Università degli studi di Torino
Dipartimento di Scienze Oncologiche

Dottorato di Ricerca in:

“Sistemi complessi applicati alla Biologia Post-Genomica”

The miR-126 regulates angiopoietin-1 signaling and vessels maturation by targeting p85beta

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Anni Accademici 2008/2010

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ABSTRACT

MicroRNAs are short non-coding RNA molecules that post-trascriptionally regulate gene expression by translational suppression or in some instances cleavage of the respective mRNA target. We found that microRNA-126 is highly expressed on human and mouse endothelial cells. Interestingly, the expression of miR-126 in zebrafish blood vessels start late during embryonal vascular development, when vasculogenesis has already taken place. In order to characterize the functional role of miR-126 we performed knock-down experiments on Zebrafish embryos injecting morpholinos anti-miR126. The morphants exhibited a not completely formed vascular tree with leaky vessels. To identify the miR-126 target genes we performed a gene expression analysis on endothelial cells transfected with an oligo anti-miR126. One of the most regulated mRNA in treated cells was PIK3R2/p85β. The p85β protein is the regulatory subunity of the PI3K which has been demonstrated to be involved in several aspects of the vascular system physiology. We demonstrated that miR126 controls p85β which in turn regulates the PI3K signalling. Actually, over-expression of p85β in endothelial cells reduces Akt phosphorylation and the apoptosis protective effect induced by angiopoietin-1. The striking similarity of the miR126 morphant phenotype with that of Angiopoietin1 or Tie2 KO mice, toghether with our in vitro results, indicates that miR-126 is mainly involved in the process of vessels maturation and stabilization mediated by Angiopoietin1

INTRODUCTION

Vascular development is a complex, tightly regulated, multi-step process . A number of secreted factors regulate endothelial cell (EC) migration, proliferation and differentiation into tubular structures.

Vascular endothelial growth factor (VEGF) and Angiopoietin 1 (Ang1) play essential and complementary roles in vessel formation during embryogenesis. Both VEGF and Ang1 share the ability to promote endothelial survival, proliferation, and migration, by acting on their cognate cell-surface tyrosine-kinase receptors, VEGFR2 (Flk1, KDR) and Tie2 (Tek), respectively. Whereas VEGF is required for the formation of the initial vascular plexus early in development, Ang1 is necessary for subsequent vascular remodeling into mature blood vessels (Ferrara et al., 1996; Suri et al., 1996). It appears however that whilst both Ang-1 and VEGF initiate angiogenesis, only Ang-1 leads to maturation of the vessels. In addition, VEGF can cause vascular instability in the form of permeability leading to tissue oedema, whereas Ang-1 stabilizes existing vessels causing decreased vascular leakage and thereby less tissue oedema (Augustin et al., 2009; Thurston et al., 1999) Despite their opposing actions on permeability, Ang-1 and VEGF act in a coordinated way during embryogenesis for vascular development (Gavard et al., 2008). There is therefore a competitive balance that must be weighed up between VEGF and Ang-1. Concerning the activation of angiogenic program, the initial budding is induced by both VEGF and Ang-1 followed by stabilization of the neovessel for maturation by Ang-1 alone (Thurston et al., 1999). Once the complete maturation of the new vessels is achieved, the low ratio between VEGF and Ang-1 doesn't allow the formation of new budding (Fig. 1). Findley et al. (Findley et al., 2007)

demonstrated that VEGF induces Tie-2 receptor shedding and once cleaved.ò- sTie-2 binds to both Ang-1 and Ang-2 to inhibit ligand-mediated Tie-2 receptor activation and downstream signaling (Findley et al., 2007). This suggests a mechanism by which VEGF down-regulates the maturation and remodeling effects of Ang-1 causing resultant neovascularization.

Recently it has been demonstrated that a new class of regulatory molecules-microRNA- is actively involved in the processes of vascular development and angiogenesis. MiRNAs generally repress target mRNAs through an antisense mechanism. In animals, miRNAs typically target sequences in the transcript 3' untranslated regions (3'UTRs) that are only partially complementary to the miRNA, causing translational inhibition, or in some instances cleavage of the respective mRNA target (Fig. 2).

In this thesis we show that mature vessels express high levels of miR126, which enhances the remodeling and stabilization effect of Ang-1. MiR126 potentiates PI3K-mediated Ang-1 signaling by reducing levels of the PI3K regulatory subunit p85beta

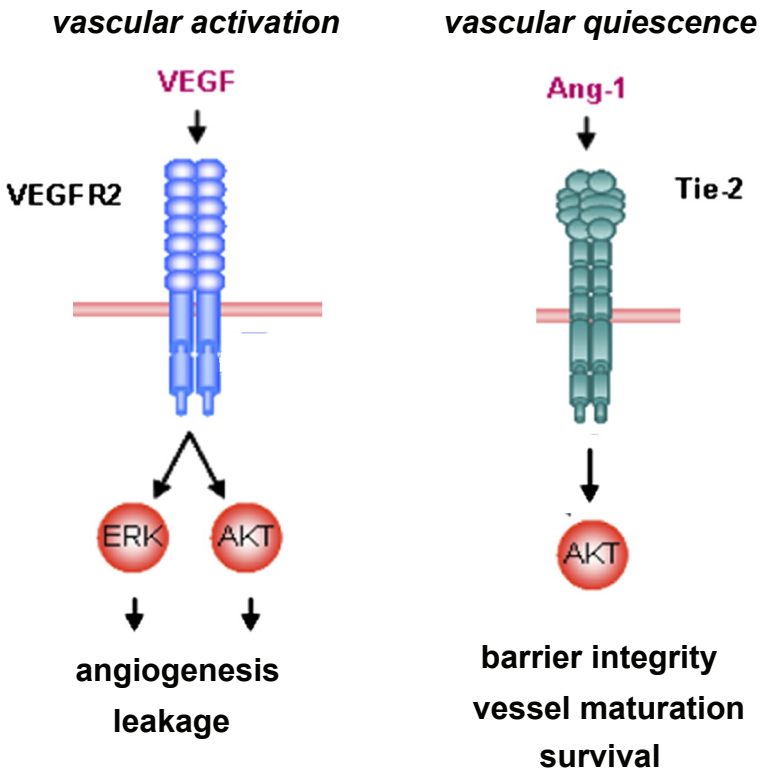


Figure 1. Model depicting how VEGF and Ang-1 stimulate endothelial cells

Different effects of VEGF-A and Ang-1 on endothelial cells. Whereas VEGF-A is required in the early steps of vasculogenesis during development and also to trigger adult angiogenesis, Ang-1 is necessary for the subsequent remodelling and maturation of vessels, also by counteracting the leakage effect of VEGF-A.

Endothelial microRNAs

MicroRNAs are short non-coding RNA molecules that post-trascriptionally regulate the gene expression (He and Hannon, 2004) (Fig. 2). They have been implicated in a growing number of diseases (Dalmay and Edwards, 2006). MicroRNAs are specifically expressed in many tissues and from the literature it is well enstablished that a particular microRNA is active in certain tissues at certain times (Aravin et al., 2003) (Wienholds et al., 2005). Deep sequences technologies have delivered a sharp rise of novel microRNA discovery; currently there are around 1212 annotated Human microRNAs sequences in last miRBase release (Kozomara and Griffiths-Jones). Despite the big potential of this new class of short non-coding RNA to regulate the gene expression, only few of them have been experimentally demonstrated to regulate a specific gene. Different approaches have been attempted in order to validate targets, initially through experimental procedure, after using microarray techniques and computational tools (Lai, 2004) (Lewis et al., 2003)

Since from the early studies on microRNAs, it has been observed that the genome location of their sequences was frequently matching with already noted cromosome fragile sites involved in malignancy (Zhang et al., 2006) (Gaur et al., 2007) (Calin et al., 2004). Alterations of microRNAs were initially identified in tumors such as the most common form of adult leukemia, the B cell chronic lymphocytic leukemia (CLL) (Calin and Croce, 2006) and colorectal cancers (Michael et al., 2003). Nowadays it is well established that differential expression of microRNAs occurs between normal and tumors counterpart. An increasing number of evidences

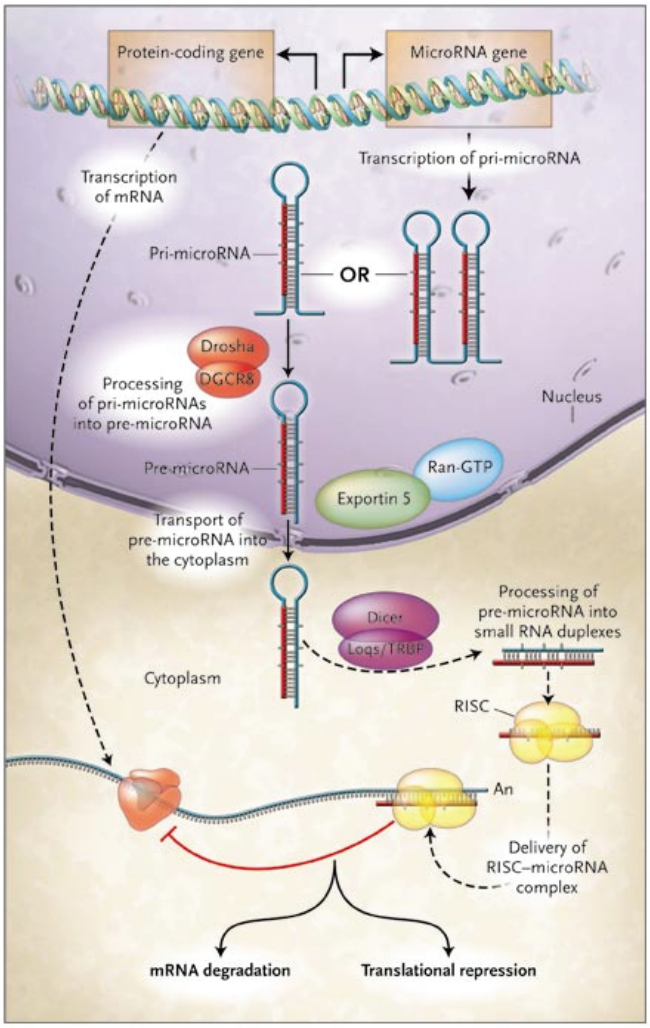
demonstrate how microRNAs can correlate with well-defined clinico-pathological features and disease outcome. MicroRNA signature in tumor is capable of distinguishing between indolent and aggressive ones and their expression has been proposed to be useful in term of prognosis (Calin et al., 2005). Considering that microRNA profiling correlates with cell differentiation and development better than gene profiling, it could be reasoned that microRNAs are useful in classifying tumor according to specific bio-phatological features and therefore can be used as cancer biomarkers (Iorio et al., 2005).

From the present scenario it appears more clear the important role of microRNAs in tuning the development and tissue differentiation. Some publications shed a light on the molecular mechanism behind these processes. Generally, microRNAs appear to promote the differentiation meanwhile repressing the pluripotency of steam cell, this feature was demonstrated for stratified epithelial tissues in which miR-203 defines the molecular boundary between the basal progenitors and terminally differentiating suprabasal cells (Yi et al., 2008). Others works focused on neural development finding that microRNAs regulate the mitotic exit of neural progenitor and therefore allowing the cells lose multipotency (Yoo et al., 2009) (Shi et al.). It is clear that microRNAs also regulate many aspects of the immune response and the differentiation of the pluripotent hematopoietic steam cells into the various blood cells lineages. Many publications have provided compelling evidence that a reange of microRNAs are involved in the regulation of the vascular system. In particular the response of the vascular edothelium to angiogenic stimuli is regulated by microRNAs (Suarez and Sessa, 2009) (Suarez et al., 2008). The first evidence

implicating the involvement of microRNAs in the formation of the cardio-vascular system came from mice homozygous for a hypomorphic allele of the *Dicer* enzyme, which is necessary for the maturation of microRNA molecules; these embryos died between days 12.5 and 14.5 of gestation and they showed blood vessel formation and maintenance severely compromised (Yang et al., 2005). In another paper it was shown that ablation of *Dicer* from the endothelium resulted in a diminished angiogenic response in tumor models and in response to ischemia (Suarez et al., 2008). Finally, *in vitro* experiments on endothelial cells also demonstrated a role for *Dicer* in several angiogenic processes, including proliferation, migration and capillary sprouting (Poliseno et al., 2006).

Up to date several microRNAs have been described to be enriched in endothelial cells; a list of them is presented in Table 1. However, one should not only focus on highly expressed microRNAs, because some of the microRNAs expressed at a lower level under physiological condition might be upregulated under certain conditions such as after angiogenic activation.

In summary, regarding the biological role of this new class of short non-coding RNAs, they provide an elegant mechanism that affords finely tuned control of protein level and ultimately of gene expression in differentiation and development. Moreover, the potential impact of few microRNAs on gene expression, due to the flexibility of target recognition, make them good targets in cancer therapy, reverting and modulating the disregulated pathways involved in the onset of malignancy.



Chen, C.Z. N Engl J Med. 2005 Oct 27;353(17):1793-801.

Figure 2. microRNA biogenesis

MicroRNAs are transcribed by RNA polymerase II as long precursors (pri-miR). Precursors are processed twice, once in the nucleous by the Drosha enzyme that gives rise to the pre-microRNA, and finally in the cytoplasm by the Dicer enzyme in order to obtain the short duplex microRNA molecule. One of the two strands of the miR duplex is loaded in the multiprotein RISC complex and is able to recognize the target sequence.

Table 1

Summary of the known microRNAs involved in vascular biology

MicroRNA	Targets	Function
Pro-angiogenic microRNAs		
MiR-130a(Chen and Gorski, 2008)	GAX, HOXA5	Expression of miR-130a antagonized the inhibitory effects of GAX or HOXA5 on endothelial cell tube formation *in vitro*
MiR-17-92 cluster(Dews et al., 2006)	TSP-1 (miR-19), CTGF (miR-18)[a,b]	MiR-17-92-transduced tumour cells formed larger, better-perfused tumours *in vivo*
Let-7f and miR-27b(Kuehbacher et al., 2007)	ND	Inhibition of miR-27b and let-7f reduced *in vitro* sprout formation
MiR-378(Lee et al., 2007)	Sufu, Fus-1b[b]	Expression of miR-378 promotes tumourigenesis and angiogenesis *in vivo*
MiR-210 (Fasanaro et al., 2008)	EphrinA3	miR-210 overexpression stimulates tubulogenesis and migration
Anti-angiogenic microRNAs		
MiR-221 and miR-222 (Suarez et al., 2008)	c-kit, eNOS[a]	Expression of miR-221/miR-222 reduces tube formation, migration,and wound healing in response to SCF *in vitro*
MiR-15, miR-16, miR-20a, and miR-20b (Cimmino et al., 2005)	VEGF, Bcl2[b]	Expression of miR-15/miR-16 induces apoptosis in leukaemic cells *in vitro*
MicroRNAs involved in vascular diseases and inflammation		
MiR-155(Martin et al., 2007)	AngII type 1 receptor	ND
MiR-21	PTEN, Bcl2[a]	Inhibition of miR-21 decreases proliferation and increases apoptosis of VSMCs *in vitro* and in injured rat carotid artery *in vivo*
MiR-126(Harris et al., 2008)	VCAM-1	Inhibition of miR-126 increases leukocyte adherence to TNFa-stimulated endothelial cells

a:This target has not been shown to be directly regulated by the corresponding miRNA.

b:This target has not been identified in vascular cells.

ND, not determined.

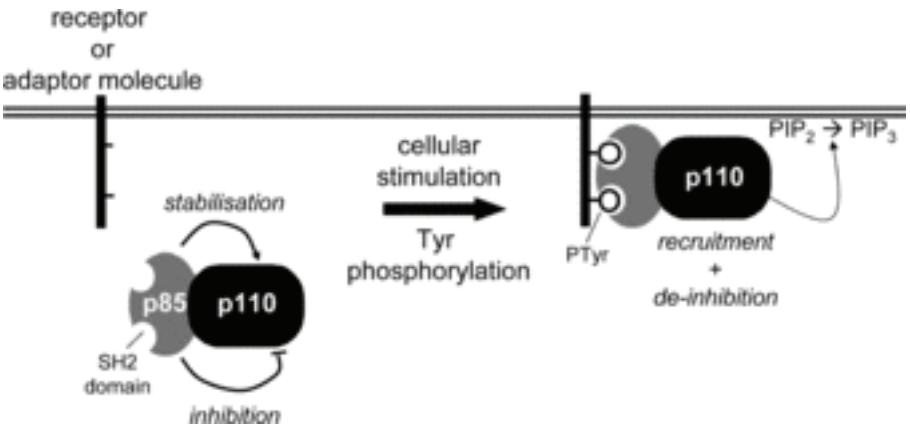
Phosphoinositide 3-kinase and Angiogenesis

Phosphoinositide 3-kinase (PI3K) enzyme generates lipids from the inner side of the cytoplasmic membrane and controls a wide variety of intracellular signalling pathways(Vanhaesebroeck and Waterfield, 1999) (Hawkins et al., 2006). Phosphatidylinositol 3,4,5-triphosphate (PIP3) is a major product of PI3K enzyme and it acts as second messenger activating downstream serine/threonine kinases, such as Akt, small GTPases, and scaffolding proteins. The class IA subset of PI3Ks signals downstream of receptors tyrosine kinases and Ras; the proteins are heterodimers consisting of a p110 catalytic subunit and a smaller p85 regulatory subunit with Src-homology 2 (SH2) domains. In mammals each of the two subunits exist in different isoform (Hawkins et al., 2006). P85s have a dual effect on the p110 subunits because they stabilize the termally labile p110s but also conformationally inhibit their catalytic activity. Upon cellular stimulation p85s is engaged by phospho tyrosine residues (pY) through the SH2 domain and relieves the inhibition on p110, thus increasing the enzymatic activity of p110 (Geering et al., 2007b) (Geering et al., 2007a) (Fig. 3). Many hypotheses have been proposed in order to explain how p85 subunit exerts its regulatory function on PI3K enzyme. The observation that different lines of mice with targeted deletions of p85s exhibit enhanced PI3K signaling in insulin-responsive cells and tissues prompted for a negative role of p85 in the regulation the catalytic activity (Ueki et al., 2003). This hypothesis was corroborated by other experiments in which forced overexpression of p85 in skeletal muscle cells was found to decrease PI3K-dependent

phosphorylation of Akt (Ueki et al., 2002). In particular, due to the phenotype observed in p85 KO mice it was initially believed that p85 could exist without p110 in cells thus competing with heterodimeric p85/p110 complexes for pY binding (Ueki et al., 2003). Many observations, coming from studies of Vanhaesebroeck's lab, argue against the "free p85 hypothesis" in which the regulatory subunit doesn't bind to p110. These observations demostrated that the two subunits are obligate heterodimers (Geering et al., 2007a). In order to explain how p85s could negative regulate the PI3K activity, loss or gain – of expression of p85 has been performed. In these experiments p85α KO mouse showed in liver cells an alter lipid phosphatase activity in particular a decreased in activity of 3-phosphoinositide phosphatase PTEN (Taniguchi et al., 2006). It seems likely that p85α enhances PTEN function by modulating some post-translational modification or altering the subcellular localization and therefore improved Akt activation at the molecular level.

PI3K is important in many cellular responses, such as the regulation of growth, proliferation and survival, but this enzyme is also implicated in angiogenesis (Jiang et al., 2000) (Geng et al., 2004). Conditional knockout experiments on mouse vasculature indicate that p85s play critical role in vascular integrity during development. Double knockout of both p85α and p85β shows that PI3K activity in the endothelium is not necessary for endothelial cell differentiation or the formation of primitive vascular network. The embryonic death of these knockout mice is concomitant with the maturation of the endocardium. Adult heterozygous mice for p85α exhibit localized vascular abnormalities, including vessel leakage and the

inability to maintain large vessels (Yuan et al., 2008). Concerning the catalytic subunit p110, p110α knockout mice display multiple vascular defects (Graupera et al., 2008). In particular these defects consiste in dilated vessels in the head, lack of hierarchical order of large and small branches in the yolk sac and impaired development of anterior cardinal veins and recapitulate on those of mice defective in the Tie-2 signalling (Lelievre et al., 2005).



Geering, B. Biochem Soc Trans. 2007 Apr;35(Pt 2):199-203.

Figure 3. General mechanism of PI3K function

The p110 catalytic subunit is stabilized by p85 protein which also inhibits its kinase activity. Upon growth factor stimulation the protein complex p110-p85 is recruited to the membrane by activated receptor and consequently the catalytic activity of the p110 subunit is de-inactivated. Knocking-down or over-expressing the regulating subunit p85 leads to iperactivation or repression of the signalling downstream the PI3K enzyme.

MATERIALS AND METHODS

Cell cultures

Human ECs (HUVEC) were isolated from umbilical cord veins, characterized and grown in EGM-2 medium (Cambrex) as previously described (Bussolino et al., 1992). 293T, Hela and H-end cell lines were grown in DME (Cambrex) supplemented with 10% FCS, 2 mM L-Glutamine (Cambrex) and antibiotics.

Vector construction

The plasmid construct used for the identification of the target of miR-126 by microarray experiments was made starting from pEGFP-N1 (Clontech). The sequence bearing three miR-126 consensus response elements (GCATTATTACTCACGGTACGA) was cloned downstream of the EGFP coding sequence into XbaI-NotI sites. The sequence introduced into the plasmid was: gaatctagaaGCATTATTACTCACGGTACGAgatactGCATTATTACTCACGGTACGAactagtGCATTATTACTCACGGTACGAta; with the miR-126 complementary sequence showed in upper case. Human p85β coding sequence was cloned in the third-generation of lentivirus vector #304.pCCL.sin.cPPT.polyA.CTE.eGFP.minhCMV.hPGK.deltaNGFR.Wpre and inserted into SalI-PstI sites for over-expression in HUVECs.

Luciferase Report Construct

p85β 3' UTR sequence was cloned into the pIS1 plasmid between SacI sites (Derived from pRL-TK AddGene) down-stream from the Renilla coding sequence. The cloning procedure was performed by recombination using the In-Fusion^{TM} 2.0 Dry-Down PCR Cloning Kit (Clontech Laboratories) with the following primers:

Fw aacaataattctaggagctcaactcccccaccccatatc and Re tcgagaccggtatagagctccgacgtttgatgttttatatcatatc. Mutated p85β 3' UTR was derived from wild-type p85β 3' UTR deleting the predicted miR-126 binding site with Phusion Site-Directed Mutagenesis Kit (Finnzymes) using the following primers: Fw [Phospho]cgaccatcaaacgtcggagc Re [Phospho] cactgcctcccagctcgtg. Renilla luciferase activity was quantified in lysates using the Dual Luciferase Report Assay kit (Promega) according to the manufacturer's recommendations. Luminescence readings were corrected for background and Renilla luciferase values were normalized to the co-transfected control oligos.

Transfection

HUVECs cells were transiently transfected with Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions.

Lentivirus vector construct and EC infection

The cDNA of p85β protein was cloned into a p304 lentiviral vector. In brief, 293T cells were co-transfected with the vector of interest, packing plasmid (pCMV-R 8.74) and the envelope plasmid (pMD2.VSVG) with the ProFection Mammalian Transfection System – Calcium Phosphate (Promega) according to the manufacturer's instructions. Virus particles were collected 24 and 48 hours post transfection, concentrated and used to infect HUVEC cells for 24 hours in the presence of 4 μg/ml Polybrene.

Quantitative RT-PCR

Total RNA was harvested from different cell lines using Trizol reagent (Invitrogen). Expression of 154 different miRNA sequences were then checked by TaqMan miR assays, with stem-looped primers specific for miR amplification (Applied Biosystems).

The expression of p85β was analyzed by quantitative real-time reverse trascription-PCR (RT-PCR) using TaqMan Gene Expression Assay from Applied Biosystems. mRNA quantities were analyzed in triplicate, normalized against human GAPDH or human TBP (Tata Binding Protein) as control genes, and expressed in relation to calibrator samples. PCR reactions were performed on the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) using fluorescent taqMan methodology. Results are expressed as relative gene expression using the ΔΔCt method (Giulietti et al., 2001).

Apoptosis assays

Apoptosis was induced in HUVECs by the serum deprivation. Briefly, HUVECs were seeded onto 1% gelatin-precoated six-well dishes to a density of $1{,}75X10^5$ cells per well. Cells were cultured in EGM-2 medium (Cambrex) washed extensively with EBM-2 serum-free medium before incubation for 24-48 hours in EBM-2 serum-free medium or with recombinant VEGF-125 50 ng/ml or Ang 300 ng/ml (R&D System) stimulation. Cytoplasmic histone-associatedDNAfragments were determined by Cell Death Detection ELISA^{PLUS} (Roche) in accordingly to the manufacturer's instruction. Briefly, $4x10^4$ HUVEC cells were cultured in 24 wells and incubated in EBM-2 serum free medim or with VEGF 50 ng/ml and Ang-1 300

ng/ml stimulation. Cell lysates from $1X10^4$ were placed in a streptavidin-coated micro-plate. A mixture of anti-histone-biotin and anti-DNA-POD (mouse monoclonal antibody conjugated with peroxidase) was added followed by incubation for 2 hours at room temperature. After removal of unbound antibodies by washing, the peroxidase activity of the POD complex was determined photometrically at 405 nm with ABTS as a substrate of the Horse Radish Peroxidase.

Western blot analysis

To obtain total cel lysates, cells were serum deprived for 2.5 hours and stimulated with VEGF (50 ng/ml) or Ang-1 (300 ng/ml) for the indicated times. Total protein were extracted in Laemmli buffer (62.5 mM Tris-HCL, pH 6.8, 2% SDS and 10% glycerol) and quantified; an equal amount of each sample was then resolved by SDS-PAGE and transferred to PVDF membrane. After blocking with TBS/0.1% Tween 20/5% BSA, membranes were incubated with primary antibody overnight ar 4°C. The following primary antibodies were used: rabbit α-pS473Akt, mouse α-γTubulin, α-p85β (all from Cell Signaling Technology). Lysates were subjected to immunoblotting, and visualized by the enhanced chemiluminescence system.

Zebrafish experiments

Wild Type and *Tg(flk:GFP)*^{s843} embryos were injected at the one-cell stage with 1 nl of 10 mM Morpholino (Gene-Tools) against the miR-126 sequence or standard control with scrambled sequence. Embryo development was assessed at 24-72 hours post-fertilization. For *in situ* hybridization the probe matching with the miR-126

sequence, was Digoxigenin labelled (Exiqon). Embryos were treated with proteinase K 10 μg/ml and fixed in 4% paraformaldehyde solution for 20 min at room temperature. Hybridization was performed in 50% formaldehyde solution with SSC 5X buffer, 0,1% Tween, 1M citric acid, pH 6.0, heparin 50 μg/ml, tRNA 500 μg/ml. As pre-incubation, embryos were first soaked in the hybridization buffer for 2 hours, after which they were incubated overnight in the presence of Digoxigenin labelled probe (Exiqon) to a final concentration of 40 nM (incubation temperature 53°C). The day after several washes were performed, initially with hybridization buffer and subsequently with SSC/PBS-0,1% Tween, embryos were then incubated overnight with an antibody against Digoxigenin. The antibody was alkaline phosphatase (AP) conjugated and the AP activity was detected by NBT/BCIP colour reaction.

Bioinformatics identification of miRNA targets

The analysis was made by searching for putative seed sequences for miR-126 in the 3'UTR of up-regulated genes. MiR-126 target enrichment analysis was performed on the basis of the Miranda database, as provided by Diana miRGen. Significance was estimated on the basis of hyper geometric distribution p-values comparing the occurrence of miR targets in the signature with respect to the universe, defined as the genes expressed in HUVEC. Frequency of log 2 ratio between the subset of mir-126 targets and the genes which are not targets of mir-126 were plotted, highlighting enrichment of the target of miR in differentially regulated genes.

Mouse Aortic Ring Angiogenesis assay

The mouse aortic ring assay was performed as previously described (Roca et al., 2003) with modification, Briefly, thoracic aortas were removed from 8- to 12-week old wild-type C57/BL6 mice (Charles River) and fibroadipose tissue was dissected away. Aortas were sectioned in 1-mm-long aortic rings and incubated for 2 days in serum-free medium with antibiotics, polybrene and lentiviral supernatant. Forty-eight-well dishes were coated with 100 μl of Basement membrane Extract (BME) and allow to solidify. Then aortic rings mixed with BME were loaded into the coated wells together with growth factor (VEGF-A 50 ng/ml, Ang-1 300ng/ml).

Endothelial Spheroid Assay

EC spheroids were generated as previously described (Korff and Augustin, 1998). Briefly, endothelial cells at concentration of 4000 cells/ml were resuspended in 20% methiocell and placed over-night in nonadhesive multi well plate. Then spheroids were collected and placed in collagen gel together with growth factors (VEGF-A 50 ng/ml, Ang-1 300ng/ml) for 24 hours. Lengths of sprouts outgrowth were quantified with the imaging software winRHIZO Pro (Reagent Instruments).

RESULTS

miR-126 is specifically expressed in the endothelium

In order to identify the microRNAs (miRNA) expressed by the vascular endothelium we analyzed miRNA expression levels in cells derived from endothelium and other tissues. By this screening we identified miR126 as the most specifically expressed miRNA in endothelial cells (EC). MiRNA126 was highly expressed in human umbilical vein EC (HUVEC), dermal microvascular EC and murine immortalized EC, whilst it was almost absent in fibroblasts, and smooth muscle cells (Fig. 1a).

To confirm the restricted endothelial expression of miR126, in situ hybridization analysis was performed on zebrafish embryos using LNA (Locked Nucleic Acid) probes. At 5 day post-fertilization (5dpf) we observed the expression of miR-126 in the vascular tree. High levels of miR126 were detected in large vessels as posterior cardinal vein (PCV) and dorsal aorta (DA), as well as in intersomitic vessels (ISV), head vasculature and endocardium (Fig. 1b).

This restricted expression pattern allowed us to examine the miR126 expression level in zebrafish embryos at different stages of development by qPCR (Fig. 1c). MiR-126 was detectable from the 8th somite stage (around 13 hpf) and further increased in later stages of development, with a maximal expression level after 5 dpf.

Notably, the differentiation of angioblasts from the mesodermal layer, with the early steps of vasculogenesis occurs before the 8th somite stage, whilst the highest expression of miR-126 is concurrent with a completely developed vascular system(Isogai et al., 2001).

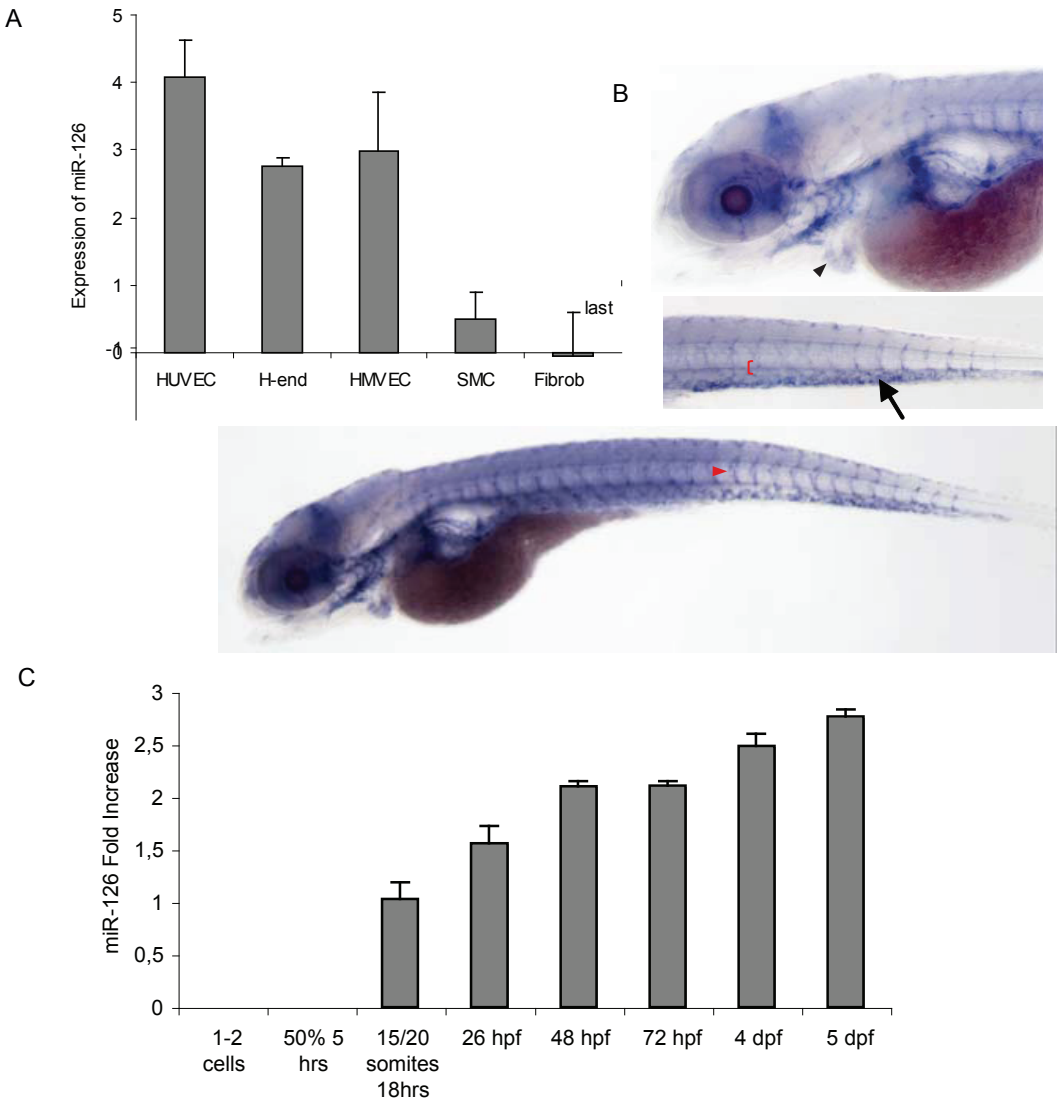


Figure 1. miR-126 is specifically expressed in endothelial cells

(A) Expression of miRNA miR-126 assayed by RT-PCR carried out on different cell types. Level of expression is presented as fold-increase with respect to MCF-10A miR-126 expression level.

(B) Specific detection of miR-126 using miRCURY LNATM microRNA Detection Probe for in situ hybridization in whole mount zebrafish embryo. The main vessels are labeled by the probe: Venus plexux (black arrow). Aorta (red brace), Heart (blach arrowhead) and Intersomitic Vessels (red arrowhead).

(D) miR-126 expression during different stages of zebrafish development. The miRNA expression is presented as fold-increase with respect to 1-2 cells stage of development

miR-126 modulates vascular maturation in zebrafish embryos

The specific spatio-temporal expression of miR-126 suggested that it could be involved in the process of vascular remodeling. Therefore, we investigated the role of miR-126 in this process by loss-of-function experiments in zebrafish embryos injected at the one cell stage with anti-miR-126 morpholinos.

Gross observation of anti-miR126 morphant embryos showed evident abnormalities in the whole body shape likely caused by pronounced oedema (Fig. 2a). The number of living miR-126 injected embryos progressively decreased from 4 dpf reaching 10% in adult animals. Nevertheless, experiments with anti-miR126 performed on transgenic fli-GFP zebrafish did not display any macroscopic defects of vasculature, such as lack of intersomitic vessels or dorsal aorta and cardinal vein. A more detailed analysis by confocal microscopy highlighted maturation defects of the vascular tree (Fig. 2b). In particular, dorsal aorta and intersomitic vessels appeared to have a reduced diameter (Fig. 2c). Besides vascular defects, cardiac development was also impaired with a reduction of the size, in particular of the atrium (Fig. 2d). Although the circulation was functional (data not shown), the heart abnormalities were likely to be the main cause of embryo mortality.

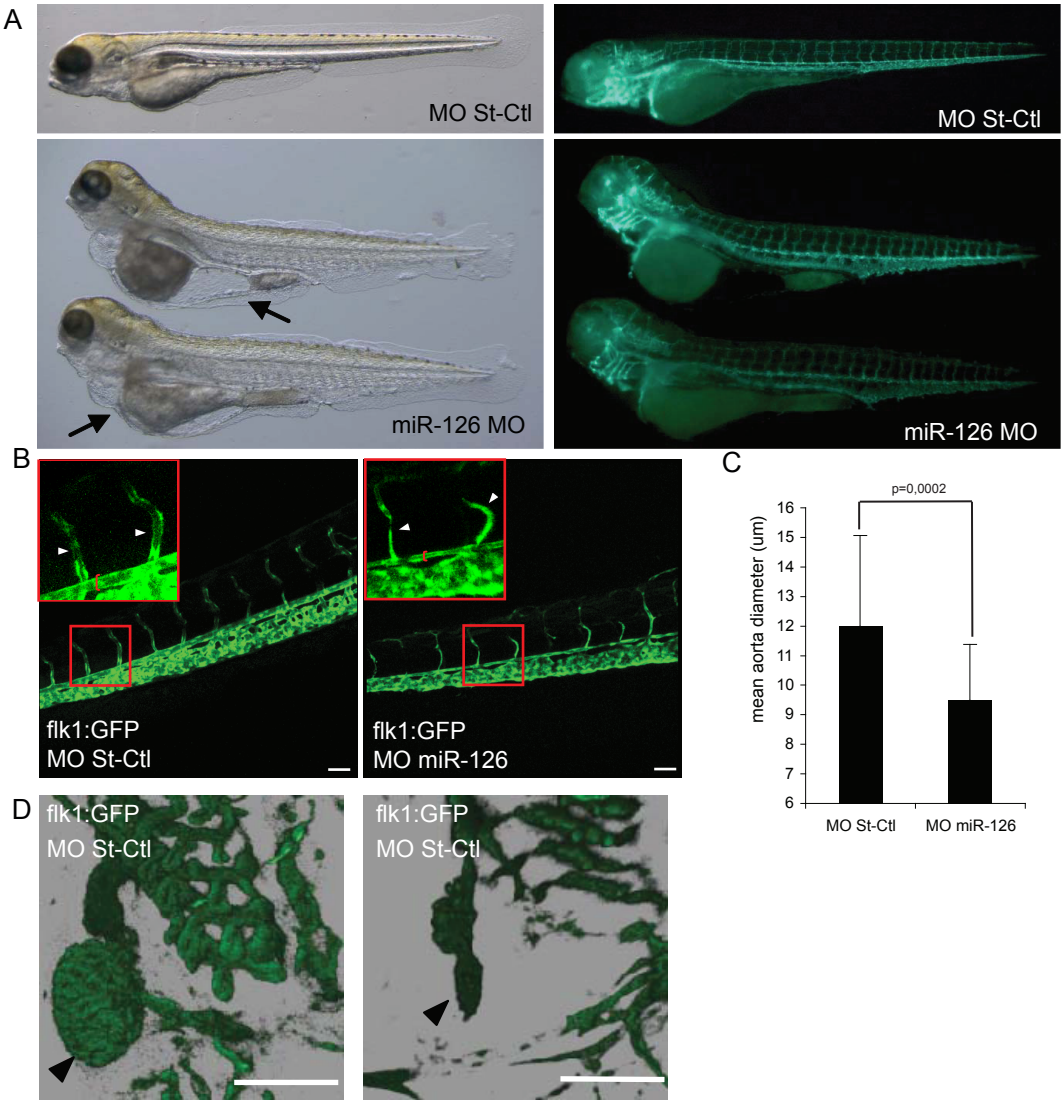


Figure 2. miR-126 modulates vascular maturation in Zebrafish embryos

(A) Lateral view of control and miR-126 MO injected Tg(flk:GFP)s843 embryos. Brightfield microscopy (left) and flk1:GFP (right). Mir-126 morphants (5dpf) present abnormal body shape with oedema (black arrow). (B) Tail lateral view by confocal microscopy of control (left) and miR-126 MO (right) Tg(flk:GFP)s843 embryos 7dpf (backward to the left), magnification of the tail vessels is shown in the red boxes. Morphants show reduced DA diameter (red braces) and improper ISV (white arrowhead). (C) The Graphics show the quantification of the mean aorta diameter. (D) Impairment of heart formation in morphant embryos compared to the normal heart shape in control (black arrowhead).

Identification of mRNAs regulated by miR-126

In order to identify genes targeted by miR126, we developed an assay to detect the mRNAs differently expressed in cells transfected with anti-miR-126. ECs were co-transfected with anti-miR-126 and a plasmid vector bearing three complementary sites for miR-126, downstream the GFP-coding sequence. As a consequence of the presence of miR-126 binding sites the GFP expression resulted to be under the control of miR-126 and therefore repressed in ECs. The expression of anti-miR-126 allowed GFP expression by antagonizing the basal level of miR-126 (Fig. 3a). GFP-positive cells were sorted and their expression profile was compared to GFP negative cells transfected with scrambled anti-miR (Fig. 3b). The differentially expressed genes were filtered through bioinformatics analysis to find out potential miR-126 targets. A list of fifteen putative targets was carried out and one of the most up-regulated genes was PIK3R2 (p85β). To validate the regulatory effect of miR126 on p85β, qPCR analysis was performed on different RNAs. PIK3R2 mRNA was up-regulated in cells transfected with the anti-miR-126 oligo (Fig. 3c)

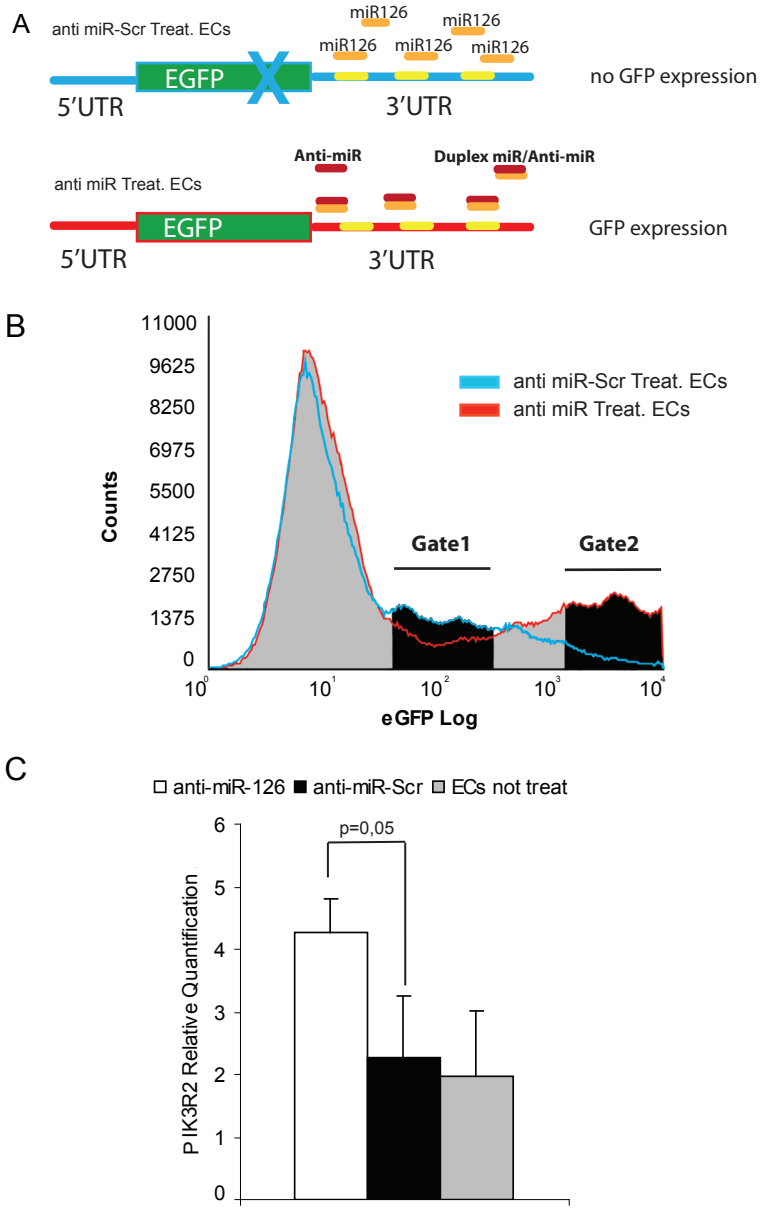


Figure 3. Identification of miR-126 targets by microarray analysis

(A) Scheme of the experimental design carried out to perform the microarray study: eGFP plasmid vector bearing the CDS three complementary sites for miR-126 downstream. In ECs this eGFP construct is under the repression of miR-126, resulting in low fluorescent cells. Conversely, if it is introduced with an oligo anti-miR-126 it leads to higher fluorescente cells. (B) Cytofluorimetric analysis of the EC cells transfected with eGFP constructs together with anti-miR-126 or control oligo with scrambled sequences. The two populations were then sorted accordingly to the gates indicated in the graph in order to isolate fluorescente cells against not fluorescence ones. (C) p85b mRNA expression level assayed by qPCR on the same RNA samples exploited for the microarray.

p85β is downregulated by miR-126

To better define the role of miR-126 on p85β regulation at post-transcriptional level, Hela cells were co-transfected with pre-miR-126 together with p85β cDNA containing the 3'UTR sequence. Pre-miR-126 strongly reduced p85β expression compared to cells transfected with pre-miR-control (Fig. 4a). As a mirrored experiment, ECs transfected with the anti-miR-126, showed a higher amount of p85β protein compared to EC transfected with control (Fig. 4b).

The specificity of miR-126 for the 3' UTR region of p85β was investigated by co-transfecting Hela and endothelial cells with pre-miR-126 and Renilla coding sequence under the control of p85β 3'UTR sequence. In the presence of miR-126, the activity of the reporter gene was significantly reduced compared to cells transfected with control oligos. Moreover, mutations inserted into the putative 3'UTR target sequence completely abolished this effect, in particular the mutation consisted in the ablation of the sequence within the 3'UTR of the p85β messanger predicted to be recognized by the seed sequence of the miR-126. Similarly, ECs co-transfected with the gene reporter and anti-miR-126 showed a specific effect of anti-miR126 on the 3'UTR sequence of p85β (Fig. 4c).

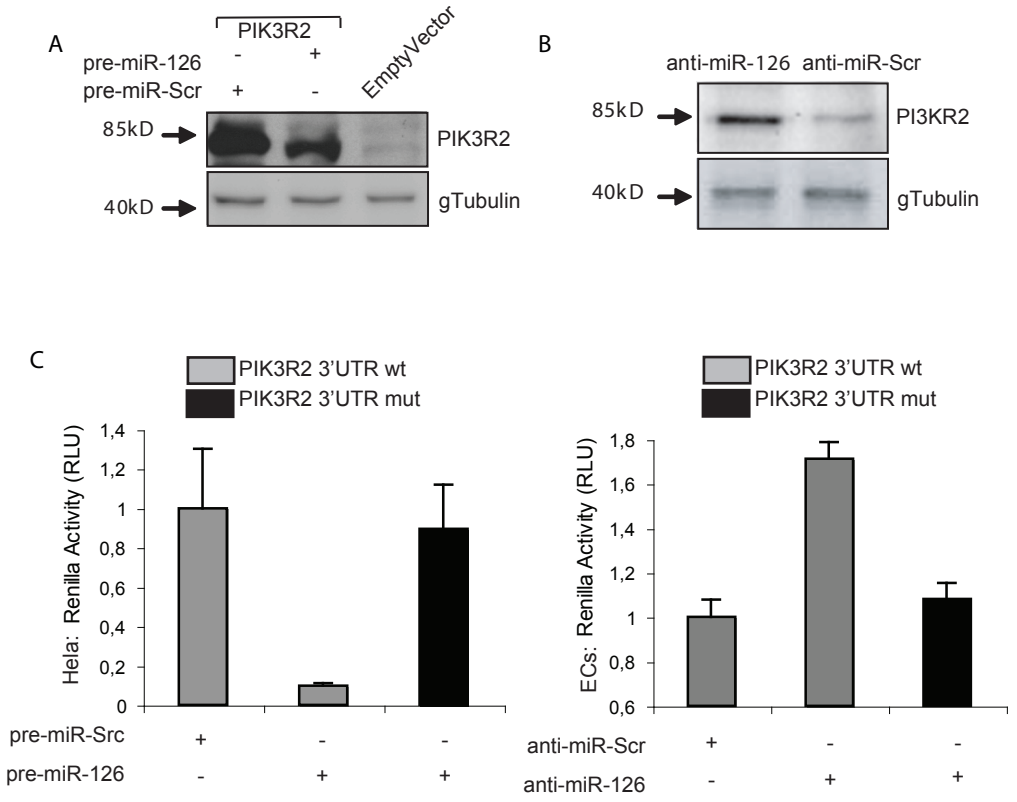


Figure 4. Identification of miR-126 targets by microarray analysis

(A) Immunoblot analysis for p85b protein in Hela cells lysates transiently transfected with pre-miR-126 together with the construct over-expressing p85b full length mRNA. (A) p85b detection by immunoblotting in lysates from ECs transiently transfected with LNA-anti-miR126. (C) Relative luciferase activity of constructs containing the p85b 3'UTR downstream the Renilla CDS and cotransfected with pre-miR-126 in Hela cells (left) and with LNA-miR-126 in ECs (right). In both experiments the assay was also performed with a 3'UTR of p85b mutated in the miR-126 predicted matching sequence (black bars).

High levels of p85b inhibit Ang1-dependent Akt activation

Since p85β is one of the regulatory subunits of the class IA PI3K enzyme which plays a pivotal role in transducing signals from tyrosine kinase receptors, we focused our attention on the two most important signaling pathways in EC: the vascular endothelial growth factor (VEGF)/VEGF Receptor 2 and Angiopoietin-1 (Ang-1)/TIE2. Both signaling pathways activate the serine/threonine kinase Akt, which is phosphorylated upon engagement with PIP3. For this reason we analysed the effect of miR-126-downregulation on Akt phosphorylation upon stimulation with VEGF-A and Ang1. Down-regulation of miR126 with anti-miR increased the expression of p85beta and slightly reduced serum- and VEGF-A-induced Akt phosphorylation (Fig. 5a). However, ECs stimulated with Ang1 showed a more pronounced Akt inhibition (Fig. 5a). To demonstrate that the level of p85β was determinant for modulation of Ang1 signaling, we over-expressed the p85β protein by lentiviral infection in EC. We evaluated whether p85β over-expression modified Akt activation in ECs stimulated with VEGF-A or Ang1. The level of Akt phosphorylation was completely impaired in p85β over-expressing ECs when they were stimulated with Ang-1 (Fig 5b). Moreover, upon Ang-1 stimulation p85β associated with Tie2 and was tyrosine phosphorylated (Fig 5c).

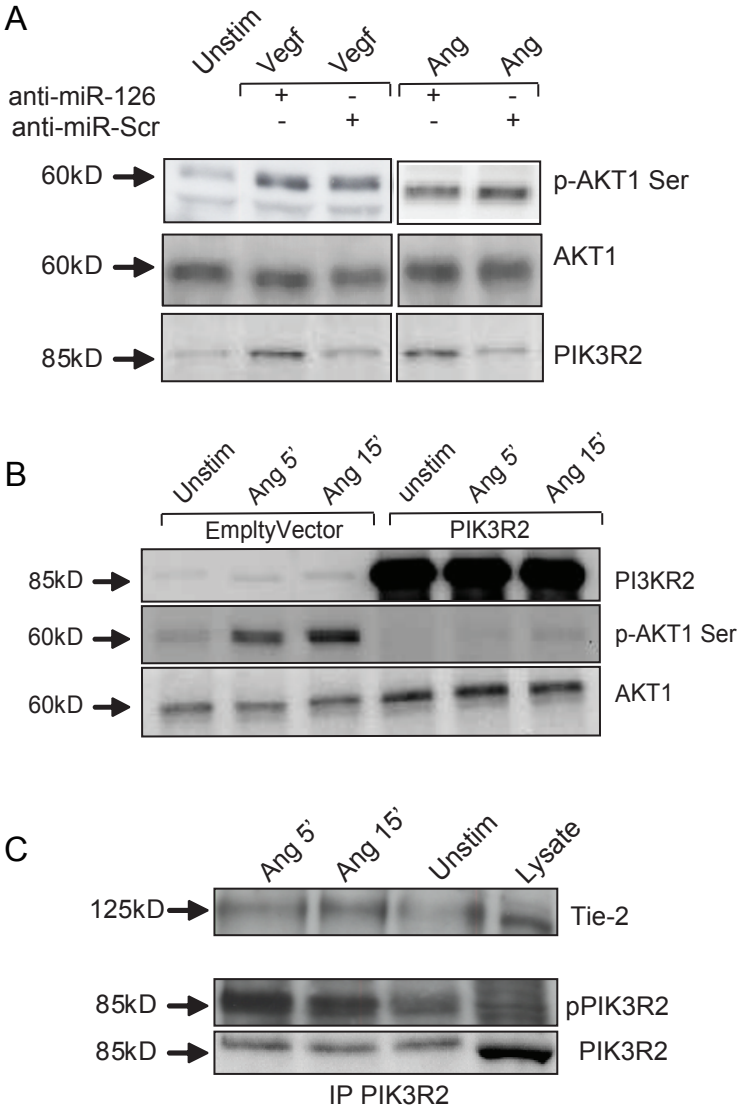


Figure 5. High levels of PIK3R2 inhibit Ang-1 dependent Akt activation

(A) ECs transfected with anti-miR-126 or anti-miR-Scr oligos and stimulated with VEGF or Ang-1, each lysate were immunoblotted as indicated. (B) ECs infected with lentivirus construct over-expressing p85b and stimulated with Ang-1, lysates were immunoblotted with the indicated antibody. (C) Lysates from ECs stimulated with Ang-1 were immunoprecipitated with antibody against p85b and immunoblotted with the indicated antibody.

Over-expression of p85β inhibits endothelial cells survival and sprouting angiogenesis induced by Ang1

To determine whether the modulation of Tie2 signaling by p85beta also affected the biological response of ECs to Ang-1, we performed apoptosis experiments based on DNA fragmentation in which ECs were maintained in serum-deprived medium, as an apoptotic stimulus, in the presence of VEGF or Ang-1, as anti-apoptotic factors. Both VEGF-A and Ang1 exerted their anti-apoptotic effect on ECs transduced with a control vector. In contrast, when p85β over-expressing ECs were treated with Ang-1, there was a higher level of apoptosis compared to EC transduced with the mock vector (Fig. 6a). However, VEGF-A treatment was still able to offer protection from apoptosis.

In addition to its anti-apoptotic role, Ang1 was previously described to be involved in vascular sprouting, remodeling and maturation. However, the availability of few in vitro assays complicated its functional characterization during these processes. We addressed this issue by performing experiments with endothelial spheroids and a modified version of the mouse aortic ring angiogenesis assay (MAR).

Endothelial spheroids experiment was used as three-dimension angiogenesis system in order to assay the sprouting capability of over-expressing p85b cells in response to VEGF-A and Ang-1. In normal condition a 12 hours of stimulation with both growth factors resulted in an increase sprouting which is more pronounced and massive with VEGF-A and with few, but frequently more elongate capillary like structure with Ang-1. The quantification of sprouting length in three independent experiments using endothelial cells infected with lentivirus expressing the p85b

protein showed a less sprouting phenotype with both VEGF-A and Ang-1. However the there is a significant more pronounced down modulation in spheroids treated Ang-1 (Fig. 6b), indeed as can be noted from the quantification graph, the fraction of effective sprouted p85b over-expressing spheroids is completely abolished.

Then, we moved to Mouse Aortic Ring. Even though in this assay Ang-1 is not able to activate endothelial sprouting, it potentiates the capability of VEGF-A to induce sprouting angiogenesis. We over-expressed p85β by lentivirus infection of aortic rings and we stimulated the formation of capillary-like structures in type-I collagen gel with VEGF-A or VEGF-A/ang1.

The lentivirus transduction efficiency on MAR was evaluated by fluorescence microscopy looking at the GFP expression while the expression of p85β was determined by real-time PCR on cells extracted from the gel. An increase of p85β mRNA was observed in MAR infected with the lentivirus carrying the cDNA compared to those infected with GFP alone (Fig. 7a). In the presence of VEGF, MAR infected with control vectors sprouted forming a network of tubular structures. The addition of Ang1 to culture medium significantly increased the length of these endothelial structures. While p85β over-expressing MAR responded to VEGF-A without severe impairment in sprouting angiogenesis, the stimulation with Ang1 did not potentiate the angiogenic effect of VEGF-A (Fig. 7b). These results were not completely unexpected, and together with the severe impairment of Ang-1 sprouting in spheroids support the extensively demonstrated concept that the PI3K/Akt signaling pathway is required for the Ang1/Tie2 biological effect (DeBusk et al., 2004; Fujikawa et al., 1999; Kim et al., 2000) while the effect triggered by VEGF-A is only partially dependent by this signaling pathway.

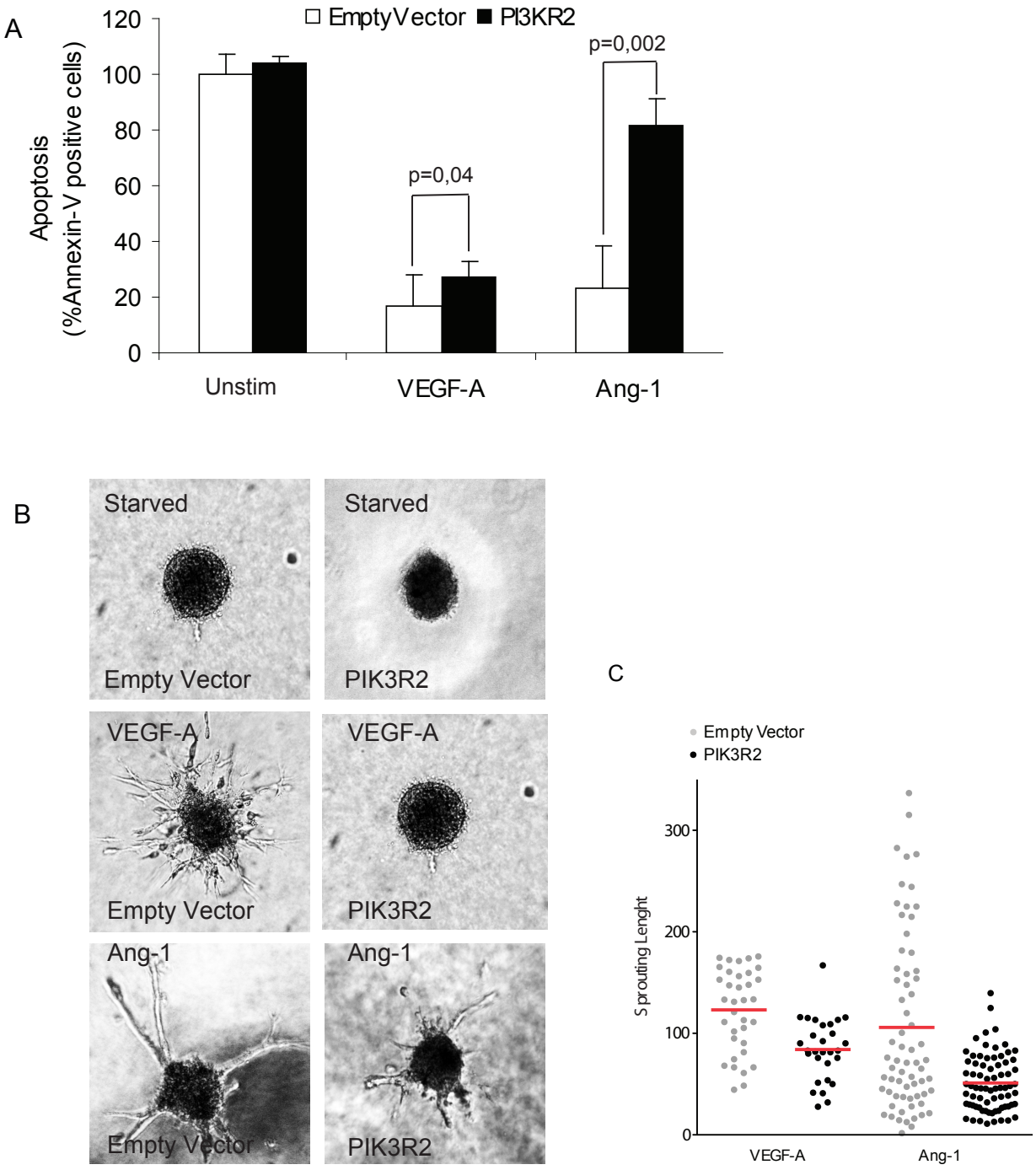


Figure 6. Over.expression of PIK3R2 inhibits endothelial cells survival and sprouting

ECs overexpressing p85b were overnight stimulated with Ang-1, VEGF or maintained in serum deprived medium, apoptosis was then assayed with Nucleosome-associated DNA fragments detection by ELISA assay (A). (B) Spheroids of ECs, embedded in collagen give rise to radial outgrowth capillary sprouts in presence of VEGF-A and Ang-1 after 24 hours of stimulation (left). Spheroids over-expressing p85b are partially inhibited in the formation of sprouts upon growth factors stimulation. Capillary sprouts outgrowth was quantified with imaging software analysis (C).

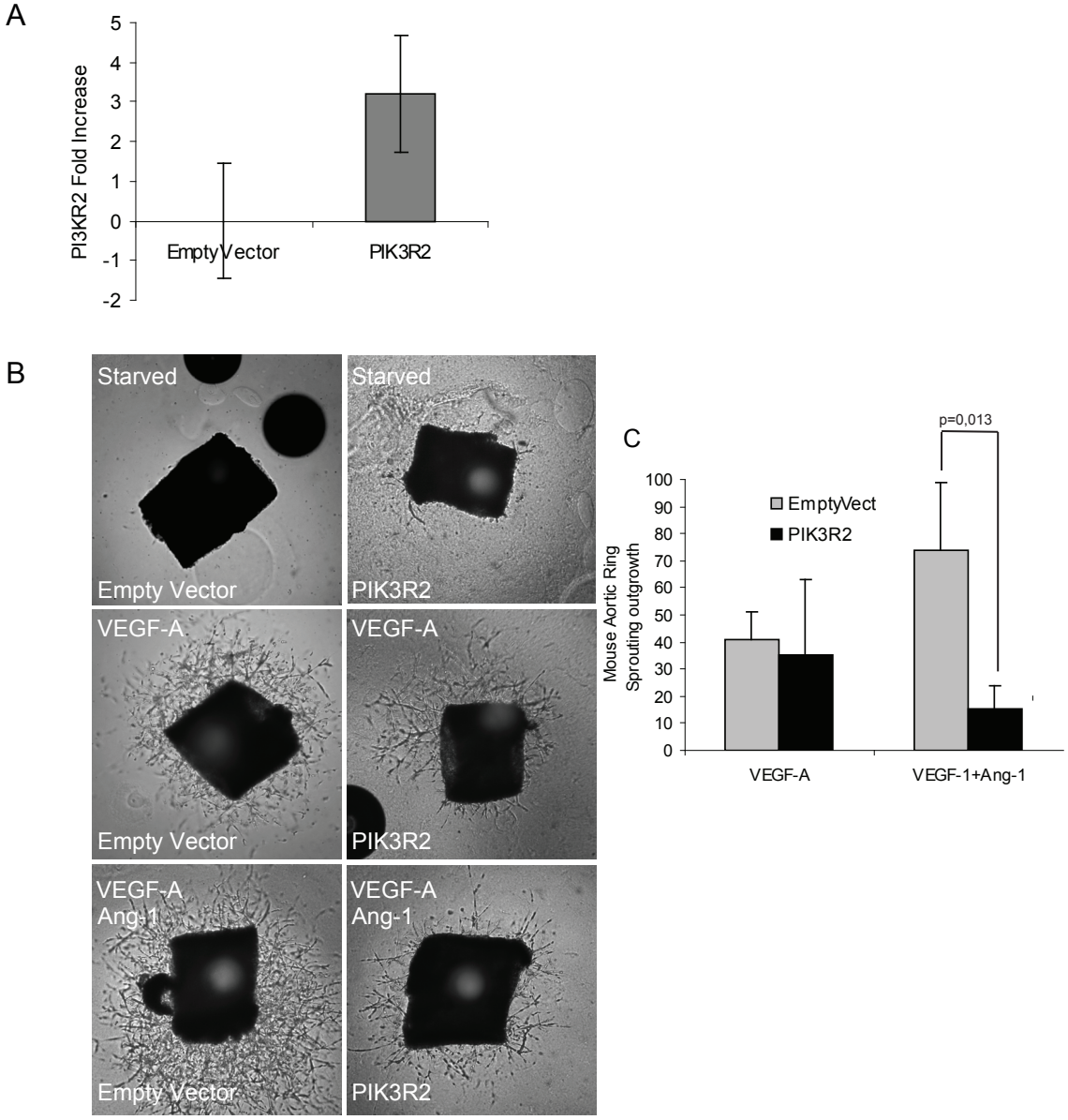


Figure 7. PIK3R2 over-expression affects sprouting of Mouse Aortic Ring assay induced by Ang-1

(A) Quantification of p85b expression in infected MAR by qPCR experiment on sprouting cells extracted from type I collagene gel. (B) Bright field images of MAR infected with lentivirus over-expressing p85b or control vector and stimulated with VEGF-A alone or in combination with Ang-1. (C) Quantification of the sprouts out growth from aortic rings infected and stimulated as showed in figure 7.c

DISCUSSION

The relevance of miRNA in the regulation of developmental processes and particularly in the definition of endothelial differentiation and vascular remodeling is now well demonstrated(Urbich et al., 2008; Wang and Olson, 2009). Specific miRNAs, required for the correct formation of vascular systems, are being defined and some of them have been functionally characterized (Anand et al.; Bonauer et al., 2009; Chen and Gorski, 2008; Dews et al., 2006; Poliseno et al., 2006; Wurdinger et al., 2008). Among these, miR-126 has been identified as one of the miRNA mostly expressed in endothelial cells(Fish et al., 2008; Wang et al., 2008). We found that miR126 is specifically expressed in several cells of endothelial origin whilst it is almost completely absent in cells derived from others tissues. Moreover, the high expression level in endothelial cells matches with the presence of miR126 in vessels of adult zebrafish. However, in zebrafish embryos, miR126 only begins to be detectable by sensitive methods, such as qPCR after 18 hpf, and reaches maximal expression in adult animals. This relatively late pattern of expression is in agreement with the phenotype observed with anti-miR-126 morpholinos embryo-injection, which displays vasculature defects at day 5 post-fertilization. These embryos showed a completely formed vascular system with defects related to maturation of the vessels, such as vascular leakage and maturation of large vessels. Cardiac developmental defects have also been observed in embryos with down-regulation of miR-126. The described phenotype is in agreement with previous observations of Fish et al.(Fish et al., 2008), and similar to the phenotype of miR126 knockout mice (Wang et al., 2008). These authors suggested that miR126 enhances the signaling activated by VEGF-A stabilizing and maintaining vessel integrity. However, it is not clear why VEGF-A signaling is enhanced, as this

pathway plays a fundamental role in endothelial differentiation, vascular sprouting and tube formation, and at this stage, these processes are almost completed. Moreover, it has been recently demonstrated that miR-126 expression is induced by blood flow through the zinc finger transcription factor *klf2a(Nicoli et al., ,)*, suggesting that miR126 is only involved in the final vascular shaping. This process of vascular maturation apparently requires blocking excessive angiogenesis and stabilizing vessels by pericyte coverage, effects which are not normally mediated by VEGF-A.

Our results show that miR-126 prevalently regulates signaling activated by Ang1, enhancing the response of ECs to this growth factor. Down-regulation of miR126 almost completely abolishes endothelial responses to Ang1, whilst signals due to VEGF-A stimulation, (and therefore its biological effects) are minimally affected.

Notably, the phenotype shown in zebrafish experiments with miR126MO is strikingly similar to those recently described for Ang1 morphant (Lamont et al.). Moreover, vascular leakage, reduced diameter of large vessels and cardiac defects are common alterations detected both in miR126-/- and Ang1-/- mice, suggesting a genetic interaction of these molecules (Suri et al., 1996; Wang et al., 2008).

A possible explanation for the ability of miR126 to target Ang1/tie2 signaling comes from the analysis of genes down-regulated by this miRNA. We identified several mRNAs regulated by the miRNA in ECs and, among these, we focused our attention on PI3KR2, the mRNA for the regulatory subunit beta of PI3K (p85β). In resting cells, p85β stabilizes the thermally labile catalytic subunit (p110) and conformationally inhibits its lipid kinase activity. Growth factor stimulation relieves the inhibition of p85β on p110, leading to the production of PIP_3 by p110. Many

different targets have been identified for miR-126, such as VCAM(Harris et al., 2008), EGFL7(Musiyenko et al., 2008), IRS-2(Zhang et al., 2008), HOXA9(Shen et al., 2008), but conclusive results have only been obtained for SPRED1(Fish et al., 2008; Nicoli et al.; Wang et al., 2008), while experiments with p85β produced contrasting evidence(Fish et al., 2008; Nicoli et al.).

We demonstrated that both p85β mRNA and protein are regulated by miR126, which targets the 3'UTR sequence of PIK3R2. Interestingly, several evidences have indicated that Tie2 activation by Ang1 requires PI3K to carry out any biological effect (DeBusk et al., 2004; Fujikawa et al., 1999; Kim et al., 2000) while it is dispensable for the several functions of VEGF-A. We clearly demonstrate that by altering expression levels of p85β, the signaling initiated by Ang1 is severely affected. High levels of p85β inhibit both the Ang1 anti-apoptotic effect and sprouting of endothelial spheroids and aortic ring, while the response to VEGF was less severe. Indeed, we noted that VEGF was still able to rescue from apoptosis the p85β over-expressing cells. It is worth noting that VEGF-A downstream signaling consists of multiple pathways and each of them triggers different effect in a strikingly context depend manner. We can speculate that this difference in the response to VEGF-A and Ang1 could be explained by the dependency on the PI3K/Akt signaling. The survival effect conferred by VEGF-A is partially overlapped between the PI3K/Akt and the MAPK/ERK signaling, this was demonstrated inhibiting the VEGF-induced ERK activation and endothelial cells survival blocking the ERK activity by the specific inhibitor PD98056 (Gupta et al., 1999) (Franklin and McCubrey, 2000). We can infer that VEGF-A induces cells survival with multiple and redundant pathways and that in the survival anti-

apoptotic context a deficiency of the PI3K/Akt pathway can be compensated by the MAPK/ERK.

In conclusion, we suggest that miR126 fine-tunes the opposite effects of VEGF-A and Ang-1 on vessel maturation and stabilization, enhancing Ang-1 signaling. Taking in consideration that PI3K enzyme is involved in many growth factor receptors downstream signaling, it not only influences the Ang1 cell responce, but it can affect signaling downstream the VEGFR2. We propose future prospects about the possibility that miR-126 acts in the balance between different signaling pathways downstream VEGFR2. One of these possibilities relies on the crosstalk between PI3K-ERK1/2 in the response to VEGF-A. Evidences from the literature pointed at the PLCγ-ERK1/2 a decisive role in arteriogenesis and early steps of cardio-vascular development (Deindl, 2007) (Isogai et al., 2003) (Lawson et al., 2003). Moreover, partial inhibition of PI3K signaling activates the MAPK pathway by suppressing Akt activity and promote arteriogenesis (Ren et al.). The crosstalk between these two pathways is mainly based on the suppressing role of the active form of Akt-1 on Raf-1 (Zimmermann and Moelling, 1999). Since miR-126 regulates the activity of PI3K enzyme we postulate that it can be involved in the switch between the PLCγ-ERK1/2 and PI3K/Akt signaling, thus promoting stabilization and maintenance of vessels network through the enhancement of the latter cascade. Moreover, the delayed expression of miR-126 during vascular development matches with the maturation of vessels and it is in agreement with this hypothesis. Further experiments focused on in-vivo models for vascular development are required to address these aspects concerning the balance between the VEGF-A and Ang1 in endothelial response.

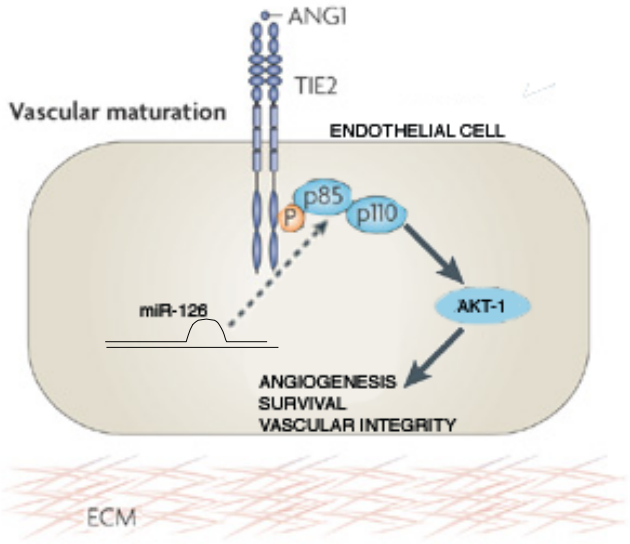


Figure 8. Model depicting how miR-126 regulates PIK3R2 expression level in order to enhance Ang-1 signalling and promote vessel maturation and stabilization.

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