5. Regulation of MiRNA-206 by MRFs

5.1. Testing RCAS-MRFs

Gallus gallus (White Leghorn) embryos were used for all the experiments described in this chapter.

RCAS-MRF virus particles (existing stock, concentrated and stored at -80° C) were injected into the chick embryo to verify the efficiency of the infections. The viral (MRF) transcripts were then detected using the method of whole mount *in situ* hybridisation, which also allowed us to verify the quality of the RNA probes used in the method.

5.1.1. Testing RCAS-MyoD

Chick embryos at stage HH14-15 were injected with concentrated viral particles of RCAS-MyoD in the coelom on the right side (of the embryo). As the limbs develop from the lateral plate mesoderm, injection of viral particles at this location lead to subsequent infection of all limb tissue, as it developed, on just the injected side (of the embryos). The embryos were then incubated and allowed to develop for 48 hours, to embryonic stage HH-25. A whole mount in-situ hybridisation was carried out on these embryos using the chick-MyoD (c-MyoD) antisense RNA probe, to visualise any ectopic expression of the MyoD transcript.

This can be seen in figure 48. Panel (a) shows the injected side of the embryo. Ectopic expression of MyoD can be seen in the forelimb compared to panel (b), which shows the control side of the embryo, (with no ectopic, only endogenous expression of MyoD).

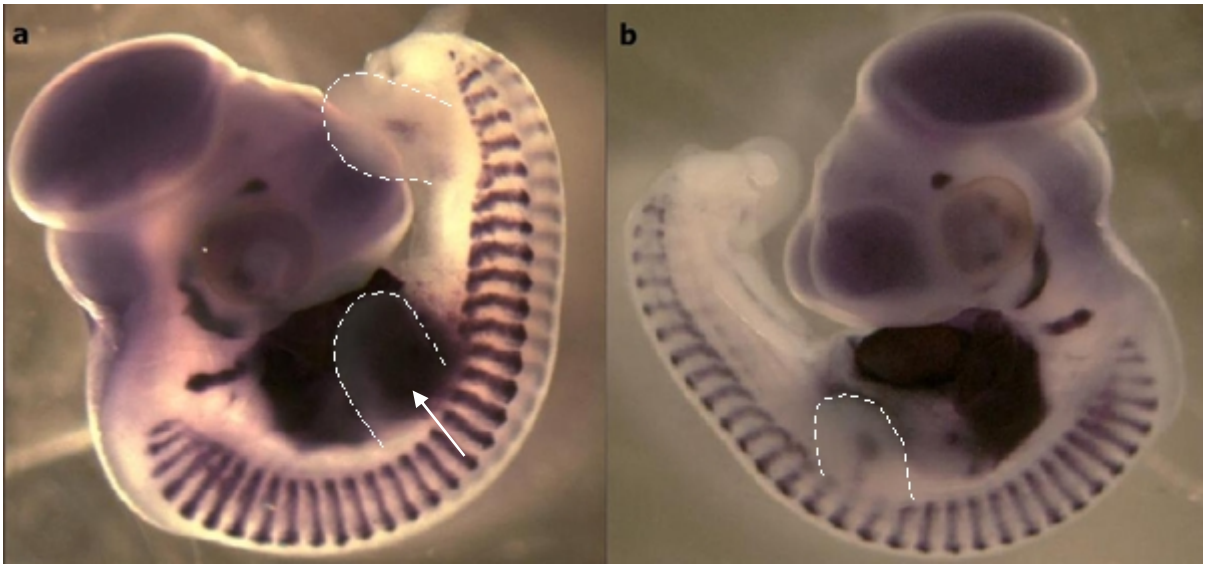


Figure 48: RCAS-MyoD infected chick embryo at embryonic stage HH25, showing **(a)** ectopic MyoD expression in the forelimb and **(b)** only endogenous (no ectopic) expression on the control side.

5.1.2. Testing RCAS-Mgn

Chick embryos at stage HH14-15 were injected with concentrated viral particles of RCAS-Mgn in the coelom on the right side (of the embryo). The embryos were then incubated and allowed to develop for 48 hours, to embryonic stage HH-25. A whole mount *in situ* hybridisation was carried out on these embryos using the chick-Myogenin (c-Mgn) antisense RNA probe, to visualise any ectopic expression of the Myogenin transcript.

The RCAS-Mgn infected and control side of an embryo are shown in figure 49. Panel (a) shows the injected side of the embryo. Ectopic expression of Myogenin can be seen in the forelimb compared to panel (b), which shows the control side of the embryo, (with no ectopic), only endogenous expression of Myogenin.

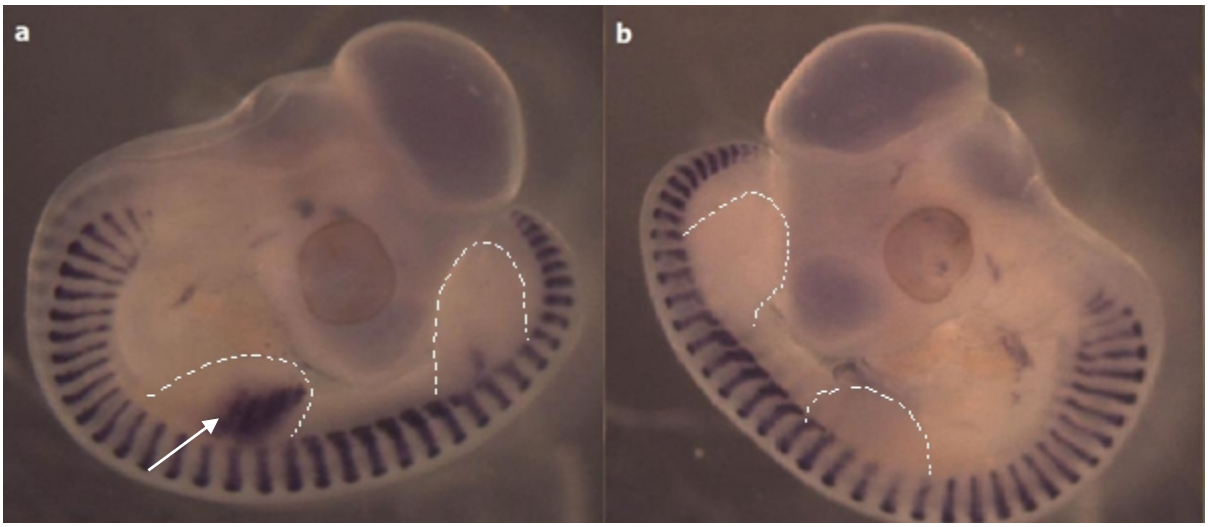


Figure 49: RCAS-Mgn infected chick embryo at embryonic stage HH25, showing **(a)** ectopic Myogenin expression in the forelimb and **(b)** only endogenous (no ectopic) expression on the control side.

5.1.3. Testing RCAS-Myf-5

Chick embryos at stage HH14-15 were injected with concentrated viral particles of RCAS-Myf-5 in the coelom on the right side (of the embryo). The embryos were then incubated and allowed to develop for 48 hours, to embryonic stage HH-25. Whole mount in-situ hybridisation was carried out on these embryos using the chick-Myf-5 (c-Myf-5) antisense RNA probe, to visualise any ectopic expression of the Myf-5 transcript.

The RCAS-Myf-5 infected and control side of an embryo are shown in figure 50. Panel (a) shows the injected side of the embryo. Ectopic expression of Myf-5 can be seen in the forelimb and hindlimb compared to panel (b), which shows the control side of the embryo, with (no ectopic) only the endogenous expression of Myf-5.

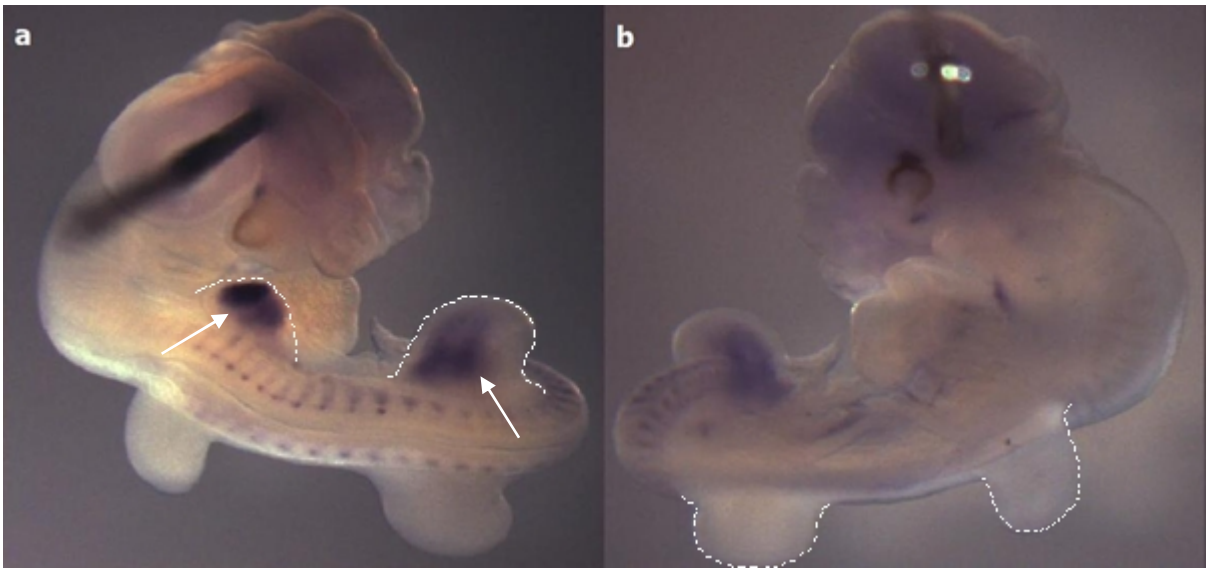


Figure 50: RCAS-Myf-5 infected chick embryo at embryonic stage HH25, showing **(a)** ectopic Myf-5 expression in the forelimb and hindlimb **(b)** only endogenous (no ectopic) expression on the control side.

5.1.4. Testing RCAS-MRF4

Chick embryos at stage HH14-15 were injected with concentrated viral particles of RCAS-MRF4 in the coelom on the right side (of the embryo). The embryos were then incubated and allowed to develop for 48 hours, to embryonic stage HH 25. Whole mount *in situ* hybridisation was carried out on these embryos using the chick-MRF4 (c-MRF4) antisense RNA probe, to visualise any ectopic expression of the MRF4 transcript.

The RCAS-MRF4 infected and control side of an embryo are shown in figure 51. Panel (a) shows the injected side of the embryo. Ectopic expression of MRF4 can be seen in the inter-limb region compared to panel (b), which shows the control side of the embryo, with (no ectopic) only endogenous expression of MRF4.

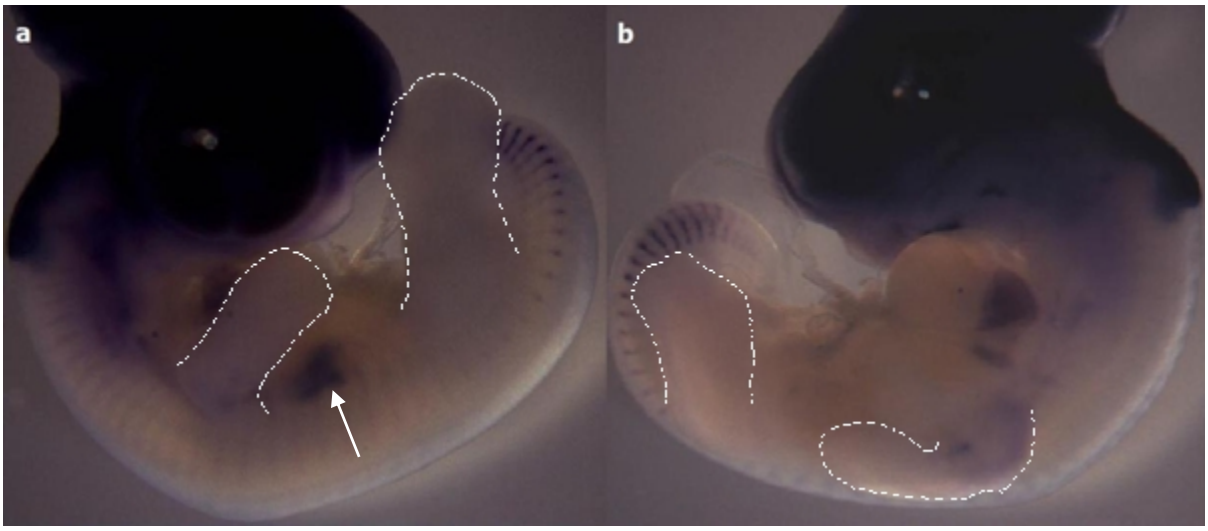


Figure 51: RCAS-MRF4 infected chick embryo at embryonic stage HH25, showing **(a)** ectopic expression in the inter-limb region **(b)** only endogenous (no ectopic) expression on the control side.

5.1.5. Conclusion

In summary, the last four experiments detailed (in section 5.1.1-5.1.4) show that the four sets of embryos showed ectopic expression of the MRFs on the injected sides with no such expression on the control side. Thus, we can conclude that the RCAS viruses were able to infect the limbs where they were being targeted during the injection process and expression for transcripts for each the four different MRFs were seen.

The observations made in the last four experiments also suggested that all four RNA probes (c-MyoD, c-Myogenin, c-Myf-5 and c-MRF4) used in the whole mount *in situ* hybridisation procedures were working efficiently to produce strong visible signals.

Once the quality of the concentrated and frozen RCAS virus (existing stocks; produced in the lab) was verified, DF1 cells were transfected with cDNA containing the RCAS-MRF (RCAS-Mgn and RCAS-MyoD) sequences, which had initially been used to produce the (stored) concentrated viral samples. The RCAS virus was then harvested, concentrated and stored at -80°C. These viral particles were then used in the experiments detailed in the latter part of this chapter.

5.2. Verifying the Quality of the MiR-206 Locked Nucleic Acid (LNA) probes

A locked nucleic acid (LNA) is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra methyl bridge connecting the 2' oxygen and 4' carbon. This is illustrated in figure 52. The LNA nucleosides contain the common bases and are able to form base pairs according to standard Watson-Crick base pairing rules. However, this 'locked' conformation enhances base stacking and backbone pre-organization, which significantly increases the thermal stability of the oligonucleotides. LNA nucleotides are used to increases the sensitivity and specificity various molecular biology techniques based on oligonucleotides. For the *in situ* detection of miRNA the use of LNA is the only efficient method. *(Kaur et al 2006)*

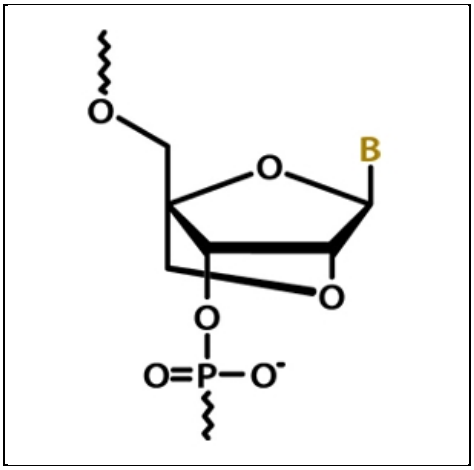


Figure 52: Structure of Locked Nucleic Acid monomer. *(geneworks.com.au)*

To visualise the endogenous MiR-206 expressed in the chick embryo, a whole mount *in situ* hybridisation was carried out on wild type chick embryos at embryonic stage HH 23-24 with a MiR-206 LNA probe.

Figure 53 shows the observed endogenous expression patter of the Mir-206 compared to an image available from a public database. The Mir-206 can be seen in the myotome and somites. This suggests that the LNA probe was able to detect the mature MiR-206 sequence and produce a visible signal.

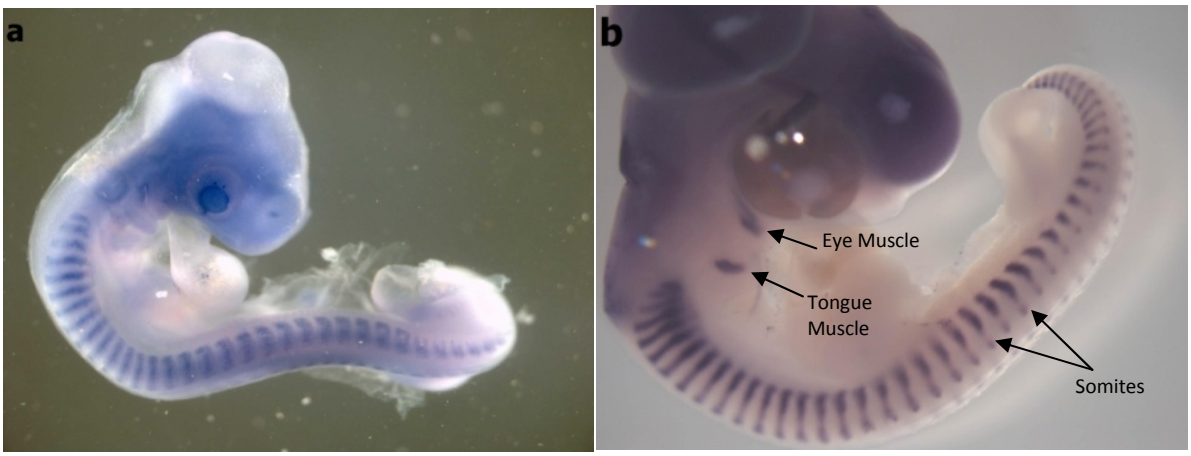


Figure 53: Wild type embryo at embryonic stage **(a)** HH 23 showing endogenous expression of miR-206 *(geisha.arizona.edu)* **(b)** HH 25 showing endogenous expression of miR-206

5.3. Effect of Ectopic Myogenin on MiR-206 Expression

To determine whether ectopic myogenin could induce the expression of miRNA-206 in the developing chick limb, the following method was applied. Chick embryos at stage HH14-15 were injected with concentrated virus particles of RCAS-Mgn in the coelom on the right side (of the embryo). The embryos were incubated until they reached embryonic stage HH 28, to allow for the RCAS-Mgn to propagate and infect all limb tissue (as they developed) on (one) the injected side.

The embryos collected were divided into three parts, each containing 11-12 embryos. The embryos in each of these sections were then subject to a whole mount *in situ* hybridisation procedure with one of the following probes:

- RNA probe to detect RCAS Gag transcripts
- RNA probe to detect Myogenin transcripts
- LNA probe to detect MicroRNA miR-206.

5.3.1.Test for RCAS Gag

To determine if the RCAS virus (RCAS-Mgn) had successfully infected the limb tissue, a whole mount *in situ* hybridisation was carried out on the embryos with the RCAS-Gag probe, which detects the viral Gag transcripts. A positive visible signal in this case allows us to conclude the presence of Gag transcripts in the marked tissue, hence, the presence of active RCAS virus indicating a successful infection.

Seven out of twelve (7/12) embryos, injected with RCAS-Mgn that were tested for the RCAS Gag transcripts, showed a positive signal.

This can be seen in Figure 54. Panel (a) shows the injected side of the embryo. RCAS Gag can be seen in the forelimb compared to panel (b) which shows the control side.

This suggested that the RCAS-Mgn virus was active and able to infect the limbs which were targeted with efficiency (during the injection process). It also shows that the RNA probe for RCAS Gag was working efficiently to produce clear visible signals.

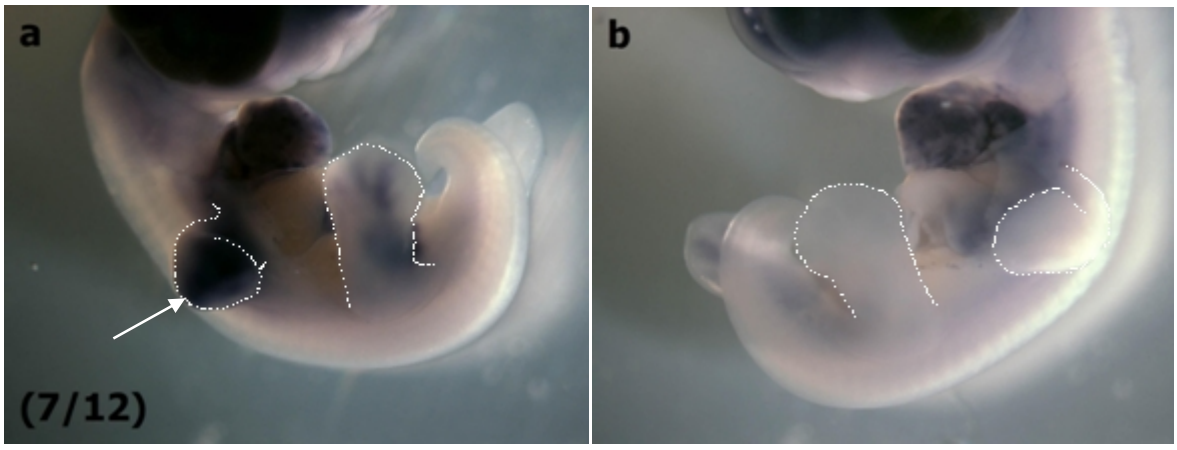


Figure 54: RCAS-Mgn infected chick embryo at embryonic stage HH28, showing **(a)** ectopic expression of RCAS Gag in the forelimb on the injected side and **(b)** the control side.

5.3.2. Test for Ectopic Myogenin

To determine if the RCAS-Mgn virus (that had infected the limb tissue) was able to express Myogenin, a whole mount *in situ* hybridisation was carried out on the embryos with the c-Mgn probe, which detects both the endogenous and viral Myogenin transcripts. A positive visible signal in this case allows us to conclude the presence of ectopic Myogenin expression in the marked tissue.

None of the twelve (12) embryos, injected with RCAS-Mgn and tested for Myogenin, showed ectopic myogenin expression.

This can be seen in figure 55. Panel (a) shows the injected side of the embryo. No Myogenin was detected (unexpected) and panel (b) which shows the control side.

The endogenous expression of Myogenin could be seen on both the injected and control side in the myotome, somites, wing and hindlimb. This suggests that the RNA probe for Myogenin was working efficiently. Both RCAS-Mgn and RCAS-MyoD were being used in parallel. A possible explanation for the negative signal is a potential mix-up, whereby viral particles of RCAS-MyoD was used for the infection instead of RCAS-Mgn.

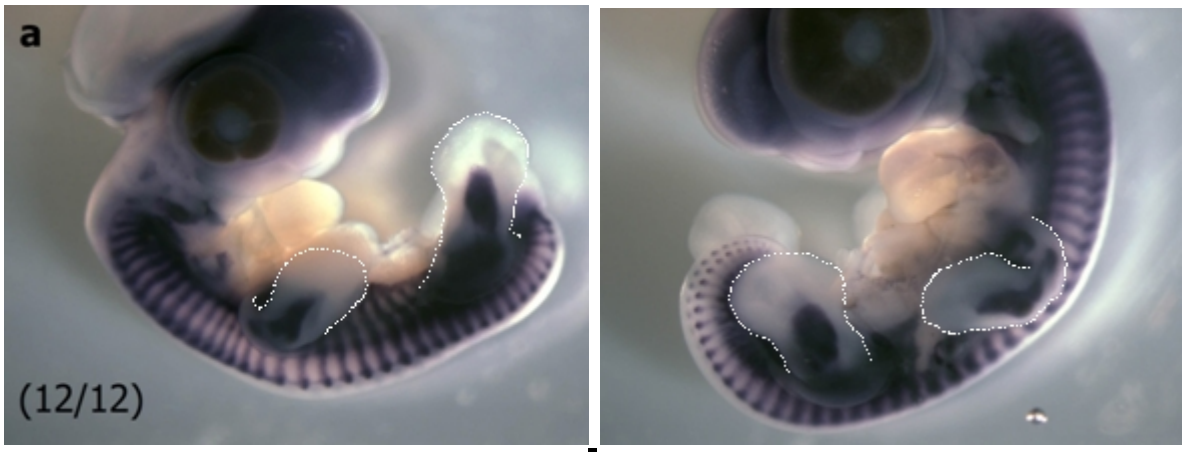


Figure 55: RCAS-Mgn infected chick embryo at embryonic stage HH28, showing **(a)** no ectopic expression of Myogenin in the limbs on the injected side and **(b)** the control side.

5.3.3 Test for Ectopic MiRNA-206

To determine if the RCAS-Mgn virus (that had infected the limb tissue) was able to affect the miRNA-206 expression, a whole mount *in situ* hybridisation was carried out on the embryos with the miR-206 LNA probe, which detects miR-206 molecules.

None of the eleven (11) embryos, injected with RCAS-Mgn that were tested for miR-206, showed any ectopic expression.

This can be seen in figure 56. Panel (a) shows the injected side of the embryo. No ectopic miR-206 was detectable and panel (b) shows the control side.

The endogenous expression of miR-206 could be seen on both the injected and control side in the myotome, somites, wing and hindlimb. This suggests that the LNA probe for miR-206 was working efficiently. Ectopic expression of miR-206 would be expected in the presence of either the Myogenin or the MyoD protein in the injected limb, therefore, a positive signal could be expected despite the mix-up mentioned in the previous section. However, due to the lack of detectable MRF transcripts, no further conclusion could be drawn.

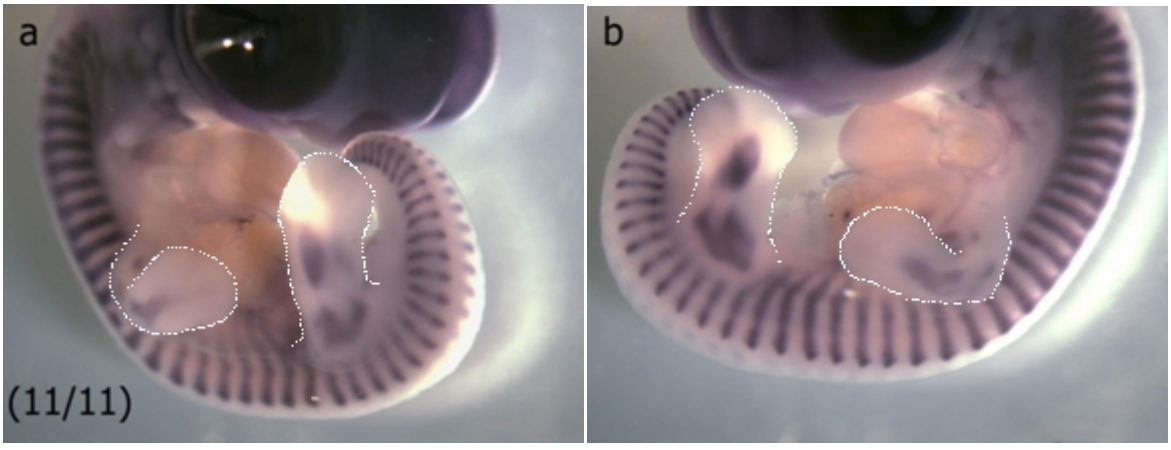


Figure 56: RCAS-Mgn infected chick embryo at embryonic stage HH28, showing **(a)** no ectopic expression of miR-206 in the limbs on the injected side and **(b)** the control side.

5.3.4 Conclusion

In conclusion, thirty-five (35) embryos were injected with RCAS-Mgn particles in the coelom at HH14-15 and allowed to develop till HH28. Postitive signal for RCAS Gag suggested successful infection of the forelimb. However, we could not detect any ectopic Myogenin transcripts or miRNA-206 in the injected limbs.

The absence of ectopic Myogenin in the limb in the presence of the RCAS Gag was due to a possible mix-up, where RCAS-MyoD was used for the injection in place of the RCAS-Mgn as they were being used in parallel.

It was suggested that the embryos tested for ectopic Myogenin transcripts were in fact possibly injected with the RCAS-MyoD virus particles and therefore not expressing any Myogenin.

This would explain the presence of the RCAS Gag transcripts in the limb tissue but the absence of ectopic Myogenin.

The embryos tested for miR-206 also showed no ectopic miR-206 expression in the injected limb. While we would have expected ectopic miR-206 in the presence of both MyoD and Myogenin, the lack of a positive signal for either MRF makes it difficult to comprehend.

The embryos that were injected in the similar way with RCAS-MyoD (discussed in section 5.4) were then tested for ectopic expression of both the MRFs, being used in parallel, MyoD and Myogenin. This measure was taken to try and understand the unexpected results obtained from this set of experiments (in section 5.3).

5.4. Effect of Ectopic MyoD on MiR-206 Expression

To determine whether ectopic MyoD could induce the expression of miRNA-206 in the developing chick limb, the following method was applied. Chick embryos at stage HH14-15 were injected with concentrated virus particles of RCAS-MyoD in the coelom on the right side (of the embryo). The embryos were then incubated until they reached embryonic stage HH 28, to allow for the RCAS-MyoD to propagate and infect all limb tissue (as they developed) on (one) the injected side.

The embryos collected were divided into four parts, each containing 6-7 embryos.
The embryos in each of these sections were then subject to a whole mount *in situ* hybridisation procedure with one of the following probes:

- RNA probe to detect RCAS Gag transcripts
- RNA probe to detect MyoD transcripts
- RNA probe to detect Myogenin transcripts
- LNA probe to detect MicroRNA miR-206

5.4.1. Test for RCAS Gag

To determine if the RCAS virus (RCAS-MyoD) had successfully infected the limb tissue, a whole mount *in situ* hybridisation was carried out on the embryos with the RCAS-Gag probe, which detects the viral Gag transcript. Visible signal in this instance suggests the infection was successful.

Four out of six (4/6) embryos, injected with RCAS-MyoD that were tested for the RCAS Gag, showed a positive signal.

This is shown in Figure 57. Panel (a) shows the injected side of the embryo. RCAS Gag can be seen in the forelimb, hindlimb and inter-limb region compared to panel (b) which shows the control side.

This suggested that the RCAS-MyoD virus was active and able to infect the limbs which were targeted with efficiency (during the injection process).

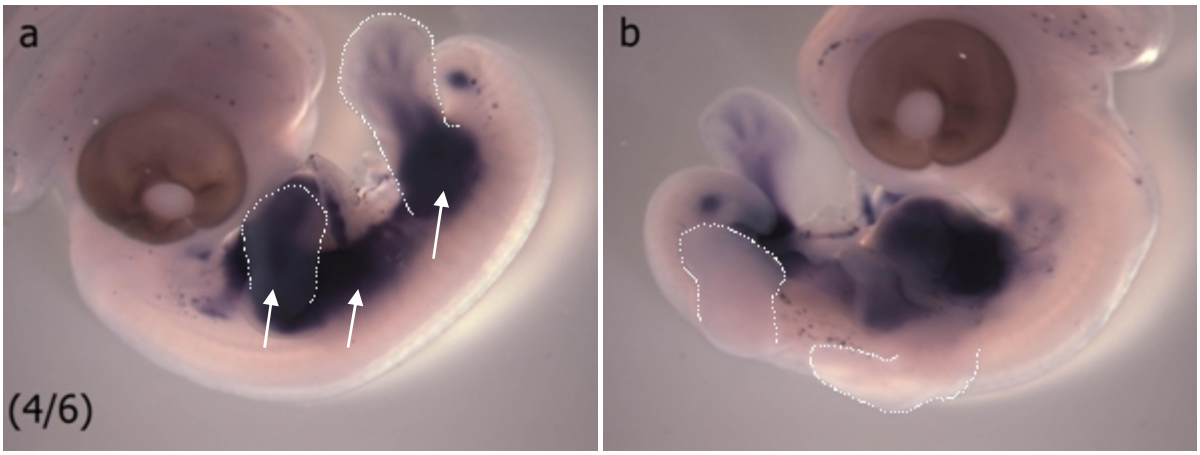


Figure 57: RCAS-MyoD infected chick embryo at embryonic stage HH28, showing **(a)** ectopic expression of RCAS Gag in the forelimb, hindlimb and inter-limb region on the injected side and **(b)** the control side.

5.4.2 Test for ectopic MyoD

To determine if the RCAS-MyoD virus (that had infected the limb tissue) was able to express MyoD, a whole mount *in situ* hybridisation was carried out on the embryos with the c-MyoD probe, which detects both the endogenous and viral MyoD transcripts. A positive visible signal in this case allows us to conclude the presence of ectopic MyoD expression in the marked tissue.

None of the six (6) embryos, injected with RCAS-MyoD and tested for MyoD, showed ectopic MyoD expression.

This can be seen in figure 58. Panel (a) shows the injected side of the embryo. No ectopic MyoD was detectable (unexpected) and panel (b) which shows the control side.

The endogenous expression of MyoD could be seen on both the injected and control side in the myotome, somites, wing and hindlimb. This suggests that the RNA probe for MyoD was working efficiently. Both RCAS-MyoD and RCAS-Mgn were being used in parallel. A possible explanation for the negative signal is a potential mix-up, whereby RCAS-Mgn was used for the infection instead of RCAS-Mgn (as the two viruses were being used in parallel experiments).

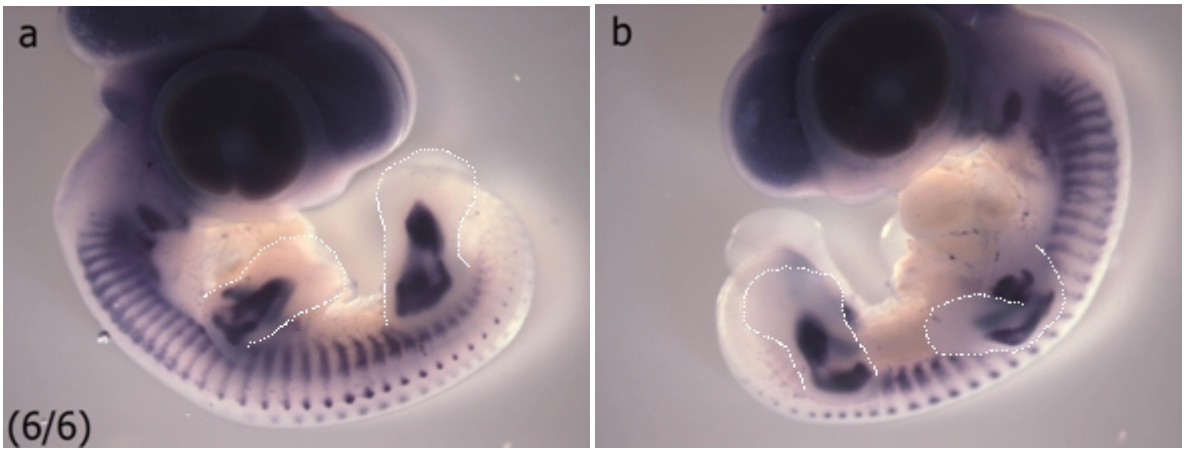


Figure 58: RCAS-MyoD infected chick embryo at embryonic stage HH28, showing **(a)** no ectopic expression of MyoD in the limbs on the injected side and **(b)** the control side.

5.4.3. Test for Ectopic Myogenin

To determine if the RCAS-MyoD virus, that was used to infected the limb tissue, had in fact been mixed up with the RCAS-Mgn virus sample, a whole mount in situ hybridisation was carried out on the embryos with the c-Mgn probe, which detects both the endogenous and viral Myogenin transcripts. A positive visible signal in this case allows us to conclude the presence of ectopic Myogenin in the marked tissue, hence confirming the source of the unexpected observations.

None of the six (6) embryos, injected with RCAS-MyoD and tested for Myogenin, showed ectopic myogenin expression.

This can be seen in figure 59. Panel (a) shows the injected side of the embryo. No Myogenin was detectable and panel (b) shows the control side.

The endogenous expression of Myogenin could be seen on both the injected and control side in the myotome, somites, wing and hindlimb. This suggested that the unexpected results discussed (so far in section 5.4 and section 5.3) were not caused by a potential mix-up between the RCAS-Mgn and RCAS-MyoD that were being used in parallel.

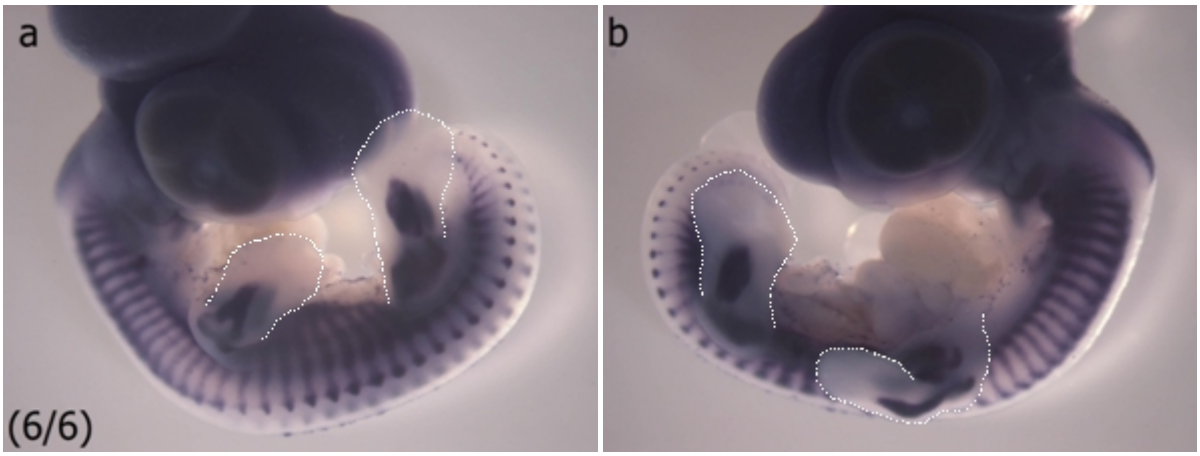


Figure 59: RCAS-MyoD infected chick embryo at embryonic stage HH28, showing **(a)** no ectopic expression of Myogenin in the limbs on the injected side and **(b)** the control side.

5.4.4 Test for Ectopic MiR-206

To determine if the RCAS-MyoD virus (that had infected the limb tissue) was able to affect the miRNA-206 expression, a whole mount *in situ* hybridisation was carried out on the embryos with the miR-206 LNA probe, which detects miRNA-206 molecules.

None of the seven (7) embryos, injected with RCAS-MyoD that were tested for miR-206, showed any ectopic expression.

This can be seen in figure 60. Panel (a) shows the injected side of the embryo. No ectopic miR-206 was detectable and panel (b) shows the control side.

The endogenous expression of miR-206 could be seen on both the injected and control sides in the myotome, somites, wings and hindlimbs. As the earlier experiments showed there were no ectopic MRFs in the injected limbs, no ectopic miR-206 was expected to be seen as we believe the MRF proteins would be responsible for the induction of the miRNA expression.

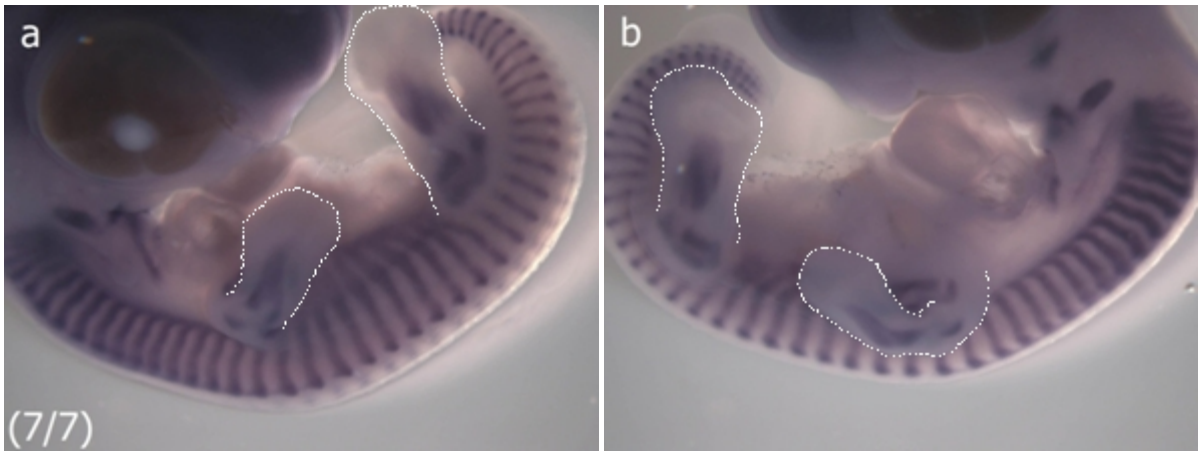


Figure 60: RCAS-MyoD infected chick embryo at embryonic stage HH28, showing **(a)** no ectopic expression of miR-206 in the limbs on the injected side and **(b)** the control side.

5.4.5 Conclusion

In conclusion, twenty-five (25) embryos were injected with RCAS-MyoD particles in the coelom at HH14-15 and allowed to develop till HH28. Positive signal for RCAS Gag suggested successful infection. However, we could not detect any ectopic MyoD. We also tested for ectopic expression of Myogenin to decipher if the lack of MRF expression (discussed in section 5.3) was due to a possible mix-up between the RCAS-Mgn and RCAS-MyoD. However, we could not detect any ectopic Myogenin or miR-206 in the limbs.

The absence of ectopic MRF expression in the limbs could not be explained with our hypothesis.

To avoid any further possibilities of potential mix-ups between different RCAS-MRFs, we decided to only work with one viral particle at a time.

We decided to continue with the experiments using RCAS-Mgn particles alone. Fresh aliquots, stored at -80^{o} C were used for future injections.

To avoid possible confusion with the RNA probes, both the RNA probes for MyoD and Myogenin were also freshly made up and tested on wild type chick embryos at stage HH 25. Both the probes provided a clear signal in the myotome, somite wing and leg muscle mesenchyme, verifying that they were working efficiently.

5.5. Test For Ectopic Myogenin Expression as a Result of RCAS-Mgn Infection

The experiments (detailed in sections 5.3 and 5.4) showed a lack of ectopic MRF expression in the limbs even in the presence of a successful RCAS-MRF infection. To investigate the unexpected results, we injected chick embryos at stage HH14-15 with concentrated virus particles of RCAS-Mgn into the coelom on the right hand side (of the embryo). The embryos were then incubated until they reached embryonic stage HH-25, to allow for the RCAS-Mgn to propagate and infect all limb tissue (as they developed) on (one) the injected side.

The embryos collected were divided into two parts, each containing 8 embryos.
The embryos in each of these sections were then subject to a whole mount *in situ* hybridisation procedure with one of the following probes:

- RNA probe to detect RCAS Gag transcripts.
- RNA probe to detect Myogenin transcripts.

5.5.1 Test for RCAS Gag

To determine if the RCAS virus (RCAS-Mgn) had successfully infected the limb tissue, a whole mount *in situ* hybridisation was carried out on the embryos with the RCAS-Gag probe, which detects the viral Gag transcript. Visible signal in this instance suggests the infection was successful.

Three out of eight (3/8) embryos, injected with RCAS-Mgn that were tested for the RCAS Gag, showed a positive signal.

This is shown in Figure 61. Panel (a) shows the injected side of the embryo. RCAS Gag can be seen in the forelimb and hindlimb compared to panel (b) which shows the control side.

This suggested that the RCAS-Mgn virus was active and able to infect the limbs which were targeted with efficiency (during the injection process).

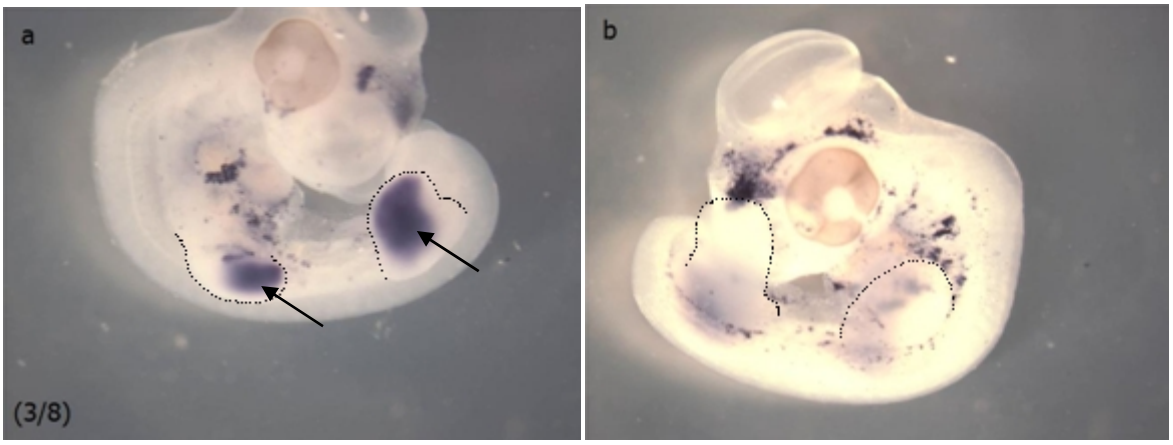


Figure 61: RCAS-Mgn infected chick embryo at embryonic stage HH25, showing **(a)** ectopic expression of RCAS Gag in the forelimb and hindlimb on the injected side compared to panel **(b)** which shows the control side.

5.5.2 Test for Ectopic Myogenin

To determine if the RCAS-Mgn virus (that had infected the limb tissue) was able to express Myogenin, a whole mount *in situ* hybridisation was carried out on the embryos with the c-Mgn probe, which detects both the endogenous and viral Myogenin transcripts. A positive visible signal in this case would allow us to conclude the presence of ectopic Myogenin expression in the marked tissue. A positive signal for Myogenin in this instance would suggest that the unexpected observations seen in the previous experiments (section 5.3 and 5.4) were due to faults in the RNA probes used.

None of the eight (8) embryos, injected with RCAS-Mgn virus and tested for Myogenin transcripts, showed ectopic Myogenin expression.

This can be seen in figure 62. Panel (a) shows the injected side of the embryo. No ectopic Myogenin was detectable compared to panel (b) which shows the control side.

The endogenous expression of Myogenin could be seen on both the injected and control side. This suggests that the RNA probe for Myogenin was working efficiently. However, the lack of an ectopic signal also suggests that the unexpected results were not due to any faults in the RNA probe used in the whole mount in situ procedure, as both the probes (previously used and freshly prepared) provided the same observation.

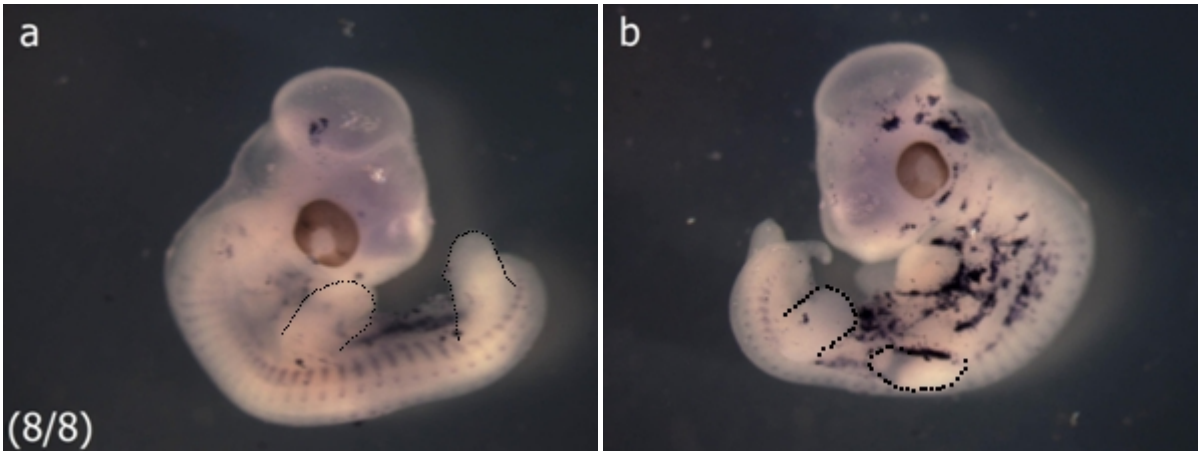


Figure 62: RCAS-Mgn infected chick embryo at embryonic stage HH25, showing **(a)** no ectopic expression of Myogenin in the limbs on the injected side and **(b)** the control side (precipitate on the outside of the embryo make the signals difficult to visualise; however, we can see no signal in the limbs)

5.5.3 Conclusion

In conclusion, sixteen (16) embryos were injected with RCAS-Mgn particles in the coelom at HH14-15 and allowed to develop to HH25. Positive signal for RCAS Gag suggested successful infection. However, we could not detect any ectopic Myogenin.

As we could see endogenous Myogenin in the somites, we concluded that the lack of an ectopic signal was not due to any mix-ups in the RNA probes used in the whole mount *in situ* hybridisation procedure.

We turned our attention to other potential sources of error. As only three out of eight (3/8) embryos showed a positive expression for RCAS Gag, we decided to explore the efficiency at which the limb was being targeted as the source of these unexpected results. Targeting the limb at a low efficiency could mean that none of the eight embryos tested for ectopic Myogenin were in fact infected by the RCAS-Mgn, therefore not displaying an ectopic expression.

To test this theory the experiment (detailed in section 5.5) was repeated.

5.6. Testing For Ectopic Expression of Myogenin on RCAS-Mgn Infected Embryos

The determine whether the a lack of ectopic MRF was a result of inefficient targeting of the limbs, chick embryos at stage HH14-15 were injected with viral particles of RCAS-Mgn in the coelom to the right hand side (of the embryo). The embryos were then incubated until they reached embryonic stage HH-28, to allow for the RCAS-Mgn to propagate and infect all limb tissue (as they developed) on (one) the injected side.

The embryos collected were divided into two parts, each containing 9 embryos.
The embryos in each of these sections were then subject to a whole mount *in situ* hybridisation procedure with one of the following probes:

- RNA probe to detect RCAS Gag transcripts.
- RNA probe to detect Myogenin transcripts.

5.6.1 Test for RCAS Gag

To determine if the RCAS virus (RCAS-Mgn) had successfully infected the limb tissue, a whole mount *in situ* hybridisation was carried out on the embryos with the RCAS-Gag probe, which detects the viral Gag transcript. Visible signal in this instance suggests the infection was successful.

Seven out of nine (7/9) embryos, injected with RCAS-Mgn that were tested for the RCAS Gag, showed a positive signal.

This is shown in Figure 63. Panel (a) shows the injected side of the embryo. RCAS Gag can be seen in the forelimb and hindlimb compared to panel (b) which shows the control side.

This suggested that the RCAS-Mgn virus was active and able to infect the limbs which were targeted with efficiency (during the injection process).

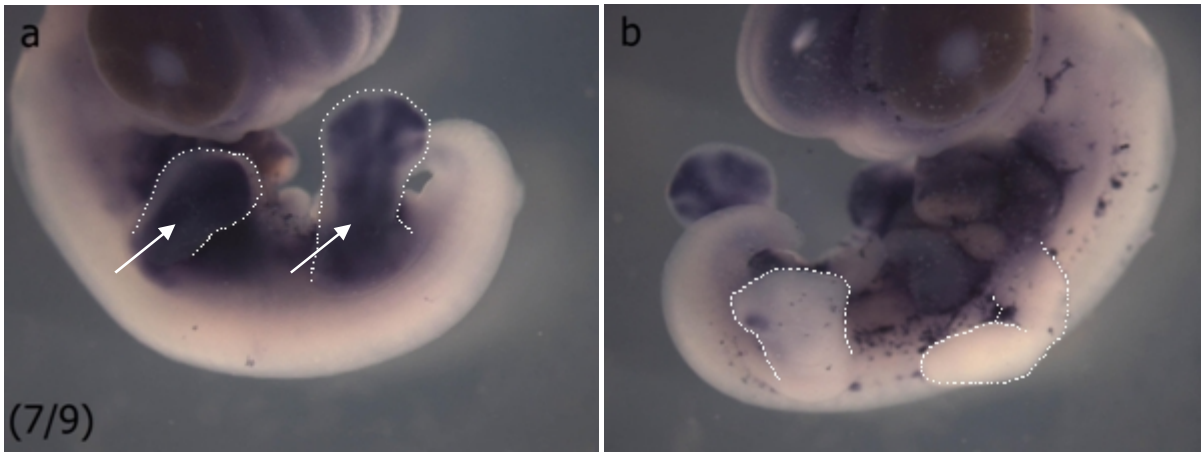


Figure 63: RCAS-Mgn infected chick embryo at embryonic stage HH28, showing **(a)** ectopic expression of RCAS Gag in the forelimb and hindlimb and **(b)** the control side.

5.6.2 Testing for Ectopic Myogenin

To determine if the RCAS-Mgn virus (that had infected the limb tissue) was able to express Myogenin, a whole mount *in situ* hybridisation was carried out on the embryos with the c-Mgn probe, which detects both the endogenous and viral Myogenin transcripts. A positive visible signal in this case would allow us to conclude the presence of ectopic Myogenin expression in the marked tissue. A positive signal for Myogenin in this instance would suggest that the unexpected observations seen in the previous experiments (section 5.3, 5.4 and 5.5) were due to inefficient targeting during the injection process.

None of the eight (8) embryos, injected with RCAS-Mgn virus and tested for Myogenin transcripts, showed ectopic Myogenin expression.

This can be seen in figure 64. Panel (a) shows the injected side of the embryo. No ectopic Myogenin was detectable compared to panel (b) which shows the control side.

The endogenous expression of Myogenin could be seen on both the injected and control side in the myotome, somites, wing and hindlimb. This suggests that the RNA probe for Myogenin was working efficiently. However, the lack of an ectopic signal also suggests that the unexpected result was not due to inefficient targeting of the limbs during the injection process.

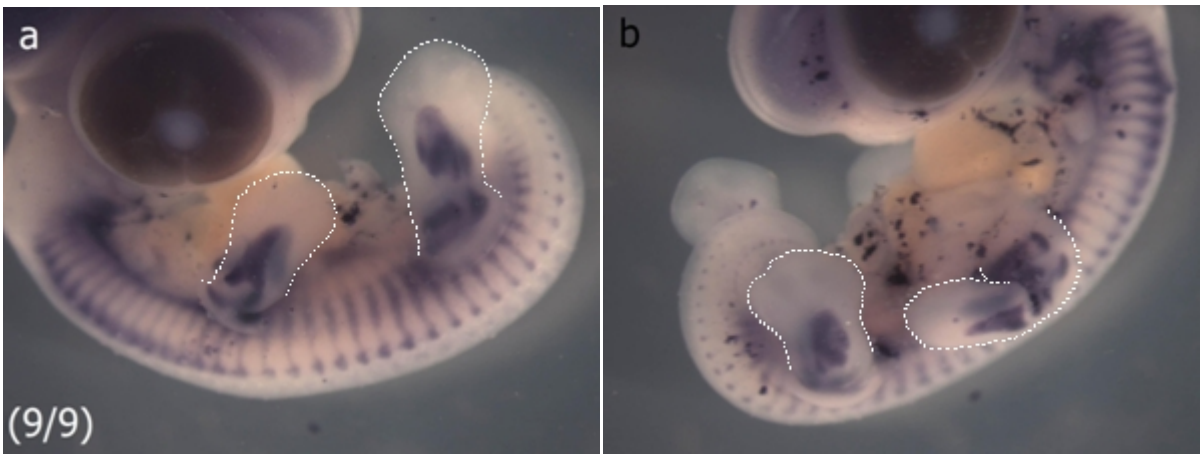


Figure 64: RCAS-Mgn infected chick embryo at embryonic stage HH28, showing **(a)** no ectopic expression of Myogenin in the limbs on the injected side and **(b)** the control side.

5.6.3 Conclusion

In conclusion, eighteen (18) embryos were injected with RCAS-Mgn particles in the coelom at HH14-15 and allowed to develop to HH28. Positive signal for RCAS Gag suggested successful infection. However, we could not detect any ectopic Myogenin.

Since seven out of nine (7/9) of the embryos injected with viral particles of RCAS-Mgn provided a positive signal for RCAS Gag expression, we had to conclude the limbs were being targeted correctly at an efficiency of seventy-eight percent (78%). The unexpected results discussed so far in this chapter (section 5.3, 5.4 and 5.5), therefore, were not caused by targeting inefficiency during the injection process.

As discussed so far, the errors mentioned were not a result of possible mix-up in the viral particles, whereby embryos tested for a certain MRF (Myogenin) were in fact injected with viral particles expressing a different MRF (MyoD). The errors were also not a result of poor quality of RNA probes used (in the whole mount *in situ* hybridisation procedure) to detect ectopic MRFs or due to mix-up in RNA probes. From the results discussed in section 5.6, we can also conclude that the unexpected results were not caused by inefficient targeting of the limbs during the injection process. With that in mind we turned out attention to testing the quality of the RCAS-MRF viral particles.

The experiments used to verify the quality of the viral particles is detailed in the following sections (5.7-5.8).

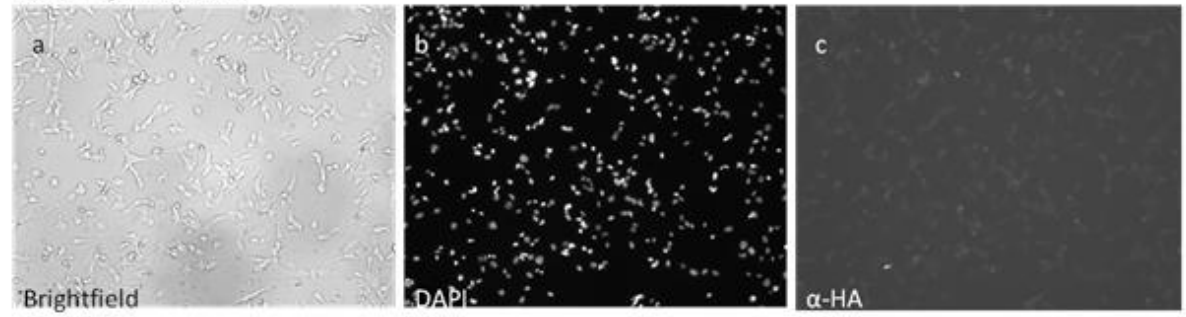
5.7. Verification of RCAS-Mgn Quality

In earlier experiments (discussed in section 5.3-5.6) chick embryos at embryonic stage HH14-15 were injected with concentrated virus particles of RCAS-MRF in the coelom on the right side (of the embryo). The embryos were then incubated until they reached embryonic stage HH 25-28, to allow for the RCAS-MRF to propagate and infect all limb tissue (as they developed) on (one) the injected side. The embryos were then tested for ectopic MRF expression. However, despite establishing the RCAS virus was able to infect the limb tissue, we failed to see any signal for ectopic MRF expression. Therefore, we decided to verify the quality of the RCAS-MRF (RCAS-Mgn) particles (as RCAS-Mgn was predominantly used for the experiments). The experimental procedures applied to test the viral particles are discussed in this section.

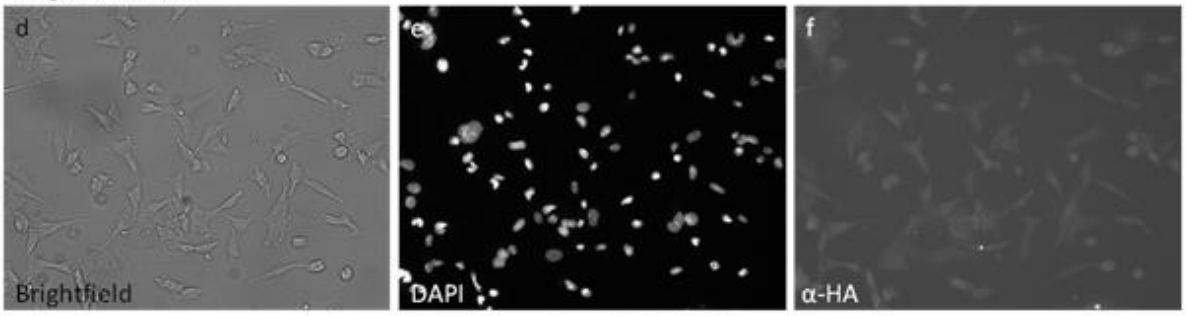
To test the RCAS- Mgn, DF1 cells growing in culture media (DMEM with 10% FBS) were added with either RCAS-Mgn-HA concentrated virus particles or RCAS-GFP concentrated virus particles. The cells were incubated allowing time for the RCAS virus to propagate and infect the adjacent (dividing) cells. The cells were then fixed and immunostained with DAPI and an HA antibody before being visualised under a fluorescent light source. Wild type DF1 cells (not infected with RCAS-Mgn) were used to establish control experiements.

The results obtained are detailed in figure **65.** Panel **(a-c)** show the DF1 cells infected with RCAS-Mgn; **(a)** bright field **(b)** DAPI stained and **(c)** α-HA stained images. Panel **(d-f)** show the negative control-wild type DF1 cells; **(d)** bright field **(e)** DAPI stained and **(f)** α-HA stained images. Panel **(g-i)** show the first set of positive control-DF1 cells infected with RCAS-GFP; (g) bright field (h) DAPI stained and **(i)** image under fluorescent light source showing GFP signal. Panel **(j-l)** show the second set of positive control-the wild type DF1 cells; **(j)** bright field **(k)** DAPI stained and **(l)** image showing staining of α-microtubes.

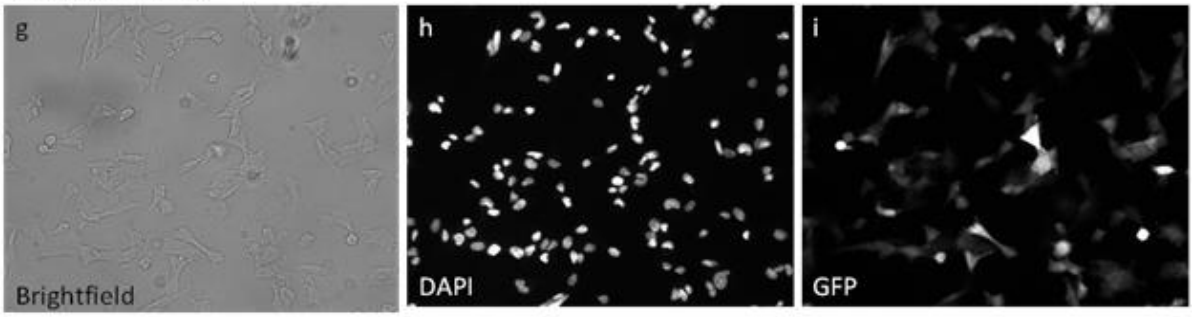
RCAS-Mgn-HA added



Negative Control



RCAS-GFP added



Positive Control

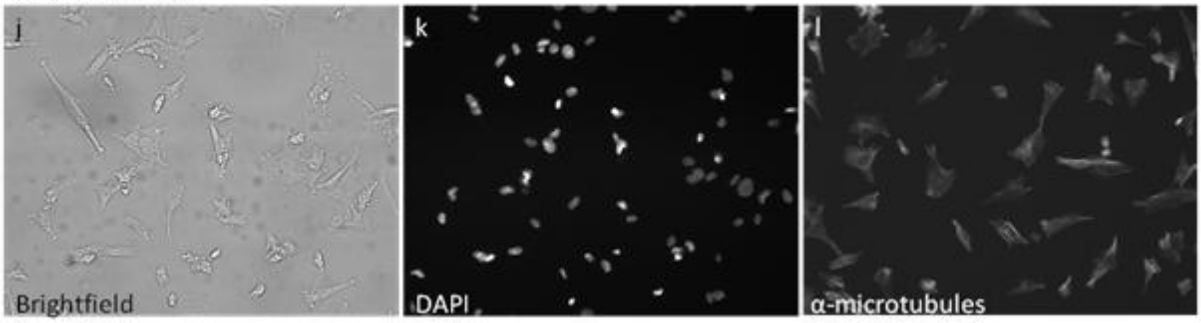


Figure 65: DF1 cells infected with RCAS-Mgn and immunostained with α-HA and controls. Panel **(a-c)** show the DF1 cells infected with RCAS-Mgn; **(a)** bright field **(b)** DAPI stained and **(c)** α-HA stained images. Panel **(d-f)** show the negative control-wild type DF1 cells; **(d)** bright field **(e)** DAPI stained and **(f)** α-HA stained images. Panel **(g-i)** show the first set of positive control-DF1 cells infected with RCAS-GFP; (g) bright field (h) DAPI stained and **(i)** image under fluorescent light source showing GFP signal. Panel **(j-l)** show the second set of positive control-the wild type DF1 cells; **(j)** bright field **(k)** DAPI stained and **(l))** image showing staining of α-microtubes. *(All images were obtained under x 20 magnification)*

5.7.1. DF1 cells infected with RCAS-Mgn

To test the RCAS- Mgn, DF1 cells were infected with concentrated virus particles of RCAS-Mgn-HA and incubated for 48 hours to allow the RCAS virus to propagate and infect the adjacent (dividing) cells. The cells were then fixed and immunostained with DAPI and α-HA antibody before being visualised under a fluorescent light source.

This can be seen in figure 65, Panel (a-c). The figure is reproduced below.

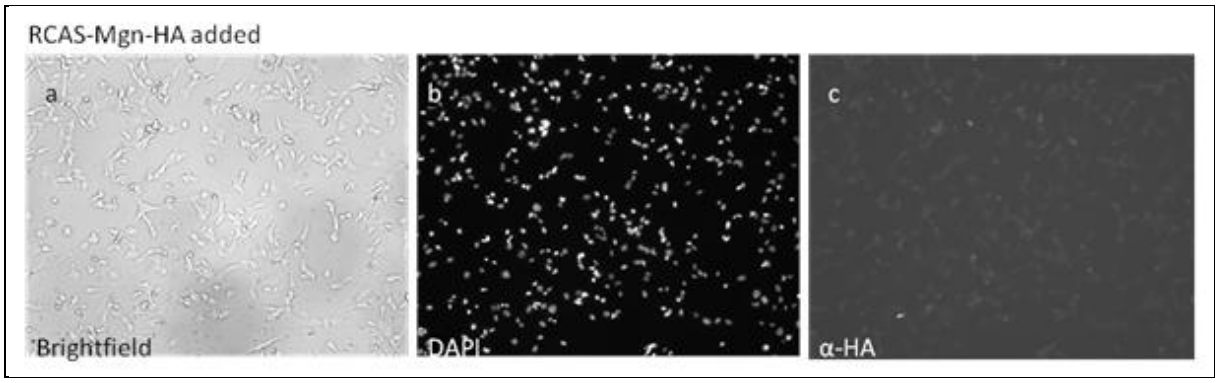


Figure 65 (a-c): DF1 cells infected with RCAS-Mgn and immunostained with α-HA.
(a) bright field **(b)** DAPI stained and **(c)** images under fluorescent light showing α-HA staining (showing no signal).

The bright field image of RCAS-Mgn-HA infected DF1 cells (shown in panel a) allows us to obtain an estimate of the total cell count. The DAPI stained image of the same RCAS-Mgn-HA infected DF1 cells (shown in panel b) allows us to obtain an estimate of the living cell count. The α-HA stained image of the same RCAS-Mgn-HA infected DF1 cells (shown in panel c) which (should) allows us to obtain an estimate of the infected cell count shows no signal.

From this we can see that despite the DF1 cells being healthy, there is no Myogenin-HA detected. We assumed one or a combination of the following reasons were responsible for these observations. We speculated this could be a result of the RCAS-Mgn virus not being able to infect the DF1 cells. Second probable cause was that the Myogenin-HA was not being

expressed despite a successful RCAS infection, therefore, no signal was seen using the α-HA antibody. Lastly, we suspected the immunostaining process was not working as expected therefore producing, misleading results.

To narrow down the reasons behind the observation to obtain the actual cause, we examined the results of the control experiments, detailed in the latter sections (5.7.2 – 5.7.4).

5.7.2 Negative Control: Wild Type DF1 Cells Stained With α-HA

To verify the quality of the RCAS-Mgn-HA, we infected DF1 cells with the RCAS-Mgn viral particles and tested it with an α-HA antibody that would detect the Myogenin-HA protein in the infected cells. However, no signal was seen. To confirm this, a negative control was set up, whereby, wild type DF1 cells were immunostained with DAPI and an α-HA antibody before being visualised under a fluorescent light source.

This can be seen in figure 65, Panel (d-f). This figure is reproduced below.

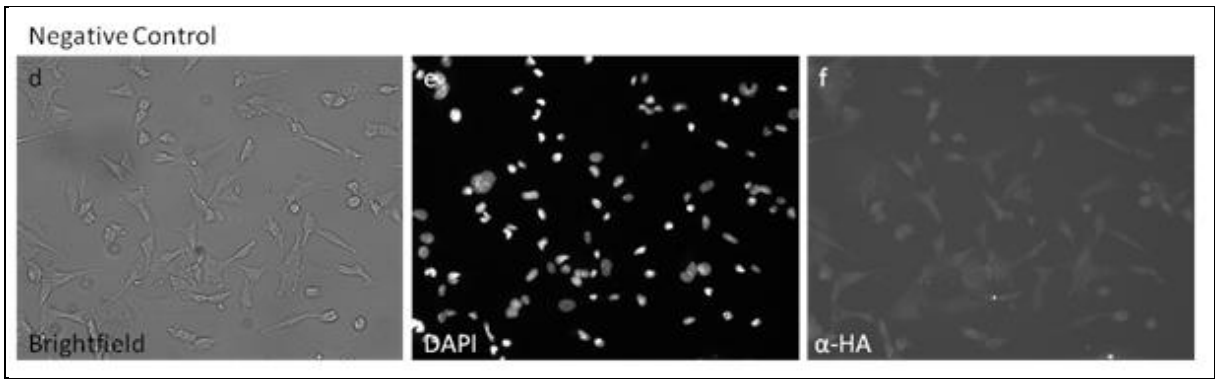


Figure 65 (d-f): Wild Type DF1 cells. **(d)** bright field **(e)** DAPI stained and **(f)** under fluorescent light showing α-HA staining.

The bright field image of the wild type DF1 cells (shown in panel d) allows us to obtain an estimate of the total cell count. The DAPI stained image of the same wild type DF1 cells (shown in panel e) allows us to obtain an estimate of the living cell count. The α-HA stained image of the same wild type DF1 cells (shown in panel f) which allows us to obtain an estimate of the infected cell count which shows no signal as expected.

From this we can see that the signal strength from the RCAS-Mgn infected DF1 cells is similar to that obtained from the wild type DF1 cells that were (both) stained with an α-HA antibody. We can also verify whatever could be seen in the image (Figure 65, Panel (c)) was merely background. At this point we still had to determine if the cause behind the lack of signal from the RCAS-Mgn infected and α-HA stained cells.

5.7.3. Positive Control: DF1 cells infected with RCAS-GFP

To verify the quality of the RCAS-Mgn-HA, we infected DF1 cells with the RCAS-Mgn viral particles and tested it with an α-HA antibody that would detect the Myogenin-HA protein in the infected cells. However, no signal was seen. To confirm this, a positive control was set up, whereby, DF1 cells were infected with concentrated virus particles of RCAS-GFP and incubated to allow the RCAS virus to propagate and infect the adjacent (dividing) cells. The cells were then fixed and immunostained with DAPI and visualised under a fluorescent light source.

This can be seen in figure 65, Panel (g-i). This figure is reproduced below.

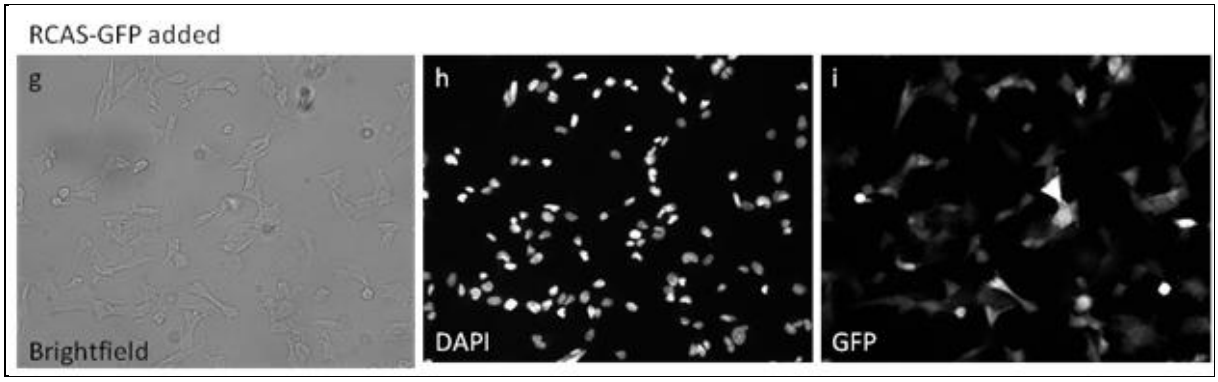


Figure 65 (g-i): DF1 cells infected with RCAS-GFP. **(g)** bright field **(h)** DAPI stained and **(i)** GFP images.

The bright field image of RCAS-GFP infected DF1 cells (shown in panel g) allows us to obtain an estimate of the total cell count. The DAPI stained image of the same RCAS-GFP infected DF1 cells (shown in panel h) allows us to obtain an estimate of the living cell count. The image showing the fluorescence from the GFP from the same RCAS-GFP infected DF1 cells (shown in panel i) which allow us to obtain an estimate of the infected cell count.

From this we can see that almost all the cells stained with DAPI also show fluorescence from the GFP. This suggested the RCAS virus was able to successfully infect the DF1 cells. Therefore, we can say the lack of a signal observed in the RCAS-Mgn infected DF1 cells (Figure 65, Panel (c)) was not due to the RCAS virus being unable to infect the DF1 cells. Hence, we speculated, this lack of signal was a result of the Myogenin-HA not being

expressed despite a successful RCAS infection or as a result of the immunostaining process not working as expected.

To decipher the cause behind the observation, we carried out further control experiments, detailed in the next section (5.7.4).

5.7.4. Positive Control: Wild Type DF1 Cells Stained With Antibody that detects Microtubules

To verify the quality of the RCAS-Mgn-HA, we infected DF1 cells with the RCAS-Mgn viral particles and tested it with an α-HA antibody that would detect the Myogenin-HA protein in the infected cells. However, no signal was seen. To confirm this, a positive control was set up, whereby, wild type DF1 cells were fixed and immunostained with DAPI and an antibody that detects microtubules and visualised under a fluorescent light source.

This can be seen in figure 65, Panel (j-l). This figure is reproduced below.

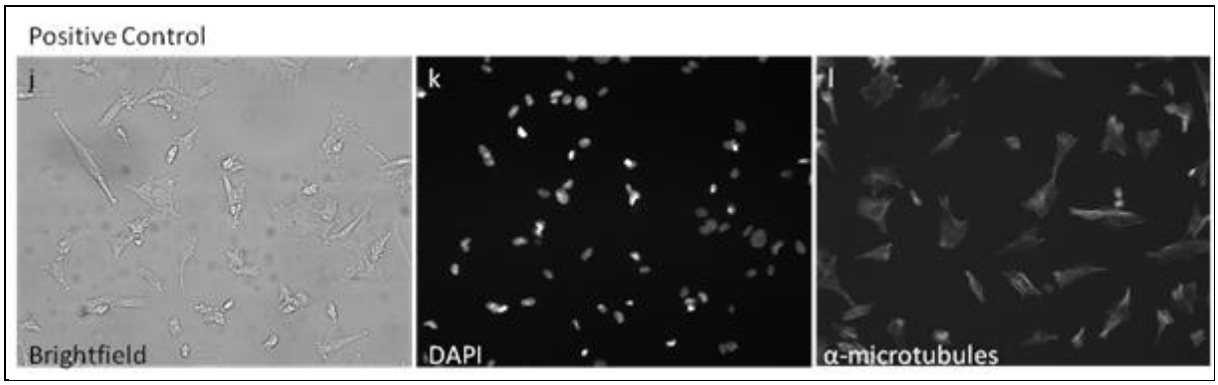


Figure 65 (j-l): Wild Type DF1 cells. **(j)** bright field **(k)** DAPI stained and **(l)** images under fluorescent light that shows staiing of microtubules.

The bright field image of the wild type DF1 cells (shown in panel j) allows us to obtain an estimate of the total cell count. The DAPI stained image of the same wild type DF1 cells (shown in panel k) allows us to obtain an estimate of the living cell count. The α-microtubule antibody stained image of the same wild type DF1 cells (shown in panel l) allows us to observe how efficiently the immunostaining procedure works. Ideally, we would have liked to set up a positive control, whereby we could detect signal using the α-HA antibody, as this approach may raise some other questions. However, we were short of time for completing another set of control experiments of that description.

From this we can see that almost all the cells stained with DAPI also show fluorescence from the antibody staining of the microtubules. This suggested that the lack of a signal observed in

the RCAS-Mgn infected DF1 cells (Figure 65, Panel (c)) was not due to the immunostaining process not working as expected.

Combination of observations described in this section (5.7.1-5.7.4) suggests that the lack of a positive signal in the RCAS-Mgn infected DF1 cells was probably a result of the Myogenin-HA not being expressed despite a successful RCAS infection. In earlier experiments (with the embryos), (detailed in sections 5.3-5.6), we also saw similar results, whereby, we could see RCAS-MRF (RCAS-Mgn and RCAS-MyoD) successfully infecting the embryonic limbs on the injected side but no ectopic MRF (Myogenin or MyoD) expression. To understand this better, we had to investigate the structure of the RCAS-MRF (RCAS-Mgn).

5.7.5.Conclusion

In earlier experiments (discussed in 5.3-5.6) we discovered RCAS-Mgn viral particles used to induce ectopic Myogenin in the chick limb tissue was unable to express any ectopic MRF even in the event of a successful infection. The quality of the RCAS-Mgn was therefore verified using DF1 cells.

DF1 cells were infected with RCAS-Mgn-HA, but no signal could be seen using the α-HA antibody, which was expected to detect the Myogenin-HA fusion protein.

A positive control, whereby, DF1 cells were infected with RCAS-GFP produced visible signals, suggesting the RCAS virus was in fact able to infect the DF1 cells.

A second positive control, whereby, wild type DF1 cells were subjected to an immunostaining using an antibody that detected the microtubules in the cell produced positive signals, suggesting, the lack of a signal from the DF1 cells infected with the RCAS-Mgn was not a result of the immunostaining protocol. Although ideally, it would be more accurate to test the positive control with the α-HA antibody, we could not do so due to time constrains.

In conclusion, we suggested a possible fault in the structure of the RCAS-Mgn construct. This was investigated next. The details of the experiments are discussed in the next section (5.8).

5.8. Verification of the RCAS-Mgn Construct

5.8.1. Verification by PCR

In earlier experiments (discussed in 5.3-5.6) we discovered RCAS-Mgn viral particles used to induce ectopic Myogenin in the chick limb tissue was unable to express any ectopic MRF even in the event of a successful infection. The quality of the RCAS-Mgn was then verified using DF1 cells. The DF1 cells also mirrored the same results, whereby, no myogenin was detected despite a successful infection. In this section we look at the RCAS-Mgn construct.

To verify the quality of the RCAS-Mgn construct, we infected chick DF1 cells with concentrated viral particles of either RCAS-Mgn or RCAS-GFP. Total RNA was extracted from the cells. A reverse transcription reaction was carried out to produce cDNA which was then used in a PCR reaction. The DNA was amplified using primers

RCAS-5': (ACGCTTTTGTCTGTGTGCTGC) and

DMF04: (ATCTCTGCAATGCGGAATTCAGTG).

As a control the primers:

chick-GAPDH-F: (CCTCTCTGGCAAAGTCCAAG) and

chick-GAPDH-R: (CATCCACCGTCTTCTGTGTG) were used.

The results of the PCR are shown in Figure 66.

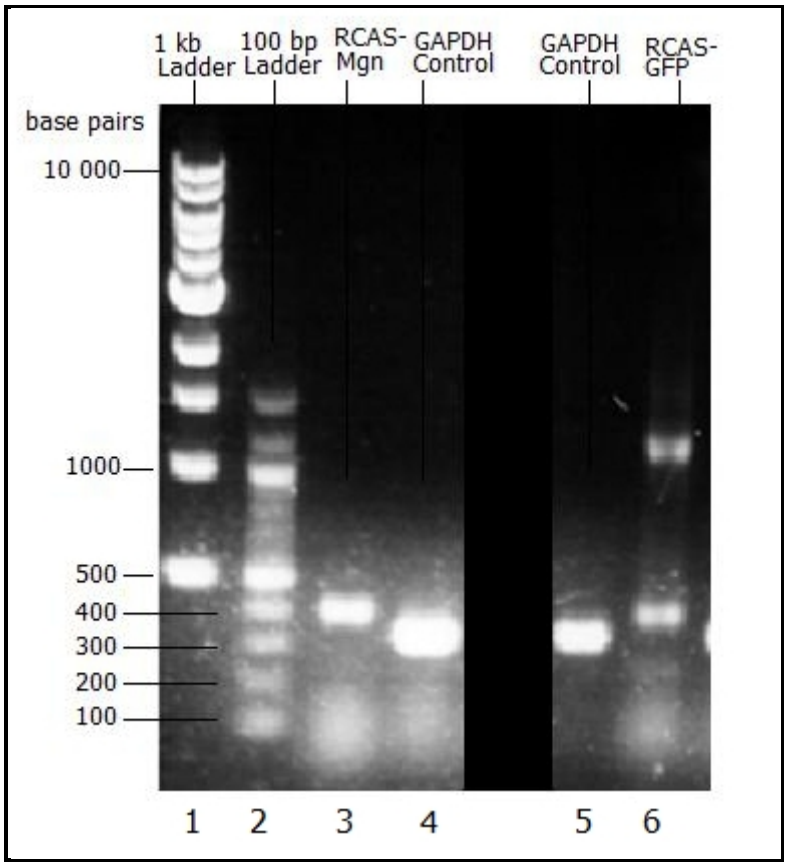


Figure 66: Results of PCR reaction, where the RCAS-Mgn and RCAS-GFP cDNA were amplified using RCAS-5' and DMF04 primers. GAPDH primers were used for the control experiments.

The 1 kb Ladder was added to Lane (1). The 100 bp Ladder was added to Lane (2). RCAS-Mgn amplified with RCAS primers from total RNA of DF1 cells infected with RCAS-Mgn virus particles was in Lane (3). GAPDH amplified from total RNA of DF1 cells infected with RCAS-Mgn virus particles was in Lane (4); it was to act as a control for Lane (3). RCAS-GFP amplified with RCAS primers from total RNA of DF1 cells infected with RCAS-GFP virus particles was in Lane (6). GAPDH amplified from total RNA of cells infected with RCAS-GFP virus particles was in Lane (5); it was to act as a control for Lane (6).

In both Lane (4) and (5) a single band of 350 bp in size could be seen. The GAPDH control is expected to generate this result, suggesting the RNA extraction, reverse transcription and

PCR procedures had been successfully carried out. In Lane (3), only one band 400 bp in size could be seen. RCAS vector with the chick-Myogenin inserted sequence (RCAS-Mgn) is expected to be 1600 bp in size. However, an empty RCAS vector is 400 bp in size. This suggests the presence of possibly an empty RCAS vector in this lane, therefore the presence of only an empty RCAS vector in the RCAS-Mgn infected DF1 cell sample. In Lane (6), two bands could be seen. One band is 1200 bp in size and the other is 400 bp in size. RCAS vector with GFP inserted sequence (RCAS-GFP) is expected to be 1200 bp in size, therefore we could deduce the 1200 bp fragment is in fact the RCAS-GFP. However, the 400 bp fragment that could also be seen in the lane may be an empty RCAS vector that had entered the sample via cross contamination as the RCAS-Mgn infection of DF1 cells were carried out at the same time (under the same sterile hood) as the RCAS-GFP infection of DF1 cells.

5.8.2 Conclusion

From this we can conclude the RCAS-Mgn virus that was used for the majority of the experiments was in fact 'faulty' and 'empty'.

When the embryos were injected with this virus particle, the infection was successful. It provided a positive signal for RCAS Gag but a negative signal for Myogenin transcripts. The 'empty' RCAS vector (thought to be the RCAS-Mgn) did express the housekeeping genes (i.e. Gag) but there was no Myogenin being transcribed (as there was no Myogenin insert present in the viral vector).

Similarly, when DF1 cells were infected with this virus particle (RCAS-Mgn) and stained with α-HA expected to bind to the Myogenin-HA protein, it provided no signal. Due to the absence of the Myogenin insert in the viral vector no Myogenin was being transcribed by the DF1 cells that could be detected.

To further verify that the RCAS-Mgn being used in these experiments was indeed just an empty RCAS vector, we would have liked to carry out a DNA sequencing procedure. Due to constrain in time, it was not possible to complete this experiment.

5.9. Future Work

The aim of the experiments detailed in chapter 5 was to determine if the MRF proteins could induce the expression of ectopic miRNA expression in the limb tissue and also to see if they induced the ectopic expression of the other members of the MRF family.

Chick embryos at stage HH14-15 were injected with concentrated viral particles of RCAS-MRFs in the coelom on the right side (of the embryo). As the limbs develop from the lateral plate mesoderm, injection of viral particles at this location lead to subsequent infection of all limb tissue, as they developed, on just the injected side (of the embryos). The embryos were then incubated and allowed to develop for 48 hours, to embryonic stage HH 25. Whole mount *in situ* hybridisation experiments were carried out on these embryos using the antisense RNA probe to detect RCAS Gag, chick-MRF transcripts and miR-206. Detection of RCAS Gag trascripts suggest expression of viral housekeeping genes, therefore, a successful viral infection of the tissue. Detection of MRF transcripts suggests the viral insert is capable working successfully to generate expression of MRFs in the tissue. Lastly, detection (or absence) of miR-206 in the tissue would allow us to deduce if the ectopic MRFs can in fact induce ectopic expression of miRNAs in the limb tissue.

During our experiments despite a strong signal in the limbs for RCAS Gag we failed to observe any signal from the MRFs. Further trouble-shooting led us to discover that the RCAS vector that was used for the injection experiments were infact missing the MRF insert and was 'empty'. As a result, we were not able achieve the aims we set out to look at due to constrains in time left.

If more time was available, we would have liked to produce new RCAS vectors with the MRF inserts with a HA tag. Carry out a PCR reaction to verify the length of the viral genetic material corresponds to that of it's own DNA and the MRF insert. On detecting bands of the right size, the DNA would then be sequenced to ensure that the MRF insert has been added to the viral vector correctly.

Once the DNA sequencing confirms correct assembly of the DNA, it would be transformed into *E. Coli* DH5-α cells and cloned. Once cloned, we would transfect it into chick DF1 cells, collect the

media containing the RCAS viral particles containing the MRF insert and concentrate the viral suspension.

The viral particles would then be used to infect wild type DF1 cells and the cells would be immunostained using an α-HA antibody. If cells are visible under florescent light once treated with this antibody, we could conclude the viral particles were working to test if the viral particles can successfully infect the cells and also express the inserted MRF.

If this experiment generated a positive result, we could then proceed to carry out the experiments on chick embryos to determine if the ectopic expression of the MRF can induce ectopic miRNA expression in the limbs.

To solidify the findings it would also be useful to obtain embryos that have each of the MRFs or a combination of the MRFs knocked out and test them for expression of endogenous miRNA using a whole mount *in situ* procedure using LNA probes. Once this data was obtained we could deduce if MRFs can infact induce miRNA in the limb tissue.