Tissue Engineering for the Neonatal and Pediatric Patients

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ABSTRACT

Of all the surgical specialties, the remit of the pediatric surgeon encompasses the widest range of organ systems and includes disorders from the fetus to the adolescent. As such, the recent emergence of tissue engineering is of particular interest to the pediatric surgical community. The individual challenges of tissue engineering depend largely on the nature and function of the target tissue. In general, the main issues currently under investigation include the sourcing of an appropriate cell source, design of biomaterials for guided tissue growth, provision of a biomolecular stimulus to enhance cellular functions and the development of bioreactors to allow for prolonged periods of cell culture under specific physiological conditions. This review aims to provide a general overview of tissue engineering in the major organ systems, including the cardiovascular, digestive, urinary, respiratory, musculoskeletal, nervous, integumentary and lymphatic systems. Special attention is paid to pediatrics as well as recent clinical applications.

Keywords: Tissue engineering, regenerative medicine, research, pediatric surgery, clinical

1. INTRODUCTION

Current treatment of tissue loss, including transplantation, surgical reconstruction, medical devices and replacement therapies, have all been associated with a range of problems and complications. Transplantation is of particular concern, with a serious shortage of organ donors, especially in the pediatric age group [1]. In the event that suitable donors are identified, organ rejection and toxic effects of life-long immunosuppressants present further limitations on the treatment of patients [2, 3, 4]. Tissue engineering has been proposed as an alternative strategy for the treatment of tissue loss. Tissue engineering is a multidisciplinary field, combining the principles of engineering and biological sciences with the aim of regenerating diseased or damaged tissues and organs. Generally, in tissue engineering, a patient's own cells are isolated

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and proliferated *in vitro* and combined with a supportive scaffold material. The resulting cell/scaffold construct is then cultured *in vitro* within a bioreactor, a device which mimics normal physiological conditions by providing physical stimulation to encourage cell growth and tissue-specific organization. Alternatively, the construct can be implanted into an *in situ* bioreactor, such as the omentum, to promote vascularization. Finally, the construct may be transplanted to the defect site [5, 6, 7].

Within the basic framework, there exist numerous alternative strategies of tissue engineering. The principle differences among those approaches lie in the choice of cell source and scaffold material. In some approaches, allogeneic cells may be applied instead of autologous cells, with the advantage of availability of larger cell numbers and the ability to create an *off-the-shelf* tissue engineering product, however, with the disadvantage of potential immunological rejection and disease [8]. With autologous cells, immune rejection would not be an issue; however, potentially, available cells may be too few, the required time for culturing (in order to achieve the required seeding density) may be too long, or the patient's own cells may be unsuitable due to disease. For both approaches, the proliferation of cells to obtain sufficient cell densities for therapeutic applications is a common difficulty. Mature cells derived from biopsies often are reluctant to proliferate *in vitro*, and may be subject to de-differentiation. Therefore, the use of stem cells has gained increasing popularity in tissue engineering research.

Stem cells benefit principally from a high proliferative capacity, allowing for the isolation of large numbers of cells from relatively small biopsies. In recent times, mesenchymal stem cells (MSCs) have received a great deal of attention [9]. MSCs can be isolated from a wide variety of tissue types and have the ability to differentiate into mesenchymal lineages [10]. Traditionally, MSCs have been isolated from bone marrow; however, MSCs derived from alternative sources, such as adipose tissue, are more easily isolated and may result in a more effective expansion of cell population [11]. The plasticity of MSCs is not as good as that of embryonic stem cells (ESCs); however, the use of ESCs is complicated by ethical issues and uncertainty as to whether such cells can be consistently differentiated into a target cell type [12]. Other promising sources of stem cells are those of the umbilical cord and amnion. Such cells are believed to show greater plasticity in comparison to adult MSCs, yet avoid the ethical dilemmas associated with the use of ESCs [13, 14].

The second factor of importance in tissue engineering is the selection of an appropriate scaffold type. A scaffold material primarily provides a supportive structure onto which cells can attach and be confined. The employment of a scaffold material has a number of benefits over simple injection of cells into a defect site. Firstly, the scaffold material localizes the cells to the defect site and restricts cell loss [15]. A scaffold material also provides mechanical support to the defect site, which is of particular importance in the regeneration of structural tissues. As of such, it is desirable that a scaffold material mimics the biomechanical properties of the native tissue with regards to, for example, tensile strength and elasticity [16]. A scaffold material must also be biocompatible, not illicit an inflammatory reaction, and have a rate of biodegradation which matches the rate of new tissue formation. Scaffold materials are generally categorized as natural or synthetic. Natural materials include decellularized tissue, in

which cellular and nuclear materials have been removed from a donor tissue leaving only the extracellular matrix, or processed extracellular matrix scaffolds in which a naturally occurring protein, such as collagen, is purified and fabricated into a porous 3D material suitable for the infiltration of cells [17, 18]. Synthetic materials include polymers, bioceramics and bioglass. Polymers, in particular, benefit from the increased flexibility in design of novel materials with specific degradation rates, biomechanics, and their ability to be combined with other polymers to modify the resulting properties [19]. Scaffold materials can also be combined with a variety of growth factors to promote tissue growth and regulate tissue formation [20]. Finally, numerous studies have also demonstrated that tissues can be formed without a supportive scaffold, thus avoiding the issue of foreign body reactions. Examples of such approaches include self-assembling blood vessels and cell sheet technology for the formation of cardiac tissue [21, 22].

As with any new therapeutic technique, there are certain ethical issues that need to be addressed. The ethical issues that dominate debate on tissue engineering tend to be focused on the use of cells, in particular embryonic stem cells [22]. Other issues of importance include the use of adequate animal models in the experimental phase and weighing the risks and benefits for the patient during the clinical phase [23]. It has also been suggested that the inherent complexity of the tissue engineering processes could present a challenge to the informed consent process [24].

The true potential of tissue engineering is only beginning to be realized as research starts to be transferred into the clinical setting. The tissue engineering approach has already been applied in patients for the treatment of vessels, bladder, bronchus, cartilage and bone [26, 27, 28, 29, 30]. It is hoped that the next decade will see a significant increase in such clinical trials in which the actual benefit of tissue engineering can be assessed. This review will encompass current research in tissue engineering related to the main systems of the body with special emphasis on state-of-the-art research and clinical trials.

2. TISSUE ENGINEERING IN PEDIATRIC SURGERY

The broadest spectrum of patients and pathologies are managed by the pediatric surgical community. Pediatric surgery involves surgical procedures on the fetus through the adolescent age groups, and is performed on a large range of organ systems. Congenital defects in particular, present a challenging task for the pediatric surgeon. The significant loss of functional tissue mass, in such cases, is often beyond the natural regenerative processes of the body. Current techniques for the treatment of disorders, such as short bowel syndrome, suffer from many well-known limitations. Critically, the shortage of donor tissues for transplantation is a large problem for the pediatric age groups. In particular, waiting times for organ donation in the 0-5 year age range are considerably longer than other age groups [1]. For these reasons, the emergence of tissue engineering technology is of particular interest to the pediatric surgical community.

2.1. The Cardiovascular System

Congenital heart disease encompasses a large group of disorders involving the muscles, valves, septum and vessels of the heart, and is the most common major birth defect,

with a prevalence of 11.89 per 1000 children [31, 32]. In the last few decades, due to improved management, mortality rates have fallen, yet remain significantly high. Correction of heart defects often requires surgical intervention and the application of allografts, xenograft and prosthetic materials. The use of such materials is limited by well-known factors including a shortage of supply and a requirement for immunosuppression with allografts and an association of disease transmission with xenografts [33]. In addition, prosthetic materials often have suboptimal degradation rates and fail to grow with the child, necessitating multiple surgeries [34]. In comparison, a tissue-engineered construct containing self-cells would avoid problems of donor shortages, immunosuppression, and disease transmission, and could potentially adapt and remodel as the surrounding environment develops.

In an early clinical case, the peripheral pulmonary artery of a 4-year old patient was reconstructed using the patient's own venous cells [26]. Cells sourced from the peripheral vein were cultured *in vitro* followed by seeding onto a caprolactone/poly lactide co-polymer conduit, reinforced with polyglycolic acid (PGA). The cell/conduit construct was cultured for another 10 days *in vitro* before implantation. The post operative angiography was satisfactory with good patency with no dilation or constriction. It was concluded that such an application was a promising strategy for reconstruction of low pressure systems [26].

Disadvantages identified with the aforementioned approach were the length of time required for *in vitro* culture and the use of xenoserum. Therefore, in subsequent clinical trials, bone marrow cells and patient's own serum were employed. In one clinical trial, 23 patients received tube-like grafts and 19 patients received a sheet-type patch [33]. The scaffold material was a copolymer of lactide acid and ϵ -caprolactone with a porosity of 80%, a pore diameter in the range of 20-100 μm and a degradation rate of a few months *in vivo*. Bone marrow samples were aspirated from the anterior superior iliac spine and passed through a nylon cell strainer followed by centrifugation with Histopaque-1077 to isolate mononuclear bone marrow cells (BMC). The BMCs were seeded onto the polymers, after which the outer surface of the scaffold was sprayed with a fibrin glue. The construct was cultured in the patient's own serum for 4 h followed by implantation. Resulting constructs showed excellent hemodynamic performance without serious complications.

In subsequent studies, the ability of BMCs to differentiate into mature vascular cells of developing tissue-engineered vascular grafts was tested [35]. Synthetic vascular grafts seeded with human mononuclear BMCs were implanted into mice. Although BMC-seeded biodegradable scaffolds did transform into functional mature blood vessels, it was shown that after 1 week of implantation, no human-derived BMCs were detected in the neovessel. The result therefore suggests that rather than differentiation into vascular cells, BMCs contribute to vascular development through a paracrine role contributing to an inflammation-mediated process of vascular remodelling.

Tissue engineering approaches have also been applied to valve replacement. Bioprosthetic and mechanical valves suffer from an inability to grow or remodel, thrombogenesis, and a susceptibility to infection. In preliminary experiments, endothelial cells and fibroblasts isolated from ovine arteries were seeded onto PGA fibers, resulting in a tissue-engineered leaflet which was subsequently implanted as a

replacement for the right posterior leaflet of the pulmonary valve [36]. Post-operatively, no signs of stenosis were observed and the function and histological appearance were adequate.

The first successful tissue engineered heart valve for clinical application was produced through seeding of autologous vascular endothelial cells, isolated from a forearm vein segment, onto decellularised, cryo-preserved pulmonary allograft [37]. The construct was used to reconstruct the right ventricular outflow tract in a 43-year-old patient suffering from aortic valve stenosis [37]. In a further study, the mid-term clinical results for 23 patients undergoing reconstruction of the right ventricular outflow tract were reported. The tissue engineered heart valves showed excellent hemodynamic performance and no signs of calcification up to 5 years post-operatively [38].

Tissue-engineered heart valves have also been applied to pediatric cases. Pulmonary valve replacement was reported in two pediatric patients diagnosed with tetralogy of Fallot [39]. Tissue-engineered heart valves were produced using decellularized pulmonary allografts seeded with autologous endothelial progenitor cells isolated from peripheral blood samples. The patients recovered well from the operation with only mild to trivial regurgitation. No dilation, stenosis or valve degradation was observed after 3.5 years of follow-up.

Despite the apparent success of heart valve tissue engineering, studies in the ovine model question the necessity for *in vitro* seeding of decellularized valve scaffolds with endothelial cells prior to implantation [40]. After six months of implantation, the recellularization densities of seeded and non seeded valve scaffolds were the same, suggesting that there is no need for an *in vitro* cell seeding step.

In treating severe heart failure, tissue engineering using cell-sheet technology has emerged as one of the most promising potential treatments. Cell-sheet technology does not need a supportive scaffold material and therefore also avoids associated complications, including poor cell migration and inflammatory reaction. In this technique, autologous cardiomyocytes are grown to confluence on a thermoresponsive culture surface. The intact cardiomyocyte cell sheet can then be released from the culture surface simply by altering the temperature. Multiple cell sheets can be placed upon a myocardial defect with the overlapping cell sheets forming electrical communications, resulting in simultaneous beating [22]. Such layered cell sheets have been reported to treat one patient suffering from dilated cardiomyopathy, with promising outcomes [41].

The formation of new capillaries and blood vessels is also a key issue in tissue engineering for all organs and tissues including cardiovascular systems. Once implanted, a tissue-engineered organ must rapidly form contact with the vascular system to enable mass transport of nutrients and gases in order to remain viable and prevent necrosis. In theory, vascularization can be achieved rapidly *in vivo* through the incorporation of angiogenic molecules in the scaffold matrix or through prevascularization *in vitro* or *ex vivo* [42, 43, 5]. Nonetheless, vascularization of tissue engineered constructs remains one of the greatest obstacles.

Experimental and clinical trials suggest that tissue engineering of cardiac tissues is a promising approach for the treatment of congenital heart defects in the pediatric population. Short term results indicate that tissue-engineered cardiac tissues provide

short-term improvements to cardiac functions. A better understanding of the cellular mechanisms behind the generation of tissue-engineered cardiac tissue and techniques for the differentiation of stem cells towards cardiac lineages are required for the continued development of this exciting field of regenerative medicine.

2.2. The Digestive System

2.2.1. The Esophagus

Esophageal atresia is a relatively common congenital malformation, occurring with 1 in 3,000-5000 births [44]. In most cases, the defect can be successfully bridged through primary anastomosis; however, in long-gap atresia, a conduit, for example a colon graft, may be required. Employment of such grafts is associated with high risk of complications including leakage, stricture and gastro-esophageal reflux [45, 46, 47]. Therefore, tissue engineering of the esophagus has been suggested as an alternative approach in esophageal repair [48].

Tissue engineering of the esophagus is complicated by the anatomical complexity of this muscular organ [49]. The muscularis externa consists of a longitudinal and circular layer, containing primarily striated muscle cells in the upper regions with a gradual transition to smooth muscle cells (SMCs) in the lower regions. The contractions of these outer muscular layers are precisely coordinated via the enteric nervous system for the generation of peristaltic waves. In addition, the esophagus contains epithelial cells, glandular cells, fibroblasts, vascular cells, interstitial cells of Cajal and a third muscular layer, the muscularis mucosa, which functions to agitate the epithelium and aid expulsion of glandular secretions.

Early experimental studies investigated the application of cell-free biomaterials for esophageal replacement. In the canine model, tubular collagen scaffolds was employed to replace 5 cm cervical and thoracic defects [50, 51]. The implant was well tolerated in the cervical defect; however, in studies of the thoracic defect, formation of the muscularis mucosae was weak, whilst after 24 months, the outer layer of skeletal muscle failed to extend towards the middle of the regenerating esophagus. These results highlight the importance of anatomical site in esophagus repair and demonstrate how biomaterials implanted without prior combination with a cellular component may result in delay or failure of tissue regeneration.

The generation of complex organs consisting of more than one cell type can be achieved through a hybrid approach, whereby different tissues are cultured independently followed by assembly prior to implantation, or via a co-culture approach where the different cell types are cultured together on a single scaffold. A preliminary study showed that ovine esophageal epithelial cells (EECs) could be isolated from ovine esophagus via a modified explant technique; however, cultures were prone to fibroblast overgrowth and the epithelial cells displayed poor proliferative capacity [49]. As an alternative technique for the isolation of esophageal epithelial cells, it was demonstrated that enzymatic separation of the epithelial sheet from the underlying mucosa, followed by mechanical disruption, minimized fibroblast contamination and resulted in the isolation of high cell densities [52]. The poor proliferative capacity of EECs was then addressed through selection of a highly proliferative subpopulation [53]. A characterization of the ovine EEC population identified that 50% of the cells were in

a proliferative state. Further investigation revealed that the subpopulation of cells expressing markers for pan cytokeratin 26 (PCK 26), expressed a significantly higher percentage of proliferative cells in comparison to the total population. By isolating PCK26+ cells through fluorescence-activated cell sorting and seeding on to collagen scaffolds, it was demonstrated that this high proliferating subpopulation produced a more uniform distribution of cells with a higher attachment rate in comparison to unsorted cells.

Once sufficient cells have been procured, cells must be seeded onto suitable scaffold materials which provide structural support as well as guiding tissue orientation. Esophageal epithelial cells were demonstrated to require a 2D surface to permit organization of a stratified epithelial layer. Rat EECs seeded onto natural Alloderm® scaffolds were shown to organize as stratified epithelium with a proliferative basal layer and a keratinized layer after 18 days of *in vitro* culture [54]. In comparison, EECs seeded onto highly porous synthetic scaffolds were unable to form a continuous epithelial layer. Likewise, EECs seeded onto 3D collagen scaffolds failed to show organized epithelium, whilst EECs seeded onto 2D collagen scaffolds formed a singular epithelial sheet layer after 3 weeks of *in vitro* culture [52]. With regard to muscle tissue formation, a scaffold material should ideally encourage the formation of orientated muscle fibres. Rat SMCs isolated from the aorta were seeded onto non-organised and unidirectional collagen fibers [49]. After 8 weeks of *in vitro* culture, SMCs were shown to retain their phenotype, as detected by α -smooth muscle actin staining. Significantly, SMCs seeded onto unidirectional collagen fibers generated orientated smooth muscle strands in contrast to those cells seeded onto non-organized collagen, which generated non-organized smooth muscle tissue.

An alternative strategy to the isolation of individual cell types is the use of organoid units. Organoid units are multicellular units, containing a mesenchymal core surrounded by epithelium, which may be obtained through enzymatic digestion and mechanical agitation. In the rat model, organoid units derived from esophagus tissue were seeded onto polymers and cultured in an omental fold, followed by interposition into an esophageal defect [48]. The resulting tissue possessed a keratinized stratified epithelial layer similar to that of native tissue. A muscularis layer, positive for α -smooth muscle actin, was also present; however, it consisted of separate slips of muscle rather than a continuous layer.

Relatively few studies of esophageal tissue engineering have been conducted in large animal models. In the canine esophageal ulcer model, oral mucosal epithelial cell sheets were shown to adhere to the underlying muscle layer and result in complete healing without stenosis [55]. In comparison, in models of full thickness esophageal defects, a scaffold material is almost certainly required. Small intestine submucosa (SIS), human amniotic membrane and collagen scaffolds have all been used as scaffold materials in large animal models [56, 57, 58]. SIS seeded with oral mucosal cells was used to repair esophageal defects in the canine model [56]. Patch defects of 5 cm in length and 50% of the circumference were created in the cervical esophagus. After 1 week of *in vitro* culture, oral mucosal cells seeded onto SIS scaffolds were implanted into the defect site. After implantation, no serious complications were observed in either the cell-seeded SIS groups or the cell-free SIS control groups. Dogs treated with cell-seeded SIS

constructs showed smoother luminal surfaces and regained weight quicker than those treated with cell-free SIS [56]. After 4 weeks, histological examination revealed a well-developed epithelial lining with only slight inflammation in the cell seeded SIS group and numerous skeletal muscle bundles extending from surrounding muscle onto the graft after 8 weeks. In comparison, the cell-free SIS constructs showed the formation of many new blood vessels, yet few skeletal muscle bundles had extended onto the graft.

Human amniotic membrane seeded with canine oral keratinocytes and fibroblasts were applied to repair a 3 cm gap defect in the canine model [57]. Decellularized amniotic membrane was cultured with cells for 1 week *in vitro*, followed by placing on a sheet of PGA felt containing minced smooth muscle tissue resected from the anterior wall of the stomach. The scaffolds were wrapped around a stent within the canine omentum. After 3 weeks of abdominal implantation, the construct was moved up through the diaphragm, as a pedicle graft, into the thoracic space and used to repair the esophageal defect. After 1 week of *in vitro* culture, keratinocytes were seen to organize into stratified layers upon the amniotic membranes, with fibroblasts penetrating within. Post abdominal implantation, the majority of scaffolds showed well differentiated luminal surfaces and formation of smooth muscle-like tissue; however, in a few cases, desquamation was observed. After 1 week post esophageal replacement, animals treated with cell-free amniotic membranes developed strictures whilst the animals treated with pre-seeded amniotic membranes showed no problems with passage and feeding, except that in those constructs which showed desquamation, strictures also occurred. Whilst the transport of food to the stomach via peristalsis was observed in the tissue engineered group, peristaltic activity in the tissues engineered segment itself was absent.

In an adult sheep model, collagen sponges pre-seeded with fibroblasts and EECs were used to create a rudimentary, hollow, tubular esophageal conduit [58]. The pre-seeded sponge sheets were draped over a sterile stent of similar size to the lumen of native esophagus and gently closed by vicryl suture loops. The construct was implanted into the adult sheep omentum. The omentum is a highly vascular, fatty tissue, capable of providing blood vessel ingrowth to avascular grafts, and therefore acting as an *in situ* bioreactor. After a period of 8 – 12 weeks, a good integration within the omentum was observed, with vasculature branches developing around the construct. The implant displayed a hollow tubular morphology with a smooth inner lining similar to that of the native esophagus. Cellular and vascular in-growth was also observed within the porosity of the collagen scaffolds with no evidence of inflammation. Finally, patches of EECs were observed along the construct.

Whilst many advances have already been made in the field of esophageal tissue engineering, a number of obstacles remain to be overcome. There is a need for a new generation of hybrid scaffolds to provide for the requirements of the different cell types and the application of stem cell technology. The continued increase in *in vitro* studies should also be matched by an increase in large animal studies. Finally, for functional integration into the peristaltic activity of the esophagus, future research must focus on the muscular and neurogenic component, in order to produce an active organ rather than simply a passive conduit.

2.2.2. The Intestine

Tissue engineering of the intestine is beset by problems caused by the complex anatomy and numerous functions of the intestine. This is unfortunate considering the potential benefit of a tissue engineered intestine for pediatric surgery. Intestinal tissue loss results from surgical resection in the treatment of a variety of diseases and disorders, including necrotising enterocolitis, cancer and irritable bowel syndrome, which may result in short bowel syndrome. The current option of treatment is transplantation, which, despite significant improvements in the last decade, has a 5 year survival rate of just 75% in the best centers [59].

Synthetic conduits are largely unsuitable for rectifying small bowel syndrome as it is not just a case of loss of intestinal length, but more of a case of loss of functional tissue capable of nutritional uptake. The goal of a tissue engineered intestinal tissue, therefore, is the provision of a functional tubular intestinal replacement containing a mucosa and associated vasculature capable of nutrient absorption and a muscular layer for participation in peristalsis. Early attempts at intestinal tissue engineering involved the seeding onto scaffolds of epithelial organoid units [60]. Organoid units can be produced by partial disruption of intestinal tissue through mechanical and enzymatic means. The units benefit from retaining the epithelial-mesenchymal cell-cell interaction which may be critical for cell survival and intestinal development. Organoid units attached to PGA scaffolds were implanted into the omentum of rats. The implanted organoid units showed proliferation and regeneration of larger complex structures with columnar epithelium, goblet cells, Paneth's cells and a crypt-villus-like morphology. Evidence that tissue engineered intestine can restore functionality has also been reported in the small animal model. Tissue engineered colon and small intestine were implanted in the rodent model, resulting in improved physiological and biochemical function and regaining of weight [61, 62].

More recently, the application of organoid units in the large animal model has shown promising results [63]. A 10 cm jejunum section was resected from swine piglets and closed by anastomosis. The resected tissue was processed for the isolation of organoid units, which were seeded onto nonwoven, PGA tubular scaffolds coated with 5% poly-L-lactic acid (PLLA) and type I collagen. The scaffolds were 3.5 cm in length with a diameter of 84 mm. The constructs were then implanted into the omentum or mesentery for a period of 7 weeks. Tissue engineered intestine was shown to possess a morphology similar to that of native tissue, with comparable villi length and crypt depth. The intestinal epithelial cells of tissue engineered intestine contained enterocytes, goblet cells and enteroendocrine cells. Both native and engineered tissue showed smooth-muscle muscularis and the presence of ganglion cells.

A concern over the use of organoid units is the poor survival rate after periods of prolonged *in vitro* culture. An alternative cell source which can be expanded *in vitro* to provide sufficient cells for therapeutic use may still be required. One such cell type which has shown promise is the amniotic stem cell which has shown to have a protective effect in animal models of necrotising enterocolitis [64]. In general, further studies are warranted for the development of intestinal tissue engineering, in particular, in the determination of suitable cell sources and strategies for differentiation into various cell types which constitute the intestinal wall. Furthermore, future tissue

engineered constructs will have to undergo a series of biochemical and physiological tests to prove functionality.

2.2.3. The Pancreas

Diabetes in the pediatric population places a great burden on healthcare systems [65]. In addition, the increased incidences of childhood obesity are resulting in a rise in type 2 diabetes mellitus, representing 45% of new pediatric diabetes cases in America [66]. Diabetes can be treated through minimally invasive pancreatic islet transplantation; however, the widespread clinical application of such a technique is limited by the availability of islet donors and the need for immunosuppression [67]. Issues of tissue match and immunosuppression can potentially be circumvented by the technique of microencapsulation, whereby transplanted islet cells are protected from the host immune system by a semi-permeable membrane, and is the subject of much research [68].

For tissue engineering of pancreatic tissue, an autologous source of pancreatic β -cells is required. Pancreas islet cells themselves have a poor proliferative capacity; therefore, a number of other potential cell sources have been suggested. For example, insulin-secreting clones selected from ESCs were shown to normalize blood glucose levels in the diabetic mouse model [69]. In addition, reprogramming of human liver cells into pancreatic β -cells has also been demonstrated [70]. An alternative approach is to improve proliferation of native pancreatic β -cells through gene transduction [71].

The provision of a scaffold material may ultimately be required for the 3-dimensional growth of engineered pancreatic tissue. For example, ESC-derived islet-like cells seeded onto poly(lactic-co-glycolic acid) (PLGA) were shown to reverse hyperglycaemia in diabetic mice [72]. Currently, much attention is focused on islet transplant via microencapsulation and pancreas tissue engineering. The tissue engineering approach is appealing in that it promises the potential of regeneration of the diseased pancreas with a patient's own cells. Future research will continue to investigate techniques for the generation of functional pancreatic β -cells from a variety of cell sources and develop the use of biomaterials that can provide a 3D environment for pancreas tissue growth and maturation.

2.2.4. The Liver

End-stage liver disease in children requires liver transplantation; however, donor shortage and issues of immunosuppression present major limitations. Previous studies have demonstrated that transplantation of hepatocytes with a mass, equivalent to 10% of the patient's liver, may be sufficient for the correction of many enzyme deficiencies [73]. Currently, there exist two main approaches for hepatocyte transplantation [74]. The first involves transplantation of hepatocytes into the spleen or portal vein, resulting in engraftment of the cells within the host liver. Despite encouraging results, such a technique presents a risk of portal vein thrombosis and pulmonary embolism [75]. The second approach involves transplantation of hepatocytes beneath the kidney capsule or in other heterotopic sites [76].

To optimize the delivery and survival of hepatocytes, research has investigated the application of a tissue engineered hepatic transplantation. In this approach, a matrix is used as a carrier for the hepatocytes, allowing for the formation of a three-dimensional

neo-tissue. Such a matrix could be coated with extracellular matrix (ECM) molecules or bound with growth factors to enhance engraftment and vascularization [77, 78]. Another benefit of localizing cells is that hepatocytes are dependent on cell-cell contact and ECM compositions for the maintenance of liver-specific function [79]. Combination of hepatocytes with a scaffold material also allows the cells to be cultured within an *in vitro* bioreactor. The application of bioreactors for hepatocyte culture has an important role in future developments of hepatic tissue engineering. Bioreactors are able to mimic the *in vivo* conditions present in the liver lobule including perfusion and oxygen tension, and studies have shown that culturing hepatocytes under flow conditions results in significant increases in cell number and enhanced cell function [77, 80].

The potential drawbacks with the use of synthetic scaffolds for hepatic tissue engineering are inflammation and fibrosis. By applying cell-sheet technology, a uniform and continuous sheet of hepatic tissue can be created without the requirement for a supportive scaffold. Furthermore, the resulting sheets can be layered *in vivo* to produce a three-dimensional construct, providing increased opportunities for cell-cell contact [81]. In such a study, primary hepatocytes were isolated from mouse livers and cells with high viabilities, determined by trypan blue dye exclusion test, were selected. The high-viability hepatocytes were plated onto temperature-responsive poly(*N*-isopropylacrylamide) (PIPAAm) polymers, cultured until confluent and subsequently harvested by temperature reduction. Ten days before implantation in the mouse, a subcutaneous space was prepared by implantation of a basic fibroblast growth factor (bFGF)-releasing mesh for the creation of a highly vascularized platform. The mesh was later removed and the hepatocyte cell-sheet was transplanted onto the vascularized platform. Additional sheets were then implanted directly on top of the first sheet to create a bilayer or 4-layer hepatic tissue.

The implanted cell-sheets showed prolonged survival for at least 235 days. In comparison to cells transplanted in the conventional method via the portal vein, subcutaneously implanted hepatocyte cell-sheets showed enhanced function and improved survival rates. The hepatocyte cell-sheets were shown to maintain phenotype and were capable of metabolizing circulating chemical compounds. In addition, the engineered tissue was shown to proliferate and grow in response to regenerative stimulus from resection of two-thirds of the liver. The strategy of implantation into a pre-vascularized subcutaneous site has notable advantages. Subcutaneous implantation is minimally invasive and allows for relatively easy multiple transplantations, whilst the vascularized platform increases engraftment and persistence of viability [81]. Such technology holds great promise for future therapies in the treatment of liver diseases. In the current status, engineered liver tissue cannot replace whole organ transplantation, as the current engineered tissue is unable to generate biliary connections to the intestine. Furthermore, the problem of hepatocyte proliferation remains unresolved. The use of stem cells differentiated towards a hepatic lineage may offer an alternative to primary hepatocytes. A variety of stem cells including bone marrow derived MSCs, hematopoietic stem cells and fetal liver progenitor cells have shown to be effective in the treatment of liver disease [82, 83, 84]. The development of such strategies will be a focus of future research.

2.3. The Urinary System

2.3.1. Bladder

Bladder defects including bladder exstrophy, meningocele and non compliant bladders may be treated by cystoplasty [85]. Cystoplasty traditionally involves the use of a gastrointestinal segment; however, such tissue may result in infection or malignancy [85,86]. Likewise, the use of synthetic materials is limited by complications related to mechanical problems, fibrosis and urinary stone formation [27].

Tissue engineering of bladder tissue, as an alternative to the use of bowel tissue, has been applied to the treatment of pediatric patients with end-stage bladder disease requiring cystoplasty [27]. The clinical trial included seven patients aged 4-19 years suffering from a poorly compliant bladder caused by myelomeningocele, which was unresponsive to pharmacotherapeutic intervention. Bladder biopsies of 1-2 cm² were taken from the bladder dome. Muscle and urothelial cells were expanded in number in culture. Once sufficient cell numbers had been obtained, the muscle cells were seeded onto one side of a collagen/PGA composite scaffold. After 48 h, the urothelial cells were seeded onto the opposite side of the scaffold. Implantation of the tissue engineered construct occurred at approximately 7-8 weeks post biopsy. The engineered construct was anastomosed to the bladder using polyglycolic sutures and fibrin glue. The augmentation cystoplasty was tolerated in all patients and the mean duration of hospital stay was 28.5 days. One patient developed a urinary yeast infection which was appropriately managed. At 3 weeks postoperatively, a radiographic cystograph did not reveal any signs of urinary leakage. Improved bladder compliance and capacity, similar to that seen with the use of gastrointestinal tissue, was reported in the bladders treated with engineered tissue. Urinary continence improved and tissue biopsy revealed an adequate structural architecture. The use of an omental wrap at the time of implantation, to enhance vascularization, was proven to be of importance to the success of the engineered tissue. The functional durability of the engineered bladder tissue was confirmed through follow-up for over 5 years.

The use of stem cells as an alternative cell source in bladder tissue engineering may reduce the duration of *in vitro* culture and allow for the generation of larger constructs. Bone marrow-derived MSCs have been shown capable of differentiating into both smooth muscle and urothelium-like cells [87]. MSCs differentiation was induced by culturing cells in the presence of SMCs and urothelial cells. Differentiation was likely induced by the secretion of specific growth factors. In media conditioned by SMCs, numerous growth factors were detected including hepatocyte growth factor, platelet-derived growth factor-BB, transforming growth factor-beta1 and vascular endothelial growth factor. In media conditioned by urothelium, epidermal growth factor, platelet-derived growth factor-BB, transforming growth factor-beta1 and vascular endothelial growth factor were detected.

The tissue engineering approach has also been applied to urethral reconstruction. During the period between 2004 and 2007, five pediatric patients were treated for urethral defects using expanded muscle and epithelial cells seeded onto tubularised polyglycolic acid:poly(lactide-co-glycolide acid) scaffolds [88]. Patients were followed up until 2010. Patients showed a median end maximum urinary flow rate of 27.1 mL/s, maintenance of a wide urethral caliber without stricture and the development of normal

architecture as revealed by biopsy, 3 months after implantation.

In addition to the tissue engineering approach, studies have also suggested that conditions such as urinary incontinence may be treated with injection of stem cells or muscle cells. For example, 8 pediatric patients with persistent urinary incontinence were treated with injections of autologous myoblasts [89]. After follow up of 12 to 18 months, patients showed increased periods of dryness, bladder capacity, detrusor leak point pressure and maximum urinary flow, although it remained unclear as to the contribution made by the injected cells in comparison to concurrent treatments such as electrical stimulation.

Whilst significant progress has been made in this branch of tissue engineering, the broader clinical application will depend on determining an appropriate cell source and the continued development of biomaterials and bioreactor technology [90]. In addition, further clinical results will provide a greater understanding as to the challenges that lie ahead.

2.3.2. *Kidney/Renal Tissue*

At present, dialysis and transplantation are the only successful treatments for end-stage renal disease. Shortage of donor tissue for transplantation and the demanding nature of life with dialysis indicate a requirement for an alternative approach. A tissue engineered approach is complicated by the complex anatomy and physiology of the kidney; however, notable success with cell-based therapies is encouraging. Currently in clinical trials are renal tubule cell assist devices (RAD) [91]. These extracorporeal devices consist of a hemofiltration cartridge containing allogeneic human renal tubule cells grown on the inner surface of hollow fibers. The hollow fibers act both as a supportive scaffold for cell attachment and as a barrier against the host immune system. Addition of renal tubule therapy to continuous venovenous hemofiltration was applied for the treatment of ICU patients with acute kidney injury. Over 180 days of follow-up mortality risk was significantly reduced in comparison to patients treated by conventional continuous renal replacement therapy.

For reconstruction of whole organs, researchers have investigated the use of decellularized kidney tissue [92]. Decellularized kidney tissues have an advantage over synthetic and biological scaffolds in that they retain many of the branched structures of native kidney tissue along with specific growth factors of the kidney extracellular matrix. Decellularized rat kidneys were produced with intact acellular glomerular, tubular and vascular structures as well as preservation of collagen IV and laminin; extracellular matrix proteins involved in cell viability and differentiation. Mouse ESCs were then seeded intra-arterially resulting in a high percentage of retention, with cells distributed throughout the vascular structures and associated glomeruli. Seeded ESCs continued to proliferate *in vitro* within the decellularized tissue for at least 6 days. ESCs within the decellularized tissue also showed signs of differentiation including a flattened endothelial-like appearance and pan-cytokeratin, Pax-2 and Ksp-cadherin expression.

Tissue engineering of the kidney still has many hurdles to clear on the way to clinical trials. Nonetheless, current studies have built a solid base from which to move forward. The application of alternative cell source, other than ESCs, would also be beneficial for

clinical studies. For example, MSCs have shown to be safe and effective for treatment of acute kidney injury [93]. In addition, it was also shown that the production of vascular endothelial growth factor by MSCs was a critical factor in renoprotection. The determination of optimal strategies for cell differentiation and the elucidation of the role of growth factors in kidney tissue regeneration will be the focus of future research.

2.4. The Respiratory System

Defects of the airway may occur due to resection of cancerous or infected tissue. In the pediatric age group, the maximum length that can be safely resected is 30% of the total length, with larger defect sizes requiring the development of a functional tracheal replacement. Reconstruction of the airway represents one of the early success stories for tissue engineering [28]. In 2004, a 30-year old woman presented with tuberculosis infection of the cervical trachea and entire left bronchus. After successful treatment of the infection, tracheal stenosis persisted requiring a subglottic resection with primary anastomosis, followed by insertion of a stent into the bronchus. Poor toleration of the stent necessitated stent removal. The only remaining conventional option was a left carinal total pneumonectomy; however, such a procedure was associated with a high mortality rate. Consequently, the option of a tissue engineered airway was selected for replacement of the left main bronchus.

A tracheal segment from a donor was decellularized by repeat cycles of sodium deoxycholate and DNase I and seeded with autologous epithelial cells, isolated from a biopsy of the right main bronchial mucosa and chondrogenically differentiated autologous bone marrow-derived MSCs. During cell seeding, the decellularised trachea was rotated 90 degrees every 30 min to ensure a uniform distribution, and MSCs were seeded onto the outer surface whilst epithelial cells were seeded onto the luminal surface. Finally, the construct was cultured in a bioreactor for 96 h in which the construct was rotated at 1.5 revolutions per minute. After *in vitro* culture, the infected left bronchus was resected and the engineered graft was implanted. After 10 days post operative, the patient was discharged. Laser-doppler showed a healthy adjacent microvascular bed at day 4 postoperative and mucosal bleeding at day 30. The graft and patient showed excellent function after 4 month follow-up and the probability of restenosis was deemed unlikely.

In comparison to the trachea, engineering of lung tissue is vastly more complicated, due to the numerous cell types and complexity of anatomy and function. Because lung tissues lack of capacity for self-regeneration, the only option for lung replacement is transplantation. In pediatric patients, transplantation may be indicated for pediatric patients suffering from cystic fibrosis, pulmonary vascular disease, bronchiolitis obliterans, pulmonary alveolar proteinosis and pulmonary fibrosis [94]. Transplantation, however, has limited effectiveness, is expensive and is hindered by donor shortage. These problems may potentially be circumvented via adopting a tissue engineering approach.

The criteria for an engineered lung is that it should contain lung-specific cells, possess a branched geometry of airways and perfusing microvasculature, provide a barrier between blood and air, and display mechanical properties allowing for ventilation at physiological pressures [95]. In the rat model, neonatal lung epithelial

cells were seeded onto decellularized lung tissue and cultured in a bioreactor mimicking the physiological environment of the fetal lung. The engineered lung was then implanted into rats. All of the implanted lungs showed rapid perfusion with blood with only modest bleeding into the airways. Engineered lung was inflated with air, although to a lesser degree than the native lung. Finally, blood gas analysis revealed that the engineered tissue was effective in oxygen and carbon dioxide exchange. Whilst such evidence of functional engineered lung tissue is extremely encouraging, future research will need to address a number of issues including improvement of alveolar barrier function, increase of surfactant production, and enhanced formation of differentiated columnar ciliated epithelium [95].

2.5. The Musculoskeletal System

2.5.1. Bone

Bone defects may arise through trauma, disease or congenital deformities. The treatment of bone defects for conditions and scenarios including pseudoarthrosis, periodontal disease, maxillofacial surgery, spinal fusion and cancer resection often necessitate the use of a bone graft. The current gold standard for bone augmentation is the autograft. Autografts are often sourced from the iliac crest, due to the high content of resident pluripotent cells [96]. Other commonly applied bone graft materials include allografts sourced from cadavers, demineralised xenografts, and synthetic bone graft substitutes. To date, there is no one ideal material for bone augmentation, with the current options all associated with well-known limitations. Autografts suffer from donor site morbidity, bleeding, haematoma, infection, chronic pain and a limited availability [97, 98, 99]. Allografts and xenografts are associated with a theoretical risk of viral, bacterial and prion infection [100]. Sterilization of allograft bone has also been shown to reduce bone matrix osteoinductivity [101]. Bone graft substitutes, such as calcium phosphates, have become increasingly popular and now have a long track record for use as bone fillers. Porous hydroxyapatite in particular was used as early as 1983 in the treatment of tumor resections; however, synthetic bone substitutes have questionable effectiveness in compromised patients and patients with large defects due to their lack of osteoinductivity [102, 103, 104]. By combining osteoconductive synthetic bone grafts substitutes with an osteogenic cell component via tissue engineering approach, many of the aforementioned limitations may be circumvented.

The most common approach for bone tissue engineering is the culturing of bone marrow-derived MSCs on porous ceramic scaffold materials, followed by induction of osteogenic differentiation resulting in the generation of an osteoinductive extracellular matrix. Bone marrow-derived MSCs can be differentiated along the osteogenic lineage by culture with ascorbic acids, dexamethasone and β -glycerophosphate [10]. Other methods for osteogenic differentiation include exposure to bFGF and bone morphogenic protein-2 (BMP-2). In addition to MSCs, other cell sources for bone tissue engineering have been proposed including ESCs, “universal donor” cell lines and hypertrophic chondrocytes [105, 106, 107].

Numerous studies in both the ectopic bone growth model and large animal non-healing defect model have shown that scaffolds seeded with MSCs produce greater bone growth in comparison to cell-free scaffolds [108, 109, 110]. Relatively few

studies, however, have investigated the effect of scaffolds combined with osteogenically differentiated cells in the non-healing defect model. An example of such a study was the combination of osteogenically induced bone marrow-derived MSCs within coral scaffolds which were then implanted into a 25 mm defect in the goat femur [111]. The bone defect treated with tissue engineered bone showed bony union after 4 months and was fully remodelled after 8 months. In contrast, the cell-free controls showed no bone formation within the duration of the experiment.

To date, few studies of bone tissue engineering have been conducted in human. Four patients, including one adolescent, were treated with MSC loaded porous ceramics after failure of treatment by conventional methods [104]. The adolescent case was a 16-year old girl presenting with an exposed fracture of the left ulna. The patient underwent reduction and immobilization; however, the fracture was complicated by osteomyelitis and a sequestrectomy was performed resulting in 4 cm bone loss. Hydroxyapatite scaffolds, with a porosity of $60 \pm 5\%$ and a mean pore diameter of $613.63 \mu\text{m}$, were prepared to fit the target bone defect. Patient's BMCs were harvested from aspirates of the iliac crest. Cells were cultured for approximately 3 weeks, followed by seeding onto the scaffolds. The construct was then shipped to the orthopaedic center overnight in a thermal box. Finally, the constructs were implanted into the patient's bone defect and the soft tissue, fascia and skin were closed according to standard procedures.

Post operatively, no major complications, pain, swelling or infection were reported associated with the implantation site. Callus formation was detected between the construct and host bone after 1-2 months and consolidation was complete after 5 to 7 months. At this stage, the external fixation apparatus was removed from two of the patients, including the adolescent case, and patients were allowed to regain limb function. The ceramic implants showed signs of disintegration over time along with progressive bone formation. By follow up at 6 to 7 years, constructs showed good integration and vascularization of the grafted zone.

It has been observed that much of the osteoinductivity attributed to bone grafts originates from the proteins of the extracellular matrix [112]. It is therefore logical to conclude that a tissue engineered construct should also include a homogeneously distributed bone-like extracellular matrix. Such a matrix can be generated by inducing osteogenic differentiation of cells seeded within a scaffold, followed by a prolonged period of *in vitro* culture prior to implantation. Such an approach was recently applied in a clinical study involving intra-oral defects in which new bone was required in preparation for receiving dental implants [30]. MSCs were seeded onto hydroxyapatite particles at a density of 4×10^8 cell/cc and cultured under osteogenic conditions for 7 days. The scaffolds containing osteogenically differentiated MSCs were implanted into 6 patients. Bone formation was observed in 3 of the patients, with only 1 of these patients showing bone growth attributed to the implanted cells. The lack of success in this trial was suggested to be caused by a poor degree of vascularization.

The challenge of vascularization is critical in the progression of bone tissue engineering, with current research exploring the fabrication of biomaterials able to provide a controlled release of angiogenic growth factors, such as VEGF [42]. The development of novel materials with improved biodegradation rates and enhanced ability to guide osteogenic differentiation and increase bone formation are also

required. In combination with advances in techniques such as BMP-2 gene transfection, future bone tissue engineering research aims to prolong the viability of the engineered tissue and increase the rate of integration and new bone formation, in order to reduce the duration of rehabilitation.

2.5.2. *Cartilage*

The reconstruction of cartilage structures may be required during the treatment of congenital defects such as congenital microtia, diseased tissues such as tracheal replacement after resection, or traumatic injury, e.g., for treatment of damage to articular cartilage. Current techniques for the repair of full-thickness chondral defects include microfracture, which is a minimal invasive technique with good short-term results, however, can suffer from deterioration after 18 months [113]. Therefore, cartilage tissue engineering presents a promising alternative strategy for cartilage repair.

In articular joints, the role of cartilage tissue is to allow for movement under load-bearing conditions. The tribological properties of engineered cartilage tissue are therefore of paramount importance to subsequent performance [114]. Such properties are dependent on the composition of the cartilage matrix, wherein hydrophilic proteoglycans, embedded in a network of collagen fibers, function to absorb high levels of water. Current research has investigated the ability of mature chondrocytes or chondrogenic progenitor cells to interact with a wide range of scaffold materials and to generate cartilage-like matrix tissue. Scaffold materials studied for cartilage tissue engineering include sponges, electrospun fibers and hydrogels [115, 116, 117]. In a number of cases, tissue engineered cartilage has already been applied to the treatment of patients.

The tissue engineering approach was taken for the treatment of lateral compartment osteoarthritis of the knee after meniscectomy in a 14-year old girl [29]. Autologous cartilage was harvested during abrasion chondroplasty and chondrocytes were isolated through enzymatic digestion. The resulting cell suspension was then mixed with an atelocollagen solution. After 3 weeks of *in vitro* culture, the atelocollagen gel containing the chondrocytes hardened into an opaque, jelly-like construct ready for implantation. Post-surgery, full weight bearing was allowed after 6 weeks. After one year, the tissue engineered implant was covered with smooth cartilaginous tissue, which appeared to display similar hardness in comparison to normal cartilage.

In adults, the tissue engineered product, Neocart, was applied to the treatment of eight patients with symptomatic grade III full thickness cartilage lesions [118]. Neocart combines a 3D collagen type-I scaffolds with autologous chondrocytes, cultured together in a bioreactor system, resulting in a proteoglycan- and glycosaminoglycan-rich tissue. Autologous cartilage biopsies were taken from non-weight bearing portions of the femoral condyle or femoral notch. On implantation, securing of the construct to the defect bed via sutures was shown to damage the construct; therefore, in subsequent patients, the construct was secured using an adhesive polymer consisting of collagen and polyethylene glycol. During 24 months of follow-up, pain decreased and range of motion and knee function improved in the majority of patients. No arthrofibrosis was detected.

Tissue engineering of cartilage is a promising option for the treatment of chondral defects, able to reduce patient symptoms and hopefully prevent further osteoarthritic

developments. For wider acceptance, certain issues still need to be addressed, including inter donor variability, reproducibility, determination of optimal scaffold materials and scaling-up of graft size. Perhaps the most pressing issue is that of a suitable cell source. Autologous cells can only be isolated from relatively small biopsies and are known to dedifferentiate during *in vitro* cell expansion. MSCs have been proposed as an alternative source of chondrogenic cells; however, studies have also suggested that such cells may show hypertrophic differentiation leading to matrix calcification and vascularization [119]. Therefore, further clinical studies are needed to determine the real benefit of the cartilage tissue engineering approach.

2.5.3. Skeletal Muscle

Skeletal muscle comprises approximately 48% of the body mass and is largely responsible for the voluntary control and active movement of the body. In addition to protecting the abdominal viscera, skeletal muscle also aids the process of respiration. Functional muscle deficiency and loss can be attributed to a wide range of causes, including muscular dystrophies, congenital anomalies such as Prune-belly syndrome and developmental defects such as hypoplasia.

Muscle transposition is a well established technique for the treatment of large muscle defects; however, such a technique may result in donor site weakness and deformity [120]. Recent studies have investigated the plausibility of treating muscle defects using myoblast transplantation. Whilst transplanted myoblasts have been shown to contribute to muscle regeneration, the low survival rates are a major limitation for application of this technique [121]. Such problems may be rectified by prior combining of myoblasts with a scaffold material [122]. Myoblasts, isolated from skeletal muscle through mechanical and enzymatic methods, were cultured *in vitro* followed by seeding onto nonwoven PGA fiber meshes with 97% porosity. After 3 hours of culture to allow for cell adhesion, constructs were implanted into the omentum of rats. After 6 weeks of implantation, myoblasts remained viable and were positive for alpha sarcomeric actin and desmin. Furthermore, the organization of neomuscle along the polymer fibers was observed.

Further evidence for the superiority of a biomaterial-based approach to skeletal tissue engineering over cell-only injections was produced in the murine model of Duchenne muscular dystrophy [123]. Implanted muscle precursor cells (MPCs) seeded onto 3D collagen scaffolds showed lower rates of apoptosis, higher proliferation and a greater ability for dystrophin restoration in comparison to MPCs delivered by cell injection. In addition, for the creation of functional tissue, it is also of importance that a tissue engineered skeletal muscle should possess orientated cells and myotubes. Such alignment can be achieved through orientated topography of the biomaterial or through electrical stimulation [124, 125].

Studies in the large animal model are relatively few. Promising results have been obtained in the repair of diaphragmatic defects in the new born lamb model. Myoblasts harvested from fetal lamb skeletal muscle were expanded in number *in vitro* and seeded within collagen hydrogels. After birth of the lamb, autologous cells were implanted between supporting membranes, spanning the diaphragmatic defect. The resulting regenerated tissue was similar in thickness and histological appearance in comparison

to native diaphragm tissue, whilst the acellular control was thin, fibrotic and contained a low cell density [126].

Skeletal muscle tissue engineering is still in its developmental stage, but current experimental results are promising. Current research suggests that the *in vitro* culture step in skeletal muscle engineering is of great importance, potentially requiring both electrical and mechanical stimulation. For transfer to the clinic, further studies are required, including the determination of an optimal cell source and addressing the issues of vascularization and reinnervations.

2.6. The Nervous System

The regeneration of injured nerves is a complex challenge, yet potential improvement to a patient's quality of life would be vast. Spinal cord injuries in the pediatric population are relatively rare, with an average annual incident of 1.9 per 10⁶ children below the age of 18; however, the consequences can be devastating [127]. Traumatic injury to the peripheral nervous system, most commonly occurring through obstetric and iatrogenic lesions, traffic accidents and lacerations, can also result in severe disability [128]. In addition to traumatic injuries, numerous congenital disorders result from abnormalities of the peripheral and enteric nervous system, such as Hirschsprung's disease, in which regeneration of the nervous tissue would be of great advantage [129].

Regeneration of a severed spinal cord is complicated by the degenerative processes which follow severance. After cutting of the spinal cord, a massive release of lysosomal hydrolases causes autolysis of the cord stump, resulting in cavitation [130]. If the gap is too wide, or filled with scar tissue, spontaneous reinnervations will not occur. Therefore, to aid reinnervation across the defect site, researchers have developed biomaterial conduits with topographical and chemotactic signals, aimed at guiding nerve growth [131].

Tissue engineering has gained increasing popularity in the research of nerve repair. In particular for the peripheral nerve injuries, tissue engineering is seen as the most important field of future research, ahead of new surgical and medical techniques, and the use of xenografts [132]. In the tissue engineering approach, regenerative cells may be seeded within the biomaterial conduit. Numerous cell types have been proposed for use in nerve regeneration of both the peripheral and central nervous systems, including Schwann cells, Olfactory ensheathing cells, neural stem cells and MSCs [133, 134, 135, 136].

Some of the most promising studies involve the use of conduits filled with Schwann cells, taking advantage of the cell's ability to remyelinate axons and secrete neurotrophic factors which promote nerve survival and axonal regeneration [137, 138]. In such a study, Schwann cells were seeded onto collagen gel sheets, which were subsequently rolled to form a conduit [139]. Neuronal cells were seen to migrate into the center of the conduit through the inter-layered space. Significantly, the inward migration of the neuronal cells did not occur in conduits without Schwann cells. Other studies have attempted to enhance the function of Schwann cells through transfection. For example, Schwann cells transfected with glial cell line-derived neurotrophic factor (GDNF), were shown to increase survival of motor neurons and improve nerve regeneration in a rat sciatic nerve defect [133].

In addition to glial cells, tissue engineered conduits containing neuronal cells have also been developed, which are of particular interest in the repair of longer nerve defects [140]. To produce a neuronal construct bridged by longitudinally orientated axons, recent research has utilized the ability of axons to grow in length in response to a continuous mechanical tension. Neurons from the dorsal root ganglia were isolated from fetal rats and cultured in media supplemented with mitotic inhibitors to limit the proliferation of glial cells. The explants were then plated as two populations at either end of two overlapping collagen-coated aclar membranes. Axonal outgrowth across the membranes integrated the two explants. Next, the membranes were gradually separated over a period of 7 days. The mechanical tension induced stretch-growth of the axons, resulting in axonal tracts of 12-13 mm in length. The cell culture was embedded in a collagen-based matrix and inserted into a PGA Neurotube™. The constructs were then implanted into a 1-cm defect in the rat sciatic nerve and sutured to the epineurium and left for 6 to 16 weeks. The neurons were shown to survive implantation and the construct architecture of longitudinally aligned axonal tracts was preserved. The maintenance of a neuronal phenotype was also verified through expression of neuronal cytoskeletal protein NF-200. The transplanted nerve processes were observed to penetrate at least 5 mm into the host nerve. Most importantly, host axons were identified growing into the proximal ends of the construct resulting in intimate contact between host axons and transplanted neurites. A similar approach was also applied to spinal cord injuries in the rodent model [141]. In this study, mechanically elongated dorsal root ganglia neurons, embedded in a collagen hydrogel, were used to bridge a 10 mm defect. The implanted cells were shown to survive for 4 weeks and implanted axons were observed extending into host spinal cord tissue.

The major concern with nerve tissue engineering is safety. It is important that no adverse reactions are produced by implanted biomaterials or cells. The threat of tumorigenesis from the use of stem cells is of particular concern; however, whilst the risk is very much real, it has also been suggested that this can be reduced through a rigorous selection of cells displaying regulated growth and cell cycle control, and a correct pattern of gene expression [142]. In 2009, Geron Inc. was granted permission to enter clinical trials with a human ESC therapy for the treatment of thoracic-level spinal cord injury [143]. The product approved for trials, contains human ESC-derived oligodendrocyte progenitor cells. Oligodendrocytes are able to remyelinate axons and also to produce neurotrophic factors which enhance the survival and function of neurones [144]. The results of this trial will be eagerly awaited by the tissue engineering community.

2.7. The Integumentary System

Large skin defect is a significant clinical problem in pediatric surgery. The treatment of burns and closure of congenital abdominal wall defects often necessitates the use of a skin graft or skin substitute. Tissue engineering of the skin is one of the earliest and most extensively studied fields of tissue engineering [12]. Several tissue engineering products have been approved for clinical use, including *Epicel*, *Dermagraft*, *Transcyte* and *Apligraf* [145].

In a clinical trial of pediatric burns covering more than 7% of the total body surface, 20 children were treated with the bioactive skin substitute, *TransCyte*, and another 20 children underwent standard treatment [8]. *TransCyte* is a polymer membrane containing newborn human fibroblasts, covered with a synthetic nylon mesh epidermis. In the control group, seven children eventually required autograft, in comparison to 1 child in the *TransCyte* group. Length of stay in hospital was reduced by 50% in the *TransCyte* group in comparison to standard treatment. The use of *TransCyte* was also seen to require less direct nursing and therefore to reduce discomfort to patient as well as to achieve significant cost savings. A specific drawback mentioned for the use of *TransCyte* was an ineffectiveness of adherence in deep burns.

In adults, fibroblast and keratinocytes were combined with a collagen matrix and applied to a 51-year old man with burns [146]. Three sheets of 6 cm × 9.5 cm were grafted onto the wound. Matrix was seen to disappear after 1 week and stratified keratinocytes were seen firmly attached to the underlying tissue. After 5 weeks, a cornified epidermal layer was seen. After 10 months, a mature epidermis and well differentiated papillary and reticular dermis were observed. Similar results were observed in a 30-year old treated with 8 sheets of 10 cm × 18 cm after tattoo removal. Biopsy at 3 months revealed mature epidermis and well differentiated reticular dermis. At 14 months, the engineered skin showed excellent results.

In addition to fibroblasts, the dermal layer also contains glands, hair follicles, nerves and capillaries. The engineering of such structures is of importance to the integration and function of a tissue engineered skin. For example, functional recovery of cutaneous sensory perception was achieved by enriching a tissue engineered skin construct with Schwann cells, resulting in increased nerve fiber migration and myelin sheath formation [147].

The main drawback of skin tissue engineering is failure due to delayed vascularization [145]. In the treatment of full thickness wounds, providing a vascular network to promote the survival and integration of grafted engineered skin is essential for a successful outcome and is a major obstacle for clinical application [148]. Current strategies for vascularization include cell-based approaches and the combination of angiogenic growth factors to matrices [148, 149]. Cell types proposed for vascularization include dermal microvascular endothelial cells, umbilical vein endothelial cells, early endothelial progenitor cells, MSCs, ESCs and induced pluripotent stem cells. Where possible, an autologous cell source is preferred; however, in acute cases, an allogenic cell source may be required. Harvesting cells should also result in a sufficient number of safe cells with limited donor-site morbidity. The optimal cell source and biomaterial for promoting vascularization of tissue engineered skin is still the subject of long-term preclinical studies [148].

2.8. The Lymphatic System

Lymphatic organs, including the spleen and lymph nodes, are highly organized structures, consisting of lymphocytes and phagocytes enmeshed in a fibrous network. The spleen and lymph nodes function to monitor the blood and lymph for infection. Removal of lymphatic organs may be required after trauma or in the treatment of cancer, and such patients are subsequently more susceptible to infection, especially in the pediatric age group [150, 151, 152].

In comparison to other organ systems, studies of tissue engineering of the lymphatic system are few. One such study is tissue engineering of the spleen using multicellular splenic units, seeded onto a polymer scaffold [153]. Splenic units were produced from resected spleens of 6 day old rats through digestion with dispase and collagenase type I, and 100,000 units were then loaded onto 1-cm PGA polymers tubes, sealed with PLLA, with a fiber diameter of 15 μm and a porosity >95%. The tissue engineered spleen was then implanted into the omentum of splenectomy rats. After 16 weeks, the rats were challenged by intraperitoneal injection of *streptococcus pneumoniae*, and animals were observed closely for the next 21 days. Two months after implantation, the tissue engineered spleen had a deep purple coloration, and histological evaluation revealed an organized spleen parenchyma with white and red pulp, and the polymer completely absorbed. Finally, tissue engineered spleen was shown to protect against *streptococcus pneumoniae* with a survival proportion of 85.7% in comparison to 41.17% seen in the splenectomy group.

Investigations into the engineering of lymph node tissue *in vitro* has also produced promising results [154]. Monocytes isolated from samples of human peripheral blood mononuclear cells were differentiated into dendritic cells by sequential exposure to specific cytokines and primed by incubation with human cytomegalovirus lysate. The dendritic cells were then seeded onto agarose gels and nonwoven polyamide fibers before inserting into a membrane-based perfusion bioreactor system. During the period of culture, lymphocytes were inoculated into the bioreactor and allowed to pass through the porous membranes and interact with the immobilized dendritic cells. Analysis of the resulting constructs showed spreading of the dendritic cells and clusters of antigen-specific leukocytes, whilst interleukin-2 and lipopolysaccharide responses indicated early T-cell activation.

In further investigations of lymphatic tissue engineering, a thymus-derived stomal cell line and bone marrow-derived dendritic cells were absorbed to sponge-like collagen scaffolds with pore sizes estimated at 50-300 μm [155]. The constructs were then immediately transplanted into the renal subcapsular space in mice. The resulting tissue engineered structures showed similarity to secondary lymphoid organs with distinct compartmentalised B- and T-cell clusters scattered with CD11c+ dendritic cells. The ratio of CD4+ and CD8+ T cells was also similar to normal secondary lymphoid organs, and there was evidence of follicular dendritic cell network formation and germinal center B-cell-like cells. As well as restoring loss lymphatic tissue, it was also suggested that such a technique could find application as a therapy for immunodeficiency diseases.

In addition to lymphatic organs themselves, connection by lymphatic vessels is also required. The passage of lymph may require more than a passive conduit, and engineered lymphatic vessels may be required to respond to lymph flow and provide active contraction [156]. In general, the complexity of lymphatic tissue is not to be underestimated and tissue engineering of the lymphatic system remains a promising, yet challenging proposal.

3. CONCLUSION

Many efforts and funds have been invested over the last two decades into tissue engineering as a solution for tissue and organ loss. At present, data from early clinical trials are beginning to emerge and the results are promising. The success of tissue engineering depends largely on the complexity of the target organ in terms of anatomy and function. It is therefore not surprising that the tissue types targeted by early tissue engineering clinical trials are relatively simple in form and function. Tissue engineering of the more complex tissues, consisting of multiple cell types and tissue components, are still relatively far away from clinical trials. A major challenge in tissue engineering is the improved proliferation of cells in order to produce sufficient cell numbers. One potential solution is the use of stem cells which have relatively high proliferative capacities; however, delivering consistent differentiation towards a target cell type remains a challenge.

The recent advances in tissue engineering are of special interest to the pediatric surgical community. Clinical trials in the pediatric population have already commenced. However, it should be noted that the efficacy and safety of the tissue engineering approach have not yet been fully understood and remain to be investigated. Tissue engineering presents a potential solution to the shortage of organs for organ transplant in the pediatric community and is therefore predicted to create a strong impact on the future of pediatric surgery.

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