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A Role for Tbx2 in the Regulation of the α2(1) Collagen Gene in Human Fibroblasts

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Abstract The T-box gene family encodes highly conserved transcription factors that play important roles in embryonic development and have been implicated in carcinogenesis. One member of the family, Tbx2, is generally regarded as a transcriptional repressor but appears to be capable of functioning as an activator depending on the cellular context. This study shows that Tbx2 is expressed in normal human fibroblasts but is drastically reduced in several transformed fibroblast cell lines. This pattern of Tbx2 expression correlated with that observed for the human α2(1) collagen gene (*COL1A2*). Interestingly, stable expression of transfected Tbx2 in transformed fibroblast cell lines further reduces expression of the human endogenous *COL1A2* gene. This ability of Tbx2 to repress the human *COL1A2* gene was confirmed in luciferase reporter assays and shown to be independent of the consensus T-box binding element. J. Cell. Biochem. 102: 618–625, 2007.

Key words: T-box factors; Tbx2; α(2)1 collagen gene

The T-box family of transcription factors plays a central role in embryonic development and is an important aspect of developmental biology. T-box members are expressed in specific cell types and are required for the development of their associated tissues and organs [Dobrovolskaïa-Zavadskaa, 1927]. Their important regulatory roles in development have been demonstrated by mutational studies where mutant alleles, including heterozygotes, commonly give a phenotype. In humans for example, ulnar-mammary syndrome is caused by mutations in *TBX3* [Bamshad et al., 1997], Holt-Oram syndrome is linked to haplo-insufficiency of *TBX5* [Li et al., 1997] and mutations within *TBX1* have been associated with DiGeorge syndrome [Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001].

Members of the T-box family all share a homologous region called the T-box which corresponds to their DNA binding domain. In the past decade several family members have been identified in a variety of organisms [Papaioannou and Silver, 1998] and all members tested to date recognize the palindromic response element TTT(G/C)ACACCTAGGTGTGAAA [Colon et al., 2001] with their target specificity thought to depend on accessory proteins. T-box proteins function as both transcriptional activators and repressors [Carreira et al., 1998; Tada and Smith, 2001] and their regulatory activities require sequences located in the carboxy-terminal portion of the protein.

Human *TBX2* has not yet been linked to any known genetic syndrome but has been implicated in limb, heart and mammary gland development [Rowley et al., 2004; Plageman and Yutzey, 2005; King et al., 2006]. Targeted mutagenesis to investigate *Tbx2* function in mice have shown that heterozygous mutants appear normal while homozygous mutants die between 10.5 and 14.5 days post-coitum of

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cardiac insufficiency [Plageman and Yutzey, 2005]. TBX2 has also been implicated in tumorigenesis because its expression is deregulated in many melanoma, breast and pancreatic cancers [Mahlamaki et al., 2002; Sinclair et al., 2002; Packham and Brook, 2003; Vance et al., 2005] and it can suppress senescence through a mechanism involving its ability to repress expression of the cell cycle regulatory genes, $p19^{ARF}$ and $p21^{WAF1/CIP1/SD11}$ [Jacobs et al., 2000; Prince et al., 2004]. Understanding the molecular role of Tbx2 in embryonic development and its impact on cell cycle control continues to represent a major challenge, in part, because its target genes are still poorly characterized.

Recent studies have implicated the mouse type 1 collagen gene as a potential Tbx2 target [Chen et al., 2001]. Type I collagen synthesis is crucial for normal embryonic development and in maintaining tissue integrity and its aberrant expression has deleterious effects on several biological processes [Bornstein and Sage, 1989]. Mice lacking the α1(I) collagen gene for example, display defective angiogenesis [Lohhler et al., 1984], while in humans, dominant negative mutations in any of the type I collagen genes result in diseases such as osteogenesis imperfecta and Ehlers Danlos syndrome [Kuivaniemi et al., 1991; Prockop et al., 1993, 1994]. Abnormal collagen synthesis is also associated with diseases such as arthritis, fibrosis and with tumor cell invasion and metastasis [Fenhalls et al., 1999; Fusenig and Mueller, 2004]. In an attempt to identify genes that may be regulated by Tbx2, DNA microarray analysis was performed on mouse NIH3T3 fibroblasts overexpressing Tbx2 and the results revealed that the type 1 collagen gene was upregulated [Chen et al., 2001]. Interestingly, a parallel investigation in which Tbx2 was overexpressed in the rat ROS17/2.8 osteoblastic cell line showed downregulation of type I collagen [Chen et al., 2001]. While this study suggests that Tbx2 may function as both activator and repressor, the data does not address whether Tbx2 directly mediates the transcriptional effect on the type 1 collagen gene. However, these contrasting results do suggest that the cell context and/or the species may be important in determining the effect of Tbx2 on the expression of the type I collagen genes. Here, we report a correlation between the expression patterns of endogenous Tbx2 and *COL1A2* in several human fibroblast cell lines and provide compelling evidence that Tbx2 represses expression of the human *COL1A2* gene. This study suggests that Tbx2 is involved in the regulation of the human *COL1A2* gene which has important implications for our understanding of the role of Tbx2 in development and cancer.

MATERIALS AND METHODS

Cell Culture

Human embryonic lung fibroblast WI-38 cells (ATCC CCL-75), SV40 transformed WI-38 fibroblast cells (SVWI-38) [de Haan et al., 1986), γ-radiation transformed WI-38 fibroblast cells (CT-1) [Namba et al., 1980], and human fibrosarcoma cells HT1080 (HT) (ATCC CCL-120) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2.

Generation of Stable Cell Lines Expressing Tbx2

To generate stably transfected cell lines, CT-1 and HT1080 cells were seeded in duplicate in 60-mm dishes at a density of 3.2×10^5 cells per dish, 24 h before transfection. Cells were transfected with either the empty expression vector pcDNA3.1 (+) or with this vector containing the full-length human Tbx2 cDNA [Lingbeek et al., 2002] using the standard calcium–phosphate precipitation method. Transfected cells were allowed to recover for 48 h and CT-1 and HT1080 transfectants were selected in medium containing 400 and 800 μg/ml G418, respectively. Colonies were subcloned and selected based on positive Tbx2 immunoreactivity in the case of pcDNA3.1-Tbx2 transfectants. Among subcloned cell lines, two from both the CT-1 and HT1080 cell lines were chosen for subsequent analysis. CT-Tbx2 and HT-Tbx2 contained the pcDNA3.1-Tbx2 construct while CT-E and HT-E contained pcDNA3.1 empty vector.

Plasmid Constructs

The human *COL1A2* promoter luciferase reporters were generated by inserting the EcoRV(−2389)-Hind III(+58), Xho 1(−721)-Hind III (+58), Bgl II (−375)-Hind III (+58), and Sma I (−107)-Hind III (+58) fragments of the *COL1A2* gene [Parker et al., 1992] into the

appropriately cleaved luciferase reporter vector pRL-CMV-basic (Promega) (refer to Fig. 3A). The pRL-CMV vector (Promega) was used as an internal control reporter to test for transfection efficiency. The pcDNA3.1-Tbx2 expression vector was kindly provided by Dr. Merel Lingbeek [Lingbeek et al., 2002].

Transient Transfection Assay

Cells were plated at 1.5×10^5 cells/ml in six-well plates 1 day before transfection. Non-liposomal mediated gene transfer was performed using FuGENE®6 (Roche Applied Science) according to manufacturer's instructions using 1 μg of DNA consisting of the reporter construct, the Tbx2 expression vector and the internal control vector. Thirty hours after transfection, cells were analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay (Promega) following manufacturer's instructions and quantified with a Luminoskan Ascent Luminometer (Thermo LabSystems).

Western Blot Analyses

Cells were harvested and solubilized at 4°C with lysis buffer (40 mM Tris-HCl, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1% Nonidet P40, 0.1% SDS, and protease inhibitors), incubated on ice for 30 min and centrifuged at 12,000 rpm for 20 min at 4°C. Protein concentrations in lysates were determined using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Twenty micrograms of protein extract were separated on a 10% SDS–PAGE gel and then transferred onto nitrocellulose Hybond-C membrane (Amersham). Following blocking for 1 h at room temperature, the membranes were probed with mouse monoclonal anti-Tbx2 primary antibody (62–2; 1:2,500). Immunoreactive bands were visualized with a horseradish peroxidase-conjugated secondary goat anti-mouse serum (1:4,000) (Biorad) and detected with enhanced chemiluminescence (ECL) (Pierce). Monoclonal mouse anti-α-tubulin (1:500, Santa Cruz Biotechnology) and rabbit polyclonal anti-p38 (1:5,000, Cell Signaling Technology, Inc., Beverly, MA) primary antibodies were used for normalization.

Northern Blot Analyses

Total RNA was extracted from cultured cells using Trizol reagent (Life Technologies). RNA concentration was determined by spectrophotometric absorbance at 260 nm, and 5 μg was separated by electrophoresis in 1% formaldehyde–agarose gels. Gels were then transferred by capillary action to nylon membranes (Amersham). Membranes were probed with random-primed ^{32}P-radiolabeled (Amersham) *Col1A2* and β-actin cDNA fragments. Hybridization was carried out in the ULTRAhyb buffer (Ambion, UK) following the manufacturer's instructions.

Microscopy

Cells grown on glass coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min and permeabilized in 0.2% Triton-X-100 in phosphate buffered saline (PBS) for 10 min. Cells were incubated overnight with mouse Tbx2 monoclonal antibody (62–2) at a dilution of 1:750 and then incubated with the appropriate secondary antibody coupled to alexa 488 (Molecular Probes) at a 1:1,000 dilution. Cells were incubated in the dark with 1 μg/ml DAPI in PBS for 10 min, mounted on a slide and visualized by fluorescence microscopy.

Real Time (RT)-PCR

Total cellular RNA was extracted as described for Northern blot analyses. Reverse transcription was carried out using Superscript III Reverse Transcriptase (Invitrogen), with oligo(dT)$_{20}$ primers. PCR was conducted using 2 μl of a 1 in 10 dilution of the cDNA and primers (*COL1A2*: forward 5′-GATTGAGACCCTTCTTACTCCTGAA-3′; reverse 5′-GGGTGGCTGAGTCTCAAGTCA-3′; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5′-GAAGGCTGGGGCTCATTT-3′; reverse 5′-CAGGAGGCATTGCTGATGAT-3′) with LightCycler FastStart DNA Master^{PLUS} SYBR green 1 kit (Roche) according to the manufacturer's protocol. Real-time PCR was carried out on a LightCycler Version 3 (Roche) with the following protocol: 95°C for 10 min; and 45 cycles of 95°C for 10 s (denaturation), 55°C for 5 s (annealing), and 72°C for 10 s (extension), followed by a standard melting curve program. Each DNA sample was quantified in duplicate and a negative control without cDNA template was run with every assay to assess the overall specificity. Melting curve analyses was carried out to ensure product specificity and data was analysed using the $2^{-\Delta\Delta Ct}$ method. COL1A2 mRNA levels were normalized to GAPDH with

PCR efficiency correction calculated using the formula ratio $= (E_{target})^{CP_{target}(control-sample)} / (E_{ref})^{CP_{ref}(control-sample)}$; E: real-time PCR efficiency, CP: crossing-point (Pfaffl, 2001).

RESULTS

Correlation Between Tbx2 and COL1A2 Expression Patterns in Fibroblast Cell Lines

To determine whether Tbx2 can regulate collagen gene expression, we firstly investigated whether Tbx2 was expressed in normal and transformed fibroblast cell lines. The results of Western blot analyses (Fig. 1A) show that Tbx2 was expressed at high levels in the normal WI-38 fibroblast cell line, was dramatically reduced in WI-38 cells transformed by exposure to cobalt radiation (CT-1) and was almost undetectable in the SV40-transformed WI-38 cells (SVWI-38) and in human fibrosarcoma cells (HT1080). Interestingly, this pattern of Tbx2 expression followed the same trend as that obtained for COL1A2 mRNA (Fig. 1B). This direct correlation initially suggested that Tbx2 may play a role in activating the human *COL1A2* promoter.

Overexpression of Tbx2 Reduces COL1A2 mRNA Levels

To determine whether Tbx2 regulates endogenous *COL1A2* gene expression, we established CT-1 and HT1080 cell lines that stably express exogenous Tbx2. The CT-1 cell line was selected because it had reduced Tbx2 protein levels and reduced COL1A2 mRNA compared to the normal parental WI-38 cells. The HT1080 cell line, on the other hand, was chosen to determine whether the introduction of Tbx2 would be sufficient to induce *COL1A2* gene expression because it lacked detectable levels of COL1A2 mRNA. A number of G418-resistant clones were tested for Tbx2 expression. Figure 2A shows the presence of Tbx2 protein in representative clones (CT-Tbx2(2), CT-Tbx2(3), HT-Tbx2(5), and HT-Tbx2(6)) that is undetectable in cells transfected with the empty vector (CT-E and HT-E). This result was confirmed by immunocytochemistry (Fig. 2B) where predominant staining was seen in the nuclei of CT-Tbx2 and HT-Tbx2 cell lines while low levels were detected in CT-E cells and no immunostaining was observed in the control HT1080 cell line harboring empty vector pcDNA (data not shown). We selected the CT-Tbx2(2), CT-Tbx2(3), HT-Tbx2(5), and HT-Tbx2(6) cell lines to investigate the effect of Tbx2 on endogenous *COL1A2* using qRT-PCR. Our results, shown in Figure 2C, indicate that the COL1A2 mRNA levels were repressed by approximately twofold in the two CT-Tbx2 cell lines compared to the control CT-E cells. This suggests that overexpression of Tbx2 leads to downregulation of *COL1A2* gene expression. Compared to CT-1 cells, the HT-E and parental HT1080 cell lines displayed 2000-fold less *COL1A2* expression levels (data not shown) and there was a negligible change in expression in the HT-Tbx2(5) and HT-Tbx2(6) cell lines. These results confirmed that the *COL1A2* gene is repressed independently of Tbx2 in the

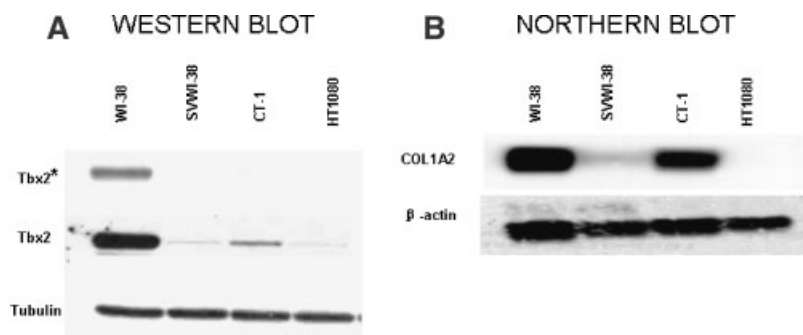

Fig. 1. Tbx2 protein and COL1A2 mRNA exhibit similar patterns of expression in normal and transformed human fibroblast cell lines. **A**: Western blot analyses were used to compare the relative levels of Tbx2 protein in normal embryonic lung fibroblasts (WI-38 cell line), two of its transformed counterparts (SVWI-38 and CT-1) and a naturally occurring fibrosarcoma cell line (HT1080). Protein blots were probed with antibodies specific for Tbx2 and tubulin and detected by enhanced chemiluminescence as described in Materials and Methods. (*) Indicate a short exposure of the blot. **B**: Northern blot analyses, using total RNA (5μg) from cell lines described in (A) were used to compare the levels of COL1A2 mRNA. Blots were hybridized with probes to COL1A2 and β-actin.

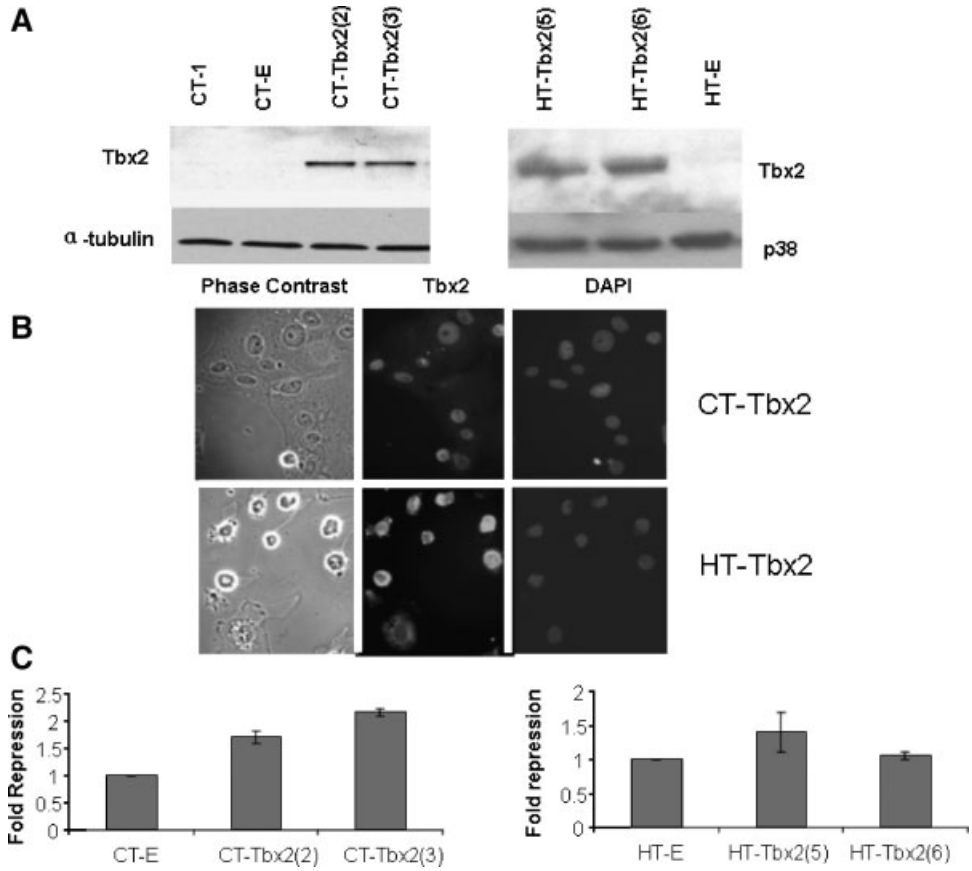

Fig. 2. Tbx2 represses endogenous *COL1A2*. CT-E, CT-Tbx2, HT-E, and HT-Tbx2 cell lines were established by stable transfection of CT-1 and HT1080 cells with either an empty vector or a Tbx2-expression plasmid followed by G418 selection. **A**: Western blot and (**B**) immunohistochemical analyses were used to confirm that the CT-Tbx2 and HT-Tbx2 cell lines express readily detectable levels of Tbx2 protein. Both tubulin and total p38 levels were used as loading controls as similar studies have shown that both markers give comparable results. **C**: Quantitative real-time PCR was performed to establish the effect of stably expressing Tbx2 on *COL1A2* in CT-1 and HT1080 cells. C: Total RNA was extracted from CT-1, CT-E, CT-Tbx2(2), CT-Tbx2(3), HT-E, HT-Tbx2(5), and HT-Tbx2(6). Quantitative real-time PCR was then performed on reverse transcribed RNA using primers specific to COL1A2 and mRNA levels were normalized to GAPDH.

HT1080 cells. Taken together these results raised the question of whether Tbx2 was able to directly regulate expression of the *COL1A2* gene.

Tbx2 Represses COL1A2 Promoter Activity

Tbx2 has been shown to regulate target gene expression by binding the consensus T-box binding site GTGTGA as well as the GTGTTA and GGGTGA sequences. We therefore screened the 5′ upstream regulatory region of the human α2(I) collagen gene for these sites and located a GTGTGA sequence at position −1400. To determine whether Tbx2 downregulates the *COL1A2* gene directly through this site, we performed transient transfection assays using a luciferase reporter driven by 5′ deletion constructs of the human *COL1A2* gene promoter (Fig. 3A). HT1080 cells were initially used in these assays because they do not have detectable levels of endogenous Tbx2 protein or collagen mRNA. The transfection data showed that wild-type Tbx2 acts as a strong dose-dependent repressor of the *COL1A2* promoter (Fig. 3B). All four human *COL1A2* promoter-deletion constructs tested appear to be strongly repressed by Tbx2 suggesting that Tbx2 acts through an element located in the region −107 to +50 of the human *COL1A2* promoter and not through the putative binding site at position −1400. Similar results were obtained in CT-1 cells that have detectable levels of COL1A2 mRNA and Tbx2 protein (Fig. 3C). Further investigation of the Tbx2 response element by deletion mapping was prevented due to loss of basal promoter activity.

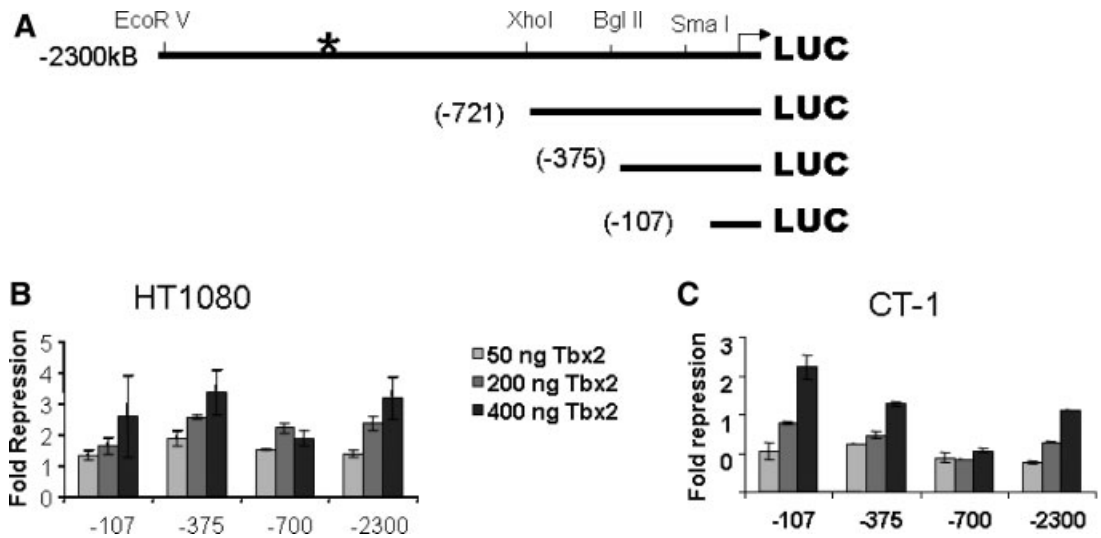

Fig. 3. Tbx2 represses *COL1A2* promoter activity. **A**: Schematic representation of *COL1A2* 5′ deletion constructs generated by restriction enzyme digestion. The arrow indicates the transcription start site at +1 and the (*) indicates the putative Tbx2 binding site (GTGTGA) at −1400. The plasmids containing sequentially deleted fragments of p(−2300)Luc were transiently transfected into the HT1080 (**B**) and CT-1 (**C**) cell lines together with increasing amounts of the Tbx2 expression plasmid pCMV-Tbx2. Total amount of plasmid DNA transfected was held constant using the corresponding empty vector, pCMV. The plasmid pRL-TK containing the Renilla luciferase reporter gene was also introduced to normalize transfection efficiency. Promoter activity is indicated as fold repression which represents the ratio of the luciferase activity generated by the pCMV empty vector (without Tbx2) to that obtained in the presence of pCMV-Tbx2.

DISCUSSION

Collagen is the most abundant protein in nature and plays a very important role during development as it forms the basis for skin, cartilage, bone, and connective tissue [Kadler et al., 1996]. The transcription factor, Tbx2, is essential for normal embryonic development, most probably by regulating the expression of developmentally important genes such as collagen. Although the expression patterns of Tbx2 during development have been widely studied, only a few of its target genes have been identified to date [Carreira et al., 1998; Jacobs et al., 2000; Chen et al., 2001; Prince et al., 2004]. This study shows that Tbx2 is expressed in human lung fibroblasts and is downregulated in transformed fibroblasts in a manner which mimics the expression pattern for the *COL1A2* gene. This initial observation suggested that Tbx2 may be acting as a positive regulator of the *COL1A2* gene. However, further investigations show that Tbx2 is in fact a negative regulator of the *COL1A2* gene and that it is able to directly repress its expression in the absence of the published consensus T-element. This result is consistent with that obtained by Jacobs et al. (2000) who also found that Tbx2 was able to repress the $p19^{ARF}$ promoter in the absence of a putative T-box binding site. Taken together, these results suggest that Tbx2 may be functioning as a co-repressor of the *COL1A2* gene which is in keeping with the suggestion that the target specificity of T-box family members depends on their association with different cofactors.

In addition to its importance in embryonic development, Tbx2 has also been implicated in melanoma, breast, and pancreatic cancers [Mahlamaki et al., 2002; Sinclair et al., 2002; Packham and Brook, 2003; Vance et al., 2005]. Its involvement in tumorigenesis is, however, poorly defined but is possibly associated with its anti-senescence function, through its ability to repress cyclin dependent kinases, $p19^{ARF}$ and $p21^{WAF1/CIP1/SD11}$ [Jacobs et al., 2000; Prince et al., 2004]. Additionally, it is not clear whether Tbx2 is involved in the initiation and/or progression of cancer. Interestingly, tumor invasion and metastasis require breakdown of collagen and possibly a reduction in collagen production [Liotta et al., 1979; Christner and Ayitey, 2006; Tanjore and Kalluri, 2006]. It would be important to clarify whether the ability of Tbx2 to repress *COL1A2* has any significance in these processes. We cannot, however, rule out the possibility that the repression of the endogenous *COL1A2* gene in

the CT-Tbx2 cell lines may have resulted from other alterations induced by Tbx2. The effect of Tbx2 on the *COL1A2* promoter in luciferase assays would however suggest that at the very least Tbx2 is able to directly regulate the expression of this gene as a co-repressor. Identifying candidate Tbx2 accessory proteins involved in regulating *COL1A2* gene expression may provide important clues as to the precise mechanism by which Tbx2 regulates this important gene.

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