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ROLE OF P73 IN ENDOTHELIAL CELL DIFFERENTIATION, VASCULOGENESIS AND ANGIOGENESIS

Ph.D. Dissertation

Rosalía Fernández Alonso

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ESTUDIO DE LA FUNCIÓN DE P73 EN LOS PROCESOS DE DIFERENCIACIÓN ENDOTELIAL, VASCULOGÉNESIS Y ANGIOGÉNESIS

Tesis Doctoral

Rosalía Fernández Alonso

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Abbreviations

2D	two-dimensional
3D	three-dimensional
ALK	activin receptor-like kinase
ANG	angiopoietin
BAMBI	BMP and activin membrane bound inhibitor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BMPER	BMP endothelial progenitor cell-derived regulator
BMPR	bone morphogenetic protein receptor
BSA	bovine serum albumin
CAM	choriallantoic membrane
CD31	cluster of differentiation 31
CFC	colony forming cell
DAPI	4´,6´-diamidino-2-phenylindole
DBD	DNA-binding domain
DEPC	diethyl pyrocarbonate
Dll4	delta-like 4
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EB	embryoid body
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchimal transcription
EPH	erythropoietin-producing human hepatocellular carcinoma
EPO	erythropoietin
ERK	extracellular signal-regulated kinase
ES	embryonic stem
FBS	fetal bovine serum
FGF	fibroblast growth factor
Flk-1	fetal liver kinase 1
Flt-1	Fms-like tyrosine kinase
GFAP	glial fibrilary acidic protein
GFP	green fluorencent protein
GFs	growth factors
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
HSPG	heparan sulfate proteoglycan
HUVEC	human umbilical vein endothelial cells
IB4	isolectin B4
ID	inhibitor of DNA binding
IGF	insulin-like growth factor

IL	interleukin
KDR	kinase insert domain receptor
KO	knockout
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein quinase
MEF	mouse embryonic fibroblast
mES	mouse embryonic stem
NG2	neuron-glial 2
NICD	Notch intracellular domain
NRP	neuropilin
o/n	overnight
OD	oligomerization domain
OIR	oxygen-induced retinopathy
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PECAM	platelet endothelial cell adhesion molecule 1
PEDF	pigment epithelium-derived factor
PI3K	phosphatidylinositol 3 kinase
PKC	protein kinase C
PlGF	placental growth factor
RE	response elements
RNA	ribonucleic acid
RNAi	RNA interference
ROP	retinopathy of prematurity
RT	room temperature
RTK	receptor tyrosine kinase
RT-PCR	real time- polymerase chain reaction
SAM	sterile alpha-motif
siRNA	small interfering RNA
SMC	smooth muscle cells
TAD	trans-activation domain
TBS	Tris buffered saline
TGF-β	transforming growth factor beta
Tie	tyrosine kinase with Ig and EGF homology
TSP	thrombospondin-1
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPF	vascular permeability factor
WT	wild type

Introduction

1. FORMATION OF BLOOD VESSELS

In higher vertebrates, the complex body architecture is dependent on efficient transport of oxygen, nutrients, signaling molecules and circulating cells between the different organs and tissues. This task is fulfilled by the vascular system, which consist of two highly branched, hierarchical tree-like networks of blood vessels and lymphatic vessels.

The ancient Greek physician Galen already described that blood vessels carried blood and identified venous (dark red) and arterial (brighter and thinner) blood, each with distinct and separate functions. He originally proposed that the arterial blood originated in the heart was distributed and consumed by all organs of the body and was then regenerated in the liver. Only in 1628, William Harvey, according to what Miguel Servet had proposed, discovered that the heart pumps the blood around the body through arteries and that veins return the blood to the heart (Jin and Patterson, 2009). A few decades later, in 1661, Marcelo Malpighi identified the capillaries as the smallest vessels that close the circulatory loop between arteries and veins. Around the same time, Caspar Aselius discovered the lymphatic vessels which return the leaked blood plasma to the blood circulation.

Since then researchers have tried to explain how blood vessels are formed and to elucidate the cellular events and the molecular mechanisms underlying these processes. This has led to a better understanding of the vascular system formation and the mechanisms related to vascular diseases.

In the developing embryo the formation of the vascular system is one of the earliest events during organogenesis (Heinke et al., 2012). Blood vessels provide the growing organs with the necessary oxygen and nutrients to develop as well as with the necessary trophic signals to promote organ morphogenesis. Therefore, the formation of new blood vessels is essential for development. Two distinct processes termed vasculogenesis and angiogenesis lead to the formation of the complex vasculature covering the entire body (Figure 1).

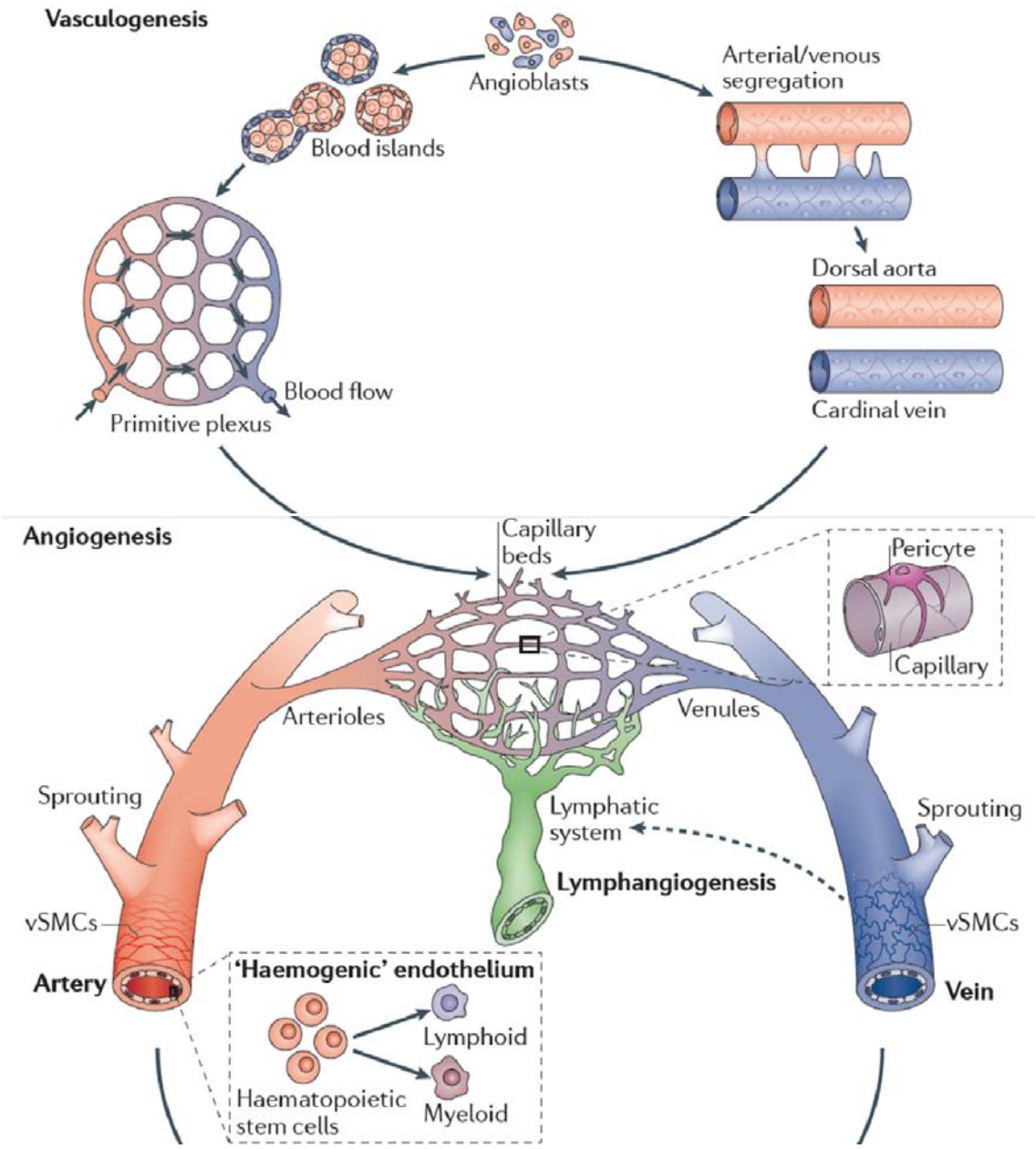

Figure 1: Development of a functional circulation from endothelial progenitors. Endothelial progenitors differentiate from mesodermal cells during early vertebrate development. Intra-embryonic angioblasts migrate and acquire arterial or venous fates. Angioblasts also aggregate to form blood islands that then fuse to generate a primary capillary plexus (From Herbert, 2011).

1.1. Vasculogenesis

Vasculogenesis is the development of new blood vessels via differentiation and assembly of mesodermal precursor cells (Risau and Flamme, 1995), which contributes to form a primary vascular labyrinth of small capillaries. Vasculogenesis was supposed to be restricted to early embryonic development but postnatal vascular development may also involve vasculogenesis, as bone marrow-derived endothelial progenitor cells have been shown to incorporate into the endothelium of new vessels in adults (Ribatti et al., 2001). This so-called neovascularization (George et al., 2011) is critical for revascularization of ischemic tissues and wound healing, but also plays a pathological role in cancer and retinopathies.

Around day 6-6.5 during mouse embryonic development (E6-6.5), primitive endothelial cells begin to arise, first from mesodermal tissue located in the extra-embryonic yolk sac (Ferkowicz and Yoder, 2005) and, afterwards, in the embryo (Drake and Fleming, 2000), allantois (Downs et al., 1998) and placenta (Yamaguchi et al., 1993). The earliest marker for endothelial progenitor cell lineage is fetal liver kinase 1 (Flk-1) which is expressed in a subset of Brachyury-positive cells in the primitive streak (Yamaguchi et al., 1993). In response to endoderm derived signals, including basic fibroblast growth factor (bFGF) and bone morphogenetic protein 4 (BMP4), these progenitor cells migrate to the extra-embryonic yolk sac. Once there, these cells proliferate, aggregate, differentiate and contribute to the formation of blood islands. The outer cells of the blood island flatten and subsequently differentiate into endothelial cells, whereas the inner cells become hematopoietic progenitors (Gonzalez-Crussi, 1971; Kessel and Fabian, 1985). Upon fusion of blood islands, a primitive capillary plexus arise. Thereafter, in the embryo itself, mesodermal angioblast precursors, marked by Flk-1 expression, migrate to the head mesenchyme and other areas. Then, they aggregate to form the dorsal aorta or the cardinal vein without an intermediate capillary plexus. In other areas, local vascular plexus develops, slowly remodel and refine and, together with the extra-embryonic plexus, form the mature vessels and capillary beds of the embryo.

The spatio-temporal proximity of nascent endothelial cells with blood cells in the blood islands led to postulate that both cell populations derive from common precursors (Sabin, 1920). The term hemangioblast was coined years later to name such precursors, (Murray, 1932). Several findings link endothelial and hematopoietic development through a possible common precursor. Indirect evidence came from *Flk-1* deficient mice which showed lack of blood islands and organized blood vessels as well as only few hematopoietic progenitors (Shalaby et

al., 1997; Shalaby et al., 1995). Formal proof of the hemangioblast was provided by a study using Flk-1-positive cells sorted from differentiating embryonic stem (ES) cells. These cells gave rise to single-cell-derived blast colonies (BL-CFCs) able to produce both endothelial and hematopoietic cells (Choi et al., 1998). This result was supported by Huber and coworkers, using isolated hemangioblast cells from E7.5 mouse embryos that could also give rise to both lineages (Huber et al., 2004), as well as by the *in vivo* cell fate tracing studies in zebrafish and chicken (Vogeli et al., 2006; Weng et al., 2007).

Despite a long history of studies in several model organisms, the hemangioblast has remained somewhat elusive and several studies have challenged the concept of a bipotential precursor (Gordon-Keylock and Medvinsky, 2011). This other theory assumes that the hematopoietic lineage arises from an endothelial cell intermediate with hematopoietic potential called hemogenic endothelium, and not directly from a mesodermal precursor. The existence of an endothelium with hemogenic properties was first described at the beginning of 1900´s, when the hemoblast was defined as a cell of hematopoietic lineage in close connection with the endothelium of the developing aorta in embryos of some vertebrates (Adamo and Garcia-Cardena, 2012). By 1916 H. Jordan wrote "there seems to be no escape from the interpretation of such clusters of hemoblast as derivatives from the ventral endothelium of the aorta" (Jordan, 1916).

The first *in vitro* experimental evidence supporting the existence of hemogenic endothelial cells came from the work of Nishikawa and coworkers in 1998. By culturing murine ES cells on type IV collagen-coated dishes, these authors showed that cells expressing both Flk-1 and the endothelial specific marker vascular endothelial cadherin (Flk^+/$VE\text{-}Cadherin^+$ cells) were multipotent and endowed with hematopoietic potential. They also reported that $VE\text{-}Cadherin^+$ cells isolated from murine E9.5 yolk sacs had hemogenic potential (Nishikawa et al., 1998). By the use of time-lapse microscopy and cell tracking methods coupled with an ES cell model, the work of Lancrin (Lancrin et al., 2009) demonstrated that hematopoietic progenitors are produced through a haemogenic endothelium intermediate stage. In the same direction, continuous live imaging of murine ES cells by Eilken (Eilken et al., 2009) showed hematopoietic cell emergence from mesodermal-derived differentiated endothelial colonies. More recently, some independent research groups provided additional data supporting this concept, both in murine embryo sections (Boisset et al., 2010) and in developing zebrafish (Bertrand et al., 2010; Boisset et al., 2010; Lam et al., 2010).

There is now overwhelming evidence supporting the notion that hematopoietic precursors come from a hemogenic cellular precursor (Adamo and Garcia-Cardena, 2012) with an endothelial identity (Chen et al., 2009), which needs to be resident at least for some time in the innermost layer of the vasculature in order to be exposed to shear stress (Adamo et al., 2009). Therefore, although some concepts regarding the origin of these precursors and the dynamics of hemangioblast differentiation *in vivo* remain unclear, there is definite evidence in support of the tight relationship between vascular and hematopoietic development.

1.2. Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing ones. During development, the original vascular plexus originated by vasculogenesis, is extended and gradually modified into a functional circulatory network through a series of morphogenic processes that include: formation of new capillaries, remodeling of preexisting vascular plexi into small and large vessels and pruning of unnecessary blood vessels (Risau, 1997). In addition, to ensure the stability of the newly formed vessels, it is necessary the recruitment of supporting cells and the deposition of a specific basement membrane.

Two principal mechanisms of angiogenesis take place during embryonic development: sprouting angiogenesis and intussusceptive angiogenesis. The first is mainly responsible for vascular growth, whereas the later can also involve vascular remodeling through pruning of excessive blood vessels (Carmeliet and Jain, 2011). The major difference between both is that sprouting angiogenesis is an invasive mechanism into primarily unvascularized tissues, like in the central nervous system. In contrast, intussusceptive angiogenesis requires blood vessels being divided and split by ingrowth of the surrounding tissue, as in the case of lung and skeletal muscle. In some organs, such as the yolk sac, intussusception and sprouting occur side by side (Flamme and Risau, 1992).

During adulthood, most blood vessels remain quiescent and angiogenesis is restricted to a limited number of physiological conditions such as wound healing, cycling ovary and endometrium, and in placenta during pregnancy. Despite this quiescent state, endothelial cells retain the capacity to sense and respond to angiogenic signals. Vascular homeostasis requires a balance between pro- and anti-angiogenic factors, a balance that, when disrupted, leads to the pathogenesis of numerous disorders (Schmidt and Carmeliet, 2011).

1.2.1 Sprouting angiogenesis

Sprouting angiogenesis is the growth of new capillary vessels out of preexisting ones. It is a slow mechanism and it is dependent on cellular proliferation (Semenza, 2007) (Figure 2). The first step during angiogenic sprouting is the selection of the site where the sprout formation is initiated (2a). Only certain endothelial cells within the capillary wall will respond to angiogenic stimulation, denoted tip cells. These cells will provide local guidance cues to their neighbors (stalk cells) and help direct the emerging sprout away from the parent vessel (Chappell et al., 2011). This cell specification allows selecting some cells for migration and some others for proliferation. Otherwise, the indiscriminate migration of all the vessel wall cells would lead to vessel disintegration and, conversely, the proliferation of all the cells would result only in an increased diameter.

Imaging of angiogenic sprouts has shown that endothelial cells dynamically compete for the tip cell position, and so, tip cell is continuously challenged and replaced by migrating cells from the stalk region (Jakobsson et al., 2010). The selection of tip- and stalk-cells depends on delta-like 4 (Dll4)−Notch signaling. Tip cells have higher levels of DLL4, compared with stalk cells, which are subject to higher levels of Notch signaling (Hellstrom et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007). It has been also described that Notch signaling influences the levels of vascular endothelial growth factor receptors (VEGFRs) (Williams et al., 2006) suggesting that relative differences in VEGFRs levels between adjacent cells may explain how Notch signaling limits sprouting (Suchting et al., 2007). In turn, VEGFR activity affects expression of Dll4 (Liu et al., 2003) , implying that the two pathways integrate in an intercellular feedback loop (Jakobsson et al., 2010).

In response to pro-angiogenic molecules such as vascular endothelial growth factor (VEGF), tip cells change their apical-basal polarity and become motile. Besides, in order to be able to migrate outwards from the parent vessel, the basement membrane must be degraded, cell contacts must be loosened and pericytes detached. Different matrix-degrading enzymes will facilitate this step including matrix metalloproteases that are expressed by endothelial or by stromal cells. In response to gradients of pro-angiogenic factors and environmental guidance cues, the tip cell, using their filopodia, guides the stalk out of the sprout, which becomes extended by endothelial cell proliferation (2b) (Eilken and Adams, 2010).

The last step in the angiogenic process is called anastomosis, which is the fusion of two sprouts to establish a new connection between two branches of the growing vascular plexus. Lumen formation has been shown to occur by assembly of vacuoles (Kamei et al., 2006), although other mechanism can be involved (2c). Finally the establishment of blood flow through the vessel provides integrity and promotes vessel maturation processes, such as mural cells (perycite and smooth muscle cells) recruitment and attachment. This event provides anti-angiogenic signals, deposition of extracellular matrix components and reduces endothelial cell proliferation. Furthermore, VEGF expression is down-regulated due to an increased oxygen supply (Adams and Alitalo, 2007).

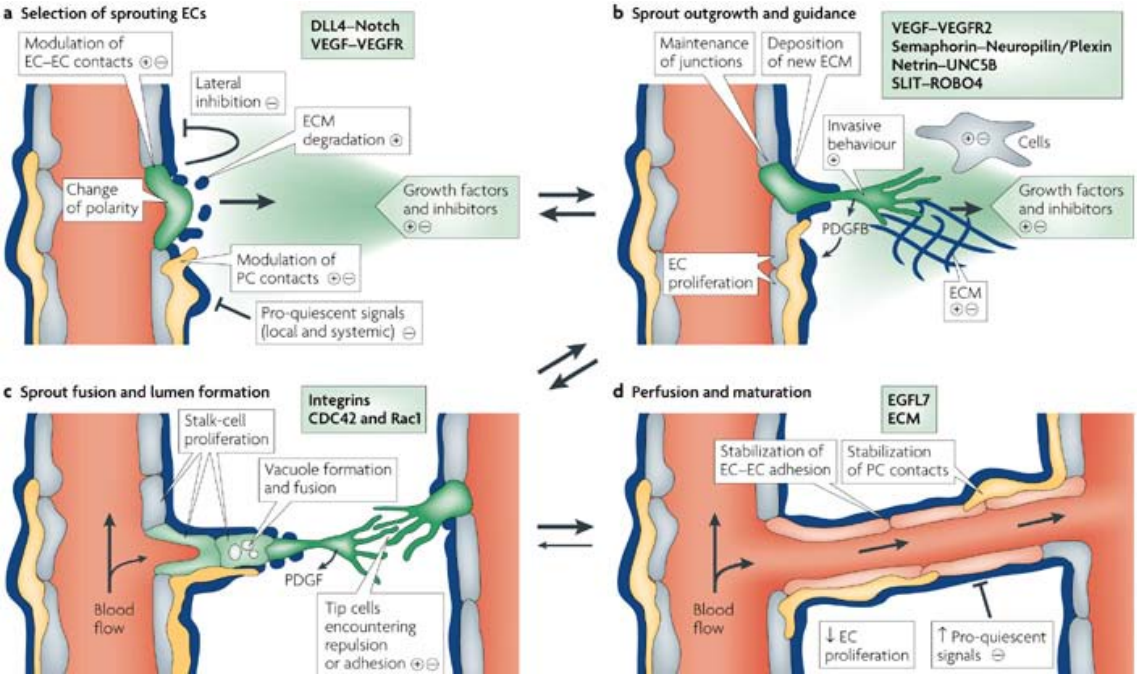

Figure 2: Sprouting angiogenesis. a) In the absence of pro-angiogenic stimuli, ECs are retained in a quiescent state. During angiogenesis pro-angiogenic signals predominate over pro-quiescent ones and some cells are selected as tip-cells. b) The growing tip cell is guided by pro-angiogenic cues. c) Adhesive or repulsive interactions regulate the fusion of adjacent sprouts. d) Finally fusion processes establish a continuous lumen and blood flow promotes maturation. (From Adams, 2010).

1.2.2 Intussusceptive angiogenesis

Intussusceptive angiogenesis, also called splitting angiogenesis or nonsprouting angiogenesis, is a novel mode of blood vessel formation and remodeling, which occurs by internal division of the preexisting capillary plexus without sprouting (Burri and Tarek, 1990; Djonov et al., 2000). It was discovered in the late eighties (Caduff et al., 1986) as a new

mechanism playing a major role in the growth and remodeling of most vascular beds, including that of tumors.

There are some evident differences between sprouting and intussusceptive angiogenesis. As mentioned before, sprouting has the advantage of being invasive; however, it is a relative slow process relying largely on cell proliferation. On the contrary, intussusceptive angiogenesis is a fast process (it can occur within hours or even minutes) and it does not primarily need cell proliferation since it takes place on existing capillary networks. A typical characteristic of intussusceptive angiogenesis is the formation of so called intraluminal tissue pillars that are created by and invagination of the capillary walls into the vascular lumen (Figure 3). The formation of these pillars proceeds through a multistep process (Burri and Djonov, 2002) finally leading to splitting up the initial capillary into two new ones.

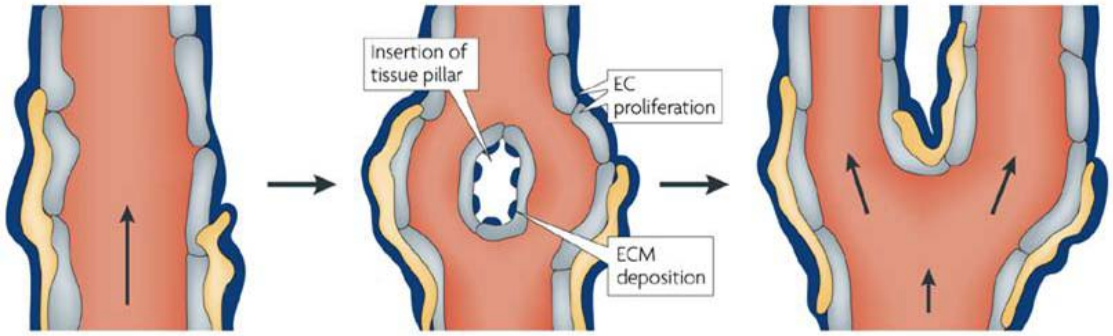

Figure 3: Intussusceptive angiogenesis. The splitting of vessels through the insertion of tissue pillars is a mechanism that leads to the expansion of blood vessels. (From Adams, 2010).

In the majority of vascular beds, the initial network is formed through sprouting angiogenesis and intussusceptive angiogenesis gradually takes over (Makanya et al., 2005). Since its discovery in developing lungs (Caduff et al., 1986), evidence for ongoing intussusceptive angiogenesis has been detected in a wide range of developing tissues such as bone, (De Spiegelaere et al., 2010a), retina (Djonov et al., 2000), muscle (Egginton et al., 2001), kidney (De Spiegelaere et al., 2010b) , mammary gland (Andres and Djonov, 2010) and many more. The current conception is that intussusceptive angiogenesis predominates in the later stages of vascular growth and remodeling, as it is quicker and it is assumed to use less metabolic energy (De Spiegelaere et al., 2011).

Research on the molecular pathways regulating intussusceptive angiogenesis is hampered by the limited availability of experimental models and analytical tools. Signaling molecules like

VEGF, angiopoietins (ANGs), FGF2 and platelet-derived growth factor B (PDGFB) have been described as possible regulators. Erythropoietin (EPO), induced by the hypoxia-inducible factor 2 alpha (HIF2α) under hypoxia conditions, could also act as a regulator (De Spiegelaere et al., 2011). Apart from this, it is known that hemodynamic forces play an important role during the process (Djonov et al., 2002).

1.3 Arteriovenous differentiation

One of the events taking place during maturation of the primary capillary plexus is the specification of arteries *versus* veins. It was formerly assumed that this was a late event in development which was instigated by hemodynamic-flow differences in the two types of vessels. This view changed when specific markers of venous *versus* arterial fate were detected on subsets of developing blood vessels before the onset of circulation (Herzog et al., 2005). Thus, signaling contributes to the fate of blood vessel identity, and arteries and veins can be distinguished by the expression of specific proteins such as Ephrin-B2 and Ephrin receptor B4 (Eph-B4) (Wang et al., 1998), or neuropilin 1 (NRP1) and NRP2 (Herzog et al., 2001; Moyon et al., 2001; Yuan et al., 2002). Although these studies point out to an intrinsic specification of arterial *versus* venous fate, the early vasculature presents considerably plasticity (Moyon et al., 2001) and, therefore, the overall vasculature architecture is probably refined by the hemodynamic of circulatory-flow patterns on top of a genetic programming (le Noble et al., 2004; Zhong et al., 2001).

1.4 Endothelial cell migration during blood vessel formation

Migration is a dynamic, cyclical process whose driving force is provided by the reorganization of the actin cytoskeleton, directing protusion at the front of the cell and retraction at the rear (Sablina et al., 2003). At the same time, cell motility involves making and breaking of attachments between cells and the extracellular matrix (ECM). Migration plays a central role in development, by orchestrating morphogenesis, and contributes to maintain tissue homeostasis; it is also involved in several pathological processes including vascular disease (Ridley et al., 2003). As mentioned in previous sections, migration of endothelial cells is a key process of blood vessel formation.

Cells migrate directionally in response to a variety of cues. Chemotaxis is originated by soluble chemoattractants; in endothelial cells is mainly originated by VEGF, bFGF and angiopoietins. Haptotasis involves migration towards immobilized ligands; in endothelial cells is triggered mainly by intregrins and ECM components. In addition, endothelial cells, which line the interior of blood vessels, are constantly in contact with shear stress which promotes mechanotaxis, migration in response to mechanical forces (Klemke et al., 1997; Lamalice et al., 2007; Li et al., 2005).

Endothelial cells integrate migration stimuli through activation of several molecular pathways that coordinately lead to cytoskeleton remodeling and eventually result in cell polarization and extension of protusions. Three main classes of Rho GTPases, Rac, Cdc42 and Rho, control the formation of different types of cell protusions during migration. Lamellipodia are large and broad extensions situated at the leading edge of the cell (Small et al., 2002). The formation of lamellipodia is associated with an elongated mode of cell motility and is controlled by Rac and Arp2/3 complex. Filopodia are spine-like membrane projections that presumably act as sensors of motile stimuli. Classically, the formation of filopodia is regulated by activation of the small GTPase Cdc42. Stress fibers are actin filaments of inverted polarity linked by α-actinin and myosin and distributed along contractile fibers (Small and Resch, 2005). Stress fibers are anchored at focal adhesions, sites of tight adhesion between the membrane and the ECM on one hand, and the membrane and the cytoskeleton on the other (Lamalice et al., 2007). The formation of actin stress fibres and focal adhesions, which is essential to drive a rounded-bleb-associated mode of motility similar to amoeboid movement, is promoted by RhoA and RhoC (Roger et al., 2006; Sahai and Marshall, 2003). All three structures are involved in endothelial cell motility (Figure 4).

Focal adhesion kinase (FAK) is a converging signaling point between VEGFR2 and integrins and it controls the assembly/disassembly of focal adhesions (Ren et al., 2000; Webb et al., 2004) for endothelial cell migration (Avraham et al., 2003). FAK functions both at the trailing (Iwanicki et al., 2008) as well as the leading edge (Owen et al., 2007) of the cell allowing endothelial cell contraction.

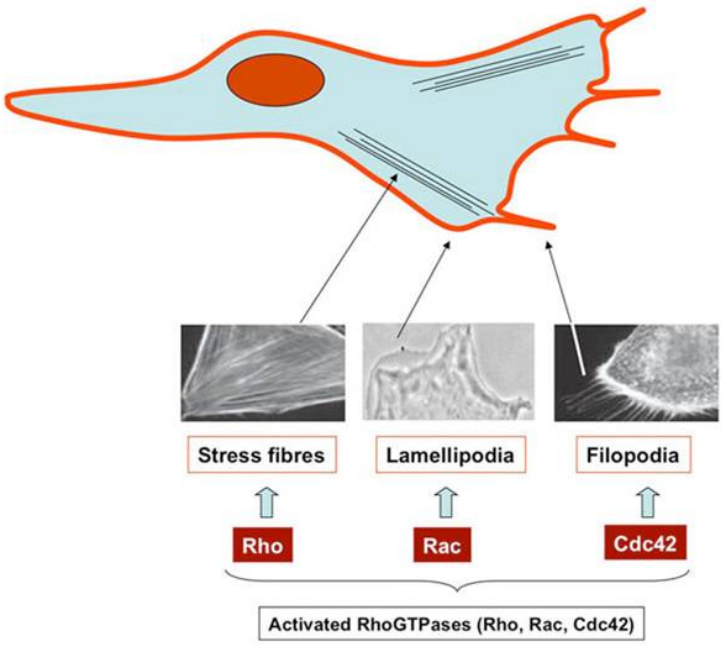

Figure 4: Distinct cellular protusions regulated by RhoGTPases. Three main classes of Rho GTPases, Rac, Cdc42 and Rho, control the formation of different types of cell protusions during migration (From Khan 2009).

1.5 Vascular morphogenesis in disease

Angiogenesis has been involved in more than seventy disorders so far, although due to its incidence and importance, special interest has been focused on tumor angiogenesis. The notion that tumor growth and angiogenesis are related is relatively old. Already in 1945, Glenn Algire and coworkers suggested that tumor progression would depend on sufficient induction of a vascular supply (Algire, 1945). Later on, it was hypothesized that inhibition of angiogenesis would be an effective strategy to treat human cancer (Folkman, 1971) and extensive search for angiogenesis regulators began.

Small tumor lesions can remain dormant for a long time because pro- and anti-angiogenesis factors are in balance. However, at some point during tumor development small clusters of cells will acquire the ability to provoke neoangiogenesis, i.e. "the ability to induce blood vessels at will" (Weinberg, 2007). This so called "angiogenic switch" is clearly an important step in tumor progression. Several stimuli can contribute to this switch. For example, tissue hypoxia can induce VEGF expression when the tumor mass reaches a certain

size; also oncogene activation and tumor-suppressor mutation can increase pro-angiogenic gene-expression.

Among all the molecular regulators of these processes, VEGF has generated particular interest; high levels of VEGF are capable of initiating angiogenesis in a quiescent vasculature. Afterwards, many other molecular (bFGF, ANGs…) and cellular players (tumor cells, infiltrating pro-inflammatory cells, fibroblast…) are involved in the formation and maturation of tumor vasculature, which is characteristically abnormal both structurally and functionally (Figure 5). It is highly irregular, tortuous and chaotic resulting in irregular and inefficiently oxygen and nutrient supply and hence further contributing to induce angiogenesis (Jain, 2005). This vicious cycle led Dvorak to describe tumors as "wounds that do not heal" (Dvorak, 1986).

The formation of blood vessels in tumors may require mobilization of endothelial precursor cells from the bone marrow to become incorporated in the growing vessels (Rafii et al., 2002), which is vasculogenesis. However, most neovascularization seems to occur via angiogenesis either by sprouting angiogenesis or intussusception angiogenesis. In addition, cancer cells can engulf existing blood vessels (vascular co-option), can *trans* differentiate (vasculogenic mimicry) and they can be incorporated into the vessel wall (mosaic vessel formation).

It has become increasingly clear that tumor vessel abnormalities contribute to create a tumor microenvironment that facilitates tumor progression. Therefore, from 1971, the antiangiogenic therapy in oncology has represented a paradigm shift. Nowadays, some antiangiogenic treatments have been used efficiently but still, for antiangiogenic medicine to have a lasting impact on patient survival, an integrated understanding of the molecular principles of vessel growth is needed. Therefore, it is highly relevant to identify novel molecules involved in angiogenesis and to integrate them in the complicated molecular network that orchestrate vascular morphogenesis, with particular emphasis in those differences between normal and tumor vasculature.

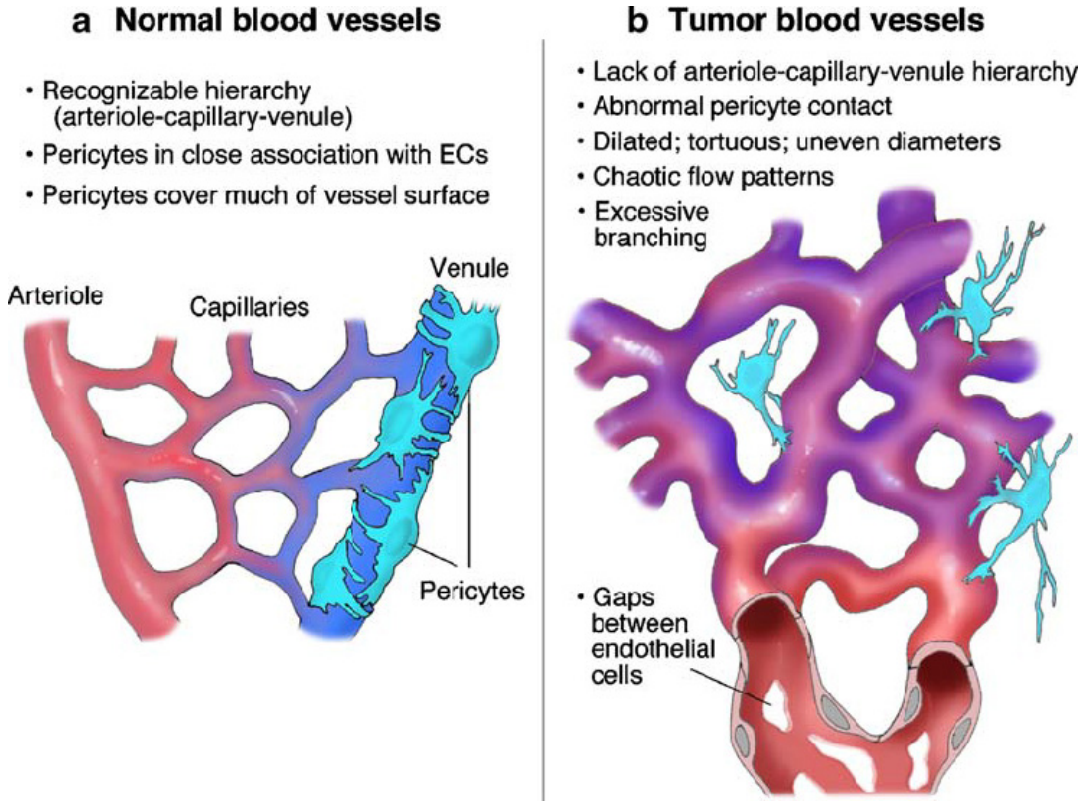

Figure 5: Phenotype of tumor blood vessels. Tumor blood vessels are characterized by loss of hierarchy, abnormal pericyte coverage, tortuosity and excessive branching. (Aird, 2009).

Pathologies characterized by an uncontrolled vessels growth are tumors and many ocular and inflammatory disorders; besides many additional processes are affected such as obesity, asthma, diabetes, cirrhosis, multiple sclerosis, endometriosis, AIDS, bacterial infections and autoimmune disease. On the other hand, some diseases are characterized by a deficiency of angiogenic factors leading to endothelial cell dysfunction, vessel malformation or regression, or preventing revascularization. This is the case in ischaemic heart disease, preeclampsia and many others (Carmeliet, 2005).

2. MOLECULAR REGULATION OF ANGIOGENESIS

The initial process of vasculogenesis seems to be genetically programmed. Characterization of vascular-mutant phenotypes in mice, fish and chick has supported that idea, showing that the vascular system is highly sensitive to genetic disruption (Coultas et al., 2005). In addition, the patterning processes leading to the establishment of the complex vascular system need to be spatio-temporally activated and coordinated. These processes have been shown to depend on a combination of intrinsic pre-patterning and extrinsic responses to environmental parameters. So far, a high variety of signalling molecules (Figure 5) and their corresponding pathways have been identified to be involved in the processes of vascular morphogenesis (Yancopoulos et al., 2000). These include growth factors, chemokines, receptors, adhesion molecules, transcription factors and intracellular signaling cascades that need to be tightly orchestrated in a spatiotemporal manner to achieve the physiologic role of the blood vessels.

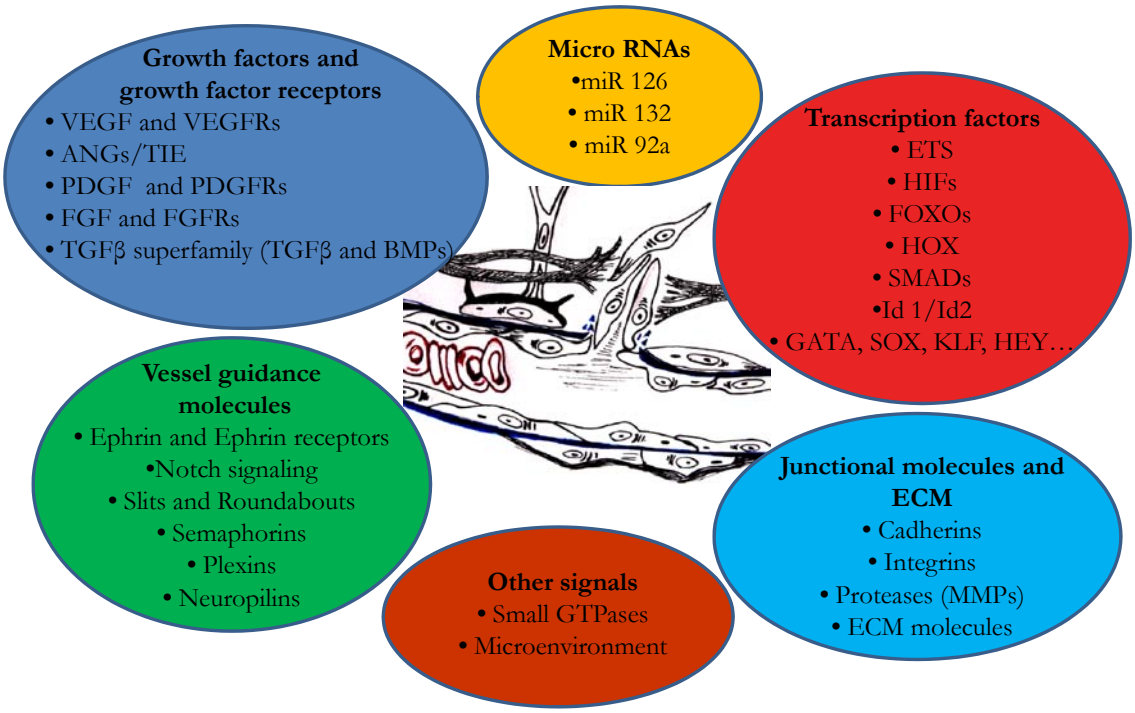

Figure 6: Molecular mechanism involved in vasculogenesis and angiogenesis. There are myriad of molecules involved in regulation of angiogenesis ranging from growth factors to molecules from the vessel microenvironment. (Adapted from http://www.unil.ch/deo/page62557.html).

Besides, it has been shown that signaling pathways that are responsible for physiological vessel formation during embryonic development are reactivated in situations of neoangiogenesis (Bikfalvi and Bicknell, 2002). Therefore, understanding the signaling pathways that are necessary and active during vascular development opens up new possibilities for a more rational therapeutics in pathological conditions like cancer or ischemic diseases in the adult.

2.1 The VEGF (Vascular Endothelial Growth factor) family

The VEGF family of growth factors comprises five members with partially overlapping functions during angiogenesis and lymphangiogenesis: VEGFA (the prototype), VEGFB, VEGFC, VEGFD and placenta growth factor (PlGF) (Ferrara et al., 2003). The complexity is further increased by alternative splicing (VEGFA, VEGFB and PlGF) and alternative processing (VEGFA, VEGFC and VEGFD) (Ferrara, 2010; Koch et al., 2011). The VEGF ligands are usually homodimeric polypeptides, produced by most parenchymal cells and act in a paracrine manner on adjacent endothelial cells to regulate angiogenic response. In addition, autocrine VEGF signaling is required for endothelial cell survival under non-pathological conditions in a cell-autonomous manner which does not contribute to the angiogenic response (Lee et al., 2007).

VEGFA, originally identified as vascular permeability factor (VPF), was the first member to be discovered (Senger et al., 1983)and it has been shown to be indispensable for vascular development; as a matter of fact, deletion of just one *Vegfa* allele leads to embryonic lethality due to strong vascular defects (Carmeliet et al., 1996; Ferrara et al., 1996). VEGFA is expressed as multiple alternatively spliced isoforms, namely VEGFA-121, VEGFA-145, VEGFA-165 and VEGFA-189 (Ladomery et al., 2007). In addition, there are a series of VEGFA splice variants (denoted VEGFA-xxxb) that are competent for binding but not able to efficiently activate VEGFRs; they have been described as anti-angiogenenic factors (Harper and Bates, 2008).

VEGFA variants differ in their ability to bind VEGFRs, heparan sulfate proteoglycans (HSPGs) and NRPs (Grunewald et al., 2010; Uniewicz and Fernig, 2008). The larger VEGF isoforms, VEGFA-165 and VEGF-189 bind to HSPGs, which are abundantly present on the plasma membrane of most cells as well as in the extracellular matrix. Therefore, these isoforms are retained in the tissue creating concentration gradients that guide migrating endothelial cells.

Additionally, VEGFA-165 and VEGFA-189 bind to NRP1 which acts as co-receptor for signaling via VEGFR2. On the other hand, VEGF121 which does not bind to either NRPs or HSPGs is freely diffusible in the tissue and activates VEGFR2 although less efficiently than other co-receptor binding forms. Invasive angiogenesis *in vitro* is preferentially induced by VEGFA-165 (Jakobsson et al., 2007).

VEGF ligands act through three receptor tyrosine kinases (RTKs), VEGFR 1-3. VEGFA mainly transmits its pro-angiogenic activity by binding to VEGFR2, also known as kinase insert domain receptor (KDR) in humans, and Flk-1 in mice. VEGFR2 is highly expressed in vascular endothelial cells and their embryonic precursors although it is also found in a range of non-endothelial cells such as pancreatic duct cells, retinal progenitor cells, megakariocytes and hematopoietic cells (Oelrichs et al., 1993). VEGFR2 is alternatively spliced to a soluble VEGFR2 isoform (sVEGFR2) which is present in various tissues such as the skin, heart, spleen, kidney, ovary and in plasma (Koch and Claesson-Welsh, 2012). It can also bind VEGFC, preventing its binding to VEGFR3 and inhibiting lymphatic endothelial cell proliferation (Albuquerque et al., 2009).

VEGFR2 is known to transduce the full range of VEGF responses, i.e. regulating endothelial survival, proliferation, migration and formation of the vasculature tube. Accordingly, knockout of *Vegfr2* in mice is, similarly to *Vegfa*-/-, lethal at day E8.5 because of impaired development of hematopoietic and endothelial cells (Shalaby et al., 1995). Binding of VEGFA to VEGFR2 promotes receptor dimerization and subsequent tyrosine phosphorylation of the two receptor chains, creating docking sites for several adaptor molecules (Olsson et al., 2006). Consequently several key signaling molecules are activated triggering three main pathways (Figure 6): phosphatidylinositol 3 kinase (PI3K), protein kinase C (PKC), and mitogen activated protein kinases (MAPKs) p42/44 and p38, which regulate endothelial survival, proliferation, migration and vascular permeability (Koch and Claesson-Welsh, 2012).

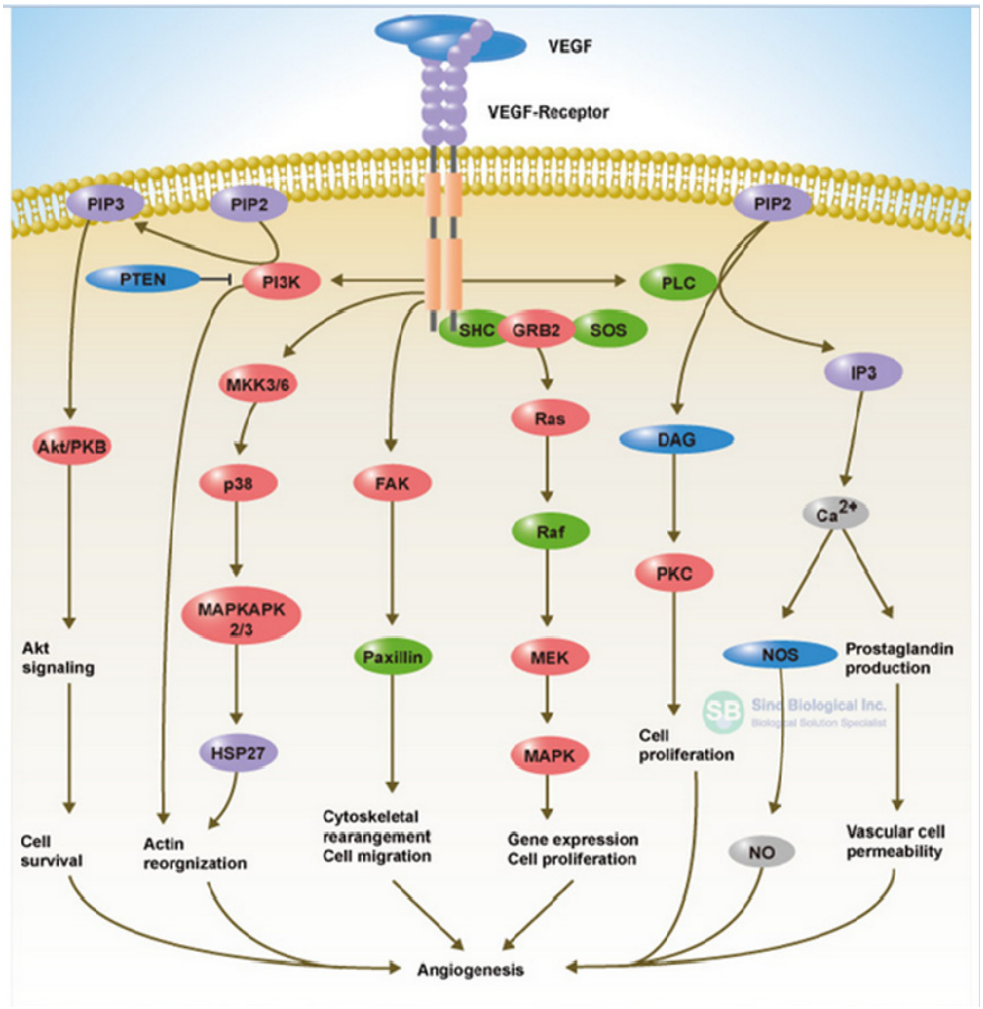

Figure 7: VEGF signaling. Upon VEGF binding, several downstream mediators are activated. The complex network of intracellular signals eventually leads to a coordinated biological response. (Downloaded from Sino Biological Inc www.sinobiological.com).

VEGFA binds also to VEGFR1, alternatively denoted Fms-like tyrosine kinase (Flt-1) in mice. VEGFR1 is widely expressed; its kinase activity is weak and is not required for endothelial cell function. VEGFR1 also exists as an alternatively spliced soluble isoform (sVEGFR1) (Kendall and Thomas, 1993). Full length VEGFR1 and sVEGFR1 both bind VEGF with much higher affinity than VEGFR2 does; therefore they have been suggested to act as decoy receptors limiting angiogenesis (Fong et al., 1995). In fact, *Flt1*-/- embryos die at day E9.0 due to increased proliferation of endothelial cells and a severe disorganization and dysfunction of the vascular system (Fong et al., 1999) .

VEGFB and PlGF can also mediate pro-angiogenic signals by binding to VEGFR1, although to a tenfold less extent when compared to VEGFA binding to VEGFR2 (Shibuya,

2008). VEGFB has been implicated in the development of cardiac vasculature, endothelial survival and uptake of fatty acids (Bellomo et al., 2000; Hagberg et al., 2010; Zhang et al., 2009). PlGF has been involved in regulating angiogenesis in pathological conditions, including tumor growth. *Vegf*b and *Plgf* knockout mice are viable (Aase et al., 2001; Bellomo et al., 2000) (Carmeliet et al., 2001).

VEGFC and VEGFD bind to VEGFR3 and stimulate lymphangiogenesis (Karkkainen and Alitalo, 2002). Besides, VEGFR3, alternatively denoted Flt-4, is highly expressed in tip cells and positively regulates angiogenic sprouting (Nilsson et al., 2010).

2.2 Angiopoietins/Tie Signaling

Other RTKs members of the endothelial cell lineage are TIE1 and TIE2 (tyrosine kinase with Ig and EGF homology receptors) and their secreted corresponding ligands, the angiopoietins: ANG1and ANG2 (Augustin et al., 2009). This is a binary system that allows vessels to maintain quiescence while remaining able to respond to angiogenic stimuli (Saharinen et al., 2010; Seegar et al., 2010).

ANG1 is expressed by mural cells and fibroblast and activates TIE2 in endothelial cells acting as a TIE2 agonist *in trans*. This leads to the stabilization of endothelial junctions, the recruitment of pericytes and to basement membrane deposition, thereby promoting vessel tightness and ultimately leading to vascular quiescence (Fukuhara et al., 2008). In addition, Saharinen and coworkers have described a novel mechanism of how ANG1 differentially activates the TIE2 receptors at endothelial cell-cell junctions and at cell-extracellular matrix (ECM) contacts, mediating endothelial stability and migration, respectively (Saharinen et al., 2008) (Figure 7).

ANG2 is expressed by endothelial cells and stored in specialized granules (Weibel-Palade bodies). In the presence of angiogenic or inflammatory activation, sprouting endothelial cells release ANG2, which antagonizes ANG1 and TIE2 signaling to enhance mural detachment and vascular permeability. ANG2 sensitizes endothelial cells to angiogenic and pro-inflammatory signals, which may lead to vessel sprouting or regression dependent on tissue and biological context (Fiedler et al., 2006; Holash et al., 1999).

TIE1 signaling is less well known but it has been shown to dynamically interact with TIE2 and modulating its function upon binding of different angiopoietins (Seegar et al., 2010). TIE1 and VEGF signaling are functionally linked. TIE1 is cleaved by gamma secretases following VEGF stimulation; the cleaved form, remaining in the cell membrane, interacts with TIE2 and is hypothesized to support TIE2 signaling (Marron et al., 2007; Tsiamis et al., 2002).

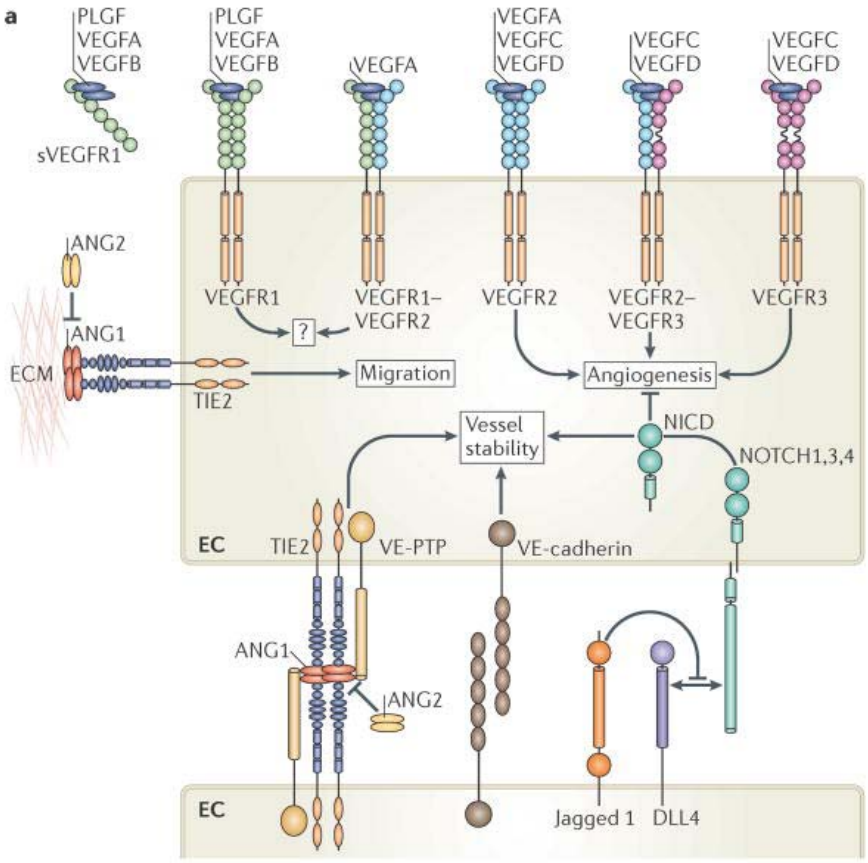

Figure 8: Signaling pathways that control angiogenesis. This figure shows some of the general signaling pathways that control endothelial cell behavior: VEGFs bind to homodimers and heretodimers of VEGFRs; signaling via VEGFR2, VEGFR3 or VEGFR2/VEGFR3 is pro-angiogenic whereas binding of VEGFA to VEGFR1 limits the availability of VEGFA to activate VEGFR2. The interaction of TIE2 receptor with ANG1 at ECM induces migration, while at cell-cell contact ANG1-TIE2 interaction promotes quiescence. ANG2 antagonizes ANG1 to destabilize vessels. VE-Cadherin interactions maintain cell-cell junctions. DLL4-mediated activation of NOTCH promotes vessel stability; Jagged 1 competes with DLL4 to decrease Notch signaling. (From Herbert, 2011).

2.3 The fibroblast growth factor (FGF) family and other pro-angiogenic factors

FGFs represent a large protein family with 22 members identified so far, some of them having potent pro-angiogenic functions (Murakami and Simons, 2008). FGFs activate their receptors (FGFRs) on endothelial cells or indirectly stimulate angiogenesis by inducing the release of angiogenic factors from other cell types (Beenken and Mohammadi, 2009). For instance, in the heart, FGF-mediated signaling sustains vessel growth by stimulating the release of ANG2 and VEGFB.

Also the epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin like growth factor (IGF) have been reported to exhibit pro-angiogenic function, mainly by inducing endothelial proliferation and survival but also by up-regulating factors like VEGF (Shibuya, 2008).

2.4 The platelet derived growth factor (PDGF) family

PDGFs are growth factors comprising a family of four structurally related members (A-D) binding to their appropriate RTKs (PDGFR-α or -β) (Cao et al., 2008). During embryonic development, PDGF signaling has been shown to be indispensable for proliferation and migration of pericytes and smooth muscle cells (SMCs). PDGF-B is secreted by endothelial cells, probably in response to VEGF stimulation, to recruit pericytes which express PDGFR-β (Holmgren et al., 1991; Lindahl et al., 1997). Additionally, pericytes themselves can secrete PDGF-B, thereby amplifying the signal (Hellstrom et al., 2001). Deficiency of either PDGF-B or PDGFR-β in mice leads to severe vascular defects as leakiness of vessels or overproliferation of endothelial cells due to the lack of pericytes (Hellberg et al., 2010; Hellstrom et al., 2001; Lindahl et al., 1997; Soriano, 1994).

2.5 TGF-β superfamily

TGF-β (transforming growth factor-β) superfamily is composed by four major subfamilies, each one playing different roles in development: the Mullerian inhibitory substance (MIS) family, the inhibin/activin family, the TGF-β family and the bone morphogenetic protein (BMP) family. The ligands of the TGF-β superfamily mediate their effect by binding to a complex of type I and type II serine/threonine kinase transmembrane receptors (Goumans et

al., 2009). Seven different type I receptors (including activin receptor-like kinase-ALK 1 to 7), and five different type II receptors (including TGF-β receptor II-TGF-βRII and BMPRII) have been identified. The BMP and activin membrane bound inhibitor (BAMBI) is a pseudoreceptor for the TGF-β pathway (Onichtchouk et al., 1999). It has the extracellular ligand-binding and trans-membrane domains, resembling type I receptors, but it lacks the intracellular kinase activity. This special structure allows BAMBI to compete with type I receptors for ligand interaction, blocking the downstream signaling of the TGF-β family (Grotewold et al., 2001) (Guillot et al., 2012).

Upon ligand binding, type I TGF-β receptors are trans-phosphorylated by the constitutively phosphorylated type II receptor. The activated type I receptor phosphorylates receptor-regulated SMADs (R-SMADs), which form complexes with other SMADs and move into the nucleus, where they regulate transcription of target genes (Miyazono, 2000). In addition to the SMAD-dependent signaling pathway, the TGF-β-activated kinase 1 (TAK1) pathway is one of the SMAD-independent signaling pathways that transduce TGF-β downstream effects (Yumoto et al., 2013; Zhang, 2009).

2.5.1 TGF-β subfamily

TGF-β subfamily regulates yolk sac vasculogenesis, hematopoiesis as well as cardiac development and lung morphogenesis. Endothelial cells release TGF-β which induces attraction and differentiation of mesenchymal progenitor cells into pericytes and smooth muscle cells, as well as synthesis of extracellular matrix (Chen et al., 2007). Besides its role in recruitment of mural cells, gene knockout studies revealed a relevant role of TGF-β1 during hematopoiesis and vasculogenesis. *Tgf-β1* knockout mice showed defective endothelial differentiation resulting in inadequate capillary tube formation and weak vessels with reduced cellular adhesiveness (Dickson et al., 1995). *In vitro* studies demonstrated that TGF-β1 has a context dependent biphasic effect on endothelial cells: at low concentrations, extracellular TGF-β1 induces pro-angiogenic effects in endothelial cells, while at high concentrations it leads to cytostasis, synthesis of extracellular matrix and vessel muscularization, thereby controlling the status of quiescent endothelium (ten Dijke and Arthur, 2007). Furthermore, it has been reported that TGF-β family members could additionally affect angiogenesis in a paracrine manner, via stimulation of pro-angiogenic molecules like VEGF (Deckers et al., 2002).

TGF-β signaling activated through ALK-5 is mainly transduced by SMAD2/3 whereas TGF-β signaling activated through ALK-1 is mainly mediated by SMAD1/5/8 and leads to activation of endothelial cell migration and proliferation (Goumans et al., 2002).

2.5.2 BMP subfamily

BMPs are another family of signaling molecules. They were originally found to induce ectopic bone formation when implanted into muscle and later described as regulators of anterio-posterior axis formation, neurogenesis and gonadogenesis (Balemans and Van Hul, 2002; Hogan, 1996; Shimasaki et al., 2004). In mammals, the BMP family is composed of the BMP group (BMP2 to BMP17) and the growth and differentiation factor (GDF) group. BMP signaling is initiated by BMP type I receptors (ALK1, ALK2, ALK3, BMP type IA receptor -BMPR-IA- and ALK6 -BMPR-IB-) and type II receptors: BMP RII, activin type II A receptor (ACTR-IIA) and ACTR-IIB. The R-Smads for BMP downstream signaling are SMAD1/5/8. BMP-dependent functions are mediated by a series of direct target genes in different cells and tissues, including *Id1, Id2, Id3, Gata2* and *Fgfr2* (Miyazono et al., 2005).

BMP4 was shown to be indispensable for the generation of $VEGFR^+$ embryonic endothelial progenitor cells in murine and human ES cells models cells (Little and Mullins, 2009; Park et al., 2004). BMP2/4 expression has also been observed in endothelial progenitor cells derived from adult peripheral blood, being related to the capacity to promote neoangiogenesis .

More insights come from deletion studies in mice; homozygous mutant *Bmp4* embryos die between E6.5 and E9.5 with a variable phenotype, but most of them do not express the mesodermal marker Brachyury and show little or no mesodermal differentiation (Winnier et al., 1995). Mice lacking either *Alk2* or *Alk3* or *BmrpII* fail to complete gastrulation and die around E7.5-E9.5. Likewise, deletion of intracellular effectors *Smads* leads to some defects in vascular development that vary depending on the affected Smad (Heinke et al., 2012).

BMP endothelial progenitor cell-derived regulator (BMPER) was identified as a BMP-binding protein expressed by endothelial cell precursors that played a role in endothelial cell differentiation by modulating local BMP activity (Moser et al., 2003). Later on, BMPER was described to be necessary for endothelial cell sprouting and to have a dose dependent stimulating effect on sprouting and migration, being involved in SMAD1/5 and ERK1/2 signaling (Heinke et al., 2008). Some other studies showed evidences supporting anti-BMP

activity for BMPER. A hypothesis proposed by Hammerschmidt's group suggests that BMPER activity on BMP signaling is dependent on its proteolytic processing. In that scenario, uncleaved BMPER behaves as an anti-BMP regulator, whereas cleaved BMPER plays a pro-BMP role since only uncleaved BMPER can bind to the extracellular matrix through heparin (Rentzsch et al., 2006).

All the above signaling pathways interact to orchestrate the different cellular events involved in the complex physiological process of blood vessel formation (Figure 9).

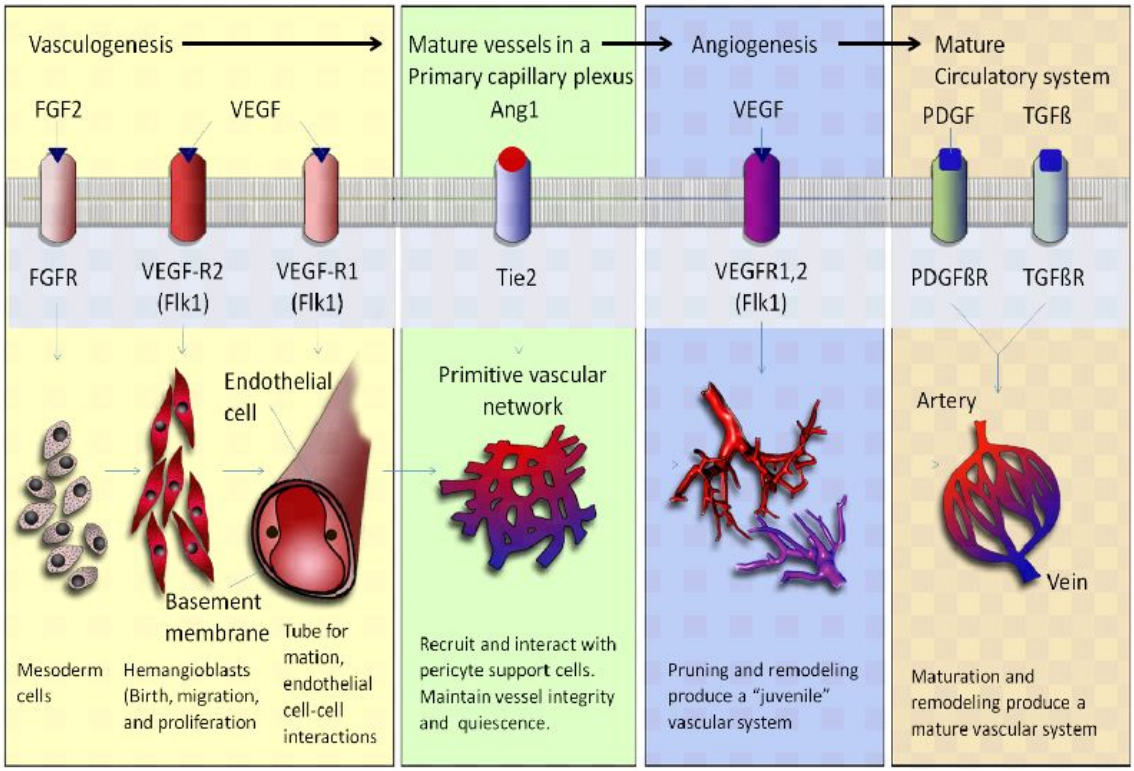

Figure 9: Molecular regulation of vasculogenesis and angiogenesis. This figure summarize some of the molecular mechanism referred in this section together with the physiological processed that they **regulate** (From Li, 2008).

2.6 Vessel guidance molecules

Endothelial tip cells sense guidance cues, similar to how axonal growth cones explore their surroundings. Nowadays, evidence is emerging that blood vessels, which arose later in evolution than nerves, recruit analogous mechanism than neurons to control directional migration (Carmeliet and Tessier-Lavigne, 2005).

2.6.1 Ephrins and Ephrin receptors

Erythropoietin-producing human hepatocellular carcinoma (EPH) receptors are RTKs that bind and are activated by cell surface proteins called ephrins (Kuijper et al., 2007). They were initially described as neuron-specific molecules that could mediate guidance signals on growing neurons (Flanagan and Vanderhaeghen, 1998). Ephrin B2 and its cognate receptor EPHB4 have key roles during blood vessel morphogenesis and arterio-venous specification (Herbert et al., 2009). In addition, it has been reported that Ephrin B2 may directly interact with VEGFRs acting as a positive regulator of VEGFR endocytosis in tumors, which then activates downstream signaling required for VEGF-induced tip cell filopodia (Sawamiphak et al., 2010).

2.6.2 Notch signaling pathway

Notch signaling is an evolutionarily conserved pathway that has well-established roles in cell fate determination in all metazoans. Ligand binding induces NOTCH receptor cleavage and the release of an intracellular fragment, Notch intracellular domain (NICD) which functions as a key transcriptional regulator during cell fate specification (Schroeter et al., 1998). As mentioned before, several studies have implicated Dll4 mediated Notch signaling in the selection of tip- and stalk-cells (Jakobsson et al., 2010). While in tip cells, in response to VEGF, activation of VEGFR2 up-regulates *Dll4* expression, in neighbouring stalk cells DLL4 activates NOTCH which in turn down-regulates *Vegfr2* but up-regulates *Vegfr1*. Therefore, Dll4-Notch signaling efficiently suppress tip cell fate by negatively regulating VEGF signaling. Consequently, the reduced Dll4-Notch signaling *in vivo* is accompanied by excessive tip cell formation, uncontrolled sprouting and disordered vessel branching (Suchting et al., 2007). Besides, Notch activation blocks also VEGFR3 expression in stalk cells such that they become less responsive to VEGF (Siekmann and Lawson, 2007).

Another Notch ligand, JAGGED1, is expressed by stalk cells and promotes tip cell formation and angiogenesis (Benedito et al., 2009). JAGGED1-NOTCH interactions do not induce productive Notch signaling. Indeed, JAGGED1 competes with DLL4 for binding to NOTCH receptor on tip cells and thereby suppressing tip cell Notch signaling.

A feedback loop has been also described between NOTCH and WNT. NOTCH activates WNT signaling in proliferating stalk cells during vessel branching (Jakobsson et al., 2010), explaining how NOTCH, which usually suppresses proliferation and promotes quiescence,

stimulates proliferation of stalk cells *in vivo*. WNT also activates NOTCH and so WNT signals in endothelial cells induce a NOTCH like phenotype characterized by branching defects and aberrant vascular remodeling (Corada et al., 2010).

2.6.3 Other axonal guidance molecules involved in angiogenesis

Apart from the ones already mentioned, three families of axon guidance cues and their receptors are involved in angiogenic signaling: Slits and Roundabouts, Netrins and UNC5B, and Semaphorins, Plexins and Neuropilins (Adams and Eichmann, 2010).

2.7 Junctional molecules and role of extracellular matrix (ECM)

The ECM plays an important role in angiogenesis. It provides the structural support necessary for blood vessel formation and supply multiple endogenous pro- and anti-angiogenic factors that regulate endothelial cell survival and vessel stability (Davis and Senger, 2005). During angiogenesis, the ECM basement membrane is degraded by proteases such as matrix metalloproteases (MMPs) that promote endothelial cell migration. These proteases also liberate immobilized angiogenic factors from the ECM, including VEGF and bFGF (Bergers and Coussens, 2000). On the other hand, proteins or protein fragments that inhibit angiogenic processes are also associated with the ECM. These endogenous inhibitors include proteins such as Perlecan, Endostatin, DANCE/Fibulin-5, Thrombospondins (TSP), and fragments of Collagen and Fibronectin. The levels of pro- and anti-angiogenic factors in the ECM are critical for determining whether new vessels can be stably formed.

Quiescent endothelial cells form a monolayer of interconnected cells whereas angiogenic endothelial cells dissociate their junctions to migrate. Endothelial cells have at least two specialized adhesive junctional regions that are comparable to adherens junctions and tight junctions found in epithelial cells. Adherens junctions and tight junctions have different functions. Adherens junctions play an important role in contact inhibition of endothelial cell growth, paracellular permeability to circulating leukocytes and solutes and they are required for a correct organization of new vessels in angiogenesis. Tight junctions provide a barrier within the membrane, by regulating paracellular permeability and maintaining cell polarity (e. g. brain blood barrier) (Bazzoni and Dejana, 2004).

Endothelial cells express cell-type specific transmembrane adhesion proteins such as vascular endothelial cadherin (VE-Cadherin) at adherens junctions (Dejana et al., 2009). Loss of VE-Cadherin does not prevent vessel development but induces defects in vascular permeability, remodeling and integrity (Carmeliet, 2003). Junctional complexes trigger intracellular signals in endothelial cells in a variety of different ways. For instance, VE-Cadherin, can form a multiprotein complex with VEGFR2 and limits its internalization and proliferative signals while activating TGF-β signaling. During sprouting, the adhesive function of VE-cadherin between adjacent cells is reduced by endocytosis in response to VEGF and angiogenic factors (Dejana et al., 2009). At the same time, the localization of VE-Cadherin at filopodia allow tip cells to establish new cell contacts.

Integrins are heterodimeric receptors (α, β) that mediate adhesion of endothelial cells to ECM components. Heterodimeric pairing of integrin α and β subunits confers specificity of binding to one or more substrates such as vitronectin, fibrinogen and fibronectin (Humphries et al., 2006). Hence the integrins expressed on the surface of a cell will determine whether it can adhere and survive in a particular microenvironment. These adhesive interactions provide survival cues and traction for invading endothelial cells. Besides, integrins regulate angiogenesis by binding to growth factors (VEGF, FGFs and ANG-1) or their receptors (VEGFRs, FGFRs) stimulating vessel growth. They also up-regulate zymogen proteases in invading tip cells and promote vessel maturation. Some intregrins play an important role in tumor angiogenesis and, in fact, integrin blockers are being evaluated in the clinic (Carmeliet and Jain, 2011).

2.8 Transcription factors

Whereas numerous growth factors, receptors and signaling molecules involved in angiogenesis have been identified, the transcriptional regulation of such angiogenic molecules is less explored. Numerous transcription factors seem to have important roles for differentiation of endothelial cells and for the formation of blood vessels; members of the E-twenty six (ETS) family play a central role in vascular development (Dejana et al., 2007). ETS binding motifs are present in virtually all analyzed endothelial promoters and enhancers including *VEGFR1*, *VEGFR2*, *TIE1* and *TIE2*. However, no ETS factor is unique for vasculature and ETS binding sites are not specific to endothelial-expressed genes (Hollenhorst et al., 2004). It has

been hypothesized that ETS proteins may achieve tissue specific activation through binding to lower affinity sites in cooperation with other proteins (Hollenhorst et al., 2007).

Members of the hypoxia induced factor (HIF) family are activated by hypoxia. They initiate a broad transcriptional response to increase the oxygen supply by angiogenesis through the up-regulation of angiogenic factors such as VEGF (Fraisl et al., 2009). HIFs are also activated in non-hypoxic conditions by oncogenes and growth factors, allowing tumor cells to stimulate angiogenesis.

Members of the Forkhead (FOX) transcription factor family also play important roles in vascular endothelial development as well as in arterio-venosus differentiation. These transcription factors have also a tumor suppressor potential: as a matter of fact, mice lacking all three FOXOs developed hemangiomas and angiosarcomas (Paik et al., 2007).

The homeobox (HOX) proteins interact with numerous regulatory pathways related to angiogenesis like FGF or BMP (Coultas et al., 2004). They are also reported to regulate expression of different angiogenesis related molecules: *Ephb4*, *Vegfr2* and integrins (Bruhl et al., 2004; Wu et al., 2003) .

It has been exposed before that SMAD transcription factors are components of the TGF-β superfamily signaling pathway. Regarding angiogenesis, SMAD5 can be considered indispensable since null embryos reveals multiple vascular defects (Chang et al., 1999). Furthermore SMAD3 is reported to stimulate VEGFA expression (Sanchez-Elsner et al., 2001). In contrast, SMAD2 and SMAD4 have been shown to inhibit angiogenesis by promoting expression of anti-angiogenic factors such as TSP-1 (Hamik et al., 2006).

The inhibitors of DNA binding (ID) 1-3 are expressed during development in endothelial cells and in tumors blood vessels, controlling the expression of different molecules such as integrins (Benezra et al., 2001).

Other transcription factors families being relevant in angiogenesis are GATA, sry-related HGM box (SOX), kruepple-like factor (KLF) and the hairy and enhancer of split-related (HEY) (Heinke et al., 2012).

2.9 microRNAs

miRNAs are a large family of small (≈22 base pairs) non-coding single-stranded RNAs that are key post-transcriptional regulators of gene expression. miRNAs function by imperfect binding to the 3´-untraslated regions of different mRNAs and act to fine tune gene expression (Lewis et al., 2005). The key enzymes to process miRNAs, Drosha and Dicer, have been targeted in endothelial cells by RNA interference resulting in decreased angiogenesis *in vitro* (Kuehbacher et al., 2007; Suarez et al., 2007). Interestingly, the tripartite motif-containing protein 32 (TRIM-32), which is involved in regulation of some miRNA has been identified as a target of p73 for our group (Gonzalez-Cano et al, 2013).

Of interest some miRNAs are highly expressed in endothelial cells, namely miR-221/222, miR-21, the let-7 family, the miR-17-92 cluster, the miR-23-24 cluster and the endothelial cell specific miR-126. miR-126 has been shown essential for normal angiogenesis in mice and zebrafish (Fish et al., 2008; Kuhnert et al., 2008; Wang et al., 2008). Actually, deletion of miR-126 in mice results in severe vascular abnormalities: systemic edema and multifocal hemorrhages. *In vitro* or *ex vivo* experiments revealed miR-126 to be involved in endothelial cell sprouting, migration, proliferation and cytoskeleton organization (Heinke et al., 2012). Expression of miR-126 positively influences VEGF-induced signaling by translational inhibition of negative regulators of the Akt and MAPK signaling pathways, PI3K and SPRED1 respectively. Recently, a connection between hemodynamics and miR-126 has also been described in zebra fish mediated by Klf2a (Nicoli et al., 2010).

Other vascular miRNAs seem to have critical roles during pathological angiogenesis such as miR-132 (Anand et al., 2010) and miR-92a (Bonauer et al., 2009).

3. MODELS OF ANGIOGENESIS

The formation of blood vessels is a systemic process in which different cell types and several biological mechanisms are involved. In order to understand the cellular mechanisms and molecular pathways comprising the formation of blood vessels, the development of models that recapitulate the spatio-temporal events that occur during vascular morphogenesis is crucial. The availability of relevant models of angiogenesis is also important to ensure rigorous preclinical testing and validation of new agents for the treatment of angiogenic-related diseases.

Some of the widely used models of angiogenesis are summarized in Figure 10. In order to address different biological questions involved in blood vessel formation, we have used several *in vitro* and *in vivo* models during the completion of this Thesis. Those ones are described below with more detail.

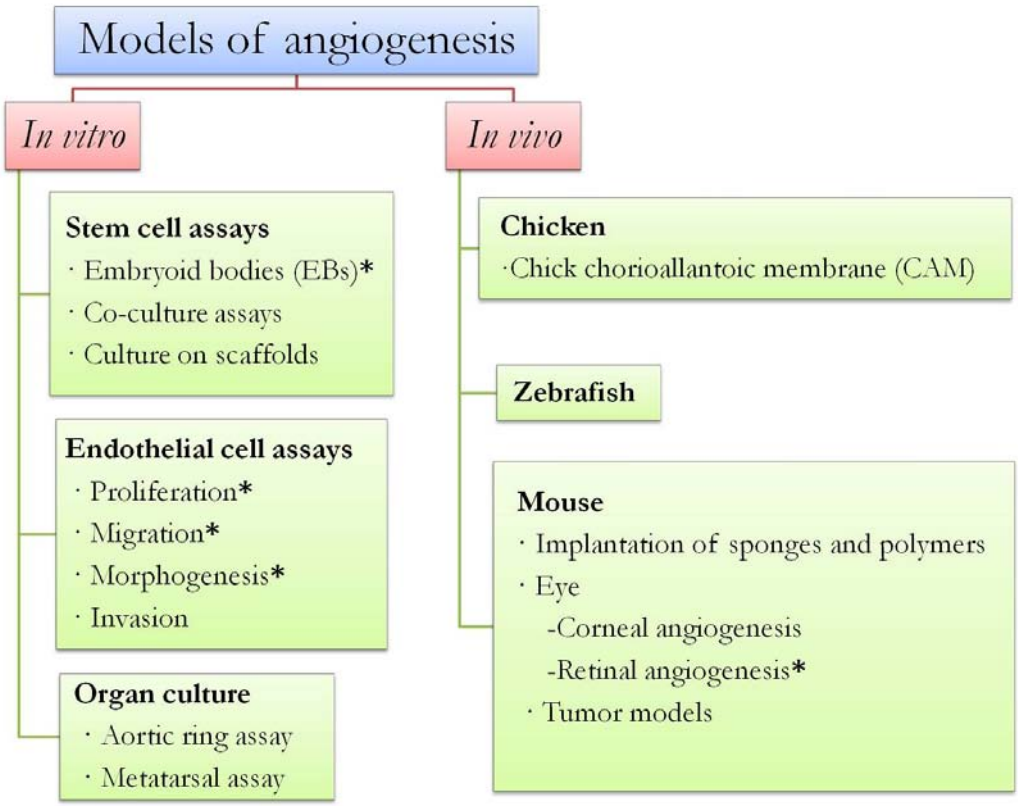

Figure 10: Scheme of the models widely used in angiogenesis. This scheme resumes some of the models that are used to model vasculogenesis and angiogenesis. Asterisks indicate the ones that have been used for this thesis.

3.1 *In vitro* assays

3.1.1 Assessment of vasculogenesis and angiogenesis using mES cells

Embryonic stem (ES) cells can be established in culture from the inner cell mass of mouse blastocysts (Evans and Kaufman, 1981). The unique nature of ES cells is defined by two properties: their self-renewal and pluripotency. ES cells are capable to undergo an unlimited number of symmetrical cell divisions in culture, while maintaining a normal karyotype. In addition, they are pluripotent, which allows them to give rise to any cell-type from the three germ layers: ectoderm, mesoderm and endoderm (Smith, 2001). These remarkable features make ES cells an excellent tool to study cell differentiation and to model embryonic development *in vitro*. Thus, ES cell differentiation holds great promise in both regenerative medicine and basic biological research.

There are currently three widely used approaches to differentiate ES cells (Keller, 2005): (1) by aggregation into three dimensional cellular aggregates called embryoid bodies (EBs), (2) by co-culture with stromal cell lines which mimick the stem cell niches conditions, (3) by culture on acellular matrices in the presence of chemically defined media (Noghero et al., 2010). EBs method is the one used for the purpose of this thesis so it is described in more detail in the following lines.

mES cells are routinely cultured in the presence of leukemia inhibitory factor (LIF) and/or feeder layers to maintain their stemness. When cultured in suspension in the absence of LIF, ES cells form three-dimensional multicellular aggregates (EBs) that eventually contain ectodermal, mesodermal and endodermal tissues which resembles the mouse embryo. This three-dimensional structure facilitates multicellular interactions and hence provides a proper microenvironment that is known to be determining for cell fate. Regarding endothelial differentiation, the first indication that endothelial cell development and vascular morphogenesis in EBs proceeds in an *in vivo* like fashion was provided by Doetschman (Doetschman et al., 1985). All these features make EBs model an attractive method to study vasculogenesis and angiogenesis.

Three methods are usually used to form EBs (Figure 11):

a) Suspension culture in bacterial-grade dishes: Basically ES cells are seeded in a bacterial-grade dish with a hydrophobic non-treated polystyrene surface at which ES cells do

not attach and, therefore, they naturally stick to each other and form aggregates (Doetschman et al., 1985). Since this process is spontaneous, the number of cells incorporated into each aggregate varies; consequently the size and shape of the resulting EBs is very heterogeneous. The homogeneity of EBs has been improved by introducing rotating suspension culture (Zweigerdt et al., 2003).

b) Methylcellulose culture: Methylcellulose was initially employed to form cell aggregates of a clonal origin. ES cells seeded onto semisolid methylcellulose media tend to remain single and these single ES cells will develop into EBs. Thus, this method, different from the other two, allows the formation of EBs from single cells. As a handicap, handling of semisolid solution is not easy. Besides, the consistency of methylcellulose medium makes very difficult the homogeneous distribution of factors added during the experiment. EBs made in methylcellulose medium has been extensively and successfully used to achieve hematopoietic differentiation (Wiles and Keller, 1991) as well as endothelial differentiation (Vittet et al., 1996).

c) Hanging drop culture: It is probably the most widely used method to differentiate ES cells into a variety of cell types. ES cells are aggregated in drops that hang from a Petri dish lid; due to gravity, ES cells in the drop fall and accumulate in the bottom forming an EB. The hanging drop culture proceeds for a few days to allow EB growth and differentiation, following by seeding them into different media or matrices. The aggregation of ES cells in hanging drops is advantageous because it allows to reproducibly forming homogenous EBs from a predetermined number of cells, an important factor in EBs development. EBs formed by the hanging drop method have been used to generate a broad spectrum of cell types including neuronal cells (Bain et al., 1995; He et al., 2006), astrocytes (Fraichard et al., 1995), oligodendrocytes (Brustle et al., 1999), lymphoid cells (Potocnik et al., 1997), hematopoietic cells (Dang et al., 2004), cardiomyocytes (Takahashi et al., 2003), smooth muscle cells (Drab et al., 1997), chondrocytes (Kramer et al., 2000), renal cells (Kramer et al., 2006), adipocytes (Dani et al., 1997), hepatocytes (Hamazaki et al., 2001), insulin-producing cells (Lumelsky et al., 2001), (Shiroi et al., 2002) and gametes (Geijsen et al., 2004). Concerning endothelial cell differentiation, ES cells have been used to generate functional endothelial cells that contribute to a stable vasculature that connects to the host circulation (Yurugi-Kobayashi et al., 2003).

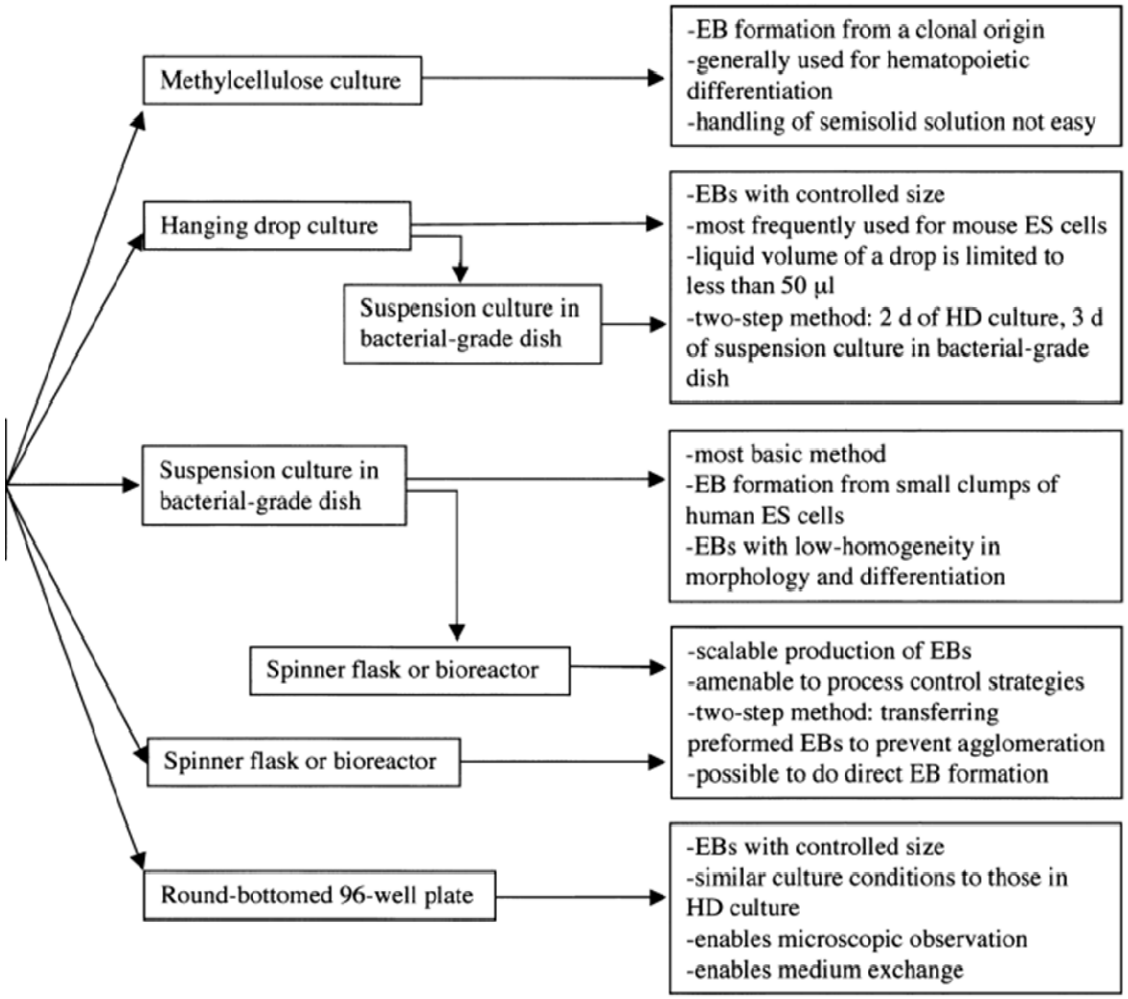

Figure 11: Outline of methods for inducing EB formation (From Kurosawa, 2007).

Several EBs-based methods have been described to achieve endothelial cell differentiation in a context that resembles the formation of blood vessels in the embryo. Indeed, at day 3 of EBs differentiation, the presence of precursors which express T cell acute leukemia/stem cell leukemia (TAL/SCL), VEGR2 and brachyury indicates the onset of vasculogenesis (Choi et al., 1998). These precursors undergo sequential maturation and eventually express a set of markers characteristic for mature endothelial cells such as VEGFR2, CD31, VE-Cadherin, TIE1 and TIE2 (Vittet et al., 1996). There are even signs of arterial/vein specification of ES-derived angioblasts (Muller-Ehmsen et al., 2006). The primitive vascular plexus in the EB is remodeled from day 6 onwards by sprouting angiogenesis. Invasive angiogenesis in three-dimensional (3D) collagen gels is manifested around day 8 by formation of sprouts emerging from the core of the EB. Subsequently, the sprouts branch and endothelial cells become surrounded by mural cells. Furthermore, the sprouts are embedded in a vascular basement membrane (Jakobsson

and Claesson-Welsh, 2008). Lumen formation has been detected at about day 10 and some of the EBs develop a mature lumen by day 12 (Jakobsson et al., 2007; Li and Claesson-Welsh, 2009). All these steps in EBs differentiation closely mimic the *in vivo* processes of blood vessel formation during embryogenesis.

3.1.2 Assessment of endothelial cell functions using endothelial cells

The isolation of endothelial cell lines and the establishment of their culture conditions represented a milestone in vascular biology research (Gimbrone et al., 1973). Human umbilical vein endothelial cells (HUVEC) (Figure 12B and 12C) are one of the standards for cell-base assays in the angiogenesis field. They can be easily isolated by perfusion of the umbilical vein with trypsin or collagenase and have been successfully cultured since 1973 (Jaffe et al., 1973). HUVEC provide an appropriate model for studying biological processes such as proliferation, migration and morphogenesis. As more sophisticated isolation techniques have become available, further primary endothelial cultures have been derived from different organs, with over 19 different types of endothelial cell now available as primary cell cultures, including pulmonary, uterine, cardiac, bladder, dermal and lymphatic microvascular cells (Staton et al., 2009).

Endothelial cells are flat cells, 1-2 μm thick and about 10-20 μm in diameter. From the morphological point of view, they can be defined as the innermost layer of blood vessels in continuous contact with blood (Gimbrone et al., 1995). Adjacent endothelial cells are tightly connected through intercellular junctions forming a pavement-like structure (Figure 12A), the endothelium, which lines the entire circulatory system. At the molecular level, endothelial cells are characterized by the expression of a set of specific markers such as VE-Cadherin (Breier et al., 1996) (Figure 12C), platelet endothelial cell adhesion molecule (PECAM-1 or CD31) and VonWillibrand Factor.

Apart from maintaining vessel integrity, endothelial cells play unique primordial roles in vascular biology. They are the primary constituents of new blood vessels and endothelial cell functions are required for angiogenesis, including matrix degradation, cell migration, proliferation and morphogenesis. Several techniques are used to asses these functions as described below.

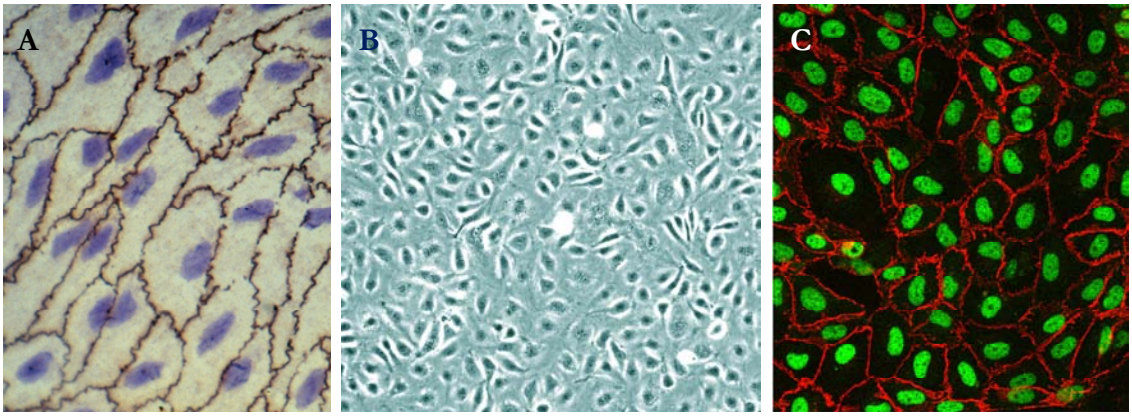

Figure 12: Endothelial cells. A) Arterial endothelium; nuclei are in blue and cell boundaries are stained in black with silver nitrate (www.daviddarling.info). B) Phase contrast micrograph of HUVEC culture (www.sciencellonline.com). C) VE-Cadherin immunostaining of HUVEC; VE-Cadherin is in red and nuclei are in green (www1.imperial.ac.uk).

A) Proliferation assays

Sprouting angiogenesis requires stalk cells proliferation. Proliferation assays are highly reproducible, easy to perform and generate precise quantifiable data; therefore, they are commonly used (Staton et al., 2009). The easiest approach for measuring this parameter is by determining the increase in cell number over time, which can be done by cell counting using hemocytometers or automated counters (Coulter counter, Vi-cell counter®, which measure viability as well as number, etc.). Another option to assess cell proliferation is by analyzing DNA synthesis, usually by quantifying the incorporation of a labeled reagent during S-phase of the cell cycle: [^{3}H]-thymidine or bromodeoxyuridine (BrdU) are frequently used. An alternative is to use antibodies detecting proliferation markers such as Ki67 or proliferating cell nuclear antigen (PCNA). In addition, cell proliferation can also be determined by evaluating endothelial cells metabolic activity. Within these methods, MTT is one of the most popular ones and measures mitochondria activity. Active mitochondria express a dehydrogenase enzyme which converts the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals, which accumulate in living cells. These crystals are dissolved by the addition of DMSO and the solubilized formazan product is quantified by spectrophotometry and correlated to the cell number (Denizot and Lang, 1986).

B) Migration assays

During angiogenesis, and following matrix degradation, endothelial cells migrate into the surrounding stroma in response to angiogenic stimuli. Assays that allow quantitative assessing of endothelial cells migration include transfilter and wound healing assays. Transfilter assays are

a modification of the Boyden chamber (Boyden, 1962). They are based on the migration of endothelial cells plated on one side of a porous membrane towards a solution containing the potential migratory factor, placed on the opposite side of the membrane (Alessandri et al., 1983). For endothelial cells, a pore size of 3 μm is the most appropriate, and the membrane is coated with extracellular matrix components like fibronectin, collagen or Matrigel™ (Albini et al., 2004) prior to plating the cells. Migrated cells are then stained and counted, either microscopically or using automated techniques, based on fluorescent staining or light-blocking membranes.

An alternative type of migration assay is based on the premise that endothelial cell migration into a denuded area is a pivotal event in wound healing *in vivo* (Staton et al., 2009). In the wound healing or scratch assays, endothelial cells are grown to confluence and a scraping tool, usually a pipette tip, is used to clear an area which provides a margin from which the endothelial cells migrate to fill the denuded area (Wong and Gotlieb, 1984). The rate and extent of endothelial cell migration is then monitored microscopically over time and the fraction of the wound repaired by endothelial cells is evaluated (Figure 13).

C) Morphogenesis assays

Angiogenesis requires the assembly of endothelial cells into vessels. Cell migration and alignment of activated endothelial cells is followed by the formation of tube-like structures, sprouting of new capillaries, and finally the development of the cellular networks. This stage of angiogenesis can be modeled *in vitro* by plating endothelial cells with extracellular matrix components, commonly collagen, fibrin or Matrigel™.

It has been shown that HUVEC plated on Matrigel™ at low densities form a network of branching structures in a manner that simulates the *in vivo* situation (Lawley and Kubota, 1989) (Figure 11). Cultures are then photographed and morphogenesis quantified by measuring different parameters, like covered area, number of tubes, average tube length and number of branching points. This can be assessed manually or by using image analysis programs such as Wim Tube (Wimasis), which perform quantitative analysis of tube formation (Figure 13).

Assays using endothelial cells alone are likely to be reproducible, readily quantifiably and rapid. However, these endothelial cell based assays also have certain limitations as they do not provide a proper microenvironment. This would involve three-dimensional interactions

between ECs and adjacent supporting cells and matrix, which are known to be absolutely crucial in the regulation of vascular processes.

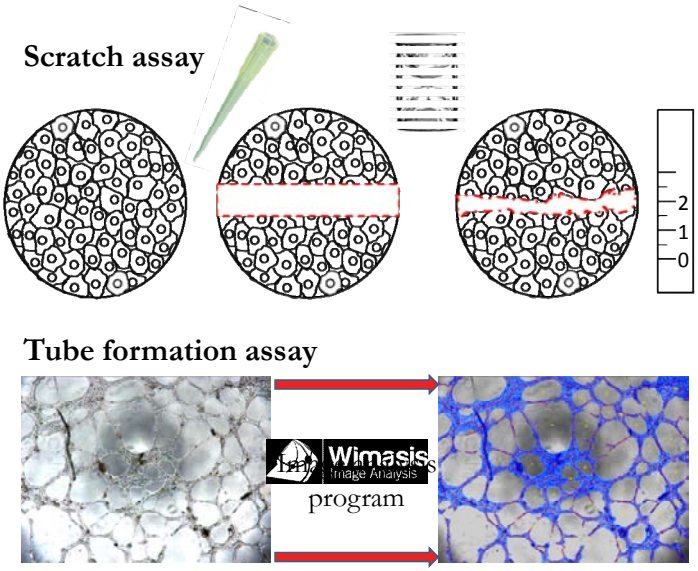

Figure 13: Endothelial cell based assays. Schematic view of a scratch assay allows determining migration and tube formation assay to assess vascular morphogenesis.

3.1.3 Organ culture (*ex vivo* assays)

The *ex vivo* approaches involve the use of explants to study angiogenesis. Segments, discs or sections of specific tissue are *in vitro* cultured in a three dimensional matrix and the growth of microvessels is monitored for a period of 10-14 days. Among them, the aortic ring assay is the most commonly used. It was described in rat (Nicosia and Ottinetti, 1990) although it was adapted to other species including chick (Auerbach et al., 2000) and mouse (Masson et al., 2002). The mouse aortic ring assay allows analysis of cellular proliferation, migration, tube formation, microvessel branching, perivascular recruitment and remodeling, all in transgenic and knockout mice.

3.2 *In vivo* assays

3.2.1 Chick choriallantoic membrane (CAM) assay

The CAM is a vascular extraembryonic membrane formed on day 4 of incubation by fusion of the chorion and the allantois. Since it has a dense capillary network, it has been commonly used *in vivo* to study both angiogenesis and antiangiogenesis in response to normal tissues and cells, to tumor bioptic specimens and cells, or to soluble factors (Ribatti, 2008).

3.2.2 Zebrafish

Zebrafish (*Danio rerio*) is a small tropical freshwater fish which constitute a valuable model for angiogenesis research. The optical clarity embryos make it possible to visualize the entire developing vasculature and therefore to evaluate the effects of drugs, injected cells or to assess gene function. In this regard, specific genes are knocked-down by antisense morpholino oligonucleotides (Currie and Ingham, 1996).

3.2.3 Mouse retina

The vascular system of the mouse retina provides a useful model for analyzing the molecular and cellular mechanism regulating angiogenesis (Roca and Adams, 2007). The main advantages of using this model rely on the fact that hierarchical vascular networks in mouse retina are formed only after birth. Hence, the entire developmental angiogenesis can be imaging and even manipulated without the difficulties associated with investigating embryonic development. Therefore, it allows for reliable detection of any vascular developmental abnormalities both in transgenic mice and pharmacologic studies (Uemura et al., 2006). Besides, the use mouse retina provides a model to investigate neurovascular relationship which may be very relevant.

During embryonic stage, the mouse retina is avascular and all gas and material exchange is carried out by diffusion from the hyaloids and choroidal vascular system (Saint-Geniez and D'Amore, 2004). Immediately after birth the retinal vascular system starts to develop at the same time that hyaloids vessels undergo pruning. This pruning of hyaloids vessels has been a very useful tool in the study of the mechanisms of physiological vessel regression (Lobov et al., 2005). Vascularization of mouse retina starts in the optic nerve and radiates outwards forming a two-dimensional structure that reaches the periphery during the first week of life. This is a scheduled process involving different cell types. First, astrocytes precursors enter the retina through the optic nerve around embryonic day 17, guided by axon bundles of ganglion cells, and form a fine meshwork extending towards the periphery. Two days after (P0), vascular endothelial cells start to sprout, guided by the preformed astrocyte network and in response to a VEGF-A gradient (Figure 14). Sprouting endothelial cells are followed by the migration of mural cells which eventually cover most vascular networks (Stahl et al., 2010). Around (P7) secondary sprouting in a perpendicular direction leads to the vascularization of deeper layers of the retina, which finally leads to the formation of a three-layered vascular system. While the

deeper vascular layers are not remodeled, the superficial layer establishes a hierarchical architecture consisting of distinct arteries, veins, and capillaries. This two-dimensional network can be flat-mounted allowing the monitoring of the entire process during retinal angiogenesis (Uemura et al., 2006).

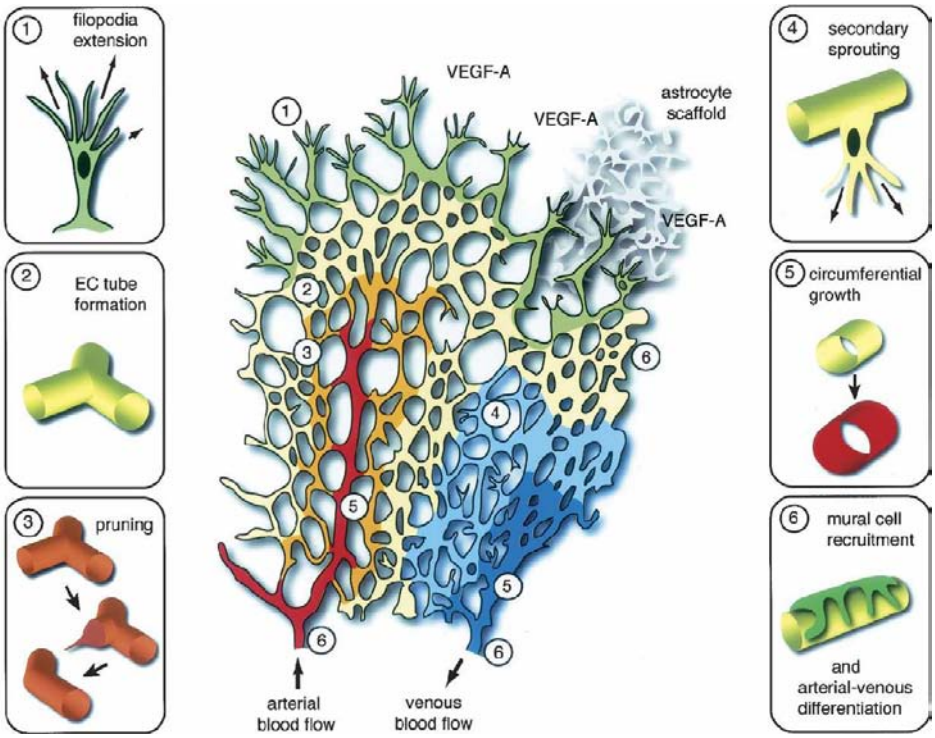

Figure 14: Growth of retinal blood vessels. (1) Endothelial tip cells extend filopodia in response to a VEGF-A released astrocytes. (2) Sprout tip cells interact and anostomous to generate a plexus (3) Periarterial zone showing pruning. (4) Secondary sprouting in a perpendicular direction. (5) Differentiation of arteries and veins. (6) Stabilization of vasculature (From Roca et al., 2007).

Besides normal development, the mouse retina can be used as a tool to investigate conditions in which normal vascular development becomes unbalanced as occurs in human retinopathy of prematurity (ROP), a major cause of acquired blindness in children (Visser et al., 2013). One of the most widely used animal models of ROP is the mouse model of oxygen induced retinopathy (OIR) (Smith et al., 1994). The OIR model along with some other models of pathologic angiogenesis have been used to investigate many relevant pathways involved in pathologic retinal angiogenesis leading to important contributions to *in vivo* angiogenic research. For example, they have helped to lay the bases for the clinical application of anti-VEGF therapies in patients (Shih et al., 2003a; Shih et al., 2003b) or to explain the neurovascular cross-talk (Akula et al., 2007; Downie et al., 2007).

4. THE P53 FAMILY AND ITS ROLE IN ANGIOGENESIS

The p53 family of transcription factors in vertebrates is constituted by p53, p63 and p73 and it is one of the most important families in vertebrate biology. p53 was identified in 1979 (Lane and Crawford, 1979; Linzer and Levine, 1979) and initially considered as an oncogene. Later experiments showed that the wild type allele of *p53* really functions to suppress cell proliferation and it was eventually categorized as a tumor suppressor gene. By 1987 it became apparent that point-mutated alleles of *p53* were common in the genomes of a wide variety of human cancers. Indeed, among all the genes examined to date in human cancer cells, *p53* is the gene found to be most frequently mutated, being present in mutant form in the genomes of almost half of all human tumors (Weinberg, 2007). The role of p53 in the maintenance of genomic stability largely resides in its capacity to sense potentially oncogenic and genotoxic stress and to coordinate a complex set of molecular events leading to growth restraining responses and, ultimately to induce senescence and/or apoptosis (Levine, 1997).

The other vertebrate p53 family members, p73 and p63, were cloned in 1997 (Kaghad et al., 1997) and 1998 (Yang et al., 1998), respectively and they all share a similar basic structure and sequence identity. Like p53, p63 and p73 also function in response to DNA damage and in apoptosis (Jost et al., 1997; Murray-Zmijewski et al., 2006; Yang et al., 2002) but they have some additional roles in development.

Members of the p53 family are not exclusive to vertebrates but they can be found in invertebrates including mollusks, insects and worms. Structural and functional studies revealed an evolutionary path from ancestral p53-like proteins, that monitor the genetic stability in germ cells of invertebrates, to p63 and p73 forms in mammals, that mainly have developmental functions, and finally to the tumor suppressor p53 (Dotsch et al., 2010). Nevertheless, recent studies suggest that the ancestral p53 ortholog in planaria, *Smed-p53*, already played a role in stem cell lineages consistent with both tumor suppressor-like activity and self renewal. This finding opens the evolutionary possibility that the ancestral molecule had already all the functions of p53, p63 and p73 which were split up among the paralogs after the gene duplication that gave rise to these homologs (Pearson and Sanchez Alvarado, 2010).

In vertebrates, the emerging picture is that of an interconnected network in which p63 and p73 share many functional properties with p53 (Figure 15). In addition, they also exert distinct and unique biological functions in development playing a role in processes like control of cell growth, cellular differentiation and morphogenesis. Indeed, p63 is essential for maintaining epithelial stem cells (Mills et al., 1999; Yang et al., 1999) and for protecting the genomic stability of oocytes (Suh et al., 2006) whereas p73 is involved in neurogenesis and development of nervous and olfactory systems (Yang et al., 2000). Moreover, all the members of this family play an important role in maternal fertility. In this regard, whereas p63 is important for maturation of the egg, p73 ensures normal mitosis in the developing blastocyst and p53 regulates implantation of the embryo through transcriptional control of leukaemia inhibitory factor (Levine et al., 2011).

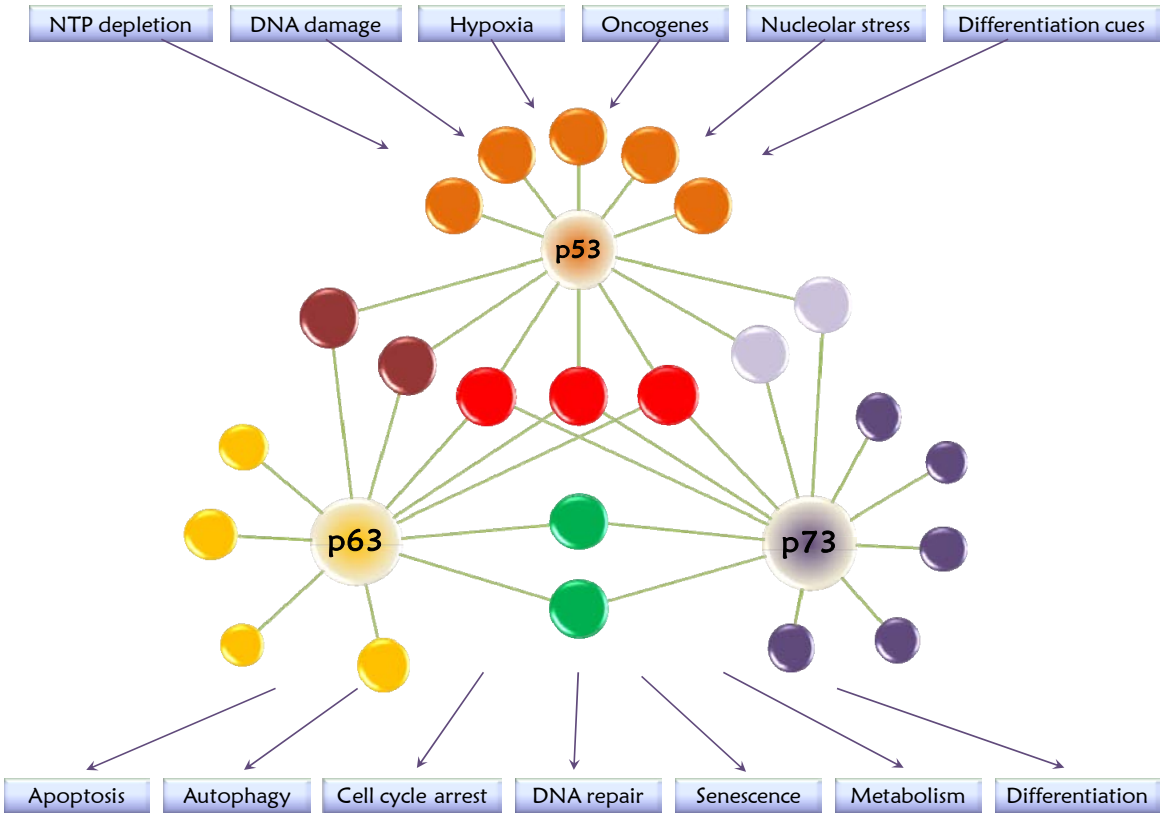

Figure 15: The p53 family as a network. The p53-family pathway is activated by a wide array of signals, including potentially oncogenic stresses, as well as physiological cues. Once activated, the pathway induces diverse cellular outcomes (Collavin et al., 2010).

4.1 Structural organization of p53 family

The p53 family members are modular proteins with similar basic structure. The overall structure is highly conserved and consists of three major domains: an amino terminal transactivation domain (TAD), a central immunoglobulin-like DNA binding domain (DBD), and a C-terminal oligomerization domain (OD) (Dotsch et al., 2010).

The TAD is responsible for transactivation of target genes. The TAD contacts the basal RNA polymerase II transcriptional machinery and transcriptional regulators such as p300 and CREB-binding protein (Zeng et al., 2000). The TAD is the least conserved among the family members with 30% between p73 and p53 (Figure 16).

The DBD is responsible for sequence specific DNA binding. Among all p53 family members, including paralogs and those orthologs from invertebrate species, the DBD is the most conserved domain, with 63% sequence identity between p73 and p53 (Figure 16). Moreover, the residues from the p73 DBD that contact the DNA bases are conserved in p53 and p63 and, consequently, both p63 and p73 can bind to canonical p53 DNA-binding sites and transactivate p53-responsive promoters (Jost et al., 1997; Yang et al., 1998). Nevertheless, growing evidence indicates that many target genes respond differently to the various family members, conferring them distinct, but sometimes overlapping functions.

The carboxy-terminal OD is indispensable for the biological function of all p53 protein family members since it is the structural domain that permits to form a tetramer, the prototype structure for all the transcriptionally active members of the p53 family. This tetramer is a dimer of dimers; ODs of p73 and p63 strongly interact resulting in heterotetramers whereas no heterooligomerization between wild type p53 and p73 or p63 was observed, supporting the notion of functional orthogonality within the p53 family (Coutandin et al., 2009). Interestingly, p53 mutants can bind and inactivate p73 and p63 by interacting through the DBD (Marin et al., 2000).

The full length proteins (denoted as α isoforms) of p63 and p73 also contain a carboxy-terminal sterile α-motif (SAM) domain involved in protein-protein interaction (Figure 16), found in many signaling proteins and transcription factors. In addition, they present a transcription inhibition domain (TID), that decreases their transcriptional activity (Arfaoui et al., 2010; Ozaki et al., 1999) by enforcing a closed conformation through the interaction with the amino-terminal TAD (Serber et al., 2002).

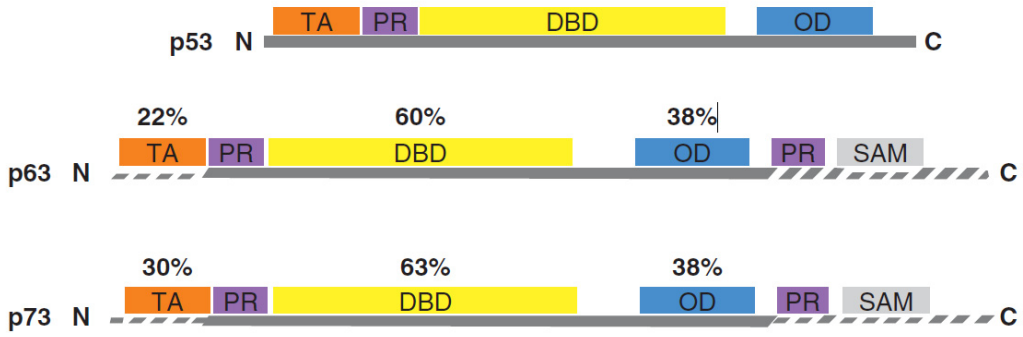

Figure 16: Structural organization of p53 family members: Structural domains of p53 family members with the percentage of homology of p63 and p73 referred to p53 (Dotsch et al., 2010).

TP73 has two promoters, P1 and P2 (on intron 3), that originate two opposing classes of proteins: the TA isoforms (containing the transactivation domain), and ΔN variants with truncated N- terminus (Ishimoto et al., 2002; Yang et al., 2000). Additionally, due to alternative splicing the p73 gene gives rise to at least seven alternatively spliced C-terminal isoforms (α, β, γ, ε, η, η1and ϕ,) that differ in their potential to activate target genes and induce growth suppression (De Laurenzi et al., 1998; Moll and Slade, 2004). Amino-terminal splicing of mRNA produced from P1 promoter isoforms can give rise to ΔEx2, ΔEx2/3 and ΔN´ transcripts (Stiewe et al., 2002; Stiewe et al., 2004).

The differential expression of TA/ΔN isoforms is determined by various sets of regulatory elements within each promoter, such as E2F induction of p73 P1 promoter (Irwin et al., 2000), while differential expression of carboxy-terminal splicing variants seems to be determined by tissue-specific mechanism of alternative RNA splicing (Morgunkova, 2005). There are also a set of degradation mechanisms that regulate protein levels.

The combinatorial effect, since each N-terminal variant can potentially exist as any of the C-terminal variants, makes p53 family very complex in terms of structure. In addition, the p53 family proteins share some common post-translational modifications, physical interactions with other proteins and reciprocal regulatory networks which may affect stability, cellular localization and ability to transactivate specific targets, thus contributing to increase functional complexity.

Interestingly, the *p53* gene has also been found to generate multiple protein variants (Bourdon et al., 2005) that derive from the use of three different promoters and alternative

splicing at the 5´end, resulting in a total of nine potential proteins that can lack the TA domain but also the OD (Bourdon, 2007).

In conclusion, the structural complexity of p53 family proteins contributes molecular flexibility that, together with their functional interactions, confers diversification to these proteins that are involved in the regulation of multiple and, sometime antagonistic, biological processes.

4.2 Biological functions of p53 family members

Life of multicellular organism requires a balance between the cellular processes of proliferation, differentiation and death, processes which are important throughout the whole life of the organism. Proliferation is required to increase the number of cells during development and to replace cells that are lost due to injury or a high turnover. Differentiation of stem cells is needed to generate and replace the various types of specialized cells that constitute tissues and organs. Programmed cell death allows controlling cell numbers and it is essential to eliminate compromised or damaged cells. These cellular processes (proliferation, differentiation and cell death) can be considered independent in molecular terms: proliferation requires CDKs activation, differentiation implies tissue-specific genes expression and is characterized by caspase activation. However these responses are linked by upstream signal transduction pathway (Blagosklonny, 2003). In this context, p53 family as a whole could be considered one of these "linkers" since different isoforms are involved in controlling one or more of these cellular processes.

4.2.1 Dual function of p73 in cellular processes: TAp73 *vs* ΔNp73

The dual nature of *TP73* resides in the existence of TA and ΔNp73 protein variants. The full-length TAp73 proteins (TAp73α and β) are capable of binding to p53 response elements (RE), transactivating p53 target genes (Jost et al., 1997), including *p21Waf1/Cip1*, *Bax*, *Mdm2* and *GATA1*, among others. Thus they promote cell cycle arrest, differentiation, senescence and apoptosis (De Laurenzi et al., 2000b; Fang et al., 1999; Marques-Garcia et al., 2009). Conversely, ΔN isoforms have been shown to act as dominant negative inhibitors of p53 and TAp73. The ability of ΔNp73 isoforms to antagonize TAp73 and p53 function may be through at least two different mechanisms: ΔNp73 can act as a dominant negative over TAp73 and p53 through the formation of inactive hetero-oligomers or it can act competing for DNA binding

by direct interaction with target promoters on p53 RE (Ishimoto et al., 2002; Zaika et al., 2002). However, several new lines of evidence have revealed that ΔNp73 (α and β) can be active in transactivation of specific target genes in a p53 dependent and independent manner (Alvarez et al., 2006; Kartasheva et al., 2003; Liu et al., 2004; Tanaka et al., 2004; Tanaka et al., 2006). Moreover, ΔNp73α may either inhibit or stimulate wild type p53 transcriptional activity depending on both, the p53 target gene and the cellular context. This suggest that ΔNp73α not only acts as an inhibitor of p53/TAp73 functions in certain tissues, but also could cooperates with wild type-p53 in playing a physiological role through the activation of specific gene targets (Goldschneider et al., 2005). That is the case in erythroid differentiation (Marques-Garcia et al., 2009). Interestingly, both p53 and TAp73 induce ΔNp73 creating an auto-regulatory feedback loop (Grob et al., 2001). These facts imply that the balance of various isoforms may determine cell fate. In addition, the activity of p63 and p73 and the expression of different isoforms may also depend on the intracellular context. Thus, the functional interaction between TA and ΔNp73 would depend upon the TA/ΔN ratio and will be tissue specific. Therefore, elucidating the differential regulation of each isoform and their tissue-specific roles will undoubtedly lead to a better understanding of their unique functions in development and disease.

A) Role of p73 in proliferation *vs* cell cycle arrest

Cell cycle regulation is highly conserved in evolution from simple unicellular eukaryotes to complex metazoans. The precision with which cell cycle events are executed ensures the survival of living organisms while loss of this precision increases genomic instability, an important factor in oncogenesis (Nurse, 2000). The cell cycle is a temporally, spatially organized and tightly regulated sequence of events leading to cell division. The existence of "checkpoints" ensures not only that the cell cycle proceeds properly but also that it does according to internal and external cell signals. In this context, p73 and p53 can be considered as "checkpoint proteins" and, indeed they can exert cell cycle control by different mechanisms.

TAp73 can induce a G1 cell cycle arrest through transcriptional up-regulation of either *P21* (Jost et al., 1997), which in turn inhibits G1-Cdk complexes, or *p57/Kip2*, which coordinates mitotic exit and transition to G1 (Balint et al., 2002; Merlo et al., 2005). Transcription of these promoters is repressed by ΔNp73 that thus promote cell cycle progression (Allocati et al., 2012).

p73 also acts at G2/M checkpoint via inhibition of the Cyclin B/Cdk1 complex. TAp73 can induce the expression of *Gadd45* and *14-3-3σ*. Both induce G2/M arrest by two different mechanisms: 14-3-3σ by sequestering the Cyclin B/Cdk1 complex in the cytoplasm and Gadd45 causing dissociation of Cdk1 from Cyclin (Damia and Broggini, 2004). In addition, p73 itself can decrease the levels of Cyclin B by attenuating expression from *Cyclin B* promoter (Innocente and Lee, 2005). It has been demonstrated recently that p73 bind to and cooperates with Flice-Associated Huge Protein (FLASH/CASP8AP2) in the regulation of histone gene transcription and its loss results in a block at the G2/M checkpoint (De Cola et al., 2012).

A p73 role in mitosis has also been demonstrated. Although p73 is hyper-phosporylated during this phase, it retains the ability to activate transcription of some genes such as *p57/Kip2*. More interestingly TAp73 interacts with spindle assembly checkpoint proteins (Bub1, Bub3 and bubR1) and cooperates in their activity. Therefore, altered p73 expression results in genomic instability (Tomasini et al., 2009; Vernole et al., 2009).

B) Role of p73 in apoptosis

Apoptosis is a regulated process leading to cell death mediated by caspase cascade activation and marked by a series of morphological changes like chromatin condensation, DNA fragmentation, cell shrinkage and formation of apoptotic bodies (Lodish, 2003). Apart from cell cycle arrest, apoptosis represents a fundamental roadblock to tumorigenesis. One common mechanism for disabling apoptosis involves direct or indirect inactivation of p53. Like p53, p73 also plays a role in apoptosis. The ability of p73 to induce apoptosis is determined by the relative levels of expression of TAp73 and its dominant negative ΔNp73 isoforms. TAp73 (α and β) isoforms are considered pro-apoptotic, while ΔNp73 isoforms hold anti-apoptotic functions (Allocati et al., 2012).

It was first established that p73, at least when overexpressed, was able to induce apoptosis irrespective to p53 status (Jost et al., 1997). TAp73 has also been shown to induce apoptosis in response to DNA damage (Agami et al., 1999; Yuan et al., 1999). Moreover, transcriptional activation of p53 apoptotic targets in response to DNA damage requires p63 or p73 to be present on the promoters in certain cellular contexts (Flores et al., 2002). Interestingly, it has been demonstrated that in response to DNA damage, ΔNp73 isoforms are degraded thus allowing the TA forms to exert their effect (Maisse et al., 2004).

In the context of neurological development, neuronal apoptosis in the formation of hippocampus during embryonic development was associated to p73 deficiency (Yang and McKeon, 2000). In addition, it has been shown that ΔNp73 can inhibit neuronal cell death through direct binding to the c-Jun N-terminal kinase (JNK) reducing Bim-EL expression and therefore inhibiting the apoptotic mitochondrial pathway (Lee et al., 2004).

Nowadays some of the molecular mechanisms by which TAp73 as well as TAp63 induce apoptosis have been elucidated (Ramadan et al., 2005). They control the mitochondrial pathway through up-regulation of the pro-apoptotic Bcl-2 family member Bax as well as promote its translocation to the mitochondria by controlling the expression of the BH3-only protein PUMA (p53 up-regulated mediator of apoptosis) (Melino et al., 2004). It has been also demonstrated that p73 can activate the BH3-only member of the Bcl-2 family NOXA in the absence of a functional p53 (Flinterman et al., 2005). TAp73 can modulate the extrinsic death receptor pathway by inducing *CD95* expression and it can induce cell death also through the endoplasmic reticulum stress pathway by up-regulation of SCOTIN, a trans-membrane protein located in the endoplasmic reticulum. Anti-apoptotic ΔNp73 isoforms confers resistance to chemotherapy via the inhibition of *CD95, Bax* and *Scotin* gene transactivation (Damia and Broggini, 2004; Muller et al., 2005).

C) Role of p73 in differentiation

Differentiation is the process by which a cell becomes specialized in order to perform a specific function. Differentiation usually involves asymmetric division of stem cells and gradual acquisition of differentiated traits. These fully differentiated cells have the ability to maintain their specialized traits (Pasque et al., 2011), although there is not an irreversible lock in this mature identity (Yamanaka and Blau, 2010) . In recent years, many of the signaling pathways and transcriptional mechanism that underlie important cell fate decisions and the establishment of differentiated traits have been elucidated. Also knowledge has increased about the mechanisms that ensure the loss of acquired cell identities (Holmberg and Perlmann, 2012).

The result of a distorted differentiation program is frequently related to disease. Terminal differentiation is a crucial developmental process in which cell cycle arrest is temporally and spatially coordinated with the expression of specialized cellular functions. Actually, it has been described as mechanism of tumor suppression (Sherr, 2004). In fact, several studies have revealed a correlation between the grade of tumor malignancy and the expression of

transcription factors that are associated with ES cell pluripotency and enhanced self renewal (Ben-Porath et al., 2008; Holmberg et al., 2011). In addition, the consequences of loss of neuronal phenotype or the difficulties in securing the differentiated state have been related to neurodegenerative diseases such as Parkinson´s and Alzheimer´s or to schizophrenia. Indeed, early symptoms in several neurodegenerative disorders are likely to be caused by loss of neuronal function rather than by cell death (Bergman et al., 2010; Liu et al., 2010).

p53 family members have been described as regulators of several differentiation processes. p53 was shown to play a regulatory role in differentiation of neurons and oligodendrocytes (Eizenberg et al., 1996) and it is involved in neural precursor differentiation (Beltrami et al., 2004). Furthermore, p73 was proved to be sufficient to induce neuroblastoma cell differentiation *in vitro*, independently of p53 (De Laurenzi et al., 2000a) and to induce differentiation of oligo-dendrocytes (Billon et al., 2004). In addition, recent data stated that p73 deficiency, most likely TAp73 induced premature neuronal differentiation (Gonzalez-Cano et al., 2010). TP73 has also been related to myeloid (Tschan et al., 2000) monocytic (Morena et al., 2002) and erythroid differentiation (Marques-Garcia et al., 2009).

A reasonably assumption is that factors with tumor suppressor capacity can exert their role by either controlling differentiation or guarding against re-entry into an undifferentiated, proliferative state. In this context, p53 has been shown to serve as a barrier in reprogramming (Hong et al., 2009; Kawamura et al., 2009).

Altogether, these data indicate that p73 plays a role in regulating differentiation, which is essential for development, but also constitute a mechanism for the tumor suppression.

D) Role of p73 in cell migration and adhesion

Cell migration is a key aspect of many physiological and pathological processes including embryonic development, angiogenesis and metastasis of tumor cells (Ridley et al., 2003).

There are controversial reports regarding the role of p53 and p73 in cellular migration. On one hand, some authors indicated that TAp73α and p53 positively regulate cell migration by regulating the activity of the PI3K/Rac1 pathway (Sablina et al., 2003); on the other hand, Zhang and coworkers argued that TAp73 is a negative regulator of this process (Zhang et al., 2012). Similarly, several studies link deficiency of p53, rather than loss, to increased cell motility

in different cell types, including neurons (Qin et al., 2009), by controlling activity of the RhoA signaling (Muller et al., 2011) and Cdc42 (Gadea et al., 2002).

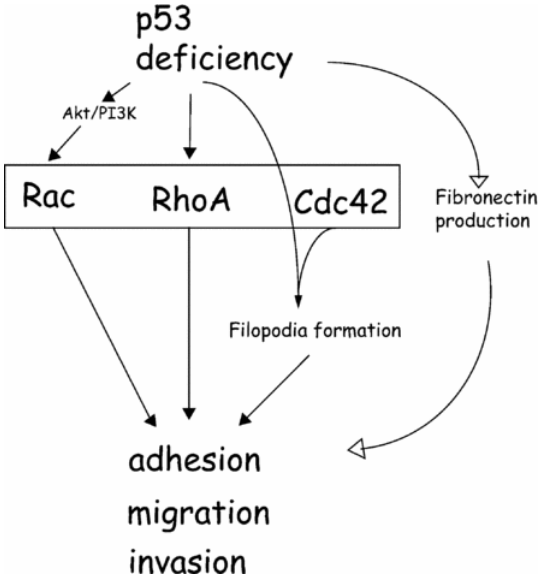

Figure 17: Effects of p53 on the Rho GTPases and cell migration. A deficiency in p53 promotes cell migration by up-regulating Rho GTPase activities and fibronectin production. From (Roger et al., 2006).

Special interest in the context of p53 family of transcription factors, which regulate developmental process and play roles in tumorigenesis, may deserve the process of epithelial-mesenchymal transition (EMT). EMT can be defined as a process that produces complete loss of epithelial traits accompanied by total acquisition of mesenchymal features (Kong et al., 2011). This process is characterized by loss of cell adhesion, repression of E-cadherin expression and increased cell motility (Weinberg, 2007). EMT is essential for numerous developmental processes including mesoderm and neural tube formation but it is also a relevant process in tumor metastasis. EMT is regulated by expression of many distinct genes including some transcription factors that orchestrate key steps of embryogenesis: Slug/Snail and Twist families (Yang et al., 2004). These proteins are master regulators during embryogenesis as they convert the epithelial cells into the migratory mesenchymal cells that form the mesoderm (Shiota et al., 2008). SLUG/SNAIL and TWIST have been shown to down-regulate molecules that stabilizes cell-cell junctions (E-cadherin) and to up-regulate components of the migratory machinery in order to become invasive (Bolos et al., 2003; Shih et al., 2005). Interestingly, there is a functional interaction between these master regulators and p53 family members: p53 (Wang et al., 2009), p63 (Herfs et al., 2010) and p73 (Zhang et al.,

2012). It has been reported that p53 (Shiota et al., 2008) and more recently TAp73 (Zhang et al., 2012) contribute to maintain a transcriptional program which prevent EMT by regulating *Slug/Snail* and *Twist*. Furthermore, p53 controls EMT by additional mechanisms, such are the regulation of extracellular matrix proteins (Guo and Zheng, 2004), extracellular proteases (Mukhopadhyay et al., 2009) and microRNAs, especially miRNA-143 (Quintavalle et al., 2010).

Therefore, p53 family members are involved in the regulation of cellular migration and EMT. This concept links with the notion that EMT represents a reactivation of latent biological programs, many of which are normally active in early mammalian embryonic development (Thiery, 2002).

4.2.2 p73 in cancer

A classical view of cancer led to classify genes as proto-oncogenes or tumor suppressor-genes. p53 is considered nowadays the tumor suppressor gene *par excellence* and, as a matter of fact, p53 knockout mice show a high rate of spontaneous tumors (Donehower et al., 1992). Moreover, p53 is characteristically mutated or inactivated during tumorigenesis. p53 is activated upon many different stress signals (radiation, hypoxia, oncogene signaling) potentially affecting genome integrity. Once activated, p53 promotes a complex cellular response that leads to cell cycle arrest, DNA repair, senescence or apoptosis. Therefore, p53 is crucial in the maintenance of genomic stability and it was so dubbed "guardian of the genome" (Lane, 1992).

The fact that both, TAp63 and TAp73, can induce cell cycle arrest, senescence, DNA repair and apoptosis in response to chemotherapic drugs independently of p53, supports the tumor suppressor role of these genes (Gressner et al., 2005; Guo et al., 2009; Irwin et al., 2003; Lin et al., 2009). However, one of the most obvious differences between the members of the p53 family is that p63 and p73, in contrast to p53, are not classic Knudson-type tumor suppressor genes. They are rarely mutated in human cancers (<1%) and their respective knockout mice, lacking all the isoforms, die tumor-free from developmental defects at early age (Mills et al., 1999; Yang et al., 1999; Yang et al., 2000). Nevertheless, a wealth of emerging data shows that both p63 and p73 have a role in tumor suppression. A careful analysis of the tumor predisposition of p63 and p73 aging heterozygous mice showed that *p63*+/- and *p73*+/- mice developed a broad spectrum of malignant lesions and the median survival time was only a few months longer than *p53*+/- mice (Flores et al., 2005). Later on, phenotypical characterization of TAp73-specific knockout mice revealed that predisposition to spontaneous and carcinogen-

induced tumorigenesis is increased by specific TAp73 loss *in vivo* (Tomasini et al., 2008a). A second major phenotype of the TAp73 knockout mice is infertility because of genomic instability of the oocyte, further revealing a role for TAp73 as a tumor suppressor maintaining genome integrity. Thus, these results indicate that TAp73 mediate the tumor-suppressive function of p73 and that loss of TAp73 alone is sufficient to promote oncogenic transformation.

In contrast, ΔNp73 is overexpressed in a wide range of tumors in which it correlates with poor prognosis (Deyoung and Ellisen, 2007) but whether ΔNp73 is physiologically relevant in tumors has been controversial. On one hand, it was stated that ΔNp73 overexpression did not confer any growth advantage to tumor cells although upon genotoxic stimuli or acquisition of a Ras mutation, high ΔNp73 expression led to increased cell survival (Ishimoto et al., 2002; Kartasheva et al., 2002; Nakagawa et al., 2002; Petrenko et al., 2003). On the other hand, some reports showed that ΔNp73 overexpression nor confer any growth advantage to cells treated with genotoxic agents (Marrazzo et al., 2006) neither generate the development of tumors in xenografs any faster than those expressing physiological levels of ΔNp73 (Sabatino et al., 2007). Originally, this observation led to the assumption that ΔNp73 was not an oncogene (Ishimoto et al., 2002) but more recent research has highlighted the potential of ΔNp73 as an oncogene in a variety of cell types depending on particular scenario (Bailey et al., 2011).

Recently it has been predicted that p53 family members could act as regulators of tumor suppressor miRNAs network. They are in control of most of the known tumor suppressor miRNAs, such as let-7 and miR-34 (Boominathan, 2010). Recent studies have implicated the miR-34 family of miRNAs in the p53 tumor suppressor network. The expression of miR-34a, miR-34b, and miR-34c is robustly induced by DNA damage and oncogenic stress in a p53-dependent manner. Thereby, miRNAs can affect tumorigenesis by working within the confines of well-known tumor suppressor pathways (He et al., 2007).

In addition, as already described, p53 family members regulate EMT which is a key step in cancer development and hence indicate that transformation and invasiveness depend on many of the same regulatory circuits and effector proteins (Weinberg, 2007).

Therefore, p53 family members play a role in tumor suppression and the mechanisms of tumor suppression are mainly based on their regulation of cellular functions like cell cycle

arrest, apoptosis, differentiation, migration or a combination of them. However, in the absence of stress, the most important role of p63 and p73 family proteins is regulation of developmental processes.

4.2.3 p73 in development

Development can be generally defined as the overall process by which a fertilized egg gives rise to an adult organism (Lodish, 2003). The four essential processes by which a multicellular organism is made are cell proliferation, cell differentiation, cell interaction, and cell movement (Alberts, 2002). As it has been shown p53 family members are involved in the regulation of these processes and hence they are implicated in development.

TP63 and *TP73* respective knockout mice have revealed that both genes are required for normal embryogenesis (Mills et al., 1999; Yang et al., 1999; Yang et al., 2000). *TP73* knockout mice, which lack all p73 isoforms, survive at birth but pups have a runting phenotype and high rates of mortality with an average life span of 2-4 weeks. Most commonly, death follows massive gastrointestinal and cranial hemorrhages. Data from our group showed a mild anemia in *Trp73* -/- E14.5 embryos and P15 mice, suggesting a defect in erythroid system, as well as impaired development of other hematopoietic compartments (Marques-Garcia et al., 2009).

However, the most striking phenotype of *Trp73*-/- animals is the neurological one; these animals show hippocampus dysgenesis and enlarged ventricles (hydrocephalus) together with reduced cortical tissue (Pozniak et al., 2000; Yang et al., 2000). Interestingly, most of the neurological abnormalities can be explained by either the absence or the loss of neurons. It was reported that the predominant p73-isoforms expressed in the developing brain were ΔN variants (Pozniak et al., 2000; Yang et al., 2000) and this leads to propose that ΔNp73, but not TAp73, was an essential pro-survival protein whose lack cause neurological defects (Jacobs et al., 2006). However, TAp73 knockout mice (Tomasini et al., 2008b) also present hippocampus dysgenesis indicating that this particular phenotype is due to TAp73 loss while ΔNp73 seems to prevent neural tissue loss due to p53 and TAp73 dependent apoptosis (Tomasini et al., 2009; Walsh et al., 2004). Therefore, p73 plays a multifunctional role in the maintenance of neurogenesis (Killick et al., 2011). In addition, supporting a developmental role of p73, it has been demonstrated that loss of one p73 allele makes mice susceptible to neurodegeneration as a consequence of aging or Alzheimer´s disease (Wetzel et al., 2008).

More recently, three independent groups, including ours, highlighted the role of p73 as a positive regulator of neural stem cell self-renewal in embryonic and adult neurogenesis (Fujitani et al., 2010; Gonzalez-Cano et al., 2010; Talos et al., 2010); moreover, data from our laboratory suggest a role of p73 in the development of the architecture of the neurogenic niches (Dr. González-Cano, PhD dissertation thesis).

4.2.4 p73 in angiogenesis

Tumor progression and metastasis is dependent on oxygen and nutrients, which are supplied by tumor blood vessels. Therefore, it is not surprising that genes considered as oncogenes, that drive tumor formation, also induce expression of pro-angiogenenic factors whereas tumor suppressors, that negatively regulate tumor growth, are potent inhibitors of angiogenesis (Bergers and Benjamin, 2003). In this context, it is very interesting to investigate the role of p53 family members in angiogenesis.

The role of p53 as a tumor suppressor go far beyond controlling cell cycle and apoptosis; there was growing evidence that activation of p53 may inhibit tumor angiogenesis by several mechanisms: by interfering with central regulators of hypoxia (e.g. HIF) (Ravi et al., 2000), by directly increasing the production of angiogenesis inhibitors such as TSP-1 (Dameron et al., 1994), endostatin (Folkman, 2006; Teodoro et al., 2006), tumstatin (Folkman, 2006), or arresten (Wei et al., 2006), or by repression of pro-angiogenic factors such as bFGF (Ueba et al., 1994) or VEGF (Mukhopadhyay et al., 1995; Pal et al., 2001). Until 2005, p53 was considered as a single protein. However, the discovery of several p53 protein variants (Bourdon et al., 2005) requires re-considering the role of p53 in several processes. One of such processes is angiogenesis, as recent studies showed that Δ133p53, which has been suggested to play an oncogenic role (Wei et al., 2012), stimulates angiogenesis (Bernard et al., 2013). This finding raises the possibility that angiogenesis can be regulated by varying the expression ratio of Δ133p53 and p53.

Concerning functional interaction between p53 and VEGF, as a growth factor involved in several aspects of cancer progression, it was accepted until recently that p53 regulated VEGF through interaction and inhibition of Specificity protein (SP1) (Pal et al., 2001) and E2F (Qin et al., 2006) transcription factors (Figure 18). In addition, p53 can indirectly repress VEGF expression during continued hypoxic challenge dependent on the presence of p21 (Farhang Ghahremani et al., 2013a). Conversely, p53 can also positively regulate VEGF expression

during the initial phases of hypoxia by direct binding to conserved sites in the VEGF promoter (Farhang Ghahremani et al., 2013b).

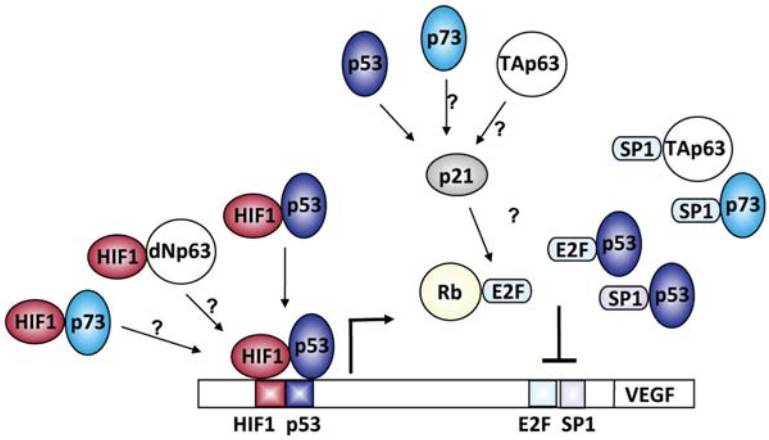

Figure 18: Schematic representation of VEGF promoter regulation by p53 family members. p53 regulate VEGF expression by several mechanisms: p53 can positively regulate VEGF expression by binding directly to VEGF promoter; p53 represses VEGF indirectly either by a process which is dependent on p21 presence or by sequestering SP1 and E2F away from VEGF promoter. The later has been proved for p73. It is also possible that p73 is able to bind to p53 binding sites within the VEGF promoter or modulates VEGF expression via p21 (From Farhang et al., 2013).

Much less is known about the role of p73 in tumor angiogenesis; just a few publications highlight a controversial role of p73 in regulating VEGF expression and angiogenesis. Salimath and coworkers demonstrated that ectopically expressed p73 acts as a repressor of VEGF transcription, in the absence of p53 function, by interacting with SP1 factors (Salimath et al., 2000) (Figure). In the same line of argument, p73 has been described as an inducer of the pigment epithelium-derived factor (PEDF) (Sasaki et al., 2005), which is a neurotrophic factor inhibitor of angiogenesis in prostate and pancreas (Doll et al., 2003), suggesting an anti-angiogenic role for p73. In contrast, p73 has been found to play a pro-angiogenic role in several contexts. In human ovarian carcinoma cells with functional p53, overexpression of p73 induces VEGF expression (Vikhanskaya et al., 2001), as well as the production of other angiogenic factors such as PlGF, FGF-2 and PDGF-B, at the same time that decreases the expression of TSP-1. In this regard, Guan and coworkers demonstrated a correlation between p73 and vascularization in colorectal tumors and they speculate that p73 is involved in tumor angiogenesis by regulating angiogenic factors (Guan et al., 2003). Therefore, it seems that p53 family members play a role in tumor angiogenesis although the molecular mechanisms remain unclear.

The role of p53 family members in the formation of blood vessels during development has been scarcely investigated and remains largely unknown. Angiogenesis is mainly a matter of proliferation, migration and differentiation. Therefore, to the extent that p53 family members contribute to regulate these processes, they might be also involved in regulation of developmental angiogenesis.

Previous data from our laboratory indicate that p73 deficiency impairs erythropoiesis (Marques-Garcia et al., 2009) but whether this was affecting the development of hematopoietic precursors was unclear. Since hematopoietic and endothelial lineages derive from common precursors, one possibility was that p73 deficiency impaired also endothelial differentiation from these precursors and this was one of the hypothesis underlying this thesis.

Recently, in order to identify angiogenesis-associated proteins the human interactome was integrated with known angiogenesis annotated proteins using bioinformatics to generate "The Angiome" (Rivera et al., 2011), the set of proteins currently annotated and topologically associated with angiogenesis. Interestingly both p63 and p73 are included in such network (Fig 12). As it has been described before and as it is reflected in "The Angiome", angiogenesis is a tightly regulated process in which several molecular pathways converge originating a real network. Therefore, factors affecting these molecular pathways can be considered as potential regulators of angiogenesis. It could be the case with p73 which is involved in the regulation of multiple genes either by direct transcriptional modulation or by cooperation with other factors, such as TGF-β (Niemantsverdriet et al., 2012). To assess the molecular pathways regulated by p73 (TAp73 *vs* ΔNp73) in a physiological angiogenic context and to place p73 within this regulatory network constitute one of the long-term goals of this thesis.

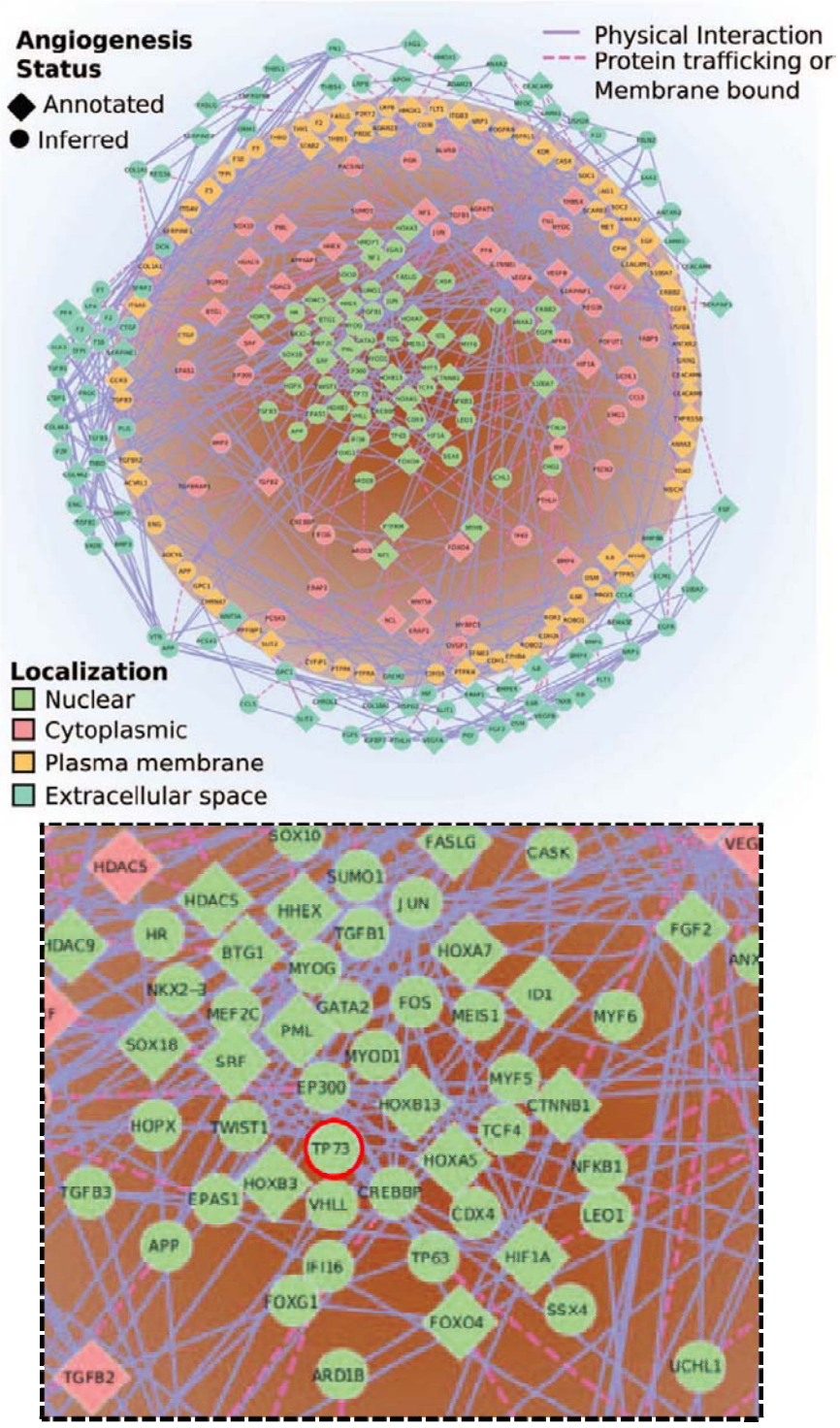

Figure 19: "The Angiome" . The Angiome represents all proteins associated with angiogenesis. p73 (red circle) is included in this complex network (From Rivera, 2011).

Aims

Our working hypothesis is that p53 family members, and in particular p73, play an important role in the regulation of endothelial cell differentiation and morphogenesis of blood vessels. Therefore, the general goal of this thesis is to evaluate the role of p73 in the formation of vascular structures *in vitro* and in the development of blood vessels *in vivo,* using different biological model of angiogenesis.

The specific aims of this thesis are:

1. To develop an endothelial differentiation model using the mouse embryonic stem cell line E14Tg2α by a comparative analysis of differentiation methods.

2. To determine the role of p73 in endothelial cell differentiation, vasculogenesis and angiogenesis *in vitro*.

   2.1 Analysis of the effect of p73 functional inhibition in endothelial cell commitment and vascular morphogenesis in the mES cell line E14Tg2α.

   2.2 Analysis of p73 deficiency in endothelial cell biology in human umbilical vein endothelial cells (HUVEC).

3. To address the role of p73 in vasculogenesis and angiogenesis *in vivo*.

   3.1 Evaluation of the effect of p73 deficiency in the developing vasculature of Trp73 knockout embryos.

   3.2 Analysis of retinal vasculature in Trp73 knockout mice.

Materials and Methods

1. ANIMAL WORK

1.1 Mice strains and animal breeding

Housing and experimental animal procedures were performed according to the European and Spanish regulations (Council Directive 86/609/CEE and RD 1201/2005, respectively) on the protection of animals used for scientific purposes, with the appropriate institutional committee approval.

Mice heterozygous for *TP73* on a mixed background C57BL/6 _ 129/svJae18 were backcrossed to C57BL/6, at least five times, to enrich for C57BL/6 background. As previously described (Yang et al., 2000), *Trp73* homozygous mutant mice (*Trp73*-/-) usually die before reaching sexual maturity so heterozygous mutants were bred to generate *Trp73*-/- mice.

1.2 Mouse genotyping

Genotypes of each offspring were determined at 10-21 days of age. For that purpose, a tail biopsy (the distal ≈2 mm end) was obtained using sterile scissors, and genomic DNA was extracted following a modification of the protocol from Laird (Laird et al., 1991). Briefly, tissue samples were incubated overnight (o/n) at 55ºC in lysis buffer (100 mM Tris-HCl pH 8.5, 200 mM NaCl, 5 mM EDTA pH 8, 0.2% SDS) with 150 μg/mL proteinase K (Sigma#P2308). To remove undigested fragments and cell debris, samples were centrifuged at 14000 g and pellets were discarded. Supernantants were transferred into clean tubes and DNA was precipitated by adding one volume of isopropanol and centrifuging for 5 min at 14000 g. Pellets were resuspended in TE (10 mM Tris-HCl, 1 mM EDTA pH 8) and DNA concentration was measured using a Nanodrop™ spectrophotometer (ND, 1000).

Genotype was determined by PCR analysis as previously described (Yang, 2000). PCR mix was prepared in a final volume of 20 μL containing 500 ng DNA, 0.25 mM dNTPs, (Biotools#20037), 0.4 μM each primer (Table 1) and 1 U of DNA polymerase (Biotools#10222) in 1X reaction buffer. PCR was run in a Gene Amp®PCR System 2700 (Applied Biosystems) under the following conditions: 94ºC for 5 min, followed by 30 cycles (94ºC for 1 min, 60ºC for 1.5 min and 72ºC for 2 min) and a final elongation step at 72ºC for 5 min.

Table 1. Primer sequences for TP73 genotyping.

Primer name- Target Gene	Sequence (5´→3´)
p73·g1 (I1)- TPp73 exon 5	GGG CCA TGC CTG TCT ACA AAG AA
p73·g2 (I2)-TPp73 exon 6	CCT TCT ACA CGG ATG AGG TG
p73·g3 (I3)- pGK·Neo	GAA AGC GAA GGA GCA AAG CTG

PCR products were loaded into 2% agarose gels with etidium bromide and separated by electrophoresis at 90 V for approximately 40 min. The gel was then visualized in a UV transiluminator (Bio-Rad) and images were recorded using the Quantity One software (Bio-Rad) (Figure 18).

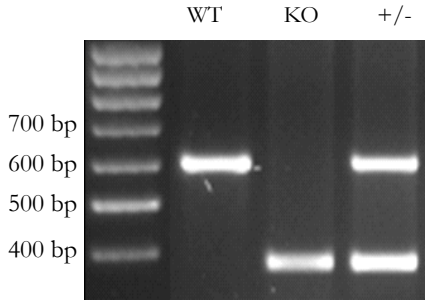

Figure 20: PCR genotyping for Trp73. Typical agarose gel electrophoresis patterns of PCR products from different genotypes. p73WT (600 bp), p73KO (375 bp).

2. CELL CULTURE

2.1 Mouse embryonic fibroblasts (MEFs)

Primary cultures of MEFs were derived from E13.5 mouse embryos to generate feeder cells. The feeders serve as a coating and support the growth of ES cells in an undifferentiated state, providing them with stemness factors.

To prepare feeder cells we adapted the protocol described in http://www.molgen.mpg.de/~soldatov/protocols/protocol/02/02_03.htm. Shortly, pregnant females were euthanized, the uterus was extracted and embryos were dissected free of the maternal and extraembryonic tissues. Embryos were washed in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4) supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B (1% antibiotic-antimycotic solution Sigma#A5955). Internal dark red organs were removed and the remaining tissues were minced carefully with scalpels. The tissue was then incubated in

Trypsin-EDTA (Sigma#T3924) for 30 min at 37°C and 5% CO_2. Finally, the mashed tissue was mechanically dissociated into a single cell suspension and cells were seeded in complete medium (DMEM, 10% FBS, 2 mM L-Glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin). Cells were grown to confluence and then split 1 to 3. When cultures were again confluent, they were treated with 10 μg/mL mitomycin C (Sigma#M4287) for 2 h at 37ºC to induce cell cycle arrest. After washing with PBS, the mitomycin C treated cells were frozen until subsequent use for ES culture.

2.2 Mouse embryonic stem (mES) cells

E14Tg2α (Doetschman et al., 1987) is a derivative of the wild type embryonic stem cell line E14, which was obtained from strain 129 blastocysts by Martin Hooper in 1987. E14Tg2α was isolated as a spontaneous hypoxanthine phosphoribosyltransferase (HPRT)-deficient derivative (Magin et al., 1992).

2.2.1 Culture conditions

mES cells must be cultured under conditions that prevent their differentiation. These conditions require either culturing mES cells on feeder layers and/or the presence of recombinant leukemia inhibitory factor (LIF) in the culture medium.

A) Culture on gelatin coated surfaces

Cells were grown on 0.1% gelatin (Sigma#G2500) coated cell culture plates with ES medium (Table 2) at 37ºC and 5% CO_2. For routine culture, medium was changed every day and cells were split 1 to 7 every second day. Stably transfected cell lines were maintained under selection medium containing 200 μg/mL geneticin sulphate (G-418, Gibco#11811).

Table 2. Media composition for E14Tg2α cells growing on gelatin coated surfaces.

Reagents (final concentration)	Catalog number
Glasgow minimal essential medium	Sigma #G5154
10% fetal bovine serum (FBS)	Sigma #F7524
2 mM L-Glutamine	Gibco# 25030
1 mM non-esential amino-acids (NEAAs)	Gibco # 11140
1 mM Sodium pyruvate	Gibco #11360
0.1 mM β-mercaptoethanol (BME)	Gibco#31350
500 U/ml ESGRO/LIF	Chemicon #ESG1107

B) Culture on feeders

Cells were grown on a mouse embryonic feeder layer with ES medium (Table 3) at 37ºC and 5% CO_2. For routine culture they were split every second day 1 to 20 and medium was changed every day.

Table 3. Media composition for E14Tg2α cells growing on feeders.

Reagents (final concentration)	Catalog number
DMEM/glutamax	Gibco #61965-026
15% FBS	Gibco #10270-106
25 mM HEPES buffer	Gibco # 15630-056
1.2 mM Sodium pyruvate	Gibco #11360-039
19 mM Monothioglycerol	Sigma #M-6145
1000 U/mL ESGRO/LIF	Chemicon #ESG1107

2.2.2 Differentiation conditions

A) EB formation from mES cells growing on gelatin coated surfaces

ES cells were trypsinized and counted to prepare a cell suspension (30000 cel/mL) in EB medium (Table 4). Cell suspension was dispensed using a multichannel pipette in 20 μL drops (600 cell/drop) hanging from a lid of a Petri dish filled with PBS to humidify the environment. Hanging drops were incubated at 37ºC, 5% CO_2, during 3 days. At day 3, EBs were flushed down from the lid and transferred to a Petri dish with EB medium, where they grew in suspension until day 7.

Table 4. EB medium (hanging drops from mES cells growing on gelatin).

Reagents (final concentration)	Catalog number
Glasgow minimal essential medium	Sigma#5154
15 % fetal bovine serum (FBS)	Sigma#F7524
2 mM L-Glutamine	Gibco #25030
1 mM NEAAs	Gibco #11140
1 mM Sodium pyruvate	Gibco #11360
0.05 mM BME	Gibco#31350

At day 7, EBs were collected and seeded in gelatin coated cell culture plates for differentiation. Several medium were assayed to improve endothelial differentiation (Table 5). Medium was changed the following day, and then every second day until EBs were collected or fixed for staining.

Table 5. EB differentiation media.

Medium A	Medium B	Medium B+GF	
DMEM	IMDM	Medium B	
20% FBS	15% FBS	+Growth factors:	50 ng/mL $VEGF_{165}$
2 mM L-Gln	450 μM MTG		2 U/mL EPO
1 mM NEAAs	10 μg/mL Insulin		100 ng/mL FGF2
0.05 mM BME	0.5 % P/S		10 ng/mL IL-6
0.5 % P/S			

• DMEM (Dulbecco's Modified Eagle's Medium, Sigma#D5796); P/S (Penicillin-Streptomycin, Sigma#P4433); IMDM (Iscove's Modified Dulbecco's Medium, Sigma#I3390); MTG (Monothyoglycerol, Sigma#M6145); $VEGF_{165}$ (Peprotech#100-20); EPO (Roche#11120166001), FGF2 (Sigma#F0291) and IL-6 (Peprotech#216-16).

B) EB formation from mES cells growing on feeders

To remove feeders, ES cells were split, transferred to 1% gelatin coated dishes and incubated in ES medium. After two days, cells were trypsinized and counted using a cell counter (Beckman Coulter). Cell suspension was diluted in EB medium (ES medium from Table 3 but without LIF) to 60000 cel/mL and 20 μL (1200 cel) drops were placed into a lid of a Petri dish filled with PBS. Hanging drops were incubated at 37ºC, 5% CO_2, during 4 days. At day 4, EBs were flushed down from the lid with EBs medium and transferred to either gelatin coated surfaces for two-dimensional (2D) culture or into a collagen I matrix for three-dimensional (3D) differentiation.

- **2D Embryoid bodies assay**

EBs were cultured in EB medium with 50 ng/mL $VEGF_{165}$; medium was changed every second day until EBs were processed. For immunofluorescence, EBs were placed individually in each well of a 8 well-chamber slide (BD Biosciences). For further RNA or protein extraction, EBs (50 to 70) were transferred to 10 cm Petri dishes.

- **3D Embryoid bodies assay**

For sprouting assays EBs were embedded between two collagen layers (Jakobsson et al., 2006). Collagen I suspension is composed of Ham's F12 medium, 6.26 mM NaOH, 12.5 mM HEPES, 0.073% NaHCO3, 1% glutamax I, and 1.5 mg/ml collagen I. To prepare the collagen I suspension, reagents were mixed as shown in table 6 in a Falcon tube placed on ice and 600

μL of the solution were added to each well of a 12-well plate. We let the collagen lower layer polymerize at least 3 hours and preferably o/n at 37ºC.

Table 6: Collagen I medium composition.

Reagents (stocks)	V from stock	Catalog number
0.1 M NaOH	0.5 mL	
10X F12	0.5 mL	PromoCell #C96211
1 M HEPES buffer	100 μL	Gibco # 15630-056
7.5% bicarbonate	78 μL	
Glutamax	50 μL	Gibco#35050-038
1XF12	2.772 mL	Gico#31765
3 mg/mL collagen	4 mL	Advanced Biomatrix#5005-B

About five EBs (in not more than one drop of medium) were seeded with a Pasteur pipette on top of the collagen first layer in each well. Immediately after, freshly prepared collagen solution was added (600 μL to each well) and it was allowed to polymerize for 4 h at 37ºC. Finally, 800 μL EB medium with $VEGF_{165}$ (50 ng/mL in a final volume of 2 mL/well) were added to each well. This 2 ml account for the 1.2 ml of collagen plus the 800 μl of EB medium.

2.2.3 Genetic modification

Stable cell lines with functional inhibition of p73 were established in our laboratory by Dr. Gonzalez-Cano as part of her Diploma of Advanced Studies (DEA) (Gonzalez-Cano, 2008). Functional inhibition of p73 was achieved through the constitutive expression of a p73 dominant negative mutant (DDp73β, Figure 21.A), which only contains the oligomerization domain (OD) and is able to functionally inhibit all p73 isoforms (Irwin et al., 2000). Figure 22.B shows the experimental procedure followed to generate the stable mES cell lines: E14Tg2α·pcDNA3 (vector control) and E14Tg2α·pcDNA3·DDp73β (DDp73β from now on). Several parameters were analyzed to ensure that, after the selection process, transgenic cell lines maintained the major characteristic features of the original cell line.

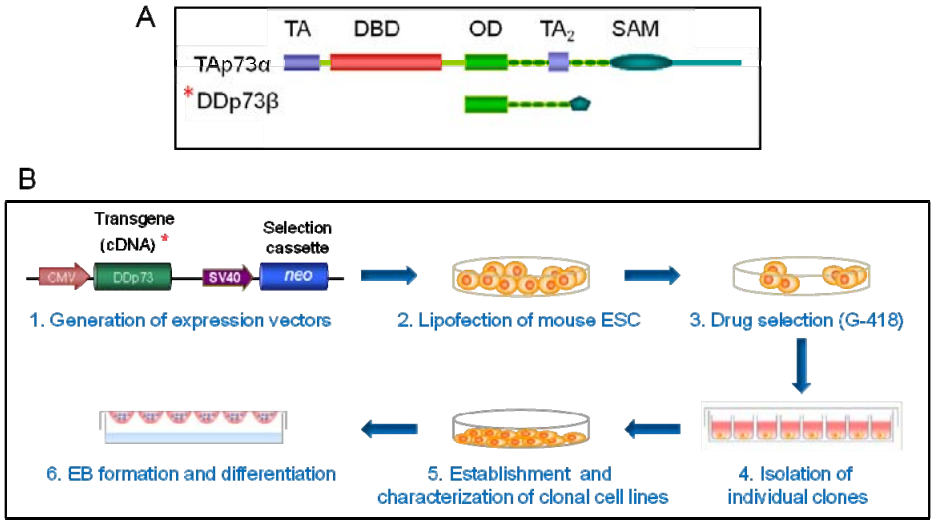

Figure 21. Generation of transgenic lines expressing dominant negative isoforms. A) Structure of TAp73 and DDp73β isoform which binds to wild-type p73 generating transcriptionally inactive hetero-oligomers. B) DDp73β cDNA was cloned into pcDNA3 expression vector (pcDNA3·DDp73β) which was transfected into E14Tg2α cells.Clones were selected with G-418 and isolated to establish clonal cell lines. These cell lines were characterized and subsequently used for differentiation studies.

First, the number of viable colonies was evaluated by staining some of the plates with 10% Giemsa (Figure 22A). Considering that p73 is involved in cell cycle regulation, apoptosis and differentiation, it was feasible that p73 functional inhibition was impairing the selection of viable clones. However, number of DDp73β colonies was not significantly different from the control, indicating that constitutive expression of DDp73β was compatible with the generation and maintenance of stable cell lines.

The morphology of DDp73β colonies was also assessed. Microscopic observation showed that DDp73β clones exhibited a typical ES morphology (round colonies with smooth bright borders) that was roughly indistinguishable from control ones (Figure 22B). Therefore, neither the selection process nor the expression of DDp73β had an effect on morphological characteristics of mES colonies.

Alkaline phosphatase (AP) activity, which is considered a stemness indicator, was next analyzed. Staining patterns were similar in DDp73β and control colonies. In addition, expression of the pluripotency marker Nanog was shown by western blot with no difference being observed between the control and the DDp73β transgenic cell lines (Figure 22C). These results corroborate that selected clonal cell lines expressing DDp73β maintained their stemness.

Finally, transgene expression was checked after several passages. As shown in the western blot in figure 22D, transgene expression was maintained even after 40 passages. Altogether, these data indicate that DDp73β cell line could be used to perform further experiments to determine the role of p73 in vasculogenic and angiogenic responses *in vitro*.

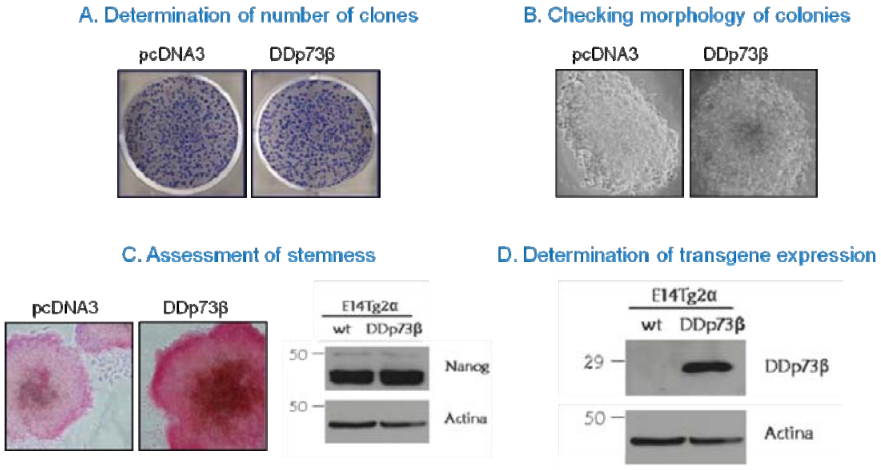

Figure 22. Characterization of E14Tg2α pcDNA3·DDp73β clonal cell lines: A) Determination of the number of clones by Giemsa staining. B) Morphological analysis through microscopical observation and phase contrast images. C) Assessment of stemness though alkaline phosphatase (AP) staining and Nanog expression. D) Checking transgene expression by western blot.

2.3 Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC are isolated from the vein of the umbilical cord and are commonly used as a model for physiological and pharmacological investigations. HUVE cells were kindly provided from Biomar Microbial Technologies (Leon, Spain).

2.3.1 Culture conditions

HUVEC were maintained on gelatinized culture dishes in endothelial basal medium, EBM (Lonza#CC3121), with full supplements (Lonza#CC4133), including bovine brain extract (BBE), hydrocortisone, human epidermal growth factor, ascorbic acid, FBS and gentamicin/ amphotericin-B. Cells were used from passage 2 to passage 8.

2.3.2 Genetic modification

A) Transfection of expression vectors

Cells were transfected with pcDNA3·DDp73 and the pertinent pcDNA3 control using Lipofectamine™2000 Transfection Reagent (Invitrogen#11668). At the time of transfection,

HUVEC were 60-80% confluent. Trasfection was performed following the manufacturer´s instructions. For each well of a 24-well plate 0.8 μg of DNA were used combined with 1 μL of Lipofectamine™. The indicated amounts of Lipofectamine and DNA were diluted in 50 μL Opti-MEM® I Reduced Serum Medium (Invitrogen#31985) and incubated for 5 min at room temperature (RT). Then, DNA and Lipofectamine were combined, mixed gently and incubated for an additional 20 min at RT to allow the DNA-Lipofectamine complexes to form. The complexes were added to cells in 500 μL Opti-MEM®. After 5 hours incubation, transfection complexes were removed and replaced with complete growth medium.

B) Transfection of small interference RNA (siRNA)

For gene silencing experiments, the indicated RNA interference oligos were used (Table 7). Cells (60-70% confluency) were transfected with Lipofectamine® RNAiMAX (Invitrogen#13778) using the manufacturer´s instructions. Briefly, for each well in a 24-well plate, 1 μL Lipofectamine® RNAiMAX was diluted in 50 μL Opti-MEM® and incubated for 5 min at RT. ,Oligos (0.6 μL from a 100 μM stock) were first diluted in 50 μL Opti-MEM® and then combined with Lipofectamine® RNAiMAX. Complexes were incubated for 15 min at RT and added to the cells growing in EBM medium. Final concentration of RNA oligos was 100 nM. Medium was changed 36 h after siRNA transfection.

Table 7: siRNA oligos..

Oligo name-Reference	Sequence
Scrambled-	5´ UAGCCACCACUGACGACCUdTdT 3´ 5´dTdTAUCGGUGGUGACUGCUGGA 3´
p73i.3- (Irwin *et al*, 2003)	5´CGGAUUCCAGCAUGGACGUdTdT 3´ 5´ dTdTGCCUAAGGUCGUACCUGCA 3´
p73i.4- (Irwin et al, 2003)	5´CCAUCCUGUACAACUUCAUGU G-3´ 5´CAUGAAGUUGUACAGGAUGGU G-3´
ΔNp73i- (Papoutsaki *et al*, 2004)	5´CGUCGGUGACCCCGCACGGUU3´ 5´CCGUGCGGGGUCACCGACGUU3´

2.3.3 Angiogenic assays

A) Proliferation

Assessment of cell proliferation was performed according to the MTT assay protocol. HUVEC (15000 cells/well) were seeded in 96-well tissue culture plates coated with gelatin and allowed to adhere o/n. The next morning (considered as time point 0h), MTT solution (3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) was added to each well (50 μL of a 1 mg/mL stock solution in PBS), and cells were incubated for 2 h at 37°C. After supernatants were discarded, residual formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO). Absorbance was measured at 595 nm on a microplate reader (Synergy HT, Biotek). The same procedure was repeated after 24 and 60 h.

B) Migration

Wound healing assays, or scratch assays, were performed on confluent monolayers, either 48 h after plasmid transfection or 72 h after RNA interference. The monolayers were wounded with a micropipette tip (yellow) making a straight scratch to form a cell-free zone into which cells at the edges of the wound can migrate. After the scraping, cells were washed with PBS and replaced with fresh medium. Cell cultures were imaged at 0 and 10 h after the scratch using a phase contrast microscope (Leica DMI 3000B) and a digital camera (Leica DFC 310FX). Wound width was measured from the images using Image J software (NIH). Wound closure rate was calculated as follows: (wound $width_{0h}$ – wound $width_{10h}$) x 100/ wound $width_{10h}$.

C) Tube formation

To perform the tube formation assay a 96 well-plate was coated with 100 μL Matrigel™ matrix (BD#354234) per well, and incubated at 37ºC for 30 min to allow gelling. Cells were seeded on top at a density of 125000 cel/mL in 200 μL of EBM medium. After 6 h, images were taken with a phase contrast microscope (Leica) and analyzed using WimTube software (Wimasis GmbH).

3. RNA ANALYSIS

3.1 RNA isolation

Total RNA was extracted from cultured cells using TRI®Reagent (Ambion), which was directly added to the cell monolayer (1 mL/10 cm^2). In the case of EBs in suspension culture, they were collected by centrifugation and resuspended in TRI-Reagent. Homogenates were transferred to a microfuge tube and incubated for 5 min at RT: then, 200 μL of chloroform per sample were added and, after vigorous shaking, they were incubated for 15 min at RT. Samples were centrifuged at 12000 g for 15 min at 4ºC and aqueous phase was transferred to a clean tube. RNA was precipitated by adding 500 μL of isopropanol; samples were vortexed for 10 s,

incubated for 10 min at RT and centrifuged at 12000 g for 8 min at 4ºC. Supernatants were discarded and RNA pellets were washed with 1 mL of 75% ethanol, prepared in 0.1% diethyl pyrocarbonate (DEPC, Sigma#D5758) water. After centrifugation at 7500 g for 5 min at 4ºC, ethanol was removed and RNA pellets were resuspended in 30 μL of 0.1% DEPC water. RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and RNA quality was assessed in an agarose-formaldehyde gel.

3.2 cDNA synthesis

cDNA was synthesized from RNA using the High Capacity RNA-to-cDNA™ Kit (Applied Biosystems#4377406) following the manufacturer´s instructions. RNA (up to 2 μg in a volume up to 9 μL) was mixed with 10 μL 2X RT Buffer and 1 μL of 20X Enzyme Mix, in a final volume of 20 μL. Reactions were carried out into a thermal cycler under these conditions: 37ºC for 60 min and 95ºC for 5 min.

3.3 Semi-quantitative RT-PCR (reverse transcription-polymerase chain reaction)

Semi quantitative RT-PCR was performed to analyse gene expression in EBs cultured in different media. One microliter of the cDNA synthesis reaction (equivalent to 100 ng reverse-transcribed RNA) was used for PCR (modified from Vittet *et al.,* 1996). cDNAs were amplified in a final volume of 25 μL with 0.75 U Taq Polymerase, 0.375 pmol of each primer (Table 8) and 1.875 mM dNTPs. The amplification parameters were: 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min, for 30 cycles followed by 5 min at 72ºC for final extension. PCR products were analyzed in 1.8% agarose gels with ethidium bromide and separated by electrophoresis at 90 V for approximately 40 min. The gel was then visualized as previously indicated for DNA electrophoresis.

Table 8. Primer sequences for RT-PCR analysis

Target mRNA	Forward 5´to 3´	Reverse 5´to 3´	Size
CD31 †	GTCATGGCCATGGTCGAGTA	CTCCTCGGCATCTTGCTGAA	260 bp
Flk-1 †	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCCAACCACCCT	269 bp
VE-Cadherin †	GGATGCAGAGGCTCACAGAG	CTGGCGGTTCACGTTGGACAT	226 bp
GAPDH ‡	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	454 bp

† Vittet *et al.* (1996); ‡ Megiorni *et al.* (2004).

3.4 Quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed to analyse gene expression in HUVEC as well as in EBs. For PCR reactions, a mix containing 1X FastStart Universal SYBR Green (Roche#04913850001) and 70 nM of each primer was added to 1 µL of cDNA to a final volume of 20 µL. Sequences of primer sets are detailed in Table 10. All reactions were performed in duplicate in a StepOnePlus™ real-time PCR instrument (Applied Biosystem). PCR program was as follows: 50°C for 2 min, a denaturing step at 95°C for 10 min, followed by 40 cycles (95°C for 15 s, 60°C for 30 s and 72°C for 30 s) and a final extension step at 72°C for 10 min.

For gene expression analysis, relative expression values were calculated according to the formula: relative expression of gene= $2^{(Ct_{\text{internal reference}}-Ct_{\text{gene}})}$ and the mean expression and standard deviation for each duplicate was calculated.

Table 9. Primer sequences for qRT-PCR analysis.

Target mRNA	Forward 5´to 3	Reverse 5´to 3´
m18S	AGTTCCAGCACATTTTGCGAG	TCATCCTCCGTGAGTTCTCCA
mCD31	TACTGCAGGCATCGGCAAA	GCATTTCGCACACCTGGAT
mVE-Cadherin	AGGACAGCAACTTCACCCTCA	AACTGCCCATACTTGACCGTG
mVEGFR-2	ACAGACCCGGCCAAACAA	TTCCCCCCTGGAAATCCTC
mVEGFR-1	GGGCAGACTCTTGTCCTCAACT	CAGCTCATTTGCACCCTCGT
mTGFβ1	TGGAGCAACATGTGGAACTC	ACGCCAGGAATTGTTGCTAT
mTAp73	GCACCTACTTTGACCTCCCC	GCACTGCTGAGCAAATTGAAC
mΔNp73	ATGCTTTACGTCGGTGACCC	GCACTGCTGAGCAAATTGAAC
mTAp63	TGAAGATCCCTGAACAGTTCC	CTTGTTGCGACGAGAATCCAT
mp53	GTCACAGCACATGACGGAGG	TCTTCCAGATGCTCGGGATAC
h18S	GGCGCCCCCTCGATGCTCTTA	GCTCGGGCCTGCTTTGAACAC
hVEGFR-2	GACTTGGCCTCGGTCATTTA	ACACGACTCCATGTTGGTCA
hVEGFR-1	TCCAAGAAGTGACACCGAGA	TTGTGGGCTAGGAAACAAGG
hTAp73	GCACCTACTTCGACCTTCCC	GTGCTGCTCAGCAGATTGAAC
h ΔNp73	ATGCTGTACGTCGGTGACCC	GTGCTGCTCAGCAGATTGAAC

* m: mouse; h: human;

4. IMMUNODETECTION

4.1 Isolation of CD31 positive cells

CD31 positive cells were isolated from E14Tg2α, E14Tg2α·pcDNA3 and E14Tg2α·DDp73β EBs at day 12 in basal culture conditions (without VEGF). About 5 Petri dishes with 50-70 EBs per dish were used for each cell line. The previous day, DYNAL beads (Invitrogen DYNAL#110.35) were coated with rat anti-mouse CD31 (BD#553370) by incubation in PBS, 0.5% BSA, 2 mM EDTA. (50 μL beads and 3 μg antibody per sample in 1 mL buffer) at 4ºC o/n.

Next morning EBs were washed with HBSS (Gibco #24020) and subsequently incubated with 2.5 mg/mL collagenasa (Sigma#C2674) and 100 U/mL DNaseI (Roche#10104159001) in HBSS for 30-40 min at 37ºC using a rocking shaker. Dishes were placed on ice and cell dissociation solution (Sigma#C5914) was added to each Petri dish. EBs were disrupted by gently pippeting to get a single cell suspension. This suspension was filtered using a 40 μm cell strainer (BD#352340), centrifuged 7 min at 900 g, 4ºC and re-suspended in PBS, 0.5% BSA, 2 mM EDTA. At this stage cells were counted.

Cells were centrifuged again, resuspended and incubated with 50 μL anti-CD31 coated beads. CD31 positive cells were collected using a magnetic particle concentrator (Dynal MPC® 120-20). Supernatants, which contains the CD31 negative cells, was also stored to be used as control. Cells were washed several times with PBS, 0.5% BSA, 2 mM EDTA and collected by centrifugation at 300 g for 3 min. At this stage, cells were counted again to get an approximate percentage of CD31 positive cells. Finally cells were collected by centrifugation and pellet was lysed in the appropriate buffer for RNA isolation. Samples were stored at -80ºC for further processing.

4.2 Immunocytochemistry

4.2.1 2D EBs (differentiation day 8-14)

EBs were washed once with Tris buffered saline (TBS: 25 mM Tris base, 137 mM NaCl, 2.7 mM KCl) and fixed with Zn-Fix (100 mM Tris HCl, 37 mM zinc chloride, 23 mM zinc

acetate, and 3.2 mM calcium acetate) containing 0.2% Triton-X100 at 4ºC o/n. The next day EBs were washed with TBS and further processed.

For immunofluorescence, EBs were washed two more times with TBS-0.1% Tween-20. Then, to block non-specific binding, EBs were incubated with TBT (3% BSA, 0.1% Tween-20 in TBS) for 45 min at RT. Subsequently, EBs were incubated with a single primary antibody (400 ng/mL goat anti-mouse VECadherin, R&D AF1002), or a combination of them (usually 500 ng/mL rat anti mouse CD31 and 200 ng/mL goat anti-mouse Flk-1 (R&D AF644). Incubation was performed in TBT at 4ºC o/n.

After three washes with TBS, incubation with secondary antibody was performed in TBT for 45 min at RT. Usually, 2 μg/mL donkey anti-rat Alexa594 and donkey anti-goat Alexa488 (Molecular Probes) were used. Cells were whased three times with TBS-0.05% Tween-20 and then incubated with 1 μg/mL Hoechst 33342 (Molecular Probes) for 10 min at RT. The plastic part from the slide was removed following the manufacturer's instructions (BD Biosciences) and slides were mounted with Fluoromount G (Southern biotechnology).

For immunocytochemistry, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 min. Then, EBs were washed twice with TBS and incubated in TBT for 45 min at RT to block unspecific binding. Incubation with primary antibody (rat anti-mouse CD31, 500 ng/mL) was performed at 4ºC o/n. Next day, EBs were washed three-times with TBS-0.05% Tween-20 and incubated with biotynilated secondary antibody for 1h at RT. After three more washes (TBS-0.05% Tween-20), incubation with streptavidin horseradish peroxidase (Vector Laboratories #SA5004) was carried out for 30 min at RT. EBs were washed four times with TBS-0.05% Tween-20 and AEC kit (Vector Laboratories #SK4200) was used to develop the results. Slides were mounted with Ultramount mounting media (DAKO) and dried for 30 min at 70ºC in the oven.

4.2.2 3D EBs (differentiation day 14-21)

EBs in collagen were washed twice with PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at RT. Upper layer of the collagen was removed using forceps and placed into a well of a 24-well plate with PBS for further processing.

After two washes with PBS for 15 min, blocking and permeabilization were carried out with 3% BSA, 0.2% Tx-100 in PBS for 2 h at RT. Primary antibody (usually, rat anti-mouse

CD31) was added in PBT (3% BSA, 0.1% Tween-20 in PBS) and incubated at 4ºC o/n;. On the second day, EBs were washed at least three times for 1h with PBS-0.01%Tween-20. Incubation with secondary antibody was done in PBT at 4ºC o/n. On the third day, EBs were washed again at least three times for 1 h with PBS-0.05%Tween-20. Nuclei were stained with 1 μg/mL Hoechst 33342 and extensively washed with PBS. For confocal microscopy, the collagen layer was placed on an adaptor glass device in PBS. Several Z-stacks were obtained for individual EBs using a Zeiss 510 confocal microscope provided with Zen software.

4.2.3 HUVEC

HUVE cells were fixed in 3.7% PFA for 15 min. Permeabilization was carried out with 0.2% Tx-100 in PBS for 20 min. To block non-specific binding, EBs were incubated with 3% BSA, 0.1% Tween-20 in PBS for 45 min at RT. After PBS washing, cells were incubated o/n with the primary antibody (Table 10) in blocking solution. Next morning, cells were gently washed and incubated with the appropriated secondary antibody diluted in blocking solution for 1 h at RT. After three washes, actin filaments were stained with 50 μg/mL TRITC-conjugated phalloidin (Sigma#P1951) for 40 min at RT. After several washes with PBS to remove unbound phalloidin, nuclei were counterstained with 1μg/ml DAPI in PBS for 1 min. Finally, cells were washed and coverslips were mounted with VECTASHIELD® Mounting media (Vector Laboraties#H-1000).

4.3 Western Blot

4.3.1 Cell lysis and protein extracts preparation

Either HUVEC or E14Tg2α cells were dispersed mechanically using a cell scraper and collected by centrifugation at 7500 g for 5 min at 4ºC. Pellets were washed with PBS and cells collected again by centrifugation at 14000 g for 5 min at 4ºC. Pellets were then resuspendend in EBC lysis buffer (50 mM Tris pH 8, 120 mM NaCl and 0.5% NP-40) containing proteases inhibitors (Aprotinin 10 μg/mL, Leupeptin 20 μg/mL, Sodium Orthovanadate 1 mM and PMSF 0.1 mg/mL), and incubated in rotation for 30 min at 4ºC. Subsequently, cell lysates were centrifuged at 14000 g for 12 min at 4ºC to remove cellular debris and supernatants were transferred to clean microfuge tubes. These lysates were either loaded immediately or stored at -80ºC for further processing.

Protein concentration was measured using the colorimetric method described by Bradford (Bradford, 1976), using bovine serum albumin (BSA) as standard protein. Samples were prepared (equal amounts of protein ranging between 20 and 200 μg) and denatured by heating them to 100ºC for 3 min in SDS gel-loading buffer (50 mM Tris·Cl pH 6.8, 100 mM dithiothreitol, 2% SDS 0.1% bromophenol blue and 10% glycerol).

4.3.2 Protein electrophoresis and immunoblot

Protein samples were resolved by denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE). using essentially the discontinuous buffer system originally described by Ornstein and Davis (Davis, 1964; Ornstein, 1964) with SDS (Laemmli, 1970). Gel casting was performed in a Mini-PROTEAN3 Cell (BioRad) following recommendations; 10% polyacrilamide resolving gels were commonly used. The gels were placed into the electrode assemby in the electrophoresis apparatus and the minitank was filled with electrophoresis buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS). Samples (up to 60 μL) were loaded into the wells and run at 70-80 V until the dye front moved into the resolving gel. Then, voltage was increased to 110-120 V and electrophoresis continued until bromophenol blue reached the bottom of the gel.Proteins separated by SDS-PAGE were transferred to a nitrocellulose filter (Burnette, 1981) using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Briefly, one side of the gel was placed in contact with a nitrocellulose filter and both were then "sandwiched" between Whatman 3MM paper and two porous pads. The entire sandwich, with the nitrocellulose filter facing the anode, was then immersed in the electrophoresis tank containing Transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol). Transfer was carried out in the cold room (4ºC) and at a constant current of 350 mA for 80 min.

For the detection of proteins with specific antibodies, nonspecific binding was prevented by incubation with blocking solution (5% nonfat dried milk, 1% goat serum in TBS-0.05% Tween-20) for 1 h at RT. Membranes were then incubated with the primary antibody directed against the target protein, diluted in 2.5% nonfat dried milk, at 4ºC o/n with gentle agitation on a platform shaker.

Membranes were washed three times for 5 min with TBS-0.05% Tween-20 and subsequently incubated with 25 ng/mL horseradish peroxidase-coupled secondary antibody (Pierce) in 2.5% nonfat dried milk/TBS-0.05% Tween-20 for 1 hour at RT. Membranes were

washed three times for 5 min with TBS-0.05% Tween-20 and incubated with Super Signal West Pico Chemiluminescent Substrate (Pierce). Chemiluminescent signal was detected by exposing the membrane to autoradiography films (GE Healthcare) that were manually developed.

Tabla 10: Antibodies used for immunodetection.

Application	**Antigen**	**Host**	**Dilution**	**Reference**
IF/IHC/	CD31	Rat	1/1000	BD#553370
IF	VE-Cadherin	Goat	1/500	R&D# AF1002
IF	Flk-1	Goat	1/500	R&D#AF644
IF	NG-2	Goat	1/500	R&D#AF2585
IF	GFAP	Rabbit	1/200	Thermo Scientific#RB087
IF	pFAK (pY397)	Rabbit	1/100	Abcam#ab4803
IF	p73 (ER15)	Mouse	1/50	Calbichem#OP109
IF	GFP	Rabbit	1/500	Santa Cruz#sc-8334
WB	Flk-1 (A3)	Mouse	1/500	Santa Cruz#sc-6251
WB	pSMAD1/5/8	Mouse	1/1000	Cell signaling #9511
WB	pERK	Mouse	1/1000	Santa Cruz#sc-32577
WB	ERK	Rabbit	1/10000	Santa Cruz#sc-154
Application	**Antibody**	**Host**	**Dilution**	**Reference**
IF	anti-rat Alexa594	Donkey	1/1000	Molecular Probes#A-21209
IF	anti-goat Alexa488	Donkey	1/1000	Molecular Probes#A-11055
IF	anti-rabbitAlexa594	Donkey	1/1000	Molecular Probes#A11037
IF	anti-rabbit-FITC	Donkey	1/100	Jackson Lab#711-095-152
IF	anti-mouse-FITC	Donkey	1/100	Jackson Lab#715-095-150
IF	anti-rabbit-Cy3	Donkey	1/1000	Jackson Lab#715-166-151
WB	anti-mouse HRP	Goat	1/20000	Pierce#31430
WB	anti-rabbit HRP	Goat	1/20000	Pierce#31460

*WB: western blot; IF: immunofluorescence; IHC: immunohistochemistry

5. *IN VIVO* ANALYSIS

5.1 Embryo analysis

Pregnant females were euthanized at 11.5 days post-coitum, uterus was extracted and embryos were dissected away from the maternal and extraembryonic tissues. Embryos were collected in PBS and fixed in 4% PFA at 4ºC o/n. Immunostaining with anti CD31 antibody was performed following the protocol for 3D EBs.

5.2 Retina dissection and flat mount preparation

Postnatal mice on day 5 and 7 were decapitated, and eyes were enucleated and fixed in 4% PFA o/n. Retinas were dissected and washed with PBS for further processing. For whole mount staining, retinas were blocked for 1h at RT in PBLEC buffer (0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, 0.1 mM $MgCl_2$, 1% Tx100 in PBS pH 6.8), followed by incubation with 2 μg/mL biotinylated isolectin B4 (Sigma#L-2140) in PBLEC, at 4ºC o/n. Retinas were washed five times with PBS at RT (each washing for 30 min) and then incubated with 4 μg/mL Streptavidin-Alexa488 (Invitrogen#s-11223) in 5% FBS, 3% BSA, 0.1% TX-100 in PBS, for 2 h at RT. Two more PBS washes were performed before proceeding to either mount the retinas or block them with 3% BSA, in case a primary antibody was used afterwards.

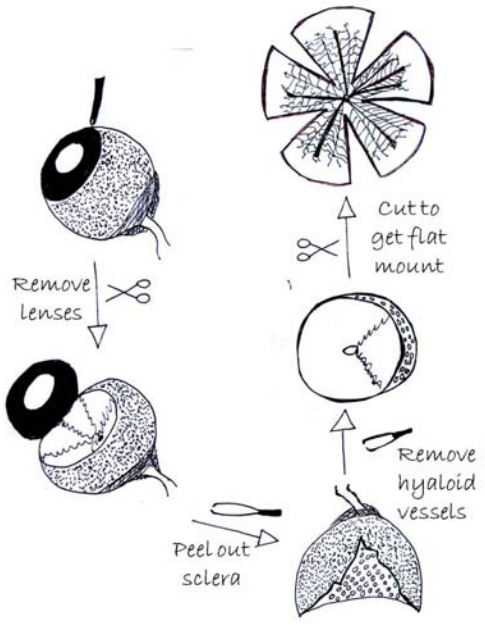

Figure 23: Retinal dissection process. Scheme showing the dissection of mouse retina.

For staining with primary antibodies (anti GFAP; Thermo Scientific#RB087), retinas were blocked with 3% BSA for 2 h at RT and subsequently incubated with primary antibody diluted 1/200 in PBS, 3% BSA, 5% FBS, at 4ºC o/n. On the next morning, retinas were washed five times with PBS at RT (each washing for 30 min) and incubated thereafter with secondary antibody (donkey anti-rabbit Alexa594, Molecular Probes) in PBS, 3% BSA, 5% FBS. After PBS washing, retinas were mounted with Fluoromont G.

Representative images were analysed independently using the AngioTool software (http://angiotool.nci.nhi.gov). At least three images from each individual and three individuals from each genotype were used for the statistical analysis by Student t-test.

6. STATISTICAL ANALYSES

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). All values are given as means and error bars indicate standard deviation (Mean±SD). Significant differences were determined using the Student´s unpaired *t* test with p values lower than 0.05 considered significant. Experiments were repeated at least twice.

Results

1. Development of an endothelial differentiation model using the mES cell line E14Tg2α: comparative analysis of differentiation methods

Embryoid bodies (EBs) are tridimensional aggregates of embryonic stem cells in which the three-dimensional structure of the EB microenvironment enables differentiation and morphogenesis which yields microtissues that are similar to native tissue structures. This is a unique model, as it represents vasculogenesis and angiogenesis at different stages of the process in the culture medium. Differentiation of mES cells using EBs involves the maintenance of mESC in an undifferentiated state using LIF and/or feeder cells followed by withdrawal of these factors and culture of EBs in some specific differentiation medium. Our main objective was to establish a cellular model that allowed us to study the cellular processes and molecular pathways involved in endothelial cell differentiation and blood vessels morphogenesis. There are several protocols to differentiate ES cells into endothelial cells using EBs; however, the competence and efficiency of these protocols to recapitulate vasculogenesis and angiogenesis have never been compared systematically. Therefore, to determine the best experimental conditions that would recapitulate vasculogenesis and improve endothelial cell differentiation and vascular morphogenesis in the mES cell line E14Tg2α, we decided to perform a comparative analysis of various protocols to differentiate mES through EBs formation.

As indicated in Figure 24, the comparative analysis was performed in three steps: first we compare three different media in two-dimensional (2D) EBs culture (Figure 25); next we compare two different protocols previously described in the literature for three-dimensional (3D) EBs culture in collagen gels, which recapitulates sprouting angiogenesis (Feraud et al., 2001; Jakobsson et al., 2006); and finally we took advantage of the already described systems to combine differentiation in 2D and 3D cultures (Jakobsson et al., 2006).

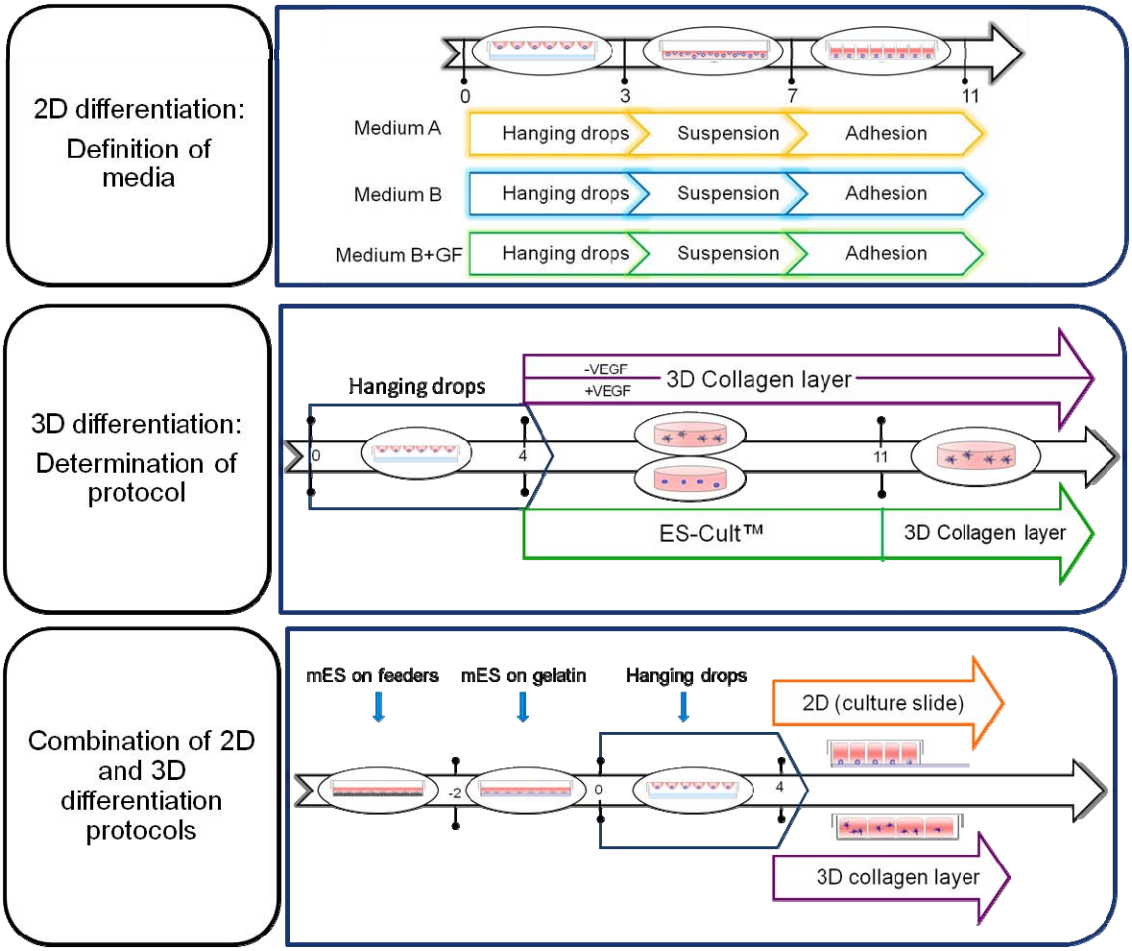

Figure 24: General outline of the comparative analysis of mES cells endothelial differentiation protocols using an EB-based model. First, three different media (with or without growth factors, GFs) were compared to optimize differentiation in 2D (upper panel). Then, two protocols were evaluated to optimize sprouting (middle panel). Finally, 2D and 3D differentiation were combined into the same protocol (lower panel).

1.1 Comparative analysis of endothelial differentiation media

The protocol previously used in our laboratory for 2D EBs differentiation consists of three steps: first, ES cells are trypsinized and let to aggregate in hanging drops; then, EBs are transferred to a Petri dish where they grow in suspension culture; finally, EBs are transferred to gelatinized tissue culture plates where they adhere to the surface and differentiate into several cell types. This is a quite versatile protocol which allowed us to obtain cardiomyocytes and endothelial cells as well as neurons depending on the specific medium used. However, we were interested in directing differentiation towards endothelial cell lineage and to promote the *in vitro* formation of vascular-like networks.

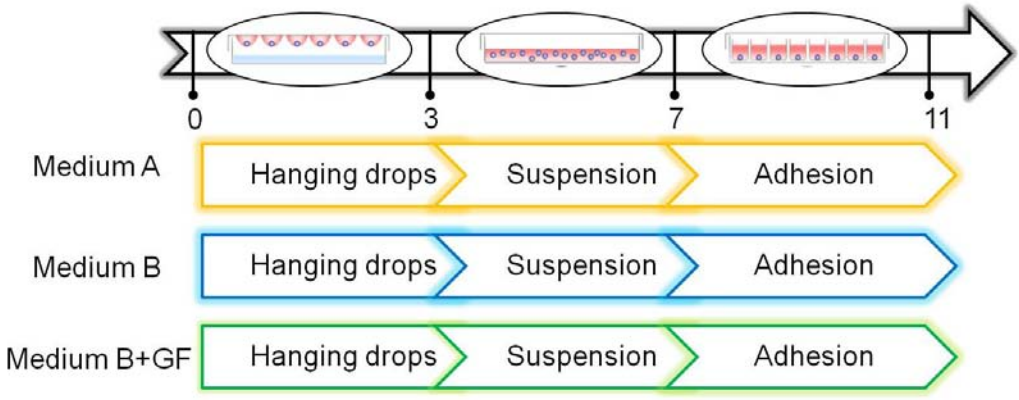

Figure 25: Schematic view of the comparative analysis of three differentiation media. EBs culture was conducted in parallel with three different media: at day 0, mES were trypsinized and cultured as hanging drops until day 3; from day 3 to day 7, EBs were grown in suspension culture, and from day 7 onwards EBs were cultured in gelatin coated surfaces.

Several groups have reported the endothelial cells enrichment in EBs cultures by using media containing some specific growth factors. Vittet and coworkers optimized endothelial cell differentiation using a cocktail of growth factors (GFs) including VEGF, FGF2, IL-6 and EPO in a semisolid medium, different from our hanging drop based medium. We postulated that using this combination of growth factors we would increase the EBs size and the differentiation rate of the EBs with respect to our original protocol. Therefore, we decided to combine our initial method, based in EB formation in hanging drops, with the utilization of different media (with or without the cocktail of GFs).

Based on all the above, we established three sets of EBs in parallel, each one with a specific medium (Figure 25): medium A, medium B and medium B+GFs (see materials and methods for details), and performed a comparative analysis using three parameters: morphology, size and expression of endothelial markers.

We first evaluated the morphology of the EBs along the differentiation process. At day 3, while growing in hanging drops, all the EBs looked as quite homogeneously-shaped spheres settled at the bottom of the drops. While the EBs grown in medium A were characterized by a more irregular surface, with cells that become detached from the EB, the edge of the EBs cultured in medium B seemed smoother independently of the presence of GFs (Figure 26).

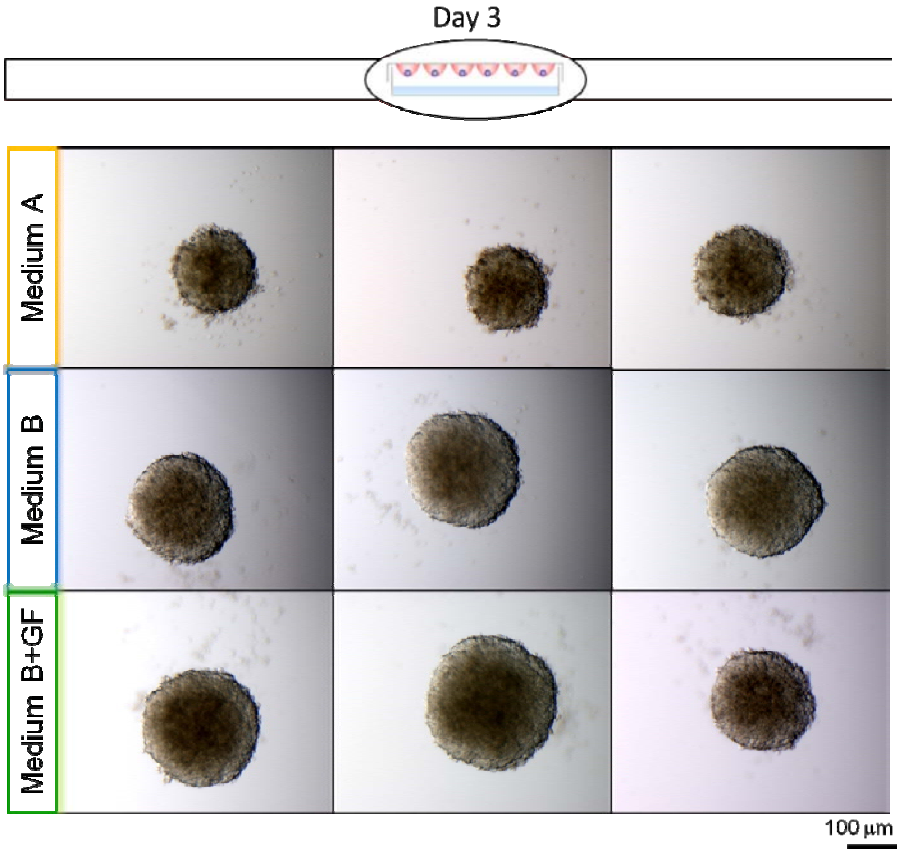

Figure 26: EBs morphology in hanging drops. EBs at day 3 appeared as quite homogeneous spheres of different sizes, settled at the bottom of every hanging drop. To reflect variability, three phase contrast images for each condition are shown. Objective: 10X.

As expected, EBs cultured in suspension in medium A at day 6 formed individual floating spheres. However, some EBs growing in medium B started to adhere to the surface of the Petri dish and became flat presenting an enhanced size. Furthermore, EBs growing in medium B+GFs attached to each other and form aggregates (Figure 27).

On the same line, by day 10, EBs in medium A appeared as individual regular spheres with homogeneous size and morphology, while EBs in medium B and medium B+GFs continued forming EBs aggregates with abnormal shapes (Figure 28).

By day 14, only EBs cultured in medium A maintained the expected morphology, whereas most of the EBs in medium B, and medium B+GFs, started to disaggregate (not shown).

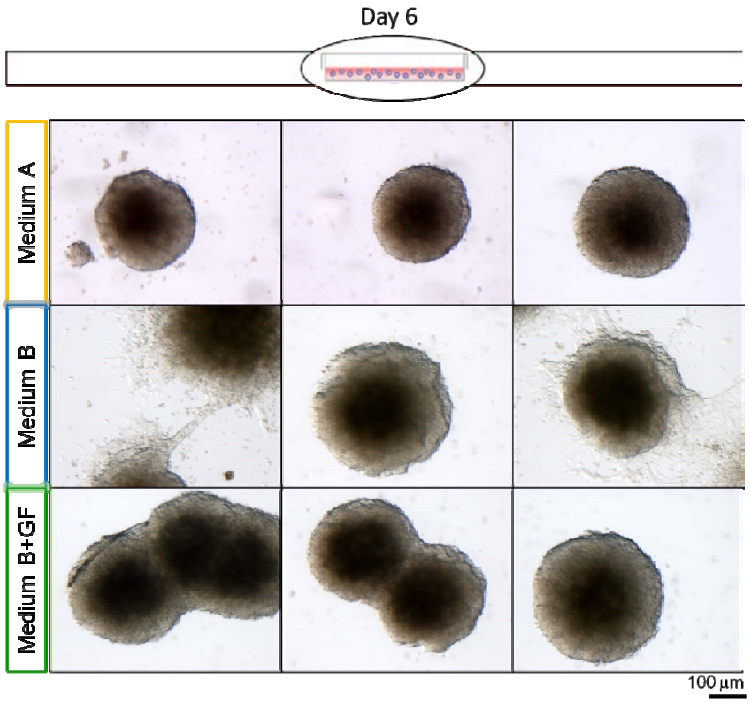

Figure 27: EBs morphology in suspension culture. EBs at day 6 appeared like floating spheres when growing in medium A, but they started to adhere to the plate surface or to each other when growing in medium B and medium B+GFs, respectively. To reflect variability three phase contrast images for each condition are shown. Objective: 10X.

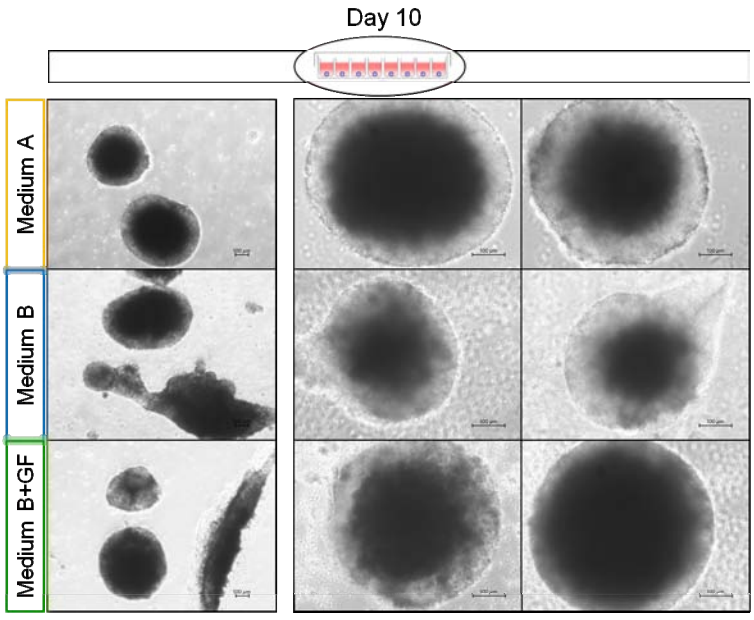

Figure 28: EBs morphology in gelatin coated surfaces. By day 10, the overall aspect of the EBs culture (left panel) showed that EBs in medium A were floating regular spheres while EBs in medium B, either with or without GFs, attached together generating aggregates with abnormal shapes. A closer look to individual EBs is shown in the right panel. Phase contrast images; objective: 4X, left panel and 10X, right panel. Scale bars: 100 µm.

Along the process we evaluated the size of the EBs cultured in the different media. EBs diameter was measured at different time points: day 3, day 7 and day 11, and plotted against time (Figure 29). Since EBs are not perfect spheres, average diameter was calculated and considered for each individual EB. Mean value is referred as the arithmetic mean of at least 10 EBs for each condition and time point.

After 3 days in hanging drops the EBs size was significantly different among the cultures. EBs cultured in medium B and medium B+GFs were bigger than those grown in medium A. In addition, the use of GFs increased the size variability within the culture (Figures 26 and 29). This difference was maintained on day 6, after 3 days in hanging drops followed by 3 days in suspension (Figures 27 and 29). However, after 10 days of culture there was no significant difference between the size of the EBs in medium A and the size of the EBs in medium B+GFs. However, EBs in medium B were significantly smaller than the other two groups (Figures 28 and 29).

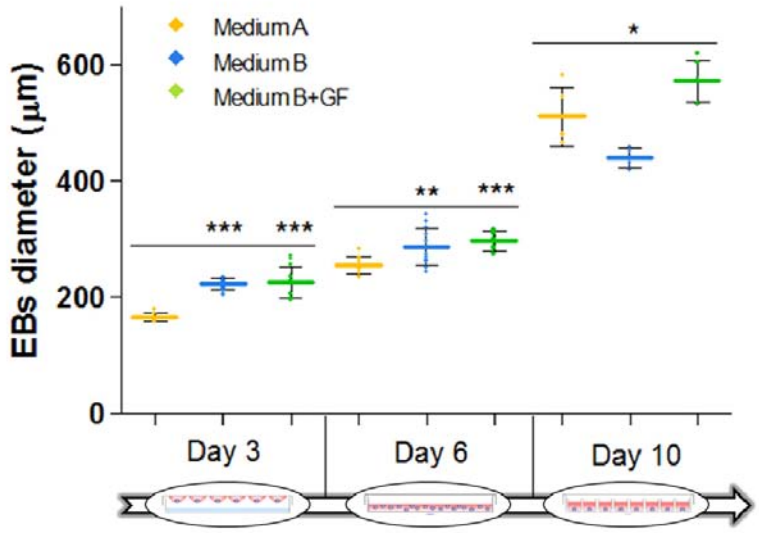

Figure 29: Comparative EBs size throughout the differentiation process. Average diameter of the EBs was determined at different time-points during the process (3, 6 and 10 days) and plotted against time. Data represent mean values ± SD; Equal variance t-test was performed to evaluate statistical differences: *p<0.05; **p<0.01; ***p<0.001.

These results suggest that, while growing in hanging drop and suspension cultures, EBs in medium B and medium B+GFs, increment their size quicker than those in medium A, probably due to the fact that medium B is richer than medium A. Once growing in adhesion on gelatin coated surfaces, EBs in medium A and medium B+GFs incremented their size

exponentially, while this increase was less pronounced for EBs in medium B, probably influenced by their premature adhesion to the dish surface when they were expected to be grown as floating spheres in suspension culture (Figures 27 and 28).

In order to evaluate the endothelial differentiation process, we analyzed the expression kinetics of some endothelial markers (Figure 30). For this purpose, RNA was isolated from the EBs at different time points (day 0, 3, 7 and 11) and expression of Platelet Endothelial cell Adhesion Molecule-1 (PECAM-1 or CD31), Vascular-Endothelial Cadherin (VE-Cadherin) and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2, Flk-1 in the mouse) were determined by semi-quantitative RT-PCR.

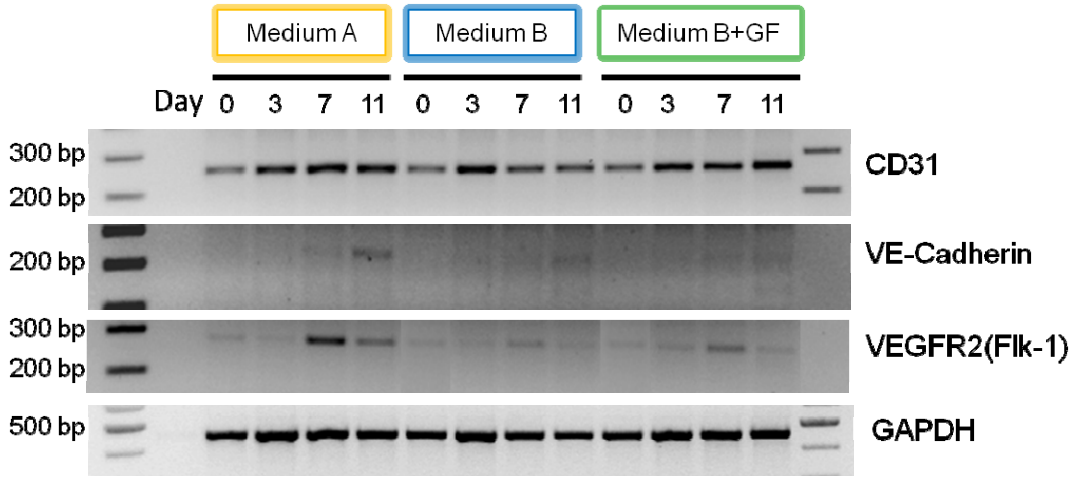

Figure 30: Expression kinetics of endothelial cell markers. Expression was evaluated by RT-PCR at the indicated time-points (0, 3, 7 and 11 days) using medium A, B or B+GFs. CD31, VE-Cadherin and VEGFR2 were analyzed as markers of the angiogenic phenotype and GAPDH was used as the internal control. The amplicon sizes were CD31:260 bp, VE-Cadherin: 226 bp, VEGFR2: 269 bp and GAPDH 454 bp.

CD31 expression increased during the hanging drop culture (day3) independently of the media used. After this point, CD31 expression remained high until day 11 in medium A and B+GFs, whereas its expression decreased in EBs cultured in medium B. CD31 expression in undifferentiated ES cells was in agreement with previous observations and may reflect a possible expression of CD31 in the inner cell mass of the blastocysts (Vittet et al., 1996). Flk-1 was also found to be expressed at low, but detectable levels, in undifferentiated cells, having an expression peak at day 7 and decreasing later. The expression was higher when culturing the EBs in medium A. With regard to VE-Cadherin expression, we only detected it clearly on EBs

grown in medium A for 11 days, while it was hardly detected when using medium B, or medium B+GFs.

We also analyzed the *in vitro* formation of vascular-like networks by evaluating the expression of CD31 by immunofluorescence at day 14 of the EBs culture. As it is shown in Figure 31, EBs growing in medium A showed CD31-positive vascular like-structures. Remarkably, only the EBs culture in media A formed such structures.

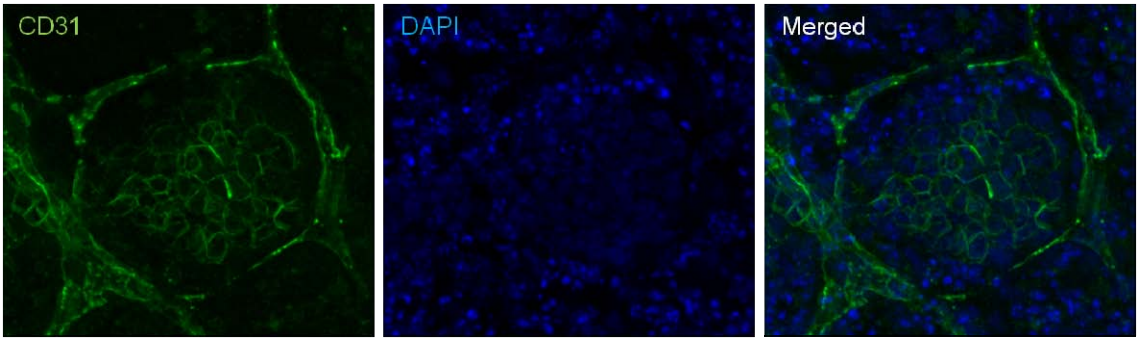

Figure 31: CD31 expression in 14 days old EBs cultured in medium A. Immunofluorescence against CD31 revealed the organization of endothelial cells as vascular-like networks. CD31 is shown in green and DAPI in blue. Objective: 20x.

Up to here, our comparative analysis revealed differences among the experimental conditions in size and morphology of the EBs, as well as in the expression kinetics of some endothelial cell markers.

An important aspect of this Thesis is the assessment of the role of p73 in the process of vascular vessel formation. Therefore, we analyzed by qRT-PCR the expression profile of the p53 family members -p73 (TA and ΔN isoforms), p53 and TAp63- during the *in vitro* differentiation of mES cells under our experimental conditions. As it is shown in Figure 32, while the expression of p53 and ΔNp73 presented a similar profile under all the experimental conditions, TAp73 appeared to be modulated in medium A, but not in the other media, while differences in TAp63 modulation were not clear. In general, p53, TAp73 decreased during the differentiation process in media A, while ΔNp73 appears to get up-regulated at day 3 and was turned off later on. It is feasible then to speculate that ΔNp73 might have a role in the initial activation of the differentiation process.

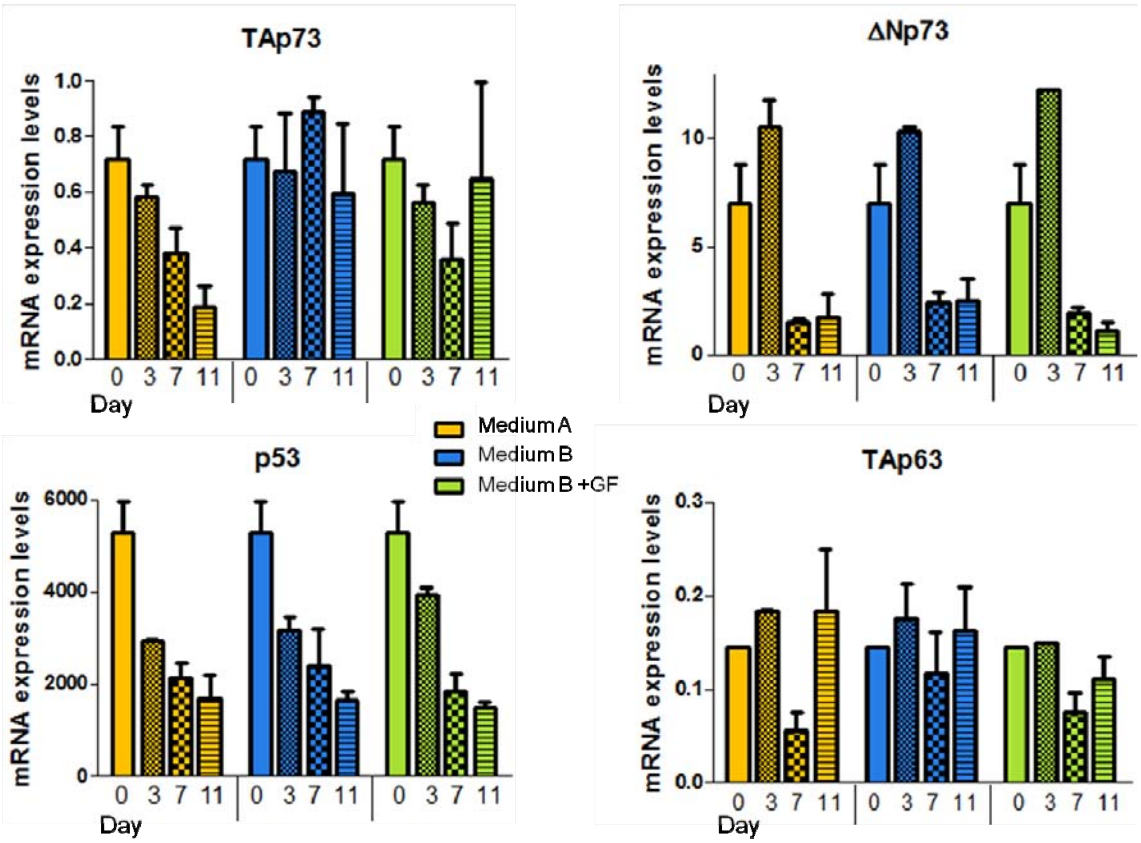

Figure 32: Expression kinetics of p53 family members. Expression of TAp73, ΔNp73, p53 and TAp63 was analyzed by qRT-PCR at day 0, 3, 7 and 11 of the EBs differentiation process.

It has been shown that the differentiation potential of EBs is influenced by their size and morphology. Indeed, EB size is considered an important parameter that influences human ES cell differentiation (Bauwens et al., 2008) and the main reason for the heterogeneity in EB differentiation (Koike et al., 2007; Leahy et al., 1999). Moreover, the initial size of the aggregate is an important factor that can affect germ layer selection as well as the differentiation scheme (Park et al., 2007). Here we have used hanging drops with a defined number of cells, which is a reported manner to avoid EBs heterogeneity. Nonetheless, at day 3, EBs growing in different media were already different in size (bigger in the presence of medium B and B+GFs with respect to medium A), probably due to the richness of medium B. However, we cannot conclude that the size was the only determinant feature leading to an inefficient differentiation, since the EBs growing in medium B and medium B+GFs showed irregular and rather abnormal shapes and started to attach each other or to the plate surface from day 6 onwards, probably contributing to the differentiation failure. Based on our observations it is probable that a gradual increase in size throughout the differentiation process, rather than an initial large

size, could be an important determinant of differentiation. In fact, EBs grown in medium B+GFs (that were bigger at day 10) did not differentiate as expected, while EBs grown in medium A (smaller at the beginning of the process), gradually increased in size and differentiate to the endothelial lineage. In agreement, expression analysis of endothelial cell markers revealed a more efficient endothelial differentiation process from EBs grown in medium A, compared to the others, which barely expressed VE-Cadherin and Flk-1.

In conclusion, EBs cultured in medium A completed a 14-days long differentiation process, eventually presented a typical two-dimensional morphology with CD31-positive cells assembled as vascular-like networks and showed detectable expression of endothelial cell markers during the process. On the contrary, in medium B and medium B+GFs, EBs disaggregated and they did not even complete the differentiation process. Therefore, we did not achieve any improvement neither in EBs size nor in expression of endothelial markers by using medium B or medium B+GFs and hence we decided to continue using medium A.

1.2. Comparative analysis of three-dimensional (3D) protocols

The EB model has been used to further recapitulate events of angiogenic processes such as vascular sprouting. Indeed, EBs embedded into collagen gels rapidly develop a network of branching endothelial outgrowths which mimic the *in vivo* processes of endothelial cell migration and proliferation within the collagen-rich extracellular matrix (Magnusson et al., 2004; Vittet et al., 1996). To asses EB-derived endothelial-like sprouting in E14Tg2α cells, two different protocols were tested, using the previously described hanging drop method to form the EBs. In Protocol 1 (Figure 33, purple outline), after 4 days in culture, the formed EBs were seeded between two collagen I layers (Jakobsson et al., 2006). We performed this experiments using medium with or without VEGF, which has been described to be responsible for sprouting. Protocol 2 (Figure 33 green outline) was a modification of the one described by Feraud and coworkers (Feraud et al., 2001). Instead of forming the EBs in methylcellulose, we used the hanging drop method. Subsequently, the EBs were transferred into ES-Cult™ and, at day 11, they were embedded into a 3D collagen I gel to obtain sprouts.

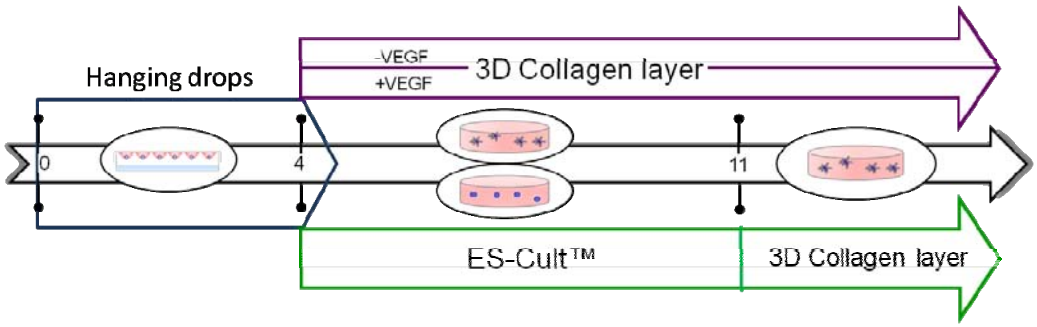

Figure 33: Outline of the comparative analysis of two protocols to induce sprouting in 3D EBs culture. mES cells were trypsinized (day 0) and seeded into?? hanging drops. At day 4, EBs were either embedded into a 3D collagen gel, where they proceed till the end of the process (Protocol 1, purple outline), or in an ES-Cult™ medium (Protocol 2, green outline) where they remained until day 11 to be subsequently transferred into a 3D collagen layer.

Under all the experimental, conditions EBs showed a spherical shape with sharp edges and they gradually increased in size from day 5 (Figure 34) to day 7 (not shown). However, by day 9, we already observed the first outgrowths coming from the EBs embedded in collagen I with VEGF (Figure 35, black arrows), while EBs in ES-Cult™ showed a spherical shape with regular edges.

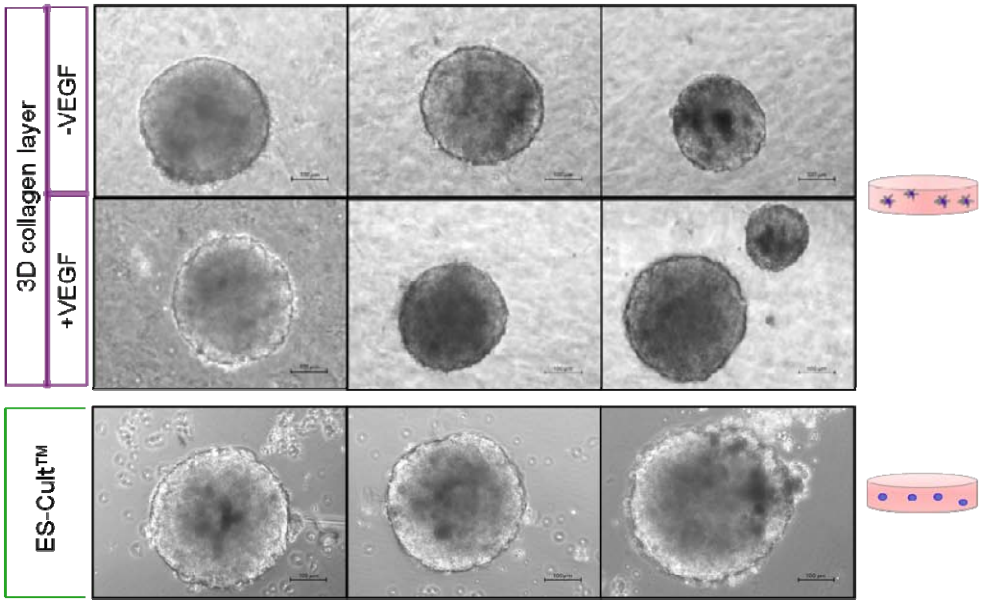

Figure 34: EBs morphology at day 5 in 3D culture. EBs in a 3D collagen layer appeared spherical and homogeneous while EBs in ES-Cult™ showed some signs of cellular disaggregation in their surface. To reflect variability, three phase contrast images for each condition are shown. Scale bars: 100 µm.

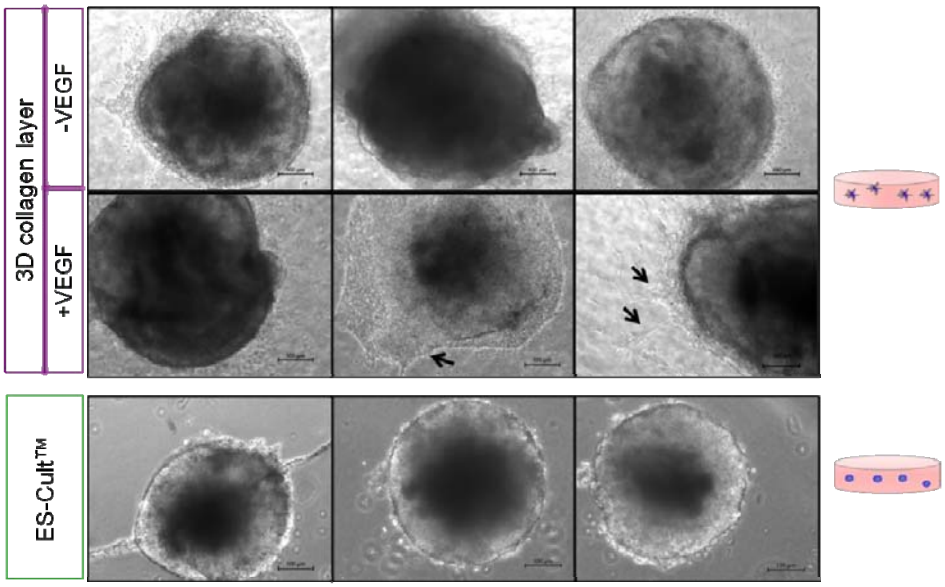

Figure 35: EBs morphology at day 9 in 3D culture. Arrows indicate cellular outgrowths. To reflect variability, three phase contrast images for each condition are shown. Scale bars: 100 μm.

At day 11 (Figure 36), EBs in collagen with VEGF showed thicker and branched cellular outgrowths which resembled vascular sprouts (arrows) protruding from the core of the EB, while EBs in the collagen gel without VEGF showed abundant and very thin cellular prolongations resembling neurite extensions (arrow heads). EBs embedded in ES-Cult™ maintained the morphology they showed on day 9 (Figure 36 lower panel).

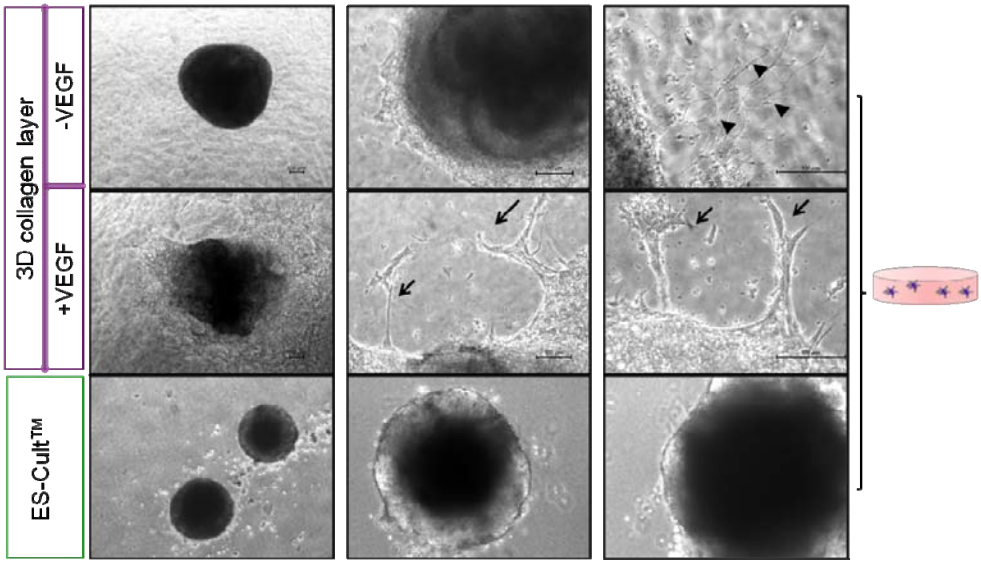

Figure 36: EBs morphology at day 11 in 3D culture. Representative phase contrast images are shown. Objective: 4X, left panel; 10X, middle panel and 20X, right panel. Scale bars: 100 μm.

At day 11, EBs embedded in ES-Cult™ were transferred into a 3D collagen gel containing a cocktail of angiogenic growth factors (VEGF, FGF2, EPO, IL-6). Remarkably, several EBs were lost at this step due to the difficulties to collect embedded EBs. At day 14, EBs from both protocols showed sprouting; however, this was more abundant in EBs from Protocol 1 with VEGF (Figure 37, upper panel). EBs without VEGF did not present sprouting, but instead some cells started to disaggregate from the EBs, highlighting the indispensable role played by VEGF in this process (data not shown). Morphological analysis of EBs from Protocol 2 revealed few sprouts but irregular morphology and even disaggregation of some of the EBs (Figure 37, lower panel).

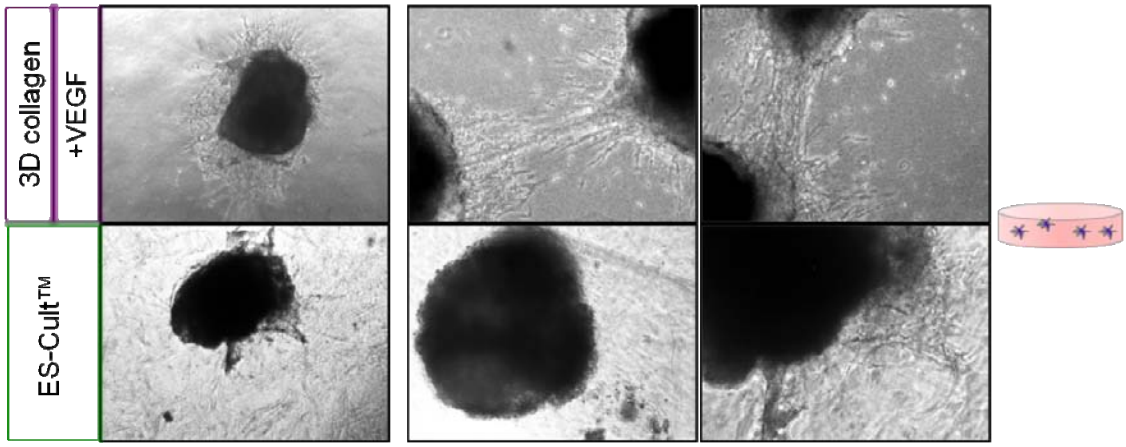

Figure 37: Sprouting EBs at day 14. Phase contrast images showing the morphology of the 14-days-old sprouting EBs. Objective: 4X, left panel and 10X, right panel.

It has been reported that non-endothelial cells can also form sprouts in a collagen matrix (Montesano et al., 1997; Uyttendaele et al., 1998). Therefore, although the observed outgrowths presented the characteristic morphology of vascular sprouts, their endothelial nature was assessed.by CD31 immunostaining (Li et al., 2008). This basically involves separating the collagen layers and performing immunofluorescence as if the collagen layer were a tissue. As shown in Figure 15, the procedure was accomplished successfully in the case of EBs from Protocol 1 with VEGF, and it confirmed the endothelial nature of the outgrowths. The EBs from Protocol 1 without VEGF were not analyzed since they did not show any outgrowths. Concerning EBs from Protocol 2, we tried to perform the immunostaining as described by Feraud and coworkers (Feraud et al., 2001). However we fail to do so, due to the difficulties in handling the viscous ES-Cult™-collagen medium that made extremely problematic to collect the embedded EBs.

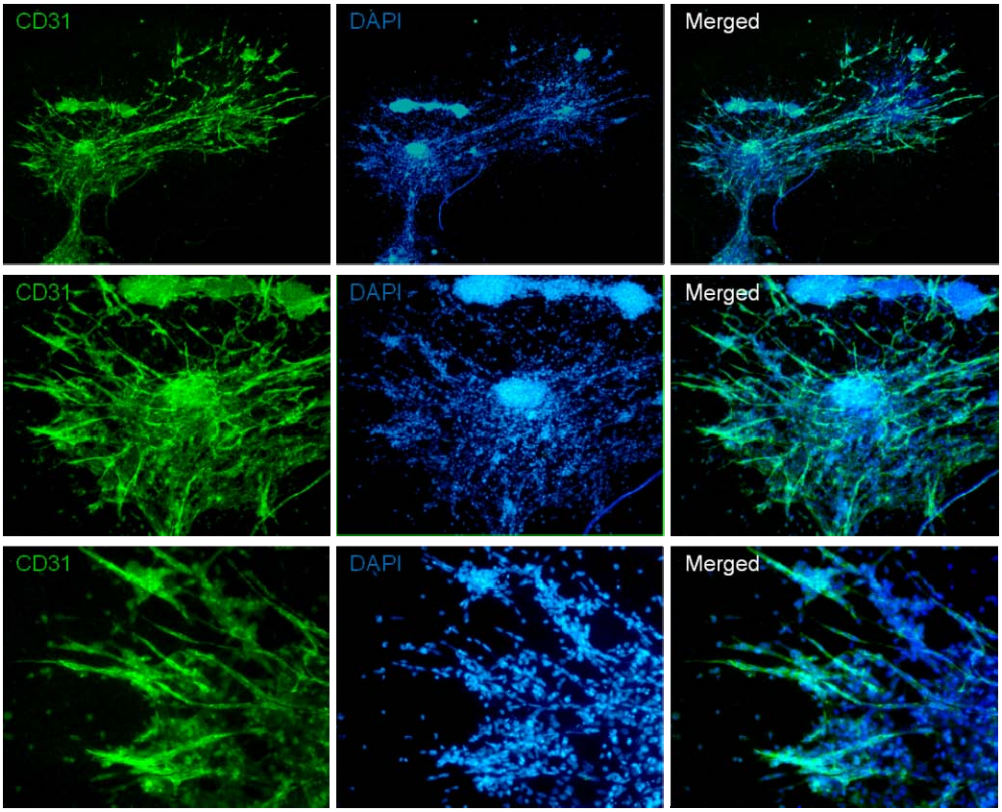

Figure 38: CD31 whole mount immunostaining of 3D sprouting EBs. CD31 immunostaining showed that 3D EBs from Protocol 1 in the presence of VEGF presented true endothelial outgrowths. CD31 is shown in green and DAPI in blue.

In conclusion, we succeed to obtain endothelial sprouts by using the hanging drop protocol described by Jakobsson (Jakobsson et al., 2006) (Protocol 1 +VEGF). However, the alternative Protocol 2 was not as effective as expected, probably due to the complications in manipulating the EBs during the process. It is also possible that the hanging drop culture was not the appropriate manner to establish the EBs but they rather have to be established from the beginning into a semisolid medium as described in the original protocol (Feraud et al., 2001).

1.3 Combination of 2D and 3D cultures

The third and last step of this analysis was to combine the 2D and 3D cultures in order to perform them in parallel (Figure 39). Therefore, we adapted the protocol previously used in our laboratory (Figure 25, medium A), including modifications from the one described by Jakobsson (Jakobsson et al., 2006). Interestingly Jakobsson and coworkers, as well as many

other authors, use mES cells cultured on MEFs feeder layers. The E14Tg2α cell line is a feeder-free cell line. Nevertheless, since the effect of feeder layers in the efficiency of mES cells differentiation was fairly unknown, we cultured E14Tg2α cells on feeder layers for these experiments. We observed right away that the cells grew faster under these conditions. After some passages, feeders were removed by seeding the cells over gelatin coated plates (day -2) for one additional passage before EB formation.

At day 0, mES cells were aggregated as hanging drops in medium A. Four days later (day 4), EBs were seeded either on gelatin coated surfaces (2D culture) or on collagen I gels (3D culture). Then, we followed the outline reported in the above lines and showed in Figure 39. We observed a clear improvement in the extent of the vascular network formed in 2D cultures. Regarding 3D culture, a minor improvement in the sprouting was also detected. Nevertheless, to further demonstrate this observation, it would be necessary to repeat this process, under identical experimental conditions, with ES cells previously grown with or without feeders.

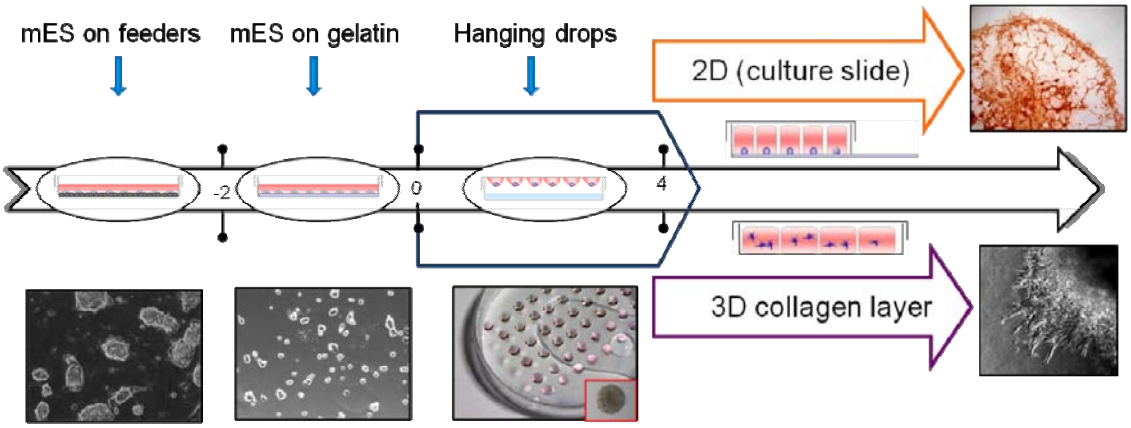

Figure 39: Schematic view of 2D and 3D EB culture. mES cells were grown on feeders for some passages and then switched to gelatin for one additional passage to remove feeders before EB formation. Then, mES were trypsinized and aggregated to create EBs (day 0) in hanging drops. At day 4, EBs were transferred either to gelatin coated surfaces for 2D culture or to collagen I gels for 3D culture. Images show the appearance of the cell culture in every step.

2. Determination of the role of p73 in endothelial cell differentiation, vasculogenesis and angiogenesis *in vitro*

The formation of blood vessels is a complex biological process that proceeds by two related but distinct mechanisms: vasculogenesis and angiogenesis (Risau, 1997). These processes are regulated by a variety of genetic and extrinsic factors that exert their effects on many cell types, including endothelial cells, which constitute the linings of the interior surface of the blood vessels. However, although intensively studied, the molecular mechanism involved in the regulation of both vasculogenic and angiogenic responses are still not completely understood. p53, in accordance with its tumor suppressor function, has been described as an inhibitor of tumor angiogenesis by several mechanisms (Bergers and Benjamin, 2003). However, the role of p53 and p53 family members in the formation of blood vessels in developmental processes has not been addressed yet. The common origin of endothelial and hematopoietic cells is today well documented. Furthermore, accumulating evidence supports the idea that the embryonic vasculature gives raise to the hematopoietic progenitors through a hemogenic endothelium (Adamo and Garcia-Cardena, 2012). The transcription factors RUNX and GATA-1 have been implicated in the regulation of this process (Iacovino et al., 2011). Since previous studies in our laboratory had identified p73 as a regulator of erythroid differentiation, and specifically of GATA-1 (Marques-Garcia et al., 2009), we hypothesized that p73 could be involved in the regulation of endothelial differentiation and/or in the processes of vasculogenesis and angiogenesis. This hypothesis led us to introduce the second aim of this thesis: to investigate the role of p73 in endothelial cell differentiation, vasculogenesis and angiogenesis *in vitro*.

To address this question we have used two *in vitro* models: mES cells and HUVEC. The mES cell model, by studies on EBs formation and directed endothelial cell differentiation, allowed us to determine whether p73 played a role during endothelial differentiation and the *in vitro* formation of vascular structures, such processes evoke the formation of a primitive vascular network. It also let us to analyze p73 role in the development of vascular outgrowths (experiments in 3D collagen gels), which is representative of sprouting angiogenesis. On the other hand, the HUVEC model was used to investigate whether p73 deficiency specifically affected endothelial cell biology, particularly proliferation, migration and morphogenesis of endothelial cells as key cellular events in the formation of blood vessels.

2.1 Analysis of the effect of p73 functional inhibition in endothelial cell commitment and vascular morphogenesis in the mES cell line E14Tg2α

Functional inhibition of p73 was achieved through the constitutive expression of a p73 dominant negative mutant, named as DDp73β, which only contains the oligomerization domain (OD) (Figure 21, Material & Methods section) and is able to functionally inhibit all p73 isoforms (Irwin et al., 2000). This stable cell line was generated in our laboratory by Dr. Gonzalez-Cano as part of her Diploma of Advanced Studies (DEA) (Gonzalez-Cano, 2008).

Previous differentiation experiments performed with this stable cell line and applying our initial protocol, showed that while some cells within E14Tg2α-DDp73β EBs displayed CD31 staining, they did not constitute vascular networks as the WT cells did (Figure 40). This could be pointing at p73 as a possible regulator of the organization of endothelial cells to form vascular structures within the EBs. It was also mentioned in the context of these experiments, and so reported by Gonzalez-Cano (2008), that DDp73β-EBs presented difficulty to adhere to plastic surfaces, suggesting that p73 functional inhibition could affect the adhesion properties of differentiating EBs. These preliminary findings meant the beginning of a series of assays designed to clarify the role of p73 in endothelial differentiation and formation of vascular structures.

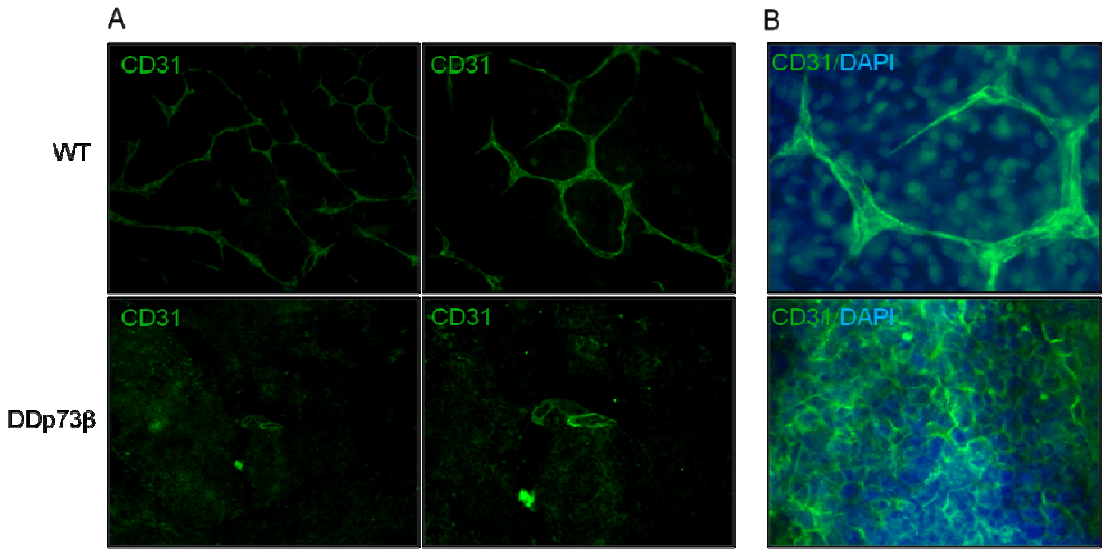

Figure 40: CD31 immunostaining of 13-day-old 2D EBs. DDp73β expressing cell lines did not constitute vascular networks as WT although individual cells expressed CD31. A) CD31: green. Objective: 10X, left; 20X, right. B) CD31: green; DAPI: blue. Objective: 60X.

2.1.1 p73 functional inhibition impairs the formation of vascular structures in 2D EB cultures

As a first approach to assess the role of p73 in endothelial differentiation, we repeated the mentioned experiments following the improved protocol described in Figure 39 and we evaluated the capacity of the E14Tg2α-DDp73β cells (DDp73β from now on) to give rise to CD31 positive endothelial cells and to assemble into vascular structures. Using this approach, EBs at day 4 in hanging drops were directly seeded on gelatinized surfaces with endothelial differentiation medium supplemented with VEGF (50 ng/mL). We analyzed size, morphology and expression of endothelial cell markers in the EBs.

As shown in Figure 41A, p73 functional inhibition consistently affected EBs size such that DDp73β-EBs were significantly smaller than WT and pcDNA3 controls.

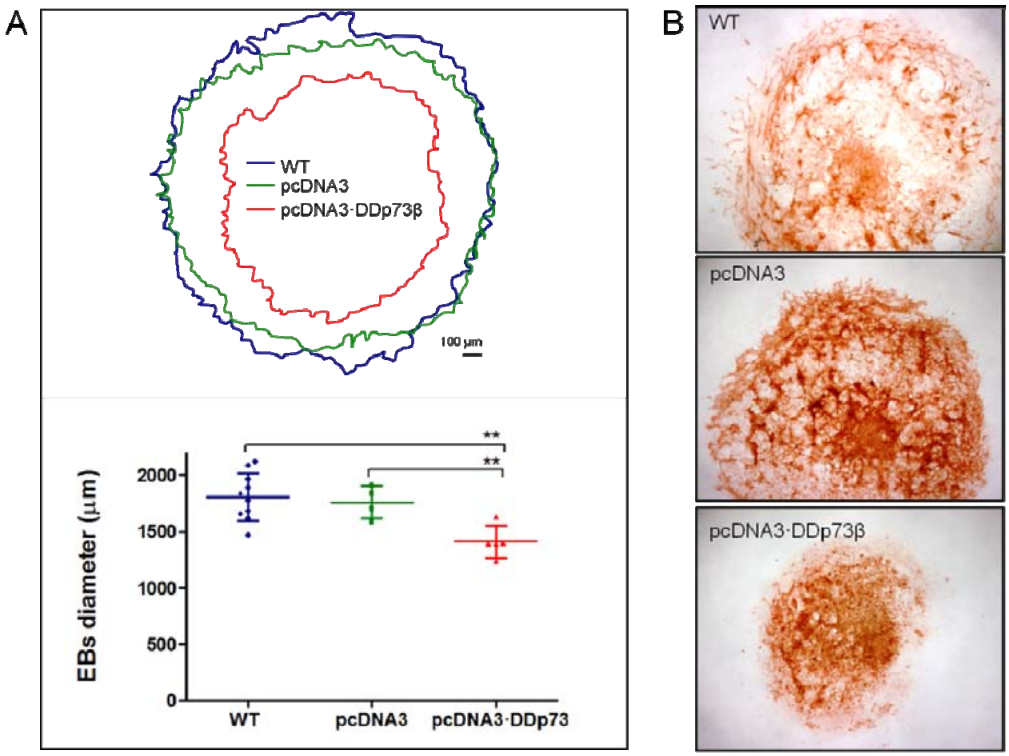

Figure 41: EBs size, morphology and formation of vascular structures in DDp73 EBs. A) EBs outline was sketched and average diameter was calculated for every individual 7-day-old EB (upper panel). EBs diameter was significantly smaller in DDp73β than in WT and pcDNA3. Data represent mean values ± SD; n≥10; equal variance Student t-test was performed to evaluate statistical differences: **p<0.01 (lower panel). B) CD31 immunocytochemistry revealed than endothelial cells are located forming a branching vascular-like network from the center to the outer rim in WT and pcDNA3 EBs while in DDp73β EBs endothelial cells did not form vascular-like structures.

As mentioned before, EBs size could act as a determinant of cellular fate (Park et al., 2007). In addition, it could be considered as an indicator of a deficient migration and, therefore, it would be suggesting that functional inhibition of p73 would have affected migration capacity. We also corroborated that DDp73β-EBs presented a defective adhesion to plastic surfaces, to the point that some of these EBs never attached to the culture dish surface and so, they were lost when the media was changed (data not shown).

As expected, E14Tg2α-WT- and pcDNA3 EBs contained CD31 positive cells forming a branching network from the center of the EB to the outer rim, which is constituted mainly by CD31 positive cells (Figure 41B and 42). However, in DDp73β-EBs, CD31 positive cells were distributed throughout the EB, but they did not assemble to form these vascular-like networks from the centre to the rim. This phenotype, characterized by the visible absence of vascular structures, was even more evident when VE-Cadherin was used as endothelial marker and the overall morphology of the EBs was observed (Figure 42A). We also analyzed the location of the mouse VEGFR2 (Figure 42B), which has been shown to be expressed in cells forming the vessel-like structures of 2D differentiating EBs. In WT and pcDNA3-EBs most of the CD31-endothelial cells forming vascular structures expressed VEGFR2, and co-localization of CD31 and VEGFR2 (yellow staining) was observed. However, in DDp73-EBs only some of the cells expressing VEGFR2 also expressed CD31, but we detected many cells expressing VEGFR2 that were neither CD31 positive nor formed vascular structures.

These data indicated that p73 deficiency impaired the formation of vascular structures in 2D differentiating EBs. Nevertheless, with these experimental settings we could not assess differences in the expression of endothelial cell markers like, CD31, VE-Cadherin and VEGFR2. Thus, in order to quantitatively assess the extent of endothelial cell commitment in 2D differentiating EBs, we decided to first isolate CD31-positive cells from 12-days-old EBs cultures using magnetic beads, and then sought to analyze the expression of these endothelial cell markers by qRT-PCR on the isolated cells.

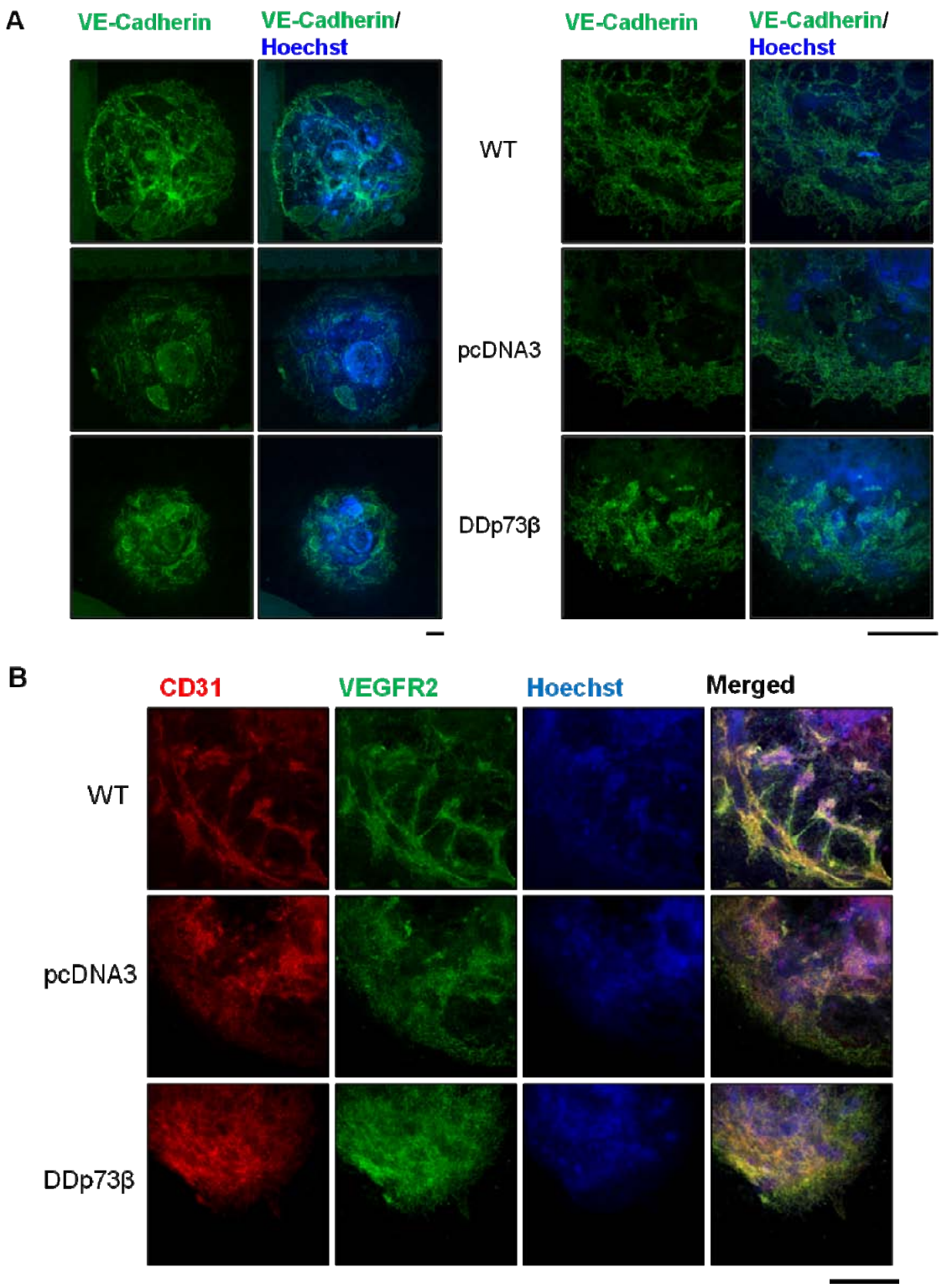

Figure 42: Expression of endothelial cell markers in DDp73 EBs. A) The overview of 11-day-old EBs immunostained for VE-Cadherin revealed that p73 deficiency leads to defective vascular structures (left panel). This defective formation was already observed in 8-day-old EBs (right panel). B) VEGFR2 was expressed by endothelial cells in 8-day-old differentiating EBs. Scale bars: 500 μm.

We first observed that the number of CD31-positive cells obtained from DDp73β-EBs was significantly smaller than those obtained from WT or pCDNA3 EBs, suggesting that lack of p73 was affecting the efficiency of the differentiation process (Figure 43A). In addition, as it is shown in figure 43B-D, p73 functional inhibition resulted in a significant decreased expression of the analyzed markers, VE-Cadherin and VEGFR2.

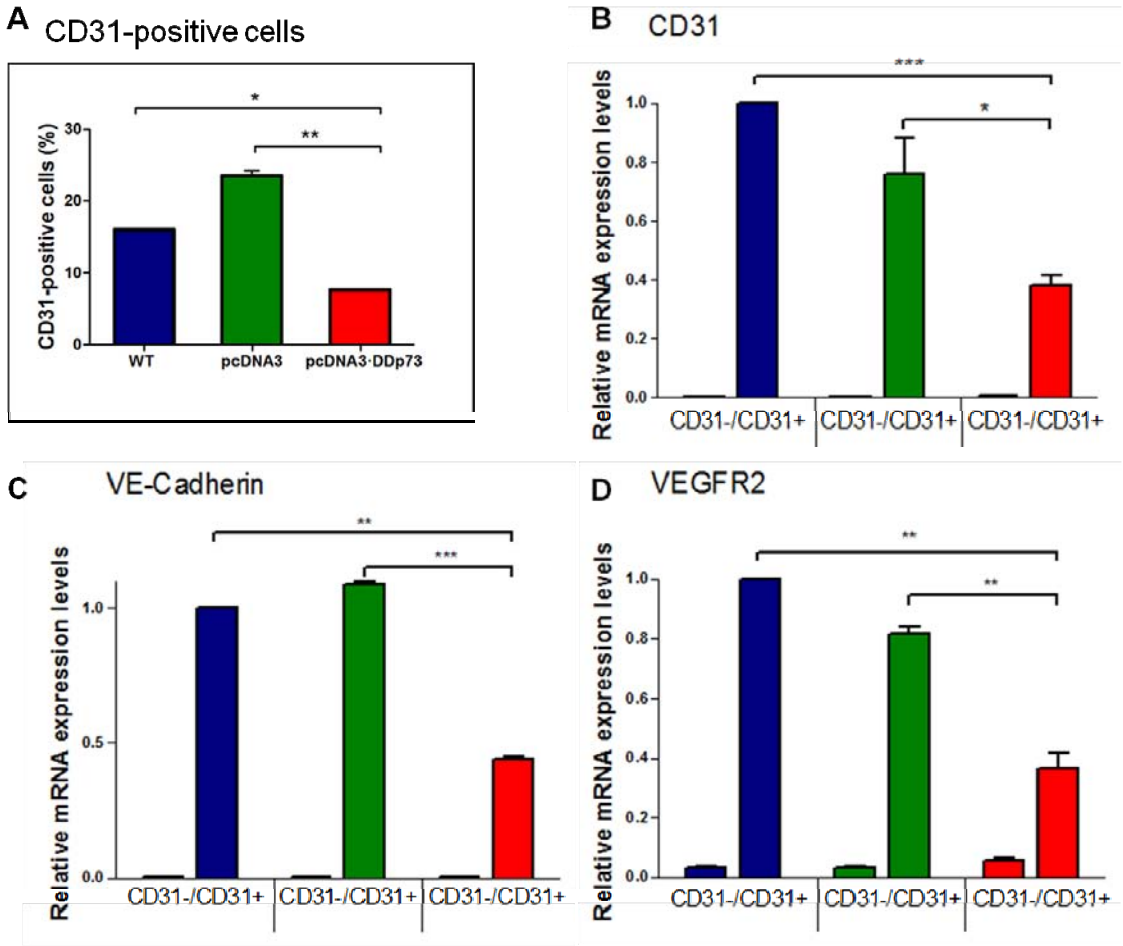

Figure 43: Endothelial cell markers expression in isolated CD31-positive cells. A) CD31-positive cells were isolated with magnetic beads and expression of B) CD31, C) VE-Cadherin and D) VEGFR2 was quantified by qRT-PCR. CD31-positive cells from DDp73 EBs expressed lower levels of every analyzed marker. Data represent mean values ± SD; experiments were repeated twice; equal variance t-test was performed to evaluate statistical differences: *p<0.05, **p<0.01, ***p<0.001.

Therefore the consequences of p73 functional inhibition were not only restricted to the capacity of endothelial cells to create vascular structures, but they also affected the expression of endothelial cell markers in CD31-positive endothelial cells, supporting the idea that p73 function is required to effectively fulfill the endothelial differentiation process.

Similar altered vascular patterns have also been described in EBs upon disruption of genes encoding growth factors, membrane receptors and proteins involved in signaling pathways like endothelial cell specific mitogen *Vegfa* (Bautch et al., 2000; Ng et al., 2004) , its cellular receptor *Vegfr2* (Hidaka et al., 1999; Jakobsson et al., 2006) or the endothelial specific adhesion molecule *VE-Cadherin* (Vittet et al., 1997), among others. Therefore, organization of endothelial cells in vascular structures can be impaired in a hierarchical manner at different key stages, including: (a) *in situ* differentiation in the center of the EB (where endothelial precursors concentrate and they appear as a sheet or primitive plexus of endothelial cells), (b) sequential maturation of endothelial precursors (associated to the expression of a set of markers characteristic for mature endothelial cells such as VEGFR2, CD31 and VE-Cadherin), and (c) migration to the periphery of the circular 2D EBs and establishment of interconnections that lead to a correct vascular morphology.

Our results indicated that p73 functional inhibition affected both the differentiation capacity and the ability to assemble into vascular networks. Based on that, two possibilities can be considered when trying to place a transcription factor like p73 in the context of molecular pathways regulating endothelial differentiation and vascular morphogenesis. On one hand, p73 could be involved in some of the molecular pathways that directly regulate expression of CD31, VE-Cadherin or VEGFR2, in which case p73 deficiency would lead to decreased expression of these markers causing a deficient endothelial differentiation. The other possibility is that p73 could be regulating processes such as the ability to respond to growth factors or the migration capacity of endothelial precursors and, therefore, p73 deficiency would lead to a deficient differentiation and, consequently, to a decreased expression of endothelial cell markers.

Neovascularization processes are characterized for changes in the genetic program of endothelial cells. p73, as a transcription factor, may be modulating these changes as well. We analyzed two of the major signaling pathways controlling endothelial differentiation: VEGFRs and TGFβ1 signaling pathways. VEGFRs regulate most of the endothelial cell angiogenic responses, including endothelial cell proliferation, migration and survival. VEGFR2 is known as the transducer of the full range of VEGFA-induced responses, whereas VEGFR1 is considered as a decoy receptor limiting angiogenic signaling (Koch et al., 2011). Our data showed that expression of VEGFR1 in DDp73β cells is more than three-fold higher than in WT (Figure 44A). Furthermore, VEGFR2/VEGFR1 ratio, which could be considered as a

pro-angiogenic indicator, is about five times lower in DDp73β than in WT cells (Figure 44B). This indicates than p73 functional inhibition significantly affects the pro-angiogenic signaling through VEGFRs and suggests a possible role of p73 as an inducer of VEGFR pro-angiogenic signaling.

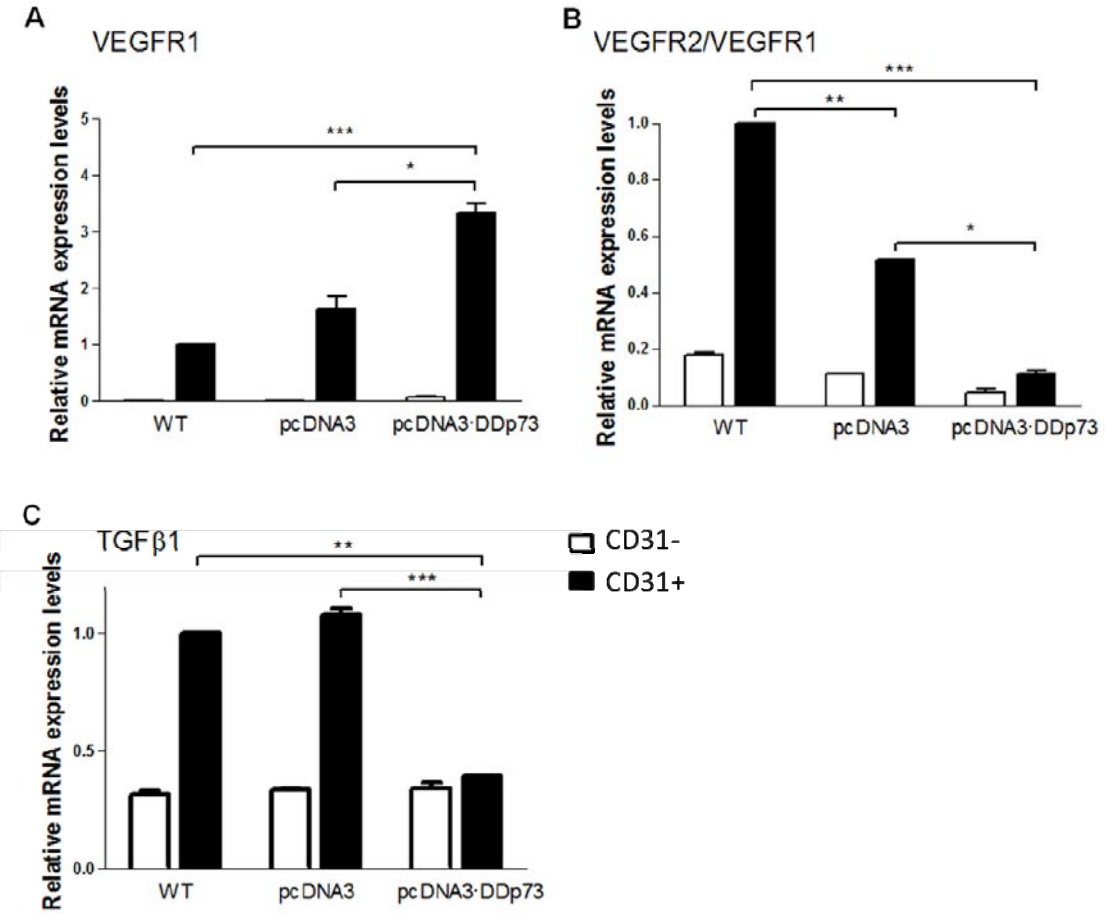

Figure 44: Expression of some angiogenic signaling molecules. The status of two signaling pathways relevant in angiogenic processes was analyzed by A) Expression of VEGFR1 in DDp73β and WT EBs. B) Ratio between VEGFR2/VEGFR1 expression. C) TGFβ1. Data represent mean values ± SD; experiments were repeated twice; equal variance t-test was performed to evaluate statistical differences: *p<0.05, **p<0.01, ***p<0.001.

TGFβ1 is a multipotent morphogen that has been shown to be involved in the regulation of vasculogenesis and angiogenesis (Pepper, 1997; Roberts and Sporn, 1989). TGFβ1 is able to increase the number of cells committed to the endothelial lineage, but inhibits endothelial angiogenic sprouting (Mallet et al., 2006). The quantitative analysis of TGFβ1 expression in CD31-positive cells from DDp73β EBs revealed a highly significant reduction when compared to controls (Figure 44C). The predominant process in 2D differentiating EBs is vasculogenesis,

although sprouting angiogenesis (but not invasive sprouting), could take over from day 6 onwards. Therefore, considering that the context in which we analyzed TGFβ1 expression was primarily vasculogenic (12 days old 2D EBs), our finding is consistent with the previous conclusion that describes TGFβ1 as an inducer of vasculogenesis (Mallet et al., 2006).

Taken altogether, we can conclude that p73 functional inhibition hinders endothelial cell differentiation and the formation of vascular structures in a 2D EB context, thus indicating a role of p73 in endothelial cell differentiation and vascular morphogenesis *in vitro*. p73 functional inhibition resulted in a predominantly anti-angiogenic molecular context where several pathways seemed to be affected. This suggests that p73 could function "upstream" of these pathways rather through direct regulation of them.

2.1.2 p73 functional inhibition results in defects in endothelial cell sprouting

EBs are used as a model for sprouting angiogenesis since they form vascular sprouts when they are embedded into type I collagen gels in the presence of angiogenic factors. Therefore, we have used this assay to evaluate the effect of p73 functional inhibition in sprouting angiogenesis and examined the development of DDp73β-EBs in collagen I gels compared to controls. Morphological observations revealed that WT and pcDNA3-EBs started to develop cellular sprouts from day 8 and onwards (Figure 23A). These sprouts gradually grew and branched until they constituted a more complex network. At day 18, the percentage of EBs with sprouts from WT cultures was higher than 75%, whereas only 30% of DDp73β-EBs showed sprouting (Figure 23B). Therefore, functional inhibition of p73 impaired sprouting in 3D differentiating EBs. Although the development of cellular outgrowths can still be observed in some DDp73β-EBs, sprouts were fewer, shorter, usually thickened and they fail to form a network of branched endothelial sprouts comparable to that found in case of WT-EBs (Figure 45).

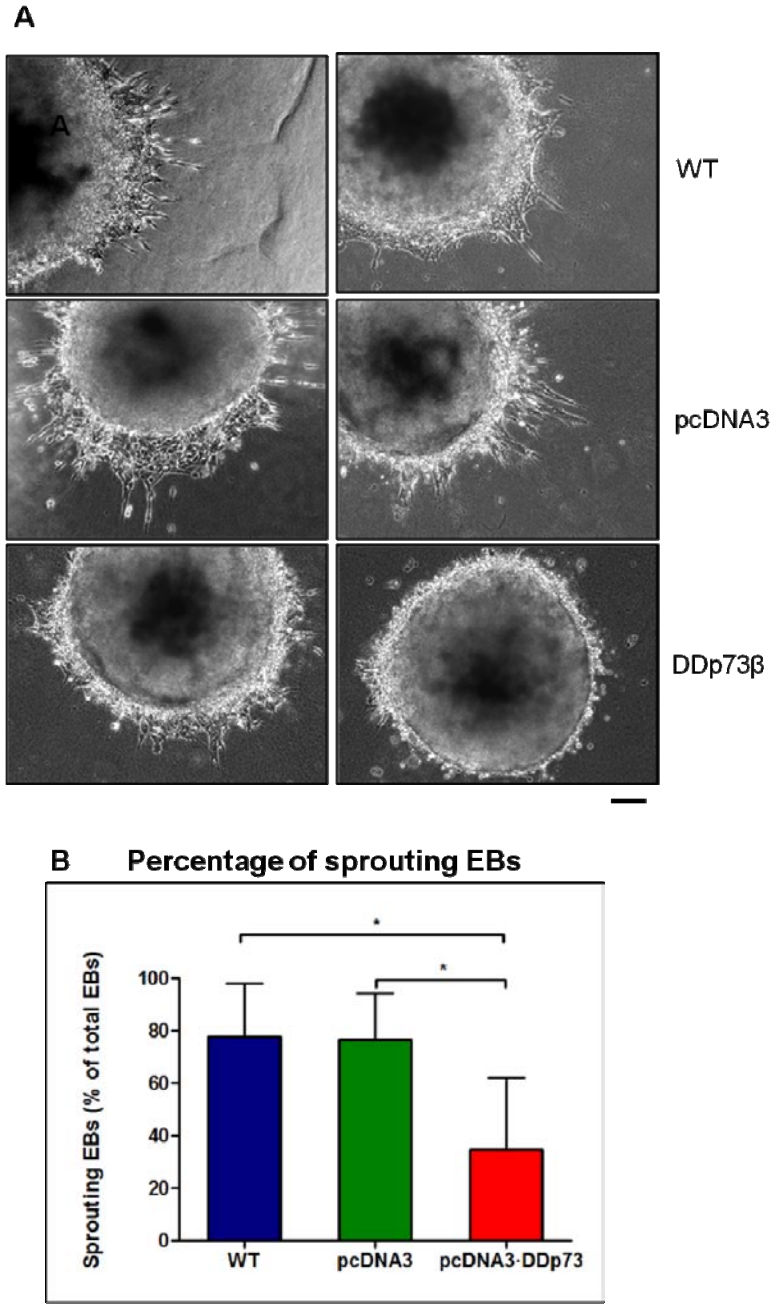

Figure 45: Sprouting EBs in 3D collagen gels. A) Phase contrast images of 8-day-old EBs show sprouts protruding from the core of the EBs. Scale bar: 100μm. B) Graph showing percentage of EBs with sprouting at day 18. Bars represent mean ± SD; *p<0.05.

The endothelial phenotype of the sprouts was examined by whole mount EB immunostaining for CD31 expression (Figure 46). Most of the sprouts in WT and pcDNA3 EBs showed CD31 staining, although several outgrowths were not positive indicating that non-endothelial cells can also form sprout-like structures, as previously described (Montesano et al., 1997; Uyttendaele et al., 1998). DDp73β-EBs showed preferential CD31 immunostaining in

the core of the EB but also in the few sprouts that they presented, even though this CD31 staining seemed weaker (Figure 46). This could reflect an impaired endothelial differentiation in addition to the abated sprouting, altogether in agreement with our previous findings in 2D EBs. Nonetheless, quantitative analyses should be performed to confirm CD31 expression levels.

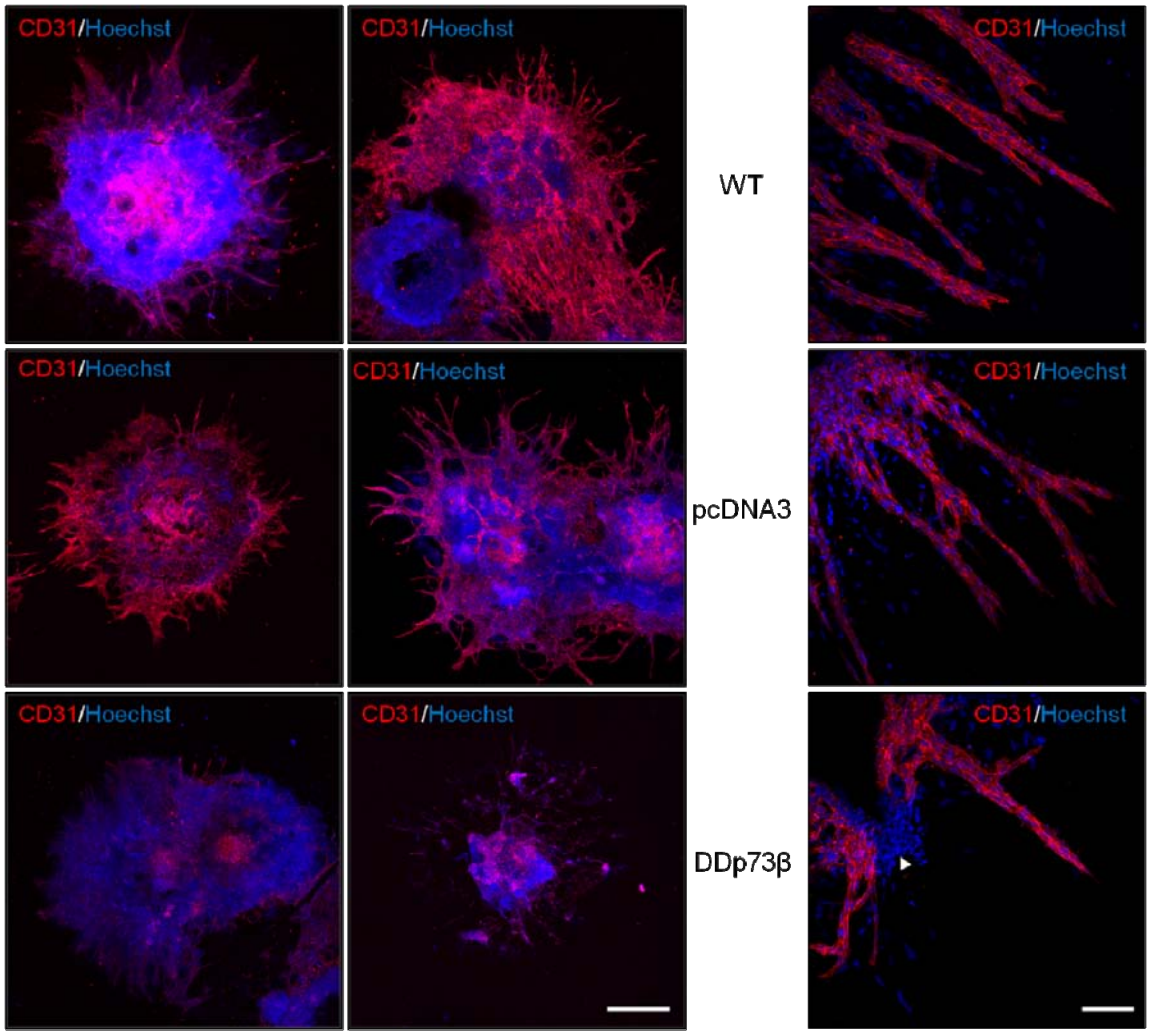

Figure 46: CD31 immunostaining of 3D EBs. CD31 immunostaining, in red, indicated vascular phenotype of the sprouts. DDp73 EBs showed an impaired formation of vascular sprouts and defective CD31 staining (arrowhead). Scale bars: 500 μm (left panel) and 100 μm (right panel).

The endothelial phenotype of the sprouts was further examined by VE-Cadherin immunoreactivity.VE-Cadherin was detected in cells forming the sprouts in WT and pcDNA3-EBs, confirming their endothelial nature (Figure 47, arrows). However, in DDp73β-EBs, VE-Cadherin is expressed in the cells that constitute the core of the EB, but was not detected in cells close to the periphery (Figure 47, arrows). The presence of VE-Cadherin-positive cells in

the core of the EB could be indicating that these cells were unable to migrate into the collagen failing to form the characteristic thin and tapered cellular outgrowths. This is consistent with our observations in 2D EBs, were VE-Cadherin positive cells were located in the central part of the EB (Figure 42).

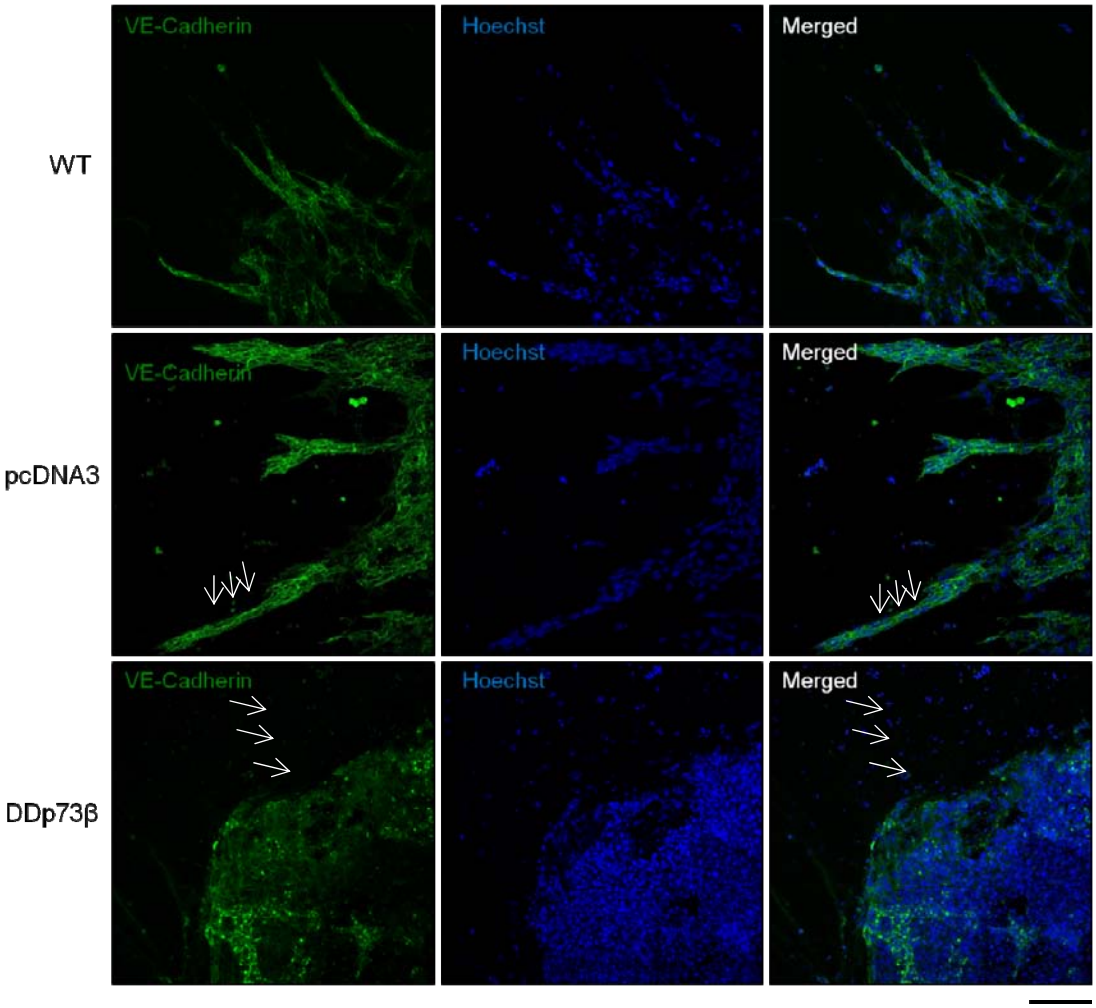

Figure 47: VE-Cadherin immunostaining in 3D EBs. p73 functional inhibition led to an impairment of the formation of a branched network of endothelial outgrowths. However, VE-Cadherin positive cells remained into the core of the EB. Scale bar: 250 μm.

VE-Cadherin is selectively expressed in endothelial cells (Lampugnani and Dejana, 1997) specifically associated with the adherens junctions and has to be loosened to allow cell migration and sprouting formation during angiogenesis. This transition from a quiescent stationary state to a dynamic migratory endothelial state is mediated in part by VEGF signaling, which induces tyrosine phosphorylation of VE-Cadherin and its binding partners (Esser et al., 1998; Lambeng et al., 2005). Additional analysis of adherens junctions would help us to

elucidate whether p73 could be directly involved in the weakening of cell-cell junctions and, thereby, acting as a positive regulator of endothelial cell migration and angiogenesis.

During angiogenesis, nascent blood vessels are further stabilized by the recruitment of mural cells like pericytes, which are essential for vascular maturation and function (Carmeliet and Collen, 2000). Characteristically, pericytes wrap around the endothelial cells in blood capillaries and serve not only as scaffolding cells, but they communicate with endothelial cells by direct physical contact and paracrine signals, which is critical for vascular remodeling and stabilization (Armulik et al., 2005; von Tell et al., 2006). To analyze the pericyte coverage of the endothelial sprouts we performed NG-2 immunostaining in 3D EBs. Our data from WT and pcDNA3-EBs revealed that the NG-2 positive cells were on top of the sprout, wrapping around the endothelial cells (Figure 48). However, in DDp73β sprouts, the NG2 positives were mingling with the endothelial cells that formed the sprout instead of wrapping them around. These p73 deficient sprouts were shorter and thicker and do no present an identifiable tip cell.

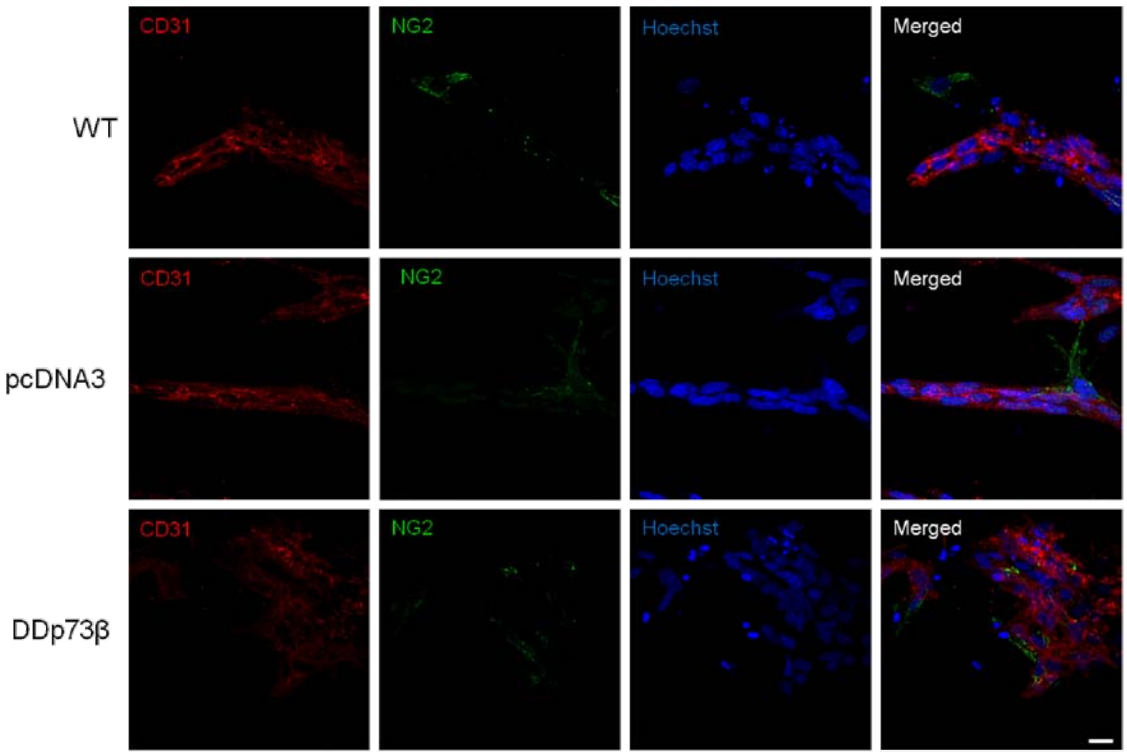

Figure 48: Tip regions of vascular sprouts. p73 functional inhibition resulted in thicker and shorter angiogenic sprouts. CD31 immunostaining, in red, indicates vascular phenotype and NG2 immunostaining, in green, revealed the presence of pericytes. Scale bar: 20 μm.

Here we show that DDp73β-EBs are capable of generating endothelial cells, as well as pericytes. However, these pericytes do not wrap around the endothelial cells but they are next to them in these fattened and misshaped sprouts. This could be probably due to the impaired capacity of endothelial cells to migrate and establish the appropriate cellular interactions, which are necessary to constitute elongated sprouts.

In summary, we found that p73 functional inhibition attenuated endothelial differentiation and impaired the formation of vascular structures in 2D EBs as well as hindered sprouting in 3D EBs. These results suggest that p73 plays a role in the formation of vascular structures *in vitro*. As stated above, defective vascular phenotype in EBs has been found also in EBs derived from mES with specific gene targeting. *Vegfr2-/-* showed defective endothelial cell development and vascular remodeling (Jakobsson et al., 2006). Similarly, *VE-Cadherin-/-* EBs showed defective vascular formation and morphogenesis (Vittet et al., 1997) and *Vegfa-/-* were characterized by attenuated endothelial cell development and vascular remodeling (Bautch et al., 2000) . Our results suggest a possible involvement of p73 in some of these molecular pathways. However, further experiments will be required to elucidate the molecular mechanisms by which p73 exerts its role in vascular morphogenesis.

2.2 Analysis of p73 deficiency in endothelial cell biology in human umbilical vein endothelial cells (HUVEC)

Once we had demonstrated that p73 functional inhibition affected the formation of vascular structures and, to some extent, expression of endothelial markers in a model based on directed endothelial differentiation from mES cells, we wanted to determine whether p73 deficiency affected endothelial biological properties, like proliferation, migration and tube formation in a gel matrix. For that purpose we used human umbilical vein endothelial cells (HUVEC) as a model.

2.2.1 p73 functional inhibition impaired proliferation, migration and tube-formation in HUVEC

To address the role of p73 in endothelial cell biology, p73 function was inhibited in HUVEC at two different levels: first, at post-translational level by transient expression of dominant negative isoforms, DDp73, and second, at post-transcriptional level by means of

RNA interference, using specific short/small interfering RNA (siRNA). These siRNA oligos have been previously reported to specifically downregulate all p73 human isoforms, without affecting the other members of the p53 family (Marques-Garcia et al., 2009). The first approach was performed by Lipofectamine™2000-mediated transient transfection of pcDNA3·DDp73 -or pcDNA3 as a control-. For the second approach, knockdown of gene expression was achieved by Lipofectamine™RNAiMAX-mediated transfection of siRNAs against all p73 isoforms (p73i.4). We then performed proliferation, migration and tube formation assays to analyze the consequences of p73 functional inhibition in the endothelial cell properties (Figure 49).

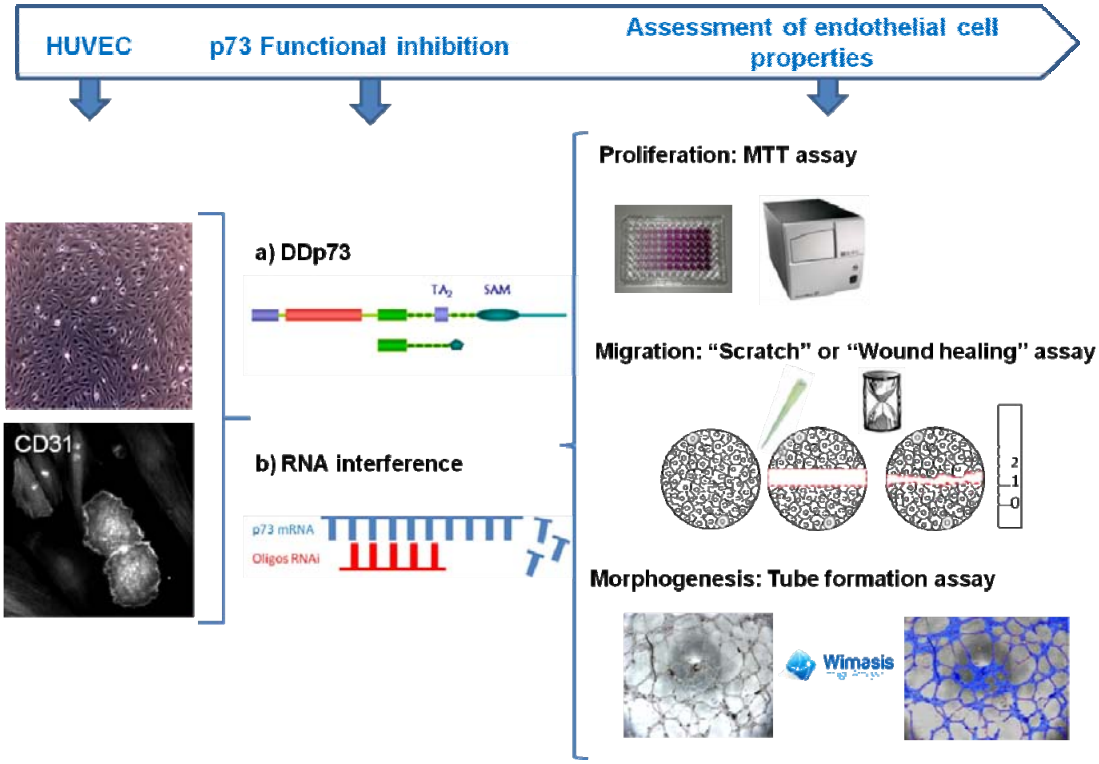

Figure 49: Experimental outline using HUVEC as cellular model. p73 function was inhibited in HUVEC by either expression of dominant negative proteins or specific RNA interference. Endothelial cell properties were then analyzed by MTT (proliferation), wound healing or scratch (migration) and tube formation (morphogenesis) assays.

In order to ensure that p73 was expressed in proliferating HUVEC, we analyzed p73 expression by qRT-PCR. Very low levels of TAp73 were detected and ΔNp73 expression was about 2.1 times higher (Figure 50). As previously reported, the p73i.4 siRNA comprehensively knocked-down TAp73 as well as ΔNp73. However, when using a previously validated ΔN-specific siRNA oligonucleotides with no sequence homology to TAp73 (Papoutsaki et al.,

2004), we detected a strong decrease of ΔNp73 expression concomitant with a moderated abatement of TAp73 (Figure 50). We have previously demonstrated that siRNA-ΔNp73 oligos did not directly affected TAp73α neither had it affect the viability of the transfected cells (Marques-Garcia et al., 2009). Nevertheless, our group and others have previously reported that TAp73α stabilization is dependent on ΔNp73 expression (Slade et al., 2004). Therefore, the mild TAp73 downregulation observed with this oligo could be an indirect effect due to the lack of ΔNp73.

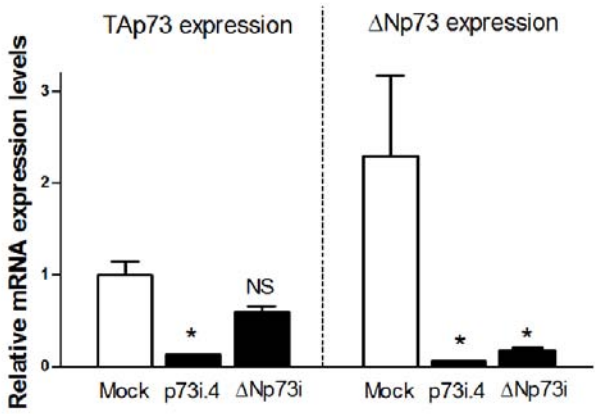

Figure 50: TA and ΔNp73 expression upon siRNA. p73i.4 knocked-down TAp73 and ΔNp73 whereas ΔNp73i was specific against ΔNp73.

- **Proliferation**

As stated in the introduction, p73 is involved in control of the cell cycle and apoptosis. In order to analyze whether p73 functional inhibition affected proliferation of endothelial cells in culture, we performed an MTT assay in HUVEC transfected with the siRNA p73i.4, which inhibits all p73 isoforms. Previous experiments in our laboratory have indicated that the maximal effect of siRNA occurs about 72 hours after transfection. Thus, 48 hours after transfection, cells were seeded in 96-well plates and 12 hours later (60 hours total after transfection), the MTT assay was carried out (Figure 51A). For each condition, this time point was considered to reflect the number of viable cells at the beginning of the experiment (0 hours) and subsequent time point values were normalized to it (Figure 51). While we did not find any difference between mock and scrambled HUVEC, p73i.4 HUVEC presented significantly lower number of viable cells, suggesting that inhibition of all p73 isoforms in HUVEC results in slower growth kinetics. It is noteworthy that in HUVE cells, the predominant p73 isoform expressed is ΔNp73, having a ΔN/TA ratio of 2.1. Thus, it is

possible that this result could be reflecting the inhibition of the predominant role of ΔNp73 function in these cells. ΔNp73 functional inhibition has been previously associated with decreased cell growth (Emmrich et al., 2009). Another possibility is that the presence of ΔNp73, which holds anti-apoptotic functions, could be necessary to protect against apoptosis in endothelial cells *in vitro*. Moreover, p73 loss has been reported to impair proliferation and induce premature senescence in primary cells due to a compensatory activation of p53 (Talos et al., 2007).

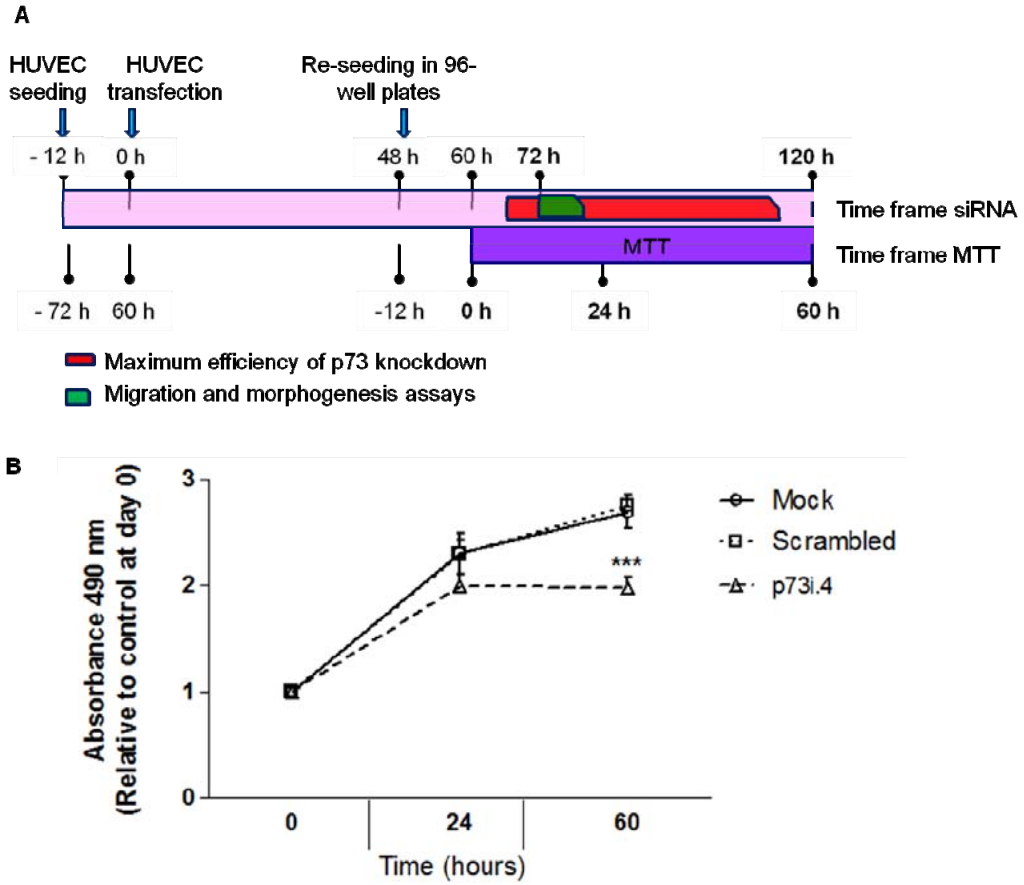

Figure 51: Proliferation in p73 interfered HUVEC. A) Timing outline of the experiments performed in HUVEC. B) Proliferation was analyzed by MTT assay. Data represent mean values ± SD; n=3; experiments were repeated twice; equal variance t-test was performed to evaluate statistical differences: ***p<0.001.

- **Migration**

During the process of angiogenesis, endothelial cells are stimulated to degrade the basement membrane and migrate into the perivascular stroma in response to a gradient of angiogenic factors. Here, we have used the "Scratch" or "Wound Healing" assay to evaluate

migration capacity of endothelial cells. It basically involves making a straight scratch simulating a wound in the confluent endothelial cell monolayer to provide a margin from which the endothelial cells migrate to fill the denuded area (Wong and Gotlieb, 1984). The rate and extent of endothelial cell migration was monitored microscopically at various time-points after the scratch.

To assess whether the migration capacity was affected by functional inhibition of p73, we took advantage of the described models, DDp73 expression and RNA interference with the siRNA p73i.4. Results showed that the percentage of wound closure after 10 hours was significantly reduced upon functional inhibition of p73 (Figure 52), regardless of the system used for the inhibition, pointing to p73 as a positive regulator of endothelial cell migration.

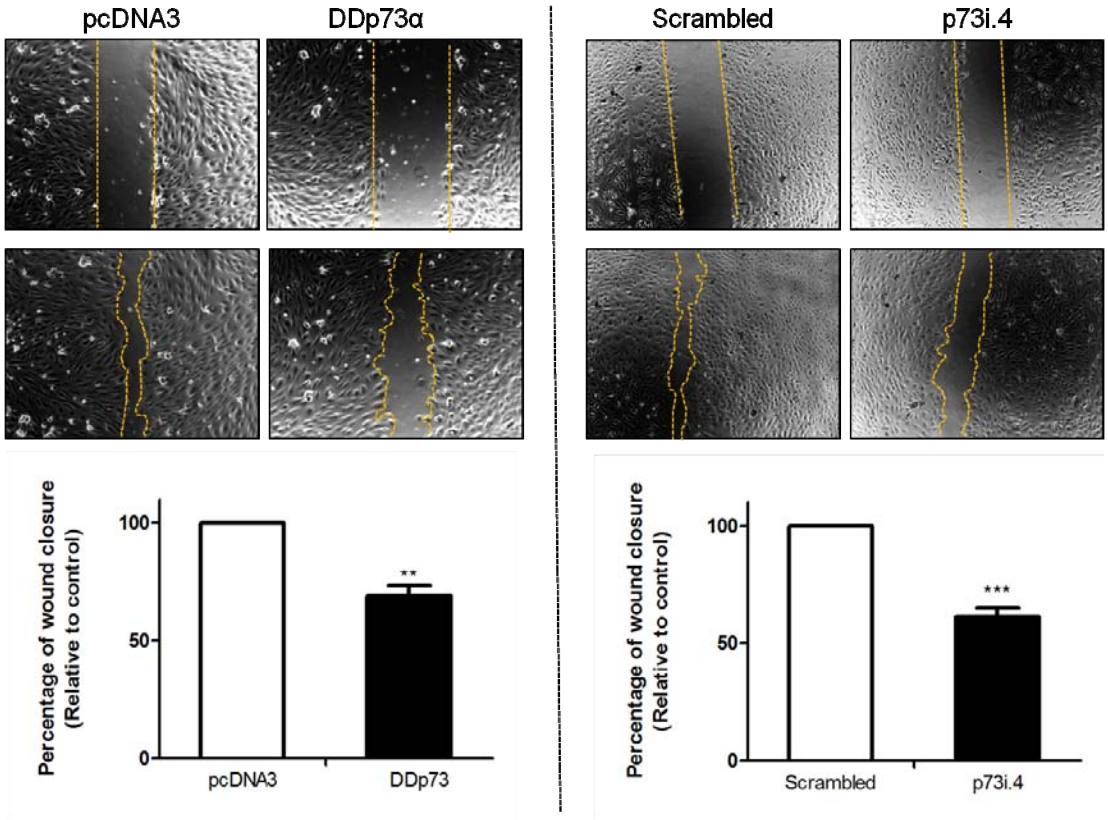

Figure 52: Effect of p73 functional inhibition in HUVEC migration. p73 functional inhibition resulted in reduced migration capacity. p73 function was inhibited either by DDp73 (left panel) or RNA interference (right panel). Endothelial cell migration into the "wound" denuded area was monitored microscopically and the percentage of wound closure after 10 hours was calculated relative to the control. Data represent mean values ± SD; n=3; experiments were repeated at least three times; equal variance t-test was performed to evaluate statistical differences: $^{**}p<0.01$, $^{***}p<0.001$.

To further analyzed cell migration, transfected migrating HUVEC were stained with phalloidin-TRITC or anti- p73 antibody after wounding. Cells transfected with empty vector migrated together from opposite sides of the wound edge. Consistent with our previous results, cell expressing DDp73 had decreased migratory rates. Furthermore, we observed that control and DDp73 cells had distinct morphological phenotypes. Most of the control transfected cells where oriented towards the wound (arrows) and presented a fusiform shape; whereas DDp73-expressing cells appeared disoriented and presented a more rounded and flattened morphology (arrowheads) (Figure 53).

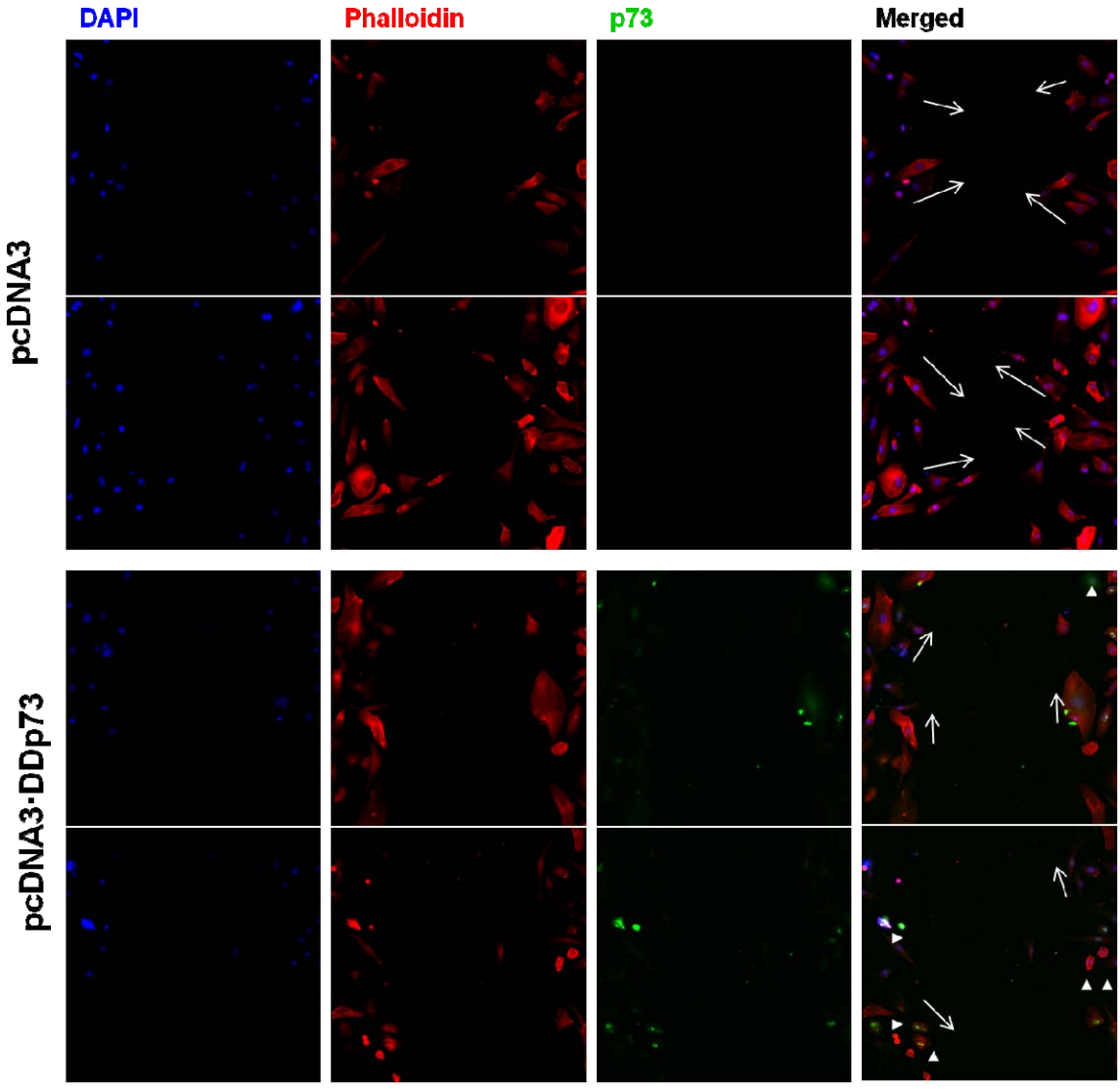

Figure 53: Actin cytoskeleton of DDp73 expressing HUVEC. HUVEC transfected either with pcDNA3 or pcDNA3·DDp73 were stained with phalloidin-TRITC (red) to observe actin cytoskeleton and cellular morphology during the wound healing. An anti-p73 antibody was used to identify DDp73-expressing cells (green). **Objective**: 20X

Cell migration is a complex and regulated process which requires the integrated activities of cytoskeleton reorganization and cell-matrix interaction. During migration, the assembly and disassembly of focal adhesions and the movement of stress fibers coordinately lead the cells to migrate (Hall, 1994; Izzard, 1988; Ridley and Hall, 1992). Focal adhesions are protein complexes that serve to transduce mechanical signals and to link the actin cytoskeleton to the extracellular matrix. Phosphorylation of focal adhesion kinase (FAK) mediates the focal adhesion dynamics and signaling in response to growth factors, like VEGF (Lamalice et al., 2004; Matsumoto and Claesson-Welsh, 2001; Rousseau et al., 1997) regulating cell motility, gene expression and cell proliferation. VEGF-induced activation of FAK regulates the proper turnover of focal adhesions that is required to allow the dynamic adhesion and de-adhesion processes inherent to cell migration (Le Boeuf et al., 2004). Therefore, we sought to analyze focal adhesion plaques by visualizing activated FAK (pY397) in cells with functional inhibition of p73. HUVEC maintained with 2% FBS were transfected with the indicated vector and immunostained with anti FAK-pY397 (p-FAK) together with either anti-GFP or anti-p73 (ER-15). As observed in figure 54, most of the p-FAK was localized to the ventral adhesion plaques in control cells. However, in DDp73-expressing cells p-FAK was recruited to the periphery of the cell, suggesting that functional inhibition of p73 alters the focal adhesion dynamics of the endothelial cells, hindering their migration capacity.

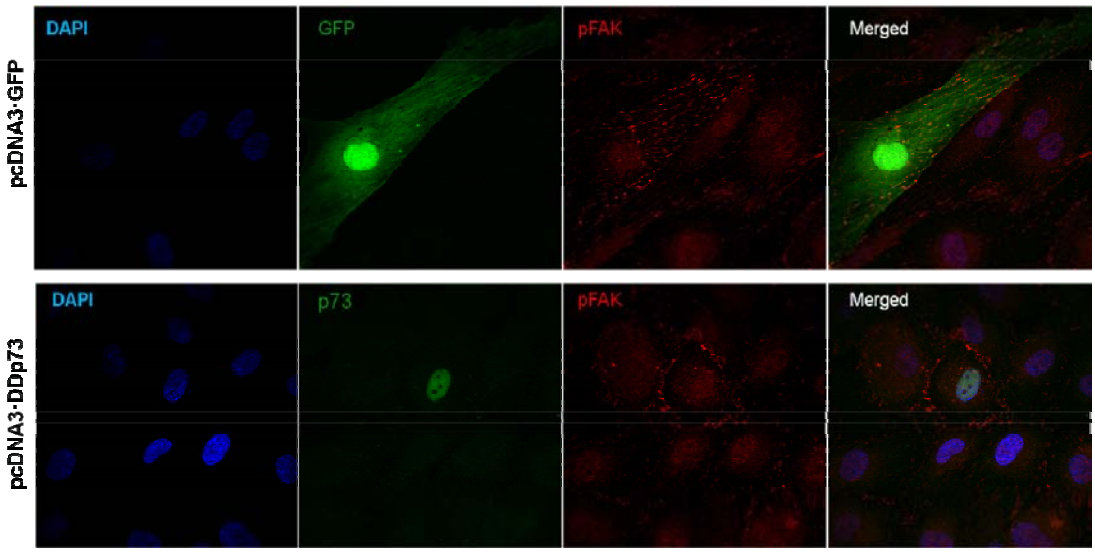

Figure 54: Focal adhesions in DDp73 expressing HUVEC. HUVEC transfected either with pcDNA3·GFP or pcDNA3·DDp73 were immunostained with anti pFAK (Y397) to visualize focal adhesions during wound healing process. Anti-GFP or anti-p73 were used to identify transfected cells (green). In control cells, pFAK was localized to the ventral adhesion plaques while in DDp73-expressing cells pFAK was recruited to the periphery of the cell. Objective: 100X.

- **Morphogenic potential: capillary tube formation assay**

Assays that stimulate the formation of capillary-like tubes are regarded as representative of the later stages of angiogenesis (differentiation and morphogenesis). The basic tube formation assay involves plating endothelial cells onto a layer of gel matrix, commonly collagen, fibrin or Matrigel. Here, in order to assess the role of p73 in late stages of angiogenesis *in vitro*, HUVEC with functional inhibited p73 via transient expression of DDp73 or siRNA were submitted to tube formation assay in Matrigel. Formation of tube was monitored microscopically taking images at several time points. Images were then quantitatively analyzed using WimTube (Wimasis GmbH). HUVEC transfected with pcDNA3 or scrambled siRNA controls showed robust tube network formation, whereas HUVEC expressing DDp73 or transfected with siRNA p73i.4 showed fewer and shorter tubes which constituted incomplete networks (Figure 55). The quantitative analysis revealed a significant decrease in the parameters analyzed: covered area, total tube length, number of branching points and number of loops in DDp73 and p73i.4 when compared with pcDNA3 and scrambled controls. Consequently, p73 functional inhibition hindered the formation of tubes and, therefore the angiogenic potential of HUVEC, indicating for the first time that p73 function is required for endothelial cell differentiation and morphogenesis.

The role of p73 in cell migration has been controversial. On one hand, Sablina and coworkers reported that p53 deficiency decreased cell motility in different cellular contexts (human foreskin fibroblasts, mouse fibroblast from lung and spleen, peritoneal macrophages, keratinocytes and human colon and lung carcinoma cell lines) and that p73α overexpression stimulated cell migration in human colon and lung carcinoma cell lines (Sablina et al., 2003). On the hand, several groups had demonstrated that TAp73 is a negative regulator of cell migration in the mammary epithelial cell line MCF10A (Zhang et al., 2012). Recently, Rhode and coworkers claimed that TAp73α and TAp73β exert opposite roles in regulating cellular migration in HeLa cells (Rodhe et al., 2013). These discrepancies could be due to the specific cellular contexts, since different cell lines would express different combination of TA and ΔNp73 isoforms, and therefore they will present different p73 functional readouts.

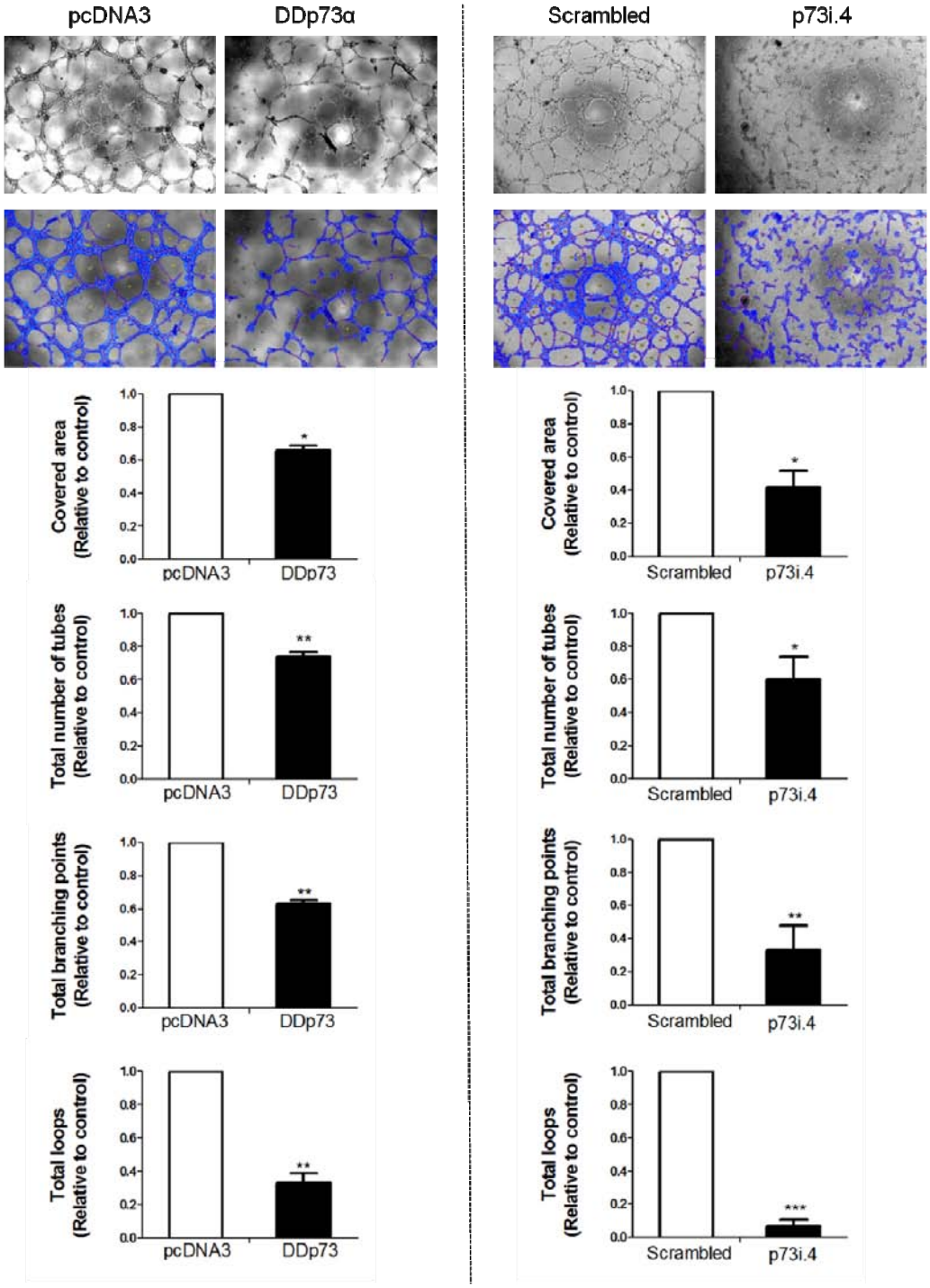

Figure 55: Tube formation assay on Matrigel in HUVEC upon p73 functional inhibition. p73 functional inhibition, either by DDp73 expression (left) or by RNA interference (right), significantly inhibited *in vitro* tube morphogenesis. HUVEC with functional inhibition of p73 were submitted to tube formation assay in Matrigel. Tube formation was monitored microscopically and images were analyzed using WimTube software. Covered area, total number of tubes, total branching points and total number of loops are shown as representative parameters. Experimental data were normalized to the control in every case. Data represent mean values ± SD; n≥3, experiments were repeated at least three times; equal variance t-test was performed to evaluate statistical differences: *p<0.05, **p<0.01, ***p<0.001.

So far, our data demonstrate that functional inhibition of total p73 affects endothelial cell migration and morphogenesis. Therefore, we hypothesize that p73, most likely ΔNp73 since it is the predominant isoform in HUVEC, plays a role in endothelial cell migration and morphogenesis.

2.2.2 ΔNp73 isoforms, but not TAp73 are involved in endothelial cell migration and morphogenesis

We have shown that p73 functional inhibition impairs the angiogenic potential of HUVEC; however, due to the existence of TA and ΔN isoforms of p73, which exert opposite functions in many biological processes, it is important to elucidate which one is responsible for the observed phenotype: TAp73 or ΔNp73. To address this question, siRNAs which specifically inhibited TA (TAp73i), ΔN (ΔNp73i) or both TA and ΔN (p73i.4) were used to carry out the different angiogenic potential assays.

- **Proliferation**

TA and ΔNp73 were independently knocked-down by transfection of either TAp73i or ΔNp73i siRNAs in HUVEC; both isoforms were inhibited by p73i.4 transfection. As described before, an MTT was carried out at different time points to assess cellular proliferation. Our data indicated that TAp73, but not ΔNp73, knockdown led to a decrease in proliferation kinetics (Figure 56), suggesting that TA is the p73 isoform responsible for the reduced proliferation that we reported in the preceding lines. This was a shocking result because, due to its pro-apoptotic and growth suppression function, TAp73 inhibition would be expected to result in increased cell proliferation. Nonetheless, it has been described that TAp73 knockout MEFs show propensity to undergo replicative senescence thereby showing growth retardation (Rufini et al., 2012). Therefore, it could be a possibility that TAp73 knockdown in HUVE cells results in induction of long term senesce with a concomitant decrease in cell growth although we have never noticed morphological changes related to *in vitro* senescence at that time point.

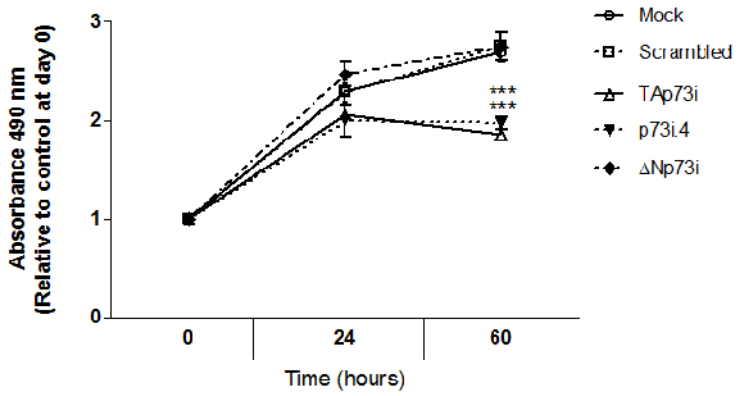

Figure 56: Proliferation of HUVEC upon TAp73 or ΔNp73 specific knockdown. TAp73 but not ΔNp73 knockdown led to a reduced proliferation in HUVEC assessed by MTT. Data represent mean values ± SD; n=3; experiments were repeated twice; equal variance t-test was performed to evaluate statistical differences: ***p<0.001.

- ## Migration

Wound healing assays were carried out as previously described, but using the isoform specific siRNAs. Upon knockdown of total p73 or ΔNp73, but not TAp73 alone, the migration capacity was hindered (Figure 57).

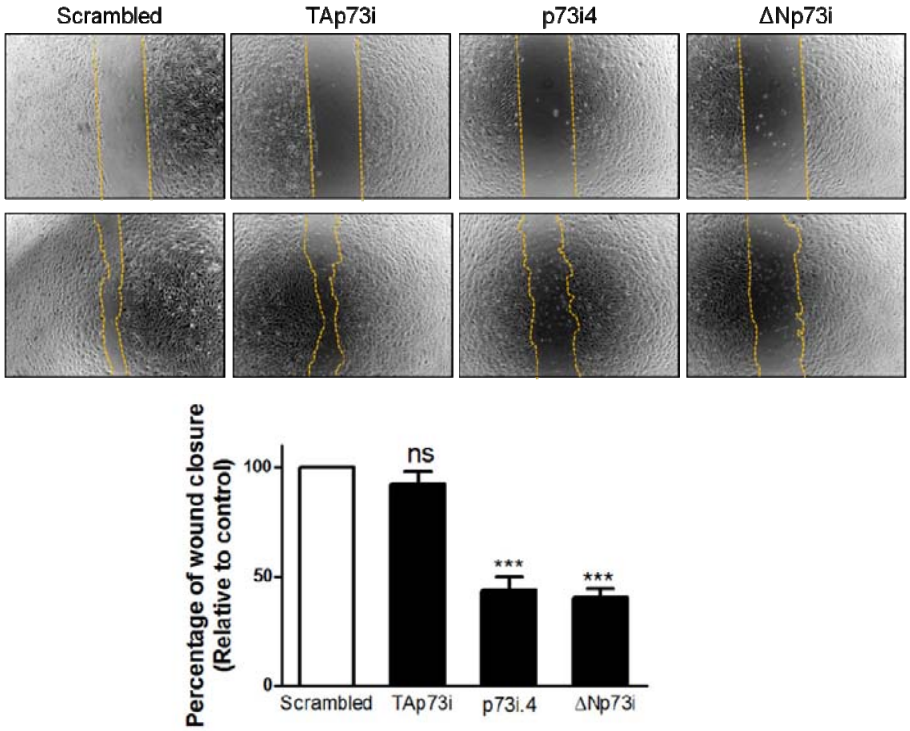

Figure 57: Effect of TAp73 or ΔNp73 specific knockdown in HUVEC migration. Endothelial cell migration into the "wound" denuded area was monitored microscopically and the percentage of wound closure after 10 hours was calculated relative to the control. Data represent mean values ± SD; n=3; experiments were repeated at least three times; equal variance t-test was performed to evaluate statistical differences: **p<0.01, ***p<0.001.

This finding demonstrates that knockdown of ΔNp73 affected endothelial cell migration, indicating a role for ΔNp73 as positive regulator of endothelial cell migration, which is coherent with previous report from Zhang and coworkers. These authors, using mammary epithelial cells demonstrated that TAp73 was a negative regulator of cell migration (Zhang et al., 2012). In their system, knockdown of all p73 isoforms gave the same result, suggesting that in that cellular model TAp73 is the functional isoform. However, in our system, where ΔNp73 is predominantly expressed, inhibition of either total p73, or ΔNp73 in particular, demonstrates that ΔNp73 acts as a positive regulator of cell migration. Thus, as in other cellular processes, TAp73 and ΔNp73 seem to be performing opposite regulatory functions in cell migration.

- **Morphogenic potential: capillary tube formation assay**

We next assessed the role of TAp73 or ΔNp73 in a tube formation assay in HUVEC by specifically knocking down these isoforms. Quantitative analysis showed a significant effect of total p73 and ΔNp73 inhibition on several parameters including covered area, total tube length, number of branching points and number of loops (Figure 58). These parameters revealed a defective capacity of endothelial cells to migrate and assemble into tubular structures in a matrix that resembles the complex extracellular environment, indicating that p73 deficiency, specifically ΔNp73, lead to an impaired endothelial morphogenesis. On the contrary, although we observed a tendency to an increased morphogenesis with inhibition of TAp73, this effect was not significant in any of the parameters analyzed in our assay. This result supports the hypothesis that ΔNp73 but not TAp73 is the isoform involved in endothelial cell differentiation and morphogenesis.

In summary, the results achieved in HUVEC provide evidence that p73 and specifically ΔNp73 regulates endothelial cell processes such as migration and tube formation, whereas TAp73 seems to be involved in endothelial cell proliferation, possibly by inducing cell growth-arrest or senescence. p73 is known to be involved in cell cycle arrest and apoptosis and it has been recently suggested that the induction of apoptosis via increased p73 expression may be partly mediating the anti-angiogenic effects of a WEHAD water extract (Bang et al., 2011), probably emphasizing the role of TAp73 pro-apoptotic isoforms. However, in our HUVEC model, TAp73 inhibition did significantly affect neither migration nor tube formation, suggesting that the role played by ΔNp73 is independent of TAp73 and, to some extent, independent on cell cycle or apoptosis regulation.

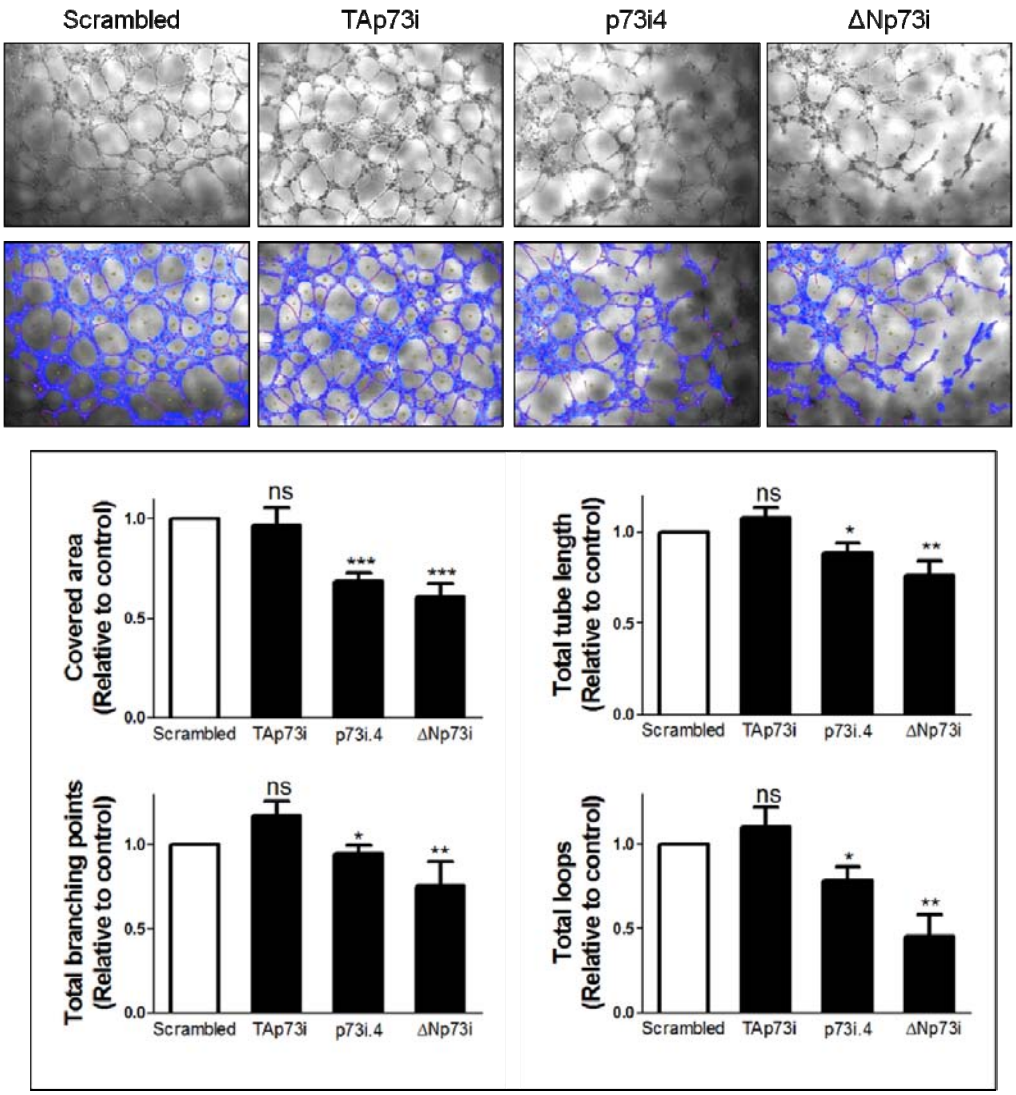

Figure 58: Tube formation assay in HUVEC upon specific knockdown of TA or ΔNp73. p73 and ΔNp73 knockdown significantly inhibited *in vitro* tube morphogenesis. HUVEC with specific TA or ΔNp73 were submitted to tube formation assay in Matrigel. Tube formation was monitored microscopically and images were analyzed using WimTube software. Covered area, total tube lenght, total branching points and total number of loops are shown as representative parameters. Experimental data were normalized to the control (scrambled siRNA). Data represent mean values ± SD; n≥3, experiments were repeated twice; equal variance t-test was performed to evaluate statistical differences: *p<0.05, **p<0.01, ***p<0.001.

2.2.3 ΔNp73 knockdown affects VEGFRs and TGF-β signaling pathways

Among the multitude of molecules that regulate angiogenesis, VEGF plays a pre-eminent role. VEGF exerts its actions by binding mainly to two cell surface receptors, VEGFR2 and VEGFR1, which have different biological functions and utilize some distinct signal transduction cascades (Koch and Claesson-Welsh, 2012). In order to determine whether p73

deficiency affected VEGFRs at transcriptional levels, we analyzed VEGFR2 and VEGFR1 mRNA expression levels by qRT-PCR upon p73 knockdown. We found that while VEGFR2 mRNA expression was not significantly affected by p73 knockdown, VEGFR1 expression was significantly increased (not shown). Moreover, the ratio between VEGFR2/VEGFR1 that could give an idea of VEGF mediated angiogenic signaling was significantly decreased upon p73 knockdown, specifically upon ΔNp73 inhibition (Figure 59).

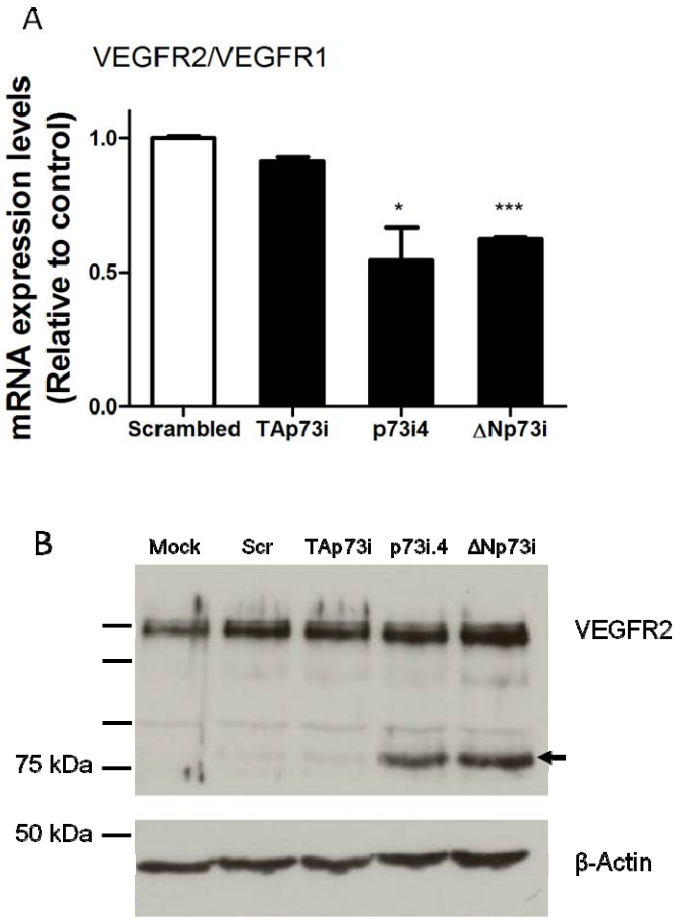

Figure 59: VEGFR signaling upon p73 knockdown. A) VEGFR2/VEGFR1 mRNA expression ratio was calculated from the mRNA expression levels of VEGFR2 and VEGFR1. VEGFR2/VEGFR1 ratio was decreased as a consequence of p73 and specifically ΔNp73 knockdown. B) VEGFR2 expression was analyzed by western-blot. There is no difference in VEGFR2 expression level but a band about 75 kDa is appeared to be highly overexpressed upon p73 and ΔNp73 knockdown.

We also analyzed the expression levels of VEGFR2 at protein level (Figure 59B) and we did not find difference in expression of VEGFR2 (about 200 kDa), which is consistent with our mRNA expression data (not shown). Unexpectedly, we found a band about 75 kDa highly overexpressed upon total p73 or ΔNp73 knockdown. This size correspond with the molecular

size of the VEGFR2 cytoplasmic domain (Bertuccio et al., 2011) so we hypothesized that it may be a truncated VEGFR receptor without VEGF binding capacity and therefore, unable to trigger angiogenic response.

It has been described that VEGF is so effective in eliciting endothelial cell responses probably due to its capacity to engage pleitropic signaling pathways. In HUVEC, VEGF signaling through VEGFR2 induced activation of MAPK pathway (Abedi and Zachary, 1997). To check the status of MAPK signaling pathway we examined the influence of p73 inhibition on phosphorylation of extracellular signal-regulated kinases (ERK). As it is shown in Figure 60, p73 and ΔNp73 inhibition led to reduced pERK levels, indicating a decreased activation of VEGFR2 downstream signal transduction. It has been previously reported for our group and others the existence of a feedback loop between p73 and the MAPK signaling cascade (Fernandez-Garcia et al., 2007; Jones et al., 2007). While p73 expression resulted in enhanced pERK levels and activity, inactivation of TAp73 by siRNA attenuates Ras-induced ERK activation and differentiation in PC12 cells (Fernandez-Garcia et al., 2007). Herein, we report that ΔNp73 inhibition leads to a decrease in ERK activation in HUVEC cells suggesting a defective VEGF signaling.

The transforming growth factor-β (TGF-β) superfamily signaling has important roles in regulating vascular biology. TGF-β signaling has a dichotomous effect which is thought to be mediated by the balance of signaling through two different responsive signaling pathways: TGF-β signaling activated through ALK-5 is mainly transduced by Smad2/3 leading to inhibition of cell migration and proliferation, whereas activation through ALK-1 is mainly mediated by Smad1/5/8 and leads to activation of endothelial cell migration and proliferation (Goumans et al., 2002). Apart from TGF-β1, members of BMP family such as BMP-9 can bind and signal through ALK1. Here, we examined the effect of p73 inhibition in TGF-β downstream signaling through Smad1/5/8 and we found a decreased phosphorylation of Smad1/5/8 upon p73 and ΔNp73 knockdown (Figure 60). Furthermore, VEGF has been identified as a putative target of BMP-9-ALK1 signaling and it has been suggested that the promotion of angiogenesis by BMP-9 is dependent on VEGFR2 activation (Suzuki et al., 2010), Thus, these results are consistent with our previous data demonstrating that functional inhibition of p73 affects endothelial cell migration and morphogenesis, even in the presence of 2% serum, which contains growth factors, including VEGF and TGF-β (Oida and Weiner, 2010).

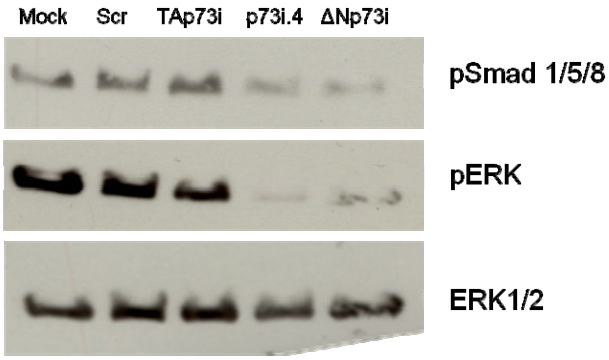

Figure 60: Downstream signaling activation upon p73 knockdown. The activation of two signaling pathways involved in angiogenic responses was analyzed by western-blot. Antibodies against pSMAD1/5/8 and pERK were used to analyze TGF-β and MAPKs signaling, respectively, and we found that knockdown of p73 and ΔNp73 resulted in a decreased expression of both pSMAD1/5/8 and pERK.

In summary, the present findings reveal a molecular scenario in which at least two signaling pathways involved in angiogenesis resulted attenuated as a consequence of ΔNp73 knockdown. Since these pathways are interconnected, it is possible to hypothesize that the impairment of TGF-β signaling mediated by ALK-1 due to ΔNp73 functional inhibition, would lead to a decreased VEGFR2 signaling and in turn into an impaired angiogenesis. Nevertheless, *Vegfr*1 expression seemed to be also affected as a result of p73 deficiency. Therefore, we could not rule out the possibility that p73 function affects both signaling pathways independently.

3. **Analysis of the role of p73 in angiogenesis *in vivo***

Results obtained from our *in vitro* experiments suggest that p73 fulfills a relevant function in endothelial differentiation and vascular morphogenesis and angiogenesis. The next objective was to investigate whether p73 was also involved in the physiologic development of the vascular system *in vivo*. For this purpose, we have used the *Trp73*-/- mouse and sought for vascular defects using two models: whole-mount CD31 immunostaining of 11.5 days post coitum embryos (E11.5) and analysis of retinal blood vessels in P5 postnatal mice.

3.1 Evaluation of the effect of p73 deficiency in the developing vasculature of Trp73 knockout embryos

Trp73-/- embryos (p73KO from now on) which lack all p73 isoforms, exhibit developmental abnormalities. We have previously reported that 60% of E14.5 p73KO embryos presented a pallor phenotype, suggesting an anemic state. Moreover, the erythroid differentiation master gene *Gata1* is a direct transcriptional target of TAp73 and the TA/ΔNp73 ratio is crucial for the transcriptional regulation of this gene. In addition, other erythropoiesis related genes have been shown to be down-regulated in p73KO liver embryos, supporting the p73 physiological role in erythroid differentiation (Marques-Garcia et al., 2009). Endothelial and hematopoietic cells share a common progenitor, the hemangioblast. There is compiling evidence that indicates that embryonic vasculature give raise to hematopoietic progenitors through the hemogenic endothelium (Adamo and Garcia-Cardena, 2012). Furthermore, it has been proposed that factors like GATA-1 or RUNX exert a reversible regulation between the two stages: endothelial and hematopoietic (Iacovino et al., 2011). Thus, we hypothesized that a *Gata-1* regulator like p73 must be required for the appropriated development of the endothelial compartment of the embryonic circulatory system.

To ascertain the physiological function of p73 in vascular formation during development, we examined the vasculature in E11.5 embryos (Figure 36). We found that vasculature of p73KO embryos developed grossly normal with proper formation of major vessels as well as small inter-somitic (red arrows) and cranial vessels (red square). Vessels were lined by CD31 positive cells indicating that p73KO embryos contained differentiated endothelial cells that were capable of assembling a primitive vasculature. This is not surprising since p73 deficiency is not embryonic lethal and a severe deficiency in the embryonic vascular system would lead to

embryonic death. Nevertheless, primary head veins (HV) seemed narrower (arrows) and less branched (circle) in some of the p73KO embryos. These findings suggest that p73 is not required for embryonic vasculogenesis but it could be required for subsequent vascular morphogenesis. However, further studies of the vasculature at earlier embryonic stages comprising also yolk-sac and placental vessels would be needed to finally elucidate the role of p73 in vasculogenesis, angiogenesis and hematopoiesis during embryonic development.

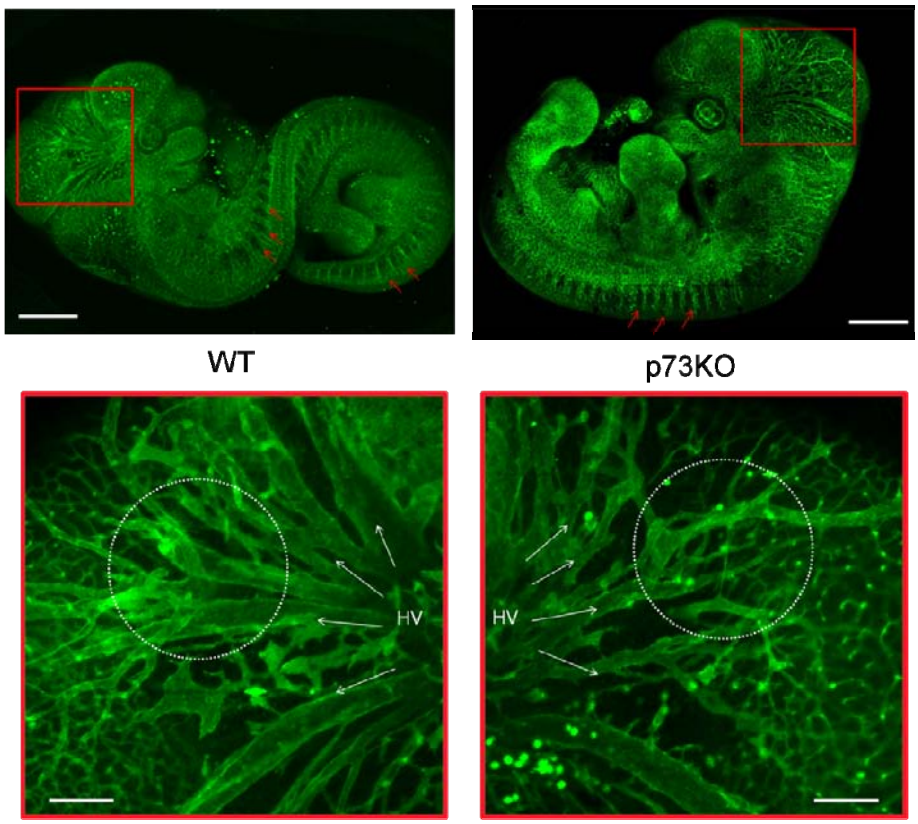

Figure 61: Whole-mount CD31 immunostaining of 11.5 embryos. Embryos were isolated and stained using an antibody against CD31. Networks of large blood vessels were seen in the head regions of both WT and p73KO embryos (red square) and intersomitic vessels (red arrows) were also apparent in both genotypes. However, the blood vessels in the head region (HV) of WT embryos had started to undergo angiogenesis to give rise to branched networks of smaller vessels (circle). Reduced caliber vessel and less branching was apparently seen in the head vessels of the p73KO embryos at E11.5. Scale bars: 1mm (upper) and 200 μm (lower).

3.2 Analysis of retinal vasculature in Trp73 knockout mice

To address whether the impaired angiogenesis potential observed upon p73 functional inhibition *in vitro* could be recapitulated in a physiological *in vivo* system, we have used the mouse retina, which is one of the most used models for studying angiogenesis. Expression of

TP73 in the postnatal retina has been reported to increase from P1 to P3, when it reached the maximum expression, and dropped precipitously thereafter to reach a minimum in P7, indicating that p73 may play a role in early retinal development (Vuong et al., 2012). For our analysis, retinas from P5 wild type (WT) and p73 deficient (p73KO) mice were isolated, labeled with isolectin B4 to visualize vasculature and flat-mounted.

The cup-like retina of the mouse starts out as an avascular tissue and only in the early neonatal period endothelial cells and pericytes proliferate and migrate along astrocytes to form a primary vascular plexus. This highly directional growth of blood vessels is controlled by a spatial concentration gradient of matrix-anchored vascular endothelial growth factor A (VEGF-A), released by astrocytes in response to local hypoxia (Roca and Adams, 2007). At the leading edge of the vascular plexus, the sprouting zone, a directional extension of tip cells and filopodia promotes vascular growth leading to an extension of the endothelial network. In WT mice, inspection of leading edge of retinas showed filopodia-like processes with and organized orientation that extended from the tips of endothelial cells at the developing vascular front. However, p73KO retinas showed disoriented tip cells (Figure 62, arrows) with long and thin filopodia (Figure 62, circles and amplified image.). In endothelial cells, filopodia may serve several functions including sense growth factors such as VEGF gradients (Kater and Rehder, 1995; Ribeiro et al., 2002). We have previously shown that p73 deficient cells present a defective VEGF signaling (Figure 59), Thus, this supernumerous filopodia may respond to a need for extending more filopodia to maintain adequate VEGF signaling, supplying somewhat a defective guidance originated by p73 absence.

We also observed abundance of cells that we identified morphologically as macrophages (Figure 62, arrowheads) in p73KO retinas. Tissue macrophages accumulate at sites of vessel fusion and, due to their affinity for tip cell filopodia, are thought to help endothelial cells to establish contacts, so they act as cellular chaperones for endothelial cell fusion to increase vascular complexity (Fantin et al., 2010). We observed, however, that macrophages seem isolated rather than interacting with endothelial tip-cell filopodia and, thus, macrophages do not align tip cells in preparation for fusion, suggesting a possible impairment of cellular communication between endothelial cells and macrophages. This would explain how despite the presence of supernumerous filopodia the complexity of the vascular network resulted limited.

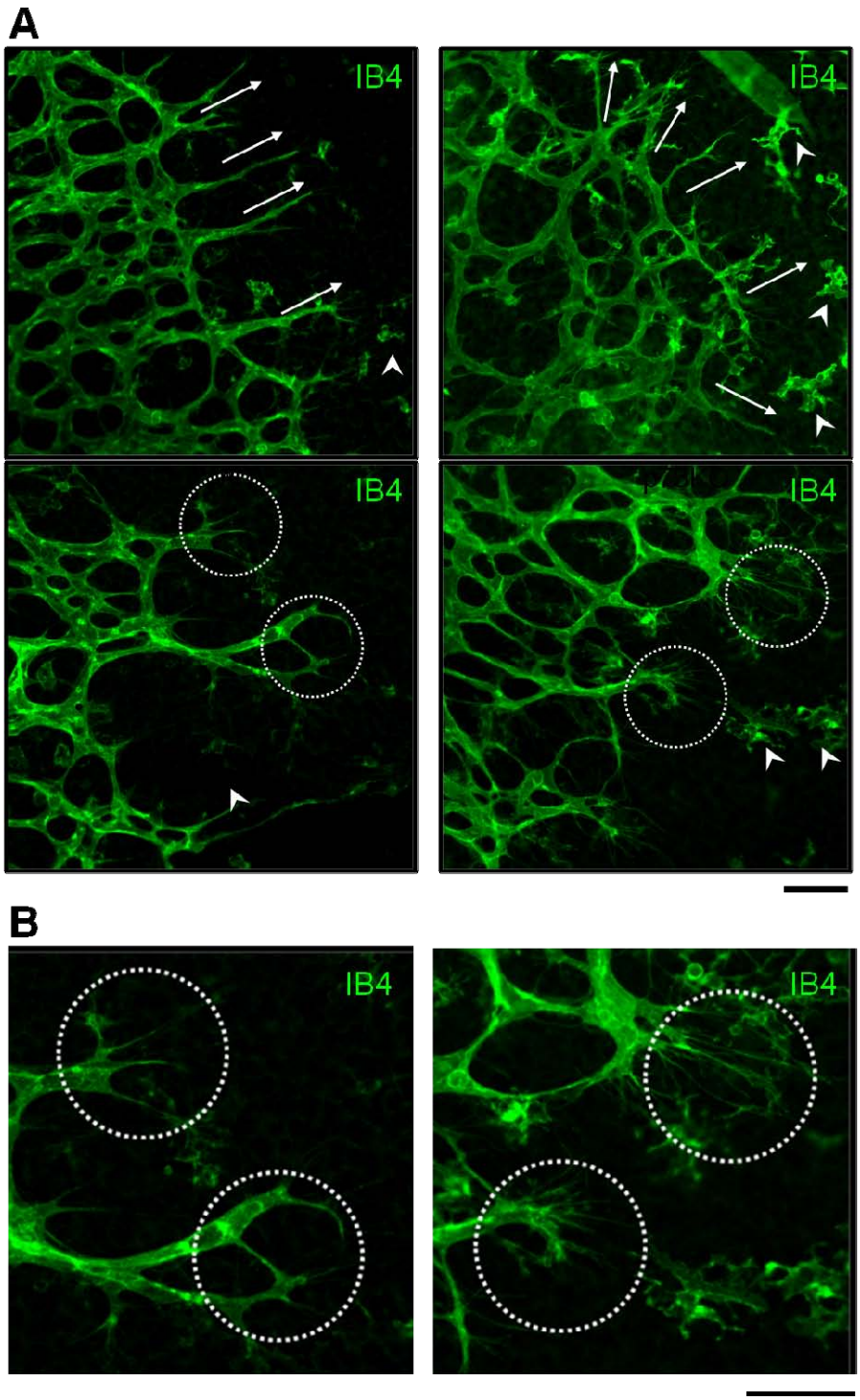

Figure 62 Leading edge of the retina. A) Leading edge of vascularization showed abundant and disorientated filopodia (arrows and circles) and higher numbers of tissue macrophages (arrow head). Two images for each genotype are shown. B) Magnification of A to show the morphology of filopodia. Scale bars: 50 μm.

Filopodia stimulate migration in several cell types. Therefore, we assessed whether the presence of more filopodia affected directed endothelial tip cell migration by measuring the spreading of vascular plexus over the retina. We averaged distance from the optic nerve to the leading edge of vascularization. At least ten distances (red arrow) were measured for each retina. A tendency to a decreased spreading of the retinal plexus was found in the p73KO, although difference was not significant (Figure 63). This lack of statistical significance could be the reflection of the partial penetrance of the p73KO phenotype, since for example, only 60% of the mice present hematopoietic deficiencies (Marques-Garcia et al., 2009). To avoid the handicap derived from the partial penetrance, a higher number of mice need to be analyzed. There is also possible that other compensatory mechanisms take place in the absence of p73.

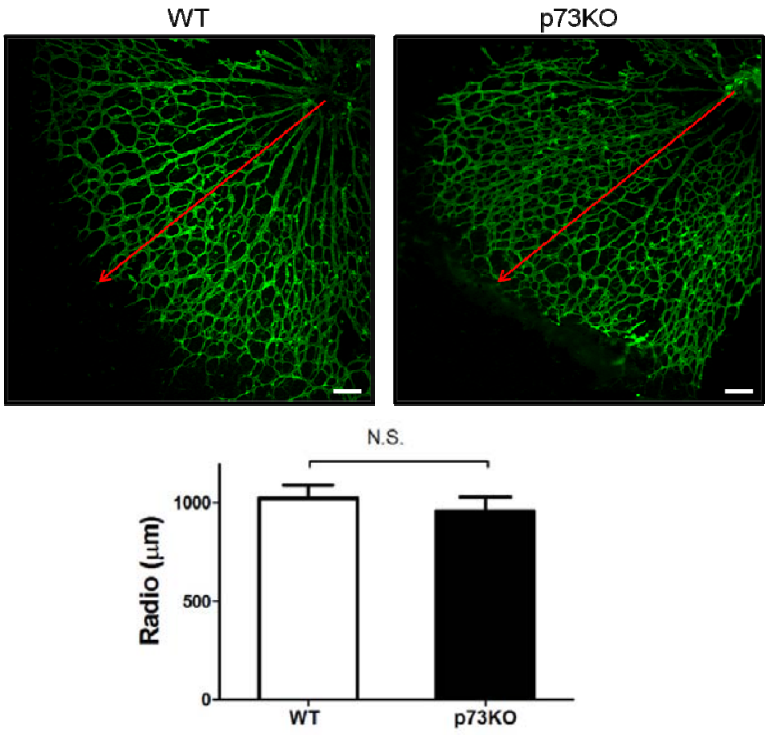

Figure 63: Spreading of vascular plexus over the retina. Migration was assessed as the spreading of the vascular plexus. Distance from the optic nerve to the leading edge of vascularization (red arrow) was averaged for each retina (n≥10). Data represent mean values ± SD; n=3, equal variance t-test was performed to evaluate statistical differences. Scale bars: 100 μm.

While the expansion of the tip cells leads to an extension of the vascular network, a second process, tubulogenesis, is required for the generation of blood vessels. It is believed that tip cells interact with other tips cells through the filopodial processes, and this interaction subsequently leads to bridge-like structures in which endothelial cells lack long filopodia and no

longer display tip cell features. It may be at this stage that sprouts are converted into new tubules, form anastomoses, and become part of a simple, plexus-like network at the vascular perimeter (Dorrell et al., 2002; Gerhardt et al., 2003). A detailed observation of the retinal vasculature at this zone showed a notably disorganized distribution of blood vessels (Figure 64) with high number of small aberrant structures constituted by accumulation of endothelial cells, that morphologically resemble neovascular tufs (Figure 64, arrows).

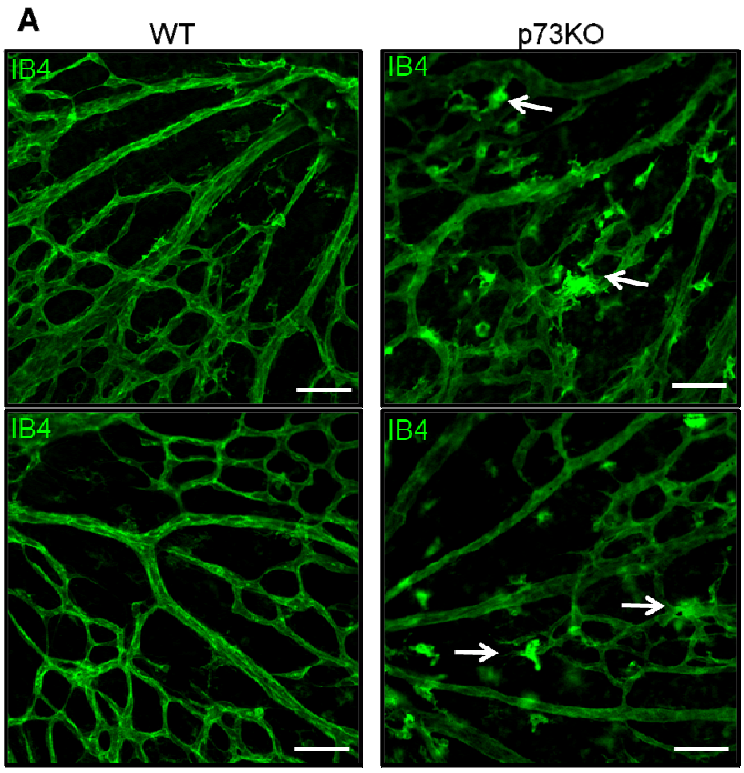

Figure 64 WT and p73 KO retinas labeled with isolectin B4 (IB4). Retinas from p5 mice were stained with IB4 to observe vasculature. A) Proximal to optic nerve photographs showed that p73 KO mice have a notably disorganized vasculature with several small "tufts" (arrows)

To further characterize the retinal phenotype of p73KO, microscopic images of the retinal capillary plexus were analyzed using Angiotool (US National Cancer Institute), software for the quantitative analyses of angiogenesis. The overall analysis of retinal parameters reveled moderated deficiencies in the retinal vasculature of p73KO mice. p73 deficiency had its most striking effect on the elaboration of the primary retinal capillary plexus, which was less dense than in wild-type littermates (Figure 65). Higher power views showed that the peripheral plexus in the retinas of p73KO mice consisted of capillaries that were shorter in diameter, less interconnected, and with more lacunarity (Fig xx). Taken together the above findings indicate

that p73 could be an endogenous positive regulator of vessel sprouting and tubulogenesis, such that p73 deficiency results in formation of a defective vasculature in p73KO mice retina characterized by a sparser vascular network (vessel area and vessel length), attenuated angiogenic sprouting (branching index) and limited uniformity of vessels (high lacunarity).

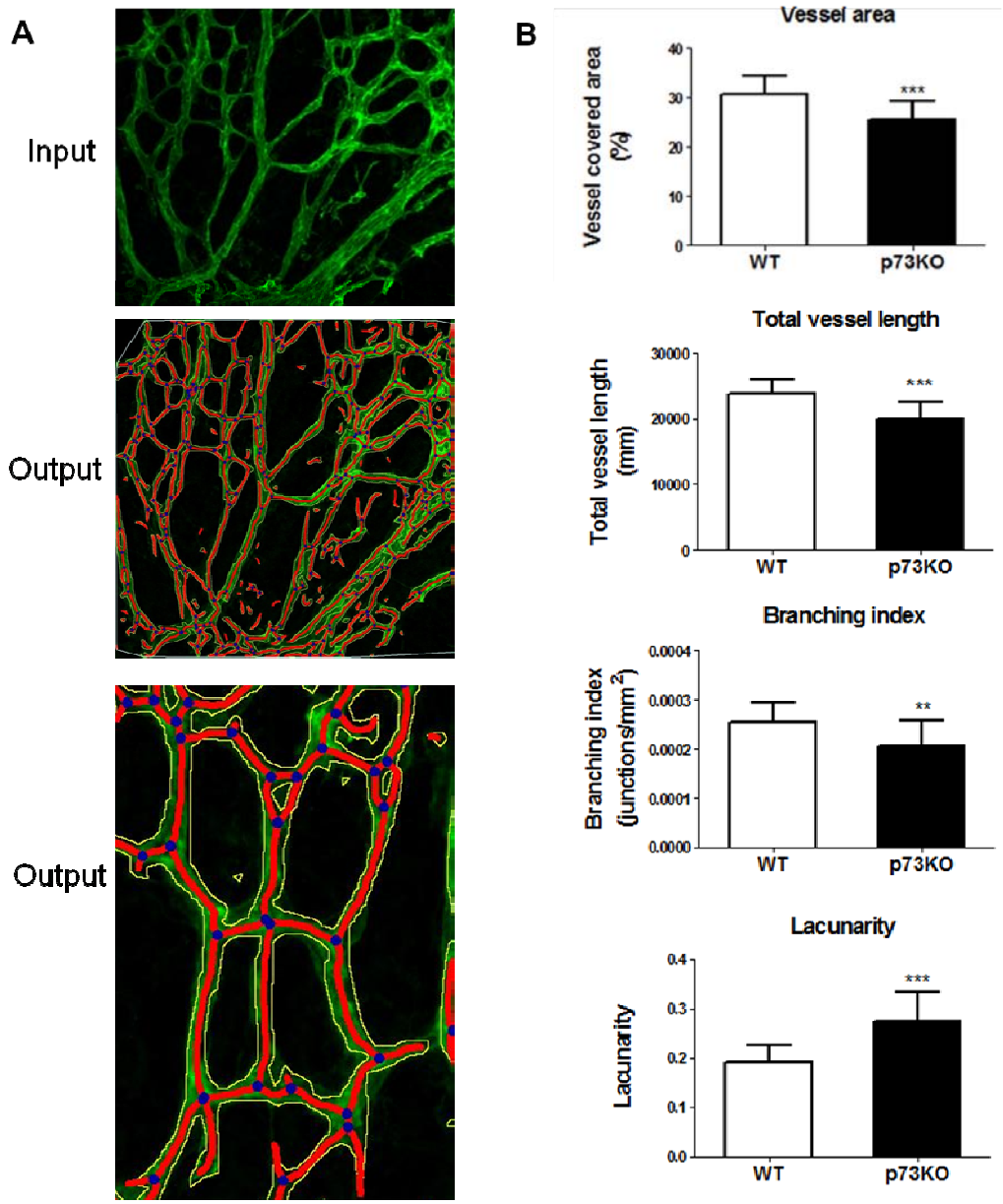

Figure 65: Quantitative analysis of vascular parameters in WT and p73 KO mice retina. A) Representative photographs were used as input images (upper) for the quantitative analysis of retinal vasculature performed with AngioTool software. The resulting images after analysis (middle) and a representative enlarged part (lower) showed the vessel outlines in yellow, the skeleton in red and branching points in blue. B) Graphical representation of the analysis with AngioTool. Data represent mean values ± SD; n=3, experiments were repeated twice; equal variance t-test was performed to evaluate statistical differences: *p<0.05, **p<0.01, ***p<0.001

One important aspect of vascular development in the retina is the interaction of endothelial cells with the pre-existing scaffolding laid down by the astrocytic network. To determine whether the changes observed in the retinal vasculature of p73KO were related to alterations in guidance cues provided by other cells, we examined the relationship between these newly formed vessels and the resident astrocytic network in both p73KO and WT mice. Astrocytes are dominant in retinal vessel pattern formation (Fruttiger et al., 1996). Angiogenic blood vessels invade the retina along a pre-formed network of glial fibrillary protein (GFAP)-expressing astrocytes (Fruttiger et al., 1996; Kubota and Suda, 2009; Stone and Dreher, 1987) which, in turn have invaded retina via the axons of retinal ganglion cells in response to PDGFA. During retinal angiogenesis, it is suggested that endothelial cells and astrocytes mature simultaneously in a mutually dependent manner.

GFAP labeling revealed that, while in WT retinas astrocytes and endothelial cells closely follow one to another in an orderly vascular network pattern, in p73KO retinas astrocytes constitute a notably chaotic reticulation forming clumps of astrocytes (white arrows) that underlie the morphological aberrant structures resembling neovascular tufts mentioned above for the endothelial cells (Figure 66).

These disturbances in the astrocyte network have been found previously to strongly affect vascular patterning (Fruttiger et al., 1996). The stabilization of the retinal vascular network would depend upon reciprocal feedback between blood vessels and astrocytes. In this way, the development of the retinal vasculature is controlled by a hierarchy of molecular interactions: retinal neurons release PDGFA to stimulate proliferation of astrocytes, which, in turn stimulate blood vessel growth by secreting VEGF. Then, the developing vessels provide feedback signals that trigger astrocyte differentiation, which implies cell cycle arrest and down-regulation of VEGF (West and Schein, 2005). Therefore, it is impossible to determine whether the observed retinal phenotype of the p73KO is due to a direct effect over the astrocytes network, caused indirectly by the impaired angiogenic potential of the p73 deficient endothelial cells. In any case, this endothelial-astrocytic correlation provides a link between neurologic and vascular compartment that could shed light on p73 role during development

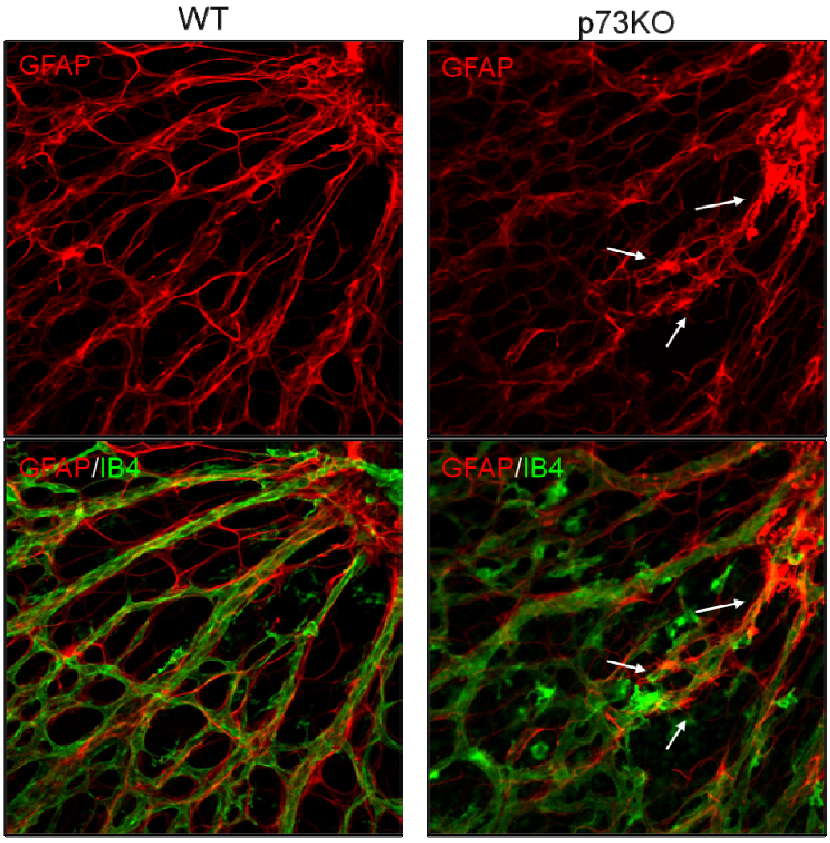

Figure 66: IB4/GFAP double staining of p5 WT and p73KO retinas. P5 mice retinas were stained with IB4 (green) and GFAP (red) to visualize vasculature and astrocytes, respectively. p73 KO retinas showed a disorganized astrocyte network underlying a chaotic vasculature.

Here we described an *in vivo* vascular phenotype in P5 p73KO mice retinas which is characterized by a sparser vascular network and a chaotic distribution of blood vessels and astrocytes. This impaired vascular network is consistent with our *in vitro* findings where we described that p73 functional inhibition impaired the formation of vascular structures in 2D EBs, hindered sprouting in 3D EBs and that p73 deficient HUVEC formed incomplete networks in a tube formation assay. This correspondence supports the idea that p73 is necessary for the appropriated formation of blood vessels both *in vitro* and *in vivo*. However, the *in vivo* phenotype seemed milder than the *in vitro* effect, probably due to the existence of redundant molecular mechanisms in physiological conditions that cannot function in the simplistic *in vitro* models that we have used. Nevertheless, the retina analysis data may indicate a restrained migration of endothelial cells as well as a deficient capacity to assembly into an organized vasculature due to p73 absence, although these defects did not turn into significant changes in the spreading of the vascular network.

According to our findings in HUVEC, the observed phenotype reflects most likely the ΔNp73 function as a positive regulator of endothelial cell migration and morphogenesis, since ΔNp73 is the predominant isoform in endothelial cells. In a pathological context, ΔNp73 overexpression would lead to an increased vasculature, in agreement with its tumor-promoting function. It is noteworthy to point out that p73 deficiency leads to a disorganized retinal vasculature, characterized by an increased lacunarity, which reminds pathological and tumor vasculature (Zudaire et al., 2011). This entropic tendency is also observed in the presence of clusters of endothelial cells and in the disorientation of tip cell filopodia.. Altogether, this lack of pattern suggests that p73, either TA or ΔN, has a role in the organization and cellular polarization during migration and morphogenesis. In this regard, Zhang and colleges had reported that TAp73 deficiency alters mammary epithelial cell polarity during differentiation and migration (Zhang et al., 2012). Again, we should emphasize the importance of the cellular context and the TA/ΔNp73 ratio in determining p73 function. Therefore, due to the existence of regulatory loops between TA and ΔN isoforms and the opposite roles that they play in some developmental processes, it is possible that the ratio between both isoforms rather than the expression of one of them is what finally determines the phenotype.

In summary, we report here that p73KO mice showed moderate defects in retinal blood vessels. This retinal phenotype provides insight into the possible connection between a gene with a dual tumor suppressor/oncogene function and the formation of blood vessels in physiological conditions. Therefore, in the future, it will be interesting to explore the functional interaction of the p53 family members in regulating molecular mechanism that underlie the formation of retinal vasculature.

Conclusions

First: Under our experimental conditions, the best method to model vasculogenesis and angiogenesis in mouse embryonic stem cells is through the embryoid body formation by hanging drop procedure, followed by either a two dimensional or a three dimensional differentiation strategy.

Second: The *TP73* gene is a positive regulator of endothelial cell differentiation, angiogenic sprouting and vascular morphogenesis in a mouse embryonic stem cell model.

Third: The *TP73* gene is required for the full angiogenic potential of human umbilical vein endothelial cells (HUVEC).

Fourth: p73 is a modulator of the angiogenic signal transduction through the VEGF and TGF-β pathways.

Fifth: ΔNp73 deficiency, but not TAp73, is responsible for the defective migration and lack of tube morphogenesis observed in HUVEC.

Sixth: Although not required for embryonic vasculogenesis, p73 function is necessary for the appropriated formation of the mice retinal vasculature.

Seventh: p73 deficiency alters both the astrocyte pattern and the vascular network in the mouse retina.

Eighth: In a physiological *in vivo* model, p73 is a regulator of endothelial cell migration, vessel sprouting and tubulogenesis.

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Summary in Spanish

UNIVERSIDAD DE LEÓN

Departamento de Biología Molecular

ESTUDIO DE LA FUNCIÓN DE P73 EN LOS PROCESOS DE DIFERENCIACIÓN ENDOTELIAL, VASCULOGÉNESIS Y ANGIOGÉNESIS

Tesis Doctoral

Rosalía Fernández Alonso

León, Junio 2013

Índice

Resumen

1. INTRODUCCIÓN

Los vasos sanguíneos constituyen una extensa red de tubos organizada de forma jerárquica que asegura el transporte de oxígeno, nutrientes y productos de desecho de forma eficiente entre los diferentes tejidos y órganos de un organismo, haciendo posible la complejidad estructural de los animales superiores.

La formación de vasos sanguíneos es un proceso esencial durante el desarrollo así como en determinadas condiciones fisiológicas del organismo adulto como son la cicatrización de las heridas y el restablecimiento del flujo sanguíneo en tejidos dañados. Durante el desarrollo, la formación de vasos sanguíneos tiene lugar mediante dos mecanismos: vasculogénesis, que es la formación de vasos sanguíneos a partir de células endoteliales progenitoras, y angiogénesis, que es el proceso de formación de vasos sanguíneos a partir de otros preexistentes. En sentido amplio se puede considerar que estos procesos están regulados por el balance entre factores pro- y anti-angiogénicos, que ha de ser adecuado para cada condición. El desequilibrio de estos factores, hacia un lado o hacia otro, conduce al desarrollo de numerosas enfermedades. Así, en el cáncer, en la artritis reumatoide, o en algunas enfermedades oculares, la excesiva formación de vasos sanguíneos facilita el crecimiento de tejido dañado y la destrucción del tejido sano. Por el contrario, la enfermedad coronaria, la isquemia o los problemas de cicatrización se caracterizan por una formación de vasos sanguíneos que resulta insuficiente, derivando en escaso flujo sanguíneo y produciendo la muerte tisular. En ambos casos, se trata de enfermedades con una frecuencia muy alta en la población occidental y en consecuencia, la investigación encaminada al descubrimiento de nuevos fármacos capaces de promover o reducir, en definitiva capaces de regular, la formación de vasos sanguíneos concentra grandes esfuerzos e ingente inversión económica. En este contexto adquiere gran relevancia la investigación básica que conduzca al descubrimiento de nuevos factores implicados en la formación de vasos sanguíneos y esto acrecienta la necesidad de desarrollar modelos celulares que nos permitan extraer conclusiones fiables acerca de los mecanismos que regulan la formación de vasos sanguíneos.

En la actualidad se utilizan varios modelos angiogénicos, tanto *in vivo* como *in vitro*, entre los que se encuentran los sistemas de diferenciación endotelial a partir de células troncales embrionarias (ES), las células de vena de cordón umbilical humanas (HUVEC), y el análisis de la vasculatura de la retina.

En particular las células ES, por su capacidad de auto-renovación ilimitada y de pluripotencia, son una fuente muy atractiva para su uso en medicina regenerativa, lo cual las hace un modelo muy interesante de estudio. En la actualidad, con la terapia regenerativa se abren nuevas esperanzas de reparar tejidos dañados, mediante la utilización de mecanismos similares a los que, de forma natural, usa el organismo para el mantenimiento y renovación de las poblaciones celulares dañadas o envejecidas. Sin embargo, independientemente de las células utilizadas, para que la terapia regenerativa sea una realidad, es necesario que ofrezca garantías de seguridad y ello va a depender del conocimiento adquirido sobre los mecanismos que controlan la capacidad regenerativa de estas células troncales.

Los factores que regulan los procesos de formación de vasos sanguíneos pueden ser extrínsecos, como la hipoxia o el flujo sanguíneo, e intrínsecos de naturaleza muy variada incluyendo, entre otros, factores de crecimiento y sus receptores, moléculas de adhesión, micro RNAs (miRNAs) o factores de transcripción.

TP73 es un factor de transcripción que pertenece a la familia de p53, el gen supresor tumoral por excelencia. p73 tiene gran homología estructural y comparte algunas funciones con p53 pero, además, lleva a cabo funciones propias relacionadas con el desarrollo. La estructura del gen *TP73*, se caracteriza por la presencia de dos promotores. Esto determina la existencia de dos tipos de isoformas: las isoformas completas, transcritas a partir del promotor P1, contienen una región de transactivación y se denominan TA mientas que las isoformas ΔN, transcritas a partir del promotor P2, carecen de la región de transactivación. Estas isoformas pueden sufrir maduración alternativa, generando proteínas distintas en su región C-terminal y con diferente capacidad de transactivación. Esta estructura de "dos genes en uno" confiere versatilidad funcional y, de hecho, las isoformas TA y ΔN suelen llevar a cabo funciones opuestas en la regulación de la proliferación, la apoptosis o la diferenciación celular. En general, las isoformas TAp73 son capaces de transactivar dianas transcripcionales de p53 y se consideran promotoras de la apoptosis e inhibidoras del ciclo celular. Por el contrario, las isoformas ΔNp73 actúan como dominantes negativos sobre la función de p53 y TAp73 a través de diferentes mecanismos. La expresión de estas isoformas y su interacción funcional es dependiente del contexto y tipo celular. En términos generales, se considera que el ratio entre las isoformas TA/ΔN de p73 es un condicionante de la función de p73 y del destino celular.

El desarrollo de tumores es un proceso dependiente del aporte de oxígeno y nutrientes y, por tanto, dependiente de la formación de vasos sanguíneos. En este contexto, es muy interesante el estudio de la posible implicación de los miembros de la familia de p53 en la regulación de la angiogénesis, ya que implica relacionar la función oncogén-gen supresor tumoral con la angiogénesis. p53 ha sido considerado clásicamente como un represor de la angiogénesis tumoral y de la producción de VEGF; sin embargo, datos recientes indican que p53 y, hipotéticamente p73, pueden actuar tanto como inductores como represores de la angiogénesis, dependiendo del ratio de expresión entre las diferentes isoformas.

Si bien parece que los miembros de la familia de p53 están implicados en la regulación de la angiogénesis tumoral, su función en la formación de vasos sanguíneos durante el desarrollo ha sido escasamente analizada. Por eso, teniendo en cuenta resultados previos publicados por nuestro grupo, que relacionan la función de p73 con la diferenciación eritroide, y considerando la vinculación existente entre los procesos de diferenciación endotelial y hematopoyética durante el desarrollo, planteamos la siguiente hipótesis:

Los miembros de la familia de p53, y específicamente p73, desarrollan una función importante en la regulación de la diferenciación endotelial y en la formación de los vasos sanguíneos en condiciones fisiológicas. Por lo tanto, el objetivo general de esta tesis fue evaluar la función de p73 en la formación de estructuras vasculares *in vitro* y en la formación de vasos sanguíneos *in vivo*, utilizando diferentes modelos angiogénicos. Para ello planteamos los siguientes objetivos específicos:

2. OBJETIVOS ESPECÍFICOS

Los objetivos de esta tesis fueron:

1. Desarrollar un modelo de diferenciación endotelial en las células troncales embrionarias murinas E14Tg2α, realizando un análisis comparativo de varios métodos de diferenciación

2. Determinar la función del gen TP73 en diferenciación endotelial, vasculogénesis y angiogénesis in vitro.

    2.1. Analizar el efecto de la inhibición funcional de p73 en la diferenciación endotelial y morfogénesis vascular de las células troncales embrionarias murinas E14Tg2α.

2.2. Analizar el efecto de la falta de p73 en la biología de las células endoteliales humanas de vena umbilical (HUVEC)

3. Identificar la función de p73 en la vasculogénesis y angiogénesis *in vivo*.

3.1. Evaluación del efecto de la falta de p73 en el desarrollo de la vasculatura de los embriones *knockout* Trp73.

3.2. Análisis de la vasculatura de la retina en los ratones *knockout* Trp73.

3. RESULTADOS

3.1 Establecimiento de un sistema de diferenciación endotelial a partir de células troncales embrionarias (ES) murinas

El primer objetivo de esta tesis fue la elección del modelo más adecuado para la diferenciación endotelial utilizando la línea de células troncales embrionarias E14Tg2α. Para ello, realizamos un estudio comparativo de diferentes modelos descritos previamente en la bibliografía, incluyendo en algunos casos pequeñas modificaciones sobre los mismos. Aunque existen otros métodos de diferenciación a partir de células troncales embrionarias, en esta tesis se han utilizado aquellos basados en la formación de agregados de células troncales embrionarias que, finalmente dan lugar a derivados de las tres capas germinales y que se asemejan al embrión en muchos aspectos, denominados cuerpos embrioides (EBs). Para formar estos EBs utilizamos el método de gota colgante (hanging drop) previamente mencionado.

Entre los múltiples factores que condicionan el proceso de diferenciación celular *in vitro*, tienen especial importancia los componentes del medio de cultivo. Normalmente, la presencia de factores de crecimiento específicos de un determinado linaje celular conlleva una diferenciación más eficiente hacia ese linaje. Para establecer un medio de diferenciación endotelial adecuado para nuestra línea celular, llevamos a cabo el proceso de diferenciación mesodérmica que se había utilizado previamente en nuestro laboratorio pero utilizando tres medios diferentes de forma paralela: el medio de rutina (medio A) y otros dos (medio B y medio B+GF); este último contenía presencia de varios factores de crecimiento como VEGF, FGF2, IL-6 y EPO. Durante el proceso de diferenciación, valoramos la eficiencia de los distintos medios analizando tres características: tamaño y morfología de los EBs y expresión de

los marcadores endoteliales CD31, VE-Cadherina y VEGFR2. Los resultados pusieron de manifiesto que los EBs cultivados en el medio A se diferencian para dar lugar, entre otras, a células endoteliales, que expresan el marcador CD31, y que son capaces de unirse y formar estructuras vasculares. Sin embargo, los EBs cultivados en el medio B y el medio B+GF mostraron tendencia a desagregarse y no llegaron a completar el proceso de diferenciación. Por lo tanto, ya que ninguno de los medios probados produjo mejoras significativas en la diferenciación endotelial, continuamos usando el medio A.

Según lo descrito en la bibliografía, los EBs incluidos en una matriz tridimensional de colágeno I y en presencia de VEGF emiten brotes vasculares de características comparables a las estructuras vasculares *in vivo*. Existen varios modelos para la obtención de brotes vasculares a partir de EBs. En esta tesis, se han comparado dos de los más utilizados en la bibliografía adaptándolos a nuestro protocolo inicial: en ambos casos los EBs se formaron como "gotas colgantes" y, posteriormente, se transfirieron bien a geles de colágeno I (protocolo 1) o bien a una matriz semisólida de Methocult como paso previo a la inclusión en geles de colágeno (protocolo 2). La proyección de brotes vasculares fue evaluada mediante la observación microscópica de los EBs durante el proceso de diferenciación. Observamos que los EBs cultivados siguiendo el protocolo 1 originaron mayor número de brotes que los EBs cultivados siguiendo el protocolo 2. Además, estos brotes mostraban morfología típica de brotes vasculares y estaban formados por células endoteliales CD31 positivas. Por lo tanto, concluimos que el protocolo 1 era más eficiente en la formación de brotes vasculares y decidimos llevar a cabo la diferenciación tridimensional de los EBs siguiendo dicho protocolo.

En la mayoría de los protocolos de formación de EBs, las células ES que se utilizan como material de partida han crecido sobre una capa de fibroblastos embrionarios de ratón (MEFs), que ejercen la función de "feeders" y previenen su diferenciación. Sin embargo, el efecto sobre la capacidad de diferenciación de las células ES ejercido por la presencia de feeders en los pasos previos a la formación de EBs, es prácticamente desconocido. Las células E14Tg2α pueden mantenerse indiferenciadas en ausencia de feeders, siempre que el medio contenga factor inhibidor de la leucemia (LIF). Cultivamos las células ES en presencia de feeders durante los pasos previos a la inducción de la diferenciación y observamos que en estas condiciones las células proliferaban más rápido. Con respecto a la diferenciación, obtuvimos ligeras mejoras en la capacidad de formación de estructuras vasculares en un contexto bidimensional y en la

capacidad de formación de brotes vasculares, todo ello indicando que el cultivo de E14Tg2α en presencia de *feeder*s mejora las condiciones de diferenciación.

2. Evaluación de la función de p73 en diferenciación endotelial, vasculogénesis y angiogénesis *in vitro*.

2.1 Estudio del efecto de la inhibición funcional de p73 sobre la capacidad de diferenciación endotelial y la formación de estructuras vasculares de la línea de células troncales embrionarias murinas E14Tg2α

El segundo objetivo de esta tesis fue el estudio de la función de p73 en los procesos de diferenciación endotelial, vasculogénesis y angiogénesis. Estos procesos son recapitulados *in vitro* mediante la formación de EBs y su diferenciación dirigida hacia linaje endotelial. Por lo tanto, para determinar la relevancia de p73 en procesos de diferenciación endotelial y morfogénesis vascular, llevamos a cabo varios protocolos de diferenciación endotelial con clones de la línea celular E14Tg2α en los que la función de p73 se encuentra inhibida por la expresión de una isoforma dominante negativa de p73 (DDp73). Como control utilizamos clones control seleccionados paralelamente tras la transfección con el vector vacio (pcDNA3) y células E14Tg2α no transfectadas (WT).

Según lo establecido en el objetivo 1, analizamos el proceso de diferenciación endotelial de los EBs tanto en un contexto bidimensional (2D) como tridimensional (3D). Durante el proceso de diferenciación 2D analizamos el tamaño, la morfología y la expresión de marcadores endoteliales y durante el proceso de diferenciación 3D evaluamos la formación de brotes, su naturaleza endotelial y su morfología, tomando siempre como referencia las líneas control: WT y pcDNA3.

La inhibición funcional de p73 afectó al tamaño de los EBs, que resultaron ser significativamente menores, a la formación de estructuras vasculares en 2D, que resultaron incompletas, y a la expresión de marcadores endoteliales como CD31, VE-Cadherina y VEGFR2, que resultó significativamente reducida en los EBs formados con los clones DDp73. Además, la inhibición funcional de p73, produjo una disminución de la capacidad de producción de brotes vasculares manifestada, tanto en una disminución significativa del número de EBs capaces de formar brotes, como en una morfología aberrante de los mismos en

los casos en los que éstos se formaron. En conjunto, nuestros resultados indican que la función de p73 es necesaria, al menos en parte, para la correcta diferenciación endotelial y la formación de estructuras vasculares *in vitro* y, por lo tanto, podría desarrollar funciones relacionadas con la diferenciación endotelial, la vasculogénesis y la angiogénesis.

Para determinar el mecanismo mediante el cual p73 regula estos procesos, analizamos algunas vías de señalización importantes en angiogénesis. La vía VEGF/VEGFRs es determinante en todas las respuestas angiogénicas. La inhibición funcional de p73 condujo a una disminución de la expresión de VEGFR2 sumada a un incremento de la expresión de VEGFR1. El VEGFR2 se considera la isoforma capaz de promover una respuesta pro-angiogénica global, en tanto que, la isoforma VEGFR1 es considerada en algunos contextos como inhibidora de la angiogénesis. Por lo tanto, el cociente entre VEGFR2/VEGFR1 nos puede dar una idea de la señalización pro-angiogénica. La inhibición funcional de p73 resultó en una disminución significativa de este cociente. TGFβ1 es un factor de crecimiento implicado en numerosas respuestas angiogénicas, entre las cuales destaca su papel inductor de la vasculogénesis. La expresión de TGFβ1 también resultó disminuida debido a la inhibición funcional de p73. Así pues, en términos moleculares, podemos decir que la inhibición funcional de p73 genera un contexto predominantemente anti-angiogénico que afecta a más de una vía de señalización.

2.2 Estudio del efecto de la inhibición funcional de p73 sobre la biología de las células endoteliales humanas de la vena umbilical (HUVEC).

Los vasos sanguíneos están formados por células de distinta naturaleza. Las células que revisten todo el sistema circulatorio son las células endoteliales que, a parte de una labor mecánica, regulan activamente la morfogénesis vascular y contribuyen a la homeostasis. Por lo tanto, aquellos factores que regulen la biología de las células endoteliales pueden estar implicados en la formación y remodelación de estructuras vasculares. Con el objetivo de determinar si p73 regula solamente el proceso de diferenciación endotelial, o si por el contrario también regula procesos biológicos característicos de las células endoteliales, analizamos el efecto de la carencia de p73 en células HUVEC. Para ello, llevamos a cabo ensayos de proliferación, migración y morfogénesis en células en las que p73 fue inhibido a dos niveles diferentes: inhibición funcional mediante expresión transitoria de DDp73 o inhibición a nivel de RNA mediante RNA pequeño de interferencia (siRNA).

La proliferación se determinó mediante un ensayo de MTT. El silenciamiento parcial de p73 no afectó la proliferación celular durante las 72 horas posteriores a la interferencia, periodo en el que se realizaron los demás ensayos. Sin embargo, a largo plazo, la inhibición de p73 afectó negativamente la proliferación de las células HUVEC. La migración, evaluada mediante un ensayo de "scratch" o "wound healing", se redujo significativamente, tanto cuando p73 fue inhibido funcionalmente como cuando se utilizó el sistema siRNA. El proceso de morfogénesis fue evaluado mediante el ensayo de formación de tubos en Matrigel y analizado cuantitativamente con la herramienta WimTube de Wimasis. Los tubos que se formaron en ausencia de p73 cubrían una superficie menor, presentaban una longitud menor y originaron una red incompleta. En conjunto, los resultados de estos ensayos nos permiten concluir que p73 es un regulador de la biología de las células endoteliales y, en consecuencia, su ausencia determina ineficiencia en los procesos de proliferación, migración y morfogénesis de las células endoteliales.

Como se ha dicho en la introducción, p73 se caracteriza por su dualidad funcional en virtud de la existencia de dos tipos de isoformas: TAp73 y ΔNp73. El siguiente paso fue, pues, determinar qué isoformas se expresaban en este modelo y cuál de ellas era responsable del fenotipo observado. Mediante un análisis de RT-PCR cuantitativo determinamos que la isoforma ΔNp73 se expresaba de manera prioritaria en estas células, aunque también expresaban, a niveles bajos, TAp73. Los ensayos de proliferación, migración y formación de tubos realizados tras la oligofección de siRNA específicos para TAp73 o ΔNp73, revelaron que la expresión de ΔNp73 era necesaria para la correcta migración y morfogénesis endotelial de las HUVEC.

A nivel molecular, evaluamos la repercusión de la inhibición de p73 sobre la vía de señalización de VEGF utilizando, como en el caso de los EBs, la proporción de los niveles de expresión de RNA de VEGFR2/VEGFR1 como un parámetro que estima la capacidad pro-angiogénica. Dicho cociente resultó significativamente disminuido en las HUVEC con inhibición de p73, específicamente con inhibición de ΔNp73. El análisis de la expresión de VEGFR2 a nivel de proteína no reveló cambios en la expresión de VEGFR2. Sin embargo, detectamos la presencia de una banda de 75 kDa en las HUVEC en las que p73, y específicamente ΔNp73, había sido inhibido. El tamaño corresponde con el tamaño de la parte citoplasmática del receptor por lo que es posible que ese banda corresponda a una isoforma truncada de VEGFR2, incapaz de unir VEGF y por tanto, incapaz de originar respuesta

angiogénica. También analizamos la señalización inducida por TGF-β y BMPs a través de los efectores Smad1/5/8. La fosforilación de estos efectores resultó disminuida en las HUVEC en las que p73, específicamente ΔNp73 había sido inhibido, lo cual indica menor activación de la cascada de señalización.

En resumen, nuestros datos indican que la expresión de ΔNp73 es necesaria para mantener el potencial angiogénico de las células HUVEC, y su falta atenúa al menos dos vía implicadas en la regulación del potencial angiogénico de estas células.

3. Análisis del efecto de la ausencia de p73 en el proceso de angiogénesis *in vivo*.

Los resultados obtenidos *in vitro* sugieren que p73 cumple una función relevante en el proceso de diferenciación endotelial, así como en la formación de estructuras vasculares. El siguiente paso fue comprobar que esta función de p73 *in vitro* tiene una relevancia fisiológica durante el desarrollo del sistema circulatorio. Para ello, utilizamos el ratón *Trp73*-/- (p73KO), que carece de todas las isoformas de p73, y buscamos la presencia de defectos vasculares en dos sistemas: el sistema circulatorio de embriones E11,5 (día embrionario 11.5) y la red de vasos sanguíneos de la retina de ratones P5 (5 días).

La vasculatura de los embriones p73KO resultó aparentemente normal ya que, tanto los grandes vasos como los vasos inter-somíticos y craneales fueron muy semejantes a los del WT; solamente en el caso de algunos embriones observamos que los vasos de la cabeza eran más estrechos y estaban menos ramificados. Esto implica que p73 no es necesario durante la vasculogénesis embrionaria aunque no descarta que pueda ser necesario en etapas posteriores de la formación de vasos sanguíneos.

El análisis de la red vascular de la retina es uno de los modelos *in vitro* más usados para el estudio de la angiogénesis. Representa un sistema fisiológico en el que se pueden analizar muchos de los procesos implicados en la formación de vasos sanguíneos. La retina es avascular en P0 y después del nacimiento se empieza a constituir una red de capilares a partir de células endoteliales que migran desde el nervio óptico, guiadas por la existencia de una red astrocítica previa. El análisis de la red vascular de las retinas del ratón p73KO reveló la existencia de deficiencias significativas. La red capilar de las retinas en los p73KO es menos densa y está formada por capilares más estrechos, de menor longitud y menor uniformidad. Estos

resultados indican que p73 es necesario para la formación adecuada de la red vascular de la retina y, por lo tanto, sugieren que p73 desarrolla una función en angiogénesis en un contexto fisiológico.

Además, los resultados obtenidos mediante el análisis de la retina son consistentes con los resultados obtenidos *in vitro* y apoyan la hipótesis inicial de que p73 es necesario, no solo durante el proceso de diferenciación, sino también para la formación de estructuras vasculares *in vitro* e *in vivo*.

Conclusiones

Primera: En nuestras condiciones experimentales, el método más adecuado para generar un modelo en células troncales embrionarias murinas que permita analizar la vasculogénesis y la angiogénesis, es aquel en el que se combina la formación de cuerpos embrioides por el procedimiento de gota colgante con estrategias de diferenciación en cultivo bi- o tri-dimensional.

Segunda: El gen *TP73* es un regulador positivo de la diferenciación endotelial, la formación de brotes y la morfogénesis vascular a partir de células troncales embrionarias murinas.

Tercera: El gen *TP73* es necesario para la plena adquisición de un potencial angiogénico en células endoteliales humanas de vena umbilical (HUVEC).

Cuarta: p73 es un modulador de las vías de señalización mediadas por VEGF y TGF-β.

Quinta: La falta de ΔNp73, pero no de TAp73, es responsable de las alteraciones en la migración y en la formación de tubos observadas en células HUVEC.

Sexta: La función de p73 es necesaria para la correcta formación de la red vascular de la retina, aunque no para la vasculogénesis embrionaria.

Séptima: La falta de p73 altera la organización de la redes astrocítica y vascular en la retina del ratón.

Octava: El gen *TP73* es un regulador fisiológico de la diferenciación endotelial, la migración y la formación de tubos y brotes vasculares en un modelo *in vivo*.