The IGF Signalling Factors during Chick Limb Muscle Development

By

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Declaration

I hereby declare that this thesis entitled ‘’The IGF signalling factors during chick limb muscle development” has been composed, written and submitted by myself to the school of Bioscience (Nottingham University), and all work of other authors are duly acknowledged. I further declare that this work has not been submitted to any other university for the award of any other degree. The work was done under the guidance of Dr. Dylan Sweetman and Dr. John Brameld.

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List of abbreviations

Abbreviations	Description
AER	Apical Ectodermal Ridge
ALS	Acid Labile Subunit
AP	Anterior–Posterior
APC	Adenomatous polyposis coli
Ask	Apoptosis signal-regulating kinase
ATP	Adenosine Triphosphate
BCIP	Bromo-4-chloro-3-indolyl-phosphate, 4-toluidine
BHLH	Basic helix-loop-helix
BMPs	Bone Morphogenetic Proteins
BSA	Bovine serum albumin
Ca2+	Calcium ions
CIMPR	Cation-independent mannose 6-phosphate receptor
CNS	Central Nervous System
CRD	Cysteine-Rich Domain
Crk	Cytoplasmic Protein
CSF	Cerebrospinal fluid
CXCR	C-X-C chemokine receptor type
D	Distal
Dach-2	Dachshund family transcription factor-2
Dm	Dermomyotome
DML	Dorsomedial lip
DMM	Dorsal Muscle Mass
Dmrt2	Doublesex and mab-3 related transcription factor 2
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DSW	Deionized distilled water
DTA	Diphtheria toxin A fragment
DV	Dorsal–Ventral
E-box	Enhancer Box
ECDs	Extracellular regions
ECM	Extracellular Matrix
EcoRI	Escherichia coli RI
EGFR	Epidermal growth factor receptor
EMT	Epithelial Mesenchymal Transition
En	Engrailed homologue
EOM	Extraocular muscles
EtBr	Ethidium Bromide
Eya	Eyes absent homolog
FGF	Fibroblast Growth Factor
FGFR	Fibroblast growth factor receptor
FoxO	Forkhead box PDKs
GDFs	Growth and differentiation factors
GH	Growth Hormone

HH	Hamburger and Hamilton
IGFBP	Insulin-like growth factor binding proteins
IGFBP-rP	IGFBP-related protein
IGFR	Insulin Like Growth Factor Receptor
IGFs	Insulin Like Growth Factors
IR	Insulin Receptor
IRR	Insulin Receptor Related Receptor
IRS	Insulin Receptor Substrate
JNK	Jun N-terminal kinases
KDa	KiloDalton
LB	Luria-Bertani medium
LBX	Ladybird Homeobox
LPM	Lateral Plate Mesoderm
M6P	Mannose 6-phosphate
MABT	Maleic acid-Tween
MAPK	Mitogen-activated protein kinase
Meox	Mesenchyme Homeobox
MgCl2	Magnesium Chloride
MHC	Myosin Heavy Chain
MKK	MAP kinase kinase
MPCs	Muscle Precursor Cells
MRFs	Myogenic Regulatory Factors
mRNA	Messenger Ribonucleic Acid
My	Myotome
NBT	Nitro blue tetrazolium chlorid
NC	Neural Tube
NCCs	Neural crest cells
NMR	Nuclear Magnetic Resonance
NSILA	Non-suppressible insulin-like activity
NT	Notochord
NTMT	NaCl- Tris- MgCl2- Tween
OB	Oligonucleotide Binding
OVA	Ovalbumin
OVM	Ovomucoid
P	Proximal
Pax	Paired Box
PBS	Phosphate buffered saline
PBGS	PBS,BSA, Goat Serum
PC3	Prostatic cancer cell line
PD	Proximal–Distal
PDGDb	Platelet derived growth factor subunit b
PDGFR	Platelet-derived growth factor receptor
PDKs	Phosphoinositide-dependent kinases
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase

Pitx	Pituitary homeobox
PSF	Prostacyclin-Stimulating factor
PSM	Presomitic Mesoderm
PZ	Progress zone
RA	Retinoic Acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic Acid Receptor
RLP	Relaxin-like peptide family
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room Temperature
RTKs	Receptor tyrosine kinases
RXR	Retinoid X Receptor
S	Sclerotome
SCs	Satellite Cells
SDF	Stromal-derived-factor
SF/HGF	Scatter Factor/Hepatocyte Growth Factor
sFRP	Secreted Frizzled-Related Protein
SH2	Sineoculis homeobox homolog
Shh	Sonic Hedgehog
Sim	Single-minded homologue
siRNA	Small interfering RNA
Six	Sineoculis homeobox homolog
TAE	Tris-acetate-EDTA
TAF	Tumor adhesion factor
Tak-1	Transforming Growth Factor Beta-Activated Kinase-1
TBX	T-box transcription factor
TCF	T-cell transcription factor
TGFB	Transforming Growth Factor Beta
UV	Ultraviolet light
VLL	Ventrolateral lip
Vmm	Ventral Muscle Mass
WISH	Whole-mount *in situ* hybridisation
ZPA	Zone of Polarising Activity
α	Alpha
β	Beta
β-catenin	Beta-catenin
γ	Gamma

Abstract

In vertebrates, muscle precursor cells delaminate and migrate from the somites into the limb, where they proliferate and differentiate to muscle masses. Many transcription factors, including Pax and the myogenic regulatory factors (Mrfs) play a vital role in regulating limb muscle growth and development. In the limb, the progenitors begin the process of myogenesis by expressing the myogenic determination factors *Myf5* and *MyoD*, and differentiation factors *Myog* and *Mrf4* then eventually form dorsal and ventral skeletal muscle masses. However, the role of insulin-like growth factors (IGFs) in regulating the early processes of limb myogenesis, and the functional relevance of IGF-1 and IGF-2 in myogenic determination remains poorly defined.

By whole-mount *in situ* hybridisation (WISH), the present study first characterised the expression of both *Mrfs* and components of the *IGF* in chick limbs at precise stages during embryonic development. The analysis of the spatial and temporal expression patterns of both *Mrfs* and *IGFs* allowed further interventions to be precisely targeted. Therefore, to determine the mechanism of how these factors interact, application of IGF-soaked beads into chick limb during the development was followed by analysis of muscle specific marker genes. Both *Pax3* and *MyoD* were selected as markers for undifferentiated and differentiated myogenic cells respectively.

First, the present study demonstrated both IGF-1 and IGF-2 as novel players in early limb myogenesis *in vivo* by stimulating the activation of *Pax3* and *MyoD* expression. IGF-1 was also shown to increase numbers of both $Pax3^{+ve}$ and mitotic cells, but not the numbers of mitotic $Pax3^{+ve}$ cells, suggesting the increase in *Pax3* expression was not simply via increased proliferation. IGF signalling was then examined using picropodophyllotoxin (or PPP) as a specific inhibitor of the IGF-1 receptor. The present data showed that blocking of IGF-1R with PPP inhibited both IGF-1 and IGF-2 induced expression of both *Pax3* and *MyoD.*

To confirm the mechanisms for the effects of the IGFs, the current study also used a panel of pharmacological inhibitors, such as U0126 and LY294002 to block the mitogen activated protein kinase (MAPK) and AKT signalling pathways, respectively as well as Nocodazole to inhibit cell proliferation. Results showed that the effects of both IGF-1 and IGF-2 on *Pax3* and *MyoD* expression were mediated by the MAPK pathway. In contrast, inhibition of the AKT signalling pathway inhibited IGF-2 (but not IGF-1) stimulated *Pax3* expression, as well as IGF-1 (but not IGF-2) stimulated *MyoD* expression. Inhibition of cell proliferation with Nocodazole had no effect on IGF-1 stimulated *Pax3* or *MyoD* expression. Similarly Nocodazole had no effect on IGF-2 stimulated *Pax3* expression, whereas it inhibited IGF-2 stimulated *MyoD* expression.

SU5402, an inhibitor of FGF receptor, showed no effect on IGF-1 and IGF-2 stimulated *Pax3* expression whereas it inhibited their stimulation of *MyoD*. These findings led to the proposal of a model where IGF signalling directs the timing of the early steps of myogenic cells by stimulating *Pax3* expression, whereas the upregulation in *MyoD* expression is indirectly controlled via Fgf. Together, these results give new insights into the early embryonic development of limb muscle in birds. Additionally, a deeper knowledge of a specific interaction between IGFs and Mrfs may well provide a molecular basis for preventing the loss of muscle and muscle diseases in animals and humans. The present study may well also shed a light to improve the production of chicken meat.

Publications

1- Mok, G.F., **Mohammed**, R.H. and Sweetman, D., 2015. Expression of myogenic regulatory factors in chicken embryos during somite and limb development. *Journal of anatomy*, 227(3), pp.352-360.

2- **Mohammed**, R.H. and Sweetman, D., 2016. Grafting of Beads into Developing Chicken Embryo Limbs to Identify Signal Transduction Pathways Affecting Gene Expression. *Journal of Visualized Experiments*, (107), pp.e53342-e53342.

Chapter 1: Literature review

1.1 Introduction

The development of limb skeletal muscle has been well studied in vertebrates. Numerous studies have provided information showing the limb muscle growth and development, and have detected a variety of important factors involved. Transcription factors are known to play a fundamental role in establishing the early aspects of limb myogenesis, such as the commitment of mesoderm progenitor cells to the myogenic lineage followed by myoblast proliferation and differentiation into post-mitotic myocytes, which in turn fuse to form myotubes. In addition, several intrinsic and extrinsic signalling pathways regulate the sequence of cellular events required for limb myogenesis. However the interactions between these transcriptional factors and signalling events are not fully understood.

1.2 Chick embryos as a model system for the study

Avian embryos, chiefly the quail (Coturnix japonica) and chicken (Gallus gallus) are important models in developmental biology (Douarin and Dieterlen-Lièvre, 2013). Embryos in avian eggs are simple to obtain and easily accessible for better understanding the neurobiology, physiology, pattern formation, toxicology as well as pathophysiology of embryonic diseases (Park et al., 2013, Smith et al., 2012). In addition, chicken embryos are easy to manipulate during development, as the immune system has not yet fully developed (Busch et al., 2013). There are also several other advantages and potential applications of genetic modification in chickens, for example, enhancement of meat and egg production as well as production of therapeutic proteins specifically in egg white (Oishi et al., 2016). Targeted gene knockout of egg white allergen genes, including ovalbumin (OVA) and ovomucoid (OVM) results in reducing the allergenicity in eggs (Oishi et al., 2016).

The general ontogeny and similarity in gene expression patterns between bird embryonic development and other vertebrates, such as mouse shows that chicken can serve as key model for genetic study and biochemical mechanisms in humans (Abu-Bonsrah et al., 2016). In chickens genetic modification has lagged behind that of other organisms due to the difficulty

in accessing the zygote and modifying its genome (Mizushima et al., 2010). Consequently, transgenesis in chickens has chiefly been achieved by viral vector infection of embryos during early stages of development (Nishijima and Iijima, 2013, Petitte and Mozdziak, 2007). However, recently, birds have been produced with specific modification such as knock-ins and knockouts for practical studies in vertebrates (Doran et al., 2016). Knockout genes in chicken somatic cells during embryogenesis has been applied using CRISPR/Cas9 system (Véron et al., 2015). This system is a powerful tool for *in vivo* genome editing of many species (Long et al., 2016, Nelson et al., 2016). CRISPR/Cas9 editing has been demonstrated in chicken primordial germ cells (PGCs), which can then be used to produce transgenic birds for studies using chicken models (Dimitrov et al., 2016).

The chick limb has been a particularly important model in following developmental pathways, from tracking cell movements during vertebrate morphogenesis to dissecting the genetic and signalling pathways to regulate its growth and patterning. The use of the limb bud as a development paradigm, particularly in chick and mouse, is mainly because the limb can be easily manipulated *in vivo* to define the essential cellular processes and molecular interactions underlying patterning and skeletal formation (Niswander, 2003, Zuniga, 2015). Loss and gain of function mutations are available for numerous experiments on the genetic determinants of limb development, in both normal and congenital malformation (Lopez-Rios, 2016).

1.3 Formation of mesodermal organs

In vertebrate embryos, at early developmental stages, the mesoderm is sectioned into four regions from medial to lateral. The midline zone is occupied by the notochord. Adjacent to that is the paraxial mesoderm, which will later become the somites, followed by the intermediate mesoderm that forms the kidneys and gonads then at the most lateral parts the lateral plate mesoderm (LPM) (Slack, 2006). The LPM is split by the coelom into two layers: an upper layer and lower layer. The upper layer, also called somatic mesoderm, forms a series of structures along the rostrocaudal axis, such as the limbs and flank body wall (Yonei-Tamura et al., 2005), whereas the lower layer, known as splanchnic mesoderm, gives

rise to the head muscle, mesenteries and heart (Nathan et al., 2008). Additionally, the vertebrate skeleton arises from three regions: the neural crest, somites and the lateral plate. The neural crest forms most of the skull; the somites give rise to the vertebrae, while the limb-girdles and bones originate from the limb buds as well as associated lateral plate (Christ and Ordahl, 1995, Huang et al., 2000, Tam and Trainor, 1994).

1.4 Origin of muscle system

In vertebrates, the most significant source of embryonic skeletal muscles and their precursors are the somites. These mesodermal structures develop in anteroposterior sequence from the paraxial mesoderm and are segmentally formed in pairs on either side of the neural tube and the notochord (Figure 1.1A) (Andrade et al., 2007, Christ and Ordahl, 1995, Dequéant and Pourquié, 2008, Keynes and Stern, 1988, Ordahl, 2000b, Ordahl, 2000a, Pourquié, 2001, Pourquié, 2011, Tam et al., 1999).

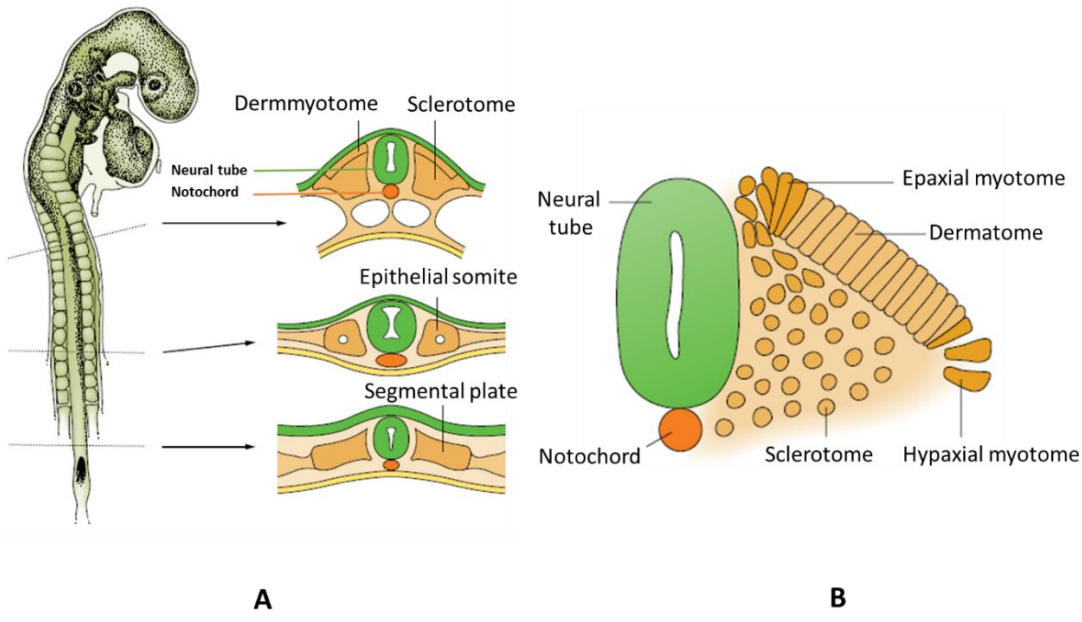


Figure 1. 1: Primary somitogenesis in the chick embryo. A, the process begins in the anterior region then a series of segments are sequentially added at the posterior end for a considerable time. The morphology is initially exhibited specific epithelial forms and it is subsequently changed to an inner sclerotome and outer dermamyotome. B, Somite is located on either side of the neural tube. It has three compartments, dermomyotome, myotome and sclerotome. The expansion of the dermomyotom results in cells extension from the dorsomedial lip (DML) to a region beneath the dermomyotome. This labels the growth of the epaxial myotome. Similarly, extension also occurs at the ventrolateral lip (VLL) producing the hypaxial myotome (Slack, 2006).

On differentiation, in response to local environmental signals, the somite is eventually specified into four compartments: the ventral mesenchymal sclerotome the syndetome, intermediate myotome and dorsal epithelial dermomyotome (Figure 1.1B) (Andress and Birnbaum, 1992, Capdevila and Johnson, 1998, Christ and Wilting, 1992, Hirsinger et al., 1997, Le Douarin, 1973, Marcelle et al., 1997, Mauro, 1961, McMahon et al., 1998, Pollak et al., 2004, Reshef et al., 1998).

The sclerotome and the syndetome form the vertebral column, meninges, ribs, connective tissues and tendons (Bailey et al., 2001, Chen and Goldhamer, 2004, Christ et al., 2004). The dermomyotome is a transitory epithelial sheet situated in between the ectoderm and the mesodermal sclerotome (Christ and Scaal, 2009). It is responsible for proliferating precursors of all skeletal muscles of the trunk, the endothelial dorsal dermis, brown fat lineages and vascular smooth muscle cells (Atit et al., 2006, Ben-Yair and Kalcheim, 2005, Brand-Saberi and Christ, 1999, Buckingham et al., 2003, Evans et al., 2006, Esner et al., 2006, Yvernogeau et al., 2012). The myotome is the first skeletal muscle to develop and form in the central part of the somite (Tajbakhsh and Buckingham, 1999). This occurs when dermomyotomal cells delaminate from the epaxial lip of the dermomyotome, near to the neural tube, and subsequently from the dermomyotomal hypaxial lip, near to the lateral mesoderm (Christ and Ordahl, 1995, Gros et al., 2004, Kahane et al., 1998, Kato and Aoyama, 1998).

The epithelial cells in the epaxial and hypaxial lips of the dermomyotome undergo an epithelial to mesenchymal transition, releasing myogenic precursors into the underlying myotome (Buckingham, 2006). Two alternative fates of these cells have been observed: they begin quickly leaving the dermomyotome and then undergo differentiation as well as elongation to form the myocytes (Figure 1.2) (Hollway and Currie, 2005, Venters et al., 1999) or they continue to proliferate for myotome growth and later for muscle masses. In addition, some of these cells will become satellite cells, adult muscle stem cells (Gros et al., 2005).

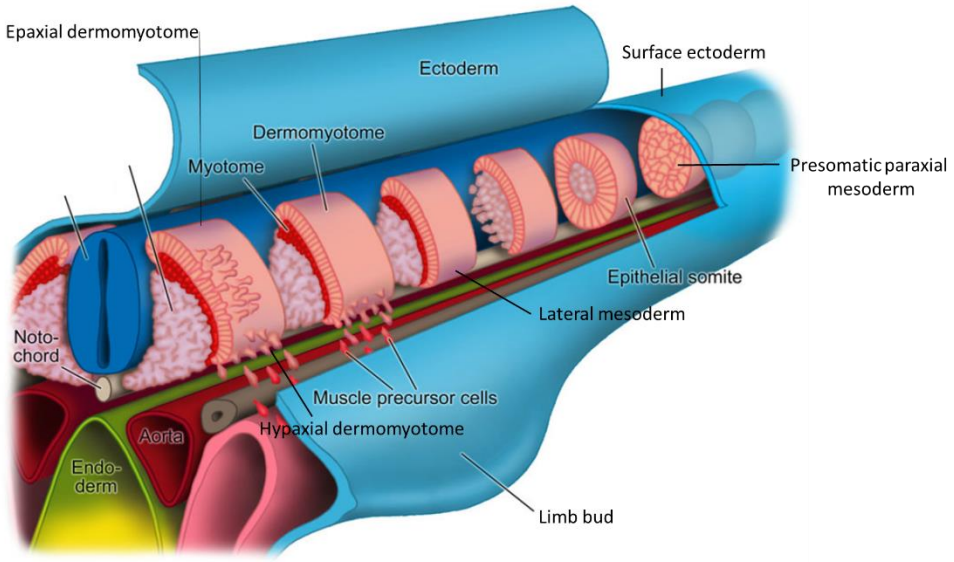


Figure 1. 2: Schematic representation of embryonic origin of somite's compartments and the migration of myogenic precursor cells in vertebrates. The presomitic paraxial mesoderm is located between the notochord sides. The lateral-plate mesoderm is seen between the intermediate mesoderm, which is laterally positioned to the paraxial mesoderm. The latter segments into epithelial ball-like structures, called somites, which are formed following a rostrocaudal gradient in response to different signals from neighbouring tissues. Somite differentiates to form dermomyotome and the sclerotome. From the epaxial domain of dermomyotome, myogenic progenitor cells delaminate and then migrate under the dermomyotome to make the epaxial myotome that will form back muscles. Similarly, from the hypaxial region, progenitor cells delaminate and migrate under the dermomyotome to make the hypaxial myotome that forms body muscles, including limb muscle (Yusuf and Brand-Saberi, 2012).

Vertebrate muscles are morphologically distributed into epaxial and hypaxial muscles. The epaxial muscles compose deep back muscles, which are situated dorsally and innervated by the dorsal rami of the spinal nerves, while the ventral ramus innervates muscles which are derived from the hypaxial domain that includes shoulder, limb and body wall muscles (Braun and Gautel, 2011, Tajbakhsh and Buckingham, 1999). In amniote embryos, however, the dorsal ramus does not innervate the epaxial myotome, but it initially enters the muscle masses during transformation of the myotome into the epaxial muscle masses (Deries et al., 2008).

1.5 The important signals that control somite myogenesis

The somites are regulated by signals from surrounding tissues, including notochord, neural tube and the overlying ectoderm, which also pattern the formation of skeletal muscle (Borycki et al., 1997, Borycki and Emerson Jr, 1999, Christ et al., 2007, Šošić et al., 1997). These tissues produce Shh, Bmps and Wnts that can specifically trigger or antagonise epaxial myogenesis (Deries and Thorsteinsdóttir, 2016). Although *Wnt-1* and *Wnt-*

3a are expressed in the dorsal neural tube, their role in myogenesis has been questioned by experiments that suggest activation of beta-catenin takes place through Notch signalling rather than the canonical Wnt pathway (Sieiro et al., 2016). The dorsal somite also secretes Noggin (Marcelle et al., 1997), while the surface ectoderm produces Wnt7a to activate myogenesis in the hypaxial domain (Figure 1.3). Furthermore, the myotome secretes Fgf4 and Fgf8 to regulate the polarization of somites and sclerotome formation (Capdevila and Johnson, 1998, Ontell et al., 1993). Surgical removal of the notochord or/and the neural tube causes a striking lack of vertebral axial muscles, but it does not impair hypaxial musculature differentiation in limbs and body wall (Rong et al., 1992). Signals from the notochord and neural tube can either induce or repress the expression of myogenic basic helix-loop-helix (bHLH) genes in the somite (Münsterberg et al., 1995, Pirskanen et al., 2000, Reshef et al., 1998).

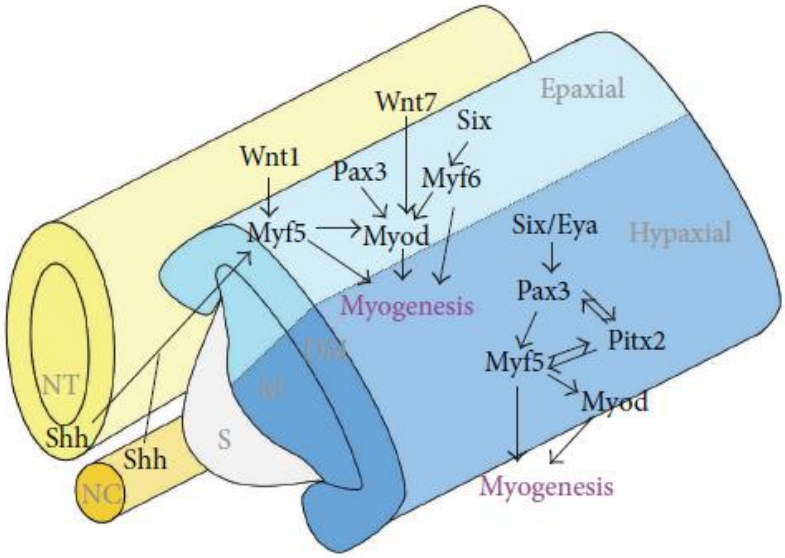


Figure 1. 3: Schematic representation of stimulation of somite myogenesis. The adjacent (NC) and (NT) give signals to the mature somite, which in turn undergoes division to form three compartments the (DM), (M) and (S). Shh signals from the floor plate of the notochord and from the neural tube; Wnt1 signals from the dorsal neural tube stimulate *Myf5* expression, which in turn activates of *MyoD*. *MyoD* is also activated by *Pax3* and *Wnt7*, both from the dorsal neural tube. For initiation epaxial somites myogenesis, *Pitx2* and *Six*/*Eya* activate *Pax3* expression, which leads to stimulation of *Myf5* and *Myod* expression (Eng et al., 2013). Abbreviation: (NC), notochord; (NT), neural tube; (DM), dermomyotome; (M), myotome; (S), sclerotome

1.5.1 Sonic hedgehog (Shh)

(Shh) is a homologue of the Drosophila hedgehog protein (Echelard et al., 1993, Riddle et al., 1993), a prototypical morphogen that has important functions during embryogenesis (Ribes and Briscoe, 2009). Shh signals are mediated through two transmembrane proteins, smoothened and patched, and they culminate with their putative downstream effectors, Gli transcription factors (Borycki et al., 2000, Marcelle et al., 1999). Gli proteins have been detected in the specification of cells fate determination, proliferation and in patterning of several cell types and organs through embryogenesis (i Altaba, 1999). Gli proteins operate either as transcriptional activators or inhibitors depending on Shh ligand concentration (Marigo et al., 1996, Ribes and Briscoe, 2009). In addition, it has also been shown that *Patched* expression occurs in response to *Shh* expression (Marigo et al., 1996).

Shh is responsible for the activity of the notochord and the floor plate in the neural tube as well as ZPA (Cohn and Tickle, 1996, Jessell, 2000). Interestingly, Shh can stimulate its own activation in the ventral neural tube where it induces the floor plate differentiation (Roelink et al., 1994). Shh antagonises the promotion of dermomyotomal markers by the dorsal neural tube (Fan and Tessier-Lavigne, 1994, Maroto et al., 1997). This factor also has a crucial role in somite myogenesis by inducing-myogenic factors (Münsterberg et al., 1995). It can directly stimulate ventral muscle precursor cells to initiate the expression of *Myf5* and then myogenesis by binding to Gli-binding domain in the Myf5 early epaxial enhancer (Anderson et al., 2012). Shh also indirectly affects myogenesis by stimulating *MyoD* expression (Francetic and Li, 2011). The loss of Shh results in the absence of *Myf5* transcription, but not that of *MyoD* (Chiang et al., 1996). However, loss of *Myf5* leads to the absence of Shh ability to activate *MyoD* expression (Borycki et al., 1999a, McDermott et al., 2005). Shh has been reported as a survival factor for sclerotomal cells, but not for proliferation of epaxial muscle progenitors (Borycki et al., 1999a).

1.5.2 Wnt family memebers (Wnts)

The Wnt family of genes comprise 19 members in vertebrates. They regulate a number of cellular physiological processes, including proliferation, differentiation, adhesion and determination of cell fate (Borello et al., 2006, Brisken et al., 2000, Kim et al., 2006, Moon et al., 2002, Moon et al., 2004, Nishizuka et al., 2008, Park et al., 2007, Willert and Nusse, 1998). Wnt signalling molecules also maintain homeostasis of tissue in the adult (Vincan, 2008); thus loss of its signalling causes different cancers, such as carcinomas, melanoma, leukaemia and colon (Gregorieff and Clevers, 2005, Moon et al., 2004, Radtke and Clevers, 2005, Reya and Clevers, 2005, Vincan, 2008).

Wnt family members, including *Wnt1* and *Wnt3a*, which are transcribed in the dorsal neural tube, have been shown to regulate myogenesis in somites (Münsterberg et al., 1995, Pollak et al., 2004, Tajbakhsh et al., 1998). Forced expression of *Wnts* in the dorsal neural tube or in surface ectoderm leads to inhibiting myogenesis in chick embryos (Tzahor et al., 2003). The Wnts and the signalling molecules within the somite precisely orchestrate the progression of myogenic progenitor cells along the muscle lineage (Brack et al., 2008, Wagner et al., 2000). In co-culture experiments, Wnts have been established to be required for muscle development in the paraxial mesoderm (Fan et al., 1997, Reshef et al., 1998, Stern and Hauschka, 1995). Enhanced Wnt signalling in the paraxial mesoderm by retroviral mediated overexpression enlarges the expression domain of *Pax3* in the dermomyotome (Pollak et al., 2004). Furthermore, the neural tube-derived-Wnts signalling inhibits *Myf5* expression, whereas ectodermal-derived-Wnts induces *MyoD* expression (Tajbakhsh et al., 1998).

Soluble Frizzled-related proteins (sFRPs) have a Frizzled (Fzd)-type, cysteine-rich domain (CRD) that acts as a binding site for Wnts due to the similarity to the ligand binding domain of Frizzleds, the Wnt receptors (Figure 1.4) (Kawano and Kypta, 2003). sFRPs can modulate Wnt signals as antagonists by preventing the ligand binding from the receptor (Kawano and Kypta, 2003, Rubin et al., 2006). However, binding of Wnts to their

receptors, the Frizzled, can activate three main pathways: the canonical, the non-canonical and the Calcium ions (Ca2+) pathway (Cadigan and Nusse, 1997, Church and Francis-West, 2002, Veeman et al., 2003). The canonical Wnt pathway precisely regulates the concentration of a cytoplasmic protein called Beta-catenin (β-catenin), which in turn controls cell adhesion as well as stimulation of Wnt target genes in the nucleus (Clevers, 2006, Sieiro et al., 2016). β-catenin is normally regulated and degraded via the β-catenin-destruction-complex, and particularly by the adenomatous polyposis coli protein (APC) (Sieiro et al., 2016). This protein in the cytoplasm enters the nucleus to support DNA-bound T-cell tran-

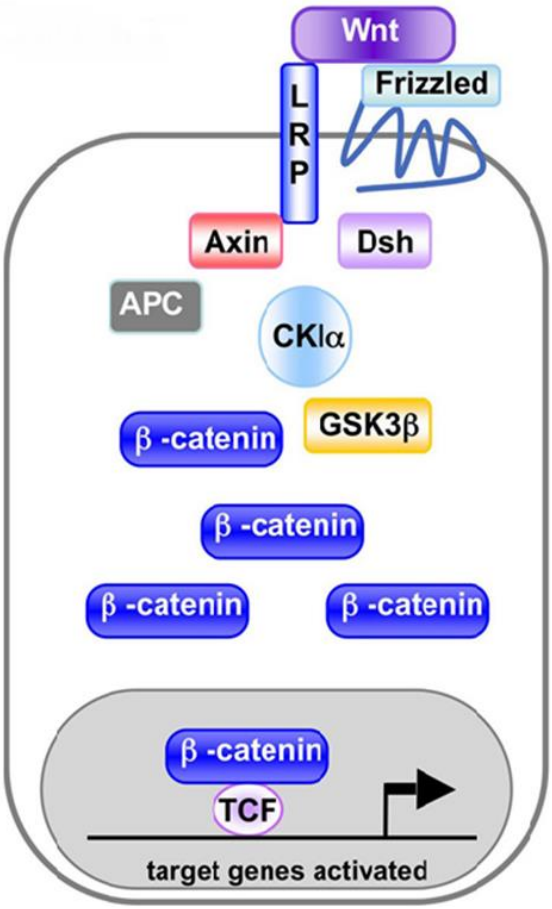


Figure 1. 4: A canonical Wnt signalling pathway. Wnt ligand binds a Frizzled/LRP-5/6 receptor complex and results in hypo-phosphorylated β-catenin stabilization, which interplays with TCF/LEF in the nucleus to induce transcription. All components of A canonical pathway, accept Axin, CKIa, GSK3b, APC, have a positive role in the cells regulation (Eisenmann, 2005).

-scription factor (TCF) for activation their targets, the Wnt/β-catenin pathway (Sieiro et al., 2016) to the cellular response. As the Myf5 promoter has TCF binding sites (Borello et al., 2006) that is required for *Myf5* activation in the DML. TCF and β-catenin also regulate the expression of *Myf5* in somites (Abu-Elmagd et al., 2010, Gros et al., 2009). This indicates the important role of Wnt/β-catenin pathway in regulating somite myogenesis (Sieiro et al., 2016).

1.5.3 Bone morphogenetic proteins (Bmps)

Bmps, also known as growth and differentiation factors (GDFs), constitute a group of growth factors that belong to the multigene family of transforming growth factor β (TGFβ) (Hogan, 1996). In the vertebrate, Bmps play a fundamental role in establishing the body plan and in organs development (Kingsley, 1994). During organogenesis, the expression of Bmp can be seen in the skin and hair as well as heart, tooth, kidney, gut, lung and testes (Bitgood and McMahon, 1995). BMPs is also involved in the peripheral nervous system and in the eye during the development (Dudley et al., 1995). High level of Bmps activity is seen in early stages of somitogenesis that then decline progressively. At these stages, the lateral plate mesoderm-(LPM)-derived Bmp acts to establish the somite medio-lateral polarity through antagonistically regulating transcription of *MyoD* and *Sim-1* laterally (Capdevila and Johnson, 1998, Hirsinger et al., 1997, Kahane et al., 2007, Tonegawa and Takahashi, 1998).

Bmps initiate signalling by binding to their receptors type-1 and 2 serine-threonine kinases that subsequently activate BMP-specific signalling pathway MAPK pathway or Smad pathway (Derynck and Zhang, 2003, Feng and Derynck, 2005). It has been reported that the receptor type-1 is the predominant Bmp receptors in the limb ectoderm (Robert, 2007). However, the extracellular antagonists such as Gremlin, Chordin, Follistatin and Noggin can bind with high affinity to Bmps and inhibit BMP signalling (Balemans and Van Hul, 2002). Noggin is essential for normal development of the neural tube and somite. Inhibition of BMP signalling by Noggin is important for the body elaboration in the vertebrate (McMahon et al., 1998). In the chick, *Noggin* expresses in the dorsal lip of the somite and it regulates myogenesis (Marcelle et al., 1997, Reshef et al., 1998). In mice,

the loss of *Noggin* expression results in massive overgrowth of cartilage (Brunet et al., 1998, Capdevila and Johnson, 1998) and leads to the fusion of limb bones (McMahon et al., 1998) showing that BMPs regulate chondrocyte proliferation and skeletal growth (Brunet et al., 1998).

1.5.4 Fibroblast growth factors (FGFs)

FGFs are a family of proteins comprised of at least 20 polypeptides involved in many different biological responses (Martin, 1998). FGF molecules regulate cell proliferation, differentiation, survival as well as cells motility (Baird, 1994). In addition, they act as general mediator in embryonic development, formation of the mesoderm and neural tissues and in regulating patterning of the trunk and tail of vertebrates (Doniach, 1995, Mahmood et al., 1995, Marics et al., 2002, Martin, 1998, Slack, 1994, Yamada and Modak, 1998, Yamaguchi et al., 1994). The loss of FGFs function shows several developmental defects in humans (Chen and Deng, 2004).

The first *in vivo* identification of FGF signalling involved in muscle development was by Itoh et al. (1996). They have found that blockade of FGF signalling suppresses the migration of somatic cells and Pax3-positive cells into the limb (Ciruna et al., 1997). Genetic evidence has also indicated a role of FGF signalling in the migration of Caenorhabditis elegans (C. elegans) myoblasts (Boilly et al., 2000, Montell, 1999), suggesting a function of FGF in migration and delamination of the myogenic progenitors (Ciruna et al., 1997, Marics et al., 2002).

FGF-ligands bind with four transmembrane tyrosine kinase receptors (Martin, 1998). A role for FGF-receptor 1 (FGF-R1) has been demonstrated in myogenesis (Moore et al., 1991, Olwin and Hauschka, 1990), in which its signalling is required to maintain the density of myoblast and organises myofiber *in vitro*. The loss of FGF-R1 signalling accelerates differentiation of muscle cells; subsequently disrupts myofiber numbers and decreases muscle size in limbs (Flanagan-Steet et al., 2000). FGF-receptor 4 (FGFR-4 also known as Frek) has a particular function in skeletal formation. It is expressed in the avian limb myogenic cells at HH stage 20 (Edom-Vovard et al., 2001, Marcelle et al., 1995) and it also associates with myoblast

proliferation (Halevy et al., 1994). Blockade of FGFR-4 signalling results in a dramatic reduction in limb skeletal muscle (Marics et al., 2002).

1.6 Initiation of the Limb bud

Limb formation and regeneration requires the coordinated development and patterning of many different tissues, including soft connective, peripheral nerve, epidermis, bone, muscle and blood vessels (Nacu et al., 2013). The initial formation of limb bud emerges as small lumps of mesenchymal cells originated from the somatic layer of the body wall (LPM) and from the somites (Figure 1.5), following an inductive signal derive from the paraxial mesoderm (Abarca-Buis et al., 2011, Gros and Tabin, 2014, Nishimoto et al., 2015, Schaller et al., 2001, Zuniga, 2015). Lateral plate mesoderm-derived cells are the dominant cells that direct pattering of limb during development (Christ et al., 1977, Hasson et al., 2010, Nacu et al., 2013, Vinciguerra et al., 2010). The LPM of the limb field begins out as an epithelium and eventually produces the mesenchyme of the limb-bud through a process known epithelial-mesenchymal transition (EMT) (Gros and Tabin, 2014).

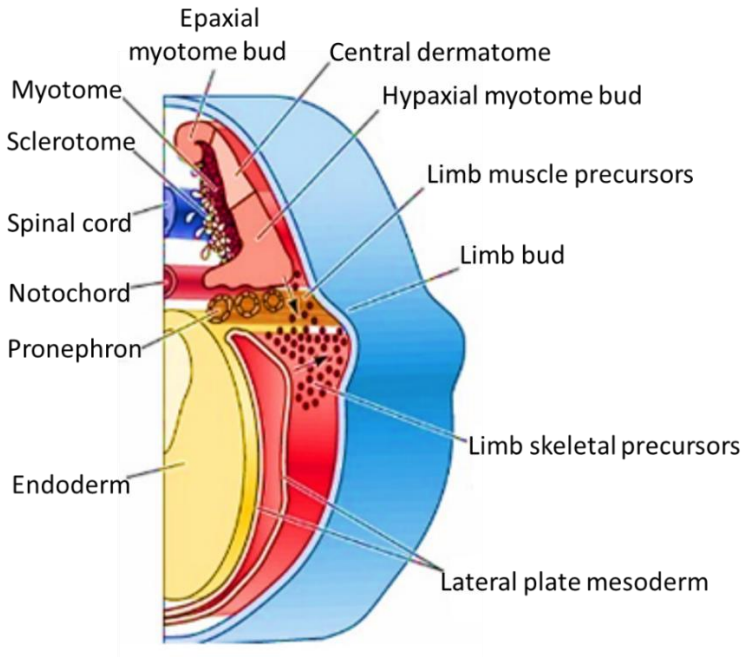


Figure 1. 5: Schematic representation of the early limb development. Mesenchymal cells from the somatic layer of the lateral plate mesoderm and from the somites are migrated into the limb field. These cells accumulate beneath the epidermal tissue and proliferate to form the limb bud (Gilbert, 2010).

Morphologically, the onset of the developing limb in chick embryo appears after 51-56 hours incubation (about HH stage 16 according to Hamburger and Hamilton, 1951) (Fernández-Terán et al., 2006, Fernandez-Teran and Ros, 2008) on the embryo's flank between somites 16-21 for the forelimb and 26-30 for the hindlimb (Houzelstein et al., 1999, Niswander, 2003). Similar swellings appear in the mouse at day 9.5 between somites 7 to 12 for the forelimb and somites 23 to 28 for the hindlimb (Fernandez-Teran and Ros, 2008).

Limb identity and morphology are specified before overt limb formation. Genetic studies have defined genes important for positioning and initiation of limb bud. Therefore, the position at which the forelimb and hindlimb buds develop is regulated by transcriptional factors, including Hox proteins, which determine the A-P body axis during embryogenesis (Kmita and Duboule, 2003, Kawakami et al., 2009, Nishimoto and Logan, 2016). Four Hox gene clusters have been identified in mammals, such as *HoxA*, *HoxB*, *HoxC* and *HoxD* (Duboule, 2007). Absence of Hox genes alters the A-P position of the hindlimb bud (van Den Akker et al., 2001).

T-box family transcription factors, *Tbx5* and *Tbx4*, a LIM-homeodomain, *Islet1* and a paired type homeodomain as well as Pituitary homeobox1 (*Pitx1*) also initiate the identity of limb-type during definition (Lamonerie et al., 1996, Yang et al., 2006). Loss of *Hoxc9* in the forelimb LPM promotes ectopic expression of *Pitx1*. Hox proteins can also directly bind to the regulatory element of Tbx5 and then affect *Tbx5* activation and inhibition (Nishimoto and Logan, 2016). *Tbx5* controls the formation of the forelimb in vertebrates (Agarwal et al., 2003, Koshiba-Takeuchi et al., 2006, Takeuchi et al., 2003), while *Tbx4 Islet1* and *Pitx1* regulate the presumptive hindlimb (Nishimoto and Logan, 2016, Takeuchi et al., 2003). Absence of mouse *Tbx5* and *Tbx4* leads to truncation of all fore and hind limbs skeletal elements respectively (Agarwal et al., 2003, Naiche and Papaioannou, 2003, Rallis et al., 2003). Although *Tbx* genes were thought to regulate fore- and hindlimb identity, it now appears that are primarily regulators of growth and the molecular basis of this remains unclear (Duboc and Logan, 2011, Minguillon et al., 2012). *Tbx-5* also induces *Fgf10*

expression (Figure 1.6) which, in turn, regulates *Fgf8* production by the apical ectodermal ridge (discussed in detail in the following sections).

Another major regulator of *Tbx5* expression is Wnt-β-catenin, which specifies cell fate and induces cell proliferation through regulating the target gene transcription (Sieiro et al., 2016). The pivotal role of the Wnt-β-catenin pathway has been seen in zebrafish and chick embryos during early limb development (Ng et al., 2002). Over time, the limb bud morphologically grows and elongates to be specified along three intrinsic axes: proximal–distal (P-D), anterior–posterior (A-P) and dorsal–ventral (D-V) (Duboc and Logan, 2009, Martin, 1998, Pascoal and Palmeirim, 2007). Primary determination of the anteroposterior axis is probably specified by the expression of the transcription molecules *Gli-3* and *Hand-2* in the anterior and in the posterior parts of the limb respectively (te Welscher et al., 2002a, te Welscher et al., 2002b), as both factors mutually oppose the action of each other. The expression of *Hand2* also identifies the LPM (Charité et al., 2000, Yelon et al., 2000).

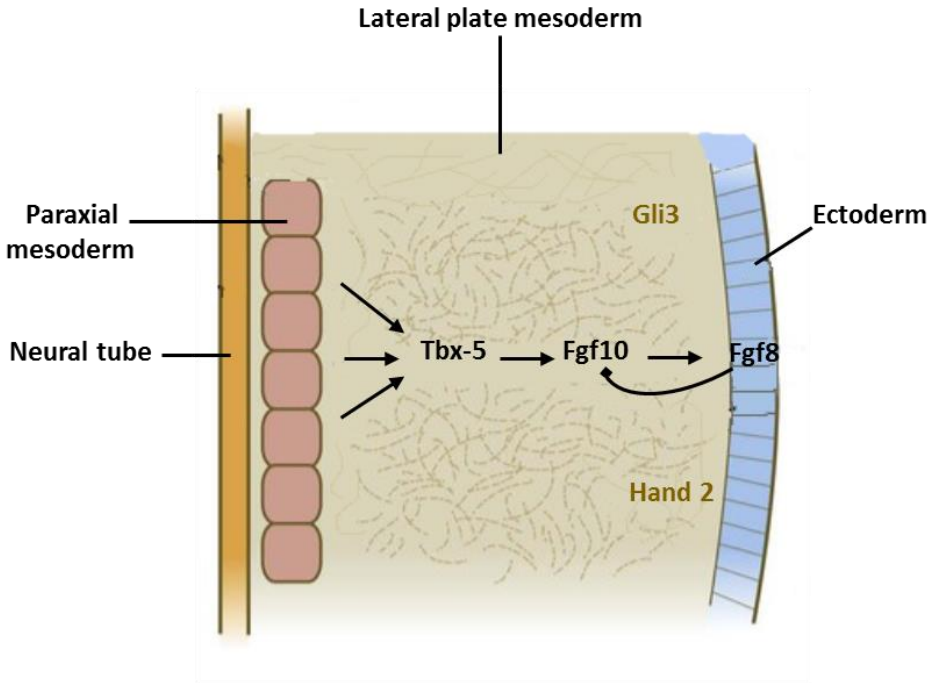


Figure 1. 6: Schematic representation of molecular interactions during the onset of limb development. The *Tbx* transcription factors seem to be the first local factors that direct forces of limb formation. *Tbx-5* stimulates the secretion and expression of *Fgf10* by the mesodermal cells. *Fgf10* in turn induces the overlying ectoderm to secrete *Fgf8*. *Gli3* and *Hand2* are expressed in the anterior and in the posterior part of the limb. They specify primary fixation of the anteroposterior axis of the limb (Carlson, 2014).

1.6.1 Apical Ectodermal Ridge and the Progress Zone Theory

1.6.1.1 Apical Ectodermal Ridge

Three important limb skeletal segments characterise the proximal-distal axis: stylopod, zeugopod and autopod (Fernandez-Teran and Ros, 2008, Zuniga, 2015). The proximal segment, the stylopod, of the forelimb and hindlimb gives rise to the humerus and femur, in the zeugopod (the middle segment) two elements will be established (radius and ulna or tibia and fibula), whereas the distal part, the autopod, has the precursors for the wrist carpalbones (carpals/tarsals), the palm metacarpals/metatarsals and digits (Figure 1.7) (Lopez-Rios, 2016, Niswander, 2003, Tamura et al., 2008). These processes are directed by multiple central signalling systems, which are established in the bud during development (Niswander, 2003, Tickle, 2003). Among these signalling centres is the apical ectodermal ridge (AER), which plays a major role in stimulating and regulating the growth of the limb and patterning of the P-D axis (Casanova et al., 2011, Fallon et al., 1994, Niswander et al., 1993).

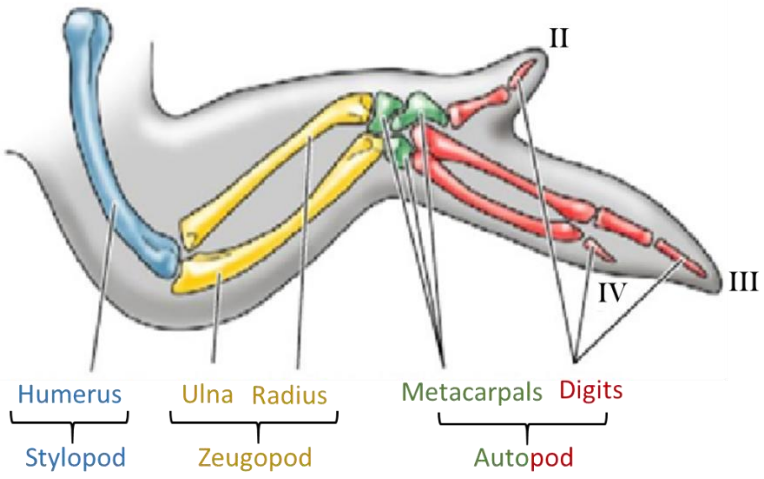


Figure 1. 7: Schematic representation of the limb bones. The proximal element, the stylopod, gives rise to the humerus and femur in the forelimb and hindlimb respectively. The middle element, zeugopod, forms the ulna and radius in the forelimb and the fibula and tibia in the hindlimb. The distal elements includes the autopod gives rise carpals-fingers and digits. These structures are originally cartilaginous, but during the development, the bone replaces most of the cartilage (Gilbert, 2010).

The AER is a specialized structured epithelium; its precursors derive from the ectodermal cells located at the distal end of limb buds (Fernandez-Teran and Ros, 2008). Cell labelling experiments in the chick have established that AER progenitors arise within the ectoderm in both the dorsal and the ventral regions (Altabef et al., 1997). In contrast, studies on mouse have indicated that the AER precursors are derived from ventral ectoderm (Kimmel et al., 2000). The AER plays a fundamental role in limb development. In chicken, microsurgical experiments have established that removal of the AER results in a developmental arrest, which amputates the wing skeleton of chicken (Saunders, 1948). On the other hand, grafts of an AER into to the limb bud promotes ectopic formation of a P-D axis (Fallon et al., 1983). This study identified the relevant signals as FGFs, produced by the AER and provided insights into how AER and FGF signalling regulates the extension and patterning of the limb bud P–D axis (Niswander et al., 1993). In addition to inducing distal fates, AER-FGF plays a fundamental role in mediating different processes throughout limb development, such as regulation of cell movement, survival and sculpting morphology (Boulet et al., 2004, Cooper et al., 2011, Sun et al., 2002, Verheyden et al., 2005).

The AER cells express four Fgf genes (*Fgf4*, *Fgf8*, *Fgf9*, *Fgf17*); these genes are functionally redundant (Mariani et al., 2008), but are essential for maintenance of limb outgrowth. It has been proposed that FGF2-soaked beads could rescue the development of P–D in the chick limb (Fallon et al., 1994, Martin, 1998, Niswander et al., 1993). However, *Fgf8* is the predominant Fgf ligand *in vivo* expressed during normal outgrowth of limbs. It is also expressed in the posterior aspect of the AER, and thus it can compensate for the absence of the *Fgf4*, *Fgf9* and *Fgf17* and sustain relatively limb development (Lewandoski et al., 2000, Mariani et al., 2008). In contrast, *Fgf4*, *Fgf9* and *Fgf17* are restricted to the posterior and distal axis of AER (Niswander, 2003). The AER is first promoted by Fgf10 signalling, which is derived from the lateral plate mesoderm of the prospective limb bud (Casanova et al., 2011). *Fgf10* expression plays an important role in establishing AER-FGF signalling, which is also required for maintain the expression of *Fgf10* itself (Boulet et al., 2004, Ohuchi et al.,

1997, Sun et al., 2002). In Fgf10-deficient mice, the AER does not initiate and this leads to the loss of all fore and hind limbs (Min et al., 1998, Sekine et al., 1999, Xu et al., 1998).

In addition to the role of Fgfs, genetic studies in mouse and embryological manipulation experiments in chick have identified other essential signals from the mesenchyme to regulate limb-bud outgrowth. For example, *Wnt2b* and *Wnt8c* are transiently transcribed in the LPM during the early stages of limb initiation to initiate the formation of forelimb and hindlimb respectively (Kawakami et al., 2001). At later stages, the AER cells express *Wnt3a* and *Wnt5a* while the dorsal ectoderm expresses *Wnt7a* (Kawakami et al., 1999, Riddle et al., 1995). Expression of Engrailed1 (*En1*), a transcription factor, inhibits *Wnt7a* expression in ventral ectoderm (Niswander, 2003). *Wnt7a* is required to maintain *Shh* expression and regulate patterning of the D–V axis through *Lmx1*, which in turn is expressed in the underlying mesenchyme of the limb (Parr and McMahon, 1995, Riddle et al., 1995, Vogel et al., 1995).

In chick embryos, gain- and loss of function studies have shown that Wnt signalling activates *Fgf10* expression in the limb mesenchyme (Kawakami et al., 2001). *Fgf10*, therefore, could act with *Wnt3a* in the ectoderm (Kawakami et al., 2001, Kengaku et al., 1998) to control AER formation through a particular signalling cascade (Niswander, 2003). In mouse embryos, however, the related *Wnt3* gene is activated in the limb ectoderm instead of *Wnt3a* (Roelink and Nusse, 1991, Takada et al., 1994).

1.6.1.2 P-D patterning Models and the outgrowth of limb bud

The AER role of providing for limb development is achieved by maintaining underlying mesenchymal cells in an undifferentiated, mitotically active and highly proliferative state called collectively as the progress zone (PZ) (Niswander, 2003). Several models have been proposed to explain the role of the AER in P-D patterning. One of these, the progress zone model (Summerbell, 1974), suggests that immediately underneath the AER is a group of proliferating cells that drive limb outgrowth (Figure 1.8A).

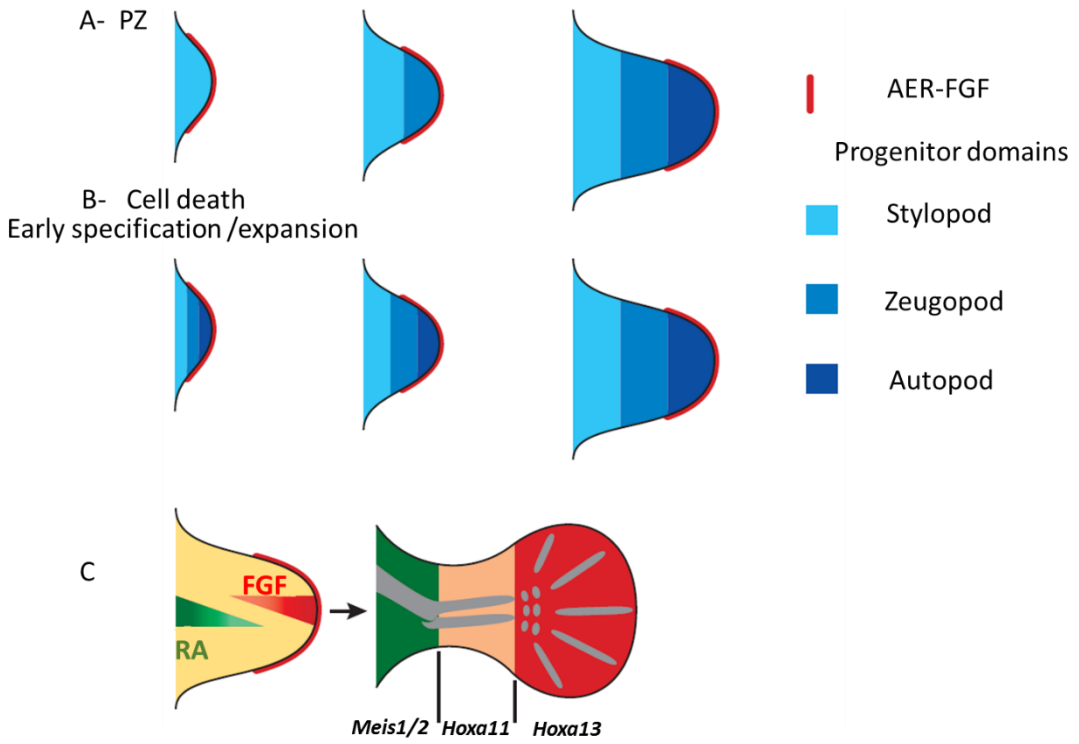


Figure 1. 8: Models and mechanisms of proximodistal limb axis patterning. (A) The old progress zone model. Removal of the AER leads to the loss of distal structures, and the information of P-D positional relays on the time cells have stayed in the progress zone underneath the AER influence. The specification of Stylopod is acquired early compared to zeugopod and autopod which are identified later. (B) Cell death and early specification and expansion model. The positional information of P-D is identified very early through the outgrowth of limb bud and the identified territories sequentially expand through distal progression of limb-bud development. (C) RA-FGF signal model. The specification of cells occurs by emanating and expanding a proximal to distal RA gradient from the early flank and also by emanating AER-FGF signalling from distal to proximal gradient. Integration of both signals over time and space provides the cells as well as cells positional values. The expression of *Meis1*/2 and *Hoxa11/13* transcription factors mark the three territories of PD. Abbreviation: PZ, progress zone (Bénazet and Zeller, 2009, Galloway and Tabin, 2008).

As cells drop out of this zone they acquire successively more distal identities. This model explains the progressive truncations seen in AER removal experiments as the failure to maintain the progress zone. Cells that have already left this region have been specified and are able to grow normally, producing relatively normal proximal structures. For the first time, the progress zone model has introduced the notion of time as an essential component of morphogenetic signalling pathway. It has been, therefore, identified FGFs as the important signals secreted by the AER to stimulate the formation of P-D limb axis (Niswander et al., 1993). The

outgrowth of P-D is rescued by exogenous of FGFs on AER removal, which results in the rapid stimulation of cell death in underlying mesenchyme (Fallon et al., 1994, Niswander et al., 1993).

An alternative model, the early specification model, suggests that P-D identity is established early and the main role of the AER is to maintain survival of limb bud cells (Figure 1.8B) (Dudley et al., 2002). The successive truncations following ridge removal in this model are explained as regions of cell death. Following ridge removal cells underlying the ridge undergo apoptosis. In earlier embryos this extends through the whole limb bud and results in loss of the entire limb. In older limbs only distal cells are affected and thus only this region of the limb is lost. Another model proposes that the P-D axis is patterned by a combination of retinoic acid (RA) signalling from the flank and FGF signalling from the AER (Figure 1.8C) (Capdevila et al., 1999, Mercader et al., 1999, Mercader et al., 2000). These two signals then act together to define the P-D pattern of the developing limb. Although there is support from various experimental systems for these different models none of them can completely explain the formation of the P-D pattern and it is still unclear how this is regulated at the cellular and molecular level.

Although RA has no a direct function in the A-P axis specification, studies in chick and mouse experiments have revealed that ectopic RA signalling alters the expression domains of the *Meis1/2* and *Hoxa11* and *Hoxa13* (Tabin and Wolpert, 2007). These transcription factors are exclusively expressed in the PZ where can also control the clock function (Fernández-Terán et al., 2006, Lallemand et al., 2005, Pizette et al., 2001). *Meis1*/2, mark the future stylopod, while the prospective zeugopod and autopod is marked by *Hoxa11* and *Hoxa13* (Figure 1.8C) (Tabin and Wolpert, 2007). RA can induce Shh when grafted into the posterior limb and thus produce an ectopic ZPA. RA is a vitamin A derived non-peptide molecule that acts as a ligand for RA receptors (RARs) (Abarca-Buis et al., 2011, Rhinn and Dollé, 2012). RA has a low molecular weight (about 300Da) and has two receptors that mediate its signalling: retinoid X receptor (RXR) and retinoic acid receptor (RAR) (Bastien and Rochette-Egly, 2004). Each of these receptors composes three isoforms: Alpha, α; Beta, β; and Gammaα, γ

(Chawla et al., 2001, Rochette-Egly and Germain, 2009). RA signalling affects pattern development in vertebrate embryos. It regulates the initiation and formation of limb in zebrafish, chick and mouse embryos (Begemann et al., 2001, Maden, 2002, Stratford et al., 1996). At early stages of the developing limb, the level of RA is highest proximally and it controls myoblast migration into the limb (Reijntjes et al., 2009). In chick embryos, blocking of RA signalling indicates its important role in the transcriptional regulation of *Tbx4* (Nishimoto et al., 2015). In addition, RA is synthesised by Retinaldehyde dehydrogenase (RALDH); absence of this enzyme leads to the complete disruption in limb bud development, but it does not affect Shh signalling (Niederreither et al., 1999). However, RA clearance from the distal mesenchyme requires AER-FGF signalling system (Probst et al., 2011).

1.6.2 The Anterior-Posterior Axis and the Zone of Polarizing Activity

In the limb bud, Fgf signalling also functions to maintain the activation of another important secreted factor, Sonic hedgehog (Shh) which is mainly produced in the posterior limb mesenchyme called the zone of polarizing activity (ZPA) (Laufer et al., 1994, Niswander et al., 1994, Riddle et al., 1993, Riddle et al., 1995). In the chick, formation of ZPA roughly initiates at the same stage when limb bud becomes visible (Martin, 1998). The ZPA acts as a signalling centre and secretes the diffusible morphogen, Shh, which in turn controls the patterning of A–P axis during limb development (Riddle et al., 1993). Therefore, grafting the ZPA into the anterior margin of limb bud leads to mirror image duplication of the limb posterior (Saunders and Gasseling, 1968). Shh is also responsible for maintaining *Fgfs* expression in the overlying AER, such as *Fgf4* (Laufer et al., 1994, Niswander et al., 1994), *Fgf9*, and *Fgf17* (Sun et al., 2000). This role of Shh occurs through up-regulating Gremlin (Grem1) (Khokha et al., 2003, Michos et al., 2004, Zúñiga et al., 1999), a bone morphogenetic protein (Bmp) antagonist (Hsu et al., 1998). Grem1 prevents Bmps from downregulating *Fgf4*, and regulates limb size by breaking down the Shh-Fgf4 loop in later development (Figure 1.9) (Gañan et al., 1998, Khokha et al., 2003, Pizette and Niswander, 1999, Scherz et al., 2004).

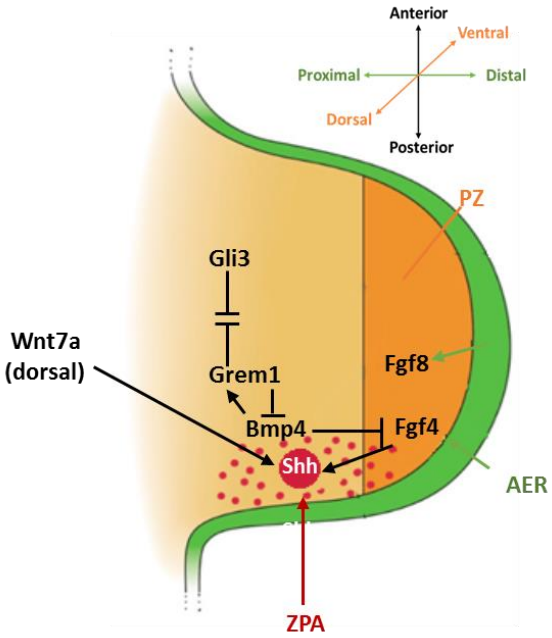


Figure 1. 9: Schematic representation of molecular interactions coordinating the developing limb bud along the three limb axes. The AER expresses fibroblast growth factors *Fgfs* that regulate the proximal–distal (P–D) axis. The posterior mesenchyme region is ZPA. This region produces Shh, which organizes the A-P axis (anterior-posterior). The dorsal–ventral (D–V) axis is regulated by the interaction between bone morphogenetic proteins (BMPs), Grem1 and Gli3 under the control of *Wnt7a* from the dorsal ectoderm. Abbreviations: PZ-Progress zone (orange), AER- apical ectodermal ridge (green), ZPA- zone of polarising activity (red). Figure modified from (Niswander, 2003, Slack, 2006).

Although *Fgf8* is not dependent on *Shh* to maintain its expression (Chiang et al., 2001), absence of Grem1 results in disorganization of the AER itself and downregulation of *Fgf8* (Khokha et al., 2003). This, therefore, affects expansion and specification of the zeugopod as well as autopod (Chiang et al., 2001, Michos et al., 2004, Khokha et al., 2003, Kraus et al., 2001). Consequently, the signalling centres in the AER and ZPA are interdependent for their activity in the limb bud, and this positive-feedback loop controls a normal structure of the limb. In addition, AER formation requires signalling of Bmp4 from the mesenchyme as well as the Bmp receptor-1 (Bmpr-1) from the ectoderm. Early inactivation of these factors completely prevents the formation of AER and leads to loss of the limb bud (Ahn et al., 2001, Pajni-Underwood et al., 2007). However, inactivation after limb bud formation leads to an upregulation of AER-FGF, including *Fgf4* and *Fgf8* (Pajni-Underwood et al., 2007). This in turn stimulates the Shh production from ZPA to maintain the mesenchymal signalling cascade, which suppresses the production of BMP, and thereby continues to induce

Fgf8 production. *Fgf8* also maintains ZPA activity particularly Shh secretion, hence establishing a positive-feedback loop (Scherz et al., 2004). The Shh-FGF signalling loop operates during limb formation until day 6 (E6) in mouse and HH stage 27 in chick (Sanz-Ezquerro and Tickle, 2000), when *Gremlin* and *Fgf4* cease expression and Shh activity is downregulated (Riddle et al., 1993).

1.7 Formation of limb skeletal muscle

1.7.1 Delamination and migration of myogenic progenitor cells

A prerequisite for the migratory myogenic progenitors into the limb buds is the delamination of these cells from the origin site, the lateral dermomyotome lip, in response to several signals emanating from the lateral plate mesoderm (Buckingham et al., 2003, Francis-West et al., 2003). At limb levels, the ventrolateral edges of the dermomyotome de-epithelialize; then individual mesenchymal progenitors delaminate and migrate to invade the limb somatopleural mesoderm (Figure 1.10) (Baker et al., 1988, Marics et al., 2002).

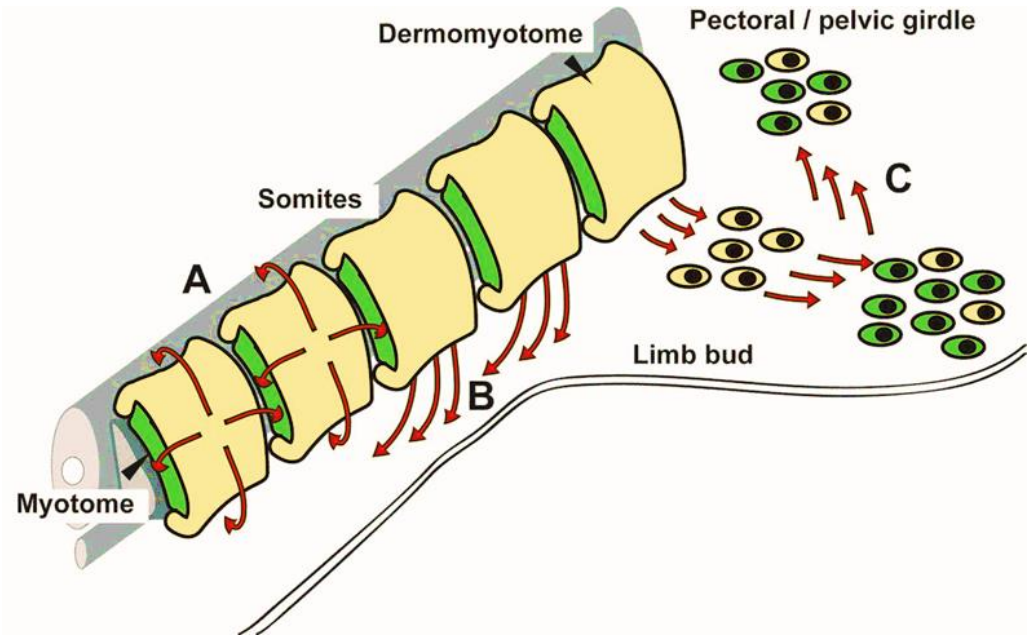


Figure 1. 10: Schematic representation of the origin of limb muscle. (A) Distribution of somatic cells to form other compartments. (B) and (C), delamination and migration of progenitor cells from the hypaxial lip of the dermomyotome to form body muscle, deep back muscle and limbs (Sinclair et al., 2016).

The migration relies principally on the activities of the tyrosine kinase receptor c-met with the ligand scatter factor/hepatocyte growth factor (SF/HGF) and on Paired Box 3 (*Pax3*) as well as *Lbx1* transcription factor (Buckingham and Rigby, 2014, Masyuk and Brand-Saberi, 2015, Tajbakhsh and Buckingham, 2000, Vasyutina and Birchmeier, 2006). In addition to SF/HGF, it has been demonstrated that BMPs secreted by the lateral mesoderm act to keep Pax3-positive myogenic precursor cells (MPCs) as undifferentiated cells for migration and reaching their target (Linker et al., 2003, Scaal et al., 1999). Migrating MPCs also express secreted frizzled-related protein 2 (Sfrp2*)*, an antagonist of Wnt (Anakwe et al., 2003), for preventing MPCs precocious differentiation since Wnt enhances the differentiation of muscle cells. Once cells are in the limb, they initiate the proliferation and differentiation to eventually constitute individual muscle (Christ and Brand-Saberi, 2002, Hutcheson et al., 2009, Kardon et al., 2002). During myogenesis, MPCs are become specified as myoblasts, which subsequently differentiate into mononuclear myocytes and then later fuse together to form multinucleated myofibers (Emerson and Hauschka, 2004, Guntur and Rosen, 2013). During these processes, myogenic cells are segregated to dorsal and ventral masses on either side of the limb (Figure 1.11) (Schramm and Solursh, 1990).

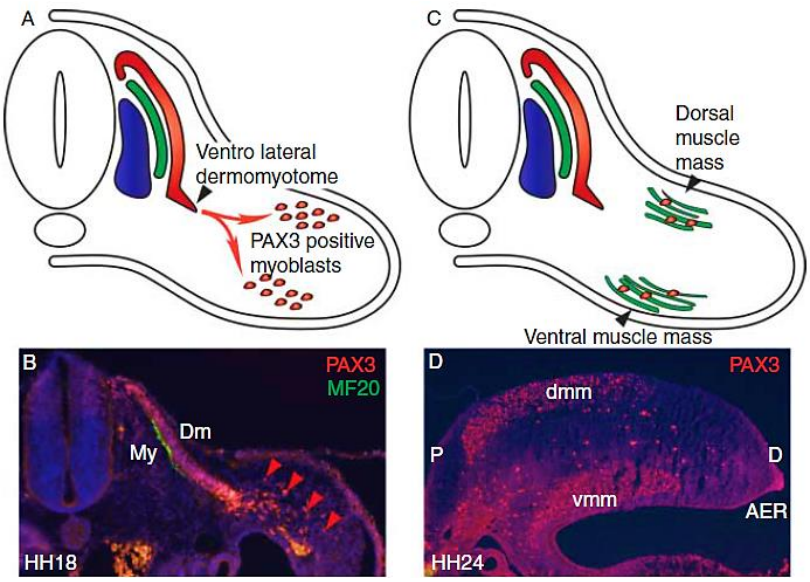


Figure 1. 11: Schematic representation of migrating myogenic progenitor cells from the ventrolateral dermomyotome into the limb bud. (A). Migration of muscle precursor from the ventrolateral dermomyotome into the limb bud and forming dorsal and ventral muscle masses. (B), immunostaing through chick embryo at HH stage 20. Pax3 antibody (red), Red arrows indicate the delamination of

myogenic positive cells into the limb. (C), schematic representation of muscle masses in the dorsal and ventral parts of the limb. (D) immunostaing section (labelled with Pax3 antibody) through chick limb at HH stage 24 shows the dorsal and ventral muscle masses (red). Abbreviations: Dm, dermomyotome, My, myotome; AER, apical ectodermal ridge; P, proximal; D, distal; vmm, ventral muscle mass; dmm; dorsal muscle mass (Mok and Sweetman, 2011).

1.7.2 Signals by the ectoderm and limb mesenchyme

Loss of function studies in mice (Harel et al., 2012, Kelly et al., 2004, Lu et al., 2002, Rudnicki et al., 1993, Tajbakhsh et al., 1997) and grafting either tissues or signalling molecules into chick embryos (Hacker and Guthrie, 1998, Harel et al., 2009, Mootoosamy and Dietrich, 2002, Noden et al., 1999, Tzahor et al., 2003) have revealed different extrinsic and intrinsic regulatory pathways that regulate limb myogenesis. The extrinsic pathways include external factors, such as Wnt, BMPs, Transforming growth factor beta (TGFβ), Notch, Noggin, sFRP, Shh, myostatin, HGF/c-met, FGFs, RA, Insulin and insulin-like growth factors (IGFs) (Duprez et al., 1996, Goldspink et al., 2001, Mok et al., 2014, Rubin et al., 2006, Valdés et al., 2013). Additionally, the earliest intrinsic pathways and lineage-specific genes that express during myogenesis are Six proteins, Pax and Mrfs genes (Fedon et al., 2012). Most of these signals have been implicated in the control of myoblast proliferation. For example, Notch and Wnt signalling play crucial roles in proliferation and differentiation of muscle cells as well as controlling satellite cell fate (Cisternas et al., 2014, von Maltzahn et al., 2012). Furthermore, myostatin, a member of the TGF-β superfamily is also an important regulator for muscle development (Amirouche et al., 2009, McPherron et al., 1997). Myostatin stimulates quiescence of cells in the limb bud during embryogenesis; in the myotome, it promotes myogenic differentiation as well as depletion of myogenic precursors (Manceau et al., 2008, Otto and Patel, 2010).

Shh, Wnts and BMPs, which are known to control axial muscle differentiation, all are expressed by the limb mesenchymal cells. Although Shh is not required for the expression of *Mrfs* during limb muscle growth and maturation (Kruger et al., 2001), it directly controls *Myf5* in the ventral muscle mass (Anderson et al., 2012, Hu et al., 2012). Therefore, Shh is

required for MPCs to make the proximo-distal muscles in the ventral limb (Deries and Thorsteinsdóttir, 2016).

Wnt genes are expressed in many embryonic tissues, such as the ectoderm and in the neural tube (McMahon, 1992, Parr et al., 1993, Schubert et al., 2002). These genes play a central role in regulating embryonic development, including cell epithelialisation process, polarity, migration, proliferation and differentiation (Geetha-Loganathan et al., 2008, Parr and McMahon, 1995, Schmidt et al., 2004). Wnt signalling also influences the initiation of limb bud, limb formation and early limb patterning (Geetha-Loganathan et al., 2008). *Wnt6* produced by the ectoderm can activate *Myf5* expression (Geetha-Loganathan et al., 2005), although other studies have demonstrated that Wnt signalling is required in later limb development and morphogenesis (Anakwe et al., 2003, Hutcheson et al., 2009). Anakwe and colleagues have found that limb mesenchymal cells express *Wnt5a* and *Wnt11* to determine specification of fibre type (Anakwe et al., 2003) and canonical β-catenin signalling induces foetal myoblast differentiation (Hutcheson et al., 2009).

BMPs are further regulators of cell differentiation. Disruption of Bmp signals results in total absence of myofibers, as the pool of proliferative presumptive myogenic cells is depleted (Pizette and Niswander, 2000). In this model, BMPs can induce proliferation and/or suppress differentiation of muscle precursor cells in the somites and limbs (Amthor et al., 1999, Amthor et al., 1998). BMPs produced by ectoderm of the limb inhibit muscle development in a similar way to their effects on development of axial muscle (Amthor et al., 1998) but are later counteracted by the effect of Noggin in the limb mesenchymal core (Amthor et al., 1998). Experiments in adult myogenesis have demonstrated that activation of BMP signalling is associated with the activation of satellite cells (SCs) (Ono et al., 2011), undifferentiated mononuclear myogenic progenitors that lie in between the basal lamina and the plasma membrane of mature myofibres (Mauro, 1961).

c-met is also required for limb muscle outgrowth (Bladt et al., 1995). It encodes a transmembrane tyrosine kinase receptor for the ligand, SF/HGF (Bottaro et al., 1991). The transcription of SF/HGF is regulated through signals involved in chick limb bud patterning, such as the ZPA signals. Removing this region leads to ectopic and enhancement of *Hgf* expression in posterior limb mesenchyme (Scaal et al., 1999). *In vitro* study has revealed that the c-met promoter possesses a specific binding site of *Pax3*, which in turn can modulate the expression of *c-met*. Consequently, *Pax3* can regulate the release of the migratory progenitors *in vivo* through c-met activity (Christ and Brand-Saberi, 2002). As *SF/HGF* is mainly expressed in the limb bud, it drives c-met activity and then promotes migration of myogenic progenitors. In chick embryos, implanting HGF soaked beads into the limb promotes migration of myoblasts-expressing *Pax3* from somites (Brand-Saberi et al., 1996) and maintains undifferentiated state of myoblast in the older stage of the developing limb (Scaal et al., 1999). Mice deficient for c-met lack skeletal muscle in the limb (Bladt et al., 1995, Maina et al., 1996) due to the disruption of myogenic progenitors populating in the limb.

1.7.3 Development of Limb Tissues

It is difficult to distinguish several types of cells in the mesenchyme of the early developing limb bud by only depending on their morphology. However, a range of mesenchymal cells, which derive from different origins, are present (see figure 1.12). Initially, The LPM contains the precursors for all population tissues of limb, including skeletal structures and connective tissues, tendon, ligaments, cartilage, dermis and muscle sheaths (Slack, 2006, Stricker and Mundlos, 2014) with the exception of muscle tissue. Mesenchymal cells originating from somites migrate into the limb bud to provide myogenic progenitors and endothelial cells. Another important migrating cell population derives from the neural crest. These cells eventually contribute sensory nerves, the myelinating glia (Schwann cells) and melanocytes (pigment cells) (Figure 1.12). In the head and neck neural crest cells (NCCs) play an important role in controlling the myogenic pattern in the branchiomeric and cucullaris muscles, although they do not affect their early myogenesis (Pu et al., 2015).

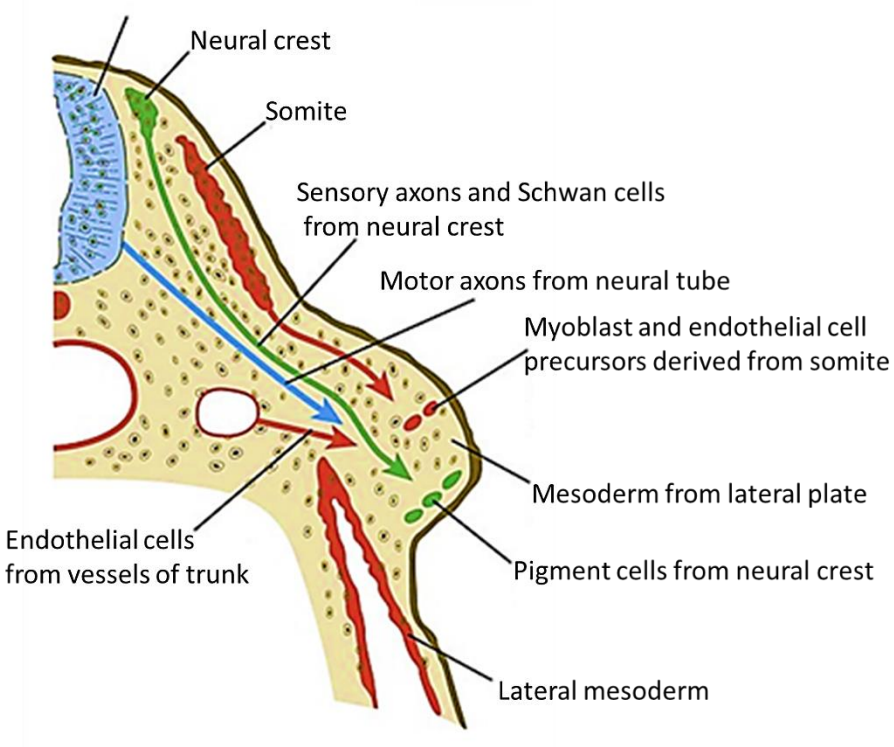


Figure 1. 12: Schematic representation of several types of mesenchymal cells that destined to enter the limb bud during development. Somite and the neural crest form most of epithelial and myogenic cell progenitors. These cells migrate into mesenchymal limb bud before starting differentiation and prior to receiving the essential pattern information leading them to producing an elaborate network of vasculature and muscles (Carlson, 2014).

Blood and nerves play an important role during limb initiation and at the early muscle differentiation. As the limb grows, blood vessels indirectly regulate the morphogenesis of skeletal by producing platelet derived growth factor subunit b (PDGFb), which influences the outgrowth of muscle connective tissue (Tozer et al., 2007). PDGFb promotes the growth of muscle connective tissue prior to segregation of muscle masses. This suppresses the development of muscle in the segregated areas and thus leads to splitting the mass (Tozer et al., 2007). In addition, nerves migrate into the dorsal and ventral muscle masses from the developing spinal cord (Hurren et al., 2015). It is worth mentioning that muscle growth is heavily influenced by the presence of the nerve and its electrical activity (Ashby et al., 1993, Ross et al., 1987). In addition, the innervation promotes proliferation of MPCs as fewer mononucleated cells are seen in muscles of denervated embryos (Ross et al., 1987). It seems likely that nerves have a role during the differentiation of muscle progenitors cells by secretion of particular molecules, including Acetylcholine and Neuregulins (Deries and

Thorsteinsdóttir, 2016). Neuregulin1, which induces the proliferation of myogenic cells, is produced from the ventral NCCs (Van Ho et al., 2011). Loss of the migrated NCCs leads to a loss of fore and hind limb muscles of mouse embryos at day 12.5 (Van Ho et al., 2011). The NCCs, which originate in the dorsal region of the neural tube, are transient migratory and multipotent population of cells that play a crucial role in the developing embryo (Manderfield et al., 2015, Noden, 1975). During embryogenesis, NCCs convert from an epithelial to mesenchymal form then migrate to particular regions of the embryo, where they adopt different cell types, such as melanocytes (Dupin and Le Douarin, 2003, Lallier, 1991), cartilage, sensory neurons and glia (Barraud et al., 2010, Chai et al., 2000, Grim and Christ, 1992, Li et al., 2007, Marmigère and Ernfors, 2007).

Two populations of crest cells have been identified in the developing limb. The first cell population migrates into the limb bud at stages before the motor axons invade the limb. In this population, two types of cells have been shown: Merkel cells and melanoblasts (Grim and Christ, 1992, Thomas and Erickson, 2008). At later stage of limb development, the second crest cell population invades the limb bud and migrates along a dorsal and a ventral route by following the path of ingrowing nerves. The second population of crest cells also forms terminal glia as well as Schwann cells (Grim and Christ, 1992). In chick embryos, NCCs trigger myogenesis process in the DML by inducing Notch signalling (Rickmann et al., 1985, Rios et al., 2011). In addition, neural crest cells have another important role by transporting the expression of both *Wnt1* and *Wnt3a* from the dorsal neural tube into the DML (Serralbo and Marcelle, 2014).

1.8 Muscle transcription factors

During myogenesis cells progress from myogenic precursors through myoblasts, then myocytes that eventually become mature myofibres. These processes require the activity of Pax genes and members of the MRF family (Buckingham and Relaix, 2007) along with several other transcription factors.

1.8.1 Pax genes

The paired box (Pax)-containing transcription factors *Pax3* and its paralogue *Pax7* are transcribed in a population of myogenic progenitors that are maintained as uncommitted embryonic cells (Buckingham and Relaix, 2007, Kassar-Duchossoy et al., 2005, Relaix et al., 2005, Tremblay and Gruss, 1994). Both *Pax3* and *Pax7* have been implicated the development of muscle (Lang et al., 2007). In chick and mouse both *Pax3* and *Pax7* are expressed in the dermomyotome (Bober et al., 1994, Buckingham and Relaix, 2007, Galli et al., 2008, Goulding et al., 1991, Goulding et al., 1994).

Signals from the lateral plate and the intermediate mesoderm regulate *Pax3* expression in the ventrolateral dermomyotome of somites (Pourquié et al., 1995). Otto and colleagues have also shown that the overlying ectoderm and lateral plate mesodermal cells both are important for the expression of *Pax3*, but *Pax7* requires the overlying ectoderm signals in addition to the neural tube (Otto et al., 2006). Shh from the notochord negatively regulates *Pax7* expression (Goulding et al., 1994, Otto et al., 2006). However, Wnt6 signalling from the surrounding tissues activates both *Pax3* and *Pax7* (Otto et al., 2006).

Pax3 plays a vital role in delamination and migration of muscle precursor cells into the limb, as Splotch mice deficient for *Pax3* function fail to develop limb muscle (Bober et al., 1994, Goulding et al., 1994, Tremblay et al., 1998). *Pax3* has an important role in activation of c-met, which is responsible for migration of muscle precursor cells from the lateral dermomyotome into the limb (Bladt et al., 1995, Epstein et al., 1996). In chick limb, *Pax3* is expressed at early stages of development (Galli et al., 2008) and then is downregulated in both limbs during myogenic differentiation (Figure 1.13). By HH stage 26, limb progenitors have completed their migration and the expression of *Pax3* will then be restricted in muscle subdomains of the developing limb (Otto et al., 2006).

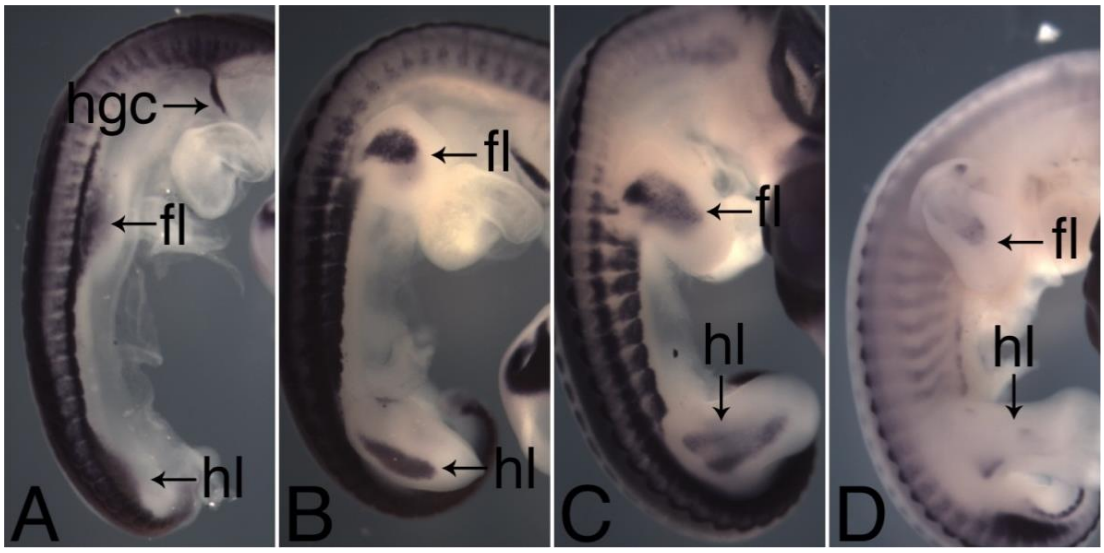


Figure 1. 13: The whole-mount *in situ* hybridisation in chick embryos (HH stages 20-26) stained by *Pax3* myogenic marker. *Pax3* is strongly expressed in both limbs from HH stage 20 to 24 (A-C). *Pax3* is downregulated in the hindlimb than in the forelimb at the differentiation stage, HH 26 (D). Abbreviation: fl, forelimb; hl, hindlimb. Images provided by D Sweetman (not published).

As well as the regulation of migratory behaviour of muscle precursor cells, *Pax3* controls their survival (Borycki et al., 1999b) at the dermomyotome edges; specifically cells located in the hypaxial lip where muscle progenitors delaminate and then migrate into the limb (Bober et al., 1994, Goulding et al., 1994, Schubert et al., 2001).

Disruption of *Pax3* expression, using antisense oligonucleotides, impairs *MyoD* expression and is accompanied by a significant increase in programmed cell death (Borycki et al., 1999b). Knockout and the loss of function studies in mice showed that in the absence of *Myf5* expression, *Pax3* is required for *MyoD* activation to stimulate the myogenic determination program (Tajbakhsh et al., 1997) in the trunk, but not in the head (Tzahor, 2015). This is because myogenic precursor cells in the head do not express *Pax3* (Hacker and Guthrie, 1998, Harel et al., 2009, Tzahor, 2015).

The expression of *Pax7* initiates later in somites, and it is widely expressed in the dermomyotome central region (Applebaum et al., 2014, Gros et al., 2005, Horst et al., 2006, Jostes et al., 1990, Relaix et al., 2004). In mouse, *Pax7* expression is required for the survival of fetal myocytes and for the maintenance of adult satellite cells (Lepper et al., 2009, Oustanina et al., 2004, Relaix et al., 2005); the loss of this gene severely affects muscle

regeneration as satellite cells require *Pax7* for self renewal (Seale et al., 2000). Some adult satellite cells also express *Pax3* (Relaix et al., 2006, Wang and Rudnicki, 2011).

1.8.2 Myogenic regulatory factors (MRFs)

The members of the MyoD family (MRFs) play a central function in determination and differentiation of skeletal myogenic precursor cells. Together with another essential regulator, the myocyte-specific enhancer factor 2 (MEF2) and E proteins, they coordinate the expression of muscle-specific genes in embryogenesis (Figure 1.14) (Berkes and Tapscott, 2005, Edmondson and Olson, 1993, Estrella et al., 2015, Moncaut et al., 2013, Rudnicki and Jaenisch, 1995, Sabourin and Rudnicki, 2000, Weintraub, 1993, Watabe, 2001).

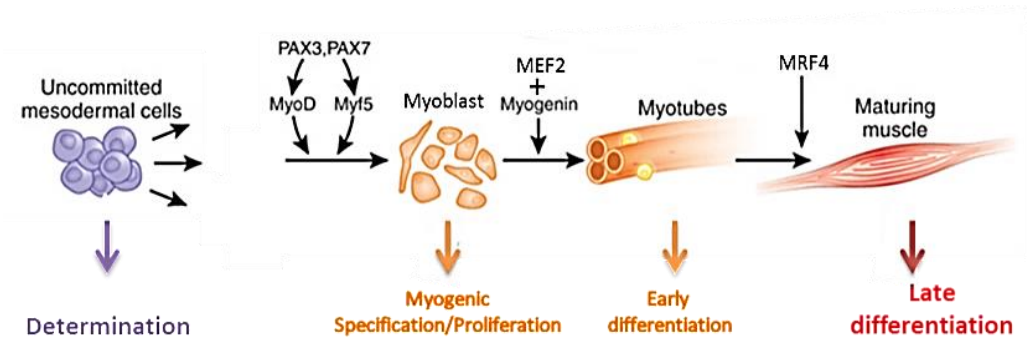


Figure 1. 14: The specification of skeletal muscle from the mesoderm requires myogenic transcription factors including Pax and MRFs. During myogenesis, mesodermal progenitor cells proliferate and differentiate to form myoblast, which is the first form of muscle through myogenesis. The differentiated form depends on the interaction between *Pax3* and *Pax7* and determination myogenic factors, *Myf5* and *MyoD*. The early differentiation of myoblasts forms multinuclear myotubes, depending on the *Myog* regulatory factors and myocyte enhancer factor-2 (*Mef2*). *Mrf4* regulates the late form of mature muscle. Figure modified from (Hettmer and Wagers, 2010).

MRFs belong to a family of basic helix-loop-helix transcription factors that form a regulatory network initiating the outgrowth of muscle fiber and controls the expression of muscle-specific genes (Olson, 1992, te Pas et al., 2007). MRFs form heterodimers with E proteins (Olson, 1992), which bind to a DNA sequence (CANNTG) also known as the enhancer box (E-box) (Moncaut et al., 2013). This DNA motif is present in the promoters of several specific skeletal muscle genes, and it regulates gene expression by MRFs (Emerson Jr, 1993, Olson and Klein, 1994, Weintraub et al., 1991). The MRF members include Myf5 (Braun et al., 1989), MyoD (Mf3) (Davis

et al., 1987), Myogenin (Myog, Myf1) (Edmondson and Olson, 1990, Wright et al., 1989) and Mrf4 (Myf6, Herculin) (Braun et al., 1990, Rhodes and Konieczny, 1989).

1.8.2.1 Myf5

Myf5 is the first MRF to be expressed in muscle precursors, initiating in the dorsal medial lip (DML) of the dermomyotome at day 8 of mouse embryogenesis (Ott et al., 1991). In the developing mouse limb, *Myf5* is the earliest gene MRF expressed at day 10.5 in embryonic myoblasts and then maintained in fetal and adult myoblasts (Biressi et al., 2007, Fujisawa-Sehara et al., 1990, Kuang et al., 2006, Ott et al., 1991). Furthermore, *Myf5* is also expressed in chick limb by HH stage 20 (Delfini et al., 2000) and in most quiescent satellite cells (Beauchamp et al., 2000, Cornelison and Wold, 1997). Indeed, this gene coordinates the other MRFs and promotes their expression (Delfini and Duprez, 2004, Sweetman et al., 2008).

1.8.2.2 MyoD

The first identification of the MRF transcription factors was in a subtractive hybridisation procedure, showing the *in vitro* ability of *MyoD* gene to convert 10T1/2 fibroblasts to the myogenic fate (Davis et al., 1987, Ren, 2009, Sabourin and Rudnicki, 2000, Weintraub et al., 1991). In the mouse limb, *MyoD* is first expressed at day 10.5 in embryonic myoblasts and myofibers (Sassoon et al., 1989); then in both fetal and adult myoblasts and myofibers (Cornelison and Wold, 1997, Kanisicak et al., 2009, Voytik et al., 1993, Yablonka-Reuveni and Rivera, 1994). Amthor and colleagues identified *MyoD* expression in chick limb by HH stage 20 (Amthor et al., 1998). Unlike *Myf5*, the expression of *MyoD* is rarely observed in quiescent satellite cells (Cornelison and Wold, 1997, Zammit et al., 2002).

1.8.2.3 Myog

Myog is required for myoblasts to enter the differentiation pathway (Andrés and Walsh, 1996). In the mouse embryo, *Myog* is expressed in the limb about day 11.5 (Sassoon et al., 1989) principally in differentiated myocytes (Ontell et al., 1993, Cornelison and Wold, 1997, Voytik et al., 1993,

Yablonka-Reuveni and Rivera, 1994). Mice lacking *Myog* are deficient in mature muscle fibres (Hasty et al., 1993). Knockout of *Myog* in mice results in the loss or reduction of the skeletal muscle in the tongue, diaphragm and limb (Hasty et al., 1993, Nabeshima et al., 1993, Ren, 2009). Therefore, mono-nucleated muscle myoblasts predominate rather than multi-nucleated mature myotubes, indicating faulty terminal differentiation of myoblast (Mastroyiannopoulos et al., 2012).

1.8.2.4 Mrf4

Mrf4 has a distinctive biphasic expression pattern (Bober et al., 1991, Hinterberger et al., 1991, Ren, 2009). For a long time, this gene has been classified as a differentiation gene, but in fact, it acts as a determination factor during myogenesis (Buckingham and Rigby, 2014, Kassar-Duchossoy et al., 2004). In mouse, *Mrf4* is expressed early in the somitic bud; in the absence of *MyoD* and *Myf5*, *Mrf4* can activate myogenesis. This indicates that *Mrf4* functions in differentiating and determinating activities (Kassar-Duchossoy et al., 2004, Summerbell et al., 2002). However, the highest level of *Mrf4* expression has been detected in the nuclei of adult muscle (Gayraud-Morel et al., 2007).

Overall, it is generally accepted that MRFs are very important for muscle cell specification and determination, as observed via genetic loss of function-analyses. *Myf5* and *MyoD* have been proposed to fulfil similar roles during skeletal muscle growth and development, as mice lacking either *Myf5* or *MyoD* are able to generate skeletal muscles (Rudnicki et al., 1993). Because *Myog* is only expressed as myoblasts differentiating into myocyte, the early stages of myogenesis mainly depend on the three other regulatory factors, *Myf5*, *MyoD* and *Mrf4* (Kassar-Duchossoy et al., 2004). The absence of *Myf5* and *Mrf4* leads to the ablation of the early myotome and mislocated delaminated cells (Chen and Goldhamer, 1999). Knockout studies have shown that the loss of *Myf5*, *MyoD* and *Mrf4* together disrupts formation of all skeletal muscles (Kassar-Duchossoy et al., 2004, Rudnicki et al., 1992). This, therefore, suggests that *Myog* is a critical factor in cell fusion and terminal differentiation, but not in commitment to myogenic lineage (Hasty et al., 1993). *MyoD* and *Myog* also bind to regulatory regions

of muscle specific-genes whose expression is controlled during the differentiation of skeletal muscle (Blais et al., 2005, Cao et al., 2006). Interestingly, *MyoD* binding is seen at different sites before the beginning of differentiation (Blais et al., 2005). This suggests that *MyoD* regulates the epigenetic reprogramming of myogenic cells (Cao et al., 2010).

1.8.3 Myocyte enhancer family 2 transcription factors (Mef2)

Mef2 proteins are homo-dimers that bind to the A/T rich sequence presented in a variety of enhancers and promoters in muscle specific genes (Gossett et al., 1989). Different isoforms of Mef2, such as Mef2a, Mef2b, Mef2c and Mef2d have been identified in vertebrates, and all contain MADS-box and DNA-binding domain (Potthoff and Olson, 2007, Shore and Sharrocks, 1995, West et al., 1997). In Drosophila, only one *mef2* gene is present to play a crucial role in the growth of all muscle lineages in somatic and cardiac muscles (Lilly et al., 1995).

Mef2 can synergistically act with MRFs to activate the expression of muscle-specific genes in mice (Molkentin et al., 1995, Naya et al., 1999, Olson, 1992). *Mef2* and *MyoD* interact genetically through their transcriptional activities (Black et al., 1998). Specifically, transfection of *Myog* into mouse fibroblasts stimulates *Mef2* DNA binding activity, which in turn induces *Myog* expression (Ridgeway et al., 2000) and results in regulation the differentiation of myogenic cells (Neuhaus and Braun, 2002). On the other hand, *Mef2* cannot activate the expression of *Myf5*, *MyoD* and *Mrf4*, and these factors in turn do not activate *Mef2* expression (Ridgeway et al., 2000).

Mef2 proteins are expressed during the formation of smooth and cardiac muscles as well as in skeletal muscle differentiation *in vivo* (Black and Olson, 1998, Naya et al., 1999, Rhodes and Konieczny, 1989). They are also expressed in the secondary heart field to regulate looping of the heart and formation of the right ventricular chamber (Dodou et al., 2004). Furthermore, Mef2 can regulate diverse intracellular signalling pathways that control muscle development and hypertrophy (Black and Olson, 1998). It also acts as a co-regulator to mediate the expression of myogenic bHLH proteins (Molkentin et al., 1995).

1.8.4 *Six*, *Lbx* and *Meox* genes

The Six homeodomain family genes interact with other transcriptional factors, such as Dachshund family transcription factor-2 (*Dach-2*) and Eyes absent homologue-2 (*Eya-2*) to generate a regulatory module for *Pax3* expression (Figure 1.15) (Heanue et al., 1999, Kawakami et al., 2000, Laclef et al., 2003) with an essential upstream function in myogenesis. Experiments performed in chicken embryos showed that ectopic Six1 and Eya activate the expression of *Pax3* and myogenic regulatory genes (Heanue et al., 1999). In addition, *Six1* and *Six4* homogenes control the initial delamination and migration steps of muscle precursors from the somite into the limb bud by regulating *Pax3* expression (Grifone et al., 2005). Both *Six1* and *Six4* also control *Myog* expression (Spitz et al., 1998). *Six1* null mutant mice lack limb skeletal muscle (Laclef et al., 2003); also *Six1*/*Six4* double mutants have a more severe phenotype in the limb and the trunk, where skeletal muscles in mice are absent (Grifone et al., 2005).

The homeobox gene, Ladybird Homeobox 1 (*Lbx1*) is exclusively expressed in the lateral dermomyotome from where muscle progenitors migrate (Dietrich et al., 1998, Jagla et al., 1995). In mice and chicken embryos, *Lbx1* is expressed in migratory myoblasts into the cervical somites as well as in fore and hind limbs (Dietrich, 1999, Jagla et al., 1995). In mice, the absence of *Lbx1* prevents migration of myogenic precursors from entering the limb buds, and just a subset of flexor muscle in the forelimb forms under the effect of local cues (Gross et al., 2000, Lours-Calet et al., 2014, Ochi and Westerfield, 2009). In contrast, *Lbx1* overexpression results in enhancement myogenesis in chick embryos (Mennerich and Braun, 2001). *Lbx1* regulates the migration of myogenic precursor cells through the transcription of C-X-C chemokine receptor type 4 (CXCR-4), the receptor of stromal-derived-factor-1 (SDF1) (Caruz et al., 1998, Vasyutina et al., 2005). It is also a target of *Pax3* (Lagha et al., 2010); deficiency of *Lbx1* in mouse results in mislocation of Pax3-positive myoblast cells adjacent to the somites and absence of limb muscles (Schäfer and Braun, 1999).

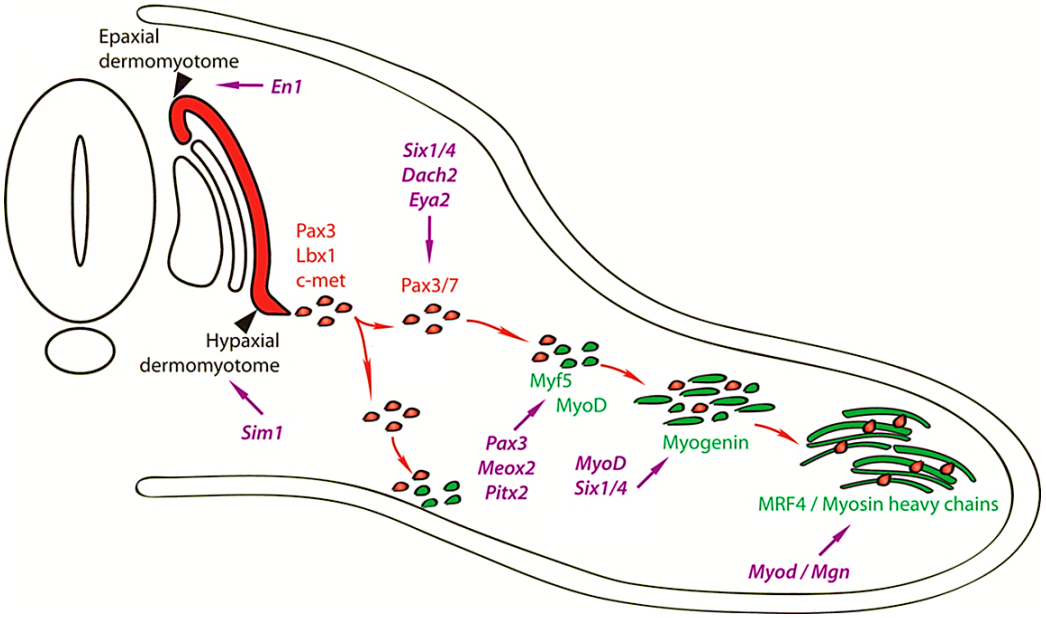


Figure 1. 15: Schematic representation of several factors that regulate the formation of limb skeletal muscle. Transcription factors in purple. Cells delaminating from the ventro-lateral lip of the dermomyotome express *Pax3*, *C-met* and *Lbx1* (red). *En1* and *Sim1* are specified epaxial and hypaxial muscle respectively. *Six1*/4, *Dach2* and *Eya2* regulate *Pax3* expression. *Pax3*, *Meox2* and *Pitx2* regulate muscle proliferation by regulating MRFs (green). Both *MyoD* and *Myogenin* regulate *Six1/4* as well as structural proteins. Figure provided by D Sweetman published in (Mok, 2014).

In vitro the Pax homeodomain physically interacts with other homeodomains, including Mesenchyme Homeobox 1(*Meox1*) associating with *Pax1* as well as Mesenchyme Homeobox (*Meox2*) that associates with *Pax3* (Stamataki et al., 2001). *Meox1* and *Meox2* are additional transcriptional factors expressed in somites and in migrating muscle precursors. Although *Meox2* does not affect the cells migration, the *Meox2* mutant disrupts the forelimb skeletal muscle (Mankoo et al., 1999), by reducing the size of limb muscle and also other pattering influences.

1.9 Insulin-like growth factor system (IGFs)

The insulin family of peptide growth factors include insulin and the insulin-like growth factors (IGFs) (Bell et al., 1984, Le Bouc et al., 1986) as well as Relaxin-like peptide family (RLP). The latter in turn is divided into seven peptides: gene 1 Relaxin (H1), gene 2 Relaxin (H2), gene 3 Relaxin (H3), (Bathgate et al., 2002, Hudson et al., 1984, Hudson et al., 1983), insulin-like peptides INSL3, INSL4, INSL5 (Adham et al., 1993, Chassin et al., 1995, Conklin et al., 1999) and insulin-like peptide 6 (INSL6), which is also known as Relaxin /Insulin factor-1 (Hsu, 1999, Lok et al., 2000). The Relaxin members have a low sequence homology, but they share high structural similarity with insulin; this is due to the presence of six cysteines that confer three disulphide bonds (Sherwood, 2004). Therefore, these genes are known as the insulin superfamily (Figure 1.16) (Wilkinson et al., 2005). Despite this similarity in the structure, insulin family ligands have different binding affinities to their receptors, which include the tyrosine kinase receptors that mediate signalling by insulin and IGFs (Nakae et al., 2001). Unlike insulin and Relaxin, IGFs are not produced within dedicated cells in given tissues, but are synthesised and released in virtually every tissue in a given organ system (Jones and Clemmons, 1995).

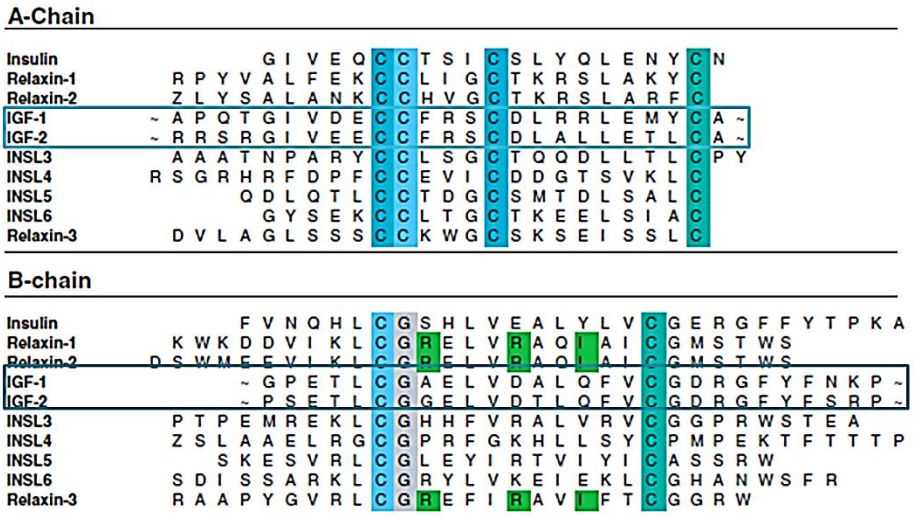


Figure 1. 16: A predicted or identified structure of insulin family members. Each of which contains sequence that gives a very low similarity except for the six cysteine that form three disulfide bridge and a single glycine amino acid within the B-chain. Figure modified from Shabanpoor et al. (2009).

The liver is the major source of circulating IGF-ligands in adult (Bolander, 2004). In addition, pituitary growth hormone (GH) and insulin at high levels in blood induce the biosynthesis of IGF-1 in the liver and other tissues (Humbel, 1990, Pollak et al., 2004). Consequently, IGFs can act as endocrine hormones through the blood as well as having local paracrine and autocrine roles (Annunziata et al., 2011, Humbel, 1990). In fact, the discovery of IGFs has led to the realisation that most, if not all, of the effects of GH involve stimulation of IGF-1 synthesis. It is now, however, accepted that both GH and IGF-1 have a combination of individual and common roles in growth and development (Chowen, 2005). Interestingly, studies have proposed that IGF-1 expression in the chicken muscle could be independent of GH, unlike mammals (Rosselot et al., 1995, Tanaka et al., 1996).

The IGF family comprises a multifunctional regulation system with a pair of structurally related secreted proteins (IGF-1 and IGF-2), cell surface receptors, including insulin receptor (IR) and type 1 and 2 insulin-like growth factor receptors IGF-1R and IGF-2R, six binding proteins (insulin-like growth factor binding proteins (IGFBP)1-6, IGFBP-proteases and other IGFBP-interacting molecules (Figure 1.17) (Abbott et al., 1992, Amaral and Johnston, 2011, Annunziata et al., 2011, Armstrong et al., 2000, Chen et al., 2015, Collett-Solberg and Cohen, 1996, Duan et al., 2010, Durzyńska et al., 2013, El-Shewy and Luttrell, 2009, Ewton et al., 1987, Gleeson et al., 2001, Hawkes and Kar, 2004, Hjortebjerg and Frystyk, 2013, Holly, 2004, Humbel, 1990, Hwa et al., 1999, Jiao et al., 2013, Jones and Clemmons, 1995, Kelley et al., 1996, Le Bouc et al., 1986, Le Roith, 1997, LeRoith and Roberts, 2003, Pavelic et al., 2007, Rosenfeld, 2005, Salmon Jr and Daughaday, 1957).

In muscle, IGFs play a key role in the formation of muscle by stimulating proliferation, differentiation and hypertrophy (Duan et al., 2010, Vinciguerra et al., 2010, Zanou and Gailly, 2013). Moreover, they are essential signalling molecules that regulate the terminal steps of satellite cells lineage progression (Vinciguerra et al., 2010). IGFs also regulate cells mitogenesis (Reiss et al., 1998), migration (Baserga, 1999, Kooijman, 2006), adhesion and motility (Mauro et al., 2003) as well as stimulating

normal maturation and growth of many different cells during foetal and adult life (Barbieri et al., 2003, Pavelic et al., 2007, Yakar et al., 2004). They are also important for the reproductive organs, skeletal tissues and development of the central nervous system (CNS) (Clemmons, 2009, Duan et al., 2010).

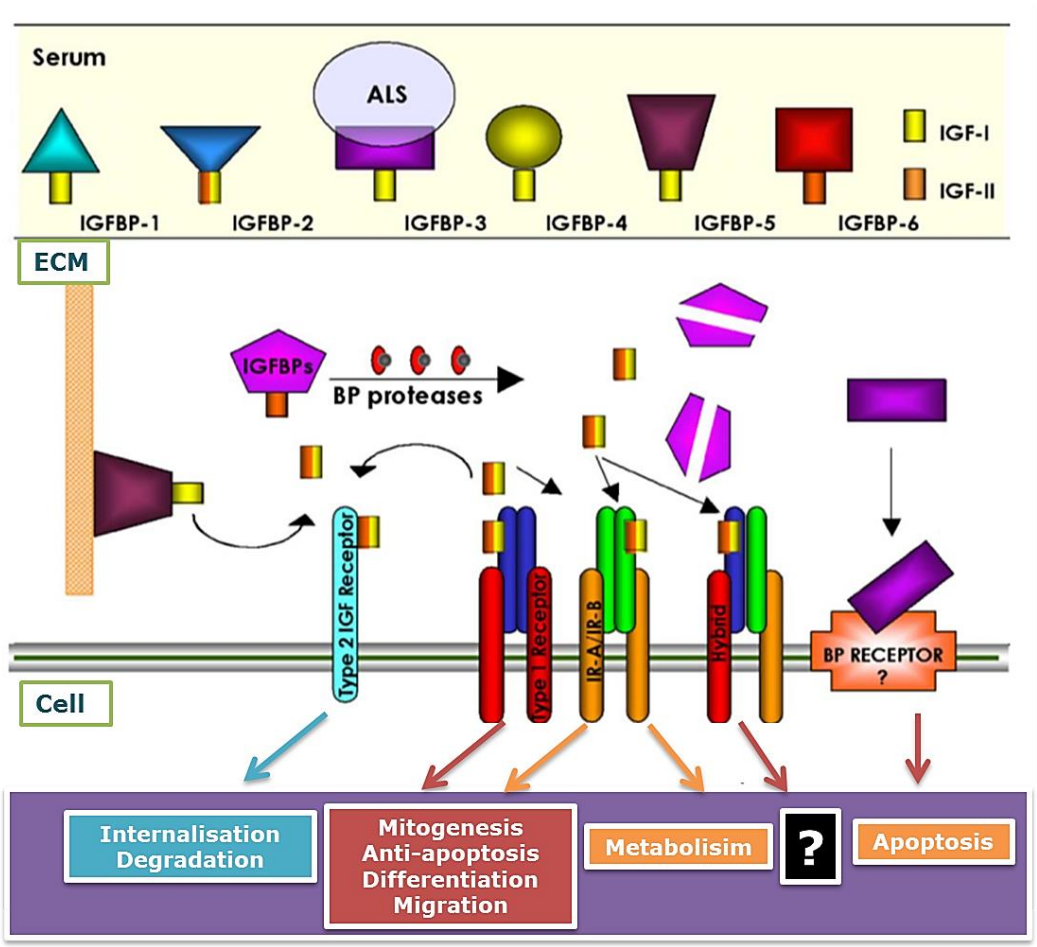


Figure 1. 17: Schematic representation of the insulin-like growth factor components. The IGF system comprises ligands (IGF-1/IGF-2), receptors (IGF-1R/IGF-2R), insulin receptor (IR) the hybrid receptor (IR/IGF-1R) and the six of IGF binding proteins (IGFBP-1-6) with their proteases. IGFBP-3: (ALS) complex is the main circulatory binding protein transported in serum. Release of IGFs interaction with IGFBPs occurs by (ECM) binding or by IGFBP proteolysis. IGF-2 is modulated by IGF-2 receptor. Figure modified from (Annunziata et al., 2011). Abbreviation: ALS, acid labile subunit; ECM, extracellular matrix.

1.9.1 Insulin-like growth factor ligands

1.9.1.1 Insulin-like growth factor-1 (IGF-1)

In 1963, a polypeptide was discovered in human serum with insulin-like activity, but it was not neutralized via anti-insulin antibodies and was therefore termed to Non-suppressible insulin-like activity (NSILA) (Froesch et al., 1963). In the 1970, IGF-1 was called sulfation factor and subsequently changed to the name somatomedin C' in the 1980s (Salmon Jr and Burkhalter, 1997). The three dimensional structures of IGF-1 have been determined by X-ray crystallography and by nuclear magnetic resonance (NMR) approaches (Cooke et al., 1991, Schaffer et al., 2003, Siwanowicz et al., 2005, Vajdos et al., 2001).

IGF-1 in humans is a single chain peptide of 70 amino acids with a linear organization including four contiguous domains called B, C, A and D (in order from N to C terminus) (Figure 1.18A and B) (Denley et al., 2005, Rotwein, 1999). Domains B and A have a crystal structure similar to that of insulin (Baker et al., 1988, Pavelic et al., 2007). It contains triple a-helices one within the B-domain equivalent of the B-chain in insulin and two in the A-domain similar to the insulin A-chain helices. The single chain of the IGF-1 is folded into a tertiary shape which is held together via triple intramolecular disulfide bonds (Denley et al., 2005).

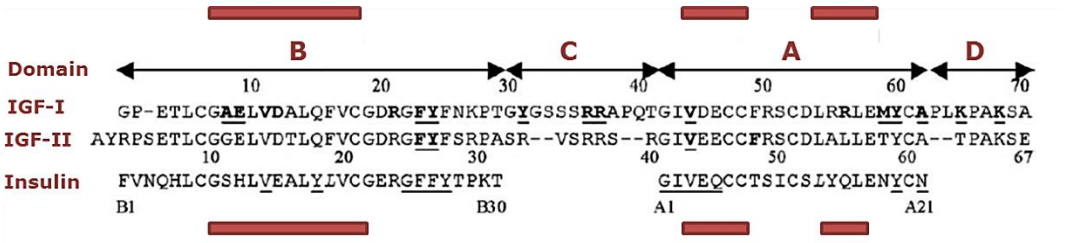


Figure 1. 18A: The molecular structure of insulin, IGF-1 and IGF-2. Sequence bias shows domains and helices (boxes). The residues of both IGF-1 and IGF-2 contains 5-fold (underlined and bold) or 2 (bold) lower IGF-1 receptor binding sites affinity are displayed. Insulin domain in the classic site 1 (underlined) or site 2 binding sites (italics) are also shown. Figure modified from Denley et al. (2005).

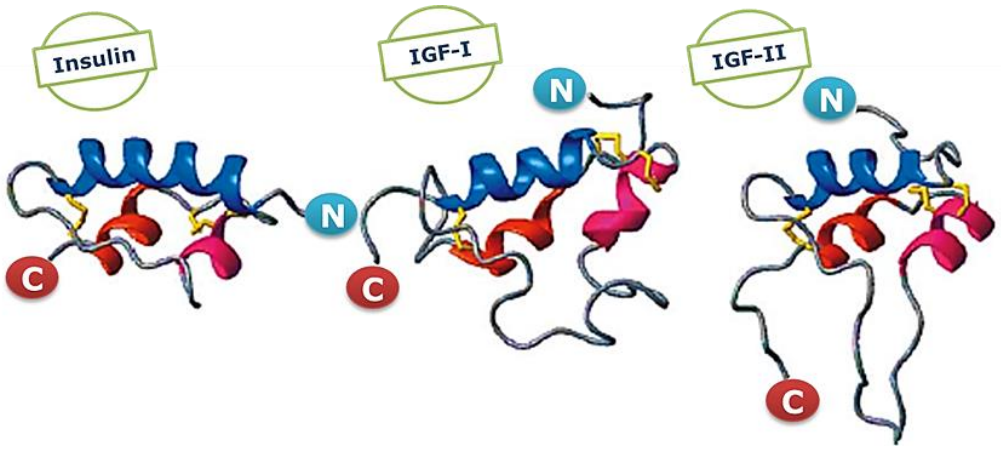


Figure 1. 18B: Ribbon structures of insulin, IGF-1 and IGF-2. It shows the contiguous domains B- helix 1 (blue), A-helix 2 (pink) and A- helix 3 (red) (in order from N to C terminus). Figure modified from Denley et al. (2005).

IGF-1 is GH dependent involved in many different cellular processes, such as differentiation (Tureckova et al., 2001), maintenance and repair of muscle (Nguyen et al., 2007, Yang et al., 1997). Furthermore, in *IGF-1* null mice embryonic development is delayed and defected in bone maturation and mineralisation of bone matrix are seen (Zhang et al., 2002). In addition, *IGF-1* overexpression increases the risks of various cancers including breast, prostate, bladder, lung and colon (Yakar et al., 2004).

1.9.1.2 Insulin-like growth factor-2 (IGF-2)

IGF-2 is a single chain polypeptide with 67 amino acids (Denley et al., 2005, Shabanpoor et al., 2009). The regulation of *IGF-2* is complex; its transcription can be controlled by multiple promoters, stability of mRNA, alternative translation initiation and genomic imprinting (DeChiara et al., 1991, Erbay et al., 2003, Tang et al., 2006). Like *IGF-1*, the tertiary structure of *IGF-2* has been investigated using NMR spectroscopy (Denley et al., 2005) which showed that *IGF-2* also contains four domains (B, C, A and D) (Figure 1.18A) (Denley et al., 2005, Rinderknecht and Humbel, 1976). Both B and A domains have about 50% sequence identity to B- and A-domains of insulin (Rinderknecht and Humbel, 1976). Unlike IGF-1, GH does not regulate IGF-2 (Pierce et al., 2010, Yu and Hung, 2009). Nevertheless, *IGF-2* plays a fundamental role in the development and growth during fetal life (Chao and D'Amore, 2008). In rodent foetuses, *Igf2* circulates at high levels then rapidly declines after birth. Targeted

disruption of this gene causes a clear deficiency in growth and development (Kiess et al., 1994). IGF-2 acts by binding to several cell surface receptors, including the IGF-1R and IGF-2R, but with variety of affinities (Chao and D'Amore, 2008, LeRoith et al., 1995). IGF-2 has also been shown to activate and bind with high affinity to the insulin receptor-A (IR-A) (Frasca et al., 1999, Louvi et al., 1997). This has been demonstrated in human when IR-A or IR-B cDNAs were transfected into fibroblasts from mice where the *IGF-1R* gene was deleted by homologous recombination. The absence of *IGF-1R* results in binding of IGF-2 to IR-A with a very high affinity (Frasca et al., 1999).

1.9.2 Insulin-like growth factor receptors

The activity of the IGF system is mediated by binding to a family of receptor tyrosine kinases (RTKs) including the IR, IGF-1R, IGF-2R and the insulin receptor related receptor (IRR) (Figure 1.19) (Benyoucef et al., 2007, Slaaby et al., 2006, Taguchi and White, 2008, Werner, 1999).

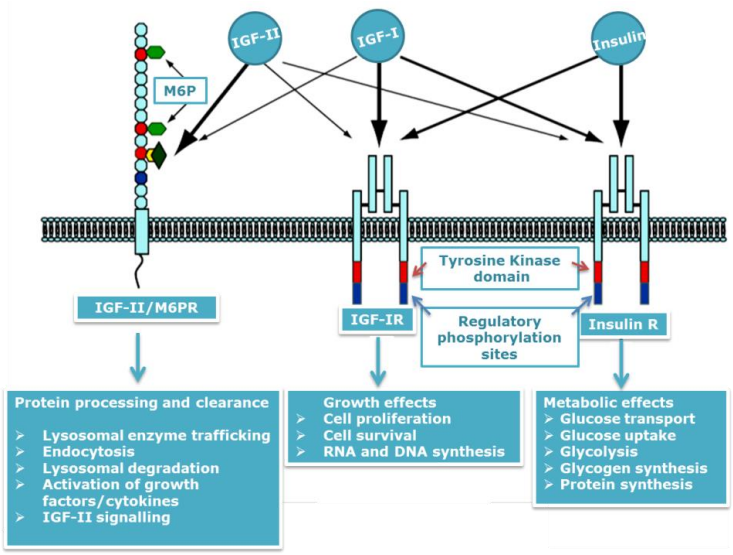


Figure 1. 19: Structure of mature molecules of the insulin, IGF-1 and IGF-2 /M6P receptors. The insulin and IGF-1 receptors are tyrosine kinases receptors that have high homology in the structure. Each of which possesses a hetero-tetrameric structure, which consists of two α and two β subunits bonding by disulphide-bridge. The IGF-2 receptor is a transmembrane glycoprotein, which has a large extracellular residue, a short cytoplasmic tail and a single transmembrane domain. The insulin, IGF-1 and IGF-2 have different binding affinity to their relative receptors as demonstrated using the arrow thickness. Figure modified from (El-Shewy and Luttrell, 2009).

Most, if not all, the impacts of both IGF ligands (IGF-1 and IGF-2) on growth and differentiation are mediated through IGF-1R (Collett-Solberg and Cohen, 2000, Duan et al., 2010, Jones and Clemmons, 1995, Werner, 1999).

1.9.2.1 Type 1 insulin-like growth factor receptor (IGF-1R)

The IGF-1R is a transmembrane tyrosine kinase receptor that mediates biological actions of IGFs (LeRoith et al., 1991, Samani et al., 2007). This receptor is widely expressed by many tissues and organs (Baserga, 2000, Grimberg and Cohen, 2000); only mature B hepatocytes and lymphocytes do not express the *IGF-1R* (Rosenthal, 1999, Valentinis and Baserga, 2001). Several studies performed in Zebrafish and Xenopus have shown that the IGF-1R structure and role are conserved evolutionarily (Eivers et al., 2004, Maures et al., 2002, Pera et al., 2001, Schlueter et al., 2007). IGF-1R has a high sequence identity and structural similarity to the insulin receptor (Abbott et al., 1992, De Meyts and Whittaker, 2002, LeRoith et al., 1995). It is formed as a single progenitor polypeptide chain composed of 1367 amino acid residues, and at residue 706, the receptor is cleaved into an extracellular domain NH-signal peptide, α-subunits and two β-subunits-COOH linked by disulfide bonds (Jacobs et al., 1983, Nissley, 2003 , Ullrich et al., 1986).

In the mature IGF-1-receptor, the α subunit resides extracellularly and contains a cysteine-rich region (comprising 24 Cys residues) as well as 11 potential N -linked glycosylation sites (Asn-X-Ser/Thr motifs) that are critical for high-affinity IGF-1 binding (Kjeldsen et al., 1991). The intracellular region of the β subunits, which includes a 24-amino-acid hydrophobic sequence located between 906 and 929 residues, has tyrosine kinase activity (Duan et al., 2010) that relies on tyrosine phosphorylation and an adenosine triphosphate (ATP) binding site. The β subunits extracellular portion is located between 711 and 905 residues (Figure 1.20), and contains five potential N-linked glycosylation region (Backer et al., 1990, Prager et al., 1994).

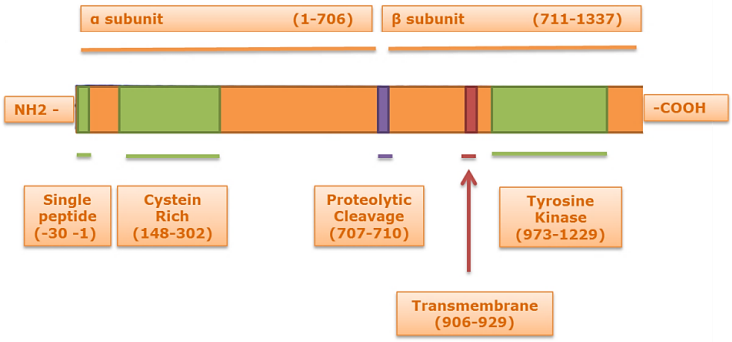


Figure 1. 20: The IGF-1 receptor progenitor molecule is schematically represented. The 1367-amino-acid IGF receptor progenitor has a 30-amino-acid signal peptide that act to transfer of the embryonic polypeptide chain into the endoplasmic reticulum. Between 707 and 710 residues, the (Arg-Lys-Arg-Arg) site will be cleaved by endopeptidase digestion to release α and β subunits that together join by disulfide bonds to form the final configuration of the mature heterotetrameric receptor. Figure modified from (Werner, 1999).

The two receptors (IGF-1R and IR) play important roles in multicellular organisms (Drakas et al., 2004). Although IGF-1R and IR have a high degree of homology in their structure, both maintain separate distinct roles, such as the involvement IGF-1R in cell proliferation, whereas IR maintains glucose homeostasis (Frasca et al., 2008). In mammals, IGF-1R has a key function in cell metabolism, formation, and differentiation of different tissues and organs (De Meyts, 2004, Siddle, 2012). It is also important for normal growth and development (Accili et al., 1996, Liu et al., 1993), thus abnormal IGF-1R signalling may lead to a variety of disorders, including diabetes and cancer (De Meyts and Whittaker, 2002, Pollak, 2012).

1.9.2.2 Type 2 insulin-like growth factor receptor (IGF-2R)

In 1980, a photo-affinity crosslinking study detected a 250 kDa species that bind IGF-2. Meanwhile, cation-independent mannose 6-phosphate receptor (CI-MPR) was identified as a similar sized receptor that binds mannose 6-phosphate (M6P) (Morgan et al., 1986, Polychronakos et al., 1990). Subsequently, the molecular cloning of IGF-2R and CIMPR established that they were the same molecule, called the IGF-2/M6P receptor (Szebenyi and Rotwein, 1994). IGF-2R/M6P is also known as cytokine receptor CD222 and is structurally, biologically and chemically distinct from the IGF-1R or IR (El-Shewy and Luttrell, 2009, Harris and Westwood, 2012). IGF-2R is a type 1 integral-membrane protein that

contains a single transmembrane domain with a large N-terminal extra-cytoplasmic region and a short C-terminus located in the cytoplasmic region of lipid membranes with a short cytoplasmic tail (Lobel et al., 1988). Unlike IGF-1R, IGF-2R does not contain an intracellular signalling domain, thus it does not possess intrinsic signal transduction capability (Pavelic et al., 2007).

The type 2 receptor does not bind insulin, but has greater affinity than IGF-1R in binding IGF-2 (El-Shewy and Luttrell, 2009), which is then sequestered from interactions with the IGF-1R through internalization and degradation (Denley et al., 2005, Kornfeld, 1992, Pavelic et al., 2007, Scott and Firth, 2004). A mouse lacking *Igf-2R* function showed that this receptor acts as a sink for IGF-2 to avoid abnormal growth during fetal development (Wylie et al., 2003); thus the loss of IGF-2/M6P receptor imprinting causes fetal overgrowth (Lau et al., 1994).

1.9.3 Insulin-like growth factor binding proteins (IGFBPs)

Unlike insulin, which freely circulates in the blood stream, IGFs are restricted in complexes with a family of six IGFBP-1 to -6 (Clemmons, 2001, Denley et al., 2005, Duan, 2002, Duan and Xu, 2005, Firth and Baxter, 2002, Kelley et al., 2002). The IGFBPs with molecular mass of 24–45kDa share a similar domain structure (Figure 1.21) containing highly conserved N- and C- terminal domains, which are essential for high affinity binding of IGFs. Each of these domains has internal disulfide links (L-domain) (Bach and Parker, 2003, Denley et al., 2005, Duan et al., 1999, Maures and Duan, 2002).

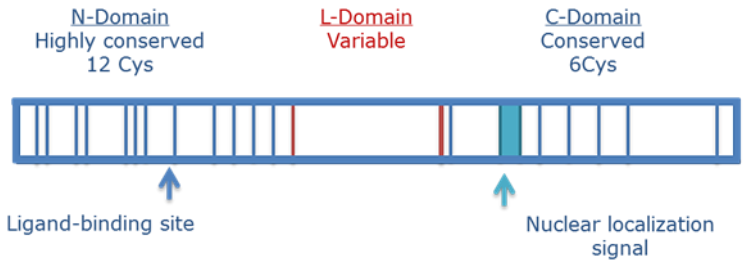


Figure 1. 21: The IGFBPs structure. IGFBP-5 has been schematic represented to be as a candidate represents other IGFBP members. The mature IGFBP protein has 252 amino acid residues, and it is divided into three domains. The amino-terminal domain (N-domain) composes 12 conserved cysteine residues. L-domain consists of the central binder domain that has multiple posttranslational medication sites. The C-domain contains a nuclear localization signal (NLS). Figure modified from (Duan and Xu, 2005).

The L-domain is a region of posttranslational modification including phosphorylation, glycosylation and areas of IGFBP proteolysis. The N- and C-terminal domains of the IGFBPs contain highly conserved cysteine residues, which makes intra-domain disulfide bonds within the N- and C-domains to subsequently determine their overall final globular structure (Bach and Parker, 2003, Clemmons, 2001). The N-domain has the high affinity IGF-binding region, whereas the C-domain shows lower affinity (Bach and Parker, 2003, Clemmons, 2001, Hobba et al., 1998, Żesławski et al., 2001). The variable central L-region acts as a flexible linker domain connecting both N- and C- domains (Chelius et al., 2001, Forbes et al., 1998, Neumann and Bach, 1999). IGFBP-C-domain can mediate its activity and interact with other proteins. An example, a glycoprotein ALS binds IGFBP-3 together with IGF-1 forming a trimeric complex (Baxter, 1994a, Firth and Baxter, 2002, Jones and Clemmons, 1995).

IGFBP activity has been thoroughly investigated by knockout approaches and overexpression using mice as a model. In transgenic mice, overexpression of IGFBP1, 2, 3 and 5 caused retardation in growth (Silha and Murphy, 2002, Salih et al., 2004). The six IGFBPs bind to the IGFs and control their availability (Bach and Parker, 2003, Ferry Jr et al., 1999, Hwa et al., 1999), but the interactions between IGFs and their binding proteins are modulated through proteases (Collett-Solberg and Cohen, 2000). This leads to the regulation of the interaction between IGF-ligands, IGFBPs and IGFRs; these interactions modulate normal and abnormal proliferation of tissue (Bunn and Fowlkes, 2003, Jones and Clemmons, 1995, Vasylyeva and Ferry, 2007). Alterations in IGFBPs levels in serum can affect IGF bioavailability for interaction with cell transmembrane receptors (Lemozy et al., 1994). IGFBPs also act to prolong the IGF half-life in circulation and cell survival, but probably suppress mitogenesis and cell differentiation (Denley et al., 2005, Duan and Xu, 2005, Ferry Jr et al., 1999, Firth and Baxter, 2002, Jones and Clemmons, 1995, Rajaram et al., 1997, Ueki et al., 2000).

1.9.3.1 Insulin-like growth factor binding protein-1 (IGFBP-1)

IGFBP-1 is a 25-kDa protein (Shimasaki et al., 1991a) specifically bound to α5- β1 integrin by the RGD recognition sequence in its C-domain (Jones et al., 1993). The integrin and IGFBP-I binding as well as the resultant signalling play a crucial role in the migration and implantation of trophoblast (Gleeson et al., 2001). IGFBP-1 is serine phosphorylated in its central domain as IGFBP-3 and 5 (Coverley and Baxter, 1997). Both IGF-1 and IGF-2 bind to IGFBP-1 with equal affinity, but the phosphorylated form has a higher affinity for IGF-1 and therefore blocks its mitogenic actions (Rechler, 1993).

IGFBP-1 is predominantly expressed in the liver (Chen et al., 2015, Duan and Xu, 2005, Ooi et al., 1993) and kidney (Chen et al., 2015). Even though IGFBP-1 null mice grow normally, they have impaired liver regeneration after hepatectomy (Leu et al., 2003). IGFBP-1 is also expressed in breast cancer (Yee et al., 1989) and found at very low level in adult serum, meanwhile it is the predominant IGFBP in human amniotic fluid (Póvoa et al., 1984). *In vivo* IGFBP-1 concentrations are controlled by nutritional status. For instance, during fasting states, the high level of IGFBP-1 curbs hypoglycaemia through binding IGF and restricting the free IGF-1 levels (Collett-Solberg and Cohen, 1996). Overexpression of IGFBP-1 in cultured cells causes inhibition of IGF-dependent growth (Jones and Clemmons, 1995). This suggests that high levels of IGFBP-1 may restrict IGF-1 and prevent IGF-1 binding to its receptor (Collett-Solberg and Cohen, 1996).

1.9.3.2 Insulin-like growth factor binding protein-2 (IGFBP-2)

IGFBP-2 is a 34-kDa protein (Yang and Rechler, 1993); like IGFBP-1, it has an RGD sequence but it does not bind integrin membrane receptors and undergoes glycosylation or phosphorylation. IGFBP-2 expression is distinct and complementary to IGF-2 expression in rat (Wood et al., 1990, Wood et al., 1992). Mouse embryos have a similar expression pattern of IGFBP-2 (Schuller et al., 1993). Large quantities of IGFBP-2 are found in the neonatal circulation and the Osteoblast-like oligonucleotide binding (OB) cells from rat and human; such cells secrete this protein (Clemens and

Rosen, 2003). Overexpression of recombinant human IGFBP-2 inhibits IGF-1-stimulated OB replication and matrix synthesis of fetal rat (Okazaki et al., 1994). In human serum and cerebrospinal fluid, IGFBP-2 has been shown to be the predominant IGFBPs at mid-gestation (Guan et al., 2005, Lassarre et al., 1991). It also has higher affinity for IGF-2 than IGF-1 (Guan et al., 2005). IGFBP-2 concentrations increase more at birth to levels about fourfold higher than in human adulthood (Bang et al., 1994, Blum et al., 1990). In rodents, high levels of IGFBP-2 are observed in fetal serum and throughout early postnatal life; then it disappears in the adult animal (Babajko et al., 1993, Donovan et al., 1989). IGFBP-2 expression has been also detected in numerous endodermal and ectodermal derivatives (Wood et al., 1990). Northern analysis showed that IGFBP-2 is expressed in the intestines, kidney, liver and lungs by the end of fetal life (Ooi et al., 1990). *IGFBP-2* knockout mice have a normal total body weight, but larger livers and smaller spleens (Wood et al., 2000).

In chick embryos, *IGFBP-2* is expressed as early as HH stage 3 in the primitive streak. This expression then increases and localises in somites and branchial arches at HH stage 19 (Allan et al., 2003). In chick and mouse limbs (van Kleffens et al., 1998), *IGFBP-2* is abundantly expressed in the AER (Allan et al., 2003, McQueeney and Dealy, 2001). The AER in turn controls *IGFBP*-2 expression. This indicates that cessation of the expression of *IGFBP*-2 is probably important for proliferation and inhibition of subridge mesoderm differentiation that is necessary for the ongoing limb growth (McQueeney and Dealy, 2001). During early chick embryonic development, a broad overlap of *IGFBP-2* mRNA expression has been observed with *IGF-1* and *IGF-1R*, while *IGFBP-5* overlaps with *IGF-2* (Allan et al., 2003).

1.9.3.3 Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3)

IGFBP-3 is a 29 kDa molecular protein in the non-glycosylated form, but in serum glycosylated, IGFBP-3 has a molecular weight of 40 to 44 kDa (Jones and Clemmons, 1995). The highest concentration of IGFBP-3 is found in postnatal animals (Baxter, 1994b). At birth, IGFBP-3 concentration is about one third of the level in mature humans (Donovan

et al., 1989). *IGFBP-3* is expressed in endothelial cells of different tissues, including rat white adipose tissue, placenta and liver as well as shown in human testis (Chin et al., 1994, Zhou and Bondy, 1993). In mouse embryos, *IGFBP-3* expression is undetectable at day 12, but it is expressed in the vertebrae and liver at day 18 (Schuller et al., 1993). *In situ* hybridisation in rat embryo has shown that *IGFBP-3* is expressed during the development of cerebral cortex and urogenital system, and it is also seen in the kidney, liver and muscle (Cerro et al., 1993). In addition, biochemical analysis has established that IGFBP-3 is linked with the extracellular matrix and cell surface (Clemmons, 2001, Firth and Baxter, 2002). In rodents, it has been suggested that IGF-1 acts as a direct regulator to elevate the IGFBP-3 serum concentration (Camacho-Hubner et al., 1991, Zapf et al., 1989). Overexpression of IGFBP-3 in culture media blocks and potentiates IGF-1–induced synthesis of the DNA in human skin fibroblasts, while the isolation of IGFBP-3 from the culture results in stimulation of IGF-1 action (De Mellow and Baxter, 1988).

1.9.3.4 Insulin-like growth factor binding protein-4 (IGFBP-4)

IGFBP-4 is a 24 kDa protein, or 28 kDa in its glycosylated state (Kelley et al., 1996). Like IGFBP-3, IGFBP-4 has been purified from many sources based on its action in suppressing cell outgrowth, and this factor is abundant in human serum (Kiefer et al., 1991a), osteosarcoma cells (Mohan et al., 1989), and the PC3 prostatic cancer cell line (Perkel et al., 1990) as well as in neural cells (Martin and Baxter, 1999). Furthermore, IGFBP-4 is detected in the B104 rat neuro blastoma cell line, rat serum (Cheung et al., 1991, Shimonaka et al., 1989), porcine ovarian follicular fluid (Shimasaki and Ling, 1991) and bovine plasma (Walton et al., 1990).

IGFBP-4 consistently blocks IGF action (Clemmons, 1997b). It inhibits IGF-1 induced growth of chick pelvic cartilage (Mohan et al., 1989) and suppresses IGF-2-induced DNA synthesis in human vascular smooth muscle cells, osteoblast and mouse bone cells (Amarnani et al., 1993, Duan and Clemmons, 1998, Hsieh et al., 2003, Mohan et al., 1995); this probably occurs by preventing the ligand binding to its receptor (Mohan et al., 1995).

1.9.3.5 Insulin-like growth factor binding protein-5 (IGFBP-5)

IGFBP-5 has a molecular weight of 29 kDa and also detected at 32 kDa (Katz et al., 1995). IGFBP-5 acts as a pivotal regulatory factor through the development of different key cell lineages, including neural cells and myoblasts (Cheng et al., 1999, James et al., 1996, Pera et al., 2001). IGFBP-5 was first identified in human placenta and osteosarcoma (Kiefer et al., 1991b, Shimasaki et al., 1991b) as well as in rat ovary (Shimasaki et al., 1991b). Moreover, IGFBP-5 is found in various tissues including connective tissues, cerebrospinal fluid (CSF), osteoblasts (Katz et al., 1995, Kelley et al., 1996), and it is also localised in the nuclei of skeletal muscle of mouse embryos (Duan et al., 2010, Zhao et al., 2006).

During early mouse embryonic development, *IGFBP-5* is seen in the myotome and in the central nervous system (Green et al., 1994). High levels of IGFBP-5 are found in the extracellular matrix and in peripheral tissues (Jones et al., 1993, Schneider et al., 2002), while it is weakly expressed in the liver (Schneider et al., 2000). *IGFBP-5* expression is also detected in the ectoderm of the developing limb (van Kleffens et al., 1998). Unlike in mice, no expression of *IGFBP-5* is seen in the ectoderm or the AER in chick embryos, but it is highly expressed in the musculature during limb development (Allan et al., 2003, McQueeney and Dealy, 2001); then in skeletal elements (McQueeney and Dealy, 2001).

IGFBP-5 can bind both IGF-1 and IGF-2 with high affinity, mainly via an N-terminal motif (Kalus et al., 1998) and it blocks IGF-1-induced DNA synthesis (Hsieh et al., 2003) by preventing the interaction between the ligand and IGF-1R. In addition, IGFBP-5 inhibits the growth of many different types of cells, such as granulosa, renal and osteosarcoma cells (Kelley et al., 1996). It is also controlled by posttranslational modifications that induce conformational changes, which reduce IGFBP-5 affinity for IGF-ligands; this then activates their effective transmission to the IGF-1R (Clemmons, 2001). IGFBP-5 potentiates IGF-1-stimulated cell migration (Hsieh et al., 2003). Although knockout of *IGFBP-5* in mice reduced mammary cell number, these mice had normal growth in body composition and weight (Ning et al., 2007). The level of IGFBP-5 in circulation is about

10% or less of total IGFBPs (Rajaram et al., 1997). High concentration of IGFBP-5 in murine bone tissues leads to a transient blocking in osteoblastic function and reducing the volume of trabecular bone (Devlin et al., 2002).

1.9.3.6 Insulin-like growth factor binding protein-6 (IGFBP-6)

IGFBP-6 is an O-glycosylated protein (Bach et al., 1992) with a molecular weight of 34 kDa (Cohen et al., 1994). IGFBP-6 is expressed in multiple cells, such as fibroblasts, ovarian cells, breast cancer cells and prostate cells (Bach et al., 1994). It is also seen in adult rat serum (Shimasaki et al., 1991b), porcine follicular fluid and serum (Shimasaki et al., 1991a), and in human CSF (Roghani et al., 1991, Zapf et al., 1990). IGFBP-6 can bind to IGF-2 (Bach et al., 1992, Bach et al., 1994, Baxter and Saunders, 1992) and inhibit IGF-2-dependent myoblast proliferation and differentiation (Bach et al., 1994, Bach et al., 1995). In bone cells, addition of IGFBP-6 inhibits IGF-2-induced DNA synthesis, but it has a weak effect on blockade of IGF-1 actions (Baxter and Saunders, 1992).

1.9.3.7 Insulin-like growth factor binding protein-7 (IGFBP-7)

IGFBP-7 also called IGFBP-related protein 1 (IGFBP-rP1), prostacyclin-stimulating factor (PSF), mac25 and tumor adhesion factor (TAF) shares about 30% similarity with other IGFBPs in its N-terminal domain (Burger et al., 2005). The main difference between IGFBP-7 and the other six IGFBP members is that IGFBP-7 lacks the C-terminus (Yamanaka et al., 1997) and has lower affinity for IGF-1 (Hwa et al., 1999, Oh et al., 1996). Furthermore, IGFBP-7 can directly bind to IGF-1R then suppress its activity (Evdokimova et al., 2012). In contrast, the six IGFBPs decrease the activation of IGF-1R through sequestering IGFs (Firth and Baxter, 2002). IGFBP-7 has been found to enhance insulin (Ruan et al., 2016) and IGF-1 activities through inducing formation of mouse fibroblasts (Akaogi et al., 1996). In addition IGFBP-7 acts as a potential tumor inhibitor gene for many cancers, such as Hepatocellular carcinoma, mammary tumour and others (Chen et al., 2011, Swisshelm et al., 1995, Vizioli et al., 2010).

1.9.4 The sequential functions of IGFs in skeletal muscle cells

Muscle growth and development are highly ordered processes (Buckingham et al., 2003). The proliferation and differentiation of skeletal myoblasts are strongly associated with the cellular environment; these processes are affected by cell-cell and cell-matrix interactions (Heino and Massague, 1990, Rosen et al., 1992) as well as by soluble growth factors (Florini et al., 1991a). Although growth factors in general suppress myogenic differentiation (Florini et al., 1991a), IGFs are the only mitogenic factors that can sequentially induce proliferation and differentiation of skeletal myogenic cells (Barbieri et al., 2003, Duan et al., 2010, Engert et al., 1996, Ewton et al., 1994, Rosenthal and Cheng, 1995, Stewart and Rotwein, 1996, Valentinis and Baserga, 2001, Zanou and Gailly, 2013).

1.9.4.1 IGF action on proliferation and differentiation

All IGF components are expressed in skeletal muscle (Florini et al., 1996); they regulate muscle cell proliferation and differentiation both *in vivo* and *in vitro* (Brown et al., 1992, Collett-Solberg and Cohen, 2000, Duan et al., 2010, Ferry Jr et al., 1999, Hill et al., 1985, Jones and Clemmons, 1995). Manipulation experiments performed in chicken embryos and mice have shown the crucial function of IGFs in the normal formation of skeletal muscle. Genetic studies have also demonstrated that skeletal muscle cells produce endogenous IGFs that prolong survival and initiate the differentiation in tissue culture (Florini et al., 1991c, Stewart and Rotwein, 1996). Removing serum from proliferating myoblasts in culture results in spontaneous muscle differentiation following the stimulation of IGF peptide production by these cells (Florini et al., 1991c, Tollefsen et al., 1989b).

IGF-2 levels dramatically increase during myogenesis (Florini et al., 1991a); overexpression of IGF-1 (Coleman et al., 1995) or IGF-2 (Stewart et al., 1996) in murine skeletal myoblasts leads to acceleration of myoblast differentiation (Ren et al., 2008, Stewart et al., 1996). Moreover, treatment with IGFs induces myoblast proliferation and differentiation in rodents (Brown et al., 1992, Hill et al., 1985). Overexpression of *IGF-1* in mice enhances muscle strength and increases the size of muscle fibres

(Barton-Davis et al., 1998, Barton et al., 2002, Kaspar et al., 2003). Both IGF-1 and IGF-2 antisense oligonucleotides and cRNAs abolish muscle cell differentiation (Florini et al., 1991c, Montarras et al., 1993, Stewart and Rotwein, 1996).

1.9.4.2 The intracellular mediators involved in IGF-1R effects on myogenic cell proliferation and differentiation

The IGF-1R is thought to have an important function in myogenesis following binding of either IGF-1 or IGF-2 (Valentinis and Baserga, 2001). This receptor mediates all actions of IGF-1 and IGF-2 during myoblast proliferation and differentiation, and it shows the specificity IGF-1> IGF-2> insulin (Ewton et al., 1987). IGF-1R is expressed in murine skeletal myoblasts (Rosenthal et al., 1991, Tollefsen et al., 1989a), such as C2C12 cells (Palmer et al., 1997). Even though mice overexpressing IGF-1R or IGF-2R have muscle hypoplasia, they die due to respiratory failure (Liu et al., 1993, Powell-Braxton et al., 1993). Ligand binding to the α-subunit of IGF-1R stimulates receptor kinase activity, which is intrinsic to the β-subunit, leading to phosphorylation of signalling adaptor proteins including insulin receptor substrate (IRS) (Hubbard, 1997, Wei et al., 1995, White, 2003, Siddle, 2012) and the SRC homology containing protein (Shc) (p46/p52/p66) (Figure 1.22) (Dupont and LeRoith, 2001, Florini et al., 1996, Nissley, 2003 , Samani et al., 2007). Six members of IRS family protein have been identified (Cai et al., 2003, Fantin et al., 1998, Glasheen et al., 1995, Sun et al., 1991), among them IRS-1 and IRS-2, which are extensively expressed compared to the other IRS members that are restricted to certain tissues (Ren, 2009). Both IRS-1/-2 can directly interact with the IR and IGF-1R (Myers Jr et al., 1993, White, 1997), but IRS-2 substrate acts with IR better than IGF-1R (Kim et al., 2001, Rother et al., 1998).

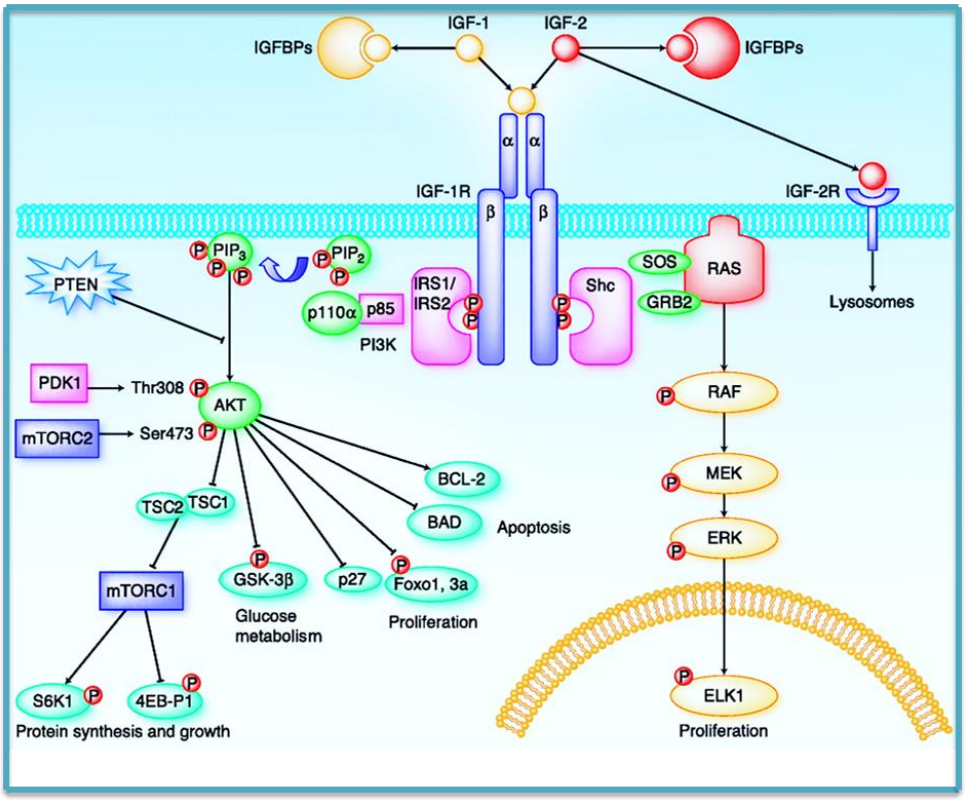


Figure 1. 22: The intracellular signalling pathways derived from induced-IGF-1 receptor are schematically represented. The induced IGF receptor undergoes auto-phosphorylation in response to the binding of its ligands. This stimulates number of endogenous intracellular adaptor proteins. The adaptor proteins, Tyrosyl-phosphorylated, have an ability to join the following substrates in the signalling cascade. The IGF-1R is molecularly phosphorylated several of adaptor proteins, such as SHC and IRS proteins. Tyrosyl-phosphorylated (SHC) binds the SOS and Grb2 complex, which in turn associates with Ras, leading to membrane association and Raf activation. Dual tyrosine/threonine kinases, subsequently, phosphorylate MEK kinase. This pathway is essential for activation cellular proliferation through mediating the IGF-1. The regulatory PI3K subunit, p85 tyrosine phosphorylation, stimulates the catalytic pliO activity. Stimulated PI3-K is catalysed bisphosphonate of phosphorylation. Akt phosphorylation with triphosphate mediates signals linked with apoptosis, proliferation, glucose metabolism and protein synthesis (Zha and Lackner, 2010).

More details have been provided by several studies about IRS-1 (Lavan et al., 1997, Patti et al., 1995, Sun et al., 1991) and its role as a multisite 'docking' protein through binding to downstream signal transduction molecules (Youngren, 2007). Upon phosphorylation of tyrosine, IRS binds and interacts with the two SH2 (Src Homology 2) domains of the p85 adaptor protein leading to recruitment and activation of phosphoinositide 3-kinase (PI3K) (Harrington et al., 2005) and Forkhead box (FoxO) to the plasma membrane (Samani et al., 2007, Yanase and Fan, 2009). These molecules, subsequently, recruit and trigger phosphoinositide-dependent kinases (PDKs), which in turn phosphorylate and stimulate protein kinase

Cξ, p70rsk (PKCξ) and Akt/protein kinase B (Cianfarani et al., 2007, Sun and Liu, 2009, Vanhaesebroeck and Alessi, 2000).

Akt mediates cell growth and survival in many tissues and cells in response to growth factors, including IGF-1 (Datta et al., 1999, Vivanco and Sawyers, 2002). Several studies have also suggested that the PI3Kinase–Akt pathway is the important IGF-activated signalling cascade in muscle growth (Bodine et al., 2001, Glass, 2003, Lai et al., 2004). The absence of Akt causes a muscle disruption phenotype in mice (Pelosi et al., 2007), but transgenic mice expressing active Akt show hypertrophy in muscle (Lai et al., 2004). In addition, activation of Akt is crucial for insulin metabolic effects, such as glycogen synthesis, glucose uptake and synthesis of proteins and fatty acids (Efeyan and Sabatini, 2010, Harrington et al., 2005).

Activation of the Akt pathway leads to the phosphorylation of FoxO transcription factors, a family of transcription factors that play a key role in cellular proliferation, differentiation, survival and in protein degradation (Accili and Arden, 2004, Carlsson and Mahlapuu, 2002, Carter and Brunet, 2007). FoxOs are crucial transcriptional targets of IGF-1. Three isoforms of FoxOs, including FoxO1, FoxO3 and FoxO4 (Lam et al., 2006) have been identified in regulating autophagy and proteasomal lysosomal degradation in muscle (Mammucari et al., 2007, Sandri et al., 2004). In addition, it has also recently been shown that both IR and IGF-1 receptor control FoxO-mediated signalling in proteostasis of muscle (O'Neill et al., 2016).

PI3K/Akt pathway phosphorylates FoxO proteins on multiple sites, resulting in the exclusion of phosphorylated FoxO transcription factors from the nucleus and hence repression of their activities (Brunet et al., 1999). It has also been reported that Akt-dependent FoxO protein regulation is involved in the differentiation of myoblasts (Bois and Grosveld, 2003, Hribal et al., 2003). Deregulation of PI3K/Akt signalling pathway causes activation of FoxO proteins, which occurs during skeletal muscle atrophy (Stitt et al., 2004). Overexpression of FoxO3, therefore, results in muscle atrophy (Sandri et al., 2004). Importantly, stimulation of IGF-1 or over-expression of Akt both lead to a perturbation in the atrophy IGF-1/Akt

pathway by down-regulating the FoxO transcription, indicating their direct role in suppressing the atrophy pathways of skeletal muscle (Sandri et al., 2004, Stitt et al., 2004).

Several genetic studies have shown that distal DNA elements regulate *MyoD* transcription during muscle differentiation (Asakura et al., 1995, Chen et al., 2002, Kucharczuk et al., 1999) and that *Pax3* and *Pax7* in myogenic precursors (Fujisawa-Sehara et al., 1992) are essential transcription factors for regulation the Mrfs expression (Bajard et al., 2006, McKinnell et al., 2008). Evidence for a possible link and interaction between *Pax3* and *FoxOs* transcription factors is underscored by the discovery of a naturally occurring chromosomal translocation between *Pax3* and *FoxO1* that produces fusion protein PAX3/FKHR, which presents in Rhabdomyosarcoma (Mercado and Barr, 2007). Alveolar Rhabdomyosarcoma is identified by deficiencies in the differentiation of muscle cells and a failure to exit the cell cycle (Tapscott et al., 1993). Cells originated from these tumors express myogenic regulatory factors, including *MyoD* and *Myog*, but cannot differentiate into myotubes (Tapscott et al., 1993). Thus, the link between Pax and FoxO transcription factors provides myogenic cells with a powerful direct mechanism for utilizing co-dependent activators expressed in various tissues to regulate cell type-specific gene transcription (Hu et al., 2008). Hu and colleagues have also identified that FoxO3 reveals a possible connection between signalling cascades regulation of *MyoD* transcription in adult muscles (Hu et al., 2008). Furthermore, overexpression of *FoxO1* stimulates proteasome-dependent degradation of mTOR signalling components and mTOR itself, leading to inhibition of *IGF-2* transcription (Wu et al., 2008). Exogenous IGF-2 rescues the differentiation of myocyte from FoxO1 effects (Wu et al., 2008). This suggests that the mTOR/IGF2 pathway is required in mediating of FoxO1 inhibitory role in skeletal myogenesis. However, it should be noted that FoxO is not fully dependent on mTOR signalling in myogenesis. FoxO1 can interact with and activate *Notch* genes, resulting in an inhibition of differentiation (Kitamura et al., 2007).

IRS-1 and IRS-2 are able to recruit the Shc of the guanine-nucleotide-exchange factor Sos through the SH2, the adaptor of growth factor receptor bound protein 2 (Grb2) (Ceresa and Pessin, 1998, Dupont and LeRoith, 2001). This complex leads to the activation of the Raf-1/ mitogen-activated protein kinase-(MAPK)-(ERK) downstream nuclear factors, which is essential for insulin signalling to the nucleus, controlling gene transcription and stimulation of cellular proliferation (Ceresa and Pessin, 1998, Chang and Karin, 2001, Samani et al., 2007). Activation of IGF-1R is coupled to the induction of MAP kinases, including ERK-1 and ERK-2, Jun N-terminal kinases -1 and -2 (JNK-1/2) as well as p38 MAP kinases (Dérijard et al., 1994, Han et al., 1994, Rouse et al., 1994). JNKs phosphorylate the amino terminus of c-Jun and increase its ability to stimulate transcription (Dérijard et al., 1994).

The p38 MAP kinase also has important functions in biology of skeletal muscle. p38 MAP kinase is a signal transducer that *in vitro* stimulates the differentiation of muscle and *in vivo* affects muscle formation and repair (Cuenda and Cohen, 1999, Lassar, 2009, Puri et al., 2000, Zetser et al., 1999). This pathway is induced by cell-cell contact (Krauss, 2010) via upstream kinases, including transforming growth factor beta-activated kinase-1 (Tak-1), apoptosis signal-regulating kinase-1 (Ask-1) and activation of the mitogen activated protein kinase kinase, also called MAP kinase kinase, such as MKK-3 and MKK-6 (Han et al., 1996, Hattori et al., 2009, Tran et al., 2012). The most important p38 forms in skeletal muscle are p38α and p38β (Keren et al., 2006, Lluís et al., 2006). In cultured mouse myoblasts, the loss of p38α decreases differentiation and delays growth and development (Park et al., 2010), whereas signalling via p38β elevates the activity of myogenic regulatory factors (Lluís et al., 2005, Wu et al., 2000, Zetser et al., 1999). Recently, it has been shown that IGF-1 can promote skeletal muscle differentiation in the presence of SB202190 that inhibits p38 MAP kinase activation (Gardner et al., 2015).

Many targets have been identified downstream of the MAP kinases, such as MAPKAP, ribosomal S6 kinase (Rsk 90), phospholipase A2 and several transcription factors (Davis, 1993). IGF-1 can stimulate tyrosine phosphorylation of the cytoplasmic protein Crk (Beitner-Johnson and LeRoith, 1995). Crk in turn interacts with mSOS (Matsuda et al., 1994) and is probably involved in IGF-1R-mediated Ras-MAP kinase signalling pathway to enhance IGF-1R dependent mitogenesis (Beitner-Johnson et al., 1996, Okada and Pessin, 1997).

1.9.4.3 Function of IGF binding protein-5 in skeletal myogenic cell proliferation and differentiation

IGFBP-5 is more highly expressed in muscles than the other IGFBPs (Duan et al., 2010). It is expressed in the myotome during early embryogenesis of zebrafish and rodents (Green et al., 1994, Dai et al., 2010). *IGFBP-5* expression is stimulated in differentiating myoblast cultures (Bayol et al., 2000, James et al., 1993, Ren et al., 2008, Rotwein et al., 1995). Blocking endogenous IGFBP-5 action in C2 cells with antisense RNA augments the differentiation processes (James et al., 1996). In contrast, overexpression of IGFBP-5, using a constitutive promoter in cultured myoblasts, inhibits their differentiation (James et al., 1996, Cobb et al., 2004, Mukherjee et al., 2008).

Addition of purified exogenous IGFBP-5 into the proliferating myoblast culture, induced by IGF-1, activates cell differentiation (Ewton et al., 1998). Although local IGFBP-5 is important in muscle development and differentiation, knockout mice have normal muscle growth and development (Ning et al., 2007). This is probably due to the compensatory interaction among IGFBP-5 and other IGFBP members in regulating muscle growth (Ning et al., 2007). Triple knockout *IGFBP-3*, *-4*, and *-5* in mice reduces quadriceps muscle weights (Ning et al., 2006). Using siRNA knockdown of *IGFBP-5* leads to inhibition of *IGF-2* expression and subsequent disruption of muscle growth and development (Ren et al., 2008).

1.10 Aims of thesis

Although previous experiments have determined many of the molecules and inductive signals implicated in myogenesis, there remain many unanswered questions about this process. Therefore, the main aim of this project was to investigate how the IGFs control the initial steps that regulate the myogenic programme *in vivo* during chick limb growth and development. Myogenic progenitors that have delaminated and migrated from somites into the limb express *Pax3* and *Pax7* (Buckingham and Relaix, 2007). These cells then together form muscle fibres by expressing myogenic genes, including myogenic regulatory factors (Murphy and Kardon, 2011). The chick embryo is well-suited to investigate the molecular mechanisms regulating these initial steps of limb muscle formation.

- The first aim of the thesis was to characterise the *Mrfs* expression patterns with a particular emphasis on the developing limb. This work has been published by Mok et al. (2015) (see appendix). It was very important to focus on when *Mrfs* are transcribed in the limb, because this helps to investigate how they interact with IGF signalling in the developing limb.

- The second aim of the thesis was to determine the expression of IGFs signalling components in the developing chick limb. Although Allan et al. (2003) have identified IGF mRNA expression during embryonic chick development, IGF component expression in the developing limb has not been so far characterised in detail. In this thesis, the expression profile of *IGF-1, IGF-2, IGF-1R, IGF-2R* and *IGFBP*-5 in the chick limb was precisely characterised using whole-mount *in situ* hybridisation. This analysis was important to perform in order to understanding when the IGFs are expressed prior to determine their role in providing inductive signals that occur in the limb.

- The third aim of the present study was to determine the role of IGF signalling in the regulation of myogenic transcription factors during limb myogenesis *in vivo*. Recently, it has been reported that IGFs interact with MRFs in hypertrophy and regeneration where IGFs can stimulate MRFs, which in turn induce the expression of IGF (Zanou and Gailly, 2013). However, how IGF specifically regulates early limb myogenesis is still unknown. For this purpose, IGF soaked beads were grafted into the chick forelimb mesenchyme at precise stages of myogenesis. Subsequently, *in situ* hybridisation analysis was performed for *Pax3* and myogenic regulatory factors to investigate IGF effects on muscle specific genes. These data determined that IGFs play a key role in regulating the initial steps of limb myogenesis. Analysis of these data was also important to investigate the downstream pathways of IGF-1 and IGF-2 signalling.

- The fourth aim of the thesis was to determine the IGF signalling pathway involved in limb myogenesis. To this purpose, specific pharmacological inhibitors were loaded onto beads and co-grafted along with IGF soaked beads into the chick forelimb mesenchyme (also at precise stages of muscle development); then *in situ* hybridisation analysis was again performed for *Pax3* and *MyoD*.

Chapter 2: General materials and methods

2.1 Growing and staging of experimental animals

Fertilised white leghorn chicken (*Gallus gallus*) eggs were purchased from Henry Stewart Limited (Norwich, UK). Eggs were incubated at 15°C in (Sanyo incubator MIR-154) until the day of use, but no more than 5 days; then transferred to 38°C (Forma scientific CO2 water incubator) for growing until they reached the required stages. Embryos were staged according to Hamburger and Hamilton (1951). HH prefix is located prior to each embryo's stage (i.e. HH stage X). All the embryos work was performed in strict conformity with the Nottingham University Guide. Experiments were performed taking into account the Animals (Scientific Procedures) Act A (SP) A, 1986 legislation. In this project, chicken embryos do not need full ethical review as the classification of these animals is not under A (SP) A, which specifies two thirds of the way through the development (14 days in chickens). This matter was discussed by the project supervisor with ethics officer of the school.

2.2 Molecular Biology Techniques

2.2.1 Plasmid Preparation and transformation into DH5α cells

The pGEM plasmids were made according to the protocol in (Mok, 2014). A single colony of Escherichia coli MAX Efficiency DH5α (E. coli DH5α, Invitrogen) was picked up from a fresh plate and then incubated in 5 ml of LB-broth (Luria-Bertani) media subsequently grown in 100 ml flask at 37°C overnight in a shaker at 250 rpm.

LB media (1 L) was prepared as follows:

- 10 g tryptone with 10 g NaCl and 5 g yeast extract were dissolved in 950 mL Deionized Sterile Water (DSW).
- pH of the medium was adjusted to 7.0 by 1N NaOH, then volume was elevated to 1 litre.
- Solution was immediately autoclaved for 20 minutes at 15 psi.
- Solution was then allowed to cool to 50°C, and antibiotic was added (50µg / mL of Amp).
- Stock was stored at room temperature (RT).

LB agar-plates:

- As LB media prepared, 15 g/L agar was added before autoclaving.
- The autoclaved solution was left to cool and then antibiotic was added.

The bacterial pre-culture was then transferred and diluted in a 500 of media to have a significant ratio between volume and surface and was grown until OD600 (2-2.5 hours). Then bacterial suspension was then poured in falcon tubes (50 ml), chilled on ice for 10 minutes and centrifuged at 5000 rpm for 5 minutes at 4°C. All other steps were performed on ice. The bacterial pellet was re-suspended in 50 ml of ice cooled storage buffer (FSB; 50 mM CaCl2, 100 mM KCl, 10 mM potassium acetate and 10% glycerol) with gentle shaking and kept on ice for 2 hours. After that, the bacterial suspension was centrifuged at the sample at 5000 rpm for 5 minutes at 4°C. The obtained cells were re-suspended in 10 ml of FSB solution. Aliquot of 200 µl was placed in Eppendorf tubes and directly stored at -80°C.

For transformation, a 200 µl aliquot of competent cells was thawed on ice then mixed with 4 µl of ligation mixture. Tubes containing plasmid DNA were swirled then incubated on ice for 30 minutes. After incubation, the cells were then heat-shocked for 30-60 seconds at 42°C water bath and then directly transferred on ice for 5 minutes. Then, cells were added into 1 ml of LB media and incubated for 1 hour at 37°C with shaking incubator at 225-250 rpm. Subsequently, cells were centrifuged for 5 minutes at 6000 rpm, and this step led to removing about 400 µl of supernatant. 10 µl of Promega X-gal was added to the cells and then re-suspended. After that, cells were plated on LB plates containing the appropriate antibiotic (10 µg/ml of ampicillin) and incubated at 37°C overnight.

2.2.2 Isolation of a plasmid and purification of plasmid DNA via midi prep

Isolation of DNA is purification of DNA from an appropriate sample by a combination of chemical and physical methods. The QIAGEN plasmid plus midi prep kit (cat no. 27104) was performed according to the manufacture's procedure (www.qiagen.com) for large-scale plasmid DNA preparation. A single colony of DH5a E coli was picked up from a fresh plate and then incubated in 5 ml of LB media containing ampicillin 5ug/ml; then grown in 95 ml flask containing ampicillin 95ug/ml at 37°C overnight in a shaker at 250 rpm. The next day, Bacterial culture was transferred to 100 ml of LB media containing ampicillin 100ug/ml and incubated at 37°C overnight in a shaker at 250 rpm. Bacterial cultured was then harvested using the centrifuge (Sorval instruments 9000088) at 6000x g for 15 minutes at 4°C. Pelleted bacteria were re-suspended in 2ml of buffer P2 and incubated 3 minutes at RT. 2ml of Buffer S3 and 2 ml of buffer BB were added after 10 minutes incubation at RT between each other.

DNA was washed by 700 µl buffer ETR and buffer PE with vacuum for few minutes between both steps. To further washing, column was centrifuged at 10,000 x g (9,700rpm) for 1 minute in Eppendorf table top microcentrifuge. Column was placed in to a fresh Eppendorf tube and 200µl water was added into the centre of the QIAGEN Plasmid Plus spin column to elute the DNA. Plasmids were then digested with Escherichia coli RI (EcoRI) at 37°C overnight. The following reaction was used:

- 1 µl Plasmid DNA
- 1 µl EcoRI enzyme (Promega)
- 1 µl Buffer H (Promega)
- 7 µl Sigma water
- 10 µl Total volume

The following day, the reaction was run on a 1% agarose gel for 30-40 minutes then visualised under the ultraviolet light (UV light) (UVP GelDoc-IT-imaging system).

2.2.3 Gel elution sequencing and linearisation of plasmid DNA

2.2.3.1 Gel electrophoresis

Whole isolated DNA or RNA as previously described was checked by agarose gel electrophoresis. A gel was prepared with a 1% agarose concentration diluted in 1X Tris-acetate (1x TAE) buffer. Ethidium Bromide (EtBr) was added into a final concentration of 1 µg/ml. The DNA\RNA samples with loading buffer (0.1% bromophenol blue/50% glycerol) were mixed and loaded into the sample wells. 1kb DNA ladder (NEB) was used to estimate the size of each band. Samples were loaded into wells, run at a voltage 90-120V for 30-40 minutes and then directly visualized under illumination by the UV light.

- Tris-EDTA buffer solution (TE buffer): It was made from 1M stock of Tris with 0.1 mM EDTA.
- 1M Tris (hydroxymethyl) aminomethane, FW 121.4 g/mol: prepared by adding 60.57 g in 0.5L mq dissolved in water at pH 7.5 by using HCl.
- 0.1M EDTA (Diaminoethane tetraacetic acid), FW 372.2 g/mol: prepared by adding 18.61 g in 0.5L DSW. adjust the pH to 8.0 using NaOH.

2.2.3.2 Sequencing

To determine the primary structure of DNA fragments, they were sequenced using Geneservice Sequencing (Nottingham, UK). 100 ng of plasmid DNA was sent and was analysed by reverse (SP6 promoter) or T7 (promoter) universal primers provided by Geneservice Sequencing. The obtained data from the sequences were analysed using sequence analysis software for molecular biology (for downloading this program follow this webpage http://informagen.com/SA/).

2.2.3.3 Plasmid DNA linearisation

Linearised isolated plasmid DNA for synthesising RNA probes was performed using particular restriction enzymes for SP6 or T7 RNA polymerase promoter sites. The different sites in the vector were selected according to a pGEM Teasy vector (Promega) that contains an ampicillin

resistant gene (Ampr) and β-galactosidase (lacZ) gene (Figure 2.1). The reaction was incubated at 37°C (Mini/18/Clad MI8Z) for 2 hours then run on a standard agarose gel (1%) and visualised using the UV light.

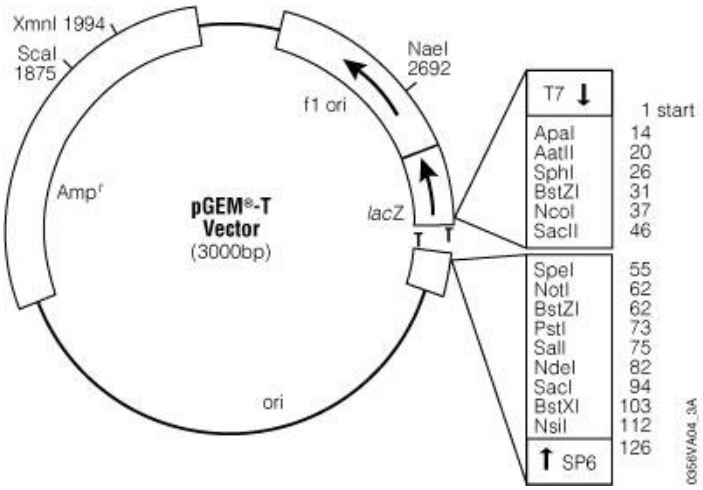


Figure 2. 1: pGEM-T vector (Promega) 3000 bp in size. The vector contains ampicillin-resistant gene, T7 and SP6 promoter regions as well as many different sites of restriction. For more details see Promega website: https://www.promega.co.uk /resources/product-guides-and-selectors/protocols-and-applications-guide/cloning/

2.2.4 Purification of plasmid DNA

In this study, two protocols were used to purify the plasmid DNA:

2.2.4.1 Qiaex II Gel Extraction Kit

Plasmid DNA was purified using the Qiaex® II Gel Extraction kit (150) (cat no. 20021). According to manufacturer's instructions, 100 µl of the large linearisation reaction was picked up from 37°C. After that, 300 µl of buffer QG and 100 µl of isopropanol were gently added to the suspension. The total volume of the suspension, 500 µl, was applied into the spin column then centrifuged for 30 seconds at 13000 rpm. The flow-through was discarded; then 750 µl of buffer PE was added into the remaining lysate and applied into the spin column. The solution was then centrifuged at 13000 rpm for 1 minute and the flow-through was discarded. For further wash to remove unappropriated residual wash buffer, the column was centrifuged at 13000 rpm for 1 minute then 50 µl of DEPC-treated water was added to the top of the column directly onto the mini spin column membrane. Then, the column was centrifuged at 13000 rpm for 1 minute;

the eluate was collected in RNase-free Eppendorf tube. Purified DNA was stored at -20°C for transcription reaction.

2.2.4.2 Phenol-chloroform extraction

100 µl of the large linearisation reaction was picked up from 37°C then 10 µl of 3M sodium acetate (pH5.5) and 100 µl phenol (pH 8.0) were added. The reaction was vortexed and centrifuged at 13K rpm for 5 minutes. The aqueous layer was transferred into the new Eppendorf tube then 100 µl chloroform and 4 µl isoamyl-alcohol was added and centrifuged at 13K for 5 minutes. A further extraction was performed by 330 µl of 100% ethanol then 500 µl of 70% ethanol was centrifuged at 13K for 30 minutes. The supernatant and residual liquid was discarded and DNA pellet was incubated for 15 minutes at RT then re-suspended in 20 µl of RNAse free water.

2.2.5 *In situ* hybridisation analysis

2.2.5.1 Preparation of Labelled RNA probes

The standard *in situ* hybridisation process was performed as described in (Nieto et al., 1996). Linearised plasmid DNA was used to generate Dig labelled antisense RNA probes for *in situ* hybridisation.

1- To synthesise Dig-labelled RNA probes 1ug of linearised DNA was added to and mixed with the following reaction at RT:
   - 10µl sterile distilled water
   - 4µl 5X transcription buffer (5x)
   - 2µl T7, SP6 or T3 RNA polymerase (Promega)
   - 2µl DTT (Promega)
   - 1µl nucleotide Dig-mix
   - 1µl RNasin (Promega)

All constructed plasmids DNA were added to the following restriction enzymes:

Plasmid DNA Constructs	**Restriction enzymes, buffer and RNA polymerase promoter**	**Origin of constructs**
pGEM-cMyf5	Sac II, C, SP6 (Promega)	Andrea Münsterberg
pGEM-cMyoD	Sac II, C, SP6 (Promega)	Andrea Münsterberg
pBS-cMgn	Sal I, D, T7 (Promega)	Andrea Münsterberg
pGEM-cMRF4 Apa I, multicore	SP6 (Promega)	Andrea Münsterberg
pBS-cPax3	Sac II, A, T3 (Promega)	Andrea Münsterberg
pGEM-cIGF-1-HA full length	SacII, C, SP6 (Promega)	Dylan Sweetman
pGEM-cIGF-2-HA full length	SacII, C, T7 (Promega)	Sweetman, Mok
pGEM-cIGF-1R	Sall, D, T7 (Promega)	Dylan Sweetman
pGEM-IGF-2 R ISH	Sacl, J, T7 (Promega)	Sweetman, Mok
pGEM-cIGFBP-5	Sall, D, T7 (Promega)	Dylan Sweetman

2- Reaction was incubated at 37°C for 2 hours.
3- 1 µl aliquot was removed then run on an agarose gel with 0.5 ethidium bromide for estimation the amount made.
4- 2 µl from ribonuclease-free DNase I (1 UIpl) was added, the reaction then was incubated at 37°C for 15 minutes.
5- 130 µl distilled water was then added into 50 µl 10 M ammonium acetate, and 400 µl ethanol. Solution was then mixed and incubated for 30 minutes at -20°C. Do not incubate at a lower temperature as this may precipitate unincorporated nucleotides.
6- The reaction was then spanned for 10 minutes at 750 rpm. Pellet was washed with 70% ethanol then left to air-dry.
7- Pellet was dissolved in 200 µl hybridisation buffer.
    - The probe should not be left to dry for long time, this probably make it hard to resuspend. The probe was dissolved on ice for

a time, subsequently mixed carefully by pipetting using a p200 and then vortexing.

- The purified probe was then added into 20 ml of hybridisation buffer and stored at -20 for the use.

2.2.5.2 Collection, fixation, pre-treatment and hybridisation of embryos

1- Embryos were dissected in 1X PBS, eliminating as many extraembryonic membranes and unappropriated tissues as possible.

- 1x PBS (Phosphate Buffered saline): prepared by adding 4 tablets Dulbecco "A" tablets (Oxoid) (BR00146) to 400 ml deionised water. The solution then should be incubated in a fume hood overnight then autoclaved and stored for the use.

2- Embryos were fixed in 4% PFA at 4°C overnight on rocker.

- After this step, samples can be stored for several days.
- 4% Formaldehyde fixative (4% PFA): prepared by adding 16g of paraformaldehyde powder to the heated (65°C) 400 ml PBS solution under a ventilated hood until dissolved; then solution was filtered and used on the prepared day or stored at 20°C.
- Extreme care should be taken because Paraformaldehyde fumes are toxic take.

3- Prior to hybridisation, embryos were rinsed and washed twice in 1X PBSTw for 10 minutes at RT on rocker.

- PBSTw: Prepared by using 400 ml sterilised PBS with 400 µl Tween 20 (10%).

4- Embryos were dehydrated at RT for 5 minutes on rocker by washing them in following ascending concentrations of methanol:

- 25% MeOH in 1X PBSTw
- 50% MeOH in 1X PBSTw
- 75% MeOH in 1X PBSTw
- 100% MeOH in 1X PBSTw

5- Embryos were washed twice in 100% MeOH for 5 minutes on rocker, and then stored in 100% MeOH at -20°C.
   - In this step, embryos can be stored at -20°C for several months.

6- Rehydration and washing were carried out at RT by progressive concentrations of MeOH/PBSTw
   - 75% MeOH in 1X PBSTw
   - 50% MeOH in 1X PBSTw
   - 25% MeOH in 1X PBSTw

7- Embryos were washed twice for 5 minutes in PBSTw on rocker.

8- Embryos were then treated in proteinase K in PBSTw for 30 minutes at RT.
   - This step was prepared by adding 10µl of 10mg/ml Prot. K into 10ml 1X PBSTw.
   - Proteinase K: For chicken embryos between HH stages 20 to 25 use 10 mg/ml stock. For old embryos, HH stages 25 to 28, use 15mg/ml stock, whereas for embryos over HH28, use 20mg/ml.
   - Proteinase K is diluted in PBSTw.

9- Embryos were rinsed and washed in PBSTw and then re-fixed in 4% PFA containing 0.1% glutaraldehyde for 20 minutes.
   - Glutaraldehyde, 25% stock solution (Sigma): Prepare using 80 µl Glutaraldehyde in 20 ml 4%PFA.

10- Pre-hybridisation was carried out at RT. Embryos were rinsed and washed twice in PBSTw at RT on rocker, and then in mix of solution containing PBSTw and hybridisation buffer (1:1) for 10 minutes at RT.
   - Hybridisation buffer to 50ml: Prepared using 25ml of 50% formamide, 3.25 ml of 1.3X SSC (20X pH with citric acid), 0.5 ml of 5mM EDTA (0.5M pH8), 125 µl Yeast RNA (sigma R6750, 20mg/ml), 1 ml Tween 20 (10%), 2.5 ml CHAPS (10%), 100 µl Heparin (sigma, H3149, 50mg/ml) all were mixed under the fume hood in 17.5ml DEPC-water.

- 20X SSC stock solution: Prepared by adding 3 M NaCl to 0.3 M sodium citrate at pH 7.0
- DEPC-water: Prepared using 100 µl diethyl pyro-carbonate (DEPC) in 400 ml deionized distilled water.

11- Embryos were again washed in hybridisation buffer at RT.

- At this step, embryos can be stored overnight at 4°C, and re-heated to 65°C the next day.

2.2.5.3 Post-hybridisation, Probe, washes and incubation

Hybridisation and post-hybridisation processes were carried out at 65°C overnight with a precise probe.

1- Hybridisation solution was warmed to 65°C, embryos were washed twice in this solution for at least 2 hours per wash.

2- Next, the hybridisation solution was replaced with the required probe, embryos were incubated overnight at 65°C in probe oven.

- Enough probe should be added for covering the entire embryos.

3- The next day, the probe was re-stored at -20 for other uses; embryos were washed in hybridisation buffer for 10 minutes at 65°C.

4- Embryos were washed twice in wash-buffer at 65°C (30 minutes per wash step).

- At this step, embryos can be left longer than 30 minutes and can also be stored overnight if necessary.
- Washing Buffer to 50ml: Prepared using 25 ml Formamide 50%, 2.5 ml SSC (20X pH with citric acid), 0.5 ml Tween 20 (10%) all were mixed under the fume hood in 22 ml deionized distilled water.

5- Embryos were washed in mix solution containing wash-buffer and MABT (1:1) for 10 minutes at RT, and then rinsed three times in MABT.
   - 5X MAB stock: Prepared by dissolving 100mM Maleic acid (Sigma M0375), 150mM NaCl, then pH was adjusted to 7.5.
   - Maleic acid Molecular, FW 116.07g/mol: It was made by dissolving 290.175 mg MAB in 500 ml DSW at pH 7.5 using NaOH.
   - 1X Maleic acid-Tween (1X MABT): It was made by adding 40 ml 5x MAB, 2 ml Tween-20 to 160 ml deionized distilled water.

6- Embryos were washed twice in MABT at RT on rocker (30 minutes per wash step).

7- Embryos were blocked in Roche blocking reagent/20% goat serum (Roche Cat. No. 11096176001) and were then re-blocked in solution containing either anti-Dig-AP Fab fragments (Roche Cat. No. 11093274910) or Anti-Fluorescein-AP Fab Fragment (11940700) at 4 °C on rocker overnight.
   - To dilute AP conjugated anti-DIG Ab (Roche Cat. No. 11093274910) add 1 anti-DIG: 2000 blocking solution.
   - To dilute anti-fluorescein antibody (11940700), add 1 anti-fluorescein: 5000 blocking solution.
   - Blocking solution is prepared as follows:
     10 ml BBR
     10 ml goat serum
     30 ml MABT
   - Blocking solution: 2% Blocking Reagent (Roche, 1096176001) and 20% goat serum were dissolved in 1XMABT, aliquots then was stored at 25 °C.
   - Working solution: blocking reagent was dissolved in 1XMABT to a final concentration of 10% (w/v) with shaking and heating.

8- The next day, embryos were washed in MABT three times (1 hour per wash) at RT on rocker then at 4°C on rocker overnight.

9- Embryos were washed twice in NTMT for 10 minutes on rocker, and then transferred to the plastic embryos chambers for visualisation by staining them in the dark in NTMT containing 9 µl NBT and 7 µl BCIP per ml.

- Some probes are strong, therefore embryos should not be left too long.
- Staining reaction buffer (NTMT) can be performed by making a fresh stock shortly before use, as follows: NTMT: 1ml of 100mM NaCl, 2.5 ml of 100mM Tris pH 9.5, 1.25 ml of 50mM MgC12, 5 ml of Tween-20 (10%) all solutions in 40.25 ml deionized distilled water.
- NBT stock solution: 4-Nitro blue tetrazolium chloride (Roche Cat. No. 11087479001), stock at 75 mg/ml NBT (Boehringer) in 70% dimethylformamide. Aliquot and store at -20°C in dark.
- BCIP stock solution: 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Roche Cat. No. 10760994001), stock at 50 mg/ml BCIP (Boehringer) in dimethylformamide. Aliquot and store at -20°C in dark.

10- Embryos were washed twice in TBSTw at RT on rocker, and then overnight at 4 °C on rocker to remove purple.

- 5X Tris-buffered saline (5X TBS): It was made by adding 40 g NaCl, 1 g KCl and 125 ml 1 M Tris pH 7.5, make to 950 ml with DSW the solution then was autoclaved.
- Tris-buffered saline with detergent (TBSTw): Prepared by adding 50 ml Tween-20 10% into the autoclaved 950 ml TBS.
- The selected embryos were incubated in 4% PFA with Na-ZIDE at 4°C. This step is prepared by adding 10 µl Na-AZIDE in to 20ml 4% PFA).

2.3 Paraffin embedding, sectioning and photography of chick embryos

2.3.1 Materials

- Ethanol: 25%, 50%, 70%, 95%, 100% (diluted in DSW).
- 1X PBS
- Xylene (Cat. No. NC-13133)
- Steel or plastic wax moulds
- Marker for labelling wax blocks
- Plastic cups using for paraffin melting
- Hot water (30-40 °C).
- Hot plate for draying and embedding
- Microtome and blades for trimming and sectioning
- Forceps and brushes
- Microscope slide and cover slips

2.3.2 Paraffin Embedding

On the embedding day, paraffin should be melted in the oven for the use. Embryos were embedded as follows:

- They were washed twice in 1X PBS at RT and in sterile distilled water.
- Dehydration was carried out by progressive concentrations of ethanol 25%, 50%, 70%, 90% and 100% for 30 minutes each time. Care should be taken during changing solutions to avoid drying and damaging the chicken embryos.
- Embryos were then washed in xylene and later transferred into hot paraffin wax at 65°C for 2 hours.
- Embryos were appropriately orientated in paraffin wax solution and cooled for 2 hours at 4°C. For cross sections, embryos should be oriented to upright position and their bodies axes to the bottom surface

2.3.3 Embryos sectioning and photography

- To begin with, embryos were set up onto the right position of a HM 355 microtome.
- The sections were cut slowly to check whether the block of wax on the chuck requires readjustment.
- Sections were carried cut at 7 µm thickness and then placed onto glass microscope slides.
- Sections were mounted in Omnimount (Histological Mounting Medium HS-110).
- Clarified slides were photographed by using Olympus BH-2 microscope.

2.4 OCT embedding, sectioning, immunofluorescence staining and photography

2.4.1 OCT embedding

- Treated embryos were harvested in a cold PBS then fixed in 4% PFA at 4°C overnight.
- Followed day, embryos were washed three times in cold PBS for 15 minutes (5 minutes per wash step).
- Embryos were washed in 30% filter sterilised sucrose and incubated at 4°C overnight.
- Embryos were then embedded into Optimal Cutting Temperature compound (OCT 361603E) mounting medium in order to frozen sectioning on a microtome-cryostat.

2.4.2 Sectioning, immunofluorescence staining and photography

- Cryo-sectioned embryos were cut at a thickness of 15µm on a freezing microtome.
- Sections were then transferred onto the slides. Slides were then washed 5-6 minutes in PBS only; then in PBS 0.1 % TritonX-100 (053K0027) and incubated for 10 minutes at RT.
- Slides were blocked in PBS, 5% BSA, 5% normal goat serum (PBGS) followed by incubation with PAX3-antibody for $Pax3^{+ve}$ cells according to (Mok and Sweetman, 2011) diluted in PBGS 1/250 µl .

- Slides were then incubated overnight at 4°C.
- Followed day, three times washes in PBS were applied for 30 minutes (10 minutes per wash step), slides were then incubated in secondary antibody 1/500 µl, Goat anti-Mouse IgG1, Alexa Fluor 568 (A-21124) for an hour at RT.
- Slides were washed in PBS for 30 minutes (10 minutes per wash step), then incubated with the specific antibody 1/250 µl (Phospho-Histone H3 (Ser10) antibody (44-1190G), for mitotic cells staining according to (Abu-Elmagd et al., 2015), and incubated overnight at 4°C.
- Slides were washed in PBS for 30 minutes (10 minutes per wash step), then incubated with its particular secondary antibody 1/500 µl (Goat anti-Rabbit IgG (H+L), Alexa Fluor 488 (A-11034) and incubated for 1 hour at RT.
- Slides were also washed for 30 minutes (10 minutes per wash step) and mounted in hydro-mount (Floroshield with DAPI SLBK4338V) then coverslips were added.
- Slides were stored at 4°C thereafter in the dark for staining intensity for several days. Samples were visualised and photographed by using immunostaining microscope (Leica DMP).

2.5 Preparation of beads for grafting

2.5.1 IGF-beads

Grafting beads was carried out as previously described according to (Mohammed and Sweetman, 2016). All implanting experiments were performed *in vivo*.

- Heparin-immobilized Acrylic beads (Sigma H-5263) were saturated for 1 hour at RT with recombinant human IGF proteins at 1µg\ml diluted in PBS with 0.1% Bovine Serum Albumin (1% BSA) (Cat. No. A-3983-500G).
- Beads were ectopically manipulated into slits cut into the forelimb using a sharpened tungsten wire needle.

- Grafted embryos were visualised by injection of specific Windsor and Newton Black India Ink diluted in PBS. This step can be prepared as follows:
- 300 ml PBS
- 300µl Penicillin-streptomycin (1:1)
- 5-6 drops of the biological ink
- Embryos were grafted between HH stage 17 and HH stage 22.
- Eggs were then covered using a tape and incubated at 38°C for 18 or 24 hours.

2.5.2 Pharmacological inhibitor beads

AG-1 X2 beads (BioRad) were incubated in the following pharmacological drugs:

- 10mM Picropodophyllotoxin (Tocris, 2956)
- 10mM U0126 (Merck)
- 10mM LY294002 (Calbiochem)
- 10mM SU5402 (Calbiochem)
- 10mM Nocodazole.

According to the manufacturers, all inhibitors were dissolved in Dimethyl Sulfoxide 99.9% EC 200-664-3 (DMSO).

- Inhibitors were incubated with beads for an hour in a dark condition at RT.
- Inhibitors-beads were visualised using a red phenol 2% (dissolved in water).
- Prior to manipulation, beads were washed in PBS and then the AG-1 X2 control beads were saturated in DMSO.

Chapter 3: Characterisation of myogenic regulatory factors in the developing chick limb

3.1 Introduction

The myogenic regulatory factors (MRFs) are a family of basic helix-loop helix (bHLH) transcription factors (Pownall et al., 2002), including myogenic factor 5 (*Myf5*), Myogenic determining factor 1, (*MyoD*), *Myog* and *Myf6* (also known as *Mrf4*) (Bryson-Richardson and Currie, 2008). *MyoD* was the first member of MRFs to be identified. It has been characterised and isolated by subtractive hybridisation from myoblast-specific cDNA libraries (Davis et al., 1987). Homologues of the MRFs have been identified in numerous species, and *in situ* hybridisation has determined their expression patterns in fish, quail, frogs, chicken and mice (Amali et al., 2004, Armand et al., 2008, Hopwood et al., 1991, Kablar et al., 2003, Miner and Wold, 1990, Mok and Sweetman, 2011).

The four MRFs are transcribed first in somites (Mok et al., 2015, Sassoon et al., 1989, Tajbakhsh and Buckingham, 2000). In chick embryos, the expression of *Myf5* is detected first in somites and paraxial mesoderm at HH stage 9, while early *MyoD* expression is seen at HH stage 12 followed by *Myog* then *Mrf4* at HH stage 14 (Mok et al., 2015). At day 8 of mouse embryo, *Myf5* is also expressed first in the rostral somites; then downregulated after 2 weeks of development (Ott et al., 1991). In contrast, no expression of *MyoD* can be seen in newly formed somites (Sassoon et al., 1989). However, about day 8.5, *Myog* is clearly expressed in the myotome compartment followed by *Mrf4*, which is transiently elevated between days 9 and 12 (Bober et al., 1991). In the limb bud, *Myf5* is transiently expressed between days 10 and 12 of mouse embryo followed by *MyoD* which is co-expressed with *Myog* after day 10.5; subsequently, *Mrf4* is the last MRF expressed at day 16 (Bober et al., 1991, Ott et al., 1991).

As MRFs are well established master regulators of muscle development they can be used as markers of muscle cell commitment and differentiation in the limb (Bajard et al., 2006, Berkes and Tapscott, 2005, Olson et al., 1986, Pownall et al., 2002, Sweetman, 2012, Tajbakhsh and Buckingham,

1994). During myogenesis, these genes are expressed at specific phases. For instance, *Myf5*, *MyoD* and *Mrf4* are expressed in myoblasts (Biressi et al., 2007, Kassar-Duchossoy et al., 2004, Sassoon et al., 1989), while *Myog* expression is seen in myocytes / myotubes (Sassoon et al., 1989). Moreover, *MyoD*, *Myog* and *Mrf4* are expressed in myofibers and mature muscle (Sassoon et al., 1989, Voytik et al., 1993).

MyoD, *Myog* and *Mrf4* are also expressed in the nuclei of differentiated myofibres (Bober et al., 1991, Hinterberger et al., 1991, Fujisawa-Sehara et al., 1990, Ontell et al., 1993, Sassoon et al., 1989). *Myf5* and *MyoD* have redundant effects in the determination of myoblast; loss of either gene does not lead to any dramatic effect on myogenesis (Braun et al., 1992, Rudnicki et al., 1992). Loss of function experiments, particularly mutant studies performed in mice, have found that *Myf5*, *MyoD* and *Mrf4* are myogenic determination factors and the loss of the three genes cause a complete absence of skeletal muscle (Kassar-Duchossoy et al., 2004, Rudnicki et al., 1993, Rawls et al., 1998). Although these studies and others have identified the Mrfs and their transcription during myogenesis, a comparative analysis of their expression patterns during limb muscle formation has not been published. To fully understand the effects of signalling molecules in limb bud myogenesis, it is necessary to know this analysis. Therefore, this chapter examined in detail the spatiotemporal expression of *Myf5*, *MyoD*, *Myog* and *Mrf4* in the developing limb of chick embryos. This work was published in Mok et al. (2015).

3.2 Results

3.2.1 *Myf5* expression

Whole-mount *in situ* hybridisation and sections through embryos at HH stage 20 did not detect expression of *Myf5* in limbs (Figures 3.1A; 3.2A and 3.3A). Early *Myf5* expression was seen only in the proximal region of the forelimb at HH stage 22 embryos (Figure 3.1B). Sections through this stage showed that *Myf5* was the first Mrfs to be expressed in the dorsal and ventral muscle masses of both fore and hind limbs (Figures 3.2B and 3.3B). At HH stage 23 (Figure 3.1C), *Myf5* expression increased and extended from the proximal to distal region of the forelimb. At the same stage, *Myf5* was expressed in the central region of the hindlimb. *Myf5* expression subsequently maintained from HH stages 24 to 26 during limb muscle development (Figure 3.1D and E). Transverse sections through embryos at HH stages 24 and 26 showed that *Myf5* expression localised to the dorsal and ventral muscle masses of both limbs (Figures 3.2C, D and 3.3C, D). Although the level of *Myf5* expression reduced in both limbs at HH stage 30 (Figure 3.1F), the expression maintained and outlined the differentiated muscle.

3.2.2 *MyoD* expression

Whole-mount *in situ* hybridisation and sections through chick embryos at HH stage 20 and 22 did not show any expression of *MyoD* in the forelimb or hindlimb (Figures 3.1G, H; 3.2E, F and 3.3E, F). By HH stage 23 embryos, faint expression of *MyoD* was seen in the forelimb and hindlimb, where the expression localised to the central zone of the hindlimb mesenchymal core (Figure 3.1I). At HH stage 24 embryos (Figure 3.1J), *MyoD* expression increased in the central forelimb and it also extended in the hindlimb from the proximal to the distal region. Sections through HH stage 24 showed a clear expression of *MyoD* in the dorsal and ventral muscle masses of both limbs (Figures 3.2G and 3.3G). The highest level of *MyoD* expression was shown in the forelimb and hindlimb at HH stage 26 (Figure 3.1K). In addition, sections through this stage showed *MyoD* expression localised to the dorsal and ventral muscle masses of limbs

(Figures 3.2H and 3.3H). Later, at HH stage 30, the expression of *MyoD* was distinctly seen in muscle of both forelimb and hindlimb (Figure 3.1L).

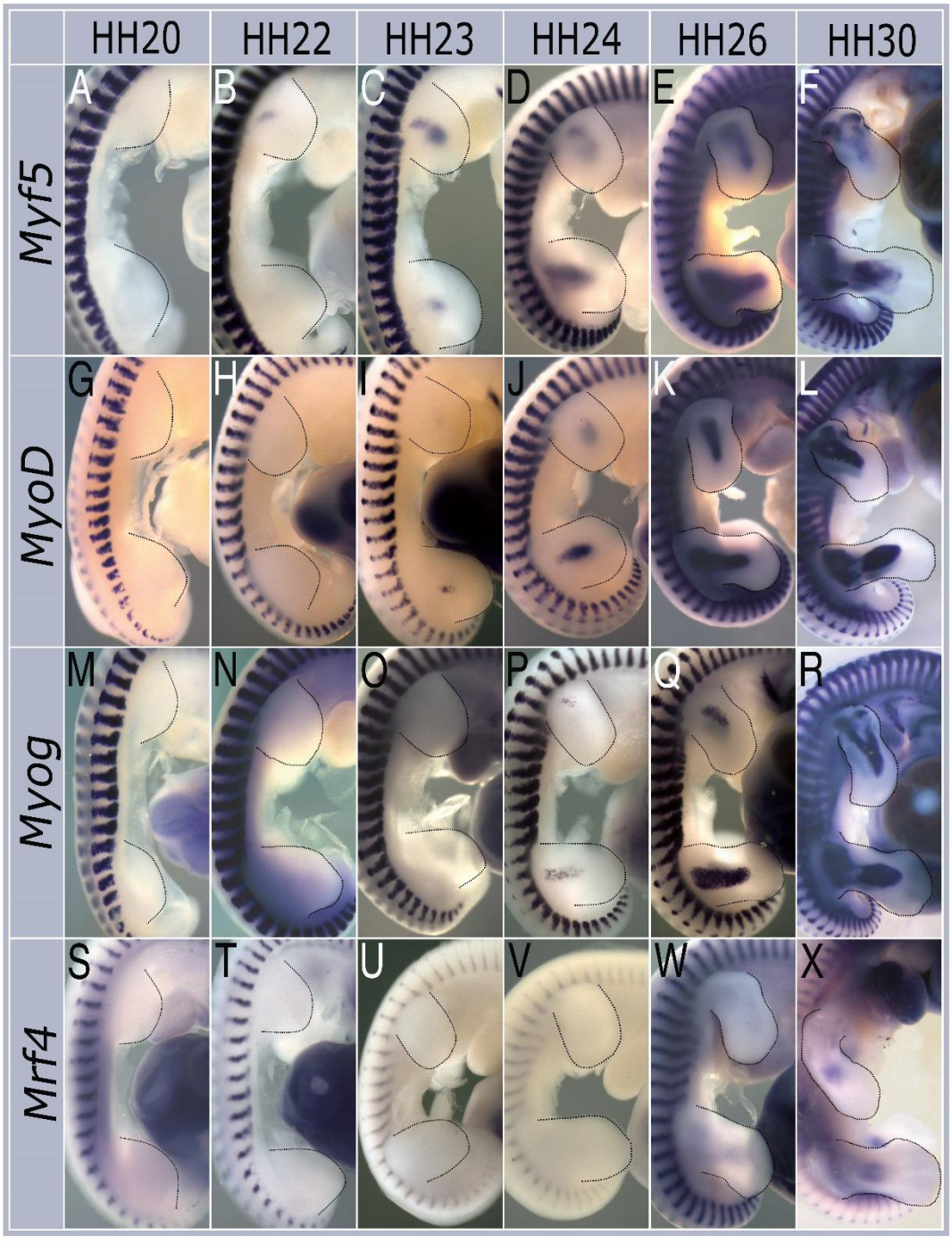


Figure 3. 1: mRNA distribution patterns of *Mrfs* in the developing chick limb. No expression of *Myf5* at HH stage 20 (A), but it is primarily localized in the central forelimb at HH stage 22 (B). *Myf5* is expressed in the hindlimb at HH stage 23 (C), thereby continued to express strongly in both limbs between HH stage 24 to HH stage 30 (D-F). No expression of *MyoD* shows at HH stages 20 and 22 (G and H), but it is detected in the central region of the hindlimb at HH stage 23 (I). The expression of *MyoD* then increases and extends from the proximal to the distal aspects of both limbs (J-L). *Myog* does not show activation from HH stage 20 to 23 (M-O). Weak expression of *Myog* is detected in the proximal region of the forelimb along with strong expression in the hindlimb at HH stage 24 (P). Q and

R show the increase and extension of *Myog* expression from proximal to the distal regions of fore and hind limbs at HH stages 26 and 30. No expression of *Mrf4* is detected from HH stages 20 to 26 (S-W). The first expression of *Mrf4* can be seen adjacent to the distal region of the forelimb as well as hindlimb at HH stage 30 (X).

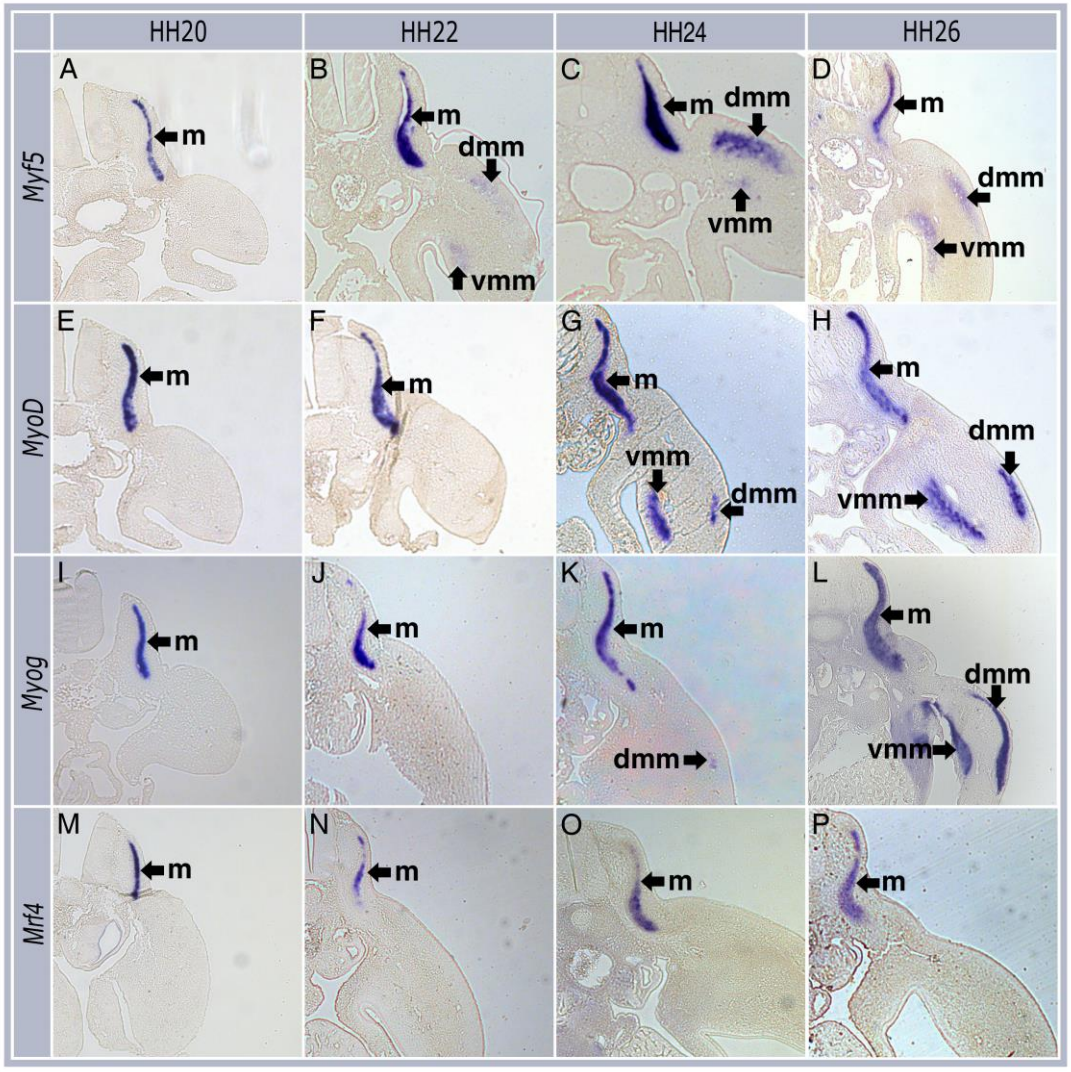


Figure 3. 2: Transverse sections in the developing forelimb of chick embryos. No expression of *Myf5* at HH stage 20 (A), but it is weakly expressed in the dorsal and ventral regions of the limb at HH stage 22 (arrows) (B). Strong expression of *Myf5* is shown in the dorsal limb at HH stage 24 (arrows) (C), then the expression is maintained in the dorsal and ventral regions at HH stage 26 (arrows) (D). *MyoD* does not express at HH stage 20 and 22 (E-F); then it is clearly expressed in the dorsal and ventral parts of the limb from HH stage 24-26 (arrows) (G and H). No expression of *Myog* is detected between HH stages 20 and 22 (I-J). K shows a weak expression of *Myog* in the dorsal region of the limb at HH stage 24 (arrow); then it is strongly expressed in the dorsal and ventral limb at HH stage 26 (arrows) (L). *Mrf4* does not show expression from HH stages 20 to 26 (M-P). Abbreviation: m, myotome; dmm, dorsal muscle mass; vmm, ventral muscle mass.

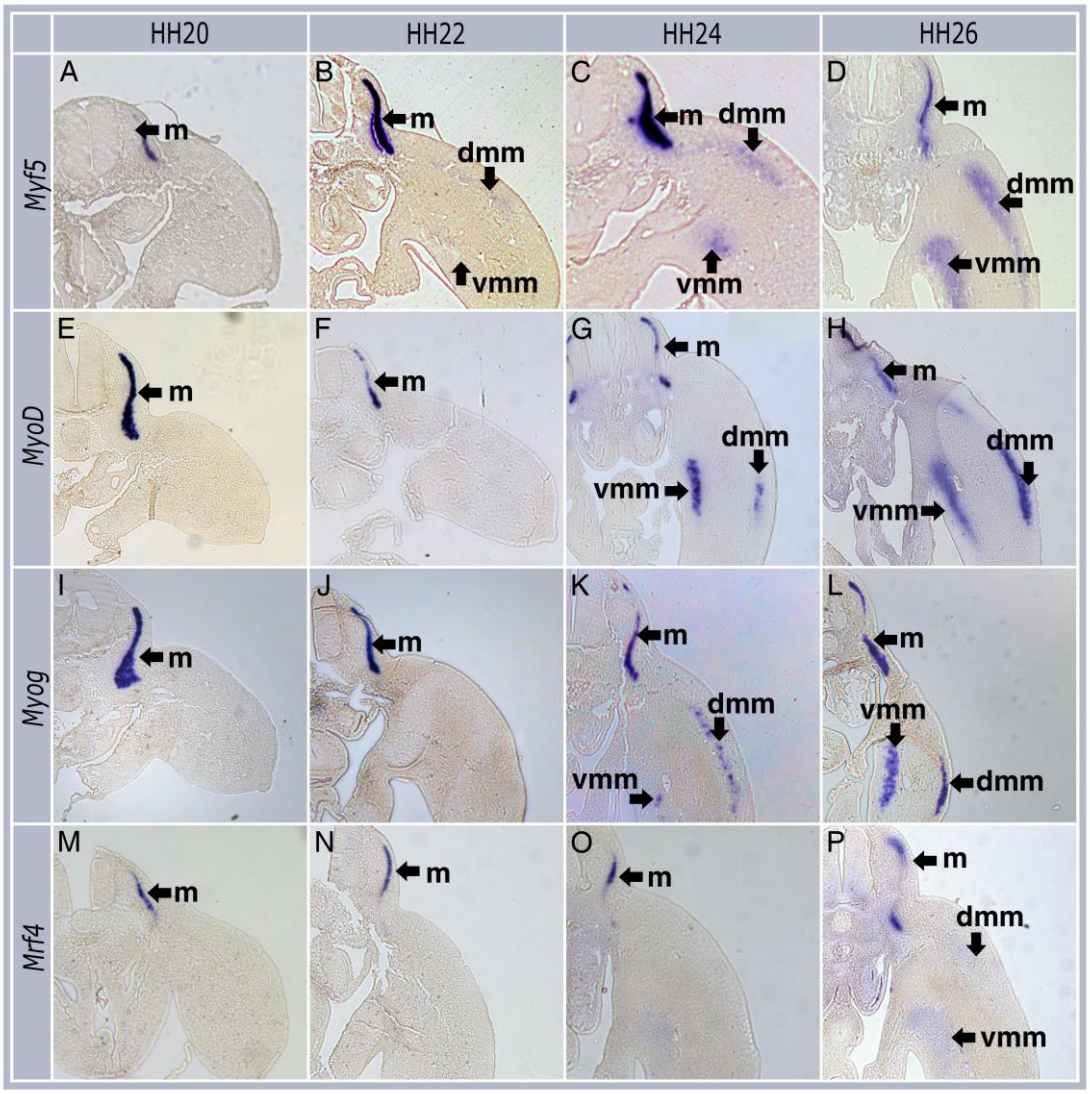


Figure 3. 3: Transverse sections in the developing hindlimb of chick embryos between HH stage 20 to 26. No expression of *Myf5* can be seen at HH stage 20 (A), but weak expression is demonstrated in the dorsal and ventral parts of the limb at HH stage 22 (B). *Myf5* is strongly expressed in both regions of the limb at HH stages 24 and 26 (C and D). No expression of *MyoD* shows at HH stages 20 and 22 (E and F); then *MyoD* is strongly expressed in the dorsal and ventral limb at HH stages 24 and 26 (G and H). No expression of *Myog* is detected at HH stage 20 (I) and HH stage 22 (J). Clear expression of *Myog* is shown in the dorsal and ventral parts of the limb at HH stages 24 (K) and 26 (L). The expression of *Mrf4* is not observed between HH stages 20 to 24 (M-O). At HH stage 26 (P), a weak expression of *Mrf4* is seen in the dorsal and ventral parts of the limb. Abbreviation: m, myotome; dmm, dorsal muscle mass; vmm, ventral muscle mass.

3.2.3 *Myog* expression

Whole-mount *in situ* hybridisation of embryos from HH stages 20 to 23 did not detect any expression of *Myog* in limb buds (Figure 3.1M-O). Sections of embryos through HH stage 20 and 22 also did not show any expression of *Myog* in the dorsal or ventral muscle masses of either limbs (Figures 3.2I, J and 3.3I, J). *Myog* was detected first in the proximal region of the forelimb at HH stage 24 (Figure 3.1P). At this stage, *Myog* was strongly expressed in the hindlimb from the proximal to the distal regions. Transverse sections through HH stage 24 showed some weak staining of *Myog* in the dorsal muscle mass of the forelimb (Figure 3.2K) and strong staining was shown in the dorsal and ventral muscle masses of the hindlimb (Figure 3.3K). At HH stage 26, the expression of *Myog* increased in both limbs (Figure 3.1Q). Sections of embryos at HH26 showed strong staining of *Myog* in the dorsal and ventral muscle mass of the forelimb (Figure 3.2L) and hindlimb (Figure 3.3L). The highest level of *Myog* expression was seen in both limbs at HH stage 30 (Figure 3.1R) in the muscle masses.

3.2.4 *Mrf4* expression

Mrf4 was the last Mrfs to be expressed through the developing limb. Whole-mount *in situ* hybridisation followed by sectioning of embryos from HH stages 20 to 24 did not show any expression of *Mrf4* in either fore- or hindlimbs (Figures 3.1S-V; 3.2M-O and 3.3M-O). Some expression of *Mrf4* was seen in the limbs at HH stage 26 (Figure 3.1W), sections through this stage showed some weak staining only in the hindlimb, but not forelimb, localised in the dorsal and ventral muscle masses (Figures 3.2P and 3.3P). By HH stage 30 embryos, however, weak staining of *Mrf4* was observed from the proximal to distal regions of both limbs (Figure 3.1X).

3.3 Discussion

The limb skeletal muscle is generated from muscle precursor cells originating in the dermomyotome of the somite compartment. Once these cells are in the limb, they proliferate then form two types of cells: endothelial or muscle (Hutcheson and Kardon, 2009, Mayeuf-Louchart et al., 2014, Vinciguerra et al., 2010). During myogenesis, myogenic

precursors are specified to be myoblasts then differentiate into myocytes, which in turn form myofibers (Emerson and Hauschka, 2004). These processes are principally controlled by the expression of transcription factors, particularly the MRFs, which play a crucial role in regulating myogenesis (Buckingham and Relaix, 2007). In the present study, whole-mount *in situ* hybridisation analysis demonstrated that the expression of Mrfs mRNA in the developing chick limb follows this pattern: *Myf5* -> *MyoD* -> *Myog* -> *Mrf4*.

3.3.1 The expression domains of *Myf5* and *MyoD* in the limb bud

Because myogenic precursors, myoblasts, myocytes, and myofibers express both Pax genes and the MRF family of transcription factors, interaction between these is possible; and consequently, could be important in the developing chick limb muscle. The expression of *Pax3* has been well defined during chick embryonic development (Otto et al., 2006). *Pax3* is detected first in the pre-segmented mesoderm, then the expression is localised in the dermomyotome (Goulding et al., 1994). *Pax3* can directly regulate *Myf5* expression in the limb bud by binding to a limb specific *Myf5* enhancer (Bajard et al., 2006). The expression of *Myf5* is also indirectly controlled through the epaxial enhancer, where *Pax3* directly activates *Dmrt2* expression which in turn regulates *Myf5* expression (Sato et al., 2010). Absence of *Pax3* in mice results in no diaphragm and no limb skeletal muscles (Bober et al., 1994, Goulding et al., 1994). Therefore, it appears that *Pax3* is required for maintenance of *Myf5* expression, which in turn is important to regulate chick limb myogenesis. In adult muscle, however, *Myf5* can control myogenesis independently of *Pax3* (Relaix et al., 2006).

The present study, therefore, was able to detect the first expression of *Myf5* in the developing chick limb muscle by HH stage 22. No expression of *Myf5* was shown at HH stage 20 when these cells express *Pax3*. Nevertheless, the present study agrees with other studies that a possible overlap between both genes occurs during limb development. However, the *in situ* hybridisation of the present study showed a different spatiotemporal mRNA expression pattern of *Myf5* to that reported by

Delfini and colleagues who detected *Myf5* expression in the limb bud by HH 20 (Delfini et al., 2000). The analysis of gene expression using reverse transcription–polymerase chain reaction (RT–PCR) also detected *Myf5* expression in the developing limb at very early stage, HH 15 (Lin-Jones and Hauschka, 1996), where muscle progenitor cells have been found following their spatial migration from the somite. These differences may well reflect differing sensitivity of these techniques. RT-PCR is more sensitive than the *in situ* hybridisation and thus it is not surprising that this approach detects earlier *Myf5* expression than in the present study (Lin-Jones and Hauschka, 1996). However, the differences between the present data and the expression pattern in Delfini *et al* is less easy to explain. The most likely reason is that differing protocols have differing sensitivity. In our protocol we wash embryos extensively with 5xTBST between colour reactions. This removes background staining very effectively and makes it possible to identify mRNA expression in the migratory limb myoblasts unambiguously. However, it may be that this approach also reduces the sensitivity of the experiment and that the earliest stages of expression are lost. Delfini et al. (2000) show that *Myf5* is present at early stages of the developing limb including stage 20 embryos. In chick, Pax3-expressing myoblasts reach their destination in the limb at HH stage 20 (Amthor et al., 1998, Chevallier et al., 1977). Since *Myf5* is a determination factor, its expression in $Pax3^{+ve}$ cells marks the first stage of their commitment to the myogenic lineage which is consistent with their data and the idea that our technique sacrifices sensitivity for specificity.

The current study also demonstrated that *Myf5* is expressed in chick limb earlier than those of *MyoD*. This result is confirmed the differential spatiotemporal mRNA expression patterns of both *Myf5* and *MyoD* during avian somitogenesis, limb development (Delfini et al., 2000, Hirsinger et al., 2001) and mice skeletal myogenesis (Ott et al., 1991, Sassoon et al., 1989, Tajbakhsh et al., 1997). In mice somitogenesis, *Myf5* is also detected first in both epaxial and hypaxial myotome (Tajbakhsh and Buckingham, 1999), while *MyoD* is activated the hypaxial domain (Braun and Arnold, 1996, Tajbakhsh and Buckingham, 1999). These findings together have been already demonstrated by gene targeting study in mice,

when double *Pax3*/*Myf5* mutant led to no *MyoD* expression and no skeletal muscle forms (Tajbakhsh et al., 1997). Nevertheless, in the absence of *MyoD,* muscle normally forms and the fact that is probably due to the activity of *Myf5,* which can predominate *MyoD* (Rudnicki et al., 1992, Rudnicki et al., 1993, Tajbakhsh and Cossu, 1997).

In the present study, *Myf5* expression was then maintained in both limbs at HH stages 23, 24 and 26 embryos. Significant expression of *Myf5* was seen in the proximal region of limb buds by HH stage 26. This was expected, because differentiation of muscle in proximal limb portion is obvious at this stage. Therefore, the present study examined transverse sections of the limb buds and detected that *Myf5*-expressing cells were found in the dorsal and ventral pre-muscle masses. This pattern occurred progressively from HH stage 22, suggesting that in fore and hind limbs muscle development is equivalent by this stage. Myogenic progenitors expressing *Pax3* accumulate in the dorsal and ventral limb mesenchyme by HH stage 20 (Amthor et al., 1998). The data presented here is consistent with the expression of *Myf5* being linked to proliferation and determination of limb muscle cells.

The earliest expression of *MyoD* seen in this study in the limb was at HH stage 23 which then increased in both limbs during development. No expression of *MyoD* was seen in migratory progenitors of limb skeletal muscle at HH stage 20 or stage 22. In agreement, Lin-Jones and Hauschka (1996) detected the first expression of *MyoD* in both limbs of chick embryos by HH stage 23. Although the authors have examined *MyoD* expression using RT–PCR and this technique is probably more sensitive in detection of gene sequences, the result is consistent with the current study. However, the expression of *MyoD* has been shown in the proximal centre of the limb earlier than HH stage 23, as forelimb mesenchymal cells express *MyoD* by HH stage 20 which is then maintained to HH stage 22 (Amthor et al., 1998). Alternatively, the use of *in situ* hybridisation testing limb tissue sections shows no *MyoD* mRNA by HH stages 20 or 21 (Delfini et al., 2000). Williams and Ordahl (1994) also did not detect *MyoD* in the chick limb bud until stage 24. However, the likely explanation for these contrasting results is that the mRNA probe used for the *in situ* hybridisation

in Amthor *et al* was much more sensitive than the probe and protocol used here. Using *in situ* hybridisation for mRNA detection is greatly dependent on the signal-to-noise ratio (SNR) and background noise of radioactive *in situ* probes probably because the combination of some probes could result from leaky gene expression leading to nonspecific hybridisation (Lin-Jones and Hauschka, 1996). As a result, the protocol used here contains extensive washes to remove background staining. As with the discrepancies in different reports of *Myf5* expression it is probable that this reduces sensitivity in favour of specificity and this may be why the earlier expression reported by others was not observed here. Although these differences in timing across these studies may argue that the data presented here is incomplete, it is worth noting that in these *in situs* the timings observed are highly consistent. Very little variability is observed across stage matched embryos. Given that the temporal pattern of Mrf expression seen here is consistent with other studies we believe that the lower sensitivity of this protocol does not significantly alter our conclusions about the process of myogenesis.

The present study then examined serial transverse sections of both fore and hind limbs using also *in situ* hybridisation. MyoD-expressing cells were organised in the mesenchymal core of both limbs by HH stages 24 and 26. This gave similar results in terms of the stages when Amthor et al. (1998) observed *MyoD* transcripts in central mesenchymal core at HH stage 25 of limb bud. Comparison of the presence of *Myf5* and *MyoD* expression in sections of limbs reveals no major discrepancy in spatiotemporal patterns, as both genes were in myoblasts situated in the dorsal and ventral muscle masses. It seems likely that *Myf5* might regulate commitment of myogenic precursors to the muscle cell lineage; then *MyoD* starts the differentiation process. While *Myf5* determines embryonic myoblasts, its inability to regulate the differentiation results in a delay in limb muscle formation in compound mutants of *MyoD* and *Mrf4* (Murphy and Kardon, 2011). In mice, *MyoD* expression follows *Myf5* activation and starts at day 10.5 in the myoblasts and myofibers (Ontell et al., 1993, Sassoon et al., 1989). Then, it continues to be expressed in fetal and adult myoblasts as well as

myofibers (Hinterberger et al., 1991, Kanisicak et al., 2009, Voytik et al., 1993).

Delfini et al. (2000) have already detected temporal differences in the *Myf5* and *MyoD* expression domains within the dorsal and ventral muscle masses of the limb bud similar to what we observe. The loss of function experiments have established that there are Myf5-independent, but not MyoD (Gensch et al., 2008, Haldar et al., 2008, Wood et al., 2013) myogenic lineages within the embryo. These findings had been challenged by (Comai et al., 2014, Haldar et al., 2014). Comai et al. (2014) have argued that the survival of cells in the Myf5 driven Diphtheria toxin A fragment (DTA) mice is due to incomplete expression rather than because there is a *Myf5* independent population. Therefore, it may well be that in fact all cells express both *Myf5* and *MyoD*. However, the *in situ* hybridisation of the present study cannot identify distinct populations as single cell resolution cannot reliably be characterised, thus the question needs further work to resolve.

3.3.2 *Myog* and *Mrf4* mark differentiation of the limb muscle

The present study also examined *Myog*, the third *Mrfs*, which first expressed in both fore and hind limbs at HH stage 24 chick embryos. *Myog* expression then continued through later stages, HH26 and 30. Activation of *Myog* is partly driven by *MyoD* expression, which binds to the *Myog* promoter; thus the myogenic program activation requires them to reach a critical threshold (Thayer et al., 1989). The expression of *Myog* occurs as single-nucleated myoblasts initiated fusion with each other to form multi-nucleated myotubes (Hasty et al., 1993, Sassoon et al., 1989). Therefore, terminal differentiation of muscle fibers depends on *Myog* expression (Rehfeldt et al., 2011, Venuti et al., 1995). In mouse, *Myog* is detected in the limb by day 11.5 and is specifically expressed in myocytes and myofibers of adult muscle (Cornelison and Wold, 1997, Hinterberger et al., 1991, Sassoon et al., 1989, Yablonka-Reuveni and Rivera, 1994). The presence of *Myog* in myocyte or myofibers confirms that this factor plays a key role during chick limb terminal differentiation.

Mrf4 is the last myogenic regulatory factor to be expressed in the limb at HH stage 26 embryos. No expression of *Mrf4* was seen before this stage compared to other Mrfs. Although *Mrf4* expression was not examined at HH stage 25 in the present data, Mok (2014) detected a weak expression of *Mrf4* in the limb at HH stage 25 embryos. This clearly demonstrates that *Mrf4* has no role during the early proliferation of the developing limb, but it functions during late differentiation of limb muscle cells. *Mrf4* expression has been associated with mature myotubes and myofibres outgrowth (Hinterberger et al., 1991, Rehfeldt et al., 2011). Although clear expression of *Mrf4* was detected in both limbs at HH stage 30 embryos, it was weak compared to *MyoD* and *Myog* at the same stage. This suggests that upregulated *Mrf4* may correlate with downregulation of *MyoD* and/or *Myog*. In this case, *Mrf4* regulates the late myogenic program and affects myofibers formation.

In mice, *Mrf4* is involved in regulating the maintenance and maturation of myofibres (Miner and Wold, 1990, Zhang et al., 1995, Zhu and Boone Miller, 1997). However, the expression of *Mrf4* is seen first by day 13.5 of mice development; then it is strongly expressed in fetal myoblasts at day 16.5 to be the predominant myogenic factor in differentiated myofibers (Bober et al., 1991, Hinterberger et al., 1991, Voytik et al., 1993). Although the loss of function study has demonstrated that absence of *Mrf4* expression has no effect on myotome and on the formation of limb skeletal muscle (Zhang et al., 1995), *Mrf4* acts as a determination gene and compensates somite myogenesis in *Myf5*/*MyoD*-null mutant mice (Kassar-Duchossoy et al., 2004). However, whether *Mrf4* can play this role in chicken limb myoblasts is open to question as we do not see it expressed early enough for this. It is notable that *Mrf4* expression in chicken somites is also not seen until after the other Mrfs (Berti et al., 2015, Mok et al., 2015). This may be either because chicken and mouse embryos have distinct roles for *Mrf4* or because it has only been seen to play this role in mice lacking both *Myf5* and *MyoD*. *Mrf4* could only be acting as a determination gene in this situation while its normal role is in later differentiated myotubes.

3.4 Conclusion

These data show a clear progression of Mrfs expression in the developing limb with *Myf5*, *MyoD*, *Myog* and *Mrf4* being expressed in that order. Having carefully defined these spatial and temporal expression patterns, the next study then went on to determine how Mrfs are regulated by IGF signalling during limb development.

Chapter 4: Characterisation of IGF expression patterns through chick limb development

4.1 Introduction

The insulin-like growth factor (IGF) system comprises two ligands (IGF-1 and IGF-2), two receptors (IGF-1R and IGF-2R) and six binding proteins, IGFBP-1-6 (see chapter 1.9 for detail) (Jones and Clemmons, 1995, Stewart and Rotwein, 1996, Stewart et al., 1996). The localization of IGF mRNA expression pattern has been extensively studied during vertebrate embryogenesis, using immunohistochemistry and *in situ* hybridisation (Allan et al., 2001). In mice and rats, most components of the IGF axis have been detected in muscle, the skeletal system, central nervous system as well as reproductive organs (Allan et al., 2001, Armstrong et al., 2000, Annunziata et al., 2011, Jianmin et al., 2014, Dubey et al., 2015, Dupont and Holzenberger, 2003, Richards et al., 2005). IGF signalling is also involved in limb development to regulate its morphogenesis (Agrogiannis et al., 2014, Geduspan et al., 1992, van Kleffens et al., 1998). *In vivo*, IGFs stimulate the development of limb bud like structures from the prospective limb area (Dealy and Kosher, 1996). In this context, IGF-1 is expressed in the progress zone in mice (van Kleffens et al., 1998), and it promotes proliferation of limb mesenchymal cells in response to the AER and ZPA regions (Agrogiannis et al., 2014, Kaplowitz et al., 1982).

However, there are few studies focused on the expression of IGFs in birds. Only (McMurtry et al., 1997, McMurtry, 1998) have studied the effect of nutritional status and the development on the transcription of IGF axis in avian tissues. The IGF-1 receptor (Lu et al., 2009), but not IGF-2 receptor (M6P-R) (Lu et al., 2009, Zhou et al., 1995) has been characterised in chicken myogenic satellite cells and in the limb during early stages of chick embryogenesis (Allan et al., 2003). In addition, the mRNA expression pattern of IGFBPs has also been well established in chicken. *IGFBP-2* is expressed in many different areas during embryogenesis, including somites, pharyngeal clefts, surface ectoderm, brain, eye and the AER during limb formation (McQueeney and Dealy, 2001, Schoen et al., 1995). Allan et al. (2003) have also described the expression of *IGFBP-2* and

IGFBP-5 during chick embryogenesis, using *in situ* hybridisation. *IGFBP-5* expression in chicken tissue is similar to that of mammals during fetal and postnatal stages (Allander et al., 1997). *IGFBP-5* is not activated in the ectoderm or in the AER, but it is localised in osteoblast progenitors and strongly expressed in musculature of the developing limb (McQueeney and Dealy, 2001). In the limb, *IGFBP-5* is expressed in the central region; then is strongly expressed in the zeugopod and autopod at HH stage 26 (Allan et al., 2003). Although all of these studies have provided a wealth of information about IGF distribution in chick embryos, IGF expression patterns during limb development are not fully characterised. Therefore, the aim of this chapter is to characterise the expression of IGF axis components in detail at the stages of limb development when myogenesis is taking place. Wild type chick embryos were staged according to Hamburger and Hamilton (1951) and then analysed using *in situ* hybridisation.

4.2 Results

The expression patterns of IGF members were obtained using whole-mount *in situ* hybridisation (WISH) over the embryonic development of chick limb. Expression profiles were examined in whole limbs from HH stage 20 until HH stage 26 embryos.

4.2.1 *IGF-1* expression

At HH stage 20, weak expression of *IGF-1* was observed between somites and strong staining was seen in the forelimb adjacent to the distal end (Figure 4.1A). Between HH stages 21 to 22, strong expression of *IGF-1* localised in the posterior forelimb (Figure 4.1B and C). In contrast, no labelling was seen in the hindlimb at these stages. However, by HH stages 23 and 24 (Figure 4.1D and E respectively), *IGF-1* expression was observed in the forelimb and very weak expression was detected in the proximal hindlimb. At HH stage 26, the expression of *IGF-1* extended into the regions where the zeugopod and stylopod will form (Figure 4.1F).

4.2.2 *IGF-2* expression

Like *IGF-1*, *IGF-2* was expressed between somites and also detected in the posterior forelimb by HH stage 20 (Figure 4.1G). At this stage, no labelling of *IGF-2* was observed in the hindlimb. *IGF-2* expression then disappeared from the forelimb between HH stages 21 to 23 (Figure 4.1H-J). At HH stage 24, however, *IGF-2* expression returned in the proximal part of forelimb, but still no expression was seen in the hindlimb (Figure 4.1K). Strong staining of *IGF-2* was seen in both limbs from proximal to near the distal regions at HH stage 26 to highlight the future stylopod (Figure 4.1L).

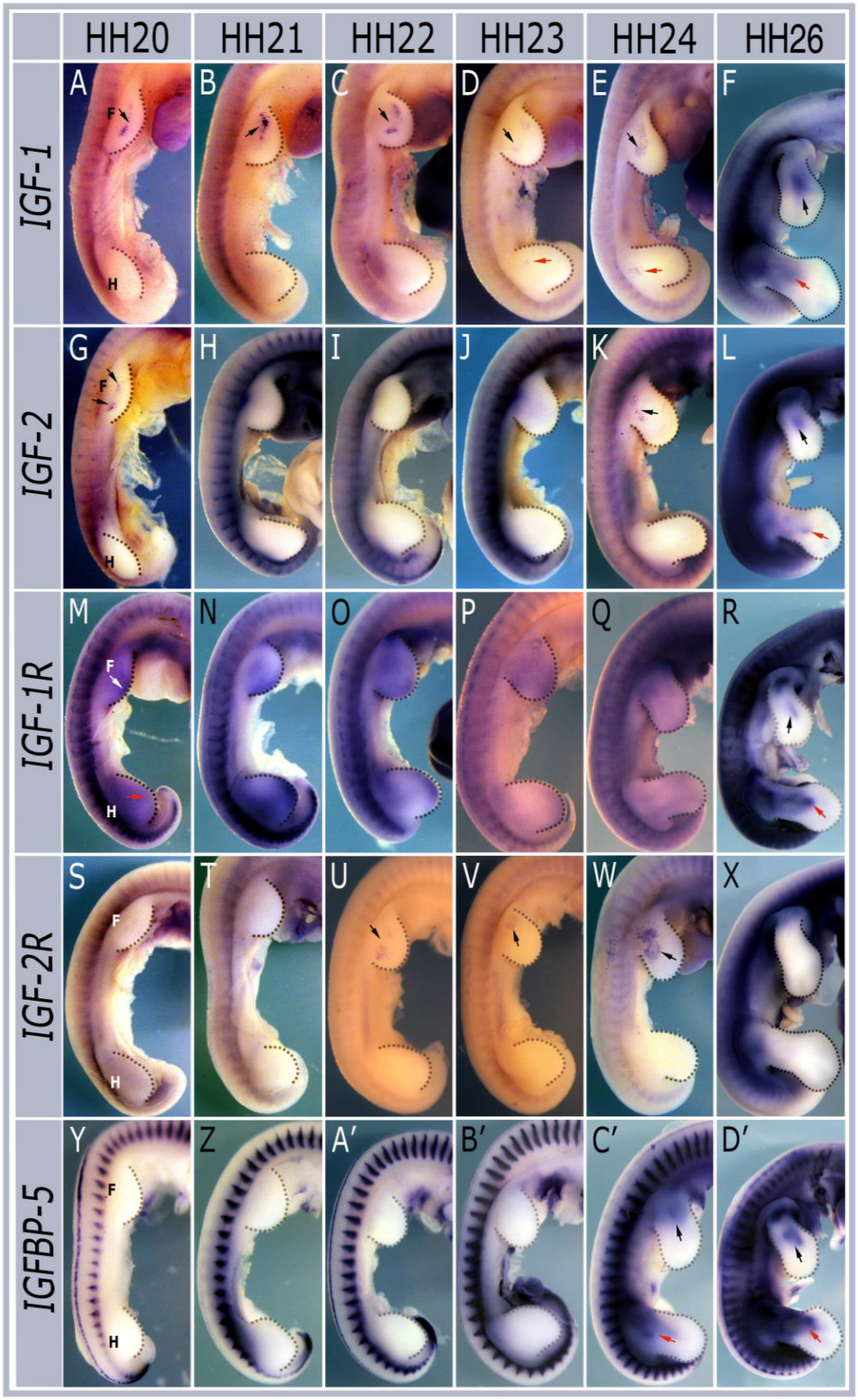


Figure 4. 1: Lateral view of the whole-mount *in situ* hybridisation between days 3-5 chicken embryos after treatment with *IGFs* probes. At HH stage 20 (A), weak expression of *IGF-1* is seen between somites; it is expressed first in the forelimb (black arrow) then maintained the expression to HH stage

22 embryos (B-C) (black arrows). D and E, weak expression of *IGF-1* in the posterior hindlimb (red arrows) is shown between HH stages 23 to 24. By HH stage 26 (F), strong expression of *IGF-1* extends in both limbs and highlights where the zuegopod will form in the future (black and red arrows). G shows first weak expression of *IGF-2* between somites, but it is strongly expressed in the forelimb at HH stage 20 embryos (black arrow). This expression then disappears from HH stage 21 to HH stage 23 embryos (H-J). K demonstrates *IGF-2* expression in the proximal region of the forelimb at HH stage 24 (black arrow). Strong expression of *IGF-2* is seen at HH stage 26 (black and red arrows) (L) in both limbs to highlight the future zuegopod region. *IGF-1R* expression is shown in the tissue immediately proximal to the ridge (white arrow) at HH stage 20 (M-Q). Strong expression of *IGF-1R* is seen in both limbs at HH stage 26 embryos (R) (black and red arrows). No expression of *IGF-2R* can be seen in both limbs at HH stage 20 and 21 embryos (S and T). *IGF-2R* is expressed in the posterior forelimb, but not the hindlimb from HH stage 22 to HH stage 24 (U-W) (black arrows). No expression of *IGF-2R* is shown in limbs at HH stage 26 (X). No expression of *IGFBP-5* is observed between HH stages 20-23 embryos (Y-B'). At HH stage 24 embryos (C'), clear expression of *IGFBP-5* is seen in the proximal region of both limbs (black and red arrows). D' demonstrates an increase in the *IGFBP-5* expression, which highlights where the future of the zuegopod is developed. Abbreviation: F, forelimb; H, hindlimb.

4.2.3 *IGF-1R* expression

At HH stage 20, low levels of *IGF-1R* expression were observed in both limbs localised in the tissue immediately proximal to the ridge (Figures 4.1M and 4.2B). Subsequently, from HH stages 21 to 24, *IGF-1R* was expressed in the limb bud mesenchyme (Figure 4.1N-Q). At HH stage 26 embryos (Figure 4.1R), *IGF-1R* was expressed in the proximal and distal forelimb. In the hind limb, however, the expression then expanded distally and domain widened to highlight the stylopod.

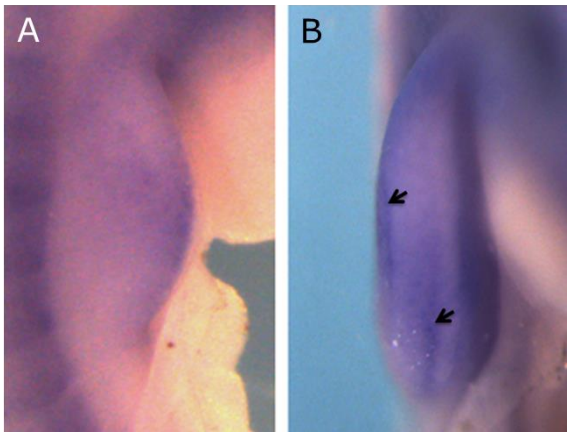


Figure 4. 2: Expression of *IGF-1R* in the limb at HH stage 20. Lateral (A) and frontal views (B) of the limb bud show the location of *IGF-1R* expression in the tissue immediately proximal to the ridge (black arrows).

4.2.4 *IGF-2R* expression

No expression of *IGF-2R* was seen in either forelimb or hindlimb from HH stages 20 to 21 (Figure 4.1S and T). Although weak expression of *IGF-2R* was observed in the anterior and posterior forelimb between HH stages 22 to 24 (Figure 4.1U-W), no later expression was shown in either fore or hind limbs at HH stage 26 (Figure 4.1X).

4.2.5 *IGFBP-5* expression

IGFBP-5 was strongly expressed in somites at all limb bud stages. However, no expression of *IGFBP-5* was observed in the forelimb or hindlimb from HH stages 20 until 24 (Figure 4.1Y-B'). Expression for *IGFBP-5* was first seen adjacent to the proximal mesenchyme of fore and hind limbs at HH stage 24 (Figure 4.1C'). Subsequently, this expression then increased and expanded to outline where the future stylopod will develop (Figure 4.1D').

4.3 Discussion

The IGF axis mRNA expression pattern during early chick embryogenesis has been well-documented by Allan et al. (2003), utilising *in situ* hybridisation. However, the *IGFs* expression has not so far been examined in detail during chick limb muscle development. Also, little is known about the precise expression of *IGF-2R*. The present study, therefore, determined the spatial and temporal expression patterns of *IGF-1*, *IGF-2*, *IGF-1R*, *IGF-2R* and *IGFBP-5* in developing chick limb muscle; whole-mount *in situ* hybridisation was performed to monitor expression. This is related to the main purpose of the project, because it was essential to know when the *IGFs* expression occurs prior to determine inductive signals could possibly proceed.

4.3.1 The expression of *IGF-1* and *IGF-2* in the limb bud

The present study detected low levels of *IGF-1* expression between somites, but strong expression in the forelimb at HH stage 20. By this stage, *IGF-2* was also weakly expressed in somites, but strongly in the forelimb. This similarity reinforced the hypothesis that both IGF-ligands

work in parallel and overlap each other to regulate the embryonic development of limb muscle. Early expression of *IGF-1* has been detected in the mesoderm during mouse and chick embryogenesis (Allan et al., 2003, van Kleffens et al., 1998). The presence of both *IGF-1* and *IGF-2* in the posterior mesenchyme of the limb suggests that they are probably involved in stimulating the proliferation and formation of the limb mesoderm in response to signals from the AER and the ZPA. The posterior aspect expresses genes that are mainly induced by the ZPA, including Shh (for detail, see chapter 1.5.1) (Allan et al., 2003, Li and Muneoka, 1999).

The present work showed a strong expression of *IGF-1* in the forelimb at HH stage 22, but was weak at HH stage 23. Allan and colleagues have demonstrated that *IGF-1* is strongly expressed by HH stage 23 embryos then downregulated during limb development (Allan et al., 2003). However, sections through embryos at HH stage 23 wing bud display low level of *IGF-1* expression in the limb bud, but it increases in the distal region (Allan et al., 2003). Unlike *IGF-1*, the expression of *IGF-2* disappeared in the limb bud from HH stage 21 to HH stage 23 embryos. It is possible that IGFs can travel through the blood and have non-local activities. By HH stage 24, however, both *IGF-1* and *IGF-2* were obviously expressed in the forelimb.

Having the mRNA expression of *IGF-1 and IGF-2* at HH stage 20 and later stages of the developing limb (HH24) suggests a role for these ligands during both early and late events occurring in myogenic cells. Both *IGF-1* and *IGF-2* have been implicated in the proliferation and differentiation of myogenic cells (Engert et al., 1996). Bach et al. (1995) have also confirmed the role of *IGF-2* in stimulating these processes in rat. However, Stewart and Rotwein (1996) have suggested that *IGF-2* acts only as a differentiation factor to regulate muscle formation. In HH stage 26 embryos both ligands were highly expressed in the forelimb and hindlimb highlighted future stylopod. This data implies that *IGF-1* and *IGF-2* could work in parallel for stimulating cell differentiation and subsequently regulating the development of skeletal system. Mouse models have shown that normal bone development and bone health throughout the life require the circulation of IGF-1 (Giustina et al., 2008, Guntur and Rosen, 2013,

Locatelli and Bianchi, 2014, Yakar et al., 2002). Expression of both ligands can increase the stimulation of the postnatal growth of muscle tissues, as Song et al. (2013) detected the highest expression of *IGF-1* and *IGF-2* in the duck hindlimb after 3 weeks of hatching.

4.3.2 The expression of IGF-receptors in the limb bud

The present study examined both *IGF-1R* and *IGF-2R* as main receptors for biological action and as members of IGF system. At HH stage 20, *in situ* hybridisation detected early expression of *IGF-1R* in the tissue immediately proximal to the ridge, whereas *IGF-2R* expression was not seen. Allan et al. (2003) detected weak expression of *IGF-1R* in the chick limb bud at HH stage 20 that then increased by HH stage 24 to include the posterior aspect of the AER. Because *IGF-1R* is thought to mediate IGF-ligand actions (Ewton et al., 1994), it might be important for this receptor to possess precise transcription patterns and period with IGF-ligands for their role in limb myogenesis (Figure 4.1A, G and M).

Immunohistochemistry and *in situ* hybridisation have shown that *IGF-1R* is expressed *in vivo* through the sub ridge mesodermal cells of the developing chicken and rat limb buds (Streck et al., 1992) (reviewed in Agrogiannis et al. (2014)). Similar to *IGF-1* and *IGF-2*, the present study showed an increase and expansion of *IGF-1R* expression in both limbs by HH stage 26, (Figure 4.1R). At this stage, *IGF-1R* did express in the proximal and distal mesenchyme where it showed the forming of zeugopod (future tibia, fibula) and stylopod (future femur). In accordance with other studies, the present data also confirmed the expression of *IGF-1R* at late stage, when it regulates chick osteogenesis, of limb development. In mouse, normal bone growth not only depends on *IGF-1*, but also requires *IGF-1R* to mediate ligand function (Guntur and Rosen, 2013).

IGF-2R did not expressed at early stages of limb myogenesis (HH stage 20 or 21). However, this receptor was detected in the anterior and posterior forelimb at HH stages 22, 23 and 24 embryos. The M6P/IGF-2R is important in mediating lysosomal enzymes and cell proliferation (Kornfeld, 1992, Scott and Firth, 2004). The main function of this receptor is to bind as well as internalize *IGF-2* (Pavelic et al., 2007) for clearing from

circulation. However, it is difficult here to determine the biological action of *IGF-2R* during chick limb growth and development, because this receptor has no intracellular signalling domain and thus it lacks the capability of signalling transduction (for detail, see chapter 1.9.2.2) (Pavelic et al., 2007).

4.3.3 The expression of *IGFBP-5* in the limb bud

IGFBP-5 is a key part of the IGF system (Beattie et al., 2006). Allan's study characterised the expression of *IGFBP-5* in developing limbs at HH stages 22 and 25 (Allan et al., 2003). The authors found that *IGFBP-5* is strongly expressed in HH stage 25 embryos. The present study investigated further *IGFBP-5* expression during chick limb development. It showed that *IGFBP-5* is expressed first at HH stage 24 and the expression then increases and expands from the proximal to the distal both limbs at HH stage 26 (Figure 4.1C' and D'). It seems clear from these studies that *IGFBP-5* probably has no role in early limb growth and development, because its expression only appeared at the later stages of the developing limb. This confirms Allan's work, and it could be that *IGFBP-5* is locally expressed during limb differentiation and regulates the activity of IGF-2 signalling (Ren et al., 2008). For instance, in myogenic cells, *IGFBP-5* is preferentially expressed and is important for differentiation of skeletal muscle stimulated by IGF-2 signalling, as *IGFBP-5* knockdown impairs myogenesis (Ren et al., 2008). A number of studies have shown that *IGFBP-5* expression is significantly increased during myogenesis to negatively or positively regulate differentiation, depending on the conditions of the experiments (James et al., 1993, Ren et al., 2008). In contrast, Henningsen et al. (2010) did not see any dramatic regulation of released IGFBP-5 through differentiation. However, *IGFBP-5* also has an important cellular function in regulating apoptosis and survival during limb development (Beattie et al., 2006).

Like other IGF members, *IGFBP-5* was strongly expressed in the forelimb and hindlimb at HH stage 26 for the future zuegopod and stylopod. The localization of *IGFBP-5* in bone and extracellular matrix could locally regulate bone metabolism (Hakeda et al., 1996). Furthermore, proteolytic activity of *IGFBP-5* could modulate the action of IGFs in bone (Kanzaki et

al., 1994), cited from (Akkiprik et al., 2008). Adding IGFBP-5 promotes IGF-stimulated differentiation of myoblasts in rat myogenic cells (Ewton et al., 1998). In addition, *IGFBP-5*, *IGF-2* and *Myog* are all stimulated during myogenic cell differentiation (Bayol et al., 2000, Rotwein et al., 1995); *IGFBP-5* is associated well with *Myog* expression (Bergstrom and Tapscott, 2001). Together these findings make it plausible that the IGF signalling system has an important role in limb bud myogenesis.

4.4 Conclusion

Because IGF-ligands and their receptors are expressed during limb development at the times when myogenic cells are undergoing proliferation and differentiation, next their effects on these processes were directly examined.

Chapter 5: The effects of IGFs on chick limb skeletal muscle

5.1 Introduction

The insulin-like growth factors (IGFs) are known to interact with myogenic regulatory factors (MRFs) in hypertrophy and regeneration where IGFs can induce MRF expression. Conversely, MRFs can also stimulate IGF expression (Zanou and Gailly, 2013). However, their precise roles in regulating myoblast commitment and specification are not well understood. Since the expression patterns of myogenic markers of commitment and differentiation are well characterised (see chapter 3), and components of *IGFs* are expressed in developing limbs (see chapter 4), this chapter aimed to determine how IGFs can affect limb bud myogenesis *in vivo*. The hypothesis tested here is that IGFs can influence both myogenic commitment and differentiation during limb development. To understand and investigate the role of IGFs in early developing limb muscle, IGF soaked beads (1mg/ml) were grafted into limb buds; then *Pax3* and three myogenic regulatory factors (*Myf5*, *MyoD* and *Myog*) were used as muscle markers and then analysed by *in situ* hybridisation, paraffin sectioning and immunostaining.

5.2 Results

To determine the effects of IGFs on limb specific gene expression, beads were grafted at different stages as summarised in (Figure 5.1).

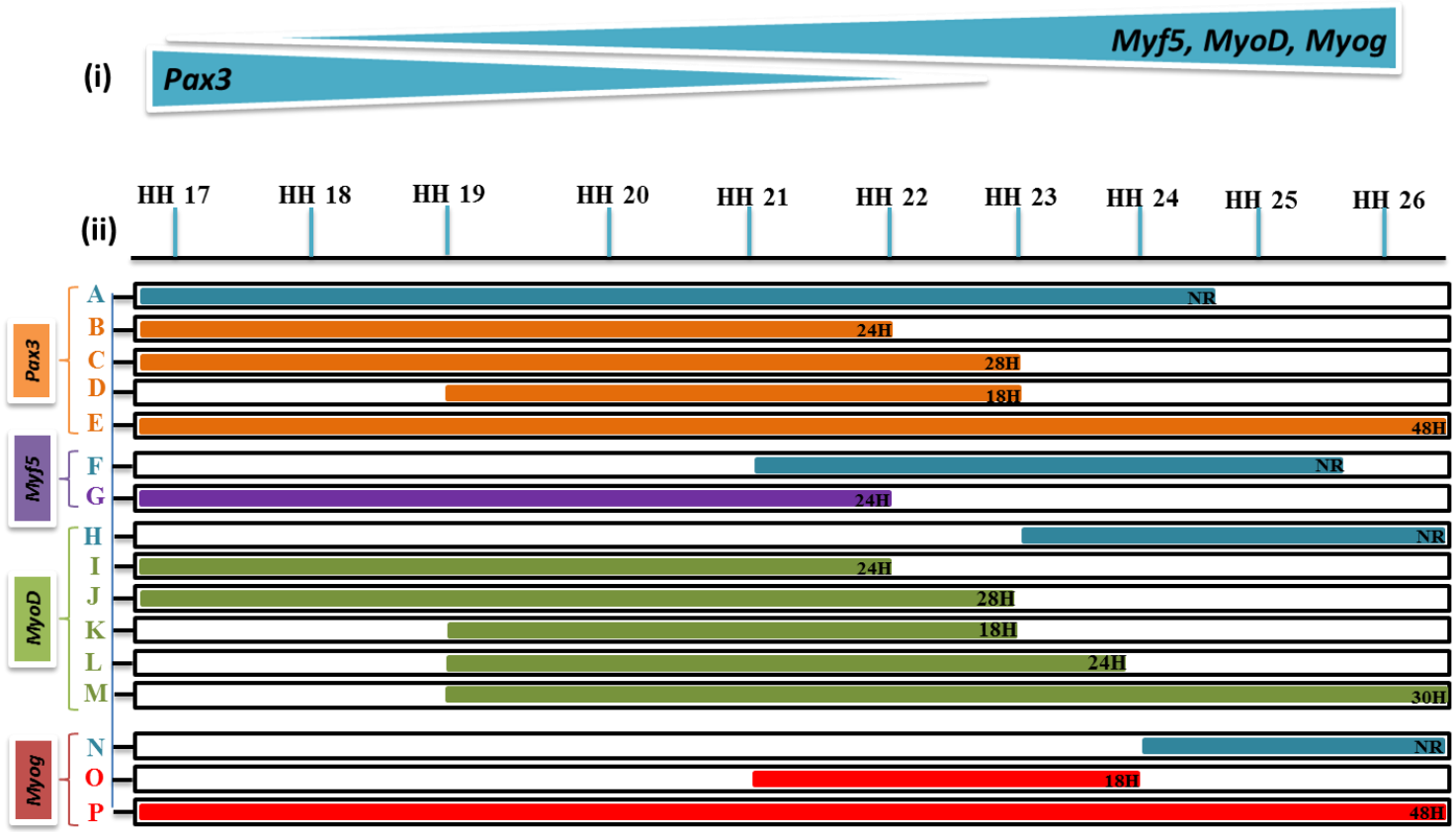


Figure 5. 1: Schematic summarising experiments that were performed with beads soaked in IGF-1 or IGF-2. (i-blue) explains the downregulation of *Pax3* is followed by switching *Myf5*, *MyoD* and *Myog*. (ii)-(A, F, H and N, blue) Highlight the phenotype expression of *Pax3* and myogenic regulatory factors according to (Mok, 2014, Mok et al., 2015).

(B,C,D and E, orange) Show grafting IGF-soaked beads and testing *Pax3* marker with various times of incubation. Embryos were first grafted into the limb at HH stage 17 (B and C) and were incubated for 24 hours to HH stage 22 and for 28 hours to HH stage 23 respectively. (D) Highlights grafting IGF-soaked beads into the limb at HH stage 19; embryos were incubated about 18 to HH stage 23, whereas (E) shows the long term of incubation when IGF-soaked beads were grafted at HH stage 17; embryos were incubated 48 hours to HH stage 26. (G, violet), shows testing IGF-soaked beads with *Myf5*, where IGF soaked beads were manipulated into the limb at HH stage 17; embryos were incubated for 24 hours. (I, J, K, L and M, Avocado) demonstrate testing the role of IGFs with *MyoD*. Here, embryos were treated with IGF soaked beads and were examined at early and later stages of the developing limb. Embryos were first grafted at HH stage 17 followed by 24 and 28 hours incubation to HH stages 22 and 23 respectively. Subsequently, embryos were grafted at HH stage 19 then incubated for 18 to HH stage 23, and for 24 and 30 to HH stages 24 and 26 respectively. (O, red) IGF soaked beads were tested with *Myog*, as differentiation marker. Embryos were grafted with IGF soaked beads at HH stage 21-22; followed by 18 hours incubation to HH stage 24. (P, red) the long term of incubation by grafting IGF soaked beads at HH stage 17 embryos followed by 48 hours incubation to HH stage 26.

5.2.1 IGF beads grafted at HH17 upregulate *Pax3* but less efficiently induce *Myf5* and *MyoD*

To test whether IGF can affect *Pax3*, *Myf5* and *MyoD* expression, IGF soaked beads (1µg/ml) were implanted adjacent to the somites at the level of limb development in HH stage 17 embryos (Figure 5.2i). Embryos were then incubated for 24 hours to reach HH stage 22. In limbs grafted with IGF-1 beads, *Pax3* expression increased in 28 out of 34 (28/34) embryos compared to their non-grafted contralateral limbs (Figure 5.2A and B). Likewise, IGF-2 induced *Pax3* expression in 22/30 embryos (Figure 5.2C and D). BSA control beads, which were similarly grafted into the level of forelimb development, showed no effect on *Pax3* expression (8/8 embryos, Figure 5.2E and F). Sections through the forelimb at HH stage 22 showed strong expression of *Pax3* stimulated by IGF-2 in the dorsal and, in some cases, the ventral muscle domains (Figure 5.3A). This result was compared to sections through limbs grafted with BSA control beads, which showed normal expression of *Pax3* in the dorsal and ventral muscle mass (Figure 5.3B).

In contrast to *Pax3*, whole-mount *in situ* hybridisation analysis showed less effect on *Myf5* expression following grafts of either IGF-1 (2/8 embryos showed upregulation, Figure 5.2G and H) or IGF-2 (3/10 embryos showed upregulation, Figure 5.2I and J) compared to both non-grafted limb bud and to BSA control beads (4/4 embryos, Figure 5.2K and L). In addition,

limbs grafted with IGF-1 soaked beads did not efficiently stimulate *MyoD* expression (2/11 embryos, Figure 5.2M and N). Likewise, IGF-2 only affected the expression of *MyoD* in small number of grafted embryos (4/12 embryos**,** Figure 5.2O and P); all results were compared to the non-grafted contralateral limb bud. BSA control beads also had no effect on *MyoD* expression (6/6 embryos, Figure 5.2Q and R).

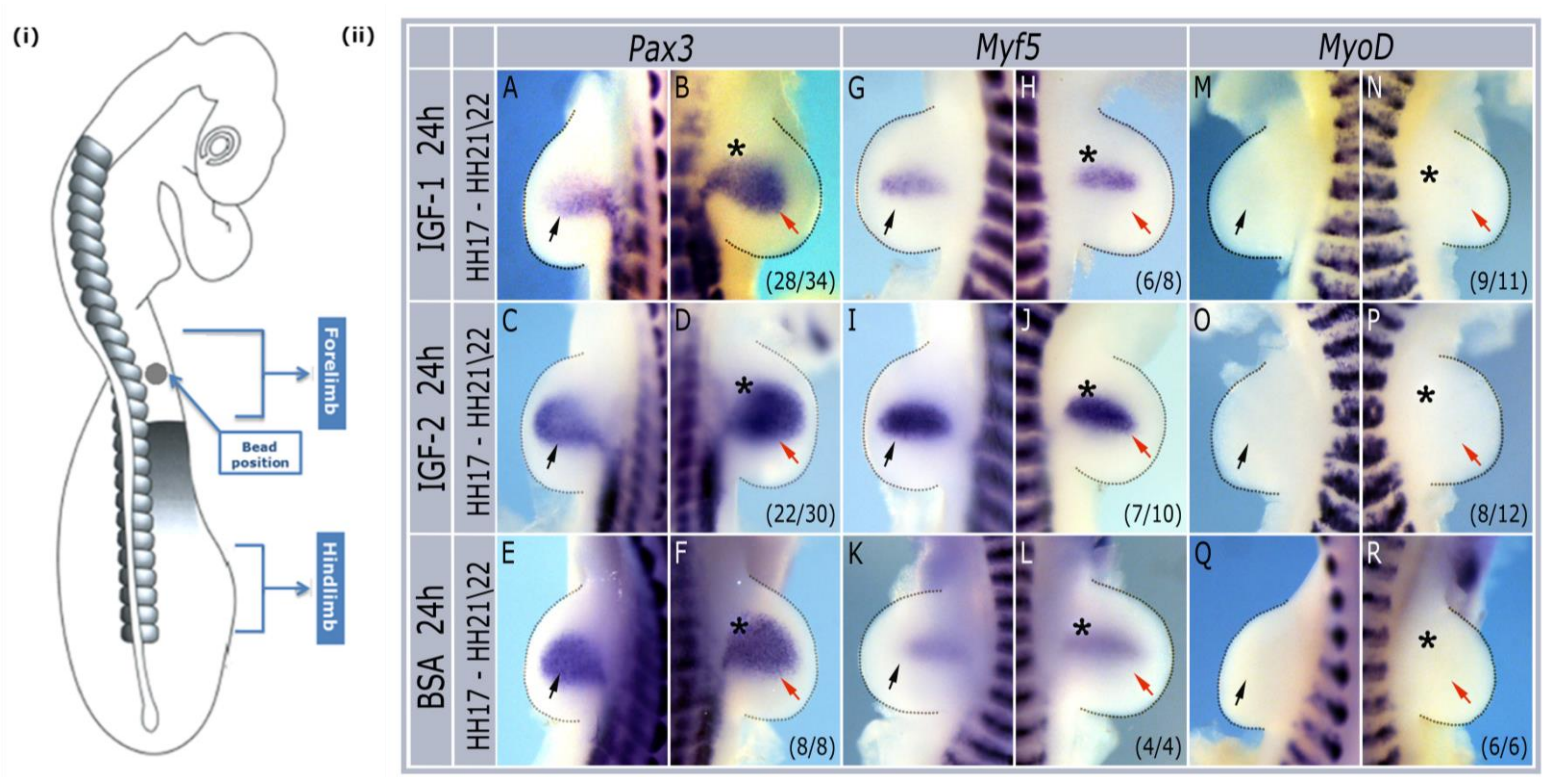


Figure 5. 2: The effect of IGF-ligands on *Pax3*, *Myf5* and *MyoD* expression. (i) Schematic figure of chick embryos at about HH stage 17 shows the position of grafted bead adjacent to the somite at the level of limb development before the growing. (ii) Ectopic IGF-1 and/or IGF-2 is able to induce the expression of *Pax3*, but it less effectively stimulates *Myf5* and *MyoD* at HH stage 22 embryos. IGF-1 and IGF-2- soaked beads were manipulated into the level of the forelimb at HH stage 17. Embryos were incubated for 24 hours to HH stage 22; then were harvested and hybridised with DIG-labelled antisense probes. (A and B) show upregulation of *Pax3* expression stimulated by IGF-1. Likewise, (C and D) show the induction of *Pax3* expression by IGF-2. Both IGF-1 (G and H) and IGF-2 (I and J) have less effect on *Myf5*. Likewise, IGF-1 and IGF-2 (M;N and O;P respectively) do not efficiently stimulate *MyoD* expression. All results were compared to non-grafted contralateral limbs from the same embryos. Control beads were soaked with BSA (0.1% in PBS) and used as control to *Pax3* (E and F), *Myf5* (K and L) and *MyoD* (Q and R). Black asterisks represent beads position. Dotted black lines highlight limb buds. Magnifications: 25x. (i) has been modified from (Tickle, 1995).

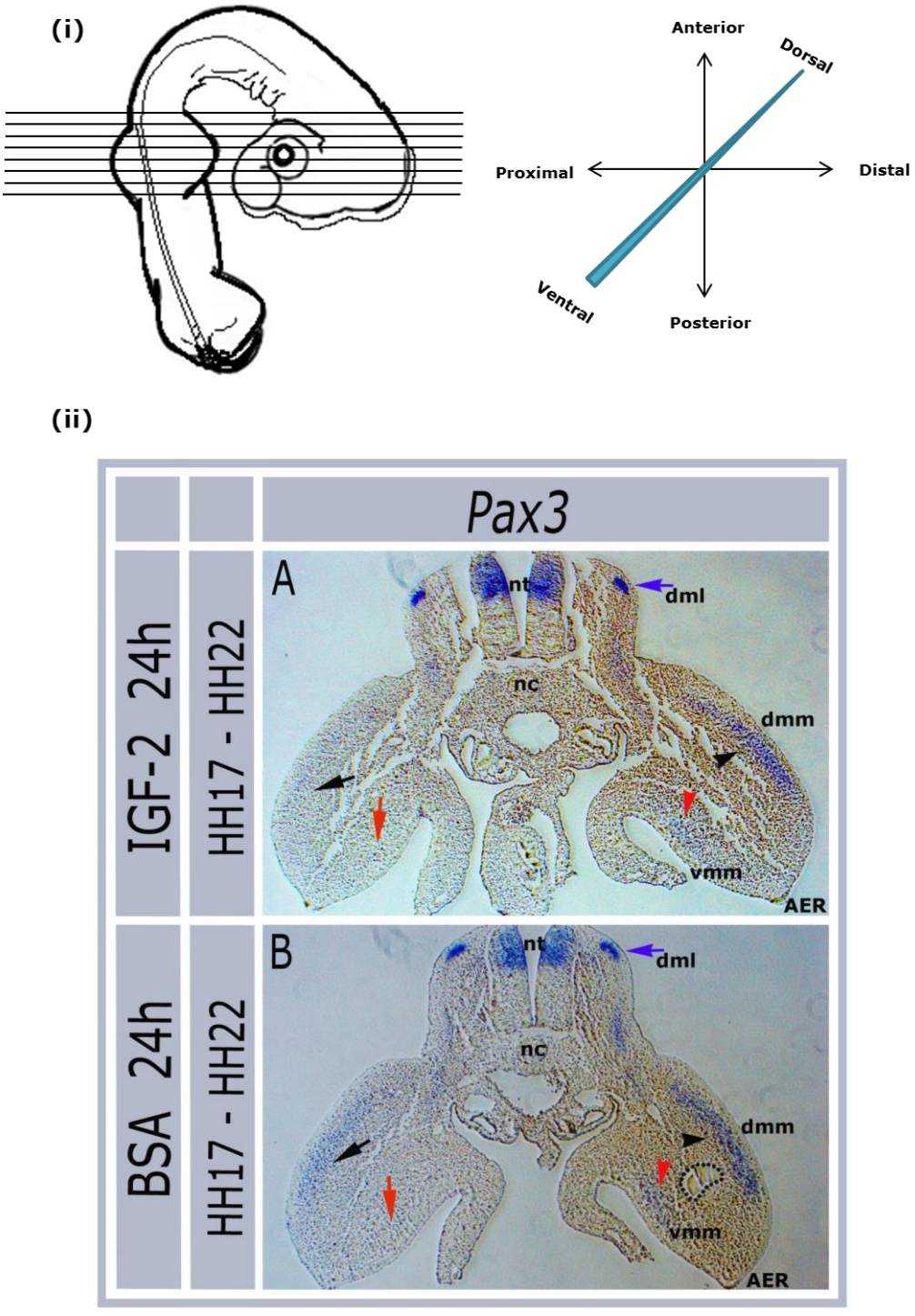


Figure 5. 3: Sectioning of chick embryo through the forelimb at HH stage 22. (i) Schematic representing how embryos were sectioned from the head to the end of the forelimb and representing the direction of sectioning from the anterior to the posterior limb. (ii) Transverse section through chick forelimb at HH stage 22. (A) Demonstrates upregulation of *Pax3* expression (by IGF-2) in the dorsal (black head arrow) and ventral (red head arrow) muscle mass of the limb, compared to the non-grafted contralateral limb (black and red arrows). (B) Shows no effect on *Pax3* expression treated with BSA control beads (black and red head arrows). Dotted circle represents the bead position. Abbreviation: nt, neural tube; nc, notochord; dmm, dorsal muscle mass; vmm, ventral muscle mass; m, myotome; AER, apical ectodermal ridge. Figure (i) has been modified form http://www.cmrbarcelona.eu

5.2.2 IGF beads grafted at HH17 upregulate *Pax3* and *MyoD*

As in the previous experiment, IGF soaked beads were grafted into the forelimb level at HH stage 17, but here, embryos were incubated for 28 hours to HH stage 23 (see Figure 5.1). Both IGF-1 (6/8 embryos, Figure 5.4A and B) and IGF-2 (7/10 embryos**,** Figure 5.4C and D) strongly up-regulated *Pax3* expression compared to the non-grafted contralateral limb bud. Once again, BSA control beads did not affect *Pax3* expression (6/6 embryos**,** Figure 5.4E and F). Although IGF-1 only induced *MyoD* expression after 28 hour of incubation in 1/7 embryos (Figure 5.4G and H), 60% of embryos showed a weak increase in *MyoD* expression stimulated by IGF-2 (6/10 embryos, Figure 5.4I and J). No effect was seen on *MyoD* expression by BSA control beads (7/7 embryos, Figure 5.4K and L).

5.2.3 IGF beads grafted at HH19 upregulate *Pax3* and *MyoD*

IGF soaked beads were placed into the forelimb at HH stage 19; embryos were then harvested at HH stage 23 after 18 hours incubation (see Figure 5.1). At this stage, HH23, both IGF-1 (6/13 embryos, Figure 5.5A and B) and IGF-2 (9/14 embryos, Figure 5.5C and D) upregulated *Pax3* expression in the grafted forelimb. BSA control beads had no effect on *Pax3* expression (7/7 embryos, Figure 5.5E and F).

In contrast to beads grafted at HH stage 17, a dramatic increase in the expression of *MyoD* induced by IGF-1 was observed in 76% of manipulated forelimb (19/25 embryos, Figure 5.5G and H) compared to the non-grafted control side. Likewise, IGF-2 induced upregulation of *MyoD* expression in 68% of embryos (24/35 embryos, Figure 5.5I and J). BSA control beads showed no effect on the grafted limb (9/9 embryos, Figure 5.5K and L). Sections through embryos grafted with IGF-1 and IGF-2 soaked beads at HH stage 23 (Figure 5.6A and B respectively) showed high levels of *MyoD* expression in the dorsal and ventral muscle mass, compared to the control side. Sections through embryos grafted with BSA control beads had no effect on *MyoD* expression (Figure 5.6C).

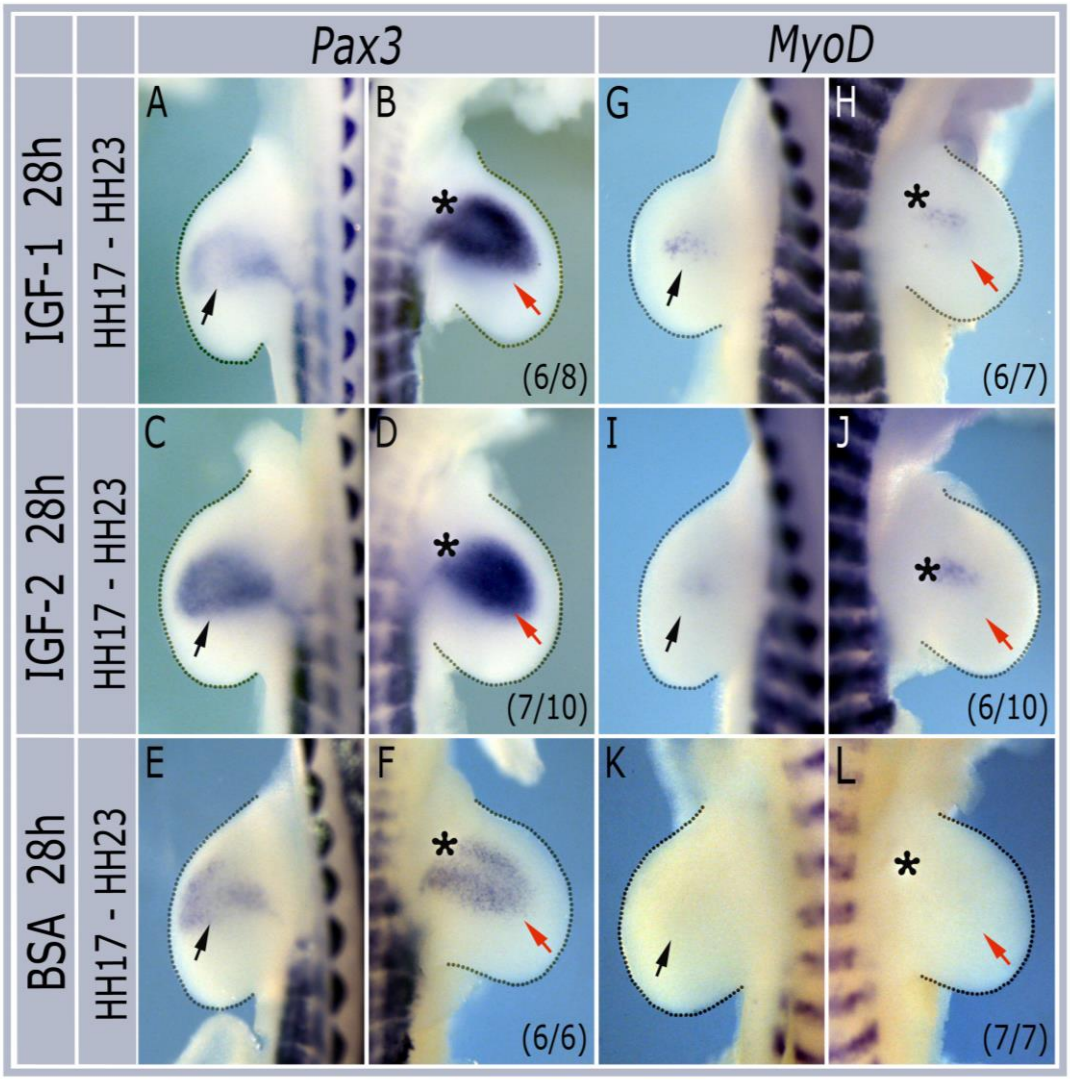


Figure 5. 4: Ectopic IGF-1 or IGF-2 is able to induce the expression of *Pax3* in the limb, but it has less effect on *MyoD* at HH stage 23 embryos. IGF-1 and IGF-2 soaked beads were grafted into level of forelimb at HH stage 17. *In situ* hybridisation was performed to test *Pax3* and *MyoD* after 28 hours of incubation. IGF-1 (A and B) and IGF-2 (C and D) clearly upregulate *Pax3* expression compared to the non-grafted contralateral limb. BSA control bead shows no effect (E and F). No induction on *MyoD* expression by IGF-1 is seen (G and H). IGF-2 slightly increases *MyoD* expression (I and J) compared to the counterpart. BSA control bead shows no effect on the expression of *MyoD* (K and L). Black asterisks represent beads position. Dotted black lines highlight limb buds. Magnifications: 20x

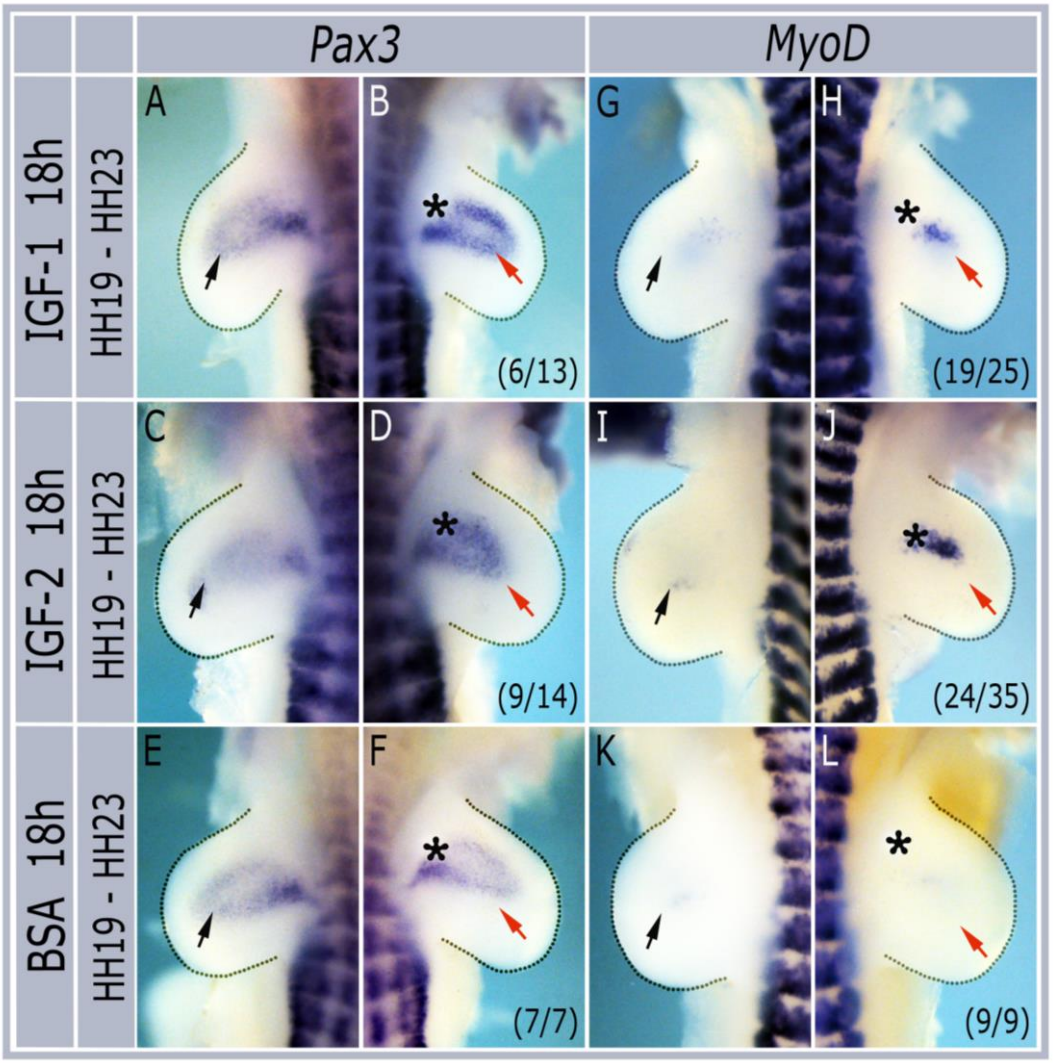


Figure 5. 5: Ectopic IGF-1 or IGF-2 is able to induce the expression of both *Pax3* and *MyoD* in the limb at HH stage 23. Beads were soaked with IGFs and then grafted into the mesenchyme of the right forelimb at HH stage 19 embryos, followed by 18 hours incubation to HH stage 23. Both IGF-1 (A and B) and IGF-2 (C and D) (red arrows) upregulate *Pax3* expression compared to the contralateral limb bud (black arrows). BSA control bead shows normal expression (E and F). Also, strong induction by IGF-1 (G and H) and IGF-2 (I and J) (red arrows) is seen on *MyoD* expression compared to the contralateral limb bud (black arrows). BSA control bead has no effect on *MyoD* expression (K and L). Black asterisks represent beads position. Dotted black lines highlight limb buds margin. Magnifications: 20x

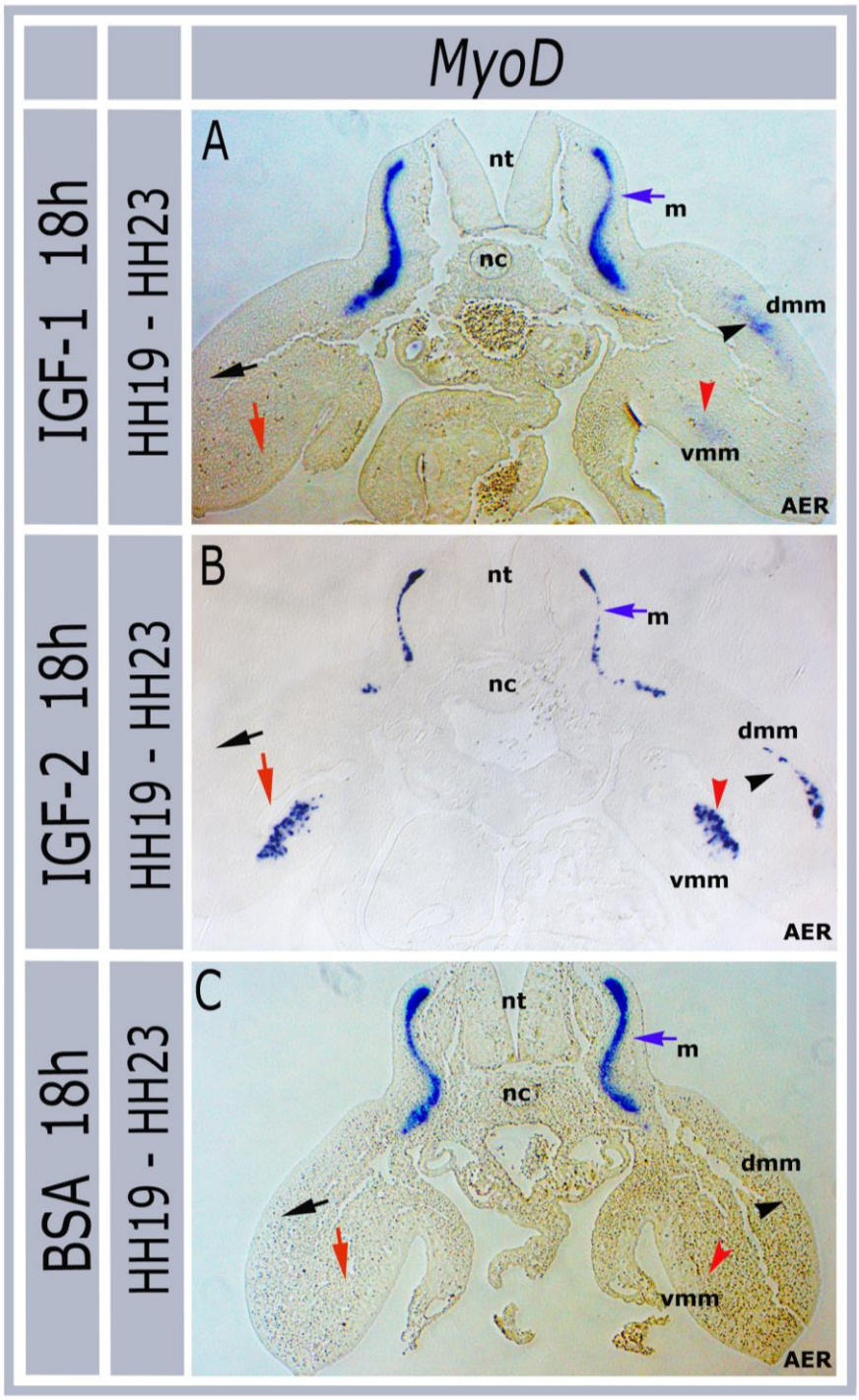


Figure 5. 6: Transverse section through chick forelimb at HH stage 23. (A) Induced-IGF-1 upregulates *MyoD* expression in the dorsal (black head arrow) and ventral (read head arrow) of forelimb compared to the non-grafted contralateral limb (black and red arrows). (B) Shows IGF-2 stimulates *MyoD* expression in the dorsal (black head arrow) and ventral (read head arrow) of forelimb compared to the un-manipulated counterpart (black and red arrows). (C) BSA control bead has no effect on *MyoD* expression (black and red head arrows) Abbreviation: nt, neural tube; nc, notochord; dmm, dorsal muscle mass; vmm, ventral muscle mass; m, myotome (blue arrow); AER, apical ectodermal ridge.

5.2.4 IGF beads grafted at HH19 and HH21-22 upregulate *MyoD* and *Myog*

To test the role of IGF during the late stage of limb myogenesis using later myogenic markers, IGF soaked beads were first grafted into the mesenchyme forelimb at HH stage 19; embryos were harvested after 24 hours incubation at HH stage 24 (see Figure 5.1). Both IGF-1 (8/18 embryos, Figure 5.7A and B) and IGF-2 (10/19 embryos, Figure 5.7C and D) upregulated *MyoD* expression compared to the non-grafted contralateral limb. BSA control beads showed no effect on *MyoD* expression (6/6 embryos, Figure 5.7E and F).

To examine the effect of IGFs on other later myogenic markers, *Myog* was used as a probe. In this case, IGF soaked beads were grafted into the mesenchyme forelimb between HH stages 21 to 22; embryos were then harvested at HH stage 24 after 12-18 hours incubation (see Figure 5.1). Ectopic IGF-1 (7/9 embryos, Figure 5.7G and H) and IGF–2 (12/21 embryos, Figure 5.7I and J) upregulated *Myog* expression at HH stage 24, compared to the non-grafted contralateral limb bud. No transcription of *Myog* was seen when BSA soaked beads were used as control (9/9 embryos, Figure 5.7K and L).

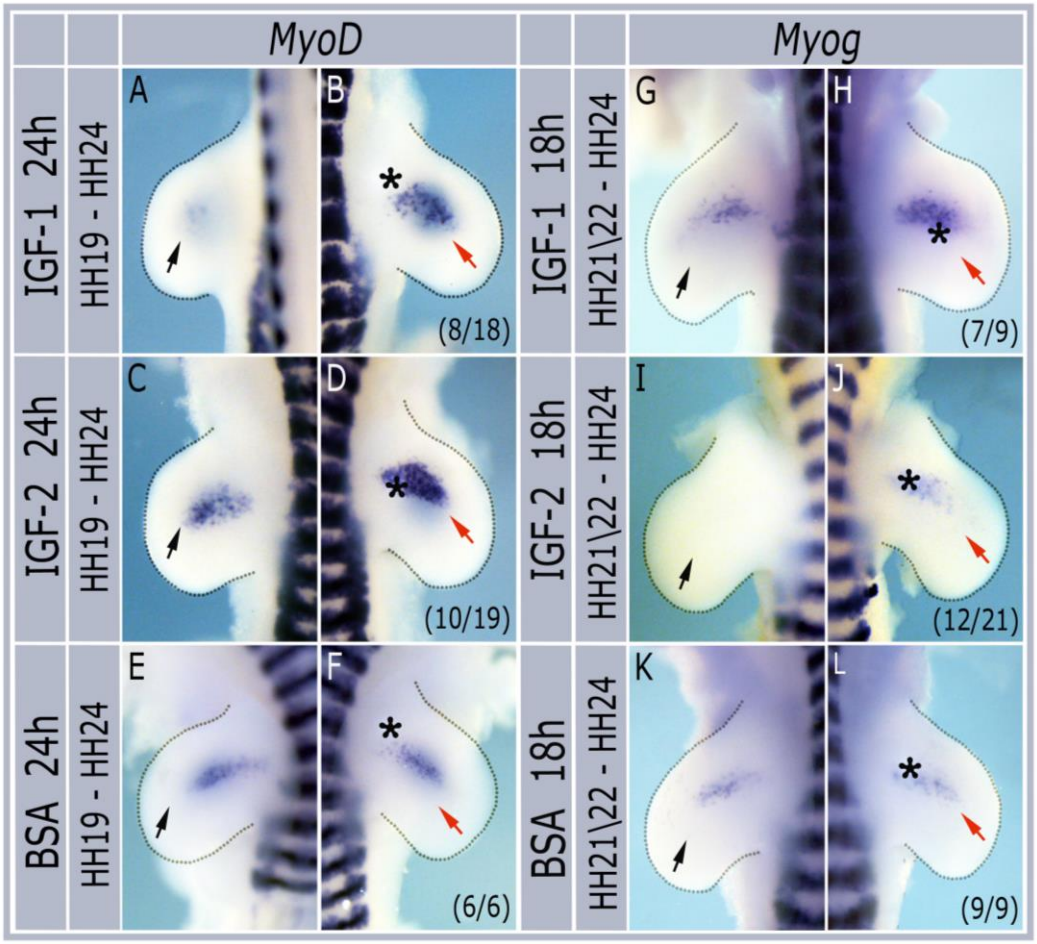


Figure 5. 7: Ectopic IGF-1 or IGF-2 is able to induce the expression of *MyoD* and *Myog*at HH stage 24 embryos limb buds. IGF soaked beads were grafted at HH stage 19 for *MyoD*, followed by 24 hours incubation to HH stage 24, and between HH stage 21-22 for *Myog* followed by 18 hours incubation. IGF-1 (A and B), likewise IGF-2 (C and D) both stimulate *MyoD* expression, compared to the non-grafted limb. BSA bead has no effect (E and F). Both IGF-1 and IGF-2 also upregulate *Myog* expression (G,H and I,J respectively). Once again BSA control bead shows normal expression and has no effect on *Myog* (K and L). Black asterisks represent beads position. Dotted black lines highlight limb buds. Magnifications: 20x.

5.2.5 The effect of IGFs on *Pax3*, *MyoD* and *Myog* after long term of incubation

In order to examine the effect of longer term of incubation, IGF soaked beads were grafted into the level of the forelimb at HH stage 17. Embryos were then incubated for 48 hours to HH stage 26 (see Figure 5.1). 55% of embryos showed upregulation of *Pax3* expression stimulated by IGF-1 (5/9 embryos, Figure 5.8A and B), while only 20% of embryos showed upregulation of *Pax3* expression stimulated by IGF-2 (2/10 embryos, Figure 5.8C and D). BSA control beads did not affect *Pax3* expression (9/9 embryos, Figure 5.8E and F).

To analyse *MyoD* expression at these time points, IGF soaked beads were grafted into the forelimb mesenchyme at HH stage 19, and embryos were incubated for 30 hours to HH stage 26 (see Figure 5.1). There was no alteration in *MyoD* expression by ectopic IGF-1 (12/12 embryos, Figure 5.8G and H) or by IGF-2 (10/10 embryos, Figure 5.8I and J). BSA control beads had no effect and showed normal *MyoD* expression in both limbs (13/13 embryos, Figure 5.8K and L). To assess longer term effects on *Myog* expression, IGF soaked beads were grafted into the level of the forelimb at HH stage 17 followed by 48 hours incubation to HH stage 26. Both IGF-1 (7/7 embryos, Figure 5.8M and N) and IGF-2 (8/8 embryos, Figure 5.8O and P) did not affect the expression of *Myog*. Once again, BSA control beads had no effect and showed normal expression of *Myog* in grafted limbs (4/4 embryos, Figure 5.8Q and R).

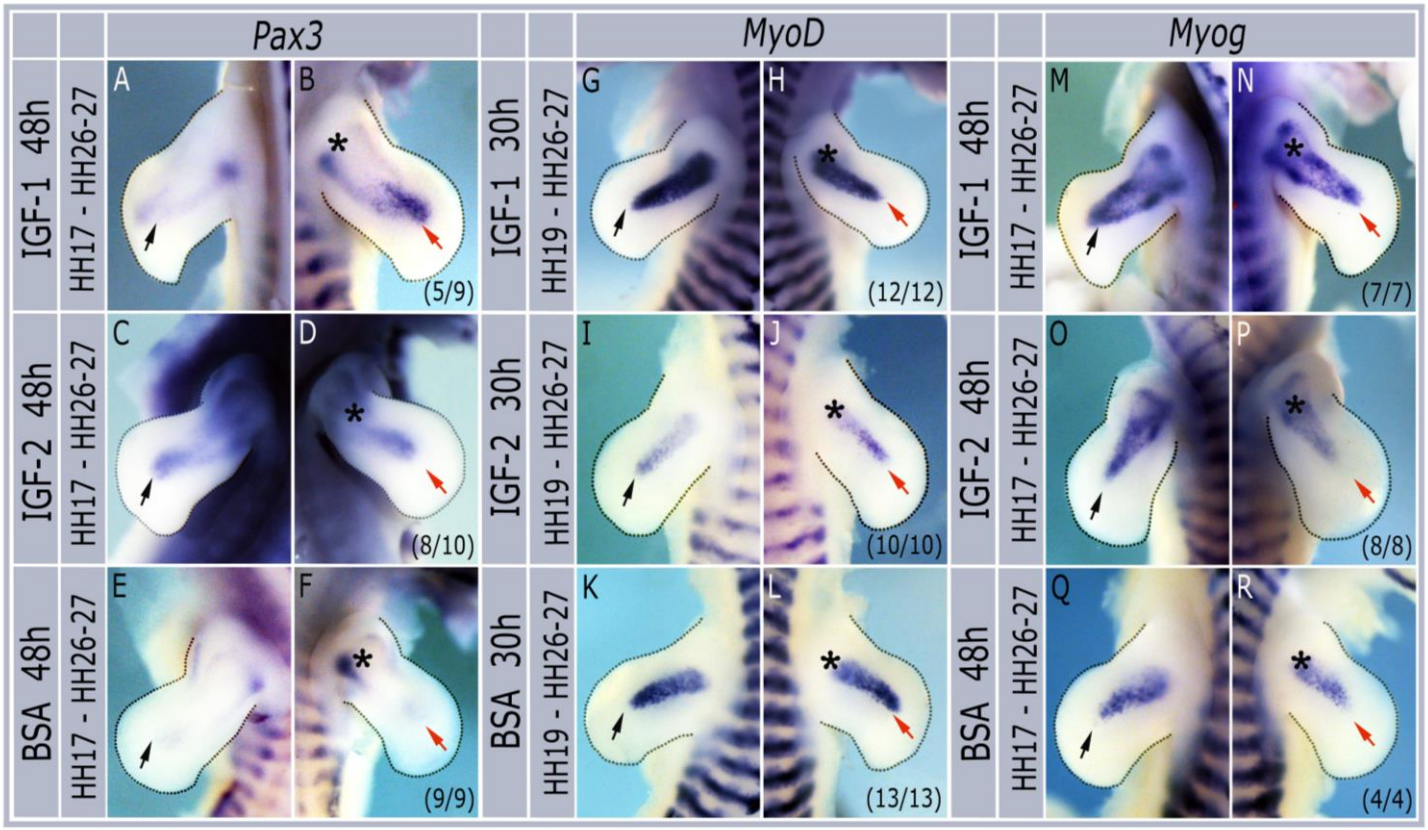


Figure 5. 8: Ectopic IGF activates *Pax3* expression, but not *MyoD* and *Myog* in HH stage 26 embryos. Beads were soaked in IGF-1 and IGF-2; then grafted into the position of forelimb level at HH stage 17, followed by 48 hours incubation to HH stage 26. *Pax3* expression is induced by IGF-1 (A and B), but not by IGF-2 (C and D) compared to the non-grafted limb. BSA control bead shows no effect (E and F). When IGF soaked beads were grafted into the mesenchyme forelimb at HH stage 19 embryos, followed by 30 hours incubation to HH stage 26, both IGF-1 and IGF-2 did not induce *MyoD* expression (G;H and I;H respectively). BSA control bead shows no effect on *MyoD* transcripts (K and L). At early grafting of IGF soaked beads, IGF-1 (M and N) and IGF-2 (O and P) also do not stimulate *Myog* expression at HH stage 26. BSA control bead (Q and R) shows no effect on *Myog* expression. Black asterisks represent beads position. Dotted black lines highlight limb buds. Magnifications: 15x

5.2.6 Ectopic IGF significantly increases Pax3 cells and mitotic cells in early limb myogenesis

To determine the influence of IGF-1 and IGF-2 on myoblast proliferation of embryonic development of chicken limb muscle *in vivo*, IGF-1 soaked beads were grafted into the forelimb at HH stage 17. Embryos were then harvested at HH stage 22 after 24 hours incubation. Sections through this stage were simultaneously stained with immunofluorescence using Pax3 and Phospho-Histone-H3 antibodies. DAPI staining was used to stain all nucleii and also to determine the limb boundary in each section. Across a series of sections cells positive for Pax3 (red), phospho-Histone-H3 (green) and both markers (yellow) were counted to identify the effect of IGF.

$Pax3^{+ve}$ cells in the limbs treated with IGF-1 after 24 hours incubation were compared with contralateral non-grafted counterparts (n=21 paired readings). An increased number of $Pax3^{+ve}$ cells (red, 67.6) were seen in the grafted limb compared with non-grafted counterpart (24.29) (Figure 5.9A and B). In addition, the staining in these cells was uniformly brighter than in control limbs, suggesting higher protein levels in these cells. The number of $Pax3^{+ve}$ cells was analysed by Graph pad prism 7 using Student's T test to investigate whether $Pax3^{+ve}$ cells were significantly increased and gave a $P < 0.0001$ (Figure 5.10A).

Following grafting of IGF-1 beads, there was also an increase in the number of mitotic cells in the grafted limb compared with the contralateral control side (Figure 5.9C and D). The average number of mitotic cells in each section was 60.8 compared to an average of 44.6 in contralateral non-grafted limb, and the analysis then detected an increase of 60% ($P < 0.0138$, two tailed T test) (Figure 5.10B). Although there was an increase in mitotic $Pax3^{+ve}$ cells (12.95), this was not significant compared to the non-grafted contralateral limb (9.90) ($P = 0.9821$) (Figures 5.9G,H and 5.10C).

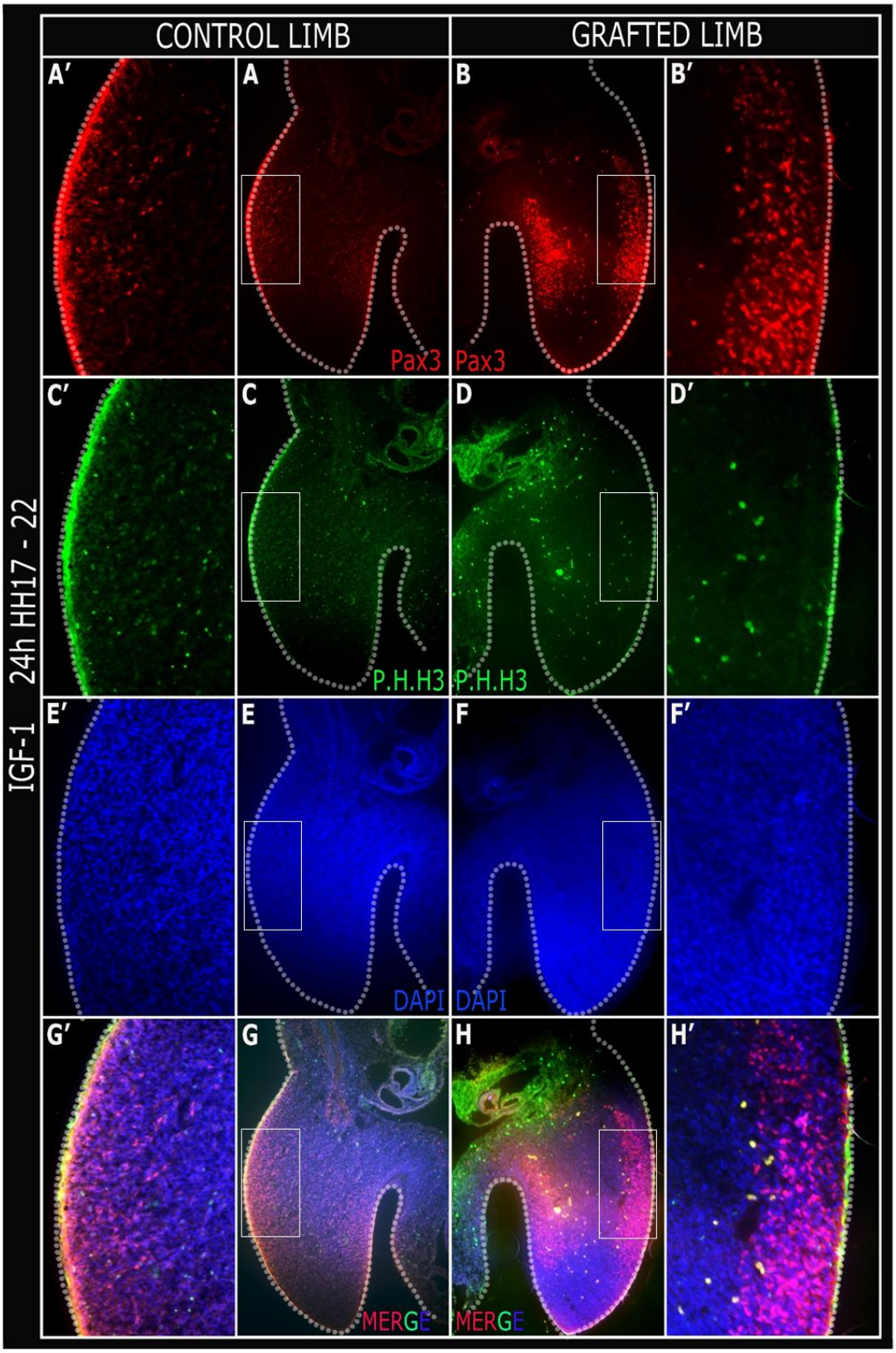


Figure 5. 9: Immunostaining analysis of Pax3^{+ve} cells and mitotic cells in chick limb muscle at HH stage 22 embryos. (A, C,E and G) fluorescent panels of the non-grafted contralateral limb. (B,D, F and H) fluorescent images of grafted IGF-1 soaked beads into the right forelimb. (B, B') Immunostaining with Pax3 antibody specifies migrated Pax3^{+ve} cells and demonstrates an increase in their level, compared to the control side (A,A'). (D,D') Sections were stained with P.H.H3 antibody shows an increase in the

number of mitotic cells, compared to the counter side (C,C') (E,E' and F,F') Sections were labelled with DAPI to realise cells populations as well as bounder the limb. (G,G' and H,H') Merged immunostaining sections of Pax3, P. H.H3 and DAPI. Abbreviation: Pax3^{+ve}, Pax3 positive cells, P.H.H3, Phospho-Histone-H3. Magnification: 500X in (A', B', C', D', E', F, G' and H'), 50X in all other sections.

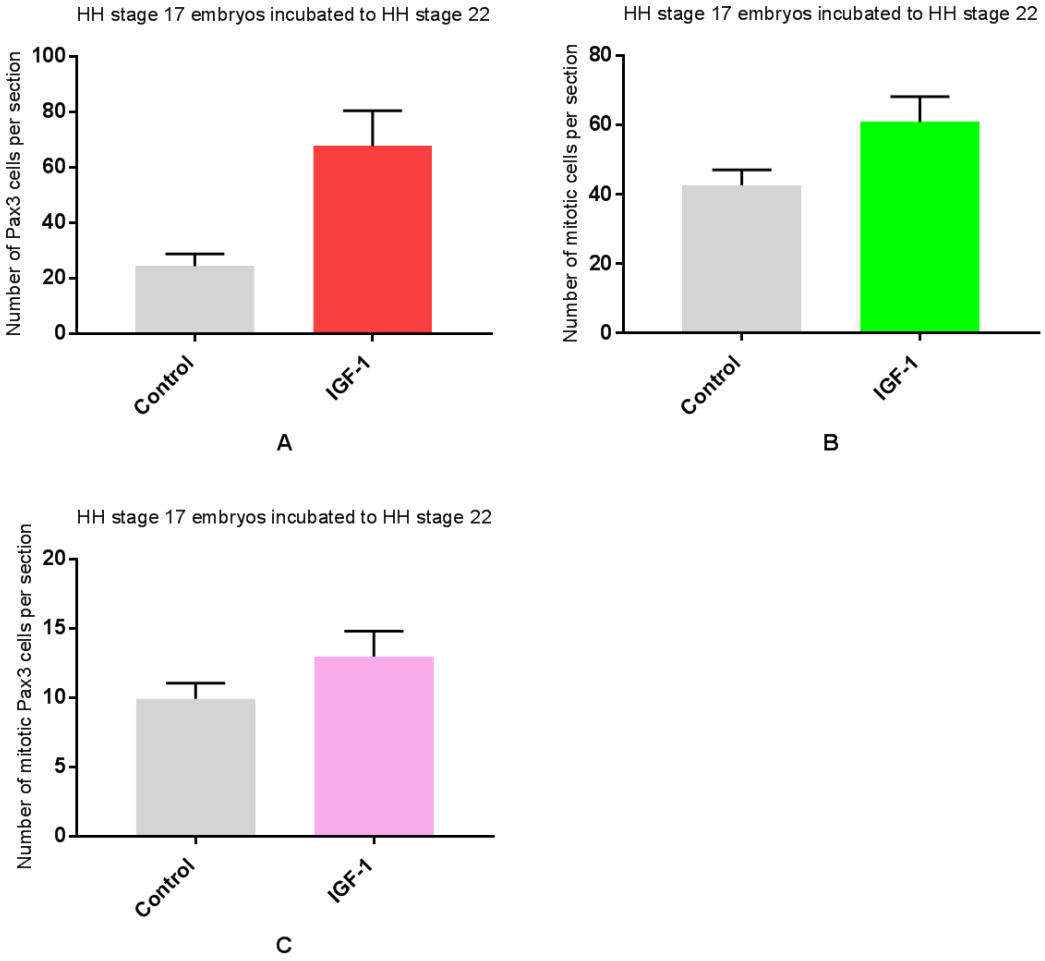


Figure 5. 10: Statistical analysis of the Pax3^{+ve} and P. H. H3 antibodies assay for IGF-1 effects. The columns provide results of the GraphPad Prism 7 statistical analysis of the Pax3^{+ve} (A, red column) and mitotic cells (B, green column) in the limb grafted with IGF-1 soaked beads, (grey columns represent the control). The analysis indicates that Pax3^{+ve} cells and mitotic cells are statistically significant ($P < 0.0001$ and 0.0138 respectively). In contrast, cells stained with both Pax3 and P. H.H3 antibodies (C, violet column) show no significant ($P = 0.9821$, two tailed T test). Abbreviation: Pax3^{+ve}, Pax3 positive cells. P.H.H3, Phospho Histone H3.

5.3 Discussion

This study was designed to investigate the effects of IGFs on myogenic transcription factors at early stages of limb myogenesis in chick embryos. The data demonstrates that IGF beads, grafted into chicken embryo limbs, can induce the expression of *Pax3,* a marker of proliferative muscle precursors, and both *MyoD and Myog* which induce myoblast differentiation but these effects are highly dependent upon the timing for both the grafting and the incubation time following the graft.

5.3.1 Induction of *Pax3*, *Myf5* and *MyoD* by IGF

In chick embryos, $Pax3^{+ve}$ cells are derived from somites that migrate into the limb buds between HH stages 18-22 (Otto et al., 2006, Tajbakhsh and Buckingham, 2000). Application of IGF soaked beads into the limb level at HH stage 17 can effectively upregulate *Pax3* expression at HH stage 22 and 23 (Figure 5.11A and B respectively). However, at HH stage 17, *Pax3* expressing cells do not yet express differentiation markers. Therefore, *Myf5* and *MyoD* are much less likely to be activated. This can be explained by the presence of signals in the early limb that repress myogenic differentiation such as BMPs and RA. Previous work has demonstrated that FGF can only induce *MyoD* expression when RA signalling is blocked (Mok et al., 2014), and that BMPs can also repress *Myf5* and *MyoD* (Amthor et al., 1998, Hirsinger et al., 1997). Like IGFs, signals such as Wnt (Brunelli et al., 2007, Maroto et al., 1997) and Shh (Maroto et al., 1997) can induce *Pax3* prior to the regulation of other myogenic factors. However, FGF8 signals*,* which stimulates muscle growth within the somite, inhibits *pax3*, but induces *myf5* and *myod* transcripts in myogenic progenitor populations in zebrafish (Hammond et al., 2007). Interaction of IGFs with *Myf5* is less well characterised and only a few publications have documented stimulation of *Myf5* expression by IGF-1 (Wimmer et al., 2014). This project, therefore, focused primarily on the IGFs role with *Pax3*, as a myogenic marker expresses during the early migration of progenitors, and *MyoD* as a determination and proliferation myogenic marker.

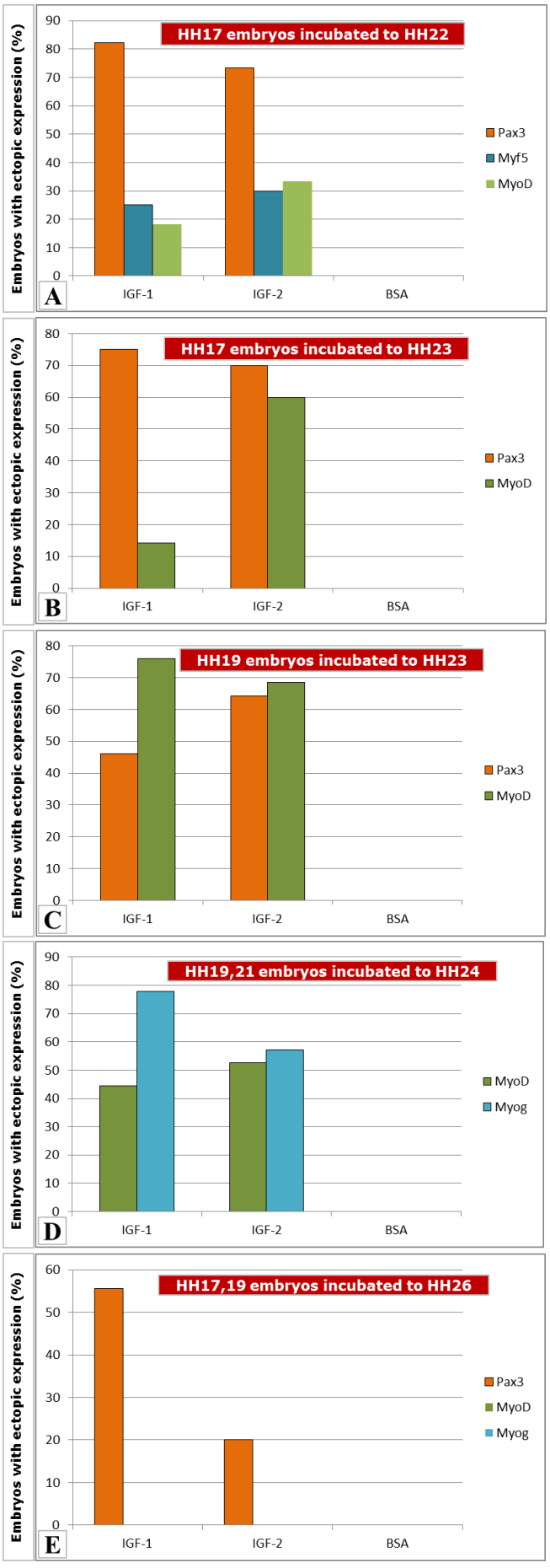


Figure 5. 11: The percentage of embryos with ectopic expression It can be seen from (A) that the highest number of treated embryos with IGF shows when IGF loaded beads grafted at HH stage 17 followed by 24 hours incubation to HH stage 24, wherein 82%, 73% of embryos show upregulation in *Pax3* stimulated by IGF-1 and IGF-2 respectively compared to *Myf5* and *MyoD*. BSA control shows no effect. B, shows that both ligands also activate *Pax3* expression at HH stage 23. The early grafting of IGF-1 soaked beads to HH stage 23 also upregulates *MyoD* expression in 10% embryos, compared to IGF-2. However, it can be seen from C that both IGF-1 and IGF-2 effectively stimulate *MyoD* expression at HH stage 19 where embryos incubated for 18 hours to HH stage 23 However, less effect of IGF ligands on *MyoD* expression at HH stage 24 compared to *Myog* (D). E, shows high induction in *Pax3* expression induced by IGF-1 compared to IGF-2 after long term of incubation to HH stage 26, but both ligands have no effect on *MyoD* and *Myog*. In all cases, BSA control shows no effect.

Although application of IGFs at HH19 can effectively activate early steps of limb myogenesis by stimulating both *Pax3* and *MyoD*, at HH stage 23 (Figure 5.11C), IGF-1 increased *Pax3* expression in only 46% of embryos compared to IGF-2. Muscle precursor cells that activate *MyoD* delaminate from the dermomyotome edges and they, as then differentiate, rapidly downregulate *Pax3* expression, partly through induction of muscle specific microRNAs (Buckingham and Relaix, 2015, Goljanek-Whysall et al., 2011). The reduced effectiveness of IGFs in inducing *Pax3* in these later stages may be due to the more advanced state of the limb myoblasts in the myogenic program. It is also likely that induction of *MyoD* expression is more effective at HH stage 19 because the signals that block myogenic differentiation are downregulated. Mok et al. (2014) have demonstrated that blocking RA signalling results in stimulation of *MyoD* expression by FGF18 soaked-beads-grafted into the limb at HH stage 19. Using the *in situ* hybridisation protocol described here normal *MyoD* expression was seen in the limb at HH stage 23 embryos (see chapter 3 Figure 3.1I) at which point both IGF-1 and IGF-2 strongly upregulated *MyoD* expression. This data supports the idea that IGFs are present in the limb mesenchyme, and that they regulate the expression of muscle specific genes.

In situ hybridisation also showed that IGFs increase *MyoD* expression at a later stage of limb myogenesis, HH stage 24 (see Figures 5.7A-D and 5.11D). The strong induction of *MyoD* by IGFs also indicates that this stimulation acts as a pro-differentiation transcription factor. Growing evidence also demonstrates that IGFs are a potential effector in regulating *MyoD* expression through muscle growth and regeneration (Clemmons, 2009, Florini et al., 1991b, Hsu et al., 1997, Noguchi, 2005, Rotwein et al., 1995, Sun et al., 2004, Wilson and Rotwein, 2006, Wilson and Rotwein, 2007, Yu et al., 2015). Indeed, stimulation of the *MyoD* transcriptional activity, by IGF-1, has been shown in C2C12 cells (Xu and Wu, 2000).

5.3.2 IGFs stimulate later stage of limb myogenesis

A number of investigations have examined *Myog* as a target of IGF action as *Myog* expression causes myoblasts to leave the cell cycle and start differentiation (Ott et al., 1991, Olson, 1992, Rocheteau et al., 2015, Sassoon, 1993). *Myog* was expressed first in both limbs at HH stage 24 (see chapter 3 Figure 3.1P). To examine the effect of IGF on the later markers of myogenesis, IGF soaked beads were grafted into the developing limb between HH stages 21-22 followed by 24 hours incubation to HH stage 24. As shown in figure 5.11D, Both IGF-1 (77.7% embryos) and IGF-2 (57.1% embryos) upregulated *Myog* expression. IGF-1 induces terminal muscle cell differentiation in rodent L6A1 myogenic cells by stimulating *Myog* expression after 24 hours of treatment or more (Florini et al., 1991b, Silverman et al., 1995). In contrast, a shorter time (less than 24 hours) treatment with equivalent IGF peptides decreases *Myog* mRNA level in L6E9 myoblast cultured cells (Engert et al., 1996, Rosenthal and Cheng, 1995).

Several studies have also established that IGF can induce muscle differentiation through upregulating the *Myog* transcription (Florini et al., 1991b, Tiffin et al., 2004). Zanou and Gailly (2013) have proposed that the IGF-1 pathway plays a critical role in regulating expression of MRFs, particularly *MyoD* and *Myog*. Additionally, endogenously produced IGF-2 induces myog expression in cultured myogenic cells (Wilson et al., 2004). However, in some cases IGF-1 inhibits myog production, thereby allowing increased proliferation of myoblasts (Tureckova et al., 2001). The different effects may be due to a biphasic action, because the expression of myog is increased by prolonging the duration of exposure to IGF-1, which in turn facilitates differentiation (Tureckova et al., 2001).

Both IGF-1 and IGF-2 were able to induce expression of myogenic transcription factors. They switched from activation of proliferation (as measured by *Pax3* expression) to stimulation of differentiation (*MyoD* and *Myog* expression) depending upon stage of limb muscle development. However, the early grafting of IGF soaked beads (HH stage 17 embryos) followed by long term incubation (48 hours) showed that IGF-1 less

efficiently stimulates *Pax3* expression at later times (Figure 5.11E). This probably indicates the ability of $Pax3^{+ve}$ cells for proliferation as well as retaining long term self-renewal (Yang et al., 2016). However, no induction of *MyoD* and *Myog* expression by either IGF-1 or IGF-2 was shown after longer term incubation. It is likely that IGF activity has been depleted after these longer incubations and this may explain why their effects are harder to detect. However, the data presented here demonstrated that IGF-signals regulate early stages of limb myogenesis in a time-dependent manner.

5.3.3 More myoblasts but not more proliferation caused by IGF treatment

Myoblast proliferation is a critical step that contributes to muscle growth during development. Skeletal muscle cells respond to different growth factors, among them IGF-1 and IGF-2 that are involved in regulating proliferation and differentiation (Coleman et al., 1995, Noguchi, 2005). The early myogenic precursors are identified by the expression of *Pax3* and *Pax7* both act to regulate muscle cell fate and ensure myoblast cell survival during development (Relaix et al., 2005). The Pax gene family also plays an important role in determining the shape and size of whole organs (Dahl et al., 1997), including limb muscle. As shown in sections 5.2.1 and 5.2.3 both IGF-1 and IGF-2 increased *Pax3* and *MyoD* expression. There are two possible explanations for these observations; either the recently migrated myoblasts are expressing higher levels of these genes or IGF induces proliferation and the higher levels seen are due to higher cell numbers. This cannot be resolved by using *in situ* hybridisation as it does not give single cell resolution. Therefore immunostaining was used to quantify numbers of Pax3 expressing and proliferative cells.

There was a significant increase in $Pax3^{+ve}$ cells and mitotic cells subjected to IGF-1 *in vivo*. Although the IGF-1 beads clearly stimulated both *Pax3* expression and cell proliferation, the lack of a statistically significant in mitotic Pax3 cells (see figure 5.10C) suggests that they are separate effects and that the majority of cells proliferating are not $Pax3^{+ve}$ and similarly the majority of $Pax3^{+ve}$ cells are not proliferating.

In vitro and *in vivo* studies have reported that IGF-1 has a profound stimulatory influence on skeletal muscle size by elevating the number of proliferating myoblasts (Clemmons, 2009, Florini et al., 1996, Jacquemin et al., 2004, Yu et al., 2015), as *IGF-1* null mice have retardation in growth and reduced skeletal muscle mass (Baker et al., 1993, Liu et al., 1993). Engert and colleagues have also reported that IGF-1 promotes both proliferation and differentiation (Engert et al., 1996). However, the present study did not see proliferation in the limb subjected to IGF-1 probably because that was the initial response which has now finished and the cells are moving towards the second phase of their response which is to differentiate.

In chick embryos, over-expression of *IGF-1* has been shown to increase myoblast proliferation, consequently expanding the population of undifferentiated myoblasts and elevating numbers of muscle fibres (Mitchell et al., 2002). However, that study used the RCAS retroviral system to deliver IGF, thus it was over a longer time of exposure and the embryos were much older than the ones examined here. It is possible that there is a stage specific difference with older myoblasts responding by proliferating while the younger ones in the present study embryos do not.

The data presented here raises a question that the additional Pax3 cells observed after IGF-1 bead grafting have to come from somewhere. There are several possibilities that could explain this: either the cells are proliferating more but the numbers in the sections are too variable to draw that conclusion. This could be compounded by the fact that Histone H3 phosphorylation is only seen during mitosis. Other markers of proliferation that marks cells for longer, for example BrdU incorporation, would make these results more robust. It may also be the case that an initial burst of proliferation occurs following the graft but, because the IGF has been used up after 24h they have returned to their normal proliferation rate. Another alternative is that the IGF is inducing more cells to migrate into the limb bud and this, not proliferation, explains the increased cell numbers. There is no evidence of this in these sections but an earlier increase in migration cannot be ruled out.

Although the present study showed that IGF-1 can control the number of Pax3 expressing cells and mitotic cells during limb skeletal development, a requirement of IGF-1 for stimulating myoblast proliferation has yet to be established in this data. Based on this data, it is not possible to conclude that IGF is regulating myoblast proliferation during early limb myogenesis. Nevertheless, IGF does upregulate Pax3 at both mRNA and protein levels. These findings agree with other studies reporting that IGF-1 probably alters transcription and translation of myogenic factors by increasing protein synthesis to control muscle proliferation or differentiation (Coleman et al., 1995, Jacquemin et al., 2007, James et al., 1996).

5.4 Conclusion

The data in this chapter supports the hypothesis that IGF signalling regulates early myogenesis in the chick limb. There are more myoblasts in the limb but they are not obviously more proliferative. Either the effect is hidden because of the relatively small numbers of cells, the IGF has been used up or more cells migrated into the limb buds. The next objective was to determine the IGF signalling pathway involved, to give a better understanding of the molecular basis for the effects of IGFs on myogenic transcription.

Chapter 6: The IGF signalling pathway

6.1 Introduction

The IGFs are implicated in maintaining and activating protein turnover, as well as exerting mitogenic, myogenic and pro-differentiation roles in most cell types (Laviola et al., 2007). These biological actions of IGFs are mediated via IGF receptors, particularly the IGF-1R (see chapter 1.9.2.1). IGF binding stimulates the IGF-1R kinase, resulting in autophosphorylation of the receptor and tyrosine phosphorylation of multiple intracellular protein substrates (see chapter 1.9.4.2). Through these adaptors, IGF signals are transduced to a cascade of signalling networks, which are ultimately responsible for the various cellular responses, including migration, proliferation and differentiation as well as protection from apoptosis (Pavelic et al., 2007). Although both IGF-1 and –2 are able to stimulate the expression of myogenic transcription factors (see chapter 5), the question of which cellular mechanisms and IGF signal transduction pathways are involved in chick limb myogenesis remains unknown. Therefore, this chapter aimed to determine the precise signal transduction pathways through which this happens *in vivo*. For this purpose, IGF soaked beads were co-grafted into limb buds with specific pharmacological inhibitors, and the effects were analysed using *in situ* hybridisation testing *Pax3* and *MyoD* as myogenic markers.

6.2 Results

6.2.1 Nocodazole as a mitotic cell inhibitor

One possible reason for the effect of IGF on *MyoD* expression is that this is merely a secondary effect caused by the increased numbers of myoblasts in the limbs. To determine if this is the case, beads soaked in Nocodazole (10mM), a microtubule disruptor that blocks mitosis (Castedo et al., 2011, Zarrilli et al., 1999), were grafted alongside IGF soaked beads at HH stage 17 followed by 24-28 hours of incubation. This inhibitor did not affect the induction of *Pax3* stimulated by IGF-1 (12/12 embryos, Figure 6.1A and B) or IGF-2 (9/13 embryos, Figure 6.1C and D). DMSO control beads showed no effect on *Pax3* expression stimulated by IGF-1 (8/10 embryos, Figure 6.1E and F) and IGF-2 (6/6 embryos, Figure 6.1G and H). In addition, grafting of Nocodazole soaked beads adjacent to IGF soaked beads at HH 19 followed by 18-22 hours incubation showed no effect on *MyoD* expression stimulated by IGF-1 (5/7 embryos, Figure 6.2A and B). However, the inhibitor blocked *MyoD* expression stimulated by IGF-2 (5/6 embryos, Figure 6.2C and D). In control embryos treated with DMSO, normal induction of *MyoD* stimulated by IGF-1 (11/16 embryos, Figure 6.2E and F) and by IGF-2 (12/19 embryos, Figure 6.2G and H) was observed.

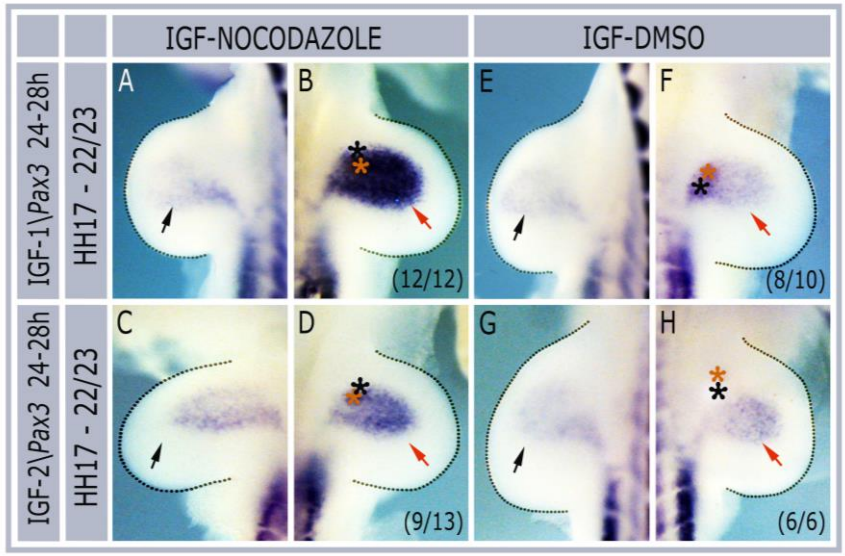


Figure 6. 1: The inhibitory effect of the Nocodazole on IGF-1 and IGF-2 stimulated function. Nocodazole soaked beads were co-grafted with IGF soaked beads at HH stage 17 followed by 24-28 hours of incubation. Nocodazole does not block *Pax3* expression stimulated by IGF-1 (A,B) or by IGF-2 (C,D) compared to the contralateral limb bud. DMSO control beads show no effect on *Pax3* expression stimulated by IGF-1 and IGF-2 (E,F and G,H respectively)**.** Black asterisks label the position of grafted either IGF-1 or IGF-2 beads, while brown asterisks mark the position of Nocodazole and DMSO soaked beads. Red arrows indicate the effect patterns in the grafted forelimb and black arrows label none-grafted contralateral limb buds. Dotted black lines highlight the border of limb buds. Magnifications: 20X Abbreviation: DMSO, Dimethyl Sulfoxide.

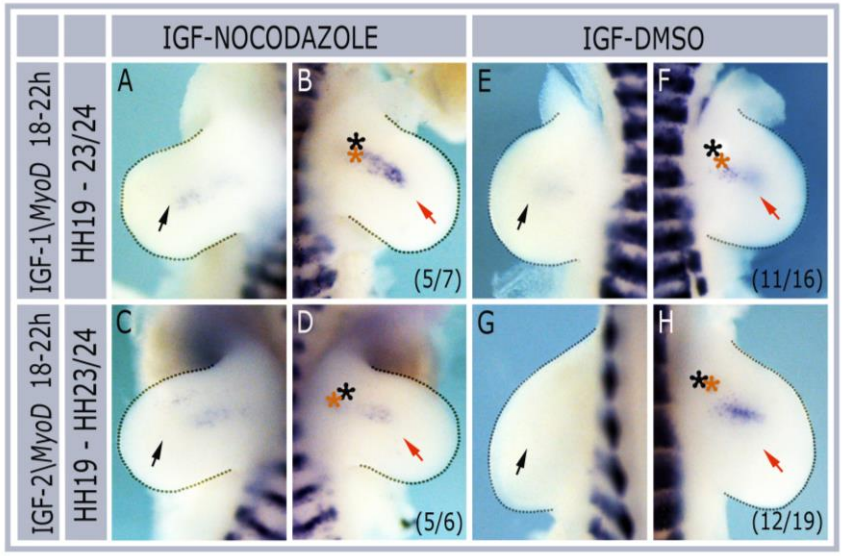


Figure 6. 2: The inhibitory effect of the Nocodazole on IGF-1 and IGF-2 stimulated function. IGF-Nocodazole soaked beads were co-grafted into the forelimb at HH stage 19 followed by 18 hours of incubation. The inhibitor does not block *MyoD* expression stimulated by IGF-1 (A,B), but it blocks the stimulated *MyoD* expression by IGF-2 (C,D). DMSO control beads show no effect on *MyoD* expression stimulated by IGF-1 and IGF-2 (E,F and G,H respectively)**.** Black asterisks label position of IGFs soaked beads, while brown asterisks mark position of Nocodazole and DMSO soaked beads. Red arrows indicate the effect patterns in the grafted forelimb, whereas black arrows indicate none-grafted contralateral limb buds. Dotted black lines highlight limb buds. Magnifications: 20X. Abbreviation: DMSO, Dimethyl Sulfoxide.

6.2.2 IGF-1R is crucial for IGF activity

To ensure that the effects seen with IGFs were specific, beads soaked in Picropodophyllotoxin (PPP) (10mM), a specific IGF-1R inhibitor (Economou et al., 2008, Inzunza et al., 2010, Ohshima-Hosoyama et al., 2010) were grafted alongside IGF soaked beads. PPP and IGF beads were co-grafted into the limb level at HH stage 17 followed by 24 hours incubation to HH stage 22, the stage where *Pax3* was upregulated by IGF-1 and IGF-2 (Figure 6.3A,B and C,D respectively). The comparison between the grafted limbs and contralateral ones showed that PPP blocked the induction of *Pax3* by IGF-1 (12/18 embryos, Figure 6.3I and J). Likewise, PPP blocked IGF-2 induced *Pax3* expression in 8/11 embryos (Figure 6.3K and L). DMSO soaked beads were used as a control; no effect was shown on *Pax3* induction by either IGF-1 (8/10 embryos Figure 6.3H' and H'') or by IGF-2 (6/6 embryos, Figure 6.3I' and I'').

The effect of PPP on *MyoD* expression stimulated by IGFs was also examined by co-grafting PPP soaked beads adjacent to IGF soaked beads into the forelimb of embryos at HH stage 19. Embryos were then incubated for 18 hours to HH stage 23, the stage where *MyoD* was upregulated by IGF-1 and IGF-2 (Figure 6.3E,F and G,H respectively). PPP blocked induction of *MyoD* stimulated by both IGF-1 (7/9 embryos Figure 6.3M and N) and by IGF-2 (8/11 embryos, Figure 6.3O and P). DMSO control beads did not affect *MyoD* stimulation by IGF-1 (11/16 embryos, Figure 6.3J' and J'') and IGF-2 (12/19 embryos, Figure 6.3K' and K''). Sections through embryos at HH stage 23 showed that PPP blocked the induction of *MyoD* expression by IGF-1 in the dorsal limb bud, but some staining remained ventrally (Figure 6.4A). However, PPP efficiently blocked *MyoD* expression that was stimulated by IGF-2 in both dorsal and ventral limb bud (Figure 6.4B). Together these results confirm that the effects of the IGFs are being mediated by the IGF-IR and that they are not off target effects caused by high levels of protein.

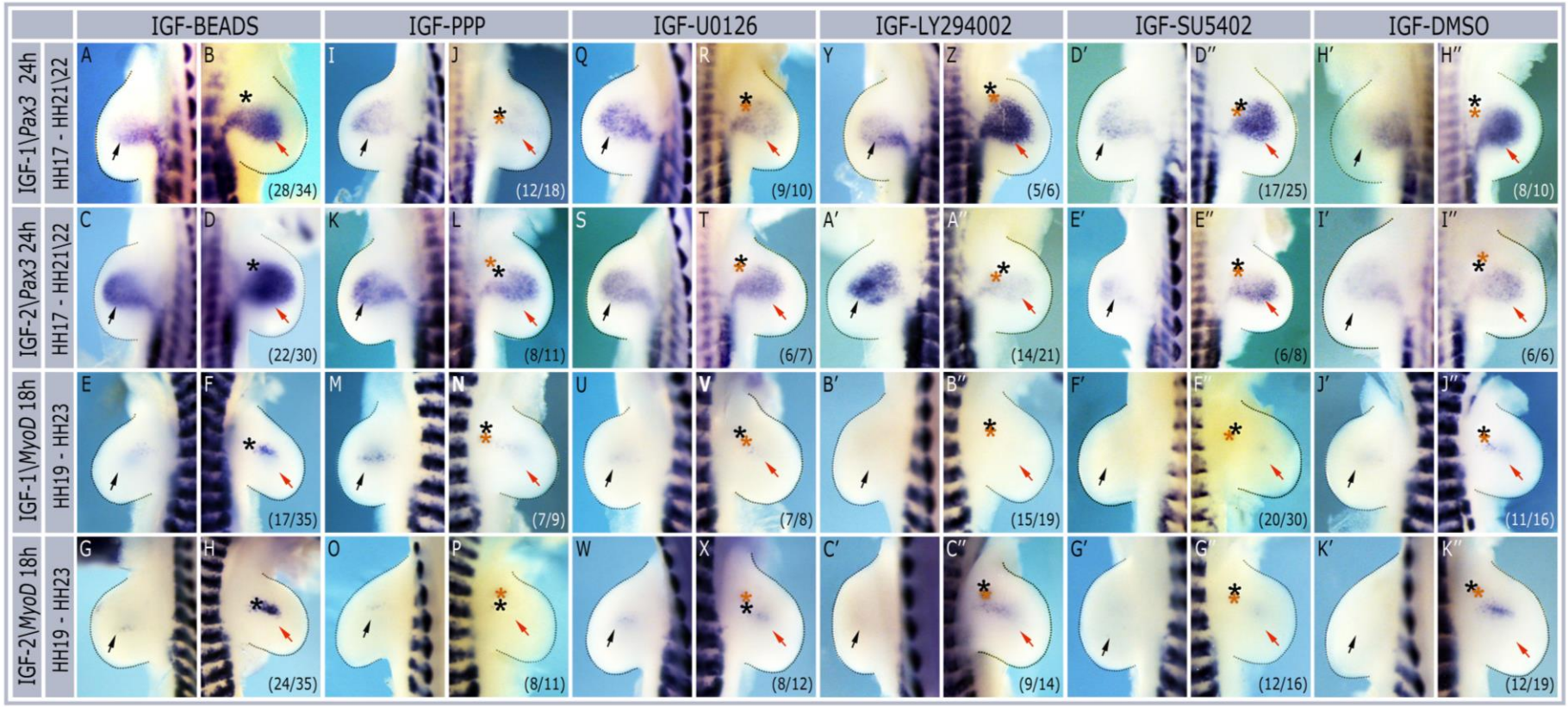


Figure 6. 3: Determination of IGFs signals involved in the chick limb myogenesis. Blocking of IGF-1R and MEK inhibits IGF-1 and IGF-2 stimulated expression of both *Pax3* and *MyoD* but Akt inhibition shows alternative role. A-D show upregulation in *Pax3* expression induced by both IGF-1 and IGF-2. Upregulation of *MyoD* expression stimulated by IGF-1 and IGF-2 (E-H). PPP inhibitor beads, which were co-grafted with IGF soaked beads (at HH stage 17 followed by 24 hours), block and downregulate *Pax3* expression induced by IGF-1 (**I,J**) and IGF-2 (K,L). (M-P) show the inhibitory role of PPP in blocking the induction of *MyoD* expression by IGF-1 and IGF-2, compared to the non-grafted cont-

ralateral limb bud. U0126 soaked beads block the stimulated role of IGF-1 (Q,R) and IGF-2 (S,T) on *Pax3* expression at HH stage 22 after 24 hours of incubation. (U-X) show that U0126 blocks *MyoD* expression induced by IGF-1 and IGF-2 at HH stage 23 after 18 hours of incubation. LY294002 soaked beads do not block *Pax3* expression induced by IGF-1 (Y,Z). LY294002 blocks *Pax3* expression stimulated by IGF-2 (A',A''), compared to the non-grafted contralateral limb bud. LY294002 blocks *MyoD* expression stimulated by IGF-1 (B',B''), but not by IGF-2 (C,C''). SU5402 soaked beads do not block *Pax3* expression induced by IGF-1 (D',D'') and by IGF-2 (E',E''). Co-grafting of SU5402 soaked beads with IGF soaked beads into the forelimb at HH stage 19 followed by 18 hours incubation block *MyoD* expression stimulated by IGF-1 (F',F'') and by IGF-2 (G',G''). DMSO soaked beads were used as control, no effect can be seen on *Pax3* expression induced by IGF-1 (H',H'') and IGF-2 (I',I''). DMSO also does not affect *MyoD* expression stimulated by IGF-1 and IGF-2 (J',J'' and K',K'' respectively). Black asterisks label the position of grafted IGF-1 and IGF-2 beads, while brown asterisks mark the position of pharmacological inhibitors grafted beads and DMSO soaked beads. Red arrows indicate the effect patterns in the grafted forelimb, whereas black arrows indicate non-grafted contralateral limb buds. Dotted black lines highlight the border of limb buds. Magnifications: 20X

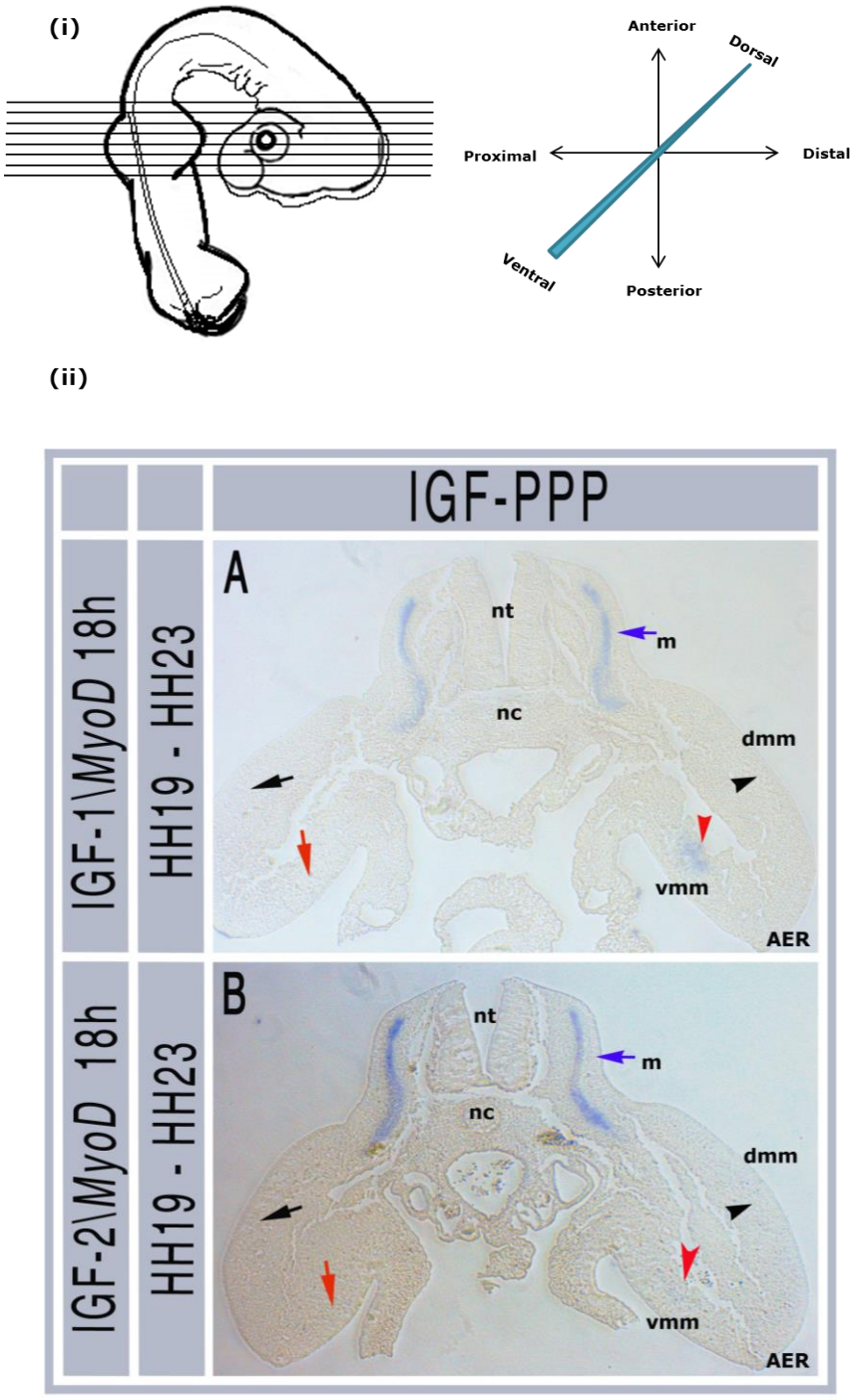


Figure 6. 4: Transverse section through chick limb at HH stage 23. (i) Schematic representation the direction of sectioning of chick limb bud. (ii) Section through the forelimb at HH stage 23. PPP soaked beads were co-grafted with IGF-1 and IGF-2 at HH stage 19, embryos were incubated for 18 hours. (A), PPP blocks the induction of *MyoD* expression by IGF-1, accept little stains in the ventral part of the forelimb. PPP efficiently blocks *MyoD* expression (B) stimulated by IGF-2 and no expression is observed at all in the dorsal and ventral parts of the forelimb. Abbreviation: nt, neural tube; nc, notochord; dmm, dorsal muscle mass; vmm, ventral muscle mass; m, myotome; AER apical ectodermal ridge. Black head arrows indicate the dorsal part of the grafted limb, while the red head's arrows indicate the ventral part. Black arrows indicate the dorsal part of the non-grafted contralateral limb bud, while red arrows indicate the ventral part.

6.2.3 IGF dependant *Pax3* and *MyoD* activation requires ERK phosphorylation

In order to determine whether the effect of IGF-ligands on *Pax3* and *MyoD* expression depends on ERK phosphorylation, beads soaked with 10mM U0126, an inhibitor of MEK, the kinase that phosphorylates ERK (Favata et al., 1998, Mok et al., 2014), were used. U0126 beads were placed into the forelimb adjacent to IGF-soaked beads (1 mg/ml) at HH stage 17. Embryos were incubated for 24 hours to HH stage 22. *In situ* hybridisation showed that U0126 efficiently blocked the induction of *Pax3* expression by IGF-1 (9/10 embryos, Figure 6.3Q and R). Likewise, 6 out of 7 embryos showed blocking of *Pax3* expression induced by IGF-2 (Figure 6.3S and T). To test whether the inhibitor can also affect *MyoD* expression, U0126 soaked beads were co-grafted adjacent to IGF soaked beads into the forelimb at HH stage 19 followed by incubation for 18 hours to HH stage 23. In limbs grafted with IGF-1 and U0126 soaked beads, 7 out of 8 embryos showed blockage in *MyoD* induction (Figure 6.3U and V). Likewise, U0126 blocked the induction of *MyoD* expression activated by IGF-2 (8/12 embryos, Figure 6.3W and X).

6.2.4 The roles of PI3K in IGF dependant *Pax3* and *MyoD* induction

To determine the specificity of MEK in blocking the expression of both *Pax3* and *MyoD* through IGF, PI3K activity was blocked. LY294002 (10mM) was employed as a specific inhibitor that blocks phosphoinositide 3-kinase (PI3K) (Mok et al., 2014, Sanchezmargalet et al., 1994, Vlahos et al., 1994). Beads were soaked with this inhibitor then co-grafted with IGF soaked beads at HH stage 17. Embryos were then incubated for 24 hours to HH stage 22. In the grafted limbs with IGF-1 and LY294002 soaked beads, strong induction of *Pax3* was shown in the limb (5/6 embryos, Figure 6.3Y and Z). However, LY294002 clearly blocked the induction of *Pax3* expression by IGF-2 (14/21 embryos, Figure 6.3A' and A''). To assess the role of IGFs and LY294002 in *MyoD* expression, beads were co-grafted into the forelimb at HH stage 19; embryos were incubated for 18 hours to HH stage 23. Interestingly, LY294002 blocked the induction of *MyoD*

expression by IGF-1 (15/19 embryos, Figure 6.3B' and B''), but not by IGF-2 (9/14 embryos, Figure 6.3C' and C'').

To confirm the specificity of the receptor interactions following IGF bead grafting, SU5402 (10mM) soaked beads were co-grafted in to the forelimb similar to all experiments at HH stages 17 and 19 for testing effects on *Pax3* and *MyoD* respectively. SU5402 was not expected to block IGF induced gene expression as it is an FGFR inhibitor (Mok et al., 2014). After 24 hours of incubation, 17 out of 25 embryos showed no effects of SU5402 on *Pax3* expression stimulated by IGF-1 (Figure 6.3D' and D''). Likewise, the inhibitor did not block *Pax3* expression stimulated by IGF-2 (6/8 embryos, Figure 6.3E' and E''). Surprisingly, after 18 hours incubation (HH stage 23 embryos), SU5402 blocked the expression of *MyoD* stimulated by IGF-1 in 20 out of 30 embryos (Figure 6.3F' and F'') and by IGF-2 in 12 out of 16 embryos (Figure 6.3G' and G'').

6.3 Discussion

In this chapter, different pharmacological inhibitors were used to determine the IGF signalling pathways involved in early stages of chick limb skeletal muscle development.

6.3.1 Implications for the role of mitosis

In order to determine whether the increase in *Pax3* and *MyoD* expression could be due to either increased proliferation of myogenic precursors or differentiation, Nocodazole was used as a reversible suppressor of microtubules that can inhibit karyokinesis and induce mitotic arrest (Castedo et al., 2011, Zarrilli et al., 1999). Nocodazole is effective suppressor for the intracellular transport processes (Melnitskaya et al., 2014). Disruption of microtubules using antimitotic agents decreases the impacts on Na+ trans-epithelial transport of various hormones (Verrey et al., 1995); it strongly prevents several cellular impacts of insulin (Eyster et al., 2006). Nocodazole did not prevent the upregulation of *Pax3* stimulated by both IGF-1 and IGF-2. It also showed no effect on *MyoD* expression induced by IGF-1, but blocked the expression of *MyoD* stimulated by IGF-2.

As observed previously that the majority of cells proliferating are not $Pax3^{+ve}$ and similarly the majority of $Pax3^{+ve}$ cells are not proliferating (see Figures 5.9 and 5.10). The lack of effect of Nocodazole on IGF-stimulated *Pax3* expression, as Nocodazole stops cells going into mitosis by disrupting the microtubules (Castedo et al., 2011, Zarrilli et al., 1999), indicates that the increase seen is presumably due to increased transcription and translation of Pax3 and is not just an artefact of cell number.

As regards the discrepancy in the effects of Nocodazole on IGF-1 and IGF-2 stimulated *MyoD*, indeed, there is no clear explanation of this situation since both IGFs are working through the same receptor. However there has been a long history of debate about how the IGFs can stimulate both cell proliferation and cell differentiation, given that in order to differentiate a cell must withdraw from the cell cycle while stimulation of cell proliferation normally inhibits cell differentiation. Some of the literature does suggest that IGF-1 has a greater effect on proliferation (Davison et al., 2011, Duleba et al., 1997, Giustina et al., 2008, LeRoith and Raizada, 2012, Yu et al., 2015) as well as on differentiation (Broussard et al., 2004, De Arcangelis et al., 2003, Engert et al., 1996, Miyata et al., 2016), whereas IGF-2 has a greater effect on differentiation (Ewton et al., 1994, LeRoith and Raizada, 2012, Piecewicz et al., 2012, Stewart et al., 1996). The discrepancy seen here could relate to IGF-2 having greater effects on proliferation of $MyoD^{+ve}$ cells (thus blocked by Nocodazole), whereas IGF-1 has bigger effects on differentiation (thus not blocked by Nocodazole), which could include IGF-1 inducing more precursor ($MyoD^{-ve}$) cells to become $MyoD^{+ve}$ (i.e. more cells committing to the myogenic lineage).

6.3.2 IGF-1R mediates the majority of IGF signalling

It is generally accepted that IGF-ligands bind with different affinity to their receptors (IGF-1R and IGF-2R). However, IGF action depends on the integrity of the IGF-1R signal transduction pathway (Laviola et al., 2007). In order to determine the signalling pathway, this chapter determined first the precise receptor by using the cyclolignan, Picropodophyllotoxin (PPP) (also known as Axenar AB or AXL171) (Economou et al., 2008, Inzunza et al., 2010, Ohshima-Hosoyama et al., 2010). PPP is a small molecule, highly selective for IGF-1R, that acts as a potent inhibitor of tyrosine

phosphorylation and does not cross-react with other receptors, such as insulin receptor (IR), beta-type platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR) and epidermal growth factor receptor (EGFR) (Girnita et al., 2004, Ohshima-Hosoyama et al., 2010). PPP blocks the phosphorylation of Tyr1136 of IGF-1R (Vasilcanu et al., 2004) and accelerates IGF-1R ubiquitination for degradation (Vasilcanu et al., 2008). Significant reduction in phospho-MAPK has been shown in PPP-treated cells (Girnita et al., 2004, Ohshima-Hosoyama et al., 2010, Zhang et al., 2015). PPP also affects phospho-Akt (Thr308) (Ohshima-Hosoyama et al., 2010), but does not inhibit phospho-Akt (Ser473) in multiple cell lines of myeloma (Ohshima-Hosoyama et al., 2010, Strömberg et al., 2006). However, Vasilcanu et al. (2004) tested melanoma cell lines and they have reported that PPP can also block phospho-Akt (Ser473).

Loss of function experiments using the PPP inhibitor co-grafted with IGF-ligands soaked beads strongly blocked the induction of both *Pax3* and *MyoD*. Although IR and IGF-2R are present on cells surfaces, the results with PPP showed that IGF-ligand interactions with these receptors do not mediate the IGF-1 and IGF-2 signalling. These results confirmed that both IGF-1 and IGF-2 activate IGF-1R and their signal is exclusively via this receptor. El-Shewy et al. (2007) have demonstrated that knockdown of the expression of *IGF-1R* by RNA interference also decreases the IGF-1 response, but more than that of IGF-2 (El-Shewy et al., 2007). This suggests that IGF-1R does not mediate all IGF-2 effects. It seems, therefore, likely that the PPP probably reduce the number of proliferating cells stimulated by IGFs as well as blocking the induction of differentiation.

6.3.3 ERK signalling mediates stimulated IGF-1 and IGF-2

In mammals, three distinct types of MAPKs have been detected, including ERK1/2, p38α, β, γ and δ and the JNK family (Chang and Karin, 2001). All of these types are crucial in cell proliferation, survival and apoptosis (Widmann et al., 1999). Both ERK1 and ERK2 are required for the proliferation of myoblasts, but are dispensable for cell fusion and myogenic gene expression (Jones et al., 2001). The growth factors that activate

ERK1/2 are associated with cell proliferation and differentiation processes (Pearson et al., 2001). Genetic studies performed in mouse and bovine have established the crucial function of MAP kinase signalling in regulating proliferation and differentiation of myogenic progenitor cells *in vitro* (Kook et al., 2008, Volonte et al., 2005). In mouse, ERK signalling is preferentially stimulated by initiating terminal myogenic cell differentiation in C2 myoblasts cell line (Gredinger et al., 1998). In rat muscle cells line, induction of p38 MAP kinase in L6E9 and activation of ERK1/2 kinase signalling lead to terminal differentiation (Khurana and Dey, 2002).

In order to directly address whether Akt activation by PI3K or ERK activation by MEK is required for IGF ligand induced upregulation of *Pax3* and *MyoD* expression, specific inhibitors that eliminate the phosphorylation of Akt and ERK by alteration of the kinase pathways were used. MAPK activation by the IGF-1R is very well studied (Peruzzi et al., 1999). Therefore, U0126 (a highly specific suppressor for the MEK cascade) that can block the activation of ERK by MEK (Favata et al., 1998) was tested. Blocking ERK activation resulted in downregulation of *Pax3* and *MyoD* expression stimulated by IGF-ligands. It seems likely that both IGF-1 and IGF-2 stimulate ERK MAP Kinase phosphorylation in chick limb; the interference with the IGF-ligands signalling by ERK inhibitor prevents myogenic cells proliferating from entering the skeletal muscle determination program. *In vitro* induction of IGF-1 results in phosphorylation of MAPKs (ERK1 and ERK2) (Peruzzi et al., 1999), which is associated with cell proliferation (Lee et al., 2006). El-Shewy et al. (2007) have demonstrated that both IGF-1 and IGF-2 can induce ERK1 and ERK2 cascade via triggering sphingosine kinase, depending on G protein-coupled sphingosine-1-phosphate receptors.

Mok et al. (2014) have also established that U0126 blocks *MyoD* expression stimulated by FGF18 at HH stage 21, and this inhibitor not only blocked FGF18-induced *MyoD* expression but also the onset of endogenous *MyoD* expression (Mok et al., 2014). However, manipulating FGF4 or FGF8 soaked beads close to newly formed somites elevated the activity of MAPKs ERK kinase, but blocked *MyoD* expression induced by growth factors after 24 hours incubation of chick embryos (Smith et al., 2005). It is apparent

that myoblasts, in different tissues, e.g. somites and limbs, response differently to ERK activation. In limbs, they switch on *MyoD*, while in somites induce scleraxis protein and downregulate *MyoD*. This data is consistent with the previous work performed in limb myogenesis.

6.3.4 Inhibition of PI3K suggests distinct functions for IGF-1 and IGF-2

The PI3K/AKT pathway is important for proliferative signalling (Patruno et al., 2015), and has a critical function in IGF-1 induced myogenic and mitogenic signalling (Coolican et al., 1997, Kaliman et al., 1998). In cardiac muscle, IGF-1 can stimulate both PI3-kinase-AKT and ERK pathways (Liu et al., 2001, Ren et al., 1999). To determine if this pathway was required for IGF function, LY294002 was employed as a specific inhibitor to block PI3K (Sanchezmargalet et al., 1994, Vlahos et al., 1994), which is upstream of Akt protein kinase (Bellacosa et al., 1991). LY294002 did not block induction of *Pax3* expression but did block *MyoD* induced by IGF-1. In contrast, LY294002 downregulated *Pax3* expression, but not *MyoD* induced by IGF-2. The reasons and interpretation for the contradictory actions of this inhibitor on IGF-1 and IGF-2 effects remain somewhat unclear. One possible explanation for this difference is that IGF-1 or/and IGF-2 can independently activate Akt signalling through other receptors and thereby they showed a different function. The insulin receptor can mediate IGF-2 signalling during fetal growth (Louvi et al., 1997). Targeted mutagenesis of the IR and IGF-1R genes has demonstrated that loss of IGF-2 and IGF-1R mice are severely growth-retarded compared to embryos lacking single IGF-1R (Baker et al., 1993, Liu et al., 1993, Louvi et al., 1997). Double *IGF-1R/IGF-2* mutant mice show a similar phenotype to mice lacking both *IGF-1R/IR*, suggesting that IGF-2 effects are also mediated via IR (Liu et al., 1993, Louvi et al., 1997), more precisely, IR-A (Sciacca et al., 2002). In addition, knockdown of *IGF-2R* expression markedly decreases IGF-2-induced ERK1/2 phosphorylation, but does not affect the IGF-1 response, suggesting that IGF-2R can be as mediator for IGF-2 response (El-Shewy et al., 2007). However, the cross-talk between IGF-2 and other receptors requires further work to identify the mechanism.

Because ERK activation is also negatively regulated by the Akt signalling pathway (Lee et al., 2006), the present study also proposed a cross-talk between signalling pathways of the ERK/MAPK and PI3K/Akt in IGF-1 induced myogenic factors. Cooperative effects and/ or cross-talk between the Ras/ Raf/ MEK/ ERK1/ ERK2 and PI3K/Akt have been shown at different levels to exert antagonistic or cooperative impacts based on cellular background and stimuli (Chang et al., 2003, Choudhury et al., 1997).

6.3.5 SU5402 inhibitor effectively blocks *MyoD* expression stimulated by IGF

SU5402 is a fibroblast growth factor receptor (FGFR) specific tyrosine kinase inhibitor (Mohammadi et al., 1997); it inhibits signalling from FGFR1 to FGFR3 (Mok et al., 2014). This inhibitor was used as a negative control to determine the precise role of IGF-1R. SU5402 did not block the induction of *Pax3* stimulated by IGF-1 and IGF-2 at HH stage 22 after 24 hours incubation. It was not surprising to obtain this result, because SU5402 is a potent suppressor for fibroblast growth factor receptor (FGFR) and does not block IGF signalling. Surprisingly, SU5402 inhibited the induction of *MyoD* expression stimulated by IGF-1 and IGF-2 at HH stage 23 embryos (see Figure 6.3F'-G''). In contrast, grafting SU5402 soaked beads into HH stage 21 embryos have no effect on *MyoD* expression induced by FGF18 after 6 hours incubation (Mok et al., 2014). This therefore suggests a cross-talk between IGF and FGF signalling in regulating limb myogenesis. The stimulatory role of FGF in the limb depends on IGF-1 activity (Dealy and Kosher, 1996, Dealy et al., 1996). However, Karam et al. (2012) have demonstrated interactions between EGF/ FGF and IGF. The authors have established that EGF and FGF suppress the IGF-1-stimulated IRS-1 tyrosine phosphorylation through PI3-kinase dependent and MEK/ERK independent signalling pathways respectively (Karam et al., 2012).

6.4 Conclusion

Application of specific pharmacological inhibitors of downstream signalling pathways (e.g. PPP) showed that IGF-1 and IGF-2 signals are mediated through the IGF-1R. In addition, U0126 showed that IGF signalling is important to directly control *Pax3* expression of limb myogenesis via ERK1/2 MAP kinase. Moreover, IGF signalling may indirectly regulate *MyoD* expression via FGF and then through ERK1/2 MAP kinase pathway. It is also probable that IGF-1 and IGF-2 can be mediated through other pathways including the PI3K\Akt.

Chapter 7: General discussion, summary and further work

7.1 Introduction

In vertebrates, all the myogenic precursor cells that form the skeletal muscles of the trunk and limb originate from somites (Buckingham et al., 2003, Duprez, 2002). During the formation of limb skeletal muscle, myogenic cells delaminate from the lateral part of the dermomyotome then migrate to enter the limb (Haines and Currie, 2001, Neyt et al., 2000) depending on c-met and *Pax3* activity. (Tajbakhsh and Buckingham, 2000). In the limb, the muscle progenitor cells express the myogenic regulatory factors, *Myf5*, *MyoD*, *Myog* and *Mrf4* as they proliferate and differentiate into mature myofibers (see chapter 1.8.2 and Mok et al. (2015)). Several growth factors are secreted during muscle growth and development among which are insulin-like growth factors (IGFs) that stimulate both myogenic cell proliferation and differentiation (see chapter 1.9) (Zanou and Gailly, 2013). However, how IGFs interact with Mrfs to regulate the early processes of limb myogenesis remains poorly understood. In this thesis, the spatial and temporal expression patterns of both Mrfs and IGFs were characterised in chick limb development. This project also focused on the effects of IGF-1 and IGF-2 on myogenic transcription factors and identified some of the signalling pathways that mediate these effects. This chapter will highlight the final discussions and summaries from the project, and it will also suggest further work for future studies.

7.2 Characterisation of myogenic regulatory factors and insulin like growth factors in chick limb

The chick limb is a well studied model, regulated by many different transcription factors and signals, that provides an excellent system to determine the biological functions of particular gene products (Schwabe et al., 1998). During limb myogenesis, myogenic cells express members of the MyoD family of transcription factors (Weintraub, 1993). Four of MRFs have been characterised known Myf5, MyoD, Myog and Mrf4 (Braun et al., 1989, Edmondson and Olson, 1989, Rhodes and Konieczny, 1989, Weintraub et al., 1991). The present study characterised the expression of these factors in the developing chick limb and showed that *Myf5* was expressed first in the forelimb at HH stage 22 then the expression maintained in both limbs to HH stage 26 embryos. *MyoD* followed *Myf5* and was expressed in the hindlimb at HH stage 23 and the expression increased in both limbs at HH stages 24, 26 and 30 embryos. However, the present study observed a different spatiotemporal mRNA expression pattern of *Myf5* and *MyoD* to that reported by other studies (Amthor et al., 1998, Delfini et al., 2000) probably due to the sensitivity of the probes that used in the experiment (see chapter 3.3.1 for detail). Along with *MyoD*, *Myog* was detected in both limbs at HH stages 24 to 30. The last myogenic regulatory factor was *Mrf4* transcribed in the limb at HH stage 26 embryos to HH30. This thesis offers clear evidence that the MyoD family is expressed in a set order during limb development, starting with *Myf5*, then *MyoD*, *Myog* and finally *Mrf4*. Detecting of where and when these factors are expressed allowed the present study to determine the correlation between the IGF axis and Mrfs during limb development.

In this project, the IGF axis was also characterised in the developing chick limb. Using *in situ* hybridisation, Allan et al. (2003) have described the expression pattern of IGFs in chick embryos. However, the spatial and temporal expression patterns of *IGF-1*, *IGF-2*, *IGF-1R*, *IGF-2R* and *IGFBP-5* in the embryonic development of chick limb are not fully described. The present study, therefore, aimed to characterise IGF axis expression in detail using the *in situ* hybridisation technique. The results showed that

IGF-1 was first localised in the forelimb at HH stage 20, then the expression maintained to HH stages 22,23 and 24. At HH stage 26, IGF-1 was clearly expressed in both limbs. At HH stage 20, *IGF-2* also had a spatio-temporal profile similar to that of IGF-1 in the forelimb. This data suggests an overlap between both ligands to regulate the early stages of limb development. In contrast to IGF-1, however, IGF-2 did not express between HH stages 21 to 23. At HH stage 24 and 26, IGF-2 was seen in the forelimb then expressed in both limbs at HH stage 26. Having IGF-1 and –2 at early and later stages of limb development indicates possible roles in regulating cell proliferation and differentiation, especially as both ligands have shown to regulate these processes of myogenic cells (Bach et al., 1995, Engert et al., 1996, Stewart and Rotwein, 1996). Although there is not a complete overlap between the Mrfs and IGFs this does not preclude an interaction as IGFs can be transported through the bloodstream and have systemic, as well as local, effects.

The IGF-receptors (IGF-1R and IGF-2R) were also measured as the main receptors that mediate IGF biological functions. IGF-1R expressed in the tissue immediately proximal to the ridge at HH stage 20. IGF-1R then maintained its expression in both limbs to HH stage 26. Allan et al. (2003) detected *IGF-1R* expression in the AER at HH stage 24. The presence of *IGF-1R* expression in the limb at early and later stages of the development indicates that this receptor binds with both IGF-1 and IGF-2 to induce proliferation and differentiation. In contrast, *IGF-2R* did not express at HH stage 20, but the expression was observed in the forelimb by HH stages 22, 23, and 24; then it disappeared at HH stage 26. Because IGF-2R does not bind to IGF-2 (Canfield and Kornfeld, 1989, Yang et al., 1991), plus this receptor has no intracellular signalling domain and therefore no signal transduction (Pavelic et al., 2007). It is unlikely that IGF-2R has a role in mediating IGF activity from HH stage 22 to 24 However, El-Shewy et al. (2007) have proposed that IGF-2R can mediate IGF-2 signalling which might explain some of the distinct effects of IGF-1 and -2 seen in this study. In addition, it is generally accepted that IGF-2R works to clear IGF-2 by degradation to the lysosomal enzymes (Kornfeld, 1992, Scott and Firth, 2004).

IGF-ligands also bind with high affinity to six binding proteins (IGFBPs) (Bach and Parker, 2003, Clemmons, 1997a). The present study focused on IGFBP-5 due to its interaction with IGF-ligands during myogenesis (Ren, 2009). The results observed the first expression of *IGFBP-5* in both fore and hind limbs at HH stages 24 and 26, possibly to act as a differentiation regulator in the developing chick limb. IGFBP-5 correlates well with IGF-2 and *Myog* expression during the differentiation of muscle cells (Bayol et al., 2000, Bergstrom and Tapscott, 2001, Ren, 2009, Rotwein et al., 1995). Therefore, IGFBP-5 possesses an important role in regulating cellular differentiation, apoptosis and survival in the developing limb (Beattie et al., 2006). Altogether, the presence of IGF expression during early stages of limb development makes them good candidates for testing a role with myogenic transcription factor regulation.

7.3 The role of IGF with myogenic transcription factors and signalling pathways involved

Several studies have shown that the IGFs can regulate the myogenic regulatory factors, particularly *MyoD* and *Myog*. Zanou and Gailly (2013) have revealed that IGFs can regulate *MyoD* during the hypertrophy and regeneration of skeletal muscle. IGF can also stimulate myoblast differentiation in chick embryos by stimulating *Myog* expression (Mitchell et al., 2002, Musarò and Rosenthal, 1999, Yu et al., 2015). However, the role of the IGF axis in the developing limb skeletal muscle of chick embryos is still undefined, and how signals affect early processes of muscle development remains largely mysterious. The present study, therefore, hypothesised a cross-talk occurs between the IGFs and myogenic transcription factors to regulate limb myogenesis. Examining this hypothesis, IGF soaked beads were grafted into the level of forelimb at HH stage 17. Embryos were incubated for 24 hours subsequently harvested at HH stage 22. At this stage, both IGF-1 and IGF-2 upregulated *Pax3* expression but had less effect on *Myf5* and *MyoD* expression. However, when IGF soaked beads were grafted into HH stage 19 embryos followed by 18 hours incubation, both IGF-1 and IGF-2 stimulated both *Pax3* and *MyoD* expression. It is apparent that upregulation of *Pax3* expression

results in stimulation of other myogenic regulatory factors, as pax3 is required upstream of myod for lateral dermomyotome to form myogenic cells in the amniotes (Hammond et al., 2007). It seems also possibly that the presence of signals in the early limb, such as BMPs and RA block the myogenic differentiation. BMPs can repress *Myf5* and *MyoD* expression (Amthor et al., 1998, Hirsinger et al., 1997); thus blocking of RA signalling leads FGF to inducing the expression of *MyoD* (Mok et al., 2014).

Myog, the late marker of myogenesis, was also tested with IGFs by grafting IGF soaked beads into the forelimb between HH stages 21-22; embryos were incubated for 24 hours. Both IGF-1 and IGF-2 activated *Myog* expression at HH stage 24. Because *Myog* induces differentiation, and is expressed first in chick limbs at HH stage 24 expressed (see chapter Figure 3.1P and Mok et al. (2015)), the present data proposed that both IGF-1 and IGF-2 regulate pro-differentiation of limb muscle growth and development by stimulating *Myog* transcription. Treatment with IGF in cultured cells for 24 hours (Engert et al., 1996, Rosenthal and Cheng, 1995), leads to upregulation of *Myog* mRNA levels (Florini et al., 1991b, Silverman et al., 1995).

In addition, to quantify numbers of Pax3 expressing and proliferative cells, the present study performed immunostaining experiment and showed a significant increase in $Pax3^{+ve}$ cells and mitotic cells subjected to IGF-1 *in vivo*. However, there was a lack of a statistically significant in mitotic Pax3 cells. This suggests that they are separate impacts and that the high number of cells proliferating are not $Pax3^{+ve}$, likewise the majority of $Pax3^{+ve}$ cells are not proliferating (see 5.2.6 and 5.3.3 for detail).

To determine whether IGFs effects on *MyoD* are secondary to the effects on proliferation, Nocodazole was used as a microtubule disruptor that blocks mitosis (Castedo et al., 2011). The inhibitor did not affect the induction of *Pax3* by IGF-1 or IGF-2, but it blocked the induction of *MyoD* by IGF-2. These results indicate that the increase seen is apparently due to elevated Pax3 transcription and translation and it is not just an artefact of cell number. The present study, consequently, proposed that IGF-2 has a great effects on proliferation of $MyoD^{+ve}$ cells (thus blocked by

Nocodazole), compared to IGF-1 which affects differentiation (thus not blocked by Nocodazole) and IGF-1 probably stimulate more precursor ($MyoD^{-ve}$) cells to become $MyoD^{+ve}$.

The cyclolignan Picropodophyllotoxin (PPP) was employed as an antagonist of IGF-1R (Ohshima-Hosoyama et al., 2010). This inhibitor prevented IGF-1R activation and led to the downregulation of both *Pax3* and *MyoD* expression. As both IGF-1 and IGF-2 bind with high affinity to IGF-1R (Laviola et al., 2007), this that the ligands interact with IGF-1R, which in turn mediates the biological actions of IGF-1 and IGF-2 in regulating limb myogenesis. Binding of either IGF-1 or IGF-2 to IGF-1R extracellular regions (ECDs) induces the activity of receptor kinase, and results in phosphorylation of multiple substrates as well as initiation of particular signalling cascades (Siddle, 2012). Therefore, to directly address whether Akt via PI3K or ERK via MEK phosphorylation mediates IGFs dependant *Pax3* and *MyoD* activation, U0126 was applied as a specific inhibitor of the MEK cascade. This inhibitor blocks the activation of ERK by MEK and MEK by Raf (Favata et al., 1998). Treatment with IGF-1 or –2 and together with U0126 downregulated *Pax3* and *MyoD* transcripts, suggesting that the effects of IGF-ligands on regulating myogenic transcription factors in chick limb myogenesis require ERK phosphorylation.

Because PI3K/AKT is the important proliferative pathway (Patruno et al., 2015) that mediates IGF myogenic and mitogenic action (Coolican et al., 1997, Kaliman et al., 1998), the present study tested LY294002, a phosphoinositide 3-kinase (PI3K) antagonist (Sanchezmargalet et al., 1994, Vlahos et al., 1994). Application of this inhibitor along with IGF-ligands showed that LY294002 blocked *MyoD* expression, stimulated by IGF-1, rather than blocking *Pax3* expression. In contrast, the inhibitor blocked the expression of *Pax3*, but not *MyoD* expression stimulated by IGF-2. Here, the present work proposed that IGF-1 and IGF-2 can collaborate to maintain myogenic action and cell viability. Another possibility is that IGF-ligands could bind to more than one receptor, such as insulin receptor A (IRA), which can mediate IGF-2 effects during fetal growth (Louvi et al., 1997). IGF-1R and IGF-2R can independently activate Erk/MAPK signalling in HEK293 cells (El-Shewy et al., 2007), suggesting a

potential role of IGF-2R in mediating IGFs action. The present data also suggested a relationship between both Akt and ERK, since it has been suggested by different studies that a crosstalk may occur between signalling pathways wherein one pathway can stimulate or inhibit signalling by another (Dent et al., 1999, Dent, 2014, Lee et al., 2006, Reardon et al., 1999, Toulany et al., 2014).

To confirm the precise role of IGF-1R, SU5402, a pharmacological inhibitor that binds FGFR (Mohammadi et al., 1997) and supresses signalling from FGFR1 to FGFR3 (Mok et al., 2014) was also tested. This inhibitor did not block *Pax3* expression, but it blocked *MyoD* stimulated by both IGF-1 and IGF-2. This data led to a model where the stimulation of *Pax3* and *MyoD* by treatment of either IGF-1 or IGF-2 acts through two modes to control early myogenesis in the limb. First, both IGF-1 and IGF-2 directly activate *Pax3* expression via ERK MAP kinase pathway (Figures 7.1 and 7.2A). The ability of an FGFR inhibitor to block *MyoD* induction led to the suggestion that IGFs then also activate FGF expression in the limb bud via the ERK MAP kinase pathway; this then induces *MyoD* expression (Figures 7.1 and 7.2B).

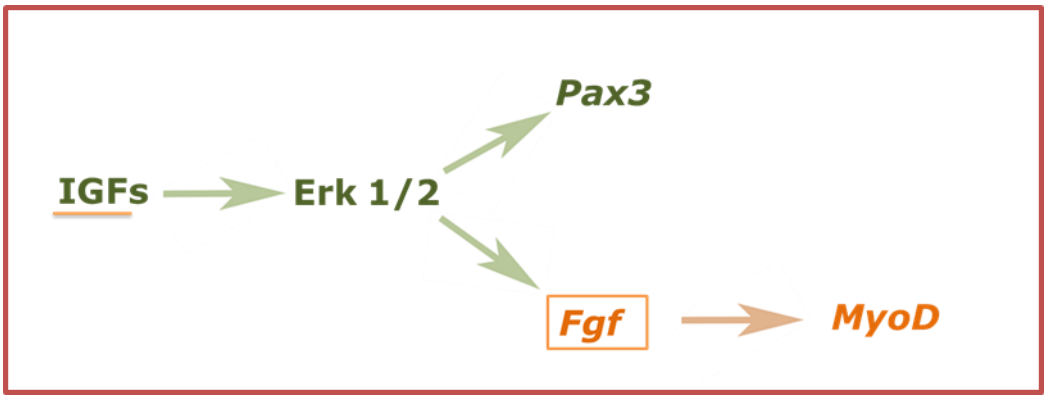


Figure 7. 1: Model for stimulated role of IGF signalling by ERK MAP kinase in regulation early limb skeletal muscle. IGF-ligands directly activate *Pax3* expression (green) via ERK1/2, and indirectly activate *MyoD* expression by stimulating *Fgf* via ERK MAP kinase pathway (orange).

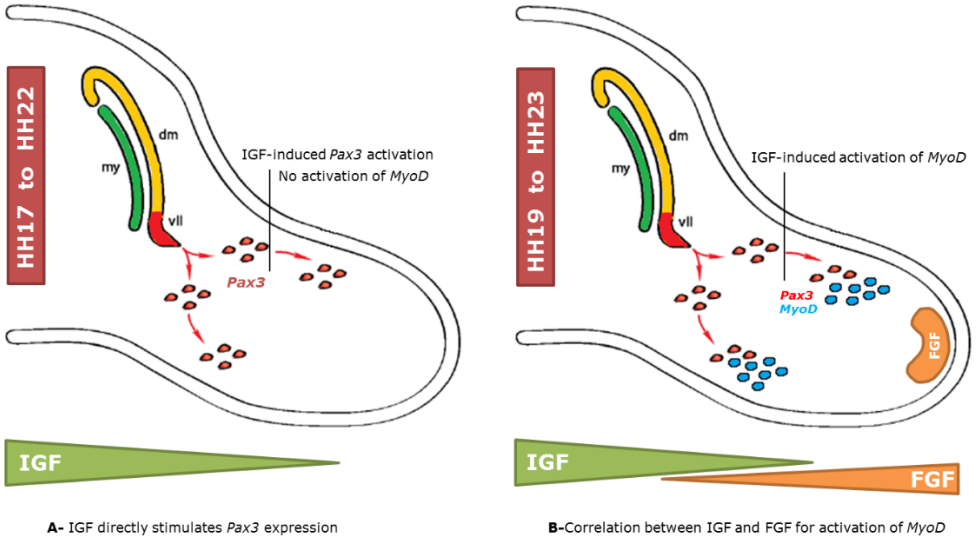


Figure 7. 2: Schematic representation of the present study's model showing IGF-1 induced activation of myogenic transcription factors in regulating limb myogenesis. (A) Grafting IGF-soaked beads at HH stage 17 embryos directly activate *Pax3* expression after 24 hours incubation to HH stage 22. At this stage, no activation of *MyoD* is shown. At HH stage 23 (B), IGFs signalling activates *Fgf*, which in turn stimulates *MyoD* expression. This data was performed by grafting IGF soaked beads at HH19 embryos followed by 18 hours of incubation. Abbreviations: dm, dermomyotome; my, myotome; vll, ventro-lateral lip.

To test this hypothesis, IGF-1 soaked beads were grafted into the forelimb at HH stage 17 embryos followed by 24 hours of incubation, and *Fgf18* expression measured by *in situ* hybridisation. IGF-1 upregulated *Fgf18* mRNA expression in 9\19 (Figure 7.3) while other embryos showed no effect (Figure 7.4), both compared to the non-grafted contralateral limb bud. However, this experiment needs further work to confirm this result. The results together show that IGFs play a pivotal role as stimulators of the early phases of myogenesis, and also offer insight into the mechanisms responsible for IGFs inducing skeletal muscle growth and development.

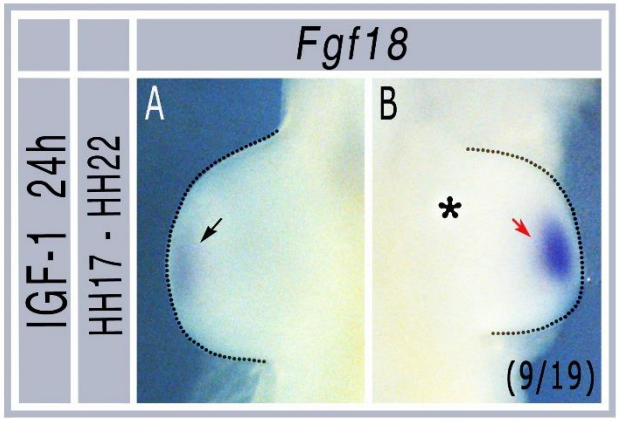


Figure 7. 3: The effect of IGF-1 on *Fgf18* expression. Ectopic IGF-1 stimulates the expression of *Fgf18* at HH stage 22 embryos limb buds. Beads were soaked with IGF-1 and then grafted into the level of the forelimb at HH stage 17 embryos, followed by 24 hours incubation to HH stage 22. IGF-1 induces the activation of *Fgf18* (B, red arrow) compared to the contralateral limb bud (A, black arrows). Black asterisk represents the position of IGF-1 soaked bead. Dotted black lines highlight limb buds. Magnifications: 20x

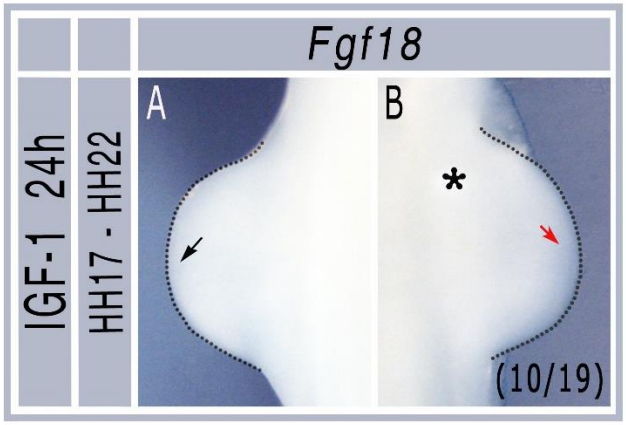


Figure 7. 4: The effect of IGF-1 on *Fgf18* expression. No change is seen on *Fgf18* expression induced by IGF-1 (B, red arrow) similar to that in the counterpart (black arrow). Black asterisk represents the position of IGF-1 soaked bead. Dotted black lines highlight limb buds. Magnifications: 20x

7.4 Summary

There has been a long history of debate about how the IGFs have a fundamental role in stimulating cellular growth, proliferation and differentiation (which is different to most other growth factors) (Engert et al., 1996, Coolican et al., 1997, Duan et al., 2010, Duclos, 2005, Erbay et al., 2003, Foulstone et al., 2001, Huat et al., 2014, Liu et al., 2015, Reckenbeil et al., 2017), given that in order to differentiate a cell must withdraw from the cell cycle while stimulation of cell proliferation normally inhibits cell differentiation. The data presented here suggests one way of resolving this contradiction that the effects of IGF on proliferation are direct but the ability to induce differentiation requires the induction of *Fgf18* in the surrounding limb bud mesenchyme.

7.5 Further work

Chapter 3 characterises the myogenic regulatory factors (Mrfs) at precise stages of chick limb during the development. Further investigations on the expression profile of other muscle markers, such as the myosin heavy chains (MHC) and *Pax7* in the older stages of chick limb will be essential areas for future research, utilising whole-mount *in situ* hybridisation and immunostaining.

Age-related study of limb myogenesis is vital not only to this project's goal, but also to general health concerns in human and animals; future analysis that will possibly make essential contributions to this field. Given the role of the Mrfs in transcriptional activation, future work could also concentrate on identifying the role of *Mrf4.* As this gene acts as a differentiation regulator, but was not expressed at early stages in the developing limb. *In vitro* studies will also be needed to understand the co-ordinated role of the MyoD family, and to show the mechanistic intercellular changes which occur for MRF proteins.

The results demonstrated in chapter 4 identified the expression of IGF axis in the developing chick limb between HH 20 to 26, of Hamburger and Hamilton staging series. These data showed that all IGF system members, except *IGF-2R*, have precise spatio-temporal expression patterns. Future characterisation study on the older stages of chick limb will also be needed

to elucidate how IGFs are involved in ongoing limb development. Little is known regarding the localisation of IGF-2R during the late stage of limb development, and this will be further analysis and study. In the future researches, it may well be necessary to elucidate the interactions between IGF axis during limb development and effects on apoptosis.

Chapter 5 showed the effect of IGF-1 and IGF-2 signalling on the limb muscle by the inducing of myogenic transcription factors. An important aim for further study includes resolving the interaction between IGF and *Myf5* in limb skeletal muscle. This will give a much more complete picture of IGF function during muscle proliferation and differentiation. As the chick limb is easy to manipulate, and since IGFs induced *Myog*, the later myogenic differentiation regulator, further work will test whether grafting of IGF soaked beads target *Mrf4* or not. Future research could also focus on the effects of IGFs on somite expression of myogenic regulatory factors, because the myogenic regulatory factors were characterised in somites during chick embryogenesis (Mok et al., 2015). Other further work will also examine whether IGF can regulate tyrosine kinase receptor *c-met*, as a regulator for migration of myogenic progenitors into the limb (Tajbakhsh and Buckingham, 2000).

In vivo and *in vitro* studies have demonstrated that IGF-1 induces myoblast expansion and the development of myofiber in the limb (Gardner et al., 2015, Mitchell et al., 2002, Yu et al., 2015), future work will hopefully shed light on the effect of IGF-2 signals that differentially regulate the different phases of muscle development. This is based on the result that we have obtained when IGF-2 upregulated *Myog* expression after long term of incubation (5 days) (see Figure 7.5, page 153). This data may well be novel in the field of muscle research. However it needs further experiments using immunostaining and *in situ* hybridisation analysis and probably targeting gene. Further work also needs to investigate the effect of IGF-2 on satellite cells, as these cells increase muscle regeneration and hypertrophy induced by IGF-1 (Barton-Davis et al., 1999). Because *Pax7* might also be useful as it should mark future satellite cells, the role of IGFs needs further characterising, particularly their *in vivo* and *in vitro* effects on *Pax7* and MHC. In addition, most studies have demonstrated that IGF-1 can

significantly increase bone resorption formation (Guntur and Rosen, 2013, Locatelli and Bianchi, 2014, Yakar et al., 2002), but how IGF-2 specifically affects bone muscle growth and development is largely unknown. Thus, determining the IGF-2 full range that controls osteogenesis from embryonic to adult will be a new area for future research.

Results of chapter 6 showed how IGF signal is involved in limb myogenesis via ERK\MAPK pathway. The data of the present study also showed that IGF-1R mediates IGF-1 and IGF-2 signalling that induced *Pax3* and *MyoD* transcripts, thus testing the later myogenic marker (*Myog*) is an area for further experiments. Furthermore, not much is known about the possible correlation between IGF-ligands and the type 2 receptor during limb muscle development; this will be an interesting area for further experiment. The identification of IGF-2R interactions is important for understanding the mechanical action of IGF-2R, and may well lead to development of specific therapies against cancer in the future. Further study is still needed to elucidate the interactions between IGF-1/IGF-2 and Akt pathway during early myogenesis.

Since the present study hypothesised that IGFs indirectly upregulate *MyoD* expression in response to *FGF* via ERK, it will be necessary for future work to focus on elucidating whether *Fgf4*, *Fgf8*, *Fgf18* or another signal altogether, maintains the populations of myogenic precursors. This step may well also be confirmed by applying other modern technique, such as CRISPR/Cas9 as a system for Knockout gene used in chicken somatic cells during embryogenesis. Knockout of FGFR probably help to understand and confirm the indirectly role of IGFs in stimulating *MyoD* expression. Future study will also focus on treating mesenchymal cells of chick limb bud with virus, such as RCAS or bacteria. This may well develop a new vaccine and then play a crucial role in immunity. Another important study for future is direct transfection and injection limb bud by gonocytes or cultured PGCs *in vivo*. This probably results in obtaining genes knockouts and then disruption of chicken gene, since few studies are available on this technique. Important aims for further work include *in vitro* clarification of how IGF signals influence the cellular downstream effectors, and how they differentially regulate the different phases of muscle development. This

may offer a novel method to manipulate IGF for regulation of muscle development. As some studies showed increases in the number of myoblast and myotubes stimulated by IGFs (Jacquemin et al., 2004, Yu et al., 2015). It will be important for future study to test other markers of proliferation, which marks cells for longer, such as BrdU incorporation. This because that the present study did not detect a significant number in Pax3-mitotic cells. BrdU may also help to investigate whthere IGF-1 or IGF-2 can stimulate premature satellite cell.

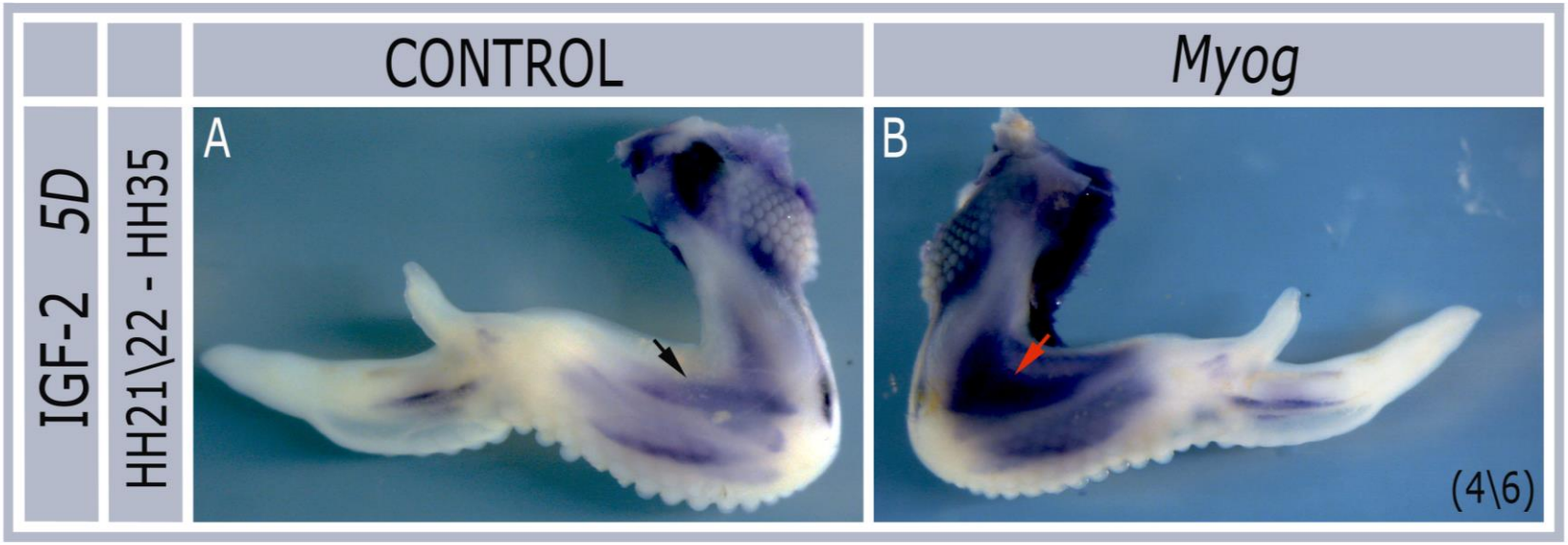


Figure 7. 5 The effect of IGF-2 on myogenic differentiation factor after 5 days of incubation. IGF-2 soaked beads were grafted into the forelimb between HH stages 21 to 22 and embryos were harvested at HH stage 35. IGF-2 stimulates the activation of *Myog* (B, red arrow) compared to the non-grafted contralateral limb bud (A, black arrows). Magnifications: 16x

Chapter 8: References

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