# Activation of the Retinoid X Receptor Augments the Expression of Akt2 to Enhance Myogenic Differentiation

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**Abstract**

Cachexia or muscle atrophy is a condition that is associated with a variety of diseases such as chronic heart failure and cancer. In North America, Europe and Japan, more than 8 million patients suffer from cachexia, and it is estimated that cachexia is the cause of death in 30% of cancer patients. Unfortunately, there is no available treatment for cachexia. Bexarotene, a retinoid X receptor (RXR) agonist, is a FDA approved drug used to treat cancer and is able to induce myogenic differentiation in embryonic stem cells. In this study, we investigated the mechanism by which bexarotene enhances myogenic differentiation. The Akt signaling pathway is required for myogenesis and thus we examined its involvement in bexarotene-enhanced myogenic differentiation. We showed that bexarotene, through the activation of RXR signaling, regulates Akt2 expression to enhance myoblast differentiation and fusion. Additionally, we showed that Akt2, but neither Akt1 nor Akt3, is required for bexarotene-enhanced differentiation. Furthermore, we showed that the activation of RXR signaling by bexarotene correlates with a specific histone acetylation mark at the Akt2 locus. More importantly, we demonstrated that bexarotene is able to rescue myoblast differentiation in an *in vitro* cachexia system. Taken together, our data revealed the significance of Akt2 in bexarotene-enhanced myogenic differentiation and the potential of using bexarotene as a treatment for cachexia.

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**List of Abbreviation**

bHLH: Basic helix-loop-helix

ChIP: Chromatin Immunoprecipitation

ChIP-seq: Chromatin Immunoprecipitation sequencing

DM: Differentiation medium

DMEM: Dulbecco's Modified Eagle Medium

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FDA: US Food and Drug Administration

FoxO: Forkhead box O

GCN5: General Control of Amino Acid synthesis 5-Like 2

GEO: Gene Expression Omnibus database

GM: Growth medium

H3K18ac: Histone 3 lysine residue 18 acetylation

H3K27ac: Histone 3 lysine residue 27 acetylation

H3K27me1: Histone 3 lysine residue 27 monomethylation

H3K27me3: Histone 3 lysine residue 27 trimethylation

H3K4me1: Histone 3 lysine residue 4 monomethylation

H3K4me3: Histone 3 lysine residue 4 trimethylation

HAT: histone acetyltransferases

HMT: histone methyltransferase

IGF: Insulin-like growth factor

K: lysine

Kb: kilobase

LBD: Ligand binding domain

LLC: Lewis lung carcinoma

Mrf4: Muscle regulatory factor 4

MRFs: Muscle Regulatory Factors

mTOR: Mammalian target of rapamycin

Myf5: Myogenic factor 5

MyHC: Myosine heavy chain

MyoD: Myogenic differentiation antigen

NF-κB: Nuclear factor kappa B

NP-40: Nonidet P40

P38 MAPK: p38 mitogen-activated protein kinases

PBS: Phosphate buffered saline

PBST: Phosphate buffered saline with Tween

PCAF: p300/CBP-associated factor

PI3K: Phosphatidylinositol 3-kinase

PIP3: Phosphatidylinositol-3,4,5-triphosphate

PKB: Protein kinase B

PMSF: Phenylmethylsulfonyl fluoride

PPAR: Peroxisome proliferator-activated receptor

qPCR: Quantitative Polymerase Chain Reaction

RAR: Retinoic acid receptor

RARE: Retinoid acid responsive element

RNA-seq: RNA sequencing

RNAi: RNA interference

RPMI 1640: Roswell Park Memorial Institute 1640 medium

RT-PCR: Reverse-transcription Polymerase Chain Reaction

RXR: Retinoid X receptor

RXRE: Retinoid X responsive element

SDS: Sodium dodecyl sulfate

Ser473: Serine 473

shRNA: Small hairpin RNA

Thr308: Theronine 308

TNFα: Tumor necrosis factor alpha

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## 1. Introduction

Skeletal muscle is the major type of muscle tissue in mammals and its main functions are controlling body movement and generating body heat. Skeletal muscle atrophy, also known as wasting or cachexia, is a condition characterized by a muscle mass loss that is often associated with an underlying primary illness such as AIDS, cancer, and sepsis (Argiles, Moore-Carrasco, Fuster, Busquets, & Lopez-Soriano, 2003; Tisdale, 1997). It is also associated with normal physiological changes like aging, referred to as sarcopenia (Evans, 2010). The consequences of skeletal muscle atrophy range from poor quality of life to increased mortality rate (Fanzani, Conraads, Penna, & Martinet, 2012). It is estimated that cachexia is the cause of death in 30% of cancer patients (von Haehling & Anker, 2010). Moreover, over 50 % of cancer patients die while being diagnosed with cachexia (von Haehling & Anker, 2010). Unfortunately, there is currently no treatment for muscle wasting because the molecular regulation of muscle development and regeneration is not well understood. Understanding the molecular mechanisms underlying myogenesis is key to finding a treatment for muscle wasting and was therefore the focus of our study. We investigated the involvement of the Retinoid X receptor (RXR) signaling in myogenic differentiation by using bexarotene, a RXR agonist.

### 1.1 Skeletal myogenesis and its regulatory factors

Skeletal muscle development is controlled by muscle regulatory factors (MRFs), a family of four genes that include MyoD, myogenin, Myf5 and Mrf4 (Weintraub et al., 1991). These MRFs are basic helix-loop-helix (bHLH) proteins that bind to a specific DNA motif, the E-box (CANNTG), at target muscle gene promoters and enhancers (Sartorelli & Caretti, 2005). The overexpression of any of these genes

in non-myogenic cells converts these cells into skeletal muscle (Emerson, 1990). The first MRF to be discovered was MyoD and its dominance was characterized after successfully converting fibroblasts into myogenic cells (Davis, Weintraub, & Lassar, 1987; Tapscott et al., 1988). The ability of the rest of the MRFs to convert fibroblasts into myogenic cells has been subsequently identified; myogenin (Thayer et al., 1989), Myf5 (Braun, Buschhausen-Denker, Bober, Tannich, & Arnold, 1989) and then Mrf4 (Rhodes & Konieczny, 1989).

While MyoD and Myf5 are expressed during myoblast proliferation, the expression of myogenin appears specifically during differentiation (Edmondson & Olson, 1989; Emerson, 1990). In satellite cells, which are the myogenic precursors in adult muscle, Myf5 and MyoD are the first to be activated after muscle injury, followed by the expression of myogenin (Cornelison & Wold, 1997). Satellite cells are positioned between the plasmalemma and the basal lamina at the muscle fiber surface (Mauro, 1961) and they proliferate and differentiate to form muscle fibers *in vivo* and *in vitro* (Bischoff, 1975).

Knockout studies of MRF genes showed that MyoD, Myf5 and Mrf4 regulate the myogenic specification process while myogenin, Mrf4 and MyoD regulate the differentiation stages. MyoD knockout mice showed normal skeletal muscle development with an elevated expression of Myf5 (Rudnicki et al., 1993), however isolated satellite cells from MyoD knockout mice showed a reduction in their ability to differentiate (Cornelison, Olwin, Rudnicki, & Wold, 2000). Myf5 knockout mice also showed normal skeletal muscle formation without any changes in the expression of the other MRFs, though these mice died perinatally due to rib deformation (Braun, Rudnicki, Arnold, & Jaenisch, 1992). Interestingly, in MyoD and Myf5 double-knockout mice, embryos die after birth due to the lack of skeletal muscle (Rudnicki et

al., 1993). These results, which suggest a redundancy in the function of MyoD and Myf5, have been challenged in one study where double-mutant mice for MyoD and Myf5 showed normal skeletal muscle formation when Mrf4 is intact (Kassar-Duchossoy et al., 2004). This contradiction was justified by the fact that Mrf4 is located upstream of Myf5, so that knocking out Myf5 may affect the regulation of Mrf4 (Olson, Arnold, Rigby, & Wold, 1996). Myogenin knockout mice showed the ability to form myoblasts but not myotubes, and these mice die perinatally (Hasty et al., 1993; Nabeshima et al., 1993; Venuti, Morris, Vivian, Olson, & Klein, 1995). Mrf4 knockout mice showed normal skeletal muscle development with an increased expression of myogenin, which suggest that myogenin can compensate for the loss of Mrf4 but not the other way around (W. Zhang, Behringer, & Olson, 1995). MyoD and Mrf4 double-knockout mice showed the same phenotype as myogenin knockout mice, which suggests that MyoD, myogenin, and Mrf4 are important for the differentiation of myoblasts (Rawls et al., 1998). Myf5 has no function at the stage of differentiation as null mutations in all of MyoD, myogenin, and Mrf4 fail to form muscle fibers (Valdez, Richardson, Klein, & Olson, 2000).

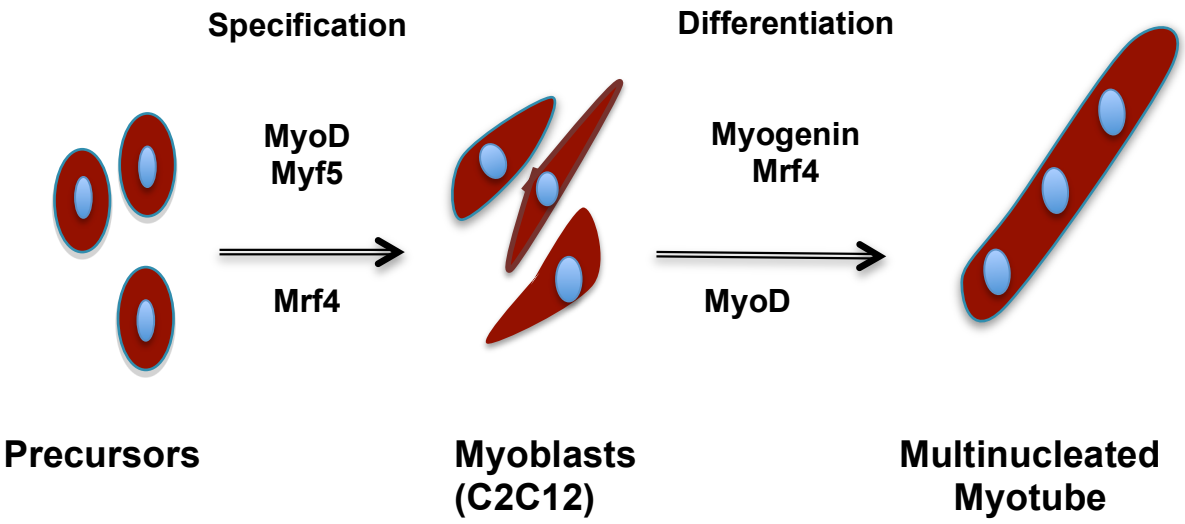


**Figure 1: The involvement of MRFs in myogenic differentiation.** Myf5 and MyoD are involved in the commitment of myogenic precursors into myoblasts, while myogenin and Mrf4 are involved in the process of differentiation. Mrf4 and MyoD also participate in both specification and differentiation processes.

### 1.2 The Retinoid X receptors

The Retinoid X receptors (RXR) are members of the nuclear receptor superfamily, which is a group of transcriptional factors that bind DNA directly at specific sequences, known as response elements, to control target genes (Mangelsdorf et al., 1995). They regulate a variety of cellular functions involved in development, homeostasis and metabolism (Chung & Cooney, 2003). In human and mouse, the nuclear receptor family encompasses 48 and 49 members respectively (Chung & Cooney, 2003). They share a common four-domain structure, which from the C-terminus to N-terminus are the ligand-binding domain (LBD, AF-2), the flexible joint between DBD and LBD (domain D), DNA-binding domain (DBD) and ligand-independent transactivation domain (AF-1) (Burris, Busby, & Griffin, 2012; Robinson-Rechavi, Escriva Garcia, & Laudet, 2003). The ligand-binding domain contains 12 alpha-helices named H1 to H12. In response to ligand binding, the helix

12 moves to create conformational changes that lead to the recruitment of coactivators and/or the release of corepressors proteins (C. K. Glass & Rosenfeld, 2000). The recruitment of coactivators leads to chromatin reorganization that promotes the transcription of target genes (Rochette-Egly, 2003).

RXR has the unique ability of forming heterodimers with 16 other nuclear receptors (Mangelsdorf et al., 1995). This distinct ability makes RXR a potential candidate in diverse signaling pathways. RXR can form both permissive and non-permissive heterodimers, as well as homodimers. RXR ligands activate RXR homodimers and RXR permissive heterodimers (e.g., PPAR-RXR), but not RXR non-permissive heterodimers (e.g., RAR-RXR) (Tanaka & De Luca, 2009). RXR and its partners bind to the DNA-specific consensus sequence 5'-PuGGTCA-3' in the promoters and enhancers of the genes they regulate (Umesono & Evans, 1989). The number of spacer nucleotides in between the two direct repeats of 5'-PuGGTCA determines the DNA binding specificity (Umesono & Evans, 1989). RXR homodimers bind with high affinity to the retinoid X response element (RXRE), which is two TGGTCA repeats separated by one nucleotide (DR1), whereas the RXR-RAR heterodimers bind to the retinoic acid response element (RARE) with 2 or 5 nucleotide separators (DR2 or DR5) (Tanaka & De Luca, 2009). It is possible for RAR-RXR to bind to direct repeats that are separated by DR1, but with a lower affinity as compared to RXR homodimers (X. K. Zhang et al., 1992).

### 1.3 The Retinoid X receptor subtypes

RXR has three different subtypes (α, β, and γ) that are encoded by different genes (Chiba, Clifford, Metzger, & Chambon, 1997). In the mouse, RXRα is on chromosome 2, RXRβ on chromosome 17 and RXRγ on chromosome 1 (Nagata et al., 1995). During mouse development, while RXRα and RXRβ are expressed at high levels, RXRγ is expressed at a low level (Mangelsdorf et al., 1992). In adults, while both RXRα and RXRβ are expressed in most tissues, RXRγ is restricted to only a few tissues (Mangelsdorf et al., 1992). RXRα is expressed largely in the liver, muscle, lung, kidney and spleen; RXRβ is highly expressed in all tissues with the exception of the intestine, testes and liver; and RXRγ is expressed mainly in the skeletal and heart muscles (Mangelsdorf et al., 1992). More recently, RNA sequencing (RNA-seq) data from five different tissues of adult mouse showed that RXRα is the predominant subtype in the liver, skeletal muscle and kidney whereas RXRβ is the predominant in the brain (Barbosa-Morais et al., 2012) (figure 2). The three subtypes are expressed at comparable levels in the heart (Barbosa-Morais et al., 2012).

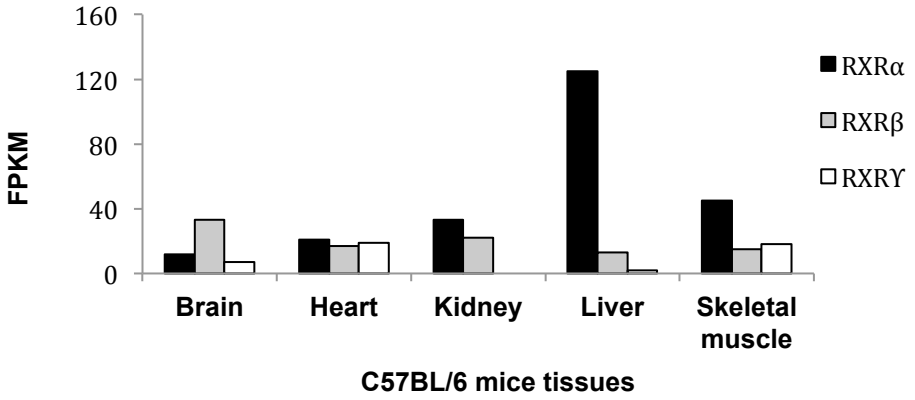


**Figure 2: The expression of RXR subtypes in mouse tissues.** Processed RNA-seq data were obtained from Expression Atlas database (EMBL-EBI) (Kapushesky et al., 2010). The original raw data are available on Gene Expression Omnibus database, GSE41338 (Barbosa-Morais et al., 2012). RXR subtypes expression from brain, heart, kidney, liver and skeletal muscle tissue of adult mice is plotted as the fragments per kilobase per million mappedreads (FPKM), the normalized value for gene expression in RNA-seq data.

Knockout studies showed the importance of RXR in development and also revealed the dominance of RXR subtypes. *RXRα* $^{-/-}$ mice die in utero with ocular and cardiac deformities and *RXRα* $^{-/+}$ show growth deficiencies (Kastner et al., 1994). On the other hand, RXRβ and RXRγ mutations are not as severe as RXRα mutations, which may imply that RXRα can compensate for their loss (Kastner et al., 1994; Krezel et al., 1996; Sucov et al., 1994). More interestingly, a single RXRα allele in *RXRγ* $^{-/-}$ and *RXRβ* $^{-/-}$ mice allowed for all of the fundamental development processes to occur and mice lived to adulthood (Krezel et al., 1996). This compensation by RXRα may explain the normal muscle formation in RXRγ knockout mice (Krezel et al., 1996).

### 1.4 The Retinoid X receptor in development and muscle differentiation

In muscle development, RXR signaling is involved in a ligand-independent and -dependent fashion. Exogenous expression of RXRα enhanced myogenic differentiation in mouse myoblast progenitor cells in a ligand-independent manner (Zhu et al., 2009). It has been shown that RXR as a partner with thyroid hormone receptor binds to the promoters of myogenin and MyoD (Downes, Griggs, Atkins, Olson, & Muscat, 1993; Muscat, Mynett-Johnson, Dowhan, Downes, & Griggs, 1994). A study from our laboratory has shown that bexarotene, a synthetic RXR ligand, is able to enhance skeletal myogenesis in pluripotent P19 cells and mouse embryonic stem cells (Le May et al., 2011). Additionally, we established in our laboratory that bexarotene enhances myogenic differentiation in C2C12 cells, committed for the skeletal muscle lineage (Yaffe & Saxel, 1977) and in primary myoblasts isolated from mice. The molecular mechanism by which bexarotene enhances myogenic differentiation is yet to be determined.

The ability of RXR to form heterodimeric complexes with a variety of nuclear receptors suggests its involvement in the transcriptional regulation of a broad range of genes involved in different cellular functions such as growth and differentiation (Leid, Kastner, & Chambon, 1992; Mangelsdorf et al., 1995). This ability could imply a crosstalk between RXR receptors and different signaling pathways. For example, it has been shown that retinoids, via RAR-RXR heterodimers, target the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in mouse embryocarcinoma cells to induce their differentiation and inhibit their proliferation (Bastien, Plassat, Payrastre, & Rochette-Egly, 2006).

### 1.5 The role of Akt isoforms in development

Protein kinase B (PKB or Akt) is a serine/threonine kinase that is involved in a variety of cellular functions (E. Gonzalez & McGraw, 2009). Akt needs to be at the plasma membrane to be activated and this translocation requires phosphatidylinositol 3,4,5-triphosphate (PIP3), which requires PI3k to be formed (Vivanco & Sawyers, 2002). At the plasma membrane, the phosphorylation of Akt at Thr308 and Ser473 leads to its activation (Alessi et al., 1997; Sarbassov, Guertin, Ali, & Sabatini, 2005), allowing Akt to phosphorylate its substrates and resulting in the regulation of different cellular activities (Manning & Cantley, 2007) (figure 3).

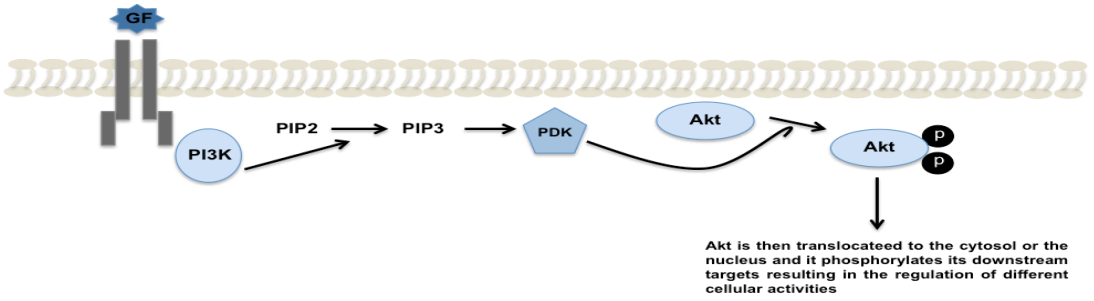


**Figure 3: The activation of Akt.** The activation of phosphatidylinositol 3-kinase (PI3K) by growth factors leads to the formation of phosphatidylinositol 3,4,5-triphosphate (PIP3), which is required for the phosphorylation of Akt by PI3K-dependent kinase (PDK).

Three isoforms of Akt protein, encoded by different genes, are found in mammals: Akt1, Akt2 and Akt3 (also known as PKBα, PKBβ, and PKBγ respectively) (E. Gonzalez & McGraw, 2009). These proteins are more than 80% identical in their sequences with a similar structural organization (Kumar & Madison, 2005). In general, Akt1 and Akt2 are highly expressed in most tissues while Akt3 is hardly expressed (Buzzi et al., 2010). RNA-seq data from brain, heart, kidney, liver and skeletal muscle tissues of adult mice showed that Akt1 is the dominant isoform in all tissues except skeletal muscle, in which Akt2 is the dominant isoform, while Akt3 is expressed at a very low level in all tissues except in the brain (Barbosa-Morais et al., 2012) (figure 4). The Akt isoforms have overlapping functions in bone and muscle development, adipogenesis, the regulation of body weight and size, and embryonic development and survival (E. Gonzalez & McGraw, 2009). Additionally, each Akt isoform has specific functions such as cellular growth and angiogenesis by Akt1, glucose homeostasis by Akt2 and neuronal development by Akt3 (E. Gonzalez & McGraw, 2009).

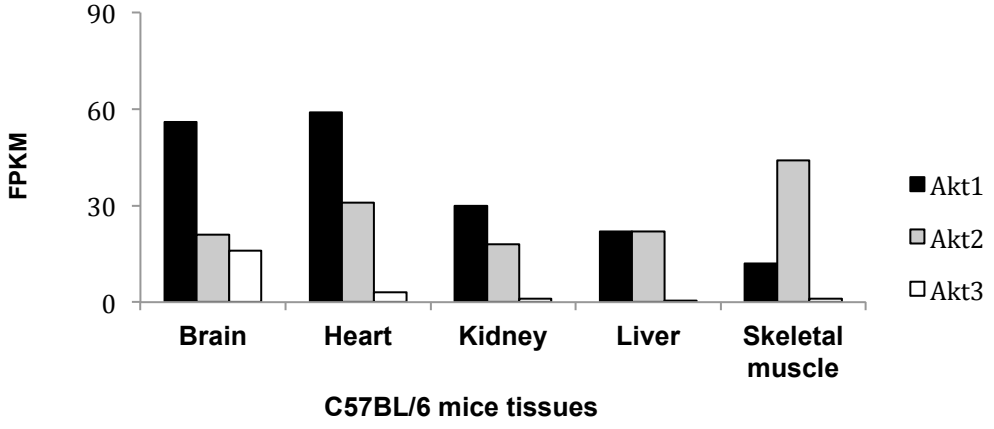


**Figure 4: The expression of Akt isoforms in mouse tissues.** Processed RNA-seq data were obtained from Expression Atlas database (EMBL-EBI) (Kapushesky et al., 2010). The original raw data are available on Gene Expression Omnibus database, GSE41338 (Barbosa-Morais et al., 2012). Akt isoforms expression from brain, heart, kidney, liver and skeletal muscle tissue of adult mice is plotted as the fragments per kilobase per million mappedreads (FPKM), the normalized value for gene expression in RNA-seq data.

Gene knockout experiments in mice shed light on the role of each isoform during development. Akt1 knockout mice exhibited a high rate of mortality in utero and survivors showed reduced body weight with normal glucose homeostasis (Chen et al., 2001; Cho et al., 2001; Yang et al., 2003). Akt2 knockout mice exhibited normal growth with fasting hyperglycemia (Cho et al., 2001; Garofalo et al., 2003). Akt3 knockout mice exhibited a reduced brain size and weight, but body size and glucose homeostasis were normal (Easton et al., 2005; Tschopp et al., 2005). In order to examine the ability of Akt isoforms to compensate for one another, combined Akt isoform knockouts in mice were generated. Mice lacking both Akt1 and Akt2 died after birth with growth deficiencies, muscle atrophy, impaired skin development, and delayed ossification and adipogenesis (Peng et al., 2003). Mice with Akt1/Akt3 double knockouts died in utero with defective development of their cardiovascular and nervous systems (Yang et al., 2005). More interestingly, a single allele of Akt1 in mice lacking both Akt2 and Akt3 was sufficient to carry out developmental processes (Dummler et al., 2006). These mice were viable with reduced body size, a 60% reduction compared to control 12 week-old mice, and had glucose and insulin intolerance (Dummler et al., 2006).

### 1.6 The role of Akt isoforms in muscle

Among the factors regulating myogenesis are the insulin-like growth factors (IGFs). IGF signaling is involved in muscle development and regeneration, mainly via the PI3K-Akt pathway (Bodine et al., 2001; D. J. Glass, 2010a; Lai et al., 2004; Pallafacchina, Calabria, Serrano, Kalhovde, & Schiaffino, 2002). PI3K activity is induced during myogenesis and Akt is one of its downstream targets (Fujio et al., 1999). Inhibition of the PI3K pathway in myoblasts blocks myotube formation and

myogenic protein expression, and the effect can be completely rescued by the overexpression of Akt (B. H. Jiang, Aoki, Zheng, Li, & Vogt, 1999). The roles of the Akt isoforms, mainly Akt1 and Akt2, have been extensively studied in muscle *in vivo* and *in vitro*. Akt1/Akt2 double-knockout mice suffer from muscle atrophy and die after birth (Peng et al., 2003), while overexpressing Akt1 in mice leads to muscle hypertrophy (Lai et al., 2004). *In vitro* studies comparing the role of Akt1 and Akt2 in myogenesis showed that Akt1 is required for differentiation whereas Akt2 is not as crucial as Akt1 at this stage (Gardner, Anguiano, & Rotwein, 2012; Rotwein & Wilson, 2009). While Akt1 knockdown in MyoD-induced fibroblasts showed a reduction in myogenin protein expression and myotube formation in differentiating cells, Akt2 knockdown did not affect myogenin expression or myotube formation (Wilson & Rotwein, 2007). Similar results were observed in the C2 muscle cell line (Rotwein & Wilson, 2009). Overexpressing Akt1 and Akt2 independently caused an increase in myofiber area and fusion index but with no significant effect on the expression of muscle proteins (Gardner et al., 2012). The role of Akt3 in myogenesis has not been extensively studied.

Akt signaling controls muscle development and muscle atrophy by promoting protein synthesis and reducing protein degradation (D. J. Glass, 2005; D. J. Glass, 2010b; Sandri, 2008). It stimulates protein synthesis by activating S6 kinase 1 via mTOR (Bodine et al., 2001). Likewise, Akt phosphorylates the Forkhead box O (FoxO) transcription factors to prevent the entry of FoxO3 into the nucleus, which results in the prevention of protein degradation pathways (Fanzani et al., 2012). Akt signaling is inhibited by two muscle atrophy inducers: TNFα (Sishi & Engelbrecht, 2011) and myostatin (Amirouche et al., 2009; Trendelenburg et al., 2009).

Studying the role of Akt isoforms in myogenic differentiation, and whether bexarotene enhances myogenic differentiation via the regulation of Akt will provide further insight into the potential of using bexarotene as a protective drug from muscle atrophy.

### 1.7 Akt2 in skeletal muscle

While the knockdown of Akt2 *in vivo* and *in vitro* did not result in muscle development defects, studies have suggested that it might have a distinct role in myogenesis. The expression of Akt2 is induced during myogenic differentiation at both the transcriptional and translational levels whereas the expression of Akt1 remains constant (Altomare, Lyons, Mitsuuchi, Cheng, & Testa, 1998; Vandromme et al., 2001). Moreover, the expression of Akt2 in the skeletal muscle tissue of adult mice is 3-fold greater than that of Akt1 (Barbosa-Morais et al., 2012). These observations suggest an important and specific role for Akt2 in skeletal muscle. As previously mentioned, the overexpression of either Akt1 or Akt2 in myogenic cell lines enhances differentiation (Gardner et al., 2012). Another study showed that overexpressing Akt2 rather than Akt1 is more efficient in enhancing myogenic differentiation (Vandromme et al., 2001). In the same study, it was shown that microinjecting antibody that inhibits Akt2 protein in mouse, rat and human myoblasts blocks differentiation while injecting an antibody that inhibits Akt1 has no effect on differentiation. This data led to an interest in the mechanisms by which Akt2 is regulated during myogenic differentiation. MyoD was shown to bind the promoter of Akt2, leading to Akt2 upregulation during myogenic differentiation (Kaneko et al., 2002). The same study showed that overexpressing Akt2 transactivates a myogenin proximal promoter, which has MyoD binding sites (Kaneko et al., 2002). They concluded that a positive feedback loop existed between Akt2 and MyoD. More

interestingly, Akt2 was found to be regulated by two important pathways during differentiation; the p38 mitogen-activated protein kinase (p38 MAPK) and PI3K pathways (I. Gonzalez et al., 2004). The activation of p38 induces Akt2 expression and the PI3K pathway mediates the activation of Akt2 by phosphorylation (I. Gonzalez et al., 2004). Both pathways are essential for myoblast differentiation since the inhibition of either one leads to a reduction in myoblast differentiation (I. Gonzalez et al., 2004). It has been suggested that p38 is directly involved in myogenesis as it transactivates MyoD (Wu et al., 2000). The activation of p38 MAPK or PI3K enhances myogenic differentiation and the differentiation induced by one requires that the other pathway to be functional (Y. Li, Jiang, Ensign, Vogt, & Han, 2000). A reduction in phosphorylated Akt2 was observed in myogenic cells when differentiation was inhibited by p38 MAPK inhibitor or by dominant negative p38α, which suggests that p38 regulates Akt2 (I. Gonzalez et al., 2004). Interestingly, overexpressing Akt2 but not Akt1 overcomes the inhibited differentiation that was caused by the blocking of the p38 MAPK pathway (I. Gonzalez et al., 2004). While Akt1 is required for cell survival and differentiation (Chen et al., 2001), Akt2 seemed to be more involved in muscle differentiation and maintenance.

### 1.8 Enhancers and gene expression regulation

Eukaryotic DNA is wound around structural proteins called histones, and the resulting DNA-histone complexes, known as nucleosomes, form the structural units of chromatin. Each nucleosome is formed from a histone octamer, consisting of the histone proteins H2A, H2B, H3 and H4. DNA packaging was thought to be the only function of histones until their role in the regulation of gene transcription was revealed in the 1990's (Grunstein, 1992). The N-terminal tail of each histone is

subjected to various modification processes, such as acetylation and methylation, that reorganize the chromatin structure to control the accessibility of specific gene loci to transcriptional machinery (Fischle, Wang, & Allis, 2003). Histone modifications are carried out by histone modifying enzymes, such as histone acetyltransferases (HAT) and histone methyltransferases (HMT) that respectively acetylate or methylate histones at specific lysine (K) residues (Brownell et al., 1996; Trievel, 2004). Studies have shown that both histone acetylation and methylation play a role in the regulation of gene expression (Kouzarides, 2007; B. Li, Carey, & Workman, 2007). Generally speaking, histone acetylation is associated with gene activation (Wang et al., 2008), while histone methylation is associated with both gene activation and repression (Barski et al., 2007; Kouzarides, 2007). For example, the acetylation of H3 at K27 (H3K27ac) and the monomethylation of H3 at K4 (H3K4me1) are associated with active genes (Heintzman et al., 2007; Shlyueva, Stampfel, & Stark, 2014), while the trimethylation of H3 at K27 (H3K27me3) is associated with repressed genes (Simon & Kingston, 2009). Some coactivators, which are recruited to DNA indirectly by transcription factors, possess histone-modifying enzymes. For example, the GCN5 protein was identified as the first coactivator with HAT activity that directly acetylates histones to activate gene expression in yeast (Brownell et al., 1996).

Transcription is a highly regulated process that requires protein interactions at specific DNA sequences to activate or repress the expression of specific genes (Maston, Evans, & Green, 2006). One type of regulatory DNA sequence are enhancers, which are *cis*-acting DNA sequences that interact with promoters over short or long ranges to enhance the transcription of target genes (Banerji, Rusconi, & Schaffner, 1981; Bulger & Groudine, 2011). Enhancer activity is regulated by the binding of transcription factors to specific DNA motifs within the enhancer region

(Lee, Haslinger, Karin, & Tjian, 1987). Enhancers are reported to be within 50 kilobases, on average, from the transcription start site of genes they regulate (Blum, Vethantham, Bowman, Rudnicki, & Dynlacht, 2012; T. K. Kim et al., 2010; Rada-Iglesias et al., 2011). Enhancer-promoter interactions are mediated by protein complexes made up of transcription factors and coactivators (Kagey et al., 2010; Roeder, 1998) (figure 5). Often associated with enhancers are coactivators with HAT activity, like p300, allowing histone acetylation at specific lysine residues and thus enhancing the expression of target genes (Wang et al., 2009). In muscle, the HAT activity of p300 is required for Myf5 and MyoD expression and thus for normal skeletal muscle formation (Roth et al., 2003). The histone marks that are associated with active enhancers include increased levels of H3K27ac, H3K18ac and H3K4me1 and the absence of H3K4me3 and H3K27me3 (Blum, 2014; Heintzman et al., 2007; Shlyueva et al., 2014; Simon & Kingston, 2009; Wang et al., 2008). These marks are used to study the relationship between gene expression and enhancer activity.

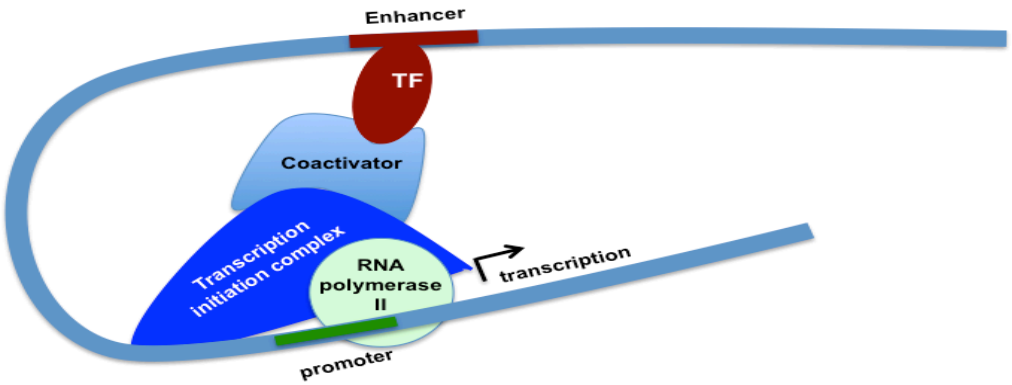


**Figure 5: Enhancer-promoter interaction**. Enhancers interact with promoters over short or long range to promote gene's transcription. A coactivator acts as a mediator for the interaction between transcription factor (TF)-bound enhancer and the transcriptional machinery at the promoter region.

### 1.9 In vitro cachexia assay

Cachexia or muscle wasting is triggered by a variety of signaling pathways in response to cytokines that are secreted by cancerous cells. The complexity of the molecular mechanism underlying muscle atrophy, as it is a multifactorial condition, makes it hard to be studied properly *in vitro*. Fortunately, in 2006, a group presented an *in vitro* cachexia system that allows the investigation of this condition in a system that resembles some of the *in vivo* molecular events of cachexia (Z. Jiang & Clemens, 2006). They showed that using the supernatant of different cancer cell lines, referred to as conditioned media, inhibits the ability of myoblast cells to differentiate. Exposure of proliferating primary myoblasts to conditioned media from human prostate cancer (PC-3) or human melanoma cells subsequently inhibited myoblast differentiation (Z. Jiang & Clemens, 2006). The cancer cell lines that used in this study express interleukin-1β, tumor necrosis factor alpha (TNF-α) and proteolysis-inducing factor, all of which are known to contribute to muscle wasting *in vivo* and *in vitro* (Espat, Copeland, & Moldawer, 1994; Z. Jiang & Clemens, 2006; Pfitzenmaier et al., 2003; Tisdale, 1997; Todorov, Field, & Tisdale, 1999). The activation of NF-κB was proposed as the mechanism by which cancer cell line secretions inhibit myogenic differentiation (Z. Jiang & Clemens, 2006). The activation of NF-κB inhibits MRFs expression leading to myogenic differentiation inhibition. This system resembles some of the *in vivo* mechanisms of cachexia and thus provides a simpler approach to evaluating potential therapeutic agents. In our study, this system was used to evaluate bexarotene as a treatment for muscle wasting.

## 2. Hypothesis

Since bexarotene, a RXR agonist, is able to enhance myogenic differentiation and since Akt is involved in cell proliferation and differentiation and is a major regulator of myogenic differentiation, I hypothesized that bexarotene enhances myogenic differentiation by the regulation of the Akt expression.

## 3. Objectives and significance

The aim of this study was to identify bexarotene's mechanism of action in enhancing myogenic differentiation and whether it is via the regulation of Akt expression. This would help to understand the molecular mechanisms underlying myogenesis, which is crucial to finding a treatment for muscle atrophy. Bexarotene is a FDA approved drug to treat cutaneous T-cell lymphoma (Gniadecki et al., 2007). Thus in addition to studying the mechanism of bexarotene-enhanced differentiation, its ability to rescue myogenic differentiation in an *in vitro* cachexia system was tested.

# 4. Material and methods:

## 4.1 Cell culture and differentiation

C2C12 myoblasts (American Type Culture Collection, Manassas, VA, USA) were grown in growth media (GM), Dulbecco's Modified Eagle Medium (DMEM 4.5g/L glucose, 110 mg/L sodium pyruvate and 584 mg/L L-glutamine) (Wisent, Saint-Bruno, QC, Canada) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). At confluence, cells were switched to differentiation media (DM), DMEM supplemented with 2% horse serum (Gibco, Life Technologies, Grand Island, NY, USA), and differentiation was induced for 1-4 days.

For the *in vitro* cachexia system, human prostate cancer cells (PC-3, American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 (Gibco, Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (HyClone, Logan, UT, USA). At 90% confluence, fresh medium was added and the medium was collected after 48 hours. This conditioned media was used to treat proliferating C2C12 myoblasts in GM (1:1 ratio) for 48 hours before inducing differentiation. For control conditioned medium, C2C12 myoblasts were treated with conditioned media from C2C12 myoblasts that were grown in the same way as PC-3 cells. All cells were grown at 37°C with 5% $CO_2$. All media used were supplemented with 1% penicillin/streptomycin. Cells were treated with or without bexarotene (LC Laboratories, Wobum, MA, USA) or UVI 3003 (Tocris, Bristol, UK) at the indicated concentrations during differentiation.

**4.2 shRNA knockdown**

For knockdown experiments, shRNA lentiviral particles targeting *RXRα, Akt1, Akt2, Akt3* and control shRNA were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). 1.5 X $10^5$ infectious units of shRNA lentiviral particles were used to transduce 30% confluent C2C12 myoblasts grown in GM with 5ug/ml Polybrene (Santa Cruz Biotechnology, Dallas, Texas, USA). Following the manufacturer's instruction, cells were incubated overnight before the media was changed to Polybrene-free GM for another day. To select pooled stable clones, puromycin (Santa Cruz Biotechnology, Dallas, Texas, USA) was then used with GM at 2 μg/ml for a minimum of 7 days.

**4.3 Immunofluorescence microscopy**

Cells were grown and differentiated for 3 or 4 days on glass coverslips and fixed with cold methanol. Cells were air dried, rehydrated in phosphate buffered saline (PBS) and incubated overnight with anti-myosin heavy chain primary antibody (MF20, DSHB, Iowa, USA) in PBS at 4°C. Cells were washed three times with PBS before incubation with Alexa Fluor® 594 secondary antibody (Life Technologies, Eugene, OR, USA) for 2 hours in the dark and then washed again three times with PBS. To stain the DNA, cells were incubated in 2 ml of 0.1 μg/ml Hoechst stain (Molecular Probe, Eugene, OR, USA) for 5 minutes, and then washed three times with PBS. The coverslips were then mounted on slides with 70% glycerol. A fluorescence microscope (Zeiss AxioImager M2) was used to visualize the cells through a 10X objective. Five random images of each condition were captured for downstream quantification. Two parameters were used to evaluate myoblast differentiation: differentiation percentage and fusion index. Differentiation percentage

was calculated as the number of nuclei expressing myosin heavy chain divided by the total nuclei number multiplied by 100, while fusion index was specified as the number of nuclei inside myofibers divided by the total number of myofibers. Cells were counted using ImageJ software.

**4.4 Western blotting**

To prepare whole cell extract, cells were incubated with whole cell extract buffer (10% glycerol, 50 mM Tris-HCl pH 7.6, 5 mM EDTA, 400 mM NaCl, 1 mM PMSF, 1 mM DTT, and 1% NP-40) on a shaker for 30 minutes at 4°C. Samples were then centrifuged for 10 minutes at 14,000 rpm and supernatants were collected. Protein concentration was determined by Bradford assay using Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) and a Multiscan Spectrum photospectrometer (Thermo). Equal amounts of protein in each experimental condition were diluted in 2X Laemmli buffer (25% glycerol, 125 mM Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 5 minutes at 95°C and resolved on a 8-10% sodium dodecyl sulfate polyacrylamide gel, followed by a 1 hour transfer to an immunoblot PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were then blocked for 1 hour with 5% non-fat dry milk in PBS with 1% Tween (PBST). Next, membranes were incubated with diluted primary antibody in 1% milk in PBST overnight at 4°C. Membranes were then washed three times with PBST before and after a 30-60 minutes incubation with secondary antibody. To visualize the proteins, membranes were treated with Western Lightning$^{TM}$ chemiluminescence substrates (Perkin Elmer, Waltham, MA, USA) prior to film development. Membranes were incubated with stripping buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8 and 100 mM β-mercaptoethanol) at 50°C for 35 minutes

followed by 30 minutes blocking with 5% skimmed milk and then re-probed as described previously. Scion Image software (Scion Corporation) was used to quantify the blots. Antibodies against Akt1, Akt2 and Akt3 were purchased from Cell Signaling (Danvers, MA, USA). Anti-RXRα (D-20) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Anti-myogenin (F5D), myosin heavy chain (MF20) and β-tubulin (E7) were prepared from hybridoma cell lines (DSHB, Iowa, USA).

**4.5 Real Time PCR**

Cells were harvested at the indicated time points and a small fraction was used for RNA isolation. Total RNA was extracted using E.Z.N.A® Total RNA Kit I (Omega bio-tek, Norcross, GA, USA) according to manufacturer's instructions. An optional on-column DNase I digestion was performed using DNase I (Omega bio-tek, Norcross, GA, USA). Total RNA was quantified by Nanodrop (Thermo) and 1 μg was reverse transcribed with a high capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using SYBR green with ROX as a reference dye on the Mx3000p qPCR system (Strategene). Results were analyzed using the threshold cycle (Ct) comparative method with GAPDH as an internal control. The $2^{-\Delta\Delta Ct}$ was calculated, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$ in which $\Delta Ct = Ct_{gene} - Ct_{GAPDH}$. The primers sequences are:

myogenin fwd- ATCCAGTACATTGAGCGCCTAC.

myogenin rev- AGCAAATGATCTCCTGGGTTGG.

Akt1 fwd- CCTGAAGCTGGAGAACCTCA

Akt1 rev- TTCATAGTGGCACCGTCCTT

Akt2 fwd- GCGCAAGGAGGTCATCATT

Akt2 rev- GCATACTTGAGGGCTGTAAGG

Akt3 fwd- AGTATGACGACGACGGCAT

Akt3 rev- GTAGAGATGTCCAGGAATCAGTC

GAPDH fwd- TCGGTGTGAACGGATTTG.

GAPDH rev- GGTCTCGCTCCTGGAAGA.

**4.6 Chromatin immunoprecipitation assay (ChIP)**

At indicated time points, cells were fixed with 1% formaldehyde for 30 minutes on a shaker at room temperature. Crosslinking was quenched with 125 mM glycine for 5 minutes. After two washes with cold PBS, the cells were harvested and then incubated on ice for 10 minutes with ChIP lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS and 1X protease inhibitors). Cells were then sonicated for 38 cycles, 30/40 seconds ON/OFF per cycle, using the Diagenode bioruptor® followed by 15 minutes centrifugation at 14,000 rpm. Chromatin DNA, the supernatant, was reverse-crosslinked and purified with Omega Cycle Pure Kit (Omega bio-tek, Norcross, GA, USA) and quantified by Nanodrop (Thermo). For immuneprecipitation, equal amounts of chromatin were used for each condition and diluted in ChIP dilution buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 1X protease inhibitors). Diluted chromatin was pre-cleared with Dynabeads protein A (novex, Life Technologies, Oslo, Norway) for 2 hours at 4°C. Pre-cleared samples were incubated with 2 μg of the indicated antibodies overnight at 4°C. Samples were then incubated with Dynabeads protein-A for 2 hours at 4°C to pull down the immunoconjugates. For RXRα ChIP, samples were incubated with RXRα antibody for 2 hours at 4°C and then Dynabeads protein-A were added for overnight co-incubation at 4°C. The beads were washed with buffers of different salt

concentration at 4°C. The buffers used were wash buffer A (20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA pH 8, 1% Triton X-100 and 0.1% SDS), wash buffer B (20 mM Tris-HCl pH 8, 500 mM NaCl, 2 mM EDTA pH 8, 1% Triton X-100 and 0.1% SDS) and wash buffer C (20 mM Tris-HCl pH 8, 250 mM LiCl, 1 mM EDTA pH 8, 1% sodium dioxycholate and 1% NP-40) for 10 minutes each and then for 5 minutes twice with TE buffer (10 mM Tris-HCl pH 8 and 1 mM EDTA pH 8). The DNA chromatin was eluted with elution buffer ($T_{50}E_{10}S_1$, 50 mM Tris-HCl pH 8, 10 mM EDTA pH 8 and 1% SDS) by rotating at room temperature for 30 minutes. The samples were then incubated for 16 hours at 65°C for reverse-crosslinking. To eliminate RNA from the samples, RNase A (Roche, Mannheim, Germany) was added to each sample and incubated at 37°C for 1 hour. Then Proteinase K (Roche, Mannheim, Germany) and $CaCl_2$ were added to each sample and incubated at 65°C for 30 minutes to digest proteins. The DNA was then purified using Omega Cycle Pure Kit (Omega bio-tek, Norcross, GA, USA) and qPCR was performed on the Mx3000p platform (Strategene) using SYBR green. The quantity of the immunoprecipitated DNA was determined based on a standard curve that was created by using an input DNA for each immunoprecipitate (enrichment was calculated as the percentage of target DNA over input). Antibodies that were used are H3K18ac, H3K27ac (Abcam, Cambridge, UK), RXRα (D-20) (Santa Cruz Biotechnology, Dallas, Texas, USA) and normal rabbit IgG (Invitrogen Corporation, Frederick, MD, USA) as negative control. Primer sequences used are:

*Akt2* locus

fwd- CTTACTGTGGTCCCTAAGCAGG

rev- GGCAAGCCAAGATCACAAGC

Negative primer (no binding of histone acetylation, on chromosome 7)

fwd- CCTGAGTATCTGGTAGGGT

rev- GCATTTAAGAGGGCCCAGAGT

**4.7 Bioinformatics analysis**

For ChIP-seq signal tracks, raw data reads were obtained from publically available data in the Gene Expression Omnibus database. Reads were then mapped to the mouse genome assembly mm9 using the short read aligner Bowtie (Langmead, Trapnell, Pop, & Salzberg, 2009) that is integrated in the Galaxy website, a web-based platform for bioinformatics analysis. Files were then processed and visualized in the Integrative Genomics Viewer (IGV), Broad institute (Robinson et al., 2011). ChIP-seq data for H3K18ac (GSE25308) and H3K27ac (GSE37525) in undifferentiated myoblasts and differentiated myotubes were generated by the Brian Dynlacht group. ChIP-seq data for RXRα from wild-type C57BL/6 mouse liver tissue treated with or without bexarotene (GSE35262) were generated by the Susanne Mandrup group.

## 5. Results

### 5.1 Rexinoid enhances myoblast differentiation and fusion

In our laboratory, we established that bexarotene enhances the differentiation of C2C12 myoblasts and we sought to investigate the molecular mechanism of this enhancement. C2C12 myoblast is a well-established model to study myogenesis (Yaffe & Saxel, 1977) and thus these cells are more relevant to study the involvement of RXR in skeletal muscle. They proliferate when grown sparely with a high concentration of serum, but differentiate when they reach confluency with serum withdrawal. The expression of myogenin appears by day 1 of differentiation and it is used as a differentiation marker through out this study. The differentiated myoblasts, cells that are expressing myosin heavy chain, form multinucleated myotubes by day 3 of differentiation (figure 6A).

C2C12 myoblasts were differentiated with or without 50 nM of bexarotene, based on previous results in our lab, and results were compared to an untreated control. To assess myoblast differentiation, cultures were subject to real time RT-PCR, Western blotting and immunofluorescence microscopy.

For the immunofluorescence staining analysis, cells were grown on coverslips and induced to differentiate for 3-4 days in DM with or without bexarotene. After 3 and 4 days of differentiation, cells were immunostained for myosin heavy chain expression. Bexarotene-treated cells showed a higher myosin heavy chain expression than untreated controls (figure 6A). Additionally, the myotubes were larger and more mature in bexarotene treated cells. In order to measure the efficacy of myoblast differentiation and fusion, the differentiation percentage, the percentage of cells expressing myosin heavy chain, and the fusion index, number of cells expressing myosin heavy chain divided by number of myotubes, were calculated for each

condition (figure 6B). Bexarotene enhanced the differentiation percentage by 1.5 fold on day 3 and 4 of differentiation. Also, it increased the fusion index on both days of differentiation with a greater effect observed on day 4 (figure 6B).

To characterize the observed enhancement of myogenic differentiation by bexarotene, we examined the mRNA levels of myogenin on day 1 of differentiation. Bexarotene increased the transcript levels of myogenin by more than 3-fold as compared to the untreated control (figure 6C). Additionally, myogenin and myosin heavy chain protein levels on days 1 and 2 of differentiation were analyzed by Western blotting (figure 6D). Myogenin expression was detected on days 1 and 2 of differentiation and there was more than a 2-fold increase in its expression in bexarotene treated cells (figure 6E). Bexarotene also enhanced the expression of myosin heavy chain on day 2 of differentiation only, as it was not detected on day 1 in either the treated or untreated condition (figure 6E).

These results indicate that bexarotene acts as a molecular enhancer for myogenic differentiation possibly through RXR signaling. Also, it showed that C2C12 myoblasts are a valuable model to study the mechanism by which bexarotene enhances myogenic differentiation.

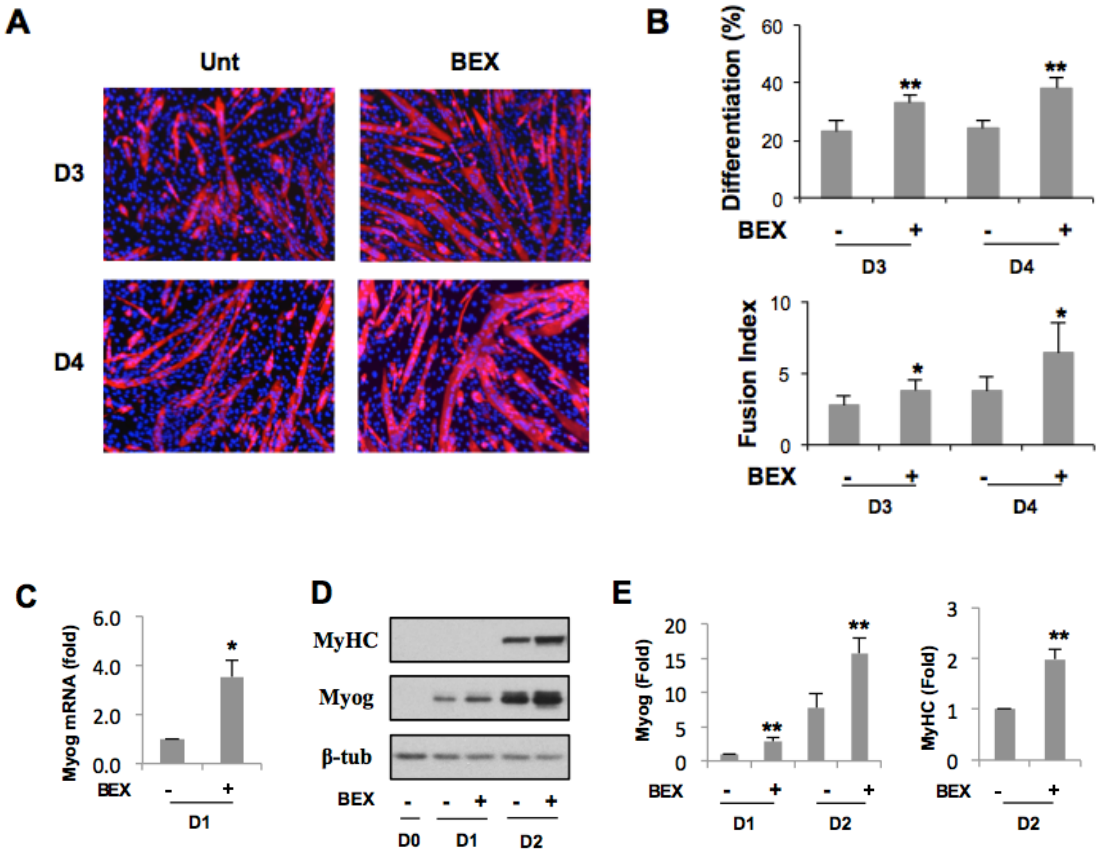


**Figure 6. Bexarotene enhances myoblast differentiation and fusion.** C2C12 cells were grown in growth medium (GM) and then, at 80% confluence, the medium was changed to differentiation medium (DM) without treatment (unt) or with 50 nM bexarotene (BEX). **(A)** Representative images of immunofluorescence staining of myosin heavy chain (red) with Hoechst nuclear stain (blue) on days 3 and 4 of differentiation. **(B)** The average differentiation percentage and fusion index are calculated from five random pictures for each condition. The differentiation percentage = (the number of nuclei inside myofibers) / (the total nuclei number) X 100, while fusion index= (the number of nuclei inside myofibers) / (the total number of fibers) (mean + SD, n=5). **(C)** The relative myogenin mRNA levels were determined by RT-PCR and normalized to GAPDH then presented as fold difference in relation to the untreated condition (mean + SD, n=2). **(D)** Myogenin (Myog) and myosin heavy chain (MyHC) protein levels were analyzed by Western blotting on days 0 to 2 of differentiation and β-tubulin was used as a loading control. **(E)** Quantification of Myog and MyHC protein levels on day 1 and/or 2 of differentiation is presented as a fold difference in relation to the untreated condition (mean + SD, n=3). *P value ≤ 0.05 **P value ≤ 0.01 in relation to the untreated condition.

### 5.2 RXR antagonist attenuates bexarotene-enhanced myogenic differentiation

In order to determine if bexarotene enhances myogenic differentiation via RXR activation, the RXR antagonist UVI3003 (Nahoum et al., 2007) was used. C2C12 myoblasts were differentiated for 4 days with or without bexarotene and in the absence or presence of increasing concentrations of UVI3003 (figure 7A). As shown in figure 7B, UVI3003 had no significant effect on cell differentiation or fusion as compared to the untreated condition. However, co-treatment of UVI3003 with bexarotene resulted in attenuation of the effect of bexarotene on cell differentiation and fusion (figure 7B). Both concentrations of UVI3003 were able to keep the differentiation percentage and fusion index at the same level as the untreated myoblasts when co-treated with bexarotene (figure 7B). In addition to the immunofluorescence staining analysis, myogenin protein levels were analyzed by Western blotting on day 1 of differentiation with the undifferentiated condition as a negative control (figure 7C). While bexarotene treatment increased myogenin protein expression by more than 2-fold in comparison to the untreated cells, co-treatment with UVI3003 prevented this enhancement (figure 7D). Similar to their effects on differentiation percentage and fusion index, both concentrations of UVI3003 were able to attenuate bexarotene-enhanced myogenic differentiation, with the lower concentration of UVI3003 being more efficient (figure 7D). These results indicate that bexarotene enhances myogenic differentiation via RXR and imply that a release of corepressor and recruitment of coactivator is required for this enhancement.

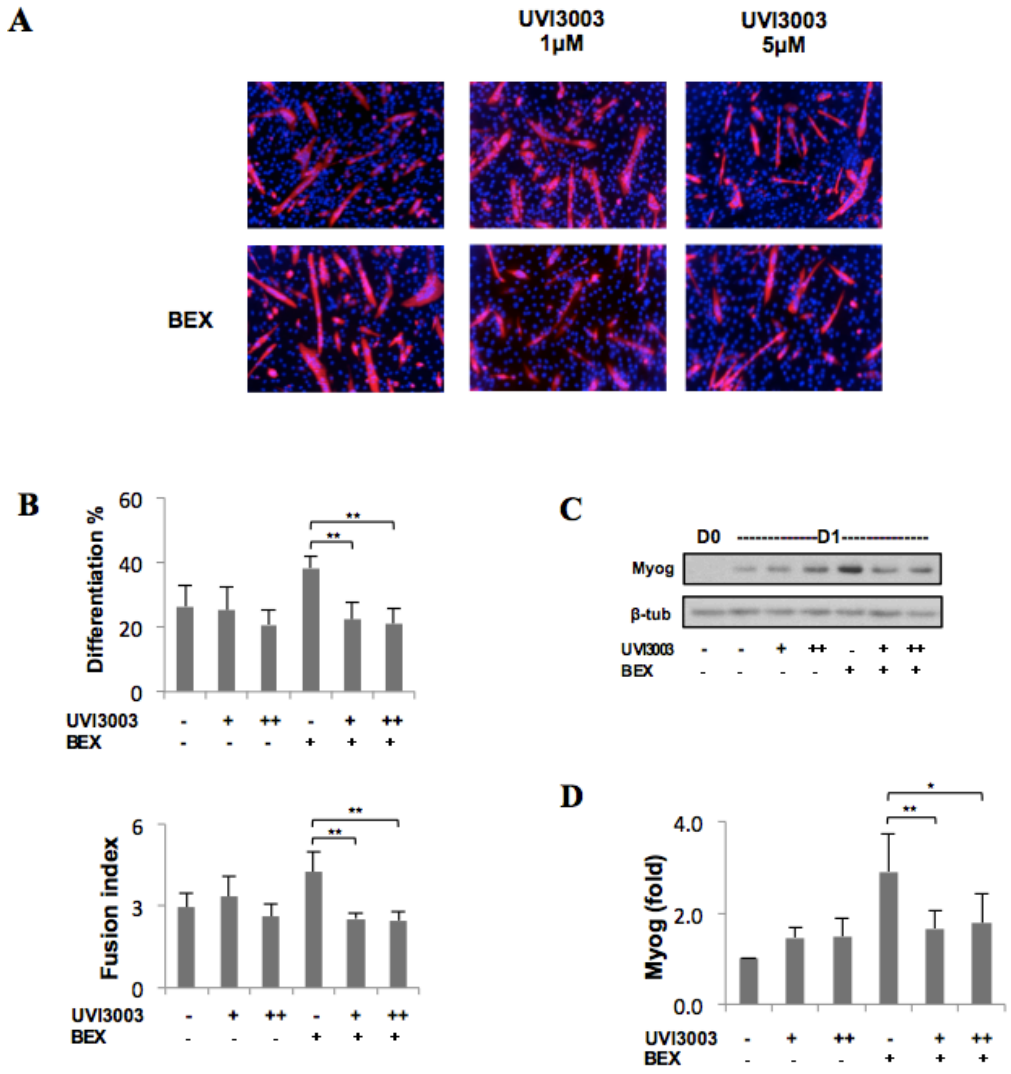


**Figure 7. RXR antagonist attenuates bexarotene-enhanced myogenic differentiation.** C2C12 cells were grown to 80% confluence in GM and then medium was changed to DM. Three controls were used: untreated, 1 µM of UVI3003 and 5 µM of UVI3003. Cells were treated with 50 nM bexarotene (BEX) alone or with 1µM (+) or 5µM (++) of UVI3003. **(A)** Representative images of immunofluorescence staining of myosin heavy chain (red) with Hoechst nuclear stain (blue) on day 4 of differentiation. **(B)** The average differentiation percentage and fusion index as in figure 1. (mean +SD, n=4) **(C)** Myogenin protein level was analyzed by Western blotting on day 1 of differentiation and β-tubulin was used as a loading control. **(D)** Quantification of myogenin protein levels is presented as a fold difference in relation to the untreated condition. (Mean + SD, n=5). *P value ≤ 0.05 **P value ≤ 0.01.

### 5.3 Bexarotene-enhanced myogenic differentiation is inhibited in RXRα knockdown cells

Since RXRα is the main subtype of RXRs as a single allele of RXRα in *RXRγ* $^{-/-}$ and *RXRβ* $^{-/-}$ mice was able to carry out all the required developmental processes (Krezel et al., 1996), and to demonstrate that bexarotene enhances myogenic differentiation through RXR, an RNAi knockdown approach was used. C2C12 cells were transduced with RXRα shRNA lentiviral particles (sh-RXRα) or with a non-target shRNA (Ctl). Puromycin was used to select pooled stable clones. Cells were then conditioned to differentiate for 1-4 days with or without bexarotene, and myoblast differentiation was assessed using immunofluorescence staining and Western blotting. Western blotting analysis for RXRα protein showed efficient RXRα knockdown in proliferating myoblasts as compared to the control (figure 8A).

RXRα knockdown showed no significant effect on the morphology or the differentiation of myoblasts (figure 8B). Differentiation and fusion of sh-RXRα myoblasts on day 4 of differentiation were comparable to the control condition, however the bexarotene effect on differentiation and fusion was attenuated in sh-RXRα cells (figure 8C). To confirm the effect of RXRα knockdown on bexarotene treatment, myogenin protein levels were analyzed by Western blotting on day 1 of differentiation (figure 8D). Results showed that bexarotene failed to enhance the expression of myogenin in sh-RXRα cells (figure 8E). While the RXRα knockdown showed no effect on cell differentiation on day 4, the myogenin protein levels were reduced as compared to the control (figure 8E). The data shows that RXRα is required for the bexarotene-enhanced differentiation.

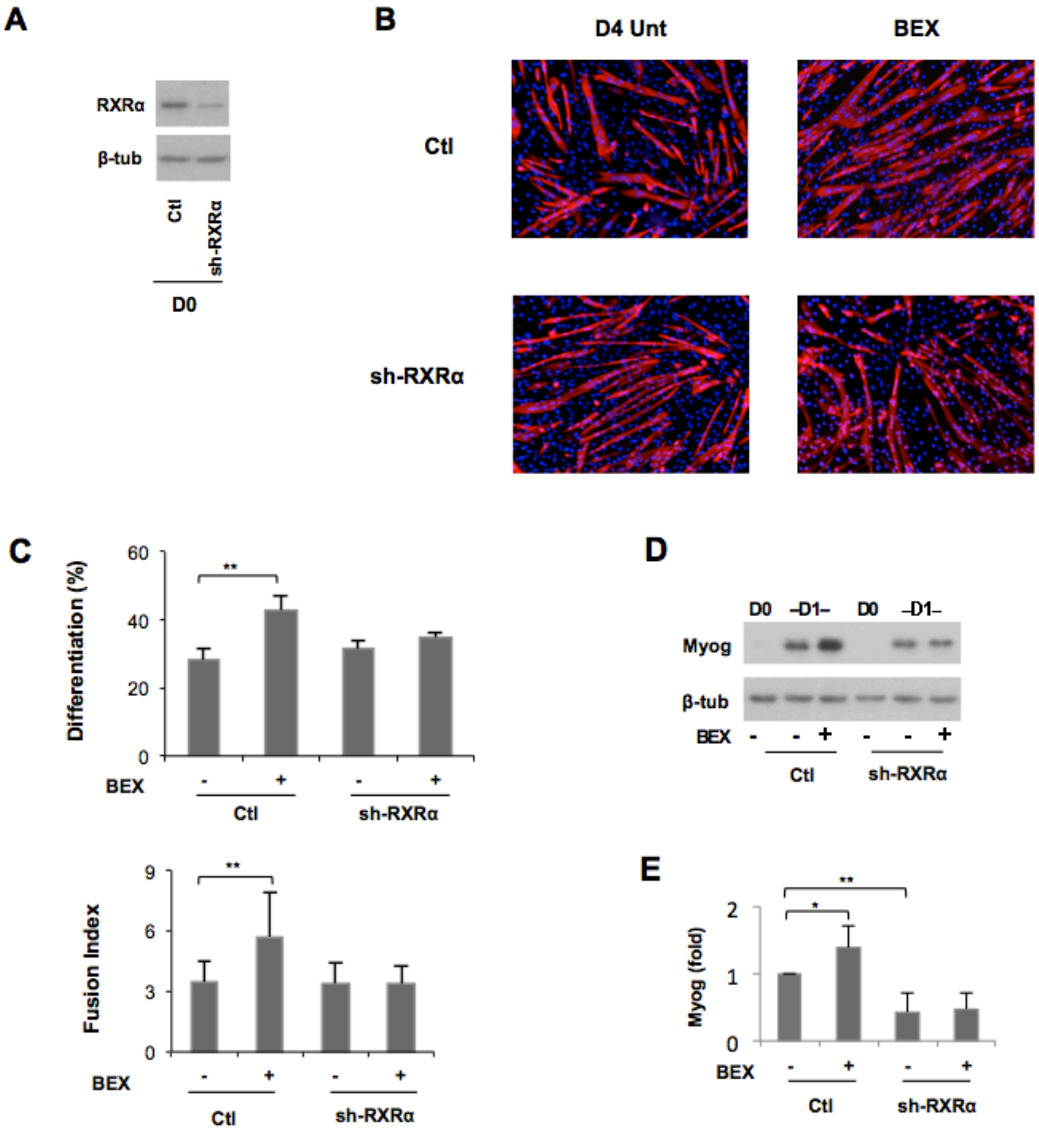


**Figure 8. Bexarotene-enhanced myogenic differentiation is inhibited in RXRα knockdown cells.** C2C12 cells were transduced with RXRα shRNA lentiviral particles (sh-RXRα). A non-target shRNA was used as a control (Ctl). Cells were grown in GM and then, at confluence, medium was changed to DM with or without 50 nM bexarotene (BEX or Unt). **(A)** Protein level of RXRα in confluent myoblasts was analyzed by Western blotting and β-tubulin was used as a loading control. Cropped blot images are presented. **(B)** Representative images of immunofluorescence staining of myosin heavy chain (red) with Hoechst nuclear stain (blue) on day 4 of differentiation. **(C)** The average differentiation percentage and Fusion index as in Figure1. **(D)** Myogenin protein level was analyzed by Western blotting on day 1 of differentiation and β-tubulin was used as a loading control. **(E)** Quantification of myogenin protein levels is presented as a fold difference in relation to the untreated Ctl. (mean + SD, n=minimum of 3). *P value ≤ 0.05 **P value ≤ 0.01.

### 5.4 Bexarotene enhances Akt2 expression during myogenic differentiation

Since the Akt pathway is one of the main signaling pathways in cell proliferation and differentiation (E. Gonzalez & McGraw, 2009) and it is required for skeletal muscle development, we asked if bexarotene regulates the expression of Akt isoforms during differentiation. C2C12 myoblasts were differentiated for 2 days with or without bexarotene and cells were harvested to examine the expression pattern of Akt isoforms during differentiation. The expression levels of all Akt isoforms during differentiation were examined by real time RT-PCR analysis on day 1 of differentiation along with the undifferentiated condition. Akt2 was the only isoform that showed an upregulation in mRNA during differentiation and with the addition of bexarotene (figure 9A). Similarly to RT-PCR, Western blotting analysis showed that among the Akt isoforms, Akt2 was the only one to be upregulated during all days of differentiation (figure 9B and 9C). Additionally, while the expression of Akt1 and Akt3 did not change in response to bexarotene treatment, Akt2 expression levels were significantly increased with bexarotene (figure 9B and 9C).

These results indicate that Akt2, but not Akt1 or Akt3, is involved in bexarotene-enhanced myogenic differentiation. To verify this, Akt2 protein expression was examined in cells from the previous antagonist experiment (figure 9D). Western blotting analysis showed that using RXR antagonist along with bexarotene prevents the enhanced expression of Akt2 by bexarotene (figure 9E). Similar results were observed in sh-RXRα knockdown cells where bexarotene failed to enhance the expression of Akt2 (figure 9F). Taking together, these findings demonstrate that bexarotene-enhanced myogenic differentiation is associated with Akt2 upregulation.

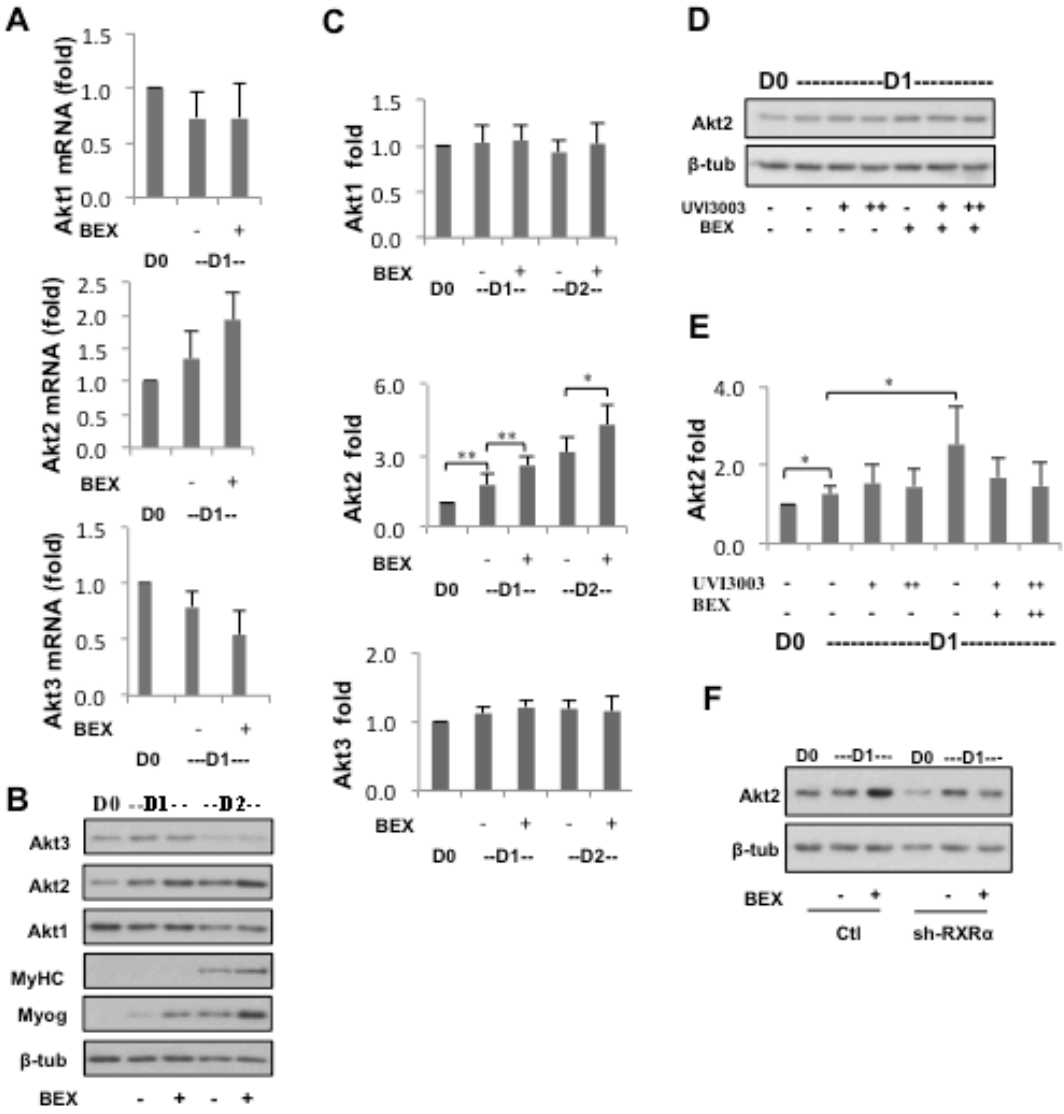


**Figure 9. Bexarotene enhances Akt2 expression during myogenic differentiation.** (**A)** C2C12 cells were differentiated for 1 day with or without 50 nM bexarotene (BEX) and the transcript levels of Akt1, Akt2 and Akt3 were determined by RT-PCR and normalized to GAPDH then presented as fold difference relative to day 0 (D0). **(B)** The protein levels of myogenin (Myog), myosin heavy chain (MyHC), Akt1, Akt2 and Akt3 in differentiating C2C12 cells with or without BEX were analyzed by Western blotting. **(C)** Quantifications of Akt1, Akt2 and Akt3 protein levels in differentiating C2C12 cells are presented as a fold difference in relation to D0. **(D)** C2C12 cells were differentiated for 1 day with or without BEX in the presence or absence of an RXR antagonist (UVI3003) and Akt2 protein levels were analyzed by Western blotting. **(E)** Quantification of Akt2 protein levels of cells that were grown as in D is presented as a fold difference in relation to D0. **(F)** Akt2 protein levels in RXRα knockdown cells (sh-RXRα), as in figure 3, were analyzed by Western blotting on day 0 and 1 of differentiation. β-tubulin was used as a loading control for all Western analysis. (mean + SD, n=minimum of 3). *P value ≤ 0.05 **P value ≤ 0.01.

### 5.5 The effect of Akt isoform knockdowns on bexarotene-enhanced myogenic differentiation

In order to uncover the mechanism by which bexarotene enhances myogenic differentiation and whether Akt2 is required, we sought to examine the effect of Akt2 knockdown on bexarotene-enhanced differentiation. Additionally, since it has been shown in the literature that Akt1 is vital for myogenic differentiation (Gardner et al., 2012; Lai et al., 2004; Rotwein & Wilson, 2009) and the involvement of Akt3 in myoblast differentiation is not well studied in muscle, we aimed to study the effect of Akt1 and Akt3 knockdown on myogenic differentiation and whether bexarotene is able to enhance the differentiation in these cells. Thus, in this study we tested all three isoforms of Akt in order to investigate their involvement in bexarotene-enhanced myogenesis.

C2C12 myoblasts were transduced with Akt1, Akt2 or Akt3 shRNA lentiviral particles (sh-Akt1, sh-Akt2 and sh-Akt3 respectively) or with a non-target shRNA. Pooled stable clones were selected using puromycin and the efficacy of the knockdown was evaluated by Western blotting analysis in proliferating myoblasts. Akt1, Akt2 and Akt3 protein levels in the sh-Akt1, sh-Akt2 and sh-Akt3 knockdown cells, respectively, were compared to respective controls and showed efficient knockdown of targeted proteins (figure 10A). Cells were grown in growth medium until they reached confluence, and the medium was then switched to differentiation medium with or without bexarotene for 3 days. Cultures were subjected to myosin heavy chain immunofluorescence staining.

Immunofluorescence analysis showed that while knockdown of Akt1 caused a reduction in the size and the number of cells expressing myosin heavy chain during differentiation, Akt2 knockdown showed normal differentiation with thinner multinucleated myotubes (figure 10B). Akt3 knockdown showed a similar phenotype

as the Akt1 knockdown (figure 10B). These results support previous findings on the role of Akt1 and Akt2 on myogenic differentiation and also shed light on the importance of Akt3. The differentiation percentage and fusion index were calculated for each condition, where both sh-Akt1 and sh-Akt3 cells showed a significant reduction in the two parameters compared to controls (figure 10C). On the contrary, the myoblast differentiation and fusion in the sh-Akt2 cells were similar to the control (figure 10C). We then asked if bexarotene could restore or enhance the level of myoblast differentiation and fusion in all Akt knockdown cells. As shown in figure 10C, while bexarotene enhanced the defective differentiation in sh-Akt1 and sh-Akt3 cells, it did not enhance the differentiation in sh-Akt2 cells. To quantify this effect, the differentiation percentage and fusion index were calculated. This showed that bexarotene was able to restore the level of differentiation percentage and fusion index close to the normal level in both sh-Akt1 and sh-Akt3 cells with more efficiency in sh-Akt3 cells (figure 10C). Interestingly, bexarotene enhanced neither the differentiation percentage nor the fusion index in sh-Akt2 cells (figure 10C). These findings demonstrate that bexarotene-enhanced myogenic differentiation requires Akt2.

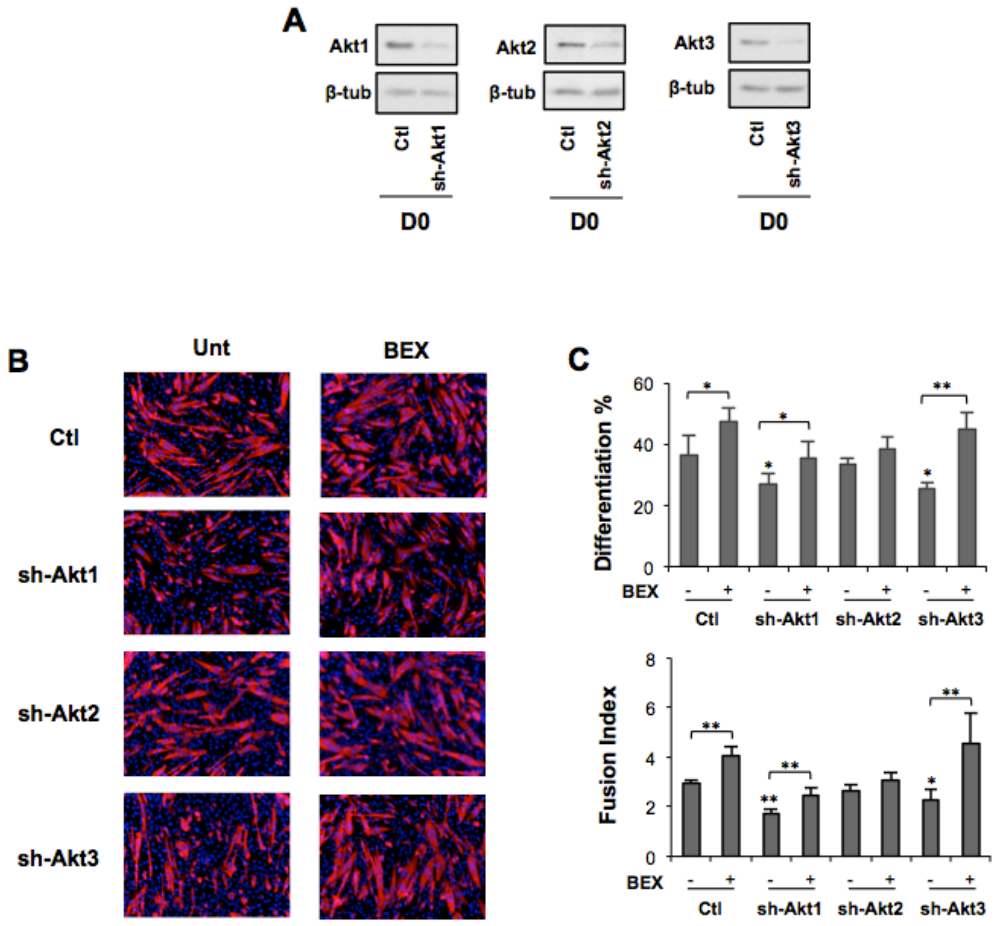


**Figure 10. Effect of Akt isoform knockdowns on bexarotene-enhanced myogenic differentiation.** C2C12 cells were infected with Akt1, Akt2 or Akt3 shRNA lentiviral particles (sh-Akt1, sh-Akt2 and sh-Akt3). A non-target shRNA was used as a control (Ctl). Cells were grown as in figure 3. (**A)** Protein levels of Akt1, Akt2, and Akt3 in confluent myoblasts were analyzed by Western blotting and β-tubulin was used as a loading control. Cropped blot images are presented. **(B)** Representative images of immunofluorescence staining of myosin heavy-chain (red) with Hoechst nuclear stain (blue) on day 3 of differentiation. **(C)** The average differentiation percentage and fusion index as in figure1. (Mean + SD, n=4). *P value ≤ 0.05 **P value ≤ 0.01 in relation to the untreated Ctl or as indicated.

### 5.6 Histone acetylation at the Akt2 locus

Since bexarotene enhances the expression of Akt2 during differentiation and the presence of Akt2 expression is required for bexarotene-enhanced differentiation, we asked if RXR regulates Akt2 expression. To do so, we first used published ChIP-seq signal tracks for H3K18ac (GSE25308) (Asp et al., 2011) and H3K27ac (GSE37525) (Blum et al., 2012) of C2C12 myoblasts and myotubes, and RXRα in liver tissue (GSE35262) (Boergesen et al., 2012) to examine the Akt2 locus. From the available data sets, the liver RXRα ChIP-seq track was the closest to our experiment because Akt2 is also expressed in liver tissues (figure 4) (Barbosa-Morais et al., 2012).

As shown in figure 11A, both H3K18ac and H3K27ac were induced with differentiation at the Akt2 locus. Aligning the RXRα signals with those of the histone acetylation marks pointed out a region upstream of the Akt2 promoter that has clear RXRα binding and overlaps with H3K18ac and H3K27ac. It is located about 12kb upstream of the transcription start site (TSS) of Akt2 and thus this makes it a potential enhancer region (figure 11A).

To verify the observed profiles from the ChIP-seq data and to examine the effect of bexarotene treatment on histone acetylation at the potential enhancer region of Akt2, C2C12 cells were differentiated for 1 day with or without bexarotene and a ChIP assay was performed to quantify the levels of H3K18ac and H3K27ac at the specified region. Undifferentiated myoblasts were also included in the assay. As shown in figure 11B, the level of H3K18ac was increased significantly with differentiation and bexarotene further augmented this level. While there was an increase in the level of H3K27ac with differentiation, bexarotene did not cause a significant increase as compared to the untreated condition (figure 11B). To verify the

binding of RXRα at the potential enhancer region, RXRα ChIP analysis showed that RXRα occupies the region during proliferation and differentiation (figure 11C). Taken together, our data suggests that the RXR binding region is a potential RXR-dependent enhancer for Akt2.

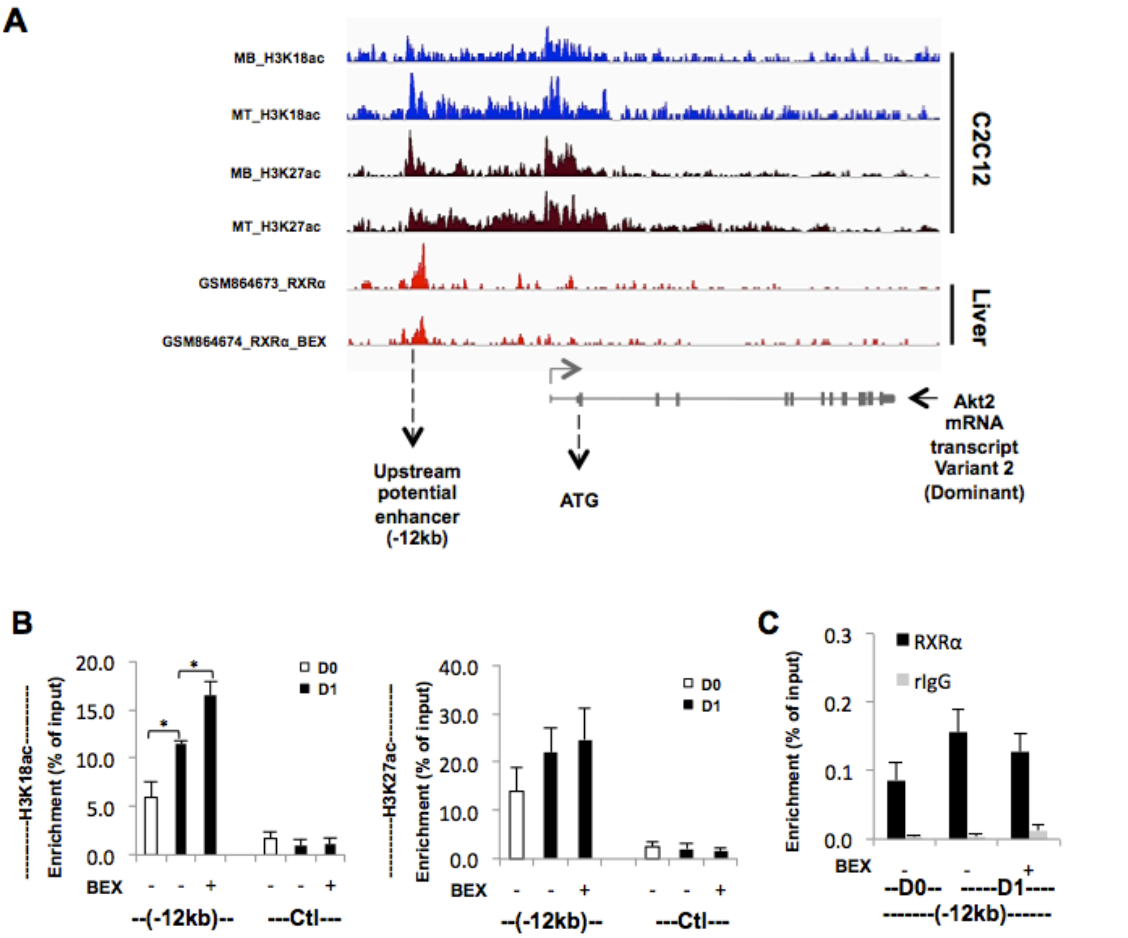


**Figure 11. Histone acetylation at the Akt2 locus. (A)** ChIP-seq signal tracks at the Akt2 locus show acetylated H3K18 (H3K18ac) and H3K27 (H3K27ac) in myoblasts and myotubes C2C12 (MB and MT), and RXRα tracks from mouse liver cells. Tracks were obtained from Gene Expression Omnibus (H3K18ac, GSE25308; H3K27ac, GSE37525; and RXRα, GSE35262). See material and methods. The region that was subject to ChIP analysis is highlighted (-12kb). **(B)** C2C12 cells were differentiated for 1 day with or without 50 nM bexarotene (BEX) and cultures were subject to ChIP analysis using antibodies against H3K18ac and H3K27ac and the quantification is plotted as the enrichment percentage in relation to the input chromatin DNA. A negative locus in the upstream region of Akt2 was used in the ChIP analysis (Ctl) (mean + SD, n= 3). **(C)** ChIP analysis was performed using RXRα antibody and normal rabbit IgG antibody was used as a negative control. Enrichment percentage is presented as in B (mean + SD, n=3).

### 5.7 Bexarotene enhances myogenic differentiation in an *in vitro* cachexia system

In order to investigate the potential of using bexarotene as a therapeutic agent for muscle wasting, an *in vitro* cachexia assay was used in this study. Proliferating C2C12 myoblasts were exposed to PC-3-conditioned media or C2C12-conditioned media (mock control) for 2 days and then differentiation was induced by switching to differentiation media for 1-3 days with or without bexarotene. The effect of factors secreted by PC-3 on myoblast differentiation and the effect of bexarotene treatment were evaluated by immunofluorescence staining and Western blotting.

Immunofluorescence staining of myosin heavy chain showed that exposing C2C12 myoblasts to PC-3 conditioned media inhibited the expression of myosin heavy chain and the formation of myotubes as compared to the mock control (figure 12A). Interestingly, bexarotene treatment of cells that were conditioned with PC-3 media showed more expression of myosin heavy chain and more mature myotube formation compared to bexarotene untreated cells (figure 12A). To quantify the observed phenotypes, differentiation percentage and fusion index were calculated for each condition. Conditioned media from PC-3 cells caused more than 50% reduction in differentiation percentage and fusion index compared to the mock control (figure 12B). Bexarotene had the ability of partially rescuing the differentiation of PC-3-conditioned myoblasts by restoring the differentiation percentage to a level that is close to the mock control (figure 12B). Additionally, bexarotene improved the fusion index in defected myoblasts, but did not fully restore the fusion index to the control level (figure 12B).

To further characterize the observed ability of bexarotene to enhance the defective myogenic differentiation in PC-3-treated cells and also to find if the bexarotene-mediated rescue of myogenic differentiation is via Akt2 in this system,

Western blotting analysis for myogenin, Akt1, Akt2, and Akt3 was performed on day 1 of differentiation (figure 12C). PC-3-conditioned media inhibited the expression of myogenin by more than 70% compared to the mock control and bexarotene corrected this defect by enhancing myogenin expression by over 50% (figures 12C and D). Among the Akt isoforms, Akt2 was downregulated in cells that were exposed to PC-3-conditioned media and bexarotene enhanced its expression (figure 12C).

These data showed the ability of bexarotene to partially rescue myogenic differentiation in an *in vitro* cachexia system, however the mechanism by which bexarotene is rescuing the defective differentiation requires more investigation. The data suggests a role for Akt2, however more experiments should be done at different time points. This system could also be applied on sh-Akt2 cells to test the possibility of the existence of another bexarotene-rescue mechanism distinct from bexarotene-Akt2 mediated enhancement.

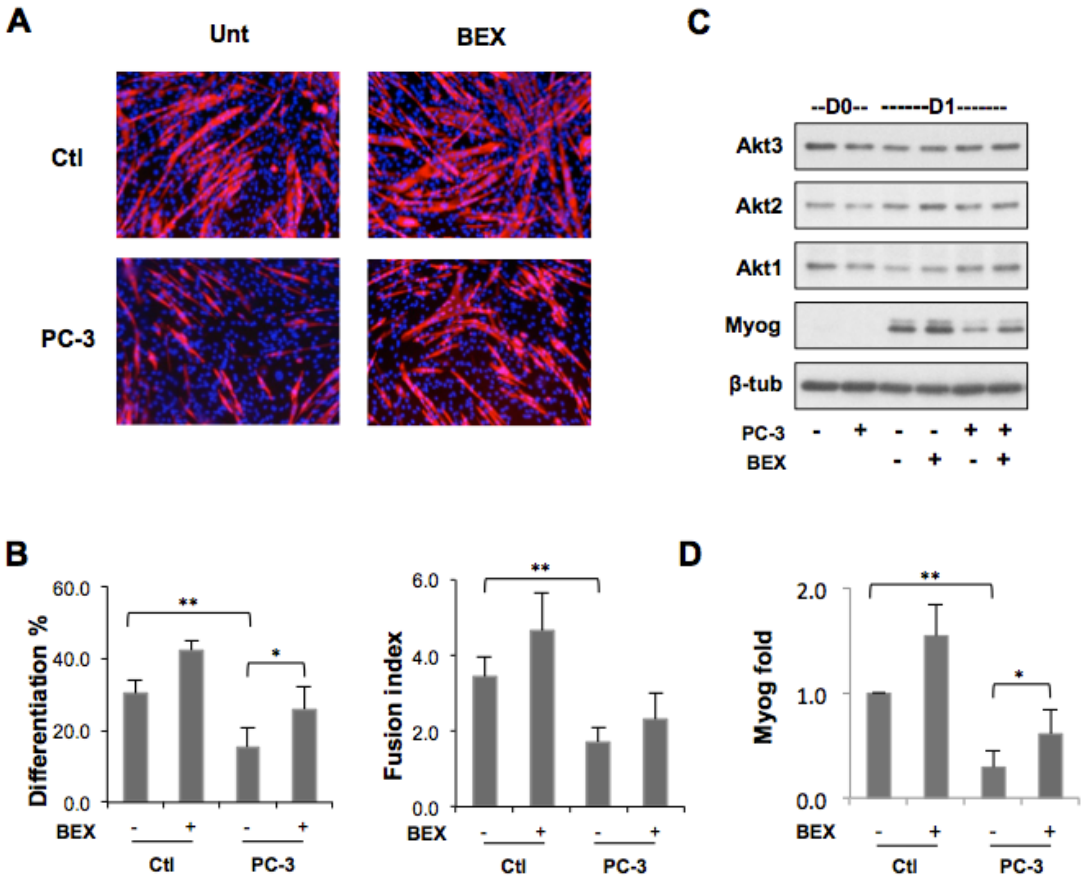


**Figure 12. Bexarotene enhances myogenic differentiation in an *in vitro* cachexia system.** C2C12 myoblasts were grown in growth media (GM) with conditioned media (C2C12, as control, or PC-3) in a 1:1 ratio for 48 hours, and at 90% confluence, media was changed to differentiation media (DM) with or without 50 nM bexarotene (BEX). **(A)** Representative images of immunofluorescence staining of myosin heavy chain (red) with Hoechst nuclear stain (blue) on day 3 of differentiation. **(B)** The average differentiation percentage and fusion index as in figure 1. (**C)** Myogenin (Myog), Akt1, Akt2 and Akt3 protein levels were analyzed by Western blotting on days 0 and 1 of differentiation and β-tubulin was used as a loading control. **(D)** Quantification of myogenin protein levels is presented as a fold difference in relation to the untreated control. (mean + SD, n= minimum of 4).

## 6. Discussion

In this study we investigated the molecular mechanism by which bexarotene, a RXR agonist, enhances myogenic differentiation. We showed that bexarotene, via RXR, enhances myoblast differentiation by augmenting the expression of Akt2, but not Akt1 or Akt3. We identified a potential RXR-dependent enhancer at the Akt2 locus and showed the potential of using bexarotene as a treatment for muscle wasting.

Previous studies in our laboratory established that bexarotene promotes the differentiation of C2C12 myoblasts and hence we used this model to identify bexarotene's mechanism of action in these cells. We first reproduced our previous findings (figure 6), as observed by the upregulation of myogenin and myosin heavy chain proteins in bexarotene-treated myoblasts. Additionally, we showed that bexarotene enhances cell fusion, the formation of multinucleated cells.

We also demonstrated that bexarotene-enhanced myogenic differentiation occurs in a RXR-dependent manner (figure 7 and 8). This was shown when the co-treatment of RXR antagonist with bexarotene attenuated bexarotene-enhanced myoblast differentiation. Additionally, we used a RNA interference approach to knockdown RXRα in C2C12 cells to demonstrate that the enhanced differentiation requires the function of RXRα as a transcription factor. Bexarotene failed to enhance myogenic differentiation in RXRα knockdown myoblasts. Our result showed that while RXRα knockdown inhibited bexarotene enhancement, it showed no effect on normal myoblast differentiation. The normal differentiation in RXRα knockdown cells could be explained by the fact that the full expression of RXRα is not required to carry out all the developmental processes in mice (Krezel et al., 1996). Our data indicated that the normal expression level of RXRα is required to enhance differentiation by bexarotene. This may suggest that the enhancement of myogenic

differentiation by bexarotene activates RXR in a different dimeric complex than during normal differentiation. It has been shown that *9-cis*-retinoic acids induce the formation of RXR homodimers and also enhance the binding of these homodimers to some retinoid acid responsive elements, which activates new pathways (X. K. Zhang et al., 1992). Our data suggest that bexarotene treatment might enhance differentiation by changing the binding of RXR complexes, RXR homodimers and permissive heterodimers, to the rexinoid or retinoid responsive elements, which may lead to the activation or upregulation of myogenic genes.

We next investigated the potential pathways involved in myogenic differentiation that might lead us to the mechanism by which bexarotene is able to enhance differentiation. Insulin-like growth factor is one of the factors that regulate myogenesis, mainly via the PI3K-Akt pathway (Bodine et al., 2001; D. J. Glass, 2010a; Lai et al., 2004; Pallafacchina et al., 2002). The inhibition of PI3K in myogenic cells obstructs myogenic differentiation, and the overexpression of Akt restores the normal differentiation (B. H. Jiang et al., 1999). Interestingly, the exogenous expression of Akt1 or Akt2 enhances myogenic differentiation (Gardner et al., 2012).

We therefore examined the expression pattern of the Akt isoforms, Akt1, Akt2 and Akt3, during differentiation under normal conditions and following bexarotene treatment (figure 9). Our data recapitulate the previously observed pattern of Akt expression where Akt2 is the only isoform that is upregulated during myogenic differentiation. More importantly, bexarotene enhanced the expression of Akt2 during differentiation, which implies the involvement of Akt2 in the bexarotene-enhanced mechanism. We also showed that bexarotene failed to upregulate Akt2 in cells that were co-treated with a RXR antagonist and also in RXRα knockdown cells. An

observation that shows the association between bexarotene-enhanced myogenic differentiation and the enhanced expression of Akt2.

Akt1 and Akt2 have been extensively investigated in muscle, in which the majority of studies have reported that Akt1 but not Akt2 is essential for myogenic differentiation (Gardner et al., 2012). Our data confirmed previously published data (Altomare et al., 1998) and showed that Akt1 knockdown inhibited myogenic differentiation, while Akt2 knockdown did not (figure 10). More importantly, our findings showed that bexarotene failed to enhance differentiation in Akt2 knockdown myoblasts but not in Akt1 or Akt3 knockdown cells (figure 10). This indicates that Akt2 upregulation is required to enhance myogenic differentiation by bexarotene.

During myogenic differentiation, Akt2 was shown to be regulated by two major pathways; p38 and PI3K (I. Gonzalez et al., 2004). While p38 regulates the transcription of Akt2, PI3K regulates the activity of Akt2 by controlling Akt2 phosphorylation (I. Gonzalez et al., 2004). At the same time, PI3K regulates p38 activity, which suggests the existence of a positive feedback loop between these proteins (I. Gonzalez et al., 2004). As previously mentioned, RXRα knockdown did not affect Akt2 expression but bexarotene failed to enhance Akt2 expression in these cells, which suggests that bexarotene upregulates Akt2 by activating a RXR-dependent enhancer region at the Akt2 locus.

Enhancers are *cis*-regulatory elements that enhance gene expression by interacting with target gene promoters (Banerji et al., 1981; Bulger & Groudine, 2011). The binding of transcription factors to enhancer regions does not necessarily represent active enhancers, though histone marks indicate enhancer activity. For example, H3K18ac and H3K27ac are well-established marks for active enhancers (Heintzman et al., 2007; Shlyueva et al., 2014; Wang et al., 2008). We examined the

Akt2 locus to look for potential RXR-dependent enhancers using published ChIP-seq data for H3K18ac (Asp et al., 2011) and H3K27ac (Blum et al., 2012) in C2C12 myoblasts and myotubes and for RXRα in liver tissue (Boergesen et al., 2012). We identified RXR binding about 12 kb upstream of the Akt2 TSS that overlaps with signals of H3K27ac and H3K18ac, which suggests that this locus is a potential enhancer region. Using ChIP analysis, we found that this region is responsive upon the induction of differentiation based on the induction of both H3K18ac and H3K27ac. We also showed that bexarotene significantly enhanced the levels of H3K18ac but not H3K27ac, which suggests different regulating mechanisms of Akt2 during normal differentiation and with bexarotene treatment. We also confirmed that RXR binds to this region in proliferating and differentiating myoblasts with and without bexarotene by ChIP assay. Thus, RXR appears to be involved in the regulation of Akt2, and the activation of RXR by bexarotene occurs at the identified Akt potential enhancer. Further studies are required to determine the importance of the potential enhancer region on Akt2 expression during normal and bexarotene-enhanced myogenic differentiation and which coactivators might be involved in the regulation of that region.

To demonstrate the disease relevance of our findings, we showed that bexarotene could be a potential treatment for muscle atrophy as it had the ability to enhance myogenic differentiation in an *in vitro* cachexia system (figure 10). Our data showed that the previously described *in vitro* cachexia system using primary myoblasts (Z. Jiang & Clemens, 2006) works in the C2C12 myoblast system. The most important finding is the ability of bexarotene to enhance defective myoblast differentiation in this system. Bexarotene partially restored myogenin expression and improved the formation of myotubes in cells that were exposed to tumour-secreated

factors. Whether bexarotene enhances differentiation in this system by Akt2 upregulation or by a different pathway requires further investigation. However it has been reported that muscle cachexia in some diseases such as diabetes and in physiological conditions like aging is associated with reduced Akt signaling (Penna et al., 2010; Price et al., 1996).

In cancer, one of the strongest identified proatrophic factors is myostatin, a member of the transforming growth factor beta (TGFβ) family, which was found to induce muscle atrophy mainly by targeting Akt signaling (Amirouche et al., 2009; Trendelenburg et al., 2009). Another well established proatrophic factor that inhibits Akt signaling is TNFα (Sishi & Engelbrecht, 2011). In the *in vitro* cachexia system that we used in this study, TNFα is expressed in PC-3 cells and the administration of TNFα alone was shown to inhibit myoblast differentiation (Z. Jiang & Clemens, 2006). Our data showed that the expression of Akt2 was reduced in PC-3-treated cells and bexarotene restored Akt2 expression. Additionally, based on our findings that Akt2 expression is essential for bexarotene-enhanced myogenic differentiation and Akt2 is upregulated by bexarotene, one could assume that Akt2 is involved in the rescue of the differentiation following treatment with bexarotene. Nevertheless, due to the complexity of the cachexia system, more studies are required to address the mechanism by which bexarotene rescued the cachectic cells. The ability of bexarotene to enhance differentiation via upregulation of Akt2, the predominant Akt isoform in adult skeletal muscle (Barbosa-Morais et al., 2012), suggests a promising approach to treat muscle atrophy as it is frequently associated with reduced Akt signaling (Fanzani et al., 2012). It has also been shown that the activation of Akt attenuates muscular degeneration and enhances muscle hypertrophy in Duchene's muscular dystrophy, a

recessive X-linked disease caused by malfunction of the dystrophin gene that leads to muscular dystrophy (M. H. Kim et al., 2011; Peter & Crosbie, 2006).

Bexarotene's capacity to enhance differentiation in cachectic myoblasts is significant since there is no available treatment for cachexia, and it is estimated that at least nine million patients in Japan, North America and Europe suffer from this syndrome (von Haehling & Anker, 2010). Bexarotene is a FDA approved drug (Gniadecki et al., 2007), and it has been tested on different cancer types in human and mice subjects. However, the focus of these studies was on the effect of bexarotene on the progression of cancerous cells rather than on muscle mass. Including an evaluation of skeletal muscle mass in such studies may address the possibility of using bexarotene as a protective treatment from cachexia in cancer patients.

## 7. Future directions

We showed that the activation of RXR enhances myogenic differentiation via the regulation of Akt2 expression. In addition, RXR activation is able to rescue cachectic myoblasts, and together these findings revealed the potential of using bexarotene as a molecular enhancer for myogenic differentiation. This work showed that our *in vitro* system could be used to study the RXR signaling pathway, although *in vivo* studies would be required to confirm this.

As a common next step in myogenic studies, results need to be confirmed using primary cultures. In our laboratory, we found that bexarotene is able to enhance the differentiation of mouse primary myoblasts. However, the role of Akt2 needs to be addressed during bexarotene-enhanced differentiation in these cells, which can be accomplished by isolating primary myoblasts from *Akt2*-knockout and evaluating their response to bexarotene.

Additionally, *in vivo* studies need to be performed to assess bexarotene's ability to enhance myogenesis when given as a drug. These experiments would examine bexarotene's capacity to promote normal skeletal muscle development and also regeneration after muscle injury. Similarly, prevention of muscle atrophy, by bexarotene, can be examined using well-established *in vivo* cachexia models such as Lewis lung carcinoma (LLC)-induced cachexia (Ohe et al., 1993). In LLC-induced cachexia, C57BL/6 mice develop muscle atrophy within four weeks post-cancer cells injection, and the capacity for bexarotene to prevent or attenuate atrophy can be tested at this time. Including *Akt2*-knockout mice in all of these *in vivo* experiments would strengthen our *in vitro* findings that Akt2 is required for bexarotene-enhanced differentiation.

Moreover, next-generation RNA sequencing would be helpful in identifying novel genes that are targeted by bexarotene to enhance myogenic differentiation or prevent muscle cachexia. This would enrich our knowledge about muscle development and muscle cachexia, allowing for the development of new approaches to treat muscle diseases. Furthermore, such studies would provide insight into the complex RXR signaling network that governs cell differentiation.

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## 9. Appendix

### 9.1 Supplementary Table 1. Reagents

| Reagent | Catalogue number and supplier |
|---|---|
| 37% formaldehyde | BDH0500-1LP, BDH |
| Bexarotene | B-2422, LC Laboratories |
| Bio-Rad protein Assay Dye Reagent | 500-0006, Bio-Rad |
| Cycle Pure Kit I | D6492-02, Omega bio-tek |
| DMEM | D6429, Sigma |
| DMEM | 319-005-CL, Wisent |
| Dnase I set | E1091-02, Omega bio-tek |
| dNTP mix | U151B, Promega |
| Dynabeads protein A | 10002D, Invtrogen |
| Fatal Bovine serum | F1051, Sigma |
| Fatal Bovine serum | SH30396.03, HyClone |
| Glycerol | 4750, EMD |
| High Capasity cDNA RT Kit | 4368813, Applied biosystems |
| Hoechst 33258 pentahydrate | H-21491, Molecular Probes |
| Horse serum | 16050-122, Gibco |
| HotStar Taq Polymerase | 203205, Qiagen |
| Immun-Blot PVDF membrane | 162-0177, Bio-Rad |
| NP-40 | 9036-19-5, Bio Basic Inc. |
| Penicillin G potassium salt | P7794-100MU, Sigma |
| Polybrene | Sc-134220, Santa Cruz |
| Precision Plus Protein Dual Color Standards | 161-0374, Bio-Rad |
| Protease inhibitor cocktail | 539134, Calbiochem |
| Proteinase K | 03115879001, Roche |
| Puromycin dihrochloride | sc-108071, Santa Cruz |
| RNase A | 10109169001, Roche |
| Rox Reference dye | 54881, Invitrogen |
| RPMI 1640 | 31800, Gibco |
| Streptomycin Sulfate | 11860-038, Gibco |
| SYBR green | S7563, Invitrogen |
| Total RNA Kit I | R6834-02, Omega bio-tek |
| Triton X-100 | H5142, Promega |
| Tween 20 | TWN510, Bioshop |
| UVI3003 | 3303, Tocris |
| Western Lightning Chemiluminescence plus-ECL | NEL105001EA, Perkin Elmer |
| β-mercaptoethanol | O3446I, Fisher Scientific |
| sh-RXRα | sc-36448-V, Santa Cruz |
| sh-Akt1 | sc-29196-v, Santa Cruz |
| sh-Akt2 | sc-38910-v, Santa Cruz |
| sh-Akt3 | sc-38912-v, Santa Cruz |
| Control shRNA | sc-108080, Santa Cruz |

### 9.2 Supplementary Table 2. Antibodies

| Antibody | Catalogue number and supplier |
|---|---|
| Anti-MyHC (MF20) | DSHB, homemade |
| Anti-Myogenin (F5D) | DSHB, homemade |
| Anti-β-tubulin (E7) | DSHB, homemade |
| Anti-Akt1 (C73H10) | 2938, Cell Signaling |
| Anti-Akt2 (D6G4) | 3063, Cell Signaling |
| Anti-Akt3 (L47B1) | 8018, Cell Signaling |
| Anti-RXRα (D-20) | Sc-553, Santa Cruz |
| Anti-H3K27ac | Ab4729, Abcam |
| Anti-H3K18ac | Ab1191, Abcam |
| Anti-mouse IgG II Ab | NA931, GE healthcare UK limited |
| Anti-rabbit IgG II Ab | AP132P, Millipore |
| Alex Fluor 594 donkey anti-mouse II Ab | A21203, Invitrogen |
| Normal Rabbit IgG | 10500C, Invitrogen |