FINAL REPORT MARIE CURIE ACTIONS - IEF

Project: Netrin-Angio; Researcher: Inma Segura

The central aim of this project was to identify candidate molecules involved in the guidance of vascular sprouts by using zebrafish and mouse as animal models and taking advantage of the striking similarities between the vascular and nervous systems. Indeed, a remarkable recent finding is that well-known axon guidance cues have been involved in vessel pathfinding as well. Molecules belonging to the netrin/DCC/UNC, Slit/Robo, semaphorin/NRP/plexin and Eph/ephrin families were originally described as axon guidance cues and can act on endothelial cells (ECs) [1, 2]. However, a role for netrin molecules in developmental vessel navigation or pathological angiogenesis is on debate, since it has been reported that they exhibit pro- and anti-angiogenic activities [3-5]. Although this controversial response has been linked to cell survival [6], it is not clear whether other mechanisms could be also involved.

In the nervous system, netrins induce attraction, repulsion, adhesion, proliferation and survival. These different cellular effects of netrin signaling depends on (*i*) the subset of receptors that is expressed by the target cell and (*ii*) the intracellular levels of second messengers. However, these two aspects of netrin-mediated signaling have not been studied in detail in ECs. Upon netrin stimulation, intracellular signaling involves tyrosine phosphorylation, phosphatidylinositol signaling and regulation of small Rho GTPases. In addition, downstream signaling regulates gene transcription and protein translation, although these effects remained poorly characterized, both in neurons and ECs.

The aim of this project was to analyze the effects triggered by netrins on ECs at the molecular and *in vivo* functional level.

Therefore, we studied modifications on gene expression patterns in cultured ECs after stimulation with two different netrin ligands: Netrin1 and Netrin4. We have performed mRNA microarrays to determine the gene expression profile of primary cultures of human venous and arterial ECs, upon Netrin1 or Netrin4 stimulation. In venous ECs, we found that the expression of 165 genes was regulated by Netrin1, 115 genes by Netrin4, while 98 genes were regulated in a opposed manner by both ligands ($p\text{-val}<0.001$). In arterial ECs, we found that 165 genes were regulated by Netrin1, 386 genes by Netrin4 and 121 genes were differentially regulated by both ligands ($p\text{-val}<0.001$). This differential regulation was further confirmed by quantitative RT-PCR for some of the candidates. Taking together, these results indicate that netrins induce a considerable shift in EC expression pattern, in both arteries and veins. Moreover, this genetic program is not common for both types of ECs, as only 7-13% of the regulated genes are shared by arterial and venous ECs.

To further characterize the role of these candidate genes during angiogenesis, we performed an unbiased selection of 34 candidates. These candidates have been implicated in different biological processes, including proliferation, cell signaling, gene regulation, adhesion, mRNA processing, or they had an unknown function. To determine the *in vivo* relevance of these candidates in the developmental formation of blood vessels, we used the larvae of Zebrafish as animal model. To determine the expression of the zebrafish orthologous genes of the candidates, we synthesized complementary DNA from a pool of different stages of zebrafish embryos and cloned probes to perform *in situ hybridization* (ISH) in zebrafish embryos. Analysis of the vasculature on zebrafish embryos revealed that 65% of these candidates (22/34) were expressed in the blood vessels (see figure 1 for expression pattern of 14 of the positive candidate genes). This list includes both known and previously unknown genes.

To study more in detail the role of these candidate genes during developmental angiogenesis, we microinjected morpholino antisense oligomers (morpholinos) into 1-2 cell stage of zebrafish embryos to specifically knockdown the expression of the candidates. The study particularly focused on the sprouting of stereotyped intersomitic vessels (ISVs) from the dorsal aorta.

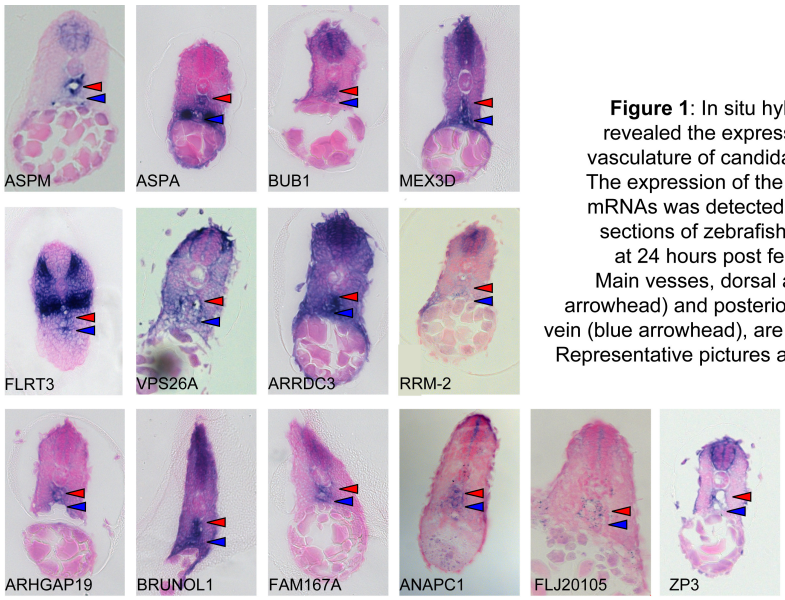

Figure 1: In situ hybridization revealed the expression in the vasculature of candidate genes. The expression of the indicated mRNAs was detected in sagital sections of zebrafish embryos at 24 hours post fertilization. Main vesses, dorsal aorta (red arrowhead) and posterior cardinal vein (blue arrowhead), are indicated. Representative pictures are shown.

Out of the 22 genes, positive for vascular expression, we selected 10 genes for functional evaluation during developmental angiogenesis in the zebrafish: FLRT3, RRM2, ZP3, PBK, FLJ20105, BrunoL1, ARHGAP19, ARRDC3, FAM167A and TTYH1. Morpholino injections revealed that half of the tested candidates (ZP3, FLJ20105, FLRT3, BrunoL1 and FAM167A) were involved in developmental angiogenesis, as defective ISVs were detected upon morpholino injection. We are currently evaluating the characteristics of the defects. Moreover, these 5 genes were never before indicated as molecules involved in the angiogenic process, revealing that the screening that we have performed is a powerful technique to identify relevant novel players during blood vessel formation.

We selected FLRT3 for further characterization of its role during developmental and pathological angiogenesis, using the mouse as animal model. FLRT3 is a transmembrane protein without catalytic activity that is required for early embryonic development [7]. It has been recently shown that FLRT3 is a binding partner of UNC5B [8], one of the canonical receptors of netrin, but the function of this interaction is not fully understood, and its role in the vasculature has not been previously reported. Indeed, we have observed that FLRT3 is expressed in certain vessels, both in the mouse and in zebrafish. We have generated conditional knockout mice (constitutive or inducible) for FLRT3 in the vasculature (FLRT3cKO). These mice are viable and fertile. We are currently evaluating the effect of FLRT3 deletion on both physiological and pathological angiogenesis. Preliminary analysis revealed that FLRT3cKO are protected to melanoma progression (B16F10 tumor model), as they have reduced tumor metastasis number and better survival after tumor inoculation. We are currently evaluating vessel morphology and density in these tumors.

Taking together, the studies of this project have revealed that our strategy to screen for genes regulated by netrin signaling is an effective tool to identify new molecules involved in physiological and pathological angiogenesis. Unraveling the molecular mechanisms that rule these processes may have socio-economical impact in the long term, as they will allow the identification of new therapeutic targets for diseases associated with deregulated angiogenesis, such as cancer.

References

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