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Applying elastic fibre biology in vascular tissue engineering

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For the treatment of vascular disease, the major cause of death in Western society, there is an urgent need for tissue-engineered, biocompatible, small calibre artery substitutes that restore biological function. Vascular tissue engineering of such grafts involves the development of compliant synthetic or biomaterial scaffolds that incorporate vascular cells and extracellular matrix. Elastic fibres are major structural elements of arterial walls that can enhance vascular graft design and patency. In blood vessels, they endow vessels with the critical property of elastic recoil. They also influence vascular cell behaviour through direct interactions and by regulating growth factor activation. This review addresses physiological elastic fibre assembly and contributions to vessel structure and function, and how elastic fibre biology is now being exploited in small diameter vascular graft design.

Keywords: elastic fibres; blood vessels; tissue engineering; elastin; fibrillin; fibulin

1. INTRODUCTION

Vascular disease is the major cause of death in Western society. In the USA alone, more than 600 000 arterial bypass operations using autogenous grafts are conducted per annum, while valvular dysfunction results in well over 60 000 valve replacement operations per annum (American Heart Association 2005 update). Coronary artery bypass operations relieve the symptoms of angina and life-threatening vascular disease, but have significant associated morbidity and cost. Many patients do not have suitable autologous vessels (usually internal mammary artery or saphenous vein), there is compliance mismatch between veins and arteries, and thrombosis is a serious problem. At the cellular level, major problems associated with small diameter vessels include endothelial cell (EC) detachment leading to thrombosis, altered smooth muscle cell (SMC) phenotype and limited deposition of functional vascular extracellular matrix (ECM). Valve replacement surgery currently uses mechanical valves that are rigid and do not 'grow' with young patients, require chronic anti-coagulant therapy and can fail suddenly; or bioprosthetic valves such as fixed xenografts or cryopreserved homografts that have poor long-term durability due to tissue calcification and mechanical damage and risk of thromboembolism, and generally require re-replacement. Thus, there is an urgent need for stable tissue-engineered patent cardiovascular prostheses that retain long-term biological function and are biocompatible.

Vascular tissue engineering of small calibre artery substitutes that restore and sustain biological function involves the development of compliant synthetic or biomaterial scaffolds, incorporating vascular cells and ECM, with long-term stability and functional properties. Since elastic fibres are major structural elements of arterial walls that also strongly influence vascular cell behaviour through direct interactions and natural growth factor delivery, elastic fibre biology is now being exploited in vascular tissue engineering. This review focuses on how elastic fibre molecules can enhance vascular graft design and patency. First, elastic fibre assembly, structure and function, and contributions to normal vessel walls are outlined, and then applications of elastic fibres in vascular tissue engineering are addressed in the context of small diameter vascular graft design.

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One contribution of 21 to a Theme Issue 'Bioengineering the heart'.

2. ELASTIC FIBRES

Elastic fibres are major insoluble ECM assemblies that are deposited in blood vessel walls during early postnatal life. They are multimolecular complexes comprising a cross-linked elastin core surrounded by a mantle of fibrillin-rich microfibrils and several other associated molecules such as fibulin-5 (Kielty *et al.* 2002; Kielty 2006; figure 1).

(a) *Molecular components*

Elastin, the most abundant component of elastic fibres, is secreted as soluble tropoelastin (70 kDa; Mecham & Davis 1994; Mithieux & Weiss 2005; figure 2). It comprises alternating hydrophobic and lysine-rich cross-linking domains that are critical for extracellular assembly and elastic function.

Fibrillins, the principal structural molecules of microfibrils, are large multidomain glycoproteins (approx. 350 kDa) comprising calcium-binding epidermal growth factor (cbEGF)-like domain arrays interspersed with eight-cysteine (TB) motifs (Pereira *et al.* 1993;

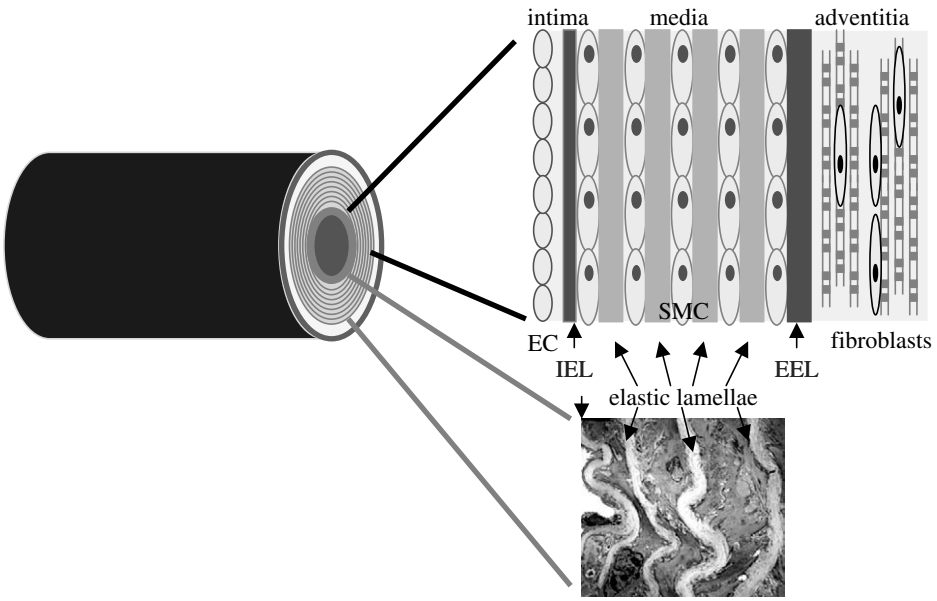

Figure 1. Cartoon of a cross section through an artery. The tunica intima, tunica media and tunica adventitia, and positions of the internal elastic lamina (IEL), external elastic lamina (EEL) and medial elastic lamellae are shown, together with a transmission electron micrograph of an arterial wall.

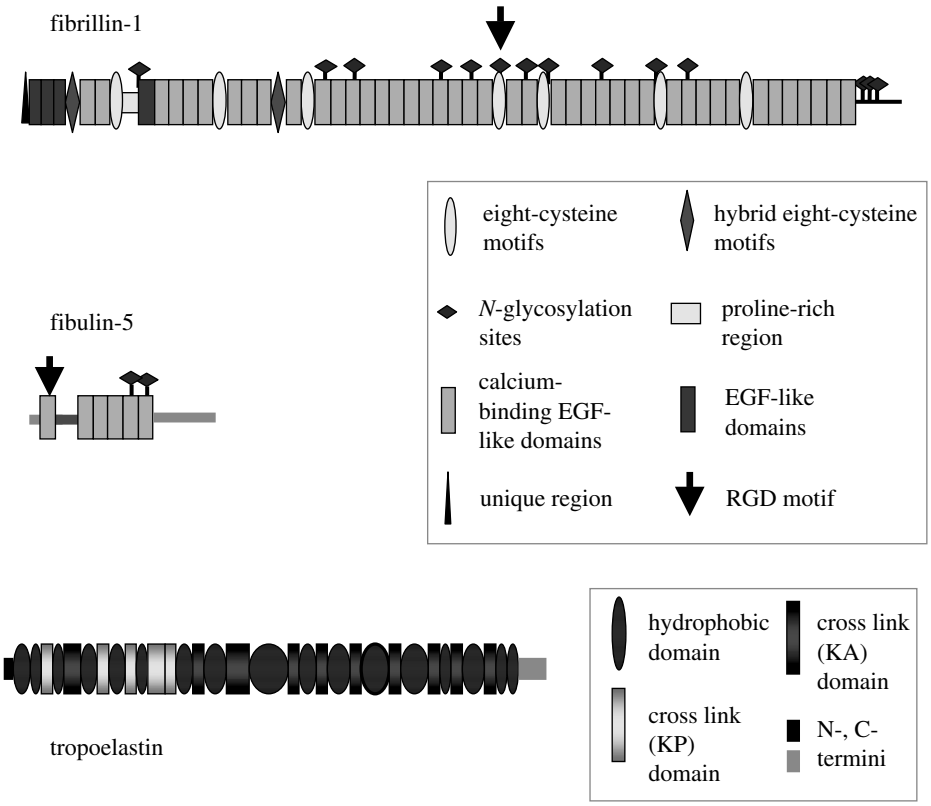

Figure 2. Domain structures of fibrillin-1, fibulin-5 and tropoelastin.

Zhang *et al.* 1994; Corson *et al.* 2004; Kielty *et al.* 2005*a*), and containing an arg-gly-asp (RGD) cell adhesion motif (figure 2). Furin processing of fibrillin-1 precedes extracellular microfibril deposition. Genetic linkage of fibrillin-1 mutations to Marfan syndrome (Robinson *et al.* 2002), its abundance in developing and adult tissues (Quondamatteo *et al.* 2002) and proteomic analysis of isolated microfibrils (Cain *et al.* 2006) confirm its major contribution to elastic fibres.

There are several other molecules that co-localize or co-purify with microfibrils (microfibril-associated glycoproteins 1 and 2, MAGP-1 and MAGP-2; latent TGFβ-binding proteins, LTBPs), or occur at elastin–microfibril or elastic fibre–cell interfaces (fibulin-5, fibulin-2, emilin-1, decorin) or within the elastin core (biglycan), and stabilize elastic fibres (tissue transglutaminase, TG2; lysyl oxidase, LOX; Kielty *et al.* 2002; Kielty 2006). Collagen VIII also co-localizes with

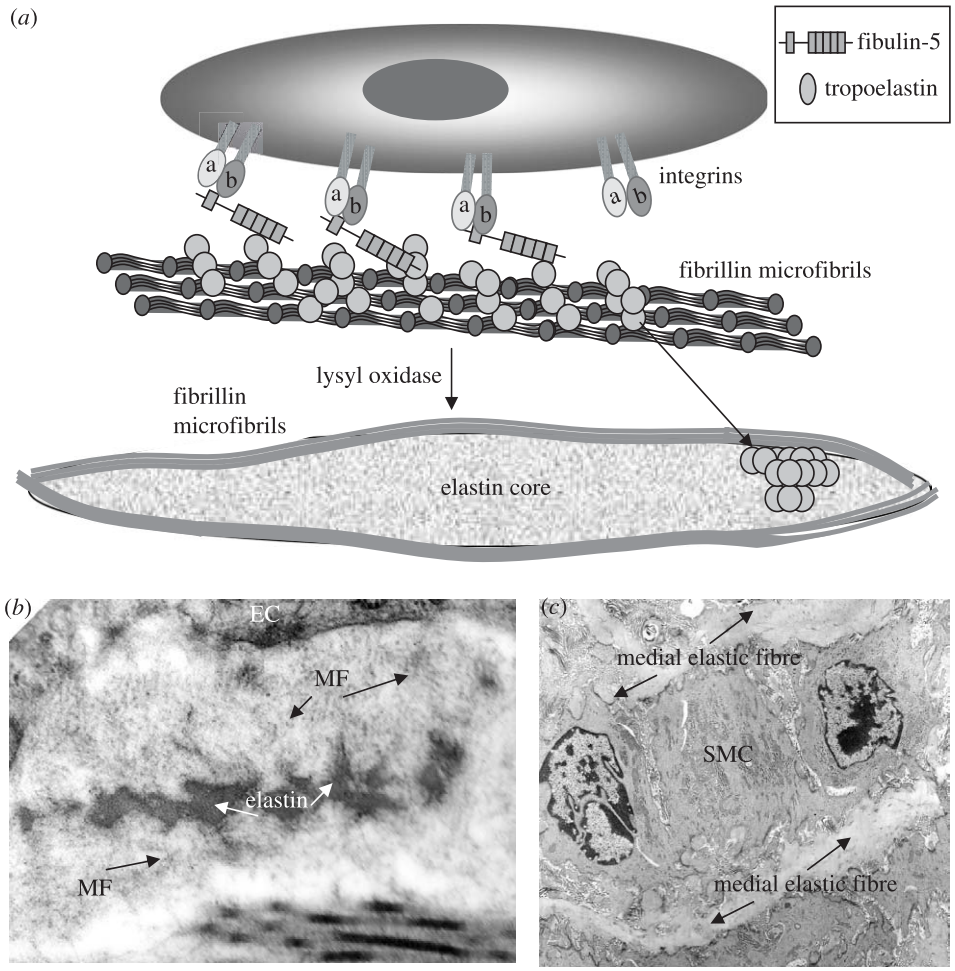

Figure 3. Elastic fibre formation (*a*) Cartoon depicting the deposition of tropoelastin on fibrillin microfibrils in the pericellular space, in potential association with fibulin-5. (*b*) Transmission electron micrograph showing elastic fibre formation in the subendothelium (MF, microfibrils; EC, endothelial cell). (*c*) Transmission electron micrograph showing mature elastic fibre lamellae juxtaposed to medial smooth muscle cell (SMC).

elastic fibres; this collagen forms specialized hexagonal basement membrane-associated arrays and is strongly expressed by SMC following arterial injury (Sinha *et al.* 2001).

Fibulin-5, a glycoprotein of approximately 55 kDa containing an RGD motif, is expressed by vascular SMC and EC, mediates vascular cell adhesion through integrin receptors, influences cell growth and motility in development and wound healing, and regulates elastin fibrillogenesis (Nakamura *et al.* 2002; Yanagisawa *et al.* 2002; Chu & Tsuda 2004; figure 2). It antagonizes angiogenesis and localizes on elastic lamina surfaces adjacent to the EC and in the aortic media. Its expression is markedly downregulated in adult arteries, but is highly induced in SMC and EC in response to injury in atherosclerotic cells and neointimal cells following balloon angioplasty (Kowal *et al.* 1999). Fibulin-5 interacts with apolipoprotein(a) and superoxide dismutase in arterial walls (Nguyen *et al.* 2004). Overexpression of fibulin-5 promotes wound healing, and its expression is upregulated by transforming growth factor beta (TGFβ; Jean *et al.* 2002).

(**b**) ***Deposition***

Vascular elastic fibres are deposited developmentally to reinforce the high pressure circulation (Faury 2001). An early step in elastic fibre formation is the pericellular deposition of fibrillin-1 rich microfibrils as a template for soluble tropoelastin (Kielty *et al.* 2002; figure 3). In foetal development, parallel arrays of fibrillin-rich microfibrils are apparent in the extracellular space juxtaposed to cell membranes. Elastin is then deposited as small aggregates of amorphous material within microfibril arrays, and subsequently coalesces to form mature elastic fibres (Mecham & Davis 1994). Recent studies indicate that an early step in elastic fibre formation is the appearance of small cell surface-associated elastin globules that coalesce hierarchically into larger fibres over time, coupled with cell motion (Czirok *et al.* 2006; Kozel *et al.* 2006). In the extracellular space, elastin crosslinking by LOX and lysyl oxidase-like 1 (LOXL1) is required for normal elastic fibre integrity (Maki *et al.* 2005; Thomassin *et al.* 2005). The proportion of microfibrils to elastin declines with age, with adult elastic fibres often having only a sparse peripheral mantle of microfibrillar material. Tissue-specific elastic fibre architectures, such as arterial medial lamellae, reflect the organization of the microfibril template, in turn dictated by cells and the strength and direction of forces put upon the tissue. In developing aorta, subendothelial microfibril bundles

run parallel to the direction of blood flow and provide elastic anchorage for ECs in flow conditions (Davis 1993*a*).

At molecular and cellular levels, the assembly of fibrillin microfibrils is a poorly understood process. Secreted furin-processed fibrillin molecules are thought to accrete both head-to-tail and laterally, to form beaded microfibrils (Reinhardt *et al.* 1996; Marson *et al.* 2005) which are then stabilized by transglutaminase cross links (tissue transglutaminase, TG2). We have shown that tropoelastin binds strongly to fibrillin-1 and that transglutaminase can be cross-linked to a specific fibrillin-1 sequence (Rock *et al.* 2004; Clarke *et al.* 2005). Elastin may also interact with microfibril-associated molecules such as microfibril-associated (MAGP-1) or biglycan (Trask *et al.* 2000; Jensen *et al.* 2001; Rock *et al.* 2004). These interactions may be regulated by cell surface receptors. Our model of elastic fibre assembly is that tropoelastin first binds periodically along microfibrils at TG2 cross-link sites, and then further tropoelastin molecules accrete and are covalently linked by LOX. Heparan sulphate binds and profoundly influences microfibril and elastic fibre formation (Tiedemann *et al.* 2001; Cain *et al.* 2005). The roles of the other elastic fibre-associated molecules remain unclear. Fibrillin-rich microfibrils probably integrate into vascular basement membranes since they can strongly interact with perlecan (Tiedemann *et al.* 2005).

A 67 kDa elastin-binding protein (EBP) which acts as a recyclable chaperone facilitates the secretion of tropoelastin (Privitera *et al.* 1998). EBP forms a cell surface-targeted molecular complex with lysosomal sialidase, the activity of which is needed for elastogenesis. It is thought to remove terminal sialic acids from microfibril glycoprotein carbohydrates, thereby unmasking galactosugars to act with the galactolectin domain of EBP, allowing the release of tropoelastin onto microfibrils (Hinek *et al.* 2006).

The critical role of fibulin-5 in elastic fibre and blood vessel formation is poorly defined. LOXL1 isoform, which is important in elastic fibre renewal in adult tissues, co-localized with fibulin-5 and bound a C-terminal region of fibulin-5 (Liu *et al.* 2004). This juxtaposition of fibulin-5 to tropoelastin and LOXL1 may allow efficient elastin core cross-linking. Recombinant rat fibulin-5 bound tropoelastin in a calcium-dependent manner, but no significant binding was observed for fibrillin-1, laminin or collagen I (Yanagisawa *et al.* 2002). In another study, recombinant fibulin-5 associated with pre-formed elastic fibres deposited by cultured cells (Nakamura *et al.* 2002). Fibulin-5 also interacts with emilin-1 (Zanetti *et al.* 2004). In our pilot studies using recombinant human fibulin-5, fibulin-5 bound to tropoelastin in a calcium-independent manner and to a conformation-sensitive N-terminal fibrillin-1 fragment (PF1; Freeman *et al.* 2005). Scanning transmission electron microscopy mass mapping revealed that fibulin-5 binds microfibrils, confirming a physiological role. Our data thus suggest a role for fibulin-5–microfibrillar interactions during elastic fibre formation. Fibulin-5-deficient mice also reveal a role of fibulin-5 in SMC proliferation and migration (Spencer *et al.* 2005).

3. ELASTIC FIBRES AND ARTERIAL WALL ORGANIZATION

Elastic fibres are major structural elements of arterial walls. Their abundance, structure and functions are outlined below, in the context of normal vessel wall organization.

(a) *Arterial wall organization*

Arteries comprise three major layers: the tunica intima, tunica media, and tunica adventitia (Quaglino & Pasquali-Ronchetti 2002); elastic fibres are components of all three layers (figure 1). The intima comprises an EC monolayer underpinned by subendothelial ECM. It provides an anti-thrombogenic inner lining to blood vessels, a barrier to mononuclear blood cells and a diffusion gradient to systemic cytokines and growth factors. A thick continuous elastic fibre (internal elastic lamina, IEL) forms a physical boundary between intimal EC and medial SMC. We have shown that, *in vitro*, direct EC contact profoundly disrupts the cytoskeletal organization of precursor SMC (Ball *et al.* 2004), and a major function of the IEL may be to prevent direct contact between these cells. The media comprises alternating layers of contractile SMC interspersed with concentrically arranged elastic fibres, as well as some collagen fibre bundles and proteoglycans. The elastic fibre and SMC lamellae endow elastic recoil and contractility on vessel walls. The number of elastic lamellae varies depending on the vessel type. The human aortic and elastic (conducting) arteries have between 40 and 70 elastic lamellae; they expand in response to blood flow and then have passive elastic recoil that allows for the maintenance of diastolic pressure. Coronary, peripheral and other muscular arteries have fewer (3–40) elastic lamellar layers. The external elastic lamina is a thick elastic fibre layer delineating the outer boundary of the tunica media. The outer adventitial layer is a thin dense connective tissue containing smooth muscle-like myofibroblasts, abundant collagen fibres, dispersed elastic fibres and vasa vasorum. It exerts homeostatic control on the vessel, limits wall extensibility and integrates the blood vessel into the surrounding tissue.

(b) *Vascular cells*

Three major cell types populate the vessel wall, regulate its physiology and deposit its ECM. Intimal ECs exhibit classic cobblestone morphology in culture but, in vessels, are somewhat more elongated with their long axis parallel to blood flow. EC monolayers line the lumen and provide a barrier between the blood and the vessel wall, control vessel tone, and regulate leucocyte adhesion and migration through the vessel wall. They also contribute synthetically to the deposition of the IEL that prevents direct interactions between SMCs and EC. During development, SMCs are responsible for the deposition of most of the vessel wall ECM, especially the medial elastic lamellae and collagen fibrils. In the mature media, SMCs are spindle-shaped cells that adopt a distinctive contractile phenotype characterized by SMC-specific cytoskeletal elements such as SM α-actin, SM myosin, smoothelin and SM22α

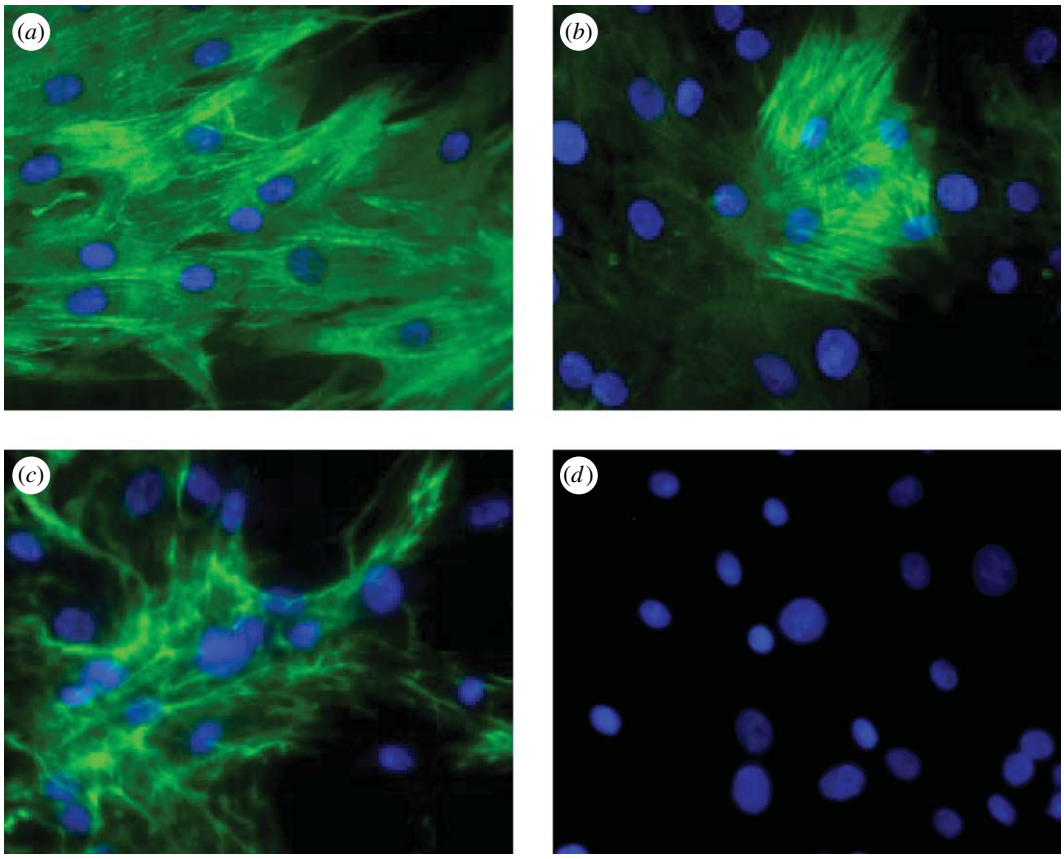

Figure 4. SMC: immunofluorescence analysis of cytoskeletal and ECM elements. Cultured porcine aortic SMCs immunostained for (*a*) smooth muscle α-actin, (*b*) SMC myosin heavy chain, (*c*) fibronectin and (*d*) control omitting primary antibody. Images are 400× magnification.

(Owens 1995; Hungerford & Little 1999) as well as vascular matrix molecules (figure 4). They are surrounded by a specialized basal lamina except where they are connected to one another by gap junctions and to elastic fibres at prominent dense plaques. SMCs often have a 'feathered' appearance indicating that they are 'tethered' to the medial elastic fibre lamellae that intercalate the SMC layers (Davis 1993*b*). In culture, SMC phenotype is much more variable, and they usually adopt more synthetic proliferative 'hills and valleys' phenotype. Adventitial myofibroblasts express SM α-actin (Shi *et al.* 1996; Gao *et al.* 2003), deposit abundant collagen fibres and some elastic fibres and, following vascular injury, orchestrate adventitial remodelling. In vascular graft design, their growth, differentiation and functional characteristics need to be tightly regulated and monitored.

(c) *Vascular extracellular matrix*

Elastic fibres represent a major component of the vascular ECM. They comprise a cross-linked elastin core surrounded by an outer mantle of fibrillin microfibrils (figure 3). Other key components, many of which interact with elastic fibre molecules, include various collagens and proteoglycans. Collagen fibres (mainly types I and III) represent more than 30% of the dry weight of blood vessels (Quaglino & Pasquali-Ronchetti 2002). Subendothelial and SMC basal laminae contain network-forming collagens IV and VIII. Collagen types XV and XVIII are associated with subendothelial basement membranes; their C-terminal 'endostatin' fragments influence angiogenesis (Morbidelli *et al.* 2003; Ricard-Blum *et al.* 2003). Collagen VI microfibrils are major cell–matrix linking polymers present throughout the vessel wall (Kielty & Grant 2002). Vascular cell adhesion glycoproteins include laminin in basement membranes, fibronectin and vitronectin. Proteoglycans function as structural elements of the vessel wall, and modulate cell adhesion and vascular growth factor availability. Their biological roles arise from their protein and carbohydrate (glycosaminoglycan) components. They form a hydrated scaffold and are especially abundant in the subendothelium. The heparan sulphate proteoglycan, perlecan, is an intrinsic component of subendothelial and SMC basement membranes. Heparin (a highly sulphated form of heparan sulphate) can inhibit SMC proliferation and migration, and it binds the growth factor FGF, fibrillin-1 (Cain *et al.* 2005) and elastin (Broekelmann *et al.* 2005; Gheduzzi *et al.* 2005), and influences elastic fibre deposition. The small leucine-rich proteoglycans decorin and biglycan are directly implicated in the process of elastic fibre formation (Reinboth *et al.* 2002) and also regulate collagen packing (Danielson *et al.* 1997; Wiberg *et al.* 2002). Some elastic fibre molecules bind TGFβ growth factors and regulate their bioavailability. Transmembrane proteoglycan receptors (syndecans) present on the medial SMC contribute, together with integrins, to elastic fibre-mediated cell–matrix communication and signalling.

(d) *Elastic fibres in vascular development*

Fibrillin microfibrils and elastic fibres are important players in blood vessel formation. In early vasculogenesis, primitive EC tubes are formed (Hungerford & Little 1999; Jain 2003); then a flexible subendothelial matrix is deposited that supports the functional EC monolayer. Mesenchymal smooth muscle precursor cells are recruited from surrounding tissues, through the actions of vascular growth factors such as platelet-derived growth factor (PDGF)-BB and TGFβ1 (Hellstrom *et al.* 1999); they deposit abundant vascular wall ECM, including elastic fibres. In mature vessels, medial SMC intercalated between elastic lamellae are contractile and quiescent. Abundant fibrillin microfibrils, or 'connecting filaments', are laid down by EC during vasculogenesis, in the direction of blood flow (Davis 1993*b*). These filaments are closely associated with the EC subluminal surface and may act as a long-range biomechanically flexible anchorage for EC. Microfibril bundles act as a template for tropoelastin deposition throughout the vessel wall. Developmental thickening of the tunica media and tunica adventitia reflects abundant deposition of elastic fibres and also collagen fibres (mainly collagens I, III), and occurs during the first years of life. During this time, microfibrils may also act as a conduit for LTBPs that sequester TGFβ and regulate its availability during vessel formation and repair (Isogai *et al.* 2003).

4. BIOLOGICAL ROLES OF ELASTIC FIBRES

Elastic fibres perform at least three critical functions in arteries. An obvious major role is the provision of elastic recoil to blood vessels. Fibrillin microfibrils and elastic fibres also regulate the activity of TGFβ family growth factors in vascular and other elastic tissues. Furthermore, elastic fibre molecules contain specific cell attachment motifs that mediate cell attachment, migration, survival and differentiation, and elastin profoundly influences cells in vascular pathology (Brooke *et al.* 2003). For these reasons, we and others are now exploiting elastic fibre biology in vascular tissue engineering.

(a) *Elastic recoil*

Fibrillin-rich microfibrils and elastic fibres profoundly influence vessel wall mechanics. Invertebrate and mammalian microfibril studies have shown that isolated microfibrils and microfibril bundles have elastic properties (McConnell *et al.* 1997; Thurmond *et al.* 1997; Megill *et al.* 2005). Molecular combing revealed that individual microfibrils are relatively stiff reinforcing fibres with Young's modulus of approximately 70 mPa when compared with that of elastin (approx. 1 mPa; Sherratt *et al.* 2003). Tropoelastin comprises alternating hydrophobic and lysine-rich domains which, in their cross-linked polymerized state, impart the physical properties of extensibility and elastic recoil on arteries (Faury 2001).

(b) *Growth factor sequestration and activation*

Genetic evidence in humans and mice, and biochemical studies strongly implicate fibrillin microfibrils in the extracellular control of TGFβ and bone morphogenetic protein (BMP) activation and signalling in cardiovascular and other elastic connective tissues (Charbonneau *et al.* 2004). These growth factors are potent regulators of cell survival and differentiation, tissue morphogenesis and homeostasis, and of cellular responses to injury (Massague & Chen 2000). Fibrillin-1 mutations cause Marfan syndrome and related fibrillinopathies, with symptoms ranging from tall to short stature, hypermobile joints to joint contractures and stiffness, and severe to mild or no cardiovascular defects (Robinson *et al.* 2002; Dietz *et al.* 2005). TGFβ family growth factor dysregulation may contribute to this phenotypic variability. Mitral valve defects in a murine model of Marfan syndrome were rescued by TGFβ neutralizing antibodies, confirming a causal relationship between mutant fibrillin-1 and TGFβ dysregulation (Ng *et al.* 2004; Weyman & Scherrer-Crosbie 2004). Fibrillin-1 deficiencies due to TGFβ dysregulation also caused failure in distal alveolar septation, and apoptosis in the developing lung, leading to emphysema and pneumothorax (Neptune *et al.* 2003). Mutations in *TGFBR1* or *TGFBR2* cause a new syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development (Loeys *et al.* 2005). Null mice models also revealed that fibrillin-2 and BMPs 4 and 7, a member of the TGFβ superfamily, are functionally and genetically linked (Chaudhry *et al.* 2001). Fibrillin-1 may regulate TGFβ1 availability through tissue and development-specific associations with LTBPs (Charbonneau *et al.* 2004). We recently showed that fibrillin-1 directly regulates TGFβ1 bioavailability (Chaudhri *et al.* 2007). Elastin has also been referred to as a matrikine (Duca *et al.* 2004).

TGFβ activity is regulated by small and large latent complexes (Rifkin 2005). LTBP-1 intracellularly forms a large latent complex with TGFβ1, which is stabilized by a disulphide bond between the latency-associated peptide (LAP) of TGFβ and the third TB module of LTBP-1 (Hyytiäinen *et al.* 2004; Rifkin 2005). The large latent complex is efficiently secreted, and LTBP-1 has been shown to bind the N-terminal region of fibrillin-1 (Isogai *et al.* 2003). Active TGFβ1 can then be released by the actions of integrins, protease, heat or thrombospondin-1. The nuclear magnetic resonance (NMR) structure of the TB3 domain in LTBP-1 has revealed the amino acid residues responsible for interactions with LAP; they include two hydrophobic and five acidic residues (Chen *et al.* 2005). The third TB module of LTBP-3 has TGFβ1-binding residues similar to those of LTBP-1, and it also strongly binds TGFβ (Saharinen & Keski-Oja 2000). However, LTBP-4 binds LAP–TGFβ only weakly, and LTBP-2 not at all. Significantly, although fibrillin microfibrils and LTBP-1 often co-localize, particularly in developing tissues, they are not always juxtaposed. Nevertheless, this interaction is proposed to underlie fibrillin-1-mediated TGFβ1 regulation (Rifkin 2005). Another member of the TGFβ superfamily BMP-7 can bind fibrillin-1 directly (Charbonneau *et al.* 2004).

(c) *Cell adhesion and signalling*

Elastic fibres have important vascular cell adhesion functions. Electron microscopy and biochemical studies have highlighted that ECs interact strongly

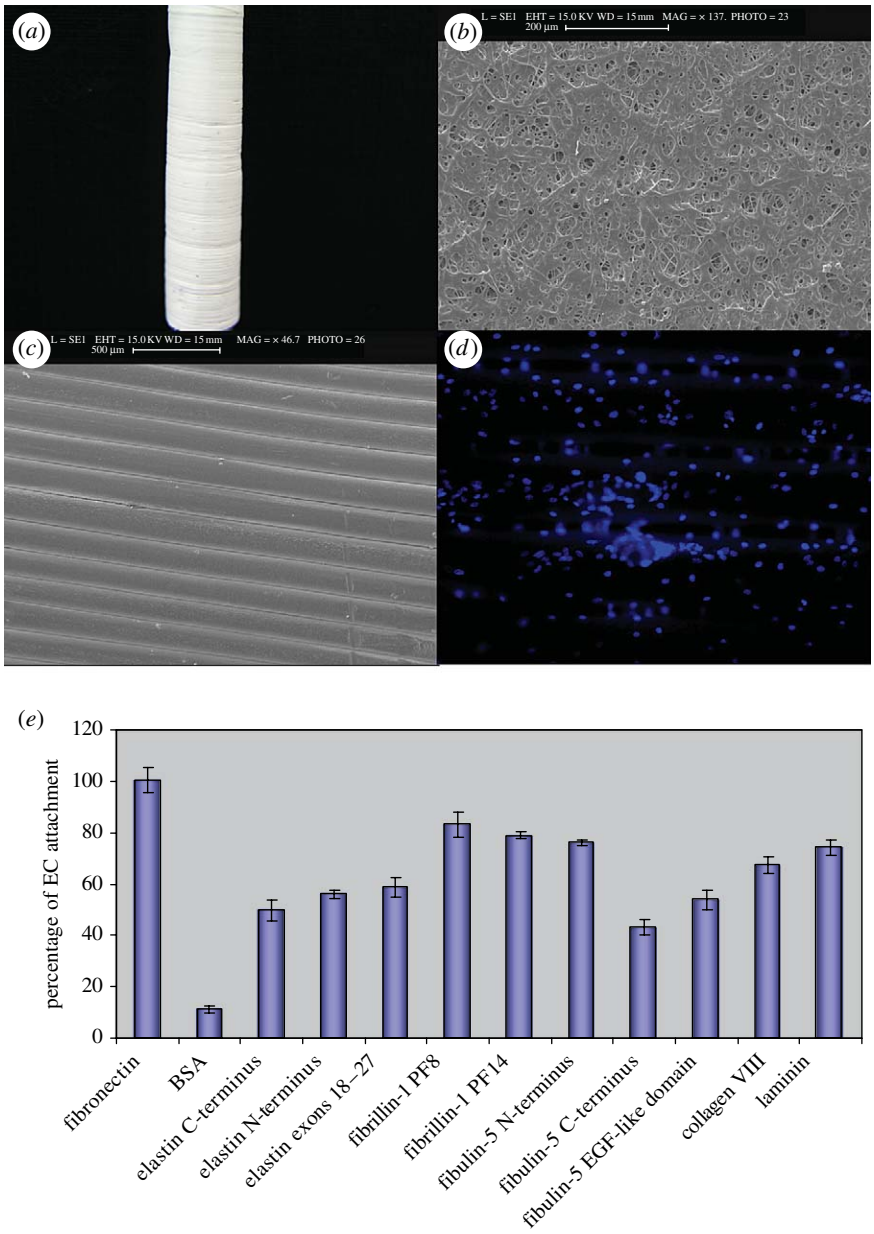

Figure 5. (*a–d*) Images of our PU–PCL composite scaffold. (*a*) Composite scaffold on electrospinning mandrel. (*b*) Scanning electron microscopy (SEM) image of the anti-lumen surface; (*c*) SEM image of the lumen surface. (*d*) Human umbilical vein endothelial cells (HUVECs) cultured on the lumen surface for seven days, stained with DAPI; (*e*) HUVECs attached well to recombinant vascular matrix molecules coated onto the composite scaffold. Maximal percent cell attachment occurred after 45 min. The fragments used were (i) tropoelastin N-terminal fragment encoded by exons 1–18 and C-terminal fragments encoded by exons 18–36 and 18–27; (ii) fibrillin-1 fragments encoded by exons 30–38 and 33–40; (iii) fibulin-5 N- and C-terminal halves, and the N-terminal calcium-binding epidermal growth factor-like domain that contains the RGD motif. Recombinant collagen VIII (a product of EC and SMC) and laminin (from Sigma Chemical Co., UK) were also tested.

with their subendothelial elastic fibre-containing matrix, and SMCs interact with juxtaposed elastic fibre lamellae at cell surface dense plaques (Davis 1993*a*,*b*). These interactions are mediated mainly through integrins, which are heterodimeric transmembrane receptors (Mould & Humphries 2004), and are critical both for a stable endothelium and normal EC vasoactive functions, and for regulating SMC contractile function within the media. Following integrin ligation of vascular ECM molecules, the integrin β-subunit cytoplasmic tail interacts with talin, which in turn binds vinculin that is associated with α-actinin, actin stress fibres and focal adhesions. In this way, the vascular ECM is linked directly, through integrins, to the cellular cytoskeletal framework. Thus, integrin-mediated cell–matrix interactions regulate vascular cell survival, phenotype proliferation, migration and ECM expression and deposition. Integrins expressed by EC and SMC include integrins α1β1, α2β1, α5β1, α6β1 and αvβ3 (Vorp *et al.* 2005). The major elastic fibre molecules that mediate cell adhesion are outlined below.

(i) *Elastin*

Cells interact directly with elastin through at least two different mechanisms. The EBP (an alternatively spliced form of β-galactosidase; 67 kDa) has the repeat hexapeptide in tropoelastin, VGVAPG, as its main

ligand, and signalling through this receptor profoundly influences SMC proliferation and phenotype (Mochizuki *et al.* 2002). The C-terminus of elastin also interacts with integrin αvβ3 in a saturable, divalent cation-dependent manner; however, this is not an RGD-mediated mechanism since elastin lacks this motif (Rodgers & Weiss 2004). Certain elastin proteolytic fragments are also highly chemotactic (Bisaccia *et al.* 1994; Uemura & Okamoto 1997). Using recombinant human tropoelastin (full-length and overlapping fragments), we have demonstrated strong SMC and EC adhesion to elastin-coated substrata, with associated increases in proliferation (Williamson *et al.* submitted; figure 5).

(ii) *Fibrillin-1*

We were the first to show that human SMCs exhibit RGD and cation-dependent adhesion to microfibrils (Kielty *et al.* 1992). We and others have since shown that fibrillin-1 is a major cell adhesion molecule, mediating adhesion mainly through a single active RGD motif in its sixth TB module (Pfaff *et al.* 1996; Sakamoto *et al.* 1996; Bax *et al.* 2003; Lee *et al.* 2004). The corresponding RGD motif in fibrillin-2 is also active. Fibrillin-1 was shown to interact with cells through αvβ3, and we have shown that it also ligates the α5β1 receptor. These interactions profoundly influence cell adhesion and spreading. Cell adhesion to fibrillin-1 specifically modifies gene expression levels—we showed at mRNA and protein levels that there is enhanced fibrillin-1 self-expression when cells are attached to the fibrillin-1 RGD motif but not to fibronectin, which also ligates α5β1 (Bax *et al.* 2003). Another group recently showed enhanced matrix metalloproteinase (MMP) expression in cells plated on fibrillin-1 RGD-containing peptides (Booms *et al.* 2005). The molecular bases of these phenotypic effects are unknown, but certainly reflect integrin signalling. We and others have now demonstrated a requirement for domains upstream to the RGD motif for optimal cell adhesion (Lee *et al.* 2004; Bax *et al.* 2007); we are conducting domain-swop experiments to establish whether enhanced cell adhesion reflects engagement of a synergy sequence. By defining these cell adhesion mechanisms, we have been able to optimize fibrillin-1 fragments that can now be exploited in vascular tissue engineering.

(iii) *Fibulin-5*

Fibulin-5 interacts directly with vascular cells, a function that may contribute to its critical role in elastic fibre deposition. Recombinant fibulin-5 expressed in a bacterial system was reported to interact directly with integrins αvβ3 and αvβ5 (RGD-dependent) and α9β1 (not RGD-dependent; integrin subgroup with α4β1) on integrin-overexpressing Chinese hamster ovary cells (Yanagisawa *et al.* 2002). We have conducted extensive human SMC adhesion studies using recombinant full-length human fibulin-5 (Freeman *et al.* 2005). We have identified the receptors used by SMCs and ECs on fibulin-5, and associated cellular organization and phenotype associated with low proliferation and migration (Lomas *et al.* in press).

5. TISSUE-ENGINEERED VASCULAR GRAFTS

Most current blood vessel tissue engineering approaches are based on the *ex vivo* generation of living prostheses, colonized with contractile SMC and with vasoactive anti-thrombotic endothelium, which mimic natural vessels and can undergo growth, remodelling and repair (L'Heureux *et al.* 1998; Niklason *et al.* 1999; Mitchell & Niklason 2003; Daly *et al.* 2004; Borschel *et al.* 2005; Swartz *et al.* 2005). Most grafts are, in effect, tissue engineered in 'reverse' of normal vessel development, since they start with a strong 'tunica media equivalent' with appropriate elastic and non-porous properties essential for immediate graft patency. During graft pre-conditioning, SMCs are encouraged to deposit abundant ECM with native architecture and the essential biomechanical properties of elastic recoil (elastic fibres) and tensile strength (collagen fibres), so as to gradually replace biodegradable scaffolds.

A fundamental requirement of tissue-engineered grafts is a compliant polymer scaffold to which attached ECs provide an anti-thrombogenic luminal surface and vasoactive properties, and within which SMC can migrate and deposit functional elastic fibre-rich vascular ECM. Strong cell attachment is essential, and it can be enhanced by coating scaffolds with cell–matrix motifs; however, scaffold surface chemistry profoundly influences the adsorption, conformation and functional properties of biological molecules. Since developmental biomechanical forces direct SMCs to lay down an elastic matrix during vasculogenesis, their phenotype within pre-compliant scaffolds often requires experimental modulation through vascular growth factor signalling. Elastic fibre molecules are central to these strategies. As outlined above, they deliver elastic recoil to arterial walls; profoundly influence cell survival, migration and differentiation through specific cell–matrix interactions; and play a central role in regulating the availability of TGFβ growth factors (Kielty *et al.* 2002).

(a) *Polymer scaffolds*

Several small diameter graft models are being developed that use polymer scaffolds to provide mechanical integrity to ensure that they will not rupture when subjected to physiological arterial pressure and flow. Existing synthetic scaffold polymers include inert polyester Dacron and expanded polytetrafluoroethylene (Kannan *et al.* 2005; Kielty *et al.* 2005*b*; Vorp *et al.* 2005). These materials, however, are not particularly suitable for small diameter grafts owing to a tendency for thrombus induction, embolism and occlusion of the graft lumen, lack of compliance and excessive intimal hyperplasia at anastomoses. They do not allow vessel remodelling or vascular physiological responsiveness, and are prone to infection. Polyurethane (PU) is a promising biocompatible synthetic scaffold material, since it is elastomeric with suitable compliance and controlled degradation characteristics (Zhang *et al.* 2004; Kannan *et al.* 2005; Vara *et al.* 2005). In its unmodified state, PU is uncharged, but can be modified to become cationic, anionic or zwitterionic. PU has a tendency to degrade, causing

aneurysm formation. However, carbonate-based PU with no ester linkages has improved stability and endothelialization characteristics (Kannan *et al.* 2005). Poly(ester-urethane)ureas are more flexible and stronger but have reduced cell adhesion and oxidative biodegradation. We have developed a composite PU–polycaprolactone (PU–PCL) scaffold that shows good EC attachment to the PCL luminal surface (Williamson *et al.* submitted; figure 5). These synthetic scaffolds are often treated with biological molecules such as collagen, heparin, laminin, synthetic RGD cell adhesive peptides (see below), fibrin–gelatin, growth factors, anti-coagulant peptides and dextran derivatives (Krijgsman *et al.* 2002; Rashid *et al.* 2004; Kannan *et al.* 2005) and with antibiotics (Cagiannos *et al.* 2005) to regulate cell behaviour through integrin-mediated cell adhesion signalling and to prevent infection and intimal hyperplasia. Biodegradable biomaterials such as polylactic acid, poly(D,L-lactide-*co*-glycolic acid) (PLGA) and poly(ester-urethane)urea may be used as temporary graft scaffolds but require ingrowth of cells and deposition of crosslinked matrix (Kannan *et al.* 2005; Kielty *et al.* 2005*a*,*b*; Vorp *et al.* 2005).

In addition to synthetic polymer scaffolds, decellularized arteries and veins which retain elastic lamellar structure have been tested with some success, although such grafts are susceptible to degradation and aneurysm formation (Walles *et al.* 2003). Burst strengths of such grafts are often low. However, recent studies of decellularized vein allografts have shown such scaffolds to have burst strengths similar to native vessels (Schaner *et al.* 2004; Martin *et al.* 2005). A recent development is decellularized scaffolds based on pure elastin or collagen which were both populated by host cells *in vivo* and remodelled to contain numerous collagen and elastic fibres (Simionescu *et al.* 2006). Tissue-engineered vessels based on rolled up sheets of SMC and fibroblasts embedded within their own matrix to form tubes of media coated in adventitia have higher burst strength but take at least three months to prepare (L'Heureux *et al.* 1998, 2001). Autologous artificial vessels generated by the formation of a matrix-rich granulation tissue capsule surrounding biocompatible tubing placed within the peritoneal cavity also have high burst strengths, can be prepared within two to three weeks and are not rejected (Thomas *et al.* 2003; Chue *et al.* 2004).

Our small diameter vascular graft model is based on elastomeric porous scaffolds fabricated from slowly biodegradable PU or PU–PCL composite grafts (figure 5), with SMC seeded within the scaffolds and ECs on the luminal surface. Using a caprine carotid PU interposition graft, we have demonstrated that delivery of TGFβ3 attenuates myointimal hyperplasia and reduces elastin in anastomoses (Ghosh *et al.* 2006). We are also exploiting elastic fibre molecules to control SMC and EC attachment, migration, proliferation and differentiation within the scaffold. To this end, we have developed a library of recombinant elastic fibre molecules, and characterized SMC and EC integrin-mediated cell adhesion, spreading, proliferation and migration on these molecules (Bax *et al.* 2003; 2007; Lomas *et al.* in press; Williamson *et al.* 2006). We are investigating whether specific elastic fibre motifs can be used for slow release of TGFβ1, and how mechanical forces (cyclic stretch, shear stress) influence SMC proliferation and elastic fibre deposition.

(b) *Adsorption of vascular adhesion motifs on scaffolds*

Synthetic polymers such as PU do not bear epitopes that directly bind cell adhesion receptors, so many studies have focused on modifying polymers with cell–matrix adhesion motifs to ensure cell and tissue biocompatibility. Since integrin receptors are major vascular cell–matrix receptors (Mould & Humphries 2004), attention has been focused on the attachment of the RGD tripeptide cell adhesion motif to scaffold materials. Adhesion peptides can be stably adsorbed by grafting to the termini of surface-active polymers that adsorb to hydrophobic or anionic surfaces; the surface can then be passivated against non-specific interactions. As well as achieving good surface density of peptides, clustering of peptides is advantageous for cell adhesion. An aqueous-based process has been reported for surface functionalization of RGD moieties onto the luminal surface of a prefabricated cardiovascular graft made of poly(carbonate-urea)urethane (Salacinski *et al.* 2003; Tiwari *et al.* 2003). Other immobilized cell adhesion peptides (KQAGDV from fibrinogen, recognized by integrin αIIbβ3; VGVAPG from elastin) have been adsorbed onto scaffolds. In general, cells adhere more strongly to surfaces modified with these adhesive ligands than to control surfaces (Mann & West 1999). Given the exquisite specificity of integrin-RGD recognition and ligation in native molecular context, and the highly specific resultant cellular signalling responses, there is significant potential to exert much greater regulation of cell behaviour by controlling the conformation of selected cell–matrix adhesion motifs on scaffold surfaces.

(c) *Surface effects on ECM protein conformation and function*

Different scaffold surfaces have distinct chemistries, roughness indices and abilities to adsorb ECM molecules in native conformation and to support cell adhesion. Several recent studies provide critical insights into how adsorption of proteins to different surfaces affects their conformation and biological functions, especially cell adhesion.

Owing to their amphiphilic nature, proteins adsorb to most surfaces over a wide range of solution conditions both *in vitro* and *in vivo* (Vandulm & Norde 1983). Such non-specific physiosorption, driven by van der Waals' forces, the electrostatic double layer force and the hydrophobic effect, is influenced by substrate topography and charge, the concentration and valency of the solute and the unique charge distribution of each protein (Haynes & Norde 1995; Muller *et al.* 1997). Substrate chemistry and topography-dependent conformational changes have been demonstrated for many proteins including the ECM components fibronectin, type I collagen, fibrillin microfibrils (see below) and recombinant elastin peptides (Denis *et al.* 2002; Yang *et al.* 2002; Bergkvist *et al.* 2003; Sherratt *et al.* 2004). Conformational effects may be limited to alterations in relative domain

positions, masking or revealing biologically active sites, or may extend to the secondary structure disrupting α-helical content (Krammer *et al.* 2002; Bergkvist *et al.* 2003). The ability of adsorbed fibronectin to bind specific antibodies and to support cell attachment and spreading cell is dependent on the hydrophilicity/hydrophobicity of the substrate (Garcia *et al.* 1999; Michael *et al.* 2003).

Substrate hydrophobicity/hydrophilicity is commonly quantified by measuring water drop contact angles (Sherratt *et al.* 2004; figure 6). For a sessile liquid drop on a substrate, the angle formed between the liquid–solid and liquid–vapour interfaces is called the wetting or contact angle (θ). Surfaces are defined as hydrophilic if $\theta < 90°$ and hydrophobic if $\theta > 90°$. Although contact angle measurements may be made on sessile drops, on most solid surfaces the measured contact angle as the drop advances differs from the angle measured as the drop recedes (Bachmann *et al.* 2000). This contact angle hysteresis may be induced by surface roughness, which is also known to influence both protein adsorption and cellular adhesion (de Gennes 1985; WojciakStothard *et al.* 1996; Denis *et al.* 2002). Nanoscale surface roughness is readily quantified using atomic force microscopy (AFM; Bottomley 1998). Substrate roughness and protein conformation can be determined from AFM height maps before and after protein adsorption, respectively.

Scaffold surface-patterning techniques, such as inkjet printing of ECM molecules in different geometries, offer a powerful means of controlling the cell-adhesive properties of grafts. Self-assembled networks of the basement membrane molecule laminin-1 have recently been obtained on glass substrates by physisorption-assisted microcontact printing (Sgarbi *et al.* 2004). Recent advances in AFM technology have led to the development of dip-pen nanolithography which employs an AFM tip as a 'nib', a solid substrate as the 'paper' and molecules with affinity for the substrate as the 'ink' (Piner *et al.* 1999). In this way, substrates can be patterned with linewidths in the range 10–100 nm, generating biologically active multicomponent nanostructures and arrays (Zhang *et al.* 2002; Lee *et al.* 2003).

Exploiting elastic fibre molecules in vascular tissue engineering requires detailed molecular knowledge of their assembly and biological interactions. This information throws light into scaffold surface design, supports the development of scaffold coatings of template molecules such as fibrillin-1 to encourage elastin deposition in grafts and support cell adhesion, and provides insights into how vascular cells *in vivo* can be encouraged to deposit ordered elastic fibres.

6. ELASTIC FIBRES IN VASCULAR TISSUE ENGINEERING

Several groups are exploiting elastin and microfibrillar molecules to address a major challenge in tissue-engineered graft design, namely, to regulate vascular cell phenotype within the construct. We and others have developed recombinant and purified resources of human elastic fibre molecules that we are using to understand the molecular basis of elastic fibre assembly and function, and to exploit directly in vascular grafts as elastin-based materials and cell-seeding gels (Kielty *et al.* 2005*b*).

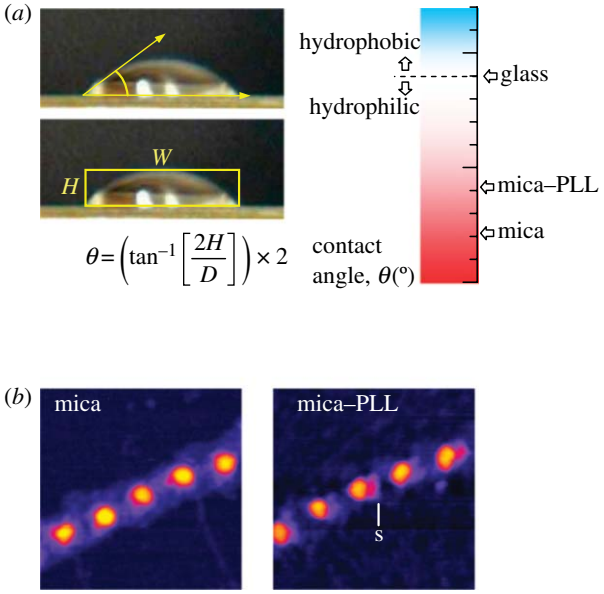

Figure 6. Substrate hydrophilicity/hydrophobicity and fibrillin microfibril morphology. (*a*) Drops of liquid on a surface assume a shape which depends on the relation between the free energies of the three involved surfaces. The contact angle (θ), determined from the height (H) and width (W) of the drop, increases with decreasing substrate hydrophilicity. (*b*) Foetal bovine aorta fibrillin microfibrils adsorbed on hydrophilic mica and less hydrophilic mica + poly-L-lysine (mica–PLL). Intermittent contact mode atomic force microscopy (AFM) height images, 250 × 250 nm, *Z*-scale 10 nm. Fibrillin microfibrils are more diffuse on mica and lack the shoulder region (s) which is present in microfibrils adsorbed on mica–PLL.

(a) *Understanding elastic fibre formation*

In order to better exploit elastic fibre molecules in tissue engineering, there is a need to understand the molecular interactions that normally occur during elastic fibre formation *in vivo*. For initial microfibril assembly, homotypic interactions of fibrillin-1 appear to be critical. Our recent studies have demonstrated specific high affinity binding between amino- and carboxy-terminal fragments that may form the basis of head-to-tail linear assembly of fibrillin-1 (Marson *et al.* 2005). MAGP-1 showed calcium-dependent binding to the fibrillin-1 N-terminal region (encoded by exons 1–8), which localizes to the beads. These fibrillin-1 interactions may regulate pericellular microfibril assembly.

The next stage in elastic fibre assembly is the deposition of tropoelastin on microfibrils. The molecular basis of fibrillin-1 interactions with tropoelastin and MAGP-1 has also been defined (Jensen *et al.* 2001; Rock *et al.* 2004). Binding assays revealed high affinity calcium-independent binding of two overlapping fibrillin-1 fragments (encoded by central exons 18–25, 24–30) to tropoelastin, which, in microfibrils, map to an exposed 'arms' feature adjacent to the beads. A further binding site within an adjacent fragment (encoded by exons 9–17) was within an eight-cysteine motif

designated TB2 (encoded by exons 16 and 17). A novel transglutaminase (TG2) cross link between tropoelastin and fibrillin-1 fragment (encoded by exons 9–17) was localized by mass spectrometry to a sequence encoded by exon 17. The high affinity binding and cross-linking of tropoelastin to a central fibrillin-1 sequence imply that this association is fundamental to elastic fibre formation. MAGP-1 may form secondary interactions with adjacent microfibril-bound tropoelastin.

Fibrillin-1 has also been shown to interact with LTBP-1 (Isogai *et al.* 2003), fibulin-2 (Reinhardt *et al.* 1996), versican (Isogai *et al.* 2002), small chondroitin sulphate proteoglycans (Kielty *et al.* 1996) and with heparin and heparan sulphate. Heparin inhibits microfibril assembly and was shown to interact with fibrillin-1 at three sites (Tiedemann *et al.* 2001). Using BIAcore 3000 technology to investigate fibrillin-1 interactions with heparin, and with heparin saccharides analogous to S-domains of heparan sulphate, we have now shown that there are four high-affinity heparin-binding sites on fibrillin-1, located in three of these sites and defined their binding kinetics (Cain *et al.* 2005). Heparin does not inhibit fibrillin-1 N- and C-terminal interactions, but heparin and MAGP-1 compete for binding to the fibrillin-1 N-terminus, and heparin and tropoelastin compete for binding to a central fibrillin-1 sequence. By modulating these interactions, heparin may regulate microfibril and elastic fibre assembly.

(b) *Effects of surface chemistry on microfibril organization*

Current models of fibrillin-1 alignment within microfibrils are based on the prominent 56 nm periodic beaded morphology of isolated, often apparent by rotary shadowing, microfibrils on mica and by negative staining microfibrils on carbon-coated grids (Baldock *et al.* 2001). However, surface chemistry, especially hydrophobicity/hydrophilicity, has a profound effect on microfibril beaded morphology and presumably also functional interactions (Sherratt *et al.* 2004, 2005; figure 6). We showed that microfibrils adsorbed to a hydrophilic mica substrate adopted a diffuse morphology, but adsorbed to mica coated with poly-L-lysine (PLL) or to borosilicate glass substrates had a more compact morphology and a directional asymmetry to the bead, which was not present on mica alone. Intermediate morphologies were observed along a substrate gradient. We also examined the morphology of microfibrils on topographically similar, chemically homogeneous substrates with a larger amphiphilic range, silicon oxide and silicon oxide coated with polymer films of PLGA and self-assembled monolayers of octadecyl trimethylchlorosilane. Extremes of substrate amphiphilicity profoundly altered periodicity and curvature and induced lateral spreading, implying the disruption of domain structure. Microfibrils adsorbed to a substrate with an intermediate amphiphilicity (PLGA) were similar in dimensions and morphology to hydrated microfibrils. Substrate-induced conformational changes are thus highly likely to direct the biological properties of elastic fibre components adsorbed to artificial surfaces for tissue engineering purposes (Yang *et al.* 2002; Sherratt *et al.* 2004, 2005).

(c) *Elastin coacervates*

Tropoelastin is a highly non-polar molecule, consisting essentially of 34 alternating hydrophobic and cross-linking domains (Kielty *et al.* 2002; Mithieux & Weiss 2005). The cross-linking domains contain the lysine residues destined to form the covalent intermolecular cross links through the actions of LOXs that stabilize the assembled polymer. The hydrophobic domains are thought to be sites of interactions that contribute to the juxtaposition of lysine residues in preparation for cross link formation. *In vitro*, tropoelastin has an intrinsic capacity to form an ordered assembly through a process of hydrophobic self-aggregation or 'coacervation', in which the protein comes out of solution as a second phase on an increase in solution temperature. The kinetics of the transition appears to be that of a nucleation process. The temperature at which this transition takes place is dependent on elastin concentration, ionic strength and pH (figure 7). Analysis of the ability of individual elastin domains to coacervate and self-assemble has revealed that most domains can self-assemble but only a few (especially domains encoded by exons 18, 20 and 24) can coacervate (Pepe *et al.* 2005; Tamburro *et al.* 2005*a*). New evidence for contact points between tropoelastin monomers (domains encoded by exons 19–25) has been obtained using cross-linking, protease digestion and mass spectrometry (Mithieux *et al.* 2005; Wise *et al.* 2005).

The coacervation behaviour of short recombinant fragments of tropoelastin has been described in detail. As few as three hydrophobic domains flanking two cross-linking domains of human tropoelastin are sufficient to support a self-assembly process that aligns lysines for zero-length cross-linking, resulting in the formation of the cross links of native elastin and with solubility and mechanical properties similar to native elastin (Bellingham *et al.* 2001; Keeley *et al.* 2002; Miao *et al.* 2003). The structures of both cross-linking and hydrophobic domains have significant effects on the assembly of these short elastin polypeptides (Miao *et al.* 2005). This process provides the basis for fabrication of vascular polymeric elastin-like matrices for incorporating into vascular grafts. Interestingly, in a variable temperature *in situ* AFM study, these elastin peptides self-assembled in a substrate-dependent manner. On hydrophilic mica surfaces, the peptides were adsorbed as discrete, rounded aggregates. Adsorption to hydrophobic highly ordered pyrolitic graphite (HOPG) induced a fibrillar arrangement (Yang *et al.* 2002). The order observed on HOPG substrates may be due to hydrophobic peptide–substrate interactions, which form an energetically close-packed arrangement acting as a template for fibril growth.

Another group has recently described the production and properties of massive synthetic elastin assemblies formed by chemically cross-linking recombinant human tropoelastin with bis(sulphosuccinimidyl)-suberate, permitting the construction of elastic sponges, sheets and tubes (Mithieux *et al.* 2004). These synthetic elastin constructs also had similar extensibility properties to those of native elastin, with Young's modulus ranging from 220 to 280 kPa with linearity of extension to at least 150%. The constructs

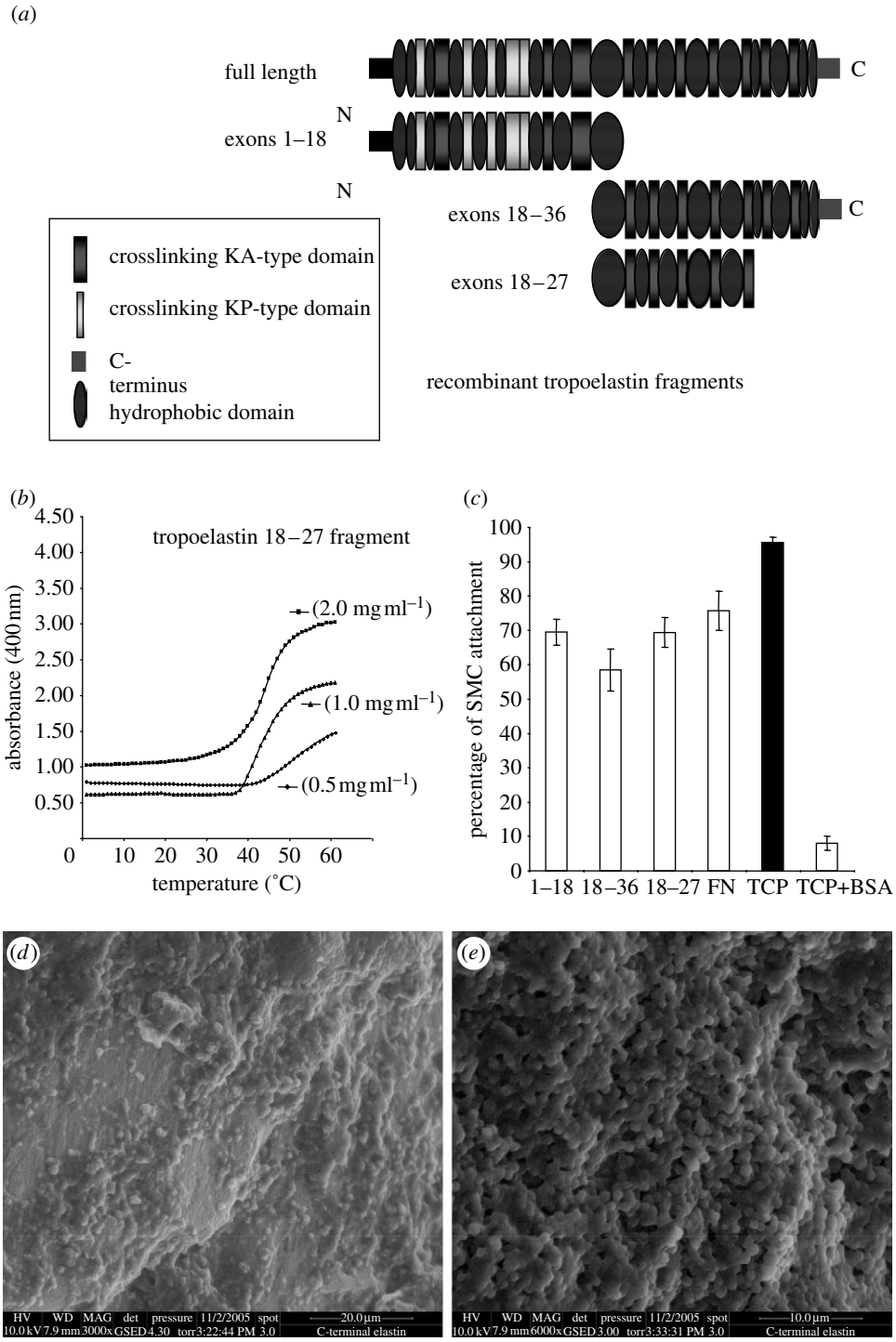

Figure 7. (*a*) Schematic of full-length human tropoelastin, and recombinant tropoelastin fragments that we have expressed and purified for vascular tissue engineering. (*b*) Concentration-dependent coacervation, at increasing temperature, of a recombinant human tropoelastin fragment encoded by exons 18–36, monitored at OD_{400}. (*c*) Human aortic SMC attached well to tropoelastin fragments (encoded by exons 1–18, 18–36 and 18–27) that were coated onto tissue culture plastic (TCP). Fibronectin (FN) and untreated TCP were positive controls; cells blocked with bovine serum albumin (BSA) were negative controls. (*d*,*e*) Environmental scanning electron microscopy images of 50°C coacervate of recombinant human tropoelastin fragment encoded by exons 18–36 (2 mg ml^{-1}). The coacervate had been crosslinked using 3,3′-dithiobis-(sulphosuccinimidylpropionate).

behaved as hydrogels and displayed stimuli-responsive characteristics towards temperature and salt concentrations. Growth and proliferation of cells were supported *in vitro*, while *in vivo* implants were well tolerated. A separate study revealed that the polypeptide sequence encoded by human tropoelastin exon 30 assembles with an ultrastructural organization similar to amyloid networks, with anti-parallel β-sheet conformation predominant in the exon 30 fibres (Tamburro *et al.* 2005*b*).

We have produced recombinant human tropoelastin molecules and fragments, and have characterized their coacervation and cross-linking characteristics in the absence or presence of other elastic fibre molecules including fibrillin-1 (figure 7). We are now developing elastin–fibrillin composites for cell-seeding gels

and scaffold coatings. In a collaborative study with A. S. Weiss (Sydney, Australia), we have shown that elastin coacervation occurs at a lower temperature in the presence of a fibrillin-1 fragment which contains the transglutaminase cross link site to elastin (Clarke *et al.* 2005).

(d) *Elastin-based vascular graft coatings and cell-seeding gels*

Since the ECM is the authentic substrate for connective tissue cells including SMCs and ECs, cells cultured within such a biological matrix experience a richer, more complex physical environment and markedly different geometry than cells on flat surfaces (Cukierman *et al.* 2001, 2002). Cells sense their surrounding ECM, are directly anchored to it, respond to ECM through cell–matrix adhesion signals that influence cell growth, migration, differentiation, survival, tissue organization and remodel their surrounding matrix. Three-dimensional vascular ECM materials are thus powerful tools for modulating grafts and regulating vascular cell phenotype within them (Gobin & West 2003; Grinnell 2003; Hubbell 2003).

There is currently much interest in the engineering of polypeptides incorporating elastin motifs to generate novel hydrogels and drug-delivery polymers (Bandiera *et al.* 2005; Haider *et al.* 2005; Herrero-Vanrell *et al.* 2005; Junger *et al.* 2005). We are developing cell-seeding gels based on cross-linked elastin matrices (figure 7) that can be used to deliver cells onto and within our vascular graft scaffolds. Such gels may be used to direct cell proliferation, regulate cell migration and ECM deposition within the scaffold, and control the delivery and release of TGFβ1 growth factors. Elastin composite gels can be formed by incorporating other vascular cell–matrix motifs, including fibrillin-1 and fibulin-5 fragments.

Recombinant vascular matrix molecules generated in our laboratory have been used to coat vascular scaffolds, in order to enhance SMC and EC attachment and to regulate their function. We have shown that SMCs adhere well to tropoelastin (figure 7), fibrillin-1 (Bax *et al.* 2003) and fibulin-5 (Lomas *et al.* in press), while ECs adhere to tropoelastin fragments, as well as to fibrillin-1 and fibulin-5 in an RGD-dependent manner (figure 5).

(e) *Elastic fibres in the controlled delivery of vascular growth factors*

In natural vessels, growth factors influence vascular cells and ECM deposition, and their activation is, in turn, tightly regulated by ECM. Crosstalk between growth factor and integrin receptor signalling pathways profoundly influences growth factor effects. Thus, it is important that strategies are devised which allow the controlled release of vascular growth factors during graft engineering. Bioactive polymers are now being engineered which not only provide physical support and a template for cells but also closely mimic the *in vivo* release mechanisms of vascular growth factors from the ECM (Zisch 2004).

TGFβ family growth factors are particularly important in the vasculature and in vascular tissue engineering (Ghosh *et al.* 2005). TGFβ1 stimulates SMC proliferation and ECM deposition, and roles required of SMCs during graft pre-conditioning. TGFβ3 has anti-scarring properties, and we have shown that it reduces scarring at carotid artery anastomotic sites after grafting (Ghosh *et al.* 2006). Elastic fibre molecules have great potential for regulating TGFβ activity in grafts. The molecular mechanism of LTBP-1 (which interacts with fibrillin microfibrils) binding to the TGFβ1 latency-associated peptide (LAP) has recently been resolved (Chen *et al.* 2005). We are investigating whether LTBP-3 can be used for controlled delivery of TGFβ isoforms within grafts. Using recombinant fragments, we have shown that that it interacts with a fibrillin-1 sequence, and can, in this way, be stably incorporated into elastic fibre materials. It may also be possible to exploit the heparan sulphate-binding properties of elastin and fibrillin-1 to regulate the delivery of vascular endothelial growth factor and FGF-2 in grafts.

(f) *Elastin products in vascular graft fabrication*

Purified elastin products can be used directly in vascular graft fabrication. Elastin blends, containing collagen and PLGA, have been electrospun (Stitzel *et al.* 2006). This technology controls composition, structure and mechanical properties of the resulting biomaterial. These scaffolds possess biocompatibility and mechanical properties similar to native vessels (Stitzel *et al.* 2006). Controlling the shape and size (diameter or width) of electrospun fibres by varying solute concentration and polymer delivery rate yielded α-elastin and tropoelastin fibres that were several microns in width (Li *et al.* 2005). These fibres supported attachment and growth of human embryonic mesenchymal cells. Intact purified elastin was incorporated into collagen-based tissue-engineered blood vessels, forming hybrid constructs that mimicked arterial physiology and exhibited improved mechanical properties (Berglund *et al.* 2004). Porous scaffolds composed of elastin and collagen prepared by freeze drying showed good strain recovery; these products could be chemically cross-linked, which gives increased stiffness while preserving open porous structure during cross-linking (Buttafoco *et al.* 2006).

(g) *Role of elastin as a non-thrombogenic coating*

It has been shown that a recombinant human elastin polypeptide can act as an anti-thrombogenic coating on synthetic scaffolds (Woodhouse *et al.* 2004). Three commercially available synthetic materials coated with adsorbed elastin all demonstrated reduced platelet activation and adhesion in platelet rich plasma *in vitro*. When compared with non-coated controls, there was a significant decrease in platelet microparticle release and P-selectin expression for the polypeptide-coated surfaces. Scanning electron microscopy showed fewer adhering platelets on coated surfaces when compared with non-coated controls. *In vivo* evaluations of PU catheters coated with the polypeptide showed a marked increase in catheter patency and a significant decrease in fibrin accretion and embolism when compared with uncoated controls. Thus, this and

other tropoelastin polypeptides show a strong potential for use as a non-thrombogenic coating for small diameter vascular grafts.

7. ELASTIC FIBRE DEPOSITION IN VESSEL REGENERATION

The deposition of elastic fibres is tightly developmentally regulated, and in adult tissues elastic fibre regeneration is limited. However, several recent strategies suggest new approaches to stimulate the deposition of ordered functional elastic fibres for vascular repair.

(a) *Stimulation of adult vascular elastic fibre deposition by versican V3 isoform*

Retroviral-mediated overexpression of the versican proteoglycan variant V3, which lacks chondroitin sulphate chains, has been shown to alter arterial SMC phenotype in short-term cell culture. These cells exhibited significantly increased expression of tropoelastin and increased formation of elastic fibres in long-term cell cultures (Merrilees *et al.* 2002). When V3-overexpressing SMCs were seeded into ballooned rat carotid arteries, by four weeks they had produced a highly structured neointima significantly enriched in ordered elastic fibre lamellae containing elongated SMC arranged in parallel arrays and separated by densely packed elastic fibres and collagen bundles. Reduction of versican using an antisense approach caused flattened SMC morphology, reduced cell proliferation and migration, increased tropoelastin synthesis, increased EBP and increased the deposition of elastic fibres in long-term cultures, but incorporation of chondroitin sulphate reversed these effects. Thus, versican and its constituent chondroitin sulphate chains play a central role in controlling cell phenotype, elastogenesis and intimal structure (Huang *et al.* 2006). The V3 variant of versican thus offers a powerful new therapeutic approach for the deposition of elastic fibres in vascular conduits.

(b) *Using fibrin gels to enhance elastic fibre deposition*

It was recently reported that SMCs seeded within fibrin gels remodel their ECM environment over four weeks and deposit abundant elastic fibres (Long & Tranquillo 2003). This was the first observation of ordered, cross-linked elastic fibre deposition in tissue-engineered replacements fabricated *in vitro* with SMC, and indicates that *in vitro* elastogenesis can be achieved for three-dimensional elastic structures.

Recently, small diameter vessels based on ovine SMCs and ECs embedded in fibrin gels have been described (Swartz *et al.* 2005). Vessels implanted into lamb jugular veins demonstrated patency and similar blood flow rates as native vessels. By 15 weeks post implantation, they exhibited remarkable matrix remodelling with the production of abundant elastic and collagen fibres and orientation of SMC perpendicular to the direction of blood flow. Thus, fibrin-based graft models hold significant promise for vascular graft design.

(c) *Biomechanical forces to regulate elastic fibre molecular expression*

In the healthy artery, radial stretch of the vessel wall is the predominant physical variable that influences SMC phenotype, since shear forces are separated from the tunica media by an intact intima (Baguneid *et al.* 2004; Hamilton *et al.* 2004). Although SMCs are not normally exposed directly to the shear stresses of flowing blood, EC desquamation in diseased or injured arteries exposes underlying SMCs to uncharacteristically high shear forces. Interstitial flow across the vessel wall, driven by the transmural pressure differential, also influences SMCs (Wang & Tarbell 1995; Civelek *et al.* 2002). Although its superficial velocity is typically very low, the interstitial spaces in the tissue are small and the shear stress on SMCs can be significant, up to 1–3 dyn cm^{-2} in rabbit aortas (Wang & Tarbell 1995; Civelek *et al.* 2002). Fluid flow of blood through the vascular system has been variously reported to affect SMCs in terms of gene expression, increased proliferation (Kim *et al.* 1999), decreased proliferation (Ueba *et al.* 1997; Liu *et al.* 2003), increased contraction (Civelek *et al.* 2002), alignment perpendicular to flow (Lee *et al.* 2002), alignment parallel to flow (Mcintire *et al.* 1998), increase in apoptosis (Apenberg *et al.* 2003) and increase in nitrate production. These various flow-induced changes to SMCs may be dependent on differences in tissue origin, species, culture conditions, and the type and intensity of shear stress applied.

Since shear stress influences SMC phenotype and may be exploited to modify SMC in grafts, we have examined the influence of *in vitro* shear stress on the expression and deposition of elastic fibre components by human coronary artery SMC, and the role of TGFβ1 in regulating these effects (Ghosh *et al.* 2005). In static culture, elastin and fibrillin-1 mRNAs were in very low abundance, but after exposure to 3 h of shear stress at 15 dyn cm^{-2} both mRNAs were readily detected. These effects were mediated by TGFβ since neutralizing antibodies blocked the upregulation. We are currently investigating the effects of cyclical stretch on elastic fibre deposition by SMC.

8. CONCLUSION

Tissue-engineered vascular graft design has advanced greatly in recent years, but several major issues remain to be addressed. These include improving scaffold compliance match, strong luminal attachment of ECs that retain anti-thrombotic and vasoactive properties, population of grafts with contractile SMCs and deposition of an ordered functional elastic fibre-rich vascular matrix that contributes to long-term graft contractile function. This review has outlined how elastic fibre biology can be used to address all of these concerns, since elastic fibres are major elastomeric components of vascular walls, prominent vascular cell adhesion macromolecules and natural delivery systems for vascular growth factors. Moreover, the unique aggregation characteristics of elastin itself are now providing the basis for many novel cell-seeding and drug-delivery hydrogels, and composite gels incorporating specific cell–matrix signals are being developed.

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