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TWIST, a Basic Helix-Loop-Helix Transcription Factor, Can Regulate the Human Osteogenic Lineage

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Abstract Basic helix-loop-helix (bHLH) transcription factors have been shown to play an important role in controlling cell type determination and differentiation. *TWIST,* a member of the bHLH transcription factor family, is involved in the development of mesodermally derived tissue, including the skeleton. We examined the role of human *TWIST* in osteoblast metabolism using stable expression of sense and antisense *TWIST* in human osteoblast HSaOS-2 cells. Changes in morphology and osteogenic phenotype characterized these stable clones. Cells that overexpressed *TWIST* exhibited a spindle shaped morphology, reduced levels of alkaline phosphatase, a reduced proliferation rate, and failed to respond to basic fibroblast growth factor (bFGF). In contrast, those that underexpressed *TWIST* demonstrated a cuboidal epithelial-like morphology characteristic of differentiated osteoblasts. *TWIST* antisense cells exhibited increased levels of alkaline phosphatase and type I collagen mRNA, initiated osteopontin mRNA expression, and had a reduced proliferation rate. These results indicate that *TWIST* overexpressing cells may de-differentiate and remain in an osteoprogenitor-like state, and antisense *TWIST* cells progress to a more differentiated mature osteoblast-like state. Therefore, the level of *TWIST* can influence osteogenic gene expression and may act as a master switch in initiating bone cell differentiation by regulating the osteogenic cell lineage. J. Cell. Biochem. 75:566–577, 1999.

Key words: transcription factor, helix-loop-helix, *TWIST*, osteoblast differentiation, SaOS-2

Our understanding of mesodermal cell differentiation has greatly increased in the last several years with the discovery of basic helix-loop-helix (bHLH) transcription factors. The *TWIST* gene, originally identified in *Drosophila,* is an evolutionarily conserved bHLH transcription factor that is necessary for the development of embryonic gastrulation and the formation of mesoderm [Leptin, 1991; Thisse et al., 1987]. Vertebrate *TWIST* is initially expressed throughout the somatic mesoderm in embryos and its expression is maintained in mesenchymal stem cells as the somites develop [Gitelman, 1997; Glackin et al., 1994; Rose and Malcolm, 1997; Wolf et al., 1991]. *TWIST* null mice die at embryonic day 11.5 due to defects in the cranial neural fold, head mesenchyme, somites and limb buds [Chen and Behringer, 1995].

The mechanism of bHLH molecules require that they either homo- or heterodimerize with other bHLH molecules, and bind to a conserved E-box region (CANNTG) on the promoter of genes which activate or inhibit transcription [Murre et al., 1989]. During development, combinations of multiple positive- and negative-acting bHLH transcription factors are involved in activating genes for cell type specification and differentiation. Positive acting bHLH transcription factors (e.g., MyoD) dimerize to a ubiquitous E protein (e.g., E12, E47) and bind to an E-box, which trans-activates the expression of a target gene [Olson, 1992]. Members of the *Id* family, a well-characterized negative acting HLH factor, do not have a DNA binding basic region. Therefore, *Id* blocks cellular differentiation by sequestering positive bHLH molecules away from binding to an active promoter region [Benezra et al., 1990; Sun et al., 1991]. Mouse *TWIST* has been demonstrated to be a negative transcription factor in muscle and bone cell development [Glackin et al., 1992; Hebrok et

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al., 1997; Hebrok et al., 1994; Murray et al., 1992]. However, negative regulation of mouse *TWIST* is more efficient and complex than mouse *Id* during myogenesis [Spicer et al., 1996]. Mouse *TWIST* can not only sequester away positive regulators and titrate ubiquitous E proteins, but it can also occupy the E-box and inhibit other non-bHLH trans-activators (e.g., MEF2) [Cripps et al., 1998; Spicer et al., 1996]. Mechanisms of the *TWIST* transcription factor have been well characterized in muscle cells, but much less is known about the function of bHLH factors in bone or cartilage development.

Osteoblast development is categorized in a presumed linear sequence of osteoprogenitor, preosteoblast, osteoblast, mature osteoblast, and osteocyte [Martin et al., 1987; Nijweide and Mulder, 1986]. Bone-forming osteoblasts are cells responsible for the synthesis of bone marker genes that regulate deposition and mineralization of extracellular matrix (ECM) in newly forming bone tissue. Three distinct stages are observed in osteoblast differentiation, proliferation, matrix maturation, and mineralization [Stein et al., 1989]. These developmental stages involve several tightly regulated expression patterns of bone and cell cycle related genes [Stein and Lian, 1993]. Osteogenic precursor or osteoprogenitor cells are thought to reside in periosteal tissues and in bone marrow stroma [Aubin et al., 1995]. These cells are clonal, spindle shaped fibroblast-like cells with an elongated nuclei [Scott, 1967]. During embryonic mesoderm development, osteoprogenitor cells arise from pleuripotent mesenchymal stem cells that express high levels of *TWIST*. These pleuripotent stem cells are capable of giving rise to a number of committed and restricted cell lineages, including adipocytes, fibroblasts, myoblasts and osteoblasts [Owen, 1988]. *TWIST* expression is excluded from the forming myotome but it continues to be expressed in other mesodermal structures such as the dermomyotome, sclerotome, lateral plate mesoderm, and cranial neural crest. The level of *TWIST* expression gradually decreases as mesenchymal stem cells further differentiate [Wolf et al., 1991]. The expression of this transcription factor has been implicated in the partitioning of the somatic mesoderm into bone or cartilage formation. Furthermore, high levels of *TWIST* expression are also detected in ectoderm derived cranial neural crest cells [Wolf et al., 1991], which also have the capacity to form the bone, cartilage, connective tissue, and tooth odontoblasts of the facial skeleton. The major phenotype of the *TWIST* null mice showed failure of the cranial neural fold to fuse [Chen and Behringer, 1995]. Recently, mutations in human *TWIST* gene have been identified in Saethre-Chotzen syndrome, a bone disease characterized by premature fusion of the cranial sutures [el Ghouzzi et al., 1997; Howard et al., 1997].

Based on these observations, we hypothesize that expression levels of human *TWIST* are involved in regulating and maintaining osteogenic precursor cell phenotype. Down regulation of *TWIST* leads to the differentiation of osteoprogenitor cells. To test this hypothesis, we examined the effects of human *TWIST* overexpression and reduced expression on osteoblast differentiation in an osteoblast-like cell line (HSaOS-2) [Farley et al., 1991]. *TWIST* was expressed under a human cytomegalovirus (CMV) promoter driven mammalian expression vector (pcDNA3, Invitrogen) in both sense and antisense orientations. Our observations reveal that human *TWIST* expression can regulate the osteoblastic lineage and influence bone cell morphology. In addition, the expression of bone matrix proteins and alkaline phosphatase and the rate of proliferation and responsiveness to growth factors are also dependent on *TWIST* levels during human bone cell development.

MATERIALS AND METHODS

Vector Construction

A 1.1-kb human *TWIST* cDNA clone was isolated from a custom bone library prepared from cDNAs of the femur from a 55-year-old woman (Lambda ZAP®II, Stratagene). Both *TWIST* sense and antisense orientation fragments were subcloned into a CMV promoter driven mammalian expression vector (pcDNA3, Invitrogen). In the sense construct, an 849-bp *SmaI* and *EcoR1* fragment was removed and ligated into the multiple cloning site of the expression vector (Figure 1, panel A). Overexpressing *TWIST* in the sense orientation results in a 1.1kb mRNA transcript representing 849bp of the *TWIST* fragment and 250bp of the BGH poly A and linker regions from the vector (Figure 3, panel D). In the antisense construct, the entire 1.1kb *EcoR1* and *BamH1* fragment was ligated in a reverse orientation relative to the *CMV* promoter (Figure 1, panel B). Sequencing analysis

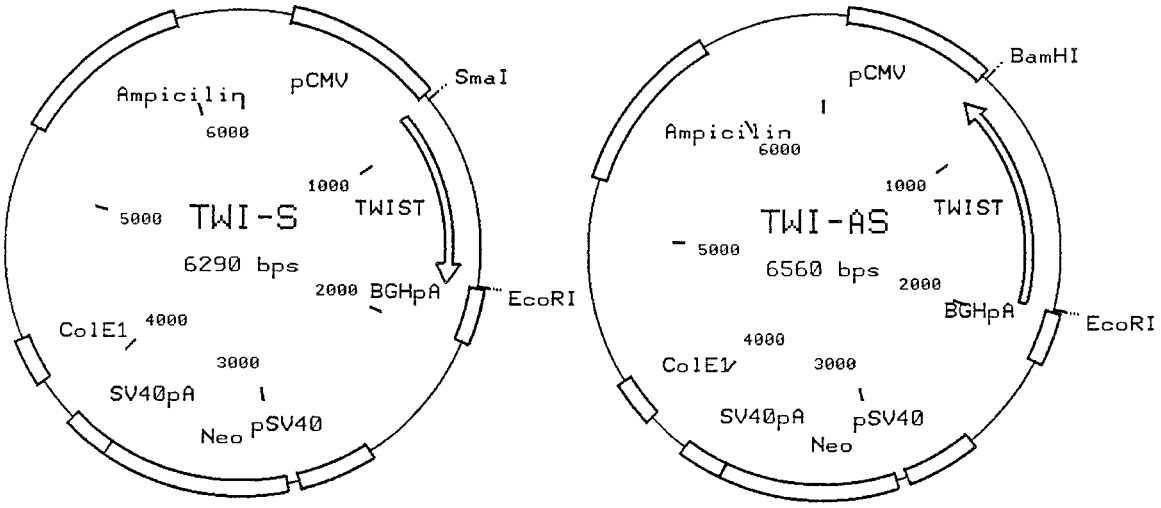


Fig. 1. Schematic diagrams of the constructs used for stable HSaOS-2 transfections. Human *TWIST* cDNAs were cloned in both sense and antisense orientations into the pcDNA3 (Invitrogen) mammalian expression vector. **A:** 849 bp of *TWIST* cDNA was subcloned into the *Sma*I and *EcoRI* polylinker sites in the sense orientation with respect to the CMV promoter (TWI-S). **B:** 1,118 bp of *TWIST* was ligated into the *EcoRI* and *BamHI* polylinker sites in the antisense orientation with respect to the CMV promoter (TWI-AS).

confirmed the orientation of *TWIST* DNA constructs.

Cell Culture

HSaOS-2 [Farley et al., 1991], subculture of SaOS-2 (ATCC; HTB-85) [Rodan et al., 1987], human osteoblast-like sarcoma cells were maintained by weekly passaging in low glucose Dulbecco's Modified Eagles Medium (DMEM, Gibco BRL) containing 10% fetal calf serum (FCS, HyClone). Cells were grown in a humidified incubator in 5% CO_2 / 95% air at 37°C.

Stable Transfection

HSaOS-2 cells were plated in six-well plates and maintained in 10% FCS DMEM until reaching 70–80% confluence. Before transfection, the cells were washed once with serum-free medium. Transfections were performed with LipofectAMINE reagents (Gibco BRL). 1µg of plasmid DNA containing the pcDNA3-*TWIST* expression vector and 5µl of LipofectAMINE in OPTI-MEM (Gibco BRL) was mixed for 45 minutes. Serum-free medium was added to the mixture and the complexes overlaid onto cells. Cells were incubated with the complexes for 5–6 hours at 37°C. At end of this incubation, 1ml of 20% FCS DMEM was added to the cells and the incubation continued for up to 24 hours. The medium was replaced with fresh medium containing 10% FCS DMEM and incubated for another 24 hours. At 72 hours after transfection, the cells were passaged at a 1:10 dilution into medium containing Geneticin (Gibco BRL) for neomycin (400µg/ml) selection. After about two weeks of culture in Geneticin, positive clones were selected and expanded for further characterization.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared using an acid-guanidinium-thiocyanate-phenol-chloroform [Chomczynski and Sacchi, 1987] or Trizol (Gibco BRL). Poly(A)$^+$ RNA was prepared by affinity chromatography on oligo (dT) cellulose (Sigma) [Aviv and Leder, 1972; Edmonds et al., 1971]. About 15µg of total RNA and/or 4µg of poly(A)$^+$ RNA were separated for Northern analysis on a 1.5% agarose-formaldehyde gel and transferred to a Hybond-N (Amersham) nylon membrane overnight. After cross-linking, the blot was prehybridized 2 hours and hybridized in 10ml 50% formamide hybridization buffer (5X SSC, 5X Denhardt's, 0.5% SDS, 0.1mg/ml denatured salmon testes DNA, 50% formamide) at 42°C overnight. After washing under stringent conditions in high salt (2X SSPE, 0.1% SDS) at room temperature, and low salt (0.1X SSPE, 0.1% SDS) at 50°C, the blots were exposed to X-ray film and quantitated with a phosphoimager (Molecular Dynamics). ^{32}P-labeled probes were prepared by random priming (Amersham) of gel isolated cDNA fragments. The blots were then stripped and re-hybridized with other

probes including human glyceraldehyde-2-phosphate dehydrogenase (GAPDH) for normalization [Ercolani et al., 1988].

TWIST Polyclonal Antibody

Sixteen amino acids (RKIIPTLPSDKLSKIQ) of the *TWIST* antigen were chemically synthesized near the loop region of the bHLH domain and conjugated with a carrier, keyhole limpet hemocyanin (KLH). After high-performance liquid chromatography (HPLC) analysis to determine KLH binding efficiency and peptide purification, the protein antigen was injected into a rabbit for immunization and serum was prepared from whole blood three times after primary and booster immunization. The serum was characterized by Western, immunohistochemistry, and enzyme-linked immunoabsorbent assays (ELISA).

Western Blot Analysis

40µg of total protein samples were electrophoresed in a 12% SDS-PAGE gel and transferred to Immobilon-P PVDF membrane (Millipore) by electro-blotting. After drying, the membrane was blocked with 5% non-fat milk in phosphate buffered saline-Tween20 (PBST) for 2 hours at room temperature with gentle shaking. Primary *TWIST* anti-serum, diluted 1:500 in 5% non-fat milk in PBST, was hybridized for 2 hours at 4°C. The membrane was then washed 3 times for 15 minutes in PBST. After washing, the membrane was incubated for 1 hour at room temperature with a 1:5000 dilution of secondary antibody (alkaline phosphatase conjugated anti-rabbit IgG, Tropix) in 5% non-fat milk in PBST. Bands were visualized by staining with nitro blue tetrazolium (NBT) and 5-bromo 1-chloro 3-indolyl phosphate (BCIP) for 10 minutes in alkaline phosphate buffer (100mM Tris HCl, pH9.5; 100mM NaCl; 5mM $MgCl_2$).

RT-PCR Method

In order to confirm the expression of antisense (AS) *TWIST* overexpressing cells, we performed RT-PCR analysis using primers that distinguished endogenous from exogenous *TWIST* expression. 1µg of mRNA from *TWIST* AS transfected HSaOS-2 cells was reverse transcribed using 1µl of Moloney murine leukemia virus (M-MLV) reverse transcriptase (5 units/µl) using random primers (500ng/µl) at 42°C for 1 hour in a 20µl reaction mix. 5µl of reverse transcribed cDNA was amplified in the PCR reaction using a Taq polymerase amplification kit (Qiagen Inc.) and the following primers. The first primer used was designed at the carboxy terminal region of the *TWIST* gene (ATTAATAATAACGTCACACTTG) and the other primer was designed to anneal at the linker region of the pcDNA3 vector (CGAAATTAATACGACTCACTAT). Results indicate that a 237-bp fragment, representing reverse orientation exogenous *TWIST* (AS), was detected in a 1.5% agarose gel. GAPDH primers (GGCAAATTCCATGGCACCGTCAA) and (CAGCAGAGGGGGCAGAGATGAT) were also used to generate a 390-bp product as a control for the RT-PCR with and without reverse transcriptase as positive and negative controls.

Alkaline Phosphatase Biochemical Assay

Total cell extracts were prepared from transfected cell lines by sonication in 1ml lysis buffer (50mM Tris-HCl pH 8.0, 5mM EDTA, 150mM NaCl, 0.5% Nonidet-P40, 1mM phenylmethylsulfonyl fluoride). After centrifugation, supernatants were transferred and the protein concentration was determined by protein assay kit (Bio-Rad). Alkaline phosphatase activity was measured spectrophotometrically at 410nm by release of p-nitrophenol from p-nitrophenyl phosphate at 37°C using an alkaline phosphatase diagnostic kit (Sigma). Activity was then converted into units according to a standard calibration curve.

Basic FGF Treatment

TWIST transfected bone cells were passaged and grown to 70–80% confluence. Cells were then grown in serum free (SF) DMEM for 24 hours to adapt to serum free conditions. The medium was replaced with fresh SF DMEM medium containing 10ng/ml of recombinant human basic fibroblast growth factor (bFGF, Promega) diluted in 1X phosphate-buffered saline (PBS). Total RNA was extracted at various time points after bFGF addition for Northern blot analysis.

RESULTS

HSaOS-2 Cells Express the Transcription Factor *TWIST* and Other Bone Markers

HSaOS-2 cells, a subpopulation of SaOS-2 cells that have a high level of alkaline phosphatase activity, possess several osteoblastic features such as the production of alkaline phos-

phatase and parathyroid-stimulated adenylate cyclase, and the synthesis and secretion of type I collagen and mineralized matrix [Farley et al., 1991; Rodan et al., 1987]. To characterize the osteogenic properties of HSaOS-2 cells, we performed Northern blot analyses using several bone marker cDNA probes. We found that HSaOS-2 cells express high alkaline phosphatase (ALP) and low type I collagen (Coll-I), but no osteopontin (OP) or osteocalcin (OC) mRNA under basal conditions (Figure 2, panel A). These characteristics, together with the slightly elongated cuboidal morphology (Figure 3, panel C), suggest that the HSaOS-2 cell has a differentiating osteoblast phenotype. In addition, using a human *TWIST* specific carboxy region probe, which does not cross hybridize to other bHLH transcription factors, we found that low levels of *TWIST* mRNA (1.3, 1.6 and 4.9kb transcript) were detected in HSaOS-2 cells in the poly(A)$^+$ RNA Northern blot (Figure 2, panel B). Western blot analysis with *TWIST* polyclonal antibody resulted in a protein of approximately 26 kDa (Figure 3, panel D).

TWIST Expression Changes Osteoblastic Morphology

Based on morphological and histological studies, osteoblast cells exhibit a linear sequence of cellular differentiation events progressing from osteoprogenitors to preosteoblasts, to osteoblasts, and then to lining cells or osteocytes [Martin et al., 1987; Nijweide and Mulder, 1986]. Osteoprogenitor cells demonstrate a fibroblas-

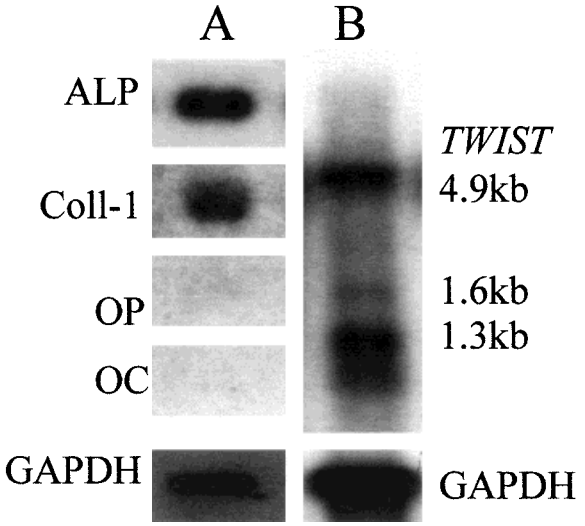


Fig. 2. Northern blot analysis of bone markers (panel A) and *TWIST* mRNA levels (panel B) in HSaOS-2 human osteosarcoma cell lines. **A:** Expression levels of alkaline phosphatase (ALP), type I collagen (Coll-I), osteopontin (OP) and osteocalcin (OC) genes. 15μg total RNA was loaded in each lane. **B:** Three distinct *TWIST* transcripts (1.3kb, 1.6Kb and 4.9kb) are expressed in HSaOS-2 cells. 4μg poly (A)$^+$ RNA was loaded. GAPDH mRNA served as a control. The data represent one of two independent experiments with similar results.

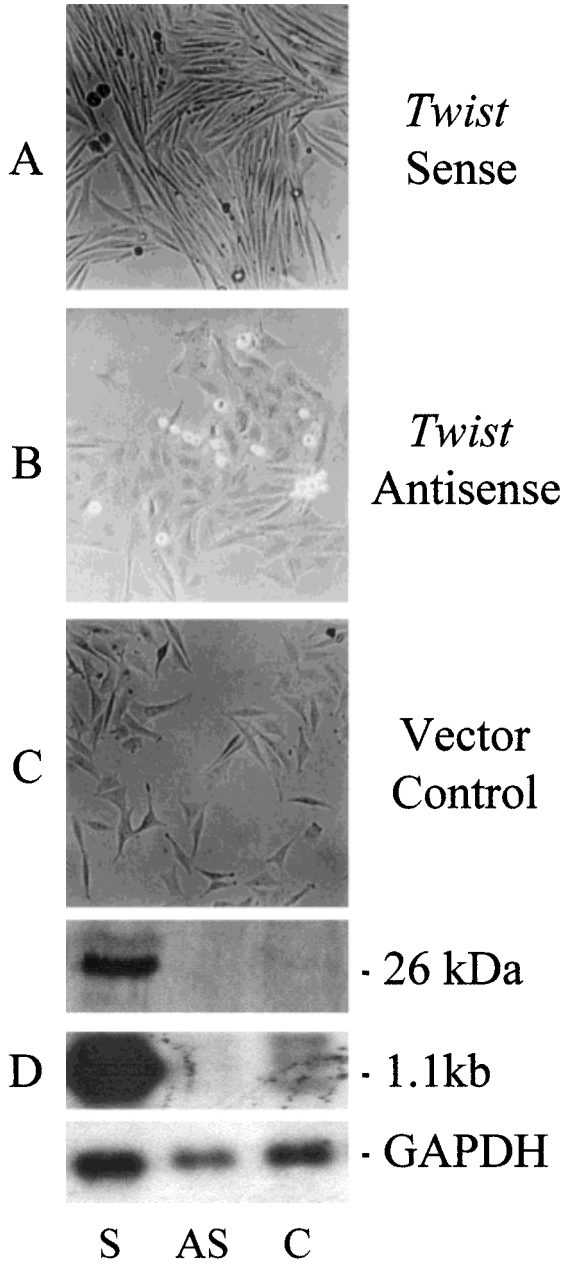


Fig. 3. Morphological features of HSaOS-2 cells that express different levels of human *TWIST*. **A:** *TWIST* overexpressing HSaOS-2 cells exhibit a spindle shape fibroblast phenotype (S). **B:** *TWIST* antisense expressing HSaOS-2 cells have a cuboidal osteoblast phenotype (AS). **C:** pcDNA3 vector control HSaOS-2 cells (C). **D:** Western and Northern blot analyses of *TWIST* expression in these three cell lines (top panel: *TWIST* 26kDa protein, middle panel: *TWIST*-S 1.1kb mRNA, bottom panel: GAPDH 1.3kb mRNA). Two independent clones were tested from each stably expressing cell line with similar results.

tic or spindle shape morphology with oval or elongated nuclei, whereas active osteoblast cells are cuboidal and polar [Scott, 1967]. We have made stable transformed (HSaOS-2) human osteoblast-like sarcoma cell lines containing human *TWIST* in both sense and antisense orientations. We have confirmed through Northern blot and RT-PCR analyses, stable transfected cell lines that overexpress sense and antisense *TWIST* transcripts. Overexpressing *TWIST* in the sense orientation results in a 26kDa protein product and a 1.1kb mRNA transcript. Although *TWIST* protein expression level was not changed as dramatically as the mRNA level, increased and decreased protein levels were demonstrated in Western blotting analyses (Figure 3, panel D).

We found that both overexpressing cells and cells that express antisense *TWIST* demonstrate significant morphological differences compared to control cells typically observed in osteoblast development. When cells overexpress *TWIST*, they remain in an undifferentiated state and their morphology changes from a cuboidal osteoblast phenotype to a spindle shape fibroblast phenotype characteristic of osteoprogenitor cells (Figure 3, panel A). By contrast, cells which express antisense *TWIST* retain a highly differentiated state. Their morphology is cuboidal, representing a mature osteoblast phenotype (Figure 3, panel B) when compared to controls (Figure 3, panel C). No morphological difference was seen in pcDNA3 vector only transfected and parental HSaOS-2 cells. These results indicate that human *TWIST* expression levels in osteoblast cells may affect osteogenic differentiation.

TWIST Regulates Bone Marker Gene Expression in Osteoblasts

In addition to the morphological changes observed, HSaOS-2 cell lines expressing varying levels of human *TWIST* exhibit distinct expression patterns of several osteoblastic marker transcripts indicative of the osteogenic lineage. We found that when HSaOS-2 cells overexpress *TWIST*, they strongly suppress the expression of alkaline phosphatase and type I collagen. However, cells expressing antisense *TWIST* express increased levels of alkaline phosphatase (about 5-fold) and type I collagen (about 10–fold). Overproduction of antisense *TWIST* induces the expression of osteopontin, a late marker of osteoblast differentiation. Untransfected HSaOS-2 and pcDNA3 vector-only control cells have high alkaline phosphatase and low type I collagen, but no osteopontin and osteocalcin expression detected by Northern blot analysis (Figure 4). These results demonstrate that *TWIST* overexpressing cells alter osteogenic differentiation by suppressing bone marker gene expression, whereas those expressing *TWIST* antisense promote differentiation by increasing alkaline phosphatase and type I collagen, and initiating osteopontin expression.

TWIST Changes Alkaline Phosphatase Activity in Osteoblasts

Alkaline phosphatase (ALP) is an ectoenzyme that can hydrolyze monophosphatase on the osteoblast plasma membrane [Register and Wuthier, 1984]. ALP enzyme activity increases as preosteoblasts differentiate, but is absent from the osteocytes [Doty and Schofield, 1976]. Therefore, the level of osteoblastic ALP activity has been routinely used as a marker of preosteoblast differentiation [Rodan and Noda, 1991].

To provide further evidence for the role of human *TWIST* in osteogenic differentiation, we

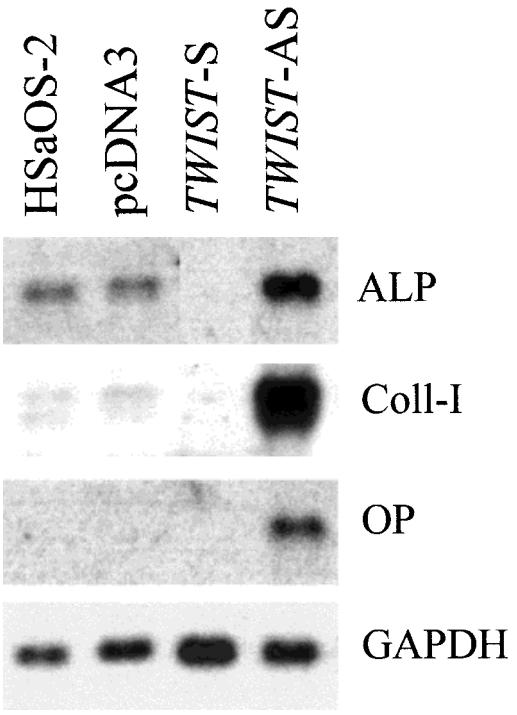


Fig. 4. Expression of bone marker genes in HSaOS-2 cell lines that express different levels of human *TWIST*. *TWIST* sense overexpressing cell lines (*TWIST*-S) suppress expression of all of the bone markers shown, whereas antisense (*TWIST*-AS) cell lines increased alkaline phosphatase (ALP), type I collagen (Coll-I) and osteopontin (OP) compared to controls (HSaOS-2 and pcDNA3). GAPDH served as a control. The data represent at least two independent experiments with similar results.

measured ALP enzyme activities in the osteogenic cell lines expressing various *TWIST* levels. We found that cells which overexpress *TWIST* dramatically reduce ALP enzyme activity (about 10–fold), whereas those with the antisense *TWIST* increased the enzyme activity (about 2–fold) compared to the vector only control (Figure 5).

TWIST Changes the Proliferation Rate in Osteoblasts

To test whether differential levels of human *TWIST* expression in osteoblastic cells could change the cell proliferation rates, we counted total cell numbers and measured the levels of histone-4 (H4) mRNA. Histone genes are transcriptionally upregulated in response to a series of cellular regulatory signals that mediate competency for cell cycle progression of the G1/S-phase transition. Therefore, the level of H4 transcript has been used as a marker for cell proliferation. During osteoblast differentiation, the level of H4 mRNA is high during the period of active proliferation (preosteoblast and early osteoblast) and gradually decreases as the cells mature [Stein et al., 1996]. On the other hand, early osteoprogenitor cells go through 6–7 slow doublings to yield functional bone forming preosteoblasts and increase proliferative capacity [Bellows et al., 1990]. Other studies show that the earliest cells in the osteogenic lineage are relatively quiescent, have a long G0 time, a low turnover rate, and have a low probability of entering into the cell cycle [Potten et al., 1979].

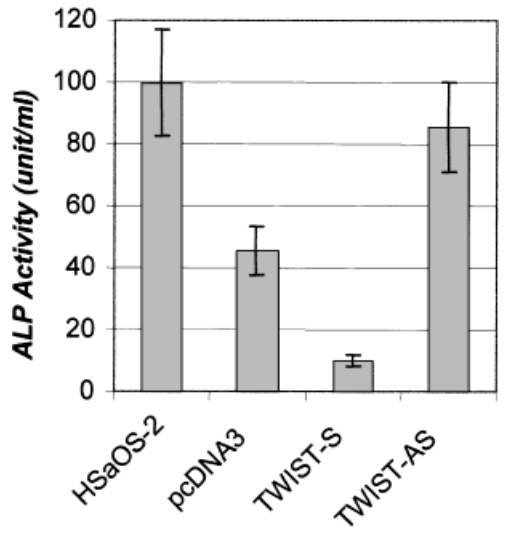


Fig. 5. Alkaline phosphatase (ALP) activity in HSaOS-2 cell lines that differentially express human *TWIST*. High *TWIST* expression (*TWIST*-S) dramatically suppresses ALP activity, and reduced *TWIST* expression (*TWIST*-AS) slightly increases activity compared to the control (pcDNA3). We found a pcDNA3 vector effect in ALP activity compared to the non-transfected parental HSaOS-2 cells. The data represent three independent experiments with similar results.

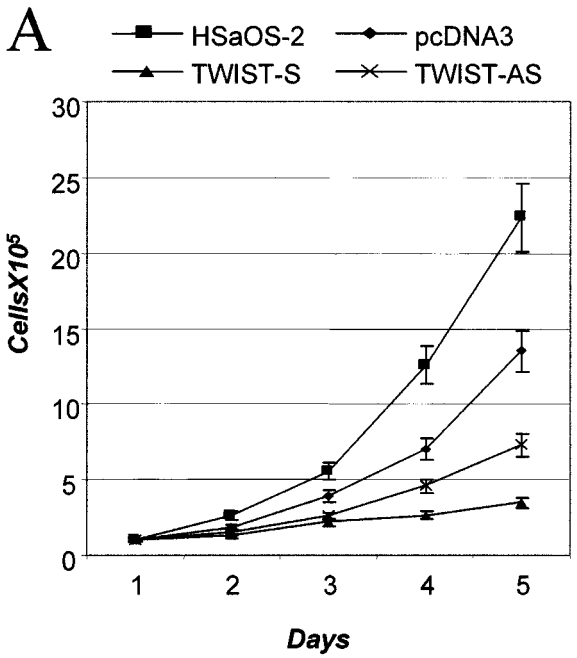
Our results demonstrate that both HSaOS-2 cell lines which express sense and antisense *TWIST* have decreased proliferation rates and levels of H4 mRNA (Figure 6, panels A and B) when compared to controls. Therefore, we hypothesize that the *TWIST* sense overexpressing cells are proliferating very slowly and phenotypically resemble early osteoprogenitor cells, resulting in low levels of the proliferation marker H4. On the other hand, *TWIST* antisense cells are more mature osteoblasts that have a reduced proliferation capacity, allowing the cells to differentiate.

Overexpressing *TWIST* Fails to Respond to the bFGF in Osteoblasts

Osteoblastic cells respond to various bone growth factors such as transforming growth factor-beta (TGF-β), bone morphogenetic proteins (BMPs), 1,25 dihydroxyvitamin D_3, and fibroblast growth factors (FGFs). The growth factor responsiveness of the osteogenic cells is known to depend on their relative stage of differentiation. Basic fibroblast growth factor (bFGF) is a heparin binding growth factor derived from neuroectodermal and mesodermal tissue and deposited in the extracellular matrix [Gospodarowicz, 1990]. bFGF acts as a potent mitogen in osteoblast cells, and stimulates bone cell population, which results in an increased pre-osteoblastic cell population and an increase in bone formation [Nakamura et al., 1995]. It has been shown that bFGF exerts a growth factor response by inducing its own expression through an increase in endogenous bFGF mRNA [Lee et al., 1995]. This autoregulated transcriptional response uses a signal transduction pathway that involves the activation of the early growth response element (egr-1), also known as Krox24, Zif268 and NGFI-A zinc-finger transcription factor [Wang et al., 1997a]. bFGF responsiveness has been shown to differ in heterogeneous populations of bone precursor cells. Early populations of osteoprogenitor cells do not respond to bFGF while the more differentiated osteoblasts do respond to bFGF [Long et al., 1995].

We demonstrate that *TWIST* overexpressing cell lines treated with 1nM bFGF do not induce

the expression of Egr-1 transcription factor even after 2 days (Figure 7, panel C). In contrast, bFGF treated *TWIST* antisense and control cell lines respond with an increase in the expression of Egr-1 by bFGF mRNA within 1 hour (Figure 7, panel A, B, and D). These results indicate that human *TWIST* overexpressing cells represent an earlier stage of osteogenesis



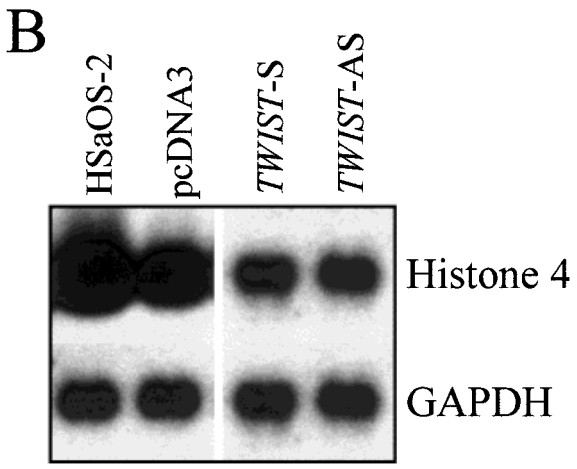


Fig. 6. Comparison of cell proliferation in HSaOS-2 cells that express different levels of human *TWIST*. **A:** Number of total cells in 10cm culture flask. The proliferation rate decreases in cell lines that overexpress (*TWIST*-S), and in those that express antisense (*TWIST*-AS) *TWIST*. **B:** Northern blot of Histone 4 (H4) transcripts. The H4 mRNA level down regulates about three to four fold in both *TWIST-S* and *TWIST-AS* transfected cell lines compared to controls (HSaOS-2 and pcDNA3). GAPDH mRNA served as a control. All data represent at least two independent experiments with similar results.

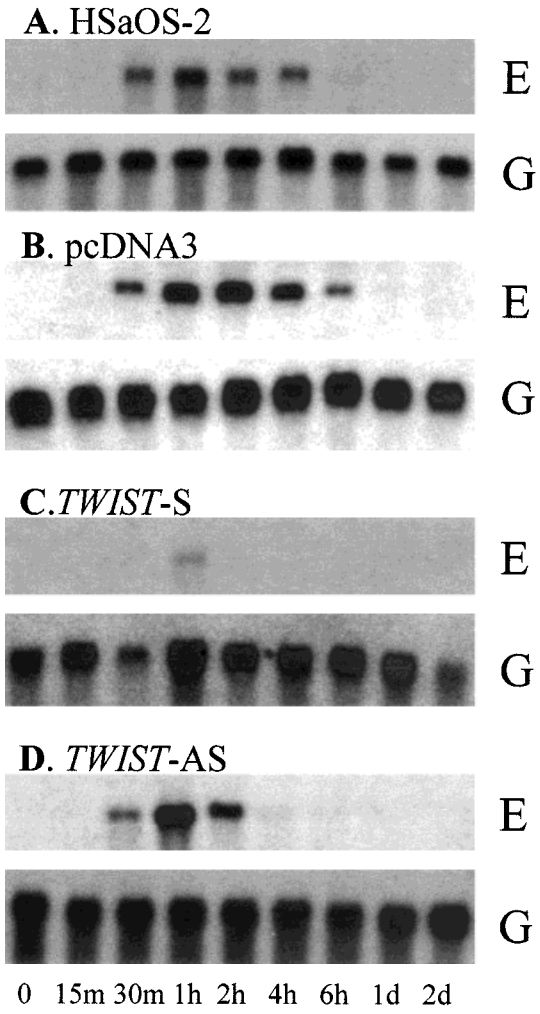


Fig. 7. Time course of bFGF effects on Egr-1 **(E)** mRNA expression in HSaOS-2 cell lines that differentially express human *TWIST*. The *TWIST* overexpressing cell line (*TWIST*-S) fails to increase accumulation of Egr-1 mRNA in response to bFGF. The time course without bFGF treatment did not accumulate Egr-1 mRNA (data not shown). GAPDH **(G)** mRNA served as a control. The data represents two independent experiments with similar results. (m: minute, h: hour, d: day)

in which the bFGF growth factor is unable to elicit an Egr-1 response. In addition, these results also demonstrate that altered expression of human *TWIST* can change the osteoblast lineage and, in turn, change the internal biochemical responsiveness of osteoblasts to external growth factors.

DISCUSSION

The results demonstrate that expression levels of human *TWIST* can alter osteoblastic differentiation patterns in bone development. *TWIST* overexpressing cells are more progeni-

tor-like and cells with *TWIST* in the antisense orientation represent a more differentiated osteoblast phenotype compared to the parental cells (Figure 8). This study indicates for the first time that overexpression of a single transcription factor can block osteoblast differentiation, and can also reverse osteoblastic cells to a more progenitor or stem cell-like state. This is a novel finding since no bHLH transcription factor so far has been implicated in the reverse or de-differentiating regulation of mesodermal tissue development.

Role of *TWIST* in Cellular Aging

In cellular aging studies, dramatic differences in human *TWIST* expression were observed in young versus senescent human diploid fibroblasts (WI-38) [Doggett et al., 1992; Wang et al., 1996]. *TWIST* was found to be overexpressed in young quiescent cells [Wang et al., 1997b] and underexpressed in senescent fibroblasts. Young diploid fibroblast cells may be derived from mesodermal stem cells, which have more precursor-like cell populations than their senescent counterpart. As cells are passaged, they gradually lose their stem cell population that expresses *TWIST*, causing a limited proliferation capacity in culture. Therefore, *TWIST* may be involved in this type of cellular aging process by causing some cell populations to remain in a precursor-like or stem cell state.

Effects of *TWIST* in Cell Proliferation

Our results demonstrate that human *TWIST* expression in osteogenic HSaOS-2 cells decreases their proliferation rate and suppresses the proliferation marker histone-4 mRNA in both sense and antisense orientations (Figure 6). We hypothesize that *TWIST* overexpressing cells may resemble a slowly proliferating early osteoprogenitor phenotype, and *TWIST* antisense cells are highly differentiated osteoblasts that have a reduced proliferation. The effects of *TWIST* overexpressing cells on proliferation were surprising, since the undifferentiated early osteogenic cells were expected to exhibit significant proliferation potential. However, some populations of early osteoprogenitor cells have been shown to have slow growth and proliferation rates [Bellows et al., 1990; Potten et al., 1979]. We also demonstrate that expression of the pcDNA3 vector alone in the HSaOS-2 cells decreases their proliferation rate and histone-4 mRNA levels (Figure. 6). However, this vector effect was moderate when compared to the *TWIST* expressing cell lines. The mechanism of action of *TWIST* as a suppressor of osteoblast proliferation is not clearly understood. One hypothesis is that *TWIST* suppresses the effects of growth factors (e.g., bFGF) which may be involved in cell proliferation and histone-4 production. FGF receptors (FGFR) play a functional role during osteoblast differentiation [Wang et al., 1994], and *TWIST* has been suggested to be an upstream regulator of FGFRs in craniofacial and limb development [Howard et al., 1997]. Therefore, the characterization of *TWIST* expression on other aspects of growth factor signaling, including FGFR expression pattern, will be important for future studies. In

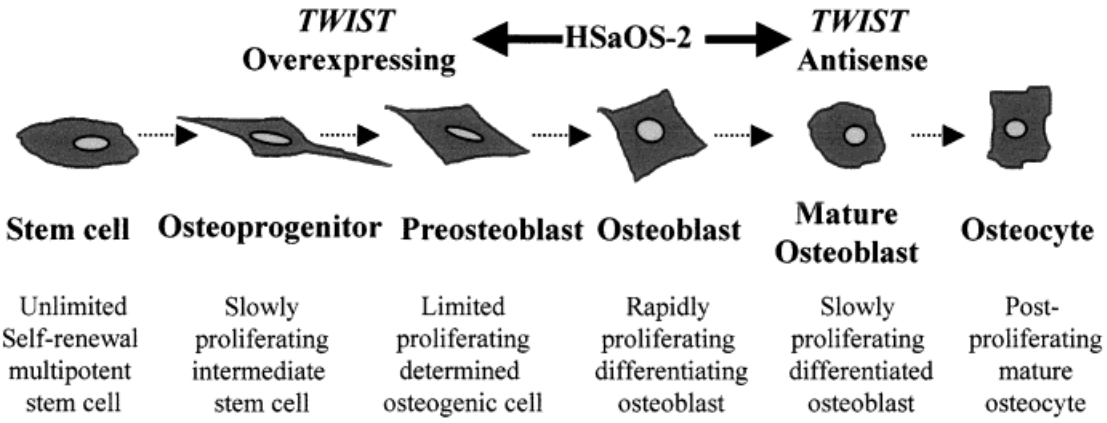


Fig. 8. Proposed model of *TWIST* function in bone cell development. Differential levels of *TWIST* can regulate the osteoblastic lineage in the human osteoblast-like cell line (HSaOS-2). Ectopic overexpression of *TWIST* can de-differentiate cells into an osteoprogenitor-like stem cell state, whereas decreased levels of *TWIST* (*TWIST* Antisense) can shift cells to a more differentiated osteoblast cell phenotype.

addition, we do not know whether *TWIST* overexpressing cells globally decrease osteoblast response to all mitogens or only selectively abrogate subsets of FGF signaling. Furthermore, growth regulation and the general characteristics of transformed osteosarcoma cells may differ significantly from those of the normal osteoblasts. It has been reported that overexpression of mouse *TWIST* in myogenic C2C12 cells demonstrates no change in proliferation rates during growth and differentiation conditions [Hebrok et al., 1997]. This apparent contradiction may be explained by the fact that the role of bHLH proteins in other tissue types and species may be different. In addition, this could also be explained by differences in the vector integration events and vector copy number in the transfected cell lines.

Vector Effects in Osteogenesis

We used a pcDNA3 mammalian expression vector (Invitrogen) system that has a neomycin-resistant gene to select the ectopic expression of *TWIST*. The neomycin phosphotransferase (neo) gene is one of the most common marker genes used in gene transfer experimentation. Several studies have suggested that neo gene expression could have deleterious effects on hematopoiesis [Brenner et al., 1993; Kohn et al., 1987], but potential effects in osteogenic cells have not been systematically investigated. Our data demonstrate that the pcDNA3 vector-only transfection with the neomycin resistance gene had vector effects in HSaOS-2 osteogenic cells. We found a moderate decrease in alkaline phosphatase activity, cellular proliferation rate and histone-4 mRNA expression in control cell lines that had only pcDNA3 vector compared to the parental HSaOS-2 cells (Figures 5,6). Therefore, we are careful to compare data from parental HSaOS-2 cells and pcDNA3 vector only cell controls.

Overall, results indicate that human *TWIST* overexpressing cells may de-differentiate and remain in an early osteoprogenitor-like state, and antisense *TWIST* cells progress to a more differentiated mature osteoblast-like state (Figure 8). Therefore, the level of *TWIST* can influence osteogenic gene expression and may act as a master switch in initiating bone cell differentiation by regulating the osteogenic cell lineage. The observations of human *TWIST* regulation during osteogenesis of osteoblast-like cells have added further understanding not only to the basic molecular mechanisms for tissue type growth and differentiation but also to their possible clinical applications. We are currently interested in testing whether *TWIST* can increase stem cell or progenitor populations in marrow stromal-derived stem cells. Enriching for stem cells or progenitor populations is important for success with respect to gene therapy applications. Furthermore, *TWIST* sense and antisense expressing osteoblast cell lines could provide useful tools to study the regulation of bone cell growth and differentiation.

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