SWITCHING OFF ANGIOGENIC SIGNALLING: CREATING CHANNELLED CONSTRUCTS FOR ADEQUATE OXYGEN DELIVERY IN TISSUE ENGINEERED CONSTRUCTS

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Abstract

A major question in biomimetic tissue engineering is how much of the structure/function of native vasculature needs to be reproduced for effective tissue perfusion. O_2 supplied to cells in 3D scaffolds *in vitro* is initially dependent upon diffusion through the scaffold and cell consumption. Low O_2 (3%) enhances specific cell behaviours, but where O_2 is critically low (pathological hypoxia) cell survival becomes compromised. We measured real-time O_2 in 3D scaffolds and introduced micro-channelled architecture to controllably increase delivery of O_2 to cells and switch off the hypoxic response. Simple static micro-channelling gives adequate perfusion and can be used to control cell generated hypoxia-induced signalling.

Keywords: 3D collagen scaffolds, channelled architecture, physiological hypoxia, bone marrow stromal cells, hypoxia-inducible factor-Iα.

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Introduction

Within the fields of tissue engineering (TE) and tissue modelling (TM), the formation of 3D cell-seeded constructs poses problems with metabolite permeability, including O_2 across longer, cell-dense matrix pathways, as delivery of factors is mainly through diffusion. Given the nature of an engineered cell scaffold, a native vasculature does not exist, potentially leading to damaging deep hypoxia with changed cell behaviour, even cell death, due to superficial cell consumption and long diffusion pathways, especially within the core of 3D constructs. Whilst synthetic polymer scaffolds have the advantage of pre-determined architecture, including porosity, which can be controlled to aid free-circulating culture media to supply cells throughout the 3D structure, they are poorly biomimetic at the meso-scale level (Rouwkema *et al*., 2008). Synthetic scaffolds cannot mimic the mechanical and protein properties of native materials, and therefore collagen type I is an ideal biomimetic support for engineering living tissues (Howard *et al*., 1996).

The dependence on O_2 (as well as other nutrients) for cells and tissues is evident by the extensive vascular network throughout the mammalian body (Fraisl *et al*., 2009), and mimicking this vasculature is critical for successful tissue engineering. The development of a vascular network is dependent upon multiple cell types (including endothelial cells (ECs), smooth muscle cells and pericytes) and a well-orchestrated cascade of angiogenic factors regulating vessel assembly (Hirschi *et al*., 2002). The majority of these are under the control of the transcription factor hypoxia-inducible factor 1α, which is a master O_2 sensor, effectively initiating cells responses to low O_2 environments (Semenza, 1999). Specific angiogenic factors act at precise points along the process of vessel assembly. Fibroblast growth factor 2 (FGF2) has a role in stimulating endothelial cell migration and proliferation in early vessel assembly (Beenken and Mohammadi, 2009). In addition to this, for smooth muscle cell migration and vessel wall assembly (maturation and stabilisation of the vessel) FGF2 is also critical (Kenagy *et al*., 1997). Vascular endothelial growth factor (VEGF) signalling is essential during the early stages of vessel assembly and this growth factor is tightly regulated. VEGF not only promotes the survival of ECs, but also acts as a chemo-attractant for ECs, implying a role in the migration and invasion of ECs (Byrne *et al*., 2005). In particular tip cells (i.e. the leading cells in a growing vessel sprout) are navigated along gradients of factors including VEGF (Fraisl *et al*., 2009). Transforming growth factor β (TGF

β) and platelet derived growth factor (PDGF-B) have also been implicated in the process of angiogenesis, however it is fair to say that there may be further factors critical to the process of angiogenesis which as of yet are unknown (Hirschi *et al*., 2002).

The host of factors involved in the process of angiogenesis and their tightly regulated concentrations are challenging to administer using current technologies, and we have therefore employed a technique to manipulate cells in 3D by the creation of low O_2 environments, so that they are induced to manufacture the entire angiogenic cascade in cell-regulated concentrations (Hadjipanayi *et al*., 2010). Due to the lack of vasculature, gradients of O_2 exist from the surface to the core of 3D cell-seeded constructs, and are dependent upon perfusive properties of the scaffold material and cell consumption of O_2 (Cheema *et al*., 2008; Malda *et al*., 2004). By manipulation of cell density and path-length these gradients can be controlled and in some experimental cases, provide good models to test the effects of varying O_2 concentration on cell behaviour. However, for the survival of a 3D TE model, an adequate, even and continuous supply of O_2 is critical for viability of the core cells.

Tailoring the material properties of 3D tissue constructs to the O_2 demand of resident cells is a challenge. This is even more so when O_2 itself can be used to influence cell behaviour, and control the signals which resident cells produce. As an example, cell exposure to low physiological O_2 can stimulate the upregulation of angiogenic factors, which *in vivo* would induce angiogenesis (Semenza, 1999). This is advantageous when building a model tissue, as along with the incorporation of ECs (the building blocks of vessels) these angiogenic proteins stimulate the production of primitive capillary networks (Simon and Keith, 2008). TE would benefit hugely if simple flow micro-channels, introduced into tissue constructs, could substitute for endothelialised tubes, until the construct integrated with the host vasculature.

O_2 is a particularly good cue for angiogenic host production, as it is one of the major factors upregulating angiogenic factors *in vivo*, but also because employing a continuous O_2 monitoring system in 3D allows precise readout of what cells were exposed to over 7 days (Cheema *et al*., 2008). In adult bone marrow, progenitors are exposed to O_2 levels of ~20 mmHg (equivalent to just under 3% O_2), this must be important in maintenance of multi-potency (Fraisl *et al*., 2009). However the precise levels of O_2 required for either inducing differentiation or inducing specific angiogenic responses remain elusive. Within the developing embryo (~25mmHg) low levels of O_2 stimulate the early mesoderm into haemangioblasts (Ramirez-Bergeron *et al.* 2004). Even within the vessel wall cells are exposed to different O_2 levels dependent upon their location, and as such when cells are removed from their distinct location they have differing O_2 requirements (Cheema *et al*., 2009).

Here we describe a method to produce dense cell-seeded collagen constructs, where resident cells in the core up-regulate a host of angiogenic factors when exposed to low O_2. This up-regulation can be effectively 'switched off' by incorporating phosphate-based glass fibres (PGFs) which dissolve over 24 hours to leave channels in the 3D construct, hence supply O_2 to core cells. Previous work has shown the continuous nature of such channels created in collagen, when microbubbles were tracked through the channel lumen (Nazhat *et al*., 2007). We compare cell viability and regulation of angiogenic signals in constructs with/without channels. Our main hypothesis is that dissolution of PGFs will leave channels to increase O_2 to core cells, and switch off angiogenic cell signalling, which is normal when cells are cultured at defined levels of physiological hypoxia. Therefore this approach provides a method to control when angiogenic signalling is switched on by cells in 3D (when exposed to low O_2 in the core), then switched off by the introduction of channels (hence increase O_2), and potentially leave architectural features, in which a new cell type (ECs) can be seeded to form a primitive capillary network. In this study the changing expression of angiogenic signals is used as a read-out of deep cell perfusion. We have used Human Bone Marrow derived stromal cells (HBMSCs) as the cell type studied due to the multi-potency of such cells and the clinical relevance as they are widely used in clinical practise. As well as this HBMSCs have been shown to be stem-cell like in nature and may the capability to differentiate to become cell relevant for angiogenic engineering.

Materials and Methods

Cell culture

Human bone marrow-derived stromal (or stem) cells (HBMSCs) were plated from whole blood (obtained from the operating theatre, with ethical approval from the local joint RNOH/IOMS ethics committee), as previously described (adapted from Igarashi *et al*., 2007). Cells were maintained in low glucose (1000mg/L) Dulbecco's Modified Eagles Medium (DMEM, Gibco, Paisley, UK), supplemented with 20% foetal calf serum (FCS), (First Link, Wolverhampton, West Midlands, UK), 2mM glutamine and penicillin/streptomycin (1000u/ml; 100μg/ml, Gibco). For removal of cells from monolayer culture (all <70% confluence), flasks were washed with 0.1M Phosphate Buffered Saline (PBS), and incubated with trypsin (0.5%) for 5 minutes at 37°C. The media specifications (i.e., low glucose and 20% FCS) were taken from published literature for optimising HBMSC growth.

Collagen scaffold preparation

0.5 ml of 10x Eagle's MEM solution (Gibco) was added to 4ml of rat-tail type I collagen (2.04mg/ml, First Link) in 0.1M acetic acid and neutralised with 1M NaOH. Once neutralised, $2x10^6$ cells were added, and gels were set for 30 minutes at 37°C. Collagen gels were then placed on layers of blotting paper, between nylon mesh, and loaded with a 120g weight for 5 minutes at room temperature, giving flat collagen sheets (50-60μm thick) protected between 2 nylon meshes. Gels were routinely compacted by this combination of compression and blotting (with no effect to cell viability (Brown *et al*., 2005). Dense single sheets of collagen were then rolled to produce a tight spirally wound rod, 2.3 mm diameter and 21 mm length.

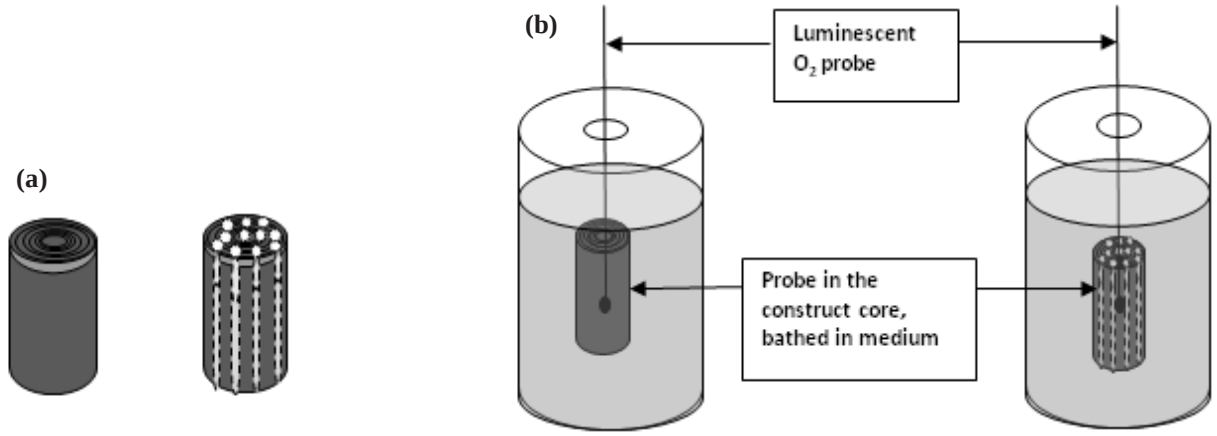

Fig. 1. (a) 3D Collagen construct schematic, with/without fibres. Over 24 hours the fibres dissolve, leaving continuous channels. **(b)** Diagrammatic representation of culture set-up with real-time O_2 monitoring.

The collagen density after compaction was 11%. Unidirectional fast degrading PGFs having composition (in mol fraction) of 0.50 P_2O_5-0.25 CaO-0.25 Na_2O, diameter of ~40μm (inter-fibre spacing of ~170 μm) were prepared and incorporated into collagen construct by laying on top of the gel prior to compression, aligned parallel to axis of spiralling (fig. 1a) as previously described (Nazhat *et al*., 2007).

Scanning electron microscopy

Constructs were fixed in 4% paraformaldehyde (in 0.1M sodium cacodylate buffer) and 1% tannic acid (in 0.05M sodium cacodylate buffer) both for 1 hour, followed by dehydration through an alcohol series to hexamethyldisilazane with air drying. Samples were then gold-palladium sputter-coated, and then viewed using a JEOL (Tokyo, Japan) 5500LV scanning electron microscope (SEM).

Real-time O_2 measurement

Fibre-optic oxygen probes (Oxford Optronix, Oxford, UK) were inserted into the centre of the 3D constructs to place the measuring tip halfway along their long axis (Fig. 1b). This gave a diffusion length of >1 mm (from surface to core, perpendicular to the long axis, here termed lateral diffusion). The collagen constructs are spiralled and form robust 3D structures. These were not tethered, however when a fibre-optic probe was inserted in the core, it was possible to position the probe (with an external clamp) in a pot of media, so the construct was bathed within the media (and essentially held in place by the probe running through its core). The sensor probe (280 μm diameter) incorporates an oxygen sensitive luminescent probe, which is quenched in the presence of molecular oxygen so that the luminescence emission lifetime is extended longer at lower local oxygen tensions. The calibration of the probe (accurate to 0.7 mmHg), correlating the luminescence lifetime *versus* the oxygen concentration (Seddon *et al*., 2001), gives an exceptionally stable and calibrated response. After each experiment, the probe reading was checked in the external medium to confirm that there was no drift in the response. The fibre-optic probes were used in conjunction with an OxyLab (Oxford Optronics, Oxford, UK) pO2 E™ system coupled to an analogue/digital converter (12 bit).The results were recorded on a standard laptop using Labview (National Instruments, Austin, TX, USA) and are presented as partial pressure values, i.e., pO_2 in mmHg (e.g., 7.6 mmHg corresponds to 1% O_2).

Quantitative PCR

RNA was extracted from the spiral construct, by unrolling and dissecting out the core. Total cellular RNA was isolated using the Qiagen RNeasy method (Qiagen UK, Crawley, West Sussex, UK). RNA was measured using the Nanodrop™.

First strand cDNA synthesis was performed using Amplitaq Reverse Transcriptase (Applied Biosystems, Roche, Basel, Switzerland). Total RNA (0.5 μg RNA in 40 μl) was added to 9.15 μl of mastermix (dNTP, RNase inhibitor, MgCl, Oligo DT Random primers), and heated at 70°C for 10 minutes. Then 2 μl of Reverse Transcriptase was added to each tube and incubated at 40°C for 1.5 hours, followed by heating at 90°C for 2 minutes.

Relative quantitative PCR was performed using Applied Biosystems 7300 Real-time PCR system (Carlsbad, CA, USA), with the Taqman universal PCR Master Mix. 9 μl of the cDNA, 1μL of the required gene probe (Assay IDs: 18S RNA: HS99999901_s1; VEGF: Hs00900057_m1; HIF-Iα: HS00936366_m1; FGF2: HS00266645_m1) and 10 μl of Mastermix were mixed per sample for cycling and analysis in the Applied Biosystems 7300 Real-time PCR machine. The primer sequences are kept confidential by Applied Biosystems. The combined thermal cycling and amplification-specific software enabled detection of the PCR products as cycle-by-cycle accumulation in a single-tube reaction.

CT (cycle threshold) values, indicating when the PCR product was amplified in a logarithmic manner, were ascertained using the Applied Biosystems software. Values for each sample were normalised to the corresponding 18S RNA result according to the following equation and then values were directly compared.

$$\frac{1}{2^{(\text{CT test} - \text{CT controlgene})}}$$

Signalling experiments were performed independently of the cell viability, and a sample size (n) of 4 per time point and construct type were analysed.

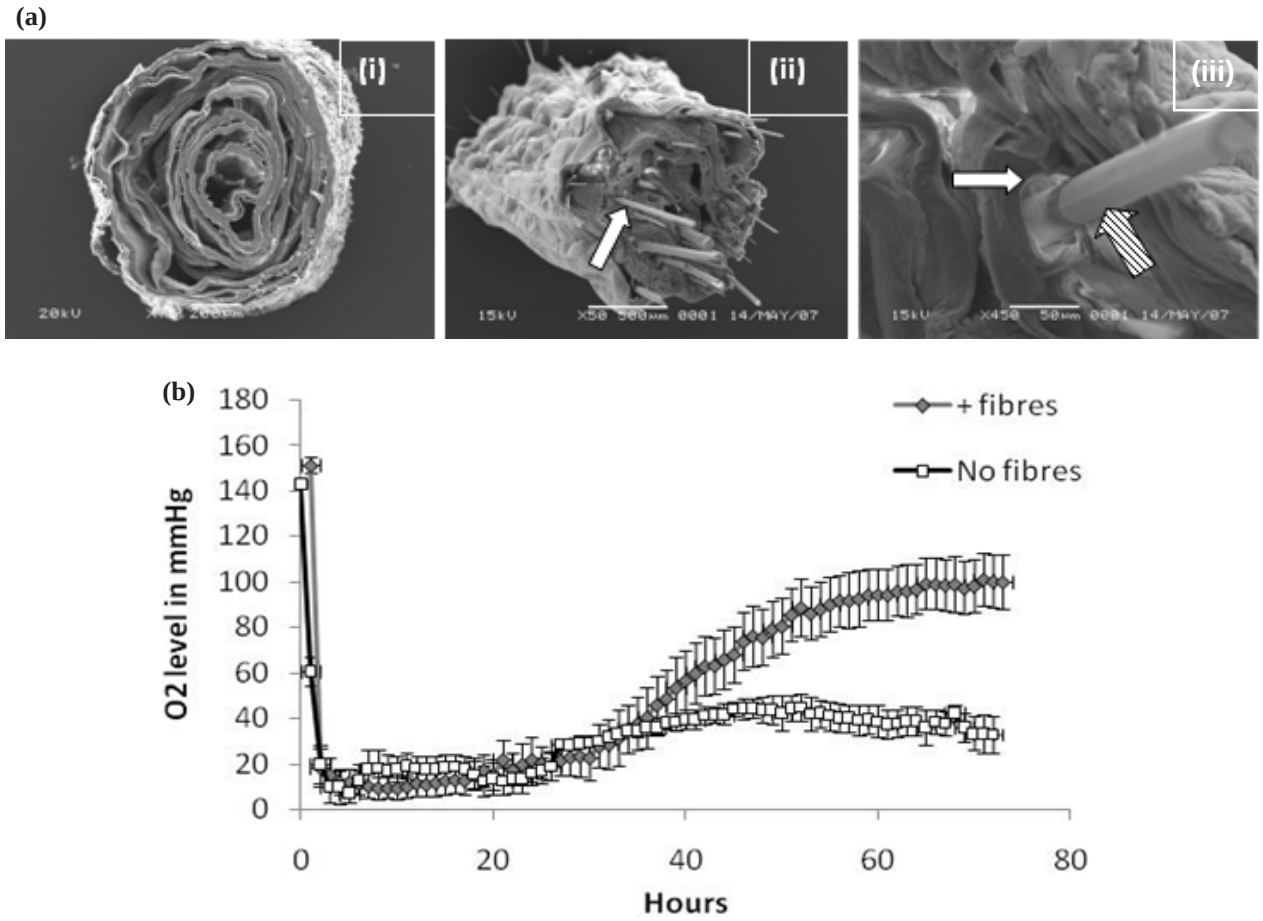

Fig. 2. (a) (i) SEM micrograph of collagen construct, **(ii)** with glass fibres. **(iii)** Construct containing dissolving glass fibres, as the fibre is gradually dissolving the channel forming is visible. Striped arrow indicates the incorporation of a PGF, and plain arrow denotes the channel wall which is left following dissolution of the fibre after 4 hours. **(b)** O_2 levels in the core of cell-collagen constructs with/without dissolving PGFs. By 24 hours the fibres have completely dissolved, and levels of O_2 increase in the core, compared to control constructs without fibres, up to 3 days.

Cell viability

Quantitative analyses were carried out with Live/Dead Reduced Biohazard Viability/Cytotoxity Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA, L-7013) according to the manufacturer's protocol. SYTO® 10, a green fluorescent nucleic acid stain for live cells, and Ethidium homodimer-2, a red stain for dead cells, were used to stain constructs after which images were taken in 10 random fields within the core region using confocal microscopy (BioRad Radiance 2100, Carl Zeiss, Welwyn Garden City, UK). Live/dead nuclei were counted to ascertain percentage viability. Cell viability experiments were performed independently of the signalling, and a sample size (n) of 3.

Results and Discussion

SEM images show the collagen construct without and with embedded PGFs (Fig. 2a (i) and (ii)), with the detail of a single glass fibre gradually dissolving at 4 hours (Fig. 2a (iii)). The dissolution of glass fibres takes 24 h, however, at 4 h it is possible to see some degradation of the fibre, and the channel that is gradually being created, which will be complete when the fibre fully dissolves by 24 h. Continuous O_2 plots show that levels decrease within the initial 2 h of culture time. These low levels (~20 mmHg) were maintained such that resident HBMSCs in the core were exposed to this physiological hypoxia for the first 24 h. There was a gradual increase in O_2 from 40 h onwards, to ~40 mmHg (Fig. 2b), where cells were exposed to a range of O_2 from between 20-40 mmHg (2.6-5.3% O_2). In constructs containing PGFs, however, an increase in core O_2 tension was evident after the 24 h dissolution period (Fig.2b). This produced an increase in O_2 to ~80 mmHg (10.5%) and ~100 mmHg (13.1%) by 48 and 72 h, respectively. The formation of channels therefore increased supply of O_2 to cells within the core. The introduction of microchannel architecture into 3D collagen constructs can be easily controlled by adjusting PGF's chemistry (Abou-Neel *et al*., 2005).

A direct comparison of HBMSC cells with pulmonary artery smooth muscle cells (PASMCs), within this system can be made where at the same cell density O_2 levels dropped to well below 10mmHg in the core of seeded constructs (Cheema *et al*., 2009). The high O_2 demand of PASMCs is interesting as they themselves are already part of the vascular network, whereas EC cells, the first cells

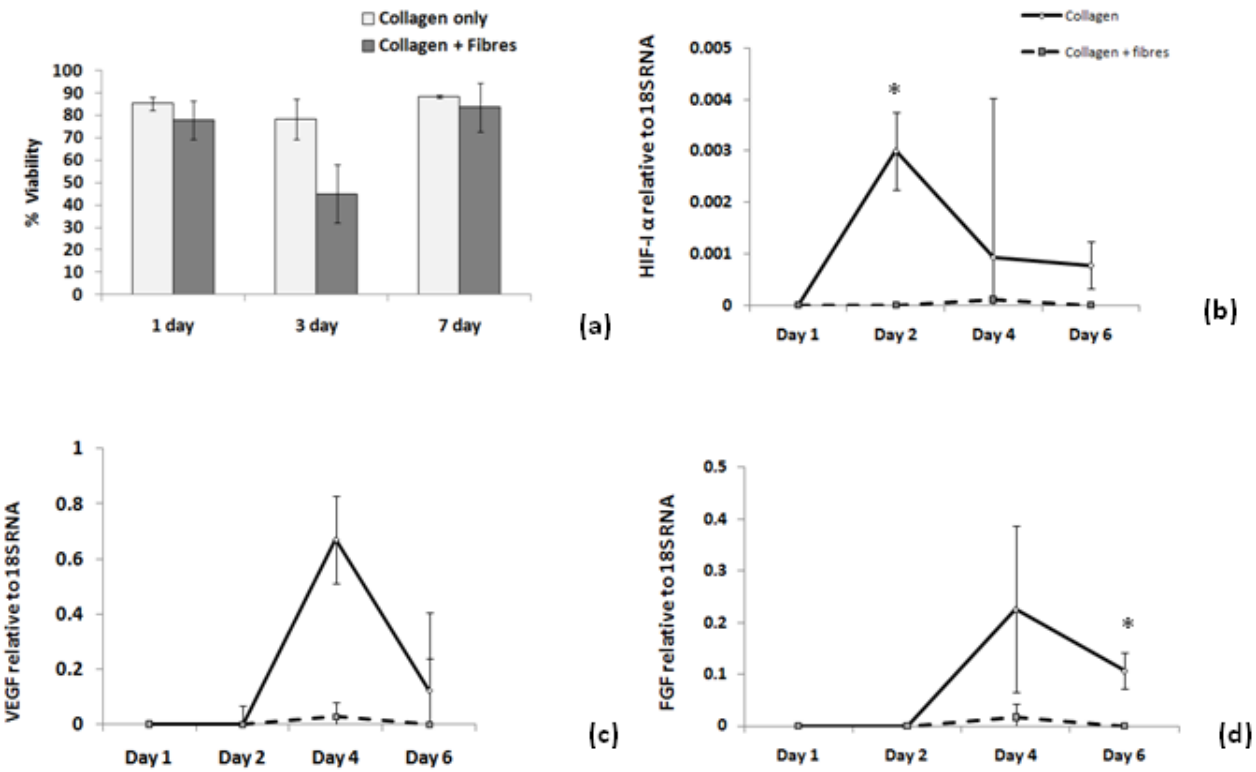

Fig. 3. (a) Viability of Human Bone-marrow derived stromal cells in 3D constructs with and without fibres. **(b)** VEGF in constructs with and without fibres. **(c)** HIF-I **a** in constructs with and without fibres. **(d)** FGF in constructs with and without fibres. *denotes statistically significant differences between the 2 groups using students *t*-test, $p<0.05$.

to come into contact with oxygenated blood in the vascular system have a relatively low O_2 demand, and instead derive most of their energy anaerobically through glycolysis (Quintero *et al*., 2006). A study comparing the angiogenic factor output of these cells should provide further insight into how vascular networks can be ideally engineered in culture.

Viability of cells was established to correlate O_2 exposure to cell survival. There was a decrease in cell viability in collagen/PGFs constructs by day 3 (Fig. 3a). This was due to the release of dissolution products from the glass fibres, as no comparable fall was seen without fibres. Interestingly though, this fall was noticeable not at 24 h, but at 72 h. We hypothesise this to be due to the fact that as it took 24 h for the fibres to completely dissolve, at this point there was a maximal exposure of the known dissolution products to cells in the constructs. As viability wasn't checked at 48 h, the reading at 72 h is simply a late indication of the reduced viability on day 2. The drop in viability can only be directly influenced by the dissolution of PGF fibres, as there was no comparable fall in identical cultures without the fibres. Cell death due to low O_2 in collagen only was minimal. In PGF/collagen constructs and the transient nature of the reduced cell viability was seen by day 7, when all the glass products had disappeared, and viability returned to ~80% (Fig. 3a). This increase in viability must have been due to an increased proliferation rate of cells in the PGF/collagen composite cultures.

Angiogenic signalling was up-regulated when cells were exposed to 20-40 mmHg or 2.6-5.2% O_2. Peak expression of HIF-Iα was at day 2, followed by VEGF at day 4 and FGF2 at day 6, in collagen scaffolds, in response to physiological hypoxia (Fig. 3b-d). However no significant elevation in of any of these angiogenic genes was seen in channelled collagen/PGF constructs despite cell death at day 3 (Fig. 3). This is clear evidence that hypoxia was the stimulus for angiogenesis factor expression that the presence of PGF channels prevented this stimulation.

In addition since cells in both types of constructs were exposed to O_2 levels of ~20 mmHg over the initial 24 h of culture, it would seem that HIF-Iα signalling is not triggered by cells in 3D by short-term exposure to this level of low O_2. Rather, exposure for 24-48 h at this level is needed to initiate angiogenic response and, further, that the response is effectively reset or stopped by rising O_2 during this time period.

Although there was a decrease in cell viability by day 3 in collagen/PGF constructs, qPCR expression data was relative to 18S RNA, and so was corrected for the reduced cell numbers. HIF-Iα is a master O_2 sensor within cells, and a transcription factor for a multitude of downstream factors, including VEGF (Semenza, 1999). Consequently, without HIF-Iα upregulation in collagen/PGF constructs, the up-regulation of VEGF was also prevented. VEGF levels were higher in collagen only constructs by day 4, where O_2 levels remained low (Fig. 3c). Hence, the upregulation of VEGF at day 4 is likely to be HIF-Iα mediated. The upregulation kinetics of HIF-Iα in this 3D system are quite different from studies looking at cells

grown in medium, where HIF-Iα upregulation has been measured within minutes rather than hours or days (Jewell *et al*., 2001). This difference is likely to be due to the differences in cell types studied and O_2 exposure: whereas other studies subject cells to O_2 levels from 0-0.5%, we stayed within above a threshold of 2.5%. The current study is specifically looking at cell behaviour in a 3D native matrix environment, and therefore will have some physiological advantages over studies looking at cells outside of this geometric environment. If a hypoxic response was required, i.e., to produce angiogenic signals to form a primitive vascular network, O_2 levels would either need to be lower in the core (increase cell O_2 consumption), or the fibres chemistry would need to be adjusted to keep core cells exposed to 20-40mmHg O_2 for up to 48 h.

Levels of FGF2 were significantly higher in collagen-only constructs (Fig. 3d) by day 6. FGF2 is a more potent angiogenic factor than VEGF. As it is up-regulated through a different mechanism, it may be suggested that prolonged hypoxia was again the trigger. It can therefore be postulated that many such factors (probably much of the angiogenic cascade) will have been affected and regulated through the low O_2 environment. The role of FGF2 in the process of angiogenesis is two-fold. During the formation of a vessel, the early lineage commitment of ECs is regulated by FGF2 and later on the recruitment of other mural cells (i.e., smooth muscle cells) is also regulated by FGF2 to stabilise the forming vessel (Hirschi *et al*., 2002).

Addition of these PGFs to create channelled architecture in 3D scaffolds is a simple method to deliver O_2 to cells throughout the 3D structure. This study specifically targeted O_2 delivery; however, other nutrients, including glucose, may also be delivered through diffusion *via* the same channels. Any angiogenic proteins produced will diffuse along the path of least resistance, i.e. along the channel tracks. Architectural features in 3D scaffolds can, therefore, be used to both control specific cell behaviour and aid media perfusion. It may be necessary to produce fibres with longer dissolution periods, so resident cells within the core optimally upregulate angiogenic factors, before O_2 is gradually delivered. The degradation of the PGF within the construct is an active process and depends on several factors: (i) Fibre diameter; (ii) Fibre composition; (iii) Spacing between them; (iv) Flow rate of the surrounding medium into the construct (density of collagen construct). These fibres are completely degradable into ionic products (e.g., Ca, Na and PO_4) according to their compositions. These ions can be utilised by the body or easily eliminated by the normal physiological mechanism. Altering the chemistry or diameter of such fibres will allow introduction of the channelled architecture in a controlled, predictable manner.

Conclusion

Timed introduction of a gradual channelled architecture into 3D cell-seeded collagen tissue constructs results in the switching off of angiogenic signalling by resident cells, dependent upon the ambient O_2 tension in the surrounding media. Therefore, simple channelling is an effective means of engineering deep cell perfusion, without the aid of media flow. By monitoring core O_2 levels, and correlating them with cell viability and angiogenic gene upregulation, cell growth in 3D and specific cell signalling can be controlled and optimised. The addition of architectural features within 3D scaffolds, therefore, influences cell behaviour in terms of signalling.

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Discussion with Reviewers

Reviewer I: What is the long term of this study? Can the probe be used *in vivo*? What are the clinical implications of this model?
Authors: The long term implications for this study are two-fold. The first is focused on *in vitro* engineering. In 3D constructs (not necessarily just collagen) the formation of O_2 gradients provides a means to upregulate angiogenic cascades by resident cells, in the form of hypoxia-induced signalling. The cell density can be controlled to optimise this signalling, and the addition of real-time O_2 monitoring means cells behaviours can be attributed to differing O_2 tensions. Once signalling is optimal (i.e. prior to any detrimental affect the hypoxia may have, i.e. enter levels of pathological hypoxia) this can be fed into the design of PGF fibres, so they dissolve at this precise time to leave continuous channels throughout the construct. The presence of angiogenic factors may then be used to introduce a further cell type, endothelial cells, to line the channel lumen, to form a base vascular network.

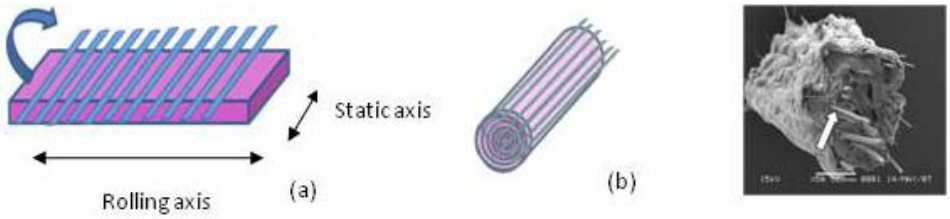
The second implication is *in vivo*, such constructs have the capacity to provide channelled architectural features along which vascular networks and neural networks can anastomose.

Real-time O_2 monitoring using this system has been possible *in vivo* (Hadjipanayi *et al.*, 2010) and this real time measurement of changing *in vivo* local O_2 levels, deep inside the implant, is probably the most effective/relevant functional outcome of angiogenic engineering currently available. This provides direct readout of the integration and likely survival of constructs, making close analysis of the core O_2 data particularly important.

Reviewer II: Please comment on the actual perfusion improvement for a collagen gel by introducing a channelled architecture under static conditions.
Authors: The improved delivery of O_2 in a scaffold where no perfused media system is in place is interesting. It may be expected that without actual flow this delivery would be limited, with little affect on cells in the core of 3D scaffolds. In contradiction to this, we have found significant changes in cell signalling in response to the introduction of simple channels without the aid of perfusion. This finding is of interest to the broad spectrum of 3D scaffold engineers.

Reviewer III: The manuscript describes linear fibres aligned parallel to the axis of spiralling in the construct-would it be possible to have smaller fibres perpendicular to these fibres, to mimic, for instance, the presence of capillaries between arteries and veins? Given time, would endothelial cells line these channels?
Authors: The fibre arrangement within a 3D scaffold is of significant importance, as these channels are designed to try and mimic the vascular network within tissues. Indeed a range of fibre diameters (mimicking larger arteries and veins with smaller capillaries) would be an ideal scenario, and work in this area is currently underway. The arrangement of fibres is slightly more complex given the configuration of the 3D scaffold. By creating a dense 3D sheet of cells and collagen, we rely upon spiralling of this sheet for 3D configuration. However, this only allows relatively rigid PGF fibres to be aligned along the 'static' axis, and not the 'rolling' axis (Supplementary Fig. 1.).

Reviewer III: Does the diameter of these micro-channels correspond to those of new vessels formed during angiogenesis? Would it be possible for cells to travel along with them, as well as the diffusion of oxygen and nutrients?
Authors: The diameter of these dissolving PGF fibres is around 40μm, which does fall into the range of a newly forming vascular network. As this parameter can be modified by altering the diameter of the PGF fibres, different vascular networks can be formed dependent upon



Supplementary Fig. 1. (a) PGF fibres placed onto 3D cell-seeded collagen sheet, and (b) spiralled along the rolling axis, schematic and micrograph. It is only possible to place fibres along the static axis, as they are rigid and therefore will not bend.

the size of tissue engineered construct under development. Of particular importance is the point raised regarding the introduction of endothelial cells (ECs) into these channels. ECs are the primary cell type attracted by angiogenic gradients, particularly VEGF. Our aim is to optimally engineer hypoxia in 3D constructs, and as the fibres dissolve, ECs be introduced (possibly by means of a rolling bioreactor system), which will preferentially travel to line the channel walls. As ECs are quite resistant to hypoxia, it may be further possible to continue the entire construct (even with continuous channels) under physiological hypoxia to maintain hypoxia-induced signalling by cells seeded in the collagen to continue to attract ECs.

Additional Reference

Hadjipanayi E, Brown RA, Mudera V, Deng D, Liu W, Cheema U (2010) Controlling physiological angiogenesis by hypoxia-induced signaling. J Control Release **146**: 309-317.