

# 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). Committed 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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A	Xt	1	MKVQLRQLFIMCCSLPLLLFSKEITNSEYPAKNSRRVKGWIBWNQMFIIQEQQPNLPH	60
	Xt	1	M---KW---I----.-----F---A---TH---D---TT-----S---RN----	58
	Xt	50	YVGKLQINSSNVHENAKFAIQGESANTIFVKVNERNGDIYCFERLDREKKIEYHMLMALLVD	120
	Xt	61	-----NSSLHQ.-----K-----S-----	116
	Xt	110	KRTNKTLIEHPSNFIIKVLINDNAPEFTQKAFCNGSVNEMSDRGIVFTKVNADVDKDDPTIG	180
	Xt	119	-----K-A-----V-R-----T-----T-----T-----N-----	176
	Xt	170	GNADVNRYRIIQGGEYFTIDNNNGAIYATVPNLDREQKDTYEVLEARDSPGRTLYLASTAI	240
	Xt	179	-----E-T-K-----I-T-----S-----R-----V-----QNM-MS-----T	236
	Xt	230	VTIRLIDINDNPFTEERFKFNVPETGSWGRS.GRLKVEDIDEQPQRNNTKYSFLKERFQ	299
	Xt	239	-----I-N-----SQ-----D-----LKV-GEV-----	296
	Xt	289	EMFAVTTNAITNEGILILKKPLDYESVKQYKMDIEATDPLIDLVRVARQPRPKSITNVIIN	359
	Xt	299	-----I-----VN-----NV-----K-T-----	356
	Xt	349	VLDVDEPPVFSKPFYKFEISENSKSLTNIIGFSAKDPAANRNIRYSMRNFKDEPIKVTS	419
	Xt	359	-----D-----N-----	416
	Xt	409	TGNIINVKTLDRETADWHNFTVVAAEVDPSPNPPIKKESLGLVFIKVLDVNDNAPEFAEHY	479
	Xt	419	N-----	476
	Xt	469	APRVCENAAHQHTVIANISATDKDEMKGPKTFKTYSSAKKENNFTVQDNHDNTATILVKYGY	539
	Xt	479	-----T-----KM-----	536
	Xt	529	FNREVAKFHYLPIVISDNGQPEQSSTNLITIVCKCNEGEFTFCEP.AKLAASVPTI	598
	Xt	539	-----	595
	Xt	588	IIILVSLFLIIILVVAIALVLRMMQKKDTNLGKNTAEIHEQLVTYDEEGGEMDTNSYDV	658
	Xt	598	-----V-F-----T-----N-----	655
	Xt	635	SVLNSVRRNVQPRPQDMETDPLYAHVQKPARNGDMSFMIEVKKDEADNNGEGLPYDTLH	718
	Xt	658	-----E-----AS-----T-----D-----	715
	Xt	635	IFYGYEGSESIVESLSSIESGSSESIDYDVLNWNNGPRFKMLAELYGLEPIGDFPY	773
	Xt	718	-----E-----	770

**Fig. 1. Sequence alignments 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 Ca<sup>2+</sup>-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.

Signal peptide		EC1	
Xtr 1	MVKWKLQVIMICSSLFLSKEFTNAET	DKTTRVRKGRWIWNQMFSEER. NGNLPHYV	GKLNSLSSILHQNAFKFAIQGESANTIFKVNEKNQDGYCFER
Hsa 1	MQLMLMLLATSACGLG--AVAAVAGAN	PADQDTHSLP-HR-Q-D-----H-D-K.-TS-----	-IK--VSRK--YLLK--YVGKV-R-DAET--VFAI-
Mmu 1	MQRLLTELALGAFLG--AVAMAGPN	FQPIDTPNMLPAHH-Q-D-----H-D-K.-ES-----	-IK-NVNR--YVL--FAGK-G-DANT-NVLAY--
Gga 1	MKKL1-L-SLFQPSYKSENQ	KINQNFNSNTSHK-L-D-----R-H-R-I-.DSP-----	-T--VGNK-MY-E-Y--QGDY---A--
Dre 1	MKMQCQARQTMPEVPRVAVALLALSLSI	GVDVHQAKQTPSISAAQH-L-D-----H-D-K-LKLYA-	-TRPK-P-EKI--ENTFFSSSTRYILK-DG-KDK-T,D--VVLAK
Gac 1	MARLL-WT-GL-AIMSVALAVAVDFVLP	VAEGHH..EIVKKEHSPILS-Q-D-----ALYVE--K.PAPVAYRI	--K--KTVDVK--E-S-G--R--D.SK--LFVNQT
EC2		EC2	
89	LDREKKSEYHMLLVLDKKTAKTLEHPNSNFI	VIRVIDINDNAQFTQKA	FNGSVNEMSDRTFTVTKTAVVNDDPPTIGGNAEV
107	-----T-VI----D-GEN--T-S-T-K-H-VNDNW	-----HRL--A-P-S-AV--S-IS-----	TYKTIQGQFYFTIDNIG...TIYTAVSNLD
106	-----V-F-T--I--N--NKN--Q--S--TVK-H--W--	-----A--V-DH-S-M-Q-LK-K--A-S--S--R-I-ITKS--	-----V-FH--T-L-Q-VK-N--S--S--L--F-KIKK--
101	-----KA--E-T--HII--RRNNRS--P--K--I-K-S--A--I--V--I--	-----P--M--RL--S-TK-----E--A--V--HA--T--Q--K--N--V--DS-R.GV-S--RAD--	-----V--A--V--I--P--M--RL--S-TK-----E--A--V--HA--T--Q--K--N--V--DS-R.GV-S--RAD--
112	-----TQSV-N-S-S-LNIH--GELVDKDES--V-L--I--V--	-----V--DS.DQS--IS-S-RA-TIMK	. GRIDFKLNL-TDL-KIKPN...GDLITALK-D--
110	-----NSM-K-T-KMFDGN.GELI-DSGD--VQ-T--I--V--	-----K-T-A--SSTE.	-----NSM-K-T-KMFDGN.GELI-DSGD--VQ-T--I--V--PR.TYN--IM-R-PI--E-VE--K-T-A--N-TA--GDLR-SLT-REDFAAF-IDSICKV-SRINTN--
EC3		EC3	
198	REQRDITYEVVE	EARSPGPQNNMMSTATVI	NLIDINDNPFDTESKYFK.....FDVPETLKVGGEVGR
216	--KQAR--I--A--Q--LRGD-G--LVT-Q--F--QT--T--	-----KVEDIDEPQNRNNTKYSFLKERQFQEIFI	VTDPTKQFEEVLTNVNTNEG
215	--KQAB-KI--TQ--AL--LRGE-G--M-R--E--V--Q--T--T-----	-----F--S--P-----M--I--R-GDY-DA-TIE--PAH--	-----F--S--P-----M--I--R-GDY-DA-TIE--PAH--
212	--SOSA--II--K-K--AL--LTGE-S--I--R-T--V--KHR--N-----	-----S--DIR--KPL--F--T--V--P-----M--I--M--IMQGEYDRD-TIE-DPKRM--	-----S--DIR--KPL--F--T--V--P-----M--I--M--IMQGEYDRD-TIE-DPKRM--
219	--KQSQ-LIA--Q-K-M--EHLTGN-A-TV-T--IK--IA--KKN--I	-----V--NIS-----VK-----H-----V--VRGDYRDT-EIIA-PETT--	-----V--NIS-----VK-----H-----V--VRGDYRDT-EIIA-PETT--
219	-----ILKLNCLMLLYQ-T-K-D--P-S-I-L-E--K--I--K-DPTFALQSKFN.DV-DIKRTK.EKD--	-----T-KSQS--V--K--Q--MRGMPPGSSTS-TS-TVG-T--HAS-----Q--ERT--E--LN--R-DH-LNEKI--T--Q--D--R--IR--KVPVF--PDKPTKWP--G-EVSKPNKND--	-----T-KSQS--V--K--Q--MRGMPPGSSTS-TS-TVG-T--HAS-----Q--ERT--E--LN--R-DH-LNEKI--T--Q--D--R--IR--KVPVF--PDKPTKWP--G-EVSKPNKND--
EC4		EC4	
301	IILKKPLDYEVSKQYQNV	DIATEATDPLIDLRAVAKQTRPKS	ITNVIINVLVDVDEFPVFSKPYKF
317	--IKPMK--I-YQ--SFIV--T--YMSPPAGRN.AQ--I--IT--	-----FEISEDSKLNNII	GPAKDPDAANRNIRYSMR..NFKDEPIKVTNNGNTI
316	--IKPTKS--VIQ--TFY--T--R--YEYLSS--SG--NKA--T-----	-----G--I--RTSD--GQFR--KK--D	-----G--I--RTSD--GQFR--KK--D
313	--IRP--F-K-AE-RF--HNVPNAPPY--PGGSR--STIT-EVT-----	-----QRFH--H--KLP--NQK--KPL--T--V-----	-----QRFH--H--KLP--NQK--KPL--T--V-----K-Q-S-G--I--KTSDRGQFFRI--KQ--N--
329	M-S--V--KE--THKPIVIVEEHTH--T--PDPNKGELLK--E--DVT-----	-----T--LS--E--KVR--NDPEIKTL--S--W--H-----	-----T--LS--E--KVR--NDPEIKTL--S--W--H-----K--K--FAR--RASPNGDYVR--SDS--I--
321	D-V--QA--TMSS--TFNVKLHES.L--VTDVUVNSATTAR--TI--VL--A-----	-----I--NQTE--T--SVF--GPFP--PV--A--S-----S--SYK--T--TENIA--CPVDPD--N--Y--	-----D--V--QA--TMSS--TFNVKLHES.L--VTDVUVNSATTAR--TI--VL--A-----I--NQTE--T--SVF--GPFP--PV--A--S-----S--SYK--T--TENIA--CPVDPD--N--Y--
EC5		EC5	
411	INVKTLDRETADWHNFTVVAEEVDPNSNPKKES	ISLGLVFIKV	LVDVNDNAEFAE
427	Y-E--E--V--Y--L--E--K-L-STGT--TG--IVQ--H--E--E--	-----V--G--I--QVLO--I--IPRNVN--KFLNNTEN--	-----V--G--I--QVLO--I--IPRNVN--KFLNNTEN--
427	Y-E--E--V--Y--L--E--N--L--SRGN--VG--IVQ--Y--E--E--	-----T--V-----K--Q--S--G--I--KTSDRGQFFRI--KQ--N--	-----T--V-----K--Q--S--G--I--KTSDRGQFFRI--KQ--N--
425	QLP--P--FSSLY--I--A--Q--ILEDDE--R--HAQ--HVI--T--E--A--	-----T--Q--E--K--QG-----	-----T--Q--E--K--QG-----
438	SLKRK--QESLYTFQ--T--H--D.VL--GLK--STM--SL--L--I--	-----KLVQV--I--V--E--K--Q--E--K--QG-----	-----KLVQV--I--V--E--K--Q--E--K--QG-----
386	-----L--I--L--N--L--V--S--D-----	-----T--Q--E--K--QG-----	-----T--Q--E--K--QG-----
519	TILVKGGYFNRN	EVRAFKHLPIV	DISNQPCQSTNTLIT
534	N-T--Q--D--H--T--V--F--V-----M--SRTG--S--VA-----	-----V--Q-----DMAAQGVG--I	-----V--Q-----DMAAQGVG--I
535	N-T--Q--D--H--T--V--F--V-----M--SRTG--S--VA-----	-----Q-----IQLA--A--LCILT--T--I--L--I--	-----Q-----IQLA--A--LCILT--T--I--L--I--
533	N-T--D--Q--L--I--L--I--V--I-----N--L--V--S-----	-----R-----RAKQVG--QAL--A--ICI--T--TA--IAL--IL--	-----R-----RAKQVG--QAL--A--ICI--T--TA--IAL--IL--
540	--V--L--Q--G--ST--NSEEYV--E--A--G--T--K--V--L--Q--K--T--Q--SQRVEY--MSYAR.TGM-----	-----KRH--DLSG--RR--VA-----	-----KRH--DLSG--RR--VA-----
425	DLT--O--P--SLDDPADYPSVDVH--G--A--T--VTK--A--KS--R--DARRP--Q--KA	-----D-----ER--D--GTR--A--DQP--AS--SFSVQ--SS--S--RSRNGN--S	-----D-----ER--D--GTR--A--DQP--AS--SFSVQ--SS--S--RSRNGN--S
Transmembrane domain		Cadherin C	
519	TILVKGGYFNRN	EVRAFKHLPIV	DISNQPCQSTNTLIT
534	N-T--Q--D--H--T--V--F--V-----M--SRTG--S--VA-----	-----V--Q-----DMAAQGVG--I	-----V--Q-----DMAAQGVG--I
535	N-T--Q--D--H--T--V--F--V-----M--SRTG--S--VA-----	-----Q-----IQLA--A--LCILT--T--I--L--I--	-----Q-----IQLA--A--LCILT--T--I--L--I--
533	N-T--D--Q--L--I--L--I--V--I-----N--L--V--S-----	-----R-----RAKQVG--QAL--A--ICI--T--TA--IAL--IL--	-----R-----RAKQVG--QAL--A--ICI--T--TA--IAL--IL--
540	--V--L--Q--G--ST--NSEEYV--E--A--G--T--K--V--L--Q--K--T--Q--SQRVEY--MSYAR.TGM-----	-----KRH--DLSG--RR--VA-----	-----KRH--DLSG--RR--VA-----
425	DLT--O--P--SLDDPADYPSVDVH--G--A--T--VTK--A--KS--R--DARRP--Q--KA	-----D-----ER--D--GTR--A--DQP--AS--SFSVQ--SS--S--RSRNGN--S	-----D-----ER--D--GTR--A--DQP--AS--SFSVQ--SS--S--RSRNGN--S
Ident. [%]			
734	DVLNNWGPFRKMLADLYGLE	PIEDFPY	
757	-F--D-----E--SD--R--ELL--	52, 4	
758	-F--D-----E--SD--Q--ELII--	51, 5	
752	-F--D-----E--SD--K--KGDDDS	53, 5	
750	-FIHE----RT--Q--V--VDSDDSSY	36, 9	
536	-F--E--F--FRT--LAELYGVADPYHQY	36, 1	
760			

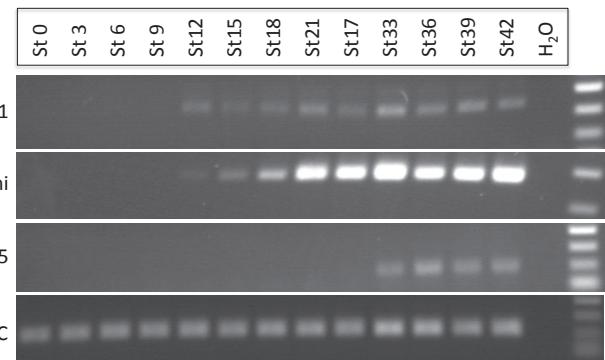
Prot A 1	MKVQLQFLMIMCCSLPLLLFSKEITNSESY-PAKNSRRVKRGWIWNQMFQ-EEQPGNL	58
Prot B 1	:     :     :     :     :     :     :     :     :     :     :	59
Prot A 59	PHYVGKINSSNVHENAKFAIQGESANTIFVKVNERNGDIYCFERLDREKKIEYHLMALLVD	118
Prot B 60	:     :     :     :     :     :     :     :     :     :     :	119
Prot A 119	KRTNKTLLEHPSNFIIKVLDINDNAPEFTQKAFNGSVNEMSDRGIFVTKVNAVDKDDPTIG	178
Prot B 120	:     :     :     :     :     :     :     :     :     :     :	179
Prot A 179	GNADVNRYRIIQQGQEYFTIDNNNGAIYTAVPNLDRDREQKDTYEVLVEARDSPGRTLYLASTAI	238
Prot B 180	:     :     :     :     :     :     :     :     :     :     :	239
Prot A 239	VTIRLIDINDNPFTEFKEFVNPNENLKVGAEVGRLKVEDIDEQPNRNTKYSFLKERFQ	298
Prot B 240	:     :     :     :     :     :     :     :     :     :     :	299
Prot A 299	EMFAVTTNAITNEGILILKKPLDYYESVKQYKMDIEATDPLIDLRVARQPRPKSITNVIIN	358
Prot B 300	:     :     :     :     :     :     :     :     :     :     :	359
Prot A 359	VLDVNEPPVFSKPFYKFEISEDSKLNNIIGFVSAKDPDAANRNIRYSMRNFKDEPIKVTS	418
Prot B 360	:     :     :     :     :     :     :     :     :     :     :	419
Prot A 419	TGNIINVKTLDRRETADWHNFTVVAEVDPSNPPIKKESELGLVFIKVLVDVNDNAPEFAEHY	478
Prot B 420	:     :     :     :     :     :     :     :     :     :     :	479
Prot A 479	APRVCENAAHQTVIANISATDKDEMKGKFTYYSSAKKENNFTVQDNHDNTATILVKYGY	538
Prot B 480	:     :     :     :     :     :     :     :     :     :     :	539
Prot A 539	FNREVAKFHYPPIVSDNGQPEQSSTNTLTITVCKCNEKGEFTCEEPAKLAASVPTII	598
Prot B 540	:     :     :     :     :     :     :     :     :     :     :	599
Prot A 599	IILVSLFLIIILVVAILAVLRRMQKKDTNILGKNTAEIHEQLVTYDEEGGGEMDTNSYDV	658
Prot B 600	:     :     :     :     :     :     :     :     :     :     :	659
Prot A 659	VLNSVRNVQRPRQDMETDPYLYAHVQKPARNGDMSFMIEVKKDEADNNGEGLPYDTLHI	718
Prot B 660	:     :     :     :     :     :     :     :     :     :     :	719
Prot A 719	FGYEGSESIVESLSSIESGSSESIDYDVLNWGPFRFKMLAELYGLEPIGDFPY	772
Prot B 720	:     :     :     :     :     :     :     :     :     :     :	773

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



**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 *f1t-1* and *ami* by semi quantitative rt-PCR. Expression of *f1t-1* 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.

EC5), a transmembrane domain, a cytoplasmic domain, four conserved cysteine residues, three putative calcium binding motifs (DxDNxDP), 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 homologous 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%) (Fig.1b). Since two paralogous sequences are contained in the *Xenopus laevis* genome, we compared the putative amino acid sequences of both paralogs. Both putative proteins are highly conserved sharing 97.5% similarity, 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 *f1t-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 *f1t-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**

Locus A	474	TGTGGATAAAGATGATCCTACAATAGGTGGAAATGCA <b>GACGTGA</b> ACTATAGAATTCATCCA	533
Locus B	477	TGTGGATAAAGATGATCCTACAATAGGTGGAAATGCAGATGTAACCTACAGAATAATCCA	536
Locus A	534	AGGACAAGAGTACTTTACAATTGATAACAATGGGCAATTATGCACTAACCTAATT	593
Locus B	537	AGGACAAGAGTACTTTACAATTGATAACAGTGGGACAATTATGCAATACCTAATT	596
Locus A	594	AGACAGAGAGCAGAAAGATACTATGAAAGTTCTGGTAGAGGCCAGAGATTCTCCAGGGCG	653
Locus B	597	AGACAGAGAGCAGAAAGATACTATGAAAGTTCTGGTAGAGGCCAGAGATTCTCCAGGGAC	656
Locus A	654	AACTCTTACTTGGCAAGCACAG <b>CCATAGTGACCATTGCTTG</b> ATAGACATTAATGACAA	713
Locus B	657	AACCATTACCTGGCAAACACAGCCACAGTAACCATCCACTTGACAGACATTAATGACAA	716

**B**

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

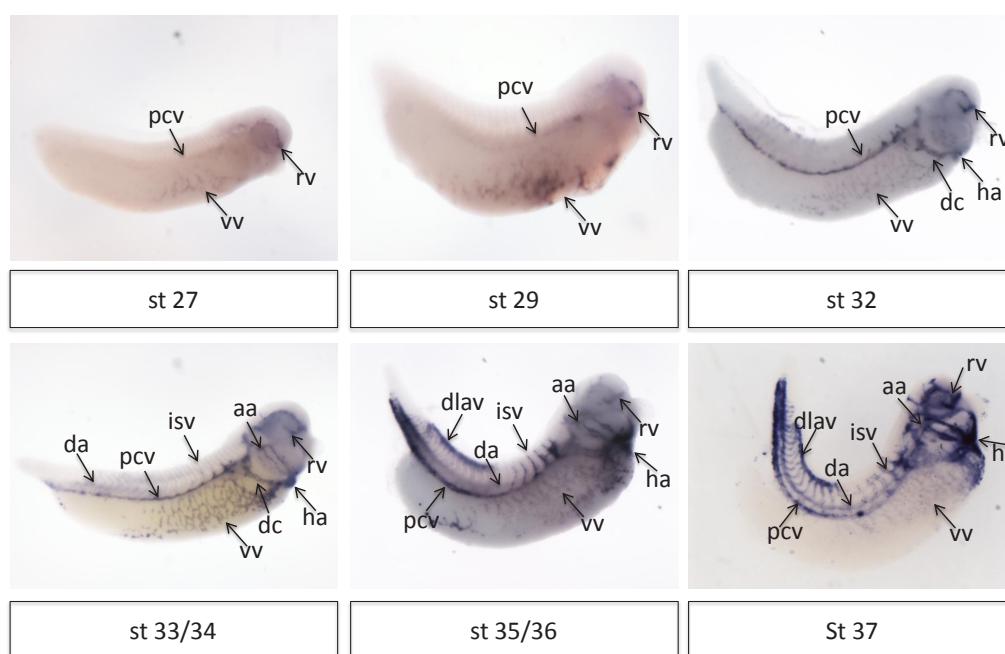
Spatial expression of *cdh5* was analyzed by *in situ* hybridization on whole-mount and sectioned early *Xenopus* embryos. Transcripts were first detectable at NF stage 29/30 in the ventrolateral region, where the first vitelline veins form and in vessels around the developing eye. (Fig. 3). A few hours 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

**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 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.

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 *fli-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 (Holleman 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 (Holleman *et al.*, 1998). To generate antisense RNA probes, corresponding plasmids were digested and transcribed as follows: *cadherin-5*, Sall 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-GCCATTGTGAAGACTCTCTCCATT,
XL-ODC1R	5-TTCGGGTGATTCCTGCCAC, 26
Cycles;	
XL-ami-rt-f202	5-TAAATGGGTGCTGAGTCAG
XL-ami-rt-r577	5-GTCCGGCGATTACAGACAT; 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-CGCCCTCCTCATCATAGTG; 28 Cycles
XL-cdh5-locusA-550-forw	5-GACGTGAACATAGAACATCC
XL-cdh5-locusA-735-rev	5-CAAGCGAATGGTCACTATGG; 34

Cycles

XL-cdh5-locusB-689-forw	5-CAGGACAAACCATTACCTG
XL-cdh5-locusB-935-rev	5-AGTATCGCATTGTGGTAAC; 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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