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Injectable system for spatio-temporally controlled delivery of hypoxia-induced angiogenic signalling

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ABSTRACT

While chronically ischaemic tissues are continuously exposed to hypoxia, the primary angiogenic stimulus, they fail to appropriately respond to it, as hypoxia-regulated angiogenic factor production gradually undergoes down-regulation, thus hindering adaptive angiogenesis. We have previously reported on two strategies for delivering on demand hypoxia-induced signalling (HIS) *in vivo*, namely, implanting living or non-viable hypoxic cell-matrix depots that actively produce factors or act as carriers of factors trapped within the matrix during *in vitro* pre-conditioning, respectively. This study aims to improve this approach through the development of a novel, injectable system for delivering cell-free matrix HIS-carriers. 3D spiral collagen constructs, comprising an inner cellular and outer acellular compartment, were cultured under hypoxia (5% O_2). Cell-produced angiogenic factors (e.g. VEGF, FGF, PLGF, IL-8) were trapped within the nano-porous matrix of the acellular compartment as they radially diffused through it. The acellular matrix was mechanically fragmented into micro-fractions and added into a low temperature (5 °C) thermo-responsive type I collagen solution, which underwent a collagen concentration-dependent solution-to-gel phase transition at 37 °C. Levels of VEGF and IL-8, delivered from matrix fractions into media by diffusion through collagen sol–gel, were up-regulated by day 4 of hypoxic culture, peaked at day 8, and gradually declined towards the baseline by day 20, while FGF levels were stable over this period. Factors captured within matrix fractions were bioactive after 3 months freeze storage, as shown by their ability to induce tubule formation in an *in vitro* angiogenesis assay. This system provides a minimally invasive, and repeatable, method for localised delivery of time-specific, cell-free HIS factor mixtures, as a tool for physiological induction of spatio-temporally controlled angiogenesis.

1. Introduction

Current strategies targeting therapeutic angiogenesis in chronic wounds (e.g. ulcers, burns) and ischaemic tissues (e.g. peripheral, myocardial, cerebral tissues), as well as vascularisation of grafts and tissue engineered implants largely rely on exogenous delivery of single or few angiogenic factors (e.g. recombinant factor proteins, gene transfer etc.). However, the limited success of such strategies in clinical trials has highlighted how difficult it is to mimic the spatio-temporal complexity of an angiogenic growth factor response, solely by isolating and delivering certain factors (e.g. VEGF) [1–4]. It is therefore not surprising that strategies targeting the process at its onset by utilizing hypoxia, the primary angiogenic stimulus [5], have thus far provided promising results in *in vivo* studies [6–8]. A large area of research, for example, focuses on stabilisation of hypoxia-inducible factor 1 alpha (HIF-1a) (e.g. pharmacologically, via gene transfer etc.) in order to recapitulate hypoxia-induced signalling [9–11].

Utilisation of hypoxia as a tool for angiogenic induction harnesses the innate biological mechanism that naturally generates angiogenesis in the body, in physiological (e.g. during embryogenesis), as well as pathological states (e.g. cancer), thus overcoming limitations associated with an incomplete understanding of complex angiogenic factor cascades. It also provides a rational starting point for extending our knowledge of such intricate mechanisms. Exposing cells to hypoxia to induce production of angiogenic factors has widely been investigated [12–14]. Indeed, conditioned media from hypoxic cell cultures have previously been described to induce angiogenesis *in vitro* and *in vivo* [14]. Furthermore, pre-conditioning cells (e.g. bone marrow stem cells) to hypoxia has been shown to increase their survival and angiogenic potency upon transplantation [15], while grafting adipose-derived stromal cells as spheroids to ischemic limbs improves therapeutic efficacy due to enhanced cell survival and paracrine effects, which are mediated

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by cell preconditioning to the hypoxic environment within spheroid cultures [16].

While chronically ischaemic tissues are constantly exposed to hypoxia, they have a limited capacity to appropriately respond to hypoxic stress [17]. The proposed mechanism for the inadequate amount of compensatory angiogenesis seen in many chronic ischemic/hypoxic conditions involves a blunting of the ability of cells to upregulate angiogenic factors (e.g. VEGF, angiopoietins) in response to prolonged/repeated hypoxic episodes [17–19], but this effect might be further complicated by the fact that the spatial and temporal distribution patterns of endogenously produced angiogenic growth factors in ischaemic tissues are, to a great extent, influenced by inflammation [20]. Furthermore, it has been suggested that angiogenic responses by endothelial cells to certain factors, such as VEGF, are impaired under chronic hypoxia [21]. Exposing selected cell types to hypoxia *in vitro*, in order to stimulate production of multiple angiogenic factor proteins, with subsequent localised delivery of such complex, yet physiological, factor mixtures *in vivo* could therefore provide a solution to overcoming the limited ability of ischaemic tissues (and tissues at a distance, where collateralization/arteriogenesis must primarily occur [20]), to effectively upregulate angiogenic signalling, despite prolonged exposure to hypoxia. Effectively, this strategy then aims at overriding the habituated response of cells within an ischaemic tissue to the constant O_2 microenvironement, thus restarting the angiogenic process and driving it to completion.

As well as controlling the onset of an angiogenic response, it is also important to spatio-temporally regulate it, i.e. control when and where it is induced, for how long and in what direction. For example, control of the directionality of angiogenesis could be achieved by localised delivery of angiogenic signalling at a target site (e.g. ischaemic area, implant site etc.), while allowing diffusion of these factors to generate spatial factor gradients that chemo-attract host endothelial cells towards the factor source [22]. The ability to locally deliver factors, as opposed to systemically administer them or deliver them in liquid media (that rapidly and widely leak into tissues), is therefore key for controlling the directionality of angiogenesis. Local factor delivery would also help prevent unwanted side effects, such as ectopic angiogenesis, vascular leakage, tumour formation etc. Furthermore, recent studies have highlighted the importance of using physiological temporal patterns of angiogenic growth factor presentation (e.g. sustained vs. bolus delivery) for successful induction of angiogenesis [23,24]. This has motivated the development of injectable polymeric systems that allow controlled release of multiple recombinant angiogenic factors [25,26].

Previous work by this group has shown that exposing normal human dermal fibroblasts, seeded within 3D collagen matrices, to cell-mediated physiological hypoxia (the reduction in O_2 tension to 3% O_2 within constructs was achieved by cellular O_2 consumption) results in upregulation of angiogenic factor signalling (HIF-1a, VEGF) [13], while active production of hypoxia-induced factor proteins by hypoxic cell-matrix depots can induce directional angiogenesis *in vitro* and *in vivo* [27]. Importantly, the *in vivo* angiogenic response was both rapid (within 1 week), as well as functional, as shown by improvement in deep implant oxygenation compared to acellular constructs. Utilisation of implantable hypoxic cell-matrix depots, as factory units that actively produce angiogenic factors *in vivo*, could therefore prove a useful future strategy for improving vascularisation of engineered constructs and ischaemic tissues. However, the reliability on living cell implantation could be a setback for immediate clinical application, due to safety and ethical concerns. In addition, since it is difficult to characterise the *in vivo* behaviour of implanted cells (e.g. levels/duration of angiogenic factor production, cell survival etc.), process control in living cell implants still remains problematic. In an effort to overcome such limitations, we designed an implantable device for delivering hypoxia-induced signalling (HIS) without having to rely on living cells [28]. Here, dermal fibroblast-seeded nanoporous collagen matrices were cultured under cell-mediated hypoxia to upregulate production of angiogenic proteins (e.g. VEGF), before snap-freezing the matrices to kill all cells. Subcutaneous implantation of such pre-conditioned, non-viable depots in rabbits induced a directional angiogenic response within 1 week, through release of trapped angiogenic factor proteins. While this study provided a paradigm of how hypoxia-induced signalling can be delivered *in vivo*, on-demand, without relying on ongoing production of factors by living cells [29], it still faces certain limitations; 1) since the strategy is based on implanting an angiogenic depot, it is highly invasive and not only frustrating for patients, but also accompanied by the common complications of surgery (e.g. bleeding, infection, thrombosis), 2) since it has been shown that stabilisation of a newly formed vascular network requires long-term release of angiogenic factors (note; exogenous VEGF has a short half-life (~50 min) *in vivo*) [3], and that physiological angiogenesis critically depends on tight temporal regulation of factor release through differential gene expression at different time points [30–32], it is likely that any therapeutic approach will have to rely on multiple applications, which is difficult to carry out with an implantable device, and 3) frozen implants contained dead cells (in addition to the produced factors), which raises concerns about possible immunogenic reaction to allogeneic cells (which would be the likely cell source in an off-the-shelf preparation).

In this study we describe the development of a novel, injectable (i.e. minimally invasive) system for localised and temporally-controlled delivery of cell-free matrix carriers loaded with hypoxia-induced angiogenic factor proteins (Fig. 1). The current design addresses its predecessors' limitations, while providing the possibility of time-dependent capture, as well as repeatable HIS delivery.

2. Materials and methods

2.1. Characterization of type I collagen solution-to-gel transition

Acid soluble type I bovine collagen solutions (*c*: 2 or 4 mg/ml) were neutralised with drop-wise addition of alkali (NaOH 1 M). Typically total collagen solution volume was 5 ml, with a composition of 80% collagen, 10% 1 M NaOH and 10% 10xMEM with phenol red as pH-indicator. After neutralisation collagen solutions were stored either at +5 °C for 3 weeks or frozen at −20 °C for 1 h or 3 weeks, or immediately used for gel preparation. Gel formation was performed by incubation at 37 °C and examined every 4 min with a phase contrast microscope to determine gelling times (n = 4). Gelling time was defined as the minimum time required for observation of a homogeneous, interconnected network of branched clusters [33], in at least 5 visual fields (100 fold magnification) within the same focal plane.

2.2. Engineering of cell-free matrix carriers for hypoxia-induced cellular factors

A cell-seeded type I collagen gel (2×10^6 primary rabbit dermal fibroblasts, RDFs) was set into a 3.3×2.2 cm plastic mould (5 ml gel volume) and allowed to set for 30 min at 37 °C before undergoing plastic compression under fixed mechanical loading on a blotting paper support (for detailed description of collagen hydrogel plastic compression (PC) see Brown et al. [34]; note PC does not significantly reduce cell viability). The compressed cell-seeded collagen sheet was spiraled along its long axis and then a second, acellular compressed collagen sheet (produced by same PC method, dimensions; 3.3×2.2 cm) was spiraled (along its long axis) around the cell-seeded spiral. The two ends of the acellular collagen wrap were sealed to prevent lateral diffusion of cell-produced factors into media. The resulting 3D spiral construct, comprising an inner cellular and an outer acellular compartment (Fig. 1), was then cultured at 5% O_2, in medium (DMEM containing 4 g glucose/l, stable glutamine and 10% FCS) for 2 weeks. Following this incubation period, the acellular sheet was unwrapped and cut in two halves along its long axis (i.e. parallel to the radial factor diffusion axis

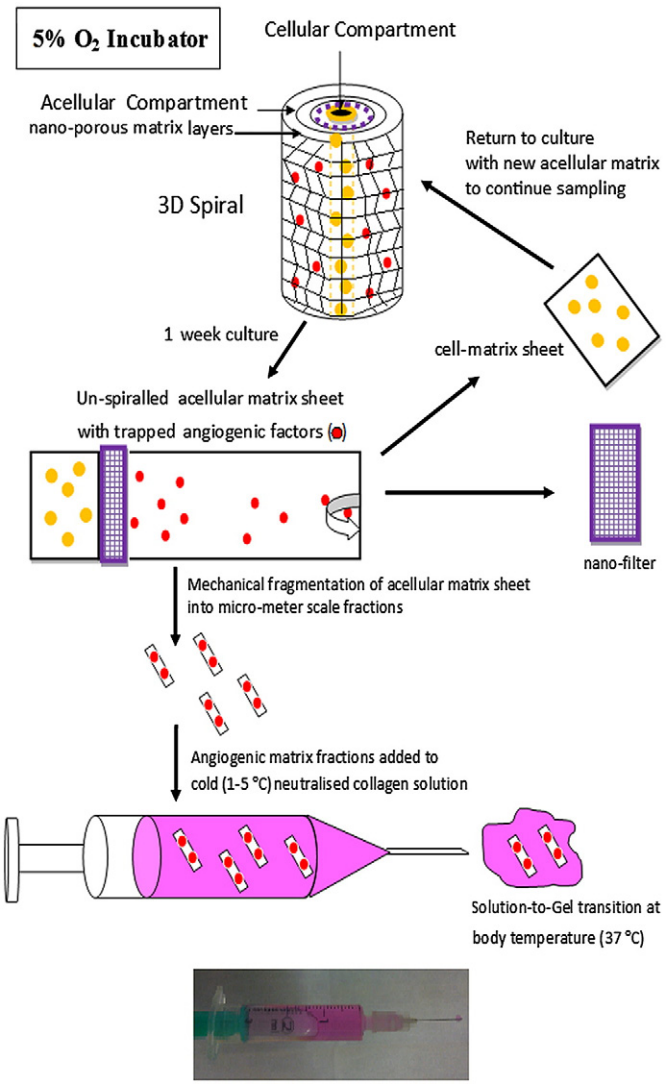

Fig. 1. System design. Schematic of system setup for exposing cells, seeded within a central matrix compartment, to hypoxia in order to upregulate expression of angiogenic factor proteins, which can be time-dependently collected within a peripheral acellular matrix compartment as they radially diffuse through it. The acellular nano-porous matrix wrap is sealed at both ends (e.g. with bio-glue) to prevent lateral diffusion of factors into media, and allows free diffusion of O_2 and nutrients (e.g. glucose) into the central cellular compartment. Optionally, it can be isolated from the cellular compartment through an intervening nano-porous filter to prevent movement of cells/pathogens into the acellular matrix. By removing acellular matrix layers at specified points during culture, freeze-storing them, and replacing them with fresh layers around the same cellular compartment, continuous sampling of temporally-specific factor mixtures can be achieved. Factor-loaded acellular matrix is subsequently mechanically fragmented into micro-fractions which are added to low temperature collagen sol–gel delivery vehicle for injection.

in the spiral) to produce two identical samples (i.e. both strips had same factor composition/concentration), which were then mechanically fragmented into micro-meter scale fractions with a micro-dismembrator (Fig. 1). Fractions from each sample were added to 500 μl medium (DMEM + 10% FCS, note; the BSA in FCS is important for factor stability) and stored in vials at −20 °C for either 4 weeks or 3 months. Following the storage period, fractions were thawed at +20 °C for 1 h and added into a neutralised type I bovine collagen solution (*T*: +5 °C, *c*: 4 mg/ml, *v*: 5 ml).

2.3. Quantification of angiogenic factors trapped within matrix fractions

Neutralised type I bovine collagen solutions (*T*: +5 °C, *c*: 4 mg/ml, *v*: 5 ml) containing matrix fractions (obtained from 2 weeks hypoxic culture, see above), and control collagen solutions without matrix fractions, were pipetted into 6-well plates and allowed to set into gels at 37 °C for 30 min. After setting, 1 ml of medium (DMEM + 10% FCS) was added into each well and incubated overnight, to allow diffusion of factors from collagen gels into media. Media were then sampled from each well and analysed with an Angiogenesis Proteome Profiler assay (R&D, USA), according to manufacturer's instructions. Quantification of relative (sample/control medium) amounts of factors present was carried out by image analysis of array signals (quantification of mean spot pixel densities) from scanned x-ray film images (4 and 6 min exposures) using an imaging software (Image J, NIH, USA). Four samples were tested for each condition (n = 4).

2.4. Visualization of HIF-1a and VEGF within matrix fractions

Matrix fractions were obtained by mechanical fragmentation of the cell-seeded compartment and acellular matrix wrap of 2 week hypoxia-cultured 3D collagen spiral constructs, stored for 3 months at −20 °C and added to collagen gels in 6 well plates. Once gels were set, they were incubated with primary antibody rabbit anti-human VEGF or HIF1a overnight at 4 °C, followed by secondary antibody goat anti-rabbit Alexa-568 or Alexa-488. Counterstaining of nuclei was done with DAPI. After staining procedure 4 fields of each sample were examined with a fluorescent microscope (Nikon) at 100 fold magnification (n = 4 for each condition). Fields were also viewed under phase contrast microscopy for visualization of collagen matrix fractions.

2.5. Analysis of the temporal profile of hypoxia-induced factor expression

A 4 ml type I collagen gel seeded with 8×10^6 RDFs was set into a 3.3 × 2.2 cm plastic mould. Following setting and PC (see above) the gel was cut into 4 equal strips (7 mm wide, 2.2 cm long each). Each strip was placed onto a compressed acellular collagen sheet (this was produced by setting a 5 ml collagen gel in the same 3.3 × 2.2 cm mould for 30 min, followed by 5 min PC); the cellular strip was placed at one end of the acellular sheet (long axis of cellular strip parallel to short axis of acellular sheet, both 2.2 cm), then the acellular sheet was spiraled along its long axis around the cellular strip. The acellular sheet was spiraled 6 times, i.e. there were 6 layers from the spiral surface to the cellular strip in the core. A single layer cell-seeded matrix was used here to prevent formation of O_2 consumption gradients (which form in thick 3D cellular matrices [13]), thus ensuring that all cells were exposed to the same O_2 level, and to maintain efficient capture of produced factors, since these diffused directly into the acellular compartment without first having to traverse cell-seeded matrix layers. The composite spiral construct (comprising inner cellular and outer acellular compartments) was cultured in 4 ml media (DMEM + 10% FCS) at 5% O_2 for a set period: at 2,4,8,12,16 and 20 days the construct was removed from culture, the acellular collagen wrap was unspiralled from the cellular strip and a fresh acellular collagen sheet was spiraled around the same cellular strip, before returning the construct into culture (Fig. 1). Media were changed with every acellular wrap change. Sampled acellular collagen wraps and media were stored at −20 °C. At the end of the experiment, collected acellular collagen sheets and the cellular strip were mechanically fragmented and added to collagen gels in 6 well plates as described above. After setting, 1.5 ml of medium (DMEM + 10% FCS) was added into each well and incubated overnight, to allow diffusion of factors from collagen gels into media. ELISAs for VEGF, PLGF, FGF and IL-8 (all from R&D, USA) were then carried out on samples obtained from these solutions, according to manufacturer's instructions. Four samples were tested for each factor, per time point (n = 4).

2.6. In vitro Matrigel assay for assessing freeze-stored matrix-bound factor biological activity

Matrix fractions containing hypoxia-induced factors, obtained from 2 week hypoxic cultures, were frozen at −20 °C for 3 months. Thawing, gel formation and diffusion of factors into media was performed as described above. The biological activity of hypoxia-induced factors was assessed by testing their ability to induce formation of capillary-like networking structures by endothelial cells seeded on Matrigel. Growth factor-depleted Matrigel (BD Biosciences, Germany) was thawed at 4 °C overnight. Using pre-cooled pipette tips and plates, the Matrigel was then distributed in 24-well plates (1 ml/well) or 12-well plates (1.5 ml/well) and allowed to solidify at 37 °C for at least 1 h. HUVECs (purchased from Cell Systems, Germany) were cultivated in VascuLife Basal Medium (Cell Systems, Germany) in 5% CO_2 at 37 °C, and used at passage 2. HUVECS were seeded at low density in 24-well plates (20 000 cells/well) and at high density in 12-well plates (150 000 cells/well), and left to attach for 4 h before adding 0.5 ml medium M-199 supplemented with 10%FCS. Factor-containing media (*v*; 1 ml) or control media (DMEM, *v*; 1 ml) were added to HUVEC-seeded Matrigel (3 samples per condition). Plates were incubated at 37 °C for 48 h. Tubule formation was observed with phase contrast microscopy, and digital images were captured using a Nikon digital camera. Assessment of the extent of capillary-like network formation in high cell density cultures was carried out by counting the number of tubules and nodes (a node was defined as the point of intersection of two or more tubules). A minimum of 5 fields (100 fold magnification) were analysed per sample, with 3 samples tested for each condition.

2.7. Statistical analysis

For each experimental condition $n \geq 3$ was used. Data is expressed as mean ± standard deviation. Statistical analysis was carried out using *t*-test where a maximum of 2 groups was used per analysis or oneway ANOVA accompanied with multiple comparison tests for analysis of 2 or more groups, using SPSS 14 software. Differences were considered significant when $p<0.05$, unless otherwise noted.

3. Results

3.1. Characterisation of collagen sol–gel as injectable delivery vehicle

The proposed delivery vehicle for cell-free matrix carriers of hypoxia-induced angiogenic factors in this system is alkali-neutralised (acid-soluble) type I collagen, which can undergo a temperature-sensitive solution-to-gel (sol–gel) phase transition. We therefore first investigated the correlation between collagen concentration and rate of sol–gel transition. Fig. 2A shows that 4 mg/ml solutions gelled approximately twice as fast as 2 mg/ml solutions, suggesting that a near-linear co-relation exists, at least in the range of concentrations tested.

The effect of cold-storing neutralised collagen solution, prior setting, on sol–gel transition rate was then tested. No significant difference in gelling time was observed between collagen solutions (4 mg/ml) set immediately after neutralisation or pre-stored at + 5 °C for 3 weeks (Fig. 2B), indicating that cold-storing neutralised collagen solution prior setting does not interfere with the gelling process. The effect of freezing neutralised collagen solution was also tested by storing it at −20 °C prior thawing and setting. While short-term (1 h) freeze storage did not significantly affect gelling time, solutions that had been stored frozen for a longer period (3 weeks) appeared to gel significantly faster than both solutions stored at −20 °C for 1 h or not stored at all ($p<0.05$) (Fig. 2C).

Dynamic changes occurring within the collagen fibrillar network during gelling could be analysed with phase contrast microscopy, as previously described [33]. In solutions of collagen concentration 4 and 2 mg/ml branched fibrillar structures first appeared at 8 min and 40 min of incubation at 37 °C, respectively (Fig. 3B, G arrowed). In 4 mg/ml solutions more filament clusters appeared at various sites of polymerisation between 8 min (B) and 12 min (C), while the branched filament bundles started to intersect at 16 min (D). After this time the structure of the network no longer changed rapidly, such that no significant difference was observed between images at 16 min (D) and 20 min (E). By this time, a more homogeneous assembly of randomly distributed branched clusters had formed, and the clusters were partly interconnected. Collagen solutions (4 mg/ml) that had been stored at +5 °C following neutralisation, for 3 weeks, exhibited normal polymerization by 12 min at 37 °C (I, J). In contrast, collagen solutions (4 mg/ml) that were frozen at −20 °C following neutralisation, and stored for 3 weeks, showed accelerated polymerization at 37 °C (M–O), compared to collagen solutions that were not frozen (A–E) or briefly frozen at −20 °C for 1 h following neutralisation (K, L). In this case, clusters of fibers (clumps) appeared at an early stage (4 min, M), while individual fibers were

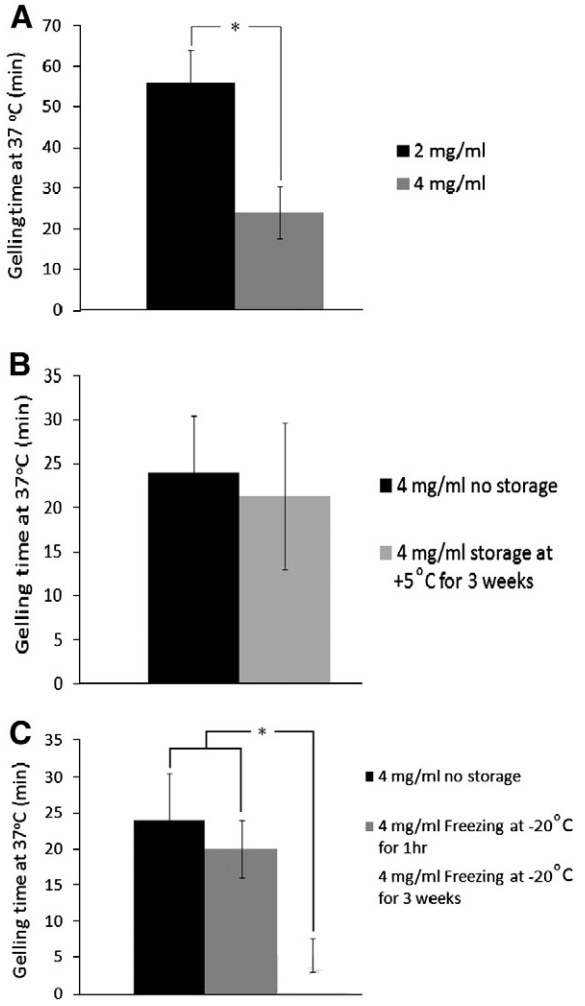

Fig. 2. Effect of collagen concentration and cold/freeze storage on collagen sol–gel transition rate. A) Plot showing *in vitro* gelling times for alkali-neutralised collagen (bovine type I) solutions (*v*; 1 ml) of concentration 2 and 4 mg/ml, at +37 °C. Four collagen solutions were tested for each condition (n = 4), *$p<0.05$. B) Plot comparing gelling times for neutralised collagen solutions (*v*; 1 ml, *c*; 4 mg/ml) that were either incubated at +37 °C immediately after neutralisation or stored at +5 °C for 3 weeks before incubation at + 37 °C. Four collagen solutions were tested for each condition (n = 4). C) Plot showing the effect of freezing a neutralised collagen solution at −20 °C on gelling time. Neutralised collagen solutions (*v*; 1 ml, *c*; 4 mg/ml) were either incubated at +37 °C directly after neutralisation or frozen at −20 °C and stored for 1 h or 3 weeks, then thawed at +20 °C for 1 h and incubated at +37 °C. Four collagen solutions were tested for each condition (n = 4), *$p<0.05$.

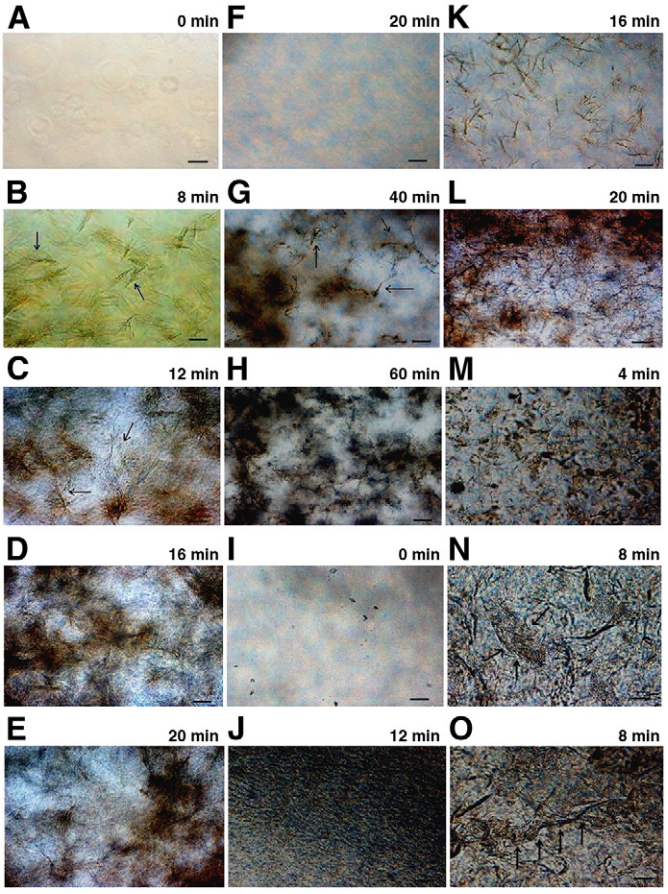

Fig. 3. Visualization of collagen polymerization during sol–gel phase transition. Time series of phase contrast micrographs of collagen solutions (same sample analysed over time for each condition) at concentration 4 mg/ml (A–E) and 2 mg/ml (F–H), that were incubated at +37 °C directly after neutralisation with alkali. Collagen solutions of concentration 4 mg/ml were also pre-stored for 3 weeks at +5 °C (I, J), and at −20 °C for 1 h (K, L) or 3 weeks (M–O), prior to incubation at +37 °C (frozen samples stored at −20 °C were thawed at +20 °C for 1 h prior to incubation at +37 °C). Arrows show branched bundles of filaments (B, C, G) or larger fibre-cluster structures (N–O). Bars = 100 μm.

difficult to identify (compared with B and K, where individual fibers were visible). As gelling progressed, larger fibre-cluster structures that did not exhibit significant branching could be seen (N, O; arrowed), with complete polymerisation being observed at 8 min. This suggests that while freezing a neutralised collagen solution for a prolonged period (i.e. weeks) does not inhibit its thermo-responsive sol–gel property, it might have an effect on the polymerisation process at the fibrillar level.

3.2. Development of cell-free carrier system for hypoxia-induced factors

The system setup employed here for production and capture of hypoxia-induced angiogenic factors is shown in Fig. 1; cells (rabbit dermal fibroblasts) seeded within the central compartment up-regulated factor expression in response to hypoxic exposure (5% O_2), while produced factors were captured while radially diffusing through the peripheral acellular compartment comprising nano-porous collagen matrix (note; this allowed free diffusion of O_2/nutrients to the cellular compartment [35,36]) (Fig. 1). Following culture, the acellular matrix underwent mechanical fragmentation to obtain micro-meter scale fractions loaded with angiogenic factors, that were then incorporated into a type I collagen sol–gel for testing (Fig. 1).

An angiogenesis proteome profiler assay was used to rapidly screen the expression profile of angiogenesis-related proteins present within matrix fractions obtained from 2 week hypoxic cultures, and stored at −20 °C for 1 or 3 months prior testing. Factors trapped in matrix fractions were delivered into media (DMEM + 10%FCS) by diffusion through a type I collagen sol–gel. Fig. 4 shows that for a range of angiogenic factors their level in media, which were incubated with collagen gels containing matrix fractions, was significantly elevated relative to that in control media incubated with empty gels. The levels of VEGF, FGF, PLGF and IL-8 were found to be particularly elevated. Importantly, for all factors detected there was no significant difference in factor relative (sample/control medium) level between samples stored for 1 or 3 months, suggesting that factors remained stable after 3 months of storage at −20 °C.

HIF-1a could be visualized by immunofluorescence within matrix fractions obtained from the cell-seeded compartment of constructs that had been cultured under 5% O_2 for 2 weeks, which confirmed its upregulation in response to hypoxia in this system (Fig. 5). Immunofluorescent staining was also used to detect VEGF within matrix fractions obtained from the cellular and acellular compartments (Fig. 5), which confirmed the previous quantitative findings (Fig. 4).

In order to dissect out the temporal profile of angiogenic factor expression in this system, the acellular collagen compartment (peripheral wrap) containing trapped factors was removed and replaced by a fresh one at set time intervals during culture, thus allowing continuous sampling of hypoxia-induced cellular factors over 20 days. Fig. 6a shows that production of factors was not linear over time. Indeed, VEGF and IL-8 expression initially decreased from day 2 to day 4, and thereafter increased, peaking at day 8. From that point on expression showed a gradual decline, reaching significantly lower levels ($p<0.05$) compared to the day 8 peak by day 16, and falling below initial levels by day 20. FGF expression, on the other hand, showed a small initial decrease from day 2 to 4 (similar to VEGF and IL-8), but thereafter remained relatively stable. PLGF was also present within acellular matrix, reaching a concentration of 9.5 ± 1.19 pg/ml at day 2, but levels were too low to be measurable after this time point. However, this apparent down-regulation in PLGF expression appeared to follow the same pattern as for VEGF and IL-8. There was a higher accumulation of FGF in the cellular compartment, relative to the acellular compartment, compared to VEGF and IL-8 over the 20 day culture period (Fig. 6B), which was likely the result of FGF binding to FGF receptors on fibroblasts within the cellular compartment. A similar difference (~15 fold) in FGF and VEGF/IL-8 concentration gradient was also observed between the acellular compartment and medium (16 to 20 day period) (Fig. 6C). Nonetheless, retention-release ratios (ratio of factor concentration in acellular compartment to that in medium) greater than 1, for all factors tested, confirmed the ability of the nano-

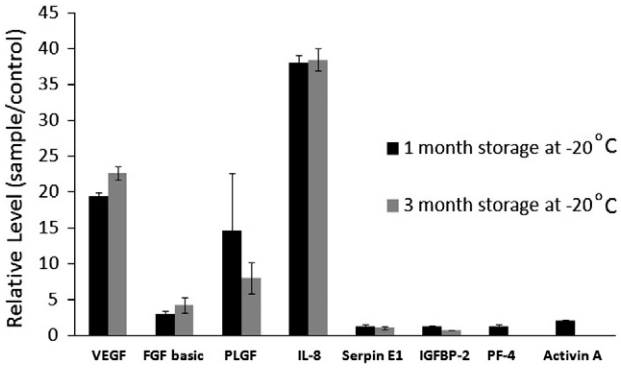

Fig. 4. Matrix-bound hypoxia-induced factors remain stable over 3 months freeze storage. Plot showing the profile of angiogenic factors that were trapped in 1 and 3 month freeze stored (at −20 °C) matrix fractions, and delivered into media (DMEM) by diffusion through a type I collagen sol–gel (*c*; 4 mg/ml). Y-axis represents the ratio of factor level in sample medium (i.e. medium added to collagen gels containing matrix fractions) over control medium (i.e. medium added to empty collagen gels). 3 samples were tested for each condition. Bars indicate standard deviation of means.

porous collagen matrix to efficiently trap hypoxia-induced cellular factors diffusing through it.

3.3. Test of the biological activity of freeze stored hypoxia-induced factors

Since this system is primarily targeted towards an off-the-shelf clinical therapy, able to deliver hypoxia-induced angiogenic factors 'on-demand', the ability to store factors prior use is important. Having shown that 3 month freeze storage does not interfere with factor stability (Fig. 4), we moved on to test the ability of 3 month freeze-stored matrix-bound factors to induce tubule formation *in vitro*, as a test of factor biological activity. Factors were delivered from matrix fractions (obtained after 2 weeks hypoxic culture) into media (by diffusion through type I collagen sol–gel), which were then added onto Matrigel seeded with endothelial cells (HUVECs). Fig. 7A shows that in low cell density cultures (~10×10^3 HUVECs/cm^2), where cell-cell contact was minimal, addition of factor-containing media (VEGF concentration; 2 ± 0.3 ng/ml) resulted in endothelial cells with an elongated/sprouting morphology, within 48 h, which is considered to be the *in vitro* counterpart of *in vivo* angiogenic endothelial cells [37]. In contrast, in cultures where control media (DMEM) were added, endothelial cells maintained a cuboidal/cobblestone appearance, reminiscent of resting endothelial cells lining vessel lumens [37]. In high cell density cultures (~40×10^3 HUVECs/cm^2), capillary-like network formation was evident at 48 h when factor-containing media were added, compared with minimal tubule formation observed with control media (Fig. 7A). Indeed, the difference in number of capillary-like tubules formed in high cell density cultures with factor-containing media versus control media was statistically significant both at 24 h and 48 h ($p<0.05$ and $p<0.01$, respectively), although the difference in number of nodes (defined as points of intersection of two or more tubules) only became significant at 48 h (Fig. 7B). These findings confirm that matrix-bound factors maintain their biological activity after at least 3 months of freeze storage.

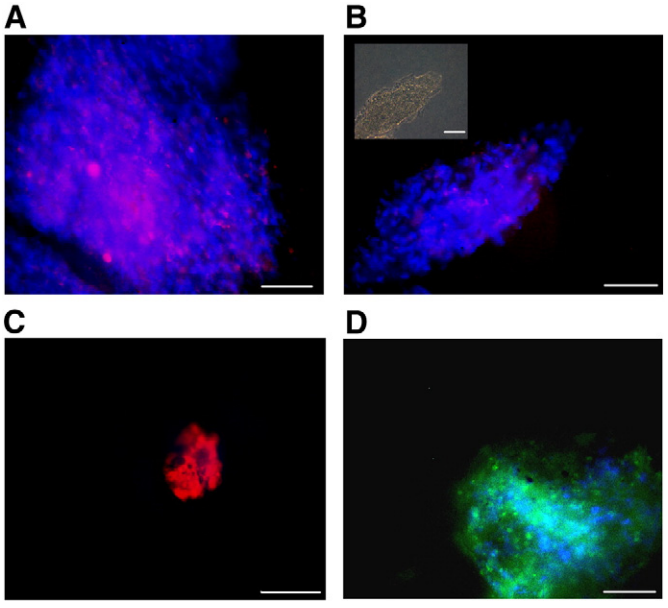

Fig. 5. Visualization of hypoxia-induced factors trapped within matrix fractions. Immunofluorescent staining of VEGF (red: A, B, C) and HIF-1a (green: D), within cell-seeded (A, B, D) and acellular (C) matrix fractions obtained from mechanical fragmentation of the cellular or acellular matrix compartments of constructs cultured under hypoxia (5% O_2) for 2 weeks (constructs comprised a central cellular compartment and outer acellular matrix wrap). Matrix fractions were stored for 3 months at −20 °C before being added into a type I collagen solution (4 mg/ml), which was allowed to set into gel at 37 °C. DAPI (blue) nuclear staining was positive in cellular matrix fractions. Inset in image B shows corresponding field (only matrix fraction visible) viewed under phase contrast. Bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

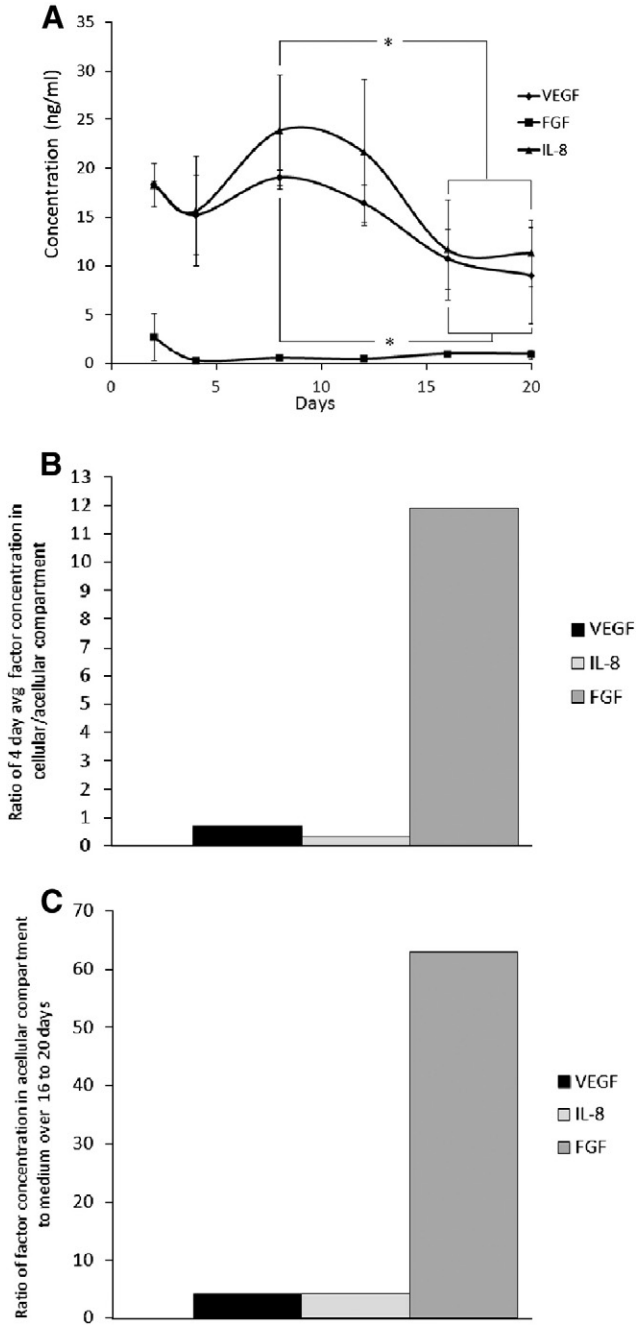

Fig. 6. Analysis of the temporal profile of hypoxia-induced factor expression. A) Plot of the temporal profile of factor (VEGF, IL-8, FGF basic) concentration in the acellular matrix compartment at 2, 4, 8, 12, 16 , and 20 days of *in vitro* culture at 5% O_2. Four samples were tested for each factor, per time point (n = 4). Bars represent standard deviation of means, *$p<0.05$. B) Plot showing the ratio of the 4 day average factor concentration in the cellular compartment to that in the acellular compartment, over the 20 day culture period (n = 4). C) Plot showing the ratio of factor concentration in the acellular compartment to that in the medium over 16 to 20 days of culture (n = 4).

4. Discussion

Recent work from this group, and others, has highlighted the effectiveness of hypoxia-induced signalling as a tool for inducing physiological angiogenesis in ischaemic tissues and tissue engineered implants [14,16,27,28,38]. Here we have developed a novel system for optimizing the delivery of hypoxia-induced angiogenic growth

factor mixtures. The current system is cell-free, thus overcoming potential safety and ethical concerns associated with living/dead cell-based therapies. Furthermore, the ability to deliver factors through an injectable sol–gel would allow spatio-temporally controlled delivery, which is a pre-requisite for regulated angiogenic induction [4,24].

The proposed system comprises two primary components; cell-free matrix fractions as carriers of cell-produced, hypoxia-induced factors, and a thermo-responsive collagen sol–gel as injectable delivery vehicle (Fig. 1). Therefore, factor release kinetics can be controlled at two levels, by tuning the nano-/micro-scale structure (i.e. density/porosity/pore size) and biodegradability of the matrix in which factors are trapped, as well as of the resulting gel in which they are delivered. These properties could be adjusted by selecting suitable biomaterials and, optionally, with additional chemical modification (e.g. cross-linking) [39]. The nano-porous matrix structure forms an important design criterion in selecting suitable biomaterials for the acellular compartment, since this effectively ensures that macro-molecular cellular factor proteins can be efficiently retained as they diffuse through it, while O_2 and nutrients (e.g. glucose) can rapidly reach the cellular compartment [35,36], in order to maintain high cell viability during culture (this is especially relevant under conditions of reduced O_2 tension). A nano-porous internal structure also provides a large surface area for optimizing factor binding and retention. The functionality of an injectable sol–gel, as delivery vehicle for factor-loaded matrix fractions, is based on the idea that upon injection, the solution undergoes a solution-to-gel phase transition at body temperature, and therefore it remains (together with the contained matrix fractions) localised at the injection site. The gel then effectively acts as a reservoir that sequesters the factors at the injection site, while releasing them in a sustained/controlled manner (i.e. avoiding spikes in factor concentration and short-lived effects). This would also help prevent unwanted systemic side effects (e.g. ectopic angiogenesis, vascular leakage, mitogenic effects etc.) that are common with systemic factor delivery or injection of liquid media that leak into tissue [2,40].

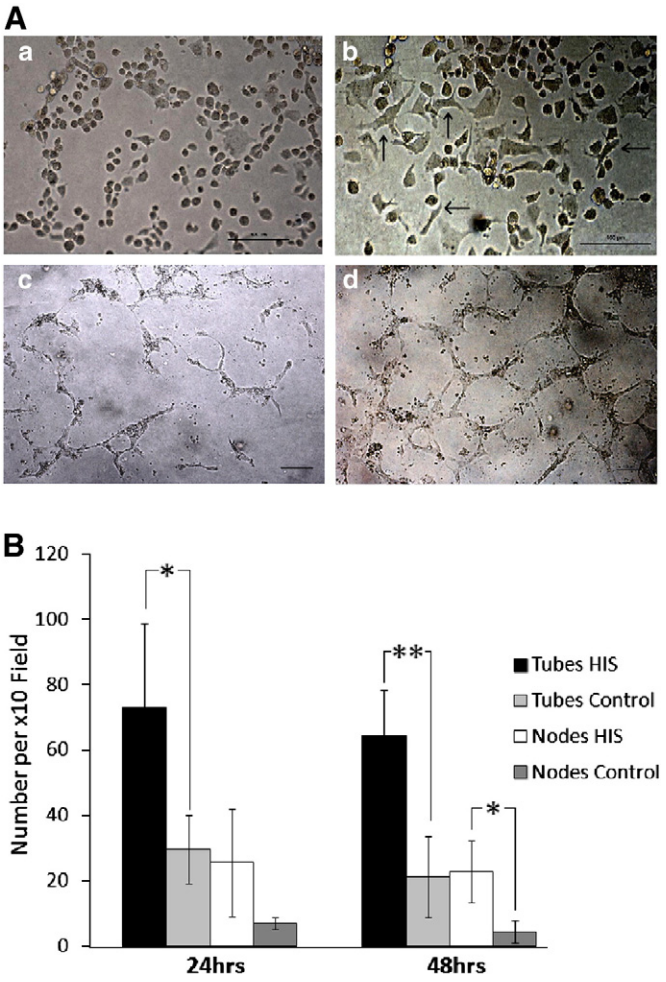

Fig. 7. Media containing 3 month freeze-stored hypoxia-induced factors, delivered from 2 week culture matrix fractions by diffusion through type I collagen sol–gel, induce tubule formation *in vitro*. a) HUVECs were seeded at low density (~10×10^3 cells/cm^2; a,b) and high density (40×10^3 cells/cm^2; c, d) on Matrigel-coated plates and cultured with control media (DMEM) (a,c) or factor-containing media (b, d) for 48 h. Phase contrast microscopy images show elongated cells in B (arrowed) and network tube formation in D, bars = 100 μm. B) Plot of the number of tubules and nodes formed in high cell density cultures with media containing hypoxia-induced signalling (HIS) factors and control media, at 24 h and 48 h. A minimum of 5 fields (×10) were analysed per sample, with 3 samples tested for each condition, *$p<0.05$ and **$p<0.01$.

Here we show that a biomimetic nano-porous matrix, such as plastic compressed collagen matrix [34], configured into a 3D spiral (i.e. a multiplayer 360° wrap) around the cellular compartment, has the ability to efficiently retain hypoxia-induced cellular factors, radially diffusing through it. While it would be possible to carry out the loading of factors onto matrix carriers (e.g. from pre-conditioned media) at a later stage, our system employs simultaneous loading of factors as they are being produced, which ensures physiological levels of factor concentration, since these will effectively only be determined by the matrix biomimetic 3D structure (i.e. pore diameter, pore size distribution, and especially total pore volume) [41]. Importantly, our findings indicate that the variable temporal trace of factor expression could be faithfully captured within the matrix over time, suggesting that matrix porosity/total pore volume was a match for (i.e. sensitive to) the range of factor levels encountered. In addition to natural polymers, synthetic polymers (in various forms, e.g. nano-/micro-fibers, micro-spheres) can also be used as factor-binding carriers, as these can be highly tailored in terms of biodegradability and factor release kinetics [42–44].

Our findings show that collagen concentration is a good controller of the rate of sol–gel phase transition in alkali-neutralised type I collagen solutions, at 37 °C. Additionally, it has previously been shown that incorporation of collagen-binding micro-particles (e.g. polystyrene latex beads) within the collagen solution, above a critical number density, can accelerate the sol–gel transition [45]. This is important, as control of the rate in which injected solution gels within the body would allow control of the extent of factor localisation/spread at a preferred site. We also found that neutralised collagen solution maintains its thermo-responsive property after prolonged periods of cold or freeze storage. Since matrix-bound hypoxia-induced factors remained stable and biologically active at low temperature (for a minimum of 3 months at −20 °C, as shown by their ability to induce tubule formation *in vitro*), the complete formulation (i.e. collagen solution with factor-loaded matrix fractions) could be stored (at liquid or frozen state) for significant periods before application, which would be useful for off-the-shelf clinical use. Collagen hydrogels are preferred candidate delivery vehicles as they are thermo-responsive, biocompatible (i.e. non-immunogenic, FDA approved) and biomimetic (e.g. they undergo natural cellular remodelling). However, other biodegradable temperature-sensitive polymers could be used to locally deliver angiogenic matrix fractions, including polyesters, polyphosphazenes, polypeptides, and chitosan [46,47]. In addition to an injectable thermo-responsive solution, a sustained delivery vehicle could be a semi-solid material, such as a conventional gel of an appropriate viscosity, that can be injected (e.g. alginate gel [48], fibrin gel [49]).

Detection of angiogenic factors within media, incubated for 24 h with collagen gels containing matrix fractions, indicated that matrix-bound factors were successfully released and diffused through the collagen sol–gel used as delivery vehicle. The ability of hypoxia-induced angiogenic factors (e.g. $VEGF_{165}$), trapped within a biomimetic matrix, to diffuse and form a spatial gradient over time through a type I collagen hydrogel *in vitro* has already been described [28], which is

critical for this system's utility as a tool for inducing directional angiogenesis. Furthermore, we showed that conditioned media containing hypoxia-induced factors were effective not only in stimulating tubule formation in high endothelial cell density cultures, but also in encouraging morphological changes, characteristic of early angiogenesis, at the single cell level (low density cultures). Together, these findings suggest that localised delivery of HIS factor mixtures could successfully drive angiogenesis even in tissue areas distant from a perfusing vascular source, where endothelial cell migration towards that site would occur slowly.

Injection of factor-loaded, cell-free carriers through a sol–gel/conventional gel represents a minimally invasive, repeatable form of administration, which is necessary for temporal process control. Here we show that factor (VEGF, IL-8) protein levels within matrix fractions varied over time. Importantly, stable FGF levels confirmed the relatively stable cell viability in this system [13], suggesting that temporal variations in the level of VEGF and IL-8 were a result of changes in gene expression, and not just a consequence of varying cell number. Indeed, similar temporal changes in VEGF gene expression (i.e. early up-regulation followed by down-regulation) were previously observed in engineered 3D fibroblast-seeded collagen constructs cultured under hypoxia [13], in endothelial cell/fibroblast co-cultures in 3D nanofibres [50], as well as in neonatal rodent retinas during early vascularisation [30]. It is possible that down-regulation of growth factor expression over time is the result of cellular habituation to the oxygen micro-environment, which could explain why chronically ischaemic/hypoxic tissues have a limited angiogenic capacity despite prolonged exposure to hypoxia [17,18]. By allowing continuous sampling of matrix-bound factors the current system provides, for the first time, the possibility to obtain cell-free carriers loaded with 'time-specific' hypoxia-induced factor mixtures (i.e. corresponding to different phases of gene expression). These could be injected at pre-determined, suitable time intervals, for example matching the *in vivo* temporal profile of factor expression during a pre-habituated state, in order to temporally recapitulate a physiological angiogenic factor response, thus overriding cellular habituation to hypoxic stress.

In addition to culture duration, the composition/concentration of hypoxia-induced factor mixtures could be controlled by selecting specific cell types, as it is well known that different cells respond differently to hypoxia. While here only one cell type was tested, the cellular compartment could comprise multiple cell type-specific matrix layers (e.g. inner endothelial cell layer, middle vascular smooth muscle cell layer, and outer fibroblast layer, thus mimicking a vascular wall structure). Such organotypic co-cultures could provide tissue-specific, physiological factor mixtures that could be delivered to corresponding *in vivo* tissue sites, that as a result of habituation to chronic hypoxic stress, have lost the ability to appropriately respond by up-regulating angiogenic signalling.

5. Conclusion

This study describes the development of a novel injectable (i.e. minimally invasive), cell-free system for spatio-temporally controlled delivery of hypoxia-induced angiogenic factor mixtures that can provide a useful tool for stimulating physiological angiogenesis in ischaemic tissues and aid the vascularisation of grafts and tissue engineered implants.

Disclosure Statement

In the past 5 years AFS has provided consulting services to IPB and has received institutional support by Biomet, Curasan, Eucro, Heraeus, and Johnson & Johnson. No royalties. Parts of this work have been submitted as a patent application by E. Hadjipanayi, H.G. Machens and A.F. Schilling.

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