Regenerative Medicine- from gene to cell and tissue therapies (II): postnatal approaches

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Abstract

This review article of paediatric regenerative medicine focuses on recent advances in postnatal approaches. New gene-, cell-, and niche-based technologies, and their combinations allow structural and functional reconstitution/simulation of complex postnatal cell, tissue and organ hierarchies. Organoid and tissue engineering advances provide human disease models and novel treatments for both rare paediatric diseases and common diseases affecting all ages such as Coronavirus Disease 2019. Preclinical studies for gastrointestinal disorders are directed towards esophageal replacement, short bowel syndrome, enteric neuropathy, biliary atresia and chronic end-stage liver failure. For respiratory diseases, beside the first human tracheal replacement, more complex tissue engineering represents a promising solution to generate transplantable lungs. Genitourinary tissue replacement/expansion usually involves application of biocompatible scaffolds seeded with patient-derived cells. Gene- and cell- therapy approaches seem appropriate for rare paediatrics disease of the musculoskeletal system such as spinal muscular dystrophy, whereas congenital diseases of complex organs such as the heart continue to challenge new frontiers of regenerative medicine.

Search Strategy

We searched Web of Science and PubMed for reports in English from October 1, 2011 to September 1, 2021 using the search terms “congenital diseases”, “paediatric diseases”, “monogenic diseases”, “regenerative medicine “, “stem cell therapy”, “gene therapy”, “gene editing” “cell-free therapy”, “tissue engineering” and “organoids”. Some older references were also included owing to their importance. Because of restrictions in the number of references allowed, review articles were chosen where appropriate to provide readers with more details and further references to some worthy, but older, original articles.

Introduction

Congenital malformations and acquired disorders in children lead to significant morbidity and mortality. Therapies targeted at correcting tissue damages in children are less effective, when compared to adults. This is due to children's longer life expectancy. With scars, tissue damage, and organ failure occurring during and beyond childhood, the potential for needing complex treatments including recurrent surgeries and transplantation increases. In the last two decades, Regenerative Medicine has emerged as a concrete alternative for tissue and organ replacement.

While the current clinical strategy focuses primarily on treating the symptoms, Regenerative Medicine holds the potential to substitute or heal tissues damaged by disease or trauma, as well as to normalize congenital defects, hence trying to restore structure and function of a healthy organ. The ultimate goal of this approach is to find a way to cure previously untreatable injuries and diseases.

Admittedly, most attention has been given to adult degenerative diseases, while little has been reported in childhood. The current Regenerative Medicine toolkit is constantly evolving, and the new knowledge on cell and gene therapies, stem cell biology, medical devices and artificial organs, biomaterials and polymer design, and tissue engineering could help design new treatment for children. Interestingly, paediatric physicians and surgeons often deal with rare conditions and are therefore familiar with designing a customised treatment that is at the basis of regenerative medicine applications. Crucially there have been several paediatric clinician-scientists who have been involved in this field of research from its early development.

This review focuses on postnatal paediatric regenerative medicine. First, we briefly define the basis of regenerative medicine, and subsequently we critically examine the advancement in various tissues and organs with special focus on paediatric applications.

Technological platforms

Tissue engineering

Tissue engineering emerged from the unmet need for replacement or repair of diseased tissues and organs. The modern era began in the 1980s and has emerged as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function"(1). There are now multiple strategies of tissue generation, such as synthetic or biologically active scaffolds that induce a regenerative response in healing, scaffold-free approaches using cells capable of self-assembly, delivery of tissue growth-enhancing factors, decellularization of living organs while maintaining the vascular scaffolding, and three-dimensional printing and bioprinting.

Stem cells and organoids

Stem cells are capable of both self-renewal and differentiation, which are required for durable future therapies. Tissue engineering may capitalize on native regenerative populations or *in vitro* protocols that reproduce aspects of embryonic development. Tissue-engineered organs or components thereof can be generated from native stem/progenitor (pluripotent/multipotent) populations, terminally differentiated cells, or their combination from multiple sources.

Tissue-engineered small intestine (TESI) serves as an example of progress that has been made for other tissues(2–6). Historically, initial experiments employed organoid units (OU), which are clusters of adult stem cells derived in a rudimentary way from primary tissues. OU are distinct from organoids or enteroids, in that they are comprised of epithelium and mesenchyme(7). Organoids and enteroids may be derived from primary tissues or pluripotent cells such as embryonic stem cells (ES) or induced pluripotent stem cells (iPSC) from healthy or diseased primary tissues and may be genetically altered to correct the phenotype or enhance specific functions (**Figure 1**).
Pluripotent stem cells can also be differentiated into all of the layers of the small intestine, except the enteric nervous system (ENS), and these are termed human intestinal organoids (HIO)(8). For other tissues there are existing adult stem cell and iPSC correlates.

Scaffolds: polymers, extracellular matrix, bioreactors, three-dimensional (3D) bioprinting
The second component which allows the organisation of engineered tissues and organs is the biomaterials which play an essential role to support cell self-renewal, migration, proliferation, and functional differentiation within a three-dimensional environment. To obtain efficient regeneration such biomaterials must be biocompatible, non-immunogenic and biodegradable, providing initial scaffolding and allowing gradual new tissue substitution. Extracellular matrix (ECM) derived from decellularized tissue has the advantage of replicating the highly complex signal of the native tissue. Natural polymers (alginate, collagen, hyaluronan, hydroxyapatite and polyethylene-glycol) can serve as alternative to ECM, but engineering sophisticated materials that are able to mimic the *in vivo* performance of natural scaffolds remains challenging. Three-dimensional (3D) bioprinting has the capability to fabricate complex structures that provide an artificial environment for cell self-organization and cell development. However, the paradigm of 3D bioprinting is still linked to producing customized constructs implanted through invasive open surgery. *In situ in vivo* 3D bioprinting uses light to penetrate tissue and shape constructs. Laser-assisted technology for *in vivo* bioprinting may allow minimally invasive implantation in paediatric patients or prenatally. There remain challenges in developing proper biomaterials and coupling of imaging and bioprinting technology for 3D tissue reconstruction within a specific anatomical site.

Organoids for COVID-19 and novel infectious diseases research
The COVID-19 pandemic has caused havoc worldwide since December 2019 and urgent efforts are needed to understand disease biology and to design optimal treatment. In this respect, tissue-specific organoids have provided us a unique research platform for disease modelling(9). Organoids are 3D structures which contain a full complement of differentiated cell types in the organ of interest and can be established either from induced pluripotent stem cells (iPSCs) or more commonly, from multipotent adult tissue stem cells. Recent studies have confirmed the promise of both airway and gastrointestinal organoids for COVID-19 research(10–12). These tools will definitely prepare us better for the next pandemic.

Microfluid and in vitro system for disease and preclinical modelling
Microfluidic and organ-on-chip technology are rapidly emerging as informative platforms for developmental studies, disease modelling and drug screening, reducing animal use and increasing human research models. Microfluid-based technology integrates biophysical stimuli with biological components to aid in recreating the dynamic microenvironment of cells, tissues, and organs constituting a micro-

physiological system. Organs-on-a-chip technologies mimic the dynamic conditions of the complex systems such as cardiovascular, liver, kidney and intestine.

Microfluidic fabrication enables the integration of sensors and actuators within organ-on-a-chip platform for monitoring and coupling electrical and mechanical stimuli, and for generating accurate profiles of pulsatile flow and chemical stimuli(13). Microfluidic organ-on-a-chip models can recapitulate important organ-level functions, multicellular microarchitecture, and environment dynamics. For instance, an immature engineered iPSC-derived cardiac tissue can achieve tissue maturity and relevant physiological responses by physical conditioning and increasing electromechanical stimulations(14). Similarly, microfluidic research revealed ECM-associated proteins enhancing functional hepatic organoid formation from iPSC(15). Micro-design guided epithelial organoids to self-organize and differentiate into intestinal organoids mimicking more physiological micro-intestines(16). The integration of patient-specific cells within micro-physiological system will generate data on genetic impacts on drug response, aetiology of polygenic diseases and genetic-environmental interaction.

Ex-vivo gene therapy for inherited disorders

The field of gene therapy has expanded hugely over the past two decades encompassing a growing range of inherited disorders and delivery platforms, from highly personalised therapies such as autologous ex-vivo haematopoietic stem cell therapy (HSC) to in vivo delivery of AAV therapeutics. Although the path has not always been smooth with setbacks including immune responses to adenoviral and AAV vector treatments and the development of leukaemias as a result of gammaretroviral vector site insertion across several clinical trials for primary immunodeficiencies, gene therapy is experiencing renewed enthusiasm with the advent of safer technologies and an improving understanding of vector biology. Retroviral and lentiviral gene therapies are now licensed for specific monogenic disorders (Zynteglo for beta-thalassaemia, Libmeldy for Metachromatic leukodystrophy and Strimvelis for ADA-SCID). Many early phase clinical trials using autologous lentiviral gene corrected HSCs are underway for immunodeficiencies, metabolic and haematological disorders with a comprehensive review provided in Ferrari et al(17). Longer term follow-up studies are demonstrating the durability of this approach for many conditions supporting their therapeutic potential as a one-off curative therapy(18–23). Gene edited HSC therapies, using targeted engineered nuclease platforms such as the CRISPR/Cas system, offer precise correction which could allow for more regulated gene expression which may be beneficial over lentiviral gene addition for specific conditions where protein expression is tightly controlled (eg CD40 Ligand deficiency). Although the low efficiency of homology directed repair strategies using an AAV virus to deliver a donor template may currently limit progression to clinical trial, very positive results have been recently reported using CRISPR/Cas9 to edit the *BCL11A* enhancer in patients with sickle cell disease and transfusion dependent beta-thalassaemia(24). *BCL11A* represses foetal haemoglobin and gamma-globulin expression and when disrupted leads to increase in foetal haemoglobin expression and transfusion independence. Undoubtedly targeted gene editing will be increasingly used in future clinical trials.

In vivo gene therapies

AAV-based gene therapy has been used to successfully treat several metabolic, haematological, neurological, neuro-muscular, cardiac and retinal disorders, again leading to three licensed medicines; Glybera for LPL deficiency, Luxturna for retinal dystrophy and Zolgensma for SMA. Multiple other viral and non-viral delivery systems are also being investigated in pre-clinical and translational studies, beyond

the scope of this review. For some conditions, such as cystic fibrosis and mucopolysaccharidoses, more than one strategy (eg AAV, lentiviral, adenoviral) is available or in clinical trial, further demonstrating how far the field has progressed.

Gastrointestinal tract

Short Bowel Syndrome

Tissue-engineered small intestine (TESI) can potentially restore intestinal function in patients with short bowel syndrome (SBS), a devastating condition of extensive loss of the epithelial absorptive capacity, leading to life-long dependence on intravenous nutrition. A complex network of signals sustains and terminally differentiates stem cell populations in the intestinal crypts. TESI invokes the regenerative capacity of these stem/progenitor cells and chemical messengers that maintain the cell niche.

TESI recapitulates the key components of the intestine. Specialization in the gastrointestinal regions produces tissue-engineered esophagus (TEE), tissue-engineered liver (TELi), tissue-engineered stomach (TES), and tissue-engineered colon (TEC)(3,25–28). The biologic constructs must function and support the key cell populations for absorption and peristalsis. Macroscopically, TESI must recapitulate native intestinal layers enough to be robust and regenerative. Intestinal function also requires reconstitution of an enteric nervous system (ENS), which is derived independently from the neural crest, an adequate vascular and lymphatic supply(29), and correct localization of enzymes and membrane proteins(30). To overcome the obstacles of engineering a complex intestine, an alternative is to concentrate on the mucosa function by replacing the colonic mucosa with *in vitro* engineered small intestinal mucosa or small intestinal organoids(31). This is particularly relevant in children where SBS is often associated with the existence of normal large bowel.

Adult Stem Cell Approaches

Strategies to generate TESI have evolved from initial studies by Vacanti et al to protocols generating epithelial and mesenchymal cell clusters which grow to various intestinal cell types after being delivered on biodegradable or decellularized scaffolds(2,3,26–28,32).

TESI derived from adult stem cell clusters implanted *in vivo* develops with a luminal facing epithelium and surrounding mesenchyme. The vascular supply is shared between donor and host contributions and the lymphatic system is relatively rudimentary. The epithelial surface is variable(28). The OU can restore iPSCs, secretory and absorptive epithelial cell types, and numerous mesenchymal support cells.

Improved protocols enhance TESI growth and function. Concurrent small intestinal resection stimulates intestinal adaption with increased TESI size and diameter. Anastomosis of TESI to native small intestine results in improved absorptive surface, suggesting mechanoluminal signalling factors may drive additional intestinal development. The as-yet-to-be identified stimulatory signals may include VEGF, EGF, GLP-1/2, and other growth factors such as FGF10.

The goal of TESI is to generate a sizeable construct that can be successfully anastomosed to the native intestine and provide enough digestive and absorptive surface. Rats with anastomosed TESI regained weight more quickly than control rats without TESI. TESI generated from both murine and human OU recapitulated the structure and digestive of mature intestine(28). Furthermore, both human and murine OU can be maintained in long term culture without growth factor supplementation and can generate TESI with all mature intestinal epithelial and mesenchymal cell types and ENS(33).

Pluripotent Stem Cell Approaches including Human Intestinal Organoids

All the non-ENS components of the intestine can be derived from either human embryonic or induced pluripotent stem cells (PSCs) with growth factors to form human intestinal organoids (HIO), which are spherical organoid structures with a luminal epithelium and surrounding mesenchyme(34,35). *In vitro*, HIO contain immature villus-like structures that lack lamina propria and fail to develop crypts and mesenchyme(34). However, implantation into the kidney capsule led to significant maturation of transplanted HIO (tHIO)(4) with increased crypt proliferation, mature absorptive/secretory epithelium, and increased mesenchyme. Importantly, the vasculature and lymphatics supporting tHIO are solely of host origin.

Significant intestinal adaptation occurred in tHIO following post-ileocecal resection suggesting that HIO respond to intrinsic cues for intestinal adaptation. Although tHIO are more mature than HIO, tHIO gene expression more closely resembles fetal than adult intestine(36). Implantation of HIO-seeded PGA/PLLA scaffolds into the omentum of immunosuppressed mice results in HIO-TESI resembling native intestine(2). Although HIO provide a potential platform for generating limited amounts of patient-specific intestinal tissue, they lack the ENS. ENS cells can be derived from primary intestinal biopsies or directed differentiation of hPSCs(37–39). Co-implantation of HIO-TESI with murine OU yields nerves and glia adjacent to HIO-derived epithelium and within the myenteric layers(2,40). HIO co-cultured with human pluripotent stem cell-derived vagal neural crest cells and implanted into the kidney capsule demonstrate immature restoration of ENS components(41) by inhibitory but not excitatory neurons, and synaptic formation is deficient. An alternate differentiation protocol of HIO-TESI restored key components of the ENS including neuroepithelial connectivity and neuron-dependent smooth muscle contractility and relaxation(40).

Esophageal Atresia

Esophageal atresia may be accompanied by an airway fistula or may stand alone as a long gap anomaly. Very long-gap esophageal atresia requires interposition of the stomach, jejunum or colon. However, these reconstructions are complex and not always durable. Additionally, esophageal defects may result after cancer surgery, or accidental ingestions in children. Implantation of tissue-engineered esophagus containing some but not all components of a native esophagus resulted in extrusion of the inert material. Alternate approaches included wrapping layers of cells around nonabsorbable scaffolds that required future removal and generation of muscular tubes or epithelial layers that lacked the other components of a native esophagus(42).

Tissue-engineered esophagus (TEE) has been generated from mouse and human cells. Early esophageal OU contained epithelium and mesenchyme, with differentiated suprabasal and proliferative basal layers of esophageal epithelium, muscle, and nerve, and lineage tracing identified multiple possible cellular interactions that can lead to the TEE formation(26,43). More recently, (32,44,45) prevascularized decellularized TEE grafts were reconstituted with mesangioblasts and fibroblasts that formed a muscle layer after dynamic culture prior to implantation. Further input included mucosal reconstitution with epithelial progenitor cells. Enteric neural cells could functionally engraft into the correct anatomic location when provided as neural crest cells(32).

Enteric neuropathy

The enteric nervous system (ENS) derives embryologically from vagal and sacral neural crest (NC) cells that migrate along the length of the intestine to create densely interconnected and richly varied ENS cell types that underpin proper intestinal function. Improper development, or disruption of the ENS leads to intestinal obstruction, life-threatening infection, chronic pain, frequent hospitalizations, and death. Congenital or acquired enteric neuropathies can occur in multiple regions of the gastrointestinal tract.
Hirschsprung's disease is a congenital enteric neuropathy in which the neural crest cells fail to migrate to all or some of the total intestine(46). Typically, multiple surgeries are required for newborns, who present with intestinal obstruction and sometimes life-threatening enterocolitis. In severe cases (10-15%), most or all the intestine lacks the ENS and the intestine is therefore not functional. Although some of these children will survive until intestinal transplantation, which is expensive, morbid, and has a 50% failure rate at 5 years while requiring maximal immunosuppression, many will not.
As described above, there are multiple methods for deriving possible ENS progenitors such as adult stem cell NC cells or by direct differentiation from iPSC. Cell-based therapies, supplemented by gene-editing for underlying genetic mutations, are highly promising(47).
For advanced TESI, NC cells were added to a multi-stage TESI with correct integration. Adult stem cell derived NC cells can also form ENS structures in conjunction with HIO, which lack an ENS whether delivered contemporaneously with transplant or in a delayed fashion(2,40,48)

Liver and pancreas

Liver diseases
Liver disease affects 1 in 2,500 live births. Congenital and progressive/chronic hepatobiliary diseases such as biliary atresia and Alagille syndrome constitute a major healthcare burden. Neonatal liver dysfunction leads to cholestasis. Unresolved cholestasis triggers inflammation, fibrosis, hepatic dysfunction and ultimately liver failure. Ideally paediatric liver disorders should be diagnosed early and effectively treated to avoid end-stage liver failure(49).
The liver develops through a process of liver progenitor induction and proliferation, hepatocyte and cholangiocyte differentiation, and organogenesis of vascularized hepatic lobules connected to the intrahepatic and extrahepatic bile duct system. Understanding the underlying molecular and cellular mechanisms is the key to the development of novel functional therapies, or/and liver regeneration for paediatric liver disorders. Relevance of laboratory and animal research findings to clinical application is often questioned. Regenerative medicine now provides human surrogates for disease modelling and drug screening for pre-clinical testing, capable of diagnostic and therapeutic breakthroughs.
Inborn errors of liver metabolism (IELMs) are caused by a single enzyme defect resulting in severe hepatic dysfunction. IELMs collectively account for 10-50% of paediatric liver transplantation. Gene/cell-based therapy represents an attractive novel therapeutic approach. Human heterologous hepatocyte transplantation can potentially restore hepatic enzyme activity but is limited by shortage of suitable donors and progressive loss of the non-proliferating transplanted hepatocytes. Genome-edited hepatocytes derived from patient-specific iPSCs can be an alternative strategy, but non-proliferation of derived hepatocytes remains an issue. A promising approach is the use of mesenchymal stem cells (MSCs) which have greater regenerative and immune-tolerogenic properties and can be obtained with large-scale production and storage. A Phase I/II Trial of Liver-derived MSCs (HepaStem) infusions in 20 paediatric

patients with urea cycle disorders (UCDs) or Crigler-Najjar (CN) syndrome showed safety and preliminary efficacy(50).

The employment of gene transfer viral vectors is another promising approach for liver-targeted gene therapy for IELMs. More specifically, adeno-associated virus (AAV) offers a natural hepatic tropism, stability of vector genome, and persistent high-level expression without associated human pathology after infection, making them ideal vector candidates(51,52). The first clinical trial using AAV for an IELM liver disease was for the treatment of acute intermittent porphyria (AIP) in 2016. To date, Phase I/II Trials are ongoing or about to start for a multitude of IELMs, including CN, phenylketonuria (PKU), and Wilson's disease (WD)(51).

Multifactorial and heterogeneous liver diseases present even greater challenges(49). Biliary atresia (BA), a fibroinflammatory obliterative disease of the extrahepatic and intrahepatic biliary tree cannot be eradicated by Kasai portoenterostomy, a surgical procedure to relieve extrahepatic bile duct obstruction. Yet, few novel non-surgical therapies have been evaluated systematically or shown to be effective. Apart from poor understanding of BA pathogenesis, current paradigms tend to pool all BA patients in clinical testing even though it is unlikely that they will be equally responsive to new therapeutics(53).

Liver organoids derived from BA patients serve as a valuable human disease model to elucidate the complex pathobiological mechanisms. Beta-amyloid deposition around liver bile ducts in BA organoids represents a promising biomarker and novel therapeutic target(**Figure 2**)(54). Patient-derived organoids (livers biopsies or iPSCs) could become a clinically tractable strategy for BA precision medicine.

Organoids have greatly enhanced the capacity to isolate and expand human hepatocytes and cholangiocytes for regeneration/repair of diseased parts of the liver(55). Normal cell phenotype may also be restored through genome-editing(56–58) and niche modification in patient-derived organoids. Although cholangiocytes represent only 5% of all liver cells, cholangiopathies account for 70% of paediatric liver transplantation. Repair of human intrahepatic ducts(59) by transplantation of human cholangiocyte organoids provides proof-of-principle for regenerative therapies for intractable paediatric cholangiopathies(60).

Alternatives to liver transplantation for end-stage liver failure

Orthotopic liver transplantation remains the only definitive treatment for end-stage liver disease, with major limitations including donor organ shortage, high cost and expertise, and long-term immunosuppression(61). Alternative cell-based and tissue engineering liver therapies aim to support hepatic function during a patient's wait for a suitable organ or to permanently provide specific missing biochemical functions in existing organs and eventually to create tissue-engineered alternatives to donor organs. All approaches are based on allocating adult hepatocytes or stem cell-derived hepatocyte-like cells within a three-dimensional structure that ensures their survival and maintains their function(62). There are three main strategies: extracorporeal bioartificial livers (BALs), injection of liver cell suspensions, and *in vitro* organ recreation for subsequent implantation (**Figure 3**)(61).

BALs are extracorporeal devices that utilize cartridges of primary hepatocytes or cell lines to process patient plasma. The goal is to remove blood toxins which damage the brain and kidney, supplement liver function and provide time for liver regeneration or organ transplantation. Several BALs (e.g. HepatAssist, extracorporeal liver assist device (ELAD)) evaluated in clinical trials showed no adverse events, but no definitively increased survival(61). The parenchymal cells within these devices developed necrosis with a negative functional impact. The implementation of a microfluidics-based vascular network has supported

viability and function of liver cells in a short-term *ex vivo* model. Liver cell transplantation aims to foster organ regeneration or provide a missing metabolic function arising from a genetic defect. Primary hepatocytes are delivered to the liver via the portal vein or spleen. Long-term clinical trials indicate safety but low efficiency of cell engraftment (10%). This limits treatment utility to a few pathologies where only a minimal replacement of a missing hepatic function will have a noticed clinical effect(63). Ultimately, approaches to develop a tissue-engineered whole liver *in vitro* for transplantation continue to unfold, with a current focus on decellularization-recellularization technology or three-dimensional bioprinting(62).

Stem cell derived beta cell therapy for type 1 diabetes

Diabetes is a major chronic disease worldwide. Allogeneic islet transplantation has proven to be a major advance over the traditional use of insulin injections. Nonetheless, its widespread adoption is limited by insufficient donor source, small number of harvested cells, long-term immunosuppression, and islet loss from ischaemia in the peri-transplant period. The use of stem cells as the source of beta cells may provide a feasible solution in clinical islet transplantation(64,65). Recently, viable and functional insulin-secreting organoids have been generated and help protect the islets from inflammatory insults and promote engraftment(66,67). Advances in regenerative medicine technology could one day mean unlimited sources of insulin-producing cells as therapy for type 1 diabetes.

Airways and lungs

Airway and lung regeneration would be extremely relevant in children affected by congenital and acquired diseases. Rarely, infants and children are affected by airway disease with insufficient trachea (>30% total tracheal loss) to perform a reconstruction (slide tracheoplasty) without tension. However, there are also a number of congenital tracheal pathologies where surgical options are limited, such as tracheobronchomalacia and extensive laryngotrachesophageal clefts, tracheal agenesia and congenital high upper airway obstruction syndrome (CHAOS). In those extreme situations, ideally an autologous engineered tissue should be ready to be implanted in case of failure of conventional therapies. Indeed tracheal tissue engineering using decellularized cadaveric tissue was first successfully adopted in a child affected by congenital tracheal stenosis who previously failed a series of conventional surgeries(68).

More complex lung engineering is a promising solution to generate functional and transplantable lungs, and is a powerful research tool to study lung development, repair and regeneration both in health and disease. Lung tissue engineering mainly relies on a two-step process based on a scaffold preparation and the subsequent cell repopulation. The scaffold can be obtained by decellularization of non-human (small and large animal) or human organs(69–71). Different physical or chemical approaches are used to remove the cellular component from the organ while preserving the extracellular matrix (ECM) composition and structure. Most of the decellularization protocols rely on a series of detergents-based treatments used to lyse and remove cells and residual DNA which are delivered by perfusion-based protocols (solutions are perfused through the vasculature and/or airways). The respective protocols differ in their efficiency, matrix preservation and cellular material removal. Mechanical properties of the extracellular matrix influence various biological and physiological processes (e.g. stem cell differentiation, perfusion etc.). Methods for their assessment are needed to control the quality and biocompatibility of the lung scaffolds as cells also use mechanical cues to guide attachment, differentiation, migration, and proliferation. Repopulation of the scaffold is the next step of the *in vitro* lung model development. Different cells and

their mutual combinations have been used to successfully repopulate lung scaffolds – the most promising being primary tissue-isolated progenitor cells(72) and human induced pluripotent stem cells (hiPSC)-derived lung progenitors derived through the activation of various signalling pathways that recapitulate lung development(73,74). Clinical translation is on the way thanks to progress in microvasculature engineering making it possible to produce and transplant bioengineered lung in an autologous porcine model(71) and to scale up the engineering and transplantation of viable lung grafts based on decellularized porcine lung scaffolds and human endothelial and epithelial cells(75).

Heart and vascular

New approaches for incurable/complex congenital heart disease

Complex congenital heart disease (CHD) is largely palliative and as of today, rarely curable. Despite decades of study, the pathogenesis and treatment of this heterogeneous group of diseases remain unresolved. Although the annual global prevalence of CHD is decreasing, the relative importance of CHD as a cause of child mortality is rapidly increasing. There are over 250,000 deaths per year from CHD(76), with the most rapid declines coming from increasing socio-demographic index (SDI). Persistent wide variability in patient outcomes across hospitals is incompletely understood, although in several countries, centralization of resources has resulted in considerable improvements(77). Notably, in middle or high SDI, mortality from other diseases has declined to a greater degree than CHD, thus mortality from CHD accounts now for a larger percentage of infant deaths.

A focus on the pathogenesis of CHD has helped define a vast genetic landscape, with contributions of Mendelian and rare genetic variants becoming more completely described(78). However, the contribution of noncoding variants and epigenetics remain barely explored. The combination of machine learning approaches, with advances in natural language processing will allow associations with otherwise overlooked variants informing novel therapeutics. The interpretation of pathogenic variants in the context of sub-primate animal models has proven incompletely predictive of human phenotypes. Thus, the directed differentiation of human pluripotent stem cells has provided insights and opportunities to understand the timing and mechanisms of early disease development(79).

The majority of important cardiac developmental steps are complete by six weeks of gestation, complicating prenatal therapy. There have been bold attempts using catheter-based approaches for prenatal intervention of ventricular hypoplasia. However, the variability in responses suggests a heterogeneous pathogenetic topography, requiring robust patient stratification. Stem cell therapies may eventually provide a potent approach for heart muscle regeneration, though failures and misdirection within the field have obfuscated progress(80). Delivery of intraoperative gene therapy or modified mRNA could be considered for a subset of patients with well-described monogenic disorders, or for modulation of predictable interventional effects (e.g. impaired function, fibrosis). Despite the development of advanced technologies, global impact may best be instituted by public health and economic measures. Yet, the plateau in patient outcomes is clear, suggesting an acute need for new treatments. Thus, an appropriate focus is a deeper understanding of the molecular and genetic pathogenesis of CHD enabling development of personalized therapies.

Vascular biology for regenerative medicine

Vascularization of tissue engineered constructs is essential to supply nutrients and oxygen to cells, in order to survive and function *in vivo*(81). *In vivo* neovascularization aims at creating vascular networks following cell transplantation, or to connect host vasculatures with prefabricated *in vitro* vascular networks in tissue engineered constructs. Neovascularization can be achieved with the ability to form vascular networks *in vivo*, by surgical techniques including muscle flaps and omentum to engineered organs or tissue such as esophageal patch(82) or engineered airway tissue from decellularised cadaveric trachea(81).
Alternatively, neovascularization is achieved through the building of a vascular structure in the construct *in vitro* by the controlled assembly of different vascular cells, such as endothelial cells and pericytes, to generate engineered construct with vascular structures seen in pathological tissues, and quantitatively recapitulate the *in vivo* dynamic behaviour in *in vitro* conditions(83). Vascular endothelial cells (VECs) manifest distinct structural and functional attributes that regulate angiogenic, metabolic, inflammatory and regenerative functions in tissue-resident stem cells(84). VECs supply angiocrine signals that modulate expansion and maturation of adult stem cells(85). Thus, VEC-driven vascularization of the stem cells/organoids to be transplanted for regenerative purposes will not only augment the expansion of stem cells/organoids, but also facilitate anastomosis and engraftment to host vasculature. While generating tubulogenic endothelium has been technically difficult in the past, recent advances in reprogrammed human vascular endothelial cells brought to the development of an adaptable and tubulogenic state in defined extracellular matrix (Reset-VECs, R-VECs)(86). R-VECs self-assemble into functional non-leaky vasculature within microfluidic devices capable of transporting human blood under physiological normoxia and fluid shear stress, thereby establishing vascularized engineered tissues. While building a fully functional vascularised tissue before transplantation remains the most attractive approach, functionally anastomosing the *in vitro* engineered vascular network with the existing host vasculature remains a major hurdle(87).

Genitourinary tract

Various regenerative medicine technologies involving the genitourinary organs have been used in patients, including bladders, urethras, vaginal organs, and the kidney, with penile tissue currently entering clinical trials. The initial protocols developed for engineered bladder tissue involved studies to achieve primary cell expansion and development of biodegradable scaffold systems using a canine model. The human study was in paediatric patients requiring augmentation cystoplasty. The technique involved a tissue biopsy from the patient's organ, expanding and seeding the cell types in the biodegradable scaffold, stabilizing the structure in a bioreactor, and implantation of the engineered construct into the patient. These patients showed normal bladder pressures on 5-year follow-up. Tissue engineered urethras were created using the same strategy. Children with urethral injury had a 5 cm tubularized scaffold seeded with the patient's cells. The implanted urethral constructs showed maintenance of normal flow rates, wide calibers without strictures, and normal tissue architecture on 6-year follow-up. Likewise, engineered vaginal organs were implanted and assessed in patients aged 13–18 years with vaginal aplasia caused by the Mayer-Rokitansky syndrome(88). The engineered vaginal organs showed normal functional variables on 8-year follow-up (**Figure 4**).
Solid organs are most complex. Over 80% of patients requiring organ transplantation are waiting for a kidney. Experimentally, functioning renal units were generated that produced urine-like fluid. Examination of the renal devices showed formation of organized glomeruli- and tubule-like structures.

Using the same expansion techniques, kidney cells can be combined with a hydrogel, and inserted back into the same patients. This technology recently completed phase II FDA human clinical trials for the prevention of kidney function decline, and phase III trials are being planned. Engineered penile tissue was implanted in a rabbit model(89). The bioengineered corpora demonstrated normal functional parameters, and male rabbits receiving the implants successfully impregnated females who delivered live pups. This technology was recently approved by the FDA for phase I clinical trials for patients with penile defects. More recently, rabbits underwent a subtotal uterine excision and were reconstructed with autologous cell-seeded constructs(90). The cell-seeded engineered uteri developed native tissue-like structures, including organized luminal/ glandular epithelium, stroma, vascularized mucosa and two-layered myometrium, leading to normal pregnancies, fetal development to term and live birth.

Skin, neural and musculo-skeletal systems

The skin

Skin, the largest organ of the human body and barrier between the internal and external environment, was the first engineered organ that went from laboratory research to patient care.

Now, in addition to traditional wound healing strategies and tissue regeneration, such as skin grafting, tissue-engineered skin is a major treatment alternative for patients with extensive full-thickness burns, acute traumas, genetic skin disorders, large chronic wounds, or surgical interventions. Tissue-engineered skin preparation involves two major components: cells and/or extracellular matrix(91). A cell-based strategy to develop skin substitutes involves differentiated, embryonic, or induced pluripotent stem cells, such as human dermal fibroblasts, foreskin derived keratinocytes, and keratinocyte stem cells. By co-culturing these cells, skin models are being developed with the goal of making engineered tissues similar to their natural counterparts(91). A scaffold approach is the main basis of skin tissue regeneration, as scaffolds are the best materials for restoring, maintaining, and improving tissue function(92). There are several scaffold types, such as porous, fibrous, microsphere, hydrogel, composite and acellular, each with discrete advantages and disadvantages(92). These scaffolds are either made up of highly biocompatible and biodegradable natural biomaterials or synthetic materials. Biomaterials, such as collagen, cellulose, and chitosan, closely resemble natural extracellular matrix (ECM) and are best suitable for skill cell growth, while synthetic materials, such as polycaprolactone (PCL), poly-ethylene-glycol (PEG), or poly lactic acid (PLA), enhance the strength of the scaffold material. Applying more efforts towards developing composite scaffolds of natural and synthetic biomaterials will provide an optimized environment for skin tissue growth(92). Though skin tissue engineering has evolved tremendously in recent years, several limitations, such as reduced vascularization and failure to integrate, persist, and a complete functional skin substitute is not available(91).

The spinal cord

Spinal cord injuries (SCI) are direct trauma to central nervous system tissue, which result in functional and sensory deficits caudal to the site of injury due to damage to or severance of axons, loss of neurons and glia, and demyelination. Approximately 11,000 Americans sustain SCI each year, 14% being children(93). Spontaneous regeneration, and thus recovery of neural function, after SCI is limited due to lack of CNS plasticity and neural guidance. Improvement in our understanding of the pathophysiology of SCI coupled with advances in stem cell technology, biomaterials, and regenerative medicine over the last decade have

resulted in promising preclinical advances in neuroregeneration(93). A tissue engineering approach simulates the architecture of a healthy spinal cord via an implant consisting of a polymer scaffold seeded with neural stem cells. These units have been implanted into rat and monkey SCI models promoting long-term (i.e. months) degree of recovery with no negative consequences(1). In a canine SCI model, implantation of neural network tissues resulted in significant motor recovery of paralyzed pelvic limbs, and the neural network tissue improved synaptic integration with host neural circuits, transmitting excitatory electrical signal across the injured area to the caudal spinal cord, providing just one promising strategy for SCI clinical therapy(94).

The musculo-skeletal systems

Gene and cell therapy approaches in the musculoskeletal system are at the forefront of experimental therapeutics in paediatric diseases because of the plethora of genetic disorders affecting muscle, cartilage and bone, and the nervous system with secondary problems in the musculoskeletal system. Cell therapies based on tissue engineering are potential treatments of congenital disorders in which tissue replacement is the primary therapeutic outcome. Approval for gene therapies for spinal muscular atrophy (SMA) caused by mutations in the survival motor neuron (SMN) 1 gene has recently be granted by the FDA and EMA (95). These include the gene replacement strategy using Adeno-associated virus (AAV) to deliver a wild-type SMN gene to motor neurons. In addition, an orally available small molecular alternative to AONs that can also modify splicing has been approved in the US and Europe. AON therapy for exon skipping in Duchenne muscular dystrophy (DMD) has also been granted approval. These are applicable to approximately 13% of DMD patients based upon the specific genetic mutations in the dystrophin gene. A wide variety of other gene therapy approaches to DMD, such as stop codon read-through, AAV-mediated gene delivery, and AAV-mediated CRISPR/Cas9 gene editing, are under active investigation(96). An enterally administered small molecule for stop codon read-through has been approved in Europe but not yet in the US and is applicable to approximately 10% of DMD patients with nonsense mutations. Clinical trials of stem cell therapies for DMD have revealed limited success(97) with a major hurdle of cell delivery. Gene and stem cell therapies have entered clinical trials for several rare, genetic bone disorders such as osteogenesis imperfecta (OI)(98).

Areas of ongoing consideration

When considering regenerative medicine applications to medicine, there are some practical limitations which are relative to optimization, scalability and standardization of the process(99). While engineering functional organs from scratch will be the ultimate goal, applications in human may take different directions and various options may become available. Bioengineered human organs could be produced in host animals such as pigs or monkeys(100). Producing human-animal chimeras could indeed offer the possibility of obtaining transplantable human organs using autologous stem cells and overcome the donor organ shortage problem by offering patient-specific and immune-matched organs for transplantation(101). Interspecies chimera can be obtained by introducing human pluripotent stem cells into the animal embryo which has key regulators for organogenesis knocked out. Human cells injected into the blastocyst or in the fetus via in utero transplantation will be able to integrate and generate the human organ which will not be rejected(102).

On the other hand, xenotransplantation of genetically modified organs has been discussed as a potential alternative(103). Genetically multi-modified pigs that lack galactose-α1,3-galactose epitopes and express a human membrane cofactor protein (CD46) and human thrombomodulin have survived for up to 945 days after heterotopic abdominal transplantation in baboons. In 2021, surgeons at New York University Langone Health transplanted kidneys from genetically modified pigs into two legally dead people with no discernible brain function, and more recently the first human patient received a new xenogenic heart, opening the way to xenogenic transplantation(104).

Looking into the near future

It is likely that progress in the field of regenerative medicine will see an increased number of applications of engineered tissues and organs to congenital disorders. To address the complexity of the congenital and acquired disorders in children, we should focus on the innate capacity of tissue to regenerate in children (wound healing), which will ultimately lead to better outcomes in this age group when compared to the attempt of transplantation to adults who have poorer regenerative capacity and impaired local tissue vascularization. In order to safely introduce these innovative technologies, long term outcome studies should be encouraged with translation focusing on restoring some functionality of the organ, rather than its entire replication. Indeed, engineering functional tissues such as epithelium for cornea, trachea, esophagus, or intestine rather than re-building the entire organ can be safer, more effective, and offer a real therapeutic advantage. We envision that delivering technology to patients will require full integration of regenerative medicine into clinical practice, with physicians and surgeons being involved in the process from the beginning. Specific training programmes may be required to prepare the next generation of regenerative medicine specialists while regulatory bodies should help translating these technologies into children by creating safe framework. It is possible that rather than waiting for randomized controlled trial (RCT) we would have to build cumulative experience through international registries.

Contributors

PKHT & PDC designed and orchestrated the review. Sections were written respectively by KKYW & PKHT (Liver, COVID-19), AA (Genitourinary), NE & GGG (Technologies), PJG & MM (Heart), SR & PDC (Vascular), TAR (Musculoskeletal), JV & CDC (Tissue engineering, Liver, Skin, Neural), TG (Gastrointestinal), PDC, GGG & NE (Airways and lungs), CB (Gene therapy), PKHT, GGG & PDC (Introduction, conclusion). PKHT & PDC critically reviewed and redrafted the whole report. All authors approved the final version of the report for publication.

Declaration of interests

Professor Tam reports fee from BlueRock Therapeutics LP and Xellera Therapeutics. Dr. Mononen reports grants from Knut och Alice Wallenbergs Stiftelse, grants from Swedish Research Council, during the conduct of the study and outside the submitted work. Prof. Raffi reports being a non-paid consultant and co-founder of Angiocrine Bioscience. Dr. Vacanti reports personal fees from 3D BioLabs, LLC, outside the submitted work; In addition, Dr. Vacanti has a patent at Partners Healthcare and MIT pending, and a patent at Partners Healthcare and MIT issued. Dr. Atala has patents issued in the fields of bladder, urethral, vaginal, penile and uterine tissue enigneering. Dr. Comer reports personal fees from 3D BioLabs, LLC, outside the submitted work. Dr. Gruber reports grants from NIH during the conduct of the study; In

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Figure legends

Figure 1. Examples of human organs to be modelled and their corresponding *in vitro* models: human induced pluripotent stem cell derived liver organoids (left), gastric organoids derived from paediatric stomach biopsy (centre) and small intestinal organoids derived from paediatric surgical specimen (right). The organoid models are characterised for specific marker expression. The immunofluorescence panels show hiPSCs liver organoid expressing hepatocyte

nuclear factor 4 alpha in green, albumin in red, and nuclei in blue (lower left); a human gastric organoid expressing mucin 5ac in green, and nuclei in blue (lower centre); a human small intestinal organoid expressing epithelial cadherin in green, mucin 2 in red, and nuclei in blue (lower right).

Figure 2. Compared to control, Biliary Atresia (BA) organoids displayed aberrant morphology and positive staining for β-amyloid. Upper row: representative bright field images of the organoid cultures from liver biopsies of a hepatoblastoma patient (control) and a BA patient. Lower row: representative images of fluorescent staining of organoids from control and BA with β-amyloid (red). Live cells are stained blue with 4',6-diamidino-2-phenylindole (DAPI).

Figure 3. Alternative strategies to liver transplantation for end stage liver disease.

Figure 4. 18 year old patient with Type I MRKH=Mayer-Rokitansky-Kuster-Hauser who received an engineered vaginal organ. MRI Studies (A-C): (A) Preoperative MRI image shows absence of vaginal organ. (B) MRI 1 year after surgery shows engineered vaginal organ. (C) MRI image 5 years after surgery (boxes within the MRIs show engineered vaginal organs). Tissue analyses (D-H): Histological and immunohistochemical results of engineered vaginal segment biopsy samples at 1 year (top column) and 5 years after surgery(middle column), and from a normal control tissue (bottom column); scale 25 μm. (D&G) Hematoxylin and eosin. (E&H) Myosin. (C&F) AE1/AE3. Figure adapted from (88).