Molecules in motion: unravelling the dynamics of vascularization control in tissue engineering.

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Significant progress has been made in tissue engineering (TE), aiming at providing personalized solutions and overcoming the current limitations of traditional tissue and organ transplantation. Three-dimensional (3D) bioprinting has emerged as a transformative technology in the field, able to mimic key properties of the natural architecture of the native tissues. However, most successes in the area are still limited to avascular or thin tissues due to the difficulties in controlling the vascularization of the engineered tissues. To address this issue, several molecules, biomaterials, and cells with pro- and anti-angiogenic potential have been intensively investigated. Furthermore, different bioreactors capable to provide a dynamic environment for in vitro vascularization control have been also explored. The present review summarizes the main molecules and TE strategies used to promote and inhibit vascularization in TE, as well as the techniques used to deliver them. Additionally, it also discusses the current challenges in 3D bioprinting and in tissue maturation to control in vitro/in vivo vascularization. Currently, this field of investigation is of utmost importance and may open

doors for the design and development of more precise and controlled vascularization strategies in TE.

1. Introduction

Tissue engineering (TE) represents a multidisciplinary field that merges principles of engineering, biology, chemistry, and materials science to create functional biological tissues.(Ashammakhi et al., 2021) At its core, TE aims to develop native-like tissue and organ constructs that can seamlessly integrate with the human body, offering transformative solutions for organ failure, injury, and degenerative conditions.(Sanchez-Rubio et al., 2023) By providing tailored and patient-specific solutions, TE holds the promise of overcoming the limitations of traditional transplantation methods, reducing the dependency on donor organs, and mitigating issues related to rejection.(Mandrycky et al., 2017) Moreover, three-dimensional (3D) bioprinting has emerged in TE as a transformative technology, offering unprecedented possibilities and addressing critical challenges.(Zhang et al., 2021) Its importance lies in its ability to precisely deposit living cells, molecules, and biomaterials, layer by layer, mimicking the intricate architecture of the native tissues.(Dey & Ozbolat, 2020) This level of precision enables the creation of complex and functional structures that have allowed researchers to design and develop artificial tissues with defined sizes, geometries, and different microenvironment complexities.(Murphy & Atala, 2014)

However, in the field of TE, achieving control over vascularization remains one of the pivotal problems to achieve functional engineered tissues capable of integrating into the human body. Appropriate vascularization of the tissues is what ensures their vitality and function, and abnormalities can originate from a variety of pathological conditions. For instance, insufficient blood supply can compromise nutrient and oxygen delivery, leading to several issues, such as stroke, ulcerative disorders, and tissue necrosis.(Carmeliet & Jain, 2011a) On the contrary, abnormal vessel growth can give rise to other situations, namely tumour formation and growth, inflammatory responses, and osteoarthritis.(Carmeliet & Jain, 2000) Therefore, effective control of both the promotion and inhibition of vascularization is a crucial step in producing functional lab-grown tissues that merge properly with the complex human body.

The biology behind the vascular system relies upon two distinct processes aiming to generate blood vessels: (1) vasculogenesis and (2) angiogenesis.(Patan, 2004) Vasculogenesis refers to the formation of new blood vessels, and it happens predominantly in embryonic development, even though it can also occur in adults under specific conditions. The embryonic stage starts with the differentiation of the mesoderm into angioblasts, which respond to local cues, namely vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), to aggregate

into a primitive vascular plexus. This aggregation supports the differentiation of angioblasts into endothelial cells (ECs) that self-organize and give rise to new blood vessels.(Patel-Hett & D'Amore, 2011) On the other hand, angiogenesis refers to the formation of new blood vessels by sprouting from a pre-existing vascular network, and it can occur in every phase of life. Typically, it is triggered by specific stimuli indicating the need for blood supply, such as wound healing, inflammation, or embryogenesis. In these cases, ECs can enter an activated state, which leads to increased motility and proliferation, while secreting proteinases to degrade the basement membrane.(Hirschi et al., 2002) At this point, ECs start to migrate, guided by a concentration gradient of chemotactic factors such as VEGF, leading to the formation of long capillaries. ECs return to their normal state, and a new basement membrane is synthesised. Mural cells, namely pericytes for capillaries and smooth muscle cells (SMCs) for larger vessels, are then recruited to ensure proper function to the new blood vessels.(Jain, 2003; Kamei et al., 2006) However, when comparing the two processes in the vascularization field of TE, it is far more common to find works reproducing the angiogenesis process, mainly due to the difficulty of mimicking the elevated biological complexity involved in vasculogenesis.(Kargozar et al., 2022; Moon & West, 2008)

Although these two biological processes have been comprehensively described in the literature, the control of vascularization is still a significant hurdle in TE. Researchers are actively exploring several strategies to address vascularization by incorporating numerous angiogenic factors into scaffold materials or using 3D bioprinting technologies to enable the creation of vascular networks within the engineered tissues.(Devillard & Marquette, 2021) However, for cartilage, the challenge is exacerbated, primarily attributed to the coexistence of vascular and avascular regions within a relatively dense tissue. Cartilage must be surrounded by blood vessels to maintain its function.(Sophia Fox et al., 2009) Conversely, vascular invasion of the tissue results in matrix breakdown, apoptosis of chondrocytes, and, ultimately, ossification.(Y. A. Pei et al., 2022) This delicate balance of vascularization has been one of the hardest features to replicate in TE. (Chen et al., 2017; Zhou et al., 2023)

This review focuses on a comprehensive outlook on specific molecules that have been employed to either promote or inhibit vascularization (**Figure 1**). Furthermore, it will discuss the latest advancements in TE,3D bioprinting and bioreactor systems as viable approaches to control vascularization within engineered tissues.

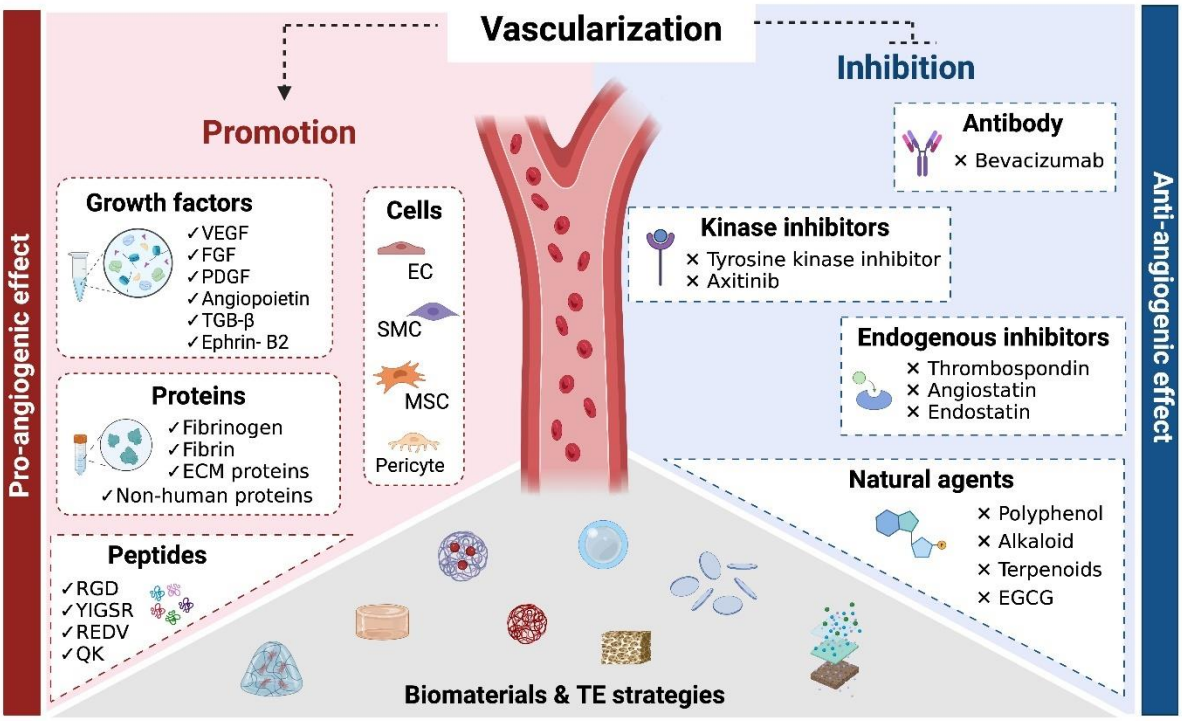

Figure 1 - Diagrammatic illustration delineating the array of molecules and cellular entities recognized for their roles in either facilitating or impeding vascularization. These elements may be employed singularly or in conjunction with a variety of biomaterials and tissue engineering methodologies. VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor; PDGF: platelet-derived growth factor; TGB-□: transforming growth factor-β; ECM: extracellular matrix; RGD: (Arg-Gly-Asp) sequence; YIGSR: (Try-lle-Gly-Ser-Arg) sequence; REDV: (Arg–Glu–Asp–Val) sequence; QK: (KLTWQELYQLKYKGI) sequence; EC: endothelial cells; SMCs: smooth muscle cells; MSC: mesenchymal stem cells; EGCG: epigallocatechin gallate. This image was created with Biorender.com.

2. Promotion of vascularization: molecules and TE strategies

The combination of molecules, especially the use of endogenous human proteins, with cells and biomaterials has emerged as an attractive strategy in TE. These molecules provide a microenvironment much more adapted to cell growth, proliferation, differentiation, and migration and can play a role in angiogenesis by interacting with several cell receptors that promote vascularization.(Mastrullo et al., 2020)

2.1. Growth Factors

Growth factors are natural diffusible proteins secreted by cells or glands, which can stimulate cell growth, differentiation, inflammation, and tissue repair.(Ren et al., 2020) For this reason, these molecules have been extensively explored in TE and regenerative medicine.(Atienza-Roca et al., 2018) Focusing on angiogenesis, specific growth factors play important roles in

both inducing and guiding the angiogenic process by exerting influence at different phases.(Ucuzian et al., 2010) Among these factors, VEGF is notably one of the most extensively studied and applied. VEGF is essential for activating a signalling cascade that culminates in the proliferation and migration of ECs.(Simons, 2012) Numerous studies have demonstrated the incorporation of VEGF into diverse biomaterial matrices, including collagen and alginate.(Koch et al., 2006; Azarpira et al., 2021; Geng et al., 2021; Liu et al., 2022) These endeavours have yielded outcomes such as the differentiation of stem cells into ECs and the facilitation of vascularization, thereby underscoring the crucial role of VEGF in promoting events conducive to angiogenic processes.(Barati et al., 2016; Ikuno et al., 2017; Koch et al., 2006; Y. Li et al., 2021; M. Song et al., 2018) Another well-studied growth factor with angiogenic capabilities is the basic FGF (bFGF). In addition to activating the ECs, bFGF upregulates the expression of other factors like VEGF. Consequently, bFGF has been used in several TE strategies, where it was verified that its addition stimulates the vascularization process.(Miyanaga et al., 2018) In addition, two other growth factors that have garnered attention for their roles in promoting vascularization are platelet-derived growth factor (PDGF) and angiopoietins.(J. Lee et al., 2020; Saharinen et al., 2017) Recognized as stabilizers and guiders of newly formed vessels, these factors are frequently co-administered with VEGF and bFGF.(Rufaihah et al., 2017) This approach aims to promote the maturation of the newly formed vessels, which without a stabilization step, can regress or undergo apoptosis.(Awada et al., 2015; Chiu & Radisic, 2010; Gümüşderelioğlu et al., 2015; Kim et al., 2011)

Furthermore, other growth factors, namely transforming growth factor-β (TGF-β) and ephrin-B2 have demonstrated efficacy in enhancing vascularization.(Y. Wang et al., 2010; Zonneville et al., 2018) TGF-β and ephrin-B2 have a key role in the maturation and remodelling of vessels through the recruitment of cells such as pericytes and SMCs, as well as the generation of extracellular matrix (ECM).(Adams & Eichmann, 2010; Carmeliet & Jain, 2011b) While these growth factors are crucial, there is a notable gap in the literature regarding their application in angiogenesis. This is primarily because their function is more associated with the stabilization of already formed vessels, rather than actively promoting their formation.

2.2. Other Proteins

Fibrinogen is a protein synthesized in the liver which is involved in blood clotting and platelet aggregation.(Pieters & Wolberg, 2019) In TE, this protein has been impregnated into different materials, especially synthetic polymers, to improve their biological qualities. When combined with polyethylene glycol (PEG) hydrogels, fibrinogen allowed the 3D culture of ECs and

SMCs, which is an essential step for the angiogenesis process.(Almany & Seliktar, 2005) Additionally, it was also combined with polylactic acid (PLA) to produce nanofibers with improved biological properties.(Gugutkov et al., 2017) The produced scaffold supported the culture of human umbilical vein endothelial cells (HUVECs). Additionally, the scaffold showed potential for implant endothelization and guided neovascularization, depending on the fibres' orientation. Furthermore, when combined with thrombin, fibrinogen undergoes an enzymatic crosslinking reaction, mirroring the physiological blood clotting cascade and resulting in its conversion into fibrin.(Pieters & Wolberg, 2019) Fibrin has been widely investigated for various TE approaches due to good biocompatibility, rapid biodegradability, and easy fabrication.(C. H. Park & Woo, 2018) This protein has been widely combined with other materials. For instance, the conjugation of fibrin with alginate was evaluated for bone TE.(Kohli et al., 2021) The construct was submitted to an *ex ovo* chick embryo chorioallantoic membrane (CAM) assay. Results showed blood vessel infiltration into the middle of the scaffold, confirming its angiogenic potential. Porous PEG hydrogels loaded with fibrin, after implanted in a rodent subcutaneous model, showed higher vascular density when compared with PEG alone.(Jiang et al., 2012)

Another interesting application of fibrin involves its role in the delivery of other molecules.(Luyendyk et al., 2019) In a study by Sacchi *et al*., a recombinant VEGF protein was incorporated into an optimized fibrin platform to overcome the stability issues of the direct use of this molecule.(Sacchi et al., 2014) This highly tunable fibrin platform allowed the controlled delivery of VEGF protein in both ischemic hind limb and wound-healing models, resulting in improved angiogenesis and tissue perfusion. This study underscores the versatility of fibrin not only as a protein that promotes vascularization but also as a carrier for bioactive molecules.

Moreover, diverse studies have evaluated the effect of various ECM proteins, including laminin, collagen, and elastin, on promoting vascularization. Laminin, for instance, has been conjugated with materials such as alginate and expanded poly(tetrafluoroethylene) (ePTFE).(Kidd et al., 2005; Oskarsdotter et al., 2023) Notably, the combination of laminin with alginate demonstrated the *in vitro* differentiation of stem cells into mature ECs, while *in vivo* implantation revealed an improvement in the neovascularization of ePTFE implants. Collagen type I (Col-I), the most abundant protein present in ECM, has also been widely used in TE due to its suitable physical, chemical, and biological properties for scaffold fabrication.(Radhakrishnan et al., 2020) Additionally, Col-I exhibits inherent potential in promoting vascularization, which can be further augmented by incorporating growth factors or proteins like elastin.(Chan et al., 2016; Daamen et al., 2008; H. Lin et al., 2006)

Apart from the proteins discussed earlier, proteins from non-human sources have also been explored. For example, silk fibroin, derived from the silk produced by some insects, offers great properties for scaffold fabrication, namely biodegradation and good mechanical properties.(Kundu et al., 2013) In an *in vitro* coculture environment, ECs and primary human osteoblast cells were embedded within silk fibroin scaffolds.(Unger et al., 2010) This setup demonstrated that ECs could autonomously organize into microcapillary-like structures featuring a distinct lumen. The constructs were implanted into immunodeficient mice, and the results 14 days after implantation showed anastomosis of the microcapillary structures with the host vasculature, presenting a perfused lumen with red blood cells. Furthermore, when cultured with mesenchymal stem cells (MSCs), silk fibroin can also stimulate and enhance angiogenic differentiation.(Y. Li et al., 2020) Besides its intrinsic angiogenic activity, silk fibroin can be further impregnated with other proteins or growth factors to boost angiogenesis.(L. Wang et al., 2021)

2.3. Peptides

An additional strategy that has been explored to promote angiogenesis is the use of small peptides typically derived from ECM proteins.(Ren et al., 2015a) These peptides contain solely amino acids required for the specific bioactivity of interest, such as cell adhesion or promoting angiogenesis. This approach can mitigate the complexity associated with the interaction involving ECM proteins once ECM proteins can engage in multiple cellular functions. These peptides are also known for their enhanced stability and ease of delivery.(Lamers, 2022)

One of the most used peptides is RGD (Arg-Gly-Asp), a sequence found in several ECM proteins, namely fibronectin, collagen, and laminin.(Bellis, 2011; Yang et al., 2021) RGD motif is responsible for the cell-adhesion process through its binding with integrins. This property has been widely explored for surface modification and applied in the coating of various materials, including PEG and hyaluronic acid, enhancing the adhesion of ECs.(Gallagher et al., 2020; X. Wang et al., 2012) For example, the incorporation of RGD in PEG hydrogels demonstrated a positive impact on the differentiation of ECs.(Claxton et al., 2023; Schukur et al., 2013) This study emphasizes the capacity of RGD to promote *in vitro* differentiation of ECs, highlighting its potential role in directing cellular processes within engineered microenvironments. Additionally, RGD has been employed as a coating in polycaprolactone (PCL) scaffolds to further evaluate its ability to recruit ECs *in vivo.*(Kang et al., 2015) Once again, the results revealed the potential of RGD-modified materials to facilitate the recruitment and integration of ECs. This was concluded after implantation of the scaffold in a rabbit carotid artery

consistently led to enhanced re-endothelization by *in situ* recruitment of ECs and reduced the possibility of graft failure.

Analogous to the RGD motif, another peptide that demonstrates affinity for integrins across a diversity of cell types to facilitate cell adhesion is YIGSR (Tyr-Ile-Gly-Ser-Arg). Originating from the larger laminin protein, YIGSR has garnered attention for its adhesive properties.(Morwood et al., 2023) This peptide sequence has been investigated for its capacity to engage with integrins on various cell surfaces, promoting robust cell adhesion.(Ren et al., 2015b) In a recent study, the presence of YIGSR peptide in scaffold free microtissues (SFMs) promoted enlargement, proliferation and viability of HUVECs.(Yarali Çevik et al., 2022) Similar results have been reported with collagen hydrogels, poly (L-lactide-co-glycolide) (PLGA) nanofibers, and even decellularized blood vessels.(Koh et al., 2013; J. S. Lee et al., 2014; J. Yu et al., 2014) Another interesting study created a gradient of YIGSR on 3D printed fibres and demonstrated the directional migration of EC to the areas with a higher density of YIGSR.(Zeng et al., 2021)

REDV (Arg–Glu–Asp–Val) is derived from fibronectin and presents an interesting characteristic for vascularization, once it binds to a specific integrin that is widely present on ECs but very rare on SMCs and fibroblasts.(Devalliere et al., 2018) Due to its ability, REDV has been used to modify the surface of several biomaterials to improve endothelization and promote and drive angiogenesis. In fact, the functionalization of polymers with REDV, such as polyurethane, promoted the spreading of HUVECs while retarding the human umbilical artery SMCs (HUASMCs), leading to a formation of HUVECs layer within 3 days.(Ding et al., 2018) Additionally, REDV immobilization in a heparin/chitosan multilayer showed a selective cell attachment for ECs.(Q.-K. Lin et al., 2012) Furthermore, a broader study functionalized different alginate scaffolds with RGD, YIGSR, and REDV.(W. Wang et al., 2015) *In vitro* analysis showed that REDV exhibited a superior capability of selective adhesion for HUVECs, while *in vivo* assays demonstrated an angiogenic potential with new vessel formation.

QK (KLTWQELYQLKYKGI) is another emerging peptide that has gained a lot of attention. Distinguished from previous peptides, this sequence is not derived from an ECM protein but rather from the VEGF.(De Rosa et al., 2021) The selection of QK from VEGF, a growth factor with substantial influence in the angiogenesis process, underscores the strategic extraction of bioactive sequences to improve angiogenic response. The increased attention on QK peptide led to several studies showing its similar influence on the VEGF in the angiogenesis process.(D'Andrea et al., 2005) These studies demonstrated comparable effects both *in vitro* and *in vivo*. For example, *in vitro* assays using Matrigel seeded with ECs showed that QK binds

to VEGF receptors, activating the ECs and consequently promoting the formation and organization of capillary structures.(D'Andrea et al., 2005) When tested *in vivo*, it induced neovascularization in ischemic hindlimb and wound healing models, suggesting that the peptide is able to mimic the functions of the full VEGF protein.(Santulli et al., 2009) Due to its unique properties, this peptide has been used in TE research in combination with different materials. For example, QK has been successfully absorbed into a chitosan surface of mesoporous silica nanoparticles.(Sun et al., 2019) This functionalization demonstrated a pro-angiogenic effect *in vivo*, as confirmed by a subcutaneous embedding test. In an alternative application, QK was tethered chemically to elastin hydrogels, and after *in vitro* and *in vivo* studies, it increased EC proliferation and the formation of functional capillaries, respectively.(Flora et al., 2019)

3. Inhibition of vascularization: molecules and TE strategies

On the other side of the spectrum, there are molecules that inhibit the vascularization. In fact, extensive research has been dedicated to the discovery and synthesis of molecules with this role, primarily to fight pathological angiogenesis commonly observed in diseases such as cancer and age-related macular degeneration.(Bressler, 2009; Lopes-Coelho et al., 2021) However, a paradigm shift has been observed in the use of anti-angiogenic molecules, making them great candidates for the control of vascularization in TE. Recent research has demonstrated the possibility of interplay between pro and anti-angiogenic molecules to fine-tune the vascularization processes and mimic the vasculature of the native tissues.(Centola et al., 2013; Forouzideh et al., 2020; Herrera Millar et al., 2022)

Bevacizumab, commercially known as Avastin, is an antibody with anti-angiogenic properties that has been approved by the Food and Drug Administration (FDA) to treat several types of cancer.(Jain et al., 2006) Besides that, this drug has also been used in TE research. For instance, Centola *et al.* used bevacizumab to functionalize a fibrin/hyaluronan scaffold seeded with nasal chondrocytes.(Centola et al., 2013) After subcutaneous implantation in nude mice, the functionalized scaffold blocked host vessel ingrowth. This effect resulted in enhanced survival of the constructs after 3 weeks, whereas the control was infiltrated by the host vasculature, which led to the degradation of the engineered cartilage tissue. *In vitro* assays were also performed to have a better understanding of the processes. Interestingly, in scaffolds without bevacizumab, the presence of fibrin notably amplified proliferation and facilitated macrophage migration. In contrast, bevacizumab-functionalized scaffolds demonstrated a mitigating effect, effectively countering the proliferative impact on ECs and limiting the migratory tendency of macrophages.

A class of molecules that also exhibits anti-angiogenic properties includes kinase inhibitors, in particular tyrosine kinase inhibitors. Usually, this class of kinase inhibitors can block the signalling pathways of VEGF.(Gotink & Verheul, 2010) For this reason, several of these molecules have already been FDA-approved for the treatment of cancer.(Ji et al., 2021; Roskoski, 2021) Notably, axitinib, commercially known as Inlyta, has been loaded into nanofibrous membranes of PCL and collagen.(Ji et al., 2021) These membranes served as encapsulating matrices for engineered cartilage tissue derived from MSCs. Subsequently, the respective engineered cartilages, with and without the axitinib membrane, were subcutaneously implanted in nude mice. The sustained release of axitinib successfully inhibited vascular invasion, leading to better cartilage maturation with a stable cartilaginous phenotype, while the constructs without axitinib were more prone to ossification due to the infiltration of the host vasculature.

Endogenous inhibitors of angiogenesis, particularly thrombospondins (TSP), angiostatin, and endostatin, are another distinct class of molecules that have been studied as an alternative approach for the treatment of cancer and other diseases.(Dimberg & Sund, 2014; Ribatti, 2009) Whereas, in the context of cancer research, these inhibitors have been well explored as potential anti-angiogenic agents, in TE they are still poorly investigated. TSP-1 and TSP-2 are glycoproteins found in the ECM and pericellular matrix.(Dimberg & Sund, 2014) Antagonizing the activity of VEGF, they inhibit ECs survival, proliferation, and migration, induce ECs apoptosis, and activate TGF.(Lutty, 2010) In a TE strategy, the anti-angiogenic effect of TSP-1 suppressed the osteogenic differentiation of adipose-derived stem cells (ADSCs) *in vitro* and inhibited ossification of engineered cartilage constructed by ADSCs cells *in vivo*.(Xie et al., 2017)

Angiostatin is a fragment of plasminogen that inhibits the proliferation of ECs.(Cao, 1999) In this sense, to investigate the effect of angiostatin on the inhibition of angiogenesis and promotion of chondrogenesis, collagen scaffolds with and without MSCs were functionalized and implanted subcutaneously in rats.(Helgeland et al., 2020) In comparison to scaffolds without angiostatin, scaffolds functionalized with angiostatin demonstrated a significant decrease in the expression of inflammatory (interleukin 1 *alpha* and *beta*) and angiogenic genes (platelet and ECs adhesion molecule 1) after 2 weeks of implantation. Additionally, after 8 weeks, the scaffolds with angiostatin had fewer inflammatory cells and more collagen matrix formation, but fibrocartilage formation was not detected. Together, these results emphasize that although angiostatin suppressed angiogenesis, it did not stimulate ectopic chondrogenesis in tissue-engineered constructs in vivo.

Endostatin is a fragment from collagen type XVIII that inhibits EC proliferation and migration, and it has been studied in the production of engineered cartilage tissue.(Walia et al., 2015) Taking advantage of these properties, endostatin was added to fibrin scaffolds containing swine neonatal meniscal cells.(Herrera Millar et al., 2022) After molecular analysis of different markers, such as Col-I and II, the results showed that the presence of endostatin promoted differentiation into a fibro-chondrogenic phenotype and improved cell efficiency. These findings underscore the potential of endostatin as a valuable molecular modulator for TE applications, particularly for the differentiation of avascular tissues.

Research on natural anti-angiogenic products has gained emphasis in recent years due to the need to find less toxic and cost-effective alternative molecules. Some of these have been found in nature, namely polyphenols, alkaloids, terpenoids, and saponins, which have demonstrated anti-angiogenic properties and are presently being tested to target various tumour types.(R. Li et al., 2021) Among those molecules, epigallocatechin gallate (EGCG) has been investigated for TE. EGCG, which is a catechin, is widely abundant in green tea leaves.(Talib et al., 2024) This natural compound has been loaded into silk-fibroin scaffolds to serve as an anti-angiogenic for corneal TE.(Forouzideh et al., 2020) In fact, EGCG inhibited the *in vitro* proliferation of HUVECs seeded in silk-fibroin scaffolds, suggesting the combination of scaffold/EGCG could have the same effect when translated to *in vivo* models.

4. Strategies for controlled release of pro and anti-angiogenic molecules

Drug delivery systems can play a critical role in modulating vascularization, facilitating the controlled release of both angiogenic and anti-angiogenic molecules. The precise regulation of these molecules is crucial for promoting a well-balanced angiogenic response, essential for the successful integration of engineered tissues.(Bhise et al., 2011) Among these systems, hydrogels, and microparticles have been extensively explored in TE applications. Hydrogels provide a 3D network that can encapsulate and release drugs in a controlled manner. They have the capacity to protect molecules from degradation and can be engineered in terms of degradability to serve as a platform to release angiogenic and anti-angiogenic molecules gradually, aiming to control vascularization.(J. Li & Mooney, 2016)

Recently, hydrogels of PEG-Maleimide, used to deliver pancreatic islets, were biofunctionalized with VEGF to promote graft vascularization.(Phelps et al., 2015) The findings demonstrated an extended *in vivo* release of VEGF through enzymatic degradation and an augmented vascularization of the islets. According to the authors, this strategy can be used as a vascular-inductive cell delivery vehicle and warrants further investigation in diabetic

animal models. Similar approaches have been taken to improve the delivery of anti-angiogenic molecules. For example, to overcome the stability issues of bevacizumab when applied to tumour microenvironments, this molecule was loaded into an alginate hydrogel.(Ferreira et al., 2017) The biological performance of the construct was investigated by the CAM assay. Results *in ovo* demonstrated an improved anti-angiogenic activity compared to free bevacizumab due to a slow controlled release from the hydrogel (**Figure 2 A1**). Moreover, the loaded hydrogel was applied to microtumours, and 5 days after treatment, there was a considerable regression in tumour size, and immunohistochemistry showed a reduced number of blood vessels compared to the control (Figures 2 A2 and 2 A3).

Besides of hydrogels, microparticles and microspheres composed of proteins or other polymeric structures can also encapsulate molecules and release them over time in a controlled manner. For example, these systems offer a larger payload capacity compared to nanoparticles, making them suitable for sustained release applications.(da Silva et al., 2023) VEGF immobilized on the surface of synthetic magnetic microparticles has shown increased angiogenic activity. Both *in vitro* and *in vivo* experiments demonstrated that the immobilized VEGF improved the proliferation and survival of outgrowth endothelial progenitor cells when compared to soluble VEGF (Figures 2 B1 and 2 B2).(Aday et al., 2017) Another approach that has gained a lot of attention is the use of siRNA due to its the ability to suppress gene expression.(Dana et al., 2017) However, its effect is temporary. As an alternative to overcome this issue, the encapsulation of anti-VEGF siRNA in PLGA microspheres has shown some promising results.(Murata et al., 2008) Notably, *in vitro* assays presented a sustained release of siRNA for over a month, and an intra-tumour injection of the microspheres in mice bearing S-180 tumours successfully suppressed tumour growth.

Nanomaterials, including liposomes, polymeric nanoparticles, nanofibers, and dendrimers, have also emerged as promising vehicles for the targeted and sustained delivery of angiogenic and anti-angiogenic molecules.(Kargozar et al., 2020) Nanomaterials, characterized by their nanoscale dimensions, offer advantages such as increased surface area, enhanced cellular uptake, and the ability to cross biological barriers.(Mitchell et al., 2021) Furthermore, the controlled and targeted release provided by these nanomaterials allows for sustained exposure to molecules, promoting optimal conditions to control vascularization. As an example, PLGA has been widely used as a carrier of different types of molecules. PLGA nanoparticles coated with pro-angiogenic molecules on their surface, namely VEGF and apelin, were tested to evaluate the ability of such a strategy to promote angiogenesis both *in vitro* and *in vivo*.(J. S. Park et al., 2016) Results showed that the coated nanoparticles could easily enter human MSC

without cytotoxicity, leading to their differentiation into ECs and vascular formation in Matrigel. Injection of human MSC transfected with the PLGA nanoparticles into an ischemic hind limb mouse model had the same effect on MSC differentiation and induced accelerated neovascularization. Moreover, nanoparticles of PEG-co-PCL were functionalized with CGKRK, a peptide with anti-angiogenic properties that binds to both neovascular ECs and tumour cells.(Hu et al., 2013) *In vitro* assays showed that the modified nanoparticles effectively penetrated into U87MG tumour spheroids, reducing cell proliferation, and triggering apoptosis both in HUVECs and U87MG cells in a dose/time-dependent manner (Figure 2 C). Interestingly, when subcutaneously injected in U87MG tumour-bearing mice, loaded nanoparticles targeted blood vessels of the tumour leading to a decrease in tumour growth and weight when compared with the controls. The observed results were correlated with the presence of CGKRK peptide-functionalized nanoparticles, which demonstrated their anti-angiogenic effects.

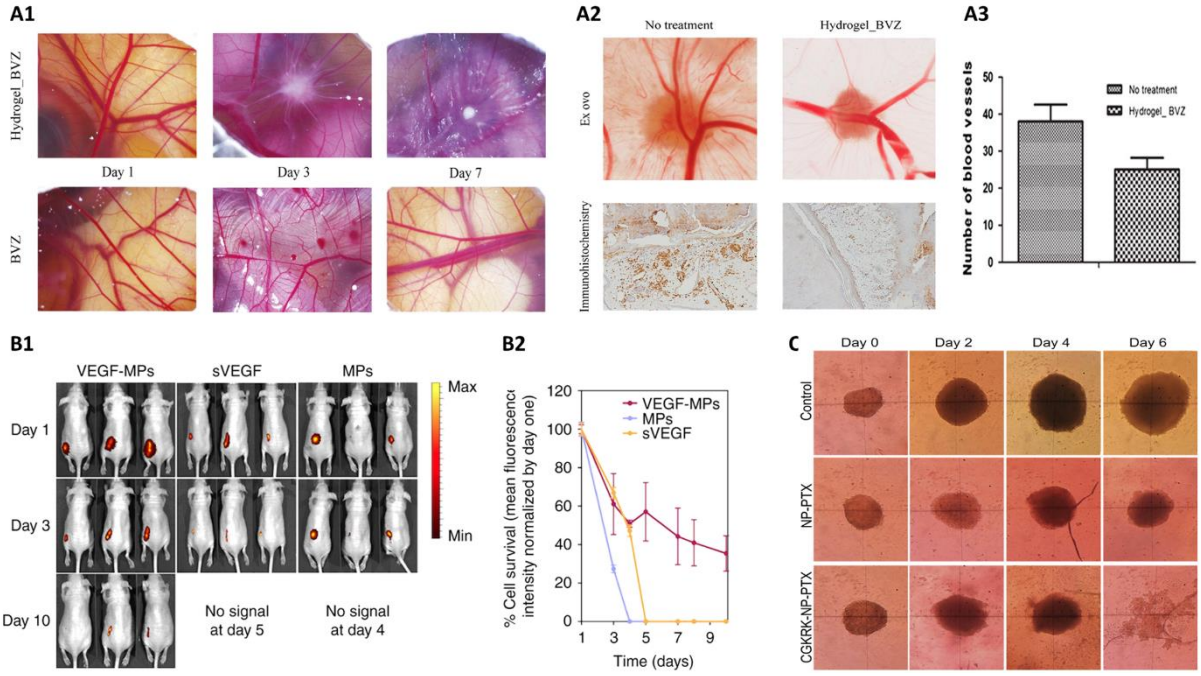

Figure 2 - TE strategies for controlled release of pro and anti-angiogenic molecules. (A1) Stereomicroscopy images acquired *in ovo* show vascular changes of groups treated with free bevacizumab and bevacizumab loaded alginate hydrogels after 1, 3 and 7 days. (A2) Photographs of 3D xenograft microtumours taken *ex ovo* 5 days after the application of bevacizumab loaded alginate hydrogels and no treatment. Immunohistochemical staining from excised CAM containing microtumours. Sections were stained with SNA-lectin to highlight blood vessel. (A3) *Ex ovo* quantification of macroscopic blood vessel. Reproduced with permission.(Ferreira et al., 2017) Copyright 2017, Elsevier. (B1) Representative *in vivo* images of outgrowth endothelial progenitor cells aggregates prepared with VEGF immobilized microparticles (VEGF-MPs), soluble VEGF (sVEGF) and blank microparticles (MPs). Images were obtained after 1, 3 and 10 days of the injection. (B2) Fluorescence intensity measurements over the experimental procedure are shown. The fluorescence intensities were normalized by

day one. Results are average ± SEM (n = 7). Reproduced with permission.(Aday et al., 2017) Copyright 2017, Nature. (C) Morphology of U87MG tumour spheroids treated with serum-free DMEM (Control), nanoparticles loaded with paclitaxel (NP-PTX) and CGKRK loaded nanoparticles (CGKRK-NP-PTX) at day 0, 2, 4, and 6 after applied treatment- Reproduced with permission.(Hu et al., 2013) Copyright 2013, Elsevier.

5. Vascularization control through 3D bioprinting

3D bioprinting emerges as a pivotal technology to control vascularization within a tissue-engineered scaffold. The essence of 3D bioprinting lies in its ability to enable the spatial configuration of cells, biomaterials, and bioactive agents. This precision allows the generation of intricate vascular networks that closely mimic the complex vasculature of native tissues.

Beyond structural complexity, tissue engineers can finely modulate the insertion of pro and anti-angiogenic molecules, thereby regulating the angiogenic response within the scaffold. A noteworthy attribute of 3D bioprinting is its dynamic material deposition capabilities, easing the establishment of gradients within the scaffold. This characteristic can influence the hierarchical organization of vascular structures, allowing tissue engineers to dynamically control material deposition and guide the formation of vascular networks with spatially defined patterns, closely mirroring the intricate complexity observed *in vivo*.

Presently, two primary methodologies are employed for the integration of blood vessels into bioprinted materials. The first methodology focuses on the controlled release of angiogenic factors to induce blood vessel growth within 3D-printed tissues.(Datta et al., 2017) A notable example is the work of Lee *et al*., who employed the Freeform Reversible Embedding of Suspended Hydrogels (FRESH) technique to 3D bioprint collagen infused with VEGF.(A. Lee et al., 2019) This strategy enhanced vascularization *in vivo*, highlighting the potential for controlled angiogenesis. Concurrently, a ground-breaking integration of 3D projection bioprinting with orthogonal photo-conjugation has been explored.(C. Yu et al., 2020) This approach involves bioprinting soft hydrogels into complex geometries using a photoreactive thiol-ene gelatin bioink and digital light projection technology. The combination of these techniques allowed for immobilization of proteins and peptides, namely QK, with high spatiotemporal precision that induced guided growth of ECs and human cardiomyocytes (**Figure 3** A).

The second strategy involves the direct bioprinting of vascular scaffolds.(Jia et al., 2016) Pioneering this approach, Smith *et al*. used a commercial 3D printer with distinct cold cellular solutions to bioprint vascular tissue.(Smith et al., 2004) Kolesky *et al*. advanced this methodology, developing a multi-material 3D bioprinting method for creating thick human

tissues with embedded vasculature and multiple cell types (Figure 3 B).(Kolesky et al., 2016) Demonstrating stability, constructs with parallel channels lined with HUVECs maintained their endothelial phenotype after prolonged perfusion. Furthermore, a coaxial extrusion printing technique has been employed to fabricate vessel-like tubes with tuneable diameters using a polyelectrolyte-based material.(Zeng et al., 2021) The surface functionalization of these tubes with heparin and YIGSR enhanced scaffold biocompatibility and endothelialisation.

Despite the advances made in using pro-angiogenic molecules in 3D bioprinting, a noticeable gap persists in the literature concerning the application of anti-angiogenic molecules. Recent efforts have looked to address this gap by developing bioinks with both pro and anti-angiogenic traits, revealing spatially confined vascular networks within an anatomically shaped meniscus construct.(Terpstra et al., 2022) These matrices were developed using endothelial cell-laden bioinks supplemented with microfibers from Col-I and decellularized cartilage ECM (Figure 3 C1 and 3 C2). HUVECs-driven capillary networks formed two days post-bioprinting. Cartilage-derived microfibers induced a reduction in neo-microvessel length by 29% and microvessel junctions by 37% after 14 days, compared to pro-angiogenic collagen microfibers. This study introduces an innovative approach for zonal biomimetic meniscal constructs, using ECM-derived microfibers to modulate capillary networks in tissue biofabrication.

3D bioprinting stands as a driving force in advancing precision vascularization control within tissue-engineered scaffolds. Its ability to manipulate spatial arrangements and finely tune scaffold designs holds immense promise for regenerative medicine, opening avenues for the development of clinically relevant tissue constructs endowed with unprecedented vascular intricacy.

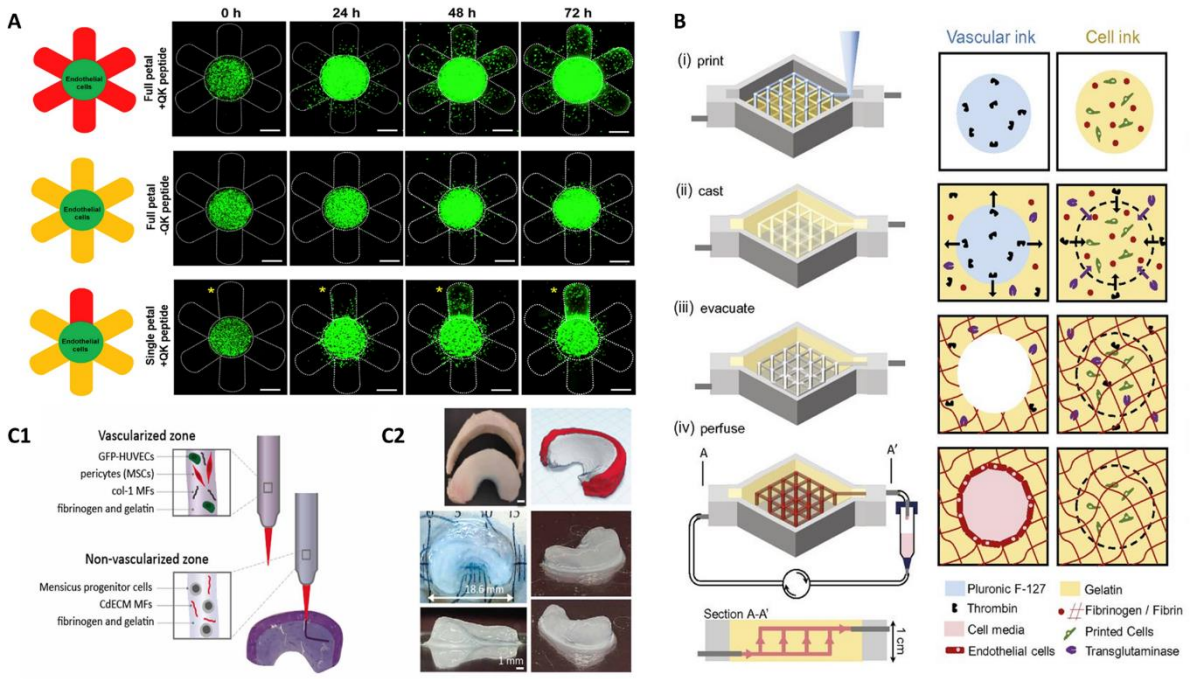

Figure 3 - The use of 3D bioprinting technology for controlling vascularization. (A) Photopatterning of the QK peptide for guidance of endothelial cell migration, proliferation, and directionality. Representative fluorescent images of 3D bioprinted daisy constructs showing the migratory response of encapsulated fluorescently stained HUVEC from the central floral disc to the petal regions over 72 h (red zones represent the immobilized QK peptide). Scale bars = 500 µm. Reproduced with permission.(C. Yu et al., 2020) Copyright 2020, Elsevier. (B) Schematic illustration of the tissue manufacturing process. (i) Fugitive (vascular) ink, and cell-laden inks are printed within a 3D perfusion chip. (ii) ECM material is then cast over the printed inks. After casting, thrombin induces fibrinogen cleavage and rapid polymerization into fibrin. Similarly, transglutaminase diffuses from the molten casting matrix and slowly cross-links the gelatin and fibrin. (iii) Upon cooling, the fugitive ink liquefies and is evacuated, leaving behind a pervasive vascular network, which is (iv) endothelialized and perfused via an external pump. Reproduced with permission.(Kolesky et al., 2016) Copyright 2016, PNAS. (C1) Schematic of the meniscus-shaped construct was bioprinted with a pro-angiogenic outer zone consisting of HUVECs, MSCs, and COL-I microfibers, and an anti-angiogenic inner zone, consisting of meniscus progenitor cells with cartilage decellularized ECM microfibers. (C2) Images of the meniscus file obtained from an equine meniscus micro-CT scan, and of the bioprinted meniscus construct of 24 layers with the developed bioinks. Reproduced with permission.(Terpstra et al., 2022) Copyright 2022, Biofabrication.

6. Bioreactors for Growing Vascularized and Non-Vascularized Tissues

The advancement of 3D printing technologies opens new opportunities for fabricating complex structures combining hierarchical engineered tissues such as vascular/non-vascular mimetic constructs. However, new challenges emerge on the side of the maturation of these living materials.(Martin et al., 2004) The use of bioreactors, widely established in tissue engineering and providing a controlled and dynamic environment for *in vitro* development, must be rethought for constructs that merge several engineered tissues. These can present different cell composition zones which requires bioreactors with compartmentalization during maturation.

This calls for advanced bioreactor technologies that completely challenges biomedical engineers.(Castro et al., 2020; Zhang et al., 2021b)

Bioreactors provide controlled environments for optimal cell growth and tissue development. These systems include spinner-flask, rotating-wall, perfusion-based, compression, and double-chamber stirred bioreactors.(Castro et al., 2020; Niu et al., 2023; Zhang et al., 2021b; Zhao et al., 2016) Spinner-flask bioreactors offer continuous stirring for mixing oxygen and nutrients, but can introduce turbulence and shear gradients.(Ismadi et al., 2014) Nevertheless, they have proven effective for cultivating osteoblasts and promoting the formation of vascularized bone tissue constructs.(Gaspar et al., 2012; Kedong et al., 2014) Additionally, they have been used to support the growth and differentiation of chondrocytes for the development of functional cartilage structures.(K. Song et al., 2015) Rotating-wall bioreactors have been also widely used as they present reduced turbulence and shear stress by simulating microgravity.(Hammond & Hammond, 2001) By using this bioreactor modality DiStefano *et al.* have efficiently differentiated pluripotent stem cells into 3D retinal organoids, demonstrating improved growth, well-defined morphology, and better neuronal differentiation.(DiStefano et al., 2018) The use of rotating-wall vessels accelerated and improved the development of these organoids which can be utilized to better model retinal diseases towards therapy assessment.

Perfusion-based bioreactors improve mass transfer and are effective for stimulating shear stress, demonstrating success in improving cell viability and promoting cell differentiation.(Bijonowski et al., 2013) This has been widely demonstrated in case of bone tissue engineering where osteogenic differentiation has been achieved through mechanotransduction.(Montorsi et al., 2022) These types of bioreactors are particularly relevant for vascular approaches, as they provide consistent nutrient and oxygen transfer by simulating conditions closer to the vascular environment.(Niu et al., 2023) While these bioreactors have proven to be also highly effective in bone tissue engineering, they are especially suitable for engineering of vascular tissue.(Gaspar et al., 2012) For example, arterial tissue has been engineered using flow perfusion leading to the creation of what the Janke, *et al.* referred to as the "Artificial Artery".(Janke et al., 2013)

Compression bioreactors are another modality able to introduce controlled mechanical stimulation to improve structural and functional properties for tissue regeneration.(Zhao et al., 2016) These are used, for example, in cartilage tissue engineering, as demonstrated in the study by Mauck *et al.* which applied a dynamic compression load to agarose discs.(Mauck et al., 2000)

New processing methods are available to create complex tissue engineering constructs combining vascular and non-vascular tissues and to allow the scale-up to reach functional dimensions. This brings new difficulties in getting these living materials to mature properly. In this context bioreactors need to be adapted to host different tissues and cell compositions. This requires new modular bioreactor designs. Double-chamber stirred bioreactors, featuring two or more independent chambers, have been designed for simultaneous yet separate cultivation of different cell types. This bioreactor modality has been useful for engineering interface tissues such as vascular/non-vascular osteochondral constructs. Chang *et al.* introduced an innovative model for osteochondral tissue engineering, utilizing a silicon membrane to divide the bioreactor into two chambers.(Wendt et al., 2005) Each chamber has its own media recirculation and stirring systems, allowing independent control and customization of culture conditions. This design proves invaluable for coculturing chondrocytes and osteoblasts within a single scaffold, enabling tailored growth environments with distinct media compositions and no cross-contamination. Such bioreactors play a crucial role in advancing tissue engineering, offering precise control for the coculture and differentiation of stem cells, particularly beneficial in the cultivation of interface tissues.(Y. Pei et al., 2014)

The multifaceted role of bioreactors in the vascularised and non-vascularised tissue engineering underlines their importance in creating personalised microenvironments for different types of tissues.(Kannan et al., 2005; Niu et al., 2023) In fact, they are essential tools for unravelling the dynamics of vascularisation control in tissue engineering. Their versatility in creating controlled environments, stimulating growth, and improving tissue properties positions them as key components shaping the future of engineering vascularised and non-vascularised functional tissues in a wide number of applications.

7. Future Perspectives

The field of TE has seen rapid evolution, marked by notable advancements in recent years aimed at enhancing the control of vascularization within engineered tissues. The future trajectory of this field is likely to comprise the integration of different approaches to achieve optimal control over vascularization (**Figure 4**). However, the integration of multiple technologies, such as 3D bioprinting and bioreactor systems, and ensuring their efficient interaction requires overcoming technical and biological hurdles.

New bioinks that better mimic the ECM and support the controlled differentiation of cells is a pivotal step toward achieving better engineered tissues. Recent advances in smart biomaterials,

incorporating various molecules responsive to environmental cues, are opening new horizons for vascularization control in TE. The use of 3D bioprinting allows precise spatial deposition of these biomaterials within engineering tissues, enabling the promotion or inhibition vascularization as needed. In addition, innovative 3D bioprinting techniques, such as coaxial and in-bath bioprinting, can significantly advance the field by replicating the cylindrical and hollow morphologies characteristic of vascularized tissue.

Furthermore, novel bioreactors capable to deliver precise mechanical and biological cues, can enhance tissue maturation and functionality of engineered vascular tissues. The development of programmed bioreactors for controlled release of angiogenic or anti-angiogenic molecules can play a critical role in vascularization control.

The complexity of vascularization control and the numerous parameters associated with promising technologies including 3D bioprinting and bioreactor systems can be addressed by future applications of artificial intelligence (AI) algorithms. By leveraging collected datasets, AI can simulate complex biological processes, facilitate protocol design, and potentially reduce both the cost and time required to identify suitable molecules, materials, and processing parameters. Furthermore, AI can identify patterns and relationships that might not be evident through traditional analysis, leading to new insights and innovations.

Finally, as TE continues to advance, the establishment of regulatory frameworks for clinical translation is imperative. Developing standardized processes to ensure the safety and scalability of TE products is crucial. Although this is not a research and innovation process, it is of extreme importance, to ensure that new technologies and advancements in the field successfully reach the market.

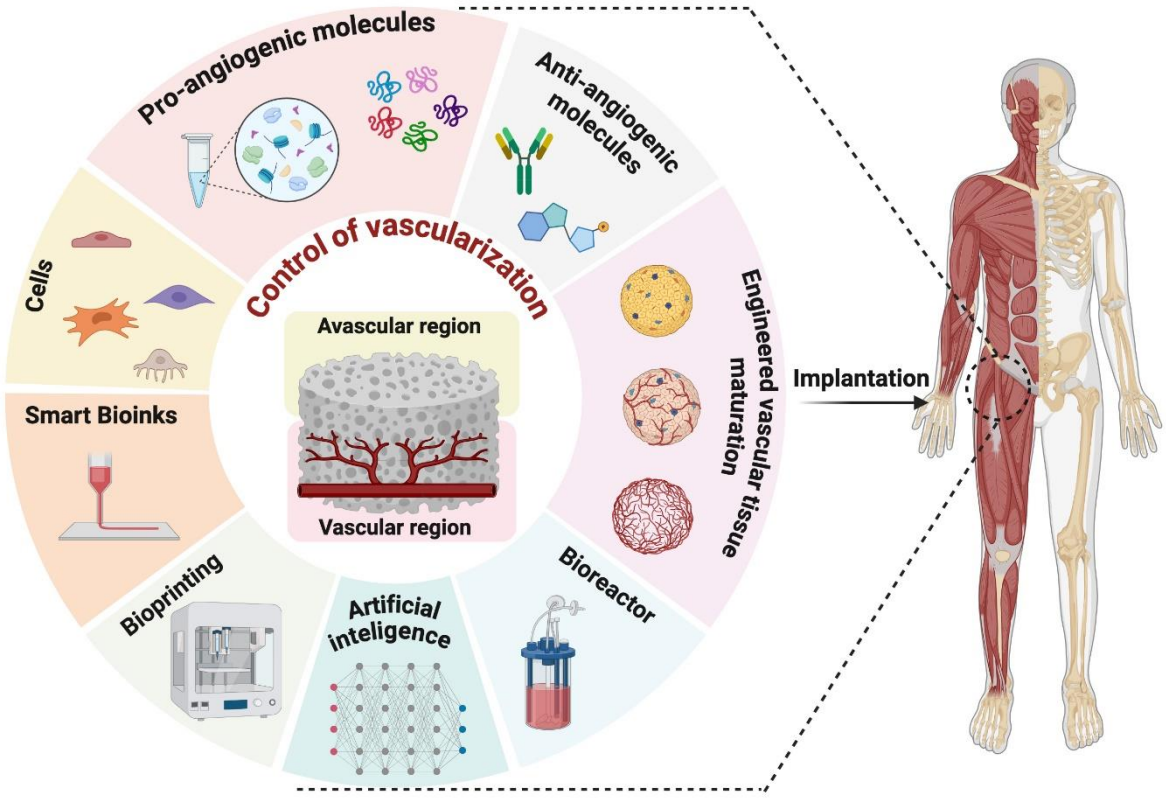

Figure 4 – Future perspectives for vascularization control in TE. Schematic illustration showing an overview of the methods and technologies anticipated to advance vascularization control in TE.

8. Conclusions

TE strategies can play a critical role in modulating vascularization to produce functional human tissues for *ex vivo* and *in vivo* applications. However, intensive investigation is still necessary to design and develop strategies that facilitate the release of both angiogenic and anti-angiogenic molecules in a controlled manner and according to the specificities of each tissue. Although 3D bioprinting has emerged as a promising technology for controlling the vascularization within TE constructs, several challenges and limitations remain, including i) the multiple cell players, the cell culture conditions, their maintenance and stability during and after printing; i) the complex interactions and crosstalk between molecules and cells; iii) the real-life tissue biological complexity and scales; iv) scaling-up the vascularization of tissues; and vi) the difficulties associated with translating this type of technologies to human clinical trials. Finally, is the appropriate selection of pro- and anti-angiogenic factors, as well as the best strategy to

control the release of such factors in a dose and time-dependent manner when considering tissues that span from vascularized to non-vascularized zones.

Addressing these limitations and exploring innovative and precise technologies are fundamental steps for enhancing the 3D bioprinting of complex, functional, and clinically relevant tissue constructs.

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