The Evolution of Vascular Tissue Engineering and Current State of the Art

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Key Words

Tissue engineering · Small-diameter vascular graft · Blood vessel · Hemodialysis access · Self-assembly · Human · Bioreactor · Mechanical strength · Extracellular matrix · Review

Abstract

Dacron® (polyethylene terephthalate) and Goretex® (expanded polytetrafluoroethylene) vascular grafts have been very successful in replacing obstructed blood vessels of large and medium diameters. However, as diameters decrease below 6 mm, these grafts are clearly outperformed by transposed autologous veins and, particularly, arteries. With approximately 8 million individuals with peripheral arterial disease, over 500,000 patients diagnosed with end-stage renal disease, and over 250,000 patients per year undergoing coronary bypass in the USA alone, there is a critical clinical need for a functional small-diameter conduit [Lloyd-Jones et al., *Circulation* 2010;121:e46–e215]. Over the last decade, we have witnessed a dramatic paradigm shift in cardiovascular tissue engineering that has driven the field away from biomaterial-focused approaches and towards

more biology-driven strategies. In this article, we review the preclinical and clinical efforts in the quest for a tissue-engineered blood vessel that is free of permanent synthetic scaffolds but has the mechanical strength to become a successful arterial graft. Special emphasis is given to the tissue engineering by self-assembly (TESA) approach, which has been the only one to reach clinical trials for applications under arterial pressure.

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Abbreviations used in this paper

AV	arteriovenous
ECM	extracellular matrix
ePTFE	expand polytetrafluoroethylene
ESRD	end-stage renal disease
FBS	fetal bovine serum
FDA	Food and Drug Administration
HSFs	human skin fibroblasts
ID	internal diameter
IEL	internal elastic lamina
IgG	immunoglobulin G
IM	internal membrane
MDSCs	muscle-derived stem cells
PGA	polyglycolic acid
SBTE	sheet-based tissue engineering
SMCs	smooth muscle cells
TBTE	thread-based tissue engineering
TEBV	tissue engineered blood vessel
TESA	tissue engineering by self-assembly

Cytograft Tissue Engineering sponsored clinical trials discussed in this review. Marissa Peck, David Gebhart, Nathalie Dusserre, Nicolas L'Heureux, and Todd McAllister are employees and own stocks/options of Cytograft Tissue Engineering.

Introduction

In 1974, Howard Green and his team were the first to develop culture conditions to mass-produce normal human diploid cells (keratinocytes) [Rheinwald and Green, 1974]. This key advancement was translated, less than a decade later, into what could be considered the first clinical use of a tissue-engineered product – a completely biological, living, autologous epithelium [O'Connor et al., 1981]. Around the same time, in the vascular field, progress in cell biology led to the idea of seeding endothelial cells onto the lumen of grafts in order to mitigate the thrombogenic nature of the synthetic surface [Mansfield et al., 1975]. Herring et al. [1978] first used an intraoperative technique (without culture) to seed the lumen of small-diameter synthetic vascular grafts in order to improve their less than satisfactory patency rate. However, this approach proved clinically disappointing [Herring et al., 1984]. In the mid-80s, taking advantage of the recently developed culture conditions that allowed for the serial propagation of endothelial cells [Maciag et al., 1981; Thornton et al., 1983], Zilla et al. [1987] combined *in vitro* cell expansion and postseeding culture to create a confluent autologous human endothelium in fibrin-coated small-diameter expanded polytetrafluoro ethylene (ePTFE) grafts. Today, with over 15 years of clinical use, this approach has clearly demonstrated that a biological component can lead to superior outcomes [Deutsch et al., 2009].

During the same period, Bell's group wanted to go further and create a completely biological blood vessel to avoid the many complications resulting from the use of synthetic materials (inflammation, stenosis, and infection) [Weinberg and Bell, 1986]. By endothelializing the lumen of concentric tubular collagen gels containing bovine smooth muscle cells (SMCs) and fibroblasts, he effectively pioneered the field of cardiovascular tissue engineering. However, this construct had poor mechanical strength and could not be used clinically.

In the ensuing years, multiple groups attempted to combine purified proteins and cells to create blood vessels, but all were mechanically weak [Matsuda et al., 1988; L'Heureux et al., 1993; Hirai et al., 1994; Girton et al., 2000; Berglund et al., 2003; Orban et al., 2004; Swartz et al., 2005; Yao et al., 2008]. As a result, in the late 1990s, the prevailing view was that the presence of a permanent synthetic scaffold was a prerequisite for the design of an implantable tissue-engineered blood vessel (TEBV) or other tissues with mechanical functions. It is in this context that a series of seminal papers were published pro-

posing various approaches to avoid the detrimental presence of permanent synthetic scaffolds [L'Heureux et al., 1998; Campbell et al., 1999; Niklason et al., 1999]. In this paper, we look at the evolution of these and other approaches (table 1) as well as how, over the last decade, the concept of a mechanically relevant yet completely biological tissue-engineered graft is no longer a preposterous idea but a clinical reality.

Purified Proteins as Scaffolds

Matsuda and coworkers followed in the footsteps of Bell by developing a graft composed of a combination of purified collagen and cells [Kanda et al., 1993]. For the first time, they implanted such grafts (cultured for 12 days) in the posterior vena cava of rabbits and dogs and ultimately performed an arterial implant [Hirai et al., 1994; Matsuda and Miwa, 1995; Hirai and Matsuda, 1996]. But, as Bell did, Matsuda had to reinforce the graft with Dacron®. Despite reasonable outcomes at 6 months and positive tissue remodeling, the presence of the permanent synthetic scaffold suggested that potential benefits of this approach did not appear to outweigh its complexity.

As an alternative to collagen scaffolds, a number of groups have also researched the use of cell-impregnated fibrin gels to create vascular grafts. While most studies have been performed with isolated/processed components, a possible advantage of using fibrin is that fibrinogen and thrombin, the precursors to fibrin gel formation, can be readily obtained from a patient's own blood [Haisch et al., 2000]. Like collagen gel-based grafts, constructs created from fibrin-gels have a typically low mechanical strength. Swartz et al. [2005] have implanted this type of graft, after a 24-day culture period, in the external jugular vein of chronically heparinized lambs. After 12 weeks, these allogeneic endothelialized grafts showed considerable remodeling, including some elastic fiber development, and their strength increased more than 3-fold upon integration with the host tissue. Despite apparent dilatation, both grafts were fully patent at the time of explantation. Efforts to translate their approach to the use of human cells is under way [Peng et al., 2011].

The group of Tranquillo, who initially worked on collagen approaches [Barocas et al., 1998], has significantly moved forward fibrin-based vascular engineering by creating vessels that can be used for arterial reconstruction. This was achieved through culture condition improvement and by recognizing, like Zilla and his group, that

Table 1. Milestones in the evolution of cell-based approaches to tissue engineer blood vessels

Approaches	Group leaders	In vitro: animal cells	In vivo: animal models	In vitro: human cells	In vivo: human in animal	Clinical use
EC seeded and cultured in a synthetic vascular graft	Zilla	Yes ^a	Yes ^a	[Kadletz et al., 1987], adults.	No	[Zilla et al., 1987; Deutsch et al., 2009], ID: 6–7, BP: ? (high), lower limb bypass, (adults).
Cell-seeded collagen gels	Bell/Matsuda/ L'Heureux	[Weinberg and Bell, 1986], + Dacron mesh ID: 6, BP: 323, B.	+ Dacron mesh [Hirai et al., 1994], ID: 3, BP: 110, vena cava, B × L. [Matsuda and Miwa, 1995], ID: 4, BP: ?, 26 weeks, carotid artery, C.	[L'Heureux et al., 1993], ID: 6, BP: ?, neonatal.	–	–
Cell-seeded bioresorbable scaffold (low pressure)	Shin'oka	[Shin'oka et al., 1998], ID: fifteen, 24 weeks, pulmonary artery, O.		No	No ^b	[Shin'oka et al., 2001; Hibino et al., 2010], ID: 12–24, pulmonary artery defects (pediatric).
Completely biological grafts produced using the TESA principle	L'Heureux	No	No	[L'Heureux et al., 1998], ID: 3, BP: 2,594, diseased adults.	[L'Heureux et al., 2006], ID: 1.5, BP: 3,688, 7.5 months, aorta, M ID: 4.2, BP: 3,468, eight weeks, aorta, P.	[L'Heureux et al., 2007; McAllister et al., 2009], ID: 4.8, BP: 3,340, hemodialysis access (adults).
Cell-seeded bioresorbable scaffold (high pressure)	Niklason	[Niklason et al., 1999], ID: ?, BP: 2,150, saphenous artery, 24 days, B in P. ID: ?, BP: ?, saphenous artery, 24 days, P.		[McKee et al., 2003] ID: 3.1, BP: 356 ^d , infant. [Poh et al., 2005], ID: 3, BP: 246, diseased adults.	[Dahl et al., 2011], decellularized, ID: 6, BP: 3,337, six months, axillary art. to brachial vein shunt, P.	–
	Vorp	[Nieponice et al., 2008], ID: 4, BP: 2,127 ^d , M.	[Nieponice et al., 2010], ID: 1.2, BP: ?, 8 weeks, M.	[He et al., 2010], ID: 0.95, BP: 4,462 ^d , 8 weeks, aorta, M, adult.		–
In vivo bioreactor	Campbell	NA	[Campbell et al., 1999], ID: 3, BP: ?, 4 months, aorta, M ID: 5, BP: ?, 4 months, carotid art., L. ^c	NA	–	–
Cell-seeded fibrin gels (high pressure)	Tranquillo	[Grassl et al., 2003], UTS: 1.4 MPa (we estimate BP: ≈ 1,500 at ID: 3).	Under rats [Tranquillo, pers. commun.]	[Syedain et al., 2011], ID: 2.4, BP: 1,600, neonatal.	Under way in nude rats, [Tranquillo, pers. commun.]	–
Cell-seeded fibrin gels (low pressure)	Andreadis	[Swartz et al., 2005], ID: 4, vena cava, 15 weeks, O.		–	–	–
Cell-seeded fibrin + bioresorbable scaffold	Jockenhoevel	[Tschoeke et al., 2009], ID: <5, BP: 460, O.	[Koch et al., 2010], ID: 6, BP: ?, 6 months, carotid artery, O.	–	–	–

Burst pressures are reported in millimeters of Hg. Inner diameters are reported in millimeters. NA = Not applicable; ? = not disclosed; BP = burst pressure; UTS = ultimate tensile stress; ID = inner diameter; C = canine; B = bovine; L = lapine (rabbit); M = murine; O = ovine; P = primate.

^a Studies published after clinical use [Zilla et al., 1989; Muller-Glauser et al., 1993].

^b Some studies done in mice after clinical use [Mirensky et al., 2009].

^c Later study in dogs gave BP of over 2,500 mm Hg and ID = 4.75 mm [Chue et al., 2004].

^d Burst pressure estimated from tensile testing.

biological approaches often need significant culture periods to be successful [Grassl et al., 2003]. A fibrin-based graft seeded with rat SMCs had a burst pressure of 1,122 mm Hg, with an internal diameter (ID) of 3 mm, after 6 weeks of culture [Isenberg et al., 2006]. Recently, this approach was translated to the use of human cells [Syedain et al., 2011]. Seeded with human skin fibroblasts (HSFs) and cultured for 7–9 weeks in a pulsating bioreactor, grafts with ID of 2 and 4 mm displayed burst pressures of up to 1,600 mm Hg, which is in the range of human veins. While these grafts still had a problematic suture retention strength, aortic implants in nude rats are giving promising results [Tranquillo, pers. commun.].

The recent work of Jockenhoevel and colleagues combines the use of fibrin gels with biodegradable scaffolds (knitted polylactide) [Tschoeke et al., 2009]. After 21 days of dynamic conditioning, these grafts seeded with ovine vascular cells had only a burst pressure of 466 ± 78 mm Hg (ID ≈ 5 mm), which was $\approx 1/3$ that of the ovine carotid artery. Nonetheless, in their most recent report, autologous and endothelialized vessels (ID 6 mm, length 4–5 cm) were successfully implanted in the carotid artery of aspirin-fed sheep [Koch et al., 2010]. After 6 months, all 6 of the ovine grafts were patent showing positive remodeling and no evidence of thrombus, calcification, or aneurism in the mid-section of the graft. Endothelial coverage was confirmed at 3 and 6 months. Remodeling was still incomplete after 6 months as the synthetic scaffold was still present and the collagen content (hydroxyproline) was 40% of the native artery.

Synthetic Biodegradable Scaffolds

As an alternative to permanent synthetic scaffolds, many groups have explored the use of biodegradable synthetics. Popularized by the laboratory of Robert Langer, polyglycolic acid (PGA)- and polylactic acid-based constructs have dominated the field of tissue engineering in the last 10 years. In the mid 80s, resorbable grafts were initially used without cells in the hope that they would be gradually remodeled and repopulated by the host's cells. However, balancing scaffold degradation with tissue deposition to maintain appropriate mechanical strength can be a considerable challenge affected by polymer design, indication, and interpatient variability. These cell-free approaches for arterial applications were susceptible to aneurism [Fox et al., 1999] and never reached clinical use. Still, this approach continues to be explored yielding promising preclinical data [Torikai et al., 2008; Yokota et

al., 2008; Zavan et al., 2008]. In 2001, Shin'oka et al. [2011] reported the first clinical use of a TEBV. Mindful of its mechanical limitation, they used an autologous cell-seeded biodegradable scaffold to repair a pediatric congenital heart defect in the low-pressure circulation. The grafts consisted of a porous scaffold of the copolymers L-lactide and ϵ -caprolactone reinforced with a PGA-woven fabric and seeded with autologous cells. Cells were initially from vascular tissue and cultured *in vitro* but later studies were performed with cells isolated from the bone marrow and directly seeded in the operating room [Shin'oka et al., 2005]. The resulting grafts were used for extracardiac total cavopulmonary connection or as tissue-engineered patches in 42 patients. Though these grafts lack the mechanical strength to function in a high-pressure system, the long-term efficacy, the ability of this graft to grow, and the limited rate of stenosis observed at a mean follow-up of 5.8 years confirm the resounding success of this approach [Hibino et al., 2010].

In an important evolution of this approach, Niklason et al. [1999] reported the use of a tubular synthetic biodegradable scaffold composed of PGA and seeded with bovine or porcine SMCs as a vascular graft under arterial circulation. To achieve sufficient mechanical strength, the grafts were cultured for 8 weeks under pulsatile flow conditions to allow cells to synthesize a substantial extracellular matrix (ECM). The bovine grafts had a burst pressure of $2,150 \pm 709$ mm Hg (diameter undisclosed) and suture retention strength of 91 ± 26 gf. For reference, native human saphenous veins have a burst pressure of $\approx 2,000$ mm Hg while arteries yield at over 3,000 mm Hg [Lamm et al., 2001; Konig et al., 2009]. One xenogeneic graft and one autologous graft were endothelialized with autologous endothelial cells and then implanted in the right saphenous artery of Yucatan miniature pigs for 4 weeks. The grafts remained open for the duration of the experiment, although the autologous graft had a diminished flow at explantation. Over the last decade, this group has struggled to translate these preliminary animal results to the use of human cells. Despite using retrovirally driven telomerase reverse transcriptase expression to avoid senescence, passaged human neonatal cells [McKee et al., 2003] and clinically relevant adult cells [Poh et al., 2005] produced vessels with unacceptably low mechanical properties (burst pressure 356 ± 64 mm Hg and 246 ± 56 mm Hg, respectively, at an ID of 3 mm). The use of adult human bone marrow-derived mesenchymal stem cells also resulted in vessels with burst pressures below 500 mm Hg [Gong and Niklason, 2008]. While extrapolating scientific results from animal models to hu-

mans has always been problematic in general, this series of studies exemplifies how these limitations may be particularly relevant to tissue engineering product development. When significant culture periods are needed, one can hypothesize that a seemingly minimal difference in cell behavior can be compounded over time and lead to drastically different outcomes.

In a very recent study published by the company Humacyte, Niklason and coworkers reported an exciting evolution in their TEBV model [Dahl et al., 2011]. Using cadaveric aortic SMCs, human TEBVs were produced with a 6-mm ID and a clinically relevant burst pressure ($3,337 \pm 343$ mm Hg, $n = 10$ segments). The vessels were produced by culturing the cells in a PGA scaffold for 10 weeks in a pulsating bioreactor and then decellularized. While the suture retention strength of the human grafts highlighted the donor-to-donor variability of the mechanical properties (range 80–275 g, or 3.4-fold, $n = 1-4$), pooling the cells from 2, 3, 4, or 6 cadavers significantly decreased this variability to a range that was within values for native tissue (range 140–265 g, or 1.9-fold, $n = 1-7$). The variability of burst pressure was not presented but would have likely shown similar results. These un-endothelialized human vessels were implanted in non-immunosuppressed aspirin-fed baboons as 12-cm-long arteriovenous (AV) conduits. Three out of 3 grafts were patent after 6 months. Grafts showed no changes in mechanical properties and only limited intimal hyperplasia at explantation. These results, however, may not be predictive of success as a hemodialysis graft. Indeed, the grafts were punctured only 3 times over 6 months, which is at least 30-fold less than the clinically relevant puncture frequency. This suggests that eventual clinical trials should plan for a very conservative enrolment rate in order to evaluate the risk of rupture due to the stress of repeated puncture (2 needles, 3 times per week). In our own studies, we staggered patients by more than 3 months, taking nearly 5 years to enroll our first 14 patients.

The xenogeneic grafts did not elicit calcification, giant cell formation, or immunological sensitization of the animals. The latter may be the most significant result from this study and underlines the fundamental differences between the immunological potential of allogeneic tissue-engineered products and allogeneic cadaveric tissues or isolated/processed ECM. Humacyte postulated that this improved TEBV model might be suitable for widespread clinical use given the ‘off-the-shelf’ availability implied by the allogeneic approach and the positive patency and shelf life data [after 12 months at 4°C in PBS, burst pressure and compliance decreased by only 20%

which did not reach statistical significance ($n = 5$ segments)].

The use of low passage ($p = 2$) aortic SMCs entails that only a small cell batch can be produced from each cadaver. The authors estimate that 37 vessels can be produced from a full-length cadaveric aorta but, in a commercial setting, the effective yield would be significantly decreased by cell loss associated with the banking process (freeze/thaw), cell testing (viability, identity, sterility, mycoplasma, endotoxin, karyology, tumorigenicity, and for a series of viruses), and final product testing (biomechanical testing and, at least, sterility, mycoplasma, and endotoxin). The clinically relevant vessel length (greater than 30 cm) would similarly reduce the number of vessels that could be produced from a single cadaver. This low amplification capability will create challenges that are not faced by typical allogeneic approaches (e.g. organogenesis, Osiris) where a single biopsy can produce a large well-characterized master cell bank. In these classic allogeneic models, the cost of the starting material, which is often negligible, and the cost of extensive characterization testing of the allogeneic cell line are distributed over tens and even hundreds of thousands of ‘units’.

Finally, while pooling cells from multiple cadavers can mitigate the large batch-to-batch variability, this raises significant regulatory issues. Indeed, in its latest relevant guidance document, the Food and Drug Administration (FDA) clearly states that Good Tissue Practices prohibit pooling of human cells or tissues during manufacturing in accordance to the Code of Federal Regulations title 21, part 1271, section 220(b) [FDA-CBER, 2009]. While provisions exist to negotiate an exception with the FDA when necessary to obtain ‘a therapeutic dose’ (CRF21.1271.155), if granted, this will likely be associated with additional quality control requirements to compensate for the increased risk of disease transmission and likely require some testing of each individual cell line before pooling. Niklason has also reported in vitro results with human bone marrow-derived cells [Gong and Niklason, 2011]. This approach would eliminate the manufacturing and regulatory challenges associated with cadaveric SMCs. Indeed, this approach could be an interesting variation of Shin’oka’s landmark clinical approach and bring a cell-seeded PGA scaffold to high-pressure arterial bypass indications.

Synthetic biodegradable scaffolds can also be produced from other polymers. Vorp and colleagues have recently shown impressive results with a bilayered, biodegradable graft composed of poly(ester-urethane) urea [Nieponice et al., 2008, 2010]. Using a rotational vacuum

seeding device, rat muscle-derived stem cells (MDSCs) were bulk seeded into the graft wall without compromising the integrity of the cells. The inner layer of the construct was created using thermally induced phase separation, producing a substrate suitable for cellular growth, and the outer layer of the graft was electrospun to provide the graft with the requisite mechanical strength to withstand physiological conditions. The grafts had an estimated burst pressure just over 2,000 mm Hg although reaching clinically relevant mechanical strength is typically not a challenge for electrospun grafts. After 8 weeks as aortic interposition grafts, vessels were significantly remodeled but the cell-seeded constructs had a considerably higher patency than the acellular control (65 vs. 10%, respectively). A similar rodent model was developed by Li and coworkers [Hashi et al., 2007]. In a recent report, Vorp's group translated this technology to human cells [He et al., 2010]. Pericyte-seeded constructs implanted as aortic grafts in nude rats showed positive remodeling and 100% patency at 8 weeks while cell-free grafts were poorly remodeled and thrombosed. Despite an estimated burst pressure of $\approx 4,500$ mm Hg, the authors noticed that the graft showed a very significant dilatation unlike that in the rat MDSC model.

The in vivo Bioreactor Approach

The original promise of tissue engineering was to create living biological autologous tissues to avoid the introduction of a foreign body and the ensuing scarring and encapsulation. Schillings [1961, 1964], and later Sparks [1969, 1973], pioneered a very innovative surgical method that takes advantage of this inflammatory reaction to create autologous living vascular grafts. In this approach, often called the Sparks Mandril, a piece of synthetic tubing is implanted in the patient (under the skin) to create a tubular capsule in a few weeks. This tissue casing can then be removed from the mandril, resulting in an autologous conduit populated by myofibroblasts. Clinical use revealed that these grafts had a propensity for early thrombosis and late aneurisms [Guidoin et al., 1984]. In the late 90s, Campbell et al. [1999] reinvigorated this method by publishing results from 4-month autologous arterial implants in rats (65% patency with 3-mm ID grafts) and in rabbits (70% patency with 5-mm ID grafts). Their approach used the peritoneal cavity as the 'in vivo bioreactor' and everted the construct before implantation to use the outer mesothelial cell monolayer as an endothelium surrogate. Unfortunately, this method produced

a good graft on only 50% of the implanted mandrils on average and, despite the introduction of 4 mandrils per animal, sometimes did not produce any viable implants. In a dog study, various mandril materials were tested to improve tissue formation [Chue et al., 2004]. While adding a sheet or mesh over the mandril drove tissue formation in 100% of cases, the resulting grafts had poor surgical outcomes (60% patency at 6.5 months). The best uncoated mandril material was polyethylene tubing, which still produced graftable vessels in only 50% of cases. These completely biological vessels had an impressive burst pressure (above 2,500 mm Hg at 4.8-mm ID) and a suture retention strength that exceeded that of native arteries. Interpositional implants in the femoral artery displayed a patency rate of 83% at 6.5 months and impressive remodeling of the cell population towards vessel-like phenotypes (vessels were not everted). Nakayama and colleagues are pursuing a similar approach, using subcutaneous implants, in rats and rabbits, with promising results for up to 3 months with a graft called a 'biotube' [Nakayama et al., 2004; Watanabe et al., 2010; Ma et al., 2011].

In their most recent publication, Campbell's group reported the use of a device specifically designed to produce tissues that would not adhere to the peritoneal wall, a common cause of failure to produce a good graft [Stickler et al., 2010]. This device is anchored subcutaneously and also has a pulsatile core that is pressured by a transcutaneous catheter. In the sheep, 100% of implants produced vessels but pulsation was required to achieve significant mechanical strength. The circumferential ultimate tensile stress of the vessels, which is proportional to the burst pressure, was 63-fold weaker than that of native arteries and varied by 36-fold (0.02–0.72 MPa, average 0.07 MPa). Not surprisingly, carotid artery interpositional grafts ruptured upon implantation and carotid patches developed aneurism by 2 weeks. The important variability of tissue formation and of the quality of the resulting grafts highlights the manufacturing and regulatory challenges associated with this approach. It is also unclear how such an approach would be regulated.

Tissue Engineering by Self-Assembly

Inspired by the clinical success of epithelial cell sheets in the treatment of burn patients, we imagined that a similar process could be developed for mesenchymal cells. We used the well-known effect of ascorbate supplementation to drive cells to produce large amounts of ECM. In a

Fig. 1. a The workhorse of TESA: a sheet of HSFs. The sheets can become very robust depending on the culture time and conditions. **b** Sheet holding 500 g.

Color version available online

little more than a month, a 'tissue sheet' composed of live cells embedded in a complex, natural ECM could be lifted from the culture substrate (fig. 1a). These sheets lacked the potentially detrimental effects of synthetic scaffolds, while they displayed the advantageous mechanical properties of naturally constructed collagen. Unlike fragile sheets produced by epithelial cells, mesenchymal cells produced robust tissues (fig. 1b) that would later prove to be the essential building blocks of the tissue engineering by self-assembly (TESA) principle. TESA can generate completely biological constructs by using the tissue that is naturally assembled by mesenchymal cells. As is often the case in the discovery process, this 'self-assembly' phenomenon had been well described in many academic laboratories [de Clerck and Jones, 1980; Murad et al., 1983; Grinnell et al., 1989], but its potential for clinical tissue engineering had yet to be realized in the mid-90s.

Development of a Small-Diameter Blood Vessel Using TESA

Sheet-based tissue engineering (SBTE) was the first platform developed from the TESA principle. In SBTE, self-assembled sheets are rolled into the many distinct layers that compose a natural blood vessel. This method was used to construct the first tissue-engineered human blood vessel that displayed physiological mechanical properties without the need for an exogenous scaffold [L'Heureux et al., 1998]. The blood vessel was composed of three distinct biological layers: a functional endothelium seeded onto an 'internal membrane' (IM), a 'media' made of SMCs, and an 'adventitia' made of living HSFs. To produce the graft, the IM was created by rolling an HSF sheet around an inert cylindrical support and culturing it until the individual revolutions fused together, forming a homogenous tissue. The IM was then devitalized by dehydration. The IM plays a vital role in the graft

by mimicking the internal elastic lamina (IEL) of a native vessel. Though lacking the elastin of the IEL, the IM was designed to be a barrier to prevent the migration of SMCs into the lumen of the graft that leads to intimal hyperplasia [Hsiang et al., 1990; Chapman et al., 2009]. In addition, it provides a cell-free surface for endothelial cell seeding and proliferation. After IM devitalization, a sheet composed of SMCs was rolled around the IM, forming the medial layer. After a week of maturation, a sheet of HSFs was rolled around the SMCs to produce an adventitia layer. The layers were then allowed to fuse for another 8 weeks. After this maturation period, the support mandrel was removed and the lumen was lined with endothelial cells.

In vitro analysis of the vessel confirmed structural similarity to native tissue, with the presence of numerous ECM proteins (collagen types I, III, and IV, laminin, fibronectin, and chondroitin sulfates) and a functional endothelium [L'Heureux et al., 1998]. More importantly, the vessel exhibited burst strengths that were significantly higher than that of human saphenous vein, a vessel commonly used for coronary bypass ($2,594 \pm 501$ mm Hg and $1,680 \pm 307$ mm Hg, respectively). The medial layer of SMCs did not form a mechanically strong layer but, on the other hand, it was shown to have the expected contractile properties [Stoclet et al., 1996; L'Heureux et al., 2001]. The unendothelialized human TEBV was evaluated in vivo as an interpositional arterial femoral graft in a canine model to assess handling characteristics and its ability to withstand physiological pulsatile pressure and flow. This test confirmed that the grafts handled well and were mechanically stable for 7 days. This study proved the feasibility of using cell-generated matrix and finally challenged the dogmatic view that an exogenous scaffold is a prerequisite to build a successful tissue-engineered graft.

Fig. 2. **a** An age- and risk-matched human TEBV for implantation in the aorta of nude rats (ID 1.5 mm). **b** After 180 days in a nude rat, Verhoff-Masson staining reveals a collagen-rich (blue) and acellular IM as well as well-developed elastic lamellae in the 'neo-media' on the luminal side of the IM. The arrow indicates vasa vasorum in the 'adventitia'. Scale bar = 20 μ m. **c** SMC-specific α -actin staining of the vessel after 90 days confirms that the cells in the 'neo-media' are SMC-like cells. The arrow indicates cells

around the IM that are positive, suggesting a phenotypic transformation of the implanted fibroblasts or a recruitment of cells from the surrounding tissue. Comparison of the thickness of the 'neo-media' in **b** and **c** shows that it does not thicken with time. Scale bar = 100 μ m. **d** At the anastomotic region at 90 days, Movat staining shows a smooth interface (large arrow) and the difference in elastin (black) between the native aorta and the remodeling graft. Small arrows indicate sutures. Scale bar = 500 μ m.

Preclinical Studies with the Lifeline™ GRAFT

Although the first sheet-based graft represented an important scientific milestone, much work was to be done to transform TESA into a clinical reality. Cytograft Tissue Engineering, Inc., was founded in 2000 with the purpose of rapidly driving this new technology to the clinic. An important part of this translational effort was to progressively simplify the complex in vitro model without compromising its many biological advantages.

As a first step, the medial layer of SMCs was eliminated. In the native vasculature, the SMC layer provides contractility. Contractility plays an important role in very small vessels (ID <1 mm) to control the systemic blood pressure, but it has an inconsequential effect on graft patency. Additionally, by minimizing the number of cell types in the graft, cytografts could eliminate the need for multiple culture conditions and additional quality control measures. Further, removing the medial layer removes the concern that the hyperproliferative serum-fed

SMCs would increase the risk of intimal hyperplasia despite the inclusion of the IM [Pauly et al., 1994].

As discussed earlier, the successful transition from animal to human cells poses a daunting challenge. To eliminate this difficult transition, we have always used human cells throughout our initial research. However, multiple studies have clearly demonstrated that donor age and health status can dramatically affect cell behavior [Phillips et al., 1994; Becerra et al., 1996; Michel et al., 1996; Mogford et al., 2002; Heeschen et al., 2004; Zhang et al., 2005; Garolla et al., 2009; Krenning et al., 2009; Westerweel and Verhaar, 2009]. Thus, the second translational step was to use age- and risk-matched human cells. These cells were sourced from elderly patients who suffered from cardiovascular disease, i.e. our target population. To overcome the challenges of our difficult cell source, we increased culture time and optimized culture conditions. This led to a TEBV with mechanical characteristics in line with those previously achieved using young healthy donor cells [L'Heureux et al., 2006].

In 2006, we successfully validated our simplified Lifeline graft using a series of animal studies performed with age- and risk-matched human TEBVs [L'Heureux et al., 2006]. These grafts were constructed using cells taken from patients aged 57–79 years who had undergone vascular bypass surgery. Initial mechanical stability was confirmed by a short-term canine study. Next, a long-term study was performed in nude rats using 1.5-mm ID human grafts lined with syngeneic endothelium (fig. 2a). These grafts had an average burst pressure of $3,688 \pm 1,865$ mm Hg ($n = 9$). The overall patency during the 225-day study was 85%. Macroscopically, long-term implants were free of thrombus (both on the lumen and in the wall) and were well embedded in the surrounding tissue.

Histological evaluation confirmed complete tissue integration of the graft and showed an acellular IM after 6 months (fig. 2b). This suggests that the IM effectively inhibited cell migration towards the lumen. The sustained integrity of the IM also suggests that the cell-synthesized collagen of the TESA principle is not recognized as damaged or foreign by the body, unlike structures composed of solubilized and reassembled collagen, which typically degrade within a few weeks [Morimoto et al., 2005]. The *in vivo* stability of ECM grafts created using the TESA principle is critically important for clinical application and one of the method's key advantages over synthetic or modified biomaterial approaches. We observed the formation of an SMC-specific α -SMC-actin-positive layer and a confluent endothelium on the luminal side of the IM by 90 days (fig. 2c). While these luminal cells were probably the result of cell migration from the anastomotic regions (fig. 2d), we also observed a thin layer of SMC-like cells on the outside of the IM suggesting that implanted fibroblasts or cells from the implantation bed were developing vascular phenotypes (fig. 2c). Based on Movat staining, this 'neomedia' contained well-defined thin elastic lamellas and abundant glycosaminoglycans. At 180 days, the medial layer thickness remained unchanged, the elastic lamellas appeared thicker, and the glycosaminoglycans were largely absent. These changes indicate a mature and balanced remodeling process which may be linked to the stable and noninflammatory nature of the IM [Wight, 1996].

Finally, a primate study to assess the performance of grafts with a more clinically relevant ID (4.2 mm) and biomechanical environment was performed. Three immunosuppressed cynomolgus macaques received unendothelialized human TEBVs made from age- and risk-matched cells (average burst pressure $3,468 \pm 500$ mm Hg, $n = 5$) as interpositional arterial grafts. Though there

was an expected, moderate immune response to the xenogeneic grafts, when the vessels were explanted between 6 and 8 weeks, all grafts were patent, free of aneurysm or narrowing of the lumen, and re-endothelialization of the IM was observed. Histological analysis of the explanted primate grafts revealed less pronounced remodeling compared to that observed in the rat model; this was possibly a result of the shorter implant time, aggressive immunosuppression, or intrinsic differences in the animal models.

Reaching the Clinic

In 2007, encouraging preliminary results from human trials of the Lifeline graft were reported for the first 6 patients [L'Heureux et al., 2007]. This initial clinical trial focused on the use of the graft as AV shunts for hemodialysis access. Participants were end-stage renal disease (ESRD) patients ranging in age from 29 to 89 years. During hemodialysis, a patient's blood must be filtered through an external dialyzer 3 times weekly. Each dialysis session requires the patient to be punctured by two large-diameter needles, one to and one from the dialyzer (fig. 3). To accelerate blood filtration, a vein is connected directly to an artery (AV fistula), which creates supra-physiological blood flow (≥ 1 liter/min) in the vein. The repetitive trauma of needle puncture accompanied by nonphysiological flow dynamics inevitably leads to vein obstruction. Once all native veins have been exhausted, an AV shunt is made using an ePTFE graft. Using ePTFE carries with it increased risks of thrombosis, infection, and rapid stenosis due to intimal hyperplasia. The patients enrolled into this study were in later stages of ESRD and had a previous access failure.

This model was chosen because frequent hemodialysis visits allow for close surveillance of the implant. In addition, graft failure in this patient population is not usually life or limb threatening. However, this model is associated with significant challenges. Dialysis requires supra-physiological flow rates and repeated puncture, each of which puts considerable mechanical stress on the graft. Extensive biomechanical testing suggested that the Lifeline graft had mechanical properties that could cope with this difficult environment. With an average burst pressure of $3,490 \pm 892$ mm Hg ($n = 230$ segments, 25 patients), the graft exceeded the average rupture strength of human saphenous vein ($1,599 \pm 877$ mm Hg, $n = 7$) and matched that of the human internal mammary artery ($3,196 \pm 1,264$ mm Hg, $n = 16$) [Konig et al., 2009]. Dynamic, static, and stepwise fatigue *in vitro* testing were used to confirm the vessel's

Fig. 3. Before beginning the clinical trials, we tested the resistance of the human grafts punctured in vitro under physiological pressure (**a–c**) versus an ePTFE graft (**d–f**). While both grafts seal well once the needle is inserted (**a, d**), the ePTFE graft leaks aggressively after removal of the 16-gauge hemodialysis needle while our graft self-seals like a native vessel. Note the poor compliance of the ePTFE around the needle in **d**. Leaking grafts are clinically difficult to manage because they require pressure to stop the bleeding, which can also cause graft thrombosis.

mechanical stability prior to implantation. It is also noteworthy that production of the graft has proven to be a reproducible process across age, gender, health status, and time. Comparison of the average burst strengths of the first 6 patients with the cumulative average of 25 patients verifies the reproducibility of the graft ($3,238 \pm 366$ mm Hg, $n = 6$, vs. $3,561 \pm 435$ mm Hg, $n = 25$ segments).

In 2009, expanded findings from the study were published [Garrido et al., 2009; McAllister et al., 2009]. Of the 10 patients enrolled, 9 were had successful implantations. One patient withdrew prior to implantation because of poor health. Another patient died of unrelated causes 39 days postimplantation, but the implanted graft was functional at the time of death. Another patient experienced an acute immune response leading to aneurysmal failure of the graft at the 3-day time point. This immune response was likely the result of high levels of immuno-

globulin G (IgG) present in a new batch bovine serum used during culture of this set of grafts. The validity of this hypothesis has been reinforced by the recent success of an implanted vessel which had been cultured with IgG-depleted serum. Two other grafts failed within the first 90 days (thrombus and aneurism). The remaining grafts were patent with only one intervention required to maintain patency with time points to 20 months. At this time, with a total of 13 grafts, we have observed 4 failures within the first 90 days. This is on the low end of the expected ‘failure-to-mature’ rates for AV fistulas [Gibson et al., 2001; Dember et al., 2008]. The remaining 8 grafts required only 3 graft-related interventions to maintain patency in 124 patient-months of use. This event rate of 0.7 events/patient-year is between what is seen with synthetic grafts and native fistulas in the general ESRD patient population [Perera et al., 2004]. However, it has been re-

Fig. 4. Evolution of our tissue-engineered graft. We have followed a strategy that initially favors a positive clinical outcome over ease of production. Only when some level of clinical success is demonstrated do we introduce cost-effective design changes. If early re-

sults are confirmed in follow-up studies, we could produce an allogeneic, serum-free, off-the-shelf, and completely biological graft in about 10 weeks by using TBTE.

ported that late-stage patients have about a 3-fold increased event rate [Rosas et al., 2003]. Indeed, our own population experienced an event rate of nearly 3 events per year in the 6 months immediately prior to implantation. While still a small study, the 4.2-fold decrease in event rate observed with the use of the Lifeline graft bodes well for the future.

The Next Generation

Having established the clinical feasibility of a scaffold-free tissue-engineered vascular graft, we believe it is an appropriate time to study further rationalization of the manufacturing process in order to reduce the cost and complexity of this process (fig. 4). For example, an endothelium may not be necessary for the AV shunt application considering the ultrahigh flow. Removing the endothelium from the process would significantly decrease

the cost and complexity by eliminating the need for a blood vessel biopsy, specific media additives, endothelial-specific identity/purity testing, endothelialization-specific bioreactors, and coordination of the endothelialization with the surgical date. Similarly, living fibroblast may not be needed if the remodeling process relies on recruitment of local peripheral or circulating cells. Clearly, they are not needed to maintain the mechanical strength of the graft. Eliminating the need to keep the tissue alive allows for ‘unlimited’ storage time, simpler shipping conditions, storage at the surgical site, and elimination of a quality control viability test. We recently published the human implant of a devitalized frozen graft [Wystrychowski et al., 2011]. Finally, and most importantly, allogeneic fibroblasts could likely be successfully used considering that they have not caused immune responses in thousands of patients that have received commercially sold allogeneic tissue-engineered dermal constructs (Ap-ligraf® or Dermagraft®). The use of a bank of allogeneic

fibroblasts would significantly reduce production costs by eliminating the need for skin biopsies for each patient, avoiding a culture system segregating cells of different patients, and it would reduce quality control costs by improving reproducibility and allowing for large batches to be produced and validated.

An allogeneic, nonliving, nonendothelialized process would not only be less complex or costly but it would effectively transform Lifeline into an off-the-shelf product, which would make it available for patient populations and applications requiring urgent treatment. This strategy is also pursued by Niklason [Dahl et al., 2011] but it should be noted that our approach has the added advantage of being able to easily produce tens of thousands of grafts from a single skin biopsy. We recently implanted a devitalized/allogeneic Lifeline graft in a hemodialysis patient. The graft is currently used for hemodialysis access and functions well 5 months postoperatively.

Another improvement in development is the adoption of a serum-free culture medium. The Lifeline graft, like practically all tissue-engineered products, is currently produced in medium containing fetal bovine serum (FBS), a relatively undefined and highly variable additive. However, FBS has been linked to rare acute immune responses and poses a risk for the transmission of diseases (most notably the prion that causes bovine spongiform encephalopathy) [Selvaggi et al., 1997; Tuschong et al., 2002; Moques et al., 2005]. As a result, most regulatory agencies have enacted restrictions on the use of FBS, which complicates international clinical trials and market authorizations [Asher, 1999a, b; von Rheinbaben and Schmidt, 2004]. From a manufacturing point of view, the well-known lot-to-lot variability of FBS requires thorough, costly, and lengthy validation testing of new batches of FBS. Animal-free alternatives have emerged for the culture of continuous (transformed) cell lines but few products exist for normal adult human cells and they typically provide suboptimal results. Through years of collaborative research efforts, we have developed a defined medium that supports the production of strong sheets [unpubl. data].

Finally, we have also explored more radical modifications of the manufacturing process to shorten the production time. We have focused our efforts on eliminating the 'maturation' phase, i.e. the culture period during which the layers of the rolled sheet fuse together, since maturation of the IM and the adventitia accounts for a majority of the overall time and cost of production. These efforts led to the development of a new approach termed thread-based tissue engineering (TBTE). Using the TESA principle, we can grow cell-synthesized threads of vary-

ing lengths and diameters. Taking advantage of medical textile technologies, the threads can be assembled into complex 3-dimensional structures by methods like weaving, braiding, or knitting (fig.4). Because the maturation phase has been eliminated, a vascular graft woven from biological threads can be produced in about 8 weeks without the need for a custom bioreactor. These grafts show burst pressures and suture retention strengths that far exceed those of SBTE grafts and are in preclinical trials [unpubl. data].

Conclusion

In 1996, when we presented our first results in a poster at a Keystone Symposium on Tissue Engineering and Wound Healing, the idea of a strong but scaffold-free graft was so inconceivable that the question we were most often asked was: 'What scaffold are you using?' [L'Heureux et al., 1996]. Over the last decade, we have witnessed a clear paradigm shift from purely synthetic approaches, primarily preoccupied by the simplicity of the manufacturing process, to biological approaches that prioritize a positive outcome. Today, we are joined by multiple teams that are also pursuing approaches that require long, and costly, culture times to allow cells to produce a robust natural ECM. Undeniably, economical feasibility is a key factor in the clinical availability of a technology but, ultimately, a technology that outperforms the standard of care will evolve to become more affordable with time (e.g. MRI) whereas an inexpensive technology that marginally works will be abandoned.

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References

- Asher, D.M. (1999a) Bovine sera used in the manufacture of biologicals: current concerns and policies of the U.S. Food and Drug Administration regarding the transmissible spongiform encephalopathies. *Dev Biol Stand* 99: 41–44.
- Asher, D.M. (1999b) The transmissible spongiform encephalopathy agents: concerns and responses of United States regulatory agencies in maintaining the safety of biologics. *Dev Biol Stand* 100: 103–118.

- Barocas, V.H., T.S. Girton, R.T. Tranquillo (1998) Engineered alignment in media equivalents: magnetic prealignment and mandrel compaction. *J Biomech Eng* 120: 660–666.
- Becerra, J., J.A. Andrades, D.C. Ertl, N. Sorgente, M.E. Nimni (1996) Demineralized bone matrix mediates differentiation of bone marrow stromal cells in vitro: effect of age of cell donor. *J Bone Miner Res* 11: 1703–1714.
- Berglund, J.D., M.M. Mohseni, R.M. Nerem, A. Sambanis (2003) A biological hybrid model for collagen-based tissue engineered vascular constructs. *Biomaterials* 24: 1241–1254.
- Campbell, J.H., J.L. Efendy, G.R. Campbell (1999) Novel vascular graft grown within recipient's own peritoneal cavity. *Circ Res* 85: 1173–1178.
- Chapman, S.L., F.X. Sicut, E.C. Davis, J. Huang, T. Sasaki, M.L. Chu, H. Yanagisawa (2009) Fibulin-2 and fibulin-5 cooperatively function to form the internal elastic lamina and protect from vascular injury. *Arterioscler Thromb Vasc Biol* 30: 68–74.
- Chue, W.L., G.R. Campbell, N. Caplice, A. Muhammed, C.L. Berry, A.C. Thomas, M.B. Bennett, J.H. Campbell (2004) Dog peritoneal and pleural cavities as bioreactors to grow autologous vascular grafts. *J Vasc Surg* 39: 859–867.
- Dahl, S.L., A.P. Kypson, J.H. Lawson, J.L. Blum, J.T. Strader, Y. Li, R.J. Manson, W.E. Tente, L. Dibernardo, M.T. Hensley, R. Carter, T.P. Williams, H.L. Prichard, M.S. Dey, K.G. Begelman, L.E. Niklason (2011) Readily available tissue-engineered vascular grafts. *Sci Transl Med* 3: 68ra69.
- de Clerck, Y.A., P.A. Jones (1980) The effect of ascorbic acid on the nature and production of collagen and elastin by rat smooth-muscle cells. *Biochem J* 186: 217–225.
- Dember, L.M., G.J. Beck, M. Allon, J.A. Delmez, B.S. Dixon, A. Greenberg, J. Himmelfarb, M.A. Vazquez, J.J. Gassman, T. Greene, M.K. Radeva, G.L. Braden, T.A. Ikizler, M.V. Rocco, I.J. Davidson, J.S. Kaufman, C.M. Meyers, J.W. Kusek, H.I. Feldman (2008) Effect of clopidogrel on early failure of arteriovenous fistulas for hemodialysis: a randomized controlled trial. *JAMA* 299: 2164–2171.
- Deutsch, M., J. Meinhardt, P. Zilla, N. Howanietz, M. Gorlitzer, A. Froeschl, A. Stuempflen, D. Bezuidenhout, M. Grabenwoeger (2009) Long-term experience in autologous in vitro endothelialization of infrainguinal ePTFE grafts. *J Vasc Surg* 49: 352–362, discussion 362.
- FDA-CBER (2009) Guidance for industry: current good tissue practice (CGTP) and additional requirements for manufacturers of human cells, tissues, and cellular and tissue-based products (HCT/PS) (draft). Rockville, FDA-CBER, p 59.
- Fox, D., D.A. Vorp, H.P. Greisler (1999) Biore-sorbable grafts: a counterintuitive approach; in Zilla, P., H.P. Greisler (eds): *Tissue Engineering of Vascular Prosthetic Grafts*. Austin, R.G. Landes, pp 489–503.
- Garolla, A., R. D'Inca, D. Checchin, A. Biagioli, L. De Toni, V. Nicoletti, M. Scarpa, E. Bolzonello, G.C. Sturniolo, C. Foresta (2009) Reduced endothelial progenitor cell number and function in inflammatory bowel disease: a possible link to the pathogenesis. *Am J Gastroenterol* 104: 2500–2507.
- Garrido, S., T. McAllister, N. Dusserre, A. Marini, N. L'Heureux (2009) Haemodialysis access via tissue-engineered vascular graft. *Lancet* 374: 201.
- Gibson, K.D., D.L. Gillen, M.T. Caps, T.R. Kohler, D.J. Sherrard, C.O. Stehman-Breen (2001) Vascular access survival and incidence of revisions: a comparison of prosthetic grafts, simple autogenous fistulas, and venous transposition fistulas from the United States Renal Data System Dialysis Morbidity and Mortality Study. *J Vasc Surg* 34: 694–700.
- Girton, T.S., T.R. Oegema, E.D. Grassl, B.C. Isenberg, R.T. Tranquillo (2000) Mechanisms of stiffening and strengthening in media-equivalents fabricated using glycation. *J Biomech Eng* 122: 216–223.
- Gong, Z., L.E. Niklason (2008) Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs). *FASEB J* 22: 1635–1648.
- Gong, Z., L.E. Niklason (2011) Use of human mesenchymal stem cells as alternative source of smooth muscle cells in vessel engineering. *Methods Mol Biol* 698: 279–294.
- Grassl, E.D., T.R. Oegema, R.T. Tranquillo (2003) A fibrin-based arterial media equivalent. *J Biomed Mater Res A* 66: 550–561.
- Grinnell, F., H. Fukamizu, P. Pawelek, S. Nakagawa (1989) Collagen processing, cross-linking, and fibril bundle assembly in matrix produced by fibroblasts in long-term cultures supplemented with ascorbic acid. *Exp Cell Res* 181: 483–491.
- Guidoin, R., A. Thevenet, H.P. Noel, H. Mary, M. Marois, C. Gosselin, M. King (1984) The Sparks-Mandril arterial prosthesis: an ingenious concept, a total failure. What can we learn from it? *J Mal Vasc* 9: 277–283.
- Haisch, A., A. Loch, J. David, A. Pruss, R. Hansen, M. Sittlinger (2000) Preparation of a pure autologous biodegradable fibrin matrix for tissue engineering. *Med Biol Eng Comput* 38: 686–689.
- Hashi, C.K., Y. Zhu, G.Y. Yang, W.L. Young, B.S. Hsiao, K. Wang, B. Chu, S. Li (2007) Antithrombotic property of bone marrow mesenchymal stem cells in nanofibrous vascular grafts. *Proc Natl Acad Sci USA* 104: 11915–11920.
- He, W., A. Nieponice, L. Soletti, Y. Hong, B. Gharaibeh, M. Crisan, A. Usas, B. Peault, J. Huard, W.R. Wagner, D.A. Vorp (2010) Pericyte-based human tissue engineered vascular grafts. *Biomaterials* 31: 8235–8244.
- Heeschen, C., R. Lehmann, J. Honold, B. Assmus, A. Aicher, D.H. Walter, H. Martin, A.M. Zeiher, S. Dimmeler (2004) Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. *Circulation* 109: 1615–1622.
- Herring, M., A. Gardner, J. Glover (1978) A single-staged technique for seeding vascular grafts with autogenous endothelium. *Surgery* 84: 498–504.
- Herring, M., A. Gardner, J. Glover (1984) Seeding human arterial prostheses with mechanically derived endothelium: the detrimental effect of smoking. *J Vasc Surg* 1: 279–289.
- Hibino, N., E. McGillicuddy, G. Matsumura, Y. Ichihara, Y. Naito, C. Breuer, T. Shinoka (2010) Late-term results of tissue-engineered vascular grafts in humans. *J Thorac Cardiovasc Surg* 139: 431–436, 436.e1–2.
- Hirai, J., K. Kanda, T. Oka, T. Matsuda (1994) Highly oriented, tubular hybrid vascular tissue for a low pressure circulatory system. *ASAIO J* 40: M383–388.
- Hirai, J., T. Matsuda (1996) Venous reconstruction using hybrid vascular tissue composed of vascular cells and collagen – tissue regeneration process. *Cell Transplant* 5: 93–105.
- Hsiang, Y., R.A. White, G.E. Kopchok, C.B. Guthrie, S.K. Peng (1990) The role of internal elastic laminae damage in the development of canine arteriosclerosis. *J Invest Surg* 3: 11–21.
- Isenberg, B.C., C. Williams, R.T. Tranquillo (2006) Small-diameter artificial arteries engineered in vitro. *Circ Res* 98: 25–35.
- Kadletz, M., R. Moser, P. Preiss, M. Deutsch, P. Zilla, R. Fasol (1987) In vitro lining of fibronectin coated PTFE grafts with cryopreserved saphenous vein endothelial cells. *Thorac Cardiovasc Surg* 35 Spec No 2: 143–147.
- Kanda, K., T. Matsuda, T. Oka (1993) In vitro reconstruction of hybrid vascular tissue: hierarchical and oriented cell layers. *ASAIO J* 39: M561–565.
- Koch, S., T.C. Flanagan, J.S. Sachweh, F. Tanios, H. Schnoering, T. Deichmann, V. Ella, M. Kellomaki, N. Gronloh, T. Gries, R. Tolba, T. Schmitz-Rode, S. Jockenhoevel (2010) Fibrin-poly(lactide)-based tissue-engineered vascular graft in the arterial circulation. *Biomaterials* 31: 4731–4739.
- Konig, G., T.N. McAllister, N. Dusserre, S.A. Garrido, C. Iyican, A. Marini, A. Fiorillo, H. Avila, W. Wystrychowski, K. Zagalski, M. Maruszewski, A.L. Jones, L. Cierpka, L.M. de la Fuente, N. L'Heureux (2009) Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery. *Biomaterials* 30: 1542–1550.
- Krenning, G., P.Y. Dankers, J.W. Drouven, F. Waanders, C.F. Franssen, M.J. van Luyn, M.C. Harmsen, E.R. Poca (2009) Endothelial progenitor cell dysfunction in patients with progressive chronic kidney disease. *Am J Physiol Renal Physiol* 296: F1314–F1322.

- Lamm, P., G. Juchem, S. Milz, M. Schuffenhauer, B. Reichart (2001) Autologous endothelialized vein allograft: a solution in the search for small-caliber grafts in coronary artery bypass graft operations. *Circulation* 104(suppl 1): I108–I114.
- L'Heureux, N., N. Dusserre, G. Konig, B. Victor, P. Keire, T.N. Wight, N.A. Chronos, A.E. Kyles, C.R. Gregory, G. Hoyt, R.C. Robbins, T.N. McAllister (2006) Human tissue-engineered blood vessels for adult arterial revascularization. *Nat Med* 12: 361–365.
- L'Heureux, N., L. Germain, R. Labbe, F.A. Auger (1993) In vitro construction of a human blood vessel from cultured vascular cells: a morphologic study. *J Vasc Surg* 17: 499–509.
- L'Heureux, N., L. Germain, R. Labbé, F.A. Auger (1996) A graftable blood vessel produced exclusively from human cells (poster). Keystone Conf on Tissue Eng and Wound Heal, Taos.
- L'Heureux, N., T.N. McAllister, L.M. de la Fuente (2007) Tissue-engineered blood vessel for adult arterial revascularization. *N Engl J Med* 357: 1451–1453.
- L'Heureux, N., S. Paquet, R. Labbe, L. Germain, F.A. Auger (1998) A completely biological tissue-engineered human blood vessel. *FASEB J* 12: 47–56.
- L'Heureux, N., J.C. Stoclet, F.A. Auger, G.J. Lagaud, L. Germain, R. Andriantsitohaina (2001) A human tissue-engineered vascular media: a new model for pharmacological studies of contractile responses. *FASEB J* 15: 515–524.
- Lloyd-Jones, D., R.J. Adams, T.M. Brown, M. Carnethon, S. Dai, G. De Simone, T.B. Ferguson, E. Ford, K. Furie, C. Gillespie, A. Go, K. Greenlund, N. Haase, S. Hailpern, P.M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lackland, L. Lisabeth, A. Marelli, M.M. McDermott, J. Meigs, D. Mozaffarian, M. Musolino, G. Nichol, V.L. Roger, W. Rosamond, R. Sacco, P. Sorlie, T. Thom, S. Wasserthiel-Smoller, N.D. Wong, J. Wylie-Rosett (2010) Heart disease and stroke statistics – 2010 update: a report from the American Heart Association. *Circulation* 121: e46–e215.
- Ma, N., Z. Wang, H. Chen, Y. Sun, H. Hong, Q. Sun, M. Yin, J. Liu (2011) Development of the novel biotube inserting technique for acceleration of thick-walled autologous tissue-engineered vascular grafts fabrication. *J Mater Sci Mater Med* 22: 1037–1043.
- Maciag, T., G. Hoover, M. Stemberman, R. Weinstein (1981) Serial propagation of human endothelial cells in vitro. *J Cell Biol* 91: 420–426.
- Mansfield, P.B., A.R. Wechezak, L.R. Sauvage (1975) Preventing thrombus on artificial vascular surfaces: true endothelial cell linings. *Trans Am Soc Artif Intern Organs* 21: 264–272.
- Matsuda, T., T. Kitamura, H. Iwata, H. Takano, T. Akutsu (1988) A hybrid artificial vascular graft based upon an organ reconstruction model: significance and design criteria of an artificial basement membrane. *ASAIO Trans* 34: 640–643.
- Matsuda, T., H. Miwa (1995) A hybrid vascular model biomimicking the hierarchic structure of arterial wall: neointimal stability and neoarterial regeneration process under arterial circulation. *J Thorac Cardiovasc Surg* 110: 988–997.
- McAllister, T.N., M. Maruszewski, S.A. Garrido, W. Wstrychowski, N. Dusserre, A. Marini, K. Zagalski, A. Fiorillo, H. Avila, X. Mangano, J. Antonelli, A. Kocher, M. Zembala, L. Cierpka, L.M. de la Fuente, N. L'Heureux (2009) Effectiveness of haemodialysis access with an autologous tissue-engineered vascular graft: a multicentre cohort study. *Lancet* 373: 1440–1446.
- McKee, J.A., S.S. Banik, M.J. Boyer, N.M. Hamad, J.H. Lawson, L.E. Niklason, C.M. Counter (2003) Human arteries engineered in vitro. *EMBO Rep* 4: 633–638.
- Michel, M., N. Török, M.J. Godbout, M. Lussier, P. Gaudreau, A. Royal, L. Germain (1996) Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. *J Cell Sci* 109: 1017–1028.
- Mirensky, T.L., G.N. Nelson, M.P. Brennan, J.D. Roh, N. Hibino, T. Yi, T. Shinoka, C.K. Breuer (2009) Tissue-engineered arterial grafts: long-term results after implantation in a small animal model. *J Pediatr Surg* 44: 1127–1132; discussion 1132–1123.
- Mogford, J.E., N. Tawil, A. Chen, D. Gies, Y. Xia, T.A. Mustoe (2002) Effect of age and hypoxia on TGFβ1 receptor expression and signal transduction in human dermal fibroblasts: impact on cell migration. *J Cell Physiol* 190: 259–265.
- Mogues, T., J. Li, J. Coburn, D.J. Kuter (2005) IgG antibodies against bovine serum albumin in humans – their prevalence and response to exposure to bovine serum albumin. *J Immunol Methods* 300: 1–11.
- Morimoto, N., Y. Saso, K. Tomihata, T. Taira, Y. Takahashi, M. Ohta, S. Suzuki (2005) Viability and function of autologous and allogeneic fibroblasts seeded in dermal substitutes after implantation. *J Surg Res* 125: 56–67.
- Muller-Glauser, W., P. Zilla, M. Lachat, B. Bisang, F. Rieser, L. von Segesser, M. Turina (1993) Immediate shear stress resistance of endothelial cell monolayers seeded in vitro on fibrin glue-coated ePTFE prostheses. *Eur J Vasc Surg* 7: 324–328.
- Murad, S., S. Tajima, G.R. Johnson, S. Sivarajah, S.R. Pinnell (1983) Collagen synthesis in cultured human skin fibroblasts: effect of ascorbic acid and its analogs. *J Invest Dermatol* 81: 158–162.
- Nakayama, Y., H. Ishibashi-Ueda, K. Takamizawa (2004) In vivo tissue-engineered small-caliber arterial graft prosthesis consisting of autologous tissue (biotube). *Cell Transplant* 13: 439–449.
- Nieponice, A., L. Soletti, J. Guan, B.M. Deasy, J. Huard, W.R. Wagner, D.A. Vorp (2008) Development of a tissue-engineered vascular graft combining a biodegradable scaffold, muscle-derived stem cells and a rotational vacuum seeding technique. *Biomaterials* 29: 825–833.
- Nieponice, A., L. Soletti, J. Guan, Y. Hong, B. Gharaibeh, T.M. Maul, J. Huard, W.R. Wagner, D.A. Vorp (2010) In vivo assessment of a tissue-engineered vascular graft combining a biodegradable elastomeric scaffold and muscle-derived stem cells in a rat model. *Tissue Eng Part A* 16: 1215–1223.
- Niklason, L.E., J. Gao, W.M. Abbott, K.K. Hirschi, S. Houser, R. Marini, R. Langer (1999) Functional arteries grown in vitro. *Science* 284: 489–493.
- O'Connor, N.E., J.B. Mulliken, B.S., Kehinde, H. Green (1981) Grafting of burns with cultured epithelium prepared from autologous epidermal cells. *Lancet* 1: 75–78.
- Orban, J.M., L.B. Wilson, J.A. Kofroth, M.S. El-Kurdi, T.M. Maul, D.A. Vorp (2004) Cross-linking of collagen gels by transglutaminase. *J Biomed Mater Res A* 68A: 756–762.
- Pauly, R.R., A. Passaniti, C. Bilato, R. Monticone, L. Cheng, N. Papadopoulos, Y.A. Gluzband, L. Smith, C. Weinstein, E.G. Lakatta, et al (1994) Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. *Circ Res* 75: 41–54.
- Peng, H.F., J.Y. Liu, S.T. Andreadis, D.D. Swartz (2011) Hair follicle-derived smooth muscle cells and small intestinal submucosa for engineering mechanically robust and vasoreactive vascular media. *Tissue Eng Part A* 17: 981–990.
- Perera, G.B., M.P. Mueller, S.M. Kubaska, S.E. Wilson, P.F. Lawrence, R.M. Fujitani (2004) Superiority of autogenous arteriovenous hemodialysis access: maintenance of function with fewer secondary interventions. *Ann Vasc Surg* 18: 66–73.
- Phillips, C.L., S.B. Combs, S.R. Pinnell (1994) Effects of ascorbic acid on proliferation and collagen synthesis in relation to the donor age of human dermal fibroblasts. *J Invest Dermatol* 103: 228–232.
- Poh, M., M. Boyer, A. Solan, S.L. Dahl, D. Pedrotty, S.S. Banik, J.A. McKee, R.Y. Klinger, C.M. Counter, L.E. Niklason (2005) Blood vessels engineered from human cells. *Lancet* 365: 2122–2124.
- Rheinwald, J.G., H. Green (1974) Growth of cultured mammalian cells on secondary glucose sources. *Cell* 2: 287–293.
- Rosas, S.E., M. Joffe, J.E. Burns, J. Knauss, K. Brayman, H.I. Feldman (2003) Determinants of successful synthetic hemodialysis vascular access graft placement. *J Vasc Surg* 37: 1036–1042.

- Schilling, J.A., H.M. Shurley, W. Joel, K.M. Richter, B.N. White (1961) Fibrocollagenous tubes structured in vivo: morphology and biological characteristics. *Arch Pathol* 71: 548–553.
- Schilling, J.A., H.M. Shurley, W. Joel, B.N. White, R.H. Bradford (1964) Abdominal Aortic Grafts: Use of in Vivo Structured Autologous and Homologous Fibrocollagenous Tubes. *Ann Surg* 159: 819–828.
- Selvaggi, T.A., R.E. Walker, T.A. Fleisher (1997) Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. *Blood* 89: 776–779.
- Shin'oka, T., Y. Imai, Y. Ikada (2001) Transplantation of a tissue-engineered pulmonary artery. *N Engl J Med* 344: 532–533.
- Shin'oka, T., G. Matsumura, N. Hibino, Y. Naito, M. Watanabe, T. Konuma, T. Sakamoto, M. Nagatsu, H. Kurosawa (2005) Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells. *J Thorac Cardiovasc Surg* 129: 1330–1338.
- Sparks, C.H. (1969) Autogenous grafts made to order. *Ann Thorac Surg* 8: 104–113.
- Sparks, C.H. (1973) Silicone mandril method for growing reinforced autogenous femoropopliteal artery grafts in situ. *Ann Surg* 177: 293–300.
- Stickler, P., G. De Visscher, L. Mesure, N. Famaey, D. Martin, J.H. Campbell, H. Van Oosterwyck, B. Meuris, W. Flameng (2010) Cyclically stretching developing tissue in vivo enhances mechanical strength and organization of vascular grafts. *Acta Biomater* 6: 2448–2456.
- Stoclet, J.C., R. Andriantsitohaina, N. L'Heureux, C. Martinez, L. Germain, F. Auger (1996) Use of human vessels and human vascular smooth muscle cells in pharmacology. *Cell Biol Toxicol* 12: 223–225.
- Swartz, D.D., J.A. Russell, S.T. Andreadis (2005) Engineering of fibrin-based functional and implantable small-diameter blood vessels. *Am J Physiol Heart Circ Physiol* 288: H1451–1460.
- Syedain, Z.H., L.A. Meier, J.W. Bjork, A. Lee, R.T. Tranquillo (2011) Implantable arterial grafts from human fibroblasts and fibrin using a multi-graft pulsed flow-stretch bioreactor with noninvasive strength monitoring. *Biomaterials* 32: 714–722.
- Thornton, S.C., S.N. Mueller, E.M. Levine (1983) Human endothelial cells: use of heparin in cloning and long-term serial cultivation. *Science* 222: 623–625.
- Torikai, K., H. Ichikawa, K. Hirakawa, G. Matsumiya, T. Kuratani, S. Iwai, A. Saito, N. Kawaguchi, N. Matsuura, Y. Sawa (2008) A self-renewing, tissue-engineered vascular graft for arterial reconstruction. *J Thorac Cardiovasc Surg* 136: 37–45, 45 e31.
- Tschoeke, B., T.C. Flanagan, S. Koch, M.S. Harwoko, T. Deichmann, V. Ella, J.S. Sachweh, M. Kellomaki, T. Gries, T. Schmitz-Rode, S. Jockenhoevel (2009) Tissue-engineered small-caliber vascular graft based on a novel biodegradable composite fibrin-poly lactide scaffold. *Tissue Eng Part A* 15: 1909–1918.
- Tuschong, L., S.L. Soenen, R.M. Blaese, F. Candotti, L.M. Muul (2002) Immune response to fetal calf serum by two adenosine deaminase-deficient patients after T cell gene therapy. *Hum Gene Ther* 13: 1605–1610.
- von Rheinbaben, F., A. Schmidt (2004) Regulatory aspects of BSE and CJD with special emphasis on Germany. *Contrib Microbiol* 11: 193–207.
- Watanabe, T., K. Kanda, H. Ishibashi-Ueda, H. Yaku, Y. Nakayama (2010) Autologous small-caliber 'biotube' vascular grafts with argatroban loading: a histomorphological examination after implantation to rabbits. *J Biomed Mater Res B Appl Biomater* 92: 236–242.
- Weinberg, C.B., E. Bell (1986) A blood vessel model constructed from collagen and cultured vascular cells. *Science* 231: 397–400.
- Westerweel, P.E., M.C. Verhaar (2009) Endothelial progenitor cell dysfunction in rheumatic disease. *Nat Rev Rheumatol* 5: 332–340.
- Wight, T.N. (1996) The vascular extracellular matrix; in Fuster, V., R. Ross, E. Topol (eds): *Atherosclerosis and Coronary Artery Disease*. New York, Raven Press, pp 421–440.
- Wystrychowski, W., L. Cierpka, K. Zagalski, S. Garrido, N. Dusserre, S. Radochonski, T.N. McAllister, N. L'Heureux (2011) Case study: first implantation of a frozen, devitalized tissue-engineered vascular graft for urgent hemodialysis access. *J Vasc Access* 12: 67–70.
- Yao, L., J. Liu, S.T. Andreadis (2008) Composite fibrin scaffolds increase mechanical strength and preserve contractility of tissue engineered blood vessels. *Pharm Res* 25: 1212–1221.
- Yokota, T., H. Ichikawa, G. Matsumiya, T. Kuratani, T. Sakaguchi, S. Iwai, Y. Shirakawa, K. Torikai, A. Saito, E. Uchimura, N. Kawaguchi, N. Matsuura, Y. Sawa (2008) In situ tissue regeneration using a novel tissue-engineered, small-caliber vascular graft without cell seeding. *J Thorac Cardiovasc Surg* 136: 900–907.
- Zavan, B., V. Vindigni, S. Lepidi, I. Iacopetti, G. Avruscio, G. Abatangelo, R. Cortivo (2008) Neoarteries grown in vivo using a tissue-engineered hyaluronan-based scaffold. *FASEB J* 22: 2853–2861.
- Zhang, H., S. Fazel, H. Tian, D.A. Mickle, R.D. Weisel, T. Fujii, R.K. Li (2005) Increasing donor age adversely impacts beneficial effects of bone marrow but not smooth muscle myocardial cell therapy. *Am J Physiol Heart Circ Physiol* 289: H2089–2096.
- Zilla, P., R. Fasol, M. Deutsch, T. Fischlein, E. Minar, A. Hammerle, O. Krupicka, M. Kadletz (1987) Endothelial cell seeding of polytetrafluoroethylene vascular grafts in humans: a preliminary report. *J Vasc Surg* 6: 535–541.
- Zilla, P., R. Fasol, P. Preiss, M. Kadletz, M. Deutsch, H. Schima, S. Tsangaris, P. Groscurth (1989) Use of fibrin glue as a substrate for in vitro endothelialization of PTFE vascular grafts. *Surg* 105: 515–522.