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Xenopus cadherin 5 is specifically expressed in endothelial cells of the developing vascular system

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ABSTRACT Vasculogenesis is an important, multistep process leading to the formation of a functional primary network of blood vessels in the developing embryo. A series of interactions between secreted growth factors and their specific receptors leads to the specification of mesodermal cells to become hemangioblasts, which then differentiate into angioblasts. These subsequently proliferate, coalesce into cords and finally form tubular vascular structures. For proper function of these primary blood vessels, the close connection of endothelial cells is required. This is conferred by the interaction of an endothelium specific cadherin (Cadherin-5), starting during early vascular development. However, this interaction remains important throughout life and ageing. Therefore, cadherin-5 is a useful marker for late stages of vasculogenesis in several vertebrate species. To establish cadherin-5 as a marker for vascular studies in *Xenopus,* we cloned the *Xenopus laevis* ortholog and analyzed its expression pattern during embryogenesis.

KEY WORDS: *vasculogenesis, cadherin, early embryogenesis, ageing, paralogs*

Identification and cloning of the Xenopus laevis cadherin-5 *ortholog*

The cardiovascular system is the first organ system whose function is required for efficient exchange of nutrients, gases and waste products and in consequence for continuous growth of a developing embryo (Risau, 1995). A primary vascular network is established by the process of vasculogenesis (Pardanaud *et al*., 1996). In a first step mesodermal cells differentiate into precursors of the endothelial and blood cell lineage (hemangioblasts) short after gastrulation (Turpen *et al*., 1997). Commited angioblasts proliferate as a response to VEGF signals from regions adjacent to the mesoderm. VEGF ligand binds to three tyrosine kinase receptors, VEGFR-1 (FLT-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4) (Holmes *et al*., 2007) (Dumont *et al*., 1995) (Cleaver *et al*., 1997). Flt-1 and Flk-1 both bind VEGF-a and functional studies demonstrated that they are required for vasculogenesis during embryonic development, probably due to the activity of Etv2, an ETS-protein related transcription factor (Flamme *et al*., 1995) (Fong *et al*., 1995) (Neuhaus *et al*., 2010) (Salanga *et al*., 2010). Corresponding to their expression in vascular endothelial cells, Flt-1 and Flk-1 are the earliest known endothelial marker genes (Breier *et al*., 1996) (Fong *et al*., 1995). Subsequent to their proliferation the angioblasts coalesce into continuous cords. The formation of open spaces between two cells of these endothelial cords starts the development of endothelial vascular tubes (Houser *et al*., 1961). Cells forming these tubular structures now express junctional proteins as vascular-endothelial cadherin (cadherin-5, cdh5, CD144), which is frequently used as a marker for advanced stages of vasculogenesis and angiogenesis (Breier *et al*., 1996). However, in mice *cdh5* expression and *flk-1* expression were both detected by *in situ* hybridization in mesodermal cells of the yolk sac mesenchyme forming the first blood islands already at embryonic day 7.5 (Breier *et al*., 1996; Yamaguchi *et al*., 1993). Mouse embryos carrying a homozygous null mutation of *VE-cadherin* showed severe vasculogenic defects in the yolk sac and the embryo. From E9.5 those embryos were abnormal and died at E11.5 (Gory-Faure *et al*., 1999). In zebrafish embryos *cdh5* expression is already detectable in anterior and trunk mesodermal cells at 12 hpf before formation of vessel primordia (Larson *et al*., 2004). A knockdown of VE-cadherin in zebrafish did not affect vascular development and vessel sprouting, but cardiac looping and circulation were impaired. Additionally, the separation of the myocardial and endocardial layer were abnormal (Mitchell *et al*., 2010). To establish *cdh5* as a marker for the analysis of blood vessel development in the frog we cloned the *cdh5* ortholog from

Abbreviations used in this paper: VEGF, vascular endothelial growth factor.

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web: http://www1.medizin.uni-halle.de/ag-hollemann/hollemannhome.html

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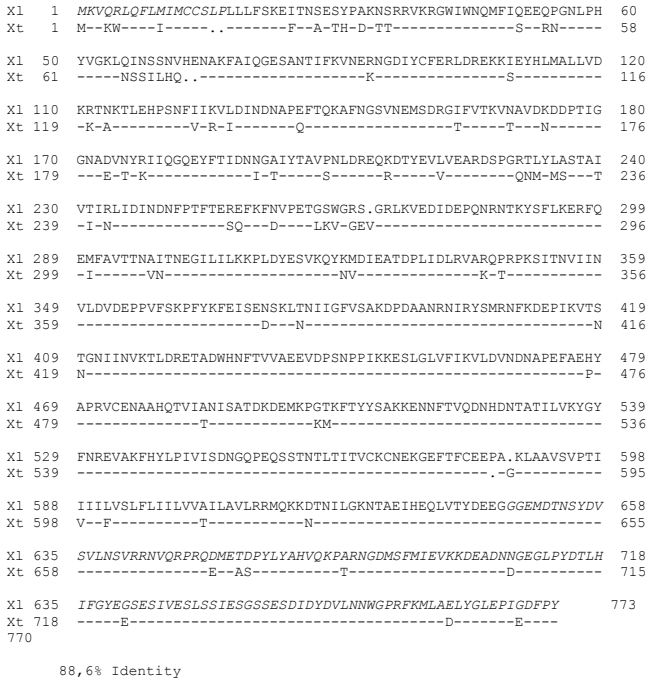
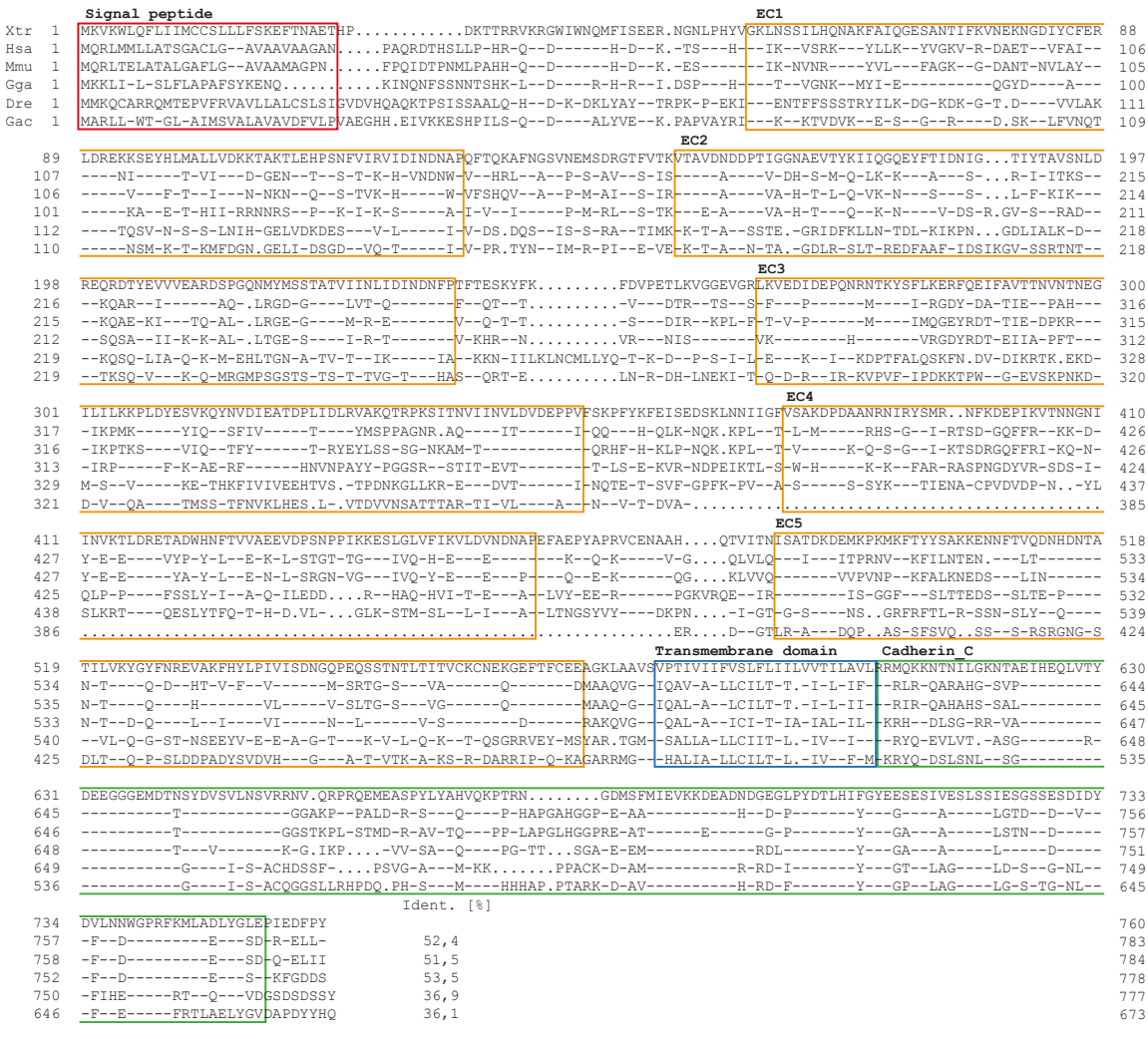


Fig. 1. Sequence alignements of vertebrate CDH5 proteins. (A) *Comparison of the putative amino acid sequence of the newly cloned cdh5 cDNA from* Xenopus laevis *with the cdh5 amino acid sequences from* Xenopus tropicalis *demonstrates that cdh5 is well conserved between the two frog species, sharing 89% identity to each other.* **(B)** *Comparison of the deduced cdh5 amino acid sequences from* Xenopus laevis *(Xla; GenBank Accession no. KF279630),* Homo sapiens *(Hsa; GenBank Accession no. NP_001786),* Mus musculus *(Mmu; GenBank Accession no. AAH54790),* Gallus gallus *(Gga; GenBank Accession no. AAN33002),* Danio rerio *(Dre; GenBank Accession no. AY496430). The N-terminal signal peptide is boxed in red, the five typical extracellular Ca2⁺-binding Cadherin repeats are boxed in yellow, the transmembrane region is boxed in blue and the intracellular, catenin binding domain is boxed in green. Percentage identities are indicated at the end of the aligned sequences.*

B



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Prot B  1    MKVQRLQFLIIMCCSLPLLLFSKEITNSE-TPPHKNSRRVKRGWIWNQMFISEEQKTGNL  59

Prot A  59   PHYVGKINSSNVHENAKFAIQGESANTIFKVNERNGDIYCFERLDREKKIEYHLMALLVD  118
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Prot A  239  VTIRLIDINDNFPTFTEREFKFNVPENLKVGAEVGRLKVEDIDEPQNRNTKYSFLKERFQ  298
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Prot A  359  VLDVNEPPVFSKPFYKFEISEDSKLNNIIGFVSAKDPDAANRNIRYSMRNFKDEPIKVTS  418
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Prot A  419  TGNIINVKTLDRETADWHNFTVVAEEVDPSNPPIKKESLGLVFIKVLDVNDNAPEFAEHY  478
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Prot A  539  FNREVAKFHYLPIVISDNGQPEQSSTNTLTITVCKCNEKGEFTFCEEPAKLAAVSVPTII  598
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Prot A  659  VLNSVRRNVQRPRQDMETDPYLYAHVQKPARNGDMSFMIEVKKDEADNNGEGLPYDTLHI  718
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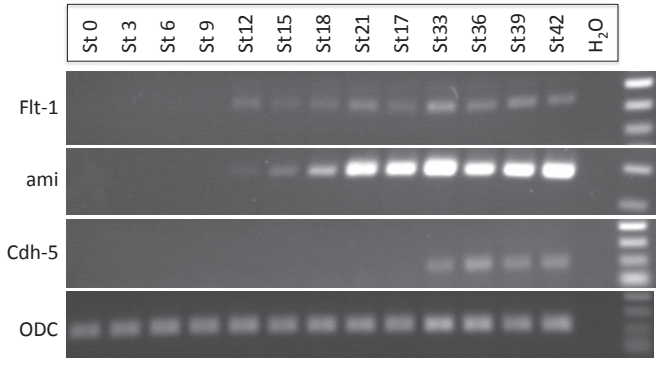

Fig. 2 (Left). Alignment of the putative amino acid sequence from both *cadherin-5* paralogs. *Both sequences are highly conserved, showing 97.5% similarity.*

Fig. 3 (Right). Temporal analyses of *cadherin-5* expression. Expression of xl cdh5 *mRNA was compared to the expression of early vasculogenesis marker genes* flt1 *and* ami *by semi quantitative rt-PCR. Expression of* flt1 *and* ami *could be detected already at low levels at NF stage 12, whereas expression of* cdh5 *was not detectable before stage 27, demonstrating that* cdh5 *expression in* Xenopus *is restricted to later stages of vessel formation. To exclude false results from genomic DNA contamination we used intron spanning primer pairs.* ODC1 *was used to control the amount of input RNA.*

Xenopus laevis and analyzed the temporal and spatial expression of *cdh5* during *Xenopus* embryogenesis.

Cloning of Xenopus laevis cadherin-5

Surprisingly no *Xenopus laevis* sequences, which are orthologs to *cadherin-5*, could be found in cDNA-based sequence databases. We used the *cdh5* sequence of *Xenopus tropicalis* to design a number of different primer pairs covering the entire open reading frame to clone the *Xenopus laevis* cDNA. Using these primer combinations on NF stage 36 *Xenopus laevis* cDNA, we were able to clone a 1,9 kb cDNA fragment containing a continuous reading frame of 634 aa. Despite several efforts, we were not able to clone the 5'- and 3'-part of the *xl-cdh5* cDNA.

To obtain the potential full open reading frame we made use of the recently released genomic sequences of *Xenopus laevis*. *Xenopus laevis* contains two genes for cdh5 of which one is identical to our cloned cDNA. Short extensions of the 5'- and 3'-end allowed to deduce a full length putative cadherin 5 protein, which showed all the typical characteristics of a type 2 cadherin e.g., a signal peptide, five internal homologous repeats (EC1 to EC5), a transmembrane domain, a cytoplasmic domain, four conserved cysteine residues, three putative calcium binding motifs (DxNDNxP), and three four-amino acid repeats of unknown function (LDRE) (Vestweber, 2008). Comparison of the deduced amino acid sequence of the *Xenopus laevis* cDNA with cdh5 sequences of *Xenopus tropicalis* showed that both sequences are 88,6% identical and 94% similar to each other (Fig.1a). *Xenopus laevis* cdh5 was highly homologues to all other vertebrate cadherin-5 proteins included in this analysis. Highest homology was found with the cdh5 sequence of *Gallus gallus* (52,5%) and lowest homology was found with *Danio rerio* cadherin-5 sequence (36,2%*l*) (Fig.1b). Since two paralogous sequences are contained in the *Xenopu laevis* genome, we compared the putative amino acid sequnces of both paralogs. Both putative proteins are highly conserved sharing 97.5% similaritiy, suggesting that both genes might be expressed and have a highly similar function.

Temporal expression of cadherin-5 *during* Xenopus *development*

To analyze the temporal expression of *cdh5* during embryogenesis we prepared cDNA from mRNA collected from a series of *Xenopus* embryos at different developmental stages. To evaluate the onset of *cdh5* expression in the process of vasculogenesis we compared the expression pattern of *cdh5* with the expression of *ami* and *flt-1, which* are already expressed at the early stages of vascular endothelial cell differentiation (Inui and Asashima, 2006) (Fong *et al*., 1996). Our rt-PCR analysis showed that earliest *ami* and *flt-1* expression could be detected in late gastrula stage embryos at NF st 12 whereas *cdh5* expression could not be detected before the embryos reached tailbud stages (Fig.2). To analyze the expression of the two different genomic loci we designed two

A

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Locus A  474  TGTGGATAAAGATGATCCTACAATAGGTGGAAATGCAGACGTGAACTATAGAATCATCCA  533
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Locus B  477  TGTGGATAAAGATGATCCTACAATAGGTGGAAATGCAGATGTAACCTACAGAATAATCCA  536

Locus A  534  AGGACAAGAGTACTTTACAATTGATAACAATGGGGCAATTTATACTGCAGTACCTAATTT  593
              |||||||||||||||||||||||||||| |||| ||||||||||||| ||||||||||
Locus B  537  AGGACAAGAGTACTTTACAATTGATAACAGTGGGACAATTTATACTGCAATACCTAATTT  596

Locus A  594  AGACAGAGAGCAGAAAGATACCTATGAAGTTCTGGTAGAGGCCAGAGATTCTCCAGGGCG  653
              |||||||||||||||||||||||||||||| |||| |||||||||||||| ||||| |
Locus B  597  AGACAGAGAGCAGAAAGATACCTATGAAGTTGTGGTGGAGGCCAGAGATTCCCCAGGACA  656

Locus A  654  AACTCTTTACTTGGCAAGCACAGCCATAGTGACCATTCGCTTGATAGACATTAATGACAA  713
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Locus B  657  AACCATTTACCTGGCAAACACAGCCACAGTAACCATCCACTTGACAGACATTAATGACAA  716
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B

Fig. 4. Comparison of the temporal expression of the two *cadherin-5* paralogs. *Two primer pairs, specific for either locus A or locus B were used to compare the temporal expression of both paralogs. In figure 4a a part of the nucleotide sequence is shown, demonstrating the high degree of sequence conservation on the nucleotide level. Sequences used for primer pair A are marked in bold letters. <semi-quantitative rt-PCR in figure 4b shows that transcripts from both paralogs could be detected as early as st 27.*

primer pairs that are specific for only one of the two loci (A or B) and used them for rt-PCR on RNA from different developmental stages. Expression of both loci could be detected as early as st 30 and showed an identical temporal pattern. The identity of the PCR products was confirmed by sequence analysis.

Spatial expression of cadherin-5 *during* Xenopus *development*

Spatial expression of *cdh5* was analyzed by *in situ* hybridization on whole-mount and sectioned early *Xenopus* embryos. Transcripts were first detectable at NF stage 29/30 in the ventrolateral region, where the first vitelline veins form and in vessels around the developing eye. (Fig. 3). A few hours later at NF st 31 additional expression could be detected in the posterior cardinal vein, which starts to form at this stage of development. In swimming tadpoles at NF st. 36 *cdh5* becomes expressed in all vascular structures that are subsequently formed e.g.: the anterior aorta, the vessels of the branchial arches, the duct of cuvier, the retinal vein, the heart and the intersomitic veins (Fig. 3). Analysis of sectioned embryos demonstrated that expression of *cdh5* expression is strictly restricted to endothelial cells and could not be detected in surrounding tissues (e. g. the myocardial wall in the heart) (Fig. 4).

In this report we describe the cloning and the expression pattern of *Xenopus laevis cdh5*. The high degree of sequence similarity to other vertebrate cdh5 proteins in combination with the described spatial expression pattern strongly suggests that the cadherin gene we identified, is the *cdh5* ortholog of *Xenopus laevis*. Since the genome of *Xenopus laevis* contains two highly conserved paralogs of *cdh* we analyzed the temporal expression of both paralogs and could show that both genes are expressed in an identical temporal pattern. However, it is possible that the spatial expression patterns of the two paralogs are different. Unfortunately, due to the high degree of nucleotide sequence conservation it is impossible to distinguish the potentially different spatial expression of both paralogs by *in situ* hybridization.

Interestingly, in mice and zebrafish the onset of *flt-1* expression, as an early marker of vasculogenesis and *cdh5* expression, as a marker for differentiated vascular cells, coincides with the early

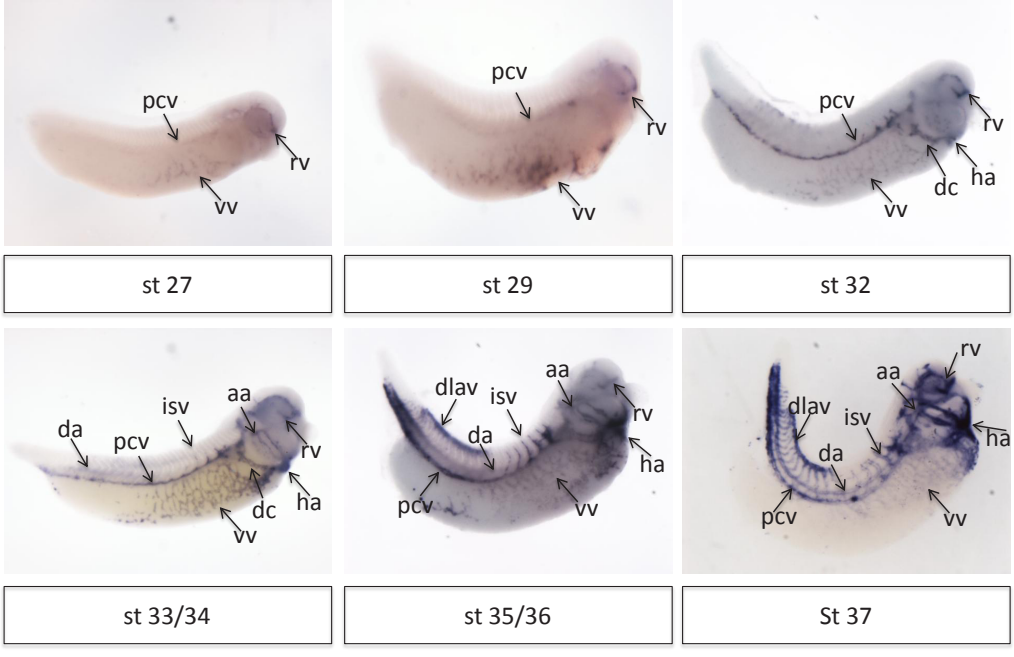

Fig. 5. Spatial analyses of *cadherin-5* expression. *Whole-mount* in situ *hybridization of wild type embryos at developmental stages 27 to 37. Earliest* cdh5 *expression was detectable at NF stage 27 when first vascular structures developed. Subsequently* cdh5 *expression could be detected in all newly formed vascular structures. The tissue of a NF stage 37 embryo was cleared before pictures were taken. Abbreviations: (aa) aortic arches, (da) dorsal aorta, (dc) duct of cuvier, (dlav) dorsal longitudinal anastomosing vessel, (ha) heart anlage, (isv) intersomitic veins, (pcv) posterior cardinal vein, (rv) retinal vein, (vv) vitelline veins.*

appearance of blood islands or mesodermal precursors of the vascular system, whereas in *Xenopus cdh5* expression was detectable solely much later in clearly formed vascular structures. This could allow an easier dissection of the developmental program leading from early vascular progenitor cells to differentiated endothelial cells forming functional blood vessels in *Xenopus*.

Materials and Methods

Animals

Pigmented and albino *Xenopus laevis* were obtained from Nasco (Ft. Atkinson, WI). Production and rearing of embryos was as described (Hollemann and Pieler, 1999). Staging of embryos was done according to Nieuwkoop and Faber (1967).

Whole mount in situ *hybridization*

In general, whole-mount *in situ* hybridization was carried out as described (Hollemann *et al*., 1998). To generate antisense RNA probes, corresponding plasmids were digested and transcribed as follows: *cadherin-5*, SalI and T7. For the analysis of *cadherin-5* expression on sectioned embryos whole mount ISH embryos were embedded in technovit (Kulzer) and 10 μm section were made using a microtome (Leica, Germany) and mounted on glass slides.

RNA preparation and reverse transcription

RNA was prepared from whole *Xenopus* embryos using a Qiagen RNeasy Kit following the instructions provided by the manufacturers. First strand cDNA was prepared from 500 ng total RNA using oligo–dT- or random primer and reverse transcriptase (Gibco).

RT-PCR

RT-PCR was performed with the following intron spanning primers and PCR-Cycles:

XL-ODC1-F 5-GCCATTGTGAAGACTCTCTCCATTC,
XL-ODC1R 5-TTCGGGTGATTCCTTGCCAC, 26 Cycles;
XL-ami-rt-f202 5-TAAATGGGTGCTGAGTGCAG
XL-ami-rt-r577 5-GTTCCGGCGATTACAGACAT; 28 Cycles
XL-flt1-rt-f433 5-GCCATCTACGAACCAGGTGT
XL-flt1-rt-r770 5-AAATGTGGGATTGGGAATGA; 28 Cycles
XT-ve-cadherin-Ex10 5-ATTCTGTGAGGAGGCTGGAA
XT-ve-cadherin-Ex11 5-CGCCTTCCTCATCATAGGTG; 28 Cycles
XL-cdh5-locusA-550-forw 5-GACGTGAACTATAGAATCATCC
XL-cdh5-locusA-735-rev 5-CAAGCGAATGGTCACTATGG; 34 Cycles
XL-cdh5-locusB-689-forw 5-CAGGACAAACCATTTACCTG
XL-cdh5-locusB-935-rev 5-AGTGATCGCATTTGTGGTAAC; 30 Cycles

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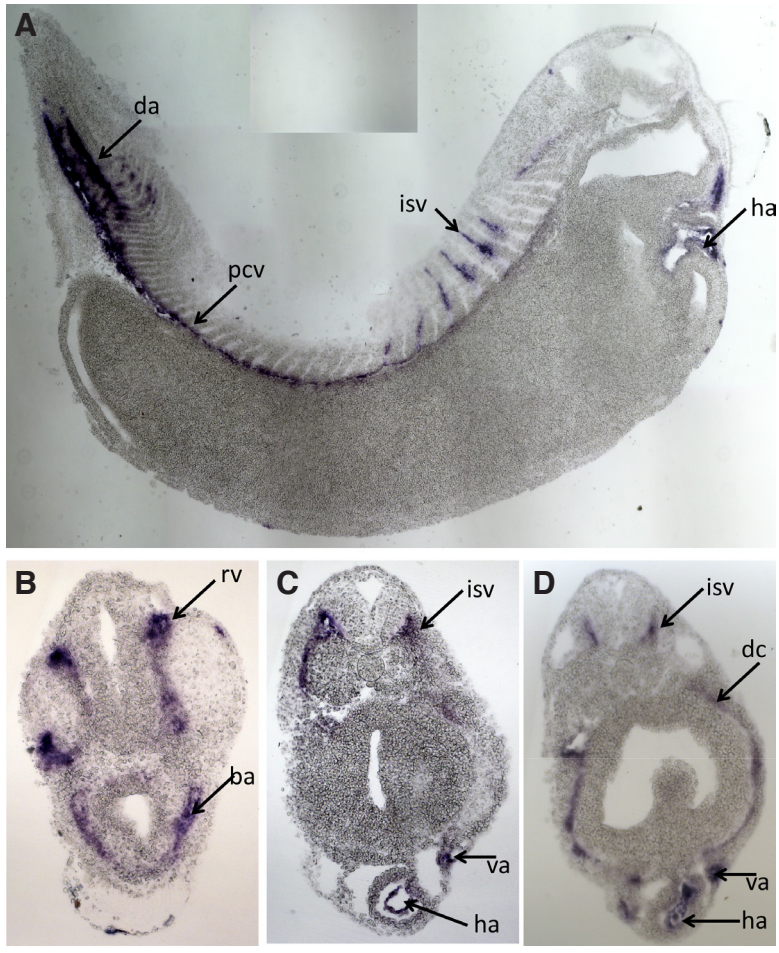

Fig. 6. Spatial analyses of *cadherin-5* expression on sectioned embryos. *Sagittal section* **(A)** *and coronary sections at different positions of st 36 embryos* **(B,C,D)** *show, that cdh5 expression is restricted to the endothelial linings of the developing vascular structures. Abbreviations: (aa) aortic arches, (ba) branchial arch, (da) dorsal aorta, (dc) duct of cuvier, (ha) heart anlage, (isv) intersomitic veins, (pcv) posterior cardinal vein, (rv) retinal vein, (va) ventral aorta, (vv) vitelline veins.*

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