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MyoD TRANSCRIPTION FACTOR INDUCES MYOGENESIS BY INHIBITING TWIST-1 THROUGH miR-206

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DEMETRIS KOUTALIANOS

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DEMETRIS KOUTALIANOS

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Demetris Koutalianos

……..

ABSTRACT (in Greek)

Η Twist-1 εκφράζεται ως επί το πλείστον κατά την ανάπτυξη και έχει προηγουμένως συνδεθεί με τον έλεγχο της μυογένεσης. Δεδομένου ότι ο ρόλος της στη μυογένεση δεν έχει αξιοποιηθεί πλήρως, ο στόχος της μελέτης ήταν να προσδιορίσει miRNAs στο μυ που ρυθμίζουν τη Twist-1. Ένα από τα πιο σημαντικά myomiRs, το miR-206, είναι αναγνωρισμένο ως ένα πιθανό υποψήφιο για το Twist-1 mRNA. Προσδιορισμοί λουσιφεράσης και επιμολύνσεις σε εμβρυϊκούς ανθρώπινους μυοβλάστες, έδειξαν ότι η Twist-1 είναι ένας άμεσος στόχος για το miR-206 και μέσω αυτής της οδού προωθείται η διαφοροποίηση των μυϊκών κυττάρων. Επίσης διερευνήθηκε εάν το MyoD, ένας σημαντικός μεταγραφικός παράγοντας της μυογένεσης, ρυθμίζει τη Twist-1, αφού είναι γνωστό ότι επάγει την γονιδιακή έκφραση του miR-206. Βρήκαμε ότι η υπέρ-έκφραση του MyoD προκαλεί αύξηση του miR-206 και μείωση της έκφρασης της Twist-1, μέσω του miR-206 και προάγει την αύξηση της διαφοροποίησης των μυϊκών κυττάρων. Τέλος, τα πειράματα πραγματοποιήθηκαν σε μυϊκά κύτταρα από ασθενείς με μυοτονική δυστροφία τύπου 1, τα οποία αποτυγχάνουν να διαφοροποιηθούν προς μυοσωληνάρια. Η υπέρ-έκφραση του MyoD ανέστειλε την έκφραση της Twist-1 μέσω του miR-206, που επίσης ακολουθείται από μια αύξηση στη διαφοροποίηση των μυϊκών κυττάρων. Τα αποτελέσματα αυτά αποκαλύπτουν ένα νέο μηχανισμό που εμπλέκεται στη μυογένεση, που θα μπορούσε επίσης να διαδραματίσει σημαντικό ρόλο στη μυϊκή νόσο.

ABSTRACT (in English)

Twist-1 is mostly expressed during development and has been previously shown to control myogenesis. Since its regulation in muscle has not been fully exploited, the aim of the project was to identify miRNAs in muscle which regulate Twist-1. miR-206, one of the most important myomiRs, was identified as a possible candidate for Twist-1 mRNA. Luciferase assays and transfections in human foetal myoblasts showed that Twist-1 is a direct target for miR-206 and through this pathway muscle cell differentiation is promoted. We next investigated whether MyoD, a major myogenic transcription factor regulates Twist-1, since it is known that MyoD induces *miR-206* gene expression. We found that forced MyoD expression induces miR-206 up-regulation and Twist-1 down-regulation through miR-206 promoter binding, followed by increase in muscle cell differentiation. Finally, experiments were performed in muscle cells from patients with congenital Myotonic Dystrophy type 1 which fail to differentiate to myotubes. MyoD overexpression inhibited Twist-1 through miR-206 induction, followed by an increase in muscle cell differentiation. These results reveal a novel mechanism of myogenesis which might also play an important role in muscle disease.

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TABLE OF CONTENTS

ABSTRACT (in Greek) ..... **iii**

ABSTRACT (in English) ..... **iv**

ACKNOWLEDGEMENTS ..... **v**

1. INTRODUCTION **1**

1.1 Muscle 2

1.2 Myogenesis 3

1.2.1 Myogenesis during embryogenesis 3

1.2.2 Myogenesis in adult 6

1.2.3 Identification of satellite cells 7

1.3 Regulation of myogenesis by Transcription Factors 8

1.3.1 The Basic Helix-Loop-Helix transcription factors 8

1.3.2 Myogenic regulatory factors (MRFs) 9

1.3.3 The role of the MRFs in skeletal muscle development and differentiation 9

1.3.4 Expression of MRFs during embryonic development 9

1.3.5 Regulation of skeletal muscle cell differentiation 10

1.4 Twist transcription factor 13

1.4.1 Twist discovery 13

1.4.1.1 Human TWIST (H-TWIST) gene homologue 13

1.4.2. Twist transcriptional activity 14

1.4.3. Expression of Twist in different tissues 15

1.4.3.1 Twist expression during embryonic development 16

1.4.3.2 Role of Twist in cell differentiation 16

1.4.4 Twist's function during muscle differentiation 17

1.4.5 Implication of Twist in diseases 20

1.5 Non-coding RNA 21

1.5.1 RNA interference (RNAi) 21

1.5.1.1 Small interfering RNA (siRNA) 22

1.5.1.2 microRNAs 23

1.5.1.2.1 Biogenesis of miRNAs 24

1.5.1.2.2 Target recognition 27

1.5.1.2.3 Inhibition or cleavage by the RISC 28
1.5.1.2.4 MicroRNA stability 29
1.5.2 miRNAs in skeletal muscle differentiation 29
1.5.2.1 Non muscle specific miRNAs in muscle 29
1.5.2.2 Muscle specific miRNAs 30
1.5.2.3 mRNA targets of myomiRs 32
1.5.2.3.1 miR-206 32
1.5.2.3.2 miR-1 32
1.5.2.3.3 miR-133 32
1.5.2.4 Transcriptional regulation of myomiRs 33
1.6 Muscular diseases 34
1.6.1 Myotonic Dystrophy Type 1 (DM1) 35
1.6.1.1 Mechanisms of DM1 pathogenesis 36
1.6.1.1.1 RNA pathogenesis 36
1.6.1.1.2 Mis-regulation of alternative splicing in DM1 36
1.6.1.1.3 Myogenic defects 39
1.7 Hypothesis and specific objectives 41
1.7.1 Hypothesis 41
1.7.2 Objectives of the proposed research project 41
1.7.3 Importance and innovation 42

2. MATERIALS & METHODS 43
2.1 Primary mouse muscle satellite cell isolation 44
2.1.1 Removal of Extensor digitorum longus (EDL) muscle from mouse 44
2.1.2 Isolation of single muscle fibers 45
2.1.3 Plating of satellite cells 46
2.2 Culture of Cell lines 47
2.2.1 Cell propagation 47
2.2.1.1 Passaging of cultured cells 48
2.2.1.2 Freezing of cell lines for storage 48
2.2.1.3 Recovery of cultured cells from liquid nitrogen 48
2.2.2 Transfections of cultured cells. 49
2.2.3 Transductions of cell lines with adenoviruses 50
2.2.4 Cell harvesting 50

2.3 Immunocytochemistry 50
2.4 Protein analysis 51
2.4.1 Protein extraction 51
2.4.2 Measurement of protein concentration 52
2.4.3 Western Blotting 52
2.4.3.1 Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) 52
2.4.3.2 Protein transfer to a polyvinylidene difluoride (PVDF) membrane 53
2.4.3.3 Blocking and antibody incubation of the membrane 53
2.4.3.4 Visualization of the target protein 54
2.5 Chromatin Immunoprecipitation (ChIP) Assay 55
2.5.1 Coupling of Dynabeads® to primary antibody 55
2.5.2 Crosslinking of chromatin 55
2.5.3 Cell lysis 56
2.5.4 Chromatin shearing 56
2.5.5 Chromatin dilution 56
2.5.6 Chromatin binding to the Antibody-Dynabeads® 57
2.5.7 Chromatin-Antibody-Dynabeads® washing 57
2.5.8 Reversing the crosslinking of the chromatin 57
2.5.9 Magnetic purification of the un-cross-linked DNA 58
2.5.10 Polymerase Chain Reaction (PCR) analysis of the eluted DNA 58
2.5.11 Vertical polyacrylamide gel electrophoresis 60
2.6 Production of human TWIST (H-TWIST) 3 UTR/luciferase plasmid 61
2.6.1 Cloning of H-TWIST 3 UTR/luciferase plasmid 61
2.6.1.1 PCR Amplification of Twist 3' UTR 61
2.6.1.2 Insertion of the PCR product to TOPO Vector 62
2.6.1.3 Transformation of the DNA plasmid in DH5α competent cells 62
2.6.1.4 Small scale preparation of DNA plasmids 63
2.6.1.5 Digest of Luciferase plasmid and TOPO plasmid/Ligation 63
2.6.1.6 Large scale preparation of DNA plasmids 64
2.6.2 Site-directed mutagenesis of DNA plasmids 65
2.6.3 Luciferase Assay 68
2.7 Nucleic acid isolation 68
2.7.1 RNA extraction from mammalian cells 68

2.7.1.1 RNA quantification 69
2.7.1.2 Reverse transcription (RT) 69
2.7.1.3 Polymerase Chain Reaction (PCR) analysis of the cDNA 69
2.7.2 MiRNA extraction 71
2.7.2.1 Reverse transcription (RT) for miRNA 71
2.7.2.2 Real-Time PCR 72
2.8 Statistical analysis 73
2.8.1 Statistical analysis of Real time PCR 73

3. RESULTS 74
3.1. Prediction of Twist-1 miRNAs 75
3.1.1 Introduction 75
3.1.2 Results 76
3.1.2.1 Predicted miRNAs target human Twist-1 3' UTR 76
3.1.3 Conclusions 78
3.2 Experimental validation of the binding of miR-206 on Human Twist-1 3'UTR 79
3.2.1 Introduction 79
3.2.2 Results 79
3.2.2.1 Cloning of Twist-1 3'UTR into luciferase reporter plasmid 79
3.2.2.2 miR-206 represses Twist-1 through its 3'-UTR. 85
3.2.3 Conclusion 87
3.3 miR-206 regulates Twist-1 expression and muscle cell differentiation. 88
3.3.1 Introduction 88
3.3.2 Results 88
3.3.2.1 miR-206 regulates differentiation through the regulation of Twist-1 88
3.3.3 Conclusion: 95
3.4 MyoD promotes muscle cell differentiation through upregulation of miR-206 and downregulation of Twist-1 95
3.4.1 Introduction: 95
3.4.2 Results 95
3.4.2.1 MyoD overexpression induces miR-206 Levels 95
3.4.2.2 MyoD induces miR-206 levels through its promoter 96
3.4.2.3 MyoD promotes differentiation through Twist-1 inhibition 98

3.4.2.4 MyoD inhibits Twist and promotes differentiation through miR-206 101

3.4.3 Conclusion 106

3.5 MyoD/miR-206/Twist-1 mechanism is involved in Myotonic Dystrophy type 1 cells which exhibit defective differentiation. 106

3.5.1 Introduction 106

3.5.2 Results 107

3.5.2.1 MyoD protein and miR-206 Levels are inhibited and Twist-1 protein levels are induced in DM1 myoblasts 107

3.5.2.2 MyoD/miR-206/Twist-1 pathway is inhibited in DM1 myoblast 107

3.5.3 Conclusion 110

4. DISCUSSION 111

5. REFERENCES 118

6. APPENDIX 150

LIST OF FIGURES

Figure 1.1 Structure of skeletal muscle **3**
Figure 1.2 Origin of myogenic progenitor cells. **5**
Figure 1.3 Skeletal muscle cell differentiation. **12**
Figure 1.4 Structure of H-TWIST. **14**
Figure 1.5 MiRNA biogenesis and function **26**
Figure 1.6 Genomic locations of the human myomiRs and their sequence homologies. **31**
Figure 1.7 Consequences of the CUG repeat expansions. **38**
Figure 2.1 Diagram of the mouse hind leg with major muscles shown **45**
Figure 2.2 Diagram of muscle fiber with satellite cells. **46**
Figure 2.3 Transfer of proteins from gel to membrane. **54**
Figure 2.4 Overflow of ChIP assay **60**
Figure 3.1 Predicted miRNA candidates for human Twist-1 3'UTR. **76**
Figure 3.2 Predicted conserved miRNA for human Twist-1 3'UTR. **77**
Figure 3.3 Twist 3' UTR map. **78**
Figure 3.4 mir-206 binding site conservation. **78**
Figure 3.5 The pmiR-luciferase-Twist-1 3'UTR construct. **80**
Figure 3.6 Genomic sequence of Twist-1 3'UTR. **81**
Figure 3.7 DNA amplification for Twist-1 3'UTR. **81**
Figure 3.8 Screening for positively TOPO cloned plasmids. **82**
Figure 3.9 Digested of the TOPO and Luciferase plasmid. **83**
Figure 3.10 Screening for positively cloned plasmids. **84**
Figure 3.11 Sequencing of Twist-1 3'UTR in pmiR-report luciferase. **84**
Figure 3.12 miR-206 repress Luciferase expression. **85**
Figure 3.13 Mutation on predicted miR-206 binding site. **86**
Figure 3.14 miR-206 specific binds to the Twist-1 3'UTR. **86**
Figure 3.15 miR-206 reduces the Luciferase levels. **87**
Figure 3.16 Twist-1 inhibits muscle cell differentiation. **89**
Figure 3.17 Twist-1 inhibition promotes muscle cell differentiation. **90**
Figure 3.18 miR-206 downregulates Twist-1 expression. **91**
Figure 3.19 Overexpression of miR-206 downregulates Twist-1 expression. **91**
Figure 3.20 Overexpression of miR-206 promotes muscle cell differentiation. **92**
Figure 3.21 Downregulation of miR-206 upregulates Twist-1 expression. **93**

Figure 3.22 Downregulation of miR-206 inhibits muscle cell differentiation. 94
Figure 3.23 MyoD overexpression. 96
Figure 3.24 MyoD overexpression induces miR-206 levels. 96
Figure 3.25 miR-206 promoter and E-boxes. 97
Figure 3.26 MyoD binds to E-boxes on miR-206 promoter. 97
Figure 3.27 MyoD promotes miR-206 expression. 98
Figure 3.28 MyoD overexpression inhibits Twist-1 expression. 99
Figure 3.29 Overtake the Twist-1 inhibition. 99
Figure 3.30 MyoD regulates muscle cell differentiation through inhibition of Twist-1. 100
Figure 3.31 MyoD regulates downregulation of Twist-1 by miR-206. 101
Figure 3.32 MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206. 102
Figure 3.33 Twist-1 is expressed in 4 weeks-old mouse. 103
Figure 3.34 MyoD regulates downregulation of Twist-1 by miR-206 in primary myoblast. 104
Figure 3.35 MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206 in primary mouse myoblasts. 105
Figure 3.36 Suggested pathway. 106
Figure 3.37 MyoD and miR-206 are supressed in DM1 while Twist-1 levels are decreased. 107
Figure 3.38 MyoD regulates downregulation of Twist-1 by miR-206 in DM1 myoblasts. 108
Figure 3.39 MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206 in DM1 myoblasts. 109

LIST OF TABLES

Table 2.1 Primers used in PCR after ChiP assay..... 59
Table 2.2 PCR- thermal cycle used for PCR after ChiP assay. 59
Table 2.3 Primers for amplification of human Twist-1 3'UTR. 61
Table 2.4 Thermal cycle of PCR for amplification of human Twist-1 3' UTR. 62
Table 2.5 Reagents used in mutagenesis reaction. 66
Table 2.6 Primers used in mutagenesis reactions. 66
Table 2.7 Thermal cycle PCR used for mutagenesis. 67
Table 2.8 Recombination reaction of mutated plasmid before transformation. 67
Table 2.9 Primers of PCRs. 70
Table 2.10 Thermal-cycle reaction of PCR. 70
Table 2.11 Annealing Temperatures for PCRs. 70
Table 2.12 Thermal cycle for Reverse transcriptase of miRNA. 72
Table 2.13 Thermal cycle for Real-Time PCR. 73

ABBREVIATION LIST

3’ UTR	3’ Untranslating Region
amiR	Antagomir
ATP	Adenosine triphosphate
bHLH	Basic Helix-Loop-Helix
BIN1	Bridging integrator-1
BMD	Becker muscular dystrophy
bp	Base pair
Cdks	Cyclin-dependent Kinases
CDM1	Congenital muscular dystrophy type 1
ChIP	Chromatin Immunoprecipitation
CIC-1	Chloride channel-1
cTNT	Cardiac troponin T
CUGBP1	CUG binding protein 1
Cx43	Connexin43
DGCR8	DiGeorge syndrome critical region gene 8
DM	Differentiation medium
DM1	Myotonic Dystrophy type 1
DM2	Myotonic dystrophy Type 2
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco’s Modified Eagle Medium
DMPK	Dystrophia myotonica protein kinase
Dpc	Days postcoitum
D-Twist	Drosophila Twist
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factor
Foxo3a	Forkhead box O3
FSHD	Facioscapulohumeral
GM	Growth medium
H_20_2	Hydrogen peroxide
HAT	Histone acetyltansferase
HGF	Hepatocyte growth factor
HIV-1	Human immunodeficiency virus 1
H-Twist	Human Twist
IGF	Insulin growth factor
IL-6	Interleukin-6
IR	Insulin receptor
lncRNA	Long non-coding RNA
miRNA	microRNA
MOI	Multiplicity of infection
MRE	miRNA response element
MRFs	Myogenic regulatory factors
MSC	Mesenchymal stem cell
MTMR1	Myotubularin-related 1
M-Twist	Mouse Twist
MyCH	Myosin heavy chain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B

	cells
NO	Nitric Oxide
OPMD	Oculopharyngeal muscular dystrophy
PCR	Polymerase Chain Reaction
piRNA	Piwi-interacting RNA
PKC	Putative protein kinase C
PLB	Passive Lysis Buffer
pRb	Retinoblastoma protein
PVDF	Polyvinylidene difluoride
RISC	RNA inducing silencing complex
rRNA	Ribosomal RNA
RT	Reverse Transcription
RT-PCR	Real-Time PCR
RyR1	Ryanodine receptor 1
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl PolyAcrylamide Gel Electrophoresis
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SIP	Sphingosine-1-phosphate
siRNA	Small interfering RNA
snoRNA	Small nuclear RNA
SRF	Serum response factor
TA	Tibialis anterior
TBE	Tris-borate
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
tRNA	Transfer RNA
UV	Ultraviolent
WWS	Walker-Warburg syndrome
X-Twist	Xenopus Twist
3’ UTR	3’ Untranslating Region
amiR	Antagomir
ATP	Adenosine triphosphate
bHLH	Basic Helix-Loop-Helix
BIN1	Bridging integrator-1
BMD	Becker muscular dystrophy
bp	Base pair
Cdks	Cyclin-dependent Kinases
CDM1	Congenital muscular dystrophy type1
ChIP	Chromatin Immunoprecipitation
CIC-1	Chloride channel-1
cTNT	Cardiac troponin T
CUGBP1	CUG binding protein 1
Cx43	Connexion43
DGCR8	DiGeorge syndrome critical region gene 8
DM	Differentiation medium
DM1	Myotonic Dystrophy type 1
DM2	Myotonic dystrophy Type 2
DMD	Duchene muscular Dystrophy
DMEM	Dulbecco’s Modified Eagle Medium

DMPK	Dystrophia myotonica protein kinase
Dpc	Days postcoitum
D-Twist	Drosophila Twist
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factor
Foxo3a	Forkhead box O3
FSHD	Facioscapulohumeral
GM	Growth medium
H202	Hydrogen peroxide
HAT	Histone acetyltansferase
HGF	Hepatocyte growth factor
HIV-1	Human immunodeficiency virus 1
H-Twist	Human Twist
IGF	Insulin growth factor
IL-6	Interleukin-6
IR	Insulin receptor
lncRNA	Long non-coding RNA
miRNA	microRNA
MOI	Multiplicity of infection
MRE	miRNA response element
MRFs	Myogenic regulatory factors
MSC	Mesenchymal stem cell
MTMR1	Myotubularin-related 1
M-Twist	Mouse Twist
MyCH	Myosin heavy chain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric Oxide
OPMD	Oculopharyngeal muscular dystrophy
PCR	Polymerase Chain Reaction
piRNA	Piwi-interacting RNA
PKC	Putative protein kinase C
PLB	Passive Lysis Buffer
pRb	Retinoblastoma protein
PVDF	Polyvinylidene difluoride
RISC	RNA inducing silencing complex
rRNA	Ribosomal RNA
RT	Reverse Transcription
RT-PCR	Real-Time PCR
RyR1	Ryanodine receptor 1
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl PolyAcrylamide Gel Electrophoresis
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SIP	Sphingosine-1-phosphate
siRNA	Small interfering RNA
snoRNA	Small nuclear RNA
SRF	Serum response factor
TA	Tibialis anterior
TBE	Tris-borate

TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
tRNA	Transfer RNA
UV	Ultraviolent
WWS	Walker-Warburg syndrome
X-Twist	Xenopus Twist

1

INTRODUCTION

1.1 Muscle

Muscle is a type of soft tissue found along the animal kingdom. It is one of the four major types of tissues in animals and is divided in three types of muscle tissues: skeletal muscle, cardiac muscle and smooth muscle. Muscle tissues contain filaments of actin and myosin that slide one over the other and contribute to muscle contraction thus changing its shape and length. The main function of muscle is to create motion and force.

Cardiac muscle, also known as myocardium, is an "automatic muscle" found in the heart. “Automatic muscle” is an involuntary muscle that regulates the contraction of the muscle in an autonomous manner. Cardiac muscle controls the involuntarily contraction of the ventricles of the heart, thus forcing blood to circulate through the arteries to the whole body. Smooth muscle is also an "automatic muscle" found within the walls of organs and structures, such as the intestines, blood vessels, oesophagus, bronchi, urethra, uterus, stomach and the arrector pili in the skin.

The skeletal muscle is the only voluntary muscle tissue. The most of the skeletal muscles are connected to at least two bones by tendons, a fibrous connective tissue to efficiently promote movement (Figure 1.1). There are however, other muscles that are not attached to bones such as some facial muscles that attached to skin. Skeletal muscle is controlled via the nervous system by sending electrical impulses to the muscle and promotes its contraction. Under physiological conditions, an adult male contains approximately 42% of skeletal muscle of total body mass and a female approximately of 36%.

Skeletal muscle consists of plenty of individual components known as muscle fibers, which are the largest cells in the animal body. Mononucleated cells fuse together to form the multinucleated muscle fibers (Figure 1.1). Muscle fibers consist of myofibrils which are rod-like elements, which are in turn composed of repeated fundamental units known as sarcomeres (Brooks, 2003). The two main proteins that make up the sarcomeres are: actin, which is the thin filament, and myosin, which is the thick filament. These two proteins are responsible for the mechanism necessary for muscle contraction. Muscle fibers have been divided into two categories based on histochemical analysis: type I, which are the slow muscle fibers, and type II which are the fast muscle fibers (Schantz, 1986). Skeletal muscle tissue, like all other tissues, is formed during embryo development through a process known as myogenesis, which is also induced upon stimulation in adults during the regeneration of skeletal muscle.

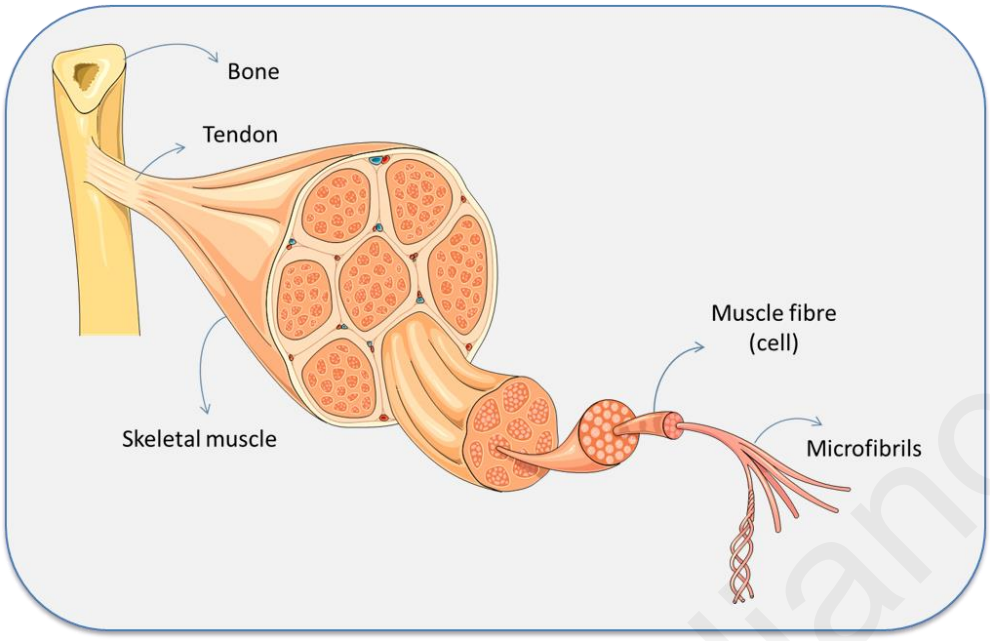


Figure 1.1 Structure of skeletal muscle. Skeletal muscle is made up of hundreds or thousands of individual elongated components called muscle fibers, which are composed of hundreds of rod-like elements, known as myofibrils. Most of the skeletal muscles are attached to bones by tendons. Adapted from http://training.seer.cancer.gov/anatomy/muscular/structure.html.

1.2 Myogenesis

Myogenesis is the process that forms the muscular tissue. Myogenesis is a complex and tightly regulated procedure. During myogenesis, the myoblasts are pulled out from the cell cycle by the stimulation of the expression of the myogenic differentiation-specific genes and fused together to form multi-nuclei fibers called myotubes (Sabourin and Rudnicki, 2000). Myogenesis occurs during different stages of human development. Specifically, myogenesis takes place during the embryogenesis in order to form the muscle tissue, and in adults to replace damaged or lost muscle.

1.2.1 Myogenesis during embryogenesis

After the fertilization of the oocyte, the embryo is divided in two lineages, the embryonic and the extra-embryonic. After the 7th cleavage of the cells division, the embryo is called blastula and it is a spherical layer of cells with a yolk-filled or fluid-filled cavity. Mammals at this stage form the blastocyst, which has an inner cell mass. At this stage, the outer layer of blastula forms the trophoblast, which later forms the placenta. The inner cell mass is called embryoblast and forms the embryo. The embryoblast then arises to the epiblast, which is an epithelial disc with a thin layer of cells called the hypoblast. All the tissues that will form the embryo derive from the epiblast. The hypoblast will form the extra-embryonic structure which includes the yolk sac and also provides three-dimensional signals for the epiblast as it forms its primary axes. The next step is the

gastrulation. At this stage, the epiblast cells move into the primitive streak, where they change from epithelial to mesenchymal cells, thus losing their epithelial characteristics, for example the cell-cell adhesion. This mass of cells is divided into the three germ layers of the embryo (endoderm, mesoderm and ectoderm) forming the structure called the trilaminar embryo (Narasimha and Leptin, 2000, Nakaya and Sheng, 2008).

Each of the three layers gives rise to specific tissues and organs during the embryonic development. For example, ectoderm layer produces: neural tube, epidermis, neural plate and other tissues that will later form the nervous system (Chang and Hemmati-Brivanlou, 1998). The endoderm layer, produces the epithelial tissue of the stomach, intestines, oesophagus and lungs, as well as important parts of many glands such as the pancreas, thyroid, liver and thymus (Grapin-Botton and Constam, 2007). The mesoderm layer begins to segment in order to form somites, while at the posterior of the embryo, the tail spot, remains extending, generating more mesoderm (Parker et al., 2003). Somites develop and form different compartments of the embryo depending on their location according to the axial neural tube, the notochord and the signals received from their environment (Parker et al., 2003). These compartments are called the sclerotome. From the sclerotome arise the vertebrae, and the dermomyotome, from which dermis and muscle are formed. The dermomyotome, a naturally 'C'-shaped transient epithelial structure, consists of a mixture of myogenic and dermal progenitor cells. These cells form the dermatome and the myotome by subsequent separations (Christ et al., 2007) (Figure 1.2).

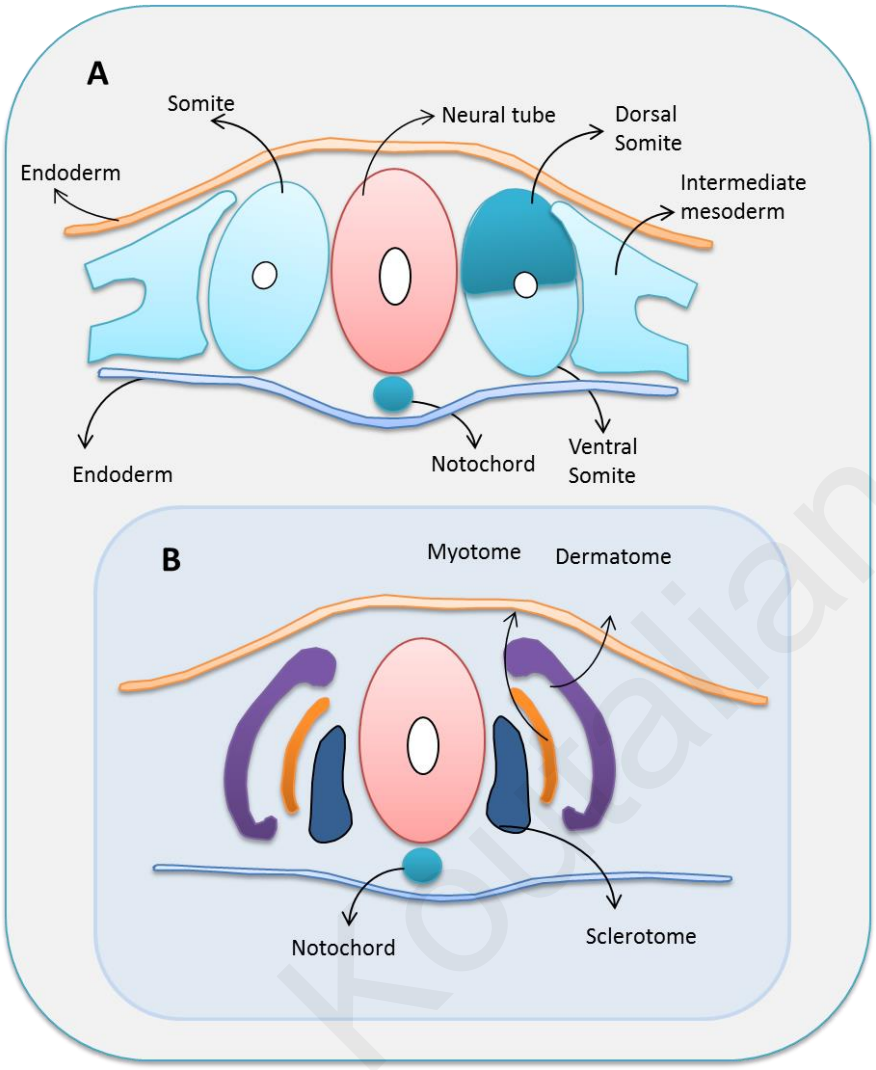


Figure 1.2 Origin of myogenic progenitor cells. (A) The spatial arrangement of mesodermal derivatives (blue), ectodermal derivatives (orange) and endoderm (yellow). The dorsal somite will give rise to the dermomyotome, and the ventral somite will produce the sclerotome. (B) The spatial organization of somite compartments in the embryo as somites undergo differentiation into the dermomyotome (red), which contains undifferentiated myogenic progenitor cells, and the sclerotome (blue). Somites maturation will produce the myotome (green), where myogenesis takes place for the first time. Adapted from Fedon et al 2012.

During embryogenesis the myogenic progenitor cells arise from the somites. Somites are consensually combined blocks of mesoderm that form along the anterior-posterior axis of the developing embryo (Buckingham, 2001, McKinnell and Rudnicki, 2005). Somites differentiate along the dorsal–ventral axis to give rise to the dorsally located epithelial dermomyotome and the ventrally located mesenchymal sclerotome (Parker et al., 2003). The dermomyotome gives rise to dermis and musculature, whereas the sclerotome forms the bone and cartilage. Myogenic precursors are restrained in the epithelium of the dermomyotome and express Pax7, Pax3 and low levels of the myogenic determination factor Myf-5. Following induction, muscle progenitor cells de-epithelialise and migrate to the distal region of the dermomyotome to form the myotome, which is the third somitic compartment that contains the first differentiated myofibers (Relaix, 2006).

During the late stages of embryogenesis, a specific population of myogenic stem cells, called satellite cells, are formed in order to provide most of the myonuclei to adult muscles during the postnatal growth of muscle tissue.

1.2.2 Myogenesis in adult

In adults, myogenesis occurs in order to replace damaged muscle caused by injury or exercise, a process known as muscle regeneration. The mechanisms underlying the process of muscle regeneration in adults have many similarities with the corresponding mechanisms involved in muscle development during embryogenesis. During muscle repair, an inflammation is initially formed followed by the degeneration of the damaged muscle. Furthermore, the satellite cells that are mitotically inactive and exist in a niche under the basal lamina of adult skeletal muscle surrounding each myofiber, are activated (Mauro, 1961, Hollway and Currie, 2005). The satellite cells show limited gene expression and protein synthesis. They are able however, to self-renew and contribute to muscle growth and regeneration when they are activated (Mok and Sweetman, 2011). Satellite cells become activated in response to stress induced by weight-bearing exercise or by a trauma, such as injury (Le Grand and Rudnicki, 2007). Following their activation, the satellite cells proliferate and form a number of myoblasts, which differentiate and finally fuse to produce the myonuclei fibers required to repair or replace the damaged tissue (Moss and Leblond, 1971, Snow, 1978).

The mechanisms that control the transition between the silenced and activated satellite cells are not well-established. Many factors however, have been revealed to be involved in the activation of satellite cells. One of the stimuli which have been proposed to activate the satellite cell is the extract from the defragmented muscle fibers; which is composed by molecules that are released by invading macrophages and soluble factors of the other tissues surrounding the muscle. *In vitro* experiments showed that a number of trophic factors are involved in the regulation of the proliferation and the differentiation of the satellite cells. These factors include fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), insulin growth factor (IGF), tumor necrosis factor-α (TNF-α), nitric oxide (NO), the interleukin-6 (IL-6) family of cytokines, neural-derived factors and adenosine-5'-triphosphate (ATP) (Hawke and Garry, 2001, Charge and Rudnicki, 2004). Only few of these factors however, have been shown to be involved in the regeneration of muscle *in vivo* (Charge and Rudnicki, 2004). Another essential factor that was found to induce the process of muscle regeneration is the sphingosien-1-phosphate (SIP) that is released from the inner leaflet of the plasma membrane. The role of the S1P is to induce the satellite cells to enter the cell cycle and proliferate. Inhibition of SIP reduces the number of satellite cells able to proliferate *in vitro* and reduced the amount of regenerated muscle *in vivo* (Nagata et al., 2006).

1.2.3 Identification of satellite cells

Quiescent satellite cells that are located in adult skeletal muscle are related to the expression of a variety of proteins which are used as markers to identify and purify the satellite cells (Dhawan and Rando, 2005). Some of the most well studied proteins as markers for the satellite cells are: M-cadherin, CD34, c-met, syndecan-4, syndecan-3 and Pax7. (Dhawan and Rando, 2005). None of these proteins however, is specifically associated only to quiescent satellite cells.

Satellite cells were not found to express any of the myogenic regulatory factors (MRFs) (myogenic determination factor (MyoD), Myf-5, myogenin and Myf-6), which are the most important muscle-specific transcription factors necessary for myogenesis (Dhawan and Rando, 2005, Smith et al., 1994, Grounds et al., 1992). Some quiescent satellite cells however, were found to express Myf-5 (Beauchamp et al., 2000). The activated satellite cells were determined to express *MyoD* and afterwards Myf-6 in order to differentiate and form the muscle fibers. The specification of satellite stem cells must therefore occurs prior to the MRFs expression and probably are regulated by other developmentally defined genes (Dhawan and Rando, 2005).

Quiescent satellite cells express *Pax3* and *Pax7* genes. These two genes work as a pair for the transcriptional regulation of the myogenic progenitors during development. Pax7 however, is expressed by almost all the quiescent satellite cells of the mouse limb whereas Pax3 is expressed only in satellite cells of certain muscle groups (Seale et al., 2000, Relaix et al., 2006). Pax3 performs crucial functions during the development and its expression begins to be decreased before birth. In adults, muscle growth or regeneration after injury, quiescent satellite cells are activated and start to proliferate. This process is linked to the co-expression of *Pax* and *MyoD* genes (Buckingham, 2006). MyoD is one of the most necessary transcription factors of myogenesis and it belongs to the MRFs family. In order for the satellite cells to be differentiated into the new muscle fibers, *Pax* genes must be silenced. (Buckingham, 2006, Seale et al., 2000). Satellite cells that continue to express *Pax* genes rebuild the satellite cell compartment and return to a state similar to quiescence accompanied by the downregulation of *MyoD* (Seale et al., 2000, Buckingham, 2006). *MyoD* expression in postnatal satellite cells depends on Pax proteins, while the expression of *Myf-5*, which is already transcribed in many quiescent satellite cells, does not depend on Pax proteins. In some cases, Myf-5 is able to promote adult skeletal muscle differentiation even in the absence of MyoD (Tajbakhsh et al., 1997).

1.3 Regulation of myogenesis by Transcription Factors

Myogenesis is a multistep complex and tightly regulated procedure. During myogenesis, the myoblasts are pulled out of the cell cycle by the stimulation of the expression of the myogenic differentiation-specific genes and are fused together to form the multi-nuclei fibers called myotubes (Sabourin and Rudnicki 2000). This process is almost the same in both embryonic and postnatal myogenesis. The main difference of the process between the two stages is that during the embryonic myogenesis there are the precursor cells that are forced to determinate as myoblasts, while during the myogenesis in adults there are the quiescent satellite cells that mimic the role of the precursor cells. A second difference is that in adults the fusion occurs initially between the new myoblasts and pre-existing myotubes, while in embryonic myogenesis the fusion occurs between post mitotic myocytes.

1.3.1 The Basic Helix-Loop-Helix transcription factors

MRFs belong to the superfamily of transcription factors called basichelix-loop-helix (bHLH). bHLH transcription factors have intrinsic protein structure which consists of a basic amino acid region and two α-helices connected by a region forming a loop (Voronova and Baltimore, 1990). bHLH proteins retain two highly conserved domains: the basic domain and the helix-loop-helix domain, which are responsible for protein-protein interactions and DNA binding interactions, respectively (Atchley and Fitch, 1997). The bHLH transcription factors form either homodimer complexes or heterodimers with other transcription factors (Murre et al., 1994). The DNA binding domain recognizes a specific DNA sequence called the E-box (5'-CANNTG-3'). These E-boxes are found in promoter and enhancer regions of many lineage specific genes including muscle-specific genes, and are responsible for the regulation of many pathways (Kyriacou and Rosato, 2000).

The bHLH transcription factors are divided into two subgroups. The first group consists of proteins that are universally expressed in all types of tissues, such as E12 and E47. (Benezra, 1994, Bain et al., 1993). The second group consists of proteins that are expressed in a tissue-specific manner, such as the MRFs that are specifically expressed in muscle tissue and play a crucial role in tissue specific gene expression, cell lineage determination and cell differentiation (Massari and Murre, 2000a). bHLH transcription factors of the second group play a critical role during the regulation of developmental processes, such as myogenesis (Olson and Klein, 1994), pancreas formation (Krapp et al., 1998), haematopoesis (Baer, 1993) and neurogenesis (Guillemot, 1999). bHLH proteins can act both as transcriptional inducers such as MyoD, or as repressors such as MyoR (Lu et al., 1999b).

1.3.2 Myogenic regulatory factors (MRFs)

Skeletal myogenesis is initiated and regulated mainly by the expression of the myogenic regulatory factors (MRFs). MRFs belong to the family of skeletal muscle-specific transcription factors. MRFs are important for both determination and maintenance of skeletal muscle and belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors.

1.3.3 The role of the MRFs in skeletal muscle development and differentiation

The first member of the MRFs identified is the MyoD. This discovery revealed a new age in research in myogenesis (Davis et al., 1987). MyoD was found through its ability to convert non-muscle cells to stable myoblasts at high frequency (Davis et al., 1987, Blau et al., 1983, Wright, 1984). Following MyoD discovery, three additional MRFs were identified: Myf-5 (Braun et al., 1989), myogenin (Edmondson and Olson, 1989, Wright et al., 1989) and Myf-6 (or MRF4 or Herculin) (Rhodes and Konieczny, 1989, Braun et al., 1990, Miner and Wold, 1990). In all the cases, overexpression of the MRFs converted a massive number of non-muscle cell lines, such as fibroblasts, to myoblasts, implying their essential role in myogenic lineage determination and differentiation (Perry and Rudnick, 2000).

MRFs form heterodimers with E proteins, both *in vitro* and *in vivo*. The MRF-E protein complex binds to E-boxes present in the promoters of many skeletal muscle-specific genes thus initiating and promoting myogenesis (Lassar et al., 1991). MRFs also form homodimers or heterodimers with other bHLH transcription factors, such as the myocyte enhancer factor (MEF), and Id family of transcription factors, thus promoting the myogenic programme (Lassar et al., 1991).

1.3.4 Expression of MRFs during embryonic development

MRFs are expressed in all embryonic muscles in a temporary and distinct spatial pattern (Sabourin and Rudnicki, 2000).

In situ hybridization experiments showed that Myf-5 is expressed during murine embryogenesis. More specifically, Myf-5 is initially detected in the earliest somites in the dermomyotome, approximately 8 days postcoitum (dpc), before the expression of the other MRFs (Ott et al., 1991). The downregulation of Myf-5 begins at 11.5 dpc and at 14 dpc Myf-5 is not detectable (Ott et al., 1991). Following the expression of Myf-5, myogenin is expressed at around 8.5 dpc in the myotome compartment of the somites. The expression of myogenin is increased by 9.25 dpc in mouse embryos (Sassoon et al., 1989). MyoD is expressed at about 10.5 dpc in somites

and muscle myotome in parallel with the expression of markers of terminal differentiation, such as myosin heavy chain (MyHC) (Sassoon et al., 1989). The fourth member of the MRFs, the *Myf-6,* is expressed in biphasic pattern. Firstly, Myf-6 is momentarily expressed in the somites of the mouse embryo at 9 dpc until 12 dpc (Bober et al., 1991a). Secondly, Myf-6 is expressed in all skeletal muscles at 16 dpc, and its expression is continued at a high level after birth (Bober et al., 1991a). Moreover, in the limb bud, Myf-5 is expressed transiently between 10 and 12 dpc and this is followed by the expression of both MyoD and myogenin after 10.5 dpc, whereas Myf-6 is induced after 16 dpc (Ott et al., 1991, Sassoon et al., 1989, Bober et al., 1991b). The pattern of expression of the MRFs in the precursor cells for myotomal muscle is different to that in the precursor cells for the muscle of the limb, indicating that they function in a spatially and temporally different manner during development (Sassoon et al., 1989).

1.3.5 Regulation of skeletal muscle cell differentiation

During the process of myogenesis the mononucleated proliferating muscle cells called myoblasts, are stimulated to permanently exit the cell cycle and fuse together to form the multinucleated cells called myotubes. Myotubes are finally organized into myofibers. Various studies have discovered a strong relationship between the proteins that regulate the cell cycle progression and the myogenic transcription factors (Molkentin and Olson, 1996). The cell cycle is a tightly regulated process that takes place in cells and controls the cell division and the proliferation rate of the cells. The cell cycle is mainly regulated by the cyclins and cyclin-dependent kinases (cdks). Different cyclins and cdks are expressed constitutively throughout the cell cycle and form active complexes, thus regulating the progress of the cell cycle (Molkentin and Olson, 1996). During the cell cycle, cdk4 activated by cyclin D1, force the transition of the cells from the G1 phase of the cell cycle to the S phase. At this stage, DNA synthesis begins (Molkentin and Olson, 1996). In differentiated myotubes, the expression levels of the cdk4 and most of the cyclins, including Cyclin A, Cyclin B2, Cyclin C and Cyclin D1 are reduced to negligible (Rao et al., 1994, Rao and Kohtz, 1995). Overexpression studies showed that Cyclin D1 negatively regulates the myogenic bHLH regulators in a dose-depended manner (Rao et al., 1994). The other Cyclins, such as Cyclin A, Cyclin B1, Cyclin B2, Cyclin C, Cyclin D3 and Cyclin E that are expressed in different stages of the cell cycle than the Cyclin D1, do not show this negative regulation (Rao et al., 1994, Skapek et al., 1995). Remarkably, the expression of Cyclin D3 was found to be induced during muscle cell differentiation (Rao et al., 1994, Rao and Kohtz, 1995).

The expression of the MRFs is suppressed during the proliferation of myoblasts. Taking into consideration that the ectopic expression of Cyclin D1 during the proliferation of myoblasts inhibits MyoD activity on muscle-specific gene activation and that overexpression of bHLH transcription factors repress cell cycle progression, it can be suggested that there is an alternative

regulation between the myogenic transcription factors and the cell cycle regulatory proteins (Molkentin and Olson, 1996). The upregulation of cyclin/cdk inhibitors and retinoblastoma protein (pRb) is associated with the exit of myoblasts from the cell cycle. Upregulation of cdk inhibitors is also found to be crucial for the terminal myogenic differentiation. These inhibitors were found to play a critical role firstly in the cell cycle exit, as well as in the cells resistance to apoptosis (Wang and Walsh, 1996b) and in the stability of MyoD (Reynaud et al., 1999).

A well-established cyclin/cdk inhibitor that is involved in muscle cell differentiation is p21. P21 is upregulation during muscle cell differentiation at mRNA and protein levels as well as in its activity levels (Guo et al., 1995, Andres and Walsh, 1996). Moreover, p21 was found to form complexes with cdks, such as cdk2 and cdk4, in mature myotubes (Wang and Walsh, 1996a, Guo et al., 1995). P21 overexpression studies showed that p21 promotes the expression of muscle-specific proteins in cells kept in high concentrations in the serum (Skapek et al., 1995). P21 was determined to be induced by MyoD transcription factor. Specifically, MyoD expression was shown to activate p21 expression during differentiation of murine muscle cells and also in non-myogenic cell lines (Halevy et al., 1995, Guo et al., 1995). A trademark of the regulation of p21 during skeletal myogenesis is the preservation of its expression and activity during re-stimulation with mitogen-rich growth media, which is consistent with the permanent nature of cell cycle exit and the terminal skeletal muscle cell differentiation (Guo et al., 1995, Halevy et al., 1995, Andres and Walsh, 1996).

Other cyclin/cdk inhibitors have been identified to be possibly involved in the initiation and/or retention of the post-mitotic state of muscle cells. Such a cdk inhibitor is p18, which was found to be overexpressed in skeletal muscle cells and its expression was confirmed to be increased during muscle cell differentiation during *in vitro* experiments (Franklin and Xiong, 1996). This inhibitor therefore, may play a critical role in the initiation and/or maintenance of the permanent cell cycle exit associated with the terminal skeletal muscle cell differentiation (Franklin and Xiong, 1996).

A complex that was found to be involved in muscle cell differentiation is the E2F-pRB complex. The *Rb* gene is a tumor suppressor gene, that inhibits the cell cycle progression and maintains the post-mitotic state of the cells (Gu et al., 1993). Cdks are regulators of Rb activity. Specifically, cdks phosphorylate Rb and do not allow the Rb to interact with other proteins and therefore its cell cycle inhibitory activity is repressed and cell proliferation is promoted (Weinberg, 1995). One of the proteins that bind to Rb protein is the transcription factor E2F whose function is to activate a variety of genes required for cell proliferation (Weinberg, 1995). During cell division, the Rb protein is dephosphorylated and as a result a complex with E2F transcription factor is formed thus inhibiting cell cycle progression (Molkentin and Olson, 1996). In fully differentiated myotubes of the C2C12 mouse cell line, the presence of E2F-pRB complexes was confirmed by

using electrophoretic mobility shift assays (Corbeil et al., 1995). In addition, *in vivo* experiments performed in mice indicated that Rb protects myotubes from apoptosis (Zacksenhaus et al., 1996).

The permanent withdrawal of the proliferating myoblasts from the cell cycle is followed by a series of events to finally form the mature muscle fibers. During myogenesis the four MRFs play the main role in the regulation of these events. In order to understand the function of the MRFs in myogenesis, *in vivo* studies were performed using gene targeting experiments against the four MRFs, MyoD, Myf-5, myogenin and Myf-6 (Sabourin and Rudnicki, 2000). Mice with null mutation of individual MRFs, as well as combination of the MRFs were generated in order to provide evidence of a graded association between the four MRFs (Sabourin and Rudnicki, 2000).

The results from gene targeting experiments discovered a hierarchical role of the four MRFs during the process of skeletal muscle formation (Rudnicki et al., 1992, Donoviel et al., 1996, Braun et al., 1992, Rudnicki et al., 1993, Hasty et al., 1993, Nabeshima et al., 1993, Wang et al., 1996, Wang and Jaenisch, 1997, Mastroyiannopoulos et al., 2012, Braun and Arnold, 1995, Zhang et al., 1995). MRFs can be divided into two categories based on their function and time of expression. The primary MRFs, MyoD and Myf-5 are expressed in the early stage of myogenesis and are responsible for the establishment of muscle identity. The later expressed secondary MRFs, myogenin and Myf-6 play a crucial role as differentiation factors (Sabourin and Rudnicki, 2000) (Figure 1.3). Moreover, gene targeting experiments have provided evidence for the presence of potential redundancy among the MRFs, since some MRFs can substitute for the function of other MRFs (Weintraub, 1993, Sabourin and Rudnicki, 2000).

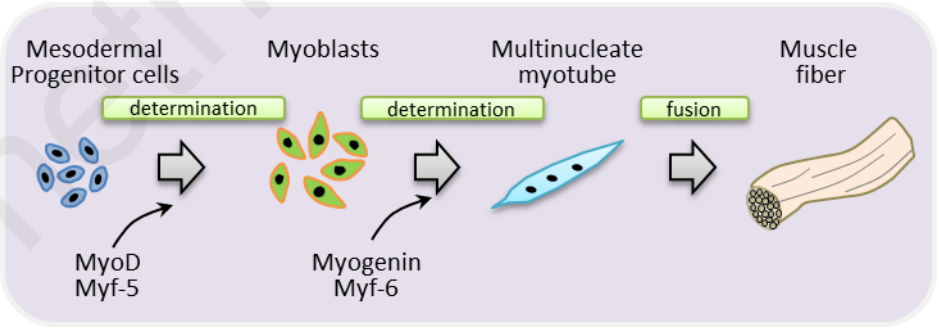


Figure 1.3 Skeletal muscle cell differentiation. The myogenic determination MRFs, MyoD and Myf-5, are required for the commitment of the proliferating mesodermal progenitor cells to the myogenic lineage. The committed cells (myoblasts) proliferate and differentiate into multinucleated myotubes under the action of the differentiation MRFs, myogenin and Myf-6. Multinucleated myotubes eventually fuse into muscle fibers. Adapted from Sabourin and Rudnicki 2000.

1.4 Twist transcription factor

MRFs and cell cycle regulators are not the only proteins that regulate the process of myogenesis. Other transcription factors are also involved in muscle cell differentiation, either by promoting or inhibiting the differentiation process. One of these transcription factors is the Twist which belongs to the superfamily of bHLH transcription factors.

1.4.1 Twist discovery

Twist was initially discovered in *Drosophila* (D-Twist). D-Twist was identified as one of the zygotic genes necessary for the establishment of dorso-ventral patterning in the early embryo (Thisse et al., 1987, Thisse et al., 1988). After *Drosophila*, *Twist* was identified in *Xenopus, X-twist, during the* early stage of the development of the mesoderm (Hopwood et al., 1989). The mouse *Twist*, *M-Twist*, gene was cloned and sequenced in 1991. The *M-Twist* is about 48% homologous to the *D-Twist*, and was found to be expressed the most at 9.5 days in the cephalic and branchial mesectoderm, in some derivatives of the mesodermal layer and in the limb buds (Wolf et al., 1991). In 1997 the human *Twist, H-TWIST*, gene was cloned and characterized (Wang et al., 1997).

1.4.1.1 Human TWIST (H-TWIST) gene homologue

The human *TWIST* (*H-TWIST*) gene was cloned in 1997 by Wang and colleagues (Wang et al., 1997). The sequencing of the clone H-Twist, which was about 2.9 Kb long, is homologous in high level with other species in both non coding and coding regions of the gene (Wang et al., 1997). For example, mouse and human Twist coding region is up to 92 % homologous (Wang et al., 1997, Wolf et al., 1991). Furthermore, the promoter regions of mouse and human *Twist* genes share about 71% nucleotide sequence homology (Wang et al., 1997, Wolf et al., 1991). The putative promoter sequence of *Twist* gene is located about -824 bp upstream of the transcription initiation site. The promoter of *H-Twist* gene contains consensus DNA sequence motifs to which known regulatory proteins might bind, such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which is 100% conserved in the corresponding region of the *M-Twist* gene (Wang et al., 1997, Wolf et al., 1991). Twist is very homologous among *Drosophila*, mouse, *Xenopus* and human species in protein level (Hopwood et al., 1989, Thisse et al., 1988, Wang et al., 1997, Wolf et al., 1991). The H-TWIST protein consists of 201 amino acids, whereas those of the *Xenopus*, mouse and *Drosophila* consist of 166, 206 and 490 amino acids respectively (Wang et al., 1997, Hopwood et al., 1989, Thisse et al., 1988, Wolf et al., 1991). In more detail, conservation of the amino acid sequences can be found throughout the entire length of the proteins (Wang et al.,

1997). There is a 100% conservation among the four species in the DNA-binding domain of the Twist protein suggesting that its binding to E-boxes may be the same in different cell type-specific genes (Wang et al., 1997, Hopwood et al., 1989, Thisse et al., 1988, Wolf et al., 1991) (Figure 1.4). The loop region of the HLH domain is also 100% conserved among the four species. The HLH region is however, 68% conserved among the species (Wang et al., 1997, Hopwood et al., 1989, Thisse et al., 1988, Wolf et al., 1991). Through sequence analysis a number of potential phosphorylation sites were identified in H-Twist protein. Six of them were detected as putative protein kinase C (PKC) sites and four of the six were found to be conserved among the three of the four species (human, mouse and *Xenopus)* implying that phosphorylation processes may regulate the function of H-TWIST (Wang et al., 1997).

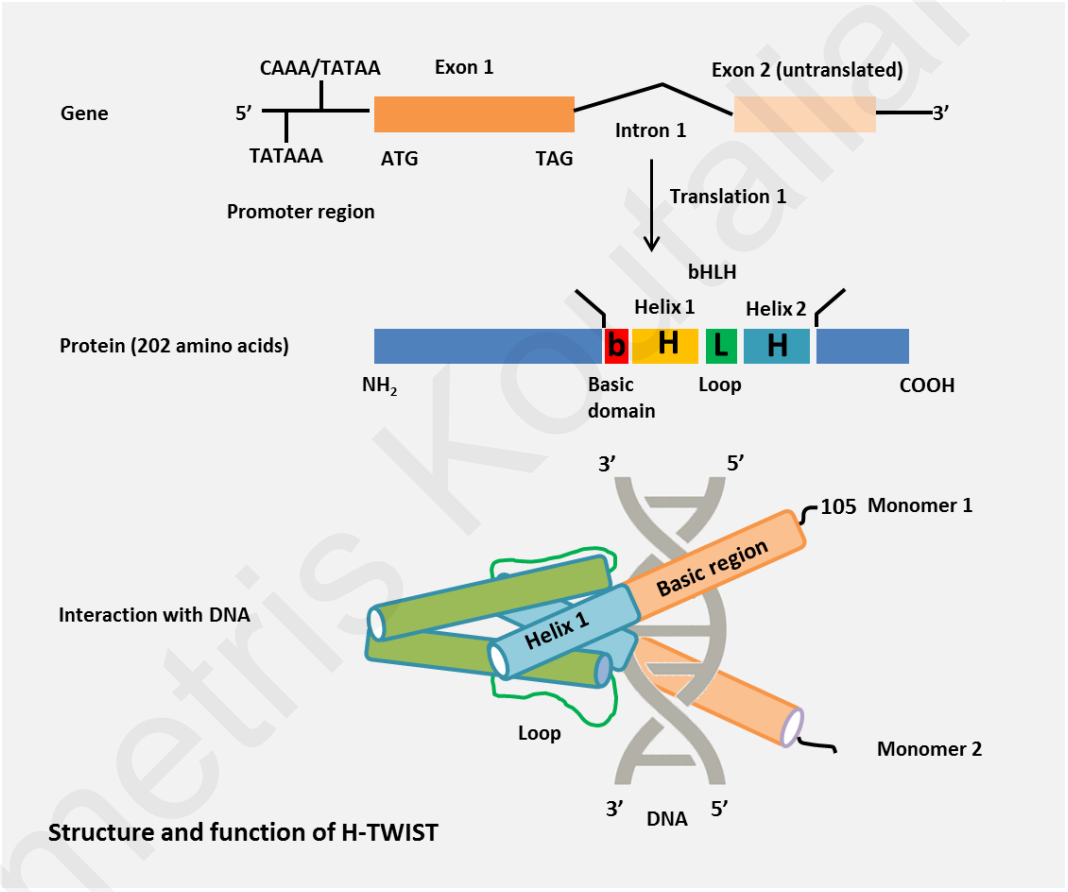


Figure 1.4 Structure of H-TWIST. The *H-TWIST* gene consists of two exons separated by an intron and is translated into a bHLH protein. H-TWIST protein dimerises through its HLH domain and binds to DNA via its basic domain. Adapted from Bonaventure and El Ghouzzi 2003

1.4.2. Twist transcriptional activity

Twist is involved in many transcriptional mechanisms which are multifaceted (Laursen et al., 2007, Miraoui and Marie, 2010). Twist belongs to the superfamily family of the second group of bHLH transcription factors. In mammals, there are six Twist orthologs, Twist (or Twist-1)

(Thisse et al., 1987, Thisse et al., 1988), Twist-2 (or Dermo-1) (Li et al., 1995), Hand1 (or eHAND, Hxt, Thing1) (Cserjesi et al., 1995b, Cross et al., 1995, Firulli, 2003), Hand2 (or dHAND, Hed, Thing2) (Cross et al., 1995, Firulli, 2003), Paraxis (Wilson-Rawls et al., 2004) and Scleraxis (Cserjesi et al., 1995a) which can form stable and functional homodimers or heterodimers with other bHLH transcription factors of the same group or with the broadly expressed bHLH E proteins. The formed complexes of Twist bind to E-boxes located in the regulatory elements upstream of target genes (Castanon et al., 2001, Connerney et al., 2008). The ability of Twist to form different dimers affects its function as either an activator or a repressor of gene transcription (Castanon et al., 2001, O'Rourke and Tam, 2002). Moreover, the formation of different types of dimerised complexes, show the wide range of functional properties of Twist clarifying its diverse activity detected in separate organisms (O'Rourke and Tam, 2002). As the other members of the bHLH superfamily, Twist's function is being reversed by the HLH Id proteins that inhibit the activity of bHLH proteins (Miraoui and Marie, 2010, Massari and Murre, 2000b). The relative levels between Twist and Id proteins regulate the formation of Twist homodimer or heterodimer, and the balance between the formation of dimers with its different associated proteins is crucial for its function (Connerney et al., 2008, Connerney et al., 2006, Miraoui and Marie, 2010). For example, Twist forms heterodimers with E proteins in the lack of Id proteins, whereas when Id levels are increased, Twist forms homodimers (Connerney et al., 2008, Connerney et al., 2006).

Furthermore, the choice of Twist dimerisation partners is affected by the phosphorylation state of conserved residues within the helix I (Firulli et al., 2005, Firulli and Conway, 2008). Specifically, B56δ-containing protein phosphatase 2A (PP2A) and protein kinase A (PKA) were shown to regulate phosphorylation of Twist and Hand2 at the conserved helix I residues (Firulli et al., 2005).

1.4.3. Expression of Twist in different tissues

Twist is expressed in many tissues at different times of development and is involved in many processes that regulate mesenchymal stem cell (MSC) fate determination, morphogenesis and differentiation (Gitelman, 1997). MSCs are multipotent stem cells able to differentiate into many cell types including myoblasts, osteoblasts, adipocytes and chondrocytes (Garcia-Gomez et al., 2010). The process of differentiation of MSCs to the different lineages is strongly regulated by numerous key transcription factors such as TGFβ (Lian et al., 1998, Karsenty and Wagner, 2002, Augello and De Bari, 2010).

Twist was found to be expressed in high levels in MSCs and to be involved in their regulation. Twist is is therefore implicated in diverse developmental systems such as myogenesis (Hebrok et al., 1994, Hamamori et al., 1997), skeletogenesis (Wu et al., 2008), neurogenesis (Verzi

et al., 2002), cardiogenesis (Conway et al., 2010), mesoderm formation (Fuchtbauer, 1995) and cranial tube morphogenesis (Chen and Behringer, 1995).

1.4.3.1 Twist expression during embryonic development

Twist was firstly identified in *Drosophila* embryos as a gene regulating the dorso-ventral pattern of zygotic development and the development of all mesoderm-derived lineages (Thisse et al., 1987). *In vivo* experiments in *twist*-null mutated *Drosophila* showed that the embryo was partially dorsalised. Moreover, there was abnormal gastrulation and the embryo failed to perform mesoderm differentiation (Thisse et al., 1987). On the contrary, *Twist*-null experiments on mouse showed that embryos performed normal gastrulation but died at embryonic days E10.5-11 showing failure to form cranial neural folds fusion and faults in cranial mesenchyme, somites and limb bud development (Chen and Behringer, 1995). The differences found between the two different species propose that Twist perhaps acts at earlier stages of development in *Drosophila* than in mice during mesoderm development., suggesting that in mice Twist possibly acts earlier during mesoderm specification and it is necessary for its differentiation into separate cell lineages including muscle, osteogenic and cartilage cell lineages (O'Rourke and Tam, 2002, Ansieau et al., 2010). Twist was detected to promote the epithelial-to-mesenchymal transition which is associated with mesoderm, through the stimulation of mesenchymal genes (Onder et al., 2008). During the embryo gastrulation, Twist was found to be primarily expressed in the somatic mesoderm (Fuchtbauer, 1995, Tavares et al., 2001, Wolf et al., 1991). The expression of Twist was found to be restricted in sclerotome and dermomyotome as the somite matures (Wolf et al., 1991, Fuchtbauer, 1995, Tavares et al., 2001). When myotome is formed, the expression of Twist was found to be silenced thus suggesting that Twist plays a role as a repressor of myogenic differentiation (Wolf et al., 1991, Hopwood et al., 1989, Ansieau et al., 2010).

1.4.3.2 Role of Twist in cell differentiation

Twist was identified as a regulator of differentiation of different cell types. Specifically, Twist was found to regulate the chondrocyte differentiation through the inhibition of the expression of chondrocyte genes (Reinhold et al., 2006). For the differentiation of the chondrocytes the Runx2 is induced, which is a target of Twist, thus promoting the expression of chondrocyte genes (Reinhold et al., 2006, Dong et al., 2007). Additionally, Twist possibly regulates chondrocyte maturation also through indirect mechanisms. Twist expression is controlled in the perichondrium, in mouse cartilage. Perichondrium is a structure made of undifferentiated mesenchymal cells surrounding the growth plate cartilage and its function is to control the chondrocyte maturation

(Hinoi et al., 2006). *In vivo* studies in mice models with loss- and gain-of-function showed that Twist regulates chondrocyte maturation indirectly in a Runx2-dependent manner, which further promotes the perichondrial expression of FGF18, a regulator of chondrocyte maturation (Hinoi et al., 2006).

Twist is also a regulator of osteogenesis, at all the stages of the osteoblast development, from the commitment of MSCs to osteoblasts to proliferation, differentiation and cell death (Miraoui and Marie, 2010). The expression of Twist was found to be decreased during osteogenic differentiation *in vitro* (Murray et al., 1992, Rice et al., 2000, Miraoui and Marie, 2010) and bone development *in vivo* (Alborzi et al., 1996). These findings underpin that Twist acts as an inhibitor of osteoblast differentiation and holds the osteoblastic cells in an osteoprogenitor-like state (Lee et al., 1999, Oshima et al., 2002, Miraoui and Marie, 2010). *In vitro* and *in vivo* Twist overexpression inhibited osteoblast differentiation (Oshima et al., 2002, Isenmann et al., 2009, Funato et al., 2001, Bialek et al., 2004). Twist was also found to be involved in both direct and indirect mechanisms in order to regulate the complex process of osteoblast differentiation (Connerney et al., 2006, Zhang et al., 2008).

Another process that Twist is known be involved the differentiation of MSCs into adipocytes during the early stages of development. In murine MSCs culture, Twist is downregulated and this leads to the reduction of the expression of adipocyte genes and adipogenic differentiation occurs. The mechanisms involved in this regulation however appear to be indirect. The adipogenic differentiation is reduced by the downregulation of Twist through upregulation of FGF Receptor 2 (FGFR2) signaling (Miraoui et al., 2010), which is the inhibitor of adipogenic differentiation in murine MSCs (Miraoui et al., 2009). The fact that adipocyte genes are downregulated from Twist silencing could also be an indirect consequence of Twist downregulation on MSC differentiation towards osteoblast differentiation (Miraoui et al., 2010, Miraoui and Marie, 2010). Moreover, Twist was shown to be expressed in adipose tissue and act as a negative-feedback regulator of PGC-1a/PPARδ-mediated brown fat metabolism (Pan et al., 2009). Even though Twist was found to be involved in many mechanisms of the regulation of adipogenesis, its exact role is not clarified yet.

1.4.4 Twist's function during muscle differentiation

Twist has an opposite function in *Drosophila* and vertebrate myogenesis. Specifically, Twist in *Drosophila* was found to promote muscle cell differentiation (Baylies and Bate, 1996), in contrast to vertebrates where Twist inhibits the process of myogenesis (Hebrok et al., 1994). Overexpression of D-Twist induced somatic myogenesis and inhibited the development of other mesodermal derivatives in *Drosophila* embryos. Moreover, overexpression of D-Twist in the

ectoderm germ layer of the embryo drove these cells into myogenesis process (Baylies and Bate, 1996). Furthermore, D-Twist was shown to directly regulate the *Mef2* gene transcription in adult somatic muscle precursor cells (Cripps et al., 1998). Specifically, D-Twist protein was identified to bind on an evolutionarily conserved E-box present in an enhancer element of the *Mef2* gene thus promoting the transcription of this gene (Cripps et al., 1998).

M-Twist was identified to be transcribed primarily in the early somites. M-Twist is downregulated at the myotome where the expression of the myogenic bHLH factors, MyoD and Myf-5, is upregulated in order the newly formed somites to be differentiated to diverse compartments of the embryo (Sassoon, 1993, Castanon and Baylies, 2002, Hebrok et al., 1994). The downregulation of M-Twist when the myogenic factors were upregulated in the early stages of development underpins that M-Twist is involved in the regulation of premature muscle differentiation and inhibition of myogenesis (Castanon and Baylies, 2002). Overexpression of M-Twist in myogenic mouse cell line C2C12 reversibly repressed muscle cell differentiation with an associated decrease in transcript levels of Myf-5 and myogenin, and increase in Id transcript levels (Hebrok et al., 1994). Furthermore, overexpression of M-Twist in the mouse embryonic stem cells of line BLC6 which have the ability to differentiate into skeletal muscle, caused delayed and decreased muscle differentiation in a dose- dependent manner (Rohwedel et al., 1995). The delay in skeletal muscle cell differentiation caused by M-Twist overexpression was accompanied by a delay in the expression of muscle-specific genes, including *MyoD, Myf-5* and *myogenin* (Rohwedel et al., 1995). The fact that Twist acts as an inhibitor in mammalian muscle cell differentiation is extensively established. There is however, limited information about the mechanisms by which Twist inhibits the process of myogenic differentiation in mammals.

M-Twist was found to inhibit MyoD transactivation and DNA binding activity by attracting the E proteins away from the myogenic bHLH transcription factors. This causes the decrease of the formation of functional E protein-MyoD complexes (Spicer et al., 1996). Furthermore, M-Twist was determined to block MEF2 activation (Spicer et al., 1996). The Mef2 was found to directly interact with the HLH domain and the carboxyl-terminus of M-Twist, however a residue in the basic domain of M-Twist is required for the inhibition of MEF2 transactivation (Spicer et al., 1996). The direct interaction between MEF2 and M-Twist does not allow the MEF2 to bind with MyoD and promote myogenesis. Overexpression of both E protein and MEF2 however, did not overtake the inhibition of MyoD transactivation caused by M-Twist, showing that there are also other mechanisms for the inhibition of muscle differentiation by M-Twist (Hamamori et al., 1997). There is evidence that M-Twist interacts directly with MyoD *in vitro* as well as *in vivo* experiments and this interaction is necessary for M-Twist inhibition of MyoD transactivation (Hamamori et al., 1997). Usually, the heterodimerisation between bHLH transcription factors is facilitated through the HLH domains, the interaction however between the M-Twist and MyoD was found to involve the basic domains of both proteins (Hamamori et al., 1997). The involvement of the basic DNA-

binding domain and the carboxyl-terminal domain of Twist during myogenic inhibition was further established by Hebrok and colleagues (Hebrok et al., 1997). Although, Twist do not have a role in the regulation of muscle cell proliferation and cell cycle exit, it inhibits muscle cell differentiation through different mechanisms during the process of myogenesis in a cell-autonomous manner (Hebrok et al., 1997). Moreover, Twist was found to be involved in the inhibition of the activation of muscle-specific genes through the formation of complexes, instead of sequestering E proteins (Hebrok et al., 1997). Particularly, by using gel mobility shift assays it was shown that Twist binds to E-box present on the *MCK* gene, thus preventing E12-MyoD heterodimers and E12 homodimers to bind on the *MCK* promoter (Hebrok et al., 1997). Furthermore, the inhibitory role of Twist during muscle cell differentiation was also supported by the overexpression of M-Twist which caused maturely differentiated myotubes to reversely differentiate accompanied by an increase in Cyclin D1 expression (Hjiantoniou et al., 2008). Furthermore, it was shown that overexpression of M-TWIST in C2C12 cell line can reverse muscle cell differentiation in the presence of growth factors by binding and downregulating myogenin, as well as reversing cellular morphology in the absence of growth factors (Mastroyiannopoulos et al., 2013).

Moreover, Twist was identified to inhibit myogenesis by inhibiting the catalytic activities of histone acetyltransferase (HAT) during chromatin modifications. The regulation of gene expression in eukaryotic cells is achieved through the restricted access of transcriptional machinery to DNA by the repressive structures of chromatin (Grunstein, 1997). The process by which the repression of chromatin is released is the histone deacetylation. This process involves, firstly, the recruitment of p300/CBP (CREB-binding protein) transcriptional activators, which have HAT domains, which bind to the promoters of target genes through interactions with sequence-specific activators such as MyoD (Grunstein, 1997, Yuan et al., 1996, Eckner et al., 1996, Puri et al., 1997, Sartorelli et al., 1997). Next, the p300/CBP recruits a second coactivator such as PCAF (p300/CBP-associated protein) which, has also HAT activity (Grunstein, 1997). MyoD transcription factor was found to act with p300 to activate the transcription of muscle-specific genes (Franco et al., 2011, Puri et al., 1997, Yuan et al., 1996). Twist was also found to interact with p300 and PCAF and reduce the acetyltransferase activity of p300 and PCAF N-terminal domain, thus resulting to the inhibition of transcriptional activation (Hamamori et al., 1999).

The mechanisms that regulate *Twist* gene expression in skeletal muscle cell differentiation remain largely unknown. An association of Twist expression with Insulin-like Growth Factor-1 (IGF-1) was determined (Dupont et al., 2001). Pathways such as the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) are initiated by the binding of IGF-1 to its specific receptor, the IGF-1 receptor (IGF-1R), which exists in many tissues. *In vivo* experiment where mice were injected with IGF-1 showed an increase in Twist expression, suggesting that IGF-1 signaling may be involved in the activation of *Twist* gene expression in skeletal muscle (Dupont et al., 2001). The HGF signaling pathway was also found to probably play a role in the activation

of Twist expression during the development, growth and regeneration of skeletal muscle (Leshem et al., 2000). *In vitro* experiment showed that satellite cells transfected with HGF significantly increased the expression of *Twist* gene. The effect of HGF on muscle cell proliferation and differentiation might therefore occur through the regulation of Twist expression (Leshem et al., 2000).

H-TWIST was found to be endogenously expressed in high levels in human foetal myoblasts, and its expression levels decreased during late stages of development. The differentiation capacity of the myoblasts was increased during the development. This study shows that there is an inversely proportional relation between the differentiation capacity of myoblasts and Twist expression thus suggesting that Twist is involved in the regulation of muscle development (Koutsoulidou et al., 2011a).

In conclusion, Twist is an important transcription factor that is involved in muscle development during early developmental stage with opposite role between mammals and *Drosophila* during myogenesis. Twist was found to be implicated in the inhibition of myogenesis in mammals and found to inhibit the transactivation of MyoD through protein-protein interaction and inhibit the expression of myogenin. Moreover, the expression and the function of Twist was found to be regulated through other signaling pathways as well as other regulatory elements, such as the microRNAs (miRNAs) (Sokol and Ambros, 2005).

1.4.5 Implication of Twist in diseases

The important role of Twist in mesenchymal cell differentiation and its function in various tissues during development involve Twist to several disorders. One such disorder is the Saethre-Chotzen syndrome which is an autosomal dominant hereditary craniosynostosis syndrome. This disorder is characterized by premature fusion of coronal suture and limb and craniofacial abnormalities (Reardon and Winter, 1994, Bonaventure and El Ghouzzi, 2003).

DNA sequence analyses in Saethre-Chotzen syndrome patients showed multiple mutations in the *H-TWIST* gene. Most *H-TWIST* gene mutations that were discovered showed to cause truncated or degraded H-TWIST protein (El Ghouzzi et al., 2000, El Ghouzzi et al., 1999), loss of dimerization function H-TWIST with E proteins (El Ghouzzi et al., 2000) and decrease DNA binding affinity of H-Twist to bind to the target E-boxes (El Ghouzzi et al., 2001). Furthermore, some mutations caused the disruption of PKA-mediated phosphorylation of H-TWIST thus causing the phenotypes observed that led to Saethre-Chotzen syndrome (Firulli et al., 2005).

Twist is known to promote the transition of epithelial-to-mesenchymal, due to the downregulation of the epithelial markers, such as E-cadherin and upregulation mesenchymal

markers, such as N-cadherin. Loss of the regulation of epithelial-to-mesenchymal transitions and cellular senescence processes result in a fully malignant phenotype and the formation of tumors (Smit and Peeper, 2008). In many cancers, such as rhabdomyosarcoma, melanomas, lung cancer and breast cancer, Twist was found to be upregulated (Ansieau et al., 2008, Kwok et al., 2005, Maestro et al., 1999). In human cancers, Twist was found to be commonly activated and this leads to the induction of the transcription of oncogenes and overrides pathways involving Rb and p53 Rb and p53 which are tumor suppressors, thus inducing epithelial-to-mesenchymal transitions (Ansieau et al., 2008).Twist was also found to be involved in the metastasis of tumor through its ability to induce epithelial-to-mesenchymal transitions (Yang et al., 2004, Mironchik et al., 2005, Shi et al., 2014). Finally, Twist was found to be involved in the regulation of angiogenesis in oxygen-induced retinopathy (OIR) retinas (Li et al., 2014a). Twist-1 was also identified to be necessary for the maintenance of mesenchymal characters and the initiation of tumour of synovial sarcoma (Lee et al., 2014).

1.5 Non-coding RNA

The central dogma theory in biology is that DNA is transcribed into RNA in the nucleus and then exported in the cytoplasm where it is translated into protein. The most well studied sequences in the human genome are those of the protein coding genes. There was an assumption that all of the genetic information necessary for the complexity found in multicellular organisms are present in the protein coding genes, in the eukaryotes, like prokaryotic organisms (Mattick and Makunin, 2006).

The protein coding genes cover only the 1.5% of the whole human genome (Alexander et al., 2010) and the large amount of non-coding RNA that is present in eukaryotic organisms was believed to be consists of evolutionary debris. It was believed that the complexity of the multicellular organisms is proportional to the number of protein coding genes expressed in the cells. Moreover, multicellular organisms have a comparatively the same stationary number of protein coding genes. Although, the worm *C. elegans* which consists of only 1000 cells it has more than 70% similarity of its protein coding genes with the human, which consists of more than 10^{14} cells, and human genome is more complex in both development and physiology than *C. elegans* (Frith et al., 2005). It was found that the non-coding region of DNA produces non-coding RNAs that are proportional to the complexity of an organism (Mattick, 2001). Comparing with the coding-DNA the region that transcribes the non-coding RNAs; does not contain an open reading frame, which makes their prediction very difficult. Non coding RNA genes include microRNA (miRNA), small interfering RNA (siRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), piwi-interacting RNA (piRNA), small nuclear RNA (snoRNA) and long non-coding RNA (lncRNA).

1.5.1 RNA interference (RNAi)

The tight regulation of gene expression is necessary for the suitable development and physiology of an organism particularly in complex multicellular organisms such as human. RNA interference is a biological process that eventually represses gene expression where double-stranded RNA is cleaved into small fragments thus degrading the target mRNA. Initially, RNAi was discovered in plant, back in 1990. Napoli and colleagues tried to introduce a coding gene for the pigmentation of the petunia plant (chalcone synthase) in order to produce flowers with more colors however, the flowers had fewer colors. This was found to be a result of silencing by RNA of both the introduced and the endogenous gene but the mechanism was not clarified fully (Napoli et al., 1990). In few years later, in 1998, Andrew Fire and Craig Mello published their work about the mechanisms of RNA interference and they were awarded with the Nobel Prize in physiology and medicine in 2006 for this work. They found that insertion of double stranded RNA in cells causes a decrease of the target mRNA. Their experiments performed in the nematode worm *C. elegans* showed that efficient silencing is achieved with the introduction of double stranded RNA, as opposed to single stranded sense and antisense RNA which has little or no effect. Furthermore, the silencing detected was as a result of RNA-mRNA homology and therefore it was specific. The double stranded RNA produced efficient silencing only when it matched to mature sequences of mRNA, suggesting that RNAi acts as a post transcriptional regulator. Moreover, when the RNAi was inserted the targeted mRNA was not detectable showing that the mRNA was degraded and the silencing was sufficient, even with few molecules per cell. Finally, it was found that the activity of the RNAi was transmitted between cells as the effect of the RNAi was found in the neighboring tissues and in the next generation (Fire et al., 1998).

Following this discovery, Fire and collages made it strong that double stranded RNA targeted mature RNA and degraded mRNA before being translated into protein and this made stronger the statement that RNAi acts as post transcriptional regulator (Montgomery et al., 1998). After *C. elegans,* RNAi was identified in several other organisms including plants and animals (Tuschl et al., 1999). After this huge discovery, it was found that the small interfering RNAs (siRNAs) are approximately 20-25 nucleotide (Elbashir et al., 2001).

1.5.1.1 Small interfering RNA (siRNA)

siRNAs are a class of small double stranded RNA molecules about 20-25 nucleotides base pairs long. siRNAs can be divided into two categories depending on their origin: the endogenous and the exogenous. Endogenous siRNAs are encoded by the genome of the host organism. These siRNAs were identified in plants and are located in regions between protein coding genes,

transposons and repetitive DNA sequences (Llave et al., 2002, Hamilton et al., 2002). siRNAs that are located between transposons were found to play a role in the regulation of the transposon that derived from (Waterhouse et al., 2001, Aravin et al., 2003, Sunkar et al., 2005). The trans-acting siRNAs regulate other genes using mechanisms similar to that used by microRNAs (miRNAs) (Vazquez et al., 2004). For the synthesis of the endogenous, transposon derived siRNAs, the activity of DNA-dependent RNA-polymerase-4, Dicer-like3 and RNA-dependent RNA-polymerase 2 is necessary (Xie et al., 2004, Hamilton et al., 2002, Herr et al., 2005). In contrast, the synthesis of trans-acting siRNAs involves only the RNA dependent RNA-polymerase-6 (Vazquez et al., 2004).

Endogenously derived siRNA is considered to be transcribed in both directions letting the formation of the double stranded RNA (Volpe et al., 2002). Following the transcription, the produced double stranded RNA is cleaved in the nucleus and is then either exported to the cytoplasm (Volpe et al., 2002) or remains in the nucleus where t acts as a transcription silencer (Schramke and Allshire, 2003). It was found that when long double stranded RNA was introduced in cells was able to downregulate a corresponding gene. It has been next demonstrated however, that there is an anti-viral interferon response that stops all protein synthesis in mammalian cells (Reynolds, Anderson et al. 2006). On the other hand, synthetic siRNAs did not show this interferon response and are used until now as a powerful tool for studying mammalian gene silencing (Bantounas et al., 2004).

Furthermore, stable siRNA expression was successfully performed using plasmid and viral-based small hairpin RNAs. The small hairpin RNA transcribed forms a precursor miRNA like structure which is processed similarly to miRNAs. The hairpin structure is moved out of the nucleus into the cytoplasm. In the cytoplasm hairpin RNA is processed by Dicer to give rise to the small double stranded RNA fragments containing the siRNA. The siRNA then becomes incorporated with RISC where it binds to its complementary mRNA sequence to induce degradation (Bantounas et al., 2004).

1.5.1.2 microRNAs

An additional class of small RNAs that was identified in all organisms, from plant to human is the microRNAs (miRNAs) (Lee and Ambros, 2001, Lau et al., 2001, Lagos-Quintana et al., 2001).

miRNAs were initially discovered in 1993 in *C. elegans*. The first reported miRNA was the lin-4 (Lee et al., 1993). Analyzing the genomic sequence of the lin-4, it was found that there is no open reading frame suggesting that the *lin-4* gene does not encode for a protein. It was found

however, that *lin-4* locus transcribes two RNA products: a 22 nucleotides long form, which is the mature form, and a 61 nucleotides long form which is the premature form (Lee et al., 1993) (Sayed and Abdellatif, 2011). Lee and colleagues also determined that the lin-4 miRNA was complementary to a sequence located on the 3'-untranslated region (3'UTR) of the lin-14 mRNA. When the lin-4 miRNA was present, the translation of lin-14 was significantly decreased proposing a model by which miRNAs inhibit protein synthesis at the post transcriptional level through an antisense mechanism by which they bind to 3'UTR of their target mRNA (Lee et al., 1993). A similar regulatory miRNA, let-7, was next found, to play a role in the temporal developmental of *C. elegans*, using a similar regulatory mechanism like lin-4 (Reinhart et al., 2000). Specifically, let-7 is found to be expressed during the later stages of *C. elegans* development and it negatively regulates the lin-41 protein, adaptable to change into the adult stage (Reinhart et al., 2000).

The discovery of these first members of the family of the miRNAs showed that miRNAs can function as an effficient regulatory switch for the timing control of a cellular event. Moreover, miRNAs act as regulators of many different and functionally diverse mRNAs (Sayed and Abdellatif, 2011).

1.5.1.2.1 Biogenesis of miRNAs

In animals, the biogenesis of miRNAs takes place in two stages and involves two cleavage processes, one in the nucleus and one in the cytoplasm. The cleavage processes are controlled by two ribonuclease III endonucleases, Drosha and Dicer (Denli et al., 2004, Du and Zamore, 2005, Gregory et al., 2004, Lee et al., 2003). miRNAs can be transcribed either by intergenic or intragenic loci (Rodriguez et al., 2004, Ying and Lin, 2009). Firstly, the miRNAs are transcribed as the primary miRNA (pri-miRNA) which is next cleaved to produce the precursor miRNA (pre-miRNA). Pre-miRNA is then further processed to produce a small 21-25 nucleotide long mature miRNA (Lee et al., 2002).

More specifically, intergenic miRNA is primarily transcribed by RNA polymerase II or RNA polymerase III to give rise to the pri-miRNA, a stem-loop structure with extended both the 5' and the 3' ends (Han et al., 2006, Borchert et al., 2006). The pri-miRNAs with the suitable stem length are selected and further processed. Only the pri-miRNAs with more than 10 base pairs length in both 5' and 3' terminal will be processed, in order to form the mature miRNAs (Lee et al., 2003, Zeng et al., 2005, Zeng and Cullen, 2003, Zeng and Cullen, 2005). The transcribed pri-miRNA is processed in the nucleus by a protein complex microprocessor, which consists of RNase III endonuclease Drosha and the double stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8), to form the pre-miRNA (Denli et al., 2004, Han et al., 2004). In some circumstances however, an associated protein is required to act with the microprocessor. For

example, the protein factor hnRNPA1 is involved in miR-18a production (Michlewski et al., 2008, Guil and Caceres, 2007).

It was suggested that DGCR8 recognizes the ssRNA-dsRNA site of the pri-miRNA and drives Drosha to a cleavage site approximately 11 base pairs (bp) from the ssRNA-dsRNA site where Drosha cleaves and release the pre-miRNA which is a hairpin structure approximately 60-70 bp long (Zeng and Cullen, 2003, Han et al., 2006, Zeng et al., 2003). The site where Drosha cleaves defines one end of the pre-miRNA and keeps the mature miRNA in either the 5' or 3' arm (Du and Zamore, 2005).

The intragenic miRNAs which are present within introns of protein coding genes are transcribed by RNA polymerase II (Rodriguez et al., 2004). There are two possible mechanisms for the excision of the intragenic miRNAs which are believed to occur together or individually (Kim and Kim, 2007). One of the two mechanisms involves splicing process during the production of miRNA which are initially excised out by spliceosomal components (Ying and Lin, 2004, Lin et al., 2003).

It is not clarified yet in the case of intragenic miRNAs, if the splicing process releases pre-miRNA or pri-miRNA. In the case that pre-miRNA is released, it will be exported in the cytoplasm for the maturation procedure, while in the case of pri-miRNA production, the pri-miRNA will be proceeded in the same manner as intergenic pri-miRNA to be processed by the microprocessor complex (Ying and Lin, 2004, Lin et al., 2003). The spliced introns that contain the miRNAs are called mirtrons. Mirtrons have been found in both invertebrates and mammals (Berezikov et al., 2007, Ruby et al., 2007). Experiments performed in invertebrates showed that the mirtrons skip the cleavage process by Drosha and are exported directly from the nucleus and proceed to miRNA maturation stage. This has not been shown in mammals.

The second mechanism of the intragenic miRNA excision hypothesis is that pre-mRNA splicing is not essential for pri-miRNA processing. Specifically, miRNAs are excised after the splicing decision has been taken and the miRNA is excised before the intron is excised. . Based on this hypothesis, there are splice sites in the pre-mRNA that attract a splicing commitment complex which binds to and drives the microprocessor to perform the excision, releasing the pre-miRNA, while the splicing of host gene continues as normal in order to form mature mRNA. This scenario has not been investigated yet (Kim and Kim, 2007, Ruby et al., 2007, Lin et al., 2006).

Once the pre-miRNA is formed, it is transported from the nucleus to the cytoplasm for further process. The nucleocytoplasmic transporter factor Exportin-5 and the nuclear GTP binding protein RanGTP are the proteins that form complex with the pre-miRNA and are responsible for its translocation from the nucleus to the cytoplasm and thereby preventing nuclear degradation (Bohnsack et al., 2004, Lund and Dahlberg, 2006, Okada et al., 2009, Zeng and Cullen, 2004).

There is a direct binding of Exportin 5 and the pre-miRNA, which makes use of more than 16 nucleotides and 4 nucleotides of the terminal loop (Zeng and Cullen, 2004). Mirtron translocation from nucleus to the cytoplasm was found to take place in species such as *Xenopus* and *Drosophila.* Whether this occurs in mammals however, remains unclear (Berezikov et al., 2007, Okamura et al., 2007).

As soon as the pre-miRNA hairpin structure is transported to the cytoplasm, is further processed in order to remove the stem loop and create a double stranded duplex (Figure 1.5).

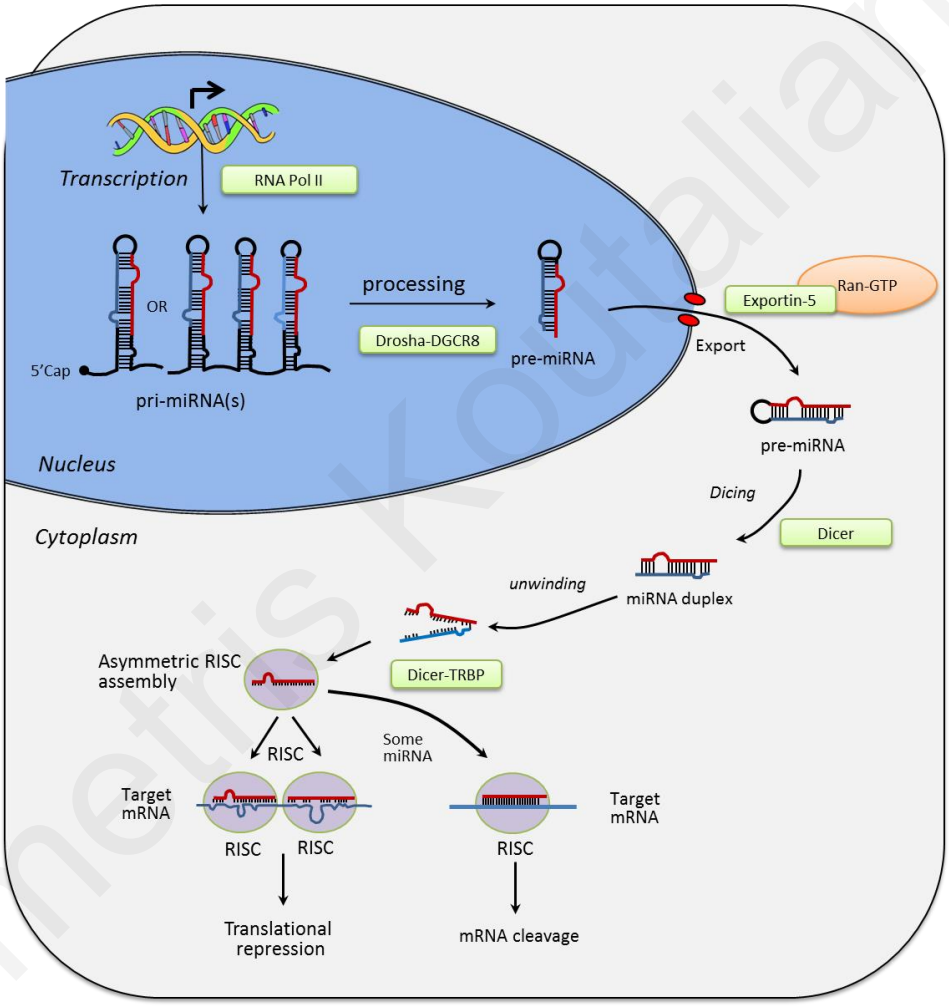


Figure 1.5 MiRNA biogenesis and function. MiRNAs are transcribed in the nucleus by RNA pol II as pri-miRNAs, which are processed by Drosha-DGCR8 complex into pre-miRNA. Pre-miRNA is exported to the cytoplasm, via Exportin-5 in a Ran-GTP-dependent manner, where it is diced by Dicer to generate a miRNA duplex. Following processing, the mature miRNA duplex is preferentially incorporated into the RISC where it binds its target mRNA causing either repression of translation or degradation of target mRNA. Adapted from He and Hannon 2004

Dicer is the protein responsible for the further cleavage of pre-miRNA in order to produce the double stranded duplex. Dicer is a multi-domain protein which contains a double stranded RNA-binding domain near the C-terminal, two tandem RNaseIII nuclease domains, a central

Piwi/Ago/Zwille (Novello et al.) domain, a DEAD box helicase domain which is located near the N-terminal (Provost et al., 2002) and a domain of unknown functions (DUF283). The silencing of the *Dicer* gene in mice implied the significance of this protein. Stem embryonic cells lacking Dicer expression showed severe failings in differentiation both *in vivo* and *in vitro* (Kanellopoulou et al., 2005).

Experiments performed in zebrafish showed that if the Dicer is inactivated, there is no production of miRNAs and this has as a result the inhibition of development and the overall termination of cell growth in larvae (Wienholds et al., 2003). An important domain of Dicer is the PAZ domain, which recognizes the 2-nucleotide 3'overhang resulted by Drosha processing and removes the two helical turns in order to form the double stranded RNA molecule, containing at both ends a 2-nucleotide 3'overhangs (Bernstein et al., 2001, Hutvagner et al., 2001, Starega-Roslan et al., 2011).

Dicer interacts with the proteins of the RNA Induced Silencing Complex (RISC) including members of the Argonaute (AGO) family, and especialy with the Ago2 which is resposnisble for the cleavage of the target mRNA (Maniataki and Mourelatos, 2005, O'Carroll et al., 2007, MacRae et al., 2008), PACT (Lee et al., 2006), the human immunodeficiency virus 1 (HIV-1), the transactivation response (TAR) RNA-binding protein (TRBP), which is essential for the assembly of Ago2 to the RISC (Chendrimada et al., 2005, Haase et al., 2005), and PW182 which is a P-body protein (Liu et al., 2005a). After the formation of the double stranded miRNA molecule from the cleavage process the duplex is unwound and the miRNA strand that will be incorporated into RISC is selected by how tightly paired the 5' end is (Khvorova et al., 2003), permitting the guide strand to be bound with the AGO protein, generating the programmed miRNA induced silencing complex (miRISC) (Shruti et al., 2011).

1.5.1.2.2 Target recognition

When the miRNA corporates with the RISC and forms the active form of the complex, complementary base pair sequence of the miRNA force the RISC to bind to the corresponding 3'UTR of the target mRNA (Wightman et al., 1993, Lee et al., 1993). The target mRNA is recognized by the complementarity to 2-8 nucleotides at the 5' end of the mature miRNA which comprise the seed region of the miRNA (Brennecke et al., 2005, Lewis et al., 2003). The main silencing mechanism factors for the activity of miRNAs are the degree and type of complimentary sites between the seed region of the miRNA and the target mRNA (Bartel, 2004, Yekta et al., 2004).

Almost perfect complementarity between the guide strand and the target mRNA usually results in the degradation of the target by Ago2 protein. On the other hand, an imperfect complementarity will result in inhibition of translation (Bartel, 2004, Yekta et al., 2004). The key

region of miRNAs for the control of the specificity and the activity of the reaction, is the seed region which is important for the thermal stability of the reaction between the miRNA and its target mRNA (Doench and Sharp, 2004, Stark et al., 2003). It was initially, believed that mismatch base pairing would be resulted to a non-functional miRNA. The interaction with mismatch base pairing however, is still likely to take place, even though it affects the specificity and activity (Brennecke et al., 2005, Doench and Sharp, 2004). miR-196 for example, is almost fully complementary to the HOXB8 mRNA except a G: U mismatch in the seed region, and is able to downregulate HOXB8 via mRNA cleavage (Yekta et al., 2004).

1.5.1.2.3 Inhibition or cleavage by the RISC

Many different miRNAs can bind to a single mRNA target as well as a single miRNA is able to bind to many different mRNA targets (Bartel, 2004). It is widely accepted that miRNAs inhibit the process of translation through two silencing mechanisms, mRNA cleavage and translation repression (Wu et al., 2006, van Dijk et al., 2002, Coller and Parker, 2005, Macfarlane and Murphy, 2010).

In the first mechanism by which the mRNA is cleaved, a slicer dependent process occurs and depends on the endonuclease activity of Ago2 protein. High levels of base pairing between the miRNA and its target mRNA are necessary in order for Ago2 to be functional (Meister et al., 2004, Liu et al., 2004, Valencia-Sanchez et al., 2006). The result of both mechanisms is the same, the down regulation of the gene expression at post transcription level. There is however, a significant difference. In the situation of the repression of translation the inhibition is reversible and the mRNA is stable and can be translated when the inhibition is released. On the contrary, in the situation of the mRNA cleavage the inhibition is irreversible and translation cannot be performed (Valencia-Sanchez et al., 2006, Maroney et al., 2006). Perfect complementarity between the miRNA guide strand and the mRNA allows Ago2 to facilitate the mRNA cleavage causing the deadenylation of mRNA by protein complexes including Ccr4, Not1 and Pop2 which remove the poly (A) tail of the target mRNA (Valencia-Sanchez et al., 2006, Parker and Song, 2004).

Following the deadenylation, mRNA is degraded either from 5' to 3' by the Xrn1p exonuclease or from 3' to 5' by the exosome complex following decapping by the Dcp1 and Dcp2 enzymes (Valencia-Sanchez et al., 2006, Coller and Parker, 2004). Low complementarity between the miRNA and its target mRNA form bulges, preventing Ago2 mRNA cleavage and promotes the slicer independent mechanism.

It has been shown that slicer independent repression inhibits the initiation and elongation stages of translation (Pillai, 2005). Small RNAs block translation after initiation stage in mammalian cells with the exact mechanisms to remain unclear. Kong and colleagues showed that the translation repression mechanism is based on the promoter of the target mRNA (Kong et al.,

2008). It has been also suggested that miRNA can suppress translation indirectly by physically attracting away the mRNA from ribosomes to cytoplasmic foci known as P-bodies (Brengues et al., 2005, Liu et al., 2005b, Teixeira et al., 2005). In addition, the deadenylation and decapping processes accelerate independently of slicer activity thus effecting translation initiation (Wu et al., 2006, Coller et al., 2001).

1.5.1.2.4 MicroRNA stability

Both exogenous and endogenous miRNAs are integrated into the mechanisms of cell's processing. Dicer1 genetic ablation was used in immortalized embryonic fibroblasts, to measure the miRNA decay. miRNAs were found to have a half-life of 5 days in mammals which is ten times more stable than mRNAs (Gantier et al., 2011). Regardless that miRNAs are stable in cells, the dysregulation of miRNAs enables active miRNA expression patterns which are involved in many processes such as development and can be associated with different diseases (Ruegger and Grosshans, 2012, Chang and Mendell, 2007).

1.5.2 miRNAs in skeletal muscle differentiation

In order to identify the role of miRNAs in skeletal muscle differentiation, Dicer was deactivated. Dicer is the enzyme involved in the biogenesis of miRNAs and more specifically is responsible for miRNA maturation. Through these experiments it was shown that the miRNAs are necessary for the myogenesis since the deletion of the Dicer suppressed the differentiation of muscle cells (O'Rourke et al., 2007). miRNAs that are involved in the regulation of skeletal muscle cell differentiation can be divided into two categories. The first category consists of the muscle specific miRNAs, miR-1, miR133a, miR-133b and miR-206, which are miRNAs that are only expressed in muscle tissues and are also called myomiRs. The myomiRs were found to be regulated by the MRFs (Chen et al., 2006b). The second category includes the non-muscle specific miRNAs which are also expressed in other tissues rather than muscles (Granjon et al., 2009, Callis et al., 2008). The miRNAs expressed in muscle have as main function the regulation of muscle mRNAs and transcription factors which are involved in the regulation of the process of myogenesis. The miRNAs in muscle are expressed in patterns showing a regulation of skeletal muscle cell differentiation (Sjogren et al., 2014).

1.5.2.1 Non muscle specific miRNAs in muscle

Several miRNAs highly but not specifically expressed in muscle tissue were found to play a critical role in muscle cell differentiation. Some of these miRNAs were found to promote muscle cell differentiation and other to inhibit the process through their action in all the stages of myogenesis. miR-31 was identified to regulate the expression of Myf5 in the quiescent satellite

cells (Crist et al., 2012). Another important miRNA in the regulation of myogenesis is miR-181 which was detected in both C2C12 cell line and embryonic stem cells (Naguibneva et al., 2006). Specifically, miR-181 was found to regulate MyoD expression as well as the expression of homeobox protein Hox-A11, a protein found to be involved in skeletal muscle development (Naguibneva et al., 2006, Yamamoto and Kuroiwa, 2003).

miR-148 promotes muscle cell differentiation by targeting ROCK1 mRNA, an inhibitor of myogenic differentiation (Zhang et al., 2011). The expression of miR-221 and miR-222 is decreased during muscle cell differentiation suggesting a role of these miRNAs in the development of muscle and in the establishment of the differentiated muscle myotubes (Cardinali et al., 2009). Another miRNA that was found to promote muscle cell differentiation is miR-24 (Sun et al., 2008). miR-24 was found to be inhibited at the transcriptional level by the transforming growth factor-β (TGF-β) which is an inhibitor of myogenesis. In vitro experiments in C2C12 cell lines showed that miR-24 downregulation inhibited myogenic differentiation whereas the overexpression of miR-24 induced the muscle cell differentiation (Sun et al., 2008). miR-214 was found to be highly expressed during the muscle differentiation of C2C12 muscle cell line, as well as in primary cells. The overexpression of miR-214 upregulated the expression of muscle gene and induced the muscle cell differentiation (Juan et al., 2009).

Some miRNAs expressed in muscle tissues were found to be regulated by MRFs. miR-378 for example, was identified to be regulated by MyoD and its levels were found to be upregulated during C2C12 differentiation. Furthermore, overexression of miR-378 induced the muscle cell differentiation by inhibiting MyoR, an antagonist of MyoD (Gagan et al., 2011, Lu et al., 1999a). Another important non-muscle specific miRNA is the miR-27. miR-27 was identified to downregulate the paired box family of transcription factor 3 (PAX3) mRNA, which is important for the entry of the skeletal muscle stem cells into the myogenic program (Crist et al., 2009), and the regulation of the known inhibitor of myogenesis myostatin, thus promoting myoblast proliferation (Huang et al., 2012).

1.5.2.2 Muscle specific miRNAs

Muscle specific miRNAs, miR-1, miR-133a, miR-133b and miR-206, are miRNAs that are expressed only in muscle tissues, and are called myomiRs. MyomiRs have many related structural and functional characteristics (Luo et al., 2013). The transcription of the four myomiRs is regulated by the MRFs which are crucial for the regulation of all the steps in muscle development (Naya and Olson, 1999). The myomiRs are transcribed in pairs as bicistronic clusters and are divided into two families: miR-1/miR-206 and miR-133a/b. miR-206 and 133b are transcribed from a locus on chromosome 6, miR-1-2 and 133a-1 are transcribed from chromosome 18 and miR-1-1 and133a-2 are transcribed from chromosome 20 (Chen et al., 2009). Both miR-1-1 and miR-1-2 produce the

same identical mature miRNA but they differ in their pre-miRNA sequences. The mature miR-206 and miR-1 sequences have only four nucleotides different and they have the same seed region (Kim et al., 2006). miR-133a and miR-133b differ only by two bases. miR-133a and miR-1 are transcribed in both skeletal and cardiac muscle, while miR-133b and miR-206 are expressed only in skeletal muscle (Chen et al., 2006a, Kim et al., 2006) (Figure 1.6).

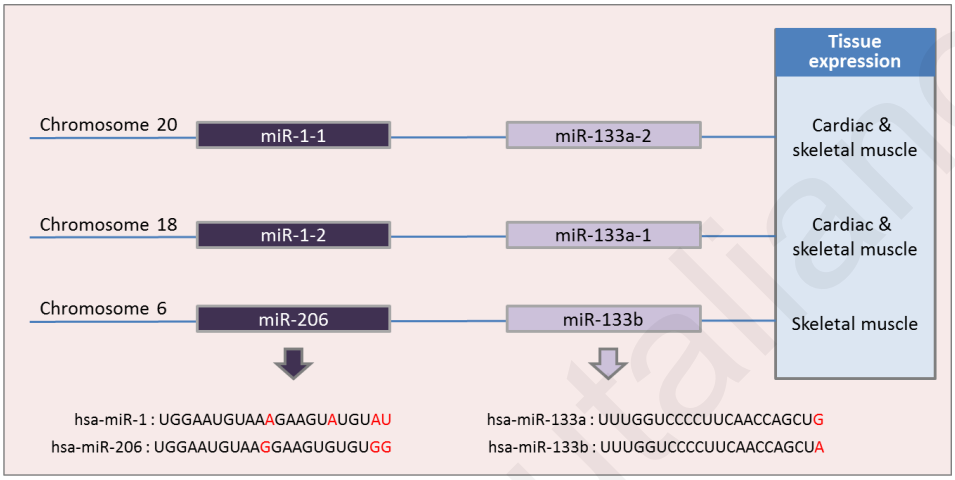


Figure 1.6 Genomic locations of the human myomiRs and their sequence homologies. Three bicistronic clusters encode for the human myomiRs in cardiac and/or skeletal muscle tissues. The mature sequences of miR-1, miR-133a, miR-133b and miR-206 and their sequence similarities are also indicated. Adapted from Townley-Tilson et al., 2010

The great similarity in the origin and sequence of the four myomiRs implies that they may have similar role through skeletal muscle differentiation. The myomiRs however, appear to have opposing effects during myogenesis. *In vitro* experiments showed that miR-1 and miR-206 induce muscle cell differentiation, while miR-133a and miR-133b regulate the myoblast proliferation (Chen et al., 2006a, Kim et al., 2006). Furthermore, overexpression of miR-1 and/or miR-206, showed that they inhibit myoblasts proliferation and promotes muscle cell differentiation *in vitro* (Chen et al., 2010). The mechanisms of these opposing roles were ascribed to the different mRNAs that are targeted by the myomiRs. miR-206/133b cluster is dispensable for development, function and regeneration of skeletal muscle, probably due to the overlapping functions of the related miR-1/133a clusters, which are strongly expressed in skeletal muscle. It was however found that the miR-206/133b cluster is not a sufficient regulator of skeletal muscle regeneration (Boettger et al., 2014).

1.5.2.3 mRNA targets of myomiRs

1.5.2.3.1 miR-206

Some targets of miR-206 are well established. Pax3 and Pax7 inhibit the early differentiation of myoblasts during skeletal muscle development. Overexpression of miR-206 during early stage of skeletal muscle development caused a reduction in the expression levels of Pax3 and Pax7 and promotes the muscle cell differentiation (Chen et al., 2010), Hirai, Verma et al. 2010).

The connexin 43 (Cx43) is another experimentally verified target of miR-206. Cx43 is a gap junction channel, which is necessary for the fusion of myoblasts in order to differentiate into mature myotubes by allowing the passage of signaling molecules and metabolites necessary for the maturation (Kalderon et al., 1977, Anderson et al., 2006). miR-206 therefore, inhibits myoblast proliferation and induces myoblast fusion through the downregulation of Cx43 protein (Anderson et al., 2006).

Another experimentally verified target of miR-206 is Pola1 which is the largest subunit of DNA polymerase α and is necessary for the DNA synthesis. Pola1 was found to be directly inhibited by miR-206 during muscle cell differentiation and is therefore associated to cell quiescence and DNA synthesis (Kim et al., 2006). Moreover, two additional mRNAs were found to be negatively regulated by miR-206, follistatin-like 1 and utrophin, through binding to their 3'UTR regions (Rosenberg et al., 2006).

1.5.2.3.2 miR-1

Taking into consideration that miR-1 and miR-206 have identical seed sequences it was expected that they would have the same function and target mRNAs. They have however, their own unique targets, roles and characteristics. One of the targets of miR-1 is the class II histone deacetylase, HDAC4 which is a transcriptional repressor of muscle gene expression such as MEF2, an important transcription factor of muscle development. miR-1 was found to regulate HDAC4 by directly binding to 3'UTR (Chen et al., 2006b, Naya and Olson, 1999). Moreover, miR-1 was found to regulate the zinc finger transcription factor YY1, a negative regulator of the transcription of muscle genes, by binding to the 3'UTR of YY1 mRNA (Lu et al., 2012).

1.5.2.3.3 miR-133

The other muscle specific miRNAs is the miR-133. miR-133 was also found to regulate a number of proteins that are involved in myogenesis. miR-133 was found to negatively regulate the MAML, a key regulator of Notch signaling pathway. Notch is associated with MEF2 and miR-133 was identified to regulate the connection of Notch and MEF2 thus inducing muscle differentiation

(Shen et al., 2006). Another target of miR-133 is the cell surface receptor, IGF-1R, which plays an important role in post-natal growth and muscle development through the activation of IGF-1 and IGF-2 (Huang et al., 2011a). In addition, miR-133 was found to negatively regulate the nPTB, a regulator of the pre-mRNA splicing in muscle differentiation (Boutz et al., 2007). miR-133 was also established to regulate the serum response factor (SRF) by binding to its 3'UTR and inhibiting its mRNA translation without degradation (Chen et al., 2006a). I*n vivo* and *in vitro* experiments showed that SRF plays a critical role in muscle proliferation and differentiation (Soulez et al., 1996, Li et al., 2005).

1.5.2.4 Transcriptional regulation of myomiRs

Myogenin and MyoD were found to directly bind to upstream regions of the myomiRs by using Chromatin immunoprecipitation (ChIP) analysis in C2C12 cells indicating that these MRFs are involved in the transcriptional regulation of myomiRs (Mok and Sweetman, 2011, Rao et al., 2006).

Myogenin and MyoD were found to bind only in the region upstream of miR-206 locus, but were not found to bind to any region between the loci of miR-206 and miR-133 (Rao et al., 2006). The direct binding between myogenin and MyoD on the upstream regions of the myomiRs suggests that the two MRFs are transcriptional regulators of miR-1-1, miR-1-2, miR-133a-1, miR-133a-2 and miR-206 during muscle cell differentiation (Rao et al., 2006). ChiP analysis showed that MyoD binds to an E-box element present nearby to the transcription start site of the miR-206 (Rosenberg et al., 2006). Furthermore, overexpression of the four MRFs individually found to promote the expression of miR-206 and miR-1 *in vivo* in the neural tube of chicken embryos (Sweetman et al., 2008).

The overexpression of Myf-5 and myogenin was found to promote the expression of both miR-1 and miR-206, while the overexpression of Myf-6 and MyoD promoted only the expression of miR-206. (Sweetman et al., 2008). Myf-5 probably compensate for lack of MyoD in terms of the regulation of the myomiRs. *In vivo* experiments performed in mouse suggested that Myf-5 is essential for the regulation of miR-206 and miR-1 in somites, while MyoD downregulation did not affect the expression of myomiRs implying the possible mechanism of functional redundancy (Sweetman et al., 2008, Rudnicki et al., 1993).

Additionally to the MRFs, other transcription factors have been involved during the transcriptional regulation of the four myomiRs. MEF2 was found to activate the transcription of miR-1-2 and miR-133a-1 through a muscle specific intragenic enhancer (Liu et al., 2007). Moreover, Forkhead box O3 (Foxo3a) was found to bind directly on the promoter of miR-1 and induce its expression (Elia et al., 2009).

The complexity of the miRNA pathways is further supported by the feedback loops in which miRNAs participate to control cellular proliferation and differentiation. For example, miR-133 directly regulates the SRF mRNA; however, SRF was identified to directly activates the transcription of the miR-1 gene in cardiogenesis (Zhao et al., 2005). Additionally, miR-133 was found to bind to the 3'UTR of the IGF-1R and negatively regulate its expression. In overexpression studies performed in C2C12 it was found that IGF-1, the ligand of IGF-1R, induced the expression of miR-133 in differentiated myotubes, possibly through myogenin induction (Huang et al., 2011b). These observations show that there is an excessive control necessary for the regulation of the expression of myomiRs and the transcription factors that regulate them in order for a complex processes, such as myogenesis, to be controlled.

1.6 Muscular diseases

Muscle tissue accounts for almost half of the body mass in adults and more than 80 related monogenic muscle diseases exist (Negroni et al., 2011, Kaplan, 2009). Among these diseases, there is a heterogeneous group of genetic muscle disorders that affect both adults and children and are called muscular dystrophies (Negroni et al., 2011). This group of diseases is characterized by skeletal muscle wasting, weakness and degeneration. Various mutations of different genes were identified and associated with the phenotype of muscular dystrophy disorders (Wallace and McNally, 2009). At present, more than 30 genetically different forms of muscular dystrophies were determined (Kaplan, 2009, Negroni et al., 2011).

Mutation on the X-linked *dystrophin* gene was the first mutated gene that was linked to a muscular dystrophy disorder. *Dystrophin* gene encodes a cytoskeletal protein that maintains the transmission of cytoplasmic signals, the myofiber integrity and the function of muscle (Koenig et al., 1988, Kumar et al., 2004, Ghahramani Seno et al., 2010, Negroni et al., 2011). The mutated *dystrophin* gene is associated with the most common muscular dystrophy disorder, the Duchene Muscular Dystrophy (DMD), and the Becker Muscular Dystrophy (BMD) which is the milder form of DMD (Davies and Nowak, 2006). Different mutations on *dystrophin* gene are associated with DMD and BMD (Davies and Nowak, 2006). In DMD patients, the phenotype is due to the absence of dystrophin protein or the production of fully deactivated protein, while the phenotype of BMD patients is due to the reduction of the levels of the normal dystrophin or to the expression of a truncated but partially functional protein (Davies and Nowak, 2006).

The most common muscular dystrophies in adults are the myotonic dystrophy type 1 and type 2 (DM1 and DM2). Many other muscular dystrophies are also exist including:1) the facioscapulohumeral (FSHD) muscular dystrophy, 2) the lethal congenital muscular dystrophy Walker-Warburg syndrome (WWS), 3) the Fukuyama congenital muscular dystrophy, which is a

severe autosomal recessive disorder, 4) the Emery-Dreifuss muscular dystrophy, which is an autosomal dominant disorder caused by mutations in the *lamin* gene or X-linked mutations in the *emerin* gene, 5) the late-onset oculopharyngeal muscular dystrophy (OPMD), and many others (Davies and Nowak, 2006).

1.6.1 Myotonic Dystrophy Type 1 (DM1)

Myotonic Dystrophy type 1 (DM1) was firstly described in 1909 by Steinert and Batten and Gibb, independently. DM1 is the most common form of muscular dystrophy in adults, and the second most common type of muscular dystrophy after DMD. Statistics show that 1 in 8000 individuals globally is affected by DM1 (Harper, 1989). It is an autosomal dominant inherited neuromuscular disorder. The severity of the disease increases through the generations a characteristic known as genetic anticipation. The disease affects primarily the skeletal muscle tissue and is characterized by skeletal muscle weakness, wasting and myotonia.DM1 however, is a multi-systemic disorder affecting many tissues such as the heart and the central nervous system (CNS) (Larkin and Fardaei, 2001). DM1 patients are determined to have congenital, juvenile or adult-onset form of the disease according to the age of symptom onset. Congenital form of Myotonic Dystrophy type 1 (CDM1) is the most severe phenotype of the disorder with 25% neonatal mortality rate (Harper, 1989).

The gene that is responsible for DM1 is the *dystrophia myotonica protein kinase* (*DMPK*) gene that is found on the small arm of the chromosome 19. DM1 is associated with a trinucleotide CTG repeat expansion located in the 3′UTR of the *DMPK* gene (Aslanidis et al., 1992). In the normal population the CTG repeats size ranges for 5 to 35, whereas in DM1 patients it increases from 50 to several thousand repeats. Individuals with 38 to 49 repeats are considered to have the pre-mutation of the disease and they have no symptoms of the disorder, but they face a high risk to have children with larger, pathologically expanded repeats (Foff and Mahadevan, 2011). The CTG repeat size however, does not associate with the age of onset and the disease severity (Turner and Hilton-Jones, 2010). Furthermore, there is no association between the muscle weakness and the number of CTG repeats (Zatz et al., 1995). The length of CTG repeats in the *DMPK* gene is mitotically unstable in DM1 patients thus leading to the somatic mosaicism in the size of the CTG expansion (Turner and Hilton-Jones, 2010).. More specifically, the size of the repeats found in a tissue of DM1 patient, for example blood, is not associated with the severity of the disorder, and the size of the repeats of other affected tissue such as the muscle (Turner and Hilton-Jones, 2010). CTG repeat size is frequently stable in some perinatal tissues, for example blood but it is unstable in others, such as cardiac and skeletal muscle (Turner and Hilton-Jones, 2010). The CTG repeats that are measured in clinical practice are from blood and there is no any further clinical advantage to measure the number of repeats in muscle (Turner and Hilton-Jones, 2010). The methods that are

used for the detection of the number of repeats at molecular genetic level is the PCR analysis for the repeats of less than 100, and the Southern blot analysis for greater repeats expansions (Turner and Hilton-Jones, 2010).

1.6.1.1 Mechanisms of DM1 pathogenesis

DM1 mutation is present in the 3'UTR of the *DMPK* gene and therefore the expanded repeats are transcribed in mRNA but not translated into the DMPK protein. The DMPK mutation was found to cause a reduction in the production of DMPK protein (DMPK haploinsufficiency) (Reddy et al., 1996, Jansen et al., 1996), which however, is not correlated with the disease symptoms (Mankodi et al., 2000, Foff and Mahadevan, 2011). Other mechanisms have been suggested to correlate the repeats expansions in the *DMPK* gene and the DM1 phenotype (Tapscott and Thornton, 2001).

1.6.1.1.1 RNA pathogenesis

The transcription of the mutated *DMPK* gene produces an mRNA with expanded 3'UTR containing the corresponding CUG repeats. Due to free energy the expanded CUG transcript has the ability to fold back on itself and form stable duplex hairpin structures (Napierala and Krzyzosiak, 1997, Koch and Leffert, 1998, Tian et al., 2000, Michalowski et al., 1999). The mutated containing the CUG repeat expansions imposes dominant-negative effects through the sequestration of RNA-binding proteins. As a result, the mutant transcript remains to the nucleus (nuclear retention) and forms ribonuclear inclusions known as RNA foci (Mastroyiannopoulos et al., 2010). Nuclear retention of the mutated DMPK transcripts was determined in myoblasts and myotubes isolated from CDM1 patients (Furling et al., 2001a), as well as in fibroblasts from muscle biopsies from DM1 patients (Taneja et al., 1995).

1.6.1.1.2 Mis-regulation of alternative splicing in DM1

One of the best characterized trans-dominant effects caused by the mutated DMPK RNAs is the mis-regulation of the process of alternative splicing of a subset of pre-mRNAs, known as spliceopathy, (Lee and Cooper, 2009). Alternative splicing is a process during of which different isoforms of proteins are formed through different reconnections of the exons of the pre-mRNA that produce different mRNAs. Spliceopathy is associated with variable symptoms in DM1 patients. In DM1, mis-regulation of alternative splicing caused by altered levels and functions of specific RNA-binding proteins that participate in the regulation of RNA splicing has as a result the consequent abnormal splicing of many downstream targets. Currently, the number of mis-spliced transcripts found to be associated with DM1 patients is more than twenty (Klein et al., 2011).

In 2000, Miller and colleagues identified the muscleblind (MBNL) family of proteins which were found to bind and associated with the CUG repeat expansions (Miller et al., 2000).

MBNL1 alternative splicing factor binds to the specific motif YGCY present in pyrimidine-rich pre-mRNAs and induces either the exclusion or inclusion of alternative exons based on *cis*-regulatory elements and their 5′ or 3′ localization (Goers et al., 2010). There are three isoforms of MBNL family proteins: MBNL/MBNL1, MBLL/MBNL2 and MBXL/MBNL3. All three isoforms bind to the foci of the mutant RNA present in the nucleus of DM1 cells preventing them to proceed to their normal function (Wojciechowska and Krzyzosiak, 2011, Fardaei et al., 2001, Mankodi et al., 2003, Kino et al., 2004, Houseley et al., 2005). Since MBNL proteins interact with the mutant DMPK RNA the alternative splicing is disrupted, a characteristic of DM1 pathogenesis (Wojciechowska and Krzyzosiak, 2011, Mankodi et al., 2005, Jiang et al., 2004, Houseley et al., 2005). Overexpression studies performed *in vivo* showed that MBNL1 expression restored disease-associated clinical features, confirming the hypothesis that deactivation of MBNL1 is the main pathogenic event in the development of the DM1 disorder (Kanadia et al., 2006, de Haro et al., 2006).

In addition to the MBNL family of proteins, there is a second group of proteins that was found to be associated with DM1 pathogenesis, the CELF family of proteins which includes the CUG binding protein (CUGBP) and the ETR-3-like factors. These proteins belong to a family of structurally related to RNA-binding proteins and participate in various stages of RNA processing such as mRNA stability, alternative splicing and translation. CELF1 which is also called CUG binding protein 1 (CUGBP1), was discover through experiments performed to investigate the molecular mechanisms of DM1 disorder (Gallo and Spickett, 2010). It was reported that the CUGBP1 binds precisely to CUG repeats (Timchenko et al., 1996a, Timchenko et al., 1996b). Additionally, it was found that in a yeast three-hybrid system CUGBP1 was able to associate with long CUG repeats ($(CUG)_{(11)}(CUG)_{(12)}$), and not with the short version ($(CUG)_{(12)}$) (Takahashi et al., 2000). In later studies however, it was reported that the CUGBP1 does not bind directly to the RNA foci present in the DM1 fibroblasts (Fardaei et al., 2001). *In vitro* experiments and analysis of DM1 patients showed that the mutated RNA is correlated with the expression of CUGBP1 in skeletal and heart muscle tissues, as well as in myoblasts isolated from DM1 patients (Timchenko et al., 2001a). More specifically, the production of CUGBP1-CUG RNA complexes was found to be associated with elevated CUGBP1 protein stability and thereby elevated levels of the protein (Timchenko et al., 2001a). Moreover, in DM1 patients, the nuclear CUGPB1 protein levels were found increased compared to normal individuals thus implying that the DM1 pathogenesis is correlated with the abnormal activation of CUGBP1 (Timchenko et al., 2001b). *In vivo* experiments performed in transgenic animal models, showed that overexpression of CUGBP1 produced the same symptoms like DM1 such as retention of the extended transcripts in nucleus as RNA foci, wasting phenotypes in eye and muscle tissues, mis-splicing, arrhythmias and distended cardiomyopathy (Ho et al., 2005, de Haro et al., 2006, Wang et al., 2007, Koshelev et al., 2010). The exact molecular mechanisms that lead to the increase of CUGBP1 levels in DM1 are not

clarified yet. It was reported however, that the mutant DMPK RNA causes the hyperphosphorylation and stabilization of CUGBP1 by activating the protein kinase C (PKC) pathway, (Kuyumcu-Martinez et al., 2007) (Figure 1.7).

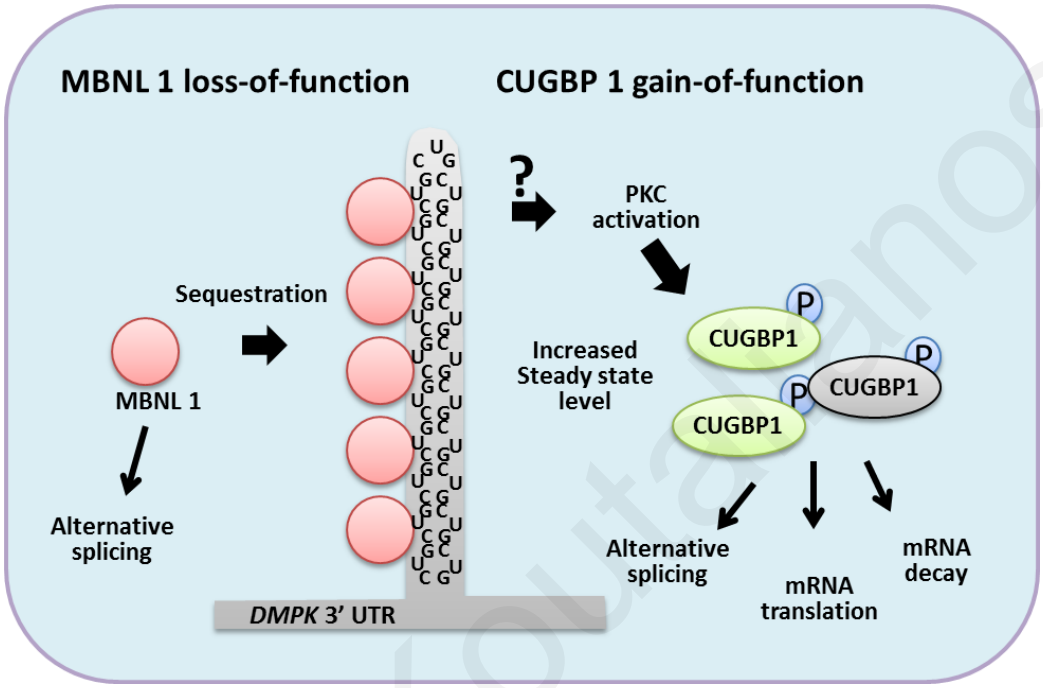


Figure 1.7 Consequences of the CUG repeat expansions. The mutated DMPK mRNA contains an unstable CTG repeat expansion within its 3′UTR. The expanded DMPK mRNA binds and sequesters the MBNL1. Another pathogenic consequence of the mutation is the upregulation of CUGBP1, possibly due to an inappropriate activation of PKC. MBNL1 loss-of-function and CUGBP1 gain-of-function leads to mis-regulation of alternative splicing, abnormal mRNA translation and mRNA decay. Adapted from Lee and Cooper 2009.

MBNL1 and CUGBP1 have significant roles in RNA alternative splicing events. The two proteins are thought to be antagonistic to each other in a number of mis-splicing events detected in DM1 pathogenesis (Foff and Mahadevan, 2011). The abnormal alternative splicing of pre-mRNA is correlated with different symptoms, such as muscle weakness and wasting, as well as insulin resistance and heart problems. The first mis-regulated alternative splicing event that was found to be associated with DM1 is that of cardiac troponin T (cTNT) (Philips et al., 1998, Kanadia et al., 2003, Ho et al., 2004). Another mis-splicing event associated with DM1 pathogenesis is that of insulin receptor (IR) which causes the insulin resistance detected in DM1 patients (Savkur et al., 2001, Ho et al., 2004, Dansithong et al., 2005). The mis-splicing of chloride channel-1 (CIC-1) pre-mRNA was found to play a critical role in DM1 patients. CIC-1 is the main chloride channel of muscle cells and loss of ClC-1 protein results in myotonia, a very common characteristic of DM1 patients. (Mankodi et al., 2002, Charlet et al., 2002). Alternative splicing can give rise to products

that are muscle specific. For instance, one of the transcripts that result from the alternative splicing of myotubularin-related 1 (MTMR1) phosphatase pre-mRNA is muscle specific and is produced during myogenesis, both *in vitro* and *in vivo* (Buj-Bello et al., 2002). The alternative splicing of MTMR1 pattern was found altered in both *in vitro* and *ex vivo* experiments (Santoro et al., 2010, Buj-Bello et al., 2002). Another symptom of DM1, dementia, is associated with the mis-splicing of Tau pre-mRNA, which encodes for an essential protein in neurons (Sergeant et al., 2001). Another pathway that was found to be affected in skeletal muscle of DM1 patients due to mis-splicing is the intracellular Ca^{2+} homeostasis through sarcoplasmic reticulum. The mis-splicing of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) 1 or 2 and Ryanodine receptor 1 (RyR1) are responsible of the impaired function of Ca^{2+} homeostasis in DM1 muscle (Kimura et al., 2005). In DM1 patients, skeletal muscle degeneration is observed, which can be associated with the mis-regulation of the alternative splicing of Mef2 transcription factor (Lee et al., 2010). Furthermore, myomesin 1 (MYOM1), which is a structural essential protein of muscle (Lange et al., 2005), was also found to be abnormally spliced in DM1 and this mis-splicing event may contribute to muscle impairment of DM1 patients (Koebis et al., 2011). Moreover, mis-spliced events in the bridging integrator-1 (BIN1) pre-mRNA were reported. BIN1 is a protein involved in tubular invaginations of membranes and is essential for the biogenesis of muscle T tubules (specialised skeletal muscle membrane structures essential for excitation-contraction coupling). Mis-spliced BIN1pre-mRNA was identified in skeletal muscle samples of patients with CDM1 and DM1, and is associated with muscle weakness (Fugier et al., 2011).

1.6.1.1.3 Myogenic defects

Molecular events that are correlated with DM1, such as mis-splicing and the abnormal recruitment of RNA-binding proteins into the ribonuclear RNA foci give rise to alterations in cell function. Remarkably, a characteristic that differs in DM1 compared to other muscular dystrophy disorders is the defects in muscle differentiation. Muscle development is altered in CDM1 fetuses, whereas muscle regeneration is compromised in adults with DM1 (Amack and Mahadevan, 2004).

In vitro experiments performed in C2C12 cell line demonstrated that overexpression of *dmpk* gene causes a significant inhibition of both fusion and terminal differentiation of the myoblasts (Okoli et al., 1998). Additional *in vitro* experiments performed on C2C12 cell line showed that overexpression of human DMPK mRNA inhibits the terminal process of differentiation and inhibits the expression of myogenin suggesting that DMPK 3'UTR overexpression possibly interferes with the expression of muscle specific genes and consequently the muscle cell differentiation is delayed (Sabourin et al., 1997) Moreover, the expression of DMPK 3'UTR with 200 CUG repeats in C2C12 myoblasts selectively repressed myogenic differentiation (Amack et al., 1999). In another study, overexpression of DMPK 3'UTR with large number of repeats or the normal number of repeats caused the inhibition of muscle development *in*

vivo with mice showing muscle atrophy at three months of age. Furthermore, in *ex vivo* experiments performed in primary myoblasts isolated from both normal and expanded CTG repeat mice showed that the expression of the mutated CTG repeats inhibits more the differentiation compared to the normal myoblasts (Storbeck et al., 2004). Remarkably, export of the expanded DMPK transcripts to the cytoplasm, using woodchuck post-transcriptional regulatory element (WPRE), restored myogenic differentiation in C2C12 cells stably expressing the mutant DMPK 3′-UTR mRNA with $(CUG)_{200}$ (Mastroyiannopoulos et al., 2008, Mastroyiannopoulos et al., 2005). The defect in myogenic differentiation was also verified in *ex-vivo* experiments using muscle cells isolated from DM1 fetuses and patients (Furling et al., 2001b, Timchenko et al., 2001b).

Molecular experiments were performed in order to discover the effect of the DMPK mRNA with expanded CUG repeats on the inhibition of myogenesis. Many reports correlate the CUGBP1 protein with the myogenic impairment observed in DM1 since CUGBP1 is an important regulator of translation of proteins involved in muscle cell differentiation and muscle development. Cultured myoblasts isolated from DM1 patients were unable to permanently exit from the cell cycle when forced to differentiate (Timchenko et al., 2001b). Normal differentiated muscle cells accumulate CUGBP1 in cytoplasm, whereas muscle cells isolated from patients with DM1failed to induce the export of the protein to the cytoplasm (Timchenko et al., 2001b). Under physiological conditions, CUGBP1 was found to promote the expression of p21 translation during differentiation by binding to a GC-rich sequence found in the 5' region of p21 mRNA (Timchenko et al., 2001b). In DM1 cultured cells CUGBP1 does not accumulate in the cytoplasm thus inhibiting the translation of p21 and other proteins responsible for cell cycle exit (Timchenko et al., 2001b). In DM1 cells cdk4 activity was found to be increased during myogenic differentiation whereas in normal cells the activity of cdk4 is decreased during differentiation (Timchenko et al., 2001b). Moreover, in normal differentiated muscle cells, the Rb/E2F repressor complexes are in abundance whereas DM1 cells fail to produce these complexes (Timchenko et al., 2001b). These findings imply that DM1 muscle cells fail to exit the cell cycle and propose that changes in CUGBP1 activity lead to the interruption of p21-dependent regulation of cell cycle withdrawal (Timchenko et al., 2001b). Further experiments showed that CUGBP1 is phosphorylated by a number of kinases during myoblast proliferation and differentiation. Phosphorylation of CUGBP1at different sites is responsible for its different mRNA targets (Salisbury et al., 2008). In particular, CUGPB1 was found to be phosphorylated during proliferation, by Akt kinase and during differentiation by cyclinD3-cdk4/6 (Salisbury et al., 2008). During differentiation, the phosphorylation of CUGBP1 by cyclin D3-cdk4 was found to promote the interaction of CUGBP1 with eIF2 under normal conditions, whereas in DM1 this process is reduced (Salisbury et al., 2008). Furthermore, overexpression of cyclin D3 in DM1 cells was determined to promote the fusion of the myoblasts thus restoring the differentiation (Salisbury et al., 2008). More recently, it was shown that human skeletal muscle satellite cells isolated from fetal CDM1 patients with large CTG expansions

(>3000) release prostaglandin E2 (PGE(2)). PGE2 is known to prevent the fusion of normal myoblasts by reducing intracellular levels of calcium (Beaulieu et al., 2011). It was therefore suggested that the delay in muscle cell differentiation observed in CDM1 patients possibly results, at least in part, from an altered autocrine mechanism (Beaulieu et al., 2011).

1.7 Hypothesis and specific objectives

1.7.1 Hypothesis

MiRNAs play a criticlal role in post-transcriptional regulation of different processes, including myogenesis, and they are also potential regulators of the transcription of genes that are involved in the process. The purpose of this proposal is to investigate the post-trancriptional regulation of Twist, an inhibitor of myogenesis, through miRNAs, in muscle cells.

1.7.2 Objectives of the proposed research project

Twist is a bHLH transcription factor that inhibits muscle cell differentiation. Twist was determined to be downregulated during myogenesis.The mechanisms however, that regulate the expression of Twist are not clarified yet. The proposed project aims to investigate the post transcriptional regulation of Twist by miRNAs.

The specific objectives are as follows:

1. Identify potential miRNAs for Twist 3' UTR by bioinformatics analysis.
2. Investigate the potential of miRNAs to bind to Twist 3' UTR by luciferase assays.
3. Validate miRNAs as regulators for Twist in muscle cell lines and primary mouse cells.
4. Confirm the post-transcriptional regulation of Twist by miRNAs *in vivo*
5. Investigate the Twist expression and regulation in muscle cell lines isolated from Myotonic Dystrophy type 1 (DM1) patients that show impaired muscle cell differentiation

1.7.3 Importance and innovation

Skeletal muscle tissue is important mainly because it is responsible for motion. The process by which skeletal muscle tissue is formed is highly complex and tightly regulated. It is very important to discover as much as possible about the mechanisms that take place during the formation of the muscle. Myogenesis is the process by which myoblasts are differentiated into mature myotubes, which eventually fuse to form the muscle fibers. A series of pathways and molecules are involved during myogenesis. *Twist* is an early developmental gene that inhibits the muscle cell differentiation in mammals. The process of myogenesis is also regulated by the miRNAs, the small non-coding RNA molecules. MiRNAs inhibit the translation of their target mRNA by binding to their 3'UTR in the cytoplasm and play a role in modulation of cellular phenotypes. MiRNAs have been also determined to function during the degenerative process of muscle in muscle diseases (Eisenberg et al., 2007). Although some information exists regarding the transcriptional regulation of Twist, to date, there is no evidence on the post-transcriptional regulation of Twist. The present project therefore aims to investigate for the first time the regulatory role of miRNAs in the expression of the Twist in muscle, as well as in muscle disease.

2

MATERIALS & METHODS

2.1 Primary mouse muscle satellite cell isolation

2.1.1 Removal of Extensor digitorum longus (EDL) muscle from mouse

C57 black 6 mice were taken from an onsite mouse facility. They were chosen to be about 4 weeks old. The method that was used to kill the mice was cervical dislocation. The mice were soaked with 70% ethanol and hind limbs were shaved using scalpel blades (Martin, Germany) to remove fur and leave naked skin. After being shaved and washed with ethanol, the mouse was pinned onto a dissecting board with one forelimb pinned through the palm, and the contralateral hind limb thorough the dorsal side of the paw. The tail was placed to one side and the other hind limb directly opposite so that the hind leg was facing upwards. The mouse was placed under a light microscope. A scratch was made with the blade from above the knee to the paw. Afterwards, the skin was removed without disrupt the muscle by fascinating the skin with a pair of forceps. The four tendoms of the EDL that located in the lower part of the leg, on the paw, were spotted out. Those tendoms were cut near to their insertions on the base of the third phalanx. Above the EDL is the tibaialis anterior (TA) (Figure 2.1). The TA was then cut at its tendon near to its attachment on the first cuneiform and near end of the first metatarsal. The epimysium which covers the TA muscle was cut without damaging the muscle. The TA was carefully removed by holding it through tendoms and pulling it away using forceps followed by a scratch at the proximal end to remove it finally. The EDL was then kept by all four tendons and pulled away gently form the underlying musculature and bone. Each EDL muscle was placed in a 7 ml Bijoux (Sterilin, U.K.) containing with 5 ml of 0.2% collagenase Type 1 (Sigma-Aldrich, U.S.A.) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, U.S.A) and incubated for 1 hour at 37ºC. Collagenase is an enzyme that breaks down the native collagen which grips the tissues together. This allows the single fibers to be isolated from the entire muscle.

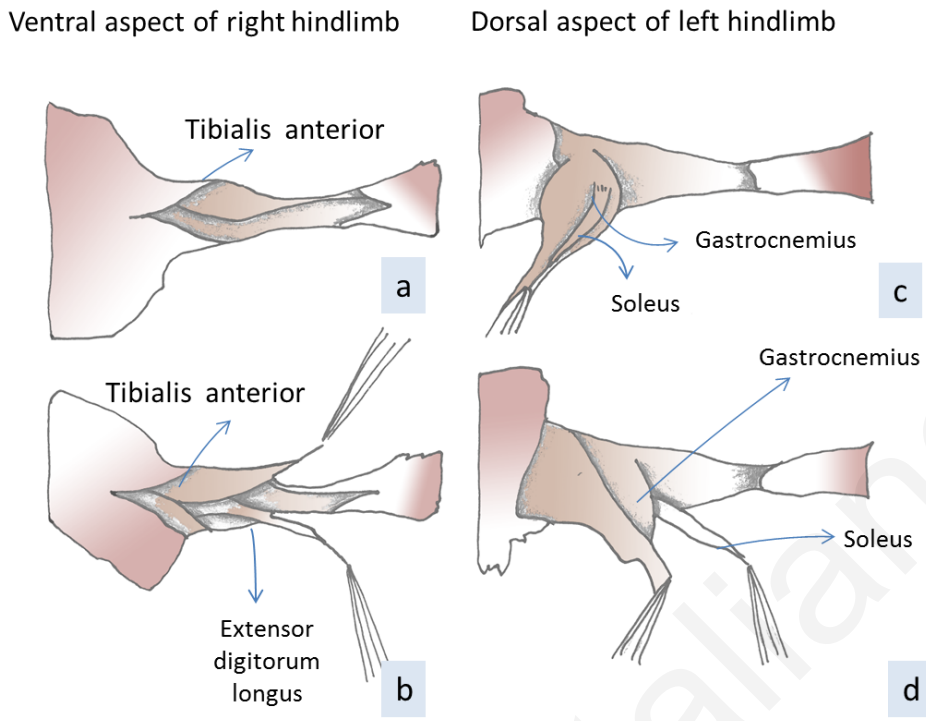


Figure 2.1 Diagram of the mouse hind leg with major muscles shown. A-B) The anatomical position of the TA muscle and the location of the EDL. An incision of the four tendons and a gentle pull of the two tendons holding the TA reveal the EDL.C-D the anatomical position of the gastrocnemius muscle and the location of the soleus. A cut of the tendons holding the gastrocnemius reveals the soleus muscle which can then be carefully removed after removing the gastrocnemius.

2.1.2 Isolation of single muscle fibers

During the 1 hour that the muscle is in the collagenase, the preparation for the purification of the single muscle fibers must be done. In detail, glass pasteur pipettes were cut using a diamond pen (VWR international, U.S.A.) in order to form pipettes of different openings with diameters from 0.5 mm to 2.5 mm. In order to be sure that there are no sharp edges in the pipettes which may damage the fibers the pipettes were heat polished and tested on aluminium foil and placed in UV for sterilization. For each muscle removed (depending on how many mice were used) 3-4 petri dishes were treated with H horse serum (Gibco) and DMEM was added to them and kept at 37 ^{o}C for at least 30 minutes. The horse serum is used in order to prevent the fibers from sticking to the petri dishes. Once the 1 hour incubation period was over, the muscles in collagenase were poured into a horse serum treated sterile dish containing 10 ml DMEM with 1% (v/v) penicillin/streptomycin solution (Sigma) and left at 37 ^{o}C for 30 minutes to rest. After the half hour, the separation of the single fibers was began by gently blowing DMEM from the dish onto the muscle. This released all the fibers from muscle to the medium. The separation of the fibers did not last for more than 10 minutes and the muscle was placed back into the 37^{o}C incubator because the fibers star to be destroyed if stay for long time out of the incubator. For the whole process, the pipettes that were used are continually flooded with horse serum in order not to harm the fibers.

The fibers passed through a total of 4 rounds of purification using pipette with smaller diameter each time. In the final round, single fibers were transferred using the smallest pipette, to the last sterile dish, one for each muscle. The muscle fibers were then removed from the last petri dish using the narrowest pipette. Those fibers were placed into non-treated Corning® 6-well plates coated with DMEM containing 1 mg/ml Matrigel (Collaborative Research) in a 1:10 dilution. Due to the fact that the matrigel is solidified at room temperature, the Matrigel dilutions and treatment of plates must be done at 4 ºC. The matrigel coated plates were placed into the 37ºC incubator for 30 minutes and then activation media was added (DMEM with 20% Foetal Bovine Serum (FBS) (Gibco), 10% (v/v) horse serum (Gibco), 0.5% (v/v) chick embryo extract (ICN Flow), 1% (v/v) penicillin/streptomycin solution (Sigma) and 0.01 % murine FGF- basic (Peprotech) prior the plating of the fibers. After plating, the plate with the fibers was placed in the incubator at 37°C for 48 hours. The fibers were stuck on the matrigel due to the adhesion properties of the matrigel and the plates left untouched in activation media for 48 hours for in the incubator in order for the satellite cells to migrate from the fibers to the matrigel.

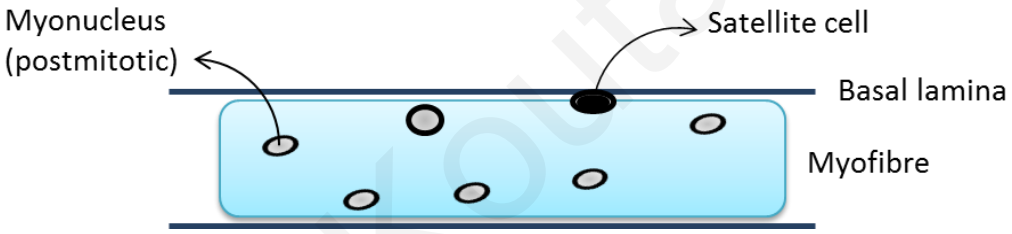


Figure 2.2 Diagram of muscle fiber with satellite cells. Following incubation in activation medium, the satellite cells become activated and migrate from the myofiber surface to the matrigel coating the base of the cell culture plate.

2.1.3 Plating of satellite cells

After 48 hours, another matrigel was formed as prepared before. The muscle fibers were removed by gentle blowing with horse serum treated pipettes in order for the plates to contain fibroblasts and satellite cells. The cells were then washed with preheated at 37ºC Dulbecco's Phosphate-Buffered Saline (PBS) and each well treated with 100µl Trypsin-LE (Gibco®, U.S.A.), for 5 minutes at 37°C. The cells were then distanced from the matrigel monolayer. Following this, the cells were harvested by pipette disturbance with 2 ml of DMEM and plated into non-treated petri dish and incubated for 30 minutes at 37°C. At this stage, the population of the cells contained both satellite cells and fibroblasts. During the 30 minute incubation time, the fibroblasts stuck to the bottom of the non-treated petri dish leaving only the satellite cells in suspension. The suspension containing only the satellite cells was plated into the new matrigel treated Corning® 6-well plate containing activation media and placed in the incubator at 37°C and 5% CO2. Every 24 hours the satellite cells were washed with PBS and fresh activation media was added.

2.2 Culture of Cell lines

Human myoblast cell lines were used. Human myoblasts were isolated from muscle biopsies under antiseptic conditions. The biopsies were originally obtained from the Tissue Bank for Research (BTR) of the Association Francaise contre les Myopathies in agreement with the French legislation on ethical rules and the local Bioethics Committee and donated to our laboratory by Dr Denis Furling, Sorbonne University,Paris, France. For cell lines from foetuses (14, 31 and 37 weeks of development), cell originated from biopsies of proximal-ventral region of the limb, which form the quadriceps (Edom-Vovard et al., 1999). For the newborn cell line, cells arise from biopsies that were taken from the quadriceps (Decary et al., 1997). For DM1 cell lines, cells arise from biopsies of DM1 unborn or newborn foetus (31 and 37 weeks respectively). Morphometric analysis of the head, femur and foot was used in order to specify the stage of development of the foetuses and for the newborn; the age was calculated from birth.

The human skeletal muscle cells were grown in 20% growth medium (20% GM) composed of DMEM, (High Glucose, Pyruvate, no Glutamine) (Gibco®, U.S.A.) complemented with 20% Foetal Bovine Serum (FBS) (Heat Inactivated) (Gibco®, U.S.A.), 2% GlutaMAXTM (Gibco®, U.S.A.) and 1% Penicillin-Streptomycin 100X Solution (Gibco®, U.S.A.) in T75 flasks (Iwaki) or 6-well plates (Corning, U.S.A.). The DMEM was changed every second day. To differentiate cells to myotubes, they were grown until the confluency stage and the media was changed to differentiation medium (DM) (DMEM complemented with 2% Horse Serum (Heat Inactivated) (Gibco®, U.S.A.), 2% GlutaMAXTM (Gibco®, U.S.A.) and 1% Penicillin-Streptomycin 100X Solution (Gibco®, U.S.A.)). After 5 days in differentiation medium the fully differentiated muscle cells were observed. DM was also refreshing every 2 day.

HeLa cell line was proliferated in DMEM complemented with 10% FBS (Heat Inactivated) (Gibco®, U.S.A.), 2% GlutaMAX^{TM} (Gibco®, U.S.A.) and 1% Penicillin-Streptomycin 100X Solution (Gibco®, U.S.A.).

All the cell lines were cultured in an incubator at 37°C with 5% CO2 (BINDER, U.S.A.) and all the processes concerning cell cultures were carried out in a sterile cabinet (MICROFLOW ADVANCED BIOSAFETY CABINET, U.K.).

2.2.1 Cell propagation

Cells were sub-cultured when reached around 80% confluence. The GM was removed and the cells were washed with PBS, (Gibco®, U.S.A.) to avoid any traces of FBS existing in the remaining culture media. After the removing of PBS, 1 ml of 0.25% Trypsin-EDTA (1X) with phenol red (Gibco®, U.S.A.) was added for detachment of the cells, and left for approximately 3

minutes in the incubator at 37 ºC. Cells were collected using the corresponding GM, and transported in a 15 ml RNase-free centrifuge tube (Corning, U.S.A.). For each experiment the proper amount of the cells were placed in a new T75 flask or 6-well plates containing the corresponding GM.

2.2.1.1 Passaging of cultured cells

Cells were subcultured until the cells reached around 80% confluence. At this stage the GM was aspirated from the flask and the cells were washed with phosphate buffered saline (PBS, Gibco, Invitrogen) in order to remove any FBS left in the flask. PBS was then remove and 1 ml of 0.25% Trypsin-EDTA (1X) with phenol red (Gibco®, U.S.A.) was added and the cell were incubated for 3 minutes at 37°C in order the cell to be detached from the base of the flask. Cells were collected using 9 ml of GM. The GM has the property to deactivate the trypsin also. Harvested cells were transferred to a 15 ml RNase-free centrifuge tube (Corning, U.S.A.). 1-2ml of the harvested cells, depending on how fast the cells were proliferating, were left in the flask and fresh GM was added to the total volume of 10ml allowing for the cells to proliferate. The harvest cells were then plated into 6-well plated containing GM for further experiments or being discarded.

2.2.1.2 Freezing of cell lines for storage

Cells were harvested as above in the passaging of cultured cells. In this case, the remaining cells were transferred in RNase-free centrifuge tube (Corning, U.S.A.) and centrifuged for 5 minutes at 1,500 rpm. With the centrifugation the cells were collected at the bottom of the tube in form of pellet. The supernatant was removed and the pellet of cells were re-suspended in freezing medium (90% of 20% GM and 10% Dimethyl Sulfoxide (DMSO, Sigma)). The suspension was then aliquoted into freezing vials (cryovials tubes, Thermo Scientific, USA) and placed into a freezing container (Sigma) with isopropanol and placed into an -80°C freezer for 24 hours. Finally the vials were stored in liquid nitrogen tanker.

2.2.1.3 Recovery of cultured cells from liquid nitrogen

The cells were kept freshly and well proliferated by keeping the passaging below 30. When the passaging reached the limit, fresh cells were then defrosted from storage in liquid nitrogen. One aliquot containing 1 ml of freezing cells was quickly defrosted by dipping the vial into a beaker with water at room temperature and immediately after the defrost, cells were placed directly into a T75 flask with 9 ml pre-warmed 20% GM at 37 ºC. The 20% GM is used since this is the identical

media that was used during freezing with DMSO. In the next day, the medium was changed to the corresponding medium for each cell as described above.

2.2.2 Transfections of cultured cells.

Human myoblasts, HeLa and primary mouse muscle cells were regularly transfected with DNA and RNA, including plasmids, microRNA mimics, microRNA inhibitors and siRNA.

Lipofectamine® 2000 (Life Technologies) transfection reagent was used for the plasmid transfection. 5 μl of the transfection reagent was added to 100 μl of Opti-MEM® reduced serum media (Gibco), mixed well by pipetting and left to stand for 5 minutes. At the same time the appropriate amount of plasmid DNA was added to another 100 μl of Opti-MEM®. Lipofectamine®- Opti-MEM® and the plasmid- Opti-MEM® are mixed together and incubated in room temperature for 30 minutes in the dark, due to the fact that Opti-MEM® is light sensitive, and mixed by pipetting thoroughly. The 200 μl mixture was then added, dropwise onto the cells in the 6 well plates containing 1.8 ml Opti-MEM®. After 6 hours the Opti-MEM® wasremoved and fresh GM was added.

For microRNA mimic and inhibitor transfections into human myoblast cells, X-tremeGENE 9 DNA Transfection Reagent was used. 6 μl of the transfection regent was added to Opti-MEM® reduced serum media (Gibco) as well as the appropriate amount of 100 pmoles of either miRNA mimics or antimiRs (final concentration 0.05 μM) , mixed well by pipetting and incubated for 20 minutes at room temperature in the dark. The mixture was added drop wise in the 6 well plates containing 2 ml GM and left to incubate for 24 hours.

For transfections into the HeLa cell line and mouse primary muscle cells with miRNA mimics, inhibitors and siRNA, Lipofectamine® RNAi MAX was used. 5 μl of the transfection reagent was added to 100 μl of Opti-MEM® reduced serum media (Gibco), mixed well by pipetting and left to stand for 5 minutes. At the same time the appropriate amount of plasmid DNA (400 ng) was added to another 100 μl of Opti-MEM®. Lipofectamine®- Opti-MEM® and the plasmid- Opti-MEM® were mixed together and incubated at room temperature for 30 minutes in the dark, due to the fact that Opti-MEM® is light sensitive, and mixed by pipetting thoroughly. The 200 μl mixture was then added in the cell drop wise onto the cells in the 6 well plates containing 1.8 ml Opti-MEM®. After 6 hours the Opti-MEM® was removed and fresh GM was added

2.2.3 Transductions of cell lines with adenoviruses

2 days before reaching confluency, the cell lines were transduced with 100 M.O.I. (multiplicity of infection) of human MyoD adenovirus under the control of the cytomegalovirus (CMV) promoter (Vector Biolabs, U.S.A.) (AdM), or 100 M.O.I. of human Twist-1 adenovirus under the control of the CMV promoter or control virus (Vector Biolabs, U.S.A.), or (AdC), containing an empty vector with a CMV promoter and an intact viral backbone of serotype 5 adenovirus or combination of the two viruses (100 M.O.I AdM and 70 M.O.I AdT) The cells were left to grow for two days until reaching confluency and then induced to differentiate or processed according to the experiments needs.

2.2.4 Cell harvesting

Cells were grown in either GM or DM and transfected or not as above and left to reach the required state. The medium was removed and cells washed with PBS. After the removal of the PBS 0.25% Trypsin-EDTA (1X), phenol red (Gibco®, U.S.A.) was added, approximately 150 μl for one well of a 6-well plate or 1 ml for a flask, in order the cell detached the plate or the flask. Cells were harvested using 2 ml of GM and transferred in a 15 ml RNase-free centrifuge tube (Corning, U.S.A.). The suspension was centrifuged at 1500 rpm for 5 minutes at room temperature. The supernatant was removed and the pellet consisting of the cells was re-suspended in 2 ml of PBS in order to be washed from any treatment left from GM. The new suspension was again centrifuged for 5 minutes at 1500 rpm at room temperature, and the PBS was removed leaving the pellet and place it in the ice until further use.

2.3 Immunocytochemistry

After leaving the skeletal muscle cells to differentiate for 5 days, they were washed with PBS. 1 ml of 4% (w/v) paraformaldehyde (Merck, Germany), diluted in PBS, was added in each well in order to fix the cells and incubated for 30 minutes at 4 ^{o}C. The fixation process, force the cellular and subcellular structures to retained and in the same time immobilize the antigens allowing the antibody to enter all the cells and the subcellular components. After the half hour the 4% paraformaldehyde was removed and the cells were washed 3 times, for 5 minutes each, with 2 ml of PBS. The next step was to permealise the fixed cells for 15 minutes with 1 ml 0.1% (v/v) Triton® X-100 (Merck, Germany) in 1% (w/v) bovine serum albumin (BSA) (GE Healthcare, U.K.) diluted in PBS. This process is necessary in order to improve the antibody penetration. Paraformaldehyde (cross-linker) may reduce the antigenicity of some cell components and therefore by permealising the cells, is enhanced. Afterwards the cells were washed for 5 minutes

with 2 ml PBS. PBS was removed and 2 ml of 1% (w/v) BSA, diluted in PBS, was added for 10 minutes in order to prevent unspecific binding of the antibodies. The antibody used for the Immunocytochemistry experiments in this project was the mouse monoclonal primary antibody against myosin heavy chain (MyHC) (Skeletal, Fast) (MY-32; Sigma-Aldrich, U.S.A.). This antibody was diluted in 1:400 in 1ml of 1% (w/v) BSA in PBS and added in the cells and incubated for 2 hours at 37 ºC in the dark. Until the end of the procedures of the immunocytochemistry experiments the cells were kept in the dark. The solution with the antibody was aspired and the cells were washed 3 times for 5 minutes with 2 ml of PBS. 1 ml of Alexa Fluor 594 goat anti mouse antibody (ReadyProbes®, life Technologies, USA) in 1:500 dilutions in PBS was added to the cells and incubated in room temperature for 1 hour in dark. The secondary antibody solution was removed and the cells were washed twice with 2 ml of PBS for 5 minutes each wash. 1 ml of trihydrochloride, trihydrate (Hoechst) (Invitrogen) of 1:10000 dilutions in PBS was added to the cells. The Hoechst solution was removed and the cells were then left to dry. Hoechst binds to the minor groove of double stranded DNA preferring mostly region rich of adenine and thereby staining the cell nuclei. Images were captured using Cell Observer microscope (Zeiss, Germany) and AxioVision Digital Image Processing Software version 4.8.2 (Zeiss, Germany). The images were accumulated using Adobe Photoshop software. Each experiment was repeated at least three times and myotubes and the number of fuses cells were counted from 5 different cellular areas.

2.4 Protein analysis

2.4.1 Protein extraction

Protein extractions were obtained at different stage of differentiation of the cells lines including proliferating and confluent myoblasts (day 0 of differentiation) and differentiated muscle cells (days 2, 3 and 5) or 2 days after transfection. The cells were cultured until the required stage and then were harvested and exposed to protein extractions using lysis buffer. The lysis buffer composing by 1 M NaCl, 10 mM Tris-HCl (pH=7.6) (Sigma-Aldrich, U.S.A.), 10% of glycerol (BDH Laboratory Supplies, U.K.), 1% of Tween 20 (Affymetrix, U.S.A.), 10 mM β-Mercaptoethanol (Merck, Germany) and 1X complete, EDTA-free protease inhibitor cocktail (Roche, Germany). The protease inhibitor was added to the buffer just before used and used in order to inhibit the degradation of the protein in the extraction. Protein extracts were exposed to three cycles of 10 seconds sonication with 10 seconds incubation on ice between cycles in order to disrupt cell membranes and release the cellular contents including the proteins. The sonicated solution was then centrifuged at 13,200 rpm for 30 minutes at 4 ºC. The resulting supernatant was collected without the pellet that contains the cell debris and transferred to a new 1.5 ml RNase-/DNase-free tube and stored at -80 ºC until further use.

2.4.2 Measurement of protein concentration

For each extraction sample, the concentration was determined by using the colorimetric protein assay, Bradford Protein Assay. For each sample, one 1.5 ml tubes was prepared and 300 μl of Coomassie Plus (Bradford) Protein Assay reagent (Thermo Scientific, U.S.A.) was added with 7 μl of distilled water and 3 μl of protein extract, mixed well and left to stand for 10 minutes on the bench at room temperature. The brown acidic coomassie-dye reagent when come in contact with a protein solution, changes colour to blue and the density of the colour is proportional to the concentration of protein present in the sample. Also a blank sample was prepared by mixing 300 μl of Bradford and 10 μl of water. By using Bradford with water as blank in order to set the spectrophotometer (Eppendorf BioPhotometer, Germany). The samples were placed in the cuvette (Eppendorf, Germany) and then in the spectrophotometer. Then the machine was re-blanked using 200 μl of the blank sample. After each sample was measured 200 μl of each sample were placed in a cuvette and the absorbance measured at 595 nm. A pre-prepared standard curve was used in order to clarify the concentration of the proteins by using a computational program GraphPad Prism 4 Software. The standard curve was prepared using a series of known dilutions of BSA.

2.4.3 Western Blotting

2.4.3.1 Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

30-40 μg of protein extracts were prepared with 1X Sodium Dodecyl Sulfate (SDS) gel-loading buffer composing of 0.25 M Tris-HCl (pH=6.8), 10% SDS, 50% glycerol, 0.005% Bromophenol Blue sodium salt (Sigma-Aldrich, U.S.A.) and 0.5 M β-mercaptoethanol. The prepared samples were incubated for 5 minutes at 95 ^{o}C in order to denature the proteins. SDS is a strong detergent that detaches the proteins and binds to the denatured polypeptides and loads a negative charge on the protein that helps for the migration through the gels using voltage current. The method is called Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and helps to separate proteins on polyacrylamide gels containing SDS. Each gel was made of two different layers. The stacking and the resolving gel. The stacking gel was composed of 500 μl (5%) of 30% Acrylamide/Bis Solution 29:1 (BIO-RAD. U.S.A.), 750 μl of 4X stacking gel buffer (0.5 M Tris-HCl and 0.4% of SDS) pH=6.8, 30 μl of 10% ammonium peroxodisulfate (APS) (Scharlau, Spain),(molecular biology grade diluted in distilled water), 3 μl of N,N,N',N'-Tetramethylethylenediamine (TEMED) (BDH Laboratory Supplies, U.K.) and distilled water until the volume was 3 ml. Resolving gel was used to separate the denatured proteins regarding to their molecular size. Resolving gel consisted of 2400 μl (12%) of 30% Acrylamide/Bis Solution 29:1 (BIO-RAD, U.S.A.), 750 μl of 4X resolving gel buffer (1.5 M Tris-HCl and 0.4% of SDS) pH=8.8, 60 μl of 10% APS, 6 μl of TEMED and distilled water up to 6 ml. The samples were ran using the Mini-PROTEAN Tetra Cell electrophoresis system (BIO-RAD. U.S.A.) filled up with 1X running

buffer composed of 0.025 M Tris-HCl, 192 M glycine and 1% SDS at approximately 70 Volts until the sample came in a line through the stacking gel and enter the resolving gel and the voltage was increased to 90 Volts and run for 1.5-2 hours. With the sample, ladder was also been run in order to compare the sizes of the proteins, SpectraTM Multicolor High Range Protein Ladder was used (Fermentas-Thermo Scientific, U.S.A.).

2.4.3.2 Protein transfer to a polyvinylidene difluoride (PVDF) membrane

A Hybond-P hydrophobic polyvinylidene difluoride (PVDF) membrane, optimized for protein transfer (Amersham, GE Healthcare, U.K.) was inactivated by suction in methanol ≥99.9% (Sigma-Aldrich, U.S.A.) for 3 minutes in a shaker at room temperature. Methanol was removed and the membrane washed with water for 5 minutes in a shaker at room temperature. After the removal of the water the membrane was incubated in transfer buffer, consisting of 200 mM glycine, 25 mM Tris base and 10% of methanol, for 10 minutes in a shaker at room temperature. Following SDS-PAGE, gels were detached and incubated in transfer buffer for at least 5 minutes in a shaker at room temperature. By using a transfer machine (Trans-Blot® SD Semi-Dry Transfer Cell, BIO-RAD. U.S.A.) the proteins transferred in a PVDF membrane. The gel was placed on the top of activated membrane and both between two pieces of Extra Thick Blot Paper (BIO-RAD. U.S.A.) soaked in transfer buffer and then on the transfer machine. 14 volts was applied on the machine for 50 minute and the proteins were transferred from the gel to the membrane.

2.4.3.3 Blocking and antibody incubation of the membrane

Following transfer, membranes were blocked with 2-5% non-fat milk (Regilait, France) in 0.1% Tween-PBS for 30 minutes. The concentration of blocking solution varies depending on the non-specific binding of each primary antibody that was used. Following blocking, the membranes were washed with 0.1% Tween-PBS and incubated with primary antibodies diluted in 0.1% Tween-PBS overnight at 4 ^{o}C on a shaker. Primary antibodies used were Twist antibody (mouse monoclonal IgG1 - 100 μg/ml) in 1:150 dilution (Twist 2C1a; Santa Cruz Biotechnology, U.S.A.), MyoD antibody (rabbit polyclonal IgG - 200 μg/ml) in 1:300 dilution (M-318; Santa Cruz Biotechnology, U.S.A), Troponin antibody (rabbit polyclonal IgG - 200 μg/ml) in 1:300 dilution (H-170; Santa Cruz Biotechnology, U.S.A), Muscle Actin antibody (mouse monoclonal IgG - 200 μg/ml) in 1:300 dilution (4i346; Santa Cruz Biotechnology, U.S.A) or GAPDH antibody (mouse monoclonal IgG1 - 100 μg/ml) in 1:3000 dilution (6C5; Santa Cruz Biotechnology, U.S.A.). The next day, membranes were washed 3 times with 0.1% Tween-PBS, 8 minutes each and incubated with the corresponding secondary antibodies for 2 hours on a shaker at room temperature. The

secondary antibodies used are: goat anti-mouse IgG (200 µg/0.5 ml) in 1:2000 (1:4000 for GAPDH) dilution in 0.1% Tween-PBS or donkey anti-rabbit IgG (200 µg/0.5 ml) in 1:7000 dilutions in 0.1% Tween-PBS.

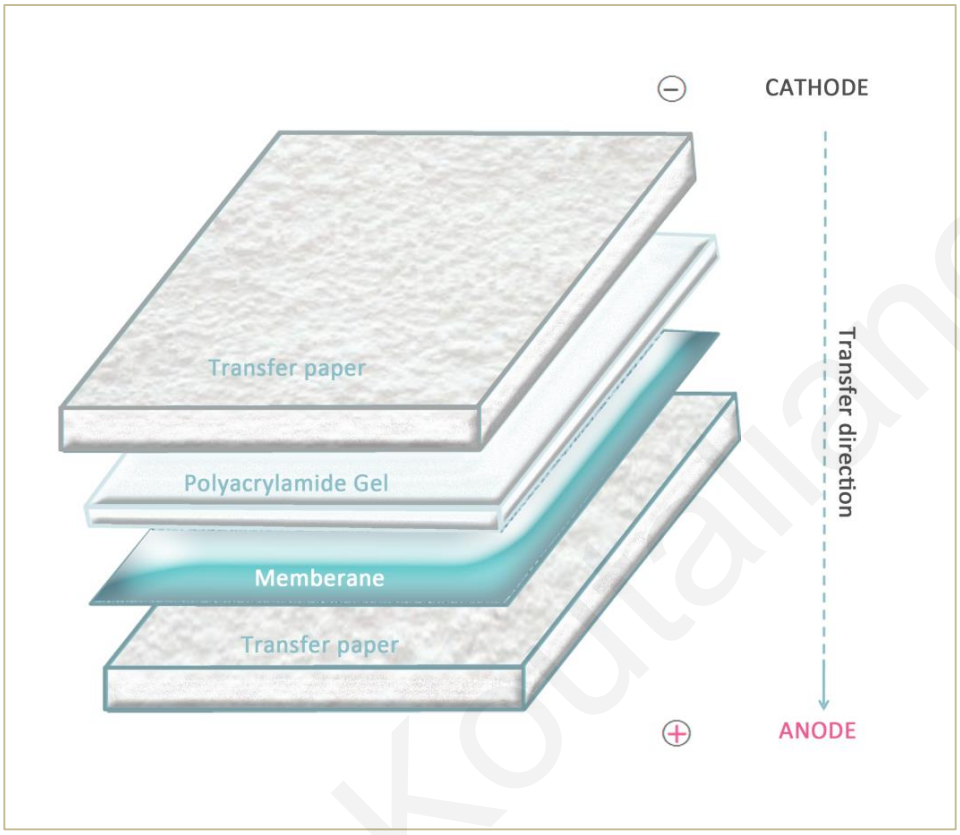


Figure 2.3 Transfer of proteins from gel to membrane. The membrane was placed beneath the gel and sandwiched between the soaked transfer paper as the proteins are negatively charged and will move downwards towards the anode.

2.4.3.4 Visualization of the target protein

Membranes were then washed with 0.1% Tween-PBS three times for 5 minutes each and once with PBS for 1 minute. Reagent A, consist of 4.5 ml distilled water, 500 µl of 0.1 M Tris-HCl, 50 µl of luminol and 30 µl of p-Coumaric acid, was mixed with Reagent B consist of 4.5 ml of distilled water, 500 µl of 0.1 M Tris-HCl and 30 µl of hydrogen peroxide (H2O2) solution 30% (Merck, Germany). The mixer was added directly to the membranes and incubated for 1 minute at room temperature. The Luminol was prepared by diluting 221 mg of luminol, for chemiluminescence, ≥98.0% (HPLC) (Fluka) in 5 ml of 100 mM Tris-HCl. p-coumaric acid was prepared as 74 mg of p-coumaric acid ≥98.0% (HPLC) (Sigma-Aldrich, U.S.A.) diluted in 5 ml of Dimethyl sulfoxide (DMSO) ≥99.5% (Sigma-Aldrich, U.S.A.). Both Luminol and p-coumaric were aliquoted and stored at -20ºC. In the presence of H_2O_2 the HRP protein that is found on secondary antibodies undergoes a chemiluminescent reaction resulting in luminol oxidation. When luminol comes back to its ground state, light is emitted and therefore the bands can be visualized. p-

coumaric acid is a phenol acid that improves the quantity and duration of the light emitted by the reaction. The membranes were then transferred to a HypercassetteTM Autoradiography Cassette (Amersham) and exposed on medical Super RX X-Ray films (Fuji, Japan). The film was then passed through a film processor (KODAK M35 X-OMAT Processor) and the bands were visualized. All western blots were repeated three times. The bands were transfer through scanner to computer their density was measured using the software Image J.

2.5 Chromatin Immunoprecipitation (ChIP) Assay

MAGnifyTM Chromatin Immunoprecipitation System (Invitrogen, U.S.A.) was used for the Chromatin Immunoprecipitation (ChIP) Assay, according to the company's instructions.

2.5.1 Coupling of Dynabeads® to primary antibody

The Dynabeads® Protein A/G provided by the kit was gently re-suspended. From the fully re-suspended Dynabeads® 10 μl was taken and added to 100 μl of Dilution Buffer which was placed in a 0.2 ml DNase-/RNase-free PCR tube. The tube was placed in DynaMagTM-PCR Magnet until the beads formed a close-fitting pellet. The liquid was discarded by pipetting and the pelleted magnetic beads were re-suspended in appropriate antibody dilution in 100 μl of Dilution Buffer. For MyoD binding, 10 μg of MyoD antibody (mouse monoclonal IgG1 – 0.5 mg/ml) (MoAb 5.8A; BD PharmingenTM, U.S.A.) was added in 100 μl of Dilution Buffer. For control experiments in ChIP assay, two controls were used, a negative control antibody and a positive control antibody. 1 μg Mouse IgG antibody in 100 μl of Dilution Buffer was used as a negative control and 2.5 μg of unconjugated polyclonal antibody specific to human histone H3, trimethylated at lysine 9 [K9me3] (H3-K9Me3) (Invitrogen, U.S.A.) in 100 μl of Dilution Buffer was used as a positive control. The tubes containing the samples with he antibodies were placed in end-over-end rotor and left for rotating at 4^{o}C for 1 hour and then stored at 4 ^{o}C until further process.

2.5.2 Crosslinking of chromatin

After the preparation of the Antibody/Dynabeads® mixtures, cells which were grown in 6-well plates were harvested and crosslinked. The medium was removed and the cells washed with 2 ml PBS. PBS was then removed and 150 μl of Trypsin was added in each well to detach the cells from the plate. By using PBS, the cells were harvested and placed in 15 ml centrifuge tube and spun for 5 minutes at 200xg. The supernatant was aspired and the pelleted cells formed were re-

suspended in roughly 300 μl of PBS. 10 μl of the re-suspended cells was used in order to measure the number of cells by using the Countess® Automated Cell Counter (Invitrogen, U.S.A.). After the cell measure, the volume of re-suspended cells was adjusted up to 500 μl of room temperature PBS. Next step was to add 13.5 μl of Formaldehyde solution 37% (Merck, Germany) to the 500 μl of re-suspended cells in order to obtain a final concentration of 1%. The sample was mixed by invertion and then incubated for 10 minutes at room temperature. In order to stop the crosslinking reaction, 57 μl of 1.25 M Glycine (provided by the kit) was added. The tube was also mixed by invertion and incubated at room 5 minutes. The cross-linked cells were centrifuged at 200 x g for 10 minutes at 4 ^{o}C. The supernatant was aspired and the pelleted cells were re-suspended in 500 μl of cold PBS and centrifuged again at 4 ^{o}C for 10 minutes at 200 x g. the pellet was re-suspended again in 500 μl of cold PBS and centrifuged again at 4 ^{o}C for 10 minutes at 200 x g. The supernatant was aspired and the pelleted cells were immediately taken to the cell lysis step.

2.5.3 Cell lysis

According to the number of cells, the pellet was re-suspended to the appropriate amount of Lysis Buffer. For every million cells, 50 μl of Lysis Buffer was prepared containing Protease Inhibitors (to a final concentration of 1X). The cell pellet was re-suspended in 50 μl of Lysis Buffer containing Protease Inhibitors and incubated on ice for 5 minutes. This step was carried out in order to lyse the cells and release the chromatin from the nuclei.

2.5.4 Chromatin shearing

The chromatin was then cut into DNA fragments, approximately 200-500 bp long according to the protocol, using sonication. During this step, the cell lysate was kept on ice, in order to overtake the heat released by the sonication probe and kept the sample cool. The heat may reverse the crosslinks. Therefore the cell lysate was sonicated for 30 seconds and then placed immediately on ice for additional 30 seconds. This cycle was repeated for 16 times. After sonication the cell lysate was centrifuge for 5 minutes at 13,200 rpm at 4 ^{o}C. At this stage the cell debris was pelleted. The supernatant containing the sonicated chromatin was transferred to a fresh 1.5 ml DNase-/RNase-free tube.

2.5.5 Chromatin dilution

By using the Dilution Buffer containing 1X Protease Inhibitors, the Cell Lysate was diluted after sonication in order to proceed to immunoprecipitation reactions. For each

immunoprecipitation reaction, 200,000 cells were used. Assuming that the starting concentration was 1 million cells per 50 μl of Lysis Buffer, 10 μl of sheared chromatin (200,000 cells) was diluted in 90 μl of Dilution Buffer containing 1X Protease Inhibitors to get a total volume of 100 μl. The solution was gently mixed. For each sample to be analysed, an extra 100 μl was arranged and 10 μl of this dilution was shifted in a new 0.2 ml RNase-/DNase-free PCR tube. This was used for reverse-crosslinking and DNA purification without proceeding to immunoprecipitation procedure and used as input control, a positive control at the end of the process.

2.5.6 Chromatin binding to the Antibody-Dynabeads®

The tubes with the Antibody/Dynabeads® mixtures pass through a short spin to remove any liquid trapped on the caps and placed for about 30 seconds in the DynaMagTM-PCR Magnet until the beads formed a tight pellet. The liquid was removed and the pelleted magnetic beads holding the appropriate Antibody/Dynabeads® mixtures were re-suspended in the corresponding 100 μl of the diluted chromatin shearing mixture. The beads were re-suspended into the diluted chromatin mixture by gentle flicking of the tube and were rotated end-over-end at 4 ^{o}C for 2 hours. In that stage the cross-linked proteins present in the sheared chromatin samples bind with the beads containing the equivalent antibody.

2.5.7 Chromatin-Antibody-Dynabeads® washing

The tubes were next briefly centrifuged and placed in the DynaMagTM-PCR Magnet for about 30 seconds until the beads formed a tight pellet and the liquid was removed. By using 100 μl of IP Buffer 1 the bead pellet was re-suspended for washing, flicked to mix and rotated end-over-end for 5 minutes at 4 ^{o}C. These beats were passed through the Buffer 1 three times in total and three times through the IP Buffer 2 as described above.

2.5.8 Reversing the crosslinking of the chromatin

After the washed steps, the tubes were briefly centrifuged and placed in the DynaMagTM-PCR Magnet for about 30 seconds until the beads formed a tight pellet and the liquid was removed. 53 μl of Stock Reverse Crosslinking Buffer and 1 μl of Proteinase K, (provided by the kit), were added in each tube and also 43 μl of Stock Reverse Crosslinking Buffer in the 10 μl of input control for reversing the formaldehyde crosslinking of the chromatin. The tubes were vortexed briefly to mix and the beads were re-suspended and incubated for 15 minutes at 55 ^{o}C in a thermal cycler. The tubes were placed in the DynaMagTM-PCR Magnet for about 30 seconds until the beads

formed a tight pellet and the liquid was transferred to fresh 0.2 ml DNase-/RNase-free PCR tubes without disturbing the pellets. The tubes were briefly spun and further incubated for 15 minutes at 95 ºC in a thermal cycler. Next the tubes were placed on ice for cooling for about 5 minutes, centrifuge and continued to magnetic DNA purification step.

2.5.9 Magnetic purification of the un-cross-linked DNA

The DNA Purification Magnetic Beads provided by the kit were briefly vortexed to re-suspend. For each sample, 20 µl of re-suspended DNA Purification Magnetic Beads were mixed gently by pipetting with 50 µl of DNA Purification Buffer (including input control). These 70 µl of the re-suspended DNA Purification Magnetic Beads prepared with DNA Purification Buffer were added to each sample of un-cross-linked DNA and gently mixed by pipetting and incubated at room temperature for 5 minutes. The tubes were placed back to the DynaMagTM-PCR Magnet for at least 1 minute until the beads formed a constricted pellet and the liquid was removed. The DNA Purification Magnetic Beads were re-suspended by gentle pipetting in 150 µl of DNA Wash Buffer twice. The DNA Purification Magnetic Beads were finally re-suspended in 150 µl of DNA Elution Buffer for eluting the DNA. The re-suspended DNA Purification Magnetic Beads were incubated for 20 minutes at 55ºC in a thermal cycler. The tubes were then shortly centrifuge and placed for at least 1 minute in the DynaMagTM–PCR Magnet until the beads formed a tight pellet and the liquid containing the purified DNA sample was collected and transferred in fresh 0.2 ml DNase-/RNase-free PCR tube without disturbing the pellet. The DNA samples were stored at -20 ºC until further use.

2.5.10 Polymerase Chain Reaction (PCR) analysis of the eluted DNA

The DNA eluted from ChIP assay stored from the previews step, was amplified by Polymerase Chain Reaction (PCR) using primers specific for the target region. The primers were designed using Primer3Plus software and ordered by Eurofins MWG Operon (U.S.A.).

For the PCR, into a 0.2 ml DNase-/RNase-free PCR tube 3 µl of the eluted DNA (including input control and negative control) was mixed with 1 µl of 100 ng/µl of forward miR-206 promoter E-box primer (table 2.1), 1 µl of 100 ng/µl of reverse miR-206 promoter E-box primer, 2 µl of 2 mM solution of dNTPs mix (dATP, dCTP, dGTP, and dTTP), 2 µl of 10X PCR Buffer (Qiagen, Germany), (Invitrogen, U.S.A.), 1µl of 5X Q-Solution (Qiagen, Germany), 9.5 µl of DEPC-treated water and 0.5 µl of Taq DNA Polymerase (Qiagen, Germany) and (Applied Biosystems, U.S.A.). The PCR tube was loaded into the PCR machine (Veriti® 96-Well Thermal Cycler; Applied Biosystems, U.S.A.) and run the program seen on the (table 2.2).

Table 2.1 Primers used in PCR after ChiP assay.

Primer Sequences	
Forward miR-206 promoter E-box 1	TGA ACA ATG GTG CTT GG
Reverse miR-206 promoter E-box 1	TTC CAC ATT CAC GCA GAG AG
Forward miR-206 promoter E-box 2	AAC CCC ATC TCC CTC CAG
Reverse miR-206 promoter E-box 2	GAT CCT TTT GTC GGG CTT CT

Table 2.2 PCR- thermal cycle used for PCR after ChiP assay.

Step		Temperature	Time
Enzyme activation		95 ºC	5 minutes
PCR (35 cycles)	Denaturation	95 ºC	1 minutes
	Annealing	58 ºC	1 minutes
	Extension/elongation	72 ºC	1 minutes
Final elongation		72 ºC	5 minutes
Final step		4 ºC	∞

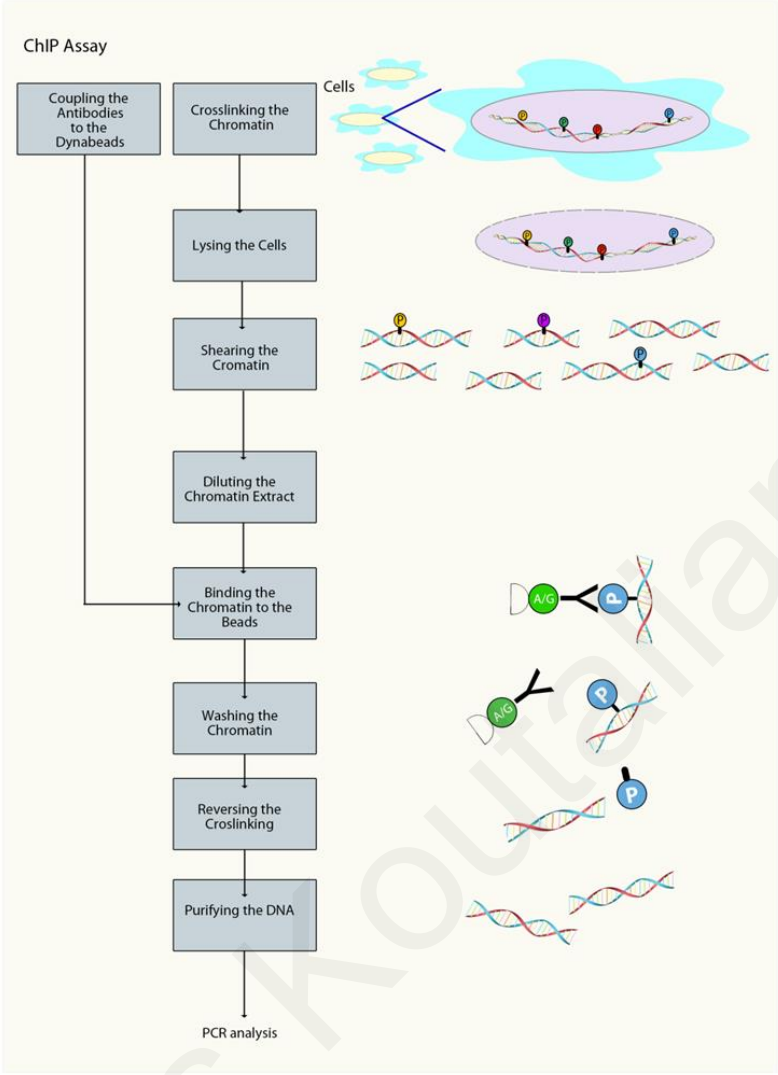


Figure 2.4 Overflow of ChIP assay. ChIP assay was used to determine endogenous direct protein-DNA binding occurring within the cells.

2.5.11 Vertical polyacrylamide gel electrophoresis

The PCR product was run on a polyacrylamide gel that made of 1.5 ml of 40% Acrylamide/Bis Solution 29:1 (BIO-RAD), 1 ml of 10X Tris-borate (TBE) buffer, 60 µl of 10% APS, 9 µl of TEMED and 7.5 ml of distilled water. The 10X TBE consist of 890 mM Tris Base, 890 mM Boric Acid (Scharlau, Spain) and 20 mM Ethylenediaminetetraacetic acid (EDTA) (Merck, Germany). The polyacrylamide gel was placed into a vertical electrophoresis system (Thermo Scientific, U.S.A.) with a comb and left to set before used. The gel was next plunged into the vertical electrophoresis system (Thermo Scientific, U.S.A.) and shielded by 1X TBE buffer. The DNA samples were set by adding 3 µl of 0.4% (w/v) bromophenol blue loading dye and loaded into the wells of the gel in addition to molecular weight ladder, ΦX174 DNA-HaeIII Digest ladder (New England BioLabs, U.S.A.). The gel was ran for about an hour at 100-120 Volts. The

gel was then incubated in 1X TBE buffer complemented with 10 μl of 10 mg/ml ethidium bromide (EtBr) (Amersham, GE Healthcare, U.K.). The bands in the gel were visualized and saved as images, using a UV instrument (Vilber Lourmat, France).

2.6 Production of human TWIST (H-TWIST) 3 UTR/luciferase plasmid

2.6.1 Cloning of H-TWIST 3 UTR/luciferase plasmid

2.6.1.1 PCR Amplification of Twist 3' UTR

Sequences needed for downstream cloning were PCR amplified from Human genomic DNA. The PCR primers used were designed and ordered from Eurofins MWG Operon (U.S.A.) and are shown in table 2.3. For the PCR reaction, 4 μl of 1:3 diluted human genomic DNA was mixed with 2 μl of 10X PCR buffer (Qiagen, Germany), 2 μl of 2 mM solution of four dNTPs (dATP, dCTP, dGTP, and dTTP) (Invitrogen, U.S.A.), 0.5 μl of 100 ng/μl of forward primer, 0.5 μl of 100 ng/μl of reverse primer, 1 μl of 5X Q-Solution (Qiagen, Germany), 0.5 μl of Taq DNA Polymerase (Qiagen, Germany) and 9.5 μl of DEPC-treated water (Applied Biosystems, U.S.A.) into a 0.2 ml DNase-/RNase-free PCR tube. The PCR tube was loaded into the PCR machine (Veriti® 96-Well Thermal Cycler; Applied Biosystems, U.S.A.) and the program present in Table 2.4 was run.

Table 2.3 Primers for amplification of human Twist-1 3'UTR.

Primer Sequences	
Forward Twist 3'UTR	ACA TGC TTG TGC CTG TCA GT
Reverse Twist 3'UTR	CTG GGT TTT TGC AGA TGT CA

Table 2.4 Thermal cycle of PCR for amplification of human Twist-1 3' UTR.

Step		Temperature	Time
Enzyme activation		95 ºC	5 minutes
PCR (30 cycles)	Denaturation	95 ºC	1 minutes
	Annealing	58 ºC	1 minutes
	Extension/elongation	72 ºC	1 minutes
Final elongation		72 ºC	5 minutes
Final step		4 ºC	∞

2.6.1.2 Insertion of the PCR product to TOPO Vector

The TOPO® cloning vector (Invitrogen, U.S.A.) was used to clone the PCR product into the vector of interest. Briefly, 4 µl of purified PCR product were mixed together with 1 µl salt solution, and 1 µl of the TOPO vector. The reaction was mixed well and incubated at room temperature for 5 minutes before proceeding for transformation.

2.6.1.3 Transformation of the DNA plasmid in DH5α competent cells

The TOPO vector, containing the PCR product was then subjected to transformation. 50 µl of MAX Efficiency® DH5αTM Competent Cells (Invitrogen, U.S.A.) were thawed on ice. Meanwhile a falcon tube was placed on ice to be chilled. Once the cells were thawed, they were transferred into the falcon tube and 3 µl of the TOPO vector was added into the tube and mixed gently by pipetting once. The mixture was incubated on ice for 30 minutes. Next, the mixture was heat shocked at 42 ºC in a water bath for 25 seconds. The sample was then incubated on ice for 2 minutes. following incubation, 900 µl of 2.5% Luria Broth Base (Miller's LB Broth Base)® (LB) pre-warmed at 37 ºC was added into the tube which was incubated in a shaker/incubator (MRC ORBITAL SHAKER INCUBATOR, Israel) at 37 ºC for 2 hours. Meanwhile, a selection plate consisting of 1.5% Agar, 2.5% LB and 1 µg/ml of ampicillin (Sigma-Aldrich, U.S.A.) was warmed at 37oC. After incubation, 200 µl of the cells and ligation mixture was spread onto the plate and incubated at 37 ºC incubator (Memmert, Germany) overnight for forming colonies.

2.6.1.4 Small scale preparation of DNA plasmids

Some of the colonies were grown in bacterial cells, initially at low amounts. The selected colonies were picked using a pipette tip and transferred into a falcon tube containing 5 ml of 2.5% LB (Invitrogen, U.S.A.) dissolved in water, which was previously autoclaved and supplemented with 100 μg/ml of ampicillin antibiotic. The plasmid encodes an ampicillin resistance gene and therefore ampicillin can be used for the selection of cells containing the plasmid. All the other bacterial cells would die due to the addition of the antibiotic. The bacterial cells were left to grow overnight in a shaker/incubator (MRC ORBITAL SHAKER INCUBATOR, Israel) at 37ºC. Next day, plasmid DNA was isolated from 1.5 ml of bacterial cells using the QuickLyse Miniprep kit (Qiagen) according to the manufacturer's instructions. Briefly, 1.5 ml of bacterial cells were centrifuged and pelleted in a 2 ml QuickLyse Lysis Tube (provided by the kit) at 13,200 rpm for 1 minute at room temperature. The supernatant was aspirated and the pelleted bacterial cells re-suspended in 400 μl of cold Complete Lysis Solution composed of the Buffer QLL (provided by the kit), supplemented with the re-suspended RNase A (provided by the kit), by vortexing for 30 seconds or until the pellet was fully re-suspended. The lysate was incubated for 3 minutes at room temperature and then transferred to a QuickLyse spin column, supplied by the kit. The column was centrifuged at 13,000 rpm for 30-60 seconds and then washed with 400 μl of Buffer QLW, diluted in isopropanol, by centrifugation at 13,000 rpm for 30-60 seconds. The flow-through was discarded and the column was further centrifuged at 13,000 rpm for 1 minute to dry. Finally, the QuickLyse spin column was transferred into a new 1.5 ml DNase-/RNase-free collection tube and the DNA was eluted by adding 50 μl of Buffer QLE directly onto the column's center and centrifuging at 13,000 rpm for 1 minute. The eluted DNA was digested using enzyme on table and for 2 hours at 37 ºC and analysed on a 0.8% agarose gel.

2.6.1.5 Digest of Luciferase plasmid and TOPO plasmid/Ligation

The TOPO plasmid contains the correct PCR product and the Luciferase plasmids were digesting with the enzymes: The luciferase plasmid was digested with PmeI and SpeI and TOPO plasmid containing the insertion with EcoRV and SpeI. The digested plasmid run on a 0.8% agarose gel and the insertion PCR product with the Luciferase plasmid were gel purified using GEL Purification KIT (PrepEase) (USB-affmetrix, Santa Clara, CA, USA). Having created one large DNA fragment called the vector and a smaller PCR fragment called the insert, with compatible sticky ends, the two were ligated together using the T4 DNA Ligase enzyme (New England BioLabs, U.S.A.). Specifically, the insert and vector were added to the mixture at a ratio of 7:1 (insert: vector) and mixed with 1 μl of 10X T4 DNA Ligase Reaction Buffer (New England BioLabs, U.S.A.) to reach a final concentration of 1X, and 1 μl of T4 DNA Ligase enzyme for a final volume of 10 μl in a 0.2 ml DNase-/RNase-free PCR tube. The reaction was mixed well by

pipetting and incubated at 16ºC overnight or 18 hours. T4 DNA ligase catalysis the formation of a phosphodiester bond between the adjacent 5' phosphate and 3' hydroxyl termini in double stranded DNA.

Then proceed to Transformation reaction in order to insert the DNA plasmid in DH5α competent cells as described in 2.6.1.3 and moved to small scale preparation of DNA plasmids as described in 2.6.1.4.

2.6.1.6 Large scale preparation of DNA plasmids

The positive construct holding the H-TWIST 3'UTR and luciferase gene was further grown in larger amounts in order to get sufficient yield for use in transfections. For this, perseverance the remaining bacterial cells after mini-prep used for the detection of the positive clone, approximately 3.5 ml of the bacterial were added in 250 ml of autoclaved LB comprising 250 μg/ml of ampicillin under sterile conditions. The bacterial cells were left to grow overnight in a shaker/incubator at 37 ºC. The grown bacterial cells were aliquoted in four 50 ml tubes at next day, and harvested by centrifugation at 6,000 x g for 15 minutes at 4 ºC. The supernatant was aspirated and the HiSpeed Plasmid Maxi Kit (Qiagen, Germany) was used for the purification of the plasmid according to the manufacturer's instructions. Specifically, the four bacterial pellets were re-suspended in 10 ml of cold re-suspension buffer, Buffer P1 (supplied by the kit) containing 100 μg/ml of RNase A, a ribonuclease that cleaves the RNA. 10 ml of lysis Buffer P2 (supplied by the kit) was then added to the tube and mixed thoroughly by vigorous inversion of the tube. The sample was left for 5 minutes at room temperature for lysis of the cells. 10 ml of cold neutralization Buffer P3 (supplied by the kit) was added to the lysate for precipitating the genomic DNA, proteins and cell debris, and mixed immediately and thoroughly by vigorous inversion of the tube. The lysate was poured into the barrel of the QIAfilter Maxi Cartridge (supplied by the kit) to which the cap was screwed onto its outlet nozzle previously. The QIAfilter Maxi Cartridge containing the lysate was incubated for 10 minutes at room temperature in order for the precipitate to form a layer on the top of the solution. Meanwhile, a HiSpeed Maxi Tip (supplied by the kit) was equilibrated by adding 10 ml of equilibration Buffer QBT and the column was allowed to completely drain by gravity flow in a QIArack (Qiagen, Germany). The cap from the QIAfilter outlet nozzle containing the lysate was removed and a plunger was inserted into the QIAfilter Maxi Cartridge and the cell lysate was filtered into the equilibrated HiSpeed Maxi Tip. The cleared lysate was allowed to enter the resin by gravity flow. The HiSpeed Maxi Tip was next washed with 60 ml of wash Buffer QC and allowed to pass through the HiSpeed Maxi Tip by gravity flow. The HiSpeed Maxi Tip was then placed in a 50 ml tube and 15 ml of high salt elution Buffer QF (supplied by the kit) was added to elute the DNA by gravity flow. The eluted DNA was precipitated by adding 10.5 ml of room

temperature isopropanol. Isopropanol precipitation is required for concentrating and de-salting the DNA. The sample was mixed and incubated for 5 minutes at room temperature for minimizing salt precipitation. Next, the plunger was removed from a 30 ml syringe (supplied by the kit) and the QIAprecipitator Maxi Module (supplied by the kit) was gently and carefully attached onto the outlet nozzle. The eluate/isopropanol mixture was transferred into the QIAprecipitator Maxi Module, the plunger was inserted and the eluate/isopropanol mixture was filtered through the QIAprecipitator Maxi Module using constant pressure. The QIAprecipitator Maxi Module was removed from the 30 ml syringe and the plunger was pulled out. The QIAprecipitator Maxi Module was attached to the 30 ml syringe again and 2 ml of 70% ethanol was added to the syringe. The plunger was inserted into the syringe and the ethanol was passed through the QIAprecipitator Maxi Module using constant pressure for washing the DNA. The QIAprecipitator Maxi Module was removed from the 30 ml syringe and the plunger was pulled out. The QIAprecipitator Maxi Module was re-attached to the 30 ml syringe, the plunger was added and the membrane of the QIAprecipitator Maxi Module was dried by pressing air through the QIAprecipitator Maxi Module quickly and forcefully. This step was repeated until the QIAprecipitator Maxi Module was completely dried. The outlet nozzle was also dried to avoid ethanol carryover. The plunger from a 5 ml syringe (supplied by the kit) was removed and the QIAprecipitator Maxi Module was attached onto the outlet nozzle. The outlet of the QIAprecipitator Maxi Module was holding over a 1.5 ml DNase-/RNase-free collection tube and 1 ml of eluting Buffer TE (supplied by the kit), containing EDTA, was added to the 5 ml syringe. The plunger was inserted into the syringe and the DNA was eluted into the 1.5 ml DNase-/RNase-free collection tube using constant pressure. The QIAprecipitator Maxi Module was removed from the 5 ml syringe, the plunger was pulled out and the QIAprecipitator Maxi Module was re-attached to the 5 ml syringe. The 1 ml of eluted DNA was transferred to the 5 ml syringe and eluted by inserting the plunger, for a second time into the same 1.5 ml DNase-/RNase-free collection tube for ensuring the recovery of the maximum amount of DNA and store at -20 oC for further used

2.6.2 Site-directed mutagenesis of DNA plasmids

The produced plasmid was mutated at the selected site using GENEART®Site-Directed Mutagenesis System (Life Technologies, U.S.A.). As a first step, a mixture was created as described in the table 2.5 in a 0.2 ml DNase-/RNase-free PCR tube. All the reagents were provided by the kit, except the prime, which was designed according to the kit specification and ordered by Eurofins MWG Operon (U.S.A.) table 2.6.

Table 2.5 Reagents used in mutagenesis reaction.

Component	Volume	Final Concentration
10X AccuPrime™ Pfx Reaction mix	5 µl	1X
10X Enhancer	5 µl	1X
Primer mix (10 µM each)	1.5 µl	0.3 µM each
Plasmid DNA (20 ng/µl)	1 µl	20 ng
DNA Methylase (4U/µl)	1 µl	4 units
25X SAM	2 µl	1X
AccuPrime™ Pfx (2.5 U/µl)	0.4 µl	1 unit
PCR water	34.1 µl	

Table 2.6 Primers used in mutagenesis reactions.

Primer	Sequence
Forward mutated primer Twist 3 UTR	CCT CAA TAA ATA AAT ACC GGC TTG TTT TTT GTA TTT
Reverse mutated primer Twist 3 UTR	AAA TAC AAA AAA CAA GCC GGT ATT TAT TTA TTG AGG
Forward mutated primer miR-206 promoter E1	ACC CTG AGT GGC TCA TGT CGA GCC AAT GTC CCT CAT
Reverse mutated primer miR-206 promoter E1	ATG AGG GAC ATT GGC TCG ACA TGA GCC ACT CAG GGT
Forward mutated primer miR-206 promoter E2	TGG AAT GCT GGA TGG CGT CGA GCT GCC CAT CAA CAA
Reverse mutated primer miR-206 promoter E2	TTG TTG ATG GGC AGC TCG ACG CCA TCC AGC ATT CCA

The PCR tube was loaded into the PCR machine (Veriti® 96-Well Thermal Cycler; Applied Biosystems, U.S.A.) and the program present in Table 2.7 was run.

Table 2.7 Thermal cycle PCR used for mutagenesis.

Cycles	Temperature	Time
1	37 ºC	20 minutes
	94 ºC	2 minutes
18 cycles	94 ºC	20 seconds
	57 ºC	30 seconds
	68 ºC	4 minutes
1	68 ºC	5 minutes
1	4 ºC	∞

After the program the following reaction is set up as shown in the table 2.8.

Table 2.8 Recombination reaction of mutated plasmid before transformation.

Component	Volume	Final Concentration
5X Reaction Buffer	4 μl	1X
PCR water	10 μl	-
PCR sample	4 μl	-
10X Enzyme mix	2 μl	1X

The mixture was mixed well and incubated for 10 minutes at room temperature. After this, the reaction was stopped by adding 1 μl of 0.5M EDTA (provided by the kit). The mixture was mixed well and placed on ice. The procedure was continued with the transformation of the mutated DNA plasmid in DH5α competent cells as described in 2.6.1.3. Finally, DNA sequencing was performed in order to confirm the mutagenesis.

2.6.3 Luciferase Assay

The Dual-Luciferase® (DLR™) Reporter Assay (Promega, U.S.A.) was used to find out if miRNAs could bind to and negatively regulate the myogenin 3'UTR. After transfections described in section 2.2.2, the cells were harvested and lysed in 500 µl of 1X Passive Lysis Buffer (PLB) (supplied by the kit). PLB was diluted using distilled water, as supplied by the kit at a 5X concentration. The lysate mixture was mixed well by vortexing and incubated for 15 minutes at room temperature. During this time, 100 µl of aliquoted LAR II, which was made by mixing lyophilized Luciferase Assay Substrate with Luciferase Assay Buffer II (provided by the kit), was added into luminometer glass tubes (FL Medical, Italy), Then 20 µl of the lysate mixture was added and mixed by pipetting. The tube was then placed into the single tube luminometer (Berthold Detection Systems, Germany) which was set up to take an average reading of luminescence over a 10 second period. The tube was removed from luminometer and 100 µl of 1X Stop and Glo reagent was added, which was made by diluting the 50X Stop and Glo Substrate with Stop and Glo Buffer (provided by kit) and mixed by pipetting. The tube was then placed again into the luminometer and luminescence was then read and noted again. The Stop and Glo reagent quenches the initial firefly luciferase reactions and initiates the renilla luciferase reaction. The firefly luciferase luminescence reading was divided by the renilla luciferase luminescence reading for normalization.

2.7 Nucleic acid isolation

2.7.1 RNA extraction from mammalian cells

For extraction of RNA the RNeasy® Mini kit (Qiagen) was used according to the manufacturer's instructions. In more details, cells were harvested as in section 2.2.4 and collected as a cell pellet before lysis. The pelleted cell was disrupted by adding 600 µl buffer RLT consists of 0.01% β-mercaptoethanol (Merck Millipore, Germany) to Buffer RLT (provided by kit) and mixed by pipetting. The lysate was then homogenized by being passed through a 0.9 mm diameter needle fitted to an RNase-free syringe 5 times and 600 µl of 70 % ethanol was added and mixed by pipetting. Up to 700 µl of the mixture at a time was added to an RNeasy spin column placed in a 2 ml collection tube provided by kit, after which the lid was closed carefully and centrifuged at 10 000 rpm for 15 seconds at room temperature. The flow through was discarded in a waste bottle and the procedure was repeated with the remaining volume of lysate mixture. Following this, 700 µl of Buffer RW1 was added to the RNeasy spin column and again centrifuged for 15 seconds at 10 000 rpm and the flow through was discarded. Next step, 500 µl of buffer RPE (containing ethanol) was then added to the spin column, centrifuged at 10 000 rpm and the flow through was discarded the flow through. Then the column was centrifuged for additional 2 minutes at 10 000 rpm and discarded the flow through. As ethanol was added to the RPE buffer in preparation for usage of the kit, the column was left to dry for a 1 minute and ensured that no ethanol was carried over during

RNA elution as residual ethanol may interfere with downstream reactions. The RNeasy spin column was then placed in a new fresh 1.5 ml collection tube and 30 μl of RNase free water was added directly to the spin column surface. The spin column was then centrifuged at 10 000 rpm for 1 minute to elute the RNA. The RNA was then stored at -20°C until further use.

2.7.1.1 RNA quantification

Stored RNA was thawed on ice and its concentration was measured using NanoDrop spectrophotometer (ND-1000, U.S.A.). NanoDrop was blanked with 1.5 μl of water before 1.5 μl of the RNA was added to be measured at wavelengths of 260 nm (A260), 280 nm (A260) and 230 nm (A230). The reading at 260 nm allows the calculation of the RNA concentration. The readings at 280 nm specified the presence of protein contamination which absorbs light at 280 nm (in particular, the aromatic amino acids) whereas the reading at 230 nm shown the contamination by phenol ion, thiocynates and other organic compounds. In general, one unit of optical density (OD) corresponds to approximately 40 μg/ml of RNA. The ratio between the readings at 260 nm and 280 nm (OD260:OD280) and the readings at 260 nm and 230 nm (OD260:OD230) provide an estimate of the RNA purity with respect to any contaminants present in the RNA. An OD260:OD280 ratio of 1.8-2.0 and an OD260:OD230 ratio very close to 2.0, were indicative of highly purified RNA.

2.7.1.2 Reverse transcription (RT)

500 ng of total RNA was mixed with 3 μl of 6N primer mix (MWG) and filled to 30 μl with water. It was then incubated at 65 ^{o}C for 5 minutes and then cooled down to 4 ^{o}C for 3 minutes. 10 μl of the master mix was added, which consist of 5 μl of 2nM of dNTPs mix, 4 μl of Mo MULV buffer (10X) and 1 μl of Reverse Transcriptase and incubated for 1 hour at 37 ^{o}C. The cDNA was stored at -20 oC until further used.

2.7.1.3 Polymerase Chain Reaction (PCR) analysis of the cDNA

The cDNA eluted from RT reaction stored from the previews step, was amplified by Polymerase Chain Reaction (PCR) using primers specific for the target region. The primers were designed using Primer3Plus software and ordered by Eurofins MWG Operon (U.S.A.).

For the PCR, the procedure was the same as described at 2.5.10 with the programs and the primers of tables 2.9, 2.10 and 2.11.

Table 2.9 Primers of PCRs.

Primer Sequences	
GAPDH Mouse F	TCA TCA TCT CCG CCC CTT CT
GAPDH Mouse R	GAG GGG CCA TCC ACA GTC TT
GAPDH Human F	GAG TCA ACG GAT TTG GTC GT
GAPDH Human R	TTG ATT TTG GAG GGA TCT CG
luciferase gene F	AAT CTG ACG CAG GCA GTT CT
luciferase gene R	CCA GGG ATT TCA GTC GAT GT
Twist-1 Mouse F	CCC AAG CTT GTC GTA CGA GGA GCT GCA GA
Twist-1 Mouse R	CGC GGA TCC CTC CAG ACG GAG AAG GCG TA

Table 2.10 Thermal-cycle reaction of PCR.

Step		Temperature	Time
Enzyme activation		95 ºC	5 minutes
PCR (35 cycles)	Denaturation	95 ºC	1 minutes
	Annealing	Depend on the primer set (table)	1 minutes
	Extension/elongation	72 ºC	1 minutes
Final elongation		72 ºC	5 minutes
Final step		4 ºC	∞

Table 2.11 Annealing Temperatures for PCRs.

Genes	Annealing Temperature
GAPDH Mouse	60 ºC
GAPDH Human	58 ºC
Luciferase gene	54 ºC
Twist mouse	58 ºC

2.7.2 MiRNA extraction

Total RNA enriched with small RNAs, including miRNAs, was isolated using mirVana™ miRNA Isolation Kit (Ambion, U.S.A.), according to the manufacturer's instructions. Briefly, the cells were grown in 6-well plates (Corning, U.S.A.). One well of the plate was sufficient for RNA extraction. The cell pellet from the harvesting was re-suspended in 600 μl of the denaturing Lysis/Binding Solution (supplied by the kit). 60 μl of miRNA Homogenate Additive (supplied by the kit) was added to cell lysates and mixed by vortex and let to stand on ice for 10 minutes. The lysate mixture was transferred in a 1.5 ml DNase-/RNase-free tube. Lysis/Binding Solution stabilizes RNA and also inactivates RNases. Next, the lysate was mixed with Acid-Phenol: Chloroform extraction which removes most of the other cellular components, such as DNA, and provides a strong front-end purification. Specifically, 600 μl of Acid-Phenol: Chloroform was added to the lysate which was then vortexed for 1 minute to mix and centrifuged at maximum speed (13,200 rpm) for 5 minutes at room temperature to separate the aqueous and organic phases. The aqueous (upper) phase was carefully removed and transferred in a new fresh 1.5 ml DNase-/RNase-free tube without disturbing the lower phase and its volume was measured. 1.25 volumes of the aqueous phase, of room temperature 100% ethanol was added to the sample and mixed. A glass-fiber Filter Cartridge (supplied by the kit), which is able to immobilize RNA, was placed into a Collection Tube (supplied by the kit) and 700 μl of the lysate/ethanol mixture was transferred in the Filter Cartridge and centrifuged at 10,000 rpm for 15 seconds at room temperature for the sample to pass through the filter. The flow-through was discarded and the step was repeated until all of the lysate/ethanol mixture was passed through the Filter Cartridge. Next, 700 μl of miRNA wash Solution 1 (supplied by the kit), composing of 41% (v/v) of 100% ethanol, was added into the Filter Cartridge and centrifuged for 10 seconds at 10,000 xg at room temperature to wash the filter. The flow-through was discarded from the Collection Tube. 500 μl of Wash Solution 2/3 (supplied by the kit), containing of 44% (v/v) of 100% ethanol, was added into the Filter Cartridge, centrifuged for 10 seconds at 10,000 xg at room temperature and the flow-through was discarded. The last wash was repeated twice. The Filter Cartridge was further centrifuged at 10,000 xg for 1 minute at room temperature to remove any remaining fluid from the filter. The Filter Cartridge was transferred into a fresh Collection Tube (supplied by the kit) and 100 μl of 95 ºC pre-heated Elution Solution was applied to the center of the filter which was then centrifuged at maximum speed (13,200 rpm) for 30 seconds at room temperature to elute the RNA. The RNA was stored at -80 ºC until further use.

2.7.2.1 Reverse transcription (RT) for miRNA

For reverse transcription of the isolated total RNA the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, U.S.A.) was used according to manufacturer's instructions.

During the preparation of the RT reactions all the tubes and reagents were kept on ice. Specifically, 10 ng of each of the RNA samples to be reverse transcribed was mixed with DEPC-treated water (Applied Biosystems, U.S.A.) in a 0.2 ml RNase-/DNase-free PCR tube for a final volume of 9.16 μl. Next, 3 μl of 5X RT primer, 0.15 μl of 100 mM dNTPs (with dTTP), 1.5 μl of 10X Reverse Transcription Buffer, 0.19 μl of RNase Inhibitor (20 U/μl) and 1 μl of MultiScribeTM Reverse Transcriptase (50 U/μl) were added in each reaction tube and mixed gently. The RT primer used was (hsa-miR-206 TaqMan® MicroRNA Assay (Applied Biosystems, U.S.A.). MiRNA expression was normalized to the RNA U6B small nuclear (RNU6B) using RNU6B Control miRNA Assay (Applied Biosystems, U.S.A.). The PCR tubes were centrifuged and incubated on ice for 5 minutes. The reactions were loaded into a thermal cycler. The reaction program is present in Table 2.12

Table 2.12 Thermal cycle for Reverse transcriptase of miRNA.

Step	Temperature	Time
1	16 ºC	30 minutes
2	42 ºC	30 minutes
3	85 ºC	5 minutes
4	4 ºC	∞

2.7.2.2 Real-Time PCR

For detection and quantification of the miRNA levels the TaqMan® MicroRNA Assays (Applied Biosystems, U.S.A.) were used according to manufacturer's instructions. Each reaction volume was 20 μl and was ran in triplicate in a MicroAmp® Optical 96-Well Reaction Plate with Barcode (Applied Biosystems, U.S.A.) containing: 1 μl of 20X TaqMan® Small RNA Assay, 1.33 μl of product from RT reaction, 10 μl of TaqMan® Universal PCR Master Mix, No AmpErase® UNG and 7.67 μl of DEPC-treated water (Applied Biosystems, U.S.A.) were added in each reaction. The Real-Time PCR primer was hsa-miR-206 TaqMan® MicroRNA Assay (Applied Biosystems, U.S.A.). MiRNA expression was normalized to the RNU6B using RNU6B Control miRNA Assay (Applied Biosystems, U.S.A.). The plate was sealed with Optical Adhesive Films (Applied Biosystems, U.S.A.), briefly centrifuged and loaded into the Real-Time PCR system (7900HT Fast Real-Time PCR System; Applied Biosystems, U.S.A.). The thermal cycling conditions used are presented in Table 2.13. All the Real-Time PCR experiments were repeated three times.

Table 2.13 Thermal cycle for Real-Time PCR.

Step		Temperature	Time
Enzyme activation		95 ºC	10 minutes
PCR (40 cycles)	Denature	95 ºC	15 seconds
	Anneal/extend	60 ºC	60 seconds

2.8 Statistical analysis

ANOVA and Student's t test statistical tests were used to determine whether specific group mean differences were significant. The level of significance was set at 0.01. Data are presented as mean ±S.D

2.8.1 Statistical analysis of Real time PCR

Statistical analysis was performed as described before (Yuan et al., 2006), using SAS, v 9.1 (SAS Institute Inc., Cary, NC, USA) software. ΔCt values were calculated as the Ct (miRNA) - Ct (RNU6B). Exact Wilcoxon tests were used to compare the ΔCt parameters. A p-value of below 0.05 was considered statistically significant. All probabilities were two-tailed.

3

RESULTS

3.1. Prediction of Twist-1 miRNAs

3.1.1 Introduction

Since 1993 that the first miRNA was discovered, thousands of miRNA were identified in both animal and plant kingdoms. Modern technology including microarrays were used in order to associate expression of identified miRNAs between specific tissues in different species and demonstrated differential expression patterns between normal and models of disease (Lagos-Quintana et al., 2002, Trang and Liu, 2008). miRNAs belong to the family of small, non-coding RNAs which have as main function to negatively regulate gene expression at the post transcriptional level. miRNAs act through the RISC complex where miRNAs are directed to not fully bind sequences in target mRNAs in order to inhibit its translation through blocking translation mechanism or through the cleavage of mRNA. The 5' end, called the seed region, of the miRNA is the part that is responsible for the interaction of miRNA with the 3' UTR of the target mRNA. In animals, it is not necessary to have full complementarity between the seed region and the mRNA in order for the miRNA to be functional. Many algorithms have been created to predict the interaction between the miRNA and the mRNA. Most of the prediction software based on algorithms that have as main criteria the complementarity between the seed sequence of the miRNA and the 3'UTR of the possible mRNA. Apparently those algorithms use also other principals than the complementarity, including thermodynamic properties of the miRNA-mRNA duplex and conservation of miRNA target sites among different species.

One of the most commonly used software for predicting possible miRNA binding on target mRNA is the miRanda-algorithm software. The first criteria that is used by miRanda is the sequence alignment of the whole sequence of miRNA to the target mRNA's 3'UTR and secondly the thermodynamic stability of the miRNA-mRNA duplex.

One miRNA could bind to several mRNA targets, but one mRNA can be targeted also by several miRNAs. Even that the algorithms use such criteria. Moreover the conservation among species, there is always a possibility for false positive outcome. It is therefore necessary to confirm bioinformatics findings in the laboratory.

The aims of the first part of this project was to identify possible miRNAs which may target Twist-1 3'UTR by using computational algorithms and tools for conservation among the species.

3.1.2 Results

3.1.2.1 Predicted miRNAs target human Twist-1 3' UTR

In order to find possible miRNAs regulating Twist-1, the computational algorithm approach was first used to predict possible miRNAs that could target Twist-1 3'UTR,

The database http://www.microrna.org/microrna was used in order to predict the possible miRNAs. This is an online tool used to predict miRNA binding sites on the 3'UTR in rat mouse and human genes. Specifically this online tool is integrated with the miRanda algorithm and calculates a score (mirSVR) which gives information about the possibility of a miRNA-mRNA interaction.

Having set the parameters of the prediction at a high level (high mirSVR scores), miRanda software produced a number of possible miRNAs (Figure 3.1).

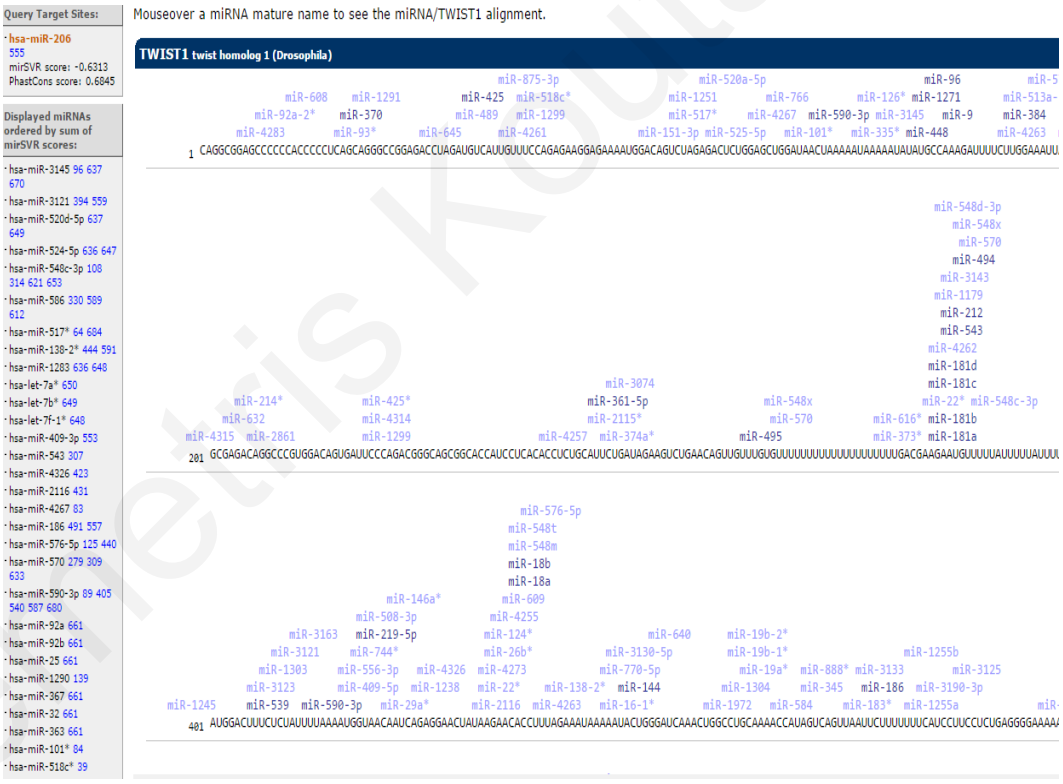


Figure 3.1 Predicted miRNA candidates for human Twist-1 3'UTR. Microrna.org online tool was used to predict possible miRNAs that could bind to the Twist-1 3'UTR. The software was set to show only miRNAs with high scores. The miRNAs are listed based on mirSVR score on the left panel and the binding position on the 3'UTR where it is predicted is stated.

In order to narrow down the list of the predicted miRNAs, the software tool was set to show conserved microRNAs with good mirSVR scores, as shown in the figure 3.2.

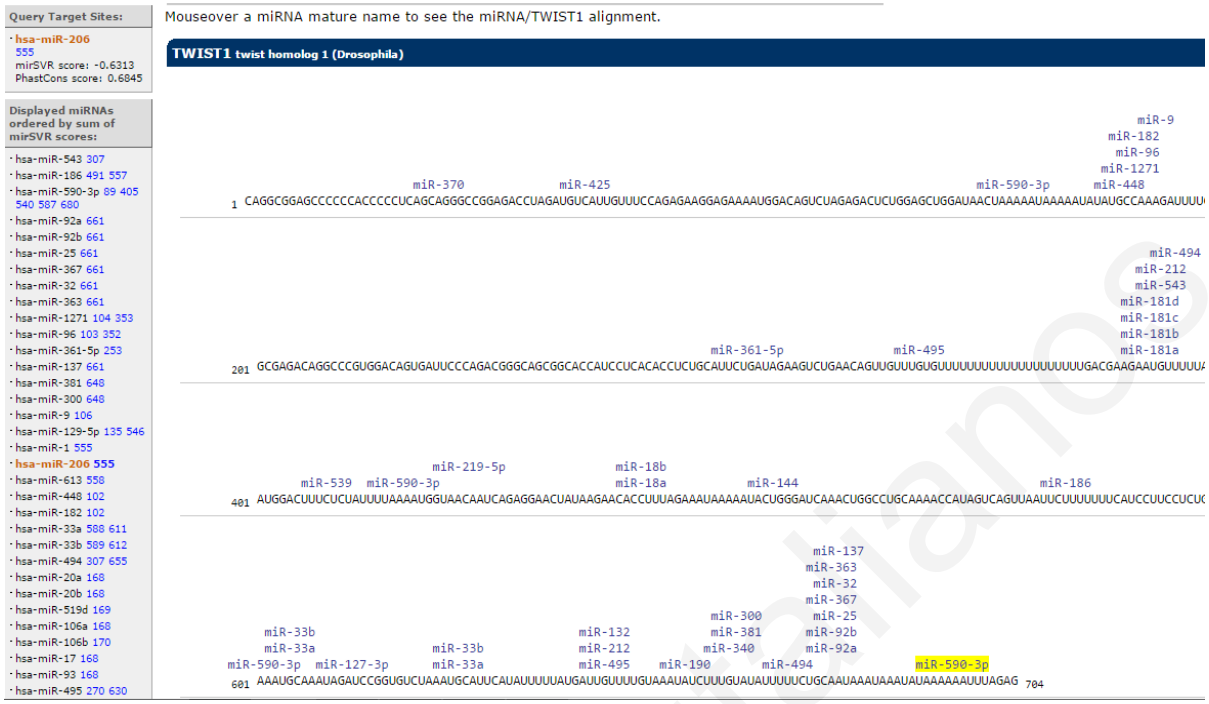


Figure 3.2 Predicted conserved miRNA for human Twist-1 3'UTR. The search was carried out only for miRNAs with high scores. Only conserved miRNAs are displayed and are ranked based on score in the left panel.

This online tool does not take into concern the co-expression of miRNAs and the target in the tissue of interest which in our case is the skeletal muscle. If miRNA is conserved and produces a high mirSVR score, it has no value if miRNA and target mRNA are not co-expressed in the same tissue.

Among the possible candidate miRNAs one of the known myomiRs, miR-206 was identified. The human *Twist-1* transcript was predicted to contain one canonical miRNA response element (MRE) for miR-206 (figure 3.3). The binding site of miR-206 to the human Twist-1 3'-UTR was more conserved between closely related species compared to divergent species, indicating possible evolutionary importance (Figure 3.4).

1 CAGGCGGAGCCCCCCACCCCCUCAGCAGGGCCGGAGACCUAGAUGUCAUUGUUUCCAGAGAAGGAGAAAAUGGAC 75

76 AGUCUAGAGACUCUGGAGCUGGAUAACUAAAAAUAAAAAUAUAUGCCAAAGAUUUUCUUGGAAAUUAGAAGAGCA 150

151 AAAUCCAAAUUCAAAGAAACAGGGCGUGGGGCGCACUUUUAAAAGAGAAAGCGAGACAGGCCCGUGGACAGUGAU 225

226 UCCCAGACGGGCAGCGGCACCAUCCUCACACCUCUGCAUUCUGAUAGAAGUCUGAACAGUUGUUUGUGUUUUUUU 300

301 UUUUUUUUUUUUUGACGAAGAAUGUUUUUAUUUUUAUUUUUUUCAUGCAUGCAUUCUCAAGAGGUCGUGCCAAUC 375

376 AGCCACUGAAAGGAAAGGCAUCACUAUGGACUUUCUCUAUUUUAAAAUGGUAACAAUCAGAGGAACUAUAAGAAC 450

451 ACCUUUAGAAAUAAAAAUACUGGGAUCAAACUGGCCUGCAAAACCAUAGUCAGUUAAUUCUUUUUUUCAUCCUUC 525

miR-206

526 CUCUGAGGGGAAAAACAAAAAAAAACUUAAAAUACAAAAAACAACAUUCUAUUUAUUUAUUGAGGACCCAUGGUA 600

601 AAAUGCAAAUAGAUCCGGUGUCUAAAUGCAUUCAUAUUUUUUAUGAUUGUUUUGUAAAUAUCUUUGUAUAUUUUUC 675

676 UGCAAUAAAUAAAUAUAAAAAAUUUAGAG 704

Figure 3.3 Twist 3' UTR map. Map of the human Twist-1 gene locus indicating the length of the 3' UTR and putative binding position of the miR-206

```
Human 5′ ataaataaataGAATGTtgttttt 3′
Chimp 5′ ataaataaataGAATGTtgttttt 3′
Rhesus 5′ ataaataaataGAATGTtgttttt 3′
Rat 5′ ataaataaataGAATGTtgtcttt 3′
Mouse 5′ ataaataaataGAATGTtgtcttt 3′
Rabbit 5′ ataaataaataGAATGTtgttttt 3′
Dog 5′ ataaataaataGAATGTtgttttt 3′
```

Figure 3.4 mir-206 binding site conservation. Conservation of the miR-206 putative binding site among species as shown by the sequence homology (highlighted).

3.1.3 Conclusions

The initial aim of this work was to identify miRNAs which regulate the expression of Twist-1 gene. The 3'UTR of human Twist-1 was screened for potential miRNA binding sites using miRanda software. Using the online tool microrna.org, a number microRNAs were identified as candidates for binding to the human Twist -1 3'UTR.The criteria that were used in order to choose the candidate miRNA were: 1) the conservation of the miRNA binding sites between species, 2) miR-206 expression in muscle tissue and 3) the opposite expression profiles of the miR-206 and Twist-1 protein. Among the possible candidate miRNAs one of the known myomiRs, miR-206 was identified. The human Twist-1 transcript was predicted to contain one canonical miRNA response element (MRE) for miR-206. The binding site of miR-206 to the human Twist-1 3'-UTR was more conserved between closely related species compared to divergent species, indicating possible evolutionary importance. The next step was to assess the experimental efficacy of the miR-206 in cells.

3.2 Experimental validation of the binding of miR-206 on Human Twist-1 3'UTR

3.2.1 Introduction

It is known according to the literature that the percentage of the predicted false positive miRNAs through prediction programs is between 24 and 70 (Bentwich, 2005, Sethupathy et al., 2006). In order to confirm that the predicted miRNA is a negative regulator of the target, this needs to be validated in the laboratory. For the validation of the miRNA-mRNA interaction a series of experiments are required. Firstly the 3'UTR sequence of Twist must be cloned into a reporter construct and contransfected with candidate miRNA mimics into a cell line independent of both the miRNA and mRNA. In order to confirm the binding specificity of the candidate miRNA and the target mRNA, mutagenesis analysis must also be performed. Also, the endogenous miRNA-mRNA interaction must also be confirmed through overexpression and inhibition experiments in the tissue of interest and assayed using protein or Immunofluorescence analysis.

AIMS

- To create a reporter construct including the 3'UTR of Human Twist-1.
- To experimentally confirm candidate miRNAs and Twist-1 interactions.

3.2.2 Results

3.2.2.1 Cloning of Twist-1 3'UTR into luciferase reporter plasmid

Firstly, in order to validate that candidate miRNA negatively regulate target mRNA, a construct must be created with a reporter gene and the 3'UTR of Twist-1. The Twist-1 3' UTR was inserted in the pmiR report luciferase DNA plasmid, downstream the luciferase gene located in (Figure 3.5).

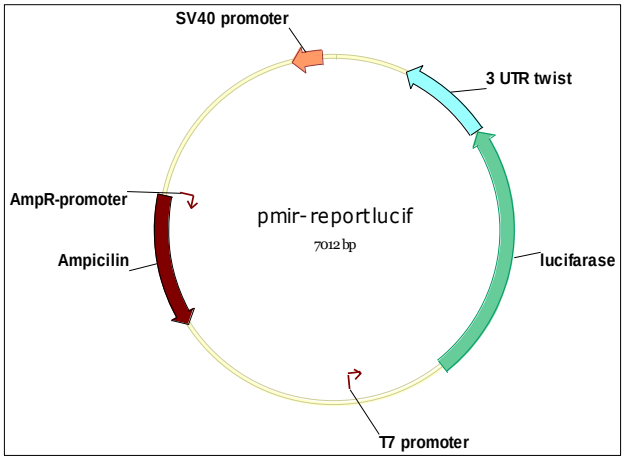


Figure 3.5 The pmiR-luciferase-Twist-1 3'UTR construct.. For cloning the Twist-1 3'UTR, pmiR-report luciferase plasmid was used. This plasmid consists of a luciferase open reading frame (McCabe et al.) under the T7 promoter and the ampicillin open reading frame under the SV40 promoter for antibiotic selection.

In order to amplify the human Twist 3'UTR, a gradient PCR was performed with genomic DNA extracted from human blood cell as a template in order to optimize the PCR conditions (Figure 3.6). From the gradient PCR it was found that the optimum annealing temperature in order to give the maximum and the most pure amplicon was 60ºC (Figure 3.7). The PCR product was inserted into TOPO 2.1 vector and transformation was performed in DH5α competent cells. A selection process was performed in order to select positive plasmids that contain the insertion in the correct orientation. The plasmids were isolated and digested independently with 2 enzymes (EcoRI and NcoI). EcoR1 cuts outside the insertion and if the correct insertion is present, two bands appear one around 620 bp and one around 4000 bp (Figure 3.8 A). The plasmids were digested in the same time also with the NcoI enzyme in order to find the correct orientation (Figure 3.8 B). Then, the chosen plasmid was digested with EcoRV and SpeI enzyme in order to create inserts with cohesive ends compatible with the SpeI / PmeI openings in the luciferase construct. Following this, the linearization of the pmiR report luciferase plasmid was performed by digesting the plasmid with PmeI and SpeI and the TOPO plasmid with EcoRV and SpeI, digests were ran on an agarose gel to verify the sizes and to predict the DNA concentration of each based on the intensity of bands in the agarose gel before proceeding to the ligation reaction (Figure 3.9). The vector and insert were then ligated together to create the pmiR-report luciferase-Twist-1 3'UTR construct (Figure 3.5). The construct incorporates a T7 promoter on 5' of the luciferase gene meaning that the luciferase gene is constitutively on, and will give a maximum reading unless the luciferase gene expression is inhibited by miRNAs.

ACATGCTTGTGCCTGTCAGTAGCTGCTTTATTTGAGTTTTTCATTGCTTT
GTTTATATTAGTGCCCCCTCCCTCCTGGGTGCCTCTAGAATTAAACAAGC
AGGTATTTACCACCAACTTAATCTTTAGTTTTAAAAATATAGACCAAACT
CTAAGGTTCTCTAAATTTTTTATATTTATTTATTGCAGAAAAATATACAA
AGATATTTACAAAACAATCATAAAAATATGAATGCATTTAGACACCGGAT
CTATTTGCATTTTACCATGGGTCCTCAATAAATAAATAGAATGTTGTTTT
TTGTATTTTAAGTTTTTTTTTGTTTTTCCCCTCAGAGGAAGGATGAAAAA
AAGAATTAACTGACTATGGTTTTGCAGGCCAGTTTGATCCCAGTATTTTT
ATTTCTAAAGGTGTTCTTATAGTTCCTCTGATTGTTACCATTTTAAAATA
GAGAAAGTCCATAGTGATGCCTTTCCTTTCAGTGGCTGATTGGCACGACC
TCTTGAGAATGCATGCATGAAAAAAATAAAAATAAAAACATTCTTCGTCA
AAAAAAAAAAAAAAAAAAACACAAACAACTGTTCAGACTTCTATCAGAAT
GCAGAGGTGTGAGGATGGTGCCGCTGCCCGTCTGGGAATCACTGTCCACG
GGCCTGTCTCGCTTTCTCTTTTAAAAGTGCGCCCCACGCCCTGTTTCTTT
GAATTTGGATTTTGCTCTTCTAATTTCCAAGAAAATCTTTGGCATATATT
TTTATTTTTAGTTATCCAGCTCCAGAGTCTCTAGACTGTCCATTTTCTCC
TTCTCTGGAAACAATGACATCTGCAAAAACCCAG

Figure 3.6 Genomic sequence of Twist-1 3'UTR. The Twist-1 3'UTR is displayed as the genomic sequence. Primers (indicated in grey) were designed for the amplification of genomic DNA, including the position of the miR-206 binding side as shown in red.

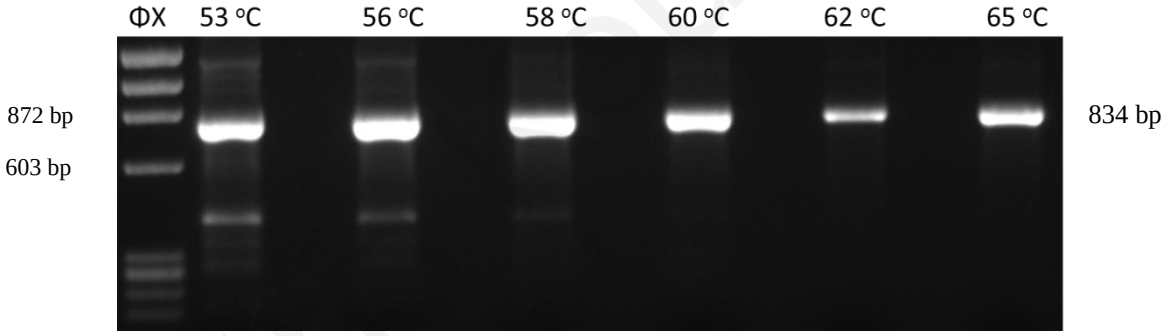


Figure 3.7 DNA amplification for Twist-1 3'UTR. Primers indicated in figure 3.6 were used to amplify the Twist-1 3'UTR from mouse genomic DNA. The expected band size was 834 bp. The gradient PCR using annealing temperatures ranging from 53°C to 65°C indicated that the ideal annealing temperature was 60°C.

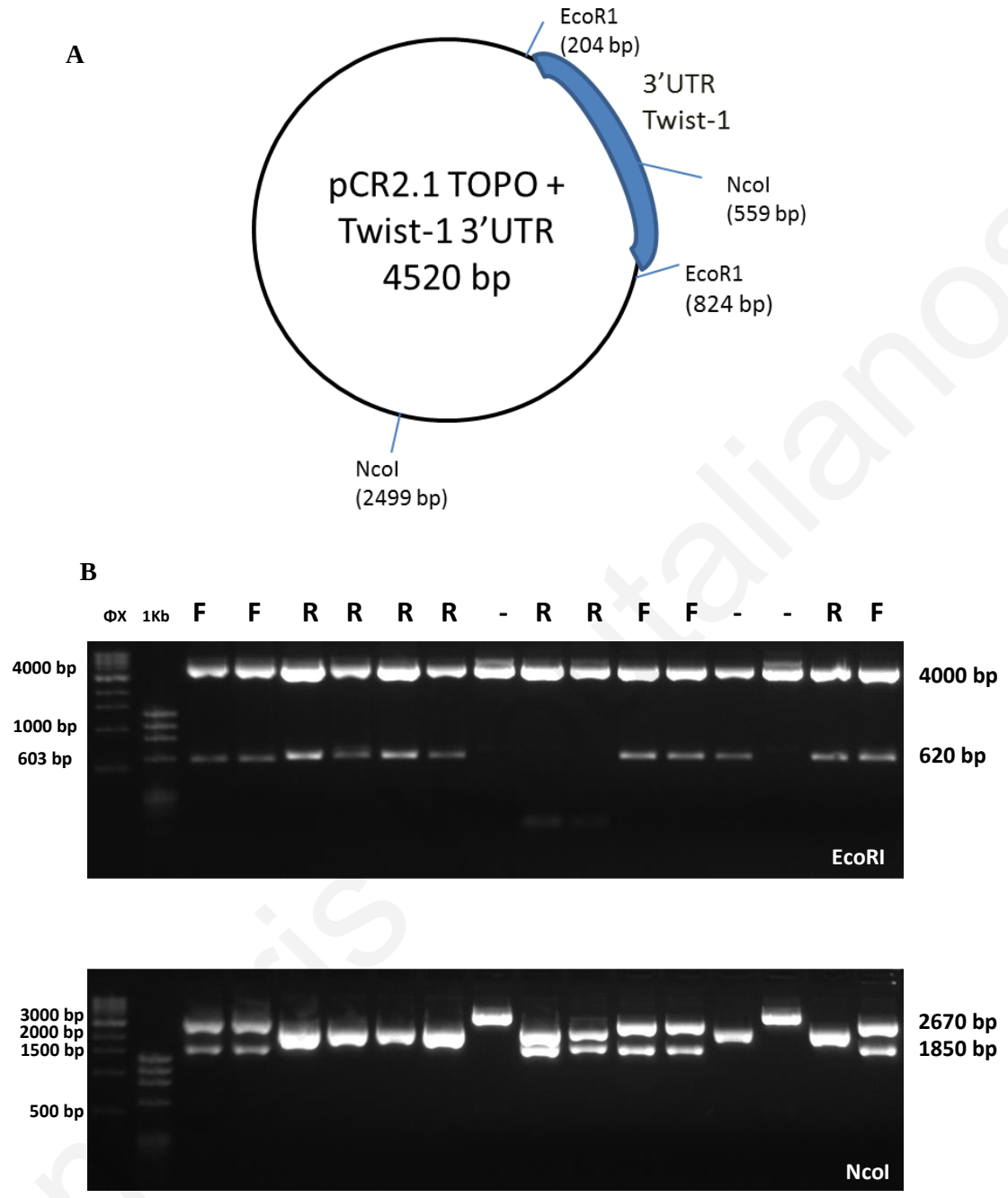


Figure 3.8 Screening for positively TOPO cloned plasmids. Colonies collected following transformation were grown and harvested for screening purposes. A) Map of the expected Topo plasmid with the Twist 3' UTR showing the restriction sites B) The screening was performed with EcoRI and NcoI plasmid digests producing bands of different sizes, the expected bands was 620 bp with EcoRI and 2670 and 1850 bp with NcoI. 5 out of the 15 colonies screened displayed successful insertion of the Twist-1 3'UTR into the TOPO in correct Forward orientation (F); 7 colonies displayed successful insertion but in the reverse orientation (R); 3 colonies displayed no correct insertion (-). The 1Kb and ΦX ladders was used for size verification.

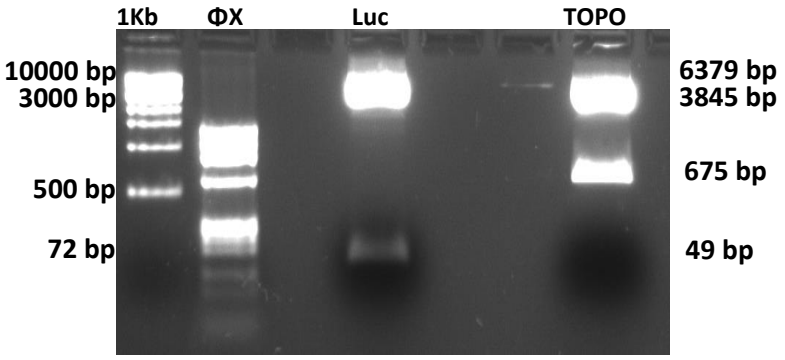


Figure 3.9 Digested of the TOPO and Luciferase plasmid. Linearisation of the luciferase plasmid with PmeI and SpeI (6421 bp) (Luc) and digestion of TOPO plasmid with EcoRV and SpeI (TOPO) living the PCR product with complementary sticky ends similar to the linearized Luciferase plasmid (675 bp).

Ligation was performed followed by transformation into DH5α competent cells, each colony was screened for correct orientation and insertion using the restriction enzyme PstI which would produce for the correct orientation produced 2 bands one around 800 bp and another at 6200 bp. In this case, the desired orientation was the majority of the colonies selected (Figure 3.10). Sequencing was then performed to verify the presence of the Twist-1 3'UTR (Figure 3.11)

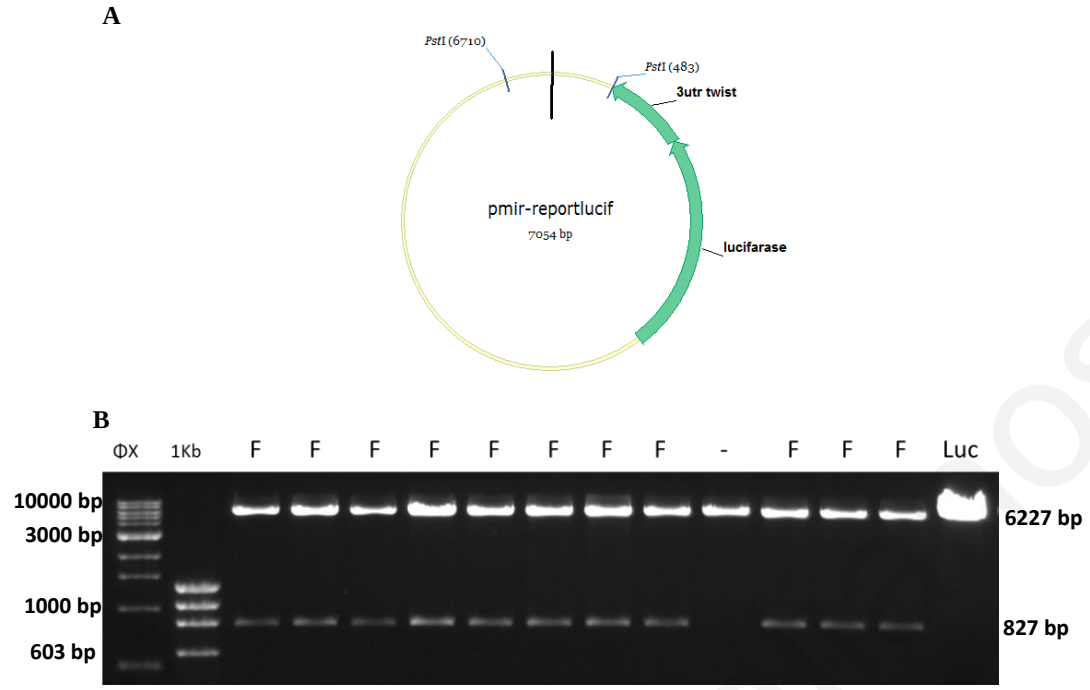


Figure 3.10 Screening for positively cloned plasmids. Colonies collected following transformation were grown and harvested for screening purposes. The screen was performed using a PstI digest producing bands of different sizes. A) Maps pf the pmir-report luciferase with 3'UTR Twist-1. B) Digesting with PstI the plasmids from transformation. 11 out of the 12 colonies (F) screened displayed successful insertion of the Twist-1 3'UTR into the Luciferase vectors As a negative control (Luc) the pmiR report luciferase vector was digested with the same restriction enzyme. The 1Kb and ΦX ladder was used for size verification.

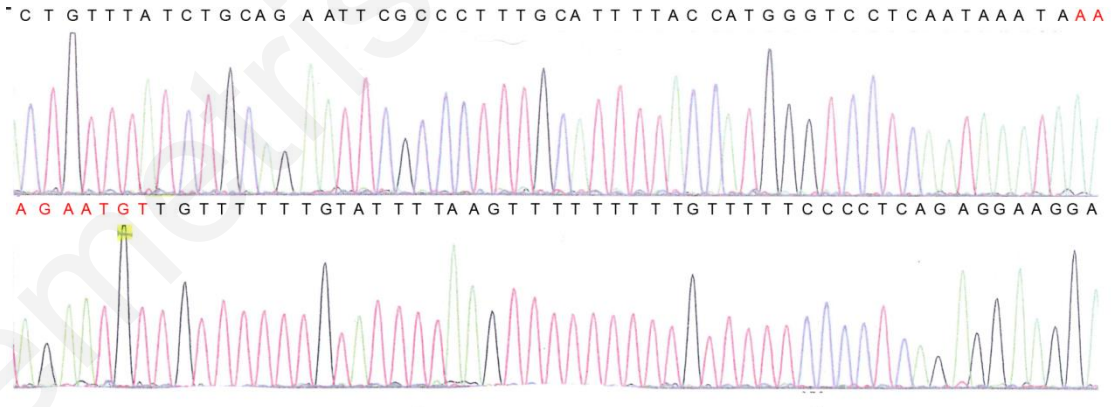


Figure 3.11 Sequencing of Twist-1 3'UTR in pmiR-report luciferase. The cloned plasmid was sequenced to verify the presence and orientation of the inserted Twist-1 3'UTR with the binding side of miR-206 to be in red colour.

3.2.2.2 miR-206 represses Twist-1 through its 3'-UTR.

The initial aim of this work was to identify miRNAs which regulate the expression of Twist-1 gene. The 3'UTR of human Twist-1 was screened for potential miRNA binding sites using miRanda software. The criteria that were used in order to choose the candidate miRNA were: 1) the conservation of the miRNA binding sites between species, 2) miR-206 expression in muscle tissue and 3) the opposite expression profiles of the miR-206 and Twist-1 protein. Among the possible candidate miRNAs one of the known myomiRs, miR-206 was identified. The human Twist-1 transcript was predicted to contain one canonical miRNA response element (MRE) for miR-206. The binding site of miR-206 to the human Twist-1 3'-UTR was more conserved between closely related species compared to divergent species, indicating possible evolutionary importance. To investigate if the candidate miRNA binds on the 3'-UTR of the human Twist-1, the full human Twist-1 3'-UTR was cloned downstream of the luciferase gene and assayed in HeLa cells. The luciferase assay indicated that miR-206 repressed luciferase activity efficiently (50%) (Figure 3.12). Introduction of mutations at the Twist-1 binding site of miR-206 (Figure 3.13) and transfections with a miR-206 mutant variant brought the luciferase activity back to the original levels, indicating miRNA-mRNA binding specificity (Figure 3.14). Moreover, the levels of Luciferase are decrease indicating that there is some degradation of Luciferase mRNA (Figure 3.15).

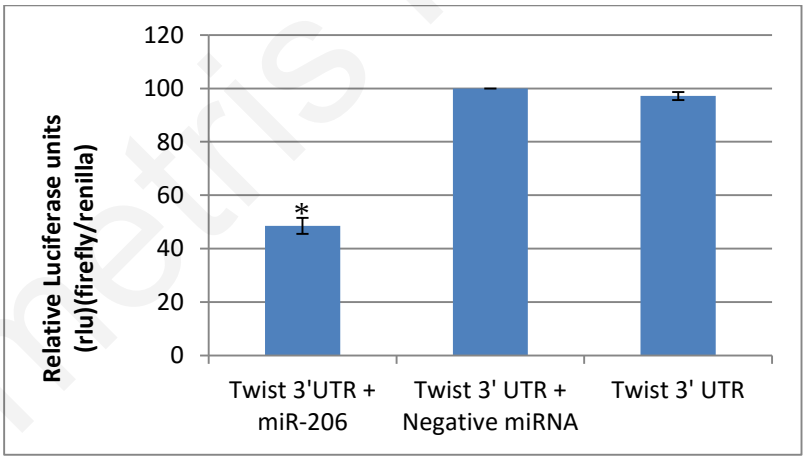


Figure 3.12 miR-206 repress Luciferase expression. The luciferase assay indicated that miR-206 repressed luciferase activity efficiently (50%) compare to the controls cells

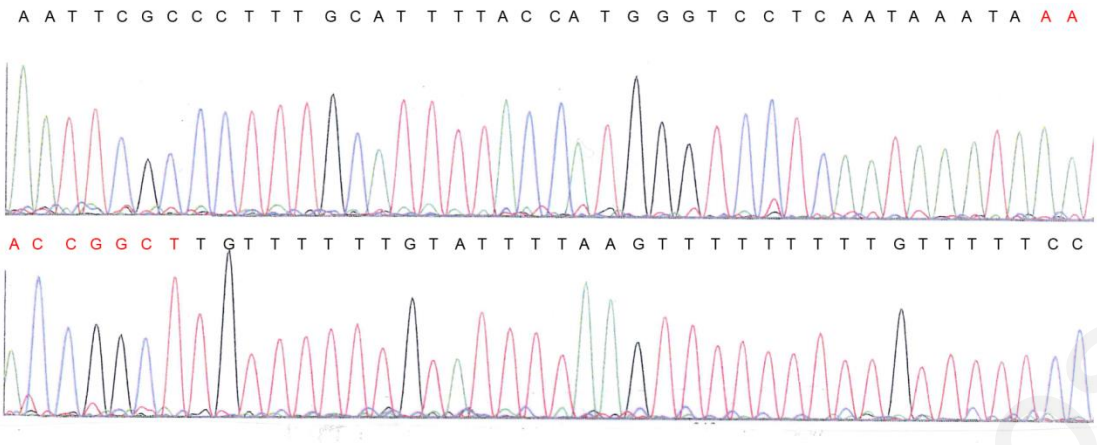
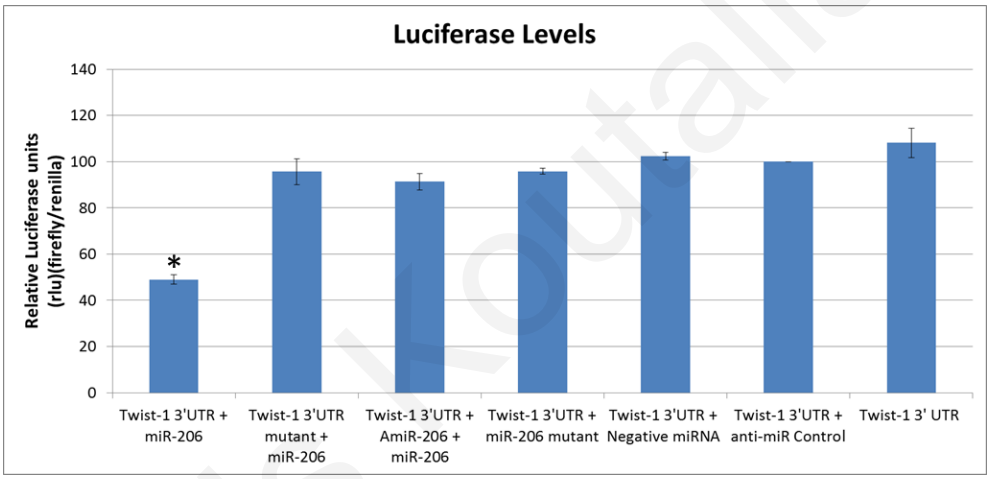


Figure 3.13 Mutation on predicted miR-206 binding site. Sequence confirm the mutagenesis (in red colour) on the predicted binding site of miR-206 on 3'UTR of Twist-1



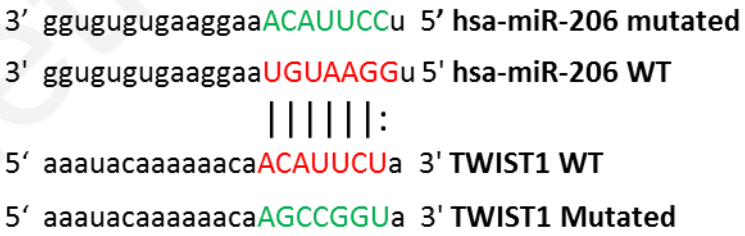


Figure 3.14 miR-206 specific binds to the Twist-1 3'UTR. Transfections of HeLa cells with miR-206 mimic and the plasmid expressing the luciferase gene with the Twist-1 3' UTR cDNA (Twist-1 3' UTR) caused a significant decrease in luciferase/human Twist-1 3' UTR activity compared to cells transfected with the Twist-1 3' UTR or transfected with the Twist-1 3' UTR and a non-specific miRNA (negative miRNA). Introduction of mutations in both miR-206 (miR-206 mutant) and 3' UTR binding site (Twist-1 3' UTR mutant) showed no decrease in luciferase activity, indicating specificity of miR-206 for Twist-1 3' UTR. Similarly, addition of miR-206 antagomir (amiR-206) to cells co-transfected with the miR-206 mimic reversed the decrease of the mimic alone. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

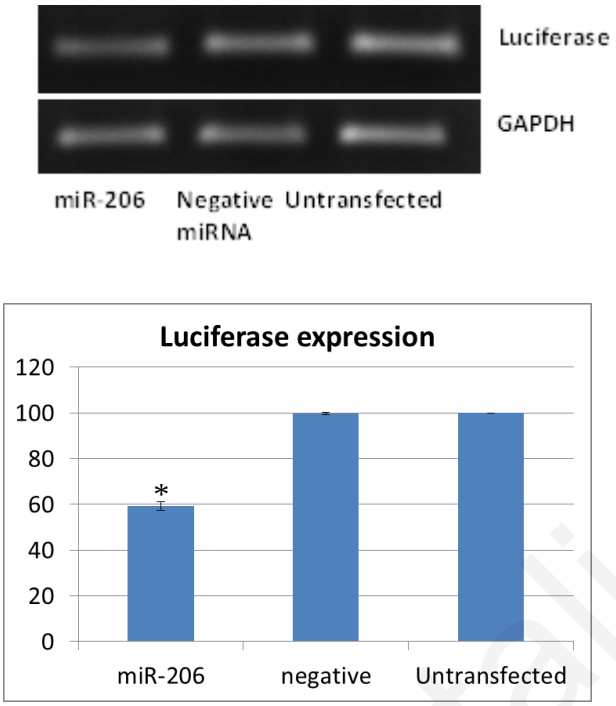


Figure 3.15 miR-206 reduces the Luciferase levels. Transfections of HeLa cells with miR-206 mimic and the plasmid expressing the luciferase gene with the Twist-1 3' UTR cDNA (Twist-1 3' UTR) caused a significant decrease in luciferase/human Twist-1 3' UTR levels compared to cells transfected with the Twist-1 3' UTR or transfected with the Twist-1 3' UTR and a non-specific miRNA (negative miRNA). Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

3.2.3 Conclusion

The first aim of this section was to experimentally validate miRNA mediated down regulation of Twist-1. Co-transfections of the luciferase reporter plasmid containing the human Twist-1 3'UTR along with the miR-206 were performed in HeLa cells and assayed using the Dual Glo® luciferase assay. miR-206 caused a ~50% decrease in relative luciferase activity compared to the negative controls. This repression of luciferase activity was specific to the particular binding site on the Twist-1 3'UTR as indicated when the transfection was repeated using the mutated Twist-1 3'UTR. The initial repression by miR-206 was reversed when contransfected with the mutant version, indicating that miR-206 represses Twist-1 through a specific binding site on the Twist-1 3'UTR. This result was also confirmed when no result was obtained when attempting to align to miR-206 with the mutated Twist-1 3'UTR binding site in-silico using the miRanda software.

3.3 miR-206 regulates Twist-1 expression and muscle cell differentiation.

3.3.1 Introduction

Muscle differentiation (myogenesis) is a complex and tightly regulated process involving the commitment of embryonic precursors to the myogenic lineage, myoblast proliferation and exit from the cell cycle, and finally fusion into multinucleated myotubes (Weintraub, 1993, Lassar et al., 1991, Sabourin and Rudnicki, 2000, Charge and Rudnicki, 2004). This multistep process is orchestrated by a handful of genes coding the myogenic regulatory factors (MRFs). These MRFs belong to the basic-helix-loop-helix (bHLH) transcription factor family and consist of Myf5, MyoD, Myf6 and myogenin. Each MRF plays an important role in the process. MYf5 and MyoD are responsible for regulating the formation, proliferation and longevity of myoblasts whereas Myf6 and Myogenin play a critical role in the regulation of the final stage of differentiation (Lassar et al., 1994, Yun and Wold, 1996, Pownall et al., 2002). An important molecule involved in myogenesis is Twist-1 which belongs to the family of bHLH transcription factors. It is expressed during embryonic development and it was found to be involved in developmental processes such as myogenesis. In Human Embryonic Stem Cell-Derived embryoid bodies, Twist-1 was found to inhibit muscle cell differentiation, as well as human myoblast the Twist-1 was also found to inhibit cell differentiation (Hebrok et al., 1994, Rohwedel et al., 1995, Cao et al., 2008, Koutsoulidou et al., 2011a). To date, not much information exists regarding the regulation of Twist-1 expression in muscle. In cancer, Twist-1 was shown to be regulated by a series of miRNAs such as miR-543, miR-720 and miR-181a (Li et al., 2014b, Liu et al., 2013a, Bing et al., 2014). There is no information however, regarding Twist-1 regulation by miRNAs in muscle. Our investigation focused on the identification of miRNAs that could bind to and regulate Twist-1 expression during myogenesis.

3.3.2 Results

3.3.2.1 miR-206 regulates differentiation through the regulation of Twist-1

It has been previously shown that overexpression of the Twist-1 resulted in the inhibition of muscle cell differentiation in adult cells (Hebrok et al., 1994, Rohwedel et al., 1995). Before proceeding to evaluating Twist-1 as a possible target for miR-206, we overexpressed Twist-1 through an adenoviral vector in human myoblast cells isolated from a newborn, that have the ability to differentiate and express low levels of Twist-1. High levels of exogenous Twist-1 demonstrated a decrease in the differentiation capacity of cells when stained for myosin heavy chain (MyHC), a late muscle cell differentiation marker (Figure 3.16). Also inhibition of Twist-1 foetus myoblast shows the reverse effect. Thus the inhibition of Twist-1 with siRNA shows increase in the differentiation capacity of the cells (Figure 3.17)

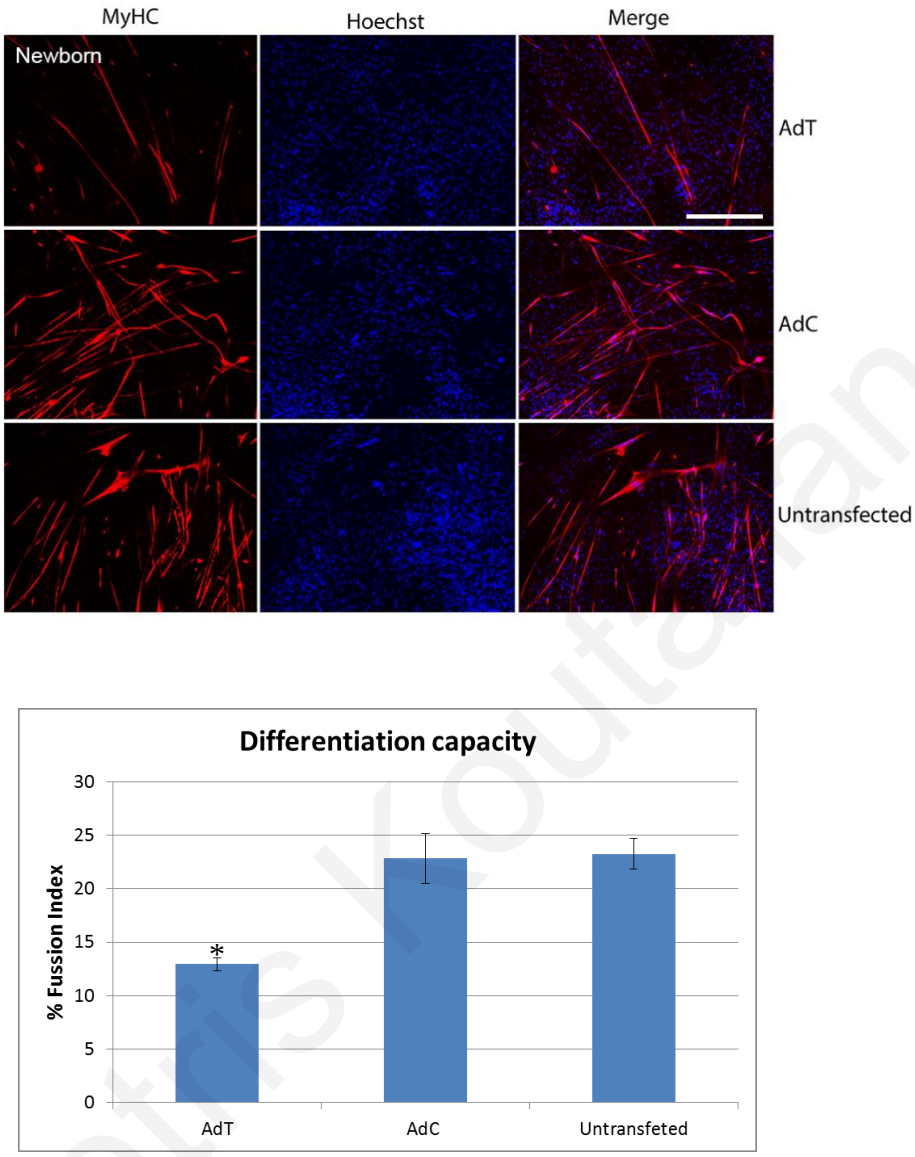


Figure 3.16 Twist-1 inhibits muscle cell differentiation. Newborn human myoblasts were transfected with an adenovirus that expressed human Twist-1 (AdT) and left to differentiate for 6 days. Myotubes were fixed and stained with MyHC and nucleus-directed Hoechst. AdT transduction caused inhibition of muscle cell differentiation compared to cells transfected with a control adenovirus (AdC) or compared to untransfected cells (Scale bar, 200 μm) and Inhibition of muscle cell differentiation by AdT compared to untransfected cells or to cells transfected with AdC by the calculation of fusion index.

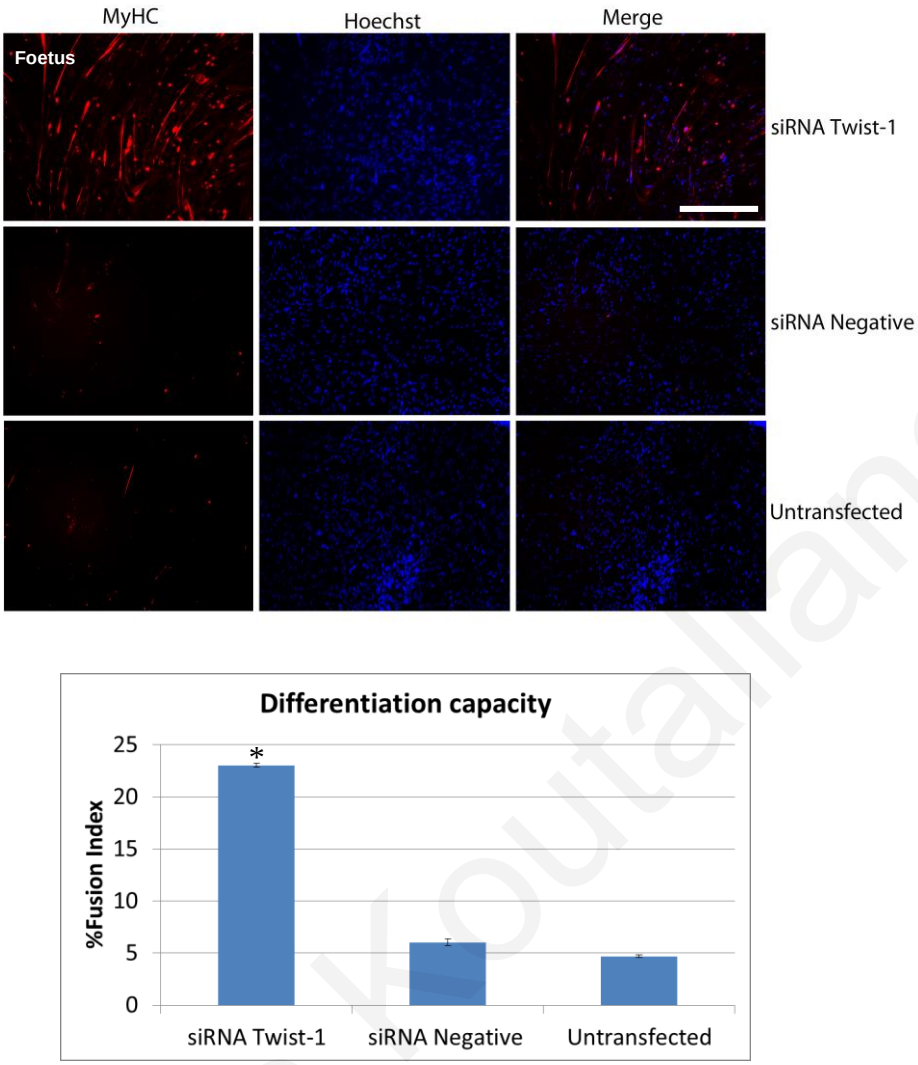


Figure 3.17 Twist-1 inhibition promotes muscle cell differentiation. Foetus human myoblasts were transfected with siRNA Twist-1 and left to differentiate for 6 days. Myotubes were fixed and stained with MyHC and nucleus-directed Hoechst. Twist-1 siRNA transfection caused an increase of muscle cell differentiation compared to cells transfected with a control siRNA (siRNA negative) or compared to untransfected cells (Scale bar, 200 μm). The increase of muscle cell differentiation by Twist-1 siRNA compared to untransfected cells or to cells transfected with siRNA negative is shown through the calculated fusion index.

As a next step, a human myoblast cell line isolated from a 14-week-old foetus which expresses high levels of Twist-1 and has low differentiation capacity, was used to determine the cellular activity of miR-206 on Twist-1 (Koutsoulidou et al., 2011b). The endogenous levels of the miR-206 were increased following overexpression of miR-206 and as a result the levels of Twist-1 were decreased (Figure 3.18-9). Muscle differentiation markers, troponin and muscle actin protein levels were also increased in myoblasts that overexpressed miR-206 compared to control cells (Figure 3.18). The same cells transfected with miR-206 were then induced to differentiate to form myotubes. Myoblasts that overexpressed miR-206 showed an increased capacity to differentiate in vitro compared to control cells (Figure 3.18). This was also shown by the increased Fusion Index (Figure 3.20).

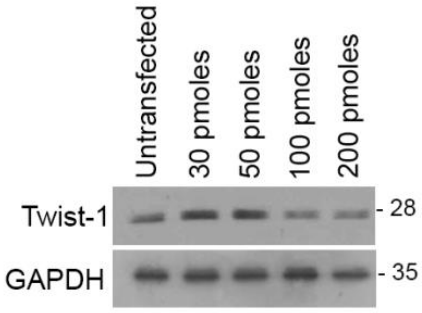


Figure 3.18 miR-206 downregulates Twist-1 expression. Titration of miR-206 shows that 100 pmoles of miR-206 (final concertation 0.05 μM) are enough to reduce the endogenous levels of miR-206

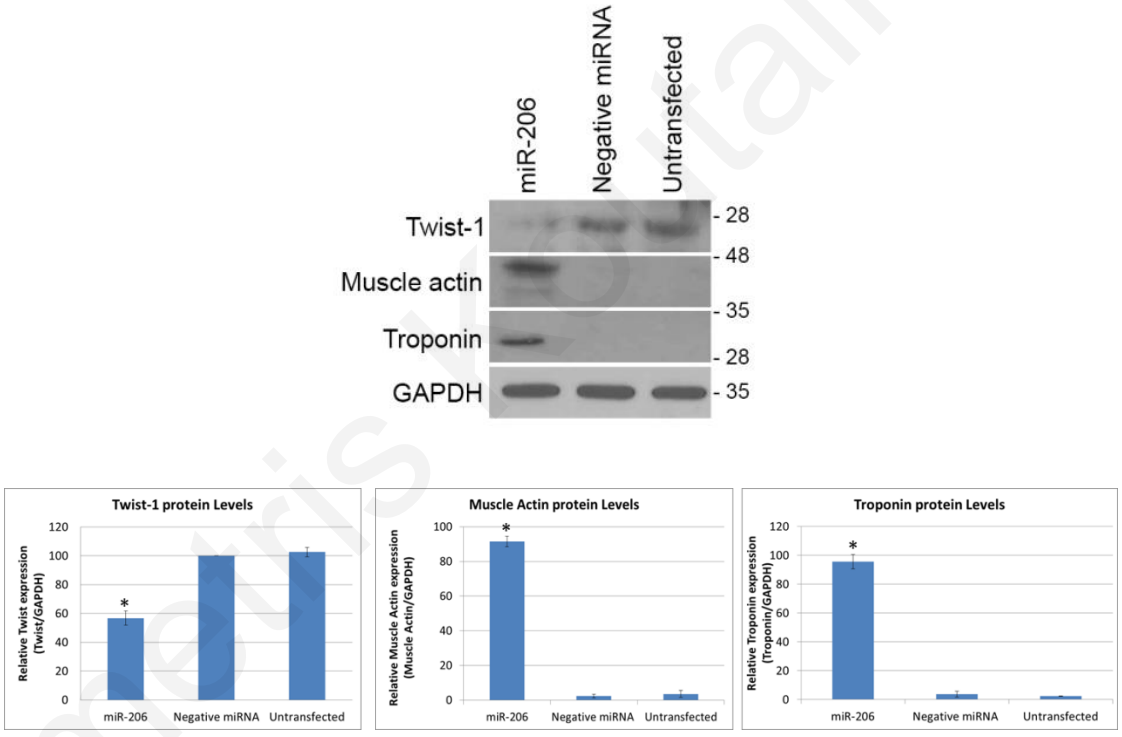
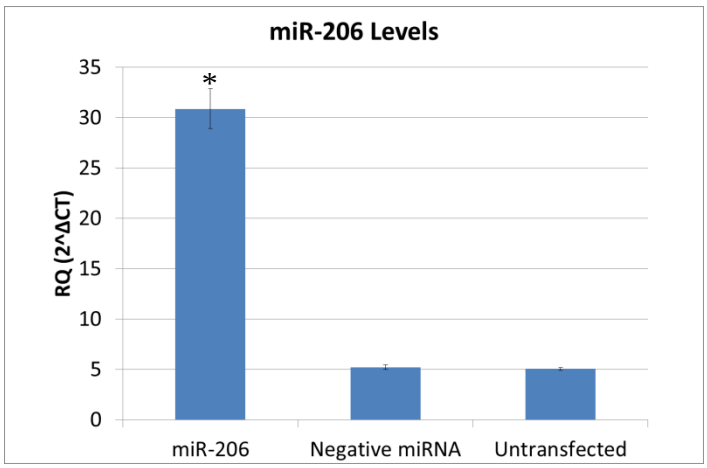
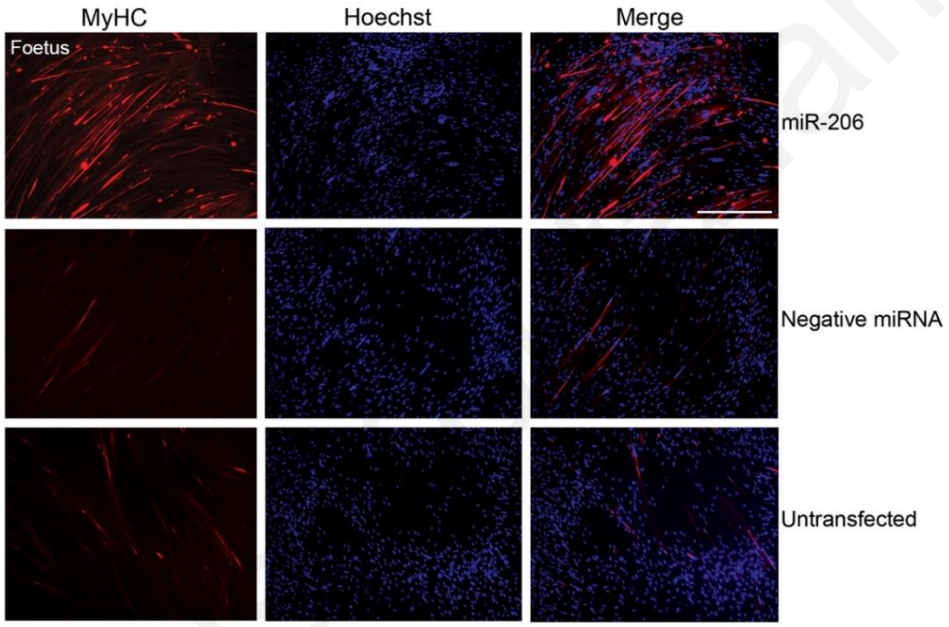


Figure 3.19 Overexpression of miR-206 downregulates Twist-1 expression. Transfection with miR-206 mimic resulted in decrease in the endogenous Twist-1 protein levels by Western blotting, as compared to cells transfected with a scrambled miRNA (negative miRNA) or untransfected cells. In parallel, miR-206 mimic caused an increase in the myogenic markers Muscle Actin and Troponin in 14-week-old foetus human myoblasts. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.





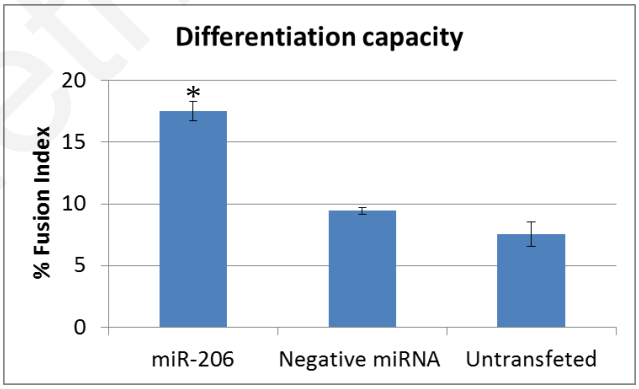


Figure 3.20 Overexpression of miR-206 promotes muscle cell differentiation. 14-week-old foetus myoblasts were transfected with miR-206 mimic and left to differentiate for 6 days. Myotubes were fixed and stained with the late differentiation marker Myosin Heavy Chain (MyHC) and nucleus-directed Hoechst. miR-206 mimic increased muscle cell differentiation compared to transfection with the negative control or to untransfected cells (Scale bar, 200 μm) and Calculation of Fusion Index demonstrated the increase in muscle cell differentiation in miR-206 mimic transfected cells compared to control cells.

Human muscle cell line isolated from a newborn, which expresses low levels of Twist-1 and has high differentiation capacity, was then used for transfections with miR-206 inhibitor (amiR-206). Following inhibition of miR-206, the levels of human Twist-1 protein were increased whereas the levels of the markers of differentiation, muscle actin and troponin, were decreased (Figure 3.21). The differentiation capacity of the transfected cells with amiR-206 was decreased compared to control cells (Figure 3.22).

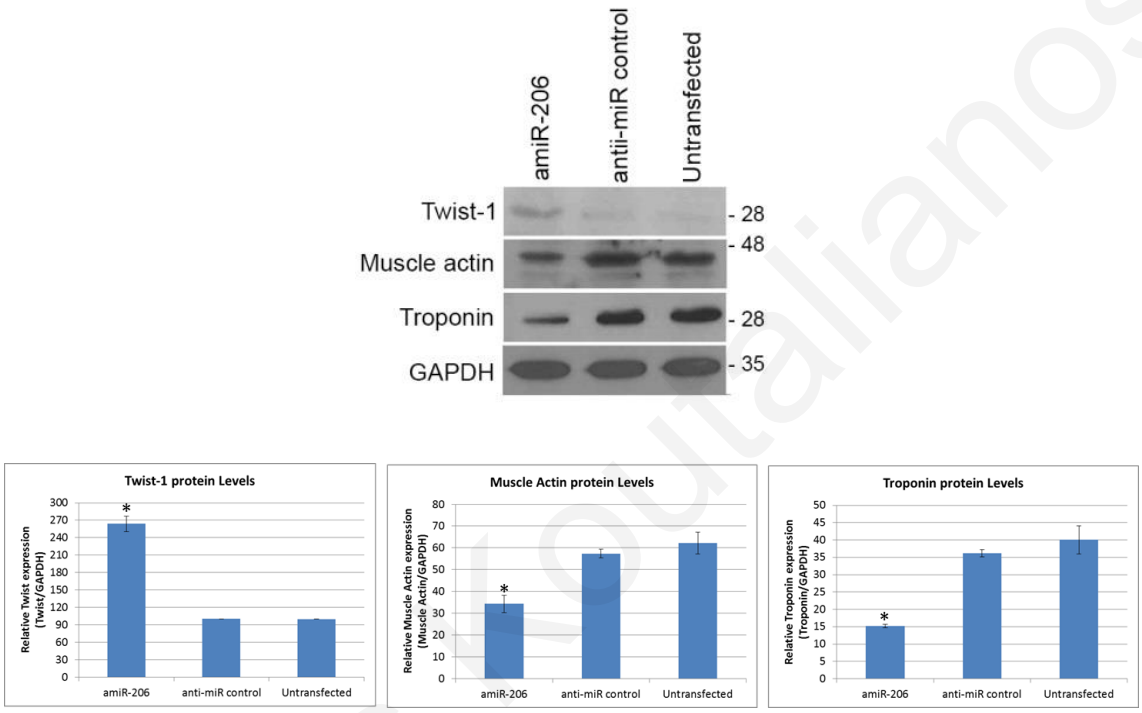
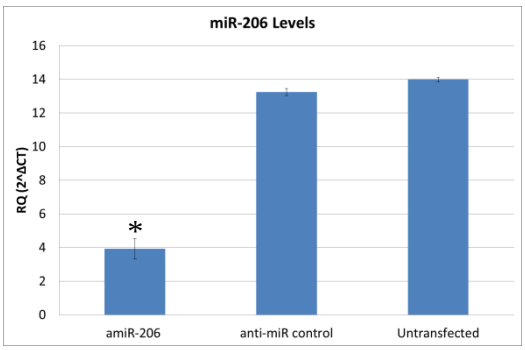
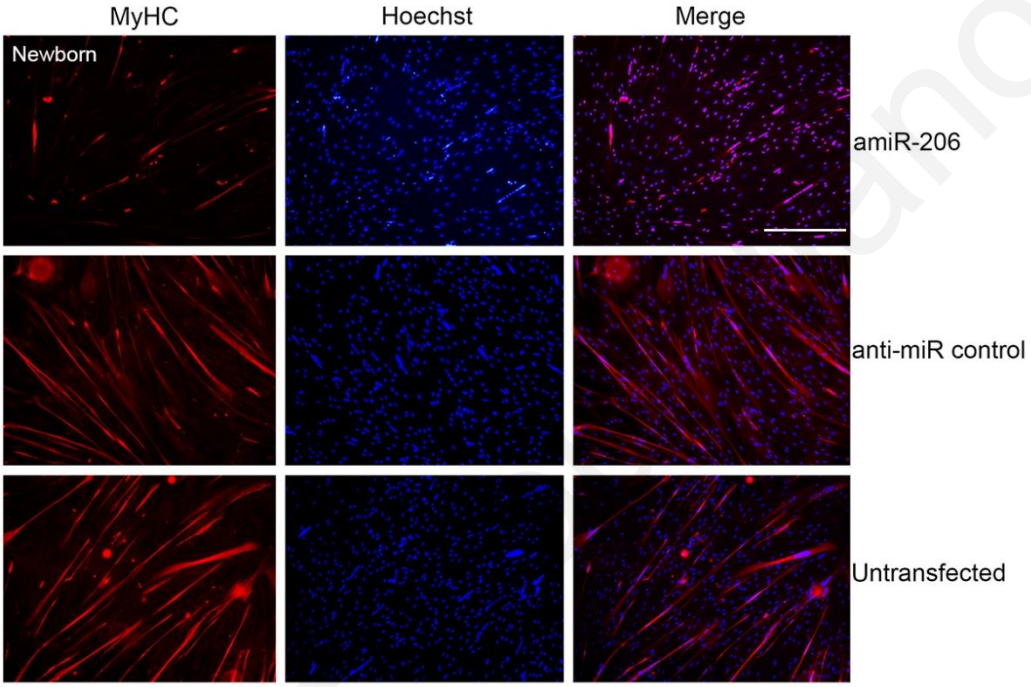


Figure 3.21 Downregulation of miR-206 upregulates Twist-1 expression. miR-206 antagomiR (amiR-206) transfection resulted in increase in the endogenous Twist-1 protein levels by Western blotting, as compared to cells transfected with scrambled anti-miRNA (anti-miR control) and untransfected cells. This increase was accompanied by a decrease in the myogenic markers Muscle Actin and Troponin in newborn human myoblasts. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.





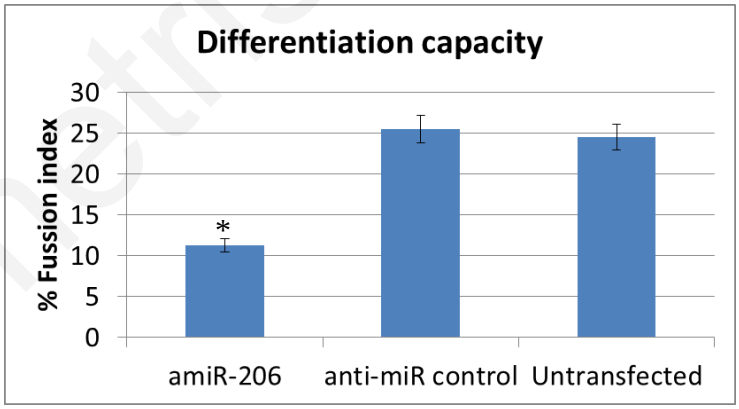


Figure 3.22 Downregulation of miR-206 inhibits muscle cell differentiation. Newborn myoblasts were transfected with miR-206 antagomiR (amiR-206) and left to differentiate for 6 days. Myotubes were fixed and stained with MyHC and nucleus-directed Hoechst. miR-206 antagomiR transfection caused an increase in muscle cell differentiation compared to transfection with the anti-miR control or to untransfected cells (Scale bar, 200 μm) and Calculation of Fusion Index demonstrated the decrease in muscle cell differentiation in miR-206 mimic transfected cells compared to control cells.

These results support the earlier experiments that Twist-1 is a target for miR-206 in order to induce muscle cell differentiation.

3.3.3 Conclusion

The aim of this part was to investigate the effect of miR-206 on muscle cells and its effects on Twist-1 expression levels during differentiation. Foetus myoblasts were there are low levels of endogenous miR-206 levels and low differentiation capacity, transfected with miR-206 mimics caused decrease in Twist-1 levels and increase in differentiation capacity of the cells. In Newborn myoblasts, where the endogenous levels of miR-206 were increased during differentiation, transfection with miR-206 inhibitors showed increase in Twist-1 levels and decrease in the differentiation capacity of the cells.

There is enough evidence in this study to show that miR-206 downregulates Twist-1, and increases significantly the levels of differentiation as indicated by the differentiation indexes muscle cells.

3.4 MyoD promotes muscle cell differentiation through upregulation of miR-206 and downregulation of Twist-1.

3.4.1 Introduction

One of the main positive regulators of miR-206 is the transcription factor MyoD (Rosenberg et al., 2006). MyoD was found to regulate miR-206 expression by direct binding to its promoter. In an attempt to further elucidate the mechanism of Twist-mediated regulation of myogenesis, experiments were performed in order to investigate the possible implication of MyoD.

3.4.2 Results

3.4.2.1 MyoD overexpression induces miR-206 Levels

As a first step, a 14-week-old foetal myoblast cell line was transduced with an adenovirus expressing the MyoD transcription factor (AdM) (Figure 3.23). MyoD overexpression increased the capacity of the cells to differentiate and simultaneously increased the markers troponin and muscle actin (Figure 3.23) and the levels of miR-206 (Figure 3.24).

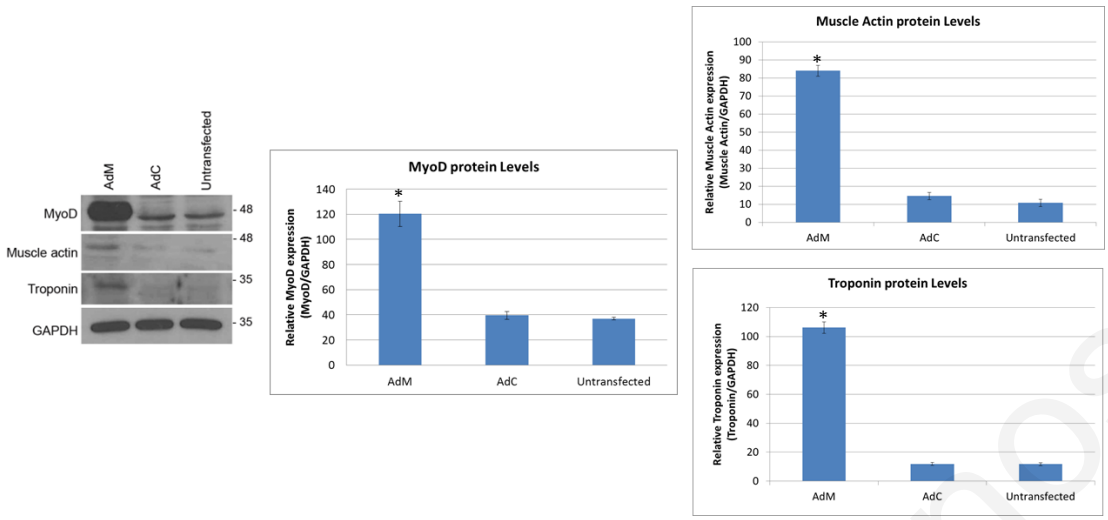


Figure 3.23 MyoD overexpression. Transduction of 14-week-old foetus human myoblasts with an adenovirus which expresses MyoD (AdM) caused an increase in cellular MyoD levels compared to untransfected cells or to cells transduced with a control adenovirus (AdC) as well as the troponin and muscle actin levels were increased.

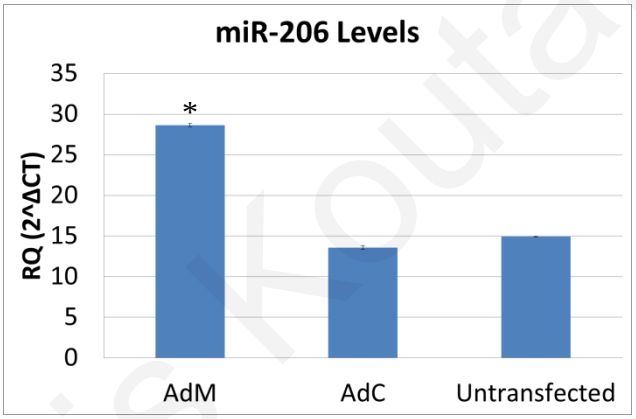


Figure 3.24 MyoD overexpression induces miR-206 levels. Similarly, AdM transduction caused an increase in the endogenous miR-206 compared to untransfected cells or to cells transduced with a control adenovirus (AdC). Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

3.4.2.2 MyoD induces miR-206 levels through its promoter

As a next step, the possibility of MyoD direct binding to the miR-206 promoter and its subsequent regulation of expression in 14-week-old human myoblasts were investigated. miR-206 promoter contains two E-boxes which are highly conserved among species (Figure 3.25). Chromatin Immunoprecipitation assay showed that endogenous MyoD binds to both E-boxes located on miR-206 promoter in 14-week-old human myoblasts (Figure 3.26). Luciferase assays were next performed in order to investigate the effect of MyoD on miR-206 expression. Overexpression of MyoD in HeLa cells transfected with a plasmid containing the promoter of miR-206 upstream of a luciferase gene showed that MyoD induces the expression of miR-206 (Figure 3.27). The direct binding of MyoD on human miR-206 promoter was further investigated using

mutagenesis experiments. Single mutations were introduced on each of the E-boxes located on the promoter of miR-206. MyoD overexpression in HeLa cells transfected with the mutated plasmids showed a dramatic reduction on luciferase activity. Introduction of mutations on both E-boxes located on the promoter of human miR-206 and overexpression of MyoD supressed luciferase activity (Figure 3.27).

Figure 3.25 miR-206 promoter and E-boxes. A) Map of the human miR-206 promoter and putative MyoD binding E-boxes. B) Conservation among species of E-box 1 and E-box 2 based on sequence homology.

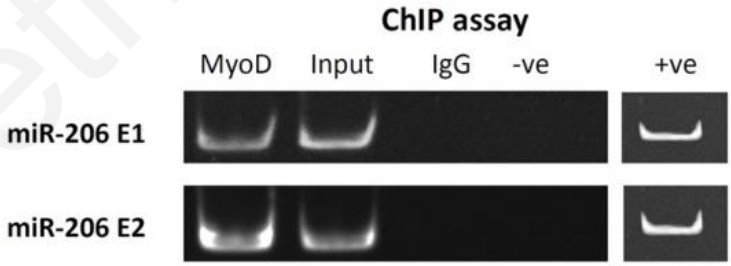
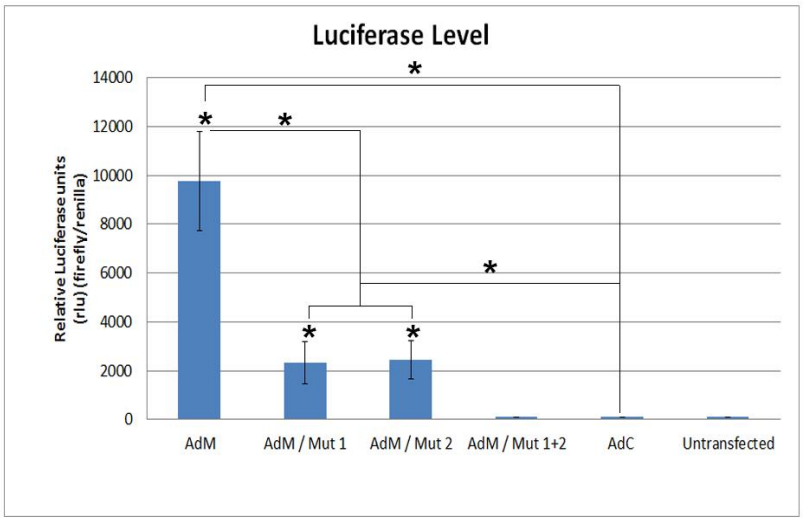


Figure 3.26 MyoD binds to E-boxes on miR-206 promoter.

ChIP assay revealed that following MyoD overexpression, MyoD bound to both E-boxes on the promoter of miR-206.



E-box 1

ACCCTGAGTGGCTCAACAGCTGCCAATGTCCCTCAT **WT**

ACCCTGAGTGGCTCATGTCGAGCCAATGTCCCTCAT **Mutated**

E-box 2

TGGAATGCTGGATGGGCAGCTGCTGCCCATCAACAA **WT**

TGGAATGCTGGATGGCGTCGAGCTGCCCATCAACAA **Mutated**

Figure 3.27 MyoD promotes miR-206 expression. HeLa cells transfected with an adenovirus expressing MyoD (AdM) showed an increase in Luciferase activity compared to cells transfected with a control adenoviral vector (AdC) or untransfected cells. Introduction of mutations in either E-boxes showed a large reduction of Luciferase activity while introduction of both mutations showed no activity, similar to the one seen in AdC-transfected or untransfected cells. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

3.4.2.3 MyoD promotes differentiation through Twist-1 inhibition

MyoD was found that inhibit the expression of Twist-1 (figure 3.28). The effect of MyoD overexpression on Twist-1 expression was next investigated. The levels of human Twist-1 protein were found significantly decreased in muscle cells overexpressing MyoD compared to control muscle cells (Figure 3.28). In order to investigate whether MyoD induces muscle cell differentiation through inhibition of Twist-1, we overexpressed MyoD and at the same time overexpressed Twist-1 using adenoviruses in 14-week-old human myoblasts. By overexpressing both MyoD and Twist-1, the inhibition of Twist-1 caused by the overexpression of MyoD was abolished (figure 3.29). Following the double overexpression of the two transcription factors, MyoD and Twist-1, the cellular differentiation capacity was reduced compared to the overexpression of MyoD only. Specifically, the double transduced cells had similar capacity to differentiate compared to the control cells (Figure 3.30).

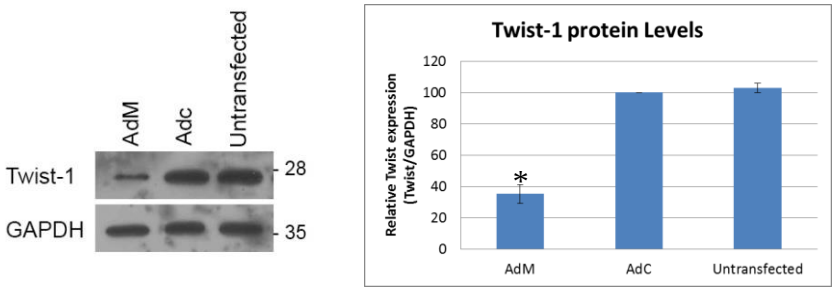


Figure 3.28 MyoD overexpression inhibits Twist-1 expression. Overexpression of MyoD with an adenoviral vector (AdM) caused a decrease in the endogenous Twist-1 protein levels by Western blotting, as compared to the control adenovirus (AdC) and untransfected cells in the 14-week-old foetus human myoblasts.

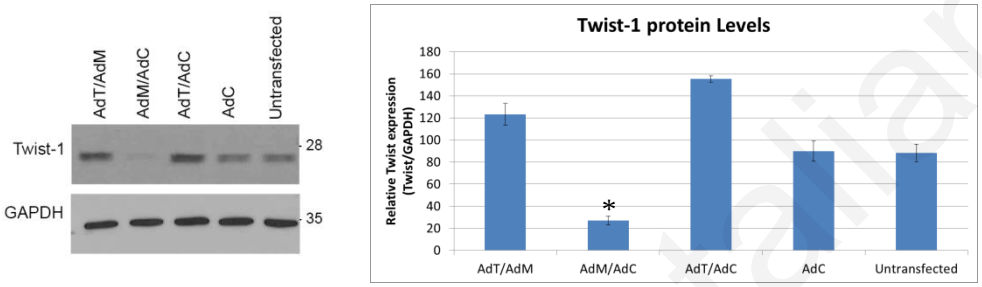


Figure 3.29 Overtake the Twist-1 inhibition. Competition co-transductions with both overexpressions of MyoD (AdM) and Twist-1 (AdT) reversed the decrease in endogenous Twist-1 levels, thus proving the effect of MyoD on Twist-1. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

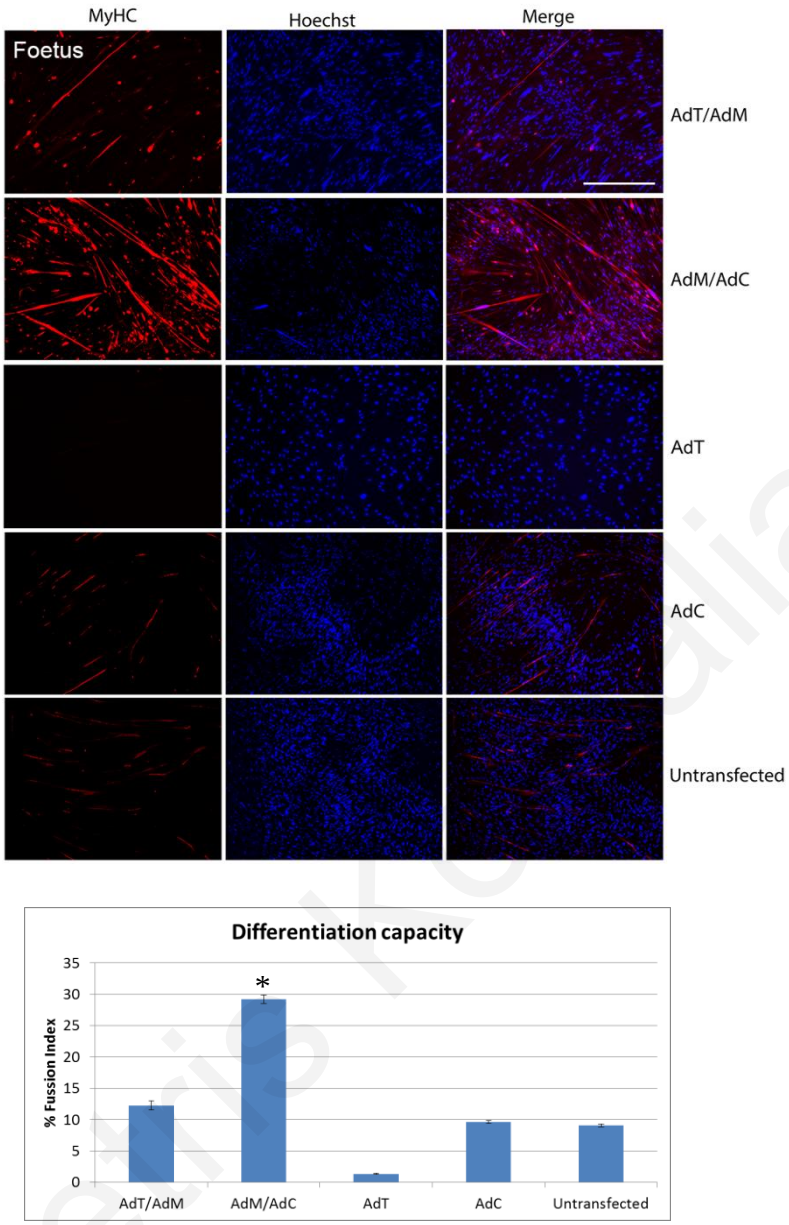


Figure 3.30 MyoD regulates muscle cell differentiation through inhibition of Twist-1. Error! Bookmark not defined. 14-week-old foetus myoblasts were co-transduced with AdM and AdT, or AdM and AdC alone and left to differentiate for 6 days. Myotubes were fixed and stained with MyHC and nucleus-directed Hoechst. MyoD induced differentiation while combination of overexpression of MyoD and Twist-1 reversed this increase and behaved similarly to untransduced cells or to cells transduced with control adenovirus (AdC) (Scale bar, 200 μm) and the above results were confirmed by the calculation of Fusion Index.

3.4.2.4 MyoD inhibits Twist and promotes differentiation through miR-206

Based on our results, MyoD promotes muscle cell differentiation, induces the expression of miR-206 and inhibits Twist-1 expression. In order to investigate whether MyoD inhibits Twist-1 expression through miR-206 induction, MyoD was overexpressed in 14-week-old human myoblasts followed by inhibition of miR-206 by an antagomiR. Protein analysis showed that the levels of Twist-1 protein were increased compared to the cells that overexpress either MyoD or miR-206 (Figure 3.31). Furthermore, the levels of Twist-1 protein in double transfected cells were similar to the control cells (Figure 3.31). Myogenesis markers muscle actin and troponin levels were elevated when either MyoD or miR-206 were overexpressed (Figure 3.30)Moreover, double transfected cells showed a decrease in their capacity to differentiate in vitro compared to the overexpression of either MyoD or miR-206 alone and a similar differentiation capacity to the control cells (Figure 3.32). The differences in muscle cell differentiation were also confirmed by fusion index (Figure 3.32).

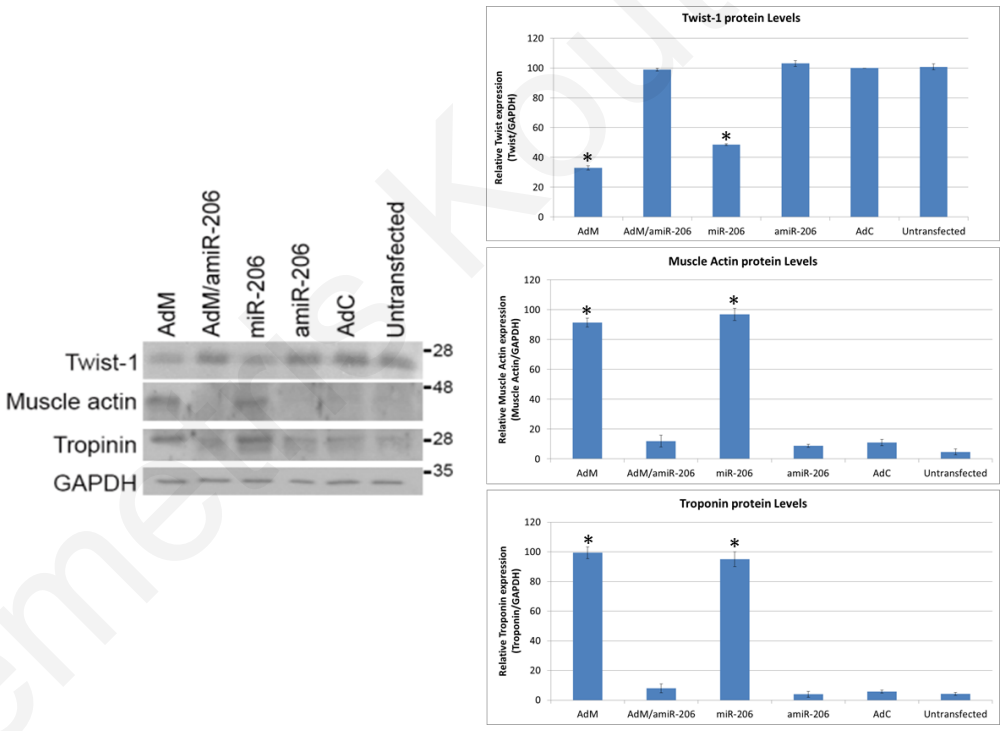


Figure 3.31 MyoD regulates downregulation of Twist-1 by miR-206. Competition co-transductions with both overexpressions of MyoD (AdM) and miR-206 antagomiR (amiR-206) reversed the decrease in endogenous Twist levels by MyoD. Overexpression of amiR-206 alone had no effect on Twist-1 levels, presumably because of the low miR-206 endogenous levels. The effect of AdM and miR-206 on the induction of differentiation was shown by the increased levels of the myogenesis markers muscle actin and troponin. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

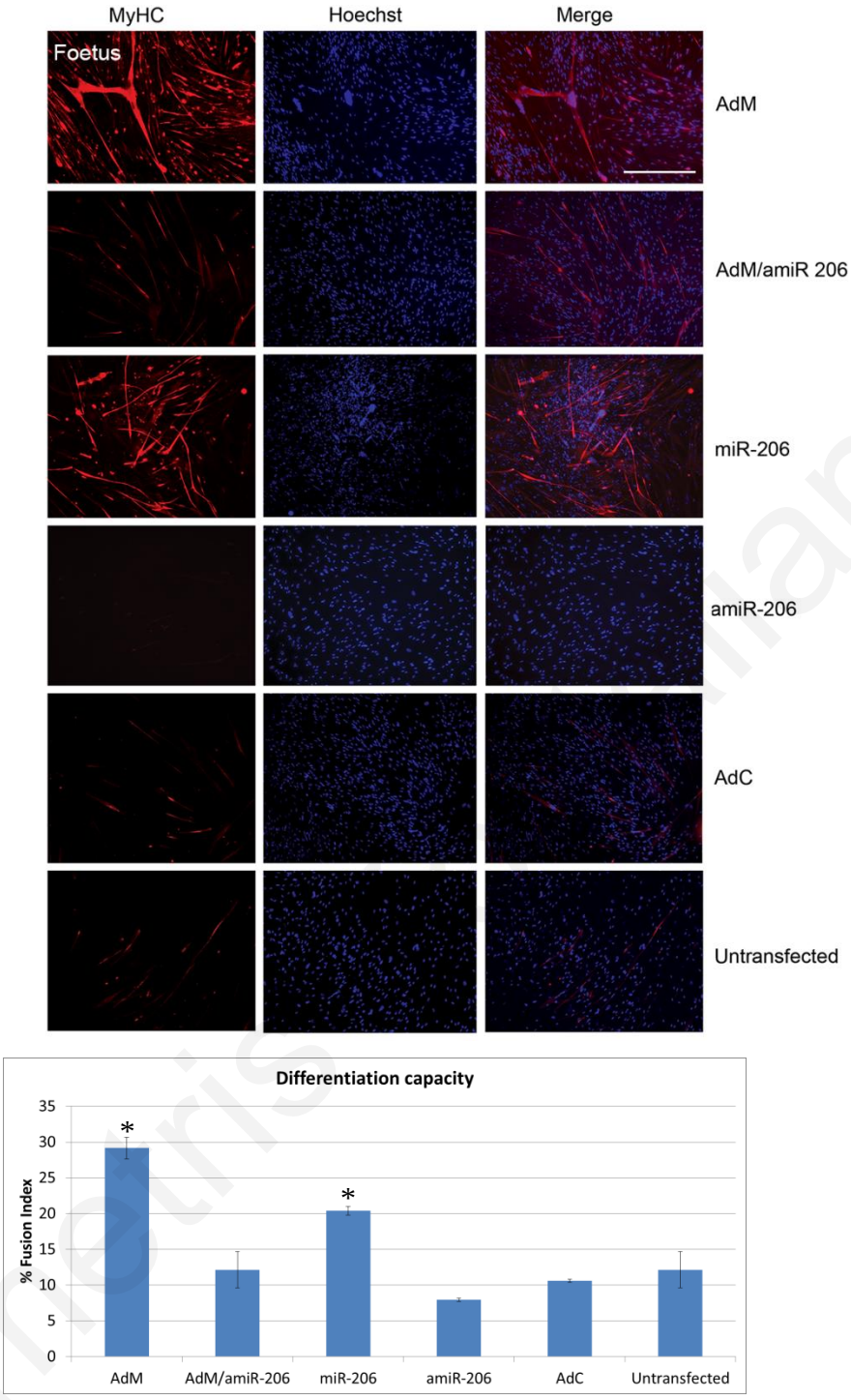


Figure 3.32 MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206. 14-week-old foetus myoblasts were co-transduced with AdM and amiR-206, or miR-206 mimic and AdC alone and left to differentiate for 6 days. Myotubes were fixed and stained with MyHC and nucleus-directed Hoechst. Combination of overexpression of MyoD and amiR-206 reversed the increase in muscle cells differentiation with AdM or miR-206 alone and behaved similarly to untransduced cells, to cells transfected with amiR-206 alone or to cells transduced with control adenovirus (AdC) (Scale bar, 200 μm) and C) The above results were confirmed by the calculation of Fusion Index

These results indicate, therefore, that MyoD can induce muscle differentiation by inhibiting Twist-1, through miR-206.

To further evaluate these findings in more physiological setting, experiments were performed in primary mouse cells. This is possible since (1) the binding site of miR-206 on Twist-1 3'UTR is conserved between human and mouse, (2) the expression profile of miR-206 in mouse is inversely proportional to the expression of Twist-1 (Kim et al., 2006, Koutsoulidou et al., 2011b), (3) Twist-1 overexpression in mouse has the same negative effect on muscle cell differentiation (Hebrok et al., 1994, Rohwedel et al., 1995) and (4) the E-boxes bound by MyoD and found in the promoter region of miR-206 are conserved between mouse and human (Figure 3.25). Primary cells were isolated from a 4-week-old mouse which expresses Twist-1 gene at low levels (Dupont et al., 2001) (Figure 3.34). Inhibition of MyoD or miR-206 caused an increase in Twist protein levels in primary cells in agreement with human muscle cell lines (Figure 3.35). Furthermore, inhibition of MyoD or miR-206 reduced the capacity of primary cells to differentiate in vitro compared to the control cells (figure 3.35). In order to investigate whether MyoD inhibits Twist-1 expression through miR-206, MyoD was inhibited and at the same time miR-206 was overexpressed in primary muscle cells. As a result, Twist-1 levels were decreased compared to the cells subjected to inhibition of MyoD or amiR-206 alone (Figure 3.34). Similarly, Twist-1 levels were similar to the control primary cells and the differentiation capacity of the cells was restored (Figure 3.35).

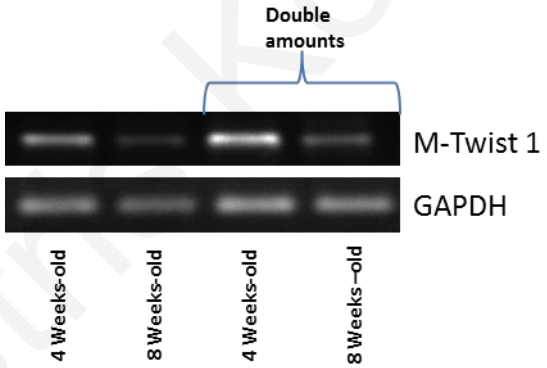


Figure 3.33 Twist-1 is expressed in 4 weeks-old mouse. RNA was extracted from primary mouse myoblast pf 4 weeks and 8 weeks old mice. cDNA from the extraction was used for PCR in order to verify that Twist-1 is expressed. 4 weeks-old mouse expressed more Twist-1 than 8 weeks-old mouse.

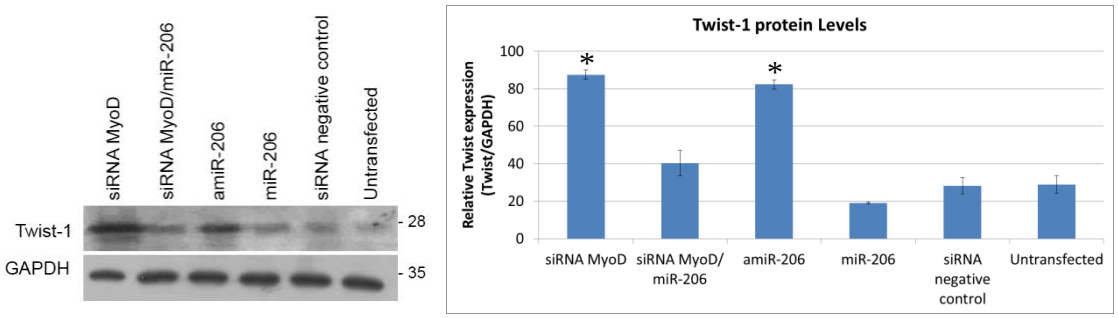


Figure 3.34 MyoD regulates downregulation of Twist-1 by miR-206 in primary myoblast. Transfections of 4-weeks old mouse primary myoblasts with a siRNA for MyoD and amir-206 resulted in an increase in the endogenous Twist-1 protein levels by Western blotting, as compared to the scrambled siRNA (siRNA negative control) and to untransfected primary mouse myoblasts. On the other hand, co-transfection of siRNA for MyoD and miR-206 mimic reversed the increase of Twist-1 back to normal levels. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

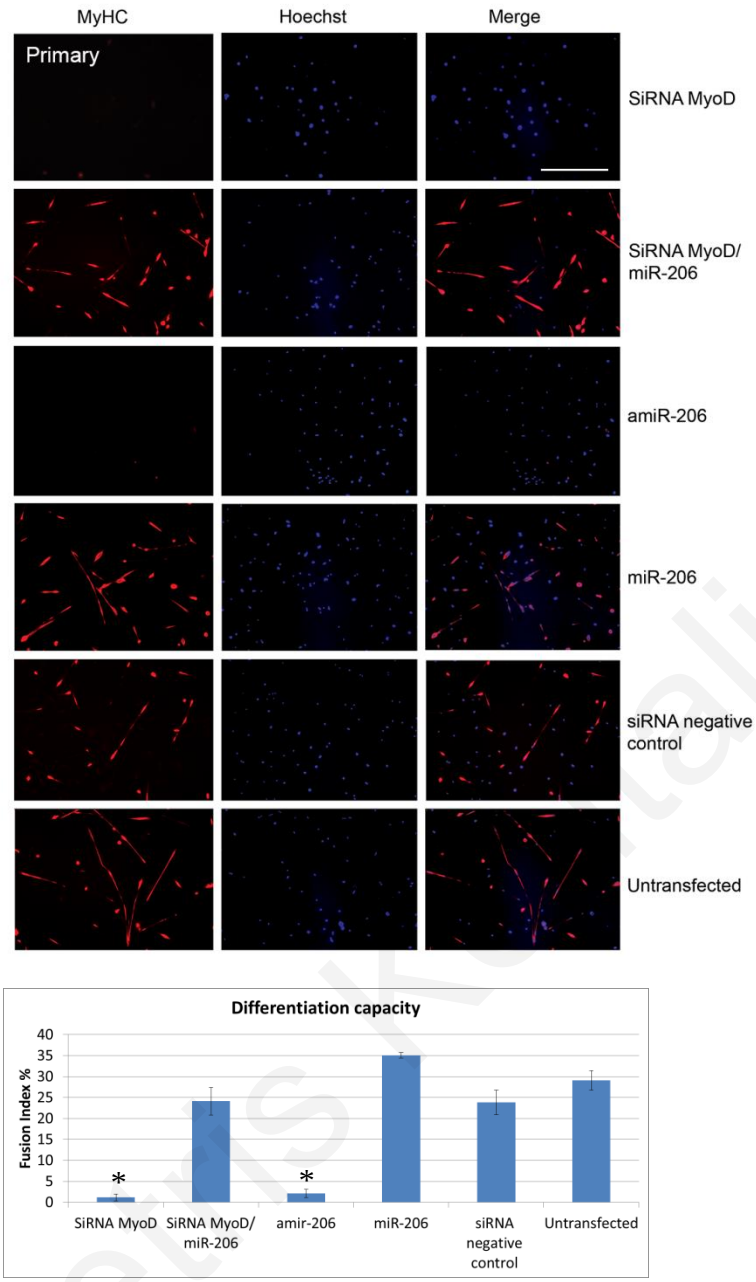


Figure 3.35 MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206 in primary mouse myoblasts. Primary mouse myoblasts were co-transfected with siRNA for MyoD and miR-206 mimic, or with siRNA for MyoD and amiR-206 alone and left to differentiate for 3 days. Myotubes were fixed and stained with MyHC and nucleus-directed Hoechst. siRNA for MyoD and amiR-206 transfections inhibited muscle cell differentiation while co-transfections with siRNA for MyoD and amiR-206 showed almost similar effect on differentiation compared to control miRNA transfection or compared to untransfected (Scale bar, 200 μm) and Calculation of Fusion Index confirmed the above results.

3.4.3 Conclusion

From the results shown above, it could be concluded that MyoD promotes differentiation through the inhibition of Twist-1 via miR-206. It is well known from the literature that MyoD induces myogenesis through several mechanisms. It is also known that MyoD induces myogenesis through the promotion of miR-206 gene expression. The results above suggest and prove the existence of a novel mechanism MyoD induces myogenesis by inhibiting Twist-1 through miR-206 (Figure 3.36).

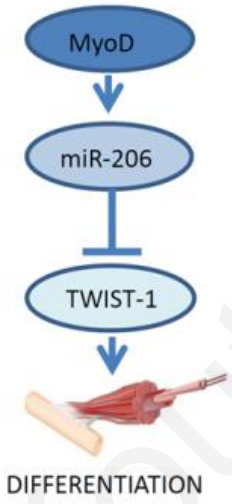


Figure 3.36 Suggested pathway. Suggested a novel mechanism MyoD induces myogenesis by inhibiting Twist-1 through miR-206

3.5 MyoD/miR-206/Twist-1 mechanism is involved in Myotonic Dystrophy type 1 cells which exhibit defective differentiation.

3.5.1 Introduction

The results of this study so far showed that MyoD may exert its positive effect on myogenesis through an additional pathway and more specific through miRNA-mediated inhibition of Twist-1. The final step of this project aimed at investigating the role of this new pathway in a disease setting. Myotonic Dystrophy type 1 (DM1) is an inherited neuromuscular disease which usually manifests during adulthood and is caused by a CTG expansion in the 3 UTR of the Dystrophia myotonica protein kinase (DMPK) gene (Miller et al., 2000). A rarer and more severe congenital form also exists (Meola, 2013). Defects in the differentiation program of DM1 have been reported in numerous cases and certain mechanisms responsible identified (Amack and Mahadevan, 2004).

DM1 human myoblast cells with low differentiation capacity were used to evaluate the role of the new pathway in the defective differentiation program of the disease.

3.5.2 Results

3.5.2.1 MyoD protein and miR-206 Levels are inhibited and Twist-1 protein levels are induced in DM1 myoblasts

MyoD protein levels were found inhibited in cells expressing DM1 myoblast compared to wild type cells (Figure 3.37). Twist-1 was found to have an inversely proportional expression profile compared to MyoD during differentiation both in DM1 and the healthy control muscle cells (Figure 3.37). Also the miR-206 levels were increased during the differentiation of healthy cells but inhibited in DM1 cells (Figure 3.37).

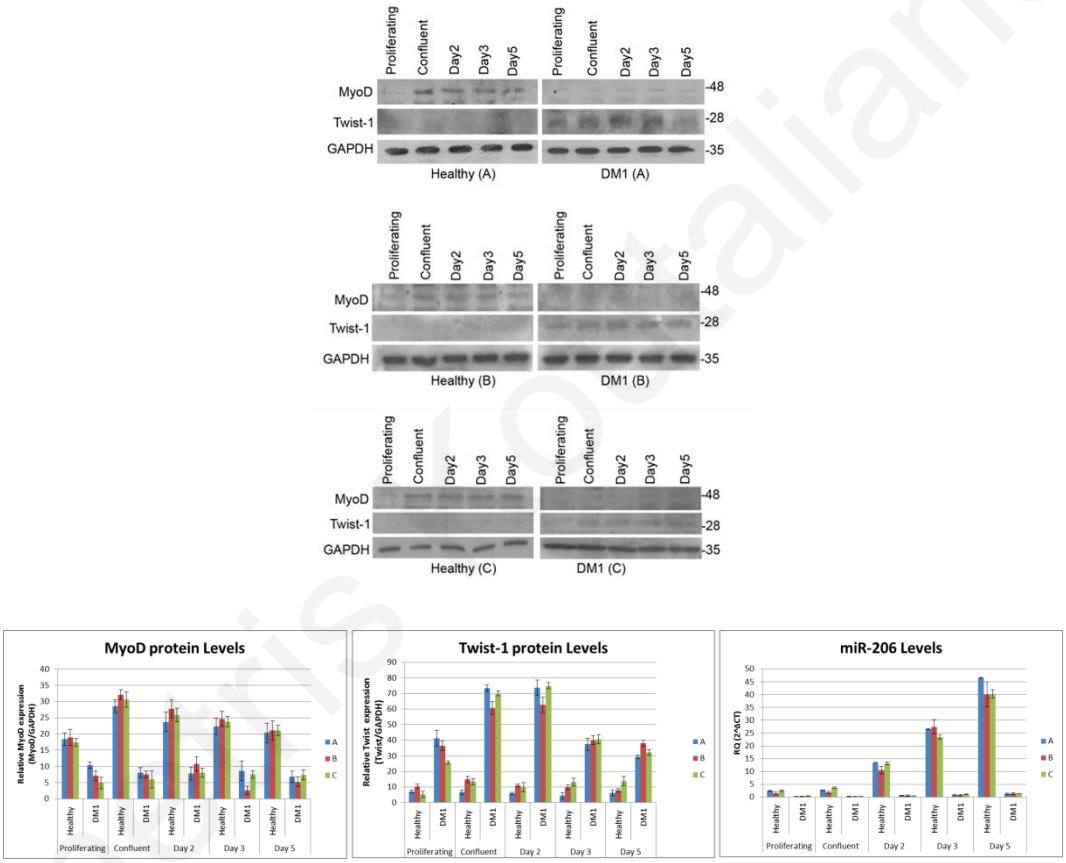


Figure 3.37 MyoD and miR-206 are supressed in DM1 while Twist-1 levels are decreased. Protein analysis by Western blotting of 3 different DM1 and healthy cell lines (A-C) showed that MyoD protein levels were decreased in DM1 cells compared to the healthy cells, whereas Twist-1 protein levels were increased in DM1 cells compared to healthy ones. Protein expression profile for MyoD and Twist-1 and RNA levels for miR-206 in 3 different healthy and DM1 cells from proliferation to differentiation (Days 2-5). MyoD levels and miR-206 levels were found to be decreased in DM1 cells compared to normal cells, whereas Twist-1 levels were increased in DM1 cells.

3.5.2.2 MyoD/miR-206/Twist-1 pathway is inhibited in DM1 myoblast

Protein analysis showed that the levels of Twist-1 protein were increased in the DM1 cells co-transfected with MyoD and amiR-206 compared to the cells that overexpressed either MyoD or

miR-206 alone (Figure 3.38). Furthermore, the levels of Twist-1 protein in the double transfected cells were similar to the control cells (Figure 3.38). Moreover, overexpression of MyoD together with amiR-206 revealed a decrease in the capacity of cells to differentiate in vitro compared to either MyoD or miR-206 alone. Co-transfection of MyoD and amir-206 showed similar differentiation capacity compared to control cells (Figure 3.39).

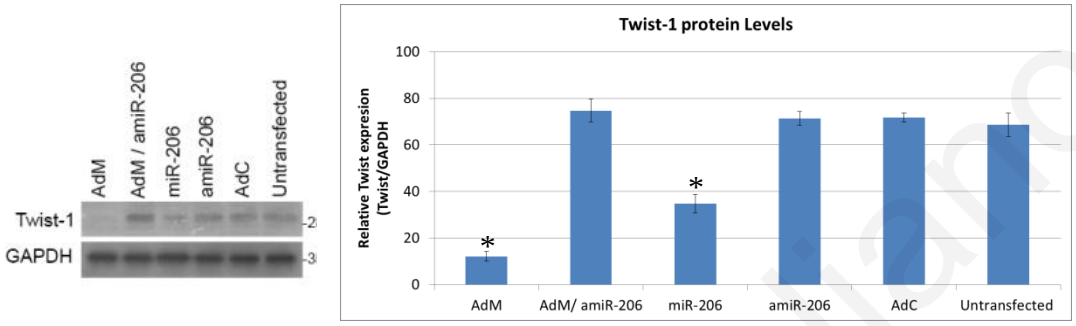


Figure 3.38 MyoD regulates downregulation of Twist-1 by miR-206 in DM1 myoblasts. Overexpression of MyoD (AdM) or transfection with miR-206 mimic in DM1 cells caused a decrease in the endogenous Twist-1 levels compared to control adenovirus transduction (ADC) or compared to untransfected cells. Twist-1 endogenous levels were increased back to the original levels when DM1 cells were co-transduced with AdM and miR-206 antagomiR (amiR-206). Overexpression of amiR-206 alone had no effect on Twist-1 levels, presumably because of the low miR-206 endogenous levels. Experiments were performed in triplicate, and the data represent the mean ± S.D. * represents a significance of ($p < 0.01$) using Student's t test.

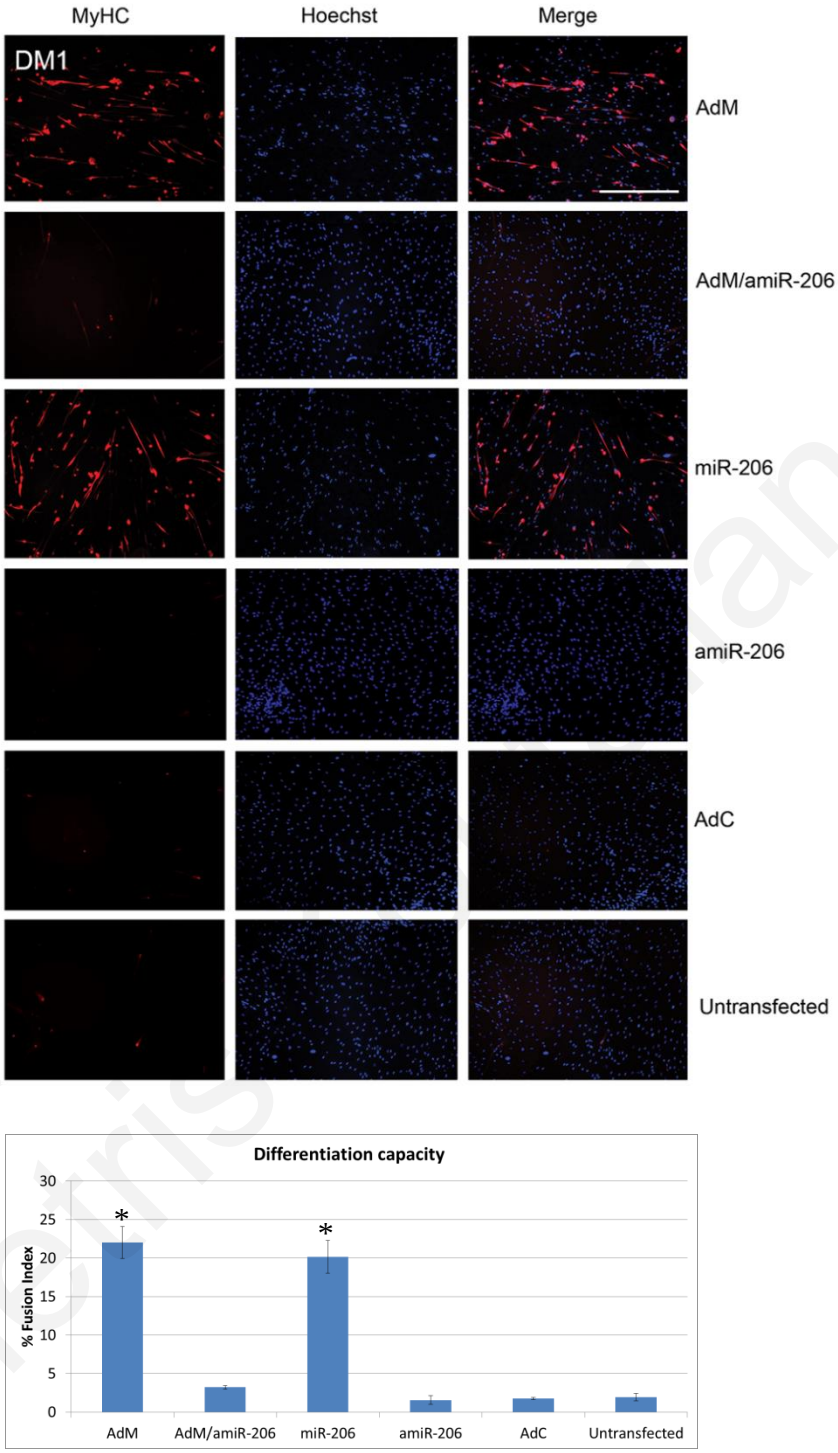


Figure 3.39 MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206 in DM1 myoblasts. Following differentiation of DM1 human myoblasts for 6 days, myotube formation was seen only in those cells which were transduced with AdM or miR-206 compared to control transduced cells (AdC) to cells transfected with amiR-206 alone or compared to untransfected cells. Myogenesis was reversed when amiR-206 was contransfected with AdM. Myotubes were fixed and stained with MyHC and nucleus-directed Hoechst. Scale bar, 200 μm and Calculation of Fusion Index confirmed the above results.

3.5.3 Conclusion

Therefore, these results further support that this novel myogenic pathway which implicates MyoD, Twist-1 and miR-206 may induce the defective differentiation program in Myotonic Dystrophy type 1 and result to an increase in the formation of mature muscle cells. The novel pathway identified in this project was found to be compromised in cells from Myotonic Dystrophy type I patients, which are known to have a defective differentiation program. This was based on experimental evidence indicating that the three key molecules studied in this project (MyoD, miR-206, Twist-1) were altered together with the inhibition of differentiation of muscle cells. Moreover, the important implication of this novel pathway in the differentiation program of Myotonic Dystrophy type I was shown when myogenesis was reversed following interference and correction of the said pathway.

4

DISCUSSION

Myogenesis is a complex tightly regulated procedure that forms the muscular tissue. During myogenesis, myoblasts are pulled out from the cell cycle while stimulation of the expression of the myogenic differentiation-specific gene expression occurs. Furthermore, these myoblasts fused together to form multi-nuclei fibers called myotubes (Sabourin and Rudnicki, 2000). Myogenesis occurs during different stages of human development. Specifically myogenesis takes place during the embryonic stage for the formation of muscle tissue and in adult stage for the replacement of damaged or lost muscle. These processes are almost the same in both embryonic and postnatal myogenesis. The main difference between these two stages of myogenesis is that during embryonic stage, the new myoblasts arise from the precursor cells while in the adult stage from the quiescent satellite cells. One other difference is that in adults the fusion occurs initially between the newly formed myoblasts with the preexisting myotubes. While in the embryonic stage, myotube formation occurs only by the fusion of myoblast arised from post mitotic myocytes.

Myogenesis is initiated and regulated mainly by the expression of the myogenic regulatory factors (MRFs). MRFs belong to the family of skeletal muscle-specific transcription factors. The MRFs (MyoD, Myf5, Myf-6 and myogenin) are important for both determination and maintenance of skeletal muscle. They belong to a superfamily of basic helix-loop-helix (bHLH) transcription factors which bind to the promoter of muscle specific gene and promote their expression. MyoD was the first member of the MRFs that was identified (Davis et al., 1987). MyoD was found through its ability to renovate non-muscle cells to stable myoblasts at high frequency (Davis et al., 1987, Blau et al., 1983, Wright, 1984). After MyoD, the other three MRFs were discovered: Myf-5 (Braun et al., 1989), myogenin (Edmondson and Olson, 1989, Wright et al., 1989) and Myf-6 (or MRF4 or Herculin) (Rhodes and Konieczny, 1989, Braun et al., 1990, Miner and Wold, 1990). The MRFs can be divided into two groups. The first group includes the MyoD and the Myf-5, both of these genes are expressed during the early stages of myogenesis and are involved in the determination of myoblasts. The second group includes myogenin and Myf-6 which are involved in the latest stage of differentiation and force myoblast to fuse together in order to formed myotubes.

Other regulatory elements are the microRNAs (miRNAs), which belong to a class of small RNAs identified in all organisms (Lee and Ambros, 2001, Lau et al., 2001, Lagos-Quintana et al., 2001). miRNAs play critical role during muscle cell differentiation. In order to identify the role of miRNAs in skeletal muscle differentiation, Dicer was deactivated. Dicer is the enzyme involved in the biogenesis of miRNAs and more specifically is responsible for miRNA maturation. As Dicer was deactivated, maturation of miRNAs was significantly compromised. Through these experiments it was shown that the miRNAs are necessary for myogenesis, due to the fact that through the deletion of Dicer, muscle cell differentiation was suppressed (O'Rourke et al., 2007). MicroRNAs involved in the regulation of skeletal muscle differentiation are divided into two categories. The first category includes the muscle specific miRNAs. These miRNAs were found to

be expressed only in muscle. These miRNAs, miR-1, miR-133a, miR-133b and miR-206, also called myomiRs, were found to be regulated by the MRFs (Chen et al., 2006b). The second category includes non-muscle specific microRNAs also expressed in other tissues (Granjon et al., 2009, Callis et al., 2008). These miRNAs regulate in an orchestrated way muscle specific genes and transcription factors and as a result the process of myogenesis (Sjogren et al., 2014).

Initially, the aim of this study was to identify miRNAs that regulate Twist-1 in muscle cells. Twist-1 is a transcription factor that belongs to the bHLH family and was previously found to act as an inhibitor of muscle cell differentiation during embryonic development (Hebrok et al., 1994, Rohwedel et al., 1995, Spicer et al., 1996, Hjiantoniou et al., 2008, Koutsoulidou et al., 2011a, Liu et al., 2013b). Results from this study show that MyoD induced the expression of miR-206, followed by the post-transcriptional regulation of Twist-1 during skeletal myogenesis.

Twist-1 is an important transcription factor involved in muscle development during early developmental stage. Initially, Twist-1 was discovered in drosophila in 1987 and in mouse and human back to 1991 and 1997 respectively. Interestedly, Twist-1 shows an opposite effect during muscle cell differentiation in mammals compare to drosophila. Twist-1 was found to promote muscle differentiation in Drosophila while was found to be implying the inhibition of myogenin expression and hence of myogenesis in mammals. Moreover, Twist-1 regulates other signaling pathways involved in muscle cell differentiation, as well as the regulation of various miRNAs during the same process.

Bearing in mind the important role of Twist-1 in mesenchymal cell differentiation and its effect in various tissues during development, Twist-1 could be associated with many disorders. One of those disorders is the Saethre-Chotzen syndrome which is an autosomal dominant hereditary craniosynostosis syndrome. This disorder is characterized by premature fusion of coronal suture and limb and craniofacial abnormalities (Reardon and Winter, 1994, Bonaventure and El Ghouzzi, 2003).

In cancer, Twist-1 was shown to be regulated by a series of miRNAs such as miR-543, miR-720 and miR-181a (Bing et al., 2014, Yu et al., 2014, Li et al., 2014b). There is no information however, regarding Twist-1 regulation by miRNAs in muscle. Through bioinformatics analysis of the 3'UTR of Twist-1, one of the four myomiRs, miR-206 was predicted to be a post-transcriptional regulator of Twist-1. miR-206 was shown to target the 3' UTR of Twist-1 mRNA, demonstrating that it is a strong candidate for binding to endogenous human Twist-1 mRNA.

Some targets of miR-206 are well established in myogenesis. Pax3 and Pax7 are two of the experimental verified targets of miR-206. Pax7 and Pax3 prevent the early differentiation of

myoblasts during myogenesis. Overexpression of miR-206 during early stages of skeletal muscle development reduced the expression levels of Pax3 and Pax7 thus promoting muscle cell differentiation (Chen et al., 2010). Connexin43 (Cx43) is another experimentally verified target of miR-206. It as an important gap junction channel which is necessary for the fusion of myoblasts to differentiated mature myotubes. More specifically, Cx43 allows the passage of signalling molecules and metabolites which are necessary for the maturation of myotubes (Kalderon et al., 1977, Anderson et al., 2006). miR-206 was found to inhibit myoblast proliferation and to promote myoblast fusion through the down-regulation Cx43 protein (Anderson et al., 2006). Another experimentally verified target of miR-206 that was identified is Pola1. Pola1 is the largest subunit of DNA polymerase α active during DNA synthesis (Pellegrini, 2012). miR-206 was determined to directly inhibit Pola1 expression during differentiation thus associated with the cell cycle silencing during differentiation (Kim et al., 2006). miR-206 was also found to negatively regulate follistatin-like 1 and utrophin through the binding to the 3' UTR. Both of these were found to be implicated in muscle cell differentiation (Rosenberg et al., 2006).

Apart from scoring highly using target predicting software, miR-206 was chosen because of its importance in promoting muscle cell differentiation but also because it is known that its expression levels are inversely proportional to the expression levels of Twist-1 during differentiation (Koutsoulidou et al., 2011a, Koutsoulidou et al., 2011b). In order to confirm the binding of miR-206 to Twist-1 3' UTR, a series of experiments were performed. Firstly luciferase assays using mutant plasmids for the seed matched region of miR-206 located on the 3'UTR of Twist-1 along with a miR-206 mimic, as well as mutated version of miR-206 and the plasmid containing the Twist-1 3'UTR, confirmed that only co-transfection with the wild type reporter and the miR-206 mimic could reduce luciferase activity. This indicates that there is a specificity of miR-206 on the inhibition of Twist-1 through a specific region on its 3' UTR.

Secondly, overexpression of miR-206 suppressed Twist-1 translation and increased the differentiation capacity in a human myoblast cell line isolated from a 14-week-old foetus which expresses high levels of Twist-1 and has low differentiation capacity. In the other hand, inhibition of miR-206 in a human muscle cell line, isolated from a newborn, which expresses low levels of Twist-1 and has high differentiation capacity, increased the endogenous Twist-1 protein level and decreased the differentiation capacity of the cells. Those results show that miR-206 endogenously act as a post transcriptional regulator of Twist-1.

The luciferase assays as well as the in vitro experiments in muscle cells reveal a new target for miR-206 through which muscle cell differentiation is promoted.

Moreover, through the luciferase assay experiments, it was concluded that miR-206 regulated Twist-1 expression. This was proven by the ability of miR-206 to partially cleave the Luciferase mRNA that contained the Twist-1 3' UTR region.

Since it is known that MyoD promotes the expression of miR-206 directly by binding to its promoter in mouse, we next investigated the possibility that this is also implicated in the regulatory pathway of Twist-1. (Rao et al., 2006, Sassoon et al., 1989, Sassoon, 1993, Berkes and Tapscott, 2005). MyoD was found to regulate many pathways during muscle cell differentiation. Specifically, it was shown to activate p21 expression during differentiation of murine muscle cells (Halevy et al., 1995). MyoD was also found to interact with several proteins such as Cyclin-dependent kinase 4, HDAC1, STAT3 and many other in order to promote the muscle cell differentiation (Zhang et al., 1999a, Zhang et al., 1999b, Puri et al., 2001, Mal et al., 2001, Kataoka et al., 2003, Weintraub et al., 1994, Brook et al., 1992).

MyoD is known to bind to E-box sequences and regulate the expression of muscle-specific genes (Shklover et al., 2007, Weintraub et al., 1994). By scanning the promoter of human miR-206, two highly conserved E-boxes were found to be present. The binding of MyoD on both E-boxes on the promoter of miR-206 was confirmed by chromatin immunoprecipitation and by luciferase assays. This confirmed that binding of MyoD on miR-206 promoter induces miR-206 expression.

Overexpression of MyoD promotes the muscle cell differentiation and miR-206 expression (Rao et al., 2006, Crescenzi et al., 1990). From our results it was found that overexpression of MyoD also reduced the expression level of Twist-1 in 14-week-old foetus myoblasts. Competition experiments with MyoD and Twist-1 overexpression proved this specific association. Overexpression of MyoD induced muscle cell differentiation while the overexpression of both Twist-1 and MyoD brought the levels of cell differentiation almost back to normal suggesting that MyoD regulates muscle cell differentiation through downregulation of Twist-1. Furthermore, in a similar to the above experimental approach, it was shown that MyoD inhibits Twist-1 through miR-206 induction and subsequently promotes muscle cell differentiation. The implication of this new pathway in myogenesis was also demonstrated in primary mouse muscle cells. Primary cells were isolated from a 4-week-old mouse which expresses *Twist-1* gene at low levels (Dupont et al., 2001). Inhibition of MyoD or miR-206 caused an increase in Twist protein levels in primary cells in agreement with human muscle cell lines. Furthermore, inhibition of MyoD or miR-206 reduced the capacity of primary cells to differentiate *in vitro* compared to the control cells. In order to investigate whether MyoD inhibits Twist-1 expression through miR-206, MyoD was inhibited and at the same time miR-206 was overexpressed in primary muscle cells. Furthermore, Twist-1 levels were decreased compared to the cells subjected to inhibition of MyoD or amiR-206 alone. As a result of the decrease of Twist-1 levels, which matched those in control primary cells, differentiation capacity was restored.

Finally, we wanted to investigate whether this novel pathway is implicated in a disease with defective muscle cell differentiation and most specifically in Myotonic Dystrophy type 1 (DM1). DM1 was firstly pointed out independently by Steinert and Batten and Gibb, back in 1909. DM1 is the most common form of muscular dystrophy in adults, and the second most common type of muscular dystrophy after Duchene Muscular Dystrophy. Statistics show that 1 in 8000 individuals globally is affected by DM1 (Harper, 1989). It is an inherited autosomal dominant, neuromuscular disorder. The severity of the disease increases through the generations showing genetic anticipation characteristic. The diseases affect firstly the skeletal muscles through a progressive skeletal muscle weakness, wasting and myotonia. Moreover, DM1 is a multi-systemic disorder with many clinical symptoms including the affecting of the heart and the central nervous system (CNS) (Larkin and Fardaei, 2001). Some patients with DM1 observe congenital, juvenile or adult-onset form of the disease and this in dependable on the age of symptom onset. Congenital form of Myotonic Dystrophy type 1 (CDM1) demonstrates the most severe phenotype of the disorder with 25% neonatal mortality proportion (Harper, 1989). Symptoms of DM1 include myotonia, muscle weakness and progressive muscle atrophy (Meola, 2000). DM1 is caused by an unstable expansion of CTG trinucleotide repeats found in the 3'UTR of *DMPK* gene (Brook et al., 1992, Fu et al., 1992, Mahadevan et al., 1992). C2C12 containing transcripts of mutant *DMPK* 3′-UTR forced into nuclear foci and inhibited C2C12 differentiation (Amack et al., 1999). This differentiation deficiency may represent muscle development abnormalities found in congenital DM1 patients (Sarnat et al., 1976, Farkas-Bargeton et al., 1988). Differentiation inhibition was confirmed in cultured myogenic satellite cells taken from DM1 patients (Furling et al., 2001a, Timchenko et al., 2001b). Mutant *DMPK* 3′-UTR transcripts were shown to disrupt myoblast differentiation by reducing MyoD levels (Amack et al., 2002). Increasing MyoD levels were shown to be sufficient to rescue the differentiation defect in DM1 myoblasts (Amack and Mahadevan, 2004)

Our results showed that congenital DM1 myoblast cells which have a defective differentiation program have low levels of MyoD and miR-206 but high Twist-1 levels. This seems rational based on the properties which characterise these three molecules during muscle cell differentiation. In an attempt to prove that this pathway plays a critical role in the defective differentiation in those cells, we overexpressed MyoD and determined the downstream effects. As expected, MyoD overexpression did correct muscle cell differentiation and moreover both miR-206 and Twist-1 levels were upregulated and downregulated, respectively. Moreover, competition with an antagomiR for miR-206 demonstrated the specificity and effectiveness of the pathway to cell differentiation. Therefore, based on these results, the pathway MyoD/miR-206/Twist-1 is compromised in DM1 cells with defective differentiation program.

In summary, our results suggest a novel mechanism by which MyoD induces muscle cell differentiation through the induction of miR-206 expression and the subsequent inhibition of Twist-1. This mechanism might play a valuable role in myogenesis and also in diseases where differentiation is defective.

In the present study the role of a novel mechanism implicating MyoD/miR-206/Twist-1 in myogenesis has been identified in normal muscle cells and in a disease model with myogenic defects. The discovered pathway can be manipulated and be used as a therapeutic approach to eliminate the myogenic defects observed in muscular dystrophies such as DM1. Transgenic mice for DM1 such as HSA^{LR} with myogenic defect phenotype can be used as animal models (Kanadia et al., 2006). Primary DM1 myoblasts will be isolated from the diseased animal model primarily to verify that the proposed mechanism is suppressed in primary DM1 myoblasts. As therapeutic approach, the transgenic mice will be next manipulated in order to induce the altered MyoD/miR-206/Twist-1 pathway and overtake the myogenic defect, either by overexpressing MyoD or miR-206. MyoD overexpression can be achieved through the introduction of a virus expressing the MyoD transcription factor in the muscle of the transgenic mouse model. This method may not be muscle specific targeted and therefore MyoD overexpression may affect other tissues. Furthermore, MyoD ectopic expression may cause toxicity for muscle and other tissues, Overexpression of miR-206 can be performed through direct injection of miR-206 mimic in muscle tissue. To achieve the necessary specificity and the targeted injection of the mimic to muscle, a muscle specific signalling vehicle attach on the mimic such as aptamer can be used. Aptamers are oligonucleotides that bind to a specific target molecule in order to drive them direct to a specific type of tissue (Yan and Levy, 2009). MiRNA mimics are very small and therefore it is easy to be manipulated and used in combination with an aptamer. miR-206 can be therefore used attached to an aptamer and directly injected to the muscle without affecting other tissues. Twist expression will therefore be downregulated in DM1 myoblasts and the differentiation will be induced thus overtaking the myogenic defects observed in DM1.

5

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APPENDIX

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RESEARCH ARTICLE

MyoD transcription factor induces myogenesis by inhibiting Twist-1 through miR-206

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ABSTRACT

Twist-1 is mostly expressed during development and has been previously shown to control myogenesis. Because its regulation in muscle has not been fully exploited, the aim of this project was to identify micro (mi)RNAs in muscle that regulate Twist-1. miR-206, one of the most important muscle-specific miRNAs (myomiRs), was identified as a possible regulator of *Twist-1* mRNA. Luciferase assays and transfections in human foetal myoblasts showed that Twist-1 is a direct target of miR-206 and that through this pathway muscle cell differentiation is promoted. We next investigated whether MyoD, a major myogenic transcription factor, regulates Twist-1 because it is known that MyoD induces expression of the *miR-206* gene. We found that forced MyoD expression induced miR-206 upregulation and Twist-1 downregulation through binding to the *miR-206* promoter, followed by increased muscle cell differentiation. Finally, experiments were performed in muscle cells from subjects with congenital myotonic dystrophy type 1, in which myoblasts fail to differentiate into myotubes. MyoD overexpression inhibited Twist-1 through miR-206 induction, which was followed by an increase in muscle cell differentiation. These results reveal a previously unidentified mechanism of myogenesis that might also play an important role in muscle disease.

KEY WORDS: Twist-1, MiR-206, MyoD, Myogenesis

INTRODUCTION

Skeletal muscle tissue is derived from the differentiation of myoblasts into myotubes through a process known as myogenesis. Myogenesis is a complex and tightly regulated procedure. During myogenesis, the myoblasts exit the cell cycle through the orchestrated expression of specific genes that are important for muscle cell differentiation, and this is followed by their fusion to form multi-nuclei fibres known as myotubes (Sabourin and Rudnicki, 2000). Myogenesis occurs during different stages of human development. Specifically, myogenesis takes place during embryogenesis in order to form muscle tissue and occurs in adults to replace damaged or lost muscle (Wang and Conboy, 2010). During embryogenesis, the myogenic progenitor cells arise from the somites, which are sequentially combined blocks of mesoderm that form along the anterior–posterior axis of the developing embryo (Parker et al., 2003). Somites differentiate along the dorsal–ventral axis to give rise to the dorsally located epithelial dermomyotome and the ventrally located mesenchymal sclerotome (Parker et al., 2003). The dermomyotome gives rise to dermis and musculature, whereas the sclerotome forms the bone and cartilage. Myogenic precursors restrained in the epithelium of the dermomyotome express Pax3, Pax7 and low levels of the myogenic determination factor Myf-5. During the late stages of embryogenesis, a specific population of myogenic stem cells, called satellite cells, arise in order to provide most of the myonuclei to adult muscles during the postnatal growth of muscle tissue (Parker et al., 2003). At the molecular level, myogenesis is regulated by the basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs) Myf-5, MyoD, Myf-6 and myogenin. Genetic studies in mice have demonstrated that the genes encoding for the four MRFs are expressed in pairs. Myf-5 and MyoD are the first MRFs required for the commitment of progenitor cells to the myogenic lineage. Myf-6 and myogenin are the second group of MRFs, which are responsible for the downstream regulation of muscle differentiation (Relaix, 2006). MyoD is also expressed in proliferating myoblasts throughout the cell cycle in the absence of differentiation (Rudnicki et al., 1993).

MicroRNAs (miRNAs) are small non-coding regulatory RNA molecules, and their function is to negatively regulate gene expression at the post-transcriptional level (Bartel, 2004). miRNAs are incorporated with the RNA-induced silencing complex (RISC) and bind to the 3′ untranslated region (3′-UTR) of its target mRNA, causing either inhibition of protein translation or mRNA cleavage (MacFarlane and Murphy, 2010).

Many miRNAs are found to be expressed in muscle tissue. The most studied miRNAs in muscle are miR-1, miR-133a, miR-133b and miR-206, which are also called myomiRs owing to their muscle-specific expression. miR-1 and miR-133a are highly expressed in both skeletal and cardiac muscle, whereas miR-206 and miR-133b are specifically expressed in skeletal muscle (Chen et al., 2006; Kim et al., 2006). The expression of the four myomiRs is induced during muscle cell differentiation, indicating that they have a crucial role in regulating the process (Chen et al., 2006; Kim et al., 2006). Their expression is regulated by the MRFs MyoD and myogenin, as well as serum response factor (SRF) and myocyte enhancer factor 2 (MEF2) (Chen et al., 2006; Rao et al., 2006; Liu et al., 2007). MyomiR levels are found to be increased during the late stages of human foetal muscle development, and increases in their expression levels are proportional to the capacity of myoblasts to form myotubes (Koutsoulidou et al., 2011b).

An important molecule involved in myogenesis is Twist-1, which belongs to the family of bHLH transcription factors. Twist was initially identified in *Drosophila* (Thisse et al., 1987). Twist isoforms have been identified in other species, including human and mouse (Wolf et al., 1991; Wang et al., 1997). Twist-1 forms either homodimers or heterodimers with different bHLH protein partners that bind to specific DNA sequences called E-boxes (CANNTG) located in the promoters of target genes (Castanon et al., 2001).

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These complexes can act as repressors or activators of the target genes (O'Rourke and Tam, 2002). Twist-1 plays a crucial role during the development of the embryo. It is expressed during embryonic development, and it has been found to be involved in developmental processes such as myogenesis, neurogenesis, cardiogenesis, cranial tube morphogenesis and mesoderm formation (Miraoui and Marie, 2010). Twist-1 has also been found to be involved in the process of epithelial-to-mesenchymal transition, which plays an essential role in cancer metastasis (Yang et al., 2004). Regarding myogenesis, in *Drosophila*, Twist has been shown to promote myogenesis. However, in mouse C2C12 myoblasts and in human embryonic stem cell (HESC)-derived embryoid bodies, Twist-1 is found to inhibit muscle cell differentiation (Hebrok et al., 1994; Rohwedel et al., 1995; Cao et al., 2008; Koutsoulidou et al., 2011a). Interestingly, overexpression of Twist-1 reverses the process of muscle cell differentiation (Hjiantoniou et al., 2008; Mastroyiannopoulos et al., 2013). *Twist-1* is transcribed primarily in the early somites, and its expression is downregulated when the myogenic factors MyoD and Myf-5 are upregulated in order to localise the newly formed somites to diverse compartments of the embryo (Sassoon, 1993; Hebrok et al., 1994; Castanon and Baylies, 2002). The downregulation of Twist-1 when the myogenic factors are expressed in early stage of development suggests that Twist-1 inhibits myogenesis and that it is involved in a process that prevents premature muscle cell differentiation (Castanon and Baylies, 2002). Overexpression of Twist-1 in the myogenic mouse cell line C2C12 reversibly represses muscle differentiation with an associated decrease in transcript levels of Myf-5 and myogenin (Hebrok et al., 1994). Moreover, it has been shown that overexpression of Twist-1 in the C2C12 cell line can reverse muscle cell differentiation in the presence of growth factors by binding and downregulating myogenin, as well as reverse cellular morphology in the absence of growth factors (Mastroyiannopoulos et al., 2013). Human Twist-1 is expressed endogenously at high levels in human foetal myoblasts, and its expression levels decrease during the late stages of development (Koutsoulidou et al., 2011a). The differentiation capacity of the myoblast increases during development. This shows that there is an inversely proportional relationship between the differentiation capacity of myoblasts and expression of Twist-1, and this indicates that Twist-1 is involved in the regulation of muscle development (Koutsoulidou et al., 2011a).

To date, not much information exists regarding the regulation of Twist-1 expression in muscle. In cancer, Twist-1 has been shown to be regulated by a series of miRNAs, such as miR-543, miR-720 and miR-181a (Liu et al., 2013; Bing et al., 2014; Li et al., 2014). There is no information, however, regarding Twist-1 regulation through miRNAs in muscle. Our investigation focused on the identification of miRNAs that could bind to and regulate Twist-1 expression during myogenesis. Our results show that miR-206 is a negative regulator of Twist-1 and promotes muscle cell differentiation. Moreover, we reveal that MyoD induces muscle cell differentiation by inhibiting expression of Twist-1 through miR-206, both in normal and myotonic dystrophy type 1 (DM1) muscle cells.

RESULTS

miR-206 represses *Twist-1* through its 3′-UTR

The initial aim of this work was to identify miRNAs that regulate the expression of the *Twist-1* gene. The 3′-UTR of human *Twist-1* was screened for potential miRNA-binding sites using miRanda software. The criteria that were used in order to choose the candidate miRNA were (1) the conservation of the miRNA-binding sites between species, (2) specific miRNA expression in muscle tissue and (3) opposing expression profiles of specific miRNAs and the Twist-1 protein. Among the possible candidate miRNAs, one of the known myomiRs, miR-206, was identified. The human *Twist-1* transcript was predicted to contain one canonical miRNA response element (MRE) for miR-206 (Fig. 1A). The binding site of miR-206 to the human *Twist-1* 3′-UTR was more conserved between closely related species compared to divergent species, indicating possible evolutionary importance (Fig. 1B). To investigate whether the candidate miRNA binds to the 3′-UTR of human *Twist-1*, the full human *Twist-1* 3′-UTR was cloned downstream of the luciferase gene and assayed in HeLa cells (Fig. 1C). The luciferase assay indicated that miR-206 repressed luciferase activity efficiently (50%) (Fig. 1D). Introduction of mutations at the Twist-1-binding site of miR-206, and transfections with a miR-206 mutant variant, did not alter the luciferase activity levels, indicating miRNA–mRNA binding specificity (Fig. 1D).

miR-206 regulates Twist-1 expression and muscle cell differentiation

It has been previously shown that overexpression of Twist-1 results in the inhibition of muscle cell differentiation in adult cells (Hebrok et al., 1994; Rohwedel et al., 1995). Before proceeding to evaluating Twist-1 as a possible target of miR-206, we overexpressed Twist-1 through an adenoviral vector in human myoblast cells isolated from a newborn; these cells have the ability to differentiate and to express low levels of Twist-1. High levels of exogenous Twist-1 resulted in a decrease in the differentiation capacity of cells when stained for myosin heavy chain (MyHC), a late muscle cell differentiation marker (supplementary material Fig. S1A). Moreover, reducing the endogenous levels of Twist-1 caused an increase in the differentiation capacity of the cells when stained for MyHC (supplementary material Fig. S1B).

As a next step, human myoblasts isolated from a 14-week-old foetus, which express high levels of Twist-1 and have low differentiation capacity, were used to determine the cellular effects of miR-206 acting on Twist-1 (Koutsoulidou et al., 2011a). The levels of miR-206 were increased following overexpression of miR-206 (supplementary material Fig. S2A) and, as a result, the levels of Twist-1 decreased (Fig. 2A, supplementary material Fig. S2B). The protein levels of muscle differentiation markers – troponin and muscle actin – were also increased in myoblasts that overexpressed miR-206 compared to those of control cells (Fig. 2A). The same cells that had been transfected with miR-206 were then induced to differentiate to form myotubes. Myoblasts that overexpressed miR-206 showed an increased capacity to differentiate *in vitro* compared to control cells (Fig. 2B). This was also shown by the increased fusion index (Fig. 2C).

Human muscle myoblasts isolated from a newborn, which express low levels of Twist-1 and have a high differentiation capacity, were then used for transfections with a miR-206 inhibitor (amiR-206) (supplementary material Fig. S2C). Following inhibition of miR-206, the levels of human Twist-1 protein increased, whereas the levels of the markers of differentiation, muscle actin and troponin, were decreased (Fig. 3A, supplementary material Fig. S2D). The differentiation capacity of the cells that had been transfected with amiR-206 was decreased compared to control cells (Fig. 3B,C).

These results support the findings of earlier experiments that Twist-1 is a target for miR-206 in order to induce muscle cell differentiation.

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

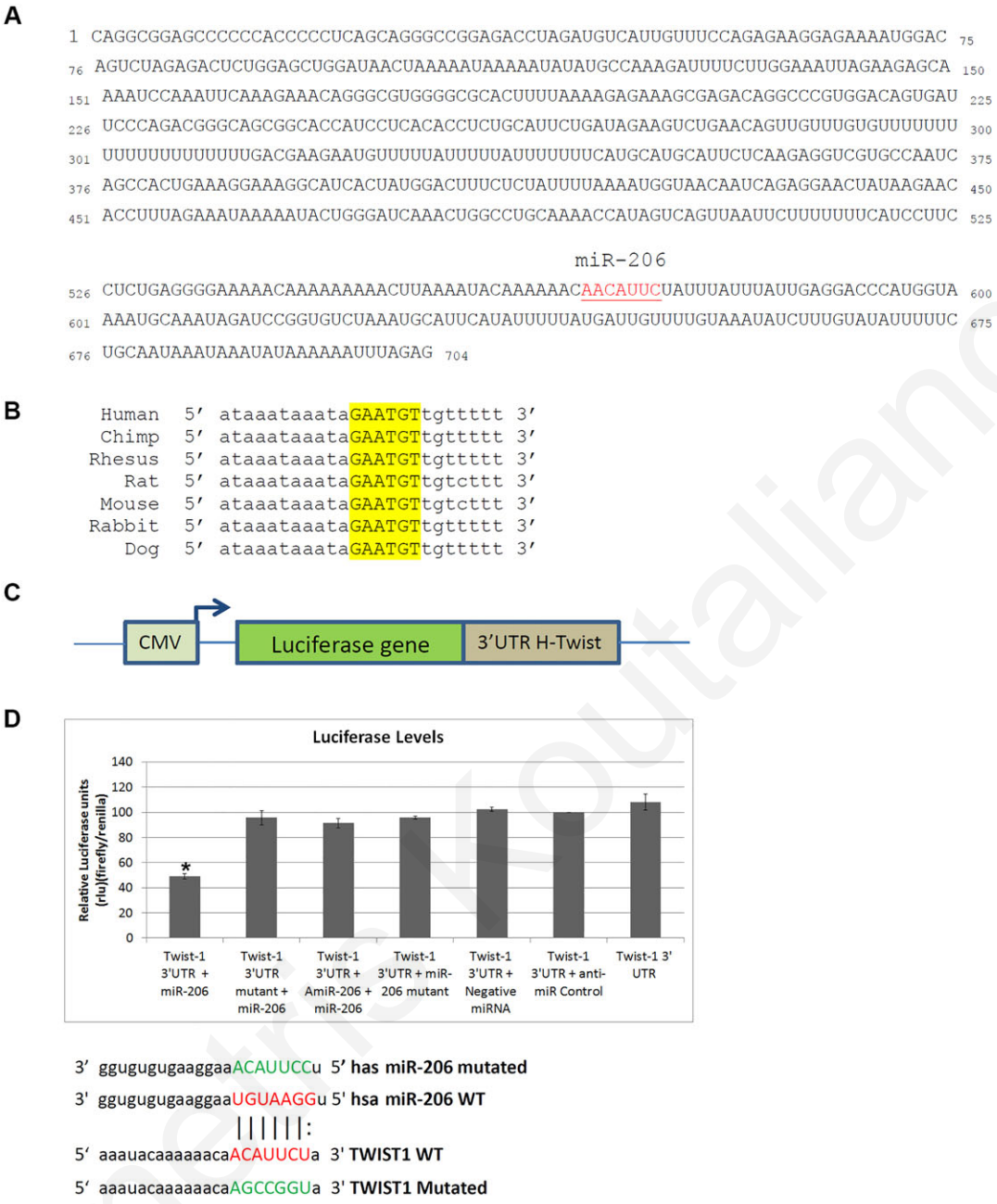

Fig. 1. Identification of miRNAs binding to the *Twist-1* 3′-UTR. (A) Map of the human *Twist-1* gene locus indicating the length of the 3′-UTR and putative binding position of the miR-206 (red). (B) Conservation of the miR-206 putative binding site among species, as shown by the sequence homology (highlighted). (C) A plasmid construct of human *Twist-1* 3′-UTR downstream of the luciferase gene under the control of a CMV promoter was generated to determine the effect of miR-206 on the *Twist-1* 3′-UTR. (D) Transfections of HeLa cells with a miR-206 mimic and the plasmid expressing the luciferase gene with the *Twist-1* 3′-UTR cDNA (Twist-1 3′-UTR) caused a significant decrease in luciferase and human Twist-1 3′-UTR activity compared to cells transfected with the Twist-1 3′-UTR, or transfected with the Twist-1 3′-UTR and a non-specific miRNA (negative miRNA). Introduction of mutations in both miR-206 (miR-206 mutant) and 3′-UTR binding site (Twist-1 3′-UTR mutant) did not decrease luciferase activity, indicating specificity of miR-206 for the *Twist-1* 3′-UTR. Similarly, addition of miR-206 antagomir (amiR-206) to cells that had been co-transfected with the miR-206 mimic reversed the decrease observed with the mimic alone. Schematic underneath shows miR-206 and Twist-1 base interaction (red) and their mutant version (green). Experiments were performed in triplicate, and the data represent the mean±s.d. *P<0.01 compared with controls using Student's t-test. WT, wild type.

MyoD promotes muscle cell differentiation through upregulation of miR-206 and downregulation of Twist-1

One of the main positive regulators of miR-206 is the transcription factor MyoD (Rosenberg et al., 2006). MyoD was found to regulate miR-206 expression through direct binding to its promoter. In an attempt to further elucidate the mechanism of Twist-mediated regulation of myogenesis, experiments were performed in order to investigate the possible implication of MyoD. As a first step, 14-week-old foetus myoblasts were transduced with an adenovirus expressing the MyoD transcription factor (AdM) (supplementary material Fig. S3A). MyoD overexpression increased the capacity of the cells to differentiate, as reflected by the myogenesis markers (muscle actin and troponin). At the same time, MyoD overexpression caused an increase in the levels of miR-206 (supplementary material Fig. S3B).

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

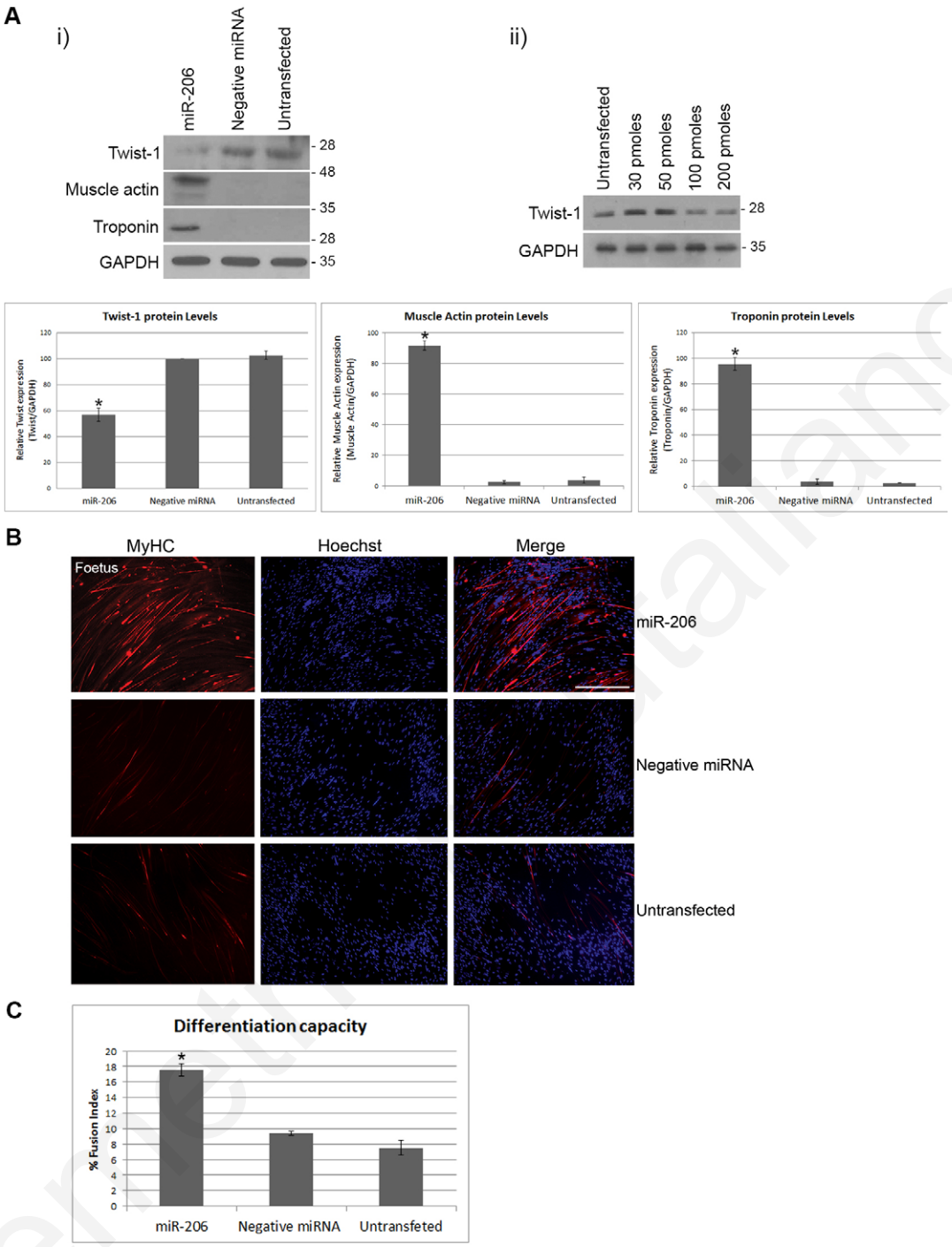

Fig. 2. Overexpression of miR-206 downregulates Twist-1 expression and promotes muscle cell differentiation. (A) (i) Transfection with a miR-206 mimic resulted in a decrease in the endogenous Twist-1 protein levels by western blotting, as compared to cells transfected with a scrambled miRNA (negative miRNA), or untransfected cells. In parallel, the miR-206 mimic caused an increase in the myogenic markers muscle actin and troponin in 14-week-old foetus human myoblasts. Graphs show quantification of the western blots. (ii) Confirmation of optimum miR-206 amount used in downregulating Twist-1 expression. (B) 14-week-old foetus myoblasts were transfected with a miR-206 mimic and left to differentiate for 6 days. Myotubes were fixed and stained for the late differentiation marker myosin heavy chain (MyHC, red) and nucleus-directed Hoechst 33342 (blue). The miR-206 mimic increased muscle cell differentiation compared to transfection with the negative control or to untransfected cells. Scale bar: 200 µm. (C) Calculation of the fusion index demonstrated the increase in muscle cell differentiation in miR-206-mimic-transfected cells compared to control cells. All experiments were performed in triplicate, and the data represent the mean±s.d. *P<0.01 compared with controls using Student's t-test.

As a next step, the possibility of MyoD directly binding to the miR-206 promoter and its subsequent regulation of expression in 14-week-old human myoblasts were investigated. The *miR-206* promoter contains two E-boxes that are highly conserved among species (Fig. 4A,B). A chromatin immunoprecipitation assay showed that endogenous MyoD binds to both E-boxes located on the *miR-206* promoter in 14-week-old human myoblasts (Fig. 4C). Luciferase assays were next performed in order to investigate the

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

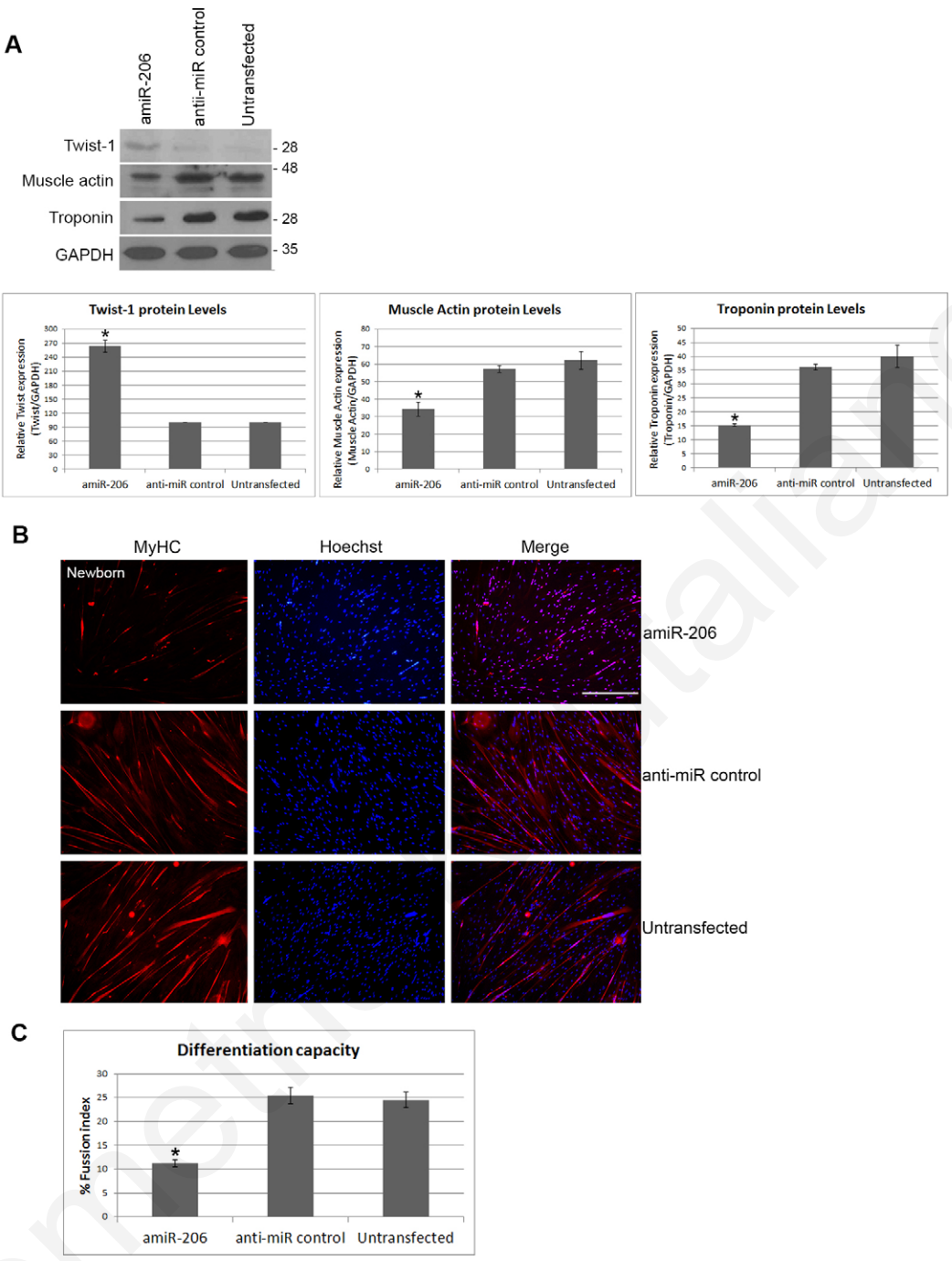

Fig. 3. Downregulation of miR-206 upregulates Twist-1 expression and inhibits muscle cell differentiation. (A) miR-206 antagomiR (amiR-206) transfection resulted in an increase in the endogenous Twist-1 protein levels by western blotting, as compared to cells transfected with scrambled anti-miRNA (anti-miR control) and untransfected cells. This increase was accompanied by a decrease in the myogenic markers muscle actin and troponin in newborn human myoblasts. Graphs show quantification of the western blots. (B) Newborn myoblasts were transfected with miR-206 antagomiR (amiR-206) and left to differentiate for 6 days. Myotubes were fixed and stained for MyHC (red) and nucleus-directed Hoechst 33342 (blue). miR-206 antagomiR transfection caused a decrease in muscle cell differentiation compared to transfection with the anti-miR control or to untransfected cells. Scale bar: 200 µm. (C) Calculation of the fusion index demonstrated the decrease in muscle cell differentiation in amiR-206-transfected cells compared to control cells. Experiments were performed in triplicate, and the data represent the mean±s.d. **P*<0.01 compared with controls using Student's *t*-test.

effect of MyoD on miR-206 expression. Overexpression of MyoD in HeLa cells that had been transfected with a plasmid containing the promoter of *miR-206* upstream of a luciferase gene showed that MyoD induces the expression of miR-206 (Fig. 4D,E). The direct binding of MyoD onto the human *miR-206* promoter was further investigated using mutagenesis experiments. Single mutations were introduced into each of the E-boxes located on the promoter of *miR-206*. MyoD overexpression in HeLa cells that had been transfected with the mutated plasmids showed a dramatic reduction in luciferase activity. Introduction of mutations into both E-boxes located on the promoter of human *miR-206* and overexpression of MyoD supressed luciferase activity (Fig. 4E).

The effect of MyoD overexpression on Twist-1 expression was next investigated. The levels of human Twist-1 protein were found to be

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

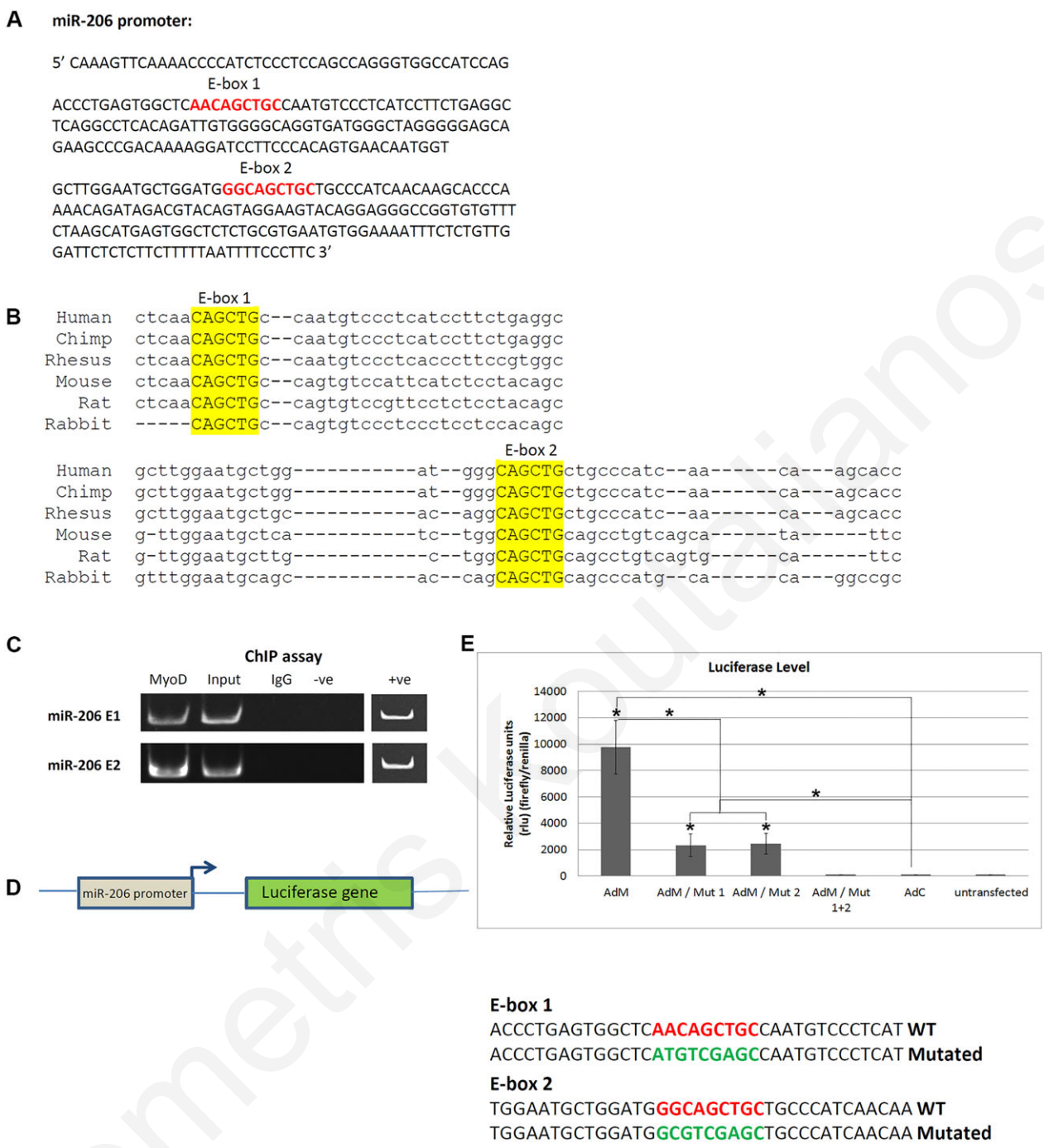

Fig. 4. MyoD binds to the *miR-206* promoter and induces its expression. (A) Map of the human *miR-206* promoter and putative MyoD-binding E-boxes (red). (B) Conservation among species of E-box 1 and E-box 2 based on sequence identity. (C) A chromatin immunoprecipitation (ChIP) assay revealed that, following MyoD overexpression, MyoD bound to both E-boxes on the promoter of *miR-206*. For positive control (+ve), unconjugated polyclonal antibody specific to human and mouse histone H3, trimethylated at lysine 9 (K9me3) (H3-K9Me3) was used. For negative control (–ve), IgG antibody was used. (D) A plasmid construct of the human *miR-206* promoter upstream of the luciferase gene was used to evaluate binding of MyoD to the *miR-206* promoter. (E) HeLa cells were transfected with an adenovirus expressing MyoD (AdM) and showed an increase in luciferase activity compared to cells transfected with a control adenoviral vector (AdC), or untransfected cells. Introduction of mutations into either E-box showed a large reduction in luciferase activity, whereas introduction of both mutations resulted in no luciferase activity, similar to that seen in AdC-transfected or untransfected cells. Experiments were performed in triplicate, and the data represent the mean± s.d. *P<0.01 using Student's t-test for the indicated comparisons. Mut 1 and Mut 2, mutation of E-box 1 and E-box 2, respectively; WT, wild type.

significantly decreased in muscle cells overexpressing MyoD compared to control muscle cells (Fig. 5A). In order to investigate whether MyoD induces muscle cell differentiation through inhibition of Twist-1, we overexpressed MyoD and at the same time overexpressed Twist-1 using adenoviruses in 14-week-old human myoblasts. By overexpressing both MyoD and Twist-1, the inhibition of Twist-1 caused by the overexpression of MyoD was abolished (Fig. 5B). Following the co-expression of the two transcription factors MyoD and Twist-1, the cellular differentiation capacity was reduced compared to that upon overexpression of MyoD only. Specifically, the double-transduced cells had a similar capacity to differentiate to that of the control cells (Fig. 5C,D).

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

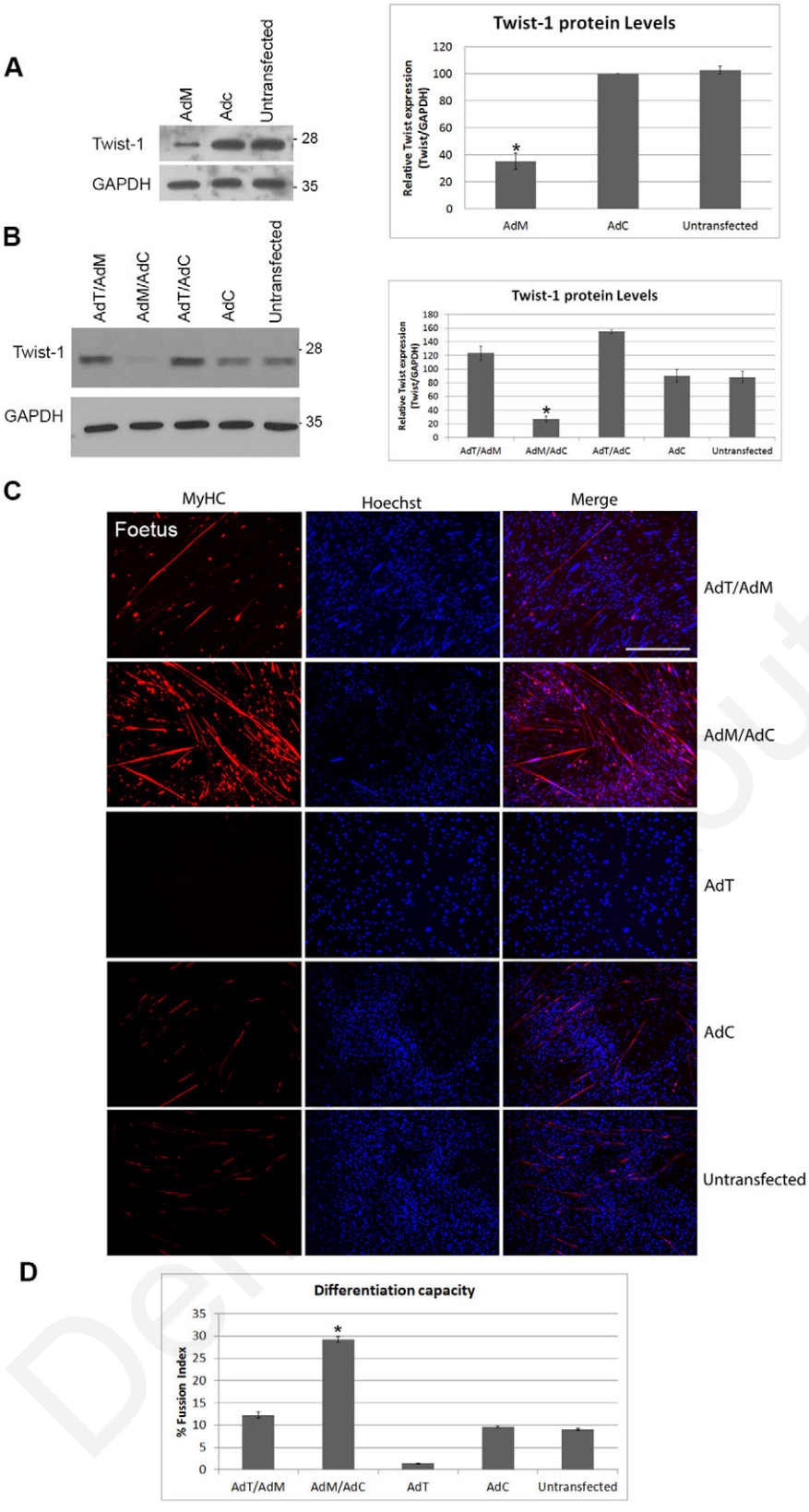

Fig. 5. MyoD regulates muscle cell differentiation through inhibition of Twist-1. (A) Overexpression of MyoD with an adenoviral vector (AdM) caused a decrease in the endogenous Twist-1 protein levels by western blotting, as compared to the control adenovirus (Adc) and untransfected cells in the 14-week-old foetus human myoblasts. Graph shows quantification of the blot. (B) The indicated competition co-transductions of MyoD (AdM) and Twist-1 (AdT) reversed the decrease in endogenous Twist-1 levels, thus proving the effect of MyoD on Twist-1. Overexpression of Twist-1 increased the endogenous levels of Twist-1. (C) 14-week-old foetus myoblasts were co-transduced with AdM and AdT (AdT/AdM), or AdM (AdM/AdC), AdT and AdC alone, and left to differentiate for 6 days. Myotubes were fixed and stained for MyHC (red) and nucleus-directed Hoechst 33342 (blue). MyoD induced differentiation, whereas combination of overexpression of MyoD and Twist-1 reversed this increase and resembled untransduced cells or cells transduced with control adenovirus (AdC). Finally, AdT alone inhibited muscle cell differentiation. Scale bar: 200 µm. (D) The above results were confirmed by the calculation of fusion index. All experiments were performed in triplicate, and the data represent the mean±s.d. **P*<0.01 compared with controls using Student's *t*-test.

Based on our results, MyoD promotes muscle cell differentiation, induces the expression of miR-206 and inhibits Twist-1 expression. In order to investigate whether MyoD inhibits Twist-1 expression through miR-206 induction, MyoD was overexpressed in 14-week-old human myoblasts, and miR-206 was inhibited through expression of antagomiR. Analyses of the proteins showed that the levels of Twist-1 were increased compared to that in the cells that overexpressed either MyoD or miR-206 (Fig. 6A). Furthermore, the

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

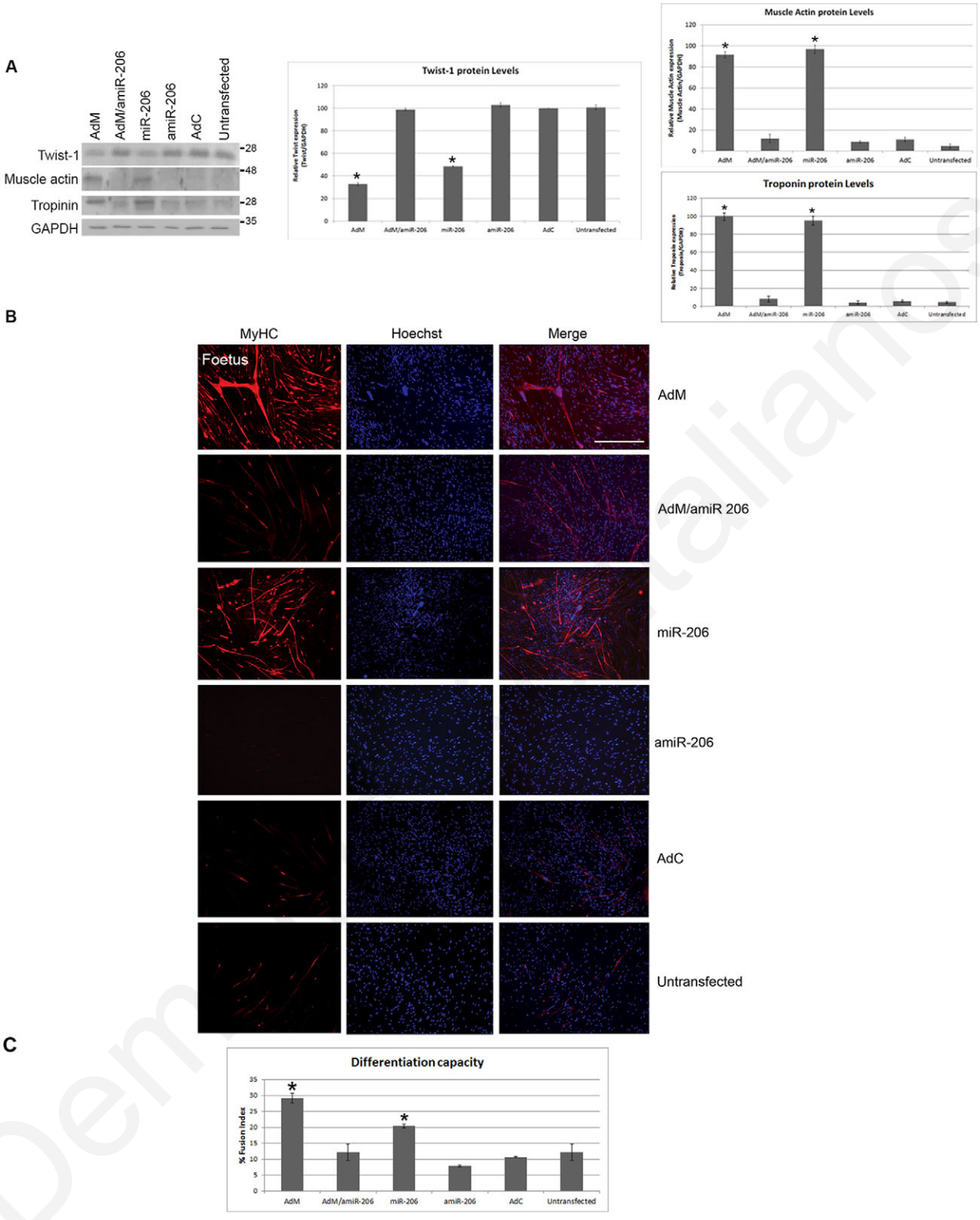

Fig. 6. MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206. (A) Competition co-transduction with MyoD (AdM) and miR-206 antagomiR (amiR-206) reversed the decrease in endogenous Twist-1 levels associated with overexpression of MyoD. Overexpression of amiR-206 alone had no effect on Twist-1 levels, presumably because of the low miR-206 endogenous levels. The effect of AdM and miR-206 on the induction of differentiation was shown by the increased levels of the myogenesis markers muscle actin and troponin. Graphs show quantification of the protein levels. (B) 14-week-old foetus myoblasts were co-transduced with AdM and amiR-206 (AdM/amiR 206), or miR-206 mimic (miR-206) and AdC alone, and left to differentiate for 6 days. Myotubes were fixed and stained for MyHC (red) and nucleus-directed Hoechst 33342 (blue). Combination of overexpression of MyoD and amiR-206 reversed the increase in muscle cells differentiation observed with AdM or miR-206 alone, and resulted in the cells behaving as untransduced cells, as cells transfected with amiR-206 alone or as cells transduced with control adenovirus (AdC). Scale bar: 200 µm. (C) The above results were confirmed through the calculation of the fusion index. All experiments were performed in triplicate, and the data represent the mean±s.d. *P<0.01 compared with controls using Student's *t*-test.

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

levels of Twist-1 protein in double-transfected cells were similar to those in the control cells (Fig. 6A). The levels of the myogenesis markers muscle actin and troponin were elevated when either MyoD or miR-206 was overexpressed (Fig. 6A). Moreover, double-transfected cells showed a decrease in their capacity to differentiate *in vitro* compared to that upon the overexpression of either MyoD or miR-206 alone, and a similar differentiation capacity to the control cells (Fig. 6B). The differences in muscle cell differentiation were also confirmed by fusion index analysis (Fig. 6C).

These results indicate, therefore, that MyoD can induce muscle differentiation by inhibiting Twist-1 through miR-206.

To further evaluate these findings, experiments were performed in primary mouse cells. This is possible because (1) the binding site of miR-206 on the *Twist-1* 3′-UTR is conserved between human and mouse (Fig. 1B), (2) the expression profile of miR-206 in mouse is inversely proportional to the expression of Twist-1 (Kim et al., 2006; Koutsoulidou et al., 2011b), (3) Twist-1 overexpression in mouse has the same negative effect on muscle cell differentiation (Hebrok et al., 1994; Rohwedel et al., 1995) and (4) the E-boxes bound by MyoD and found in the promoter region of *miR-206* are conserved between mouse and human (Fig. 4B). Primary cells were isolated from a 4-week-old mouse; these express the *Twist-1* gene at a low level (Dupont et al., 2001). Inhibition of MyoD or miR-206 caused an increase in Twist-1 protein levels in primary cells, in agreement with the results from human muscle cells (Fig. 7A). Furthermore, inhibition of MyoD or miR-206 reduced the capacity of primary cells to differentiate *in vitro* compared to that of the control cells (Fig. 7B,C). In order to investigate whether MyoD inhibits Twist-1 expression through miR-206, MyoD was inhibited and, at the same time, miR-206 was overexpressed in primary muscle cells. As a result, Twist-1 levels were decreased compared to those in the cells that had been subjected to inhibition of MyoD or amiR-206 alone (Fig. 7A). Furthermore, Twist-1 levels were similar to those in the control primary cells, and the differentiation capacity of the cells was restored (Fig. 7B,C).

Therefore, these results support the findings of earlier experiments performed in cultured cells and suggest that, through this newly identified mechanism, MyoD induces myogenesis by inhibiting Twist-1 through miR-206 (Fig. 7D).

The MyoD–miR-206–Twist-1 mechanism is involved in myotonic dystrophy type 1 cells, which exhibit defective differentiation

The results of this study so far have shown that MyoD might exert its positive effect on myogenesis through an additional pathway, and more specifically through miRNA-mediated inhibition of Twist-1. The final step of this project aimed to investigate the role of this newly identified pathway in a disease setting. DM1 is an inherited neuromuscular disease that usually manifests during adulthood and is caused by a CTG expansion in the 3′-UTR of the dystrophia myotonica protein kinase (*DMPK*) gene (Miller et al., 2000). A rarer and more severe congenital form of DM1 that is associated with a large CTG expansion also exists (Meola, 2013), and defects in the myogenic differentiation program have been reported in numerous DM1 cases (Amack and Mahadevan, 2004).

DM1 human myoblast cells, which have a low differentiation capacity, were used to evaluate the role of the newly identified pathway in the defective differentiation program of the disease. Three different DM1 muscle cell cultures derived from congenital DM1 individuals were examined. In all three cases, MyoD protein levels were inhibited compared to those in healthy cells (Fig. 8A). Twist-1 was found to have an inversely proportional expression profile compared to that of MyoD during differentiation both in DM1 and the healthy control muscle cells (Fig. 8A, supplementary material Fig. S4). Furthermore, the miR-206 levels were increased during the differentiation of healthy cells but inhibited in DM1 cells (Fig. 8A). Protein analysis showed that the levels of Twist-1 protein were increased in the DM1 cells that had been co-transfected with MyoD and amiR-206 compared to those in the cells that overexpressed either MyoD or miR-206 alone (Fig. 8B). Furthermore, the levels of Twist-1 protein in the double-transfected cells were similar to those in the control cells (Fig. 8B). Moreover, overexpression of MyoD together with amiR-206 resulted in a decrease in the capacity of cells to differentiate *in vitro* compared to expression of either MyoD or miR-206 alone. Co-transfection of MyoD and amiR-206 resulted in a similar differentiation capacity to that of control cells (Fig. 8C,D).

Therefore, these results further support the notion that this new myogenic pathway involving MyoD, Twist-1 and miR-206 might be used to overcome the defective differentiation program in DM1 in order to promote the formation of mature muscle cells.

DISCUSSION

The aim of this study was to identify miRNAs that regulate Twist-1 in muscle cells. Twist-1 is a transcription factor that belongs to the bHLH family, and it has been previously found to act as an inhibitor of muscle cell differentiation during embryonic development (Hebrok et al., 1994; Rohwedel et al., 1995; Spicer et al., 1996; Hjiantoniou et al., 2008; Koutsoulidou et al., 2011a). Results from this study show that MyoD induces the expression of miR-206, which post-transcriptionally regulates Twist-1 during skeletal myogenesis.

In cancer, Twist-1 has been shown to be regulated by a series of miRNAs, such as miR-543, miR-720 and miR-181a (Liu et al., 2013; Bing et al., 2014; Li et al., 2014). There is no information, however, regarding Twist-1 regulation through miRNAs in muscle. By using bioinformatics analysis of the 3′-UTR of Twist-1, one of the four myomiRs, miR-206, was predicted to be a post-transcriptional regulator of Twist-1. miR-206 was shown to target the 3′-UTR of *Twist-1* mRNA, demonstrating that it is a strong candidate for binding to endogenous human *Twist-1* mRNA.

Some targets of miR-206 are well established in myogenesis. Pax3 and Pax7 are two of the experimentally verified targets of miR-206. Pax7 and Pax3 prevent the early differentiation of myoblasts during myogenesis. Overexpression of miR-206 during the early stages of skeletal muscle development reduce the expression levels of Pax3 and Pax7, thus promoting muscle cell differentiation (Chen et al., 2010). Connexin 43 (Cx43) is another experimentally verified target of miR-206. It is an important gap junction channel that is necessary for the fusion of myoblasts to form differentiated mature myotubes. More specifically, Cx43 allows the passage of signalling molecules and metabolites that are necessary for the maturation of the myotubes (Kalderon et al., 1977; Anderson et al., 2006). miR-206 inhibits myoblast proliferation and promotes myoblast fusion through the downregulation of the Cx43 protein (Anderson et al., 2006). Another experimentally verified target of miR-206 that has been identified is Pola1. Pola1 is the largest subunit of DNA polymerase α active during DNA synthesis (Pellegrini, 2012). miR-206 has been shown to directly inhibit Pola1 expression during differentiation, and is thus associated with inhibition of the cell cycle during differentiation (Kim et al., 2006). miR-206 also negatively regulates follistatin-like 1 and utrophin through binding to the 3′-

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

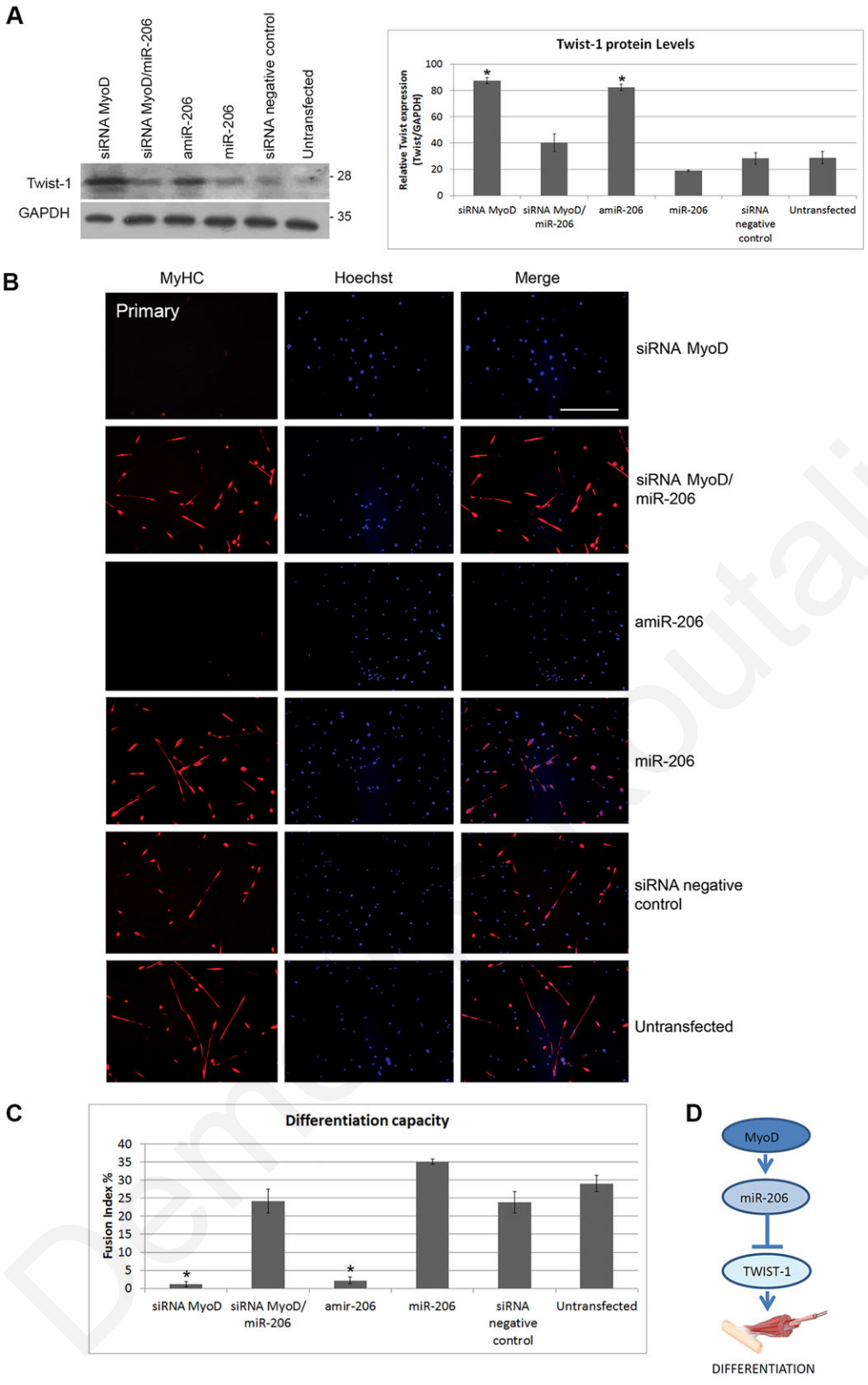

Fig. 7. MyoD regulates muscle cell differentiation through downregulation of Twist-1 by miR-206 in primary mouse myoblasts. (A) Transfections of 4-week-old mouse primary myoblasts with an siRNA against MyoD (siRNA MyoD), or amir-206 resulted in an increase in the endogenous Twist-1 protein levels by western blotting, as compared to the scrambled siRNA (siRNA negative control), mir-206 and to untransfected primary mouse myoblasts. By contrast, co-transfection of the siRNA against MyoD with the miR-206 mimic reversed the increase of Twist-1 back to normal levels. Graphs show quantification of the protein levels. (B) Primary mouse myoblasts were co-transfected with siRNA against MyoD, and the miR-206 mimic (siRNA MyoD/ miR-206), or with siRNA against MyoD (siRNA MyoD) or amiR-206 (amiR-206) alone, and left to differentiate for 3 days. Myotubes were fixed and stained for MyHC and nucleus-directed Hoechst 33342. siRNA against MyoD and amiR-206 transfections inhibited muscle cell differentiation, whereas co-transfections with siRNA against MyoD, and amiR-206 showed almost similar effects on differentiation compared to control miRNA transfection or to untransfected cells. Cells transfected with miR-206 alone showed a slight increase in differentiation capacity. Scale bar: 200 µm. (C) Calculation of the fusion index confirmed the above results. (D) Proposed novel myogenesis pathway implicating MyoD, miR-206 and Twist-1. All experiments were performed in triplicate, and the data represent the mean±s.d. **P*<0.01 compared with controls using Student's *t*-test.

UTR of these mRNAs. Both of these are implicated in muscle cell differentiation (Rosenberg et al., 2006).

Apart from scoring highly using target-predicting software, miR-206 was chosen because of its importance in promoting muscle cell differentiation and because it is known that its expression levels are inversely proportional to those of Twist-1 during differentiation (Koutsoulidou et al., 2011a,b). In order to confirm the binding of miR-206 to the *Twist-1* 3′-UTR, a series of experiments were performed, including (1) luciferase assays using mutant plasmids for the seed-matched region of miR-206 located on the 3′-UTR of

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

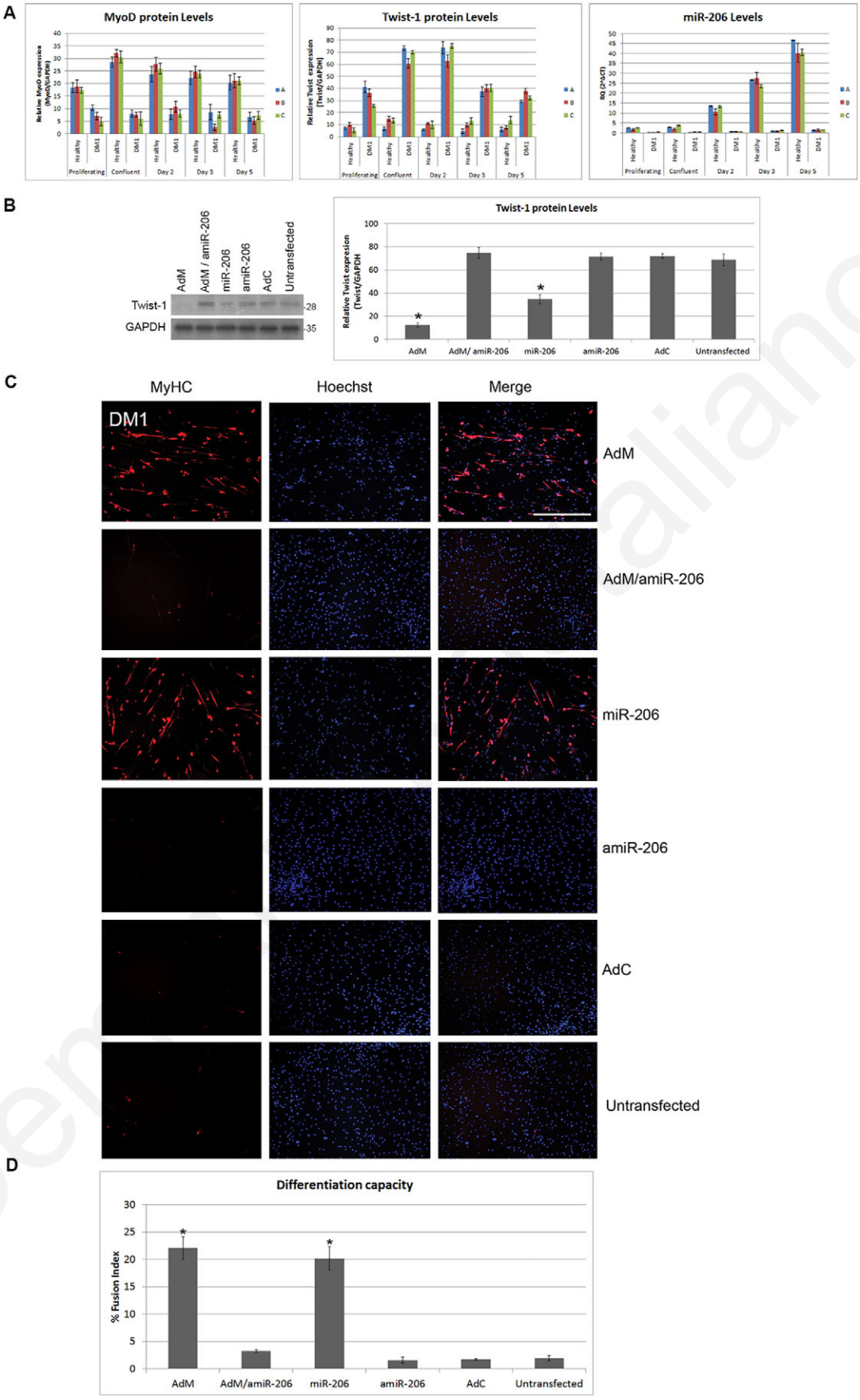

Fig. 8. See next page for legend.

Journal of Cell Science (2015) 128, 3631-3645 doi:10.1242/jcs.172288

Fig. 8. The MyoD–miR-206–Twist-1 pathway is implicated in the defective differentiation pathway in DM1 muscle cells. (A) The protein expression profile for MyoD and Twist-1, and RNA levels for *miR-206* in three different samples of healthy and DM1 cells from the proliferating phase through to differentiation (days 2–5). MyoD levels and miR-206 levels were found to be decreased in DM1 cells compared to normal cells, whereas Twist-1 levels were increased in DM1 cells. (B) Overexpression of MyoD (AdM) or transfection with miR-206 mimic (miR-206) in DM1 cells caused a decrease in the endogenous Twist-1 levels compared to control adenovirus transduction (AdC) or the untransfected cells. Twist-1 endogenous levels were increased back to the original levels when DM1 cells were co-transduced with AdM and miR-206 antagomiR (amiR-206) (AdM/amiR-206). Overexpression of amiR-206 alone had no effect on Twist-1 levels, presumably because of the low miR-206 endogenous levels. (C) Following differentiation of DM1 human myoblasts for 6 days, myotube formation was seen only in those cells that had been transduced with AdM or miR-206, compared to control transduced cells (AdC), cells transfected with amiR-206 alone or untransfected cells. Myogenesis was reversed when amiR-206 was co-transfected with AdM. Myotubes were fixed and stained for MyHC (red) and nucleus-directed Hoechst 33342. Scale bar: 200 µm. (D) Calculation of the fusion index confirmed the above results. All experiments were performed in triplicate, and the data represent the mean±s.d. **P*<0.01 compared with controls using Student's *t*-test.

Twist-1 with a miR-206 mimic, which confirmed that only co-transfection with the wild-type reporter and the miR-206 mimic could reduce luciferase activity; (2) overexpression studies of miR-206 in which *Twist-1* translation was suppressed and the differentiation capacity of human myoblasts that had been isolated from a 14-week-old foetus (high levels of Twist-1, low differentiation capacity) was increased; and (3) inhibition studies of miR-206 in a human muscle cell line that had been isolated from a newborn (low levels of Twist-1, high differentiation capacity), which showed increased endogenous Twist-1 protein levels and a decreased capacity for cell differentiation. These above findings reveal a new target for miR-206 through which muscle cell differentiation is promoted.

Because it is known that MyoD promotes the expression of miR-206 directly by binding to its promoter, we next investigated the possibility that this is also implicated in the regulatory pathway of Twist-1 (Sassoon, 1993; Berkes and Tapscott, 2005; Rao et al., 2006). MyoD regulates many pathways during muscle cell differentiation. Specifically, it has been shown to activate p21 expression during differentiation of murine muscle cells (Halevy et al., 1995). MyoD also interacts with several proteins, such as cyclin-dependent kinase 4, HDAC1, STAT3 and many others, in order to promote muscle cell differentiation (Zhang et al., 1999a,b; Mal et al., 2001; Puri et al., 2001; Kataoka et al., 2003).

MyoD is known to bind to the E-box sequence and to regulate the expression of muscle-specific genes (Weintraub et al., 1994; Shklover et al., 2007). By scanning the promoter of human *miR-206*, two highly conserved E-boxes were found to be present. The binding of MyoD to both E-boxes on the promoter of *miR-206* was confirmed by chromatin immunoprecipitation analyses, which confirmed the direct binding of MyoD to the promoter of *miR-206* to both sites, and by using luciferase assays, which confirmed that binding of MyoD to the *miR-206* promoter induces miR-206 expression.

Overexpression of MyoD promotes muscle cell differentiation and the expression of miR-206 (Crescenzi et al., 1990; Rao et al., 2006). From our results, it was found that overexpression of MyoD also reduced the expression level of Twist-1 in 14-week-old foetus myoblasts. Competition experiments with MyoD and Twist-1 overexpression proved this specific association. Overexpression of MyoD induced muscle cell differentiation, whereas the overexpression of both Twist-1 and MyoD brought the levels of cell differentiation almost back to normal, suggesting that MyoD regulates differentiation through downregulation of Twist-1. This was further investigated, and our results showed that MyoD inhibited Twist-1 through miR-206 induction and subsequently promoted muscle cell differentiation. The implication of this new pathway in myogenesis was also demonstrated in primary mouse muscle cells.

Finally, we wanted to investigate whether this newly identified pathway is involved in a disease causing defective muscle cell differentiation. DM1 is the most common form of muscular dystrophy in adults. Symptoms of DM1 include myotonia, muscle weakness and progressive muscle atrophy (Meola, 2000). DM1 is caused by an unstable expansion of CTG trinucleotide repeats found in the 3′-UTR of the *DMPK* gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). C2C12 cells containing mutant DMPK 3′-UTR transcripts assemble to nuclear foci and do not undergo differentiation (Amack et al., 1999). This differentiation deficiency might represent the muscle development abnormalities found in congenital DM1 individuals (Sarnat and Silbert, 1976; Farkas-Bargeton et al., 1988). Differentiation inhibition has been confirmed in cultured myogenic satellite cells taken from DM1 individuals carrying a large CTG expansion (Furling et al., 2001; Timchenko et al., 2001). Mutant *DMPK* 3′-UTR transcripts have also been shown to disrupt myoblast differentiation by reducing MyoD levels (Amack et al., 2002), and an increase in MyoD levels is sufficient to rescue the differentiation defect in DM1 myoblasts (Amack and Mahadevan, 2004). Our results showed that congenital DM1 cells, which have a defective differentiation program, have low levels of MyoD and miR-206 but high Twist-1 levels. This seems rational based on the properties that characterise these three molecules during muscle cell differentiation. In an attempt to prove that this pathway plays a role in the defective differentiation in those cells, we overexpressed MyoD and determined the downstream effects. As expected, MyoD overexpression did correct muscle cell differentiation and, moreover, miR-206 and Twist-1 levels were upregulated and downregulated, respectively. Moreover, competition with an antagomiR for miR-206 demonstrated the specificity and effectiveness of the pathway with regards to the promotion of cell differentiation. Therefore, based on these results, the MyoD–miR-206–Twist-1 pathways is compromised in DM1 cells that exhibit a defective differentiation program.

In summary, our results newly identify a mechanism by which MyoD induces muscle cell differentiation through the induction of miR-206 expression and the subsequent inhibition of Twist-1. This mechanism might play a valuable role in myogenesis and also in diseases where differentiation is defective.

MATERIALS AND METHODS

Cell culture

Human myoblasts were isolated from muscle biopsies obtained during autopsies of normal foetuses (14, 31, 37 weeks old) and congenital DM1 foetuses (31, 37 weeks old and newborn) in accordance with French legislation on ethics rules. Informed consent was obtained and the study conformed to the Declaration of Helsinki. Myoblasts were isolated as previously described, and grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 20% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA) (Edom et al., 1994; Furling et al., 2001). When cells reached confluence, they were differentiated with DMEM supplemented with 2% horse serum (HS; Invitrogen, Carlsbad, CA). Human myoblasts were culture under 5% CO_2 at 37°C. HeLa cells were grown to 90% confluence before being subjected to transfections in growth medium using DMEM (Invitrogen, Carlsbad, CA) supplemented

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with 10% (v/v) FBS (Invitrogen, Carlsbad, CA), 2 mM glutamine (Invitrogen, Carlsbad, CA) and penicillin-streptomycin (100 mg/ml-100 units/ml) (Invitrogen, Carlsbad, CA). For primary mouse cell extraction, 4-week-old mice were euthanized through cervical dislocation, and the muscles were carefully removed from the hind limb in accordance with Cyprus legislation on ethics rules. Myofibres were isolated from the extensor digitorum longus (EDL) muscle as described previously and digested in 0.2% collagenase type 1 for 1 h at 37°C (Rosenblatt et al., 1995). Muscle fibres were next purified from fibroblasts and cell debris. The washed muscle fibres were next placed in Corning 6-well plates coated with 1 mg/ml Matrigel (Corning, Tewksburym, MA) and incubated in activation medium [DMEM with 20% (v/v) foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 10% (v/v) horse serum (Invitrogen, Carlsbad, CA), 1% (v/v) chick embryo extract (MP Biomedicals, France), 0.01% murine FGF-basic (Peprotech, Rocky Hill, NJ), and 1% (v/v) penicillin-streptomycin solution (Sigma-Aldrich, St Louis, MO)] at 37°C for 48 h. The muscle fibres were removed from the plate by gentle blowing, leaving the satellite cells attached to the plate. The satellite cells were further purified by re-plating the remaining population onto non-treated plates, where the satellite cells remained in suspension. The suspended satellite cells were then plated into Corning 6-well plates coated with 1 mg/ml Matrigel and maintained in activation medium at 37°C in 5% CO_2. After 4 days of proliferation, the medium of the primary cells was changes to differentiation medium (used for culture of human myoblasts).

Plasmid and RNA oligonucleotide transfection, and adenoviral vector transduction

For HeLa and human myoblasts, 100 pmol of each miRNA mimic (Qiagen, Limburg, The Netherlands), antimiR (Ambion, Carlsbad, CA), negative miRNA (Qiagen, Limburg, Netherlands), mutated miRNA (Eurofins Genomics, Ebersberg, Germany), or anti-miR control (Ambion, Carlsbad, CA) was transfected using X-tremeGENE 9 transfection reagent (Roche, Penzberg, Upper Bavaria, Germany) in Opti-MEM reduced serum medium (Invitrogen, Carlsbad, CA). After 24 h, cells were then transfected with the wild-type or mutated Twist-1 3′-UTR luciferase plasmid or miR-206 promoter luciferase plasmid (400 ng), and CMV-pRLRenilla (5 ng) (Promega, Madison, WI) plasmid in complex with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For transfections into primary mouse muscle cells, the same transfection procedure was repeated. For small interfering (si) RNA transfections, 100 pmol of MyoD siRNA (Invitrogen, Carlsbad, CA) and siRNA negative control (Invitrogen, Carlsbad, CA) were combined with Lipofectamine RNAi or RNAiMAX (Invitrogen, Carlsbad, CA). MyoD (AdM), (Vector Biolabs, Malvern, PA) and Twist-1 (AdT) (Vector Biolabs, Malvern, PA) adenoviruses were used to overexpress MyoD and Twist-1. AdC (Vector Biolabs, Malvern, PA) was used as a control virus, containing an empty vector with a CMV promoter. Cells were transduced with the adenoviruses a day before confluence for 48 h.

Real-time miRNA expression assays

Total RNA enriched for small RNAs, including miRNAs, was isolated using mirVana™ miRNA Isolation Kit (Ambion, Carlsbad, CA) according to the manufacturer's protocol. 10 ng of the extracted RNA was subjected to reverse transcriptase PCR using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). miRNA levels were measured by using real-time PCR amplification using TaqMan® MicroRNA Assays specific for miR-206 (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instructions. miRNA expression was normalized to that of the RNA U6B small nuclear (RNU6B) (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed using the Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA).

Plasmid construction and site-directed mutagenesis

Human *Twist-1* 3′-UTR was amplified by using PCR from genomic DNA extracted from human myoblasts, and this was then cloned into the multiple cloning site of the pMIR-Report luciferase miRNA expression reporter (Applied Biosystems, Carlsbad, CA). The PGL3 plasmid with the promoter of *miR-206* was kindly provided by the laboratory of Dr Stephen Tapscott Fred Hutchinson Cancer Research Center, Seattle, WA.

The predicted miR-206-binding site located in the *Twist-1* 3′-UTR and the predicted MyoD sides on the promoter of *miR-206* were mutated by a substitution of 6 bp (Twist-1 3′-UTR: CATTCT to GCCGGT, miR-206 promoter: ACAGCT to TGTCGA and GCAGCT to CGTCGA) using the GeneArt® Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA). All of the cloned vectors were verified by sequencing.

Dual luciferase reporter assays

Following transfections, HeLa cells were harvested, and assays were performed 24 h after the last transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was normalized to *Renilla* luciferase expression (internal control). Cells were subsequently lysed with commercial cell lysis buffer (Promega, Madison, WI), and luciferase activity was measured using a luminometer (Berthold, Bad Wildbad, Germany) according to kit protocols.

Chromatin immunoprecipitation

One day before inducing differentiation, cells were transfected with AdM (MyoD-overexpressing adenoviral vector). The chromatin immunoprecipitation assay was performed on myotubes that had been differentiated for 2 days using MAGnify™ Chromatin Immunoprecipitation System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, dynabeads were coupled to the anti-MyoD primary antibodies (Abcam, Cambridge, UK) before the crosslinking of chromatin. Following cell lysis, samples were subjected to chromatin binding to the antibody–dynabead complexes in order to isolate only the DNA of interest after a series of washes. The cells were immunoprecipitated using anti-MyoD antibodies (Abcam, Cambridge, UK). For chromatin immunoprecipitation assay, two controls were used – a positive- and a negative-control antibody. For positive control, 2.5 µg of unconjugated polyclonal antibody specific to human and mouse histone H3 trimethylated at lysine 9 (K9me3) (H3-K9Me3) (Invitrogen, Carlsbad, CA) was added. For negative control, 1 µg of mouse IgG antibody was used. Primers for the chromatin immunoprecipitation assay were design for the promoter of *miR-206*, E1 forward primer, 5′-CAGTGAACAATGGTGCTTGG-3′; E1 reverse primer, 5′-TTCCACATTCACGCAGAGAG-3′; E2 forward primer, 5′-AACCCCATCTCCCTCCAG-3′; and E2 reverse primer, 5′-GATCCTTTTGTCGGGCTTCT-3′.

Western blot analysis and immunofluorescence

Proliferating myoblasts or differentiated muscle cells were used for protein extractions. 30–50 µg protein extracts were incubated with primary antibodies against Twist-1 (1:100, Santa Cruz Biotechnology, Dallas, TX), troponin (1:200, Santa Cruz Biotechnology, Dallas, TX), skeletal actin (1:200, Santa Cruz Biotechnology) or GAPDH (1:1500, Santa Cruz Biotechnology, Dallas, TX), followed by incubation with goat anti-mouse IgG or donkey anti-rabbit IgG secondary antibodies. Differentiated human myoblasts or primary mouse myoblasts were fixed in 4% paraformaldehyde and incubated with a monoclonal antibody against myosin heavy chain (MyHC) (Sigma-Aldrich, St Louis, MO) at a concentration of 1:400 in 1% BSA in PBS or Twist-1 (Abcam, Cambridge, UK) at a concentration of 1:50 in 1% BSA in PBS and Alexa-Flour-conjugated anti-mouse secondary antibody (Life Technologies, Carlsbad, CA). Nuclei were stained with Hoechst 33342 (Invitrogen, Carlsbad, CA). Images were taken using a Zeiss Axiovision digital camera (Zeiss, Oberkochen, Germany) and then accumulated using Adobe Photoshop Software. Cells were counted at least three times from each pool of clones in ten different cellular areas to determine the fusion index (percentage of nuclei located under myotubes divided by the total number of nuclei).

Statistical analysis

ANOVA and Student's *t*-test were used to determine whether specific group mean differences were significant. The level of significance was set at 0.0. Data are presented as mean±s.d.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
D.K. carried out human skeletal muscle cell culture, isolation and culture of mouse primary muscle cells, adenovirus and plasmid transfections, miRNA analysis, immunocytochemistry, western blotting, luciferase assays, mutagenesis and drafted the manuscript. A.K. participated in chromatin immunoprecipitation assays, immunocytochemistry, western blotting and adenovirus transfections. N.P.M. participated in isolation of mouse primary muscle cells and immunocytochemistry. D.F. isolated myoblasts and established cell lines. L.A.P. conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

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