Chapter 8

Discussion

Summary

To determine the function of the interaction between Jamb and Jamc during embryonic development, I studied the expression and biochemistry of both proteins, characterized the phenotype of loss-of-function mutations of both genes and determined the mechanism and necessity of physical interaction between Jamb and Jamc *in trans* for myoblast fusion *in vivo*.

In this chapter I will discuss the importance and implications of these findings in the context of vertebrate myogenesis, unanswered and open questions and future directions.

8.1 Novel regulation of myoblast fusion in vertebrates

The central aim of this project was to discover a biological function for the interaction between Jamb and Jamc during embryonic development of zebrafish. I examined the orthology and evolutionary conservation of all the members of the zebrafish *jam* family, identifying two novel paralogues, *jama2* and *jamc2* (Chapter 3). Once aware of the extent of duplication of the gene family in teleosts, I sought to explore the possibility of redundancy between paralogues through a thorough analysis of their respective expression patterns and biochemical properties. I determined that the *JAM-B*-like and *JAM-C*-like paralogues were expressed in different tissues in different developmental stages, suggesting little conservation of regulatory elements between them (Chapter 4). In addition, the *JAM-B*-like and *JAM-C*-like paralogues had retained similar binding specificity but with varied relative strengths of interaction, suggesting that they are not equivalent biochemically (Chapter 5). I then characterised the phenotypes of embryos containing heritable mutations in *jamb* and *jamc* (Chapter 6). The mutant embryos displayed a near-complete block in myoblast fusion, resulting in a striking arrangement of nuclei positioned centrally with respect to myotome boundaries in each somite. Interestingly, there was a concomitant increase in the number of fast muscle fibres of approximately 1.8-fold in both mutants compared to wild-type embryos. This suggests that the majority, if not all, myoblasts within the somite are able to form a muscle fibre in the absence of fusion. Through transplant experiments, I determined that Jamb and Jamc do not interact *in cis* or with any other ligand, but instead interact between cells *in trans*, and this interaction is essential for myoblast fusion (Chapter 7).

These findings are not consistent with the currently held paradigm of muscle development, first identified in grasshopper (Ho *et al*, 1983) and thoroughly characterised in *Drosophila* (recently reviewed in Rochlin *et al*, 2010; Haralalka and Abmayr, 2010). The founder cell model posits that a muscle fibre is pre-figured by specification of a rare sub-population of myoblasts as founder cells. These cells contain all the information necessary to form any of the 30 possible body wall muscles in each hemisegment. In the absence of myoblast fusion, the founder cells still continue to develop, forming mononucleate and differentiated muscle fibres (Ruiz-Gomez *et al*, 2002). The remaining myoblast population, the fusion-competent myoblasts (FCMs), remain rounded, weakly express myosin and are cleared by macrophages (Rushton *et al*, 1995). Vertebrate myoblast fusion has largely been explored by comparison to this model. The conservation of activity of orthologues of

Drosophila proteins in vertebrate models, for example *kirrel*, has implied a conservation of mechanism. However, the phenotypes of disruption of these genes have suggested otherwise; a possibility that has not been acknowledged in the literature. For example, genetic disruption of the vertebrate homologues of *myoblast city*, *Dock1* and *Dock5*, does result in a block in myoblast fusion – the involvement of the orthologues in this process is clearly conserved. However, loss-of-function of these genes does not result in a small number of elongated muscle fibres and a numerous population of rounded myoblasts that undergo apoptosis. In contrast, each muscle contains elongated and aligned differentiated muscle fibres (Laurin *et al*, 2008). The loss-of-function of Jamb and Jamc illustrate this clearly and unequivocally. In addition, *jamb* and *jamc* represent a novel, vertebrate signalling pathway, suggesting innovation of the process during evolution, at least at the cell surface.

Identification and characterisation of the critical cell surface proteins involved in myoblast fusion in *Drosophila* has been of key importance to the understanding of the invertebrate mechanism of myoblast fusion, primarily because loss-of-function of these genes results in a complete block of the process. An important aspect of the founder cell model is the mutually exclusive expression of the key cell surface receptors, *dumbfounded* expressed by founder cells (Ruiz-Gomez *et al*, 2000; Artero *et al*, 2001) and *sticks and stones* expressed by fusion-competent myoblasts (Bour *et al*, 2000). Biochemical interaction between *sticks and stones* and either *dumbfounded* (or *roughest*, a paralogue expressed by all myoblasts) is necessary for fusion (Strunkelnberg *et al*, 2001; Bour *et al*, 2000; Galletta *et al*, 2004). Restricted expression of the receptors to the different cell types presumably prevents fusion within the two different populations. In contrast to this, *jamb* and *jamc* are co-expressed by myoblasts (Chapter 4). However, *jamc* is expressed very dynamically in comparison to its binding partner, *jamb*. It is first expressed in a small medial sub-population of *jamb* expressing myoblasts in rostral somites, after approximately 10-13 somites have formed. The *jamc* expression domain expands throughout the transitional myotome during segmentation and is then attenuated by the end of primary myogenesis. This expression pattern is reminiscent of *sox6* (von Hofsten *et al*, 2008), a transcription factor that reinforces the fast muscle fate of somitic myoblasts (Hagiwara *et al*, 2005; von Hofsten *et al*, 2008) and myogenin (Weinberg *et al*, 1996), a transcription factor linked with the terminal differentiation of myoblasts (reviewed in Pownall *et al*, 2002). Whether or not *jamc* is a direct target of either of these transcription factors remains to be determined. In addition, *jamc* is mis-

expressed in the adaxial cells of *prdm1* mutant embryos, but *jamb* and *kirrel* are not. The expression of this critical myoblast fusion cell surface receptor is carefully regulated during differentiation of the primary axial fast muscle fibres, whilst its binding partner is present throughout.

From these results I propose a new model for vertebrate myogenesis (figure 8.1). I hypothesise that differentiation of the fast muscle myoblasts is initiated by local dynamic signalling. Myoblasts nearest to this signal respond by elongating and expressing *sox6* and *jamc*, making them fully competent for fusion with nearby myoblasts that are primed for fusion by expression of *jamb*.

In the zebrafish axial musclulature, a subset of myoblasts must elongate and connect to myotome boundaries before they can fuse to other rounded cells (Snow *et al*, 2008b). This process is similar to that of avian primary myogenesis (Gros *et al*, 2004), suggesting it is a general characteristic of vertebrates. How does a myoblast decide between elongation or fusing to a nearby elongated fibre? In *Drosophila*, this decision is made by early specification into two distinct cell types. In chick, this process is regulated by temporal separation of elongation of myocytes into a primary myotome, followed by fusion (Gros *et al*, 2004). The phenotypic consequences of a complete block of fusion, outlined in this thesis, demonstrate that this decision is likely to be stochastic in zebrafish. I propose that local signalling directs a limited population of nearby myoblasts to elongate. The remaining myoblasts do not perceive this signal, but are primed for fusion through the expression of *jamb*.

8.2 Determining candidate signalling pathways

The model I have proposed defines some of the characteristics of the signalling necessary for the development of the primary axial fast muscle fibres, based upon observations of this process in the literature and those of the function of *jamb* and *jamc*.

Firstly, the signal should at least regulate transcription of *jamc,* and other genes that induce aspects of the differentiation programme, such as elongation. The transcription factor *sox6* is a possible target of this signal. Secondly, fast muscle myoblasts differentiate in a medio-lateral wave (Henry and Amacher, 2004) and elongation begins from the most posterior border of the somite (Stellabotte *et al*, 2007). Therefore, the differentiation signal should begin in the medio-posterior region of each somite, and propagate laterally. Thirdly, this process begins in early segmentation, seemingly simultaneously in the rostral somites of 10-13 somites stage embryos (see Chapter 4). The signal must be regulated in a timely fashion.

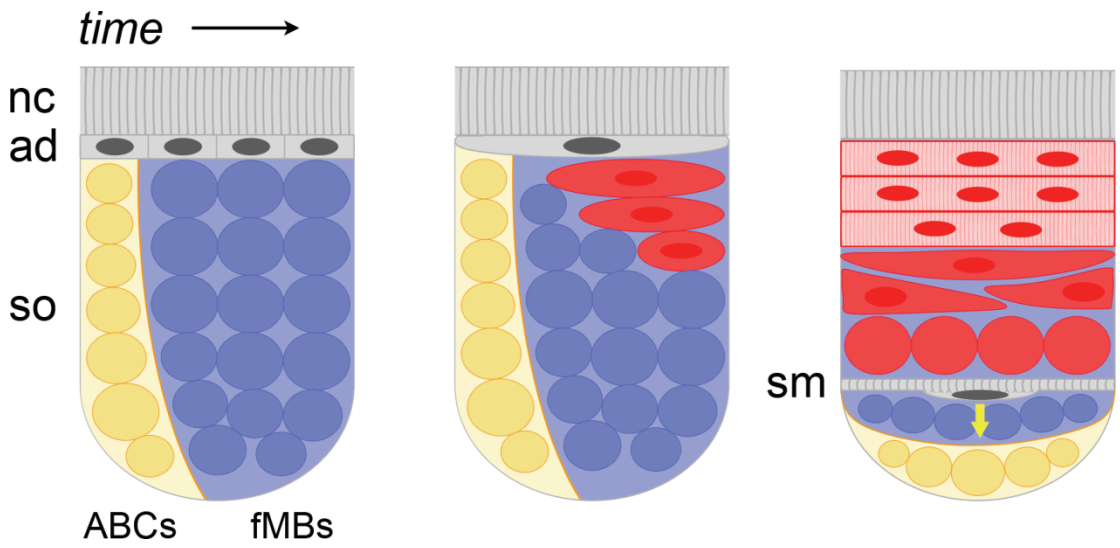

Figure 8.1 Proposed model of primary fast muscle development.

Diagram illustrating proposed regulatory model depicting key developmental changes in a single somite (so). Jamb^{+}, Kirrel^{+} fast muscle myoblasts (fMBs) are specified shortly after somite formation and are primed for fusion (left). Fast muscle myoblast differentiation starts in medio-posterior cells which begin to elongate and express *jamc* (red cells; middle). Fully elongated myocytes start to fuse with other myoblasts to form myofibres (right). This process progresses medio-laterally as slow muscle fibres (sm) migrate towards their superficial position (yellow arrow). nc: notochord; ad: adaxial cells; ABCs: anterior border cells (yellow cells).

Fourthly, myocytes elongate towards the anterior boundary of the somite, in parallel, and stop elongating upon reaching a defined myotome boundary (Henry *et al*, 2005). These elongated fibres do not undergo fusion until such contact is made (Snow *et al*, 2008b). There must therefore be a directional cue that also regulates competence of elongated myocytes for fusion.

I hypothesise that these features are regulated by combinatorial input of different signalling pathways. There are a wide range of secreted signals involved in the development of the musculature (discussed in Chapter 1) and any combination could be responsible for these behaviours. There may also be spatio-temporal redundancy amongst the signals. Disruption of individual pathways at different timepoints may not be sufficient enough to disrupt the process of differentiation, which may explain why no individual signal has previously been identified. I will outline several possible approaches to begin to characterise the molecular nature of these differentiation signals.

I believe that studying slow muscle development is of great importance to understanding the process of differentiation of fast muscle, as exemplified by studying the expression of critical receptors for myoblast fusion in *prdm1* mutants (Chapter 4). The mutant adaxial cells are able to fuse to nearby myoblasts, suggesting they are competent for fusion. This context provides an opportunity to test if *jamb*, *jamc* or *kirrel*, or the interaction between Jamb and Jamc, is sufficient for myoblast fusion. Movement of the slow muscle has also been demonstrated as important for differentiation for fast muscle fibres (Henry and Amacher, 2004). The signal(s) that trigger the migration of slow muscle through the myotome might also trigger fast muscle differentiation. Indeed, the migrating slow muscle cells may form an important part of the morphogenetic signal itself by virtue of its medio-lateral progression. A suitable test of this hypothesis would be to look at the position of slow muscle fibres during their migration and the expression domain of *jamc;* if slow muscle is part of the signalling process, then *jamc* expression must be limited by the extent of its migration. It would also be interesting to assess fast muscle differentiation in zebrafish embryos lacking *m-* or *n-cadherin*, as loss of either gene disrupts migration, but not specification of slow muscle (Cortés *et al*, 2003). Hedgehog signalling has been shown to play a role in the elongation of fast muscle myocytes. Inhibition of hedgehog signalling in *laminin γ1* mutants enhances the elongation defect observed in fast muscle (Peterson and Henry, 2010). Interestingly, this effect appears to be indirect and dependent on the correct development of slow muscle. Slow muscle cannot be the only source of signalling however, as loss of slow

muscle does not block fast muscle differentiation or myoblast fusion (Ingham and Kim, 2005).

Another means of elucidating the molecular signalling involved in this process is a thorough analysis of the regulatory elements that control *jamc*, in comparison to *jamb* and *sox6*. I propose a reporter gene assay performed *in vivo* in which different non-coding regions of the *jamc* loci be placed upstream of a reporter gene in a plasmid and injected in to wild-type embryos. This could be used to determine the regulatory regions responsible for spatial and temporal activation of *jamc*. These could then be compared to the *sox6* and *jamb* loci to identify informative similarities and differences. It would be also interesting to determine the effect of disruption of known signalling pathways, such as FGF or Hedgehog, on the reporter gene constructs containing regulatory elements of interest, *in vivo*, in addition to any effect on the expression patterns of *jamb* and *jamc*.

A whole transcriptome approach applied to dissociated myoblasts could also be informative. $Jamb^+$ single positive and $Jamb^+$, $Jamc^+$ double positive cells could be isolated by dissociation of early segmentation embryos followed by fluorescence-activated cell sorting (FACS). Purified mRNA from these cells could then be used in transcript-counting or microarray experiments to identify differentially regulated genes between undifferentiated $Jamb^+$ cells and differentiated $Jamb^+$, $Jamc^+$ cells. This process might help identify intracellular effectors of differentiation and help determine the function of signalling pathways in this context.

8.3 Relative roles of cell surface receptors in myoblast fusion

Whilst many cell surface receptors have been identified as important for myoblast fusion in vertebrates, very few cell surface proteins have been demonstrated to have a significant effect on myoblast fusion in mouse (Krauss, 2010), leading to the suggestion that there is significant functional redundancy between different proteins. This is in stark contrast to myoblast fusion in *Drosophila*, in which only four receptors (*dumbfounded/roughest* and *sticks and stones/hibris*) are critical for fusion (Haralalka and Abmayr, 2010). The strength of the phenotype of loss-of-function of *jamb* and *jamc* and the necessity of interaction between the two proteins in the zebrafish myotome demonstrate that I have identified a critical receptor:ligand pair for the initiation of myoblast fusion. Whether or not these proteins also play a critical role in mammals remains to be determined.

Both Jam-B and Jam-C are expressed in the developing skeletal muscle of mouse embryos (Visel *et al*, 2004). Several mutant mouse lines for *Jam-B* (Sakaguchi *et al*,

2006; Tang *et al*, 2010) and *Jam-C* (Gliki *et al*, 2004; Praetor *et al*, 2008) have been reported. No myogenesis phenotype has been described in reports of any of the *Jam* mutants, although no direct attempt to analyse muscle development has been made. *Jam-C* mutant mice die very shortly after birth, are cyanotic and unable to breathe (Praetor *et al*, 2008), a phenotype consistent with previously reported myoblast fusion defects, for example *Dock1* knockout mutants (Laurin *et al*, 2008). However, this may be confounded by defects in the immune system (Imhof *et al*, 2007). Surviving mutant *Jam-C* mice exhibit growth retardation (Imhof *et al*, 2007; Ye *et al*, 2009) which may suggest a general defect in myogenesis. Significant reduction in stride length and grip strength has also been described, although the authors attribute this to defective nerve conduction (Scheiermann *et al*, 2007). Jam-C knockout mice also present with megaoesophagus which the authors attribute to dysfunctional smooth muscle cells (Imhof *et al*, 2007). However, it is worth noting that the oesophageal muscle is unusual, because it contains a mixture of striated and smooth muscle (Shiina *et al*, 2010). Surprisingly, no phenotype has been identified for *Jam-B* mutant mice (Sakaguchi *et al*, 2006), even though spermatogenesis was expected to be as defective as in the *Jam-C* mutant (Gliki *et al*, 2004).

Previously, orthologues of *Drosophila* myoblast fusion cell surface proteins have been studied in order to understand the process of vertebrate myoblast version. For example, morpholino knockdown of *kirrel,* a zebrafish orthologue of the paralogues *dumbfounded* and *roughest*, has revealed a near-complete block in myoblast fusion, with an equivalent degree of mononucleate fibres to that of *jamc*^{sa0037} mutant embryos, approximately 80% (Srinivas *et al*, 2007). In contrast to the phenotype of both *jamb* and *jamc* mutants, a ‘large’ number of rounded, unfused myoblasts are observed in the embryo. It is unclear what proportion of these cells, if any, remain as rounded cells and are destroyed by phagocytosis, or elongate into mononucleate fibres at a later stage. What the relative roles of *kirrel*, *jamb* and *jamc* are in zebrafish myoblast fusion remains to be assessed.

Identification of any interacting partners of Kirrel at the cell surfaces would be of great interest. Srinivas *et al* (2007) used transplant experiments to establish if *kirrel* was required cell-autonomously. If so, Kirrel must therefore interact with an unknown ligand for myoblast fusion. The authors were unable to draw any sound conclusion from the results of these experiments. I believe that the reason for this is flaws in the experimental procedures. Firstly, any donor-donor fusion events they identified were categorised as ‘unfused’, biasing their results heavily in the wild-type donor, morpholino-injected host transplants. In addition, it is unclear how the authors could

reliably identify any such donor-donor fusion events. A transgenic strain expressing a nuclear-localised histone2A.F/Z-GFP fusion protein (H2A.F/Z-GFP) was used as the donor strain. Within the results, it appears that any binucleate fibres in which both nuclei are labelled with GFP are referred to as donor-donor cell fusions, with the presumption that only donor cell nuclei are labelled with GFP. This runs contrary to the syncytial nature of myofibres. The contents of the donor cell, including the mRNA encoding H2A.F/Z-GFP, must diffuse throughout the syncytia formed between the fused cells. This has been demonstrated quite elegantly through time-lapse studies of *MAZe* embryos (Collins *et al*, 2010). Myoblasts, containing the recombined transgene, expressing a nuclear-localised RFP (nlsRFP) and GFP and are observed fusing to other myoblasts, which do not express nlsRFP or GFP, to form a fibre. Subsequently, other nuclei within the syncytia are labelled with nlsRFP, and the cytoplasmic GFP spreads throughout the fibre. I would improve the *kirrel* transplant experiment in much the same manner as I have proposed for my own transplants: labelling of donor cell DNA through nucleotide analogues, or use of a bi-partite reporter gene expression system (Chapter 7). Kirrel has been used in a recent AVEXIS screen for potential ligands and found to interact homophilically, but no other binding partners were identified (Martin *et al*, 2010). One proposed binding partner for Kirrel is Nephrin, based upon its orthology to *sticks and stones* (Sohn *et al*, 2009). It has been previously associated with severe forms of nephritic syndrome (reviewed in Hauser *et al*, 2009), but no muscle phenotype has been described for *Nephrin* knockout mice mutants. The role of *nephrin* in myoblast fusion has been poorly characterised in zebrafish.

8.4 Intracellular effectors of Jamb and Jamc signalling

The precise functional role of the interaction between Jamb and Jamc in myoblast fusion remains to be characterised. For example, binding between Jamb and Jamc might be necessary for adhesion between myoblasts, cellular recognition between primed myoblasts and elongated myocytes, or to activate signalling pathways between myocytes for fusion. Both proteins are thought to play important roles in tight junctions and are known to interact with cytoplasmic proteins through a C-terminal PDZ domain-binding motif. For example, JAM-B and JAM-C have been shown to interact with the well-known cell polarity protein PAR-3 (Ebnet *et al*, 2003). Of particular interest is the possibility of interactions between Jamb and Jamc and Cdc42, through Par3, Par6 and aPKC (Gliki *et al*, 2004), as Cdc42 is known to play a vital role in myoblast fusion (Vasyutina *et al*, 2009). Interaction between Jamb and Jamc might lead to enrichment of Cdc42 activity to sites of fusion. Both JAM-B and

JAM-C interact with the tight junction protein ZO-1 (Ebnet *et al*, 2003), a membrane-associated guanylate kinase (MAGUK; Stevenson *et al*, 1986) that is known to bind F-actin (Fanning *et al*, 2002). This suggests a direct link between the JAM proteins and the actin cytoskeleton in epithelia, but this requires further investigation in the context of myoblast fusion. Jam-C has also previously been shown to regulate the expression of β1-integrins (Mandicourt *et al* 2007) which are known to be critical for muscle differentiation (Schwander *et al*, 2003; Conti *et al*, 2009).

To test the functions of both proteins it would be possible to use splice-blocking morpholinos to truncate the cytoplasmic domains. If the interaction between Jamb and Jamc is necessary for adhesion and recognition, but not signalling, then one might expect myoblast fusion to occur normally. It would be interesting to test the function of Kirrel in the same manner.

8.5 Future directions

The issues discussed above represent outstanding long-term questions relating to the deeper understanding of the process of myogenesis. Before submission, I began to prepare reagents for future experiments to explore the function of Jamb and Jamc further and a possible role of both genes in other muscle tissues and in later development. These avenues of investigation remain incomplete.

Gain-of-function and rescue experiments can provide new functional data and generate new hypotheses. To perform these experiments I designed plasmids to simultaneously express membrane-targetted RFP and a gene of interest, either enhanced green fluorescent protein (eGFP), full-length *jamb* or full-length *jamc*, from separate CMV promoters. I prepared these plasmids in collaboration with Dr Céçile Wright-Crosnier, and injected the control plasmid containing mRFP and eGFP into 1-2 cell stage embryos. I observed co-expression of mRFP and eGFP in injected embryos, suggesting that both CMV promoters are active in the same cells (figure 8.2). In the future I intend to attempt rescue of $jamb^{HU3319}$ and $jamc^{sa0037}$ myoblasts by injecting dual promoter plasmids containing full length wild-type *jamc* or *jamb*. This can be extended to a functional dissection of the proteins by replacing the full length wild-type genes with truncated or mutated versions.

At later stages of development, *jamb* and *jamc* were found to be co-expressed in the presumptive craniofacial mesoderm, hypaxial, epaxial and pectoral fin (see Chapter 4), suggesting that Jamb and Jamc may play a role in myoblast fusion in the development of craniofacial and limb musculature. To address this possibility, I intend to observe the morphology and number of nuclei in these muscles using

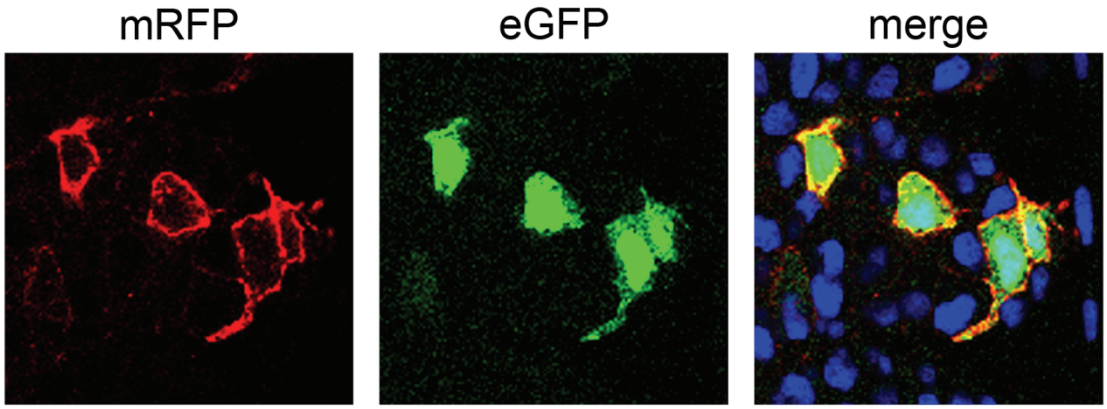

Figure 8.2 Co-expression of fluorescent reporter genes in transfected zebrafish embryos.

Confocal microscopy images of myoblast cells co-expressing membrane-targetted RFP (red) and eGFP (green) in a zebrafish embryo transfected with a dual reporter construct. Both promoters are active within the same cells (merge). Images from a fixed 14 somites stage embryo counterstained with DAPI to highlight nuclei (blue).

immunohistochemistry.

Given the near-complete absence of fusion in both *jamb*^{HU3319} and *jamc*^{sa0037} mutants and the persistence of this phenotype until at least 120 h. p. f., it was a surprise to find that homozygous mutant embryos are viable and fertile. In collaboration with Dr Céçile Wright-Crosnier, we attempted to isolate muscle fibres from dissected adult fish muscles to see if they remained mononuclear. This remains ongoing.

8.6 Concluding Remarks

With thorough application of many different techniques, I believe I have succeeded in characterising an important interaction between a vertebrate-specific receptor:ligand pair previously unknown to be necessary for myoblast fusion. I believe that my research has the potential to help other scientists elucidate the general principles and molecules that govern how muscle tissue forms in vertebrates.

There are many outstanding questions and many new avenues of research made possible by these discoveries. I hope that in the future my efforts contribute in some small way to improved treatment and even prevention of the painful, debilitating and often terminal muscular diseases that blight the lives of many, sufferers and carers alike.