NR4A1 ANTAGONISTS TARGET THE ONCOGENIC PAX3-FOXO1A GENE AND INDUCE INTERLEUKIN -24 IN RHABDOMYOSARCOMA (RMS)

A Dissertation

by

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ABSTRACT

Rhabdomyosarcoma (RMS) is a pediatric cancer for which common therapeutics include cytotoxic chemotherapeutics that result in adverse health outcomes later in adulthood. Previous studies have found NR4A1 expression contributes to the oncogenicity of several solid tumors and this study aims to determine if the established NR4A1-dependent mechanisms are observed in RMS.

The orphan nuclear receptor NR4A1 is expressed in tumors from RMS patients and RMS cell lines. NR4A1 knockdown/antagonism also decreases the expression of growth promoting/pro-survival genes, inhibits mTOR signaling and induce oxidative stress. 1,1-Bis(3-indolyl)-1-(p-substituted phenyl)methane (C-DIM) analogs are NR4A1 ligands in RMS cells and inhibit RMS cell growth in vitro and in vivo.

We also report overexpression of the nuclear receptor NR4A1 in rhabdomyosarcoma that is sufficient to drive high expression of the oncogenic PAX3-FOXO1A protein. RNAi for NR4A1 or C-DIM treatment decreased expression of PAX3-FOXO1A and its downstream effector genes. NR4A1 also regulated expression of β1-integrin, along with PAX3-FOXO1A, contributed to tumor cell migration that was blocked by C-DIM/NR4A1 antagonists.

RNASeq studies using NR4A1 knockdown and antagonism and PAX3-FOXO1A antagonism in RMS cells revealed several genes that are commonly
regulated by PAX3-FOXO1A and NR4A1, including the tumor suppressor-like factor interleukin-24 (IL-24). Studies show IL-24 expression is repressed in ARMS tumors and PAX3-FOXO1A positive tumors and transfection of ARMS cells with siPAX3-FOXO1A or siNR4A1, or treatment with C-DIM/NR4A1 antagonist result in IL-24 induction. In cells cotransfected with siPAX3-FOXO1A and siIL-24 or in IL-24 KO Rh30 cells, effects of siPAX3-FOXO1A were significantly abrogated. These data show that the oncogenic activity of PAX3-FOXO1A was due to, in part, IL-24 repression. Therefore, IL-24 induction contributes to the anti-carcinogenic actions observed upon PAX3-FOXO1A suppression.

Previous studies have demonstrated the ability of C-DIMs to inhibit NR4A1-dependent pathways, yet these compounds exhibit low serum half-life, making them unsuitable for therapeutic applications. The current studies using selected p-hydroxyphenyl-substituted analogs (second-generation C-DIMs) show up to 10 times more potent than the parent compound in vitro and in vivo. This new class of potent NR4A1 antagonists is being developed for future clinical applications for treating RMS, a cancer which has a poor prognosis with current treatments.
DEDICATION

I would like to dedicate this thesis to my Father, Terry David Reeder, who passed away when I was a child, but continues to serve as an aspiration for the type of person I hope to become. I would not be where I am today if not for him. I also want to thank my mom Diane Reeder and my brother David Reeder. I also appreciate the support I have received from my in-laws as well as extended family. I would also like to thank my husband, Jason Lacey, for his continue love, support, patience, and laughs throughout this process. Your support and encouragement has pushed me to where I am today.
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

CANCER OVERVIEW

Cancer is a complex, multifaceted disease and it is estimated that 14 million individuals are diagnosed with this disease. 8.2 million people per year will die from cancer [1]. Furthermore, the estimated cost of cancer in the United States, including treatment and loss of productivity, is approximately $226 billion annually [1]. These statistics point to the continued need for cancer research and the development of new methods for diagnosis and treatment. Breast, colorectal, and lung cancer are the most prevalent cancers in the United States and together, account for approximately 175,000 new cases and 250,000 deaths every year [1]. While a cancer diagnosis in adulthood can be life-altering, it is even more tragic when the diagnosis is in children. In 2016 alone, an estimated 10,000 new cases of cancer were diagnosed along with 1,200 deaths among children from birth to age 14 [2]. The most commonly diagnosed childhood cancers include acute lymphocytic leukemia (ALL), neuroblastoma, and other central nervous system (CNS) cancers [2].

Tumors can arise from various specialized cells throughout the body, yet most human cancers arise from epithelial cells. Epithelial cells are sheets of cells that line the walls of cavities or channels in the body and skin cells, are epithelial in origin and protect tissues from external insults. Furthermore, these specialized cells give rise to the most common cancer type, carcinomas, which
are responsible for more than 80% of cancer-related deaths in the Western world [3]. Carcinomas affect various tissues, including the esophagus, stomach, intestines, lung, liver, and mammary glands [3]. The remaining tumor types arise from nonepithelial tissues throughout the body and are divided into three major classifications; neuroectodermal, hematopoietic, and stromal [3]. Neuroectodermal tumors are derived from components of the central and peripheral nervous system [3]. This cancer type includes gliomas, neuroblastomas, medulloblastomas, and glioblastomas [3]. The second cancer type includes hematopoietic, or blood-forming, tissues, and cells of the immune system, including erythrocytes, plasma cells, and T and B lymphocytes [3]. The common cancer types within this group include leukemias, which are malignancies of circulating immune cells, such as white blood cells, and also include lymphomas, which encompass tumors of lymphoid lineage, such as B and T lymphocytes [3]. The latter will commonly aggregate into solid tumors in lymph nodes, while leukemias are usually dispersed, single cell populations of malignancies. The third subtype includes cancers that are derived from connective tissues throughout the body and have developed from the mesoderm layer of the early embryo. Common cell types include collagen-secreting cells or fibroblasts, bone-forming cells or osteoblasts, and muscle forming cells or myocytes [3]. The most common sarcomas are osteosarcomas, liposarcomas, rhabdomyosarcomas, and Ewing sarcomas.
Indeed, some cancers are associated with the 3 germ cell layers that are formed during the gastrulation phase of embryonic development, including the ectoderm, mesoderm, and endoderm [3]. The ectoderm layer is the basis for both the outer skin layer and the nervous system; this cell type is primarily associated with the development of neuroectodermal malignancies that arise from the outer cell layer of the early vertebrate embryo [3]. The mesoderm layer develops into connective tissues, such as muscle, cartilage, and blood vessels and this layer is implicated in the development of various sarcomas [3]. The endoderm gives rise to epithelium of the digestive and respiratory system as well as the liver and pancreas; this cell type can develop into adenocarcinomas [3]. During the last phase of gastrulation, the cells in the embryo undergo epithelial to mesenchymal transition (EMT) to form germ cell layers [3]. This process allows three germ cell layers to form from a single layer of cells. During this process, the cells must lose their epithelial characteristics, such as cell-cell adhesion, and become mobile. Various signaling pathways are key for this process to occur. Fibroblast growth factor (FGF) signaling is required for the upregulation of snail family transcriptional repressor 1 (SNAI1), which then downregulates E-cadherin, resulting in a loss of cell adhesion [3]. Mesenchymal cell formation is important in cancer development because they are the basis for the formation of connective tissue as well as lymphatic and circulatory systems [3]. Therefore, sarcomas are derived from mesenchymal cell types.
Over the years, research has shown that a normal cell requires multiple heritable changes to evolve into a cancer cell and this process is called carcinogenesis. Carcinogenesis is a multistage process involving several genes and includes three major phases, initiation, promotion, and progression [4]. The underlying premise of this multistep model of carcinogenesis is the role of genetic or epigenetic alterations of multiple, independent genes. The initiation step usually requires some type of DNA damage and may involve a mutational event associated with an oncogene or tumor suppressor gene [5]. Evidence for this step is supported by findings showing that ras proto-oncogene activation is the result of mutational activation in various animal models, such as mouse skin papillomas and rat mammary carcinomas [6, 7]. Furthermore, initial studies in rat liver observed the immediate progeny of initiated cells form altered hepatic foci [5]. The mechanisms by which initiation occurs can vary across tissue types, but it is understood that most initiated cells do not progress through the following stages of cancer, but rather remain quiescent in the organism [5]. This has been confirmed by the observation of numerous initiated cells in most organs of the body with only a select few that undergo promotion [5].

The next step in the process is promotion, during which the initiated cells undergo clonal expansion into a benign lesion, such as a papilloma or foci of preneoplastic cells. This step does not involve direct genomic changes that occur during initiation, but is rather characterized by altered expression of the genome of the initiated cell [4]. During this step, the precancerous cell may
exhibit decreased expression of tumor suppressor genes such as p53 or retinoblastoma protein (RB1), among others. An alternate mechanism is through upregulation of oncogene expression, which can result in the activation of survival signals and growth factors, such as Ras, mitogen activated protein kinase kinase (MAPKK), and vascular endothelial growth factor (VEGF) [3], among others.

The third step, cancer progression, occurs following promotion and is an irreversible step during which malignant neoplasms and major genetic and structural changes occur within the cells, including karyotypic alterations. These karyotypic alterations can result in the evolution of cancerous characteristics, such as invasion, increased growth rate, and increased angiogenesis [8]. While normal cells can regulate the structure of their genome and karyotype, malignant cells are unable to do this [8], resulting in the genomic instability that is a hallmark of cancer.

Cancer Hallmarks

During the last 50 years there have been remarkable advances in cancer research. From identifying the link between cigarette smoking and lung cancer, oncogene and tumor suppressor discovery, identifying the \textit{P53} gene, to the establishment of cancer hallmarks, these discoveries have been integral to our current understanding of cancer and how it develops. Most notably, cancer research has revealed that cancer is a dynamic disease involving multiple stages and various alterations to the genome. These genetics changes are the
basis for the transformation of normal cells to highly malignant ones. While the genetic changes that occur are diverse, they normally fall into one of six categories that represent the major hallmarks of cancer. These six hallmarks, put forth by Weinberg and Hanahan, include self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis (Figure 1) [4]. Each of these steps represents a bypass of the cell’s normal defense against pathways/genes leading to the formation of transformed cells.

**Figure 1. Cancer Hallmarks.** Six cancer hallmarks as put forth by Hanahan and Weinberg. Reprinted with permission: Hanahan Cell Volume 144; 5 March 2011.
Self-sufficiency in Growth Signals

All cells rely on mitogenic growth signals to move from a dormant state to a proliferative one and these signals are transmitted via transmembrane receptors. These receptors bind several ligands including cell-to-cell adhesion molecules, diffusible growth factors, and extracellular matrix components [4]. These molecules activate growth-stimulatory signaling pathways that cause the cell to proliferate. One of the ways malignant cells can take advantage of this process is by producing their own growth signals and thereby become autonomous and eliminate their need for external growth signals [4]. Additionally, many oncogenes act by mimicking normal growth signaling molecules. While growth factor dysregulation is a mechanism by which cells can acquire self-sufficiency, the cell surface receptors that bind growth factors can also be hijacked. Growth factor receptors are frequently overexpressed in a variety of cancers. For example, the epidermal growth factor receptor (EGFR) is overexpressed in breast and stomach cancer [9] and the HER2/neu receptor is upregulated in both breast and stomach carcinomas [10]. Furthermore, highly overexpressed receptors often exhibit ligand-independent signaling due to constitutively active receptor signaling [4]. Malignant cells can also alter the expression of extracellular matrix receptors, known as integrins, and these also enhance transmission of growth signals. For example, the α5β1 integrin heterodimeric receptor can induce the expression of the anti-apoptotic protein Bcl-2 [11], thereby enhancing cell survival and inhibiting apoptosis. Yet another
integrin αvβ3, promotes melanoma and endothelial cell survival via suppression of the p53 pathway and activation of the transcription factor nuclear factor kappa B [12].

**Insensitivity to Anti-growth Signals**

Normal tissues have two major mechanisms by which they maintain cellular homeostasis and block proliferation, both of which are associated with the cell cycle. Cells may transition from an active proliferative state to the quiescent (G0) state from which they may re-enter to activate proliferation if the proper signals are present [4]. Alternatively, cells may be induced to enter a post-mitotic state. Normal cells sense the external environment for signals that dictate whether cells will proliferate, be quiescent, or enter a post-mitotic state [4]. While the process by which this occurs is complex, there are a few key components. Many of the antiproliferative signals are mediated by pocket protein family members, which consists of the retinoblastoma protein (Rb) as well as p130 and p107 [13], all of which act together to regulate the cell cycle. pRb acts as a tumor suppressor protein that inhibits cell cycle progression until the cell is ready to divide, thereby inhibiting excessive cell growth [13]. pRb normally blocks proliferation by sequestering E2F transcription factors that control expression of genes that determine progression through the cell cycle [14]. When pRb is mutated or disrupted, during carcinogenesis, E2F transcription factors are released from pRb-dependent inactivation results in enhanced
expression of E2F-dependent genes required for DNA replication and for production of cyclins E and A [13].

For cancer cells to grow, they must be able to bypass these anti-proliferative signals, and particularly the pRb pathway. When this pathway is disturbed, it allows for E2F transcription factors to be liberated, leading to cell growth and progression through the G1 phase of the cell cycle [4]. This also allows for proliferative actions that are independent of the growth inhibitory actions of the pRb pathway [4]. One of the best documented cases of interactions between pRb and growth inhibitory factors is that of TGFβ. While the complete activity of TGFβ is not fully understood, studies have shown that TGFβ inhibits the inactivating phosphorylation of pRb, thereby blocking the advance of cells through the G_1 phase of the cell cycle [15]. TGFβ also suppresses c-myc expression, the latter of which plays a role in regulating the cell cycle machinery [15]. Additionally, TGFβ plays a more direct role by enhancing synthesis of p15^{INK4B} and p21, both of which block the cyclin:CDK complexes that are responsible for pRb phosphorylation [16, 17]. TGFβ signaling can be inhibited in cancer cells via downregulation or mutation of TGFβ receptors [18]. The Smad4 transcription factor transduces signals from TGFβ receptors to downstream targets and Smad4 is commonly lost in colorectal cancer and pancreatic ductal adenocarcinoma due to loss of chromosome 18q21[19, 20]. Furthermore, CDK4, one of the cyclin:CDK complexes, can become unresponsive to the inhibitory action of p16^{INK4a}, due to missense mutations affecting INK4A, and this mutation
is commonly found in melanoma cases [21]. While disruptions can occur
upstream to pRb, pRb can also be lost through mutations, which is the case in
retinoblastoma, a highly aggressive malignancy of the eye primarily observed in
young children; Rb can also be sequestered by viral oncoproteins, as is the case
with HPV associated cervical carcinomas [22]. These results indicate that
various disruptions that occur with the pathways upstream and downstream of
Rb, and this results in cell cycle dysregulation.

Acquired Capability: Evading Apoptosis

Uncontrolled cell proliferation is one mechanism by which cancer cells
grow and this can be enhanced or complemented by alterations of pathways
associated with cell survival and apoptosis. Apoptosis is a precisely planned
program of cell death that can be triggered by certain physiological signals. The
process results in the breakdown of cellular membranes and cytoskeleton as
well as chromosome and nucleus degradation [4]. Intracellular and extracellular
sensors are responsible for monitoring the external and internal cell environment
and for conveying the appropriate signals to cells. Many of these pro-apoptotic
signals converge on the mitochondria, which responds with the release of
cytochrome C, a hemeprotein found in the inner membrane of the mitochondria
[4]. The release of cytochrome C, activates caspase 9, which then activate
caspases 3 and 7, both of which are responsible for activating various
downstream effector caspases and cell death pathways [23, 24]. Members of the
Bcl-2 family that either have proapoptotic (Bax, Bak, Bim) or antiapoptotic (Bcl-2,
Bcl-XL, Bcl-W) function also regulate mitochondrial death signaling through cytochrome C release. One mechanism by which p53 can elicit apoptosis is via upregulation of proapoptotic Bax in response to DNA damage; Bax in turn stimulates mitochondrial release of cytochrome C [4].

**Acquired Capability: Limitless Replicative Potential**

While growth signal autonomy, resistance to apoptosis, and insensitivity to antigrowth signals together can dysregulate growth of normal cells, tumor formation requires the alteration of the cell autonomous program that normally limits replicative potential. Early studies showed that cells in culture have a finite replicative potential which results in senescence. These studies found that senescence can be circumvented by disabling the pRb and p53 tumor suppressor proteins, which allowed for additional generations [25]. This “crisis” state is usually categorized by chromosomal fusion and genetic abnormalities and variants (1 in $10^7$) that have acquired limitless replicative potential, termed immortalization [26]. Chromosomal fusion usually occurs as a result of progressive erosion of telomeres through many cycles of replication, leaving the chromosomes unprotected and allowing them to participate in end-to-end chromosomal fusions [27], which in most cases leads to cell death. Alternatively, almost all malignant cells have sustained telomere maintenance and usually do so by upregulation of telomerase enzyme [28, 29]. Both *in vitro* and *in vivo* studies have demonstrated the importance of telomerase activity and when telomerase is ectopically expressed in late passage cells about to crisis, the
cells continue to proliferate without any sign of crisis [30, 31]. Furthermore, tumor susceptible mice exposed to carcinogens develop tumors with elevated telomerase activity [32]. Telomere maintenance is crucial in obtaining immortalization and is a key mechanism by which cancer cells bypass the normal mechanisms for growth limitations.

**Acquired Capability: Sustained Angiogenesis**

All cells require oxygen and nutrients which are supplied by the vasculature and nearby capillaries. The process of forming and developing new blood vessels is termed angiogenesis. Research has found that the initial proliferative lesions that develop in cancer lack angiogenic ability, which hampers their ability to expand; as tumors begin to develop and grow to a larger size, these neoplasias must develop angiogenic ability in order to obtain the proper vasculature needed for growth [33, 34]. Early research in tumor development using *in vivo* bioassays demonstrated the need for angiogenesis for tumor development [35] and this was confirmed by showing that VEGF antibodies impaired new vascularization and tumor growth [36]. These results are further supported by the increasing number of antiangiogenic substances that impair tumor growth in various mouse models [37], leading to development of a class of anticancer drugs known as angiogenic inhibitors that target factors such as VEGF and the VEGF receptor (VEGFR).

Angiogenic signals are transmitted via receptor-ligand complexes on the cell surface. The ability to acquire and sustain signaling necessary for
angiogenesis occurs in steps during the midstages of lesion development and before tumors develop [4]. Some of the most common angiogenesis-initiating signals include VEGF and fibroblast growth factor (FGF) [34], both of which bind to transmembrane tyrosine kinase receptors [38]. Integrin signaling is also an important aspect of vasculature development. Quiescent vessels express one class of integrins and while growing capillaries express a different class of integrins, demonstrating the importance of cell adhesion to the angiogenic processes [4]. In addition to altered integrin signaling, cancer cells can also modulate transcription of angiogenesis-associated genes, resulting in increased VEGF and/or FGF expression compared to normal tissues [4]. Alternatively, some cells acquire the ability to downregulate endogenous angiogenesis inhibitors such as β-interferon or thrombospondin-1 [4]. While the regulation of many of these angiogenic factors is not fully understood, there are some well-documented examples, including thrombospondin-1 regulation. The angiogenesis inhibitor thrombospondin-1 is positively regulated by the p53 tumor suppressor, which is commonly mutated or inactive in cancer. Suppression of p53 activity is suppressed, it results in decreased levels of thrombospondin-1, leading to the loss of its inhibitory activity [39]. These studies indicate some of the various mechanisms by which cancer cells can hijack normal angiogenic signaling.

*Tissue Invasion and Metastasis*

Tumor metastasis accounts for approximately 90% of all cancer deaths [40], and emphasizes the importance of tumor cells to acquire the ability to
detach from the primary tumor and invade adjacent tissues. The mechanism of tumor metastasis is not completely understood, but several classes of proteins that are integral to this process include proteins involved in cell adherence and extracellular structure maintenance such as cell-cell adhesion molecules (CAMs), integrins, and cadherins. Studies focused on CAMs have identified N-CAM as an important factor in neuroblastoma as well as small cell lung cancer during which N-CAM undergoes a switch in expression from a highly adherent form to a poorly adherent one [4]. Furthermore, studies in pancreatic and colorectal cancers have shown an overall decrease in the adherent form of N-CAM, leading to a decrease in pro-adherent factors [41]. Functional studies on N-CAM support these observations and have established its role in metastasis suppression in mouse models since overexpression of N-CAM prevents tumor metastasis [41]. In addition to CAMs, integrins also play an important role in cancer cell invasion and metastasis. In order to adapt to the new invading environment, cancer cells sometimes demonstrate a shift in integrin \( \alpha \) and \( \beta \) subunit expression, and this can also change the integrin substrate preference. Cancer cells typically shift integrin expression from those that favor the extracellular matrix present in normal epithelium to those that bind the degraded stromal component produced by extracellular proteases [42]. Furthermore, forced expression of integrins in cultured cells can induce integrin-specific inhibition or enhancement of this behavior, identifying integrins as key determinants of this process [42].
Genomic Instability

Cell cycle checkpoints pathways and tumor suppressors are key players in the maintenance of genomic stability and their disruption leads to instability. One mechanism by which genomic instability ensues is when these genetic “caretakers” are malfunctioning. While many cell cycle checkpoints and tumor suppressors are known, the most prominent member is the p53 tumor suppressor protein which plays an integral role in DNA damage repair in most human cancers [4]. Upon sensing DNA damage, the p53 protein either induces apoptosis or arrest of cells in G\(_1\) of the cell cycle, and also induces cyclin dependent kinase inhibitors [4]. The study of tumor suppressor genes and their behavior identified one-hit and two-hit mechanisms that explain how tumor suppressor genes malfunction and can drive cancer. Some tumor suppressors require that both alleles are lost in tumors (two hits) and this results in failure of cells to produce the correct protein [43]. Conversely, the one-hit hypothesis describes a dominant negative or haploinsufficiency expression indicating one mutated allele can prevent the function of normal protein from the un-mutated allele [43]. This mechanistic hypothesis accounts for the action of various tumor suppressor genes including, p53 in many cancers, the Adenomatous polyposis coli (APC) in colon cancer, and Von hippel-Lindau (VHL) in renal cell carcinoma [43]. These observations demonstrate the relative “ease” by which a single mutation can cause genomic instability. The loss of function of p53 and other
tumor suppressors allows for genome variability and mutant cells with selective advantages that contribute to the hallmarks of cancer.

**RHABDOMYOSARCOMA (RMS) BACKGROUND**

Rhabdomyosarcoma (RMS) is a malignant tumor of mesenchymal origin arising from cells that normally develop into skeletal muscle [44] with common sites including the head and neck, urinary and reproductive organs, trunk, arms, and legs [44]. RMS is part of a large group of soft tissue sarcoma cancers that affect connective tissues throughout the body while RMS specifically affects skeletal muscle cells [44]. Soft tissue sarcomas comprise about 7% of all malignancies in children and adolescents under the age of 20, and RMS accounts for 40% of these cases [45, 46]. Overall, in the United States, there are approximately 350 cases of RMS in children/adolescents annually with more than 50% of cases occurring in the first decade of life [46]. RMS is commonly observed as a systemic disease, and it is likely to spread to lymph nodes, bone marrow, and spaces adjacent to the primary site [47]. There are three major subtypes of RMS, embryonal RMS (ERMS), alveolar RMS (ARMS), and anaplastic (undifferentiated) RMS; this thesis will focus on ERMS and ARMS.

**RMS Subtypes**

*Embryonal RMS*

Embryonal RMS (ERMS) accounts for approximately 65% of all cases of RMS and most commonly occurs in young patients, from birth to age 10. Tumors of this type are primarily observed in the head/neck region as well as the
genitourinary tract [47]. Additionally, there is a subtype of ERMS called boytroid RMS which represents approximately 7-10% of RMS cases and occurs commonly in the bladder, vagina, or nasopharynx [47]. Boytroid tumors exhibit a similar appearance to a cluster of grapes and therefore received its name for the Greek word for grapes, or botrys [47]. Boytroid tumors also occur during the same life stages as ERMS. ERMS and boytroid RMS do not have characteristic abnormalities, but often exhibit loss of heterozygosity or imprinting in the short arm of chromosome 11 [47].

**Alveolar RMS**

Alveolar RMS (ARMS) is common in children and adolescents and comprises approximately 25% of all RMS cases [47]. ARMS tumors are commonly found in the extremities or on the trunk of the body [47]. This tumor type gets its alveolar name because it has a similar histological appearance to lung tissue [47]. ARMS is also distinguished from ERMS and boytroid RMS because of a characteristic chromosomal abnormality in the form of a chromosomal translocation. This translocation is present in the majority of ARMS tumors and is the result of the translocation of chromosomes 13 and either 2 or 1, to form the PAX3-FOXO1A and PAX7-FOXO1A fusion genes, respectively. Those patients with the t(2;13) translocation are usually older than 5 years of age and exhibit a worse prognosis than younger patients that commonly have the t(1;13) translocation [48, 49]. While the histology and chromosomal characteristics of each RMS subtype are different, one common
feature is that they stain positive for vimentin and desmin as well as myosin, MyoD1, and myogenin upon immunohistochemical analysis [47].

**RMS Etiology & Risk Factors**

There are a variety of factors that are associated with the development of RMS. Childhood cancers usually have a relatively short lag time between exposure and disease onset [50] which means early childhood diseases such as RMS could manifest after prenatal or early infancy exposures. Previous studies have found that individuals that have undergone radiotherapy for a previously treated cancer have been known to develop soft tissue sarcomas in the irradiated area [47]. While evidence of environmental exposure has not been shown to result in RMS, there is evidence demonstrating that most cases are sporadic and only 7-33% of RMS results from a familial predisposition, such as Li-Fraumeni syndrome and neurofibromatosis [51]. In addition, pleuropulmonary blastoma with DICER1 mutations [52, 53], Beckwith-Widemann syndrome [54], as well as Noonan syndrome [55, 56] have all been associated with predisposition to RMS.

Studies have shown that some genetic aberrations are associated with RMS, including TP53 mutations [57] and RMS tumors are often characterized by overexpression of insulin-like growth factor 2 (IGF2). Very little information has been gathered regarding environmental factors that lead to increased susceptibility to childhood cancers such as RMS but recent studies have found parental use of marijuana, maternal stillbirth history, prenatal X-ray exposure,
higher maternal age at childbirth, and high birth weight as contributing factors in RMS development [51, 58].

**Diagnosis & Staging**

There are a variety of ways to diagnose a case of RMS since clinical presentations vary with age at diagnosis, tumor site, and other factors. The presence of abnormal chromosomes and genetic markers can inform or confirm diagnosis when microscopic and immunohistochemical analysis tests are inconclusive [47]. Such techniques also can provide additional information on ensuing disease behavior and biology as each RMS subtype has a distinct histological and chromosomal abnormalities [47]. ERMS has the appearance of embryonic muscle tissue upon microscopic evaluation [47]. Patients with RMS will usually present with an enlarging mass that may obstruct the sinus, nasal cavity, or blood vessels, and can also cause nerve, bowel, and urinary tract compression [47]. Additionally, a painless mass on the trunk of the body may cause a patient to seek medical attention. An MRI for the patient is usually the first test to determine the presence of a tumor. Furthermore, an open incision or needle biopsy is needed to establish a diagnosis and it will also be used for chromosomal analysis if the lesion is malignant [47]. RMS also has a tendency to spread to lung parenchyma [47].

**RMS Patient Staging**

Patient outcome and risk stratification for RMS patients is based on both pretreatment staging as well as surgical groupings established by the Intergroup
Rhabdomyosarcoma Study Group (IRSG), now called Children’s Oncology Group (COG). Pretreatment staging is performed before any therapeutic intervention occurs while surgical or clinical groupings are determined following initial surgical intervention but before chemotherapeutic intervention; the latter is based on the extent of residual tumor and lymph node involvement after surgery [59]. The COG represents the most comprehensive and up to date resource for RMS therapeutics and outcomes and they commonly recruit patients for long-term treatment studies. Furthermore, the classifications set forth by the COG are widely used by facilities that commonly see RMS patients. Furthermore, the COG has put forth staging groups from 1-4, which specifies tumor site and presence of metastasis [47] (Figure 2).

![Figure 2. Children's Oncology Group (COG) Pretreatment Staging Classifications](image)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Sites</th>
<th>( T )</th>
<th>Size</th>
<th>Nodes</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Orbit, head and neck (non-PM), GU (non-B/P), biliary tract</td>
<td>( T_1 ) or ( T_2 )</td>
<td>a or b</td>
<td>N0 or N1 or Nx</td>
<td>M0</td>
</tr>
<tr>
<td>2</td>
<td>B/P, extremity, PM, other (includes trunk, retroperitoneum, etc.)</td>
<td>( T_1 ) or ( T_2 )</td>
<td>a</td>
<td>N0 or Nx</td>
<td>M0</td>
</tr>
<tr>
<td>3</td>
<td>B/P, extremity, PM, other (includes trunk, retroperitoneum, etc.)</td>
<td>( T_1 ) or ( T_2 )</td>
<td>a</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>4</td>
<td>Any</td>
<td>( T_1 ) or ( T_2 )</td>
<td>b</td>
<td>N0 or N1 or Nx</td>
<td>M0</td>
</tr>
</tbody>
</table>

PM, parameningeal; B/P, bladder/prostate. \( T \): \( T_1 \), confined to anatomic site of origin; \( T_2 \), extension and/or fixation to surrounding tissue; \( T \): Size: \( a \), \( \leq 5 \) cm in diameter; \( b \), \( > 5 \) cm in diameter; \( T \): Nodes: N0, regional nodes not involved; N1, regional nodes involved; Nx, regional node status unknown; \( T \): Metastases: M0, no distant metastases; M1, metastases present (includes positive cytology in CSF, pleural, or peritoneal fluid).

**Figure 2. Children’s Oncology Group (COG) Pretreatment Staging Classifications.** COG staging includes 4 stages that uses tumor site, size, lymph node involvement, and metastasis status to determine staging and subsequent treatment. Reprinted with permission: Denes FT Pediatric Genitourinary Oncology Frontiers in Pediatrics 1:48 2013.

Stage 1 includes orbit, head/neck, and biliary tract tumor sites with no metastasis, and tumor size ranging from 0 cm to >5 cm. Stage 2 includes bladder/prostate, extremity, parameningeal tumor sites with no metastasis and a
tumor size of <5cm. Stage 3 encompasses the same tumor sites, size, and metastasis status as Stage 2, but differs in lymph node involvement. Stage 4 includes any tumor site, tumor size from 0 cm to >5 cm, various lymph node involvement, and metastases present. In addition to the use of staging, the COG also uses surgical/clinical Group classifications to produce a comprehensive risk stratification scheme [47]. Group I describes local, completely resected tumor. Group II defines total resection with evidence of regional spread. Group III describes a biopsy or incomplete resection and residual disease present, while Group IV describes distant metastatic disease. The majority of patients (approximately 50%) have Group III disease while the remaining patients have Group I (15%), Group II (20%), or Group IV (15%) [60].

Using both the staging information as well as the surgical/clinical group classifications, the COG has developed four risk groups, Low (subset 1), Low (subset 2), Intermediate, and High. Each risk stratification group is associated with a Stage, Group, and RMS subtype (either ARMS or ERMS). The 5-year failure free survival (FFS) of Low risk (subset 1) is 90%, while that of Low (subset 2) is 87%. Moving from Low to Intermediate decreases the 5 year FFS to 65-73%, while High risk has a FFS of less than 30%.

**RMS Treatment Strategies**

Most cases of RMS require a multimodal therapeutic approach which usually will include a combination of systemic chemotherapy with either surgery, radiation therapy, or both, to maximize tumor control with surgical resection
performed first [61, 62]. Furthermore, treatment regimens are usually determined by risk group (I,II,II,IV) with surgery and radiation therapy used for local control management while chemotherapy is used for systemic control. Yet another variable factor in a treatment regimen depends on the coordinating group with the major groups including Children’s Oncology Group (COG), Intergroup Rhabdomyosarcoma Study Group (IRSG), and International Society of Pediatric Oncology Malignant Mesenchymal Tumor (MMT) Group, all of which differ in management and treatment philosophies [62]. For example, the primary goal of the COG is to utilize local therapy following surgical resection to obtain event free survival as the target endpoint, while MMT employs chemotherapy as a front line therapeutic and the use of surgical resection only when the patient demonstrates a poor clinical response to initial chemotherapy; the goal of the latter approach is to avoid major surgical procedures and the damaging effects of radiation therapy [62].

Recent observations of clinical groups have found local recurrence as the primary site of treatment failure in those patients initially presented with localized RMS. While surgical removal as an initial therapy has shown promise, this approach has had minimal success in patients with metastatic disease [63]. Furthermore, patients with only microscopic residual tumor following surgery generally have an improved prognosis over those that undergo debulking surgery (leaving macroscopic residual tumor) followed by chemotherapy or radiation therapy [63].
As local control and recurrence is a significant problem in children with RMS, radiation therapy is commonly used to gain local control. A recent IRSG study found those patients that achieve complete remission with chemotherapy and surgical intervention had a 20% relapse rate if in Groups I to III, while the relapse rate for patients in Group IV was 30% [64]. As an alternative, radiation therapy has been successful in achieving local control for patients with a lower relapse rate [64]. There has been some success in ERMS patients that undergo radiation therapy whereas in most ARMS patients radiation therapy does not achieve local control of the tumor site [65]. Furthermore, radiation therapy is recommended at all COG risk levels (Groups I, II, III, IV) regardless of ARMS or ERMS classification, with the exception of Group I ERMS patients [66]. In addition to gaining local control, metastasis to extremity sites is a common problem in RMS, especially in high risk groups. Pooled analysis from four RMS study groups showed regional lymph node involvement was two times higher in ARMS when compared to ERMS [67], and intensity modulated radiation therapy can successfully manage extremity RMS as well as provide optimal soft tissue targeting [68].

Chemotherapeutic treatment options after surgery for patients can vary depending on staging, tumor site, and age, among other factors. Across all of the major study groups, chemotherapy is recommended at some point during the course of treatment with the intensity and duration dependent upon the risk group. It is the general trend that adolescents treated for RMS experience
decreased adverse side effects than younger patients [69]. A recent COG study for low risk RMS patients achieved close to 90% survival rate after treatment with a combination of vincristine and dactinomycin followed by radiation therapy [70]. Previous studies have found vincristine, dactinomycin, and cyclophosphamide (VAC) chemotherapy for 43 weeks to be successful in obtaining a 5-year FFS of 93% compared to previous treatments of vincristine and dactinomycin (VA) for 54 weeks resulting in an 83% FFS in a previous study [70]. Furthermore, there has been success and improvements in 5 year FFS with the addition of cyclophosphamide to VA therapy [70]. Treatment of metastatic and or intermediate risk group disease are commonly administered VAC therapy which has been established as the standard regimen and has been successful in eliminating residual tumor from metastatic sites [52]. Despite these efforts, a curative outcome is achieved in less than 30% of those patients with a high-risk classification [71].

While there has been treatment success with the addition of cyclophosphamide, it has been advised to keep the dose as low as possible due to the side-effects which may include myelosuppression, infectious complications, as well as infertility in all men and some women [72, 73]. A comprehensive cohort study published in 2013 investigated a variety of clinical outcomes to determine the prevalence of chronic health conditions of adults treated for childhood cancer. The study found a high incidence of cardiomyopathy from anthracyclines, such as cyclophosphamide, which is a
common component of the multi-modal treatment strategy [74]. Thyroid disorders from radiation are also a common adverse effect as a result of the common area of tumor occurrences of the head and neck [74]. Additionally, male and female infertility and reproductive dysfunctions were observed in patients treated with radiation therapy, a common treatment modality for RMS tumors occurring in the bladder and reproductive region [74]. In addition to these effects, neurocognitive impairment from cranial irradiation or neurosurgery are also observed and these treatments are commonly used with RMS patients [74].

The currently used treatments for RMS are also associated with various adverse health outcomes. It is estimated that one-third of childhood cancer survivors report severe or life-threatening complications up to 30 years after primary diagnosis and 95% of adults treated for childhood cancer have chronic health condition while 80.5% develop a life threatening chronic condition [75]. A large cohort study of survivors of young adult cancers found a 2-fold increased hospitalization risk in those that achieve 5-years of recurrence free disease. This increased risk does not decrease in 20-year survivors, indicating the health burden of young adults that have been previously treated for cancer [76]. Several large studies have observed childhood cancer survivors to have a 6-fold increased risk in developing a secondary cancer and this risk continues from remission into adulthood [75, 77]. Furthermore, long term childhood cancer survivors have an estimated 8.4-fold increased risk of premature death when compared to age and sex-matched controls in the general population [78], and
also have a 6-fold increased risk for developing a second cancer [79]. Late stage treatment associated mortalities often occur as a result of the development of respiratory, circulatory, and second cancers, with an increased relative risk of 14.8, 12.7, and 11.6, respectively [80].

To aid in the characterization and evaluation of adverse events, the National Cancer Institute has established a new set of guidelines called, the National Cancer Institute’s Common Terminology Criteria for Adverse Events (CTCAE)), providing a common system for grading severity of adverse outcomes in cancer patients, and is widely used in clinical trials [81]. This system has established guidelines for the evaluation of late stage adverse events, including growth, developmental, and cognitive disorders, as well as cancer development. These guidelines are applicable for late stage adverse events, while there is still a need for guidelines for acute stage adverse events [81], which will aid in system harmonization and collaborative work.

**NOVEL DRUG TARGETS**

Due to the various adverse outcomes that have been associated with the current RMS treatments as well as the minimal 5-year survival of ARMS patients, recent studies have brought to light various novel drug targets that can be utilized for treating this decrease. Recently the National Cancer Institute Pediatric Oncology Branch conducted a pilot study utilizing cryoreductive treatment followed by T-cell therapy in addition to a tumor vaccine which resulted in minimal toxicity and improved outcomes in those with the most
aggressive ARMS subtypes, including PAX3-FOXO1 fusion positive cases [82].
There also is a current clinical trial that is studying the effectiveness of
etoposide, an established chemotherapeutic, in combination with vorinostat, a
histone deacetylase (HDAC) inhibitor [83].

HDAC inhibitors were initially used as mood stabilizers and anti-epileptics
but recently have been investigated for their effectiveness as chemotherapeutics
for treating acute myeloid leukemia, acute lymphoblastic leukemia, lung cancer,
and breast cancer, among others [84, 85]. HDACs are a part of the epigenetic
machinery that are responsible for the accessibility of DNA for transcription.
Histone acetyl transferases act to acetylate lysine residues on histones, resulting
in less compact and more transcriptionally active chromatin, whereas histone
deacetylases remove acetyl groups from lysine residues, making the chromatin
condensed and transcriptionally silent [86]. Previous studies have found that
HDAC inhibitors exhibit antiproliferative activity, cell cycle arrest, induction of
apoptosis and tumor growth inhibitory activity [86].

Previous studies have utilized genomic analysis of patients and have
reported that skeletal muscle or RMS exhibit higher levels of ROS than other
cancer cells and are therefore more highly sensitive to inducers of oxidative
stress [87]. This observation is thought to occur because of high basal levels of
ROS, leaving minimal tolerance for additional oxidative stress. This observation
has been confirmed in studies showing RMS tumor growth inhibition after
treatment with ROS inducers [87], which also inhibit growth of pancreatic cancer
cells and tumors [87]. This mechanism is believed to be due to a novel epigenetic mechanism by which ROS-inducing HDAC inhibitors decrease RMS cell and tumor growth by first targeting cMyc, resulting in downregulation of microRNAs and induction of ZBTB transcriptional repressors, which in turn downregulate Sp transcription factors; the latter of which are overexpressed in many cancers and regulate numerous pro-oncogenic genes [88, 89] (Figure 3).

**Figure 3.** ROS agents regulate Sp-regulated genes. ROS agents downregulate cMyc and Myc regulated miR-27a and miR-20a/miR-17, resulting in induction of miR-regulated transcriptional repressor ZBTB10/ZBTB34 and ZBTB4. Reprinted with permission: Safe S MicroRNA-Specificity Protein (Sp) Transcription Factor Interactions and Significance in Carcinogenesis Current Pharmacology Reports 1:2; 2015.

Sp transcription factors, including Sp1, Sp3, and Sp4, are overexpressed in RMS cell lines [90], and Sp1 is overexpressed in a high proportion of human RMS tumors. These transcription factors are important because some Sp-
regulated genes are themselves individual drug targets for treating RMS, and these include genes such as CXCR4, cyclin D1, epidermal growth factor receptor (EGFR), bcl-2, hepatocyte growth factor receptor (c-MET), and platelet-derived growth factor receptor α (PGFRα), which are important for RMS cell growth, survival, and angiogenesis [88]. Furthermore, Sp transcription factors serve as a promising drug target as evidenced by results of a phase I/II clinical trial evaluating the effectiveness of mithramycin in solid tumors, such as RMS [91]. Mithramycin acts by decreasing chromatin accessibility by binding to GC-rich promoter sequences, resulting in decreased Sp binding to oncogenic promoters [90]. Furthermore, studies in this laboratory show that ROS-inducing anticancer agents downregulate cMyc (epigenetic) and Myc-regulated miR-27a and or miR-20a/miR-17 resulting in increased expression of the miR-regulated transcriptional repressors ZBTB10/ZBTB34 and ZBTB4 [92-94] (Figure 3). The ZBTB family of proteins competitively bind and displace Sp transcription factors from Sp-regulated gene promoters and GC-rich sites and thereby decrease gene expression [95]. Previous studies using panobinostat as an ROS inducer in RMS cells resulted in activation of this pathway (Figure 3) and demonstrate that Sp transcription factors (Sp1, Sp3, Sp4) are a novel drug target for RMS treatment [96]; The results also show that Sp transcription factors are also important for maintaining the RMS phenotype and exhibit properties of non-oncogene addiction genes [97].
One of the most promising drug targets at the forefront of RMS therapeutics is the fusion protein PAX3-FOXO1. The PAX3-FOXO1 fusion gene results from the chromosomal translocation between chromosomes 2 and 3 and is a genetic signature of ARMS [98]. This translocation fuses the DNA binding domain of PAX3 with the transactivation domain of FOXO1 [98]. PAX3 and FOXO1 are transcription factors and, this fusion gene results in the generation of a novel transcription factor with altered transcriptional targets as well as post-translational regulation [98]. While a relatively small number of tumors harbor a PAX7-FOXO1 translocation from the rearrangement of chromosomes 1 and 13, the clear majority of patients harbor the PAX3-FOXO1 fusion gene [98]. PAX3-FOXO1 also has clinical relevance as a prognostic factor since studies have found those patients expressing the PAX3-FOXO1 fusion gene are classified as a high risk subgroup [99]. This high-risk group also has a high rate of relapse, metastasis, and drug resistance [98]. Furthermore, initial studies on PAX3-FOXO1 found its expression to result in anchorage-independent growth and when expressed as a single genetic change, failed to cause tumorigenesis \textit{in vivo} [100]. These early observations suggest this fusion gene contributes to oncogenesis but cannot alone cause tumorigenesis, which requires additions genetic lesions. Later studies using ectopically expressed PAX3-FOXO1 in cells that do not normally express PAX3-FOXO1 found it to contribute to tumorigenesis by several mechanisms, including increased proliferation, support of cell survival, and inhibition of differentiation [98].
While initial studies identified the role of PAX3-FOXO1 in ARMS tumorigenesis, later studies identified the transcriptional targets by which PAX3-FOXO1 exerts its tumorigenic effect. While a variety of genes have been identified, they can be clustered into groups that are involved in myogenic differentiation, myogenic signaling, and transcription factors involved in mesoderm development [101], and these include MyoD1, DAPK1, Nmyc, and RASSF4, among others. Furthermore, genomic analysis revealed that the genes expressed in RMS are closer to that of fetal muscle than of normal muscle, which suggest that the embryonic transcriptional programs are illegitimately reactivated [102]. PAX3-FOXO1 also has the ability to promote several characteristics of cancer hallmarks. Studies have shown PAX3-FOXO1 expression results in stimulation of cell proliferation, as evidenced by an increased proliferation rate and growth in low-serum conditions [103]. Also, ectopic expression of PAX3-FOXO1 accelerated the transition of cells from G₀/G₁ to S phase by increasing the degradation of cyclin dependent kinase inhibitor 1B as well as cyclin dependent kinase inhibitor 1C [104]. In addition to modulating cell proliferation, PAX3-FOXO1 also plays a major role in promoting cell survival and this conclusion is supported by downstream targets of PAX3-FOXO1 that are involved in cell survival. Inhibition of PAX3-FOXO1 also causes decreased expression of the anti-apoptotic protein Bcl-XL which is regulated by both PAX3 and PAX3-FOXO1 [98]. Furthermore, PAX3-FOXO1 suppresses terminal differentiation which is an important characteristic since the ability to
ignore or suppress differentiation signals contributes to tumorigenesis [105]. Evidence to support this comes from studies in murine myoblasts transfected with *MyoD1* that showed PAX3-FOXO1 inhibition of low serum-induced myogenic differentiation [106]. This mechanism is thought to be due in part to an indirect loss of function of cyclin dependent kinase inhibitor 1C, which is a cell cycle regulator known to effect terminal differentiation [107]. In addition to these characteristics, there is some evidence suggesting that PAX3-FOXO1 plays a role in tumor angiogenesis. Subcutaneous injection of RMS cells that do not express PAX3-FOXO1A develop fewer blood vessels in the xenografts. This process is thought to be controlled by regulation of VEGF signaling as a potential target of PAX3-FOXO1 [108]. However, comparable experiments with ARMS cells lines that express PAX3-FOXO1 exhibit upregulation of VEGFR1, and increased angiogenesis [109]. One of the most widely studied characteristics of ARMS cells includes their propensity to metastasize and there is evidence suggesting that PAX3-FOXO1 is promotes a metastatic phenotype. For example, when PAX3-FOXO1A is ectopically expressed in ERMS cells, these cells exhibit heightened invasion and increased matrix metalloproteinase MMP-2 activity [103, 110]. In terms of this mechanism, upregulation of chemokine receptor CXCR4 by PAX3-FOXO1 is thought to be a major player due to its role in regulating the cell microenvironment [111]. These observations demonstrate the important role of PAX3-FOXO1 in the growth, invasion, and
metastasis of ARMS which is consistent with the poor prognosis of patients with ARMS.

The mTOR pathway is often dysregulated in RMS, and has been linked to reduced survival and promotion of cell growth; therefore, it represents a therapeutic target in RMS. The Preclinical Pediatric Testing Program (PPTP) has utilized an mTOR inhibitor, AZD8055, that exhibits antitumor activity \textit{in vivo} [112]. They have also utilized another drug, ABT-737, that is a novel inhibitor of the antiapoptotic proteins Bcl-2 and Bcl-XL and has greater efficacy than previous Bcl-2 inhibitors because it has a higher affinity for Bcl-2 [112]. Recently, the synergistic effect of both ADZ8055 and ABT-373 has been reported and this combinatorial therapy induces apoptosis by triggering loss of mitochondrial membrane potential, and activation of caspases [112].

Receptor tyrosine kinases also represent potential RMS drug targets. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase responsible for cell survival and proliferation and is inversely correlated with patient survival in head/neck, ovarian, cervical, bladder, and oesophageal cancer [113]. EGFR is commonly overexpressed in cancer and is mutated in 47% of RMS cases [113]. PDGFR\(\alpha\) is mutated in more than 40% of RMS cases and has been shown to be a transcriptional target of PAX3-FOXO1A [114]. Previous studies have used RNA interference and PDGFR\(\alpha\) neutralizing antibodies to decrease tumor volume in \textit{in vivo} mouse studies [114]. In addition to PDGFR\(\alpha\), cMET is a receptor tyrosine kinase involved in growth, invasion,
and metastasis [114]. cMet is also a downstream target of PAX3-FOXO1A and is overexpressed in more than 50% of RMS patient samples and also in RMS cell lines [115]. Treatment with MET inhibitors, such as SU11274, inhibited RMS cell proliferation and migration via G1 cell cycle arrest in vitro [115].

**NUCLEAR RECEPTORS**

**Structure, Function and Location**

The nuclear receptor (NR) superfamily of transcription factors include nuclear hormone receptors, enigmatic receptors, adopted orphan and orphan receptors. Nuclear hormone, enigmatic, and adopted orphan receptors have endogenous ligands whereas ligands for orphan nuclear receptors have not yet been identified. Nuclear hormone receptors are ligand-activated transcription factors that bind glucocorticoids, mineralcorticoids, sex steroids, thyroid hormones, and vitamin-derived ligands [116]. One of the unique properties of nuclear receptors is their ability to directly interact with DNA and regulate expression of genes that play a role in embryonic development as well as adult homeostasis. Nuclear receptors regulate expression of target genes through recruitment of various coactivators, corepressors, and other nuclear factors [117]. Coactivation recruitment is an integral step in ligand-induced transcription whereas recruitment of corepressors mediates repression of non-ligand bound nuclear receptors [117] and these interactions regulate various physiological responses in the cell.
While their ligands may vary, nuclear receptors have common structural features, which include an amino-terminal activation domain (AF-1) a DNA binding domain (DBD), a hinge region, a conserved ligand binding domain, and a second activation domain (AF-2), which is located at the carboxy-terminal end [116]. DBD of NRs are highly conserved zinc finger proteins and responsible for targeting the receptor to specific DNA sequences, known as response elements [116]. The ligand binding domain recognizes and binds specific ligands that dictate the biological response. Nuclear receptors can exist as homo or heterodimers and each partner can bind to specific response element sequences that are represented by half-sites separated by nucleotide spaces between direct or inverted half-site repeats [116].

NRs can be categorized by their interactions with other nuclear receptors to form homo- or heterodimers. Class 1 receptors function as homodimers that bind to half site response element inverted repeats and include the steroid hormone receptors [116]. In the absence of ligand, these receptors are primarily in the cytosol bound to heat shock proteins; and upon ligand binding, they translocate into the nucleus, bind to hormone response elements and recruit coactivators and other nuclear factors to initiate transcription of target gene sequences [116]. Class 2 receptors exist as heterodimers that act in combination with RXR receptor partners and function in a ligand dependent manner. This class of receptors remain in the nucleus regardless of ligand binding [116], in the absence of ligand, these receptors are often bound to
corepressor proteins. Ligand binding induces both dissociation of the bound receptor from corepressors and recruitment of coactivators for initiation of transcription [116]. The remaining two NR classes include orphan nuclear receptors that either function as homodimers that bind direct response element repeats (Class 3), or monomers that bind single site response elements (Class 4) [116]. Nuclear receptors play a role in almost all aspects of human physiology and therefore, represent important therapeutic targets for various diseases. This underscores the importance of studying these systems for new drug development across many areas of study. The 48 NRs have been extensively investigated; this review will focus on selected receptors that are important in various disease processes and are drug targets.

Nuclear receptors can also be divided into groups based on their endogenous ligands. The endocrine receptors include steroid hormone receptors that bind steroid hormones such as 17β-estradiol (E2) and also heterodimeric receptors that partner with retinoid X receptor (RXR) and bind thyroid hormones, retinoids, and vitamin D [116]. Research on endocrine receptors and their ligands has led to the development of selective receptor modulators (SRMs) for the endocrine receptor that exhibit tissue specific agonist or antagonist activity and are commonly used in treating hormone-dependent diseases, such as ER positive breast cancer [116]. The second type of nuclear receptors are adopted orphan receptors that initially were not associated with an endogenous ligand, but were identified in subsequent studies [116]. The
adopted orphan receptors are divided further into two major groups one of which include the lipid sensor receptors such as the retinoid X receptor (9-cis-retinoic acid), peroxisome proliferator-activated receptors (PPARs) (fatty acids), liver X receptor (oxysterols), farnesoid X receptor (bile acids), and pregnane X receptor, which binds cholesterol derivatives [118]. The other subgroup are the enigmatic orphan receptors and these include constitutive androstan receptor (androstan and drugs/xenobiotics), hepatocyte nuclear factor-4, steroidogenic factor-1/liver receptor homolog 1(LRH-1) (phospholipids), and retinoid acid-related orphan receptor (cholesterol and retinoic acids) [116, 118]. The third class of nuclear receptors are orphan receptors for which endogenous ligands have not yet been identified.

Estrogen Receptors

**ER Structure Binding and Activation**

There are several well defined nuclear receptor (NR) systems that have been studied for their biological relevance as well as their clinical implications. One such example is the estrogen receptor (ER) which belongs to the Class 1 category of the nuclear receptor superfamily of ligand activated nuclear receptors and ER has also been classified as an endocrine type nuclear receptor. Two subtypes of ER have been identified, namely ERα (NR3A1) and ERβ (NR3A2) (Figure 4).
The ER binds to cis-acting estrogen response elements (EREs) to facilitate transactivation and the DBD is the most conserved region of the ER [119]. The ER acts in the nucleus as a homodimer to regulate expression of genes through interactions with and recruitment of coactivators and corepressors on EREs or modified/imperfect EREs. [119].

ERs regulate gene transcription via two major pathways which include direct recruitment of the ER to ERE promoter region or through protein-protein interactions with other DNA-bound transcription factors where the ER acts as a ligand-activated cofactor. In the absence of a hormone/ligand, the ER is sequestered in the cytosol as an inhibitory complex with heat shock protein 90 (Hsp90) and Hsp70 [119]. The addition of a ligand induces a conformational change in the ER, nuclear import and homodimerization and binding to specific DNA response elements. These EREs are palindromic half sites separated by a 3-base pair spacer and are commonly found in promoters of the EBAG9,
ZNF147 (Efp), and COX7RP (5'-AGGTCAnnTGACCT-3’) genes [120]. In addition to this canonical ERE sequence, there are REs that are slightly different from the canonical sequence and the ER binds these “imperfect” EREs identified in promoters of several genes including TERT, TGFα, and Lactoferrin [119]. The liganded ER binds with the highest affinity to canonical EREs and less efficiently to imperfect EREs, and this can influence ER-mediated transactivation with respect to the magnitude of the response and sensitivity to ligand [119]. In addition to direct ER/DNA interactions, ER can interact with other DNA-bound transcription factors and ER-protein/DNA interaction can also be activated by estrogens [119]. For example Specificity protein 1 (Sp1) binds to GC-rich promoter regions and ER can enhance Sp1 binding to DNA and estrogen induced transactivation [121]. Nuclear factor κB (NFκB) also interacts with ERα, and this inhibits NFκB/DNA binding and instead of NFκB-dependent induction of interleukin-6, ligand/ER interactions decreases interleukin-6 expression [122].

In both direct and indirect DNA mechanisms of ER signaling, the ER forms a complex with various cofactors that are required for ER-mediated gene regulation. One of the most well-characterized coactivator family includes SRC1, SRC2, and SRC3, of which SRC1 and -2 have the highest affinity for the AF2 domain of agonist bound ERα [119]. SRC-family coactivators contain two transcription activation domains, AD1 and AD2, and they are involved in recruitment of CBP/p300 coactivators as well as acetyltransferases and protein modifying enzyme coactivator-associated arginine methyltransferase (CARM1).
[119], respectively. These coactivators serve to recruit chromatin-modifying proteins to ligand activated ERs. Other factors such as steroid receptor RNA activator (SRA), Med-20, and Bgr/Brm, function as a protein binding scaffolds and serve as a bridge between ligand bound ER and the coactivator complex [123]. In addition, several ER corepressors have been identified, including receptor interacting protein-140 (RIP-140) and short heterodimer partner (SHP), both of which compete for ER binding and antagonize SRC1 coactivator activity [123]. Both NCoR and SMRT are corepressors that inhibit ER-mediated transactivation and these corepressors are involved as repressors for other NRs [123]. In addition, sin-associated polypeptide 30 (SAP30) is important for NCoR mediated repression of ERα while SMRT/HDAC1 associated repressor protein (SHARP) indirectly represses ligand induced ERα activity by repression of the SRA coactivator [123]. ER and other NRs interact with multiple nuclear cofactors and the specific set of cofactors required for ligand-induced transactivation is ligand-, gene-, and cell context-dependent.

Differential Expression of ERα and ERβ and Physiological Function

ERα and ERβ have distinct tissue distributions and this provides some insight on ER-isoform targeted effects in specific tissues. ERα is expressed in testis, ovary, kidney, bone, and adrenal tissues, with moderate amounts present in the prostate gland, bladder, liver, pituitary gland, and various brain sections [124]. High levels of ERβ are found in the prostate gland, bone, and ovary [124]. The uterine tissue and pituitary gland are distinctive in that ERβ is expressed
during development whereas ERα is expressed upon tissue maturation [124]. In the brain, ERα is found in cholinergic neurons of the basal forebrain and enhances cognition by modulating acetylcholine production [125]. ERα and ERβ knockout mouse studies have been essential for determining the normal function of ER during all life stages.

While ERα and/or ERβ loss results in physiological alterations, this loss is not lethal. ERα knockout (KO) adult female mice have a hyperplastic uterus and are unresponsive to ERα ligands [126]. These females also demonstrate a lack of sexual behavior or responses, indicating a role for ERα in mediating nervous system effects [126]. ERα KO in male mice results in decreased sperm motility, sperm counts, and testis weight, resulting in infrequent ejaculations and reduced fertility [127]. In bone, ERα KO females also show decreased femoral length, diameter, and density [128]. ERα is also expressed in adipose tissue and in KO mice this results in a 2-fold increase in mouse weight compared to wild type controls [129]. ERβ KO mice also exhibit physiological changes; female mice have decreased corpora lutea and exhibit follicular development arrest and decreased follicular maturation, but they exhibit normal sexual behavior and mammary gland structure [130]. ERβ KO male mice are fertile but show epithelial hyperplasia in the prostate and bladder wall [130]. These mice also show no altered effects in bone characteristics [130], emphasizing the importance of ERα in bone.
Pathophysiological Differences of ERα and ERβ

In humans, the ER has been implicated in several disease states. Low ERα expression is associated with the development of coronary artery disease in females since ERα is expressed in normal arteries but not in those individuals with coronary artery disease [124]. This observation is supported by an increase in silencing methylation epigenetic marks on the gene encoding ERα in human atherosclerotic plaques compared to normal aortic tissue. In addition, ERα polymorphisms are associated with increased blood pressure in men while ERβ polymorphisms are associated with ventricular wall thickness and high blood pressure in post-menopausal women [124]. This observation is supported in animal models showing that ERβ KO mice develop hypertension at a later age [131].

In addition to these non-cancer endpoints, differential ER expression is also associated with several cancer endpoints. In both human and rodent normal prostate tissue, ERβ expression is higher than ERα whereas ERα expression is reduced in prostate tumors while epithelial hyperplasia of the prostate is observed in ERα KO mice [132]. In addition, ERα expression was observed in 65% of high grade prostate tumors while ERβ expression was decreased in both local and metastatic tumor sites when compared to normal prostate tissue [132]. Furthermore, aromatase-deficient mice that do not express ERβ display prostate hyperplasia and administration of an ERβ agonist suppresses that effect [132].
Reintroduction of ERβ signaling in prostate carcinoma cells results in antiproliferation, invasive and pro-apoptotic responses [132].

ER expression is also important in development of cervical cancer. A cervical cancer mouse model using HPV transgenic mice deficient in ERα showed that these mice fail to develop any of the progressive lesions that lead to cervical cancer and did not develop cervical hyperplasia when administered estrogen, which is common in cervical cancer development [133], indicating the requirement for ERα in cervical cancer development. ERα and ERβ expression is observed in 2/3 of human ovarian tumors, and several studies show that expression of ERα is a predictor of overall survival compared with patients with ERα negative tumors [134, 135]. ERβ expression was not associated with overall survival, but instead with chemosensitivity, treatment response, and decreased lymph node infiltration [134].

Several studies using cell culture and animal models have demonstrated the role of ER in breast cancer development. ERβ expression is high in normal mammary tissue and decreases as the tumor progresses with high ERβ expression associated with low grade tumor, higher disease free survival rate, decreased metastasis and angiogenesis [136]. This indicates ERβ may have a tumor suppressor role and loss of ERβ promotes breast cancer progression [137]. ERα is an important biomarker in breast cancer, since approximately 70% of all primary breast tumors are ERα positive [136]. Breast tumors lacking ERα often demonstrate an aggressive phenotype while ERα expression is a favorable
predictor of prognosis in patients treated with endocrine therapy [136]. However, ERα seems to loses its prognostic potential with a longer (>5 years) follow-up [136].

**ER Ligands**

Several endogenous and exogenous ligands have been identified for the ER. The most potent estrogen produced in the body is 17β-estradiol (E2) and its metabolites, estrone and estriol, have weaker agonist activity than E2. Both ERα and ERβ have high binding affinity for E2 with $K_D$ values of .04 nM and .11 nM, respectively [138]. Treatment of human breast cancer cells with E2 induced anchorage-dependent growth and invasiveness, demonstrating its carcinogenicity and ER agonist activity [138]. In addition, administration of E2 to HPV transgenic mice results in cervical cancer development [133].

E2 represents a classic ER agonist that activates ER signaling and faslodex (ICI 182,780) is a prototypical ER antagonist that binds, inhibits, and promotes degradation of ERs [139]. ICI 182,780 binds both ERα and ERβ with $K_D$ values of .42 nM and 1.3 nM, respectively [139]. ICI 182,780 treatment is effective in postmenopausal women with advanced breast cancer or patients who have become tamoxifen-resistant, which is a common first line treatment for breast cancer [138]. In addition, ICI 182,780 has no estrogenic effects in target tissues such as the uterus and breast cancer cells and tumors that are tamoxifen-resistant remain sensitive to growth inhibition by ICI 182,780 [138]. In randomized human studies of post-menopausal women with advanced breast
cancer treatment with ICI 182,780 did not induce any estrogen agonist activity and increased the rate of remission [140].

Selective estrogen receptor modulators (SERMs) represent a class of synthetic ER ligands that exhibit tissue-selective effects. SERMs can act as antagonists that oppose estrogen action in certain tissues, while mimicking the effects of estrogen in other tissue [138]. This class of compounds was initially developed as contraceptives, until their use as a hormonal treatment for breast cancer. Tamoxifen is the most studied SERMs to date and exhibits clinical efficacy in treating several diseases. Tamoxifen is a common first-line treatment for patients with ERα-positive breast cancer and demonstrates antagonist activity in breast [141]. Tamoxifen also exhibits agonist activity in bone and has been used in the treatment for bone density diseases in postmenopausal women [141]. One of the adverse side-effects of tamoxifen use for breast cancer is the increased risk of endometrial cancer development in both mice and humans [141]. A recent cohort study found a 69% increased incidence of endometrial cancer in patients treated with tamoxifen, with a 4-fold increased risk in patients over the age of 35 [142]. The overall risk-benefit for using tamoxifen is the low overall rate of endometrial cancer and this risk from can be decreased by decreasing the duration of treatment [142] In addition, tamoxifen administered to postmenopausal women prevents bone loss and results in loss of bone mineral density in premenopausal women [143, 144]
Glucocorticoid Receptor

GR Binding and Activation

Glucocorticoids are steroid hormones that bind the glucocorticoid receptor (GR) and they are synthesized and released by the adrenal cortex in a circadian manner in response to various stimuli. This process is controlled by the hypothalamic-pituitary-adrenal (HPA) axis. The actions of glucocorticoids are mediated by glucocorticoid receptor (GR) binding, which is estimated to target 10-20% of the human genome. In the absence of hormone, GR remains in the cytoplasm as a complex with chaperone proteins, such as hsp90, hsp70, and p23 [145]. These chaperone proteins help maintain a receptor conformation that is transcriptionally silent, but still favors ligand binding. Glucocorticoid binding induces a conformation change with results in the dissociation of chaperone proteins and exposure of its nuclear localization signal, resulting in nuclear translocation [146] and binding to its cognate response elements (GGAACAnnnTGTCT) [147] to regulate target gene expression. The GR exerts its function via a glucocorticoid response element that consists of two 6-base pair half-sites. GR binds to its response element as a homodimer with a three nucleotide space [147]. Recently, a negative GR response element that is different from the classic GR response element has been identified (CTCC(n)GGAGA) and binding to this element mediates glucocorticoid-dependent repression of specific genes [146, 147]; this finding is relatively new and more studies are needed to elucidate the details of this mechanism.
The location of GR response elements and negative response elements have been observed to be at a considerable distance from the transcription start site [148], suggesting a potential for the response elements to form loops that facilitate interactions with the promoter area of target genes. Once bound to DNA, GR undergoes further conformational changes that allow for recruitment of nuclear cofactors and chromatin remodeling complexes. These coregulators include histone acetyltransferases and steroid receptor coactivators, which aid transcriptional activation, while NCoR and SMRT [149] are recruited for transcriptional repression.

GR activation is dependent on ligand availability and chromatin accessibility. Some GR response elements are occupied at low concentrations of glucocorticoids, while others require high doses for GR binding and transactivation [150]. GR activation can occur via binding of endogenous steroid hormones such as cortisol or by synthetic ligands, including glucocorticoid drugs, such as dexamethasone (Figure 5) [150] and GR antagonists include mifepristone (RU-486) and ketoconazole. Both mifepristone and ketoconazole compete with GR agonists for receptor binding and result in repression of GR-mediated gene expression [151]. In addition, selective GR modulators (SGRM), function in a similar matter to SERMs. SGRMs preserve the anti-inflammatory and immunosuppressive (agonist) functions of glucocorticoids but do not exhibit the adverse effects, such as osteoporosis, diabetes, susceptibility to infection, and muscle atrophy [152].
**Figure 5. Structure of GR ligands.** Cortisol is an endogenous ligand for GR while dexamethasone and RU-486 are synthetic ligands for GR. Reprinted with permission: Weikum E Glucocorticoid receptor control of transcription: precision and plasticity via allostery *Nature Reviews Molecular Cell Biology* 18; 2017

**GR Physiological and Pathophysiological Functions**

Glucocorticoids regulate various physiological functions and homeostatic balances, including metabolism, immune function, skeletal growth, reproduction, cognition, and carcinogenesis [153]. In addition to direct interactions with DNA, GR also interact with other transcription factors to induce physiological changes. Cooperative binding with members of the STAT family enhances target gene transcription [154]; the GR also directly interacts with proinflammatory transcription factors such as NFκB to suppress target gene transcription, and this is a mechanism for GR-mediated anti-inflammatory activity [155]. Glucocorticoids regulate various immune cells, such as dendritic cells, T-cells, and macrophages and alter or depress the immune response [155]. GR also acts in a sex-specific way, since glucocorticoids induce a stronger antiinflammatory response in male compared to female rats [156]. Additionally,
GR signaling enhances neutrophil phagocytosis and inhibits neutrophil infiltration of inflammatory sites, both of which also result in a depressed immune response [155, 157]. GR signaling also plays a role in inflammatory respiratory conditions such as asthma. These respiratory conditions are commonly aggravated by proinflammatory transcription factors, such as NFκB, which can be inhibited by glucocorticoid binding, resulting in decreased production of cytokines, chemokines, and cell adhesion molecules of the respiratory tract [158]. Due to their role in anti-inflammatory and immunosuppressive functions, glucocorticoids are among the most widely used drugs in the world for disease such as inflammatory bowel disease, asthma, and rheumatoid arthritis [152]. Despite their many applications, some major adverse effects of these drugs include osteoporosis, diabetes, obesity, glaucoma, hypertension, and growth retardation in children [147].

In addition to these physiological effects, GR signaling also exerts anti-proliferative and anti-apoptotic activity via its ligand-binding interactions. Studies have found that GR is expressed at high levels early during breast cancer development with expression levels decreasing during progression [159]. Furthermore, GR expression is associated with a favorable outcome [159]. In addition to breast cancer, glucocorticoids have also been used to treat lymphoid malignancies, such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, and Hodgkin's lymphoma [160]. The synthetic glucocorticoid drug dexamethasone is widely used and induces apoptosis in hematopoietic
malignancies. GR-mediated apoptosis occurs through various mechanisms, including activation of pro-apoptotic genes such as Bim and via NF-κB mediated downregulation of survival cytokines [161]. Recent studies have also identified a new role for GR signaling in mitosis since GR signaling is necessary for mitotic progression and knockdown of GR results in increased genetic aberrations and increased time to complete mitosis [162]. Furthermore, GR haploinsufficient mice have increased tumor formation [162]. These results indicate a role of GR and GR dependent signaling in tumorigenesis as well as in tumor formation.

Retinoid X Receptors (RXR)

RXR Structure and DNA-binding

RXR belongs in the adopted orphan nuclear receptor subclass and includes three members, RXRα, RXRβ, and RXRγ, all of which have structural similarities. RXR exerts its function by heterodimerization with several other nuclear receptors including Constitutive androstane receptor (CAR), peroxisome proliferator-activated receptor (PPAR), and retinoic acid receptor (RAR) and functions as ligand activated transcription factors. Furthermore, RXR binds to specific hormone response elements (‘5-AGGTCA-3’) to regulate target gene transcription. This receptor primarily functions as a heterodimeric partner for other nuclear receptors that regulate retinoid and other signaling pathways [163].

Mutations to the amino acid sequence, such as tyrosine 402 in the helical structure of RXR, results in diminished capacity for form heterodimers, in which case homodimer formation is enhanced [164]. RXR interactions with other
nuclear receptors are linked to its potential to induce pleiotropic effect on various physiological pathways.

**RXR Ligand Binding and Dimerization**

Ligands for RXR have been identified and include 9-\textit{cis} retinoic acid (RA) synthetic ligands called rexinoids that are RXR selective, phytanic acid, and decosahexaenoic acid [165, 166]. While 9-\textit{trans} and \textit{-cis} RA can bind all RXR isoforms, 9-\textit{cis} RA has the highest affinity for RXR\textalpha{} [167]. Despite identification of these ligands, none have proven to be endogenous ligands for RXR. RXR is subject to ligand induced conformational change [168] which allows for homo or heterodimer formation. The conformational changes induced by ligand binding also repositions the protein into an active conformation that facilitates coactivator binding [169]. RXR signaling by rexinoid binding has been shown to increase oxidation and uptake of saturated fatty acids in diabetic skeletal muscle cells, which is important since insulin resistance is associated with intramuscular saturated fatty acid accumulation [170].

Some RXR target genes, such as fatty acid transporter (FAT/CD36) are upregulated in response to RXR ligand binding and increased FAT/CD36 expression has been associated with increased uptake of saturated fatty acids into skeletal muscle cells [171]. In addition to FAT/CD36 upregulation, binding of other synthetic ligands to RXR resulted in induced expression of UCP3 (Uncoupling protein 3) and PDK4 (pyruvate dehydrogenase lipoamide kinase isoenzyme 4) in skeletal muscle cells of mice and both of these genes lead to
increased uptake of saturated fatty acids [172, 173]. This demonstrates a mechanism by which RXR ligands can increase fatty acid uptake and oxidation and insulin sensitization.

**RXR Physiological and Pathophysiological Function**

RXRα is expressed in liver, kidney, spleen, and placenta tissues while RXRβ is widely expressed and found in almost all tissues throughout the body [167]. In contrast, RXRγ localization is limited to the muscle and brain [167]. While all three members of the RXR subfamily have similar structural homologies, their expression patterns can vary, and this may have implications for their respective functions. This diverse expression patterns observed for RXR is demonstrated by its varied role in pathophysiological and metabolic functions.

Other nuclear receptors that regulate gene expression in cardiac metabolism, namely PPARα and LXR, require RXR as a heterodimeric partner [174]. One example of this interaction is the role of PPAR, LXR, and RXR in atherosclerosis and cholesterol metabolism. Common lipid components of atherosclerotic plaques, such as 9- and 13-hydroxyoctadecadienoic acid, serve to activate PPAR, which leads to the transcriptional activation of LXR [175, 176] and thereby induction of key lipid transporters, ABCA1 (Cholesterol efflux regulatory protein) and ABCG1 (ATP binding cassette subfamily G member 1) [176]. This is indicative of a coordinated role of both LXR and PPAR in lipid signaling. RXR can play a role in this process via heterodimeric binding to LXR, resulting in formation of a complex that can be activated by oxysterol ligand
binding to LXR or rexinoid binding to RXR [177]. This suggests that rexinoids have the ability to regulate lipid homeostasis implicated in cardiovascular disease.

In addition to their role in cardiovascular disease and metabolic processes, RXR signaling is also important in cancer. For example, RXR can cooperate with RAR to induce differentiation and apoptosis, which have direct protective effects on cancer development and growth. RXR activation induces apoptosis in immature acute promyelocytic leukemia cells while RXR antagonism inhibits this process [178]. Apoptosis of immature hematopoietic cells is regulated by RXR while apoptosis of mature hematopoietic cells is triggered by RAR agonists via induction of TRAIL (TNF-related apoptosis inducing ligand) [178], indicating distinctive roles of RXR and RAR signaling in triggering apoptosis. In addition to leukemia, RXR signaling also induces apoptosis in the retinoic acid resistant breast cancer cell line MDA-MB-231 via RXR/Nur77 heterodimers [179] and an unknown downstream mechanism. In addition to leukemia and breast cancer, RXRα knockout in an F9 murine embryonal carcinoma cell line resulted in impaired apoptosis and decreased proliferation as evidenced by chromatin condensation along the nuclear membrane, apoptotic bodies and endocytosis by nearby cells [180].
Peroxisome Proliferator-activated Receptors

*PPAR Structure and DNA-binding*

Peroxisome proliferator-activated receptors (PPAR) also have diverse roles and regulate multiple biological pathways, including cellular differentiation, diabetes, atherosclerosis and cancer. Studies on this receptor have shown that PPAR exerts its function through heterodimer formation with one of the retinoid X receptors (RXR) [181]. There are several PPAR isoforms, including PPAR\(\beta/\delta\), PPAR\(\alpha\), and PPAR\(\gamma\) (Figure 6), all of which bind to a PPAR response element (‘5-AGGTCANAGGTCA-3’) and have a structure similar to other NRs [181].

![Figure 6. Structural homology of PPAR isoforms.](image)

The N-terminal A/B domain contains ligand-independent transcriptional activation (TA). The C domain is the DNA binding domain (DBD). The D domain includes the hinge (H) region. The E/F domain consists of the LBD, including the ligand-dependent activation function, and RXR interaction. Percentages are in reference to PPAR\(\delta\) isoform structure.

Reprinted with permission: Ehrenborg E Regulation of Skeletal Muscle Physiology and Metabolism by Peroxisome Proliferator-Activated Receptor \(\delta\) *Pharmacological Reviews* 61;3 2009
PPARs interact with various coactivators and corepressors that interface the PPAR-RXR heterodimer with chromatin and transcriptional machinery, allowing for repression or activation of target genes [181]. Common coactivators observed for this interaction include members of the CBP/p300 and DRIP/TRAP families, which regulates chromatin acetylation, and transcriptional activity [182].

*Role of PPARs in Physiology and Disease*

PPARs regulate multiple genes/pathways, including adipogenesis, metabolism, and cardiomyocyte development. Ectopic expression of PPARγ promotes adipogenesis in nonadipoegenic fibroblast cells, furthermore, when combined with a PPARγ agonist and the pro-adipogenic protein C/EBPα myoblasts can be transdifferentiated into adipocytes; this points to a major role of PPARγ in the generation of adipose tissue. Mouse studies have also demonstrated this effect in PPARγ−/− mice which fail to develop brown adipose stores [182]. *In vitro* studies demonstrate that this receptor is integral for differentiation of ES cells into adipose cells [183]. In addition to its role in fat storage, PPARγ is also crucial for placental and heart development. Previous studies have found PPARγ to be essential as early as embryonic day 10 and plays a major role in establishment of trophoblast lineage cells [182]. Additionally, PPARγ KO mouse placentas have major structural differences when compared to normal placental tissue, and loss of PPARγ has a deleterious effect on the placental vasculature as well as maternal-fetal nutrient exchange [182]. Additionally, histological examination of PPARγ null embryos revealed
degeneration of myocardial cell structures and ventricular defects when compared to normal mouse embryos. Furthermore, the PPARγ null phenotype also includes premature cardiomyocyte differentiation, leading to developmental defects [182].

In addition to PPARγ, PPARα and PPARβ/δ are also required for lipid homeostasis and to breakdown fatty acid for energy production, primarily during starvation [184]. This observation pointed to endogenous fatty acids as activators of PPARα. PPARα also improves insulin resistance in high fat diet rodent models of diabetes by altering gene expression of LPL and leptin, resulting in weight gain prevention [185]. Like the other PPAR isoforms, PPARβ/δ also regulates lipid and glucose homeostasis, with expression highest in the intestine, colon, and skin with some studies indicating its colocalization with RXR [169]. Ligand activation of PPARβ/δ decreases serum triglycerides, inhibits high-fat diet-induced obesity, and increase insulin sensitivity, primarily through regulation of genes that encode fatty-acid metabolizing enzymes and lipogenic proteins in the liver [186]. Furthermore, PPARβ/δ inhibits liver inflammation as a result of genetic, dietary, or chemical stimuli, and there is some evidence showing that this represses NFκB signaling and decreases inflammatory cytokine expression [185]. These results demonstrate a variety of potential PPAR targets that can be exploited in development of pan-PPAR and specific PPAR isoform specific ligands. Endogenous ligands for PPAR include free fatty acids, eicosanoids, and arachidonic acid metabolites [186]. These
endogenous ligands act as PPAR agonists that activate genes involved in lipid and glucose homeostasis and this includes liver fatty acid binding protein, long chain fatty acyl-CoA synthetase, and peroxisomal acyl-CoA oxidase [187]. Additionally, various high affinity synthetic ligands have also been identified as PPAR ligands, which include thiazolidinedione drugs and aryl tyrosine derivatives, both of which have metabolic targets and have applicability to diabetes treatments [188]. Recent studies have found PPARα-active drugs to treat hypolipidemia, including benzafibrate, clofibrate, and fenofibrate [186].

PPAR and Cancer

In addition to these biological functions, a number of studies have shed light on the role of all three PPARs in the development of cancer. Several studies have implicated PPARα in liver cancer development in humans, since long term administration of PPARα agonists induces liver cancer in rodents, a mechanism which is PPARα dependent, since PPARα null mice do not develop hepatocarcinoma when administered PPARα agonists [189]. Other studies have also found PPARβ/δ expression is higher in ovarian, endometrial, and breast tumors compared to non-transformed tissue [184]. Unlike PPARα, the evidence for the role of PPARβ/δ in cancer development has been controversial. There is a lack of consensus on its role in cancer due to contradictory studies in the literature. Two hypotheses have emerged from the published reports; (1) PPARβ/δ is overexpressed in tumors and can promote anti-apoptotic activity and increased cell proliferation and (2) PPARβ/δ can promote terminal differentiation
and inhibit pro-inflammatory signaling, which has antitumorigenic implications [184].

In contrast, there are also data that supports the anti-tumorigenic effects of PPARβ/δ. There have been reports of higher PPARβ/δ expression in normal colonic epithelium in both human and mouse tissues [184], directly conflicting with other studies [184, 190-193]. There is some evidence that the differential expression can be attributed to APC/β-catenin mediated transcription [184]. Key concerns regarding these conflicting results is the lack of positive and negative controls to compare expression and the low number of samples examined was relatively low. In addition, there is evidence showing that PPARβ/δ promotes terminal differentiation in multiple cell types, including keratinocytes, intestinal epithelium, osteoblasts, and oligodendrocytes [184]. This mechanism is thought to occur via upregulation of genes required for terminal differentiation, an observation that is not seen in cells lacking PPARβ/δ expression [184]. Additionally, PPARβ/δ also inhibits expression of various proinflammatory signaling molecules, including NFκB, TNF, IL-6, and IL-1β [194]. This observation could have clinical impact on the role of PPARβ/δ in tumor development, and the application of PPARβ/δ ligands since inflammation is a key component of tumor development.

PPARγ is also implicated in carcinogenesis and several studies have demonstrated a role for PPARγ as an inhibitor of colon, breast, prostate, and lung cancer via various mechanisms, including terminal differentiation, inhibition
of tumor promotion and cell growth, and induction of apoptosis. Tumor expression profiles indicate positive outcomes in colon cancer patients expressing high levels of PPARγ [195]. Additionally, PPARγ agonists have demonstrated modest efficacy in clinical trials as a chemoprevention agent in colon cancer [195, 196]. Furthermore, ligand activation of PPARγ in cancer cell lines is associated with cell cycle arrest and induction of genes/proteins required for terminal differentiation, including E-cadherin, keratins, carcinoembryonic antigen [184]. PPARγ also acts in concert with co-activators, such as HIC5 (TGFB111), to cooperatively increase expression of proteins known to induce terminal differentiation, such as kruppel-like factor 4 and keratin 20 [188].

In addition to its role in induction of differentiation, PPARγ agonists can also modulate the expression of cell cycle and apoptosis regulators. Such actions include decreased expression of cyclin D1 and increased expression of cyclin-dependent kinase inhibitors p21 and p27 [196]. PPARγ also plays a role in increased apoptotic signaling and PPARγ agonists modulate the expression of apoptosis regulating genes, including increasing expression of pro-apoptotic BAX and BAD and decreased expression of anti-apoptotic proteins BCL-XL and Bcl-2 [188]. Ligand-bound PPARγ also inhibits PI3K activity, AKT phosphorylation, and activation of JUN N-terminal protein kinase [184] and these activities are also linked to induction of apoptosis. Studies on all three PPARs demonstrate their diverse functions in disease processes, including insulin resistance, diabetes, obesity, chronic inflammation, and cancer risk. Therefore,
all forms of this nuclear receptor represent promising targets for receptor agonist or antagonists in treating a variety of diseases.

**ORPHAN NUCLEAR RECEPTORS**

**Introduction**

Endogenous ligands have been identified for several members of the nuclear receptor superfamily, however, ligands have not yet been identified for those NRs designates or orphan NRs. While their structures and functions are similar to that of other nuclear receptors, there is evidence that some orphan nuclear receptors have target regions outside of the conserved ligand binding pocket that facilitate receptor-cofactor interactions and receptor functions. One such example is nuclear receptor 4A2 (NR4A2, Nurr1) and its agonist, 6-mercaptopurine (6-MP) that activates NR4A2 through its AF1 domain [197]. Identification of ligands for orphan receptors can be achieved by various methods and the most frequently used approach is a cell-based assay in which cultured cells are transfected with an NR4A-responsive promoter construct linked to a reporter gene, such as luciferase or green fluorescent protein [198]. These transfected cells are then treated with ligands and activity (decreased or increased) of the reporter gene is assessed. This screening assay has resulted in identification of ligands for various receptors, including RAR, RXR, PXR, and PPAR, among others [198]. Another screening method uses an immobilized target protein on a solid support and mixtures containing possible ligands are passed over the immobilized target protein, washed, and the putative ligand is
eluted and analyzed by methods such as mass spectrometry [198]. Additionally, crystal structures of ligand binding domains have been important for identifying ligands for orphan receptors. These crystal structure provide a detailed picture of the ligand binding domain, which facilitates the design of pharmacologically active ligands for these receptors. For example, analysis of the crystal structure for RAR related orphan receptor α (RORα) identified cholesterol as a potential ligand [199]. While crystal structure analysis can be beneficial, the emergence of virtual screening of molecular compound libraries to identify ligands has emerged as a powerful tool in this field. This process utilizes high-throughput molecular docking and allows for the filtering of a larger number of proteins, based on the crystal structure of the target protein, to screen a large number of proteins [200]. This technique has identified antagonists for RAR as well as selective estrogen receptor modulators (SERMs) [200]. As more ligands are identified, the field of orphan receptor drug discovery will progress and this is important since orphan receptors also play a major role in disease and are potential drug targets.

**NR0B Subfamily**

Members of the NR0B [201] subfamily include DAX1 (NR0B1) and SHP (NR0B2), which contain a classical LBD but not a DBD, and therefore their functions are dictated by receptor-protein interactions and not receptor-DNA interactions. The adamantly-derived retinoid CD437 and its derivatives bind SHP resulting in retinoid induced nuclear translocation of SHP that is
characteristic of retinoid binding [202, 203]. While ligands have been identified for SHP, identification of ligands for DAX1 has not yet been successful. Indeed, mithramycin was identified as a DAX1 ligand in a screening assay, however, the mithramycin-induced effects could very well be due to other functions of this drug, including its ability to inhibit transactivation from GC-rich promoter regions [204, 205]. The crystal structure analysis of DAX1 shows that the ligand binding pocket is filled with amino acid side chains [206], suggesting that identification of a classical nuclear receptor-ligand is unlikely.

DAX1 is primarily expressed in the hypothalamic-pituitary-adrenal axis and mutations result in adrenal hypoplasia, hormone deficiencies, and gonadal dysfunction [201]. Conversely, SHP is highly expressed in the liver and small intestine, where it plays a role in cholesterol regulation and bile acid and glucose metabolism [118]. While both members have tissue specific expression, both SHP and DAX1 have been characterized as transcriptional repressors [206]. SHP has been identified as a corepressor of NF-κB during inflammatory signaling and as a repressor of OCT4, indicating its role in inflammatory signaling and embryonic stem cell pluripotency [118].

In addition to these established physiological roles, both members of this subfamily are prognostic factors for cancer or overexpressed in some tumors. While DAX1 expression is limited in normal tissues, it is highly expressed in mammary, endometrial, ovarian, pituitary, lung, prostate, and Ewing’s sarcoma [201]. Several studies have correlated the expression level of DAX1 with cancer
prognostic factors. Higher levels of DAX1 expression were observed in endometrial tumors compared to normal tissues and this pattern was also observed in prostate tumors. In node-negative breast cancer cases, low DAX1 expression is associated with poor survival while high DAX1 expression was associated with greater survival [207]. High DAX1 expression in lung cancer tumors is associated with lymph node metastasis and decreased disease-free survival [208]. Mechanistic studies on DAX1 have demonstrated that silencing in lung cancer decreases growth, survival, and invasion, pointing to its pro-oncogenic role [201]. Furthermore, DAX1 cooperates with NR4A1 to mediate OCT4 activation, which is a stem cell marker and indicates a role for DAX1 in stem cells and cancer stem cell function [209].

Limited reports on SHP indicate high expression in hepatocellular carcinomas compared to normal liver and higher expression in intestinal precancerous lesions when compared with normal gastric mucosa [210]. Evidence of a role for SHP in hepatocellular carcinoma comes from observations that SHP null mice develop heptacellular tumors at one year of age [211], suggesting an inhibitory role for SHP in liver tumorigenesis. In contrast, SHP plays a tumor-promoting role in intestinal metaplasia by regulating expression of caudal-related homeobox gene (CDX1), which has a known pro-oncogenic role in intestinal precancerous lesions [210]. While there is limited data for the use of SHP as a cancer prognostic, one study found high SHP expression correlated with greater disease-free survival in breast cancer patients [212]. In addition,
high SHP expression is correlated with greater survival of hepatocellular carcinoma patients [211]. In addition, SHP is known to interact with various transcription factors, nuclear receptors, cofactors, and oncogenes [213]. SHP overexpression has been shown to enhance the ubiquitin ligase activity of Mouse double minute 2 homolog (MDM2), which induces p53 instability [214]. Taken together, these results demonstrate a role for NR0B orphan receptors in tumorigenesis and tumor development.

**RAR-related Orphan Receptor (ROR) Subfamily**

The ROR subfamily of orphan receptors includes three isoforms, RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3) that bind to their respective response elements as monomers to specific ROR response elements (ROREs) consisting of the consensus core motif AGGTCA preceded by a 5-bp A/T-rich sequence [215]. RORα is expressed in specific areas of the brain, including the cerebellum and SCN of the hypothalamus and also in the spleen and thymus [201]. Mutations of the RORα gene in mice result in an ataxia-like phenotype which is thought to be due to neurodegeneration in the cerebellum, resulting in major developmental defects [216]. RORβ is expressed in areas of the CNS involved in sensory information processing and components of the mammalian circadian system, such as the suprachiasmatic nuclei and the retina. Suggesting a role for RORβ in regulation of circadian rhythm and sensory information processing. RORβ knockout mice display an altered gait, abnormal circadian behavior, as well as retinal degeneration [217]. RORγ expression is
concentrated in skeletal muscle and thymocytes and RORγ knockout mice have dysregulated thymocyte development and altered lymphoid organogenesis [218].

While ligands for RORγ have not been described, they have been identified for RORα and RORβ. It has been reported that RORβ transactivation is inhibited by all-trans retinoic acid, suggesting a role for retinoids in RORβ-related CNS diseases. Also, melatonin and cholesterol have been reported as potential endogenous ligands for RORα [216]. Studies supporting this conclusion found a reduction in RORα activity after depletion of cellular cholesterol, whereas high levels of cholesterol reactivated RORα [216]. This points to a role for RORα in cholesterol homeostasis and as a potential drug target for cholesterol-related diseases.

In addition to these physiological roles, ROR also play a role in cancer. RORα expression is high in breast cancer tumors and is associated with higher relapse rates and metastasis [219]. RORα is also highly expressed in colorectal cancer and is associated with lymph node metastasis and decreased survival rates [220]. RORα mediates inhibition of Wnt signaling and Wnt target genes in colon cancer [221] and this is due to displacement of coactivators that bind to β-catenin [221] by RORα, resulting in decreased expression of Wnt target genes such as cyclin D1 and c-myc. This provides evidence for a mechanism associated with RORα-induced tumor suppressive activity. Recent studies show
that activation of RORα by a synthetic agonist resulted in stabilization of p53 protein expression via Mouse double minute 2 homolog (MDM2) repression [222], which has obvious implications for increased apoptosis in cancer. RORγ is highly expressed in castration-resistant prostate cancer and metastatic prostate cancer [223]. Conversely, RORγ expression is associated with increased disease-free survival of breast cancer patients [224], indicating its tumor type specific role.

**NR1D Subfamily**

The NR1D subfamily include NR1D1 (Rev-erbα) and NR1D2 (Rev-erbβ) and have a protein structure similar to other NRs and exhibits high sequence homology (Figure 7).

Figure 7. *Structure homology of Rev-Erb subfamily.* (A) The general organizational structure of members of the nuclear receptor superfamily compared to the (B) Rev-Erb family. Numbers above each receptor represent the amino acid position. Percentages indicate amino acid identity within a particular domain relative to REV-ERBα. A/B, C, D, E and F refer to classically defined regions in the nuclear receptor domain structure as previously described. DNA-binding domain (DBD); ligand-binding domain (LBD). Reprinted with permission: Kojetin D REV-ERB and ROR nuclear receptors as drug targets *Nature Reviews Drug Discovery* 13; 2014
This receptor subfamily is unique because they lack an alpha helix located in the AF-2 domain that plays a role in receptor-coactivator interactions, which results in a protein conformation that favors corepressor recruitment [225]. Members of this subfamily primarily exhibit transcriptional repressor activity and bind to response elements identical to that of ROR (NR1F) orphan receptors [226]. In the search for NR1D ligands, studies have identified heme as a ligand for both Rev-erbα and Rev-erbβ and heme-binding enhances cofactor recruitment as well as repression of circadian genes, such as BMAL1 and CLOCK [227, 228]. Furthermore, synthetic ligands for both Rev-erbα and Rev-erbβ have been developed [229], several with promising agonist or antagonist activity. The NR1D subfamily has also been implicated in various physiological processes, including cerebellar development, osteoarthritis, adipogenesis, and circadian rhythms. Mice lacking Rev-erb have decreased development and increased apoptosis in the cerebellum, and [230] Rev-erb has been shown to play an integral role in adipocyte differentiation [231]. While the localized expression of the NR1D members, if any, has not been determined yet, these receptors play a role in gene regulation, including that of Nmyc and deleted in breast cancer 1 (DBC1) via promoter binding or protein-protein interactions [232-234]. Additionally, Rev-erbα has been implicated as a negative prognostic factor in breast cancer, and its expression is correlated with poor clinical outcomes and was a significant predictor of breast tumor recurrence within 5 years in patients
with ER positive and negative tumors [235]. These results contrasted to a later study that found lower expression of Rev-erbα in ER positive and negative breast tumors when compared to normal breast tissue [236]; this contradiction could be due to the variation in the number of patient samples taken in both studies, since the latter study was significantly more robust.

NR2F Subfamily

The NR2F subfamily includes NR2F1 (COUP-TFI), NR2F2 (COUP-TFII), and NR2F6 (EAR2) and are widely expressed in several tissue and tumor types. While the structure of these receptors is similar, COUP-TF1 and COUP-TFII exhibit the highest sequence homology (Figure 8).

![Figure 8. Structure of COUP-TFI and COUP-TFII. Sequence homology between COUP-TFI and COUP-TFII. The DBD and LBD have the highest level of homology between them and significant homology in their AF1 domains. Reprinted with permission: Boudot A Involvement of COUP-TFs in Cancer Progression Cancers 3;1 2011](image)

These orphan receptors have the highest affinity for the direct repeat sequence GGTCA motif with a 2-bp spacing and recognize direct and inverted half site
response elements of other NRs, including RAR, RXR, and vitamin D receptor (VDR) [237, 238], which gives them promiscuity and the ability to antagonize the action of other nuclear receptors. This subfamily generally exhibits transcriptional repressor activity which is mediated by heterodimerization with other transcription factors or with RXR [239]. While ligands have not been identified for all members of this family, 9-cis and all-trans retinoic acid bind COUP-TFI [201]. EAR2 also exhibits repressor activity and the ability to not only heterodimerize with other nuclear receptors, but also its other subfamily member, COUP-TF [240]. Most of the physiological roles of the NR2F subfamily have been elucidated via loss of function models. Both COUP-TFI and COUP-TFII have essential functions in neural and retinal development, and in many cases, their functions overlap [239, 241]

COUP-TFII expression is higher in prostate tumor samples compared to nontumor tissue and also predicted earlier recurrence of the disease [242]. In addition to prostate cancer, COUP-TFII expression was a negative prognostic factor for patient survival and was also associated with ERα expression [243], which points to the pro-oncogenic function of COUP-TFII. In addition to COUP-TFII, EAR2 is also expressed in various tumor types. This receptor is highly expressed in both ER positive and ER negative breast tumors when compared to normal breast tissue [244] and lower levels of EAR2 conferred enhanced sensitivity to anticancer drugs [244]. Furthermore, EAR2 plays a role in development of lymphoma and colorectal tumors.
NR2E Subfamily

The NR2E subfamily includes TLX also known as NR2E1, as well as NR2E3, or PNR. TLX can act as a monomer that binds to 5′-AAGTCA-3′ half-sites in the promoter regions of target genes, such as the retinal development gene Pax2 [245, 246] while PNR binds as a homodimer to direct repeats of 5′-ANGTCA-3′ sites separated by 1 bp [247]. Recent studies show that PNR forms homodimers and heterodimers with PPARγ and not other members of the PPAR subfamily [248]. Ligands for PNR have been identified using transactivation assays [249] but direct binding studies are necessary to confirm these interactions. PNR and PPARγ are coexpressed in human retinal tissue while alterations in the PNR LBD result in human retinopathies and disruption of PNR/PPARγ complex formation [248], which is congruent with the localization of PNR in the retina and in retinal cells [250]. These observations suggest a role for PNR/PPARγ interactions in retinal development and disease. Additionally, PNR correlated with ERα expression in breast tumor and breast cancer cell lines [251] and expression in breast tumors has been associated with recurrence-free survivals of breast cancer patients [251]. Furthermore, PNR knockdown resulted in decreased estrogen induced cell proliferation and expression of estrogen responsive genes [201].

TLX is an orphan receptor that is mainly expressed in the brain and plays an important role in neural development. It is also required for the formation of cortical layers in the embryonic brain and for timing of neurogenesis during
development [117]. TLX knockout mice also show an altered phenotype in embryonic stages as well as in adulthood, and mature mice have reduced cerebral hemispheres as well as severe retinopathy [252]. Additionally, TLX mutants also exhibit deficits in their limbic system, resulting in increased aggressiveness and violent behavior, reduced learning abilities, and decreased copulation [253]. TLX also plays a major role in retinal development and is critical in controlling the generation of retinal cells. TLX knockout mice have neural retinas that are significantly thinner than normal counterparts [254]. Furthermore, TLX plays an important role in neural stem cell maintenance and self-renewal capabilities since it keeps adult neural stem cells in a self-renewable and undifferentiated state [117]. TLX-expressing cells from TLX-heterozygote brains are able to proliferate, self-renew, and differentiate into all neuronal cell types in vitro, while TLX-null cells lack the ability to proliferate and reintroduction of TLX to TLX-null cells rescues this characteristic [252]. Furthermore, in vivo, neural stem cells of TLX mutant mice loose their ability to proliferate and show reduced neural precursors in adult brains [117], which again points to the role of TLX in regulating neuronal stem cell population characteristics.

Both TLX and PNR play a role in cancer development, and TLX overexpression results in increased cell proliferation, tumorigenesis enhancement, and glioma formation [252]. The mechanisms responsible for this activity are thought to be associated with TLX regulation of vascular endothelial
growth factor (VEGF) and activation of cyclin D1 [255]. Mechanistic studies on PNR have demonstrated that PNR works cooperatively with signal transducer and activator of transcription 3 (STAT3) to bind the ERα promoter and regulate changes in ERα gene expression [251]. Furthermore, genetic screening has showed that PNR interacts with p53 and the cofactor p300, further indicating its role in cancer signaling [256].

NR4A SUBFAMILY OF ORPHAN NUCLEAR RECEPTORS

NR4A Structure and DNA-Binding

The NR4A subfamily of nuclear receptors is made up of three members, Nur77 (NR4A1), Nurr1 (NR4A2) and Nor1 (NR4A3) all of which have a similar structure to NRs (Figure 9).
Figure 9. Structure and domains of NR4A subfamily members. Sequence homology is shown above each receptor relative to NR4A1. Members of this family are highly conserved in the DBD (C domain; ~90%) and LBD (E domain ~60%), but divergent in the N-terminal A/B region (~20%).

They were initially identified as immediate-early genes induced by nerve growth factors in PC12 cells [257], their expression and activation is cell-type specific and they respond to a variety of signals, including mitogenic, growth factors, cytokines, hormone, neurotransmitters, and apoptotic signals [216]. Members of this subfamily bind to specific Nur-responsive elements (NuRE) as homo or heterodimers and to NGFI-B response element (NBREs), as monomers; NR4A1 also forms a heterodimer with RXR (Figure 10) [216].
While NR4A1 and NR4A2 bind as heterodimers with RXR to mediate retinoid signaling, NR4A3 is unable to do this [216]. The classical understanding of nuclear receptor signaling includes a hydrophobic surface in the LBD, which acts to recruit cofactors for transcription. In contrast, NR4A subfamily receptors have a hydrophilic surface in the LBD, rather than the classical hydrophobic cleft, which mediates coactivator recruitment [258]. Crystallography studies show that the LBD of NR4A2 can adopt a folding pattern that resembles an agonist activated LBD [259], which can result in ligand-independent recruitment of cofactors and transcriptional activation.
The NR4A subfamily can be induced by various physiological signals, including growth factors, calcium, inflammatory cytokines, neurotransmitters, and fatty acids [216], indicating its ability to sense and respond to changes in the cellular environment. Recent studies have identified cytosporone B (CsnB) as a ligand for NR4A1 and 6-mercaptopurine, substituted benzimidazole, and pyridinone derivatives as Nurr1 ligands [197, 260-262]. While the activity of most of these ligands involves nuclear localization of the receptor, CsnB induces both nuclear retention and export and this directly affects the function of NR4A1 [260]. CsnB can induce proapoptotic activity by inducing nuclear export of NR4A1 to the mitochondria [260] and can also decrease expression of antiapoptotic genes, such as brain and reproductive organ expressed (BRE) via nuclear NR4A1 dependent repression [263].

**Physiological Role of NR4A**

The NR4A subfamily members are expressed throughout the body and play a tissue/organ-specific role in homeostasis and disease. NR4A2 is expressed in nervous tissue, and is a drug target for nervous system disorders, such as Parkinson’s Disease (PD) [264]. Studies have shown that Nurr1 knockout mice have impaired dopaminergic function and increase apoptosis of dopaminergic neurons, which are cells that are lost as PD progresses [265]. Furthermore, NR4A2 activates tyrosine hydroxylase, an enzyme involved in dopamine synthesis, and it is essential for dopaminergic neuron development in the midbrain [266]. Furthermore, PD midbrains have decreased expression of
NR4A2, especially in cell types that are associated with neuronal degeneration in PD [266]. Nurr1 mutations have also been associated with the development of PD [264].

NR4A receptors regulate genes in the hypothalamus-pituitary-adrenal axis, that are associated with steroidogenesis, inflammation, stress response, and energy storage, among others. Studies focused on steroidogenesis show that NR4A1 regulates steroid 17-hydroxylase and the 20α-hydroxysteroid dehydrogenase promoters and enhances gene expression of adrenocorticotropic hormone precursors, indicating its role in adrenal steroidogenesis [216]. Recent studies show that NR4A1 is elevated in macrophages after lipopolysaccharide or cytokine stimulation [216]. Specifically, NR4A1 is induced in response to inflammatory cytokines in rheumatoid arthritis [216]. In addition, NR4A1 also plays a role in atherosclerosis, and is expressed in human atherosclerotic lesions in various stages of atherosclerosis development and also in smooth muscle cells [216].

NR4A3 is also expressed in vascular smooth muscle cells and Nor1 inhibition decreases LDL-induced mitogenic proliferation of these cells [267]. In addition, mitogenic stimulation with PDGF results in induction of NR4A3 expression in smooth muscle cells of atherosclerotic lesions, indicating its role in smooth muscle cell proliferation and atherosclerosis [268]. In addition, NR4A3 expression in adipocytes has been linked to insulin signaling by stimulating insulin-dependent glucose uptake and is induced by insulin and
thiazolidinedione drugs used to treated type 2 diabetes [269]. NR4A3 inactivation by lentiviral short hairpin RNA resulted in attenuated ability of insulin to stimulate glucose transport [269].

**NR4A Expression and Prognostic Significance in Cancer**

NR4A receptors are expressed in many tumors and cancer cell lines, however, there is limited data on direct comparisons of all 3 receptors and their expression and prognostic significance in the same tumor type. NR4A1 is highly expressed in cancer cell lines and high expression groups have been observed in tumors from ER-positive and ER-negative breast cancer, colon, lung, and pancreatic cancer patients [236, 270-272]. Moreover, NR4A1 is a negative prognostic factor for lung and breast cancer patients [236, 270, 273]. High NR4A1 expression in colorectal cancer cells is also predictive of the resistance of cells to various chemotherapeutic agents [274], overall contributing to a negative prognosis.

NR4A2 overexpression has been observed in tumors from bladder, prostate, and ER positive breast cancer patients [275-277]. NR4A2 expression in tumors is a negative prognostic factor for bladder, prostate, colon, and gastric cancer patients [274-276, 278] and potentially a positive prognostic factor for relapse free survival of breast cancer [277]. High levels of NR4A2 in the cytoplasm is also indicative of high tumor grade, decreased survival, and increased metastasis in bladder cancer patients [275].
The studies on NR4A3 expression in cancer cells and tumors are limited, however, NR4A3 is overexpressed in ER positive and negative breast cancer compared to normal breast tissue [236]. In addition, NR4A1 and NR4A3 double knockout mice rapidly develop acute myeloid-type leukemia (AML) [279] and levels of NR4A1 and NR4A3 are low in AML [280] while low NR4A1 expression is associated with decreased survival [281] and NR4A3 expression is positively correlated with therapy success in AML patients [282].

**Novel Role of NR4A1 in Solid Tumors**

The role of NR4A members can be tissue and disease specific, and this is evidenced by NR4A1 expression which is decreased in acute myeloid leukemia patients [283], and this is in contrast to the high expression and pro-oncogenic role of NR4A1 in solid tumors [201]. Knockdown of NR4A1 in acute myeloid leukemia cell lines resulted in increased growth and decreased apoptosis while this same treatment in pancreatic, colon, lung, melanoma, cervical, and ovarian cancer cell lines resulted in inhibition of cell growth, survival, migration, and invasion [270, 284-287]. Furthermore, many of these effects in solid tumors involve nuclear NR4A1 [201]. Retinoid induced proapoptotic responses that are NR4A1 mediated are blocked leptomycin B, a nuclear export inhibitor [288], indicating that NR4A1 mediated apoptosis induced by specific apoptosis-inducing agents require nuclear export of the receptor. The mechanism behind this apoptotic pathway involves formation of a novel NR4A1/bcl-2 proapoptotic complex that results in cytochrome c release [288] and thereby activates the
intrinsic apoptosis pathways. Providing further evidence for this mechanism is the increased survival of liver cancer cells and platinum drug resistance as a result of CHD1L (chromodomain helicase/adenosine triphosphatase (ATPase) DNA-binding protein 1-like) expression which inhibits platinum drug-induced nuclear export of NR4A1 [203]. Taken together, these results demonstrate the diverse and tissue specific mechanisms behind the action of NR4A1 subfamily orphan receptors in normal and disease states.

Development of NR4A1 Ligands

Cruciferous Vegetables and Their Indole-Derived Anticancer Agents

The effects of cruciferous vegetable consumption in mediating diseases, such as cancer, has been investigated showing that cruciferous vegetables are protective and therapeutic against cancer in human and in laboratory animal studies. The anticarcinogenic actions of cruciferous vegetables, such as brussel sprouts, cabbage, kale, and broccoli are due to breakdown products of sulfur containing compounds called glucosinolates, and more specifically glucobrassicin (3-indolylmethyl glucosinolate) [289] (Figure 11). When taken orally, the enzyme myrosinase is activated and catalyzes hydrolysis of glucobrassicin to give glucose and Indole-3-carbinol (I3C) [290] (Figure 11). In the acidic environment of the stomach, I3C forms several condensation products, one of which is 3,3’-diindolylmethane (DIM) [290] (Figure 11). While this hydrolysis predominates when cruciferous vegetables are consumed raw, cooking methods such as boiling, decreases myrosinase activity and
Glucobrassicin hydrolysis is decreased. This does not entirely preclude hydrolysis of glucobrassicin, since there is myosinase activity in human intestinal bacteria. Cooking Brussel sprouts and broccoli for 9-5 minutes decreases glucobrassicin levels in the vegetables due to leaching into water [289].

![Structure of DIM and its precursors](image)

**Figure 11. Structure of DIM and its precursors.** Glucobrassicin is found in cruciferous vegetables and consumption results in myrosinase catalyzed hydrolysis into indole-3-carbinol and a condensation reaction results in DIM. Reprinted with permission: Fujioka, N Harnessing the Power of Cruciferous Vegetables: Developing a Biomarker for Brassica Vegetable Consumption Using Urinary 3,3'-Diindolylmethane Cancer Prevention Research 9;10 2016.

DIM has been used to treat a variety of diseases but exhibits poor biodistribution and absorption in animal tissues [291]. The pharmacokinetics and bioavailability of several DIM formulations have been investigated and the highest bioavailability was achieved using liquid DIM in a solution of cod liver oil when compared to crystalline DIM formulations [291]. Previous studies have estimated DIM clearance in mice to be approximately 7 mL/h while blood concentrations peak from 45 to 60 minutes [291]. Physiologically based pharmacokinetic modelling (PBPK) has also been used to determine the pharmacokinetics of DIM. A phase I trial administered a range of doses of I3C.
(200 and 400 mg/day) to women with cervical dysplasia and found that the that the time to max blood concentrations was 2 hours with max concentration levels near 15 ng/mL [292]. Doses in the same range have been used in studies to treat recurrent respiratory papillomatosis [293]. These clinical levels are significantly higher than dietary levels from cruciferous vegetable consumption which can range between 20 to 120 mg daily [292].

**Diindolylmethane (DIM)**

DIM is a major metabolic product of glucobrassicin via indole-3-carbinol (I3C) and studies have demonstrated its role in cancer and autoimmune disorders. The mechanism behind the anticarcinogenic effects of DIM are due, in part to modulation of carcinogen metabolism into inactive and nontoxic metabolites. For example, DIM binds the aryl hydrocarbon receptor (AhR) which activates Ahr-dependent transcription of phase I and phase II enzymes, and the subsequent increased rates of toxicant and carcinogen elimination [294]. DIM induced hepatic levels of CYP1A2 upon initial exposure [294], and also induced CYP3A4 after chronic administration [295], demonstrating the role of DIM in induction of CYPs that enhance metabolism of carcinogens. Indeed, CYP3A4 is responsible for the metabolism of the majority of therapeutic drugs [296], and this may have implications for DIM induced adverse reactions or drug interactions. Additionally, DIM also regulates cell cycle progression, Akt/NFκB signaling, cyclin dependent kinase activity, caspase activation, estrogen receptor signaling, and estrogen metabolism, among others [297, 298]. Platelet derived
growth factor D overexpressing PC3 prostate cancer cells exhibit rapid growth and enhanced cell invasion that involves mammalian target of rapamycin (mTOR). In these cells, DIM inhibited mTOR and Akt, resulting in decreased cell invasion and proliferation [299]. In the same cell line, I3C induced G1 cell cycle arrest due to upregulation of p21 and p27 CDK inhibitor upregulation [300]. While this treatment utilized I3C to demonstrate cell cycle arrest, this effect is likely to be mediated by DIM, since I3C is converted into DIM under cell culture conditions [289]. Additionally, in breast cancer cells, DIM treatment inhibited DNA synthesis and Bcl-2 expression and induced chromatin condensation as well as DNA fragmentation in both MCF-7 and MDA-MB-231 breast cancer cell lines [301]. Furthermore, DIM also inhibited cell adhesion and invasion via upregulation of the tumor suppressor PTEN and cell adhesion regulator E-cadherin in breast cancer cells [302]. DIM induced ER calcium release in pancreatic cancer cells which was associated with increased expression of C/EBP homologous transcription factor (CHOP) [303]. This ER stress response was accompanied by apoptosis as evidenced by cleavage of caspase 8, caspase 3, Bid, and PARP [303]. These effects are comparable to those observed by treating these cells with known ER stress inducers, such as thapsigargin [92].
1,1-Bis (3’-indolyl)-1-(p-substitutedphenyl) methane (C-DIMs) as an NR4A1 Ligand

1,1-Bis (3’-indolyl)-1-(p-substitutedphenyl) methane (C-DIMs) are derivative of DIM that contain an aromatic ring and these compounds did not exhibit AhR activity but were potent anticancer agents [304]. Their role as receptor ligands was investigated using GAL4-receptor chimeras and a subset of these compounds containing p-trifluoromethyl, p-tbuty, and p-phenyl groups were identified as PPARδ ligands. Their activity as PPARδ ligands was investigated in several cancer cell lines and it was concluded that with the exception of colon cancer that PPARδ was not a major factor in solid tumor-derived cancer cell lines [304-312]. Several PPARδ-inactive C-DIMs such as p-methoxy- and p-hydroxyphenyl (DIM-C-pPhOH) analogs were also potent anticancer agents and a second round of GAL4-receptor screening assays identified NR4A1 as a target for C-DIMs [313].

Recently, our lab has synthesized and investigated a series of 1,1-bis(3-indolyl)-1-(p-substituted phenyl)methane (C-DIM) analogs that bind NR4A1 and exhibit NR4A1 antagonist activity in cancer cells and inhibit cancer cell and tumor growth [271]. These are synthetic triaryl methane derivatives of DIM, which is a diaryl methane [271]. More recent studies have focused on two particular C-DIMs, namely C-DIM [1,1-bis(3-indolyl)-1-(p-hydroxyphenyl)methane] (DIM-C-pPhOH) and [1,1-bis(3-indolyl)-1-(p-carboxymethylphenyl)methane] (DIM-C-pPhCO₂Me) which bind and inactivate
NR4A1 and acts as a NR4A1 antagonist [272, 303, 313]. C-DIMs have a substituted phenyl or another aromatic group and may also have ring indole substituents. They the ability to modulate nuclear NR4A1-dependent transactivation, similar to that reported for CsnB [260]. Previous studies have investigated binding and interactions of C-DIM analogs with the LBD of NR4A1 using fluorescence assays. While binding was not observed for some of the analogs, $K_D$ values of compounds that bound NR4A1 ranged from 0.1 to 0.74 µM [314]. These studies identified DIM-C-pPhOH as the most active ligand ($K_D = 0.11$ µM) that bound the LBD of NR4A1 [314]. Previous crystallography studies identified two separate binding sites on the surface of the NR4A1 LBD [315], which correspond to a LBD and a cofactor binding site. C-DIM analogs exhibit a low affinity for the cofactor binding site, suggesting that these analogs bind to the ligand binding site [314] and modeling studies found all C-DIM analogs are capable to interacting with the LBD of NR4A1. These interactions include hydrogen bond interactions with Glu445 and His516 and $\pi$ interactions with Arg515, with a configuration similar to that of DIM-C-pPhOH [314]. Furthermore, mutation of His516 in the NR4A1 LBD resuled in the inability of DIM-C-pPhOH to bind the receptor [314], indicating the importance of this residue in NR4A1 ligand binding interactions.

Functional results of C-DIM treatment has been established in several studies in cancer cell lines and in vivo [270, 272, 316, 317]. A subset of C-DIMs have also been shown to activate PPAR$\gamma$ to induce growth inhibition and
transactivation in colon cancer cells [305]. Furthermore, the p-chlorophenyl C-DIM analog (DIM-C-pPhCl) activates Nurr1 and also inhibited bladder cancer cell and tumor growth [316]. Specifically, the p-methoxyphenyl analog (DIM-C-pPhOCH₃) can also activate NR4A1 dependent transactivation and also inhibited cancer cell and tumor growth and induced apoptosis in lung and pancreatic cancer cells [270, 272]. In addition, another set of C-DIMs, DIM-C-pPhOH and DIM-C-pPhCO₂Me, inactivate NR4A1, resulting in cancer cell apoptosis and inhibition of cell and tumor growth [318].

Subsequent studies using both NR4A1 silencing via siRNAs and DIM-C-pPhOH have identified several NR4A1 dependent pathways that are also important chemotherapeutic targets. NR4A1 regulates expression of survivin and bcl-2 via interactions of NR4A1 and p300 with the DNA-bound transcription factor Sp1 bound to GC-rich promoter elements [272]; treatment of pancreatic cancer cells with DIM-C-pPhOH or transfection with siRNAs targeted to NR4A1 (siNR4A1) decreased survivin and bcl-2 expression in these cells [272]. A second NR4A1-regulated pathway that has been identified is p53-dependent and is only observed in p53 positive cancer cell lines. Mechanistic studies of this pathways found that NR4A1 binds and inactivates p53 and upon treatment with DIM-C-pPhOH or transfection with siNR4A1, these cells respond by activating p53, which induces sestrin2, and in turn activates phospho-cAMP activated protein kinaseα (AMPKα), resulting in the inhibition of mTOR signaling [270]. Thus, NR4A1 regulates mTOR which plays an important role in cancer cell
growth, proliferation, and protein synthesis and NR4A1 antagonists act as a novel class of mTOR inhibitors. A more recently identified pathways involves the regulation of oxidative stress by NR4A1. NR4A1 regulates expression of genes, such as isocitrate dehydrogenase 1 (IDH1) and thioredoxin domain-containing protein 5 (TXNDC5) and keeps their expression high, resulting in higher overall “reductant” levels and low oxidative stress levels in pancreatic cancer cells [303]. Treatment with DIM-C-pPhOH or transfection with siNR4A1 in pancreatic cancer cells decreased expression of these genes, resulting in induction of oxidative and endoplasmic reticulum stress and stress-induced apoptosis [303].
NR4A1 regulates apoptotic, stress, and mTOR pathways in solid tumors. NR4A1 binds and inactivates p53, thereby inhibiting sestrin and AMPKa, allowing for mTOR signaling. NR4A1 activates transcription of the reductants TXNDC5 and IDH1 that keep oxidative stress low. NR4A1 also forms a complex with Sp1 and p300 to transcriptionally activate pro-growth and pro-apoptotic genes such as surviving, bcl2, cyclin D1, and EGFR. Reprinted with permission: Safe S, The orphan nuclear receptor NR4A1 (Nur77) regulates oxidative and endoplasmic reticulum stress in pancreatic cancer cells Molecular Cancer Research 12(4); 527-38 2014

Recently, these mechanisms have been identified in breast, colon, pancreatic, and kidney cancer cell lines [317]. The results of RNAi studies demonstrate that NR4A1 regulates multiple pro-oncogenic pathways (Figure 12) in solid tumors, and C-DIM/NR4A1 antagonists, such as DIM-C-pPhOH and DIM-C-pPhCO₂Me are inhibitors of these pathways. Previous studies have shown NR4A1 is overexpressed in RMS, therefore I hypothesize that NR4A1 will contribute to the oncogenicity of RMS and serve as a driver of the more aggressive ARMS
In this thesis, I will investigate the function of NR4A1 in RMS (ARMS and ERMS), the anticancer activities of C-DIM/NR4A1 antagonists and the development of a new set of more potent “second generation” of C-DIM/NR4A1 ligands for treatment of RMS.
CHAPTER II

NUCLEAR RECEPTOR 4A1 (NR4A1) AS A DRUG TARGET FOR TREATING RHABDOMYOSARCOMA (RMS)*

INTRODUCTION

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma that is primarily observed in children and adolescents and accounts for 5% of all pediatric cancers and 50% of soft tissue sarcomas in children [319, 320]. Embryonal RMS (ERMS) and alveolar RMS (ARMS) are the two major classes of RMS in children and adolescents and differ with respect to their histology, genetics, treatment, and prognosis [319-322]. ERMS accounts for over 60% of RMS patients and is associated with loss of heterozygosity at the 11p15 locus. ERMS patients have a favorable initial prognosis; however, the overall survival of patients with metastatic ERMS is only 40% [321]. ARMS occurs in a lower percentage of RMS patients and is associated with translocations resulting in formation of pro-oncogenic gene products resulting from the fusion of PAX3 or PAX7 with the Forkhead gene FOXO1A [323, 324]. ARMS patients have a poor diagnosis and patient survival is <10% for metastatic ARMS.

RMS patients are treated with radiotherapy, surgery, and chemotherapy using cytotoxic drugs and/or drug combinations, and successful treatment varies with tumor type (ARMS vs. ERMS) and extent of metastasis.

However, a recent study on adults treated for childhood cancers showed that over 90% of these individuals exhibited chronic adverse health conditions later in life [74], demonstrating that there is a critical need for development of new mechanism-based drugs for treatment of RMS.

The orphan nuclear receptor 4A1 (NR4A1, Nur77/TR3) does not have an endogenous ligand; however, this receptor plays a key role in cellular homeostasis and in several diseases including cancer [201, 325]. NR4A1 is overexpressed in lung, breast, pancreatic and colon cancer patients [201, 270, 271, 326, 327], and functional studies show that NR4A1 is pro-oncogenic and plays a role in cancer cell proliferation, survival, migration and invasion [reviewed in 201]. Several structurally-diverse ligands that directly bind NR4A1 have been characterized [260, 314, 315, 328] and studies in this laboratory have shown that among a series of 1,1-bis(3-indolyl)-1-(p-substituted phenyl)methanes (C-DIMs), several compounds including the p-hydroxy (DIM-C-pPhOH) and p-carbomethoxy (DIM-C-pPhCO$_2$Me) analogs directly bind NR4A1 (Figure 13A). Results of RNA interference (RNAi) studies show that NR4A1 activates mTOR by binding and inactivating p53 [270], regulates genes such as isocitrate dehydrogenase 1 (IDH1) and thioredoxin domain-containing 5 (TXNDC5) to decrease cellular stress [303], and regulates expression of growth promoting/survival genes such as survivin and epidermal growth factor receptor (EGFR) through NR4A1-Sp1 interactions with their proximal GC-rich promoter elements [272]. The pro-oncogenic NR4A1-regulated activities have previously
been characterized in colon, lung and pancreatic cancer cells [270, 272, 303, 314], and the C-DIM/NR4A1 antagonists inhibited these pathways (Figure 13B) and gave results comparable to those observed for RNA interference (RNAi). In preliminary data mining studies, we observed that NR4A1 was also overexpressed in RMS tumors compared to normal tissue and high levels were observed in prototypical ARMS (Rh30) and ERMS (RD) cell lines. This study also demonstrates that NR4A1 regulates pro-oncogenic pathways (Figure 13B) in RMS cells and C-DIM/NR4A1 antagonists inhibit these responses, demonstrating that NR4A1 is a potential novel target for RMS chemotherapy.

MATERIALS AND METHODS

Cell Lines, Antibodies, Chemicals, and Other Materials

Rh30 and RD human RMS cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were maintained at 37°C in the presence of 5% CO₂ in RPMI-1640 Medium or Dulbecco's Modified Eagle’s Medium, respectively, both supplemented with 10% fetal bovine serum and 5% antibiotic. Dulbecco’s Modified Eagle’s Medium, and RPMI-1640 were purchased from Sigma-Aldrich (St. Louis, MO), glutathione (GSH) reduced free acid were purchased from Millipore (Temecula, CA), and Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were subsequently viewed using a filter set for FITC, rhodamine, and DAPI on an Advanced Microscopy EVOS fl, fluorescence microscope. RGB-4103
GelRed nucleic acid stain was used in place of Ethedium Bromide from Phoenix Research Products (Candler, NC). The C-DIM compounds were prepared as previously described [314] and a summary of the antibodies are provided in Supplemental Table B-1. A summary of oligonucleotide for RNAi and real time PCR and ChIP primers are summarized in Supplemental Table B-2.

**Total RNA Expression Analysis**

Patient sample data of total RNA was acquired from NCBI GEO dataset GSE28511 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28511) and was previously analyzed for quality control, quantile normalized. In addition, multi-probe genes were averaged by the submitter. Expression values were listed into non-tumor and RMS tumor groups in JMP® and a box plot was generated, from which a t-test was performed; significance was determined as a p-value less than 0.01, shown by an asterisk (Figure 13C).

**Cell Proliferation and Tumor Growth Assay**

Rh30 and RD cells were plated in 12-well plates at 1.0 x 10^5 and allowed to attached for 24 hr before treatment with DIM-C-pPhOH, DIM-C-pPhCO_2Me, or transfected with siNR4A1, with DMSO (dimethyl sulfoxide) as empty vehicle or siCtl siRNA (with lipofectamine vehicle) as controls, respectively. Cells were then trypsinized and counted at indicated times using a Coulter Z1 cell counter. Female athymic nude mice (6-8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions, and housed at Texas A&M University in accordance with the
standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The protocol of the animal study was approved by the Institutional Animal Care and Use Committee, Texas A&M University. Rh30 cells (4x10^6 cells) grown in RPMI media containing 10% FBS were detached, resuspended in 100 µl of phosphate-buffered saline with matrigel (BD Bioscience, Bedford, MA) (75:25), and implanted subcutaneously in the mice. When tumors reached about 40-50 mm^3 size, the animals were randomized into control and treatment groups (6 animals per group) and mice were treated with placebo or DIM-C-pPhCO₂Me (40 mg/kg/d) in corn oil by oral gavage every second day for 20 days. Tumor volumes and weights, and body weight were determined; the tumor size was measured using Vernier calipers, and the tumor volume was estimated by the formula: tumor volume (mm^3) = (L x W^2) x ½, where L is the length and W is the width of the tumor.

**Annexin V Staining**

Rh30 and RD cells were seeded in 2-well Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hr before treatment with C-DIMs or DMSO for 48 hr and with siNR4A1 (100 µM) or siCtl for 72 hr, and Annexin V staining was determined as described [314].
**Immunofluorescence**

Rh30 and RD cells were seeded at 1.0 x 10^5 in 2-well Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hr in DMEM/Ham F-12 containing 5.0% charcoal-stripped fetal bovine serum and treated with C-DIM compounds for 24 hr. Cells were then treated with fluorescent NR4A1 antibody [Nurr77 (D63C5) XP®] and the manufacturer's protocol (Cell Signaling Technologies, Danvers, MA) was used to observe immunofluorescence. Hoechst staining from the apoptotic and necrotic cells assay (Biotium, Hayward, CA) was used to visualize nuclear DAPI staining, while NR4A1 localization was determined by green fluorescence. Images were taken using an EVOS fluorescence microscopy from Advance Microscopy; NR4A1 and DAPI images were subsequently merged.

**Western Blot**

Rh30 and RD cells were seeded in 6-well plates at 1.0 x 10^5 and allowed to attach for 24 hr before treatment with DIM-C-pPhOH, DIM-C-pPhCO₂Me, or transfected with siNR4A1, with DMSO as empty vehicle or siCtl siRNA (with lipofectamine vehicle) as controls, respectively. Cells were treated with C-DIMs or DMSO for 48 hr or transfected with siNR4A1 (100 μM) or siCtl for 72 hr, and Western blots of whole cell lysates were determined as described [314].
Transactivation, real-time PCR, and chromatin immunoprecipitation (ChIP) assays

Real time PCR and ChIP assays using RMS cell lines transfected with oligonucleotides or treated with C-DIMs were carried out essentially as described [270, 272, 303, 314]. Transactivation studies were carried out in RD cells transfected with two NR4A1-responsive constructs, NuRE₃-luc and NBRE₃-luc, that bind NR4A1 as a homodimer or monomer, respectively, or transfected with a GAL4-NR4A1 (chimera) and a GAL4-responsive construct (UASₓ₅-luc) essentially as described [329]. Real-time PCR and chromatin immunoprecipitation assays were carried out essentially as described [272].

Generation and Measurement of ROS

Cellular ROS levels were measured utilizing a cell permeable probe, CM-H2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen (Grand Island, NY). CM-H2DCFDA diffuses into the cell, where its acetate groups are cleaved by intracellular esterases and upon oxidation, yields a fluorescent adduct that is measured by flow cytometry using Accuri’s C6 Flow Cytometer (Ann Arbor, MI). Cells were plated in a 6-well culture plate and allowed to attach for 24 hr and treated for the indicated time with DIM-C-pPhOH, DIM-C-pPhCO₂Me, or siNR4A1. Subsequently, cells were trypsinized, neutralized, then loaded with 10 μM of probe for 20 min incubation, and were washed with serum free media for ROS quantification.
Statistics

Results for each treatment group were replicated (at least 3X) and expressed a means ± SE. Statistical comparisons of the treated groups vs. a control for each treatment were determined using Student's t-test.

RESULTS

NR4A1 Expression and Transactivation

Examination of publically-available RMS array data show that NR4A1 mRNA is more highly expressed in RMS tumors compared to non-tumor tissue (Figure 13C). Previous studies show that the C-DIM compounds DIM-C-pPhOH and DIM-C-pPhCO₂Me bind NR4A1 and act as NR4A1 antagonists for transactivation assays in colon cancer cells [314] and therefore these compounds were also used in this study on RMS cells. RD cells were transfected with constructs containing the DNA binding domain of the yeast GAL4 protein fused to NR4A1 and the UAS₁₅ luc construct containing 5 GAL4 response elements, and treatment with DIM-C-pPhOH or DIM-C-pPhCO₂Me decreased luciferase activity (Figure 13D). DIM-C-pPhOH and DIM-C-pPhCO₂Me also decreased luciferase activity in RD cells transfected with NBRE₃-luc and NuRE₃-luc constructs containing 3 binding sites for NR4A1 monomer and homodimer, respectively (Figure 13D).
Figure 13. NR4A1 expression and transactivation by C-DIMs. (A) Structure of C-DIMs and (B) NR4A1-regulated pro-oncogenic pathways in cancer cells. (C) Analysis of NR4A1 gene expression in patient-derived mRNA acquired from the NCBI GEO dataset GSE2851. (D) C-DIMs inhibit NR4A1-dependent transactivation. RD cells were transfected with pGAL4-NR4A1/UASx5-luc, NBREx3-luc or NuREs3-luc, treated with DMSO or 15 µM DIM-C-pPhOH or DIM-C-pPhCO2Me, and luciferase activity was determined as outlined in the Materials and Methods. Results are expressed as means +/- SE for at least 3 separate experiments and significantly (p < 0.05) decreased activity is indicated (*). (E) Cellular localization of NR4A1. Rh30 (A) and RD (B) cells were treated with DMSO or 20 µM DIM-C-pPhOH for 24 hr and cells were stained with DAPI and a fluorescent NR4A1 antibody. The individual and merged staining was determined as outlined in the Materials and Methods.
Basal activity was low for both constructs but significantly enhanced by cotransfection with a FLAG-TR3 expression plasmid in RD cells. These results were comparable to those previously observed in colon cancer cells [314] and demonstrate that the two C-DIM compounds exhibit antagonist activity for transactivation in RD cells. Immunostaining of Rh30 and RD cells with DAPI and NR4A1 antibodies showed that NR4A1 was nuclear in these RMS cell lines (Figure 13E). Moreover, after treatment with 20 µM DIM-C-pPhOH for 24 hr, we did not observe any nuclear export of NR4A1 which was comparable to observations in other cancer cell lines [270, 272, 303, 314].

**Role of NR4A1 in RMS Cell Growth and Survival**

Transfection of Rh30 and RD cells with siNR4A1 significantly decreased proliferation of Rh30 and RD cells and comparable results were observed for two different siRNAs (Figure 14A). Treatment of Rh30 cells with 7.5 to 22.5 µM DIM-C-pPhOH and 5 to 15 µM DIM-C-pPhCO2Me of the NR4A1 antagonists for 24 hr also inhibited growth of RH30 (Figure 14B) and RD (Figure 14C) cells with IC50 values ranging from 6.6 to 29 µM. Figure 14D also shows that although inhibition of RD cell growth after treatment with 15 µM DIM-C-pPhCO2Me was only 20-25%, after prolonged treatment (48 and 72 hr), more complete growth inhibition was observed. In addition, we also observed that DIM-C-pPhOH (40 mg/kg/d) inhibited tumor growth in athymic nude mice bearing Rh30 cells as xenografts (Figure 14E).
Figure 14. NR4A1 regulates growth of RMS cells which can be inhibited by C-DIM/NR4A1 antagonists. (A) Rh30 and Rd cells were transfected with two different oligonucleotides targeted to NR4A1 [siNR4A1(1) and siNR4A1(2)], and after 72 hr, the cells were counted and compared to the number of cells observed after transfection with a non-specific control (siCtl) oligonucleotide. Rh30 and RD cells were treated with different concentrations of DIM-C-pPhOH (B) or DIM-C-pPhCO$_2$Me (C) for 24 hr, and (D) RD cells were treated with 15 µM DIM-C-pPhCO$_2$Me for 24, 48 or 72 hr. Cells were counted and compared to the number observed after treatment with the solvent control (DMSO, set at 100%). (E) In a preliminary in vivo study, we observed that after treatment of athymic nude mice with DIM-C-pPhOH (40 mg/kg/d for 28 days), there was a small but significant inhibition of tumor growth and future studies will use a higher dose of this compound. Results (A – E) are expressed as means ± SE for at least 3 separate treatments for each group and significant (p < 0.05) growth inhibition is indicated (*).
We also investigated the role of NR4A1 in mediating survival of Rh30 and RD cells, and Figure 15A shows that transfection of these cells with siNR4A1 resulted in the induction of Annexin V staining. Moreover, transfection of Rh30 and RD cells with siNR4A1 also induced PARP cleavage, another marker of apoptosis in these cells (Figure 15B). Treatment of Rh30 and RD cells with the NR4A1 antagonists DIM-C-pPhOH and DIM-C-pPhCO₂Me also induced Annexin V staining (Figure 15C) and PARP cleavage (Figure 15D), thus confirming the pro-survival activity of NR4A1 in RMS cells and effects of C-DIM/NR4A1 antagonists as inhibitors of cell growth and survival.
Figure 15. NR4A1 regulates RMS survival that can be inhibited by C-DIM/NR4A1 antagonists. Rh30 and RD cells were transfected with siNR4A1 or siNR4A2, and induction of Annexin V staining (A) or enhanced PARP cleavage (B) were determined as outlined in the Materials and Methods. Rh30 and RD cells were treated with DIM-C-pPhOH or DIM-C-pPhCO₂Me and effects on Annexin V staining (C) or enhanced PARP cleavage were determined as outlined in the Materials and Methods. Results (A and C) were expressed as means ± SE for at least 3 replicate determinations per treatment group and significant (p < 0.05) induction is indicated (*).
NR4A1 Antagonists Inhibit Growth/Survival Pathways and Gene Products in RMS Cells

Previous studies show that NR4A1 acts as a coactivator of genes with GC-rich promoters (Figure 13B, pathway 3) that play a role in cancer cell proliferation and survival, and these include survivin, bcl-2, cyclin D1, epidermal growth factor receptor (EGFR) and the oncogene cMyc [272, 314]. Knockdown of NR4A1 by RNA in Rh30 and RD cells decreased expression of several genes with GC-rich promoters including EGFR, bcl2, c-Myc and cyclin D1, and this was accompanied by minimal effects on expression of Sp1 (Figure 16A). Treatment of Rh30 and RD cells with the NR4A1 antagonists DIM-C-pPhOH (Figure 16B) and DIM-C-pPhCO2Me (Figure 16C) also decreased expression of survivin, bcl-2, cyclin D1, EGFR and cMyc, and these results paralleled those observed after knockdown of NR4A1 in these cells lines (Figure 16A). DIM-C-pPhCO2Me was used to further investigate the mechanism of downregulation of Sp-regulated genes at the transcriptional level. In a ChIP assay, DIM-C-pPhCO2Me decreased binding of NR4A1 and p300 (but not Sp1) at the GC-rich region of the survivin promoter and pol II binding was also decreased (Figure 16D). These results are comparable to previous studies in pancreatic cancer cells showing that p300/NR4A1 coregulated survivin expression by interacting with DNA-bound Sp1 (Figure 13B) [272]. In addition, DIM-C-pPhCO2Me also decreased expression of survivin, cyclin D1 and EGFR mRNA levels (Figure 16E). Thus,
NR4A1 also coregulates expression of Sp-regulated pro-survival/growth promoting genes with GC-rich promoters in RMS cells.
Figure 16. NR4A1 regulation of pro-survival/growth promoting genes and their inhibition by C-DIM/NR4A1 antagonists. (A) Rh30 and RD cells were transfected with siNR4A1 or siCtl, and whole cell lysates were analyzed by Western blots as outlined in the Materials and Methods. Rh30 and RD cells were treated with DMSO (solvent control), DIM-C-pPhOH (B) or DIM-C-pPhCO$_2$Me (C), and whole cell lysates were analyzed by Western blot as outlined in the Materials and Methods. (D) Rh30 and RD cells were treated with DIM-C-pPhCO$_2$Me, and binding of NR4A1, Sp1, p300 and pol II to the survivin promoter was determined in a ChiP assay. (D) Cells were treated with DIM-C-pPhCO$_2$Me, and survivin, cyclin D1 and EGFR mRNA levels were determined by real time PCR. Results are expressed as means ± SE (3 replicates) and significant (p < 0.05) changes in gene expression are indicated (*).
NR4A1 also regulates expression of \textit{TXNDC5} and \textit{IDH1} to maintain low oxidative and endoplasmic reticulum (ER) stress [303, 314], and transfection of Rh30 and RD cells with siNR4A1 induced ROS as determined using the cell permeable fluorescent indicator CM-H2DCFDA (Figure 17A) and similar results were observed after treatment with the NR4A1 antagonists (Figure 17B). Knockdown of NR4A1 (Figure 17C) or treatment of Rh30 and RD cells with the NR4A1 antagonists (Figure 17D) decreased expression of \textit{TXNDC5} and \textit{IDH1} and this was accompanied by induction of several markers of ER stress including phosphorylated PERK (pPerk), ATF4 and CHOP. Both TXNDC5 and IDH1 have GC-rich promoter sequences at -22 and -112, respectively, in untreated cells, and a ChIP assay showed binding of NR4A1, Sp1 and p300 to the GC-rich regions of the promoter in Rh30 cells (Figure 17E). Treatment of these cells with the NR4A1 antagonist DIM-C-pPhCO$_2$Me resulted in decreased interactions of NR4A1, p300 and pol II with the GC-rich TXNDC5 and IDH1 promoters and also some loss of Sp1 from the TXNDC5 promoter, suggesting that like survivin, expression of these genes also involves interaction of the p300/NR4A1 complex with Sp1 at GC-rich elements (Figure 13B). In addition, DIM-C-pPhCO$_2$Me also decreased expression of TXNDC5 and IDH1 mRNA levels (Figure 17F). The induction of ROS by inactivation of NR4A1 also has functional significance since DIM-C-pPhOH-induced cleavage of PARP, caspases 3 and 7 (markers of apoptosis), and growth inhibition were significantly reversed after cotreatment with 5 mM glutathione (GSH) (Suppl. Figure A-1).
NR4A1 binds and inactivates p53 (Figure 13B) and knockdown of NR4A1 or treatment with NR4A1 antagonists results in p53-dependent induction of sestrin 2, an upstream regulator of AMPKα in lung and colon cancer cells [270, 314]. Even though Rh30 and RD cells are p53-negative; knockdown of NR4A1 in Rh30 cells or treatment with DIM-C-pPhOH induced sestrin 2 and increased phosphorylation of AMPKα and this resulted in decreased activation of mTOR-dependent phosphorylation of both 4EBP1 and 6SRP which are kinases downstream from mTOR (Figure 18A). Similar results were observed in RD cells (Suppl. Figure A-2A) and after treatment with DIM-C-pPhCO₂Me (Suppl. Figure A-2B). Sestrin 2 is also induced in response to ROS [330] and since C-DIM/NR4A1 antagonists induce ROS (Figure 17B), the effects of the antioxidant GSH as an inhibitor of sestrin 2 induction after NR4A1 inactivation was investigated. Sestrin 2 induction in RD and Rh30 cells treated with DIM-C-pPhOH or DIM-C-pPhCO₂Me was attenuated after cotreatment with GSH (Figure 18B) and similar results were observed after NR4A1 knockdown (Figure 18C). DIM-C-pPhCO₂Me also induced sestrin 2 gene expression in Rh30 and RD cells (Figure 18D), and the induction response was attenuated in cells cotreated with the antioxidant GSH (Figure 18E).
Figure 17. Role of NR4A1 in regulating oxidative stress. Rh30 and RD cells were either transfected with siNR4A1(1)/siNR4A1(s) (A) or treated with DIM-C-pPhOH or DIM-C-pPhCO₂Me (B), and ROS was determined using the cell permeable fluorescent probe CM-H₂DCFDA as outlined in the Materials and Methods. Rh30 and RD cells were either transfected with siNR4A1 (C) or treated with DIM-C-pPhOH or DIM-C-pPhCO₂Me (D), and whole cell lysates were analyzed for TXNDC5, IDH1 and various ER stress genes by Western blot analysis as outlined in the Materials and Methods. (E) Rh30 cells were treated with DMSO or 20 μM DIM-C-pPhCO₂Me, and binding of NR4A1, p300, Sp1 and pol II to the GC-rich regions of the TXNDC5 and IDH1 gene promoters were determined in a ChIP assay as outlined in the Material and Methods. (F) Relative TXNDC5 mRNA Levels and Relative IDH1 mRNA Levels were determined by real-time PCR. Results are expressed as means ± SE (triplicate determinations) and significant (p < 0.05) changes in gene expression are indicated.
Thus, the NR4A1 antagonists block at least three NR4A1-regulated pro-oncogenic pathways (Figure 13B) in RMS cells indicating that NR4A1 is a potential new drug target for treatment of RMS.

DISCUSSION

The NR4A orphan nuclear receptors are immediate early genes induced by multiple stressors and there is increasing evidence that these receptors play a critical role in maintaining cellular homeostasis in multiple tissues and organs [325]. There is evidence that NR4A1 is important in metabolism and metabolic disease, cardiovascular and neuronal function, and inflammation in multiple tissues [325]. The function and mechanism of action of NR4A1 in cancer cells is complex; transgenic mice in which both NR4A1 and NR4A3 (Nurr1) have been knocked out rapidly develop an acute myeloid leukemia (AML) type of leukemia and there is evidence that NR4A1 is a tumor suppressor for AML [283, 331]. In contrast, NR4A1 is overexpressed in most solid tumors and is a negative prognostic factor for lung, breast and colon cancer patients and knockdown studies show that NR4A1 plays a role in cancer cell proliferation, survival, migration and invasion [201, 285-287, 326, 332, 333].
Figure 18. NR4A1 regulates sestrin 2 and mTOR in RMS cells. (A) Rh30 cells were either transfected with siNR4A1 or treated with DIM-C-pPhOH, and whole cell lysates were analyzed as outlined in the Materials and Methods. Rh30 and RD cells were either treated with DIM-C-pPhOH or DIM-C-pPhCO₂Me alone or in combination with GSH (B) or transfected with siCtl/siNR4A1 alone or in combination with GSH treatment (C). Whole cell lysates were analyzed for sestrin 2 (SESN2) by Western blots as outlined in the Materials and Methods. Rh30 and RD cells were treated with DMSO and DIM-C-pPhCO₂Me alone (D) or in combination with GSH (E), and expression of sestrin 2 mRNA levels were determined by real time PCR as outlined in the Materials and Methods. Results (D and E) are expressed as means ± SE (3 replicates) and significant (p < 0.05) induction (*) or inhibition of induction (**) are indicated.
Early studies on drug-mediated effects of NR4A1 demonstrated that many apoptosis-inducing drugs that do not directly bind NR4A1 induce nuclear export of this receptor which subsequently binds to mitochondrial bcl-2 to form a pro-apoptotic complex that disrupts mitochondria in cancer cells, resulting in increased cell death (24, 25). However, more recently cytosporone B and some structurally-related compounds have been identified as NR4A1 ligands [260, 315, 328] and studies in this laboratory have also identified C-DIMs as NR4A1 receptor ligands and these compounds act as NR4A1 antagonists that inhibit cancer cell growth and survival by directly targeting nuclear NR4A1 [260, 314, 315, 328].

NR4A1 is a nuclear protein expressed in RD and Rh30 cells (Figure 13E), and there is evidence from publically available array data from RMS tumors that NR4A1 mRNA is overexpressed in tumor vs. non-tumor tissue (Figure 13C). The functional role of NR4A1 in RMS was investigated by RNAi showing that this receptor plays a role in RMS cell proliferation and survival (Figs. 14 and 15) and these results are comparable to those observed in many other solid tumors [reviewed in 201]. NR4A1 knockdown studies also demonstrated that NR4A1 also plays a role in activating mTOR and maintaining low stress levels through its regulation of TXNDC5 and IDH1 (Figure 17). Both IDH1 and TXNDC5 are regulated by NR4A1 in pancreatic and colon cancer cells, and knockdown of either NR4A1 or TXNDC5 in pancreatic cancer cells results in the induction of ROS [303]. The results illustrated in Figures 17A and 17B show that knockdown
of NR4A1 in RMS cells also results in downregulation of TXNDC5 (and IDH1) and induction of ROS and ER stress genes, confirming that NR4A1 regulation of TXNDC5 suppresses ER and oxidative stress. NR4A1 also regulates IDH1 expression which also generates cellular reductants and complements the function of TXNDC5 in terms of stress suppression [330, 334]. In contrast to glioma and other cancer cells which express IDH1 mutations [335] enhancing D-2-hydroxyglutarate production, this mutation has not been detected in RMS [336]. NR4A1-dependent maintenance of low oxidative stress levels also contributes to mTOR signaling since knockdown of NR4A1 results in oxidative stress-dependent induction of sestrin 2 [337] which in turn activates AMPKα and inhibits mTOR. Genomic analysis coupled with high throughput screening of primary RMS cultures identified ROS inducers as a therapeutically relevant approach for treating ERMS [87]. Results of our studies implicate NR4A1 regulation of TXNDC5 and IDH1 for maintaining low oxidative stress in RD and Rh30 cells and suppression of these gene by the C-DIM/NR4A1 antagonists induces ROS which in turn induces ER stress and also sestrin 2-dependent inhibition of mTOR (Figs. 17 and 18). Induction of this latter pathway may be an important contributor to the efficacy of C-DIM/NR4A1 antagonists and other ROS-inducing agents since mTOR inhibitors show promise as mechanism-based drugs for RMS chemotherapy [112, 338, 339]. Moreover, Supplemental Figure A-1 also demonstrates that DIM-C-pPhOH-mediated induction of ROS
plays a major role in growth inhibition and induction of apoptosis and this is due to the high sensitivity of RMS cells to ROS-inducing compounds [87].

Nuclear receptors not only activate gene expression through direct binding to their cognate response elements but also indirectly through interactions with DNA-bound transcription factors such as Sp1, and this has been observed for several other nuclear hormone and orphan receptors [340-346]. Similar results have previously been reported for NR4A1 which coactivates expression of growth-promoting and pro-survival genes with GC-rich promoters through interactions of p300/NR4A1-Sp1 bound to GC cis-elements [272, 314]. Knockdown of NR4A1 decreases expression of several Sp1-regulated genes (but not Sp1) including survivin, bcl-2, EGFR, cyclin D1 and c-Myc in Rh30 and RD cells as observed in other cancer cell lines and in this study, ChIP assays indicated that not only survivin (Figure 16D) but also TXNDC5 and IDH1 are regulated by interactions of p300/NR4A1 with Sp1 bound to GC-rich promoters (Figure 13B). A previous study reported that Sp1 is overexpressed in RMS tumors and cells and Sp-regulated genes such as platelet-derived growth factor receptor α, hepatocyte growth factor receptor, insulin-like growth factor receptor and CXCR4 are important for the oncogenic phenotype of RMS [90]. Current studies using RNA-seq combined with receptor knockdown are focused on the role of NR4A1 in regulating expression of these genes and other pro-oncogenic factors in RMS cells via pathway 3 (Figure 13B).
The important pro-oncogenic functions of NR4A1 in RMS cells indicate that antagonists of this receptor represent a potential novel clinical approach for treating RMS. DIM-C-pPhOH was initially characterized as an inhibitor of NR4A1-dependent transactivation and recent structure-binding studies demonstrate that DIM-C-pPhOH, DIM-C-pPhCO₂Me and other C-DIMs bind the ligand binding domain of NR4A1 and exhibit NR4A1 antagonist activity in colon cancer cells [314]. In RMS cells, we have also observed parallel effects of NR4A1 knockdown and treatment with DIM-C-pPhOH and DIM-C-pPhCO₂Me, demonstrating that C-DIM/NR4A1 antagonists represent a new class of mechanism-based drugs for treating RMS. This observation is particularly important for RMS patients since their current treatment regimens rely on cytotoxic drugs which lead to serious health problems later in life [74, 320].
CHAPTER III
PAX3-FOXO1A EXPRESSION IS REGULATED BY THE NUCLEAR RECEPTOR NR4A1 IN Rhabdomyosarcoma AND CAN BE TARGETED BY NR4A1 ANTAGONISTS*

INTRODUCTION

The orphan nuclear receptors NR4A1 (Nur77, TR3), NR4A2 (Nurr1) and NR4A3 (Nor1) play important roles in maintaining cellular homeostasis by their involvement in inflammation, immune and neuronal functions, metabolism, and differentiation [258, 325]. These receptors are early immediate genes induced by multiple stimuli and there is increasing evidence that NR4A receptors are potential drug targets for many diseases including cancer [201, 258, 283, 325]. Among the NR4A receptors, there has been extensive research on the expression and role of NR4A1 in cancer and one study found the loss of both NR4A1 and NR4A2 in mice results in hematological malignancies [347], suggesting tumor suppressor-like activity for NR4A1. In contrast, NR4A1 exhibits tumor promoter activity [201, 283] in solid tumors. NR4A1 is also overexpressed in tumors from breast, lung, pancreatic, colon and ovarian cancer patients and is a negative prognostic factor for breast, lung and ovarian cancer patients [236, 270-272, 285, 326, 327]. Although endogenous ligands for NR4A1 and other NR4A receptors have not been identified, structurally-diverse

compounds directly or indirectly target this receptor. Initial studies demonstrated that several apoptosis-inducing agents activated nuclear export of NR4A1 and formation of a pro-apoptotic complex with bcl-2 which subsequently disrupted mitochondria [288, 348, 349]. Wu and coworkers identified cytosporone B and structural analogs as NR4A1 ligands and these compounds exhibited structure-dependent activation of nuclear NR4A1 and nuclear export [260, 263, 315, 328]. In contrast, studies in this laboratory have demonstrated that among a series of 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes (C-DIMs), several compounds, including the p-hydroxy (DIM-C-pPhOH) and p-carboxymethyl (DIM-C-pPhCO₂Me) bound and activated nuclear NR4A1 and acted as NR4A1 antagonists [314].

In a series of studies, it was demonstrated that knockdown of NR4A1 (siRNA) by RNAi or treatment with C-DIM/NR4A1 ligands inhibits pancreatic, lung, kidney, colon, rhabdomyosarcoma (RMS), and breast cancer growth and induces apoptosis [270-272, 303, 314, 317, 350-352]. Moreover, in breast cancer cells, these same treatments inhibit migration through downregulation of β1-integrin, an NR4A1-regulated gene [351]. Pro-oncogenic NR4A1-regulated pathways/genes in RMS and other cancer cell lines are summarized in Figure 19A. NR4A1 activates TXNDC5 and IDH1 to decrease reactive oxygen species which facilitates activation of mTOR, and NR4A1 also regulates pro-survival and growth promoting genes through an NR4A1/Sp complex interacting with GC-rich gene promoters [270, 272, 303]. The NR4A1/Sp gene regulation pathway does
not require direct NR4A1 binding to promoter DNA and is commonly observed
for several nuclear receptors including orphan receptors [353, 354]. Sp
transcription factors (Sp1, Sp3 and Sp4) are highly overexpressed in RMS cells
[90, 96, 352], and the anticancer agent tolfenamic acid decreased expression of
Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes including PAX3-FOXO1A
[90], a critical pro-oncogenic factor in alveolar RMS (ARMS), a deadly form of
RMS. In this study, we hypothesize and subsequently confirm that PAX3-
FOXO1A is an NR4A1/Sp-regulated gene and treatment with NR4A1
antagonists decreases expression of PAX3-FOXO1A and downstream genes in
ARMS cells. Thus, NR4A1 antagonists represent a novel approach for treating
ARMS patients that overexpress this receptor.

MATERIALS AND METHODS

Cell Lines, Antibodies, Chemicals, and Other Materials

RD and Rh30 cell lines were purchased from the American Type Culture
Collection and were authenticated in 2014 (Promega Powerplex 18D) at the
Duke University DNA Analysis Laboratory. Rh18 and Rh41 cells were received
from Texas Tech University Health Sciences Center-Children’s Oncology Group
in 2015. All cell lines were maintained at 37°C in the presence of 5% CO2 with
Rh30 maintained in RPMI-1640 Medium supplemented with 10% fetal bovine
serum and 5% antibiotic. Rh41 and Rh18 cells lines were maintained in Iscove’s
Modified Dulbecco’s Medium (IMDM) supplemented with 20% fetal bovine
serum, 1X ITS (5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenous acid),
and 5% antibiotic. RPMI-1640 was purchased from Sigma-Aldrich (St. Louis, MO). IMDM was purchased from ThermoFisher Scientific (Waltham, MA) and ITS was purchased from Sigma Aldrich (St. Louis, MO). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY). The C-DIM compounds were prepared as previously described [314]. Summaries of the antibodies and oligonucleotides for RNAi, real time PCR and ChIP primers are summarized in Supplemental Table B-3.

**Total RNA Expression Analysis**

Sample data of total RNA was acquired from NCBI GEO dataset GSE2787 ([http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2787](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2787)). In addition, transcription profiles of competitively hybridized microarray samples were quantified and lowess normalization for each spot was performed and subsequently converted in logarithmic scale, with submitted expression values corresponding to a log(2) ratio of normalized intensities. Expression values were listed into PAX3-FOXO1A positive and PAX3-FOXO1A negative groups in JMP® and a box plot was generated, from which a t-test was performed; significance was determined as a p-value less than 0.01, shown by an asterisk.

**Boyden Chamber Assay**

RMS cells were seeded for 24 hr in a 24-well plate and subsequently allowed to attach for 24 hr transfection with IL-24 overexpression vector, siPAX3-FOXO1A (100 μM), or siIL-24 (100 μM) with a control of siCtl. The cells were trypsinized, counted, placed in 24-well 8.0-μm-pore ThinCerts from BD
Biosciences (Bedford, MA), allowed to migrate for 24 h, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted.

**Western Blot**

Rh30, Rh18, and Rh41 cells were seeded in 6-well plates at $1.0 \times 10^5$ and allowed to attach for 24 hr before treatment with DIM-C-pPhOH, DIM-C-pPhCO2Me, or transfected with siNR4A1, siPAX3-FOXO1A, or siIL-24 with DMSO as empty vehicle or siCtl siRNA (with lipofectamine vehicle) as controls, respectively. Cells were treated with C-DIMs or DMSO for 48 hr or transfected with siNR4A1, siPAX3-FOXO1A, or siIL-24 (all at 100 μM) or siCtl for 72 hr, and Western blots of whole cell lysates were determined as previously described [314].

**Real-time PCR, and Chromatin Immunoprecipitation (ChIP) Assays**

Real time PCR and ChIP assays using RMS cell lines transfected with oligonucleotides or treated with C-DIMs were carried out using the SYBR Green RT-PCR Kit (Bio-rad Laboratories, Hercules, CA) and the ChIP-IT Expression Kit (Carlsbad, CA) following the manufacturers protocol. Oligonucleotides and primers used are summarized in Supplemental Table S1.

**Cell Proliferation and Tumor Growth Assay**

Rh30, Rh18, and Rh41 cells were plated in 12-well plates at $1.0 \times 10^5$ and allowed to attach for 24 hr before treatment with DIM-C-pPhOH, DIM-C-pPhCO2Me, or transfected with siNR4A1, siPAX3-FOXO1A, or siIL-24 with
DMSO (dimethyl sulfoxide) as empty vehicle or siCtl siRNA (with lipofectamine vehicle) as controls, respectively. Cells were then trypsinized and counted at 24 hours using a Coulter Z1 cell counter.

Female athymic nude mice (6-8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions, housed in isolated vented cages, and allowed to acclimate for one week with standard chow diet. The animals were housed at Texas A&M University in accordance with the standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The protocol of the animal study was approved by the Institutional Animal Care and Use Committee (IACUC), Texas A&M University. Rh30 cells (4x10^6 cells) grown in RPMI media containing 10% FBS were detached, resuspended in 100 µl of phosphate-buffered saline with matrigel (BD Bioscience, Bedford, MA) (75:25), and implanted subcutaneously in the mice. When tumors reached about 40-50 mm^3 size, the mice were randomized into control and treatment groups (6 animals per group) and treated with placebo or DIM-C-pPhCO₂Me (40 mg/kg/d) in nano liquid carrier (administered in sodium carboxymethyl cellulose) by oral gavage every second day for 3 weeks. Tumor volumes and weights, and body weight were determined; the tumor size was measured using Vernier calipers, and the tumor volume was estimated by the formula: tumor volume (mm^3) = (L x W^2) x ½, where L is the length and W is the width of the tumor. All animals in the
treatment group presented with an infiltrative, densely cellular neoplasm with similar histological features as observed in the control group. Multifocal areas of necrosis were also observed within the neoplasm in this group (Suppl. S3).

RESULTS

NR4A1 Regulates PAX3-FOXO1A Expression in ARMS Cells

Previous analysis of publically available patient arrays shows that NR4A1 is upregulated in tumors from RMS patients and analysis of ARMS tumors showed that NR4A1 levels were also higher in PAX3-FOXO1A-positive vs. PAX3-FOXO1-negative tumors (Figure 19B) [352, 355]. This analysis was limited by the few studies available; however, there was a trend between expression of NR4A1 and PAX3-FOXO1A. Transfection of Rh30 cells with siCtl (non-specific oligonucleotide) and siNR4A1 resulted in decreased expression of NR4A1 and PAX3-FOXO1A proteins and in a separate experiment, siNR4A1 also decreased PAX3-FOXO1A mRNA (Figure 19C). Transfection of Rh30 cells with siPAX3-FOXO1A decreased expression of PAX3-FOXO1A but not NR4A1 protein confirming that NR4A1 regulated expression of the fusion gene in this cell line. We also carried out an identical set of experiments in Rh41 (Figure 19D) and Rh18 (Figure 19E) ARMS cell lines and the results were similar to that observed in Rh30 cells (Figure 19C), confirming that NR4A1 regulates PAX3-FOXO1A expression.
Figure 19. NR4A1 regulates PAX3-FOXO1A expression in ARMS. (A) Pro-oncogenic pathways regulated by NR4A1 in RMS and other cancer cell lines. (B) Analysis of PAX3-FOXO1A gene expression in ARMS tumors expressing high and low levels of NR4A1 in patient-derived mRNA acquired from the NCBI GSE2851 dataset. Effects of knockdown of NR4A1 by RNAi (siNR4A1) in Rh30 (C), Rh41 (D) and Rh18 (E) on PAX3-FOXO1A protein and RNA was determined by western blot analysis of whole cell lysates and real time PCR of RNA extracts, respectively, as outlined in the Materials and Methods. Results were compared to cells transfected with a non-specific control oligonucleotide, and RNA results are means ± SE for three replicated determinations and significant (p<0.05) decreases are indicated (*).
The highly pro-oncogenic activity of PAX3-FOXO1A is primarily due to regulation of downstream genes which include the oncogene NMyc, ras-association domain family 4 (RASSF4), myogenic differentiation-1 (MYOD1), gremlin 1 (GREM1) and death-associated protein kinase-1 (DAPK1) [356-359]. Knockdown of NR4A1 in Rh30 cells resulted in decreased expression of NMyc, Rassf4, MyoD1, Grem1 and DAPK1 and similar results were observed in cells transfected with siPAX3-FOXO1A (Figure 20A). Moreover, comparable results were observed in Rh41 (Figure 20B) and Rh18 (Figure 20C) cells confirming that NR4A1 downregulation results in decreased expression not only of PAX3-FOXO1A but also PAX3-FOXO1A-regulated genes in ARMS cells.
NR4A1 Antagonists Decrease Expression of PAX3-FOXO1A in ARMS Cells

Previous studies show that DIM-C-pPhOH and DIM-C-pPhCO₂Me bind the ligand binding domain of NR4A1 [314] and act as NR4A1 antagonists in pancreatic, colon, breast, kidney and RMS cells [270, 303, 314, 317, 350-352]. Treatment of Rh30 cells with DIM-C-pPhOH and DIM-C-pPhCO₂Me decreased expression of PAX3-FOXO1A protein and this was accompanied by downregulation of NMyc, Rassf4, MyoD1, Grem1 and DAPK1 proteins (Figure

\[ \text{Figure 20. NR4A1 regulates expression of PAX3-FOXO1A-dependent genes. Rh30 (A), Rh41 (B) and Rh18 (C) cells were transfected with siCtl, siNR4A1 or siPAX3-FOXO1A and after 72 hr, whole cell lysates were analyzed by western blots. Common lysates were used for western blots illustrated in Figures 19 and 20.} \]}
Similar results were observed in Rh41 (Figure 21B) and Rh18 (Figure 21C) cells, confirming that knockdown of NR4A1 by RNAi (Figs. 19 and 20) or inactivation of NR4A1 by treatment with NR4A1 antagonists resulted in the abrogation of the PAX3-FOXO1A signaling pathway in ARMS cells. Using Rh30 cells as a model, we also observed that DIM-C-pPhCO₂Me decreased expression of PAX3-FOXO1A (Figure 21D) and NMyc, Grem1, DAPK1, MyoD1 and Rassf4 (Figure 21E) mRNA levels in Rh30 cells. This is consistent with the potential role of NR4A1 as a regulator of PAX3-FOXO1A gene expression and PAX3-FOXO1A-mediated regulation of the downstream genes.
Figure 21. NR4A1 antagonists downregulate PAX3-FOXO1A. Rh30 (A), Rh41 (B) and Rh18 (C) cells were treated with the NR4A1 antagonists DIM-C-pPhOH and DIM-C-pPhCO2Me for 24 hr and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. Rh30 cells were treated with 10 or 15 µM DIM-C-pPhCO2Me for 24 hr and RNA extracts were examined by real time PCR for expression of PAX3-FOXO1A (D) and PAX3-FOXO1A-regulated genes (E). Results (D and E) are expressed as means ± SE for 3 replicate determinations and significantly (p<0.05) decreased expression compared to DMSO (control) is indicated (*).
Mechanism of NR4A1 Regulation of PAX3-FOXO1A

Previous studies show that NR4A1, in combination with p300, act as nuclear cofactors for expression of some Sp1-regulated genes including survivin, TXNDC5, IDH1, α5-integrin and β1-integrin [272, 351, 352]. The PAX3-FOXO1A promoter has several GC-rich binding sites (Figure 22A), and we therefore investigated the role of Sp1 in regulating expression of PAX3-FOXO1A and downstream genes by RNAi. Knockdown of Sp1 decreased Sp1 and p300 proteins but did not affect expression of PAX3-FOXO1A or downstream genes in Rh30, Rh41 or Rh18 cells (Suppl. Figure A-3A), suggesting that in contrast to previous studies on NR4A1/Sp1-regulated genes [272, 351, 352], neither Sp1 nor p300 were required. This was confirmed by knockdown of p300 in ARMS cells which did not affect expression of PAX3-FOXO1A and downstream genes (Suppl. Figure A-3B). Since Sp3 and Sp4 also bind GC-rich promoter sites and are overexpressed in RMS cells [90, 96], we investigated the effects of Sp3 and Sp4 knockdown and downregulation of Sp1/3/4 (combined) (Figs. 22B-22D, respectively). Knockdown of Sp3 had minimal effects on expression of PAX3-FOXO1A and downstream genes; however, knockdown of either Sp4 or Sp1/3/4 resulted in decreased expression of PAX3-FOXO1A, NMyc, Rassf4, Grem1, MyoD1 and DAPK1. Results of these RNAi experiments indicated that Sp4 interactions with NR4A1 regulated PAX3-FOXO1A expression and therefore we carried out ChIP assays on the three different GC-rich regions of the PAX3-FOXO1A gene promoter (Figure 22A) to
determine NR4A1/Sp4 promoter interactions. In untreated Rh30 cells, NR4A1, Sp4, p300 and pol II were associated with the promoter and treatment with 20 \( \mu \text{M DIM-C-pPhOH} \) for 6 hr decreased interactions of pol II, NR4A1 and Sp4 with the two distal and proximal regions of the PAX3-FOXO1A gene promoter (Figure 22E). P300 and other Sp proteins also interacted with the PAX3-FOXO1A promoter (data not shown); however, these genes did not play a functional role in regulation of PAX3-FOXO1A. We also showed by RNAi that CBP knockdown did not alter expression of PAX3-FOXO1A (Suppl. Figure A-3C) and current studies are investigating other cofactors which may coregulate NR4A1/Sp4-dependent expression of PAX3-FOXO1A.
Figure 22. Role of p300/NR4A1/Sp in regulation of PAX3-FOXO1A in ARMS cells. (A) GC-rich Sp binding sites in the proximal and two distal regions of the PAX3-FOXO1A gene promoter. ARMS cells were transfected with siSp3 (B), siSp4 (C), and siSp1/3/4 (D). Whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (E) Rh30 cells were treated with 20 µM DIM for 6 hr and association of various factors with the proximal and two distal regions of the PAX3-FOXO1A promoter were determined in a ChIP assay as outlined in the Materials and Methods. Sp1 and Sp3 also bound the PAX3-FOXO1A gene promoter (data not shown).
**Functional and in Vivo Studies**

Previous studies showed that PAX3-FOXO1A plays an important role in ARMS cell migration/invasion [360, 361] and this is confirmed in Rh30 cells where transfection of siPAX3-FOXO1A decreased migration in a Boyden chamber assay (Figure 23A). Moreover, transfection of Rh30 cells with siNR4A1 or treatment with the NR4A1 antagonist DIM-C-pPhCO$_2$Me also inhibited Rh30 cell migration, confirming that either direct (siPAX3-FOXO1A) or indirect downregulation of the fusion gene by inactivation of NR4A1 decreased Rh30 cell migration. We recently observed that knockdown of β1-integrin by siNR4A1 or treatment with DIM-C-pPhCO$_2$Me decreased migration of breast cancer cells and this involved an NR4A1/Sp1 complex binding to GC-rich elements in the β1-integrin promoter [351]. DIM-C-pPhCO$_2$Me also decreased expression of β1-integrin and phosphorylation of FAK (pFAK downstream from β1-integrin) in Rh30 cells and knockdown of NR4A1 by RNAi also gave similar results (Figure 23B). Moreover, using lysates from the Sp knockdown studies (Figure 22 and Suppl. Figure A-3), we also observed that only siSp4 and siSp1/3/4 decreased β1-integrin and pFAK (Figure 23C), suggesting that NR4A1/Sp4 regulated both β1-integrin and PAX3-FOXO1A, and knockdown of β1-integrin also decreased Rh30 cell migration (Figure 23D). We conclude that NR4A1-mediated migration of Rh30 cells is dependent on both PAX3-FOXO1A and β1-integrin (Figure 23E) which can be targeted simultaneously by C-DIM/NR4A1 antagonists.
Figure 23. PAX3-FOXO1A and β1-integrin are regulated by NR4A1/Sp4 and are both pro-migration genes. (A) Knockdown of PAX3-FOXO1A (siPF) or NR4A1 by RNAi and treatment with DIM-C-pPhCO₂Me decreased Rh30 migration in a Boyden chamber assay as outlined in the Materials and Methods. (B) Rh30 cells were treated with DIM-C-pPhCO₂Me or transfected with siNR4A1, and whole cell lysates were analyzed for β1-integrin/FAK expression by western blots. (C) Rh30 cells were transfected with oligonucleotides targeted to Sp1, Sp3, Sp4 and Sp1/3/4 (combined), and whole cell lysates were analyzed by western blots. Lysates were obtained from studies outlined in Supplemental Figure B-3 and Figures 20B-20D. (D) Rh30 cells were transfected with β1-integrin and cell migration was determined in a Boyden chamber assay. (E) Model for NR4A1/Sp4 regulation of PAX3-FOXO1A and β1-integrin. Results (A and D) are expressed as means ± SE for 3 replicate determinations and significant (p<0.05) decreases are indicated (*).
Previous studies showed that the NR4A1 antagonist DIM-C-pPhCO$_2$Me (40 mg/kg/day) inhibited tumor growth in athymic nude mice bearing Rh30 cells as xenografts [352]. We examined lysates from tumors treated with corn oil (control) or DIM-C-pPhCO$_2$Me and the treatment significantly decreased expression of PAX3-FOXO1A mRNA and protein and downstream genes nMyc, Rassf4, MyoD1 and DAPK1 (Figs 24A and 24B). This is consistent with the results of in vitro studies (Figure 21). Moreover, using human β2-microglobulin mRNA as a unique marker, we observed decreased expression in lungs of mice bearing Rh30 cells as xenografts; this was consistent with decreased lung metastasis (Figure 24C). Histological analysis of RMS xenografts of DIM-C-pPhCO$_2$Me-treated and control-treated mice showed minimal differences (Figure 24D).
Figure 24. In vivo studies. Tumors from mice treated with corn oil or DIM-C-pPhCO\textsubscript{2}Me (40 mg/kg/d) (25) were extracted for protein (A) and mRNA (B) analysis by western blots and real-time PCR, respectively. (C) Human β2-microglobulin mRNA expression in lungs from control and DIM-C-pPhCO\textsubscript{2}Me-treated mice were determined by real time PCR. (D) Representative images of rhabdomyosarcoma observed on control (a, b) and treatment groups (c, d). In both groups, the neoplasm was characterized by a population of round and pleomorphic cells arranged in sheets, with moderate to abundant cytoplasm and round nuclei. Scattered multinucleated neoplastic cells were observed. The neoplasm had multifocal necrotic areas in both the control and treatment groups. Hematoxylin and eosin; 100X (a, c), 400X (b, d).

DISCUSSION

RMS is primarily observed in children and adolescents and accounts for 5% of all pediatric cancers and 50% of soft tissue sarcomas in children [362]. Although ERMS patients respond well to current therapies which include surgery and radiotherapy combined with treatment with cytotoxic drug combinations, patients with ARMS have a poor diagnosis. Cytogenetic analysis has
demonstrated that 2;13 and 1;13 chromosomal translocations generating PAX3-FOXO1A and PAX7-FOXO1A fusion genes, respectively, are highly prevalent (55% and 22%, respectively) in tumors from ARMS patients. The PAX3-FOXO1A fusion gene is the critical prognostic marker for ARMS patients with metastatic disease, with an estimated overall 4-year survival of 8% compared to 75% survival rate of patients with PAX7-FOXO1A-expressing tumors [362-364]. Unfortunately, RMS patients that survive current cytotoxic drug therapies have an increased risk for several diseases later in life [74], emphasizing the critical need for development of new mechanism-based therapies which have fewer long term adverse effects. Results of PAX3-FOXO1A knockdown or overexpression studies in RMS and other cell lines demonstrate the functional importance of this fusion gene in maintaining the aggressive cancer cell phenotype and this is due, in part, to the pro-oncogenic PAX3-FOXO1-regulated genes [362, 365]. Development of agents that target PAX3-FOXO1A is ongoing and includes thapsigargin, fenretinide, HDAC inhibitors, and polo-like kinase inhibitors; however, the efficacy of these compounds for clinical applications in ARMS chemotherapy is not known [87, 366-369].

There is evidence that ROS-inducing anticancer agents such as HDAC inhibitors are effective anticancer agents against RMS in both laboratory rodent and cell models and that ROS decreases expression of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes [87, 96, 366]. In addition, ROS-independent downregulation of Sp transcription factors in RMS cells treated with tolfenamic
acid demonstrates that several pro-oncogenic genes in RMS including c-Met, CXCR4, insulin-like growth factor receptor (IGFR), and platelet-derived growth factor receptor α (PDGFRα) are Sp-regulated. Tolfenamic acid also decreases PAX3-FOXO1A in Rh30 cells [90]. Knockdown of Sp transcription factors or NR4A1 in RMS cells resulted in decreased cell proliferation, induction of apoptosis, and inhibition of cell migration [90, 96]. The comparable functions of Sp transcription factors and NR4A1 are due, in part, to coregulation of genes by NR4A1/Sp complexes that bind GC-rich gene promoters such as survivin, TXNDC5, IDH1 and β1-integrin [272, 351, 352]. Ongoing RNAseq and array studies also show that there is a considerable overlap between genes coregulated by NR4A1 and Sp transcription factors, and we hypothesized that PAX3-FOXO1A is also an NR4A1/Sp-regulated gene.

Knockdown of NR4A1 or treatment of Rh30, Rh41 and Rh18 ARMS cells with NR4A1 antagonists decreased expression of PAX3-FOXO1A (protein and mRNA) and downstream genes (Figs. 19-21) and similar results were observed after PAX3-FOXO1A knockdown by RNAi. In vivo studies also showed that DIM-C-pPhCO₂Me decreased PAX3-FOXO1A and downstream genes in Rh30-derived tumors (Figure 24A). The role of Sp transcription factors in mediating this response was investigated by RNAi (Figure 22) and the results indicated that Sp4 and not Sp1 or Sp3 was involved in expression of PAX3-FOXO1A. A recent study showed that Sp1, Sp3 and Sp4 regulate expression of several genes in common; however, all three transcription factors also regulate unique
sets of genes [97] and in ARMS cells PAX3-FOXO1A expression is dependent on NR4A1 and Sp4; this was confirmed in ChIP assays (Figure 22). We did not observe that p300 (or CBP) was required for NR4A1/Sp4-mediated regulation of PAX3-FOXO1A. This differed from NR4A1/Sp1-dependent genes, and current studies are investigating the identity of factors that may be involved.

In summary, this study shows that the critical pro-oncogenic PAX3-FOXO1A fusion gene is regulated by NR4A1/Sp4 interactions with GC-rich gene promoter elements. PAX3-FOXO1A gene expression can be inhibited by targeting either the receptor or Sp4 since knockdown of NR4A1 or Sp4 by RNAi blocks PAX3-FOXO1A gene expression. This study shows for the first time that C-DIM/NR4A1 antagonists represent a new class of mechanism-based agents that target PAX3-FOXO1A and other pro-oncogenic genes/pathways (Figure 19A). These antagonists also simultaneously decrease expression of PAX3-FOXO1A and β1-integrin genes (Figure 23E) which play a role in ARMS cell migration/invasion. Current studies are focused on development of more efficacious C-DIM/NR4A1 antagonists for clinical applications in ARMS chemotherapy and for combination therapies that will reduce requirements for cytotoxic drugs and thereby decrease the incidence of health effects later in life [74].
CHAPTER IV

ANTICANCER AGENTS DOWNREGULATE PAX3-FOXO1A IN ALVEOLAR RHABDOMYOSARCOMA (ARMS) CELLS AND INDUCE INTERLEUKIN-24

INTRODUCTION

Rhabdomyosarcoma (RMS) is primarily observed in children and adolescents and accounts for approximately 5% of all pediatric cancers with incidence rates of 4.5/10^6 [319, 320, 370]. Embryonal RMS (ERMS) and alveolar RMS (ARMS) are the two major classes of RMS in children and adolescents and differ with respect to their histology, genetics, treatment, and prognosis [322, 370]. ERMS accounts for over 60% of RMS patients and is associated with loss of heterozygosity at the 11p15 locus [322]. ERMS patients have a favorable initial prognosis; however, the overall survival of patients with metastatic ERMS is only 40% [371]. Cytogenetic analysis of tumor from ARMS patients have identified that 2;13 and 1;13 chromosomal translocations generating PAX3-FOXO1A and PAX7-FOXO1A fusion genes, respectively, are highly prevalent (55 and 22%, respectively) [99]. The PAX3-FOXO1A fusion gene is the critical prognostic marker for ARMS patients with metastatic disease, with an estimated overall 4 year survival of 8% compared to 75% survival rate of patients with PAX7-FOXO1A-expressing tumors [364, 372, 373].

Results of PAX3-FOXO1A knockdown or overexpression studies in RMS and other cell lines demonstrate the functional importance of this fusion gene in maintaining the aggressive cancer cell phenotype and this is due, in part, to the
pro-oncogenic PAX3-FOXO1-regulated genes [98, 356, 374]. Treatment of RMS patients include radiotherapy, surgery, and chemotherapy with cytotoxic drugs and/or drug combinations of vincristine, dactinomycin, cyclophosphamide, irinotecan, fosfamide, etoposide, doxorubicin, and others. A serious problem also exists for RMS patients that survive current cytotoxic drug therapies, since these individuals as adults have an increased risk for several diseases [74].

The orphan nuclear receptor NR4A1 is overexpressed in colon, pancreatic, breast (estrogen receptor positive and negative), and lung tumors; in breast, colon, and lung tumor patients, high expression of NR4A1 predicts decreased survival [236, 270-272, 285, 327]. The functional activity of NR4A1 in cancer has been extensively investigated in cancer cell lines by either knockdown or overexpression. NR4A1 regulates one or more of cancer proliferation, survival, cell cycle progression, migration, and invasion in lung, melanoma, lymphoma, pancreatic, colon, cervical, ovarian, and gastric cancer cell lines [271, 272, 284, 286, 287, 303, 327, 332, 333, 352, 356, 375].

Studies in this laboratory have reported that NR4A1 is also overexpressed in RMS tumor compared to normal muscle tissue [352] and NR4A1 regulated many of the same genes/pathways observed in other solid tumors [270, 272, 303, 314, 317, 350, 352, 376, 377]. The NR4A1 ligand 1,1-bis(3-indolyl)-1-(p-hydroxyphenyl)methane (DIM-C-pPhOH) which acts as a receptor antagonist to inhibit growth, survival, and migration of RMS cells and also inhibits RMS tumor growth in a xenograft mouse model [352]. These
NR4A1 antagonists are particularly effective against ARMS cells/tumors since they block expression of PAX3-FOXO1A, an NR4A1 regulated gene [377]. The present study was initiated after analysis of RNASeq data showed that after knockdown of NR4A1 or PAX3-FOXO1A or treatment with DIM-C-pPHOH, the most highly induced gene in common was the tumor suppressor-like factor interleukin-24 (IL-24). Thus, the oncogenic activity of PAX3-FOXO1A is due, in part to suppression of IL-24 while the anticancer activities observed after PAX3-FOXO1A knockdown/suppression are due primarily to induction of IL-24. We also observed that IL-24 overexpression inhibited ARMS cell growth, survival, and migration, suggesting that the clinically approved IL-24 adenoviral expression vector potentially [378] represents a promising new approach for ARMS therapy.

MATERIALS AND METHODS

Cell Lines, Antibodies, Chemicals, and Other Materials

Rh30 human RMS cancer cells were obtained from the American Type Culture Collection and authenticated in 2014 (Manassas, VA) and were maintained at 37°C in the presence of 5% CO2 in RPMI-1640 Medium and supplemented with 10% fetal bovine serum and 5% antibiotic. Rh18 and Rh41 cells were received from Texas Tech University Health Sciences Center-Children’s Oncology Group in 2015. Rh41 and Rh18 cell lines were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 20% fetal bovine serum, 1X ITS (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous
acid), and 5% antibiotic. IMDM was purchased from ThermoFisher Scientific (Waltham, MA). RPMI-1640 was purchased from Sigma-Aldrich (St. Louis, MO), and Lipofectamine 2000 for siRNA transfection was purchased from Invitrogen (Grand Island, NY). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were subsequently viewed using a filter set for FITC, rhodamine, and DAPI on an Advanced Microscopy EVOS fl, fluorescence microscope. RGB-4103 GelRed nucleic acid stain was used in place of Ethedium Bromide from Phenix Research Products (Candler, NC). SB203580 was purchased from Cell Signaling Technology (Danvers, MA). The human IL-24 cDNA clone in a pCMV-6 vector was purchased from origene (Rockville, MD). The C-DIM compounds were prepared as previously described [272] and a summary of the antibodies are provided in Supplemental Table B-4. A summary of oligonucleotide for RNAi and real time PCR and ChIP primers are summarized in Supplemental Table B-4.

**Total RNA Expression Analysis, RNASeq Analysis, IL-24 Overexpression, and CRISPR/Cas9**

Patient sample data of total RNA was acquired from NCBI GEO dataset GSE28511 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28511) and was previously analyzed for quality control, quantile normalized. In addition, multi-probe genes were averaged by the submitter. Expression values were listed into non-tumor and RMS tumor groups in JMP® and a box plot was generated, from which a t-test was performed; significance was determined as a
p-value less than 0.01, shown by an asterisk (Figure 25B). RMS cells were treated with DIM-C-pPhOH for 48 hr or transfected with siPAX3-FOXO1A or siNR4A1 for 72 hr after which RNA was extracted and sent to the Texas A&M AgriLife Sequencing Core for preparation, sequencing, and analysis. IL-24 cDNA was transfected into RMS cells using lipofectamine 2000 delivery at a concentration of 50 μM before endpoint analysis using western blot, PCR, or Annexin V staining. IL24 CRISPR guide RNA plasmid [pSp Cas9 BB-2A-GFP (PX458)] was purchased from GenScript (Piscataway, NJ). To create a stable IL-24 KO cell line, DH5α supercompetent bacterial cells were transformed using IL24 guide RNA. This included a preincubation of bacteria with plasmid for 30 mins on ice, followed by a 1min 30 sec heat shock, then cells were allowed to recuperate on ice with 200 μl SOC broth supplement. Cells were then plated on LB agar plates and allowed to grow for 18-24 hr. Cells were screened for GFP under microscope and selected to be innocuated overnight (18 hr) in LB media. IL24 CRISPR guide plasmid was then isolated using a DNA miniprep from Zymo Research (Irvine, CA) using manufacturer’s protocol. RD and Rh30 rhabdomyosarcoma cells were seeded (1.2 x 105 per well) in six well plates in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and left to attach for 24 hours. Cells were transfected with IL24 CRISPR guide RNA plasmid using Lipofectamine 2000 reagent according to the manufactures protocol. Cells were then visualized under the microscope to detect GFP fluorescence after a period of 6-12 hr. After
72 hr cells were then used for cell proliferation, apoptotic, migration, and western blot analysis.

**Cell Proliferation Assay**

Rh30, Rh18, and Rh41 cells were plated in 12-well plates at 1.0 x 10^5 and allowed to attach for 24 hr before treatment with DIM-C-pPhOH, DIM-C-pPhCO_2Me, or transfected with siNR4A1, siPAX3-FOXO1A, or siIL-24 with DMSO (dimethyl sulfoxide) as empty vehicle or siCtl siRNA (with lipofectamine vehicle) as controls, respectively. Cells were then trypsinized and counted at 24 hours using a Coulter Z1 cell counter.

**Annexin V Staining**

RMS cells were seeded at 1.0 x 10^5 in 2-well Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hr transfection with IL-24 overexpression vector, siPAX3-FOXO1A (100 μM), or siIL-24 (100 μM) with a control of siCtl (with lipofectamine vehicle) for 72 hr, and Annexin V staining was determined as described [Diindolylmethane analogs bind NR4A1 and are NR4A1 antagonists in colon cancer cells]. Hoechst staining from the apoptotic and necrotic cells assay (Biotium, Hayward, CA) was used to visualize apoptotic cells. Images were taken using an EVOS fluorescence microscopy from Advance Microscopy.

**Boyden Chamber Assay**

RMS cells were seeded for 24 hr in a 24-well plate and subsequently transfected with attach for 24 hr transfection with IL-24 overexpression vector,
siPAX3-FOXO1A (100 μM), or siIL-24 (100 μM) with a control of siCtl. The cells were trypsinized, counted, placed in 24-well 8.0-μm-pore ThinCerts from BD Biosciences (Bedford, MA), allowed to migrate for 24 h, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted.

**Western Blot**

Rh30, Rh18, and Rh41 cells were seeded in 6-well plates at 1.0 x 10^5 and allowed to attached for 24 hr before treatment with DIM-C-pPhOH, DIM-C-pPhCO₂Me, or transfected with siNR4A1, siPAX3-FOXO1A, or siIL-24 with DMSO as empty vehicle or siCtl siRNA (with lipofectamine vehicle) as controls, respectively. Cells were treated with C-DIMs or DMSO for 48 hr or transfected with siNR4A1, siPAX3-FOXO1A, or siIL-24 (all at 100 μM) or siCtl for 72 hr, and Western blots of whole cell lysates were determined as previously described [314].

**Real-time PCR, and Chromatin Immunoprecipitation (ChIP) Assays**

Real time PCR and ChIP assays using RMS cell lines transfected with oligonucleotides or treated with C-DIMs were carried out using the SYBR Green RT-PCR Kit (Bio-rad Laboratories, Hercules, CA) and the ChIP-IT Expression Kit (Carlsbad, CA) following the manufacturers protocol. Oligonucleotides and primers used are summarized in Supplemental Table B3. Transactivation studies were carried out in Rh30 cells transfected with two NR4A1-responsive constructs, NuREx3-luc and NBRE3-luc, that bind NR4A1 as a homodimer or
monomer, respectively, or transfected with a GAL4-NR4A1 (chimera) and a
GAL4-responsive construct (UASx5-luc) essentially as described [329].

Statistics

Results for each treatment group were replicated (at least 3X) and expressed a means +/-SE. Statistical comparisons of the treated groups vs. a control for each treatment were determined using Student's t-test.

RESULTS

Changes in gene expression in Rh30 cells after knockdown of NR4A1 and PAX3-FOXO1A or treatment with DIM-CpPhOH were determined by RNASeq and comparisons with controls. Figure 25A illustrates the changes in gene expression in the three treatment groups and the overlap of common genes that were induced (6) or repressed (7). Among the treatment related induced genes, IL-24 was induced by up to 27.9-fold. Therefore, we examined the relative expression of IL-24 in ARMS tumors vs normal muscle (Figure 25B) using publically available databases. IL-24 levels were significantly higher in normal muscle vs ARMS and also higher in PAX3-FOXO1A negative vs PAX3-FOXO1A positive tumors (Figure 25B). Furthermore, the expression of IL-24 and NR4A1 are inversely related in ARMS and normal muscle tissue samples (Figure 25B).
Figure 25. PAX3-FOXO1A and NR4A1 regulate IL-24. (A) RNASeq analysis identified IL-24 as a commonly regulated gene of NR4A1 and PAX3-FOXO1A. (B) Analysis of IL-24 gene expression in ARMS tumors in patient-derived mRNA acquired from the NCBI GSE2851 dataset.
RNASeq analysis shows that both NR4A1 and its ligand (DIM-C-pPhOH), which acts as an antagonist induce IL-24 in Rh30 cells and transfection of siNR4A1 or treatment with DIM-C-pPhOH induced IL-24 protein levels in this cell line (Figure 26A). Similar induction was observed after treatment with DIM-C-pPhCO₂Me, another NR4A1 ligand (Figure 26A). A similar approach was used for Rh18 (Figure 26B) and Rh41 (Figure 26C) and both siNR4A1 and NR4A1 ligands induced IL-24 expression. PAX3-FOXO1A expression is also regulated by NR4A1 [377] and results in Figure 26D show that knockdown of PAX3-FOXO1A (siPF) induces IL-24 expression. These results are consistent with the RNASeq data (Figure 25A), confirming that PAX3-FOXO1A suppressed IL-24 in ARMS cells.
Figure 26. NR4A1 antagonism or inactivation upregulates IL-24. Rh30 (A), Rh41 (B) and Rh18 (C) cells were transfected with siRNA for NR4A1 and treated with the NR4A1 antagonists DIM-C-pPhOH and DIM-C-pPhCO₂Me for 24 hr and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (D) ARMS cells were transfected with siRNA targeted for PAX3-FOXO1A.

Figure 27A shows that NR4A1 knockdown or treatment with NR4A1 antagonists DIM-C-pPhOH and DIM-C-pPhCO₂Me induce IL-24 gene expression in Rh30, Rh18, and Rh41 ARMS cells and similar results were observed after knockdown of PAX3-FOXO1A (Figure 27B).
We further examined PAX3-FOXO1A-IL24 interactions and observed that the IL-24 promoter contains proximal PAX3 binding sites at -472 and -95 (Figure 27C). Primers targeted to the PAX3 sites were used in a ChIP assay and showed PAX3-FOXO1A (PAX3), CBP, and p300 binding and in cells transfected with siPAX3-FOXO1A (siPF), the PAX3-FOXO1A interaction decreased, while the CBP and p300 was unchanged and poll interactions were enhanced (Figure 27D). Previous studies indicated that HDAC4 inhibited transcriptional activation of IL-24 [379] and results of a ChIP assay showed that HDAC4 was associated with the IL-24 promoter (Figure 27E) as previously described and after knockdown of PAX3-FOXO1A, HDAC4 promoter interactions were decreased. These results are consistent with the gene/protein expression studies showing that PAX3-FOXO1A represses IL-24 gene expression.
**Figure 27. Role of PAX3-FOXO1A in regulation of IL-24 in ARMS cells.** (A) Rh30 cells were treated with DIM-C-pPhCO₂Me or DIM-C-pPhOH for 24 hr and (B) transfected with siPAX3-FOXO1A for 72 hr and RNA extracts were examined by real time PCR for expression of IL-24 and are expressed as means +/- SE for 3 replicate determinations. (C) IL-24 promoter shows PAX3 binding sites and GC-rich sequences. (D & E) Rh30 cells were transfected with siPAX3-FOXO1A for 6 hr and association of various factors with the proximal and two distal regions of the IL-24 promoter were determined in a ChIP assay as outlined in the Materials and Methods.
The anticarcinogenic effect of IL-24 in ARMS cells was investigated by overexpression studies and Figure 28A shows the overexpression of IL-24 inhibited Rh30, Rh18, and Rh41 cell proliferation. IL-24 overexpression induced activation of caspase-3, caspase-9 and PARP cleavage in Rh30 (Figure 28B), Rh18 (Figure 28C), and Rh41 (Figure 28D) cells and also induced Annexin V staining in the 3 ARMS cell lines (Figure 28E). IL-24 also inhibited migration of ARMS cells (Figure 28F) and these results were similar to those previously observed in ARMS cells treated with C-DIM/NR4A1 antagonists or transfected with siNR4A1 or siPAX3-FOXO1A [352, 377].
Figure 28. Anticarcinogenic effect of IL-24 overexpression. (A) IL-24 overexpression decreases ARMS cell proliferation. IL-24 overexpression induced apoptosis markers in (B) Rh30, (C) Rh18, and (D) Rh41 ARMS cells. (E) Observed Annexin V staining upon overexpression of IL-24 in ARMS cells. (F) IL-24 overexpression decreases cell migration/invasion as determined by Boyden Chamber assay.
Previous studies have characterized several other IL-24 induced responses in diverse cancer cell lines, including increased phosphorylation of STAT1 and STAT3, activation or induction of stress/survival genes including, Bax, PERK, p38, GADD45, and GADD34 and downregulation of survival genes Bcl-2 and Bcl-XL [380-385]. These responses were investigated by overexpression of IL-24 in Rh30 (Figure 29A), Rh18 (Figure 29B), and Rh41 (Figure 29C) cells and all of the IL-24 mediated effects previously reported in other cancer cell lines were also observed in ARMS cells. Previous studies also showed that IL-24 mediated downregulation of Bcl-2 and Bcl-XL, induction of Bax, GADD45, and GADD34 were p-38 dependent and Figure 5D shows that the p38 inhibitor SB203580 also inhibits the same IL-24 dependent responses in Rh30 cells. Results illustrated in Figures 28 and 29 demonstrate that IL-24 exhibits multiple anticarcinogenic activities in RMS cells, which are suppressed by PAX3-FOXO1A.

Previous studies show that knockdown of PAX3-FOXO1A by RNAi or by NR4A1 antagonists inhibit ARMS cell growth, survival and migration [377] and the role of IL-24 in mediating these responses was investigated by transfecting cells with siPAX3-FOXO1A (which induces IL-24) and siIL-24. Results summarized in Figure 30A show that transfection of ARMS cells with siPAX3-FOXO1A inhibits cell growth and migration, whereas in ARMS cells cotransfected with siPAX3-FOXO1A+siIL-24, these inhibitory responses are significantly attenuated. Figure 30B confirms this as siPAX3-FOX1A decreases
expression of PAX3-FOXO1A and induces IL-24 expression while cotransfection with IL-24 decreases IL-24 but not PAX3-FOXO1A expression. Using the same treatment protocol, we observed that transfection of siPAX3-FOXO1A induced caspase-3, PARP cleavage (Figure 30C), and Annexin V staining (Figure 30D) and in cells cotreated with siPAX3-FOXO1A plus siIL-24 these same markers of apoptosis were significantly attenuated.

Figure 29. IL-24 dependent responses. Overexpression of IL-24 regulates expression of apoptosis, survival, and cellular stress genes in (A) Rh30, (B) Rh18, and (C) Rh41 ARMS cells. Inhibition of p38 by SB203580 attenuates IL-24 induced responses (D).
Figure 30. Role of IL-24 in mediating PAX3-FOXO1A activity. siPAX3-FOXO1A inhibits cell growth, migration (A), and apoptosis demonstrated by caspase-3 and PARP induction (C) and Annexin V staining (D), and cotransfection of siPAX3-FOXO1A+siIL-24 attenuates these responses in ARMS cells. (B) siPAX3-FOXO1A induces IL-24 expression and cotransfection with siIL-24 decreases IL-24 but not PAX3-FOXO1A.
We also generated Rh30-IL-24 KO cells by CRISPR/Cas9 and results in Figure 31A show that DIM-C-pPhOH and siPAX3-FOXO1A-dependent proliferation, Annexin V staining, and migration were observed in wild-type but were significantly attenuated in (Figure 31A) Rh30-IL-24 KO cells in which DIM-C-pPhOH does not induce IL-24 (Figure 31B). These data suggest that ARMS cell growth inhibition, anti-migratory and apoptotic responses triggered by downregulation of PAX3-FOXO1A are primarily due to derepression of IL-24 and this correlates with the anticancer activities of IL-24 in ARMS cells (Figs 28 and 29) and other cancer cell lines [385-388].
DISCUSSION

NR4A1 regulates PAX3-FOXO1A gene expression in ARMS cells and treatment with the NR4A1 antagonist DIM-C-pPhOH downregulated PAX3-FOXO1A expression, resulting in inhibition of ARMS cell and tumor growth. RNASeq was used to investigate the common and divergent effects of NR4A1 and PAX3-FOXO1A knockdown and DIM-C-pPhOH treatment on expression of gene in Rh30 cells and we observed induction of 6 and inhibition of 7 genes in common after all 3 treatments (Figure 25A). The most dramatic response was observed for IL-24, which is highly expressed in ARMS tumors that overexpression PAX3-FOXO1A (Figure 25B and 25C), but was induced 2.9 to 27.9-fold in the different treatment groups. IL-24, which is also known as melanoma differentiation associated gene 7 is somewhat of a unique tumor suppressor cytokine that is a member of the IL-24 subfamily. IL-24 activates signaling through the type 1 and type 2 IL-24 receptors (IL-20R), which consists of the IL20RA:IL-20RB and IL-22RA1:IL20RB heterodimeric receptors, respectively [389]. The tumor suppressor-like activities of IL-24 include inhibition of cancer cell growth and migration, induction of apoptosis, and inhibition of drug resistance, and these activities are observed in several different cancer cell lines through activation/repression of multiple genes/pathways (rev. in [385-388]).

Results of knockdown (NR4A1 and PAX3-FOXO1A) or treatment with DIM-C-pPhOH resulted in increased expression of IL-24 mRNA and protein in Rh30, Rh18, and Rh41 ARMS cells and ChIP assays confirmed that this was
accompanied by increased polIII associated with the IL-24 promoter (Figs 26 and 27). This was also associated with the loss of PAX3-FOXO1A and HDAC4 from the promoter and the loss of HDAC4 is consistent with a previous report showing that HDAC4 suppressed IL-24 expression is melanoma cells [379]. Since PAX3-FOXO1A can be targeted by NR4A1 antagonists, we therefore examined the effects of IL-24 overexpression in ARMS cells (Figure 28) and also determined the relative contributions of IL-24 to the tumor suppressor like activity observed after knockdown of PAX3-FOXO1A (Figure 30). Overexpression of IL-24 in ARMS cells inhibited cell growth and migration, activated caspase-dependent PARP cleavage and Annexin V staining, confirming that the anticarcinogenic activities of IL-24 observed in ARMS cells are similar to those reported in other solid tumors [385-388]. Overexpression of IL-24 activates or suppresses multiple genes and pathways in cancer cells that contribute to its tumor suppressor like activity. For example, IL-24 induces activation (phosphorylation) of p38 (MAPK) [383, 390], and induces the growth arrest and DNA damage (GADD)-inducible genes GADD45 and GADD34 [383], activates STAT1 and STAT3 [380, 385], increases the Bax/Bcl-2 ratio, and activates (phosphorylation) the stress gene protein kinases R-like endoplasmic reticulum kinases (PERK) [382]. We also observed these same IL-24 dependent responses in ARMS cells and their inhibition by SB203580 in Rh30 cells confirmed that p38 activation plays an important role in mediating the effects of IL-24 in ARMS cells (Figure 29).
Thus, the direct effects of IL-24 in ARMS cells are similar to those observed in other cancer cell lines and the contributions of IL-24 to the tumor suppressor like activities observed after PAX3-FOXO1A downregulation were further investigated by RNAi (Figure 30). Knockdown of PAX3-FOXO1A by RNA inhibited growth, migration, and induced apoptosis in ARMS cells as previously reported; however, cotransfection with siIL-24 significantly attenuated the siPAX3-FOXO1A mediated anticarcinogenic activity. Moreover, we also generated IL-24 KO Rh30 cells by CRISPR/Cas9 and transfection of siPAX3-FOXO1A had minimal effects on ARMS cell proliferation, survival, and migration (Figure 31), confirming that the tumor suppressor like activity resulting from PAX3-FOXO1A suppression is primarily due to induction of IL-24.

In summary, we show that PAX3-FOXO1A suppressed IL-24 gene expression in ARMS and that knockdown or drugs (NR4A1 antagonists) target PAX3-FOXO1A induced IL-24 expression. The results of both in vitro and in vivo studies demonstrate that IL-24 exhibits tumor suppressor like activity in ARMS cells and these observations are similar to results observed in other solid tumors. The safety of adenoviral-delivered IL-24 has been shown in a phase I trial in patients with advanced tumor and our results in ARMS cells suggests that IL-24 therapy may be warranted for clinical application in treating ARMS patients. This approach may be particularly efficacious in light of toxic-side effects associated with current therapies.
CHAPTER V

POTENT BIS-INDOLE-DERIVED NUCLEAR RECEPTOR 4A1 (NR4A1) ANTAGONISTS INHIBIT Rhabdomyosarcoma (RMS) TUMOR GROWTH

INTRODUCTION

Rhabdomyosarcoma (RMS) is primarily observed in children and adolescents and this represents approximately 50% of all soft tissue sarcomas in children [319, 320, 391]. Embryonal RMS (ERMS) is the most common form of RMS observed in children and represents approximately 60% of all cases. Alveolar RMS (ARMS) is observed in approximately 20% of all RMS patients and is characterized by chromosomal translocations generating PAX3-FOXO1A and PAX7-FOXO1A fusions, and patients expressing the former more prevalent fusion gene have a poor prognosis [321, 370]. ERMS and ARMS tumors are distinguished histopathologically and both types of RMS are treated with combinations of surgery, radiotherapy and cytotoxic drugs [392, 393]. ERMS patients without metastasis respond well to the various therapies, whereas ARMS patients are less responsive and the overall 4-year survival rate of metastatic ARMS patients is <8%. RMS patients and survivors of other childhood cancers suffer the consequences from their aggressive therapies since it was reported that 95.5% of these individuals at 45 suffer from some increased chronic health problem [74].

Studies in this laboratory have been focusing on development of ligands that target (and inhibit) the orphan nuclear receptor 4A1 (NR4A1, TR3, Nur77)
NR4A1 is overexpressed in estrogen receptor positive and negative breast tumors, pancreatic, colon and lung tumors and overexpression in breast, colon and lung tumors is a negative prognostic factor for patient survival [236, 270, 272, 285, 326, 327, 394]. Recent studies also show that NR4A1 is overexpressed in RMS tumors and knockdown experiments in these solid tumors demonstrate that NR4A1 is pro-oncogenic and regulates genes/pathways associated with cancer cell proliferation, survival and migration/invasion [352, 394].

Studies in this laboratory have identified a series of 1,1-bis(3-indolyl)-1-(substituted phenyl)methane (C-DIM) analogs containing para-substituents on the phenyl ring that act as NR4A1 antagonists and block the NR4A1-regulated pro-oncogenic pathways GENES in RMS and other solid tumors [270-272, 303, 314, 317, 350-352, 394]. One of the most active compounds containing the p-hydroxyphenyl moiety, 1,1-bis(3'-indolyl)-1-(p-hydroxyphenyl)methane (DIM-C-pPhOH, CDIM8), also bound with high affinity to NR4A1 [314] but exhibited low serum levels in pharmacokinetic studies [395]. Therefore, in this study we generated a series of C-DIM analogs containing a p-hydroxyphenyl group but also buttressed by one or two additional substituents ortho to the p-hydroxyl group. Our results show that some of these "second generation" substituted DIM-C-pPhOH analogs exhibited up to an order of magnitude higher potency than DIM-C-pPhOH and represent a class of second generation C-DIM/NR4A1 antagonists that are promising new agents for treatment of rhabdomosarcoma.
MATERIALS AND METHODS

Synthesis of Substituted DIM-C-pPhOH Analogs

Indole, 3,5-dimethoxy-, 3,5-dimethyl-, 3-methyl-, 3-methoxy-, 3-chloro-, 3-bromo-, 3,5-dibromo- and 3-chloro-5-methoxybenzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO) and 3-fluorobenzaldehyde was obtained from Alfa Aesar (Ward Hill, MA). Condensation of 2 mole equivalent of indole with 1 mole equivalent of the benzaldehyde derivative was carried out in 50 ml water plus 0.6 ml acetic acid at 90° C for 24-48 hr; the solid material was filtered and recrystallized from benzene or benzene/petroleum spirit. Compounds were ≥98% pure and overall yields for all the condensation reaction were 80-95%.

The compounds synthesized include 1,1-bis(3'-indolyl)-1-(3,5-dibromo-4-hydroxy-phenyl)methane (DIM-C-pPhOH-3,5-Br₂), 1,1-bis(3'-indolyl)-1-(3,5-dimethoxy-4-hydroxyphenyl)methane [DIM-C-pPhOH-3,5-(OCH₃)₂], 1,1-bis(3'-indolyl)-1-(3,5-dimethyl-4-hydroxyphenyl)methane [DIM-C-pPhOH-3,5-(CH₃)₂], 1,1-bis(3'-indolyl)-1-(4-hydroxy-3-methylphenyl)methane (DIM-C-pPhOH-3-CH₃), 1,1-bis(3'-indolyl)-1-(4-hydroxy-3-methoxyphenyl)methane (DIM-C-pPhOH-3-OCH₃), 1,1-bis(3'-indolyl)-1-(3-fluoro-4-hydroxyphenyl)methane (DIM-C-pPhOH-3-F), 1,1-bis(3'-indolyl)-1-(3-bromo-4-hydroxyphenyl)methane (DIM-C-pPhOH-3-Br), 1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxyphenyl)methane (DIM-C-pPhOH-3-Cl), and 1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl)methane (DIM-C-pPhOH-3-Cl-5-OCH₃).
Cell Lines, Antibodies, Chemicals, and Other Materials

Rh30 human RMS cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and authenticated in 2014, and were maintained at 37°C in the presence of 5% CO₂ in RPMI-1640 Medium and supplemented with 10% fetal bovine serum and 5% antibiotic. RPMI-1640 and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). The C-DIM compounds were prepared as previously described above. FOXO1 antibody was purchased from Cell Signaling Technologies (Danvers, MA); TXNDC5 antibody was purchased from GeneTex (Irvine, CA); IL-24, GDA, and DCDC2 antibodies were purchased from Abcam (Cambridge, MA).

Tumor Growth Assay

Female athymic nude mice (6-8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions, housed in isolated vented cages, and allowed to acclimate for one week with standard chow diet. The animals were housed at Texas A&M University in accordance with the standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The protocol of the animal study was approved by the Institutional Animal Care and Use Committee (IACUC), Texas A&M University. Rh30 cell lines (4x10⁶ cells) grown in RPMI media containing 10% FBS were detached, resuspended in 100 µl of phosphate-buffered saline with matrigel (BD Bioscience, Bedford, MA) (75:25), and implanted.
subcutaneously in the mice. When tumors reached about 40-50 mm$^3$ size, the mice were randomized into control and treatment groups (6 animals per group) and treated with placebo or DIM-C-pPh-4-OH-3,5-Br$_2$ (2.5, 5, 7.5, or 10 mg/kg/d) in nano liquid carrier (administered in sodium carboxymethyl cellulose) by oral gavage every second day for 3 weeks. Tumor volumes, weights, and body weights were determined; tumor size was measured using Vernier calipers, and the tumor volume was estimated by the formula (mm$^3$) = (L x W$^2$) x $\frac{1}{2}$, where L is the length and W is the width of the tumor. Tumors and lung tissue were homogenized for protein isolation for subsequent western blot and RT-PCR analysis.

**Western Blot**

Rh30 cells were seeded in 6-well plates at 1.0 x 10$^5$ and allowed to attach for 24 hr prior to treatment with C-DIMs, with DMSO as empty vehicle at indicated time points. Western blots of whole cell lysates were determined as previously described [352, 394].

**Real-time PCR and Transactivation Assay**

Real time PCR was carried out using Rh30 RMS cells and treated with indicated C-DIMs for 10 hr and RNA extraction was carried out using Quick-RNA MiniPrep Kit from Zymo Research (Irvine, CA) following the manufacturer's protocol. RT-PCR was carried out using SYBR Green RT-PCR kit (Bio-Rad Laboratories, Hercules, CA) essentially as previously described [352, 394]. Values for each gene were normalized to expression levels of TATA-binding
protein. The sequences of the primers used for reverse transcription-PCR were as follows; β2 microglobulin: 5'-GCA ATC ACC TGT GGA TGC TAA-3' (sense), 5'-TAA ATG GTT GAG TTG GAC CCG-3' (antisense); IL-24: 5'-ATG AAT TTT CAA CAG AGA GGG CTG-3' (sense), 5'-GCA GAA ATT CTA CAA GCT CTG A-3' (antisense); DCDC2: 5'-GAC CCT CAA AGA CCS CCA AG-3' (sense), 5'-AAA TGT TCT AAG CCA CGG CA-3' (antisense); GDA: 5'-ATT AGC GTG GTT CTG CAT CTC-3' (sense), 5'-TTA TGA ACC CTC TCA ACC AGA G-3' (antisense). Transactivation studies were carried out in Rh30 cells transfected with two NR4A1-responsive constructs, NuREx3-luc and NBRE3-luc, that bind NR4A1 as a homodimer or monomer, respectively, or transfected with a GAL4-NR4A1 (chimera) and a GAL4-responsive construct (UASx5-luc) essentially as described [314].

Statistics

Results for each treatment group were replicated (at least 3X) and expressed a means ± SE. Statistical comparisons of the treated groups vs a control for each treatment were determined using Student’s t-test.

RESULTS

The buttressed analogs of DIM-C-pPhOH were synthesized by coupling two mole equivalents of indole with the corresponding substituted ρ-hydroxybenzaldehyde to give the condensation products in 80-90% yield (crystallized from benzene or benzene/petroleum spirit). Figure 32A illustrates the structure of the buttressed DIM-C-pPhOH which were further screened in
Rh30 cells. Previous studies show that DIM-C-pPhOH decreased thioredoxin domain-containing 5 (TXDC5) and PAX3-FOXO1A fusion gene expression in Rh30 cells [352, 394], and these NR4A1-regulated genes play a key role in maintaining low oxidative stress and regulating multiple pro-oncogenic pathways, respectively. DIM-C-pPhOH (0 – 20 µM) significantly decreased expression of both gene products in Rh30 (Figure 32B). The buttressed derivatives of DIM-C-pPhOH were treated with 5 µM concentrations and all of the compounds significantly decreased expression of TXNDC5 and PAX3-FOXO1A and their potency (at 5 µM) was similar to that observed for 20 µM DIM-C-pPhOH (Figure 32C).

Quantitative structure activity relationships were investigated using 3 genes identified in RNAseq analysis of differences in mRNA expression in Rh30 cells treated with DIM-C-pPhOH or DMSO (control) for 6 hr. Three NR4A1-regulated genes induced by DIM-C-pPhOH were interleukin-24 (IL-24), guanine deaminase (GDA), and double cortin domain-containing 2 (DCDC2). The concentration-dependent induction of all 3 genes in Rh30 cells is illustrated in Figure 33. The fold induction responses were variable for these compounds and DIM-C-pPhOH-3,5-Br$_2$ and DIM-C-pPhOH being the most and least, respectively, potent inducers of IL-24, GDA and DCDC2 in terms of fold-induction response.
EC$_{50}$ values for induction of IL-24, GDA and DCDC2 by DIM-C-pPhOH were 5.2, 7.4 and 6.1 µM, respectively, whereas EC$_{50}$ values for the second generation analogs ranged from 0.54-1.58, 0.36-1.2 and 0.55-1.79 µM, respectively, for induction of the 3 genes. These results suggest that these buttressed derivatives of DIM-C-pPhOH were up to ten times more potent than the parent compound in terms of activation or de-repression of NR4A1-responsive genes. Previous studies showed that DIM-C-pPhOH and DIM-C-pPhCO$_2$Me inhibit tumor growth in mouse xenograft models at doses of 20-40 mg/kg/d.
Figure 33. C-DIM analogs: quantitative structure-activity relationships. Rh30 cells were treated with different concentrations of DIM-C-pPhOH (0, 1, 2.5, 5, 10, 15 and 20 µM) and related analogs (0, 0.5, 1.25, 2.5 and 5 µM) for 10 hr. RNA was isolated and expression of IL-24 (A), GDA (B) and DCDC2 (C) were determined by real time PCR using TATA binding protein as an internal control. EC50 values for induction used the maximal induction response for each compound as the 100% value and results were analyzed using Prism Software to determine EC50 induction values.

[317, 350-352, 394] and in an RMS mouse xenograft model, 40 mg/kg/d DIM-C-pPhCO2Me inhibited approximately 50-60% tumor volume compared to control animals [352, 394]. In this study, we used DIM-C-pPhOH-3,5-Br2 to test the effect of buttressing the p-hydroxyphenyl group on tumor growth inhibitory activity in athymic nude mice bearing Rh30 cells as xenografts. The results (Figure 34) of initial studies demonstrated highly significant inhibition of tumor
growth at concentrations of 10, 7.5, 5.0 and 2.5 mg/kg/d and there was almost complete tumor growth inhibition at all of these doses.

Figure 34. *In vivo* tumor growth inhibition by DIM-C-pPhOH analogs. Athymic nude mice bearing Rh30 cells as xenografts were treated with 10, 7.5, 5.0 and 2.5 mg/kg/d DIM-C-pPhOH-3,5-Br$_2$ in corn oil every second day, and tumor values were determined for the duration of the 3-week study as outlined in the Material and Methods. Significant (p<0.05) differences between tumor volumes in the treated and control mice are indicated (*).

In addition, tumor weights were also decreased at all dose levels (Figure 35A) and expression of human B2-microglobulin in the lung, a marker for lung metastasis of the human RMS cells, was also decreased (Figure 35B) compared to controls. In addition, we also observed decreased expression of NR4A1-
regulated *PAX3-FOXO1A* and *TXNDC5* and increased expression of *IL-24*, *DCDC2* and *GDA* genes in the treatment groups (Figure 35C). These results demonstrate the increased anticancer potencies of buttressed analogs of DIM-C-pPhOH compared to the parent compound and their enhanced potential for clinical applications in RMS chemotherapy.

![Graph](image)

**Figure 35. In vivo tumor growth inhibitory effects.** (A) Tumor weights. Tumor weights from mice treated with DIM-C-pPhOH-3,5-Br2 (outlined in Figure 34) were determined and are presented as means +/- SE [significant (p<0.05) inhibition is indicated (*)]. (B) Lung metastasis. Lungs from animals treated with DIM-C-pPhOH-3,5-Br2 were analyzed for expression of human β2-microglobulin to determine the relative levels of metastasis of Rh30-derived tumors that have metastasized to the lung. (C) Changes in gene product levels. Tumor lysates from mice (described in Figure 34) were analyzed by western blot analysis as outlined in the Materials and Methods.
**Figure 36.** NR4A1-regulated genes/pathways. NR4A1 regulates cell growth, survival and migration/invasion and related genes and these are also inhibited by C-DIM/NR4A1 antagonists.

**DISCUSSION**

RMS patients routinely receive cytotoxic drug therapies and most of those patients who survive into their 40s experience serious chronic health problems due to their prior cancer treatment [74]. Therefore, it is imperative that new treatment regimens using mechanism-based drugs be developed for treating RMS. Studies in the laboratory have characterized some of the pro-oncogenic pathways/genes regulated by NR4A1 in RMS and other solid tumors (Figure 36) and these can be targeted by NR4A1 antagonists [270-272, 303, 314, 317, 350-352, 394]. Many of the currently used mechanism-based anticancer agents target a single critical gene or pathway and this includes inhibition of epidermal growth factor with receptor tyrosine kinase inhibitors (e.g. lapatinib and erlotinib)
or antibodies such as Herceptin that bind and inactivate the oncogene HER2 receptor. In contrast, NR4A1 antagonists such as the C-DIMs inhibit NR4A1-dependent growth, survival and migration/invasion and many genes such as *survivin, bcl-2, EGFR* and integrins that are themselves individual drug targets (Figure 36). The effectiveness of this approach is related to the fact that NR4A1 is a nuclear transcription factor regulating multiple genes/pathways, whereas many mechanism-based drugs target a single gene product.

Previous studies showed that DIM-C-pPhOH and DIM-C-pPhCOOMe were effective inhibitors of solid tumor-derived cancer cells and tumors and this included RMS [270-272, 303, 314, 317, 350-352, 394]; however, pharmacodynamic studies indicated that DIM-C-pPhOH exhibited a short serum half-life [395]. Therefore, we synthesized a series of DIM-C-pPhOH analogs containing one or two (3- or 3,5-) substituents ortho to the p-hydroxyl group and we hypothesized that the substituents would enhance the effectiveness of the "second generation" C-DIM analogs by a buttressing effect and inhibit metabolism. The rapid metabolism of DIM-C-pPhOH and other phenolics are due, in part, to conjugation (e.g. glucuronidation) of the *para*-hydroxyl group. However, previous studies indicate that the glucuronidation rates of various substrates can be inhibited sterically (buttressing) by nearby substitutents [396] and therefore, we synthesized DIM-C-pPhOH analogs containing one or two adjacent substituents at the 3- or 3,5- positions on the phenyl ring (Figure 32). This study then focused on the enhanced anticancer activities of the C-
DIM/NR4A1 antagonists using Rh30 ARMS cells as a model since previous studies showed that DIM-C-pPhOH induced similar responses in a panel of ARMS cells (Rh30, Rh18, Rh41) [394]. The DIM-C-pPhOH analogs decreased expression of the NR4A1-regulated gene products TXNDC5 and PAX3-FOXO1A (Figure 32). The PAX3-FOXO1A fusion gene is a negative prognostic factor for ARMS patient survival and functions in ARMS cells as an oncogenic transcription factor that regulates expression of multipole genes including the oncogene NMyC [100, 358, 397-399]. Our results suggest that the new analogs of DIM-C-pPhOH are more potent than the parent compound and this was confirmed by determining EC$_{50}$ values for induction of 3 NR4A1-repressed genes in Rh30 cells, namely IL-24, GDA and DCDC2 (Figure 33). The *in vitro* quantitative structure activity relationships were complemented by *in vivo* studies showing that in initial studies DIM-C-pPhOH-3,5-Br$_2$ inhibited tumor volume and weight and modulated NR4A1-regulated genes at concentrations as low as 2.5 mg/kg/d (Figure 34). Moreover, using human β2-microglobulin as a marker gene in the lung (Figure 35), we also observed significantly lower levels of this gene in mice treated with DIM-C-pPhOH-3,5-Br$_2$ indicating that this drug also inhibited metastasis of Rh30 cells to the lung. Thus, we have identified members of a second generation set of DIM-C-pPhOH-substituted analogs that are 10 times or more potent than the parent compound. Based on results of future
pharmacokinetic, receptor binding and toxicity studies, we plan to develop one or more of these analogs for clinical applications in treating ARMS patients.
CHAPTER VI

SUMMARY AND CONCLUSIONS

NR4A1 is overexpressed in several solid tumors, including RMS, and serves as a transcription factor that mediates the expression of several genes and pathways that are involved in cancer cell proliferation, apoptosis, invasion, migration, and ROS. We have investigated a series of structurally diverse C-DIMs that have the ability to antagonize NR4A1 and decrease the pro-oncogenic activity of NR4A1. Two NR4A1 ligands used in this study, 1,1-bis (3'-indolyl)-1-(p-hydroxyphenyl)methane (DIM-C-pPhOH) and a p-carbomethoxy derivate (DIM-C-pPhCO₂Me) bind NR4A1 and do not induce nuclear export of NR4A1, which is comparable to results observed for these compounds in other cancer cell lines. The C-DIM/NR4A1 antagonists or knockdown (siRNA) decreased proliferation of Rh30 (ARMS) and RD (ERMS) cells, induced apoptosis, as evidenced by PARP cleavage and Annexin V staining, and decreased RMS xenograft tumor growth in athymic nude mice. Previous studies in this lab showed that NR4A1 acts as a coactivator of Sp-regulated genes with GC-rich promoters, such as bcl-2, cyclin D1, EGFR, and cMyc and knockdown or antagonism of NR4A1 decreased expression of these pro-growth/pro-survival genes. ChIP assays were used to investigate downregulation of NR4A1/Sp-regulated genes in RMS cells treated with DIM and we observed decreased interactions of NR4A1 and p300 on the GC-rich survivin promoter. In addition,
DIM-C-pPhCO$_2$Me also decreased mRNA levels of survivin, cyclin D1, and EGFR.

We also investigated the regulation of TXNDC5 and IDH1 expression by NR4A1 since both were previously identified by RNASeq analysis after siRNA-mediated knockdown of NR4A1 or treatment with NR4A1 antagonist. Both TXNDC5 and IDH1 maintain low oxidative and ER stress levels to enable cell proliferation and this is important for RMS cells which have high basal levels of ROS, making them sensitive to ROS-inducing agents. Knockdown or antagonism of NR4A1 in these cells induced ROS and decreased expression of both TXNDC5 and IDH1, and resulted in induction of cellular stress markers, PERK, ATF4, and CHOP. In addition, ChIP assay of the TXNDC5 and IDH1 promoters exhibit decreased levels of NR4A1 and Sp1 loss after treatment with C-DIM, indicating that both genes are regulated by NR4A1/Sp1 as described for survivin. These results point to the critical role of ROS in mediating C-DIM-induced effects in RMS cells.

C-DIMs also inhibited RMS cell growth by upregulation of sestrin 2 (SESN2), which activated AMPK$\alpha$, resulting in downregulation of mechanistic target of rapamycin (mTOR) signaling and a similar result was observed upon NR4A1 inactivation by RNAi. This result was observed in p53 mutant cell lines and demonstrated that SESN2 can be induced in a p53-independent manner. SESN2 is also induced in response to ROS and C-DIM/NR4A1 antagonists induced ROS and SESN2 and these effects were attenuated upon cotreatment
with the antioxidant glutathione (GSH). This is a novel observation demonstrates a p53-independent mechanism by which mTOR signaling may be inhibited.

PAX3-FOXO1A is a critical prooncogenic factor in the more aggressive and deadly ARMS subtype. Sp transcription factors (Sp1, Sp3, and Sp4) are overexpressed in RMS cell lines and previous studies in this lab have identified PAX3-FOXO1A as a Sp-regulated gene that can be inhibited by the ROS-inducing agent tolefemic acid. Analysis of patient tumors shows that NR4A1 is upregulated in ARMS (PAX3-FOXO1A-positive) compared to ERMS (PAX3-FOXO1A-negative) tumors and knockdown of NR4A1 decreases PAX3-FOXO1A expression in several ARMS cell lines and has no effect on normal muscle cells, confirming NR4A1 regulates PAX3-FOXO1A expression. The prooncogenic activity of PAX3-FOXO1A is due to, in part, its regulation of several genes, including myogenic differentiation-1 (MYOD1), death-associated protein kinase-1 (DAPK1), ras-association domain family 4 (RASSF4), and the oncogene nMyc, all of which play a role in myogenic differentiation, cell growth, and apoptosis. Antagonism or knockdown of NR4A1 inhibited expression of PAX3-FOXO1A-regulated genes in several ARMS cell lines. Furthermore, DIM-C-pPhCO₂Me (40mg/kg/day) inhibited ARMS tumor growth in athymic nude mice and also downregulated expression of PAX3-FOXO1A and its downstream genes, which is consistent with in vitro studies. Our observations align with previous studies demonstrating the importance of PAX3-FOXO1A as a contributor to the ARMS subtype.
Previous studies have shown NR4A1 regulates several genes, including *survivin*, *TXNDC5*, *IDH1*, \( \alpha5\)-*integrin* and \( \beta1\)-*integrin* mainly via binding to GC-rich promoters. The PAX3-FOXO1A promoter has several GC-rich binding sites and using siRNA targeted to Sp1, Sp3, and Sp4, we found Sp4 knockdown decreased expression of PAX3-FOXO1A and downstream genes. These results indicate a role for Sp4 interactions with NR4A1 in regulation of PAX3-FOXO1A. This observation was confirmed using ChIP assays showing that NR4A1/C-DIM antagonists decreased interactions of NR4A1 and Sp4 with the PAX3-FOXO1A gene promoter. Previous studies have found PAX3-FOXO1 plays a vital role in ARMS migration and we confirmed this using Boyden chamber assays showing decreased ARMS cell migration upon PAX3-FOXO1A or NR4A1 knockdown and C-DIM treatment. Studies in this lab have shown that NR4A1 and Sp1 cooperatively regulate \( \beta1\)-*integrin* expression and C-DIM treatment or knockdown of \( \beta1\)-*integrin* by siRNA decreased breast cancer cell migration. While the role of \( \beta1\)-*integrin* has not previously been established in RMS, its expression in breast cancer is associated with negative prognosis and metastasis. In ARMS cells, C-DIM treatment and NR4A1 antagonism also decreased \( \beta1\)-*integrin* expression and phosphorylation of FAK, which is downstream of \( \beta1\)-*integrin*. We also observed Sp4 knockdown decreased \( \beta1\)-*integrin*. In addition, PAX3-FOXO1A knockdown had no effect on \( \beta1\)-*integrin* expression, which demonstrates that NR4A1 and Sp4 are novel regulators of
both β1-integrin and PAX3-FOXO1A and both genes can be targeted by C-DIMs.

RNASeq studies using NR4A1 knockdown and antagonism and PAX3-FOXO1A antagonism revealed several genes that are commonly regulated (activated or repressed) by PAX3-FOXO1A and NR4A1, including the tumor suppressor-like factor interleukin-24 (IL-24). IL-24 inhibits cancer cell growth and migration, and mediates induction of apoptosis in several cancer cell lines and tumors. Analysis of gene expression results from ARMS tumors showed that IL-24 and PAX3-FOXO1A and IL-24 and NR4A1 were inversely expressed in these tumors. C-DIM/NR4A1 antagonists or PAX3-FOXO1A knockdown induced IL-24 and this was confirmed via ChIP assay showing increased pol II upon PAX3-FOXO1A knockdown. Loss of HDAC4 was also observed upon PAX3-FOXO1A knockdown, which is consistent with a previous report showing HDAC4 suppressed IL-24 expression in melanoma cells. Functional studies showed that IL-24 overexpression decreased ARMS cell proliferation and invasion induced Annexin V staining and caspase 3, and -9, and PARP cleavage, confirming the anti-oncogenic activities of IL-24 in RMS cells as previously observed in other cancers. In addition, IL-24 regulates several genes and pathways involved in cancer and we found IL-24 overexpression activated STAT1, STAT3, and p38 (MAPK), while inducing the growth arrest and DNA damage (GADD)-inducible genes GADD45 and GADD34 and increasing the Bax/Bcl-2 ratio. In addition, previous studies have reported p38 as a mediator of IL-24 responses and this
was confirmed in Rh30 cells upon IL-24 overexpression and cotreatment with the p38 inhibitor SB203580.

We further investigated the role of IL-24 in mediating the decreased growth, migration, and induced apoptosis induction in ARMS cells after PAX3-FOXO1A knockdown. In cells cotransfected with siPAX3-FOXO1A and siIL-24, effects of siPAX3-FOXO1A were significantly abrogated. In IL-24 KO Rh30 cells generated by CRISPR/Cas9 transfection with siPAX3-FOXO1A had no effect on ARMS cell survival, proliferation, and migration. These data show that the oncogenic activity of PAX3-FOXO1A was due to, in part, IL-24 repression and IL-24 induction contributes to the anti-carcinogenic actions observed upon PAX3-FOXO1A suppression. Our findings represent a novel role of IL-24 in RMS that can be targeted using C-DIM/NR4A1 antagonists.

While DIM-C-pPhOH and DIM-C-pPhCO$_2$Me are efficacious in in vivo and in vitro studies, pharmacodynamic studies showed that DIM-C-pPhOH has a short serum half-life, which is a concern for potential chemotherapeutic applications. Therefore, a second generation of C-DIM compounds have been synthesized using one or two substituents ortho to the p-hydroxyl group on DIM-C-pPhOH to buttress the p-hydroxyl group since this structural feature increase the half-life of the compound. These second-generation DIM-C-pPhOH analogs decreased expression of NR4A1-regulated genes, including TXNDC5 and PAX3-FOXO1A in RMS cells and EC$_{50}$ values for the induction of NR4A1-repressed genes (IL-24, GDA, and DCDC2) in Rh30 cells were up to 10-times
lower than observed for DIM-C-pPhOH. *In vivo* studies using one of the analogs (DIM-C-pPh-4-OH-3,5-Br₂) showed decreased tumor growth at the lowest dose of 2.5 mg/kg/day compared to high doses (20-40 mg/kg/day) required for first generation C-DIMs, confirming the increased potency of the new analogs. In addition, using β2-microglobulin as a marker for metastasis, we found decreased levels in the lungs of DIM-C-pPh-4-OH-3,5-Br₂-treated mice, indicating decreased lung metastasis. Our results demonstrate the increased therapeutic efficacy of “second generation” C-DIMs, which will be further developed for clinical applications. To further establish these second-generation C-DIMs as therapeutic agents, further studies should be carried out. The *in vivo* pharmacokinetic and pharmacodynamics should be determined to demonstrate increased serum half-life and any effects on internal organs. Furthermore, screening assays should be performed to elucidate any off-target effects of the second-generation C-DIMs.
REFERENCES


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Supplementary Figure A-1. (A) Apoptosis. Cells were treated with DIM-C-pPhOH, glutathione (GSH) alone and in combination for 24 hr, and whole cell lysates were analyzed by western blots. (B) Growth Inhibition. Cells were treated as described in (A) and cell growth was determined. [Significant (p<0.05) inhibition (*) and reversal (**).]
Supplementary Figure A-2. RD cells were transfected with siCtl or siNR4A1 or treated with DIM-C-pPhOH (A) and both RMS cell lines were treated with DIM-C-pPhCO₂Me (B) and whole cell lysates were analyzed by Western blots as outlined in the Materials and Methods.
Supplemental Figure A-3. Role of Sp1, p300 and CBP in PAX3-FOX01A expression. ARMS cells were transfected with siSp1 (A), sip300 (B) and siCBP (C) for 72 hours, and whole cell lysates were analyzed by western blots.
## Supplemental Table B-1

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<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siCtl:</td>
<td>5'-CGUACGCAGGAUACUUCG A-3'</td>
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<tr>
<td>siPAX3-FOXO1A</td>
<td>5'-CCUCUCACCCAGAAGAUCATT-3' (sense)</td>
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<tr>
<td></td>
<td>5'-UGAUAUUCUGGAGGAGGTT-3' (antisense)</td>
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<tr>
<td>siNR4A1:</td>
<td>SASI_Hs02_00333289</td>
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<tr>
<td>siSp1</td>
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<td>β1-Integrin</td>
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### Primers

<table>
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<tr>
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<tbody>
<tr>
<td>PAX3-FOXO1A</td>
<td>5'-AGACAGCTTTTGCTCCCAT-3' (sense)</td>
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<td>5'-CTCTTGCTCCCTCTGGATT-3' (antisense)</td>
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<td>PAX3-FOXO1A Distal #1 ChIP</td>
<td>5'-TCCTAGCCCAAGACTTCTGTCCGC-3' (sense)</td>
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<td>5'-TCAGCGTGGTCTCAGGAA-3' (antisense)</td>
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<td>PAX3-FOXO1A Distal #2 ChIP</td>
<td>5'-ATTCCCTGAGAACAAACGCT-3' (sense)</td>
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PAX3-FOXO1A Proximal ChIP

5'-GAGGCCTAACCCTCTCCAGTC-3' (sense)
5'-ACAGAGATTCCGATGGTGTT-3' (antisense)

β2-Microglobulin

5'-GCAATCACCTGTGGATGCTAA-3' (sense)
5'-TAATGGTTGAGTTGGACC-3' (antisense)

TATA Binding Protein

5'-GCCAGCTTCCGAGATTCTGATT (sense)
5'-CGGCCACGAAATGGCTTCTT-3' (antisense)

Nmyc

5'-GTATTAAACGAACGGGCG-3' (sense)
5'-AAGTCATCCTCCGAGGTA-3' (antisense)

Gremlin

5'-ATTTAAACCGGGAGACGCG-3' (sense)
5'-GGCCTCGCTTCTGAC-3' (antisense)

DAPK1

5'-GACTCGGCAACTCGAG-3' (sense)
5'-GTTCGGACGAGCACT-3' (antisense)

MyoD1

5'-CTTTTGCTATCTACAGCGG-3' (sense)
5'-GTCGTCATAGGAGTCGCG-3' (antisense)

RASSF4

5'-AGGATACGATATATGTAGGTGGTTTTGGATT-3' (sense)
5'-ATTATAACCGCTAAATTACTAAACAAAATA-3' (antisense)
### Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier 1</th>
<th>Supplier 2</th>
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<tbody>
<tr>
<td>β-actin</td>
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<tr>
<td>IL-24</td>
<td>Abcam (Cambridge, MA)</td>
<td>Abcam (Cambridge, MA)</td>
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<tr>
<td>PAX3</td>
<td>Abcam (Cambridge, MA)</td>
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<td>Sp1</td>
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<td>RASSF4</td>
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<td>RNA Polymerase II</td>
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<td>p/PERK</td>
<td>Biolegend (San Diego, CA)</td>
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<tr>
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### Oligonucleotides

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<tr>
<td></td>
<td>5'-UGAAUUCUGCAGUGAGAGTT-3' (antisense)</td>
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<td>IL-24</td>
<td>5'-GACTTATTCCACAGCAGCCT-3' (sense)</td>
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<tr>
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<td>5'-GTTGCCAGTTGGACACGAT-3' (antisense)</td>
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<td>siNR4A1</td>
<td>SASI_Hs02_00333289</td>
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<tr>
<td>PAX3 (-95) ChIP</td>
<td>5'- CAGTCTTTGACACATCAGCT-3' (sense)</td>
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<tr>
<td></td>
<td>5'- ACAGTATTCCACAGCAGAAG-3' (antisense)</td>
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<td>PAX3 (-472) ChIP</td>
<td>5'- GCATGTCAGGAAACACTCCT-3' (sense)</td>
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<td>5'- TTGCTCTAGTCACCACATCCT-3' (antisense)</td>
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<td>β2-Microglobulin</td>
<td>5'-GCAATCACCTGTGGATGCTAA-3' (sense)</td>
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<td>5'-TAAATGGTTGAGTTGGACCC-3' (antisense)</td>
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</table>
IL-24

5'-ATGAATTTTCAACAGAGGGCTG-3' (sense)
5'-GCAGAAATTCTACAAGCTCTGA-3' (antisense)