Reexression of hSNF5 in Malignant Rhabdoid Tumor Cell Lines Causes Cell Cycle Arrest through a p21CIP1/WAF1-Dependent Mechanism

Yasumichi Kuwahara, Aubri Charboneau, Erik S. Knudsen, and Bernard E. Weissman

Abstract

Loss of hSNF5 function is usually observed in malignant rhabdoid tumor (MRT), a highly aggressive pediatric neoplasm. Previous studies have shown that reexpression of hSNF5 in MRT cell lines causes G1 cell cycle arrest with p16INK4A, p21CIP1/WAF1, and cyclin D1 playing key roles in MRT cell growth control. However, we have shown that reexpression of hSNF5 induced cell cycle arrest in the absence of p16INK4A expression. These results indicate that the mechanism of hSNF5-induced cell cycle arrest is context dependent. Here, we investigated the relationship between p21CIP1/WAF1 and hSNF5 in the regulation of growth using several MRT cell lines. We found that G1 cell cycle arrest occurred concomitant with an increase in p21CIP1/WAF1 mRNA and protein levels and preceded p16INK4A mRNA and protein upregulation. Chromatin immunoprecipitation data confirmed that hSNF5 appeared at both p21CIP1/WAF1 and p16INK4A promoters after reexpression. We further showed that p21CIP1/WAF1 induction showed both p53-dependent and p53-independent mechanisms. We also showed that reduction of p21CIP1/WAF1 expression by RNAi significantly inhibited hSNF5-induced G1 arrest. Our results show that both p21CIP1/WAF1 and p16INK4A are targets for hSNF5 and that p21CIP1/WAF1 upregulation during hSNF5-induced G1 arrest precedes p16INK4A upregulation. These findings indicate that hSNF5 mediates a temporally controlled program of cyclin-dependent kinase inhibition to restrict aberrant proliferation in MRT cells. Cancer Res; 70(5): 1854–65. ©2010 AACR.

Introduction

Malignant rhabdoid tumor (MRT) is a rare and extremely aggressive childhood cancer. MRT was initially described as an unfavorable histologic type of pediatric renal tumor, a variant of Wilms' tumor (1). Whereas the most common locations occur in the kidney and central nervous system, MRT also arise in almost any site (2, 3). Despite significant advances in the treatment and outcome of other pediatric tumors, for MRTs diagnosed before the age of 6 months, patient survival at 4 years drops to ∼8.8% (4). Therefore, improved patient outcome requires a better understanding of malignant rhabdoid tumorigenesis and the development of novel therapeutic strategies.

In the past several years, the discovery of deletions and mutations at 22q11.2 involving hSNF5/INI1 has contributed to the clarification of pathogenesis of MRT (5). The finding that genetic alterations in MRTs are usually limited to hSNF5 mutations and deletions implicates the loss of hSNF5 function as the primary cause of these tumors. Now, hSNF5 function is recognized as being lost in almost 100% of MRTs (6, 7). Therefore, the elucidation of hSNF5 function should lead to the identification of the key molecular steps necessary for MRT tumorigenesis.

hSNF5 is one of the core subunits of the SWI/SNF chromatin remodeling complex that also includes an ATPase subunit (either BRG1 or BRM), BAF155, and BAF170. SWI/SNF complexes are ATP-dependent chromatin remodeling complexes that regulate gene transcription by causing conformational changes in chromatin structure, as well as by cooperation with histone acetylation complexes (8). In human cells, studies have shown a role for these genes in MRT cell cycle control (17–20). Kia
and colleagues reported that reexpression of hSNF5 mediates eviction of polycomb complex proteins, such as BMI-1, from epigenetically silenced promoters of the INK4a-ARF-INK4a locus followed by their activation (21). Furthermore, some reports showed that hSNF5 controls the differentiation of MRT cells (22, 23) and hSNF5 loss changes gene transcription epigenetically and contributes to oncogenesis without genomic instability (24).

Our previous study showed that reexpression of hSNF5 induced cell cycle arrest even in the absence of p16\(^{INK4a}\) expression (25). This finding suggested that other genes besides p16\(^{INK4a}\) play a critical role at early time points of G1 cell cycle arrest induced by hSNF5. Therefore, in this study, we determined the mechanism of G1 cell cycle arrest induced by hSNF5 in MRT cells within 24 hours after reexpression using adenoviral vectors. We show that induction of p21\(^{WAF1/CIP1}\) appears at the onset of hSNF5-induced growth arrest and precedes p16\(^{INK4a}\) expression. Furthermore, we show that p21\(^{WAF1/CIP1}\) knockdown inhibits hSNF5-induced G1 cell cycle arrest. We also show differences in the histone methylation changes at these two promoters after hSNF5 reexpression. Finally, we demonstrate that p21\(^{WAF1/CIP1}\) shows both p53-dependent and p53-independent mechanisms of induction after hSNF5 reexpression. Our results suggest that p21\(^{WAF1/CIP1}\) plays a key role in hSNF5 control of cell growth, and hSNF5 loss may alter p21\(^{WAF1/CIP1}\) transcription by a different mechanism than that reported for the p16\(^{INK4a}\) promoter in MRT cells.

Materials and Methods

**Cell culture and adenovirus infection.** A204.1 (American Type Culture Collection, ATCC), G401.6 (ATCC), TTC642 (Dr. Timothy Triche, Children’s Hospital of Los Angeles), and NIH3T3 (Dr. Stuart Aaronson, National Cancer Institute) cells were cultured in RPMI 1640, and UNC N3T cells were cultured in DMEM containing 10% fetal bovine serum. The Ad/pAdEasyGFPINI-SV+ adenoviral vectors expressed GFP (designated Ad-GFP) were previously published (20). For infection, cells were incubated with lentiviral particles and polybrene and then selected with puromycin. At least three puromycin-resistant colonies of A204.1 and TTC642 were isolated and expanded for further characterization.

**Protein extracts and Western blotting.** Western blotting was carried out as described previously (25). Western analyses of proteins were carried out by using anti-p21\(^{WAF1/CIP1}\) (AB1; Calbiochem), anti-p16\(^{INK4a}\) (G175-1239; BD Pharmingen), anti-pRb (DO-1; Santa Cruz), anti-cyclin A (H-432; Santa Cruz Biotechnology), anti-actin (A2066; Sigma), anti-p53 (DO-1; Dr. Weidong Wang), BMI-1 (upstate cloneF6; Millipore), normal rabbit IgG (sc-2027; Santa Cruz Biotechnology), normal mouse IgG (sc-2025; Santa Cruz Biotechnology), or p53 (DO-1; Calbiochem). DNA present in each immunoprecipitation was quantified by QT-PCR using gene-specific primers on an ABI 7500 sequence detection system. All expression values were normalized against input DNA. Antibody specificity was also determined for each cell line (Supplementary Fig. S1). The primer sequences are shown in Supplementary Table S1.

**Lentiviral procedures and small hairpin RNA.** Lentivirus was generated using 293FT cells following the protocol of Kafri and colleagues (29). Either pLKO.1, a nontarget small hairpin RNA (shRNA) control vector (SHC002; Sigma), an equal mixture of five types of NM_00039 p21 MISSION shRNA lentiviral transduction particles (TRCN0000040123, TRCN0000040124, TRCN0000040125, TRCN0000040126, and TRCN0000040127), or NM_000546 p53 MISSION shRNA lentiviral transduction particles (TRCN000003756), obtained from Sigma, were cotransfected with the packing construct ΔNRF (from Dr. Tal Kafri, University of North Carolina; ref. 29) and the VSV-G envelope expression plasmid (pMDK64; from Dr. Matthias Kaeser, Salk Institute) into 293FT cells with FuGene (Roche). pLKO.1 is a negative control containing an insert sequence that does not target any human or mouse gene but will activate the RNAi pathway. For infection, cells were incubated with lentiviral particles and polybrene and then selected with puromycin. At least three puromycin-resistant colonies of A204.1 and TTC642 cells were isolated and expanded for further characterization.

**Cell cycle analysis.** Cell cycle analyses were performed according to the procedure of Huang and colleagues (30). Percentages of cells within each of the cell cycle compartments were determined by flow cytometry (CyAn; Dako) and analyzed with ModFit software (Verity).

Results

**The effects of reexpression of hSNF5 on the growth of MRT cell lines.** We have previously shown that both hSNF5 analyzed using TaqMan (Applied Biosystems) quantitative real-time reverse transcription–PCR (QT-PCR) analysis, with β-actin as the reference gene in each reaction. Reactions were performed on an ABI 7900 HT sequence detection system (Applied Biosystems), and relative quantification was determined using the 2\(^{-ΔΔCt}\) method (27). The primers used for p16\(^{INK4a}\) QT-PCR were 5’-CTGCCAACCGCAGCGAAATA-3’ and 5’-GCCGTGCCCATCATGTA-3’. The probe used for p16\(^{INK4a}\) QT-PCR was 5’-CTGGAATCGCTTCCAGCGTA-3’. The TaqMan gene expression assay primer/probe set Hs00355782_m1 (Applied Biosystems) was used for p21\(^{WAF1/CIP1}\), the primer/probe set Hs01034249_m1 (Applied Biosystems) was used for p53, and the primer/probe set Hs99999903_m1 (Applied Biosystems) was used for β-actin.
and p16INK4A can induce G1 cell cycle arrest in MRT cell lines at 72 hours after transfection (19). However, hSNF5 also induced G1 cell cycle arrest without p16INK4A induction in the same time frame (25). Because of the extended period between hSNF5 transfection and the characterization of cell cycle arrest in these previous studies, we characterized the effects of hSNF5 expression on the growth of two MRT cell lines within 24 of infection with Ad-hSNF5 and Ad-GFP (negative control) adenoviruses.

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**Figure 1.** G1 cell cycle arrest induced by reexpression of hSNF5. A, cells were harvested at the indicated times after infection with Ad-hSNF5 and Ad-GFP. Total cell protein (30 μg) was separated on a 4% to 20% SDS-polyacrylamide gel and probed with either anti-SNF5 or anti-β-actin. un, uninfected control. B, 24 h after infection with Ad-hSNF5 or Ad-GFP, cells were harvested and analyzed by flow cytometry. Left, representative profiles; right, columns, mean of three independent experiments; bars, SD. *, p < 0.05 relative to the number of S phase of uninfected control. C, cells were harvested at the indicated times after infection with Ad-hSNF5 and Ad-GFP. Total cell protein (30 μg) was separated on a 4% to 20% SDS-polyacrylamide gel and probed with appropriate antibodies. un, uninfected control.
The induction of hSNF5 protein expression in the A204.1 and TTC642 cells by adenoviral infection is shown in Fig. 1A. No hSNF5 expression was detected in either MRT cell line in the absence of infection or after Ad-GFP infection. However, infection with Ad-hSNF5 led to hSNF5 expression as early as 12 hours postinfection, followed by a time-dependent increase in hSNF5 expression levels in both MRT cell lines.

We next tested the effects of hSNF5 reexpression on cell cycle regulation by flow cytometry. Both cell lines infected with Ad-hSNF5 showed cell cycle arrest 24 hours after infection, characterized by the presence of nearly 80% of cells in the G1 phase of the cell cycle and the presence of <10% of cells in the S phase (Fig. 1B). The percentage of Ad-hSNF5- and Ad-GFP–infected cells in the S phase was significantly different at 24 hours after infection. Similar results were found at 48 hours postinfection. These results showed that the G1-S cell cycle progression was inhibited by 24 hours after hSNF5 reexpression in these MRT cell lines.

Figure 2. hSNF5-induced p21CIP1/WAF1 expression. A, RNA was extracted at the indicated times after infection with Ad-hSNF5 and Ad-GFP. The mRNA levels were measured for each gene by QT-PCR and normalized for β-actin expression. Columns, mean of three independent experiments; bars, SD. *, P < 0.05 relative to the Ad-GFP and uninfected control. un, uninfected control. B, at 24 h after infection with Ad-hSNF5 and Ad-GFP, protein was extracted for ChIP assays. ChIP assays were performed using antibodies directed against hSNF5, BRG-1, p53, and H3K4me3 on −2,283 and −1,391 kb of p21CIP1/WAF1 promoter. Columns, mean of triplicates; bars, SD. **, P < 0.01 relative to the Ad-GFP control; ***, P < 0.001 relative to the Ad-GFP control; #, P > 0.05 relative to the Ad-GFP control.

hSNF5-induced p16INK4A and p21CIP1/WAF1 protein expression in MRT cell lines. We previously showed that hSNF5 reexpression induced the downregulation of cyclin A, the dephosphorylation of pRb, and the upregulation of p16INK4A and p21CIP1/WAF1 expression at 3 days after transfection (25). To determine whether these changes also occurred simultaneously with hSNF5-induced cell cycle arrest at 24 hours postinfection, we examined the expression of cyclin-dependent kinase (CDK) inhibitors, especially p16INK4A and p21CIP1/WAF1, as well as their downstream targets by Western blotting (Fig. 1C). We observed increased p21CIP1/WAF1 and decreased phosphorylated pRb and cyclin A levels at 24 hours after infection.
after Ad-hSNF5 infection compared with Ad-GFP control and uninfected control in both A204.1 and TTC642 cells. In A204.1, p16INK4A protein levels were increased slightly at 12 hours after Ad-hSNF5 infection and showed a further increase at 24 hours after infection compared with control cells. In contrast, p16INK4A protein expression was absent at baseline in TTC642, with a slight increase at 24 hours, followed by a marked increase at 48 hours after infection. On the other hand, p53 was not significantly changed in both MRT cell lines (Fig. 1C).

Reexpression of hSNF5 induces p21CIP1/WAF1 transcription with or without p53 recruitment in MRT cell lines. We next examined whether the increase in p21CIP1/WAF1 protein levels resulted from an increase in its mRNA levels by QT-PCR. We found the level of p21CIP1/WAF1 mRNA increased within 12 hours after Ad-hSNF5 infection in comparison with Ad-GFP infection in both MRT cell lines. In TTC642, p21CIP1/WAF1 also increased more with Ad-GFP infection than uninfected control, especially at 48 hours (Fig. 2A).

In our previous study, we showed hSNF5-regulated p21CIP1/WAF1 and p16INK4A transcription in A204.1 at 4 days after hSNF5 plasmid transfection by ChIPs (25). Therefore, we analyzed the chromatin status at p21CIP1/WAF1 promoter, −2,283 kb (p53 high-affinity binding site) and −1,391 kb (p53 low-affinity binding site) in both A204.1 and TTC642 cells (28), at 24 hours after Ad-hSNF5 infection to clarify the mechanism of p21CIP1/WAF1 activation by hSNF5. ChIP data confirmed that hSNF5 bound to both −2,283 kb site and −1,391 kb site in either cell line. Furthermore BRG1 is also recruited by hSNF5 induction to both sites in A204.1, but only at the −2,283 site in TTC642 (Fig. 2B).

Moreover, our previous reports also suggested hSNF5 recruits p53 to the p21 promoter (25). Therefore, we determined whether hSNF5 recruitment to the p21 promoter affected p53 binding. In A204.1, p53 binding is increased after reexpression of hSNF5 in p53 stable knockdown MRT cells. A, p53 knockdown cells (A204 p53KD and TTC642 p53 KD) and control cells (A204 pLKO.1 and TTC642 pLKO.1) were harvested, and RNA and protein were extracted. The mRNA levels were measured by QT-PCR for each gene and normalized for β-actin expression. Columns, mean of three independent experiments; bars, SD. Total protein (30 μg) was separated on a 4% to 20% SDS-polyacrylamide gel and probed with appropriate antibodies. B, every 24 h after infection with Ad-hSNF5 and Ad-GFP, cells were harvested and RNA was extracted. The mRNA levels were measured for each gene by QT-PCR and normalized for β-actin expression. Columns, mean of three independent experiments; bars, SD. * P < 0.05 relative to the Ad-hSNF5–infected parent cells; #, P > 0.05 the Ad-hSNF5–infected parent cells.

Figure 3. p21CIP1/WAF1 expression after reexpression of hSNF5 in p53 stable knockdown MRT cells. A, p53 knockdown cells (A204 p53KD and TTC642 p53 KD) and control cells (A204 pLKO.1 and TTC642 pLKO.1) were harvested, and RNA and protein were extracted. The mRNA levels were measured by QT-PCR for each gene and normalized for β-actin expression. Columns, mean of three independent experiments; bars, SD. Total protein (30 μg) was separated on a 4% to 20% SDS-polyacrylamide gel and probed with appropriate antibodies. B, every 24 h after infection with Ad-hSNF5 and Ad-GFP, cells were harvested and RNA was extracted. The mRNA levels were measured for each gene by QT-PCR and normalized for β-actin expression. Columns, mean of three independent experiments; bars, SD. * P < 0.05 relative to the Ad-hSNF5–infected parent cells; #, P > 0.05 the Ad-hSNF5–infected parent cells.
Figure 4. hSNF5-induced p16<sup>INK4A</sup> expression. A, cells were infected with Ad-hSNF5 and Ad-GFP. RNA was extracted at the indicated times after infection. mRNA levels were measured by QT-PCR analysis for each gene and normalized for β-actin expression. Columns, mean of three independent experiments; bars, SD. *, P < 0.05 relative to Ad-GFP and uninfected control. un, uninfected control. B and C, at 24 and 48 h after infection with Ad-hSNF5 and Ad-GFP, cells were harvested and protein was extracted for ChIP assays. ChIP assays were performed using antibodies directed against hSNF5(B), BMI-1(B), BRG-1(B), and H3K4me3(C) on −450 kb site of p16<sup>INK4A</sup> promoter. Columns, mean of triplicates; bars, SD. *, P < 0.05 relative to the Ad-GFP control; **, P < 0.01 relative to the Ad-GFP control; #, P > 0.05 relative to the Ad-GFP control. D, total protein (30 μg) was separated on a 4% to 20% SDS-polyacrylamide gel and probed with BMI-1 antibody.
hSNF5 reexpression, with a higher amount detected at the high-affinity −2,283 kb site (Fig. 2B). In contrast, we did not observe a difference in p53 recruitment between Ad-hSNF5 infection and Ad-GFP infection in TTC642, although the difference in binding between the two affinity sites remained (Fig. 2B). We next determined the effect of hSNF5 reexpression on H3K4me3, a chromatin mark associated with gene activation (31). H3K4me3 decreased after hSNF5 reexpression at both −2,283 kb and −1,391 kb sites in both A204.1 and TTC642 cells (Fig. 2B).

Reexpression of hSNF5 induces p21CIP1/WAF1 transcription through both p53-dependent and p53-independent mechanisms in MRT cell lines. Because our results indicated that hSNF5 reexpression activated p21CIP1/WAF1 transcription with p53 recruitment in A204.1 cells but without p53 recruitment in TTC642 cells, we next assessed the role of p53 in p21CIP1/WAF1 transcription in the MRT cell lines. We established two independently derived p53 stable knockdown MRT cell lines from both A204.1 and TTC642 cells using lentiviral vectors encoding a shRNA targeting p53 mRNAs. We also developed a negative control cell line using a lentiviral vector encoding a shRNA targeting a nonmammalian sequence (pLKO.1). By QT-PCR and Western blotting, all p53 knockdown cells (A204.1 p21KD and TTC642 p21 KD) showed significant decreases in the p53 mRNA levels along with a decrease in p21CIP1/WAF1 mRNA levels compared to control cells (Fig. 2B).

**Figure 5.** Inhibition of G1 cell cycle arrest by reexpression of hSNF5 in p21CIP1/WAF1 stable knockdown MRT cells. A, p21CIP1/WAF1 knockdown cells (A204 p21KD and TTC642 p21 KD) and control cells (A204 pLKO.1 and TTC642 pLKO.1) were harvested, and RNA and protein were extracted. The mRNA levels were measured by QT-PCR for each gene and normalized for β-actin expression. Columns, mean of three independent experiments; bars, SD. Total protein (30 μg) was separated on a 4% to 20% SDS-polyacrylamide gel and probed with p21CIP1/WAF1 antibodies. B, every 24 h after infection with Ad-hSNF5 and Ad-GFP, cells were harvested and analyzed by flow cytometry. Columns, mean of three independent experiments; bars, SD. *, P < 0.05 relative to each Ad-GFP control.
with the protein levels of p53 and p21<sup>CIP1/WAF1</sup> compared with the parental cells or the control cells (A204.1 pLKO.1 and TTC642 pLKO.1; Fig. 3A).

We next determined whether the reduction in p53 expression affected p21<sup>CIP1/WAF1</sup> transcription induced by hSNF5. Infection of the pLKO.1 and p53KD cells with Ad-hSNF5 or Ad-GFP resulted in increased levels of p21<sup>CIP1/WAF1</sup> mRNA at 24 hours after Ad-hSNF5 infection in pLKO.1 cells as in the parental cell lines (Fig. 3B). However, whereas the increase of p21<sup>CIP1/WAF1</sup> mRNA by hSNF5 reexpression was significantly inhibited in all A204.1 p53KD cells, the increase of p21<sup>CIP1/WAF1</sup> mRNA by hSNF5 reexpression was not significantly different among TTC642, TTC642 pLKO.1, and all TTC642 p53KD cells (Fig. 3B). These results suggested that the upregulation of p21<sup>CIP1/WAF1</sup> transcription by hSNF5 reexpression was operated through a p53-dependent mechanism in A204.1 cells and through a p53-independent mechanism in TTC642 cells.

Reexpression of hSNF5 induces p16<sup>INK4A</sup> transcription through BMI-1 eviction in the TTC642 cell line. Although A204.1 expresses p16<sup>INK4A</sup> mRNA and protein, TTC642 does not show detectable expression of p16<sup>INK4A</sup> protein. However, reexpression of hSNF5 caused upregulation of p16<sup>INK4A</sup> protein in both MRT cell lines (Fig. 1C). We, therefore, examined whether the increase of p16<sup>INK4A</sup> protein resulted from an increase in its mRNA levels by QT-PCR. We found that p16<sup>INK4A</sup> mRNA levels increased within 24 and 48 hours after Ad-hSNF5 infection in A204.1 and TTC642 cells, respectively (Fig. 4A). These results showed that the increase in p21<sup>CIP1/WAF1</sup> mRNA occurs earlier than the increase in p16<sup>INK4A</sup> mRNA.

Because BMI-1 represses transcription at p16<sup>INK4A</sup> locus (32) and an earlier report indicated that p16<sup>INK4A</sup> transcription is activated by induction of hSNF5 via BMI-1 eviction (21), we first confirmed that hSNF5 binding at the p16<sup>INK4A</sup> promoter increased at 24 hours after Ad-hSNF5 infection in both cell lines (Fig. 4B). We next determined the binding of BMI-1 to the p16<sup>INK4A</sup> promoter as an indication of polycomb complex silencing. In TTC642, we observed BMI-1 binding was also significantly less after infection of Ad-hSNF 24 and 48 hours after infection compared with control infected cells (Fig. 4B). We also found a modest increase in BRG-1 binding on the p16<sup>INK4A</sup> promoter after hSNF5 reexpression at 24 hours, followed by a dramatic increase in H3K4me3 binding at 48 hours in TTC642 cells (Fig. 3B and C). These results seem consistent with hSNF5 reexpression increasing the binding of the SWI/SNF complex to the p16<sup>INK4A</sup> promoter accompanied by polycomb eviction at 24 hours followed by H3K4 methylation and activation of p16<sup>INK4A</sup> transcription at 48 hours in TTC642 cells.

In contrast, we detected little BMI-1 binding on the p16<sup>INK4A</sup> promoter in A204.1 cells, even in the absence of hSNF5 expression (Fig. 4B). We also observed that hSNF5 reexpression had little effect on the expression of BRG-1 and the level of H3K4 methylation at this promoter (Fig. 4B and C). These results suggested the absence of polycomb complex silencing at the p16<sup>INK4A</sup> promoter in the A204.1 cells. Therefore, we examined the expression of BMI-1 in our MRT cell lines by Western blotting. The results in Fig. 4F show that the A204.1 cell line fails to express detectable BMI-1 protein compared with the TTC642 cell line (Fig. 4D). The absence

![Figure 6. Inhibition of pRb dephosphorylation after hSNF5 reexpression in p21<sup>CIP1/WAF1</sup> knockdown MRT cell lines. A and B, p21<sup>CIP1/WAF1</sup> knockdown cells (A204 p21KD and TTC642 p21 KD) and control cells (A204 pLKO.1 and TTC642 pLKO.1) were harvested, and protein was extracted. Total cell protein (30 μg) was separated on a 4% to 20% SDS-polyacrylamide gel and probed with appropriate antibodies.](image-url)
of BMI-1 may explain the basal level of p16INK4A mRNA observed in A204.1 cells.

**Reduced p21CIP1/WAF1 expression inhibits G1 arrest induced by reexpression of hSNF5 in MRT cell lines.** Because our results indicated that hSNF5 reexpression activated p21CIP1/WAF1 transcription earlier than p16INK4A transcription, we next assessed the role of p21CIP1/WAF1 in hSNF5-induced cell cycle arrest in the MRT cell lines. We established three independently derived p21CIP1/WAF1 stable knockdown MRT cell lines from both A204.1 and TTC642 cells using lentiviral vectors encoding a shRNA targeting p21CIP1/WAF1 mRNAs and a negative control cell line (pLK.O1). By QPCR and Western blotting, all p21CIP1/WAF1 knockdown cells (A204.1 p21KD and TTC642 p21 KD) showed significant decreases in the mRNA levels along with the protein levels of p21CIP1/WAF1 compared with the parental cells or the control cells (A204.1 pLKO.1 and TTC642 pLKO.1; Fig. 5A).

We next determined whether the reduction in p21CIP1/WAF1 expression affected hSNF5-induced cell cycle arrest. Therefore, we infected the pLK.O1 and p21KD cells with Ad-hSNF5 or Ad-GFP and assayed the effects on cell cycle by flow cytometry. We observed a similar G1 cell cycle arrest induced by Ad-hSNF5 infection in pLK.O1 cells after 24 hours as in the parental cell lines (Fig. 5B). However, the inhibition of the G1-S cell cycle progression by hSNF5 reexpression was significantly inhibited in all A204.1 p21KD cells and TTC642 p21 KD cells (Fig. 5B). These results indicated that p21CIP1/WAF1 upregulation contributed to the inhibition of the G1-S cell cycle progression by hSNF5 at 24 hours after Ad-hSNF5 infection.

**Reduced p21CIP1/WAF1 expression inhibits the dephosphorylation of pRb after hSNF5 reexpression in MRT cell lines.** Because p21CIP1/WAF1 knockdown caused inhibition of G1 cell cycle arrest induced by hSNF5 reexpression, we examined whether the effect occurred at the level of pRb phosphorylation. Although p21CIP1/WAF1 expression increased after hSNF5 reexpression in A204.1 pLK.O1 and TTC642 pLK.O1 cell lines, we observed limited increase of p21CIP1/WAF1 and limited reduction of phosphorylated pRb and cyclin A at 24 hours after Ad-hSNF5 infection in A204.1 p21KD cells and TTC642 p21 KD cells (Fig. 6A and B). The expression of p16INK4A was not significantly increased at 24 hours after Ad-hSNF5 infection in A204.1 p21KD cells. In TTC642 p21KD cells, the p16INK4A protein increased slightly at 24 hours after Ad-hSNF5 infection (data not shown) and then increased significantly at 48 hours, similar to the TTC642 parent cell line (data not shown). The change of p21CIP1/WAF1 mRNA was similar to the results seen with p21CIP1/WAF1 protein levels (data not shown). Taken together, these results indicated that the G1 arrest induced by hSNF5 reexpression strongly correlated with dephosphorylation of pRb through p21CIP1/WAF1 activation.

**Discussion**

By studying the mechanism of G1 cell cycle arrest induced by hSNF5 at a time point concomitant with the induction of growth arrest, our study shows three important observations. First, hSNF5 directly regulates the p21CIP1/WAF1 and p16INK4A loci through different mechanisms among MRT cell lines. Second, hSNF5 regulates p21CIP1/WAF1 through either a p53-dependent or a p53-independent mechanism. Finally, p21CIP1/WAF1 has a key role in hSNF5-induced cell growth arrest in MRT cell lines.

Reexpression of hSNF5 increased p21CIP1/WAF1 transcriptional activity immediately through the recruitment of BRG1 to the p21CIP1/WAF1 promoter. Similarly, some reports have suggested that BRG1 associates with the p21CIP1/WAF1 promoter and activates it along with hSNF5 (33, 34). Does this recruitment of the SWI/SNF complex lead to an interaction with another transcription factor? Lee and colleagues (35) suggested that the BRG-1 interacts with p53 and activates the p21CIP1/WAF1 promoter in a p53-dependent manner. In contrast, Liu and colleagues (36) and Hendricks and colleagues (33) suggested that BRG-1 activates the p21CIP1/WAF1 promoter in a p53-independent manner. Indeed, previous reports have shown that multiple mechanisms can activate the p21CIP1/WAF1 promoter, including p53-dependent, Sp1- or Sp3-dependent, or CDK8-dependent mechanisms (37). Our results in the A204.1 cell line seem consistent with the former model. We observed increased p53 levels at the p21CIP1/WAF1 promoter after hSNF5 reexpression and reduction in p53 expression inhibited upregulation of p21CIP1/WAF1 induced by hSNF5. This finding, at a minimum, supports the notion that reexpression of hSNF5 in these cells facilitates the recruitment of p53 to the p21CIP1/WAF1 promoter. However, our studies did not determine whether this occurs through a direct interaction between p53 and hSNF5. We also need to identify the upstream signal that initiates p53 binding to the p21CIP1/WAF1 promoter.

In contrast, p53 levels on p21CIP1/WAF1 did not change in the TTC642 cells after hSNF5 reexpression nor did decreased p53 levels affect the ability of hSNF5 to increase p21CIP1/WAF1 transcription. Whereas we cannot exclude the possibility that a low level of p53 protein remains at the p21CIP1/WAF1 promoter, sufficient for transcriptional activation, it seems that hSNF5 reexpression may operate through a different mechanism in these cells. Whereas activation of p21CIP1/WAF1 transcription by hSNF5 in both cell lines seems associated with recruitment of BRG-1, the transcription factors recruited to the p21CIP1/WAF1 promoter may differ. Additional ChIP analyses of the p21CIP1/WAF1 promoter in the TTC642 cell line will clarify this matter. In addition, our results showed a modest increase in p21CIP1/WAF1 transcription after Ad-GFP infection compared with the uninfected control. We believe that the high MOI of adenovirus required for infection of this cell line caused upregulation of p21CIP1/WAF1 through apoptotic induction (38).
for activation of the p16INK4A promoter by hSNF5 (21). On the other hand, the A204.1 cell line expresses low basal levels of p16INK4A consistent with a lack of expression of BMI-1. Our results seem in accord with the report that p16INK4A expression is directly regulated by polycomb proteins, such as BMI-1 and EZH2 (39). Moreover, while hSNF5 appeared at p16INK4A promoter after reexpression followed by an increase in p16INK4A mRNA in the A204.1 cell line, BRG-1 and H3K4me3 levels at the promoter did not change significantly. Therefore, in the absence of polycomb silencing, p16INK4A mRNA could increase more rapidly in the A204.1 cell line than in the TTC642 cell line. Regardless, the data still show that the hSNF5-induced increase in p16INK4A levels in the A204.1 cell line follows the p21CIP1/WAF1 upregulation regardless of BMI-1 expression. The mechanism for the increase in p16INK4A mRNA in the A204.1 cell line requires further investigation.

The difference in H3K4me3 patterns between the p21CIP1/WAF1 and p16INK4A promoters was unexpected. The significant increase in H3K4me3 at the p16INK4A promoter (−450 kb) in the TTC642 cell line after hSNF5 reexpression seems consistent with the activation of transcription from this promoter. Although the levels of this modification did not change in the A204.1 cell line, this may reflect the active p16INK4A transcription present in these cells before hSNF5 reexpression. However, in both cell lines, H3K4me3 decreased at the p53 binding sites in the p21CIP1/WAF1 promoter (−2,283 kb and −1,391 kb) after hSNF5 reexpression, although p21CIP1/WAF1 transcription increased. One possible explanation for this observation might come from hSNF5 re-expression activating SWI/SNF complex activity resulting in nucleosome repositioning by chromatin remodeling. Therefore, the subsequent change in H3K4me3 positioning on the p21CIP1/WAF1 promoter would be reflected by a decreased signal in our ChIP assay.

In our study, p21CIP1/WAF1 knockout experiments showed that inhibition of p21CIP1/WAF1 expression partially inhibited the efficiency of hSNF5-induced G1 cell cycle arrest in MRT cell lines. The failure to completely abrogate the growth arrest may result from the residual activation of p21CIP1/WAF1 protein in the RNAi-expressing MRT cell lines. In addition, the increasing levels of p16INK4A protein may also begin to affect the cells because we observed a complete cell cycle arrest at 48 hours in the p21 knockdown cells. We also cannot exclude the possibility that altered expression of other cell cycle regulatory genes may contribute to the G1 arrest induced by hSNF5 (40, 41). This result indicated that p21CIP1/WAF1 has a key role in hSNF5-induced cell growth arrest in MRT cell lines. Importantly, Smith and colleagues recently showed that cell cycle arrest and cytotoxicity induced by flavopiridol in MRT cell lines correlated with the downregulation of cyclin D1 and the upregulation of p21CIP1/WAF1 (42). Taken together, these results support p21CIP1/WAF1 as a relevant target for therapy of MRT.

Many MRTs arise in infants under the age of 6 months and in neonates (43). Therefore, it seems plausible that a significant number of MRTs arise from the loss of hSNF5 in stem cells or progenitor cells during development. The expression of p16INK4A in stem cells is restricted by the expression of BMI-1 (44). Indeed, mouse embryo cells do not display expression of p16INK4A (45) and stem cells in young mice (8–12 weeks old) do not express detectable p16INK4A mRNA (46). Thus, a strong possibility exists that p16INK4A was already silenced in the cell that gave rise to the TTC642 cell line before hSNF5 loss occurred.

Our results may indicate that a decrease in p21CIP1/WAF1 expression after hSNF5 loss may signify one key event during MRT development. For example, the absence of p21CIP1/WAF1 expression can increase hematopoietic stem cell proliferation (47) or maintain neural stem cells by playing a significant role in regulating their proliferation (48). Our recent studies showing cooperation between SNF5 loss and pRb family inactivation in the acceleration of formation of spinal cord MRTs in mice support this notion (49). These results suggest that p21CIP1/WAF1 and its downstream targets may regulate the boundary between quiescence and proliferation in stem cells and progenitor cells.

In conclusion, our results showed that, whereas hSNF5 re-expression in MRT cells increases both p21CIP1/WAF1 and p16INK4A expression during the induction of G1 cell cycle arrest, p21CIP1/WAF1 upregulation precedes p16INK4A. Although our studies firmly substantiate p21CIP1/WAF1 as a key target for hSNF5 in cell cycle regulation, the role of hSNF5 within the activities of SWI/SNF complex and gene regulation seem complex. Studies from other laboratories also implicate a role for hSNF5 in the regulation of cellular differentiation, cell migration, and DNA repair (40, 41, 50). However, the establishment that SNF5 loss alters p21CIP1/WAF1 expression during MRT tumorigenesis provides an important new target for therapy in a tumor with limited options for treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Reexpression of hSNF5 in Malignant Rhabdoid Tumor Cell Lines Causes Cell Cycle Arrest through a p21/CIP1/WAF1-Dependent Mechanism


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