FINAL RELATION

In the recent years our laboratory at the Institute of Molecular Genetic– CNR, in Pavia has started to investigate the role of alternative splicing (AS) during angiogenesis.

We found that the AS factor Nova2, until now considered neural cell-specific, is also expressed in endothelial cells (ECs) of the blood vessels and regulated during angiogenesis. In the brain Nova2 controls AS of pre-mRNAs encoding for molecules involved in synaptic development, synaptic transmission, cell-cell signaling and dynamic organization of the actin cytoskeleton (Ule J et al., 2005). Several studies have highlighted significant similarities between the nervous and vascular systems and molecules affecting both neural and vascular functions are termed "angioneurins" (Zacchigna S et al., 2008). Through gain- and loss-of function approaches, we determined that Nova2 regulates AS of target genes encoding for factors essential in the establishment of ECs apical-basal polarity, a prerequisite for correct vascular lumen formation (Iruela-Arispe ML and Davis GE, 2009). Importantly, Nova2 mis-expression causes vascular lumen formation defects *in vivo* in zebrafish embryo, a powerful model to study vertebrate cardiovascular development and physiology (Manuscript in revision in *Nature Communication*).

In order to identify novel genes with an AS modulated by Nova2 in ECs, we performed RNA-Seq of the RNA extracted from ECs over-expressing or knockdown for Nova2.

Among the newly identified Nova2 target genes, *Unc5b* had caught our attention.

Unc5b, one of the four members of UNC5 family receptors for Netrin-1, plays important roles in axon guidance and in vascular patterning (Adams RH and Eichmann A, 2010). In addition, it is able to interact with the EC-specific axon guidance receptor Robo 4 and maintain vessel integrity by counteracting the VEGF/VEGFR signalling (Koch AW et al., 2011).

Validating the RNA-seq data we have found that Nova2 modulates the AS profile of *Unc5b* exon 8 promoting the exclusion of this exon from the mature transcript. Exon 8 of *Unc5b* encodes for 11 aminoacids located between the transmembrane domain and the second thrombospondin-like repeat in the extracellular region of the protein.

The role of the Unc5b isoform lacking exon 8 (Unc5bΔ8) has not been characterized in the literature. Thus, to investigate the possible functions of Unc5bΔ8 in ECs, in collaboration with the laboratory of Dr. Daniel Nyqvist (Karolinska Institute, Stockholm, SE), we have first examined its expression during developmental angiogenesis.

During my stay (from June 5, 2015 to July 6, 2015), I took advantage of the experience of this laboratory in using *in vivo* models to identify and characterize signaling pathways involved in

vascular development. In particular, the mouse retina has been used extensively to study both physiologic and pathologic angiogenesis. Using whole-mount immunostaining, simultaneous vascular sprouting at the periphery and remodeling at the center (observable, for example, at P5), will allow the study of different aspects of vessel formation, maturation, and specialization in a single preparation.

Since specific antibodies able to distinguish between the isoform lacking exon 8 from the full-length (FL) protein are not available, I used RNA *in situ* hybridization (RNA-ISH) technique to directly detect the expression of the different *Unc5b* mRNAs. In particular, I used LNA (Locked Nucleic Acid) oligonucleotide probes (from Exiqon) that offer substantially increased affinity for its complementary strand, compared to traditional DNA or RNA oligonucleotides. This results in unprecedented sensitivity and specificity and makes LNA oligonucleotides ideal for the detection of small or highly similar DNA or RNA targets. Notably, LNA probes have been successfully used in RNA-ISH with whole zebrafish and mouse embryos, but they never been used in RNA-ISH with whole mount mouse retina. I have used two LNA oligonucleotide probes:

1) "LNA exon8 only" able to detect the *Unc5b* mRNA generated from inclusion of exon 8 (FL isoform);
2) "LNA exon8 skipping", which spans the junction site between exon 7 and 9 and is able to detect the *Unc5b* mRNA generated from skipping of exon 8 (Unc5bΔ8).

As first step, in order to set the protocol for these different type of probes, I used as a positive control a LNA probe targeting the *vascular endothelial growth factor receptor 1* (*VEGFR*1) mRNA, which is mainly expressed in the ECs of the retinal vasculature. I dissected retinas from mice at postnatal day 10 (P10), when the vasculature is completely developed and, using the *VEGFR1* LNA probe, I obtained a specific signal in artery, veins and capillaries of the retina. Next, in order to test the expression of the *Unc5b* isoforms, I dissected retinas from mice at postnatal day 4 (P4), 6 (P6) and 10 (P10). I have choosen these stages because from the literature it is known that *Unc5b* is expressed in arteries, capillaries and endothelial tip cells of retinal vessels during active angiogenesis (from P0 until P9) and it becomes progressively downregulated once these vessels reach quiescence (P21).

I have found that the mRNA *Unc5b* containing exon 8 is expressed in ECs of the retina vessels during the stages P4, P6 and P10. More interestingly, I have found that the uncharacterized *Unc5b* variant lacking exon 8 (Unc5bΔ8) is also expressed during retina development *in vivo* but further investigations are needed to understand whether it is expressed differently by retinal vessels (artery, veins and capillaries) and in the ECs at the sprouting front as compared to the central part of the retina.

In conclusion, with the Short Term Mobility Grant, I have used for the first time the LNA probes to study the expression of specific AS variant in RNA-ISH with whole mount mouse retina and I have detected the expression of an uncharacterized *Unc5b* transcript.

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