

MOLECULAR MECHANISMS OF MYOGENESIS IN STEM CELLS

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ABSTRACT

Embryonic stem cells (ESCs) represent a promising source of cells for cell replacement therapy in the context of muscle diseases; however, before ESC-based cell therapy can be translated to the clinic, we must learn to modulate cell-fate decisions in order to maximize the yield of myocytes from this systems. In order to gain a better understanding of the myogenic cell fate, we sought to define the molecular mechanisms underlying the specification and differentiation of ESCs into cardiac and skeletal muscle. More specifically, the central hypothesis of the thesis is that myogenic signalling cascades modulate cell fate via regulation of transcription factors.

Retinoic acid (RA) is known to promote skeletal myogenesis, however the molecular basis for this remains unknown. We showed that RA expands the premyogenic progenitor population in mouse stem cells by directly activating pro-myogenic transcription factors such as Pax3 and Meox1. RA also acts indirectly by activating the pro-myogenic Wnt signalling cascade while simultaneously inhibiting the anti-myogenic influence of BMP4. This ultimately resulted in a significant enhancement of skeletal myogenesis. Furthermore, we showed that this effect was conserved in human embryonic stem cells, with implications for directed differentiation and cell therapy.

The regulation of cardiomyogenesis by the Wnt pathway was also investigated. We identified a novel interaction between the cardiomyogenic transcription factor Nkx2.5 and the myosin phosphatase (MP) enzyme complex. Interaction with MP resulted in exclusion of Nkx2.5 from the nucleus and inhibition of its transcriptional

activity. Finally, we showed that this interaction was modulated by phosphorylation of the Mypt1 subunit of MP by ROCK, downstream of Wnt3a. Treatment of differentiating mouse ESCs with Wnt3a resulted in exclusion of Nkx2.5 from the nucleus and a subsequent failure to undergo terminal differentiation into cardiomyocytes. This likely represents part of the molecular basis for Wnt-mediated inhibition of terminal differentiation of cardiomyocytes. Taken together, our results provide novel insight into the relationship between myogenic signalling cascades and downstream transcription factors and into how they function together to orchestrate the myogenic cell fate in stem cells.

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LIST OF ABBREVIATIONS

ANF	Atrial natriuretic factor
ATCC	American Type Culture Collection
bHLH	Basic Helix-Loop-Helix
BMP	Bone Morphogenetic Protein
cDNA	Complementary deoxyribonucleic acid
Cx40	Connexin 40
Cyp26	Cytochrome P450 26
d.p.c.	Days post coitum
DCX	Doublecortin
DKK	Dickkopf
DML	Dorsal-medial lip
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dsh	Dishevelled
E	Embryonic Day
EC	Embryonal Carcinoma
EnR	Engrailed
epiSCs	Epiblast Stem cells
FGF	Fibroblast Growth Factor

FHF	First heart field
GSK	Glycogen synthase kinase
h	Human
HDAC	Histone Deacetylase
HES	Hairy and Enhancer of Split
hESCs	Human Embryonic Stem Cells
HMG	High mobility group
iPSCs	Induced Pluripotent Stem Cells
JNK	c-Jun N terminal kinase
Lef	Lymphoid enhancer factor
LIF	Leukaemia Inhibitory Factor
MAPK	Mitogen-Activated Protein Kinase
mESCs	Mouse Embryonic Stem Cells
MLC	Myosin light chain
MLCK	Myosin Light Chain Kinase
MP	Myosin phosphatase
MRF	Myogenic Regulatory Factor
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
MyHC	Myosin Heavy Chain
NFAT	Nuclear Factor of Activated T cells
PBS	Phosphate buffered saline

PGK	Phosphoglycerate kinase
PKC	Protein Kinase C
PP1	Protein Phosphatase 1
PSM	Presomitic Mesoderm
Puro	Puromycin
QPCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
ROCK	Rho Associated Coiled-coil forming serine/threonine kinase
RXR	Retinoid X Receptor
SHF	Second heart field
Shh	Sonic hedgehog
TAP	Tandem Affinity Purification
TCF	T cell factor
Thr	Threonine
VLL	Ventra-lateral lip

CONTRIBUTIONS OF COLLABORATORS

Some of the content of this thesis is from published or accepted manuscripts. The author contributions are as follows:

In Chapter 2 the P19[β CatEnR] and P19[BMP4] cell lines were generated by Helen Petropoulos and Christina Karamboulas respectively. Karen Kennedy performed the P19 RA and P19[BMP4] differentiations as well as the associated Northern Blots and cell counts. I performed the mESC differentiations as well as the associated gene expression analysis. I also performed the ChIP experiments and the associated *in silico* analysis to define potential RAREs. I performed the differentiations for the analysis of neurogenesis shown in Figure 5 and I performed all luciferase reporter experiments.

Virja Mehta differentiated the P19[β CatEnR] cells and performed the immunofluorescence shown in figure 3 as well as the Northern blots in figure 4 and all associated QPCR analyses. Scott Ryan performed the immunofluorescence and quantification of neurogenesis in Figure 5. Feodor Price helped with the mESC culture. Vian Peshdary assisted with the quantification of neurogenesis in Figure 5. Josee Savage carried out the microarray analysis of RA treated cells, identifying Tob-1. Thomas Drysdale participated in the interpretation of data for RA treated cells and the role of Tob-1 and revising the manuscript. Shun-Cheng Li contributed to providing lab space and reagents for acquisition of Q-PCR data. Steffany Bennett conceived and designed the neurogenesis analysis

In Chapter 3, I performed most of the hESC differentiations and the associated QPCR analysis, immunofluorescence/quantification. Jun Liu performed most of the hESC maintenance as well as some of the differentiations and expression analysis. Jun Liu also contributed to the MyHC immunofluorescence. Alphonse Chu and Alexandre Blais assisted with microarray analyses while Lisheng Wang assisted with hESC culture.

In Chapter 4 the P19[Nkx2.5TAP] and control cell lines were generated by Sophie Boisvenue. I performed the initial affinity purifications while Jean-Phillipe Lambert performed the mass spectrometry and related analyses. I subsequently performed all co-immunoprecipitation experiments, reporter assays, localization experiments and related quantifications. I also performed the mESC/Wnt3a differentiations as well as the associated gene expression analysis and immunofluorescence. Michael Shelton performed the quantification of Mlc2v^{+ve} cells in Figure 6. Daniel Figeys provided facilities and resources for all mass spectrometry experiments.

The manuscripts for chapters 3 and 4 were written by me with assistance from Ilona Skerjanc. The manuscript for chapter 2 was written by Karen Kennedy with assistance from Ilona Skerjanc and myself.

1: INTRODUCTION

Regenerative medicine currently represents an exciting therapeutic avenue for a multitude of intractable human diseases including Parkinson's, muscular dystrophy, leukemia and heart disease (1-6). The promise of stem cell therapy derives from the possibility of replacing diseased or damaged cells with healthy, functional tissue in lieu of long term pharmacological interventions that may only mediate symptoms without having a significant positive impact on patient quality of life. With respect to diseases of muscle, cell therapy is a real therapeutic possibility for muscular dystrophy, sarcopenia, cardiomyopathy and chronic heart failure (2, 3, 7-9). Many different types of cells have been studied for their therapeutic potential, including human embryonic stem cells, (hESCs), adult stem cells and induced pluripotent stem cells (iPSCs) but the question remains: which cells are the 'right' cells? Furthermore, at what stage of differentiation should cells be transplanted i.e. is it better to transplant a progenitor or a terminally differentiated cell? Finally, how do we generate sufficient yields of these cells for therapeutic purposes? In order to begin to address these questions, we must first understand and learn to modulate the pathways that govern muscle differentiation from stem cells. Knowledge gained from studies of embryonic muscle development can point us in the right direction.

1.1 Skeletal Myogenesis

1.1.1 Somite formation and patterning

Skeletal muscle in the embryo originates from paraxial mesoderm, located laterally to the neural tube. As development progresses, the paraxial mesoderm becomes segmented into epithelial block-like structures known as somites in a rostral to caudal pattern.

Somitogenesis occurs in a strict periodic fashion in response to morphogen gradients. FGF

and Wnt in the posterior portion of the presomitic mesoderm (PSM) antagonize somitogenesis while retinoic acid (RA) in the anterior PSM promotes epithelisation and somite segmentation (10-15). Within the PSM, FGF and Wnt are synthesized only in the most posterior region, such that as the body axis elongates posteriorly, the concentration of these ligands decreases in more anterior regions, resulting in downregulation of their downstream targets (14, 16). This transition from exposure to high FGF/Wnt to high RA results in differentiation. Indeed, inappropriate exposure of PSM cells to continuously high FGF levels blocks somite segmentation (12). The region of the PSM where these two gradients meet is referred to as the determination front (12). The determination front progresses posteriorly as the axis elongates and FGF/Wnt mRNA undergoes progressive decay, and cells located anterior to this position undergo a mesenchymal to epithelial transition. However, the actual segmentation of the somite from the PSM also relies on the action of a molecular clock that is triggered by Notch and Wnt signalling (17). Many members of the Notch pathway, including delta, the Hairy and Enhancer of split (HES) transcription factors, *hes1* and *hes7*, and the glycosyl-transferase *lunatic fringe* exhibit temporal pulses of expression pattern throughout the PSM (18-22). The Wnt signalling inhibitor *axin2* also cycles in the PSM, albeit out of phase with Notch pathway members (14). These temporal pulses represent the oscillations of a molecular clock, the output of which is integrated with the determination front to position somite boundaries. Specifically, when the determination front reaches a given position within the PSM, the cells become competent to respond to the stimulus of the 'clock'. During somitogenesis, the determination front progresses posteriorly by the length of one somite, in the time it takes for one oscillation of the clock, thus positioning the somite boundary at the point where the

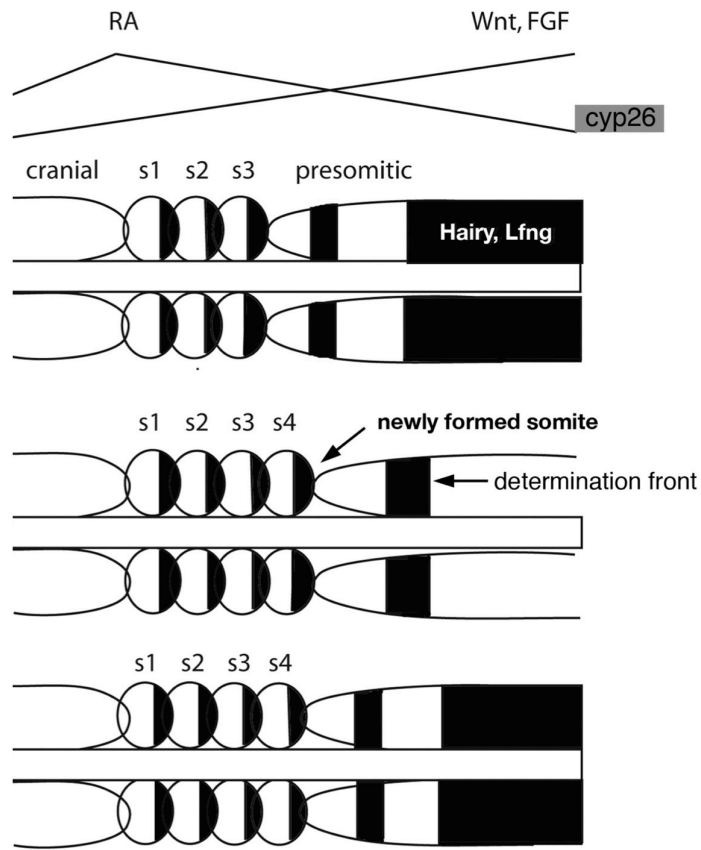
determination front and the oscillation meet. This is referred to as the clock and wavefront model of somite segmentation (Figure 1.1A).

Once the somite has budded off from the PSM, it begins a process of internal patterning (Figure 1.1B). Initially, the dorsal and ventral portions of the somite become distinct and form the dermomyotome and the sclerotome respectively. While the sclerotome will eventually form the ribs and vertebrae, the dermomyotome will segment further to form the dermatome, which will give rise to the dermis of the back, and the myotome, which will become a source of skeletal muscle. The myotome forms via translocation of cells from the dorsal-medial and ventral-lateral lips of the dermomyotome (23-26). Cells from the dorsal-medial lip will give rise to the epaxial muscles of the back and body wall while cells coming from the ventral-lateral lip will migrate and form the hypaxial muscles of the limbs. A subpopulation of cells will remain undifferentiated and proliferative and assume a position beneath the muscle basal lamina to establish the satellite cell niche, which represents the future reservoir of muscle stem cells, termed satellite cells, that will participate in regeneration (27).

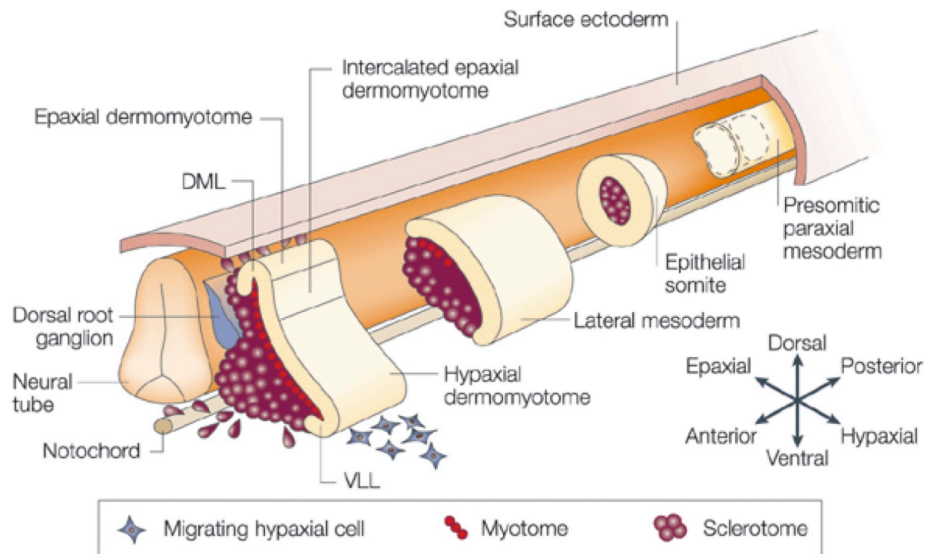
These gross anatomical movements, as well as the differentiation of skeletal myocytes, are tightly coordinated and controlled by a network of morphogens and transcription factors that ensure timely development of body muscle. These will be discussed in detail in the following sections.

Figure 1.1 Skeletal myogenesis in the embryo. A) Schematic depiction of the clock-and-wavefront model of somitogenesis. The intersection of opposing gradients of anterior RA and posterior Wnt/FGF, known as the determination front, establishes the positional information required for formation of new somites from the PSM. Reproduced from Schilling, T.F., Anterior-posterior patterning and segmentation of the vertebrate head, *Integrative and Comparative Biology*, 2008, 48 (5):658-667, by permission of Oxford University Press B) Vertebrate skeletal muscle arises from somites, which form adjacent to the neural tube. As development progresses, the somite undergoes a process of internal patterning which results in the formation of the dermomyotome and, subsequently, the myotome. The myotome forms as a result of migration of cells from the dorsal-medial and ventral-lateral lips of the dermomyotome. Cells from the dorsal-medial lip will give rise to the epaxial muscles of the back and body wall while cells coming from the ventral-lateral lip will migrate and form the hypaxial muscles of the limbs. Reprinted by permission from Macmillan Publishers Ltd: Parker, M.H., Seale, P., Rudnicki, M.A., Looking back to the embryo: defining transcriptional networks in adult myogenesis. *Nature Reviews Genetics*, 2003; 4(7):497-507, Copyright, 2003.

A



B



1.1.2 Transcriptional Regulation of Myogenesis

1.1.2.1 The Myogenic Regulatory Factors

The myogenic regulatory factors (MRF) family is composed of four basic helix loop helix (bHLH) transcription factors, Myf5, MyoD, MRF4 and myogenin, whose potent myogenic properties are evidenced by their ability to convert other cell types into skeletal muscle (28-31). The MRFs bind to E-box target sequences as monomers or as heterodimers in complex with E-proteins and activate muscle gene transcription by coordinating chromatin rearrangements in concert with transcriptional coactivators. The functions of the four MRF proteins have been extensively examined using gene targeting and these studies have revealed a complex and somewhat redundant network controlling myogenesis.

Myf5 is the first MRF to be expressed in the developing embryo and it can be detected as early as stage 3 in the PSM of developing chick embryos or 8.0 days post coitum(d.p.c.) in the mouse somite (32, 33). The somitic expression domain of Myf5 includes the dermomyotome and both the dorsal-medial lip and ventral-lateral lip regions where it plays a role in progenitor migration into the myotome and specification of the myogenic fate (34, 35). In the absence of Myf5, a subset of progenitor cells migrate abnormally to sites within the sclerotome and dermomyotome where they ultimately adopt fates specific to these regions. In spite of this, Myf5 is dispensable for normal myotomal muscle development and activation of MyoD expression, which is thought to rescue the myogenic program in the absence of Myf5 (36, 37). Myf5 null mice do however exhibit defects of epaxial muscle (37).

Like Myf5, MyoD is dispensable for normal embryonic muscle development, however, MyoD null mice exhibit elevated levels of Myf5, which is thought to compensate and rescue myogenesis in this context (38). Interestingly, loss of MyoD does impact on adult muscle regeneration as MyoD mutant mice exhibit impaired regeneration in response to injury, which results from a propensity of MyoD(-/-) satellite cells to undergo self-renewal at the expense of terminal differentiation (39, 40). A recent high-throughput study aimed at identifying MyoD binding sites revealed, unexpectedly, that MyoD was in fact enriched at thousands of sites within the genome and that binding was associated with increased histone acetylation at these sites (41). This implies that MyoD has the potential to exert wide-ranging changes to the epigenome of a cell, resulting in reprogramming to the myogenic lineage.

Until recently, Mrf4 was regarded as being important primarily for terminal differentiation. The Mrf4 knockout mouse exhibited only minimal muscle defects, likely due to compensation by elevated levels of myogenin (42). However, retrospective analysis of the original Myf5/MyoD compound mutant mouse, which exhibited a complete lack of skeletal muscle, revealed that Mrf4 expression was also disrupted as a result of the targeting strategy and the proximity of Mrf4 and Myf5 (37, 43). Further analysis of the relationship between these three genes revealed that Mrf4 was sufficient to maintain myogenesis in the absence of Myf5 and MyoD (37). This challenged the traditional view of MyoD and Myf5 as determination genes functioning upstream of Mrf4 and myogenin driving terminal differentiation and revealed a role for Mrf4 in the specification of the myogenic fate.

Myogenin is the second MRF to be activated in the embryo, after Myf5, which is thought to regulate its expression (44, 45). Interestingly, although myogenin mRNA is detected as early as 8.5 d.p.c. in the mouse, the protein product is not detected until 10.5

d.p.c., implying some level of post-transcriptional regulation or protein instability (44, 45). Despite its early expression pattern, myogenin is thought to be primarily important for the terminal differentiation of skeletal muscle. Myogenin null mice form myocytes normally but exhibit a fusion defect whereby myotubes are not formed and mice die perinatally (46, 47). Interestingly, when myogenin was knocked in to the *Myf5* locus in the original *Myf5/MyoD* compound null mouse (which is in fact also deficient in *Mrf4*), it was able to compensate to some extent for the loss of these factors, indicating the myogenin has some ability to direct cells into the myogenic lineage, albeit less efficiently than *Myf5* (48). This is in contrast to rib development, for which *Myf5* and myogenin were functionally interchangeable (49).

Although genetic analysis of the MRFs has provided a wealth of knowledge that has improved our understanding of muscle development, it has also revealed that the interplay between these factors results in a complex regulatory network of gene activation and repression controlling myogenesis that we are only beginning to comprehend.

1.1.2.2 The Pax family in Myogenesis: Pax3 and Pax7

Upstream of the MRFs in skeletal myogenesis are members of the Pax family of transcription factors, which have also been implicated in many other developmental processes (50). They are characterized by their paired box domain and paired-type homeodomain and are classified into groups based on structural characteristics and similarity of expression patterns. *Pax3* and *Pax7* are expressed in developing muscle, and are involved in the formation of both trunk and limb muscles, in addition to satellite cell specification.

Expression of *Pax3* is initiated first in the PSM and becomes progressively limited to the dermomyotome as the somite becomes patterned (51). The domain of *Pax3* expression extends to the extremities of the dermomyotome where it controls the differentiation of

hypaxial muscle as well as the delamination and migration of limb muscle progenitors (51-53). Consistent with this, *plotch* mutant mice, which are deficient in Pax3, exhibit a lack of limb and diaphragm muscle in addition to defects related to neural tube development (52, 54-57). Pax7 expression is initiated later than Pax3 in the dermomyotome and is dispensable for embryonic myogenesis (58, 59). Pax3 regulates expression of the MRF during myogenesis, however, to date, only Myf5 has been identified as a direct target regulated by binding of Pax3 to an enhancer controlling expression in limb progenitor cells (60, 61). In Myf5/Mrf4 null mice, MyoD expression is maintained, however, in Myf5/MRF4/Pax3 triple mutant mice, MyoD expression is lost, implying that Pax3 is upstream of MyoD and is required for its activation in the absence of other MRFs. Furthermore, forced expression of Pax3 leads to MRF activation in embryonic tissues and in pluripotent cells (62, 63).

Recently, a Pax3/7 positive/MRF negative progenitor population was identified in skeletal muscle (64). This population remained proliferative throughout development and contributed to the developing muscle. Moreover, in later development, these cells assumed a sublaminar position adjacent to the muscle fibre, implying that this population may in fact be a developmental source of satellite cells. Genetic ablation of both Pax3 and Pax7 resulted in a loss of skeletal muscle, with the exception of the early muscle of the myotome. These findings highlight the importance of Pax3/7 but they also indicate some level of compensation between these two factors resulting in the milder phenotypes of the Pax3 and Pax7 single knockout mice (52, 59).

In addition to their role in embryonic myogenesis, Pax3 and Pax7 are also involved in regulation of satellite cell maintenance and function (65). Although its activity is dispensable for embryonic myogenesis in the presence of Pax3, Pax7 is important for survival of satellite cells (65). Interestingly however, it appears that both Pax3 and Pax7 are

dispensable for adult muscle regeneration. Loss of Pax7 or Pax3 expression in adult mice has no impact on the ability of these mice to regenerate in response to injury, implying that while Pax7 is important for establishing the satellite cell pool, its activity is not important for self-renewal or differentiation of these cells in the adult (66).

1.1.2.3 The Meox Family: Meox1 and Meox2

Meox1 and Meox2 are homeodomain transcription factors that are expressed in the developing somite (67, 68). Meox1 expression is initiated in the PSM and later becomes segregated to the dermomyotome whereas Meox2 expression is initiated later in the developing limb bud (69). Consistent with this, Meox1 null mice exhibit sclerotomal defects in the form of fused ribs and vertebrae while Meox2 deficient mice exhibit defective limb muscle development accompanied by a loss of Pax3 and Myf5 expression (70, 71). Meox1/2 compound null mice exhibit a much more severe phenotype characterized by a loss of almost all skeletal muscles, implying that, like Pax3/7, some level of compensation exists between these two factors (71). A role for Meox factors in myogenesis was further supported by work in P19 embryonal carcinoma cells, which showed that a dominant negative Meox factor abolished skeletal myogenesis in this system (72). Interestingly, Meox factors appear to exert their influence in concert with Pax factors. Yeast two hybrid studies revealed that Meox1 and Meox2 can interact directly with Pax1 and Pax3 respectively via the Meox homeodomain (73). Although these interactions were demonstrated *in vitro*, it is tempting to speculate that Pax3 and Meox2 may cooperate to direct limb muscle development *in vivo* given the requirement of each factor for this process.

1.1.3 Signalling Pathways Impacting on Myogenesis

1.1.3.1 Wnt

Wnt ligands secreted from the neural tube, adjacent to the medial portion of the somite, are critical determinants of the myogenic lineage (74). Wnt ligands signal through three distinct pathways, the canonical pathway which functions through β -catenin, the Ca^{2+} pathway through Protein Kinase C, and the planar cell polarity pathway which utilizes the rac and rhoA pathways (Figure 1.2) (75). In the canonical pathway, Wnt factors bind to their Frizzled (Frz) receptors, resulting in activation of Dishevelled (Dsh), which in turn prevents phosphorylation of β -catenin by GSK3 β . This inhibits the proteasomal degradation of β -catenin, allowing it to translocate to the nucleus where it activates TCF/Lef dependent transcription of target genes. The Ca^{2+} pathway activates protein kinase C, as well as NFAT dependent gene transcription downstream of calcineurin. The target genes of this pathway remain largely unknown. The planar cell polarity pathway is involved in cytoskeletal reorganization and polarization of cells, rather than gene transcription. In this pathway Dsh activates rhoA and rac, resulting in activation of Rho-associated coiled coil-containing kinase (ROCK) and Jun N-terminal kinase (JNK). This in turn leads to reorganization of the cytoskeleton and also to activation of Ap1 dependent gene transcription.

In the context of myogenesis, Wnt ligands secreted from the neural tube exert a pro-myogenic effect on the adjacent somites. Specifically, Wnt1, Wnt3 and Wnt4, which are expressed in the dorsal part of the neural tube, are able to induce myogenesis in somite explants (76). Interestingly, the action of specific Wnt ligands appears to be tied to the activation of specific downstream targets. Wnt1 secreted from the neural tube preferentially activates Myf5, which is a direct downstream target, while Wnt7a emanating from the dorsal

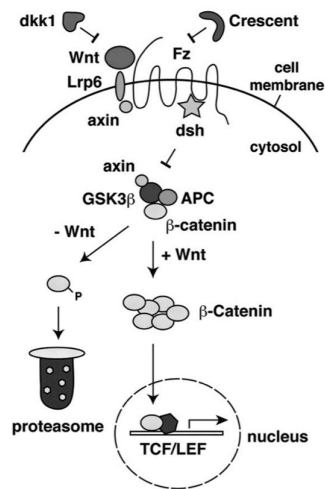
ectoderm induces expression of MyoD (77, 78). Wnt signalling acts in cooperation with Sonic Hedgehog signalling to activate Myf5 expression via binding of β -catenin/Tcf-Lef to its epaxial enhancer (78). Consistent with this, transplacental delivery of the Wnt antagonist Frz1b inhibits skeletal myogenesis and results in downregulation of Myf5, however, the expression of the premyogenic mesoderm genes Pax3 and Meox1 was unaffected, suggesting that Wnts are required for commitment and terminal differentiation of muscle, but not for specification (79).

Results from *in vitro* models support the pro-myogenic influence of Wnt/ β -catenin signalling. Treatment of rat mesenchymal stem cells with Wnt3a conditioned medium induced skeletal myogenesis (80). In P19 embryonal carcinoma cells, overexpression of β -catenin induces skeletal myogenesis while expression of a dominant negative form of β -catenin blocks myogenic differentiation, implying that β -catenin is both necessary and sufficient for myogenesis to occur (81). Furthermore, co-culture experiments of P19 cells overexpressing MRFs with P19 cells overexpressing Wnt3a resulted in enhanced myogenesis, suggesting that Wnt can precipitate MRF function in cells (82). In spite of the obvious importance of Wnt ligands for myogenesis, few direct targets of this pathway have been identified in developing muscle. In addition to Myf5, Wnts have been shown to directly regulate expression of Foxc1, a member of the forkhead/winged-helix family of transcription factors, which is involved in somitogenesis (83). Future work in this area should employ global genomic approaches, (such as CHIP-Seq) in an effort to better understand the downstream implications of Wnt signalling in skeletal myogenesis.

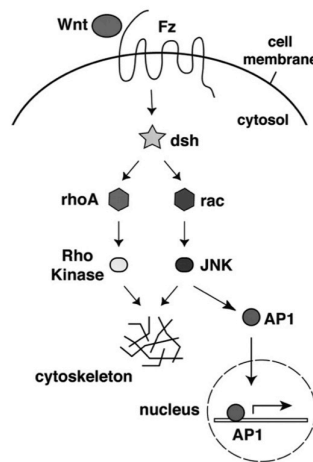
Figure 1.2 Mechanisms of Wnt Signalling. Wnt ligands can signal through three known pathways. A) The canonical pathway involves stabilization of β -catenin, downstream of GSK3 β inhibition, and results in activation of TCF/Lef dependent transcription. B) The non-canonical pathway involves mobilization of rhoA and rac downstream of Dsh, leading to activation of ROCK and JNK and ultimately to AP1 dependent transcription. This leads to reorganization of the cytoskeleton. C) The Wnt/Ca²⁺ pathway activates CamKII, PKC and Calcineurin signalling cascades and culminates in NFAT-dependent transcription.

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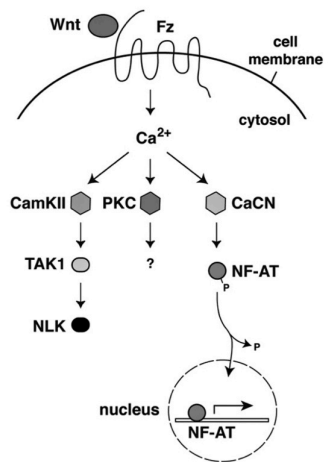
A Wnt / β -catenin pathway



B Wnt / JNK pathway



C Wnt / Ca²⁺ pathway



1.1.3.2 RA

RA is a derivative of vitamin A and plays a critical role in several developmental processes, including skeletal myogenesis (84). As such, its bioavailability is strictly regulated by RA synthesizing and degrading enzymes, such as RALDH2 and Cyp26 (Figure 1.3). RA exerts its effects by binding to its nuclear receptors (retinoic acid receptors [RARs]/ retinoid X receptors [RXRs]), which reside on retinoic acid response elements (RAREs) within the genome (85). Under RA-poor conditions, the receptors recruit transcriptional co-repressors SMRT or NCoR, which in turn recruit histone deacetylases that shut down chromatin at these sites to prevent gene transcription (86). In response to ligand binding, these co-repressors are exchanged for co-activators, resulting in activation of RA-responsive genes (Figure 1.4).

Single gene knockout analysis has not been particularly informative regarding the developmental role of RAR isoforms (α , β , γ), as most of these mice are viable and relatively normal (87-91). This is also the case for RXR β and RXR γ knockout mice, although loss of RXR α results in cardiac dysfunction during development and is lethal (92-94). Mice homozygous for loss of function of two of the RAR family members exhibit more significant phenotypes that recapitulate fetal vitamin A deficiency including abnormalities of the heart and the respiratory tract, as well as the urinary and digestive systems (95, 96). Despite the well characterized role of RA in somitogenesis, no somite-related defects were observed in RAR/RXR knockout mice (97). This may indicate a high level of compensation between receptors in this particular process.

Figure 1.3 The RA biogenesis pathway. Retinol is taken up from the extracellular environment by STRA6 and metabolized to all-trans-RA by RALDH enzymes. This results in activation of chromatin-associated RAR/RXR complexes. Alternatively, it is metabolized and eliminated by Cyp26 enzymes. The biological relevance of 9-cis-RA is not fully understood. Translated by permission from Macmillan Publishers Ltd: Niederreither K, Dollé P. Retinoic Acid in Development: towards an integrated view. *Nature Reviews Genetics*, 2008, 9(7):541-53 copyright 2008.

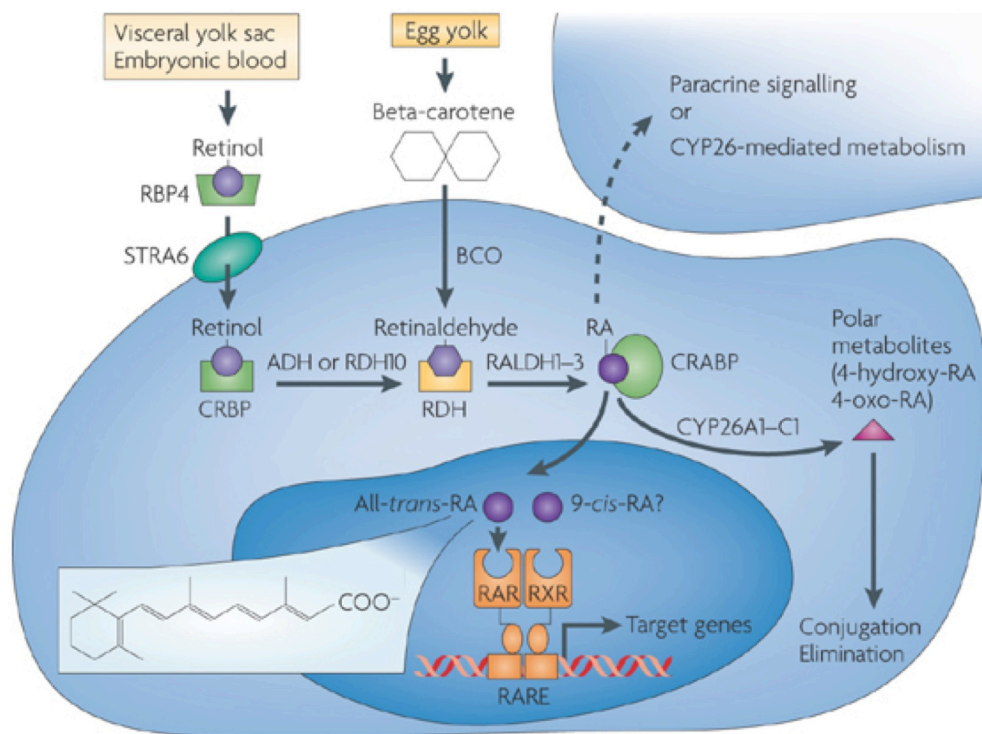
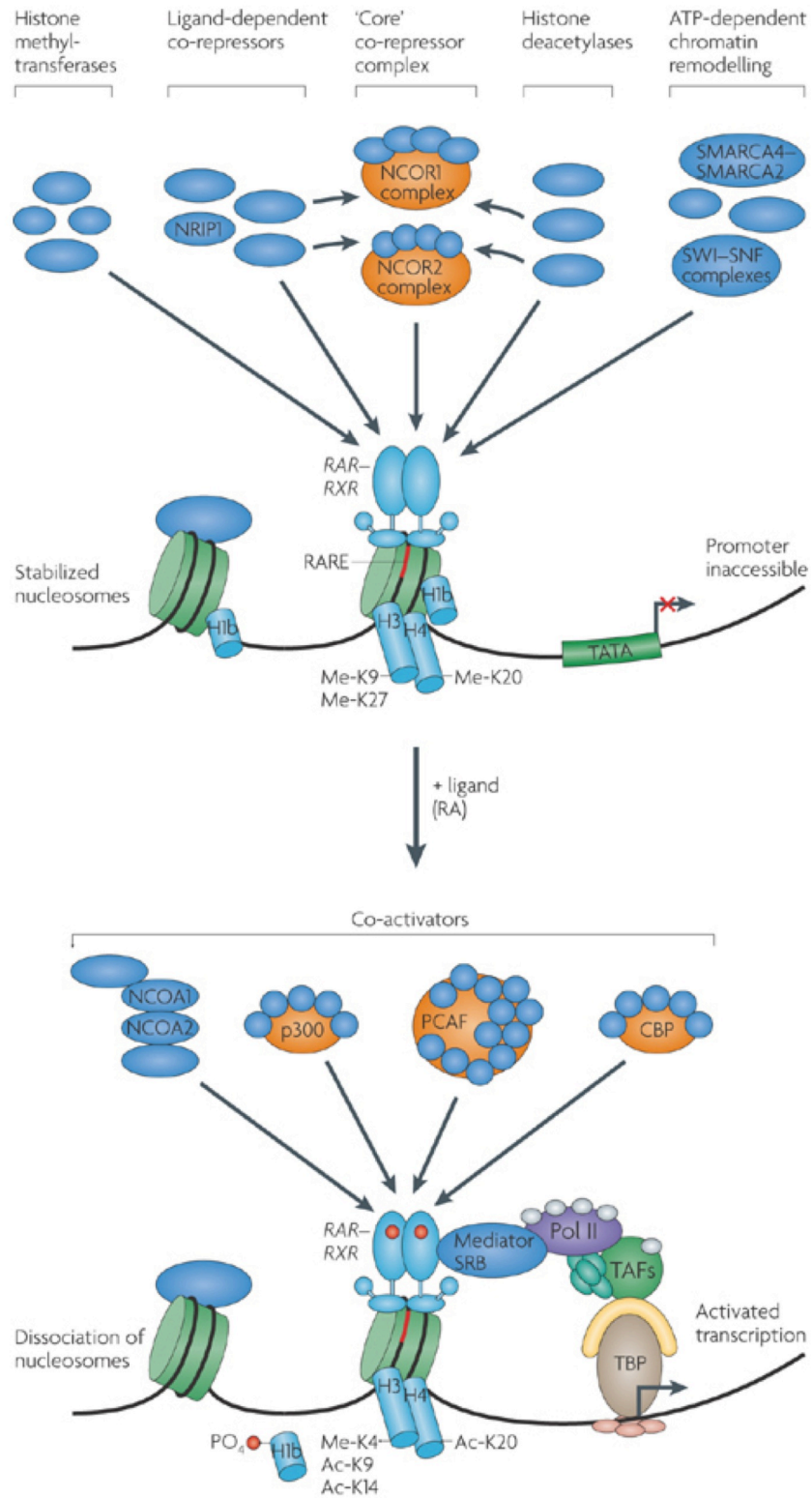


Figure 1.4. RAR/RXR-mediated chromatin remodelling. In the absence of RA ligand, chromatin-bound RAR/RXR complexes recruit transcriptional co-repressors such as NCoRs, resulting in stabilization of nucleosomes and inhibition of RA-responsive gene transcription. Alternatively, in the presence of RA, these co-repressors are exchanged for co-activators and gene transcription is initiated. Translated by permission from Macmillan Publishers Ltd: Niederreither K, Dollé P. Retinoic Acid in Development: towards an integrated view. *Nature Reviews Genetics*, 2008, 9(7):541-53 copyright 2008.



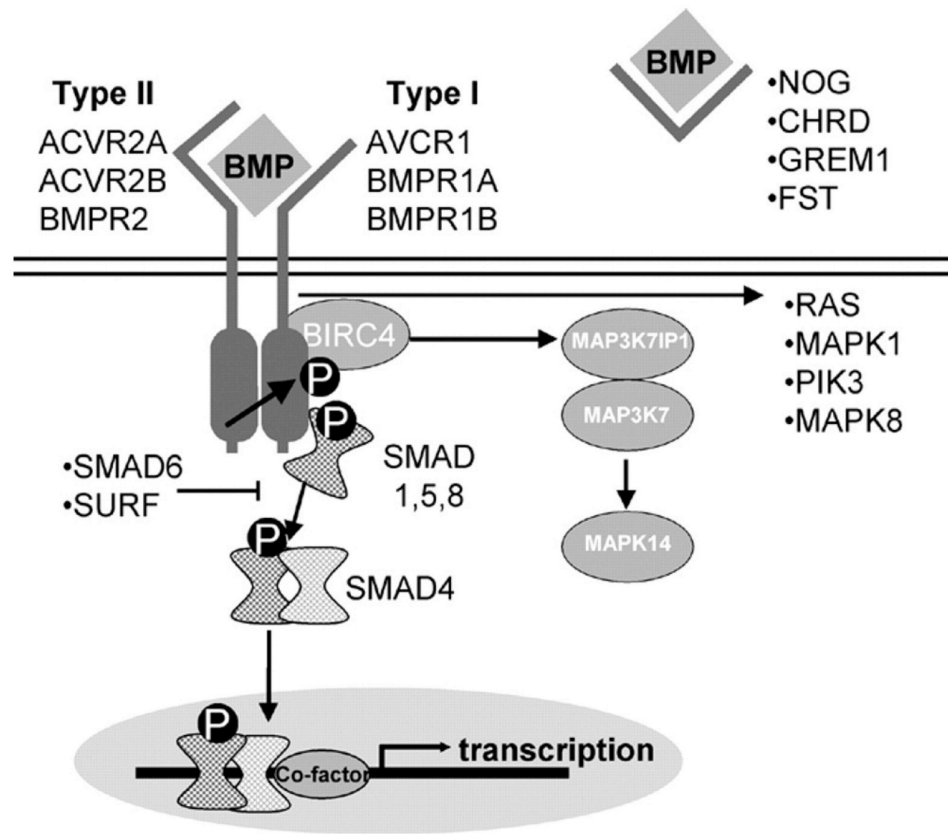
expression as well as by direct synergy between MRFs and RARs (99, 102, 103).

Interestingly, RARs and RXRs appear to play functionally distinct roles as expression of a dominant negative RXR in C2C12 myoblasts blocked expression of MyoD while expression of a dominant negative RAR did not (104). More recently, the role of RA in limb myogenesis was investigated and it was found that treatment of developing chick limbs with citral, which inhibits RA synthesis, resulted in downregulation of Meox1/2, Pax3, MyoD and Myf5 and ultimately an inhibition of myogenesis in this system (105). However, treatment of the developing limb with excess RA also resulted in downregulation of these same genes and an inhibition of myogenesis. These apparently conflicting results illustrate the complexity of RA signalling in developing muscle. Clearly the concentration and timing of exposure to this morphogen are critical determinants of cell fate. A detailed molecular analysis of RA signalling in myogenesis is required in order to better understand its role in this process.

1.1.3.3 Bone Morphogenetic Protein

Bone Morphogenic Proteins (BMPs) are members of the TGF- β family of growth factors and were named for their ability to induce the formation of ectopic bone within muscle tissue (106, 107). In addition to their role in osteogenesis, BMPs have been implicated in chondrogenesis, cardiac and skeletal myogenesis, as well as adipogenesis (108). BMP ligands signal by binding and activating their receptor serine/threonine kinases, resulting in phosphorylation of Smad1/5/8 proteins, which translocate to the nucleus and activate downstream transcription (Figure 1.5) (109).

Figure 1.5 The BMP signalling pathway. BMP ligands bind to their serine/threonine kinase receptors, resulting in their activation and downstream phosphorylation of SMAD proteins. Activated SMADS translocate to the nucleus and activate gene transcription. BMP signalling is blocked by inhibitors such as Noggin (NOG), Chordin (CHRD), Gremlin (GREM1) and Follistatin (FST). Reproduced from van Wijk, B., Moorman, A.F.M., van den Hoff, M.J.B., Role of bone morphogenetic proteins in cardiac differentiation, *Cardiovascular Research*, 2007 74(2):244-55 by permission of Oxford University Press.



The role of BMP/Smad signalling in skeletal myogenesis is extremely complex. In the chick, BMP-4 secreted from the lateral plate mesoderm acts as a lateralizing signal, inducing cells to express the hypaxial marker Sim1 while inhibiting the expression of MyoD and as a result, terminal differentiation of muscle (110). However, BMP signals derived from the dorsal neural tube direct activation of Wnt1 and Wnt3a, resulting in activation of Wnt11 in the medial lip, which is important for migration of progenitor cells into the myotome (111). BMP/Smad signalling inhibits myogenesis in myoblast cell lines and in limb micromass cultures (112-114). Furthermore, Id proteins, which are known to inhibit myogenesis by antagonizing MRF function, are direct targets of BMP/Smad signalling and likely contribute to the anti-myogenic effects of BMP (115-119).

The positional specificity of BMP signalling is, to some extent, conferred by the presence of BMP inhibitors such as Noggin. Noggin inhibits BMP signalling by binding it directly and preventing activation of the receptor. As noted above, BMP signalling is important for mediating expression of Wnt11 in the medial lip via Wnt1 and Wnt3a, however, BMP signals are prevented from actually reaching the medial lip by the presence of Noggin in the somite, which restricts the domain of BMP activity (111). Noggin signalling is believed to counteract the inhibitory effects of BMP4 on the epaxial somite (120-122). Experiments using somite explants showed that relative levels of BMP4 and noggin regulated the activity of Pax3 to control the temporal and spatial activation of the MRFs (123).

The BMP signalling network has been shown to engage in cross-talk with components of other signalling pathways during development, including the Notch, MAPK, Wnt, FGF, RA and sonic hedgehog pathways, all of which have been implicated in some.

aspect of muscle development (124, 125). However, how these cross-talk events actually contribute to myogenesis remains to be examined.

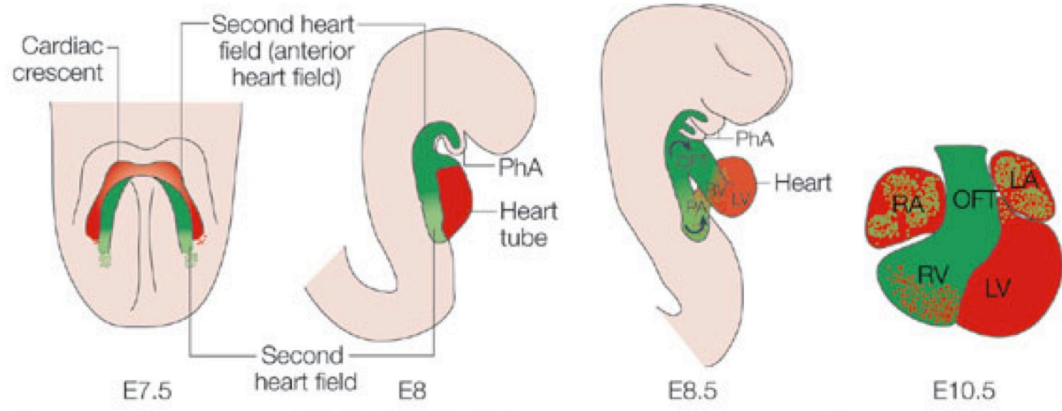
1.2 Cardiomyogenesis

1.2.1 Morphogenesis of the Vertebrate Heart

The heart is the first organ to form during embryonic development in vertebrates (126). Like skeletal muscle, the cells that will ultimately contribute to the vertebrate heart originate from the mesodermal germ layer. These progenitors, which are not yet committed to the cardiac fate, leave the primitive streak and migrate in an anterior-lateral direction to form a crescent-shaped structure below the head folds of the embryo. As development progresses, the two sides of this crescent will fuse at the embryonic midline to form the linear heart tube. This tube will undergo a series of morphogenetic movements involving looping and septation, which will give rise to the mature, four chambered heart (Figure 1.6).

Until recently, it was thought that the cardiac crescent contained a single source of progenitor cells, which gave rise to all portions of the mature heart. It has since been shown that two distinct populations of progenitors exist, termed the primary and secondary heart fields, where the secondary heart field is positioned medially to the primary heart field at the crescent stage (126). Later, as the heart tube forms, the secondary heart field is located anteriorly and dorsally to the tube. The primary heart field lineage contributes to both atria, the left ventricle, and to a lesser extent, the right ventricle (126). Gene trapping experiments using an FGF10-LacZ transgene, which marks the secondary heart field, revealed that this population is highly proliferative, differentiates later than the primary heart field, and contributes to the outflow tract and to the right ventricle of the heart (127, 128). As is the case with skeletal myogenesis, the proliferation and differentiation of cardiac progenitors, as

Figure 1.6 Morphogenesis of the vertebrate heart. At E7.5, cardiac progenitor cells found in the cardiac crescent and are segregated into two distinct groups termed the first and second heart fields (shown in red and green respectively). The two halves of the cardiac crescent will merge at E8 to form the linear heart tube, which initiates contraction. The tube will undergo subsequent looping events which result in formation of the four-chambered heart. Adapted by permission from Macmillan Publishers Ltd: Buckingham, M., Meilhac S., Zaffran, S. Building the mammalian heart from two sources of myocardial cells. *Nature Reviews Genetics*, 2005; 6(11):826-35, copyright 2005.



well as the morphogenetic events that shape the heart are regulated by transcription factors and signalling molecules emanating from surrounding tissues, such as endoderm. These are discussed in depth in the following sections.

1.2.2 Transcriptional Regulation of Cardiomyogenesis

1.2.2.1 Mesp1

The bHLH transcription factor Mesp1 (mesoderm posterior 1) is the earliest marker of cardiac mesoderm in the developing embryo (129-131). Mesp1 expression is initiated at the onset of gastrulation at E6.5 and plays a critical role in the migration of cardiac progenitors out of the primitive streak. When the Mesp1 progenitor population is traced in Mesp1^{LacZ/-} (Mesp1 null) mice, they tend to accumulate in the primitive streak and are delayed in their subsequent migration. This results in a cardia bifida phenotype that is lethal (130, 131). Consistent with this role in promoting migration, Mesp1 has been implicated in the regulation of epithelial to mesenchymal transition (132). Under wild-type conditions, Mesp1-Cre lineage tracing experiments have shown that Mesp1 expressing cells contribute to all the cell types of the heart, including myocardium, endocardium, epicardium and the cardiac conduction system and that they encompass derivatives of both the primary and secondary heart fields (130, 131, 133). These data suggest that almost all cells of the heart arise from the Mesp1^{+ve} population.

Consistent with this, Mesp1 has strong cardiac inductive properties both *in vivo* and *in vitro*. Forced expression of Mesp1 in frog embryos resulted in the formation of ectopic beating areas while overexpression in ESCs enhanced cardiomyogenesis (134). In the ESC system however, Mesp1 overexpression only induces cardiomyogenesis when it is transiently expressed early in differentiation whereas sustained expression is inhibitory toward the

cardiac gene program (135). These inductive properties can be attributed to direct or indirect regulation of the expression of cardiogenic factors such as *Dkk1*, *Nkx2.5*, *Hand2*, and *Gata-4* by *Mesp1* (135, 136). Given the multifunctional nature of the role of *Mesp1* and its inductive cardiogenic properties, *Mesp1* has been proposed as a ‘master regulator’ of cardiomyogenesis.

1.2.2.2 The NK2 Family

Nkx2.5 is a member of the NK family of homeodomain transcription factors and is expressed throughout the cardiac progenitor population in both the primary and secondary heart fields (137). The expression of *Nkx2.5* is initiated early in cardiomyogenesis, at E7.5 in the cardiac crescent. During embryonic development in the mouse, loss of *Nkx2.5* results in failure of the heart to undergo looping, leading to embryonic lethality (138, 139). Although cardiomyocytes are specified normally, genes downstream of *Nkx2.5*, including atrial natriuretic factor (ANF), the ventricular isoform of myosin light chain 2 (*Mlc2v*) and *Connexin40* (*Cx40*), are aberrantly expressed. This is in direct contrast to the role played by the *Drosophila* homologue *tinman*, loss of which results in abrogation of cardiomyogenesis (140). Studies in mouse ESCs (mESCs) have confirmed that *Nkx2.5* is dispensable for cardiomyocyte specification, although, as in the knockout mouse, the expression of downstream genes was disrupted in *Nkx2.5*^{-/-} mESC derived cardiomyocytes (141). It has been suggested that this is due to genetic redundancy with other vertebrate NK family members expressed in the developing heart. This is supported by the observation that co-expression of dominant negative *XNkx2.5* and *XNkx2.3* in frogs results in a complete inhibition of cardiomyogenesis (142). Furthermore, expression of a dominant negative form of *Nkx2.5* in P19 cells also results in inhibition of cardiomyogenesis in this system,

indicating that Nkx activity is required for this process (143). However, this relationship is not as straightforward in other systems. In the mouse, Nkx2.6 is also expressed in developing heart. Nkx2.6 null mice do not exhibit any cardiac defects and although Nkx2.5/Nkx2.6 double knockout mice exhibit morphological defects of the heart, cardiomyocytes are specified normally (144, 145).

These discrepancies have led several groups to study the function of Nkx2.5 on a finer scale. In mice, it was found that Nkx2.5 worked in a negative feedback loop to inhibit Bmp2/Smad1 signalling, ultimately regulating the balance between specification and proliferation of the progenitors within the secondary heart field (146). Importantly, hypomorphic mutants generated in this study exhibited cardiac abnormalities consistent with those observed in human patients with congenital heart disease associated with mutations in Nkx2.5. In zebrafish, morpholino mediated inhibition of *nkx2.5* and *nkx2.7* expression caused excessive specification of atrial cardiomyocytes coupled to a decrease in the number of ventricular cardiomyocytes, suggesting that Nkx genes differentially regulate progenitor populations within the developing heart (147). Furthermore, although Nkx2.5 seems to be dispensable for cardiomyocyte formation, it is sufficient to induce cardiac differentiation when overexpressed in P19 cells (148). Although progress has been made toward elucidating the role of Nkx2.5 in cardiomyogenesis, more research will be required to clarify its complicated and likely multifaceted function in this process.

Nkx2.5 regulates many of its target genes via direct interactions with other cardiogenic transcription factors including GATA-4, Tbx5 and Mef2c (149-151). These cofactor interactions confer target specificity and modulate Nkx2.5 activity. For example, Nkx2.5 synergizes with Tbx5 to activate expression of ANF while it can cooperate with Tbx2 to repress ANF transcription (150, 152, 153). This 'switch' in cofactors has been

proposed as a mechanism for blocking ANF expression and the formation of chamber myocardium in Tbx2 expressing regions of the developing heart (153). It is clear that protein-protein interactions are important for the regulation of Nkx2.5 activity and thus, of cardiomyocyte differentiation and heart development. In spite of this, there has not been a comprehensive proteomic study of Nkx2.5 protein interactions during cardiomyogenesis.

1.2.2.3 The GATA Family

GATA factors are members of the zinc finger family of transcriptional regulators. Three of these factors, GATA4, GATA5 and GATA6 have been found to be expressed in the developing heart, amongst other tissues (154-157). During murine embryonic development, expression of GATA factors is initiated in pre-cardiac mesoderm at E7-7.5, implicating them in the earliest stages of cardiomyogenesis (158). Of the three members involved in heart development, GATA4 is by far the most studied. Like Nkx2.5, GATA4 is not required for specification or terminal differentiation of cardiomyocytes *in vivo* or in ESCs (159-161). In GATA4 null mice, the cardiac crescent fails to fuse at the midline owing to defective ventral migration of progenitors, and the heart tube is not formed. This defective migration is not cell intrinsic, as GATA4(-/-) ESCs can contribute to the heart in chimeric mice (159).

Consistent with results of loss of function analyses, which have indicated that no single murine GATA factor is absolutely required for cardiomyogenic differentiation, studies of compound mutants have revealed a certain level of redundancy between family members (162, 163). GATA4/GATA6 compound mutants exhibit a complete lack of terminally differentiated cardiomyocytes and acardia (164). Interestingly, GATA4 and GATA6 exert differential effects on primary and secondary heart field progenitor populations. While the progenitors of the secondary heart field are formed normally in GATA4/GATA6(-/-) mice,

primary heart field progenitors are lost, indicating that GATA factors are essential for progenitor specification in the primary but not the secondary heart field. P19 embryonal carcinoma cells express only GATA4 and not GATA5 or GATA6 (165, 166). Antisense mediated knockdown of GATA4 in this system blocks cardiomyogenesis, consistent with requirement of GATA activity for this process (165).

Although single GATA factors are not required for cardiomyogenesis, they do possess intrinsic cardiogenic properties. Ectopic expression of GATA4 or GATA5 can induce cardiac gene expression in embryonic ectoderm explants from *Xenopus* (167). GATA6 is significantly less cardiogenic in this context and indeed showed a stronger tendency to induce formation of liver. GATA4 overexpression also induced and accelerated cardiomyogenesis in P19 embryonal carcinoma cells (168). Recently, GATA4 was also found to cooperate with Mef2c and Tbx5 to reprogram cardiac fibroblasts into cardiomyocytes, which may have important implications for cardiac cell therapy (169).

1.2.2.4 The Tbx Family

Several members of the Tbox (Tbx) family of transcription factors play important roles in cardiomyocyte differentiation and heart development. Tbx factors are characterized by their Tbox DNA binding domain and are divided into two families designated Tbx1 (members include Tbx1, Tbx18 and Tbx20) and Tbx2 (including Tbx2, Tbx3 and Tbx5) (170). Family members can be further subdivided into transcriptional activators, such as Tbx5, and repressors, such as Tbx2 (152, 153). The first member of this family to be identified, Brachyury/T is required during gastrulation for proper specification of mesoderm (171). Tbx5 was the first Tbx factor to be directly implicated in heart development, with the finding that mutations in this gene caused Holt-Oram syndrome, which is characterized by

congenital heart and limb malformations (172). To date, Tbx5 is the most studied of the Tbx factors in cardiomyogenesis. Tbx5 null mice exhibit improperly formed linear heart tubes with hypoplasia of posterior structures and ultimately fail to undergo looping (152). Like in the case of Nkx2.5, cardiomyocytes were specified but expression of downstream genes such as Mlc2v, ANF and Cx40 was also disrupted. Importantly, expression of a dominant negative Tbx5-Engrailed fusion protein in developing *Xenopus* embryos resulted in a ‘heartless’ phenotype, indicating that some Tbx factor activity is required for cardiomyogenesis to occur (173). Gene profiling studies have implicated Tbx5 in the regulation of many cardiac genes (174). Indeed, Tbx5 appears to have potent cardiogenic properties and has been shown to be able to cooperate with other factors to convert mouse mesoderm (with Gata4 and Baf60c) and fibroblasts (with Mef2c and Gata4) into cardiomyocytes (169, 175).

1.2.3 Signalling Pathways Regulating Cardiomyogenesis

1.2.3.1 Wnt

Both the canonical and non-canonical Wnt signalling cascades are essential for cardiomyocyte differentiation and morphogenesis of the heart, although the timing and nature of their effects are very dissimilar. The canonical Wnt signalling cascade exerts differential effects at different stages of cardiomyogenesis (76). Canonical Wnt signalling mediated by Wnt3a is important for Mesp1 expression and mesoderm induction *in vivo* and in mESCs (176, 177). However, following mesoderm induction, the subsequent specification of cardiac progenitor cells from Mesp1^{+ve} cells is potentiated by the Wnt inhibitor Dkk1, which is a direct target of Mesp1 dependent transcription, indicating that canonical Wnt signalling is inhibitory at this stage (132, 134). Canonical Wnts have also

been implicated in regulation of cardiac progenitor proliferation. Blocking canonical Wnt signalling in the SHF results in reduced numbers of progenitors and aberrant development of SHF derived cardiac structures while induction of this signalling cascade results in expansion of the progenitor population (178, 179). Wnt signalling is also inhibitory at the terminal stages of cardiomyogenesis as activation of Wnt/ β -catenin signalling in differentiating mESCs at a late stage results in a reduced cardiomyocyte differentiation (180, 181). Furthermore, Wnt signalling through β -catenin has also been shown to block terminal differentiation of resident cardiac progenitor cells (182, 183). Although the multiphasic nature of canonical Wnt signalling in cardiomyogenesis has been well documented, the molecular mechanisms whereby Wnt exerts these differential effects remain unknown.

Whereas canonical Wnt signalling is primarily refractory to cardiac specification, non-canonical Wnts tend to promote this process. In particular, Wnt11 has potent cardiogenic properties as evidenced by its ability to induce cardiomyogenesis in several systems, including *Xenopus* explants, P19 embryonal carcinoma cells and mESCs (180, 184, 185). Conversely, a dominant negative Wnt11 blocks cardiac differentiation (184). A more detailed analysis of this effect revealed that activation of PKC or JNK could mimic these cardiogenic properties and override the inhibitory effect of the dominant negative, indicating that in this instance, Wnt11 is in fact functioning through the non-canonical pathway.

1.2.3.2 BMP

The role of BMP signalling in cardiomyogenesis has been extensively studied. During cardiac development in *Drosophila* and in chick, BMPs (known as dpp in *Drosophila*) are secreted from the surrounding tissues and are required for induction of the cardiac gene program (186-188). More specifically, in chick, this effect is mediated by BMP2 and BMP5

originating from the endoderm as well as BMP4 and BMP7 signalling from the ectoderm (188, 189). In this system, BMP2 and BMP4 can induce expression of Nkx2.5 and GATA4 and subsequent cardiomyogenesis (188, 190). Furthermore, Nkx2.5 has been shown to be a direct transcriptional target of this pathway (191, 192). In *Xenopus*, inhibition of BMP signalling using dominant-negative receptors or intracellular antagonists, results in defective development of the heart and in some instances, acardia (193, 194). In the mouse, BMP2, BMP5 and BMP7 are expressed in the anterior mesoderm and contribute to cardiogenesis (195). The overlapping domains of expression seem to contribute to some level of functional redundancy between these factors. While BMP2-null mice have cardiac defects, heart development in BMP5- and BMP7- null mice is normal (196-198). Compound BMP5/BMP7-null mice however, exhibit significantly compromised heart development (199).

BMP signalling also plays a crucial role in cardiomyogenesis in stem cells at the levels of specification where it regulates the expression of Nkx2.5 and GATA4 as well as in terminal differentiation where it can activate expression of terminal markers (200). Interestingly, it was shown that intrinsic BMP signals emanating from hepatocyte-like cells induced cardiac differentiation in mESCs, indicating that the inductive properties of tissues surrounding the developing heart are, to some extent, conserved during endogenous ES differentiation within embryoid bodies (201). Several studies in various stem cell systems have indicated that BMP signalling is essential and sufficient for cardiomyogenesis *in vitro*. Exposure to BMP2 can enhance cardiomyogenesis in ESCs while inhibition of this pathway by ectopic expression of Noggin can prevent cardiomyogenesis in P19 and P19CL6 cells (202-204). Under the appropriate conditions however, Noggin can also induce cardiomyogenesis in stem cells. mESCs are maintained in an undifferentiated state by

treatment with leukemia inhibitory factor (LIF) and differentiation is induced by embryoid body formation, coupled to LIF withdrawal. Addition of Noggin to undifferentiated mESC cultures, 3 days prior to LIF withdrawal and embryoid body formation, resulted in a significant increase in cardiomyogenesis in this system (205). Clearly BMP-mediated regulation of cardiomyogenesis is context-dependent.

1.2.3.3 RA

RA regulates heart development at multiple levels from cell specification to organ morphogenesis, although our understanding of its role is complicated by the time and concentration-dependent kinetics of this morphogen. Most of what is known about the role of RA during cardiogenesis relates to its regulation of anterior-posterior patterning of the heart *in vivo*. During early (8.25dpc) cardiac development in the mouse, the expression of the RA synthesizing enzyme RALDH2 is confined to the posterior-most regions of the heart and correlates strongly with the zone of RA-responsiveness, as indicated by an RARE-LacZ transgenic response element (206). Treatment of developing chick and zebrafish embryos with RA results in truncated hearts where anterior structures are more severely affected than posterior ones (207, 208). These data were further supported by the finding that treatment of chick embryos with RA resulted in an anterior expansion of the atrial myosin heavy chain expressing domain, usually confined to the posterior heart tube (209). Mouse embryos exposed to RA also exhibit a cardiac phenotype consistent with posteriorization (210).

With regard to specification and differentiation of cardiomyocytes, RA has been found to inhibit and promote this process in a context-dependent fashion. Mutation of *raldh2* in zebrafish causes an expansion of the Nkx2.5^{+ve} myocardial progenitor population, resulting in an increased number of cardiomyocytes (211). Importantly, it was shown that

this expansion occurs as a result of fate transformation and is not due to increased progenitor proliferation. Treatment with an RAR-antagonist elicited a similar effect in *Xenopus* (212). In *Raldh2*-null mice, cardiac morphogenesis is compromised, such that the heart fails to loop and the atria are not formed properly, resulting in embryonic lethality. A detailed analysis of this phenotype revealed that it was caused mainly by defects in development of the secondary heart field (213). These mice exhibited a posterior expansion in the domain of expression of primary heart field markers such as *Tbx1* and *FGF8* but these progenitors failed to differentiate. In a model of partial rescue of *Raldh2*-null mice by maternal RA-supplementation, it was found that cardiac progenitor differentiation was compromised but could be rescued in explant cultures by supplementation with *FGF2*, implying a certain degree of crosstalk between these two pathways (214). Interestingly, these mice exhibited a significant increase of approximately 30% in cardiomyocyte proliferation, although the reason for this remains unclear.

In mESCs, RA can induce or inhibit cardiomyogenesis, depending on the timing of treatment. Whereas early exposure of differentiating cells to RA inhibited cardiac differentiation in this system, later exposure enhanced it (215). Consistent with its role in anterior-posterior patterning of the heart, it has been shown that modulating RA signalling can alter atrial and ventricular cell fate choices. In mESCs, treatment with RA favoured the differentiation of cardiomyocytes with a ventricular, as opposed to atrial identity (216, 217). Whereas treatment of hESCs with *Noggin* and an RAR-antagonist resulted in preferential formation of ventricular cardiomyocytes, treatment with *Noggin* and RA promoted the differentiation of atrial cardiomyocytes as evidenced by electrophysiological properties (218). Like in the case of Wnt signalling, the multiphasic nature of RA-mediated regulation

of cardiomyogenesis has been well documented but the molecular mechanisms underlying this effect remain to be identified.

1.3 Stem Cells: a model for myogenesis and a source for muscle cell therapy

In addition to their potential use for cell therapy, stem cells present a good model in which to study the molecular mechanisms of myogenesis as the fundamental pathways that regulate this process *in vivo* are conserved in these systems (Figures 1.7 and 1.8), several examples of which have been highlighted in the sections above. Many different ESC lines have been derived from various organisms but prior to the advent of highly efficient protocols for maintaining mESCs and hESCs, embryonal carcinoma (EC) cell lines such as the P19 and F9 cell lines provided a model system in which to study cellular differentiation processes. In particular, P19 cells have proven particularly useful for the study of cardiac and skeletal myogenesis (219). P19 cells are derived from a primary teratocarcinoma induced in a CH3/HC mouse and their pluripotent character was confirmed by their ability to contribute to tissues derived from all three germ layers (220). The main advantages of the P19 cell system include its amenability to genetic manipulation, its ability to grow quickly without a feeder layer and its requirement for induction by dimethyl sulfoxide for myogenesis. This last characteristic makes P19 cells an ideal system in which to determine whether a gene is sufficient to drive the myogenic program by its ability to overcome the need for drug-mediated induction (63, 82, 221). Over the years, many of the differentiation pathways or directed culture conditions identified in P19 cells or in its subclones have proven to be conserved in other cell lines and *in vivo*, supporting their validity as a model of embryonic stem cell differentiation (60, 63, 65, 222-225).

Figure 1.7 Skeletal myogenesis in stem cells. The myogenic differentiation pathway in ESCs is conserved from the embryo. Cells initially form mesoderm which in turn undergoes specification to premyogenic progenitors which express Pax3 and Meox1. These cells become committed to the myogenic lineage by forming myoblasts which express MRFs. Myoblasts undergo terminal differentiation into myocytes.

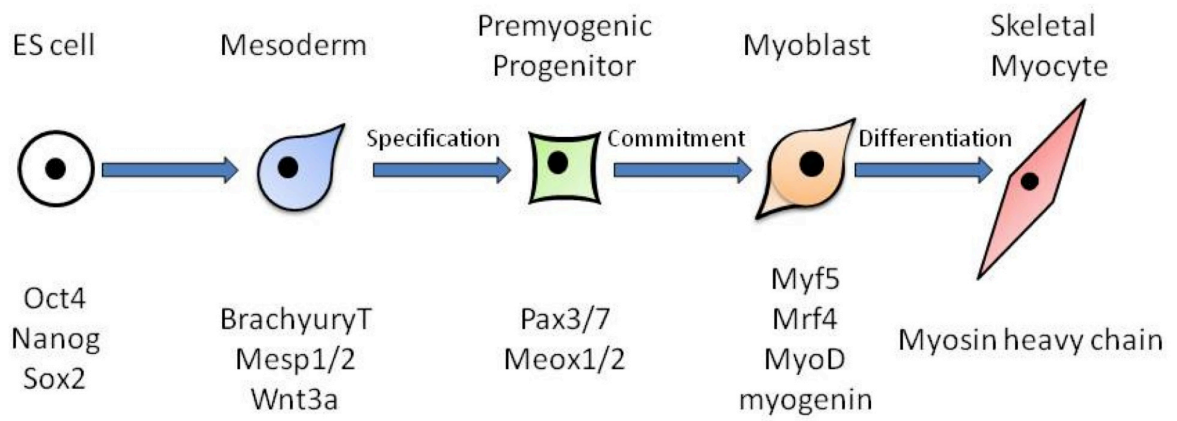
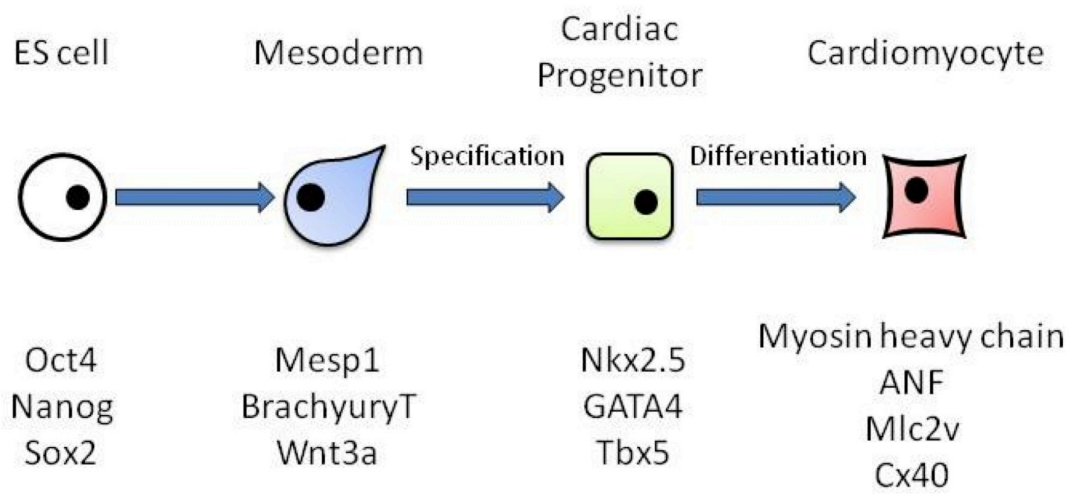


Figure 1.8 Cardiomyogenesis in stem cells. The pathways involved in differentiation of cardiomyocytes *in vivo* are recapitulated *in vitro*. ESCs form mesoderm and are subsequently specified to cardiac progenitors expressing Mesp1, Nkx2.5, GATA4 and various Tbx family members. These cells undergo terminal differentiation into cardiomyocytes.



Both mESCs and hESCs are derived from the inner cell mass of a blastocyst stage embryo, however, despite their shared developmental origin, distinct differences exist between these two systems. In both mESCs and hESCs, pluripotency is maintained by a triad of transcription factors, Oct4, Nanog and Sox2, which regulate self-renewal in these systems through activation and repression of target genes (226). In fact, these factors share a large subset of common target genes (353 shared targets in hESCs), supporting the notion that they cooperate to regulate global networks involved in pluripotency (227). However, a direct comparison of targets of these three factors in mESCs and hESCs has revealed that most targets are not conserved between these two species (226-229). In fact, studies have shown that hESCs share more common characteristics with murine epiblast stem cells (epiSCs) derived from a post-implantation mouse embryo than with mESCs (230, 231). These include global expression profiles, epigenetic signatures and culture requirements. For example, whereas it was found that Oct4 had only 27 shared targets between hESCs and mESCs, this number increased to 173 when hESCs were compared to murine epiSCs (231).

Both hESCs and mESCs have been studied extensively in order to understand the molecular mechanisms that control cell fate. Both of these cell types have been shown to readily differentiate into cardiomyocytes utilizing an embryonic pathway involving the formation of a progenitor population which may prove valuable for cell therapy purposes (232). The focus of current research is a more thorough understanding of differentiation and maturation, in order to optimize the yield of cardiomyocytes from ESCs, as well as methods of purification of cardiomyocytes or progenitors from heterogeneous populations and finally use of these cells in transplantation therapy. Work in these areas may very well lead to the use of ESCs as a source for cardiac cell therapy in the not too distant future.

In the case of skeletal myogenesis, the field has not progressed as far due primarily to the difficulty of generating skeletal muscle from ESCs. Although there has been some limited success in hESCs, current methods involved extremely long differentiation protocols which have proven not to be reproducible and do not utilize an embryonic pathway (233). Alternatives have thus far been limited to chemical modification of cells (234). Studies in mESCs have proved somewhat more promising and indeed indicate that there is potential for cell therapy mediated treatment of muscle diseases (235-237). Further research will be required to increase the yield of skeletal muscle from ESCs before cell therapy becomes a really viable option.

1.4 Thesis summary

In order to employ ESCs in the context of regenerative medicine, it is imperative that we learn to modulate cell fate decisions in order to optimize directed culture conditions that will allow us to generate high yields of specific cell types. In order to do this, we must understand the mechanisms that govern these decisions to such an extent that we are able to intercede and manipulate them. The goal of this research was to extend the knowledge base relative to molecular mechanisms of myogenesis in stem cells. The central hypothesis to be tested was that myogenic signalling cascades interface with downstream transcription factors to orchestrate muscle cell fate decisions in stem cells.

In Chapter 2, experiments were designed to test the hypothesis that RA regulates myogenesis in P19 cells and mESCs by regulating the expression of myogenic genes both directly and indirectly via other signalling cascades. We show that RA activates the expression of premyogenic mesoderm markers in both P19 cells and mESCs, resulting in an enhancement of MRF expression and of skeletal myogenesis. We go on to show that this

occurs via direct binding of RARs to regulatory regions of myogenic genes such as Pax3, Meox1 and Wnt3a, as well as by activation of canonical Wnt signalling. However, RA-mediated enhancement of myogenesis required a functional β -catenin. Taken together, these findings suggest that RA acts both upstream of and in parallel to Wnt signalling to induce myogenesis in stem cells. Conversely, we present data that RA prevents cardiomyogenesis by inhibiting expression of GATA4 and by inducing expression of the BMP inhibitor Tob1. Analysis of the intersection between these pathways revealed that RA could overcome BMP4-mediated inhibition of skeletal myogenesis but could not inhibit cardiomyogenesis as induced by BMP4. These findings illustrate the complexity of crosstalk between the RA, Wnt and BMP pathways during myogenesis and provide novel mechanistic insight into the molecular basis for the action of RA during muscle differentiation *in vitro*.

In Chapter 3 we sought to test the hypothesis that the molecular mechanisms involved in RA-mediated enhancement of myogenesis in murine stem cell systems would be conserved in hESCs. This represents a significant extension to the work in Chapter 2 as it represents a direct application of knowledge gained in P19 cells and mESCs to a clinically relevant model system. In this chapter, we show that, as in the two murine systems, RA acted early in the differentiation cascade, enhancing expression of the premyogenic mesoderm marker Meox1, and expanding the Pax3^{+ve} progenitor population, which occurred prior to MRF expression and had not been previously described in this system. This expansion leads to increased expression levels of MyoD and myogenin and ultimately to enhanced myogenesis in hESCs. The timing and concentration of treatment employed did not result in enhancement of other RA-responsive cells types, such as cardiac muscle, neurons and smooth muscle. Therefore, RA enhances myogenesis in hESCs via similar mechanisms to those employed in P19 cells and mESCs. Furthermore, myogenesis in this

system utilizes an embryonic pathway involving the formation of a Pax3^{+ve}/Meox1^{+ve} progenitor population prior to MRF expression, which may have clinical implications as these cells have been shown to be more efficient in their ability to repopulate the satellite cell niche (238).

In Chapter 4 we performed experiments to test the hypothesis that cofactor interactions regulate the function of Nkx2.5 during stem cell differentiation. We undertook a proteomic screen to search for novel binding partners of Nkx2.5 during cardiomyogenesis in P19 cells and we identified protein phosphatase 1 β and myosin phosphatase targeting subunit 1 (Mypt1), two subunits of the myosin phosphatase (MP) enzyme complex. We go on to show that co-expression with MP results in exclusion of Nkx2.5 from the nucleus and inhibition of its transcriptional activity. This exclusion is regulated via modulation of Mypt1 phosphorylation by ROCK, downstream of Wnt signalling. Furthermore, Wnt3a treatment of differentiating mESCs resulted in increased exclusion of Nkx2.5 from the nucleus and a consequent failure to undergo terminal cardiomyogenesis. This represents a novel mechanism of regulation of Nkx2.5 and for Wnt3a-mediated inhibition of cardiac progenitor differentiation.

1.5 References

1. Allan, L.E., G.H. Petit, and P. Brundin. 2010. Cell transplantation in Parkinson's disease: problems and perspectives. *Curr Opin Neurol* 23:426-432.
2. Menasche, P. 2011. Cardiac cell therapy: lessons from clinical trials. *J Mol Cell Cardiol* 50:258-265.
3. Meregalli, M., A. Farini, D. Parolini, S. Maciotta, and Y. Torrente. 2010. Stem cell therapies to treat muscular dystrophy: progress to date. *BioDrugs* 24:237-247.

4. Mozid, A.M., S. Arnous, E.C. Sammut, and A. Mathur. 2011. Stem cell therapy for heart diseases. *Br Med Bull*
5. Arenas, E. 2010. Towards stem cell replacement therapies for Parkinson's disease. *Biochem Biophys Res Commun* 396:152-156.
6. Robak, T., and A. Wierzbowska. 2009. Current and emerging therapies for acute myeloid leukemia. *Clin Ther* 31 Pt 2:2349-2370.
7. Tedesco, F.S., A. Dellavalle, J. Diaz-Manera, G. Messina, and G. Cossu. 2010. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J Clin Invest* 120:11-19.
8. Palmieri, B., J.P. Tremblay, and L. Daniele. 2010. Past, present and future of myoblast transplantation in the treatment of Duchenne muscular dystrophy. *Pediatr Transplant* 14:813-819.
9. Vinciguerra, M., A. Musaro, and N. Rosenthal. 2010. Regulation of muscle atrophy in aging and disease. *Adv Exp Med Biol* 694:211-233.
10. Moreno, T.A., and C. Kintner. 2004. Regulation of segmental patterning by retinoic acid signaling during *Xenopus* somitogenesis. *Dev Cell* 6:205-218.
11. Diez del Corral, R., I. Olivera-Martinez, A. Goriely, E. Gale, M. Maden, and K. Storey. 2003. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. *Neuron* 40:65-79.
12. Dubrulle, J., M.J. McGrew, and O. Pourquie. 2001. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* 106:219-232.
13. Vermot, J., and O. Pourquie. 2005. Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. *Nature* 435:215-220.
14. Aulehla, A., C. Wehrle, B. Brand-Saberi, R. Kemler, A. Gossler, B. Kanzler, and B.G. Herrmann. 2003. Wnt3a plays a major role in the segmentation clock controlling somitogenesis. *Dev Cell* 4:395-406.
15. Aulehla, A., W. Wiegraebe, V. Baubet, M.B. Wahl, C. Deng, M. Taketo, M. Lewandoski, and O. Pourquie. 2008. A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. *Nat Cell Biol* 10:186-193.
16. Dubrulle, J., and O. Pourquie. 2004. fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* 427:419-422.

17. Pourquie, O. 2003. The segmentation clock: converting embryonic time into spatial pattern. *Science* 301:328-330.
18. Jiang, Y.J., B.L. Aerne, L. Smithers, C. Haddon, D. Ish-Horowicz, and J. Lewis. 2000. Notch signalling and the synchronization of the somite segmentation clock. *Nature* 408:475-479.
19. Li, Y., U. Fenger, C. Niehrs, and N. Pollet. 2003. Cyclic expression of *esr9* gene in *Xenopus* presomitic mesoderm. *Differentiation* 71:83-89.
20. Jouve, C., I. Palmeirim, D. Henrique, J. Beckers, A. Gossler, D. Ish-Horowicz, and O. Pourquie. 2000. Notch signalling is required for cyclic expression of the hairy-like gene *HES1* in the presomitic mesoderm. *Development* 127:1421-1429.
21. Bessho, Y., R. Sakata, S. Komatsu, K. Shiota, S. Yamada, and R. Kageyama. 2001. Dynamic expression and essential functions of *Hes7* in somite segmentation. *Genes Dev* 15:2642-2647.
22. Holley, S.A., R. Geisler, and C. Nusslein-Volhard. 2000. Control of *her1* expression during zebrafish somitogenesis by a delta-dependent oscillator and an independent wave-front activity. *Genes Dev* 14:1678-1690.
23. Denetclaw, W.F., Jr., B. Christ, and C.P. Ordahl. 1997. Location and growth of epaxial myotome precursor cells. *Development* 124:1601-1610.
24. Denetclaw, W.F., and C.P. Ordahl. 2000. The growth of the dermomyotome and formation of early myotome lineages in thoracolumbar somites of chicken embryos. *Development* 127:893-905.
25. Ordahl, C.P., E. Berdougo, S.J. Venters, and W.F. Denetclaw, Jr. 2001. The dermomyotome dorsomedial lip drives growth and morphogenesis of both the primary myotome and dermomyotome epithelium. *Development* 128:1731-1744.
26. Venters, S.J., and C.P. Ordahl. 2002. Persistent myogenic capacity of the dermomyotome dorsomedial lip and restriction of myogenic competence. *Development* 129:3873-3885.
27. Biressi, S., and T.A. Rando. 2010. Heterogeneity in the muscle satellite cell population. *Semin Cell Dev Biol* 21:845-854.
28. Weintraub, H., S.J. Tapscott, R.L. Davis, M.J. Thayer, M.A. Adam, A.B. Lassar, and A.D. Miller. 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of *MyoD*. *Proc Natl Acad Sci U S A* 86:5434-5438.

29. Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H.H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J* 8:701-709.
30. Edmondson, D.G., and E.N. Olson. 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev* 3:628-640.
31. Miner, J.H., and B. Wold. 1990. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc Natl Acad Sci U S A* 87:1089-1093.
32. Ott, M.O., E. Bober, G. Lyons, H. Arnold, and M. Buckingham. 1991. Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development* 111:1097-1107.
33. Kiefer, J.C., and S.D. Hauschka. 2001. Myf-5 is transiently expressed in nonmuscle mesoderm and exhibits dynamic regional changes within the presegmented mesoderm and somites I-IV. *Dev Biol* 232:77-90.
34. Tajbakhsh, S., D. Rocancourt, and M. Buckingham. 1996. Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf-5 null mice. *Nature* 384:266-270.
35. Pownall, M.E., M.K. Gustafsson, and C.P. Emerson, Jr. 2002. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu Rev Cell Dev Biol* 18:747-783.
36. Braun, T., M.A. Rudnicki, H.H. Arnold, and R. Jaenisch. 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* 71:369-382.
37. Kassar-Duchossoy, L., B. Gayraud-Morel, D. Gomes, D. Rocancourt, M. Buckingham, V. Shinin, and S. Tajbakhsh. 2004. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* 431:466-471.
38. Rudnicki, M.A., T. Braun, S. Hinuma, and R. Jaenisch. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* 71:383-390.
39. Megeney, L.A., B. Kablar, K. Garrett, J.E. Anderson, and M.A. Rudnicki. 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev* 10:1173-1183.
40. Sabourin, L.A., A. Girgis-Gabardo, P. Seale, A. Asakura, and M.A. Rudnicki. 1999. Reduced differentiation potential of primary MyoD^{-/-} myogenic cells derived from adult skeletal muscle. *J Cell Biol* 144:631-643.

41. Cao, Y., Z. Yao, D. Sarkar, M. Lawrence, G.J. Sanchez, M.H. Parker, K.L. MacQuarrie, J. Davison, M.T. Morgan, W.L. Ruzzo, R.C. Gentleman, and S.J. Tapscott. 2010. Genome-wide MyoD binding in skeletal muscle cells: a potential for broad cellular reprogramming. *Dev Cell* 18:662-674.
42. Zhang, W., R.R. Behringer, and E.N. Olson. 1995. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes Dev* 9:1388-1399.
43. Rudnicki, M.A., P.N. Schnegelsberg, R.H. Stead, T. Braun, H.H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 75:1351-1359.
44. Cusella-De Angelis, M.G., G. Lyons, C. Sonnino, L. De Angelis, E. Vivarelli, K. Farmer, W.E. Wright, M. Molinaro, M. Bouche, M. Buckingham, and et al. 1992. MyoD, myogenin independent differentiation of primordial myoblasts in mouse somites. *J Cell Biol* 116:1243-1255.
45. Yee, S.P., and P.W. Rigby. 1993. The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev* 7:1277-1289.
46. Hasty, P., A. Bradley, J.H. Morris, D.G. Edmondson, J.M. Venuti, E.N. Olson, and W.H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* 364:501-506.
47. Nabeshima, Y., K. Hanaoka, M. Hayasaka, E. Esumi, S. Li, and I. Nonaka. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* 364:532-535.
48. Wang, Y., and R. Jaenisch. 1997. Myogenin can substitute for Myf5 in promoting myogenesis but less efficiently. *Development* 124:2507-2513.
49. Wang, Y., P.N. Schnegelsberg, J. Dausman, and R. Jaenisch. 1996. Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. *Nature* 379:823-825.
50. Buckingham, M., and F. Relaix. 2007. The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. *Annu Rev Cell Dev Biol* 23:645-673.
51. Williams, B.A., and C.P. Ordahl. 1994. Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* 120:785-796.
52. Bober, E., T. Franz, H.H. Arnold, P. Gruss, and P. Tremblay. 1994. Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* 120:603-612.

53. Daston, G., E. Lamar, M. Olivier, and M. Goulding. 1996. Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development* 122:1017-1027.
54. Tremblay, P., S. Dietrich, M. Mericskay, F.R. Schubert, Z. Li, and D. Paulin. 1998. A crucial role for Pax3 in the development of the hypaxial musculature and the long-range migration of muscle precursors. *Dev Biol* 203:49-61.
55. Epstein, D.J., M. Vekemans, and P. Gros. 1991. Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax-3. *Cell* 67:767-774.
56. Engleka, K.A., A.D. Gitler, M. Zhang, D.D. Zhou, F.A. High, and J.A. Epstein. 2005. Insertion of Cre into the Pax3 locus creates a new allele of Splotch and identifies unexpected Pax3 derivatives. *Dev Biol* 280:396-406.
57. Li, J., K.C. Liu, F. Jin, M.M. Lu, and J.A. Epstein. 1999. Transgenic rescue of congenital heart disease and spina bifida in Splotch mice. *Development* 126:2495-2503.
58. Jostes, B., C. Walther, and P. Gruss. 1990. The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. *Mech Dev* 33:27-37.
59. Mansouri, A., A. Stoykova, M. Torres, and P. Gruss. 1996. Dysgenesis of cephalic neural crest derivatives in Pax7^{-/-} mutant mice. *Development* 122:831-838.
60. Bajard, L., F. Relaix, M. Lagha, D. Rocancourt, P. Daubas, and M.E. Buckingham. 2006. A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev* 20:2450-2464.
61. Lagha, M., T. Sato, B. Regnault, A. Cumano, A. Zuniga, J. Licht, F. Relaix, and M. Buckingham. 2010. Transcriptome analyses based on genetic screens for Pax3 myogenic targets in the mouse embryo. *BMC Genomics* 11:696.
62. Maroto, M., R. Reshef, A.E. Munsterberg, S. Koester, M. Goulding, and A.B. Lassar. 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell* 89:139-148.
63. Ridgeway, A.G., and I.S. Skerjanc. 2001. Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. *J Biol Chem* 276:19033-19039.
64. Relaix, F., D. Rocancourt, A. Mansouri, and M. Buckingham. 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* 435:948-953.
65. Relaix, F., D. Montarras, S. Zaffran, B. Gayraud-Morel, D. Rocancourt, S. Tajbakhsh, A. Mansouri, A. Cumano, and M. Buckingham. 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* 172:91-102.

66. Lepper, C., S.J. Conway, and C.M. Fan. 2009. Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. *Nature* 460:627-631.
67. Candia, A.F., J. Hu, J. Crosby, P.A. Lalley, D. Noden, J.H. Nadeau, and C.V. Wright. 1992. Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. *Development* 116:1123-1136.
68. Reijntjes, S., S. Stricker, and B.S. Mankoo. 2007. A comparative analysis of Meox1 and Meox2 in the developing somites and limbs of the chick embryo. *Int J Dev Biol* 51:753-759.
69. Candia, A.F., and C.V. Wright. 1996. Differential localization of Mox-1 and Mox-2 proteins indicates distinct roles during development. *Int J Dev Biol* 40:1179-1184.
70. Mankoo, B.S., N.S. Collins, P. Ashby, E. Grigorieva, L.H. Pevny, A. Candia, C.V. Wright, P.W. Rigby, and V. Pachnis. 1999. Mox2 is a component of the genetic hierarchy controlling limb muscle development. *Nature* 400:69-73.
71. Mankoo, B.S., S. Skuntz, I. Harrigan, E. Grigorieva, A. Candia, C.V. Wright, H. Arnheiter, and V. Pachnis. 2003. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. *Development* 130:4655-4664.
72. Petropoulos, H., P.J. Gianakopoulos, A.G. Ridgeway, and I.S. Skerjanc. 2004. Disruption of Meox or Gli activity ablates skeletal myogenesis in P19 cells. *J Biol Chem* 279:23874-23881.
73. Stamatakis, D., M. Kastrinaki, B.S. Mankoo, V. Pachnis, and D. Karagogeos. 2001. Homeodomain proteins Mox1 and Mox2 associate with Pax1 and Pax3 transcription factors. *FEBS Lett* 499:274-278.
74. Cossu, G., and U. Borello. 1999. Wnt signaling and the activation of myogenesis in mammals. *EMBO J* 18:6867-6872.
75. Gessert, S., and M. Kuhl. 2010. The multiple phases and faces of wnt signaling during cardiac differentiation and development. *Circ Res* 107:186-199.
76. Munsterberg, A.E., J. Kitajewski, D.A. Bumcrot, A.P. McMahon, and A.B. Lassar. 1995. Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev* 9:2911-2922.
77. Tajbakhsh, S., U. Borello, E. Vivarelli, R. Kelly, J. Papkoff, D. Duprez, M. Buckingham, and G. Cossu. 1998. Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. *Development* 125:4155-4162.

78. Borello, U., B. Berarducci, P. Murphy, L. Bajard, V. Buffa, S. Piccolo, M. Buckingham, and G. Cossu. 2006. The Wnt/beta-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. *Development* 133:3723-3732.
79. Borello, U., M. Coletta, S. Tajbakhsh, L. Leyns, E.M. De Robertis, M. Buckingham, and G. Cossu. 1999. Transplacental delivery of the Wnt antagonist Frzb1 inhibits development of caudal paraxial mesoderm and skeletal myogenesis in mouse embryos. *Development* 126:4247-4255.
80. Shang, Y.C., S.H. Wang, F. Xiong, C.P. Zhao, F.N. Peng, S.W. Feng, M.S. Li, Y. Li, and C. Zhang. 2007. Wnt3a signaling promotes proliferation, myogenic differentiation, and migration of rat bone marrow mesenchymal stem cells. *Acta Pharmacol Sin* 28:1761-1774.
81. Petropoulos, H., and I.S. Skerjanc. 2002. Beta-catenin is essential and sufficient for skeletal myogenesis in P19 cells. *J Biol Chem* 277:15393-15399.
82. Ridgeway, A.G., H. Petropoulos, S. Wilton, and I.S. Skerjanc. 2000. Wnt signaling regulates the function of MyoD and myogenin. *J Biol Chem* 275:32398-32405.
83. Savage, J., A. Voronova, V. Mehta, F. Sendi-Mukasa, and I.S. Skerjanc. 2010. Canonical Wnt signaling regulates Foxc1/2 expression in P19 cells. *Differentiation* 79:31-40.
84. Rune Blomhoff, H.K.B. 2006. Overview of retinoid metabolism and function. *Journal of Neurobiology* 66:606-630.
85. Niederreither, K., and P. Dolle. 2008. Retinoic acid in development: towards an integrated view. *Nat Rev Genet* 9:541-553.
86. Nagy, L., H.Y. Kao, D. Chakravarti, R.J. Lin, C.A. Hassig, D.E. Ayer, S.L. Schreiber, and R.M. Evans. 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* 89:373-380.
87. Mendelsohn, C., M. Mark, P. Dolle, A. Dierich, M.P. Gaub, A. Krust, C. Lampron, and P. Chambon. 1994. Retinoic acid receptor beta 2 (RAR beta 2) null mutant mice appear normal. *Dev Biol* 166:246-258.
88. Lohnes, D., P. Kastner, A. Dierich, M. Mark, M. LeMeur, and P. Chambon. 1993. Function of retinoic acid receptor gamma in the mouse. *Cell* 73:643-658.
89. Lufkin, T., D. Lohnes, M. Mark, A. Dierich, P. Gorry, M.P. Gaub, M. LeMeur, and P. Chambon. 1993. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. *Proc Natl Acad Sci U S A* 90:7225-7229.

90. Ghyselinck, N.B., V. Dupe, A. Dierich, N. Messaddeq, J.M. Garnier, C. Rochette-Egly, P. Chambon, and M. Mark. 1997. Role of the retinoic acid receptor beta (RARbeta) during mouse development. *Int J Dev Biol* 41:425-447.
91. Li, E., H.M. Sucov, K.F. Lee, R.M. Evans, and R. Jaenisch. 1993. Normal development and growth of mice carrying a targeted disruption of the alpha 1 retinoic acid receptor gene. *Proc Natl Acad Sci U S A* 90:1590-1594.
92. Kastner, P., J.M. Grondona, M. Mark, A. Gansmuller, M. LeMeur, D. Decimo, J.L. Vonesch, P. Dolle, and P. Chambon. 1994. Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* 78:987-1003.
93. Kastner, P., M. Mark, M. Leid, A. Gansmuller, W. Chin, J.M. Grondona, D. Decimo, W. Krezel, A. Dierich, and P. Chambon. 1996. Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev* 10:80-92.
94. Krezel, W., V. Dupe, M. Mark, A. Dierich, P. Kastner, and P. Chambon. 1996. RXR gamma null mice are apparently normal and compound RXR alpha +/-RXR beta +/-RXR gamma -/- mutant mice are viable. *Proc Natl Acad Sci U S A* 93:9010-9014.
95. Lohnes, D., M. Mark, C. Mendelsohn, P. Dolle, A. Dierich, P. Gorry, A. Gansmuller, and P. Chambon. 1994. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development* 120:2723-2748.
96. Mendelsohn, C., D. Lohnes, D. Decimo, T. Lufkin, M. LeMeur, P. Chambon, and M. Mark. 1994. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120:2749-2771.
97. Mark, M., N.B. Ghyselinck, and P. Chambon. 2009. Function of retinoic acid receptors during embryonic development. *Nucl Recept Signal* 7:e002.
98. Edwards, M.K., and M.W. McBurney. 1983. The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev Biol* 98:187-191.
99. Halevy, O., and O. Lerman. 1993. Retinoic acid induces adult muscle cell differentiation mediated by the retinoic acid receptor-alpha. *J Cell Physiol* 154:566-572.
100. Albagli-Curiel, O., G. Carnac, M. Vandromme, S. Vincent, P. Crepieux, and A. Bonnieu. 1993. Serum-induced inhibition of myogenesis is differentially relieved by retinoic acid and triiodothyronine in C2 murine muscle cells. *Differentiation* 52:201-210.

101. Zhu, G.H., J. Huang, Y. Bi, Y. Su, Y. Tang, B.C. He, Y. He, J. Luo, Y. Wang, L. Chen, G.W. Zuo, W. Jiang, Q. Luo, J. Shen, B. Liu, W.L. Zhang, Q. Shi, B.Q. Zhang, Q. Kang, J. Zhu, J. Tian, H.H. Luu, R.C. Haydon, Y. Chen, and T.C. He. 2009. Activation of RXR and RAR signaling promotes myogenic differentiation of myoblastic C2C12 cells. *Differentiation* 78:195-204.
102. Froeschle, A., S. Alric, M. Kitzmann, G. Carnac, F. Aurade, C. Rochette-Egly, and A. Bonniou. 1998. Retinoic acid receptors and muscle b-HLH proteins: partners in retinoid-induced myogenesis. *Oncogene* 16:3369-3378.
103. Momoi, T., S. Miyagawa-Tomita, S. Nakamura, I. Kimura, and M. Momoi. 1992. Retinoic acid ambivalently regulates the expression of MyoD1 in the myogenic cells in the limb buds of the early developmental stages. *Biochem Biophys Res Commun* 187:245-253.
104. Alric, S., A. Froeschle, D. Piquemal, G. Carnac, and A. Bonniou. 1998. Functional specificity of the two retinoic acid receptor RAR and RXR families in myogenesis. *Oncogene* 16:273-282.
105. Reijntjes, S., P. Francis-West, and B.S. Mankoo. 2010. Retinoic acid is both necessary for and inhibits myogenic commitment and differentiation in the chick limb. *Int J Dev Biol* 54:125-134.
106. Urist, M.R., and B.S. Strates. 1971. Bone morphogenetic protein. *J Dent Res* 50:1392-1406.
107. Massague, J. 1992. Receptors for the TGF-beta family. *Cell* 69:1067-1070.
108. Yamaguchi, A. 1995. Regulation of differentiation pathway of skeletal mesenchymal cells in cell lines by transforming growth factor-beta superfamily. *Semin Cell Biol* 6:165-173.
109. Chen, D., M. Zhao, and G.R. Mundy. 2004. Bone morphogenetic proteins. *Growth Factors* 22:233-241.
110. Pourquie, O., C.M. Fan, M. Coltey, E. Hirsinger, Y. Watanabe, C. Breant, P. Francis-West, P. Brickell, M. Tessier-Lavigne, and N.M. Le Douarin. 1996. Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* 84:461-471.
111. Marcelle, C., M.R. Stark, and M. Bronner-Fraser. 1997. Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite. *Development* 124:3955-3963.
112. Duprez, D.M., M. Coltey, H. Amthor, P.M. Brickell, and C. Tickle. 1996. Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev Biol* 174:448-452.

113. Katagiri, T., A. Yamaguchi, M. Komaki, E. Abe, N. Takahashi, T. Ikeda, V. Rosen, J.M. Wozney, A. Fujisawa-Sehara, and T. Suda. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J Cell Biol* 127:1755-1766.
114. Murray, S.S., E.J. Murray, C.A. Glackin, and M.R. Urist. 1993. Bone morphogenetic protein inhibits differentiation and affects expression of helix-loop-helix regulatory molecules in myoblastic cells. *J Cell Biochem* 53:51-60.
115. Miyazawa, K., M. Shinozaki, T. Hara, T. Furuya, and K. Miyazono. 2002. Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells* 7:1191-1204.
116. Miyazono, K., and K. Miyazawa. 2002. Id: a target of BMP signaling. *Sci STKE* 2002:pe40.
117. Sun, X.H., N.G. Copeland, N.A. Jenkins, and D. Baltimore. 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol Cell Biol* 11:5603-5611.
118. Jen, Y., H. Weintraub, and R. Benezra. 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. *Genes Dev* 6:1466-1479.
119. Chen, B., B.H. Han, X.H. Sun, and R.W. Lim. 1997. Inhibition of muscle-specific gene expression by Id3: requirement of the C-terminal region of the protein for stable expression and function. *Nucleic Acids Res* 25:423-430.
120. Marcelle, C., M.R. Stark, and M. Bronnerfraser. 1997. Coordinate Actions Of Bmps, Wnts, Shh and Noggin Mediate Patterning Of the Dorsal Somite. *Development* 124:3955-3963.
121. Streit, A., and C.D. Stern. 1999. Mesoderm patterning and somite formation during node regression: differential effects of chordin and noggin. *Mech Dev* 85:85-96.
122. McMahon, J.A., S. Takada, L.B. Zimmerman, C.M. Fan, R.M. Harland, and A.P. McMahon. 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev* 12:1438-1452.
123. Reshef, R., M. Maroto, and A.B. Lassar. 1998. Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. *Genes Dev* 12:290-303.
124. Guo, X., and X.F. Wang. 2009. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res* 19:71-88.
125. Sheng, N., Z. Xie, C. Wang, G. Bai, K. Zhang, Q. Zhu, J. Song, F. Guillemot, Y.G. Chen, A. Lin, and N. Jing. 2010. Retinoic acid regulates bone morphogenetic protein

- signal duration by promoting the degradation of phosphorylated Smad1. *Proc Natl Acad Sci U S A* 107:18886-18891.
126. Buckingham, M., S. Meilhac, and S. Zaffran. 2005. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet* 6:826-835.
 127. Kelly, R.G., N.A. Brown, and M.E. Buckingham. 2001. The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev Cell* 1:435-440.
 128. Zaffran, S., R.G. Kelly, S.M. Meilhac, M.E. Buckingham, and N.A. Brown. 2004. Right ventricular myocardium derives from the anterior heart field. *Circ Res* 95:261-268.
 129. Saga, Y., N. Hata, S. Kobayashi, T. Magnuson, M.F. Seldin, and M.M. Taketo. 1996. MesP1: a novel basic helix-loop-helix protein expressed in the nascent mesodermal cells during mouse gastrulation. *Development* 122:2769-2778.
 130. Saga, Y., S. Miyagawa-Tomita, A. Takagi, S. Kitajima, J. Miyazaki, and T. Inoue. 1999. MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. *Development* 126:3437-3447.
 131. Saga, Y., S. Kitajima, and S. Miyagawa-Tomita. 2000. Mesp1 expression is the earliest sign of cardiovascular development. *Trends Cardiovasc Med* 10:345-352.
 132. Lindsley, R.C., J.G. Gill, T.L. Murphy, E.M. Langer, M. Cai, M. Mashayekhi, W. Wang, N. Niwa, J.M. Nerbonne, M. Kyba, and K.M. Murphy. 2008. Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs. *Cell Stem Cell* 3:55-68.
 133. Kitajima, S., S. Miyagawa-Tomita, T. Inoue, J. Kanno, and Y. Saga. 2006. Mesp1-nonexpressing cells contribute to the ventricular cardiac conduction system. *Dev Dyn* 235:395-402.
 134. David, R., C. Brenner, J. Stieber, F. Schwarz, S. Brunner, M. Vollmer, E. Mentele, J. Muller-Hocker, S. Kitajima, H. Lickert, R. Rupp, and W.M. Franz. 2008. MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. *Nat Cell Biol* 10:338-345.
 135. Bondue, A., G. Lapouge, C. Paulissen, C. Semeraro, M. Iacovino, M. Kyba, and C. Blanpain. 2008. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* 3:69-84.
 136. Bondue, A., and C. Blanpain. 2010. Mesp1: a key regulator of cardiovascular lineage commitment. *Circ Res* 107:1414-1427.

137. Akazawa, H., and I. Komuro. 2005. Cardiac transcription factor Csx/Nkx2-5: Its role in cardiac development and diseases. *Pharmacol Ther* 107:252-268.
138. Lyons, I., L.M. Parsons, L. Hartley, R. Li, J.E. Andrews, L. Robb, and R.P. Harvey. 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev* 9:1654-1666.
139. Tanaka, M., Z. Chen, S. Bartunkova, N. Yamasaki, and S. Izumo. 1999. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* 126:1269-1280.
140. Bodmer, R. 1993. The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. *Development* 118:719-729.
141. Nakashima, Y., K. Ono, Y. Yoshida, Y. Kojima, T. Kita, M. Tanaka, and T. Kimura. 2009. The search for Nkx2-5-regulated genes using purified embryonic stem cell-derived cardiomyocytes with Nkx2-5 gene targeting. *Biochem Biophys Res Commun* 390:821-826.
142. Fu, Y., W. Yan, T.J. Mohun, and S.M. Evans. 1998. Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. *Development* 125:4439-4449.
143. Jamali, M., P.J. Rogerson, S. Wilton, and I.S. Skerjanc. 2001. Nkx2-5 activity is essential for cardiomyogenesis. *J Biol Chem* 276:42252-42258.
144. Tanaka, M., M. Schinke, H.S. Liao, N. Yamasaki, and S. Izumo. 2001. Nkx2.5 and Nkx2.6, homologs of *Drosophila* tinman, are required for development of the pharynx. *Mol Cell Biol* 21:4391-4398.
145. Tanaka, M., N. Yamasaki, and S. Izumo. 2000. Phenotypic characterization of the murine Nkx2.6 homeobox gene by gene targeting. *Mol Cell Biol* 20:2874-2879.
146. Prall, O.W., M.K. Menon, M.J. Solloway, Y. Watanabe, S. Zaffran, F. Bajolle, C. Biben, J.J. McBride, B.R. Robertson, H. Chaulet, F.A. Stennard, N. Wise, D. Schaft, O. Wolstein, M.B. Furtado, H. Shiratori, K.R. Chien, H. Hamada, B.L. Black, Y. Saga, E.J. Robertson, M.E. Buckingham, and R.P. Harvey. 2007. An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell* 128:947-959.
147. Targoff, K.L., T. Schell, and D. Yelon. 2008. Nkx genes regulate heart tube extension and exert differential effects on ventricular and atrial cell number. *Dev Biol* 322:314-321.
148. Skerjanc, I.S., H. Petropoulos, A.G. Ridgeway, and S. Wilton. 1998. Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. *J Biol Chem* 273:34904-34910.

149. Durocher, D., F. Charron, R. Warren, R.J. Schwartz, and M. Nemer. 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J* 16:5687-5696.
150. Hiroi, Y., S. Kudoh, K. Monzen, Y. Ikeda, Y. Yazaki, R. Nagai, and I. Komuro. 2001. Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat Genet* 28:276-280.
151. Vincentz, J.W., R.M. Barnes, B.A. Firulli, S.J. Conway, and A.B. Firulli. 2008. Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. *Dev Dyn* 237:3809-3819.
152. Bruneau, B.G., G. Nemer, J.P. Schmitt, F. Charron, L. Robitaille, S. Caron, D.A. Conner, M. Gessler, M. Nemer, C.E. Seidman, and J.G. Seidman. 2001. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* 106:709-721.
153. Habets, P.E., A.F. Moorman, D.E. Clout, M.A. van Roon, M. Lingbeek, M. van Lohuizen, M. Campione, and V.M. Christoffels. 2002. Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev* 16:1234-1246.
154. Nemer, G., and M. Nemer. 2003. Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Dev Biol* 254:131-148.
155. Heikinheimo, M., J.M. Scandrett, and D.B. Wilson. 1994. Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev Biol* 164:361-373.
156. Morrisey, E.E., H.S. Ip, M.M. Lu, and M.S. Parmacek. 1996. GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev Biol* 177:309-322.
157. Morrisey, E.E., H.S. Ip, Z. Tang, M.M. Lu, and M.S. Parmacek. 1997. GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. *Dev Biol* 183:21-36.
158. Brewer, A., and J. Pizzey. 2006. GATA factors in vertebrate heart development and disease. *Expert Rev Mol Med* 8:1-20.
159. Kuo, C.T., E.E. Morrisey, R. Anandappa, K. Sigrist, M.M. Lu, M.S. Parmacek, C. Soudais, and J.M. Leiden. 1997. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev* 11:1048-1060.

160. Molkenin, J.D., Q. Lin, S.A. Duncan, and E.N. Olson. 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev* 11:1061-1072.
161. Narita, N., M. Bielinska, and D.B. Wilson. 1997. Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. *Development* 124:3755-3764.
162. Molkenin, J.D., K.M. Tymitz, J.A. Richardson, and E.N. Olson. 2000. Abnormalities of the genitourinary tract in female mice lacking GATA5. *Mol Cell Biol* 20:5256-5260.
163. Koutsourakis, M., A. Langeveld, R. Patient, R. Beddington, and F. Grosveld. 1999. The transcription factor GATA6 is essential for early extraembryonic development. *Development* 126:723-732.
164. Zhao, R., A.J. Watt, M.A. Battle, J. Li, B.J. Bondow, and S.A. Duncan. 2008. Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. *Dev Biol* 317:614-619.
165. Grepin, C., L. Robitaille, T. Antakly, and M. Nemer. 1995. Inhibition of transcription factor GATA-4 expression blocks in vitro cardiac muscle differentiation. *Mol Cell Biol* 15:4095-4102.
166. Hu, D.L., F.K. Chen, Y.Q. Liu, Y.H. Sheng, R. Yang, X.Q. Kong, K.J. Cao, H.T. Gu, and L.M. Qian. 2010. GATA-4 promotes the differentiation of P19 cells into cardiac myocytes. *Int J Mol Med* 26:365-372.
167. Latinkic, B.V., S. Kotecha, and T.J. Mohun. 2003. Induction of cardiomyocytes by GATA4 in *Xenopus* ectodermal explants. *Development* 130:3865-3876.
168. Grepin, C., G. Nemer, and M. Nemer. 1997. Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* 124:2387-2395.
169. Ieda, M., J.D. Fu, P. Delgado-Olguin, V. Vedantham, Y. Hayashi, B.G. Bruneau, and D. Srivastava. 2010. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142:375-386.
170. Plageman, T.F., Jr., and K.E. Yutzey. 2005. T-box genes and heart development: putting the "T" in heart. *Dev Dyn* 232:11-20.
171. Showell, C., O. Binder, and F.L. Conlon. 2004. T-box genes in early embryogenesis. *Dev Dyn* 229:201-218.
172. Basson, C.T., D.R. Bachinsky, R.C. Lin, T. Levi, J.A. Elkins, J. Soultz, D. Grayzel, E. Kroumpouzou, T.A. Traill, J. Leblanc-Straceski, B. Renault, R. Kucherlapati, J.G. Seidman, and C.E. Seidman. 1997. Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat Genet* 15:30-35.

173. Horb, M.E., and G.H. Thomsen. 1999. Tbx5 is essential for heart development. *Development* 126:1739-1751.
174. Plageman, T.F., Jr., and K.E. Yutzey. 2006. Microarray analysis of Tbx5-induced genes expressed in the developing heart. *Dev Dyn* 235:2868-2880.
175. Takeuchi, J.K., and B.G. Bruneau. 2009. Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature* 459:708-711.
176. Liu, P., M. Wakamiya, M.J. Shea, U. Albrecht, R.R. Behringer, and A. Bradley. 1999. Requirement for Wnt3 in vertebrate axis formation. *Nat Genet* 22:361-365.
177. Lindsley, R.C., J.G. Gill, M. Kyba, T.L. Murphy, and K.M. Murphy. 2006. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* 133:3787-3796.
178. Ai, D., X. Fu, J. Wang, M.F. Lu, L. Chen, A. Baldini, W.H. Klein, and J.F. Martin. 2007. Canonical Wnt signaling functions in second heart field to promote right ventricular growth. *Proc Natl Acad Sci U S A* 104:9319-9324.
179. Cohen, E.D., Z. Wang, J.J. Lepore, M.M. Lu, M.M. Taketo, D.J. Epstein, and E.E. Morrisey. 2007. Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *J Clin Invest* 117:1794-1804.
180. Ueno, S., G. Weidinger, T. Osugi, A.D. Kohn, J.L. Golob, L. Pabon, H. Reinecke, R.T. Moon, and C.E. Murry. 2007. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc Natl Acad Sci U S A* 104:9685-9690.
181. Naito, A.T., I. Shiojima, H. Akazawa, K. Hidaka, T. Morisaki, A. Kikuchi, and I. Komuro. 2006. Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc Natl Acad Sci U S A* 103:19812-19817.
182. Qyang, Y., S. Martin-Puig, M. Chiravuri, S. Chen, H. Xu, L. Bu, X. Jiang, L. Lin, A. Granger, A. Moretti, L. Caron, X. Wu, J. Clarke, M.M. Taketo, K.L. Laugwitz, R.T. Moon, P. Gruber, S.M. Evans, S. Ding, and K.R. Chien. 2007. The renewal and differentiation of Isl1+ cardiovascular progenitors are controlled by a Wnt/beta-catenin pathway. *Cell Stem Cell* 1:165-179.
183. Zelarayan, L.C., C. Noack, B. Sekkali, J. Kmecova, C. Gehrke, A. Renger, M.P. Zafiriou, R. van der Nagel, R. Dietz, L.J. de Windt, J.L. Balligand, and M.W. Bergmann. 2008. Beta-Catenin downregulation attenuates ischemic cardiac remodeling through enhanced resident precursor cell differentiation. *Proc Natl Acad Sci U S A* 105:19762-19767.

184. Pandur, P., M. Lasche, L.M. Eisenberg, and M. Kuhl. 2002. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* 418:636-641.
185. Terami, H., K. Hidaka, T. Katsumata, A. Iio, and T. Morisaki. 2004. Wnt11 facilitates embryonic stem cell differentiation to Nkx2.5-positive cardiomyocytes. *Biochem Biophys Res Commun* 325:968-975.
186. Frasch, M. 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature* 374:464-467.
187. Somi, S., A.A. Buffing, A.F. Moorman, and M.J. Van Den Hoff. 2004. Dynamic patterns of expression of BMP isoforms 2, 4, 5, 6, and 7 during chicken heart development. *Anat Rec A Discov Mol Cell Evol Biol* 279:636-651.
188. Schultheiss, T.M., J.B. Burch, and A.B. Lassar. 1997. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev* 11:451-462.
189. van Wijk, B., A.F. Moorman, and M.J. van den Hoff. 2007. Role of bone morphogenetic proteins in cardiac differentiation. *Cardiovasc Res* 74:244-255.
190. Schlange, T., B. Andree, H.H. Arnold, and T. Brand. 2000. BMP2 is required for early heart development during a distinct time period. *Mech Dev* 91:259-270.
191. Lien, C.L., J. McAnally, J.A. Richardson, and E.N. Olson. 2002. Cardiac-specific activity of an Nkx2-5 enhancer requires an evolutionarily conserved Smad binding site. *Dev Biol* 244:257-266.
192. Liberatore, C.M., R.D. Searcy-Schrick, E.B. Vincent, and K.E. Yutzey. 2002. Nkx-2.5 gene induction in mice is mediated by a Smad consensus regulatory region. *Dev Biol* 244:243-256.
193. Walters, M.J., G.A. Wayman, and J.L. Christian. 2001. Bone morphogenetic protein function is required for terminal differentiation of the heart but not for early expression of cardiac marker genes. *Mech Dev* 100:263-273.
194. Shi, Y., S. Katsev, C. Cai, and S. Evans. 2000. BMP signaling is required for heart formation in vertebrates. *Dev Biol* 224:226-237.
195. Dudley, A.T., and E.J. Robertson. 1997. Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev Dyn* 208:349-362.
196. Zhang, H., and A. Bradley. 1996. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* 122:2977-2986.

197. Dudley, A.T., K.M. Lyons, and E.J. Robertson. 1995. A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* 9:2795-2807.
198. Kingsley, D.M., A.E. Bland, J.M. Grubber, P.C. Marker, L.B. Russell, N.G. Copeland, and N.A. Jenkins. 1992. The mouse short ear skeletal morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF beta superfamily. *Cell* 71:399-410.
199. Solloway, M.J., and E.J. Robertson. 1999. Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development* 126:1753-1768.
200. Monzen, K., Y. Hiroi, S. Kudoh, H. Akazawa, T. Oka, E. Takimoto, D. Hayashi, T. Hosoda, M. Kawabata, K. Miyazono, S. Ishii, Y. Yazaki, R. Nagai, and I. Komuro. 2001. Smads, TAK1, and their common target ATF-2 play a critical role in cardiomyocyte differentiation. *J Cell Biol* 153:687-698.
201. Pal, R., and A. Khanna. 2005. Role of hepatocyte-like cells in the differentiation of cardiomyocytes from mouse embryonic stem cells. *Stem Cells Dev* 14:153-161.
202. Monzen, K., I. Shiojima, Y. Hiroi, S. Kudoh, T. Oka, E. Takimoto, D. Hayashi, T. Hosoda, A. Habara-Ohkubo, T. Nakaoka, T. Fujita, Y. Yazaki, and I. Komuro. 1999. Bone Morphogenetic Proteins Induce Cardiomyocyte Differentiation through the Mitogen-Activated Protein Kinase Kinase Kinase TAK1 and Cardiac Transcription Factors Csx/Nkx-2.5 and GATA-4. *Mol Cell Biol* 19:7096-7105.
203. Jamali, M., C. Karamboulas, P.J. Rogerson, and I.S. Skerjanc. 2001. BMP signaling regulates Nkx2-5 activity during cardiomyogenesis. *FEBS Lett* 509:126-130.
204. Behfar, A., L.V. Zingman, D.M. Hodgson, J.M. Rauzier, G.C. Kane, A. Terzic, and M. Puceat. 2002. Stem cell differentiation requires a paracrine pathway in the heart. *Faseb J* 16:1558-1566.
205. Yuasa, S., Y. Itabashi, U. Koshimizu, T. Tanaka, K. Sugimura, M. Kinoshita, F. Hattori, S. Fukami, T. Shimazaki, S. Ogawa, H. Okano, and K. Fukuda. 2005. Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. *Nat Biotechnol* 23:607-611.
206. Moss, J.B., J. Xavier-Neto, M.D. Shapiro, S.M. Nayeem, P. McCaffery, U.C. Drager, and N. Rosenthal. 1998. Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. *Dev Biol* 199:55-71.
207. Stainier, D.Y., and M.C. Fishman. 1992. Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. *Dev Biol* 153:91-101.

208. Osmond, M.K., A.J. Butler, F.C. Voon, and R. Bellairs. 1991. The effects of retinoic acid on heart formation in the early chick embryo. *Development* 113:1405-1417.
209. Yutzey, K.E., J.T. Rhee, and D. Bader. 1994. Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. *Development* 120:871-883.
210. Xavier-Neto, J., C.M. Neville, M.D. Shapiro, L. Houghton, G.F. Wang, W. Nikovits, Jr., F.E. Stockdale, and N. Rosenthal. 1999. A retinoic acid-inducible transgenic marker of sino-atrial development in the mouse heart. *Development* 126:2677-2687.
211. Keegan, B.R., J.L. Feldman, G. Begemann, P.W. Ingham, and D. Yelon. 2005. Retinoic acid signaling restricts the cardiac progenitor pool. *Science* 307:247-249.
212. Collop, A.H., J.A. Broomfield, R.A. Chandraratna, Z. Yong, S.J. Deimling, S.J. Kolker, D.L. Weeks, and T.A. Drysdale. 2006. Retinoic acid signaling is essential for formation of the heart tube in *Xenopus*. *Dev Biol* 291:96-109.
213. Ryckebusch, L., Z. Wang, N. Bertrand, S.C. Lin, X. Chi, R. Schwartz, S. Zaffran, and K. Niederreither. 2008. Retinoic acid deficiency alters second heart field formation. *Proc Natl Acad Sci U S A* 105:2913-2918.
214. Lin, S.C., P. Dolle, L. Ryckebusch, M. Nosedá, S. Zaffran, M.D. Schneider, and K. Niederreither. 2010. Endogenous retinoic acid regulates cardiac progenitor differentiation. *Proc Natl Acad Sci U S A* 107:9234-9239.
215. Wobus, A.M., Rohwedel, J., Maltsev, V., Hescheler, J. 1994. In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. *Roux's Arch Dev Biol* 204:36-45.
216. Wobus, A.M., G. Kaomei, J. Shan, M.C. Wellner, J. Rohwedel, G. Ji, B. Fleischmann, H.A. Katus, J. Hescheler, and W.M. Franz. 1997. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J Mol Cell Cardiol* 29:1525-1539.
217. Hidaka, K., J.K. Lee, H.S. Kim, C.H. Ihm, A. Iio, M. Ogawa, S. Nishikawa, I. Kodama, and T. Morisaki. 2003. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. *FASEB J* 17:740-742.
218. Zhang, Q., J. Jiang, P. Han, Q. Yuan, J. Zhang, X. Zhang, Y. Xu, H. Cao, Q. Meng, L. Chen, T. Tian, X. Wang, P. Li, J. Hescheler, G. Ji, and Y. Ma. 2011. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. *Cell Res* 21:579-587.
219. Skerjanc, I.S. 1999. Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. *Trends Cardiovasc Med* 9:139-143.

220. Rossant, J., and M.W. McBurney. 1982. The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. *J Embryol Exp Morphol* 70:99-112.
221. Savage, J., A.J. Conley, A. Blais, and I.S. Skerjanc. 2009. SOX15 and SOX7 differentially regulate the myogenic program in P19 cells. *Stem Cells* 27:1231-1243.
222. Jones-Villeneuve, E.M., M.W. McBurney, K.A. Rogers, and V.I. Kalnins. 1982. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J Cell Biol* 94:253-262.
223. Schuldiner, M., R. Eiges, A. Eden, O. Yanuka, J. Itskovitz-Eldor, R.S. Goldstein, and N. Benvenisty. 2001. Induced neuronal differentiation of human embryonic stem cells. *Brain Res* 913:201-205.
224. Manabe, I., and G.K. Owens. 2001. CArG elements control smooth muscle subtype-specific expression of smooth muscle myosin in vivo. *J Clin Invest* 107:823-834.
225. Huang, H., X. Zhao, L. Chen, C. Xu, X. Yao, Y. Lu, L. Dai, and M. Zhang. 2006. Differentiation of human embryonic stem cells into smooth muscle cells in adherent monolayer culture. *Biochem Biophys Res Commun* 351:321-327.
226. Schnerch, A., C. Cerdan, and M. Bhatia. 2010. Distinguishing between mouse and human pluripotent stem cell regulation: the best laid plans of mice and men. *Stem Cells* 28:419-430.
227. Boyer, L.A., T.I. Lee, M.F. Cole, S.E. Johnstone, S.S. Levine, J.P. Zucker, M.G. Guenther, R.M. Kumar, H.L. Murray, R.G. Jenner, D.K. Gifford, D.A. Melton, R. Jaenisch, and R.A. Young. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122:947-956.
228. Loh, Y.H., Q. Wu, J.L. Chew, V.B. Vega, W. Zhang, X. Chen, G. Bourque, J. George, B. Leong, J. Liu, K.Y. Wong, K.W. Sung, C.W. Lee, X.D. Zhao, K.P. Chiu, L. Lipovich, V.A. Kuznetsov, P. Robson, L.W. Stanton, C.L. Wei, Y. Ruan, B. Lim, and H.H. Ng. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* 38:431-440.
229. Babaie, Y., R. Herwig, B. Greber, T.C. Brink, W. Wruck, D. Groth, H. Lehrach, T. Burdon, and J. Adjaye. 2007. Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. *Stem Cells* 25:500-510.
230. Brons, I.G., L.E. Smithers, M.W. Trotter, P. Rugg-Gunn, B. Sun, S.M. Chuva de Sousa Lopes, S.K. Howlett, A. Clarkson, L. Ahrlund-Richter, R.A. Pedersen, and L. Vallier. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448:191-195.

231. Tesar, P.J., J.G. Chenoweth, F.A. Brook, T.J. Davies, E.P. Evans, D.L. Mack, R.L. Gardner, and R.D. McKay. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448:196-199.
232. Kattman, S.J., A.D. Witty, M. Gagliardi, N.C. Dubois, M. Niapour, A. Hotta, J. Ellis, and G. Keller. 2011. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 8:228-240.
233. Barberi, T., M. Bradbury, Z. Dincer, G. Panagiotakos, N.D. Socci, and L. Studer. 2007. Derivation of engraftable skeletal myoblasts from human embryonic stem cells. *Nat Med* 13:642-648.
234. Zheng, J.K., Y. Wang, A. Karandikar, Q. Wang, H. Gai, A.L. Liu, C. Peng, and H.Z. Sheng. 2006. Skeletal myogenesis by human embryonic stem cells. *Cell Res* 16:713-722.
235. Darabi, R., J. Baik, M. Clee, M. Kyba, R. Tupler, and R.C. Perlingeiro. 2009. Engraftment of embryonic stem cell-derived myogenic progenitors in a dominant model of muscular dystrophy. *Exp Neurol* 220:212-216.
236. Darabi, R., K. Gehlbach, R.M. Bachoo, S. Kamath, M. Osawa, K.E. Kamm, M. Kyba, and R.C. Perlingeiro. 2008. Functional skeletal muscle regeneration from differentiating embryonic stem cells. *Nat Med* 14:134-143.
237. Darabi, R., F.N. Santos, A. Filareto, W. Pan, R. Koene, M.A. Rudnicki, M. Kyba, and R.C. Perlingeiro. 2011. Assessment of the myogenic stem cell compartment following transplantation of pax3/pax7-induced embryonic stem cell-derived progenitors. *Stem Cells* 29:777-790.
238. Kuang, S., K. Kuroda, F. Le Grand, and M.A. Rudnicki. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell* 129:999-1010.

2: RETINOIC ACID ENHANCES SKELETAL MUSCLE PROGENITOR FORMATION AND BYPASSES INHIBITION BY BONE MORPHOGENETIC PROTEIN 4 BUT NOT BY A DOMINANT NEGATIVE B-CATENIN*

* A version of this chapter has been published.

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2.1 Abstract

Understanding stem cell differentiation is essential for the future design of cell therapies.

While retinoic acid (RA) is the most potent small molecule enhancer of skeletal myogenesis in stem cells, the stage and mechanism of its function has not yet been elucidated. Further, the intersection of RA with other signalling pathways that stimulate or inhibit myogenesis (such as Wnt and BMP4, respectively) is unknown. Thus, the purpose of this study is to examine the molecular mechanisms by which RA enhances skeletal myogenesis and interacts with Wnt and BMP4 signalling during P19 or mouse embryonic stem cell (mESC) differentiation. Treatment of P19 or mESCs with low levels of RA led to an enhancement of skeletal myogenesis by upregulating the expression of the mesodermal marker, Wnt3a, the skeletal muscle progenitor factors Pax3 and Meox1, and the myogenic regulatory factors (MRFs) MyoD and myogenin. By chromatin immunoprecipitation, RA receptors (RARs) bound directly to regulatory regions in the Wnt3a, Pax3, and Meox1 genes and RA activated a β -catenin-responsive promoter in aggregated P19 cells. In the presence of a dominant negative β -catenin/engrailed repressor fusion protein, RA could not bypass the inhibition of skeletal myogenesis nor upregulate Meox1 or MyoD. Thus, RA functions both upstream of and in parallel with Wnt signalling. In contrast, it functions downstream of BMP4, as it abrogates BMP4 inhibition of myogenesis and Meox1, Pax3, and MyoD expression. Furthermore, RA downregulated BMP4 expression and upregulated the BMP4 inhibitor, Tob1. Finally, RA inhibited cardiomyogenesis but not in the presence of BMP4. RA can enhance skeletal myogenesis in stem cells at the muscle specification/progenitor stage by activating RARs bound directly to mesoderm and skeletal muscle progenitor genes,

activating β -catenin function and inhibiting bone morphogenetic protein (BMP) signalling. Thus, a signalling pathway can function at multiple levels to positively regulate a developmental program and can function by abrogating inhibitory pathways. Finally, since RA enhances skeletal muscle progenitor formation, it will be a valuable tool for designing future stem cell therapies.

2.2 Introduction

The initiation of skeletal myogenesis involves a complex interplay of signalling molecules secreted from the tissues surrounding the somite, including Wnt, Sonic hedgehog, and Bone morphogenetic proteins 4 (BMP4) (1-5). Somites respond to the various signals by activating the expression of transcription factors that specify cells to the skeletal muscle lineage, including Pax3, Meox1 and Gli2 (6-10). Commitment into skeletal myoblasts is dependent on the expression of the myogenic regulatory factors (MRFs), including MyoD, Myf-5, myogenin and myf-6/MRF4/ herculin, and is regulated by factors in the dermomyotome (11, 12).

P19 cells are pluripotent embryonal carcinoma (EC) cells, derived from mouse embryonic stem (ES) cells, that can differentiate into cardiac and skeletal muscle in a dimethyl sulfoxide (DMSO)- and aggregation-dependent manner (13). While cells grown in monolayer maintain their stem cell phenotype, the process of cellular aggregation initiates mesoderm induction, shown by the expression of Brachyury T (14). Subsequent muscle development proceeds in the presence of DMSO. The order of transcription factors and signalling pathways for myogenesis in P19 cells appear to be similar to those during early embryogenesis. Thus P19 cells are a useful tool for examining *in vitro* myogenesis, potentially leading to novel mechanisms relevant to ES stem cell therapy.

Retinoic acid (RA) is a derivative of vitamin A and plays a crucial role in a wide variety of embryonic developmental processes (15). In the embryo, the ability of RA to bind its receptors (retinoic acid receptors [RARs]/ retinoid X receptors [RXRs]) is precisely controlled by regulating the availability of RA through proteins that synthesize RA, such as retinaldehyde dehydrogenase 2 (RALDH2), and those that metabolize RA, such as Cyp26, and other proteins that transport or bind RA. Low levels of RA are known to enhance skeletal myogenesis in stem cells and myoblast cell lines (16-18). RA can regulate MRF expression in myoblasts and chick limb (17-19), whereas RARs interact and synergize with MRFs (20). However, the exact stage(s) at which RA functions to enhance skeletal myogenesis in a stem cell context has not been clearly defined.

Altered RA signalling in vertebrates affects body patterning, generating homeotic transformations and/or segmentation defects (21). In *Xenopus* embryos, RA signalling regulates segmental patterning by promoting anterior segmental polarity and by positioning segmental boundaries (22). In mice, RA coordinates somitogenesis and left-right patterning (23). How the effect of RA on the somite impacts on the development of the myotome is not well understood.

In the canonical pathway, Wnt binds to cell surface receptors of the frizzled family, leading to the activation of Dishevelled and stabilization of cytosolic β -catenin (24). In a simplified view, β -catenin enters the nucleus, binds the T-cell factor / lymphoid enhancer factor (TCF/LEF) family of transcription factors, and activates gene expression. Several studies have shown that exogenous Wnt and/or activated β -catenin can replace the dorsal neural tube in the induction of myogenesis in somite explant cultures (25). A combination of Wnt and Shh signals regulates the expression levels of both β -catenin and Lef1 in the

myotome prior to MyoD expression in the chick (26). Further, β -catenin regulates the expression of Pax3 (27). In P19 cells, a dominant negative β -catenin inhibits the expression of Pax3, Gli2, Meox1, MyoD, and abrogates myogenesis (8). Finally, Wnt was shown to act directly on the Myf5 epaxial enhancer via β -catenin (28). Therefore, there is strong evidence that Wnt signalling regulates specification and commitment into the skeletal muscle lineage. How Wnt signalling intersects with RA signalling during myogenesis is unknown.

BMP4 belongs to the TGF- β superfamily of peptide growth factors (29). BMPs inhibit myogenesis in myoblast cell lines, limb micromass cultures, and developing somites (3, 30, 31). Noggin signals are derived from the notochord and the somite. Noggin signalling is believed to counteract the inhibitory effects of BMP4 on the epaxial somite (32-34). Further experiments using somite explants showed that relative levels of BMP4 and noggin regulated the activity of Pax3 to control the temporal and spatial activation of the MRFs (35). Therefore, extensive studies have demonstrated the inhibition of embryonic skeletal myogenesis by BMP. How BMP signalling intersects with RA signalling is unknown.

The role of BMP in cardiomyogenesis has also been extensively studied. In *Drosophila*, the BMP homologue decapentaplegic protein (dpp) is secreted from the dorsal ectoderm and maintains tinman expression in the mesoderm (36). Similarly, in chick BMP2 or -4 is expressed in tissues adjacent to the precardiac mesoderm and can induce Nkx2-5 and GATA-4 expression (37, 38). Conversely, disruption of BMP signalling with noggin or dominant negative receptors can prevent cardiomyogenesis in chick, *Xenopus*, ES, P19 and P19CL6 cells (37, 39-45). Therefore, BMP/dpp signalling is essential in controlling cardiomyogenesis.

Studies with embryonic stem and embryonic carcinoma cells have shown that RA inhibits cardiomyogenesis when added at an early stage (16, 46, 47) and enhances ES cell

cardiomyogenesis when added at a late stage of differentiation (46, 48). RA can block myocardial gene expression, including *XNkx2.5*, in *Xenopus* embryos (49) and can alter cardiomyogenesis proliferation and patterning in other model systems (50-53). Mice lacking various combinations of RXRs and RARs have shown that retinoids are required to prevent differentiation and support proliferation of ventricular cardiomyocytes (54, 55). RA deficiency in *RALDH2* *-/-* mice alters second heart field formation (56, 57). Clearly, RA affects the timing and positioning of cardiomyogenesis at multiple levels and further studies are required to dissect out the role of RA at each step of development.

Here we investigate signalling events leading to the control of stem cell entry into skeletal and cardiac muscle lineages by RA, Wnt, and BMP4. We show that low levels of RA stimulate skeletal myogenesis by accelerating and increasing the expression of *Wnt3a*, *Pax3*, *Meox1*, and MRFs. This early and enhanced activation of skeletal muscle is refractory to inhibitory signals from BMP4 but not from a dominant negative β -catenin. Furthermore, low levels of RA inhibit stem cell differentiation into the cardiac muscle lineage, as shown by the absence of *GATA-4* expression. The inhibitory activity of RA on cardiomyogenesis can be abrogated by the presence of BMP4. Therefore, BMP4 and RA function antagonistically to regulate each other's inhibition of entry into skeletal and cardiac muscle lineages, respectively. However, RA functions both upstream of and in parallel with Wnt signalling through β -catenin.

2.3 Materials and Methods

2.3.1 Plasmid Constructs

The expression construct phosphoglycerate kinase (PGK)-BMP4 contains a 1.8 Kb EcoRI BMP4 cDNA fragment, containing the complete coding sequence of human BMP4 (Wyeth Pharmaceuticals, MA, USA), driven by the PGK-1 promoter. The empty PGK vector was used as control. The constructs PGK-Puro, B17, and PGK-Lac-Z were previously described (92).

2.3.2 P19 Cell Culture and Isolation of Stable Cell lines

P19 embryonal carcinoma cells (American Type Culture Collection, VA, USA) were cultured as previously described (93) in α -minimum essential media supplemented with 5% cosmic calf serum (Hyclone, UT, USA) and 5% fetal bovine serum (Cansera, Rexdale, ON, Canada). P19[β -cat/EnR] cells were isolated and described previously (8). For P19[BMP4] cells, stable cell lines were created using similar protocols. Briefly, P19 cells were transfected with the DNA constructs PGK-BMP4 or the empty PGK vector along with PGK-puro, PGK-LacZ and B17 with the aid of the *Fugene 6* transfection kit (Roche Diagnostics Canada, Quebec, Canada) as per manufacturer's instructions. Twenty-four hours after transfection, cells were grown under puromycin selection for 10 days. Individual colonies were analysed for BMP4 expression.

To examine the role of BMP4 in P19 cell differentiation, P19 cells, P19[BMP4] cells, P19[control] cells, 1:1 mixtures of P19 and P19[BMP4] cells, and 1:1 mixture of P19 and P19[control] cells were aggregated with or without 0.8-1% DMSO for 4 days in petri dishes

(94). Subsequently cells were transferred to tissue culture plates or gelatin coated cover slips and examined for differentiation on days 6 or 9.

To examine the role of RA in P19 cell differentiation, P19 cells, P19[β -cat/EnR] cells P19[BMP4] cells, P19[control] cells, 1:1 mixtures of P19 and P19[BMP4] cells, and 1:1 mixture of P19 and P19[control] cells were aggregated with or without 0.8-1% DMSO in the presence of 0-30 nM all-trans RA. Stocks of RA were purchased and prepared every 3-4 months. Each new stock was titrated for optimal skeletal myogenesis.

2.3.3 Immunofluorescence

For analysis of muscle-specific markers, cultures were treated as described previously (95) with MF20 (96). Staining was visualized with a Zeiss Axioskop microscope and photographed with a Sony 3CCD camera. Images were formatted using Adobe Photoshop and Canvas software. Myogenic differentiation was quantified by counting five to 10 fields containing 100-200 cells.

For analysis of neuron-specific markers, cultures were plated on gelatin-coated coverslips and cultures were fixed with methanol at -20°C. Antigenic analysis was performed using mouse-anti-Tuj1 (Research Diagnostics, USA) guinea pig anti-DCX (Chemicon, USA) and Hoechst dye as a nuclear marker. To identify PAX3/7-positive cells, some cultures were also labeled with goat-anti-PAX3/7 (Invitrogen, Canada). Secondary antibodies were AMCA- or FITC-conjugated anti-mouse-IgG, Cy3-conjugated anti-guinea pig-IgG, (Jackson ImmunoResearch Laboratories, USA) and Alexa-488 conjugated anti-goat IgG (Invitrogen, Canada) where appropriate. Immunofluorescence was evaluated using OpenLab Software, version 3.4 (Improvision, MA, USA) on a Leica DMXRA2 microscope

equipped for epifluorescence. Antigen-positive cells were counted in three to 10 fields in triplicate samples performed over two independent experiments and data expressed as a percentage of the total cell number ($n=6-10$). Counts were performed by two investigators blind as to the treatment conditions of each field.

2.3.4 Northern blot analysis

Cells were harvested for RNA extraction using the Urea/lithium chloride method (97) as previously described (98). The cDNA fragments used to probe for MLC1/3 and MyoD (47) and for BMP4, GATA4, Pax3, Meox1, Myogenin, BrachyuryT, Wnt5b and 18s have been described previously (10). A 1.8 Kb Kpn1/Mlu1 full length human cDNA fragment was used to probe for Tob1 (GeneBank Accession Number: BC031406) and a 400bp HindIII fragment for mouse RALDH2 (GI Number 31982069).

2.3.5 mESC culture and differentiation

J1 or D3 mESCs were maintained in 15% fetal bovine serum in the presence of Leukemia Inhibitory Factor (LIF). Differentiation was induced by aggregating cells in the absence of LIF. Cells were aggregated in hanging drops containing 800 cells for 2 days and in suspension for a further 5 days. Aggregates were treated with increasing concentrations of retinoic acid from day 2 to day 5 or 7 of differentiation. On day 7 of differentiation, cells were plated on tissue culture plates and allowed to differentiate for a further 13 days. The efficiency of myocyte formation was enhanced by plating at a high cell density. Experiments were performed at least twice with each cell line with similar enhancement of skeletal myogenic precursors.

2.3.6 PCR Analysis

For RT-PCR southern analysis, total RNA was harvested from day 7 mES cells and 0.8 μ g was subjected to reverse transcription (Qiagen, Mississauga, Canada). The resultant cDNA was subjected to PCR with primers specific for Pax3, Gata-4 and β -actin (85, 99) and for MyoD and Meox1 (100) described previously. The PCR product was hybridized to DNA probes and visualized by autoradiography.

For quantitative real-time PCR (Q-PCR) expression analysis of mES cells, total RNA was harvested from differentiating cells using the RNeasy Micro kit (Qiagen, Mississauga, Canada). RNA harvested from P19 cells using the urea/lithium chloride extraction was further purified using the RNeasy Micro kit (Qiagen, Mississauga, Canada). The resultant cDNAs were used for Q-PCR, using protocols and primers for Pax3/7, Meox1, MyoD, myogenin and GAPDH as described previously (100). mRNA levels were normalized to β -actin or glyceraldehyde-3-phosphate dehydrogenase levels for the corresponding day and subsequently to levels in untreated samples for the corresponding day.

2.3.7 Promoter Analysis

P19 cells were transfected with 6 μ g of the super8 TOPFlash (101) or FOPFlash reporter plasmids (generous gifts from R Moon), as well as with 2 μ g of Renilla Luciferase (Promega, WI, USA), using Fugene Transfection Reagent (Roche Applied Sciences, QC, Canada). Cells were either left in monolayer culture or aggregated in the presence of 1% DMSO, 20mM LiCl, 1% DMSO with retinoic acid, or 1%DMSO with 50ng/ml BMP4. Luciferase assays were conducted using the Dual Luciferase Reporter Assay System from Promega, 24 hours after treatment. Luciferase activity was normalized to *Renilla* activity. Data from

samples treated with lithium chloride or RA were normalized to samples treated with DMSO alone.

2.3.8 Chromatin Immunoprecipitation

Protein was cross-linked to DNA and chromatin was harvested as described previously (100) from three 150 mm dishes of day 2 P19 aggregates, treated with 1% DMSO and 10nM RA. For immunoprecipitation, 2 µg of RAR antibody (Santa Cruz, CA,USA) or 2µg of rabbit immunoglobulin G antiserum (Chemicon, MA, USA) was incubated with chromatin and the immune complexes were captured by addition of protein-G sepharose beads, as described (100). After RNase A and proteinase K treatments, DNA was purified using Qiagen's PCR Purification Kit (Qiagen, ON, Canada). Relative enrichment of binding sites compared to the IgG negative control immunoprecipitation was analysed using SYBR green real-time PCR, as described above.

2.4 Results

2.4.1 RA inhibits cardiomyogenesis and enhances entry into the skeletal muscle lineage in P19 cells

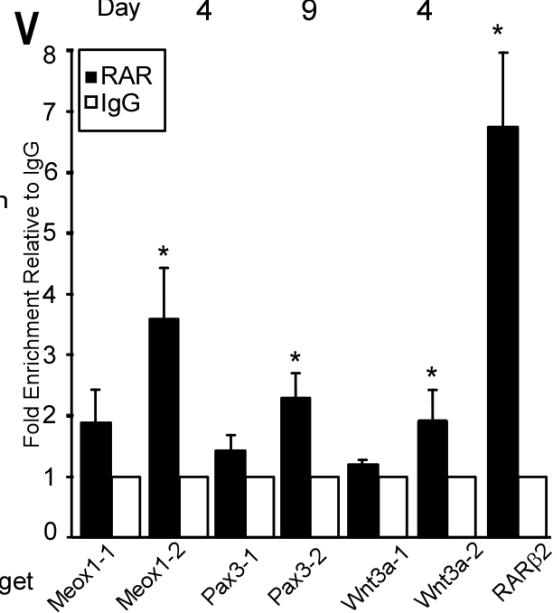
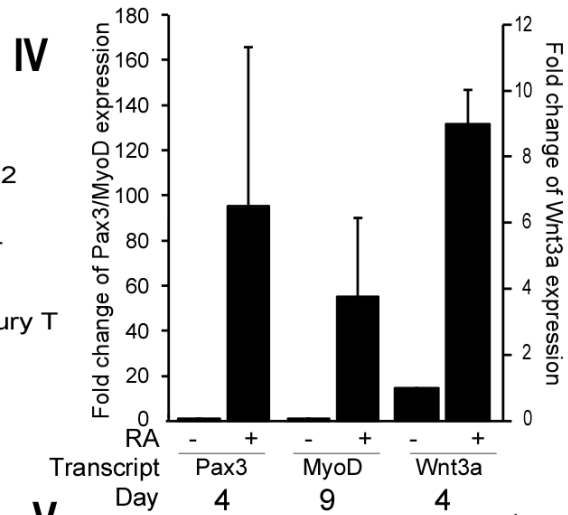
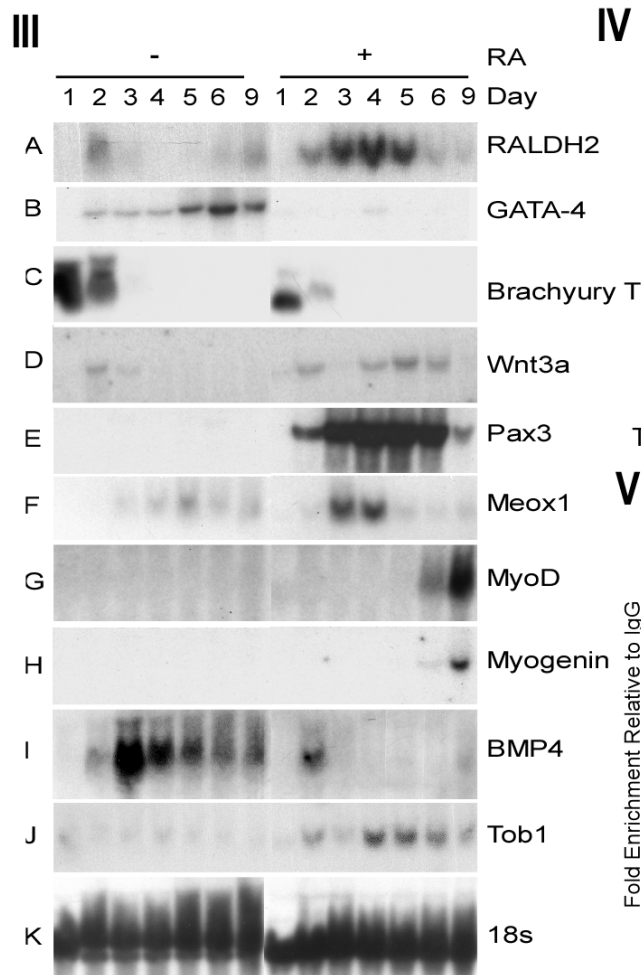
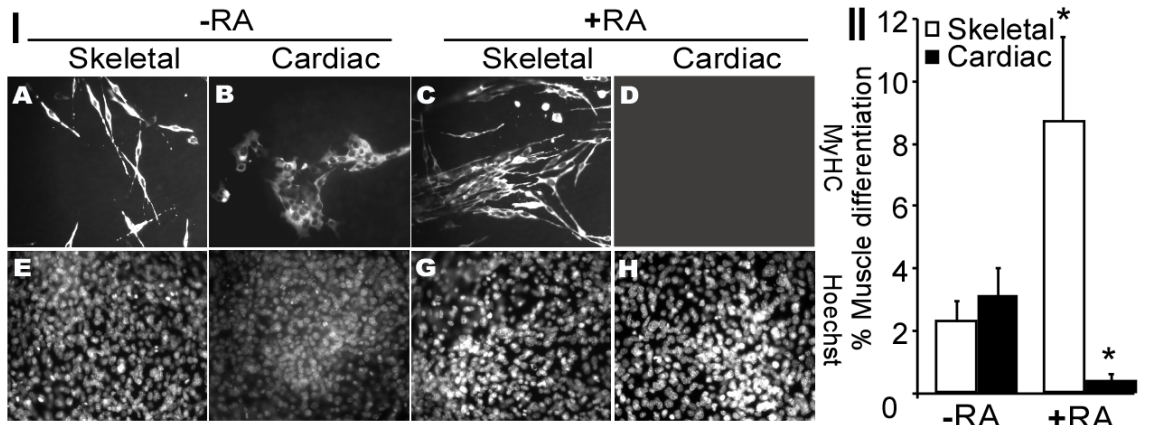
Previous studies have shown that RA can inhibit cardiomyogenesis and enhance skeletal myogenesis (16, 47), but the stage at which this occurs and the interaction with other signalling pathways have not been clearly defined. To investigate the mechanisms by which RA modulates myogenesis in P19 cells, various concentrations of RA, in the presence of DMSO, were examined. In agreement with previous results (47), it was found that skeletal myogenesis occurred in the presence of DMSO but not in its absence (summarized in Table 2.1). Furthermore, 3-30nM of RA with DMSO was to be sufficient to block cardiac and

enhance skeletal muscle development (data not shown). A time course of P19 cell differentiation was carried out in the presence of DMSO, with and without 30nM of RA. Cells were fixed on day 9 for immunofluorescence and stained with an anti-myosin heavy chain (MyHC) antibody, MF20, which identifies MyHC in both cardiac and skeletal myocytes. Cardiac and skeletal myocytes can be distinguished by their morphology and time of appearance, with rounded cardiac myocytes appearing by day 6 and elongated, bipolar skeletal myocytes by day 9 (13). Cells treated with RA did not differentiate into cardiac muscle, evidenced by the absence of rounded cardiac myocytes, expressing MyHC, compared to cells not treated with RA (Figure 2.1, panels IB and D). In contrast, significantly enhanced levels of bipolar skeletal myocytes were observed in the presence compared to the absence of RA (Figure 2.1, panels IA and C). Quantification of the number of cardiac and skeletal myocytes after treatment with RA showed that the fourfold increase in skeletal myocytes and eightfold loss of cardiac myocytes were statistically significant (Figure 2.1, panel II). Therefore, in agreement with others (58), RA inhibited cardiomyogenesis and enhanced skeletal myogenesis in P19 cells.

In order to examine the molecular basis of the effects of RA on myogenesis, total RNA was harvested from a time course of cells differentiated with and without RA and subjected to northern blot analysis. Endogenous RALDH2 levels were enhanced on day 2 of the DMSO-induced differentiation in the absence of exogenous RA (Figure 2.1, panel IIIA), indicating that endogenous RA signalling could be involved at this stage of development (59). RA-treated cells showed enhanced expression of RALDH2 from days 3-5 (Figure 2.1, panel IIIA).

Figure 2.1 RA inhibits cardiomyogenesis and enhances skeletal myogenesis in P19 cells.

P19 cells were aggregated with 0.8% dimethyl sulfoxide (DMSO) in the presence and absence of 30 nM RA. *Panel I:* Cells were fixed on day 9 for immunofluorescence with MF20 antibody (A-D) and counter stained with Hoechst dye (E-H). Magnification is 160x. *Panel II:* Cardiac ($n=3$) and skeletal ($n=4$) myogenesis were quantified by counting the number of MHC⁺ve myocytes as a percentage of the total. Average \pm standard error of mean (SEM) is shown and statistics were Student's t -test, $*P<0.05$. *Panel III:* Total RNA was harvested for northern blot analysis on the days indicated and hybridized to the cDNAs on the right. Lanes are spliced from the same autoradiogram. *Panel IV:* Quantitative polymerase chain reaction (PCR) analysis was performed on day 4 of differentiation for Pax3 and Wnt3a transcript levels ($n=2$) and on day 9 for MyoD levels ($n=4$). Results were expressed as fold change of transcript levels in the presence compared to the absence of RA treatment. *Panel V:* Chromatin immunoprecipitation experiments were performed on day 2 P19 aggregates treated with DMSO/ retinoic acid and analysed by real-time PCR using primers for sites within regulatory regions of the genes indicated. Average \pm SEM is shown, relative to IgG, and statistics were Student's t -test of each region compared to IgG, $n=3-4$, $*P<0.05$. *Panel VI:* The position and conservation of the Meox1-2 and the Pax3-2 RAREs are shown.



VI

Meox1-2	<i>H. sapiens</i> (-43887)	GCATCTTCTCTCTCTGTA AACTGGGAACAAGAAGACAT AGGTCACAGGGTCA TTG
	<i>M. musculus</i> (-33912)	GCCTGC-----GTCAACTGGGAATGGCAAGATT AGGTCACAGGGTCA TTA
Pax3-2	<i>H. sapiens</i> (+123889)	ACAATC AGTTCAGGGGTCA GGCTTAGAGAAACAGTGC AAAGTTAGCAAATGCAGG
	<i>M. musculus</i> (+114741)	ACAATC AGTTCAGGGGTCA GGCTTAGGGAAACAGTTC CAAGTGAAGGACAAGCGGG

To determine at what stage cardiomyogenesis was inhibited by RA, expression of the cardiomyoblast gene GATA-4, was examined (60). Northern blot analysis revealed a lack of induction of GATA-4 transcripts in cells treated with RA (Figure 2.1, panel IIIB), consistent with the interpretation that under these conditions RA inhibits the commitment into the cardiac muscle lineage.

In order to determine at which stage skeletal myogenesis was enhanced, the expression of genes expressed in the primitive streak (Brachyury T and Wnt3a), the dermomyotome (Pax3 and Meox1), and skeletal myoblasts (MyoD and myogenin) was examined. Mesoderm induction occurred in the presence of RA, shown by the expression of Brachyury T (Figure 2.1, panel IIIC). The levels of Brachyury T appear to be slightly decreased with RA, consistent with the increasing differentiation of mesodermal progenitors. In contrast to the results with GATA-4 expression, Wnt3a (days 4-6), Pax3 (days 2-9), Meox1 (days 3-4), MyoD (days 6-9) and Myogenin (days 6-9) transcripts were upregulated with RA treatment (Figure 2.1, panels IIID-H), which is consistent with an increase in skeletal myogenesis. Quantitative polymerase chain reaction (Q-PCR) was used to quantify the levels of Pax3, MyoD and Wnt3a transcripts, showing a 9.95-fold upregulation of these factors with RA treatment (Figure 2.1, panel IV). Hence, RA enhances skeletal myogenesis by upregulating Wnt3a, Pax3 and Meox1 (summarized in Table 2.1).

Since BMP4 is known to inhibit skeletal and enhance cardiac myogenesis (61), and RA has been shown to inhibit BMP4 expression in the limb forebud (62), we examined the expression of BMP4 transcripts in the presence and absence of RA (Figure 2.1, panel III- I). The endogenous levels of BMP4 were down-regulated in RA-treated cells relative to the controls from days 3-9. Finally, we examined the expression of the transducer of ErbB2

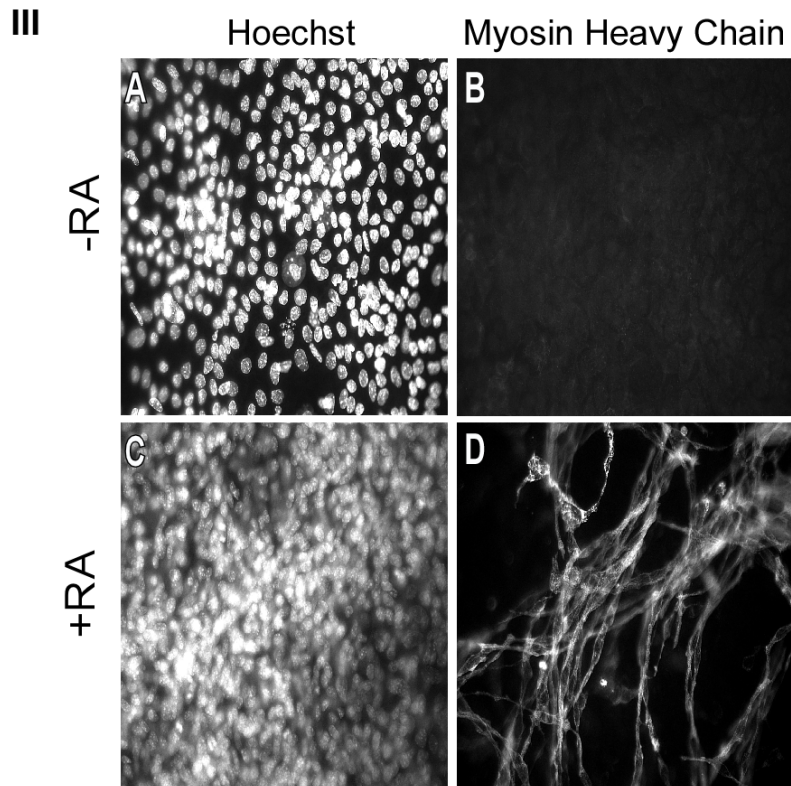
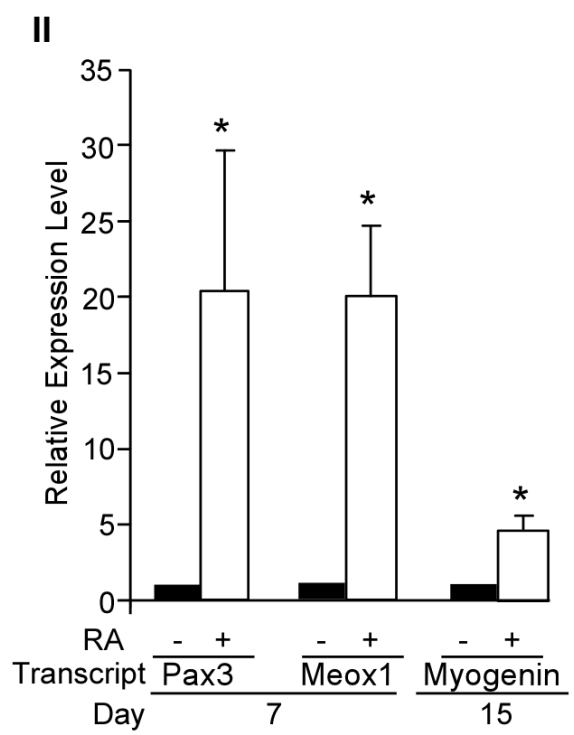
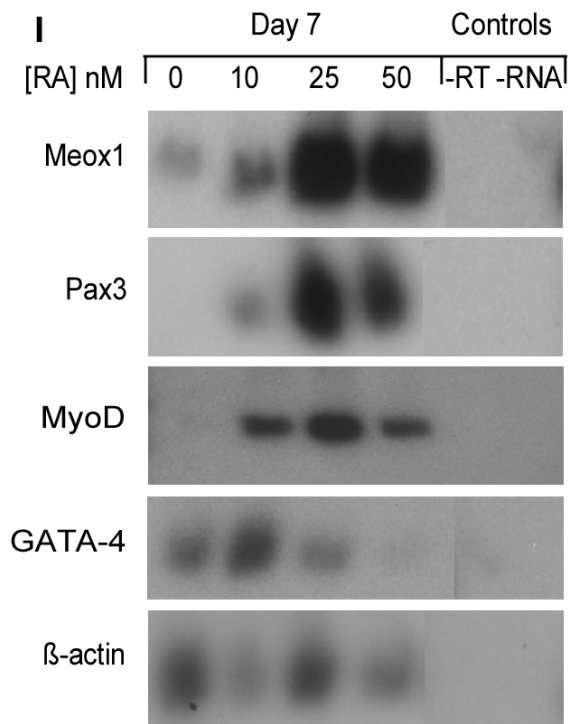
(Tob1), an intrinsic inhibitor of BMP signalling (63, 64). Tob1 was upregulated by RA from days 2-9 (Figure 2.1, panel III-J). Thus, RA may function to enhance skeletal myogenesis and inhibit cardiomyogenesis in part by inhibiting BMP4 expression and/or function.

To identify direct chromatin targets of RAR binding, we used multiple sequence local alignment and visualization (<http://mulan.dcode.org/>) to find retinoic acid response elements (RARE) sequences (defined as two tandem repeats of the RGKTCA element in DR1-7 arrangements) located within +/- 100Kb of the start site for Wnt3a, Pax3 and Meox1. While all three genes contained RARE sequences, only one of the two Meox1 sites (Meox1-2) and one of the two Pax3 sites (Pax3-2) were conserved between mouse and human, located in *Mus musculus* at -33869 bp and + 114735, respectively (Figure 2.1, panel VI). Chromatin immunoprecipitation experiments were performed on day 2 of differentiation, using an antibody that recognizes all RARs. We detected a significant \approx threefold enrichment in chromatin fragments corresponding to the Meox1-2 site, and a significant \approx twofold enrichment in chromatin fragments corresponding to the Pax3-2 site compared to immunoprecipitation with a rabbit IgG as a negative control (Figure 2.1, panel V). We also tested several of the non-conserved RAREs identified upstream of Wnt3a. One of these RAREs (Wnt3a-2 at position -36625) showed a significant \approx twofold enrichment. Several non-conserved RARE sequences in Wnt3a, Meox1, or Pax3 genes were not significantly associated with immunoprecipitated RARs, whereas a known site in RAR β 2 was associated, as expected (65). Thus, RARs bind to conserved elements in the Meox1 and Pax3 genes as well as to one non-conserved element upstream of Wnt3a in a population of differentiating P19 cells, indicating that RA functions both upstream of and in parallel to Wnt3a signalling.

2.4.2 RA enhances skeletal myogenesis in mESCs

To determine if our results in P19 cells were applicable to mouse embryonic stem cells, we differentiated mESCs in hanging drops for 2 days and in suspension culture for an additional 5 days, the latter with 0-50nM RA. After replating in tissue culture dishes, cells were harvested on days 7 and 15 for RNA and fixed on day 20 for immunofluorescence. Examination of RNA by RT-PCR indicated increasing transcript levels of Meox1, Pax3, and MyoD in the presence of RA, peaking at 25nM (Figure 2.2, panel I). Quantification of gene expression changes by Q-PCR indicated statistically significant 20-fold increases in Pax3 and Meox1 expression on day 7 and a significant fivefold increase in myogenin on day 15 (Figure 2.2, panel II). Finally, skeletal myocytes were not observed on day 20 in the absence of RA, as shown by the lack of bipolar cells reacting with MF20 (Figure 2.2, panel III-B). In the presence of 25nM RA, MyHC^{+ve} bipolar skeletal myocytes were visible (Figure 2.2, panel IIID). Thus, similar to the findings in P19 cells, RA treatment of mESCs results in the enhancement of skeletal muscle progenitor formation, shown by the increase in expression of Pax3, Meox1, MyoD, and myogenin (summarized in Table 2.1).

Figure 2.2 Mouse Embryonic Stem cells differentiate into skeletal muscle in response to RA. mES cells were aggregated in hanging drops for 2 days, cultured in suspension for a further 5 days with increasing concentrations of RA, and transferred to tissue culture dishes. *Panel I:* RNA was harvested from day 7 cultures and subjected to real-time polymerase chain reaction (PCR) followed by Southern blot analysis with the probes indicated on the left. *Panel II:* RNA was harvested from days 7 and 15 after differentiation with or without 25nM retinoic acid and examined by quantitative PCR analysis. The expression levels are expressed as fold increase in the presence, compared to the absence of RA, as the mean and standard error, $n=3$. Statistics were Student's t -test, $*P<0.05$. *Panel III:* On day 20 of differentiation, cells were fixed and reacted with Hoechst dye to detect nuclei (A and C) and with MF20 antibody to detect muscle (B and D). Magnification is 400x.



2.4.3 RA cannot bypass the inhibition of skeletal myogenesis by a dominant negative β -catenin

Since earlier studies have shown that Wnt signalling, via β -catenin, activates Pax3, Gli2 and Meox1 expression, inducing skeletal myogenesis in P19 cells (8), we were interested in determining how the Wnt signalling pathway intersects with RA signalling. Furthermore, a dominant negative β -catenin, with the transcriptional activation domain replaced by an engrailed repressor domain (β -Cat/EnR), inhibited skeletal myogenesis in P19 cells (8). This dominant negative approach identifies genes bound by β -catenin and their downstream targets. In order to determine if RA can bypass this inhibition, P19 cells expressing β -Cat/EnR were differentiated in the presence of DMSO, with and without RA and compared to control P19 cells. Cultures were fixed on day 9 and examined by immunofluorescence with MF20. In agreement with previous results (8), the overexpression of β -Cat/EnR resulted in the loss of skeletal myogenesis, shown by the lack of bipolar skeletal myocytes, compared to control cells (Figure 2.3D versus B). The addition of RA resulted in an increase in the number of skeletal myocytes observed in control cells (Figure 2.3F versus B), but not P19[β -Cat/EnR] cells (Figure 2.3H). Therefore, RA was not sufficient to circumvent the inhibition of skeletal myogenesis by β -Cat/EnR.

To examine at which time point the RA enhancement of skeletal myogenesis was inhibited by β -Cat/EnR, RNA was harvested on days 0, 5 and 9 from P19[control] and P19[β -Cat/EnR] cultures differentiated with increasing amounts of RA. Northern blots showed the expression of exogenous β -Cat/EnR in the P19[β -Cat/EnR] cells and not in P19[control] cells (Figure 2.4, panel IA). Endogenous β -Catenin expression was constitutive, as expected (Figure 2.4, panel IB). In agreement with Fig2.1, MyoD, Meox1

Figure 2.3 RA cannot override the inhibition of skeletal myogenesis by β -Cat/EnR.

P19[control] (A, B, E, F) and P19[β -Cat/EnR] (C, D, G, H) cells were aggregated in the presence of 0.8% dimethyl sulfoxide (DMSO) with (E-H) and without (A-D) 10 nM RA. Cells were fixed on day 9 of differentiation for immunofluorescence with MF20 antibody (B, D, F, H) and counter stained with Hoechst dye (A, C, E, G). Magnification is 160x.

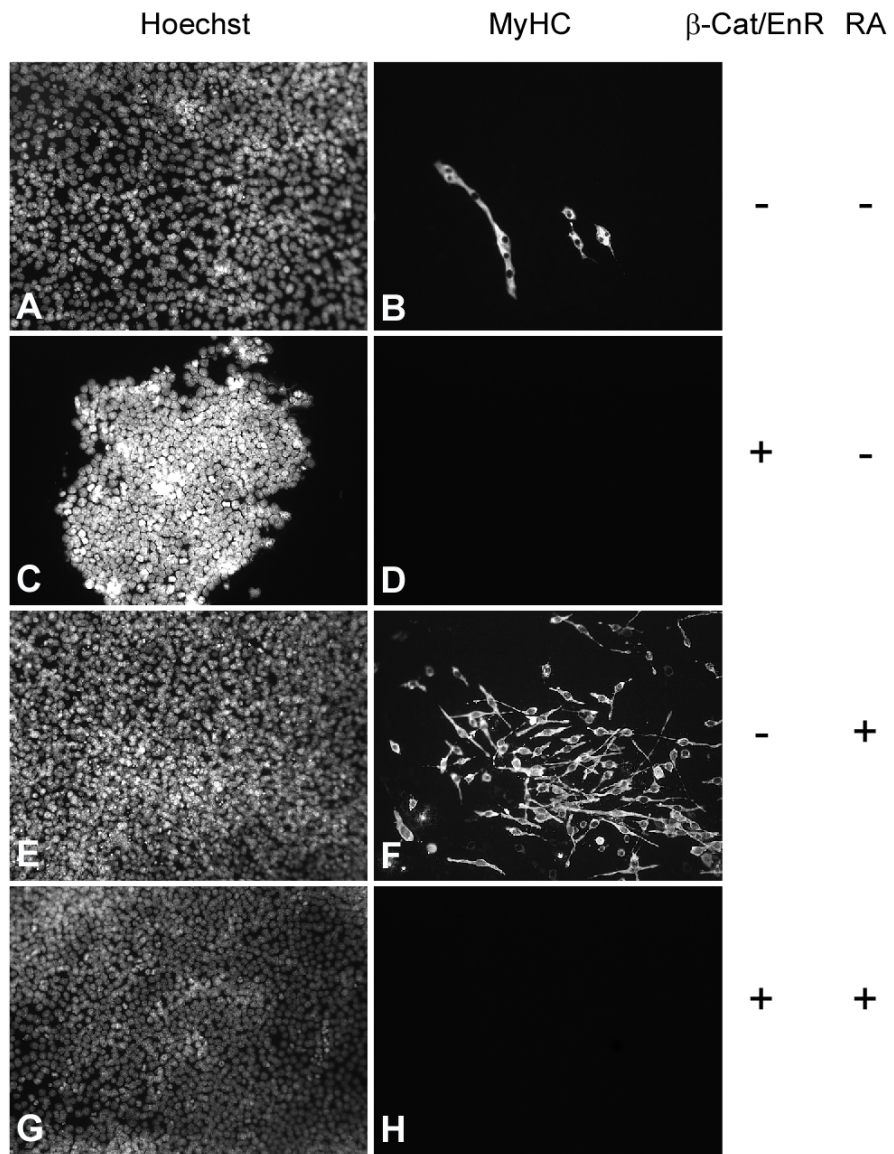
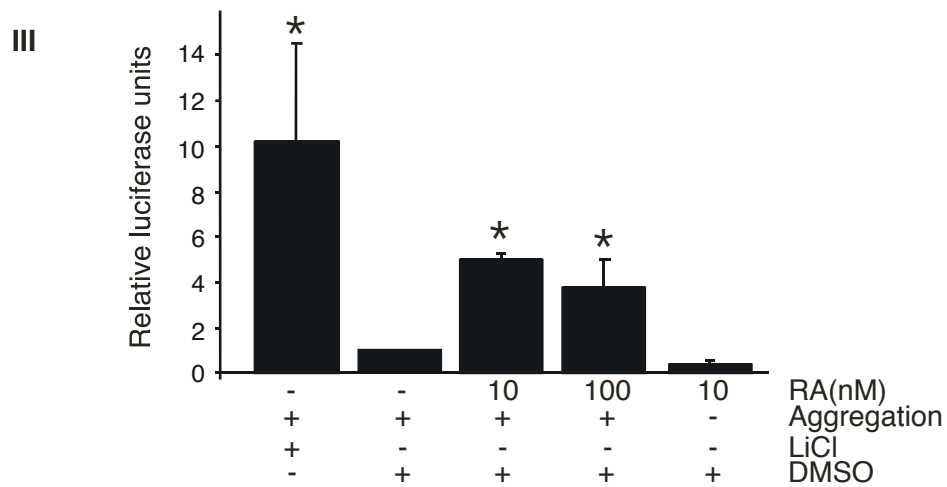
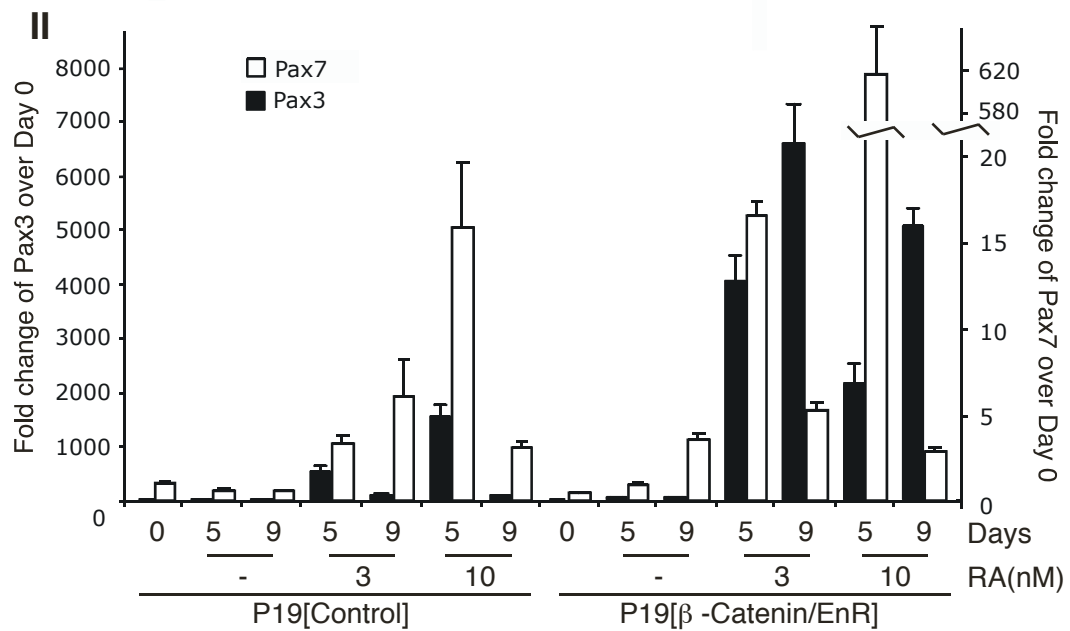
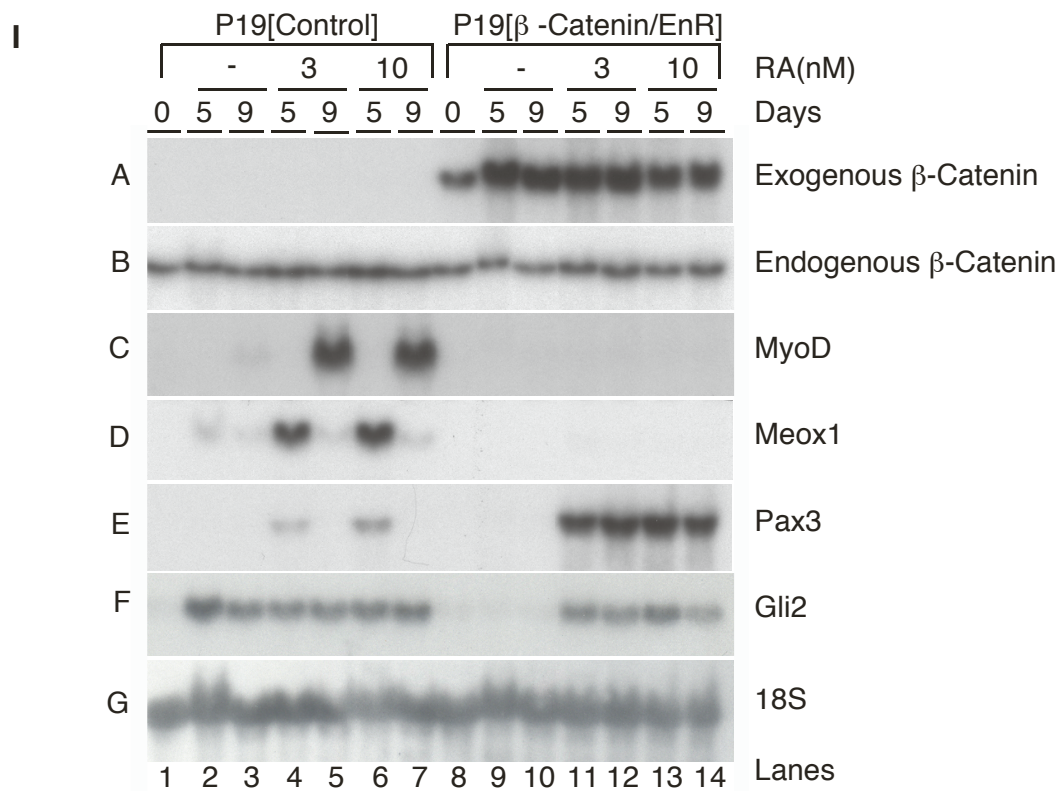


Figure 2.4 RA enhances the expression of Pax3/7 but not MyoD or Meox1 in the presence of β -Cat/EnR. *Panel I:* P19[control] and P19[β -Cat/EnR] cultures were differentiated with 0.8% dimethyl sulfoxide in the presence of 0, 3, and 10 nM RA. Total RNA was harvested and hybridized with the probes as indicated. *Panel II:* quantitative polymerase chain reaction analysis of Pax3 and Pax7 transcript levels, for each condition in Panel I, shown as one representative experiment performed in triplicate. *Panel III:* RA activates β -catenin in P19 aggregate but not monolayer cultures. P19 cells were transfected with the TOPFlash or FOPFlash reporter and treated with the compounds indicated in aggregated or monolayer cultures. Cells were harvested 24 hours later for luciferase assays ($n=2$). Numbers represent the average \pm standard error of mean and statistics were Student's t -test, $*P<0.05$.



and Pax3 were upregulated with 3 and 10nM RA in P19[control] cells, compared to no RA treatment (Figure 2.4, panels IC - E, lanes 4-7 versus lanes 2-3). Gli2 was upregulated during myogenesis on days 5 and 9, in agreement with previous results (9), but was not further upregulated by RA (Figure 2.4, panel IF). Interestingly, while MyoD and Meox1 were no longer upregulated by RA in the presence of β -Cat/EnR (Figure 2.4, panels IC - D, lanes 11-14), Pax3 transcript levels were increased by RA treatment (Figure 2.4, panel IE, lanes 11-14). Myogenin and Myf-5 were also not upregulated in P19[β -Cat/EnR] cells treated with RA (data not shown). The enhancement of Gli2 during myogenesis (Figure 2.4, panel IF, lanes 2-3) was abrogated in the presence of β -Cat/EnR (lanes 9-10), as shown previously (8). This loss of Gli2 expression was reversed in the presence of RA (Figure 2.4, panel IF, lanes 11-14). Therefore, the RA enhancement of MyoD and Meox1 was abrogated in the presence of β -Cat/EnR, but not the enhancement of Pax3. Furthermore, the Gli2 expression became responsive to RA in the presence of β -Cat/EnR (summarized in Table 2.1).

Changes in gene expression for Pax3 and Pax7 were quantified by Q-PCR (Figure 2.4, panel II). RA treatment resulted in the increased expression of Pax3 and Pax7 in P19[β -Cat/EnR] cells on days 5 and 9 (Figure 2.4, panel II). Thus, both Pax3 and Pax7 were upregulated by RA in the presence of β -Cat/EnR.

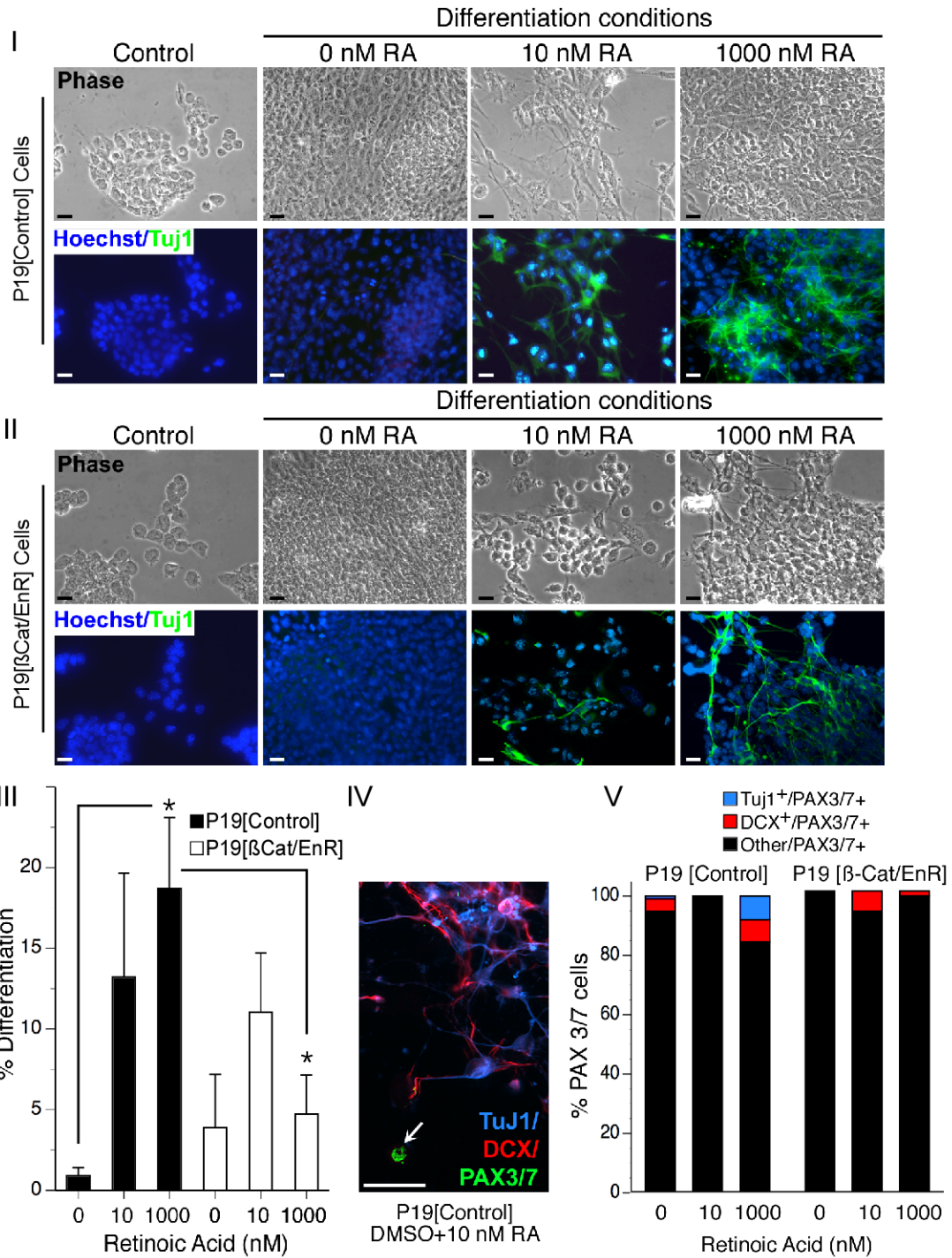
To further assess the relationship between RA and Wnt, the ability of RA to activate a β -catenin-responsive promoter – TOPflash - which contains 8 high mobility group (HMG) box sites, was examined using luciferase assays and compared to a mutated HMG box promoter, FOPflash (66). Both 10 and 100nM RA were sufficient to induce four- to fivefold increases in β -catenin activity in aggregated P19 cells (Figure 2.4, panel III). Interestingly, the activation by RA required cellular aggregation, since RA did not

significantly enhance β -catenin function in monolayer cultures. Furthermore, no evidence was obtained of synergy or inhibition of RAR β on β -catenin activity using a β -catenin-responsive TOPflash reporter (data not shown), in contrast to previous reports of inhibition (67). Thus RA can activate β -catenin function in DMSO-treated P19 aggregates, likely by enhancing β -catenin translocation to the nucleus.

2.4.4 Induction of Pax3 by RA is not due to enhanced neurogenesis

Since high levels of RA (1 μ M) can induce neurogenesis in P19 cells and both Pax3 and Shh signalling via Gli2 can regulate neurogenesis in spinal cord and brain (68-70), the increase in Pax3 mRNA may not reflect skeletal myogenesis but, rather, result from an increase in neurogenesis. To test this hypothesis, we quantified the percentage of cells that differentiated to a neuronal phenotype in P19[control] and P19[β -Cat/EnR] cells under cardiac and skeletal myogenic conditions (1%DMSO), skeletal myogenic conditions (1%DMSO+10nM RA), or neurogenic conditions (1%DMSO+ 1 μ M RA) (Figure 2.5). Differentiation to a post-mitotic neuronal phenotype was identified by labelling of neuron-specific β -III tubulin using anti-Tuj1 antibodies. As expected, 1 μ M RA triggered a significant neuronal differentiation compared to DMSO alone (Figure 2.5, panels I and III). However, neurogenesis with 10 nM RA was variable and was not significantly different compared to DMSO alone (Figure 2.5, panels I and III). Consistent with previous reports that the dominant-negative inhibition of β -catenin signalling reduces neurogenesis (71), we found that significantly fewer cells differentiated to Tuj1-positive neurons in P19[β -Cat/EnR] cultures treated with 1 μ M RA (Figure 2.5, panels II and III).

Figure 2.5 PAX3/7 expression in P19 cultures treated with DMSO and 10 nM RA is indicative of skeletal myogenesis and not neurogenesis. *Panel I:* P19[control] cells, undifferentiated in monolayer cultures (control) or differentiated with 1% DMSO, 1% DMSO+ 10 nM RA, or 1% DMSO + 1 μ M RA for 9 days, were immunolabelled with anti-TuJ1 (green) to detect terminally differentiated neurons and stained with the nuclear marker Hoechst (blue). *Panel II:* P19[β -Cat/EnR] cells were treated as in Panel I. *Panel III:* Quantitative analysis of the percentage of TuJ1-positive cells was established, expressed as the percentage of the total cell number ($n=6-10$). *Panel IV:* Immunofluorescent staining of PAX3/7 protein (green), committed neuronal precursors (double cortin-positive, red), and terminally differentiated neurons (TuJ1-positive, blue) in triple-labelled P19[control] cultures treated with DMSO + 10 nM RA, demonstrating that PAX3/7-positive cells (arrow) are not neuronal precursors or neurons. *Panel V:* Quantitative analysis indicated that the overwhelming majority of PAX3/7-positive cells in all treatments were non-neuronal. Statistics were analysis of variance, *post-hoc* Bonferroni, $*P<0.05$, Scale bars, 50 μ m.

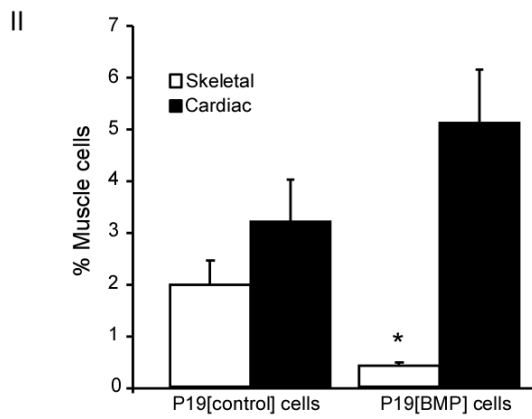
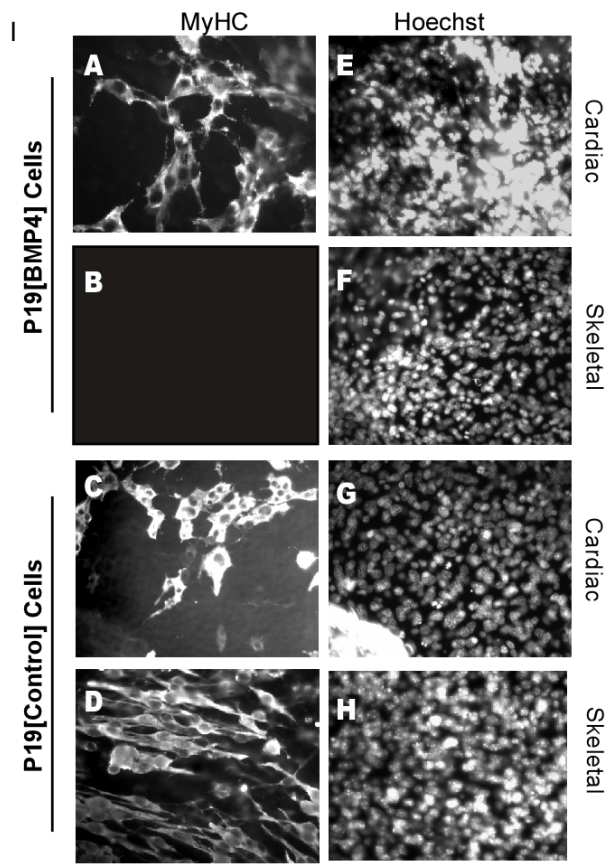


In order to determine whether Pax3/7 proteins were found in neurons, or in neural precursor cells, we used immunofluorescence to co-localize Pax3/7 proteins with neuronal markers (Figure 2.5, panels IV and V). Anti-Tuj1 antibodies were used to detect immature neurons and anti-double cortin (DCX) antibodies were used to detect committed neuronal precursor cells. DCX protein is expressed as early as 1 day after the initiation of neurogenesis in P19 cells and is the earliest known marker of neurogenesis (72). To confirm that P19 cells do not express Pax3/7 following neuronal commitment, we quantified the percentage of Pax3/7-positive neuronal precursors and immature neurons in our day 9 cultures (Figure 2.5, panels IV and V). Under all conditions tested, the overwhelming majority of Pax3/7-positive cells were non-neuronal (Figure 2.5, panels IV and V). No Pax3/7-positive neurons were detected in P19[control] cultures under conditions promoting skeletal myogenesis (DMSO + 10nM RA) (Figure 2.5, panels IV and V). Taken together, these results provide strong evidence that enhancement of Pax3 and Gli2 expression by low concentrations of RA is indicative of increased skeletal myogenesis and not neurogenesis.

2.4.5 Overexpression of BMP4 blocks skeletal myogenesis in P19 cells

Since RA enhances skeletal myogenesis, while downregulating BMP4 expression, we were interested in examining the interplay between RA and BMP4 in P19 cell myogenesis. In order to examine the effect of BMP4 on skeletal myogenesis in a stem cell context, stable cell lines expressing BMP4 were isolated and termed P19[BMP4] cells. When aggregated in the absence of DMSO and examined for immunofluorescence with MF20, neither skeletal nor cardiac myogenesis occurred in P19[BMP4] cells (data not shown). When cells were

Figure 2.6 BMP4 inhibits skeletal but not cardiac myogenesis. *Panel I:* P19[BMP4] and P19[control] cells were aggregated in the presence of 0.8% dimethyl sulfoxide (DMSO). Cells were fixed on day 9, stained with MF20 antibody (A-D), and counter-stained with Hoechst dye to show the nuclei (E-H). Magnification is 400x. *Panel II:* The number of MHC+ve cells were counted and the average +/- standard error of mean shown. Statistics were Student's *t*-test, **P*<0.05, *n*=3.



aggregated in the presence of DMSO, P19[BMP4] cells differentiated into cardiac muscle (Figure 2.6, panel IA) but not skeletal muscle (Figure 2.6, panel IB). Under these conditions, control cells differentiated efficiently into both cardiac (Figure 2.6, panel IC) and skeletal muscle (Figure 2.6, panel ID). Furthermore, a similar inhibition of skeletal myogenesis was obtained when parental P19 cells were mixed with P19[BMP4] cells in various ratios and aggregated in the presence of DMSO (data not shown). This indicated that BMP4 can function extracellularly. By counting the number of MHC+ve cells, skeletal myogenesis was inhibited an average of \approx fivefold in the presence of BMP4 and cardiomyogenesis was enhanced about 1.6-fold (Figure 2.6, panel II). Thus, overexpression of BMP4 in P19 cells is sufficient to block skeletal muscle and to slightly enhance cardiac muscle development.

2.4.6 BMP4 inhibits skeletal muscle specification

In order to investigate at what point in the pathway BMP4 inhibited myogenesis, the expression patterns of skeletal muscle-specific markers in P19[BMP4] and P19[control] cells during DMSO-induced differentiation were compared. Total RNA was harvested on days 0, 6 and 9 for northern blot analysis. P19[BMP4] cell lines expressed high levels of BMP4 (Figure 2.7, panel IA), but failed to express early markers of skeletal myogenesis such as Meox1 and Pax3 (Figure 2.7, panels IB - C) and late markers such as MyoD (Figure 2.7, panel ID) compared to P19 [control] cells. A 17 ± 5 ($n=$ four) loss of MyoD transcript levels were detected by Q-PCR in the presence of BMP4 (Figure 2.8, panel II). These findings suggest that BMP4 inhibited an early stage of skeletal muscle development by preventing proper muscle specification (summarized in Table 2.1).

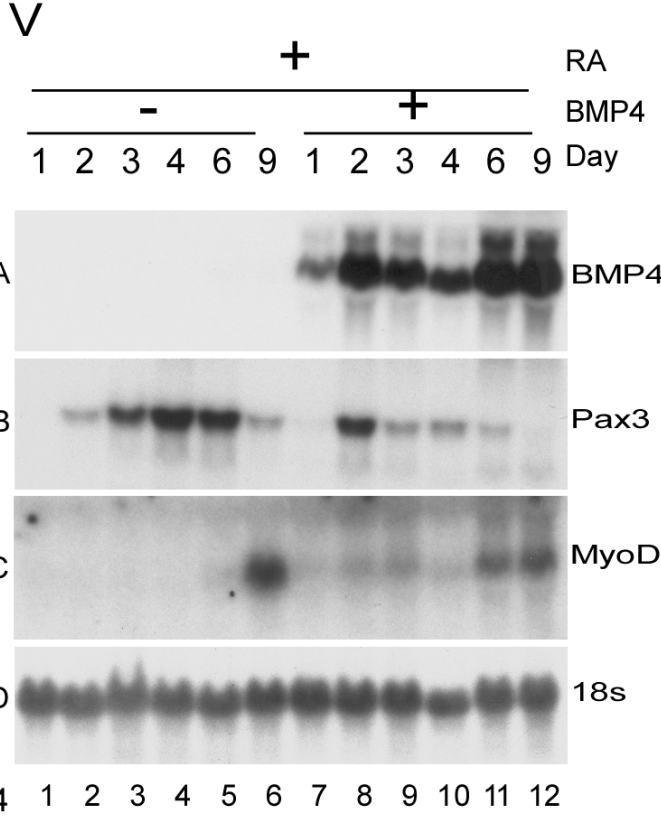
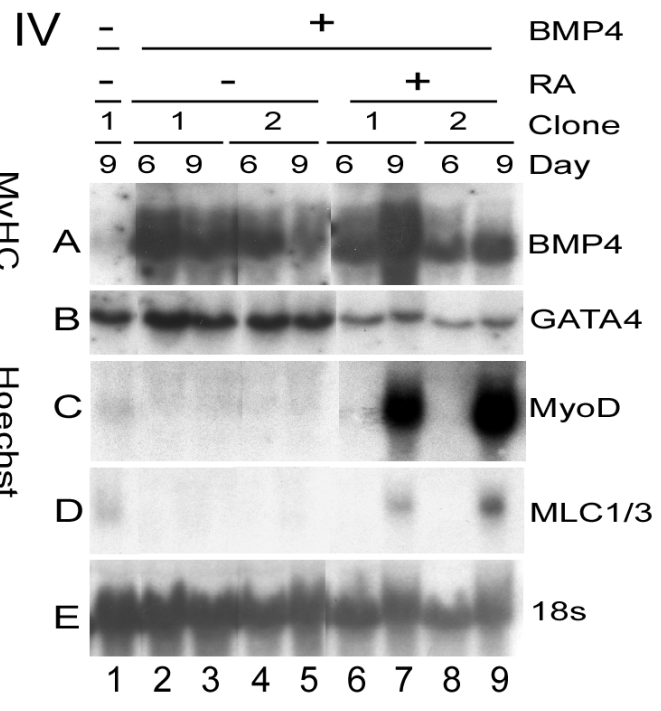
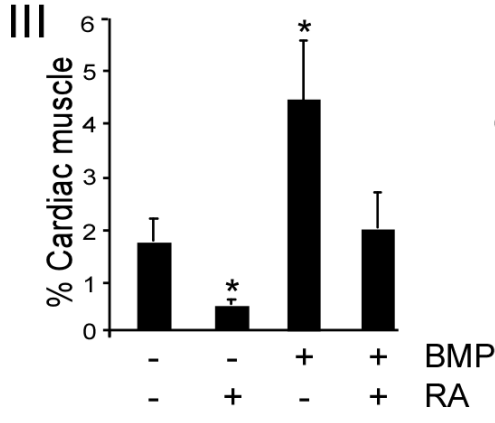
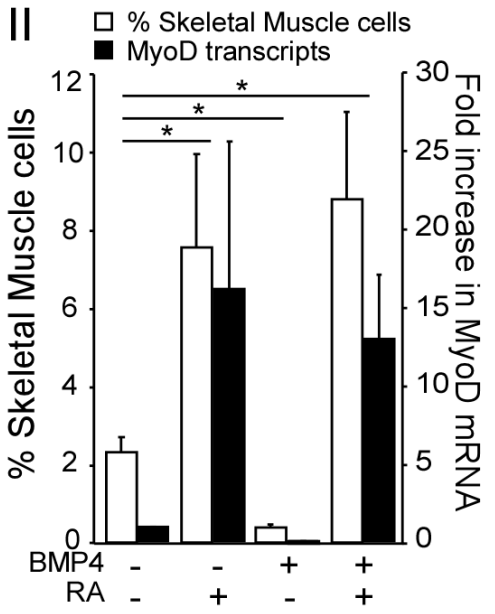
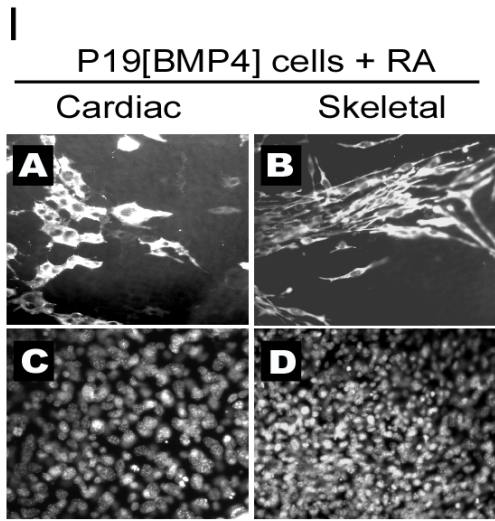
Figure 2.7 BMP4 inhibits skeletal muscle specification. *Panels I and II:* P19[BMP4] and P19[control] cells were aggregated in the presence of 0.8% dimethyl sulfoxide (DMSO). P19[control] cells were also aggregated in the absence of DMSO to serve as negative controls. On days 0, 6 and 9 (*Panel I*) and days 1-5 (*Panel II*), total RNA was harvested for northern blot analysis and hybridized with the cDNAs indicated on the right. *Panel III:* P19 cells were transfected with the TOPFlash reporter, aggregated, and treated with the compounds indicated. Cells were harvested 24 hours later for luciferase assays ($n=2$). Numbers represent the average \pm standard error of mean and statistics were Student's t -test, $*P<0.05$.

To determine if mesoderm induction, the stage prior to muscle specification, was affected by BMP4 expression, a time course of DMSO-induced differentiation was performed. P19[BMP4] and P19[control] cells were aggregated in the presence of DMSO and total RNA was harvested during the time course of differentiation for northern blot analysis. P19[BMP4] and P19[control] cell lines both expressed the mesoderm markers BrachyuryT and Wnt5b (Figure 2.7, panels IIA and B). Wnt5b expression levels appeared to be slightly increased. Finally, in order to compare the abilities of RA and BMP4 to enhance β -catenin activity, P19 cells were aggregated with DMSO in the presence and absence of BMP4 and the β -catenin-responsive TOPflash promoter was examined by luciferase assay. BMP4 was able to activate the β -catenin-responsive reporter in aggregated P19 cells treated with DMSO (Figure 2.7, panel III). Taken together, these results show that BMP4 interfered with skeletal myogenesis at a time point after mesoderm induction but before the specification of skeletal muscle.

2.4.7 Reciprocal Regulation of Myogenesis by RA and BMP4

Since BMP4 was found to inhibit, and RA to enhance skeletal muscle specification in P19 cells, we wanted to determine if BMP4 could override the enhancement of skeletal myogenesis by RA, and vice versa for cardiomyogenesis. To this end, P19 cells were mixed with P19[BMP4] or P19[control] cells and aggregated in the presence of DMSO with or without RA. As expected, P19 cells, mixed with P19[control] cells and aggregated in the presence of DMSO, differentiated readily into cardiac and skeletal muscle, as seen by positive MyHC staining. As shown in Figures 2.1 and 2.4, and quantified in Figure 2.8, RA

Figure 2.8 RA and BMP4 counteract each other's inhibition of skeletal myogenesis or cardiomyogenesis. P19 cells were mixed with P19[BMP4] or P19[control] cells in the presence of 1% dimethyl sulfoxide (DMSO), with or without RA. *Panel I:* P19[BMP4] cultures treated with RA were fixed on day 9 for immunofluorescence with MF20 antibody (A, B) and counter stained with Hoechst dye (C, D). Magnification is 160x. *Panel II:* Skeletal myogenesis was quantified for each condition by counting the number of myosin heavy chain^{+ve} bipolar skeletal myocytes, expressed as the percentage of total cells (white bars) and their standard errors ($n=3$). MyoD transcript levels (black bars) were quantified by quantitative polymerase chain reaction and expressed relative to control cultures ($n=2$), $*P<0.05$. *Panel III:* Cardiomyogenesis was quantified as described for *Panel II*, $n=4$. *Panel IV:* On days 6 and 9 total RNA was harvested for northern blot analysis and probed with the cDNAs indicated on the right. *Panel V:* A time course of P19[BMP4] and P19[control] cells aggregated in the presence of DMSO and RA. Total RNA was harvested for northern blot analysis on days 1-4, 6, and 9 and probed with the cDNAs indicated on the right.



inhibited cardiac myogenesis (3.6-fold), but not skeletal myogenesis, and BMP4 inhibited skeletal myogenesis (5.5-fold), but not cardiac myogenesis (Figure 2.8, panels II and III). However, P19 cells mixed with P19[BMP4] cells and treated with RA differentiated efficiently into both cardiac and skeletal muscle (Figure 2.8, panels I-III), indicating that RA and BMP4 could antagonize each other's inhibitory activities.

The results of immunofluorescence were confirmed by RNA analysis. Cultures of P19 cells mixed with P19[BMP4] cell lines contained high levels of BMP4 transcripts on days 6 and 9 (Figure 2.8, panel IVA, lanes 2-9). GATA-4 transcripts were present in cultures containing both BMP4 and RA, indicating that BMP4 abrogated the inhibition of cardiomyogenesis by RA (Figure 2.8, panel IVB, lanes 6-9). MyoD and myosin light chain1/3 (MLC1/3) transcripts were absent in P19[BMP4], compared to P19[control] cells (Figure 8, panel IVC - D, lanes 2-5 compared to 1). In contrast, P19 cells treated with BMP4 and RA robustly expressed MyoD and MLC1/3 transcripts (Figure 2.8, panels IVC and D, lanes 7 and 9). Quantification of MyoD transcript levels under the four conditions by QPCR was consistent with the results from counting skeletal myocytes (Figure 2.8, panel II). Therefore RA enhanced skeletal myogenesis and antagonized the inhibitory actions of BMP4 signalling.

Based on the inability of BMP4 to inhibit skeletal muscle development in the presence of RA, we predicted that the accelerated expression of Pax3 with RA treatment would still be observed in the presence of BMP4. To test this, we examined a time course of P19 cell differentiation in the presence of P19[BMP4] cells and RA. Total RNA was harvested on days 1-4, 6, and 9 for northern blot analysis. Exogenous BMP4 transcripts were present in P19[BMP4] cultures (Figure 2.8, panel VA). Pax3 and MyoD transcripts were detected in

cells treated with RA alone in a similar expression pattern compared to cultures containing both RA and BMP4 (Figure 2.8, panels VB and C, lanes 1-6 compared to 7-12). These results are in contrast to the loss of Pax3 and MyoD expression shown in the presence of BMP4 alone (Figure 2.7). Therefore, the early enhancement of Pax3 expression by RA treatment still occurs in the presence of BMP4 (summarized in Table 2.1).

Table 2.1 Summary of Gene expression Changes in cell lines treated with and without DMSO and/or RA

Cell line	Conditions	Cardiac	Skeletal Markers				Figure
		GATA-4	Pax3	Gli2	Meox1	MRFs	
P19	-	-	-	-	-	-	Fig. 2.7
P19	+DMSO	+	+	+	+	+	Fig. 2.1
P19	+DMSO&RA	-	+++	+	+++	+++	Fig. 2.1
P19[β-cat/EnR]	+DMSO	N.D.	-	-	-	-	Figs. 2.3 & 2.4
P19[β-cat/EnR]	+DMSO&RA	N.D.	+++	+++	-	-	Figs. 2.3 & 2.4
P19[BMP]	-	-	-	-	-	-	Data not shown
P19[BMP]	+DMSO	+	-	N.D.	-	-	Fig. 2.6, 2.7 & 2.8
P19[BMP]	+DMSO&RA	-	+++	+	N.D.	+++	Fig. 2.8
Mouse ES	-	+	-	N.D.	+/-	-	Fig. 2.2
Mouse ES	+RA	+/-	+++	N.D.	+++	+++	Fig. 2.2

P19 cell lines indicated on the right were aggregated under the conditions described and the induction of muscle marker gene expression was monitored.

(+++ = high expression; + = normal expression; +/- = partial expression, - = not expressed; N.D. = not determined)

2.5 Discussion

We have examined the mechanism of P19 stem cell differentiation into skeletal muscle in response to RA, Wnt inhibition, and/or BMP4. We show that BMP signalling inhibits skeletal muscle specification, via the loss of Pax3 and Meox1, while RA enhances this step. Furthermore, RA can enhance skeletal myogenesis in the presence of BMP4 but not dominant negative β -catenin. RARs bind directly to RAREs in the upstream and downstream genomic regions of Meox1, Pax3 and Wnt3a. Both RA and BMP4 can activate the function of β -catenin in a reporter assay in aggregated, DMSO-treated, P19 cells. Thus, RA functions both upstream of and in parallel to Wnt3a signalling to enhance skeletal myogenesis. Inhibition by BMP4 can be bypassed by RA, implying that RA may function downstream of BMP4 or that BMP4 inhibition occurs by affecting RA signalling/generation.

In terms of cardiomyogenesis, RA signalling inhibits GATA-4 expression, resulting in the loss of cardiomyogenesis. RA blocks the expression of endogenous BMP4 and activates the expression of Tob1, which is an inhibitor of BMP function. The positioning of RA upstream of BMP4 expression and activity explains the ability of exogenous BMP4 to compensate for the low levels of BMP4 in the presence of RA, resulting in the enhancement of cardiomyogenesis. These findings are summarized in a model in figure 2.9.

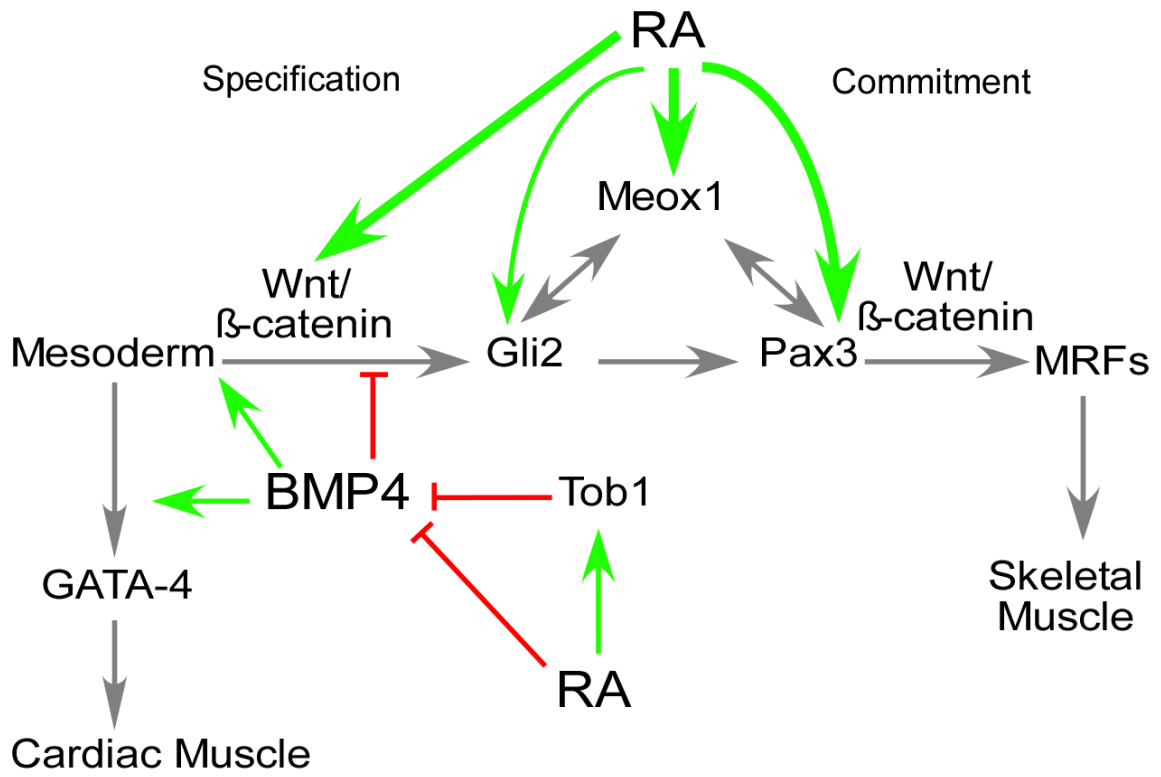
We have shown previously that β -catenin is sufficient to induce skeletal myogenesis in P19 cells, by initiating skeletal muscle specification (9). Here we show that RARs function as a positive regulator of Wnt signalling by binding directly to Wnt3a regulatory regions, upregulating Wnt3a transcript levels and activating β -catenin. In other systems,

such as F9/mouse ES cell differentiation or dorsal quail forebrain, RA can upregulate antagonists of the Wnt pathway, restrict Wnt expression, or inhibit β -catenin activity (73-75).

In contrast, RA upregulates the expression of Wnts during adult murine neurogenesis, or vertebrate limb induction (76, 77). Our observed activation of β -catenin by RA did not appear to involve synergistic interactions between RARs and β -catenin on a β -catenin-responsive promoter (data not shown). However, we have not ruled out that, once upregulated by RA signalling, β -catenin might synergize with RARs to activate RARE-responsive promoters, as described previously (78). Interestingly, BMP4 treatment also resulted in the activation of the β -catenin-responsive promoter, which is not surprising given that BMPs and Wnts cooperatively pattern mesoderm (79). It is likely that both RA and BMP4 act via canonical Wnt signalling to regulate both shared and distinct target genes, which change over time, dependent on combinatorial factors.

Pax3 expression is sufficient to initiate skeletal myogenesis in aggregated P19 cells (10) and it plays an important role in embryonic myogenesis (80). RA accelerated Pax3 expression in P19 cells, likely by activating RARs bound directly to Pax3 regulatory sequences. Although Pax3 expression was not abrogated by the presence of β -Cat/EnR, and thus likely not bound by β -Cat/EnR, its expression alone was not sufficient to initiate skeletal myogenesis under these conditions. In contrast, although Meox1 is also a direct target of RARs, it was not upregulated in the presence of β -Cat/EnR which implies that β -Cat/EnR may have bound directly to the Meox1 regulatory sequences and inhibited its activation by RARs. Future studies will include a global analysis of RAR and β -catenin binding sites. The finding that RA cannot bypass the β -Cat/EnR inhibition implies that β -catenin and RARs bind to an overlapping, essential set of genes during myogenesis.

Figure 2.9 Model of the intersection of RA, Wnt, and BMP4 signaling during cardiac and skeletal muscle development. BMP4 upregulates Wnt/ β -catenin during mesoderm induction (green arrow) and blocks skeletal myogenesis by downregulation of Meox1, Pax3 and myogenic regulatory factor expression (red inhibition arrow). This inhibition can be reversed by RA, which enhances Tob1, Wnt3a, Pax3 and Meox1 expression, activates β -catenin and inhibits BMP4 expression (green arrows). RA receptors bind directly to the Wnt3a, Meox1, and Pax3 regulatory regions (bold green arrows). RA inhibits GATA-4 expression and cardiomyogenesis, likely by inhibiting BMP4 expression and function. Grey arrows indicate previous work (6, 8-10, 35, 38, 40, 44, 102, 103).



BMP4 inhibits skeletal myogenesis *in vitro* and *in vivo* (3, 31, 35, 81). In agreement with these studies, we show that BMP4 can inhibit the specification of P19 cells into the skeletal muscle lineage, shown by the loss of Pax3 and Meox1 expression. When combined, RA signalling was able to reverse the inhibition of Pax3 expression by BMP4. RA signalling enhances the expression of Tob-1, which is an inhibitor of BMP signalling. However, it is unlikely that RA functions solely by inhibiting BMP activity. For example, P19 cells expressing the BMP inhibitor noggin (44) did not show an enhancement/acceleration of skeletal myogenesis (M Jamali and I S Skerjanc, unpublished observations). Since RA can activate myogenic progenitor genes in the presence of BMP4, it likely functions to activate transcription downstream of the BMP inhibitory signal.

Low levels of RA inhibit cardiomyogenesis in P19 cells (16), although the mechanism has not been fully characterized. Here we show that RA inhibits the expression of GATA-4, preventing mesodermal cells from becoming committed to the cardiac muscle lineage. These results are similar to the finding that continued exposure to RA disrupts heart formation in *Xenopus* and zebrafish, although early in mesodermal patterning RA may increase the proportion of cardiac progenitors (49, 53, 82). Low levels of RA may be redirecting pre-cardiac mesodermal cells into pre-skeletal mesoderm in P19 cells. Furthermore, RA may inhibit cardiomyogenesis via downregulation of BMP4 expression and upregulation of the BMP inhibitor Tob1. BMP4 functions to enhance cardiomyogenesis and can activate Nkx2-5 and GATA-4 expression (83). The finding that exogenous BMP4 can override the inhibition of cardiomyogenesis by RA suggests that BMP4 functions downstream of the RA inhibition, or that BMP4 reduces the ability of RA to signal in cardiac muscle precursors.

In P19 and P19CL6 cells, noggin inhibits cardiomyogenesis, indicating that BMP signalling is essential (40, 44). However, we found that BMP4 overexpression was not sufficient to induce cardiomyogenesis in aggregated P19 cells without DMSO treatment, although a mild upregulation of cardiomyogenesis was observed with DMSO. Indeed, BMP4 during the aggregation stage may be inhibitory to cardiomyogenesis (84). These results contrast with the role of sonic hedgehog (shh), which is sufficient to induce cardiomyogenesis in aggregated P19 cells without DMSO (85). Shh may provide an earlier signal than BMP4 in the cascade of events leading to cardiomyogenesis.

In other systems BMP and RA have also been shown to function antagonistically. For example, noggin enhanced RA function in chick chondrocyte maturation (86). In contrast, RA induced the expression of BMP-signalling molecules and enhances BMP effects in chondrocytes (87, 88). RA can induce or inhibit BMP expression, depending on the context (62, 89, 90). Finally, BMP2/4 can inhibit RA-induced neurogenesis in P19 monolayer cultures (91). Given the high degree of complexity of these signalling pathways, further studies are required to delineate the cross-talk mechanisms involved.

In conclusion, we have examined the roles of RA, BMP4 and canonical Wnt signalling in directing entry into the cardiac and skeletal muscle lineages. RA enhanced skeletal myogenesis by inhibiting BMP4 expression and function, while activating the expression of pre-skeletal mesoderm genes, and activating Wnt/ β -Catenin signalling. RA inhibited cardiomyogenesis, likely by inhibition of BMP4 function. RA and BMP4 can each reverse the other's inhibition of myogenesis. Therefore, the precise balance of these signalling molecules is necessary to regulate specification into skeletal or cardiac muscle.

2.6 References

1. Borycki, A.G., B. Brunk, S. Tajbakhsh, M. Buckingham, C. Chiang, and C.P. Emerson, Jr. 1999. Sonic hedgehog controls epaxial muscle determination through Myf5 activation. *Development* 126:4053-4063.
2. Munsterberg, A.E., J. Kitajewski, D.A. Bumcrot, A.P. McMahon, and A.B. Lassar. 1995. Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev* 9:2911-2922.
3. Pourquie, O., C.M. Fan, M. Coltey, E. Hirsinger, Y. Watanabe, C. Breant, P. Francis-West, P. Brickell, M. Tessier-Lavigne, and N.M. Le Douarin. 1996. Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* 84:461-471.
4. Tajbakhsh, S., U. Borello, E. Vivarelli, R. Kelly, J. Papkoff, D. Duprez, M. Buckingham, and G. Cossu. 1998. Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. *Development* 125:4155-4162.
5. Dietrich, S., F.R. Schubert, C. Healy, P.T. Sharpe, and A. Lumsden. 1998. Specification of the hypaxial musculature. *Development* 125:2235-2249.
6. Williams, B.A., and C.P. Ordahl. 1994. Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* 120:785-796.
7. McDermott, A., M. Gustafsson, T. Elsam, C.C. Hui, C.P. Emerson, Jr., and A.G. Borycki. 2005. Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. *Development* 132:345-357.
8. Petropoulos, H., and I.S. Skerjanc. 2002. Beta-catenin is essential and sufficient for skeletal myogenesis in P19 cells. *J Biol Chem* 277:15393-15399.
9. Petropoulos, H., P.J. Gianakopoulos, A.G. Ridgeway, and I.S. Skerjanc. 2004. Disruption of Meox or Gli activity ablates skeletal myogenesis in P19 cells. *J Biol Chem* 279:23874-23881.
10. Ridgeway, A.G., and I.S. Skerjanc. 2001. Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. *J Biol Chem* 276:19033-19039.
11. Chanoine, C., B. Della Gaspera, and F. Charbonnier. 2004. Myogenic regulatory factors: redundant or specific functions? Lessons from *Xenopus*. *Dev Dyn* 231:662-670.
12. Tapscott, S.J. 2005. The circuitry of a master switch: MyoD and the regulation of skeletal muscle gene transcription. *Development* 132:2685-2695.

13. Skerjanc, I.S. 1999. Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. *Trends Cardiovasc Med* 9:139-143.
14. Vidricaire, G., K. Jardine, and M.W. McBurney. 1994. Expression of the Brachyury gene during mesoderm development in differentiating embryonal carcinoma cell cultures. *Development* 120:115-122.
15. Rune Blomhoff, H.K.B. 2006. Overview of retinoid metabolism and function. *Journal of Neurobiology* 66:606-630.
16. Edwards, M.K., and M.W. McBurney. 1983. The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev Biol* 98:187-191.
17. Halevy, O., and O. Lerman. 1993. Retinoic acid induces adult muscle cell differentiation mediated by the retinoic acid receptor-alpha. *J Cell Physiol* 154:566-572.
18. Albagli-Curiel, O., G. Carnac, M. Vandromme, S. Vincent, P. Crepieux, and A. Bonniieu. 1993. Serum-induced inhibition of myogenesis is differentially relieved by retinoic acid and triiodothyronine in C2 murine muscle cells. *Differentiation* 52:201-210.
19. Momoi, T., S. Miyagawa-Tomita, S. Nakamura, I. Kimura, and M. Momoi. 1992. Retinoic acid ambivalently regulates the expression of MyoD1 in the myogenic cells in the limb buds of the early developmental stages. *Biochem Biophys Res Commun* 187:245-253.
20. Froeschle, A., S. Alric, M. Kitzmann, G. Carnac, F. Aurade, C. Rochette-Egly, and A. Bonniieu. 1998. Retinoic acid receptors and muscle b-HLH proteins: partners in retinoid- induced myogenesis. *Oncogene* 16:3369-3378.
21. Kessel, M., and P. Gruss. 1991. Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell* 67:89-104.
22. Moreno, T.A., and C. Kintner. 2004. Regulation of segmental patterning by retinoic acid signaling during *Xenopus* somitogenesis. *Dev Cell* 6:205-218.
23. Vermot, J., and O. Pourquie. 2005. Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. *Nature* 435:215-220.
24. Gordon, M.D., and R. Nusse. 2006. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* 281:22429-22433.
25. Brand-Saber, B. 2005. Genetic and epigenetic control of skeletal muscle development. *Ann Anat* 187:199-207.

26. Schmidt, M., M. Tanaka, and A. Munsterberg. 2000. Expression of (beta)-catenin in the developing chick myotome is regulated by myogenic signals. *Development* 127:4105-4113.
27. Capdevila, J., C. Tabin, and R.L. Johnson. 1998. Control of dorsoventral somite patterning by Wnt-1 and beta-catenin. *Dev Biol* 193:182-194.
28. Borello, U., B. Berarducci, P. Murphy, L. Bajard, V. Buffa, S. Piccolo, M. Buckingham, and G. Cossu. 2006. The Wnt/{beta}-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. *Development* 133:3723-3732.
29. Massague, J. 1992. Receptors for the TGF-beta family. *Cell* 69:1067-1070.
30. Duprez, D.M., M. Coltey, H. Amthor, P.M. Brickell, and C. Tickle. 1996. Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev Biol* 174:448-452.
31. Murray, S.S., E.J. Murray, C.A. Glackin, and M.R. Urist. 1993. Bone morphogenetic protein inhibits differentiation and affects expression of helix-loop-helix regulatory molecules in myoblastic cells. *J Cell Biochem* 53:51-60.
32. Marcelle, C., M.R. Stark, and M. Bronnerfraser. 1997. Coordinate Actions Of Bmps, Wnts, Shh and Noggin Mediate Patterning Of the Dorsal Somite. *Development* 124:3955-3963.
33. Streit, A., and C.D. Stern. 1999. Mesoderm patterning and somite formation during node regression: differential effects of chordin and noggin. *Mech Dev* 85:85-96.
34. McMahon, J.A., S. Takada, L.B. Zimmerman, C.M. Fan, R.M. Harland, and A.P. McMahon. 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev* 12:1438-1452.
35. Reshef, R., M. Maroto, and A.B. Lassar. 1998. Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. *Genes Dev* 12:290-303.
36. Frasch, M. 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early Drosophila embryo. *Nature* 374:464-467.
37. Schlange, T., B. Andree, H.H. Arnold, and T. Brand. 2000. BMP2 is required for early heart development during a distinct time period. *Mech Dev* 91:259-270.
38. Schultheiss, T.M., J.B. Burch, and A.B. Lassar. 1997. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev* 11:451-462.

39. Ladd, A.N., T.A. Yatskievych, and P.B. Antin. 1998. Regulation of avian cardiac myogenesis by activin/TGFbeta and bone morphogenetic proteins. *Dev Biol* 204:407-419.
40. Monzen, K., I. Shiojima, Y. Hiroi, S. Kudoh, T. Oka, E. Takimoto, D. Hayashi, T. Hosoda, A. Habara-Ohkubo, T. Nakaoka, T. Fujita, Y. Yazaki, and I. Komuro. 1999. Bone Morphogenetic Proteins Induce Cardiomyocyte Differentiation through the Mitogen-Activated Protein Kinase Kinase Kinase TAK1 and Cardiac Transcription Factors Csx/Nkx-2.5 and GATA-4. *Mol Cell Biol* 19:7096-7105.
41. Schultheiss, T.M., and A.B. Lassar. 1997. Induction of chick cardiac myogenesis by bone morphogenetic proteins. *Cold Spring Harb Symp Quant Biol* 62:413-419.
42. Shi, Y., S. Katsev, C. Cai, and S. Evans. 2000. BMP signaling is required for heart formation in vertebrates. *Dev Biol* 224:226-237.
43. Walters, M.J., G.A. Wayman, and J.L. Christian. 2001. Bone morphogenetic protein function is required for terminal differentiation of the heart but not for early expression of cardiac marker genes. *Mech Dev* 100:263-273.
44. Jamali, M., C. Karamboulas, P.J. Rogerson, and I.S. Skerjanc. 2001. BMP signaling regulates Nkx2-5 activity during cardiomyogenesis. *FEBS Lett* 509:126-130.
45. Behfar, A., L.V. Zingman, D.M. Hodgson, J.M. Rauzier, G.C. Kane, A. Terzic, and M. Puceat. 2002. Stem cell differentiation requires a paracrine pathway in the heart. *Faseb J* 16:1558-1566.
46. Wobus, A.M., G. Kaomei, J. Shan, M.C. Wellner, J. Rohwedel, G. Ji, B. Fleischmann, H.A. Katus, J. Hescheler, and W.M. Franz. 1997. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J Mol Cell Cardiol* 29:1525-1539.
47. Skerjanc, I.S., and M.W. McBurney. 1994. The E box is essential for activity of the cardiac actin promoter in skeletal but not in cardiac muscle. *Dev Biol* 163:125-132.
48. Hidaka, K., J.K. Lee, H.S. Kim, C.H. Ihm, A. Iio, M. Ogawa, S. Nishikawa, I. Kodama, and T. Morisaki. 2003. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. *Faseb J* 17:740-742.
49. Drysdale, T.A., K.D. Patterson, M. Saha, and P.A. Krieg. 1997. Retinoic acid can block differentiation of the myocardium after heart specification. *Dev Biol* 188:205-215.
50. Stainier, D.Y., and M.C. Fishman. 1992. Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. *Dev Biol* 153:91-101.

51. Osmond, M.K., A.J. Butler, F.C. Voon, and R. Bellairs. 1991. The effects of retinoic acid on heart formation in the early chick embryo. *Development* 113:1405-1417.
52. Yutzey, K.E., J.T. Rhee, and D. Bader. 1994. Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. *Development* 120:871-883.
53. Keegan, B.R., J.L. Feldman, G. Begemann, P.W. Ingham, and D. Yelon. 2005. Retinoic acid signaling restricts the cardiac progenitor pool. *Science* 307:247-249.
54. Kastner, P., J.M. Grondona, M. Mark, A. Gansmuller, M. LeMeur, D. Decimo, J.L. Vonesch, P. Dolle, and P. Chambon. 1994. Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* 78:987-1003.
55. Kastner, P., N. Messaddeq, M. Mark, O. Wendling, J.M. Grondona, S. Ward, N. Ghyselinck, and P. Chambon. 1997. Vitamin A deficiency and mutations of RXRalpha, RXRbeta and RARalpha lead to early differentiation of embryonic ventricular cardiomyocytes. *Development* 124:4749-4758.
56. Ryckebusch, L., Z. Wang, N. Bertrand, S.C. Lin, X. Chi, R. Schwartz, S. Zaffran, and K. Niederreither. 2008. Retinoic acid deficiency alters second heart field formation. *Proc Natl Acad Sci U S A* 105:2913-2918.
57. Sirbu, I.O., X. Zhao, and G. Duyster. 2008. Retinoic acid controls heart anteroposterior patterning by down-regulating Isl1 through the Fgf8 pathway. *Dev Dyn* 237:1627-1635.
58. Wobus, A.M., J. Rohwedel, V. Maltsev, and J. Hescheler. 1994. In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. *Roux's Arch Dev Biol* 204:36-45.
59. Moss, J.B., J. Xavier-Neto, M.D. Shapiro, S.M. Nayeem, P. McCaffery, U.C. Drager, and N. Rosenthal. 1998. Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. *Dev Biol* 199:55-71.
60. Grepin, C., L. Dagnino, L. Robitaille, L. Haberstroh, T. Antakly, and M. Nemer. 1994. A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol Cell Biol* 14:3115-3129.
61. Miller, J.B., L. Schaefer, and J.A. Dominov. 1999. Seeking muscle stem cells. *Curr Top Dev Biol* 43:191-219.
62. Mic, F.A., and G. Duyster. 2003. Patterning of forelimb bud myogenic precursor cells requires retinoic acid signaling initiated by Raldh2. *Dev Biol* 264:191-201.

63. Yoshida, Y., A. von Bubnoff, N. Ikematsu, I.L. Blitz, J.K. Tsuzuku, E.H. Yoshida, H. Umemori, K. Miyazono, T. Yamamoto, and K.W. Cho. 2003. Tob proteins enhance inhibitory Smad-receptor interactions to repress BMP signaling. *Mech Dev* 120:629-637.
64. Matsuda, S., J. Kawamura-Tsuzuku, M. Ohsugi, M. Yoshida, M. Emi, Y. Nakamura, M. Onda, Y. Yoshida, A. Nishiyama, and T. Yamamoto. 1996. Tob, a novel protein that interacts with p185erbB2, is associated with anti-proliferative activity. *Oncogene* 12:705-713.
65. Gillespie, R.F., and L.J. Gudas. 2007. Retinoic acid receptor isotype specificity in F9 teratocarcinoma stem cells results from the differential recruitment of coregulators to retinoic response elements. *J Biol Chem* 282:33421-33434.
66. DasGupta, R., A. Kaykas, R.T. Moon, and N. Perrimon. 2005. Functional genomic analysis of the Wnt-wingless signaling pathway. *Science* 308:826-833.
67. Easwaran, V., M. Pishvaian, Salimuddin, and S. Byers. 1999. Cross-regulation of beta-catenin-LEF/TCF and retinoid signaling pathways. *Curr Biol* 9:1415-1418.
68. Baker, C.V., and M. Bronner-Fraser. 2000. Establishing neuronal identity in vertebrate neurogenic placodes. *Development* 127:3045-3056.
69. Koblar, S.A., M. Murphy, G.L. Barrett, A. Underhill, P. Gros, and P.F. Bartlett. 1999. Pax-3 regulates neurogenesis in neural crest-derived precursor cells. *J Neurosci Res* 56:518-530.
70. Pozniak, C.D., and S.J. Pleasure. 2006. A tale of two signals: Wnt and Hedgehog in dentate neurogenesis. *Sci STKE* 2006:pe5.
71. Lie, D.C., S.A. Colamarino, H.J. Song, L. Desire, H. Mira, A. Consiglio, E.S. Lein, S. Jessberger, H. Lansford, A.R. Dearie, and F.H. Gage. 2005. Wnt signalling regulates adult hippocampal neurogenesis. *Nature* 437:1370-1375.
72. Bogoch, Y., and M. Linial. 2008. Coordinated expression of cytoskeleton regulating genes in the accelerated neurite outgrowth of P19 embryonic carcinoma cells. *Exp Cell Res* 314:677-690.
73. Halilagic, A., V. Ribes, N.B. Ghyselinck, M.H. Zile, P. Dolle, and M. Studer. 2007. Retinoids control anterior and dorsal properties in the developing forebrain. *Developmental Biology* 303:362-375.
74. Verani, R., I. Cappuccio, P. Spinsanti, R. Gradini, A. Caruso, M.C. Magnotti, M. Motolese, F. Nicoletti, and D. Melchiorri. 2007. Expression of the Wnt inhibitor Dickkopf-1 is required for the induction of neural markers in mouse embryonic stem cells differentiating in response to retinoic acid

doi:10.1111/j.1471-4159.2006.04207.x. *Journal of Neurochemistry* 100:242-250.

75. zur Nieden, N.I., F.D. Price, L.A. Davis, R.E. Everitt, and D.E. Rancourt. 2007. Gene Profiling on Mixed Embryonic Stem Cell Populations Reveals a Biphasic Role for β -Catenin in Osteogenic Differentiation 10.1210/me.2005-0438. *Mol Endocrinol* 21:674-685.
76. Jacobs, S., D.C. Lie, K.L. DeCicco, Y. Shi, L.M. DeLuca, F.H. Gage, and R.M. Evans. 2006. Retinoic acid is required early during adult neurogenesis in the dentate gyrus. *Proc Natl Acad Sci U S A* 103:3902-3907.
77. Mercader, N., S. Fischer, and C.J. Neumann. 2006. Prdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction 10.1242/dev.02455. *Development* 133:2805-2815.
78. Tice, D.A., W. Szeto, I. Soloviev, B. Rubinfeld, S.E. Fong, D.L. Dugger, J. Winer, P.M. Williams, D. Wieand, V. Smith, R.H. Schwall, D. Pennica, and P. Polakis. 2002. Synergistic induction of tumor antigens by Wnt-1 signaling and retinoic acid revealed by gene expression profiling. *J Biol Chem* 277:14329-14335.
79. Guo, X., and X.F. Wang. 2009. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res* 19:71-88.
80. Relaix, F. 2006. Skeletal muscle progenitor cells: from embryo to adult. *Cell Mol Life Sci* 63:1221-1225.
81. Katagiri, T., A. Yamaguchi, M. Komaki, E. Abe, N. Takahashi, T. Ikeda, V. Rosen, J.M. Wozney, A. Fujisawa-Sehara, and T. Suda. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J Cell Biol* 127:1755-1766.
82. Collop, A.H., J.A. Broomfield, R.A. Chandraratna, Z. Yong, S.J. Deimling, S.J. Kolker, D.L. Weeks, and T.A. Drysdale. 2006. Retinoic acid signaling is essential for formation of the heart tube in *Xenopus*. *Dev Biol* 291:96-109.
83. van Wijk, B., A.F.M. Moorman, and M.J.B. van den Hoff. 2007. Role of bone morphogenetic proteins in cardiac differentiation. *Cardiovascular Research* 74:244-255.
84. Angello, J.C., S. Kaestner, R.E. Welikson, J.N. Buskin, and S.D. Hauschka. 2006. BMP induction of cardiogenesis in P19 cells requires prior cell-cell interaction(s). *Dev Dyn*
85. Gianakopoulos, P.J., and I.S. Skerjanc. 2005. Hedgehog signaling induces cardiomyogenesis in P19 cells. *J Biol Chem* 280:21022-21028.

86. Shimo, T., E. Koyama, H. Sugito, C. Wu, S. Shimo, and M. Pacifici. 2005. Retinoid signaling regulates CTGF expression in hypertrophic chondrocytes with differential involvement of MAP kinases. *J Bone Miner Res* 20:867-877.
87. Drissi, M.H., X. Li, T.J. Sheu, M.J. Zuscik, E.M. Schwarz, J.E. Puzas, R.N. Rosier, and R.J. O'Keefe. 2003. Runx2/Cbfa1 stimulation by retinoic acid is potentiated by BMP2 signaling through interaction with Smad1 on the collagen X promoter in chondrocytes. *J Cell Biochem* 90:1287-1298.
88. Li, X., E.M. Schwarz, M.J. Zuscik, R.N. Rosier, A.M. Ionescu, J.E. Puzas, H. Drissi, T.J. Sheu, and R.J. O'Keefe. 2003. Retinoic acid stimulates chondrocyte differentiation and enhances bone morphogenetic protein effects through induction of Smad1 and Smad5. *Endocrinology* 144:2514-2523.
89. Qin, P., J.M. Haberbusch, Z. Zhang, K.J. Soprano, and D.R. Soprano. 2004. Pre-B cell leukemia transcription factor (PBX) proteins are important mediators for retinoic acid-dependent endodermal and neuronal differentiation of mouse embryonal carcinoma P19 cells. *J Biol Chem* 279:16263-16271.
90. Hallahan, A.R., J.I. Pritchard, R.A. Chandraratna, R.G. Ellenbogen, J.R. Geyer, R.P. Overland, A.D. Strand, S.J. Tapscott, and J.M. Olson. 2003. BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. *Nat Med* 9:1033-1038.
91. Glozak, M.A., and M.B. Rogers. 1996. Specific Induction Of Apoptosis In P19 Embryonal Carcinoma Cells By Retinoic Acid and Bmp2 or Bmp4. *Developmental Biology* 179:458-470.
92. Skerjanc, I.S., H. Petropoulos, A.G. Ridgeway, and S. Wilton. 1998. Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. *J Biol Chem* 273:34904-34910.
93. Rudnicki, M.A., and M.W. McBurney. 1987. Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In *Teratocarcinomas and embryonic stem cells. A practical approach*. E.J. Robertson, editor IRL Press, Oxford. 19-49.
94. Ridgeway, A.G., H. Petropoulos, A. Siu, J.K. Ball, and I.S. Skerjanc. 1999. Cloning, tissue distribution, subcellular localization and overexpression of murine histidine-rich Ca²⁺ binding protein. *FEBS Lett* 456:399-402.
95. Karamboulas, C., G.D. Dakubo, J. Liu, Y. De Repentigny, K. Yutzey, V.A. Wallace, R. Kothary, and I.S. Skerjanc. 2006. Disruption of MEF2 activity in cardiomyoblasts inhibits cardiomyogenesis. *J Cell Sci* 119:4315-4321.

96. Bader, D., T. Masaki, and D.A. Fischman. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol* 95:763-770.
97. Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* 107:303-314.
98. Ridgeway, A.G., S. Wilton, and I.S. Skerjanc. 2000. Myocyte enhancer factor 2C and myogenin Up-regulate each Other's expression and induce the development of skeletal muscle in P19 cells [In Process Citation]. *J Biol Chem* 275:41-46.
99. Karamboulas, C., A. Swedani, C. Ward, A.S. Al-Madhoun, S. Wilton, S. Boisvenue, A.G. Ridgeway, and I.S. Skerjanc. 2006. HDAC activity regulates entry of mesoderm cells into the cardiac muscle lineage. *J Cell Sci* 119:4305-4314.
100. Savage, J., A.J. Conley, A. Blais, and I.S. Skerjanc. 2009. SOX15 and SOX7 differentially regulate the myogenic program in P19 cells. *Stem Cells* 27:1231-1243.
101. Veeman, M.T., D.C. Slusarski, A. Kaykas, S.H. Louie, and R.T. Moon. 2003. Zebrafish prickles, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol* 13:680-685.
102. Grepin, C., G. Nemer, and M. Nemer. 1997. Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* 124:2387-2395.
103. Mankoo, B.S., S. Skuntz, I. Harrigan, E. Grigorieva, A. Candia, C.V. Wright, H. Arnheiter, and V. Pachnis. 2003. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. *Development* 130:4655-4664.

3: RETINOIC ACID ENHANCES SKELETAL MYOGENESIS IN HUMAN EMBRYONIC STEM CELLS BY EXPANDING THE PREMYOGENIC PROGENITOR POPULATION*

* A version of this chapter has been published.

Reproduced with kind permission from Springer Science + Business Media, Stem Cell reviews and Reports, 2011, **T. Ryan, J. Liu, A. Chu, L. Wang, A. Blais, and I.S. Skerjanc.** Retinoic acid enhances skeletal myogenesis in human embryonic stem cells by expanding the premyogenic progenitor population.

3.1 Abstract

Human embryonic stem cells (hESCs) are a potential source of material for cell therapy of muscle diseases. To date, it has proven difficult to generate skeletal muscle from hESCs in high yields and within a reasonable timeframe. Further, a hESC-derived Pax3/7-positive skeletal muscle progenitor population has not yet been described. Previous studies have shown that Pax3/7-positive progenitor cells can repopulate the satellite cell niche, indicating the importance of this population for therapy. We sought to optimize the differentiation of hESCs into skeletal muscle in order to characterize myogenesis at a molecular level and shorten the time course. We treated hESCs with retinoic acid (RA) and found an enhancement of skeletal myogenesis, and the expression of the myogenic regulatory factors (MRFs) MyoD and myogenin by day 25. Furthermore, we found that RA treatment expanded the muscle progenitor pool, which occurred as a distinct Pax3^{+ve} population prior to MRF expression. Non-skeletal muscle tissue types were not significantly affected. Therefore, we have identified a differentiation pathway in hESCs that provides a skeletal muscle progenitor population which can undergo myogenesis more efficiently. We propose that RA could fit into a directed culture method for deriving skeletal muscle from hESCs.

3.2 Introduction

Human Embryonic Stem Cells (hESCs) present a promising source for cell therapy of muscle diseases such as muscular dystrophy. While hESC-derived skeletal muscle can be stably engrafted into mice, the most significant barrier for hESC-based therapy is the generation of a desirable amount of muscle in a reasonable timeframe. Successful strategies have included the use of a covalent chemical modifying agent, 5-azacytidine, or derivation of multipotent mesenchymal precursors from hESCs, which give rise to a higher proportion of skeletal myocytes than the parental line (1-3). The latter approach required a lengthy time course (>45 days), starting with several weeks of low density monolayer culture to generate mesenchymal precursor cells. Furthermore, it did not utilize embryoid body formation, nor did it follow an embryonic pathway involving mesoderm induction followed by the formation of a distinct Pax3/7⁺ progenitor population prior to expression of the myogenic regulatory factors (MRFs). Such an embryonic pathway has been shown to occur during skeletal myogenesis in P19 embryonal carcinoma (EC) cells, mouse embryonic stem cells (mESCs), and mouse induced pluripotent stem cells (miPSCs) (4-6). The formation of a Pax3/7⁺ progenitor population would be valuable for therapeutic purposes, as it can replenish the satellite cell niche (7, 8).

Skeletal muscle progenitor cells are marked by the expression of Pax3, Pax7 and Meox1 (9-12). While Pax3-null mice exhibit a lack of limb and diaphragm muscle, Pax7 is dispensable for normal embryonic myogenesis (13-17). Compound Pax3/Pax7-null mice however, exhibit a much more severe phenotype characterized by a complete failure of skeletal myogenesis (with the exception of early myotomal muscle), indicating that there is

some level of functional redundancy between these two factors (9). Consistent with this, expression of a dominant-negative Pax3 protein, created by fusion to the repressor domain of engrailed, resulted in a complete inhibition of myogenesis in P19 EC cells and aberrant expression of MyoD and myogenin in cultured satellite cells (12, 18). These data indicate that Pax activity is necessary for normal myogenesis. Conversely, forced expression of Pax3 is known to induce MRF expression and myogenesis in embryonic tissue and in various cell culture models, including P19 EC cells and mESCs (12, 19, 20). Furthermore, Pax3 binds directly to a Myf5 enhancer region, activating its expression in the hypaxial somite (21). Taken together, these data show that Pax3 is required for skeletal myogenesis, upstream of the MRFs.

Our understanding of the role of Meox factors in skeletal myogenesis is complicated by a high degree of redundancy between Meox1 and Meox2. While Meox1-null mice exhibit defects in sclerotomal derivatives, Meox2-null mice have defects in limb muscle development (10, 22). However, like the Pax3/7 compound mutant, Meox1/Meox2 double knockout mice exhibit a much more severe phenotype characterized by an almost complete abrogation of skeletal myogenesis (10). Meox activity is also required for myogenesis in P19 EC cells, where expression of a dominant negative Meox1-Engrailed fusion protein disrupts expression of Pax3, Pax7, MyoD and myogenin (11). In addition to their important roles in myogenesis, the co-expression of Pax3 and Meox1 serves as a good marker for premyogenic mesoderm as the expression domains of these two factors overlap in the paraxial mesoderm, somite, and dermomyotome, while Meox1 is absent from the dorsal neural tube, which has abundant Pax3 expression (23-25). Therefore, strategies to produce a distinct Pax3^{+ve}/Meox1^{+ve} skeletal muscle progenitor population and enhance skeletal myogenesis in a concise time frame, ideally without genetic or covalent chemical

modification, would undoubtedly facilitate the application of hESCs for cell therapy of muscle diseases.

Retinoic acid (RA) is a potent morphogen and is known to direct differentiation of several tissue types in the embryo and in ES cells (26, 27). RA acts by activating its downstream effectors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), which bind to retinoic acid response elements (RAREs) within regulatory regions of RA-responsive genes. In the presence of RA, RARs and RXRs act by recruiting co-activators that promote gene transcription (28). We have previously shown that RA enhances skeletal myogenesis in mESCs and in P19 EC cells (6). In these systems, RA acted early in differentiation to enhance the expression of the precursor genes Pax3 and Meox1 by direct binding of RARs to regulatory regions of these genes (6). This ultimately resulted in enhanced MRF expression and terminal differentiation.

Although many of the genetic networks controlling pluripotency and differentiation in mESCs are conserved in hESCs, there exist fundamental differences between the two systems (29). As such, pathways that have previously been elucidated in the murine system bear a re-examination in hESCs for their ability to drive tissue specific differentiation with a view to therapeutic applications. Here, we show that both myogenesis and the expression of skeletal muscle genes were significantly enhanced by RA treatment of hESCs. Importantly, RA treatment resulted in expansion of a Pax3^{+ve} skeletal muscle progenitor population, which appeared prior to MRF expression. Addition of RA had no significant influence on the differentiation of other cell types.. Thus, RA might be useful as part of a directed culture system designed to maximize the yield of muscle progenitor cells from hESCs and/or human iPS cells.

3.3 Materials and Methods

3.3.1 Cell Culture

H9 hESCs were maintained on feeder layers as described with the exception that feeders were mitotically inactivated with 10 μ g/ml mitomycin C for 2 hours (30). Briefly, H9 hESCs were maintained in Knock-out DMEM supplemented with 20% Knock-out serum replacement, 1% non-essential amino acids, 1mM L-glutamine, penicillin-streptomycin (100U/ml and 100ug/ml) and 0.1mM beta-mercaptoethanol. Media was also supplemented with 4ng/ml basic fibroblast growth factor. Cells were maintained on a mouse embryonic fibroblast layer. Cells were differentiated by generating embryoid bodies in suspension culture for 10 days and then transferred to adherent plates for a further 29 or 15 days. The medium was changed every 2-3 days. Differentiation medium was the same as the propagation medium supplemented with 20% FBS in place of Knock-out serum replacement. All-trans RA (Sigma-Aldrich, Oakville, ON) was added to the culture medium at increasing concentrations from Day 0 to Day 10 of differentiation. Ethanol was used as the vehicle at a final concentration of 0.01%. On day 35 or day 20 of differentiation, the differentiation medium was replaced with D-MEM F12 containing N2 supplement (Invitrogen, Burlington, ON) and Insulin/Transferrin/Selenium (Invitrogen, Burlington, ON), termed N2-ITS medium, until the end point of the experiment. D3 mESCs (ATCC#CRL-1934, Manassas, VA) and P19 EC cells (ATCC#CRL-1825, Manassas VA) were cultured as described (6).

3.3.2 Gene Expression Analysis

Total RNA was harvested from hESCs using RNeasy Micro and Mini kits as per the manufacturer's instructions (Qiagen, Mississauga, ON). cDNA synthesis was performed

using the Quantitect Reverse Transcription Kit (Qiagen, Mississauga, ON) followed by quantitative polymerase chain reaction (QPCR) with either the FastStart SYBR Green Master Mix kit (Roche, Laval, QC) or GoTaq qPCR Master Mix (Promega Madison, WI). QPCR reactions were performed and analyzed on the ABI 7300 or 7500 systems using the SDS analysis software or on the Eppendorf Realplex2 using the Realplex software for analysis. GAPDH was used as a reference for all QPCR reactions and expression changes were quantified relative to Day 0 levels. Unless otherwise stated, error bars represent the standard error of the mean. Statistical analysis was performed using Student's t test.

3.3.3 Immunofluorescence

For myosin heavy chain (MyHC) and Tuj1 labelling, cells were fixed with methanol and labelled with MF20 (pan-MyHC) or Tuj1-specific antibodies (Sigma-Aldrich, Oakville, Ontario). MyHC staining was performed as previously described (6). For alpha smooth muscle actin labelling, cells were fixed with 4% paraformaldehyde and permeabilized for 15 minutes with PBS containing 0.5% TritonX-100. Cells were then blocked for one hour with PBS containing 0.1% bovine serum albumin, 0.1% TritonX-100 and 10% donkey serum. The alpha smooth muscle actin antibody was obtained from Abcam (Cambridge, MA). Pax3/MyoD, Pax3/Meox1 and Pax3/7/myogenin labelling was performed as described with the exception that cells were fixed with 4% paraformaldehyde for Pax3/MyoD labelling (31). Pax3 antibodies were obtained from R&D Systems (Minneapolis, MN) while Meox1 and Pax3/7 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Myogenin labelling was performed with the F5D hybridoma and MyoD antibodies were obtained from BD Biosciences (Franklin Lakes, NJ). Cy3-, Alexa488- and DyLight488-conjugated secondary antibodies were used for detection as appropriate (Jackson ImmunoResearch,

West Grove, PA and Invitrogen, Burlington, ON). Confocal images were captured using a Zeiss LSM 510 microscope and processed with Zen software (Zeiss). Epifluorescent images were captured on a Zeiss Axioskop. All images were processed in Canvas11.

3.4 Results

3.4.1 RA enhances skeletal myogenesis in hESCs

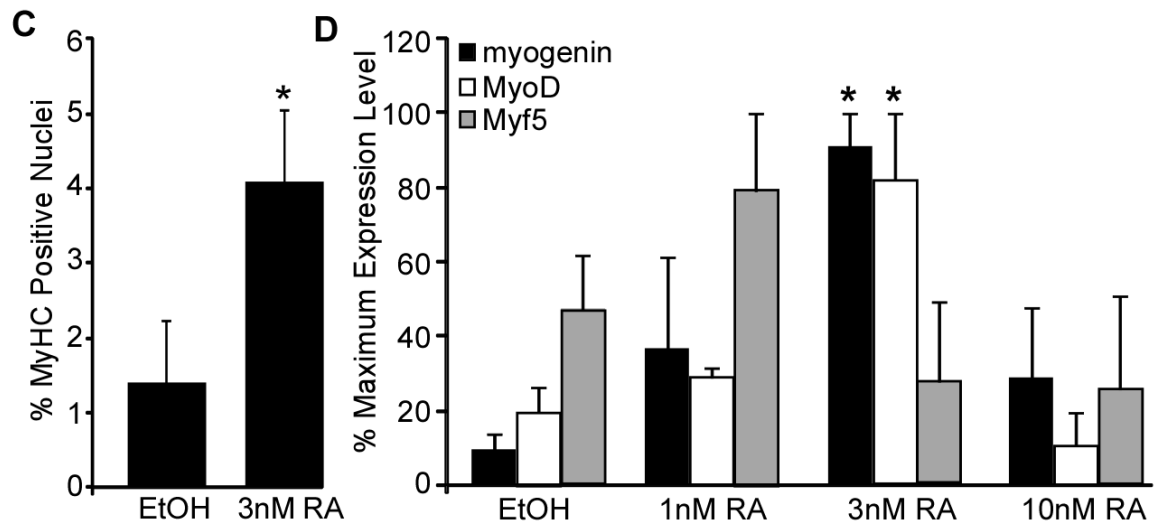
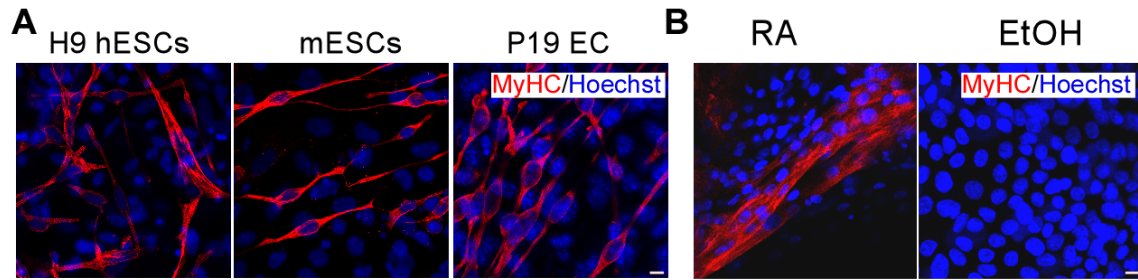
We have previously shown that treatment with low levels of RA can enhance skeletal myocyte formation in both P19 EC cells and in mESCs (6). However, since mechanisms are not always conserved between mESCs and hESCs, it is important to determine whether or not RA would enhance skeletal myogenesis in hESCs (29). Here, we treated differentiating hESCs with increasing concentrations of RA from Day 0 to Day 10 during embryoid body formation, after which the embryoid bodies were plated into tissue culture dishes until day 35. Subsequently, the medium was changed on day 35 to N2-ITS medium and the presence of skeletal myocytes was examined on day 39 by immunofluorescence with an anti-MyHC antibody. We found that treatment with 3nM RA resulted in the presence of abundant hESC-derived skeletal myocytes (Figure 3.1A&B). RA-treated hESCs differentiated into skeletal myocytes (Figure 3.1A) and multinucleated myotubes (Figure 3.1B). Quantification revealed that RA treatment resulted in a significant increase in MyHC-positive nuclei, from approximately 1 to 4% of total cells (Figure 3.1C).

To compare the myocytes generated from hESCs with those derived from mESCs and P19 EC cells, we differentiated the latter two cell lines and examined their morphology by immunofluorescence. D3 mESCs were differentiated by the formation of embryoid bodies for 7 days, followed by plating onto tissue culture plates and examining cultures on day 15. The embryoid bodies were treated with RA from days 2-7 of differentiation (6). P19

EC cells were differentiated by aggregation of cells for 4 days in the presence of RA and 1% DMSO, after which cells were plated in tissue culture plates in the absence of drug and examined on day 9 of differentiation (6). hESCs differentiated into bipolar myocytes with a morphology similar to those derived from either D3 mESCs or P19 EC cells, however, some hESC-derived myocytes appeared thicker and displayed additional cellular projections (Figure 3.1A).

To determine if changes in gene expression correlated with the observed changes in cell morphology, cultures were examined for transcript levels of the MRFs myogenin, MyoD, and Myf5 by QPCR (Figure 3.1D). hESC cultures treated with increasing amounts of RA (from 1nM to 10nM) were differentiated as described above and total RNA was harvested on day 39. Whereas treatment with 1nM RA did not significantly affect the levels of MRF expression relative to vehicle control, treatment with 3nM RA resulted in a significant enhancement of myogenin and MyoD expression levels (Figure 3.1D). Notably, treatment of differentiating cells with 10nM RA did not result in increased MRF expression, indicating that RA-mediated enhancement did not follow a dose-response pattern. Unlike MyoD and myogenin, the expression of Myf5 was not significantly upregulated by treatment with RA (Figure 3.1D). Although Myf5 expression showed a trend toward upregulation at 1nM RA, this was not statistically significant. Thus, 3nM RA enhanced skeletal myogenesis, upregulated the expression of MyoD and myogenin and was used in our subsequent experiments.

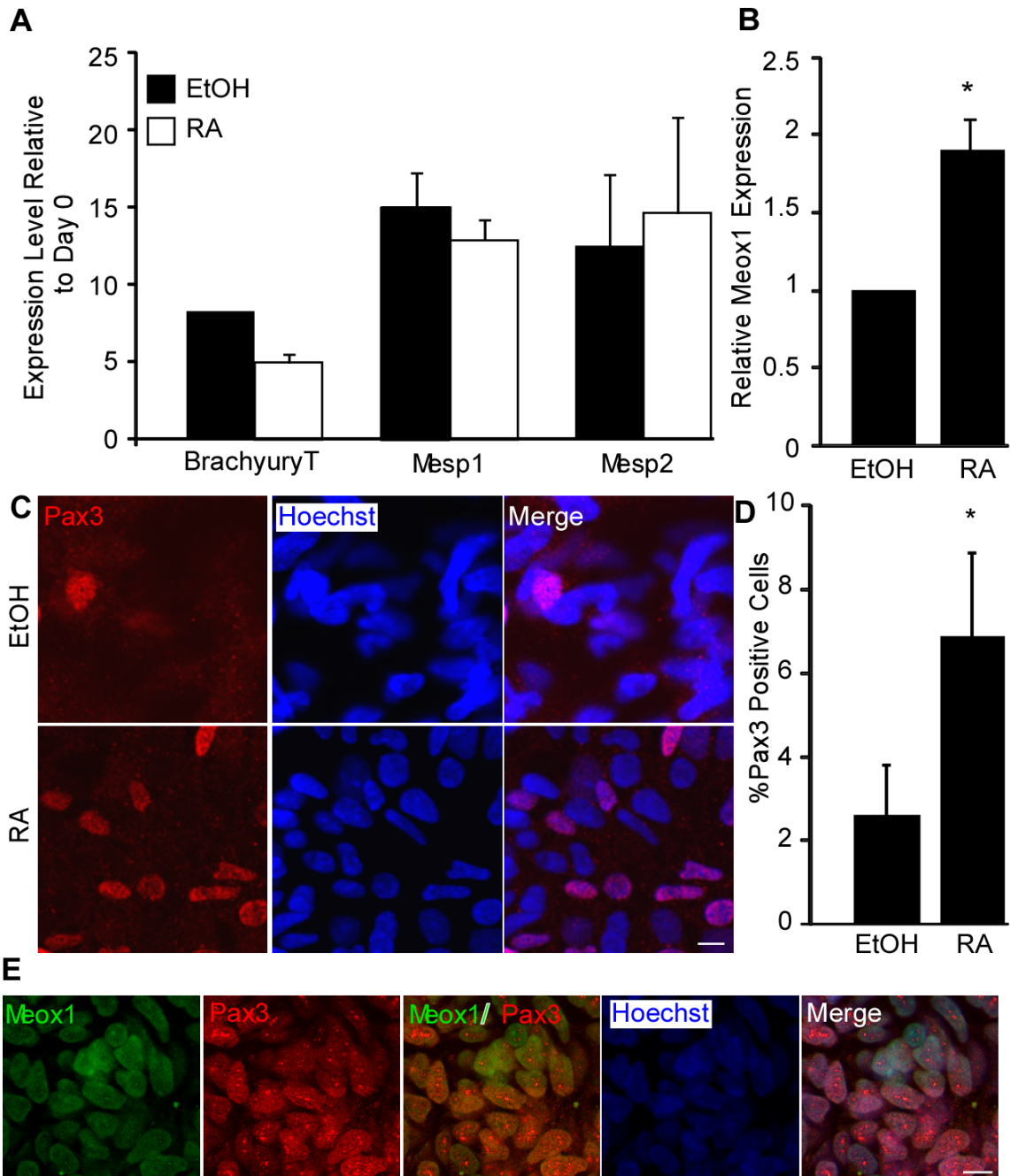
Figure 3.1 Human Embryonic stem cells differentiate into skeletal muscle in response to retinoic acid. H9 hESCs were differentiated for 10 days in the presence of increasing concentrations of RA or vehicle control, followed by growth on adherent plates. Cultures were fixed with methanol for immunofluorescence or harvested for RNA on day 39. A) hESC, P19EC, or mESC aggregates were labelled with a pan-MyHC antibody and Hoechst dye to detect nuclei. P19 cell aggregates were fixed on Day 9, while mESC aggregates were fixed on day 15 (scale bar represents 10 μ m). B) hESC derived muscle formed multinucleated myotubes(scale bar represents 10 μ m). C) Skeletal myogenesis was quantified by counting MyHC^{+ve} cells with a bipolar morphology. A minimum of 20,000 nuclei were quantified for each treatment across 30 fields of view (*p<0.05). D) RNA was analyzed by QPCR for expression of the genes indicated (n=4-5; *p<0.05) and expressed as a percent of maximum for each independent experiment.



3.4.2 Treatment with RA results in expansion of the skeletal muscle progenitor population.

We have previously shown that RA enhances myogenesis in mESCs and P19 EC cells by expanding the premyogenic progenitor population (6). We sought to determine whether the enhancement of myogenesis by RA in hESCs occurred at the same stage of differentiation. We first analyzed the expression levels of the mesodermal markers BrachyuryT, Mesp1 and Mesp2 in the presence and absence of RA by QPCR at day 3 of embryoid body formation. We found that the expression levels of these three markers remained unaffected by RA treatment, suggesting that RA does not enhance myogenesis through mesoderm induction (Figure 3.2A). We next examined control and RA-treated cultures for the expression of premyogenic progenitor markers. The expression levels of Meox1 were analyzed by QPCR on day 8 of embryoid body formation and were found to be increased in RA-treated samples (Figure 3.2B). The premyogenic progenitor population is also marked by the expression of Pax3. In order to determine whether RA treatment resulted in an expansion of this Pax3^{+ve} population, we quantified the number of Pax3^{+ve} cells in control and RA-treated cultures. hESCs were differentiated by forming embryoid bodies for 10 days with and without 3nM RA and then plated into tissue culture plates and fixed on day 15. Cultures were labelled with a Pax3-specific antibody and quantified in control and RA-treated cultures. RA treatment increased the number of Pax3^{+ve} cells by approximately 3-fold (Figure 3.2C&D). Co-staining with antibodies specific to Pax3 and Meox1 identified a population of Pax3^{+ve} cells that were also positive for Meox1 expression, demonstrating the existence of a Pax3^{+ve}/Meox1^{+ve} muscle progenitor population in hESCs (Figure 3.2E). Importantly, co-staining of Pax3 with either MyoD- or myogenin-specific antibodies at Day 15, showed that

Figure 3.2 RA Treatment expands the skeletal muscle progenitor population A) RNA was harvested from Day 3 cultures and analyzed for expression levels of BrachyuryT, Mesp1 and Mesp2 by QPCR. The change in expression is quantified relative to Day 0 levels. Data are from one representative experiment. Error bars represent standard deviation. B) RNA was harvested from Day 8 cultures and analyzed for expression of Meox1 by QPCR (n=4; *p<0.05). The change in Meox1 expression levels as a result of RA treatment is expressed relative to vehicle (EtOH) treatment. C) Differentiating cultures were fixed on Day 15 and labelled with an anti-Pax3 antibody and Hoechst dye to detect nuclei (scale bar represents 10µm). D) The number of Pax3-positive cells was quantified by counting at least 600 cells over 20 fields of view (*p<0.05). E) Differentiating cultures were fixed on Day 15 and labelled with anti-Pax3 and anti-Meox1 antibodies and stained with Hoechst dye to detect nuclei (scale bar represents 10µm).



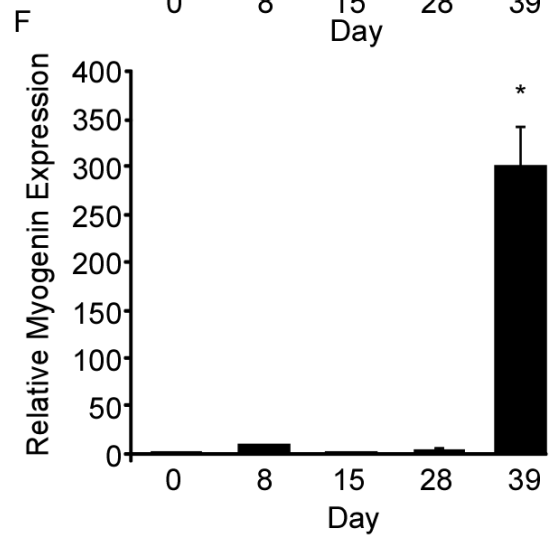
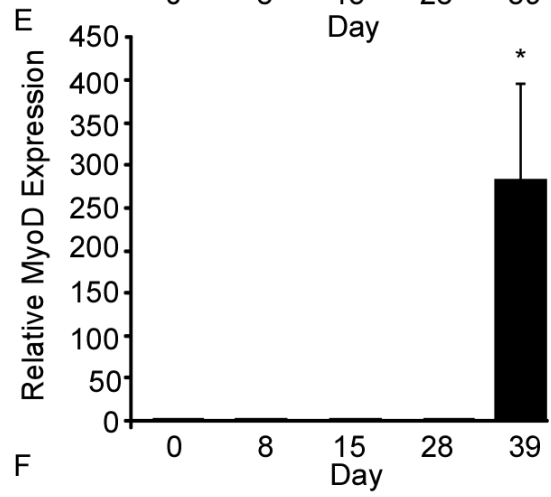
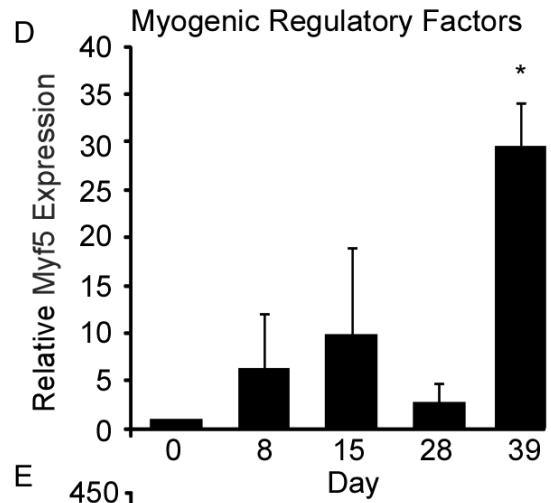
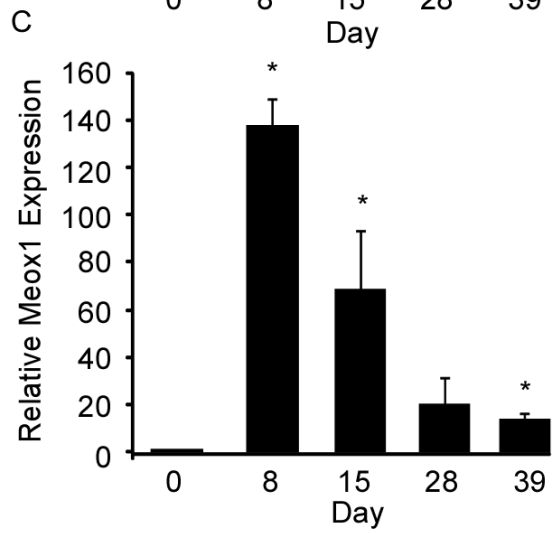
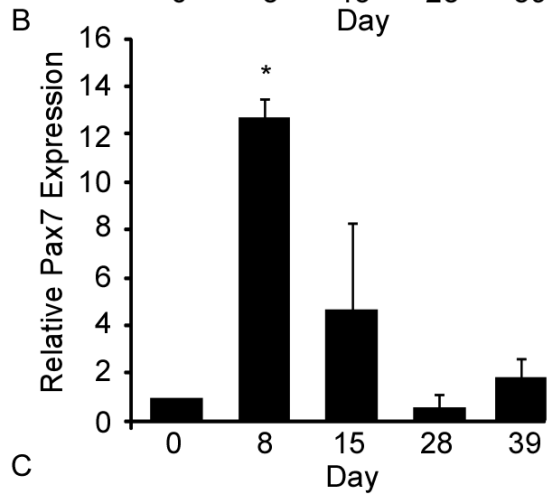
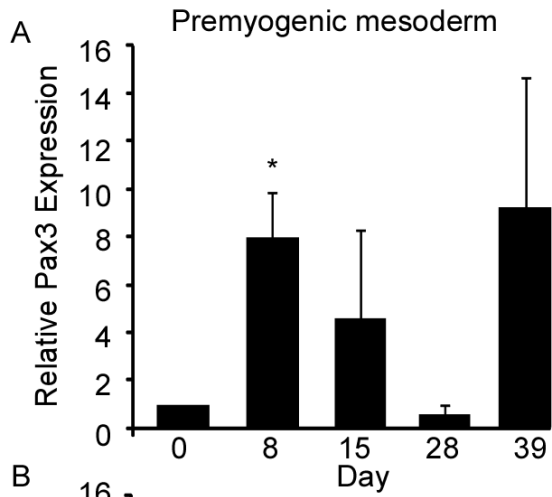
Pax3^{+ve} cells were negative for myogenin and MyoD expression (data not shown), in agreement with the lack of MyoD or myogenin transcripts present at this time (Fig. 3.3, see below). These results suggest that RA acts after mesoderm induction to upregulate muscle precursor gene expression, resulting in an expansion of the Pax3^{+ve} muscle progenitor population, leading to enhanced myogenesis.

3.4.3 Skeletal muscle gene expression is temporally regulated during RA-induced myogenesis

Studies in mESCs, miPSCs and in P19 EC cells have revealed that skeletal myogenesis in these systems proceeds via a temporal pattern of gene expression that is reminiscent of muscle formation in the embryo (4-6). The temporal pattern of gene expression was examined by QPCR using a time course of hESC differentiation. hESCs were differentiated in the presence of RA by forming embryoid bodies for 10 days as described above. Total RNA was harvested on days 0, 8, 15, 28, and 39. In RA-treated hESCs, the expression of the premyogenic mesoderm markers Meox1, Pax7 and Pax3 peaked at Day 8 of differentiation (Figure 3.3A-C). There was a trend for an increase in Pax3 expression at day 39 but this was not significant. The expression of Myf5, MyoD and myogenin was not significantly upregulated until Day 39 (Figure 3.3D-F), four days after the switch from serum-containing differentiation medium to N2-ITS medium. Interestingly, there was a trend for increases in Myf-5 expression at earlier stages, coinciding with the premyogenic mesoderm factors, but this was not significant. Thus, myogenesis proceeded via an embryonic pathway, resulting in a progenitor to myoblast transition.

Figure 3.3 Skeletal muscle genes are temporally regulated during hESC differentiation.

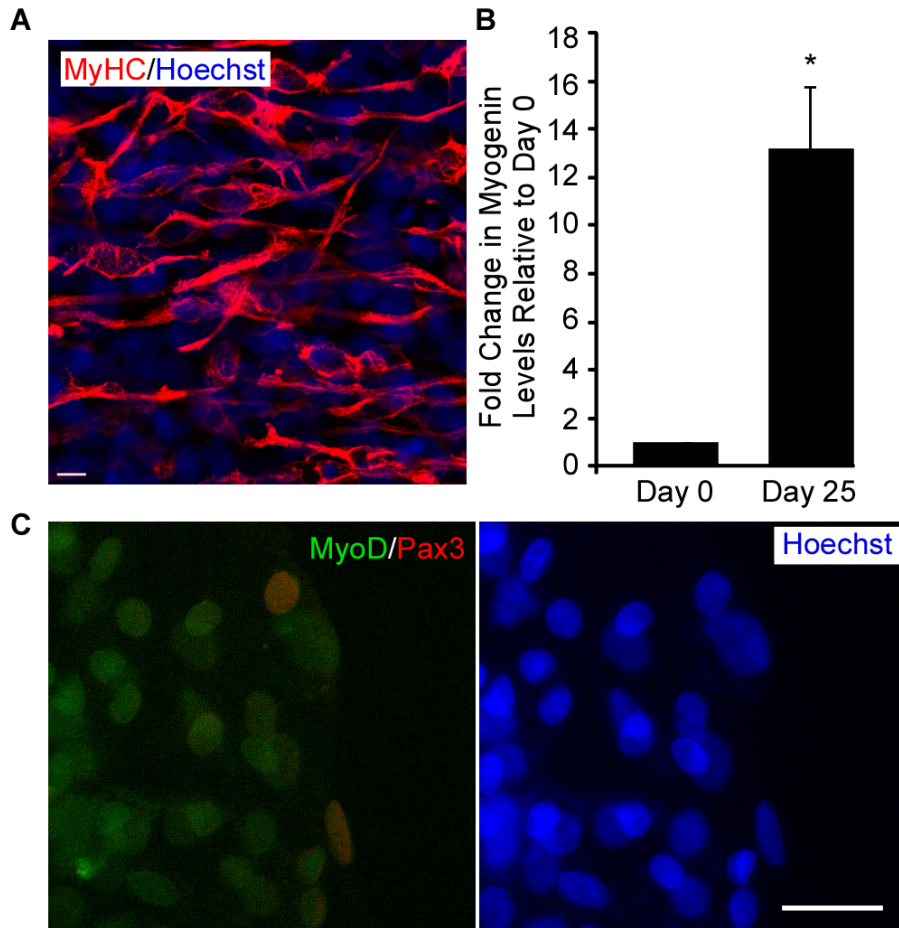
RNA was harvested from hESC cultures at the times indicated and analyzed for skeletal muscle gene expression, for the genes indicated, using QPCR. (n=4-5; *p<0.05). Changes in gene expression levels are expressed relative to undifferentiated cells (Day 0).



3.4.4 The differentiation timecourse of skeletal myogenesis in hESCs can be shortened to 25 days

Given that P19 EC cells and mESCs require 5 and 8 days respectively to form myoblasts from premyogenic mesoderm (Fig. 3.6), we speculated that our identification of 31 days for hESCs could be shortened. We therefore sought to determine whether skeletal muscle could be derived from hESCs at an earlier timepoint by lowering the serum in the differentiation medium prior to Day 35. Indeed we found that the serum could be lowered as early as Day 20 of RA-directed differentiation and that skeletal myocytes were formed by Day 25, as shown by the presence of MyHC^{+ve} myocytes by immunofluorescence (Figure 3.4A). We analyzed the day 25 transcript levels of myogenin by QPCR and found that they were significantly upregulated relative to day 0 levels (Figure 3.4B). Therefore, skeletal muscle can be derived from hESCs in a 25 day time course, which is significantly shorter than previously published protocols (1). To determine if Pax3^{+ve} cells were able to become myoblasts, we also labelled these Day 25 cultures for Pax3 and MyoD. Most MyoD^{+ve} cells at day 25 were negative for Pax3 expression which is likely due to the significant downregulation of Pax3 expression at this timepoint (Figure 3.3A). We did however observe some Pax3^{+ve}/MyoD^{+ve} cells, indicating that at least part of the Pax3^{+ve} progenitor population does give rise to myoblasts during hESC differentiation (Figure 3.4C). Therefore, hESCs can be differentiated into skeletal muscle via an embryonic pathway that can be as short as 25 days.

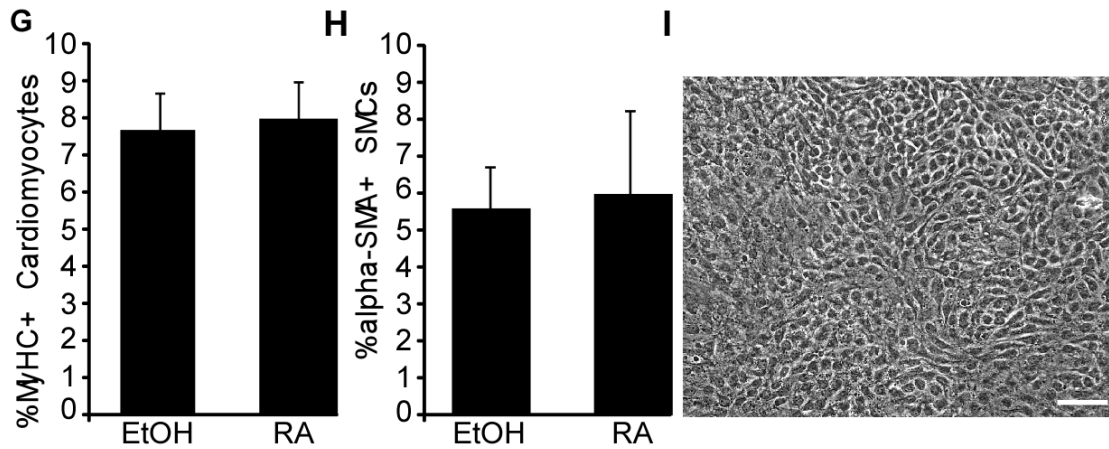
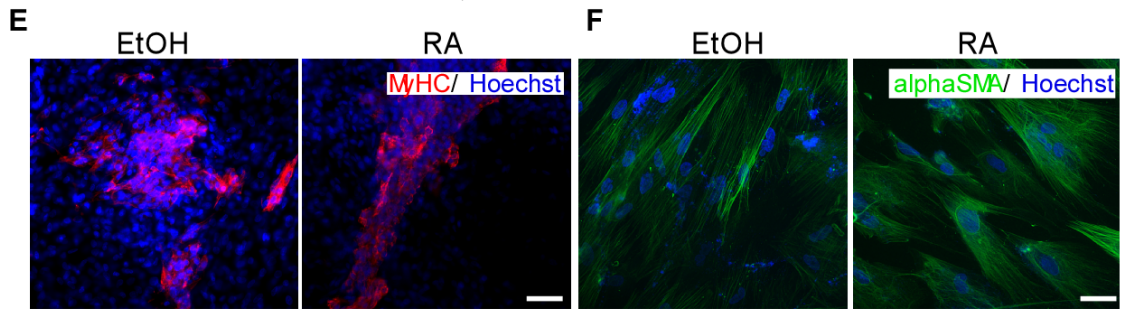
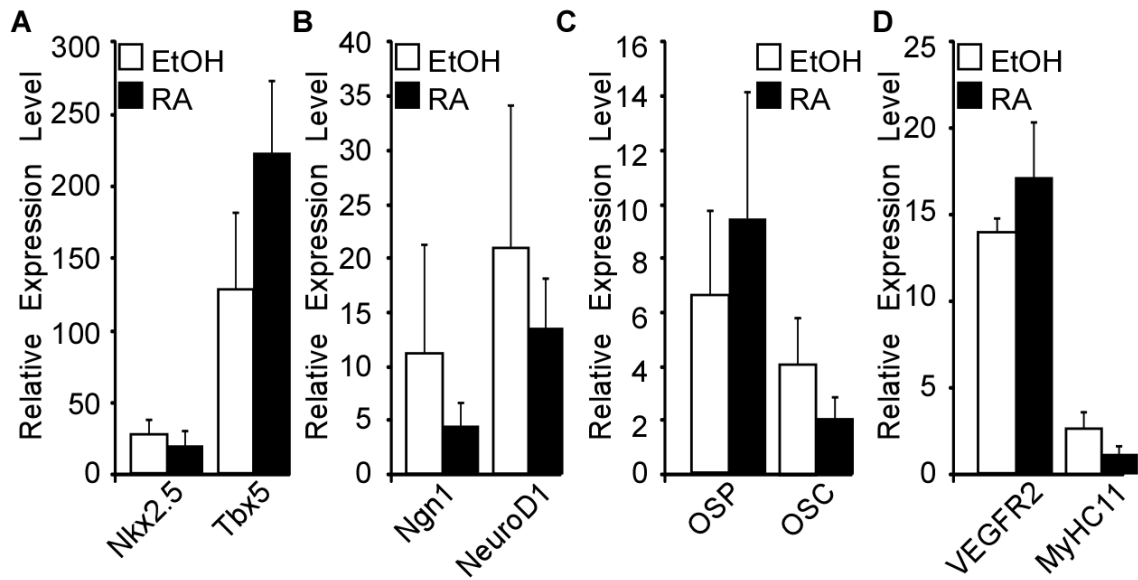
Figure 3.4 The timecourse of skeletal myogenesis in hESCs can be shortened to 25 days. A) Differentiating hESC cultures were fixed with methanol on Day 25 and labelled with antibodies against MyHC and with Hoechst dye to detect nuclei (scale bar represents 10 μ m). B) RNA was harvested from Day 0 and Day 25 RA- treated cultures and analyzed for the expression of myogenin. Day 25 expression levels are quantified relative to Day 0. C) Differentiating cultures were fixed on Day 25 and labelled with antibodies against Pax3 and MyoD and with Hoechst dye to detect nuclei. Scale bar represents 10 μ m.



3.4.5 Low levels of RA have little effect on the differentiation of non-skeletal muscle lineages

RA is known to induce or enhance the formation of many cell types during stem cell differentiation, including smooth muscle, bone, cardiac muscle and neurons, although, at least in the case of smooth muscle and neurons, this enhancement occurs at higher (μM) concentrations of RA (32-35). We therefore examined our RA-treated hESC cultures for differentiation into these other cell types. We assessed cardiomyogenesis by QPCR analysis of the genetic markers *Nkx2.5* and *Tbx5* and found no significant difference in expression levels of these two genes between control and RA cultures (Figure 3.5A). We also quantified the number of *MyHC*^{+ve} cardiomyocytes in both control and RA-treated conditions, and found that these cells accounted for approximately 8% of total cells in each context (Figure 3.5E&G). We analyzed expression levels of the smooth muscle markers *VEGFR2* and *MyHC11* and found that RA treatment did not significantly enhance the expression of these two genes, nor did it result in an increase in the number of alpha-smooth muscle actin (*alpha-SMA*)^{+ve} smooth muscle cells (SMCs), which accounted for approximately 6% of total cells in both control and RA-treated cultures (Figure 3.5D, F&H). We also examined the expression of neurogenic (*NeuroD1*, *Neurogenin1/Ngn1*) and osteogenic (*Osteopontin/OSP* and *Osteocalcin/OSC*) markers and we did not observe a significant increase in the expression of these genes in RA-treated cultures (Figure 3.5B&C). We were able to visualize very few *Tuj1*⁺ neurons in our differentiated cultures and thus the extent of terminal neurogenesis was negligible (<1% of total cells, data not shown), despite the presence of neurogenic transcripts. We were unable to detect any mature osteoblasts by

Figure 3.5 Retinoic Acid does not affect the differentiation of non-skeletal muscle lineages. (A-D) RNA was harvested from Day 39 hESC cultures and analyzed by QPCR for the markers indicated (n=5-7; *p<0.05). Changes in gene expression are expressed relative to undifferentiated cells (Day 0). E&F) Differentiated hESCs were fixed on Day 25 (α SMA, Panel F) or on Day 39 (MyHC, Panel E) and labelled with the antibodies indicated. Hoechst dye was used to detect nuclei. Scale bar represents 10um. G&H) The number of MyHC^{+ve} cardiomyocytes (G) and the number of alpha-SMA^{+ve} smooth muscle cells (H) were quantified by counting a minimum of 800 nuclei across 20 fields of view for control and RA-treated cultures. I) Differentiated hESCs were visualized on Day 39 and most cells exhibited a mesenchymal-like morphology.



Alizarin Red staining (data not shown). The majority of the cells in our cultures appeared to have a mesenchyme-like morphology (Figure 3.5I), which is difficult to identify by specific genetic markers (36). Therefore, the effects of RA were limited to the myogenic lineage and had no significant impact on the differentiation of the other cell types examined here.

3.5 Discussion

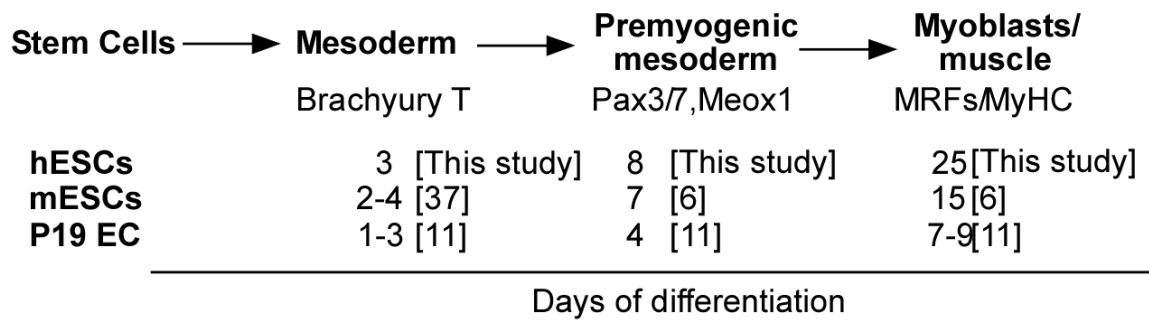
hESCs represent a promising source of material for cell replacement therapy of muscle diseases. In this study, we have shown that the treatment of differentiating hESCs with RA resulted in the enhancement of skeletal myogenesis and the upregulation of expression of the MRFs MyoD and myogenin (Figure 3.1). Furthermore, RA functioned after mesoderm induction by expanding the muscle progenitor population, shown by the upregulation of Meox1 transcript levels and an increase in the proportion of cells expressing Pax3 (Figure 3.2). The identification of muscle progenitor cells expressing both Pax3 and Meox1, and not MyoD or myogenin demonstrated that hESCs can be induced to form muscle via an embryonic pathway. The muscle progenitor cells could differentiate into skeletal myoblasts, as shown by the presence of Pax3^{+ve}/MyoD^{+ve} cells on day 25 of differentiation. Thus, this study represents the first clear demonstration of skeletal myogenesis from hESCs that utilizes an embryonic pathway, involving the formation of a Pax3^{+ve}/ Meox1^{+ve} skeletal muscle progenitor population generated prior to MyoD and myogenin expression. Finally, we have shown that our method does not significantly affect the differentiation of non-muscle tissue types that are known to be enhanced by RA.

The identification of an embryonic pathway for skeletal myogenesis in hESCs allows for a comparison of the timeline of expression of mesodermal, premyogenic, and myogenic

genes during skeletal myogenesis in P19 EC, mESC, and hESCs (Figure 3.6). In P19 EC cells, the expression of the mesodermal marker BrachyuryT is initiated on Day 1 of differentiation and maintained until Day 3 while in mESCs it is expressed from Day 2 to Day 4 (11, 37). This is followed by the expression of the premyogenic mesoderm markers Meox1 and Pax3 on Days 4 and 7 in P19 EC cells and mESCs, respectively (6, 11). While P19 EC cells begin to express MRF genes on days 7-9 of differentiation and undergo terminal differentiation by day 9, mESCs do not form skeletal myocytes until Day 15 of differentiation, in response to low-serum conditions (6, 11). Interestingly, hESCs follow a similar, albeit lengthened, pattern of expression during early differentiation (Figure 3.6). The mesoderm markers BrachyuryT, Mesp1 and Mesp2 are expressed on Day 3 of differentiation (Figure 3.2A and Figure 3.6), followed by the expression of Pax3 and Meox1 on Day 8 (Figure 3.3 and Figure 3.6) (38). Finally, MRF expression is initiated at Day 25, in response to low serum conditions. Thus, the three systems utilize a comparable embryonic pathway to achieve skeletal myogenesis and show a similar enhancement by RA of premyogenic mesoderm, leading to augmentation of the myoblast or myocyte populations (6).

The consistency observed between P19 EC cells, mESCs and hESCs supports the validity of these first two systems as models for the study of molecular mechanisms that contribute to tissue-specific differentiation of hESCs. This comparison illustrates the conservation of the pattern of gene expression during differentiation in all of these systems. In addition, it clearly shows that the similarity to embryonic myogenesis can be exploited to identify future methods to enhance myogenesis in ES systems. Finally, the differences in the timing can be explained in part by differences in the embryonic stages represented by each stem cell and are reflective of the different times of gestation between mouse and human

Figure 3.6 A timeline for myogenic differentiation. P19 cells, mESCs and hESCs share common pathways during myogenic differentiation, although the timelines are distinct. Pluripotent cells are specified to the mesodermal lineage and express Brachyury T. These cells are committed to premyogenic progenitor cells expressing Meox1, Pax3 and Pax7 which undergo terminal differentiation into skeletal muscle, expressing the MRFs MyoD and myogenin.



(39-41). Future studies will determine if lowering the serum earlier than day 20 will result in a further reduction in the time course of myogenesis from hESCs.

Interestingly, the enhancement of myogenesis in hESCs by RA did not show a standard dose-response pattern (Figure 3.1D). This has been observed previously during osteogenesis from mesenchymal stem cells and myogenesis from mESCs (42, 43). This property of RA is poorly understood but may represent a negative feedback mechanism that is activated at high RA concentrations. Alternatively, it may indicate the activation of different subsets of differentiation pathways for each concentration of RA, changing the presence of various progenitor populations. This is supported by the morphogenic nature of RA signalling in the embryo. In the developing embryo, RA is present in an anterior-posterior gradient which provides positional information that contributes to cell fate specification (44). Therefore it is not surprising that specific RA concentrations exert differential effects during stem cell differentiation.

The ability of RA to expand a Pax3^{+ve} muscle progenitor population is particularly important with regard to the use of hESCs for cell therapy, as it may facilitate purification of progenitor cells with the capacity to repopulate the satellite cell niche (7, 8, 45). It appears that as MRF expression is increased, the ability to replenish the stem cell niche decreases (8). Previous studies by others have not shown the formation of a Pax3^{+ve} or Pax7^{+ve} population formed prior to expression during skeletal myogenesis in hESCs (1).

We showed that low levels of RA did not greatly affect the development of other cell types examined in this system, including cardiac and smooth muscle, bone and neurons. Although the differentiation of these cell types is known to be enhanced by RA treatment in various systems, it is not surprising that we did not observe an increase, since RA-directed differentiation is heavily dependent on concentration as well as on timing of addition and

withdrawal (46). For example, neurogenic differentiation in hESCs requires RA concentrations in the range of 100nM to 1 μ M, while we are treating cultures at 3nM (35). Enhancement of smooth muscle differentiation by RA requires treatment of monolayer hESCs with a concentration of 10 μ M, which is orders of magnitude higher than the levels used here (32). The low RA concentrations used to induce skeletal myogenesis can also promote cardiomyogenesis in mESCs, however, the timing of treatment required is different (42). It was shown previously that while skeletal muscle differentiation is enhanced by early exposure of ES cells to RA at the onset of embryoid body formation (Day 0), cardiomyogenesis is inhibited by early exposure to RA and enhanced by later treatment (Day 5-7) (42). As we were treating our cultures from embryoid body formation at Day 0, we did not expect to see any enhancement. However, we did not observe inhibition of cardiomyogenesis by RA, which may be due to fundamental differences in the timing of origin between mESCs and hESCs (39).

In conclusion, we have devised a method that employs RA to enhance the formation myogenic progenitor cells, and consequently, of skeletal muscle from hESCs without genetic or covalent chemical modification. This method has little effect on other lineages known to be enhanced by RA. Furthermore, the time required is shorter than that previously reported and the ability to distinguish embryonic stages is unique. We propose that RA can promote skeletal myogenesis for the purposes of cell therapy.

3.6 References

1. Barberi, T., M. Bradbury, Z. Dincer, G. Panagiotakos, N.D. Succi, and L. Studer. 2007. Derivation of engraftable skeletal myoblasts from human embryonic stem cells. *Nat Med* 13:642-648.

2. Barberi, T., L.M. Willis, N.D. Socci, and L. Studer. 2005. Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PLoS Med* 2:e161.
3. Zheng, J.K., Y. Wang, A. Karandikar, Q. Wang, H. Gai, A.L. Liu, C. Peng, and H.Z. Sheng. 2006. Skeletal myogenesis by human embryonic stem cells. *Cell Res* 16:713-722.
4. Mizuno, Y., H. Chang, K. Umeda, A. Niwa, T. Iwasa, T. Awaya, S. Fukada, H. Yamamoto, S. Yamanaka, T. Nakahata, and T. Heike. 2010. Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. *FASEB J* 24:2245-2253.
5. Petropoulos, H., and I.S. Skerjanc. 2002. Beta-catenin is essential and sufficient for skeletal myogenesis in P19 cells. *J Biol Chem* 277:15393-15399.
6. Kennedy, K.A., T. Porter, V. Mehta, S.D. Ryan, F. Price, V. Peshdary, C. Karamboulas, J. Savage, T.A. Drysdale, S.C. Li, S.A. Bennett, and I.S. Skerjanc. 2009. Retinoic acid enhances skeletal muscle progenitor formation and bypasses inhibition by bone morphogenetic protein 4 but not dominant negative beta-catenin. *BMC Biol* 7:67.
7. Montarras, D., J. Morgan, C. Collins, F. Relaix, S. Zaffran, A. Cumano, T. Partridge, and M. Buckingham. 2005. Direct isolation of satellite cells for skeletal muscle regeneration. *Science* 309:2064-2067.
8. Kuang, S., K. Kuroda, F. Le Grand, and M.A. Rudnicki. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell* 129:999-1010.
9. Relaix, F., D. Rocancourt, A. Mansouri, and M. Buckingham. 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* 435:948-953.
10. Mankoo, B.S., S. Skuntz, I. Harrigan, E. Grigorieva, A. Candia, C.V. Wright, H. Arnheiter, and V. Pachnis. 2003. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. *Development* 130:4655-4664.
11. Petropoulos, H., P.J. Gianakopoulos, A.G. Ridgeway, and I.S. Skerjanc. 2004. Disruption of Meox or Gli activity ablates skeletal myogenesis in P19 cells. *J Biol Chem* 279:23874-23881.
12. Ridgeway, A.G., and I.S. Skerjanc. 2001. Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. *J Biol Chem* 276:19033-19039.
13. Bober, E., T. Franz, H.H. Arnold, P. Gruss, and P. Tremblay. 1994. Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* 120:603-612.

14. Tremblay, P., S. Dietrich, M. Mericskay, F.R. Schubert, Z. Li, and D. Paulin. 1998. A crucial role for Pax3 in the development of the hypaxial musculature and the long-range migration of muscle precursors. *Dev Biol* 203:49-61.
15. Li, J., K.C. Liu, F. Jin, M.M. Lu, and J.A. Epstein. 1999. Transgenic rescue of congenital heart disease and spina bifida in Splotch mice. *Development* 126:2495-2503.
16. Jostes, B., C. Walther, and P. Gruss. 1990. The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. *Mech Dev* 33:27-37.
17. Mansouri, A., A. Stoykova, M. Torres, and P. Gruss. 1996. Dysgenesis of cephalic neural crest derivatives in Pax7^{-/-} mutant mice. *Development* 122:831-838.
18. Relaix, F., D. Montarras, S. Zaffran, B. Gayraud-Morel, D. Rocancourt, S. Tajbakhsh, A. Mansouri, A. Cumano, and M. Buckingham. 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* 172:91-102.
19. Maroto, M., R. Reshef, A.E. Munsterberg, S. Koester, M. Goulding, and A.B. Lassar. 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell* 89:139-148.
20. Darabi, R., K. Gehlbach, R.M. Bachoo, S. Kamath, M. Osawa, K.E. Kamm, M. Kyba, and R.C. Perlingeiro. 2008. Functional skeletal muscle regeneration from differentiating embryonic stem cells. *Nat Med* 14:134-143.
21. Bajard, L., F. Relaix, M. Lagha, D. Rocancourt, P. Daubas, and M.E. Buckingham. 2006. A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev* 20:2450-2464.
22. Mankoo, B.S., N.S. Collins, P. Ashby, E. Grigorieva, L.H. Pevny, A. Candia, C.V. Wright, P.W. Rigby, and V. Pachnis. 1999. Mox2 is a component of the genetic hierarchy controlling limb muscle development. *Nature* 400:69-73.
23. Williams, B.A., and C.P. Ordahl. 1994. Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* 120:785-796.
24. Reijntjes, S., S. Stricker, and B.S. Mankoo. 2007. A comparative analysis of Meox1 and Meox2 in the developing somites and limbs of the chick embryo. *Int J Dev Biol* 51:753-759.
25. Candia, A.F., J. Hu, J. Crosby, P.A. Lalley, D. Noden, J.H. Nadeau, and C.V. Wright. 1992. Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. *Development* 116:1123-1136.

26. Ross, S.A., P.J. McCaffery, U.C. Drager, and L.M. De Luca. 2000. Retinoids in embryonal development. *Physiol Rev* 80:1021-1054.
27. Soprano, D.R., B.W. Teets, and K.J. Soprano. 2007. Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells. *Vitam Horm* 75:69-95.
28. Marletaz, F., L.Z. Holland, V. Laudet, and M. Schubert. 2006. Retinoic acid signaling and the evolution of chordates. *Int J Biol Sci* 2:38-47.
29. Schnerch, A., C. Cerdan, and M. Bhatia. 2010. Distinguishing between mouse and human pluripotent stem cell regulation: the best laid plans of mice and men. *Stem Cells* 28:419-430.
30. Xu, C., M.S. Inokuma, J. Denham, K. Golds, P. Kundu, J.D. Gold, and M.K. Carpenter. 2001. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19:971-974.
31. Savage, J., A.J. Conley, A. Blais, and I.S. Skerjanc. 2009. SOX15 and SOX7 differentially regulate the myogenic program in P19 cells. *Stem Cells* 27:1231-1243.
32. Huang, H., X. Zhao, L. Chen, C. Xu, X. Yao, Y. Lu, L. Dai, and M. Zhang. 2006. Differentiation of human embryonic stem cells into smooth muscle cells in adherent monolayer culture. *Biochem Biophys Res Commun* 351:321-327.
33. Yamashita, A., T. Takada, J. Narita, G. Yamamoto, and R. Torii. 2005. Osteoblastic differentiation of monkey embryonic stem cells in vitro. *Cloning Stem Cells* 7:232-237.
34. Niebruegge, S., A. Nehring, H. Bar, M. Schroeder, R. Zweigerdt, and J. Lehmann. 2008. Cardiomyocyte production in mass suspension culture: embryonic stem cells as a source for great amounts of functional cardiomyocytes. *Tissue Eng Part A* 14:1591-1601.
35. Schuldiner, M., R. Eiges, A. Eden, O. Yanuka, J. Itskovitz-Eldor, R.S. Goldstein, and N. Benvenisty. 2001. Induced neuronal differentiation of human embryonic stem cells. *Brain Res* 913:201-205.
36. Kalluri, R., and M. Zeisberg. 2006. Fibroblasts in cancer. *Nat Rev Cancer* 6:392-401.
37. Willey, S., A. Ayuso-Sacido, H. Zhang, S.T. Fraser, K.E. Sahr, M.J. Adlam, M. Kyba, G.Q. Daley, G. Keller, and M.H. Baron. 2006. Acceleration of mesoderm development and expansion of hematopoietic progenitors in differentiating ES cells by the mouse Mix-like homeodomain transcription factor. *Blood* 107:3122-3130.

38. Sumi, T., N. Tsuneyoshi, N. Nakatsuji, and H. Suemori. 2008. Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. *Development* 135:2969-2979.
39. Rossant, J. 2008. Stem cells and early lineage development. *Cell* 132:527-531.
40. Brons, I.G., L.E. Smithers, M.W. Trotter, P. Rugg-Gunn, B. Sun, S.M. Chuva de Sousa Lopes, S.K. Howlett, A. Clarkson, L. Ahrlund-Richter, R.A. Pedersen, and L. Vallier. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448:191-195.
41. Tesar, P.J., J.G. Chenoweth, F.A. Brook, T.J. Davies, E.P. Evans, D.L. Mack, R.L. Gardner, and R.D. McKay. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448:196-199.
42. Wobus, A.M., Rohwedel, J., Maltsev, V., Hescheler, J. 1994. In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. *Roux's Arch Dev Biol* 204:36-45.
43. Zhang, W., Z.L. Deng, L. Chen, G.W. Zuo, Q. Luo, Q. Shi, B.Q. Zhang, E.R. Wagner, F. Rastegar, S.H. Kim, W. Jiang, J. Shen, E. Huang, Y. Gao, J.L. Gao, J.Z. Zhou, J. Luo, J. Huang, X. Luo, Y. Bi, Y. Su, K. Yang, H. Liu, H.H. Luu, R.C. Haydon, T.C. He, and B.C. He. 2010. Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. *PLoS One* 5:e11917.
44. White, R.J., Q. Nie, A.D. Lander, and T.F. Schilling. 2007. Complex regulation of *cyp26a1* creates a robust retinoic acid gradient in the zebrafish embryo. *PLoS Biol* 5:e304.
45. Darabi, R., F.N. Santos, A. Filareto, W. Pan, R. Koene, M.A. Rudnicki, M. Kyba, and R.C. Perlingeiro. 2011. Assessment of the myogenic stem cell compartment following transplantation of *pax3/pax7*-induced embryonic stem cell-derived progenitors. *Stem Cells* 29:777-790.
46. Rohwedel, J., K. Guan, and A.M. Wobus. 1999. Induction of cellular differentiation by retinoic acid in vitro. *Cells Tissues Organs* 165:190-202.

**4: MYOSIN PHOSPHATASE MODULATES CARDIAC CELL
FATE DECISIONS BY REGULATING THE SUBCELLULAR
LOCALIZATION OF NKX2.5 IN A WNT/ROCK DEPENDENT
PATHWAY**

4.1 Abstract

Nkx2.5 is a transcription factor that regulates cardiac gene expression in vivo and in embryonic stem cells. Although Nkx2.5 has been implicated in the regulation of many cellular processes which ultimately contribute to cardiomyogenesis and morphogenesis of the mature heart, little is known about how it is regulated at the functional level. We have undertaken a proteomic screen to identify novel cofactors of Nkx2.5 during cardiomyogenic differentiation. We have identified the myosin phosphatase subunits PP1 β and Mypt1 as putative binding partners of Nkx2.5 in this process. The interaction with PP1 β /Mypt1 resulted in exclusion of Nkx2.5 from the nucleus and consequently, inhibition of its transcriptional activity. Furthermore, in transient transfection experiments, nuclear excluded Nkx2.5 co-localized outside the nucleus with phosphorylated Mypt1 whereas this effect was attenuated by inhibition of canonical Wnt signalling or by ROCK inhibition. Treatment of differentiating mouse embryonic stem cells with Wnt3a resulted in enhanced phosphorylation of endogenous Mypt1, increased nuclear exclusion of endogenous Nkx2.5 and a failure to undergo terminal cardiomyogenesis. Therefore, promoting the interaction of Nkx2.5 with PP1 β /Mypt1 represents a novel mechanism whereby Wnt3a mediates inhibition of cardiac progenitor differentiation.

4.2 Introduction

During development, differentiation of myocardial cells and morphogenesis of the heart are controlled by a cascade of transcription factors that are in turn regulated by the convergence of several extracellular signalling factors and morphogens. Cardiac progenitor cells originate from the splanchnic mesoderm and are organized in a crescent-like structure that ultimately fuses at the midline of the embryo to generate the linear heart tube, which in turn loops to form the mature, four chambered organ (1). Cardiac progenitors are segregated into two distinct populations, termed the first and second heart fields (FHF and SHF), each of which gives rise to particular regions of the mature heart (2-6). The differentiation of these progenitors into mature cardiomyocytes and subsequent morphogenetic events are regulated by transcription factors including GATA-4, Nkx2.5, Mef2c and several members of the Tbx family. These same mechanisms are employed during differentiation of mouse embryonic stem cells (mESCs) into cardiomyocytes (7).

Nkx2.5 is one of the earliest markers of cardiomyogenesis in differentiating cells both *in vivo* and in ESCs (8). Mutations in Nkx2.5 are a common cause of congenital heart disease in humans, with more than 40 known disease causing mutations associated with multiple different morphological phenotypes (9). In the mouse, loss of Nkx2.5 results in failure of the heart to undergo looping, leading to embryonic lethality (10, 11). Although cardiomyocytes are specified normally in these mice, the expression of genes downstream of Nkx2.5, including atrial natriuretic factor (ANF), the ventricular isoform of myosin light chain 2 (Mlc2v) and Connexin40 (Cx40) is disrupted. Interestingly, this is in direct contrast to the role played by the *Drosophila* homologue *tinman*, loss of which results in failure of cardiomyocyte specification (12). Studies in mESCs have supported the notion that Nkx2.5

is not absolutely required for cardiomyocyte specification, although, as in the null mouse, the expression of downstream genes was disrupted (13). In some organisms, this is most easily explained by functional redundancy between Nkx factors whose expression overlaps in the developing heart. For example, in frogs, coexpression of dominant negative *XNkx2.5* and *XNkx2.3* results in a complete abrogation of cardiomyogenesis (14). In the mouse, the interplay between Nkx factors is not as well understood. Although expression of Nkx2.6 overlaps to some extent with that of Nkx2.5, compound Nkx2.5/Nkx2.6-null mice exhibit normal specification of cardiomyocytes (15, 16). However, expression of a dominant negative form of Nkx2.5, fused to the repressor domain of engrailed, resulted in inhibition of cardiomyogenesis in P19 cells, suggesting that some murine Nkx activity may be required for cardiac muscle differentiation (17). Interestingly, Nkx2.5 can also induce cardiomyogenesis in this system, indicating that it is sufficient to drive the cardiogenic program under appropriate conditions (18).

This paradox has led several groups to examine the role of Nkx factors in cardiomyogenesis on a finer scale. Analysis of the role of Nkx2.5 in the SHF identified a negative feedback loop in which Nkx2.5 inhibits Bmp2/Smad1 signalling, ultimately controlling the specification and proliferation progenitors (19). In zebrafish, morpholino mediated inhibition of *nkx2.5* and *nkx2.7* expression resulted in the formation of an excess number of atrial cardiomyocytes paralleled by a decrease in the number of ventricular cardiomyocytes, suggesting that Nkx genes differentially regulate distinct progenitor populations within the developing heart (20).

Nkx2.5 regulates many of its target genes via direct interactions with other cardiomyogenic transcription factors including GATA-4, Tbx5 and Mef2c (21-23) In addition to regulating the extent of target gene activation, these interactions also confer target

specificity and modulate Nkx2.5 activity. Although Nkx2.5 acts most often as a transcriptional activator, it can repress target gene expression in the context of particular regulatory complexes. Specifically, the interaction of Nkx2.5 with Tbx5 promoted expression of ANF while interaction with Tbx2 is repressive (22, 24, 25). This ‘switch’ in cofactors has been proposed as a mechanism for blocking ANF expression and the formation of chamber myocardium in Tbx2 expressing regions of the developing heart (25). It is clear that protein-protein interactions are important for the regulation of Nkx2.5 activity and thus, of cardiomyocyte differentiation and heart development.

Cardiomyogenesis is also regulated by the action of extracellular signalling molecules derived from surrounding cells and tissue structures. In particular, the canonical Wnt signalling cascade plays diverse roles at different stages of cardiomyogenesis (26). Canonical Wnt signalling mediated by Wnt3a is important for the induction of mesoderm both *in vivo* and in mESCs (27, 28). Indeed, a lack of canonical Wnt signalling resulted in the loss of Mesp1 expression, which is perhaps the earliest marker of the cardiovascular lineage and has been suggested to act as a master regulator of the specification of these cells (28, 29). Conversely, specification of cardiac progenitor cells from Mesp1^{+ve} cells is potentiated by the Wnt inhibitor Dkk1, which is a target of Mesp1-dependent transcription, indicating that canonical Wnt signalling is inhibitory at this later stage (30, 31). Consistent with this, activation of Wnt/ β -catenin signalling in differentiating mESCs at later stages resulted in reduced cardiomyogenesis (32, 33). Furthermore, a directed differentiation protocol involving treatment of mESCs and human embryonic stem cells (hESCs) with Dkk1 (amongst other factors) promoted high yields of cardiomyocytes from these systems (34, 35). Within the SHF, Wnt signalling appears to modulate the number of progenitor cells by regulating their proliferation. Loss or inhibition of canonical Wnt signalling in the SHF

results in reduced numbers of progenitor cells and defective development of SHF derived portions of the heart while induction of this signalling cascade results in expansion of the progenitor population (36-41). Although the multiphasic and multifunctional nature of Wnt signalling has been well documented, the molecular mechanisms whereby Wnt exerts these differential effects on cardiomyogenesis remain unknown.

We have undertaken a proteomic screen to identify novel cofactors of Nkx2.5 in the context of cardiomyogenesis. The β -isoform of protein phosphatase 1 (PP1 β , also known as PP1 δ) and its regulatory subunit Myosin Phosphatase Targeting Subunit 1 (Mypt1), two subunits of the myosin phosphatase (MP) enzyme complex, were identified as putative cofactors of Nkx2.5 and validated by co-immunoprecipitation. Together with Myosin light chain kinase (MLCK), MP modulates the balance of phosphorylation of the regulatory light chain of myosinII, thereby regulating muscle contraction (42). We show here that coexpression with MP resulted in exclusion of Nkx2.5 from the nucleus and inhibition of its transcriptional activity. This occurs downstream of Wnt signalling and is modulated by ROCK-mediated phosphorylation of Mypt1. Treatment of differentiating mESCs with Wnt3a, which is known to inhibit cardiomyogenesis (32, 33), was accompanied by increased exclusion of endogenous Nkx2.5 from the nucleus. Our findings suggest a novel mechanism for Wnt3a-mediated inhibition of cardiac progenitor differentiation.

4.3 Materials and Methods

4.3.1 Plasmid constructs

The tandem affinity purification (TAP) tag, consisting of a protein A domain and a calmodulin binding domain separated by a TEV protease cleavage site, was excised from pRC/CMV by digesting with Apa1 and BamH1 (43, 44). The PGK destination vector (18,

45) was digested with Xho1 and BamH1. The Apa1 and Xho1 sites were subsequently blunted and the two resulting fragments were ligated to generate PGK-TAP. The Nkx2.5 sequence was excised by digesting with BamH1 and Not1 and ligated into PGK-TAP which had been digested with the same enzymes to generate PGK-Nkx2.5TAP, where the TAP tag was fused to the N-terminus of Nkx2.5. Flag-Nkx2.5, HA-PP1, myc-Mypt1 and myc-NIPPI have been previously described (23, 46, 47). PGK-Puro and B17 have also been previously described (48, 49).

4.3.2 Cell Culture and Transfections

P19 cells (ATCC #CRL-1825, Manassas, VA) were cultured and differentiated as previously described (17). Briefly, cells were maintained in α -minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and gentamycin (100 μ g/ml).

Differentiation was induced by aggregation and treatment of the cells with dimethyl sulfoxide (DMSO). Aggregates were maintained in DMSO for 4 days and then plated in adherent plates for a further two days. P19[Nkx2.5TAP] and P19[TAP] stable cell lines were generated as previously described (17). For transient transfection experiments, P19 cells were transfected using Fugene (Roche, Laval, QC), as per the manufacturer's instructions. Cells were treated with 150ng/ml Dkk1 (R&D Systems, Minneapolis, MN), 10nM LeptomycinB (Sigma-Aldrich, Oakville, ON) or 10nM γ -27632 (EMD Chemicals, Gibbstown, NJ) 3-5 hours after transfection and fixed for immunofluorescence one day later. For analysis of pMypt1T853 levels, total protein was harvested from P19 cells treated for 24 hours with 150ng/ml Dkk1 or 10nM γ -27632 and analyzed by western blot with antibodies specific to pMypt1T853 (Santa Cruz Biotechnology, Sanata Cruz, CA), total Mypt1 (Cell Signalling, Danvers, MA) and alpha-tubulin (Sigma-Aldrich, Oakville, ON).

D3 mESCs (ATCC #CRL-1934, Manassas, VA) were maintained as previously described (50). Differentiation was induced by LIF withdrawal and cell aggregation using the hanging drop method (800cells/drop). Cells were aggregated in hanging drops for two days then transferred to non-adherent plates for three days. On day 5, aggregates were transferred to adherent plates for a further 2 days for a total of 7 days. Differentiating cells were treated with 200ng/ml Wnt3a (R&D Systems, Minneapolis, MN) or vehicle (0.1% BSA in PBS) from day 4 to day 7 of differentiation.

HEK-293 cells were maintained in α -MEM supplemented with 10% FBS supplemented with penicillin-streptomycin (100U/ml and 100ug/ml).

4.3.3 Protein purification

P19[TAP] and P19[Nkx2.5TAP] cells were differentiated for 6 days and protein was harvested for purification on Day 6. Cells were harvested in extraction buffer containing 20mM Hepes pH 7.4, 0.1% Tween20, 2mM MgCl₂ and 200mM NaCl, supplemented with Complete EDTA Free Protease Inhibitors (Roche, Laval, QC), 1% aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride. Lysates were sonicated 4 times for 4 second bursts and centrifuged for 30 minutes at 55000rpm in a Beckman TLA120.2 rotor. 10mg of total protein was immunoprecipitated overnight at 4°C with rabbit IgG (Chemicon) coupled to magnetic Dynabeads, Tosylactivated as per the manufacturer's instructions (Invitrogen, Burlington, ON). Beads were washed three times with extraction buffer and immunoprecipitated protein was eluted by heating in 25 μ l of protein sample buffer without beta-mercaptoethanol at 65°C for 10 minutes. Beads were collected with a magnet and the eluate was removed, supplemented with 0.5 μ l of beta-mercaptoethanol and boiled for 5 minutes.

4.3.4 Mass Spectrometry analysis

Gel sections encompassing the whole sample lane were excised, reduced, alkylated, and digested as described previously (51). The resulting peptide solutions were dried in a SpeedVac and stored at -20°C until the mass spectrometric analysis. LC-MS/MS was performed by dissolving the peptide samples in 5% formic acid and loading them into a 200µm x 5-cm precolumn packed in house with 5µm ReproSil-Pur C18-AQ beads (Dr. Maisch HPLC GmbH) using a micro Agilent 1100 HPLC system (Agilent Technologies). The peptides were desalted on line with 95% water, 5% acetonitrile, 0.1% formic acid (v/v) for 10 min at 10 µl/min. The flow rate was then split before the precolumn to produce a flow rate of ~200 nl/min at the column. Following their elution from the precolumn, the peptides were directed to a 75µm x 5cm analytical column packed with 5µm ReproSil-Pur C18-AQ beads. The peptides were eluted using a one hour gradient (5–80% acetonitrile with 0.1% formic acid) into an LTQ linear ion trap mass spectrometer (Thermo-Electron). MS/MS spectra were acquired in a data-dependant acquisition mode that automatically selected and fragmented the five most intense peaks from each MS spectrum generated. Peak lists were generated from the MS/MS .raw file using Mascot Distiller 2.0.0.0 (Matrix Science) to produce a .mgf file with default parameters except that for each MS/MS individual peak lists were generated assuming a +2 and a +3 charge. All .mgf files were analyzed and matched to the 56729 *Mus musculus* protein sequences in the International Protein Index (IPI) database (version 3.68; released on the 17th of December 2009) using the Mascot 2.1.04 database search engine (Matrix Science) with trypsin as the digestion enzyme, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. Peptide and MS/MS mass tolerances were set at +/- 3 and +/- 0.8 Da, respectively, with one miss-cleavage allowed and the significance threshold set to 0.01 ($p > 0.01$). Finally, an ion

score cutoff of 30 was chosen to produce a false-positive rate of less than 1% in the MS data (52). A protein hit required at least two “bold red peptides,” i.e. the most logical assignment of the peptide in the database selected. Furthermore, when peptides matched to more than one database entry, only the highest scoring protein was considered

4.3.5 Co-immunoprecipitations

HEK 293 cells were transiently transfected with Flag-Nkx2.5, HA-PP1 and myc-Mypt1. Total protein was harvested 24 hours after transfection using RIPA buffer supplemented with Complete-EDTA free protease inhibitors (Roche, Laval, QC). Lysates were precleared and immunoprecipitations were performed with anti-Flag M2 agarose (Sigma), anti-HA (12CA5 clone, Santa Cruz Biotechnology) or anti-myc (9E10 clone, Santa Cruz Biotechnology). Mouse IgG was used as a negative control. Reactions were incubated overnight and antibody-antigen complexes were captured with Protein-A/G agarose beads (Invitrogen). Beads were washed with RIPA buffer and immunoprecipitated protein was eluted by boiling in sample buffer. Input and immunoprecipitated samples were analyzed by western blot.

4.3.6 Luciferase assays

P19 cells were transiently transfected with Flag-Nkx2.5, HA-PP1 and myc-Mypt1 or myc-NIPP1 along with ANF-luciferase and SV40-Renilla as an internal reference control. The ANF reporter has been previously described (53). Transfected cells were harvested after 24 hours and luciferase assays were conducted as per the manufacturer’s instructions (Promega, Madison, WN).

4.3.7 Immunofluorescence

P19 cells were transfected with Flag-Nkx2.5 or GATA4 along with HA-PP1 and myc-Mypt1 or myc-NIPP1. Cells were fixed one day after transfection with ice-cold acetone for 10 minutes and allowed to air dry for 10 minutes, then rehydrated with PBS for a further 10 minutes. Cells were blocked for 1 hour in PBS containing 10% donkey serum, 0.1% BSA and 0.1% Triton X-100. Cells were then labelled with antibodies against Nkx2.5 or GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA) and myc (9E10 Hybridoma) or Nkx2.5 (Abcam, Cambridge, MA) and p-Mypt1 T853 (Santa Cruz Biotechnology, Santa Cruz, CA and Millipore, Billerica, MA). D3 mESCs were processed in the same way and labelled with antibodies against Nkx2.5 (Santa Cruz Biotechnology, Santa Cruz, CA) and Mlc2v (Synaptic Systems, Goettingen, Germany). Donkey anti goat Alexa 488 (Invitrogen, Burlington, ON), donkey anti mouse Cy3 and Donkey anti rabbit Cy3 (Jackson ImmunoResearch, West Grove, PA) were used for detection as appropriate. All images were captured using a Zeiss LSM 510 confocal microscope and were processed in Zen (Zeiss) and Canvas 11 softwares.

4.3.8 Gene Expression Analysis

Gene expression analysis was performed as described previously (54). Briefly, total RNA was harvested from mESCs on day 7 of differentiation and on day 0 (undifferentiated) using the RNeasy Mini kit as per the manufacturer's instructions (Qiagen, Mississauga, ON). RNA was reverse-transcribed using the Quantitect Reverse Transcription Kit (Qiagen, Mississauga, ON) followed by quantitative polymerase chain reaction (QPCR) with the GoTaq qPCR Master Mix (Promega Madison, WI). QPCR reactions were performed and analyzed on the Eppendorf Realplex2 using the Realplex software for analysis. β -actin was used as a reference for all QPCR reactions and expression changes were quantified relative

to Day 0 levels. Data are expressed relative to vehicle control expression levels. Error bars represent the standard error of the mean. Statistical analysis was performed using Student's T Test.

4.4 Results

4.4.1 Nkx2.5 interacts with PP1 β and Mypt1

In an effort to identify novel interacting partners of Nkx2.5 during cardiomyogenesis in stem cells, we performed a proteomic screen using affinity purification of Nkx2.5 from differentiating P19 cells coupled to mass spectrometry. P19 stable cell lines were generated to overexpress PGK-Nkx2.5TAP or PGK-TAP as a control. These cell lines, termed P19[Nkx2.5TAP] and P19[TAP] respectively, were differentiated in the presence of DMSO and protein was harvested on Day 6. Lysates were subjected to immunoprecipitation with IgG and subsequently analyzed by mass spectrometry. Using this proteomic screen technique, we identified PP1 β and Mypt1 as putative binding partners of Nkx2.5 (Table 4.1). Both PP1 β and Mypt1 are subunits of the MP enzyme complex.

The validity of this interaction was supported by co-immunoprecipitation experiments, in which Nkx2.5-Flag was transiently co-expressed with HA-PP1 β and myc-Mypt1 in HEK293 cells. We immunoprecipitated each subunit individually with antibodies specific to Flag, HA or myc and we detected the presence of interacting proteins by western blot. Both HA-PP1 β and myc-Mypt1 co-immunoprecipitated with Flag-Nkx2.5, using an anti-Flag antibody (Figure 4.1). Reciprocal immunoprecipitations of HA-PP1 β or myc-Mypt1 also co-immunoprecipitated Flag-Nkx2.5. Therefore we have identified PP1 β and Mypt1, two subunits of the MP enzyme complex, as novel cofactors of Nkx2.5.

Table 4.1 Mass Spectrometry Results of Nkx2.5 affinity purification

Entrez Gene ID	Protein Name	Mascot Score	% Coverage (total # of peptides)
18091	Nkx2-5	273	14% (5)
19046	Ppp1cb	804	38% (10)
17931	Ppp1r12a (Mypt1)	289	6% (5)

Figure 4.1 Nkx2.5 interacts with PP1 β and Mypt1. HEK293 cells were transiently transfected with Flag-Nkx2.5, HA-PP1 β and myc-Mypt1. Total protein was harvested and immunoprecipitated with antibodies specific for Flag, HA and myc or with mouse IgG as a negative control. Immunoprecipitated lysates and input (30 μ g/0.02% of input) fractions were analyzed by western blots with the antibodies indicated.

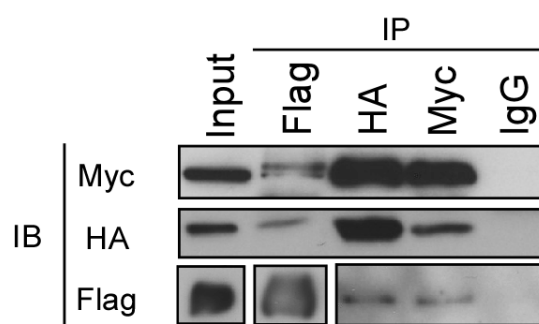
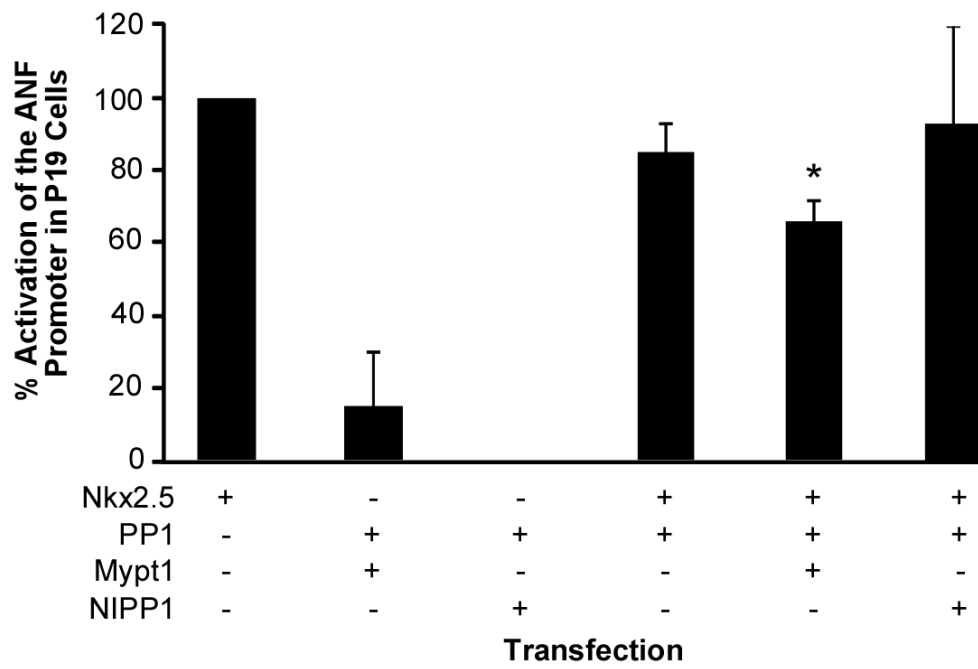


Figure 4.2 Coexpression with MP inhibits Nkx2.5 activity. P19 cells were transiently transfected with the plasmids indicated or with empty vector, along with the ANF-luciferase reporter and SV40-Renilla as an internal control. Firefly luciferase activity was normalized to Renilla luciferase and reporter activation was quantified relative to the empty vector control and data are expressed as a percent of Nkx2.5 activation, n=3, *p<0.05.

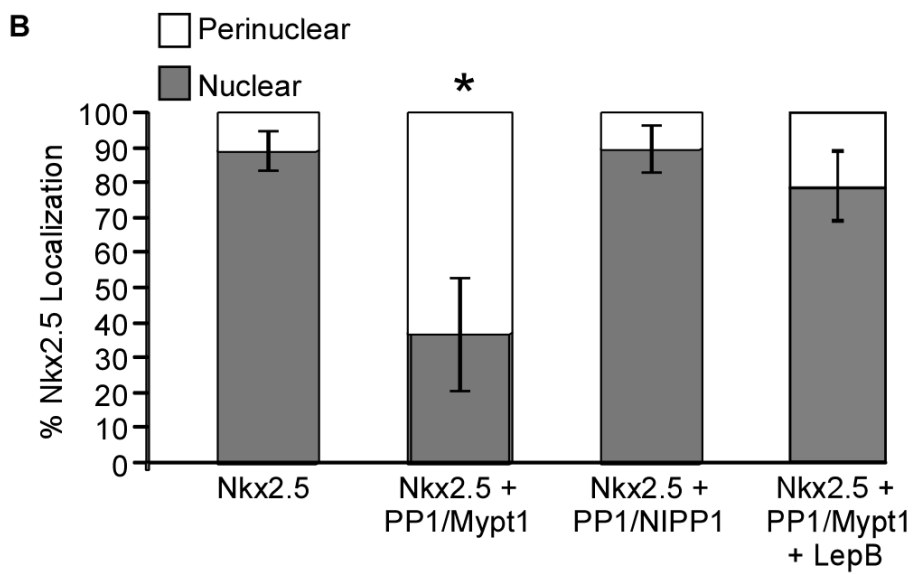
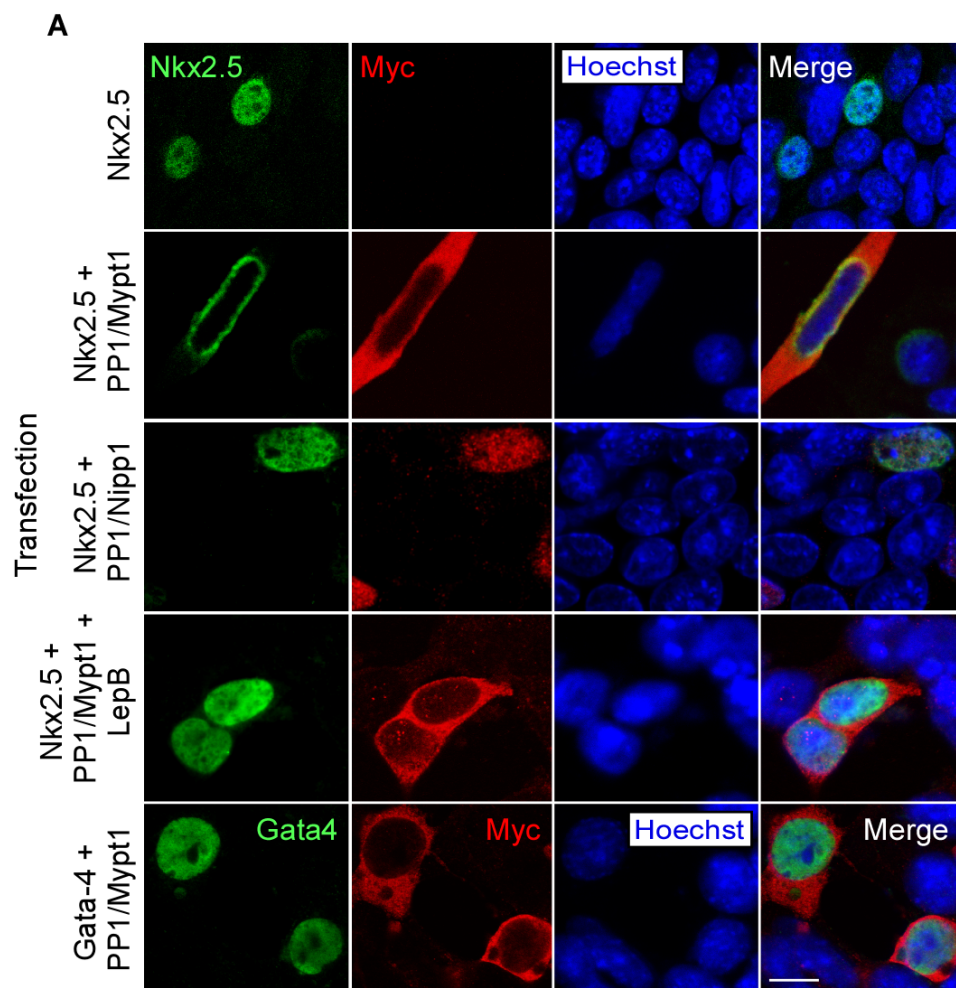


interaction occurs within the cell. Flag-Nkx2.5 was co-transfected with and without the MP subunits in P19 cells and the subcellular distributions of the proteins were visualized by immunofluorescence. When Flag-Nkx2.5 was transfected alone, it was localized almost exclusively to the nucleus (Figure 4.3A&B). However, in the context of co-expression with MP, Flag-Nkx2.5 was redistributed to the cytoplasm into a perinuclear pattern (Figure 4.3A). Quantification of this phenomenon confirmed a significant change in subcellular distribution of Nkx2.5, with more than 60% of co-expressing cells exhibiting a perinuclear distribution of Nkx2.5 (Figure 4.3B). In agreement with our reporter assay data, the redistribution of Nkx2.5 did not occur if NIPP1 was substituted for Mypt1, once again indicating that this effect is specific to MP (Figure 4.3A&B). Interestingly, treatment with LeptomycinB, an inhibitor of CRM1-dependent nuclear export, prevented nuclear exclusion of Nkx2.5 in the presence of MP, suggesting that Nkx2.5 is actively exported from the nucleus into the cytoplasm, where it engages in an interaction with MP. Finally, co-expression with MP did not result in an altered subcellular distribution of GATA4, suggesting that this phenomenon is not a general regulatory mechanism affecting all cardiogenic transcription factors (Figure 4.3A).

4.4.4 Nkx2.5 interacts with phosphorylated MP

Although MP is primarily localized within the cytoplasm, phosphorylation of human Mypt1 at T853 (T852 in the mouse) by Rho-associated kinase (ROCK) causes a redistribution of the MP complex to the perinuclear region and the nucleus (56). In addition, this phosphorylation event is known to be inhibitory to the activity of the PP1 β catalytic subunit (57, 58). The perinuclear redistribution of Nkx2.5 in response to coexpression with MP suggests an

Figure 4.3 Coexpression with MP results in exclusion of Nkx2.5 from the nucleus. P19 cells were transiently transfected with the plasmids indicated and fixed one day later for analysis of subcellular localization. A) Fixed cells were labelled with antibodies specific to Nkx2.5 and myc as well as with Hoechst dye to detect nuclei. Scale bar represents 10 μ m. B) Subcellular localization of Nkx2.5 was quantified in cells transfected cells, n=3, *p<0.05.

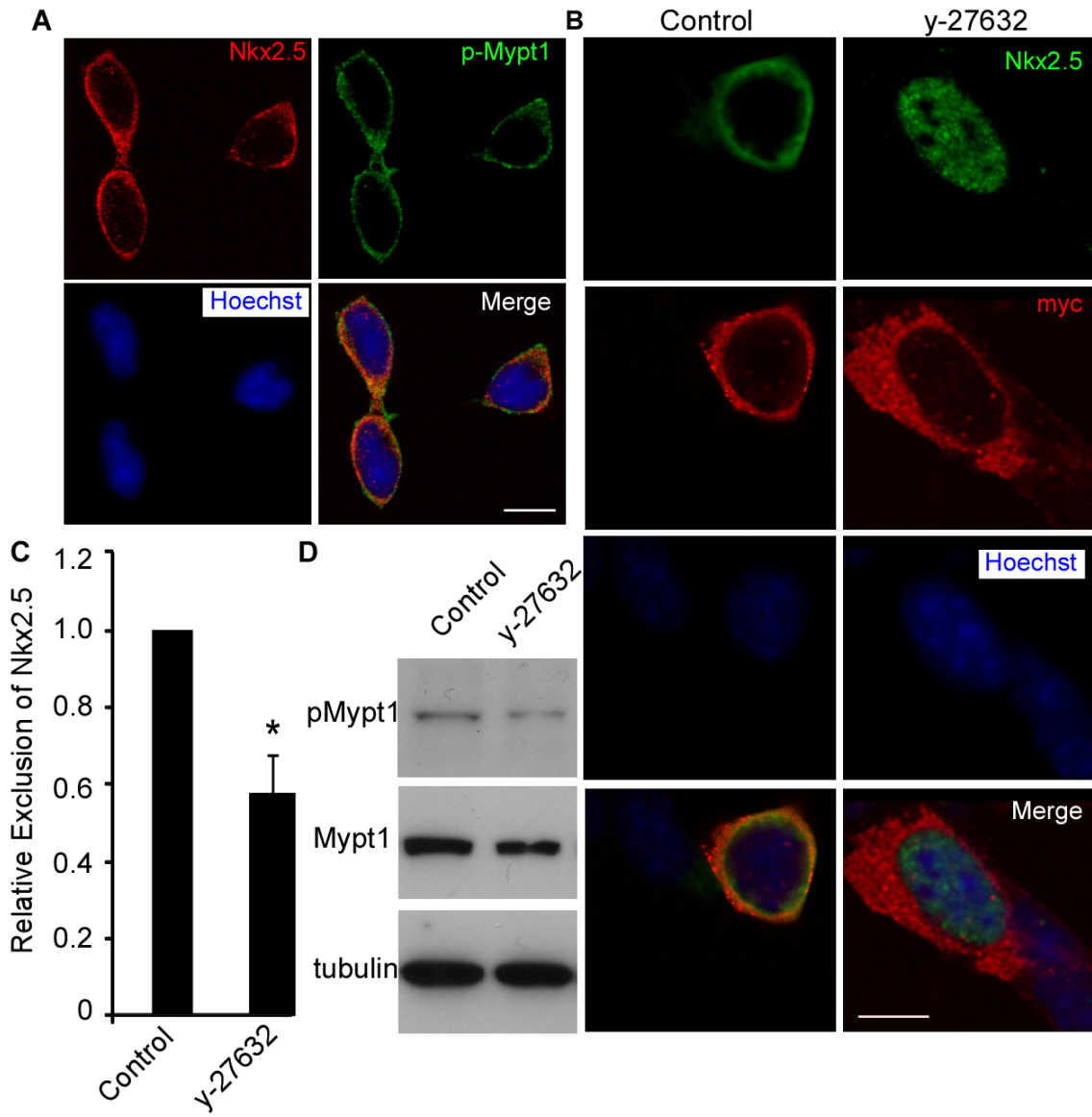


interaction with phosphorylated MP. Immunofluorescence with antibodies specific to Nkx2.5 and a p-Mypt1T853 in co-transfected cells identified co-localization of Nkx2.5 with p-Mypt1T853 in transfected cells (Figure 4.4A). To determine whether modulation of Mypt1 phosphorylation could affect the subcellular localization of Nkx2.5, cells co-transfected with Flag-Nkx2.5 and the MP subunits were treated with the ROCK inhibitor y-27632. When examined by immunofluorescence, redistribution of Nkx2.5 to the perinuclear region in response to co-expression with MP was significantly attenuated in the presence of y-27632 by approximately 40% relative to untreated control cultures (Figure 4.4B&C). Consistent with previously published reports, treatment with y-27632 resulted in a redistribution of myc-Mypt1 to a broader cytoplasmic area by analysis with immunofluorescence (Fig. 4.4B) and reduced levels of p-Mypt1T853 by western blot analysis (Figure 4.4D) when compared to untreated control cultures (57). Thus, phosphorylation of Mypt1 is required for the movement of Nkx2.5 out of the nucleus.

4.4.5 The canonical Wnt inhibitor Dkk1 blocks MP-dependent redistribution of Nkx2.5

Exclusion of Nkx2.5 from the nucleus and the consequent inhibition of its function are likely to result in downregulation of the expression of its target genes in the context of differentiation. Therefore, it stands to reason that regulation of the ROCK-mediated phosphorylation of Mypt1, and subsequent nuclear exclusion of Nkx2.5, might occur via a signalling cascade that is inhibitory toward cardiomyogenesis. The canonical Wnt pathway is a good candidate in this instance as it is known to inhibit terminal differentiation of cardiac progenitors and it also activates ROCK function (32, 33, 59). In order to investigate whether

Figure 4.4 Nkx2.5 interacts with the phosphorylated form of Mypt1. P19 cells were transiently transfected with Flag-Nkx2.5, HA-PP1 β and myc-Mypt1. A) Cells were labelled by immunofluorescence with antibodies specific to Nkx2.5 and pMypt1Thr853. Scale bar represents 10 μ m. B) Cultures were treated with the ROCK inhibitor γ -27632 and labelled by immunofluorescence with antibodies specific to Nkx2.5 and myc as well as with Hoechst dye to detect nuclei. Treatment with γ -27632 attenuated redistribution of Nkx2.5 in response to coexpression with MP when compared to untreated controls. Scale bar represents 10 μ m. C) The subcellular localization of Nkx2.5 was quantified in control and γ -27632-treated cultures. Data are expressed relative to the level of nuclear exclusion of Nkx2.5 in untreated controls, n=3, *p<0.05. D) Total protein was harvested from γ -27632-treated and control cultures and analyzed by western blots using antibodies specific to the factors indicated.



canonical Wnt signalling might play a role in this process, we co-transfected Flag-Nkx2.5 with MP and analyzed the subcellular distribution of Nkx2.5 in the presence and absence of the canonical Wnt inhibitor Dkk1. By immunofluorescence analysis, treatment with Dkk1 resulted in significant inhibition of the MP-dependent exclusion of Nkx2.5 from the nucleus when compared to vehicle control (Figure 4.5A and B). The extent of inhibition (approximately 44%) was similar to that observed in cultures treated with y-27632. In addition, Dkk1 treatment caused a decrease in the levels of p-Mypt1T853, suggesting that it may inhibit relocalization of Nkx2.5 by regulating ROCK-dependent phosphorylation of Mypt1 (Figure 4.5C).

4.4.6 Wnt3a treatment of differentiating mESCs excludes Nkx2.5 from the nucleus and inhibits cardiomyogenesis

As activation of canonical Wnt signalling at a late stage of mESC differentiation is known to inhibit their ability to form cardiomyocytes, we investigated whether this inhibition might involve nuclear exclusion of endogenous Nkx2.5 (32, 33). Differentiating mESCs were treated with and without Wnt3a from days 4-7 and the effects on cardiomyogenesis were examined by gene expression analysis and immunofluorescence. Treatment of differentiating mESCs with Wnt3a resulted in a reduced number of Mlc2v^{+ve} cardiomyocytes, from approximately 11% to 2% of total cells (Figure 4.6A&B). This was accompanied by a significant reduction in Nkx2.5 transcript levels, as well as of the transcripts for cardiomyocyte markers Mlc2v, Cx40, ANF and myosin heavy chain isoform 6 (MyHC6) (Figure 4.6C). Interestingly, the expression of GATA4 was not significantly affected by Wnt3a treatment (Figure 4.6C). We also examined levels of p-Mypt1T853 in Wnt3a and vehicle treated cultures and found that, consistent with our Dkk1 results, Wnt3a

Figure 4.5 The redistribution of Nkx2.5 in response to coexpression with MP is attenuated by treatment with Dkk1. P19 cells were transiently cotransfected with Flag-Nkx2.5, HA-PP1 β and myc-Mypt1. A) Cultures were treated with 150ng/ml Dkk1 or vehicle (PBS+0.1% BSA) and labelled with antibodies specific to Nkx2.5 and myc as well as with Hoechst dye to detect nuclei. Treatment with Dkk1 attenuated redistribution of Nkx2.5 in response to coexpression with MP when compared to vehicle-treated controls. Scale bar represents 10 μ m. B) The subcellular localization of Nkx2.5 was quantified in vehicle and Dkk1-treated cultures. Data are expressed relative to the level of nuclear exclusion of Nkx2.5 in vehicle-treated controls, n=4, *p<0.05. C) Total protein was harvested from Dkk1 and vehicle treated cultures and analyzed by western blots using antibodies specific to the factors indicated.

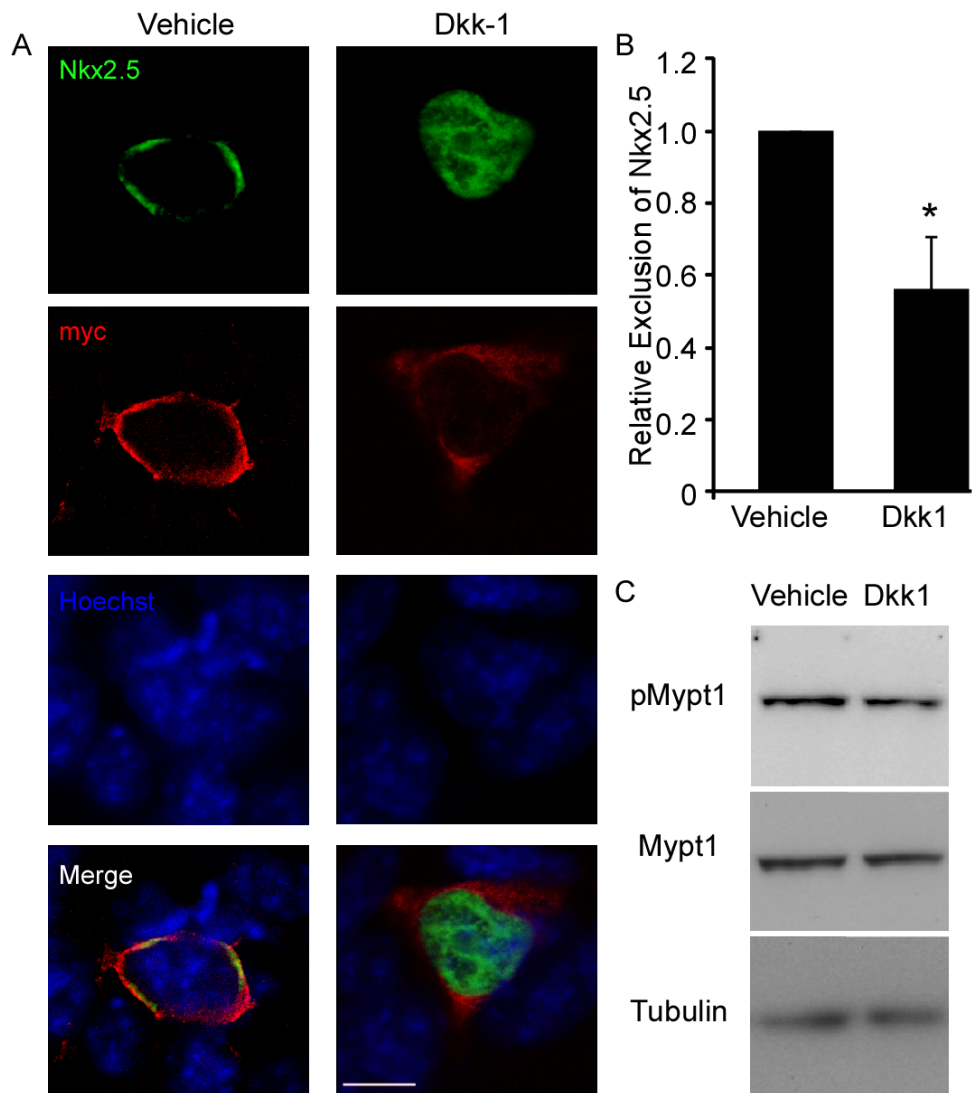
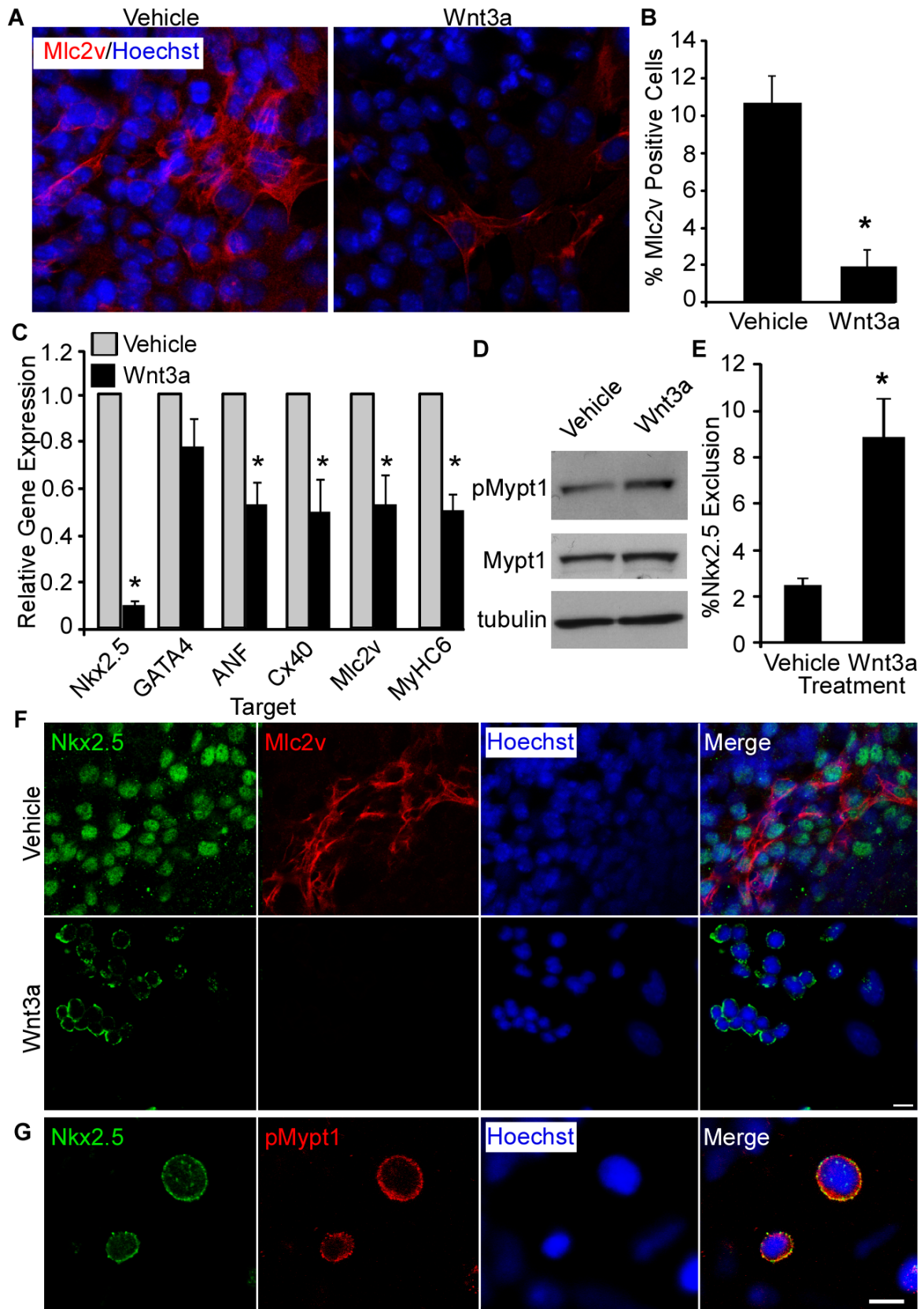


Figure 4.6 Treatment of differentiating mESCs with Wnt3a results in reduced cardiomyogenesis associated with increased exclusion of endogenous Nkx2.5 from the nucleus. Differentiating D3 mESCs were treated with Wnt3a or vehicle (PBS + 0.1% BSA) control from days 4 to 7. A) Wnt3a- and vehicle- treated cells were fixed on day 7 and labelled with antibodies specific to Mlc2v and stained with Hoechst dye to detect nuclei. Scale bar represents 10 μ m. B) The number of Mlc2v⁺ cells was quantified in vehicle- and Wnt3a- treated cultures, n=3, *p<0.05. C) The expression of genes associated with cardiomyogenesis was analyzed by QPCR in vehicle- and Wnt3a- treated cultures. Data are expressed relative to expression levels in vehicle treated cultures, n=3, *p<0.05. D) Total protein was harvested from Wnt3a- and vehicle- treated mESCs on day 7 and analyzed by western blot with antibodies specific to the factors indicated. E) The exclusion of endogenous Nkx2.5 from the nucleus was quantified in vehicle- and Wnt3a- treated mESC cultures, n=3, *p<0.05. F) Differentiated mESCs from vehicle- and Wnt3a- treated cultures were fixed on day 7 and labelled with antibodies specific to Nkx2.5 and Mlc2v. Scale bar represents 10 μ m.



treatment resulted in increased levels of p-Mypt1T853 relative to vehicle control (Figure 4.6D). We next analyzed the subcellular distribution of endogenous Nkx2.5 in Wnt3a and vehicle treated cultures. In vehicle treated cultures, endogenous Nkx2.5 was primarily found in the nucleus, with only a small number (approximately 2%) of cells displaying Nkx2.5 in the perinuclear region (Figure 4.6E&F). In Wnt3a treated cultures however, the fraction of cells in which endogenous Nkx2.5 was excluded from the nucleus increased significantly to approximately 8% (Figure 6E&F). In order to determine the differentiation status of these cells, we co-labelled them with antibodies specific to Mlc2v and Nkx2.5 (Figure 4.6F). Whereas cells which exhibited a nuclear pattern of endogenous Nkx2.5 expression differentiated into Mlc2v^{+ve} cardiomyocytes, we did not observe any Mlc2v^{+ve} cardiomyocytes in which Nkx2.5 was excluded from the nucleus, suggesting that this phenomenon is associated with a failure to undergo terminal differentiation. As in our transient transfection experiments, excluded endogenous Nkx2.5 co-localized with endogenous p-Mypt1T853 (Figure 4.6G). Therefore, endogenous Nkx2.5 is excluded from the nucleus in response to Wnt3a treatment of differentiating mESCs and this exclusion is correlated with a failure to undergo terminal differentiation into cardiomyocytes.

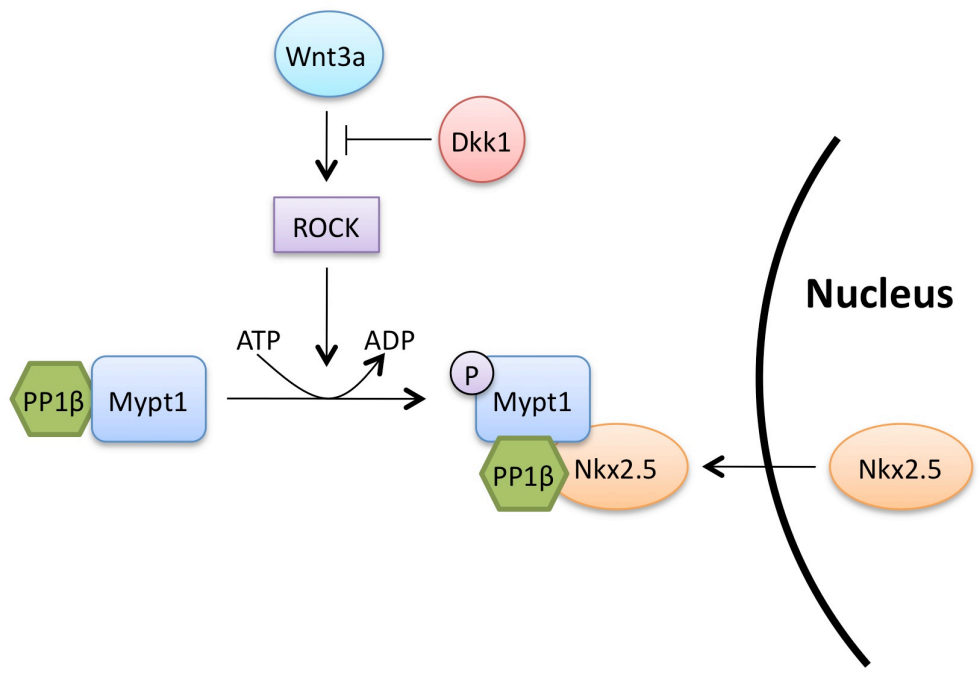
4.5 Discussion

Nkx2.5 has long been known to be an important regulator of gene expression during heart development *in vivo* and during cardiogenic differentiation in stem cells. It is one of the earliest genes to be expressed in the cardiogenic pathway and is responsible for regulating the expression of many downstream targets that are essential for proper heart development. Despite this, little is known about how the Nkx2.5 protein is regulated at a functional level. We have shown, using a proteomic screen that PP1 β and Mypt1, two subunits of the MP

enzyme complex, are novel interacting partners of Nkx2.5 and this interaction was validated by co-immunoprecipitation (Table 4.1 and Figure 4.1). More specifically, Nkx2.5 interacts with the phosphorylated form of MP, resulting in exclusion of Nkx2.5 from the nucleus, its accumulation in the perinuclear region of the cell, and a decrease in its transcriptional activity (Figures 4.2-4.4). This interaction is modulated by the canonical Wnt signalling inhibitor Dkk1, which inhibits phosphorylation of Mypt1 and the consequent relocalization of the MP complex to the perinuclear region (Figure 4.5). Consistent with this, treatment of differentiating mESCs with Wnt3a results in enhanced exclusion of endogenous Nkx2.5 from the nucleus, associated with downregulation of cardiac muscle gene expression and inhibition of cardiomyogenesis (Figure 4.6). We have summarized these findings in a model, shown in Figure 4.7.

Although the primary function of MP is usually associated with dephosphorylation of myosin, this is not the first example of a role for MP in the regulation of subcellular localization of proteins. It has previously been shown that MP interacts with, and promotes the nuclear translocation of HDAC7 resulting in inhibition of apoptosis in thymocytes (60). In this study, the authors found that dephosphorylation of HDAC7 by MP was required for the translocation event. We have shown that Nkx2.5 interacts with a form of the MP complex characterized by phosphorylation of the Mypt1 subunit at T853. This phosphorylation event has profound inhibitory effects on the phosphatase activity of the PP1 β subunit which results from docking of the phosphorylated Mypt1 residue within the active site of the catalytic subunit (57, 58). Therefore, it is unlikely that the regulation of the subcellular localization of Nkx2.5 is dependent on dephosphorylation by MP. Recently, it was shown that PP1 α interacts with the cardiomyogenic transcription factor MEF2 and that this interaction inhibits MEF2-dependent gene transcription in a phosphatase-independent

Figure 4.7 MP interacts with Nkx2.5 and controls its subcellular localization downstream of ROCK/Wnt3a. Under cardiomyogenic conditions, Nkx2.5 resides in the nucleus and activates cardiac muscle gene expression. In response to Wnt3a signalling, Mypt1 is phosphorylated at Thr853 by ROCK, causing MP to accumulate in the perinuclear region. Nkx2.5 is exported from the nucleus in response to an unknown signal and is bound by MP, causing it to remain excluded from the nucleus.



fashion (61). Taken together with our findings, this suggests that PP1-containing complexes likely regulate targets by both phosphatase-dependent and –independent mechanisms. Furthermore, we have shown that modulating ROCK activity can alter the subcellular redistribution of Nkx2.5 in response to coexpression with MP by regulating the levels of p-Mypt1T853 (Figure 4). Inhibition of ROCK activity significantly enhances survival of hESC derived cardiomyocytes after dissociation of aggregates (62). Given that Nkx2.5 has been shown to protect cardiomyocytes from stress-induced cell death, it is tempting to speculate that these two pathways might be linked by regulation of Nkx2.5 localization (63).

The mechanisms regulating the nucleo-cytoplasmic shuttling of Nkx2.5 remain to be identified. We have shown here that treatment with the CRM1-dependent nuclear export inhibitor LeptomycinB prevents nuclear exclusion of Nkx2.5 in response to co-expression with MP. This suggests that Nkx2.5 is being actively exported from the nucleus by utilizing this transport system and that accumulation of MP in the perinuclear region results in cytoplasmic retention of Nkx2.5. Further study will be required to determine why this export initially occurs or whether Nkx2.5 is in a constant state of transition between these two domains, in which case regulation of Mypt1 phosphorylation may be the limiting factor in this process.

Wnt signalling is known to inhibit terminal differentiation of cardiac progenitors both *in vivo* and *in vitro*, although a mechanistic insight into this regulation has been lacking (32, 33, 37). We have shown in this study that treatment of differentiating mESCs with Wnt3a results in exclusion of Nkx2.5 from the nucleus, which is accompanied by downregulation of gene expression associated with cardiomyogenesis and ultimately results in reduced cardiac muscle development. Wnt3a has previously been shown to activate ROCK and indeed, we observed higher levels of pMypt1T853 in our Wnt3a-treated cultures (Figure 6) (59). This

represents a novel mechanism whereby canonical Wnt signalling may inhibit terminal differentiation of cardiac muscle progenitors. Previous studies have shown that Nkx2.5 activity is not essential for mESC differentiation into cardiomyocytes, although downstream target genes are aberrantly expressed in Nkx2.5-null cells (13). Therefore, it is likely that promoting exclusion of Nkx2.5 from the nucleus represents at least part of the anti-cardiomyogenic effects exerted by Wnt.

Our group has recently shown that MLCK can modulate skeletal myogenesis by phosphorylating Mef2c (64). When considered together with the model described here, as well as work done by others (60), it seems that modulating the balance of myosin light chain phosphorylation represents only one aspect of a more diverse role played by MLCK/MP. In summary, we have identified a novel interaction between Nkx2.5 and MP. This interaction regulates the subcellular distribution of Nkx2.5 downstream of Wnt signalling and consequently modulates expression of downstream genes and cardiomyogenesis in stem cells. Our results may also have implications for cardiomyogenesis *in vivo* and for resident cardiac progenitor cells.

4.6 References

1. Vincent, S.D., and M.E. Buckingham. 2010. How to make a heart: the origin and regulation of cardiac progenitor cells. *Curr Top Dev Biol* 90:1-41.
2. Buckingham, M., S. Meilhac, and S. Zaffran. 2005. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet* 6:826-835.
3. Meilhac, S.M., M. Esner, R.G. Kelly, J.F. Nicolas, and M.E. Buckingham. 2004. The clonal origin of myocardial cells in different regions of the embryonic mouse heart. *Dev Cell* 6:685-698.
4. Zaffran, S., R.G. Kelly, S.M. Meilhac, M.E. Buckingham, and N.A. Brown. 2004. Right ventricular myocardium derives from the anterior heart field. *Circ Res* 95:261-268.

5. Kelly, R.G., N.A. Brown, and M.E. Buckingham. 2001. The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev Cell* 1:435-440.
6. Cai, C.L., X. Liang, Y. Shi, P.H. Chu, S.L. Pfaff, J. Chen, and S. Evans. 2003. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev Cell* 5:877-889.
7. Murry, C.E., and G. Keller. 2008. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 132:661-680.
8. Akazawa, H., and I. Komuro. 2005. Cardiac transcription factor Csx/Nkx2-5: Its role in cardiac development and diseases. *Pharmacol Ther* 107:252-268.
9. Reamon-Buettner, S.M., and J. Borlak. 2010. NKX2-5: an update on this hypermutable homeodomain protein and its role in human congenital heart disease (CHD). *Hum Mutat* 31:1185-1194.
10. Lyons, I., L.M. Parsons, L. Hartley, R. Li, J.E. Andrews, L. Robb, and R.P. Harvey. 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev* 9:1654-1666.
11. Tanaka, M., Z. Chen, S. Bartunkova, N. Yamasaki, and S. Izumo. 1999. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* 126:1269-1280.
12. Bodmer, R. 1993. The gene tinman is required for specification of the heart and visceral muscles in Drosophila. *Development* 118:719-729.
13. Nakashima, Y., K. Ono, Y. Yoshida, Y. Kojima, T. Kita, M. Tanaka, and T. Kimura. 2009. The search for Nkx2-5-regulated genes using purified embryonic stem cell-derived cardiomyocytes with Nkx2-5 gene targeting. *Biochem Biophys Res Commun* 390:821-826.
14. Fu, Y., W. Yan, T.J. Mohun, and S.M. Evans. 1998. Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. *Development* 125:4439-4449.
15. Tanaka, M., M. Schinke, H.S. Liao, N. Yamasaki, and S. Izumo. 2001. Nkx2.5 and Nkx2.6, homologs of Drosophila tinman, are required for development of the pharynx. *Mol Cell Biol* 21:4391-4398.
16. Tanaka, M., N. Yamasaki, and S. Izumo. 2000. Phenotypic characterization of the murine Nkx2.6 homeobox gene by gene targeting. *Mol Cell Biol* 20:2874-2879.

17. Jamali, M., P.J. Rogerson, S. Wilton, and I.S. Skerjanc. 2001. Nkx2-5 activity is essential for cardiomyogenesis. *J Biol Chem* 276:42252-42258.
18. Skerjanc, I.S., H. Petropoulos, A.G. Ridgeway, and S. Wilton. 1998. Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. *J Biol Chem* 273:34904-34910.
19. Prall, O.W., M.K. Menon, M.J. Solloway, Y. Watanabe, S. Zaffran, F. Bajolle, C. Biben, J.J. McBride, B.R. Robertson, H. Chaulet, F.A. Stennard, N. Wise, D. Schaft, O. Wolstein, M.B. Furtado, H. Shiratori, K.R. Chien, H. Hamada, B.L. Black, Y. Saga, E.J. Robertson, M.E. Buckingham, and R.P. Harvey. 2007. An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell* 128:947-959.
20. Targoff, K.L., T. Schell, and D. Yelon. 2008. Nkx genes regulate heart tube extension and exert differential effects on ventricular and atrial cell number. *Dev Biol* 322:314-321.
21. Durocher, D., F. Charron, R. Warren, R.J. Schwartz, and M. Nemer. 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J* 16:5687-5696.
22. Hiroi, Y., S. Kudoh, K. Monzen, Y. Ikeda, Y. Yazaki, R. Nagai, and I. Komuro. 2001. Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat Genet* 28:276-280.
23. Vincentz, J.W., R.M. Barnes, B.A. Firulli, S.J. Conway, and A.B. Firulli. 2008. Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. *Dev Dyn* 237:3809-3819.
24. Bruneau, B.G., G. Nemer, J.P. Schmitt, F. Charron, L. Robitaille, S. Caron, D.A. Conner, M. Gessler, M. Nemer, C.E. Seidman, and J.G. Seidman. 2001. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* 106:709-721.
25. Habets, P.E., A.F. Moorman, D.E. Clout, M.A. van Roon, M. Lingbeek, M. van Lohuizen, M. Campione, and V.M. Christoffels. 2002. Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev* 16:1234-1246.
26. Gessert, S., and M. Kuhl. 2010. The multiple phases and faces of wnt signaling during cardiac differentiation and development. *Circ Res* 107:186-199.
27. Liu, P., M. Wakamiya, M.J. Shea, U. Albrecht, R.R. Behringer, and A. Bradley. 1999. Requirement for Wnt3 in vertebrate axis formation. *Nat Genet* 22:361-365.

28. Lindsley, R.C., J.G. Gill, M. Kyba, T.L. Murphy, and K.M. Murphy. 2006. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* 133:3787-3796.
29. Bondue, A., G. Lapouge, C. Paulissen, C. Semeraro, M. Iacovino, M. Kyba, and C. Blanpain. 2008. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* 3:69-84.
30. Lindsley, R.C., J.G. Gill, T.L. Murphy, E.M. Langer, M. Cai, M. Mashayekhi, W. Wang, N. Niwa, J.M. Nerbonne, M. Kyba, and K.M. Murphy. 2008. Mesp1 coordinately regulates cardiovascular fate restriction and epithelial-mesenchymal transition in differentiating ESCs. *Cell Stem Cell* 3:55-68.
31. David, R., C. Brenner, J. Stieber, F. Schwarz, S. Brunner, M. Vollmer, E. Mentele, J. Muller-Hocker, S. Kitajima, H. Lickert, R. Rupp, and W.M. Franz. 2008. MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. *Nat Cell Biol* 10:338-345.
32. Ueno, S., G. Weidinger, T. Osugi, A.D. Kohn, J.L. Golob, L. Pabon, H. Reinecke, R.T. Moon, and C.E. Murry. 2007. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc Natl Acad Sci U S A* 104:9685-9690.
33. Naito, A.T., I. Shiojima, H. Akazawa, K. Hidaka, T. Morisaki, A. Kikuchi, and I. Komuro. 2006. Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc Natl Acad Sci U S A* 103:19812-19817.
34. Yang, L., M.H. Soonpaa, E.D. Adler, T.K. Roepke, S.J. Kattman, M. Kennedy, E. Henckaerts, K. Bonham, G.W. Abbott, R.M. Linden, L.J. Field, and G.M. Keller. 2008. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 453:524-528.
35. Kattman, S.J., T.L. Huber, and G.M. Keller. 2006. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* 11:723-732.
36. Kwon, C., J. Arnold, E.C. Hsiao, M.M. Taketo, B.R. Conklin, and D. Srivastava. 2007. Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. *Proc Natl Acad Sci U S A* 104:10894-10899.
37. Qyang, Y., S. Martin-Puig, M. Chiravuri, S. Chen, H. Xu, L. Bu, X. Jiang, L. Lin, A. Granger, A. Moretti, L. Caron, X. Wu, J. Clarke, M.M. Taketo, K.L. Laugwitz, R.T. Moon, P. Gruber, S.M. Evans, S. Ding, and K.R. Chien. 2007. The renewal and differentiation of Isl1+ cardiovascular progenitors are controlled by a Wnt/beta-catenin pathway. *Cell Stem Cell* 1:165-179.

38. Ai, D., X. Fu, J. Wang, M.F. Lu, L. Chen, A. Baldini, W.H. Klein, and J.F. Martin. 2007. Canonical Wnt signaling functions in second heart field to promote right ventricular growth. *Proc Natl Acad Sci U S A* 104:9319-9324.
39. Cohen, E.D., Z. Wang, J.J. Lepore, M.M. Lu, M.M. Taketo, D.J. Epstein, and E.E. Morrisey. 2007. Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *J Clin Invest* 117:1794-1804.
40. Tian, Y., L. Yuan, A.M. Goss, T. Wang, J. Yang, J.J. Lepore, D. Zhou, R.J. Schwartz, V. Patel, E.D. Cohen, and E.E. Morrisey. 2010. Characterization and in vivo pharmacological rescue of a Wnt2-Gata6 pathway required for cardiac inflow tract development. *Dev Cell* 18:275-287.
41. Lin, L., L. Cui, W. Zhou, D. Dufort, X. Zhang, C.L. Cai, L. Bu, L. Yang, J. Martin, R. Kemler, M.G. Rosenfeld, J. Chen, and S.M. Evans. 2007. Beta-catenin directly regulates Islet1 expression in cardiovascular progenitors and is required for multiple aspects of cardiogenesis. *Proc Natl Acad Sci U S A* 104:9313-9318.
42. Matsumura, F., and D.J. Hartshorne. 2008. Myosin phosphatase target subunit: Many roles in cell function. *Biochem Biophys Res Commun* 369:149-156.
43. Canton, D.A., M.E. Olsten, K. Kim, A. Doherty-Kirby, G. Lajoie, J.A. Cooper, and D.W. Litchfield. 2005. The pleckstrin homology domain-containing protein CKIP-1 is involved in regulation of cell morphology and the actin cytoskeleton and interaction with actin capping protein. *Mol Cell Biol* 25:3519-3534.
44. Yoon, H.J., A. Feoktistova, B.A. Wolfe, J.L. Jennings, A.J. Link, and K.L. Gould. 2002. Proteomics analysis identifies new components of the fission and budding yeast anaphase-promoting complexes. *Curr Biol* 12:2048-2054.
45. Adra, C.N., P.H. Boer, and M.W. McBurney. 1987. Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. *Gene* 60:65-74.
46. Eto, M., J.A. Kirkbride, and D.L. Brautigan. 2005. Assembly of MYPT1 with protein phosphatase-1 in fibroblasts redirects localization and reorganizes the actin cytoskeleton. *Cell Motil Cytoskeleton* 62:100-109.
47. Trinkle-Mulcahy, L., P. Ajuh, A. Prescott, F. Claverie-Martin, S. Cohen, A.I. Lamond, and P. Cohen. 1999. Nuclear organisation of NIPP1, a regulatory subunit of protein phosphatase 1 that associates with pre-mRNA splicing factors. *J Cell Sci* 112 (Pt 2):157-168.
48. McBurney, M.W., S. Fournier, K. Jardine, and L. Sutherland. 1994. Intragenic regions of the murine Pkg-1 locus enhance integration of transfected DNAs into genomes of embryonal carcinoma cells. *Somat Cell Mol Genet* 20:515-528.

49. Skerjanc, I.S., R.S. Slack, and M.W. McBurney. 1994. Cellular aggregation enhances MyoD-directed skeletal myogenesis in embryonal carcinoma cells. *Mol Cell Biol* 14:8451-8459.
50. Kennedy, K.A., T. Porter, V. Mehta, S.D. Ryan, F. Price, V. Peshdary, C. Karamboulas, J. Savage, T.A. Drysdale, S.C. Li, S.A. Bennett, and I.S. Skerjanc. 2009. Retinoic acid enhances skeletal muscle progenitor formation and bypasses inhibition by bone morphogenetic protein 4 but not dominant negative beta-catenin. *BMC Biol* 7:67.
51. Lambert, J.P., L. Mitchell, A. Rudner, K. Baetz, and D. Figeys. 2009. A novel proteomics approach for the discovery of chromatin-associated protein networks. *Mol Cell Proteomics* 8:870-882.
52. Elias, J.E., W. Haas, B.K. Faherty, and S.P. Gygi. 2005. Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nat Methods* 2:667-675.
53. Fan, C., M. Liu, and Q. Wang. 2003. Functional analysis of TBX5 missense mutations associated with Holt-Oram syndrome. *J Biol Chem* 278:8780-8785.
54. Savage, J., A.J. Conley, A. Blais, and I.S. Skerjanc. 2009. SOX15 and SOX7 differentially regulate the myogenic program in P19 cells. *Stem Cells* 27:1231-1243.
55. Kim, S.E., H. Shima, K. Nakamura, and K. Kikuchi. 2000. Broad specificity in binding of NIPP-1, nuclear inhibitor of protein phosphatase-1, to PP1 isoforms in vivo. *Tohoku J Exp Med* 191:39-45.
56. Lontay, B., A. Kiss, P. Gergely, D.J. Hartshorne, and F. Erdodi. 2005. Okadaic acid induces phosphorylation and translocation of myosin phosphatase target subunit 1 influencing myosin phosphorylation, stress fiber assembly and cell migration in HepG2 cells. *Cell Signal* 17:1265-1275.
57. Muranyi, A., D. Derkach, F. Erdodi, A. Kiss, M. Ito, and D.J. Hartshorne. 2005. Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells. *FEBS Lett* 579:6611-6615.
58. Khromov, A., N. Choudhury, A.S. Stevenson, A.V. Somlyo, and M. Eto. 2009. Phosphorylation-dependent autoinhibition of myosin light chain phosphatase accounts for Ca²⁺ sensitization force of smooth muscle contraction. *J Biol Chem* 284:21569-21579.
59. Rossol-Allison, J., L.N. Stemmler, K.I. Swenson-Fields, P. Kelly, P.E. Fields, S.J. McCall, P.J. Casey, and T.A. Fields. 2009. Rho GTPase activity modulates Wnt3a/beta-catenin signaling. *Cell Signal* 21:1559-1568.

60. Parra, M., T. Mahmoudi, and E. Verdin. 2007. Myosin phosphatase dephosphorylates HDAC7, controls its nucleocytoplasmic shuttling, and inhibits apoptosis in thymocytes. *Genes Dev* 21:638-643.
61. Perry, R.L., C. Yang, N. Soora, J. Salma, M. Marback, L. Naghibi, H. Ilyas, J. Chan, J.W. Gordon, and J.C. McDermott. 2009. Direct interaction between myocyte enhancer factor 2 (MEF2) and protein phosphatase 1alpha represses MEF2-dependent gene expression. *Mol Cell Biol* 29:3355-3366.
62. Braam, S.R., R. Nauw, D. Ward-van Oostwaard, C. Mummery, and R. Passier. 2010. Inhibition of ROCK improves survival of human embryonic stem cell-derived cardiomyocytes after dissociation. *Ann N Y Acad Sci* 1188:52-57.
63. Monzen, K., W. Zhu, H. Kasai, Y. Hiroi, T. Hosoda, H. Akazawa, Y. Zou, D. Hayashi, T. Yamazaki, R. Nagai, and I. Komuro. 2002. Dual effects of the homeobox transcription factor Csx/Nkx2-5 on cardiomyocytes. *Biochem Biophys Res Commun* 298:493-500.
64. Al Madhoun, A.S., V. Mehta, G. Li, D. Figeys, N. Wiper-Bergeron, and I.S. Skerjanc. 2011. Skeletal myosin light chain kinase regulates skeletal myogenesis by phosphorylation of MEF2C. *EMBO J*

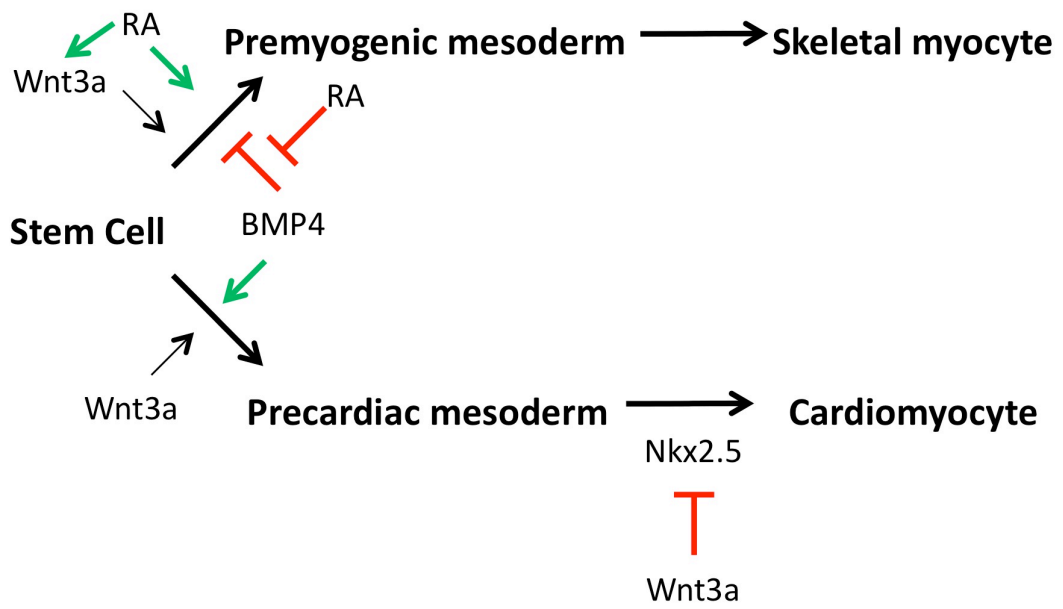
5: DISCUSSION

5.1 Stem cells as a source for muscle cell therapy

In this research project, we sought to examine myogenic cell fate decisions at a molecular level. We have shown that RA mediates enhancement of skeletal myogenesis in these systems by expanding the premyogenic progenitor population in murine and human stem cell systems. Furthermore, we have shown that this occurs through direct regulation of premyogenic mesoderm genes and also by crosstalk with other signalling cascades such as Wnt and BMP. We have also identified a novel mechanism for Wnt3a-mediated inhibition of cardiac progenitors, involving exclusion of Nkx2.5 from the nucleus as a result of direct interaction with the MP enzyme complex in a ROCK-dependent pathway. Given that RA inhibits cardiomyogenesis and activates Wnt signalling during differentiation and that Wnt inhibits cardiomyogenesis, at least in part, by promoting exclusion of Nkx2.5 from the nucleus, it is possible that these mechanisms are linked in their ability to regulate myogenic cell fate decisions (Figure 5.1).

The first hESC line (H9) was derived in 1998 (1). Since then, amazing progress has been made towards understanding cell fate decisions and indeed, the first clinical trial using hESC derived oligodendrocyte precursor cells for treatment of spinal cord injury was announced in 2010 (2). In fact, as of June, 2010, there was 68 cell-based therapies under development (3). The excitement associated with stem cell research received an additional boost in 2007 with the generation of human iPS cells by direct reprogramming of human dermal fibroblasts (4). The main advantage of these cells over hESCs is that they are autologous and as such, are less likely to induce an immune response in a transplantation setting; however, they also provided a way around the ethical issues associated with the

Figure 5.1 A model for the regulation of cardiomyogenesis and skeletal myogenesis by RA/Wnt/BMP . In the context of skeletal myogenesis, RA activates Wnt signalling to enhance muscle differentiation while simultaneously inhibiting BMP4 signalling, which favours cardiomyogenesis. RA can override the ability of BMP4 inhibit skeletal myogenesis, however, RA requires a functional β -catenin in order to enhance skeletal muscle differentiation. In the context of cardiomyogenesis, Wnt exerts differential effect at distinct stages of differentiation. Inhibition of terminal differentiation of progenitors is accomplished, atleast in part, by promoting nuclear exclusion of Nkx2.5 via interaction with MP downstream of ROCK activation.



generation of new ES lines from human embryos. In addition, they presented a novel model for the study of disease in which pluripotent cells could be generated from patient fibroblasts and differentiated into the cell types of interest, thereby recreating the 'disease in a dish'. In support of this approach, a relatively recent proof of principle study by Hanna et al. showed that a mouse model of sickle cell anemia could be rescued by transplantation with gene-corrected iPS-derived hematopoietic progenitors from autologous skin (5). Diseases of muscle, such as muscular dystrophy or various diseases of the heart, represent good candidates for cell-based therapies. Duchenne muscular dystrophy (DMD) is an X-linked disorder that affects approximately 1 in 3500 male births and is caused by a deficiency in the expression of dystrophin, which, as a component of the dystrophin glycoprotein complex, is important for linking the muscle fibre cytoskeleton to the extracellular matrix (6). As a result of this loss of structural integrity, DMD is characterized by a high degree of muscle wasting and ultimately exhaustion of the muscle satellite cell compartment, resulting in an inability to regenerate damaged fibres. Current cell-based therapy approaches that have been applied in this context include myoblast transplantation, hematopoietic stem cell transplantation via bone marrow and transplantation of vessel-associated progenitors termed mesoangioblasts (7). There have been several clinical trials of myoblast/satellite cell transplantation but this strategy appears to be hampered by limited cell migration, massive acute cell death and a failure of functional recovery of engrafted muscles. In addition, due to the limited migratory abilities of these cells, an enormous number of injections would be required in order to reach all affected areas.

Several cell types have been investigated in multiple disease backgrounds for cardiac cell therapy (8). Skeletal myoblasts and mesenchymal stem cells have been tested clinically in the context of acute myocardial infarction and chronic heart failure respectively (9, 10).

The outcomes of these studies seem to indicate that cell therapy for the heart is safe, but as to whether these cells contribute to functional repair of damaged tissue is controversial. Conversely, transplantation of autologous bone marrow derived stem cells into patients suffering from acute myocardial infarction resulted in a significant improvement in left-ventricular contractile function and was associated with a reduced risk of recurrence or death 1 year after treatment (11). Although these data are preliminary, they seem to indicate that cell therapy is a viable option for treatment of heart disease.

Although ES cells have not been tested in clinical models of skeletal muscle disease, pre-clinical proof of principle studies in animals have shown that they have the potential to functionally repair damaged cardiac or skeletal muscle tissue (12-16). Results of the Geron trial will undoubtedly provide important insight into the safety and efficacy of hESC based therapy. Therefore, it is imperative that we improve our understanding of the pathways that induce the myogenic cell fate in order to optimize the differentiation of these cell types from ES cells. Lessons learned from heart and skeletal muscle development *in vivo* can provide us with the tools required to achieve this goal.

5.2 RA mediates expansion of the premyogenic progenitor pool in stem cells

RA has long been known to be involved in multiple aspects of skeletal muscle development, from the earliest stages of somitogenesis to cooperation with MRFs in the context of terminal differentiation (17, 18). It therefore represents a good candidate molecule for promoting this process in ESCs *in vitro*. In fact, early work in mESCs indicated that RA could in fact enhance both cardiac and skeletal myogenesis in a time- and

concentration-dependent fashion consistent with its morphogenetic properties *in vivo* (19). We sought to better understand the molecular mechanisms whereby RA enhanced myogenesis in murine stem cells in order to apply this knowledge to hESCs, a more relevant system for therapeutic purposes. We found that RA enhanced the formation of premyogenic progenitors, as evidenced by increased expression levels of Meox1, Pax3 and Pax7, ultimately resulting in upregulated expression of MyoD and myogenin as well as in terminal myogenesis. With regard to the mechanism for this, we found that the myogenic properties of RA could be attributed, at least in part, to binding of RARs to regulatory regions of Meox1, Pax3 and Wnt3a. Furthermore, we found that RA could activate canonical Wnt signalling during differentiation. Taken together, these data suggest that RA functions both upstream and downstream of Wnt signalling to enhance myogenesis. Importantly however, RA required a functional β -catenin in order for this enhancement to occur. In the context of cardiomyogenesis, RA was inhibitory and could override BMP4-mediated enhancement of cardiac differentiation. This occurred, at least in part, by activation of the BMP inhibitor Tob1.

Clearly, crosstalk with other pathways plays an important role during RA-directed differentiation *in vitro*. There are several other examples of crosstalk between RA and canonical Wnt signaling during endodermal differentiation as well as during neurogenesis (20, 21). Interestingly, the effect of RA on Wnt signaling differs depending on the context and can result in either activation or inhibition of this cascade. For example, RA can inhibit Wnt activity by activating expression of antagonists, restricting the domain of expression or by inhibiting downstream effectors (22-24). Furthermore, it has been shown that RAR γ can interact directly with β -catenin in retinoid-free cultures, promoting its dissociation from LEF and thereby inhibiting Wnt-dependent gene transcription in chondrocytes (25). Conversely,

RA can activate Wnt expression during adult murine neurogenesis or vertebrate limb induction (26, 27). It can also activate the Wnt signaling cascade in chondrocytes (25). With respect to crosstalk between RA and BMP, the interaction is quite similar. RA can induce or inhibit BMP expression, depending on the context (28-30). Some mechanistic insight into this was gained when it was shown that RA promotes the interaction of phosphorylated Smad1 with E3 ubiquitin ligases, resulting in degradation of Smad1 by the proteasome and attenuation of the BMP signal (31).

Given that crosstalk between these three cascades occurs in multiple systems, it is likely to reflect fundamentally conserved molecular interactions between components of each pathway. It is also clear however, that these interactions are context-dependent, as they often involve discrete molecular components of each pathway, resulting in different outcomes. This could be explained by the abundance of ligands, receptors and downstream effectors that participate in the Wnt and BMP pathways as well as by the variety of RAR/RXR isoforms, which are differentially activated under distinct conditions, and their individual binding affinities. These interactions are likely to occur at multiple levels within the signaling cascade and are probably characterized by a high degree of complexity. Further research in this area should make use of high-throughput systems biology approaches such as location analysis (chromatin immunoprecipitation coupled to sequencing), and proteomics in order to gain a more global understanding of the interactions between these pathways and the resulting impact on differentiation.

We next sought to exploit the mechanistic insight gained in our studies of RA-mediated enhancement of myogenesis in P19 cells and mESCs to the hESC system. It has proven difficult to generate skeletal muscle from hESCs and previously published protocols

involved covalent chemical modification of cells or extremely lengthy timecourses (32, 33). Furthermore, these studies have not reported the formation of a Pax3^{+ve}/Meox1^{+ve} premyogenic progenitor population. This progenitor population could potentially be particularly valuable in the context of cell therapy as it has been shown that it can replenish the satellite cell niche more efficiently than progenitor cells expressing MRFs (34). We found that treatment of differentiating hESCs with RA resulted in a significant enhancement in the number of Pax3^{+ve} progenitor cells as well as in the number of terminally differentiated muscle cells. Furthermore, we have shown that the Pax3^{+ve}/Meox1^{+ve} progenitor population occurs prior to MRF expression.

Although the total amount of muscle generated amounted to only approximately 4% of total cells in our hESC cultures, these data indicate that RA could potentially be used as part of a directed differentiation protocol for the generation of skeletal muscle from hESCs. In the past few years protocols of this nature have been developed to generate high yields of cardiomyocytes from mESCs, hESCs and iPSCs (35-37). These protocols are modelled on knowledge gained from our understanding of the signalling networks that control cardiomyogenesis and heart development *in vivo*. Cells are treated with carefully optimized combinations of cardiogenic factors such as Dkk1, FGF2, VEGFA, BMP4 and activin A at distinct timepoints during the early stages of differentiation and this directed differentiation protocol can yield more than 50% cardiomyocytes from hESC cultures (35). An equivalent protocol remains to be designed to drive skeletal myogenesis but might include such pro-myogenic factors as Wnt3a, sonic hedgehog and noggin, in addition to RA, as well as modulation of the Notch and FGF signalling cascades. These protocols are particularly valuable because they do not require genetic modification of the cells.

The question remains, however, at what stage of differentiation should the cells be transplanted? In muscular dystrophy patients, the progressive wasting of skeletal muscle requires massive levels of regeneration and leads to exhaustion of the muscle stem cell compartment. Therefore, transplantation strategies that address this by employing a cell population that can replenish the satellite cell niche will be more likely to result in long-term engraftment and will not require repeated intervention. Muscle transplantation studies by the Perlingeiro group have shown improved contractility of muscle of dystrophic mice transplanted with myofibers derived from mESCs engineered to overexpress Pax3 (13, 14). More recent work however, has begun to take the satellite cell compartment into account. A study by this same group found that progenitors derived from mESCs overexpressing Pax3 or Pax7 could also regenerate injured muscle but in addition, these cells could repopulate the satellite cell niche upon transplantation and could maintain self-renewal *in vivo* (12). Two additional studies also found that progenitor cells derived from mESCs could generate satellite-like cells with myogenic potential *in vivo* (38, 39). Importantly, these populations were derived without genetic modification of cells. Taken together, these findings seem to indicate that the premyogenic progenitor population may in fact represent the more valuable source of cells for cell therapy, rather than terminally differentiated myofibers. Our study represents the first identification of a premyogenic progenitor in hESCs, however, it only represents approximately 6% of total cells. Future work in this area should focus on strategies to enhance the formation of this population using directed differentiation protocols coupled to purification. A homogeneous progenitor population, ideally derived without genetic modification, will undoubtedly prove to be a valuable therapeutic tool.

Expression and epigenetic profiling of hESCs has shown that these cells are in fact more similar to mouse epiSCs than they are to mESCs (40). This may seem to suggest that

mESCs and other murine stem cell models such as P19 cells might not be a good model in which to study the molecular mechanisms of myogenesis. Our findings indicate that this is not the case. The insight that we gained from our RA studies in mESCs and P19 cells held true in the hESC system. In all three of these models, RA enhanced myogenesis by causing an expansion of the premyogenic progenitor population, suggesting that the mechanism of action is likely to be similar. The implication of these results is that directed culture methods optimized in murine systems are likely to translate to human systems. Indeed, this has proven to be the case for directed cardiomyogenesis, where factors used to induce cardiac differentiation in mESCs were also employed for the same purpose in hESCs with similar success (37). Therefore, pro-myogenic factors identified in the mouse during differentiation *in vivo* or *in vitro* bear a re-examination as potential components of a directed culture system for myogenesis in hESCs.

5.3 Wnt3a modulates cardiac cell fate in stem cells by regulating the subcellular localization of Nkx2.5

Nkx2.5 is one of the earliest markers of the cardiac fate *in vivo* and *in vitro* and is expressed in almost all the lineages of the heart (41). Although its expression is not essential for differentiation of cardiomyocytes, it is required for proper morphogenesis of the heart and in its absence the primitive heart tube fails to undergo looping resulting in embryonic lethality (42-44). Nkx2.5 is a weak transcriptional activator *in vitro* and it has been proposed that this results from the presence of an inhibitory domain within the C-terminus of the protein (45). It has also been shown that Nkx2.5 can cooperate with several other important cardiomyogenic transcription factors, and that these interactions determine that nature of Nkx2.5 activity (inhibitory vs activating) and confer a higher degree of target gene activation

through synergy (46-48). Given the importance of protein interactions for proper function of Nkx2.5, we performed a proteomic screen to identify novel binding partners of this factor during differentiation in stem cells. This screen identified PP1 β and Mypt1, two subunits of the MP enzyme complex, as putative partners of Nkx2.5. We found that coexpression with MP resulted in exclusion of Nkx2.5 from the nucleus and consequently, inhibition of its transcriptional activity. This exclusion was found to be regulated by phosphorylation of Mypt1 by ROCK downstream of Wnt signalling. Consistent with this, Wnt3a treatment of mESCs resulted in increased nuclear exclusion of Nkx2.5 and a failure to undergo terminal cardiomyogenesis.

We also found that nuclear exclusion of Nkx2.5 could be prevented by treatment with the CRM1-dependent nuclear export inhibitor LeptomycinB. This is not the first example of nucleo-cytoplasmic shuttling of cardiogenic transcription factors. In fact, both GATA4 and SRF have been shown to be regulated by nucleo-cytoplasmic shuttling in a CRM1-dependent fashion (49, 50). In the case of GATA4, exclusion was mediated by phosphorylation by GSK3 β and this could be blocked by stimulation of the β -adrenergic receptor using isoproterenol, causing nuclear export of GSK3 β (49). This finding suggested a potential link to hypertrophic signalling. Moreover, RhoA/ROCK has been implicated in the regulation of subcellular localization of SRF (51). Changes in Nkx2.5 expression levels are associated with cardiac hypertrophy (52, 53). Therefore, it is possible that this mechanism has implications for more than stem cell differentiation and may represent a general mechanism whereby the activity of cardiac transcription factors is modulated by nuclear export in various contexts. However, as we did not observe nuclear exclusion of GATA4 in response to coexpression with MP, the mechanism is probably not identical in each case.

The canonical Wnt signalling pathway plays diverse roles at various stages of cardiomyogenesis. Wnt signalling is required for expression of *Mesp1*^{+ve}, the earliest marker and master regulator of cardiomyogenesis (54). It also promotes expansion of cardiac progenitor populations (55). Conversely, many groups have documented the inhibition of cardiac progenitor differentiation by canonical Wnt signalling *in vivo* and *in vitro* (56-58). Wnt3a-mediated nuclear exclusion of Nkx2.5 represents the first indication of a molecular basis for its ability to prevent terminal differentiation of progenitors. Nuclear exclusion of Nkx2.5 will likely result in less activation of target genes and contribute to a subsequent failure to differentiate. The regulation of Nkx2.5 localization can only represent part of this mechanism however, as Nkx2.5 is dispensable for cardiomyocyte differentiation *in vitro*, although downstream target genes are aberrantly expressed in this context (44). Given that both SRF and GATA4 are excluded from the nucleus and that these processes are regulated by known targets of Wnt signalling (GSK3 β and ROCK), it would be interesting to explore whether Wnt-mediated inhibition of cardiomyogenesis might be upstream of these exclusion events, albeit in an MP-independent pathway, as we have shown that co-expression with MP does not cause a redistribution of GATA4. Furthermore, we know that treatment with the ROCK inhibitor y-27632 inhibited exclusion of Nkx2.5 and that it has also been shown to improve cardiomyocyte survival after dissociation of embryoid bodies. As such, this drug should be considered as part of any directed differentiation/cardiomyocyte purification based strategy to derive cells for therapeutic purposes (59). Future directions should also concentrate on a more thorough understanding of these mechanisms, in order to exploit them in directed differentiation and purification strategies.

5.4 Conclusion

In summary, the work presented herein represents a novel contribution to the understanding of the signalling pathways regulating the molecular networks that control myogenic cell fate decisions in stem cells. Future work should aim to expand on this information in an effort to optimize myogenesis in stem cells for the purposes of cell therapy. Although there has been significant progress made toward understanding the molecular mechanisms of stem cell self-renewal and myogenic differentiation, more research will be required to gain the level of insight into these processes that will be required in order to manipulate them to our advantage.

5.5 References

1. Thomson, J.A., J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, and J.M. Jones. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.
2. Geron. 2009. World's first clinical trial of human embryonic stem cell therapy cleared. *Regen Med* 4:161.
3. McKernan, R., J. McNeish, and D. Smith. 2010. Pharma's developing interest in stem cells. *Cell Stem Cell* 6:517-520.
4. Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861-872.
5. Hanna, J., M. Wernig, S. Markoulaki, C.W. Sun, A. Meissner, J.P. Cassady, C. Beard, T. Brambrink, L.C. Wu, T.M. Townes, and R. Jaenisch. 2007. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* 318:1920-1923.
6. Palmieri, B., J.P. Tremblay, and L. Daniele. 2010. Past, present and future of myoblast transplantation in the treatment of Duchenne muscular dystrophy. *Pediatr Transplant* 14:813-819.

7. Tedesco, F.S., A. Dellavalle, J. Diaz-Manera, G. Messina, and G. Cossu. 2010. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J Clin Invest* 120:11-19.
8. Menasche, P. 2011. Cardiac cell therapy: lessons from clinical trials. *J Mol Cell Cardiol* 50:258-265.
9. Menasche, P., O. Alfieri, S. Janssens, W. McKenna, H. Reichenspurner, L. Trinquart, J.T. Vilquin, J.P. Marolleau, B. Seymour, J. Larghero, S. Lake, G. Chatellier, S. Solomon, M. Desnos, and A.A. Hagege. 2008. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. *Circulation* 117:1189-1200.
10. Hare, J.M., J.H. Traverse, T.D. Henry, N. Dib, R.K. Strumpf, S.P. Schulman, G. Gerstenblith, A.N. DeMaria, A.E. Denktas, R.S. Gammon, J.B. Hermiller, Jr., M.A. Reisman, G.L. Schaer, and W. Sherman. 2009. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J Am Coll Cardiol* 54:2277-2286.
11. Schachinger, V., S. Erbs, A. Elsasser, W. Haberbosch, R. Hambrecht, H. Holschermann, J. Yu, R. Corti, D.G. Mathey, C.W. Hamm, T. Suselbeck, B. Assmus, T. Tonn, S. Dimmeler, and A.M. Zeiher. 2006. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med* 355:1210-1221.
12. Darabi, R., F.N. Santos, A. Filareto, W. Pan, R. Koene, M.A. Rudnicki, M. Kyba, and R.C. Perlingeiro. 2011. Assessment of the myogenic stem cell compartment following transplantation of pax3/pax7-induced embryonic stem cell-derived progenitors. *Stem Cells* 29:777-790.
13. Darabi, R., J. Baik, M. Clee, M. Kyba, R. Tupler, and R.C. Perlingeiro. 2009. Engraftment of embryonic stem cell-derived myogenic progenitors in a dominant model of muscular dystrophy. *Exp Neurol* 220:212-216.
14. Darabi, R., K. Gehlbach, R.M. Bachoo, S. Kamath, M. Osawa, K.E. Kamm, M. Kyba, and R.C. Perlingeiro. 2008. Functional skeletal muscle regeneration from differentiating embryonic stem cells. *Nat Med* 14:134-143.
15. Laflamme, M.A., J. Gold, C. Xu, M. Hassanipour, E. Rosler, S. Police, V. Muskheli, and C.E. Murry. 2005. Formation of human myocardium in the rat heart from human embryonic stem cells. *Am J Pathol* 167:663-671.
16. Caspi, O., I. Huber, I. Kehat, M. Habib, G. Arbel, A. Gepstein, L. Yankelson, D. Aronson, R. Beyar, and L. Gepstein. 2007. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J Am Coll Cardiol* 50:1884-1893.

17. Moreno, T.A., and C. Kintner. 2004. Regulation of segmental patterning by retinoic acid signaling during *Xenopus* somitogenesis. *Dev Cell* 6:205-218.
18. Froeschle, A., S. Alric, M. Kitzmann, G. Carnac, F. Aurade, C. Rochette-Egly, and A. Bonnieu. 1998. Retinoic acid receptors and muscle b-HLH proteins: partners in retinoid-induced myogenesis. *Oncogene* 16:3369-3378.
19. Wobus, A.M., Rohwedel, J., Maltsev, V., Hescheler, J. 1994. In vitro differentiation of embryonic stem cells into cardiomyocytes or skeletal muscle cells is specifically modulated by retinoic acid. *Roux's Arch Dev Biol* 204:36-45.
20. Lu, J., L. Tan, P. Li, H. Gao, B. Fang, S. Ye, Z. Geng, P. Zheng, and H. Song. 2009. All-trans retinoic acid promotes neural lineage entry by pluripotent embryonic stem cells via multiple pathways. *BMC Cell Biol* 10:57.
21. Inoue, A., A. Nagafuchi, and A. Kikuchi. 2009. Retinoic acid induces discrete Wnt-signaling-dependent differentiation in F9 cells. *Biochem Biophys Res Commun* 390:564-569.
22. Halilagic, A., V. Ribes, N.B. Ghyselinck, M.H. Zile, P. Dolle, and M. Studer. 2007. Retinoids control anterior and dorsal properties in the developing forebrain. *Developmental Biology* 303:362-375.
23. Verani, R., I. Cappuccio, P. Spinsanti, R. Gradini, A. Caruso, M.C. Magnotti, M. Motolese, F. Nicoletti, and D. Melchiorri. 2007. Expression of the Wnt inhibitor Dickkopf-1 is required for the induction of neural markers in mouse embryonic stem cells differentiating in response to retinoic acid
doi:10.1111/j.1471-4159.2006.04207.x. *Journal of Neurochemistry* 100:242-250.
24. zur Nieden, N.I., F.D. Price, L.A. Davis, R.E. Everitt, and D.E. Rancourt. 2007. Gene Profiling on Mixed Embryonic Stem Cell Populations Reveals a Biphasic Role for β -Catenin in Osteogenic Differentiation
10.1210/me.2005-0438. *Mol Endocrinol* 21:674-685.
25. Yasuhara, R., T. Yuasa, J.A. Williams, S.W. Byers, S. Shah, M. Pacifici, M. Iwamoto, and M. Enomoto-Iwamoto. 2010. Wnt/beta-catenin and retinoic acid receptor signaling pathways interact to regulate chondrocyte function and matrix turnover. *J Biol Chem* 285:317-327.
26. Jacobs, S., D.C. Lie, K.L. DeCicco, Y. Shi, L.M. DeLuca, F.H. Gage, and R.M. Evans. 2006. Retinoic acid is required early during adult neurogenesis in the dentate gyrus. *Proc Natl Acad Sci U S A* 103:3902-3907.
27. Mercader, N., S. Fischer, and C.J. Neumann. 2006. Prdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction
10.1242/dev.02455. *Development* 133:2805-2815.

28. Qin, P., J.M. Haberbusch, Z. Zhang, K.J. Soprano, and D.R. Soprano. 2004. Pre-B cell leukemia transcription factor (PBX) proteins are important mediators for retinoic acid-dependent endodermal and neuronal differentiation of mouse embryonal carcinoma P19 cells. *J Biol Chem* 279:16263-16271.
29. Hallahan, A.R., J.I. Pritchard, R.A. Chandraratna, R.G. Ellenbogen, J.R. Geyer, R.P. Overland, A.D. Strand, S.J. Tapscott, and J.M. Olson. 2003. BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. *Nat Med* 9:1033-1038.
30. Mic, F.A., and G. Duester. 2003. Patterning of forelimb bud myogenic precursor cells requires retinoic acid signaling initiated by Raldh2. *Dev Biol* 264:191-201.
31. Sheng, N., Z. Xie, C. Wang, G. Bai, K. Zhang, Q. Zhu, J. Song, F. Guillemot, Y.G. Chen, A. Lin, and N. Jing. 2010. Retinoic acid regulates bone morphogenic protein signal duration by promoting the degradation of phosphorylated Smad1. *Proc Natl Acad Sci U S A* 107:18886-18891.
32. Zheng, J.K., Y. Wang, A. Karandikar, Q. Wang, H. Gai, A.L. Liu, C. Peng, and H.Z. Sheng. 2006. Skeletal myogenesis by human embryonic stem cells. *Cell Res* 16:713-722.
33. Barberi, T., M. Bradbury, Z. Dincer, G. Panagiotakos, N.D. Socci, and L. Studer. 2007. Derivation of engraftable skeletal myoblasts from human embryonic stem cells. *Nat Med* 13:642-648.
34. Kuang, S., K. Kuroda, F. Le Grand, and M.A. Rudnicki. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell* 129:999-1010.
35. Yang, L., M.H. Soonpaa, E.D. Adler, T.K. Roepke, S.J. Kattman, M. Kennedy, E. Henckaerts, K. Bonham, G.W. Abbott, R.M. Linden, L.J. Field, and G.M. Keller. 2008. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 453:524-528.
36. Kattman, S.J., T.L. Huber, and G.M. Keller. 2006. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* 11:723-732.
37. Kattman, S.J., A.D. Witty, M. Gagliardi, N.C. Dubois, M. Niapour, A. Hotta, J. Ellis, and G. Keller. 2011. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 8:228-240.
38. Sakurai, H., Y. Okawa, Y. Inami, N. Nishio, and K. Isobe. 2008. Paraxial mesodermal progenitors derived from mouse embryonic stem cells contribute to muscle regeneration via differentiation into muscle satellite cells. *Stem Cells* 26:1865-1873.

39. Chang, H., M. Yoshimoto, K. Umeda, T. Iwasa, Y. Mizuno, S. Fukada, H. Yamamoto, N. Motohashi, Y. Miyagoe-Suzuki, S. Takeda, T. Heike, and T. Nakahata. 2009. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. *FASEB J* 23:1907-1919.
40. Tesar, P.J., J.G. Chenoweth, F.A. Brook, T.J. Davies, E.P. Evans, D.L. Mack, R.L. Gardner, and R.D. McKay. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448:196-199.
41. Akazawa, H., and I. Komuro. 2005. Cardiac transcription factor Csx/Nkx2-5: Its role in cardiac development and diseases. *Pharmacol Ther* 107:252-268.
42. Lyons, I., L.M. Parsons, L. Hartley, R. Li, J.E. Andrews, L. Robb, and R.P. Harvey. 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev* 9:1654-1666.
43. Tanaka, M., Z. Chen, S. Bartunkova, N. Yamasaki, and S. Izumo. 1999. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* 126:1269-1280.
44. Nakashima, Y., K. Ono, Y. Yoshida, Y. Kojima, T. Kita, M. Tanaka, and T. Kimura. 2009. The search for Nkx2-5-regulated genes using purified embryonic stem cell-derived cardiomyocytes with Nkx2-5 gene targeting. *Biochem Biophys Res Commun* 390:821-826.
45. Sepulveda, J.L., N. Belaguli, V. Nigam, C.Y. Chen, M. Nemer, and R.J. Schwartz. 1998. GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol Cell Biol* 18:3405-3415.
46. Durocher, D., F. Charron, R. Warren, R.J. Schwartz, and M. Nemer. 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J* 16:5687-5696.
47. Habets, P.E., A.F. Moorman, D.E. Clout, M.A. van Roon, M. Lingbeek, M. van Lohuizen, M. Campione, and V.M. Christoffels. 2002. Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev* 16:1234-1246.
48. Vincentz, J.W., R.M. Barnes, B.A. Firulli, S.J. Conway, and A.B. Firulli. 2008. Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. *Dev Dyn* 237:3809-3819.
49. Morisco, C., K. Seta, S.E. Hardt, Y. Lee, S.F. Vatner, and J. Sadoshima. 2001. Glycogen synthase kinase 3beta regulates GATA4 in cardiac myocytes. *J Biol Chem* 276:28586-28597.

50. Camoretti-Mercado, B., H.W. Liu, A.J. Halayko, S.M. Forsythe, J.W. Kyle, B. Li, Y. Fu, J. McConville, P. Kogut, J.E. Vieira, N.M. Patel, M.B. Hershenson, E. Fuchs, S. Sinha, J.M. Miano, M.S. Parmacek, J.K. Burkhardt, and J. Solway. 2000. Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor. *J Biol Chem* 275:30387-30393.
51. Liu, H.W., A.J. Halayko, D.J. Fernandes, G.S. Harmon, J.A. McCauley, P. Kocieniewski, J. McConville, Y. Fu, S.M. Forsythe, P. Kogut, S. Bellam, M. Dowell, J. Churchill, H. Lesso, K. Kassiri, R.W. Mitchell, M.B. Hershenson, B. Camoretti-Mercado, and J. Solway. 2003. The RhoA/Rho kinase pathway regulates nuclear localization of serum response factor. *Am J Respir Cell Mol Biol* 29:39-47.
52. Thompson, J.T., M.S. Rackley, and T.X. O'Brien. 1998. Upregulation of the cardiac homeobox gene Nkx2-5 (CSX) in feline right ventricular pressure overload. *Am J Physiol* 274:H1569-1573.
53. Saadane, N., L. Alpert, and L.E. Chalifour. 1999. Expression of immediate early genes, GATA-4, and Nkx-2.5 in adrenergic-induced cardiac hypertrophy and during regression in adult mice. *Br J Pharmacol* 127:1165-1176.
54. Lindsley, R.C., J.G. Gill, M. Kyba, T.L. Murphy, and K.M. Murphy. 2006. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* 133:3787-3796.
55. Cohen, E.D., Z. Wang, J.J. Lepore, M.M. Lu, M.M. Taketo, D.J. Epstein, and E.E. Morrisey. 2007. Wnt/beta-catenin signaling promotes expansion of Isl-1-positive cardiac progenitor cells through regulation of FGF signaling. *J Clin Invest* 117:1794-1804.
56. David, R., C. Brenner, J. Stieber, F. Schwarz, S. Brunner, M. Vollmer, E. Mentele, J. Muller-Hocker, S. Kitajima, H. Lickert, R. Rupp, and W.M. Franz. 2008. MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. *Nat Cell Biol* 10:338-345.
57. Ueno, S., G. Weidinger, T. Osugi, A.D. Kohn, J.L. Golob, L. Pabon, H. Reinecke, R.T. Moon, and C.E. Murry. 2007. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc Natl Acad Sci U S A* 104:9685-9690.
58. Naito, A.T., I. Shiojima, H. Akazawa, K. Hidaka, T. Morisaki, A. Kikuchi, and I. Komuro. 2006. Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc Natl Acad Sci U S A* 103:19812-19817.
59. Braam, S.R., R. Nauw, D. Ward-van Oostwaard, C. Mummery, and R. Passier. 2010. Inhibition of ROCK improves survival of human embryonic stem cell-derived cardiomyocytes after dissociation. *Ann N Y Acad Sci* 1188:52-57.

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Appendix G: QPCR Primers

m: mouse; h: human

Primer Name	Forward primer sequence	Reverse Primer sequence
hPax3	CTCACCTCAGGTAATGGGACT	CGTGGTGGTAGGTTCCAGAC
hMeox1	GCAGGGGGTTCCAAGGAAAT	GTCAGGTAGTTATGATGGGCAAA
hPax7	CCCCCGCACGGGATT	TATCTTGTGGCGGATGTGGTTA
hMyoD	TGCACGTCGAGCAATCCAAA	CCGCTGTAGTCCATCATGCC
hMyogenin	GCTGTATGAGACATCCCCCTA	CGACTTCCTCTTACACACCTTAC
hMyf5	AATTTGGGGACGAGTTTGTG	CATGGTGGTGGACTTCCTCT
hNkx2.5	GCAGGACCAGACTCTGGAGC	GAGTCCCCTAGGCATGGCTT
hTbx5	CAGGGCATGGAGGGAATCAAA	TCGTGGAATTTTAGCCAAGTT
hOsteopontin	TTTACAACAAATACCCAGATGC	ATGGCTTTCGTTGGACTTAC
hOsteocalcin	GGACTGTGACGAGTTGGCTG	CCGTAGAAGCGCCGATAGG
hNeuroD	TGCTACTCCAAGACGCAGAAG	CACGTAGGACACTAGGTCTGG
hNeurogenin1	GCTCTCTGACCCCAGTAGC	GCGTTGTGTGGAGCAAGTC
hVEGFR2	GTGACCAACATGGAGTCGTG	TGCTTCACAGAAGACCATGC
hMyHC11	TGAACGCACTCAAGAGCAAG	TCTACGTCTCCAGACCTTCT
hBrachyuryT	TTCATAGCGGTGACTGCTTATCA	CACCCCCATTGGGAGTACC
hMesp1	GAAGTGGTTCCTTGGCAGAC	TCCTGCTTGCCTCAAAGTGT
hMesp2	CTTTAGCCAAGGCTCCCTTT	TGCCTCTACACCCATTCTC
hGAPDH	TGGTGCTGAGTATGTCGTGGAGT	AGTCTTCTGAGTGGCAGTGATGG
Meox1-1 (ChIP)	CATGAGTTCAAGCCTCAGCA	CCAGAGATACGCTTGGTGTC
Meox1-2 (ChIP)	GAGGCCTAGCTTCAGCTCCT	TGAAATGCCTGATCTGACACA
Wnt3a1-1 (ChIP)	GAGGGAATCAAATCCCATTATAGA	GGCAGAACCTGTAGTCAGAACT
Wnt3a-2 (ChIP)	CAGGTATTGCCATCCAGGTT	GAGAATGCTCTGTGGGGTTC
Pax3-1 (ChIP)	ACAGGGTAAAACAATGTGTGGA	TTGAAGCCAGCCTGACCTAT
Pax3-2 (ChIP)	AGTGGAGCGCACCTCTGT	CTACAAACCCTTAATGACAAACG

RAR β 2 (ChIP)	GGTTCACCGAAAGTTCACTCGCAT	CAGGCTCGCTCGGCCGATCCA
mPax3	TTTCACCTCAGGTAATGGGACT	GAACGTCCAAGGCTTACTTTGT
mPax7	CTCAGTGAGTTCGATTAGCCG	AGACGGTTCCTTTGTGCG
mMyoD	CCCCGGCGGCAGAATGGCTACG	GGTCTGGGTTCCTGTTCTGTGT
mMeox1	TGGCCTATGCAGAATCCATTCC	TGGATCTGAGCTGCGCATGTG
m β -Actin	AAATCGTGCGTGACATCAAA	AAGGAAGGCTGGAAAAGAGC
mGATA4	AAAACGGAAGCCCAAGAACCT	TGCTAGTGGCATTGCTGGAGT
mWnt3a	TGGCTGAGGGTGTCAAAGC	CGTGTCACTGCGAAAGCTACT
mNkx2.5	AAGCAACAGCGGTACCTGTC	GCTGTCGCTTGCACTTGTAG
mMlc2v	ATCGACAAGAATGACCTAAGGGA	ATTTTTCACGTTCACTCGTCCT
mCx40	GGTCCACAAGCACTCCACAG	CTGAATGGTATCGCACCGGAA
mANF	ACTAGGCTGAACAGCTTCC	TGACACACCACAAGGGCTTA
mMyHC6	CAACAACCCATACGACTACGC	ACATCAAAGGGCCACTATCAGTG

Appendix H: Mass Spectrometry Data and Protein Sequence Alignment

IPI NUMBER	PROTEIN NAME	MASCOT SCORE
IPI00133185	60S ribosomal protein L14	141
IPI00222549	60S ribosomal protein L30	170
IPI00468203	Annexin A2	125
IPI00230035	ATP-dependent RNA helicase DDX3X	128
IPI00474883	Capping protein (Actin filament) muscle Z-line	268
IPI00307837	Elongation factor 1-alpha 1	429
IPI00330063	F-actin capping protein subunit alpha-1	744
IPI00308607	F-box only protein 30	153
IPI00223714	Histone H1.4	333
IPI00153400	Histone H2A	293
IPI00114642	Histone H2B	319
IPI00230730	Histone H3	245
IPI00407339	Histone H4	405
IPI00117496	Homeobox protein Nkx-2.5	273
IPI00354819	Isoform Smooth muscle of Myosin light polypeptide 6	245
IPI00319994	L-lactate dehydrogenase A chain	512
IPI00109044	myosin regulatory light chain A	370
IPI00109044	myosin regulatory light chain A	497
IPI00127415	Nucleophosmin	294
IPI00117910	Peroxiredoxin-2	108
IPI00420814	Pol protein	133
IPI00128904	Poly(rC)-binding protein 1	106
IPI00671847	Protein phosphatase 1 regulatory subunit 12A	289
IPI00222509	Protein RCC2	88
IPI00126010	Ribosomal protein SA	114
IPI00469713	RIKEN cDNA 3732412D22 gene	92
IPI00311873	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	804
IPI00465823	similar to 60S ribosomal protein L18	149
IPI00467833	Triosephosphate isomerase	111
IPI00230044	Tropomyosin alpha-3 chain	1115
IPI00421223	Tropomyosin alpha-4 chain	655
IPI00123319	Tropomyosin beta chain	950
IPI00110753	Tubulin alpha-1 chain	672
IPI00110753	Tubulin alpha-1A chain	129
IPI00314844	Twinfilin-1	335

* Proteins identified in control purifications have been excluded

Serine/threonine-protein phosphatase PP1-beta catalytic subunit protein sequence alignment (Sequence 1: Human Sequence 2: Mouse)

```

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# 2: EMBOSS_001
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# Extend_penalty: 0.5
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# Length: 327
# Identity:      327/327 (100.0%)
# Similarity:    327/327 (100.0%)
# Gaps:          0/327 ( 0.0%)
# Score: 1763.0
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  |||
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  |||
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  |||
EMBOSS_001    301  KAKYQYGGLNSGRPVTTPRTANPPKRR      327
  |||
EMBOSS_001    301  KAKYQYGGLNSGRPVTTPRTANPPKRR      327
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Protein phosphatase 1 regulatory subunit 12A protein sequence alignment (Sequence 1:

Human Sequence 2: Mouse)

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# Aligned sequences: 2
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# 2: EMBOSS_001
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# Extend_penalty: 0.5
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# Similarity:    996/1032 (96.5%)
# Gaps:          5/1032 ( 0.5%)
# Score: 4893.0
#
#
#=====
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  |||
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  |||
EMBOSS_001     51 GDTDEVLKLLHRGADINYANVDGLTALHQACIDDNVDMVKFLVENGANIN      100
  |||
EMBOSS_001     51 GDTDEVLKLLHRGADINYANVDGLTALHQACIDDNVDMVKFLVENGANIN      100
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EMBOSS_001    301 LIESTANMDNNQSQKTFKKNKETLIIPEKNASRIESLEQEKVDEEEEGKK      350
  |||:-.
EMBOSS_001    301 LIESTANMENNQPQKAFKKNKETLIIPEKNASRIESLEHEKADEEEEGKK      350
  |||:-.
EMBOSS_001    351 DESSCSSEEEDEDDSESEAETDKTKPLASVTNANTSSTQAAPVAVTTPTV      400
  |||:
EMBOSS_001    351 DESSCSSEEEDEDDSESEAETDKTKPMASVSNAHTSSTQAAPAAVTAPT      400
  |||:

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Appendix I: Curriculum Vitae

TAMMY RYAN (PORTER)

EDUCATION

PhD Biochemistry (In progress)

The University of Ottawa, Ottawa, ON, Canada

Thesis: Molecular Mechanisms of Myogenesis in Stem Cells

Supervisor: Dr. Ilona Skerjanc

Bachelor of Medical Sciences, Honours Biochemistry (June 2005)

The University of Western Ontario, London, ON, Canada

Thesis: Mechanisms of substrate recognition by the SecA ATPase.

Supervisor: Dr. Brian Shilton

SCHOLARLY PUBLICATIONS

Peer Reviewed Articles

1. Karen A.M. Kennedy*, **Tammy Porter***, Virja Mehta, Scott D. Ryan, Feodor Price, Vian Peshdary, Christina Karamboulas, Josee Savage, Tom Drysdale, Shun-Cheng Li, Steffany A.L. Bennett and Ilona S. Skerjanc. Retinoic Acid can enhance skeletal muscle specification and bypass inhibition of Bone Morphogenetic Protein 4 but not by dominant negative β -catenin. *BMC Biology* 2009 Oct 8;7:67. ***These authors contributed equally to this work.**
2. Anastassia Voronova, Anna Fischer, **Tammy Ryan**, Al-Madhoun Ashraf, and Ilona Sylvia Skerjanc. *Ascl/Mash1* is a novel target of *Gli2* during *Gli2*-induced neurogenesis in P19 EC cells. *PLoS One*. 2011 Apr 29;6(4):e19174.
3. **Tammy Ryan**, Jun Liu, Alphonse Chu, Lisheng Wang, Alexandre Blais and Ilona S. Skerjanc. Retinoic Acid Enhances Skeletal Myogenesis in Human Embryonic Stem Cells by expanding the premyogenic progenitor population. Manuscript accepted for publication in *Stem Cell Reviews and Reports* (STCR-331).
4. **Tammy Ryan**, Jean-Phillipe Lambert, Sophie Boisvenue, Daniel Figeys and Ilona S. Skerjanc. Myosin Phosphatase interacts with *Nkx2.5* and controls its subcellular localization during cardiomyogenesis in stem cells. Manuscript in Preparation.
5. Ashraf Al-Madhoun, Christian McIntyre, **Tammy Ryan**, Anastassia Voronova and Ilona S. Skerjanc. Testosterone enhances stem cell cardiomyogenesis through the

recruitment of AR1 and histone acetyltransferases to cardiac specific genes.
Manuscript in Preparation.

Conference Abstracts

1. **Ryan, T.**, Liu, J., Chu, A., Wang, L., Blais, A., Skerjanc, I.S.. Retinoic Acid Enhances Skeletal Myogenesis in Human Embryonic Stem Cells by expanding the premyogenic progenitor population. MDA National Scientific Conference, Las Vegas, USA, March 2011.
2. **Porter, T.**, Lambert, J.P., Boisvenue, S., Figeys, D. and Skerjanc, I.S. Myosin Phosphatase interacts with Nkx2.5 and controls its subcellular localization. Mechanisms of Cardiac Death, Growth and Differentiation (Keystone Symposia) Keystone, Colorado, USA, February 2011.
3. **Porter, T.**, Lambert, J.P., Boisvenue, S., Figeys, D. and Skerjanc, I.S. Protein Phosphatase 1 interacts with Nkx2.5 and controls its subcellular localization. The Ottawa Conference on New Directions in Biology and Disease of Skeletal Muscle, Ottawa, Canada, May 2010.
4. **Porter, T.**, Lambert, J.P., Boisvenue, S., Figeys, D. and Skerjanc, I.S. Protein Phosphatase 1 interacts with Nkx2.5 and controls its subcellular localization. Cardiovascular Development and Repair (Keystone Symposia) Keystone, Colorado, USA, March 2010.
5. **Porter, T.**, Kennedy, K., Mehta, V., Price, F., Skerjanc, I.S. Retinoic Acid Regulates Myogenesis in Stem Cells by Enhancing the Commitment of Mesodermal Progenitors to the Myogenic Lineage. Making muscle in the embryo and Adult, New York City, USA, May 2009.
6. **Porter, T.**, Lambert, J.P., Boisvenue, S., Figeys, D. and Skerjanc, I.S., Protein Phosphatase 1 interacts with Nkx2.5 during cardiomyogenesis in P19 cells. Cardiac Regeneration and Repair, Boston, USA, October 2008.
7. **Porter, T.**, Lambert, J.P., Boisvenue, S., Figeys, D. and Skerjanc, I.S.. The Identification of Interacting Partners of Nkx2-5 in cardiomyogenesis. The 7th Annual Conference of the Canadian Proteomics Initiative. Ottawa, Ontario, June 2007.
8. Kennedy, K.A.M., **Porter, T.**, Mehta, V., Price, F., Karamboulas, C., Savage, J., Rudnicki, M., and Skerjanc, I.S. Retinoic Acid can enhance muscle specification and bypass inhibition by Bone Morphogenetic Protein 4 but not by a dominant negative β -catenin. Gordon Research Conference, Il Ciocco, Italy, May 2007.

INVITED PRESENTATIONS

1. **Porter, T.**, Retinoic Acid enhances skeletal myogenesis in stem cells. Signaling and Cell Biology Seminar Series. The University of Ottawa, 2007.
2. **Porter, T.** The identification of interacting partners of Nkx2.5 in cardiomyogenesis. Signaling and Cell Biology Seminar Series. The University of Ottawa, 2008.

SCHOLARSHIPS

1. Ontario Graduate Scholarship in Science and Technology. Awarded May, 2008. Duration: 2 years. Total amount of scholarship: \$30000
2. Master's Studentship, Heart and Stroke Foundation of Ontario. Awarded July 2006. Duration: 2 years. Total amount of scholarship: \$36 000.
3. University of Ottawa Excellence Scholarship. Awarded May 2006. Duration: 2 years. Total amount of scholarship: \$12 000.

TEACHING ASSISTANTSHIPS

1. Teaching Assistant, Cell Regulation and Control, Faculty of Science, The University of Ottawa, 2008-2011.
2. Laboratory Demonstrator, Undergraduate Molecular Biology Laboratory, The University of Ottawa, 2007- 2010.
3. Teaching Assistant, Intermediate Metabolism, Faculty of Science, The University of Ottawa, 2006.