Tbx2 Directly Represses the Expression of the p21\textsuperscript{WAF1} Cyclin-Dependent Kinase Inhibitor

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ABSTRACT

T-box factors play a crucial role in the development of many tissues, and mutations in T-box factor genes have been implicated in multiple human disorders. Some T-box factors have been implicated in cancer; for example, Tbx2 and Tbx3 can suppress replicative senescence, whereas Tbx3 can cooperate with Myc and Ras in cellular transformation. The p21\textsuperscript{WAF1} cyclin-dependent kinase inhibitor plays a key role in senescence and in cell cycle arrest after DNA damage. Here, using a combination of \textit{in vitro} DNA-binding, transfection, and chromatin immunoprecipitation assays, we show that Tbx2 can bind and repress the p21 promoter \textit{in vitro} and \textit{in vivo}. Moreover, small interfering RNA-mediated down-regulation of Tbx2 expression results in a robust activation of p21 expression. Taken together, these results implicate Tbx2 as a novel direct regulator of p21 expression and have implications for our understanding of the role of T-box factors in the regulation of senescence and oncogenesis, as well as in development.

INTRODUCTION

Recent work has contributed to an increasing body of evidence implicating the T-box transcription factor family in the maintenance of cell identity during development (reviewed in Refs. 1–3). For example, \textit{Tbx}1 mutations have been implicated in DiGeorge syndrome (4–6), Holt-Oram syndrome correlates with mutations in \textit{Tbx}5 (7), \textit{Tbx}4 and \textit{Tbx}5 play crucial roles in limb bud outgrowth and specification of limb identity (8–11), and \textit{T-Pit} determines cell fate in the pituitary (12).

In addition to their key function in development, evidence suggesting a role for T-box factors in breast cancer is accumulating. Tbx3 is required for normal breast development (13), with mutations in the \textit{Tbx}3 gene linked to ulnar-mammary syndrome (14). Moreover, Tbx3 can cooperate with Ras and Myc to induce cellular transformation and suppress apoptosis (15). Importantly, both Tbx3 and the highly related factor Tbx2 are transcriptional repressors (16–19) and can suppress senescence through a mechanism involving repression of the expression of the p19\textsuperscript{ARF} (p14) gene (20–22). Tbx2, like Tbx3, is also expressed in the developing breast and has been implicated in breast cancer where the \textit{Tbx}2 gene is preferentially amplified in\textit{ BRCA1} and \textit{BRCA2} mutants (23) and Tbx2 is overexpressed in breast cancer cell lines (24). These results suggest that T-box factors may contribute to cell cycle control and oncogenesis but it is not known whether their effect on proliferation extends beyond regulation of p19\textsuperscript{ARF}.

Proliferation of mammalian cells is regulated by signals acting to control the activity and expression of components of the cell cycle machinery. A combination of cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors act together to control proliferation, cell cycle arrest, apoptosis, and senescence, and deregulation of components of these key cell cycle regulators can contribute to cancer. The p21\textsuperscript{WAF1/CIP1/SDHI} cdk inhibitor (referred to as p21) is induced in differentiating cells and in response to a wide variety of cellular stresses including DNA damage, and contributes to stress-induced growth arrest (24, 25). The promoter of the p21 gene can be induced via p53 (26), and p21 expression is necessary for p53-mediated growth arrest (27–30). In contrast to the p16 cdk inhibitor, mutation of the p21 gene in human cancers occurs infrequently, and mice lacking p21 appear to undergo normal development, with no increase in the frequency of spontaneous tumor formation (27, 28). Nevertheless, some studies on the role of p21 in Ras transformation have suggested that p21 status is important. For example, in a mouse breast cancer model, the onset of Ras-induced tumors was accelerated in mice deficient in p21 (31). Any effects on the onset of tumor formation may reflect a role for p21 in replicative senescence. In senescent human fibroblasts, p21 levels are strongly elevated (32–34) and although mouse fibroblasts lacking p21 undergo normal senescence and are resistant to transformation by moderate levels of expression of oncogenic Ras (35), the cell cycle arrest associated with a high-intensity Ras/Raf signal is bypassed in p21-deficient fibroblasts (36). Additionally, a clone of human diploid fibroblasts in which the p21 gene had been disrupted exhibited a senescence bypass phenotype (37), whereas p21 expression can induce senescence in a p53-independent fashion (38, 39). Given the complex role of p21, identifying the controls regulating p21 expression is essential for understanding fully the balance between proliferation, differentiation, and senescence.

Here, we used a combination of \textit{in vitro} DNA binding, transfection, and chromatin immunoprecipitation assays together with small interfering RNA (siRNA)-mediated down-regulation of Tbx2 to identify Tbx2 as a novel negative regulator of p21 expression. These results have implications for our understanding of the role of T-box factors in the regulation of senescence and oncogenesis, as well as in development.

MATERIALS AND METHODS

\textbf{siRNA.} Suppression of Tbx2 cellular expression was achieved using siRNA that specifically targets Tbx2 mRNA. The siRNA oligonucleotide sense sequence with two uracil residues at the 3’ end was 5’-GGCGAGGCCG- GAUGGCGCUU-US3 corresponding to amino acids 302–309 of murine Tbx2 or 5’-GGACGAGGACCCAGUUUCU-US3 corresponding to amino acids 102–108 of human Tbx2. B16 cells or MCF-7 cells were transfected with anti-Tbx2 siRNA or a control (nonsilencing) 21-mer siRNA 5’-UUCUC- CGAACUGUCAGGUTT-US3 (Xeragon-Qiagen, Crawley, United Kingdom) using Oligofectamine reagent (Life Technologies, Inc./Invitrogen, Paisley, United Kingdom) according to Dharmacon Research instructions.

\textbf{Immunofluorescence Microscopy.} B16 cells grown on glass coverslips 3 days after siRNA transfection were washed three times with PBS and fixed with 3% paraformaldehyde for 20 min at room temperature before permeabilization with 0.1% Triton X-100 for 10 min at room temperature. Slides were incubated for 1 h with mouse Tbx2 monoclonal antibody (62–2) at a dilution of 1:750 and with the p21 (C-19) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250, then incubated with the appropriate secondary antibody coupled to FITC or Texas Red (Vector Laboratories, Peterborough, United Kingdom) at 1:100 dilution. Cells were

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mounted using Vectashield mounting medium and examined by confocal microscopy.

Western Blot Analysis. B16 whole-cell extracts were prepared 3 days after siRNA transfection. Proteins were resolved on 9–15% SDS-polyacrylamide gels as required and then transferred to Hybond C (Amersham, Amersham, United Kingdom). The membranes were probed with appropriate primary antibodies and detected using peroxidase-conjugated antimouse or antirabbit antibodies and visualized by enhanced chemiluminescence (Amersham). The primary antibodies used were mouse monoclonal anti-Tbx2 antibody 62–2, anti-p21(C-19) rabbit polyclonal antibody (Santa Cruz Biotechnology), and mouse monoclonal anti-α-tubulin (Clone B-5–1–2; Sigma).

Electrophoretic Mobility Shift Assays. Binding reactions were performed with double-stranded 32P-labeled oligonucleotide probes, in vitro transcribed/translated protein, B16 cell extract, antibody, and competitor oligos as described previously (16) and resolved on a 6% polyacrylamide gel.

The sequence of double-stranded oligonucleotides used as probe and competitors are as follows: p21, 5'-ctagaCTCGAGCACCTGAGTTGAGCAGCTGCGCGt-3'; p21mut, 5'-ctagaCTCGAGCACCTGAGTTGAGCAGCTGCGCGt-3'; and consensus T-element, 5'-AGGTGTGAAG-3' and 5'-CGAGCGGTCGACCTGAGACTCGTCGACGGCTTC-3'.

Chromatin Immunoprecipitation Assays. Chromatin immunoprecipitations were performed as described essentially previously (41). Briefly, mouse NIH 3T3 cells stably expressing an SV5-tagged Tbx2 cDNA were treated with formaldehyde, and DNA-containing fractions were incubated overnight with different antibodies and collected on protein G beads. Cross-linked products were reversed by heating at 65°C for 4 h and the immunoprecipitated DNA was purified. Primers for PCR amplification of the p21 promoter DNA were 5'- TAAACC GGCCGGTGTCTA-3' and 5'-AAGGCGCATGTGTTCCAGCAGGA-3'. For the HSV105 promoter, primers were 5'-CGAAAATGGCTCCCTAGGCTT-3' and 5'-CCCCTGGCAACCCGGTGCT-3'.

Generation of Stable Cell Lines Expressing Epitope-Tagged Tbx2. The parental cell line ER-NIH3T3, stably expressing the heterodimer of the eddyone receptor and the retinoid X receptor, which binds to the eddyone response element (encoded by pEGSH) in the presence of the synthetic eddyone analogue Ponasterone A (Stratagene), was purchased from Stratagene. These cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml each of penicillin and streptomycin, and 400 μg/ml of G418. A plasmid, pEGSH/SV5Tbx2, containing full-length mouse Tbx2 cDNA sequences encoding the SV5 (PK) epitope tag at its 5' end and the hygromycin-resistant gene was constructed. For stable lines, ER-NIH3T3 cells were transfected with pEGSH/SV5Tbx2 plasmid using FuGENE (Roche Molecular Biochemicals, Lewes, United Kingdom) according to the manufacturer’s instructions with 700 ng of the relevant reporter plasmid plus 200 ng of the Tbx2 expression plasmid or an equal amount of empty vector plasmid. The vector pCMV β-galactosidase containing the cytomegalovirus promoter driving the expression of a luc2 reporter was used as an internal control for transfection efficiency (100 ng/transfection). Cells were cultured for 48 h, and then the extracts were assayed for CAT and β-galactosidase activity. CAT values were normalized to the β-galactosidase activity and expressed relative to the empty vector control. All of the experiments were performed in duplicate, and at least three independent experiments were done to confirm reproducibility.

Growth and Infection of ST.HdhQ5111 Cells. Conditionally immortalized ST.HdhQ5111 mouse striatal cells (40) expressing a mutant huntingtin protein and tsA58 SV40 large T antigen were grown at 33°C in DMEM supplemented with 10% fetal bovine serum. Shift to the nonpermissive temperature (39°C) resulted in a loss of SV40 tsA58 protein and a senescence-like cell cycle arrest. To generate retrovirus-producing cell lines, Psi2 packaging cells were transfected with pBabePuro.SV5 (empty vector expressing the SV5 epitope tag only) or pBabePuroSV5-Tbx2 expression vectors. Two days later, 1 μg/ml puromycin was added. Pooled cell lines were expanded and maintained in 10% fetal bovine serum in DMEM under selective conditions.

Retroviral infections were carried out using viral supernatants from Psi2 cells stably producing SV5-Tbx2 or empty SV5 virus. On the day before infection, 4 ml of growth medium (minus puromycin) was added to a near-confluent 10-cm dish of virus-producing Psi2 cells. Twenty-four h later, the medium was removed and filtered through a 0.45 μm filter. ST.HdhQ5111 cells to be infected were plated at 5 × 10^5/cm2-dish. Twenty-four h later, the medium was removed and replaced with 4 ml of viral supernatant containing 10 μg/ml Polybrene. Cells were incubated for 5–6 h at 33°C, then the viral supernatant was removed and growth medium was added. For the senescence bypass assay, cells were moved to the nonpermissive temperature 3 days later. After 11 days, cells were fixed with acetone:methanol (1:1) for 5 min at room temperature, and colonies were visualized with 0.01% crystal violet. Alternatively, ST.HdhQ5111 cells expressing SV5 or SV5-Tbx2 at the nonpermissive temperature were selected using 1 μg/ml puromycin. Seven days later, ST.HdhQ5111 SV5-expressing cells were pooled and whole-cell lysates were prepared. SV5-Tbx2-expressing cells proliferating at the nonpermissive temperature were isolated and expanded under selective conditions. Lysates for Western blotting were prepared from each individual clone.

Site-Directed Mutagenesis of the p21 Promoter. The wild-type p21-CAT plasmid was modified as follows: the T-element (AGGTGTTA) located at the initiator region of the wild-type human p21 promoter was mutated to CTCTGA by site-directed mutagenesis using the Stratagene QuikChange system and the primer pair 5'-GCTGCCGCGTCGAAGCTGAGCGCCG-3' and 5'-CGAGGCCGTCGACCTGAGACTCGTCGACGGCTTC-3'.

RESULTS

Tbx2 Binds the p21 Promoter. Recent work suggests that both Tbx2 and the highly related T-box factor Tbx3 can suppress senescence via their ability to bind and repress the p19ARF promoter, thereby preventing the accumulation of p19ARF observed in cells undergoing senescence. Because p19ARF inhibits MDM2-mediated degradation of p53 (42, 43), one scenario that may explain, at least in part, the ability of Tbx2 and Tbx3 to suppress senescence is that inhibition of p19ARF expression prevents p53-mediated up-regulation of the promoter driving expression the p21 cdk inhibitor that has been implicated in control of proliferation, differentiation, and replicative senescence. However, the binding site for Tbx2 and Tbx3 in the p19ARF promoter AGGTGTA (22) does not match the previously identified high-affinity consensus T-box factor binding motif AGGT- GTGA (44) and may represent a relatively low-affinity site. For T-box factors to suppress senescence efficiently, it therefore may be necessary for the p19ARF gene to possess a high-affinity binding site. Western blotting of the parental ST.HdhQ5111 cell line revealed a dramatic increase in p21 expression within 8 h after shifting to the nonpermissive temperature (39°C), and this high level of expression was maintained for at least 8 days (Fig. 1A). In contrast to control cells infected with an empty retrovirus, ST.HdhQ5111 cells infected with a Tbx2-expressing retrovi-
Fig. 1. p21 expression is strongly up-regulated in ST.\(Hdh^{Q111}\) cells on shifting to 39°C. A, ST.\(Hdh^{Q111}\) cells grown at 33°C and analyzed by Western blotting using anti-p21 antibody at the indicated times (8 h to 7 days) after shifting to 39°C. A nonspecific band (NS) was used as a loading control. B, ST.\(Hdh^{Q111}\) cells infected with a retrovirus expressing Tbx2 escape senescence at 39°C. ST.\(Hdh^{Q111}\) cells grown at 33°C were infected with either an empty retrovirus vector (SV5) or a virus expressing SV5-tagged Tbx2 were grown at 33°C for 2 days and then shifted to 39°C for 10 days in the presence of paromycin before analysis by Western blotting using anti-SV5, anti-p21, or antitubulin antibodies as indicated.

Fig. 2. Tbx2 binds the p21 promoter in vitro. A, sequences of oligonucleotides used as probes and competitors. The sequences in lowercase letters indicate bases added for cloning purposes. The consensus T-element is based on that described previously for binding to Brachyury. The human p19 (p14) element is taken from that described by Lingbeek et al. (22), and the p21 sequence is taken from the human promoter extending from bases −3 to +17. B, Bandshift assay using in vitro transcribed/translated (ITT) Tbx2 together with the p21 oligonucleotide probe. The indicated competitors are used at 10, 50, and 250 ng and anti-Tbx2 antibody was included where indicated. Unprogrammed reticulocyte lysate was used as a control. C, bandshift assay using B16 cell nuclear extract and probe and competitors as in B. The effect of the anti-Tbx2 antibody is shown as an enlarged inset for clarity.
usually in the region of 4–6-fold, whereas mutation of the p21 AGGTGTGA motif to AGGcGGA abrogated the ability of Tbx2 to repress transcription. The degree of repression observed is comparable with that seen on other T-box factor target promoters (16, 18, 46).

To verify that Tbx2 could bind the p21 promoter directly in vivo, we performed a chromatin immunoprecipitation assay. Because currently available anti-Tbx2 antibodies do not immunoprecipitate Tbx2 efficiently, we established a 3T3 cell line expressing a low level of SV5 epitope-tagged Tbx2. Using this cell line, we treated cells with paraformaldehyde, extracted the chromatin, and immunoprecipitated Tbx2-bound chromatin using the SV5 antibody. After extensive washing, the cross-linking was reversed and the chromatin subjected to PCR using primers specific for either the p21 or HSP105 promoters. Nonspecific IgG or no antibody was used as a control.

Tbx2 Represses Endogenous p21 Gene Expression. To confirm that the endogenous Tbx2 regulates endogenous p21 expression, we designed a specific siRNA to down-regulate Tbx2 expression in B16 melanoma cells. The efficacy of the siRNA-mediated inhibition of Tbx2 expression was initially monitored by Western blotting using an anti-Tbx2 antibody that revealed that the levels of Tbx2 expressed were down-regulated by ~80% (Fig. 4A). No effect was observed on tubulin that was used as a loading control or if a control, nonsilencing siRNA was used. Reprobing the same Western blot with an anti-p21 antibody revealed that siRNA-mediated repression of Tbx2 expression also resulted in a substantial increase in p21 expression.

As an alternative assay, we used immunofluorescence in B16 melanoma cells to determine the effects of siRNA on both Tbx2 and p21 expression. Using an anti-Tbx2 antibody and the control siRNA, Tbx2 can be detected readily in the nucleus of all cells examined (Fig. 4B), although some variation between individual cells in the absolute level of fluorescence detected is seen. Staining with anti-p21 antibody revealed a low level of staining. In contrast, using the Tbx2-specific

Fig. 3. Tbx2 represses the p21 promoter. A, the sequence of the p21 probe and mutated site used for the DNA-binding assay. B, bandshift assay using the consensus T-element probe and in vitro transcribed/translated (ITT) Tbx2 together with the indicated consensus, wild-type (WT), and mutated (mut) p21 oligonucleotides. Only the bound DNA is shown. C, Tbx2 represses the p21 promoter. B16 cells were transfected with a WT p21-chloramphenicol acetyltransferase (CAT) reporter or a reporter in which the AGGTGTGA element was mutated to AGGcGGA in the presence of a cotransfected vector expressing Tbx2. CAT activity was determined 24 h posttransfection. D, Tbx2 binds the p21 promoter in vivo. Chromatin immunoprecipitation of Tbx2 was performed using a 3T3 cell line expressing a low level of SV5-tagged Tbx2. After cross-linking and shearing the chromatin to ~500 bp, chromatin was immunoprecipitated using the SV5 antibody. After extensive washing, the cross-link was reversed and the DNA subjected to PCR using primers specific for either the p21 or HSP105 promoters. Nonspecific IgG or no antibody was used as a control.

Fig. 4. Tbx2 represses the endogenous p21 gene. A, Small interfering RNA (siRNA)-mediated down-regulation of Tbx2 results in up-regulation of p21 expression. B16 melanoma cells were treated with siRNA specific for Tbx2 or a control siRNA. Three days after treatment, cells were analyzed by Western blotting using antibodies specific for Tbx2, p21, and tubulin. B and C, immunofluorescence using anti-Tbx2 or anti-p21 antibodies on B16 cells (B) or MCF-7 cells (C) treated for 3 days with siRNA specific for Tbx2, or a control siRNA. Cells were viewed using a 63 × Ph3 plan apochromat objective.
siRNA, Tbx2 expression is extinguished in up to 80% of the cells examined, consistent with the results of the Western blot, although in some cells Tbx2 expression is maintained at levels comparable with that seen in the control cells. Resistance to the effects of siRNA in 10–20% of the cell population is observed routinely using siRNA to repress expression of specific genes. Examination of p21 expression in the cells treated with the Tbx2 siRNA revealed that, in those cells in which Tbx2 expression had been extinguished, p21 expression was elevated to a level in excess of that observed in any cell treated with the control siRNA. p21 levels also remained low in those cells that apparently were resistant to the Tbx2-specific siRNA. Thus, the results from both Western blotting and immunofluorescence are consistent with Tbx2 acting to repress the expression of p21. Similar results using immunofluorescence were obtained using an MCF-7 breast cancer cell line that is known to overexpress Tbx2 (Ref. 21; Fig. 4C). However, in this case the efficiency of siRNA-mediated down-regulation of Tbx2 was not as elevated as that seen in B16 cells, and we were unable to achieve a decrease in Tbx2 levels that was high enough to enable the effects on p21 to be determined using Western blot analysis.

**DISCUSSION**

In addition to their role in development, there is a growing body of evidence implicating Tbx2 and Tbx3 in cancer. In particular, both factors are able to suppress senescence, with the evidence to date indicating that overexpression of Tbx2 or Tbx3 results in binding and repression of the promoter of the gene encoding p19ARF (20–22), a key regulator of the p53 pathway. We show here that in addition to p19ARF, the endogenous Tbx2 protein is a direct repressor of the p21 promoter through an element located close to the p21 transcription start site. Because p21 is implicated in the control of cellular senescence, the ability of Tbx2 to suppress expression of both p19ARF and p21 may provide an explanation for a powerful and cooperative antisenescence signal. Importantly, given that T-box factors share a highly related DNA-binding specificity, it is likely that other members of the family will also act to regulate p21 expression either positively or negatively, depending on whether the given T-box factor is a transcription activator or repressor. Although the transcription regulatory status of most T-box factors is unknown, Tbx3, the most closely related T-box factor to Tbx2, is also a transcriptional repressor and has been shown to cooperate with Ras in cellular transformation. Because the growth arrest associated with acute, high-level oncogenic Ras activity requires p21 (36), we anticipate that, like Tbx2, Tbx3 also acts to suppress p21 expression and that this, together with its ability to repress the p19ARF promoter, may explain its cooperative activity with Ras. Given the ability of Tbx2 and Tbx3 to repress the promoters of key regulators such as p19ARF and p21, the potential involvement of these factors in cancer warrant closer examination.

Given the regulation of the p19ARF and p21 promoters by Tbx2 and Tbx3 in cells in culture, an effect on proliferation in animals in which these genes are defective or disrupted might be expected. It has been suggested that in individuals haploinsufficient for Tbx3, ulnar-mammary syndrome may develop in part through hypoplasia of tissues resulting from inappropriate apoptosis or differentiation at key stages of development (13–15). Moreover, Tbx3-null mouse embryos exhibit reduced liver size and massive cell death of the yolk sac endoderm (13) that may be explained by a failure to regulate correctly the p19ARF–MDM2-p53-p21 pathway in the absence of Tbx3. Whether Tbx2 also may play a role in the control of proliferation or apoptosis during development will be revealed when the phenotype of Tbx2-null mice is examined.

Finally, the differential binding of *in vitro* transcribed/translated Tbx2 compared with that found in B16 cells is intriguing. One possible explanation is that Tbx2 DNA-binding specificity is affected by post-translational modifications that occur *in vivo* but that are absent from the *in vitro* transcribed/translated Tbx2. Alternatively, and perhaps more likely, the Tbx2 complex identified in B16 extracts may represent a Tbx2 heterodimer with an unidentified factor that can recognize efficiently sequences flanking the p21 Tbx2 AGGTGTTGA motif but that cannot bind the AGGTGTA motif itself. Thus, although Tbx2 homodimers produced from the *in vitro* transcribed/translated reaction would bind the consensus T-element better, a heterodimer with a non-T-box dimerization partner would prefer the p21 element. In this respect, the target gene specificity of VegT and Eomesodermin can be induced to resemble that of Xbra by introduction of a single amino acid substitution in the DNA-binding domain that would be predicted to mediate protein-protein interaction rather than protein-DNA interaction (47). Moreover, both Tbx3 and Tbx2 have been shown to bind cooperatively to target genes through interaction with the homeodomain factor Nkx2.5 (48, 49), whereas Tbx19 (T-Pit) activates the pro-opiomelanocortin promoter synergistically with the homeodomain protein PitX1 (50). It may be that a similar mechanism operates in the regulation of the p21 promoter, in which case only those T-box factors with the ability to interact with the appropriate factor will be able to regulate p21 expression. The ability of T-box factors to bind their target sequences with high affinity as heterodimers may explain why all of the known T-box factor target sites identified to date represent T-element half sites (51) and also would provide a mechanism for specificity because the choice of dimerization partner would dictate the nature of the target sites. It is also possible that the low levels of transcriptional repression by Tbx2 and other T-box factors achieved in transfection assays may arise through the limited availability of any dimerization partner required for efficient recruitment of Tbx2 to target promoters. The identification of T-box factor dimerization partners is clearly a priority if we are to identify the repertoire of genes targeted by this important transcription factor family.

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