3.3: Discussion

3.3.1: The expression pattern of *lama1* is conserved in vertebrates

Very few studies have performed a detailed analysis of the expression pattern of *lama1* in the zebrafish embryo, although detailed analyses of *Lama1* expression have been performed in the mouse embryo. The signalling pathways and transcription factors that regulate the expression of *lama1* in the zebrafish embryo have also not been previously addressed. In this chapter, I have reported on the expression pattern of *lama1* in the zebrafish embryo from the two-cell stage to 97hpf, and addressed the role of Hh signalling in the regulation of *lama1* expression.

In contrast to previous reports, my in situ hybridisation data reveals that *lama1* is expressed as early as the two-cell stage as maternal *lama1* transcripts. Others reported that *lama1* transcription first occurs at 3hpf (Zinkevich et al. 2006) or at 6hpf (Pollard et al. 2006). The expression of Laminin genes as maternal transcripts in the zebrafish embryo is not uncommon. Both *Lamb1* and *Lamc1* are detected as maternal transcripts, and maternal Laminin-111 protein is also produced at the blastula stage (Parsons et al. 2002). As both Laminin β and γ subunits require the presence of Laminin α chains for their secretion (Yurchenco et al. 1997), the presence of Laminin-111 protein suggests that Laminin α1 chain must also be synthesised at this stage. Therefore, my observation that *lama1* is maternally expressed is consistent with the presence of maternal Laminin-111 protein (Parsons et al. 2002).

Thus, the discrepancy observed in *lama1* expression could be due to the presence of alternative *lama1* mRNA transcripts. Indeed, alternative transcripts are common to the Laminin genes (Airenne et al. 1996; Tunggal et al. 2000; Hamill et al. 2009). Supporting this idea, the RNA probe used by Pollard et al. (2006) binds to a different region of the *lama1* mRNA in comparison to the RNA probe used in this thesis. Pollard et al. (2006) designed a probe with homology to the *lama1* transcript that includes 439 bases of 5'UTR, whereas the RNA probe used in this thesis binds to only 121 bases of 5'UTR of the *lama1* transcript. Two alternative zebrafish *lama1* transcripts are reported in Ensembl.org, although both lack annotation of a 5'UTR sequence. Human also has two *lama1* transcripts, whilst chicken has four transcripts (www.ensembl.org).

Consistent with previous reports, I found that *lama1* expression is restricted to the chordamesoderm and neural plate in embryos whilst at the tail bud stage (10hpf) (Pollard et al. 2006). *lama1* is expressed throughout the chordamesoderm, whilst Laminin-111 immunoreactivity is detected around the forming chordamesoderm (Parsons et al. 2002). From the 9-somite stage and onwards, I show that *lama1* is restricted to the posterior notochord, although by the 19-somite stage, *lama1* is no longer detected in the notochord. Like the chordamesoderm, expression of Laminin-111 protein is not observed within the notochord but is observed around the notochord, in the notochordal basement membrane (Parsons et al. 2002;

Pollard et al. 2006). Expression of *Lama1* in the notochord of the chick or mouse has not been reported (Miner et al. 1997; Zagris et al. 2000; Anderson et al. 2009).

My data reveal that *lama1* is later expressed in the somites and pre-somitic mesoderm (PSM), anterior CNS, neural tube, eye, otic vesicles, lateral line organs, notochord, hypochord, pro-nephric tubules, vasculature, and the uro-genital region. After 24hpf, I reported a decreased expression of *lama1* in the somites and neural tube, and this is in agreement with Pollard et al. (2006) and Sztal et al. (2011). However, others report that *lama1* expression is strongly maintained throughout the somites, even at 96hpf (Zinkevich et al. 2006). By 48hpf, I show that *lama1* expression remains in the eye, midbrain-hindbrain boundary, and otic vesicles, and *lama1* expression is also detected in the pectoral fins and the musculature of the jaw. This expression pattern is maintained up to 97hpf, at which time otic vesicle expression is also down-regulated. Based on the lack of *Lama1* expression in adult mice, and the fact the zebrafish expresses *lama1* in a conserved pattern, I predict that adult zebrafish would also lose expression within the anterior CNS and jaw musculature.

In this thesis, the down-regulation of *lama1* expression in the somites occurs in an anterior to posterior gradient, creating a posteriorised expression pattern. *lama1* expression is maintained in the posterior somites, which are newly generated from the PSM. As *lama1* expression is down-regulated in all somites and in the PSM by 24hpf, coinciding with the time somitogenesis is nearly complete and no new somites are produced (Stickney et al. 2000), this suggests a requirement for Laminin α1 in early but not late somitogenesis. Laminin-111 is also detected in the PSM of 24hpf zebrafish, which correlates with the expression of *lama1* (Parsons et al. 2002; Pollard et al. 2006).

Lama1 is also detected in the somites of E9.5 mouse embryos (Miner et al. 1997; Anderson et al. 2009). However, there are slight differences in the expression pattern between mouse and zebrafish somites. In zebrafish, *lama1* is initially expressed throughout both prospective fast and slow muscle domains, and by the 19-somite stage, it is down-regulated in fast muscles but maintained in the adaxial slow muscle cells. In comparison, *Lama1* expression in the mouse occurs in the sclerotome, and not in the myotome (Anderson et al. 2009).

The myotomal BM separates the sclerotome and the myotome of the mouse somite, and it is composed of Laminin-111 and -511. Its formation requires the presence of Laminin α1 (Anderson et al. 2009). Therefore, it is likely that Laminin-111 is secreted by sclerotomal cells where *Lama1* is expressed, and then assembled to form the myotomal BM at the surface of the myotomal cells, which express the Integrins and Dystroglycans (Bajanca et al. 2004; Bajanca et al. 2006; Anderson et al. 2009). In support of this, *lamb1* and *lamc1*, encoding Laminin β1 and Laminin γ1, respectively, are expressed throughout the somites of both zebrafish and mouse embryos (Yurchenco and Wadsworth 2004; Sztal et al. 2011). The requirement of Laminin α1 for

Laminin β1 and Laminin γ1 secretion (Yurchenco et al. 1997) further suggests that the Laminin-111 heterotrimer assembles at the source of *Lama1* expression, in the mouse sclerotome.

In zebrafish, Laminin-111 accumulates at the myotendinous junction (MTJ) which is the closest equivalent to the mouse myotomal BM (Parsons et al. 2002; Pollard et al. 2006). Similar to the mouse, Laminin-111 is secreted and contributes to the formation of the MTJ where Laminin receptors are located (Snow and Henry 2009). However, unlike the mouse, Laminin-111 secretion is likely to occur in the zebrafish myotome, which expresses *lama1*, and not in the sclerotome. Loss of Laminin α1 in zebrafish leads to defective formation of the MTJ, as demonstrated by the detachment of muscle fibres from the MTJ (Sztal et al. 2012). Mutations in other Laminin genes including *lama2* and *lamb2* also result in MTJ abnormalities, and lead to the retraction of muscle fibres from the somitic boundaries and their eventual apoptosis (Hall et al. 2007; Jacoby et al. 2009).

In zebrafish, expression of *lama1* in the forming notochord is likely to contribute to Laminin-111 in the notochordal BM, which is essential for survival and differentiation of the notochord (Parsons et al. 2002; Pollard et al. 2006). *lama1*, *Lamb1* and *Lamc1* in the myotome are also likely to contribute to this BM, and this is supported by evidence that all three Laminin chains can be supplied to the notochordal BM from non-notochordal sources (Parsons et al. 2002; Pollard et al. 2006). The notochordal BM also binds to adjacent myogenic cells, which become committed to the adaxial cell fate (Hirsinger et al. 2004). As development proceeds, adaxial cells stack up along the dorso-ventral axis of the somite whilst elongating in the antero-posterior axis (Devoto et al. 1996). The notochordal BM and Laminin α1 within the myotome may play a role in the regulation of cellular migration during these complex morphogenetic movements. In agreement, loss of Laminin γ1 in *sleepy* zebrafish causes a delay in slow and fast muscle elongation (Peterson and Henry 2010; Dolez et al. 2011), in addition to abnormal Engrailed expression and patterning of the myotome (Dolez et al. 2011).

Together, these results raise the possibility that *lama1* may be required directly and/or indirectly during somite formation in the zebrafish embryo. The requirement for Laminin α1 in notochord development provides an indirect control mechanism, whereby the notochord is essential for normal somite patterning and plays a role in the release of important signals such as Sonic hedgehog (Currie and Ingham 1996). Laminin α1 may be also required directly for efficient muscle morphogenesis and muscle fibre elongation, through its contribution to the MTJ. In support of this, *Lamb1* and *Lamc1* are also required for efficient MTJ formation, and their mutation in *grumpy* and *sleepy* zebrafish, respectively, leads to the elongation of fast muscle fibres across the MTJ into adjacent somites (Henry et al. 2005; Snow et al. 2008).

I find that *lama1* is also expressed in the vasculature and pro-nephric tubule region of the zebrafish embryo. This is particularly evident at the 19-23-somite stage. Laminin-111 protein is

also detected in the pro-nephric tubules (Seiler and Pack 2011). Thus, here Laminin-111 protein is deposited close to its source of production. *lama1* expression in the vasculature may also contribute to the Laminin-111 that is detected at the MTJ. Laminin-111 in the MTJ is required for the correct migration of intersegmental blood vessels (Parsons et al. 2002; Pollard et al. 2006).

Expression of *lama1* in the eye of the zebrafish closely matches the expression of Laminin-111 in the eye. I show that *lama1* is expressed in the retina and the lens up to the latest time point that I analysed, at 74hpf. In comparison, Laminin-111 immunoreactivity is detected in the lens, cornea, optic nerve, and the retinal BM (Semina et al. 2006). Semina et al. (2006) also report expression of *lama1* in the cornea. Expression of *lama1* in the lens and retina of the zebrafish eye therefore contributes to the Laminin α1 protein detected in the retinal BM, lens, and the optic nerve. In the mouse, *Lama1* is also expressed in the lens in addition to the ciliary bodies (Sarthy and Fu 1990; Dong and Chung 1991; Falk et al. 1999). In both species, *lama1* expression in these eye structures is required for the normal development of the lens, cornea, retina, and vasculature of the eye (Libby et al. 2000; Semina et al. 2006; Zinkevich et al. 2006).

The presence of Laminin-111 protein in the optic nerve (Semina et al. 2006) is likely to play a role in axonal pathfinding, through mediating interactions with the extra-cellular matrix. Strengthening this idea, a role for Laminin α1 in mediating the migration of facial branchiomotor neurons through the anterior CNS in zebrafish has previously been identified (Paulus and Halloran 2006; Sittaramane et al. 2009). This is consistent with my findings that strong expression of *lama1* is observed in the anterior CNS of the zebrafish up to 24hpf. *lama1* is then down-regulated after 24hpf, but it is still maintained in the midbrain-hindbrain boundary even up to 97hpf. Resembling *lama1* expression here, Laminin-111 protein is detected in the midbrain-hindbrain boundary and the cerebellum of the zebrafish at 32hpf (Parsons et al. 2002). It has been shown by others that Laminin expression in the basal neuro-epithelium of the anterior CNS is required for normal brain morphogenesis in the zebrafish embryo (Gutzman et al. 2008). Laminin mediates the constriction of the neuro-epithelium cells, causing the conserved folding observed in the vertebrate midbrain-hindbrain boundary (Gutzman et al. 2008). Maintenance of *lama1* expression in the zebrafish midbrain-hindbrain boundary is therefore likely needed for normal brain morphogenesis. The midbrain-hindbrain boundary contributes to the formation of the cerebellum (Louvi et al. 2003; Gutzman et al. 2008), and defects are also observed in the development of the cerebullum in mice which lack *Lama1* (Ichikawa-Tomikawa et al. 2012).

Overall, the expression of *lama1* is closely associated with the distribution of Laminin α1 in the anterior CNS, including the midbrain-hindbrain boundary and the cerebellum, in both zebrafish and mouse embryos.

Laminin α1 protein is also detected in the basal lamina of the ventral neural tube at 24hpf (Sittaramane et al. 2009). This correlates with the expression of *lama1* in the neural tube, which I

revealed is strongly expressed in the floor plate of the neural tube in 6-19-somite stage zebrafish embryos. After the 19-somite stage, I find *lama1* expression in the neural tube becomes dispersed along the dorso-ventral axis. Laminin α1 antibodies also strongly label the otic vesicle at 24hpf (Sittaramane et al. 2009). Laminin α1 in the otic vesicles is synthesised from *lama1* which I show is strongly expressed in the forming otic vesicles from the 19-somite stage, and persists until 74hpf.

The lateral line organ develops from a post-otic placode that produces a migrating sensory primordium and afferent neurons (Sarrazin et al. 2010). The identification of *lama1* expression in the zebrafish lateral line organ in this thesis is a novel finding. It is possible that Laminin α1 could also be expressed in the lateral line organ, and may play a similar role in both the otic vesicle and lateral line organ. It might be involved in the migration and organisation of sensory cells, or alternatively, Laminin α1 and other members of the extra-cellular matrix may play a role in placode development and morphogenesis. In support of this, Laminins have previously been implicated in otic primordium development in chick (Visconti and Hilfer 2002), and cell-matrix interactions are often crucial for normal cellular migration (Anderson et al. 2009; Frantz et al. 2010).

Overall, the *lama1* expression pattern is conserved in the mouse, chicken and zebrafish, with strong expression detected in the anterior CNS, neural tube, somites, eye, and pro-nephric tubules in each species (Miner et al. 1997; Zagris et al. 2000; Pollard et al. 2006; Zinkevich et al. 2006; Anderson et al. 2009). My findings that *lama1* is expressed in the pectoral fin has recently been confirmed by others (Sztal et al. 2011). Expression of *lama1* in the pectoral fins is also analogous to the *lama1* expression detected in the limb bud of E11.5 mouse embryos (unpublished data, Kalin Narov thesis).

However, some differences in the sites of *lama1* expression are observed between species. For example, unlike the zebrafish, neither the mouse nor the chick express *Lama1* in the notochord, and in both zebrafish and mouse, strong *lama1* expression is detected in the PSM whilst *Lama1* is absent from the chick PSM (unpublished data, Kalin Narov thesis). This suggests that the regulatory enhancer elements present in the *lama1* gene of mouse and zebrafish that activate expression of *lama1* in the PSM, have been lost in the chick during evolution. Alternatively, the chick PSM may not express the necessary transcription factors required to activate *Lama1*. Despite the absence of *Lama1* in the chick PSM, BMs still form around newly formed somites. Laminin α1 produced in the somites (Zagris et al. 2000) may contribute to these BMs, in addition to Laminin α5 that is produced in the lateral plate mesoderm (Coles et al. 2006). If Laminins are required for normal morphogenesis events in the PSM, then Laminin α5 produced in the lateral plate mesoderm may also compensate for the absence of *Lama1*. As in the zebrafish, *Lama1*, *Lamb1* and *Lamc1* produced in the somite of chick and mouse (Schuler and Sorokin 1995; Zagris

et al. 2000; Anderson et al. 2009; Sztal et al. 2011), is likely to form Laminin-111 and contribute to the notochordal BM of the chick and mouse. This fits with the finding that *Lama1* is not expressed in the notochord of the chick or mouse (Zagris et al. 2000; Anderson et al. 2009).

3.3.2: Hh signalling plays a role in the regulation of zebrafish *lama1* expression

Loss of Hh signalling data reveals that Hh is not required for *lama1* expression at the 12-15-somite stage, but is required for *lama1* expression by 24hpf. Specifically, Hh is needed within the PSM and the uro-genital region for normal *lama1* expression. The unaffected expression pattern of *lama1* in the PSM at earlier stages of development suggests that the reduction of *lama1* expression at 24hpf is due to defective maintenance of expression. The uro-genital region however is not structurally obvious at the 12-15-somite stage, but can be observed by 24hpf (Kimmel et al. 1995). The reduction of *lama1* expression in this structure at 24hpf could therefore be a result of improper activation of *lama1* expression caused by loss of Hh signalling, in addition to a possible requirement of Hh signalling for the maintenance of *lama1* in this structure. A role for Hh in the control of *lama1* expression in the PSM and uro-genital region is supported by the fact that Hh signalling is active in these regions, as demonstrated by the expression of the Hh-target gene *ptc1* in these tissues.

However, my data also shows that *ptc1* expression remains in the PSM, somites, and anterior CNS of 12-15-somite stage *smu* and cyclopamine-treated zebrafish embryos. This suggests that residual Hh signalling is occurring in these embryos, which could be sufficient for normal *lama1* expression at the 12-15-somite stage.

Alternatively, it is possible that other signalling factors function to regulate the expression of *lama1*. These factors may be responsible for the initiation of *lama1* expression, whilst Hh signalling may be required for the maintenance of *lama1* in the PSM and uro-genital region. Other signalling factors may also have a role in maintaining *lama1* expression and therefore compensate for the loss of Hh signalling, although only partly within the PSM and uro-genital region by 25hpf.

Not only is Hh signalling necessary for *lama1* expression in the PSM and uro-genital region, it is also sufficient. *Ptc1/2* mutant and dnPKA mRNA-injected embryos have increased levels of Hh signalling and significantly up-regulate expression of *lama1* in the PSM and uro-genital region at the 15-somite stage and at 25hpf. Therefore, data indicates that *lama1* expression in the PSM and uro-genital region is controlled by a PSM/uro-genital region specific enhancer, which is responsive to Hh signalling. However, it is unknown if Hh directly or indirectly regulates *lama1* expression in the PSM.

My results contrast with previous data in the mouse embryo (Anderson et al. 2009). Indeed, in E9.5 Hh-deficient mouse embryos, *Lama1* expression is lost in the sclerotome and the neural tube, but is unaffected in the pre-somitic mesoderm. This means that whilst Hh signalling

maintains *lama1* expression in the PSM of zebrafish, Hh is not required for *Lama1* expression in the PSM of the mouse. However, Hh signalling is required for somitic and neural tube expression of *Lama1* in the mouse, but not in the zebrafish. This suggests that a possible shuffling of Hh-responsive elements has occurred from an enhancer controlling *lama1* expression in the PSM of zebrafish, to an enhancer which controls *Lama1* expression in the somites and neural tube of the mouse, during the course of evolution (*Figure 3.23*). Alternatively, distinct molecular environments in the PSM and somites of zebrafish and mouse may account for the differences observed in *lama1* expression. In the zebrafish, signalling cues or transcription factors may be present within the somites which are capable of activating and maintaining *lama1* expression in the absence of Hh signalling, which are not present within the zebrafish PSM. In contrast, signalling cues or transcription factors could control *Lama1* expression within the PSM of the mouse which lack Hh signalling, but their absence in the somites and neural tube means a loss of *Lama1* expression from these tissues.

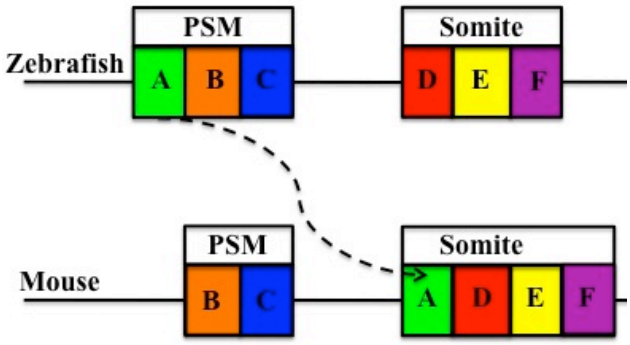

Figure 3.23: *A model to represent shuffling of enhancer elements between the zebrafish and the mouse.* In zebrafish, *lama1* in the PSM, and not the somite, is responsive to a loss of Hh signalling. In contrast, *Lama1* in the somite (and neural tube), and not the PSM, is responsive to a loss of Hh signalling in mouse embryos. It is possible that through the course of evolution, a Hh responsive element (A, green box) found in an enhancer that controls *lama1* expression in the PSM of zebrafish has translocated to an enhancer which controls *Lama1* expression in the somite (and neural tube) of the mouse embryo. A-F represent different DNA sequences capable of binding transcription factors, within tissue-specific enhancers.

In addition to the up-regulation of *lama1* expression in the PSM and uro-genital region of *ptc1/2* mutant and dnPKA mRNA-injected embryos, I find that Hh signalling is sufficient for *lama1* expression in the anterior CNS, eye, neural tube and the somite, at the 15-somite stage. By 25hpf, Hh is also sufficient for expression in the hypochord, vasculature, and pro-nephric tubules, but no longer within the somite. This suggests that although loss of Hh signalling has no effect upon *lama1* expression in these tissues, these tissues are still capable of activating *lama1* expression in response to Hh signalling. This indicates that a combination of regulatory factors controlling the expression of *lama1* in these tissues, which are unaffected by loss of Hh signalling presumably due to compensation from other signalling mechanisms. By 25hpf, it is likely that repressive signals are functioning to prevent the activation of *lama1* in the somites. As previously mentioned, tissues requiring Hh for *lama1* expression in zebrafish (PSM and uro-genital region) also up-regulate *lama1* expression in response to Hh signalling. Therefore, it is plausible to propose that, as in the zebrafish, over-expression of Hh in E9.5 mouse embryos would lead to up-

regulation of *Lama1* expression in the somites and neural tube. These tissues require Hh signalling for *Lama1* expression (Anderson et al. 2009).

Taken together, the data suggest that enhancers controlling *lama1* expression in these tissues are controlled by a variety of transcription factors. Whether Hh signalling directly affects *lama1* transcription in these tissues of both the zebrafish and mouse is unknown. For example, the changes in *lama1* expression in the zebrafish somite could be due to morphological changes that occur with alteration to Hh signalling. In 12-15-somite stage zebrafish embryos, *lama1* is expressed in both slow and fast muscle cells. Embryos lacking Hh signalling do not form slow muscle fibres and adaxial cells (Barresi et al. 2000), and fast muscle cells expand and fill the site in which adaxial cells would normally form (Barresi et al. 2000). Therefore, the expression of *lama1* could appear unchanged, as the whole somite continues to express *lama1*. Although *lama1* is expressed in both slow and fast muscle cells at this stage, *lama1* expression is more intense within the adaxial cell slow muscle population compared to the fast muscle cells. Therefore, an expansion of the adaxial cell slow muscle population, caused by increased Hh signalling such as in *ptc1/2* or dnPKA mRNA-injected embryos (Hammerschmidt et al. 1996; Barresi et al. 2000; Koudijs et al. 2008), could account for the overall increased expression of *lama1* throughout the somite that I observed at the 15-somite stage in *ptc1/2* or dnPKA mRNA-injected embryos. By 25hpf, *lama1* expression within the somites is nearly absent. Changes in the proportion of fast and slow muscle fibres caused by an alteration to the levels of Hh signalling would therefore have little effect on the expression of *lama1* in the somite.

Overall, the expression pattern of *lama1* is conserved between the zebrafish and mouse embryo. However, there is some variation within the somites, as zebrafish express *lama1* in the myotome, whilst *Lama1* is expressed in the sclerotome of the mouse. Morphological differences in the somite between zebrafish and the mouse may account for this. Whereas the zebrafish somite is mainly composed of myotome and very little sclerotome, the mouse has evolved to create a relatively smaller myotome and a larger sclerotome in the somite, to cope with the needs of supporting its own body weight (Stickney et al. 2000). Therefore, despite the fact that *Lama1* is expressed in the sclerotome of E9.5 mouse embryos, it is expressed in the medial somite close to the source of Hh signalling from the notochord and the floor plate. This is similar to the expression of *lama1* in the adaxial cells of the zebrafish somite. Although Hh signalling has a role in the regulation of *lama1* in both zebrafish and mouse embryos, some of the regulatory mechanisms controlling the gene have diverged. This could be due to changes in the enhancer sequences controlling the gene, or due to changes in the molecular environment. Alternatively, changes to the chromatin structure through epigenetic mechanisms could restrict the availability of transcription factors to *lama1* enhancers, thereby altering the regulatory networks between zebrafish and the mouse.