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TITOLO DELLA TESI

The involvement of integrins in the fine tuning of Angiopoietin-1/Tie2 signalling

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Ai “Lentiviri”.... gli Uomini lenti

ABSTRACT

Among endothelial receptor tyrosine kinases which play a pivotal role in blood vessels growth and differentiation, Tyrosine kinase with Ig and EGF homology domain (Tie2) reserves one of the most important places during embryogenesis and in adult vasculature. The activation of Tie2, subsequent to the binding of its ligand Angiopoietin-1 (Ang-1), leads to vessel assembly and maturation by mediating endothelial cells (EC)-survival and regulating the recruitment of mural cells. In the last decade it has been demonstrated that the specificity of molecular signalling in the endothelium is determined by a synergism between growth factor receptors and cell adhesion molecules, in particular integrins.

The signalling pathways triggered by Tie2 and integrins often lead to stimulation of the same downstream transducers, such as Akt and MAPK/Erk proteins. Moreover, integrin engagement can favour activation of tyrosine kinase receptors by affecting local receptor concentration at the plasma membrane. On such premises, I analyzed how cell adhesion influences Ang1-dependent Tie2 signalling in terms of receptor phosphorylation and activation of Akt and Erk.

Thus I analysed the activation of Akt and Erk as a helpful tool to evaluate how integrin ligation modulates Tie2 signalling.

The results showed that integrin engagement enhances Tie2 downstream signalling; specifically, Akt and Erk activation is maximum when $\alpha 2$ and $\alpha 5$ integrins are engaged.

My study confirms and provides a better characterization of the cross-talk between Tie2 and integrins, shedding light on the important role of $\alpha 2$ and $\alpha 5$ integrins. Further analysis will be helpful to understand which molecular mechanisms regulate Tie2-integrins co-operation.

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AIM OF THE PROJECT

Tie2 is one of the major receptor tyrosine kinases implicated in Angiogenesis. Tie2 is fundamental in embryonic Angiogenesis, as mice lacking Tie2 die for hemorrhage as a consequence of failure of the primary vascular plexus to branch and differentiate, suggesting a role in blood vessels maturation and maintenance. Tie2 also has a role in adult vasculature; it has been found upregulated and activated in the endothelium undergoing active Angiogenesis such as in the endothelium of rat ovary and in rat skin wounds. Moreover Tie2 was found expressed and activated in the endothelium of all normal tissues suggesting a role in the maintenance of adult vasculature too. It is clear that Tie2 and its agonist ligand, Angiopoietin-1 (Ang-1) have different functions during active Angiogenesis and vessel quiescence. In active Angiogenesis endothelial cells (ECs) lose cell-cell contact and Ang-1/Tie2 system preferentially activates MAPK pathway leading to ECs sprouting and migration; on the contrary, in quiescent vasculature, where ECs tightly contact each other, Ang-1/Tie2 system induces survival and anti-permeability effect by the activation of Phospho-inositol 3 kinase (PI3K)/Akt pathway. In the last decade a large amount of data from *in vitro* and *in vivo* models demonstrated that a co-operation between tyrosine receptor kinases and integrins is necessary to regulate Angiogenesis. In quiescent vasculature ECs adhere on basal membrane formed principally by laminins and collagens, while during Angiogenesis extracellular matrix (ECM) completely changes, consisting mainly of fibrinogen, fibronectin and vitronectin. Tie2 promotion of survival in quiescent mature vasculature and migration in angiogenic endothelium could be explained not only as a consequence of different confluent condition, cell-cell contact and sparse cells respectively, but also as a consequence of Tie2 modulation signalling by different integrins engagement. For this reason the aim of my project has been to evaluate how Ang-1/Tie2 signalling pathways, specifically Akt and the MAPK Erk proteins, change when different integrins are engaged. My results provide evidence that integrins enhance Ang-1/Tie2 downstream signalling and that this synergic co-operation changes according to the specific engaged integrin.

INTRODUCTION

Angiogenesis

Humans are complex multicellular organisms and all cells require a finely controlled supply of oxygen. The diffusion of oxygen through tissues is limited to 100 to 200 μm; therefore a complex vascular system has evolved to ensure that all the cells are within the distance of a supply of oxygen (Hoeben et al, 2004). Two processes are responsible for the formation of new blood vessels during embryogenesis: Vasculogenesis and Angiogenesis (Yancopoulos et al, 2000).

During early embryogenesis blood vessels formation occurs through Vasculogenesis, an *in situ* differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells that assemble into a vascular labyrinth. The subsequent growth and remodelling process of the primitive network into a complex network is called Angiogenesis (Carmeliet, 2000).

In the adult, new vessels are produced almost exclusively through Angiogenesis. The vasculature is normally quiescent in the adult mammal, except for physiological processes in the female reproductive cycles (ovulation, menstruation, implantation, pregnancy) and during wound healing. The endothelial cells are among the longest-lived in the body outside the central nervous system; in a normal adult vessel, only 1 every 10,000 endothelial cells (0, 01%) is in the cell division cycle at any given time (Hanahan & Folkman, 1996).

Appropriate stimuli, such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) can activate quiescent vasculature to form new capillaries. This process of Angiogenesis is complex and it can be divided in two phases: activation and resolution. During activation vascular permeability is increased, the basement membrane surrounding the endothelial cell tube is degraded, the endothelial cells change shape and invade the stroma. This invasion is accompanied by proliferation of endothelial cells at the leading edge of the migrating column. During resolution endothelial cells stop proliferation, cease migration, change shape and tightly adhere to each other to form a capillary lumen (Hanahan & Folkman, 1996) (Figure I).

Angiogenesis in the adult is also related to pathological situations such as tumour growth, rheumatoid arthritis, diabetic retinopathy, and psoriasis. Much of the interest in Angiogenesis comes from the notion that growing tumours need to recruit endothelial cells to form their own microcirculation system. During normal physiological Angiogenesis, new vessels rapidly mature and become stable. On the contrary tumour blood vessels fail to become quiescent, enabling the constant growth of new tumour blood vessels. Tumour blood vessels are architecturally different from their normal counterparts; they are irregularly shaped, dilated, tortuous and they can have dead ends. They are not organized into definitive venules, arterioles and capillaries but rather share chaotic features of all of them. The vascular network is often leaky and haemorrhagic, partly due to the over production of VEGF and to the fact that peri-vascular cells often become more loosely associated or less abundant (Bergers & Benjamin, 2003).

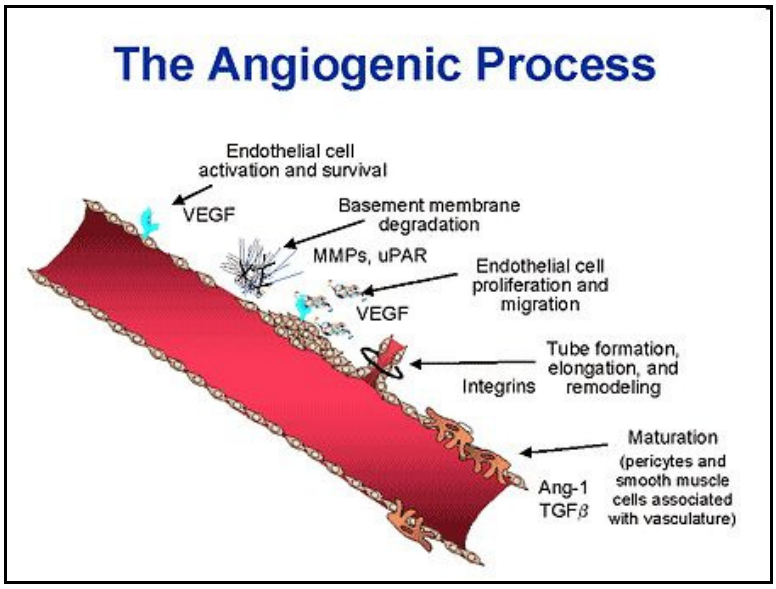


Figure I. The Angiogenic Process. Quiescent endothelial cells are activated and vascular permeability is increased; this first step is VEGF-dependent. Endothelial cells produce and release proteinases responsible for the degradation of the extracellular matrix. Matrix metalloproteinases and urokinase plasminogen activator receptor (uPAR) are involved in this phase. Endothelial cells proliferate and migrate forming a primitive tube. This primitive tube is then surrounded by perycites and a mature blood vessel is formed. Angiopoietin-1 (Ang-1) and Trasforming growth factor β (TGFβ are responsible for this late phase of Angiogenesis. By http://cme.medscape.com/viewarticle/461038

Tumour development is characterized by two phases: a prevascular phase and a vascular phase. The transition from the prevascular to the vascular phase is referred to as 'angiogenic switch'. During the prevascular phase it is visible an initial increase in tumour growth, in which the rate of tumour cell proliferation is balanced by an equivalent rate of cell death (apoptosis). On the contrary, during the vascular phase, tumour is characterized by a rapid growth, tissue invasion and haematogenous spread caused by a decreased in the rate of tumour cell apoptosis (Pepper, 1997). 'Angiogenic switch' is a finely controlled process, where the angiogenic phenotype depends on a local change in the balance between angiogenic stimulators and inhibitors (Hoeben et al, 2004). Among the factors which positively regulate Angiogenesis are FGF and VEGF, while among Angiogenesis inhibitors are angiostatin (an internal fragment of plasminogen), endostatin and antithrombin III (Figure II).

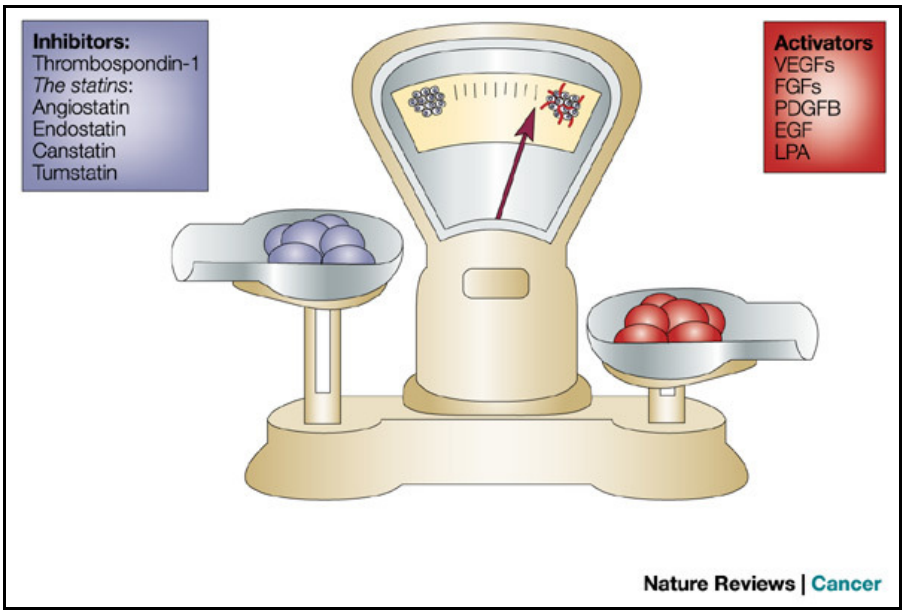


Figure II. The angiogenic balance. Angiogenesis is orchestrated by a variety of activators and inhibitors. Activators of endothelial cells are mainly receptor tyrosine kinase ligands, such as VEGF, FGF, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Among angiogenic inhibitors are thrombospondin-1 and many molecules referred to as 'statins' (Hoeben et al, 2004).

Receptor tyrosine kinases in the vascular system: VEGFR, Tie2 and ephrin receptor

The VEGF/VEGFR family

One of the most important proangiogenic factors is vascular endothelial growth factor (VEGF). The family of VEGFs comprises seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-D and Placental growth factor (PIGF) and they show differential interaction with three related receptor tyrosine kinases: fms-like tyrosine kinase (Flt-1/VEGFR-1), the kinase domain region (KDR) also referred to as fetal liver kinase Flk-1/KDR/VEGFR-2 and VEGFR-3/Flt-4. Neuropilin-1 and -2 can be involved in these receptors complexes as coreceptors (Hoeben et al, 2004).
VEGFR-1 and VEGFR-2 are principally expressed on the vascular endothelium (de Vries et al, 1992; Matthews et al, 1991; Shibuya et al, 1990; Terman et al, 1992) and they are up-regulated during Angiogenesis, accounting for a specific action of the relative growth factors in the vasculature system; while VEGFR-3 is mainly confined to lymphatic endothelial cells (Kukk et al, 1996; Lymboussaki et al, 1998). Neuropilin-1 binds VEGF-A and when coexpressed with VEGFR-2 it enhances the binding of VEGF-A to VEGFR-2 (Soker et al, 2002) and chemotaxis of Endothelial Cells (ECs). *In vitro* VEGF-A and its receptor VEGFR-2 have the ability to induce ECs proliferation, migration and sprouting and to promote ECs to form tubule-like structures. *In vivo*, VEGF-A and VEGFR-2 are indispensable for the earliest steps of Vasculogenesis, as their absence compromise blood-island, ECs and vessel tube formation (Shalaby et al, 1995). Even loss of a single VEGF-A allele results in embryonic lethality. Embryos heterozygous for VEGF-A allele allow embryos analysis in later stages of Vasculogenesis showing VEGF-A involvement also in sprouting and maintenance of vessel survival (Carmeliet et al, 1996; Ferrara et al, 1996). During Vasculogenesis, VEGFR-1 modulates ECs division, as a matter of fact embryos and differentiated ECs lacking VEGFR-1 (VEGFR-$1^{-/-}$) show increased vascularisation and ECs number (Fong et al, 1995). VEGFR-1 has the highest affinity for VEGF-A; it could be involved in down-regulating VEGF activity, dampening the angiogenic effects of VEGFR-2 and ensuring the generation of a correct ECs number (Zeng et al, 2001).

The Angiopoietin/Tie family

A second family of growth factors specific for vascular endothelium is the one constituted by Angiopoietins (Davis et al, 1996; Maisonpierre et al, 1997; Suri et al, 1996; Valenzuela et al, 1999). The Angiopoietin receptors, Tie1 and Tie2, are expressed not only on vascular endothelium (Anghelina et al, 2005; Runting et al, 1993; Schnurch & Risau, 1993) but also in non endothelial cells, including carcinoma cells, mesenchymal cells, eosinophils etc.(Feistritzer et al, 2004; Metheny-Barlow et al, 2004; Nakayama et al, 2005). The four known Angiopoietins (Ang-1, Ang-2, Ang-3 and Ang-4) are specific ligands for Tie2, acting as agonists and/or antagonists. While a specific ligand for Tie1 has been not found yet, however some studies have showed that Ang-1 and Ang-4 can phosphorylate Tie1 (Saharinen et al, 2005). *In vitro* studies have shown that Ang-1 does not induce proliferation and tubule formation, although it can promote sprouting (Davis et al, 1996; Koblizek et al, 1998) and has an anti-apoptotic effect (Takahara et al, 2004). *In vivo* studies reveal that mouse embryos lacking Ang-1 or Tie2 have no problem in the early stages of vascular development but remodelling and stabilization of this primitive vasculature is compromised and lead to embryonic death in murine model between E9.5 and E12.5 days (Dumont et al, 1994; Sato et al, 1995; Suri et al, 1996). Vessels of mouse embryos lacking Ang-1 or Tie2 show poor

association between ECs and supporting cells as smooth muscle cells (SMCs). ECs appear rounded and detached from the underlying cells and matrix, the vessels fail to undergo remodelling events as maturation and regression. Great defects are also visible in heart development. Ventricles myocardial projections, known as trabeculations, fail to form and endocardium appears detached from the underlying myocardium. Tie2 is constitutive expressed in the adult endothelium and Ang-1/Tie2 signalling is essential in the maintenance of vascular integrity (Niu et al, 2004; Wong et al, 1997). On the contrary Ang-2 binds but does not activate Tie2, resulting an antagonist of Ang-1 activity. Overexpression of Ang-2 during embryogenesis leads to embryos defects similar to the ones seen in embryos lacking Ang-1 or Tie2, accounting for an antagonist effect of this growth factor. Contrary to Ang-1, which is widely expressed in normal adult vasculature, Ang-2 is highly expressed only at sites of vascular remodelling, where it promotes vascular regression or Angiogenesis depending on the absence or presence of VEGF respectively (Hanahan, 1997; Maisonpierre et al, 1997).

The ephrins/EphR family

Ephrin (Eph) receptors and their corresponding ephrin ligands are identified as the third vascular Receptor Tyrosine Kinase (RTK) system (Pfaff et al, 2006). Originally identified as neuronal guidance molecules, Eph receptors and ephrin ligands have a role also in vascular morphogenesis processes (Palmer & Klein, 2003; Pfaff et al, 2006). Eph receptors are divided into two subgroups: class receptors A, consisting of ten receptors and class receptors B, consisting of six receptors. The ephrin ligands are correspondingly divided into a subgroup A (six members) and a subgroup B (three elements). Ephrin ligands remain bound to the membrane of the cell in which are produced; in the subgroup A ephrins are anchored to the membrane through glycosylphoshotidylinositol (GPI), while in the subgroup B ephrins are transmembrane molecules with a short cytoplasmic domain. B class Eph receptors and B class ephrin ligands have been identified as regulators of vascular morphogenesis. EphrinB2 knock out mice revealed that this ligand is expressed in arterial endothelial cells at the early stage of vascular development, while the corresponding EphB4 receptor marks only the venous endothelium (Wang et al, 1998). The embryos lacking ephrinB2 present defects in the vascular remodelling, subsequent to the initial stages of Vasculogenesis, in both arterial and venous regions. It has been suggested that these defects are due to the lacking of bidirectional signalling between arterial and venous endothelium, normally mediated by ephrinB2 and EphB4. The early proper differentiation between arterial and vascular endothelial cells seems to be a critical step for the correct vasculature development. In addiction to these defects ephrinB2 knock out mice also present defects similar to the ones seen in Ang-1 or Tie2 knock out mice: the endothelium is poorly associated to periendothelial cells in the yolk sac, venous vessels appear dilated and unorganized and heart trabeculations present abnormalities. Others B class EphR and ligands are expressed in the developing vasculature and in vessel surrounding tissues at sites of sprouting Angiogenesis, suggesting a role of Eph-ephrin system in mediating interactions between endothelium and surrounding cells (Adams et al, 1999).

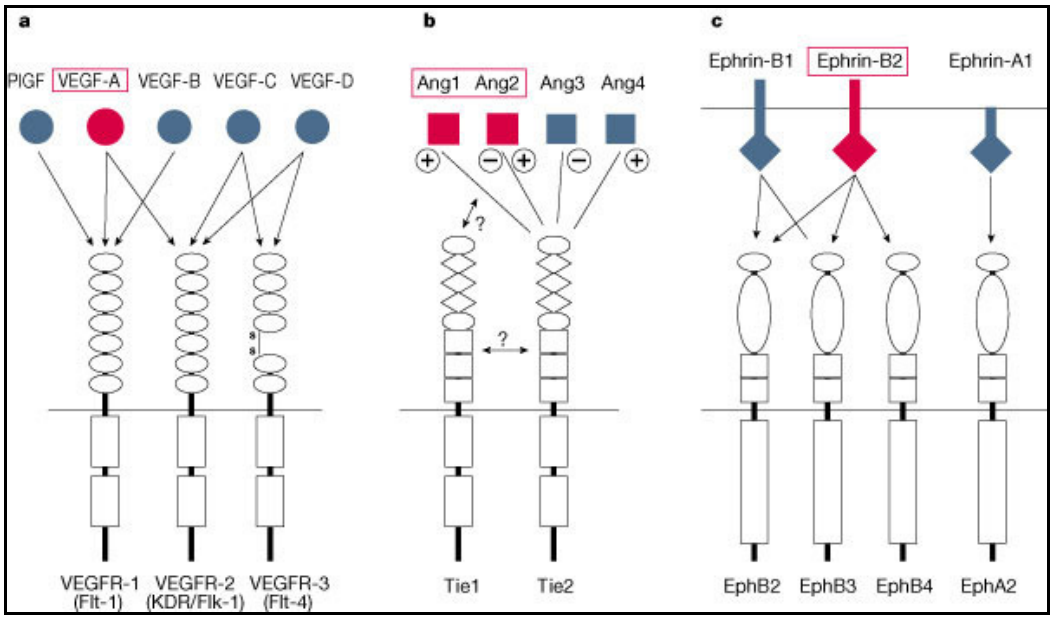


Figure III. **RTK in vascular system.** VEGF ligands and their receptors, Ang ligands and Tie receptors and ephrin ligands with Eph receptors are represented in this scheme. The four growth factors highlighted in red (VEGF-A, Ang-1, Ang-2 and ephrinB2) are the more important and studied in vascular endothelium (Yancopoulos et al, 2000).

The Angiopoietin family

The Angiopoietins have been discovered in the '90s as a family of growth factors essential for vascular system. There are four Angiopoietins: Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), Angiopoietin-3 (Ang-3) and Angiopoietin-4 (Ang-4). Ang-1 and Ang-2 are the best characterized, while Ang-3 and Ang-4 are less characterized and found in mouse and human respectively (Davis et al, 1996; Kim et al, 1999; Valenzuela et al, 1999). The Angiopoietins are ligands for the vascular RTK Tie2. Structurally, Angiopoietins contain an amino-terminal domain, a coiled-coil domain, a linker peptide and a carboxy-terminal fibrinogen homology domain. The fibrinogen homology domain is responsible for receptor binding, while coiled-coil domain and the amino terminal domain are respectively responsible for dimerization and formation of high multimeric forms of these growth factors (Figure IV).

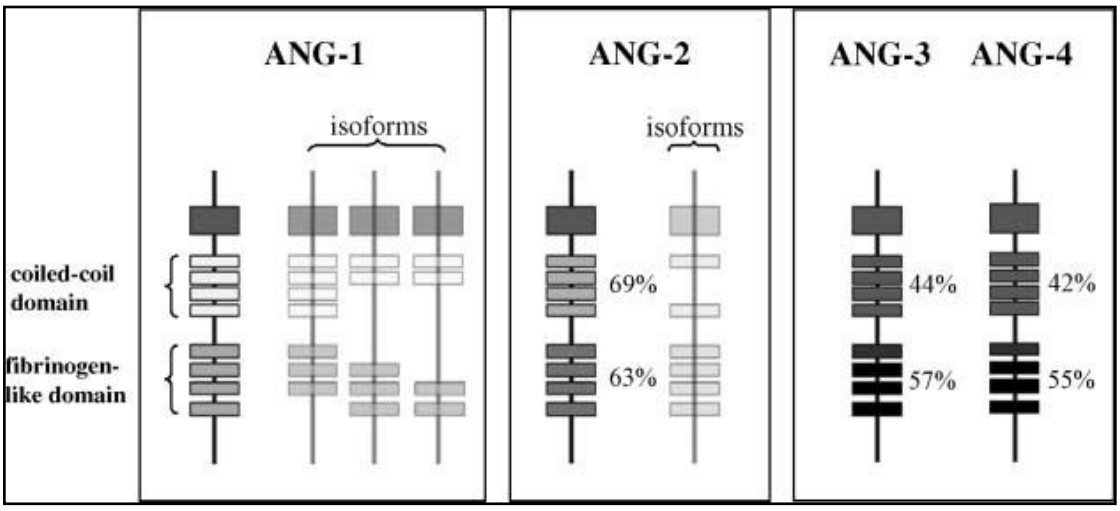


Figure IV. Structure of Angiopoietin ligands. Ang-1, the main agonist ligand of Tie2 receptor, has four isoforms with different activities on Tie2. Ang-2 is an antagonist of the Tie2 pathway and it is present in two isoforms. Ang-3 and Ang-4 are orthologues in mouse and human, respectively, and they specifically activate Tie2. The percentages indicate the homology with the different domains of Ang-1 (Shim et al, 2007).

Angiopoietin-1

Ang-1, the Tie2 agonist ligand, is secreted as a monomeric glycoprotein with a molecular weight around 70 kDa, but it can activate Tie2 receptor only as a multimer. The short amino terminal domain is responsible for high order oligomer formation allowing Ang-1 to exist as heterogenous multimers formed by trimeric, tetrameric and pentameric oligomers (Kim et al, 2005; Procopio et al, 1999). Ang-1 is principally expressed by mesenchymal cells and acts in a paracrine way on endothelial cells. It is also expressed in myocardium during development, in adult tissue, in some tumours cells and in neuronal cells (Gale et al, 2002; Maisonpierre et al, 1997; Stratmann et al, 1998; Suri et al, 1996; Zagzag et al, 1999). Different stimuli, as IL-1β, TNF-α, TGF-β, IL-10, VEGF and hypoxia, are able to regulate Ang-1 expression in different cell type (Hangai et al, 2001; Makinde et al, 2006; Park et al, 2003; Scott et al, 2002). After secretion Ang-1 is found joined to extracellular matrix (Xu & Yu, 2001). Ang-1 deficient mice die at E11-E12.5. These mice have a retard in the heart development, endocardium is collapsed and detached from underlying myocardium and ventricular

trabeculae are absent. Ang-1 deficient mice also show vascular defects, characterized by an immature primary capillary plexus: endothelial cells appear rounded and poorly associated to reduced periendothelial cells (Suri et al, 1996).

Angiopoietin-2

Angiopoietin-2 acts as an antagonist of Tie2 receptor (Maisonpierre et al, 1997), but in some case has been reported to induce receptor phosphorylation. It is no clear yet which molecular basis is responsible for agonist/antagonist function of Ang-2 but the different outcome seems to be context-dependent (Daly et al, 2006; Kim et al, 2000b). Ang-2 is almost exclusively expressed by endothelial cells and it is stored in Weibel-Palade bodies (WPB). Some cytokines, such as Histamine and Trombin, can induce the release of WPB contents and consequently Ang-2 (Fiedler et al, 2004). Ang-2 acts in an autocrine way on Tie2 by binding as homodimers or multimers (Procopio et al, 1999).
It has been found upregulated in hypoxia condition (Oh et al, 1999; Pichiule et al, 2004) and in physiological condition it is expressed in site of vascular remodelling: in site of active Angiogenesis as in vascularization of retina and in site of vessels regression as in corpus luteum (Goede et al, 1998; Maisonpierre et al, 1997). In pathological condition Ang-2 is overexpressed in the endothelium of tumour and in cell tumour themselves (Torimura et al, 2004; Zagzag et al, 1999). Transgenic overexpression of Ang-2 in mice shows a phenotype similar to the one observed in mice lacking Ang-1 or Tie2, supporting the hypothesis that Ang-2 acts as an antagonist of Ang-1/Tie2 signalling (Maisonpierre et al, 1997). Ang-2 deficient mice show different phenotype strain-dependent. Ang-2 deficient mice in 129/J background die 14 days after birth (Gale et al, 2002), while Ang-2 deficient mice in C57/BL6 background are viable, they have few vascular defects (hyaloid vessels in eye lens do not regress after birth, supporting the thesis of Ang-2 involved in remodelling vessels) and develop problem in the lymphatic system as a severe chylous ascites (Fiedler et al, 2006; Shimoda et al, 2007). The genetic Ang-1 knock in in Ang-2 locus rescues the phenotype of Ang-2 in the lympathic system, but not in the vascular remodelling defects; for this reason Ang-2 appears to be agonist ligand in the lympathic system but antagonist in the vessel one (Gale et al, 2002). Ang-2 is highly expressed during inflammation: Ang-2 level increases from a basal level of 1-2 ng/ml up to 30 ng/ml during sepsis and it also been found upregulated during neovascularization that occurs in arthritis and psoriasis (Fearon et al, 2003; Kuroda et al, 2001; Parikh et al, 2006). Ang-2 is associated to VEGF expression in the sites of vessels remodelling, together they induce Angiogenesis and matrix metalloproteases release (Etoh et al, 2001), on the contrary Ang-2 in the absence of VEGF induces vessel regression (Holash et al, 1999).

Angiopoietin-3 and Angiopoietin-4

Ang-3 and Ang-4 were found by homology cloning in virtue of the primary structure similarities to Ang-1 and Ang-2 and for their ability to bind Tie2. Mouse Ang-3 and human Ang-4 are orthologues and until now poor characterized (Valenzuela et al, 1999). Ang-3 is found tethered on the cell surface via heparin sulphate proteoglycans, it is expressed at low levels in many tissues and it can phosphorylate Tie2 in mouse endothelial cells but not in human ones. Ang-4 is highly transcripted only in lung tissue and it induces Tie2 phosphorylation in human endothelial cells (Lee et al, 2004)

Tie receptors

Structure and expression of Tie receptors

Tyrosine kinase with Ig and EGF homology domain (Tie) receptors, representing the second specific ECs surface receptors identified, are tyrosine kinase receptors involved in angiogenic processes and they are almost exclusively expressed on ECs. Tie1 and Tie2 are single membrane receptors composed by an amino-terminal extracellular region and by an intracellular region. The amino-terminal region presents: two immunoglobulin-like (Ig) domains, three endothelial growth factor (EGF)-like repeats, another Ig domain and three fibronectin type III repeats. Crystal analysis shows that the three Ig domains and the three EGF domains interact to form an arrowhead shape structure (Barton et al, 2006) and that the second Ig domain seems to be the one responsible for Ang-1 and Ang-2 binding (Fiedler et al, 2003). The intracellular region is formed by a split tyrosine kinase domain (Schnurch & Risau, 1993) (Figure V).
Tie1 and Tie2 are found co-expressed in vascular and lympathic ECs. Tie2 is constitutively expressed while Tie1 transcription seems to be subjected to regulation, as a matter of fact Tie1 mRNA is highly expressed in some cancer progression and in sites of turbulent blood flow (Kaipainen et al, 1994; Porat et al, 2004).
Tie receptors have also been found in haematopoietic cells (megakaryocytes and haematopoietic stem cells in the bone marrow), in some monocytes related to cancer known as Tie2-expressing monocytes (TEMs) and in some types of tumour cells (De Palma et al, 2005; De Palma et al, 2003).

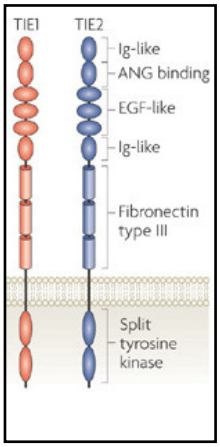


Figure V. Structure of Tie receptors. Tie receptors are single membrane receptor tyrosine kinases. The extracellular portion comprises two immunoglobulin –like (Ig) domains, followed by three endothelial growth factor (EGF)-like repeats, another Ig domain and three fibronectin type III repeats. The second Ig domain is responsible for the ligand binding. The intracellular portion is composed of a split tyrosine kinase domain. Tie1 and Tie2 share an homology of 76% in the intracellular portion and 33% of homology in their extracellular portion (Augustin et al, 2009)

Tie2 activation

The binding of Ang-1 to Tie2 leads to receptor dimerization and activation. Following dimerization the tyrosine residues present on the kinase domain of the intracellular region become phosphorylated and the adaptor proteins bind to them initiating different signal cascades. Growth factor receptor-bound protein 2 (Grb2), Grb7, Grb14, Src homology-2 domain-containing phosphatase (SHP2) and p85, which is the regulatory subunit of phosho-inositide 3-kinase (PI3K), are well known adaptor proteins which interact with Tie2 via Src homology-2 (SH2) domain (Augustin et al, 2009).
Ang-1/Tie2 signalling can induce survival in resting ECs by activating PI3K/Akt pathway but it may induce also migration in isolated non-resting ECs by activating mitogen-activated protein kinase (MAPK). In the first case Ang-1 induces the translocation of Tie2 to cell-cell junctions and the formation of in trans Tie2 complexes between neighbouring cells. These phenomena lead to stable association between Tie2 and vascular endothelial-phosphotyrosine phosphatase (VE-PTP) resulting in inhibition of permeability, additionally Akt is activated leading to survival and maturation of endothelium via endothelial Nitric Oxide Synthase (eNOS). In isolated migrating endothelial cells Ang-1 induces the translocation of Tie2 at the rear side of ECs resulting in migration via the Downstream of tyrosine kinase/ docking protein (Dok)-R (Fukuhara et al, 2008; Saharinen et al, 2008).

ECs survival in quiescent vasculature

Tie2 activation leads to the recruitment of Grb2 and p85, both of which are reported to interact with Tie2 at pTyr-1101 multisubstrate docking site (Jones et al, 1999; Kontos et al, 1998). Phosphorylation of p85, as subunit of PI3K, induces Akt activation which promotes the survival pathway through the activation of eNOS and survivin and through the suppression of proteins involved in apoptosis such as caspase-9 and BAD (DeBusk et al, 2004; Papapetropoulos et al, 2000). Moreover Akt phosphorylates the forkhead transcription factor (FOXO) 1; the phosphorylated form of FOXO1 fails to enter in the nucleus resulting in the inhibition of its transcription factor activity. FOXO1 induces Ang-2 transcription, consequently Ang-1/Tie2 signalling inhibits FOXO1-mediated Ang-2 production (Tsigkos et al, 2006) (Figure VI).

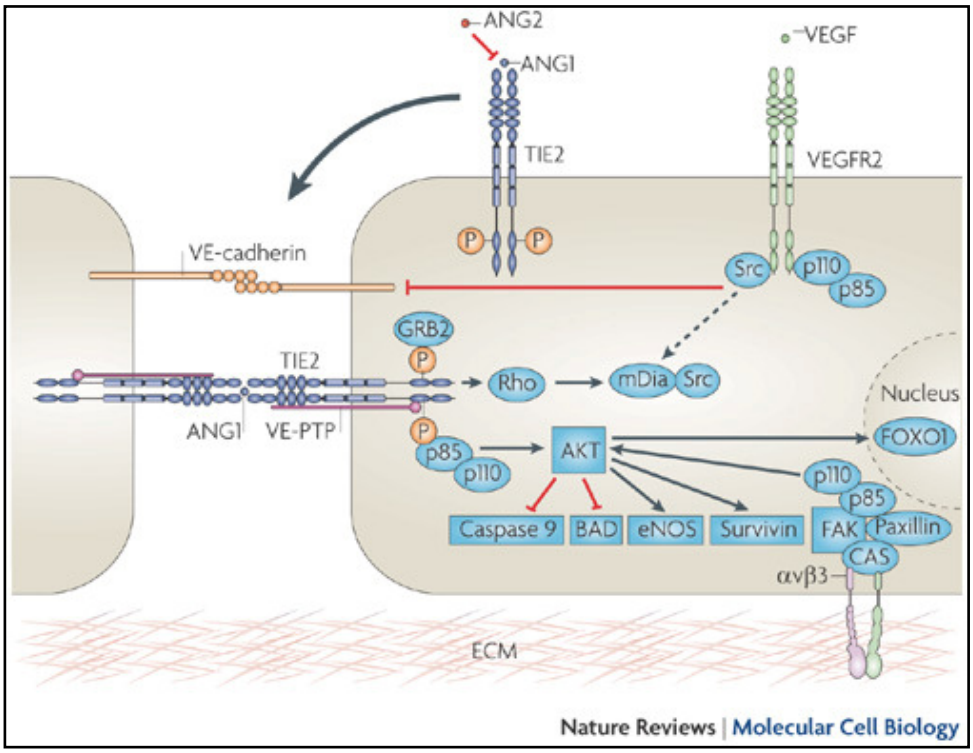


Figure VI. **Ang-1/Tie2 signal in endothelial cell quiescence.** Ang-1-bound Tie2 establishes trans complexes with Tie2 expressed on neighbouring cells. This complex leads to PI3K activation (PI3K consists of two subunits: a regulatory one p85 and a catalytic one p110) which in turn activates Akt. Akt drives an anti-apoptotic effect through activation of eNOS and survivin and inhibition of caspase-9 and BAD. Vessels integrity is maintained through sequestration of Src which is known to induce VE-cadherin internalization and subsequent vessel leakage (Augustin et al, 2009)**.**

ECs activation and migration in non-resting vasculature

PI3K phosphorylation not only leads to survival through Akt, but it may also induce sprouting and migration in non resting endothelium (DeBusk et al, 2004; Jones et al, 1999; Kim et al, 2000a) like it does on ECs activated by others cytokines (Graupera et al, 2008; Kundra et al, 1994). Ang-1 induces focal adhesion kinase (FAK) activation via PI3K and secretion of proteases. FAK phosphorylation and subsequent activation of paxillin are involved in cytoskeletal reorganization while secretions of proteases in degrading extracellular matrix; these processes are necessary for cell sprouting (Kim et al, 2000a). Ang-1/Tie2 signalling may drive ECs migration also through Dok-R. Upon Ang-1 stimulation, Dok-R interacts with Tie2 at pTyr-1107 through its phosphotyrosine binding domain (PTB) and results in tyrosine phosphorylation itself.
Phospo-Dok-R recruits rasGAP, Nck and p21-activating kinase (PAK) which increase cell motility (Jones & Dumont, 1998; Master et al, 2001).
Contrasting data on the role of mitogen-actived protein kinase (MAPK) in ECs migration are emerged: while in rat aortic ring explants MAPK inhibition attenuated Ang1-mediated angiogenesis (Zhu et al, 2002), in cell culture it had no effect on Ang1-mediated endothelial cells survival and migration (Fujikawa et al, 1999) (Figure VII a).

ECs permeability

Ang-1/Tie2 signalling acts on resting endothelium to limit permeability, consequently it counteracts VEGF/VEGFR-2 signal which causes vessel leakage. Both the signals regulate permeability through the distribution of vascular endothelial (VE) cadherin in adherens junctions. VEGF signal leads to VE cadherin phosphorylation and subsequent internalization from junctional complexes through the activity of the non receptor tyrosine kinase Src. On the contrary, Ang-1 signal leads to Src sequestration through the activity of RhoA, a small GTPase, and mammalian diaphanous (mDia) thus inhibiting VE cadherin phosphorylation and subsequent internalization from junctional structure (Gavard 2008). Additionally in confluent endothelium the trans association between Tie2 in neighbouring cells allows the interaction between Tie2 and VE-PTP, which inhibits paracellular permeability (Saharinen et al, 2008). On the contrary, Ang-2 promotes permeability by inhibiting Src sequestration (Augustin et al, 2009) (Figure. VII b).

Anti-inflammatory activity

Ang-1/Tie2 pathway also has an anti-inflammatory role by protecting ECs from microvascular leakage caused by lipopolysaccharide-induced endotoxic shock (Witzenbichler et al, 2005). Moreover Ang-1 inhibits the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, which are involved in leukocytes adhesion to ECs induced by VEGF (Kim et al, 2001). The Ang-1 mediated activation of PI3K/Akt leads to A20-binding inhibitor of NF-kB 2 (ABIN-2) recruitment which interferes with the pathway of the nuclear factor kB (NF-kB). NF-kB induces the expression of adhesion molecules responsible for leukocytes adhesion on endothelium (Hughes et al, 2003; Jeon et al, 2003) (Figure VII c)

ECs maturation

Quiescence and maturation of endothelium require tight association between ECs and periendothelial cells. Tie2 seems to be responsible for this association but the precise mechanism is still unknown. Ang-1/Tie2 signalling induces ECs production of heparin-binding epidermal-like growth factor (HB-EGF), hepatocyte growth factor (HGF) and serotonin which induce SMCs recruitment in a paracrine way (Iivanainen et al, 2003; Kobayashi et al, 2006; Sullivan et al, 2003). Platelet derived growth factor (PDGF) is produced by ECs and promotes proliferation of SMCs and pericytes during their recruitment to endothelium (Hellstrom et al, 1999). In the mouse retina vasculature, the block of pericyte recruitment by an anti-PDGF receptor β (PDGFRβ) antibody is rescued by Ang-1 injection. For this reason the activity of Ang-1 in pericyte recruitment seems to act independently from PDGF signal (Uemura et al, 2002) (Figure VII d).

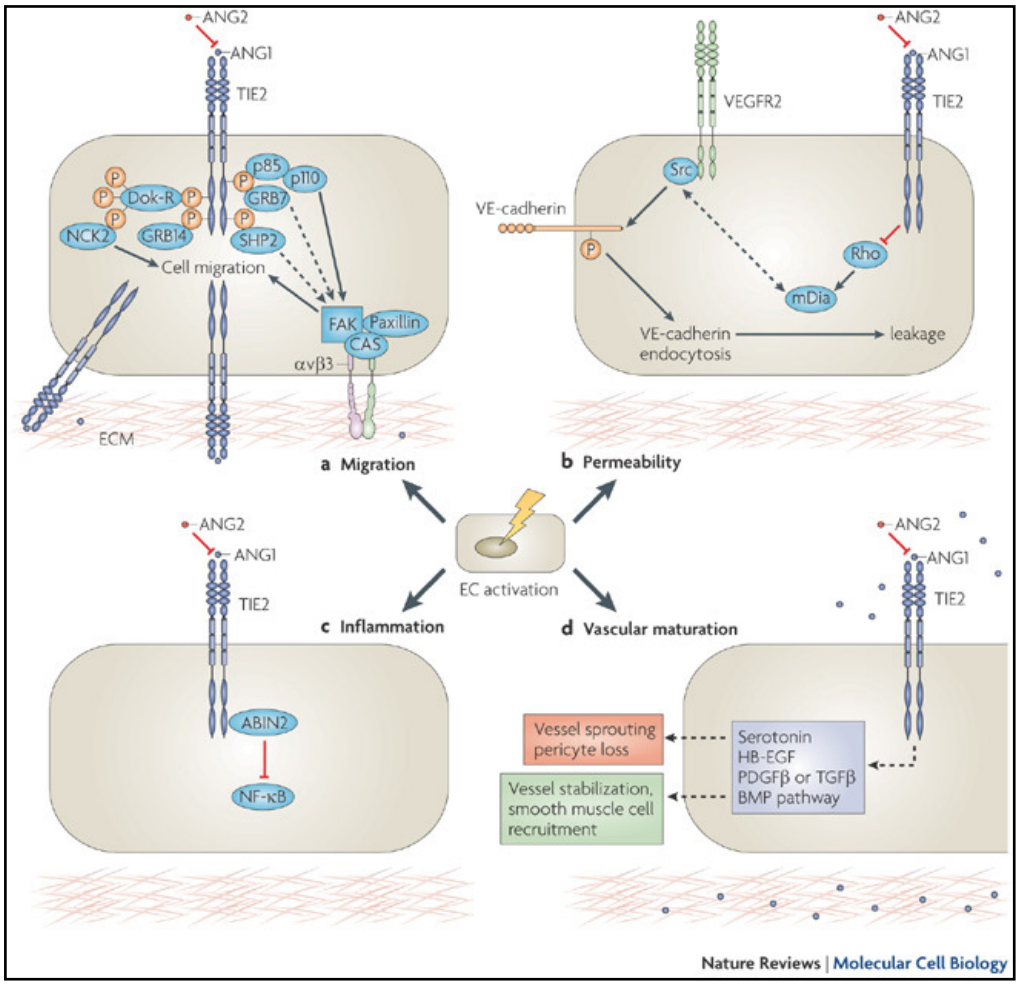


Figure VII. **Ang1/Tie2 signal in endothelial cell activation. a** EC migration is activated by PI3K through FAK phosphorylation or by Pi3K-independent Dok-R recruitment. **b** In EC the anti-permeability effect is mediated by sequestration of Src through the recruitment of mDia and subsequent inhibition of VE cadherin internalization. **c** EC inflammation is inhibited by the recruitment of ABIN-2 which blocks the NF-kB-mediated expression of adhesion molecules involved in leukocytes adhesion on endothelium. **d** Ang-1/Tie2 signalling mediates vascular maturation through the secretion of HB-EGF, HGF and serotonin which recruit SMCs and pericytes (Augustin et al, 2009; Jones et al, 1999)

Ang-1/Tie2 signalling in physiological condition

The embryonic capillary plexus begins expansion through Angiogenesis and undergoes remodelling and maturation processes between E9.5 and E12.5. These processes comprise the recruitment of pericytes, periendothelial cells, which enwrap capillary ECs and of SMCs which surround ECs in arteries, arterioles, venules and veins (Augustin et al, 2009). Ang-1/Tie2 signalling attends at these processes, controlling sprouting Angiogenesis, vascular remodelling and transition of ECs from quiescent to active state. Conversely Tie2-/- and Ang-1 -/- mice die between E10.5 and E12.5 due to hemorrhage as a consequence of reduction in the number of ECs, failure of the primary vascular plexus to branch and differentiate, reduction of pericytes and SMCs coverage around ECs, decreasing haematopoiesis and severe heart defects (Dumont et al, 1994; Patan, 1998; Sato et al, 1995; Takakura et al, 1998).

Tie2 is also found expressed in adult vascular tissues undergoing Angiogenesis as in the neovessels of corpus luteum and it has been found overexpressed during active Angiogenesis of skin wounds, but it was also found expressed in its active phosphorylated form in the mature endothelium of all adult tissues, suggesting a role of Tie2 in the maintenance of quiescent adult vasculature (Wong et al, 1997).

Moreover Ang-1/Tie2 signalling also has a non vascular function since it is required during postnatal bone marrow haematopoiesis but dispensable for embryonic one (Arai et al, 2004; Puri & Bernstein, 2003; Takakura et al, 1998). Ang-1/Tie2 system plays a role in maintaining haematopoitic stem cells in quiescent state in the bone marrow, indeed Tie2 expressing haematopoitic stem cells adhere to Ang-1 expressing osteoblast.

Besides Tie2 is expressed in some type of neuronal cells and its activation induces their survival and mitosis (Kosacka et al, 2005; Valable et al, 2003).

Ang-1/Tie2 signalling during pathology

Recently a large body of evidences has pointed out a role for Ang-1/Tie2 signalling in pathological processes.
For example, TNFα stimulation fails to induce a rapid inflammation in Ang-2 -/- mice knock-out (Fiedler et al, 2006). Inflammatory leukocytes recruitment on capillary endothelium is a multistep sequence and the absence of Ang-2 seems to affect the last part of this process: the firm adhesion and the transmigration of leukocytes. Ang-2 itself does not induce transcription of pro-inflammatory gene but it can potentiate the pro-inflammatory gene transcription induced by TNFα.
Furthermore, Angiopoietins/Tie2 system is involved in tumour Angiogenesis. In the first step of growth, primary tumour utilizes pre-existing host blood vessels, leading to ECs activation and release of Ang-2. Consequently to Ang-2 release host blood vessels around tumour regress and the resulting tumour hypoxia induces VEGF-mediated Angiogenesis. Conversely Ang-2 is found substantially up-expressed in tumour-associated endothelium and the level of circulating Ang-2 is a marker of tumour progression for different cancer (Augustin et al, 2009; Holash et al, 1999). On the contrary, the role of Ang-1 in tumour Angiogenesis is controversial. Overexpression of Ang-1 inhibits Angiogenesis and tumour growth in some types of cancers, probably by recruitment of mural cells and consequent vessel stabilization (Makinde & Agrawal, 2008; Tian et al, 2002); in other cases Ang-1 blockade leads to inhibition of tumour growth (Beecken et al, 2000; Stoeltzing et al, 2003). Tie2 and Ang-1 are found expressed not only on tumour associated vasculature but also in tumour cells, where their interaction may have an anti-apoptotic effect, as reported for neuroblastoma cells (Kosacka et al, 2005). TEMs are found associated to tumour and their depletion leads to inhibition of tumour Angiogenesis and growth. Hypoxia inside tumour induces Ang-2-mediated TEMs recruitment and this is could be the explication of TEMs clustering around vessels in some kind of tumours (Lewis et al, 2007; Venneri et al, 2007) Also Tie1 has been found in its active form in some cancer cells as breast, thyroid and gastrointestinal tumour cells (Rees et al, 2007).
In rheumatoid arthritis Tie2 and Ang-1 are expressed on synovial lining cells, endothelial cells, SMCs and fibroblasts, whereas in normal art tissue Tie2 expression is limited to the capillary endothelium (Shahrara et al, 2002). Ang-1/Tie2 signalling has a role in rheumatoid arthritis progression and arthritic joint destruction. Ang-1 and matrix metalloproteinases 3 (MMP3) are found highly expressed at the invasive front of rheumatoid pannus. Ang-1 induces synovial cells proliferation and survival through Akt and the MAPK Erk pathway and moreover it induces proMMP3 secretion, which is involved in cartilage degradation (Hashiramoto et al, 2007).
Ang-1, Ang-2 and VEGF are found highly expressed in the sputum of asthmatic patients, specifically the level of Ang-2 and VEGF positively correlates with vascular permeability and consequently it may be responsible for inflammatory cells infiltration and SMCs hyperplasia typical of asthma disease. On the contrary Ang-1 inversely correlates with vascular permeability (Kanazawa et al, 2007). The anti-inflammatory function of Ang-1 makes this growth factor a potential therapeutic candidate against asthma. As a matter of fact Ang-1 could rescue the plasma baseline leakage and inhibit the expression of adhesion molecules such VCAM-1 involved in inflammatory cells adhesion on ECs (McDonald, 2001).

Integrin

Integrins structure

Integrins are heterodimeric cell surface glycoproteins not covalently associated; the two molecules are referred as α and β integrin. There are 18 α subunits and 8 β subunits and they associate to form at least 24 different heterodimers. The α subunits are trans-membrane proteins containing 1000-1200 amino acids, while the β subunits are trans-membrane proteins too, but they are shorter, containing 700-800 amino acids. Except for β4 integrin whose cytoplasmic domain contains 1000 amino acids, normally the cytoplamic region of integrins is short, 30-40 amino acids for α subunits and 40-50 for β subunits. The interaction between integrins and extracellular matrix (ECM) is highly promiscuous, different integrins can bind to the same ligand, but in some case it has been demonstrated that different integrins, which binds the same ligand, can trigger different signals. This is the case of integrin α5β1 that, when it binds fibronectin, suppresses cells migration (Giancotti & Ruoslahti, 1990) while integrin αvβ6 when binding fibronectin it promotes migration (Thomas et al, 2001). On the other side a specific integrin can bind more than one ligand; this is the case of integrin αvβ3 that may bind fibronectin, vitronectin, thrombospondin, tenascin-C, etc (Hynes, 1992). This could result in a cell advantage when the signal internalized by the specific integrin is more important than the specific ECM encountered by the cells. Integrins may also mediate cell-cell interaction, such as when they bind to intercellular adhesion molecules (ICAMs) to mediate leukocytes adhesion to endothelium at sites of inflammation or platelet aggregation (McEver, 2001).

Integrin activation

Integrins exist in two forms: an 'inactive' form in which the integrin is not able to adhere to ECM and an 'active' form in which the integrin can bind the ligand, form clusters and associate with cytoskeleton (Woodside et al, 2001). The actual model proposes that α and β integrin interacts to form a heterodimer with a global shape of a large head on two legs. The head is formed by a β–propeller domain of the α subunit and a β–propeller domain of the β subunit. The ligand-binding pocket resides in the interface between the two β–propeller domains. The legs consist of the remaining domains, tightly packed to form straight stalks. The 'inactive' integrin has a bent conformation in which the head and consequently its ligand-binding pocket is very close to the plasma membrane. In this shape, integrin can not bind the ligand. Upon addiction of manganese, which binds the metal ion-dependent adhesion site (MIDAS) in the head region, or addiction of a high affinity ligand, integrins becomes activated and assume a stretch conformation, where ligand-binding pocket is accessible to ligands. 'Inactive' integrin have α and β cytoplasmic domains close to each others; disruption of this interaction leads to integrin activation. Talin binding to β cytoplasmic domain is a proposed mechanism involved in α and β cytoplasmic domains separation. This separation induces stretching of the extracellular domains and consequently active conformation of the head region. This integrin activation is referred to 'inside-out' signalling (Liddington & Ginsberg, 2002; Takagi & Springer, 2002) (Figure VIII). Upon activation integrins form clusters and recruit structural proteins as vinculin and α–actinin, adaptor proteins as Shc and signalling proteins as c-Src, FAK and paxillin. Integrins form focal structures (focal contacts, focal adhesion and fibrillar adhesion) and propagate tensional force between ECM and cytoskeleton (Miranti & Brugge, 2002; Sastry & Burridge, 2000). It has also been reported that growth factors can promote integrin activation. VEGF activates αvβ3 on HUVECs,

increasing adhesion and migration (Byzova et al, 2000). Physiological concentrations of PDGF can activate αvβ3 on oligodendrocytes and it can promote cell survival on vitronectin, a αvβ3 ligand. In absence of PDGF activation of integrin αvβ3 can be induced by cation Mn^{2+} (Baron et al, 2002). Integrin activation independently from the stimulus, seems to be mediated by talin, a cytoskeletal protein whose interaction with the cytoplasmic domain of β subunit activates integrins (Tadokoro et al, 2003).

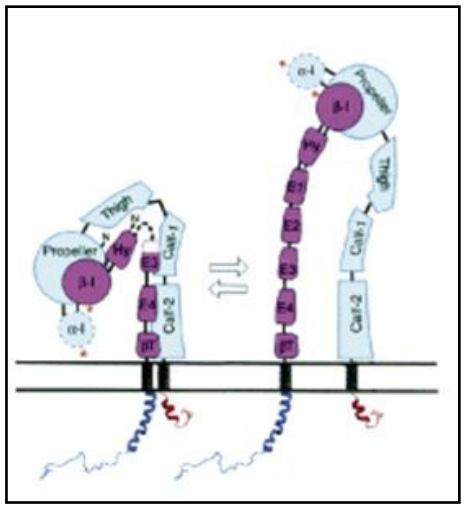


Figure VIII. **Integrin activation.** Integrin transition between 'inactive' form (bent conformation where the ligand-binding pocket is not accessible to the ligand) and 'active' form (stretch conformation where ligand-binding pocket is accessible to ligand and cytoplasmic regions are separated) (McCleverty & Liddington, 2003).

Integrins and Angiogenesis

Endothelial integrin adhesion receptors have emerged as critical mediators and regulators of Angiogenesis and vascular homeostatis. Integrins allow the interaction of endothelial cells with surrounding extracellular matrix necessary for cell adhesion, migration, positioning and signal transduction, events essential for cell survival, proliferation and differentiation which occur during Angiogenesis (Ruegg & Mariotti, 2003). Many works highlight the importance of integrins in embryonic and post-natal Angiogenesis, overall in association with the activity of growth factors receptors.

The combine signal of integrins and growth factor receptors is fundamental in endothelial activation and neovascularization (Serini et al, 2008a; Serini et al, 2008b). The majority of the signalling pathways activated by integrins are also activated by growth factor receptors and consequently an interaction between the two signals lead to an increase in specificity and control over the cell response. In many cases, the signals internalized by growth factor receptors are amplified when ECs are attached to ECM (Somanath et al, 2007).

For example integrin α5β1 has a fundamental role in Angiogenesis. α5β1−deficient mice die early during embryogenesis as a consequence of vascular and cardiac defects, moreover the deposition of fibronectin, ligand of α5β1, on basal membrane is impaired. Embryod bodies formed by embryonic stem cell α5 −/− show an impaired vascular plexus formation (Francis et al, 2002). It has also been reported a stable association between integrin α5β1 and Tie2. Engagement of integrin α5β1 by fibronectin increases integrin interaction with Tie2, resulting in receptor phosphorylation in response to low concentrations of Ang-1 (Cascone et al, 2005). ECs during Angiogenesis adhere on a provisional ECM rich in vitronectin and fibrinogen

through integrin αvβ3 (Byzova et al, 2000; Mahabeleshwar et al, 2007). Ligation of integrin αvβ3 promotes ECs proliferation, survival and motility (Eliceiri et al, 1998; Leavesley et al, 1993; Scatena et al, 1998). Stimulation of ECs with VEGF results in formation of a complex between VEGFR2 and integrin αvβ3. Two β3 cytoplasmic tyrosine residues are required for this association, consequently the mutation of both abolishes this interaction and impairs VEGFR2 activity upon VEGF stimulation (Mahabeleshwar et al, 2006). The collagen receptors, integrins α1β1 and α2β1, are implicated in Angiogenesis too. They result up-regulated by angiogenic growth factors; functional antibodies against them inhibit Angiogenesis in *in vivo* models and mice deficient for integrin α1β1 show impaired tumour Angiogenesis (Hong et al, 2004; Senger et al, 1997; Senger et al, 2002).

Integrins signalling pathways in the vascular system

Integrin signalling is fundamental to regulate Angiogenesis. Endothelial cells express at least 11 different integrins (Karaoz et al, 1996; Korhonen et al, 1990; Sobel et al, 1998). Among them α1β1, α2β1, α3β1, α6β1, α8β1, α9β1, αvβ1 and α6β4 are receptors for collagens and laminins and for this reason they mediate interaction between endothelial cells and basal membrane in mature vasculature. On the contrary α5β1 αvβ3 and αvβ5 are absent from the basal membrane of mature vasculature but they are present in endothelium undergoing Angiogenesis. Integrins have no intrinsic enzymatic activities but they trigger cell response through the recruitment of signalling molecules such as Src family kinases, FAK, PI3K, small GTPases and phosphatases (Giancotti & Ruoslahti, 1999). The main signalling pathways activated in ECs during Angiogenesis involve MAPK, NF-kB, Rho-family GTPases and PKB/Akt.

MAPK. Upon ligand binding, integrins can activate Ras-MAPK signalling leading to MAPKs translocation in the nucleus and consequently regulation of genes involved in cell proliferation, migration and survival. Integrin-mediated MAPK activation may be obtained through two different pathways: 1) FAK autophosphorylation at ^{397}Y associates with Fyn and Yes, belonging to Src-family kinases, which further phosphorylate it on ^{925}Y, allowing the binding of the complex Grb2-Sos and activation of MAPK through Ras (Schaller et al, 1994; Schlaepfer et al, 1994). 2) FAK-independent Fyn and Yes activation leads to Shc-mediated Grb2-Sos complex recruitment (Wary et al, 1996; Wary et al, 1998). The importance of integrins-mediated MAPK activation is proved in Chorion Allantoid Membrane (CAM) model where new Angiogenesis is affected by inhibition of αvβ3 through antagonist or chemical inhibitors (Eliceiri et al, 1998).

NF-kB. NF-kBis a transcription factor which regulates immune response and promotes inflammation in tumours (Karin et al, 2002). It has been reported that binding of osteopontin to integrin αvβ3 leads to NF-kB activation and consequently endothelial cells protection from apoptosis (Scatena et al, 1998).

Rho-family GTPases. Rho, Rac and cdc42 are small GTPases which regulate actin polymerization and turnover, essential processes for the formation of stress fibers, focal adhesions, lamellipodia and filopodia. Integrin ligation activates Rho GTPases: in particular integrin αvβ3 activates cell migration and Angiogenesis through Rac and its effector protein p21 activated kinase 1 (PAK1) (Kiosses et al, 1999). Rac is also involved in integrin αvβ3 recruitment to lamellipodia and consequent formation of focal adhesion at the leading edge (Kiosses et al, 2001) and in endothelial cell proliferation following integrin α5β1 binding to fibronectin (Mettouchi et al, 2001).

PKB/Akt. PKB/Akt is a serine/threonine nonreceptor kinase, activated in a PI3K dependent way and involved in cellular fundamental processes as glucose metabolism, transcription, proliferation, migration and survival.

In ECs many growth factors, such as VEGF and Ang-1, activate Akt but only when integrins are engaged (Fujio & Walsh, 1999); moreover integrin ligation activates directly PKB/Akt through FAK and Integrin-linked kinase (ILK) (Attwell et al, 2000; Sonoda et al, 2000).

MATERIALS AND METHODS

Reagents and Antibodies

Human recombinant Angiopoietin-1 was purchased from R&D Systems (923-AN/CF Minneapolis MN). For protein detection, primary antibodies anti-pSer473 Akt (Rabbit, # 4060), anti-Akt (Rabbit, # 4691), anti-Phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204) (Mouse, #9106), anti p44/42 MAPK (Erk 1/2) (Rabbit, #9102) were from Cell Signaling Technology (Danvers, MA); anti-Tie2/TEK (Mouse, #05-584) was from Upstate (Millipore; Billerica, MA); anti-Vinculin (Goat, # 7649) was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-α-Tubulin (Mouse, # T-5168), anti-α–Actinin (Mouse, A-5044) were from Sigma Aldrich (St. Louis, MO); anti-Phosphotyrosine (Mouse, 05-1050) and anti-human integrin alpha 2 (Rabbit, AB1936) were from Millipore (Billerica, MA); anti-integrin alpha 5 (Rabbit, AB1949) was from Chemicon (Millipore, Billerica, MA).
HRP-conjugated secondary antibodies used were: anti-Rabbit (#211-032-171, Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA), anti-Mouse (#AP124P, Chemicon International, Millipore; Billerica, MA) and anti-Goat (# 2020, Santa Cruz Biotechnology; Santa Cruz, CA). Extracellular matrix proteins used for plate coating: Collagen solution (C-8919), Fibronectin (F0895), Vitronectin (V 8379), Fibrinogen (F3879), Poly-DL-lysine hydrobromide (P9011) were from Sigma Aldrich (St. Louis, MO).

Cells and culture conditions

HUVECs were isolated from umbilical cord vein as previously described (Bussolino et al, 1992). HUVECs were grown on gelatin-coated plastic, in medium M199 (Sigma Aldrich; St. Louis, MO) supplemented with 20% FCS (Gibco, Invitrogen; Paisley, UK), 50 µg/ml Heparin (Sigma Aldrich; St. Louis, MO), 2% penicillin-streptomycin solution (Sigma Aldrich; St. Louis, MO) and 100 µg/ml bovine brain extract. For time course experiments, long- confluent HUVECs were seeded at a density of 20 x 10^3 cells/cm^2 and reached confluence 48 hrs before the experiments with last medium change, forming a high density monolayer with mature cell-to-cell contacts and absence of gaps between cells (Lampugnani et al, 2006). The day before the experiments, sparse cells were seeded at a density of 3,5 x 10^3 cells/cm^2, presenting rare cell contacts at the time of experiments. For experiments on ECM-bound cells, cells were seeded at a density of 20 x 10^3 cells/cm^2 1hr and 30 min before the experiments, presenting rare cell contacts at the time of experiments. HUVEC passages 3-5 were used for the experiments and they were maintained at 37°C and 5% CO_2.
293T and Hela were grown in medium DMEM (Sigma Aldrich; St. Louis, MO) supplemented with 10% FCS (Gibco, Invitrogen; Paisley, UK), 1% L-Glutamine (Sigma Aldrich; St. Louis, MO) and 2% penicillin-streptomycin solution (Sigma Aldrich; St. Louis, MO).

Time-course assay

The day of the experiment, HUVECs were first starved in M199 supplemented with 0,5 % BSA for 3 hrs and then they were properly stimulated with Ang-1. For time-course experiments, ECs were stimulated at different time points (0', 10', 20', 30') with 100 ng/ml of Ang-1 as indicated. At the end of stimulation, cells were properly processed for subsequent analysis.

Ang-1 stimulation of HUVECs upon ECM binding

To stimulate HUVECs with Ang-1, collagen type I (5 μg/mL)-, fibronectin (5 μg/mL)-, vitronectin (5 μg/mL)-, fibrinogen (10 μg/mL)-, gelatine (5 μg/mL)- and poly-DL-lysine (50 ng/mL)-coated dishes were used. HUVECs were starved in M199 supplemented with 0,5% BSA for 3 hrs, then they were detached from the culture dish and resuspended in M199 supplemented with 0,5% BSA. The cells were placed on the extracellular matrix-coated dishes for 1 hrs and 30' and then stimulated for 10' with 20 or 100 ng/mL of Ang-1. At the end of stimulation, cells were properly processed for subsequent analysis.

Western blot analysis

For whole cell lysates, after appropriate treatment, HUVECs were washed twice with cold PBS (Sigma Aldrich; St. Louis, MO) and proteins were extracted with a buffer containing 0,5 M Tris-HCl pH 6,8, 5% SDS, 20% glycerol and quantified by BCA assay (Pierce; Rockford, IL). Equal amounts of each sample were resolved by SDS PAGE and transferred to nitrocellulose membrane Hybond-C Extra (GE Healthcare; Little Chalfont, UK). Membranes were then saturated with TBS 10% BSA and incubated with specific primary antibodies and proper HRP-conjugated secondary antibodies. Immunocomplexes were then visualized by enhanced chemiluminescence (ECL) system (Perkin Elmer; Waltham, MA). The immunoreactive bands were visualized and quantified using a ChemiDoc XRS charge-coupled device (CCD) camera (Bio-Rad Laboratories; Hercules, CA) and Quantity One software.

Immunoprecipitation

Cells were washed with cold PBS and lysed in buffer with added protease and phosphatase inhibitors (50 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100 μM $ZnCl_2$, 1 mM Na_3VO_4). To immunoprecipitate Tie2, a buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 10% glycerol was used. Lysates were incubated with protein G-Sepharose and Ab indicated for 2 hrs at 4°C. After washes, immunoprecipitates were resolved by SDS Page and transferred to nitrocellulose membrane Hybond-C Extra (GE Healthcare; Little Chalfont, UK). Membranes were then saturated with TBS 10% BSA and incubated with specific primary antibodies and proper HRP-conjugated secondary antibodies. Immunocomplexes were then visualized by enhanced chemiluminescence (ECL) system (Perkin Elmer; Waltham, MA). The immunoreactive bands were visualized and quantified using a ChemiDoc XRS charge-coupled device (CCD) camera (Bio-Rad Laboratories; Hercules, CA) and Quantity One software.

Lentiviral-mediated shRNA knock-down.

Five different shRNA MISSION RNA interference vectors were obtained for each target gene from Sigma. Lentiviral particles were produced by transfecting 293T cells with hairpin-pLKO.1 vector, second generation packaging plasmid and envelope plasmid. Viral titre was calculated by infecting HeLa cells with increasing volumes of the virus preparations and by counting viable cells after puromycin selection. The optimal multiplicity of infection (MOI) for HUVECs was established at 2 MOI/cell. Cells were infected with lentiviral shRNAs at the onset of culture and afterward positively selected with 2.5 μg/ml puromycin. To measure silencing efficiency, the level expression of the target proteins were analyzed by Western blot analysis 7 days post infection and compared to the expression levels measured in cells transduced with lentiviruses carrying MISSION Non-Target shRNA control vector.

Adhesion assay

HUVECs were starved in M199 supplemented with 0,5% BSA for 3 hrs. 150 cell/μl were plated in absence or presence of 100 ng/mL Ang-1 on 96-well microtiter plates, incubated for 30' at 37°C, washed two times with PBS, fixed in 3.7% glutaraldehyde and stained with 0.1% crystal violet. The absorbance was read at 595 nm in a spectrophotometer for microtiter plate (Synergy HT, Biotek).

RESULTS

Extracellular matrix and cell-to-cell contact modulate angiopoietin-1 signalling

During vascular development Ang-1 maintains the vascular integrity exerting an anti-apoptotic effect on mature vessels by activating Tie2 signalling (Dumont et al, 1994; Sato et al, 1995). However, Ang-1 also promotes vascular sprouting and remodelling in active angiogenic endothelium (Hayes et al, 1999; Kim et al, 2000a; Koblizek et al, 1998; Witzenbichler et al, 1998).

To explain these contrasting effects it has been proposed that ECM and cell-to-cell contact might differently regulate the signalling pathways activated by Ang-1

In mature vasculature, endothelial cells tightly contact each others and upon Angiopoietin-1 stimulation, Tie2 localizes at cell-cell contacts forming Tie2 trans-association bridged by Ang-1. On the contrary, during Angiogenesis, cells lose contacts and upon Ang-1 stimulation Tie2 localizes at cell-substratum interface, where it is in contact with extracellular matrix. For this reason, I analysed Ang-1 signalling in cell confluence conditions or in absence of cell contact and adhering on different ECM proteins.

To analyze how ECM controls Tie2 activation and the downstream signalling by Ang-1, I plated ECs on different ECM proteins: collagen type I, fibronectin, vitronectin, gelatine and polylysine. Upon Ang-1 stimulation, Tie2 phosphorylation level was higher in ECs adhering on ECM that are ligands of integrins, compared to Tie2 activation of ECs adherent to polylysine, an electric charged matrix which allows cell adhesion without integrin ligation (Figure 1).

In the same experimental conditions I evaluated the activation of two downstream effectors of Tie2: Akt and the MAPK Erk 1/2. The phosphorylation level of both proteins follows the trend of Tie2: the activation is higher on ECM compared to polylysine (Figure 2).

To evaluate the effect of cell-to-cell contact I performed experiments with EC maintained at cell confluence for 48 hours termed Long Confluent (LC) compared to EC sparsely seeded for 24 hours termed as Sparse (S). The phosphorylation of Akt and Erk 1/2, as effectors proteins of Tie2 activation, has been analyzed in time course experiment.

Upon Ang-1 stimulation, the maximal activation of Akt is reached after 10-20 minutes of stimulation and slowly decreased after 30 minutes. Moreover Akt phosphorylation is higher in the confluent cells than in the sparse cells. The peak of Erk 1/2 activation is reached after 10 minutes of stimulation and then it sharply decreases after 20 minutes of stimulation. As Akt, Erk 1/2 phosphorylation is higher in the confluent cells than in the sparse cells. I did not observe changes in the kinetics of activation of Akt and Erk1/2 in confluent *versus* sparse cells, but rather a quantitative decrease of signal activity specifically in sparse cells (Figure 3).

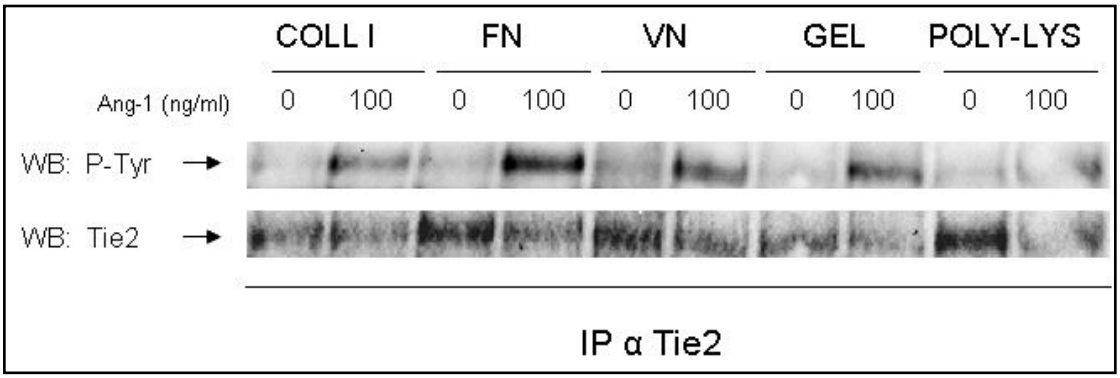


Figure 1. Activation of Tie2 on different Extracellular matrix proteins. Sparse HUVECs adherent on different ECM proteins (collagen type I –COLL I-, fibronectin –FN-, vitronectin –VN-, gelatine –GEL- and polylysine –POLY-LYS) were stimulated with 100 ng/ml Ang-1 for 10 min. Tie2 was immunoprecipitated with a specific antibody and its phosphorylation revealed by a generic phosphotyrosine antibody (p-Tyr).

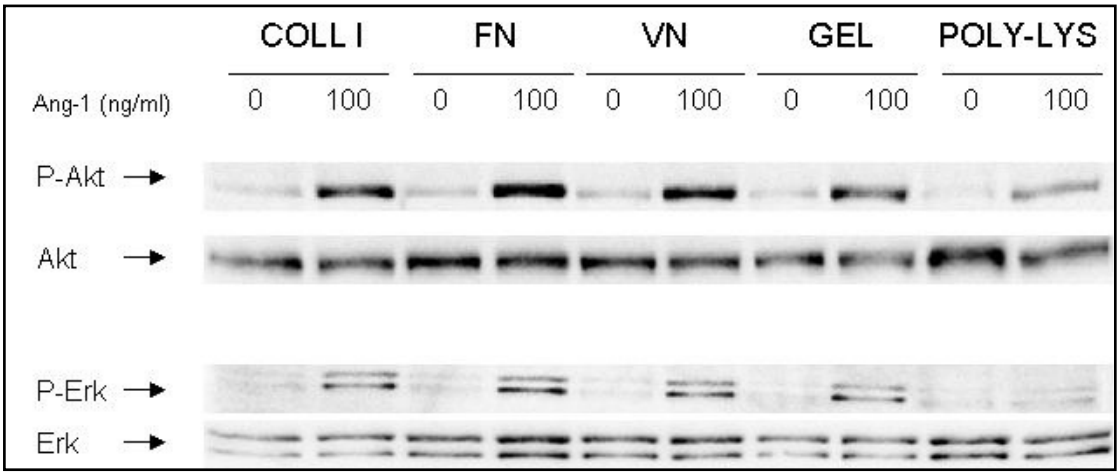


Figure 2. **Activation of Akt and Erk on different Extracellular matrix proteins.** Sparse HUVECs adherent on different ECM proteins (collagen type I –COLL I-, fibronectin –FN-, vitronectin –VN-, gelatine –GEL- and polylysine –POLY-LYS) were stimulated with 100 ng/ml Ang-1 for 10 min. Phosphorylation of Akt (p-Akt) and Erk (p-Erk) was analysed.

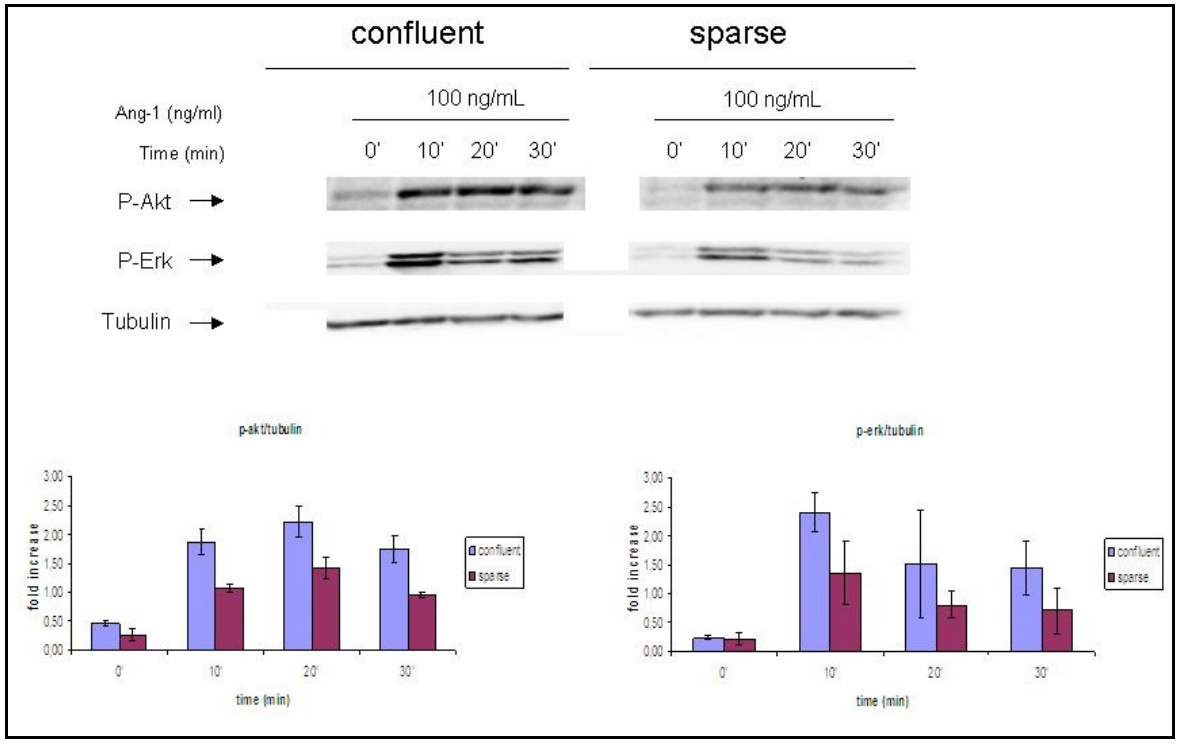


Figure 3. **Activation of Akt and ERK in confluent and sparse HUVECs**. Confluent and sparse HUVECs were stimulated with 100 ng/ml Ang-1 for the time indicated (min). Phosphorylation of Akt (p-Akt) and Erk (p-Erk) was analysed. Histograms are relative to the time course of Akt and Erk activation. Akt and Erk phosphorylation was normalized by tubulin values. The values are the average of three independent experiments ± SD.

Tie2 signalling is positively modulated by cell adhesion on collagen type I and fibronectin

The above results indicate that activation of the intracellular signalling triggered by Ang1 is enhanced in ECs adhering onto different ECM components. To get further insight into how cell adhesion may impact Tie2-regulated signal transduction pathways, we decided to expand the repertoire of matrix ligands and assess their specific contribution to Ang1-induced phosphorylation of Akt and Erk.

Firstly I evaluated Akt and Erk phosphorylation of ECs adherent to gelatine, collagen type I, fibronectin and fibrinogen. Upon stimulation with Ang-1, I observed the higher activation of Akt and Erk when HUVECs adhered on collagen type I and fibronectin. The lowest Akt and Erk activation is detected on gelatine, while on fibrinogen the phosphorylation of both proteins presents a high variability among different experiments, but substantially lower than on collagen type I and fibronectin (Figure 4). Besides I also evaluated the activation of Tie2 signalling pathways when ECs adhered on vitronectin, polylysine and when left in suspension. As before, HUVECs reach the maximum activation of Akt and Erk on collagen type I and fibronectin, Vitronectin does not enhance Akt and Erk 1/2 phosphorylation compared to polylysine, where integrins are membrane exposed but not activated. The lowest Tie2 downstream signal is detected when ECs are left in suspension and integrins are not exposed on the membrane surface (Figure 5).

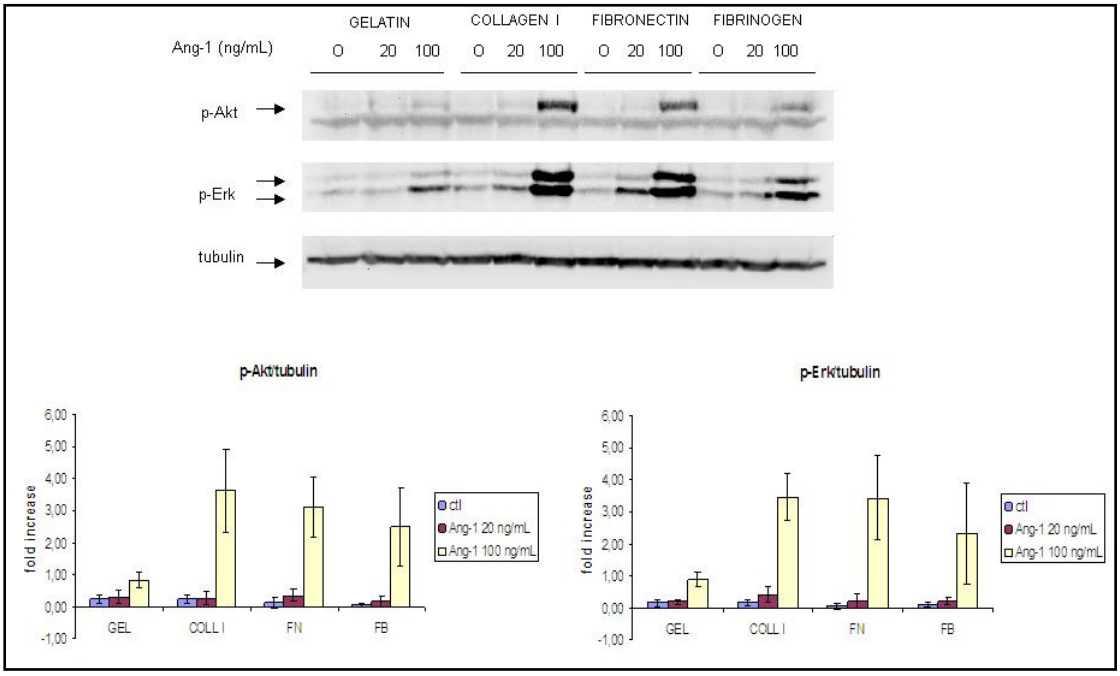


Figure 4. **Activation of Akt and ERK on different extracellular matrix proteins.** Sparse HUVECs adherent on different extracellular matrix proteins (gelatine, collagen type I, fibronectin and fibrinogen) were stimulated with 20 and 100 ng/ml Ang-1 for 10 min. Phosphorylation of Akt (p-Akt) and Erk (p-Erk) was analysed. Histograms are relative to Akt and Erk activation for increasing Ang-1 concentration. Akt and Erk phosphorylation was normalized by tubulin values. The values are the average of three independent experiments ± SD.

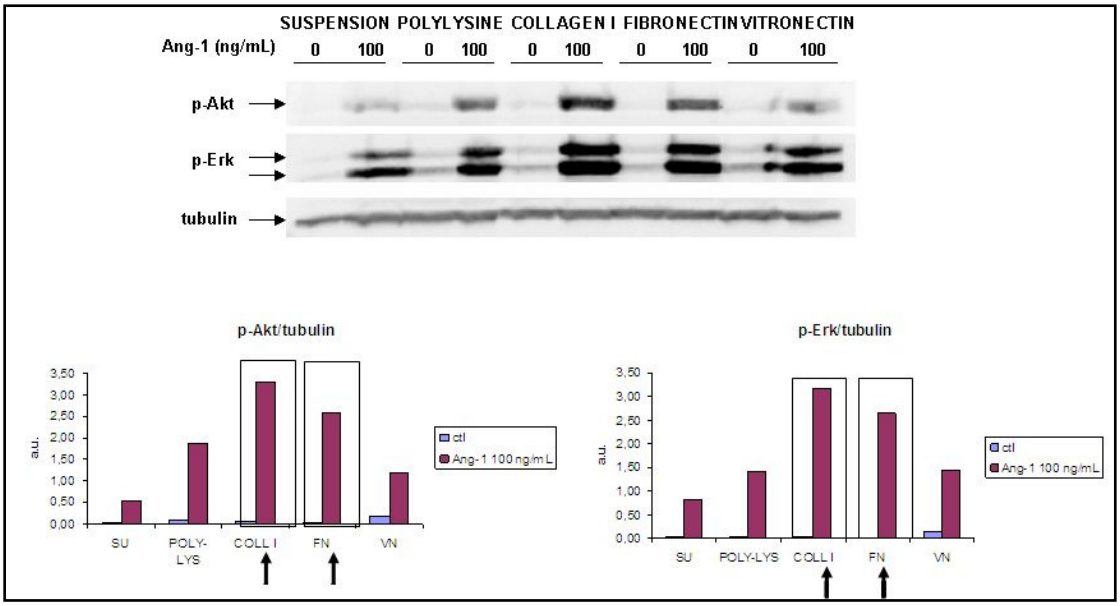


Figure 5. **Activation of Akt and ERK in different adhesion condition.** Sparse HUVECs in suspension or adherent on different extracellular matrix proteins (polylysine, collagen type I, fibronectin and vitronectin) were stimulated with 100 ng/ml Ang-1 for 10 min. Phosphorylation of Akt (p-Akt) and Erk (p-Erk) was analysed. Histograms are relative to Akt and Erk activation, the highest values are highlighted with rectangles. Akt and Erk phosphorylation was normalized by tubulin values.

Down-regulation of integrin α2 affects Tie2 signalling

Ang-1 signalling is preferentially enhanced when ECs are plated on collagen type I. The most abundant collagen receptor on EC is the integrin α2β1, then I decided to evaluate whether this integrin can affect Tie2 signalling in ECs. I silenced ECs for integrin α2 by infection with lentiviruses. I tested 5 different lentiviruses, each containing a plasmid carrying a shRNA able to block the translation of integrin α2 mRNA. Among them I decided to use shRNA Itg α2 #30 and #31; they reduce integrin α2 to 30% and 11% of the wild type expression. ECs infected with lentivirus containing scramble shRNA, a control shRNA which does not affect Itg α2 translation, present a small increase in the protein expression (107%) compare to ECs wild type. Infection of ECs with shRNA Itg α2 #28 and #32 decreases Itg α2 to 89% and 81% of the wild type expression. shRNA Itg α2 #29 increases Itg α2 up to 156% of wild type level (Figure 6).

Next I evaluated Akt and Erk 1/2 phosphorylation, as Tie2 downstream signals, in ECs silenced for integrin α2 versus control ECs. The protein activation was evaluated on collagen type I, fibronectin, vitronectin and polylysine. Upon Ang-1 stimulation, Akt phosphorylation was higher in control ECs compared to ECs silenced for integrin α2 not only on collagen type I but also on fibronectin and vitronectin. Akt phosphorylation was directly proportional to the quantity of integrin α2 (107% ctrl > 30% #30 > 11% #31). As for Akt, Erk phosphorylation was higher in control ECs compared to ECs silenced for integrin α2 on the extracellular matrix tested. Erk 1/2 phosphorylation was directly proportional to the quantity of integrin α2 expressed by the cells (107% ctrl > 30% #30 > 11% #31) (Figure 7).

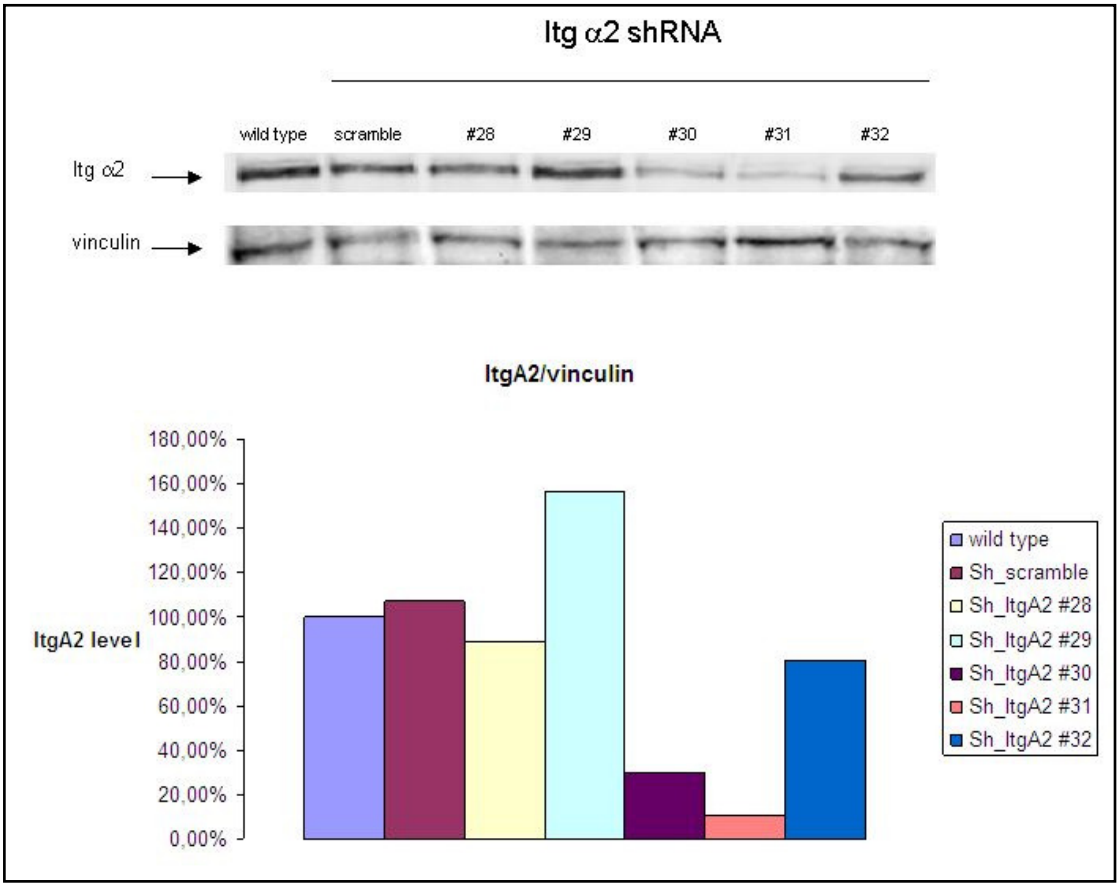
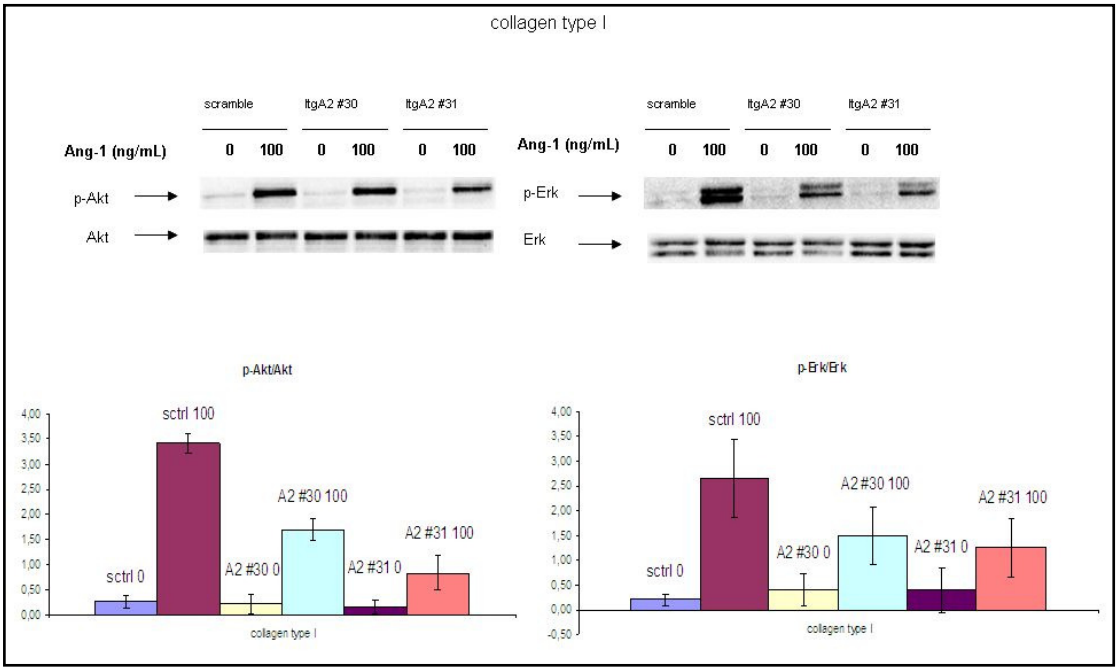
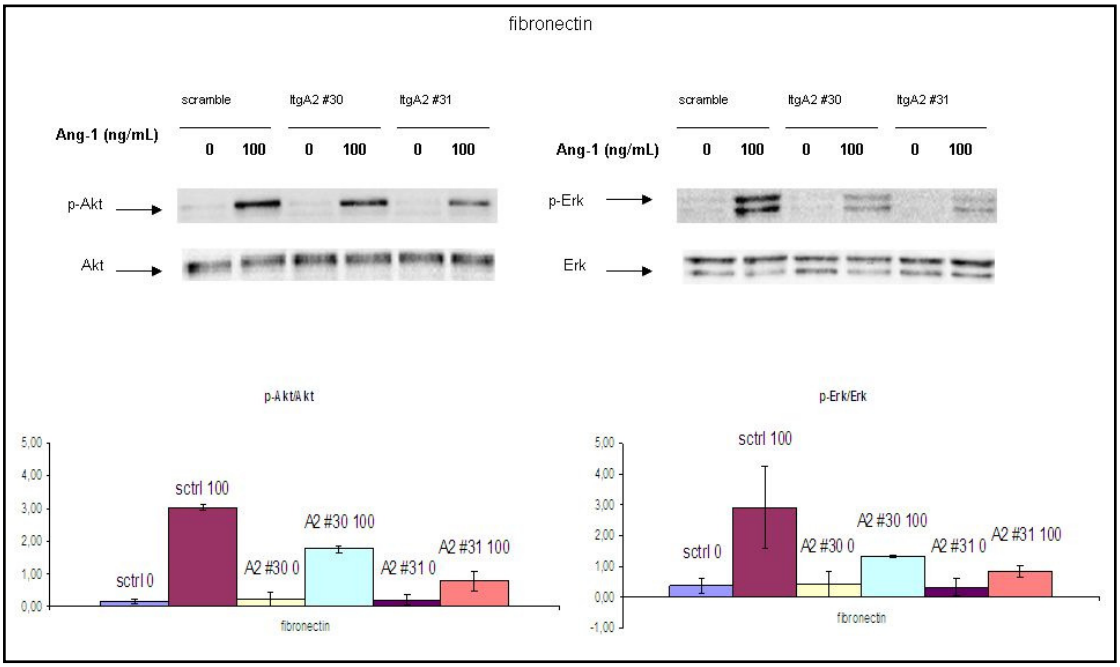


Figure 6. Integrin alpha 2 silencing. Western blotting of integrin alpha 2 in HUVECs infected with alpha 2 sh-RNA. The bar graph shows alpha 2 silencing percentage.


collagen type I
scramble
ItgA2 #30
ItgA2 #31
Ang-1 (ng/mL)
0
100
p-Akt
Akt
p-Erk
Erk
p-Akt/Akt
p-Erk/Erk
sctrl 0
sctrl 100
A2 #30 0
A2 #30 100
A2 #31 0
A2 #31 100
collagen type I


fibronectin
scramble
ItgA2 #30
ItgA2 #31
Ang-1 (ng/mL)
0
100
p-Akt
Akt
p-Erk
Erk
p-Akt/Akt
p-Erk/Erk
sctrl 0
sctrl 100
A2 #30 0
A2 #30 100
A2 #31 0
A2 #31 100
fibronectin

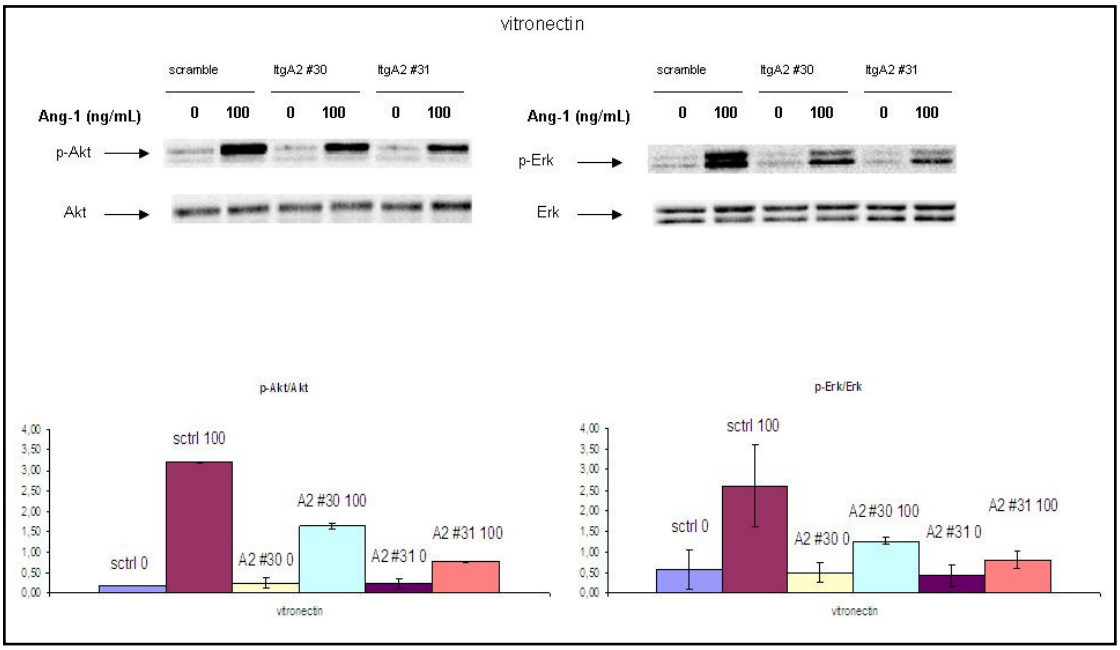


Figure 7. Akt and Erk activation in α2-silenced ECs for integrin alpha 2 versus control. Akt and Erk phosphorylation was evaluated in α2-silenced ECs versus control**.** ECs adherent on collagen type I, fibronectin and vitronectin were stimulated with 100 ng/ml of Ang-1 for 10 min. Histograms are relative to Akt and Erk activation. Akt and Erk phosphorylation was normalized by total Akt and total Erk respectively. The values are the average of three independent experiments ± SD.

Integrin α5 regulates Tie2 signalling on fibronectin

Integrin α5β1 and αvβ3 are the main receptors for fibronectin on ECs. Since integrin αvβ3 binds vitronectin too and vitronectin seems not to modulate Tie2 signalling, I decided to silence ECs for integrin α5 to evaluate how Akt and Erk 1/2 activation was affected. HUVECs were infected with 4 lentiviruses each containing a plasmid carrying a shRNA able to block the translation of integrin α5 mRNA. Among them I decided to use shRNA Itg α5 #52; this shRNA is able to reduce integrin α5 to 12% of the wild type expression. ECs infected with lentiviruses containing scramble shRNA, a control shRNA which does not affect Itg α5 translation, showed a fine increase in integrin expression (105%) compare to ECs wild type. ECs infected with shRNA Itg α5 #49, #50 and #51 showed a decrease in Itg α5 expression of 60% compared to the wild type expression (Figure 8).
Akt and Erk 1/2 phosphorylation were analysed in ECs silenced for integrin α5 versus control ECs. I evaluated protein phosphorylation not only on fibronectin but also on collagen type I and vitronectin. Akt and Erk 1/2 activation decreased in ECs silenced for integrin α5 compared to control ECs on the extracellular matrix tested an effect particularly evident for Akt and Erk 1/2 activation on fibronectin (Figure 9). These data confirm that in endothelium the complex formed by integrin alpha 5-fibronectin and Tie2 positively co-operate in signal transduction. Moreover, based on the results shown in Fig. 7, also α2 integrin appears to participate to fibronectin-dependent potentiation of Tie2 signalling.

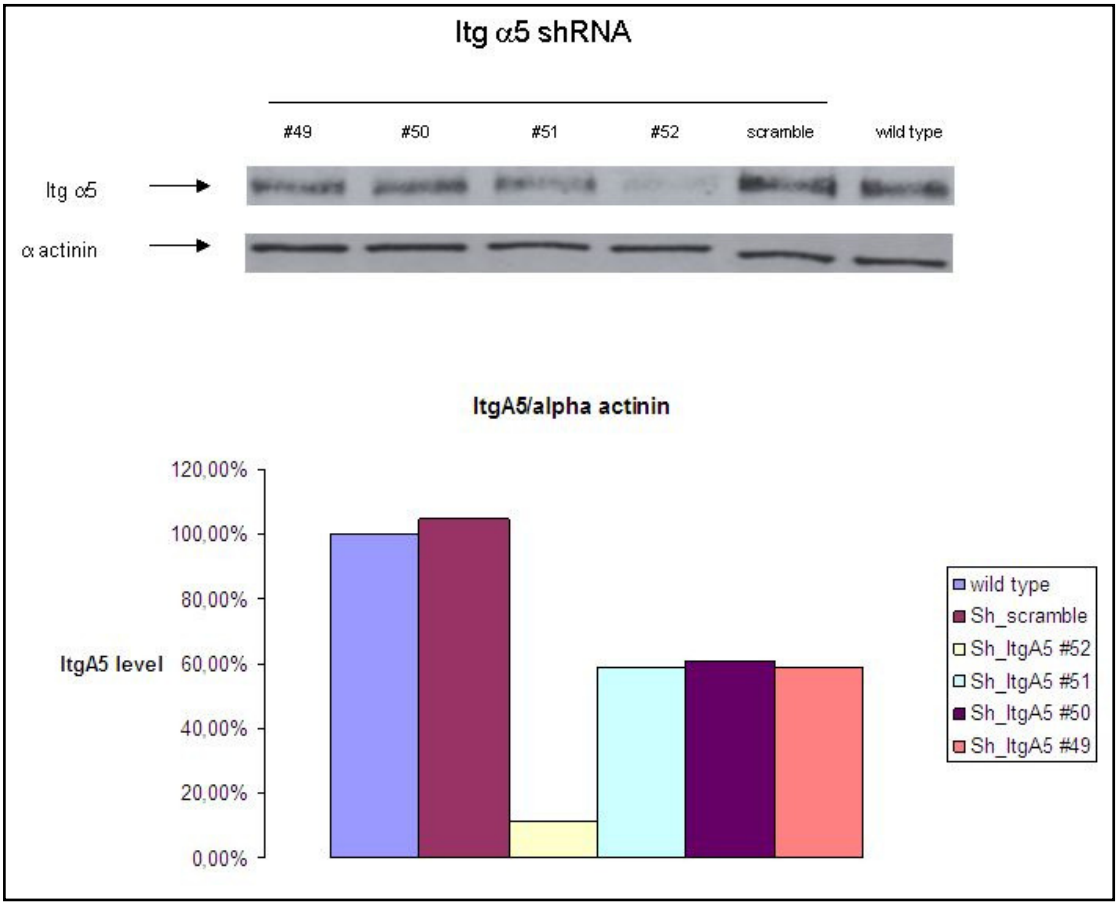


Figure 8. Integrin α5 silencing. Western blotting of α5 in HUVECs infected with shRNA α5. The bar graph shows α5 silencing percentage.

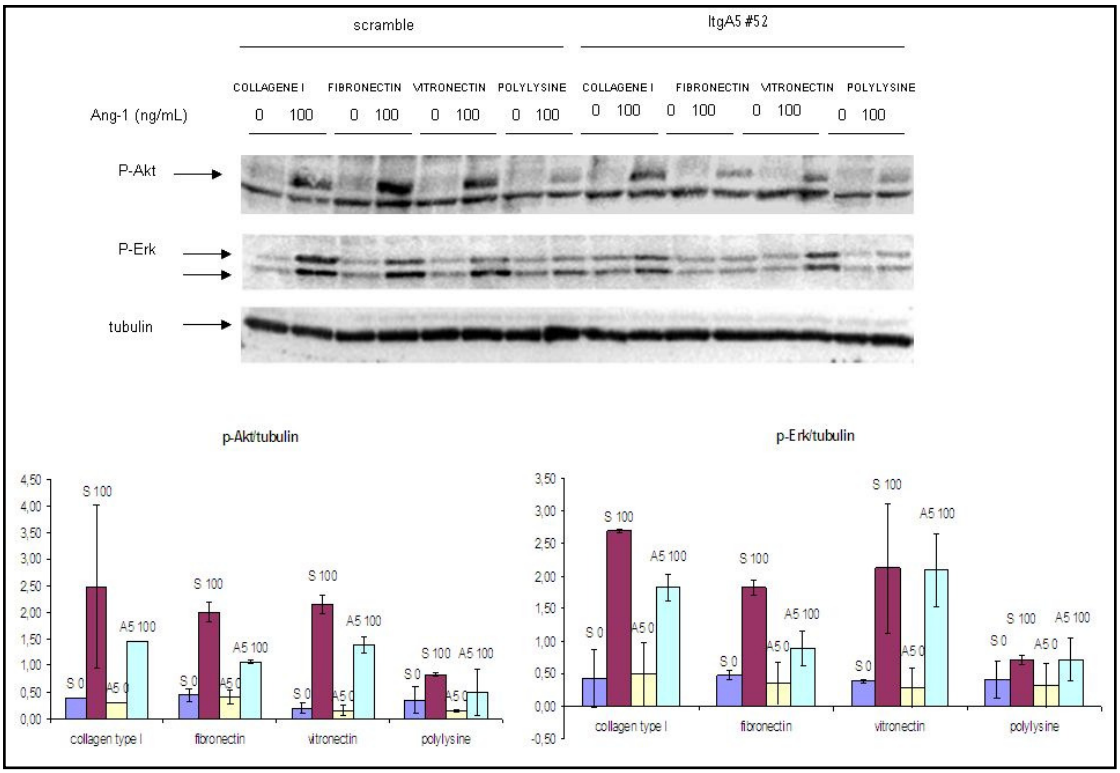


Figure 9. **Akt and Erk activation in α5-silenced ECs versus control.** Akt and Erk phosphorylation was evaluated in α5-silenced ECs versus control**.** ECs adherent on collagen type I, fibronectin and vitronectin were stimulated with 100 ng/ml of Ang-1 for 10 min. Histograms are relative to Akt and Erk activation. Akt and Erk phosphorylation was normalized by tubulin values. The values are the average of three independent experiments ± SD.

Tie2 is necessary for Akt and Erk 1/2 activation in response to Ang-1 stimulation

Integrins also can bind growth factors and trigger biological processes similar to the ones triggered by RTK. It has been described that a truncated monomeric form of the Ang-1 is not able to activate Tie2 but can bind integrin $\alpha 5\beta 1$ activating adhesion and promoting MAPK activation (Weber et al, 2005).
For this reason I decided to verify if Tie2 is dispensable for Akt and Erk activation. HUVECs were infected with 5 lentiviruses each containing a plasmid carrying a shRNA able to block the translation of Tie2 mRNA. Among 5 shRNA tested, I decide to use shRNA Tie2 #14, which reduced Tie2 expression to less than 20% of wild type. Scramble shRNA, used as control, slightly reduced Tie2 expression (80%) and shRNA Tie2 #13 and #12 reduced Tie2 expression to 45% of wild type. HUVECs infected with lentiviruses containing shRNA Tie2 #15 and #16 did not survive to the following selection with puromicin which guarantees that the infection had worked (Figure 10).
Akt and Erk 1/2 phosphorylation was evaluated in control ECs and in Tie2-silenced ECs. The protein activation was analysed on different extracellular matrix proteins (collagen type I, fibronectin, vitronectin and polylysine). The basal level of untreated cells showed that Akt and Erk 1/2 activation is higher in ECs silenced for Tie2 compared to the control. On the contrary, receptor Tie2 was indispensable to trigger Ang-1 mediated Akt and Erk phosphorylation. Akt and Erk activation did not increase over the basal level on all extracellular matrix tested in Tie2-silenced ECs (Figure 11).

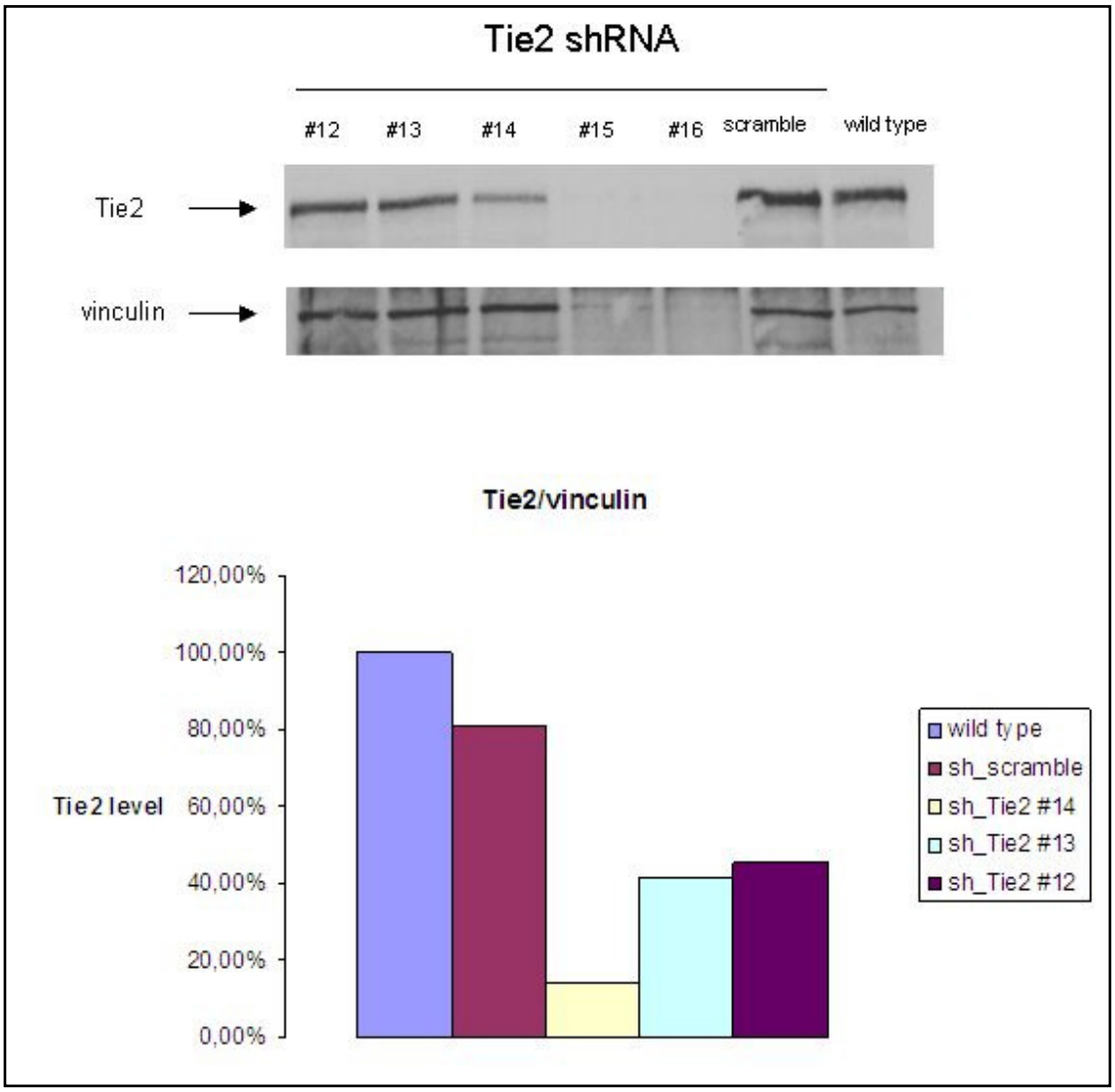


Figure 10. Tie2 silencing. Western blotting of Tie2 in HUVECs infected with Tie2 sh-RNA. The bar graph shows Tie2 silencing percentage.

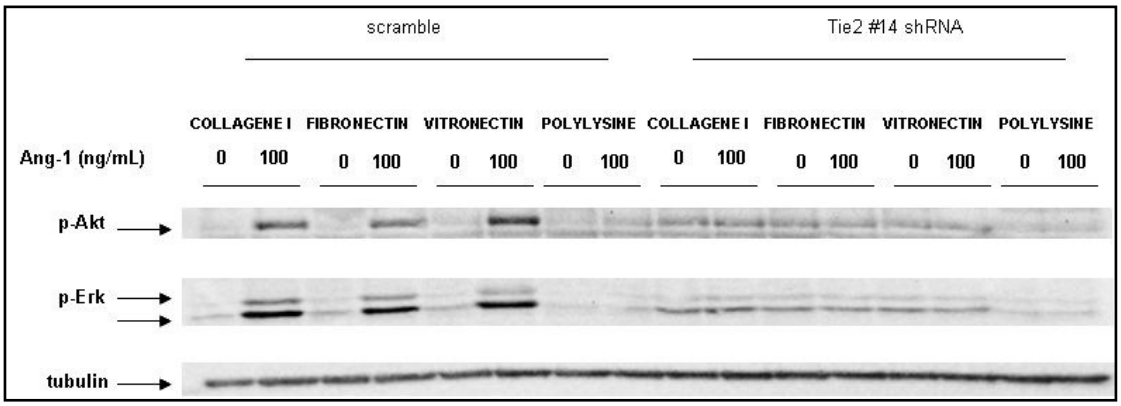


Figure 11. Akt and ERK activation in Tie2-silenced ECs versus control. The phosphorylation of Akt (P-Akt) and Erk (P-Erk) was evaluated in control and Tie2-silenced ECs. ECs adherent to different extracellular matrix proteins were stimulated with 100 ng/ml of Ang-1 for 10 min. Akt and Erk phosphorylation was normalized by tubulin values.

Ang-1 enhances HUVECs adhesion on collagen type I and fibrinogen but not on fibronectin

In endothelial cells growth factors increase integrins adhesion through a mechanism known as 'inside-out' activation, as it has been showed for integrin αvβ3 upon VEGF stimulation (Prasad et al, 2003). For this reason I verified if Ang-1enhances integrin-mediated HUVECs adhesion. Adhesion assay was performed on collagen type I, fibrinogen and fibronectin. The presence of Ang-1 enhanced HUVECs adhesion on collagen type I at the concentration of 10µg/ml ($p<0.01$), 0.5 and 5 µg/ml ($p<0.05$). Ang-1 significantly increased HUVECs adhesion also on fibrinogen at the concentration of 0.5 ($p<0.01$), 1, 10 and 20 µg/ml ($p<0.05$), while Ang-1 did not enhance HUVECs adhesion on fibronectin, except at the concentration of 0.5µg/ml. This demonstrated that at least for integrins α1β1 and α2β1, receptors for collagen type I, and for integrin αvβ3, receptor for fibrinogen, Ang-1 enhanced integrin activation and consequent ligand binding. On the contrary Ang-1 stimulation did not enhance integrin α5β1 ligation to fibronectin (Figure 12).

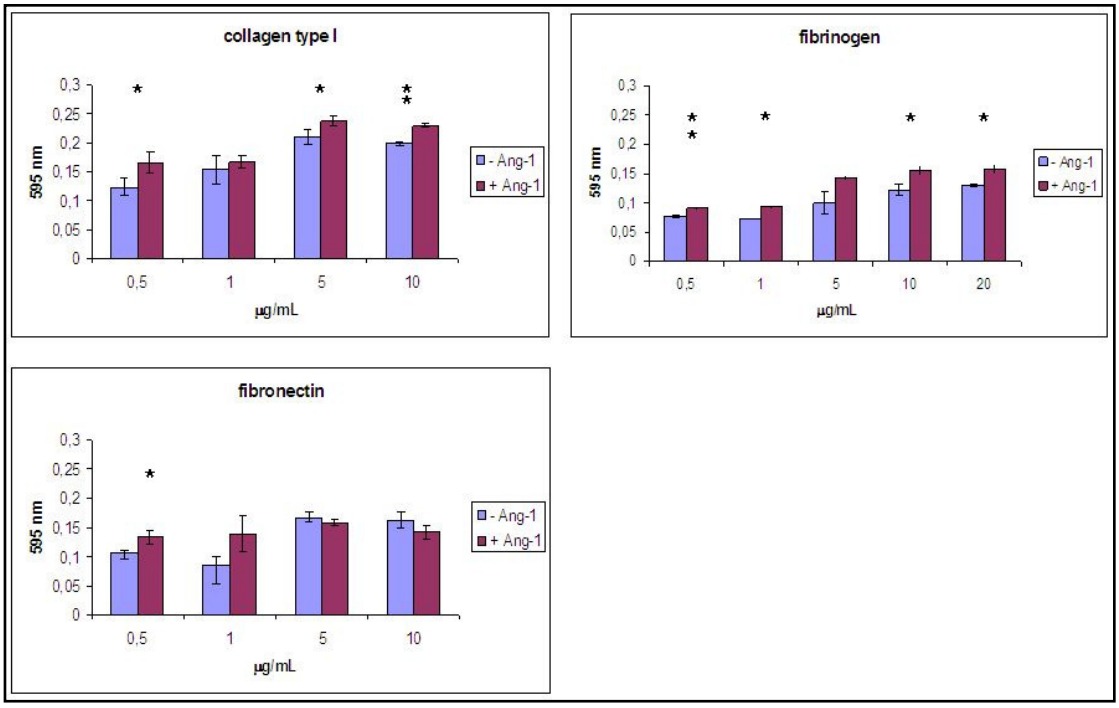


Figure 12. **Effect of Ang-1 on HUVECs adhesion.** The three panels show the increase of HUVECs adhesion induced by Ang-1 (100 ng/ml) versus unstimulated HUVECs to different concentration of collagen type I, fibrinogen and fibronectin. The experiment was performed in triplicate. Statistical significance (*, $P < 0.05$, **, $P < 0.01$) shows increased HUVEC adhesion induced by Ang-1 compared to untreated cells. The values are the average of three independent experiments ± SD.

DISCUSSION

Angiogenesis is a multistep process finely controlled by vascular specific growth factors. Vascular Endothelial Growth Factor (VEGF) is the most critical vascular growth factor and it is required to initiate the formation of immature vessels. Subsequently Angiopoietin-1 and ephrinB2 regulate the vessel remodelling and maturation. Angiopoietin-1 is a specific ligand of Tie2 receptor. Tie2 activation promotes vessel assembly and maturation by mediating survival signals and regulating the recruitment of mural cells (Thomas & Augustin, 2009). Moreover Ang-1/Tie2 system plays an active role in adult Angiogenesis in physiological condition such as in the endothelium of rat ovary and in healing rat skin wounds (Wong et al, 1997) and in pathological one such as in the endothelium of human breast cancer (Hayes et al, 2000). To shed light on the molecular mechanisms which allow Angiopoietin-1 to promote either Endothelial Cells (ECs) survival in quiescent vasculature and sprouting Angiogenesis in active endothelium, the Ang-1-dependent activation of signalling pathway was analysed in confluent ECs, an *in vitro* condition which mimics quiescent mature endothelium where mature cell-to-cell contacts are present, and in sparse and ECM-bound ECs, an *in vitro* condition which resembles endothelium during Angiogenesis.

In confluent ECs Ang-1 signalling, analysed in term of Akt and MAPK Erk 1/2 phosphorylation, was substantially higher than in sparse cells, proving that mature cell-to-cell contacts strengthen the activated pathways. VE-Cadherins are the major transmembrane component of endothelial adherens junctions and they may regulate Tie2 signalling as they do for VEGFR-2. Indeed it has been reported that when VE-Cadherin is clustered at cell-cell contacts as in confluent culture the two proteins can form a complex, resulting in a reduction in VEGFR-2 tyrosine phosphorylation. Surprisingly this reduced tyrosine phosphorylation affects MAPK-dependent proliferation but enhance the survival pathways (Lampugnani & Dejana, 2007). However, my experiments did not show a differential activation of MAPK and AKT pathways upon Ang-1 stimulation in sparse and confluent cells. Although previously evidence suggested that cell-to-cell contact preferentially activated Akt pathway following stimulation of EC with a potent Ang-1 variant (COMP-Ang-1) (Fukuhara et al, 2008; Saharinen et al, 2008), I also observed an increase of MAPK activation in confluent cells. Anyway my data are obtained with native Recombinant human (Rh)-Ang-1 and this could affect EC outcomes.

In sparse ECs Ang-1 signalling is enhanced by integrin-dependent adhesion on interstitial ECM proteins. Collagen type I, ligand of integrin $\alpha2\beta1$, and fibronectin, ligand for integrin $\alpha5\beta1$ and $\alpha v\beta3$, maxime Ang-1 signalling. For this reason I decided to verify whether and where down-regulation of integrin $\alpha2$ and $\alpha5$ affects Ang-1 signalling.

The knocking-down of integrin $\alpha2$ affects Ang-1 signalling and unexpectedly this effect is observed not only when cells adhere on its ligand collagen type I, but also when cells adhere on other unspecific ECM. These evidences demonstrated that integrin $\alpha2$ positively regulates Ang-1/Tie2 signalling, as it has already been demonstrated for VEGFR-2 where integrin $\alpha2\beta1$ activation provides an important support to VEGF signalling, endothelial cell migration and tumour Angiogenesis (Senger et al, 2002).

The reduction in integrin $\alpha5$ level decreases Ang-1 signalling and the signal detretment is more evident when cells adhere on fibronectin, its specific ligand. As for the down-regulation of integrin $\alpha2$, the absence of integrin $\alpha5$ also affects the signalling on unspecific ECM, although the effects are less important. My results provide evidence that Ang-1-dependent Tie2 signalling is increased by integrin $\alpha5\beta1$. These data are in agreement with Cascone's

work (Cascone et al, 2005) which demonstrated that Tie2 and integrin α5β1 constitutively associate and that integrin binding to fibronectin augments this association.
Although I do not have proof of a direct action of integrin α2 and α5 on regulation of Ang-1-dependent Tie2 pathways, their absence affect ECs Ang-1/Tie2 signalling either in their un-ligated form, as testified by signal down-regulation on unspecific ECM, either in their ligated form upon collagen type I and fibronectin binding.
Integrin can bind growth factor and trigger biological processes as RTK as the case for a monomeric form of Ang-1 which can not activate Tie2 but it can promote integrin α5β1−dependent ECs adhesion and MAPK activation (Weber et al, 2005).
Moreover, function-blocking antibodies against β1, α5 and αvβ5 abolish fibroblast adhesion to Ang-1 and consequent MAPK activation (Carlson et al, 2001). For this reason I tested the possibility that integrins can trigger Ang-1 signalling independently by Tie2 presence. Upon Ang-1 stimulation, Akt and Erk phosphorylation was evaluated in stable Tie2-silenced ECs compared to wild type ECs. No Akt and Erk activation was detected in Tie2-silenced ECs on any ECM tested, proving that Tie2 is indispensable to trigger Ang-1 signalling.
Thus in our *in vitro* model where HUVECs mimics angiogenic endothelium and quiescent mature vasculature the Ang-1 signalling is enhanced in presence of cell-cell contacts and in sparse ECs by integrin-mediated adhesion on ECM. Among integrins, α2β1 and α5β1 exert a positive effect on Ang-1 signalling particularly when bounded to their specific ECM, collagen type I and fibronectin respectively. The subsequent down-regulation of integrin α2 and α5 confirms their positive role in potentiating Ang-1 signalling and demonstrates that they can exert this function also in their un-ligated form. My data provide evidences that integrins alone can not trigger Ang-1 signalling, Tie2 receptor results indispensable to internalize the growth factor message.
My data confirm that Ang-1/Tie2 signalling can be modulated in confluent culture condition, which mimic quiescent endothelium, by cell-to-cell contacts and in sparse and ECM-bounded ECs, which mimic angiogenic endothelium, by integrin engagement. To confirm and validate integrin-dependent positive modulation of Ang-1 signalling, further *in vitro* and *ex vivo* angiogenic assays should be performed. For this aim it should be useful to counteract Ang-1 dependent Angiogensis with specific integrin antagonists, such as low molecular weight inhibitors and function-blocking antibodies.

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