PAX3-FOXO1 Induces Cannabinoid Receptor 1 to Enhance Cell Invasion and Metastasis

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Abstract

Alveolar rhabdomyosarcoma (ARMS) is a muscle-derived childhood tumor characterized by production of oncogenic PAX3/7-FOXO1 chimeric transcription factors. While downstream targets of the PAX3-FOXO1 oncoprotein in ARMS have been defined, the functional relevance of these targets is unclear. Here, we show that upregulation of the cannabinoid receptor 1 (Cnr1/Cb1) by PAX3-FOXO1 in mouse primary myoblasts and ARMS cell lines, contributes to PAX3-FOXO1 phenotypes, both in vivo and in vitro. In primary myoblasts, Cnr1 was dispensable for PAX3-FOXO1 to mediate cell proliferation, differentiation, or transformation; however, Cnr1 function was essential to increase the invasive capacity conferred by PAX3-FOXO1 overexpression in these cells. Genetic or pharmacologic abrogation of Cnr1 inhibited the enhanced basement membrane invasion induced by PAX3-FOXO1. Cnr1 loss by either route also dramatically reduced lung metastasis formation. Taken together, our findings strongly implicate Cnr1 as a novel tractable target to inhibit ARMS invasion and metastasis. Cancer Res; 71(24): 7471–80. ©2011 AACR.

Introduction

Rhabdomyosarcoma is a pediatric soft tissue tumor derived from the skeletal muscle lineage that expresses many muscle-specific proteins (1). Most commonly, rhabdomyosarcoma presents in one of two histologic subtypes: embryonal rhabdomyosarcoma (ERMS) or alveolar rhabdomyosarcoma (ARMS; ref. 1). Recent clinical advances have improved the outcome for patients with both ARMS and ERMS; however, patients with the more aggressive and metastatic ARMS subtype have a poorer prognosis than patients with ERMS (2).

ARMS is histologically characterized by tumors containing small, round, densely packed cells usually surrounding alveolar-like structures reminiscent of the lung (1). More than 75% of ARMS tumors possess a t(2;13)(q35;q14) translocation or the variant t(1;13)(p36;q14) translocation which generate PAX3-FOXO1 or PAX7-FOXO1 fusion proteins, respectively (3–5). These fusion proteins contain the complete paired and homeobox-DNA-binding domains of the PAX3/7 protein and the transcriptional activation domain of FOXO1 (FKHR) which is more potent than that of PAX3/7 (4, 6). Among patients with ARMS, those possessing the PAX3-FOXO1 translocation have the poorest survival rates, particularly those with metastatic disease at diagnosis, which is the case in about a quarter of patients (1, 3).

ERMS and ARMS have distinct gene expression profiles. Recent microarray studies including those of Wachtel and colleagues (7), Davicioni and colleagues (8), and Lae and colleagues (9) have identified cannabinoid receptor 1 (CNR1/CB1) upregulation as a marker of ARMS tumors. Specifically, CNR1 is overexpressed in PAX3/7-FOXO1 fusion-positive ARMS tumors (7). Moreover, microarray analysis of an osteosarcoma cell line (U2-OS) expressing PAX3-FOXO1 showed increased CNR1 expression and chromatin immunoprecipitation (ChIP) analysis showed that PAX3 and PAX3-FOXO1 bind to upstream of the human CNR1 gene (10).

CNR1 is a G-protein–coupled receptor which is predominantly expressed in the brain but is also in the testes and skeletal muscle at much lower levels (11–13). The Ch1−/− mouse lacking the gene encoding Cnr1 was developed by Zimmer and colleagues (14). These mice appear healthy and fertile but show hypoactivity, hypoalgesia, and increased mortality. No skeletal muscle phenotype has been reported in these animals, although in rat L6 skeletal muscle myotubes, treatment with Cnr1 inverse agonist/antagonist rimonabant (SR141716A) increases glucose uptake (15), indicating a possible role for Cnr1 in skeletal muscle glucose metabolism. Cnr1 can exist in 3 states; active bound to ligand, inactive not bound to ligand, and in a ligand-independent constitutively active state. Therefore, Cnr1 is capable of inducing a biological signal in the absence of ligand (16). Cnr1 is capable of signaling via a variety of different cell signaling pathways including: G-proteins; adenyl cyclase, mitogen-activated protein kinase (MAPK; p38-MAPK, extracellular signal–regulated kinase (ERK), and c-jun-NH2-kinase (JNK)) and phosphoinositide 3-kinase (PI3K; for review see ref. 17). The effect that Cnr1 signaling induces likely depends on the cell context in which
it is expressed. Cnr1 expression outside the brain has been associated with cell migration and tumor growth (18–20).

Here, we show that Cnr1 is upregulated by the ARMS fusion protein PAX3-FOXO1. The PAX3-FOXO1 upregulation of Cnr1 is required for, but not sufficient to, increase the invasive capacity of primary myoblasts through ex vivo peritoneum basement membranes. This invasion capacity can be abrogated by treatment with Cnr1 inverse agonist AM251, but only partially by neutral agonist URB447, indicating that Cnr1 ligand-independent signaling is at least partially responsible for the enhanced invasive capacity. Moreover, in vivo assays show that treatment with the cannabinoid receptor inverse agonist AM251, or loss of the Cnr1 gene, can abrogate lung metastasis formation in an ARMS metastasis model. These results show that Cnr1 expression is an important player in ARMS tumor cell propensity for invasion and metastasis. Therefore, Cnr1 represents a potential novel therapeutic target to inhibit the metastatic capacity of this aggressive pediatric sarcoma.

Materials and Methods

Cell culture

Primary myoblasts were isolated from P1-P5 C57B6, Chb<sup>−/−</sup> (14), Ink4a-Arf<sup>−/−</sup> (21), Ink4a-Abf<sup>−/−</sup>/Chb<sup>−/−</sup>, or Abf<sup>−/−</sup> (22) mice as per the protocol of Rando and colleagues (23) and Bois and colleagues (24) and cultured in Ham’s F-10 (Invitrogen or HyClone) with 100 U/mL penicillin, 100 mg/mL streptomycin (P/S; Invitrogen), 1 ng/mL basic fibroblast growth factor (bFGF; PeproTech), 20% HyClone serum lot#: AHA7632 or cell generation serum lot#: 133F05 for in vivo studies, or 10% cosmic calf serum (HyClone) for in vitro studies and 10 mmol/L HEPES, on collagen (Becton Dickenson) or gelatin (Stem Cell Technologies) coated dishes at 37°C, 5% CO<sub>2</sub>, and 6% O<sub>2</sub>. Primary myoblasts were differentiated with Dulbecco’s Modified Eagle’s Medium (DMEM), 2 mmol/L glutamax (Invitrogen), 2% horse serum (HS; Invitrogen) and P/S (23). Rhabdomyosarcoma lines RD, JR1, RH6, RH2 (ERMS) and RH30, RH4, RH41, RH3, and RH28 (ARMS) authenticated by the expression of myogenic markers (data not shown) by Western blotting and ARMS lines expressing PAX3-FOXO1 (Fig. 1B) were obtained from Dr. Peter Houghton in 2005 (Nationwide Children’s Hospital, Columbus, OH) and were cultured in DMEM, glutamax, 10% FBS, and P/S.

Proliferation assays were conducted with 10,000 cells per well on collagen-coated 24-well plates (Becton Dickenson) and counted daily for 6 days. Triplicate anchorage-independent growth assays (25) were conducted with 20,000 cells in 0.3% agar noble over 0.6% agar in complete media. Colonies were grown over 2 weeks at 37°C, 5% CO<sub>2</sub>, and 6% O<sub>2</sub>. Plates were then photographed using the Geldoc System (Bio-Rad) and colonies counted with the Quantity One 4.6.5 Software (Bio-Rad). Apoptosis assays were conducted by plating 1,000 cells in 100 µL on a 96-well collagen-coated plate (Becton Dickenson). A media change was carried out 24 hours later to contain Met-F-AEA in absolute ethanol vehicle at the indicated concentrations. After a further 24 hours, 100 µL of Apo-ONE Homogeneous Caspase-3/7 Assay reagent (Promega) was added, and caspase activity was determined according to the manufacturer’s instructions.

Figure 1. Cnr1 is upregulated by PAX3-FOXO1 in ARMS. A, real-time PCR for relative Cnr1 mRNA expression, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in primary myoblasts transduced with empty vector (GFP), PAX3, and PAX3-FOXO1 (PF). B, Western blotting using anti-PAX3 antibody to detect PAX3-FOXO1, followed by real-time PCR for relative Cnr1 expression, normalized to GAPDH in the ERMS and ARMS cell lines. C, Cnr1 mRNA expression in human ARMS and ERMS tumor samples. D, the fusion status of the human rhabdomyosarcoma tumor samples as determined by PCR.
Cnr1 Enhances Invasive and Metastatic Activity in ARMS

Constructs

PAX3, PAX3-FOXO1, Cnr1 or ΔA17-Cnr1 (26). E7 or luc2 were cloned behind the long terminal repeat (LTR) in MSCV-IRE5-GFP or YFP, MSCV-SV40-Puro, or pBabe-SV40-Puro plasmids (Supplementary Fig. S1A). Retroviruses were produced using phoenix eco cells (27) and applied to primary myoblasts with 8 μg/mL polybrene for 2 hours. Cells were then fluorescence-activated cell sorted for fluorescent label or selected with 1.5 μg/mL puromycin.

PCR

RNA was isolated with TRIzol reagent (Invitrogen) as per the manufacturer’s instructions, treated with DNase1 (Invitrogen) followed by the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer’s protocol. Real-time PCR was carried out by TaqMan Universal PCR Master Mix (Applied Biosystems), 0.3 μmol/L of primers, and 0.2 μmol/L of probes as follows: mCnr1 forward (fwd) 5’-TTGATCATCCTCTGGAAGGCTACA-3’, mCnr1 reverse (rev) 5’-GGGTTCCACGCTGGATCA-3’, mCnr1 probe 5’-CCACGCAGTCTGCGC-3’ (FAM/MGB); hCnr1 fwd 5’-CCGCTTTCCGGAGCACTGGTAGTGA-3’, hCnr1 rev 5’-TCCCCCATGCTGTTATCCA-3’, hCnr1 probe 5’-CCTCTTTCCGGAGCCTGCGCA-3’. Results were normalized to Mouse GAPDH Endogenous Control (VIC/MGB Probe, Primer Premier; Applied Biosystems). Nonquantitative reverse transcriptase PCR (RT-PCR) was performed for ARMS PAX3-FOXO1 and PAX7-FOXO1 translocation genotyping was carried out as per Barr and colleagues (28).

Western blotting

Western blotting was carried out with the following antibodies: 1:1,000 rabbit anti-PAX3 (29), 1:1,000 rabbit anti-pan-actin (Cell Signaling), 1:1,000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Jackson Laboratories). Densitometry was carried out with Quantity One Software (Bio-Rad).

Ex vivo basement membrane invasion assay

The ex vivo basement membrane invasion assay was conducted as described (30). Brieﬂy the peritonea from 8- to 12-week-old C57B6 female mice were mounted over 6.5-mm Transwell inserts (Fisher). Peritoneum constructs were then treated with 1 mol/L ammonium hydroxide for 1 hour and washed extensively. A total of 5 × 10^5 INK4a-ARF/−/− primary myoblasts were then applied to at least duplicate constructs in media for 12 days. Cells were treated daily with 300 nmol/L AM251, 2 μmol/L URB497, or DMSO vehicle; and 10 μmol/L Met-F-AEX or absolute ethanol vehicle in media. Constructs were ﬂexed, cross-sectioned, and hematoxylin and eosin (H&E) stained. The average number (± SE) of invading cells per 400× microscopic ﬁeld was counted from 6 ﬁelds, and results were normalized to empty vector control.

In vivo lung metastasis assay

Mice were pretreated one day prior to tail vein injection and continued on treatment for a further 10 days with 3 mg/kg/d intraperitonealy of AM251 in 3.6% dimethyl sulfoxide (DMSO), saline with 1% Tween-80. A total of 1 × 10^6 ARB/−/− primary myoblasts (originally isolated in HyClone serum lot #: AHA7632) or 1 × 10^6 INK4a-ARF/−/− or INK4a-ARF/−/−/Chib/−/− primary myoblasts (originally isolated in cell generation serum lot #: 13F505) expressing E7-Puro (to abrogate the pRb pathway), PAX3-FOXO1-IRES-GFP and luc2-IRES-YFP in 100-μL PBS were injected into the tail vein of NOD/SCID (nonobese diabetic/severe combined immunodeﬁcient) IL2Rα−/− mice (31). Mice were imaged using IVIS Imaging Systems (Xenogen). Animals were injected intraperitoneally with 200 μL of 15 mg/mL α-luciferin and imaged for luc2 expression on days 1, 5, 7, and 10 after tail vein injection. The luminescence in the lung region was quantitated using Live Imaging 3.1 software (Xenogen).

Lungs were ﬁxed, embedded, and sectioned. Sections were dewaxed and antigen retrieved in boiling citrate buffer, pH 6 (Invitrogen), 15 minutes. Samples were treated with 3% H2O2 in methanol, the Avidin/Biotin Blocking Kit (Vector Laboratories), and Protein Block Serum-Free (Dako). Rabbit anti-GFP (1:4,000; Abcam) diluted in antibody diluent solution (Invitrogen) for 1 hour at room temperature and detected by 1:250 biotinylated anti-rabbit antibody (Vector Laboratories) and LSAB2 Streptavidin-HRP (Dako) followed by Liquid DAB+ Substrate Chromogen system (Dako). Slides were stained with Gill hematoxylin and permounted. Micrographs were taken at 40× magniﬁcation across the whole lung section, and the number of GFP/YFP-positive metastases per ﬁeld was counted.

For comparison of Chib/−/− versus Chib−/− primary myoblast lung metastasis formation, lungs were removed from mice at day 70, ﬁxed, embedded, and sectioned. GFP/YFP and H&E staining was conducted, and micrographs of whole lung sections were taken with a dissection microscope.

Results

Cnr1 expression is induced by the PAX3-FOXO1 oncogene

Given that CNR1 has been identiﬁed as being highly expressed in fusion-positive ARMS tumors (7–10), real-time PCR analysis was conducted to determine the relative expression of Cnr1 in primary mouse myoblasts expressing either PAX3 or PAX3-FOXO1. Cnr1 was increased 3-fold in PAX3 and 170-fold in PAX3-FOXO1 samples (Fig. 1A). To conﬁrm this, Cnr1 expression was compared between ERMS and ARMS cell lines. Cnr1 expression was upregulated between 180-fold in RH30 and 150,000-fold in RH2 (the highest Cnr1 expresser among ERMS lines; Fig. 1B). Furthermore, Cnr1 expression was upregulated 175- to 330-fold in 3/4 ARMS tumor sample without CNR1 upregulation, tested negative for PAX3/7-FOXO1 translocation gene products, indicating this, Cnr1 expression is induced by the PAX3-FOXO1 oncogene. Given that Cnr1 expression is induced by the PAX3-FOXO1 oncogene.

Cnr1 upregulation does not contribute to proliferation, differentiation, or transformation effects of PAX3-FOXO1

Primary myoblasts transduced with a vector expressing PAX3-FOXO1 proliferate faster than empty vector control.
PAX3 overexpression also resulted in an increase in proliferation rate greater than empty vector or PAX3-FOXO1 (Fig. 2A), likely due to differences in expression [Supplementary Fig. S1B (i)]. Expression in Cb1−/− primary myoblasts continued to result in an equivalent increase in proliferation rate (Fig. 2A and B).

PAX3-FOXO1 expression in primary myoblasts results in the inhibition of myogenic differentiation. As seen in Fig. 2C, Cb1−/− myoblasts were indistinguishable from wild-type primary myoblasts. In addition, Cnr1 overexpression [Supplementary Fig. S1B (ii)] did not affect myoblast differentiation in either wild-type or Cb1−/− myoblasts.
PAX3-FOXO1 expression alone is not sufficient to transform primary myoblasts, additional abrogation of the p53 and pRb pathways are required (33–35). Therefore, in transformation assays, we used INK4a-ARF−/− mouse myoblasts. To determine whether Cnr1 may play a role in the transformation capacity of PAX3-FOXO1, we transduced INK4a-ARF−/− or INK4a-ARF−/−/Cb1−/− myoblasts with empty vector, PAX3, PAX3-FOXO1, or Cnr1. PAX3-FOXO1 increased colony formation by these cells compared with empty vector controls [Fig. 2D (i) and (ii)]. PAX3 or Cnr1 overexpression did not enhance colony formation, and Cb1−/− cells were not significantly different from their Cb1+/+ counterparts.

Previously, it has been reported that treatment with the Cnr1 agonist Met-F-AEA can induce apoptosis in ARMS cell lines (32). Treatment with either 3 or 30 μmol/L Met-F-AEA for 24 hours failed to induce any increase in apoptosis over that

![Figure 2](https://cancerres.aacrjournals.org/content/canres/71/24/7474/F2.large.jpg)
seen in vehicle treated wild-type or \( Ch1^{-/-} \) primary myoblasts expressing empty vector, PAX3, PAX3-FOXO1, or CNR1 [Fig. 2E (i)]. In ARMS cell lines, we only observed a small increase in apoptosis with MET-F-AEA treatment in RH41 and only after 48 hours of treatment [Fig. 2E (ii)].

These results show that although CNR1 is grossly upregulated by PAX3-FOXO1 it does not play an appreciable role in enhanced proliferation, inhibited differentiation, or enhanced transformation seen with PAX3-FOXO1 expression in primary myoblasts.

Cnr1 is required, but not sufficient, for increased cell invasion driven by PAX3-FOXO1

Patients with ARMS tumors are more likely to present with metastatic disease than ERMS (36). Moreover, PAX3-FOXO1–positive ARMS tumors have an increased propensity for localized invasiveness (3). This increased invasive and metastatic capacity contribute to the poorer prognosis associated with ARMS (1–3). Because Cnr1 has been associated with cell migration and tumor invasiveness (19, 20, 37–39), we wished to determine whether Cnr1 played a role in this PAX3-FOXO1–dependent phenotype.

\( INK4a-ARF^{-/-} \) primary myoblasts were used in an \textit{in vitro} invasion assay using \textit{ex vivo} basement membranes from the peritoneum of mice. Those cells expressing PAX3-FOXO1 showed increased invasion capacity over vector-only controls (Fig. 3A). However, \( INK4a-ARF^{-/-}/Ch1^{-/-} \) primary myoblasts expressing PAX3-FOXO1 failed to invade as well as \( Ch1^{+/+} \) counterparts and were indistinguishable from empty vector (Fig. 3A). To further confirm this result, \( INK4a-ARF^{-/-} \)

![Figure 3. Cnr1 expression and activity is required, but not sufficient, for PAX3-FOXO1–induced ex vivo basement membrane invasive capacity. A, relative number of invading cells of \( Ch1^{+/+} \) and \( Ch1^{-/-} \) primary myoblasts expressing PAX3-FOXO1 (PF). B, relative number of invading cells of \( INK4a-ARF^{-/-} \) primary myoblasts treatment with vehicle (DMSO) or 300 nmol/L AM251. C, invasion assay of \( INK4a-ARF^{-/-} \) and \( INK4a-ARF^{-/-}/Ch1^{-/-} \) primary myoblasts transduced with empty vector (Puro) or PAX3-FOXO1 (PF) and empty vector (GFP), Cnr1 or \( \Delta 417 \) Cnr1. Invasion assay of primary \( INK4a-ARF^{-/-} \) myoblasts expressing empty vector (GFP) or PAX3-FOXO1 (PF) treated with 2 \( \mu \)mol/L URB447 in DMSO (D) and 10 \( \mu \)mol/L MET-F-AEA in absolute ethanol (E). Nonsignificant differences are indicated as NS. \(* P < 0.05, \quad ** P < 0.01, \quad *** P < 0.001. \)
primary myoblasts expressing PAX3-FOXO1 or empty vector were treated with the specific Cnr1 antagonist/inverse agonist AM251, which inhibits both ligand-dependent and constitutively active Cnr1 signaling. PAX3-FOXO1 showed enhanced invasion over cells expressing vehicle alone, but this increase was completely abrogated in the presence of AM251 (Fig. 3B). Cnr1 overexpression alone was insufficient to induce this increased invasive capacity in the absence of PAX3-FOXO1 (Fig. 3C). However, reintroduction of Cnr1 in InK4a-ARF<sup>-/-</sup>/Cb1<sup>–/–</sup> primary myoblasts expressing PAX3-FOXO1 restores the ability of these cells to invade. Expression of the Δ417 Cnr1 mutant (Supplementary Fig. S1C) lacking the distal C-terminal tail of Cnr1 which is involved in repressing constitutive activity (26), results in increased invasion in InK4a-ARF<sup>-/-</sup>/Cb1<sup>–/–</sup> primary myoblasts irrespective of PAX3-FOXO1 cotransduction (Fig. 3C).

To further investigate the mechanism by which Cnr1 induces an increase in invasion in PAX3-FOXO1 expressing myoblasts, we treated cells in the invasion assay with URB474, a Cnr1 neutral agonist which inhibits only ligand-dependent signaling, and MET-F-AEA a Cnr1 ligand agonist. Compared with AM251, which completely abrogates the enhanced invasive capacity of PAX3-FOXO1 expressing primary myoblasts, the neutral agonist URB474 only reduced invasion capacity slightly (Fig. 3D). Moreover, treatment with agonist Met-F-AEA failed to further enhance cell invasive capacity and actually significantly inhibited invasion (Fig. 3E).

**Pharmacologic or genetic abrogation of Cnr1 inhibits lung metastasis formation**

To determine whether an inverse agonist such as AM251 could prevent ARMS metastasis formation in vivo, we developed an ARMS lung metastasis model. Lung metastasis occurs in 25% of metastatic ARMS cases (3). Injection of 1 × 10<sup>6</sup> ARF<sup>–/–</sup> primary myoblast cells expressing the human papilloma virus protein E7 (to abrogate the pRb pathway), PAX3-FOXO1 and luc2 into the tail vein of <i>NOD/SCID</i> mice resulted in luc2 signal in the lungs within 24 hours of administration. Over time, these cells invade into lung tissue and develop metastatic lesions, if unattended, animals die within 4 weeks due to respiratory failure (data not shown). To determine whether a Cnr1 antagonist could be used to prevent metastasis formation, we treated mice with 3 mg/kg/d intraperitoneal AM251. After one day of AM251 treatment, mice were injected intravenously with PAX3-FOXO1 expressing cells and luc2 activity in the lung area was monitored for the next 10 days (Fig. 4A).

Twenty-four hours postinjection, there was no significant difference in lung localized luciferase activity between AM251 and vehicle-treated mice. After 5 days, luciferase activity from the lung was significantly reduced in AM251-treated animals and this reduction was maintained to 10 days (Fig. 4B). The lungs from mice were sectioned and stained for GFP/YFP expression to detect lung metastasis. AM251 treatment resulted in a significant decrease in the number of metastases compared with vehicle-only–treated animals (Fig. 4C). Representative staining is shown in Fig. 4D. Thus inhibition of Cnr1 by inverse agonist AM251 prevents ARMS lung metastasis formation in this animal model.

A similar experiment was carried out, this time treating with the Cnr1 neutral agonist URB447 (data not shown). No significant reduction of lung metastases was seen, concurring with in vitro data where Cnr1 constitutive activity was required for invasive capacity.

To verify that this was the specific effect of Cnr1 affecting lung metastasis formation, we injected InK4a-ARF<sup>–/-</sup> and InK4a-ARF<sup>–/-</sup>/Cb1<sup>–/–</sup> primary myoblasts expressing E7, PAX3-FOXO1, and luc2 into the tail vein of <i>NOD/SCID Il2rg<sup>null</sup></i> mice. We were unable to generate lung metastasis with InK4a-ARF<sup>–/-</sup> loss alone, thus E7 was added to further abrogate the pRb pathway (for review, see ref. 40). Though the latency of tumor formation was longer due to a difference in the serum used to initially isolate the primary myoblasts (Fig. 5A), we could see a significant difference in lung metastasis formation comparing mice of a Cb1<sup>–/–</sup> versus a Cb1<sup>–/–</sup> genotype (Fig. 5B and C). Gross appearance of lung sections is shown in Fig. 5C. Lung metastasis can be seen by H&E and anti-GFP staining. Numerous GFP-positive tumors could be detected in the lungs of mice injected with cells of the Cb1<sup>–/–</sup> genotype, whereas only a single GFP-positive tumor could be detected among the lung sections of all 5 mice injected with myoblasts with a Cb1<sup>–/–</sup> genotype.

**Discussion**

ARMS is a particularly aggressive form of rhabdomyosarcoma and is associated with an increased propensity for metastasis (2). ARMS tumors are typically characterized by the presence of a t(2;13)(q35;q14) chromosomal translocation or variant t(1:13)(p36q14) resulting in a PAX3-FOXO1 or PAX7-FOXO1 fusion transcription factor, respectively (3–5). We and others (7–10, 32) have identified Cnr1 as a target gene of PAX3/7-FOXO1. We have shown that PAX3-FOXO1 expression results in an increased capacity for primary myoblasts to invade through an ex vivo basement membrane. Moreover, we have shown that Cnr1, upregulated by PAX3-FOXO1, is necessary for this increased invasive capacity. Treatment with AM251, an inverse agonist of Cnr1, abrogates both cell invasion in vitro and lung metastasis formation in vivo. These results show that Cnr1 expression, downstream of PAX3-FOXO1, contributes to the aggressive behavior of fusion-positive ARMS.

In primary myoblasts, a cell likely to resemble the cell of origin for ARMS, absence of Cnr1 prevents PAX3-FOXO1–induced enhanced invasive capacity. Cnr1 expression has been associated with invasive capacity in several different tumor types. In breast cancer cells, a more invasive phenotype was associated with Cnr1 expression (41). Cnr1 agonists can induce the migration of HEK-293 and normal primary bone marrow cells (19, 39) and Cnr1 antagonist rimonabant can decrease vascular smooth muscle cell migration (20). Conversely, Cnr1 agonists inhibit leukocyte and colon carcinoma cell migration (38). Therefore, it is conceivable that Cnr1 expression in other tumor cell types could also lead to increased invasive capacity, meaning drugs targeting Cnr1 could also be useful to inhibit metastatic disease of other tumor types.
Cnr1 expression has also been linked with cell proliferation. In thyroid, breast, glioma, and prostate cancer cells, Cnr1 agonists inhibited proliferation which could be abrogated by antagonists (18, 41–43). Moreover, conflicting results have been obtained in colorectal cancer where Cnr1 agonists can induce apoptosis and inhibit growth, whereas antagonist treatment can either accelerate or prevent precancerous lesion formation, depending on the animal model used (37, 44, 45). However, we could find no contribution of Cnr1 to PAX3-FOXO1–induced myoblast growth. Cnr1 expression can have different effects depending on the cell context in which it is expressed. This is most likely due to the ability of Cnr1 to activate a number of different signaling pathways including; G-proteins, adenylyl cyclase, MAPK (p38-MAPK, ERK, and JNK), and PI3K (for review see ref. 17) which can induce numerous different effects in different cell types.

Recently, Oesch and colleagues (32) reported that treatment of ARMS cell lines, RH4 in particular, with Cnr1 agonists induced apoptosis. We did not find induction of apoptosis in RH4 with treatment of MET-F-AEA [Fig. 2E (ii)]. Of the 5 ARMS cell lines tested, only RH41 showed a small increase in apoptosis after 48 hours incubation with MET-F-AEA [Fig. 2E (ii)]. In addition, apoptosis was not induced in primary myoblasts by MET-F-AEA, irrespective of PAX3-FOXO1 or Cnr1 expression [Fig. 2E (i)], suggesting that this proapoptotic effect is not universal in ARMS. It is unknown whether Cnr1 agonists will prove useful clinically for the induction of apoptosis in ARMS. In our ex vivo basement membrane invasion assay, we found that Met-F-AEA did not further induce, and in fact slightly inhibited, invasion of primary INK4a-ARF−/− myoblasts expressing PAX3-FOXO1. Therefore, agonist treatment in vitro would be unlikely to enhance cell invasive capacity. So, if MET-F-AEA or other CNR1 agonists would prove useful to induce apoptosis in ARMS, treatment with such drugs seems unlikely to further enhance invasive and metastatic capacity.

ARMS tumors metastasize to the lung in about 25% of metastatic ARMS cases (3). Consistent with this, we successfully developed a model for lung metastases. Primary ARF−/− myoblasts expressing E7, PAX3-FOXO1, and luc2 injected into the tail vein preferentially form metastases in the lung tissue. ARMS tumors have a higher propensity for metastasis than ERMS (36), and PAX3-FOXO1–positive ARMS in particular are associated with higher local tumor invasiveness and a poorer prognosis if presenting with metastatic disease (3). We have shown that Cnr1 expression downstream of PAX3-FOXO1 expression increases ex vivo basement membrane invasion capacity in vitro, a characteristic that is abrogated in Cb1−/− myoblasts or by AM251 treatment. Only D417-Cnr1, a mutant with enhanced constitutive activity, is able to enhance invasion in the absence of PAX3-FOXO1, indicating PAX3-FOXO1 expression modulates the expression of other, as yet unidentified, proteins involved in the transmission of Cnr1 signal within myoblasts.

ERMS is also known to metastasize to the lung (1). Cnr1 is not upregulated in ERMS, indicating that Cnr1 is not required
for ERMS lung metastasis and this must, therefore, be driven by
an alternative mechanism. This indicates that Cnr1-driven
metastasis is a unique feature of fusion-positive ARMS. The
most common site of metastasis for PAX3-FOXO1-positive
ARMS is the bone marrow (3). We attempted to model ARMS
bone marrow metastasis, by injecting transformed primary
myoblasts into the left ventricle of the heart. This avoids
entrainment of the cells in the lung capillary system which
leads to selective lung metastasis formation via tail vein
injection. We did find metastasis in the bone marrow, but the
frequency was below 5% of metastasis found elsewhere in the
animals (muscle, lymph nodes, and lungs). We also attempted
carry out intracardiac injections of RH30 and RH4 cell lines
but were unable to obtain any bone marrow metastases with

Figure 5. Cb1−/− primary myoblasts show a reduction in lung
metastasis formation compared with Cb1−/− counterparts. A,
comparison of the change in ROI of luciferase activity from the lung
region of mice injected with INK4a-ARF−/− or INK4a-ARF−/−/Cb1−/−
primary myoblasts expressing E7, PAX3-FOXO1, and luc2. B, number of GFP-positive tumors per lung
section comparing mice injected with INK4a-ARF−/− or INK4a-
ARF−/−/Cb1−/− primary myoblasts expressing E7, PAX3-FOXO1, and luc2. Significant differences were
determined by the Mann–Whitney U test. C, lungs isolated from mice, which were injected with INK4a-
ARF−/− or INK4a-ARF−/−/Cb1−/− primary myoblasts expressing E7, PAX3-FOXO1, and luc2, were
sectioned and stained with either H&E or anti-GFP. Gross
appearance was photographed with a dissection microscope for
comparison of lung metastasis formation. *P < 0.05, **P < 0.01, ***P < 0.001.
these ARMS cell lines. After exploring multiple different approaches to this problem, we did not observe reliable formation of bone marrow metastases with any of the cell types used. Therefore, we were unable to determine whether Cnr1 plays a role in the propensity of ARMS to form bone marrow metastases.

*In vivo* inhibition of Cnr1 with AM251 or genetic deletion can abrogate lung metastasis formation. Because this invasive capacity was less affected by treatment with the neutral agonist URB447 and not enhanced by MET-F-AEA agonist treatment, increased invasive capacity is at least partially downstream of constitutive Cnr1 signaling. Therefore a limitation of Cnr1 antagonists for ARMS treatment is that they would have to be administered early enough in disease progression to prevent the invasion of circulating, potentially metastatic, tumor cells. Moreover, these drugs would not affect the growth of the primary tumor, or micrometastases that are already established, as Cnr1 does not influence PAX3-FOXO1–induced myoblast proliferation or transformation. Despite these limitations, the combination of Cnr1 antagonists with the current standard of care for ARMS could prove useful clinically to prevent further metastasis formation, potentially prolonging the life of the patient.

One could envision that inhibition of Cnr1 activity within ARMS cells could be useful therapeutically to reduce invasive and metastatic capacity of ARMS. Use of Cnr1 antagonist rimonabant in humans, however, is associated with undesirable side effects. In November 2008, the development of rimonabant, for weight loss and smoking cessation, was abandoned by Sanofi-Aventis. This was due to failure to achieve U.S. Food and Drug Administration (FDA) approval owing to concern over depressive psychiatric side effects (46). With respect to Cnr1 expression in ARMS tumor cells, given that brain metastases are rare in rhabdomyosarcoma (2.4% ref. 47), treatment with a Cnr1 antagonist that is unable to pass through the blood–brain barrier would likely be sufficient to play a therapeutic role. Unfortunately, the only commercially available peripherally restricted Cnr1/2 antagonist, URB447, proves ineffective in our model, because it acts only as neutral agonist to Cnr1 (48) and Cnr1 constitutive activity is at least partially responsible for PAX3-FOXO1–induced invasion. The development of a peripherally restricted Cnr1-specific inverse agonist would represent a potential novel therapy for the inhibition of metastasis formation in patients with ARMS.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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