

RESULTS AND DISCUSSION

4. Expression of Muscle Specific MiRNAs in the Distal Forelimb

4.1.1 Introduction

As discussed earlier, miRNA-1/206 function to enhance the myogenic differentiation program while miRNA-133 has a role in maintaining the myoblasts in a proliferating state. Correct balance between the temporal and spatial expression and function of these miRNAs play a crucial role in ensuring proper development of skeletal muscles.

We have already encountered that ectopic expression of MRFs can induce ectopic miRNA expression in the neuraltube. To study the effect of these MRFs on muscle specific miRNA expression in the limb muscles, first we need to look at their endogenous expression pattern. Limb formation starts in the chick embryo at embryonic stage HH14, with muscle cells migrating to their required site around embryonic stage HH15-19. The cells then undergo proliferation, differentiation and splitting events. By embryonic stage HH 35 all individual muscle masses can be identified in the chick limbs.

To study the endogenous expression of MRFs, miRs-1/206 and miR-133 in the limb muscle tissues, HH 35 chick embryos were investigated. The embryos were subjected to whole mount *in situ* hybridisation process with either an antisense RNA against one of the MRF transcripts or one of the three double DIG labelled LNA probes (Exicon) that detect miRNA-1, miRNA-206 or miRNA-133.

Once the staining was complete and clearly visible, the forelimbs and hind limbs were dissected from the body, embedded and cryosectioned to generate transverse sections. The sections were then observed for specific muscle tissue staining.

4.1.2 Distal Forelimb

The forelimb can be divided into the Autopod, Zeugopod and Stylopod. This can be seen in figure 38. To study the expression pattern of MRFs and muscle specific miRNAs in the chick limb, we separated and observed the expression patterns of these genes in the distal forelimb which includes the Zeugopod and Autopod.

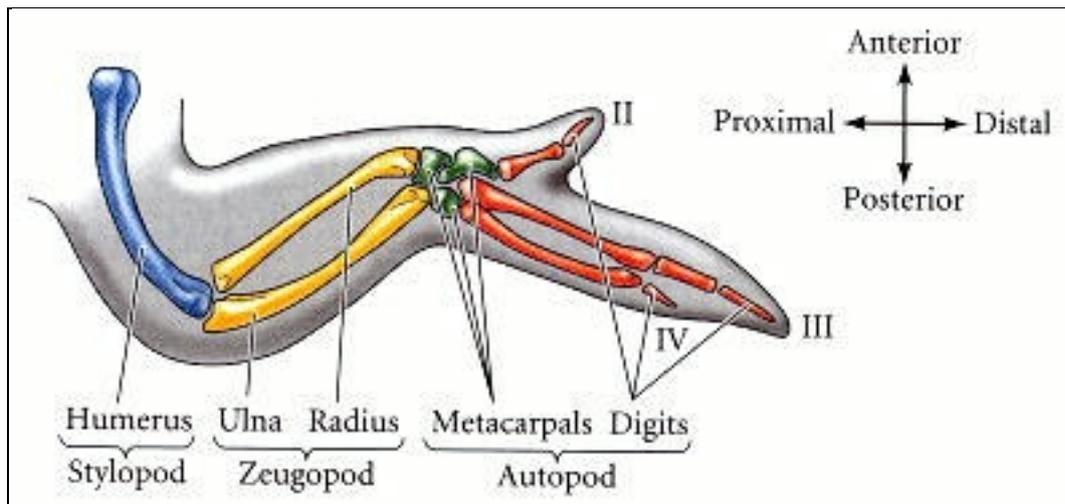


Figure 38: A diagram on the forelimb showing the Stylopod, Zeugopod and Autopod. *(Adapted from Gilbert 2000)*

Distal Forelimb (zeugopod and autopod) shows nine-teen (19) distinct individual muscles masses (Zhi *et al.*, 1996):

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis
8. Ulna Metacarpalis Ventralis
9. Flexor Digitorium Profundus
10. Flexor Digitorium Superficialis
11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis
14. Extensor Medius Longus
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
17. Extensor Digitorium Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

These muscles are shown in Figure 39, as they appear in the whole limb and transverse sections. The numbers corresponding to each muscle are used to annotate the figures in this chapter. Arrangement of all nineteen muscles can be seen by sectioning along A, B and C.

To study the expression pattern of MRFs and muscle specific miR-1, miR-206 and miR-133, we examined transverse sections obtained from embryos stained for MyoD, miR-206, miR-1 and miR-133 at (locations similar to) A, B and C (as shown in figure 39).

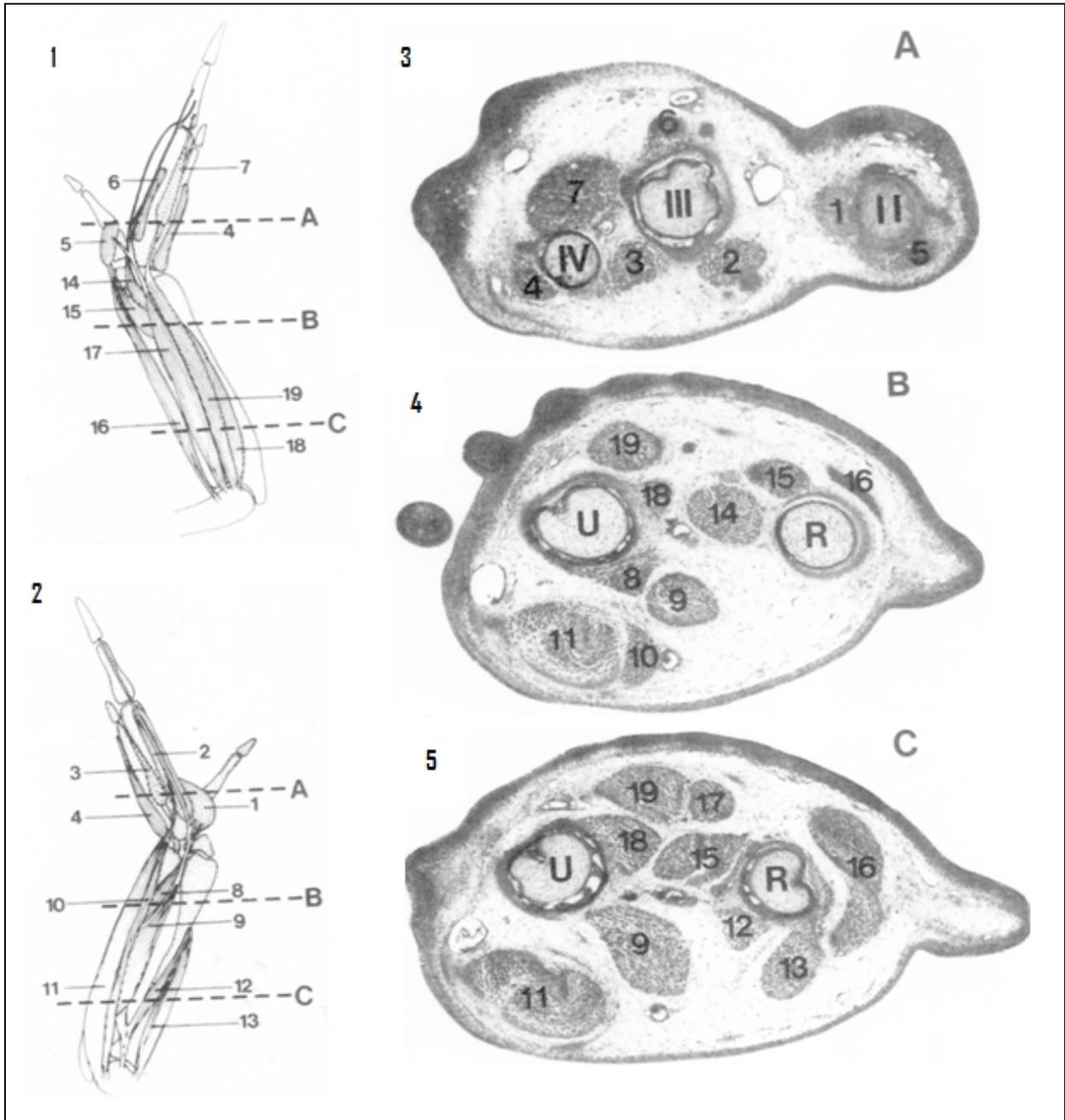


Figure 39: Identity of individual muscles in the distal forelimb (1) dorsal view of the distal limb (2) ventral view of the distal limb (3) transverse section along 'A' (4) transverse section along 'B' (5) transverse section along 'C'. 'A', 'B' and 'C' are marked in panel (1 and 2). (Zhi *et al.*, 1996)

We were also keen to see if the miRNA expressions depicted a fibre type bias. While many species have individual muscle masses made of either fast or slow fibres, chickens tend to contain a mixture of these fibre types in most muscle. We took a closer look at the forelimb zeugopod muscles.

The muscles listed below contained mostly fast fibres.

8. Ulna Metacarpalis Ventralis
9. Flexor Digitorum Profundus
12. Pronator Profundus
13. Pronator Superficialis
17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

The muscles listed below contained mostly slow fibres.

8. Ulna Metacarpalis Ventralis
9. Flexor Digitorum Profundus
11. Flexor Carpi Ulnaris
15. Extensor Indicis Longus
17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

The muscles, whose names appear in both lists seem to contain an equal distribution of fast and slow fibres.

8. Ulna Metacarpalis Ventralis,
9. Flexor Digitorum Profundus,
17. Extensor Digitorum Communis,
18. Anconeus and
19. Extensor Metacarpi Ulnaris

(Li *et al.*, 2004)

4.2 Myo-D

A whole mount *in situ* hybridisation was carried out on chick embryos at embryonic stage HH 35 with an RNA probe against transcripts of MyoD.

MyoD which is a transcription factor required for the onset of the myogenic program is known to be endogenously expressed in all myogenic cells along with other MRFs and is one of the genes responsible for determining their myogenic nature

Stained (for MyoD), limbs were photographed to observe staining of muscles before being embedded and sectioned transversely.

4.2.1. Myo-D Whole Forelimb

In this section we take a look at muscles that are visible in the forelimbs stained for MyoD.

In figure 40, we can see the limbs generated (dorsal and ventral view) once the whole mount *in situ* procedure (with RNA probe against MyoD) was completed. Distinct individual muscles can be identified from the staining. However, due to spatial arrangement of these muscles, overlap makes it difficult to distinguish all individual muscles precisely.

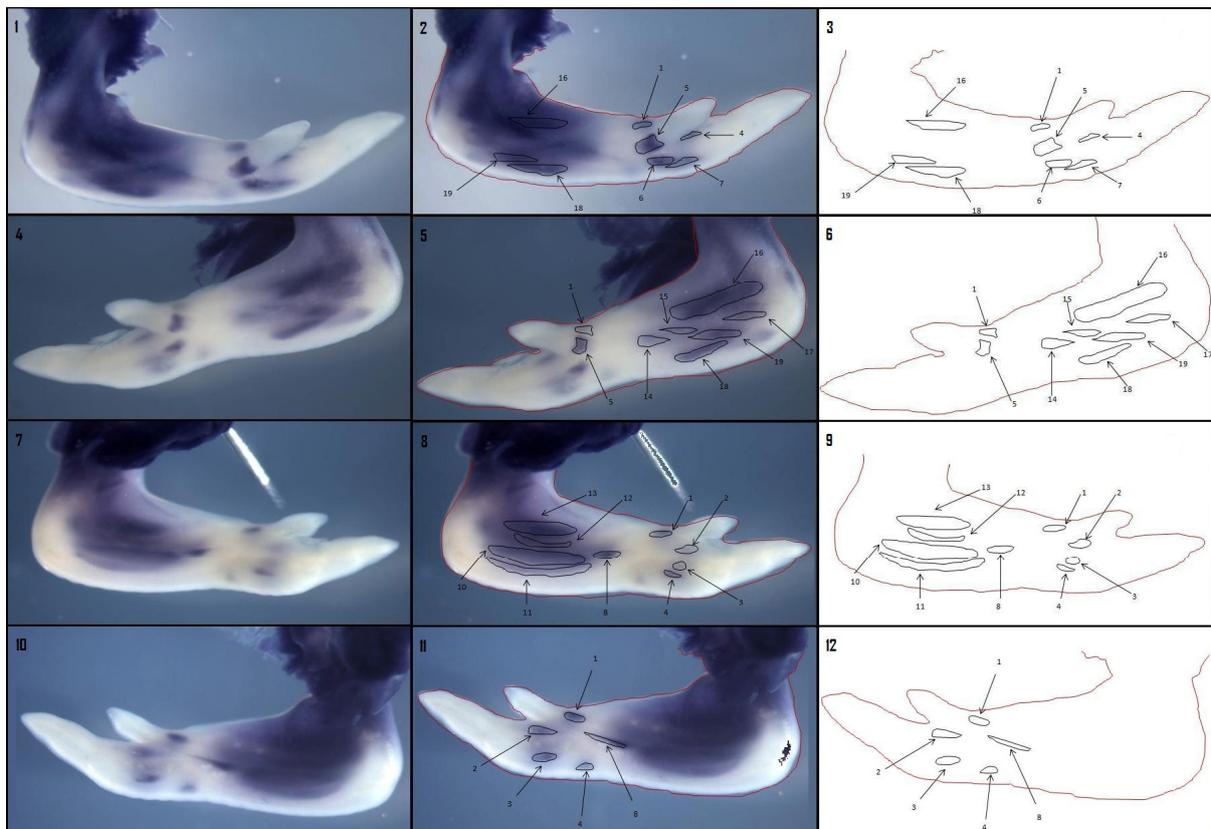


Figure 40: Forelimb from embryos at HH35, stained for MyoD. **(1-3)** Dorsal side of the right wing **(4-6)** Dorsal side of the left wing **(7-9)** Ventral side of the left wing **(10-12)** Ventral side of the right wing.

Panel (1) shows the dorsal side of the right wing, panel (2) shows the same wing with the individual muscles outlined; whilst panel (3) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (2) and (3), we can see the muscles:

1. Flexor Indicis and Abductor Indicis
4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis
16. Extensor Metacarpi Radialis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (4) shows the dorsal side of the left wing, panel (5) shows the same wing with the individual muscles outlined; whilst panel (6) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (5) and (6), we can see the muscles:

1. Flexor Indicis and Abductor Indicis
5. Extensor Indicis Brevis et. Abductor Indicis
14. Extensor Medius Longus
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
17. Extensor Digitorium Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (7) shows the ventral side of the left wing, panel (8) shows the same wing with the individual muscles outlined; whilst panel (9) diagrammatically shows the outline and position of the muscles in the same wing. In panel (8) and (9), we can see the muscles:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
8. Ulna Metacarpalis Ventralis
10. Flexor Digitorium Superficialis

11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis

Panel (10) shows the ventral side of the right wing, panel (11) shows the same wing with the individual muscles outlined; whilst panel (13) diagrammatically shows the outline and position of the muscles in the same wing. In panel (11) and (12), we can see the muscles:

5. Flexor Indicis and Abductor Indicis
6. Abductor Medius
7. Interosseus Medius
8. Flexor Digiti IV
9. Ulna Metacarpalis Ventralis

Hence, all nineteen (19) muscles are visible in this the MyoD stained distal forelimbs.

4.2.2. Myo-D Transverse Sections

In this section we take a look at muscles that are visible in the transverse sections of the limbs stained for MyoD.

In figure 41, we can see the transverse sections generated from the MyoD stained limbs.

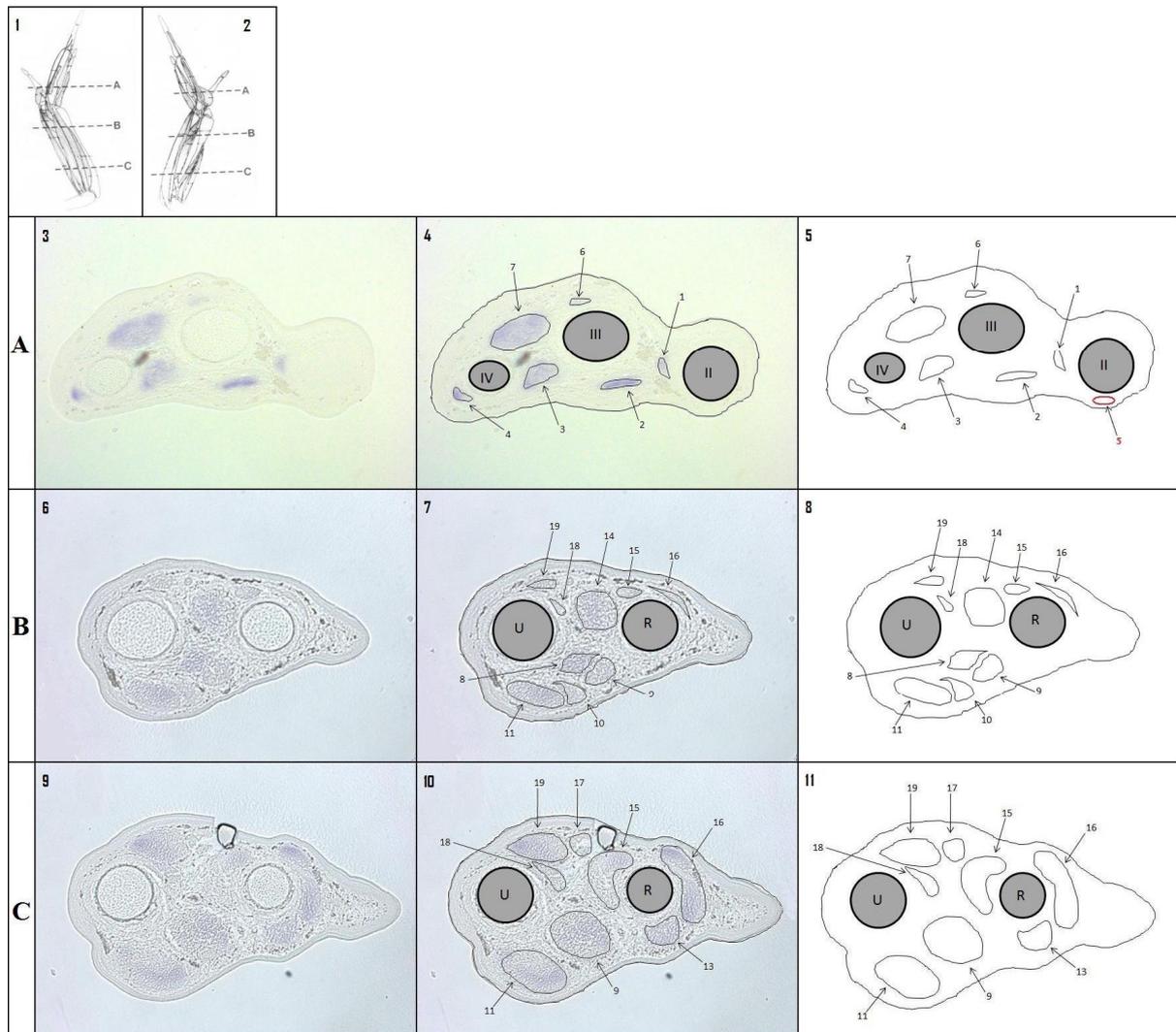


Figure 41: Transverse sections from distal forelimb (stained for MyoD). (1) dorsal and (2) ventral view of forelimb with relative locations from which the sections were obtained along the anterior-posterior axis (A, B and C) marked out (Zhi *et al.*, 1996). (3-5) Section obtained at A, (6-8) Section obtained at B and (9-11) Section obtained at C.

Panel (3) shows transverse section obtained at position 'A' from MyoD stained forelimb tissue, panel (4) shows the same section with the individual muscles outlined, whilst panel (5) diagrammatically shows the outline and position of the individual muscles in the section.

From panel (4) and (5), we can see:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
6. Extensor Medius Brevis
7. Interosseus Dorsalis

The muscle, 5. Extensor Indicis Brevis et. Abductor Indicis is shown in red in panel 5. Although it was expected to be seen at that position in the section, it could not be detected. This could be due to tissue damage caused during cryosectioning or due to the exact position of the section being slightly more anterior to the marked position of A.

Panel (6) shows transverse section obtained at position 'B' from MyoD stained forelimb tissue, panel (7) shows the same section with the individual muscles outlined, whilst panel (8) diagrammatically shows the outline and position of the individual muscles in the section.

From panel (7) and (8), we can see:

8. Ulna Metacarpalis Ventralis
9. Flexor Digitorium Profundus
10. Flexor Digitorium Superficialis
11. Flexor Carpi Ulnaris
14. Extensor Medius Longus
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (9) shows transverse section obtained at position 'C' from MyoD stained forelimb tissue, panel (10) shows the same section with the individual muscles outlined, whilst panel (11) diagrammatically shows the outline and position of the individual muscles in the section.

From panel (10) and (11), we can see:

9. Flexor Digitorum Profundus
11. Flexor Carpi Ulnaris
13. Pronator Superficialis
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Hence, eighteen (18) muscles are visible in the transverse sections obtained from the MyoD stained distal forelimbs.

4.2.3. Myo-D Conclusion

The combined observations made in the (whole) forelimbs (Figure 40) and transverse sections (Figure 41) suggest all nineteen (19) muscles express MyoD (as expected) It also suggest that the individual muscles can be distinguished and visualised (once stained using a whole mount in situ procedure) with the naked eye, without further processing.

4.3. MiRNA-206

A whole mount *in situ* hybridisation was carried out on chick embryos at embryonic stage HH 35 with an LNA probe against microRNA, miR-206.

MiR-206 is expressed endogenously only in skeletal muscle cells. They function to post transcriptionally enhance the differentiation process of myocytes whilst inhibiting their proliferation.

Stained (for miR-206), forelimbs were photographed to observe staining of individual muscles before being embedded and sectioned transversely.

4.3.1. MiRNA-206 Whole Forelimb

In this section we take a look at muscles that are visible in the forelimbs stained for miRNA-206. The whole mount *in situ* procedure was carried out to see how many of the nineteen (19) individual muscles show endogenous Mir-206 expression. We also wanted to note if the miRNA expression was (slow or fast) fibre type specific.

In figure 42, we can see the limbs generated (dorsal and ventral view) once the whole mount *in situ* procedure (with LNA probe against MiR-206) was completed. Distinct muscles could be identified from the staining

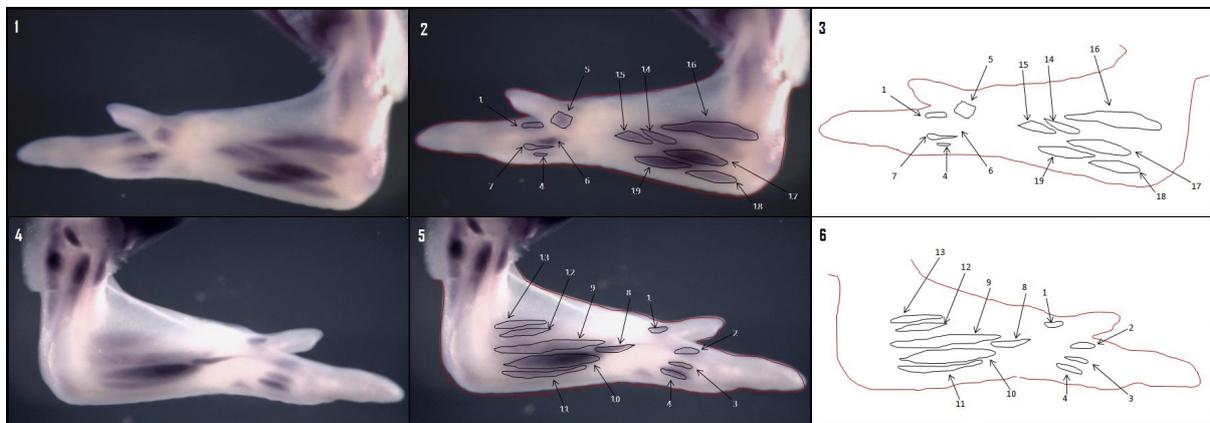


Figure 42: Forelimb from embryos at HH35, stained for MiR-206. **(1-3)** Dorsal side of the left wing **(4-6)** Ventral side of the left wing.

Panel (1) shows the dorsal side of the left wing, panel (2) shows the same wing with the individual muscles outlined whilst panel (3) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (2) and (3), we can see the muscles:

1. Flexor Indicis and Abductor Indicis
4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis
14. Extensor Medius Longus
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (4) shows the ventral side of the left wing, panel (5) shows the same wing with the individual muscles outlined whilst panel (6) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (5) and (6), we can see the muscles:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
8. Ulna Metacarpalis Ventralis
9. Flexor Digitorum Profundus
10. Flexor Digitorum Superficialis
11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis

Hence, all nineteen (19) muscles are visible in the mir-206 stained distal forelimbs.

4.3.2. MiRNA-206 Transverse Sections

In this section we take a look at muscles that are visible in the transverse sections of the limbs stained for MiR-206.

In figure 43, we can see the transverse sections generated from the MiR-206 stained limbs.

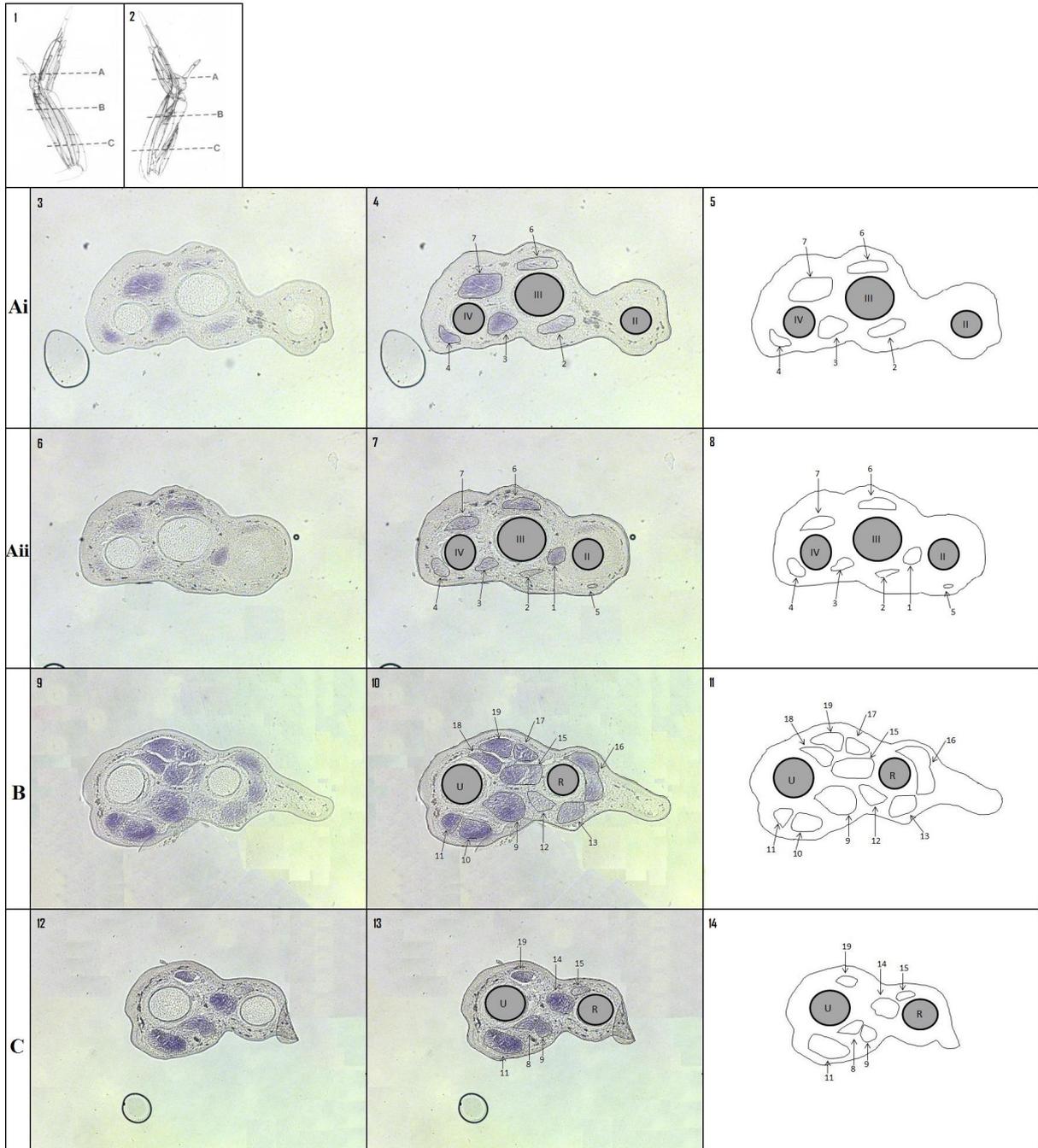


Figure 43: Transverse sections from distal forelimb (stained for miRNA-206). **(1)** Dorsal and **(2)** ventral view of forelimb with relative locations from which the sections were obtained along the anterior-posterior axis (A, B and C) marked out (Zhi et al., 1996). **(3-8)** Sections obtained at A **(9-11)** Section obtained at B and **(12-14)** Section obtained at C.

Panel (3) shows transverse section obtained at position 'A' (Ai) from MiR-206 stained forelimb tissue, panel (4) shows the same section with the individual muscles outlined, whilst panel (5) diagrammatically shows the outline and position of the individual muscles in the section. From panel (4) and (5), we can see:

2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
6. Extensor Medius Brevis
7. Interosseus Dorsalis

Panel (6) shows transverse section also obtained at position 'A' (Aii) from MiR-206 stained forelimb tissue, panel (7) shows the same section with the individual muscles outlined, whilst panel (8) diagrammatically shows the outline and position of the individual muscles in the section. From panel (7) and (8), we can see:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis

Panel (9) shows transverse section obtained at position 'B' from MiR-206 stained forelimb tissue, panel (10) diagrammatically shows the same section with the individual muscles outlined, whilst panel (11) shows the outline and position of the individual muscles in the section. From panel (10) and (11), we can see:

9. Flexor Digitorum Profundus
10. Flexor Digitorum Superficialis
11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis

17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (12) shows transverse section obtained at position 'C' from MiR-206 stained forelimb tissue, panel (13) shows the same section with the individual muscles outlined, whilst panel (14) shows the outline and position of the individual muscles in the section. From panel (13) and (14), we can see:

8. Ulna Metacarpalis Ventralis
9. Flexor Digitorum Profundus
11. Flexor Carpi Ulnaris
14. Extensor Medius Longus
15. Extensor Indicis Longus
19. Extensor Metacarpi Ulnaris

Hence, all nineteen (19) muscles are visible in this the transverse sections obtained from the Mir-206 stained distal forelimbs.

4.3.3. MiRNA-206 Conclusion

The combined observation made in the whole limbs (Figure 42) and transverse sections (Figure 43) suggest all nineteen (19) muscles express miRNA-206 at this stage of the embryos development in the proximal forelimb muscles. It shows no distinction in endogenous expression between (fast or slow) muscle fibre types.

4.4. MiRNA-1

A whole mount *in situ* hybridisation was carried out on chick embryos at embryonic stage HH 35 with an LNA probe against microRNA, miR-1.

MiR-1 is expressed endogenously in skeletal and cardiac muscle cells. They function to post transcriptionally enhance the differentiation process of myocytes whilst inhibiting their proliferation.

Stained (for miR-1), forelimbs were photographed to observe staining of individual muscles before being embedded and sectioned transversely.

4.4.1. MiRNA-1 Whole Forelimb

In this section we take a look at muscles that are visible in the (whole) forelimbs stained for miRNA-1. The whole mount *in situ* procedure was carried out to see how many of the nineteen (19) individual muscles show endogenous Mir-1 expression. We also wanted to note if the miRNA expression was (slow or fast) fibre type specific.

In figure 44, we can see the limbs generated (dorsal and ventral view) once the whole mount *in situ* procedure (with LNA probe against MiR-1) was completed. Distinct muscles could be identified from the staining

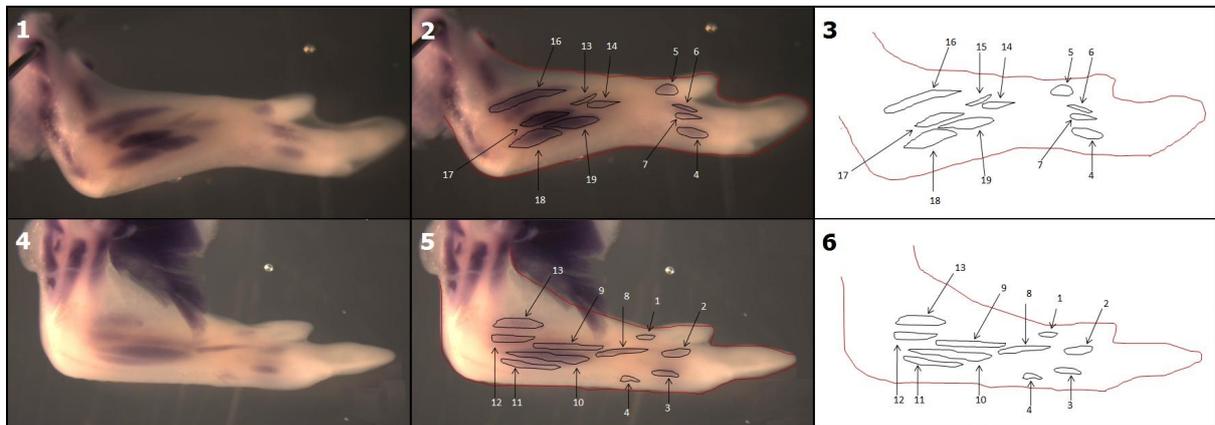


Figure 44: Forelimb from embryos at HH35, stained for MiR-1. **(1-3)** Dorsal side of the right wing **(4-6)** Ventral side of the left wing.

Panel (1) shows the dorsal side of the right wing, panel (2) shows the same wing with the individual muscles outlined whilst panel (3) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (2) and (3), we can see the muscles:

4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis
14. Extensor Medius Longus
15. Extensor Indicis Longus

16. Extensor Metacarpi Radialis
17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (4) shows the ventral side of the left wing, panel (5) shows the same wing with the individual muscles outlined whilst panel (6) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (5) and (6), we can see the muscles:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
8. Ulna Metacarpalis Ventralis
9. Flexor Digitorum Profundus
10. Flexor Digitorum Superficialis
11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis

Hence, all nineteen muscles are visible in the miR-1 stained distal forelimbs.

4.4.2. MiRNA-1 Transverse Sections

In this section we take a look at muscles that are visible in the transverse sections of the limbs stained for MiR-1.

In figure 45, we can see the transverse sections generated from the miR-1 stained limbs.

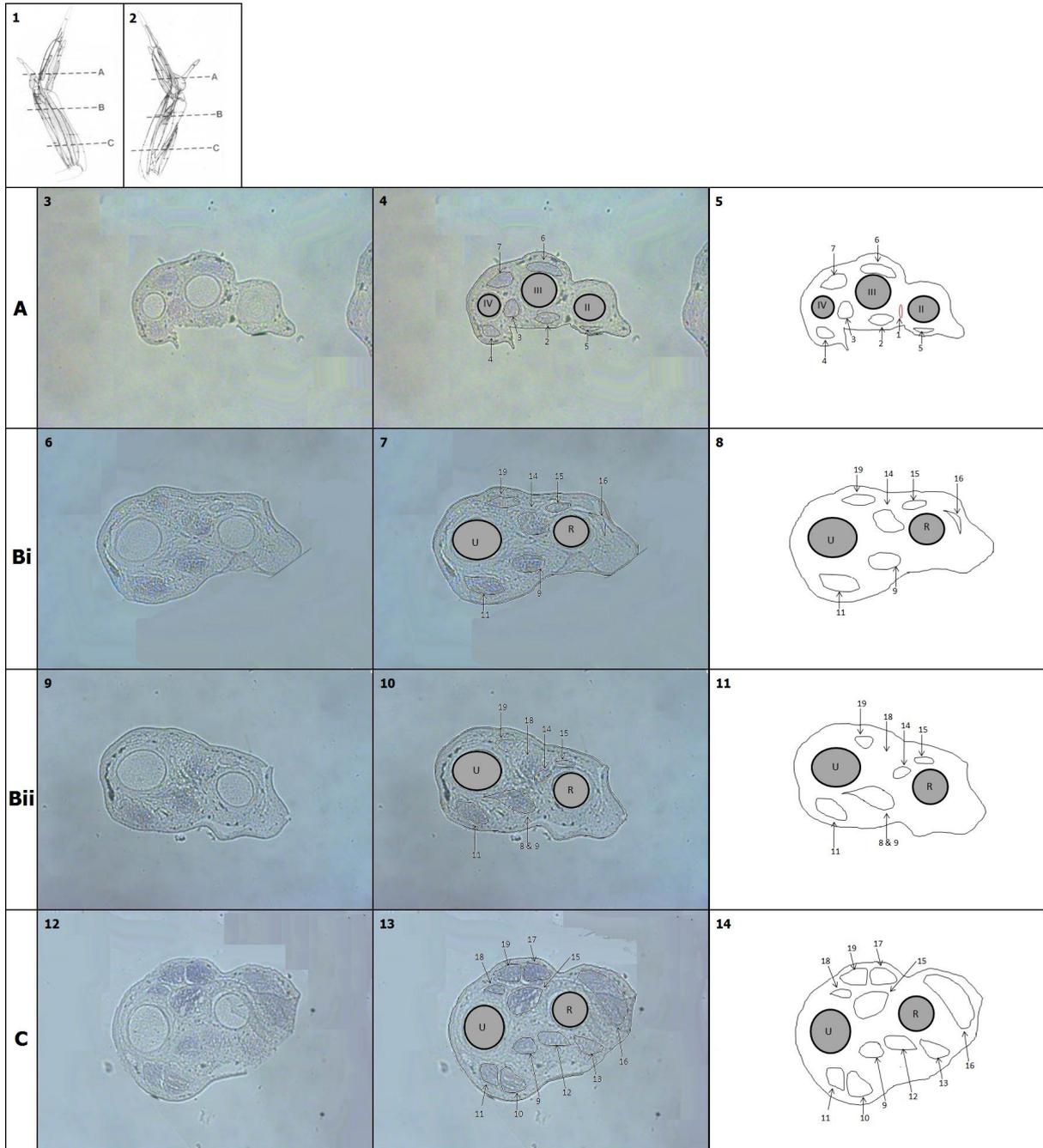


Figure 45: Transverse sections of distal forelimb tissue (stained for miRNA-1). **(1)** Dorsal and **(2)** ventral view of forelimb with relative locations from which the sections were obtained along the anterior-posterior axis (A, B and C) marked out (Zhi et al., 1996). **(3-5)** Section obtained at A **(6-11)** Sections obtained at B and **(12-14)** Section obtained at C.

Panel (3) shows transverse section obtained at position 'A' from MiR-1 stained forelimb tissue, panel (4) diagrammatically shows the same section with the individual muscles outlined, whilst panel (5) shows the outline and position of the individual muscles in the section. From panel (4) and (5), we can see:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis

Panel (6) shows transverse section also obtained at position 'B' (Bi) from MiR-1 stained forelimb tissue, panel (7) shows the same section with the individual muscles outlined, whilst panel (8) diagrammatically shows the outline and position of the individual muscles in the section. From panel (7) and (8), we can see:

9. Flexor Digitorium Profundus
11. Flexor Carpi Ulnaris
14. Extensor Medius Longus
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
19. Extensor Metacarpi Ulnaris

Panel (9) shows transverse section also obtained at position 'B' (Bii) from MiR-1 stained forelimb tissue, panel (10) shows the same section with the individual muscles outlined, whilst panel (11) diagrammatically shows the outline and position of the individual muscles in the section. From panel (10) and (11), we can see:

8. Ulna Metacarpalis Ventralis
9. Flexor Digitorium Profundus
11. Flexor Carpi Ulnaris
14. Extensor Medius Longus
15. Extensor Indicis Longus

18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (12) shows transverse section obtained at position 'C' from MiR-1 stained forelimb tissue, panel (13) shows the same section with the individual muscles outlined, whilst panel (14) diagrammatically shows the outline and position of the individual muscles in the section.

From panel (13) and (14), we can see:

9. Flexor Digitorum Profundus
10. Flexor Digitorum Superficialis
11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Hence, all nine-teen muscles are visible in the transverse sections obtained from the mir-1 stained distal forelimbs.

4.4.3. MiRNA-1 Conclusion

The combined observation made in the forelimbs (Figure 44) and transverse sections (Figure 45) suggest all nineteen (19) muscles express miRNA-1 at this stage of the embryos development in the proximal forelimb muscles. It shows no distinction in endogenous expression between (fast or slow) muscle fibre types.

4.5. MiRNA-133

A whole mount *in situ* hybridisation was carried out on chick embryos at embryonic stage HH 35 with an LNA probe against microRNA, miR-133.

MiR-133 is expressed endogenously in skeletal and cardiac muscle cells. They function to post transcriptionally enhance the proliferation of myocytes whilst inhibiting their differentiation.

Stained (for miR-133), forelimbs were photographed to observe staining of individual muscles before being embedded and sectioned transversely.

4.5.1. MiRNA-133 Whole Forelimb

In this section we take a look at muscles that are visible in the forelimbs stained for miRNA-133. The whole mount *in situ* procedure was carried out to see how many of the nineteen (19) individual muscles show endogenous mir-133 expression. We also wanted to note if the miRNA expression was (slow or fast) fibre type specific.

In figure 46, we can see the limbs generated (dorsal and ventral view) once the whole mount *in situ* procedure (with LNA probe against MiR-133) was completed. Distinct muscles could be identified from the staining.

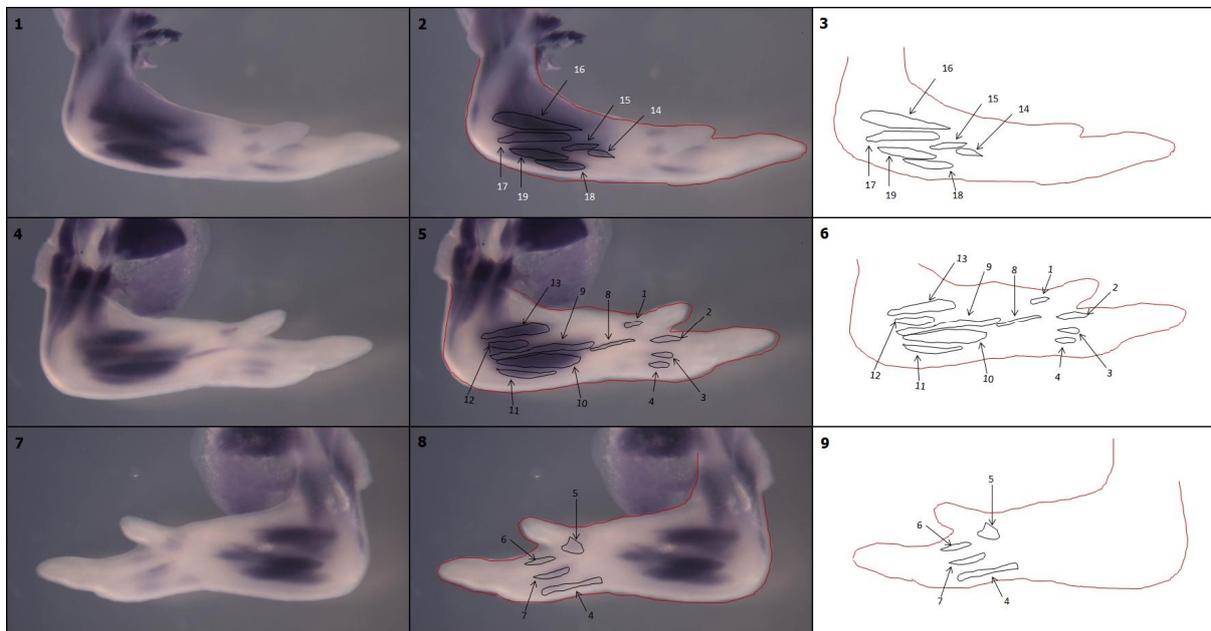


Figure 46: Forelimbs from embryos at HH35, stained for MiR-133. **(1-3)** Dorsal side of the right wing **(4-6)** Ventral side of the left wing. **(7-9)** dorsal side of the left wing

Panel (1) shows the dorsal side of the right wing, panel (2) shows the same wing with the individual muscles outlined whilst panel (3) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (2) and (3), we can see the muscles:

- 14. Extensor Medius Longus
- 15. Extensor Indicis Longus

16. Extensor Metacarpi Radialis
17. Extensor Digitorum Communis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (4) shows the ventral side of the left wing, panel (5) shows the same wing with the individual muscles outlined whilst panel (6) diagrammatically shows the outline and position of the individual muscles in the same wing. In panel (5) and (6), we can see the muscles:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
8. Ulna Metacarpalis Ventralis
9. Flexor Digitorum Profundus
10. Flexor Digitorum Superficialis
11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis

Panel (7) shows the dorsal side of the left wing, panel (8) shows the same wing with the individual muscles outlined whilst panel (9) shows the outline and position of the individual muscles in the same wing. In panel 8 and 9, we can see the muscles:

4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis

Hence, all nineteen muscles are visible in the miR-133 stained distal forelimbs.

4.5.2. MiRNA-133 Transverse Sections

In this section we take a look at muscles that are visible in the transverse sections of the limbs stained for MiR-133.

In figure 47, we can see the transverse sections generated from the miR-133 stained limbs.

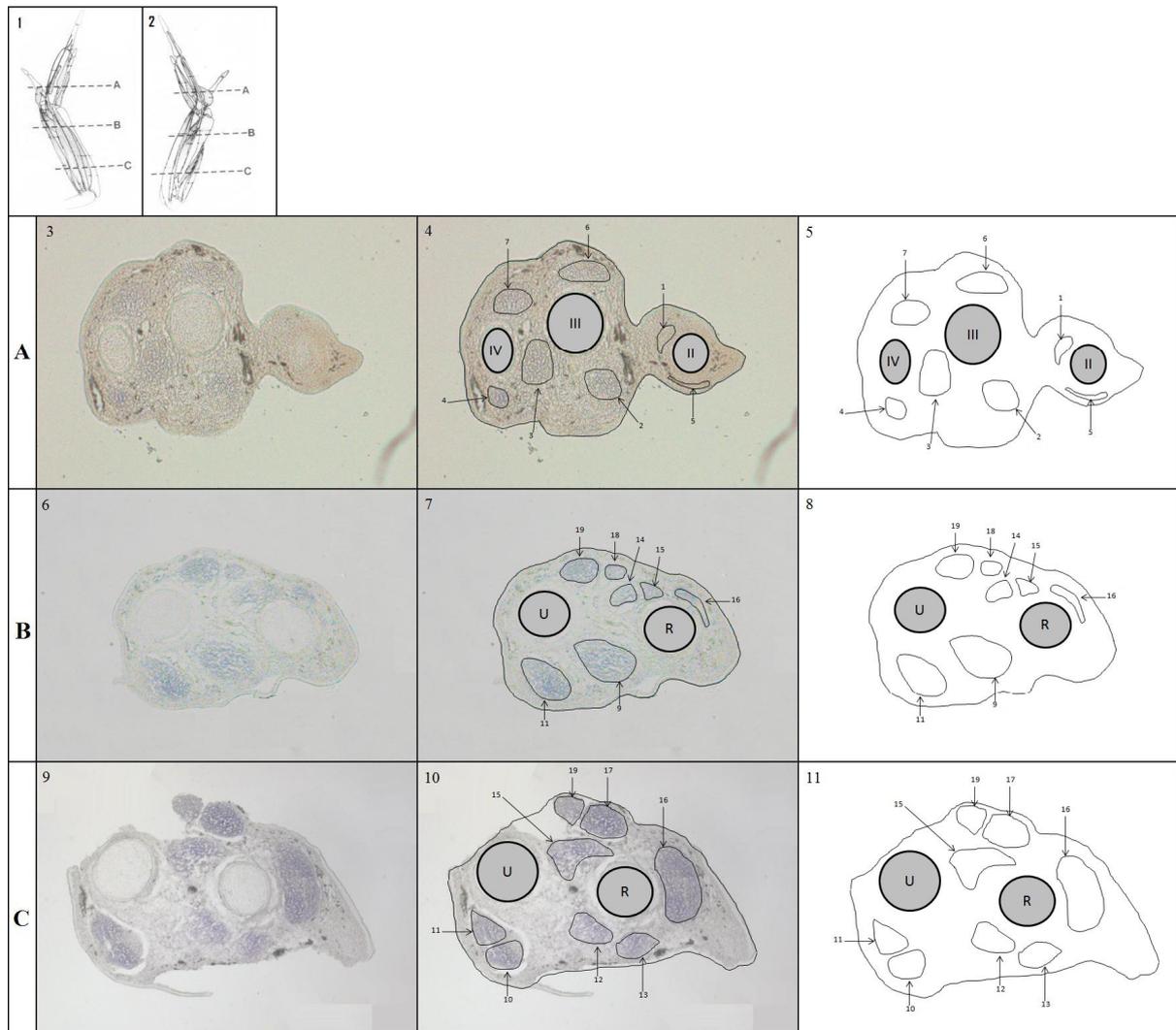


Figure 47: Transverse sections of distal forelimb tissue that is stained for miRNA-133. **(1)** Dorsal and **(2)** ventral view of forelimb with relative locations from which the sections were obtained along the anterior-posterior axis (A, B and C) marked out (Zhi et al., 1996). **(3-5)** Section obtained at A **(6-8)** Section obtained at B and **(9-11)** Section obtained at C.

Panel (3) shows transverse section obtained at position ‘A’ from MiR-133 stained forelimb tissue, panel (4) shows the same section with the individual muscles outlined, whilst panel (5) diagrammatically shows the outline and position of the individual muscles in the section.

From panel (4) and (5), we can see:

1. Flexor Indicis and Abductor Indicis
2. Abductor Medius
3. Interosseus Medius
4. Flexor Digiti IV
5. Extensor Indicis Brevis et. Abductor Indicis
6. Extensor Medius Brevis
7. Interosseus Dorsalis

Panel (6) shows transverse section obtained at position ‘B’ from MiR-133 stained forelimb tissue, panel (7) shows the same section with the individual muscles outlined, whilst panel (8) diagrammatically shows the outline and position of the individual muscles in the section.

From panel (7) and (8), we can see:

9. Flexor Digitorum Profundus
11. Flexor Carpi Ulnaris
14. Extensor Medius Longus
15. Extensor Indicis Longus
16. Extensor Metacarpi Radialis
18. Anconeus
19. Extensor Metacarpi Ulnaris

Panel (9) shows transverse section obtained at position ‘C’ from MiR-206 stained forelimb tissue, panel (10) shows the same section with the individual muscles outlined, whilst panel (11) diagrammatically shows the outline and position of the individual muscles in the section.

From panel (10) and (11), we can see:

10. Flexor Digitorum Superficialis
11. Flexor Carpi Ulnaris
12. Pronator Profundus
13. Pronator Superficialis
15. Extensor Indicis Longus

16. Extensor Metacarpi Radialis
17. Extensor Digitorum Communis
19. Extensor Metacarpi Ulnaris

Hence, eighteen (18) muscles are visible in this the transverse sections obtained from the miR-133 stained distal forelimbs.

4.5.3. MiRNA-133 Conclusion

The combined observation made in the whole limbs (Figure 46) and transverse sections (Figure 47) suggest all nineteen (19) muscles express miRNA-133 at this stage of the embryos development in the proximal forelimb muscles. It shows no distinction in endogenous expression between (fast or slow) muscle fibre types.

4.6. Summary

In this chapter we have looked at the endogenous expression of MRF- MyoD and muscle specific miRNAs - miR-1, miR-206 and miR-133 in the chick distal forelimb muscles.

Whole mount *in situ* hybridisation was carried out on wild type chick embryos at embryonic stage HH35, with either the RNA probe for MyoD or with one of three LNA probes for the muscle specific miRNAs (MiR-1, MiR-206 and MiR-133). Once the staining was visible, the limbs were dissected. We observed and noted which of the nineteen muscles in the distal forelimb (autopod and zeugopod) were stained, therefore, endogenously expressing the MRF of the miRNAs. Following that, we embedded sectioned the distal forelimbs to generate transverse sections. These transverse sections were further studied for a more detailed observation of the endogenous expression of these genes.

From our analysis (detailed in sections 4.2), we can conclude all nineteen (19) muscles in the distal forelimb showed MyoD expression as expected, since MyoD is known to be present in all muscle cells. From this we can establish all nineteen (19) muscles of the distal forelimb that we investigated could be visualised by observing transverse sections of the limb that has undergone a whole mount *in situ* hybridisation procedure

We also noted (detailed in section 4.3-4.5), all three muscle specific miRNAs – MiR-1, MiR-206 and MiR-133 were endogenously expressed in all nineteen (19) muscles of the distal forelimb in the chick embryo at embryonic stage HH35. The staining was also present in both slow and fast muscle fibres without any discrimination.