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Hypoxia-based strategies for angiogenic induction

The dawn of a new era for ischemia therapy and tissue regeneration

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Therapeutic angiogenesis promises to aid the healing and regeneration of tissues suffering from a compromised vascular supply. Ischaemia therapy has so far primarily focused on delivering isolated angiogenic growth factors. The limited success of these strategies in clinical trials, however, is increasingly forcing researchers to recognize the difficulties associated with trying to mimic the angiogenic process, due to its natural complexity. Instead, a new school of thought is gradually emerging, focusing on how to induce angiogenesis at its onset, by utilizing hypoxia, the primary angiogenic stimulus in physiological, as well pathological states. This shift in therapeutic approach is underlined by the realization of the importance of depressed HIF-1 α-mediated gene programming in non-healing ischemic tissues, which could explain their apparent habituation to chronic hypoxic stress and the limited capacity to generate adaptive angiogenesis. Hypoxia-based strategies, then effectively aim to override the habituated angiogenic cellular response, re-start the regenerative process and drive it to completion. Here we make a distinction between those strategies that utilize hypoxia in vitro as a preconditioning tool to optimize the angiogenic potential of tissue/cells before transplantation, vs. strategies that aim to induce hypoxia-induced signaling in vivo, directly, through pharmacological means or gene transfer. We then discuss possible future directions for the field, as it moves into the phase of clinical trials.

Introduction

Conditions associated with tissue ischemia (myocardial infarction, peripheral vascular disease, stroke) have a high prevalence in the ever-aging patient population of the western world, and lead to high morbidity and mortality.[1] Until recently, strategies aiming at therapeutic angiogenesis for the treatment of ischemic tissues (e.g., myocardial, cerebral, peripheral tissues) and wounds (e.g., diabetic ulcers, burns), as well as vascularisation of implants/grafts and engineered constructs have largely relied 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 the concept that the spatio-temporal complexity of an angiogenic response is difficult to mimic, by isolating and delivering certain factors (e.g., VEGF).[1-5] This has fuelled the development of a new field of angiogenesis research, focusing on inducing, rather than mimicking, the angiogenic process through hypoxia. Utilization of hypoxia-induced signaling as an angiogenic tool, harnesses the innate biological mechanism that naturally generates angiogenesis in the body, in physiological (e.g., embryogenesis), as well as pathological (e.g., ischemia, wound healing, tumor formation) states.[2,6,7] Furthermore, this approach has the advantage that it can be easily and successfully applied, even if the complex angiogenic factor cascades are currently mapped only incompeltely. Admittedly, as the field advances and gathers more universal acceptance, a broader spectrum of studies will also provide additional insight into the mechanisms facilitating hypoxia-induced angiogenesis. This knowledge can then feed back into developing more targeted therapies.

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Tissue Response to Chronic Hypoxic Stress

Ischaemia-induced angiogenesis is a physiological response to tissue hypoxia, defined as a reduction in the ambient O_2 concentration. Most tissues trigger a hypoxia response below venous pO_2 (40 mmHg or ~6% O_2),[8] and this is orchestrated by the transcriptional activator hypoxia-inducible factor 1α (HIF-1α).[2,7,8] HIF-1α stabilization 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 (PGF), angiopoietins 1 and 2, matrix metalloproteinases (MMPs) 2 and 9, plasminogen-activator inhibitor-1, stromal derived factor 1 (SDF 1) and stem cell factor (SCF).[7,8] However, while chronically ischemic tissues are constantly exposed to hypoxia, they paradoxically appear to have a limited capacity to appropriately respond to hypoxic stress. The proposed mechanism underlying the inadequate generation of compensatory angiogenesis seen in many chronic ischemic/hypoxic

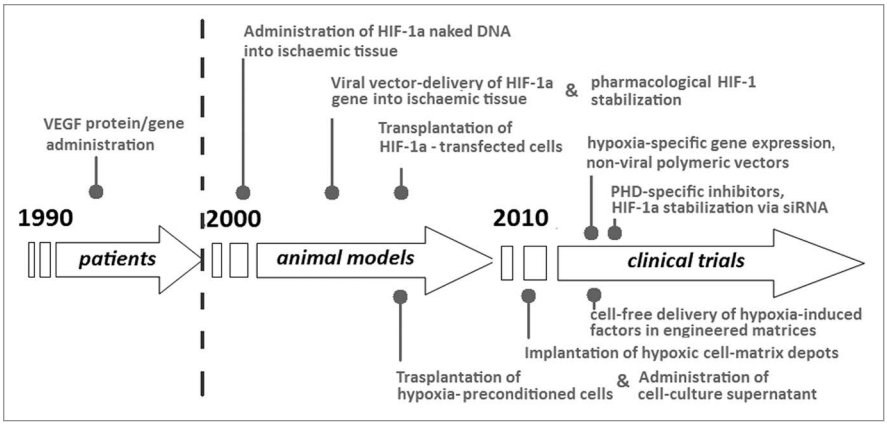

Figure 1. Flow diagram showing the chronological development of strategies targeting therapeutic angiogenesis for tissue ischemia. Following the limited success of single factor (primarily VEGF) administration in clinical trials, hypoxia-based strategies have emerged (dotted line) as a new approach that promises to provide a solution to inducing a robust, yet physiological angiogenic response, while avoiding the common side effects associated with mono-therapy. Strategies shown above the time axis represent those therapies that focus on directly inducing hypoxia-mediated angiogenic signaling in vivo, while strategies shown below the axis are those based on in vitro hypoxic pre-conditioning before in vivo cell/tissue transplantation. On the right end of the axis, a range of newer strategies are presented, which are likely to play a key role as the field moves into the phase of clinical trials.

conditions involves a blunting of the ability of cells to upregulate angiogenic factors (e.g., VEGF, angiopoietins) in response to prolonged/repeated hypoxic episodes.[9-14] Indeed, Van Weel and coworkers examined the expression patterns of VEGF, SDF-1, and CXCR4 in amputated limbs of 16 patients with peripheral arterial desease, and showed that their expression was generally decreased in ischemic muscle as compared with non-ischemic muscle in patients with chronic ischeamia, whereas substantially increased in patients with acute-on-chronic ischemia.[13] Importantly, only acute ischemic tissue displayed a high percentage of HIF-1α - positive nuclei, suggesting an inability of chronically ischemic tissues to express sufficient HIF-1α. This could explain why hypoxia-responsive genes, that are upregulated under acute ischemic conditions, gradually undergo downregulation in chronic ischemia. These findings were confirmed in another study where attenuated HIF, VEGF, and VEGFR-2 expression was observed in atrophic/regenerating myocytes from skeletal muscle samples, obtained from lower limbs that were amputated due to chronic critical ischemia, despite significant induction of these genes in samples from acute-on-chronic ischemic limbs.[15] The impairing effect of chronic ischemia/hypoxia on angiogenic factor signaling might be further complicated by the fact that the spatial and temporal distribution patterns of endogenously produced angiogenic growth factors in ischemic tissues are, to a great extent, influenced by inflammation,[16] but also because chronic hypoxia seems to attenuate VEGF-stimulated signaling in endothelial cells through specific downregulation of VEGF receptor expression.[17]

Hypoxia-Based Therapeutic Strategies

Over the last few years, with the gradual increase in awareness of the critical role that hypoxia-induced signaling could play as a tool for generating angiogenesis on demand,[7,18] two distinct approaches have emerged, as promising strategies to achieve this goal. On one hand, researchers have explored the possibility of pre-conditioning cells or grafts to hypoxia in vitro, in order to upregulate the required signaling that can then initiate angiogenesis in vivo upon transplantation. The second approach relies on direct induction of hypoxia-mediated signaling in vivo, by pharmacological means or gene therapy. A further distinction can be made on whether the therapy involves transplantation of hypoxia pre-conditioned or genetically modified cells, or if the effect is mediated directly through gene transfer or cell-free delivery of hypoxia-induced protein factors (**Fig. 1**). Nevertheless, all of these strategies aim to provide an effective solution for overcoming the limited ability of ischemic tissues to optimally upregulate angiogenic signaling, by overriding the habituated response of cells within an ischemic tissue to the constant oxygen micro-environment, supporting/re-starting the angiogenic process and driving it to completion.

Strategies Based on Hypoxic Pre-Conditioning In Vitro

As discussed above, while cells in an ischemic tissue are exposed to hypoxic stress, they increasingly respond less to it, as they habituate to constant stimulation. A sound approach to overcome this limitation would be to obtain cells from healthy tissue and expose them in vitro to controlled conditions that simulate the in vivo micro-environment, for short time periods to avoid habituation, as a means for optimizing cell properties (e.g. viability, angiogenic capacity) before transplantation. Indeed, this type of pre-conditioning is commonly encountered in nature, for example certain animals that inhabit environments where low O_2 tensions prevail (moles living underground or birds at high altitudes) have developed strategies to sustain chronic hypoxia and have become hypoxia tolerant.[18] Furthermore, it is known that hypoxic/ischemic pre-conditioning provides tissues (e.g., myocardium, cerebral tissue) with protection against subsequent lethal ischemic damage. Therapeutic strategies utilizing hypoxic pre-conditioning therefore aim to recapitulate these mechanisms and deliver their beneficial effects, on demand. One can broadly distinguish two approaches, one that relies on transplanting pre-conditioned cells into ischemic tissue directly, the other based on delivering cell-free angiogenic factor protein mixtures that the cells produce during the conditioning phase.

Cell-based pre-conditioning therapy. In 2008, Kubo et al. showed that hypoxic pre-conditioning (culture under 2% O_2 for 24 h) increased the survival and angiogenic potency of peripheral blood mononuclear cells (PBMNCs), through oxidative

stress resistance mechanisms. Three days after intramuscular implantation into the ischemic hindlimbs of mice, survival of hypoxia-preconditioned PBMNCs was higher than that of normoxia-cultured PBMNCs, while 28 d after treatment microvessel density and blood flow in the ischemic hindlimbs were significantly better in the mice implanted with hypoxia-preconditioned PBMNCs.[19] Using the same animal model, the authors showed that increased expression of CXCR4 and integrin alphaM contributed to the improved cell retention and angiogenic potency observed in hypoxia-preconditioned cells.[20] Interestingly, these effects do not appear to be limited to cells derived from young organisms. Indeed, hypoxia-preconditioned bone marrow cells (BMCs) from aged mice had enhanced adhesion, survival, and angiogenic potency in vitro, as well as upon implantation into ischemic hindlimbs in vivo.[21] Hypoxic pre-conditioning has also been shown to enhance the capacity of mesenchymal stem cells to repair infarcted myocardium, attributable to reduced cell death and apoptosis of implanted cells, increased angiogenesis/vascularization, and paracrine effects.[22] A recent study demonstrated that transplantation of hypoxia pre-conditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats, suggesting that hypoxic pre-conditioning of transplanted cells could provide an effective means of promoting their regenerative capability and therapeutic potential for the treatment of ischemic stroke.[23]

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 can be achieved by localized delivery of a cell-matrix depot to an area of interest (e.g., infracted tissue, implant site etc), while allowing diffusion of produced factors to generate spatial factor gradients that chemo-attract host endothelial cells toward the factor source.[24,25] Our group first showed that it is indeed possible to control the local O_2 microenvironment within 3D collagen constructs by adjusting the cell seeding density and spatial position, therefore total cell-depot O_2 consumption.[26] Seeding constructs with human dermal fibroblasts (HDFs) at high density resulted in rapid reduction of core O_2 tension toward the low end of the physiological hypoxic range (3% O_2), which elicited a multifold upregulation of HIF-1α and VEGF gene expression.[26] Localized implantation of such hypoxia-induced signaling cell-matrix depots, subcutaneously in rabbits, induced directional in-growth of host vessels into the constructs.[25] 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 with acellular constructs. Adopting a similar, engineering-based approach, Bhang et al. recently demonstrated that human adipose-derived stromal cells (hADSCs), cultured and grafted as spheroids, exhibited improved therapeutic efficacy.[27] In this model, spheroid cultures were more effective in pre-conditioning hADSCs to a hypoxic environment, upregulating hypoxia-adaptive signals (e.g., HIF-1α), inhibiting apoptosis, and enhancing secretion of both angiogenic and anti-apoptotic factors compared with monolayer cultures. Following intramuscular transplantation to ischemic hindlimbs of athymic mice, hADSC spheroids showed improved cell survival, angiogenic factor secretion, neovascularization, and limb survival as compared with hADSCs grafted as dissociated cells.[27] The same group showed similar effects in cord blood mesenchymal stem cell (CBMSC) and human umbilical vein endothelial cell (HUVECs) spheroid grafts, in the same animal model.[28,29] Utilization of implantable hypoxic cell-matrix depots (note; the matrix can be cell-produced and/or of exogenous origin), as factory units that continuously produce angiogenic factors in vivo, could therefore prove a useful future strategy for improving the vascularisation of ischemic tissues and engineered constructs.[30]

Recent studies have also explored the utility of hypoxic pre-conditioning in tissue regeneration and reconstruction. In vitro exposure of tissue-engineered mucosa to hypoxia resulted in increased secretion of a range of angiogenic factors, and improved its capacity to support endothelial cell proliferation and migration in vitro.[31] Furthermore, transplantation of hypoxia pre-conditioned bone marrow MSCs improved the survival of ultra-long random skin flaps, elevated in rats, via increased VEGF release and promotion of angiogenesis.[32] The effects of hypoxic pre-conditioning have also been investigated in adipose-derived stem cell (ASC) cultures, where it was shown to increase ASC viability, and reduce cell injury and apoptosis under simulated ischemic conditions, while also improving their angiogenic capacity.[33] Additionally, ASC hypoxic pre-conditioning was shown to increase VEGF release and improved the 7 d viability of ischemic flaps in rats, when cells were injected in the flap distal third.[34] These findings could have important implications for reconstructive procedures, where autologous fat grafting is commonly employed.

Cell-free pre-conditioning-based therapy. The aforementioned studies have, to a great extent, validated the therapeutic potential of cell-based hypoxic pre-conditioning strategies. However, the reliability on implanting living cells (which are likely to be of allogeneic origin) could be a setback for immediate clinical application, due to safety/ethical concerns, as well as lack of off-the-shelf availability. Furthermore, since it is difficult to characterize in vivo cell behavior (e.g., levels/duration of angiogenic factor production, cell survival), process control in living cell implants still remains problematic. Trying to address these concerns, Di Santo et al. first reported in 2009 on a novel cell-free therapy based on delivering conditioned media from hypoxic cell cultures, which were shown to induce angiogenesis in vitro and in vivo.[35] Endothelial progenitor cell-derived conditioned medium (EPC-CM), obtained from subjecting EPCs to 72 h of hypoxia, inhibited apoptosis of mature endothelial cells and promoted angiogenesis in a rat aortic ring assay. The therapeutic potential of EPC-CM was then evaluated in a rat model of chronic hindlimb ischemia, where serial intramuscular injections significantly increased hindlimb blood flow and improved muscle performance. Importantly, this effect was found to be as potent as that induced by EPC transplantation.[35] In more recent studies, hypoxic CM from cardiomyocyte hypoxic cultures was shown to protect isolated hearts against ischemia, reducing infarct size and improving the rate of contraction and relaxation,[36] while hypoxic CM from ASCs stimulated angiogenesis in subcutaneously

implanted sponges to a greater extent than normoxic CM, an effect associated with increased paracrine VEGF and ANG production by hypoxic ASCs.[37]

As mentioned in the previous section, however, the ability to locally deliver factors, as opposed to systemically administer them or deliver them in liquid media, that rapidly and widely spread into the tissue, is key for controlling the directionality of angiogenesis. Importantly, local factor delivery can help prevent unwanted side effects, such as ectopic angiogenesis, vascular leakage etc. With this in mind, our group developed a novel system for localized delivery of hypoxia-induced factor signaling, without administering living cells.[38] Here, dermal fibroblast-seeded collagen matrices were cultured under cell-mediated hypoxia (resulting from cellular O_2 consumption) for 10 d to upregulate production of angiogenic proteins (e.g., VEGF), before snap-freezing the matrices to kill all cells. We showed that the nano-porous collagen matrix could efficiently retain cell-produced proteins, while in vivo subcutaneous implantation of such pre-conditioned, non-viable depots induced a directional angiogenic response within 1 week, through release of trapped angiogenic factors. The formation of diffusion gradients for depot-released VEGF was also demonstrated in collagen gel scaffolds in vitro. This study provided a paradigm of how hypoxia-induced signaling can be locally delivered in vivo, on-demand (i.e., off-the-shelf), without relying on on-going production of factors by living cells.[38] However, certain limitations still remained. For once, since the therapy is based on implanting a depot, it is invasive and not only frustrating for patients, but also accompanied by the common complications of surgery (e.g., bleeding, infection, thrombosis). Furthermore, since it is known that stabilization of a newly formed vascular network requires long-term release of angiogenic factors (note; exogenous VEGF has a short half-life of ~50 min in vivo[39]), and that physiological angiogenesis critically depends on tight temporal regulation through differential gene expression at different time points,[40-42] a cell-free therapeutic approach will evidently have to rely on multiple applications, which is difficult to carry out with an implantable therapy. Finally, depots contained dead cells, in addition to the produced factors, which raises concerns about possible immunogenic reactions to allogeneic cellular material.

In an effort to address these limitations, we recently developed an injectable system for spatio-temporally-controlled delivery of cell-free matrix carriers loaded with fibroblast-generated, hypoxia-induced factors.[9] The central idea enabling this technology lies in the ability to engineer a composite matrix construct, in which a central cellular and a peripheral acellular compartment co-exist in culture, but remain spatially distinct through separation by a nano-porous filter. This filter prevents cell/pathogen movement into the acellular compartment. Angiogenic factor proteins, produced by cells residing in the central compartment in response to hypoxic exposure, can therefore be captured by the acellular matrix as they radially diffuse through it. Factor-loaded matrix fractions, obtained by mechanical fragmentation of the acellular matrix, can be then locally delivered using an injectable thermo-responsive sol-gel vehicle (e.g., collagen sol-gel), which also ensures sustained factor release.[9] Our quest to translate this system's utility into a device-based tool that can be used at the bed-side led us then to identify peripheral blood cells (PBCs) as the ideal factor-providing candidates, due to their autologous nature, ease of harvest and ample supply. The above described principle of combining the cell-scaffold and factor-carrier into one unit, yet keeping them spatially distinct by incorporating an intervening filter, provided the technological framework for this third-generation system.[43] The angiogenic effectiveness of cell-free collagen gel and microsphere carriers, loaded with factors derived from hypoxic PBC cultures, could be demonstrated by the ability of their releasates to induce endothelial cell tubule formation and directional migration in in vitro Matrigel assays, and microvessel sprouting in the rat aortic ring assay, where they significantly performed better than recombinant VEGF.[43] By integrating this system into a simple, one-step device that can house all steps of the process, i.e., blood collection, hypoxic cell conditioning, and factor harvest, PBC-derived angiogenic factor signaling can be readily delivered in the form of a wound dressing or an injectable preparation, making this device applicable to both open (e.g., wounds, ulcers, burns) and closed (e.g., grafts, implants) tissue sites requiring angiogenic support.[43] Such factor-loaded biomimetic (i.e., biodegradable) matrices, could potentially also serve as scaffolds for promoting tissue regeneration, as well as repair.

Strategies Based on Inducing Hypoxia-Mediated Signaling In Vivo

Given that the formation of a functional, mature, and durable vascular network is complex, the idea of switching-on hypoxia-induced angiogenesis at the onset of the process is duly justified. The ability of HIF-1, the master regulator of this process, to induce several mediators of angiogenesis, together with evidence that aging and diabetes impair HIF-1 activation in response to ischemia,[7] prompted the concept that strategies designed to increase its activity might be more efficient in inducing angiogenesis/arteriogenesis after ischemic events (e.g., hind limb, cardiac, or cerebral ischemia) than those relying on single or a few factors.[1,3,44] These strategies can largely be divided into two categories, namely HIF-1 stabilization by pharmacological means or overexpression through gene therapy.

Cell-free pharmacological therapy. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates the adaptive response to hypoxia in mammalian cells. It consists of a regulatory subunit HIF-1alpha, which accumulates under hypoxic conditions, and a constitutively expressed subunit, HIF-1beta. Under non-hypoxic conditions HIF-1α is targeted for proteasomal degradation through binding to the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex, after hydroxylation of proline residues 402 and 564 by prolyl hydroxylase domain-containing proteins (PHDs; PHD1, PHD2, PHD3), which require O_2 and 2-oxoglutarate as co-substrates, and iron and ascorbic acid as co-factors.[7,8,45] Additionally, hydroxylation of asparagine 803 of HIF-1α by FIH (factor inhibiting HIF-1) prevents binding to the transcriptional co-activators CBP and p300.[7,8] Hypoxia-induced inhibition of prolyl and asparaginyl hydroxylase activity results

in a rapid increase in HIF-1α levels and transcriptional activity. HIF-1α translocates to the nucleus, dimerizes with HIF-1β, and binds to hypoxia response elements (HREs), which function as cis-acting elements that determine the target genes for activation by HIF-1.[7,8] HIF-1α protein levels, HIF-1 DNA-binding activity, and HIF-1 transcriptional activity can be increased by exposure of cells to cobalt chloride ($CoCl_2$) or deferoxamine (DFO), agents that act through inhibition of PHDs.[7,18] Co^{2+} may induce HIF-1 activity through depletion of intracellular ascorbate,[46] which maintains iron in its active (reduced) Fe^{2+} state and is therefore essential for proper PHD function. DFO is an iron chelator that induces HIF-1 activity by reducing Fe^{2+}availability, while dimethyloxalylglycine (DMOG) is a competitive antagonist of α-ketoglutarate that inhibits PHD activity and blocks the O_2-dependent degradation of HIF-1α.[45]

Hoenig et al. discussed the importance of depressed HIF-1α-mediated gene programming as the most fundamental of all cardiovascular risk factors, linked to the dysfunctional regulation of the SDF-1/CXCR4 axis, which forms the final common pathway for endothelial progenitor cell (EPC) mobilization by hypoxia.[44] The authors proposed the use of cobalt and hydralazine, another PHD-inhibiting compound,[47] to enhance EPC function and homing to target tissues, as a strategy to overcome the limited success of studies employing EPC transplantation. An earlier study had indeed shown that intraperitoneal administration of DFO to aged mice restored HIF-1α expression and ischemia-induced mobilization of EPCs in an ischemic skin flap, which resulted in improved flap vascularization and tissue survival that was comparable to young mice.[48] In a more recent study, Takaku and coworkers treated mice, with ischemic skin flaps on their dorsum, intraperitoneally with DMOG 48hr prior to surgery.[49] There was a significant increase in the surviving area with neovascularization of the ischemic flaps, accompanied by marked increases in circulating EPCs and bone marrow proliferative progenitor cells within 48hr after DMOG treatment. Here, heterozygous HIF-1α-deficient mice exhibited smaller surviving flap areas, fewer circulating EPCs, and larger numbers of apoptotic cells than did wild-type mice, while DMOG pretreatment of the mutant mice completely restored these parameters. The angiogenic effect of hydralazine has been demonstrated in a study where exposure of transgenic zebrafish embryos, exhibiting fluorescent blood vessels, to hydralazine hydrochloride induced formation of ectopic blood vessels in the subintestinal vessel basket.[50] Local administration of $CoCl_2$, on the other hand, could correct the reduced HIF-1α and VEGF mRNA and protein expression in cutaneous wounds of leptin receptor-deficient diabetic mice.[51] Wound healing was also improved in the same animal model by local application of DFO or DMOG.[52] In another study in mice, combination of DMOG treatment with femoral artery ligation resulted in a significant increase in endogenous HIF-1α protein and a 39% increase in capillary-to-fiber ratio in ischemic muscles, whereas individual treatments produced little effect.[53] In this study, production of the VEGF receptor Flk-1 was more enhanced in ischemic + DMOG-treated muscles, which may explain the intensive growth of capillaries in those muscles.

Whereas most PHD inhibitors inhibit PHD activity via iron chelation, Nangaku et al. reported on two novel compounds that can stabilize HIF in vitro, TM6008 and TM6089, that are devoid of iron chelating activity, but instead preferentially bind to the active site of PHD.[54] In vitro Matrigel assays and in vivo sponge assays demonstrated enhancement of angiogenesis by local administration of TM6008 and TM6089. Their oral administration stimulated HIF activity in various organs of transgenic rats expressing a hypoxia-responsive reporter vector, and protected neurons in a model of cerebrovascular disease. However, it is important to note that this neuroprotection was associated with amelioration of apoptosis, but was independent of enhanced angiogenesis.

Cell-free gene therapy. Researchers started investigating the feasibility of HIF-1α delivery, at the DNA level, as early as in 2000. Vincent and coworkers constructed a hybrid protein consisting of DNA-binding and dimerization domains from the HIF-1α subunit and the transactivation domain from herpes simplex virus VP16 protein, to create a strong, constitutive transcriptional activator.[55] After transfection into HeLa, C6, and Hep3B cells, this chimeric transcription factor was shown to activate expression of the endogenous VEGF gene, as well as several other HIF-1 target genes in vitro. In vivo administration of HIF-1α/VP16 hybrid gene in a rabbit model of hindlimb ischemia was associated with significant improvements in calf blood pressure ratio, angiographic score, resting and maximal regional blood flow, and capillary density.[55] The HIF-1α/VP16 hybrid transcription factor was also shown to be effective in reducing infarct size and enhance neovascularization in a rat acute myocardial infarction model,[56] as well as confer protection to cultured neonatal rat cardiomyocytes by late-phase pre-conditioning against simulated ischemia-reperfusion injury.[57] In 2005, Kido et al. showed that constitutive overexpression of HIF-1α in the murine hearts of transgenic mice resulted in attenuated infarct size and improved cardiac function 4 weeks after myocardial infarction,[58] while in another study administration of adenovirus encoding HIF-1α increased myocardial perfusion and improved left ventricular function in a pig model of left circumflex coronary artery occlusion.[59] Furthermore, inoculation of HIF-1α naked DNA into the brain surface or the temporal muscle in a rat model of cerebral ischemia increased expression of HIF1α and VEGF, with formation of collateral circulation.[60] In the same year, Pajusola et al. showed that injection of adeno-associated virus (AAV), encoding a form of constitutively active HIF-1α, into non-ischemic skeletal muscle induced marked capillary sprouting, whereas AAV-VEGF induced only endothelial proliferation without proper vessel formation.[61] Importantly, unlike VEGF, HIF-1α overexpression did not increase vascular leakiness in the transduced muscle. These findings were in agreement with those of a previous study showing that despite marked induction of hypervascularity, HIF-1α did not induce edema, inflammation, or vascular leakage, phenotypes developing in transgenic mice overexpressing VEGF cDNA in skin.[62] In another study, Trentin et al. delivered a gene encoding a stabilized form of HIF-1α, lacking the oxygen-sensitive degradation domain (HIF-1α deltaODD), by using a non-viral, peptide-based gene delivery vector in fibrin matrix.[63] When the

peptide-DNA nanoparticles entrapped in fibrin matrices were applied to full-thickness dermal wounds in mice, the maturity of the vessels induced by HIF-1α deltaODD was significantly higher than that induced by VEGF-A165 protein, as shown by stabilization of the neovessels with smooth muscle. By showing that HIF-1α activation is not only superior to VEGF in its angiogenic effect, but also can circumvent common problems associated with overexpression of individual angiogenic growth factors, such as formation of unstable/leaky vasculature, these early studies provided the much needed evidence to the notion that engineering of physiological angiogenesis will only likely be feasible by inducing the process at its very onset using hypoxia.

In a later study, electroporation-assisted transduction into the skin of diabetic mice of a plasmid vector encoding a constitutively active form of HIF-1α, designated CA5, significantly increased cutaneous HIF-1α, ANGPT2, PDGF-B, PLGF, and VEGF mRNA levels, and the vascularization and rate of healing of excisional skin wounds.[64] This supported the findings of other studies, where improved wound healing was also observed after injection of a plasmid or adenovirus encoding various constitutively active forms of HIF-1α.[51,52] Increased expression of these factors had been already previously shown after adenoviral delivery of CA5 (AdCA5), by injection into male New Zealand white rabbits that were subjected to superficial femoral artery occlusion.[65] In that study, AdCA5-injected limbs showed improved calf blood pressure ratios, angiographic perfusion scores and distal deep femoral artery diameter ratio relative to those receiving control AdLacZ. Similarly, in diabetic mice subjected to femoral artery ligation, intramuscular (IM) injection of AdCA5 into the ischemic calf and thigh muscle, significantly increased circulating angiogenic cells (CACs) in peripheral blood, limb perfusion, tissue viability, and motor function.[66] These changes were associated with increased vessel luminal area and vessel density in the AdCA5-transduced ischemic limbs, demonstrating an arteriogenic effect. IM-administered AdCA5 was also sufficient in overcoming the impaired recovery of limb perfusion in middle-aged mice,[67] but did not improve recovery or prevent tissue damage in old mice.[68] The IM route was also used to deliver an adenovirus encoding the aforementioned HIF-1α/VP-16 fusion protein, which stimulated collateral development in diabetic rats subjected to femoral artery ligation.[69] This latter construct was also tested in a patient study, however, while well tolerated when delivered into the lower extremity of 34 patients with non-reconstructable critical limb ischemia (CLI), it only showed moderate clinical efficacy (improvement in ulcer healing and rest pain).[70] In a more recent study employing a rabbit model of acute hind limb ischemia, administration of Ad-HIF-1α-Trip, yet another form of constitutively active form of HIF-1α, improved tissue perfusion and formation of mature collateral vessels in ischemic skeletal muscle.[71]

For assessing the feasibility and efficacy of transcriptionally-controlled systemic pro-angiogenic gene therapy, Tal et al. created an adenovirus expressing a stabilized HIF-1α molecule, activated by constitutive activation of its C-transactivation domain, and regulated by PPE1–3×, a modified murine preproendothelin-1 promoter, that can target gene expression specifically to endothelial cells within ischemic muscle following systemic IV administration.[72] Systemic tail-vein administration of this adenovirus in a mouse hindlimb ischemia model resulted in enhanced blood perfusion, improved clinical outcome, and increased capillary density without systemic toxicity, in contrast to the profound systemic side effects and lack of therapeutic efficacy following cytomegalovirus (CMV)-regulated HIF-1α administration.[72]

A recent study further explored the therapeutic effect of combined gene delivery, by treating ischemic mouse hindlimbs with gene therapy of HIF-1α and/or heme oxygenase-1 (HO-1).[73] HO-1 is an enzyme that degrades heme to carbon monoxide (CO), biliverdin, and ferrous iron, and plays a major role in the protection against oxidative injury, regulates cell proliferation, modulates inflammatory response and facilitates angiogenesis.[74] Interestingly, the therapy proved superior to both single-gene therapies, resulting in rapid expression of HIF-1α gene and long-term maintenance of expressed HO-1 protein.[73] The apoptosis in the ischemic region was significantly less, while angiogenic growth factor secretion and angiogenesis were greater in the combined gene therapy than in either of the single-gene therapies. This study, therefore, highlights the scope for further exploring the concomitant delivery of two or more genes, to activate multiple targets in the angiogenic cascade and generate a more robust angiogenic response.

Cell-based gene therapy. In addition to delivering genes directly into tissue through virus- or peptide-based vectors, another strategy being explored is based on genetically modifying selected cells in vitro, prior to their transplantation. Indeed, expression of stable forms of HIF-1α and HIF-2α by retrovirally transduced bone marrow stromal cells (BMSCs) promoted differentiation of BMSCs to the endothelial lineage and induced upregulation of angiogenic proteins, which improved tube formation.[75] Interestingly, delivery of HIF-2α-transduced BMSCs induced a more robust angiogenic response, compared with HIF-1α-transduced BMSCs in the corneal micropocket angiogenesis model. In another study, EPCs modified through adenoviral transfer of HIF-1α, were administered to nude mice with hindlimb ischemia.[76] HIF-1α overexpression enhanced its mRNA and protein expression in the ischemia zone, and limb and toe necrosis was significantly reduced at 14 d after transplantation, an effect lasting up to two months of follow-up. Furthermore, neovascularization was improved and exogenous EPC homing was observed.[76]

Another study suggested that HIF-1α might also contribute to the therapeutic effect of neural stem cell (NSC) transplantation in cerebral ischemia.[77] Control NSC infected with control adenovirus (NSC-Ad), standalone recombinant adenovirus Ad-HIF-1α, or NSC infected by Ad-HIF-1α (NSC-Ad-HIF-1α), were used for intraventricular transplantion into rat brain 24 h following middle cerebral artery occlusion (MCAO). Functional improvement was accelerated in animals receiving either NSC-Ad or Ad-HIF-1α, however, improvement at all times between 7 and 28 d post MCAO was significantly greater in animals transplanted with NSC-Ad-HIF-1α. NSC-Ad-HIF-1α cells also increased the number of factor VIII-positive cells in the region of ischemic injury, indicating that HIF-1α expression could promote

angiogenesis.[77] These findings indicate that transplantation of genetically-modified NSCs, expressing HIF-1α, could have therapeutic utility in ischemic stroke.

According to recent work, HIF-1α gene therapy could potentially be used to promote bone repair. Using lentivirus-mediated delivery of a constitutively active form of HIF-1α, Zou et al. found that HIF-1α-overexpressing bone marrow-derived mesenchymal stem cells dramatically improved the repair of critical-sized rat calvarial defects, including increased bone volume, bone mineral density, blood vessel number, and blood vessel area, 8 weeks after implantation.[78]

Targeting hypoxia-mediated angiogenic induction at a more downstream level, Kim et al. introduced a hypoxia-inducible VEGF expression vector into mesenchymal stem cells (HI-VEGF-MSCs), through a non-viral delivery method, which were then used for the treatment of ischemic myocardial injury in rats.[79] In vivo transplantation of HI-VEGF-MSCs induced ischemia-responsive VEGF production, leading to a significant increase in myocardial neovascularization after myocardial infarction. When compared with unmodified-MSCs, HI-VEGF-MSCs were retained in infarcted myocardium in greater numbers and produced a substantial attenuation of left ventricular remodeling in rat myocardial infarction.[79] Thus cell-based gene therapy could also be a promising strategy for the treatment of ischemic heart disease.

Combinational therapy. In addition to the above described single-mode therapies, newer strategies have explored the possibility to combine pharmacological, cell and/or gene therapy as a multi-faceted approach to dealing with the complexity of an ischemic tissue microenvironment. For example, although AdCA5 delivery to muscle improves production of angiogenic cytokines, it does not address the functional cellular impairment that is also associated with aging. To promote vascular remodeling, cells must be recruited to, and retained in, the ischemic tissue. Rey and coworkers devised a three-component strategy for promoting recovery following femoral artery ligation in old mice;[68] first, AdCA5 was injected into the thigh and calf of the ischemic limb, which served to mobilize CACs and recruit them to the target site. Second, bone marrow-derived angiogenic cells (BMDACs) from a donor mouse were cultured for 4 d in the presence of angiogenic growth factors plus DMOG to induce HIF-1 activity. Third, IV injection of these into recipient ischemic mice was performed 24 h after femoral artery ligation and AdCA5 injection. A significant improvement in recovery of perfusion and limb salvage was observed only in mice that received IM AdCA5 + IV DMOG-treated BMDACs and was not observed in mice that received only IM AdCA5 or only IV DMOG-treated BMDACs or IM AdCA5 + IV vehicle-treated BMDACs. The authors concluded that the synergistic effect of these treatments was due to increased recruitment of BMDACs to the ischemic limb induced by IM AdCA5, whereas DMOG treatment promoted retention of recruited BMDACs by increasing the expression of cell surface CD11/CD18 (β2) integrins.[68]

As we mentioned in previous sections, the ability to provide spatially distinct signaling is paramount for successful generation of localized and directional angiogenesis. This could be achieved, for example, by employing thermo-responsive hydrogels which can provide an in situ depot for the sustained release of drugs, as well as provide protection and cohesion for encapsulated cells. In a recent study, human MSCs and DFO were combined with a thermo-responsive chitosan/β-glycerophosphate (β-GP) gel, to function as an injectable, multimodal, pro-angiogenic therapeutic for the treatment of CLI.[80] This gel provided a sustained, biologically active release of DFO over seven days, while permitting the survival, proliferation and migration of encapsulated MSCs. MSCs encapsulated in gel containing DFO displayed an upregulation in VEGF expression, while the combination of MSCs and DFO within the gel resulted in a synergistic enhancement in bioactivity, as measured by increased VEGF expression in gel-exposed human umbilical vein endothelial cells.

Future Directions

In this review we have discussed the most significant to this day developments in strategies targeting hypoxia-based therapeutic angiogenesis. The field, while it is currently rapidly advancing, is still at its infancy, and is only now getting the global attention it deserves, emerging as a promising solution to the shortcomings of single factor therapies. The studies presented here provide a solid foundation for the design of clinical trials with patients suffering from ischemic conditions. In order to successfully move into this next phase, however, strategies will need to take into consideration the importance of target specificity, controlled factor delivery kinetics, as well as the need to employ multiple therapeutic modalities so that spatiotemporally-controlled, physiological angiogenesis is timely induced and maintained. Below, we speculate on the likely thematic directions that could come to define the field in the near future, and facilitate its translational application.

When preconditioning cells to hypoxia in 3D matrices, understanding their basal O_2 and nutrient requirements is key. Any scaffold design will need to take such parameters into consideration, especially as the addition of cells introduces gradients of consumption of such molecules from the surface to the core of scaffolds.[26,81] For example, it was recently shown that the rates of O_2 consumption by human dermal fibroblasts and human bone marrow derived stromal cells, seeded in 3D native collagen type I scaffolds, are lower than previously published rates for similar cells cultured in 2D, but more representative of rates of consumption measured in vivo.[82] These values will dictate 3D culture parameters, including maximum cell-seeding density and maximum cell-matrix depot size, for optimization of cellular angiogenic factor production and delivery.

The mechanical properties and matrix structure (nano-/micro-structure, internal surface area, nano-pore volume and nano-pore distribution) of a scaffold will also determine its ability to support O_2/nutrient diffusion to seeded cells,[26,83] as well as control the retention/release of secreted macromolecular growth factor proteins. While low O_2 tension (e.g., 3% O_2) enhances angiogenic cell signaling, where O_2 is critically low (pathological hypoxia, i.e., <1% O_2), cell survival becomes compromised. In this respect, collagen matrices provide ideal scaffold candidates,

due to their ability to immerse cells in a biomimetic nano-fibrous matrix. Indeed, Cheema et al. showed that the O_2 diffusion coefficient of 11% density collagen scaffolds falls within the range of native intestinal submucosa.[84] With regards to the role of scaffolds as carriers to cell-produced factors, utilization of natural polymeric matrices is also likely to provide improved functionality. In a recent study, for example, we showed that type I collagen nano-porous gels could retain VEGF, produced by hypoxia-preconditioned PBCs, to a much greater extent than other macroporous hydrogels.[43]

The role of scaffolds could extend beyond that of carriers for cells and factors, to vehicles providing a controllable microenvironment where defined hypoxic conditions could be established, and continually exert their effect on cells during the in vitro preconditioning phase, as well as in vivo, after implantation. For example, it was shown that by introducing micro-channelled architecture in 3D scaffolds, it is possible to controllably increase delivery of O_2 to cells and switch off their hypoxic response.[85] It is foreseeable, then, that by controlling the rate of formation of such channeled structures, one could precisely control O_2 tension and the duration of hypoxic exposure. In another study pointing toward this direction, hypoxia-mimicking mesoporous bioactive glass (MBG) scaffolds were developed by incorporation of ionic Co^{2+}.[86] While low amounts of Co^{2+} (<5%) had no significant cytotoxicity, their incorporation significantly enhanced VEGF protein secretion, HIF-1α expression, and bone-related gene expression in BMSCs. Such hypoxia-mimicking scaffolds have potential utility in bone regeneration by combining enhanced angiogenesis with already existing osteogenic properties.

A disadvantage of currently available HIF-1 hydroxylase inhibitors is their lack of selectivity for PHDs and inability to specifically target different PHD isoforms, which restricts their clinical utility. A range of crystallographic analysis, sequence comparisons and modeling studies indicate that the active site, which is targeted by most of the currently used inhibitors, is highly conserved among PHDs and FIH.[18,87] This poses a challenge for the development of isoform-specific inhibitory compounds and necessitates alternative strategies. Nonetheless, several novel classes of PHD inhibitors are being developed that exhibit an improved selectivity for PHDs. Such novel compounds comprise aromatic heterocycles, related to pyridine derivatives, including pyrazolopyridines and 8-hydroxyquinolines.[88] The generation of 8-hydroxyquinolines is based on a recently described isoquinoline derivative that specifically inhibits PHDs. Co-crystallization with PHD2 revealed key interactions between this compound and the active site of the enzyme,[87] thereby serving as a basis for future design of selective inhibitors.

With regards to gene therapy, while it clearly forms a promising strategy for the treatment of tissue ischemia, caution is required in that unregulated expression of an angiogenic factor may induce pathological/ectopic angiogenesis and tumor growth. To avoid such side effects, gene expression should admittedly be tightly regulated. Working toward this goal, Kim et al. developed a hypoxia-specific gene expression plasmid, pSV-Luc-ODD, constructed with the oxygen-dependent degradation (ODD) domain for rapid degradation of a target protein under normoxia.[89] For even greater hypoxia-specific gene expression, pEpo-SV-Luc-ODD was constructed with the erythropoietin (Epo) enhancer and the ODD domain, which showed more than 1000 times increase of gene expression under hypoxia in Neuro2A cells, compared with normoxia. Reoxygenation studies after hypoxia incubation showed that gene expression was indeed decreased in response to increased oxygen concentration. In later work by the same group, the activity of the Epo enhancer-SV promoter system was further enhanced by co-transfection of the HIF1α gene (pSV-HIF1α).[90] Co-transfection of pEpo-SV-VEGF with pSV-HIF1α showed enhanced VEGF expression without loss of hypoxia specificity, while pSV-HIF1α induced endogenous hypoxia-responsive genes such as angiopoietin-1. Development of such highly hypoxia-specific gene expression systems could be useful for targeting gene therapy to ischemic tissues, and should therefore be the focus of future work.

Viral vectors offer high transfection efficiency and stable gene expression, although concerns over their ability to elicit toxic immune responses still remain.[91,92] Furthermore, this high transduction does not necessarily translate into improved efficacy.[93] Currently, polymeric non-viral gene delivery remains underrepresented.[94] In the past decade, however, several advances have been made in non-viral vector development, contributing to increased transfection efficiency.[95] Some of these polymers employ lipid modifications to improve transfection. For example, water-soluble lipopolymer (WSLP) was used to deliver hypoxia-responsive driven expression of VEGF to ischemic rabbit myocardium, and has demonstrated a significant reduction in infarct size over constitutive VEGF expression, a significant reduction in apoptotic values and an increase in capillary growth in surrounding tissue.[96] Recently, investigations have begun using bioreducible polymers made of poly(amido polyethylenimines) (SS-PAEI). SS-PAEIs degrade within the cytoplasm through inherent redox mechanisms and provide for high transfection efficiencies (upwards to 60% in cardiovascular cell types) with little to no toxicity. Moreover, in vivo transfections in normoxic and hypoxic rabbit myocardium have proven to exceed those results of WSLP transfections by 2–5-fold.[97] This new breed of polymers may thus eventually allow for decreased doses and use of new molecular mechanisms not previously available due to low transfection efficiencies.[94]

It has recently been proposed that normoxic preservation of HIF-1, by using small interfering RNA (siRNA) to silence PHD activity, could attenuate cardiac ischemia/reperfusion injury via a pre-conditioning effect.[98] In wild-type mice infused with PHD2 siRNA there was a reduction in cardiac PHD2 mRNA within 24 h, while HIF-1α protein levels and HIF-1-dependent iNOS mRNA levels were increased. Furthermore, PHD2 siRNA-transfected hearts from wild-type mice, subjected to 30 min ischemia followed by 60 min reperfusion, exhibited reduced infarct size when compared with non-targeting siRNA control, while this effect was shown to be mediated via an iNOS-dependent pathway[98] and modulation of ischemia-reperfusion-associated cardiac inflammatory responses.[99] Another study showed that in vivo angiogenesis could be observed in mice implanted with

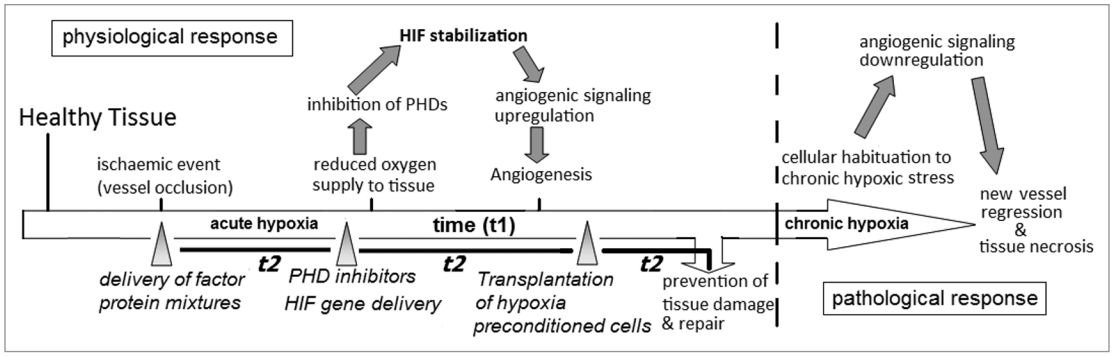

Figure 2. Schematic showing the chronological progression of the physiological, and then pathological processes, that set in following an ischemic event. A multi-modal therapeutic strategy (shown in italics) should focus at first providing immediate angiogenic support by delivering of on-demand available factor protein mixtures, obtained from in vitro pre-conditioned hypoxic cell cultures, in order to prevent the regression of newly-formed vessels and permanent tissue damage, resulting from a gradual reduction in cellular response to chronic hypoxic stress (dashed line). At the same time, chemical HIF stabilization and/or induction of HIF-mediated signaling through gene transfer will ensure a continuous supply of angiogenic factors, to sustain tissue repair/regeneration. At a later stage, transplantation of hypoxia pre-conditioned cells can aid in re-establishing a healthy, angiogenic cell population at the target site, so that similar future episodes can be prevented. The therapeutic timeline of this approach (*t2*), therefore follows in 'reverse' order the timeline of the physiological tissue response to hypoxia (t1), thus stimulating/supporting angiogenesis, and driving it to completion.

Matrigel plugs mixed with NIH3T3 cells that were transfected with a PHD2-siRNA vector,[100] suggesting that a sound strategy could be based on combining siRNA together with cell transplantation. Huang and coworkers also developed a construct that allows monitoring of gene expression non-invasively, and used it to show that inhibition of PHD2 by short hairpin RNA interference (shRNA) can lead to significant improvement in angiogenesis and cardiac contractility in a mouse model of myocardial ischemia, effects associated with higher HIF-1α expression.[101] In a recent study, it was shown that silencing of int6 gene restores function of the ischemic hindlimb in a rat model of peripheral arterial disease, an effect associated with early upregulation of HIF-2α and other angiogenic factors, including basic fibroblast growth factor and hepatocyte growth factor, in the muscles of the affected hindlimb.[102] Therefore, induction of hypoxia-regulated signaling via siRNA might be a promising future strategy for promoting therapeutic angiogenesis in tissue ischemia, and merits further investigation.

Strategies based on the combination of two or more therapeutic modalities will likely have an increasing role in the clinical trials phase, not only because they hold the potential for delivering more potent angiogenic effects, but also because they offer the possibility for greater flexibility with regards to controlling the timing of angiogenic induction. It could be envisaged that the temporal progression of the natural tissue response to hypoxic stress after an ischemic event, underlined by the timely sequence of HIF-1 stabilization, gene expression and protein release, could be followed in reverse order so that the target tissue receives immediate angiogenic factor support, while a more long-lasting hypoxia-induced signaling response is being recovered. This could be indeed achieved using currently available tools, for example first delivering off-the-shelf available factor protein mixtures, obtained from in vitro pre-conditioned hypoxic cell cultures, followed by pharmacological/gene-based stabilization of HIF-1α, while eventually transplanting hypoxia pre-conditioned (stem) cells to aid tissue regeneration by re-establishing a healthy, angiogenic cell population at the diseased site (**Fig. 2**). While the relative future contribution of these individual hypoxia-based strategies toward a multi-modal approach remains to be seen, it is now apparent that a new era for ischemia therapy and tissue regeneration has finally begun.

Disclosure of Potential Conflicts of Interest

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