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The Role of TWIST as a Regulator in Giant Cell Tumor of Bone

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ABSTRACT

Giant cell tumor of bone (GCT) is an aggressive tumor consisting of multinucleated osteoclast-like giant cells and proliferating osteoblast-like stromal cells. Our group has reported that the stromal cells express high levels of the bone resorbing matrix metalloproteinase (MMP)-13, and that this expression is regulated by the osteoblast transcription factor Runx2. The purpose of this study was to determine the upstream regulation of Runx2 in GCT cells. Using GCT stromal cells obtained from patient specimens, we demonstrated that TWIST, a master osteogenic regulator, was highly expressed in all GCT specimens. TWIST overexpression downregulated Runx2 expression whereas TWIST siRNA knockdown resulted in Runx2 and MMP-13 upregulation. Interestingly, cells obtained from a GCT lung metastasis showed a reverse regulatory pattern between TWIST and Runx2. In mutational analysis, we revealed a point mutation (R154S) at the Helix2 domain of TWIST. This TWIST mutation may be an essential underlying factor in the development and pathophysiology of these tumors in that they lead to inappropriate TWIST downregulation of Runx2, arrested osteoblastic differentiation, and the maintenance of an immature and neoplastic phenotype. J. Cell. Biochem. 112: 2287–2295, 2011.

KEY WORDS: TWIST; Runx2; GIANT CELL TUMOR; PRIMARY CELL CULTURE; siRNA; TRANSFECTION

Giant cell tumor of bone (GCT) is an aggressively osteolytic and potentially metastatic bone tumor. It typically affects the epiphyseal regions of the long bones such as the distal femur, the proximal tibia, and the distal radius, prompting the formation of a local osteolytic lesion [Turcotte et al., 1993]. High recurrence rates of 18–60% have been reported for GCT, which occasionally undergoes malignant transformation [McDonald et al., 1986; Katz et al., 1987; Rock, 1990; Ghert et al., 2002]. Histologically, GCT consists of three major cell types: a high number of osteoclast like multinucleated giant cells, monocytic round cells, and spindle-shaped mesenchymal stromal tumor cells of osteoblastic lineage [Zheng et al., 2001; Wuelling et al., 2002]. Cell culture experiments have shown that the osteoblast-like mesenchymal stromal cells are the only proliferating component of GCT [Goldring et al., 1986]. Previous work in our lab has shown that these cells express very high levels of bone-resorbing matrix metalloproteinase (MMP)-13, and that this expression is regulated by the Runx2 transcription factor [Mak et al., 2009]. The intrinsic upstream regulation of Runx2 in the stromal cell of GCT remains unknown.

Runx2 is a transcription factor that belongs to the runt domain gene family, a 128 amino acid DNA-binding domain conserved from fugu to human [Komori and Kishimoto, 1998]. Runx2 is a master osteogenic regulator and acts as an inducer and regulator of osteoblast differentiation in the osteoblast lineage [Komori and Kishimoto, 1998; Bialek et al., 2004]. Aside from the basic role in regulating bone formation through osteoblast differentiation, Runx2 is also required for osteoblast function [Ducy et al., 1999]. It has also been shown to be important in the vicious cycle of metastatic bone disease by allowing cancer cells to resorb bone [Lian et al., 2006].

The expression of Runx2 in osteoblasts is regulated by a number of transcription factors. The C-terminus of the TWIST transcription factor is a Runx2 binding site known as the "TWIST box." The binding of Runx2 to the TWIST box inhibits Runx2 function during

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skeletal development [Bialek et al., 2004]. TWIST, a helix-loop-helix transcription factor, is expressed in mesodermal and cranial neural crest cells during embryogenesis [Thisse et al., 1987; Olson, 1990]. Several studies have shown that TWIST has an important role in bone development and is expressed in primary osteoblastic cells [Murray et al., 1992] and preosteoblasts [Rice et al., 2000]. TWIST expression is reduced with maturation of these cells. TWIST may play a role during early phase osteogenic differentiation and may act as a key switch for bone cell differentiation. Although most studies have suggested that TWIST functions to maintain the osteogenic lineage by preserving more osteoprogenitor features and inhibit terminal differentiation of osteoblasts, its exact functions during osteoblast differentiation still remain elusive and may be cell-type specific [Ishii et al., 2003; Komori, 2006]. In cancer cells, TWIST has been reported as a master regulator for metastasis by promoting epithelial-to-mesenchymal transition (EMT) in an in vivo system [Yang et al., 2004].

The objectives of this study were to determine the role of TWIST in the regulation of Runx2 in the osteoblast-like stromal cells of GCT, and to identify TWIST mutagenesis in these cells. We hypothesize that abnormal TWIST regulation of Runx2 in GCT stromal cells may be responsible for arrested differentiation and an immature, neoplastic phenotype.

MATERIALS AND METHODS

PRIMARY CELL LINES CULTURE AND TRANSFECTION

We established primary cell cultures of GCT stromal tumor cells from fresh GCT tissue obtained from 11 patients following the Ethics Board approval and patient consent. The tissue was processed and maintained in DMEM containing 10% FBS, 2 mM glutamine, and 100 U/ml antibiotics. The resulting cell suspension together with macerated tissue was cultured in 37°C humidified air with 5% CO_2. Following several successive passages, the mesenchymal stromal cells became the homogeneous cell type whereas the multinucleated giant cells were eliminated from culture. GCT cells were transfected using an electroporation method. Briefly, cells were trypsinized and washed with PBS and resuspended into 1 ml FBS-free media with 35 μg of the TWIST plasmid. Cells with the TWIST plasmid mixture were electroporated using the Gene Pulser II electroporation apparatus (Bio-Rad Laboratories) with optimized combinations of voltage and capacitance. The cells were then plated in 10 cm Petridishes with supplemented DMEM. After 48-h posttransfection, cells were harvested for RNA isolation. Human fetal osteoblast (hFOB) 1.19 cells (American Type Culture Collection, ATCC# CRL-11372) were used as a control cell line. The hFOB cell line is a clonal, conditionally immortalized human fetal cell line capable of osteoblastic differentiation and bone formation.

PLASMIDS CONSTRUCTION

A TWIST open-reading frame was amplified by PCR from the Origene (Rockville, MD) using oligonucleotides cgcggatccgcgatgatgcaggacgtgtcc and ccggaattccggctagtgggacgcgacat, containing *Bam*HI and *Eco*RI restriction sites, respectively. After *Bam*HI and *Eco*RI digestion, the open-reading frame was ligated into a pcDNA3.1 vector. For immunofluorescence microscopy, TWIST cDNA was subcloned into a pDest 51-V5-HIS tag vector (Invitrogen) using the clonase enzyme and confirmed by sequencing.

siRNA TRANSFECTION

Mesenchymal stromal cells of GCT were trypsinized and transfected with TWIST small interfering RNAs (siRNAs) via electroporation. Stromal cells of GCT were washed and resuspended in Optimem reduce serum media (Gibco, Invitrogen, Canada). Subsequently, the cell suspension was mixed with 200 nM of TWIST siRNA (Invitrogen), a positive Silencer™ siRNA control against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or a non-specific negative control#1 (Ambion, Inc.). Stromal cells with the siRNA mixture were electroporated using the Gene pulser II electroporation apparatus (Bio-Rad Laboratories) under a single-pulse protocol with optimized combination of voltage and capacitance. Cells were then plated in 10 cm cell culture dishes with supplemental DMEM. After 48 h of transfection, cells were harvested for RNA isolation. β-actin was selected among other housekeeping genes for normalization in real-time PCR analysis and GAPDH was used as a positive siRNA control.

RNA ISOLATION AND RT-PCR

Total RNA extraction was performed by TRIZOL Reagent (Sigma, MO) according to the manufacturer's protocol. The resuspended RNA samples were treated with ribonuclease-A (RNase A)-free *DNase*I for 1 h at 37°C to remove residual genomic DNA. Concentration and purity of the samples were evaluated by the ratio of optical density (OD) 260:280 by a spectrophotometer. One microgram of total RNA was incubated with 2 ml primer cocktail at 68°C and subjected to reverse transcription (RT) using Superscript III reverse transcriptase (Invitrogen) for cDNA synthesis. The samples were either used directly for PCR or stored at −20°C. PCR was carried out using Prime Taq Premix and reactions were carried out in the PCR thermal cycler (Applied Biosystems, Foster City, CA). Following initial denaturation at 95°C for 5 min, PCR was performed at 95°C for 30 s, at specific annealing temperature (55–62°C) for 30 s, and 72°C for 30 s. Expression of GAPDH was used as an internal control. Primer sequences are listed in Table I.

QUANTITATIVE RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed by real-time monitoring of increases in fluorescence of the SYBR Green dye (Molecular Probes, Eugene, OR) using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). For comparison of transcript levels between samples, a standard curve of cycle thresholds for serial dilutions of a cDNA sample was established and then used to calculate the relative abundance of each gene. Values were then normalized to the relative amounts of GAPDH cDNA, which were obtained from a similar standard curve. All PCR reactions were performed in triplicate experiments.

STATISTICAL ANALYSIS

Statistical analyses for the real-time PCR were performed using the two-sample independent Student's *t*-test. The average value within each experiment was expressed relative to the expression of internal

TABLE I. Human Primer Sequences Designed for Real-Time PCR Amplification

Gene	Primers	Primer sequence	Accession #	Product size (bp)
Runx2	F	TCTGGCCTTCCACTCTCAGT	NM_004348	142
	R	AAGGTGGCTGGATAGTGCAT		
RPS18	F	GATGGGCGGCGGAAAATA	NM_022551	165
	R	GCGTGGATTCTGCATAATGGT		
GAPDH	F	CATGAGAAGTATGACAACAGCCT	NM_002046	113
	R	AGTCCTTCCACGATACCAAAGT		
β-actin	F	GGTCATCACCATTGGCAATG	NM_003019	97
	R	GGTAGTTTCGTGGATGCCACA		
TWIST	F	TACATCGACTTCCTCTACCAGGTC	NM_000474	120
	R	TAGTGGGACGCGGACATGGA		

control gene. *P*-value of <0.05 were considered to be statistically significant.

PROTEIN EXTRACTION AND WESTERN IMMUNOBLOT ANALYSIS

To confirm the overexpression and knockdown of TWIST in transfected GCT cells, we performed immunoblot analysis. Cytoplasmic fractions were isolated from these transfected cells by scraping after 24 h of incubation and then centrifuged for 5 min. The cells were lysed with NP-40 containing lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40) to disrupt the cell membrane and then the cell lysate was centrifuged at 500*g* for 5 min at 4°C. The supernatant (cytoplasmic fraction) was removed and the pellet (nuclear fraction) was resuspended in NP-40 containing cell lysis buffer. Proteins were denatured by boiling in sample buffer, separated on 12% SDS–PAGE and then transferred onto the PVDF membrane (Immobilon TM-PSQ, Millipore) and blocked overnight in 5% non-fat powdered milk in TBST (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween-20). Mouse monoclonal anti-TWIST antibody (1:1,000 diluted in TBST) (Abcam) was used for protein detection. Peroxidase conjugated goat anti-mouse IgG (1:5,000 diluted in TBST) (Sigma) was used as a secondary antibody.

MUTATIONAL ANALYSIS

Three GCT specimens were analyzed for mutational analysis. One microgram of total RNA was incubated with 2 ml primer cocktail at 68°C and subjected to RT using Superscript III reverse transcriptase (Invitrogen). For cDNA synthesis, the *TWIST* gene was amplified in two parts and sequenced directly on both strands and analyzed by the Sequencer 4.10.2 Program.

IMMUNOFLUORESCENCE ASSAY

Cells were grown on cover slips and were fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 for 5 min. Subsequently, these slides were incubated for 1 h at room temp with mouse monoclonal anti-V5 and rabbit polyclonal anti-Twist antibody. Slides were further incubated in secondary antibody (Texas red conjugated goat-anti rabbit and alexa 488 goat anti-mouse antibodies) for 1 h at room temperature. Slides were washed and incubated with DAPI for 3 min at room temperature and mounted with 50% glycerol.

RESULTS

TWIST mRNA EXPRESSION IN GCT STROMAL CELLS

We first determined the endogenous expression of TWIST in GCT stromal cells obtained from the patient samples. TWIST expression was analyzed with semi-quantitative RT-PCR using TWIST-specific primers. GAPDH was used as an internal control. TWIST expression was detected in the control cell lines hFOB and 11 GCT cell lines (Fig. 1A). We further analyzed the expression of TWIST by quantitative real-time PCR. Interestingly, quantitative real-time PCR analysis revealed relatively high expression of TWIST in all 11

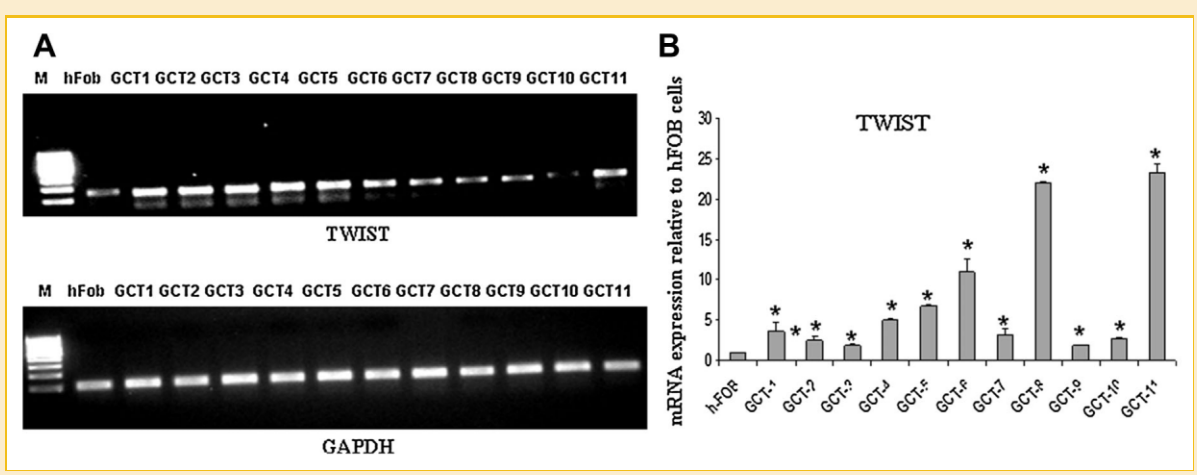

Fig. 1. TWIST expression in GCT samples by semi-quantitative and qPCR. A: Endogenous expression of TWIST in GCT stromal cells using TWIST-specific primers. B: Real-time PCR of cDNA from GCT stromal cell lysates showing expression of TWIST relative to hFOB 1.19 cells ("hFOB"). Results are the average of three replicate experiments ($^*P < 0.01$).

GCT stromal cell lines compared to hFOB 1.19 cells using the two-sample independent Student's *t*-test ($P \leq 0.01$) (Fig. 1B).

DOWNREGULATION OF Runx2 EXPRESSION IN RESPONSE TO TWIST OVEREXPRESSION

For further study, two GCT cell lines were selected from metastatic and non-metastatic cell lines. GCT-1 represents a metastatic cell line, while GCT-2 represents a non-metastatic cell line. We transfected TWIST-pcDNA3.1 and V5-HIS tagged TWIST constructs to determine the regulatory relationship between TWIST and Runx2 in primary GCT mesenchymal stromal cells. To facilitate monitoring of the cellular localization of TWIST, we generated a TWIST-V5-HIS fusion construct and electroporated as described in the Materials and Methods Section. Anti-V5 antibody was used to detect the TWIST protein in GCT cells. The recombinant nuclear TWIST protein localized in the nucleus of transiently transfected GCT stromal cells (Fig. 2A). Transfection efficiencies and anti-V5 localization were similar across both GCT cell lines. Both PCR (Fig. 2B) and Western blot data (Fig. 2C) confirmed successful transfection and over-expression of TWIST in GCT cells. TWIST mRNA levels were elevated >8-fold in GCT-1 and >3-fold in GCT-2 transfected cells when compared to GCT untransfected cells.

We transfected the TWIST mammalian expression construct into GCT mesenchymal stromal cells to observe any role that TWIST may have in Runx2 expression in these cells. TWIST overexpression significantly reduced the expression of Runx2 in GCT stromal cells (Fig. 2D, $P < 0.05$) when compared to untransfected GCT cells at the mRNA level. Runx2 expression was undetectable at the protein level in TWIST transfected and untransfected cells (data not shown). These results indicate that TWIST is an upstream suppressor of Runx2 expression in GCT stromal cells and that basal expression of the Runx2 protein is very low when TWIST is expressed.

UPREGULATION OF Runx2 IN RESPONSE TO TWIST KNOCKDOWN

RNA interference (RNAi) directed against TWIST was used to knockdown TWIST endogenous expression in both representative GCT stromal cells lines. Real-time PCR (Fig. 3A) and Western blot data (Fig. 3B) indicated successful knockdown of TWIST in both GCT

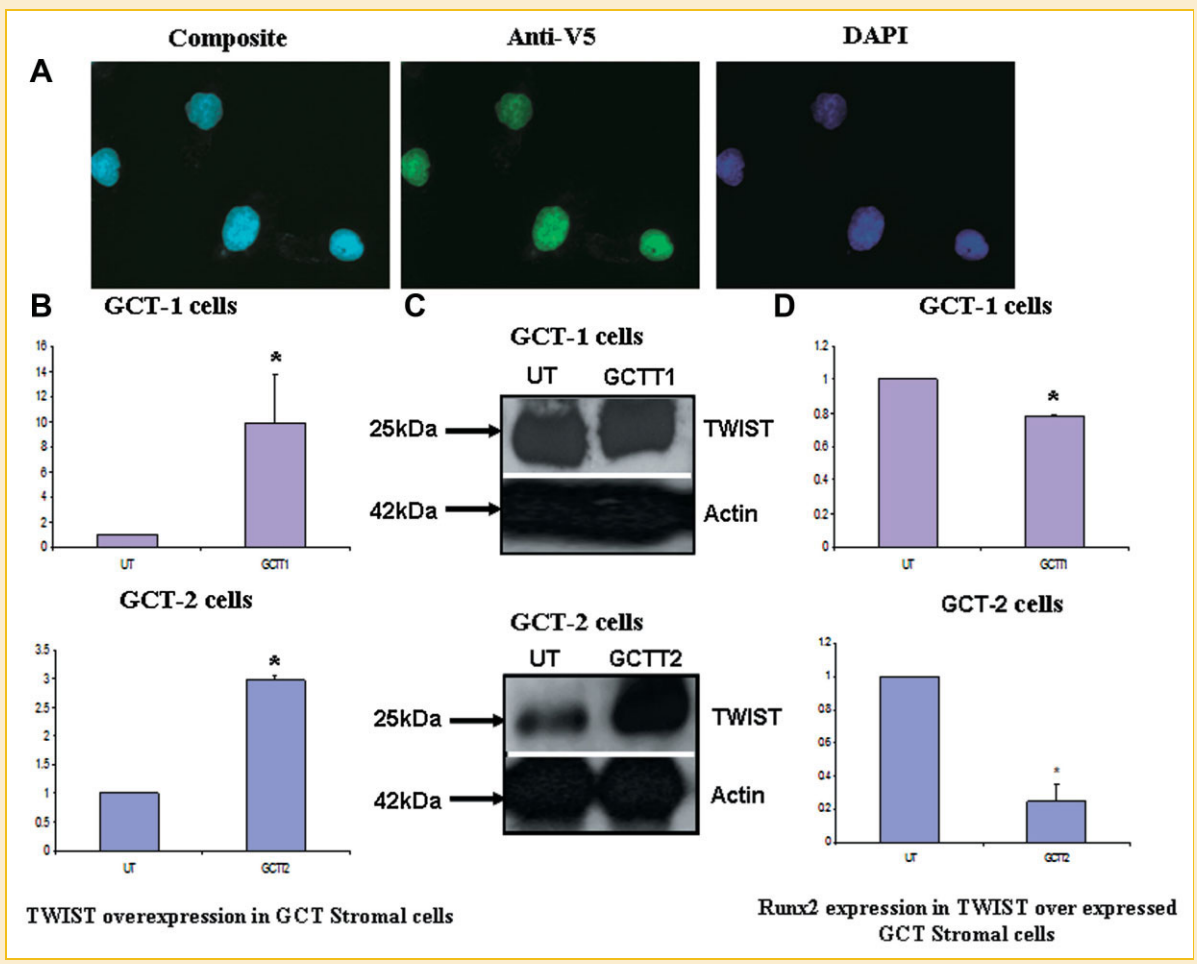

Fig. 2. A: TWIST transfection in GCT stromal cells and Runx2 expression in TWIST overexpressing cells. Expression of V5-His-TWIST protein was detected in transfected GCT of bone using anti-V5 mouse monoclonal antibody. DAPI staining (blue) indicates the nuclei (oil immersion; 100× magnification). B: Quantitative RT-PCR representing TWIST overexpression in TWIST transfected GCTT1, GCTT2 and untransfected (UT) GCT stromal cells. C: Western blot analysis representing TWIST overexpression in TWIST transfected GCTT1 and GCTT2 cell lines, and untransfected (UT) GCT stromal cells. D: TWIST overexpression resulted in a 20% decrease of Runx2 expression in GCTT1 and a 70% decrease of Runx2 expression in GCTT2 stromal cells ($^{*}P < 0.05$).

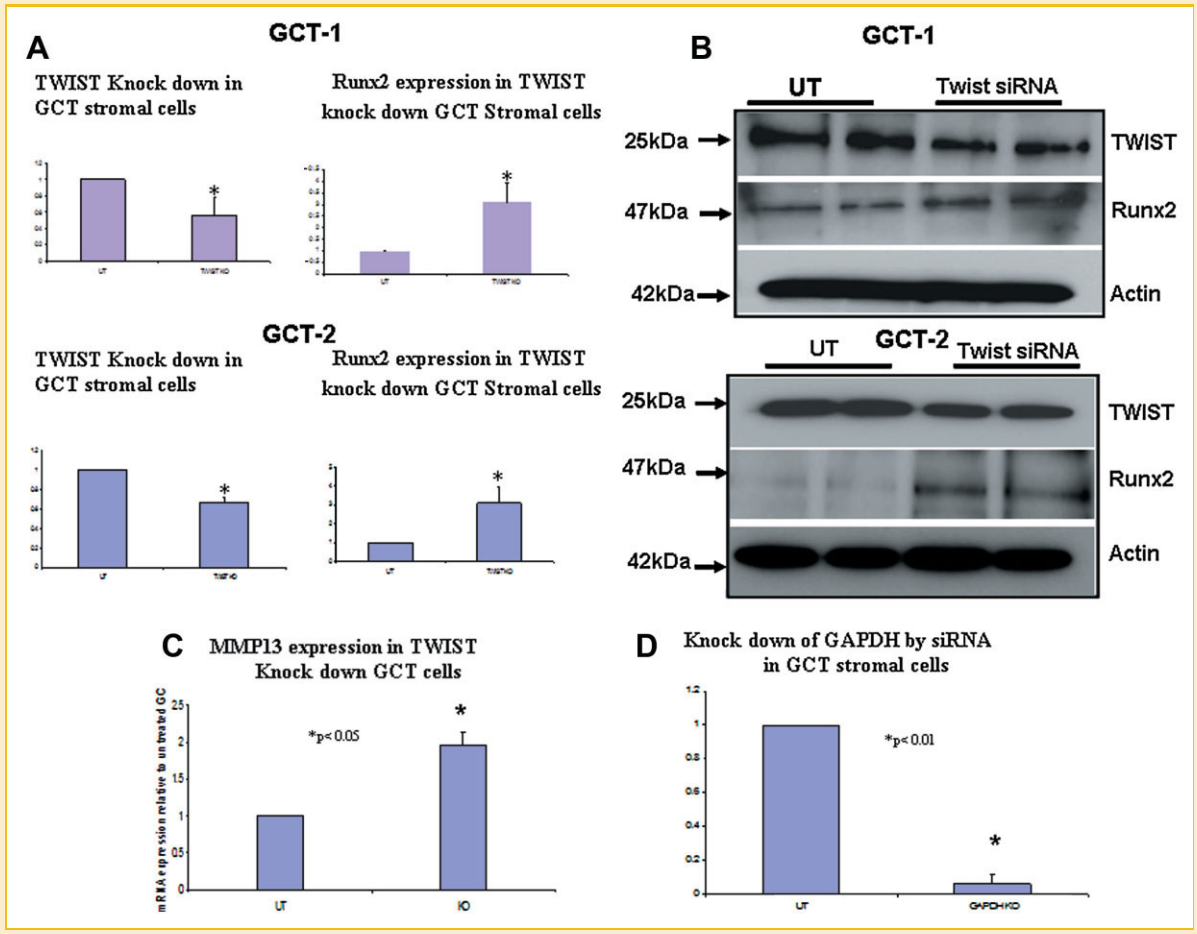

Fig. 3. A: The effect of TWIST siRNA on mRNA expression in the mesenchymal stromal cells of GCT. TWIST siRNA transfection resulted in a 50% decrease in TWIST expression in GCT1 and a 40% decrease in GCT2 cells. qRT-PCR was used to represent the expression of TWIST in untreated (UT) and TWIST knockdown (TWIST KO) in GCT cells. Results are the average of three replicate experiments ($^*P < 0.01$). TWIST knockdown resulted a threefold increase of Runx2 expression in both GCT1 and GCT2 cells. B: Western blot showing the TWIST protein levels and increased Runx2 protein levels in GCT1 and GCT2 cells transfected with TWIST siRNA compared to untreated GCT cells. C: Quantitative RT-PCR representing MMP-13 expression in a TWIST knockdown GCT stromal cell line. TWIST knockdown resulted in a twofold increase of MMP-13 expression in GCT stromal cells. D: Knockdown of GAPDH by siRNA was used as a positive control ($^*P < 0.05$).

cells. Quantitative analysis revealed that knockdown of TWIST resulted in a threefold elevation in Runx2 expression in GCT cells (Fig. 3A, $P < 0.01$) and was confirmed by Western blot analysis (Fig. 3B). We next evaluated the effect of TWIST knockdown on MMP-13 expression. We observed an increase in the expression of MMP-13 following TWIST knockdown (Fig. 3C). This generic alteration did not affect GAPDH or RPS18 mRNA levels (data not shown). GAPDH siRNA was used as a positive control to determine transfection efficiency. GAPDH mRNA was decreased approximately 80% when treated with GAPDH siRNA (Fig. 3D) but was found unaffected when treated with random and TWIST siRNA. Interestingly, stromal cells from a lung metastasis of GCT showed *decreased* Runx2 expression (Fig. 4A–C) in response to knockdown of TWIST as determined by real-time PCR and Western blot.

MUTAGENESIS AND CORRELATION TO LOCALIZATION

Localization of many transcription factors has been shown to be an important mechanism in the regulation of protein functions. TWIST gene mutations are associated with the Saethre-Chotzen syndrome phenotype characterized by premature fusion of coronal sutures. To date, all point mutations are located within the first coding exon, and most of these affect the DNA binding and the HLH domains, which until recently have been considered the main functional domains [Gripp et al., 2000]. We detected a novel mis-sense mutation within the basic HLH (bHLH) domain at amino acid 154 substituted arganine to serine (R-S) of the *TWIST* gene which may cause protein mislocalization in the two patient specimens examined (Fig. 5).

SUBCELLULAR LOCALIZATION OF TWIST IN GCT CELLS

Transcription factor proteins are nuclear proteins able to interact with gene regulatory sequence to activate gene expression. To facilitate monitoring of cellular localization of TWIST, immunofluorescence assays were performed using anti-TWIST antibody. We observed that TWIST localization in the GCT stromal cells is not restricted to the nucleus, but is also detected in the cytoplasm to varying amounts and this pattern varied among patient samples (Fig. 6).

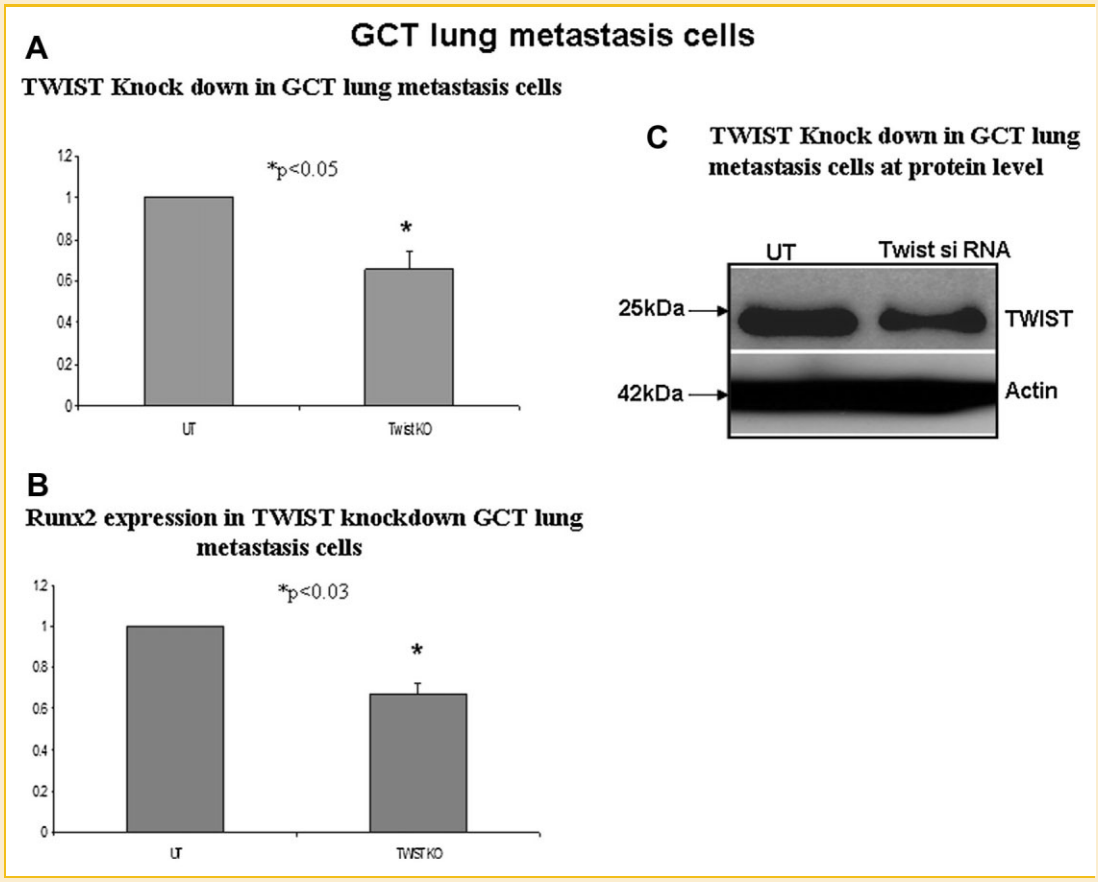

Fig. 4. The effect of TWIST siRNA on mRNA expression in the GCT lung metastatic cells. A: Quantitative RT-PCR representing Runx2 expression in a TWIST knockdown GCT lung metastatic cell line (lung metastasis from patient with GCT1) representing approximately 35% TWIST knockdown in ($^{*}P < 0.05$). B: Real-time PCR showing a similar decrease in Runx2 expression at the mRNA level in TWIST knockdown cells. C: Western blot analysis showing decreased TWIST protein levels in the GCT lung metastasis cell line when compared to GCT lung metastasis untransfected cells.

DISCUSSION

The oncogenesis of GCT is still unknown, as the neoplastic cell appears to be a preosteoblast that does not undergo terminal differentiation [Ghert et al., 2007]. In this study, we examined the role of TWIST in the regulation of Runx2 in GCT, thereby elucidating the upstream regulation of arrested osteoblast differentiation in GCT stromal cells.

Runx2 is one of the earliest and is the most specific marker of osteoblast differentiation. It is a key activator of osteogenic differentiation *via* both enchondral and intramembranous pathways [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Ducy and

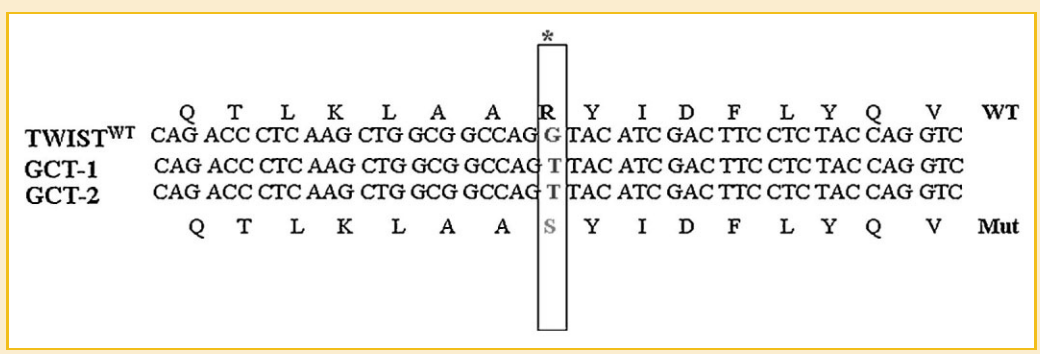

Fig. 5. Mutational analysis. A nucleotide sequencing of Helix2 domain of TWIST revealed a point mutation at 154 amino acid (R-S) substitution of arganine to serine in two GCT patients.

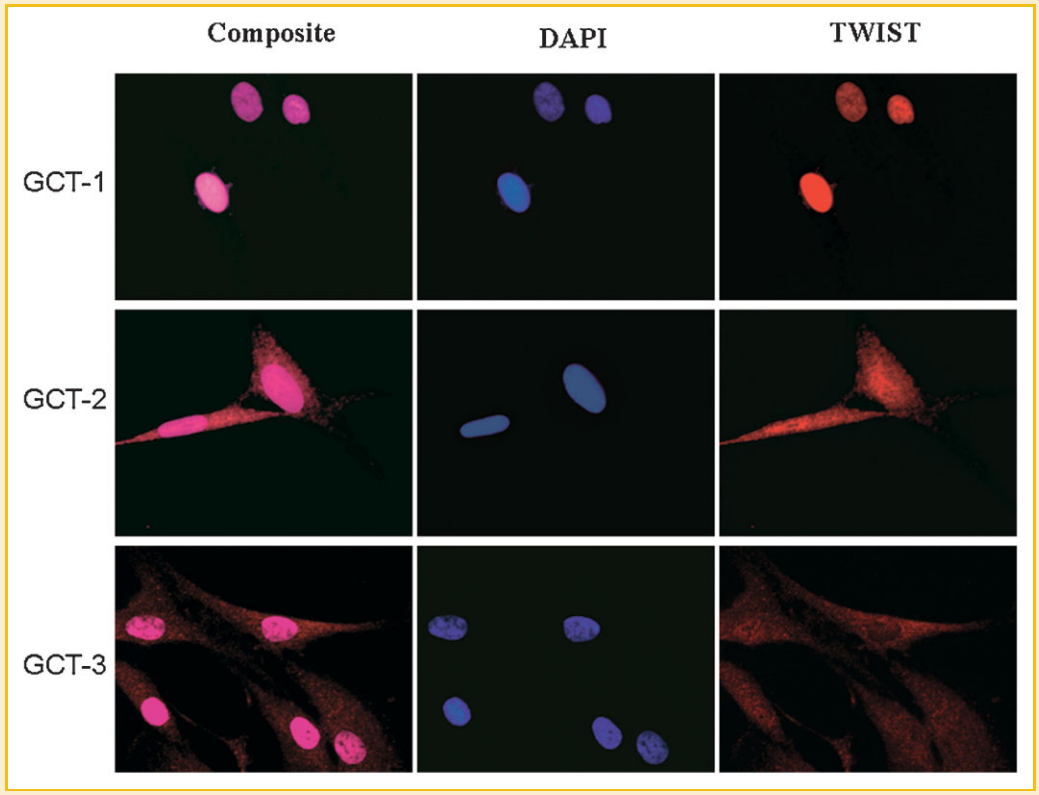

Fig. 6. Subcellular localization of TWIST in GCT stromal cells. TWIST expression in GCT-1, GCT-2, and GCT-3 was detected by immunofluorescence using anti-TWIST polyclonal antibody. DAPI staining (blue) indicates the nuclei (oil immersion; 100× magnification).

Karsenty, 1998]. Runx2 induces osteoblast differentiation by regulating expression of numerous osteoblast-specific genes [Ducy et al., 1997; Komori et al., 1997]. Mutations in the Runx2 gene result in autosomal-dominant cleidocranial dysplasia in humans, which is characterized by hypoplasia/aplasia of the clavicles, supernumerary teeth, short suture, and other defects in skeletal patterning and growth [Mundlos et al., 1997; Otto et al., 1997]. Runx2 interacts with a variety of co-regulating transcription factors and signaling proteins, forming multimeric complexes. These complexes play an important role in either transactivation or repression of target genes during osteogenic differentiation [Paredes et al., 2004; Zamurovic et al., 2004; Kobayashi and Kronenberg, 2005; Lian et al., 2006]. Thus Runx2 is a transcription factor that plays the role of the initial and terminal ''switch'' responsible for osteoblastic cell differentiation.

TWIST is a bHLH protein that plays a central role in cell type determination and differentiation and has been shown to regulate early osteogenesis [Lee et al., 1999]. The TWIST protein is negatively involved in osteoblastic differentiation by interfering with Runx2 function at early stages of osteogenesis. Mutations in the TWIST gene result in Saethre-Chotzen syndrome, characterized by increased bone formation in cranial sutures [El et al., 1997; Lee et al., 1999]. In a mouse model where the TWIST gene was overexpressed, Runx2 expression was undetectable during development [Bialek et al., 2004].

GCT cells are preosteoblastic undifferentiated precursor cells that do not undergo terminal differentiation. In semi-quantitative and qRT-PCR we found elevated TWIST expression in all GCT stromal cells when compared to hFOB 1.19 cells. Bialek et al. [2004] also reported that TWIST expression can delay osteoblast differentiation early during development. Overexpression of TWIST by transfection studies in osteosarcoma cells has been shown to inhibit osteoblast differentiation [Lee et al., 1999]. It is likely that overexpression of TWIST inhibits osteoblastic differentiation in GCT cells. Therefore TWIST functions to antagonize the action of Runx2 and may play a significant role in maintaining the neoplastic stromal cell in an undifferentiated state.

We confirmed in this study that TWIST overexpression down-regulates Runx2 expression in GCT cells. We observed that 50% TWIST gene knockdown induced a threefold increase in Runx2 in GCT stromal cells at the mRNA and protein levels. We have previously reported that expression of bone resorbing MMP-13 is elevated in GCT stromal cells compared to normal osteoblast cells, and Runx2 plays an important role in the regulation of MMP-13 expression in these cells [Cowan et al., 2009; Mak et al., 2009, 2010]. Here we also confirm that TWIST is an upstream regulator of MMP-13 through its regulation of Runx2. However, it is possible that TWIST is directly regulating MMP-13 through the TWIST E box binding site on the MMP-13 promoter region [Mengshol et al., 2001].

Furthermore, we found that in GCT cells obtained from a lung metastasis, TWIST knockdown in fact *decreased* Runx2 expression. The role played by TWIST may be cell type dependent [Komori, 2006]. Previously, similar results were obtained in a prostate cancer

cell line [Yuen et al., 2008]. These data support the conclusion that TWIST modulates Runx2 expression differently in different cell microenvironments. It is likely that TWIST interacts with proteins and signaling pathways which differ from one cell microenvironment to another to modify Runx2 expression. Since TWIST is known to play an important role in metastasis through EMT, it may be responsible for the cellular "switch" that allows GCT stromal cells to metastasize to distant sites.

TWIST is a bHLH transcription factor that is predominantly expressed in the nucleus where it binds the genome and other transcription factors. Mutations in the TWIST bHLH domain result in the Saethre-Chotzen syndrome phenotype. We found a novel mutation in the TWIST gene at the Helix2 domain of R154S. Previous reports have confirmed that the mutation in the HLH domain results in loss of dimerization with other bHLH proteins and causes mislocalization of the TWIST protein leading to pathogenic disease [El et al., 1997, 2000; Howard et al., 1997; Firulli and Conway, 2008; Barnes and Firulli, 2009; Conway et al., 2010]. We hypothesize that the R154S mutation may cause a mislocalization with variation in the localization pattern in GCT patient specimens. Further study is required to determine the effect of this mutation in GCT formation and protein signaling. However, we could not detect any mutation at the C-terminus of TWIST, which is known to be the Runx2 binding site.

In conclusion, we are the first group to successfully transfect primary GCT stromal cells and have found that TWIST is an upstream regulator of Runx2 in GCT stromal cells. We postulate that abnormal TWIST sequences lead to mislocalization of this master gene regulator and therefore downstream abnormal function of Runx2. Since GCT stromal cells are characterized as osteoblastic precursors and Runx2 is the lead transcription factor in osteoblastic differentiation, TWIST mutations may be an essential underlying factor in the development and pathophysiology of these tumors in that they lead to arrested osteoblastic differentiation and the maintenance of an immature and neoplastic phenotype. In addition, abnormal TWIST function may be involved in the metastatic process in these cells. Further studies into the subcellular interactions between TWIST and Runx2 in GCT stromal cells will further elucidate this role.

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