MATERIALS AND METHODS

3.1. Whole Mount *In Situ* Hybridization

To detect the expression of named (listed in each of the appropriate sections) genes in chicken embryos, whole mount *in situ* hybridisations were performed.

3.1.1. Synthesis of Riboprobe for *In Situ* Hybridization

Chick-MyoD antisense riboprobe: 5′-TTC TCA AGA GCA AAT ACT CAC CAT TTG GTG ATT CCG TGT AGT AGC TGC TG-3′ (Munsterberg Lab)

Chick-Myogenin antisense riboprobe: 5′- ATT TTG GGG CTC CGG TGC GGA AAT TCG ATA TCA AGCTTA TCG ATA CCG TCG -3′ (Munsterberg Lab)

3.1.1.1. Cloning of DH5α E coli Competent Cells: Heat Shock Transformation

In order to transform the competent cells, the Heat-shock protocol was applied.

1.0 µl purified plasmid was added to 20 µl competent *E.coli* cells (DH5α), (prepared and stored at -80°C) and placed in a 15ml falcon tube. It was placed on ice for 30 minutes, followed by a heat pulse at 37°C for 5 minutes before immediately being replaced on ice for 5 more minutes. 1 ml of Super Optimal broth with Catabolite repression (SOC medium) was added and the culture was incubated at 37°C for 1 hour with constant shaking. The bacterial culture was then centrifuged at 4500 rpm for 5 minutes and 1ml supernatant was taken out and the pellet was resuspended in the remaining supernatant.

The culture was plated onto a LB-Agar (Agar made in Luria Broth) plate containing Carbenicillin and incubated overnight at 37°C (elaborated in section 2.1.1.1.1).

A single colony was picked from each plate and transferred into a conical flask containing 50ml of Luria Broth (LB) broth containing 0.1mg/ml Carbenicillin and incubated on a shaker overnight at 37°C.

3.1.1.1.1. Plating and Bacterial Culture

100 µl of transformed *E.coli* (DH5α) cells were plated onto LB-Agar plates containing 0.1mg/ml Carbenicillin in a sterile environment using an ethanol-flamed glass spreader and the plates were incubated overnight at 37^{o}C.

Transformed cells were placed onto LB-Agar plates containing 0.1 mg/ml Carbenicillin (Melford laboratories, Cambridge, UK); antibiotics' resistance (to Carbenicillin) was used for selection.

When positive colonies were present, single colonies were picked using a pipette tip and added to a conical flask with 50 ml LB containing 0.1mg/ml Carbenicillin.

3.1.1.1.2. Plasmid Isolation from Bacterial Cultures

Plasmid DNA extraction was done by using the Qiagen Compact Prep Plasmid Midi Kit (Qiagen, GMBH, Germany) and manufacturer's instructions were followed.

50 ml of overnight cultures were transferred into Falcon tubes and centrifuged at 4500rpm for 15 minutes. The supernatant was removed and the pellet was re-suspended in 2.0 ml buffer P1. Next, 2.0 ml buffer P2 was added and the Falcon tubes were incubated at room temperature for 3 minutes. 2.0 ml of buffer S3 was added next and the solution was poured into the QIA-Filter midi cartridge, and incubated at room temperature for 3 minutes. The cell lysate was passed through the QIA-Filter midi cartridge and collected in another 15 ml Falcon tube. 2.0 ml of Buffer BB was added and poured into a (small) QIA-filter column. All liquid was removed through the filter over a vacuum. 700 µl of Buffer PE was added to the column and allowed to flow through the filter over a vacuum. The column was then dried over a vacuum for 10 minutes. Lastly, the DNA was eluted by adding 100 µl water (Sigma) to the (small) QIA-filter column, and collecting it in a 1.5 ml Eppendorf tube by centrifuging it at 13000 rpm for 1 minute.

3.1.1.1.3. Restriction Digestion

All restriction digests were carried out at 37^0C overnight using the recommended amount of restriction enzyme (Units/µl) for the amount of DNA used. Restriction enzymes were purchased from Promega (Promega UK, Southhampton UK) or Roche (Roche Applied Science, UK).

The example of restriction digestion reaction is digestion of pGEM-T with EcoRI restriction enzyme.

To a 5.0 µl sample of DNA solution (midi prep, extraction described in section 3.1.1.2), 2.5 µl EcoRI, 5.0 µl buffer H and 37.5 µl water (Sigma) were added. Samples were incubated at 37^oC overnight to allow the digestion to proceed.

1.0 µl of the digestion products was run on a 1.0 % agarose gel at 60V for 45 minutes.

3.1.1.1.4. Agarose Gel Electrophoresis of DNA and RNA

Agarose (Sigma) gels were made at 0.8 or 1.0% concentration by pouring a warm solution of melted agarose-TAE (Agarose melted in Tris base, acetic acid and EDTA, Ethylenediaminetetraacetic Acid Buffer) in 1x TAE (Tris base, acetic acid and EDTA, Ethylenediaminetetraacetic Acid) buffer. Ethidium bromide was added to the (running) TAE buffer at 0.01% (v/v) to visualize DNA or RNA using an UV trans-illuminator following electrophoresis.

Prior to loading the gel, sample was mixed with 1/1 volume of 1x loading buffer (50% (v/v) Glycerin and 50% (v/v) orange dye). Electrophoresis was carried out at 60 volts for 30-50 minutes on the gel.

3.1.1.2. Phenol : Chloroform Purification

50µl of water (Sigma) was added to the digested plasmid DNA. 100µl of Phenol:Chloroform/Isoamyl Alcohol (pH 8.0; Sigma) was added and vortexed. The contents were centrifuged at 13000 rpm for 10 minutes. The top phase was transferred into a new tube

and 10 µl of 3M Sodium Acetate (pH 5.2) was added. 300 µl of 100 % Ethanol was added and vortexed. The sample was centrifuged at 145000 rpm for 30 minutes at 4^{o}C. The supernatant was removed and the pellet was washed gently with 70% ethanol. The pellet was then air-dried for 10 minutes and resuspended in 10 µl water (Sigma).

1.0 µl of the purified, linearised (restriction digested) plasmid was run on a 1.0 % agarose gel at 60V for 45 minutes (as described in section 3.1.1.1.4).

2.1.1.3. Transcription Reaction

The RNA probe was next synthesized in an *in vitro* transcription reaction. The reaction mix was incubated in 37^{o}C for 3 hours.

Transcription Reaction Mix:

- 0.5 µl linearised DNA
- 4.0 µl 5X transcription buffer
- 2.0 µl DTT
- 2.0 µl FITC / DIG-labeled UTP mix
- 1.0 µl RNasin
- 2.0 µl T7 / SP6 RNA polymerase
- 10.5 µl Sigma water (volume up to 20 µl).

1.0 µl of the reaction product was run on a 1% agarose gel at 60V for 45 minutes (as described in section 3.1.1.4.1).

2.1.1.4. Purification of Transcription Product

Purification of transcription product (RNA) was done by using the Probe Quant G-50 Micro column (GE Healthcare) and manufacturer's instructions were followed.

1.0 µl of RNA probe was run on a 1.0 % agarose gel at 60V for 45 minutes (as described in section 3.1.1.1.4).

2.0 µl of the probe was added to 1 ml of hybridization buffer.

Hybridisation Buffer:

Formamide	50% (v/v),
20 x SSC (pH 5)	6.5% (v/v),
5mM EDTA (0.5M, pH 8)	1.0% (v/v)
Yeast RNA (20 mg/ml)	0.25% (v/v)
Tween-20	0.2% (v/v)
CHAPS	0.5% (v/v),
Heparin (50 mg/ml)	0.2% (v/v),
Water	35% (v/v)

3.1.2 LNA Probes

Dual-DIG labelled LNA probes were obtained from Exiqon.

3.1.3. Whole Mount *In situ* Hybridization Preparation

3.1.3.1. Embryo Removal and Dissection

Embryos were incubated in 37^0C humidified incubator for a certain period of time. Once they reached the desired stage of development (estimated from number of hours incubated), embryos were harvested from eggs by cutting away connecting tissues with fine scissors and removing the embryo using fine forceps. The serosa membrane and other attached tissues were removed. The embryos were next rinsed in sterile Phosphate Buffered Saline (PBS) and fixed in 4% paraformaldehyde in PBS (PFA)/PBS.

3.1.3.3. Fixation

Embryos were dissected in PBS-DEPC (Phosphate Buffered Saline - Diethylpyrocarbonate) and fixed overnight at 4^oC in 4% PFA/PBS.

3.1.3.4. Dehydration

Embryos were dehydrated by washing in PBT (PBS-DEPC with Tween-20, 0.1% (v/v)), 50% methanol, twice in 100% methanol, at least 5 minutes each wash. Once dehydrated, embryos could be stored in fresh methanol at -20^{0}C for several months.

3.1.3.5. Re-Hydration

Re-hydratation of embryos was done by washing in 100%, 75%, 50% methanol and 25% methanol/PBT, 5 minutes each wash, and finally two washes of PBT.

3.1.4. *In Situ* Hybridization Protocol

Following re-hydration, embryos of stage HH25 – HH35 were treated with 10-20 µg Proteinase K for 60 minutes, depending on the stage at which the embryos were collected.

Embryos were then fixed in 4% PFA added with Gluteraldehyde, 0.1% (v/v) for 20 minutes and rinsed in PBT with rocking.

Next, hybridization buffer mixed with PBT at a 1:1 ratio was added to the embryos. Once the embryos had settled, they were transferred to fresh 100% hybridization buffer.

The hybridization buffer was then replaced and the embryos were incubated for 2.5 hours (for embryos at stages HH25-28) and overnight (for embryos at HH35) at 65^{o}C (for RNA probes), 50^{o}C for LNA probe against miR-206, 60^{o}C for LNA probe against miR-133 and at 42^{o}C for LNA probe against miR-1.

After embryos had equilibrated with the hybridization buffer, they were incubated overnight with (LNA / RNA) probe in hybridization buffer at 65^{o}C for RNA probes, 50^{o}C for LNA probe against miR-206, 60^{o}C for LNA probe against miR-133 and at 42^{o}C for LNA probe against miR-1, with rocking.

Next day, the unbound probe was removed by rinsing embryos twice in hybridization buffer, followed by two washes each for 10 minutes in hybridization buffer. The embryos were then washed twice in wash buffer, for 1 hour and 2 hours respectively before being put on a third wash in wash buffer overnight. All washes were carried out at the same temperature at which the probe hybridization was carried out

Next day, the embryos were washed in wash buffer mixed with MABT (Maleic Acid, Sodium Chloride, NaCl, Tween-20) at a ratio of 1:1 for 10 minutes. Embryos were then rinsed three times in MABT, followed by then two washes in MABT, for 30 minutes each at room temperature.

MABT:

Maleic Acid	5.8% (w/v)
Sodium Chloride, NaCl	4.35% (w/v)
Tween-20	5.5% (w/v)
Water	90% (v/v)

Non-specific protein interactions were blocked by incubating embryos in 2% Boehringer Blocking Reagent (Roche) in MABT (BBR/MABT) for 1 hour and in 2% BBR/MABT with 20% goat serum (GS) for two hours at room temperature.

Antibody incubation was done overnight with Anti-Digoxigenin-Alkaline Phosphatase (DIG-AP) or Anti-Fluorescein Isothiocyanate-Alkaline Phosphatase (FITC-AP) in 2% BBR/MABT/20% GS at 4°C.

The excess of antibody was washed by six washes of MABT, at least 1 hour each wash, at room temperature. The embryos were then incubated in the final MABT wash overnight.

Next day, the embryos were rinsed in MABT mixed with NTMT (Sodium Chloride, NaCl, Tris (HCl) pH 9.5, Magnesium Chloride, $MgCl_2$, Tween-20) at a ratio of 1:1. Following which the embryos were washed in NTMT twice for 10 minutes each. Color reaction was performed after. Detection of the probe was performed by using Nitro blue tetrazolium (NBT; Roche)/ 5-Bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) in NTM (Sodium Chloride, NaCl, Tris (HCl; pH 9.5), Magnesium Chloride, $MgCl_2$)

NTMT:

5M Sodium Chloride, NaCl	2% (v/v)
1M Tris-Chloride (pH 9.5)	10% (v/v)
1M Magnesium Chloride, $MgCl_2$	5% (v/v)
Tween-20	1% (v/v)
Water	82% (v/v)

NTM:

5M Sodium Chloride, NaCl	2% (v/v)
1M Tris-Chloride (pH 9.5)	10% (v/v)
1M Magnesium Chloride, $MgCl_2$	5% (v/v)
Water	82% (v/v)

As soon as the background appeared, embryos were washed in fresh 5X Tris-Buffered Saline Tween-20 in Water (TBST/H_2O; pH 7.4). The color reaction was resumed if necessary.

TBST:

Sodium Chloride, NaCl	0.88% (w/v)
Potassium Chloride, KCl	0.02% (w/v)
Tris Base	0.3% (w/v)
Tween-20	0.05% (v/v)
Water	99% (v/v)

The microscopic analysis was carried out using an upright microscope (Leica MZ 16F).

3.2. *In Vivo* Manipulation of Chicken Embryos

Chicken embryos were used as *in vivo* model system in order to further validate the role of MRFs in inducing muscle specific microRNAs in the limb muscles.

3.2.1. Culture of Chicken Eggs

White leghorn chicken eggs (Henry Stewart & Co.Ltd., UK) were stored at 16^0C prior to incubation at 37^0C for the required period of time.

3.2.2. Microinjection

Microinjection needles were prepared by pulling glass capillaries (1.0 mm O.D. x 0.78 mm I.D.; Harvard apparatus, UK) on a Vertical micropipette puller (P-30, Sutter Instrument Co., CA). Fine forceps were used to break the tip of the capillary to generate a suitable length tip. Needles were filled with 5 µl of injectant (MRF Replication Competent Avian Sarcoma-Leukosis Virus with a Myogenic Regulatory Factor Insert**,** RCAS-MRF).

Small holes were pierced in the upper surface of the egg shell and the holes were enlarged after the embryo had been located. The shell membrane and serosa covering the embryo were removed and the micromanipulator was used to direct the needle into the coelom. Pressure was exerted to fill it with the injectant (RCAS-MRF).

RCAS (Replication-Competent Avian Sarcoma-Leukosis Virus) particles were generated, harvested and concentrated (as described in section 3.4). Fast green was added to the virus sample before injection. Embryos at embryonic stages HH 14 (22 somites-stage) were injected into the coelom. Once completed, sterile PBS was added to the egg (to prevent dehydration of the embryo) before it was sealed and placed back in a humidified incubator at 37°C (until the embryos grew to the desired developmental stage).

3.2.3. Harvesting of Treated Chick Embryos

The injected embryos were incubated for 48-72 hours and then dissected and fixed (as described in section 3.1.3.1).

3.3. Sectioning of Embryos and Microscopic Analysis

After ISH, embryos were rinsed in PBS twice, 5 minutes each wash. Next, embryos were incubated in 20% sucrose in 0.1 M PBS (cryoprotectant) for 4 hours at room temperature, with gentle rotation. The embryos were next placed in cryotubes and O.C.T. embedding medium (Miles Inc.) was added. The embryos were manually positioned with tweezers to position them correctly in relation to the plane of sectioning. The cryotubes were rapidly placed in isopentane cooled with liquid nitrogen. After the material was frozen, the tubes were stored overnight at -20^0C.

20 μm sections were cut at -15^0C. The sections were transferred to TESPA-covered slides and dried at room temperature.

Next, the slides were washed five times in PBS, 5 minutes each wash and mounted in Hydromount (National Diagnostics). The slides were dried overnight at 37°C.

Subsequent microscopic analysis was performed using an upright microscope (Zeiss). Images were captured and analysed using Axio Vision software.

3.4.Making RCAS Virus Particles

3.4.1 Cell Culture

Chick dermo-fibroblast (DF1) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 1g/ml of glucose, 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin. The cells were split 1:5 every other day.

3.4.2. Producing RCAS Virus DNA

In order to transform the competent cells, the Heat-shock protocol was applied. 1.0 µl purified plasmid (existing stock, stored at -20°C) was added to 20 µl competent *E.coli* (DH5α) cells (transformation conducted as described in section 3.1.1.1). The cells were then plated (as described in section 3.1.1.1.1). A single colony was picked and cloned. The plasmid was isolated using a Qiagen Midi Kit (as described in section 3.1.1.1.2). Plasmids were purified using the phenol: chloroform purification method (as described in section 3.1.1.2). Once the purification was complete the DNA (plasmids) were ready to be transfected.

3.4.3. Cell Transfection

Cells were split into 45 ml flasks. 10 µl Lipofectamine 2000 (Gibco) was added to 250 µl DMEM and incubated at room temperature. 150 ng plasmid DNA was added to 250 µl DMEM and added to Lipofectamine 2000 solution, then incubated at room temperature for 20 minutes. Cells were washed twice with 1 ml DMEM, which was then replaced with 10 ml DMEM per flask. The transfection mix was added to the cells dropwise and incubated for 5 hours at 37°C.

The DMEM containing the transfection mix was then replaced with 10 ml DMEM containing 10% FBS and penicillin-streptomycin solution.

3.4.4. Harvesting RCAS Virus Particles

The transfected DF1 cells were split and allowed to grow (until we had eight 75 ml flasks confluent with cells). The DMEM with 10% FBS and penicillin-streptomycin solution was replaces with 12.5 ml (per flask) of DMEM with 1% FBS and penicillin-streptomycin solution and cells were returned to incubation at 37°C for 24 hours. After that period of time the DMEM (with 1% FBS and penicillin-streptomycin solution) was collected and stored at -80°C, the cells were replaces with fresh DMEM with 1% FBS and penicillin-streptomycin solution (12.5 ml per flask). This was repeated for a third and final time.

3.4.5. Concentrating RCAS Virus Particles

The media collected was thawed slowly over ice and microfiltered. The solution was then centrifuged at 35000 rpm for 3 hours. All (almost) the supernatant was discarded. Virus collected was concentrated in a total volume of 200 µl, which was aliquotted and stored at -80°C. RCAS virus aliquots were used fresh (removed from -80°C on the day of the injection) and kept on ice once taken out of the freezer.

3.5. Viral Infection of DF1 Cells

3.5.1. Cell Culture

Chick DF1 cells were cultured (as described in section 3.4.1)

3.5.2 RCAS Virus Infection

Chick DF1 cells were split 1:4 and placed in the incubator at 37°C for 2 hours. A fresh aliquot of concentrated RCAS virus particles (section 3.4.5) was thawed over ice and added to the DF1 cells and returned to the incubator at 37°C for 48 hours.

Next, the cells were trypsinised and resuspended; half of the cells were added to gelatin-coated glass coverslips in 12- well plate dishes and the other half was returned to the flask. Both sets of cells were replaced into the incubator at 37°C for 4-5 hours.

3.5.3. Immunostaining

RCAS virus infected chick DF1 fibroblast cells were added to gelatin-coated glass coverslips in 12- well plate dishes. Wild type chick DF1 fibroblasts were used for control experiments. For the immunostaining procedure, the coverslips were moved to a fresh 12-well dish and the cells were rinsed with PBS.

Next, the cells were fixed in 4% PFA for 20 minutes at room temeperature and then rinsed three times with 1% goat serum in PBS (GS/PBS). The cells were permeablised by washing it in 0.1% Triton-200 in GS/PBS. Next, the cells were blocked with 10% goat serum in PBS (10% GS/PBS) for 30 minutes at room temperature, on a rocker.

The cells were then incubated with the primary antibody, α-mouse anti-HA (Roche) diluted at 1:1000 in GS/PBS for 1 hour at room temperature at room temperature. The cells were then washed four times for 8 minutes each with 1% goat serum in PBS (1 % GS/PBS) with gentle rocking.

The cells were then incubated with the secondary antibody, α-mouse Alexaflour 488 IgG (Invitrogen) diluted at 1:1000 in 10 % GS/PBS for 30 minutes at room temperature. The cells were then washed three times for 6 minutes each with 1% GS/PBS with gentle rocking.

Next, the cells were incubated with DAPI (diluted 1:2000) in PBS for 5 minutes, at room temperature, on a rocker. They were rinsed three times with PBS before being mounted onto slides with Hydromount.

Subsequent microscopic analysis was performed using an upright microscope (Zeiss). Images were captured and analysed using Axio Vision software.

3.5.4. Verification of Plasmid

3.5.4.1. Extraction of RNA From Cell Cultures

500 µl solution D was added to RCAS virus infected chick DF1 fibroblast cells and the cells were scratched and transferred into an Eppendorf tube. The cells were then vortexed until the tissue decomposed and then frozen at -20°C. 50 µl 2M sodium acetate (pH 4) was added and mixed. Next, 500 µl unbuffered phenol was added and mixed; followed by 180 µl chloroform/isoamyl alcohol. The samples were incubated on ice for 15 minutes and then centrifuged at 16400 rpm for 15 minutes in 4°C. The top phase was transferred into a new tube and 240 µl of 100% ethanol was added to it. This was frozen over dry ice, thawed at room temperature and centrifuged at 16400 rpm for 15 minutes at 4°C. The supernatant was removed and the pellet was washed gently with 70% ethanol before being air-dried for 10 minutes and resuspended in 100 µl DEPC-water.

Solution D:

25 g	Guanidinium thioacetate;
1.76 ml	0.75M sodium citrate (pH 7)
2.64 ml	10% Sarkosyl
38 µl	β-mercaptoethanol
29.3 ml	Water

3.5.4.2. Reverse Transcription Reaction

RNA was harvested from RCAS virus infected chick DF1 cells and cDNA was synthesized by reverse transcription from this RNA using oligodT primer. RNA, OligodT and water was mixed and incubated at 65^{o}C for 10 minutes and then transferred immediately on ice. Next, 5X Reverse Transcriptase buffer, RNasin, dNTP mix and Reverse Transcriptase were added and mixture was incubated at 65^{o}C for 30 minutes.

Reverse transcription reaction mix:

5.0 µl RNA
0.5 µl OligodT
7.0 µl Water (Sigma)
4.0 µl 5X Reverse Transcriptase buffer
0.5 µl RNasin
2.5 µl dNTP mix (10mM)
0.5 µl Reverse Transcriptase (Roche)

1.0 µl of cDNA was used for the PCR reaction.

3.5.4.3. Amplification of cDNA by Polymerase Chain Reaction

The primers used for the amplification of cDNA were:
RCAS-5': (ACGCTTTTGTCTGTGTGCTGC)
DMF04: (ATCTCTGCAATGCGGAATTCAGTG)

The primers used for the control reactions were:
Chick-GAPDH-F: (CCTCTCTGGCAAAGTCCAAG)
Chick-GAPDH-R: (CATCCACCGTCTTCTGTGTG)

A PCR reaction mix was put through reaction conditions on94^{o}C for 4 minutes; next 29 cycles of: 94^{o}C for 1 minute, 58^{o}C for 30 seconds, 72^{o}C for 1 minute, followed by 72^{o}C for 5

minutes. The success of the PCR reaction was established by using agarose gel electrophoresis (as described in section 3.1.1.1.4). Molecular weight markers (Roche) were used to establish the size of the products.

The PCR reaction mix:

1.0 µl	cDNA
1.0 µl	Forward Primer
1.0 µl	Reverse Primer
7.0 µl	Water (Sigma)
10.0 µl	BioMix (Bioline)