Journal of Controlled Release 153 (2011) 217–224

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

First implantable device for hypoxia-mediated angiogenic induction

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ARTICLE INFO

Article history:
Received 6 December 2010
Accepted 25 March 2011
Available online 31 March 2011

Keywords:
Nano-fibrillar collagen depot
Hypoxic pre-conditioning
Growth factor delivery
Angiogenesis
Vascularisation

ABSTRACT

Delayed or inadequate vascularisation is one of the major factors leading to tissue infarction and poor graft survival. Current vascularisation strategies that rely on delivering single growth factors have proved ineffective or hard to control in practise. An alternative approach has been identified by this group that relies on stimulation of physiological angiogenic factor cascades by engineering local cell-hypoxia, within a nano-fibrillar collagen material. Here we report on a novel, practical and effective implantable device for delivering engineered angiogenic signalling, on demand. Human dermal fibroblast-seeded dense-collagen depots were pre-conditioned under physiological cell-generated hypoxia to up-regulate production of key angiogenic factors, including HIF1α and $VEGF_{165}$. The level of $VEGF_{165}$ protein retained within depots (indicating general angiogenic factor production) was directly correlated to the duration of pre-conditioning. Angiogenic factor delivery from pre-conditioned, non-viable depots rapidly induced an angiogenic response within endothelial cell-seeded constructs *in vitro*, while implanted acellular 3D constructs incorporating such angiogenic depots in their core were infiltrated with perfused vessels by 1 week *in vivo*, at which stage non-angiogenic implants were minimally perfused. Depot stability, tuneability of cell/matrix composition with long clinical experience of the collagen material, together with cost effectiveness, make this angiogenic therapy a promising addition to a clinician's tool kit for improving local tissue perfusion.

1. Introduction

Promoting the growth of new blood vessels in ischaemic diseases, such as myocardial infarction, stroke or peripheral vascular disease represents an appealing approach for treating these devastating conditions. Furthermore, successful use of grafts and engineered implants to aid tissue repair/regeneration critically relies on their rapid vascularisation for optimal survival and integration within the host [1]. Consequently, the ability to control the timing, location and direction of engineered angiogenesis has become a therapeutic holy grail. Strategies to date have focused mainly on stimulating angiogenesis by delivery of commonly one or two pro-angiogenic factors (at the gene [2,3] or protein levels [4–7]), cell-based therapies [8–10], or combinations of each [11–13].

Despite successful induction of revascularization of ischemic tissues upon local delivery of single angiogenic growth factors in animal models, similar efforts have shown only modest benefit in human clinical trials [9,14]. Possible reasons for this relate to inefficient delivery of the angiogenic proteins (e.g. their short half-life *in vivo*), the risk of adverse events due to unbalanced dosage (e.g. hypotension, vascular leakage and tumour formation), and incomplete understanding of which growth factor combinations and sequences produce effective angiogenesis [9,14,15].

Ischaemia-induced angiogenesis is a physiological response to tissue hypoxia, orchestrated by the transcriptional activator hypoxia-inducible factor 1α (HIF-1α) [16]. HIF-1α stabilisation induces, directly or indirectly, a plethora of angiogenic mediators such as vascular endothelial growth factor (VEGF), platelet-derived growth factor B (PDGFB), placental growth factor (PLGF), angiopoietins 1 and 2, and matrix metalloproteinases [17]. Given that the formation of a functional, mature, and durable vascular network is complex, the ability of this master regulator to induce several mediators of angiogenesis prompted the concept that strategies designed to increase HIF-1α activity (e.g. by pharmacological stabilisation or over-expression through gene transfer) might be more efficient in inducing angiogenesis/arteriogenesis after ischemic events (e.g. hind limb, cardiac or cerebral ischemia) than those relying on single factors [9,18–22]. Indeed, the importance of switching-on hypoxia-induced angiogenesis at the onset of the process is increasingly appreciated [22,23].

In contrast to exogenous delivery of angiogenic factors, cell-generated angiogenic cascades commonly result in a more functional vasculature [1,24,25]. The efficacy of transplanting autologous bone-marrow stromal cells, mesenchymal stem cells and endothelial progenitor cells to treat patients with ischemic heart disease and

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doi:10.1016/j.jconrel.2011.03.029

ischemic limbs is currently being tested on a large scale [1,9]. This is because well-orchestrated production of angiogenic factors, in terms of combinations, sequences and concentrations, is presently only possible through the use of whole cell systems, releasing a host of angiogenic proteins under native feedback regulation. Therefore, a strategy that focuses on harnessing the natural mechanism that promotes angiogenesis in the body, i.e. physiological tissue hypoxia, while incorporating this into controlled biomimetic analogues of extracellular matrices, could not only succeed in induction of physiological angiogenesis, but also control its onset, location and direction.

Culturing cells under physiological hypoxia (1–10% O_2/pO_2 of 7.6–76 mmHg) is currently employed as a strategy to control cell behaviour, in particular up-regulation of angiogenic signalling molecules [26–30]. For example, hypoxic pre-conditioning of implanted bone marrow stem cells has been shown to increase their angiogenic potency through VEGF up-regulation [31,32]. We previously demonstrated that it is possible to control the local O_2 microenvironment within 3D collagen constructs by adjusting the seeding cell density and spatial position, therefore total cell-depot O_2 consumption [33,34]. Seeding constructs with human dermal fibroblasts (HDFs) at high density resulted in rapid reduction of core O_2 tension towards the low end of the physiological hypoxic range, which elicited a multifold up-regulation of VEGF gene expression [34]. Up-regulating hypoxia-induced signalling (*HIS*) by engineering local cell-hypoxia, then, is an important strategy for controlling physiological angiogenesis *in vitro* and *in vivo* [35]. The present study tests the concept of using the simple end of this spectrum, namely the angiogenic effectiveness of delivering *HIS* angiogenic factors, trapped within a preserved, collagen-material depot, without living cells.

Choosing appropriate vehicles for delivering angiogenic factors to clinically required locations is at least as important as the choice of factor source (e.g., genes, recombinant proteins or cell-produced factors), since the vehicle's material properties (e.g. porosity, pore size, degradation rate, factor binding affinity) critically determine the factor release kinetics [14,36]. Furthermore, in the case of cell-generated factors, the delivery vehicle must have the capacity to support the cell population, while retaining secreted proteins [37]. Therefore, the ability to precisely engineer a biomimetic depot biomaterial, with its cell population, is essential. Previous work from this group has developed a fabrication platform for biomimetic engineering of collagen [38], retaining the advantages of collagen materials (i.e., biocompatibility, low immunogenicity and ability to undergo natural cellular remodelling). Plastic compression (PC) of collagen hydrogels rapidly produces natural cellular materials with controllable cell/matrix density, tissue-like nano-/meso-scale architecture, mechanical properties and biomimetic function [38].

Here we test the hypothesis that angiogenic factors ($VEGF_{165}$ as exemplar), produced by pre-conditioning dermal fibroblasts to cell-generated physiological hypoxia within dense-collagen scaffolds, will be retained by the collagen material after freeze/thaw cell killing, to produce non-viable angiogenic depots (Fig. 1). Depot angiogenic effectiveness was tested *in vitro* by embedding them into human umbilical vein endothelial cell (HUVEC)-seeded constructs, while their ability to induce directional angiogenesis was tested *in vivo* by implanting 3D acellular collagen constructs, incorporating depots in their core, subcutaneously in rabbits. Depot angiogenic factor content was controlled by varying the duration of hypoxic pre-conditioning and the seeding cell density.

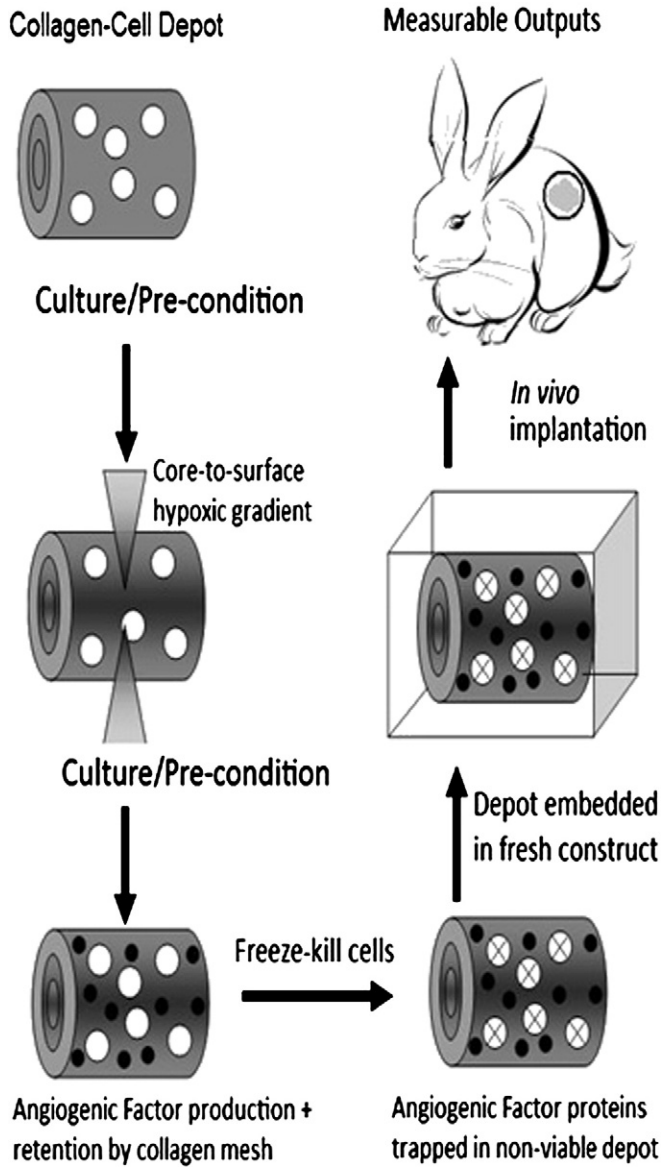

Fig. 1. Schematic of concept for development of an implantable device to deliver hypoxia-induced angiogenic signalling on demand. A dense-collagen cellular depot (of specified cell/material density) is pre-conditioned *in vitro* for a desired period. Cellular O_2 consumption generates a core-to-surface hypoxic gradient within the depot, whose level and duration can be controlled by adjusting the seeding cell density and length of culture [34]. Exposure of cells to physiological hypoxia up-regulates production of angiogenic factor proteins which are retained by the collagen material (nano-porous matrix) and remain trapped within the depot after the seeded cells are killed by snap-freezing. Non-viable depots are then embedded into fresh constructs which are implanted *in vivo*. Depot functionality (i.e. release of trapped angiogenic factor proteins) can be assessed by measuring capillary in-growth into the implant, relative to non-hypoxic baseline depots.

2. Materials and methods

2.1. Cell culture

Adult human dermal fibroblasts (HDFs) and male New Zealand white rabbit dermal fibroblasts (RDFs) were cultivated in DMEM supplemented with 10% FCS (First Link, UK), 1000 U/ml penicillin and 100 mg/ml streptomycin (Gibco, UK). Human umbilical vein endothelial cells (HUVECs) were cultured in complete endothelial cell growth medium (Promo Cell, Germany).

2.2. Scaffold fabrication and culture

2.2.1. Construct plastic compression

Acellular, HDF-, HUVEC- and RDF-seeded rat-tail type I collagen gels (5 ml) were prepared as previously described [38]. Acellular collagen gels and gels seeded with 2×10^6 HDFs, 5×10^5 RDFs or 2×10^6 RDFs were cast in rectangular moulds (size: $4.5\times1.5\times1$ cm). Following 30 min setting gels were compacted by plastic compression to produce ~200 μm thick sheets (Fig. 2a), which does not significantly reduce cell viability for HDFs, HUVECs, or human bone marrow derived stem cells

[35,38,39]. Cell densities increased proportionally to the reduction in gel volume by fixed mass loading over a porous filter paper support [38]. Post-compression cell densities (calculated based on fluid loss) were 23.2×10^6 HDFs/ml in HDF-seeded constructs and 5.8×10^6 or 23.2×10^6 RDFs/ml in RDF-seeded constructs [34].

2.2.2. Depot preparation

Compressed collagen sheets were rolled along their short axis to produce tightly wound spirals of 15 mm length, 2.3 mm diameter (Fig. 2a). HDF- and RDF-seeded spiral collagen constructs were pre-conditioned for 5 or 10 days *in vitro* by static culture in 5 ml DMEM, in a 37 °C, 5% CO_2 humidified incubator. Following pre-conditioning, spiral constructs were removed from culture and snap-frozen in liquid nitrogen (for 5 min) to kill the seeded cells. Acellular spiral constructs and one set of constructs seeded with 23.2×10^6 RDFs/ml were snap-frozen at day 0 without any pre-conditioning.

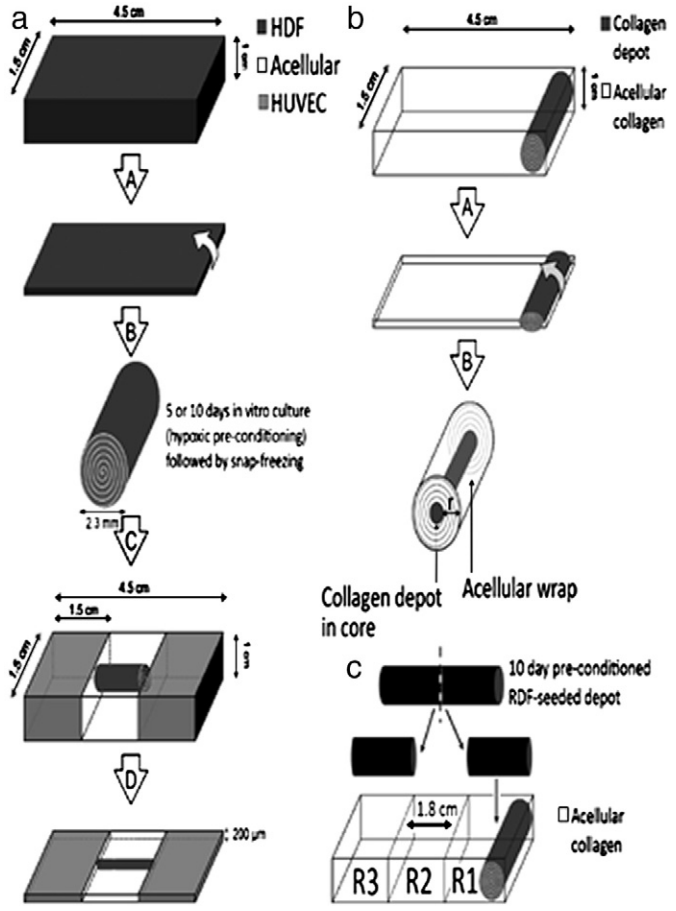

Fig. 2. (a) Schematic of collagen depot fabrication process and the *in vitro* assay for testing their ability to induce angiogenesis. Rectangular collagen gels, seeded with 2×10^6 HDFs, were compacted by plastic compression (A) to produce ~200 μm thick sheets, which were spiralled along their short axis (B) to produce tightly wound spirals that were statically cultured *in vitro* for 5 or 10 days. Cell O_2 consumption generated physiological hypoxia (~25 mmHg/3% O2) in the construct core within 24 h [34]. Following 5 or 10 days of hypoxic pre-conditioning, spiral constructs were snap-frozen in liquid nitrogen to kill the seeded cells. Non-viable constructs (depots) were embedded (C) within collagen gels, into the acellular compartment between two equal volume HUVEC-seeded compartments (each containing 1×10^5 HUVECs). Following setting, gels were compacted by plastic compression (D) to produce ~200 μm thick sheets, which were cultured *in vitro* for 4 or 8 days. (b) Schematic of the fabrication process for implanted collagen constructs. Non-viable (frozen/thawed) spiral collagen depots were embedded into acellular collagen gels, which were compacted by plastic compression (A) to produce ~200 μm thick sheets that were rolled along their short axis (B). This positioned the depot in the spiral core. The radial distance (r) from the spiral surface to the depot was 1.1 mm. (c) Setup for spatio-temporal analysis of VEGF release. Depots seeded with 23.2×10^6 RDFs/ml were hypoxia preconditioned for 10 days, then snap-frozen and sectioned in two halves. One half was analysed using ELISA to measure pre-incubation VEGF levels, while the other half was embedded into an acellular 10 ml collagen gel and incubated for 1, 3, 5 and 10 days. At these time points incubation was stopped and the half-depot was removed from the gel before dissecting it into three equal regions (R1, R2, and R3) that were assayed for $VEGF_{165}$ with ELISA.

2.2.3. Depot testing

Frozen/thawed HDF-seeded spiral constructs (depots) were embedded within the acellular compartment of collagen gels comprising three equal volume ($1.5\times1.5\times1$ cm) compartments: two HUVEC-seeded compartments (each containing 1×10^5 HUVECs) and a middle acellular compartment (Fig. 2a). Such gels were cast by transferring a HUVEC-seeded collagen gel (size: 1.5(L)×1.5(W)×1(H) cm) into each end of a rectangular mould (size: 4.5(L)×1.5(W)×1(H) cm), maintaining a 1.5 cm separation distance between them, and bathing them in 2.5 ml acellular collagen solution. Collagen gels containing only HUVECs (no depot) were cast as controls. The acellular collagen was allowed to set and integrate between the 2 other cellular gels for 30 min, prior to plastic compression. This resulted in compaction of both the collagen gel and depot, producing a sheet of uniform thickness (~200 μm) (Fig. 2a). Post-compression cell density in the HUVEC compartments was 1.16×10^6 HUVECs/ml. Compressed sheets containing HUVECs and depots or HUVECs only were cultured *in vitro* for 4 or 8 days, in 5 ml medium (50% DMEM, and 50% endothelial growth medium) in a 37 °C, 5% CO_2 humidified incubator (note: HUVECs were not exposed to hypoxia in this system [35]). Frozen/thawed acellular and RDF-seeded depots were embedded within one end of rectangular moulds (size: 4.5(L)×1.5(W)×1(H) cm) and bathed in 5 ml acellular collagen solution (Fig. 2b). For analysis of VEGF release, 10 ml acellular gels, embedded with half-depots (23.2×10^6 RDFs/ml) at one end, were incubated uncompressed for 1, 3, 5 and 10 days (1 ml media overlay was used to maintain gel moisturisation during incubation) (Fig. 2c). Otherwise gels were compacted by plastic compression to produce ~200 μm thick sheets, which were then spiralled along their short axis to produce tightly wound spirals of 15 mm length, 3 mm diameter. This positioned the depot in the spiral core and the acellular collagen wrap on the surface (Fig. 2b). The radial distance from the spiral surface to the depot was 1.1 mm. A minimum of three constructs was used to test each condition.

2.3. ELISA

$VEGF_{165}$, MMP-2 and MMP-9 concentrations were measured using Quantikine ELISA kits, while total HIF-1α concentrations were measured using Surveyor IC ELISA kit (all from R&D, USA). Triplicates of each

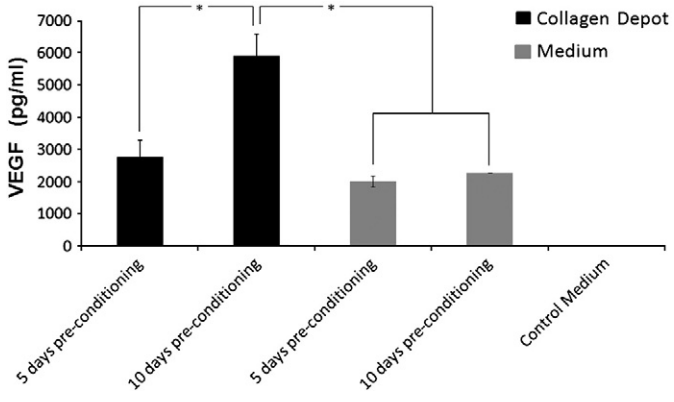

Fig. 3. VEGF was produced and retained within hypoxia pre-conditioned collagen depots. Spiral collagen constructs seeded with HDFs at high density (23.2×10^6 HDFs/ml) were pre-conditioned under cell-generated hypoxia by static culture *in vitro* for 5 or 10 days, before undergoing snap-freezing to kill the seeded cells. ELISA was used to analyse levels of $VEGF_{165}$ protein secreted in the media or retained within collagen constructs (depots). Control medium was DMEM supplemented with 5% FCS. Bars correspond ±sd of means, *$p<0.05$.Three samples were tested per time point.

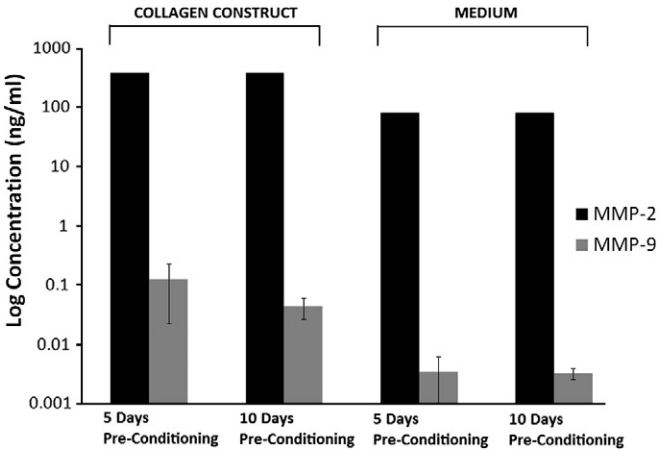

Fig. 4. Collagen depots underwent matrix remodelling during pre-conditioning, as shown by the up-regulation of the angiogenic factor-regulated MMPs, MMP-2 and -9. Spiral collagen constructs seeded with HDFs at high density (23.2×10^6 HDFs/ml) were hypoxia pre-conditioned by static culture *in vitro* for 5 or 10 days, before undergoing snap-freezing to kill the seeded cells. ELISA was used to analyse protein levels of MMP-2 and MMP-9 secreted in the media or retained within collagen depots (note logarithmic scale in y-axis). Bars correspond ±sd of means (note: no ±sd bars are shown for MMP-2 data as values exceeded the maximum detectable concentration in all samples tested, and therefore represent the minimum concentration present). Three samples per time point were analysed for each MMP.

sample were analysed using a MicroPlate reader (Biorad, UK). Readings were taken at 450 nm with 570 nm λ correction. Spiral constructs seeded with 23.2×10^6 HDFs/ml were statically cultured for 5 or 10 days in DMEM supplemented with 5% FCS, before being snap-frozen in liquid nitrogen for 5 min, pulverised with a Mikro-dismembranator (Sartorius, Germany) and dissolved in 1.2 ml medium. Media and collagen construct samples were analysed separately. For spatio-temporal analysis of VEGF release, each half (non-incubated/incubated half) of a 10 day pre-conditioned RDF-seeded depot (23.2×10^6 RDFs/ml) was pulverised and dissolved in 500 μl medium, while the collagen gels in which depots had been embedded were sectioned in three equal regions and analysed separately after 1, 3, 5 and 10 days of incubation. Three samples per time point were analysed for each factor tested.

2.4. Implantation of collagen constructs

An institutional review committee of Shanghai Jiao Tong University School of Medicine approved all animal study protocols. Six adult female New Zealand white rabbits weighing 2–2.5 kg were used. Surgery was performed under general anaesthesia. A longitudinal 10 cm skin incision was made on the rabbit's back and implants were sutured onto subcutaneous tissue. For *in vivo* studies, all constructs contained allogeneic RDFs. Three constructs containing acellular depots were sutured onto the left side and three constructs containing cellular depots (of the same series) on the right side. Cellular depots tested were low (5.8×10^6 RDFs/ml) and high (23.2×10^6 RDFs/ml) cell density 10 day pre-conditioned and high (23.2×10^6 RDFs/ml) cell density non-pre-conditioned depots. Wounds were treated with topical benzylpenicillin-sodium antimicrobial powder. Rabbits were nursed until full recovery and returned to their single cage. Rabbits were sacrificed at 1 week. Two rabbits were used to test each condition ($n=6$ for each condition tested).

2.5. Tissue processing and immuno-histochemical staining

For immunofluorescent staining *in vitro* cultured constructs were removed from culture wells, washed in 5 ml PBS and fixed in 100% ice-cold methanol for 1 h. The primary antibodies used were mouse anti-human CD31 (Dako, USA); rabbit anti-human vWF (Sigma, UK). The secondary antibodies used were anti-mouse IgG-FITC (R&D, USA); anti-rabbit IgG-FITC (Sigma), followed by DAPI (Sigma) nuclear staining. Implanted spiral constructs were washed, fixed without unrolling in 4% parafolmadehyde/PBS for 24 h, routinely processed and embedded in paraffin. Transverse and longitudinal sections (4 μm) were placed on slides for staining with H&E or immunohistochemistry. Immunohistochemical stainining was carried out using Vectastain ABC-HRP kit (Vector Laboratories, USA) according to the manufacturer's instructions. The primary antibody used was mouse monoclonal anti-rabbit CD31 (Abcam, UK) and the enzyme substrate used was DAB (brown) (Vector Laboratories). Sections were counterstained with haematoxylin.

2.6. Image analysis

Micrographs of immunofluorescent-stained specimens were captured with a fluorescent microscope (Olympus BX61) using a×10 objective, while H&E stained micrographs were captured with a light microscope (Olympus BH2) using×10, ×20 and×40 objectives. A minimum of 10 random fields were photographed per sample. An imaging software (Image J, NIH, USA) was used to determine the length of CD31 positive capillary-like structures (CLSs) with a CLS elongation index (ratio of straight line separation of CLS ends/total CLS length) of 0.8–1, the total area of red blood cell (RBC)-containing lumens, host blood vessel invasion distance (average radial distance from construct edge where RBC-containing lumens were present) and the total field area. Total number of CLSs was counted manually.

2.7. Statistical analysis

For each experimental condition a sample size of 3 or more was used. Data is expressed as mean ± standard deviation or mean ± standard error, as noted. Statistical analysis was carried out using oneway ANOVA accompanied with multiple comparison tests (Tukey's test or Dunnett's test), using SPSS 14 software. Differences were considered significant when $p<0.05$.

3. Results

3.1. Angiogenic factor production and retention within hypoxia pre-conditioned collagen depots

Previous work by this group has shown that human dermal fibroblasts (HDFs), seeded at high density within 3D collagen constructs,

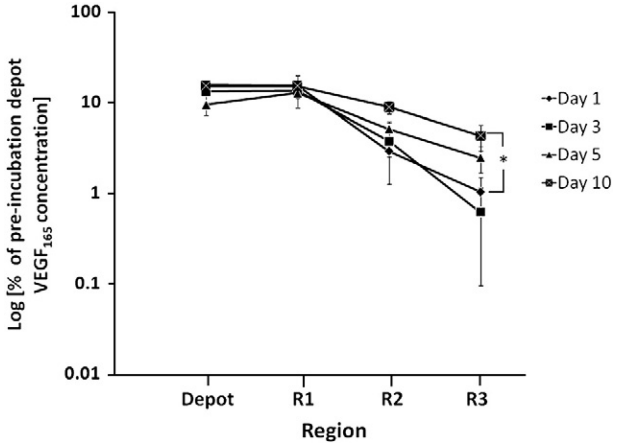

Fig. 5. Spatio-temporal profile of $VEGF_{165}$ release from hypoxia pre-conditioned RDF-seeded depots (23.2×10^6 RDFs/ml) *in vitro*. 10 day pre-conditioned depots were embedded in acellular collagen gels and incubated for 1, 3, 5 and 10 days. $VEGF_{165}$ protein levels were measured in three gel regions, proximal (R1), midway (R2) and distal (R3) to the depot at the end of each incubation period, to assess the diffusion of released VEGF. $VEGF_{165}$ levels in the depot, R1, R2 and R3 are expressed as percentage of $VEGF_{165}$ concentration in pre-incubated depots (note logarithmic scale in y-axis). Bars correspond ±se of means, *$p<0.05$.Three constructs were tested for each incubation period.

generate local physiological hypoxia (~25 mmHg/3.2% O_2), resulting in up-regulation of VEGF gene expression [34]. The first stage of this study in developing a practical angiogenic implant was then to establish a corresponding up-regulation of key angiogenic factors at the protein level. Spiral collagen constructs seeded with HDFs at high density (23.2×10^6 HDFs/ml) were pre-conditioned to cell-generated hypoxia for 5 or 10 days and then snap-frozen. HIF1α and $VEGF_{165}$ protein levels were analysed by ELISA in the media and within collagen matrices (i.e. depot factors). HIF1α (an intracellular factor) was not detected in the media, but was present within collagen depots (i.e. within the cellular component). HIF1α levels were 123 ± 8 pg/ml and 130 ± 4 pg/ml, at 5 and 10 days respectively ($p>0.05$), indicating that HIF1α expression had peaked early on during culture (showing a plateau at 5–10 days). Expression of HIF1α confirmed that HDFs were exposed to hypoxia in this system, well before the 5 day peak, which was in agreement with our previous data, obtained by real-time monitoring of core O_2 tension (using luminescence quenching), where hypoxia was reached within 24 h [34]. $VEGF_{165}$ was found both in the media and within depots at 5 and 10 days, indicating that $VEGF_{165}$ was partially retained within the depot's nano-porous collagen matrix (Fig. 3). $VEGF_{165}$ retention-:-release ratio increased from approx. 1.5:1 to 3:1 (depot-:-medium) between the 5 and 10 day stages. In addition, there was a significant 2 fold increase in the level of $VEGF_{165}$ protein retained within depots from 5 to 10 days ($p<0.05$). This was consistent with increasing levels of VEGF gene expression seen previously over 8 days [34].

3.2. Collagen matrix remodelling during depot hypoxic pre-conditioning

To test whether collagen depots underwent matrix re-modelling during hypoxic pre-conditioning we assessed the expression of two angiogenic factor-regulated MMPs, MMP-2 and MMP-9 [40]. ELISA was used to analyse protein levels of MMP-2 and MMP-9 secreted in the media or retained within collagen constructs of high cell density (23.2×10^6 HDFs/ml) depots over the 5–10 day culture period. Both MMPs were present in the media and within collagen constructs, at 5 and 10 days, indicating that the collagen matrix was actively remodelled by cells at the early stage of culture (Fig. 4). Importantly, there was an ~1000 fold difference in the level of MMP-2 and MMP-9 protein produced, suggesting that the expression of these two MMPs is differentially regulated in cells exposed to hypoxia. There was no significant difference in MMP-2 or MMP-9 levels, secreted in media or retained within constructs, from 5 to 10 day culture, indicating that MMP expression had peaked early on during culture, showing a plateau at 5–10 days.

3.3. Spatio-temporal profile of VEGF release from hypoxia pre-conditioned depots

Induction of directional angiogenesis relies on chemotactic migration of endothelial cells along angiogenic factor gradients [41]. The basic hypothesis here is that diffusion of hypoxia-induced, depot-trapped factors into host tissue would induce directional angiogenesis towards the implantation site. In order to assess the spatio-temporal release profile of depot-trapped VEGF, we embedded 10 day pre-conditioned rabbit-dermal fibroblast (RDF)-seeded depots (23.2×10^6 RDFs/ml) into acellular collagen hydrogels (note: these have matrix stiffness/density comparable to early granulation tissue[42]) and measured $VEGF_{165}$ protein levels in three regions, proximal, midway and distal to the depot (each region measured 1.8 cm) over 1, 3, 5 and 10 days of incubation (see methods). By day 1, a $VEGF_{165}$ gradient was formed from the proximal to the distal gel region, while only ~15% of trapped $VEGF_{165}$ had remained within the depot (as compared to pre-incubation depot levels, $p<0.05$) (Fig. 5). VEGF concentration ratio in the proximal to the distal region was reduced from 15-:-1 to 3.5-:-1 from day 1 to day 10, respectively, indicating ongoing diffusion and accumulation of VEGF towards the distal region. This coincided with a significant ~4 fold increase in VEGF concentration in the distal region from day 1 to day 10 ($p<0.05$).

3.4. Hypoxia pre-conditioned collagen depots induce an angiogenic response in vitro

To assess depot angiogenic potential these were tested within HUVEC-seeded 3D collagen constructs *in vitro*. Non-viable (frozen/thawed) 5 and 10 day hypoxia pre-conditioned depots were embedded within the acellular compartment, between the two HUVEC-seeded compartments (see methods). In constructs containing hypoxia pre-conditioned depots HUVECs formed CD31- and vWF-positive clusters and capillary-like structures (CLSs) after 4 days, but not in HUVEC-only seeded constructs (not exposed to hypoxia [35]) (Fig. 6a). The number of CLSs formed using 10 day pre-conditioning was 2 fold greater than for 5 days, at the 4 day culture stage ($p<0.05$) (Fig. 6b). No significant

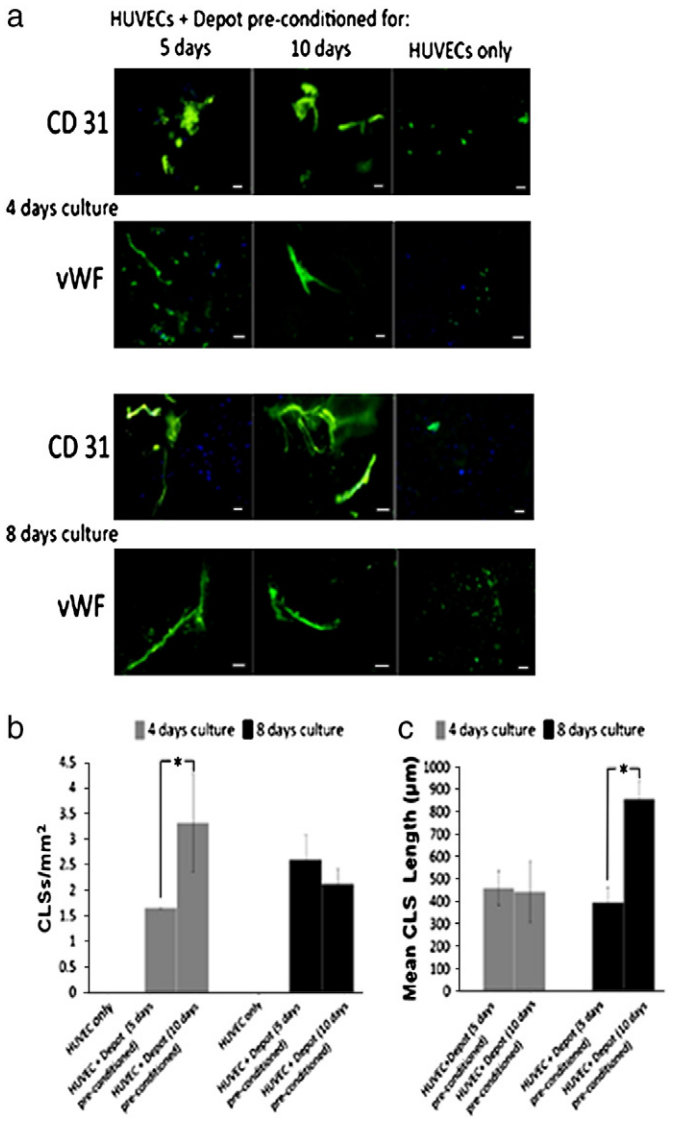

Fig. 6. Hypoxia pre-conditioned collagen depots induced an angiogenic response *in vitro*. (a) Formation of capillary-like-structures (CLSs) in HUVEC-seeded scaffolds containing hypoxia pre-conditioned collagen depots. Collagen scaffolds containing HUVECs + a 5 day pre-conditioned depot, HUVECs + a 10 day pre-conditioned depot or HUVECs only were statically cultured for 4 or 8 days *in vitro*. Construct tissue sections were stained using human-specific anti-CD31 and anti-vWF antibodies (green) and DAPI for nuclear staining (blue). Bars = 100 μm. (b) Quantification of the number of CD31 positive CLSs formed within collagen scaffolds containing HUVECs only, HUVECs + a 5 day pre-conditioned depot or HUVECs + a 10 day pre-conditioned depot, at 4 and 8 days *in vitro* culture. Note that no CLS formation was observed in HUVEC-only scaffolds at either 4 or 8 days. (c) Comparison of the mean length of CD31 positive CLSs formed in constructs containing 5 or 10 day pre-conditioned depots, at 4 and 8 days culture. Bars correspond ±se of means, *$p<0.05$. Four constructs were tested for each condition per time point.

increase in CLS number was observed between 4 and 8 days for either pre-conditioning period (Fig. 6b). CLS length appeared to follow the reverse pattern to CLS number (Fig. 6c). While there was no difference in mean CLS length between the two pre-conditioning period at 4 days, by 8 days the 10 day pre-conditioned depots generated 2 fold greater mean CLS length than 5 day depots ($p<0.05$). These results indicate that CLS number is directly related to the pre-conditioning culture period, and so to depot angiogenic factor dose (Fig. 3). However, CLS length has a much greater dependence on the assay culture period, consistent with the idea that 'vessel elongation' takes longer and so is a rate limited response.

3.5. Hypoxia pre-conditioned depots promote rapid vascularisation of collagen constructs in vivo

To test the principle that angiogenic factor delivery from hypoxia pre-conditioned depots would accelerate implant vascularisation, we tested the effect of cell seeding density on depot angiogenic potency. Depots seeded with rabbit dermal fibroblasts (RDFs) at low density (LD: 5.8×10^6 RDFs/ml) or high density (HD: 23.2×10^6 RDFs/ml) were hypoxia pre-conditioned by culturing for 10 days before being frozen to kill the resident, seeded cells. A 10 day pre-conditioning period was chosen based on our *in vitro* data to ensure maximal angiogenic factor protein up-regulation (Fig. 3). High cell density depots that were not hypoxia pre-conditioned (zero culture time) were used as controls, in addition to non-cellular depots, to isolate the effect of hypoxia-induced factors from any other released cell factors or the host inflammatory reaction to dead cells.

Test and control depots were embedded into the core of 3D acellular collagen constructs (see methods) which were then implanted subcutaneously into rabbits for 1 week. Total white blood cell count in rabbits implanted with cell-seeded constructs was 8, 15.5, 9×10^9/L on day 0, 1 and 7 respectively, indicating that the inflammatory response had subsided by 7 days. Collagen constructs containing high density (HD) pre-conditioned depots were visibly vascularised by 1 week *in vivo,* in contrast to those with low density (LD) pre-conditioned depots, high density (HD) non-pre-conditioned depots or acellular depots, each of which showed no gross signs of vascularisation (Fig. 7a). H&E staining of construct cross sections identified red blood cell (RBC)-containing vessel lumens in constructs embedded with both HD or LD pre-conditioned depots at 1 week, however vessel lumens appeared larger in constructs with HD depots (Fig. 7a). Importantly, intra-luminal RBCs indicated that infiltrating host vessels were perfused by 1 week. Such host-linked blood vessels also stained positively with anti-CD31 antibodies (Fig. 7a). Constructs with HD pre-conditioned depots had the largest mean total cross-section area of invading RBC-containing host vessels of all implants. This difference was statistically significant compared to constructs with acellular or HD non-pre-conditioned depots ($p<0.05$) (Fig. 7b). The radial distance which invading host blood vessels had penetrated into 3D spiral collagen constructs was approximately 2 times greater for HD pre-conditioned depots (0.31 ± 0.11 mm) than with LD pre-conditioned depots (0.14 ± 0.04 mm) ($p<0.05$). In both cases, however, host blood vessels had penetrated only into the acellular collagen wrap (1.1 mm radial thickness), without reaching the core depot, at 1 week implantation.

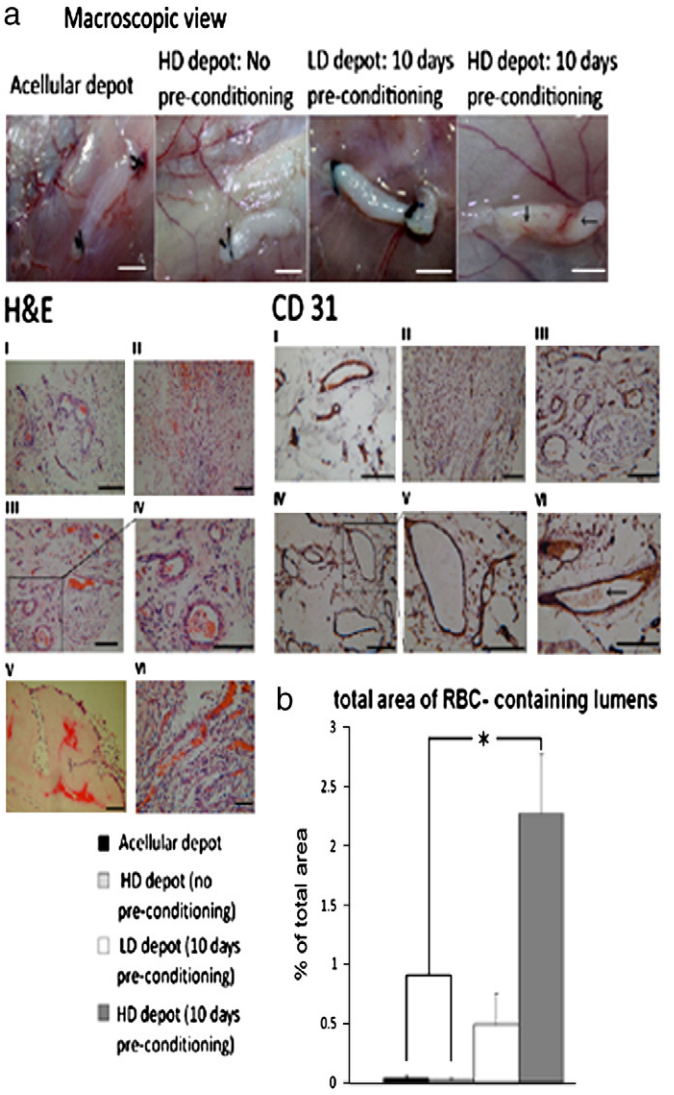

Fig. 7. Implanted collagen constructs containing hypoxia pre-conditioned depots were vascularised at 1 week *in vivo*. (a) 3D spiral acellular collagen constructs containing depots in their core were implanted subcutaneously in rabbits for 1 week. Four depot types were tested: (i) acellular depots (no pre-conditioning) (ii) high cell density (HD: 23.2×10^6 RDFs/ml) non-pre-conditioned depots, (iii) low cell density (LD: 5.8×10^6 RDFs/ml) and (iv) high cell density (HD: 23.2×10^6 RDFs/ml) 10 day pre-conditioned depots. Macroscopic images of constructs were taken at 1 week, after animals had been sacrificed, with constructs still sutured in place. Constructs containing HD pre-conditioned depots showed visible vascularisation (arrowed) (bars = 0.5 cm). Construct tissue sections were stained with H&E, showing red blood cell (RBC)-containing lumens: acellular depot (I), HD non-pre-conditioned depot (II), LD pre-conditioned depot (III) with magnification image (IV), HD pre-conditioned depot at outer (V) and inner (VI) layers of the spiral construct (bars = 200 μm). Construct tissue sections were also immunostained with anti-CD31 antibodies (brown): acellular depot (I), HD non-pre-conditioned depot (II), LD pre-conditioned depot (III), HD pre-conditioned depot (IV) with magnification image (V). CD31-stained vessels in constructs embedded with high density pre-conditioned depots contained intra-luminal RBCs (VI, arrowed) (bars = 200 μm). (b) Quantification of the mean total area of RBC-containing lumens (total area of all RBC-containing lumens in H&E stained sections as percentage of total section area) in subcutaneously implanted collagen constructs containing the four depot types. Bars correspond ±se of means, *$p<0.05$.Six constructs were tested for each depot condition.

4. Discussion

This study demonstrates the utility of cell-generated physiological hypoxia as a tool for switching on angiogenic factor signalling. The effectiveness of hypoxic pre-conditioning as a priming mechanism for induction of a physiological angiogenic cascade was confirmed here by the up-regulation of two key angiogenic factors, HIF1α and VEGF. These proteins, along with a host of other angiogenic factors (not directly measured here), were trapped and retained within the nano-fibrillar collagen matrix. The ability to 'capture' hypoxia-induced signalling within a biomimetic nano-porous matrix makes it feasible, for the first time, to engineer high quality, stable angiogenic depots. Importantly, the same cell-seeded 3D material used for the culture stage (i.e. as scaffold) was also used to retain the factors and to act directly as the delivery vehicle. While VEGF protein was only partially retained within the depot's nano-porous collagen matrix, the 3:1 retention-:-release ratio (depot-:-medium) in 10 day pre-conditioned

depots indicated that a significant proportion of synthesised factors was retained. The amount of retained VEGF protein was directly correlated to the length of pre-conditioning (doubling from 5 to 10 days), indicating that this could provide a means for controlling angiogenic depot dose and potency.

Such non-viable depots rapidly induced an angiogenic response *in vitro* and promoted vascularisation of acellular constructs *in vivo* by 1 week, in contrast to acellular (depot-free) constructs that were previously shown to require 3 weeks for significant vascular ingrowth [43]. Importantly, we previously showed that active hypoxia-induced signalling by non-pre-conditioned living cells, delivered through the same collagen material, promotes formation of functional vessels *in vivo*, confirmed by improvement in deep oxygenation of implanted constructs by 2 weeks [35]. The 10 day hypoxia pre-conditioning stage, employed in the current study, therefore must have accelerated the formation of functional angiogenesis by 1 week. While direct illustration of the functionality of formed vessels, and their stability over longer periods, was beyond the scope of this study, it could form the focus of future work.

The infiltration of perfused host vessels into the acellular collagen wrap, seen here, indicated that depot factors were bioactive following depot freezing and storage (up to 2 weeks). Diffusion of depot factors through the surrounding collagen material into host tissue must have generated spatial angiogenic factor gradients, similar to the VEGF gradients measured *in vitro*, that directionally guided endothelial cells towards the factor source [14,41,44]. These findings confirm the functionality of non-viable, hypoxia pre-conditioned depots as 'angiogenic motors', able to effectively retain and release angiogenic factor signalling. Tunable angiogenic activity, based on duration of pre-conditioning (5 vs.10 days) was confirmed by the 2 fold increase in *in vitro* angiogenic response (CLS number and length) and the proportional increase in retained VEGF protein. Interestingly, a 4 fold greater angiogenic response was seen *in vivo* with high rather than low cell density depots, which was proportional to the difference in cell density, suggesting that seeding cell density may be an additional controller of depot potency, probably also through the level of induced hypoxia [34].

A plethora of studies show that angiogenesis is a well-orchestrated, multi-step response reliant on spatio-temporal regulation of many factors [14,44,45]. Therefore, while HIF1α and VEGF up-regulation were monitored here as key marker-factors, the observed angiogenic response is likely to involve many other factors. The reasonable assumption here is that hypoxic pre-conditioning of cells is likely to up-regulate a physiological, multi-factorial angiogenic factor cascade. The rapid induction of angiogenesis seen in the absence of living cells suggests that the amount and combination of growth factors retained by depots was suitable for angiogenic induction [14]. This has important implications for trapping and releasing physiologically complex growth factor mixtures, particularly as it is known that low doses are ineffective and excess can result in pathological angiogenesis [9,14].

In contrast to other studies where cultured cells are exposed to 'global' (incubator) hypoxia [27,46,47], our model hypoxia is cell-generated by local O_2 consumption and measured directly in real time [34]. Since hypoxia is cell-generated, it is also physiologically regulated [48]. Indeed, hypoxia in this system produced only modest reduction (~20%) in core HDF viability up to 5 days [34]. The importance of fine tuning the O_2 microenvironment through cell-mediated feedback regulation is highlighted in the limited ability of chronically ischaemic tissues (e.g. in peripheral artery disease) to effectively upregulate angiogenic signalling, despite prolonged exposure to hypoxia [49]. Whilst exposure of constructs to external hypoxia (i.e., chamber incubation) may produce some comparable effects, any potential benefits of this would need to be weighed against the loss of physiological feedback regulation.

On a practical level, the ability to engineer depot composition and control matrix diffusion coefficient (and with it macromolecular retention), through control of collagen density and/or photo-chemical crosslinking [38,50], makes this system predictable and mechanistic. Furthermore, the finding that angiogenic factor-regulated MMPs (MMP-2 and -9 [40]), were up-regulated within pre-conditioned depots, indicates that the collagen matrix undergoes active remodelling during *in vitro* culture. The ability to potentially regulate this process (e.g. by cross-linking) has important implications for controlling the spatio-temporal release profile of depot factors, a pre-requisite for induction of physiological angiogenesis [14]. Knowledge of collagen degradation kinetics *in vivo* [51] is an additional useful feature for modelling growth factor release and depot function upon implantation. Using collagen as (the main) biomaterial for depot fabrication further ensures that this angiogenic therapy is minimally immunogenic and can be currently produced to clinically approved standards [52].

Work from this group and others has shown that multiple cell types (e.g. dermal fibroblasts, adipose/bone marrow-derived stromal cells, vascular smooth muscle cells) respond to physiological hypoxia by up-regulating angiogenic signalling [28–30,34]. This suggests that different cell types could be used as the source of *HIS* cells, circumventing cell source limitations and ethical concerns. Furthermore, since hypoxia-induced signalling profiles have been shown to be cell type-specific [25,28,30], it may eventually prove feasible to design tailor-made depots of unique angiogenic factor composition and potency. Since hypoxia pre-conditioned depots were angiogenic despite absence of living cells, allogeneic cells (similar to those used widely and safely in dermal substitutes [52]) could potentially be used in future clinical applications, which would be advantageous compared to using autologous cells which can be scarce and therefore require an expensive and lengthy pre-expansion stage. Such non-viable depots would also have the advantage of stability and a long shelf life, as a pre-manufactured off-the-shelf therapy, which would undoubtedly be beneficial in terms of cost effectiveness.

5. Conclusions

The challenge here has been to develop and test a novel strategy that could make hypoxia-mediated angiogenic induction a practical, device-based reality. Our findings demonstrate the feasibility of engineering high quality angiogenic depots, as potential motors for therapeutic vascularisation. The results highlight the importance of strategies combining three current approaches together (as opposed to use in isolation): (i) engineered cell therapies, (ii) multiple growth factor release and (iii) growth factor–cell–matrix interactions. To our knowledge this is the first, functional and practical implantable system for generating hypoxia-induced (i.e. physiological) angiogenesis. It is sufficiently flexible and robust to form a platform for developing therapies that target ischaemic, as well as engineered tissue vascularisation. Its reliance on engineered cell/matrix physiology makes it a promising tool for influencing local tissue perfusion.

Acknowledgements

Umber Cheema is a funded BBSRC David Phillips Fellow. We are grateful to BBSRC/EPSRC and BBSRC China Partnering Award UK, together with the National "863" Project Foundation (2006AA02A127), Shanghai Rising-Star Program grant (09QB1401400) for the animals and Donghua University "111" Project (B07024) for the scaffolds. We would like to thank Michael Ananta for help with schematic illustrations and Huiguo Chu for help with surgery.

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