Chapter 4: *The effect of functionalized self-assembling peptide scaffolds on human aortic endothelial cell function*

4.1. INTRODUCTION:

4.1.1 Vascularization and endothelialization in tissue engineering

Vascularization plays an essential role in tissue repair and regeneration. The formation of a three-dimensional vascular network is required to provide nutrients and oxygen and to remove waste products from the cells. The generation of large vascularized three-dimensional structures is today one of the major challenges in tissue engineering (Eiselt *et al*., 1998), because cells can stay alive by diffusion of nutrients only when they are within 150-200 μm from a blood supply (Colton, 1996). Unless these difficulties are overcome, tissue engineering will be restricted to thin structures such as skin, in which nutrients and oxygen can be delivered by diffusion (Cassell *et al*., 2003), or avascular tissues such as cartilage.

The general structure of a blood vessel is shown in Figure 4.1. A blood vessel is comprised by an inner lining of flattened and with a cobblestone morphology monolayer of endothelial cells (EC) separated from the underlying smooth muscle cells by a thin ECM, the basement membrane (BM).

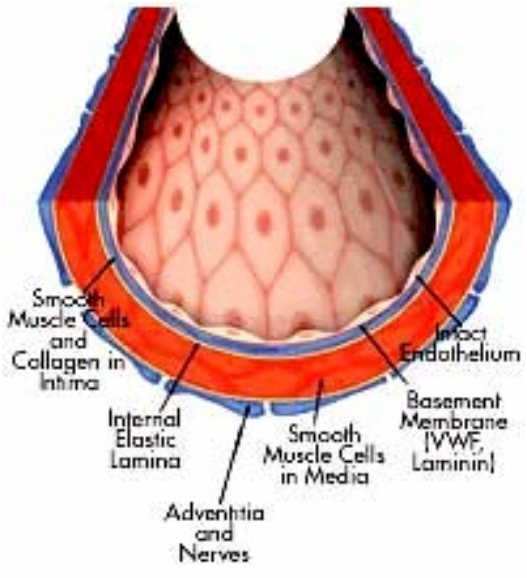


Figure 4.1. Basic blood vessel stucture.

EC possess a negative outer charge that repels platelet adherence, produce glycosaminoglycans that bind antithrombin III, as well as tissue plasminogen activator

which facilitate their anticoagulant and fibrinolytic activities. It is known that the lack of a healthy endothelium leads to vessel failure due to acute thrombosis or aneurysm formation among others. Therefore, a special effort is being made in studying *in vitro* endothelialization, as the presence of a confluent monolayer of EC could improve thromboresistance. It may also prevent the development of other diseases such as pseudointimal hyperplasia by preventing the deposition of platelets, which are the responsible cells to release bioactive factors responsible for smooth muscle recruitment and proliferation (Lanza *et al*., 2000).

4.1.2. Basement membrane of blood vessels

The BM of blood vessels is mainly composed by laminin-1, collagen IV, nidogen, and proteoglycans (See Chapter 2, Figure 2.1. for better comprehension). It is well established that the behavior of EC is critically influenced by interaction with the BM, as its components have also a role in signaling events (Tsilibary *et al*., 1988; Tashiro *et al*., 1989; Grant *et al*., 1989; Skubitz *et al*., 1990; Sakamoto *et al*., 1991; Kanemoto *et al*., 1990; Grant *et al*., 1993; Nomizu *et al*., 1992; Ponce *et al*., 1999; Malinda *et al*., 1999).

Laminin-1

Laminins represent a protein family of α, β and γ chain heterotrimers and are primarily located in BMs but also in mesenchymal compartments. Laminins create physical boundaries between stromal matrix and epithelial, endothelial, muscle, and nerve cells. So far 11 laminin isoforms have been identified giving rise to laminins 1-11. Common to all of them is a coiled-coil domain which is crucial for heterotrimer assembly.

Laminin-1 is the first laminin produced during mouse development prior blastocyst stage, contributing mainly in epithelial tissues during organogenesis (Dziadek and Timpl, 1986; Klein *et al*., 1990). Laminin-1 (M_r=900,000) is formed by subunits α1, β1 and γ1, which assemble into a cross-like structure (Engel *et al*., 1981). Its complex with nidogen forms networks with a quasi-hexagonal pattern with three-dimensional structures. The self-assembling process of the complex formation occurs in a calcium, temperature, and concentration dependent manner (Yurchenco and Cheng, 1993). Laminin-1 is involved in several important biological functions including interaction in homotypic fashion or between different isoforms. It also interacts with

other proteins of the BM forming bridges between the protein complex and cell membranes through cell-membrane receptors, or directly interacting with cells through several integrin and non-integrin receptors (Kreis and Vale, 2000). The main biological functions consist of promoting cell adhesion, cell migration, cell differentiation and proliferation, neurite outgrowth, regulation of cell shape, and establishment of cell polarity of a variety of cell types (Martin and Timpl, 1987; Timpl, 1989; Beck *et al*., 1990; Engel, 1992).

The different biological activities of laminin-1 have been characterized using proteolytic fragments, recombinant fragments or subunits and short synthetic peptides (Yamada, 1991; Yamada and Kleinman, 1992) (Table 1.1). For instance, the sequence AASIKVAVSADR derived from the laminin α chain promoted activity of neurite extention on murine PC12 cells (Yasumitsu *et al*., 1996). In addition, the peptide CSRARKQAASIKVAVSADR, induced degradation of Matrigel matrix by human umbilical vein endothelial cells (HUVEC) and zymograms analysis demonstrated active collagenase IV activity, a key enzyme in BM degradation (Grant *et al*., 1993).

The sequences YIGSR, PDSGR, RYVVLPR located on the β1 chain promoted cell adhesion and, in addition, YIGSR, promoted also cell migration and HUVEC tubular formation (Iwamoto *et al*., 1987; Sakamoto *et al*., 1991; Kleinman *et al*., 1989; Skubitz *et al*., 1990; Grant *et al*., 1989). The sequence KAFDITYVRLKF from laminin γ1 chain also promoted HUVEC adhesion and tubular formation, as well as neuronal cell adhesion and neurite outgrowth indicating that laminin-1 interacts specifically with various cellular receptors through different pathways (Ponce *et al*., 1999; Nomizu *et al*., 2001).

Collagen IV

Collagen is the most abundant protein in mammals. Collagenous proteins constitute a superfamily of ECM proteins with a structural role as their primary function, but they are also involved in cell attachment and spreading. Up to now more than 20 collagens have been identified, all of them sharing domains with a triple helical conformation (Rosso *et al*., 2004). Such domains are formed by three subunits (α chains), each containing a $(Gly\text{-}X\text{-}Y)_n$ repetitive sequence motif. Some collagens are specific for a tissue such as type II (specific for cartilage), and others like collagen I are highly conserverd and are present in many tissues.

Collagen IV is the major collagenous component of the BM which forms a network structure that involves the interaction with other proteins including laminin, nidogen, and heparan sulfate proteoglycan (Kreis and Vale, 2000).

Collagen IV molecules are composed of two α1(IV) chains and one α2(IV) chain. Collagen IV can interact with cells indirectly through laminin by direct low affinity interactions (Charonis *et al*., 1985; Yurchenco and O'Rear, 1994) or by strong binding mediated by nidogen, a glycoprotein of about 150 KDa, which binds tightly to laminin (Paulsson *et al*., 1987; Poschl *et al*., 1997) and has binding sites also for collagen IV (Timpl, 1996). Type IV collagen also binds to heparin and heparan sulfate proteoglycan (Fujiwara *et al*., 1984; Tsilibary *et al*., 1988; Koliakos *et al*., 1989; Yurchenco and O'Rear, 1994). Many cell types adhere to type IV collagen, and peptide sequences within it present specific biological activities. For instance, the peptide TAGSCLRKFSTM was found to specifically bind to heparin and intact type IV collagen in a dose dependent manner (Koliakos *et al*., 1989; Tsilibary *et al*., 1990). In addition, the same peptide was also able to promote the adhesion and spreading of bovine aortic endothelial cells (Tsilibary *et al*., 1990). The peptide sequence also inhibited collagen IV matrix assembly when both were incubated together in solution. Thus, the role of this type IV collagen derived peptide sequence is diverse, in terms that it influences matrix assembly, heparin binding and cell adhesion (Tsilibary *et al*., 1990).

Nidogen

Nidogen consists of a single polypeptide chain that binds to laminin-1 γ chain by a single module (LE) of 56 residues (Poschl *et al*., 1997). Nidogen also interacts with collagen type IV using a separate epitope and it is considered to be a linker molecule between laminin-1 and collagen IV in BMs. In addition, nidogen contains RGD sequences which are cryptic cell attachment sites via integrin molecules (Timpl, 1989).

Proteoglycans

Proteoglycans are a set of proteins found on cell surfaces, within intracellular vesicles and incorporated into ECMs. They are defined and classified by a common post-translational modification, a special type of polysaccharides, the family of glycosaminoglycans. Proteoglycans are a diverse set of macromolecules composed by a core protein which can consist of a small or large polypeptide chain (10-400 kDa) carrying from one to hundreds of glycosaminoglycan chains. There are many known activities of proteoglycans. Among those, they are known to regulate cell-cell and cell-matrix interactions by binding with other ECM proteins. They regulate extracellular matrix assembly and structure and they immobilize diffusible molecules (such as growth factors) whithin the ECM as storing and releasing compartments (Kreis and Vale, 2000).

Many researchers have focused their work on the study of the role of ECM in vascularization. Most of the studies imply observation of EC behavior on ECM derived materials such as Matrigel, obtained from the Engelbreth-Holm-Swarm mouse tumor, and collagen (Grant *et al*., 1989; Bell *et al*., 2001; Davis and Black, 2000; Davis *et al*., 2002). Thesed groups mainly study the process of tube formation or so called angiogenesis in both 2D and 3D systems (Davis and Black, 2000; Bell *et al*., 2001; Davis *et al*., 2002). The interaction of EC with several synthetic peptides (Grant *et al*., 1989; Grant *et al*., 1993; Nomizu *et al*., 2001; Ponce *et al*., 1999) has produced an extense literature about the interaction between peptide sequences and cellular activities (Table 3.1.). So far, to obtain these *in vitro* models, research has been performed mostly using primary EC isolated from different species including HUVEC and the microvascular endothelium. However, it is known that EC are a heterogeneous population, EC from small and large vessels differ in growth, propensity to from capillary-like structures, and synthesis of metabolites among others (Cines *et al*., 1998).

4.1.3. Endothelial cell function

The endothelial monolayer has multiple functions, such as facilitating blood flow by providing a nonthrombogenic surface, being a permeable barrier and transport interface for metabolites, controlling the inflammatory response, controlling the contractility of the vascular smooth muscle and the myocardium and controlling vascular tone and homeostasis, among others (Boeynaems and Pirotton, 1994; Cines *et al*., 1998). The failure of the performance of these functions can lead to several pahtological conditions, for example, atherosclerosis.

EC continuously secrete, produce and remodel their own BM and synthesize vasoactive agents, that contribute to regulate vascular tone and homeostasis (Busse and Fleming, 2003).

Nitric oxide (NO) is one of the best known vasodilators. In biological systems, NO is synthesized from L-arginine by the action of nitric oxide synthase (NOS). It is also an antiplatelet aggregation agent. Another potent vasodilator and also an inhibitor of platelet aggregation is prostacyclin (or also called prostaglandin I_2, PGI_2). PGI_2 belongs to the family of eicoisanoids, and it is formed from arachidonic acid via prostaglandin H_2 (PGH_2) by prostacyclin synthase. In addition, a 21 aminoacid peptide,

endothelin-1 (ET-1) has been shown to promote vasoconstriction. Both three substances act together as regulators of vascular tone.

It has been previously shown that EC seeded in environments that mimic physiological conditions *in vivo*, stimulate the production of these vasodilator and vasoconstrictor substances (Gloe *et al*., 1999; Busse and Fleming, 2003). Therefore, in this part of the work the effect of the functionalized self-assembling peptide scaffolds as substrates for human aortic endothelial cell (HAEC) culture was studied following the hypothesis that the new self-assembling peptide scaffolds may act as BM analogs, which mimic the extracellular microenvironment of HAEC. In this sense, the capability of these new biomimetic materials to support monolayer formation, growth, and function of HAEC was tested.

4.2. RESULTS

The main aim of this part of the work was to use the novel functionalized biomimetic scaffolds as synthetic basement membrane analogs to culture endothelial cells. In a first experiment the ability of human aortic endothelial cells (HAEC, Cambrex, CC-2535) to form a monolayer when seeded on the peptide scaffold was tested. Since aortic endothelial cells normally form monolayers that cover the internal surface matrix of arteries, we tested whether the modified peptide scaffolds would provide a better ECM analog than RAD16-I. Firstly, hydrogels were prepared diluting each peptide stock solution at a final concentration of 0.5% (w/v in deionized water). The matrices were prepared as explained in Chapter 7, cells were added at a final density of $2x10^5$ cells/cm^2 and cultures were monitored for 3 days. In this assay, only one of the modified peptides (YIG) besides the prototypic RAD16-I, was able to support the formation of a monolayer (Table 4.1., Figure 4.2.). Figure 4.2. shows HAEC grown on the peptide scaffolds. A complete monolayer was only observed for YIG; despite that in RAD16-I cells almost formed a monolayer. In the rest of the sequences cells either died or formed structures in isolated clusters (Figure 4.2.). In order to discriminate toxicity or a over-signaling of the sequence at that concentration, it was decided to blend the peptide motifs with the prototypic RAD16-I at a ratio 9:1 (v/v, RAD16-I:peptide sequence) and the experiment was repeated. In these conditions it can be observed that YIG, RYV and TAG, favoured monolayer formation (Table 4.1, Figure 4.3). These three best blending conditions together with the prototypic RAD16-I alone, were selected as the scaffolds to use in the next experiments.

Table 4.1. HAEC monolayer formation on peptide scaffolds

Peptide sequence	**Peptide content**	
	100%	**Blending 9:1***
RAD16-I	+Ψ	
AAS	-ϕ	-
YIG	+	+
PDS	-	-
RYV	-	+
KAF	-	-
TAG	-	+

* RAD16-I:Peptide sequence (9:1 ratio)

Ψ(+) scores for optimal monolayer formation and cell survival

ϕ (-) scores for poor or none monolayer formation and cell survival

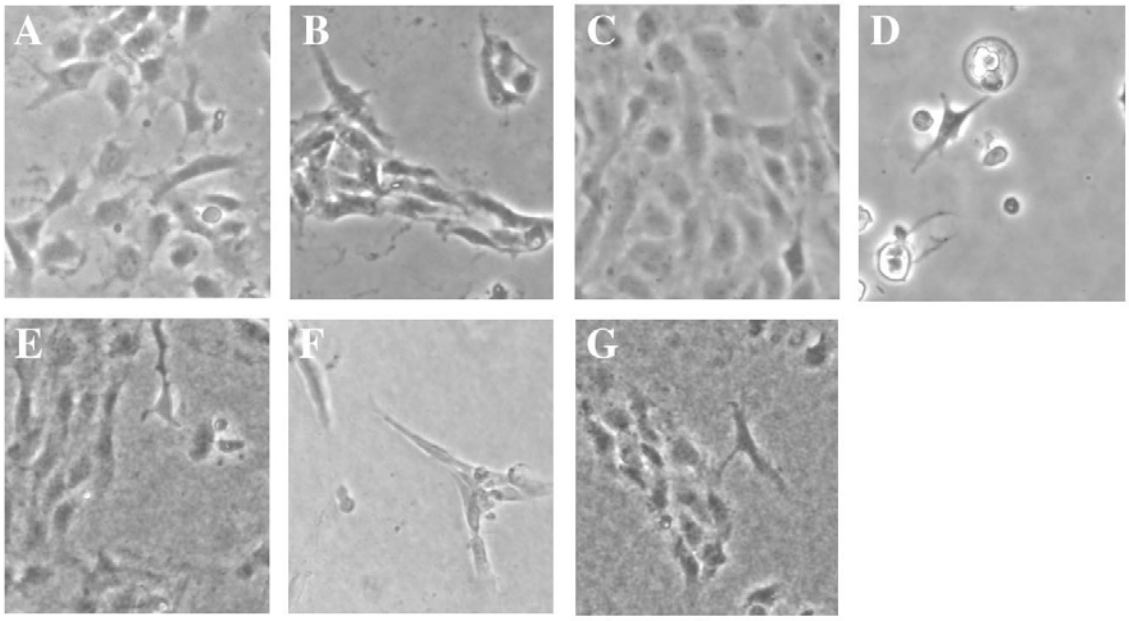


Figure 4.2. Phase contrast microscopy images of HAEC on A) RAD16-I; B) AAS; C) YIG; D) PDS; E) RYV; F) KAF); G) TAG. All peptide sequences at 100%.

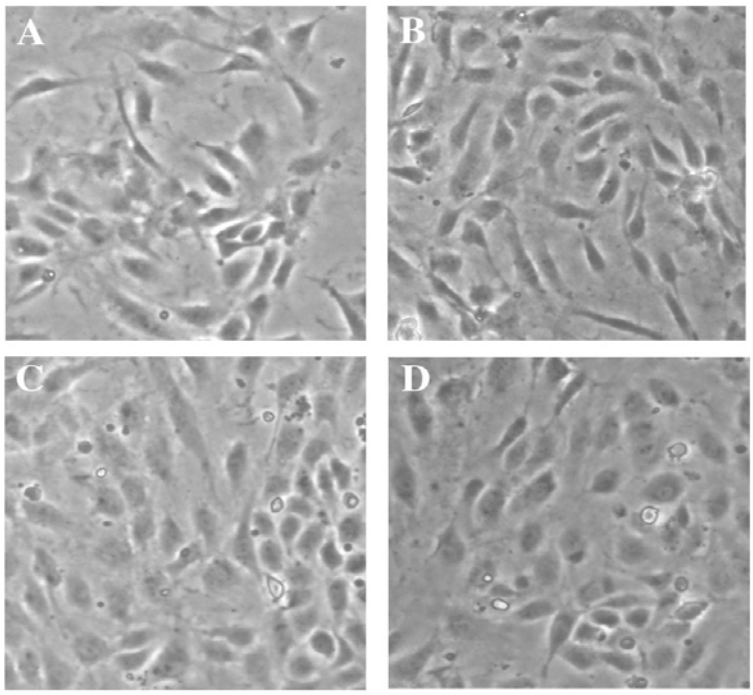


Figure 4.3. Phase contrast microscopy images of HAEC monolayer formation on A) RAD16-I; and blending 9:1 of B) YIG; C)RYV; D) TAG.

To detect differences in cell-attachment in the prepared matrices, HAEC were seeded at subconfluent density (~50%) to allow good cell-material interaction. One hour after seeding, unattached cells remaining in the supernatant media were counted. No cells were detected in the supernatants, indicating that HAEC attachment to the surface was ~100%. This result suggests that the initial interaction of the cells with the material was not affected by the functionalization. In addition, cells growing on these four surfaces maintained basic endothelial cell phenotype, as evidenced by LDL uptake (Figure 4.4.)

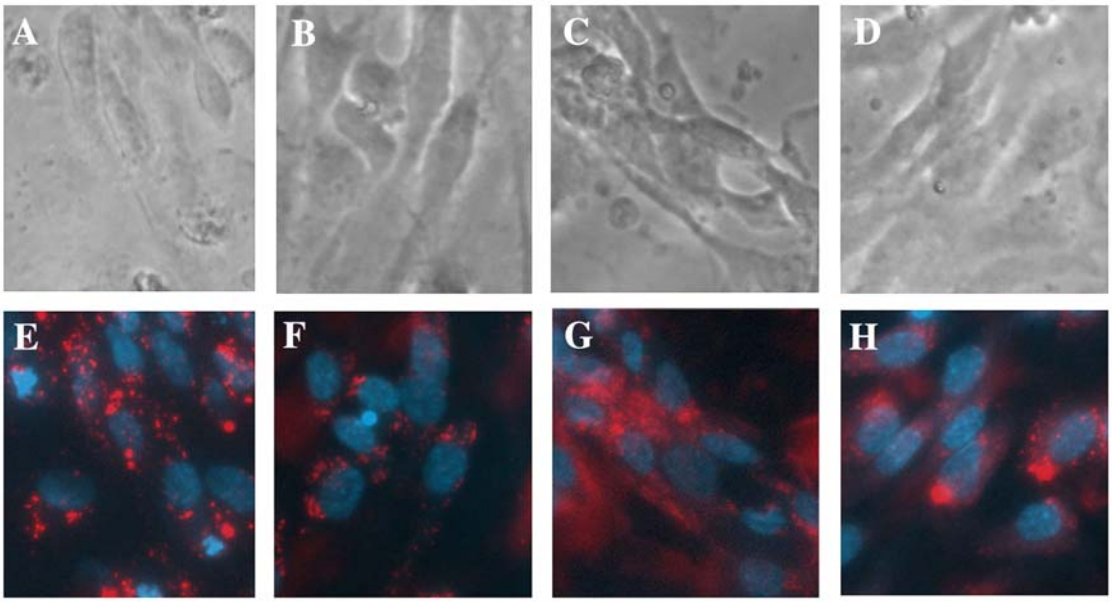


Figure 4.4. Phase contrast microscopy images of HAEC seeded on A) RAD16-I; B) YIG; C) RYV; D) TAG, and fluorescent staining with Di-Ac-LDL (red) and DAPI nuclear staining (blue) for E) RAD; F) YIG; G) RYV; H) TAG.

Moreover, the capacity of endothelial cell monolayer formation was tested after culturing the cells for 3 days on the four peptide scaffold systems described above, and two gel system controls, collagen I and Matrigel (see Materials and Methods). Monolayer formation with typical confluent cobblestone phenotype was evidenced in all the gel systems tested with the exception of RAD16-I, where cells looked clustered with extended uncovered areas (Figure 4.5.). These results suggest that the functionalized peptide scaffold surfaces present similar properties as the natural materials used (collagen and Matrigel) in terms of promoting endothelial cell monolayer formation.

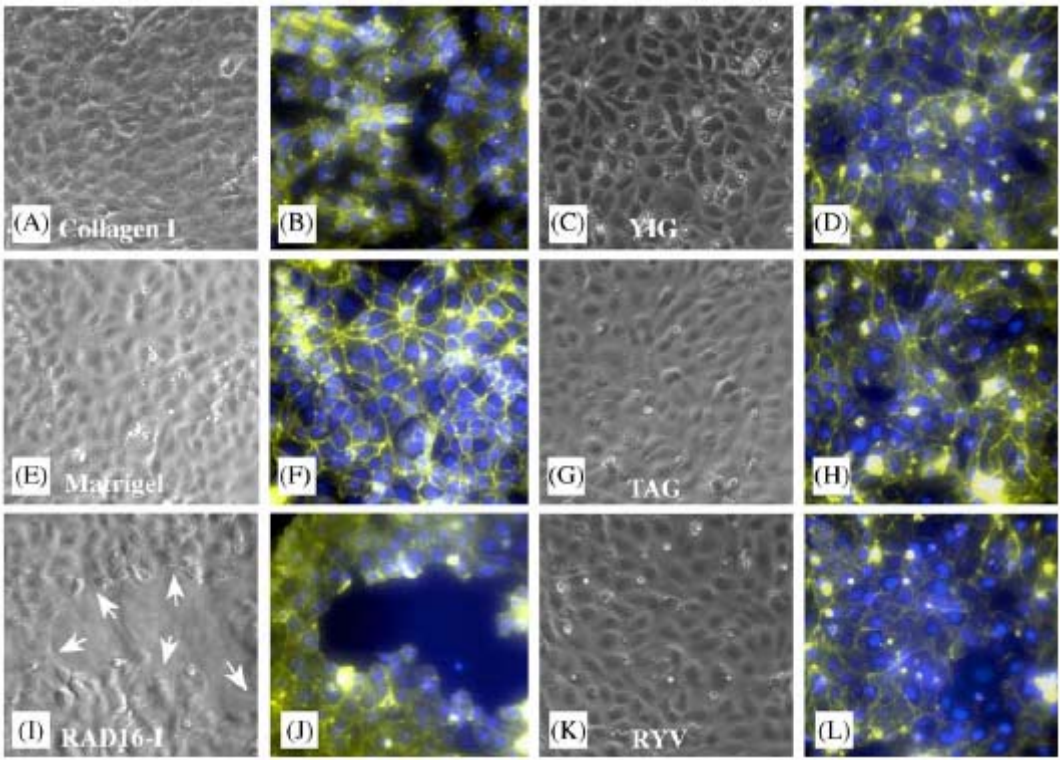
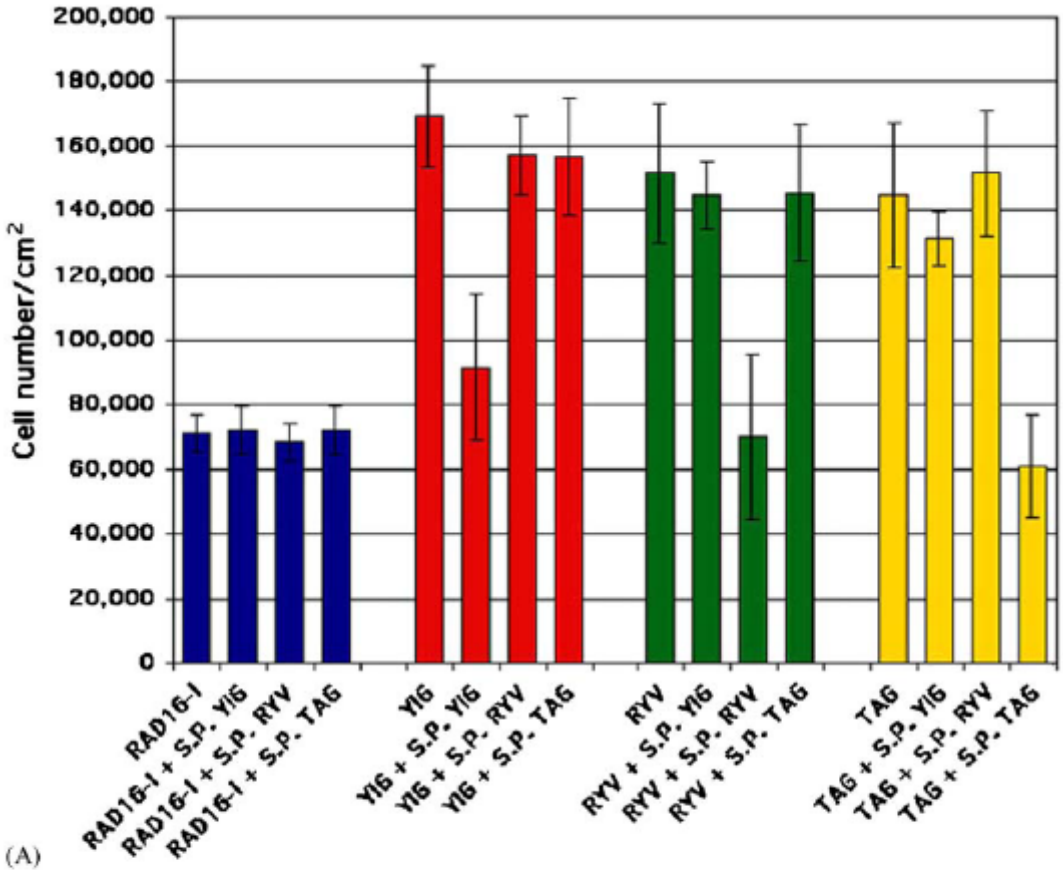


Figure 4.5. Monolayer formation of HAEC on different gel systems. Phase contrast microscopy images of HAEC cultured for 3 days on A) collagen I, E) Matrigel, 100%, I) RAD16-I, C) blending 90% RAD16-I/10% (v/v) YIG, G) blending 90% RAD16-I/10% (v/v) TAG, K) blending 90% RAD16-I/10% (v/v) RYV. Fluorescent staining of the same optical layers with TRITC-Phalloidin and DAPI in order to detec actin fibers (yellow) and nucleus (blue), respective for A(B), c(D), E(F), G(H), I(J), K(L). Phase contrast and fluorsecent images depict a typical cobblestone monolayer phenotype. White arrows in E indicate the borders of the non-confluent monolayer.

In order to analyze the growth of HAEC on each scaffold surface, cells were cultured as described above for 3 days and after that time, cell number and viability were calculated for each condition. Initial seeding number ($8x10^4$ cell/cm^2 was used to calculate fold changes in cell numbers after three days in culture. Interestingly, cell numbers increased about 2-fold on the functionalized peptide scaffolds with respect to RAD16-I control (Figure 4.6., B), and results were similar that those for collagen I and Matrigel. This change in the growth accelerated the formation of a confluent monolayer with cobblestone phenotype (Figure 4.5.), suggesting that cells may sense and respond to the motif-functionalized material. To confirm this, competition assays with each free peptide motif were performed in order to specifically block the interaction between the cell surface and the modified material. An excess (400 μM) of

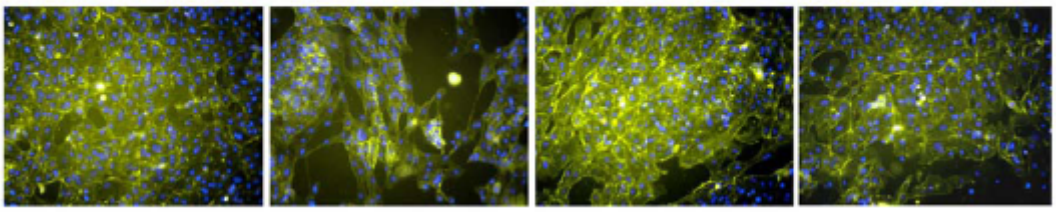
the three soluble active peptide motif was added to the culture medium independently and cells were incubated for a three-day period. Fresh media was added every day in order to maintain the cells with an excess of soluble peptide. After that time, cells were resuspended with the scaffold and counted. In all cases, soluble peptides YIGSR (S.P. YIG), RYVVLPR (S.P. RYV) and TAGSCLRKFSTM (S.P. TAG) added to the culture media of their specific functionalized peptide matrix reduced cell growth after 3 days in culture to similar levels as RAD16-I (Figure 4.6. A and B). Moreover, competition assays between the soluble peptides and the non-corresponding functionalized peptide scaffolds were also performed (i.e. soluble peptide YIGSR in the culutre media of composite scaffold RAD16-I 90% plus TAG 10%, etc…) as an additional control to demonstrate the specificity of each corresponding competitions. As expected, the non-corresponding competitions did not produce the significantly decrease in cell growth observed when cells are incubated with the corresponding soluble peptide/functionalized scaffold pair (Figure 4.6). As an example, the monolayers obtained after the competition assay with the peptide scaffold YIG shows cells covering the surface of the peptide scaffold for all cases except for the corresponding incubation with the soluble peptide YIGSR (Figure 4.6, C). Moreover, cell viability was tested using the Trypan blue exclussion assay, and it was observed, that the viability was very similar for all the cases (~80%, Figure 4.7). These results suggest that the differences in cell number observed for each case were mainly due to differential cell growth rather than cell death. Moreover, results from competition assays (Figure 4.6., A) suggest that cells physically interact with the sequence motif present in the scaffold and, in addition, this interaction can be displaced by soluble competitive peptide.



(A)

(B)

Scaffold type	No soluble peptide	S.P. YIG	S.P. RYV	S.T. TAG
RAD16-I	0.88	0.91	0.85	0.9
YIG	2.11	1.14	1.96	1.95
RYV	1.89	1.8	0.87	1.82
TAG	1.81	1.64	1.9	0.76



(C) YIG YIG + S.P. YIG YIG + S.P. RYV YIG + S.P. TAG

Figure 4.6. Competition experiment of HAEC growth with soluble peptide motifs. A) cell density of HAEC seeded on different peptide scaffolds and regular culture plates expressed in number of cells per cm^2. B) An initial seeding number (8 x 10^4 cell/cm^2) was used to calculate fold changes in cell numbers after three days in culture for each of the conditions described above. C) The competitive assay series with the scaffod YIG (blending 90% RAD16-I/10% (v/v) with YIG peptide scaffold) described in (A) is visualized after staining with TRITC-Phalloidin (actin fibers, yellow) and DAPI (nucleus,

blue). S.P. indicates soluble peptide, added to the medium at a concentration of 400 μM.

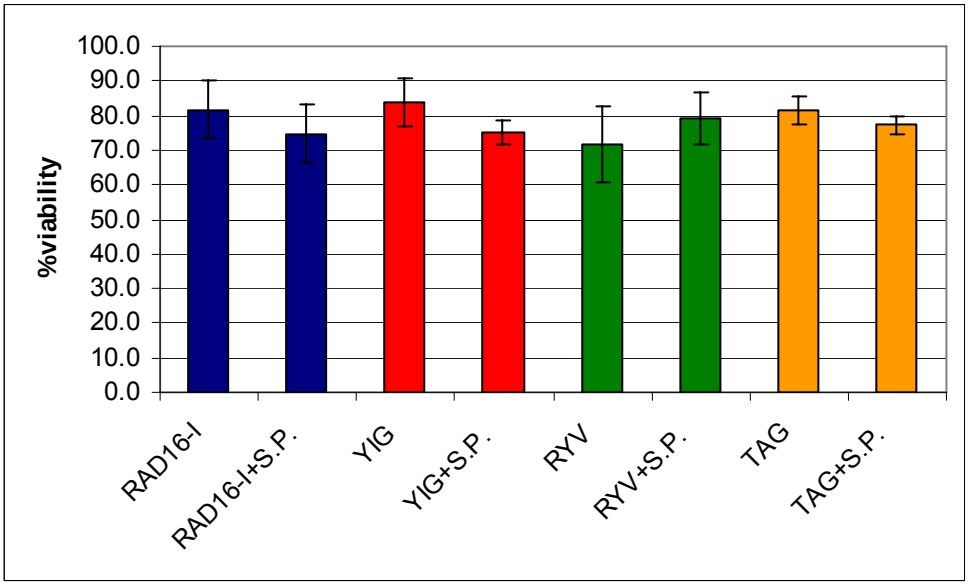


Figure 4.7. Cell viability of HAEC seeded on different peptide scaffolds in the absence or presence of soluble peptide.

In addition, the production of laminin 1 and collagen IV was analyzed using a western blot, as a way to measure the capacity of the cells to deposit their own basement membrane. Since the peptide hydrogels have a defined composition, the presence of deposited BM can be easily detected. In general, for all conditions tested, including the RAD16-I peptide, laminin-1 and collagen IV deposition was observed. In contrast using conventional 2D tissue culture plates this basement membrane deposition is not observed (Figure 4.8.). Due to the specificity of the antibody used, only one band for laminin was observed (β2 chain). In contrast, multiple bands of different molecular weights for collagen IV were detected, suggesting that the collagen molecules may be partially degraded (due to gelatinases and metalloproteases) under the culture conditions tested (Figure 4.8.), which is not observed in the case of laminin-1. These results may indicate that the basement membrane components deposited may be selectively undergoing remodeling, as show by the partial degradation of the collagen IV observed in the western blot (Figure 4.8., B).

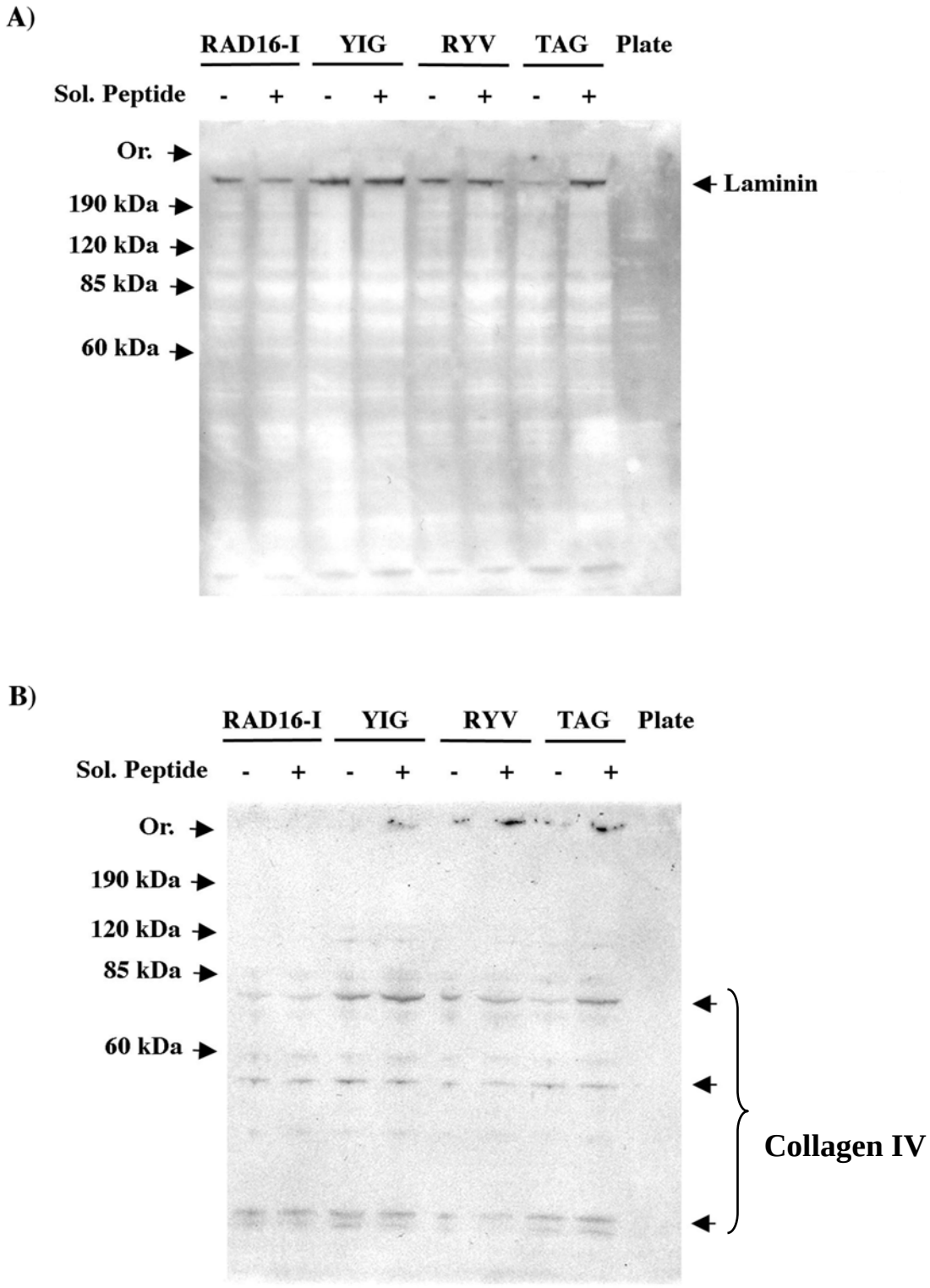


Figure 4.8. Western Blots for cell lysates from HAEC cultured on different scaffolds in absence (-) or presence (+) of soluble peptide. A) Laminin-1, B) Collagen IV. Arrows in B indicate Collagen IV fragments. All lanes contain equivalent cell numbers.

Furthermore, the secretion of vasoactive substances such as NO was evaluated. NO produced can be measured as the total amount of nitrates and nitrites present in the media (see Materials and Methods). Media samples from cells cultured on the peptide scaffolds, and media samples of cells cultured in regular tissue culture plates were analyzed and compared. The results of this assay are summarized in Figure 4.9. It is shown that, except for the functionalized peptide RYV, all the peptide scaffold cultures promote NO release (Figure 4.9.). This result shows that the peptide hydrogel is in general is an adequate substrate matrix for the culture of HAEC, as indicated by the release of NO, which can be used as a marker of endothelial cell function. Nevertheless, little difference in NO release between RAD16-I, YIG and TAG scfaffolds was observed, and it may be possible that for the case of RYV the functionalization might inhibit such activity.

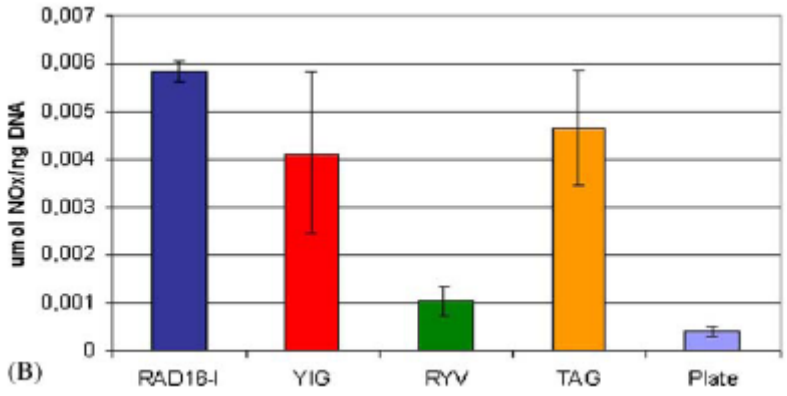


Figure 4.9. NO release by HAEC seeded on different scaffolds and regular tissue culture plates, expressed as μmol nitrates+nitrites (NOx)/ng DNA.

4.3. DISCUSSION

In this part of the Thesis, the use of the novel functionalized peptides as basement membrane analogs for the culture of HAEC was described. We decided to use three blends containing a 10% of functionalized peptides (YIG, RYV, TAG) besides the prototypic RAD16-I in order to culture and obtain HAEC monolayers. Collagen I and Matrigel were also used as positive controls, as HAEC form nice monolayers on these ECM-derived scaffolds. It was observed, that cell attachment for all the surfaces tested was close to 100% suggesting that the modifications are not playing an important role in cell attachment under the culture conditions tested. Interestingly, the functionalized

peptide scaffolds promoted the maintenance and growth of HAEC over time in culture. In particular, sub-confluent seeded cultures of HAEC developed into confluent monolayers with elevated cell number when compared with the non-functionalized scaffold (RAD16-I), suggesting their role in increasing growth rates (Figure 4.5. and 4.6.). In addition, growth rates in the non-functionalized RAD16-I scaffold did not change over time, suggesting that cells under this condition remain mainly inactive, probably they do not spread or migrate well, and, as a consequence, they do not proliferate (Figure 4.5. and 4.6.). Most importantly, competition assays with each corresponding soluble motif designed to block specific interactions between cells and the functionalized scaffolds demonstrated that growth rates were reduced to levels similar to control RAD16-I scaffold, suggesting that the cells indeed recognized the exposed adhesion motifs attached to each scaffold (Figure 4.6.). Therefore, it appeared that endothelial cell growth was modulated through the biomimetic surface matrix only when the sequence was physically attached to the nanofiber (Figure 4.6.), and that specific interaction in each case can be disrupted by adding the same soluble non-attached peptide motif. It is plausible that in this case, a mechanosignaling transduction event that regulates cell growth and monolayer formation of HAEC was involved. This is of critical importance for endothelial cells during neovascularization of multiple physiological processes, including wound healing, menstrual regeneration of endometrium, tumor angiogenesis etc… (Bogenriender and Herlyn, 2003; Ferenczy *et al*., 1979; Reed and Sage, 1996). These results are consistent with previous reports describing that the immobilization of YIGSR peptide on the synthetic polymers, PET or PTFE, promoted endothelial cell adhesion or spreading (Massia *et al*., 1993).

Moreover, and generally speaking, the peptide scaffolds enhanced endothelial cell phenotype and function, indicated in terms of basement membrane components deposition (laminin-1 and collagen IV), as well as NO synthesis. These results suggest that these peptide matrix conditions recreate better the endothelial microenvironement as it has been shown by the functional tests performed.

In addition, a bioreactor has been set up in our group in order to complement this *in vitro* system with a biomechanical environment by mimicking an interstitial flow model *in vitro*. Physiological interstitial flow can be defined as the movement of fluid through the extracellular matrix of a tissue (NG *et al*., 2004). This phenomenon is present, to some extent, in all tissues and is responsible for the convection needed to transport large proteins through the interstitial space. However, besides this role, interstitial flow also exerts a mechanical influence over interstitial cells either directly, by the induction of shear stress and hydrostatic pressure, or indirectly by imposing a

strain and elastic stress to the extracellular matrix fibers to which the cells are attached via integrin receptors (NG *et al*., 2004).

In this sense, a new bioreactor was specifically designed to study interstitial flow-mediated capillary morphogenesis (Semino *et al*., 2006; Hernández, 2006). The bioreactor was composed of a poly(dimethylsiloxane) (PDMS) fluid chamber, designed to work with interstitial flow, and allowing, at the same time, real-time microscopic monitoring of the capillary-like structures. Then a collagen layer was introduced and a human umbilical vein endothelial cell (HUVEC) monolayer was formed. After the monolayer formation, an interstitial flow normal to the monolayer was applied for different periods of time using a syringe pump. HUVECs monolayers cultured on collagen type I gels in these devices engaged in capillary morphogenesis under the synergistic effect of low interstitial flows (similar to *in vivo* interstitial flows, 0-50 μm/min) and VEGF (Figure 4.10., Figura 4.11) (Hernández, 2006). On the contrary, when monolayers were cultured in the absence of interstitial flow, no capillary-formation was observed, indicating that the presence of interstitial flow is critical to trigger the process.

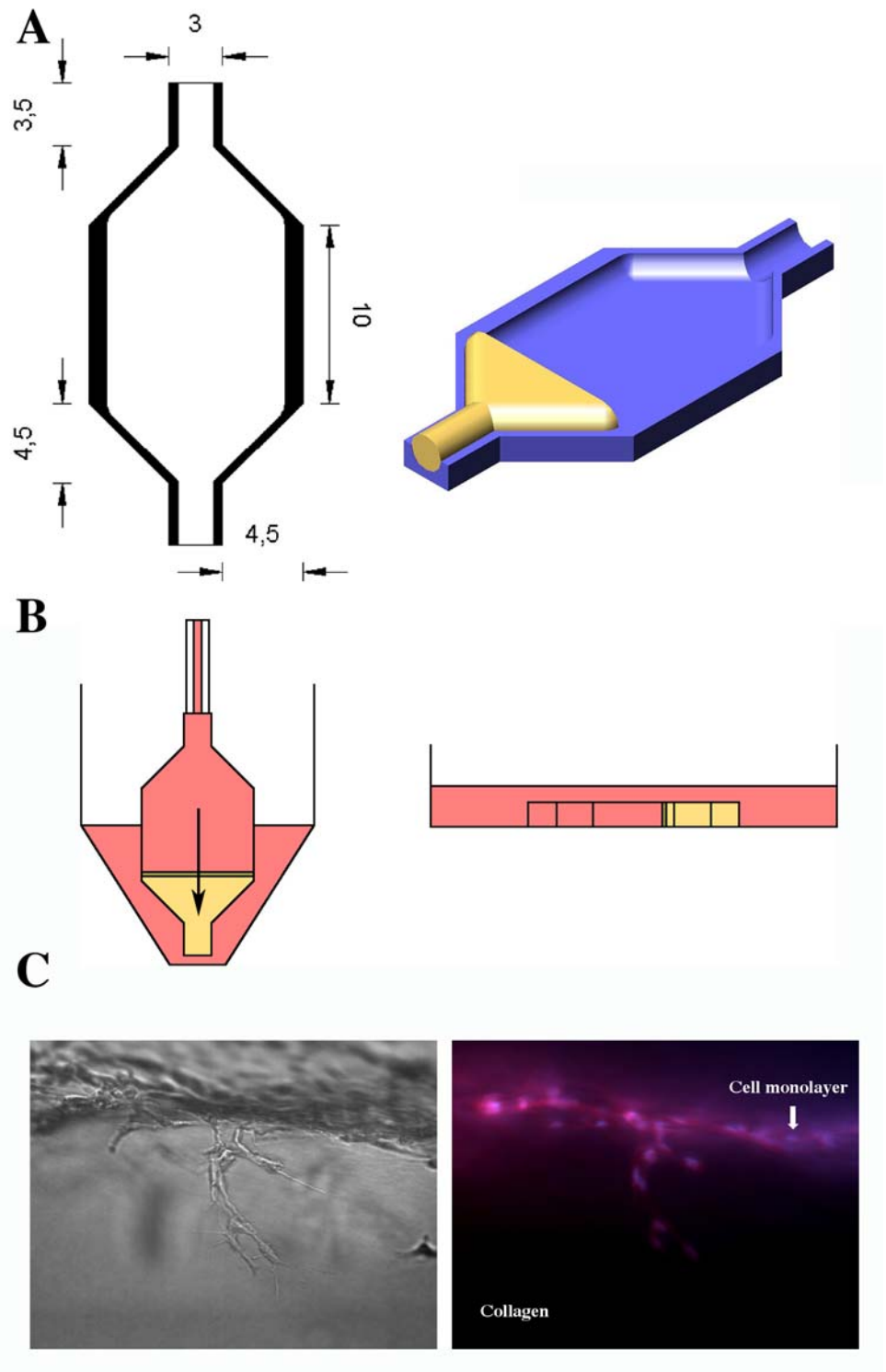


Figure 4.10. Bioreactor system designed to induce HUVEC capillary morphogenesis through interstitial flow. **A**) Scheme depicting the bioreactor. **B**) Scheme indicating the interstitial flow direction and the bioreactor position in the no-flow experiments C) Capillary-like structures formed, phase (left) and fluorescence (right) images showing in red actin fibers and in blue the nuclei.

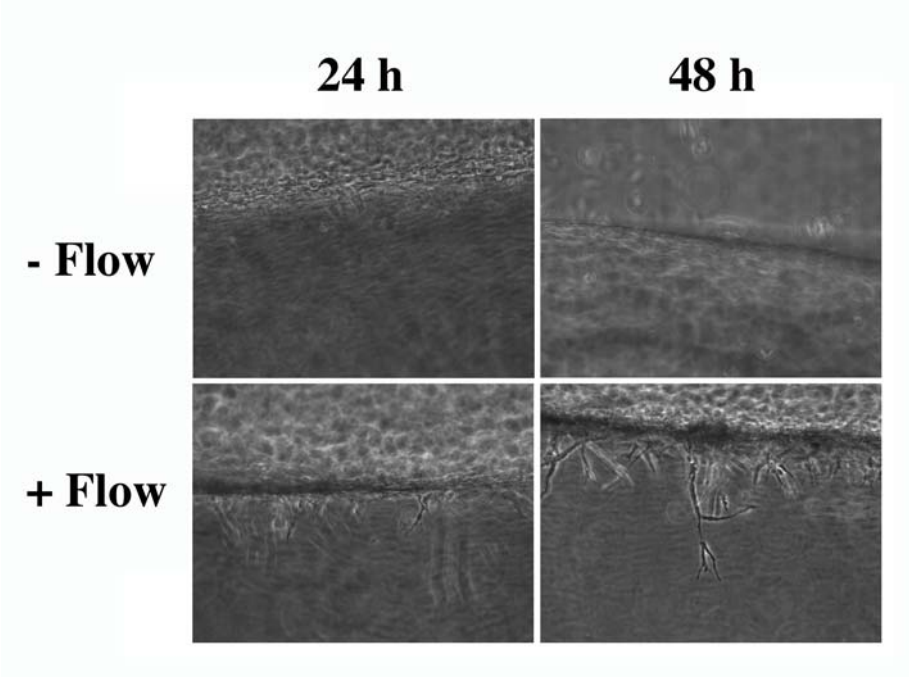


Figure 4.11. Effect of interstitial flow in the promotion of the morphogenic response. Interstitial flow was applied to 2 bioreactors, for 24 and 48 hours respectively, and two static controls were cultured simultaneously to investigate if interstitial flow was a critical parameter to trigger the morphogenic response or if it only contributed to obtain a faster response from the monolayer. As expected, neither after 24 nor 48 h (Fig. 4 B) a morphogenic response was triggered in those monolayers cultured in the absence of interstitial flow, therefore proving its key role in triggering the process of capillary morphogenesis.

This system will be adapted to work with the synthetic self-assembling peptide scaffolds, in order to combine synthetic biomaterials with a biomechanical microenvironment. This *in-vivo* like microenvironment will be used also to co-culture different cell types with endothelial cells to promote tissue-like vascularization.

We conclude that tailor-made peptide scaffolds represent a new generation of cell-responsive materials, easy to synthesize and purify and likely to have an impact on biomedical technologies. We consider that combining the functionalized surfaces with biophysical parameters could be an important approach to obtain vascularized tissues *in vitro*.

Chapter 5: *A synthetic functionalized self-assembling peptide hydrogel culture system maintains hepatocyte specific functions*

5.1. INTRODUCTION

The liver is vital and complex organ which performs many basic functions and metabolic activities including synthesis of most of plasma proteins, storage of glycogen, removal of toxins and metabolization of xenobiotics among others. Together with the pancreas, are the two organs that have both exocrine and endocrine functions. For instance as exocrine activity, it produces bile, which plays an important role in digestion and fat absorption. As an endocrine organ it produces albumin, protease inhibitors, etc… that are delivered to the blood flow. In addition, it serves as a storage of several vitamins, it incorporates toxic ammonia from the transaminase reactions in non-toxic urea, it is involved in the mobilization and export of lipids etc… Removal of liver without supporting all these metabolic functions implies loss of life within several hours (Michalopoulos and DeFrances, 2005).

5.1.1. Anatomy of the liver

As previously mentioned, the liver is a structural and functional complex organ, and it has been second only to brain in its complexity (Malarkey *et al*., 2005). It performs hundreds of vital functions and contains at least a dozen cell types. Still today, the anatomy of the liver is poorly understood. Human liver is a highly vascularized organ composed by two anatomical lobes, the right and left lobes, the right lobe being about six times the size of the left (Sherlock and Dooley, 2007). It has a double blood suply:

- The portal vein, which brings venous blood from the intestines and spleen.
- The hepatic artery, which brings to the liver highly oxygenated blood from the heart.

Both vessels enter the liver by the *porta hepatis* and together with the bile duct form the portal tract. These three vessels branch into a complex tree structure, which supply and drain the entire liver (Figure 5.1., B).

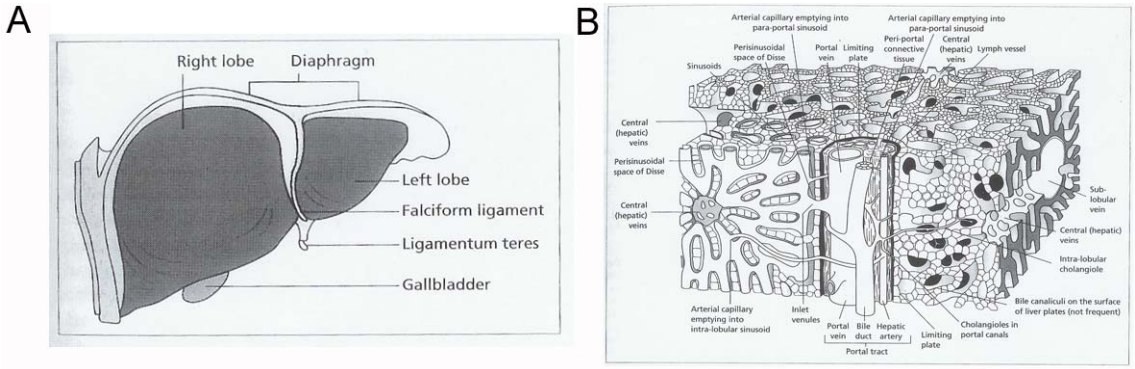


Figure 5.1. A) Anterior view of the liver. B) Structure of the normal human liver showing the portal tract composed by the portal vein, the hepatic artery and the bile duct (Sherlock and Dooley, 2007).

Even though, the basic functional architechture of the liver is still unclear. Since the first reports mainly two conflicting models are still being used: the lobular unit, proposed by Kiernan and the acinar unit proposed by Rappaport (Sherlock and Dooley, 2007; Saxena *et al*., 2000). Neither of them has been completely accepted nor discarded, meaning that there is not a definitive model yet. Although each of these models can explain some diseases and physiological processes in the liver, none can explain them all. In the first model proposed in 1833, Kiernan introduced the concept of the hepatic lobule as the basic architechture. He described hexagonal lobules consisting of the central vein located in the middle, and at the perifery a portal triad composed of the bile duct, portal vein and hepatic artery branch. Colums of liver cells and blood containing sinusoids extend between these two systems (Figure 5.2., A and B). In this model, the blood passes into the perifery from the digesive tract via the portal triad, traverses the sinusoid and then exits through the central vein. On the other hand, Rappaport, defined the liver acinus as the functional unit. In this other model, each acini is centered on the portal triad with its terminal branch of portal vein, hepatic artery and bile duct (Sherlock and Dooley, 2007) and defines zones in the hepatocytes between lobules (Malarkey *et al*., 2005) according to the depletion of oxygen and metabolites of the blood as it travels through the sinusoids.

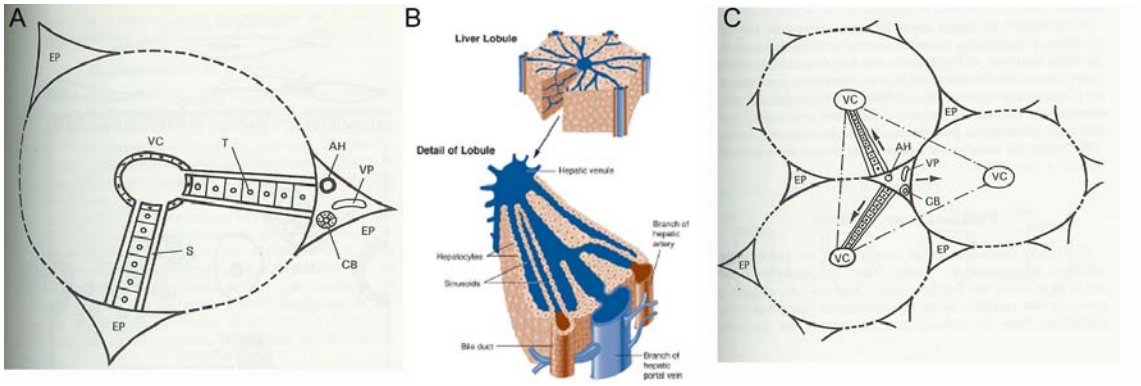


Figure 5.2.: Different models to explain liver microarchitecture A) and B) Scheme of the liver lobule (Domarus *et al*., 1973; Cunningham and Van Horn, 2004), C) Liver acinus centered on the portal triad (Domarus *et al*., 1973).

After a brief overview of the hepatic architechture, the main types of cells that conform the liver will be described below:

Hepatocytes: Hepatocytes are the main functional cells of the liver, and therefore represent a population of highly diferentiated cells. Hepatocytes are arranged in plates or laminae of cords 1 cell thick, branching and forming a continuos labrynth of plates; they are separated from the sinusoidal endothelial cells by the Space of Disse (Dunn *et al*., 1989; Malarkey *et al*., 2005) (Figure 5.3., A and B). They compose the 60% of the liver and they are responsible of most of the functions explained above, for example the synthesis of most of the plasma proteins, the detoxyfication of blood, the biotransformation of drugs, among many others. Therefore, hepatocytes contain all the machinery essential to perform vital functions. Normally, about 15% of the cell, volume is composed of smooth and endoplasmic reticulum, there are about 30 lysosomes and 500 peroxisomes per cell, and about 1000 mitochondria and thousands of enzymes. Sometimes they present multiple nuclei. Hepatocytes are highly polarized cells presenting distinct apical (bile canalicular) and basal (sinusoidal) surfaces that serve different functions (Figure 5.3., A and B). For example, bile transverses the apical surface into the bile duct, and plasma proteins are secreted through the sinusoids to enter into the circulation.

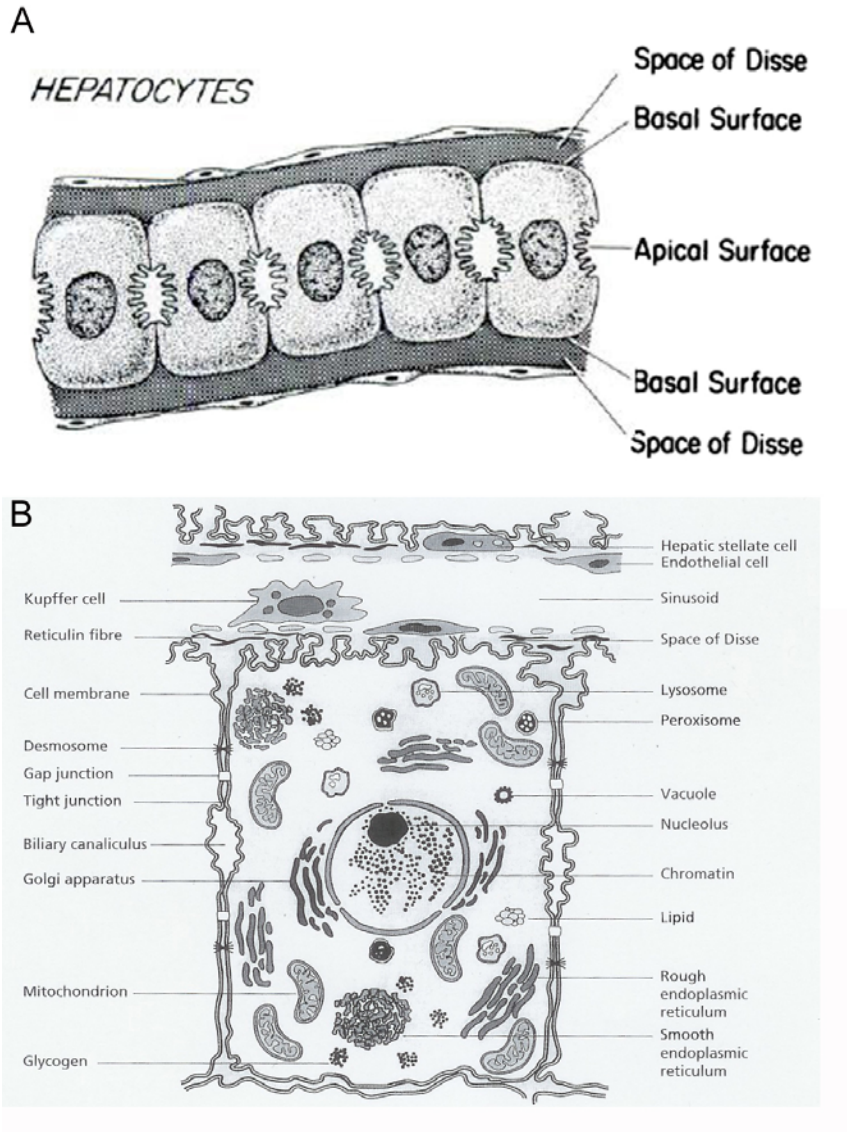


Figure 5.3. A) Hepatocyte cord showing an apical and basal surface and separated from the rest of cells by the Space of Disse. B) Image showing different cell types of the liver. The hepatocyte shows the different types of organelles, the apical surface forming bile canaliculus and gap junctions with the adjacent hepatocyte, and separated from the other cells (Kupffer cells, sinusoidal endothelial cells and stellate cells) in the sinusoids by the space of Disse (Dunn *et al*., 1989; Sherlock and Dooley, 2007)

Sinusoidal endothelial cells (SEC): These cells comprise the 20% of total liver cells. They line the sinusoids, and are the primary barrier between blood and hepatocytes. They filter fluids, solutes and particles between the blood and space of Disse. SEC are a unique type of endothelial cells, they lack an organized basement membrane and contain open fenestrae, characteristics that allow the easy transport of nutrients and macromolecules between the sinusoids and the hepatic parenchyma.