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Osteosarcoma is a common malignant bone tumor with a propensity to metastasize to the lungs. Epigenetic abnormalities have been demonstrated to underlie osteosarcoma development; however, the epigenetic mechanisms that are involved in metastasis are not yet clear. Here, we analyzed 2 syngeneic primary human osteosarcoma cell lines that exhibit disparate metastatic potential for differences in epigenetic modifications and expression. Using methylated DNA immunoprecipitation (MeDIP) and microarray expression analysis to screen for metastasis-associated genes, we identified Iroquois homeobox 1 (IRX1). In both human osteosarcoma cell lines and clinical osteosarcoma tissues, IRX1 overexpression was strongly associated with hypomethylation of its own promoter. Furthermore, experimental modulation of IRX1 in osteosarcoma cell lines profoundly altered metastatic activity, including migration, invasion, and resistance to anoikis in vitro, and influenced lung metastasis in murine models. These prometastatic effects of IRX1 were mediated by upregulation of CXCL14/NF-κB signaling. In serum from osteosarcoma patients, the presence of IRX1 hypomethylation in circulating tumor DNA reduced lung metastasis–free survival. Together, these results identify IRX1 as a prometastatic gene, implicate IRX1 hypomethylation as a potential molecular marker for lung metastasis, and suggest that epigenetic reversion of IRX1 activation may be beneficial for controlling osteosarcoma metastasis.

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IRX1 hypomethylation promotes osteosarcoma metastasis via induction of CXCL14/NF-κB signaling

Jinchang Lu,1 Guohui Song,1 Qinglian Tang,1 Changye Zou,1 Feng Han,2 Zhiqiang Zhao,1 Bicheng Yong,1,3 Junqiang Yin,1 Huaiyuan Xu,1 Xianbiao Xie,1 Tiebang Kang,4 YingLee Lam,2 Huiling Yang,5 Jingnan Shen,1 and Jin Wang1

1Department of Musculoskeletal Oncology, the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong, China. 2Division of General Orthopaedics and Oncology, Department of Orthopaedics and Traumatology, University of Hong Kong, Queen Mary Hospital, Hong Kong, China. 3Department of Pediatric Orthopedics, Guangzhou Women and Children’s Hospital, Guangzhou, Guangdong, China. 4State Key Laboratory of Oncology in South China, Sun Yat-Sen University Cancer Center, Guangzhou, China. 5Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong, China.

Osteosarcoma is a common malignant bone tumor with a propensity to metastasize to the lungs. Epigenetic abnormalities have been demonstrated to underlie osteosarcoma development; however, the epigenetic mechanisms that are involved in metastasis are not yet clear. Here, we analyzed 2 syngeneic primary human osteosarcoma cell lines that exhibit disparate metastatic potential for differences in epigenetic modifications and expression. Using methylated DNA immunoprecipitation (MeDIP) and microarray expression analysis to screen for metastasis-associated genes, we identified Iroquois homeobox 1 (IRX1). In both human osteosarcoma cell lines and clinical osteosarcoma tissues, IRX1 overexpression was strongly associated with hypomethylation of its own promoter. Furthermore, experimental modulation of IRX1 in osteosarcoma cell lines profoundly altered metastatic activity, including migration, invasion, and resistance to anoikis in vitro, and influenced lung metastasis in murine models. These prometastatic effects of IRX1 were mediated by upregulation of CXCL14/NF-κB signaling. In serum from osteosarcoma patients, the presence of IRX1 hypomethylation in circulating tumor DNA reduced lung metastasis–free survival. Together, these results identify IRX1 as a prometastatic gene, implicating IRX1 hypomethylation as a potential molecular marker for lung metastasis, and suggest that epigenetic reversion of IRX1 activation may be beneficial for controlling osteosarcoma metastasis.

Introduction

Osteosarcoma, the most common primary malignant bone tumor, occurs most frequently in children and adolescents and has a strong tendency to metastasize. Pulmonary metastasis is the main cause of medical therapy failure and death in osteosarcoma patients. Despite an intensive search for new therapeutic strategies, survival has not improved over the past 2 decades (1). Given that controlling metastasis is the key to improving survival, there is an urgent need to investigate the underlying mechanisms of osteosarcoma metastasis and to develop more effective approaches to suppress lung metastasis.

It is widely accepted that the multistep process of cancer evolution is driven by both genetic and epigenetic abnormalities (2). Unlike genetic alterations, epigenetic changes are potentially reversible, making them attractive and promising targets for therapeutic intervention. Previous studies indicated that abnormal DNA methylation, a major epigenetic modification, is involved in the dysregulation of the cell cycle and apoptosis as well as in the proliferation and differentiation of osteosarcoma (3, 4). However, the epigenetic mechanisms of osteosarcoma metastasis remain largely unknown.

Iroquois homeobox 1 (IRX1), a member of the Iroquois homeobox family of transcription factors, plays a crucial role in embryonic development (5). Recently, IRX1 was identified as a potential tumor-suppressor gene in head and neck squamous cell carcinoma (HNSCC) and gastric cancer (6, 7); however, further efforts are required to determine the exact function of IRX1 in the development of other cancers, including osteosarcoma. IRX1 is involved in limb development (8) and the etiology of kyphoscoliosis (9), suggesting that aberrant IRX1 expression may contribute to abnormal bone formation. Moreover, a gain of chromosome 5p15.33 (chromosomal location of IRX1) has been frequently detected in osteosarcoma cell lines (10). Therefore, the role of IRX1 in the pathogenesis of osteosarcoma is of interest.

Chemokine (C-X-C motif) ligand 14 (CXCL14), also known as BRAK, is a small cytokine belonging to the CXC chemokine family (11), and the gene encoding CXCL14 is commonly recognized as a tumor-suppressor gene (12–14). However, recent studies indicated that CXCL14 might actually promote tumor progression in some types of cancer (15–18). For example, CXCL14 was upregulated in pancreatic cancer tissues and enhanced the invasiveness of pancreatic cancer cells (17). CXCL14 transcript levels are markedly high in papillary thyroid carcinoma and are positively correlated with lymph node metastasis (16). We previously showed that overexpression of CXCL14 predicts poor overall survival in osteosarcoma patients (19); however, its biological function in osteosarcoma metastasis requires further assessment.
Here, we used high-throughput approaches to identify novel epigenetically regulated metastasis-related genes in osteosarcoma. We found that IRX1 is epigenetically activated in highly metastatic osteosarcoma cell lines. IRX1 expression in primary human osteosarcoma samples was positively associated with promoter hypomethylation. The downregulation of IRX1 in osteosarcoma cell lines resulted in decreased CXCL14 expression levels, inhibition of NF-κB activity, and suppression of metastasis. Moreover, the hypomethylation of IRX1 in serum from patients was correlated with a high risk of lung metastasis. Taken together, our findings represent a significant step forward in understanding the role of the epigenetically activated gene IRX1 in osteosarcoma metastasis and provide a potential target for epigenetic-based osteosarcoma therapy.

Results

Epigenetic screen for genes driving invasion and metastasis in osteosarcoma. We previously established 2 syngeneic human osteosarcoma cell lines, ZOS and ZOSM, which were derived from a primary tumor and a skip metastasis, respectively, in the same patient (20). Biologically, ZOSM cells are more invasive and metastatic than are ZOS cells. To explore the underlying epigenetic mechanisms related to metastasis, we performed a methylated DNA immuno-precipitation (MeDIP) assay in combination with expression profiling using these 2 primary cell lines. MeDIP and expression array data were then filtered and integrated. Compared with ZOS, 18 genes showed promoter hypomethylation and were upregulated in ZOSM, while 8 genes exhibited promoter hypermethylation and were downregulated in ZOSM cells (Supplemental Table 1 and Figure 1A). Some of these upregulated genes, including HOXB7, EpCAM, and CXCL6, were previously reported to be involved in cancer metastasis. Notably, high expression levels of PRAME were recently found to be associated with poor prognosis and lung metastasis of human osteosarcoma in our previous study (21). The identification of these known metastasis-related genes suggested that our high-throughput platform was effective for the discovery of genes that drive osteosarcoma metastasis.

Therefore, using real-time PCR, we determined the expression levels of the 26 differentially expressed genes selected (listed in Figure 1A). Among these genes, our results demonstrated that the greatest difference in expression levels of IRX1 was observed between ZOS and ZOSM cells (Supplemental Figure 1A). Therefore, we focused on IRX1 in this study, while other candidates such as SALL1 were investigated in separate studies. On the basis of our real-time PCR results, there was a greater than 15-fold increase in IRX1 expression in highly metastatic ZOSM cells compared with that detected in nonmetastatic ZOS cells (Figure 1B). To confirm that the IRX1 expression pattern was not specific to only the primary cell lines, we examined IRX1 expression levels in 2 other commonly used osteosarcoma cell lines, MNNG-HOS (poorly metastatic) and 143B (highly metastatic), both of which are derived from the TE85 human osteosarcoma cell line (22). As expected, higher IRX1 mRNA expression was detected in the highly metastatic 143B cells relative to the mRNA levels in MNNG-HOS cells (Figure 1B). Accordingly, IRX1 protein levels were also substantially upregulated in ZOSM and 143B cells, as indicated by Western blot and immunofluorescence analyses (Figure 1C and Supplemental Figure 1B). We next examined IRX1 mRNA expression levels in human osteosarcoma tissues and found that tissues from patients with lung metastasis showed higher IRX1 expression levels than did tissues from patients without lung metastasis (Figure 1D). To further evaluate the clinical relevance of IRX1 in osteosarcoma metastasis, we collected 16 pairs of archival samples of osteosarcoma lung metastases and their corresponding primary osteosarcoma tissues. Immunohistochemical staining showed that the IRX1 expression scores were significantly higher in the lung metastases than in the primary tumors (Figure 1, E and F). These findings suggest that IRX1 plays an important role in osteosarcoma metastasis.

Elevated IRX1 expression is linked to hypomethylation of its own promoter. We next examined the association between IRX1 expression and its promoter methylation status in osteosarcoma. As shown in Figure 2A, a decreased methylation level was detected by bisulfite-sequencing PCR (BSP) in the promoter region of IRX1 (–521 to –679 bp, which contains 5 predicted transcription factor-binding sites) in ZOSM and 143B cells compared with methylation levels detected in ZOS and MNNG-HOS cells (also see Supplemental Figure 2A–C). Next, we analyzed IRX1 promoter methylation levels in human osteosarcoma tissues. Genomic DNA isolated from 40 fresh surgical osteosarcoma specimens was treated with sodium bisulfite, amplified by PCR, and subjected to MassARRAY analysis. The results demonstrated that there was a significant correlation between increased IRX1 expression and reduced promoter methylation (Figure 2, B and C). Furthermore, treatment of ZOS and MNNG-HOS cells with the methyltransferase inhibitor 5-aza-deoxycytidine (DAC), an effective DNA demethylating agent, significantly decreased the methylation level of the IRX1 promoter and resulted in activation of IRX1 gene expression (Figure 2, D and E, and Supplemental Figure 2D). Conversely, treatment of ZOSM and 143B cells with the methyl donor S-adenosyl-L-methionine (AdoMet), which has recently been shown to inhibit demethylase activity and induce DNA methylation (23), led to suppression of IRX1 gene expression (Figure 2, F and G, and Supplemental Figure 2E).

To further explore whether DNA methylation directly regulates IRX1 promoter activity, we cloned the promoter region of IRX1 (–521 to –679 bp) into a luciferase reporter construct. The cloned fragment was methylated in vitro by SsI methylase (methylation of 12 CpGs), HpaII methylase (methylation of 2 CpGs), or HhaI methylase (methylation of 2 CpGs). IRX1 promoter activity was determined after transfection of 143B cells with methylated or mock-methylated luciferase constructs. As shown in Figure 2H, methylation repressed IRX1 promoter activity in a methylation dose–dependent manner. Taken together, these results suggest that elevated IRX1 expression in osteosarcoma is associated with loss of methylation in the IRX1 promoter.

IRX1 promotes the migration, invasion, and resistance to anoikis of osteosarcoma cells in vitro. Because osteosarcoma cells with a higher metastatic potential (ZOSM and 143B cells) exhibited elevated IRX1 expression (Figure 1, B and C), we sought to determine the role of IRX1 in osteosarcoma metastasis. It is well established that successful metastasis requires several essential steps. To evaluate the contribution of IRX1 to osteosarcoma cell migration, invasion, and resistance to anoikis in vitro, we...
anoikis is a physiological barrier to metastasis and that resistance to anoikis may facilitate distant organ metastasis (24, 25), we further examined the effect of IRX1 deletion on the anoikis of osteosarcoma cells by culturing cells on ultra-low attachment plates. After a 48-hour incubation, we found that ZOSM-shIRX1 and 143B-shIRX1 cells were more sensitive to detachment-induced apoptosis than were their scrambled controls (Figure 3D). These results suggest that IRX1 regulates the metastatic behavior of osteosarcoma cells.

first generated 2 lentiviral constructs encoding IRX1-targeting shRNAs and established cell lines in which IRX1 was stably knocked down (Figure 3A). We found that IRX1 knockdown did not influence cell proliferation (Supplemental Figure 3A). However, IRX1 downregulation inhibited the ability of ZOSM and 143B cells to fill the wound gap (Figure 3B) and migrate through Transwell membranes (Figure 3C). Suppression of IRX1 expression significantly reduced the invasive ability of both cell lines, as measured by Boyden chamber assays (Figure 3C). Given that anoikis is a physiological barrier to metastasis and that resistance to anoikis may facilitate distant organ metastasis (24, 25), we further examined the effect of IRX1 deletion on the anoikis of osteosarcoma cells by culturing cells on ultra-low attachment plates. After a 48-hour incubation, we found that ZOSM-shIRX1 and 143B-shIRX1 cells were more sensitive to detachment-induced apoptosis than were their scrambled controls (Figure 3D). These results suggest that IRX1 regulates the metastatic behavior of osteosarcoma cells.
Figure 2. Elevated IRX1 expression is associated with hypomethylation of its promoter. (A) BSP analysis of the IRX1 promoter (−521 to −679) in 4 osteosarcoma cell lines. (B) Sequenom MassARRAY analysis of the IRX1 promoter in 40 human osteosarcoma tissues. Data shown are from 8 representative samples. (C) IRX1 expression was associated with a low methylation rate. $P = 0.001$ by Spearman’s rank correlation analysis. (D) BSP analysis of the IRX1 promoter in ZOS and MNNG/HOS cells after treatment with 2 μM DAC for 3 days. (E) Western blot analysis shows that treatment with 2 μM DAC induced IRX1 protein expression in ZOS and MNNG/HOS cells. (F) BSP analysis of the IRX1 promoter in ZOSM and 143B cells after treatment with 500 μM AdoMet for 6 days. (G) Western blot analysis shows that treatment with 500 μM AdoMet suppressed IRX1 expression in ZOSM and 143B cells. (H) Methylated or mock-methylated IRX1 promoter (−521 to −679) – luciferase reporter constructs were transiently transfected into 143B cells. Luciferase activity was analyzed after a 48-hour transfection. Values shown are the mean ± SD of 3 separate determinations. *$P < 0.05$ by Student’s $t$ test (D and F) and 1-way ANOVA (H).
and Supplemental Figure 3B). Taken together, these results indicate that IRX1 positively regulates osteosarcoma cell migration, invasion, and resistance to anoikis.

**IRX1 promotes osteosarcoma metastasis to the lung in vivo.** We next examined the effect of IRX1 on osteosarcoma metastasis in vivo using mouse xenograft models. In our previous experimental metastasis assay, no metastases were found in mice injected with ZOS cells through the tail vein; however, mice injected with We further confirmed the positive effect of IRX1 on migration, invasion, and resistance to anoikis through gain-of-function studies. Here, we chose ZOS and MNNG-HOS cells that expressed low endogenous levels of IRX1 and exhibited weak metastatic ability. As expected, the ectopic overexpression of IRX1 in ZOS and MNNG-HOS cells increased their migratory and invasive abilities under suspension culture conditions (Figure 3, E–H, and Supplemental Figure 3B). Taken together, these results indicate that IRX1 positively regulates osteosarcoma cell migration, invasion, and resistance to anoikis.
ZOSM cells developed pulmonary and spinal metastases (20). To determine whether IRX1 participates in establishing lung metastases, ZOS-IRX1, ZOSM-shIRX1-A/B, and their control cells were injected into the tail veins of NOD/SCID mice ($n = 10$ per group). After 8 weeks, we checked for lung micrometastases. None of the mice injected with ZOS control cells developed lung metastases, but 2 mice in the ZOS-IRX1 group developed small metastases in the lungs (Figure 4A). Meanwhile, IRX1 knockdown in ZOSM cells caused a significant reduction in lung metastasis (Figure 4B).

To better mimic the clinical scenario, we adopted an orthotopic mouse model to further explore the effect of IRX1 on osteosarcoma metastasis. 143B-shIRX1-A/B and control cells were injected into the proximal tibia of NOD/SCID mice ($n = 10$ per group), and tumor size was measured over time. After 5 weeks, the lungs were harvested, and micrometastases were analyzed. Consistent with the in vitro results, IRX1 knockdown in 143B cells did not affect primary tumor growth (Supplemental Figure 4, A–C), but markedly inhibited lung metastasis in vivo (Figure 4C). We then examined the effect of IRX1 overexpression in MNNG-HOS cells that rarely produced spontaneous lung metastases. While there was no significant difference in primary tumor growth between MNNG-HOS-IRX1 and control cells (Supplemental Figure 4, D and E), MNNG-HOS-IRX1 exhibited an increase in the rate of metastasis (from 0% to 30%), as shown in Figure 4D. Together, these findings confirm that IRX1 is involved in osteosarcoma lung metastasis in vivo.

**Figure 4.** IRX1 promotes osteosarcoma metastasis to the lungs in a mouse model. (A) Overexpression of IRX1 increased the rate of lung metastasis after tail-vein injection of ZOS-IRX1 or control cells. (B) Suppression of IRX1 in ZOSM cells significantly decreased the number of lung metastases. (C) Mice bearing 143B-shControl (143B-shCtrl) cells developed multiple large metastases, while the suppression of IRX1 dramatically inhibited the lung metastasis of 143B cells. (D) IRX1 overexpression in poorly metastatic MNNG-HOS cells increased the metastatic rate in mice from 0% (0 of 10) to 30% (3 of 10). Representative histological images of lung sections are shown. Black arrow indicates the metastatic nodule. Scale bars: 200 μm. *$P < 0.05$ by Student’s t test.
IRX1 upregulates CXCL14 expression. To explore the underlying mechanism by which IRX1 promotes osteosarcoma progression, we used a gene expression microarray to identify genes that were differentially expressed in 143B-shIRX1-A and control cells. A 2-fold screening filter was applied, and we found 115 upregulated genes and 78 downregulated genes in the 143B-shIRX1 cells relative to what was observed in the controls. The top 15 differentially expressed genes are shown in Supplemental Table 2 and Figure 5A; we mainly focused on genes with decreased expression after IRX1 knockdown. Among them, we were specifically interested in CXCL14 (with an ~5-fold decrease), which has been reported to be associated with tumor progression and metastasis in select cancers (15, 17, 18). Importantly, our recent study demonstrated that CXCL14 expression was upregulated in osteosarcoma and predicted poor outcomes.

Figure 5. IRX1 expression upregulates CXCL14 gene expression. (A) Microarray analysis demonstrated that IRX1 suppression led to decreased CXCL14 mRNA levels in 143B cells. (B–D) CXCL14 mRNA levels (B), CXCL14 protein expression (C), and secreted CXCL14 protein levels (D) were determined in 143B-shIRX1 and MNNG-HOS-IRX1 cells. (E) CXCL14 mRNA expression levels in human osteosarcoma tissues (n = 70) were analyzed by real-time PCR. (F) IRX1 expression was correlated with CXCL14 expression in human osteosarcoma tissues (Pearson’s correlation analysis, P < 0.001). (G) CXCL14 promoter/luciferase reporter constructs (nucleotides -2000 to +515) were transiently transfected into the indicated cells, and luciferase activity was analyzed after 48 hours. (H) ChIP was performed to detect binding of IRX1 to the CXCL14 promoter in 143B cells. PCR amplification result using the primers flanking site A (ACACCTGT) is shown (positive control: BDKRB2; negative control: HEBP1). Data represent the mean ± SD of 3 separate determinations. *P < 0.05 by Student’s t test.
protein levels in the conditioned medium of 143B-shIRX1 cells, while we found increased levels of secreted CXCL14 protein in MNNG-HOS-IRX1 cells (Figure 5D). A similar IRX1-CXCL14 expression pattern was observed in ZOSM-shIRX1 and ZOS-IRX1 cells (Supplemental Figure 5). Next, we investigated the CXCL14 mRNA expression pattern in osteosarcoma tissues. Higher CXCL14 mRNA levels were found in patients with lung metastasis than in those without lung metastasis, and further analysis revealed that there was a positive correlation between IRX1 and CXCL14 expression (Figure 5, E and F). These results demonstrate that CXCL14 is positively regulated by IRX1.

To confirm the microarray data, we examined the expression of CXCL14 in 143B-shIRX1 and MNNG-HOS-IRX1 cells using quantitative real-time PCR and Western blot analyses. CXCL14 mRNA and protein levels were downregulated by IRX1 siRNA transfection and upregulated by IRX1 overexpression (Figure 5, B and C). Moreover, we detected a decrease in secreted CXCL14 protein levels in the conditioned medium of 143B-shIRX1 cells, while we found increased levels of secreted CXCL14 protein in MNNG-HOS-IRX1 cells (Figure 5D). A similar IRX1-CXCL14 expression pattern was observed in ZOSM-shIRX1 and ZOS-IRX1 cells (Supplemental Figure 5). Next, we investigated the CXCL14 mRNA expression pattern in osteosarcoma tissues. Higher CXCL14 mRNA levels were found in patients with lung metastasis than in those without lung metastasis, and further analysis revealed that there was a positive correlation between IRX1 and CXCL14 expression (Figure 5, E and F). These results demonstrate that CXCL14 is positively regulated by IRX1.
cells, while IRX1 overexpression in the MNNG-HOS cells enhanced CXCL14 promoter activity (Figure 5G). Further inspection of the CXCL14 promoter revealed 2 predicted IRX1-binding sites (26): site A at –857 bp (ACACCTGT) and site B at –1950 bp (ACATCTGT). The presence of these sites suggests that IRX1 might directly activate CXCL14 expression. We then examined whether IRX1 could bind to the CXCL14 promoter in the context of osteosarcoma cells. Because IRX1 is a transcription factor, we examined whether IRX1 upregulated CXCL14 by activating the CXCL14 promoter. A human CXCL14 promoter fragment (nucleotides –2000 to +515) was cloned into the pGL3 luciferase vector and then cotransfected with an shRNA targeting IRX1 into 143B cells or with an IRX1 cDNA construct into MNNG-HOS cells. IRX1 knockdown clearly suppressed CXCL14 promoter activity in the 143B-shIRX1 cells, while IRX1 overexpression in the MNNG-HOS cells enhanced CXCL14 promoter activity (Figure 5G). Further inspection of the CXCL14 promoter indicated that CXCL14 might have two predicted IRX1-binding sites (26): site A at –857 bp (ACACCTGT) and site B at –1950 bp (ACATCTGT). The presence of these sites suggests that IRX1 might directly activate CXCL14 expression. We then examined whether IRX1 could bind to the CXCL14 promoter in the context of osteosarcoma cells.
of ZOS cells with a CXCL14 expression vector promoted migration, invasion, and resistance to anoikis (Figure 6, A and B). Given that CXCL14 is a secreted protein, we used a neutralizing antibody to block CXCL14. CXCL14 blockade in the culture medium attenuated the metastasis-promoting effects of CXCL14 overexpression on ZOS cells (Figure 6, A and B). Conversely, transfec-
tion of ZOSM cells with CXCL14 siRNA significantly inhibited metastatic behaviors, and recombinant CXCL14 reversed the effect of CXCL14 suppression in ZOSM cells (Figure 6, C and D).

In addition, ZOS cells treated with conditioned medium from ZOSM control, but not ZOSM-siCXCL14, cells enhanced cell migration, invasion, and resistance to anoikis (Figure 6, E and F). Similar results were obtained when MNNG-HOS and 143B cells

**Figure 8. IRX1 promotes lung metastasis through CXCL14 in a mouse model.** (A) CXCL14 overexpression significantly enhanced the metastatic ability of IRX1-knockdown 143B cells. (B) Immunofluorescence analysis of IRX1 and CXCL14 in tumors from 143B cell–bearing mice. (C) Immunofluorescence analysis of IRX1 and CXCL14 expression in tumors from MNNG-HOS cell–bearing mice. (D) CXCL14 knockdown in MNNG-HOS-IRX1 cells significantly decreased lung metastases. Black arrow indicates the metastatic nodule. Scale bars: 200 μm (A and D), 100 μm (B and C). *P < 0.05 by Student’s t test.

of chromatin. ChIP analysis revealed that IRX1 could bind to site A on the CXCL14 promoter (Figure 5H); however, PCR with prim-
ers that flanked site B did not produce a detectable product when IRX1-immunoprecipitated DNA was used as a template. Taken together, these results indicate that IRX can bind specific DNA sequences (ACACCTGT) in the CXCL14 promoter and directly activate CXCL14 transcription.

**IRX1/CXCL14 signaling is required for lung metastasis of osteosarcoma cells.** Given that CXCL14 can promote the invasion of pancreatic and breast cancer cells and is associated with the metastasis of colorectal cancer (15, 17, 18), we next determined whether CXCL14 could also promote metastatic phenotypes in osteosarcoma cells. Our results demonstrated that transfection of ZOS cells with a CXCL14 expression vector promoted migration, invasion, and resistance to anoikis (Figure 6, A and B). Given that CXCL14 is a secreted protein, we used a neutralizing anti-
body to block CXCL14. CXCL14 blockade in the culture medium attenuated the metastasis-promoting effects of CXCL14 overexpression on ZOS cells (Figure 6, A and B). Conversely, transfection of ZOSM cells with CXCL14 siRNA significantly inhibited metastatic behaviors, and recombinant CXCL14 reversed the effect of CXCL14 suppression in ZOSM cells (Figure 6, C and D). In addition, ZOS cells treated with conditioned medium from ZOSM control, but not ZOSM-siCXCL14, cells enhanced cell migration, invasion, and resistance to anoikis (Figure 6, E and F). Similar results were obtained when MNNG-HOS and 143B cells
wound-healing and Transwell assays showed that CXCL14 knockdown in ZOS-IRX1 and MNNG-HOS-IRX1 cells resulted in a decrease in their migratory and invasive abilities (Figure 7, A–C). Transfection with CXCL14 siRNA also reduced anoikis resistance in ZOS and MNNG-HOS cells overexpressing IRX1 (Figure 7D). Furthermore, CXCL14 blockade in the culture medium with a neutralizing antibody could inhibit the migration, invasion, and resistance to anoikis of ZOS-IRX1 and MNNG-HOS-IRX1 cells as effectively as CXCL14 siRNA (Supplemental Figure 8). These results showed that CXCL14 could promote the metastatic behavior of osteosarcoma cells and that it likely acts in an autocrine fashion.

We then asked whether CXCL14 participates in IRX1-mediated lung metastasis. First, we examined the effect of CXCL14 impairment on IRX1 function. In proliferation assays, the siRNA-mediated downregulation of CXCL14 in ZOS-IRX1 and MNNG-HOS-IRX1 cells had no significant effects on cell growth when compared with control cells (Supplemental Figure 7A). However, these were used (Supplemental Figure 6). These results showed that CXCL14 could promote the metastatic behavior of osteosarcoma cells and that it likely acts in an autocrine fashion.

Figure 9. IRX1 activates NF-κB signaling through CXCL14. (A) Western blot analysis was performed to detect production of the indicated proteins in control and IRX1-overexpressing or -knockdown osteosarcoma cells. An NF-κB luciferase reporter assay was performed on the indicated cells. (B) Expression of the indicated proteins was determined in control and CXCL14-overexpressing or in CXCL14-knockdown osteosarcoma cells. An NF-κB luciferase reporter assay was performed on the indicated cells. (C) Expression of the indicated proteins and NF-κB luciferase reporter activity were determined in the indicated cells. Data represent the mean ± SD of 3 separate determinations. *P < 0.05 by Student’s t test.
The findings suggest that IRX1 promotes osteosarcoma progression through the autocrine action of CXCL14.

To investigate whether CXCL14 overexpression can compensate for the loss of IRX1 function, a CXCL14 cDNA expression construct was transiently transfected into ZOSM-shIRX1-A and 143B-shIRX1-A cells. Overexpression of CXCL14 increased migration, invasion, and resistance to anoikis in IRX1-knockdown ZOSM and 143B cells without impacting cell growth (Figure 7, E–H, and Supplemental Figure 7B), further confirming that CXCL14 is required for the prometastatic activity of IRX1 in osteosarcoma cells.

Next, we determined whether CXCL14 could reverse the metastatic ability of IRX1-knockdown cells in vivo. First, 143B-shIRX1-A cells were transfected with CXCL14 cDNA or an empty vector and inoculated into NOD/SCID mice. CXCL14 overexpression in 143B-shIRX1 cells promoted metastasis to the lung, and the decrease in metastatic activity caused by IRX1 deletion was abolished (Figure 8, A and B). To further confirm the effect of CXCL14 on IRX1-induced osteosarcoma metastasis, mice were injected with CXCL14 shRNA- or control shRNA-transfected MNNG-HOS-IRX1 cells. Compared with the control cells, lung metastases of the MNNG-HOS-IRX1 cells were suppressed after treatment with 500 μM AdoMet for 6 days. (B) 143B cells with control or AdoMet treatment (500 μM) were injected into the proximal tibia of NOD/SCID mice (n = 10), and the rate of lung metastasis was measured by histopathological analysis at 5 weeks. AdoMet treatment dramatically decreased the number of lung micrometastases. (C) Immunohistochemical analysis of IRX1, CXCL14, and MMP9 expression in tumors from mice that received control or AdoMet-treated 143B cells. Scale bars: 100 μm (A and C), 200 μm (B). *P < 0.05 by Student’s t test.

NF-κB pathway activation is involved in IRX1/CXCL14-induced signaling osteosarcoma metastasis. We next explored the underlying mechanisms of how IRX1/CXCL14 signaling influences the metastatic phenotypes of osteosarcoma cells. It has been demonstrated that CXCL14 enhances the invasiveness of pancreatic cancer cells by increasing NF-κB transactivation (17). Our previous studies showed that activation of NF-κB could promote the progression of osteosarcoma (27, 28), which suggests that the effect of IRX1/CXCL14 signaling on osteosarcoma cells involves the NF-κB pathway. Therefore, we aimed to determine whether IRX1 and CXCL14 overexpression promotes osteosarcoma metastasis by activating the NF-κB pathway.

To do so, we initially examined the influence of IRX1 on NF-κB signaling. Our results showed that overexpression of IRX1 in ZOS and MNNG-HOS cells increased the degradation of IκBα, the
a CXCL14 expression vector was transiently transfected into ZOSM-shIRX1 or 143B-shIRX1 cells, both nuclear NF-κB expression and NF-κB–luciferase reporter activity were completely rescued in the IRX1-deficient cells (Figure 9C), suggesting that CXCL14 is required for IRX1-induced NF-κB activation. Taken together, our findings indicate that IRX1 can activate NF-κB signaling via CXCL14 upregulation.

Finally, we determined whether blocking the NF-κB pathway using the NF-κB inhibitor BAY 11-7085, which inhibits the phosphorylation and degradation of IκBα and prevents the translocation of p65/p50 (32), attenuated the metastasis-promoting effect of IRX1- and CXCL14-overexpressing osteosarcoma cells. As shown in Supplemental Figure 9, the metastatic ability of ZOS-IRX1 and MNNG-HOS-IRX1 cells was significantly suppressed after BAY 11-7085 treatment (2.5 μM). These results indicate that NF-κB pathway activation is involved in IRX1/CXCL14-induced metastasis in osteosarcoma cells.

The DNA-methylating drug AdoMet inhibits osteosarcoma metastasis. We have shown above that AdoMet could methylate the promoter of IRX1 and hence suppress IRX1 expression in ZOSM and 143B cells (Figure 2, F and G). We thus investigated whether AdoMet could also inhibit osteosarcoma metastasis. ZOSM and 143B cells were treated with 500 μM AdoMet for 6 days prior to performing a Transwell assay. As shown in Figure 10A, AdoMet treatment decreased the migratory and invasive abilities of ZOSM and 143B cells. However, there was no significant difference in detachment-induced apoptosis after AdoMet treatment (Supplemental Figure 10A). We next examined the
The effect of AdoMet on the metastatic ability of osteosarcoma cells in vivo. AdoMet-treated and control 143B cells were injected into the proximal tibiae of NOD/SCID mice \((n = 10\) per group), and lungs were checked for metastasis at 5 weeks. The results of the examination demonstrated that mice inoculated with AdoMet-treated 143B cells developed significantly fewer metastatic tumor foci in their lungs than did control mice (Figure 10B). We then determined whether in vitro treatment with AdoMet resulted in stable silencing of IRX1 expression in tumors in vivo. Immunohistochemical staining showed strong positive expression of IRX1 in the tumors from the control group (Figure 10C). In contrast, weak staining for IRX1 was observed in the tumors formed from the AdoMet-treated cells. Furthermore, the expression levels of CXCL14 and MMP9 were also reduced in the tumors from the AdoMet-treated group. These findings suggested that the DNA-methylating drug AdoMet inhibited osteosarcoma metastasis in part through reversing hypomethylation of the IRX1 promoter and suppressing IRX1 expression.

We also examined the influence of the demethylating drug DAC, which has been demonstrated to increase IRX1 expression in ZOS and MNNG-HOS cells, on the metastatic behaviors of osteosarcoma cells. Our results showed that ZOS and MNNG-HOS cells treated with 2 \(\mu\)M DAC for 3 days exhibited greater migratory and invasive abilities in vitro than did control cells (Supplemental Figure 10, B and C). Nevertheless, DAC-treated ZOS and MNNG-HOS cells were more sensitive to detachment-induced apoptosis than were control cells (Supplemental Figure 10D), and there was no significant difference in lung metastasis in vivo after DAC treatment of MNNG-HOS cells (data not shown).

**Prognostic value of IRX1 in human osteosarcoma patients.** On the basis of our demonstration that IRX1 is prometastatic in osteosarcoma, we evaluated the clinical significance of IRX1 in primary human osteosarcoma specimens. To this end, surgical specimens from 113 cases of osteosarcoma, collected between January 2004 and December 2008, were examined for IRX1 expression by immunohistochemical staining (Supplemental Table 3). As shown in Figure 11A, IRX1 staining was mainly observed in the nuclei of tumor cells. The correlations between IRX1 expression and other clinical characteristics are presented in Supplemental Table 4. Kaplan-Meier survival analysis indicated that patients with high IRX1 expression levels had worse overall survival than did those with low expression levels (Figure 11B). Moreover, the risk of lung metastases was significantly higher in the patients expressing high levels of IRX1 than in those expressing low levels of the protein (Figure 11C). Multivariate analyses revealed that high IRX1 expression was an independent predictor of poor prognosis and lung metastasis (Supplemental Table 5).

Given that IRX1 requires CXCL14 to induce metastasis, we further examined whether there was a positive correlation between IRX1 and CXCL14 protein expression in human osteosarcoma tissues. Immunohistochemical staining for CXCL14 was performed in the same cohort of 113 patients with osteosarcoma (Supplemental Table 3). CXCL14 staining in osteosarcoma cells was mainly located in the cytoplasm (Figure 11D). \(\chi^2\) analysis indicated that there was a significant correlation between the pres-
ence of nuclear IRX1 and cytoplasmic CXCL14 (Table 1), which further confirmed that CXCL14 was essential for the prometastatic effects of IRX1 in osteosarcoma.

IRX1 promoter hypomethylation in patient serum DNA predicts the lung metastasis of osteosarcoma. Compared with tumor tissue–based biomarkers, circulating blood biomarkers are easily available and less invasive. It has been demonstrated that epigenetic changes in cell-free tumor DNA can be detected in the serum of cancer patients (33, 34). To determine whether hypomethylation of IRX1 in serum DNA is a potential marker for lung metastasis of osteosarcoma, we examined the methylation status of the IRX1 promoter by methylation-specific PCR (MSP) in 67 serum samples collected from primary osteosarcoma patients between August 2010 and September 2012. Representative results of MSP for the IRX1 promoter are shown in Figure 12A. There was no association between IRX1 promoter methylation in the serum DNA and patient age, sex, tumor site, or Enneking stage (Table 2). However, we found that the IRX1 promoter was hypomethylated in 34.2% of serum samples (13 of 38) from patients without metastases, while 62.1% of serum samples (18 of 29) from the metastatic group showed IRX1 promoter hypomethylation (P = 0.023, χ² test). Kaplan-Meier analysis indicated that patients with hypomethylated IRX1 in their serum exhibited worse lung metastasis–free survival than those with hypermethylation (Figure 12B), suggesting that the detection of IRX1 promoter hypomethylation in the serum DNA of osteosarcoma patients could be a potential predictive marker for monitoring lung metastasis in osteosarcoma.

### Discussion

Cancer metastasis is a complex process that involves several molecular and cellular changes, among which aberrant epigenetic alteration is now widely recognized as one of the most important events (35–37). In this study, we identified IRX1 as a hypomethylation-activated metastasis-driving gene in osteosarcoma. Suppression of IRX1 decreased CXCL14 expression levels and NF-κB activity and had antimetastatic effects in mouse models of osteosarcoma. We conclude that hypomethylation-mediated activation of IRX1 positively regulates CXCL14/NF-κB signaling to promote metastatic activities in osteosarcoma and that therapeutic targeting of the IRX1 pathway may be a promising strategy for suppressing osteosarcoma metastasis.

The Iroquois transcription factor family, which plays multiple roles in many metazoan developmental processes (5, 8), has recently been shown to be involved in the development of many cancers (6, 38–41). For example, IRX3 downregulation in breast cancer was linked to poor clinical outcomes, and IRX4 expression suppressed prostate cancer growth (39, 41). IRX1 was reported to be downregulated in HNSCC and gastric cancer, and it inhibited cancer cell growth, invasion, and tumorigenesis. (6, 7). However, we show here that IRX1 is overexpressed in metastatic osteosarcoma and that it functions as a prometastatic gene, suggesting that IRX1 might play dual roles in cancer development. In fact, there are many genes like IRX1 that have been demonstrated to play both tumor-suppressing and tumor-promoting roles that depend on the cancer type (42, 43). For instance, insulin-like growth factor–binding protein 5 (IGFBP5) has been shown to induce apoptosis and inhibit tumor growth in breast cancer and osteosarcoma cells (44, 45). In contrast, IGFBP5 was found to facilitate the progression of prostate cancer (46). Nevertheless, the reasons for the contradicting functions of IRX1 in different tissue contexts are unclear.

It has been reported that Iroquois transcription factors may have context-dependent dual functions as activators and repressors (47). Indeed, IRX2 can be converted from a repressor to an activator after being phosphorylated by FGF8/MAPK signaling (48). In gastric cancer, IRX1 was identified as a transcriptional repressor that could downregulate the expression of a set of tumor-promoting genes such as BDKRBI2, FGF7, and HIST2H2BE and thereby inhibit angiogenesis, cell proliferation, and invasion (6). We also found in the present study that IRX1 acted as a transcriptional activator to promote the expression of prometastatic genes like CXCL14 to facilitate osteosarcoma metastasis. Switching of IRX1 between transcriptional activation and repression may contribute to its distinct roles in different types of cancer. However, the molecular basis of such switching requires further investigation. Importantly, antimetastatic strategies that target IRX1 signaling...

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### Table 1. Immunohistochemical staining of IRX1 and CXCL14 in 113 osteosarcoma tissues

<table>
<thead>
<tr>
<th>Staining</th>
<th>CXCL14</th>
<th>IRX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>39 (34.5%)</td>
<td>27 (23.9%)</td>
</tr>
<tr>
<td>–</td>
<td>15 (13.3%)</td>
<td>32 (28.3%)</td>
</tr>
</tbody>
</table>

P = 0.004 by χ² test.

### Table 2. Association of IRX1 promoter methylation in serum DNA with the clinicopathological characteristics of 48 osteosarcoma patients

<table>
<thead>
<tr>
<th>Location</th>
<th>U⁴</th>
<th>M⁴</th>
<th>P value⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal femur</td>
<td>20</td>
<td>21</td>
<td>0.430</td>
</tr>
<tr>
<td>Proximal tibia</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Proximal humerus</td>
<td>2</td>
<td>0</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Enneking stage</th>
<th>U⁴</th>
<th>M⁴</th>
<th>P value⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIB</td>
<td>1</td>
<td>3</td>
<td>0.674</td>
</tr>
<tr>
<td>IIIB</td>
<td>24</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metastasis</th>
<th>U⁴</th>
<th>M⁴</th>
<th>P value⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>13</td>
<td>25</td>
<td>0.023</td>
</tr>
<tr>
<td>Yes</td>
<td>18</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

⁴U, hypomethylation; M, hypermethylation; χ² test.
must consider its dual roles in cancer and must include specific targeting of therapeutic agents to avoid increasing the risk of other cancers such as HNSCC and gastric cancer.

A large body of evidence has demonstrated that chemokines play key roles in several critical steps during the metastatic process, including migration, invasion, extravasation, and angiogenesis (49–51). Here, we identified CXCL14, a novel CXC chemokine, as a potential target gene of IRX1 by using a high-throughput screening method. CXCL14 has also been found to be upregulated in several cancer types and is associated with tumor progression and metastasis (15, 17–19). Consistent with these findings, we showed that CXCL14 enhanced the metastatic ability of osteosarcoma cells in an autocrine manner. Furthermore, IRX1 was able to induce CXCL14 expression by directly binding to its promoter. CXCL14 knockdown attenuated IRX1-induced metastasis in osteosarcoma, while CXCL14 overexpression partially compensated for the loss of IRX1 function. Our observations indicate that CXCL14 is a bona fide downstream effector of IRX1 in osteosarcoma metastasis. However, CXCL14 is a relatively new chemokine whose receptor is unknown and whose function is undefined, thus making it difficult to identify a pathway through which CXCL14 promotes cancer metastasis. Given that CXCL14 belongs to a chemokine family that is involved in inflammatory processes (52) and given that NF-κB signaling plays an important role in the control of inflammatory response (53), it seems reasonable that CXCL14 may activate that NF-κB pathway. In fact, it has been recently demonstrated that CXCL14 can increase nuclear NF-κB p65 levels and promote the invasiveness of pancreatic cancer cells (17). In the present study, we found that IRX1/CXCL14 signaling could activate the NF-κB pathway and increase the expression of MMP9 and c-FLIP, thus promoting osteosarcoma metastasis. Together with our earlier findings (27, 28), these results indicate that the NF-κB pathway acts as a key node in the signaling networks regulating osteosarcoma progression.

DNA methylation, a DNA modification that occurs via the covalent addition of a methyl group to cytosine by DNA methyltransferases (DNMTs), is an important epigenetic mechanism that regulates gene expression (54, 55). Recent studies have provided compelling evidence that aberrant DNA methylation plays a pivotal role in cancer initiation and progression (2, 56, 57). However, the majority of studies have focused on hypermethylation of the promoter CpG islands of tumor-suppressor genes. The impact of hypomethylation, especially the loss of promoter methylation, has been underestimated (58). In this report, we demonstrated that elevated expression of the prometastatic gene IRX1 was associated with hypomethylation of the IRX1 promoter and that DNA demethylation may be a major cause of IRX1 activation in osteosarcoma (Figure 2, A–F). This may explain why the IRX1 expression pattern in osteosarcoma is different from that observed in HNSCC and gastric cancers, in which IRX1 is silenced by promoter hypermethylation (6, 7). Given that DNA methylation is a reversible process, the pharmacological inhibition of demethylation may result in inhibition of the expression of prometastatic genes (such as IRX1) as well as metastasis (59). AdoMet, a ubiquitous methyl donor in the DNA methyltransferase reaction, is a safe and natural compound that has been used for the treatment of depression, liver disease, and osteoarthritis (60–62). Recent studies have shown that it can increase DNA methylation by either stimulating DNA methyltransferase activity or by inhibiting demethylation activity (23). Importantly, AdoMet was able to suppress uPA and MMP2 expression by reversing the hypomethylation status of their promoters, thereby blocking the associated breast and prostate cancer metastases (63, 64). Consistent with this finding, we showed that AdoMet treatment could also cause methylation of the IRX1 promoter and decrease IRX1 gene expression (Figure 2, G–I). Moreover, treatment with AdoMet suppressed the migratory and invasive abilities of osteosarcoma cells in vitro and their metastatic potential in vivo. Given that AdoMet is a broadly acting methylating drug, it may also induce hypermethylation of many other metastasis-related genes in addition to IRX1. Further studies are required to determine the mechanism through which AdoMet inhibits the invasiveness and metastasis of osteosarcoma cells. Nevertheless, one concern with using methylating drugs is that they may cause hypermethylation of tumor-suppressor genes and thereby promote tumorigenesis. It is therefore not recommended that methylating drugs be used at early stages of cancer, when tumor-suppressor genes may be easily silenced by hypermethylation (63). Thus, it is important to develop methylating drugs that are more potent and specific than AdoMet. On the other hand, it must be noted that, although the demethylating drug DAC showed no significant effect on metastasis in the in vivo mouse model studied here, use of DAC might indeed activate prometastatic genes (such as IRX1) and promote the invasiveness of osteosarcoma cells. Taken together, our observations not only indicate that reversion of hypomethylation may provide a new strategy for the effective control of osteosarcoma metastasis, but also warn against using demethylating drugs in the late stages of cancer therapy.

The molecular detection of epigenetic changes in cell-free tumor DNA in serum has been highlighted as a potential tool for cancer diagnosis and prognosis (65). Here, we used highly sensitive MSP to detect the IRX1 promoter methylation status using cell-free tumor DNA obtained from the serum of osteosarcoma patients. We found that the IRX1 promoter was hypomethylated in 46.3% (31 of 67) of serum samples and that patients with IRX1 promoter hypomethylation in their serum DNA were more likely to have lung metastasis. Given that aberrant gene methylation is one of the earliest molecular changes that occurs during cancer progression (66, 67), the detection of IRX1 hypomethylation in serum DNA could be a promising strategy for the early detection of osteosarcoma lung metastasis and might be useful for guiding individual treatment and assessing the early response to chemotherapy.

In summary, our present study identified hypomethylated IRX1 as a metastasis-driving gene that promotes osteosarcoma lung metastasis by enhancing CXCL14/NF-κB signaling (Figure 12C). Because the methyltransferase process is reversible, the pharmacologic reversion of hypomethylation activity may provide a new option for the prevention of osteosarcoma metastasis.

Methods

Microarray analysis. The MeDIP assay was performed using the Human DNA Methylation 3×720K CpG Island Plus RefSeq Pro-
moter Array (NimbleGen), and the data were deposited in the NCBI’s Gene Expression Omnibus (GEO) database (GEO GSE55961). Gene expression microarray analysis was performed using the Affymetrix Human Genome U133 Plus 2.0 Array (ZOS vs. ZOSM) and the NimbleGen Human Gene Expression 12×135K Array (143B-shCtrl vs. 143B-shIRX1), and the microarray data were deposited in the GEO database (GEO GSE55957 and GSE55958). Further details are provided in the Supplemental Methods.

Animal studies. Four- to six-week-old female NOD/SCID mice were purchased from Beijing HFK Bioscience Co. Ltd. Mice were injected with 2 × 10⁶ cells (in 100 μl PBS) into the lateral tail vein (10 mice per group). The mice were monitored 3 times per week for evidence of morbidity associated with pulmonary metastases. After 8 weeks, the mice were sacrificed by cervical dislocation. Subsequently, the lungs were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. For the orthotopic model of osteosarcoma, mice were anesthetized with 4% chloral hydrate (0.2 ml/100 g, i.p.). The right knee of the mouse was flexed beyond 90°, and 1 × 10⁶ cells suspended in 30 μl PBS were injected into the right proximal tibia using a 30-gauge needle (10 mice per group). The tumor volume was measured in 2 perpendicular dimensions (D1, D2) with an electronic digital caliper every 3 days and calculated using the formula: V = 4/3π(D1 + D2)², as described previously (68). After 5 weeks, the mice were anesthetized, and bone destruction caused by the tumor was observed using the Inveon Micro-CT/PET system (Siemens). The mice were then sacrificed, and the lungs were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. To quantify the number of pulmonary metastatic lesions, sequential serial 3-μm-thick sections of whole lungs were obtained. The sections were stained with H&E to identify the metastases by light microscopy as previously described (69).

Experimental procedures. See the Supplemental Methods for details on other experimental procedures. All primers used in this study are listed in Supplemental Table 6.


Statistics. Statistical analyses were performed using SPSS software, version 16.0 (SPSS Inc.). Measurements were analyzed using the 2-tailed Student’s t test, the Mann-Whitney U test, or 1-way ANOVA, while categorical data were analyzed with the χ² or Fisher’s exact test. Correlations between the relative expression levels of IRX1 and CXCL14 were analyzed using Pearson’s correlation method. Associations between IRX1 promoter methylation and gene expression were determined by the Spearman’s rank correlation test. Survival curves were calculated using the Kaplan-Meier method, and differences were analyzed with the log-rank test. The Cox regression model was used for multivariate survival analysis. A P value of less than 0.05 was considered statistically significant.

Study approval. This study was approved by the ethics committee of Sun Yat-Sen University, and written informed consent was obtained from the patients or their guardians before sample collection. All animal studies were approved by the IACUC of Sun Yat-Sen University.

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Address correspondence to: Jin Wang, Jingnan Shen, or Huiling Yang, Department of Musculoskeletal Oncology, The First Affiliated Hospital of Sun Yat-Sen University, No. 58, Zhongshan Road 2, Guangzhou, Guangdong, China 510080. Phone: 8620.8775766-8236; E-mail: 2004wjhf@163.com.


