INTRODUCTION

1.1. Introduction

Muscles of the limb arise from progenitor cells that originate at the somites of the developing embryo, whist the remaining tissue of the limbs have a different origin. These limb muscle cells display some crucial differences in their properties when compared to the developing muscles of the trunk of the embryo. In this chapter we will take a look at the formation of the limb tissue and some signals involved in bringing about proper formation of the limbs. We will also explore the stages involved in the limb muscle formation.

The muscle cells undergo post transcriptional regulation from muscle specific microRNAs (miRNA / miR) - miR-1/206 and miR-133. In this chapter we also detail the steps involved in the biosynthesis and function of microRNAs. The signalling pathways via which the muscle specific miRNAs exert their effect on developing muscle cells are also discussed.

Lastly in this chapter we will look at some published research that shows effect of ectopic expression of the family of myogenic regulatory factors on the miRNAs miR-1 and miR-206.

1.2. Somites

The mesoderm of the Neurula-stage embryo can be divided into five regions-

1. Chorodamesoderm
2. Paraxial Mesoderm (Somitic Dorsal Mesoderm)
3. Intermediate Mesoderm
4. Lateral Plate Mesoderm
5. Head Mesenchyme

These regions and their location with respect to each other in vivo can be seen in Figure 1.

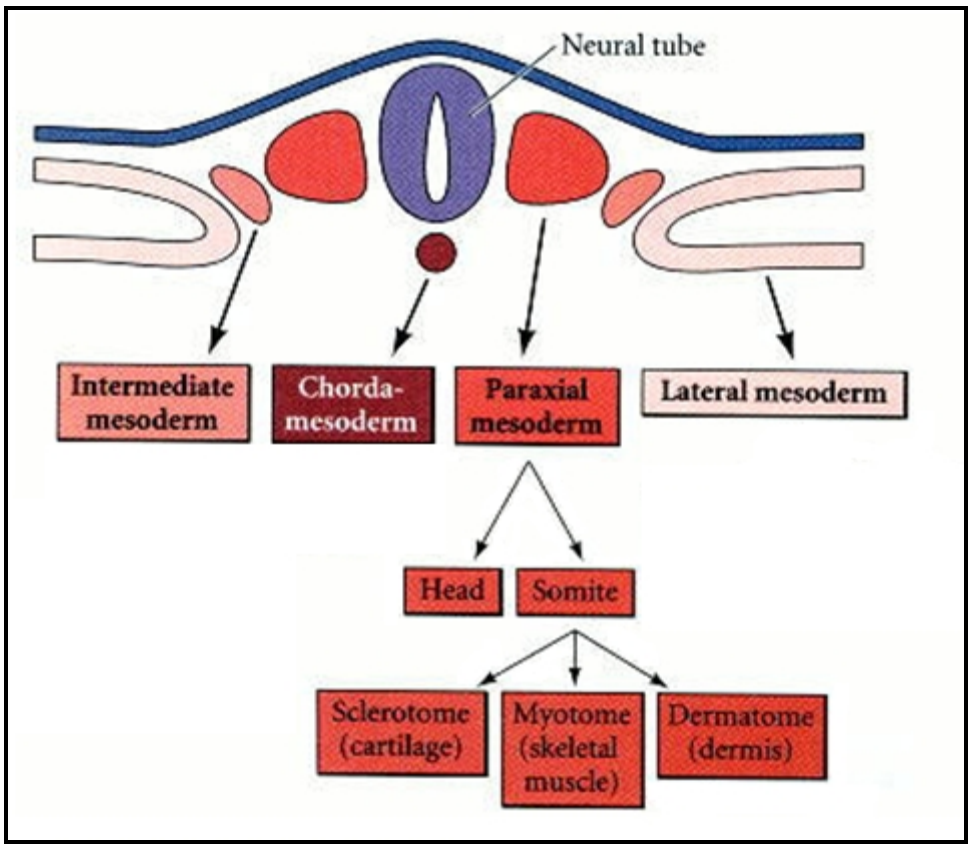


Figure 1: The major lineages of the mesoderm shown in a schematic view of a transverse section. (*Adapted from Gilbert 2000*)

The paraxial mesoderm and lateral plate mesoderm contribute to the formation of the limbs and its musculature therefore we have taken a closer look at these two regions in this section.

A mesoderm layer forms between the ectoderm and the endoderm during gastrulation. The paraxial mesoderm arises by ingression of mesoderm precursor cells through the primitive streak. It forms a uniform band of mesenchymal tissues that flanks the neural tube and the notochord. Segmentation of this tissue gives rise to the somites. Somites are transient structures but are important in organising the segment pattern of vertebrate embryos. (*Christ and Ordahl, 1995*)

Number of total somites is characteristic for individual species. There are a total of 50 somites that arise in the chick embryo. The number of somites present at a given time (until embryonic stage HH15) is usually used to identify how far the development has progressed. Following stage HH15 other criteria are used to identify the developmental progress.

The somites, despite looking identical, give rise to similar tissues but different structures along the anterior-posterior axis. This positional identity is regulated by *Hox* genes.

Somites form the cartilage of the vertebrae and ribs, the muscles of the rib cage, limbs and back and also the dermis of the dorsal skin. The commitment of the cells within a somite is established only after the somite matures. During somite maturation, the ventral part of the somite undergoes an epithelio-mesenchymal transition, allowing formation of the sclerotome, which gives rise to the skeleton and the ribs (*Scaal and Christ et al 2008*). The dorsal part of the somite, however, retains its epithelial organisation and is known as the dermomyotome, which later gives rise to the muscle and dermis. The dermomyotome gives rise to precursors to all of the epaxial (back) and hypaxial (limb and ventral body wall) musculature (*Christ et al 1977*).The region nearest to the neuraltube gives rise to the epaxial muscles, the deep muscles of the back. The region farthest from the neuraltube give rise to the hypaxial muscles, muscles of the body wall and limbs. The central portion mainly contributes to the connective tissue of the back and the skin. The dermomyotome cells divide to give rise to a

lower layer of cells called the myotome. (*Gilbert 2000; Scaal and Christ et al 2008),* The different sections of the somite and how they arise are shown in figure 2.

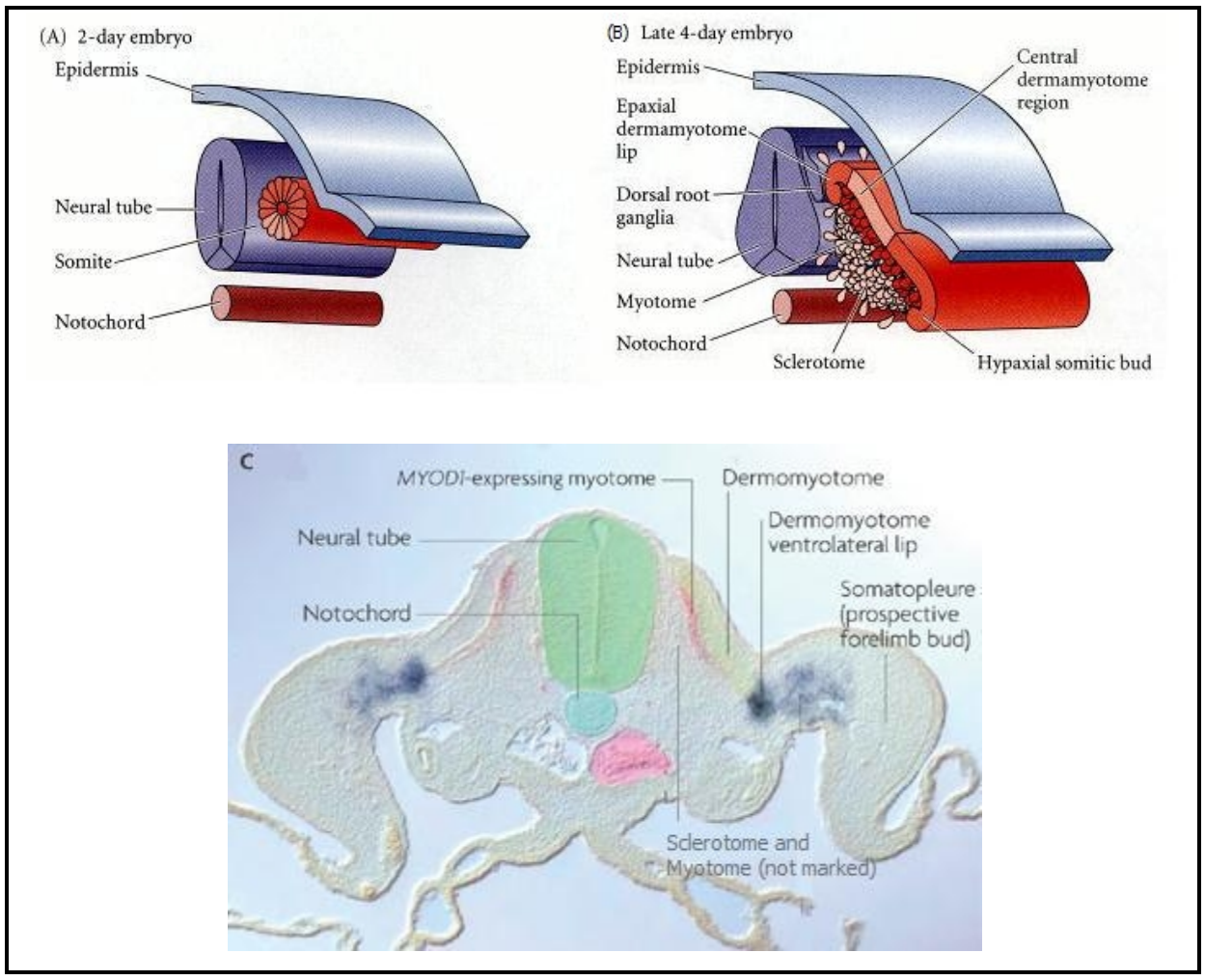


Figure 2: Diagram of transverse section through the trunk of a chick embryo showing the different parts of the somite that arise as the structure matures (and surrounding structures). **(a)** Diagram of two- day **(b)** four-day embryo trunk region. **(c)** Section through trunk region. (Panel (a & b) adapted from *Gilbert 2000*. Panel (c) adapted from *Bryson-Richardson & Currie 2008*)

1.3. Limb Tissue Formation

Vertebrate limbs originate from a dual contribution of somitic and lateral plate mesoderm. Lateral plate mesoderm cells give rise to most of the limb tissues, whereas, cells from the lateral edge of the somites (epaxial dermomyotome) are responsible for the limb muscles. (*Christ et al 1977*)

1.3.1 Limb Bud

The vertebrate limbs develop at specific coordinates along the anterior-posterior and dorsal-ventral axes of the body. In the chick embryo, during stages HH12-16 a domain comprising of ectoderm and mesoderm become competent to give rise to the limbs. A Fibroblast Growth Factor (*FGF*), *FGF8* thought to be expressed in the intermediate mesoderm, which induces the expression of *FGF10* in the lateral plate mesoderm. *Wnt* proteins, *Wnt2b* and *Wnt8c* restrict the expression of *FGF* 10 in the lateral plate mesoderm along the anterior-posterior axis to the regions where the limb buds later emerge from. The *FGF10* induces the expression of *FGF8* in the overlying ectoderm, via another *Wnt* protein *Wnt3a* (expressed in the ectoderm). This *FGF8* in the ectoderm then reinforces the expression of *FGF10* in the lateral plate mesoderm, setting up a positive feedback loop for this signal. (*Yasuhiko et al 2001*) This signalling phenomenon is further shown in figure 3.

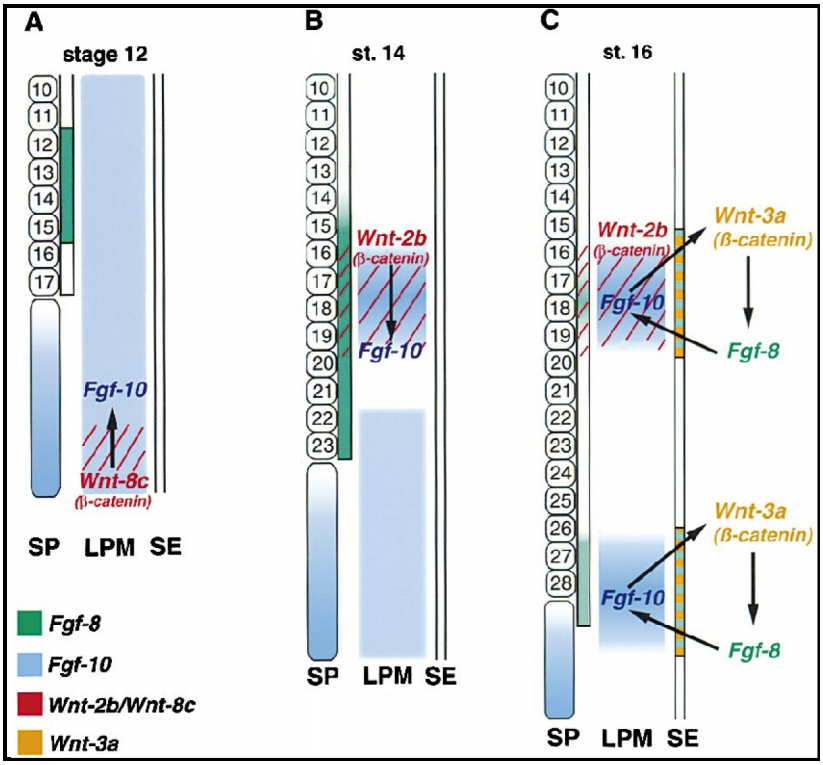


Figure 3: Diagrammatic longitudinal sections through the chick embryo in the limb competent region highlighting some key signalling processes involved in early chick limb development (stages HH12-16) (*Yasuhiko et al 2001*)

1.3.2. Apical Ectodermal Ridge (AER)

Once the limb bud emerges, it is enveloped by a layer of overling ectoderm. The distal tip of this ectoderm forms a specialised structure – the Apical Ectodermal Ridge (AER). The AER runs along the anterior-posterior axis of the limb bud at the interface of the dorsal and ventral territories. (*Fallon and Kelley, 1977*) This can be seen in figure 4.

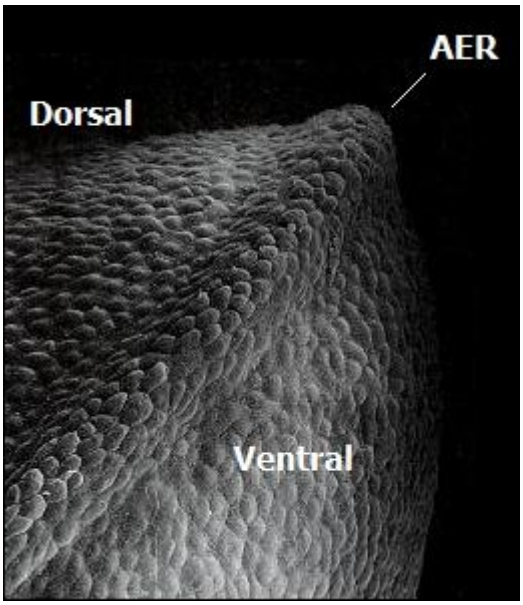


Figure 4: Electron scanning micrograph of an early chick forelimb bud, with the AER in the foreground. (*Adapted from Gilbert 2000*)

The continued outgrowth of the limb depends on the function of the AER. *FGF8* is expressed throughout the mature AER, *FGF2* can be found along the entire anterior-posterior extent while *FGF4* expression can only be detected in the posterior two-thirds. Any one of these *FGF* proteins can maintain the outgrowth of the limbs; however, the reason behind expression of the different *FGFs* is not fully understood. (*Johnson and Tabin 1997*)

Several genes are implicated in the anterior-posterior development of the AER. Engrailed-1 (*En-1*) is expressed in the ventral ectoderm and Radical fringe (*r-Fng*) is expressed in the dorsal ectoderm. The AER has been shown to emerge at the boundary of cells that express *r-Fng* and cells that do not express *r-Fng*. *En-1* functions to repress the expression of *r-Fng*, therefore, creating this boundary in the limb bud. It is also to be noted that several signals

from the mesoderm are also required to initiate the differentiation in the limb bud ectoderm to form the AER. This is illustrated in figure 5. (*Johnson and Tabin 1997*)

Many ectodermal signals specify mesodermal cell fates along the dorsal-ventral limb axis. As mentioned earlier *En-1* is expressed in the ventral ectoderm and *r-Fng* is expressed in the dorsal ectoderm. *Wnt-7a* can also be found only in the dorsal ectoderm, *En-1* functions to repress the expression of *Wnt-7a* on the ventral side. It has been suggested that the ventral pattern emerges as the default pattern and the limb bud. *Wnt-7a* induces the expression of *Lmx-1* in the dorsal mesoderm, which then instructs the dorsal patterning of the limb bud on the dorsal side (while the ventral side continues with the default). (*Johnson and Tabin 1997*) This signalling is illustrated in figure 5.

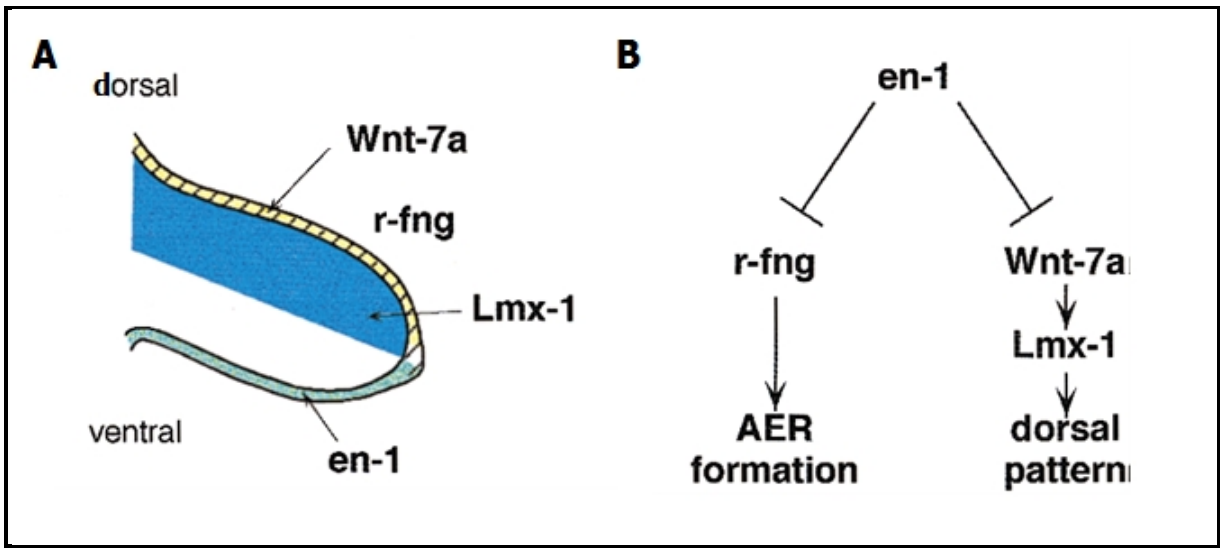


Figure 5: Mechanisms of D/V Patterning and AER Positioning. **(A)** Gene expression along the limb bud D/V axis. **(B)** Genetic interactions involved in AER formation and specification of dorsal pattern. (*Johnson and Tabin 1997*)

1.3.3. Zone of Polarising Activity (ZPA)

The Zone of Polarising Activity (ZPA) is comprised of posterior limb mesenchyme cells. It functions to promote proliferation of posterior limb bud mesenchyme and regulate the anterior-posterior patterning of the limbs as they develop.

It has been suggested that the ZPA may function through the gradient model to establish the anterior-posterior axis, whereby, the cells secrete a morphogen - Sonic Hedgehog (*Shh*) that exerts its effect in a concentration dependant manner. In this case the cells near the ZPA would be in an environment of high *Shh* concentration and therefore take more posterior fates compared to cells farther away from the ZPA (environment of lower *Shh* concentration) would adopt more anterior properties.

The gradient model is disputed by the finding that *Shh* proteins show an adherence for the cell surface, as a result of post-translational addition of a cholesterol moiety to the *Shh* protein. (Chang et al 1994) This suggests that the *Shh* is restricted to cells neighbouring the ZPA and the long range effects of *Shh* is administered through another signalling molecule – Bone Morphogeneic Protein 2 (*BMP-2*). *BMP-2* can be induced by *Shh* and has weak polarising activity, therefore, is considered to relay the effect of *Shh* to cells further away from the ZPA.

Studies suggest that *Hoxb-8* protein is responsible for localising the ZPA to discrete limb bud cells. *Hoxb-8* has also been shown to induce *Shh* under the right conditions. It is likely that the ability of the *Hoxb-8* protein being able to induce *Shh* expression is dependent on additional signals from the AER – members of the *FGF* family. Retonoic Acid (RA) directly induces *Hoxb-8*, therefore, induces the ZPA and *Shh* expression. The signalling pathway is seen in figure 6.

It has not been possible to accurately study the concentration profile of *Shh* in the limb (resulting from expression in the ZPA). Therefore, it is not fully understood whether *Shh* functions thorough the long range mechanism (gradient model) or short range mechanism. (*Johnson and Tabin 1997*)

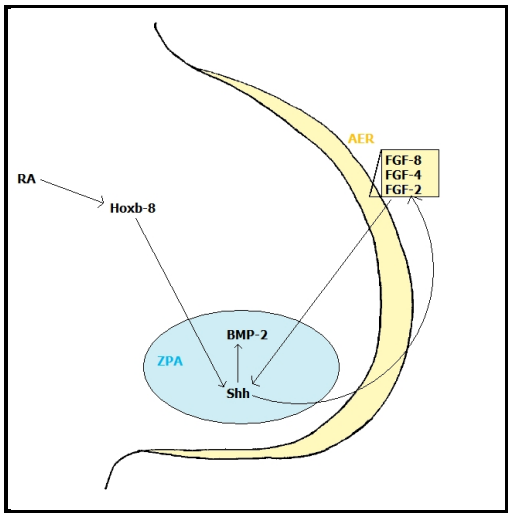


Figure 6: Signalling pathways in the ZPA those are responsible for proper anterior-posterior patterning.

The ZPA also functions to promote proliferation of posterior limb bud mesenchyme cells. This is achieved by *Shh*, expressed in the ZPA, inducing the expression of *FGF4* in the posterior mesenchyme and influencing their outgrowth. (*Laufer et al 1994*)

1.3.4. Progress Zone (PZ)

The Progress Zone (PZ) is defined as a region of distal mesenchyme that remains in close proximity to the AER. The PZ functions to establish the proximal-distal axes of the limbs. The cell fate along the proximal-distal axis is specified by the time the cell spends in the PZ; if the cell leaves the PZ early it adopts a proximal fate, whilst, cells that remain in the PZ for a longer period of time display more distal properties. (*Sumerbell et al 1973*) It has been suggested that these observations are a result of constant FGF signals that the cells in the PZ receive from the AER. Over time these signals are recorded and summed to give rise to the features of the proximal-distal axis. The progress zone model is illustrated in figure 7.

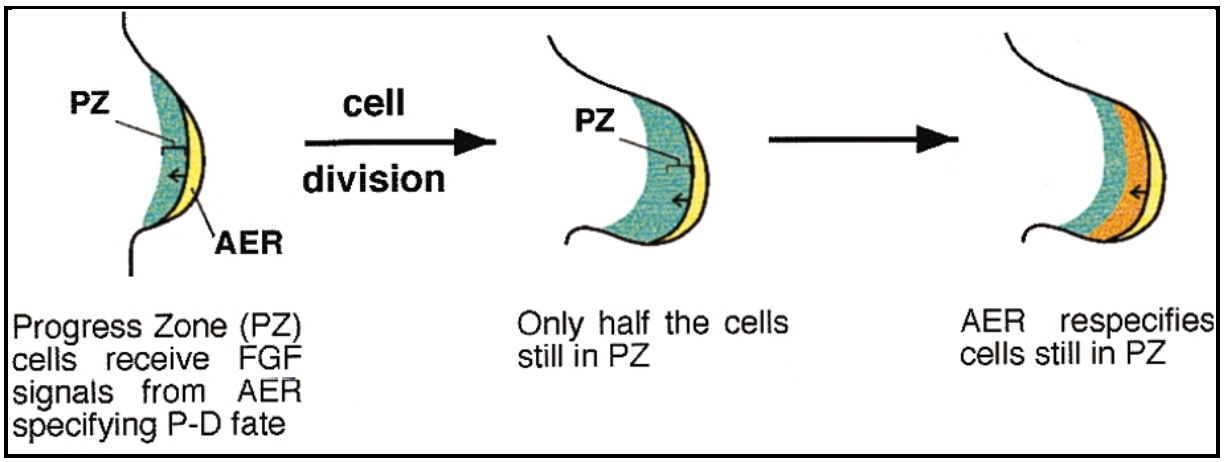


Figure 7: The Progress Zone Model. (Johnson and Tabin 1997)

1.3.5. *Hox* Genes

Specific limb primordia are restricted to their specific coordinates along the axes of the limb. This is achieved by a complex signalling mechanism and no single factor can be said to be responsible for the outcome. As the limb emerges from the body wall, some of the anterior-posterior and dorsal-ventral coordinates are retained. However, the limbs also have an autonomous set of coordinates present.

The vertebrate *hox* genes are a highly related subset of the homebox containing transcription factors that are physically linked in four chromosomal clusters (*Hoxa, Hoxb, Hoxc* and *Hoxd*). In each cluster there are upto thirteen sets of ancestrally related homologs that are referred to as paralogs of each other. The representation of these clusters and paralogs can be seen in figure 8.

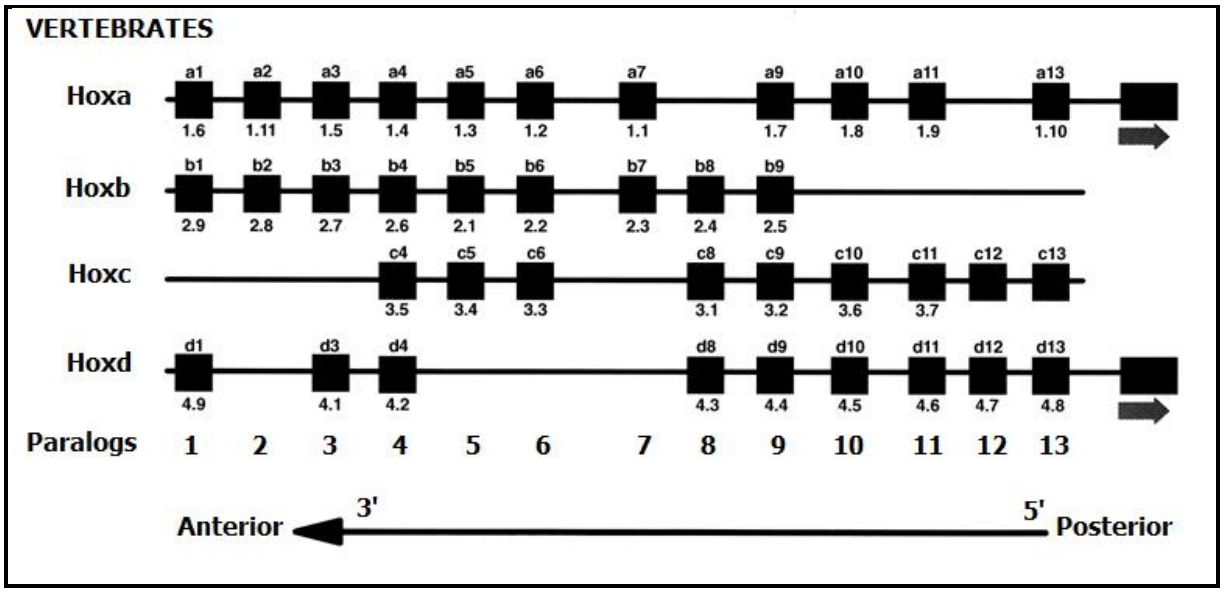


Figure 8: Diagrammatic representation of the four vertebrate *Hox* clusters with the 13 paralogs aligned. (*Adapted from* ***Kavlock and Setzer, 1996***)

To understand their role of *Hox* genes in limb patterning, we must understand the general concept of the general phenomenon of "posterior prevalence" (*Duboule, 1994*). When two or more *Hox* genes are co-expressed in the same cell, the more 5' gene(s) of the *Hox* cluster will exert a dominant effect. Thus, as the dynamic pattern of *Hox* genes unfolds during limb bud outgrowth, different *Hox* genes play dominant roles in different limb bud regions. This leads

to differential growth of limb elements, since different members of each *Hox* cluster have distinct effects on proliferation and differentiation. In contrast to *Hox* genes within a single cluster, paralogous *Hox* genes from different clusters appear to be largely redundant in function. (*Johnson and Tabin 1997*)

The hox genes are expressed in an ordered fashion along the Anterior-Posterior axis of the vertebrate body plan, regulating the development of many features along this axis. These genes are progressively activated, with genes located more 5' in the complex expressed more posteriorly. Once a gene has been activated at a particular level along the axis, it remains expressed posterior to that level. The structures along the proximal-distal axis of the limb are specified by the expression of *Hox* genes in a similar way. *Hox* gene expresson in the chick limb bud is quite dynamic pattern with three independently regulated phases of expression. In phase 1, *Hox* genes are expressed across the entire distal limb bud, during this time the upper wing is specified. Subsequently in phase 2, *Hox* genes are expressed in a posteriorly nested order, during this time the lower wing is specified, finally in phase 3, the *Hox* genes are expressed in a more distal pattern, during this time the digits are specified. (*Johnson and Tabin 1997*) These phases are shown in figure 9.

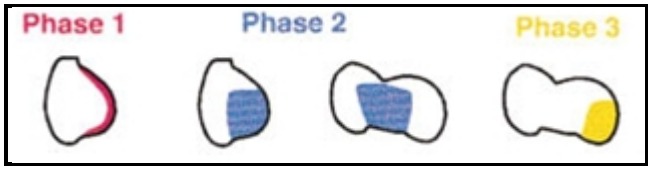


Figure 9: The three independently regulated phases of dynamic *Hox* gene expression in the chick wing bud. (*Adapted Johnson and Tabin 1997*)

A more detailed expression pattern of the *Hoxa* and *Hoxd* clusters are shown in figure 10.

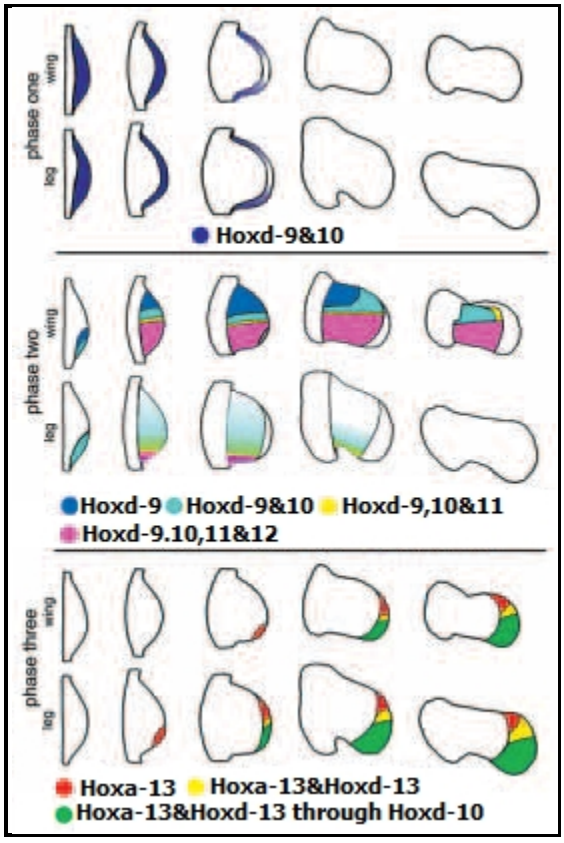


Figure 10: Schematic representation of the three phases of *Hoxd* gene expression, which have been separated schematically to illustrate their regulation and the contribution of each phase to the overall pattern of *Hoxd* gene expression in the developing limb bud. All views are oriented with anterior to the top and distal to the right. (*Nelson et al 1996*)

The digits of the limbs are brought about by a series of local interactions operating over time. Webs and pads between the digits are eliminated in certain species by apoptosis. These programmed cell deaths are achieved by action of Bone Morphogenic Proteins (*BMPs*) and their receptors.

1.3.6. Termination of Limb Development

As we know the limbs do not continue to grow indefinitely, the growth inducing activity of the AER decays over time. This is achieved by members of the Bone Morphogenic Protein (*BMP*) family, inhibiting the activity of the *FGFs*. Both *BMP2* and *BMP* antagonist, Gremlin, are expressed in the mesoderm under the control of *Shh*. Gremlin functions to ensure that the *BMP* activity does not terminate AER function too early in the process. Therefore we can conclude by saying that the balance between the *FGFs* and *Shh* interactions and *Shh* modulated *BMP* and Gremlin interactions, produces a self regulating signalling network that determines the extent and rate of limb growth. (*Gilbert 2000*) This signalling pathway is shown in figure 11.

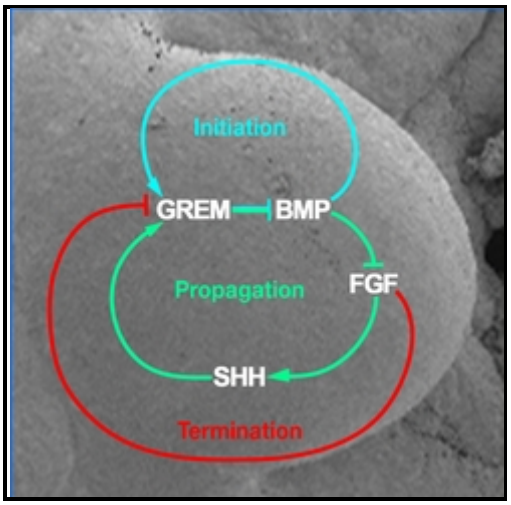


Figure 11: Shows the signalling loop that initiates, propagates and terminates limb development. (www.the-scientist.com)

1.4. Muscles of the Limb

1.4.1. Introduction

All skeletal muscles of the limb and trunk originate from the somites. The medial halves of the somites give rise to the epaxial musculature – intercostals muscles while the lateral somites give rise to the hypaxial musculature – muscles of the limb and body wall. This can be seen in figure 1.

Limb tissues arise from cells of the lateral mesoderm whilst the muscle cells of the limbs, although also of mesodermal origin, arise from the somites. In this section, we will take a look at the steps involved in the formation of these limb muscles from somitic cells –

1. Formation of muscle precursor cells in the lateral dermomyotome
2. Delamination and migration of precursor cells into the limb buds
3. Activation of the myogenic program
4. Proliferation of cells
5. Formation of dorsal and ventral muscle masses
6. Muscle differentiation
7. Muscle Splitting

and some of the signals that bring about these events (*Christ et al 1977*). A summary of the events can be seen in figure 12 and a summary of the signalling events are shown in figure 13.

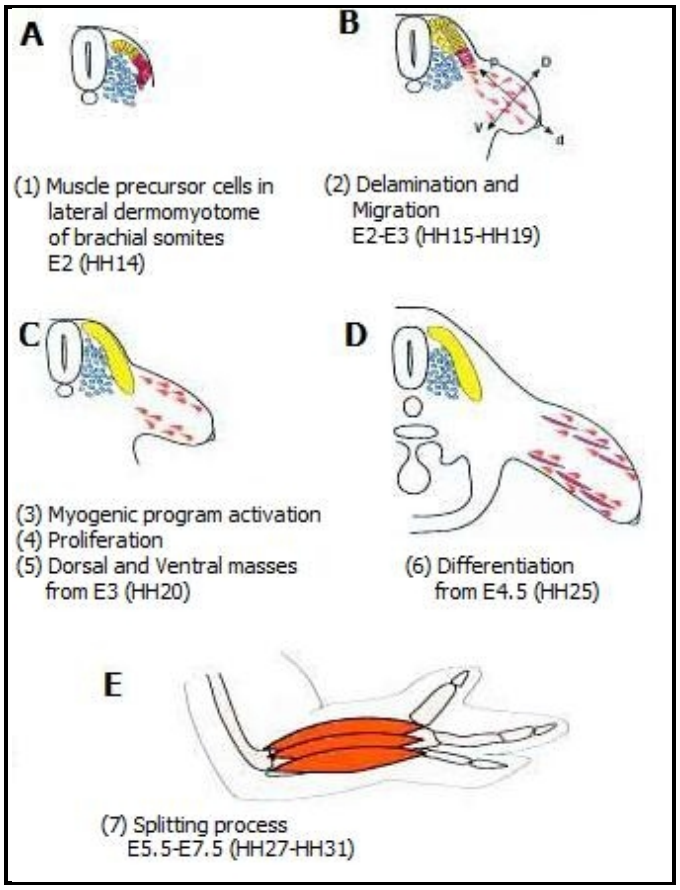


Figure 12: Events defining muscle formation in the chick wing. (*Duprez 2002*)

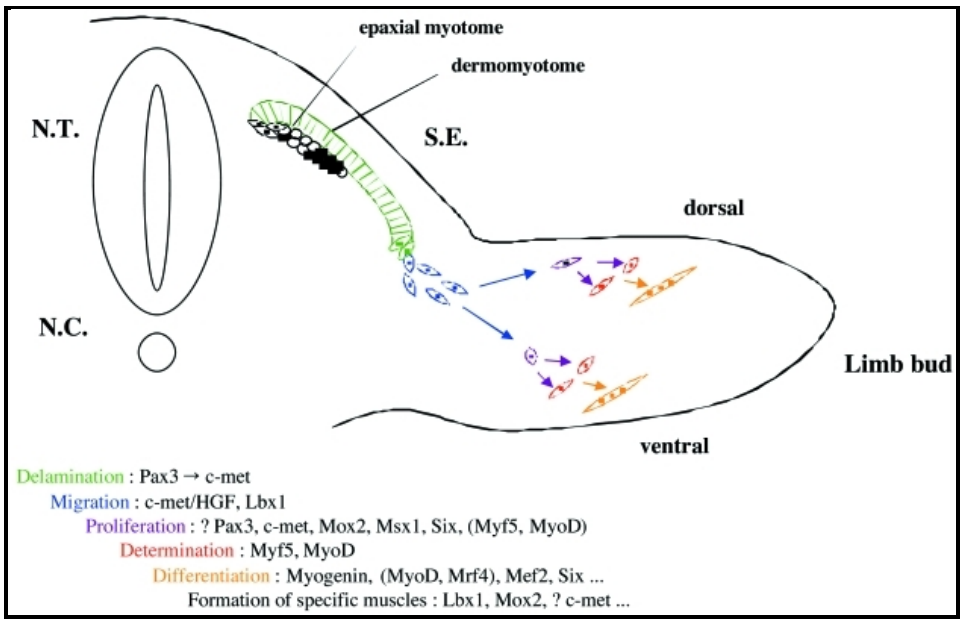


Figure 13: Schematic representation of skeletal muscle formation in the limb with the different stages and genes involved at each stage. (*Buckingham et al 2003*)

1.4.2. Formation of muscle precursor cells in the lateral dermomyotome

At embryonic stage HH-14 of the chick, lateral dermomyotome cells start to produce the muscle precursor cells of the limb. This is considered the first step in the limb muscle formation program. (*Duprez 2002*)

1.4.3. Delamination and migration of precursor cells into the limb buds

The next step involves the migration of somitic cells into the limb buds. At embryonic stage HH15-19, cells from the epithelium of the hypaxial dermomyotome of the mature somites delaminate and migrate to the limb region. This migration occurs over 24 hours and is thought to be regulated by positional cues from the mesenchymal cells of the limb bud. Cells from somites 16-21 contribute to the wing muscles while somites 26-33 contribute to the legs musculature. (*Duprez 2002*)

Hepatocyte Growth Factor (*HGF*), also called scatter factor, binds to a tyrosine kinase receptor, c-met – this interaction is essential for both delamination and migration. Transcription of c-met is controlled by the transcription factor, Paired Box 3 (*Pax 3*) as is the transcription of Ladybird Homeobox1 (*Lbx1*). *Lbx1*, a homeodomain containing transcription factor and has been implicated in the migration of limb muscle progenitor cells. *Pax 3* can be detected in presomitic mesoderm cell and its function later in the muscle progenitor cells where is can exert its effect only in the presence of essential co-activator. (*Brand-Saberi et al., 1996; Bendall et al., 1999*)

1.4.4. Activation of the myogenic program

At stage HH20, the myogenic program is activated in the wing. This is marked by the onset of MRF expression. This myogenic activation, only occurs once the cells have reached the limb, starting with the expression on *MyoD* and *Myf5*. *Wnt7a* from the dorsal surface ectoderm of the limb bud and *Shh* from the ZPA are responsible for the activation of these Myogenic Regulatory Factor (MRF) genes (*Parr and MacMahon, 1995*). *Mox2* which is also present in the muscle progenitor cells of the limb is responsible for activation of *Myf5* as

well. Transcription of *MyoD* is dependent on *Six* homeoproteins, *Six1* and *Six4*, which act through cofactors *Eya* and *Dach*. *MyoD* and *Myf5* show compensatory functions. MRFs are further explained in a later section. (*Duprez 2002*)

1.4.5. Proliferation of cells

Only a few cells migrate from the somite to the limb and therefore before skeletal muscles can be formed these precursor cells need to undergo extensive proliferation to attain the number of cells required. Pax3 can be detected in these proliferating cells and are thought to be directly or indirectly involved in maintaining the proliferative phase (*Amthor et al., 1998*). Expression of another homeodomain protein, Paraxis, can been seen in a similar pattern. Therefore, a similar role is suggested for paraxis in these proliferating cells (*Delfini and Duprez, 2000*). It is difficult to determine the exact time of the onset of proliferation as there are no known markers for this step.

1.4.6. Formation of dorsal and ventral muscle masses

Soon after the migration and proliferation of these progenitor cells, around embryonic stage HH 21, they begin to aggregate into the dorsal and ventral premuscle masses on both sides of the precondrogenic core. This division into dorsal and ventral masses however is not completed till embryonic stage HH 23. The signalling involved in this process is not very well understood yet (*Schramm and Solursh, 1990*).

1.4.7. Muscle differentiation

The first sign of terminal muscle differentiation can be observed at embryonic stage HH25 with the appearance of polynucleated cells. Skeletal muscle differentiation begins when the proliferating myoblast withdraws from the cell cycle and starts to synthesise muscle specific proteins. These post-mitotic cells fuse to form multinucleated fibres, called myotubes. The process of muscle differentiation and maturation is illustrated in the figure 14. Although several adhesion molecules such as neuronal (N-CAM) and vascular (V-CAM), cadherins, intergrins and members of the A Disintegrin and Metalloprotease Domain (ADAM) family

have been implicated to be involved in the fusion process little is understood about the actual signalling in vivo. During the remainder of the embryonic development, both myotubes and proliferating myoblasts can be observed in the limbs. These cells need to coexist to ensure continued growth of the muscles during this time (*Duprez, 2002*).

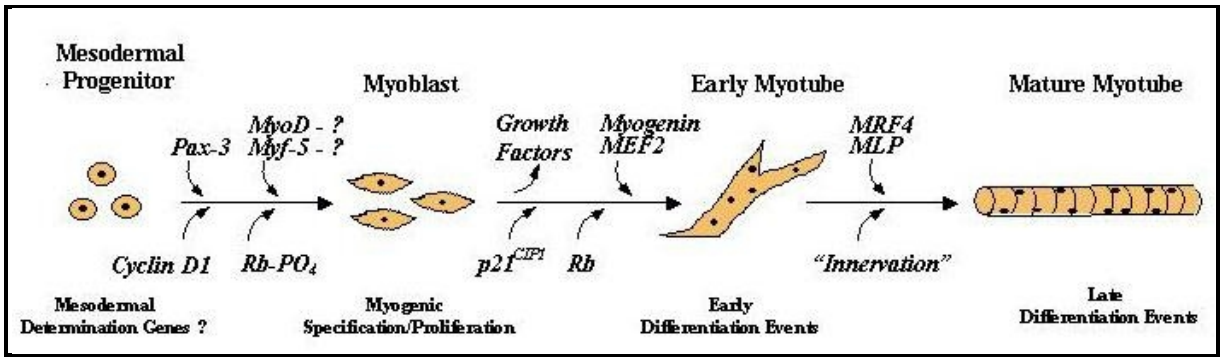


Figure 14: Stages involved in the transformation of the mesodermal progenitor cells to give rise to the mature myotubes. (*Ludolph and Konieczny, 1995*)

1.4.8. Muscle Splitting

For a period of two days following the onset of the process, whereby the dorsal and ventral muscle masses are established, there is little spatial arrangement of the muscle cells. The premuscle masses then split progressively to give rise to individual muscles. This splitting process occurs over stages HH 27-31 in the chick embryo, although the exact time the muscles undergo the splitting process differs in the wings compared to the legs. The results attained by the muscle splitting are explored in more detail in a later section (1.7). (*Pautou et al., 1982; Robson et al., 1994; Zhi et al., 1996*)

Skeletal muscle fibres are formed by two successive waves of fusion. A primary wave of fusion is observed when myoblasts begin to differentiate. This is called primary myogenesis. These primary muscle fibres then act as scaffolding for a second wave of fusion to occur. This is called secondary myogenesis. These two types of muscles have differing qualities such as metabolism and speed of contraction, leading to these fibre types being called fast and slow muscle fibres. (*Duprez, 2002*). In birds and mammals each muscle has a specific fast and slow fibre distribution. While looking at the endogenous expression of muscle

specific miRNA-1, miRNA-206 and miRNA-133, we were interested in investigating if the expressions patterns showed any co-relation to the distribution of these two fibre types.

1.5. Myogenic Regulatory Factors

Muscle differentiation (myogenesis), proceeds through irreversible cell cycle arrest of muscle precursor cells, followed by an increase in muscle function genes, leading to fusion of myoblasts into myofibres.

Myogenesis is managed through a series of transcriptional controls governed by the myogenic regulatory factors (MRFs). The MRFs are a family of basic-Helix-Loop-Helix (bHLH) transcription factors. The bLHL motif is characterized by two α-helices connected by a loop. Transcription factors including this domain are dimeric, each with one helix that facilitates DNA binding, while the other is involved in the dimerisation process. The structure of the bLHL motif and its dimer can be seen in figure 15.

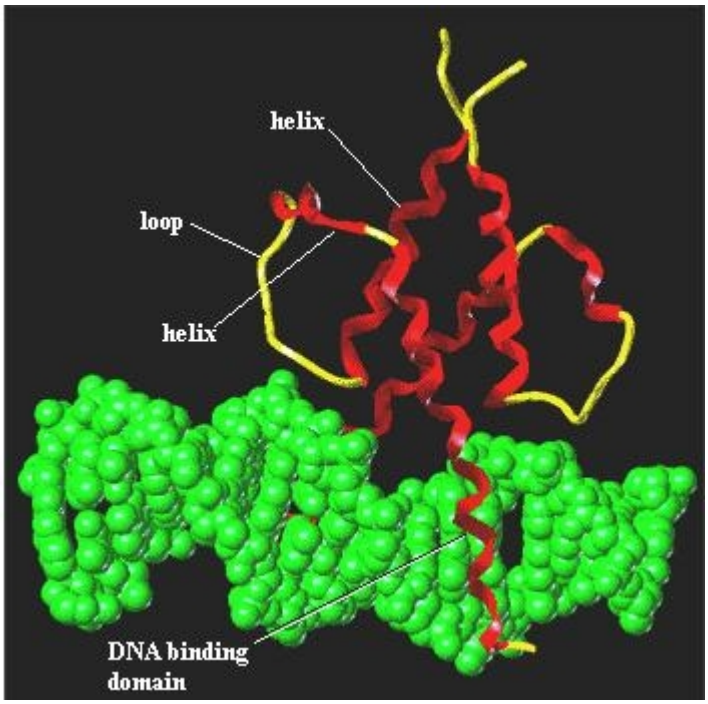


Figure 15: Transcription factors with the bLHL motif dimerises and interacts binds to DNA. (www.palaeos.com)

The larger α-helix recognises a consensus DNA sequence, CANNTG, called an E-box and binds to it (*Rao et al, 2006; Ludolph and Konieczny, 1995*). This is also illustrated in figure 16.

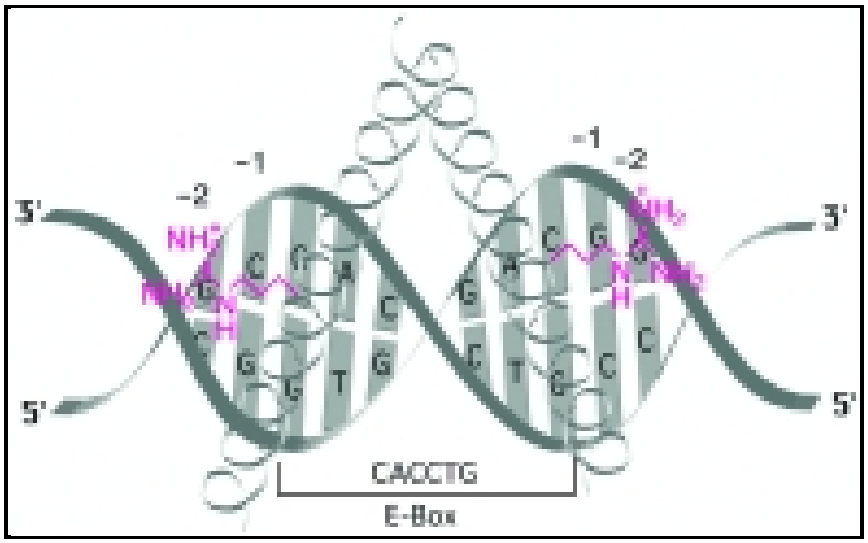


Figure 16: the two larger α-helices of a transcription factor dimer interact with DNA by binding to the E-Box. (*Beltran, et al 2005)*

MRFs are known to activate expression of muscle specific genes. Firstly *MyoD* and *Myf5* are expressed in undifferentiated myoblasts. *MyoD* and *Myf5* are potentially involved in chromatin remodelling and cell cycle regulations. Expression of *MyoD* and *Myf5* also leads to expression of Myogenin and *MEF2* in the cells promoting conversion of myoblasts to myotubes. *MyoD* expression continues to be detected in the myotubes and it collaborates with *Myogenin* to bring about expression of genes that cause terminal differentiation. *MRF4* is detected after differentiation (*Blais 2005; Buckingham 2001*).

There are unresolved functional redundancies within the MRF family. Understanding these details could provide more insight into how muscles differentiate and regenerate.

1.6. Differences Between Limb and Trunk Muscles of the Embryo

There are two different types of skeletal muscles in the body – the muscles of the axial skeleton and the muscles in the limb and ventral body wall. In this section we will look at the differences in these two muscle types. (*Ordahl and Le Douarin 1992*)

The two muscle groups are generally thought to arise from the myotome. Upon closer inspection we can see that the muscles in the axial skeleton arise from a section of the somite, where the cells differentiate in situ within the somite and sclerotome organisation centres.

On the other hand, cells originating at the edge of the dermomyotome that remain undifferentiated before migrating into the lateral parts of the body and limb bud mesenchyme. These cells are initially histologically indistinguishable from the remainder of the limb bud mesenchyme. However, later around embryonic stage HH24, they can be seen to contribute exclusively to the limb musculature. (*Chirst et al 1977*)

Therefore we can say, myoblasts that differentiate within the myotome do not participate in wing myogenesis, but rather contribute to the axial musculature. Whereas, undifferentiated cells from the ventral edge of the dermomyotome migrate into the limb bud mesenchyme to form the limb muscles. This establishes an obvious migratory difference in the progenitor cells in of the two muscle types in question. (*Ivarie 1993*)

Somites are initially composed of columnar epithelial-like cells organised around a lumen. The ventromesial part of the somite gives rise to the sclerotome while the dorso-lateral part of the somite gives rise to the dermomyotome. The dermomyotome then differentiates into the dermatome and the myotome. The myotome arises from the cells lying at the anterior edge of the dorsomedial margin. Single cells migrate ventrally along the anterior edge of the dermatome and elongate posteriorly as single mononucleated myocytes. The myocytes then go on to give rise to the axial muscles. (*Keynes and Stern 1988*)

Cells from the lateral half of the immature somite migrate to the site of the embryo, where they give rise to non axial muscles. These cells undergo muscle differentiation process (as described in section 1.3), where the myocytes first withdraw from the cell cycle and start to fuse into polynucleated cells before giving rise to multinucleated fibres called myotubes. (*Keynes and Stern 1988; Ludolph and Konieczny, 1995*)

Therefore, we can say cells that give rise to the axial musculature initially differentiate and elongate as mononucleated cells before they form muscle fibres. Cells that give rise to the muscles of the limb fuse to form polynucleated cells before they proceed to give rise to myotubes. (*Ludolph and Konieczny, 1995*)

Expression of myogenic regulatory factos is considered the earliest markers of myogenesis. Myogenin transcripts can be detected in the myotome of the somites (precursors of axial musculature) of an 8.5 days old (TS13, 8.5 days p.c.) mouse embryo and MyoD transcripts can be detected once the embryo is 10.5 days old (TS17, 10.5 days p.c.). Myogenin and MyoD transcripts can be detected in the limb buds together once the embryo is 11.5 days old (TS19, 11.5 days p.c.) Therefore, we can also note that the MRF in the limb muscles progenitor cells is detectable later compared to the body muscles progenitor cells. (*Ott et al 1991*)

Survival of the axial muscle precursor cells depend of signals the cells receive from the neuraltube and notochord. When neural primordium and notochord were removed from chick embryos at stage HH12 (E2), the sclerotomes and myotomes were observed to disappear as a result of this excision. The tissue that normally differentiates from these structures was also not seen in the embryo in later stages. (*Rong et al 1990*)

However, when the neural tube and notochord were removed at HH12 (E2), the limbs developed normally. Limb muscles were smaller in size but present at least up to embryonic stage HH34 (E10) in these neuralectomised and notochordectomised embryos. (*Tillet and Le Douarin 1983*)

Therefore, we can conclude that the signals from the neural tube and notochord directly effect the precursor cells that give rise to the axial musculature but the limb muscles are not affected as they can develop normally up to embryonic stage HH34 (E10). (*Ordahl and Le Douarin 1992*)

1.7. Muscle Splitting

The final step of muscle formation in the embryo involves a sequence of splitting events in differentiated muscle masses to give rise to individual muscle masses. (*Durpez 2002*) In this section we take a look at the steps involved in the splitting process in both the forelimb and hindlimb that give rise to the individual muscles.

1.7.1. HindLimb Muscle Splitting Events

Between embryonic stages HH 21-23, the somatic cells that migrate into the hind limb bud aggregate into a dorsal and ventral muscle masses. At stage HH 25, these two muscle masses start to further divide into thigh and shank muscle masses. (*Kardon 1998*) This is illustrated in figure 17.

Further splitting of these four muscle masses are detailed individually in figures:18 (17A) - Dorsal Thigh Muscle Mass, 19 (17B) - Dorsal Shank Muscle Mass, 20 (17C) - Ventral Thigh Muscle Mass and 21 (17D) - Ventral Shank Muscle Mass.

In this section, splitting events are recorded the following structures are observed and noted.

- Muscle masses of differentiated myotubes (MM)
- Individual muscles identifiable by orientated myotubes (IM)
- Individual segregated muscles (noted by given names of each muscle)

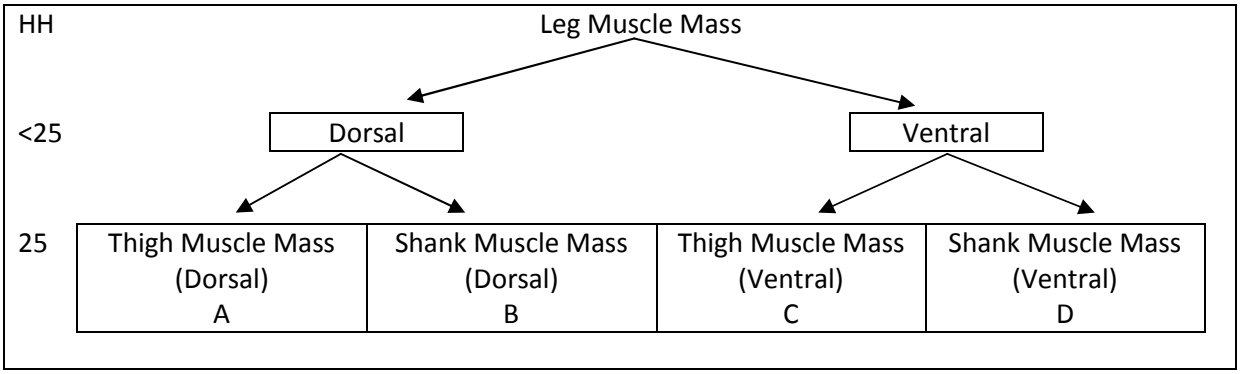


Figure 17: Splitting events of the hindlimb muscle Mass till embryonic stage HH 25.

1.7.1.1. Dorsal Muscle Mass

In this section, we discussed the splitting events that the dorsal thigh muscle mass (1.7.1.1.1), the dorsal shank muscle mass (1.7.1.1.2) and dorsal foot muscle mass (1.7.1.1.3) undergo to give rise to individual muscles are discussed.

1.7.1.1.1. Dorsal Thigh Muscles

The dorsal thigh muscle mass undergoes a series of splitting events to give rise to eleven individual segregated muscles.

- IFI - Iliofemoralis Internus
- IC – Iliotibialis Cranialis
- AMB – Ambiens
- FTI – Femorotibialis Internus
- FTE – Femorotibialos Externus
- IL – Iliotibialis Lateralis
- IF – Iliofibularis
- ITCR – Iliotrochantricus Cranialis
- ITCM – Iliotrochantericus Medius
- ITC – Iliotrochantericus Caudalis
- IFE – Iliofemoralis Externus

The muscles IF and IFI emerges as segregated individual muscles at embryonic stage HH29, whilst the rest; IL, FTE, ITCM, ITC, IFE, ITCR, IC, AMB and FTI all emerge as segregated muscles at embryonic stage HH 30. (*Kardon 1998*) This is illustrated in Figure 18 (17A).

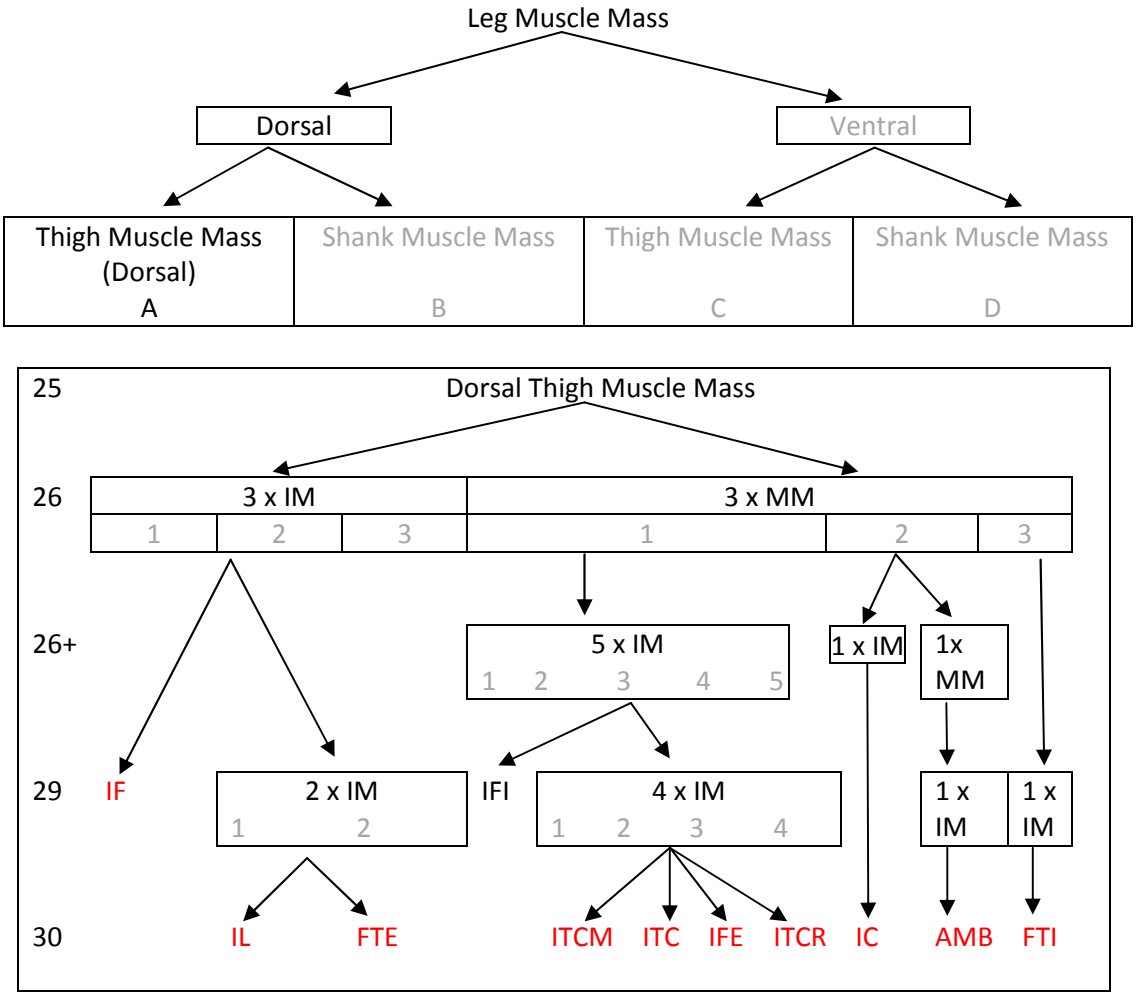


Figure 18 (17A): Splitting event of Dorsal thigh muscle mass to give rise to eleven individual segregated muscles.

1.7.1.1.2-3.Dorsal Shank and Foot Muscles

The dorsal shank muscles undergo one split to give rise to the dorsal foot muscle mass around embryonic stages HH 25- 26. (*Kardon 1998*) This can be seen in Figure 19 (17B).

1.7.1.1.2. Dorsal Shank Muscles

The dorsal shank muscle mass then goes through further sequential splitting events to give rise to four individual segregated muscles.

- EDL – Extensor Digitorum Longus
- TC – Tibialis Cranialis
- FL – Fibularis Longus
- FB – Fibularis Brevis

The EDL emerges as an individual segregated muscle at embryonic stage HH 29. The remaining three, TC, FL and FB emerge as individual segregated muscles at embryonic stage HH 30. (*Kardon 1998*) This is illustrated in figure 19 (17B).

1.7.1.1.3. Dorsal Foot Muscles

The dorsal foot muscle mass undergoes systematic splitting events to give rise to four individual segregated muscles.

- EHL – Extensor Hallucis Longus
- AB2 – Abductor Digiti 2
- EP3 – Extensor Proprius 3
- EB4 – Extensor Brevis Digiti 4

The muscles EP3 and EB4 can be seen as individual segregated muscles at embryonic stage HH 30+, whilst AB2 and EHL can only be seen as individual segregated muscles at embryonic stage HH 35. (*Kardon 1998*) This is illustrated in figure 19 (17B).

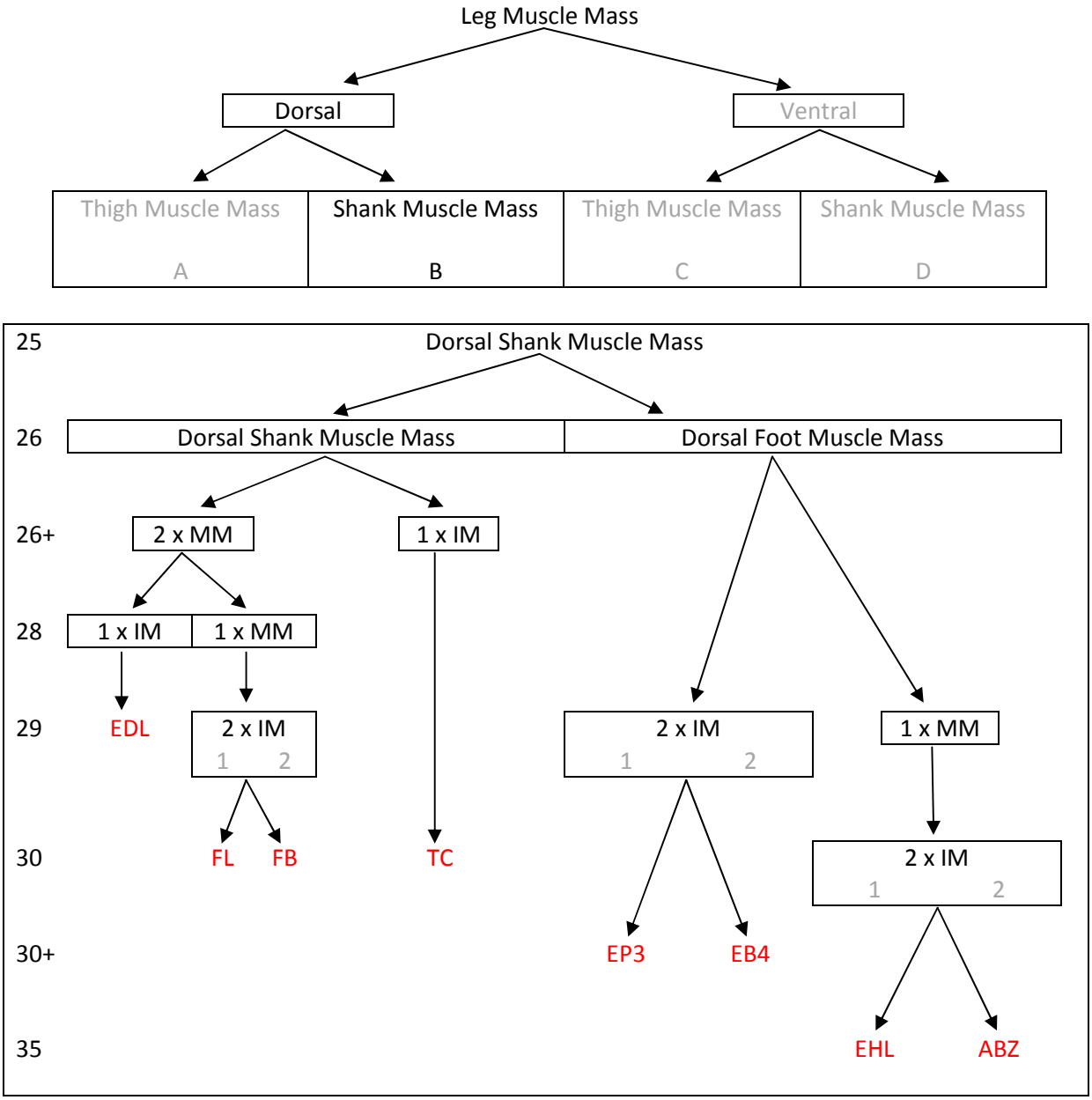


Figure 19 (17B): Splitting event of dorsal shank muscle mass to give rise to four individual segregated muscles of the shank and four more individual segregated muscle s of the foot.

1.7.1.2. Ventral Muscle Mass

In this section, we discussed the splitting events that the ventral thigh muscle mass, the ventral shank muscle mass and ventral foot muscle mass undergo to give rise to individual muscles are discussed.

1.7.1.2.1. Ventral Thigh Muscles

The ventral thigh muscle mass undergoes a series of splitting events to give rise to seven individual segregated muscles.

- OBT – Obturatorius
- PIF – Puboischiofemoralis
- ISF – Ischiofemoralis
- FCM – Flexor Cruris Medialis
- FCL – Flexor Cruris Lateralis
    - FCLA – Flexor Cruris Lateralis Pars Accessoria
    - FCLP – Flexor Cruris Lateralis Pars Pelvica
- CFC – Caudofemoralis Pars Caudalis
- CFP – Caudofemoralis Pars Pelvica

The muscle OBT can be identified as an individual segregated muscle at embryonic stage HH28. The muscles ISF and FCL can be seen as individual segregated muscles at embryonic stage HH 29. The remaining four, PIF, FCM, CFC and CFP emerge as individual segregated muscles at embryonic stage HH 30. (*Kardon 1998*) This is illustrated in Figure 20 (17C).

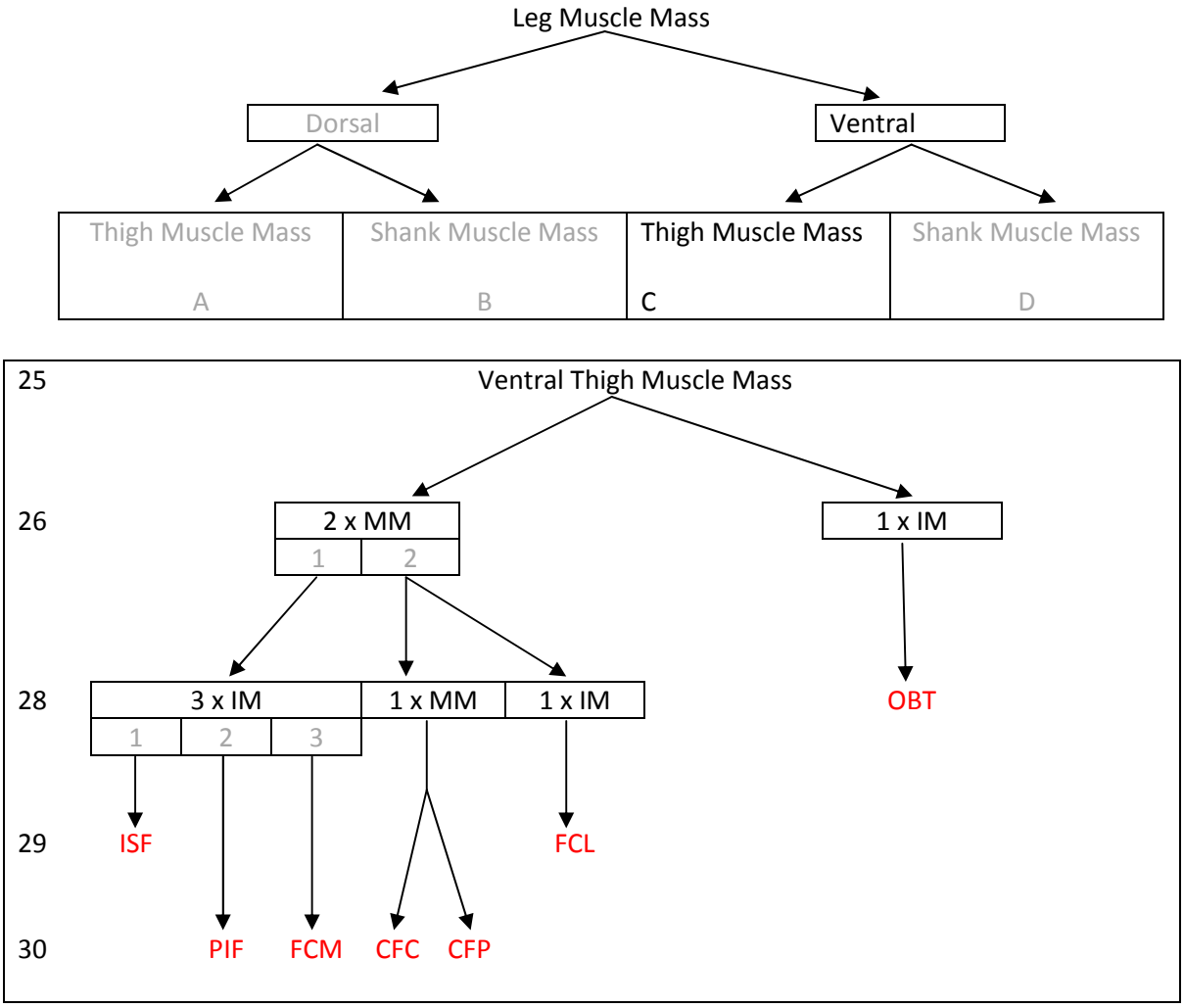


Figure 20 (17C): Splitting event of ventral thigh muscle mass to give rise to seven individual segregated muscles.

1.7.1.2.2-3.Ventral Shank and Foot Muscles

The ventral shank muscle mass splits to give rise to the foot muscle mass at embryonic stage HH26, alongside undergoing other splits that contribute towards the final individual, segregated muscles of the shank. (*Kardon 1998*) This can be seen in figure 21 (17D).

1.7.1.2.2. Ventral Shank Muscles

The ventral shank muscle mass then goes through further sequential splitting events to give rise to nine individual segregated muscles.

- GM – Gastrocnemius Intermedius
- GI – Gastrocnemius Internus
- P – Plantaris
- FDL – Flexor Digitorum Longus
- FHL – Flexor Hallucis Longus
- FP2 – Flexor Perforatus 2
- FP3 – Flexor Perforatus 3
- FP4 – Flexor Perforatus 4
- FPP3 – Flexor Perforans et Perforatus 3
- FPP2 – Flexor Perforans et Perforatus 2
- GE – Gastronemius Externus

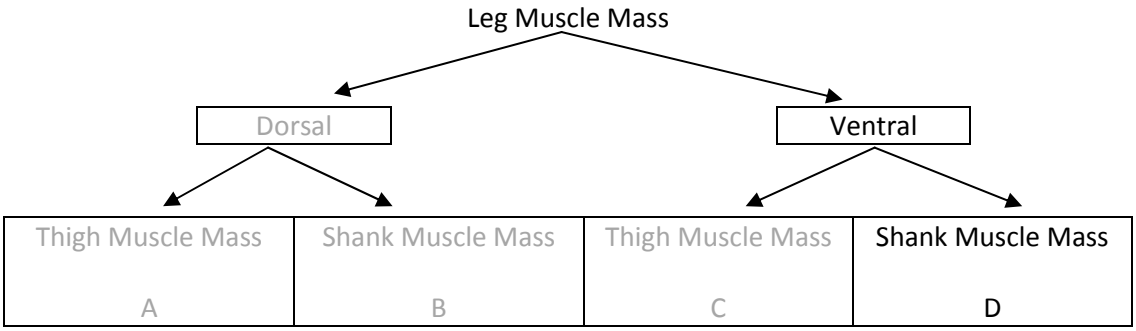
The muscles GE and FDL can be identified as individual segregated muscles at embryonic stage HH 28. The muscle FHL emerges as an individual segregated muscle at embryonic stage 29. At stage HH 30, four more muscles, FPP2, FPP3, FP4 and P can be identified as individual segregated muscles. Finally, FP2 and FP3 emerge at embryonic stage HH30+. (*Kardon 1998*) This is illustrated in figure 21 (17D).

1.7.1.2.3.Ventral Foot Muscles

The ventral foot muscle mass undergoes sequential muscle splitting events to give rise to three individual segregated muscles.

- FHB - Flexor Hallucis Brevis
- AD2 – Adductor Digiti 2
- AB4 – Abductor Digiti 4

All three muscles can only be seen as individual segregated muscles at embryonic stage HH35. (*Kardon 1998*) This is illustrated in Figure 21 (17D).


Leg Muscle Mass
Dorsal
Ventral
Thigh Muscle Mass
A
Shank Muscle Mass
B
Thigh Muscle Mass
C
Shank Muscle Mass
D

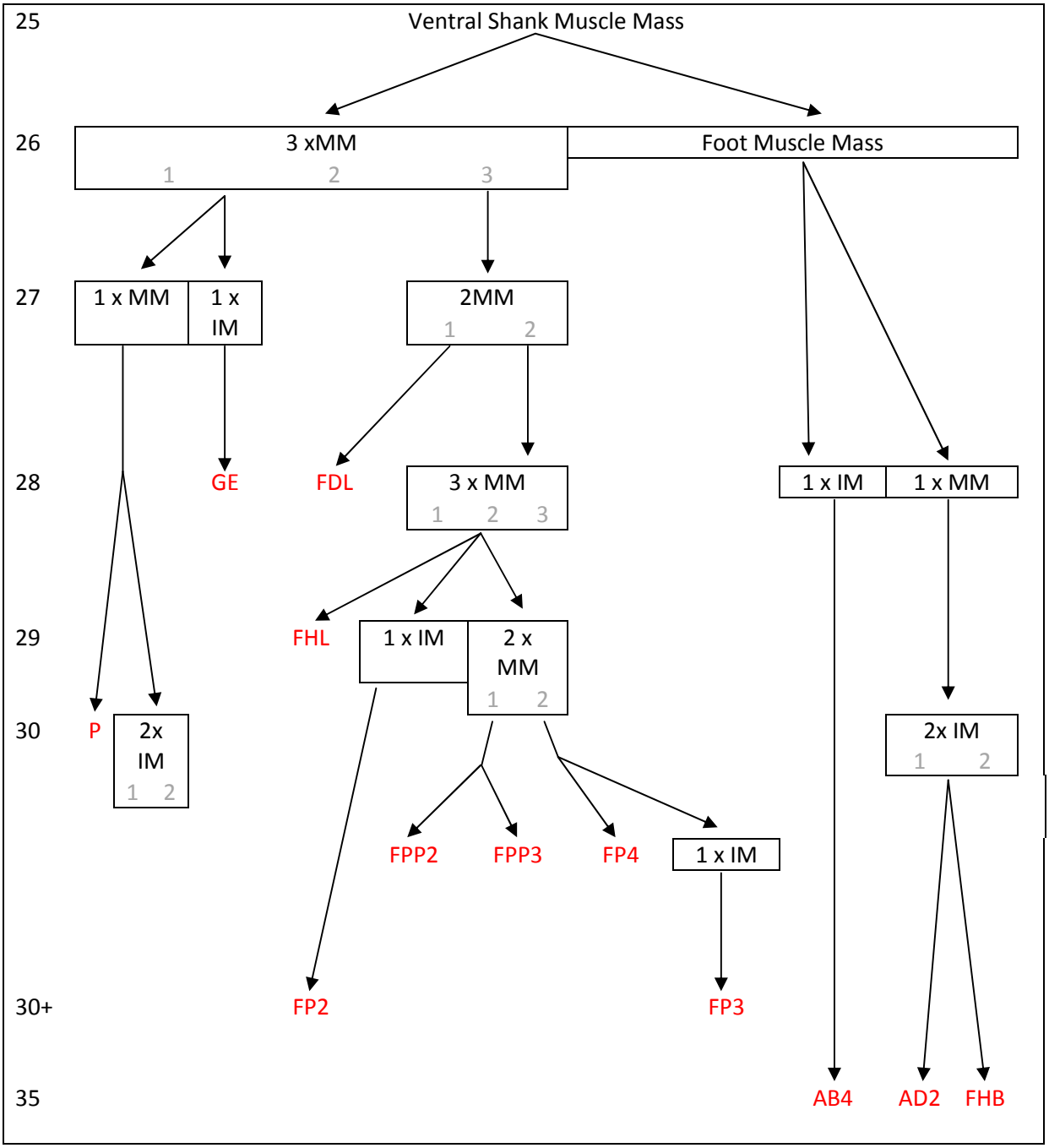

25
Ventral Shank Muscle Mass
26
3 xMM
1 2 3
Foot Muscle Mass
27
1 x MM
1 x IM
2MM
1 2
28
GE
FDL
3 x MM
1 2 3
1 x IM
1 x MM
29
FHL
1 x IM
2 x MM
1 2
30
P
2x IM
1 2
2x IM
1 2
FPP2
FPP3
FP4
1 x IM
30+
FP2
FP3
35
AB4
AD2
FHB

Figure 21 (17D): Splitting event of ventral shank muscle mass to give rise to nine individual segregated muscles of the shank and three individual segregated muscle s of the foot.

1.7.2. Forelimb Muscle Splitting Events

Between embryonic stages HH 21-23, the somatic cells that migrate into the forelimb bud, aggregate into a dorsal and ventral muscle masses. This is shown in figure 22. These two muscle masses then, start to further cleave to give rise to the individual muscles of the forelimb. Both the dorsal and ventral muscles masses contribute to individual muscles in the autopod, zeugopod and stylopod.

These are discussed in more detail individually, in figure 23 (22A) - Dorsal Stylopod (Humerus) Muscles, 24 (22B) - Dorsal Zeugopod (Radius, Ulna) Muscle Mass, 25 (22C) - Dorsal Autopod (Metacarpals, Phalange) Muscle Mass, 26 (22D) - Ventral Stylopod (Humerus) Muscles, 27 (22E) - Ventral Zeugopod (Radius, Ulna) Muscle Mass, 28 (22F) - Ventral Autopod (Metacarpals, Phalange) Muscle Mass.

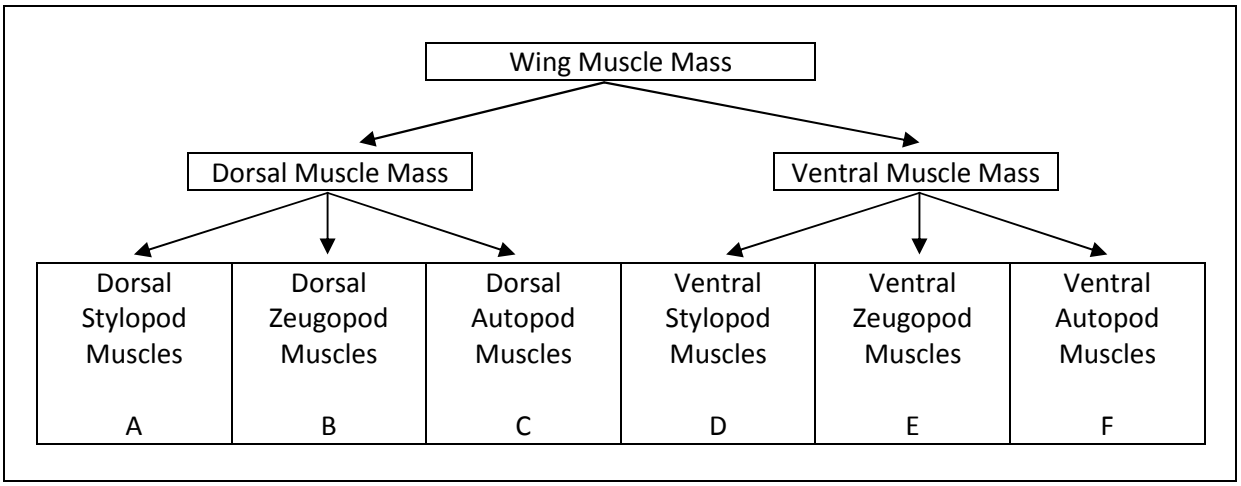


Figure 22: Early muscle splitting events in the forelimb.

1.7.2.2. Dorsal Muscle Mass

In this section, we discussed the splitting events that the dorsal muscle mass of the wing undergo to give rise to individual autopod, zeugopod and autopod muscles.

1.7.2.2.1. Dorsal Stylopod (humerus) Muscles

The dorsal stylopod muscle mass undergoes splitting events to give rise to ten individual segregated mucles.

- Triceps
- Scapulo-humeralus anterior
- Scapulo-humeralis posterior
- Coraco-brachialis posterior
- Sub-scapularis
- Sub-coracoideus
- Deltoid
- Tensor propatagium
- Latissimus dorsi anterior
- Latissimus dorsi posterior

All ten muscles were observable as individual segregated muscles by embryonic stage HH 35-36. This can be seen in figure 23 (22A). (*Lancer and Fallon, 1987)*

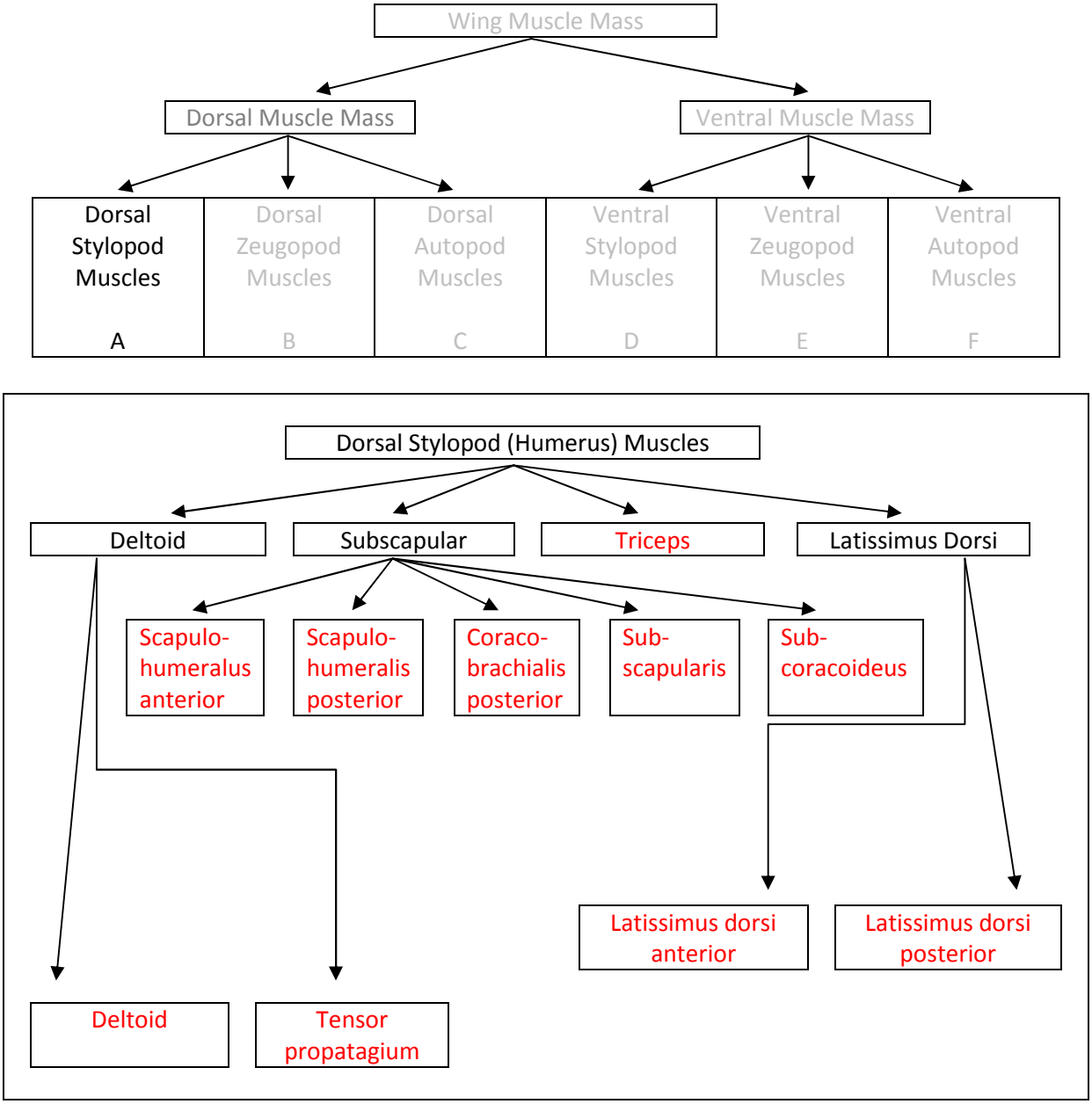


Figure 23 (22A): Splitting events of dorsal stylopd muscle mass to give rise to ten individual segregated muscles.

1.7.2.2.2. Dorsal Zeugopod (Radius, Ulna) Muscles

The dorsal zeugopod muscle mass undergoes a series of binary cleavage events to give rise to six individual segregated muscles.

- EMR - Extensor Metacarpi Radialis
- EIL - Extensor Indicis Longus
- EML - Extensor Medius Longus
- EDC - Extensor Digitorum Communis
- EMU - Extensor Metacarpi Ulnaris
- Anc – Anconeus

The muscles EMR and EDC emerge as a result of the second binary split and are identified as individual segregated muscles at embryonic stage HH-29-30. The remaining four muscles, EIL, EML, EMU, Anc emerge as a result of the next round of binary cleavage and they are identified as individual segregated muscles at embryonic stage HH 31-32. This can be seen in figure 24 (22B).

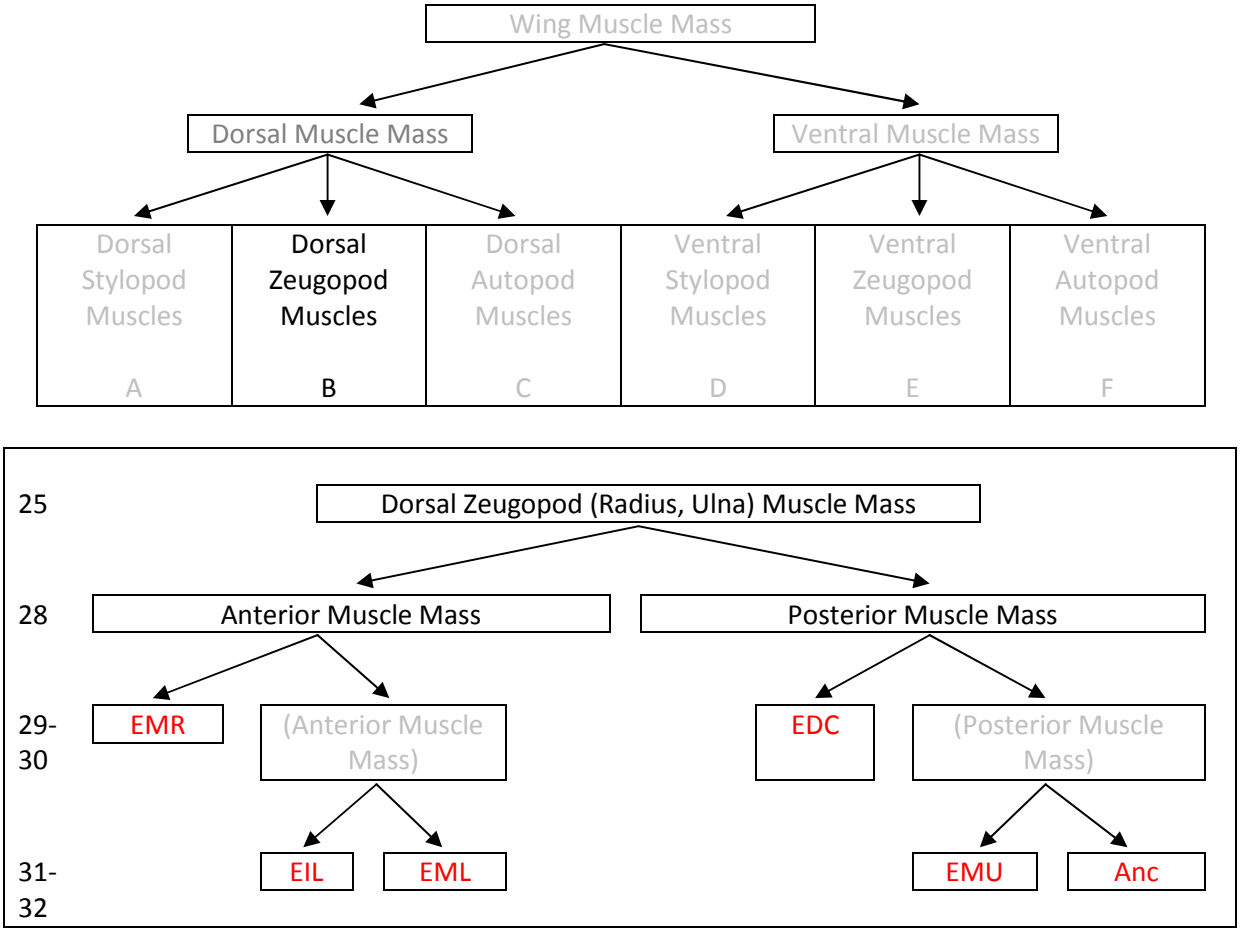


Figure 24 (22B): Splitting event of dorsal zeugopod muscle mass to give rise to six individual segregated muscles.

1.7.2.2.3. Dorsal Autopod (Metacarpals, Phalange) Muscle Mass

The dorsal autopod muscle mass undergoes splitting events to give rise to five individual segregated muscles.

- EIB – Extensor Indicis Brevis
- Ad. I – Adductor Indicis
- EMB – Extensor Medius Brevis
- IOD – Interosseus Dorsalis
- UMD – Ulnimetacarpalis Dorsalis

This can be seen in figure 25 (22C). The figure does not show any sequential cleavage the muscle mass may undergo. All individual muscles can be seen by embryonic stage HH 35.

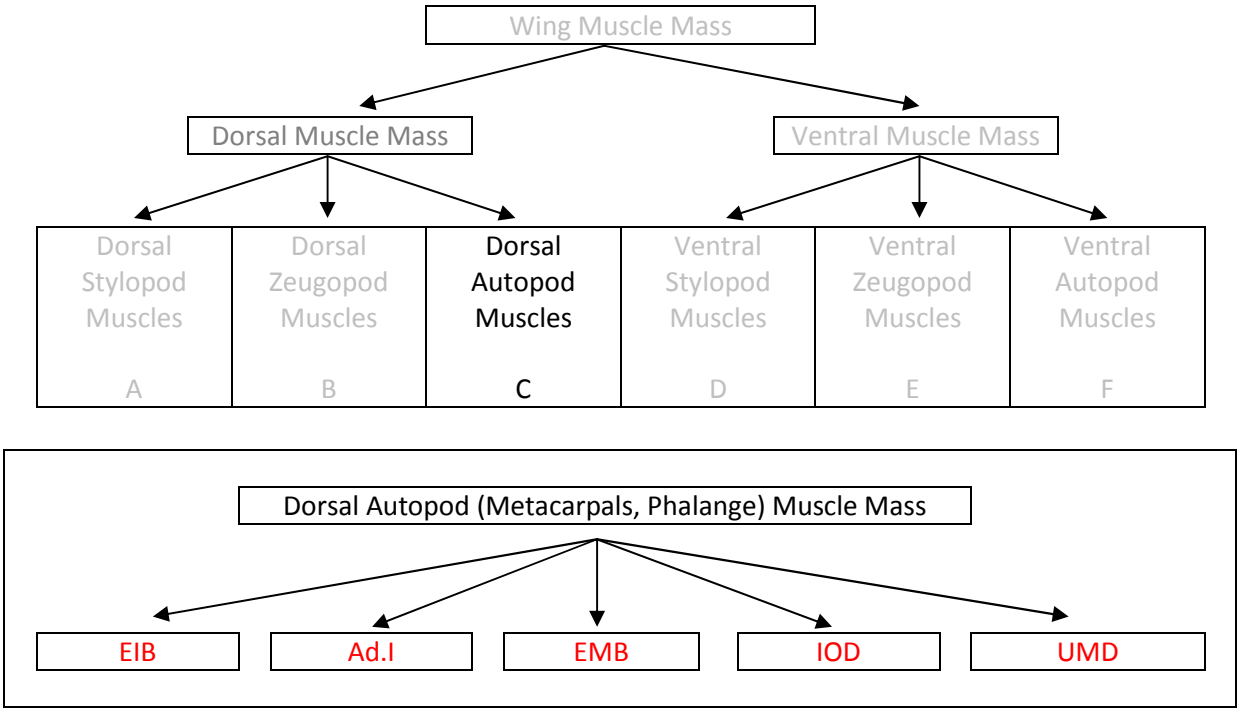


Figure 25 (22C): Splitting of dorsal autopod muscle mass to give rise to five individual segregated muscles.

1.7.2.3. Ventral Muscle Mass

In this section, we discussed the splitting events that the ventral muscle mass of the wing undergo to give rise to individual autopod, zeugopod and autopod muscles.

1.7.2.3.1. Ventral Stylopod (Humerus) Muscles

The ventral stylopod muscle mass undergoes splitting events to give rise to seven individual segregated mucles.

- Pectoralis major medial
- Pectoralis major lateral
- Brachialis
- Coracobrachialis
- Biceps
- Supracoracoideus
- Coracobrachialis anterior

All seven muscles were observable by embryonic stage HH 35-36. This can be seen in figure 26 (22D).

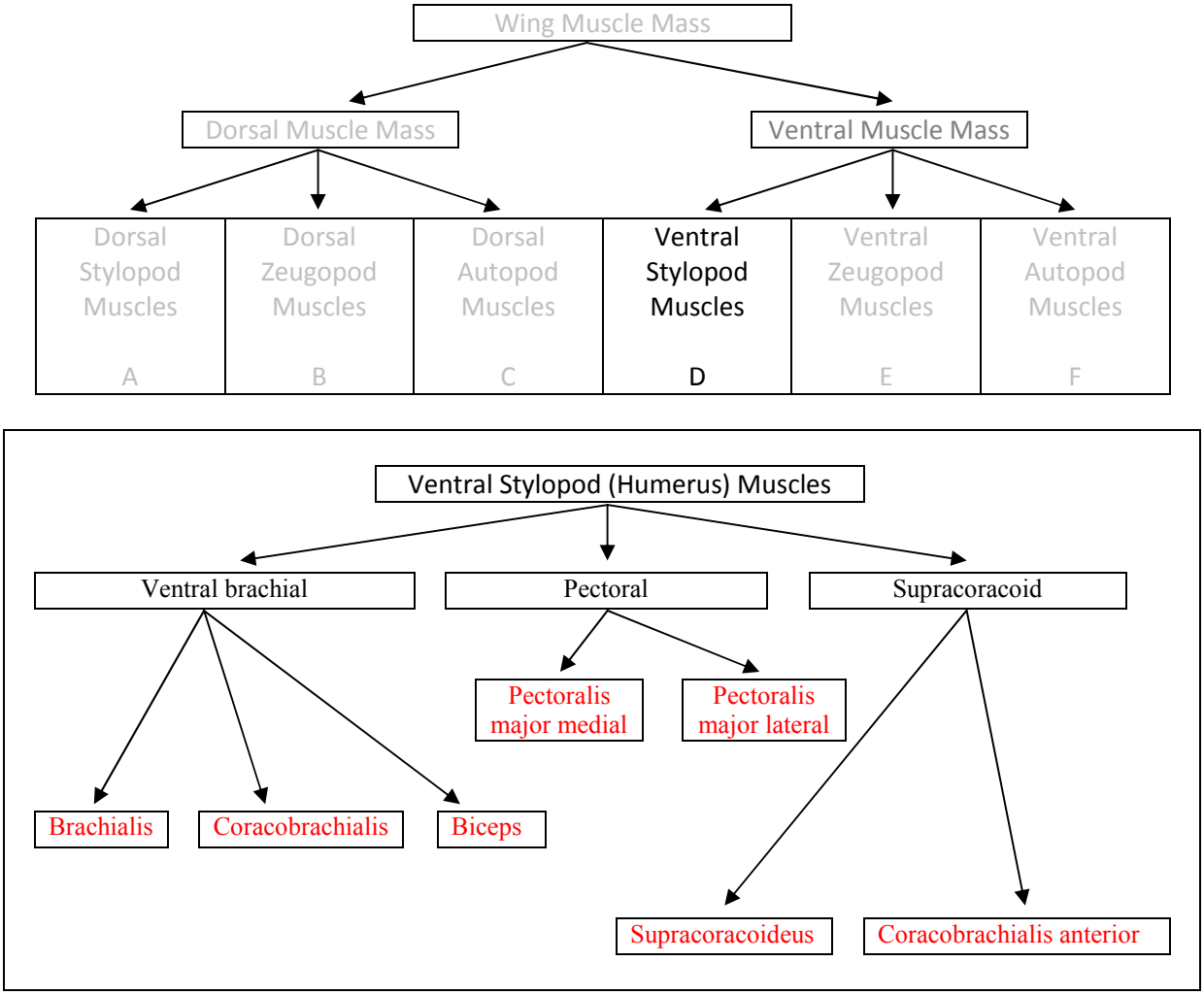


Figure 26 (22D): Splitting events of ventral stylopd muscle mass to give rise to seven individual segregated muscles.

1.7.2.3.2. Ventral Zeugopod (Radius, Ulna) Muscle Mass

The dorsal zeugopod muscle mass undergoes a series of splitting events to give rise to seven individual segregated muscles.

- PS - Pronator Superficialis
- PP - Pronator Radius
- Ent - Entepicondyloulnaris
- FDP - Flexor Digitorum Profundus
- UMV - Ulnimetacarpalis Ventralis
- FDS - Flexor Digitorum Superficialis
- FCU - Flexor Carpi Ulnaris

All seven muscles emerge as individual segregated muscles at embryonic stage HH 30-31. This can be seen in figure 27 (22E).

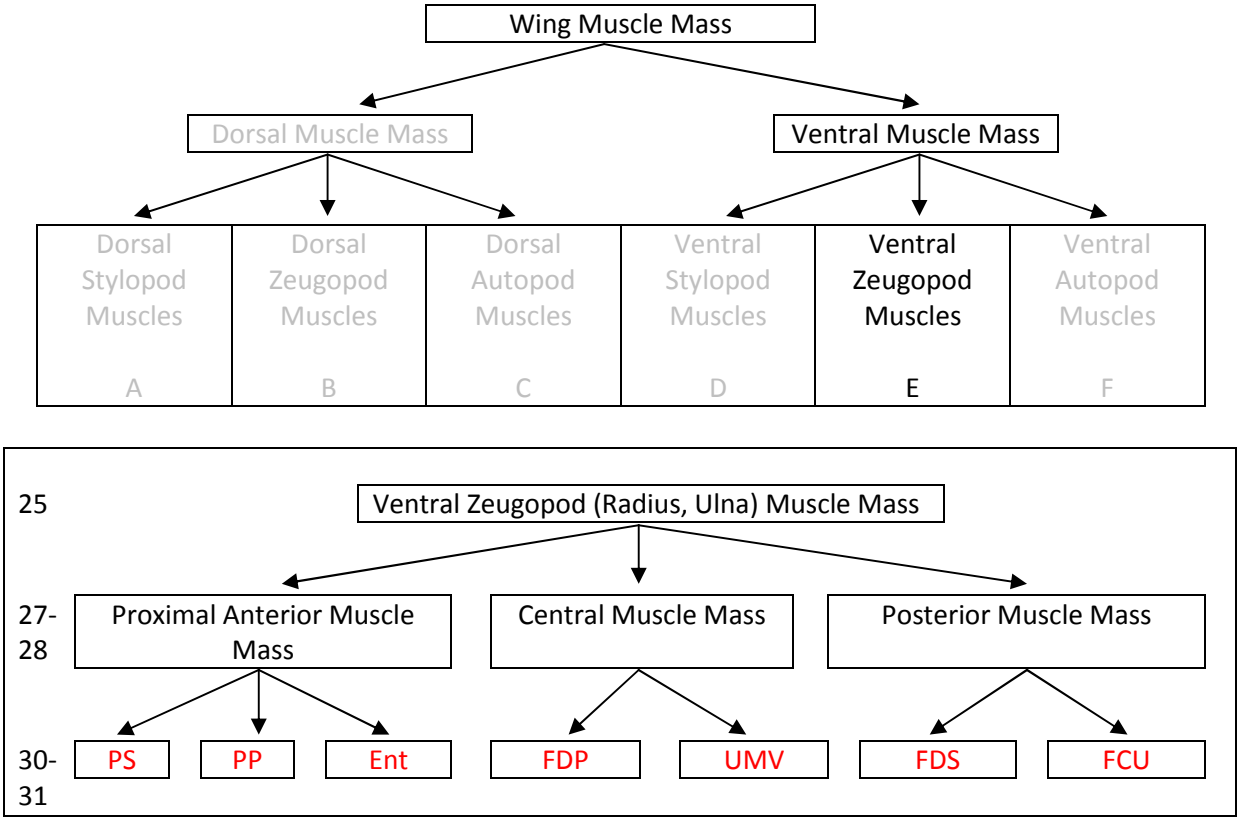


Figure 27 (22E): Splitting of ventral autopod muscle mass to give rise to seven individual segregated muscles.

1.7.2.3.3. Ventral Autopod (Metacarpals, Phalange) Muscle Mass

The ventral autopod muscle mass undergoes splitting events to give rise to five individual segregated muscles.

- Ab. I – abductor indicis
- FI – Flexor Indicis
- Ab. M – abductor medius
- IOP – interosseus Palmaris
- FDQ – Flexor Digiti Quarti

This can be seen in figure28 (22F), however the figure does not show any sequential cleavage the muscle mass may undergo. All individual muscles can be seen by embryonic stage HH 35.

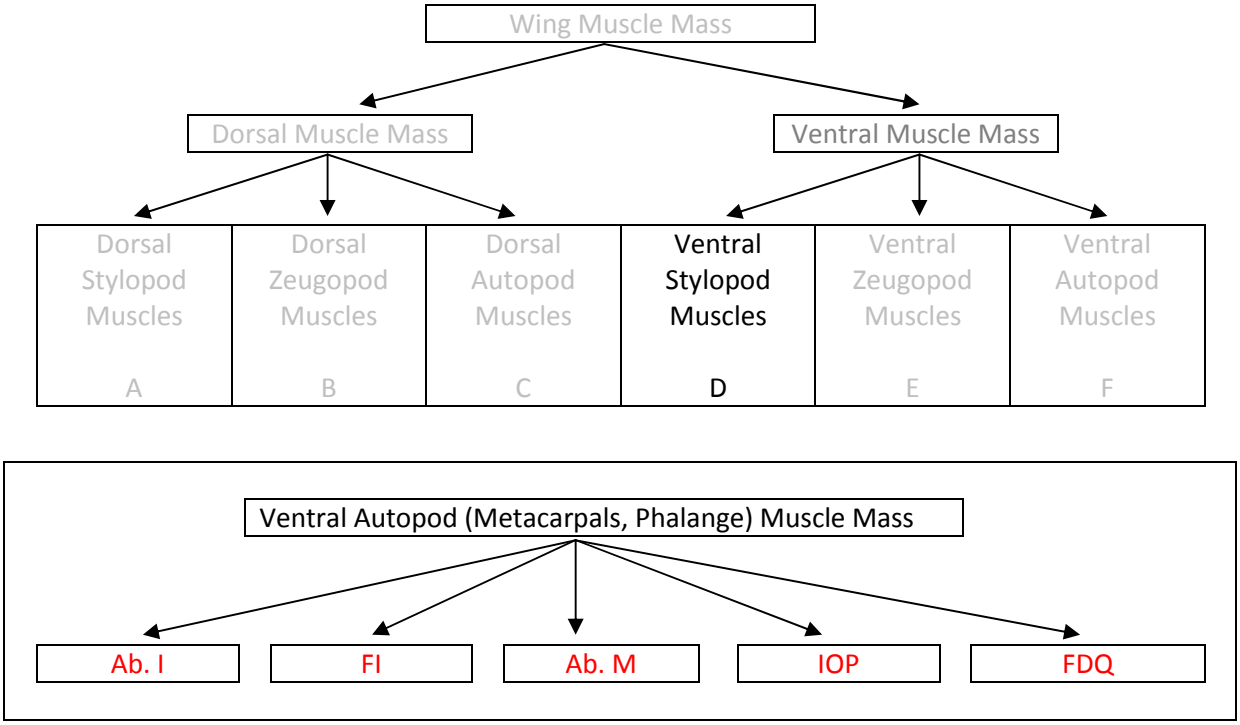


Figure 28 (22F): Splitting of ventral autopod muscle mass to give rise to five individual segregated muscles.

1.8. MicroRNAs

MicroRNAs are an abundant family of short non-coding ribonucleic acid (RNA) molecules. They are about 22 nucleotides (nt) long, single stranded RNAs that play a regulatory role in plants and animals by exerting post-transcriptional effect.

MicroRNAs were first discovered in C. Elegans. Since then, they have been implicated to have roles in various biological processes including various stages and parts of embryonic development. Every metazoan cell type, at each developmental stage might have a distinct miRNA expression profile allowing the cells to micromanage the transcripts. (*Lagos-Quintana et al 2001*)

1.8.1. Biosynthesis of MiRNA

1.8.1.1. Primary MicroRNA Transcripts (Pri-MiRNA)

The first step in miRNA biosynthesis involves a genomic fragment of >1kb being transcribed from DNA. Majority of these transcripts have their own set of promoters, but little is known of these promoter systems. These transcripts are called the primary microRNA transcripts (pri-miRNA). This transcription step is carried out most often by RNA polymerase II and in some instances by RNA polymerase III. Whilst much research has been carried out to identify the RNA polymerase involved in the transcription step, this does not have any downstream effect on the processing or function of the miRNA. This is illustrated in figure 29 (Step1). (*Bartel 2004*)

1.8.1.2. Precursor miRNAs (pre-miRNA)

The pri-miRNA is then cleaved into shorter (60-70nt) strands in length that have some internal complementarity. This complementarities cause the pri-miRNA to take the signature stem-loop structure of such molecules. These fragments are called precursor miRNAs (pre-miRNA) (*Lee et al 2002*). This cleavage if the RNA molecule is carried out by an RNA III endonuclease called Drosha. Drosha cleaves both the strands of the RNA duplex at the base of the stem-loop (*Lee-et al 2003*). RNA III Endonucleases leave a characteristic staggered 5' phosphate and a 2nt overhang at the 3' end. (*Basyuk et al 2003*)

The pre-miRNA is actively transported from the nucleus to the cytoplasm with the help of Ran-GTP and the export receptor Exportin (*Yi et al 2003*).

Once in the cytosol, a second RNA III endonuclease, Dicer comes into contact with the pre-miRNA and further processes it (*Lee et al 2003*). The Dicer molecule recognises the RNA III endonuclease cleaved 5' phosphate and 3' overhang and is thought to have a specific affinity for this region. Upon binding, Dicer cleaves the RNA molecule two helical turns away from the previously cleaved ends, chopping off the terminal base pairs and the loop of the pre-miRNA. Dicer too leaves a signature 5' phosphate end with a 3' overhang. Once the cleavage process is completed, an imperfect RNA duplex is left behind – one strand is comprised of the mature miRNA and the other is a similar sized fragment called the miRNA* sequence (*Lau et al 2001*). The miRNA:miRNA* duplex is generally short lived. This is shown in figure 29 (Step 2-4).

1.8.1.3. Mature MiRNA

Once the miRNA:miRNA* duplex forms it is loaded into an RNA induced silencing complex (RISC). Purified RISC components show that it contains a member of the Argonaut protein family, which is thought to the core component of the complex (*Hammond et al 2001*). Argaunaut proteins are about 100kDa in size and contain PAZ and PIWI domains (*Cerutti et al 2000*). The PAZ domain appears to bind weakly to single stranded RNA which are at least 5 nt in size and also to double stranded RNA (*Lingel et al 2003*). The RISC complex has also been shown to have VIG domains which bind RNA, fragile X-related proteins and the Tudor-SN which has an endonucleic activity (*Caudy et al 2002*). The roles of these domains have not been defined in the RISC complex therefore they may be part of the core complex or associated with the complex as accessory factors to modify the function of the RISC complex. Slicer, a RISC endonuclease is also said to be present in low levels and thought to be recruited once the short RNA and RISC complex have associated.

Once loaded, the miRNA:miRNA* complex is subject to the activity of a helicase like enzyme. The enzyme attempts to unwind the RNA duplex from both ends several times before proceeding to unwind the duplex from the end whose 5' end is less tightly bound and is therefore easier to unwind. As a result the RNA strand that enters the RISC complex is usually the one with the weakly paired 5' end (*Khvorova et al 2003*) This can be seen in figure MiR1 (Step 5 and 6)

1.8.2. Post-Transcriptional Regulation

MicroRNAs, once in the RISC complex, downregulate gene expression by either mRNA cleavage or translational repression. The nature of the complimentarity between the miRNA and mRNA dictate which form of control is exerted.

The complementarity sites seem to always be located in the 3' untranslated region (3'-UTR) of the mRNA molecule. This may be due to mechanistic preference allowing the bound RISC complexes to avoid the mRNA clearing the activity of the ribosome (*Kuersten and Goodwin 2003*)

It is suspected that the 5' portion of the metazoan miRNA is more important in rendering this complementarity to the 3'-UTR of the mRNA. Sequences 2-8 on the miRNA called the 'core elements' contribute to this. The 'core elements' have also been discovered to be the most conserved among homologous metazoan miRNAs. Mismatches within the core region and target mRNA can prevent cleavage or translational repression; this can often be overwritten with sufficient complementarity in the rest of the miRNA and mRNA. However, little extra help is required for the RISC complex to carry on with the translational regulation when there is perfect complimentarity between the 'core elements' and the mRNA (*Lewis et al 2003*).

If the miRNA has sufficient complementarities to the messenger RNA (mRNA), the mRNA molecule is cleaved between the sequences complementary to the 10^{th} and 11^{th} nucleotide of the miRNA (ElBashir et al 2000). Once the cleavage is complete, the miRNA remains intact and bound to the RISC complex, which then proceeds to bind more mRNA molecules (*Hutvagner and Zamore 2002*).

If the miRNA and mRNA do not show sufficient complementarity the mRNA is not cleaved, but translation is repressed (*Hutvagner and Zamore 2002*). Some metazoan miRNA show lack of complimentarity at the 12^{th} and 13^{th} nucleotide, which points to inherent sequence preference for the two respective modes of repression (*Khvorova et al 2003*).

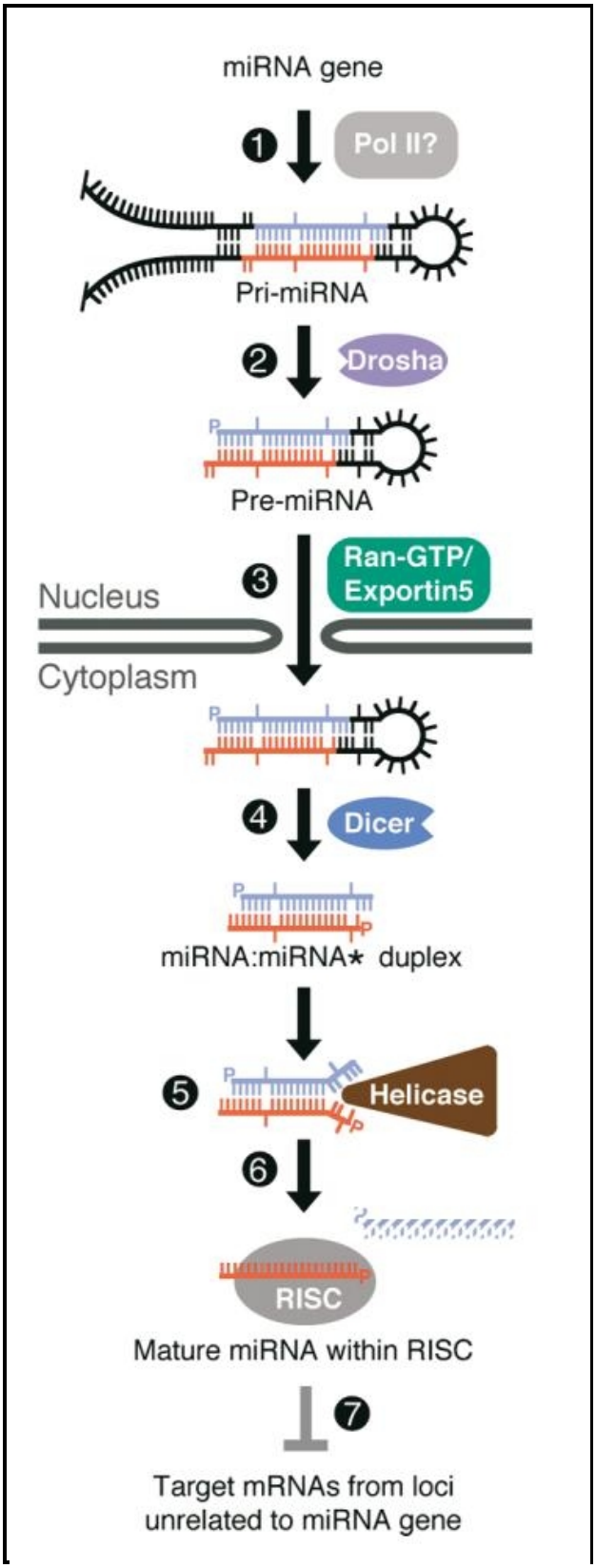


Figure 29: Diagram showing the stages of mature miRNA synthesis. (*Bartel 2004*)

1.9. Muscle Specific MicroRNAs

Six hundred (600) miRNAs have been identified in humans and it is believed many if not all gene expression is under post-transcriptional regulation of these miRNAs. Some miRNAs are ubiquitously expressed whilst others have a more tissue specific expression pattern.

MiR-1, miR-206 and miR-133 are very well characterised. They are necessary for proper skeletal and cardiac muscle development and function. Therefore these miRNAs possibly influence multiple myopathies – hypertrophy, dystrophy and conduction defects (*Townly-Tilson et al 2009*).

1.9.1 Structure and Location of MiRNA-1/206 and MiRNA-133

In-situ hybridisation studies suggest a conserved, skeletal muscle specific expression of miR-1/206 and miR-133 in Xenopus, zebrafish, chick and mouse embryo development. (*Sweetman et al 2006*). Genomic analyses have shown that there are four loci in the chicken genome, choromosomes 2, 3, 20 and 23, encoding these muscle specific miRNAs; each of which produce a transcript containing one of the miR-1/206 family and one of the miR-133 family. However, human and mouse only have three loci which produce these miR-1/206 and Mir-133 clusters. Therefore, it is believed the fourth locus has emerged as a result of further duplication (*Sweetman et al 2008*). This can be seen in figure 30. miR-1 and miR-133 are transcribed together; a highly conserved region 50 kb upstream of the cluster is responsible for their transcription. However, post-transcriptional processing separates the mature miRNAs, rendering them spatial specificity (*Chen et al 2006*).

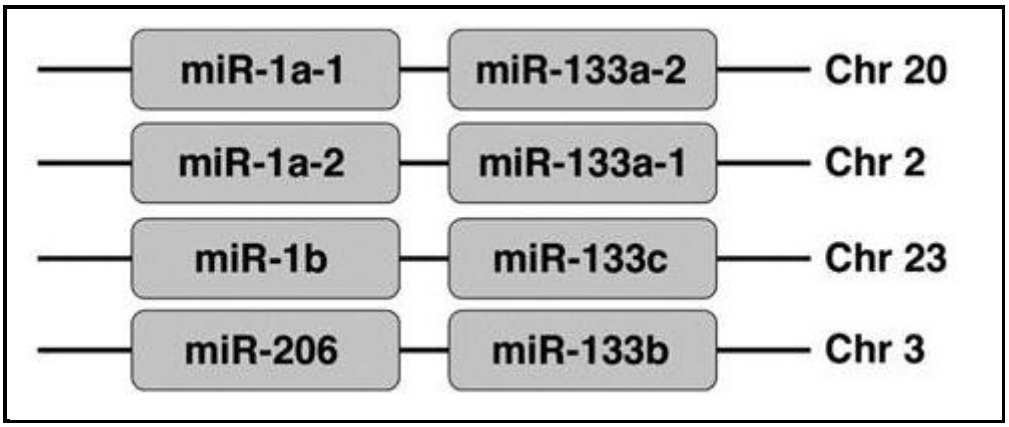


Figure 30: Alignment of the chicken muscle specific miRNAs found on chromosomes 2, 3, 20 and 23. (*Adopted from Sweetman et al 2008*)

MiR-1 and miR-206 are closely related in terms of expression and function but are found on different chromosomes, have different targets and their own transcriptional activation system. MiR-1 and miR-206 differ from each other by four nucleotides. This can be seen in figure 31. MiR-1 can be found in both skeletal and cardiac muscles but miR-206 can only be found in skeletal muscles. However, both miR-1 and miR-206 function to myoblast to myotube differentiation.

MiR-133a-1 and miR-133a-2 share identical mature sequences, while miR-133b and miR-133c differ from miR-133a and each other by a single nucleotide at the 3'-end. This can be seen in figure 31. All mature miR-133 molecules function to promote myoblast proliferation whilst inhibiting differentiation.

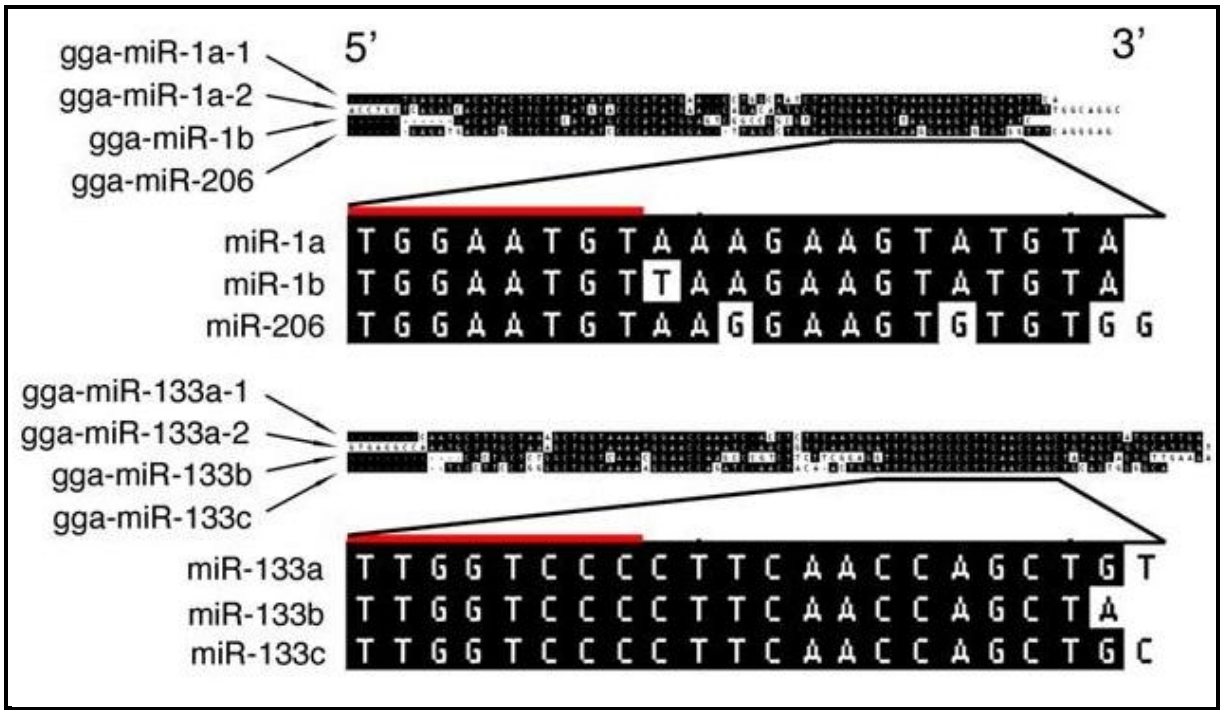


Figure 31: Alignment of pre-miRNA (produced from the chick muscle specific miRNA loci) and the mature microRNAs. The red bar indicates the 'core elements' of these miRNAs. (*Adopted from Sweetman et al 2008*)

1.9.2. Regulation and Function of MicroRNAs-1/206 and MicroRNA-133

MiR-1 and miR-133 are specifically expressed in adult skeletal and cardiac muscle tissue. It can also be found in small amounts in the developing heart and skeletal muscles. (*Chen et al 2006*). MiR-206, however, can only be found in skeletal muscles (*Kim et al 2006*). This can be seen in figure 32. In the chick embryo, mir-1, miR-206 and miR-133 are expressed in post mitotic cells in the somite myotome. At a given embryonic stage miR-1 and miR-133 can be found in more anterior, therefore more differentiated somites compared to miR-206 (*Sweetman et al 2008*). This is also illustrated in figure 32.

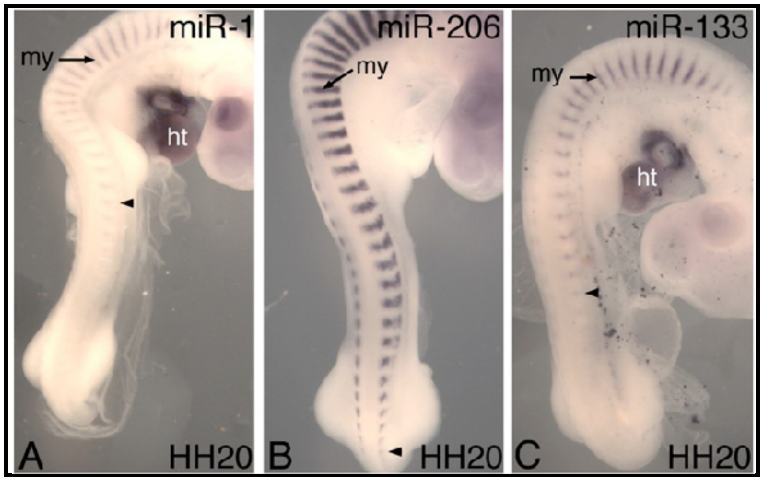


Figure 32: Expression of muscle specific miRNA-1, MiR-206 and MiR-133 in HH 20 chick embryos. (*Sweetman et al 2008*)

Muscle formation in the developing embryo requires cell proliferation and cell differentiation. It is required the correct balance is achieved between cells that remain in the form of multiplying myoblasts (to give rise to more cells) to allow for growth and cells that differentiate into myotubes to allow for development.

As discussed earlier, miR-1 strongly enhances myogenesis. Histone deacetylase 4 (HDAC4) is a transcriptional repressor of muscle specific gene MEF2. MiR-1 inhibits the function of HDAC4, therefore removing the inhibition on MEF2 and allowing muscle specific gene expression to occur. This in turn allows the myoblasts to differentiate into myotubes (*Chen et al 2006*). This can be seen in figure 33.

Mir-206, a member of the miR-1 family, also enhances myogenic differentiation. For myoblasts to differentiate into myotubes, it is crucial that DNA synthesis and the cell cycle come to an arrest. MiR-206 targets DNA polymerase α, which in turn halts the cell cycle and prevents cell proliferation and enhances differentiation. Mir-206 also targets inhibitors of myogenic transcription factors, Idl-3 and MyoR, which results in the uninhibited myogenic transcription factors to drive the myogenic differentiation to form myotubes (*Kim et al 2006*).

MiR-133, in contrast(as discussed earlier), promotes cell proliferation. Serum Response Factor (SRF) controls the expression of muscle specific genes; it binds to the DNA sequence called the CArG box and regulates transcription by recruiting numerous signal responsive and cell type restricted cofactors (*Shijie 2005*). MiR-133 inhibits the function of SRF, therefore repressing its ability to influence the expression of muscle specific genes and therefore muscle differentiation, which in turn promotes myoblast proliferation (*Chen et al 2006*). This can be seen in figure 33.

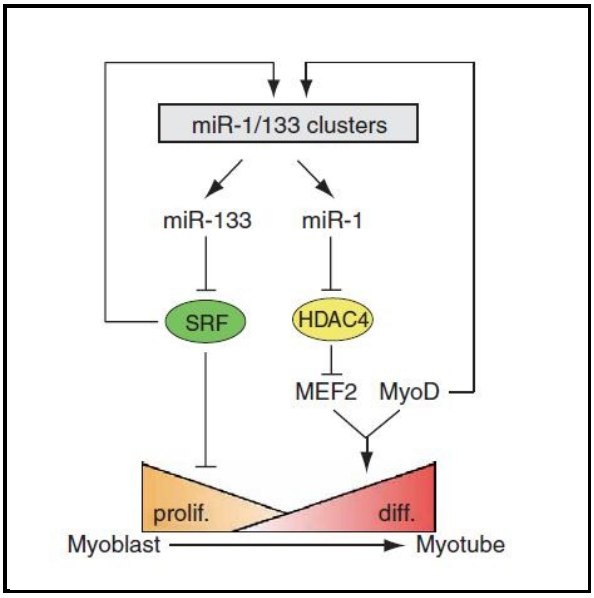


Figure 33: Model of miR-1 and miR-133 mediated regulation of skeletal muscle proliferation and differentiation. (*Chen et al 2006*)

1.10. Myogenic Regulatory Factors Induce Myogenic Genes and Ectopic MiRNA Expression

In a study to see if muscle specific miRNA can be induced in vivo, each of the members of the chicken MRF family – MyoD, Myogenin, Myf-5 and MRF-4 were cloned into pCAβ-IRES-GFP and electroporated into the neuraltube of the embryo. Expression of both endogenous and ectopic MRFs and muscle specific microRNAs (miR-1 and miR-206) were then visualised by whole mount *in situ* hybridisation to see if and how the expression patterns were affected.

1.10.1. pCAβ-Myf-5-GFP

Electroporation of pCAβ-Myf-5-GFP in the neuraltube led to an ectopic expression of MyoD, Myogenin and MRF-4 in the neural tissue. It also induced ectopic expression of miR-1 and miR-206 in the neuraltube. This can be seen in figure 34 (*Sweetman et al 2008*).

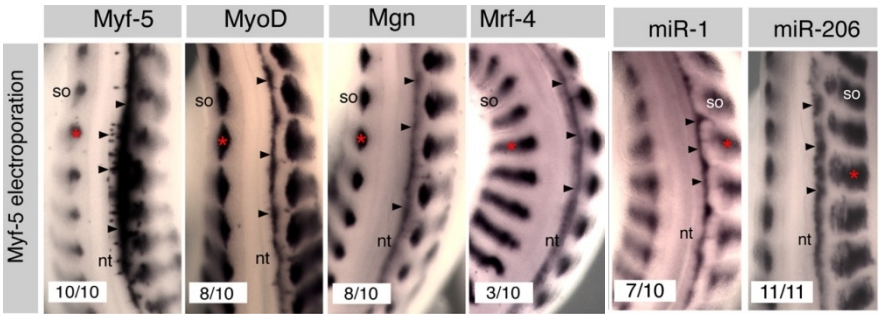


Figure 34: Expression of ectopic Myf-5 in the neural tube induces muscle specific genes and miRNA expression. (*Adapted from Sweetman et al 2008*)

1.10.2. pCAβ-Myo-D-GFP

Electroporation of pCAβ-Myo-D-GFP into the neuraltube led to an ectopic expression of Myogenin in the neural tissue. It also induced ectopic expression of miR-206 in the neuraltube. This can be seen in figure 35. Ectopic expression of Myf5, MRF4 and miR-1 were not seen in this case. (*Sweetman et al 2008*).

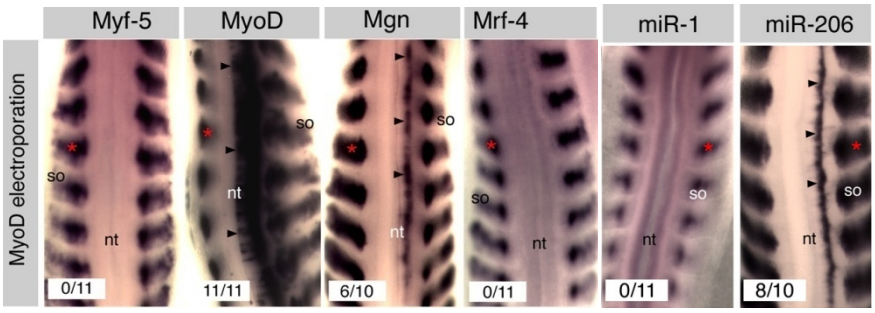


Figure 35: Expression of ectopic Myo-D in the neural tube induces some muscle specific genes and miRNA expression. (*Adapted from Sweetman et al 2008*)

1.10.3. pCAβ-Myogenin-GFP

Electroporation of pCAβ-Myogenin-GFP into the neuraltube led to an ectopic expression of MyoD, and MRF-4 in the neural tissue. It also induced ectopic expression of miR-1 and miR-206. This can be seen in figure 36. Expression of ectopic Myf5 was not seen in this instance. (*Sweetman et al 2008*)

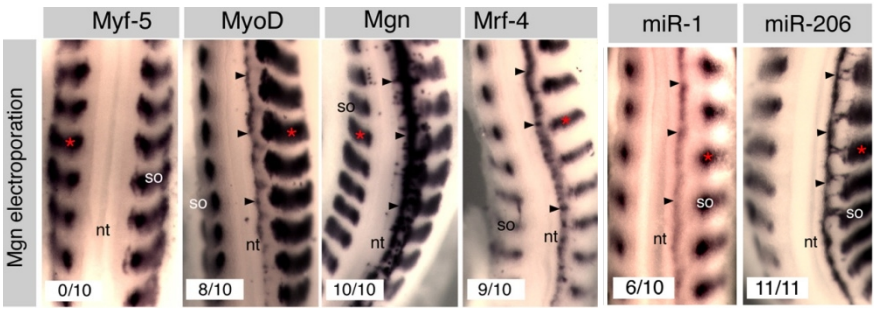


Figure 36: Expression of ectopic Myogenin in the neural tube induces some muscle specific genes and miRNA expression. (*Adapted from Sweetman et al 2008*)

1.10.4. pCAβ-MRF-4-GFP

Electroporation of pCAβ-MRF-4-GFP led to an expression of Myogeninin the neural tissue. It also induced ectopic expression of MiR-206. This can be seen in figure 37. Ectopic expression of Myf5, MyoD and miR-1 could not be seen in this case. (*Sweetman et al 2008*).

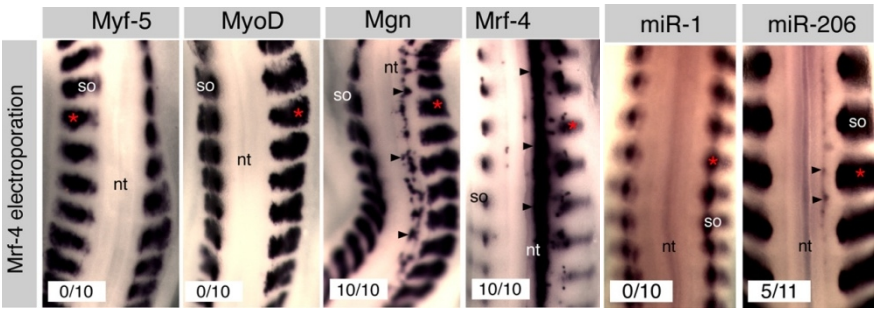


Figure 37: Expression of ectopic Myogenin in the neural tube induces some muscle specific genes and miRNAs. (*Adapted from Sweetman et al 2008*)

As discussed earlier, the limb and body muscles show several differences. In this project we tried to investigate if the MRFs played a similar role in inducing the muscle specific miR-1, miR-206 and also miR-133 in the limb musculature.