Twist2 functions as a tumor suppressor in murine osteosarcoma cells

Tomoki Ishikawa,[1,2] Takatsune Shimizu,[1,3,4] Arisa Ueki,[1] Sayaka I. Yamaguchi,[1,5] Nobuyuki Onishi,[1] Eiji Sugihara,[1,3] Shinji Kuninaka,[1] Takeshi Miyamoto,[5] Hideo Morioka,[5] Robert Nakayama,[5] Eisuke Kobayashi,[5] Yoshiaki Toyama,[5] Yo Mabuchi,[6] Yumi Matsuzaki,[6] Rui Yamaguchi,[7] Satoru Miyano[7] and Hideyuki Saya[1,3,8]

[1]Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio University,Tokyo; [2]R&D Division, Oncology Research Laboratories, Daiichi Sankyo Co. Ltd, Tokyo; [3]CREST, Japan Science and Technology Agency, Tokyo; [4]Department of Pathophysiology, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Tokyo; Departments of [5]Orthopedic Surgery; [6]Physiology, School of Medicine, Keio University, Tokyo; [7]Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan

The epithelial–mesenchymal transition (EMT) contributes to the malignant progression of cancer cells including acquisition of the ability to undergo metastasis. However, whereas EMT-related transcription factors (EMT-TF) are known to play an important role in the malignant progression of epithelial tumors, their role in mesenchymal tumors remains largely unknown. We show that expression of the gene for Twist2 is downregulated in human osteosarcoma and correlates inversely with tumorigenic potential in mouse osteosarcoma. Forced expression of Twist2 in highly tumorigenic murine osteosarcoma cells induced a slight inhibition of cell growth *in vitro* but markedly suppressed tumor formation *in vivo*. Conversely, knockdown of Twist2 in osteosarcoma cells with a low tumorigenic potential promoted tumor formation *in vivo*, suggesting that Twist2 functions as a tumor suppressor in osteosarcoma cells. Furthermore, Twist2 induced expression of fibulin-5, which has been reported as a tumor suppressor. Medium conditioned by mouse osteosarcoma cells overexpressing Twist2 inhibited expression of the *MMP9* gene as well as invasion in mouse embryonic fibroblasts, and forced expression of Twist2 in osteosarcoma cells suppressed *MMP9* gene expression in tumor tissue. Data from the present study suggest that Twist2 inhibits formation of a microenvironment conducive to tumor growth and thereby attenuates tumorigenesis in osteosarcoma. (*Cancer Sci* 2013; 104: 880–888)

The epithelial–mesenchymal transition (EMT) plays a central role in morphogenesis during embryonic development as well as in the malignant progression of cancer including acquisition of the metastatic phenotype.[1,2] Although the underlying molecular mechanisms are complex, the EMT is regulated by three major groups of transcription factors belonging to the Zeb, Snail and Twist families.[3] Upregulation of the expression of these EMT-inducing transcription factors (EMT-TF) promotes tumor invasiveness and correlates with disease relapse and poor prognosis in individuals with various types of epithelial tumor.[4] In addition, EMT-TF are associated with resistance to chemotherapy and stem-like characteristics in cancer cells.[3] The EMT-TF are implicated in the malignant progression of epithelial tumors, but their role in mesenchymal tumors remains unclear.

Osteosarcoma is the most frequent type of malignant bone tumor and primarily affects children and adolescents. Although the prognosis of individuals with osteosarcoma has improved with chemotherapy, a significant proportion of patients remains incurable as a result of the development of metastatic lesions.[5,6] While EMT-TF are thought to contribute to the maintenance of mesenchymal features of osteosarcoma, it remains largely unknown whether they might promote metastasis and malignant behavior in this tumor type as they do in epithelial tumors. Amplification or deletion of the *TWIST1* locus has been detected in pediatric osteosarcoma, with such deletion being associated with a poor clinical outcome.[7,8] Twist1 and Twist2 are highly conserved basic helix–loop–helix transcription factors that play essential roles during embryogenesis.[9] Twist proteins inhibit skeletal development through direct and indirect inhibition of Runx2,[10,11] a key regulator of osteogenic differentiation. Expression of Twist1 and Twist2 in mesenchymal cells has been suggested to promote adipogenesis.[12] Together, these various observations suggest that Twist proteins might play an important role in normal bone marrow stromal cells (BMSC) as well as in malignant mesenchymal tumors.

We recently established osteosarcoma-initiating cells by introducing the c-Myc gene into BMSC of *Ink4a/Arf* knockout (*Ink4a* KO) mice.[13,14] Mice injected with these cells develop osteosarcoma with pathological features similar to those of human osteosarcoma, including the production of osteoid and highly metastatic behavior. In addition, we established two distinct types of cell lines from the c-Myc-overexpressing *Ink4a* KO BMSC: low-tumorigenic cells, which can differentiate into osteogenic, chondrogenic and adipogenic lineages; and high-tumorigenic cells, which can differentiate into osteogenic and chondrogenic lineages. Moreover, we found that the tumorigenic capacity was suppressed by forced expression of peroxisome proliferator-activated receptor γ (PPARγ), a master regulator of adipogenesis. These findings suggest that the loss of adipogenic potential is a key factor for osteosarcoma development *in vivo*.

We have now analyzed the expression of EMT-TF in clinical specimens of human osteosarcoma and found that *TWIST2* expression is frequently downregulated in this cancer. We further found that Twist2 inhibits tumor formation in our mouse osteosarcoma model, most likely by inducing expression of fibulin-5, a tumor suppressor. Our results therefore implicate Twist2 as a tumor suppressor in osteosarcoma.

Materials and Methods

Cell culture. The BMSC from wild-type (WT) or *Ink4a* KO mice as well as AO, AOT1, AOT2, AX, AXPP and AXPPT cells were established as previously described.[13] Further details are provided in Supporting Information Data S1.

Human specimens. For the experimental use of osteosarcoma surgical samples, informed consent was obtained from patients in accordance with the Hospital Ethical Guidelines of Keio

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[8]To whom correspondence should be addressed.
E-mail: hsaya@a5.keio.jp

University Hospital and the National Cancer Center Hospital. Normal human BMSC were purchased from Lonza (Basel, Switzerland).

RT and real-time PCR analysis. Total RNA preparation and real-time PCR analysis was performed as previously described.[13] The sequences of the PCR primers are shown in Table S1. Data were normalized by the corresponding amount of *Gapdh* mRNA. All data are means ± SD from three independent experiments.

Tumor xenograft model. All animal care and procedures were performed in accordance with the guidelines of Keio University. For xenograft experiments, cells in culture were isolated by exposure to trypsin and centrifugation, suspended in PBS and injected subcutaneously (3×10^6 cells/100 μL) into 8-week-old female syngeneic C57BL/6 or nude mice.

Gene expression profiling. Analysis of gene expression in human osteosarcoma specimens was performed with a Gene-Chip Human Genome U133 plus 2.0 array (Affymetrix, Santa Clara, CA, USA). Gene expression profiling of AX-Mock and AX-Twist2 cells was performed with a 3D-Gene Chip (Toray, Tokyo, Japan) as previously described.[13]

Plasmid and retroviral gene transfer, knockdown of Twist2, differentiation assays, cell growth assay, invasion assay, immunohistochemistry, flow cytometry and immunoblot analysis. Details are described in Data S1.

Statistical analysis. Data were compared between groups with the Student's *t* test. A *P*-value of <0.05 was considered statistically significant.

Results

Downregulation of *TWIST2* expression in human osteosarcoma. We initially characterized the gene expression profiles for six EMT-TF (Snail [also known as *SNAI1*], Slug [also known as *SNAI2*], Twist1, Twist2, Zeb1 and Zeb2) in 23 human osteosarcoma specimens and nine normal human BMSC with the use of microarray analysis. The expression level of *ZEB2* was upregulated, whereas those of *ZEB1* and *TWIST2* were significantly downregulated in osteosarcoma compared with BMSC (Fig. 1). In particular, the expression

level of *TWIST2* was consistently and markedly attenuated in all osteosarcoma specimens compared with BMSC. Therefore we further evaluated the role of Twist2 in osteosarcoma.

Downregulation of *TWIST2* correlates with increased tumorigenic ability and loss of adipogenic differentiation potential in a mouse osteosarcoma model. We recently established two osteosarcoma cell lines by introducing the c-Myc gene into BMSC of *Ink4a* KO mice.[13] AO cells possess a low tumorigenic potential as well as a trilineage (adipogenic, osteogenic and chondrogenic) differentiation potential similar to that of mesenchymal stem cells (MSC). In contrast, AX cells exhibit a high tumorigenic potential as well as a bilineage (osteogenic and chondrogenic) differentiation potential similar to that of osteochondrocyte-committed progenitor cells. Therefore we then examined the expression of *Twist1* and *Twist2* using real-time PCR analysis in BMSC from WT and *Ink4a* KO mice as well as in AO and AX cells (Fig. 2a). Similar to the results obtained with human osteosarcoma, the expression of *Twist2* was significantly downregulated in AX cells compared with that in WT and *Ink4a* KO BMSC or AO cells, whereas the expression of *Twist1* did not differ among the four cell types. Immunohistochemical staining of AX-derived subcutaneous tumors revealed that nuclei of GFP-positive tumor cells were negative for Twist2, while those of the surrounding stromal cells were positive (Fig. 2b).

Twist1 and Twist2 are key regulators of osteoblast differentiation.[10–12,15] Expression of *Twist2* was previously shown to decrease markedly during osteoblastic differentiation of MC3T3 cells.[15] We observed a marked reduction in the level of *Twist2* expression during osteogenic differentiation of AO cells (Fig. S1), suggesting that *Twist2* expression is associated with a differentiation state of the osteoblast lineage. We confirmed that expression of *Twist2* correlates positively with adipogenic differentiation potential and negatively with tumorigenic ability with the use of two additional cell lines. We established AOT1 and AOT2 cells from independent late-onset osteosarcomas derived from AO cells.[13] AOT1 and AOT2 cells have a higher tumorigenic ability than AO cells and have lost adipogenic differentiation potential while retaining osteogenic differentiation potential. The
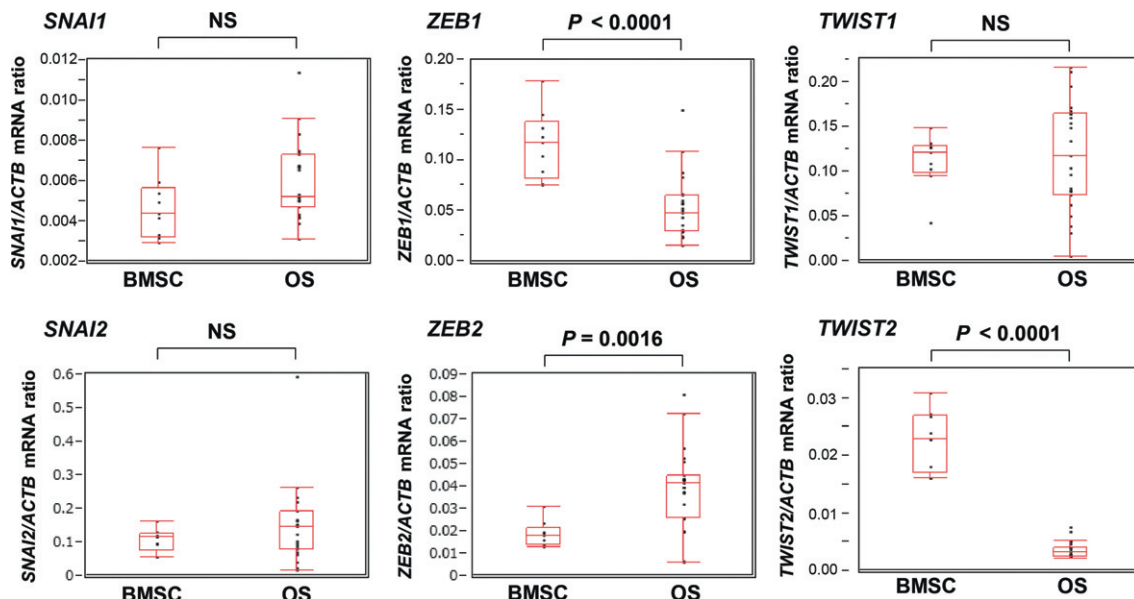

Fig. 1. Expression of EMT-TF genes in human osteosarcoma. Expression of EMT-TF in 23 human osteosarcoma (OS) specimens and nine human normal bone marrow stromal cells (BMSC) was evaluated using microarray analysis. The ratio of the abundance of each mRNA to that of β-actin (*ACTB*) mRNA is presented for each specimen as well as in box-and-whisker plots. EMT, epithelial–mesenchymal transition; NS, not significant; TF, transcription factor.
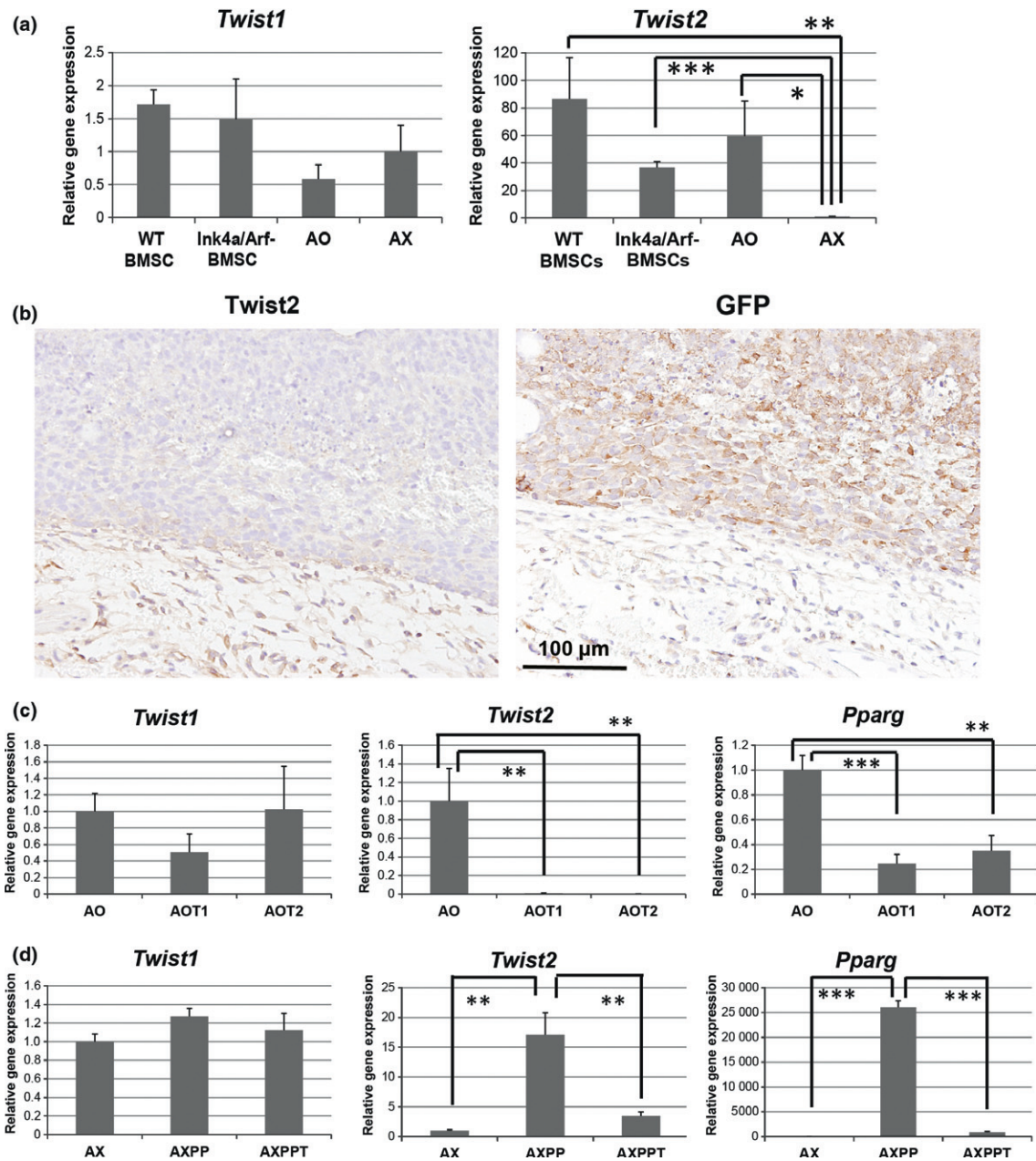
Fig. 2. Expression of *Twist2* correlates negatively with tumorigenic potential and positively with adipogenic differentiation ability in mouse osteosarcoma cells. (a) *Twist1* and *Twist2* expression in bone marrow stromal cells (BMSC) from WT or *Ink4a/Arf* knockout (*Ink4a* KO) mice as well as AO and AX cells. (b) Twist2 and GFP staining of serial sections of AX-derived subcutaneous tumor. (c) *Twist1*, *Twist2* and *Pparg* expression in AO, AOT1 and AOT2 cells. (d) *Twist1*, *Twist2* and *Pparg* expression in AX, AXPP and AXPPT cells. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

expression level of *Twist2* as well as that of the gene for PPARγ (*Pparg*) was greatly reduced in AOT1 and AOT2 cells compared with AO cells (Fig. 2c). In addition, the expression level of *Twist2* in AXPP cells (AX cells with restored adipogenic differentiation potential due to overexpression of PPARγ) was higher than that in parental AX cells (Fig. 2d). In contrast, the expression level of *Twist2* in AXPPT cells, which were established from an AXPP cell-derived osteosarcoma[13] and are highly tumorigenic, was again attenuated together with that of *Pparg* compared with that in AXPP cells (Fig. 2d). These results suggest that the expression of *Twist2* is associated with a trilineage differentiation potential and that the downregulation of *Twist2* expression is a key event in osteosarcoma tumorigenesis.

Overexpression of Twist2 attenuates tumorigenic potential in mouse osteosarcoma. To examine further the effect of Twist2 on adipogenic differentiation potential and tumorigenic ability of osteosarcoma cells, we introduced Twist2 into AX cells by retroviral gene transfer. Overexpression of Twist2 in AX cells resulted in upregulation of the mRNA for C/EBPα (*Cebpa*), a key regulator of adipogenesis,[16,17] whereas the expression of *Pparg* and adipogenic differentiation potential were not affected (Fig. 3a,c). In contrast, expression of the gene for the osteogenic marker osteocalcin (*Bglap*) as well as the osteogenic differentiation potential were attenuated by overexpression of Twist2 (Fig. 3b,c).

We then determined the effect of Twist2 on the growth of osteosarcoma cells *in vitro* and *in vivo*. The cell growth of
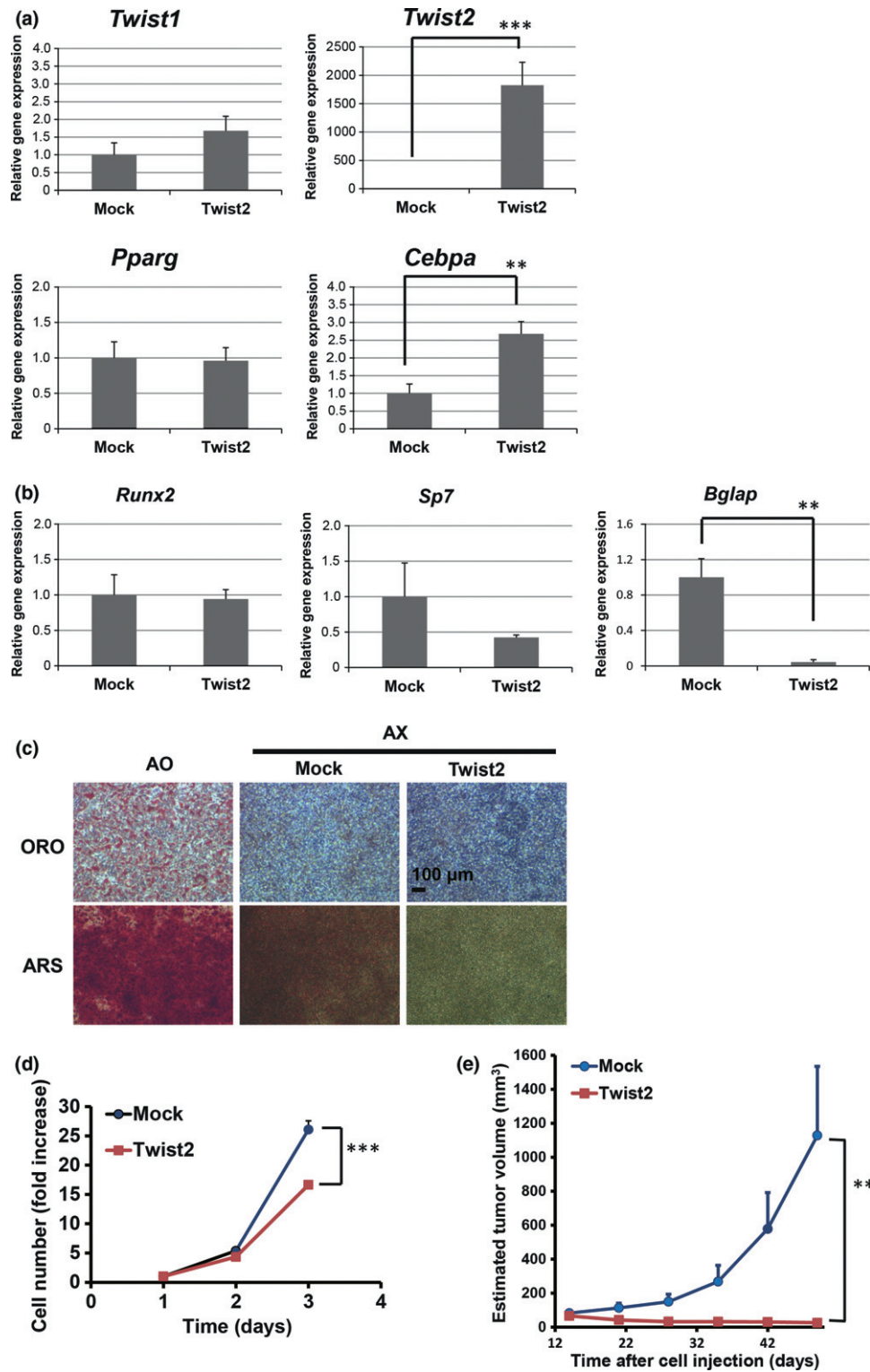
Fig. 3. Effects of Twist2 overexpression in AX cells on differentiation and tumorigenic potential. (a) *Twist1*, *Twist2*, *Pparg* and *Cebpa* expression in AX-Twist2 and AX-Mock. (b) The expression of the osteogenic differentiation marker genes *Runx2*, *Sp7* and *Bglap* in AX-Mock and AX-Twist2 cells. (c) Adipogenic and osteogenic differentiation potential in AO, AX-Mock and AX-Twist2 cells was evaluated with oil red O (ORO) and alizarin red S (ARS) staining, respectively. (d) Cell growth assay for AX-Mock and AX-Twist2 cells. (e) Growth of tumors derived from subcutaneously injected AX-Mock or AX-Twist2 cells in syngeneic mice. Data are means ± SEM for four tumors. **$P < 0.01$. ***$P < 0.001$.

AX cells was slightly but significantly inhibited by overexpression of Twist2 (Fig. 3d), whereas overexpression of Twist2 did not affect anchorage-independent growth and invasion in the *in vitro* assays (Fig. S2). In contrast, overexpression of Twist2 resulted in marked inhibition of tumorigenesis by AX cells implanted subcutaneously into syngeneic mice
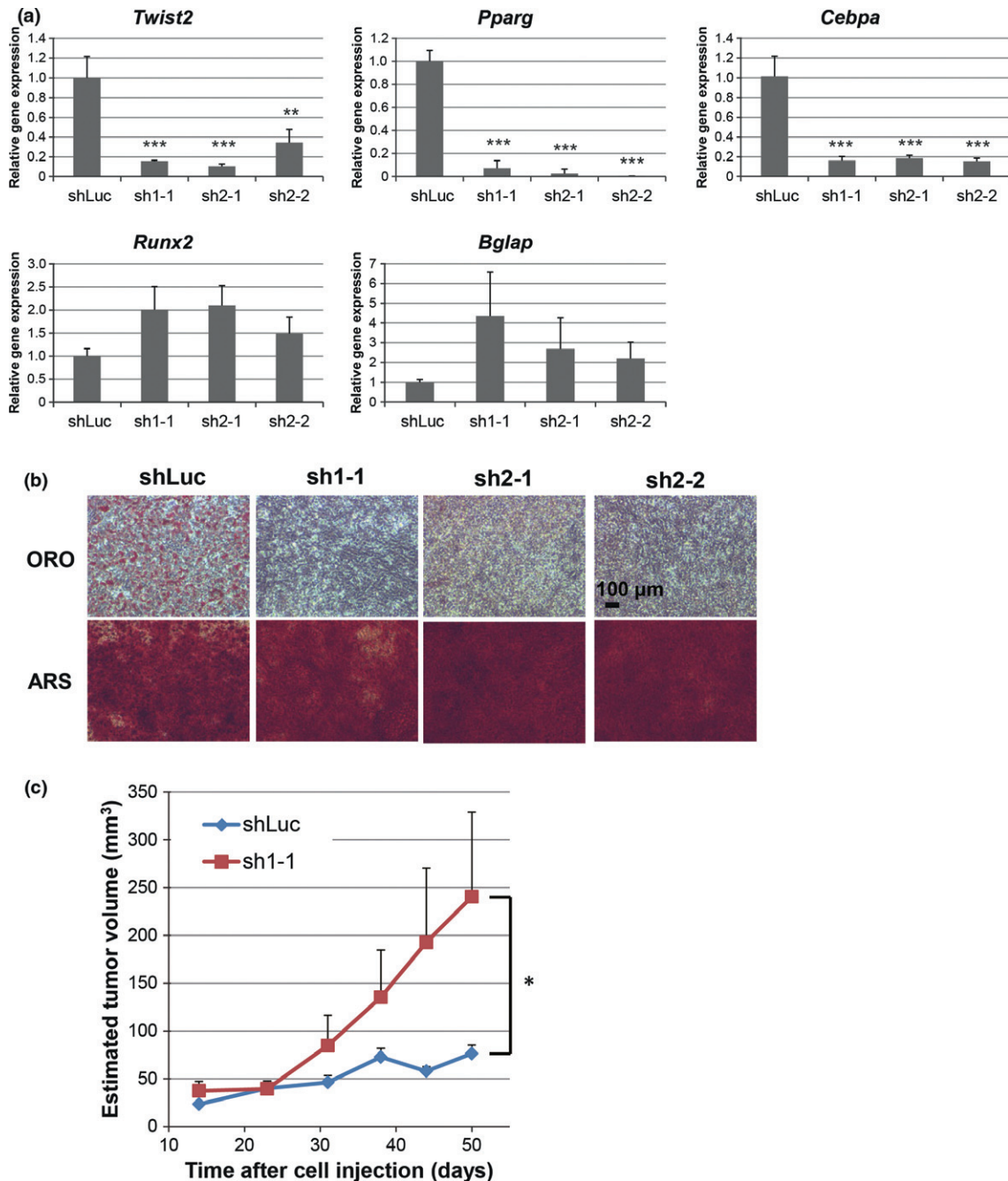
Fig. 4. Effects of Twist2 knockdown in AO cells on differentiation and tumorigenic potential. (a) *Twist2*, *Pparg*, *Cebpa*, *Runx2* and *Bglap* expression in AO cells expressing luciferase (shLuc) or Twist2 (sh1-1, sh2-1 and sh2-2) shRNA. (b) Adipogenic (ORO) and osteogenic (ARS) differentiation potential of AO-shLuc, AO-sh1-1, AO-sh2-1 and AO-sh2-2 cells. (c) Growth of tumors derived from subcutaneously injected AO-shLuc or AO-sh1-1 cells in nude mice. Data are means ± SEM for five tumors. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

(Fig. 3e). Collectively, these data suggest that the expression of Twist2 did not affect the adipogenic differentiation potential but directly inhibited tumorigenic activity in mouse osteosarcoma cells.

Knockdown of Twist2 enhances the tumorigenic potential of AO cells. To further examine the effect of Twist2 on tumor formation, we introduced a shRNA specific for Twist2 mRNA into the AO cells and established three sublines by performing single-cell cloning. The expression levels of *Pparg* and *Cebpa* as well as the adipogenic differentiation capacity were attenuated

in all sublines compared with the control AO cells (Fig. 4a,b). In contrast, osteogenic marker gene expression and osteogenic differentiation potential remained intact after depletion of Twist2. These results therefore suggest that AO cells acquired a phenotype similar to that of osteoblast-committed progenitors after knockdown of Twist2.

The AO cells expressing luciferase (control) or Twist2 shRNA failed to form tumors after subcutaneous implantation in syngeneic mice (data not shown). The Twist2-depleted cells formed tumors in nude mice, but the control cells did not

(Fig. 4c). These findings suggest that knockdown of Twist2 promotes tumorigenic activity in AO cells and that Twist2 plays an important inhibitory role in the growth of osteosarcoma cells *in vivo*.

Upregulation of *Fbln5* induced by Twist2 overexpression in mouse osteosarcoma. To gain insight into the mechanism by which Twist2 suppresses tumor growth, we examined gene expression in AX cells overexpressing Twist2 and the corresponding control (AX-Mock) cells. To investigate functional annotations enriched in upregulated genes induced in AX-Twist2 cells, we conducted a gene-enrichment analysis with Gene Ontology terms (gene-GO term enrichment analysis)

(Fig. 5a; the process is described in detail in Data S1 and Table S2). Consequently, we further analyzed a gene, *Fbln5*, which was annotated with both significant gene annotations and showed the highest fold change. Fibulin-5 was previously shown to suppress tumor growth and metastasis through inhibition of tumor angiogenesis[18–20] and the production of matrix metalloproteinase 9 (MMP9)[21], respectively. We confirmed that the expression of *Fbln5* in AX-Twist2 cells was upregulated at both the mRNA and protein levels (Fig. 5b,c). Similarly, the amounts of fibulin-5 mRNA and protein were found to be reduced in AX cells compared with BMSC or AO cells (Fig. 5b,c). Moreover, siRNA-mediated
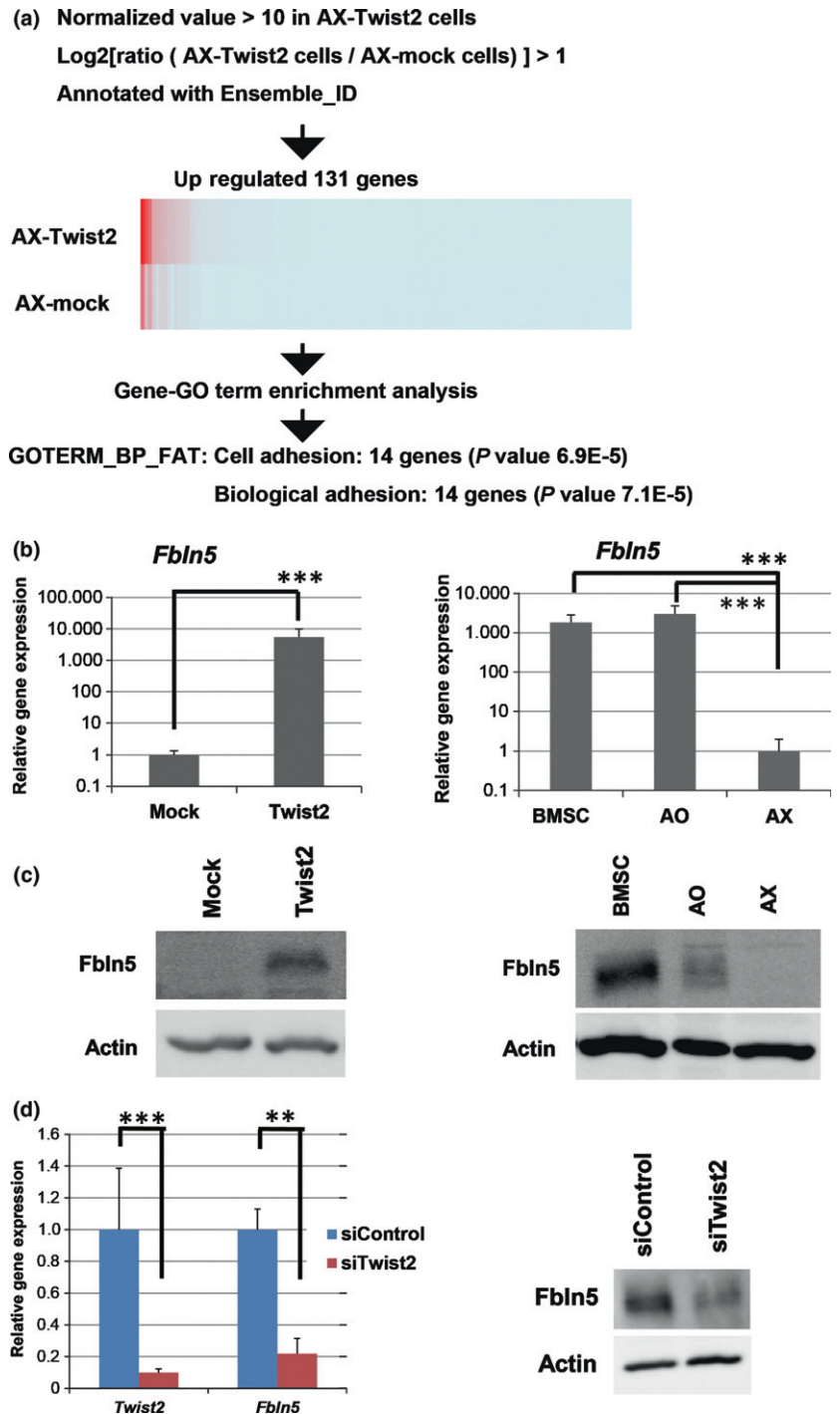

Fig. 5. Induction of *Fbln5* expression by Twist2. (a) Selection flow to identify the significant gene annotations upregulated by overexpression of Twist2. (b) Expression of *Fbln5* in AX-Mock and AX-Twist2 cells as well as in mouse bone marrow stromal cells (BMSC), AO and AX cells. (c) Immunoblot analysis of fibulin-5 expression in AX-Mock and AX-Twist2 cells as well as in mouse BMSC, AO and AX cells. β-Actin was examined as a loading control. (d) *Twist2* and *Fbln5* expression as well as immunoblot analysis of fibulin-5 in AO cells transfected with control or Twist2 siRNA. **$P < 0.01$. ***$P < 0.001$.

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knockdown of Twist2 in AO cells resulted in downregulation of the abundance of fibulin-5 mRNA and protein (Fig. 5d), again suggesting that Twist2 positively regulates expression of fibulin-5.

Overexpression of Twist2 downregulates *Mmp9* expression in osteosarcoma. Overexpression of fibulin-5 in tumor cells was previously shown to suppress metastatic organ colonization by tumor cells as well as the invasive activity of fibroblasts by blocking the induction of MMP9.[21] We therefore tested whether the forced expression of Twist2 in osteosarcoma affects the expression of *Mmp9* as well as the invasive ability in fibroblasts. Exposure of mouse embryonic fibroblasts (MEF) to the conditioned medium from AX-Twist2 cells resulted in significant suppression of the expression of *Mmp9* upregulated by TNF-α treatment (Fig. 6a), as well as MEF invasion (Fig. 6b), suggesting that overexpression of Twist2 in osteosarcoma cells affects their tumor environment.

We then analyzed the expression of *Mmp9* in subcutaneous tumors formed by AX-Mock or AX-Twist2 cells in syngeneic mice at 7 days after cell injection. The expression level of *Fbln5* in cells expressing GFP encoded by the retroviral vector used to establish AX cells was greater for tumors derived from AX-Twist2 cells than for those derived from AX-Mock cells (Fig. 7a). Furthermore, expression of *Mmp9* was downregulated in total tumor tissue derived from AX-Twist2 cells compared with that derived from AX-Mock cells (Fig. 7b). These data suggest that overexpression of Twist2 in tumor cells influences tumor-associated stromal cells and thereby inhibits tumor formation in mouse osteosarcoma.

Finally, we investigated the expression of *FBLN5* and *MMP9* in human osteosarcoma samples. The comparison of microarray data revealed that the expression level of *FBLN5* was downregulated, whereas that of *MMP9* was significantly upregulated in human osteosarcoma specimens compared with normal BMSC (Fig. 7c). These findings support our hypothesis that Twist2 functions as a tumor suppressor mediated by the induction of *FBLN5*.

Discussion

The EMT promotes malignant progression in epithelial tumors by increasing invasive capacity and conferring stem-like features, whereas the expression or function of EMT-TF in mesenchymal tumors has remained largely unexplored. We have now shown that loss of Twist2 expression is associated with progression of osteosarcoma, possibly as a result of downregulation of the tumor suppressor fibulin-5.

Signaling by transforming growth factor-β (TGF-β) plays a key role in the EMT and serves to either suppress or promote tumor growth depending on the cellular context.[22] It is possible that EMT-TF also function as tumor suppressors in specific types or at certain stages of cancer. In acute lymphoblastic leukemia, expression of *TWIST2* was found to be downregulated as a result of epigenetic inactivation, whereas overexpression of the gene resulted in inhibition of cell growth, induction of apoptosis and increased sensitivity to chemotherapeutic agents.[23] In prostate and bladder cancer cell lines, epithelial-like subpopulations possess high metastatic tumor-initiating activity, whereas mesenchymal-like subpopulations have a reduced tumor-initiating capacity, with Snail being a key EMT-TF and functioning as a tumor suppressor in these cells.[24] Thus, accumulating evidence suggests that EMT-TF play a suppressive role in tumorigenesis as well as contribute to the regulation of mesenchymal features.

The EMT-TF have recently been proposed to function as master regulators of adult stem cells. For example, Slug was found to regulate the mammary stem cell state.[25] In the present study, we focused on the role of Twist2 in osteosarcoma
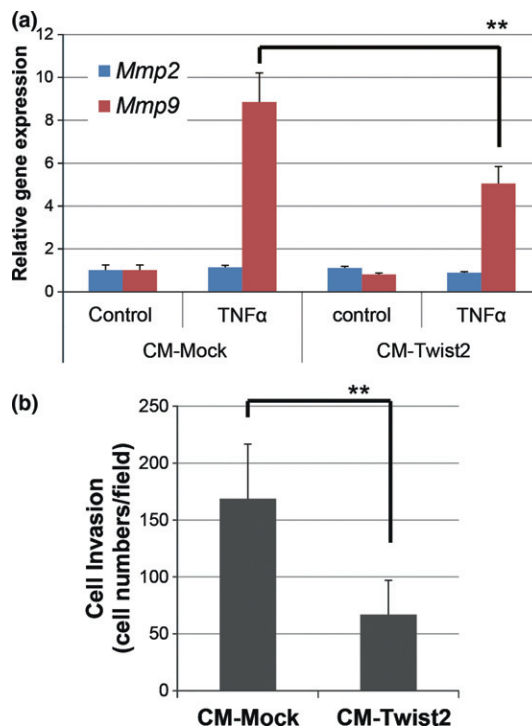

Fig. 6. Conditioned medium of AX-Twist2 cells inhibits *Mmp9* expression and invasion in MEF. (a) The expression of *Mmp2* and *Mmp9* in MEF exposed for 24 h to medium conditioned by AX-Mock or AX-Twist2 cells with or without mouse tumor necrosis factor-alpha (TNF-α) was determined using real-time PCR analysis. (b) The invasive ability of MEF treated conditioned medium (CM) of AX-Mock or AX-Twist2 cells with mouse TNF-α was analyzed using a Matrigel invasion assay. Data are means ± SD from six independent experiments. **P < 0.01.

and BMSC. Twist proteins play an essential role in osteoblast differentiation and maintenance of stem cell properties, with Twist2 being a candidate marker for MSC.[26] We found that Twist2 inhibited osteogenic differentiation and that expression of Twist2 was correlated with the MSC state and in our model.

The results of the present study suggest that loss of Twist2 expression is a key event in osteosarcoma formation, but it is unclear how expression of Twist2 is regulated in the process of tumorigenesis. In general, epigenetic changes are known to control stem cell differentiation. DNA methylation and histone modifications are critical events for MSC differentiation toward osteogenic and adipogenic lineages.[27] It was also reported that Twist2 was repressed through DNA methylation in several cancer cell lines.[28,29] Therefore, we assumed that repression of Twist2 by epigenetic modification might be essential for osteosarcoma formation from MSC.

Given that our mouse osteosarcoma model has a *Ink4a* KO background, we investigated the relationship between the expression of *TWIST2* and *INK4A/ARF* in human osteosacoma specimens. However, we could not find any significant relationship ($R = -0.189$, $P = 0.388$) between them.

It remains unclear whether inhibition of osteogenic differentiation by Twist2 is directly related to its tumor-suppressive action. However, we found that Twist2 modulates not only osteogenic and adipogenic differentiation but also expression of fibulin-5 in osteosarcoma cells. Fibulin-5 is an ECM protein that contributes to the formation and stabilization of basement membrane, elastic fibers and connective tissue.[18] It suppresses tumor formation through control of tumor cell proliferation and motility as well as angiogenic sprouting.[18–21,30] Thus, Twist2
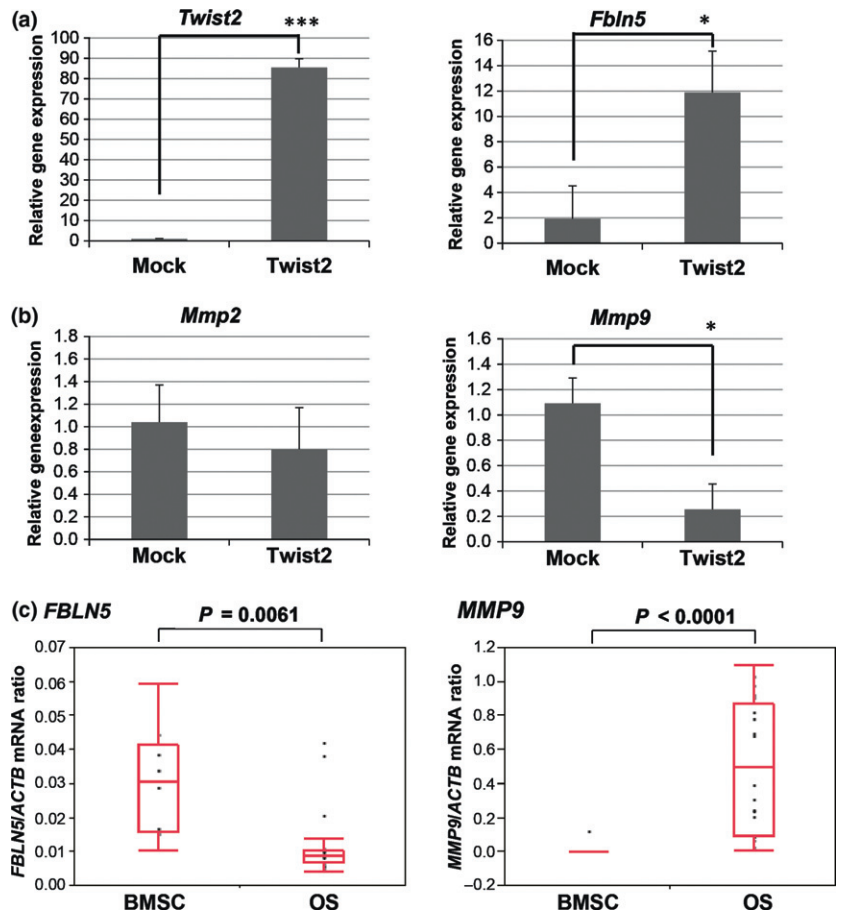
Fig. 7. Reduced *Mmp9* expression in tumors derived from AX cells overexpressing Twist2. (a) Expression of *Twist2* and *Fbln5* in GFP-positive cells isolated from tumors formed in syngeneic mice at 7 days after subcutaneous injection of AX-Mock or AX-Twist2 cells. (b) *Mmp9* expression in total tumor tissue formed as in (a). (c) The expression of *FBLN5* and *MMP9* in human osteosarcoma (OS) specimens and bone marrow stromal cells (BMSC) was evaluated using microarray analysis. The ratio of the abundance of each mRNA to that of β-actin (*ACTB*) mRNA is presented for each specimen as well as in box-and-whisker plots. *$P < 0.05$. ***$P < 0.001$.

might be expected to inhibit tumor angiogenesis or formation of an appropriate tumor microenvironment by various mechanisms. In contrast, fibulin-5 also stimulates cell growth and motility in fibroblasts and fibrosarcoma cells, suggesting that it might exhibit opposite effects on tumorigenesis in a manner dependent on cell context.[31] The fibulin-5 gene is also a target of TGF-β signaling.[32,33] Together with our data showing that Twist2 regulates the expression of fibulin-5, these various observations suggest that fibulin-5 expression might be modulated during the EMT in various cell contexts.

Twist1 and Twist2 are highly conserved and have redundant functions and Twist1 also has an important role in osteogenic differentiation.[10–12] We confirmed that overexpression of Twist1 in AX cells could also induce fibulin-5 and suppress *in vivo* tumor growth (Fig. S3). Deletion of the *TWIST1* locus has been reported in osteosarcoma patients[7] and some of our clinical samples showed a low expression of *TWIST1* (Fig. 1), suggesting that Twist1 has a similar tumor suppressive role to Twist2 in osteosarcoma formation.

The outcome of the EMT is thought to be regulated in a complex manner dependent on cell context and the microenvironment.

Further investigation into the roles of EMT-TF in various tumor types on different genetic backgrounds or in different microenvironments should provide insight into tumor formation and malignant progression as well as provide a basis for selection of patients for treatment with anticancer drugs including those that target the EMT.

Disclosure Statement

T.I. is an employee of Daiichi Sankyo Co. Ltd. H.S. received research grants from Diichi Sankyo Inc., Kyowa Hakko-Kirin Inc., LinkGenomics Inc. and Shimadzu Corp. The other authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Downregulation of *Twist2* expression during osteogenic differentiation of AO cells.

Fig. S2. Effects of Twist2 overexpression in AX cells on anchorage-independent growth and invasion potential.

Fig. S3. Effects of Twist1 overexpression in AX cells on tumorigenic potential.

Table S1. Sequences of PCR primers and sense oligonucleotides for shRNA.

Table S2. Selected genes that are more highly expressed in AX-Twist2 cells than in AX-Mock cells.

Data S1. Supplementary materials and methods.