Role of the Retinoid X Receptors in Skeletal Muscle Development

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Abstract

Pluripotent stem cells have the capacity to develop into different cell lineages and can be manipulated into certain cell types through the use of small molecule inducers. Retinoic acid (RA) signaling through retinoic acid receptors (RAR) and retinoid X receptors (RXR) has the ability to direct lineage determination but has yielded disappointing results in promoting skeletal myogenesis in embryonic stem (ES) cells. RXR is crucial in embryonic development although it is generally considered to act as a silent partner for other nuclear receptors such as RAR. Our findings demonstrate that rexinoid specific signaling enhances skeletal myogenesis and requires β-catenin but not RAR. Moreover, RXR signalling in mouse ES cells can efficiently enhance skeletal myogenesis and closely recapitulates sequential events observed *in vivo*. Since ES cells closely represent the properties of the developing embryo, efficiently generating skeletal muscle provides a means to further scrutinize signaling pathways in myogenic development in view of developing therapies for muscle related diseases.

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Abbreviations

AF-1: Transcriptional Activating Function 1

AF-2: Transcriptional Activating Function 2

α-MEM: Minimum Essential Medium α

AT: Acetyltransferase

ATRA: All-Trans Retinoic Acid

bHLH: basic Helix-Loop-Helix

BMP4: Bone Morphogenetic Protein 4

CBP: CREB Binding Protein

CRBPII: Cellular Retinol Binding Protein Type II

DBD: DNA Binding Domain

D-MEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethylsulfoxide

EC: Embryonal Carcinomal

ES cells: Embryonic Stem cells

FGF: Fibroblast Growth Factor

GSK3: Glycogen Synthase Kinase 3

HAT: Histone Acetyl Transferase

HDAC: Histone Deacetylase

ICM: Inner Cell Mass

LBD: Ligand Binding Domain

LIF: Leukemia Inhibitory Factor

LXR: Liver X Receptor

MCK: Muscle Creatine Kinase

MEF2: Myocyte Enhancer Factor 2

MHC: Myosin Heavy Chain

MRF: Myogenic Regulatory Factor

NCoR: Nuclear Receptor Corepressor

PBS: Phosphate Buffered Saline

PCAF: p300/CBP-Associated Factor

PPAR: Peroxisome Proliferator Activated Receptor

PXR: Pregnane X Receptor

RA: Retinoic Acid

RALDH: Retinaldehyde Dehydrogenases

RAR: Retinoic Acid Receptor

RARE: Retinoic Acid Response Element

RXR: Retinoid X Receptor

RXRE: Retinoid X Response Element

SDS: Sodium Dodecyl Sulfate

Shh: Sonic Hedge Hog

Sir2: Silent information regulator 2

SMRT: Silencing Mediator of Retinoic acid and Thyroid hormone receptor

TCF/LEF: T-Cell Factor/Lymphoid Enhancer Factor

TR: Thyroid Receptor

VDR: Vitamin D Receptor

Wnt: Wingless/Integrated

CHAPTER 1: REVIEW OF THE LITERATURE

Natural and synthetic retinoids are powerful regulators of cell growth and differentiation. Treatment of tissue cultures with these compounds induces differentiation into specific cell lineages. Bexarotene, a synthetic rexinoid, is a compound engineered to activate a specific nuclear receptor, the retinoid X receptor (RXR). RXR is known to act in both permissive and non-permissive partners and plays key roles in modulating a vast set of genes in different signaling systems. RXR is crucial in early development as well as in post-natal life and partakes in widespread processes from apoptosis to cholesterol metabolism (Ahuja, H.S., Szanto, A. et al. 2003). In understanding RXR signaling pathways, we may further our understanding of congenital disorders resulting from aberrant signaling. We also come one step closer to cultivating pure populations of progenitor cells as well as generating safer, more potent treatments with fewer side effects.

The Retinoid X Receptors

RXR belongs to the nuclear hormone receptor superfamily which includes steroid hormone, thyroid hormone, vitamin D receptors and nuclear receptors including RAR, PPAR, LXR and PXR (Szanto, A., Narkar, V. et al. 2004). It is a unique protein in that it has the ability to form heterodimers with one third of the 48 other nuclear receptors (Mangelsdorf, D.J., Thummel, C. et al. 1995) giving it the potential to be involved and to converge a large array of signaling pathways. The RXRs can be ligand dependant or independent and form three different types of dimers; RXR homodimers, permissive heterodimers, and non-permissive heterodimers (Tanaka, T. and De Luca, L.M. 2009).

When RXR forms homodimers or permissive heterodimers (with PPAR, LXR, PXR etc.) it is amenable to RXR ligand dependant activation. This is due to the fact that the activation domain of the partner receptor is placed in proximity to RXR helexis so that when RXR is activated by ligand, conformational changes cause direct stabilization of the activation domain of its partner (Gampe, R.T., Jr., Montana, V.G. et al. 2000b). When RXR forms non-permissive heterodimers (with RAR, VDR, TR etc.) it is not activated by ligand because binding of the other monomer to RXR allosterically inhibits it (Kurokawa, R., DiRenzo, J. et al. 1994; Tanaka, T. and De Luca, L.M. 2009) and the activation domain of the partner is not located in proximity to ligand activated residues in the RXR interface (Bourguet, W., Vivat, V. et al. 2000; Gampe, R.T., Jr., Montana, V.G. et al. 2000b).

An unusual protein-protein interaction property of RXR is that it can exist in solution as a tetramer (Kersten, S., Dawson, M.I. et al. 1996). This is due to its unique dimerization interface that is stable in both a symmetric configuration (giving rise to homodimers) and asymmetric configuration (forming heterodimers). When homodimers are formed, a new dimerization interface is formed that allows homodimer-homodimer association and thus the formation of tetramers (Ahuja, H.S., Szanto, A. et al. 2003). Since these tetramers form with high affinity, RXRs may be sequestered within the cell (Kersten, S., Kelleher, D. et al. 1995).

RXR and RAR in Development

RXR/RAR non-permissive heterodimers have been extensively studied and are well-characterized as important in development. Gene mutation studies have determined that both RXR and RAR are essential and delineated roles and tissue expression patterns for the different isoforms of the two receptors (α, β, and γ). The different RAR and RXR isotypes are encoded by different genes and their isoforms differ in their NH2-terminal regions which are generated by differential promoter usage and alternative splicing (Chiba, H., Clifford, J. et al. 1997). While RXR-α null embryos show defects in RXR/PPARγ (Peroxisome Proliferator Activated Receptor) signaling, the RARs appear to be the most important partners for RXRs (Ahuja, H.S., Szanto, A. et al. 2003).

During development, RXR-α and β are ubiquitously expressed with the highest levels of RXR-α present in the liver, heart, intestines, kidney, spleen, placenta, and the epidermis (Ahuja, H.S., Szanto, A. et al. 2003; Pratt, M.A., Crippen, C. et al. 1998). RXR-γ is expressed in all developing skeletal and cardiac muscles, the anterior pituitary, and the brain. The expression of RXRs is tissue specific and often overlaps but occasionally RXRs are uniquely expressed.(Mangelsdorf, D.J., Borgmeyer, U. et al. 1992) RXR-α is the primary isoform and supports the activity of all three RARs. Furthermore, RXR-α may be important in the expression of RXRγ since the RXRγ gene contains a Retinoid X Response Element (Barger, P.M. and Kelly, D.P. 1997).

Studies with mice lacking expression of RXR-α have found that these mice die in utero as a result of hypoplastic myocardium (Kastner, P., Grondona, J.M. et al. 1994; Sucov, H.M., Dyson, E. et al. 1994) and RXR-α null mutations exhibit growth retardation, webbed digits (Mark, M., Ghyselinck, N.B. et al. 2006) and defects in the

chorioallantoic placenta (Sapin, V., Dolle, P. et al. 1997). Loss of RXR-β and RXR-γ is not as severe since they can be compensated for by RXR-α (Tanaka, T. and De Luca, L.M. 2009) which may explain why RXRγ-/- mouse mutants are viable and have no muscular defects even in compound mutant combinations (Dolle, P. 2009).

Similarily, animals lacking RAR-α or RAR-γ result in postpartum lethality (Lohnes, D., Kastner, P. et al. 1993). In RAR knock-out studies where two RARs are deleted, the mutants display a spectrum of defects that resemble Vitamin A Deficiency Syndrome (Lohnes, D., Kastner, P. et al. 1993; Lufkin, T., Lohnes, D. et al. 1993) and the function of the residual RAR is highly dependant on RXR-α (Ahuja, H.S., Szanto, A. et al. 2003).

Even during normal development, the RARs are highly dependant on the RXRs. Homodimer formation of RARs is energetically unfavored because of limited contact between the interfaces. Pairing with RXR creates an extended area of intermolecular contact that stabilizes heterodimer formation. This substantially larger surface area and consequent stability, results in the preferential formation of heterodimers as opposed to homodimers of RAR (Bourguet, W., Vivat, V. et al. 2000; Gampe, R.T., Jr., Montana, V.G. et al. 2000a)**.**

DNA Binding

The receptor dimers of RXR and its partner, constituitively bind to specific DNA response elements in the promoters or enhancers of the genes they govern and DNA binding specificity is determined by the number of spacer nucleotides present between two direct repeats, everted repeats, or inverted repeats of the canonical binding sequence

5’-PuGGTCA (Leid, M., Kastner, P. et al. 1992; Umesono, K. and Evans, R.M. 1989). RXR/RAR heterodimers bind the Retinoic Acid Response Element (RARE) with a consensus half site separated by 2 or 5 nucleotides (DR2 or DR5) whereas RXR homodimers bind the Retinoid X Response Element (RXRE) which is separated by only one nucleotide (DR1) (Tanaka, T. and De Luca, L.M. 2009) (See Figure 1). Selective response element recognition is due to a short sequence (the P box) located at the C-terminal base of the N-terminal C1 finger of the DNA Binding Domain (DBD) which interacts with the binding motif and to a weak dimerization function which encompases the N-terminal base of the CII finger (D-box) of the DBD (Danielsen, M., Hinck, L. et al. 1989; Green, S., Kumar, V. et al. 1988; Kumar, V. and Chambon, P. 1988; Luisi, B.F., Xu, W.X. et al. 1991; Mader, S., Kumar, V. et al. 1989; Umesono, K. and Evans, R.M. 1989). While RXR/RAR heterodimers bind more effectively to the RAREs than RXR homodimers, RXRs homodimers can bind RXREs with high affinity (Zhang, X.K., Lehmann, J. et al. 1992). RAREs can overlap with RXREs and since RXR/RAR heterodimers bind with a higher affinity than RXR homodimers, (Tanaka, T. and De Luca, L.M. 2009) this may interfere with RXR signaling.

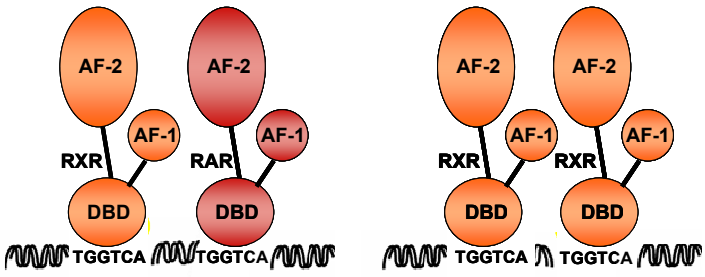

Figure 1. RXR/RAR Heterodimers and RXR Homodimers Bound to DNA. RXR/RAR heterodimers (left) and RXR homodimers (right) bind via the DNA binding domain to two direct repeats of the canonical binding sequence 5'-PuGGTCA separated by 2 or 5 nucleotides or 1 nucleotide respectively.

Receptor Ligands

RXR and RAR are ligand inducible transcription factors. While they are constituitively bound to the DNA, they require agonist binding to activate gene transcription.

RA, the active derivative of Vitamin A, is a ligand implicated in the induction and moderation of hundreds of genes; several of these being important if not key factors in the specification and development of skeletal muscle. In vitro, the expression of more than 500 genes can be altered following RA exposure, as either primary or secondary targets (Balmer, J.E. and Blomhoff, R. 2002). RA is a retinoid with important effects on vertebrate embryonic body shaping and organogenesis, tissue homeostasis, differentiation, apoptosis, regulating fertility, maintaining normal vision, and preventing neoplastic growth and neurodegenerative diseases (Blomhoff, R. 1994; Kastner, P., Mark, M. et al. 1995; Morriss-Kay, G.M. and Ward, S.J. 1999; Niederreither, K. and Dolle, P. 2008; Sporn, M.B. and Roberts, A.B. 1994).

RA exists as two isoforms: All-Trans RA and 9-cis RA (Figure 2). RARs bind and are activated by ATRA as well as its 9-cis isomer while the RXRs bind and are activated by only the bent 9-cis RA (Ricaud, S., Vernus, B. et al. 2005). However, due to the considerable difficulty of detecting 9-cis RA endogenously in embryos or adult tissue (Niederreither, K. and Dolle, P. 2008) there has been considerable debate about the in vivo role of activated RXR and has lead to the belief that RXR serves only to orient and position the heterodimers properly on the DNA (Perlmann, T. and Jansson, L. 1995; Willy, P.J. and Mangelsdorf, D.J. 1997; Willy, P.J., Umesono, K. et al. 1995).

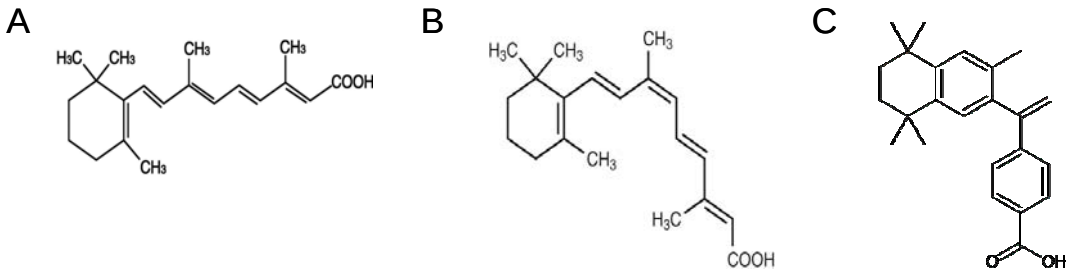

Figure 2. Structure of Ligands. (A) ATRA, (B) 9-cis RA, and (C) Bexarotene (LGD1069).

Bexarotene (LGD1069) (Figure 2) is a synthetic RXR selective compound used in the treatment of cancer. It is unable to transactivate the RXR-RAR heterodimer (Lehmann, J.M., Jong, L. et al. 1992) and will not activate RARs (Table 1). Synthetic RXR selective ligands can act as agonists and activate both homodimers and permissive heterodimers. Conversely, they can also act antagonistically of homodimers, as is the

case for the synthetic ligand LG100754, and promote only the activation of non-permissive heterodimers (Lala, D.S., Mukherjee, R. et al. 1996) (Table 2).

Compound	RARα	RARβ	RARγ	RARα	RARβ	RARγ
All-*trans* RA	15	17	17	>1000	>1000	>1000
9-*cis* RA	15.2	13.4	14.7	6.7	6.2	9.7
LGD1069	>1000	>1000	>1000	36	21	29
LG100268	>1000	>1000	>1000	3.2	6.2	9.7
LG100754	>1000	>1000	>1000	3.4	10	12.2

Table 1. K_d (nm) values for all-trans retinoic acid, 9-cis retinoic acid, LGD1069, LG100268, and LG100754. K_d values were determined by the ability of ligands to displace [^{3}H]9-cis retinoic acid from RXR or [^{3}H]all-trans retinoic acid from RAR.(Nau, H., Blaner, W.S. et al. 1999)

Compound	RXR-RXR	RXR-RAR	RXR-TR	RXR-VDR	RXR-PPAR	RXR-LXR	RXR-FXR	RXR-NGFIB
All-*trans* RA	+/-	+	-	-	-	-	-	-
9-*cis* RA	+	+	-	-	+	+	+	+
LGD1069	+	-	-	-	+	+	+	+
LG100268	+	-	-	-	+	+	+	+
LG100754	-	+	-	-	+	-	-	-

Table 2. Agonist activity of LGD1069, LG100268, and LG100754 on RXR homodimers and RXR-dependant heterodimers. +, Agonist activity; -, agonist activity is not observed.(Nau, H., Blaner, W.S. et al. 1999)

Some studies have shown that allosteric inhibition of RXR in the RXR/RAR heterodimer only occurs when the RAR is unliganded and that this inhibition is relieved once RAR is occupied (Forman, B.M., Umesono, K. et al. 1995; Lala, D.S., Mukherjee, R. et al. 1996). Other studies claim that both receptors bind their ligands independently and that their effect is additive (Kersten, S., Kelleher, D. et al. 1995). This contradiction needs further work; however, what complicates the picture is that different ligands contact different side chains within the LBD (Egea, P.F., Mitschler, A. et al. 2002) resulting in the activation or silencing of specific homodimers or heterodimers.

The effect of ligand binding on RXR tetramers depends on the identity of the ligand. The binding of agonist, such as 9-cis RA, induces a large conformational change that results in a shift of helix 12 from intermolecular to intramolecular and the resultant loss of contact between associated homodimers, resulting in the dissociation of tetramers. Interestingly, when RXR exists as tetramers, the ligand binding pocket is distorted and enlarged and can therefore accommodate ligands that do not normally bind (Gampe, R.T., Jr., Montana, V.G. et al. 2000b). As mentioned previously, ATRA cannot fit into the normal ligand binding pocket of RXR, however it does bind to the enlarged tetramer binding pocket. This binding does not induce the conformational changes required for dissociation of the tetramer and the effect of ATRA and similar ligands is therefore antagonistic and leads to sequestering of RXR (Ahuja, H.S., Szanto, A. et al. 2003). This sequestering results in transcriptional silencing of RXR since the transcriptional activating domain, AF-2, is locked in an intermolecular position (Ahuja, H.S., Szanto, A. et al. 2003).

RXR Ligand Binding Domain

The ligand binding pocket of RXR is a deep hydrophobic pocket composed of residues from multiple helixes and is characterized as a high affinity receptor. It is relatively smaller than in other nuclear receptors so ligand occupies a high fraction of the available volume and limits the number of water molecules bound within the pocket (Egea, P.F., Mitschler, A. et al. 2000; Gampe, R.T., Jr., Montana, V.G. et al. 2000b). Additionally, the bulkier side chains of the amino acid residues within the ligand binding pocket preclude the binding of ATRA and only permit the bent 9-cis RA to bind (Egea, P.F., Mitschler, A. et al. 2000). Finally, ligand binding is associated with a relatively large conformational change resulting in the partial closing of the hydrophobic channel through which the ligand gains access to the ligand binding pocket and results in a ligand that is completely removed from the exterior of the molecule (Egea, P.F., Mitschler, A. et al. 2000). This is in contrast to low affinity receptors where a larger pocket with limited ligand-receptor contact points creates an environment where the ligand has easy access to the exterior of the molecule and allows ready reversal of ligand binding (Ahuja, H.S., Szanto, A. et al. 2003)

The Interaction of Co-activators p300, CBP, and PCAF with RXR and RAR

In response to ligand, RXRs and RARs bind co-activators and the respective binding of cofactors again depends on the identity of the ligand. Agonist binding induces large conformational changes within the receptor causing helix 11 and 12 (the AF-2 domain) to close the lid of the ligand binding pocket and generate high affinity co-activator binding sites. This charged surface has a high affinity for a specific amino acid motif, LXXLL,

which mediates the binding of co-activators to nuclear receptors (Westin, S., Kurokawa, R. et al. 1998). Alternatively, if an antagonist or partial agonist binds, helix 12 is repositioned to an adjacent groove on the LBD and a charged surface that favors the co-repressor binding motif is formed (Perissi, V., Staszewski, L.M. et al. 1999).

Co-activators, as their name implies, have the ability to activate transcription and interact with the basal transcriptional machinery, bridge and direct the assembly of transcriptional pre-initiation complexes, and induce chromatin remodeling (Bastien, J. and Rochette-Egly, C. 2004; Rosenfeld, M.G., Lunyak, V.V. et al. 2006). Co-activators such as p300, CREB Binding Protein (CBP), and p300/CBP-Associated Factor (P/CAF) act as histone acetyltransferases (HATs) (Niederreither, K. and Dolle, P. 2008; Ogryzko, V.V., Schiltz, R.L. et al. 1996) and can form large multimolecular complexes.

In the absence of ligand, co-repressors such as the Nuclear Receptor Corepressor (NCoR)/ Silencing Mediator of Retinoic acid and Thyroid hormone receptor (SMRT) family bind and recruit a mulitprotein complex containing the histone deacetylase HDAC3 (Guenther, M.G., Lane, W.S. et al. 2000; Li, J., Wang, J. et al. 2000). The recruited histone methyl-transferases and histone deacetylases stabilize the nucleosome structure so that the DNA is inaccessible for transcription (Niederreither, K. and Dolle, P. 2008).

When RXR forms permissive heterodimers (i.e.: RXR/PPAR), neither receptor binds co-repressors under normal circumstances (DiRenzo, J., Soderstrom, M. et al. 1997). Ligand binding to one receptor recruits co-activators and although the other receptor may be unliganded, the high local concentration of bound co-activator favors the docking of the second LXXLL motif with the co-activator binding sites of the other

receptor. If ligand is present for both receptors of the permissive heterodimer, they can synergistically recruit co-activators (Ahuja, H.S., Szanto, A. et al. 2003).

Non-permissive heterodimers (i.e.: RXR/RAR) do bind co-repressors and this binding to unliganded RXR and its partner is stabilized by both receptors. Transactivation requires ligand binding to the RXR partner (i.e.: RAR) to convert it into the agonist conformation, displace co-repressors, and recruit co-activators (Vivat, V., Zechel, C. et al. 1997; Zhang, J., Hu, X. et al. 1999). As with permissive heterodimers, synergistic recruitment of co-activators occurs when ligands are present for both receptors (Ahuja, H.S., Szanto, A. et al. 2003).

Since RXR can form tetramers, it has one further mechanism of transcriptional repression. Unliganded RXR does not show much propensity to bind co-repressors since unliganded RXR is locked in tetramers. Yet it has a conformation distinct from either agonist or antagonist and is transcriptionally inactive since the AF-2 helix forms intermolecular interactions with the coactivator pocket of the neighboring RXR monomer which prevents coactivator binding (Bourguet, W., Ruff, M. et al. 1995; Gampe, R.T., Jr., Montana, V.G. et al. 2000b).

Interestingly, co-activators p300 and CBP are also able to acetylate proteins other than histones, such as transcription factors (Gu, W. and Roeder, R.G. 1997). CBP and p300 are heavily autoacetylated and upon recruitment to receptors, can acetylate more of themselves in an intermolecular fashion (Karanam, B., Jiang, L. et al. 2006). In addition to this, they have the ability to recruit PCAF (Yang, X.J., Ogryzko, V.V. et al. 1996). p300 influences RXR activity as RXR-α and γ are subject to p300 acetylation which promotes their binding to RXRE and increases their transcriptional activity as well (Zhao,

W.X., Tian, M. et al. 2007). Co-activators play crucial roles in gene activation, however, those recruited by particular RXR dimers at specific genetic loci in response to ligand have yet to be identified.

Therapeutic Potentials of Retinoid and Rexinoid Signaling

Pluripotent stem cells closely simulate embryonic development and present a model system with which to dissect signaling pathways of target receptors in controlled environments. They hold a tremendous potential for cell-based therapies through their capacity to grow and regenerate new tissues. Many diseases including muscular dystrophies, cancer, AIDS, and even normal conditions such as aging show prominent muscle loss that would benefit enormously from regenerative cell based therapies. However the ability to use stem cells in muscle wasting disorders has been limited due to the low frequency of myogenic precursors in Embryonic Stem (ES) cell cultures and the difficulty in identifying and isolating precursor cells. To harvest the full potential of these cells in therapies, it is imperative that we find small molecule inducers capable of efficiently directing stem cells to skeletal muscle lineage. Attempts at using RA in ES cell cultures have thus far yielded disappointing results; however, the ability of rexinoids to induce these cells has not yet been fully explored. Understanding the myogenic pathway in vivo as well as deciphering differentiation cues to culture pure populations of myogenic cells will prove a vital tool in the treatment of such devastating diseases.

Myogenesis

Skeletal muscle development is a complex interplay of processes that requires combined signaling of many different factors. In order to achieve mature skeletal muscle, pluripotent stem cells must first receive cues that specify them to myoblasts. Further signals allow them to proliferate and differentiate into myotubes which receive additional cues that allow maturation into multinucleated myofibers. However, the simple presence of the required signaling molecules at each stage does not activate transcription and does not imply differentiation as there are multiple mechanisms also in place to ensure that the myogenic program is not activated prematurely.

Skeletal muscle forms in the embryo from paraxial mesoderm, which segments into somites on either side of the neural tube and notochord (Christ, B. and Ordahl, C.P. 1995). Extracellular signals from surrounding tissues play a significant role in muscle development (Figure 3). These signals include members of the Wnt family, specifically Wnt1 and Wnt7a (Cossu, G. and Borello, U. 1999) secreted from the neural tube and surface ectoderm, Sonic Hedge Hog (Shh), secreted by notochord and floor plate cells and which acts in conjunction with Wnt1 (Cossu, G. and Borello, U. 1999), Bone Morphogenetic Protein4 (BMP4), secreted by lateral plate mesoderm cells (Borycki, A.G., Brunk, B. et al. 1999; Dietrich, S., Schubert, F.R. et al. 1998; Munsterberg, A.E., Kitajewski, J. et al. 1995; Pourquie, O., Fan, C.M. et al. 1996; Tajbakhsh, S., Borello, U. et al. 1998) and RA (Rohwedel, J., Guan, K. et al. 1999) which is under tight regulatory control for its synthesis, degradation, and transport. These act on downstream targets such as HOX genes, which controls specification of the body axis (Rohwedel, J., Guan, K. et al. 1999), Brachyury T, a protein required for posterior mesoderm and notochord

differentiation (Skerjanc, I.S. 1999), and the Myogenic Regulatory Factors (MRFs) Myf5, MyoD, myogenin, and Mrf4 which are required for the commitment and maturation of skeletal muscle (Cossu, G. and Borello, U. 1999; Rohwedel, J., Guan, K. et al. 1999; Skerjanc, I.S. 1999).

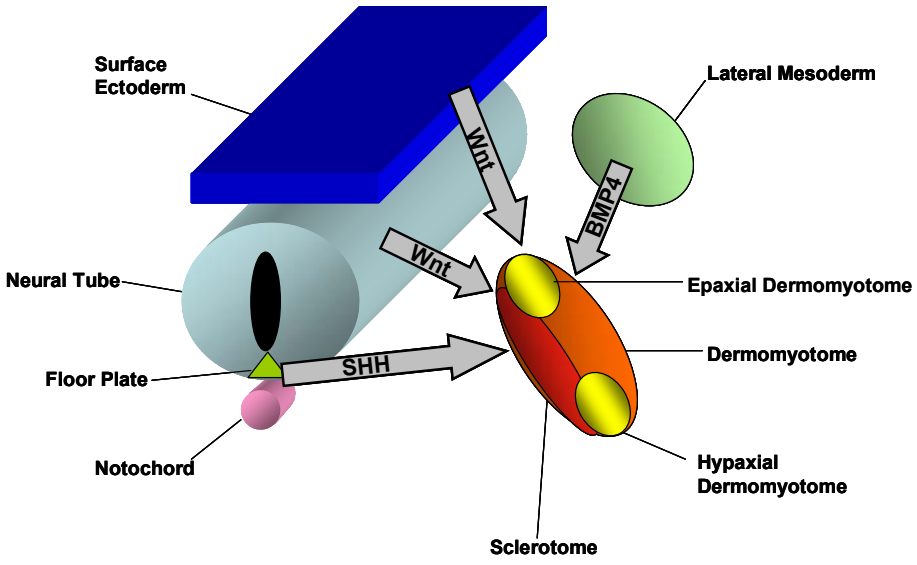

Figure 3. Regulation of Myogenesis by Signaling Molecules from Neighboring Tissues. Myogenesis is positively regulated in the epaxial dermomyotome by Wnt factors secreted from the neural tube and by Shh from the floor plate and notochord. The hypaxial dermomyotome is also positively regulated by Wnt signals from the dorsal ectoderm while BMP4 from the lateral mesoderm inhibits skeletal myogenesis.

Roles of Pax in the Specification of Myogenic Precursors

Signals from surrounding tissues activate the myogenic program and result in the expression of transcription factors such as Pax3, Meox1/2 and Gli2 that specify cells to the skeletal muscle lineage and mediate the induction of the MRFs (McDermott, A., Gustafsson, M. et al. 2005; Petropoulos, H., Gianakopoulos, P.J. et al. 2004; Petropoulos,

H. and Skerjanc, I.S. 2002; Ridgeway, A.G. and Skerjanc, I.S. 2001; Williams, B.A. and Ordahl, C.P. 1994).

Pax3, a transcription factor with homeo and paired domain motifs, is thought to be activated by Wnt6a (Fan, C.M., Lee, C.S. et al. 1997) and is responsible for both delamination and migration of muscle precursors to the limb bud (Tajbakhsh, S., Rocancourt, D. et al. 1997). Pax3 is found to be initially expressed throughout the somite before becoming restricted to the dermomyotome and subsequently the migratory muscle precursor cells (Goulding, M., Lumsden, A. et al. 1994; Williams, B.A. and Ordahl, C.P. 1994). The importance of Pax3 in the delamination and migration of muscle precursor cells is highlighted by the fact that mice which are Pax3 null have severe muscle loss (Alvares, L.E., Schubert, F.R. et al. 2003; Bladt, F., Riethmacher, D. et al. 1995; Dietrich, S., Abou-Rebyeh, F. et al. 1999; Epstein, J.A., Shapiro, D.N. et al. 1996; Grifone, R., Demignon, J. et al. 2005).

Pax3 directly regulates the expression of Myf5 through the limb bud enhancer of Myf5 (Bajard, L., Relaix, F. et al. 2006) and acts with Myf5, upstream of MyoD which cannot be properly expressed in the Pax3/Myf5 double knockout (Tajbakhsh, S., Rocancourt, D. et al. 1997). It is when the migrating cells reach the limb bud that they begin to express Myf5 and MyoD and it is both before and after activation of these genes that the cells undergo extensive proliferation (Buckingham, M., Bajard, L. et al. 2003; Tajbakhsh, S. and Buckingham, M.E. 1994). Pax3, along with additional factors such as Myf5, c-met, Msx1 and the Fibroblast Growth Factor (FGF) family of receptors promote myoblast proliferation. Proliferation is arrested by inhibitory signals which promote

differentiation by inducing cell cycle arrest proteins such as MyoD (Alric, S., Froeschle, A. et al. 1998).

Roles of Meox in the Specification of Myogenic Precursors

Meox1 and Meox2 are closely related homeobox genes with mesoderm and mesenchyme specific expression during mouse development (Candia, A.F., Hu, J. et al. 1992). Meox1 is expressed in the dermomyotome whereas after delamination and migration to the limb bud, Meox2 is predominantly expressed (Candia, A.F., Hu, J. et al. 1992; Candia, A.F. and Wright, C.V. 1996). In Meox2 deficient limb buds, Pax3 and Myf5 are downregulated and mice homozygous for a null mutation in Meox2 have defects in limb muscle differentiation resulting in an overall reduction in muscle mass and absence of specific muscles (Mankoo, B.S., Collins, N.S. et al. 1999). Interestingly, in cell cultures, overexpression of Meox1 does not induce myogenesis and while a dominant negative Meox1 has been shown to downregulate Pax3 and Gli2 expression and inhibit myogenesis in P19 cells (Petropoulos, H., Gianakopoulos, P.J. et al. 2004), Meox1 mutant mice exhibit mild defects in sclerotome-derived vertebral and rib bones (Mankoo, B.S., Skuntz, S. et al. 2003) rather than showing any overt muscle defects. It is only the compound mutant embryos of Meox1-/-; Meox2-/- that display a dramatic phenotype associated with disrupted somite development. In these embryos, the axial skeleton fails to develop and most skeletal muscles are absent or reduced in size (Mankoo, B.S., Skuntz, S. et al. 2003).

Stem Cell Systems as a Model for Study of Meox1 and Pax3

It is highly advantageous to use stem cell tissue cultures to study the importance of specification genes in a controlled environment to understand their relationship with each other and their regulation by extracellular signaling molecules. Specification factors exist in a very complex relationship and have the ability to autoregulate and cross-regulate one another (Petropoulos, H., Gianakopoulos, P.J. et al. 2004).

In P19 cell cultures, Pax3 overexpression can induce Meox1 but is unable to activate Gli2 and a dominant negative Pax3 mutation does not affect Gli2 levels. Conversely, Gli2, which also has the ability to upregulate Meox1, can upregulate Pax3 while the dominant negative Gli2 P19 cells downregulate Meox1, Pax3, and MRF expression and inhibit myogenesis. Lastly, Meox1 can activate the expression of Gli2 but overexpression of this protein is insufficient to induce Pax3 or skeletal myogenesis (Petropoulos, H., Gianakopoulos, P.J. et al. 2004). The ability of each of these factors to induce each other, or, in their absence, completely abolish myogenesis underlines the importance of these factors in the specification process.

Wnt signaling via β-catenin is also essential and sufficient for the induction of specification factors Pax3, Meox1, and Gli2 and in P19 cells, a dominant negative β-catenin inhibits Pax3, Gli2, Meox1 and MyoD expression and abolishes myogenesis (Petropoulos, H. and Skerjanc, I.S. 2002). This is not surprising since mutations of either Gli2, Meox1, or Pax3 in these cells will abrogate myogenesis (Petropoulos, H., Gianakopoulos, P.J. et al. 2004). Pax3 expression is essential and sufficient for the expression of the transcription factor Six1 and the induction of skeletal myogenesis (Ridgeway, A.G. and Skerjanc, I.S. 2001). Its overexpression induces Myf-5, MyoD, and

myogenin expression (Maroto, M., Reshef, R. et al. 1997) whereas a dominant negative Pax3 in P19 cells results in a loss of MyoD and myogenin expression and subsequent myogenesis (Ridgeway, A.G. and Skerjanc, I.S. 2001).

Roles of the Myogenic Regulatory Factors and their Cofactors in Myogenesis

After specification, the cells must further become committed to the myogenic lineage. The formation of myoblasts from myogenic precursor cells and their successive cell cycle arrest and differentiation into mature muscle cells involves two key families of transcription factors. The MyoD family of basic Helix-Loop-Helix (bHLH) proteins which includes the four master transcriptional regulators (also referred to as MRFs): Myf5, MyoD, myogenin, and Mrf4 (Arnold, H.H. and Braun, T. 2000; Braun, T., Bober, E. et al. 1990; Braun, T., Buschhausen-Denker, G. et al. 1989; Davis, R.L., Weintraub, H. et al. 1987; Edmondson, D.G. and Olson, E.N. 1990; Froeschle, A., Alric, S. et al. 1998) and the Myocyte Enhancer Factor 2 (MEF2) family of MADS-box transcription factors which includes MEF2A, -B, -C, and –D (Naya, F.J. and Olson, E. 1999).

Myf5 and MyoD are involved in muscle specification and commitment and have the capacity of remodeling chromatin and opening gene loci that participate in further muscle differentiation (Bergstrom, D.A. and Tapscott, S.J. 2001) while terminal differentiation is governed by myogenin and MRF4 (Figure 4). Each member is sufficient to dominantly induce myogenesis when introduced into a variety of non-muscle cells (Olson, E.N. 1990; Weintraub, H. 1993) and ectopic expression of MyoD can inhibit the cell cycle before the S phase independently of its DNA binding and the induction of

myogenic differentiation (Crescenzi, M., Fleming, T.P. et al. 1990; Sorrentino, V., Pepperkok, R. et al. 1990).

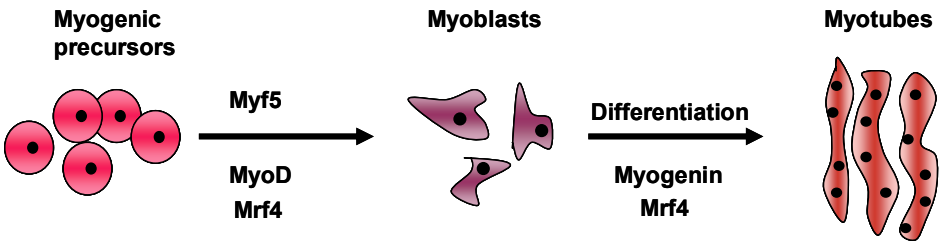

Figure 4. Involvement of Myogenic Regulatory Factors in Myogenesis. Myf5 and MyoD are involved in specification and commitment of muscle precursors to the myogenic lineage. Mrf4 also plays a role as a determination gene in addition to directing terminal differentiation along with myogenin.

Members of the MEF2 family alone are not sufficient to induce myogenesis, however the ability of the MRFs to convert cells is reliant on the function of the MEF2 family. MEF2 proteins bind as homodimers and heterodimers to the consensus sequence YTA(A/T)4TAR found in the promoter region of nearly every known muscle-specific gene (Black, B.L. and Olson, E.N. 1998) and together with the myogenic bHLH proteins, synergistically activate the transcription of myogenic genes. Unlike the MRFs, MEF2 genes are expressed outside the skeletal muscle lineage in tissues such as cardiac and smooth muscle (Edmondson, D.G., Lyons, G.E. et al. 1994; Leifer, D., Krainc, D. et al. 1993; Lyons, G.E., Micales, B.K. et al. 1995).

The bHLH domain of the MRFs is responsible for DNA binding and for dimerisation with the ubiquitously expressed bHLH E protein (Hu, J.S., Olson, E.N. et al.

1992; Murre, C., McCaw, P.S. et al. 1989; Parker, M.H., Perry, R.L. et al. 2006). The resulting myogenic bHLH-E heterodimers bind to DNA at consensus sequences known as an E-box (CANNTG), specific DNA motifs present at muscle gene enhancers and/or promoters, where they regulate gene expression (Sartorelli, V. and Caretti, G. 2005). These genes include cytoskeletal, sarcomeric, metabolic, and cell signaling proteins (Angus, L.M., Chan, R.Y. et al. 2001; Gramolini, A.O. and Jasmin, B.J. 1999; Kraner, S.D., Rich, M.M. et al. 1999; Li, H. and Capetanaki, Y. 1993; Lin, H., Yutzey, K.E. et al. 1991; Marsh, D.R., Carson, J.A. et al. 1998; Shield, M.A., Haugen, H.S. et al. 1996; Simon, A.M. and Burden, S.J. 1993; Wheeler, M.T., Snyder, E.C. et al. 1999). A requirement for the MyoD family of transcription factors in this combinatorial complex is demonstrated by the fact that E protein homodimers bind the same DNA sequences as the MyoD-E protein heterodimers but only the MyoD-E protein complex can cooperate with MEF2 factors (Naya, F.J. and Olson, E. 1999). Furthermore, the MRFs and MEF2 factors activate and repress each others transcription in a complex network (Arnold, H.H. and Winter, B. 1998; Bergstrom, D.A., Penn, B.H. et al. 2002; Cserjesi, P. and Olson, E.N. 1991; Olson, E.N. and Klein, W.H. 1994; Wong, M.W., Pisegna, M. et al. 1994). For example, expression of myogenin requires MEF2 while myogenin activates the expression of MEF2 independently of other skeletal gene products (Cserjesi, P. and Olson, E.N. 1991; Ridgeway, A.G., Wilton, S. et al. 2000). Similarily, MRFs can positively regulate their own transcription and the transcription of each other creating positive auto- and cross-regulatory loops (Braun, T., Buschhausen-Denker, G. et al. 1989; Thayer, M.J., Tapscott, S.J. et al. 1989).

Knock-out Models of MRFs

Myf5 is the first gene expressed in all muscle progenitors (Ott, M.O., Bober, E. et al. 1991). Subsequently, in the somites, myogenin, MRF4, and MyoD are expressed (Bober, E., Lyons, G.E. et al. 1991; Buckingham, M. 1992; Sassoon, D., Lyons, G. et al. 1989). In the limb bud and in head muscles, Myf5 expression is immediately followed by MyoD (Buchberger, A., Nomokonova, N. et al. 2003). The importance of each gene has been determined by mouse knock out models. Myf5 and MyoD are considered determination genes while myogenin and MRF4 are responsible for commitment to myogenesis and terminal differentiation.

In single knockout mice for either Myf5 or MyoD, skeletal muscle development proceeds without any morphological abnormalities (Braun, T., Rudnicki, M.A. et al. 1992; Rudnicki, M.A., Braun, T. et al. 1992). In Myf5 deficient mouse embryos, myotome formation is delayed until MyoD transcription begins independently of Myf5 (Braun, T., Bober, E. et al. 1994; Braun, T., Rudnicki, M.A. et al. 1992) and the levels of MyoD, myogenin and MRF4 are normal. However, the Myf5 knockout mice die perinataly due to the loss of the distal part of the ribs and inability to breathe (Braun, T., Rudnicki, M.A. et al. 1992). The MyoD deficient mice have skeletal muscle displaying normal physiology and morphology but have an increased and prolonged expression of Myf5 (Rudnicki, M.A., Braun, T. et al. 1992). The MyoD/Myf5 double knockout mice fail to generate myoblasts and display a complete loss of all skeletal muscles labeling Myf5 and MyoD with redundant and overlapping functions (Rudnicki, M.A., Schnegelsberg, P.N. et al. 1993). However, since MRF4 is located adjacent to Myf5 on the same chromosome, the Myf5 knockout can have a cis effect on MRF4 and decrease

or knockout its expression as well. Upon careful review of the double mutants, it was found that skeletal muscle is in fact present but only when MRF4 expression is not compromised.

MRF4 is known to be involved in differentiation of myoblasts, however, it is also expressed in undifferentiated cells and, as shown by the Myf5/MyoD double knockout mice, can act as a determination gene (Kassar-Duchossoy, L., Gayraud-Morel, B. et al. 2004). This new outlook places both Myf5 and MRF4 upstream of MyoD to direct embryonic multipotent cells into the myogenic lineage (Kassar-Duchossoy, L., Gayraud-Morel, B. et al. 2004). MRF4 knockout mice have an increase in myogenin expression and show deficiencies in intercostal muscle, myotomal myogenesis and deep back muscle formation (Braun, T. and Arnold, H.H. 1995; Patapoutian, A., Yoon, J.K. et al. 1995). In myogenin knockout mice, myoblasts are formed in the correct place but do not fuse into mature myofibers. These mice die perinatally with only a few myofibers observed at birth (Hasty, P., Bradley, A. et al. 1993; Nabeshima, Y., Hanaoka, K. et al. 1993; Venuti, J.M., Morris, J.H. et al. 1995). This same phonotype can be seen with the MRF4/MyoD double mutants showing that MRF4 or MyoD is necessary to activate myogenin (Kassar-Duchossoy, L., Gayraud-Morel, B. et al. 2004).

Impact of Extracellular Cues on MRF Expression

As mentioned previously, tissues surrounding the developing muscle secrete signaling factors that have a major impact on the expression of intracellular signaling molecules. Wnt signaling is essential for specification and commitment of the somatic cells to the myogenic lineage. Wnt1 expressed by neural tube preferentially activates Myf5, and

Wnt7a expressed by dorsal ectoderm activates MyoD. Wnt4, Wnt5a and Wnt6 have an intermediate effect in activation of both MyoD and Myf5 (Tajbakhsh, S., Borello, U. et al. 1998). Wnt1 acts through the classic Dishevelled→GSK3→β-catenin→Tcf pathway (Eastman, Q. and Grosschedl, R. 1999; Wodarz, A. and Nusse, R. 1998) in which Wnt signaling leads to the stabilization of β-catenin which enters the nucleus, binds the T-Cell Factor/Lymphoid Enhancer Factor (TCF/LEF) family of transcription factors and activates Myf5 (Gordon, M.D. and Nusse, R. 2006; Kennedy, K.A., Porter, T. et al. 2009) through directly binding the extended epaxial enhancer of Myf5 (Borello, U., Berarducci, B. et al. 2006). In contrast, Wnt7a appears to act via a β-catenin independent pathway (Kengaku, M., Capdevila, J. et al. 1998) and leads to activation of MyoD rather than Myf5 (Tajbakhsh, S., Borello, U. et al. 1998).

Wnt7a can also act synergistically with Shh to activate MyoD (Cossu, G. and Borello, U. 1999). In cultured somites, Shh is responsible for MyoD induction in a Myf5 dependent process through the Gli family of transcription factors (Borycki, A.G., Brunk, B. et al. 1999; Petropoulos, H., Gianakopoulos, P.J. et al. 2004) which bind to the epaxial enhancer of Myf5 (Gustafsson, M.K., Pan, H. et al. 2002) and Shh cannot activate MyoD in the absence of Myf5 (Borycki, A.G., Brunk, B. et al. 1999; McDermott, A., Gustafsson, M. et al. 2005). Shh is essential for the Myf5 and MyoD activation in epaxial muscle, however not in hypaxial myotome (Tajbakhsh, S., Borello, U. et al. 1998).

Alternatively, BMP4 signaling inhibits skeletal myogenesis and induces the expression of genes involved in cardiomyogenesis (Duprez, D.M., Coltey, M. et al. 1996; Murray, S.S., Murray, E.J. et al. 1993; Pourquie, O., Fan, C.M. et al. 1996; Schlange, T.,

Andree, B. et al. 2000; Schultheiss, T.M., Burch, J.B. et al. 1997). Noggin, produced from the notochord and somite in a Wnt dependant manner, counteracts BMP4 but it is the relative levels of both BMP4 and Noggin that regulate the activity of Pax3 and the MRFs (Kennedy, K.A., Porter, T. et al. 2009).

RA plays many roles in skeletal myogenesis. It is required for proper somite formation (Maden, M., Gale, E. et al. 1996; Maden, M., Graham, A. et al. 2000; Niederreither, K., Subbarayan, V. et al. 1999), induction of specification genes Meox1, Meox2, and Pax3, and counteracts inhibitory signals such as BMP4 (Kennedy, K.A., Porter, T. et al. 2009). RA signaling intersects with that of BMP4 as BMP4 and RA function antagonistically and have the capacity to counteract each other's inhibition of entry into skeletal and cardiac muscle lineages (Kennedy, K.A., Porter, T. et al. 2009). Low concentrations of RA can regulate the levels of Myf5 implying the existence of a RARE in the Myf5 regulatory region (Carnac, G., Albagli-Curiel, O. et al. 1993). RA also enhances MyoD and myogenin expression (Carnac, G., Albagli-Curiel, O. et al. 1993) and RA receptors and MyoD have been found to upregulate each others transcriptional activity; their transcriptional co-activation requires a RA receptor-MyoD complex that binds to MyoD DNA binding sites in muscle cells (Froeschle, A., Alric, S. et al. 1998). RA is capable of inhibiting proliferation of myoblasts through inducing cell cycle arrest proteins (Alric, S., Froeschle, A. et al. 1998) and in Vitamin A deficient embryos, myogenin is downregulated (Maden, M., Graham, A. et al. 2000) providing a link between RA and myoblast maturation.

Roles of Acetyltransferases in Myogenic Induction

Not only are extracellular signals crucial for proper induction of myogenic regulatory factors but intracellular prompts involving acetyletransferases play a fundamental role as well. CBP and p300 are required for growth arrest and apoptosis (Vo, N. and Goodman, R.H. 2001) and along with PCAF are required for terminal differentiation of myoblasts and transactivation of muscle specific promoters such as myosin heavy chain (MHC) and muscle creatine kinase (MCK) (Eckner, R., Yao, T.P. et al. 1996; Polesskaya, A., Naguibneva, I. et al. 2001; Puri, P.L., Avantaggiati, M.L. et al. 1997; Puri, P.L., Sartorelli, V. et al. 1997; Yuan, W., Condorelli, G. et al. 1996). ES cells lacking p300 or its acetyltransferase (AT) activity are strongly impaired in their ability to activate Myf5 and MyoD (Roth, J.F., Shikama, N. et al. 2003). When properly expressed, Myf5 and MyoD, in cooperation with MEF2 transcription factors and with p300 and CBP, mediate the activation of the secondary MRFs, myogenin and Mrf4. p300 has been shown to bind directly to MyoD (Sartorelli, V., Huang, J. et al. 1997; Yuan, W., Condorelli, G. et al. 1996) and p300 and PCAF play a critical role in the maximal MyoD dependant transactivation; p300 acetylates histones H3 and H4 and recruits PCAF to the promoter whereas PCAF acetylates MyoD to enhance transcription initiation, increase its affinity for DNA, and facilitate heterodimer formation with E proteins (Dilworth, F.J., Seaver, K.J. et al. 2004; Puri, P.L., Avantaggiati, M.L. et al. 1997; Sartorelli, V., Puri, P.L. et al. 1999). However, MyoD has also been found to be acetylated in proliferating myoblasts where it is inactive therefore further mechanisms besides simply acetylation are required for MyoD activation (Polesskaya, A., Duquet, A. et al. 2000).

Model systems used to investigate RXR signaling in myogenesis

The focus of the current research project is to determine how the RXRs are involved in skeletal muscle development (Figure 5). To study these effects, several embryonic stem cell lines are available that mimic embryonic development.

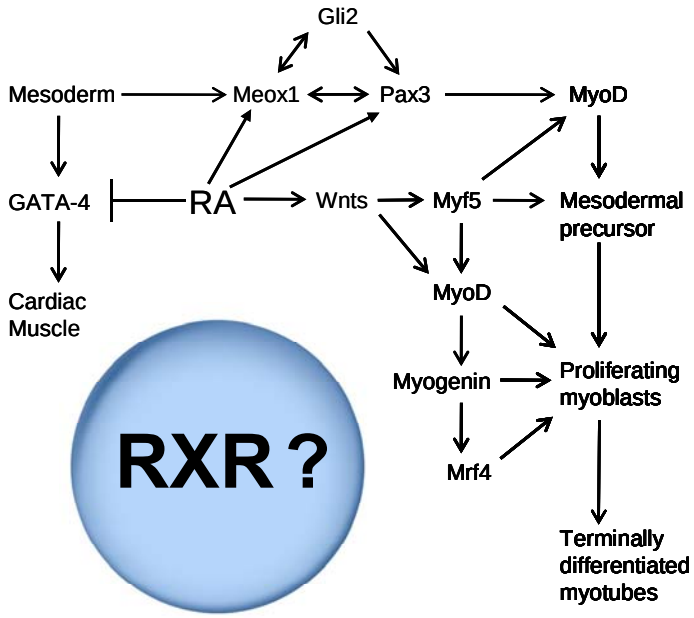

Figure 5. Involvement of RXR in Skeletal Myogenesis. Retinoic acid can induce myogenesis in pluripotent stem cells through the regulation of many genes involved in specification and commitment to the myogenic lineage. The relative contribution of RXR on specification and commitment genes for skeletal muscle has not yet been elucidated.

Embryonal carcinoma (EC) cells are the pluripotent stem cells of carcinomas (Campione-Piccardo, J., Sun, J.J. et al. 1985). The P19 EC cell line was derived from a teratocarcinoma induced in C3H/HC mice (McBurney, M.W., Jones-Villeneuve, E.M. et al. 1982) and have a stable euploid male karyotype (40:XY) (van der Heyden, M.A. and

Defize, L.H. 2003). When grown as undifferentiated monolayers, P19 cells express stem cell specific markers such as SSEA-1 (Mummery, C.L., van den Brink, C.E. et al. 1987) and Oct3 (Bain, G. and Gottlieb, D.I. 1994).

P19 cells can be induced to differentiate when exposed to small molecule inducers such as RA or dimethylsulfoxide (DMSO) (Campione-Piccardo, J., Sun, J.J. et al. 1985; Jones-Villeneuve, E.M., McBurney, M.W. et al. 1982) and can form derivatives of all three germ layers (van der Heyden, M.A. and Defize, L.H. 2003) which recapitulate the properties of the developing embryo (Skerjanc, I.S. 1999). If the cells are differentiated as monolayers, neuronal lineages are induced. On the other hand, the ability of P19 cells to undergo myogenesis is strongly dependant on unknown factors in the serum (Wilton, S. and Skerjanc, I. 1999) and the prior formation of non-adhering aggregates or embryoid bodies which resembles the inner cell mass of the embryo (Martin, G.R. and Evans, M.J. 1974; Martin, G.R. and Evans, M.J. 1975).

A key factor affecting differentiation is the concentration of RA the cells are exposed to. Cells exposed to high RA concentrations ($>10^{-7}$ M) develop into neurons and astrocytes whereas cells grown in lower concentrations of RA (10^{-9}-10^{-7} M) develop large amounts of striated muscle (Jones-Villeneuve, E.M., McBurney, M.W. et al. 1982; Jones-Villeneuve, E.M., Rudnicki, M.A. et al. 1983). RA enhances skeletal myogenesis in both P19 and ES cells by increasing the expression of Wnt3a, Pax3 and Meox1 through binding to RARs in the regulatory regions of these genes (Campione-Piccardo, J., Sun, J.J. et al. 1985; Kennedy, K.A., Porter, T. et al. 2009). Finally, another key factor in the effectiveness of differentiation is the cell density and cellular interactions between neighboring cells (Campione-Piccardo, J., Sun, J.J. et al. 1985).

Mouse ES cells were originally derived from the inner cell mass (ICM) of mouse blastocysts (Evans, M.J. and Kaufman, M.H. 1981) and later from cleavage stage embryos and individual blastomeres of two to eight cell stage embryos (Chung, Y., Klimanskaya, I. et al. 2006; Wakayama, S., Hikichi, T. et al. 2007). Like P19 EC cells, mouse ES cells are karyotypically normal (Bradley, A., Evans, M. et al. 1984). Stem cell specific markers are slightly different than P19 cells and include Oct 3/4, Nanog, Sox2, and fibroblast growth factor four (FGF4) (Boiani, M. and Scholer, H.R. 2005). Unlike P19 cells which require the presence of an inducer to differentiate, ES cells require the presence of Leukemia Inhibitory Factor (LIF) to maintain stem cell character and will otherwise spontaneously differentiate (Rohwedel, J., Guan, K. et al. 1999). Another major difference between EC and ES cells, is that ES cells do not contain the apparatus to transport retinol into the cytoplasm, and even though retinol can be transported into the cell via passive diffusion (Kawaguchi, R., Yu, J. et al. 2007), these cells do not express retinol metabolizing enzymes and cannot convert retinol into RA (Chen, L. and Khillan, J.S. 2010). That being the case, RA does influence, in a time and concentration dependant manor, the efficiency and pattern of differentiation.

P19 cells provide important advantages over mouse ES cells for several reasons: 1) they are susceptible to incorporation and expression of ectopic genes, 2) they have easy cell culture conditions and differentiate in large quantities (Hescheler, J., Fleischmann, B.K. et al. 1997) and 3) they remain undifferentiated until induced without the requirement of a feeder layer of cells or LIF, which has no parallel in its in vivo counterpart (Yu, J. and Thomson, J.A. 2008). However one disadvantage of P19 cells is

that they fail to differentiate into germ cells in vivo unlike ES cells which can generate genetically altered mice (Thomas, K.R. and Capecchi, M.R. 1987).

A P19 mutant cell line useful in the study of RA signaling is RAC65 cells. These cells do not respond to ATRA due to a dominant negative mutation in RAR-α. The RAR-α protein is truncated owing to a mutation at codon 392 (Figure 6) which results in an early translation termination signal. The resultant protein is 391 amino acids, and is missing 70 C-terminal amino acids (Pratt, M.A., Kralova, J. et al. 1990) meaning it lacks the ligand binding region, part of the AF-2 domain and part of the dimerization domain (Costa, S.L. and McBurney, M.W. 1996). The protein is still capable of heterodimerizing with RXRs and is able to bind to RAREs since the DNA-binding domain is intact, however, since the deletion affects the ligand binding domain, the receptor is unable to bind its ligand and is therefore unsusceptible to the effects of RA. Since the mutant receptor has a higher affinity for the RARE than the normal receptors, it effectively blocks access of the RARE to any normal receptor complexes and abrogates the RA response in these cells (Costa, S.L. and McBurney, M.W. 1996). This cell line represents a way to differentiate cells cultures in the presence of an RXR selective ligand so that any residual or background RAR effects that might contribute to skeletal muscle induction in P19 cells is eliminated.

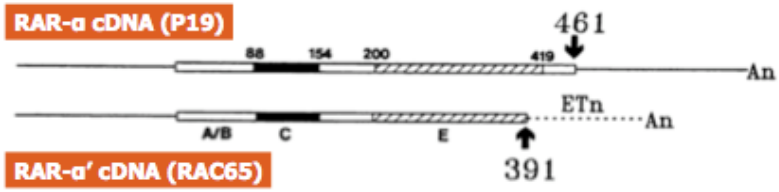

Figure 6. Schematic Diagram of RAR-α' cDNA. Letters indicate the functional receptor domains: A/B, a putative transcriptional activation region; C, the DNA-binding zinc finger region; E, the RA-binding domain. The boundaries of the domains are indicated by amino acid residue numbers. RAR-α encodes a 461-amino-acid protein, while RAR-α' terminates at amino acid 391 and is followed by an ETn-derived sequence. RAC65 cell mutation is a dominant negative mutation rendering them non-response to the effects of RA (Pratt, M.A., Kralova, J. et al. 1990).

Another cell line useful in dissecting out the molecular pathway of RXR signaling is the β-catenin-engrailed (β-Cat/EnR) cell line. β-catenin is important for cadherin dependant cell-cell adhesion, through interactions with α-catenin and cadherin proteins, and signaling via the Wnt pathway. To selectively block signaling mediated by β-catenin, the β-catenin-engrailed cell line has a chimeric fusion protein in which the C-terminal transactivation domain is replaced with the active repression domain of Drosophila Engrailed (Jaynes, J.B. and O'Farrell, P.H. 1991; Smith, S.T. and Jaynes, J.B. 1996) (Figure 7). The engrailed repression domain (EN-2) silences transcription through interactions with members of the Groucho/TLE family of transcriptional repressors (Chen, G. and Courey, A.J. 2000). The N-terminal domain, which binds α-catenin, and Armadillo domain responsible for cadherin association and which binds Lef/Tcf transcription factors harboring nuclear localizing sequences are left intact (Huber, O., Korn, R. et al. 1996). Since Wnt signaling is known to activate Myf5 directly through β-

catenin signaling, this cell line provides a means to determine if RXR signaling can bypass this block in Wnt signaling.

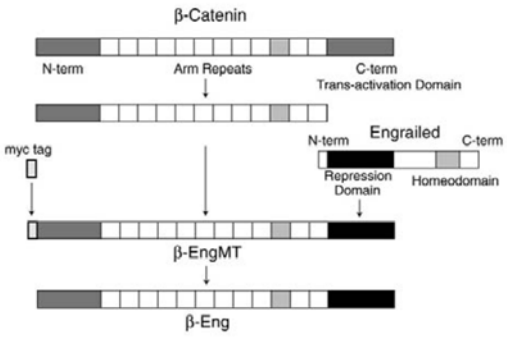

Figure 7. Construction of the β-Eng Chimera. The C-terminal transactivation domain of a stabilized Xenopus β-catenin (*, S→A, T→A mutations in the N-terminal domain) was replaced with the N-terminal repressor domain of Drosophila Engrailed (amino acids 4-300) using PCR-based subcloning techniques (β-Eng). To facilitate biochemical studies, six myc epitope tags were added to the N-terminus (β-EngMT) (Montross, W.T., Ji, H. et al. 2000).

Similar to the β-catenin engrailed mutant cells, Pax3 and Meox1 dominant negative P19 mutant cell lines are valuable in determining RXR target genes in the myogenic signaling cascade. Dominant negative Pax3 cells have the 198-amino acid N-terminal active repression domain of EN-2 fused at the first 286 amino acids of Pax3 in place of the Pax3 C-terminus activation domain which contains the DNA binding domain (Figure 8) (Ridgeway, A.G. and Skerjanc, I.S. 2001; Smith, S.T. and Jaynes, J.B. 1996; Tolkunova, E.N., Fujioka, M. et al. 1998). Meox1 mutant cells have the same repression domain fused to the C-terminus of Meox1, however, the activation domain of Meox1 is unknown and is thus unaltered (Petropoulos, H., Gianakopoulos, P.J. et al. 2004).

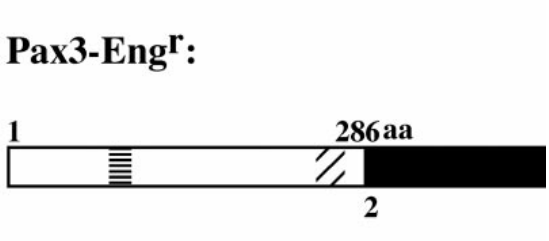

Figure 8. A Dominant Negative Pax3 Inhibits Endogenous Skeletal Myogenesis in P19 Cells. A Pax3 dominant negative protein was engineered by fusing the EN-2 repression domain (Engr), which includes amino acids (aa) 2–198 (black box) to the DNA binding domain of Pax3 (open box), and amino acids 1–286, which contains the paired domain (horizontal stripes) and the homeodomain (diagonal stripes) (Ridgeway, A.G. and Skerjanc, I.S. 2001).

Hypothesis

RA is a small molecule inducer with the capacity to enhance skeletal myogenesis in pluripotent stem cells however, thus far, it has yielded disappointing results in ES cells. Considering the potential stem cells hold for the treatment of disease and the shortcomings of RA to direct them to the myogenic lineage, it is imperative to uncover a superior inducer for this purpose. In view of the fact that RXR plays a vital role in embryonic development, we have tried selectively activating these receptors to study their contribution to skeletal myogenesis. **We hypothesize that RXR specific signaling plays an important role in the development of skeletal muscle.** We have investigated the role of rexinoids in myogenic specification and determination of pluripotent stem cells.

CHAPTER 2: MANUSCRIPT

CONTRIBUTION OF RETINOID X RECEPTOR SIGNALING TO THE SPECIFICATION OF SKELETAL MUSCLE LINEAGE

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Running title: Retinoid X receptor and skeletal myogenesis

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Pluripotent stem cells possess a tremendous potential for the treatment of many diseases because of their capacity to differentiate into a variety of cell lineages. However, they provide little promise for muscle related diseases mainly due to the lack of small molecule inducers to efficiently direct myogenesis. Retinoic acid (RA) signaling through retinoic acid receptor (RAR) and retinoid X receptor (RXR), affects stem cell fate determination in a concentration-dependent manner, but it only has modest effect on the differentiation of embryonic stem (ES) cells into myogenic lineage. RXR is important for embryonic development but generally considered to act as a silent partner of RAR in a non-permissive mode. In this study, we have examined whether the activation of RXR by rexinoid or RXR specific signaling plays a role in the commitment of stem cells into myogenic lineage. Our findings establish that mouse ES cells can effectively generate skeletal myocytes following rexinoid induction at the early stage of differentiation, and on a molecular level, rexinoid-enhanced myogenesis simulates the sequential events observed *in vivo*. Moreover, rexinoid-induced skeletal myogenic conversion requires the function of β-catenin but not RAR. Our studies establish the feasibility of applying RXR agonist in cell based therapies for muscle related diseases. The aptitude of ES cells to generate skeletal myocytes upon rexinoid induction also provides a model system to study the convergence of different signaling pathways in myogenesis.

Skeletal muscle development is a highly ordered process orchestrated by multiple myogenic regulatory factors (MRFs) including Myf5, MyoD, myogenin and Mrf4 (1). While Myf5 and MyoD initiate the transcription of muscle-specific genes and direct the cells to skeletal muscle lineage, myogenin and Mrf4 mainly regulate later stages of development such as fusion of myoblasts to myotubes (1,2). The MRFs are temporally expressed and although they regulate each other, (3) they require additional signaling factors to be induced. Early specification factors such as Meox1 and Pax3 subsequently induce the MRFs (4). Pax3 directly regulates the expression of Myf5 through the limb bud enhancer of Myf5, (5) and is also responsible for proper expression of MyoD (6). Additionally, Wnt signaling via β-catenin is essential and sufficient for the induction skeletal myogenesis and expression of the MRFs (4).

The temporal expression pattern of MRFs in embryonic stem (ES) cells reflects the sequential events observed during skeletal myogenesis *in vivo* (7). Similar to ES cells, the pluripotent embryonic carcinoma (EC) cells respond well to developmental cues *in vitro* to differentiate into cell types of all three germ layers (8). The differentiation of EC stem cells simulates the molecular and cellular processes which occur during ES cell differentiation and early embryonic development (9). Pluripotent stem cells are promising resources for cell based therapies, but have proved difficult to apply in muscle related diseases mainly due to the lack of small molecule inducers to effectively trigger skeletal myogenesis (10).

Retinoic acid (RA) is essential for a broad array of biological processes including

vertebrate embryonic body shaping, tissue homeostasis, apoptosis, and stem cell differentiation (11,12). High concentrations of RA (>10^{-7} M) enhance neuronal differentiation but inhibit myogenesis, whereas low concentrations (<10^{-7} M) enhance myogenic conversion of ES and EC cells (13-15). The diverse effects of RA are primarily mediated through retinoic acid receptors (RAR) which act as ligand-inducible transcription factors to regulate RA-responsive genes (16). The function of RAR depends on dimerization with retinoid X receptors (RXR). RAR/RXR heterodimers bind to specific DNA sequences within the genes they govern and upon ligand induction, recruit coactivator p300 complex and activate gene transcription (17,18). RAR/RXR heterodimers can bind to a number of different sequences including a DR5, which is two direct repeats of the consensus sequence 5'-PuGGTCA separated by 5 nucleotides (19,20).

In addition to RAR, RXR can dimerize with one third of the 48 known nuclear receptors (21). In permissive heterodimers or homodimers, RXR is amenable to ligand activation (22). Conversely, RAR/RXR heterodimers are non-permissive, thus ligand induction is through RAR, while RXR is generally considered a silent partner (23).

Although RA is the best characterized inducer, it only has modest efficacy on ES cells. Thus, it is imperative to comprehend on a molecular level how different signaling pathways converge to regulate the specification of muscle lineage in order to find inducers which can generate large quantities of skeletal myocytes. Bexarotene, is a RXR selective agonist that is unable to transactivate the RAR-RXR heterodimer (24) and will not bind RAR (25). Due to the vital role of RXR in early embryonic development (26) we have examined the mechanisms of rexinoid induced signaling-dependent events during myogenic conversion. Our studies have determined a role for RXR specific signaling in this process and identified RXR agonist as an effective inducer for the differentiation of ES cells into skeletal myocytes.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagent - All-trans retinoic acid was purchased from Sigma-Aldrich, LGD1069 from LC Laboratories, and Ro 41-5253 from Biomol International. P19 cells (ATCC) were grown in Minimum Essential Medium α (α-MEM) (Gibco-Invitrogen) supplemented with 5% fetal bovine serum (PAA), 5% bovine calf serum (PAA), and 1% Penicillin/Streptomycin. Cells were aggregated and treated. After 4 days aggregation, cells were transferred to tissue culture dishes or coverslips coated with 0.1% gelatin and grown for a further 5 days. D3 ES cells (ATCC) were grown in Dulbecco's Modified Eagle Medium (D-MEM) (Gibco-Invitrogen) supplemented with 15% fetal bovine serum (PAA), 1% Penicillin/Streptomycin, 1% non essential amino acids (Gibco-Invitrogen), and 1.18 mM β-mercaptoethanol (OmniPur). Undifferentiated cultures were supplemented with 1000 units/ml of Leukemia Inhibitory Factor (LIF) (Chemicon). For differentiation, cells were cultivated in hanging drops for 48 hours after which they were washed into Petrie dishes, treated, and grown for a further 5 days in suspension. Cells were then transferred to tissue culture dishes, coverslips coated with 0.1% gelatin, or harvested for Real-Time Q-PCR and Western Blotting analysis. D3 terminal differentiation medium was D-MEM F12 (Gibco-Invitrogen) supplemented with 1% N2 (Invitrogen) and 1% Penicillin/Streptomycin. Cells were again collected on day 12 and stained on day 19.

Immunofluorescence Microscopy- Cells were fixed on coverslips and incubated with primary antibodies overnight at 4°C. Subsequently, they were incubated with fluorescent secondary antibodies followed by a 3 minute incubation with 25 ng/ml of Hoechst DNA stain (Molecular Probes). Microscopy analysis was performed with the Zeiss Axiovert 200 M. Image acquisition was carried out with the AxioCam HRm monochrome camera.. Images captured through fluorescence filters (488 and 594) were processed and merged by the Zeiss

AxioVision Rel 4.6 software. Myogenic conversion was quantified as the percentage of cells staining positive for skeletal markers out of total population of cells. Primary antibodies used were anti-Myosin Heavy Chain MF20 (lab grown) and anti-MyoD (Santa Cruz). Secondary antibodies used were Alexa Flor®488 goat anti-mouse, Alexa Flor®488 goat anti-rabbit and Alexa Flor®594 donkey anti-mouse (Invtrogen).

Chromatin Immunoprecipitation (ChIP)- P19 cell aggregates were fixed on day 4, sonicated and immunoprecipitated as previously described (27). An input sample of 5% total chromatin was set aside. For immunoprecipitation, p300, RXR-α, RAR-α (Santa Cruz Biotechnology) or β-catenin (Millipore) antibody was added. IgG antiserum (Zymed Laboratories, CA) was used as a control for negative ChIP. DNA was purified using Omega Bio-tek Cycle Pure Kit and samples were analyzed using SYBR Green Real-Time Q-PCR. Primer pairs used for amplification were described previously (18).

Western Blotting- Cell pellets were lysed by incubation in whole cell extract buffer (10 % glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 % NP-40) for 30 minutes in ice. Protein concentrations were quantified by Bradford Method (Bio-Rad), using Multiscan Spectrum photospectrometer and the Multiscan software (Thermo Scientific). Protein was separated on sodium dodecyl sulphate polyacrylamide gel and transferred to Immun-Blot PVDF membrane (Bio-Rad). Membranes were incubated overnight in primary antibody followed by incubation with secondary and visualized using Westen Lightning Chemiluminescence (Perkin Elmer) reagents. Quantification was performed using Scion Image (Scion Corporation). Primary antibodies used were: anti-p300 (Santa Cruz), anti-RXR-α (Santa Cruz), anti-RAR-α (Santa Cruz), anti-myogenin (F5D hybridoma cell line), and anti-β-tubulin (E7 hybridoma cell line).

Real-Time RT-PCR Analysis- Total RNA was isolated according to the manufacture's protocol, using Total RNA Kit I (Omega) for P19 cells and RNeasy Mini kit (Qiagen) for D3 ES cells. RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-Time Q-PCR was performed using SYBR Green and ROX with HotStarTaq DNA Polymerase kit (Qiagen) and conducted on the Applied Biosystems 7500 Fast Real-Time PCR System. The amount of targets, normalized to the GAPDH endogenous reference and relative to calibrator control was calculated using the arithmetic formula $2^{-\Delta\Delta CT}$.

Cell Transfection and Luciferase Assay- Transient transfections were performed with reporter plasmid by using ExGen 500 as previously described (28). Briefly, P19 aggregates were transfected with a RAR luciferase and a RSV-β-Gal, reporter, and then induced with bexarotene or RA. Luciferase assay was performed according to the manufacturer's recommendation (Promega). The luciferase activities are expressed as fold induction relative to the untreated controls after being normalized to the β-galactosidase activity.

RESULTS

Effects of Rexinoid on Myogenic Conversion of Pluripotent P19 Cells. Pluripotent P19 cells are an excellent system to study cellular differentiation and to screen small molecule inducers for the specification of muscle lineage (8). In aggregated cultures, P19 cells can be induced to differentiate, however, in the absence of exogenous stimuli aggregation results only in the expression of markers of mesoderm but not skeletal myoblasts (29)**.** Treatment of the aggregates with DMSO induces small percentages of myoblasts while the addition of all-trans RA enhances the commitment of myogenic lineage (30). As shown in figure 1A, DMSO induced about 5% of skeletal myocytes by day 9. In contrast, co-treatment with RA enhanced myogenic conversion to about 15%, as determined by microscopic analysis of myosin heavy chain and MyoD staining (Fig. 1A). In addition, MyoD and myosin heavy chain co-

stained to the elongated bipolar myocytes while myogenin protein was also detected by day 9, which is indicative of skeletal myocyte identity (Fig. 1B-C). Thus, RA signaling is important for the specification and development of skeletal muscle lineage.

Since RXR is essential for early stages of embryonic development (26,31,32), we used bexarotene, a RXR selective agonist to determine the impact of RXR signaling on skeletal myogenesis. In the presence of DMSO, bexarotene enhanced myogenic conversion in a concentration dependent manner but with comparable efficacy as RA (Fig. 2A and B). MyoD and myogenin were also expressed at similar levels (Fig. 2B-D). Consistent with the literature (30), Meox1 and Pax3 transcripts were increased by RA on day 4 while MyoD augmented on day 9 (Fig. 2E). Intriguingly, bexarotene caused a greater increase in the Meox1 transcripts level than RA, whereas RA caused a larger increase in the Pax3 transcript level than bexarotene (Fig. 2E). The levels of MyoD transcripts appeared to reflect the efficacies of myogenic conversion (Fig. 2E). Thus the temporal expression pattern of myogenic regulatory factors induced by bexarotene and RA in the P19 model is similar to that seen *in vivo* and demonstrate that RXR agonist is an effective inducer of skeletal myogenesis.

Effects of Rexinoid on the Differentiation of ES Cells into Skeletal Myocytes. Since ES cells have proved largely unresponsive to RA induced skeletal myogenesis, we next tried rexinoid in this cell system. A hanging-drop method was used to form the embryoid bodies (EB) for ES cell differentiation and DMSO was omitted from the protocol because it is toxic to the cells. Different concentrations of bexarotene were used to treat the EBs and immunofluorescence microscopy was employed to examine the development of myoblasts. Consistent with the literature, RA alone had modest efficacy, about 3%, at converting ES cells into skeletal myocytes, (Fig. 3A). However, bexarotene was 5-fold more potent than RA and significantly increased the specification to myogenic lineage to about 16% (Fig. 3A-B). In addition, bexarotene was also more effective at inducing the expression of myogenin protein (Fig. 3C). As with the P19 cells, bexarotene increased Meox1 transcripts about 11-fold more than RA, whereas RA was more efficient at augmenting Pax3 transcripts (Fig. 3D). Again the levels of MyoD transcripts appeared to reflect the efficacies of myogenic conversion (Fig. 3D). These findings demonstrate that RXR agonist is a more effective inducer than RA to direct the differentiation of ES cells into skeletal myocytes.

Roles of β-Catenin, Meox1 and Pax3 in Rexinoid-Enhanced Skeletal Myogenesis. To delineate the molecular mechanisms of rexinoid action, we used P19 cells to take the advantage of several established stable lines. RA-enhanced skeletal myogenesis requires the function of β-catenin (30). To determine the role of β-catenin in rexinoid-enhanced myogenic conversion, we employed a clone of cells stably expressing a dominant negative β-catenin in which the transcriptional activation domain is replaced by an engrailed repressor domain (30). The engrailed repression domain (EN-2) silences transcription through interactions with members of the Groucho/TLE family of transcriptional repressors (3). Cells harbouring the empty vector were used as a control. As shown in figure 4A, the control cells were converted into skeletal myocytes with comparable efficacy as the parental cells (compare to Fig. 2A). However, the dominant negative β-catenin cells failed to differentiate into skeletal myocytes regardless of treatments and myogenin protein was not detected (Fig. 4A and B). Thus, bexarotene, just as RA, cannot bypass β-catenin to enhanced skeletal myogenesis, and the function of RXR in myogenesis depends on β-catenin.

Next, we used the Meox1 and Pax3 dominant negative cells (33,34) to study the roles of Meox1 and Pax3 in rexinoid-enhanced myogenesis. As shown in figure 4C, DMSO did not convert these cells into myocytes, consistent with previous reports (33,34). However, both bexarotene and RA restored the differentiation of these cells into skeletal

myocytes, albeit with lower efficacies (Fig. 4C). Interestingly, while Pax/EnR transcript levels were comparable (Figure 4D), stably expressing dominant negative Pax3 appeared to be less detrimental to RA-induced myogenic conversion (Fig. 4C), possibly due to RA augmenting the endogenous Pax3 expression more effectively (Fig. 2E) to titrate out the interference of dominant negative Pax3 protein. Together, our studies suggest that rexinoid enhances skeletal myogenesis through signaling pathways non-overlapping with that of RAR.

RAR-Independent RXR Signaling during Skeletal Myogenesis. Previous study has identified a DR5 binding site at the Pax3 locus (30). This site is a consensus binding motif for RAR-RXR heterodimers. We thus examined the occupancy of this locus by RXR following bexarotene induction. Chromatin immunoprecipitation (ChIP) analysis demonstrated that both RAR and RXR bound to this segment constitutively (Fig. 5A and B), in agreement with current model (27). However, the recruitment of transcriptional coactivator p300 at this region was only augmented by the addition of RA but not bexarotene, although the levels of p300 protein in the cells were consistent (Fig. 5C-D). Thus, RXR possibly acts as a silent partner of RAR at this region to upregulate Pax3 gene expression. To validate the specificity of our ChIP protocol, we also performed a ChIP analysis with a β-catenin antibody and did not detect apparent β-catenin binding to this region regardless of treatment as compared to IgG negative ChIP control (n=4).

Next, we used a reporter approach to examine the effects of rexinoid on transactivation requiring RXR acting as a silent partner of RAR. The cells were transfected with a RAR-RXR reporter (35) during aggregation and then induced with bexarotene or RA. As shown in figure 5E, RA, but not bexarotene, was able to transactivate the reporter. Taken together, our data demonstrates that rexinoid had no impact on a binding motif for RAR-RXR heterodimer, suggesting an additional role for RXR in the specification of skeletal myogenic lineage, acting through an activated or permissive mode.

To further delineate if RAR is required for rexinoid-enhanced skeletal myogenesis, we used RAC65 cells which contain a dominant negative RAR-α that effectively blocks DNA binding of normal receptors (36,37). These cells are non-responsive to RAR agonist, but respond to RXR agonist for neuronal differentiation (38,39). As shown in figure 5F, bexarotene, but not RA, enhanced the specification of muscle lineage in the RAC65 cells. We also used a RAR specific antagonist, Ro 41-5253, which selectively inhibits RAR-α signaling at low concentrations (40) to examine the role of RAR in rexinoid-enhanced myogenic conversion. As shown in figure 5G, the RAR antagonist inhibited RA-mediated myogenic conversion, whereas bexarotene-enhanced myogenesis was not compromised. Thus RXR mediates skeletal myogenesis through mechanisms independent of RAR.

DISCUSSION

We have examined whether the activation of RXR by rexinoid or RXR specific signaling plays a role in skeletal myogenesis. Our findings show that mouse ES cells can effectively generate skeletal myocytes following induction with RXR agonist at the early stage of differentiation and the molecular pathways during rexinoid-enhanced myogenesis recapitulate the sequential events observed *in vivo*. In addition, rexinoid-enhanced lineage specification is mediated through a RAR-independent mechanism. Our studies establish the feasibilities of applying rexinoid in stem cell therapies, particularly exploring RXR specific signaling to convert ES cells into myogenic lineage. The aptitude of ES cell to generate skeletal myocytes in response to rexinoid also offers a model system to delineate the complex signaling pathways involved in skeletal muscle development and to develop non-toxic protocols for generating large quantities of myocytes.

Pluripotent stem cells regardless their origin, possess the potential of developing into

skeletal myocytes among many other cell lineages (41). The central issue is how to control a specific signaling pathway to exclusively enhance myogenic conversion in an efficacy suitable for clinic therapies. RA is able to enhance skeletal myogenesis in pluripotent EC stem cells if used in combination with other small molecular inducers such as DMSO (Fig. 1). However, DMSO is toxic to ES cells and RA alone has only modest effect on the differentiation of ES cells into myogenic lineage (Fig. 3). We found that rexinoid is a more effective inducer than RA for skeletal myogenesis in the ES cell system (Fig. 3). This is significant, because to date there has been very little success at directing ES cells into skeletal muscle lineage, and thus no methods are currently available to generate sufficient population of skeletal myocytes for potential cell based therapies (42).

In addition, our studies also shed new light on the role of RXR in skeletal myogenesis. It is well known that RXR is important for development but it is generally considered to act as the silent partner of RAR in a non-permissive mode (12,26,31,32). Our studies establish a role of RXR specific signaling in the specification of skeletal myogenesis, which is independent of RAR and non-overlapping with the RAR-RXR heterodimer mode (Fig. 5). Alternatively, while certain specification events differ between rexinoid and RA-enhanced skeletal myogenesis, both inducers critically depend on the function of β-catenin (Fig. 4).

It appears that rexinoid has a significant impact on an early differentiation marker, Meox1, whereas RA may require DMSO co-treatment to activate Meox1 (Fig. 2-3). Our studies have not addressed other specific genomic targets activated by RXR specific signaling besides known MRFs. Additional systems studies are needed to determine the complex signaling pathways involved in regulating rexinoid-enhanced skeletal myogenesis and to address why RXR agonist is a more suitable inducer for the ES cell system. Understanding the molecular mechanisms of myogenic specification is essential for manipulating stem cell fate decision in cell based therapies. We have identified a potent small molecule inducer for a nontoxic protocol to direct the specification and commitment of skeletal muscle lineage. The efficacy with which rexinoid is able to convert ES cells into myogenic lineage suggests a potential to extend this strategy to human ES cells and other classes of pluripotent stem cells in view of generating functional skeletal myocytes.

REFERENCES

1. Tapscott, S. J. (2005) *Development* **132**, 2685-2695
2. Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B., and Miller, A. D. (1989) *Proc Natl Acad Sci U S A* **86**, 5434-5438
3. Chen, G., and Courey, A. J. (2000) *Gene* **249**, 1-16
4. Petropoulos, H., and Skerjanc, I. S. (2002) *J Biol Chem* **277**, 15393-15399
5. Bajard, L., Relaix, F., Lagha, M., Rocancourt, D., Daubas, P., and Buckingham, M. E. (2006) *Genes Dev* **20**, 2450-2464
6. Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997) *Cell* **89**, 127-138

7. Rohwedel, J., Maltsev, V., Bober, E., Arnold, H. H., Hescheler, J., and Wobus, A. M. (1994) *Dev Biol* **164**, 87-101
8. Yu, J., and Thomson, J. A. (2008) *Genes Dev* **22**, 1987-1997
9. Rudnicki, M. A., Reuhl, K. R., and McBurney, M. W. (1989) *Development* **107**, 361-372
10. Allsopp, T. E., Bunnage, M. E. and Fish, P. V. (2010) *Med. Chem. Commun* **1**, 16-29
11. Berbis, P. (2010) *Ann Dermatol Venereol* **137 Suppl 3**, S97-103
12. Mark, M., Ghyselinck, N. B., and Chambon, P. (2009) *Nucl Recept Signal* **7**, e002
13. Jones-Villeneuve, E. M., McBurney, M. W., Rogers, K. A., and Kalnins, V. I. (1982) *J Cell Biol* **94**, 253-262
14. Sato, Y., and Heuckeroth, R. O. (2008) *Dev Biol* **320**, 185-198
15. Hescheler, J., Fleischmann, B. K., Lentini, S., Maltsev, V. A., Rohwedel, J., Wobus, A. M., and Addicks, K. (1997) *Cardiovasc Res* **36**, 149-162
16. Niederreither, K., and Dolle, P. (2008) *Nat Rev Genet* **9**, 541-553
17. Lonard, D. M., and O'Malley B, W. (2007) *Mol Cell* **27**, 691-700
18. Hermanson, O., Glass, C. K., and Rosenfeld, M. G. (2002) *Trends Endocrinol Metab* **13**, 55-60
19. Tanaka, T., and De Luca, L. M. (2009) *Cancer Res* **69**, 4945-4947
20. Umesono, K., and Evans, R. M. (1989) *Cell* **57**, 1139-1146
21. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83**, 835-839
22. Gampe, R. T., Jr., Montana, V. G., Lambert, M. H., Wisely, G. B., Milburn, M. V., and Xu, H. E. (2000) *Genes Dev* **14**, 2229-2241
23. Kurokawa, R., DiRenzo, J., Boehm, M., Sugarman, J., Gloss, B., Rosenfeld, M. G., Heyman, R. A., and Glass, C. K. (1994) *Nature* **371**, 528-531
24. Lehmann, J. M., Jong, L., Fanjul, A., Cameron, J. F., Lu, X. P., Haefner, P., Dawson, M. I., and Pfahl, M. (1992) *Science* **258**, 1944-1946
25. Rigas, J. R., and Dragnev, K. H. (2005) *Oncologist* **10**, 22-33
26. Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dolle, P., and Chambon, P. (1994) *Cell* **78**, 987-1003
27. Higazi, A., Abed, M., Chen, J., and Li, Q. (2011) *Epigenetics* **6**, 202-211
28. Chen, J., Halappanavar, S. S., St-Germain, J. R., Tsang, B. K., and Li, Q. (2004) *Cell Mol Life Sci* **61**, 1675-1683
29. McBurney, M. W. (1993) *Int J Dev Biol* **37**, 135-140
30. Kennedy, K. A., Porter, T., Mehta, V., Ryan, S. D., Price, F., Peshdary, V., Karamboulas, C., Savage, J., Drysdale, T. A., Li, S. C., Bennett, S. A., and Skerjanc, I. S. (2009) *BMC Biol* **7**, 67
31. Sapin, V., Dolle, P., Hindelang, C., Kastner, P., and Chambon, P. (1997) *Dev Biol* **191**, 29-41
32. Sucov, H. M., Dyson, E., Gumeringer, C. L., Price, J., Chien, K. R., and Evans, R. M. (1994) *Genes Dev* **8**, 1007-1018
33. Petropoulos, H., Gianakopoulos, P. J., Ridgeway, A. G., and Skerjanc, I. S. (2004) *J Biol Chem* **279**, 23874-23881
34. Ridgeway, A. G., and Skerjanc, I. S. (2001) *J Biol Chem* **276**, 19033-19039

35. Chen, J., St-Germain, J. R., and Li, Q. (2005) *Mol Cell Biol* **25**, 525-532
36. Pratt, M. A., Kralova, J., and McBurney, M. W. (1990) *Mol Cell Biol* **10**, 6445-6453
37. Costa, S. L., and McBurney, M. W. (1996) *Exp Cell Res* **225**, 35-43
38. Jones-Villeneuve, E. M., Rudnicki, M. A., Harris, J. F., and McBurney, M. W. (1983) *Mol Cell Biol* **3**, 2271-2279
39. Yokota, Y., and Ohkubo, H. (1996) *Exp Cell Res* **228**, 1-7
40. Wang, X. J., Hayes, J. D., Henderson, C. J., and Wolf, C. R. (2007) *Proc Natl Acad Sci U S A* **104**, 19589-19594
41. Hirst, C. E., Ng, E. S., Azzola, L., Voss, A. K., Thomas, T., Stanley, E. G., and Elefanty, A. G. (2006) *Dev Biol* **293**, 90-103
42. Darabi, R., Gehlbach, K., Bachoo, R. M., Kamath, S., Osawa, M., Kamm, K. E., Kyba, M., and Perlingeiro, R. C. (2008) *Nat Med* **14**, 134-143

Foot Notes

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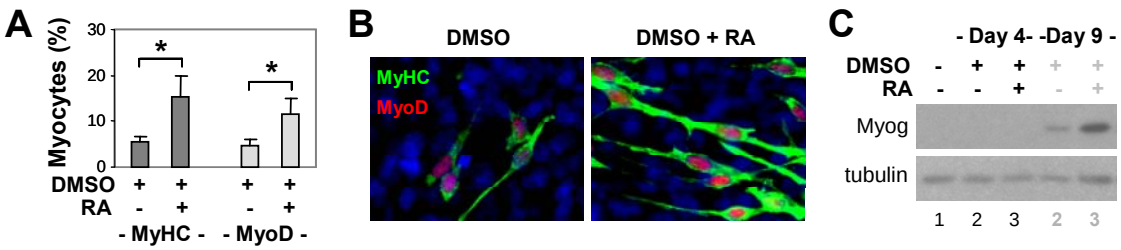

Fig 1. Effects of RA on skeletal myogenesis. (A) P19 aggregates were treated with DMSO and RA (10 nM) and co-stained for myosin heavy chain (MyHC) and MyoD. Quantification is plotted as fractions of myoblasts (*$p<0.05$). (B) Representative images of MyoD and myosin heavy chain (MyHC). (C) Western analysis of myogenin with undifferentiated cells as control.

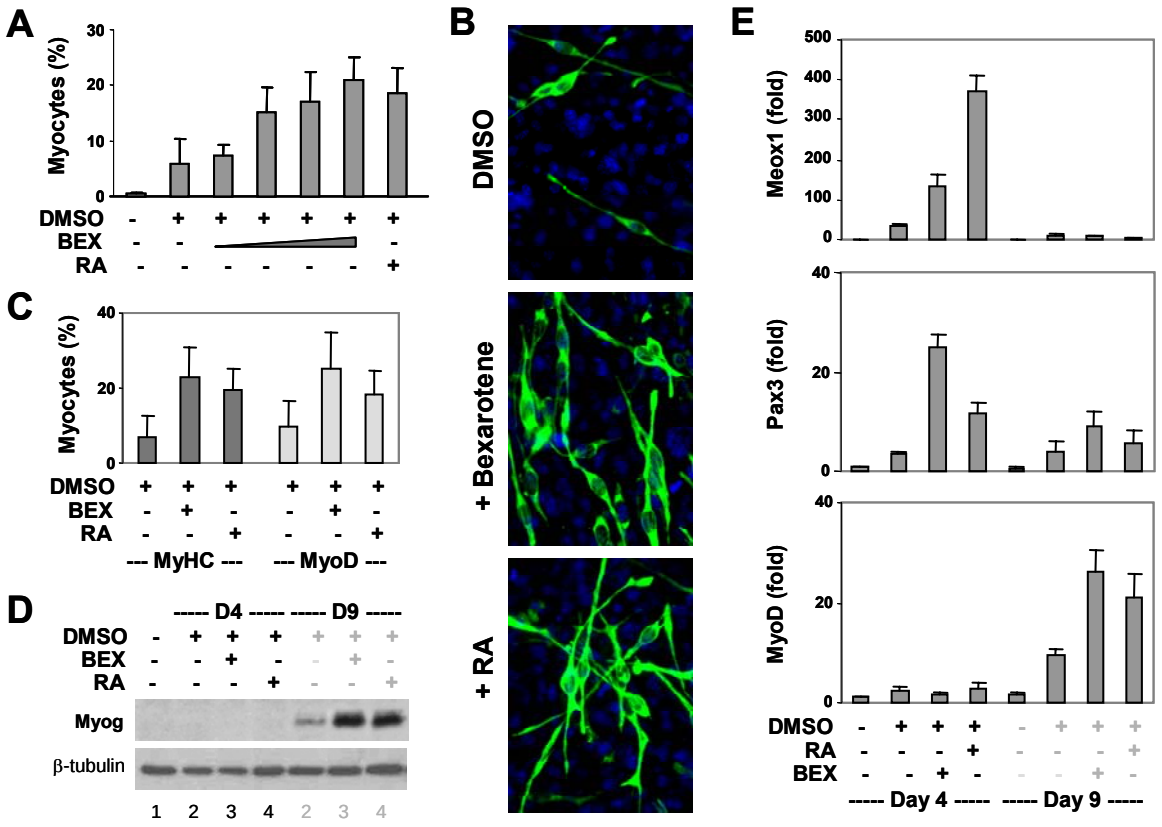

Fig 2. Roles of rexinoid in P19 cell differentiation. (A) P19 aggregates were treated with bexarotene (BEX, 1 nM, 10 nM, 100 nM, 1 μM) or RA (10 nM) in the presence of DMSO and stained for myosin heavy chain (MyHC) on day 9. Quantification is presented as fractions of myoblasts. Error bars are the standard deviations of five independent experiments. (B) Representative images. (C) The cells were also co-stained for MyoD and quantified in comparison with MyHC. (D) Western analysis of myogenin. (E) The relative mRNA levels of Meox1, Pax3 and MyoD were determined by quantitative real-time RT-PCR and plotted as fold difference in relation to untreated day 4 controls after being normalized to GAPDH. (Hymn Mach, a summer student, performed the RT-PCR).

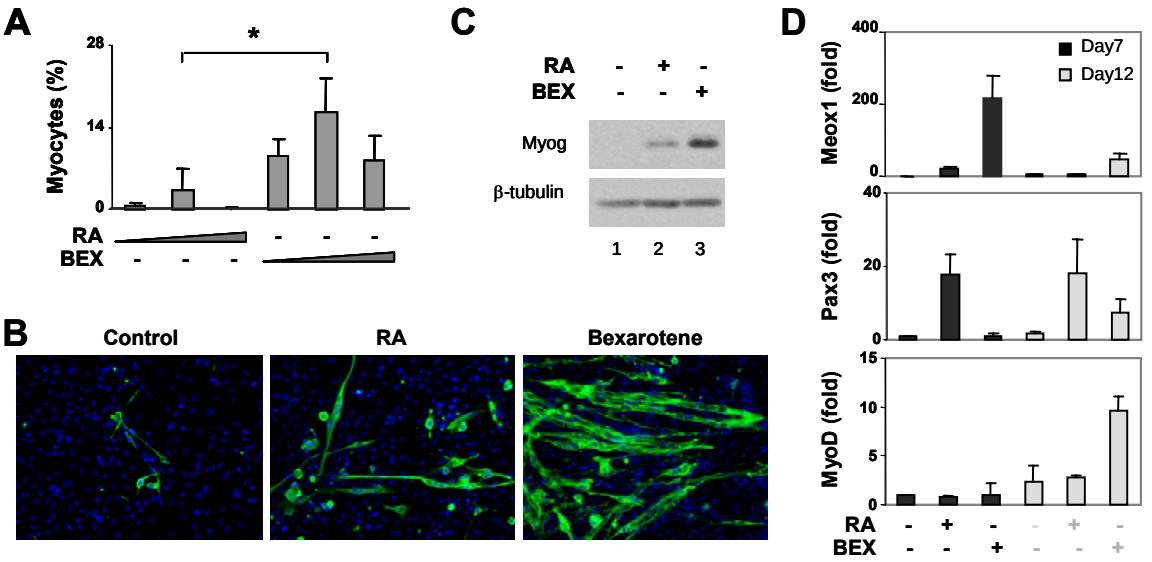

Fig 3. Effects of rexinoid on ES cell differentiation. (A) RA (5, 10, 20 nM) or bexarotene (BEX, 20, 50, 100 nM) were used during day 2 and 5 of EB formation. Cells were plated on coverslips on day 7 and stained on day 20 for myosin heavy chain (MyHC) and MyoD. Microscopic analyses were performed and plotted as fractions of myoblasts (*$p<0.05$). Error bars are the standard deviations of four independent experiments. (B) Representative images of MyHC. (C) Western analysis of myogenin. (D) The relative transcript levels of Meox1, Pax3 and MyoD were determined by real-time RT-PCR analysis and plotted as fold variance of untreated day 7 controls after being normalized to GAPDH. (Hymn Mach, a summer student, participated in the ES cell differentiation and performed the RT-PCR).

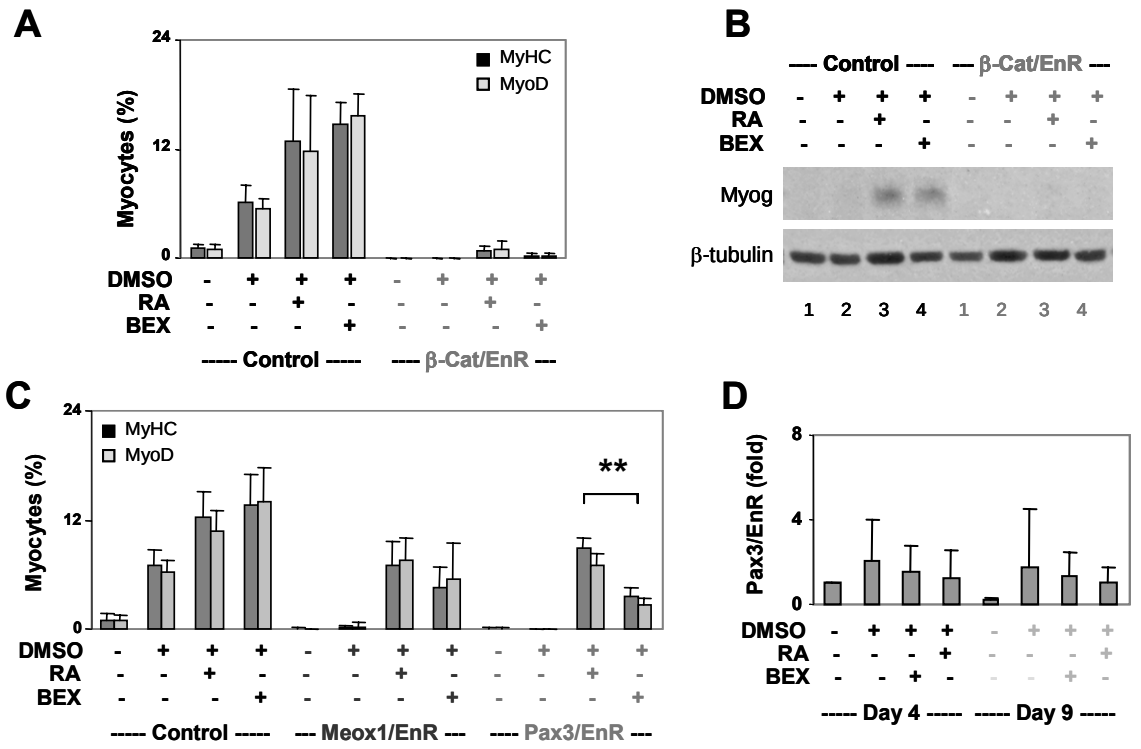

Fig 4. Roles of β-catenin, Meox1 and Pax3 in rexinoid-enhanced skeletal myogenesis. (A) A clone of P19 cells expressing a dominant negative β-catenin (β-cat/EnR) was differentiated with bexarotene (BEX, 100 nM) or RA (10 nM) and stained for myosin heavy chain (MyHC) and MyoD. Control cells harbour the empty vector. Quantification is plotted as fractions of myoblasts. Error bars are the standard deviations of three independent experiments. (B) Western analysis of myogenin in β-catenin (β-cat/EnR) cells. (C) Cells expressing dominant negative Meox1 (Meox1/EnR) or Pax3 (Pax3/EnR) were differentiated as in panel A (**$p<0.01$). Control cells harbor the empty vector. Error bars are the standard deviations of four independent experiments. (D) The relative mRNA levels EnR were determined by quantitative real-time RT-PCR and plotted as fold difference in relation to untreated day 4 controls after being normalized to GAPDH. (Natascha Lacroix, a summer student, performed the β-catenin study).

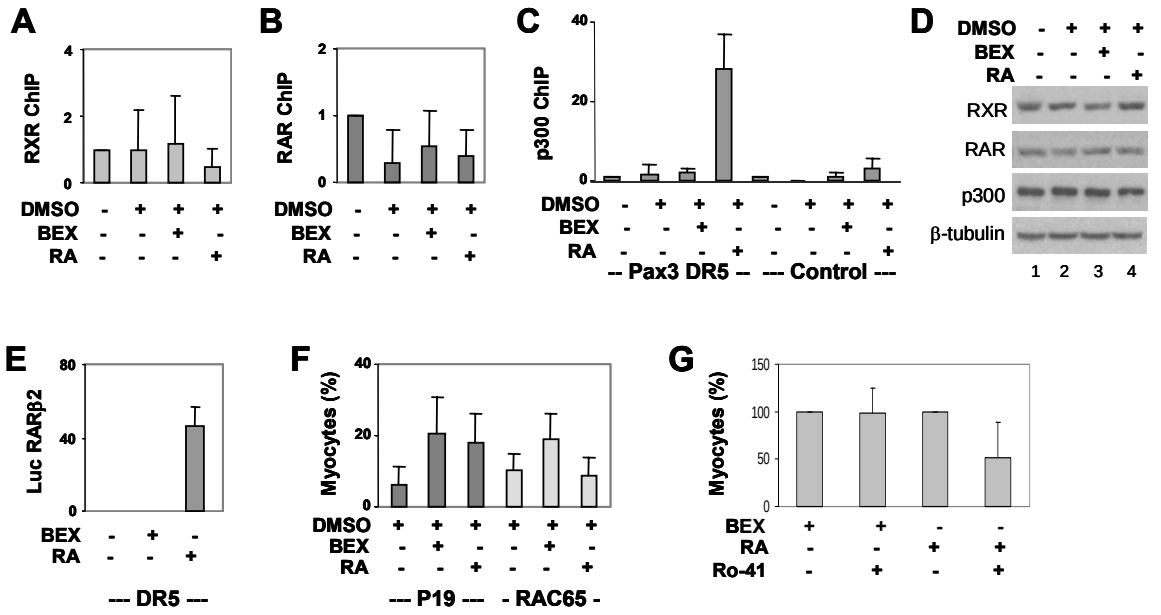

Fig 5. Role of RAR in RXR specific signaling for myogenic conversion. P19 aggregates were treated with bexarotene (BEX, 100 nM) or RA (10 nM). (A) RXR binding and (B) RAR binding to the Pax3 locus was examined by ChIP on day 4 of differentiation. Input DNA was used as internal controls. Error bars are the standard deviations of four independent experiments. (C) Occupancy by p300 was also analyzed using Meox1 as control gene. (D) Western analysis of p300, RAR and RXR. (E) P19 aggregates were transfected with a RAR luciferase reporter (0.1 μg) and a RSV-β-Gal (0.2 μg), induced with bexarotene or RA, and assayed for luciferase activity. Values are the fold induction compared to the untreated control with β-Gal as internal controls. (F) RAC65 cells were differentiated and analyzed by microscopy on day 9. Quantification is plotted as fraction of myoblasts. Error bars are the standard deviations of four independent experiments. (G) P19 aggregates treated with bexarotene (BEX, 100 nM) or RA (10 nM) were co-treated with a RAR-selective antagonist. Quantification is plotted as fractions of myoblasts. Error bars are the standard deviations of four independent experiments. (Dr. Jihong Chen is responsible for the reporter study. Chenchen Hou, an honours student, participated in RAC65 cell differentiation).

CHAPTER 3: DISCUSSION

It is well established that RXR is a bona fide receptor in vivo rather than simply a silent partner for other nuclear receptors. Additionally, there is accumulating evidence arguing against a silent partner role for RXR in the RXR/RAR heterodimer (Chen, J.Y., Clifford, J. et al. 1996; Germain, P., Iyer, J. et al. 2002; Kersten, S., Dawson, M.I. et al. 1996; Lala, D.S., Mukherjee, R. et al. 1996; Minucci, S., Leid, M. et al. 1997; Roy, B., Taneja, R. et al. 1995). We report the importance of a separate, rexinoid signaling pathway in skeletal muscle development non-overlapping with RA signal transduction. Our findings demonstrate that an RXR selective ligand, Bexarotene effectively enhances skeletal myogenesis in mouse ES cells that respond poorly to RA. We believe the difference in the two signaling pathways stems from differential activation of very early genes involved in crucial lineage specification.

RA and Rexinoid Target Different Specification Genes

While both Bexarotene and RA are dependant on functional β-catenin signaling (Figure 4), their ability to induce specification genes differs. In P19 cells, we have shown that Bexarotene primarily targets Meox1 while RA mainly targets Pax3. While both treatments are equally capable of inducing later target genes such as the MRFs, activation of different sets of early genes could explain the observed difference in morphology where RA tends to give mature, fat, multinucleated myofibers whereas Bexarotene treated cells resembled more the morphology of DMSO, being skinnier and more often mononucleated.

Based on the observed difference of the two small molecule inducers to activate specification genes, we proceeded to test the ability of Bexarotene and RA to induce skeletal myogenesis in P19 cell lines with either a dominant negative Pax3 or Meox1 protein. In the Pax3/EnR cell line, similar to previous studies, (Ridgeway, A.G. and Skerjanc, I.S. 2001) we found skeletal myogenesis was completely abrogated in aggregates and DMSO treatment while the induction of cardiomyogenesis was unaffected. Conversely, RA was able to override the dominant negative Pax3 mutation and still showed skeletal differentiation and inhibition of cardiomyogenesis. Since RA induces Pax3, it might have the capacity to increase endogenous levels of Pax3. The normal protein could compete with mutated protein for DNA binding sites thus displacing the non-functional inhibitory effects of Pax3/EnR protein and allow skeletal myogenesis to proceed; yet with lower efficiency than P19 cells (Figure 4B versus 4A). Alternatively, Bexarotene, which preferentially enhanced Meox1 expression, was still capable of inducing skeletal muscle at low levels but these levels were two fold less than that seen with RA. While Bexarotene did not induce Pax3 mRNA to the levels seen with RA, it still showed upregulation compared with DMSO which would explain the discrepancy between DMSO and Bexarotene to induce skeletal myogenesis in these cells.

In the Meox1/EnR cells we also saw inhibition of skeletal myogenesis and cariomyogenesis in aggregates and DMSO treatment as previously reported (Petropoulos, H., Gianakopoulos, P.J. et al. 2004). Skeletal and cardiac muscle would be equally affected by mutating this protein due to the fact that Meox1 is involved with mesoderm development and is not skeletal muscle specific. Our results with Bexarotene and RA were less straightforward than the Pax3/EnR results as both treatments showed an

approximately equal capacity to induce skeletal myogenesis and inhibit cardiomyogenesis, yet again, with lower efficiency than P19 cells (Figure 4B versus 4A). However, Pax3 and Meox1 differ in that stable over-expression of Pax3 in P19 cells is sufficient to induce skeletal myogenesis in aggregates while Meox1 over-expression is inadequate. Additionally, Meox1 is a mesoderm gene while the role of Pax3 in myogenesis is specific to skeletal muscle. These differences might account for our observed results. Finally, it should be noted that both Bexarotene and RA were able to induce MyoD in both Pax3 and Meox1 engrailed cell lines and this ability matched closely with that of the ability to induce skeletal myogenesis demonstrating proper development in cells directed towards skeletal muscle lineage.

RXR Induces Skeletal Myogenesis in Mouse Embryonic Stem Cells

Our results for the D3 ES cells also indicated that target genes differ between RA and Bexarotene. Again we saw that Meox1 was preferentially activated by Bexarotene while RA targeted Pax3. Yet unlike the P19 cells, which showed roughly equal enhancement of skeletal myogenesis by the two treatments, Bexarotene treatment of D3 cells gave a 5 fold greater induction than RA. Furthermore, treatment of these cells with Bexarotene gave long, mature, multinucleated myofibers.

It is intriguing that a cell type such as D3 ES cells, that has thus far been relatively resistant to RA induced skeletal differentiation develops so well in the presence of an RXR selective ligand especially considering they do not posses the machinery to synthesize 9-cis, the purported endogenous ligand. It appears that P19 cells have the ability to differentiate by both retinoid and rexinoid signaling instigated pathways while

ES cells respond only to rexinoid mediated pathways; perhaps due to different levels of RXR and RAR receptors within the cells.

The ratio of RAR to RXR receptors is known to be important in certain cases of RXR mediated signaling such as the induction of the cell cycle regulator p21 (Tanaka, T. and De Luca, L.M. 2009). Several ligands known to activate RXR homodimers also induce p21 expression and regulate cell cycle progression while ligands specific for RXR heterodimer partners do not (Tanaka, T., Suh, K.S. et al. 2007). These selective ligands do so via binding to two consecutive RXREs in the p21 promoter region and lead cell cycle arrest followed by apoptosis. However, this RXR mediated induction of p21 can be blocked by RXR/RAR heterodimers due to a RARE that exists between the two RXREs. Since the heterodimers bind with higher affinity than the homodimers, the presence of RARs can significantly inhibit this response signifying that the balance of RARs to RXRs is extremely important in dictating the outcome of this induction (Tanaka, T. and De Luca, L.M. 2009).

It remains to be resolved if the ratio of RXR to RAR receptors affects gene transcription at the promoters of other genes associated with the myogenic program. It is possible that one mechanism of action of Bexarotene in the D3 ES cells is to overcome this barrier by inducing tetramer dissociation and significantly changing the ratio of receptors. Since the receptor dimers are not statically bound to their response elements, dramatically increasing RXR numbers could competitively displace RXR/RAR heterodimer binding to DR5 in exchange for the homodimer or permissive heterodimer binding. RXR agonists dissociate tetramers and allow for an increase in the effective concentration of active RXR molecules so that RXR homodimer signaling is favored.

ATRA does not bestow the ability to dissociate the tetramers and actually acts as a competitive antagonist of this dissociation which sequesters the RXRs in the tetramers (Szanto, A., Narkar, V. et al. 2004). It is possible that unlike the P19 cells which have an alternative functional RXR/RAR signaling cascade, D3 ES cells cannot compensate in such a manner. This would explain why RA versus Bexarotene signaling in these cells gives results similar to those seen with the RAC65 cells.

RAR Independent Enhancement of Skeletal Myogenesis

RAC65 cells harbor a mutation that effectively blocks the RXR/RAR heterodimer signaling cascade (Pratt, M.A., Kralova, J. et al. 1990). They are resistant to RA induced skeletal muscle and neuronal conversion yet demonstrate efficient skeletal differentiation when treated with RXR selective ligand. The ability of rexinoid to bypass the dominant negative RAR inhibition in these cells is not unique to skeletal muscle and has also been documented for neuronal differentiation as well (Yokota, Y. and Ohkubo, H. 1996). Since ATRA cannot activate the RXRs and in these cells it is unable to signal through RARs, it was unable to enhance skeletal muscle development above levels seen with DMSO treatment. This allowed us to determine that the RXR-mediated induction is not attributable to RXR/RAR heterodimers. Similarily, we used a RAR specific inverse agonist to selectively inhibit RAR signaling. As observed with RAC65 cells, RA signaling was impaired while bexarotene induced skeletal myogenesis was not compromised (Fig. 5G). Thus RXR mediates skeletal myogenesis through mechanisms independent of RAR. Since RXR has the ability to bind with so many other nuclear

receptors, it remains to be determined whether it is acting as a homodimer or whether it is acting within a permissive heterodimer (ie: LXR, FXR, PPAR) to exert its effects.

Role of Ligand in Dictating Receptor Transactivation

Several studies report conflicting results of RXR participation in the activation of RXR/RAR heterodimers. The discrepancy between varying reports can possibly be reconciled by the fact that different ligands interact with distinct side chains in the ligand binding domain and thus mediate differential activation of the receptor complex. The exact response is therefore highly dependant on the identity of the ligand and cannot simply be classified as agonistic versus antagonistic. Although RXR can engage in ligand binding when RAR is ligand occupied and/or if a suitable synthetic ligand is present (Chen, J.Y., Clifford, J. et al. 1996; Kersten, S., Dawson, M.I. et al. 1996; Lala, D.S., Mukherjee, R. et al. 1996; Minucci, S., Leid, M. et al. 1997; Roy, B., Taneja, R. et al. 1995) it has been reported that Bexarotene is unable to transactivate the RXR/RAR heterodimer (Lehmann, J.M., Jong, L. et al. 1992) and so this would be highly unlikely to be the mechanism of activation seen in the cell systems we employed. In fact, Bexarotene has been reported to reduce interactions between RXRs and RARs whereas both RAR and RXR ligands such as 9-cis increase binding of RXRs to RARs twofold (Dong, D. and Noy, N. 1998).

Similarily, ATRA is unable to activate RXR selective pathways at the concentrations we employed. Optimal ATRA enhancement of skeletal muscle differentiation of both P19 cells and D3 embryonic stem cells required low concentrations of RA. ATRA does not bind to RXR-a (Mangelsdorf, D.J., Borgmeyer,

U. et al. 1992) and importantly, although ATRA has the ability to isomerize to 9-cis RA, pharmacological doses of ATRA are required to generate enough 9-cis to activate the RXRs (Mic, F.A., Molotkov, A. et al. 2003). In our study, the optimal concentration of RA is well below the concentration required for this and therefore isomerization is simply not a feasible explanation to the equal enhancement of RA and Bexarotene observed in the P19 cells.

Additionally, one proposed mechanism of 9-cis RA is that it induces tetramer dissociation after which some of it isomerizes to ATRA and the two isomers subsequently activate both monomers of the RXR/RAR heterodimers to generate maximum transactivation (Hewson, Q.C., Lovat, P.E. et al. 2002). Again we can rule this out based on the stability and selectivity of the synthetic ligands. Finally, while the RA metabolites such as 4-oxo-RA were originally believed to play a role in RA signaling, they have more recently been established as physiologically not required (Niederreither, K., Abu-Abed, S. et al. 2002; Pijnappel, W.W., Hendriks, H.F. et al. 1993). We can therefore conclude that RA metabolites are not responsible for the observed effects.

Two Separate Pathways Activate Skeletal Myogenesis

We have shown that activating RXRs is sufficient to induce skeletal myogenesis. If this selective activation of RXR was indeed somehow related to RXR/RAR heterodimer activation, targeting both receptors should, as previously documented, show synergistic effects. Conversely, when we tried activating both receptors by using a combination of RXR and RAR selective ligands, without the ability to isomerizes, both ligands were equally capable of enhancing skeletal myogenesis however, we did not see any further

enhancement above that seen through activating solely RXR (preliminary laboratory results). It consequently appears that two distinct yet possibly overlapping pathways exist: one in which RXR/RAR heterodimers exert the effects, and one which is governed by RXR specific signaling.

An interesting difference between ATRA treatment and Bexarotene treatment is that while Bexarotene displays classical receptor biology, as can be seen through its sigmoid dose response curve (Figure 3A), ATRA signaling appears to function through non-classical RA signaling in skeletal muscle induction as low doses of RA do not induce RAR-β (preliminary results), a known RA target gene containing a RARE. The higher doses required to increase RAR-β expression no longer induce skeletal myogenesis but on the contrary, inhibit skeletal development while inducing neurogenesis. It appears then, that these two ligands function through entirely different mechanisms.

Finally, RXR is known to be able to activate target genes involved in RA signaling that cannot be induced by RARs as is the case with the response element in the CRBPII (Cellular Retinol Binding Protein Type II) gene which contains a DR1, underscoring the possibility of RXR/RXR and RXR/RAR independent pathways (Mangelsdorf, D.J., Umesono, K. et al. 1991).

Role of Receptors in the Recruitment of Coactivators

One issue that has undermined the importance of RXR signaling is that while many RA target genes have been discovered, few RXR specific target genes have been identified. That being the case, in the presence of RXR specific ligand, RXR has been documented to bind as homodimers to PPREs dependant on specific coactivator recruitment of SRC1

and irrespective of the presence of PPAR. Liganded PPAR in the RXR/PPAR heterodimer does not recruit this coactivator (IJpenberg, A., Tan, N.S. et al. 2004). Furthermore, these PPRE bound homodimers can substitute functionally for RXR/PPAR heterodimers and regulate complex pathways in vivo. A similar scenario has been observed in S91 melanoma cells where there is evidence that RXR selective ligands promote occupancy of the RAR-β DR5 RARE (Ikeda, M., Spanjaard, R.A. et al. 1998). Selective recruitment of specific coactivators would in turn drive the assembly of specific transcription complexes and therefore result in differential activation of genes and thus signaling cascades.

It remains to be determined which specific coactivators are recruited by RXR in the enhancers or promoters of target genes in skeletal myogenesis. RXR homodimers or RXR permissive heterodimers might recruit a separate set of co-activators and therefore differentially control gene expression. It could be that the unique ability of Bexarotene versus RA to control the transcription factor's interactions with coactivators is the method by which distinct and even competing signaling pathways can be distinguished.

In our system we observed that indeed RXR/RAR heterodimers showed the distinct ability to recruit the acetyletransferase p300 to a DR5 at a locus in the Pax3 gene. At this region, RXR appears to be acting through RXR/RAR heterodimers as a silent partner and although RXR was found to constitutively bind this locus, RA but not Bexarotene was able to augment the occupancy of p300.

Unsaturated Fatty Acids Activate RXR

The physiological significance of 9-cis RA signaling is debated due to a lack of consensus on its existence in the developing embryo. However, the enzymes that contribute to its biosynthesis are well documented (Mertz, J.R., Shang, E. et al. 1997; Romert, A., Tuvendal, P. et al. 1998) in addition to its ability to induce the formation of homodimers that bind to DR1 sequences (Zhang, X.K., Lehmann, J. et al. 1992). While most studies focus on whether concentrations of 9-cis are physiologically capable of regulating RXR, they omit that other small molecule inducers may be the endogenous ligands. The lack of a known ligand is hardly reason to exclude RXR as physiologically significant in vivo and a major factor that supports the presence of an active endogenous ligand is the fact that RXR tetramers cannot dissociate without agonist binding.

Studies using RXR ligand-detector mice have identified specific regions of the spinal cord as major sites of endogenous rexinoid production and classify naturally occurring polyunsaturated fatty acids, including docosahexaenoic acid (DHA) as a major endogenous ligand for RXR in the mouse brain. (Ahuja, H.S., Szanto, A. et al. 2003; de Urquiza, A.M., Liu, S. et al. 2000) When characterized in the ligand binding domain of RXR-α, DHA has a significantly higher number of ligand-protein contacts than 9-cis and certain synthetic ligands and also has the ability to activate RXR homodimers as well as synergistically activate the RXR-RAR heterodimers in combination with ATRA (Lengqvist, J., Mata, D.U. et al. 2004). It remains to be determined if this ligand is functional in all tissues or whether there are other yet undiscovered ligands. Presently, additional unsaturated fatty acids, including docosapentaenoic, arachidonic, and oleic acids, also have been found to bind and activate RXR, suggesting that this ability is not

exclusive for DHA. Irrespective of whether an endogenous RXR ligand does indeed exist, the ability to control cell growth and differentiation through targeting RXR with highly selective ligands confers many therapeutical applications to this unique receptor.

Significance of a Separate RXR Signaling Pathway

Thus it is reasonable that in vivo, either RXR has a yet unidentified ligand or 9-cis is the actual ligand. Furthermore, it is unknown whether RXR homodimer or RXR permissive heterodimer signaling is the main mechanism governing skeletal muscle differentiation. Regardless, controlling cell processes using RXR selective ligands underlines that fact that two distinct and possibly overlapping pathways exist. Moreover, it provides another route of achieving cell cycle arrest and differentiation when RA signaling is aberrant, a situation frequently seen in cancer where differentiation often appears to result in loss of a malignant phenotype. (Gokhale, P.J., Giesberts, A.M. et al. 2000)

RXR-α overexpression sensitizes tumors to rexinoid induced anti-growth effects, cell differentiation, decreased cell proliferation, apoptosis of some cancer cell types, and prevention of angiogenesis and metastasis.(Qu, L. and Tang, X. 2010) The specific rexinoid used in our study, has been approved by the FDA for use in the treatment of refractory or persistent cutaneous T-cell lymphoma and has the ability to reduce tumor development in several other cancers (Duvic, M., Martin, A.G. et al. 2001; Wu, K., Kim, H.T. et al. 2002). However, the use of this compound in the treatment of lung and breast carcinomas has yielded disappointing results (Tanaka, T. and De Luca, L.M. 2009) demonstrating our lack of understanding of the mechanisms underlying rexinoid induced antitumor effects and RXR-induced multi-pathway activation.

One of the reasons rexinoids seem such promising chemotherapeutic compounds compared to retinoids, is that retinoids have numerous side effects which severely limit the dosage and efficacy while rexinoids display mild toxicity. Furthermore, RXR expression is rarely lost in human tumors whereas RAR expression is frequently lost or reduced in various cancers.(Sun, S.Y. and Lotan, R. 2002; Umesono, K. and Evans, R.M. 1989) Since p53 abnormalities are reported in more than 50% of human cancers, and p21 is rarely mutated. (Shiohara, M., el Deiry, W.S. et al. 1994; Tanaka, T., Suh, K.S. et al. 2007) RXR mediated induction of p21 is a promising therapeutic target for these cancers. The study of myocyte differentiation may provide some answers to new target genes as the development and progression of cancer involves aberrations in the same mechanisms that regulate cell differentiation during embryogenesis. It remains to be revealed which other genes can also be targeted by rexinoids and which specific interactions take place that we can study and apply to our development of more potent and effective therapeutics.

Future Directions

It remains to be addressed why an RXR selective ligand is a more suitable inducer in mouse ES cells. While we have examined several early specification genes known to be involved in myogenesis, genome wide analysis comparing RA and Bexarotene would be helpful to resolve other novel genomic targets of rexinoid specific signaling. Additionally, an outstanding question remains to be answered: if RXR is acting through a RAR independent pathway in skeletal myogenesis, then who is RXR's partner? Further work will be required to elucidate whether it is acting as a homodimer or as a partner in permissive heterodimers.

Interestingly, we found that 10 nM ATRA has the ability to maintain levels of p300 within the cells to day 9 of differentiation. As mentioned previously, p300 is involved with many important roles in skeletal myogenesis namely acetylating and activating Myf5, MyoD, MEF2C and also RXR. However, levels of p300 may not be indicative of its specific HAT activity. In a study on F9 cells, the levels of p300 HAT activity were found to remain constant during differentiation despite p300 protein level decrease (Brouillard, F. and Cremisi, C.E. 2003). Importantly, during this process, the HAT activity of p300 is progressively more dependant on phosphorylation. It could be that treatment of cells with RA or Bexarotene leads to increased phosphorylation of p300 and therefore increased activity of not only p300 but also its downstream targets, Myf5, MyoD, and myogenin in the process of myogenesis. Alternatively, p300 could be maintained through either altering the stability of p300 through other post-translational modifications or through altering proteins involved in its degradation such that p300 protein levels no longer decrease. It remains to be determined what the significance of p300 maintenance is on the effect of skeletal muscle differentiation and what the effect of Bexarotene treatment is on the maintenance of this crucial protein.

Determining the molecular mechanism by which rexinoid exerts its effects to enhance skeletal myogenesis is challenging due in part to the complexity of the developmental systems in which it exerts its effects as well as the intricate relationship of protein complexes and gene regulation however uncovering these details may yield rewarding outcomes.

Conclusion

In this study we have examined the ability of rexinoid to direct pluripotent stem cells into the myogenic lineage. We have identified a RXR selective ligand as an effective inducer of not only P19 pluripotent stem cells but also mouse ES cells which have thus far been relatively resistant to RA induced skeletal myogenesis. We have determined that RXR specific signaling plays a role in this process through a separate RAR independent mediated pathway. We believe that RA and rexinoid enhance skeletal myogenesis through differential activation of early developmental genes. Our findings demonstrate that activation of RXR causes an increase in the mesodermal Meox1 gene while RA induces the skeletal specific gene Pax3. While we were able to establish that p300 complex is recruited to RXR/RAR heterodimers at the Pax3 locus, further work will be required to determine which co-activators are recruited to activated RXR and at which genes within this system. Furthermore it will be interesting to uncover other novel genes targeted by rexinoid. Since ES cells closely recapitulate the properties of the developing embryo, elucidating these molecular pathways will be useful in the manipulation of stem cell progenitors with the goal of generating sufficient pure populations of skeletal myocytes used in the treatment of disease.

CHAPTER 4: REFERENCES

Ahuja, H.S., Szanto, A., Nagy, L., and Davies, P.J. 2003. The retinoid X receptor and its ligands: versatile regulators of metabolic function, cell differentiation and cell death. *J Biol Regul Homeost Agents* **17**:29-45.

Alric, S., Froeschle, A., Piquemal, D., Carnac, G., and Bonnieu, A. 1998. Functional specificity of the two retinoic acid receptor RAR and RXR families in myogenesis. *Oncogene* **16**:273-282.

Alvares, L.E., Schubert, F.R., Thorpe, C., Mootoosamy, R.C., Cheng, L., Parkyn, G., Lumsden, A., and Dietrich, S. 2003. Intrinsic, Hox-dependent cues determine the fate of skeletal muscle precursors. *Dev Cell* **5**:379-390.

Angus, L.M., Chan, R.Y., and Jasmin, B.J. 2001. Role of intronic E- and N-box motifs in the transcriptional induction of the acetylcholinesterase gene during myogenic differentiation. *J Biol Chem* **276**:17603-17609.

Arnold, H.H. and Braun, T. 2000. Genetics of muscle determination and development. *Curr Top Dev Biol* **48**:129-164.

Arnold, H.H. and Winter, B. 1998. Muscle differentiation: more complexity to the network of myogenic regulators. *Curr Opin Genet Dev* **8**:539-544.

Bain, G. and Gottlieb, D.I. 1994. Expression of retinoid X receptors in P19 embryonal carcinoma cells and embryonic stem cells. *Biochem Biophys Res Commun* **200**:1252-1256.

Bajard, L., Relaix, F., Lagha, M., Rocancourt, D., Daubas, P., and Buckingham, M.E. 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.

Balmer, J.E. and Blomhoff, R. 2002. Gene expression regulation by retinoic acid. *J Lipid Res* **43**:1773-1808.

Barger, P.M. and Kelly, D.P. 1997. Identification of a retinoid/chicken ovalbumin upstream promoter transcription factor response element in the human retinoid X receptor gamma2 gene promoter. *J Biol Chem* **272**:2722-2728.

Bastien, J. and Rochette-Egly, C. 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**:1-16.

Bergstrom, D.A., Penn, B.H., Strand, A., Perry, R.L., Rudnicki, M.A., and Tapscott, S.J. 2002. Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. *Mol Cell* **9**:587-600.

Bergstrom, D.A. and Tapscott, S.J. 2001. Molecular distinction between specification and differentiation in the myogenic basic helix-loop-helix transcription factor family. *Mol Cell Biol* **21**:2404-2412.

Black, B.L. and Olson, E.N. 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol* **14**:167-196.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376**:768-771.

Blomhoff, R. 1994. Transport and metabolism of vitamin A. *Nutr Rev* **52**:S13-S23.

Bober, E., Lyons, G.E., Braun, T., Cossu, G., Buckingham, M., and Arnold, H.H. 1991. The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. *J Cell Biol* **113**:1255-1265.

Boiani, M. and Scholer, H.R. 2005. Regulatory networks in embryo-derived pluripotent stem cells. *Nat Rev Mol Cell Biol* **6**:872-884.

Borello, U., Berarducci, B., Murphy, P., Bajard, L., Buffa, V., Piccolo, S., Buckingham, M., and Cossu, G. 2006. The Wnt/beta-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. *Development* **133**:3723-3732.

Borycki, A.G., Brunk, B., Tajbakhsh, S., Buckingham, M., Chiang, C., and Emerson, C.P., Jr. 1999. Sonic hedgehog controls epaxial muscle determination through Myf5 activation. *Development* **126**:4053-4063.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. 1995. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature* **375**:377-382.

Bourguet, W., Vivat, V., Wurtz, J.M., Chambon, P., Gronemeyer, H., and Moras, D. 2000. Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. *Mol Cell* **5**:289-298.

Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**:255-256.

Braun, T. and Arnold, H.H. 1995. Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. *EMBO J* **14**:1176-1186.

Braun, T., Bober, E., Rudnicki, M.A., Jaenisch, R., and Arnold, H.H. 1994. MyoD expression marks the onset of skeletal myogenesis in Myf-5 mutant mice. *Development* **120**:3083-3092.

Braun, T., Bober, E., Winter, B., Rosenthal, N., and Arnold, H.H. 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. *EMBO J* **9**:821-831.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H.H. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J* **8**:701-709.

Braun, T., Rudnicki, M.A., Arnold, H.H., and Jaenisch, R. 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* **71**:369-382.

Brouillard, F. and Cremisi, C.E. 2003. Concomitant increase of histone acetyltransferase activity and degradation of p300 during retinoic acid-induced differentiation of F9 cells. *J Biol Chem* **278**:39509-39516.

Buchberger, A., Nomokonova, N., and Arnold, H.H. 2003. Myf5 expression in somites and limb buds of mouse embryos is controlled by two distinct distal enhancer activities. *Development* **130**:3297-3307.

Buckingham, M. 1992. Making muscle in mammals. *Trends Genet* **8**:144-148.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., and Relaix, F. 2003. The formation of skeletal muscle: from somite to limb. *J Anat* **202**:59-68.

Campione-Piccardo, J., Sun, J.J., Craig, J., and McBurney, M.W. 1985. Cell-cell interaction can influence drug-induced differentiation of murine embryonal carcinoma cells. *Dev Biol* **109**:25-31.

Candia, A.F., Hu, J., Crosby, J., Lalley, P.A., Noden, D., Nadeau, J.H., and Wright, C.V. 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.

Candia, A.F. and Wright, C.V. 1996. Differential localization of Mox-1 and Mox-2 proteins indicates distinct roles during development. *Int J Dev Biol* **40**:1179-1184.

Carnac, G., Albagli-Curiel, O., Levin, A., and Bonnieu, A. 1993. 9-cis-retinoic acid regulates the expression of the muscle determination gene Myf5. *Endocrinology* **133**:2171-2176.

Chen, G. and Courey, A.J. 2000. Groucho/TLE family proteins and transcriptional repression. *Gene* **249**:1-16.

Chen, J.Y., Clifford, J., Zusi, C., Starrett, J., Tortolani, D., Ostrowski, J., Reczek, P.R., Chambon, P., and Gronemeyer, H. 1996. Two distinct actions of retinoid-receptor ligands. *Nature* **382**:819-822.

Chen, L. and Khillan, J.S. 2010. A novel signaling by vitamin A/retinol promotes self renewal of mouse embryonic stem cells by activating PI3K/Akt signaling pathway via insulin-like growth factor-1 receptor. *Stem Cells* **28**:57-63.

Chiba, H., Clifford, J., Metzger, D., and Chambon, P. 1997. Specific and redundant functions of retinoid X Receptor/Retinoic acid receptor heterodimers in differentiation, proliferation, and apoptosis of F9 embryonal carcinoma cells. *J Cell Biol* **139**:735-747.

Christ, B. and Ordahl, C.P. 1995. Early stages of chick somite development. *Anat Embryol (Berl)* **191**:381-396.

Chung, Y., Klimanskaya, I., Becker, S., Marh, J., Lu, S.J., Johnson, J., Meisner, L., and Lanza, R. 2006. Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. *Nature* **439**:216-219.

Cossu, G. and Borello, U. 1999. Wnt signaling and the activation of myogenesis in mammals. *EMBO J* **18**:6867-6872.

Costa, S.L. and McBurney, M.W. 1996. Dominant negative mutant of retinoic acid receptor alpha inhibits retinoic acid-induced P19 cell differentiation by binding to DNA. *Exp Cell Res* **225**:35-43.

Crescenzi, M., Fleming, T.P., Lassar, A.B., Weintraub, H., and Aaronson, S.A. 1990. MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc Natl Acad Sci U S A* **87**:8442-8446.

Cserjesi, P. and Olson, E.N. 1991. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Mol Cell Biol* **11**:4854-4862.

Danielsen, M., Hinck, L., and Ringold, G.M. 1989. Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* **57**:1131-1138.

Davis, R.L., Weintraub, H., and Lassar, A.B. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**:987-1000.

de Urquiza, A.M., Liu, S., Sjoberg, M., Zetterstrom, R.H., Griffiths, W., Sjovall, J., and Perlmann, T. 2000. Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* **290**:2140-2144.

Dietrich, S., Abou-Rebyeh, F., Brohmann, H., Bladt, F., Sonnenberg-Riethmacher, E., Yamaai, T., Lumsden, A., Brand-Saberi, B., and Birchmeier, C. 1999. The role of SF/HGF and c-Met in the development of skeletal muscle. *Development* **126**:1621-1629.

Dietrich, S., Schubert, F.R., Healy, C., Sharpe, P.T., and Lumsden, A. 1998. Specification of the hypaxial musculature. *Development* **125**:2235-2249.

Dilworth, F.J., Seaver, K.J., Fishburn, A.L., Htet, S.L., and Tapscott, S.J. 2004. In vitro transcription system delineates the distinct roles of the coactivators pCAF and p300 during MyoD/E47-dependent transactivation. *Proc Natl Acad Sci U S A* **101**:11593-11598.

DiRenzo, J., Soderstrom, M., Kurokawa, R., Ogliastro, M.H., Ricote, M., Ingrey, S., Horlein, A., Rosenfeld, M.G., and Glass, C.K. 1997. Peroxisome proliferator-activated receptors and retinoic acid receptors differentially control the interactions of retinoid X receptor heterodimers with ligands, coactivators, and corepressors. *Mol Cell Biol* **17**:2166-2176.

Dolle, P. 2009. Developmental expression of retinoic acid receptors (RARs). *Nucl Recept Signal* **7**:e006-

Dong, D. and Noy, N. 1998. Heterodimer formation by retinoid X receptor: regulation by ligands and by the receptor's self-association properties. *Biochemistry* **37**:10691-10700.

Duprez, D.M., Coltey, M., Amthor, H., Brickell, P.M., and Tickle, C. 1996. Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev Biol* **174**:448-452.

Duvic, M., Martin, A.G., Kim, Y., Olsen, E., Wood, G.S., Crowley, C.A., and Yocum, R.C. 2001. Phase 2 and 3 clinical trial of oral bexarotene (Targretin capsules) for the treatment of refractory or persistent early-stage cutaneous T-cell lymphoma. *Arch Dermatol* **137**:581-593.

Eastman, Q. and Grosschedl, R. 1999. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr Opin Cell Biol* **11**:233-240.

Eckner, R., Yao, T.P., Oldread, E., and Livingston, D.M. 1996. Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. *Genes Dev* **10**:2478-2490.

Edmondson, D.G., Lyons, G.E., Martin, J.F., and Olson, E.N. 1994. Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120**:1251-1263.

Edmondson, D.G. and Olson, E.N. 1990. 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* **4**:1450-

Egea, P.F., Mitschler, A., and Moras, D. 2002. Molecular recognition of agonist ligands by RXRs. *Mol Endocrinol* **16**:987-997.

Egea, P.F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., and Moras, D. 2000. Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. *EMBO J* **19**:2592-2601.

Epstein, J.A., Shapiro, D.N., Cheng, J., Lam, P.Y., and Maas, R.L. 1996. Pax3 modulates expression of the c-Met receptor during limb muscle development. *Proc Natl Acad Sci U S A* **93**:4213-4218.

Evans, M.J. and Kaufman, M.H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**:154-156.

Fan, C.M., Lee, C.S., and Tessier-Lavigne, M. 1997. A role for WNT proteins in induction of dermomyotome. *Dev Biol* **191**:160-165.

Forman, B.M., Umesono, K., Chen, J., and Evans, R.M. 1995. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* **81**:541-550.

Froeschle, A., Alric, S., Kitzmann, M., Carnac, G., Aurade, F., Rochette-Egly, C., and Bonnieu, A. 1998. Retinoic acid receptors and muscle b-HLH proteins: partners in retinoid-induced myogenesis. *Oncogene* **16**:3369-3378.

Gampe, R.T., Jr., Montana, V.G., Lambert, M.H., Miller, A.B., Bledsoe, R.K., Milburn, M.V., Kliewer, S.A., Willson, T.M., and Xu, H.E. 2000a. Asymmetry in the PPARgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. *Mol Cell* **5**:545-555.

Gampe, R.T., Jr., Montana, V.G., Lambert, M.H., Wisely, G.B., Milburn, M.V., and Xu, H.E. 2000b. Structural basis for autorepression of retinoid X receptor by tetramer formation and the AF-2 helix. *Genes Dev* **14**:2229-2241.

Germain, P., Iyer, J., Zechel, C., and Gronemeyer, H. 2002. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. *Nature* **415**:187-192.

Gokhale, P.J., Giesberts, A.M., and Andrews, P.W. 2000. Brachyury is expressed by human teratocarcinoma cells in the absence of mesodermal differentiation. *Cell Growth Differ* **11**:157-162.

Gordon, M.D. and Nusse, R. 2006. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* **281**:22429-22433.

Goulding, M., Lumsden, A., and Paquette, A.J. 1994. Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* **120**:957-971.

Gramolini, A.O. and Jasmin, B.J. 1999. Expression of the utrophin gene during myogenic differentiation. *Nucleic Acids Res* **27**:3603-3609.

Green, S., Kumar, V., Theulaz, I., Wahli, W., and Chambon, P. 1988. The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene specificity. *EMBO J* **7**:3037-3044.

Grifone, R., Demignon, J., Houbron, C., Souil, E., Niro, C., Seller, M.J., Hamard, G., and Maire, P. 2005. Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. *Development* **132**:2235-2249.

Gu, W. and Roeder, R.G. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**:595-606.

Guenther, M.G., Lane, W.S., Fischle, W., Verdin, E., Lazar, M.A., and Shiekhattar, R. 2000. A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. *Genes Dev* **14**:1048-1057.

Gustafsson, M.K., Pan, H., Pinney, D.F., Liu, Y., Lewandowski, A., Epstein, D.J., and Emerson, C.P., Jr. 2002. Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. *Genes Dev* **16**:114-126.

Hasty, P., Bradley, A., Morris, J.H., Edmondson, D.G., Venuti, J.M., Olson, E.N., and Klein, W.H. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**:501-506.

Hescheler, J., Fleischmann, B.K., Lentini, S., Maltsev, V.A., Rohwedel, J., Wobus, A.M., and Addicks, K. 1997. Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. *Cardiovasc Res* **36**:149-162.

Hewson, Q.C., Lovat, P.E., Pearson, A.D., and Redfern, C.P. 2002. Retinoid signalling and gene expression in neuroblastoma cells: RXR agonist and antagonist effects on CRABP-II and RARbeta expression. *J Cell Biochem* **87**:284-291.

Hu, J.S., Olson, E.N., and Kingston, R.E. 1992. HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. *Mol Cell Biol* **12**:1031-1042.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B.G., and Kemler, R. 1996. Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech Dev* **59**:3-10.

IJpenberg, A., Tan, N.S., Gelman, L., Kersten, S., Seydoux, J., Xu, J., Metzger, D., Canaple, L., Chambon, P., Wahli, W., and Desvergne, B. 2004. In vivo activation of PPAR target genes by RXR homodimers. *EMBO J* **23**:2083-2091.

Ikeda, M., Spanjaard, R.A., Noordhoek, E.W., Kawaguchi, A., Onaya, T., and Chin, W.W. 1998. Ligand-inducible retinoid X receptor-mediated protein: DNA interactions in the retinoic acid receptor beta2 gene promoter in vivo. *Mol Cell Endocrinol* **136**:109-118.

Jaynes, J.B. and O'Farrell, P.H. 1991. Active repression of transcription by the engrailed homeodomain protein. *EMBO J* **10**:1427-1433.

Jones-Villeneuve, E.M., McBurney, M.W., Rogers, K.A., and Kalnins, V.I. 1982. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J Cell Biol* **94**:253-262.

Jones-Villeneuve, E.M., Rudnicki, M.A., Harris, J.F., and McBurney, M.W. 1983. Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Mol Cell Biol* **3**:2271-2279.

Karanam, B., Jiang, L., Wang, L., Kelleher, N.L., and Cole, P.A. 2006. Kinetic and mass spectrometric analysis of p300 histone acetyltransferase domain autoacetylation. *J Biol Chem* **281**:40292-40301.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., and Tajbakhsh, S. 2004. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* **431**:466-471.

Kastner, P., Grondona, J.M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J.L., Dolle, P., and Chambon, P. 1994. Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**:987-1003.

Kastner, P., Mark, M., and Chambon, P. 1995. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* **83**:859-869.

Kawaguchi, R., Yu, J., Honda, J., Hu, J., Whitelegge, J., Ping, P., Wiita, P., Bok, D., and Sun, H. 2007. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. *Science* **315**:820-825.

Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., De La, P.J., Johnson, R.L., Izpisua Belmonte, J.C., and Tabin, C.J. 1998. Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* **280**:1274-1277.

Kennedy, K.A., Porter, T., Mehta, V., Ryan, S.D., Price, F., Peshdary, V., Karamboulas, C., Savage, J., Drysdale, T.A., Li, S.C., Bennett, S.A., and Skerjanc, I.S. 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-

Kersten, S., Dawson, M.I., Lewis, B.A., and Noy, N. 1996. Individual subunits of heterodimers comprised of retinoic acid and retinoid X receptors interact with their ligands independently. *Biochemistry* **35**:3816-3824.

Kersten, S., Kelleher, D., Chambon, P., Gronemeyer, H., and Noy, N. 1995. Retinoid X receptor alpha forms tetramers in solution. *Proc Natl Acad Sci U S A* **92**:8645-8649.

Kraner, S.D., Rich, M.M., Sholl, M.A., Zhou, H., Zorc, C.S., Kallen, R.G., and Barchi, R.L. 1999. Interaction between the skeletal muscle type 1 Na+ channel promoter E-box and an upstream repressor element. Release of repression by myogenin. *J Biol Chem* **274**:8129-8136.

Kumar, V. and Chambon, P. 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55**:145-156.

Kurokawa, R., DiRenzo, J., Boehm, M., Sugarman, J., Gloss, B., Rosenfeld, M.G., Heyman, R.A., and Glass, C.K. 1994. Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. *Nature* **371**:528-531.

Lala, D.S., Mukherjee, R., Schulman, I.G., Koch, S.S., Dardashti, L.J., Nadzan, A.M., Croston, G.E., Evans, R.M., and Heyman, R.A. 1996. Activation of specific RXR heterodimers by an antagonist of RXR homodimers. *Nature* **383**:450-453.

Lehmann, J.M., Jong, L., Fanjul, A., Cameron, J.F., Lu, X.P., Haefner, P., Dawson, M.I., and Pfahl, M. 1992. Retinoids selective for retinoid X receptor response pathways. *Science* **258**:1944-1946.

Leid, M., Kastner, P., and Chambon, P. 1992. Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends Biochem Sci* **17**:427-433.

Leifer, D., Krainc, D., Yu, Y.T., McDermott, J., Breitbart, R.E., Heng, J., Neve, R.L., Kosofsky, B., Nadal-Ginard, B., and Lipton, S.A. 1993. MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex. *Proc Natl Acad Sci U S A* **90**:1546-1550.

Lengqvist, J., Mata, D.U., Bergman, A.C., Willson, T.M., Sjovall, J., Perlmann, T., and Griffiths, W.J. 2004. Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. *Mol Cell Proteomics* **3**:692-703.

Li, H. and Capetanaki, Y. 1993. Regulation of the mouse desmin gene: transactivated by MyoD, myogenin, MRF4 and Myf5. *Nucleic Acids Res* **21**:335-343.

Li, J., Wang, J., Wang, J., Nawaz, Z., Liu, J.M., Qin, J., and Wong, J. 2000. Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. *EMBO J* **19**:4342-4350.

Lin, H., Yutzey, K.E., and Konieczny, S.F. 1991. Muscle-specific expression of the troponin I gene requires interactions between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors. *Mol Cell Biol* **11**:267-280.

Lohnes, D., Kastner, P., Dierich, A., Mark, M., LeMeur, M., and Chambon, P. 1993. Function of retinoic acid receptor gamma in the mouse. *Cell* **73**:643-658.

Lufkin, T., Lohnes, D., Mark, M., Dierich, A., Gorry, P., Gaub, M.P., LeMeur, M., and Chambon, P. 1993. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. *Proc Natl Acad Sci U S A* **90**:7225-7229.

Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R., and Sigler, P.B. 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352**:497-505.

Lyons, G.E., Micales, B.K., Schwarz, J., Martin, J.F., and Olson, E.N. 1995. Expression of mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. *J Neurosci* **15**:5727-5738.

Maden, M., Gale, E., Kostetskii, I., and Zile, M. 1996. Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr Biol* **6**:417-426.

Maden, M., Graham, A., Zile, M., and Gale, E. 2000. Abnormalities of somite development in the absence of retinoic acid. *Int J Dev Biol* **44**:151-159.

Mader, S., Kumar, V., de Verneuil, H., and Chambon, P. 1989. Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* **338**:271-274.

Mangelsdorf, D.J., Borgmeyer, U., Heyman, R.A., Zhou, J.Y., Ong, E.S., Oro, A.E., Kakizuka, A., and Evans, R.M. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev* **6**:329-344.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. 1995. The nuclear receptor superfamily: the second decade. *Cell* **83**:835-839.

Mangelsdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E.S., and Evans, R.M. 1991. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* **66**:555-561.

Mankoo, B.S., Collins, N.S., Ashby, P., Grigorieva, E., Pevny, L.H., Candia, A., Wright, C.V., Rigby, P.W., and Pachnis, V. 1999. Mox2 is a component of the genetic hierarchy controlling limb muscle development. *Nature* **400**:69-73.

Mankoo, B.S., Skuntz, S., Harrigan, I., Grigorieva, E., Candia, A., Wright, C.V., Arnheiter, H., and Pachnis, V. 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.

Mark, M., Ghyselinck, N.B., and Chambon, P. 2006. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu Rev Pharmacol Toxicol* **46**:451-480.

Maroto, M., Reshef, R., Munsterberg, A.E., Koester, S., Goulding, M., and Lassar, A.B. 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell* **89**:139-148.

Marsh, D.R., Carson, J.A., Stewart, L.N., and Booth, F.W. 1998. Activation of the skeletal alpha-actin promoter during muscle regeneration. *J Muscle Res Cell Motil* **19**:897-907.

Martin, G.R. and Evans, M.J. 1974. The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture. *Cell* **2**:163-172.

Martin, G.R. and Evans, M.J. 1975. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proc Natl Acad Sci U S A* **72**:1441-1445.

McBurney, M.W., Jones-Villeneuve, E.M., Edwards, M.K., and Anderson, P.J. 1982. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* **299**:165-167.

McDermott, A., Gustafsson, M., Elsam, T., Hui, C.C., Emerson, C.P., Jr., and Borycki, A.G. 2005. Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. *Development* **132**:345-357.

Mertz, J.R., Shang, E., Piantedosi, R., Wei, S., Wolgemuth, D.J., and Blaner, W.S. 1997. Identification and characterization of a stereospecific human enzyme that catalyzes 9-cis-retinol oxidation. A possible role in 9-cis-retinoic acid formation. *J Biol Chem* **272**:11744-11749.

Mic, F.A., Molotkov, A., Benbrook, D.M., and Duester, G. 2003. Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proc Natl Acad Sci U S A* **100**:7135-7140.

Minucci, S., Leid, M., Toyama, R., Saint-Jeannet, J.P., Peterson, V.J., Horn, V., Ishmael, J.E., Bhattacharyya, N., Dey, A., Dawid, I.B., and Ozato, K. 1997. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. *Mol Cell Biol* **17**:644-655.

Montross, W.T., Ji, H., and McCrea, P.D. 2000. A beta-catenin/engrailed chimera selectively suppresses Wnt signaling. *J Cell Sci* **113 (Pt 10)**:1759-1770.

Morriss-Kay, G.M. and Ward, S.J. 1999. Retinoids and mammalian development. *Int Rev Cytol* **188**:73-131.

Mummery, C.L., van den Brink, C.E., and de Laat, S.W. 1987. Commitment to differentiation induced by retinoic acid in P19 embryonal carcinoma cells is cell cycle dependent. *Dev Biol* **121**:10-19.

Munsterberg, A.E., Kitajewski, J., Bumcrot, D.A., McMahon, A.P., and Lassar, A.B. 1995. Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev* **9**:2911-2922.

Murray, S.S., Murray, E.J., Glackin, C.A., and Urist, M.R. 1993. Bone morphogenetic protein inhibits differentiation and affects expression of helix-loop-helix regulatory molecules in myoblastic cells. *J Cell Biochem* **53**:51-60.

Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., and . 1989. Interactions between

heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**:537-544.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I., and Nabeshima, Y. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**:532-535.

Nau, H., Blaner, W.S., Agadir, A., and . 1999. Retinoids: the biochemical and molecular basis of vitamin A and retinoid action. *Germany: Springer-Verlag Berlin HeidelBerg New York*

Naya, F.J. and Olson, E. 1999. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Curr Opin Cell Biol* **11**:683-688.

Niederreither, K., Abu-Abed, S., Schuhbaur, B., Petkovich, M., Chambon, P., and Dolle, P. 2002. Genetic evidence that oxidative derivatives of retinoic acid are not involved in retinoid signaling during mouse development. *Nat Genet* **31**:84-88.

Niederreither, K. and Dolle, P. 2008. Retinoic acid in development: towards an integrated view. *Nat Rev Genet* **9**:541-553.

Niederreither, K., Subbarayan, V., Dolle, P., and Chambon, P. 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat Genet* **21**:444-448.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., and Nakatani, Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**:953-959.

Olson, E.N. 1990. MyoD family: a paradigm for development? *Genes Dev* **4**:1454-1461.

Olson, E.N. and Klein, W.H. 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev* **8**:1-8.

Ott, M.O., Bober, E., Lyons, G., Arnold, H., and Buckingham, M. 1991. Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111**:1097-1107.

Parker, M.H., Perry, R.L., Fauteux, M.C., Berkes, C.A., and Rudnicki, M.A. 2006. MyoD synergizes with the E-protein HEB beta to induce myogenic differentiation. *Mol Cell Biol* **26**:5771-5783.

Patapoutian, A., Yoon, J.K., Miner, J.H., Wang, S., Stark, K., and Wold, B. 1995. Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121**:3347-3358.

Perissi, V., Staszewski, L.M., McInerney, E.M., Kurokawa, R., Krones, A., Rose, D.W., Lambert, M.H., Milburn, M.V., Glass, C.K., and Rosenfeld, M.G. 1999. Molecular determinants of nuclear receptor-corepressor interaction. *Genes Dev* **13**:3198-3208.

Perlmann, T. and Jansson, L. 1995. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes Dev* **9**:769-782.

Petropoulos, H., Gianakopoulos, P.J., Ridgeway, A.G., and Skerjanc, I.S. 2004. Disruption of Meox or Gli activity ablates skeletal myogenesis in P19 cells. *J Biol Chem* **279**:23874-23881.

Petropoulos, H. and Skerjanc, I.S. 2002. Beta-catenin is essential and sufficient for skeletal myogenesis in P19 cells. *J Biol Chem* **277**:15393-15399.

Pijnappel, W.W., Hendriks, H.F., Folkers, G.E., van den Brink, C.E., Dekker, E.J., Edelenbosch, C., van der Saag, P.T., and Durston, A.J. 1993. The retinoid ligand 4-oxo-retinoic acid is a highly active modulator of positional specification. *Nature* **366**:340-344.

Polesskaya, A., Duquet, A., Naguibneva, I., Weise, C., Vervisch, A., Bengal, E., Hucho, F., Robin, P., and Harel-Bellan, A. 2000. CREB-binding protein/p300 activates MyoD by acetylation. *J Biol Chem* **275**:34359-34364.

Polesskaya, A., Naguibneva, I., Fritsch, L., Duquet, A., Ait-Si-Ali, S., Robin, P., Vervisch, A., Pritchard, L.L., Cole, P., and Harel-Bellan, A. 2001. CBP/p300 and muscle differentiation: no HAT, no muscle. *EMBO J* **20**:6816-6825.

Pourquie, O., Fan, C.M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M., and Le Douarin, N.M. 1996. Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84**:461-471.

Pratt, M.A., Crippen, C., Hubbard, K., and Menard, M. 1998. Deregulated expression of the retinoid X receptor alpha prevents muscle differentiation in P19 embryonal carcinoma cells. *Cell Growth Differ* **9**:713-722.

Pratt, M.A., Kralova, J., and McBurney, M.W. 1990. A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. *Mol Cell Biol* **10**:6445-6453.

Puri, P.L., Avantaggiati, M.L., Balsano, C., Sang, N., Graessmann, A., Giordano, A., and Levrero, M. 1997. p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription. *EMBO J* **16**:369-383.

Puri, P.L., Sartorelli, V., Yang, X.J., Hamamori, Y., Ogryzko, V.V., Howard, B.H., Kedes, L., Wang, J.Y., Graessmann, A., Nakatani, Y., and Levrero, M. 1997. Differential roles of p300 and PCAF acetyltransferases in muscle differentiation. *Mol Cell* **1**:35-45.

Qu, L. and Tang, X. 2010. Bexarotene: a promising anticancer agent. *Cancer Chemother Pharmacol* **65**:201-205.

Ricaud, S., Vernus, B., and Bonnieu, A. 2005. Response of human rhabdomyosarcoma cell lines to retinoic acid: relationship with induction of differentiation and retinoic acid sensitivity. *Exp Cell Res* **311**:192-204.

Ridgeway, A.G. and Skerjanc, I.S. 2001. Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. *J Biol Chem* **276**:19033-19039.

Ridgeway, A.G., Wilton, S., and Skerjanc, I.S. 2000. Myocyte enhancer factor 2C and myogenin up-regulate each other's expression and induce the development of skeletal muscle in P19 cells. *J Biol Chem* **275**:41-46.

Rohwedel, J., Guan, K., and Wobus, A.M. 1999. Induction of cellular differentiation by retinoic acid in vitro. *Cells Tissues Organs* **165**:190-202.

Romert, A., Tuvendal, P., Simon, A., Dencker, L., and Eriksson, U. 1998. The identification of a 9-cis retinol dehydrogenase in the mouse embryo reveals a pathway for synthesis of 9-cis retinoic acid. *Proc Natl Acad Sci U S A* **95**:4404-4409.

Rosenfeld, M.G., Lunyak, V.V., and Glass, C.K. 2006. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* **20**:1405-1428.

Roth, J.F., Shikama, N., Henzen, C., Desbaillets, I., Lutz, W., Marino, S., Wittwer, J., Schorle, H., Gassmann, M., and Eckner, R. 2003. Differential role of p300 and CBP acetyltransferase during myogenesis: p300 acts upstream of MyoD and Myf5. *EMBO J* **22**:5186-5196.

Roy, B., Taneja, R., and Chambon, P. 1995. Synergistic activation of retinoic acid (RA)-responsive genes and induction of embryonal carcinoma cell differentiation by an RA receptor alpha (RAR alpha)-, RAR beta-, or RAR gamma-selective ligand in combination with a retinoid X receptor-specific ligand. *Mol Cell Biol* **15**:6481-6487.

Rudnicki, M.A., Braun, T., Hinuma, S., and Jaenisch, R. 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.

Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H., and Jaenisch, R. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**:1351-1359.

Sapin, V., Dolle, P., Hindelang, C., Kastner, P., and Chambon, P. 1997. Defects of the chorioallantoic placenta in mouse RXRalpha null fetuses. *Dev Biol* **191**:29-41.

Sartorelli, V. and Caretti, G. 2005. Mechanisms underlying the transcriptional regulation of skeletal myogenesis. *Curr Opin Genet Dev* **15**:528-535.

Sartorelli, V., Huang, J., Hamamori, Y., and Kedes, L. 1997. Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. *Mol Cell Biol* **17**:1010-1026.

Sartorelli, V., Puri, P.L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J.Y., and Kedes, L. 1999. Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. *Mol Cell* **4**:725-734.

Sassoon, D., Lyons, G., Wright, W.E., Lin, V., Lassar, A., Weintraub, H., and Buckingham, M. 1989. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature* **341**:303-307.

Schlange, T., Andree, B., Arnold, H.H., and Brand, T. 2000. BMP2 is required for early heart development during a distinct time period. *Mech Dev* **91**:259-270.

Schultheiss, T.M., Burch, J.B., and Lassar, A.B. 1997. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev* **11**:451-462.

Shield, M.A., Haugen, H.S., Clegg, C.H., and Hauschka, S.D. 1996. E-box sites and a proximal regulatory region of the muscle creatine kinase gene differentially regulate expression in diverse skeletal muscles and cardiac muscle of transgenic mice. *Mol Cell Biol* **16**:5058-5068.

Shiohara, M., el Deiry, W.S., Wada, M., Nakamaki, T., Takeuchi, S., Yang, R., Chen, D.L., Vogelstein, B., and Koeffler, H.P. 1994. Absence of WAF1 mutations in a variety of human malignancies. *Blood* **84**:3781-3784.

Simon, A.M. and Burden, S.J. 1993. An E box mediates activation and repression of the acetylcholine receptor delta-subunit gene during myogenesis. *Mol Cell Biol* **13**:5133-5140.

Skerjanc, I.S. 1999. Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. *Trends Cardiovasc Med* **9**:139-143.

Smith, S.T. and Jaynes, J.B. 1996. A conserved region of engrailed, shared among all en-, gsc-, Nk1-, Nk2- and msh-class homeoproteins, mediates active transcriptional repression in vivo. *Development* **122**:3141-3150.

Sorrentino, V., Pepperkok, R., Davis, R.L., Ansorge, W., and Philipson, L. 1990. Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. *Nature* **345**:813-815.

Sporn, M.B. and Roberts, A.B. 1994. Cervical dysplasia regression induced by all-trans-retinoic acid. *J Natl Cancer Inst* **86**:476-477.

Sucov, H.M., Dyson, E., Gumeringer, C.L., Price, J., Chien, K.R., and Evans, R.M. 1994. RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev* **8**:1007-1018.

Sun, S.Y. and Lotan, R. 2002. Retinoids and their receptors in cancer development and chemoprevention. *Crit Rev Oncol Hematol* **41**:41-55.

Szanto, A., Narkar, V., Shen, Q., Uray, I.P., Davies, P.J., and Nagy, L. 2004. Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death Differ* **11 Suppl 2**:S126-S143.

Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M., and Cossu, G. 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.

Tajbakhsh, S. and Buckingham, M.E. 1994. Mouse limb muscle is determined in the absence of the earliest myogenic factor myf-5. *Proc Natl Acad Sci U S A* **91**:747-751.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell* **89**:127-138.

Tanaka, T. and De Luca, L.M. 2009. Therapeutic potential of "rexinoids" in cancer prevention and treatment. *Cancer Res* **69**:4945-4947.

Tanaka, T., Suh, K.S., Lo, A.M., and De Luca, L.M. 2007. p21WAF1/CIP1 is a common transcriptional target of retinoid receptors: pleiotropic regulatory mechanism through retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimer and RXR/RXR homodimer. *J Biol Chem* **282**:29987-29997.

Thayer, M.J., Tapscott, S.J., Davis, R.L., Wright, W.E., Lassar, A.B., and Weintraub, H. 1989. Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58**:241-248.

Thomas, K.R. and Capecchi, M.R. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**:503-512.

Tolkunova, E.N., Fujioka, M., Kobayashi, M., Deka, D., and Jaynes, J.B. 1998. Two distinct types of repression domain in engrailed: one interacts with the groucho corepressor and is preferentially active on integrated target genes. *Mol Cell Biol* **18**:2804-2814.

Umesono, K. and Evans, R.M. 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57**:1139-1146.

van der Heyden, M.A. and Defize, L.H. 2003. Twenty one years of P19 cells: what an embryonal carcinoma cell line taught us about cardiomyocyte differentiation. *Cardiovasc Res* **58**:292-302.

Venuti, J.M., Morris, J.H., Vivian, J.L., Olson, E.N., and Klein, W.H. 1995. Myogenin is required for late but not early aspects of myogenesis during mouse development. *J Cell Biol* **128**:563-576.

Vivat, V., Zechel, C., Wurtz, J.M., Bourguet, W., Kagechika, H., Umemiya, H., Shudo, K., Moras, D., Gronemeyer, H., and Chambon, P. 1997. A mutation mimicking ligand-induced conformational change yields a constitutive RXR that senses allosteric effects in heterodimers. *EMBO J* **16**:5697-5709.

Vo, N. and Goodman, R.H. 2001. CREB-binding protein and p300 in transcriptional regulation. *J Biol Chem* **276**:13505-13508.

Wakayama, S., Hikichi, T., Suetsugu, R., Sakaide, Y., Bui, H.T., Mizutani, E., and Wakayama, T. 2007. Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. *Stem Cells* **25**:986-993.

Weintraub, H. 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75**:1241-1244.

Westin, S., Kurokawa, R., Nolte, R.T., Wisely, G.B., McInerney, E.M., Rose, D.W., Milburn, M.V., Rosenfeld, M.G., and Glass, C.K. 1998. Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature* **395**:199-202.

Wheeler, M.T., Snyder, E.C., Patterson, M.N., and Swoap, S.J. 1999. An E-box within the MHC IIB gene is bound by MyoD and is required for gene expression in fast muscle. *Am J Physiol* **276**:C1069-C1078.

Williams, B.A. and Ordahl, C.P. 1994. Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120**:785-796.

Willy, P.J. and Mangelsdorf, D.J. 1997. Unique requirements for retinoid-dependent transcriptional activation by the orphan receptor LXR. *Genes Dev* **11**:289-298.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., and Mangelsdorf, D.J. 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev* **9**:1033-1045.

Wilton, S. and Skerjanc, I. 1999. Factors in serum regulate muscle development in P19 cells. *In Vitro Cell Dev Biol Anim* **35**:175-177.

Wodarz, A. and Nusse, R. 1998. Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol* **14**:59-88.

Wong, M.W., Pisegna, M., Lu, M.F., Leibham, D., and Perry, M. 1994. Activation of Xenopus MyoD transcription by members of the MEF2 protein family. *Dev Biol* **166**:683-695.

Wu, K., Kim, H.T., Rodriquez, J.L., Hilsenbeck, S.G., Mohsin, S.K., Xu, X.C., Lamph, W.W., Kuhn, J.G., Green, J.E., and Brown, P.H. 2002. Suppression of mammary tumorigenesis in transgenic mice by the RXR-selective retinoid, LGD1069. *Cancer Epidemiol Biomarkers Prev* **11**:467-474.

Yang, X.J., Ogryzko, V.V., Nishikawa, J., Howard, B.H., and Nakatani, Y. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**:319-324.

Yokota, Y. and Ohkubo, H. 1996. 9-cis-retinoic acid induces neuronal differentiation of retinoic acid-nonresponsive embryonal carcinoma cells. *Exp Cell Res* **228**:1-7.

Yu, J. and Thomson, J.A. 2008. Pluripotent stem cell lines. *Genes Dev* **22**:1987-1997.

Yuan, W., Condorelli, G., Caruso, M., Felsani, A., and Giordano, A. 1996. Human p300 protein is a coactivator for the transcription factor MyoD. *J Biol Chem* **271**:9009-9013.

Zhang, J., Hu, X., and Lazar, M.A. 1999. A novel role for helix 12 of retinoid X receptor in regulating repression. *Mol Cell Biol* **19**:6448-6457.

Zhang, X.K., Lehmann, J., Hoffmann, B., Dawson, M.I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. 1992. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature* **358**:587-591.

Zhao, W.X., Tian, M., Zhao, B.X., Li, G.D., Liu, B., Zhan, Y.Y., Chen, H.Z., and Wu, Q. 2007. Orphan receptor TR3 attenuates the p300-induced acetylation of retinoid X receptor-alpha. *Mol Endocrinol* **21**:2877-2889.

CHAPTER 5: APPENDIX

Supplementary Table 1. Reagents and Suppliers

Reagent	Cat. No.	Supplier
P19 cells	CRL-1825	ATCC
D3 ES cells	CRL-1934	ATCC
MF20 hybridoma cells	N/A	Developmental Studies Hybridoma Bank
F5D hybridoma cells	N/A	Developmental Studies Hybridoma Bank
Minimum Essential Medium α	12000-022	Invitrogen-GIBCO
Dulbecco's Modified Eagle Medium	12800-017	Invitrogen-GIBCO
D-MEM F12	11330-057	Gibco/Invitrogen
Penicillin G	P-7794	Sigma
Streptomycin Sulfate	11860-038	Gibco/Invitrogen
Fetal Bovine Serum	095150	Multicell-Wisent
Bovine Calf Serum	074150	Multicell-Wisent
N2 Supplement	17502048	Invitrogen
Non-essential amino acids	11140	Gibco-Invitrogen
LIF	ESG1107	Chemicon-Millipore
TTNPB	T3757	Sigma-Aldrich
All-trans Retinoic Acid	R2625	Sigma-Aldrich
DMSO	D2650	Sigma
LGD1069	B-2422	LC Laboratories
Ro 41-5253	GR-110	Biomol International
β-mercaptoethanol	O3446I100	Fisher Scientific
Bio-Rad Protein Assay Dye Reagent Concentrate	500-0006	Bio-Rad
Precision Plus Protein Dual Color Standards (MW ladder)	161-0374	Bio-Rad
Immun-Blot PVDF Membrane	162-0177	Bio-Rad
Western Lightning™ Chemiluminescence	NEL105	Perkin Elmer
NP-40	9036-19-5	Bio Basic Inc
Tween 20	BP337-500	Fisher Scientific
RNeasy Mini kit (50)	74104	Qiagen
Total RNA Kit I	R 6834-01	Omega
dNTP Mix	PRU1515	Promega
GoTaq® flexi DNA polymerase PCR Kit	PRM8295	Promega
High Capacity cDNA Reverse Transcription Kit	4368841	ABI
Cycle Pure Kit	D 6492-02	Omega
Formaldehyde	BDH 0500_1LP	BDH
Protease Inhibitor Cocktail Set III, EDTA-Free	539134	Calbiochem

Proteinase K	03115879001	Roche
Triton X-100	H5142	Promega
Protein-A agarose/Salmon Sperm beads	16-157	Millipore
HotStarTaq DNA Polymerase kit	203205	Qiagen
Glycerol	4750	OmniPur/EMD
Hoechst 33258 pentahydrate	H-21491	Molecular Probes

Supplementary Table 2. Antibodies

Antibody	**Cat. No.**	**Supplier**
Anti-p300 (N-15)	sc-584	Santa Cruz
Anti-RAR-b	sc-552	Santa Cruz
Anti-RXR-a	sc-553	Santa Cruz
Anti-MyoD (M-318)	sc-760	Santa Cruz
Anti-RAR-a	sc-551	Santa Cruz
Anti-β-catenin	06-734	Millipore
Anti-mouse IgG horseradish heroxidase conjugate 2˚ Ab	NA931	GE Healthcare
Anti-rabbit IgG horseradish peroxidase conjugate 2˚ Ab	NA9340	GE Healthcare
Alexa Flour®488 goat anti-mouse 2˚ Ab	A11001	Invitrogen
Alexa Flour®488 goat anti-rabbit 2˚ Ab	A11008	Invitrogen
Alexa Flor®594 donkey anti-mouse 2˚ Ab	A21203	Invitrogen

Supplementary Table 3. Primers used for Real-Time RT-PCR

Gene	Forward Primer	Reverse Primer
Myf5	GGCATGCCTGAATGTAACAGC	CAATCCAAGCTGGACACGGA
MyoD	TGCCTTCTACGCACCTGGA	ATCATGCCATCAGAGCAGTTGG
Meox1	GAGAGGTCAGACAACCAGGAG	CGTAGCTGCTCCTTGGTGAAG
Meox2	AAAGACAGGTGAAAGTGTGGTT	TCTGACGGAAGAAGCGTTC
Pax3	GCTGCAGTCAGAGACTGGAAC	GAAAGGCACTTTGTCCATACTGC
GAPDH	TCGGTGTGAACGGATTTG	GGTCTCGCTCCTGGAAGA

Supplementary Table 4. Primers used for ChIP Assay

Gene	Forward Primer	Reverse Primer
Pax3.1	ACAGGGTAAAACAATGTGTGGA	TTGAAGCCAGCCTGACCTAT
Pax3.2	AGTGGAGCGCACCTCTGT	CTACAAACCCTTAATGACAAACG
Meox1.1	CATGAGTTCAAGCCTCAGCA	CCAGAGATACGCTTGGTGTC
Meox1.2	GAGGCCTAGCTTCAGCTCCT	TGAAATGCCTGATCTGACACA
RAR-β	GGGAGTTTTTAAGCGCTGTGA	GGAGCAGCTCACTTCCTACC
Myf5	AGAAGCGGCACACGTTGTA	TGGAGAAGAGTGAACATCCTTG