

Stem cells in vascular graft tissue engineering for congenital heart surgery

Many children with congenital heart disease need prosthetic replacement grafts in the form of new valves, conduits and patches. Owing to the lack of growth potential of the currently available grafts, a child born with congenital heart defects may typically need to undergo major cardiac surgery several times throughout their lifetime. A very promising approach to solve this problem may be the use of tissue engineering, in which stem cells are seeded to form living-tissue products that have structural and functional properties that can be used to restore, maintain or improve tissue function, and help to building the 'ideal valve and patch' to be used in congenital heart surgery. In this review, we will summarize the current knowledge and scientific progress in the field of vascular tissue engineering and stem cell differentiation with specific application to congenital heart surgery.

KEYWORDS: congenital heart surgery • stem cell • tissue engineering

Congenital heart disease (CHD) affects approximately 1% of all live births, making it the most common birth defect worldwide. CHD remains the primary cause of death among infants in North America and Europe. Moreover, improvements of cardiac surgery techniques have led to a remarkable improvement in early survival rate, with a parallel increase in the number of patients with adult CHD (5% increase annually in UK). The 2009 WHO annual report puts the disability-adjusted life year for CHD higher than diseases such as diabetes or hypertension [1,2].

Although primary repair is sometimes possible, prosthetic replacement grafts are more often required. Patients with tetralogy of Fallot, for example, require extensive opening and resection of the pulmonary artery valve (FIGURE 1A) and right ventricular outflow tract (FIGURE 1B), implantation of patches to enlarge the whole area (FIGURE 1C) and, subsequently (after several years), pulmonary valve replacement with either homografts (FIGURE 2) or other types of biological valves (FIGURE 3).

Even though these grafts may be life saving, they have limited durability and are prone to infection, immunological reactivity and thrombosis. As a result, repeat operations are often required. Of particular concern is that these currently available grafts do not grow as the patient ages. This may predicate additional procedures during childhood or adolescence. Moreover, it has been variably estimated that between 800,000 and 1 million patients with CHD are now adults, and many will require some form of

valve or valve conduit replacement due to failure of their previous grafts [3,4].

Currently available valve replacements include mechanical prostheses, several types of glutaraldehyde-fixed xenografts, homografts or autograft with the Ross procedure. The major limitation of xenografts is their relatively short lifetime, while mechanical valves require anticoagulation with an increased risk of thrombosis and hemolysis especially in the pediatric population. Using a pulmonary autograft in the aortic position (the Ross procedure) provides favorable hemodynamics, and freedom from anticoagulation and immunological reactivity. Moreover, it provides a graft that grows as the patient grows, potentially reducing the need for further surgery. However, the pulmonary autograft in the aortic position is prone to dilatation with subsequent aortic valve regurgitation. Pulmonary homografts are the most commonly used conduits for right ventricular outflow tract reconstruction in children due to their excellent characteristics; they integrate into surrounding tissue, are easily manipulated by surgeons and are stable enough to hold suture material. However, like other types of biological valve, their durability is limited by the calcification process and intimal proliferation [3,4].

Therefore, it is clear that the 'ideal' replacement heart valve, which should be durable, biocompatible, nonimmunogenic, nonthrombogenic, readily available, easy to handle, and have the potential to grow and remodel *in vivo*, does not yet exist.

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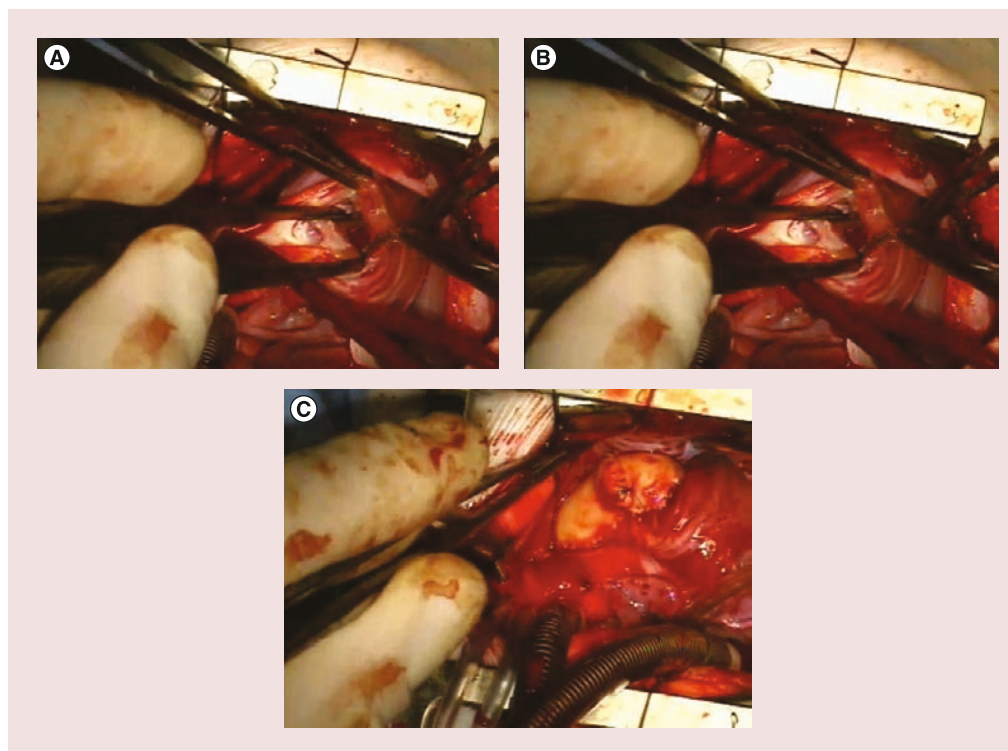


Figure 1. Corrective surgery in patients with tetralogy of Fallot. (A) The opening of the pulmonary artery up to the stenotic pulmonary valve in a 50-kg infant born with tetralogy of Fallot undergoing surgical repair. **(B)** The extension of the transannular incision into the right ventricular outflow tract with extensive subvalve muscle resection. **(C)** The transannular patch to reconstruct the right ventricular outflow tract using a pulmonary homograft patch.

Tissue engineering is a very promising approach to overcome the limitations of existing surgical repair methods [5–8]. This approach involves seeding cells in 3D matrices to form living tissue products with structural and functional properties that can be used to restore, maintain or improve tissue function. The repopulation of a decellularized matrix with a patient's own cells before surgery can potentially create a living structure with subsequent long-term preservation of mechanical and biological properties.

Scaffolds used in vascular tissue engineering

■ Prosthetic materials

Clinically available vascular prosthetic replacements have many disadvantages. Indeed, these prosthetic replacements are foreign materials that predispose the patients to life-long risks of infection and lack the ability to grow, repair and remodel, which are vital for pediatric populations. Engineering a complete 'living' autologous vascular material may overcome many of these limitations associated with the current clinically used prosthetic substitutes. These synthetic valvular and vascular prostheses have been made

from a range of materials (TABLE 1). Dacron, GORE-TEX® (WL Gore & Associates, Inc., DE, USA) and polytetrafluoroethylene (PTFE) made in various forms are commercially available and clinically used in patients with no alternative vascular structures. The use of these vascular conduits is satisfactory in large-diameter applications. However, in smaller vessel replacement they perform poorly. The blood-contacting surface and the biomechanical properties of the graft are believed to be important variables in causing patency in small vessels. Many attempts have been made to improve these prosthetic devices by lining them with autologous endothelium [9–11]. Early studies have procured endothelial cells from dogs' external jugular vein segments and seeded these autologous cells in Dacron vascular grafts immediately prior to implantation [12]. The experimental grafts studied had better clot-free surfaces than the control grafts at 14- and 28-day periods [12]. In addition, endothelial cell (EC) coverage of experimental graft surfaces after 4 weeks was up to eight-times higher than in control grafts [9]. Later investigations have shown that PTFE grafts can be lined with ECs harvested from dog jugular veins and implanted in dogs with similar results to the

Darcon grafts [13]. Other studies demonstrated that ECs cultured from human veins could be used to line PTFE grafts [14]. Furthermore, PTFE grafts lined with autologous ECs isolated from veins have been implanted in human [10,11]. However, clinical trials failed to confirm the successful results observed in animals [10,11].

■ Vascular conduits made of layers of vascular cells

In 1986, Weinburg and Bell were the first group to produce a completely biological vascular conduit made of layers of bovine vascular cells, ECs, smooth muscle cells (SMCs) and fibroblasts, and animal collagen; however, this lacked sufficient mechanical properties [15]. More recently, such a living conduit was created by wrapping a cultured sheet of human vascular SMCs cultured into a sheet around a tubular support followed by a sheet of human fibroblasts [16]. After these layers had matured, the tube was removed and the lumen was seeded with ECs, thus creating the three layers of the blood vessel (TABLE 1). The vessel showed promise in a dog model, achieving good patency, and had potential as the method used no synthetic or exogenous biological materials [16]. However, there was an increased risk of contamination as there was a delay in culturing the vessel *in vitro*.

■ Decellularized natural scaffolds

Prosthetic substitutes commonly used for vascular reconstruction are mainly synthetics and fixed tissue grafts. Within these replacements, graft infections are common, compliance mismatch is significant and handling qualities are poor [17]. Natural biological tissues that are unfixed offer a better alternative, particularly since they have been shown to resist infections and be durable and compliant. However, one disadvantage of these grafts is their potential antigenicity to the recipient.

Acellular or decellularized scaffolds (TABLE 1) have been shown to be useful as they provide the natural cues needed for recipient's cells to grow on the scaffold in terms of mechanics, composition and ultrastructure [18], and it has been shown that the extracellular matrix can still support cell growth, even though many of its components have been disrupted [19,20]. As it is hard to remove 100% of the cytoplasmic debris and nuclear materials, the consequence of remaining material in terms of host response remains unclear [21].

Early data indicated that decellularization of animal tissues is capable of producing stable

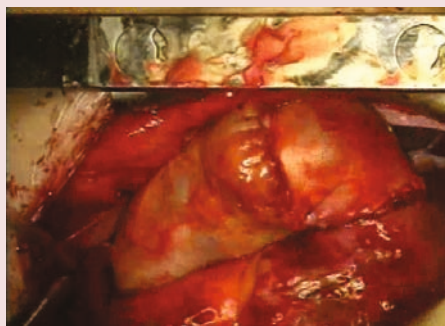


Figure 2. Right ventricular outflow tract replacement with a pulmonary homograft in a 7-year-old boy.

vascular conduits that exhibit long-term functionality in other species [22]. In this study, the SynerGraft® tissue engineering strategy was used to minimize antigenicity and produce stable unfixed vascular grafts from nonvascular bovine tissues. These grafts were used to replace the abdominal aortas of dogs that were followed for up to 10 months [22]. Early evaluation indicated rapid recellularization by recipient actin-positive SMCs, which become arranged circumferentially into the media. Arterioles were present in the adventitial areas and ECs covered luminal surfaces. After 10 months, grafts were patent and not aneurysmal.

Other forms of vascular grafts include decellularized tubular organs (TABLE 1). Examples include porcine aorta, and common carotid and iliac arteries [23–25]. A study in a dog model showed that the jejunal submucosa became endothelialized and developed a histological appearance, not dissimilar to the native vessel, when used as a vascular graft showing no evidence of long-term failure [26]. Other studies have shown similar results with bovine tissue [27].

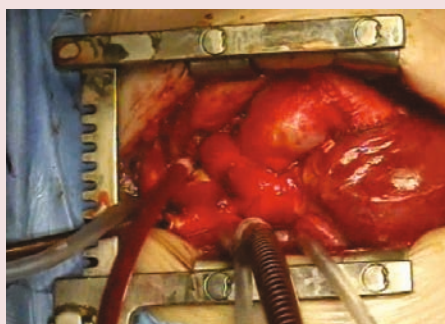


Figure 3. The use of Contegra® valved conduit (Medtronic, MD, USA) as a right ventricle to pulmonary artery connection in a 3-kg neonate born with common arterial trunk.

Table 1. Scaffolds in vascular tissue engineering.

Type of scaffold	Materials	Advantages	Disadvantages
Prosthetic materials	Dacron, Gortex, PTFE	Satisfactory in large-diameter applications	Infection, lack the ability to grow, repair and remodel
Natural protein	Collagen, elastin, fibronectin	Produce a completely biological vascular conduit	Lack sufficient physical stability and mechanical properties
Synthetic biodegradable polymers	PGA, PGA-PHO copolymer, PCL-PLA copolymer reinforced with woven PGA	Better controlled physical and mechanical properties	Challenge the host's ability to remodel the implant Side effects caused by the degradation by-products
Decellularized vessels	Small intestinal submucosa Porcine aorta carotid artery, Iliac arteries	'Off-the-shelf' graft, short maturation time Abundant supply of construct materials Natural composition and mechanical properties	Inhomogeneous distribution of cells Difficulty in retaining all extracellular matrix Immunogenicity upon incomplete decellularization

PCL: Polycaprolactone; PGA: Polyglycolic acid; PHO: Polyhydroxyoctanoate; PLA: Polylactic acid; PTFE: Polytetrafluoroethylene.

This encouraging work has led to implanting SynerGraft decellularized porcine valves into pediatric patients [28]. Unfortunately, it has been reported that three out of four children implanted with SynerGraft valves died, two of which were sudden with severely degenerated SynerGraft valves 1 year after implantation [28]. The third child died on day 7 due to SynerGraft rupture. Subsequently, the fourth graft was explanted prophylactically 2 days after implantation [28]. Macroscopically, all four grafts showed severe inflammation and degeneration of the leaflets and wall. Histology demonstrated severe foreign body-type reaction. In addition significant calcific deposits were demonstrated [28]. Surprisingly, preimplant samples of the SynerGraft revealed incomplete decellularization and calcific deposits. No cell repopulation of the porcine matrix occurred [28]. It is thought that the strong inflammatory response in humans may be due to matrix immunogenicity being conserved or may indicate insufficient decellularization to remove all xenoantigens [28]. This report resulted in the implanting the porcine SynerGraft treated heart valves being stopped.

Synthetic scaffolds seeded with cells

Many attempts have been made to create hybrid vascular grafts constructed from synthetic materials (polymers; TABLE 1) and cells, thus providing a natural interface with circulating blood. Initial results with regard to graft patency have been encouraging. However, the composite conduits are only a partial imitation of a living blood vessel. The use of biodegradable polymers as scaffolds for growing cells offered great potential to engineer a living blood conduit. Autologous cells

are expanded *in vitro* and then seeded onto the polymer. Once cells are attached to the 3D polymer structure, the resulting cell-polymer construct can be reimplanted into the experimental subject. As cells grow and the extracellular matrix develops, the polymer degrades, leaving only the 'engineered' tissue without any foreign material. The feasibility of this technique to tissue engineer a heart valve leaflet was shown in a large animal model in 1995 [29]. This study showed that a tissue-engineered valve leaflet constructed from its cellular components can function in the pulmonary valve position [29]. However, this work necessitated the sacrifice of intact vascular donor structures to harvest the autologous cells used to repopulate the polyglycolic acid fibers, a synthetic biodegradable scaffold [29]. Another study also succeeded in constructing lamb heart valve leaflets by seeding a concentrated suspension of autologous myofibroblasts onto a biodegradable synthetic polymeric scaffold composed of fibers made from polyglycolic acid and polylactic acid [30]. In both studies, the evaluation of pulmonary valve function by Doppler echocardiography demonstrated valve function without evidence of stenosis and with only trivial regurgitation under normal physiologic conditions [29,30]. Histologically, the tissue-engineered heart valve leaflets resembled native valve leaflet tissue [29,30]. A later study used autologous cells seeded onto synthetic biodegradable tubular scaffolds (polyglactin/polyglycolic acid) to replace a small segment of the pulmonary artery in lambs. Tissue-engineered grafts were patent and demonstrated an increase in diameter. The biodegradable polymer scaffold disappeared from the tissue-engineered graft by 11 weeks [31]. Collagen content

in tissue-engineered grafts was 25% lower than in the adjacent native pulmonary artery. The elastic fibers were present in the media layer of the tissue-engineered vessel wall and endothelial-specific factor VIII was identified on the luminal surface. Calcium content of tissue-engineered grafts was elevated but no macroscopic calcification was found. The acellular control graft (polymer tube) developed progressive obstruction and thrombosis [31]. Unfortunately, the use of a polyglactin–polyglycolic acid copolymer to create vascular substitutes in the systemic circulation resulted in aneurysm formation [32]. However, the polyglycolic acid–polyhydroxyalkanoate copolymer, which has a much longer degradation time, seemed to better withstand systemic pressure and offered a better option to create a vascular autograft for use in the aortic position [32]. Indeed, ovine pulmonary arteries and valve leaflets have been created from autologous cells from carotid arteries and a scaffold made from polyglycolic acid and polyglactin copolymers [32]. The scaffold was seeded with a mixture of ECs, SMCs and fibroblasts. Results showed that by 5 months, the polyglycolic acid was replaced by tissue that resembled native tissue in morphology and matrix orientation, as well as similar amounts of elastin and collagen. The cells aligned with the blood flow and von Willebrand (vWF) factor were shown to be present on the inner surface, showing endothelium formation [32]. Again, however, in all of these studies, as the polymer degraded slowly it was released in the circulation and caused damage to intact vascular structures.

Another study demonstrated the feasibility of seeding bone marrow cells onto a biodegradable scaffold (copolymer of lactic acid and caprolactone) to construct tissue engineered vascular autografts [17]. It showed that the bone marrow cells seeded onto the scaffold adhered, proliferated and differentiated into components of a new vessel wall [17]. Bone marrow cells appeared to be a promising cell source for tissue engineering. However, the use of nondifferentiated cells presents the risk of neoplasm formation and calcification [33]. In a clinical study, Shin'oka *et al.* showed that when autologous bone marrow cells, extracted on the day of surgery, were seeded onto a polymer tube scaffold composed of a copolymer of L-lactide and ϵ -caprolactone (50:50) and implanted into patients, there were no complications and the tube remained patent at 32 months [33]. In addition, it was shown that the diameter of the tube graft increased with time, indicating that it may have properties allowing it to grow, repair and remodel. In

addition, the copolymer scaffold was degraded by hydrolysis after just a few months, only leaving the living tissue by 2 years. This study highlighted the possibility of direct use of cells in tissue engineering without the need to culture them first, a useful feature in emergency cases. However, the durability of these grafts is still unknown and complications related to the long degradation time may occur.

The above studies had led to the successful reconstruction of a pulmonary artery in a 4-year-old child using their own venous cells in 2000 [34]. This patient, with a single right ventricle and pulmonary atresia had undergone pulmonary artery angioplasty and the Fontan procedure at the age of 3 years and 3 months. Angiography 7 months later revealed total occlusion of the right intermediate pulmonary artery [34]. A small segment of peripheral vein was explanted and cells from its walls were isolated. The cells were cultured and expanded, and then seeded onto a biodegradable polycaprolactone–polylactic acid copolymer tube reinforced with woven polyglycolic acid. The graft was transplanted 10 days after seeding. The occluded pulmonary artery was reconstructed with the tissue-engineered vessel graft [34]. On follow-up angiography, the transplanted vessel was completely patent. At 7 months after implantation, the patient was doing well, with no evidence of graft occlusion or aneurysmal changes on chest radiography.

Later investigations used a pulse duplicator culture environment to speed up the process of tissue formation [35,36]. The first study sequentially seeded bioabsorbable polymers (polyglycolic acid/poly-4-hydroxybutyrate) with ovine vascular myofibroblasts and ECs. After 14 days of exposure to pulsatile flow, conduits appeared to be grossly intact but without properties of a native artery. However, grafts exhibited suture retention strength appropriate for surgical implantation [35]. The second study used umbilical cord cells as a source for tissue engineering [36]. Human umbilical cord cells were harvested and expanded in culture, and then seeded onto pulmonary conduits fabricated from rapidly bioabsorbable polymers. The seeded conduits were grown *in vitro* in a pulse duplicator bioreactor [36]. Histology of the conduits revealed viable, layered tissue and extracellular matrix formation. However, extracellular matrix protein levels were significantly lower compared with native tissue. In addition, cells were positive for vimentin and α -smooth muscle actin. The mechanical strength of the pulsed constructs was comparable with native tissue [36].

■ Decellularized & cell-seeded natural scaffolds

For scaffold materials, it has been shown that natural materials have a better patency compared with artificial grafts [37]. Although biodegradable scaffold studies showed promising results, the durability of these grafts remains unknown. Decellularized tissues and organs (TABLE 1) are currently used in tissue engineering and have a favorable outcome [21]. They are thought to be promising as removal of cellular material decreases immunogenicity of the tissue caused by foreign antigens. Therefore, preventing the deterioration of the implanted grafts by the host's immune response [38,39]. It has been shown that decellularized grafts elicit significantly lower levels of class I and II HLA antibody formation at 1, 3 and 12 months after implantation than standard cryopreserved allografts [38]. There are many different methods used to decellularize scaffolds, dependent on the type of tissue. The idea of this technique is that decellularization leaves the extracellular matrix intact while removing all of the 'allogeneic and xenogeneic' cell antigens that are not tolerated and lead to an immune response [34]. A successful cell removal from the scaffold is important as this determines the body's response to the tissue after insertion. Despite the wide range of techniques used to remove cells, only a few are successful with minimum extracellular matrix disruption. Some disruption is necessary to expose cells; however, alterations such as glycosaminoglycan, removal may decrease cell migration onto the scaffold or increase the chance of *in vivo* enzymatic degradation [21].

Decellularized grafts seeded with vascular ECs

The pioneering work by Eberl and colleagues demonstrated the feasibility of endothelializing biological heart valve leaflets *in vitro* with cultured autologous ECs [40]. A decade later, Dohmen *et al.* described the first human implantation of a tissue-engineered heart valve made from a decellularized pulmonary allograft seeded with autologous vascular ECs obtained from the forearm vein 4 weeks before being implanted in patient's pulmonary position during the Ross procedure [41]. At 1-year follow-up, the valve had a normal function with no signs of calcification.

Decellularized grafts seeded with stem cells

A study by Shin'oka *et al.* confirmed that animals receiving tissue-engineered pulmonary

valve leaflets seeded with autologous bone marrow cells developed fewer infections and had less deterioration when compared with valves seeded with allogenic cells [33]. The success achieved in animals has led to clinical testing. An early investigation reported the first clinical implantation of pulmonary heart valves engineered with autologous endothelial progenitor cells (EPCs) and the results of 3.5 years of follow-up [42]. In this study, human pulmonary heart valve allografts were decellularized and resulting scaffolds were seeded with peripheral mononuclear cells isolated from human blood. Cultivation and differentiation of the seeded scaffold in a dynamic bioreactor system for up to 3 weeks resulted in the expression of endothelial markers in the seeded cells. The pulmonary valves seeded with autologous cells were then implanted into two pediatric patients (aged 11 and 13 years) with congenital pulmonary valve failure. Results showed reasonable mechanical properties and resistance. Over 3.5 years of follow-up, the valve annulus increased in both patients and there were no signs of valve degeneration observed after 3.5-year follow-up [42]. This showed that seeding decellularized tissue is a feasible and safe method for pulmonary valve replacement.

These initial data for the development of tissue-engineered valves are encouraging. However, in these cases, the tissue engineering focused on reconstitution of the endothelial lining without any attempt to repopulate the extracellular matrix of the connective tissue with cells of the appropriate phenotype. A more recent study developed a tissue-engineered artery using autologous bone marrow-derived mesenchymal stem cells (MSCs) and a decellularized arterial scaffold. MSC-derived vascular smooth muscle-like and endothelial-like cells were seeded onto decellularized ovine carotid arteries, which were inserted into the carotid arteries in an ovine host model. The scaffold retained the main structural components of the blood vessel, such as collagen and elastin. The engineered vessels were patent, antithrombogenic and mechanically stable for 5 months *in vivo*, whereas nonseeded grafts occluded within 2 weeks. Histological, immunohistochemical, and electron microscopic analyses of the engineered vessels demonstrated the existence of endothelium and smooth muscle, and the presence of collagen and elastin at 2 and 5 months, respectively [43]. In 2012, Olausson *et al.* used stem cells to grow a replacement blood vessel for a 10-year-old girl who developed portal

vein obstruction and liver failure [44]. A deceased donor vein was decellularized and subsequently recellularized with ECs and SMCs differentiated from stem cells obtained from the bone marrow of the recipient. The patient did well following the transplant and received no immunosuppressive drugs. This case report showed the great potential of tissue engineering and stem cell technology for vascular reconstruction, even though it was limited to the vein rather than the arterial system of the body.

Perinatal stem cells in vascular tissue engineering

Autologous stem cells provide a powerful source for all types of cell regeneration and would be of great advantage in cardiovascular tissue engineering. These cells are easy to obtain and can be manipulated for multiple passages. Repopulating scaffolds with patients' own stem cells before surgery can potentially create a vital conduit with a high physiological replacement of all components and subsequent long-term preservation of mechanical and biological properties.

■ Major functional cell types in vascular tissue engineering

ECs, SMCs and fibroblasts are the three major types of cells composing the vascular wall. ECs regulate the permeability, migration, remodeling, proliferation and metabolism of the blood vessel, as well as modulate the contractility of vascular SMCs by producing biochemical substances [45]. Vascular SMCs can perform both contractile and synthetic functions, which maintain the mechanical properties and radial compliance of the blood vessels. SMCs produce most of the extracellular matrix proteins. They play crucial roles for long-term adaptation, via structural remodeling by changing cell number and connective tissue composition [46]. It has been widely accepted that functional tissue-engineered blood vessels (TEBVs) cannot be achieved without ECs, SMCs, scaffolds, proper molecular signals and the unique vessel-engineering techniques [47].

It is known that the majority of the cells in the adult blood vessel are terminally differentiated, even cells isolated from umbilical cord veins still have limited proliferation potential and will lose their biological function during *in vitro* proliferation [48]. Although some TEBVs have been reported to be successfully constructed by seeding cells isolated from autologous vessels [49,50], the limited proliferation potential of harvested cells makes it

impossible to obtain a large amount of cells from a small-vessel biopsy.

Many attempts have been made to improve the proliferation potential of ECs and SMCs; for example, the genetic manipulation by introducing human telomerase reverse transcriptase subunit into human SMCs [51]. However, the safety of the cells after genetic manipulation is still a great concern. Long-term follow-up of modified cells *in vivo* is necessary before clinical application of these cells. To date, there is no promising way to solve the proliferation problem of ECs and SMCs. It is of great interest to find alternative cell sources for vessel engineering.

■ Stem cells in regenerative medicine

Stem cells are able to self-renew and differentiate into varying specialized mature cells under the correct conditions. The discovery and application of stem cells make it possible to produce a sufficient amount of targeted functional cells for tissue regeneration [52].

Besides the induced pluripotent stem cells (iPSCs), stem cells could be roughly categorized into three groups based on their origin in human: embryonic stem cells (ESCs), perinatal stem cells (PSCs) and adult stem cells (FIGURE 4).

iPSCs are reprogrammed adult somatic cells, with regained pluripotency by introducing four defined transcription factors, Oct3/4, Sox2, c-Myc and Klf4. Human fibroblast is the first and most commonly used somatic cell type in generation of human iPSCs [53,54]. The use of iPSCs could avoid the ethical concern of using ESCs and give researchers more choices in autologous transplantation.

Deriving human vascular ECs and SMCs from iPSCs has been demonstrated by different research groups [55,56]. In 2012, Cheung *et al.* reported the direct differentiation of iPSCs into SMCs by a chemically defined method via three intermediate lineages, which could benefit disease modeling [57]. Kim *et al.* demonstrated that coimplanting iPSC-derived ECs and SMCs significantly improved wound healing in a murine model 1 year later [58].

Although many studies have produced vascular-like EC and SMC from iPSCs, some researchers still question the variation in gene expression, DNA methylation and the pluripotent potential of iPSCs. Furthermore, Gore and colleagues raised questions about the somatic mutations, for example, 'protein-coding point' mutations, occurring after reprogramming [59]. Hussein's research team found a remarkable increase in copy number variations

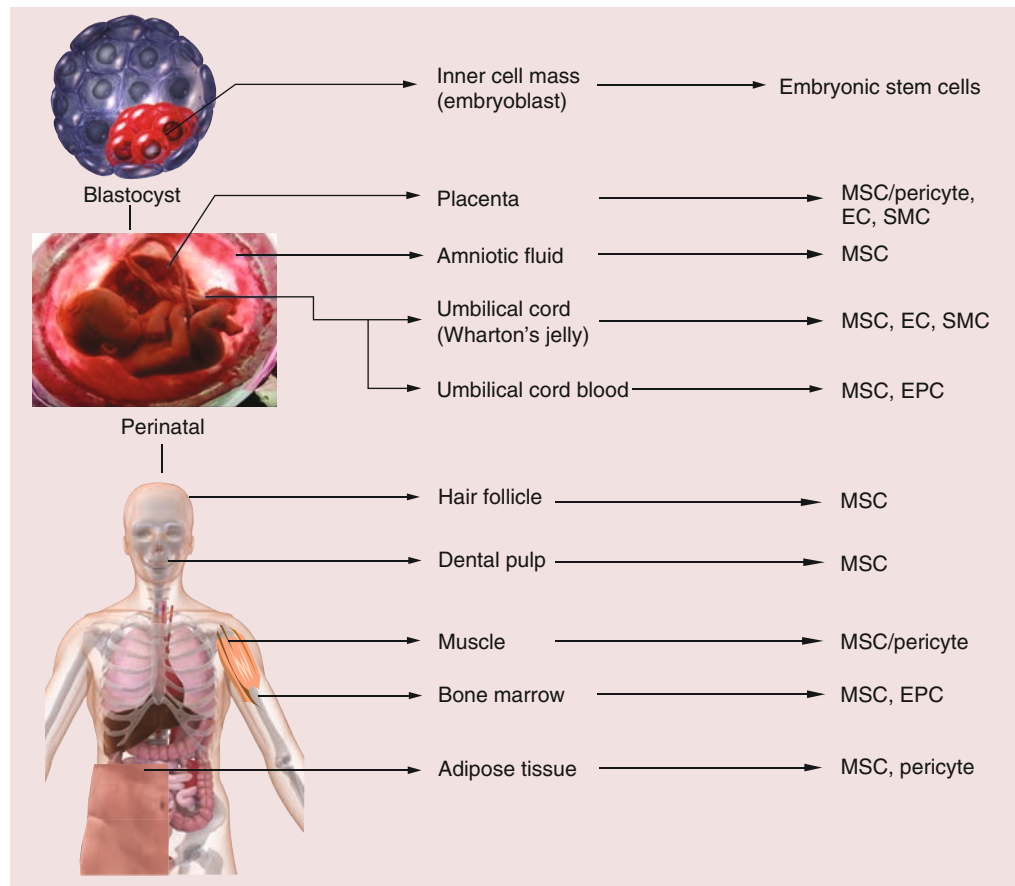


Figure 4. Vascular cells and related stem cells harvested from different human organs or tissues at different stages of development.

EC: Endothelial cell; EPC: Endothelial progenitor cell; MSC: Mesenchymal stem cell; SMC: Smooth muscle cell.

in iPSCs compared with their origin fibroblasts and ESCs [60].

ESCs are pluripotent stem cells derived from the inner cell mass of a blastocyst. Theoretically, they are able to differentiate into all cell lines derived from the three primary germ layers. Researchers have described various methodologies of differentiating human ESCs into ECs and SMCs, with or without embryoid-body intermediates [61–65]. Furthermore, other studies have indicated that ESC-engineered vessels could integrate into the recipient’s circulatory system [66,67]. However, the mechanisms of controlling ESCs to differentiate into vascular cells are poorly defined. Meanwhile, the utilization of ESCs is still under ethical controversy, as the isolating process destroys the fertilized human embryo.

Adult stem cells, also known as somatic stem cells, have been identified throughout organs and tissues from the whole body. Most of the adult stem cells are multipotent, that is, they can differentiate into all cell types existing in their original organ. Under the correct

conditions, cross-embryonic-lineage differentiation potential has also been found in some adult stem cell types, for instance, MSCs derived from adipose tissue are able to differentiate into osteocytes, skeletal muscle cells and SMCs [68]. Unfortunately, harvesting adult stem cells generally requires invasive collection; more tissue is required to obtain sufficient cells due to their limited proliferation potential compared with ESCs. These operations increase the risk to newborns and are not acceptable according to the criteria of regenerative medicine.

PSCs: the term perinatal refers to the period around birth, from the 20th week of pregnancy to the fourth week of life. The perinatal tissues, which include umbilical cord blood (UCB), the umbilical cord (including blood vessels and Wharton’s jelly matrix) and the placenta, are mostly treated as medical waste and are discarded at the time of birth. Therefore, the isolation of stem cells from these sources is non-invasive and safe to both the mothers and newborns. The stem cells obtained from those tissues are collectively called ‘PSCs’.

In the field of CHD, this perinatal tissue is of great practical and strategic importance. A detailed fetal echocardiography can detect CHD as early as 24 weeks of gestation. It is therefore possible to harvest perinatal stem cells at the time of delivery and use them for tissue engineering grafts to be used in congenital heart surgery. We will therefore concentrate in this review on the optimal stem cell populations, which can be harvested from perinatal tissues and are able to generate sufficient ECs and SMCs for the creation of tissue-engineered autologous grafts.

■ Deriving ECs from PSCs

The endothelial lining of any graft is crucial to its success, and it appears likely that only living, autologous ECs are able to meet the joint challenges of being nonthrombogenic and immune tolerated [25].

EPCs are a population of rare mononuclear cells circulating in the peripheral blood as well as residing in the bone marrow. EPCs can be isolated from UCB by gradient centrifugation followed by direct plating or further sorting by cell surface markers, such as CD34 or CD133 [69,70]. They express both hematopoietic and EC markers at an early stage.

Previous studies have demonstrated that angiogenic stimuli, such as VEGF and β -FGF, could trigger the proliferation and differentiation of EPCs into mature ECs *in vitro* [71,72]. These cells not only display a matured EC phenotype, including a 'cobblestone' morphology, expression of EC surface markers, such as CD31, Flk-1, VE-cadherin and vWF, and the uptake of LDL particles from medium; however, they also are able to form capillary-like structures when plated at subconfluent densities with extracellular matrix support *in vitro*, or migrate to incorporate into neovessels *in vivo*. This tube-formation ability indicates that these matured ECs could be functionally involved in angiogenesis [73].

Kaushal *et al.* reported that TEBVs seeded with EPCs, isolated from peripheral blood, revealed better antithrombogenic properties compared with grafts not covered with EPCs [25]. Schmidt and colleagues started to test UCB-derived EPCs (UCB-EPCs) in vascular tissue engineering 3 years later. They found a monolayer of matured ECs attached on the lumen of the polyglycolic acid scaffold after 12-days incubation. The tissue was characterized by endothelium functions [74].

UCB has been revealed to contain tenfold more EPCs compared with adult peripheral

blood, which may be sufficient for vascular tissue engineering purpose [75]. UCB EPCs have shown stable phenotype, and proliferative and differentiation potential when cultured in defined expansion and differentiation medium, which indicates that this cell type could be a promising EC candidate for the surgical treatment of pediatric CHD.

■ Deriving SMCs from PSCs

Deriving SMCs from adult MSCs

MSCs are a group of stem cells isolated from multiorigin tissues in the human body, including the bone marrow, fat, blood, liver, spleen and perinatal tissues [76–80]. The SMC differentiation potential of adult BM MSCs has been intensively studied [76,81], mainly for the purpose of understanding the mechanism and evaluating the treatment of arteriosclerosis.

These cells have been shown to be involved in angiogenesis, through the interaction with vascular cells, by releasing or responding to particular vascular growth factors [82]. Studies have shown that BM MSCs could be differentiated into endothelial phenotypic cells in the presence of VEGF and β -FGF [83], or into SMC lineage under the treatment with PDGF and/or TGF- β 1 [84–87].

In 2008, Gong and Niklason reported the first small-diameter vessel tissue engineered using human BM MSC-derived SMCs and a biodegradable scaffold in a biomimetic condition *in vitro*. The BM MSC-derived SMCs only expressed α -smooth muscle actin (α -SMA) and calponin, the early differentiation stage markers. This may be the major cause that resulted in the weak mechanical strength and improper ECM protein component ratio, although engineered vessels displayed similar morphology and histology compared with natural blood vessels [88].

Perinatal sources for MSCs & their SMC differentiation potential

UCB-derived MSCs

UCB is a rich source of stem cells including hematopoietic stem cells and MSCs, without any ethical concern owing to its autologous origin and as there is no risk from harvesting UCB. Besides using UCB as an alternative hematopoietic stem cell source [89], several research groups have reported successful isolation of MSCs from cord blood with full adult MSC multipotency [81,90,91].

The isolation of MSCs from UCB is mostly based on MSCs adherence-to-plastic property

when cultured in selective media. Mononuclear cells from fresh cord blood are cultured in medium containing fetal bovine serum, with or without growth factors, such as PDGF-BB, EGF and β -FGF, among others, and are plated on extracellular matrix protein-coated plates. Visible colonies can be observed from days 5–14. Cells grow with unified fibroblast-like morphology and display a typical ‘hill and valley’ phenomenon when reaching confluence [81,90,91].

These cells are negative for hematopoietic lineage markers CD34 and CD45, but positive for human MSC markers CD73, CD90 and CD105, indicating the MSC nature of these UCB-derived cells. These cells have a high proliferative capacity and a remarkable capacity to differentiate into multiple mesoderm lineages, for example, adipocytes, chondrocytes, osteoblasts and hepatic cells similar to BM MSCs [92–94].

The major bottleneck in the clinical application of UCB MSCs is the low collection success rate [95].

Umbilical cord stem cell (Wharton’s jelly stem cell)

Wharton’s jelly is a gelatinous substance within the umbilical cord, largely made up of hyaluronic acid and chondroitin sulfate (FIGURE 5) [96]. Since 1991, fibroblast-like cells were isolated from Wharton’s jelly by McElreavey and colleagues [97]. Weiss and other scientists’ reports indicate that those cells among the umbilical cord are multipotent stem cells displaying a bone marrow MSC-like phenotype; for example, adherence to plastic surfaces and expressing cell surface markers, including CD73, CD90 and CD105, but simultaneously show no expression for CD14, CD19, CD34, CD45 and HLA-DR [97–99]. It has been reported that

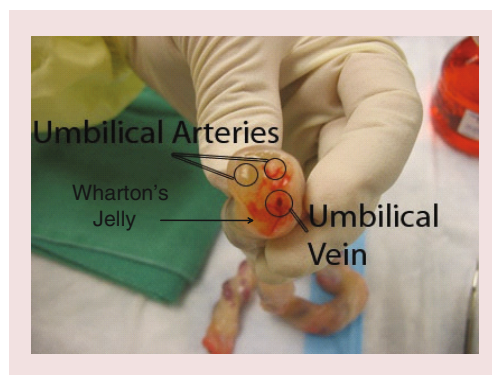


Figure 5. Cross-section of an umbilical cord. Two umbilical cord arteries and one umbilical vein are enclosed in the Wharton’s jelly.

Wharton’s jelly cells (WJCs) are capable of differentiating into all mesodermal lineage and nervous cells [100–102].

In 2002, Hoerstrup *et al.* applied WJCs in TEBVs by seeding these cells on to a biodegradable scaffold in a biomimetic growing system using a pulse duplicator bioreactor. Histological analysis showed that the seeded cells displayed some SMC markers and produced ECM proteins, for example, glycosaminoglycans and collagens [36]. Other researchers also found that cryopreserved WJCs maintain their myofibroblast-like morphology and are able to generate functional heart valves when seeded on a biodegradable trileaflet scaffold [103].

The unique advantages of WJCs are their pure fetal origin, large primary harvesting amount and nearly 100% isolation success rate by both enzymatical digestion and/or outgrowth methods. In addition, WJCs have been found to have a better proliferation capability compared with MSCs derived from bone marrow [104]. Although our unpublished data suggest that WJCs may have limited potential to give rise to contractile SMCs compared with BM MSCs and UCB MSCs, Wharton’s jelly is still a potential MSC source for vascular tissue engineering and is worth exploring further.

Placenta-derived stem cells

The placenta is an organ providing a link between the fetus and the mother to allow nutrient uptake, waste elimination and gas exchange via the maternal blood supply, while keeping the maternal and fetal circulations separated, although both exist within the placenta.

Pericyte

Perivascular cells, principally pericytes or mural cells, which exist in capillaries and microvessels, have been isolated from different human organs including, placenta, pancreas, skeletal muscle and adipose tissue. These cells express phenotypic markers, including CD146, NG2, PDGF-R β and most of the major MSC markers, but lack endothelial, hematopoietic and myogenic cell markers. Pericytes derived from tissues of different origins could retain their myogenicity in long-term *in vitro* culture [105]. Many researchers use ‘MSCs’ to name placenta-derived multipotent stem cells according to their BM MSC-like phenotype. However, considering their microvessel origin and expression of additional vascular cell-related markers, we prefer to define those cells as placenta-derived ‘pericytes’.

To isolate the pericytes from the placenta, Crisan and colleagues first obtained single cells by digesting placenta chronic villi (FIGURE 6). Pericytes were then sorted out as CD146⁺, CD34⁻, CD45⁻ and CD56⁻ cells from the single-cell suspension [105].

Pericytes are multipotent cells retaining the capacity to differentiate into vascular cells and a variety of other MSC-derived cell types. Pericytes have been reported to have the capacity to differentiate into either vascular SMCs or ECs under the influence of PDGF-BB, TGF- β 1 and/or VEGF, respectively [105–107].

He and colleagues developed a small-diameter TEBV by only seeding human skeletal muscle-derived pericytes, which were isolated by the same sorting criteria, into a biodegradable poly(ester-urethane) urea scaffold. After implanting the graft into Lewis rats for 8 weeks, they found, in the remodeled tissue multilayer, the expression of some SMC markers, such as α -SMA and calponin. The lumen monolayer was positive in endothelial marker (vWF) staining. Their results proved the feasibility of pericyte-based TEBV and indicated the pericytes functional involvement in maintaining patency of the graft and repopulating vascular cells into the TEBV [107].

Although some reports suggest that placenta-derived pericytes may have immunomodulatory effects [108], the clinical utilization of placenta-derived stem cells is still controversial because both fetal and maternal tissues are closely integrated within the placenta. There is no ready-to-use purification technique to avoid fetal or maternal cell contamination.

MSCs derived from amnion

Amnion, also named as amniotic membrane, is the extra embryonic fetal membrane derived from the ectoderm ECs and mesoderm MSCs that forms the amniotic cavity containing amniotic fluid that protects the fetus during the gestation shortly before birth [109]. This membrane can be simply peeled away from the placenta after the placenta is removed after birth (FIGURE 6).

Human amniotic membrane transplantation has been used in the treatment of skin burn wounds [110] and ocular surface reconstruction [111], before becoming an attractive stem cell source for other applications. Previous studies discovered that the amnion stem cells (ASCs) isolated from the amniotic membrane contain both amniotic ECs (AECs) and amniotic MSCs (AMSCs), which could be observed in the primary culture dishes under an optical microscope [109,112].

Amnion-derived stem cells did not express the hematopoietic (e.g., CD34 and CD45) and endothelial markers (CD31); however, they showed a varying and significantly lower positive rate of MSC surface markers, such as CD29, CD44, CD73, CD90 and CD105 [113,114]. This could be evidence to confirm that there are multiple populations with different phenotypes existing among ASCs. However, both AECs and AMSCs have been found to express some pluripotent cell markers, including OCT4, NANOG and SOX2 [115], which indicates that either AMSCs or AECs may have potential to differentiate into all three germ layer cells [115,116]. However, this hypothesis, generated based on their immune phenotype, has not been fully approved by the researchers. Compared with BM MSCs in similar differentiation conditions (beside the mesoderm lineage), the differentiated ASCs do not consistently express the characteristics of endodermal cells (e.g., hepatocytes and pancreatic cells) and/or ectodermal cells, such as neural cells [109,115,117].

Some researchers reported that AMSCs have osteocytic, chondrocytic and adipocytic differentiation potential similar to that of BM MSCs [113,114]. However, Soncini *et al.* and other researchers' data challenged the persistence of the multipotent capacity of ASCs. They found that only the freshly isolated ASCs could differentiate towards the trilineage of mesoderm with satisfying ratio. Additionally, this differentiation ability decreased sharply and the

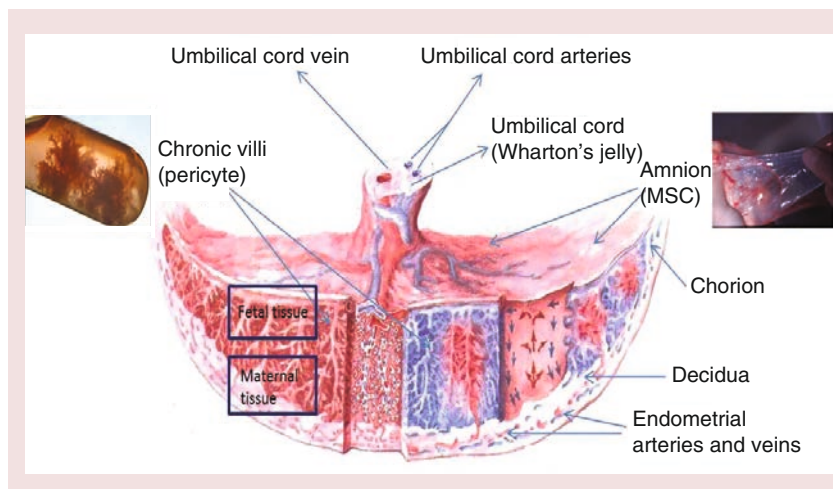


Figure 6. Cross-section of placenta anatomy. The umbilical cord arteries and vein insert into the chorionic plate and branch out over the fetal side of the placenta, which further divide to form a 'villus tree structure'. On the maternal side, endometrial arteries and veins villous are grouped into lobules from tree structures.
MSC: Mesenchymal stem cell.

proliferation speed slowed down during *in vitro* culture. Their result confirmed Portmann-Lanz and colleagues finding that most ASCs stop growing at cell passage four or five [109,118].

There has not been any significant achievement in deriving vascular SMCs from AMSCs. Considering the technical difficulty to isolate AMSCs from whole ASCs and their limited proliferation ability, ASCs can not be considered proper candidates in vascular tissue engineering based on the current research techniques.

Conclusion

Repeated operations are distressing for both young children and their families, and are increasingly risky. Since 1% of babies born have congenital defects, multiple operations are also costly. Tissue-engineered valves, conduits and vascular patches made of living tissue could function like a native structure with the potential to grow, repair and remodel; have a longer lasting therapeutic effect than acellular grafts; reduce the number of reoperations and the cost of to the National Health Service; and improve survival and quality of life.

The improved knowledge of stem cell biology and the development of the human embryo has greatly advanced our understanding of PSC's potential in the last decade. Perinatal tissues show great promise as an alternative stem cell source, and PSCs give clinical scientists more choices and the possibility of planning the building of the tissue-engineered grafts ready for when the surgical correction is required. However, there are still many limitations to be overcome to produce a well-defined, easy-to-harvest PSC population and for their seeding into appropriate matrices that are

going to be used for the treatment of newborns or infants with CHD.

Future perspective

Synthetic biodegradable and decellularized natural materials seeded with PSC-derived vessel cells may have the greatest potential and will continue to be used in tissue engineering of vascular grafts for corrective congenital heart surgery. Although it is hard to predict which approach of the two will see wider use, decellularized and cell-seeded scaffolds promise better-engineered materials for corrective congenital heart surgery. More trials will be needed to confirm the superiority of this approach. Techniques and protocols for decellularization of natural scaffolds and differentiation of PSCs into ECs and SMCs will see further optimization. The synthetic biodegradable scaffolds seeded with autologous cells approach may see a breakthrough if new polymers are developed with the advantage of not causing any damage to intact vascular structures when degraded and released into the circulation.

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Executive summary

- Many children with congenital heart disease need prosthetic and other replacement grafts in the form of new valves, conduits and patches.
- Although prosthetic and other replacement grafts may be life saving, they have limited durability and are prone to infection, immunological reactivity and thrombosis.
- The repopulation of a decellularized matrix with a patient's own stem cells before surgery can potentially create a living structure with subsequent long-term preservation of mechanical and biological properties.
- The key vessel cells, endothelial and smooth muscle cells, can be derived from perinatal stem cells (umbilical cord blood endothelial progenitor cells and mesenchymal stromal cells, respectively).
- Prosthetic materials like Dacron or Gortex predispose the patients to life-long risks of infection and lack the ability to grow, repair and remodel, which are vital for pediatric populations.
- Acellular scaffolds, made by decellularization of natural biological tissues, have been shown to resist infections and be durable and compliant in animal studies. However, human study showed that these grafts triggered a strong inflammatory response due to matrix immunogenicity being conserved.
- Synthetic biodegradable scaffolds seeded with autologous cells offer a better alternative to acellular scaffolds. However, as the polymer degrades slowly it is released in the circulation and can cause damage to intact vascular structures.
- Decellularized and cell-seeded scaffolds promise even better engineered materials for corrective congenital surgery; although more trials are needed to confirm the superiority of this approach.

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