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***laminin alpha 1* gene is essential for normal lens development in zebrafish**

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Abstract

Background: Laminins represent major components of basement membranes and play various roles in embryonic and adult tissues. The functional laminin molecule consists of three chains, alpha, beta and gamma, encoded by separate genes. There are twelve different laminin genes identified in mammals to date that are highly homologous in their sequence but different in their tissue distribution. The *laminin alpha -1* gene was shown to have the most restricted expression pattern with strong expression in ocular structures, particularly in the developing and mature lens.

Results: We identified the zebrafish *lama1* gene encoding a 3075-amino acid protein (*lama1*) that possesses strong identity with the human LAMA1. Zebrafish *lama1* transcripts were detected at all stages of embryo development with the highest levels of expression in the developing lens, somites, nervous and urogenital systems. Translation of the *lama1* gene was inhibited using two non-overlapping morpholino oligomers that were complementary to sequences surrounding translation initiation. Morphant embryos exhibited an arrest in lens development and abnormalities in the body axis length and curvature.

Conclusion: These results underline the importance of the *laminin alpha 1* for normal ocular development and provide a basis for further analysis of its developmental roles.

Background

Basement membranes play an important role in tissue development and maintenance including mechanical stability, formation of barriers between different cell types and promotion of cell adhesion, migration, growth and differentiation. Laminins are large glycoprotein heterotrimers that are found as major components of basement membranes in almost every animal tissue. To date, five *alpha*, four *beta*, and three *gamma* precursors have been

identified that can combine to form fifteen laminin isoforms with different tissue distribution [1-3]. Mutations in laminin genes have been identified in several human disorders: muscular dystrophy (*LAMA2*; [4]), epidermolysis bullosa and Laryngo-onycho-cutaneous syndrome (*LAMA3* [5,6]; *LAMB3* [7]; *LAMC2* [8], and microcoria-congenital nephrosis syndrome (*LAMB2* [9]).

lama1	MEEMRMK-----MLV	LEALVFSSVICVEAQ	QRGLFPAALINLASNA	EISTNATCGDPDFEM	FCKLVHVFVGRVIRN	PCCRICDANSONPKK	QHFYTNADIGTNLHW	QSPSIKNGRFHWHT	115
LAMA1	---MRGSG---LWVAQCR	LI-----LCVAAQCR	QRGLFPAALINLASNA	HISTNATCGEKGPEM	FCKLVHVFVGRVVRN	PCCRICDGNANPRE	RHPISHAIDGTNNW	QSPSIQNGREYHWHT	108
Lama1	---MRGSGTGAALIV	LLASV--LWVTVRSQ	QRGLFPAALINLATNA	HISANATCGEKGPEM	FCKLVHVFVGRVVRH	AQCRVLDGNSTNPRE	RHPISHAIDGTNNW	QSPSIQNGREYHWHT	115
lama1	VTLDLROVFOVAYII	IKAANSPRPGNWILE	RSLDGVNEQWQVYA	ISDTTECLTRYNITPR	IGPPTYKRDEVEICT	SYYSRLVPLEHGEIH	TSLINGRPSADDLTP	ELLEFTSARFTRLR	235
LAMA1	ITLDLROVFOVAYIV	IKAANAPRPGNWILE	RSLDGTTFSPWQVYA	VSDSECLSRYNITPR	RGPPTYRADDEVICT	SYYSRLVPLEHGEIH	TSLINGRPSADDLSP	KLLEFTSARYTRLR	228
Lama1	VTLDLROVFOVAYII	IKAANAPRPGNWILE	RSVDGVKRFQWQVYA	VSDTECLTRKXITPR	RGPPTYRADDEVICT	SYYSRLVPLEHGEIH	TSLINGRPSADDLSE	QLEFTSARYTRLR	235
lama1	QRIRTNADIMLTLSY	RDPKVDVPIVTRRYI	YSIKDISVGGMCICY	GHAQSCPWDVTKRL	QCVCEHNTCGESQNE	CCPGYHQEPWQFGLT	SDGNTCEKCNCHNKA	DDCFYINQTVADLKL	355
LAMA1	QRIRTNADIMLTLSH	REPKELEIVTRRYI	YSIKDISVGGMCICY	GHASSCPWDETKKL	QCCCEHNTCGESQNR	CCPGYHQEPWRPQVY	SSGNTCEACNCHNKA	KDCYQDESVAQKKS	348
Lama1	QRIRTNADIMLTLSY	RDLRDLDFVTRRYI	YSIKDISVGGMCICY	GHASSCPWDEEAKQ	QCCCEHNTCGESQDR	CCPGYHQEPWRPQVY	SSGNTCEACNCHNKA	KDCYQDESVAQKKS	355
lama1	MTHGQFSSGGVGVN	RRHRTAIVNCTCAB	GYYRPHKVSFVAER	CVTECDMGRVSSVY	CRDDNHNAPDALS	PQQCVKKEFGAGKCC	DRCAFETRDFVPSR	CCPNLDESHHTDFQM	475
LAMA1	LNTAGQFRGGVGVN	CLONTMGINCTCID	GYYRPHKVSFVEDEP	CRPCNCPVGSLSVY	CRKDDLSIDLHNGKQ	PQQCPKKEGYTQKCC	DRQQLGYKDYPTQVS	CCQNPVGSASDPEPT	468
Lama1	LNTAGQFSSGGVGVN	CSQNTTGINCTCID	OYRPHKVSFVDDHE	CRPCNCPVGSLSVY	CRKDDRHADLANGKW	PQQCPKRYGAGYKCC	DRQCFYGRFENQIP	CCPNLDESHHTDFQI	475
lama1	E--CYKAVNVMGSHCD	LEKGGFYNLQASNEP	GCTFCFCFVSDVQCS	STWFSSSVHRDE--	-VLRHLHQTSISFWS	PVSD--ENLISNRS	TDADPSVMTWAPE	PTLNKLLSYGSEFIN	590
LAMA1	GPCLCKNVEGKACD	RCKPFGYNLKKNR	GCSECFCFVSDVQCS	SLSHVPVGVNSMSGW	LVTDLISPRKTFPQQ	DALGGRHQVSNNTA	VMQRLAPKYWAPE	AYLGNKLLAFGGFLK	588
Lama1	GPCLCKNVEGKACD	RCKPFGYNLKKNR	GCSECFCFVSDVQCS	SLTNSISQVNTMSGW	LVTDLMSNTRKFSQ	DVLGGHRQISNNTA	VMQRLTSTYWAPE	AYLGNKLLAFGGFLK	595
lama1	YSVAYTSEINVDKT	IRSHFVITENGRNT	LRQAQASRLINNAHT	HRQVFWMLQIFIID	SHSGRSVQRDELMTV	LADVAALRVRAELED	SAEGTLRLSHVLSGV	GDSNSDVSKISLQVE	710
LAMA1	YTVSYDIPVETVDSN	LMSHADVIKNGNLT	I--STQAEGLSLQPYE	EYLNVVRLVPENFQ	FHSKQIDRDLQMTV	LANTHLLIRANYNS	AKMALYRLDESVIDI	ASNAATDVLVAADVE	704
Lama1	YTVSYDIPVETVDSN	LMSHADVIKNGNLT	I--STRAEGLSLQPYE	EYFNVRLVPENFQ	FNTREIDRDLQMTV	LANTHLLIRANYNS	AKMALYRLDESVIDI	ASNAATDVLVAADVE	717
lama1	HECPFGVSTSCSL	ILPFFIRVQGLIFGG	NELFCEDNHHTKCD	INRELCAHNTTVP	HDCQCLPGFYQDABE	GTFDCCQCESPITL	ASNNFPTCQIQAPG	FVDCQCGQSSYFQK	830
LAMA1	HECPFGVSTSCSHS	CLSGYRVDGILFFGG	ICQPCCHGHAARCN	VHGVDIACAHNTTVP	HECQCLPGFYQDABE	GTFDCCQCAPCITPI	ASNNFPTCHLNDGD	FVDCQCGQSSYFQK	827
Lama1	HECPFGVSTSCSEA	CLPGYRVDGILFFGG	ICQPCCHGHAARCN	IHGIVSVEVHTNTGD	HECQCLPGFYQDABE	GTFDCCQCAPCITSI	ASNNFPTCHITDGE	FVDCQCGQSSYFQK	834
lama1	CRCADGQYGNFTVP	QDRSFCPCNGNVDP	SEAGRCDVHTGECLK	GVGHTAPHCERKRD	GYGDAIQEKNGQAC	GGYNSGLSTICNLI	TGQCCCKHNVKETE	DRQEGYHGINSGE	950
LAMA1	CRCADGQYGNFTVP	GESCVPCDCSNGVDP	SEAGHCDVHTGECLK	CLGNTDGAHCERKRD	GYGDAVAKNCRAC	DEHVGSHSVAVCHLE	TGLDCKPMTVTCQCC	DCQLHGYGDLDSGH	947
Lama1	CRCADGQYGNFTVP	GCTVEPCDCSNGVDP	LEAGHCDVHTGECLK	CLWNTDGAHCERKRD	GYGDAVAKNCRAC	DCHENGSLGVCVHLE	TGLDCKPMTVTCQCC	DCQLHGYGDLDTGL	954
lama1	CRPCEINQSSALSS	QDEEGRCQITGVTC	DKDRCHHGYNFKK	NGCTACDCAHHTGNC	NDQTEGECIPPHTHG	LKCEQCDDEGHGHDG	VSSKQCNVCSVGGSS	SSQCDLSSGQCVCAL	1070
LAMA1	CRPCEINQSSALSS	CTDRSGQCVGVPAG	KRDRCRCAHFYAFQD	GGCTPCDCPHTGNC	DEPTGECVCPHTQD	VKCEEDCGHWGYDA	EVGQCAQCNVAVGSS	HRRCDDVTHGCKKS	1067
Lama1	CRPCEINQSSALSS	CTDEEGRCQITGVTC	KQDRCHHGYNFKK	GGCTPCDCAHHTGNC	DEPTGECVCPHTQD	LKCECEEAYWGLDP	EVGQCAQCNVAVGSS	HRRCDDVTHGCKKS	1074
lama1	QFSLHLDTRALDGLR	NFRQFDEALVDFNPF	RAQFDEALVDFNPF	EDHQSCEKQVWGR	QDEBKSSESFCQSKL	NFARCSPPCFGLSQ	VDELEHSLGRLAPVE	GSFALLVWSSGQDVI	1189
LAMA1	KFGRRADQCSLGRV	DFDQVCPDCLDRGT	SGDAENLEQGLQCVY	ETEAPECKENVFQD	QENKRETEALRAD	NFLCPCSPFCSELSH	LESELEDYVTRFVPT	GRDQFLLRVWQSNL	1187
Lama1	GFQCSQCHQCSLGR	SEFDQVCPQCDLDRGT	EDSQTSECKENVFQD	QENKRETEALRAD	QENKRETEALRAD	NFLCPCSPFCSELSH	LESELEGYVTRLITL	ASDQFLLRVWQSNL	1194
lama1	QSTLEGVYSDDEMT	LVFDQINKLSLLTGE	YYWRLEPKYKNKLL	SYGGRSISVTLTFAQ	DGVGLANQEFQVIMR	GHLRKLKLYITDASA	FANGISTQCEHALTE	HKWYKYSVSDAAS	1309
LAMA1	RTTTEGVYQAFDPT	LDAATVTRQ--HIRAEP	FYWRLEPKQFQDGLL	AYGKKLYSVAIYSL	LDGTSNFEFQVLIK	GGRRKQVYMDAPA	PENGVQCEVAMRE	NFWKYSVSEKPYT	1306
Lama1	RTTTEGVYQAFDPT	LDAEAIVRQ--HIRAEP	FYWRLEPKQFQDGLL	AYGKKLYSVAIYEST	LDGTSNFEFQVLIK	GGRRKARHVIYMDAPA	PENGVQCEVAMRE	EFWKYSVSEKPYT	1312
lama1	HADFMSVLSNLEVIY	IKASYSGLQQRIS	NISMETALEADELPE	GQDVARIVEICEYPT	GYAGLSCQECAPGYI	QAVSELNMKRNR	IIQPCVPCRESNHSQ	SCDLHTGCGLQGN	1429
LAMA1	REDFMSVLSNLEVIY	IKASYSGLQQRIS	DISMEVGRKAKVHP	EDEVASILENVCVCF	GTAFHSCQDCAPGYH	RKGLPAGSDRQ--PRE	IVAPCVPCSNHNSD	TCDDNPKCLINGDN	1425
Lama1	HSDFMSVLSNLEVIY	IKASYSGLQQRIS	NISMVGRKAKVHP	EDEVASILENVCVCF	GTAFHSCQDCAPGYH	REKLPESGGRN--PRE	IVAPCVPCSNHNSD	VCDPEVTKLISDRH	1432
lama1	TAGCHCHVCAAGYFG	KVQSVSDCSLCAFC	LRGQ--SFSSTCVLEG	AGDFRCDRSEEGYEG	RYCERAVGWFNFS	EPGGRQVCAASES	SVHSVCDAHTREEE	KPGVRHLCDQEEF	1548
LAMA1	TAGDHDVCTSRYG	KVTGSASDCALCACP	HSPFASSTPTCVLEG	DHDFRCDAAGLLGYG	KHCEKSSSYGNFQ	TFPGSCQKDDNPHQ	SVHGCDDTRSGQCVY	RLGASGLRDEEFP	1545
Lama1	HSGBHCELASGYG	KVYGLGCTPTTCE	HSPFASSTPTCVLEG	DSFRKNGALPQYEG	QYCEKRSAGHNFQ	AAFSSGCTQDENQD	SVHSDCDRASQCVY	KPGATGLRCKLPR	1552
lama1	HVIVHPCVSCNDCSC	TGVILDDIALDVSI	TSVNLTVGILVAFPSQ	MTIQNQRRELTSLM	SNWQTPDYQLTAGEE	HSRNLSCQITDLOQQ	VQSVFEDSGDILLTS	QRFTQYKGLLAL	1668
LAMA1	HILMETDVCVSCDDC	GVGLINLDEIGDVA	LSLNLTGILVFPVGI	LSNLTETKYLQESL	--KENMDLQKRI--	KLEGVAEDTDLQK	LTRMLASTQENRAT	ERFKESODLALTE	1662
Lama1	HILMESDVCVSCDDC	GVPLNLDLSDGVDA	LSLNLTGVSPVYGI	LENLENTKYPORYL	--TKENAKKIRAEI--	QLEGIAEQTENLQK	LTRVLARHQKNVAM	ERTSNQTAALTFTE	1669
lama1	NHSAHGLVQVQSS	VNVILDEGLDEANST	F--LLEETSETLEKI	RSFNLIHCRNAEQI	ESSVSVLEKVEDEI	SKRYRNNQIRRENIS	TTNTHNLSLQVQVE	SLYTAKTNNQSRRI	1786
LAMA1	RLQMSITEIMERT--T	LNQTLDEDFLLPNST	LQNMQNGTSLLEIM	QIRDFPQHQNSQVLE	DKAADLLSQIQENY	OKPLEELEVLKEAAS	HVLSKHNNEKAAEA	LVREAKLMOESNHL	1781
Lama1	QLHANKEITREKATV	LNQARKDQPPVPSA	LQSMHONISSLGLLI	KERNFTEMQNATLE	KAKKDLRSIQKRF	OKPKEKALKAEAN	--LNSNHEKQAAEE	LKPEKSGKTOENLL	1788
lama1	LQDIQSNTHLITISR	NNVSSLNHELET--	-----VQEAQDLL	TDALNIAEDMDNGAT	TDELKNDLELWSPS	LKKHVDLSVMDLTKR	DALQLVYRAEDHQA	LSKQNALNSISEV	1897
LAMA1	LILNKNANRE--	-----FSDKLHWQEE	QNLTSSELIVCGRLLI	DAAQAQTDVQDQALE	HLEDDHCKLLMSAK	IRHHIDDLVHMMSQ	NADVLYRAEDHAAE	FRLADLVISGLEMI	1892
Lama1	LILNKNANRE--	-----FQKRLVQVEE	QNVTSSELIAAGRWV	DAAGHTHAAQDQALE	QHEHRDELDLWARK	IRSHVDDLVMSMSK	RARDLVRAECHASE	LQSRGALDRDLQV	1900
lama1	SSASVNSISIRTRNA	NIRENQLQADLELA	NQNQNSALNNT--F	IRDSQFRRILQKSSSI	DRARAALLKNDHTLDM	QNVSAVTSRSLIVAA	NIQNTQMLPRLTE	IRNLFESSEAEILGV	2015
LAMA1	RNVSLNATSAAHVYS	NIQSLTEESEEARL	AHRTVT--EHSLSSES	IVSNGKAAVRSRFR	IKESVGNLKRKLPIA	LESELBNKTRNRFQ	NAVEITQTNESLIL	LHAPKTRDKOKAT	2011
Lama1	RNVSLNATSAAHVYS	NIQTLTEESEEARL	AHKTAN--KNDLISES	LASRGAVALRQSSRT	LKESVNTRRKQOQIT	MKLELDKLNLSOFQ	SVNITQKQANDSIAM	LRESFGMREKGRKA	2019
lama1	KQCTEANAALQEAAL	ERLRYRKLDESSES	ALGSADNSTORTNQL	LRDSQDTANAGSRL	SEADVADRILERIK	PLQTLGETLSRNLSD	IRELISQARKQAASI	KVAVSADRNDICIRAY	2135
LAMA1	RELATSASQSAVSTL	RDVAGLSQELLNTSA	SLSRVNTRRETRHQL	LQDSTMATLLAGRVK	KDVEIQANLFDRLK	PLKMLEENLSRNLSE	KLLISQARKQAASI	KVAVSADRNDICIRAY	2131
Lama1	RELAAAANEBAVKT	EDVLALSILRVNTSE	DLSRVNATVQETNDL	HNSTMATLLAGRVK	KMEMQANLDRLEK	PLKMLEENLSRNLSE	KLLISQARKQAASI	KVAVSADRNDICIRAY	2139
lama1	FEVTSNFTNLTITM	KTSPEPDLNLFYMGSS	SSEDFMALEMHDKV	SFLMDAGSGHNRLEY	EDVQINNDKWRINA	TRFRHATLTVQDTP	SEPLPAVKSSAA--GS	ATVMDNKHIIWVYVG	2254
LAMA1	FQISSTNYTTLTINV	KQDEPDLNLFYLGSS	TASDELAVEMRRGRV	AFELMDLGGSGTRLEF	PDFPDDNRWHSIHV	ARFGNIGLSVVKEMS	SNQKSPTKTSKSPGT	ANVLDNNSITLMMVG	2251
Lama1	FQTSSTNYTTLTINV	KQDEPDLNLFYLGSS	SSEDFLAVEMRRGRV	AFELMDLGGSGTRLEF	PEVSIINNRWHSITI	TRFGNMGSLVKEAS	AAENPFVTRTSKSPGT	SKVLDNNSITLMMVG	2259
lama1	GLGQYKSPAVKIV	HFRCGMEALLENENN	IGLWNYAERQEEQKRC	CFRSPRTEDTPEHFD	SSGFVSVKSESRMS	SVVMFKLISFPNGI	LILYASNGTRDFLSI	FVEPKVHLTFELGS	2374
LAMA1	GLGQYKSPAVKIV	HFRCGMEALLENENN	IGLWNYIEREGKRC	CFRSGSNQEDSPHFD	SSGFVSVKSESRMS	QIIMLNFISFPNGI	LILYASNGTRDFLSI	FVEPKVHLTFELGS	2371
Lama1	GLGQYKSPAVKIV	HFRCGMEALLENENN	IGLWNYIEREGKRC	CFRSGSNQEDSPHFD	SSGFVSVKSESRMS	QIIVILISFPNGI	LILYASNGTRDFLSI	FVEPKVHLTFELGS	2379
lama1	GALIMTSSEYNTYTS	WYKTLQRNRKRGHL	SYMAANSFPERVLE	AEAPQASDNLNRLD	DPYIIGGLFASRPIE	RQWARSFVGMKNV	ETARNTFDLLREAG	VKKGCVLKRFRSVV	2494
LAMA1	GPILLDRRYNNGT	WYKTLQRNRKQVIL	AVIDAYNTSNKETKQ	GETPGAASDNLNRLD	DPYIVGGLPFRSVVR	RGVTKTSFVGCIKNL	EISRSTFDLLNSYG	VRKCKLLEPFRSVV	2491
Lama1	GPLILMDRRYNNGT	WYKTLQRNRKQGLL	AVFDAYDTSKETKQ	GETPGAASDNLNRLD	DLIVVGLPFRSKAVR	RGVSSRSYVGCIKNL	EISRSTFDLLNSYG	VRKCKALEPFRSVV	2499
lama1	FGGFLPMPAVLLSS	HTEIMSTFSRRSDG	VILAGFSRSS-----	-RHTQOPLLLLIVS	GLGLRVSVDGAAV	QKAEI--SFSFDG	HEHSLILQRNKITIT	VVVDEIQASLKLPA	2605
LAMA1	LKGGYIELPKLSLSP	ESEWLVTFATTNSSG	ILIALAGGDVKEKRG	REEAHVFPFVSMVIG	GNIEVHVNPQDGTGL	RKALLHAPTCTSDG	QABSISLVNRRVIT	VQLDENNPVEMKLTG	2611
Lama1	LKGGYVEMPKLSLSP	ESLILATPATNSSG	ILVALGDKDAEAGC	-QAQHVFPFSSIMLE	GNIEVHVNSGDGTSL	RKALLHAPTGSYDG	QEHSISLVNRRVIT	IQDENSPVEMKLTG	2618
lama1	SAR--KSLALERLYE	FVPEAGDTH--FLTP	AFYGCNTRVAVGDM	DEL SARLRYEVDVDM	SLLIEPRFRRLVLED	DPEPTSDPDRPLTP	ARAEALSALFNSGSG	ASADHTQITPESHFQ	2723
LAMA1	IYVSRINVENLIVG	GFIEFGCSLLTMR	SFHCKKLIIFNLLE	LDPNSRVGHRVDLL	TWLSERKILAPDAE	DSKILPE-----	RA-----FT--EQ	VVDARLEIVGRRHF	2716
Lama1	LTFEKYIDISNLYI	GLBEDKATPMLKMT	SEHGCIKXNVVLDQAQ	LDFTHTLTSQEQVELL	TLLAEEMQSHRE	HGELPPE-----	PT-----LPQVLA	AVDTAPGVAGARHF	2725
lama1	GLSRSHHLMGFKR	TWTFSEFAKLSVTRT	SHSGVLFYMNNTQD	DYAVLQQAQRLELS	CDLGGKAASATLKT	ISDGRWHTVSAEFSK	KTVVVSVDGIESHDI	STKH--TLQVSGKL	2841
LAMA1	GILTONSHLIPFNQS	AVRKLSSVLSIRTF	ASSGLIYMAHQNSQ	DYAVLQQLGHRLEFM	FDLGGRKTRVSHPAL	LSDGKWHVTKDYK	RKGFITVDGRESPMV	TVVGDCTMLQVGRK	2836
Lama1	GILQNSHVLIPFNQS	DVYKRLIQQLSIRTE	ASSGLIYVAVHQNSQ	DYAVTLQQLGHRLEFM	FDLGGRKTRVSHPAL	LSDGKWHVTKDYK	RKAFMTVDGQESPMV	TVVGNATTLQVGRK	2845
lama1	YVGLPPTVTRKRG	NVTHSVAVQVDLTF	NGVPMNLNPLSLSHA	TGFCFSNAQDFTFN	SSGYAAMKEGVNVC	SDVTVSLEFRSTAPD	GVLGISTSKVDAIG	LLVNLQOVNENWNG	2961
LAMA1	YVGLPSPQVARKRG	NITHSIPACIGIMV	NSQDLKDKSFSVAFV	WNRKYVAQEGYTFE	SSGYAALVKEGVKQV	SDVNTLEFRSTSON	GVLGISTAKVDAIG	LLVNLQKVLPHWNG	2956
Lama1	YVGLPSPVARKRG	TTHHSIPACIGIMV	NSQDLKDKRPLSASA	VDRKYVVAQEGYTFE	SSGYAALVKEGVKRG	LDLNTLEFRSTSKN	GVLGISTSAKVAIG	LLVNLQKVLPHWNG	2965
lama1	AGRI SVS--SRSSVS	MCDGRNRLVAKKRL	HSLSLTVDVQVIMNE	NPHSSSTSAETKNPI	YVGHFADVKNQCLIS	VKQSFRCMRLRVF	RGHVVDVLDVFSRAT	LHVSPHSCFA---	3075
LAMA1	AGRI TAAEGRATV	LDCKGRVILQANKSK	HRTIILVDGNAGAN	SPHTSSTSAEDNDEI	YVGGYPAQVGRKCLR	SQTSFRGLKRLALI	KSPQVQSFVBRATF	LHGVLHSCGTFE	3075
Lama1	AGRI TATVQRARA	LDCKGWHVLAHKS	HRTIVTVDGNVRAE	SPHTSSTSAEDNDEI	YVGGYPAHVKQCLIS	SRAFSRGGVNRRLS	RGSVQVSLBRAED	LQGVFHSCEGPEP	3084

Figure 1
Alignment of laminin alpha I proteins. The amino acid residues that are identical in all three or in any two of the proteins (lama I (zebrafish), LAMA1 (human), Lama I (mouse)) are highlighted in dark or light grey, respectively.

Laminin-1, which is composed of alpha-1, beta-1 and gamma-1 chains, was first described by Timpl and co-authors in 1979 [10]. Laminin-1 shows restricted expression that is largely limited to epithelial basement membranes. Laminin-1 is detected in most embryonic tissues during early morphogenesis and remains present as a major epithelial laminin in some adult tissues [2,11-13]. Mice that are deficient in any chain that composes laminin-1 ($\alpha1\beta1\gamma1$) die during the early postimplantation period with the *Lama1*^{-/-} phenotype being the mildest of the three genes deleted [14,15]. This finding could be explained by the fact that $\beta1$ and $\gamma1$ proteins participate in multiple heterotrimers and therefore have broader functions than $\alpha1$ chain that is restricted to two laminins. Other animal models of laminin-1 deficiency include zebrafish *grumpy* ($\beta1$) and *sleepy* ($\gamma1$) mutants that were identified in a genome-wide chemical mutagenesis screen [16,17] and *lamb1* and *lamc1* (several alleles) mutants produced by retrovirus-mediated insertional mutagenesis [18,19]. The zebrafish *laminin* $\beta1$ and $\gamma1$ mutants display shortened body axes due to a failure of notochord differentiation as well as complex ocular defects ([16-19]; also see below). To date, there are no distinct human phenotypes associated with laminin-1 mutations although some studies suggested a potential involvement of *LAMB1* in a neonatal cutis laxa with a Marfan phenotype [20] and *LAMC1* in a junctional epidermolysis bullosa inversa [21].

The *laminin alpha-1* gene shows a tissue-restricted expression pattern and is considered to be the most specific of the classical laminins. Expression of *lama1* is detected in the nervous and urogenital systems, pre-somitic mesoderm, some brain blood vessels and in the embryonic and mature lens ([12,13,15], and [22]). The important role of laminins/extracellular matrix/basement membranes in eye development and in an adult ocular function has been discussed in several reports [23-26] but the specific roles of different laminin subunits are only beginning to be elucidated.

Besides laminin-1 ($\alpha1\beta1\gamma1$), laminin alpha-1 participates in one additional trimer, laminin-3 ($\alpha1\beta2\gamma1$) [11,27]. Interestingly, except for *lama1*, all other components of either laminin-1 or -3 were found to be involved in ocular developmental phenotypes. Human *LAMB2* mutations result in a complex phenotype that includes such ocular manifestations as microcoria, lenticonus, Rieger anomaly, glaucoma, cataracts and microphthalmia [9]. Mutations in *laminin* $\beta1$ and $\gamma1$ genes result in multiple eye anomalies in zebrafish: retinal blowout (expulsion of retinal cells through the RPE into the adjacent forebrain) [19], disorganized optic nerves [28], some retinal lamination defects [18,19] and lens hypoplasia, lens capsule rupture and corneal defects [19]. As laminin alpha 1 contributes to both

laminins, *lama1* mutations are likely to result in similar eye defects and may even cause more severe and/or complex ocular phenotypes due to the cumulative effect of laminin-1 and -3 deficiencies.

Zebrafish represents a valuable vertebrate model to study developmental processes. In this report, we present identification and characterization of the zebrafish *laminin alpha 1* gene including its sequence, expression pattern, and loss-of-function phenotype.

Results

Cloning of zebrafish *lama1* gene

In order to identify the zebrafish *laminin alpha 1* gene, we first performed a search for homologous sequences using the known human and mouse *laminin* $\alpha1$ sequences, zebrafish genomic database (Zv3) [29] and BLAST engine. This approach identified ten sequences homologous to the human *LAMA1* gene with the most upstream sequence corresponding to exon 4 and the most downstream one to exon 51 of the human *LAMA1* gene (the entire human gene contains sixty-three exons (GenBank accession number [NM_005559](#))). The identified sequences were used to design specific oligonucleotides that were then utilized in RT-PCR reactions using RNA isolated from 48-hpf *Danio rerio* embryos; the resultant PCR products were separated by electrophoresis, cloned into a plasmid vector and subjected to DNA sequencing. To identify the full-length *lama1* transcript, we performed 5'- and 3' RACE reactions and determined sequences for the corresponding products of these reactions. The obtained sequences were arranged into a contiguous assembly and analyzed using Vector NTI™ sequence analysis software.

The *lama1* cDNA contig comprised 9581-bp and contained a 9225-bp open reading frame that was predicted to encode 3075 amino acid protein (Figure 1), 128-bp of 5'UTR and 228-bp of 3'UTR sequence (GenBank number [DQ131910](#)). Detailed analysis of the 5' sequence identified five initiator codon trinucleotides (ATG) in the 24-bp region spanning nucleotides 128-152. Among these potential translational start sites, the second ATG appears to have the most favorable surrounding sequence GCCATGATGG with four nucleotides (underlined) being consistent with the Kozak's consensus sequence identified for vertebrate genomes [30]. As the translational site "context" sequence is not exclusive at any positions and some sites were found to be occupied by non-conserved nucleotides in all five sequences, we selected the most upstream ATG codon as a translational start site for the *lama1* protein.

The overall sequence demonstrated strong homology with laminin genes/proteins of the *alpha* family that were shown to be highly homologous to each other. In order to

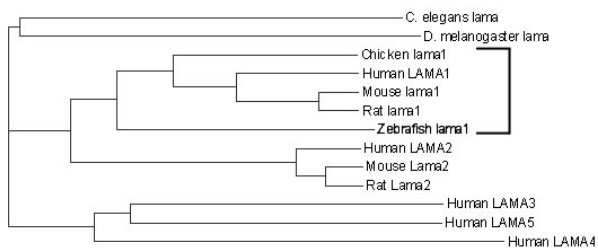


Figure 2
Phylogenetic tree analysis of the laminin alpha proteins. The zebrafish protein demonstrates close relationship with laminin alpha 1 proteins from other species (laminin alpha 1 cluster is indicated by bracket).

position the novel zebrafish gene within the *alpha* laminin family, we performed a phylogenetic tree calculation using the corresponding module of Vector NTI™ suite. This algorithm is based on a sequence distance method and utilizes the Neighbor Joining formula of Saitou and Nei [31]. This analysis demonstrated grouping of the novel transcript with the *laminin alpha 1* sequences from other species (Figure 2). The comparison of human, mouse and zebrafish laminin $\alpha 1$ amino acid sequences showed high identity level throughout the entire protein (Figure 1). Based on BLAST analysis results, the overall zebrafish lama1 sequence demonstrated ~51% identity with human, mouse and chicken laminin $\alpha 1$, and ~42% identity with human and mouse laminin $\alpha 2$ proteins. The laminin alpha 1 contains several conserved domains: short signal peptide (amino acids 1 through 17), N-terminal region (a.a. 18-269), seventeen laminin EGF-like domains and two laminin IV type A1 domains (a.a. 270-1555), and five laminin G-like domains (a.a. 2305-3070) (regions are indicated according to the human LAMA1 protein, GenBank number [P25391](#)). The N-terminal domain demonstrated the highest level of conservation (89% identity with human or mouse sequence) while the identity level in other domains varied from ~30% to 75%. The central region of the laminin alpha 1 protein encompassing amino acids 1555-2085 (this region participates in the coil-coil domain formed by three chains $\alpha 1, \beta 1$, and $\gamma 1$) demonstrated the lowest level of conservation at ~30% (Figure 1). The zebrafish *lama1* nucleotide and protein sequences were submitted to GenBank with accession number [DQ131910](#).

Identification of genomic structure of the lama1 gene

Genomic sequences of the *lama1* gene were identified either by sequence similarity search using cDNA

sequences, BLAST engine and public databases (Zebrafish Whole Genome Sequencing database; 32) or by direct sequencing of products generated by long-range PCR using exonic primers and genomic DNA. The gene was found to consist of sixty-three exons ranging from 87 to 378 bp in length. Overall, the genomic structure of the zebrafish *laminin alpha 1* gene corresponded well with the human *LAMA1*; all the donor and acceptor splicing sites contained characteristic consensus sequences conserved in vertebrates (Table 1).

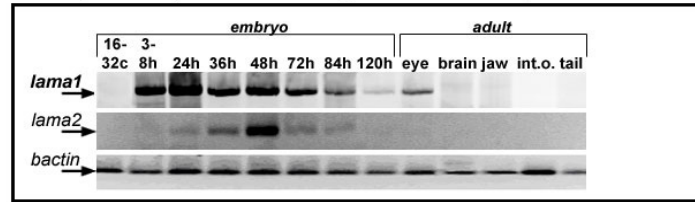
Embryonic expression of zebrafish lama1

Embryonic expression of the zebrafish *lama1* gene was studied by RT-PCR and *in situ* hybridization. Embryos ranging from the 16-32-cell stage to 120-hpf, as well as different adult tissues were examined for the presence of the *lama1* transcript. Expression of *lama1* was strong during embryonic development and depleted in most adult tissues, which is consistent with the previously reported data ([12,13], and [22]). First *lama1* transcripts were detected in 3-8 hpf embryos (encompasses embryos at 1k-cell stage of blastula to 75%-epiboly stage of gastrula) and expression continued at later embryonic and larval stages of development (Figure 3). In adult fish, expression was observed in the eye.

We also tested expression of an additional laminin transcript, *lama2*-like. The Lama1 and Lama2 proteins are highly homologous to each other and were shown to be functionally redundant [33,34]. The *Lama1&2* genes are expressed in separate as well as overlapping domains during development including ocular tissues [35-39]. The 1776-bp *lama2*-like sequence was identified from GenBank (Accession Number [XM_693031](#)) and demonstrated 61% identity with the human LAMA2 at amino acid level. Expression of zebrafish *lama2*-like gene was tested by RT-PCR with gene-specific primers. Based on RT-PCR results, *lama2* expression is detectable starting from 24-hpf embryos (pharyngula) to 84-hpf larvae and was not found in adult tissues. The *lama2* and other, not yet identified, zebrafish *alpha* transcripts are likely to be able to substitute for *laminin alpha 1* and each other during embryonic development. Identification and characterization of these genes is necessary to better understand multiple roles of different laminin isoforms during development.

Whole mount *in situ* hybridization was performed using embryos at 24-, 48-, 72-, and 96-hpf and a 590-bp antisense riboprobe that comprised *lama1* sequence corresponding to nucleotide positions 287-876 (GenBank accession number [DQ131910](#)). Expression of *lama1* was detected in the developing lens, sclera, midbrain, somites, urogenital system and notochord (Figure 3), which is consistent with the *Lama1* gene expression in other species.

I. RT-PCR analysis of *lama1* expression in embryos and adult fish.



II. *In situ* hybridization of *lama1* in zebrafish embryos.

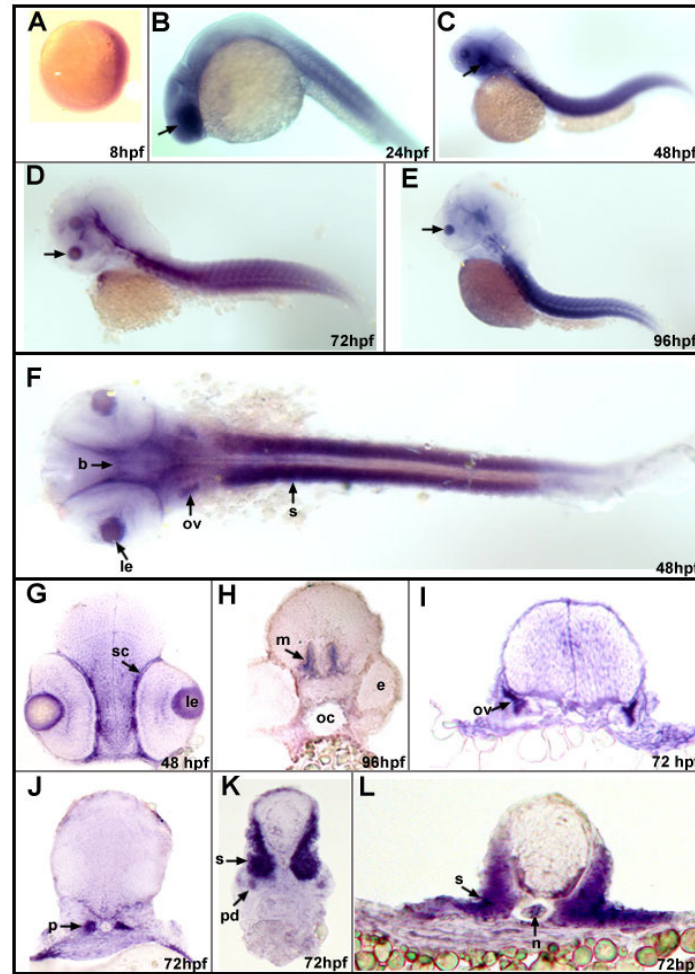


Figure 3

Expression of zebrafish laminin alpha I gene. I. RT-PCR analysis of *lama1* expression in embryos and adult fish. RT-PCR results for *lama1*, *lama2* and control *bactin* transcripts are presented as indicated. Embryonic (16-32 cells to 120-hpf) or adult (1 year old) cDNA samples employed in reactions are indicated at the top: lane 1- 16-32 cells, 2- 3-8 hpf, 3- 24 hpf, 4- 36 hpf, 5- 48 hpf, 6- 72 hpf, 7- 84 hpf, 8- 120 hpf embryos; for adult tissues- lane 9 contains products obtained with adult eye cDNA, 10- brain, 11- jaws, 12- internal organs and 13- tail. **II. *In situ* hybridization of antisense *lama1* riboprobe in zebrafish embryos.** **A-F:** 8-96 hpf whole zebrafish embryos that were hybridized with *lama1* DIG-labeled antisense riboprobe. **G-L:** Transverse sections of 48-96 hpf zebrafish embryos at the level of the eye (**G**), brain (**H**), otic vesicle (**I**), developing kidney (**J**), and trunk (**K**, **L**). Embryonic stages are indicated at the bottom of the picture. At 8-hpf, expression of the *lama1* gene was detected in all embryonic tissues; by 24-hpf, higher levels of transcript were evident in the developing lens (arrows in B-E; **le** in F and G) and sclera (**sc**) of the eye, brain (**b**), somites (**s**), and otic vesicle (**ov**), pronephros (**p**) and pronephric duct (**pd**), notochord (**n**), e-eye, **m**-midbrain.

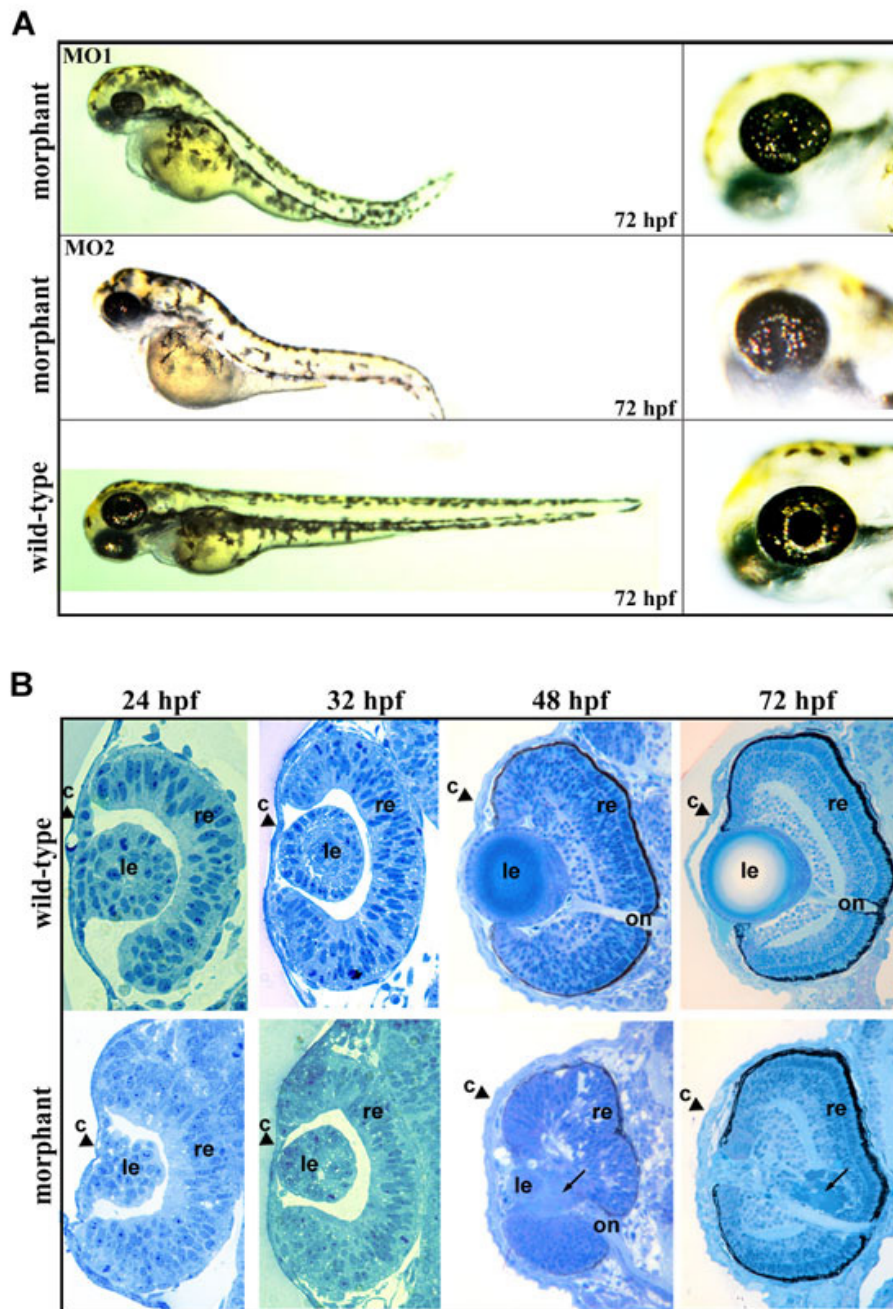


Figure 4
lama I knockdown phenotype in zebrafish. **A**, an overall view of *lama I*-morphants obtained with MO1- (top) or MO2-oligomers (middle), and control (bottom) embryos at 72-hpf. Enlarged image of a head is provided on the right. Defects in body length, axis curvature and eye structure (irregular pupil and a lack of lens) are easily detectable in *lama I* morphants. **B**, transverse sections at the eye level of control (top row) and *lama I*-morphant (bottom row) embryos at 24-, 32-, 48- and 72-hpf are presented. An obvious lens degeneration is first notable in 48-hpf morphant eyes. At 72-hpf, small eyes with missing lens and thickened cornea were observed in the morpholino-injected embryos. retina (r), optic nerve (on), lens (le) and cornea (c) are shown. Black arrows in 48- and 72-hpf eyes indicate to the products of lens degeneration.

Morpholino-mediated knockdown of zebrafish laminin alpha 1 expression

To examine the functions of laminin alpha 1 during embryonic development, we injected *lama1*-specific and control oligonucleotides into 1-2 cell stage embryos. The *lama1* morpholinos were designed to hybridize to the 5' sequence of the *laminin alpha 1* mRNA near the initiation codon (position 1): MO1 oligomer corresponds to sequence from nucleotide -65 to -39 while MO2 morpholino matches sequence between nucleotides at positions -3 and +22.

The morphological phenotype in the morpholino-injected embryos was first detected in 36-hpf embryos and became highly evident by 72-hpf. The morphants exhibited a shortened body, an abnormal body axis curvature, and malformed eyes that often lacked lenses and had misshapen pupils (Figure 4). The percentage of morphants exhibiting the phenotype positively correlated with the concentration of the injected morpholinos and ranged from 30% (0.25 mM; total number = 165) to 60% for MO1 oligomer (0.5 mM; total number = 658) or 50% for MO2 (0.5 mM; total number 650). The rate of early lethality by 24-hpf ranged from 7% (for C = 0.25 mM) to 23% for MO1 (for C = 0.5 mM) or 31% for MO2 oligomer (for C = 0.5 mM). In control experiments, zebrafish embryos that were injected with control morpholinos as well as uninjected larvae were examined for morphological phenotypes. Both groups demonstrated phenotypes indistinguishable from the wild-type fish with a lethality rate of ~10% level for un-injected larvae and embryos that were injected at C = 0.25 mM; embryos that were injected at C = 0.5 mM demonstrated 21% lethality.

To determine ocular defects in the *lama1*-morphants, we compared the histology of the *lama1* morphant eyes to wild-type in embryos ranging from 18-hpf to 72-hpf (18-hpf- data not shown; sections of 24- to 72-hpf embryos are presented at Figure 4). Since morphant fish produced by injections with either MO1 or MO2 oligomers demonstrated similar phenotypes based on visual examination, only MO1- injected morphants have been used for further histological analysis. For 48-hpf and 72-hpf stages, embryos with abnormal phenotype were selected; while for the 18-, 24- and 32- hpf time points, twelve MO1-oligomer- injected living embryos were collected before the phenotype is evident and eight (65%) of these animals were expected to be morphants (this estimate was based on the fact that 65% of embryos from the same group that were raised till 72-hpf demonstrated an ocular morphant phenotype). The stages of the establishment of the lens placode, lens delamination from the ectoderm and formation of the lens vesicle appear to be grossly normal in *lama1* deficient embryos. The cells of the lens vesicle seem to be slightly disorganized in about 20% of 24-32-

hpf embryos (Figure 4), which is below the estimated frequency of morphant embryos in this sample. Therefore, we concluded that formation of the lens vesicle appears to be mostly unaffected in *lama1* morphants. In the 48-hpf morphant eyes, a small lens vesicle remnant was found and it was surrounded by degenerating lens tissue. In the 72-hpf morphants, the lens was absent and lenticular bladder cells seem to form a deposit in subretinal space in some embryos (Figure 4). In normal 72-hpf animals, the following main ocular tissues can easily be detected: 1) laminated retina containing the photoreceptor, inner nuclear and ganglion cell layers; 2) lens that consists of a single layer of epithelial cells and mainly differentiated lens fibers and 3) cornea with three easily observed layers- surface epithelium, a thin lamellar stroma that is contiguous with sclera and flattened endothelial cells [40]. In addition to lens degradation, the eye sections obtained from 72-hpf *lama1* morphant embryos revealed different degrees of thickened cornea and reduced eye size (Figure 4). At the same time, the retina and the optic nerve are present and appear to be grossly normal in the *lama1*-knockdown fish.

Discussion

In this paper, we report identification of the zebrafish *laminin alpha 1* gene and analysis of its role during embryonic development by means of expression and knockdown studies. Our data indicate a strong conservation of *lama1* function during vertebrate development as both the predicted protein sequence and expression pattern of this gene were found to be highly conserved between zebrafish and other species. The highest level of conservation was identified in the N-terminal domain of *lama1* followed by the laminin globular (G-like) domains. The N-terminal domain was shown to be involved in laminin polymerization in vitro [41,42], and binding to integrins $\alpha 1\beta 1$ and $\alpha 1\beta 2$ [43]. The five laminin globular domains located in the C-terminus represent the main cell-adhesive sites and bind the major laminin receptor integrin $\alpha 6\beta 1$ as well as $\alpha 6\beta 4$ and $\alpha 7\beta 1$ [44], extracellular heparan proteoglycan perlecan, dystroglycan, sulfatides and heparin [45]; mice lacking the alpha 1 chain LG4-5 module were reported to die at E6.5 with failure of epiblast differentiation [46]. Preservation of the N-terminal and C-terminal domain sequences throughout vertebrate evolution suggests that the interactions mediated by these regions are of particular importance.

The zebrafish *lama1* gene was found to be strongly expressed during embryonic development. The following high-expression sites were identified in the developing embryo: lens, brain, somites, urogenital system, and notochord. This pattern is consistent with distribution of *Lama1* transcripts in other species ([12,13,22,41,47], and [48]) and suggests a high degree of conservation in *lam-*

inin alpha 1 function during embryonic development in vertebrates. The evolutionarily conserved expression of *lama1* is likely to be governed by a network of specific regulatory elements maintained in phylogenetically divergent species. Identification of *cis*-regulatory regions and *trans*-acting factors that direct the specific *lama1* expression pattern will provide important insight into mechanisms of embryonic development and ocular tissue maintenance.

Knockdown of the laminin alpha 1 expression in zebrafish resulted in a distinct phenotype characterized by anomalies in eye development as well as body axis length and curvature. This condition is different from the phenotype reported in *Lama1*^{-/-} knockout mice [15]. The *Lama1*^{-/-} null mice die prenatally around day 7 post coitus (pc) while the embryos that are deficient in either $\beta 1$ or $\gamma 1$ laminin chains do not survive past day 5.5 pc, which is the blastula stages in mice [15]. The laminin-1 is first detected around the 16-cell stage in mice and present in the two basement membranes formed before gastrulation. Mammalian embryos deficient in any component of laminin-1 ($\alpha 1\beta 1\gamma 1$) survive implantation but die before gastrulation indicating to the critical role of laminin-1 in this process [15,49]. In zebrafish, implantation does not occur as embryogenesis occurs *ex utero*. Gastrulation does not seem to require laminin-1 in zebrafish as both *lamb1*- and *lamc1*-mutants undergo normal germ-layer patterning and gastrulation movements [17]. This phenomenon may be explained by a compensation from another laminin or differences in mechanisms between fish and mammals. Identification and studies of all other zebrafish laminin isoforms are necessary to clarify this issue.

The ocular phenotype in zebrafish embryos deficient in *laminin alpha 1* is characterized by slightly smaller eyes with visible anomalies in lens and cornea development. A primary defect may be the lens degeneration due to developmental arrest that causes collapse of the surrounding ocular tissue (reduction in eye size) and abnormal patterning of the anterior segment structures (cornea defects). Similar associations between lens defects, small eye and malformed anterior chamber have been previously reported [50-58]. At the same time, the visible corneal defects may indicate a discrete function of *lama1* in the development of the anterior segment structures.

The severe ocular phenotype observed in the *lama1*-deficient fish embryos reveals a new role for this molecule during vertebrate embryonic development. The observed *lama1*- knockdown phenotype is consistent with the ocular abnormalities associated with other laminins such as *lamb1* and *lamc1* in zebrafish and *LAMB2* in humans ([9,18,19], and [28]). Interestingly, all these proteins are involved in the only two trimers that *laminin alpha 1* was

found to be a part of: laminin-1 ($\alpha 1\beta 1\gamma 1$) and laminin-3 ($\alpha 1\beta 2\gamma 1$). The *Lamb1* and *Lamc1* proteins are widely expressed in different species and can associate with any alpha laminin. Mutations in *lamb1* and *lamc1* in zebrafish result in complex phenotypes that include lens hypoplasia, lens capsule rupture and corneal defects [19]. The laminin-3 was originally identified in human placenta [27] but *Lamb2* mRNA has also been detected in lens, corneal, pigment epithelial and hyaloid cells during development [9,59-61]. Clear evidence of an important role of beta-2 and its complexes in human ocular development was provided by the discovery of *LAMB2* mutations in human patients affected with complex ocular phenotypes that include lens, iris, corneal, retinal and overall eye-size defects [9]. The exact role(s) of laminin-1 and -3 during vertebrate eye development require further investigation. Identification and functional analysis of zebrafish *lamb2* may provide an important insight into this issue.

There are several ocular phenotypes that involve lens degeneration including *aphakia* [62-64], *dysgenetic lens* [65-67], *lens aplasia* [68,69] in mice. Genes responsible for the *aphakia* and *dysgenetic lens* phenotypes have been identified as transcription factors, *Pitx3* and *Foxe3*; both genes were also shown to be involved in human ocular disorders involving abnormal lens, iris and corneal development [54,55] and zebrafish *pitx3*-morphants displayed lens degeneration similar to mammals [70]. Defects in the basement membrane and/or extracellular matrix were reported in *aphakia* and *lens aplasia* mutants [69,71] indicating a possible connection with the laminin and/or other extracellular matrix molecule pathway(s) that needs to be further investigated.

The lens is surrounded by the lens capsule that represents a thick basement membrane that includes laminins, collagen IV, heparan sulfate proteoglycans (perlecan), nidogen and fibronectin. The other components, such as type XV and type XVIII collagen, agrin, fibulins and growth factors, may be present at some stages as well. The importance of the extracellular matrix/basement membrane for lens development was proposed based on the distinctive spatio-temporal expression patterns of different extracellular matrix proteins during lens development [23,72-74], changes in the distribution of the extracellular matrix proteins during normal and aberrant lens development [23,69,71,75] as well as human and animal phenotypes associated with mutations in ECM component genes that, in addition to the above discussed, include perlecan [76] and collagen XVIII [77].

Based on the phenotype observed in zebrafish, mutations in the laminin-1 components are likely to contribute to human disorders of the lens (cataracts) and/or anterior segment development (glaucoma). Also, because the lens

plays an important role in normal ocular growth, defects in lens development may also play a role in another common ocular disease- myopia [78]. This possibility is further supported by the fact that the messenger RNA for *lama1* was detected in the developing sclera in addition to the lens in zebrafish; sclera cell development has been shown to be important for the normal eye growth in several studies [79-82]. In humans, *LAMA1* maps to the 18p11.31 region that contains a gene for high-grade myopia (MYP2; [83]). The affected individuals were characterized by an average spherical component refractive error of -9.48 diopters and an average age at diagnosis of myopia of 6.8 years; no clinical evidence of connective tissue abnormalities has been noted ([83]). Because of the essential role of laminin-1 in governing early events in mammalian development, human laminin-1 mutations in ocular phenotypes, if any, are most likely to be detected in a heterozygous state and/or to be specific to the particular interactions involved in eye development and maintenance. The expression pattern of *LAMA1* in human ocular tissues needs to be determined and the potential contribution of this gene in ocular disease should be examined.

Conclusion

The *laminin alpha 1* gene was found to play an important role in ocular development in zebrafish. Given that *Lama1* was shown to be expressed in eye tissues in mammals as well, this gene is likely to have a similar role in these higher species. Additional studies into the specific role(s) of laminin-1 and laminin-3 during eye development are necessary. The findings can then be correlated with specific human phenotypes to identify mutations that may impair different regions of this complex molecule.

Methods

Animals

Zebrafish (*Danio rerio*) were raised and maintained on a 14-hour light/10-hour dark cycle. The embryos were obtained by natural spawning and raised at 28.5°C. The developmental stage was determined by time (hours post fertilization (hpf)) and by morphological criteria [84]. All experiments were conducted in accordance with the guidelines set forth by the animal care and use committees at the Medical College of Wisconsin.

Cloning of *lama1*: RT-PCR, RACE, long-range PCR, cloning and sequencing

PCR products were generated using specific oligos, *PfuUltra* high-fidelity DNA polymerase (Stratagene, La Jolla, CA) and standard conditions described elsewhere [70]. The PCR products were separated by electrophoresis in 1% agarose gel, cloned into a pCRII-TOPO vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequencing using the ABI PRISM 373 DNA Sequencer. The 5'- and 3'

RACE (Rapid Amplification of CDNA Ends) was performed using BD SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA) and the following oligonucleotides: 5'- ACCACAGGTTGGTTCCATCGATG-3' for the 5'RACE and 5'-GCGGACCACACACAGACCATCCC-3' – for the 3' portion of the transcript. The overlapping *lama1* sequences were analyzed and arranged into contig using Vector NTI™ sequence analysis software. The long-range PCR was performed using TripleMaster™ PCR System (Eppendorf, Hamburg, Germany) and conditions suggested by the manufacturer.

Expression analysis: RT-PCR and tissue in situ mRNA hybridization

For the RT-PCR reaction: the *lama1* specific oligonucleotides complementary to sequences at positions 3031-3050, 5'-TGTCTGCGTCATGTGATGAG-3', forward primer, and positions 8440-8421, 5'-TCGCCATGTGAACAGAACG-3', reverse primer, were used to amplify 5406-bp *lama1* products from cDNA extracted from 16-cells to 120-hpf embryos. The sequence predicted to represent zebrafish *lama2* gene was identified from the database (GenBank number [XM_693031](#)) and the following primers were used to amplify 275-bp gene-specific product: forward, AAGCATCATGAACGGGATGG, and reverse, TGGAGTAGAAGGAGGTACAG. Control primers, 5'-GAGAAGATCTGGCATCACAC-3', forward and 5'-ATCAGGTAGTCTGTCAGGTC-3'- reverse primer, were used to amplify 324 -bp fragment of *beta-actin* gene. For the *lama1* *in situ* hybridization, the following probe was prepared: a 590-bp fragment that comprised *lama1* sequences corresponding to nucleotide positions 287-876 and 1475-3031 (GenBank accession number [DQ131910](#)) was subcloned into pCRII-TOPO plasmid (Invitrogen, Carlsbad, CA) and used as a template for making an antisense riboprobe. The digoxigenin-labeled antisense riboprobe was prepared using DIG RNA Labeling Kit (Roche Applied Science, Indianapolis, IN) and manufacturer protocols. Anti-DIG AP (1:2000) and NBT/BCIP substrate (Roche Applied Science, Indianapolis, IN) were used to detect the probes. Wild-type PTU-treated zebrafish embryos at 8- 96 hpf were fixed in 4% paraformaldehyde/PBS then washed in PBS and fixed in 100% MeOH. Then whole-mount *in situ* embryos were fixed in 4% paraformaldehyde/PBS and infiltrated with 2-h steps of 15% sucrose, 30% sucrose and 100% Tissue-Tek OCT (Miles Inc., Elkhart, IN). Fifteen to twenty embryos were oriented in freezing molds and stored at -20°C until sectioning. Ten-micrometer sections were cut on a cryostat and mounted on gelatin-coated glass slides.

Morpholino oligomer injections and histology

The *lama1*-specific morpholino oligomers were designed using Gene Tools (Corvallis, OR) services and purchased from the company. Two oligomers were made to hybrid-

ize to the sequence in the 5' UTR of *lama1* transcript: **MO1**, 5'-ATAAAGCTAAAGCTGTGCTGAAATC-3', and **MO2**, 5'-TCITCATCCTCATCTCCATCATCGC-3'. Control oligomer: 5'-AAACAAACCTGAGGACAGATGGA-3'. The morpholinos were resuspended in water and injected into 1-2 cell stage embryos using Nanoject II injector (Drummond Scientific, Broomall, PA) or MM33 Mircomanipulator (Stoelting Co., Wood Dale, IL) as described elsewhere [85]. Approximately eight (MM33) or fifteen (Nanoject II system) nanoliters of oligomer mixture was injected into each 1-2 cell embryo. The embryos injected with *lama1*- or control morpholino oligos as well as uninjected embryos were allowed to develop at normal temperature (28.5 °C) and examined for morphological phenotypes every 6-24 hours.

For the histological analysis of the 48-hpf and 72-hpf stages, morphant embryos that exhibited short body and abnormal eye phenotype were identified and collected. For the examination of the 18-hpf to 32-hpf embryos, twelve living embryos were collected for every stage following MO1-morpholino injection; fifty embryos from the same group were monitored till 72-hpf and 65% of these animals demonstrated mutant phenotype. Therefore a mixture of ~1/3- wild-type and 2/3- morphant embryo sections was expected to be present at 18-, 24- and 32-hpf slides. Histological specimens were processed as previously described [40]. In brief, embryos were fixed in primary fixative [2% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, 0.06% phosphate buffer (pH 7.4)] at 4 °C for 24 hours and then washed in 0.1 M phosphate-buffered saline (PBS), dehydrated through an ethanol series and propylene oxide and then infiltrated with Embed-812/Araldite resin mixture. The 1 µm- thin plastic sections were cut with a glass knife on a JB4 microtome. Sections were stained with 1% Toluidine Blue in 1% Borax buffer. Images were captured using a Nikon coolpix 995 digital color digital camera mounted on a Nikon E800 compound microscope with a 60X oil-emersion objective.

List of abbreviations used

lama1- laminin alpha 1; RT-PCR- reverse transcription polymerase chain reaction; RACE- rapid amplification of cDNA ends; hpf- hours post fertilization.

Authors' contributions

Natalia Zinkevich performed *in situ* hybridization analysis of *lama1* expression and knockdown studies. Dmitry V. Bosenko carried out *lama1* gene sequence identification and RT-PCR analysis. Brian A. Link participated in an experimental analysis of knockdown phenotype, study design and valuation. Elena V. Semina designed the study, supervised data collection, analysis and interpretation of

results. All these authors participated in drafting the paper and all authors read and approved the final manuscript.

Note added in proof

While this article was in revision, identification of the zebrafish *laminin alpha 1* gene and associated notochord and blood vessel phenotype has been described by Pollard et al. [86].

Note

Table 1. Exon- intron boundaries of laminin alpha I gene.

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