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αvβ3 and α5β1 integrin-specific ligands: From tumor angiogenesis inhibitors to vascularization promoters in regenerative medicine?

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ABSTRACT

Integrins are cell adhesion receptors predominantly important during normal and tumor angiogenesis. A sequence present on several extracellular matrix proteins composed of Arg-Gly-Asp (RGD) has attracted attention due to its role in cell adhesion mediated by integrins. The development of ligands that can bind to integrins involved in tumor angiogenesis and brake disease progression has resulted in new investigational drug entities reaching the clinical trial phase in humans. The use of integrin-specific ligands can be useful for the vascularization of regenerative medicine constructs, which remains a major limitation for translation into clinical practice. In order to enhance vascularization, immobilization of integrin-specific RGD peptidomimetics within constructs is a recommended approach, due to their high specificity and selectivity towards certain desired integrins. This review endeavours to address the potential of peptidomimetic-coated biomaterials as vascular network promoters for regenerative medicine purposes. Clinical studies involving molecules tracking active integrins in cancer angiogenesis and reasons for their failure are also addressed.

1. Introduction

Cell adhesion is a paramount feature on which many of the successful cell-based approaches using biomaterials in the field of regenerative medicine rely on. Taking this into consideration, and since most of biomaterials are essentially bio-inert, there is a need to increase their 'cell-friendliness' in order to enable cells to adhere, function normally and execute their natural occurring processes. Commonly, biofunctionalization of biomaterials is achieved by integrating a tripeptide (RGD, Arg-Gly-Asp) into the biomaterial (Assunção-Silva et al., 2015; Gomes et al., 2016; Silva et al., 2012). Linear or cyclic RGD sequences are valuable tools for this purpose since these represent the sequence of several extracellular matrix (ECM) proteins, namely fibrinogen, vitronectin and fibronectin which are known to be responsible for mediating cell adhesion to the ECM (Meyer et al., 2006; Pierschbacher and Ruoslahti, 1984a, 1984b; Ruoslahti and Pierschbacher, 1986; Suzuki et al., 1985). Specifically, this tripeptide interacts with integrins, a type of cell-surface receptors involved in the adhesion, differentiation, proliferation and migration of cells (Trabocchi and Guarna, 2014). Consequently, these heterodimeric glycoproteins are essential for homeostasis and are involved not only in the normal physiological development, maintenance and repair of tissues but also in the pathological mechanisms of diseases like cancer (Desgrosellier and Cheresh, 2010).

Regarding their structure, integrins are constituted by two different non-covalently attached subunits (α and β), for which there are 18 α and 8 β subtypes in vertebrates (Hynes, 2002). Additionally, each subunit has an extracellular domain, a single transmembrane region and a noncatalytic cytoplasmic region (Fig. 1), being the combination between subtypes accountable for the ligand affinity of a given integrin (Danhier et al., 2012).

In their resting state, integrins are bent and possess a salt bridge between both subunits in the cytoplasmic region, and can be activated by inside-out or outside-in signaling (Müller et al., 2014). Inside-out signaling relies on the action of a protein called talin (Anthis et al., 2009) which directly binds to the cytoplasmic tail of the β integrin and disrupts the salt bridge, leading to an increased affinity towards integrin ligands (Anthis et al., 2009; Ginsberg et al., 1992). This type of signaling is directly involved in adhesion strength and allows the transmission of the necessary forces to cell migration, ECM remodeling and assembly (Shattil et al., 2010). Even in their resting state, integrins have residual affinity to their ligands and so on outside-in signaling, ligand binding will cause conformational changes that in turn will increase affinity (Du et al., 1991; Schwartz et al., 1995). Therefore, since

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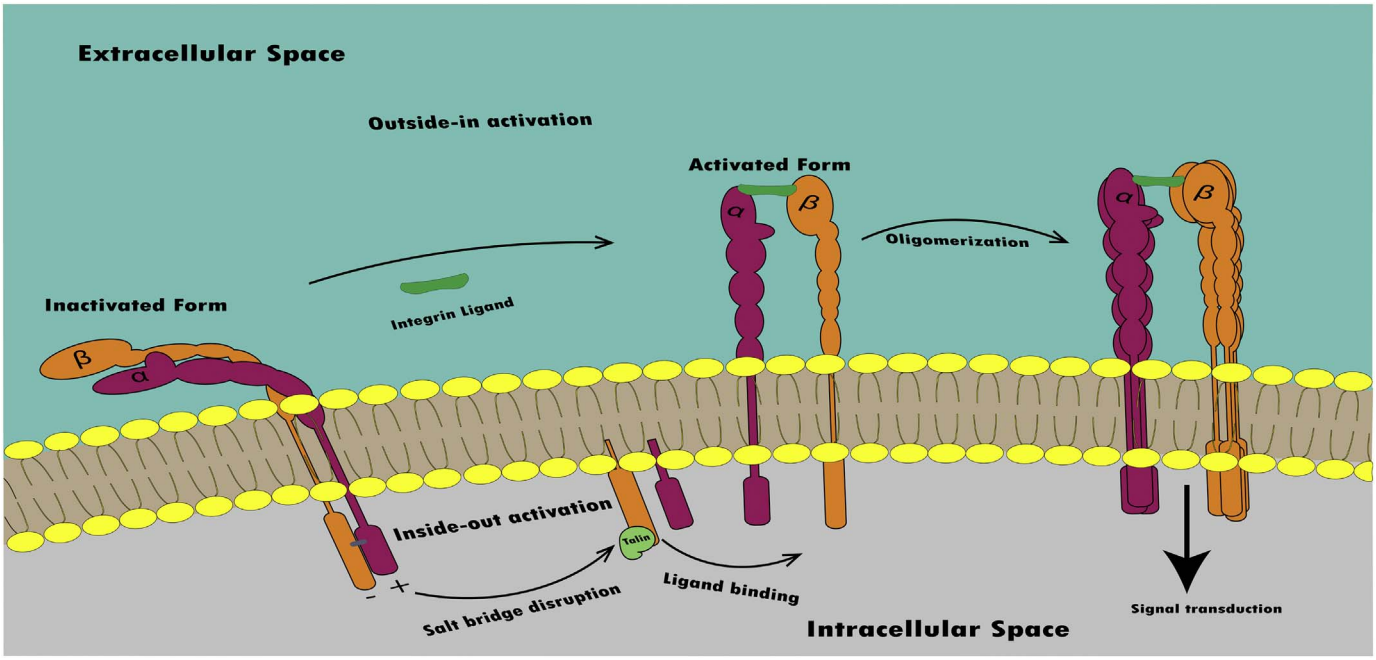

Fig. 1. Integrin activation by extracellular ligands. After ligand binding, integrins change their conformation and increase their affinity towards the ligand. This leads to their oligomerization and finally to signal transduction.

most ligands are multivalent, they participate in the oligomerization of these glycoproteins and it is by this means that signal transduction to the inside of cells occurs. With this type of signaling, it is possible to control gene expression, cell polarity, cytoskeletal structure as well as cell survival and proliferation (Shattil et al., 2010). Apart from this, it has been shown that integrins may possess intermediate states with decreased affinity to soluble ligands, but presenting strong cell adhesion capacity under applied external forces (Müller et al., 2013).

Concerning binding ligands, it is possible to divide integrins into four different groups: leucocytes, collagen, laminin and RGD receptors (Trabocchi and Guarna, 2014). RGD receptors participate in relevant physiological processes like platelet aggregation and angiogenesis and pathological processes such as cardiovascular diseases and tumor formation (Curley et al., 1999; Danhier et al., 2012; Desgrosellier and Cheresh, 2010). Accordingly, it is not surprising that this family of integrins has been the target for many studies regarding potential treatments for conditions in which they are proposed to take part. For instance, αIIβ3 is the key receptor in platelet adhesion, aggregation and thrombus formation and is implicated in ischemic heart disease and stroke (Coller, 1995; Plow et al., 1985). In fact, this integrin was the first molecular target for design of specific antagonists (Coutré and Leung, 1995; Schafer, 1996). Currently, the net outcome of this research apart from many experimental tools, is the regulatory approval by FDA of no less than three αIIβ3-targeting antagonists (abciximab in 1997, eptifibatide in 1998 and tirofiban in 1999) all with proven clinical evidence in reducing acute coronary conditions and ischemia (Blazing et al., 2004; Cohen et al., 2002; Giugliano et al., 2009; Goodman, 2003; Kastrati et al., 2006; Marzocchi et al., 2008).

These pioneering efforts have made apparent the potential for developing drugs targeting integrins involved in the pathogenesis of diseases where they have a role, or alternatively in gaining a deeper pharmacological understanding of how they conduct themselves in such pathological events (Trabocchi and Guarna, 2014). Hence, two integrins (α5β1 and αvβ3) in particular have attracted attention due to their significant up-regulation during tumor angiogenesis (Brooks et al., 1994a; Brooks et al., 1994b; Kim et al., 2000; Muether et al., 2007). Angiogenesis is the process by which new blood vessels originate from pre-existing ones and is an essential occurrence during tumor growth and metastasis because they carry oxygen and nutrients to cells, and are thus able to encourage tumor development (Carmeliet, 2005). Contrary to the situation in normal tissue, both integrins are highly expressed on tumor blood vessels, and pro-angiogenic growth factors like IL8 or bFGF stimulate their expression on endothelial cells (Brooks et al., 1994a, 1994b; Kim et al., 2000). Several selective antagonists of these integrins have since reached the clinical trial phase, including intetumumab (human antibody against α5β1 and αvβ3) (O'Day et al., 2011), etaracizumab (humanized antibody against αvβ3) (Hersey et al., 2010), volociximab (chimeric mouse-human antibody against α5β1) (Bell-Mcguinn et al., 2011) and cilengitide (cyclic RGD-containing peptide antagonist of α5β1 and αvβ3) (Reardon et al., 2008). Despite having acceptable toxicity profiles in humans, only cilengitide reached pivotal phase III safety and efficacy trials, specifically for the treatment of glioblastoma (Stupp et al., 2014). This fact demonstrates that the design of effective integrin antagonists targeting cancer angiogenesis is still to be fulfilled but the relevance of these cell adhesion receptors should not be undermined since they intervine in several physiological, mechanisms, in the disease state or otherwise.

Apart from the effect that αvβ3 has on tumor angiogenesis, this integrin also takes part both in other biological events such as apoptosis, migration of tumor cells and diseases like osteoporosis and rheumatoid arthritis (Trabocchi and Guarna, 2014). As for α5β1, some promising results show a possible contribution of this glycoprotein on the development and progression osteogenesis (Fromigué et al., 2012). This assumption is based on its up-regulation of the expression of osteogenic markers and the activity of alkaline phosphatase in vitro, while contributing to osseointegration of implants and the ectopic formation of bone (Agarwal et al., 2015; Hamidouche et al., 2009; Martino et al., 2009).

Ligand selectivity within the extensive family of integrins can be achieved through drug design strategies involving the introduction of conformational constrictions to RGD by cyclization (Haubner et al., 1996; Kessler, 1982; Trabocchi and Guarna, 2014). This strategy enabled developing cyclic RGD ligands (illustrated in Fig. 2) with high binding affinity towards αvβ3, whilst having selectivity towards it in detriment of αIIβ3 (Aumailley et al., 1991; Haubner et al., 1997). Affinity towards αvβ3 can be enhanced by developing cyclic RGD-

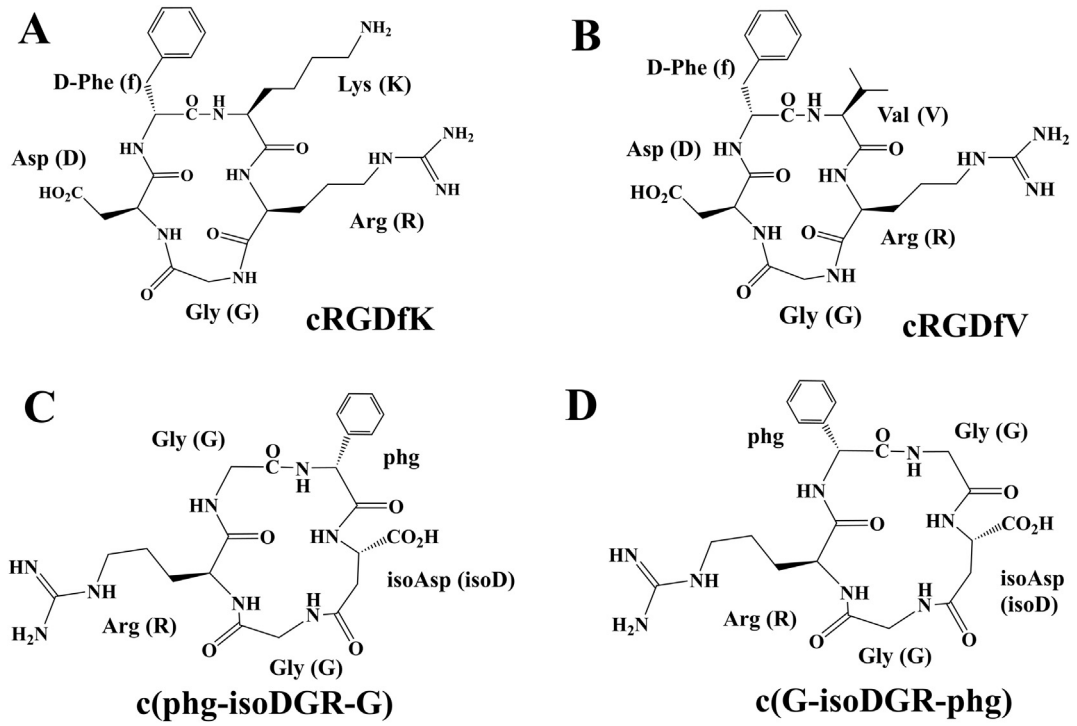

Fig. 2. Chemical structure of different cyclic RGD molecules.

containing multimeric ligands. This strategy intends to develop polyvalent ligands that interact simultaneously with several integrins (Thumshirn et al., 2003). Cyclic RGD peptides, however, were not capable of selectively distinguishing αvβ3 from α5β1 due to the close structural topography of these integrins. Among cyclic peptides some molecules bearing the *iso*DGR sequence, however, have both high affinity and selectivity towards αvβ3 and α5β1, representing one of the exceptions on this regard. This binding motif results from the deamination of the asparagine residue of NGR (a peptidic sequence present in fibronectin) and acts as a integrin binding motif (Curnis et al., 2010, 2006). Therefore, it was only after elucidating the crystal structure of αvβ3, that structure-activity relationship studies and the development of a homology model for α5β1 permitted studying the creation of such compounds (Marinelli et al., 2005; Xiong et al., 2001; Xiong, 2002). Developing ligands capable of discriminating between both of these integrins was mostly achieved by peptidomimetic ligands (Heckmann et al., 2008; Marchini et al., 2012; Smallheer et al., 2004; Stragies et al., 2007).

Targeting specific integrins is an interesting approach for regenerative medicine as most of the potential therapeutic methodologies applied in this research field are dependent on the adhesion of specific cell types into a given biomaterial. In fact, vascularization is one of the biggest challenges faced by regenerative medicine during translation from lab to clinic, which also makes it a major focus for research (Jaklenec et al., 2012). Several methods are employed to solve this problem, including RGD-functionalisation of biomaterials that promote both endothelial cell (EC) adhesion and organization, while enabling the use of pre-vascularized scaffolds for an effective connection to host circulatory system (Bidarra et al., 2011; Chen et al., 2014; Moon et al., 2009; Yang et al., 2014). Commonly, RGD incorporation consists in chemically attach the molecule to the backbone of the material. These methodologies apply different chemistries like carbodiimide (CDI) (Ferris et al., 2015), periodate oxidation (Dalheim et al., 2016) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) (D'Este et al., 2014) to form an amide bond between biomaterial and peptide or can take advantage of Diels-Alder chemistry to covalently ligate RGD to the biomaterial (Gomes et al., 2016). Even though cellular adhesion and proliferation normally increases following RGD immobilization, most studies do not take into consideration several variables that could further enhance cell behavior. Thus, studying if the peptide presents the right conformation to exert its full biological effect, understanding if there is no hindrance, being the peptide totally available to interact with cells, and its density throughout the material are variables that should be optimized in order to develop a proper vascular network by regenerative medicine methodologies. Another factor that can affect the proper creation of a vascularized construct is the specificity and selectivity of the applied RGD motif. To our knowledge, employment of ligands with specificity towards a given integrin to improve the vascularization of tissue engineering constructs has not received due attention and the coating of biomaterial surfaces is usually done with RGD-containing peptides with poor selectivity (Hersel et al., 2003). Biomaterial functionalization using molecules with enhanced integrin selectivity could prove to be a promising methodology to address the problem of vascularization in tissue engineering since, as already mentioned, αvβ3 and α5β1 play crucial roles during angiogenesis. Using specific integrin-targeting ligands could be important to specifically recruit endothelial cells (ECs) to the scaffold from complex environments while promoting their association into vascular networks and anastomosis with the vasculature of the host.

This review intends to provide an overview concerning clinical experience with integrin antagonists possessing anti-angiogenic effects. The reasons for failure to become approved therapeutic agents will be discussed. Despite these setbacks, potential applications of this type of ligands, namely peptidomimetics, in regenerative medicine therapies that aim to create optimal vascularized constructs capable of perfectly integrating with the circulatory system of the human body will also be addressed. To begin with, a brief clarification will be provided concerning the interaction between RGDs and integrins and the main features of peptidomimetic rationale design.

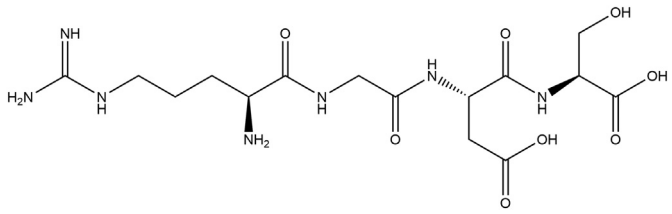

Fig. 3. Chemical organization of RGDS.

2. RGD-integrin interaction features and peptidomimetic design

2.1. Elucidation of the RGD-integrin interaction and peptidomimetic ligand advantages

Initially, the shortest molecular motif in fibronectin able of binding cells to ECM was defined by Pierschbacher and Ruoslathi as being Arg-Gly-Asp-Ser (RGDS) and its chemical structure is represented in Fig. 3 (Pierschbacher and Ruoslahti, 1984a, 1984b). Their pioneering work consisted of designing synthetic peptides endowed with this sequence and studying their effect on the adhesion of fibroblasts to surfaces displaying fibronectin. Soluble RGDS prevented fibroblast adhesion to the latter surfaces while promoting the adhesion of these cells when coated to sepharose beads. Moreover, it was proved that contrarily to the other three fragments, serine (Ser), was not essential to the bioactivity of the peptide and could be replaced by other amino acids (Pierschbacher and Ruoslahti, 1984a, 1984b). Pierschbacher and Ruoslathi also verified that the RGD sequence was also present in other proteins such as collagen type I, thrombin and fibrinogen and that these motifs had the same cell binding effect (Pierschbacher and Ruoslahti, 1984a; Pierschbacher and Ruoslahti, 1984b). These results encouraged efforts to understand if there existed a homology between different ECM proteins and the presence of RGD as the cell adhesion sequence. Indeed, the RGD sequence was discovered in laminin, von Willebrand factor, vitronectin and osteopontin and these observations put the tripeptide as a putative candidate as a universal cell adhesion motif (Grant et al., 1989; Oldberg et al., 1986; Plow et al., 1985; Suzuki et al., 1985). Curiously, this tripeptide also appears in snake venom disintegrins, a type of strong integrin inhibitors that can inhibit platelet aggregation and angiogenesis, with variable selectivity and potency towards integrins recognizing RGD (Gould et al., 1990; Swenson et al., 2007).

Notwithstanding, and despite possessing cell adhesion properties, RGD alone is unable of presenting cell-specificity and its effects are highly dependent on the conformation and spatial organization of the peptide (Ruoslahti and Pierschbacher, 1986; Trabocchi and Guarna, 2014). Complementing these features, peptides in the native state present poor pharmacokinetics, such as low metabolic stability, poor absorption after oral ingestion, rapid excretion, low diffusion in some organs and may have undesired effects due to off-target interaction with several other receptors (Giannis and Kolter, 1993). RGD, of course, is no exception and scientists have tried to improve both the biological activity and specificity of the peptide or peptide-like molecules through specific structural and functional modifications, whilst maintaining their bioactivity. This approach looks to biomolecules, in this case peptides, as a starting point to find new molecules with secondary structures, and additional fundamental structural characteristics analogous to the native peptide and are classified as peptidomimetic molecules (Trabocchi and Guarna, 2014). The final goal is to obtain a biomolecule with enhanced binding affinity towards a given receptor or target molecule. Additional advantages of peptidomimetic molecules are their extended biological activity, due to a smaller susceptibility to premature enzymatic degradation, and improved oral bioavailability (Olson et al., 1993). Therefore, these ligands have proved to be excellent cell adhesion inhibitors in their soluble form both *in vitro* and *in vivo*, even when ECM proteins are present (Henderson et al., 2013; Ravindranathan et al., 2013). Furthermore, due to their strong affinity towards integrins, in the nanomolar range, involved in cancer angiogenesis they are regarded as not only trackers of tumor vasculature but also inhibitors of tumor progression (Baum et al., 2015; Sartori et al., 2017) Additionally, RGD peptidomimetic-Paclitaxel conjugates, showing a low nanomolar affinity for the αvβ3 integrin receptor, were biologically evaluated in vitro and in vivo, and displayed a good targeting ability towards αvβ3-overexpressing cancer cell lines (Colombo et al., 2012; Dal Corso et al., 2015).

A fundamental advance towards a deeper understanding of the interaction between RGD and integrins was achieved by resolving the crystallographic structure of αvβ3 integrin (Xiong, 2002). Xiong and coworkers reported the crystal structure of the extracellular segment of this integrin complexed with cilengitide (a cyclic peptide presenting RGD and discussed in 4.5). This seminal study observed that Asp 218 and Asp150 interact with the guanidine of Arg through a salt-bridge, Asn215 and Ser121 establish hydrogen bonds with the Asp residue. The carboxylic group of this residue strongly interacts with the Mn^{2+} ion present at MIDAS (Metal-Ion-Dependent Adhesion Site) and the carbonyl group of Arg216 interacts with the amide proton of the Gly residue of RGD though hydrogen bonds. Furthermore, cilengitide includes an aromatic group able of establishing hydrophobic interactions with Tyr122 of αvβ3, thus showing that this might be an important feature to include in this type of molecules. Therefore, these findings helped establishing a general model of interaction between integrins and RGD, facilitating both the design of RGD peptidomimetics with enhanced affinity towards integrins and docking studies of these compounds.

2.2. Peptidomimetic rational design

Design of peptidomimetic ligands is focused in finding ways of mimicking the pharmacophoric elements of the original peptide. Therefore, for their proper development it is extremely important to have a profound understanding of both the peptide and its receptor and their electronic and three-dimensional conformational characteristics. Apart from this data, it is also important to meet some other considerations during the development phase of these biomolecules. Among these is the substitution of amide bonds if the biological activity remains untouched. These interactions can also be replaced if it is outside the zone of the active site. In the long term, the objective of these changes will be the substitution of the initial peptidic backbone with a non-peptidic one. Flexibility is also a very important feature to consider during the development of peptidomimetic molecules. However, this characteristic remains untouched during the development of first generation peptidomimetics as long as the molecule maintains its biological activity. Further refinements will include introducing elements that infer rigidity to side-chains of the new molecule in order to improve its initial bioactivity (Trabocchi and Guarna, 2014). Nevertheless, during the initial steps of peptidomimetic design it is preferable to preserve the side chains having biological relevance and possible adjustments to enhance activity are only included in second generation peptidomimetics. Normally, introduction of constraints into the biomolecule, chain length modifications and isosteric replacements are considered (Marshall, 1993). Moreover, the nature of the amino acid sequences flanking the bioactive sequence also impacts ligand specificity (Bochen et al., 2013). Another crucial factor to consider during the development of peptidomimetic ligands that intend to be coupled to materials lies on the utilized spacer unit. Thus, Pallarola et al. studied the effect of three different spacers [polyproline, aminohexanoic acid and polyethylene glycol (PEG)] and concluded that a simple variation on the spacer motif could determine higher or lower integrin binding affinity (Pallarola et al., 2014). A final concern during the development of peptidomimetics is to use the acquired knowledge of the three-dimensional bioactive conformation to rapidly develop the ideal peptidomimetic compound, without wasting time creating numerous molecules without relevant bioactivity (Trabocchi and Guarna,

2014).

The conversion of the initial peptide into a peptidomimetic compound is approached hierarchically, by introducing incremental chemical modifications that will additionally help elucidating the structure-activity relationship (Marshall, 1993). First, the biologically active peptide suffers alanine scanning and his biological activity is measured. As the name suggests this step is based on the substitution of different amino acids of the original peptidic sequence by alanine. Observing the bioactivity of the resulting molecule will help understanding if a given amino acid is biologically relevant or not. Therefore, if a key amino acid residue is replaced by alanine it will be observed a loss in bioactivity (Trabocchi and Guarna, 2014). Next in hierarchy is the reduction in size of the initial peptide (Marshall, 1993). With this methodology it is possible to assess the minimum sequence that interacts with the target. To achieve it a sequential removal of amino acids either from *N*- or *C*-termini and the subsequent biological activity measurement is done. Concretely, this process identifies the sequence bearing the pharmacophore (Trabocchi and Guarna, 2014). Afterwards, replacing amino acids of the parent peptide with D-amino acids and measuring the activity of the obtained molecule enlightens the structural organization of the biologically active conformation due to a change in the configuration and conformation of the side chains (Marshall, 1993; Trabocchi and Guarna, 2014). The clarification of the role each amino acid in the bioactive peptide has can also be done by creating *N*-methylated peptides. This approach creates a tertiary amide bond that contributes to a further understanding between conformation and bioactivity (Trabocchi and Guarna, 2014). Lastly, the bioactive conformation can be defined with the help of the insertion of local and/or global constraints because the initial peptide is in a loose conformation that presents low activity (Marshall, 1993).

Regarding to what specific characteristic a peptidomimetic molecule emulates, this can be fitted into three different categories: type-I, type-II and type-III mimetics (Trabocchi and Guarna, 2014). Type-I mimetics were the first peptidomimetic molecules to be described and mimic local topographic features of the native compound while still carrying all the features responsible for the interaction with the target molecule. It is frequent that these biomolecules match the peptidic backbone atom for atom by introducing isosteres into it (Ripka and Rich, 1998). Functional mimetics, or type-II mimetics, replicate the basis of the interaction between the native peptide and the target without concern for mimicking the structural arrangements of the initial molecule. When the first appeared, these peptidomimetics were thought to be equivalent to the original peptides in terms of structure, but characterization of both biomolecules found that they bind to different sub-sites in a large number of receptors (Sautel et al., 1996; Schwartz, 1994). Despite this, both types of peptidomimetics described are valuable resources to replace peptides with molecules possessing higher binding affinity or greater selectivity towards a given target, yet type-III mimetics are considered the ideal approach in designing peptidomimetic ligands. Such biomolecules present a scaffold with a different structure regarding the initial peptide and although they appear quite unrelated, they possess all the necessary groups in a well-defined spatial orientation to facilitate favourable molecular interactions. Thus, they are generally termed functional-structural mimetics (Ripka and Rich, 1998; Trabocchi and Guarna, 2014).

3. The role of integrins in angiogenesis

3.1. Brief overview of angiogenesis

Angiogenesis plays a fundamental part in fetal development, ovulation, wound healing and growth and development and is characterized as the formation of new blood vessels from pre-existing ones (Folkman, 1971). This process can be classified as sprouting angiogenesis or intussusceptive angiogenesis. As such, the former happens when ECs sprout from preexisting vessels, whereas the latter consists of the insertion of tissue pillars within capillaries to divide these vessels (Patel-Hett and D'Amore, 2011). Sprouting angiogenesis begins with the dissolution of the basement membrane and consequent detachment of pericytes from the capillary and is followed by the migration of ECs towards the extracellular space and consequent formation of an endothelial sprout. Then, downstream, the tip of the migrating edge ECs start to proliferate, the lumen of the endothelial sprout is formed and the sprout forms a closed looped with another vessel. In a final step, pericytes are recruited to the new vessel and the basement membrane involving it is formed (Carmeliet, 2000). Sprouting angiogenesis represents the process about which most information concerning angiogenesis has been gathered (Shiu et al., 2005). On the other hand, intussusceptive angiogenesis happens when endothelial walls of opposing sides of a vessel migrate towards each other and form an intraluminal pillar. Subsequently, the pillar suffers a central perforation, whereupon it is occupied with pericytes and myofibroblasts that will be responsible for the deposition of ECM. New pillars continue to form, increase in size, and finally merge to split the initial vessel into two different ones (Burri et al., 2004).

The main purpose of angiogenesis is to provide tissues with satisfactory amounts of nutrients and oxygen (Papetti and Herman, 2002). Logically, a major regulator of this physiological process is their oxygen concentration. Thus, when an oxygen deficit (hypoxia) within a tissue exists, growth factors and chemokines that will activate vascular growth and remodeling, are secreted (Fraisl et al., 2009). Acidic fibroblast growth factor (aFGF) and basic (FGF) represent two of these molecules. Both activate the production of matrix proteases in ECs which will breakdown their ECM and enable both the migration of these cells and the formation of capillary like tubes (Doi et al., 2007). One of the most important pro-angiogenic stimulators is vascular endothelial growth factor (VEGF). The production of this growth factor is up-regulated in cells with low oxygen amounts, and its effect is the proliferation and invasion of the hypoxic tissue by ECs. This grants the tissue new blood vessels which in turn will increase the oxygen level of its cells (Krock et al., 2011). Another growth factor involved in angiogenesis is platelet-derived growth factor (PDGF). PDGF is important in the maturation of blood vessels, mainly in their stabilization and integrity (Hellberg et al., 2010). Transforming growth factor-β (TGF-β) is a family of multifunctional cytokines involved in several types of cell behaviors in which angiogenesis is included. The effect of this cytokine in angiogenesis depends on its concentration. Therefore, at low concentrations, TGF-β upregulates pro-angiogenic factors, stimulates EC proliferation and migration and is also involved in the production of ECM proteinases. When this cytokine is present at high concentrations, it inhibits EC growth and acts both as a promoter of the reformation of the basement membrane and as a vessel stabilizer. Angiogenesis can also be modulated by angiopoietins (Ang-1 and Ang-2). Through the activation of a receptor of ECs named Tie-2, Ang-1 promotes the recruitment of pericytes and smooth muscle cells (SMCs) contributing to the stabilization and preservation of vascular integrity. Alternatively, Ang-2 acts as a competitor of Ang-1 for Tie-2 and its effect is the relaxation of the interactions between both pericytes and ECs and an enhanced degradation of ECM (Carmeliet, 2003).

3.2. Angiogenesis in cancer and the role of integrins in cancer growth and metastasis

Even though angiogenesis is a fundamental physiological event, it can also be nefarious in certain situations since it is essential in tumor growth and metastasis, making, therefore, this process paramount for their proliferation, spreading and infiltration within tissues (Carmeliet, 2005). Initially, tumors can survive by simply taking advantage of the available vasculature of their host and surroundings. However, tumor cells can become hypoxic if they grow beyond a distance from which both oxygen and nutrients can reach them (approximately 200 μm) (Nussenbaum and Herman, 2010). Thus, their response to hypoxia and

Table 1
Clinical trials of integrin inhibitors connected to tumor angiogenesis.

Integrins	Phase	Disease	Number of Patients	Dose	Results	Adverse events	Ref.
Vitaxin	I	Several stage IV tumors	14	0.1–4.0 mg/kg	Late reaction to the treatment: small decrease in tumor volume over 6 weeks with no sign of further tumor regression; Slow continuous tumor growth in a patient presenting metastatic breast cancer; 45% decrease in tumor volume for one patient with a leiomyosarcoma remaining stable for 93 weeks.	Generally good patient toleration Fever, chills, nausea and flushing (normal reaction against antibodies).	(Coleman et al., 1999) (Gutheil et al., 2000)
	II	Advanced leiomyosarcoma	15	0.25 mg/kg	No impact on the pathogenesis of this disease.	Well tolerated treatment.	(Patel et al., 2001)
	I	Several types of tumors	9	10 mg, 50 mg and 200 mg	No differences in clinical outcome for 50 mg or 200 mg doses; 10 mg group with worse outcome.	Normal reaction against antibodies.	(Posey et al., 2001)
Etaracizumab (Abegrin or MEDI-522)	I	Metastatic solid tumors	25	2, 6, 8 and 10 mg/Kg	3 patients with renal carcinoma remained stable for more than 34 weeks.	Grade 3 asymptomatic hypophosphatemia; Dose-liming toxicity in 2 patients (hypoxia and disseminated intravascular coagulation); One patient died from acute myocardial infarction.	(Wu et al., 1998) (McNeel et al., 2005)
	II	Metastatic melanoma	112	8 mg/Kg + 10000 mg/m^2 DCZ 8 mg/Kg	Only 2 patients remained in the treatment longer than 1 year; No differences between groups regarding the other clinical outcomes measured; Mean time to progression slightly higher for the therapeutic group.	Most frequent were infusion-related; One patient died due to pulmonary embolism; Serious cardiac disorders; Mild hypersensitivity reactions; Gastrointestinal events, most of which were not considered to be treatment-related; Grade 2 rectal hemorrhage.	(Peter Hersey et al., 2010)
Intetumumab (CNTO 95)	I	Advanced refractory tumors	24	0.1, 0.3, 1.0, 3.0, 10 mg/Kg	No maximum tolerated dose; Six patients had stable disease up until 9 months; One patient with reduction in tumor dimension presented a prolonged partial response.	Somnolence, vomiting, nausea, hematuria and abdominal pain; Grade 3 fever; Grade 3 thrombophlebitis.	(Mullamitha et al., 2007)
	II	Stage IV melanoma patients	127	5, 10 mg/Kg, 10 mg/Kg + DCZ, DCZ + placebo	Doubled 1 year survival rate for patients receiving highest intetumumab doses comparing to DCZ.	Grade 3 or 4 events for DCZ groups; Hematologic toxicity in DCZ groups; Grade 1 anemia for solo intetumumab; Low grade infusion reactions.	(O'Day et al., 2012) (O'Day et al., 2011)
	II	Castrate-resistant metastatic prostate cancer	131	10 mg/Kg + prednisone + docetaxel Placebo + prednisone + docetaxel	26% of placebo group and 21% intetumumab group abandoned treatment due to adverse events; 49 deaths; Better overall survival and progression-free disease for placebo group.	Grade > 3 Leukopenia, alopecia, neutropenia, diarrhea, fatigue asthenia, anemia, dysgeusia, paraesthesia, pulmonar embolism and pyrexia; Patients in the placebo group showed a bigger quantity of grade 3 or higher adverse effects.	(Chu et al., 2011)
Volociximab (M200)	I	Advanced solid neoplasia	21	0.5, 1, 2.5, 5, 10 and 15 mg/Kg	6 patients with benefits from the treatment; 1 had its metastasis reduced; Stable condition in 1 for 14months.	Grade < 2 fatigue and myalgias, nausea, headache, anorexia and fever.	(Ricart et al., 2008)
	II	Platinum-resistant advanced epithelial ovarian cancer; Primary peritoneal cancer	16	15 mg/Kg	No clinical advantages acquired from the treatment; 6 patients with disease progression after 1 cycle; 1 patient had disease progression following first cycle.	Grade 3 hyponatremia and pulmonary embolism; Grade 4 reversible posterior leukoencephalopathy syndrome; Grade 5 respiratory failure lead to death; Other lower grade adverse effects were arthralgia, fatigue, headache, nausea, vaginal bleeding and vomiting.	(Bell-Mcguinn et al., 2011)
Cilengitide	I	Recurrent malignant glioma	51	120 mg/m^2 incrementing to 600, then to 1200, 1800 and 2400 mg/m^2	2 patients achieved complete response to the treatment remaining without recurrence for 15 and 29 months;	Well tolerated and exhibits a very good safety profile; Grade 3 anorexia; Grade 4 intratumoral hemorrhage	(Nabors et al., 2007)

(*continued on next page*)

Table 1 (*continued*)

Integrins	Phase	Disease	Number of Patients	Dose	Results	Adverse events	Ref.
					3 patients with partial response; 16 patients with stable disease.		
	I/IIa	Newly diagnosed glioma	52	500 mg + chemotherapy and radiotherapy + TMZ	No response for patients without MGMT methylation; 15% improvement on the 6-month progression-free survival rate; 6 patients without signs of disease progression for at least 2 years; 19 patients discontinued treatment due to disease progression; 9 patients discontinued treatment due to adverse events.	No details about the adverse events observed.	(Reardon et al., 2008)
	III	Glioblastoma with MGMT methylation	545	2000 mg + TMZ + radiotherapy or TMZ + radiotherapy	No difference between groups which suggests no activity for cilengitide in the treatment of glioblastoma.	Frequent > Grade 3 thromboembolic events in the cilengitide group; Pulmonary embolism and aspiration pneumonia lead to death of patients; Most frequent treatment-emergent adverse events: headache, fatigue, constipation and vomiting.	(Stupp et al., 2014)

sub-nutrition is creating new blood vessels to fulfill their metabolic needs in a process similar to angiogenesis or by recruiting circulating bone marrow-derived endothelial progenitor cells (Lyden et al., 2001). In fact, several studies show that targeting tumor angiogenesis can stop the progression and metastasis of this disease (Ferrara and Kerbel, 2005).

Tumor angiogenesis is highly dependent on ECM disruption, the migratory capacity of ECs and their adhesion to integrins. As a natural outcome, integrins have been targeted as important actors in cancer angiogenesis (Desgrosellier and Cheresh, 2010). Accordingly, several integrins like αvβ3, αvβ5, α1β1 α2β1 α4β1, α5β1, α6β1, α6β4 and α9β1 have been implicated in angiogenesis (Avraamides et al., 2008). Despite the fact that all the former integrins have been implicated in angiogenesis, this section will only focus on those having a central role in tumor angiogenesis, namely: αvβ3, αvβ5 and α5β1 (reviewed in Bianconi et al., 2016 and Nieberler et al., 2017). Integrin αvβ3 is one of the most studied due to its role in cancer angiogenesis regulation (Nussenbaum and Herman, 2010). Its role during angiogenesis is to bind and activate MMP-2 at the migration tip of new blood vessels to disrupt ECM and facilitate their migration and infiltration. These findings put αvβ3 in the spotlight as a fundamental tumor angiogenic promotor. In accordance, αvβ3 antagonists could inhibit tumor growth in several cancer animal models (Brooks et al., 1995; Brooks et al., 1994a, 1994b). Contrarily to αvβ3, integrin αvβ5 promotes angiogenesis through a pathway involving VEGF and not bFGF (Friedlander et al., 1995). VEGF is a growth factor known for increasing vascular permeability, constituting a promotor for tumor metastasis (Weis et al., 2004). Thus, the expression of this integrin was found both in glioma cells and their vasculature, implicating it in the angiogenesis of glioma (Bello et al., 2001). Integrin α5β1 is another cell adhesion receptor implicated in tumor angiogenesis. The expression of this integrin is augmented in both the endothelium of mice and humans during tumor angiogenesis and its inhibition resulted in the repression of tumor angiogenesis and growth on animal models (Kim et al., 2000). Similarly to αvβ3, the expression of α5β1 is not induced by VEGF, but by other pro-angiogenic molecules like bFGF and IL8 (Boudreau and Varner, 2004).

Taken together, these experimental findings confirmed the potential of targeting specific integrins during tumor angiogenesis and set the ground for the development of ligands to inhibit them. A selection of clinically-tested molecules will be discussed in the next section of this review.

4. Clinical trials involving integrin ligands to inhibit tumor angiogenesis

Once the role of integrins as key mediators during tumor angiogenesis was established, several research groups started developing specific compounds targeting these cellular receptors. For this task, three different types of molecules have been used, specifically, monoclonal antibodies, RGD-containing ligands and peptidomimetics (Curley et al., 1999; Marelli et al., 2013). The objective of this therapeutic approach is straight-forward, since by inhibiting tumor vasculature, the flow of nutrients and oxygen to tumor cells is disabled, which in turn would incapacitate their growth and expansion. Contrarily to standard cancer treatments like chemotherapy, which may also indiscriminately damage healthy tissues and cause severe undesired secondary effects, integrin antagonists represent a specific means of directly targeting tumor processes without provoking non-specific interactions and side effects (Marelli et al., 2013).

The use of antibodies against specific integrins started when Cheresh et al. observed that immunoprecipitation of αvβ3 was possible by using a monoclonal antibody of M21 melanoma cells (Cheresh et al., 1987). Moreover, LM609 decreased the invasiveness and growth capacity of melanoma, lung, pancreas and larynx carcinomas implanted on CAM (Brooks et al., 1995; Brooks et al., 1994a, 1994b). These results serve as proof of concept to the possible utility of inhibiting tumor

angiogenesis with specific integrin ligands. However, the clinical viability of LM609 is limited due to its murine origin, thus raising the concern of possible immunogenicity when administered to humans. Therefore, humanization of this antibody by phage display originated vitaxin (MEDI-522) which had more suitable characteristics for human testing (Rader et al., 1998). The following section of the review intends to give a general overview about the clinical trials undertaken with molecules targeting integrins involved in angiogenesis. Clinical outcomes and adverse events found during these studies are summarized in Table 1.

4.1. Vitaxin (MEDI-522)

Initial studies with this antibody undertaken by Coleman et al. showed an inhibition of EC proliferation and angiogenesis, following a balloon injury in rabbits. Furthermore, vitaxin showed an increased antiangiogenic effect when compared to other integrin ligands, possibly due to its affinity towards αvβ3 (Coleman et al., 1999). This study proved the validity of vitaxin to inhibit angiogenesis and so this monoclonal antibody was evaluated in several clinical trials (Gutheil et al., 2000; Patel et al., 2001; Posey et al., 2001). Despite generally having a good safety profile, vitaxin proved unsuccessful changing the clinical outcome of the enrolled patients. Thus, only one patient diagnosed with a leiomyosarcoma metastatic to the liver presented a partial response to the treatment. Even though after almost 2 years the size of his measurable lesions continued stable, the tumor had progressed into the gastrointestinal system which motivated treatment cessation (Gutheil et al., 2000). The use of ^{99m}Tc-vitaxin as an imaging agent for tumor vasculature was also unsuccessful, being able to locate the tumor in just one melanoma patient. Thus, the authors correlate this failed attempt not only to the low doses applied (1 mg) but also to *in vivo* limitations in both the affinity and stability of the ^{99m}Tc label (Posey et al., 2001).

4.2. Etaracizumab

The idea of creating another humanized derivation of LM609 but increased affinity towards αvβ3 lead to the development of etaracizumab (Wu et al., 1998). This antibody proceeded to Phase I and Phase II clinical studies, either alone or in combination with FDA-approved chemotherapeutic agents, but without any significant results (Peter Hersey et al., 2010; McNeel et al., 2005). The action of etaracizumab alone did not provide any response to the treatment, but three patients having metastatic renal cancer presenting and evidences of disease progression before the clinical trial remained stable for more than 8 months (McNeel et al., 2005). Etaracizumab proved also to be ineffective when applied in combination with dacarbazine (DCZ) in metastatic melanoma patients. All the treatment responses were partial and for the combinatorial group. Generally, Hersey et al. did not observe any beneficial effects of adding etaracizumab to DCZ as most of were similar to DCZ action alone (Hersey et al., 2010). From this point onwards, interest in late-stage clinical evaluation was discontinued.

4.3. Intetumumab (CNTO 95)

Intetumumab is a human monoclonal antibody specific for both αvβ3 and αvβ5 that showed inhibition of tumor growth and angiogenesis *in vitro* and *in vivo* (Trikha et al., 2004). Like etaracizumab, intetumumab was clinically evaluated alone (Mullamitha et al., 2007) and in combination with DCZ (O'Day et al., 2011, 2012) and docetaxel and prednisone (Chu et al., 2011; Heidenreich et al., 2013). When tested alone, only one patient with angiosarcoma showed a prolonged partial response to the treatment, but after 10.5 months the tumor progressed (Mullamitha et al., 2007). The use of this antibody in combination with DCZ did not show a substantial impact on the progression of patients with melanoma concerning progression-free survival. However, it seemed to exist a tendency towards increased overall survival for patients in the highest intetumumab dose (O'Day et al., 2011, 2012). The combined use of intetumumab in combination with docetaxel and prednisone showed some potential in a Phase I clinical trial with castrate-resistant metastatic prostate cancer patients (Chu et al., 2011). However, a subsequent Phase II study in patients diagnosed with the same malignancy came into a premature end when the interim analysis revealed a tendency towards better progression-free disease and overall survival for the placebo group (Heidenreich et al., 2013). Altogether, these results led to discontinuation of the development of intetumumab as a possible therapeutic agent against cancer.

4.4. Volociximab (M200)

Volociximab is a IgG4-type chimeric monoclonal antibody with specificity towards α5β1. Preclinical data in a three-dimensional fibrin matrix showed its ability of inhibiting human umbilical vein endothelial cells (HUVECs) of forming tube-like structures independently of the administration of growth factors. When translated to animal models, volociximab showed the same anti-angiogenic properties in severe combined immunodeficient mice and cynomolgus monkeys (Ramakrishnan et al., 2006). These promising results naturally created interest in applying volociximab as an anti-angiogenic agent in human clinical trials. Therefore, this antibody was studied in patients having advanced solid neoplasia (Ricart et al., 2008), and platinum-resistant advanced epithelial ovarian cancer and primary peritoneal cancer (Bell-Mcguinn et al., 2011). The former, a Phase I study, led to the reduction in the metastasis of a patient presenting renal cell carcinoma and stable disease during 4 months and to stable condition of a patient with melanoma with visceral metastasis remained stable for 14 months (Ricart et al., 2008). A Phase II clinical trial in patients diagnosed with platinum-resistant advanced epithelial ovarian cancer or primary peritoneal cancer had unsatisfactory results regarding impact on disease progression. Consequently, only one of the patients completed the full treatment, but disease progression occurred after finishing it (Bell-Mcguinn et al., 2011). Therefore, this antibody did not present any therapeutic advantage and no clinical trial using volociximab is currently active and it seems that the interest in this antibody has weakened.

4.5. Cilengitide

Even though several ligands containing RGD, other peptides of interest (ATN-161) or mimicking the action of RGD have been developed, very few candidates have proceeded to clinical trials. Cilengitide was the first ligand mimicking the action of RGD to be reported and the one that progressed further.

Developed by the group of Horst Kessler, cilengitide is a N-methylated cyclic RGD molecule (Fig. 4) with subnanomolar activity for αvβ3 (0.58 nM) and nanomolar affinity towards αvβ5 (11.7 nM) and α5β1 (13.2 nM) (Dechantsreiter et al., 1999). Preclinical studies showed that this ligand influenced cellular adhesion to αvβ3 and exponentiated apoptosis of cells expressing both αvβ3 and αvβ5, whilst actively reducing tumor angiogenesis and retarding its growth and metastasis *in vivo* (Buerkle et al., 2002; MacDonald et al., 2001; Taga et al., 2002). Cilengitide showed potential to treat brain tumors in two Phase I studies where two patients having malignant glioma showed complete response to the treatment and one patient with refractory brain tumor presented the same outcome (MacDonald et al., 2008; Nabors et al., 2007). In addition, three glioma patients had partial responses with a mean duration of 9.3 months (Nabors et al., 2007). Cilengitide then progressed to Phase II clinical trials, either alone or in combination with chemotherapy agents, where it continued to show promising results (Fink et al., 2010; Gilbert et al., 2012; Stupp et al., 2010). Specifically, a Phase I/IIa clinical trial showed that the addition of cilengitide to TMZ/

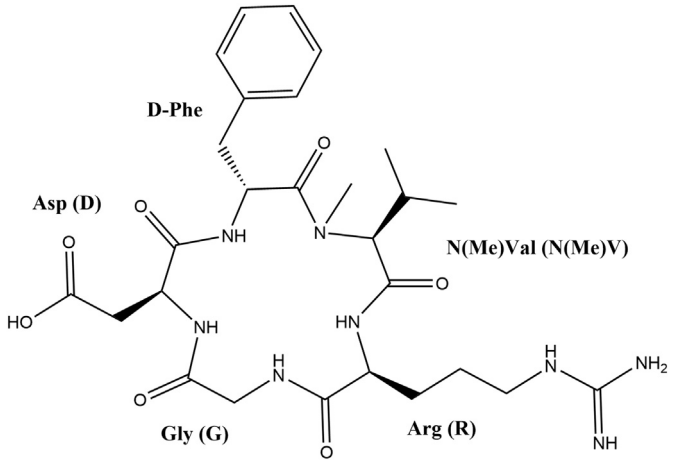

Fig. 4. Chemical structure of cilengitide [c(RGDf(NMe)V)]. This RGD-containing cyclic molecule has subnanomolar activity for αvβ3 (0.58 nM) and nanomolar affinity towards αvβ5 (11.7 nM) and α5β1 (13.2 nM). Cilengitide reached Phase III clinical trials for the treatment of glioblastoma but failed to have a significant impact on the outcome of this pathology.

radiotherapy resulted in six patients without signs of disease progression for a period of at least 2 years (Amarouch and Mazeron, 2005). Still, genetic analysis connected this improvement to patients with methylation of the promoter O^6-methylguanine-DNA methyltransferase (MGMT), a fact corroborated by another Phase II study (Amarouch and Mazeron, 2005; Reardon et al., 2008). These results motivated a Phase III clinical trial applying cilengitide, complemented by TMZ/radiotherapy, in glioblastoma patients having methylated MGMT. Contrarily to the previous reported studies, cilengitide showed no influence regarding its impact on cancer progression (Stupp et al., 2014). A probable reason could reside on the dose regimen but a Phase II study with a more intensive cilengitide regimen in individuals without MGMT methylation showed no significant differences between experimental and control groups (Nabors et al., 2015). Recently, it was found that inhibiting α5β1 activates a p53-dependent apoptotic pathway in glioma cells and that single glioma cell migration could be inhibited by α5β1 antagonists and not by αvβ3 antagonists (Ray et al., 2014; Renner et al., 2016). Hence, the lower affinity of cilengitide towards α5β1 could also provide an explanation for the lack of clinical efficacy of this molecule on glioblastoma. Paradoxically, different studies have showed that low doses of cilengitide stimulate angiogenesis, due to an agonistic effect on αvβ3 that leads to the stabilization of the vascular system (Hodivala-Dilke, 2008; Reynolds et al., 2009; Wong et al., 2015). This effect is due to the activation of the resting state heterodimeric integrin (the first step in integrin signaling). Thus, binding affinity to the ligand increases during the homo-oligomerization of integrin subunits but the blocking of cell adhesion or integrin activation requires the utilization of higher concentrations (Zhu et al., 2013). Together with the lower affinity of cilengitide against α5β1, these findings aid to explain its unsuccessful performance in the Phase III glioblastoma trial. Moreover, the agonistic effect cilengitide presents in low concentration regimens opens new applications to the RGD-based molecule. Wong et al. utilized this feature and took advantage of it to improve the delivery of chemotherapeutic drug Gemcitabine into pancreatic cancers in mice, reducing its growth, metastasis and the side effects associated to the drug while increasing animal survival (Wong et al., 2015). Even though tumor angiogenesis is highly correlated with their proliferation and spreading, this result is particularly interesting and shows that angiogenesis can be used in favor of possible experimental treatments, having cilengitide an important role on their success.

Although possible explanations for Cilengitide failure are numerous, this RGD-containing molecule represents by far up until now the integrin-targeting antiangiogenic agent with better perspective to translate into an established therapeutic agent. Adding to this, αvβ3 is expressed on melanoma, prostate, cervical, breast and pancreatic carcinoma cells which opens the potential application of cilengitide to these tumors. In fact, this molecule has been applied in several Phase I and II studies of these diseases but with no success (Alva et al., 2012; Kim et al., 2012; Manegold et al., 2013; Vansteenkiste et al., 2015; Vermorken et al., 2011).

To conclude this section, angiogenesis is a well-established hallmark of cancer and integrins are deeply involved on it. Therefore, targeting it can still be a potential approach to decrease cancer metastasis and progression. However, solely targeting angiogenesis might not be sufficient to detain the pathogenesis of cancer. Discovering possible biomarkers for tumors and then developing ligands able of only being cytotoxic towards tumor cells could prove to be a good complement to the use of molecules with high affinity towards integrins involved in angiogenesis. On this front, peptide-like ligands seem to provide the best solution as they have a safer profile and improved clinical activity than antibodies. Despite this promise, a peptide-like molecule with confirmed impact on tumor vasculature and pathogenesis seems to be still distant and so their development should face towards other areas. Consequently, regenerative medicine approaches are highly dependent on vascularization or other cellular responses that promote regeneration rather than repair. Therefore, the use of integrin-specific ligands could be extremely useful. The next section of the review intends to highlight the high potential that peptidomimetic ligands targeting proangiogenic integrins have on the improving of the vascularization of regenerative medicine constructs.

5. Importance of vascularization in Regenerative Medicine therapeutics: specific integrin-targeting biomaterials as a promising solution

5.1. Vascularization strategies in Regenerative Medicine

Regenerative medicine aims to provide therapeutic solutions to replace or regenerate damaged tissues or organs, presenting itself as an alternative to organ transplantation (Kim et al., 2016). Despite all the problems arising from tissue and organ complexity, mainly due to their intricate cellular organization and the complex interaction between cells, their natural environment and endogenous or exogenous stimulus, it remains paramount to engineer an efficient network to provide these therapeutic approaches with appropriate nutrients and oxygen *in vivo* (Rouwkema et al., 2008). In fact, prior to implantation these needs can be fulfilled by using perfusion bioreactors. However, after *in vivo* implantation the constructs will need to integrate into the host vasculature to reestablish the correct influx of nutrients to its cells. Usually, during the foreign body reaction, vascular networks are able of perfusing the implant but this event takes too long. Consequently, cells in the middle of the engineered tissue will not have appropriate access to nutrients, or become hypoxic, and may die which will impair a proper *in vivo* integration of the scaffold (Butt et al., 2007).

A natural solution to this problem is adding a vascular network to the construct before implantation, therefore accelerating its perfusion. Pre-vascularized engineered tissues can be quickly perfused with blood by inosculation with the host vasculature (Laschke et al., 2009) or by surgical anastomosis of feeding and draining blood vessels (Beier et al., 2009; Eweida et al., 2011). Such vascular networks must be highly branched in such a way that no cell is further than 200 μm from a vessel as this is broadly considered as the diffusion limit for oxygen and nutrients in tissues (Jain et al., 2005). In addition, this vascular network should be able to behave like a selective barrier to control the passage of materials from the vessels to their surrounding tissue. This will prevent an excessive drainage of fluid which otherwise would lead to

tissue edema (Rouwkema and Khademhosseini, 2016).

5.1.1. Cell seeding

In vitro pre-vascularization can be done by several techniques but the most often used is the cell seeding approach. Here, cells that will form the future vessels are seeded onto scaffolds either engineered separately or consisting of natural decellularized ECM. The use of decellularized tissue has the advantage of promptly having available the intricate 3D architecture of the vascular system (Ott et al., 2008; Song and Ott, 2011). Therefore, cells can be directly delivered into the channels that were, and will become again, the vascular network of the tissue before decellularization.

Several studies of scaffold pre-vascularization have depended on the ability of ECs to spontaneously organize and form vascular networks (Chen et al., 2009, 2012; Levenberg et al., 2005). ECs start by forming a primitive network on the initially avascular scaffold in a similar process to vasculogenesis. Then, ECs further organize in a similar way to angiogenesis. Even though these cells are able of assembling into complex networks, often without addition of growth factors or specific cues, culture conditions and the type of cells used during co-culture with ECs are of extreme importance. Thus, these factors can influence the morphology of the newly formed vascular network. Therefore, depending on these, the obtained network will vary from immature and possessing limited amounts of lumen to more mature networks with well-developed lumen (Chen et al., 2012; Kunz-Schughart et al., 2006). Levenberg et al., showed that culturing mural precursor cells like embryonic fibroblasts and mesenchymal stem cells (MSCs) with ECs helps maturating the network and its stabilization (Levenberg et al., 2005). This is also reflected by an increased vessel lumen, which will augment the quantity of blood that can be delivered to the tissue. In addition, these cells help regulating vascular permeability which results in less fluid being leaked into the tissue and lower interstitial fluid pressure (Goel et al., 2011). Pericytes are other type of cells with beneficial action towards stabilization of newly-formed endothelial tubes (Saunders et al., 2006). Stratman et al. proved that the crosstalk between ECs and pericytes induces ECs to deposit ECM proteins like collagen type IV, laminin and fibronectin contributing in turn to stabilize the vascular network (Stratman and Davis, 2012). Koike and coworkers also proved that mural cells are fundamental for obtaining stable vascular networks (Koike et al., 2004). In this seminal study, co-cultures of HUVECs and mural precursor cells developed into stable vascular networks that lasted for periods up to one year *in vivo*. In contrast, constructs engineered with HUVECs alone showed minimal perfusion and disappeared after 60 days. Even though, as mentioned, after seeding HUVECs form vascular networks and can be perfused upon implantation, these cells are difficult to harvest in large amounts under clinical conditions and have limited proliferation during the culture phase. Furthermore, this type of ECs are heterogeneous and present several different features depending on the organ from which they were harvested (molecular permeability, homeostasis, immune tolerance, angiogenic potential and vascular tone) (Aird, 2007; Baldwin et al., 2014). Despite these disadvantages, HUVECs are frequently used in *in vitro* studies. Contrarily to most ECs, HUVECs are available for extraction from unwanted umbilical cords, are easy to obtain and present an interesting expansion profile which make them an attractive source of ECs. Unfortunately, HUVECs often render unstable vessels and their transplantation is capable of inducing an immune response from the host (Baiguera and Ribatti, 2013). Several studies demonstrated that endothelial progenitor cells (EPCs) represent a promising cell population to be used on prevascularized scaffolds (Aronson et al., 2012; Duttenhoefer et al., 2013; Guerrero et al., 2013; Serrano et al., 2011; Sobhan et al., 2012). These cells represent a small population of circulating $CD34^+$ cells with the capacity of accomplishing phenotypical features of ECs *in vitro* (Asahara et al., 1997; Finkenzeller et al., 2007). Importantly, EPCs have higher proliferative potential than ECs and are easily obtained (Sales et al., 2006). Accordingly, these cells circulate in peripheral blood and can be obtained from it by non-invasive procedures. Moreover, EPCs are also present in blood from the umbilical cord, another source of high concentrations of these progenitor cells (Hristov et al., 2003; Murohara et al., 2000). Considering the time they take to appear after being cultivated *in vitro*, EPCs can be divided into two distinct groups. Thus, early EPCs appear less than 1 week after culture whereas late EPCs take 2 to 4 weeks to appear and present a cobblestone-like morphology (Asahara et al., 1997). Late EPCs are the most interesting for the development of pre-vascularized constructs since they are able of differentiating into ECs and form capillary-like structures. On the other hand, early EPCs have an indirect action towards vessel formation. Thus, by secreting angiogenic growth factors, this type of EPCs has a paracrine action on angiogenesis (Hur et al., 2004). Additional cell types with capacity to achieve complex vascular networks in scaffolds include MSCs (Almalki et al., 2017; Hsieh et al., 2016; Miranville et al., 2004; Pill et al., 2015), induced pluripotent stem cell-derived endothelial cells (Belair et al., 2015) and amniotic fluid-derived stem cells (Benavides et al., 2015; Verseijden et al., 2010b).

5.1.2. Spheroids

Spheroids represent another way of producing pre-vascularized constructs (Fig. 5) (Mishra et al., 2016; Rouwkema et al., 2009; Verseijden et al., 2010a). These cell aggregates are formed by self-assembly and can be obtained *in vitro* when cells are unable of attaching to a surface and consequently have to interact with each other (Laschke and Menger, 2017). Since spheroids represent 3D cellular structures, their organization resembles what is found physiologically. Moreover, these aggregates present high concentrations of cell-to-cell contacts, cell-matrix interactions and produce high amounts of growth factors. In addition, cells within spheroids are more resistant to hypoxia and apoptosis and have enhanced differentiation potential when compared to 2D cell culture (Bhang et al., 2012; Yoon et al., 2012). Therefore, these features make them particularly interesting to be studied as potential promoters of scaffold vascularization. Recently, Mishra et al. utilized a poly(propylene fumarate)/fibrin hydrogel to coculture HUVEC/human MSCs (hMSCs) and develop a pre-vascularized scaffold for bone regeneration (Mishra et al., 2016). These authors proved that allowing the spheroid cells to organize into vascular networks before *in vivo* implantation improves the connection within the vasculature of the host. In a different study, Laschke and coworkers proved that adipose-derived stem cells (ASCs) spheroids seeded into polyurethane scaffolds

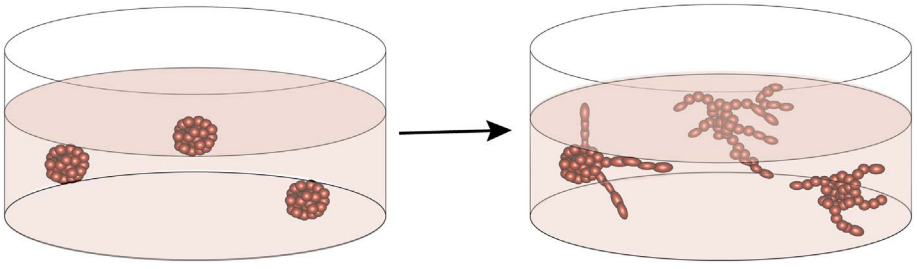

Fig. 5. Representation of the use of spheroids to develop pre-vascularized constructs. After seeding, these cell aggregates start to migrate and ultimately develop into defined vascular structures.

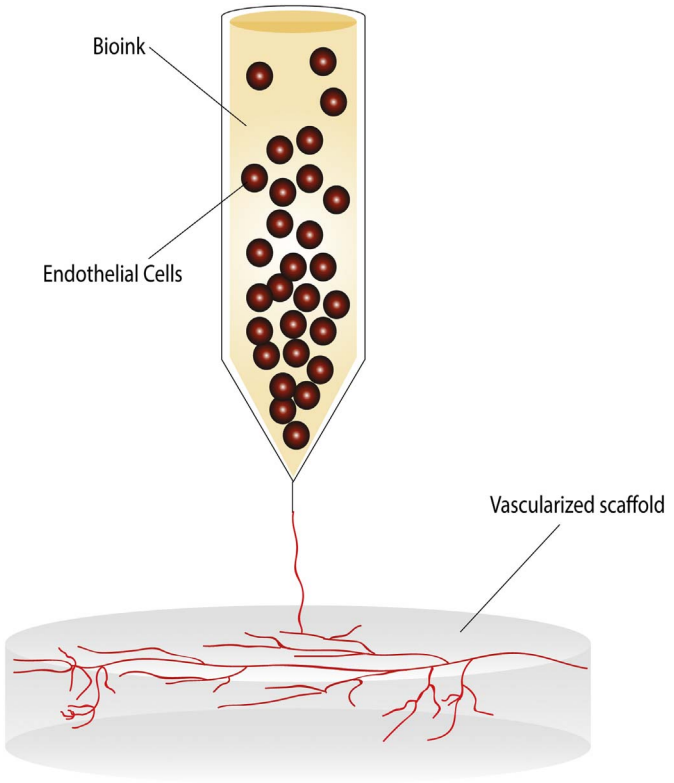

Fig. 6. Illustration of the creation of a vascularized construct by bioprinting. This technique enables the development of discrete vascular networks using additive manufacturing.

are capable of initiating blood vessel formation. Upon implantation in an animal model, this type of MSCs induced a strong angiogenic host tissue response which resulted in improved scaffold vascularization and high functional microvessel density (Laschke et al., 2013).

5.1.3. Bioprinting

3D bioprinting has enormous potential in the development of pre-vascularized structures. The application of this technique allows precise control over the location of cells in spatially defined locations within 3D environments (Mandrycky et al., 2015). Therefore, the complex architecture of a vascular network can be both addressed and controlled by directly designing it onto a scaffold. Additionally, scientists can exactly control the cellular densities of the newly patterned vessels and organize different cell types to mimic their natural assembly in blood vessels. Bioprinting is able of forming 3D vascular networks and structures by additively depositing cell suspensions containing vascular cells inside, or not, an appropriate matrix (bioink) (Fig. 6) (Jakab et al., 2006). These networks can be engineered by discretely depositing cells as droplets or spheroids adjacently to each other in the intended form. Creating a vascular network using this type of bioprinting relies on the capacity of spheroids to spontaneously join and self-assembly into blood vessels. The other way of engineering a pre-vasculature using bioprinting is by direct-writing, where cells are continuously administered as vascular cell suspensions inside an appropriate matrix. Thus, using this type of bioprinting, a vasculature more similar to the one to be replaced/needed can be obtained (Hoying and Williams, 2015). The potential of bioprinting was demonstrated in a study by Norotte *et al* (Norotte et al., 2009). These authors utilized several types of vascular cells aggregated into separated units (either multicellular spheroids or cylinders) to print layer-by-layer vessel-like structures with agarose rods as molding template. During post-printing these cellular structures started to aggregate and developed into fully biological vascular tubular structures. Using a different approach Cui and coworkers developed a 3D polylactic acid (PLA) bioprinted vascularized bone construct having a fully interconnected microvascular network that mimicked native bone (Cui et al., 2016). This innovative scaffold was then subjected to several surface modifications to optimize its capacity towards cell adhesion and smart release of growth factors. Afterwards the authors repopulated the vasculature of the scaffold with hMSCs and HUVECs under different culture conditions. Interestingly, these authors were able of modulating angiogenesis and osteogenesis through the delivery of specific growth factors entrapped on the surface of the construct with spatiotemporal coordination.

5.2. The use of molecule cues to enhance vascularization

5.2.1. Growth factors

As discussed in the section dedicated to angiogenesis, this process is able of being controlled using specific molecular cues. Therefore, it is not surprising that a great volume of research regarding the development of vascular networks in regenerative medicine constructs takes advantage of these molecules to promote vascularization. Of all these molecules, VEGF is the one which is present in most angiogenic processes. Several authors have demonstrated that better results are achieved when distinct gradients of this molecule exist within scaffolds, granting endothelial cell elongation and branching in a spatially-driven way (Bigalke et al., 2014; Poldervaart et al., 2014). Nevertheless, angiogenesis is a process with different phases in which different molecules take part. Therefore, to further enhance vascularization and have a more extensive impact on angiogenesis it is interesting to combine VEGF with other molecules involved in distinct phases of this process (Kakudo et al., 2017; Rufaihah et al., 2017; Shin et al., 2011; Sun et al., 2011). Even though the combination of growth factors provides satisfactory outcomes, it is difficult to understand their correct distribution within the construct and their availability over time. Perhaps the most complete way of inducing angiogenesis is to combine the use of these growth factors with molecules that induce their cellular expression. This way cells regulate the secretion of growth factors. It also helps promoting the formation of growth factor microgradients and also offers the possibility of expressing different growth factors at the same time, thus impacting blood vessel formation and maturation (Baiguera and Ribatti, 2013). Sonic hedgehog (Shh) is a strong tool in indirect modulation of angiogenesis. Thus, this morphogenic agent induces cells to express VEGF1, Ang-1 and Ang-2, increasing their concentration and this originates large-diameter vessels *in vivo* (Pola et al., 2001). In accordance, Rivron and coworkers took this concept and applied it for inducing the organization of a capillary network in an artificial tissue for bone regeneration (Rivron et al., 2012). In this work, vascular networks resulting from the co-culture of hMSCs and hHUVECs aggregates originated primitive 3D networks, composed of cord-like vascular structures with some lumens. In contrast, the addition of Shh to the culture media promoted vessel maturation while increasing their lumen. This led to a more functional and stable vasculature after *in vivo* implantation. Once again, the importance that having a robust vascular network can define the success of regenerative medicine approaches is proved, as only the implants exposed to Shh (having more mature and robust vascular networks) contributed to the formation of mature bone.

5.2.2. RGD-containing materials as promoters of cell adhesion and vascularization

Commonly, typical materials used in regenerative medicine approaches towards engineering vascular networks like alginate, PEG and poly-(L-lactic acid) (PLLA) are bioinert. Therefore, numerous studies produce materials that mimic the mechanisms behind the natural cell-ECM interaction. Logically, one way of creating materials promoting cell adhesion and proliferation is by functionalizing them with RGD (Chwalek et al., 2011; Hadjizadeh and Doillon, 2010; Oliviero et al., 2012; Wang et al., 2014). By utilizing the tripeptide, researchers desire mimicking the natural processes of angiogenesis and render their

biomaterials with more suitable properties for the development of matured vascular networks. Thus, Bidarra et al. utilized RGD-modified alginate hydrogels to culture HUVECs and proved that this modification improves the biofunctionality of this polymer (Bidarra et al., 2011). Indeed, these hydrogels seemed to constitute a suitable microenvironment for HUVECs. These cells maintained their viability and migrated to the outside of the matrix constituting primitive tube-like structures. In comparison, on unmodified alginate hydrogels cells quickly lost their viability and could not migrate to the outside of the artificial matrix. Additionally, RGD stimulated the production of MMP2 a type of MMP deeply involved in endothelial cell migration during angiogenesis. Biofunctionalization of otherwise bioinert materials, however, it is not sufficient *per se* to originate fully functional, matured, vascular networks. Therefore, to increase the angiogenic potential of these approaches the best option is to combine the inclusion of RGD into biomaterials with the administration of angiogenic growth factors. Designing smart materials that upon degradation are capable of releasing growth factors further stimulating cells to provide an angiogenic response, assembling and organization into vascular networks seems promising. In accordance, Phelps and coworkers used this concept developing a PEG hydrogel functionalized with RGD, presenting both MMP-sensitive spots and VEGF within it (Phelps et al., 2010). These matrices were able of sustainably deliver steady rates of this growth factor during approximately 2 weeks. On the other hand, the administration of soluble VEGF to PEG hydrogels without the growth factor lead to a 90 % decrease of its initial quantity over the same time. Subcutaneous rat implants of the hydrogel containing RGD, MMP-cleavable sites and VEGF significantly enhanced vessel ingrowth by 2 weeks and increased vasculature at 4 weeks while presenting good perfusion. On the contrary, hydrogels without VEGF or RGD presented minimal tissue invasion and the non-degradable matrix did not integrate into the host tissue. The appropriate rate of degradation of the construct is also important. In accordance, Chwalek et al. developed similar hydrogels to the previous reported study but with distinct degradation profiles (Chwalek et al., 2011). These differences were due to the inclusion of either a stable amine bond at physiological pH or an ester bond that slowly degrades in these conditions. Furthermore, these authors designed hydrogels of both types with variable elasticity and RGD density with the purpose of studying the impact of these factors in ECs growth. *In vitro* assays showed that cells growing on faster degrading materials were more prone to develop into cord-like structures and expand. Cellular invasion depth was greater for smaller RGD concentrations and higher concentrations of VEGF promoted the formation of cord-like structures. Additionally, increasing the stiffness of the hydrogels also reduced their invasion depth. These authors also studied the capacity of these constructs to induce vascularization *in vivo* in a CAM model. As in the *in vitro* studies, fast releasing VEGF hydrogels obtained the best result regarding vascularization, being in fact comparable to Matrigel® in the same model. Culver et al. demonstrated that creating specific RGD patterning within hydrogels can direct cells to create vascular networks that are similar to organs or structures of the human body. By using two photon laser scanning lithography (TP-LSL) the authors created a pattern of RGD into PEG hydrogels resembling the vascular network of mouse brain cortex (Culver et al., 2012). These materials became biodegradable by incorporating a MMP-sensitive peptide (GGPQGIWGQGK) into the backbone of the hydrogels. Then, to understand if cells would organize and form a vessel network corresponding to RGD distribution, HUVECs and mesenchymal progenitor cells were encapsulated into the patterned hydrogels. Remarkably, these cells organized into complex tubule networks in 92.3 % of the patterned PEG-RGD showing the potential of including RGD-functionalized vascular networks into the design of hydrogels. Although this methodology is somewhat complex, the evolution of techniques like bioprinting may take advantage of similar approaches to develop better bio-instructive materials. Weinandy et al. developed PLLA fibers biofunctionalized with RGD to guide vascular formation *in vitro*. HUVECs and human foreskin fibroblasts (HFFs) were then seeded onto the fibers and afterwards embedded in a fibrin gel (Weinandy et al., 2014). This co-culture originated capillary-like networks with defined lumen adjacent to the polymer fibers but not throughout the entire vascular network. Nevertheless, these capillary-like structures remained for at least 21 days of co-culture and this study demonstrated the usefulness of electrospun nanofibers as guidance for vasculature formation. Perhaps the incorporation of growth factors should have been considered to originate a more mature vascular network, at least outside PLLA fibers.

These examples show the utility of RGD-containing constructs in different applications that intend to promote vascularization in regenerative medicine applications. However, the use of synthetic RGD peptides in biomaterials has some limitations. Consequently, these peptides do not have the same potential as full ECM proteins to promote cell adhesion due to the absence of synergistic domains present in native proteins that promote optimal cell signaling. Also, the conformation of these peptides is often not the most appropriate and they do not exhibit selectivity towards a desired integrin. Finally, both linear peptides and large cyclic peptides can be easily degraded *in vivo*. Still, the use of entire ECM proteins presents several disadvantages including very limited biological half-life, induction of inflammatory responses, fast clearance and risk of infections (von der Mark and Park, 2013).

6. The potential of peptidomimetic ligands with integrin selectivity for proper development of vascular networks in regenerative medicine methodologies

To counteract the drawbacks mentioned in the preceding section, some groups have used different RGD-containing molecules like small cyclic RGD peptides and protein fragments. Notwithstanding, this type of ligands fail to target specific integrins, which represents a disadvantage if the intention is to guide cells towards a response directed by a defined integrin. Other approaches employ biomaterials functionalized with combinations of different peptidic ligands that exhibit integrin specificity. Normally, these mixtures do not fulfil their potential due to difficulties in controlling their spatial arrangement (Mas-Moruno et al., 2016). Developing peptidomimetic ligands with selectivity for specific integrins thus seems an interesting approach to enhance the angiogenic properties of biomaterials for regenerative medicine purposes (Fig. 7). Additionally, peptidomimetic molecules may be designed having the appropriate conformation to engage integrins and elicit their biological responses whilst having better pharmacokinetic parameters than proteins or peptides (Trabocchi and Guarna, 2014). Nevertheless, the majority of integrins display similar RGD binding regions making the synthesis of highly selective ligands, whilst displaying high affinity, to distinct integrin subtypes challenging. Therefore, some ligands presenting subtype selectivity have a residual, but still significant, affinity towards other integrins (Kapp et al., 2017). Although their use for regenerative medicine purposes remains undermined, some labs have considered them as promising tools in biomaterial functionalization (Fraioli et al., 2016; Guasch et al., 2015; Klim et al., 2012; Marelli et al., 2013; Mauro et al., 2017; Rahmouni et al., 2013). Consequently, in a pioneering study Marchand-Brynaert et al. developed an RGD peptidomimetic molecule based on an L-tyrosine scaffold (Fig. 8A) that was afterwards immobilized in a poly(ethylene terephthalate) film (Marchand-Brynaert et al., 1999). Interestingly, this ligand presented cell adhesion properties comparable to RGD, but inferior to the same material functionalized with fibronectin. Even though these results are not totally satisfying, it helped to demonstrate that peptidomimetic ligands could provide good alternatives for the biofunctionalization of intrinsically inert materials. Development of this peptidomimetic ligand has continued and in a subsequent study it showed higher adhesion capacity than RGD (Rerat et al., 2009). More recently, Rechenmacher and coworkers modified a peptidomimetic compound previously developed by their group with nanomolar affinity

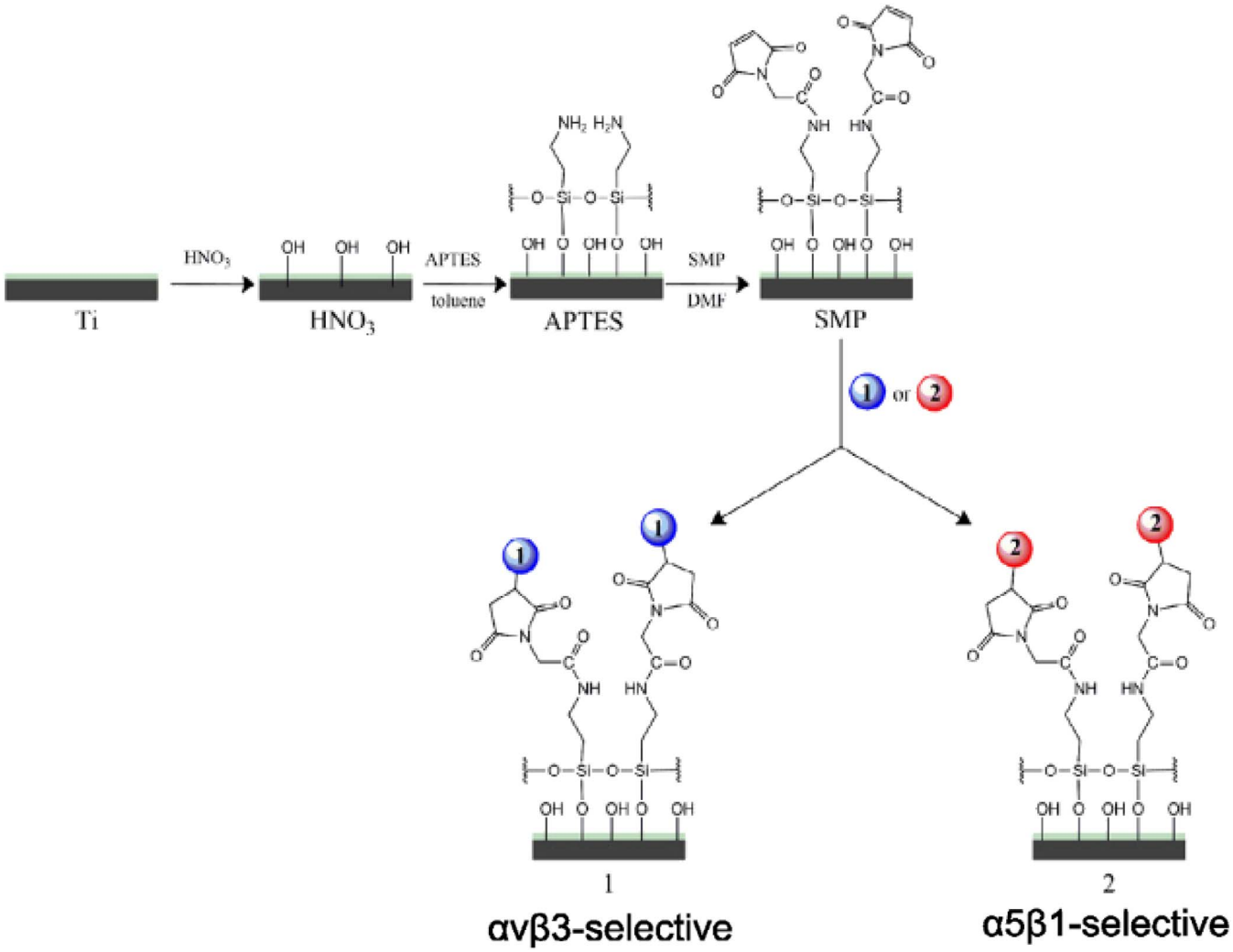

Fig. 7. Surface functionalization strategy using αvβ3- (1) or α5β1-specific (2) peptidomimetics. Integrin specificity will enable to modulate the type of integrin to be activated, and possibly to modulate the cellular response. Adapted with permission from (Fraioli et al., 2015). Copyright © 2015 Elsevier B.V.

towards either α5β1 (Fig. 8B) or αvβ3 (Fig. 8C) (Rechenmacher et al., 2013a). This modification intended to facilitate the immobilization of the molecule on materials with different surface chemistries. Initially, these authors optimized the length of the spacer motif (lysine-based) and number of anchor units (phosphonic acid). Thus, the modified peptidomimetic showed a nine-fold increase in α5β1 binding (in comparison to controls) for higher spacer lengths and seemed independent of the number of phosphonic acids (2, 3 or 4). By culturing either α5β1-expressing or αvβ3-expressing fibroblasts with TiO_2 nanoarrays that had the optimized compound immobilized, this study showed that this chemical modification did not alter affinity, nor the selectivity of the ligand. Therefore, the former cells adhered and extended their processes in culture conditions, whereas the latter cells maintained a round, typically non-adherent morphology. As a proof that this method also allowed the immobilization of a αvβ3-sellective ligand, the authors immobilized a peptidomimetic ligand with nanomolar affinity to this integrin utilizing the same strategy. After improving the solubility of this peptidomimetic molecule, αvβ3-containing fibroblasts were cultured with TiO_2-peptidomimetic nanoarrays. Contrarily to the other ligand, these fibroblasts rapidly adhered to the titanium oxide nanoparticles. On the other hand, α5β1-containing fibroblasts did not interact with the functionalized nanoparticles. This result is in line with a previous study of the same authors where they used these compounds but immobilized them into gold, via thiol (Rechenmacher et al., 2013b). These reports established that peptidomimetic ligands are able of being immobilized onto the surface of materials without losing their affinity and selectivity. Consequently, it opened excellent perspectives for the development of materials capable of equally attracting specific types of cells or guiding cellular responses of interest. Given that peptidomimetics can discriminate between integrins, immobilizing these ligands onto biomaterials may help elucidating the role that different integrins have on mechanotransduction. Likewise, Rahmouni et al. developed PEG hydrogels nanopatterned with gold nanoparticles on the surface and possessing variable bending stiffness (Rahmouni et al., 2013). Then, these hydrogels were functionalized with α5β1- or αvβ3-integrin specific ligands and by culturing fibroblasts within them the authors assessed the traction forces exerted by the adhesion mediated by each of these integrins. In this work, it is showed that cells adhering to α5β1 integrins employed higher maximum forces in the material than cells binding to αvβ3. Despite their preliminary nature, these results demonstrate that one has to not only develop integrin-specific materials but also physically tailor them to correspond to the mechanical needs of cells if the intention is to guide them towards a given response. In another effort to understanding the behavior of α5β1 and αvβ3 during cell adhesion, Guasch et al. orthogonally functionalized alternated stripes of gold and metal oxide with peptidomimetic ligands specific to αvβ3 (Fig. 8D) and α5β1 (Fig. 8E), respectively (Guasch et al., 2015). Thus, to accomplish an orthogonal functionalization these researches used an αvβ3 ligand with a thiol group and an α5β1 molecule with phosphonic acid. This work intended to correlate the focal adhesion

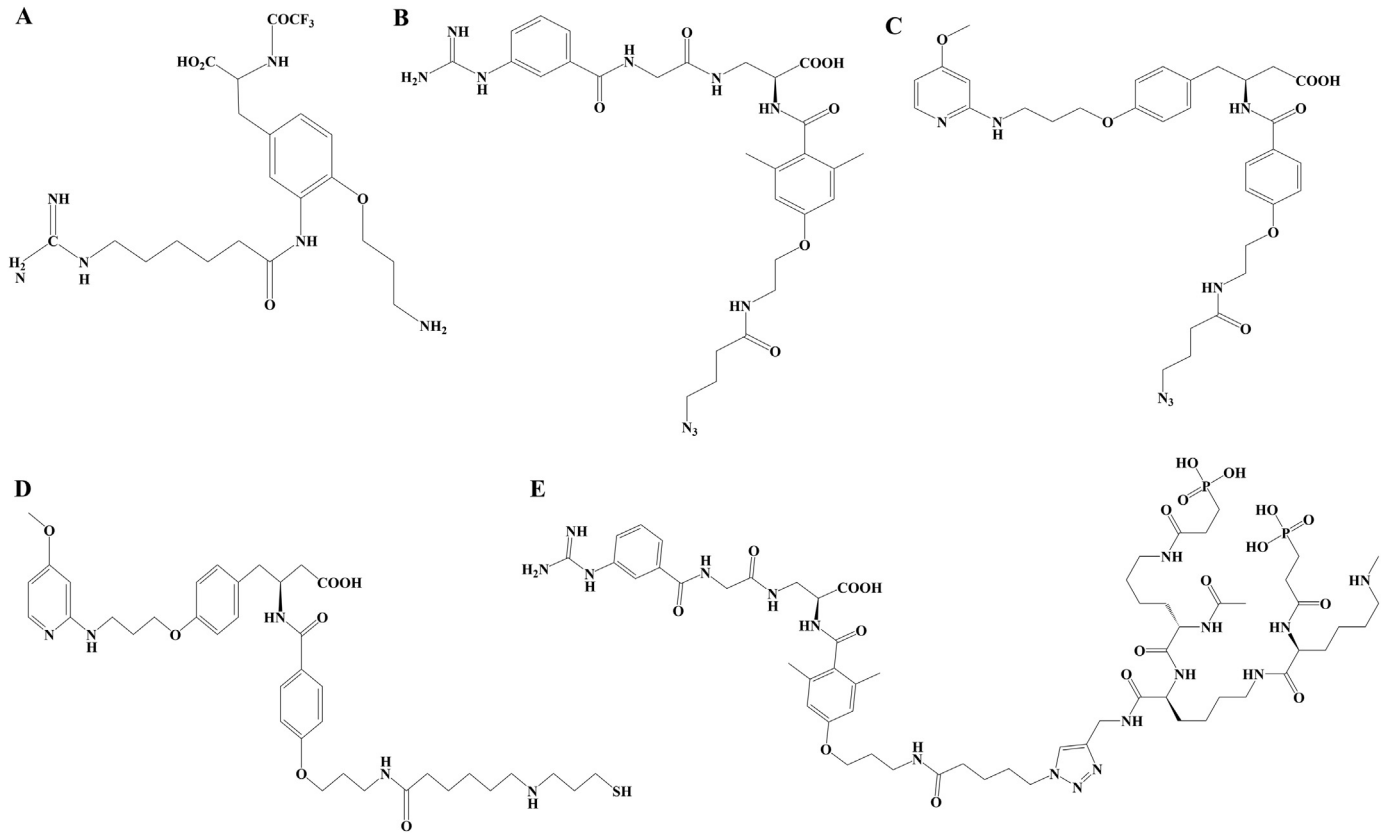

Fig. 8. Structure of peptidomimetic ligands used on the biofunctionalization of surfaces. A – Initial molecule consisting on an L-tyrosine scaffold developed by Marchand-Brynaert et al. (1999). B and C – α5β1-specific and αvβ3-specific peptidomimetics created by Rechenmacher et al., respectively (Rechenmacher et al., 2013a). D and E – Peptidomimetic ligands with specificity to αvβ3 and α5β1, respectively, used by Guasch et al. (2015).

points during cellular adhesion with the location of these integrins and assess the role cells have in positioning these receptors. Therefore, U20S osteosarcoma cells seeded in the functionalized material exhibited an expression of both integrins in the zone of the α5β1-specific ligand, i.e. independently of integrin affinity, whereas cells in the αvβ3-selective zones only presented clusters of this integrin. According to Guasch and coworkers (Guasch et al., 2015), this colocalization can be motivated by a crosstalk between the activated α5β1 and αvβ3 integrins that overcomes the affinity of the latter towards its ligand. Although the activation of αvβ3 integrins in this area is unlikely, due to the high selectivity of the peptidomimetic ligand, α5β1 might recruit αvβ3 integrins by inside-out signaling ensuring their colocalization prior to cell migration. Interestingly, the initial width of the gold stripes (7-8 μm) proved to be insufficient to motivate the expression of αvβ3 on this area. Consequently, U20S cells only started to express αvβ3 on the gold stripes after increasing the width of this surface. Hindering of the peptidomimetic ligand by its lateral confinement was discarded due to the expression of αvβ3 on metal oxide stripes with 7-8 μm. Instead, the explanation to this experimental observation might lie on the different roles α5β1 and αvβ3 integrins have during cell adhesion. Therefore, cells seemed not to sense the appropriate mechanical stimulus to express αvβ3 integrins, which again shows the importance of mechanotransduction in cell behavior, concretely in integrin expression. These results need to be explored in order to gather more knowledge about the different roles each integrin possesses during cell adhesion, if objective is developing smart-instructive materials. Consequently, peptidomimetic ligands seem the best option to ensure it as they can be tailored to have nanomolar specificity to an intended integrin. To explore the importance of mechanotransduction in bone regeneration, Mauro et al. developed RGD-like poly(amido-amine) (AGMA1) hydrogels reinforced by montmorillonite (MMT) with tunable stiffness (Mauro et al., 2017). This reinforcement originated swollen hydrogels with a shear storage modulus (G′) 20 times higher than conventional hydrogels. Thus, these hydrogels presented excellent mechanical properties to be used in bone regeneration. This was corroborated when these authors culture pre-osteoblastic MC3T3-E1 mouse cells and observed that their hydrogels fomented cell adhesion and proliferation while inducing a clear differentiation towards the osteoblastic phenotype. Fraioli et al., however, were the first to report that peptidomimetic ligands with specificity to αvβ3 and α5β1 could enhance adhesion, proliferation and differentiation of cells towards an osteogenic phenotype (Fraioli et al., 2015). Accordingly, two peptidomimetics presenting specificity to either αvβ3 or α5β1 were immobilized onto titanium surfaces. To guarantee that the effects seen on the cells were only due to the bioactivity of both ligands, these authors reduced the roughness of titanium surfaces as rough surfaces are known to positively influence of osteoblastic-like cells. Remarkably, the action of these highly specific ligands conducted SaOS-2 cells to differentiate into osteoblast-like cells while improving the cellular adhesion and proliferation in an equal extension to native ECM proteins.

The use of peptidomimetics for surface coating and regenerative medicine applications is still in its infancy but has presented promising results to date in the guidance of specific cellular responses. Although none of the reported studies is conducted with the purpose of enhancing the formation of a vascular network in a regenerative medicine therapy these works use peptidomimetic molecules specific to integrins involved in angiogenesis (αvβ3 and α5β1). The described results show that the effect of these molecules on cellular adhesion can be equivalent to the action of ECM proteins and conduct cellular differentiation to an intended cell type. Therefore, this opens excellent opportunities for the use of peptidomimetic ligands as vascularization enhancers in regenerative medicine constructs. It is important to underline, though,

that the development of these ligands is rather intricate, involving *in silico* studies to understand the spatial orientation of the chemical mimetics and deep knowledge in organic chemistry. Nevertheless, the possibility of enhancing the vascular response of cells and guiding their behavior towards the creation of complex vascular networks may help dictating the success of a therapy in this field. Another important fact to retain is that peptidomimetics do not represent the holy grail in directed vascular responses. Therefore, as it is shown in this review, there are many factors involved in the creation of vascular networks. These include: a correct spatiotemporal administration of growth factors, the use of cell types that mimic the organization of vascular structures *in vivo* and having environments that resemble the mechanical properties cells find in their habitat. Consequently, a multidisciplinary approach that addresses all these challenges will bring us closer to developing complex, functional, vascular networks that grant the success of regenerative medicine constructs upon implantation.

7. Conclusion

Angiogenesis is highly dependent on the action of integrins due to their effects on ECs adhesion, proliferation and assembly into complex networks. Accordingly, this process is fundamental for the appropriate development of vascularized constructs in regenerative medicine approaches and may help dictate their successful implantation. Thus, integrin-specific peptidomimetics can provide interesting solutions to accomplish the appropriate vascularization of these scaffolds. Peptidomimetics can help directing cells towards their assembling and maturation into complex vascular networks and ensure the success of such therapeutic approaches. These molecules present several advantages when compared to the more frequently applied proteins and peptides. Consequently, peptidomimetics present high affinity (sometimes in the subnanomolar range) towards integrins, excellent integrin selectivity, are more stable and less prone to enzymatic degradation and do not trigger immunogenic responses. However, their development is not straightforward, can be time consuming and demands specific expertise. Despite these drawbacks, they can be powerful tools in guiding cell responses and further understanding the role of integrins in cellular processes.

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