1981).

Several transcription factors, lipid modifiers, and signaling pathways (*i.e.* BMP, NKX2-1, and Nfatc3) have been described as important in the production of surfactant and its associated proteins (DeFelice et al., 2003; Ikegami et al., 2000; Luo et al., 2016; Whitsett and Weaver, 2015; Whitsett et al., 2010), however; we still know relatively little about maturation of alveolar cell types. Since there is evidence that human lung organoids possess alveolar type I cells (AECI) and AECII, it is possible that this model system may be beneficial to understand how alveolar cell types mature. Additionally, infants with mutations in genes specific to surfactant and its associated proteins (SFTPB, SFTPC, ABCA3) are rare and often left unidentified until late in presentation until all other more common conditions have been excluded (Wert et al., 2009). *Sftpb*$^{-/-}$ in particular is a fatal condition not responsive to ventilator management or surfactant replacement therapy (Clark et al., 1995) and lung organoids offer an opportunity to model these types of lethal mutations.

Premature infants not only suffer from surfactant deficiency ameliorated by surfactant replacement therapy, but also from an arrest in alveolar maturation. Alveoli in the preterm lungs are lacking in both quantity as well as quality. Alveoli present in preterm lungs, especially those prior to 28 weeks gestation are still in the saccular phase of lung development (for reviews on lung development, see: Herriges and Morrisey, 2014; Morrisey et al., 2013; Morrisey and Hogan, 2010.). These alveoli have thick walls with blood vessels located far from the minimal airspaces that are required for gas exchange (Rodriguez, 2002). Despite the availability of surfactant therapy, this ventilation-perfusion mismatch poses a significant problem for neonatologists managing extremely low gestation age infants on mechanical ventilators (Whitsett and Weaver, 2015). Mechanical ventilation, inflammation associated with preterm birth and its sequelae, and arrested pulmonary development are all likely contributors to the long term chronic condition described as bronchopulmonary dysplasia or BPD (Northway et al., 1967). The estimated economic and social costs of managing preterm infants with BPD are not limited to their stay in the neonatal intensive care unit. After discharge, infants with BPD are often managed on home oxygen or home ventilators with several pulmonary medications and frequent hospitalizations. They are more likely to be hospitalized for respiratory conditions such as viral infections, cardiovascular disease, and asthma (Bolton et al., 2012; Saarenpää et al., 2015). Therefore much interest has been focused on inducing lung maturation in extremely low gestation age infants with the hopes of avoiding some of these chronic complications.

Seminal animal model research in the late 1960s led to the discovery that steroid administration hastened lung development in preterm animals (Avery, 1995). This observation led to small clinical trials in human mothers at risk for preterm delivery (Avery, 1995). Promising results inspired a much larger randomized control trial sponsored by the National Heart Lung and Blood Institute of a maternally administered transplacentally circulating glucocorticoid, dexamethasone. At a conference held by the NIH, maternal steroid administration for the maturation of fetal lung development was made the new standard of care, effectively reducing neonatal mortality by 40% (Avery, 1995). With these guidelines, neonatologists have seen a sharp decline in the amount of RDS and BPD within each gestational age bracket. However, this also led to the ability to resuscitate babies born at younger and younger gestational ages and RDS and BPD are still quite prevalent in neonatal intensive care units around the globe.

Despite the fact that corticosteroid administration to at-risk mothers enhances fetal lung maturation and has dramatically reduced mortality rates, the mechanism of maturation is largely unclear. There is some evidence that steroid administration accelerates AECI and AECII pneumocyte development (Bonanno and Wapner, 2009). Surfactant production is potentially accelerated by induction of the surfactant proteins and enzymes necessary for phospholipid synthesis (Ballard and Ballard, 1995). Antenatal steroids also induce pulmonary beta-receptors, which play a role in surfactant release and absorption of alveolar fluid (Ballard and Ballard, 1995). By this mechanism, induction of fetal lung antioxidant enzymes and the upregulation of the epithelial Na+ channels enhance absorption of fetal lung fluid after birth (O'Brodovich, 1996). In tissue culture experiments, these biochemical changes and surfactant effects appear to be temporary,

though cytostructural benefits of maturation appear to persist in rhesus monkey models (Ballard and Ballard, 1995; Bonanno and Wapner, 2009; Bunton and Plopper, 1984). With this rationale, antenatal steroid (usually dexamethasone or betamethasone) may be repeated in pregnant women still at risk for delivery every 2–4 weeks as the exact date of delivery is often difficult to predict.

hPSC derived human lung organoids are relatively new on the scene and have yet to be used to model patient disease. However, given a high degree of similarity to the human fetal lung, they offer novel opportunities to investigate mechanisms of lung maturation, such as antenatal corticosteroid administration. Although this therapy is already widely used clinically, a better understanding of how this therapy affects the immature lung may lead to better or alternative treatments that may not carry the negative side effects associated with “big-gun” steroid delivery. Many experts in the field remain concerned about administering multiple repeat courses of steroid therapy (Crowther et al., 2015; Waffarn and Davis, 2012), citing trials suggesting that increased exposure to steroids is associated with increasing risk of adverse effects such as growth difficulty and poor neurologic outcomes (such as cerebral palsy) (Waffarn and Davis, 2012).

Pulmonary hypoplasia is a condition that can be the result of several congenital syndromes such as congenital diaphragmatic hernia (CDH) where abdominal contents are displaced into the chest, inhibiting lung formation (Langer, 1998). Pulmonary hypoplasia can also be a result of oligo- or anhydramnios (absence of amniotic fluid) due to either premature rupture of membranes or an obstructive uropathy such as posterior urethral valves (PUV) (Zingg-Schenk et al., 2008). In some situations such as congenital pulmonary airway malformations (CPAMs), large cystic structures within the thorax prevent adequate lung tissue from forming (Marchiori et al., 2015). These neonates are not only born lacking sufficient lung tissue, but many of them are born premature as well, often adding RDS and/or BPD to their list of complications. These infants would greatly benefit from tissue regenerative efforts. HLOs would offer significant advantages over traditional and rarely available or successful neonatal lung transplants. Diagnoses of these congenital conditions are often made prenatally, months before birth or need for a respiratory organ other than the placenta. This potentially leaves months of time to generate iPSCs and pulmonary tissue specific to the patient, essentially eliminating the need for harmful immunosuppressive medications. Currently, some groups have made progress in engineering lung tissue using iPSCs seeded onto de-cellularized human and rodent cadaver lung matrices (Charest et al., 2015; Ghaedi et al., 2013; Gilpin et al., 2014a, 2014b; Gilpin and Ott, 2015; Ren et al., 2015). Many of these models show promise in that they appear to have a differentiated pulmonary epithelium and are able to exchange gas for short periods of time. While this area still has far to go before applicable to premature infants, it is non-the-less an exciting area of investigation.

4. The premature intestine: potential applications for HIOs

Prenatally, the placenta is the organ not only of respiration but of nutrient delivery as well. In the preterm population, the last trimester is cut short, preventing full accretion of many critical nutrients including vitamins and minerals such as iron, calcium, and phosphorous (Hay et al., 1999). Current medical management of preterm infants usually includes total parenteral nutrition (TPN), often delivered through the use of a central venous catheter. This method of nutrition poses multiple problems, most notably the risk of complications from central venous catheters such as infection, as well as inadequate nutrient delivery due to TPN's inability to fully mimic the placenta (Cronin et al., 1990). Adequate calorie delivery is often limited by fluid volume tolerance due to edema, bronchopulmonary dysplasia, and persistent fetal vascular circulation. Additionally, many extremely low birthweight (ELBW) premature infants do not tolerate high dextrose infusion rates due to an immature insulin response. Long-term total parenteral nutrition is associated with significant cholestasis leading to a direct hyperbilirubinemia and liver damage (Lacaille et al., 2015). Lastly, TPN alone is unable to provide sufficient calcium and phosphorus alimentation due to precipitation of these solutes in solution. This puts preterm infants at risk for osteopenia, rickets, and bone fractures (Koo, 1992). For these reasons, among others, goals set by neonatal caregivers to mimic the same growth that might have been achievable with the help of the placenta are simply not obtainable. Not only is TPN significantly limited in its ability to provide complete nutrients needed for adequate growth, but also there is a growing body of literature suggestive that it may actually be harmful to the developing gut. For example, TPN has been implicated in mucosal atrophy and the loss of epithelial barrier function (Feng et al., 2009).

The limitations and risks associated with the use of TPN have lead caregivers to aim for full enteral feeds as quickly as the neonate's immature gastrointestinal tract is able to tolerate, which can ultimately lead to necrotizing enterocolitis (NEC), a serious complication that can affect the immature intestine. In its early stages NEC can mimic the signs and symptoms of feed intolerance. NEC is the most common gastrointestinal emergency in the newborn, affecting about 7% of preterm infants (Neu, 1996). The smallest of these infants (<750 g) have the highest morbidity (12%) and mortality (42%) from the disease. About 90% of NEC occurs in premature infants, suggesting prematurity as a key factor in the disease process (Neu, 1996). NEC is most often described as the “ischemic necrosis of the intestinal mucosa, which is associated with inflammation, invasion of enteric gas forming organisms, and dissection of gas into the muscularis and portal venous systems” (Fitzgibbons et al., 2009; Neu, 1996). Perhaps the most frustrating aspect of this illness is its unpredictable nature, affecting neonates at a time when they seem otherwise healthy (Kliegman et al., 1993). The average time of onset of disease is between 3 and 5 weeks of life, usually after the patient has reached relative stability in their hospital course (Uauy et al., 1991). The first clinical signs of NEC are very similar to feed intolerance: vomiting, abdominal distention and tenderness, and gastric residual fluid. Additionally, patients can develop bloody stools and other systemic signs of illness such as temperature instability, increased need for respiratory support, shock, and in the most severe cases, death (Neu and Walker, 2011). Dissection of gas into the bowel wall called “pneumatosis intestinalis” can sometimes be seen on plain radiograph, and to some degree ultrasonography. About 30% of patients will develop culture proven bacteremia (Lin and Stoll, 2006). Inflamed bowel has a propensity to perforate, leading to pneumoperitoneum. Necrotizing enterocolitis totalis is when the entire gut has been affected leaving no residual live bowel, a condition incompatible with life.

Unfortunately, the pathophysiology surrounding NEC has remained unclear and progress over the past several decades in reducing NEC incidence or severity has been modest at best. One prominent theory of NEC vulnerability suggests that defenses against invasive microbes are impaired leading to increased inflammation within the immature gut. HIOs could be a useful tool in delineating how luminal pH, Paneth cell activity, mucous production, and gap junctions not only mature, but how they interact with environmental exposures (bacteria, food products) and potential therapeutic interventions (Ford, 2006; Lin and Stoll, 2006; Neu, 1996). Gap junctions in particular have been of great interest to NEC researchers, as permeability of the mucosal barrier seems to be a key variable in the triggering an inflammatory cascade in

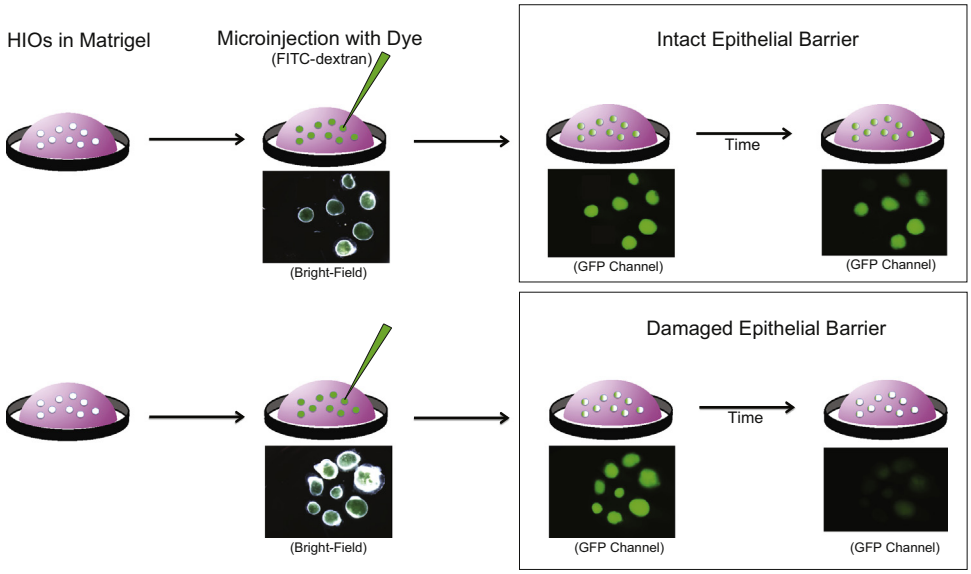

Fig. 3. HIO Barrier Function Assay using FITC-dextran Dye Microinjection. Cystic HIOs are placed in Matrigel droplets to secure them in place. Glass pulled microinjection pipettes are filled with FITC-dextran dye and carefully inserted into the cystic organoid without causing significant disturbance to the epithelium. Experimental conditions could include adding treatments, insults, or microbes to the media or the intraluminal compartment through microinjection. Intact epithelial barrier will result in dye retention over time, while a damaged epithelium will result in dye leakage and decreased luminescence over time.

the lamina propria (Ford, 2006). Antenatal steroid exposure *in vivo* matures intestinal barrier function, which is the proposed mechanism by which it reduces NEC incidence (Halac et al., 1990). However, much like antenatal steroid induced maturation of the premature lung, the exact mechanism as to how glucocorticoids are maturing or strengthening the epithelial barrier is unclear and needs further investigation. HIOs contain a luminal component lined with immature epithelium. This organization and structure has allowed researchers to study the epithelial barrier by injecting dye such as FITC-dextran into the lumen of the organoid and tracking epithelial leak over time by measuring loss of luminal fluorescence (Fig. 3) (Leslie et al., 2015). The same study demonstrated that intestinal organoids could be used to model changes in epithelial barrier function in response to exposure from different environmental stimuli such as bacterial toxins and calcium chelators (Fig. 3) (Leslie et al., 2015). Functional studies of the HIO epithelial barrier assays could be extended to further delineate the mechanisms leading to perturbed function, such as nutrient deficits, during TPN delivery (Feng et al., 2009).

The development of the intestinal microbiota also seems to be an important factor in NEC pathogenesis. Bacterial colonization is necessary for NEC development, as NEC does not occur *in utero* when the gut is sterile, nor are standardized experimental protocols able to produce NEC in germ free animals (Lin and Stoll, 2006; Morowitz et al., 2010; Patel and Denning, 2015). In the premature infant, a number of factors including virulent nosocomial pathogen exposure, prolonged antibiotic delivery, poor peristaltic motility, and the absence of human milk feedings can lead to disruption of the host microbial community (AlFaleh and Anabrees, 2014; Hunter et al., 2008; Lin and Stoll, 2006; Morowitz et al., 2010; Patel and Denning, 2015; Stewart et al., 2012). Bacteria interact with the host through toll like receptors (such as TLR2 and TLR4) to regulate gene expression through inflammatory mediators such as NF-κB (Chan et al., 2014; Lin and Stoll, 2006). Bacteria likely play a role in NEC by inducing intestinal inflammation and apoptosis through bacterial components such as lipopolysaccharide (LPS). Probiotics, which theoretically add to the pool of commensal bacteria in the host microbial community, have been shown in several clinical randomized controlled trials as well as a Cochrane Systematic Review in 2014 to significantly reduce the incidence of NEC amongst premature infants (AlFaleh and Anabrees, 2014). Routine administration of probiotics is not yet recommended, nor has it become the standard of therapy for NEC prevention due to concerns about the safety and lack of FDA regulation of a biologic agent given to functionally immune-compromised premature patients.

HIOs offer an opportunity to study host-microbe interactions in a way that has previously not been possible (Leslie and Young, 2016). A number of groups have used the microenvironment of the organoid lumen to culture bacteria such as *Helicobacter pylori*, *Salmonella*, and *Clostridium difficile* (Bartfeld et al., 2015; Forbester et al., 2015; Leslie et al., 2015; Schumacher et al., 2015). These bacteria grow, proliferate and effect host gene expression and epithelial function within the organoid. Organisms thought to be important in NEC pathogenesis and prevention, both pathogenic and commensal, could also theoretically also be introduced within the host HIO lumen as a model of the immature gut to study influence on gene expression, mucus production and other histological changes, as well as functional permeability of the epithelial barrier.

Lastly, infants born with congenital bowel malformations such as gastroschisis, omphalocele, congenital diaphragmatic hernia, or those who require large segments of bowel resection due to NEC, intestinal atresia, or spontaneous intestinal perforation could greatly benefit from PSC derived regenerative therapies in the future. Without sufficient surface area for nutrient absorption, many children suffer from chronic malnutrition and TPN dependence. They accrue the risks associated with long-term central venous access for TPN delivery (Wessel and Kocoshis, 2007). Long-term central venous access can lead to severe infections and even death. In addition to calorie malabsorption, many children are missing critical portions of the bowel responsible for vitamin and mineral acquisition. For example, neonates and children with ileal resections are predisposed to vitamin B12 (pernicious anemia) and bile acid (necessary for fat soluble vitamins A,D,E, and K absorption) deficiencies (Wessel and Kocoshis, 2007). To date, intestinal organoids, while effectively engrafting in the mouse kidney, have not been used to lengthen existing bowel (Finkbeiner et al., 2015a; Watson et al., 2014). Adult tissue-derived epithelial organoids, when introduced via enema to mice with DSS induced colitis,

remained viable and functional 25 weeks later (Yui et al., 2012). These mice also showed better weight gain at 2 weeks than mice that did not receive enemas containing tissue-derived intestinal organoids, suggesting that engrafting *in vitro* grown tissue into the native intestine is a possibility (Yui et al., 2012). Some studies have used 'organoid units' which are derived from minced mature or neonatal whole thickness intestinal fragments (Choi and Vacanti, 1997; Grant et al., 2015). These researchers were able to create tissue-engineered small intestine (TESI) after placing these units on scaffolds and transplanting them into mice. Placement of TESI in a re-anastomosed section of bowel successfully lengthened the small intestine 9 months later (Kaihara et al., 2000). However, TESIs cannot currently be expanded *in vitro*. hPSC derived intestinal organoids may hold more promise in lengthening techniques due to their self assembly with a significant outer mesenchymal component (Barthel et al., 2012; Finkbeiner et al., 2015a; Grant et al., 2015; Grant and Grikscheit, 2013; Levin et al., 2013). However, in contrast to adult tissue, significant plasticity has been demonstrated in transplanted fetal mouse intestine, suggesting that transplanted tissue loses its regional identity and can take on the characteristics of the region-specific site of transplantation (Fordham et al., 2013; Fukuda et al., 2014). With this caveat in mind, use of hPSC-derived organoids for tissue replacement therapy could present problems for infants who may have lost whole segments of bowel (such as the ileum), in which case transplanted tissue may take on characteristics of nearby tissue (such as the jejunum) rather than the desired absent tissue (ileum).

5. Future directions

hPSC-derived tissues generated through directed differentiation are inherently immature in nature and therefore make excellent models to study of diseases of the immature and premature human. Both respiratory and digestive systems of the premature infant are in great need of further investigation using human model systems such as hPSC derived human lung and intestinal organoids. We hope that highlighted areas of importance for premature neonates within this review sparks inspiration, cooperation, and innovation amongst the basic science and neonatal research communities to come together to assist this vulnerable and understudied population.

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