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ORIGINAL ARTICLE

# Nephroblastoma overexpressed *(NOV/CCN3)* gene: a paired-domain-specific PAX3-FKHR transcription target that promotes survival and motility in alveolar rhabdomyosarcoma cells

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**The CCN (Cy61, CTGF and NOV) family of proteins is a group of matricellular biomolecules involved in both physiological and pathological processes. Elevated expression of the *CCN3* (also known as NOV, Nephroblastoma overexpressed) gene has been detected in clinical samples of the skeletal muscle cancer rhabdomyosarcoma, with the highest expression found in the alveolar subtype (aRMS). Over 80% of aRMSs are characterized by a chromosomal translocation-derived fusion transcription factor PAX3-FKHR. In this study, we linked elevated CCN3 levels in aRMS cells to PAX3-FKHR expression. We found reduced CCN3 levels in aRMS cells following small interfering RNA knockdown of PAX3-FKHR, and increased CCN3 levels in C2 myoblasts following ectopic expression of PAX3-FKHR. Promoter, electrophoretic mobility shift assay and chromatin immunoprecipitation analyses confirmed that the *CCN3* gene was a direct target for PAX3-FKHR transcriptional activation through a paired-domain DNA sequence in the first intron of the *CCN3* gene. To determine the function of CCN3, we showed that knockdown and ectopic expression of CCN3 decreased survival and increased differentiation in aRMS cells, respectively. In addition, we found that exogenously supplied CCN3 protein promoted aRMS cell adhesion, migration and Matrigel invasion. Taken together, data from this study have (1) provided a mechanistic basis for the CCN3 overexpression in aRMS cells, and (2) identified CCN3 as an autocrine/paracrine factor that contributes to the aggressive behavior of aRMS cells, perhaps through a positive feedback loop. Thus, CCN3 may be an attractive target for therapeutic intervention in aRMS.**
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## Introduction

Rhabdomyosarcoma (RMS) is the most common pediatric soft-tissue sarcoma of the muscle lineage. RMS is subdivided into two major histological groups: alveolar (aRMS) and embryonal (eRMS) (Arndt and Crist, 1999). The two RMS groups exhibit distinct genetic aberrations, a frequent loss of heterozygosity in chromosome 11 in eRMS (Yun, 1992; Zhan *et al*., 1994) and gain of chromosomal translocation involving chromosomes 2 and 13 (>80%) or chromosomes 1 and 13 (<15%) in aRMS (Turc-Carel *et al*., 1986; Wang-Wuu *et al*., 1988). Significant progress has been made in treating localized RMS, yet the 5-year survival rate of metastatic RMS remains poor (<30%) (Oberlin *et al*., 2008; Davicioni *et al*., 2009). Because aggressive tumor invasion and metastasis are most prevalent in translocation positive aRMS, a better understanding of the molecular consequences of translocations in aRMS pathogenesis is essential for developing more focused treatments.

The t(2;13) translocation breaks and rejoins DNA encoding two developmental transcription factors PAX3 and FKHR. The resulting oncogenic chimeric transcription factor PAX3-FKHR contains the functional PAX3 DNA-binding domain (DBD), the paired-box domain (PD) and paired-type homeodomain (HD) at the N-terminus, and the acidic FKHR activation domain (AD) at the C-terminus (Barr *et al*., 1993; Shapiro *et al*., 1993). The oncogenic properties of PAX3-FKHR have been established through *in vitro* transformation assays (Epstein *et al*., 1995; Bernasconi *et al*., 1996; Scheidler *et al*., 1996; Lam *et al*., 1999), and verified in animal studies (Anderson *et al*., 2001; Keller *et al*., 2004; Wang *et al*., 2005; Ren *et al*., 2008; Zhang *et al*., 2009). However, the molecular basis of PAX3-FKHR-dependent oncogenesis remains poorly understood.

Considerable effort has been made to identify direct targets of PAX3-FKHR, which may lead to novel therapeutics for aRMS tumors. Initial analysis showed stronger transactivation by PAX3-FKHR than by PAX3 (Fredericks *et al*., 1995). However, the comparative expression profiles of RMS subtypes and of cells engineered to express PAX3 or PAX3-FKHR identified non-overlapping gene sets associated with the fusion protein (Khan *et al*., 1999; Begum *et al*., 2005; Mercado *et al*., 2008). This suggests that the fusion event altered

the specificity in addition to the potency of PAX3-FKHR activity. Indeed, selective transactivation by PAX3-FKHR but not PAX3 has been detected for a handful of genes (Epstein *et al*., 1998; Begum *et al*., 2005; Zhang and Wang, 2007). PAX3 can only activate transcription when both DBDs act synergistically and cooperatively (Chalepakis *et al*., 1994; Chalepakis and Gruss, 1995; Underhill and Gros, 1997; Apuzzo *et al*., 2004). Although this specificity toward PAX3-dependent targets is conserved in PAX3-FKHR, the fusion protein can use either DBD independently to activate genes that are not PAX3 targets (Epstein *et al*., 1998; Zhang and Wang, 2007). Moreover, these PAX3-independent activities are directly linked to the oncogenic and tumorogenic properties of PAX3-FKHR (Zhang *et al*., 2009). In further pursuing these genes using preliminary microarray analysis (Zhang and Wang, unpublished data), we have identified CCN3 as a novel PAX3-FKHR-specific target.

The CCN (Cyr61/Connective tissue growth factor/Nephroblastoma-overexpressed) family is made up of six core members including Cyr61/CCN1, CTGF/CCN2, NOV/CCN3, WISP1/CCN4, WISP2/CCN5 and WISP3/CCN6 (Perbal, 2001b; Perbal, 2008b). CCN proteins are secreted, matrix-associated proteins that have important roles in regulating diverse biological functions, including proliferation, differentiation, angiogenesis, survival, adhesion and motility (Perbal, 2001a; Yeger and Perbal, 2007; Holbourn *et al*., 2008). Specific CCN receptors have yet to be identified; however, several studies have indicated CCN proteins bind to cell surface integrins and modulate integrin-mediated pathways (Lau and Lam, 1999; Leu *et al*., 2002; Leu *et al*., 2003; Lin *et al*., 2005; Vallacchi *et al*., 2008; Katsube *et al*., 2009).

*CCN3* is the first CCN gene whose expression and activity are linked to cancer. It was originally discovered as an integration site of myeloblastosis-associated virus in avian nephroblastoma (Joliot *et al*., 1992). A nuclear-localized truncated CCN3 protein has also been detected in certain human cancers (Perbal, 2009), and can repress a Gal4 reporter when fused with Gal4 DBD (Planque *et al*., 2006). Elevated CCN3 expression levels have been detected in human cancers, and the level of expression has been directly linked to tumor progression (Perbal, 2006). Overexpression of CCN3 inhibits proliferation of many tumor types such as glioma (Gupta *et al*., 2001) and Ewing's sarcoma (Benini *et al*., 2005), while promoting survival, adhesion, migration and/or invasion of the same tumor cells. Thus, dysregulated CCN3 expression has been linked to an increased risk of metastasis and to a poor clinical prognosis.

Muscle is the predominant mesodermal cell type expressing CCN3 in developing embryos. High levels of CCN3 expression are detected in fusing myoblasts and in terminally differentiated myotubes (Natarajan *et al*., 2000). Ectopic expression of CCN3 in proliferating myoblasts blocks myogenesis, indicating temporal-spatial differences in CCN3 activity in muscles (Calhabeu *et al*., 2006). RMS shows elevated levels of CCN3 transcript and protein, with more uniform expression detected in aRMS (Manara *et al*., 2002). Currently, the role of CCN3 in RMS pathogenesis and how CCN3 expression is regulated in RMS cells have not yet been investigated. In this study, we establish the *CCN3* gene as a Pax3-independent target of PAX3-FKHR. Additionally, we demonstrate that CCN3 has multiple effects on human aRMS cells including altered survival, differentiation, adhesion and motility.

## Results

### *PAX3-FKHR regulation of CCN3 expression in muscle and aRMS cells*

We investigated whether PAX3-FKHR regulated CCN3 expression using the following two approaches: (1) ectopic expression of PAX3-FKHR in non-transformed C2 myoblasts (Figure 1a), and (2) small interfering RNA (siRNA) knockdown of PAX3-FKHR in aRMS cells (Figure 1b). Because CCN3 is principally a secreted protein, we measured CCN3 levels from the culture media. As shown in Figure 1a, CCN3 protein was absent in proliferating C2 cultures (GM) but was markedly increased in differentiated C2 cultures (DM). This finding is in agreement with the reports of high CCN3 levels in differentiated myotubes and mature skeletal muscle (Natarajan *et al*., 2000). When PAX3-FKHR was introduced into C2 cells, we detected a marked increase of CCN3 expression in proliferating C2 cells (GM). As expected, PAX3-FKHR-positive aRMS cells (RH4, RH30) expressed CCN3. To further explore this regulatory link in aRMS cells, we knocked down PAX3-FKHR using a siRNA (si-PF) specific for PAX3-FKHR (Figure 1b). Si-PF treatment of RH4 cells substantially reduced PAX3-FKHR levels, and was accompanied by a corresponding decrease in secreted CCN3. Similar results were obtained with other PAX3-FKHR-positive aRMS cell lines including RH28 and RH30 (data not shown). It should be noted that we only detected full-length CCN3 in these studies. We did not observe truncated forms of CCN3 that have been found in other tumors. Knockdown of PAX3-FKHR did not alter CCN2, another CCN member known to be expressed in aRMS cells (Croci *et al*., 2004; Croci *et al*., 2007). Taken together, these gain and loss of expression experiments indicated that PAX3-FKHR was an upstream regulator of the *CCN3* gene in myoblasts and in aRMS cells.

We next used a tet-inducible PAX3-FKHR expression system in C2 cells to explore the temporal relationship between PAX3-FKHR and CCN3 expression. We found that CCN3 expression, both at the protein (Figure 2a) and mRNA (Figure 2b) level, closely followed the induction of PAX3-FKHR. Interestingly, the induction of CCN3 by PAX3-FKHR was restricted to myogenic cells such as C2 and RD (PAX3-FKHR-negative eRMS line), and was absent in non-myogenic NIH3T3 fibroblasts (Figure 2c). Although PAX3 and PAX3-FKHR have the same DBDs, forced expression of PAX3 at a level several fold higher than

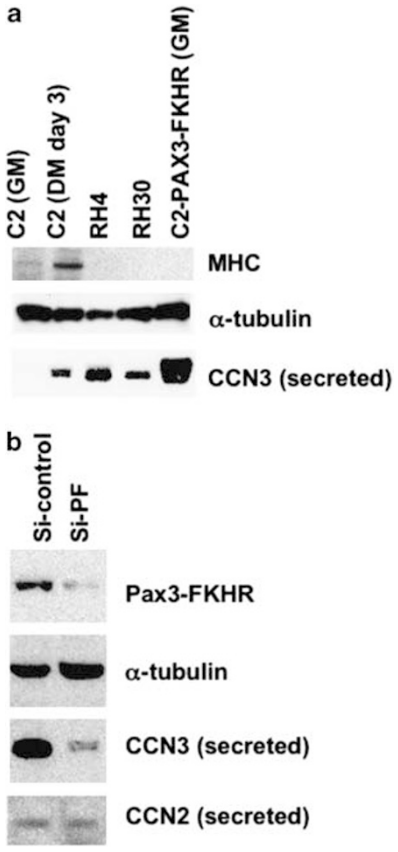


**Figure 1** PAX3-FKHR regulates *CCN3* gene expression in muscle cells. (**a**) Detection of secreted CCN3 protein in proliferating (GM) and differentiated (DM) C2 myoblast cells, aRMS (RH4, RH30) cells, and C2 cells stably expressing PAX3-FKHR (GM). For C2 cells, proliferating cell media (GM) were collected 24 h after plating, and differentiated cell media (DM) were collected 24 h after 2-days of differentiation. For aRMS cells, proliferating cell media were collected 72 h after plating without media change during this period. The results from C2 samples cannot be directly compared with the aRMS samples because of differing collection times. RIPA extract was prepared from cells for myosin heavy chain (MHC) and α-tubulin detection. (**b**) siRNA-knockdown of PAX3-FKHR reduced CCN3, but not CCN2 protein expression in RH4 cells. Transfected cells were supplemented with new media 24 h after transfection. Culture medium was collected 48 h later for CCN3 and CCN2 detection by western blot. RIPA extract was prepared from cells for PAX3-FKHR and α-tubulin detection. (**a**, **b**) Heparin-agarose samples were normalized as described in Materials and methods.

PAX3-FKHR failed to induce CCN3 expression in both non-myogenic and myogenic cells (Figures 2b and c). These observations raised the possibility that PAX3-FKHR might directly activate *CCN3* gene transcription.

### *Induction of CCN3 transcription by PAX3-FKHR involved a PD-dependent transactivation of an intronic response element of the* CCN3 *gene*

To investigate whether PAX3-FKHR directly controlled *CCN3* gene transcription, we generated several CAT reporter constructs driven by the CCN3 promoter (Figure 3a). The longest construct contained the −947–+336 region of the *CCN3* gene. The region from +1 (transcription start) to +336 contained the first exon and intron. When we compared the un-stimulated promoter activities in RD and RH4 cells, we found all constructs showed equal activity in RD cells, whereas constructs containing the exon/intron region (+47–+336) were most active in RH4 cells (Figure 3b). This suggested that the +47–+336 region might be responsive to PAX3-FKHR. To test this, we evaluated the effect of PAX3-FKHR on these promoter constructs in C2 cells. As shown in Figure 3c, PAX3-FKHR could indeed transactivate reporter constructs containing the +47/+336 region of the *CCN3* gene, thus supporting the existence of a PAX3-FKHR response element in this region.

A previous study in the Soas2 tumor cell line identified a p53 response element within the first exon of *CCN3* gene (Bohlig *et al*., 2008). As shown in Figure 3d, we did not detect transactivation of the CCN3 promoter by p53 in RH4 cells. Rather, we found that p53 antagonized the ability of PAX3-FKHR to transactivate the CCN3 promoter. Furthermore, forced p53 expression reduced endogenous CCN3 protein expression in RH4 cells (Figure 3e). The negative impact of p53 on PAX3-FKHR-dependent CCN3 promoter activation was also observed in C2 and other aRMS cell lines, for example, RH28, RH30 (data not shown). These results indicated that the p53 effect on CCN3 expression was cell type-dependent, and might provide a partial explanation as to why p53 null-RH4 cells expressed higher levels of CCN3 than the p53-positive RH28 and RH30 cells.

The +47/+336 region of the *CCN3* gene harbored a composite sequence containing PD (CACGCTT) and HD (ATTA) binding sites (Figure 4a). However, as PAX3 could neither induce CCN3 expression (Figure 2d) nor the CCN3 promoter (Figure 4b), we wondered if either or both of these sites were involved in PAX3-FKHR activation of the CCN3 promoter. We mutated the PD (nM2) or HD (nM1) binding site in the −143/336 CAT construct (Figure 4a). As shown in Figure 4c, PAX3-FKHR transactivated the HD mutated promoter (nM1) promoter to a similar extent as the wild-type (nWT) promoter; however, PAX3-FKHR failed to transactivate the PD mutated promoter (nM2). The requirement for the PD-binding site for PAX3-FKHR transactivation was similarly observed when oligonucleotides containing two copies of the wild type and mutant sequences were cloned upstream of a heterologous basal TK promoter-CAT reporter construct (Figure 4d). The PD-target site specificity was further confirmed when we tested activity of PAX3-FKHR with mutations to its two DBDs . We found that only the wild type and HD mutant (HD), but not the PD mutant (Bu35), of PAX3-FKHR was able to transactivate the CCN3 promoter (Figure 4e).

### *PAX3-FKHR directly participated in regulating* CCN3 *gene transcription*

We next investigated whether PAX3-FKHR interacted directly with the *CCN3* gene by performing *in vitro* electrophoretic mobility shift assay (EMSA, Figure 5a) and *in vivo* (chromatin immunoprecipitation, Figure 5b) DNA-protein binding assays. In EMSA analysis, purified

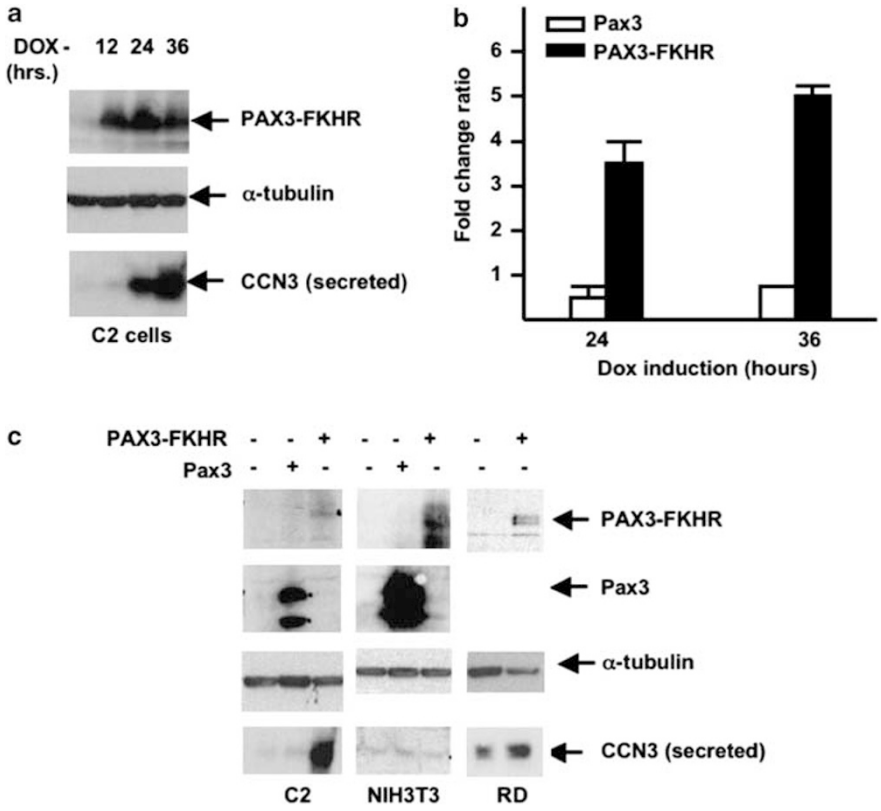


**Figure 2** Temporal activation of CCN3 protein (**a**) and mRNA (**b**) expression following PAX3-FKHR induction in C2 myoblast cells. Total RNA was collected from DOX-induced C2 cells expressing vector or vector containing PAX3 or PAX3-FKHR cDNA. Levels of CCN3 transcript were determined by quantitative real time RT–PCR. GAPDH was used for normalization of the RT samples. Fold change ratio compared GAPDH-normalized CCN3 mRNA level from PAX3 or PAX3-FKHR-expressing cells to that from vector expressing cells, and was calculated using the equation 2 to the power -delta delta Ct. (**c**) CCN3 induction by PAX3-FKHR was specific to myogenic cells. C2, NIH3T3 and RD eRMS cells were transiently transfected with vector or vector expressing PAX3-FKHR. RIPA extract and culture media were harvested 48-h post-transfection for detection of PAX3-FKHR and CCN3 proteins, respectively. PAX3 expression vector was tested in parallel in C2 and NIH3T3 cells under the same conditions. Heparin-agarose samples were normalized as described in Materials and methods.

PAX3-FKHR protein bound specifically to nWT and nM1 DNA probes, but not to the nM2 DNA probe (Figure 5a, left panel). Nuclear extracts prepared from PAX3-FKHR-expressing C2 cells formed a specific DNA–protein complex with the nWT, but not with the nM2 DNA probe (Figure 5a, right panel). Inclusion of PAX3-FKHR antibody in the binding reaction resulted in a super-shift of the DNA–protein complex, confirming the presence of PAX3-FKHR in the complex. Chromatin immunoprecipitation assays were performed on chromatin isolated from C2 cells expressing either PAX3 or PAX3-FKHR. The results showed that PAX3-FKHR was bound to the endogenous *CCN3* gene in the region containing the PD-binding site; PAX3 binding to this same region was not detected (Figure 5b).

*Both N- and C-terminal regions of PAX3-FKHR were needed to support PAX3-FKHR-regulated CCN3 transcription*

Finally, we investigated the contribution of the non-DBD regions of PAX3-FKHR to the activation of the CCN3 promoter. The regions examined included the PAX3 repressor domain (R), the FKHR AD and the FKHR linker sequence located between the PAX3 DBD and the FKHR AD. We tested a series of PAX3-FKHR deletion and replacement constructs (Figure 6a) in their ability to transactivate the CCN3 and myogenin promoters. We have previously reported that the myogenin promoter is activated by PAX3-FKHR through PD-specific binding (Zhang and Wang, 2007). We found that the FKHR AD was critical for CCN3 transactivation by PAX3-FKHR (Figure 6b). The highly acidic AD from VP16 could not substitute for the FKHR AD. Deletion of the FKHR linker region (del 23) had no effect on CCN3 transactivation. By contrast, the FKHR AD itself was not required for transactivation of the myogenin promoter, as replacement with either the PAX3 or VP16 AD had little effect on myogenin transactivation by PAX3-FKHR (Figure 6c). However, the FKHR linker region was critical for myogenin transactivation. Unlike the varying requirements for FKHR regions, the R domain of the PAX3 portion was equally required for the activation of both CCN3 and myogenin promoters by PAX3-FKHR (Figures 6d and e).

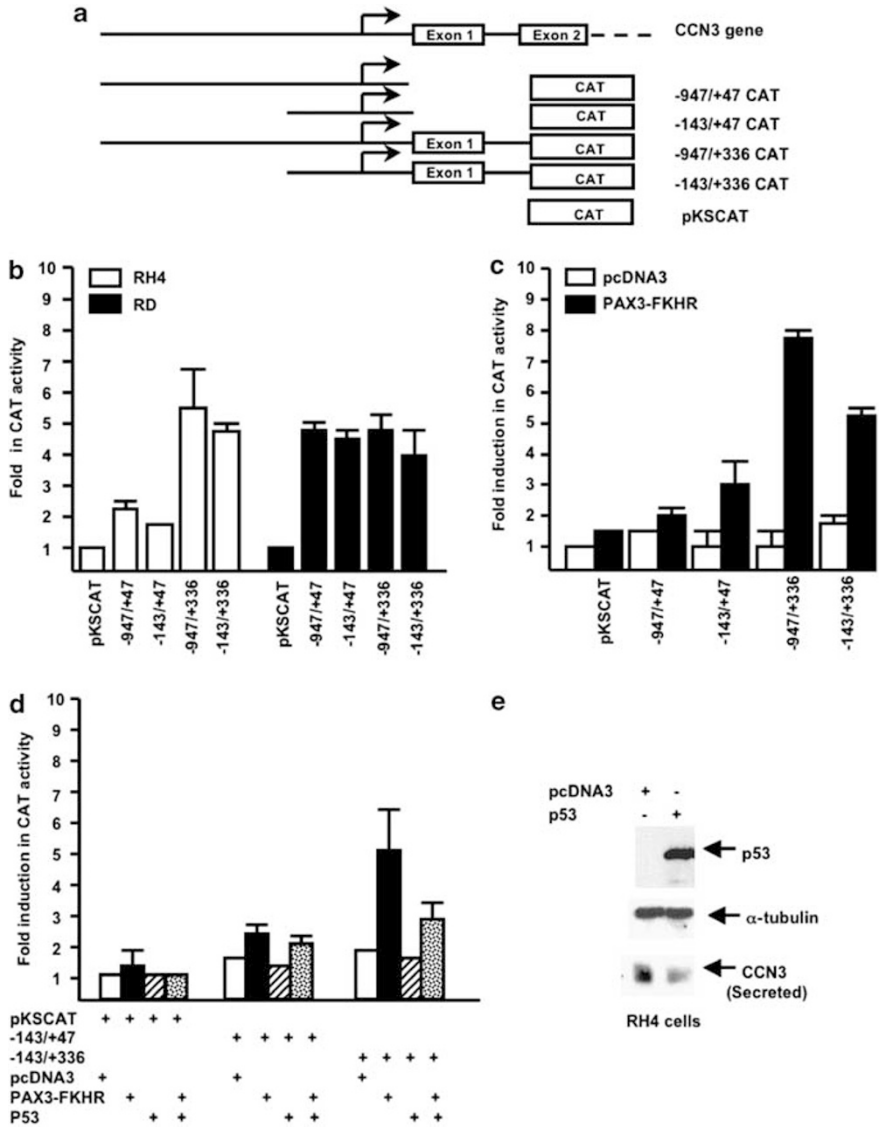


**Figure 3** (**a**) Diagrammatic illustration of the CCN3 promoter-driven CAT constructs. (**b**) Assessment of CCN3 promoter activity in RD eRMS and RH4 aRMS cells. Cells were transiently transfected with a total of 2 μg DNA containing 1.5 μg CAT DNA and 0.5 μg of CMV-lacZ DNA using lipofectamine. (**c**) PAX3-FKHR expression increased CCN3 promoter-CAT activity in C2 cells. C2 cells were transiently transfected with a total of 10 μg DNA containing 5 μg CAT DNA, 2 μg of CMV-lacZ and 1 μg plasmid pcDNA3 or pcDMA3-PAX3-FKHR using CaPO4-DNA. (**d**) p53 expression blocked PAX3-FKHR induction of CCN3 promoter. C2 cells were transiently transfected using lipofectamine with a total of 2 μg DNA containing 1 μg CAT DNA, 0.5 μg of CMV-lacZ DNA and 0.1 μg expression vector (pcDNA3, pcDNA3-PAX3-FKHR or pcDNA3-p53). (**e**) Transient expression of p53 reduced endogenous CCN3 expression in RH4 cells. Cells and culture media were collected 48-h post-transfection and analyzed for cellular p53 and secreted CCN3 content, respectively. Heparin-agarose samples were normalized as described in Materials and methods. (**b**–**d**): Lysates were prepared 48-h post-transfection for lacZ and CAT assays as described in Materials and methods. Fold in CAT activity (**b**) was presented as the ratio of CCN3 promoter-CAT activity to that of pKSCAT promoterless-CAT activity. pKSCAT activity was assigned an arbitrary value of 1 under each condition. Fold induction in CAT activity (**c**, **d**) was presented as the ratio of CCN3 promoter-CAT activity to that of pKSCAT promoterless-CAT activity co-transfected with the empty expression vector, pcDNA3, which was assigned an arbitrary value of 1. Results based on a minimum of three experiments.

### *CCN3 protein promotes aRMS cell survival and differentiation*

We used gain- (ectopic expression) and loss- (siRNA knockdown) of function approaches to assess the effects of CCN3 on aRMS cell behavior under different culture conditions. Loss of CCN3 expression only had a profound effect on RH4 cell proliferation under differentiation condition (Figure 7a, left panel). The reduced growth effect was resulted from a decrease in cell viability (Figure 7a, right panel). Similar results were obtained in another aRMS cell line, RH28 (Supplementary material). By contrast, the viability of these cells was not affected by overexpression of CCN3 protein under either culture condition (Figure 7b). On the other

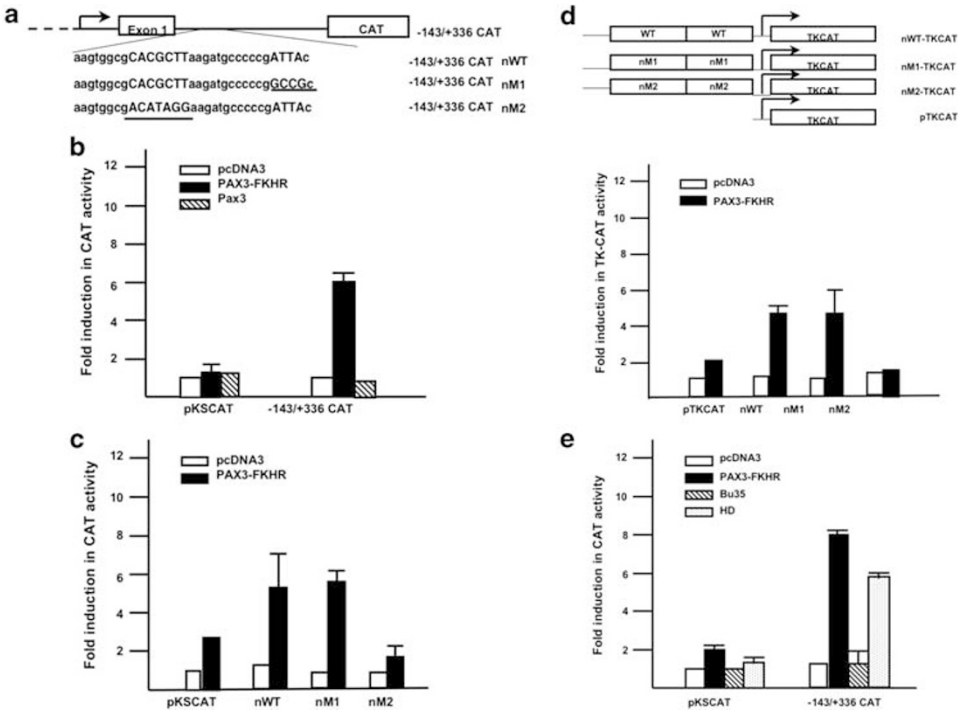


**Figure 4** (**a**) Schematic of a putative PAX3-FKHR response element located in the first intron of *CCN3* gene. The wild-type (nWT) element contained a composite PD (CACGCTT) and HD (ATTA) recognition site; mutations of the PD (nM2) and HD (nM1) recognition sites are shown. (**b**) CCN3-promoter CAT construct was selectively transactivated by PAX3-FKHR, but not by PAX3. (**c**) PAX3-FKHR activation required PD, but not HD recognition sequences, in the CCN3 promoter. (**d**) The CCN3 PD enhancer element was transactivated by PAX3-FKHR when linked to the heterologous TK promoter. Top panel: schematic representation of TKCAT constructs containing two copies of nWT, nM1 and nM2. (**e**) PAX3-FKHR transactivation of the CCN3 promoter requires an intact PD DBD, but not the HD DBD. (**b**–**e**) Transfection experiments were performed in C2 cells. Fold induction in CAT activity was defined as the level of CCN3 promoter-CAT activity over that of pKSCAT promoterless construct co-transfected with the empty expression vector, pcDNA3 (**b**, **c**) or pTKCAT basal promoter construct co-transfected with the empty expression vector, pcDNA3 (**d**, **e**). The pKSCAT or pTKCAT activity was given an arbitrary value of 1. Values based on a minimum of three experiments.

hand, excessive CCN3 expression promoted myogenic differentiation in aRMS cells as evidenced by increases in muscle-specific markers such as myosin heavy chain (MHC) and troponin-T (Figure 7c, left panel), and by increases in the number of MHC-stained myotubes in RH4 cells (Figure 7c, right panel). Gain of CCN3 expression did not alter cell proliferation under either growth condition (data not shown).

*CCN3 protein enhanced aRMS cell adhesion and motility*

Tumor formation and metastasis are facilitated through interaction between tumor cells and the extracellular matrix. To investigate whether CCN3 protein could act as an adhesive substrate for aRMS cells, we measured RH4 binding to immunological plates coated with various protein substrates. As shown in Figure 8a, RH4 cells adhered most avidly to wells coated with CCN3 than to wells coated with collagen, fibronectin or vitronectin. In fact, attachment and spreading of RH4 cells to CCN3-coated wells was seen within 1 h of plating, whereas the same level of RH4 attachment and spreading to the other protein substrates required over 2 h. Because CCN3 has been reported to interact with integrins, we examined the expression pattern of the integrins known to be associated with CCN3 function. We found that RH4, RH28 and RH30 cells expressed primarily integrin β1 and β5 whereas RD cells expressed primarily integrin β3 (Figure 8b, inset). Subsequently, we showed that integrin β1 and β5 were involved in CCN3-induced aRMS cell adhesion. Inclusion of blocking antibodies against these proteins inhibited RH4 cell attachment to the CCN3-coated plate (Figure 8b). Next, we tested how CCN3 affected aRMS cell motility using the scratch wound assay. We showed that RH4 cells exhibited increased wound closure in the presence of CCN3, suggesting CCN3 promoted cellular migration of these cells (Figure 8c). Additional evidence that CCN3 had a role in cell motility, was obtained through detection of increased number of RH4 cells migrating through a Matrigel reconstituted basement membrane (Figure 8d). These results point to a possible role of CCN3 in the invasive and metastatic properties that are prominent in aRMS tumors.

## Discussion

Modified extracellular matrix architecture is integrally linked to tumorogenesis. CCN family members are extracellular matrix-associated proteins with demon-

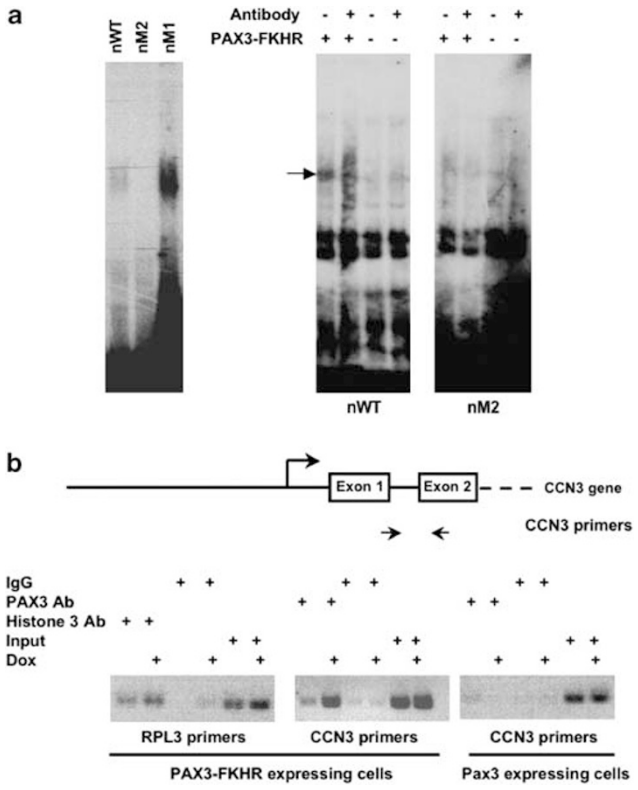


**Figure 5** (**a**) EMSA detection of direct binding of purified GST-PAX3-FKHR fusion protein (left panel) and nuclear extract from PAX3-FKHR-expressing C2 cells (right panel) to wild-type (nWT) and mutant (nM1 and nM2) DNA probes. Arrow indicates migration of PAX3-FKHR-DNA complexes. Presence of PAX3-FKHR in the bound protein complexes was detected by antibody super-shift. (**b**) Chromatin immunoprecipitation analysis detected specific *in vivo* interaction between PAX3-FKHR and *CCN3* gene. Chromatin was prepared from C2 cells expressing PAX3-FKHR (left and middle panels) or Pax3 (right panel) as described in Materials and methods, and subjected to IP using an antibody that recognizes both Pax3 and PAX3-FKHR. Histone H3 and normal rabbit IgG antibodies (provided by SimpleChIP enzymatic chromatin IP kit) were used in separate reactions as positive (RPL3, ribosomal protein L3) and negative controls for the chromatin immunoprecipitation assay, respectively. Input represented 10% of the chromatin preparation before IP. Opposing arrows indicate the locations of the CCN3 promoter primer sets. Expected sizes for RPL3 and CCN3 PCR products were 161 and 191 bp, respectively.

strated roles in various physiological processes. Changes in one or more of these activities are known to directly contribute to cancer development. The present study focuses on CCN3 expression in aRMS, an aggressive malignancy of skeletal muscle in the pediatric population. High levels of CCN3 transcript and protein are detected in aRMS cell lines and clinical samples. In this study, we provide evidence directly linking CCN3 expression to the aRMS-specific PAX3-FKHR fusion transcription factor and to aRMS tumor cell properties.

The pathways involved in PAX3-FKHR-induced tumorigenesis have only recently begun to emerge. We focused on identifying PAX3-FKHR-specific targets, as these are most likely to be factors central to the development and maintenance of aRMS tumors. Our previous and current studies have revealed a major distinction between the transcriptional targets of PAX3 and PAX3-FKHR. Although the PD and HD DBDs of PAX3, either as individual entities or in the context of the full-length protein, can bind their cognate DNA sites independently of one another *in vitro* (Underhill and Gros, 1997; Apuzzo *et al*., 2004), it is generally accepted that PAX3 transcriptional activation requires the simultaneous engagement of both DBDs *in vivo*. Those studies have identified a canonical PAX3 responsive element as a composite consensus sequence of $ATTAN_{1-13}CT(G/T)(A/C)(C/T)$, where ATTA is a HD-binding site and GT(G/T)(A/C)(C/T) is a PD-binding site (Underhill and Gros, 1997; Phelan and Loeken, 1998). Such composite sites have been identified in promoters of target genes regulated by PAX3 such as c-met (Epstein *et al*., 1996) and glut4 (Armoni *et al*., 2002). PAX3-FKHR recognizes and activates these promoters, but does so with greater efficacy than PAX3. Despite having the same DBDs, PAX3-FKHR does not always require simultaneous binding of both PD and HD DBDs. We have identified several PAX3-FKHR-specific targets that contain either a HD or PD binding site but not both. For example, PAX3-FKHR binds and transactivates a palindromic HD (5-TAAT $N_3$ATTA-3)-binding site in the PDGFRA promoter (Epstein *et al*., 1998), and PD (5-AACGCCT-3 and 5-CACGCTT-3)-binding sites in the myogenin (Zhang and Wang, 2007) and CCN3 promoters, respectively.

The PD-binding sequences (C/A/T)ACGC(C/A/T)T identified in myogenin and CCN3 promoters are particularly interesting, because they differ from the high-affinity Pax3 canonical PD-binding sites GT(G/T)(A/C)(C/T). The PAX3-FKHR consensus PD sequence closely matches a PD consensus identified through *in vitro* selection and amplification of random oligo nucleotides experiments, which was recognized by a diverse group of paired-domain-containing proteins (Epstein *et al*., 1994; Xu *et al*., 1995). For simplicity, we will refer to this sequence as the non-canonical PAX3 PD site. Thus far, such sequence has not been found in the promoters of PAX3-regulated genes, suggesting it may not be a functional PAX3 site *in vivo*. This notion is reinforced in this study. Although purified GST-PAX3 protein can bind to the nWT DNA probe by EMSA (data not shown), we do not detect PAX3 binding to this sequence *in vivo* (Figure 5b). We postulate that the PD in the PAX3 DBD has an intrinsic capability to differentiate the canonical and non-canonical PD-binding sequences *in vivo*. The specificity is likely to be controlled by its contact with other domains of the intact protein. The canonical site requires the proximal interactions between PD and HD as exemplified by PAX3; the non-canonical site requires PD autonomy as exemplified by PAX3-FKHR. The finding that PD targets of PAX3-FKHR have recognition sequences that are distinct from the PAX3 sequences may be useful in screening for PAX3-FKHR direct targets by searching DNA databases, as well as promoters of genes identified from microarray studies.

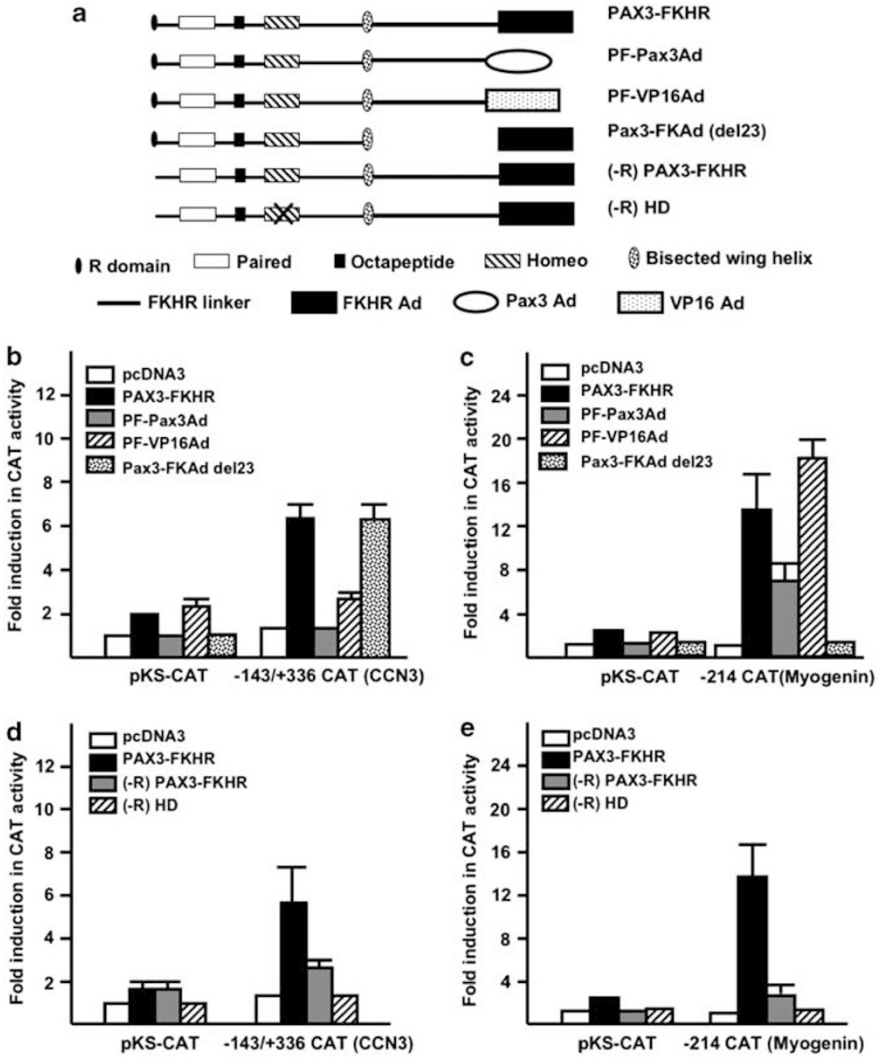


**Figure 6** PAX3-FKHR-dependent transactivation of CCN3 required both the N-terminal repressor R- domain and the C-terminal FKHR AD. (**a**) Schematic representation of PAX3-FKHR mutants with modified R and AD domains. (**b**, **c**) The effect of wild type and AD replacement mutants of PAX3-FKHR on the CCN3 promoter (−143/+336)-CAT activity (**b**) and on the myogenin promoter (−214)-CAT (**c**) constructs. (**d**, **e**) The effect of R domain deletion on wild type and HD-mutant PAX3-FKHR transactivation of the CCN3 promoter (−143/+336)-CAT (**d**) and of the myogenin promoter (−214)-CAT (**e**) constructs. (**b**–**e**) C2 cells were transiently transfected with a total of 10 μg DNA containing 5 μg CAT DNA, 2 μg of CMV-lacZ and 1 μg pcDNA3 or plasmid pcDNA3-PAX3-FKHR using CaPO4-DNA. Lysates were prepared 48-h post-transfection for lacZ and CAT assays, as described in Materials and methods. Fold induction in CAT activity was defined as the level of CCN3 promoter-CAT activity over that of pKSCAT promoterless construct, which was assigned a value of 1. Results based on a minimum of three experiments.

The DBD specificity of PAX3-FKHR also highlights the importance of intramolecular interactions between different domains in determining transcriptional activation. This is clearly demonstrated by the structural influence of the FKHR portion in activating PAX3-FKHR-specific targets. This is a complexity that is not often associated with transcription factors, where swapping of modular domains, that is, DBD and AD, has little effect on target gene specificity. The PAX3-independent activity of PAX3-FKHR requires specific intramolecular interactions that vary depending on the target promoters. In the case of HD-dependent promoters, the major structural requirement is the removal of the PAX3 AD that inhibits HD autonomy (Epstein *et al*., 1998). In the case of PD-dependent promoters, a concerted effort from both PAX3 and FKHR domains is required. The regions of FKHR involved vary depending on the target gene; the FKHR linker sequence is required for myogenin and the FKHR AD is required for CCN3. Unlike HD-dependent action where other ADs (for example, VP16) can substitute for the FKHR AD (Cao and Wang, 2000), the activation of CCN3 specifically requires the FKHR AD. This suggests the critical domain lies within either a unique sequence or configuration of the FKHR AD. The particular region within the FKHR protein needed might be related to conformational changes in response to sequence variations surrounding the PD site in target

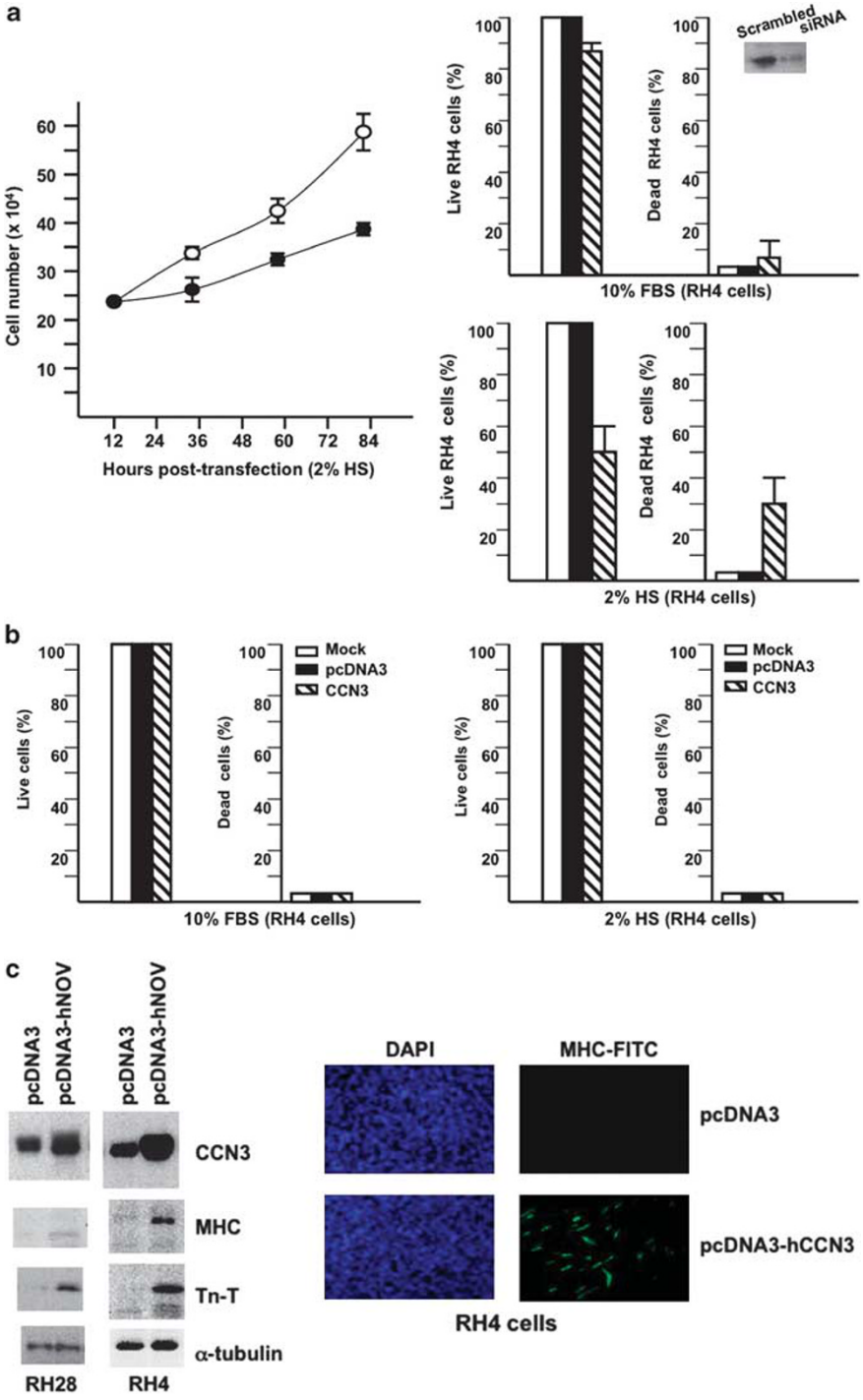


**Figure 7** CCN3 on aRMS cell viability and differentiation. (**a**) Effect of CCN3 knockdown on RH4 cell proliferation (left panel; open circle: scrambled siRNA; closed circle: CCN3 siRNA) and cell viability (right panel; open bar: mock-transfected; solid bars: scrambled siRNA; striped bars: CCN3 siRNA). (**b**) Effect of CCN3 overexpression on RH4 cell viability when grown in proliferation (10% FBS, left) or differentiation (2% HS, right) media. (**a**, **b**) For proliferation assay, cells were transfected with scrambled or CCN3 siRNA and later re-plated at $2 \times 10^5$ cells per well in 24-well culture plate in the presence of differentiation. Proliferation assay was presented as number of attached cells that were trypan blue negative at indicated time points. Cell viability was assayed in cells treated with scrambled or CCN3 siRNA for 3 days. Ratios of trypan blue positive or negative cells to the total number of attached and floating cells were calculated to determine cell viability as percentage dead or live cells, respectively. Inset: detection of CCN3 in culture medium from cells treated with scrambled or CCN3-specific siRNA. (**c**) Overexpression of CCN3 promoted differentiation markers myosin heavy chain (MHC) and troponin-T expression in RH4 and RH28 cells (left panel). Right panel: immunofluorescence image showed increased number of MHC-positive myotubes presence in RH4 cells with exogenous CCN3 expression.

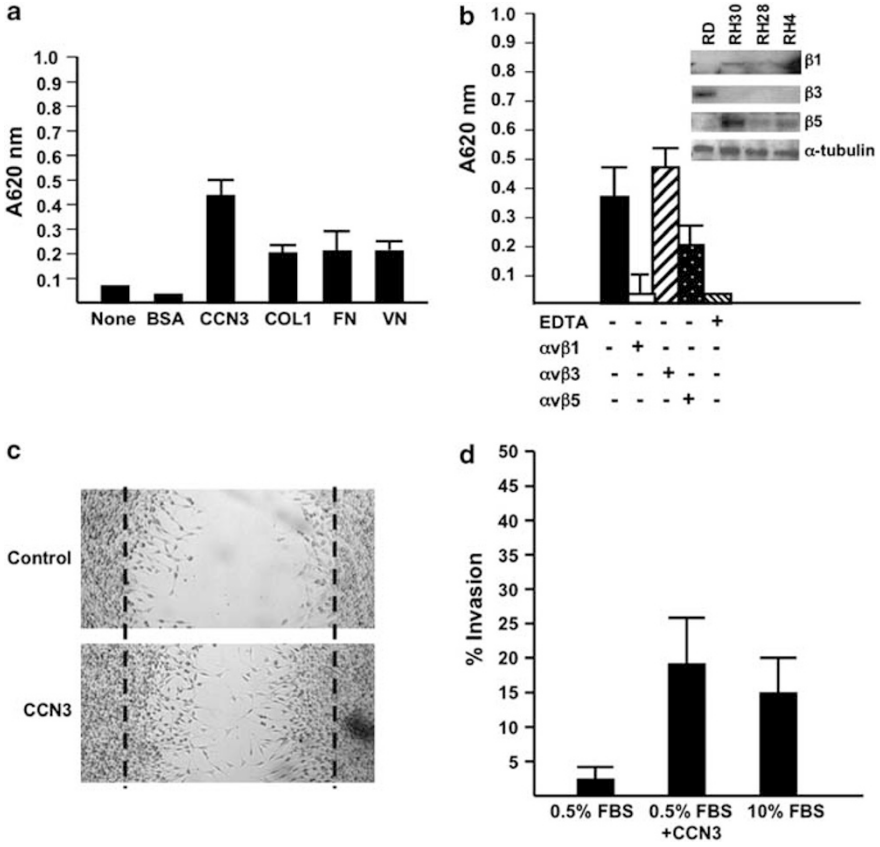


**Figure 8** Exogenously supplied CCN3 promoted aRMS cell adhesion, migration and invasion. (**a**) The adhesion assay was performed by measuring the amount of RH4 cells attached to the plastic surface of microtiter plate with or without pre-coating of indicated proteins. Col1: collagen 1; FN: fibronectin; VN: vitronectin. (**b**) RH4 cells adhesion to CCN3-coated microplate was blocked selectively by antibodies against β1 and β5 integrins. RH4 cells binding to the control bovine serum albumin-coated plates with or without antibody treatment were similar at an absorbance below 0.05 (data not shown). Inset: western blot analysis of β-integrin and α-tubulin expression in eRMS (RD) and aRMS (RH4, RH28 and RH30) cells. (**c**) CCN3 enhanced migratory activity of RH4 cells as determined by wound scratch assay. Representative light microscopic photographs (100 ×) were taken 48 h after the scratch was created. (**d**) Matrigel invasion assay was performed on RH4 cells by using Matrigel-coated filters and invasion chambers containing low (0.5%) and high (10% FBS) serum, and low serum containing 50 μg/ml CCN3 protein.

promoters. The R domain of PAX3 is a common element for both myogenin and CCN3. Prior studies have pointed to a role for the R domain in modulating PAX3 activity, but not in binding specificity (Chalepakis *et al*., 1994). Thus, we hypothesize a possible interaction between R domain and the FKHR domains, which promotes the necessary flexibility for PD-dependent activation by PAX3-FKHR.

Distinct patterns of CCN3 expression are observed in RMS subtypes. In eRMS, the immunohistochemical staining for CCN3 is not uniform, rather is concentrated in the well-differentiated cells, which led Manara *et al*. to suggest CCN3 is associated with RMS differentiation (Manara *et al*., 2002). By contrast, in aRMS, CCN3 staining is uniform and readily detected in tumor sections that lack well-differentiated cells, which seems to contradict this association. A simple explanation would be that the effect of CCN3 on RMS differentiation is subtype-specific, as aRMS and eRMS are genetically distinct. Like most CCN proteins, CCN3 function is complex as its effects on both normal and tumor cells are context-dependent (Perbal, 2004; Holbourn *et al*., 2008; Perbal, 2008a). Alternatively, CCN3 might be kept at a concentration below a certain threshold required for myogenesis, but high enough to facilitate other pro-tumorogenic activities in aRMS. The latter possibility is supported by increased differentiation seen in CCN3 overexpressing aRMS cells (Figure 7c). Regulated CCN3 expression by PAX3-FKHR provides one explanation for the uniform CCN3 expression observed in aRMS tumors.

It is noteworthy that knockdown of CCN3 in aRMS cells in our study did not affect myogenic potential. This finding differs from the previous report where knockdown of CCN2 in RMS cells is shown to reduce myogenesis (Croci *et al*., 2004). The different results may be related to the functional difference between the two CCN members in RMS cells, or most likely to the different RMS cells used in each study. The CCN2 study used RD (eRMS clones 12 and 18) and PAX7-FKHR-expressing RMZ-RC2 cell lines. Both RD/18 and RMZ-RC2 cell lines have comparatively higher intrinsic myogenesis potential than aRMS cells that express endogenous PAX3-FKHR, and have very low myogenesis

potential. Indeed, no significant myogenic effect is observed upon CCN2 knockdown in RD/12 cells that also show little intrinsic myogenic potential. It will be of interest in future studies to learn the exact relationship between CCN3 levels and myogenesis in RMS cells.

The role of *CCN3* gene in RMS development is currently unknown. Our data provide evidence that links CCN3 to activities known to contribute to the aggressive and metastatic nature of aRMS, including increased cell survival, adhesion, migration and invasion (Figures 7–8). Future studies are needed to determine the mechanistic basis of these CCN3 pro-oncogenic affects. We propose a few possibilities to be considered based on CCN3 functions in other tumors and normal cells. For example, CCN3 collaborates with PDGFRA and MMP3 in activating glioblastoma cell migration (Laurent *et al*., 2003). This link is especially intriguing because PDGFRA is a direct target of PAX3-FKHR (Epstein *et al*., 1998; Taniguchi *et al*., 2008), and elevated expression of MMPs including that of MMP3 is detected in RMS tumors (Diomedi-Camassei *et al*., 2004; Williamson *et al*., 2007; Rikhof *et al*., 2009). CCN3 has also been linked with FGF2 and IGF1 in promoting proliferation and survival of skeletal muscle cells (Lafont *et al*., 2005). IGF-1R is a direct target of PAX3-FKHR (Ayalon *et al*., 2001), and both IGF- and FGF-pathways are overactivated in RMS tumors (Williamson *et al*., 2007; Rikhof *et al*., 2009). Thus, CCN3 may work in concert with these growth factor pathways to enhance aRMS tumorogenesis. Lastly, activation of integrin-linked kinase followed by Jun Kinase (JNK) phosphorylation is specifically needed for aRMS tumorogenesis (Durbin *et al*., 2009). We have shown in this study that aRMS cell adhesion is promoted by CCN3 through integrins. It is possible that PAX3-FKHR induction of CCN3 could provide the necessary stimulus to activate the integrin-linked kinase-JNK cascade in promoting aRMS tumor cell adhesion and growth.

In conclusion, the goals of this study were to test the hypothesis of a regulatory loop between CCN3 and PAX3-FKHR expression in aRMS cells, and to examine the relevance of CCN3 expression to aRMS cell behavior. We have provided several line of evidence supporting a direct participation of PAX3-FKHR in CCN3 transcriptional activation. Through this analysis, we have expanded our knowledge of how a PD-dependent mechanism is developed in PAX3-FKHR. We have reported multiple effects of CCN3 on aRMS cells, all of which favor malignant tumor progression. A better understanding of mechanisms underlying PAX3-FKHR-dysregulated gene expression and the associated functional consequences may facilitate the development of new therapeutic strategies for aRMS.

## Materials and methods

*Materials*

Complimentary DNA constructs of PAX3-FKHR, and human CCN3 were as described (Lin *et al*., 2003; Zhang and Wang, 2007). The promoter for *CCN3* gene was obtained by PCR amplification of fibroblast genomic DNA. Primers used were: forward 5′-TCAGGCTCCTGCTCCATTC-3′, reverse 5′-GGTGATATACTGGGGCACTTG-3′. PCR accuracy was verified by comparison with the NIH database. PAX3-FKHR antibody was previously described (Zhang and Wang, 2007). Commercial antibodies were purchased for MyoD (5.8A, Pharmingen, San Diego, CA, USA), MyoG (F5D, Pharmingen), MHC (MF20, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) α-tubulin (ab1, Oncogene, Cambridge, MA, USA), hCCN3 (S21, Santa Cruz, Santa Cruz, CA, USA), mCCN3 (AF1917, R&D systems, Minneapolis, MN, USA), α5β1 (JBS5, Santa Cruz), αvβ3 (LM609, Chemicon, Temecula, CA, USA) and αvβ5 (P1F6, Chemicon). Commercial recombinant proteins were purchased for human CCN3 (R&D systems), fibronectin and vitronectin (Collaborative Biomedical, Bedford MA, USA), and collagen 1 (Sigma, St Louis, MO, USA).

*Cell culture*

Murine C2 (clone 12) myoblasts were maintained in growth medium (GM, Dulbecco modified Eagle's high glucose medium containing 15% FBS fetal bovine serum). Human RMS cell lines (eRMS: RD; aRMS: RH4, RH28 and RH30) were maintained in 10% FBS medium. To induce myogenic differentiation, cells were plated at 80% confluence. The next day, cells were rinsed with phosphate buffered saline (PBS) before adding differentiation medium (DM, 2% HS Horse serum). C2 stable cells expressing PAX3-FKHR were previously described (Zhang *et al*., 2009). Inducible C2 cells expressing PAX3 and PAX3-FKHR were established using the Tet-on advanced inducible gene expression system (Clonetech, Mountain View, CA, USA). PAX3 and PAX3-FKHR expression was induced with 1 μg/ml doxycycline.

*Immunoblotting and immunofluorescence*

Whole cell extract was prepared in RIPA buffer (137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% NP-40, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstin A, 10 μg/ml leupeptin, 500 mM orthovanadate and 1 mM phenylmethylsulfonyl fluoride). Following SDS–PAGE, proteins were detected by chemiluminescent antibody detection kit (NEN Life Science, Waltham, MA, USA). For detecting secreted CCN3, medium was harvested, clarified by centrifugation and incubated with heparin-agarose beads overnight at 4 °C. Beads were washed with PBS three times, and proteins were released by the addition of Lammeli sample buffer. Human RMS cells secreted substantially less CCN3 than the murine C2 cells. Therefore, we collected media from RMS cultures after 48–72 h incubation, whereas we collected media from C2 cultures after 24-h incubation under the same seeding density. The heparin-bound medium sample was normalized to total cell protein from the same cultures. Total cell protein was normalized to α-tubulin. A minimum of threefold higher total cellular protein equivalent was used to detect CCN3 expression in RMS cells compared with C2 cells.

For detecting MHC-positive myotubes by immunofluorescence, cells were rinsed with PBS, fixed with 1% paraformaldehyde and stained with anti-MHC antibody (MF20) followed by Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA, USA). Images were recorded using the Q-Capture Pro 5.0 image capture program (Leica DM/RB microscope, Bannockburn, IL, USA).

*Gene knockdown by RNA interference*

Cells were plated at 80% confluence a day before transfection. Cells were transfected with 25 nM PAX3-FKHR or CCN3 siRNA (Thermo Scientific, Lafayette, CO, USA) using

lipofectamaine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The specific siRNA sequences were: PAX3-FKHR forward: 5′-CUACUAUACCGAUACUCCCdTdT-3′, reverse: 5′-GGG AGUAUCGGUAUAGUAGdTdT-3′; CCN3 forward: 5′-CA CACCAACUGUCCUAAGAdTdT-3′, reverse: 5′-UCUUAG GACAGUUGGUGUGdTdT-3′. Scrambled sequences were used as controls.

*Transient transfection and CAT assays*

Transient transfection was carried out using either lipofectamine or DNA-CaPO4 precipitate method. Beta-galactosidase DNA (lacZ) driven by the CMV promoter was co-transfected for monitoring transfection efficiency. Cell lysates for lacZ and CAT assays were prepared as previously described (Zhang and Wang, 2007). The amount of cell lysate used in CAT assay was standardized by the lacZ activity. Quantitation of CAT activity was determined by scintillation counting.

*EMSA*

EMSA was performed by pre-incubating bacterial expressed GST-PAX3-FKHR (50 ng) or nuclear extract (10 μg) with non-specific salmon sperm DNA (0.05 mg/ml) in a binding buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH7.4, 0.5 mM ethylenediaminetetraacetic acid, 10% glycerol, 5 mM DTT, 1 mM MgCl2, and 50 mM KCl) for 5 min on ice. Routinely, 0.2 ng of a $^{32}$P-labeled DNA probe was added to the EMSA reaction mixture and incubated for 20-min at room temperature. For super-shift analysis, antibody was added to the DNA-protein mixture for additional 10 min incubation. The reaction complex was electrophoresed on a 5% native polyacrylamide gel at 4 °C in 0.5 × Tris-boric acid buffer at 200 V. Gel was dried and autoradiographed. EMSA DNA probe sequences were:

nWT probe, forward: 5′-GATCCAAGTGGCGCACGCTT AAGATGCCCCCGATTACA-3′,
reverse: 5′-GATCTGTAATCGGGGGCATCTTAAGCGT GCGCCACTTG-3′;
nM1, forward: 5′-GATCCAAGTGGCGCACGCTTAAG ATGCCCCCGGCCGCA-3′,
reverse: 5′-GATCTGCGGCCGGGGGCATCTTAAGCG TGCGCCACTTG-3′;
nM2, forward: 5′-GATCCAAGTGGCGACATAGGAAG ATGCCCCCGATTACA-3′,
reverse: 5′-GATCTGTAATCGGGGGCATCTTCCTATG TCGCCACTTG-3′.

*Chromatin immunoprecipitation*

Chromatin immunoprecipitation analysis was carried using SimpleChIP enzymatic chromatin IP kit, as recommended by the manufacturer (Cell Signaling, Danvers, MA, USA). In brief, a total of $1 \times 10^8$ cells were cross-linked with 1% formaldehyde and neutralized by addition of glycine before chromatin isolation. Genomic DNA from nuclei suspension was digested with micrococcal nuclease to an average of 300–900 bp followed by sonication. Lysates were pre-cleared with protein G before addition of primary antibody (1–2 μg/ml) overnight incubation at 4 °C. Antibody against histone H3 from the kit was used as the positive control. Protein A-purified rabbit IgG was used as the negative control. Immunoprecipitated protein-DNA complexes were de-crosslinked, and the genomic DNA was released by proteinase K digestion followed by purification (Qiagen PCR kit, Valencia, CA, USA). PCR primers used for detecting CCN3 sequences were: forward: 5′-AAG TGGCGACATAGGAAGATGCCCCCGATTAC-3′, reverse: 5′-GGTGATATACTGGGGCACTTG-3′. Control primers for Histone H3 were provided in the kit. These were specific to exon 3 of human *RPL30* gene.

*RNA samples and quantitative real time RT–PCR*

Total RNA was extracted from cells using Trizol reagent (Invitrogen), and complimentary DNA was prepared from DNase-treated total RNA using Affinity complimentary DNA synthesis kit (Agilent Technologies, Santa Clara, CA, USA). Quantitative PCR was performed with SYBR detection kit (BioRad, Hercules, CA, USA). Primers used were: CCN3, forward: 5′-CCCAACAACCAGACTGGCATT-3′, reverse: 5′-TACTGACAGTTCGGCTCAAAC-3′; GAPDH, forward: 5′-CATGTTCCAGTATGACTCCACTC-3′, reverse: 5′-GGC CTCACCCCATTTGATGT-3′.

*Cell adhesion assays*

96 well-microtiter plates (Nunc Maxisorp, Thermo Scientific) were coated with test proteins or bovine serum albumin overnight at 4 °C. Before use, wells were rinsed with PBS and blocked with 1% bovine serum albumin for 2 h at room temperature. Cells were trypsinized and re-suspended in medium containing 1% bovine serum albumin before plating at 6000 cells per well. Cells were allowed to attach to the plate for 3 h, before rinsing with PBS three times to remove unattached cells. Attached cells were fixed, stained with crystal violet solution, released from plates with 1% SDS solution and quantified by measuring absorbance at OD 620 nm. To test for blocking of cell adhesion, test reagents were incubated with the cell suspension for 15 min at room temperature before plating. Experiments were performed in triplicate in three independent experiments.

*Scratch wound assay*

Cells were seeded in a 12-well plate at $2 \times 10^5$ cells per well. Cells were allowed to reach confluence before the surface was uniformly scratched with a pipette tip across the center of well. Wells were rinsed twice with PBS to remove floating cells. Cells were replenished with media, with or without CCN3 protein and cultured for 48 h. The initial wound area and the movement of the cells into the scratched area was recorded. Positions of the initial wound were marked with dash lines. Experiments were performed in triplicate in three independent experiments.

*Invasion assay*

Invasion study was carried out using control and Matrigel inserts in 24-well plate (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were treated with 0.05% FBS medium for 6 h, trypsinized and re-suspended in the same medium before seeding $2 \times 10^4$ cells per insert. The lower chamber was filled with 0.05% FBS medium with or without CCN3 protein. Complete 10% FBS medium was included as positive control. After 20-h incubation, non-migrating cells from the upper membrane were removed using a wet Q-tip. Migrating cells attached to the underside of membrane were fixed in 100% methanol, stained with 0.4% crystal violet dye and counted. The effect of cell invasion was presented as percentage of the mean number of cell migrating through the Matrigel membrane over the mean number of cells migrating through the control membrane.

*Statistical analysis*
Statistical analysis was carried out by Student's *t*-test. Values shown represent mans of a minimum of three experiments. The standard deviation is defined as the root mean square deviation of n-1 determinations. Statistical significant was $P<0.05$.

## Conflict of interest

The authors declare no conflicts of interest.

## Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)