

Advancing vascular tissue engineering: the role of stem cell technology

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Atherosclerosis and heart disease are still the leading causes of morbidity and mortality worldwide. The lack of suitable autologous grafts has produced a need for artificial grafts but the patency of such grafts is limited compared to natural materials. Tissue engineering, whereby living tissue replacements can be constructed, has emerged as a solution to some of these difficulties. This, in turn, is limited by the availability of suitable cells from which to construct the vessels. The development of prosthesis using progenitor cells and switching these into endothelial cells is an important and exciting advance in the field of tissue engineering. Here, we describe recent developments in the use of stem cells for the development of replacement vessels. These paradigm shifts in vascular engineering now offer a new route for effective clinical therapy.

Introduction

Atherosclerosis and cardiovascular disease are the most common causes of morbidity and mortality in the UK [1,2], with ~2.65 million people suffering from coronary heart disease [3,4]. This includes ~390 000 patients in NHS hospitals, of which ~120 000 die per year [3]. The economic costs of the management of coronary heart disease amount to £7 billion y^{-1} [3]. Such vascular and coronary diseases are treated surgically using bypass procedures, whereby grafts of principally autogenous tissue, for example internal mammary artery or saphenous vein, are used [5]. The patency rates of internal mammary artery are high at ten years, whereas 50% of saphenous vein grafts become occluded after ten years owing to gradual deterioration and neo-intima formation [2].

In 30–50% of bypass patients, all viable autologous tissue has already been used [1,6]; under these circumstances, synthetic prostheses are utilized. In peripheral surgery, woven polyethylene tetraphthlate (Dacron) and expanded polytetrafluoroethylene (ePTFE) are used, although for coronary artery bypass ePTFE alone is suitable. After four years, the average patency is 73%,

54% and 47% for saphenous vein, ePTFE and Dacron, respectively. Saphenous vein lacks the structural qualities of an arterial wall and excessive distension is the most common cause of failure in the first year [2,7]. The internal mammary artery provides a more patent vessel [2] because it is more similar to the artery that it replaces. Longer term graft failure is due to the difference in elasticity between the native blood vessel and the graft [8–10] creating turbulence and damaging the endothelial lining. This results in thrombus formation, smooth muscle cell proliferation and intimal hyperplasia [11]. The presence of an endothelium acts as a barrier to thrombus formation [12], improving patency significantly. Artificial grafts spontaneously endothelialize in animals but not in humans [13].

Two-stage seeded ePTFE in coronary artery bypass grafts, a 91% patency rate at 2.5 years (4 mm) and 65% patency at nine years (5 mm) have been demonstrated in studies of endothelial cells (EC); this is significantly higher than non-seeded grafts. There are currently two different techniques for the seeding of EC onto artificial grafts: single- and two-stage [14,15]. Two-stage seeding requires the harvesting of EC, followed by culturing and/or amplification for 2–4 weeks and then seeding onto the ePTFE graft coated with synthetic peptide RGD (arginine–glycine–aspartic acid) or fibrin. Single-stage seeding does not require this delayed culture period and can be performed during surgery [16]. EC are harvested in the same way but are immediately seeded onto the graft. More cells must be seeded than can currently be harvested and subsequent detachment is high.

Human stem and progenitor cells have been isolated from a range of sources. Their lack of specific tissue-related characteristics and dynamics have conjured immense interest, especially their ability to differentiate into specialized cell types. Progenitor cells have potential therapeutic uses owing to their ability for self-renewal, a high proliferation capacity and potential to differentiate [17]. Difficulties are associated with finding suitable numbers of cells to create such constructs and the time required to expand them in culture, especially under

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emergency circumstances. The recent discovery of the existence of vascular endothelial stem cells or endothelial progenitor cells (EPC) [18], even in patients suffering from cardiovascular disease, has led to interest in their use in seeding and tissue engineering. The relative ease of isolating these cells and their capacity to be expanded in culture, up to as many as 1000 doublings, while retaining their capacity to differentiate, has led to their application in tissue engineering.

Here, we describe the recent developments in using these cells for the development of replacement blood vessels. We do not aim to present a comprehensive review of isolation and characterization *per se* of stem cells because several excellent reviews already exist [17,19]. Our review is intended to highlight how stem cell technology could be useful in vascular tissue engineering and highlights the latest advances in these exciting fields.

Cells types

Stem or progenitor cells can be found in the embryo or adult. Evidence suggests that embryonic stem cells have a more potent regenerative power than do adult progenitors. Human embryonic stem cells have ethical constraints and the desire for autologous grafts make adult progenitor cells more suitable for tissue engineering. Adult progenitor cells have been found to be multi-potent and to have high plasticity. They have been isolated from bone marrow, blood, brain, liver, muscle, skin and umbilical cord blood (Table 1) [20–22]. Bone marrow gives rise to what are termed haematopoietic stem cells (HSC) and marrow stromal and/or mesenchymal stem cells (MSC) [23,24]. Adult stem cells have a lower plasticity than do embryonic stem cells but have been actively studied by researchers. The review by Rezai and colleagues describes how these cells have been demonstrated to transdifferentiate into brain, gut, lung, liver, pancreas, kidney and cardiac cells [25].

Haemopoietic stem cells

Haemopoietic stem cells (HSC) are found in the bone marrow but constitute only 0.01% of bone marrow cells [26]. HSC can differentiate into multi-lineages that comprise the haematopoietic system in transplanted hosts for their entire lifespan. They are multipotent and have a high proliferation potential. HSC were the first progenitor cells reported to transdifferentiate into non-haematopoietic cells after bone marrow transplantation [27]; however, the purified cells for this study could have

been contaminated by a few MSC. HSC have been used in non-haematopoietic diseases: myocardial infarction, ischemia and cirrhosis [28,29]. HSC demonstrate sufficient plasticity that they can differentiate into bone, cartilage, neural cells, pneumocytes, muscle, skin, endothelial, epithelial cells and hepatocytes, as reviewed by Short *et al.* [19].

Heart tissue has a limited regeneration capacity; thus, the use of HSC for cardiac repair has been found to be of clinical relevance. HSC can transdifferentiate into cardiomyocytes, endothelial and smooth muscle cells in mouse models with myocardial infarction. Jackson and colleagues [27] showed that the bone marrow-derived cells had proliferated over the infarcted area, expressed cardiac specific markers and differentiated into EC [30]. Some qualifications were made by the authors; first, that cell contamination could be responsible for these findings and second, the lack of specific markers means that any donor cell might be identified even if it has not differentiated into the cell type of interest.

Mesenchymal stem cells

MSC have demonstrated the ability to differentiate into cardiomyocytes, EC and smooth muscle cells when injected into the heart of adult mice [31] using the chemical 5-azacytidine, which is not suitable for use in humans. Debate continues regarding the precise phenotype of MSC because no specific markers have yet been developed. For a fuller discussion of this topic we refer the reader to a recent review on the subject by Dimmeler and co-workers [32].

Endothelial progenitor cells

EPC can be derived from bone marrow or from the blood. In 1997, Asahara and co-workers reported the presence of EPC in human peripheral blood [18]. Increasing evidence indicates that they have the potential to differentiate into mature EC and contribute to the process of endothelium repair. EPC are reported to home to specific sites in response to endothelial injury, where they divide and have an active role in vascular repair [33–36]. They have been correlated with the cardiovascular Framingham risk score and are inversely correlated to cardiovascular risk [36]. The number of circulating EPC increase in response to local ischaemia [37], the statins [38,39], oestrogen [40–42], erythropoietin [43] and vascular endothelial growth factor (VEGF) [44]. There is a rapid mobilization of EPC after burn injury and surgical manipulation [45–48]. Raised EPC can be detected in the circulation six h after injury

Table 1. Isolation of stem cells

| Species | Cell | Isolation method | Details | Refs |
|---------|------|--------------------------------|--|------|
| Murine | HSC | HSC selection | Use monoclonal antibodies: (i) coupled to toxins; (ii) FACS; (iii) positive selection of CD34 ⁺ | [20] |
| Murine | MDSC | FACS | CD34 ⁺ Sca-1 ⁺ cells from mouse skeletal muscle | [21] |
| Human | MSC | Immunostaining | Isolation by FACS for STRO-1 bright VCAM-1 ⁺ cells | [22] |
| | BMC | MACS | BM incubated with STRO-1 supernatant, streptavidin microbeads and FITC | |
| | ESC | Isolated from inner cells mass | Immunosurgically isolating ESC from inner cell mass of human blastocyst | |
| | ASC | Isolated from liposiprate | Centrifugation of cells and separation by sedimentation | |
| | | FACS | | |
| | | MACS | | |

Abbreviations: ASC, adipose stem cell; BMC, bone marrow cells; FACS, fluorescent activated cell sorting; HSC, haematopoietic stem cells; MACS, magnetic activated cell sorting; MDSC, muscle-derived stem cell; MSC, mesenchymal stem cells.

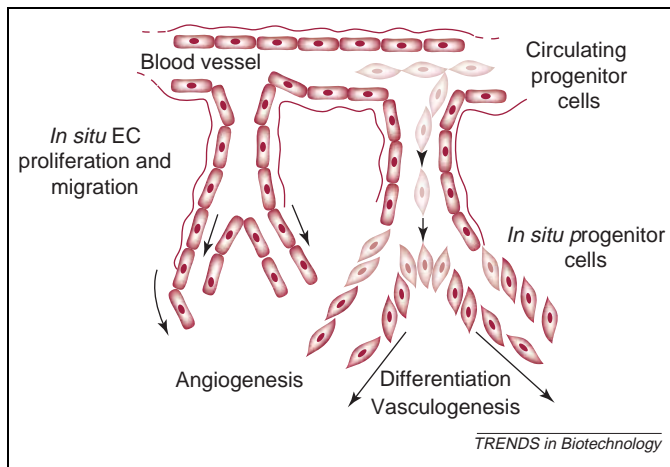


Figure 1. Revascularisation as an important event in the regenerative process. Angiogenesis and arteriogenesis depend upon the presence and activation of endothelial cells and endothelial progenitor cells. In angiogenesis, endothelial cells are believed to migrate from existing cells vessels. However, we suggest that vasculogenesis is a direct result of *in situ* progenitor cell differentiation.

and by 24 h, EPC constitute 12% of total circulating mononuclear blood cells [49].

EPC have been differentiated into EC *in vitro* and incorporated into sites of neovascularization *in vivo*. The differentiation of adult EPC to mature EC displayed for the first time that the phenomenon of vasculogenesis can occur in adults as well as embryos, as shown in Figure 1. EPC show less plasticity and demonstrate less capacity for self-renewal than earlier stem cells but retain the ability to differentiate into several cell types. EPC differentiation, *in vitro*, is dependent on culture conditions. VEGF and fibronectin promote the differentiation of EPC into EC [50]. By contrast, peripheral blood mononuclear cells cultured on collagen in the presence of platelet-derived growth factor-BB differentiate into smooth muscle-like cells [51]. EPC can differentiate into cardiomyocytes when co-cultured with neonatal rat cardiomyocytes [44].

EPC and mature EC express similar markers at different levels (detectable by immunohistochemistry and western blotting); these include VEGFR2, Tie-2, vascular endothelial (VE)-cadherin, CD34, CD146, selectin, CD31 (PECAM), von Willebrand factor and VEGFR1. EPC express CD133 (AC133), CD34 and VEGFR2, whereas mature EC are negative for CD34 and CD133 but express higher amounts of VEGF2, VE-cadherin and von Willebrand factor. CD133⁺ cells are better able to differentiate into EC than CD34⁺ cells and early expression of CD31 on cultured CD133⁺ cells during differentiation can be used as a marker of endothelial precursors [52,53]. Both EPC and EC incorporate acetylated low-density lipoprotein (Ac-LDL) and bind lectins, including *Bandeiraea simplicifolia* (BS-1) and *Ulex europaeus* [48].

Systemic administration of EPC isolated from peripheral blood and expanded *in vitro* augmented naturally impaired neovascularization in an animal model of experimentally induced limb ischemia [54]. This led to human clinical trials, which showed that bone marrow mononuclear cells, including EPC, when implanted directly to 45 ischemic lower limb muscles, improved symptoms and collateral circulation and, furthermore,

that this technique could be used safely for therapeutic angiogenesis [55].

Muscle-derived stem cells

Muscle-derived stem cells express markers of muscle cell differentiation but can self-renew and give rise to myoblasts and myofibers [56]. The progeny of these cells, the skeletal myoblast or satellite cell, is of benefit when transplanted into several animal models [35,42,57–59]. This has led to several clinical trials, which have demonstrated the positive inotropic effects of cell transplantation [60–62]. In animal models, skeletal myoblasts were found not to differentiate into cardiomyocytes: they failed to express cardiac specific markers [57,63] and were electrically uncoupled to surrounding cardiac tissue [57,63,64]. Implanted rat myoblasts form contractile myotubes and switch from fast-twitch myosin heavy chain to slow-twitch myosin heavy chain to sustain a cardiac workload [42,64].

Cardiac stem cells

The long accepted dogma has been that cardiac myocytes are unable to replicate. Over the past decade, this belief has come into question. In 1998, Kajstura and colleagues [65] discovered the presence of myocytes undergoing mitosis in the failing human heart. Such cells were found to be scarce, explaining the lack of significant myocardial regeneration after myocardial infarction. These cells have subsequently been demonstrated to be self-renewing, clonogenic and able to give rise to myocytes, smooth muscle and endothelium. These cells have subsequently been defined as Lin⁻c-kit^{PO8} cells within human hearts [66]. This work is yet to be replicated in animal models using similar systems, a fact that has been used to refute these findings [67]. The origin of these cells remains uncertain and, to date, no similar cells have been found circulating or in the bone marrow.

Subpopulations of cardiomyocytes have been identified that are able to replicate but regeneration is restricted to peri-infarcted myocardium [68]. The transplantation of skeletal myoblasts and cardiomyocytes has been investigated in animal and clinical trials, but provides limited benefit if implanted cells fail to become electrically coupled, despite surviving and differentiating [61,69]. EPC have also been found to be of importance in the mechanism of neo-vascularization in animal models of ischemic hearts, where they differentiate into EC [70]. A series of animal studies and clinical trials have been used to attempt to determine whether stem cells could be useful in myocardial infarction. The key papers of this field to date are outlined in Table 2. Readers are also referred to an excellent review of this literature by Dimmeler *et al.* [32].

Adipose-derived stem cells

Human adipose tissue has been used to extract stem cells that have been studied and differentiated to osteoid, adipose, muscle and cartilaginous cells [71]. Until recently, it was thought that these cells could not differentiate into EC. A recent study by Martinez-Estrada seems to prove that this is not the case and that such cells are indeed able

Table 2. Published studies investigating the use of stem cells in the revascularization of ischemic myocardium

| Cell | Methods | Outcome | Refs |
|-----------------------------|---|---|------|
| Mouse MSC | Mice treated with 5-azacytidine. Populations CD34 ^{low/-} , c-kit ⁺ and CD140a ⁺ selected. FACS analysis injected into ventricular myocardium or IVC | Positive for CD34, Sca-1, CD29, CD44, CD41, CD144 and Ly-6C at 1 week. Formed cardiac muscle structure with EC and pericytes or SMC | [31] |
| Rat BMC | Cells cultured with 5-azacytidine, TGFβ1, or insulin labelled with BrdU and transplanted into cryoinjured left ventricular, BMC implanted | Stained positive for troponin I and increased capillary density greatest with scar size reduced and function improved | [83] |
| Human CD34 ⁺ BMC | CD34 ⁺ cells separated. Cells tested for CD117, GATA-2 activity, VEGFR-2 (Flk-1), Tie-2 and AC133. Nude rat had LADCA ligated and cells injected | CD117 ^{Bright} /GATA-2 ^{hi} , higher proliferation than CD117 ^{Dim} /GATA-2 ^{lo} . Blast cells proliferated without differentiating | [84] |
| Porcine BMC | BMC isolated from pigs with chronic myocardial ischemia, cultured and injected transendocardially into ischemic zone | VEGF and MCP-1 increased in time-related manner. Conditioned medium enhanced PAEC proliferation | [85] |
| Mouse BMCCD34 ⁻ | Transplantation and MI- SP cells injected and 10 weeks later LADCA occluded for 60 min. Hearts removed at 2 or 4 weeks | SP cells expressed markers for angiopoietins and contributed to newly formed vessels adjacent to infarct | [27] |
| Mouse BMC | Splenectomised mice given SCF and G-CSF post coronary artery ligation | 73% survival of cytokine treated mice (27 days) 17% in control. Newly formed band of myocardium in cytokine mice | [68] |

Abbreviations: BrdU, bromodeoxyuridine; ELISA, enzyme-linked immunosorbent assay; IMDM, Iscove's modified Dulbecco's medium; IVC, inferior vena cava; LADCA, left anterior descending coronary artery; PAEC, pig aortic endothelial cells; SCF, stem cell factor; SVEC, saphenous vein endothelial cells.

to be converted to EC. Flk-1 cell populations, enzymatically released from adipose tissue and isolated by flow cytometry, were differentiated into von-Willibrand expressing EC. As such, we should also consider adipose tissue a source of EPC [72], the ease of access to fat and its abundance in making adipose tissue a potentially useful source of EC for tissue engineering.

Applications

Two cell populations, circulating EPC [1,73–75] and bone marrow cells (BMC) [25,76], have been applied in engineering based applications. The use of BMC requires the invasive techniques associated with aspiration of cells from the bone marrow. EPC can be isolated and expanded from peripheral blood using relatively small blood samples [1]. The exact cell composition remains a subject of debate within the stem cell research community. Both these cell sources possess advantageous over the surgical removal of vessels to harvest EC [1,73,77]. Progenitor cells from both sources have been used for single- and two-stage seeding [15,73]. Cells have been seeded onto a variety of graft material before implantation; the degree of differentiation in these studies has varied. The uses of grafts that will endothelialize *in vivo*, or that can be seeded at the time of implantation, are of increasing interest because they will

be suitable in emergency situations. A summary of the use of stem cells in vascular graft is given in Table 3.

In vitro studies

Shirota and co-workers isolated EPC from peripheral blood of healthy volunteers by density gradient centrifugation and cultured them. When subjected to hydrodynamic shear stress, cells aligned in the direction of flow maintaining a confluent monolayer with a high level of integrity under high flow [73]. EPC were compared to human vein EC for eNOS, PG12 and tPA. Cells were seeded onto photo-reactive gelatin coated poly(ether) urethane (1.5 mm). 82% of blood samples yielded endothelial like cells but only 20% proliferated sufficiently fast for use in further studies. Cells exposed to flow on grafts demonstrated a cobble stone morphology characteristic of statically cultured EC but not that observed *in vivo* was spindle-like, and expression of eNOS was significantly lower than human umbilical vein EC under similar conditions, despite almost complete graft coverage [73].

Wu and co-workers created tissue engineered microvessels from human umbilical vein blood isolated EPC. Cells were isolated and extensively purified using magnetic beads to CD34 and CD133. These studies further proved the ability of EPC to be expanded *in vitro* and to retain the ability to express endothelial markers.

Table 3. Published studies investigating the use of stem cells in enhancing vascular graft patency

| Cell | Vessel | Methods | Outcome | Refs |
|------------------------------|-------------------------------|--|---|------|
| Human EPC | SPU 1.5 mm <i>in vitro</i> | Graft coated with photo-reactive gelatin. Shear stress 12 h. Gene expression | 9 of 11: low proliferation. 2 of 11: high proliferation. Positive for Flk-1 and vWF. eNOS 6-keto-PGF _{1-α} and tPA | [73] |
| Ovine EPC | Porcine 4.0 mm <i>in vivo</i> | Decellularized porcine iliac arteries shear stress preconditioned anastomoses to common carotid artery. Immunofluorescence | Expressed CD31, vWF, E-selectin, a-actin. Patent at 15 and 130 days <i>in vivo</i> | [1] |
| Canine CD34 ⁺ BMC | ePTFE seeded segment, 8 mm | CD34 ⁺ were seeded and placed in canine descending thoracic artery | All patent 4 weeks. Cell coverage 92% FVIII ⁺ /vWF ⁺ and CD34 ⁺ | [79] |
| Canine BMC | P(CL/LA) with PLLA 8mm | Seeded grafts implanted inferior vena cavae Immunohistochemistry and VEGF PCR | Complete endothelialisation after 4 weeks. VEGF and SMC-positive after 2, 4 and 8 weeks. Still patent after 2 years | [76] |

Abbreviations: BMC, bone marrow cells; PAA, polyacrylamide; PBMC, peripheral blood mononuclear cells; PDT, population doubling times; P(CL/LA), porous copolymer of lactic acid and ε-caprolactone; PLLA, poly-L-lactic acid; SPU, segmented polyurethane.

A biodegradable scaffold of PGA stabilized by PLLA was generated and seeded with or without smooth muscle cells in roller bottle culture. This study demonstrated that human EPC could be expanded and seeded onto a vessel scaffold and that EC phenotype remained stable in culture. Better coverage was seen in the presence of smooth muscle cells and the authors report the formation of microvessel-like structures [78].

In vivo studies

Kaushal and co-workers showed that decellularized porcine iliac arteries seeded with EPC implanted into sheep had high patency and were structurally, functionally and morphologically similar to a native vessel [1]. The leukocyte fraction was isolated from the blood of 1–2-week old lambs and cultured for five weeks before graft seeding. Vessels were seeded for 72 h and exposed to shear stress for four days before implantation, gradual increase in shear stress from 1 to 25 dynes cm^{-2} improved cell coverage. Complete EC coverage was seen after implantation and grafts remained patent for the duration of the 130 day study. These vessels showed some smooth muscle cell in-growth in the intima, assumed to occur via the anastomosis; however, these vessels could fail as a result of intimal hyperplasia in longer studies [11]. Post-implantation organ bath studies revealed that vessels were responsive to norepinephrine and serotonin in a similar way to native vessels. The use of cells from young animals could have made this study more effective and studies using adult derived EPC would be beneficial but have still not been performed.

CD34⁺ isolated from BMC were used by Bhattacharya and co-workers [79]. Grafts were 12 cm long and consisted of a 4-cm section of crimped knitted Dacron with 4 cm of ePTFE at either end; all were 8 mm in diameter and were sealed in a silicone coat. The study focused upon the central Dacron section with ePTFE being used to prevent panus invasion into this graft material. Grafts were seeded with CD34⁺ cells isolated from canine bone marrow using magnetic beads. Cells were applied to the graft in the last stage of the normal pre-clotting procedure applied by this group. Seeded grafts were implanted into the thoracic aortae of adult dogs. CD34⁺ cell treated grafts showed significantly improved endothelialization and increased microvessels within the neointima.

Shi and co-workers [80,81] used an experimental dog model that enabled only circulating cells to reach the vascular graft surface to determine that circulating, bone marrow derived EPC were the source of EC. Circulating EPC numbers were increased by mobilization using GCSF. Dogs treated with the GCSF showed increased endothelialization of prosthetic graft (ePTFE) compared to control non-stimulated animals [80]. This supported a previous study conducted by this group, using genetic tracing methods, that the main cellular source of vascular repair is bone marrow-derived EPC [81].

Bone marrow-seeded Dacron grafts had rapidly accelerated surface endothelialization compared to control, unseeded grafts in canine studies. Fujita and colleagues used BMC from the humeral diaphysis applied to grafts in a four-stage seeding technique. This was conducted

immediately before operation. CD34⁺ cells were seen on the graft at the end of 4-week trials. All graphs remained patent and endothelialization was significantly quicker in the BMC seeded grafts than control grafts 'seeded' with systemic blood [82].

Clinical studies

To date, only one group has published data from the clinical application of progenitor cells in tissue-engineered vascular grafts. Matsumura and colleagues [76] used vascular grafts created from mature cells, which were associated with a delay of three months to create these constructs. This study detailed single-stage seeding, where BMC were aspirated and the mononuclear cells isolated or crude BMC aspirate was seeded onto biodegradable scaffolds consisting of poly-L-lactide and a copolymer of lactide and ϵ -caprolactone. The authors detail specific cases and suggest that the techniques have been a success in 22 patients with no associated morbidity. Owing to the clinical nature of these studies, no specific details are available for the graft but clinically all grafts are reported to have performed well up to the time of publication (approximately one year). Although this study shows the promise of these techniques, longer term comparisons to un-seeded prosthesis and arterial grafts are required before this can be said to have been a proven success [76].

Conclusion

The field of tissue engineering is still clinically limited owing to the demands of the highly specialist cell culture, isolation and enrichment techniques required. More understanding of the proliferation and differentiation processes that occur in transplanted stem cells populations *in vivo* will further increase the patency of tissue engineered grafts.

The future of this technology is the requirement for phenotype switches within the grafts. The combination of phenotypic switches and increased understanding of progenitor cells will provide powerful tools in advancing vascular tissue engineering. Single-stage seeding of vascular grafts of EC derived from progenitor cells has been demonstrated clinically and further advances in switch design can be expected to make *in situ* seeding more common, especially in applications such as coronary stenting. The use of progenitor cell technology in this field is, in our opinion, the most promising technology for providing sufficient cells to produce more effective vascular interventional devices.

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