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Anti–IL-20 Monoclonal Antibody Suppresses Breast Cancer Progression and Bone Osteolysis in Murine Models

Yu-Hsiang Hsu,* Chung-Hsi Hsing,†‡ Chien-Feng Li,§ Chien-Hui Chan,* Ming-Chung Chang,*§† Jing-Jou Yan,‖ and Ming-Shi Chang*

IL-20 is a proinflammatory cytokine involved in rheumatoid arthritis, atherosclerosis, and stroke. However, little is known about its role in breast cancer. We explored the function of IL-20 in tumor growth and metastasis, as well as in clinical outcome. Tumor expression of IL-20 was assessed by immunohistochemical staining among 198 patients with invasive ductal carcinoma of the breast, using available clinical and survival data. IL-20 expression was associated with advanced tumor stage, greater tumor metastasis, and worse survival. Reverse transcription quantitative polymerase chain reaction showed that clinical breast tumor tissue expressed higher levels of IL-20 and its receptors than did nontumorous breast tissue. IL-20 was also highly expressed in breast cancer bone-metastasis tissue. In vitro, IL-20 upregulated matrix metalloproteinase-9, matrix metalloproteinase-12, cathepsin K, and cathepsin G, and enhanced proliferation and migration of breast cancer cells, which were inhibited by anti–IL-20 mAb 7E. In vivo, we generated murine models to evaluate the therapeutic potential of 7E, using luminescence intensity, radiological scans, and microcomputed tomography. 7E reduced tumor growth, suppressed bone colonization, diminished tumor-mediated osteolysis, and lessened bone density decrement in mice injected with breast cancer cells. In conclusion, our results suggest that IL-20 plays pivotal roles in the tumor progression of breast cancer. IL-20 expression in breast cancer tissue is associated with a poor clinical outcome. Anti–IL-20 mAb 7E suppressed bone colonization and decreased osteolytic bone lesions. Therefore, IL-20 may be a novel target in treating breast tumor-induced osteolysis. The Journal of Immunology, 2012, 188: 000–000.
IL-20 is regulated by hypoxia and contributes to brain injury in an ischemic stroke model, which indicates that IL-20 is a promoting factor in the microenvironment of inflammatory-associated disease (28). Most solid tumor cells grow under hypoxic conditions. Thus, we hypothesized that IL-20 is upregulated in solid tumors. We also showed (23, 29) that anti–IL-20 mAb not only reduced arthritis and protected against bone loss in collagen-induced arthritis but also inhibited the differentiation of osteoclasts and protected ovariectomized mice against osteoporotic bone loss, which suggests that IL-20 is involved in the osteo-immune system. Little is known about the function of IL-20 in breast cancer and tumor-mediated osteolysis. Therefore, we investigated whether IL-20 is involved in the pathogenesis of breast cancer and bone metastasis. We also explored whether the anti–IL-20 mAb 7E has a therapeutic effect in murine models of breast cancer.

Materials and Methods

Participants and tissue specimens

This study was conducted at National Cheng Kung University Hospital and Chi-Mei Medical Center from January 1, 1999, through December 31, 2010. Tumor tissue samples were taken from 198 patients with primary localized intraductal carcinoma (IDC) of the breast and were obtained from the Chi-Mei Medical Center Tumor and Serum Bank. Another 35 women with breast cancer were enrolled at National Cheng Kung University Hospital. Tumorous and nontumorous breast tissue from 30 patients (stage I, n = 4; stage II, n = 11; stage III, n = 15) was analyzed. From the other 5 patients, we collected tissue only from bone metastases of the breast cancer. Nontumorous breast tissue was taken from nonpathological areas distant from tumors in surgical specimens and confirmed nontumorous by histological examinations. Biopsies were taken after obtaining informed consents from all participants. The study was approved by the Ethics Committee of National Cheng Kung University Hospital and Chi-Mei Medical Center Institutional Review Board.

Cell lines

Mouse 4T1, human MDA-MB-231, and human MCF-7 breast cancer cell lines were from the American Type Culture Collection. 4T1 cells were maintained in DMEM with 10% FBS (Life Technologies, Rockville, MD), 2 mM l-glutamine (Life Technologies), 100 µg/ml streptomycin, and 100 U/ml penicillin. For bioluminescence imaging experiments, Luciferase-expressing derivative of the 4T1 cell line (4T1-luc) was prepared by stable transfection of pCAG-Luc, which expresses the firefly luciferase gene under control of the β-actin promoter and the CMV IE enhancer. Transfection was followed by three rounds of single-cell cloning to establish a cell line with stably expressed luciferase activity.

Immunohistochemistry

Paraffin-embedded tissue samples were used for immunohistochemical staining with anti–IL-20 (7E), –IL-20R1, –IL-20R2, or –IL-22R1 mAb (R&D Systems, Minneapolis, MN), as previously described (26). Incubating paraffin tissue sections with mouse IgG1 isotype (clone 11711; R&D Systems), instead of primary Ab, functioned as the negative control. We used 3 µg/ml as the working concentration for each primary Ab and for control mouse IgG1. At least five sections from each primary Ab’s specimen were analyzed and examined blindly by two investigators trained in breast pathology. Two pathologists trained in breast pathology and blinded to the sample sources analyzed the histology and the IL-20 expression levels of at least five sections from each patient. The scoring of immunohistochemical stains in each specimen was determined with a histological score (H) (31) that was calculated using the following equation: H = i(1-p)i, where i is the staining intensity of the stained tumor cells (0–4+) and p is the percentage (range: 0–100%) of stained tumor cells for each intensity. The IL-20 immunostaining was labeled low-expression (H < 200) or high-expression (H ≥ 200). Immunocytochemical staining of IL-20 and its receptors in 4T1, MDA-MB-231, and MCF-7 cells was accomplished using the same protocol as described above. For apoptotic cell staining, tumor sections from 4T1-bearing mice were stained with the TUNEL agent Promega according to the manufacturer’s instructions. Immunohistochemical staining of Ki-67 in tumor sections from 4T1-bearing mice was carried out following the manufacturer’s instructions.

Cell proliferation assay

4T1 cells (2 × 104) were exposed to murine (m) IL-20 (25–200 ng/ml) for 72 h in DMEM containing 1% FBS. To demonstrate the sp. act. of IL-20, 7E was added to the culture system, either alone or together with IL-20 at a 10:1 concentration ratio (7E:IL-20). Cells were then incubated with MTT, 1 mg/ml (Sigma-Aldrich, St. Louis, MO) for 3 h and the MTT-formazan crystals dissolved in DMSO (Sigma-Aldrich). Absorbance was measured at 550 nm.

Cell migration assay

4T1 cells were assayed using a modified Boyden chamber housing a polycarbonate filter with 8-µm pores (Nuclepore, Cabin John, MD). The upper wells were loaded with 1 × 104 4T1 cells. The lower chambers were filled with mIL-20 (200 ng/ml), 7E (2 µg/ml), or mIL-20 plus 7E in DMEM containing 0.2% FBS. DMEM with 0.2% FBS was used as a negative control; 5% FBS was used as a positive control. The chambers were incubated for 5 h at 37˚C. Cells adhering to the lower side of the filter were fixed with methanol and stained in a Giemsa solution (Diff-Qick; Baxter Healthcare, Deerfield, IL) for counting. The experiment was carried out three times using quadruplicate wells; migration is expressed as the mean ± SD of the total cells counted per field.

Reverse transcription quantitative polymerase chain reaction

To examine clinical specimens, tumors and nontumorous breast tissue specimens from 30 patients were analyzed. Total RNA was isolated. Reverse transcription was done with reverse transcriptase according to the manufacturer’s protocol (Clontech, Palo Alto, CA). Human IL-20, IL-20R1, IL-20R2, and IL-22R1 expression was then amplified on a thermocycler (LC 480; Roche Diagnostics, Indianapolis, IN), with SYBR Green (Roche Diagnostics) as the interaction agent. Quantification analysis of mRNA was normalized with hGAPDH used as the housekeeping gene. To examine the expression of cathepsin K, cathepsin G, and MMP-2, -7, -9, -12, and -13, 4T1 cells were incubated with mIL-20 (200 ng/ml) in serum-free DMEM for 2–8 h. 7E was used to inhibit the activity of IL-20. 4T1 cells were treated with mIL-20 (200 ng/ml), 7E (2 µg/ml), or mIL-20 (200 ng/ml) plus 7E (2 µg/ml) for 6 h, and then total mRNA was isolated and measured. Quantification analysis of mRNA was normalized with mGAPDH as the housekeeping gene. Relative multiples of change in mRNA expression were determined by calculating 2−ΔΔCt.

Western blotting

4T1 cells (1 × 106) were stimulated with mIL-20 (200 ng/ml) (R&D Systems) for the indicated periods. Western blotting, performed using the manufacturer’s instructions, used Abs specific for phosphorylated JNK, ERK, and STAT3 (Cell Signaling Technology, Beverly, MA). Total JNK, ERK, and STAT3, used as internal controls, were detected using specific Abs (Cell Signaling Technology). To analyze the effect of IL-20 on apoptosis- and osteoclast-associated signals, Western blotting was done using Abs specific for Bcl-XL, Bad (Cell Signaling Technology), β-actin was a loading control. To analyze the effect of IL-20 on cathepsin K, cathepsin G, MMP-9, and MMP-12 production, 4T1 and MCF-7 cells (1 × 106) were stimulated with IL-20 (200 ng/ml) (R&D Systems) for 24 h. Western blotting was done using Abs specific for cathepsin K, cathepsin G, MMP-9, and MMP-12. β-actin was a loading control.

ELISA

To test the specificity of 7E, cytokines of the IL-10 family, including IL-10, -19, -20, -22, -24, and -26, were coated on the plate with various concentrations and analyzed for their binding activity with 7E (1 µg/ml) using a direct ELISA.

4T1-bearing tumor model

All animal experiments were conducted according to the protocols based on the National Institutes of Health standards and guidelines for the care and use of experimental animals. The research procedures were approved by the Animal Ethics Committee of National Cheng Kung University in Taiwan. Female 8-wk-old BALB/c mice were used in all experiments. The left mammary fat pad of each mouse was injected s.c. with 4T1 cells (2 × 106 cells). The mice were then randomly assigned to four groups (n = 6 in each group). The mice were treated with PBS, 7E (5–10 mg/kg; s.c.), or mouse IgG (10 mg/kg; s.c.) every 3 d for the duration of the treatment regimen. Healthy controls were not injected with tumor cells. At 25 d after the tumor cells had been injected, the mice were killed and the tumor tissues were harvested. To analyze the expression profile of IL-20, the tumor tissues were fixed and paraffin sectioned for immunohistochemical staining.
with anti–IL-20 polyclonal Ab. The IL-20 in tumor sections was then quantified using image analysis software (Image-Pro Plus; Media Cybernetics LP, Silver Spring, MD). To quantify the expression levels of IL-20, tumor tissue was placed in PBS solution and then homogenized, centrifuged, and analyzed using direct ELISA.

In vivo osteolytic bone metastasis model

For intracardiac inoculations, BALB/c mice were anesthetized with pentobarbital (50 mg/kg body weight) (Sigma-Aldrich). 4T1-Luc cells, at a concentration of $1 \times 10^5/100 \mu l$, were injected directly into the left ventricle of anesthetized mice. The mice were then randomly assigned to three groups ($n = 10$ in each group) and injected (i.p.) with vehicle (PBS), mIgG (10 mg/kg), or 7E (10 mg/kg) three times per week. Healthy controls were not injected with tumor cells. The survival rate in each group was monitored daily until the end of the experiment. At 17 d after treatments began, luciferase activity was used to quantify tumor growth. An in vivo imaging system (IVIS 50; Xenogen, Caliper Life Sciences, Hopkinton, MA) was used to detect luminescence from 4T1-Luc cells. The tibia metaphyses were analyzed in vivo using micro-CT with a high-resolution, low-dose x-ray scanner. BMD was analyzed in 50 consecutive slices. The results were calculated as a percentage versus values from a healthy control.

Follow-up and statistical analysis

Statistical analysis was done using SPSS 14.0 (SPSS, Chicago, IL). Associations and comparisons of IL-20 expression with various parameters were evaluated using Student t tests or Spearman correlation analysis. In this cohort, the follow-up data of 198 patients were available in August 2010 (median, 103.6 mo; mean, 89.2 mo; range, 5.6–143.6 mo). The endpoint analyzed was the metastasis-free survival or bone metastasis-free survival of patients with low- and high-grade IL-20 expression, assessed using Kaplan–Meier methods and compared using a log-rank test. A chi^2 test, Student t test, or Kruskal–Wallis one-way ANOVA test, as indicated, and then Dunn’s test, were used. The results of the expression of IL-20 and its receptors in surgical specimens are expressed as dot plots. Lines inside the figures represent the median. Other results are means ± SD. Significance was set at $p < 0.05$.

Results

Higher expression of IL-20 in primary and bone-metastatic breast cancer

To examine whether IL-20 is involved in the pathogenesis of breast cancer, we used immunohistochemical staining to analyze the

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Higher expression of IL-20 in breast tumors was correlated with clinical outcome. Clinical tissue samples were stained with anti–IL-20 mAb 7E using immunohistochemical staining. A, IL-20 was strongly stained in tumor cells and endothelial cells in three different stages of primary breast tumor specimens, but only slightly stained in endothelial cells of nontumorous breast tissue (stage I, $n = 4$; stage II, $n = 11$; stage III, $n = 15$). Original magnification ×100. B, IL-20 was also strongly expressed in breast tumors from bone-metastasis specimens ($n = 5$). Original magnification ×200. C and D, Expression of IL-20 in another 198 breast IDC tissues was determined using immunohistochemical staining. The expression levels of IL-20 in tissue samples were analyzed using H scoring and graded as low IL-20 expression (H < 200, $n = 148$) and high IL-20 expression (H ≥ 200, $n = 50$). Kaplan–Meier plots were used to stratify metastasis-free survival and bone metastasis-free survival based on IL-20 expression levels.
expression levels of IL-20 in tumorous and nontumorous breast tissue specimens from 30 patients with breast cancer. IL-20 (Fig. 1A) and its receptors (Fig. 2A) were strongly stained on tumor cells and endothelial cells of primary tumor breast tissue, but only slightly stained on endothelial cells of nontumorous breast tissue. IL-20 (Fig. 1B) and its receptors (Fig. 2C) were also strongly expressed in bone-metastatic tissue from five patients with breast cancer-mediated bone metastasis. Furthermore, reverse transcription quantitative polymerase chain reaction (RT-qPCR) showed that transcript levels of IL-20 and its receptors in tumorous breast tissue were higher than in nontumorous breast tissue, a finding consistent with the results of immunohistochemical staining (Fig. 2B). To determine whether IL-20 immunoeexpression was a prognosticator of breast IDC, another 198 IDC breast tissue samples were stained with anti–IL-20 mAb 7E. The staining intensity was high-expression in 50 samples and low-expression in 148 samples. IL-20 expression levels in breast tumors were not significantly related to patient primary tumor size (Table I). However, a strong correlation was substantiated between IL-20 immunoeexpression with nodal status (N) ( \( p = 0.019 \) ), advanced stage ( \( p = 0.017 \) ), and human epidermal growth factor receptor-2 expression ( \( p = 0.022 \) ) (Table I). IL-20 expression was remarkably correlated with both distal metastasis-free survival ( \( p = 0.0002 \), Fig. 1C) and bone metastasis-free survival ( \( p = 0.0003 \), Fig. 1D). These findings of strong associations between IL-20 expression and several adverse clinical pathological prognosticators suggested its crucial role in the pathogenesis of breast cancer-mediated bone metastasis.

**FIGURE 2.** Higher expression of IL-20Rs in breast tumors. *A,* Clinical tissue samples were stained with anti–IL-20R1, –IL-20R2, and –IL-22R1 mAb using immunohistochemical staining. The staining showed that all IL-20Rs were strongly stained in breast tumor cells, but only slightly stained in endothelial cells of nontumorous breast tissue (stage I, \( n = 4 \); stage II, \( n = 11 \); stage III, \( n = 15 \)). Original magnification \( \times 100 \). *B,* mRNA of the breast tissue was isolated for RT-qPCR analysis using IL-20–, IL-20R1–, IL-20R2–, and IL-22R1–specific primers. Lines inside the figures represent the median. Differences between the breast tumor specimens and nontumorous breast tissue are significant. *C,* mRNA of the breast tumors from bone-metastasis specimens ( \( n = 5 \)) was isolated for RT-PCR analysis, using IL-20–, IL-20R1–, IL-20R2–, and IL-22R1–specific primers. All experiments were performed three times with similar results. Data are from a representative experiment. P, patient.
ever, MMP-9, MMP-12, cathepsin K, and cathepsin G (Fig. 4A–D) expression was significantly upregulated ($p < 0.05$) by IL-20, which was inhibited by 7E (Fig. 4E). Western blotting also showed that IL-20 enhanced MMP-9, MMP-12, cathepsin K, and cathepsin G protein production (Fig. 4F). Furthermore, we used another breast cancer cell line, MCF-7 cells, to confirm the functions of IL-20 in breast tumor cells and had similar results (Supplemental Fig. 2). These results indicated that IL-20 is an upstream regulator of protease expression in breast cancer and might be involved in tumor metastasis.

Table I. Associations between IL-20 expression and other important clinical pathological variables

<table>
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<tr>
<th>Parameters</th>
<th>Category</th>
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<th>High Exp.</th>
<th>$p$ Value</th>
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</thead>
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<td></td>
<td>T2</td>
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<td>T3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>T4</td>
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<td>2</td>
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<tr>
<td></td>
<td>N1</td>
<td>46</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>9</td>
<td>5</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>II</td>
<td>78</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>HER-2 expression</td>
<td>Low exp. (0+ to ~2+)</td>
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<td>31</td>
<td>0.022d</td>
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<tr>
<td></td>
<td>High exp. (3+)</td>
<td>30</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

*Low-expression staining: H score < 200.

*High-expression staining: H score ≥ 200.

*T1: < 2 cm; T2: 2–5 cm; T3: > 5 cm; T4: spread to chest wall or skin, inflammatory carcinoma.

*Statistically significant.

*HER-2 scoring was done using standard HercepTest guidelines.

Exp., expression; HER-2, human epidermal growth factor receptor 2.

FIGURE 3. IL-20 enhanced cell proliferation and migration in breast cancer cells. Immunohistochemical staining (A) and RT-PCR (B) showed that IL-20 and its receptors were expressed in mouse 4T1 cells. Original magnification ×400. C, IL-20 enhanced cell proliferation. Cell proliferation was determined using an MTT assay. Medium alone was used as a negative control. 7E (2 μg/ml) was used to inhibit the activity of mL-20. $*p < 0.05$ versus untreated controls, $p < 0.05$ versus treatment with mL-20. Values are the means ± SD of triplicate experiments. D and E, Cell migration was evaluated using a modified Boyden chamber assay. D, Representative Giemsa stain photomicrographs are shown for each group. Original magnification ×200. E, 4T1 cells ($1 \times 10^6$) were incubated for 5 h in DMEM containing 0.2% FBS with mL-20 (200 ng/ml), 7E (2 μg/ml), or mL-20 plus 7E. Medium alone was used as a negative control. FBS (5%) was used as a positive control. 7E (2 μg/ml) was used to inhibit the activity of mL-20. $*p < 0.05$ versus untreated controls, $p < 0.05$ versus treatment with mL-20. Values are the means ± SD of triplicate experiments.
IL-20 regulated proliferation- and migration-related signals in 4T1 cells

To elucidate the mechanism of IL-20 in promoting tumor proliferation and migration, the signal molecules of JNK, ERK, STAT3, AKT, NF-κB, Bcl-XL, and Bad were assessed in mIL-20–treated 4T1 cells. Western blotting showed that IL-20 promoted the activation of JNK, ERK, STAT3, and Bcl-XL (Fig. 4G) but had not affected the activation of AKT and NF-κB (data not shown). In addition, IL-20 completely inhibited the signaling of Bad in 4T1 cells (Fig. 4G). These results suggested that IL-20 had triggered both proliferation and antiapoptosis-associated signals, and acted as a promoting factor for tumor growth.

7E reduced in vivo tumor growth in 4T1 cells

IL-20 was highly expressed in breast cancer tissue and enhanced breast tumor growth in vitro. Anti-human IL-20 mAb 7E has shown specificity (Supplemental Fig. 3) and neutralization activity in vitro and in vivo (22, 23, 28–30). Therefore, we analyzed whether 7E reduced tumor growth in vivo. 4T1 cells were injected into the left mammary fat pads of BALB/c mice. The mice were injected s.c. with PBS, mIgG, or 7E every 3 d for the next 25 d. Tumor size and weight in the 7E-treated group were smaller than in the mIgG- and PBS-treated groups (Fig. 5A,5B). Immunohistochemical staining showed that IL-20 expression was weaker in the 7E-treated group than in the mIgG-treated group (Supplemental Fig. 4). IL-20 expression in the tumor mass was lower in the 7E-treated group than in the mIgG-treated group (Fig. 5C). ELISA assay also showed that IL-20 levels in the tumor were lower in the 7E-treated group than in the mIgG-treated group (Fig. 5D). To determine whether 7E affected tumor cell proliferation or apoptosis in vivo, tumor sections from 7E-treated and control mice were prepared and stained with the TUNEL agent. Only a small number of apoptotic cells were detected in tumors of the control group (Fig. 6A), whereas a greater number of apoptotic cells were detected in tumors treated with 7E. Thus, 7E enhanced the 4T1 cell apoptosis in a dose-dependent manner. Furthermore, the tumor sections were stained with an anti–ki-67 Ab and showed that Ki-67–positive cells were decreased in the tumor section of the 7E-treated group compared with the mlgG-treated group. These data suggested that 7E inhibited 4T1 cell proliferation and enhanced tumor cell apo-
ptosis in vivo (Fig. 6B). These results are consistent with the in vitro data and confirmed that 7E reduced tumor growth in vivo by inhibiting the function of IL-20 in cell proliferation.

7E reduced 4T1-mediated bone colonization in vivo

To assess the contribution of IL-20 in breast cancer-mediated bone metastasis, 4T1/Luc cells (1 × 10^5/100 μl per mouse) were injected into the left cardiac ventricle of mice, and the mice were injected (i.p.) with PBS (controls), mIgG, and 7E, respectively, three times per week for the next 30 d. Quantification of the bioluminescent signal from the tumor cells showed that 7E reduced bone colonization in vivo (Fig. 7A). All 10 mice in the PBS group and the mIgG group died 22 d after they had been injected with 4T1 cells. Three of 10 mice in the 7E-treated group survived.
28 d after they had been injected with 4T1 cells (Fig. 7B). In addition, micro-CT scans showed less bone loss in the tibias of 7E-treated mice (Fig. 7C). They also showed a significantly lower BMD decrement of the tibias in the 7E-treated group (Fig. 7D). Lower osteoclast numbers were observed in 7E-treated mice (Fig. 7E). The results provided evidence that IL-20 was involved in breast cancer-mediated bone colonization.

**Inhibiting IL-20 reduced osteolysis in vivo**

The vicious circle of tumor–bone interaction suggests that tumor growth and survival are dependent on osteoclast-mediated bone resorption (2). In vivo inhibition of IL-20 suppressed bone colonization. Thus, we examined whether inhibiting IL-20 also reduces breast tumor-induced osteolysis in the bone microenvironment. 4T1/Luc cells (1 × 10^7/100 μl) were injected directly into the left ventricle of anesthetized BALB/c mice. PBS, mlgG (10 mg/kg), or 7E (10 mg/kg) (n = 10 in each group) was injected i.p. three times per week throughout the study. Healthy controls (n = 10) were not injected with cancer cells. A. Luciferase activity was measured on day 17 posttreatment and used to quantify tumor growth. The IVIS system was used to detect luminescence from 4T1-Luc cells. B. Survival in each group was monitored daily until the end of the experiment. Kaplan–Meier plots were used to stratify a survival curve. C. The tibial metaphysis was taken from healthy controls and from 4T1-induced osteolytic mice with different treatments. Representative micro-CT photos are shown for each group. D. Tibial BMD was measured and the results expressed in percentages relative to healthy control values. *p < 0.05 versus mlgG controls. Data are means ± SD. Data are representative of three independent experiments. E. Osteoclast numbers per bone surface in TRAP-stained sections. N.Oc/BS, number of osteoclasts per bone surface. *p < 0.05 versus mlgG controls. Data are means ± SD. Data are representative of three independent experiments.

**Discussion**

We found that IL-20 was highly expressed in primary breast tumor tissue and bone-metastasis tissue. IL-20 expression in breast cancer tissue not only is associated with a higher mitotic rate but also is correlated with advanced tumor stage and bone metastasis. IL-20 enhanced breast cancer cell growth, migration, and upregulated protease expression. The anti–IL-20 mAb 7E reduced tumor growth, suppressed bone colonization, and prevented tumor-mediated osteolysis in mice injected with breast cancer cells. These findings demonstrated that IL-20 plays pivotal roles in the pathogenesis of breast cancer and that IL-20 might be a novel target for treating breast cancer.
The IL-20 expression level was correlated with the tumor stage and bone metastasis in breast cancer patients, as revealed by immunohistochemical staining. Thus, IL-20 may be used as a biomarker for diagnosing breast cancer. All IL-20R subunits were expressed in our clinical specimens and breast cancer cell lines, which suggested that IL-20 acts in an autocrine manner in breast tumors. The local cytokine/MMP/protease milieu generated by the tumor microenvironment is important for the growth, metastasis, and immune evasion of breast tumors. In breast cancer, MMP-9 promotes tumor initiation, invasion, and metastasis (33); MMP-12 promotes invasiveness (34); and cathepsin G and MMP-9 are proteases involved in increased TGF-β signaling at the tumor-bone interface of breast tumor-induced osteolytic lesions (35). Cathepsin K is expressed in breast cancer, which has been attributed to its ability to degrade native collagen I and facilitate the expansion of tumors in the bone (36–38). We found that IL-20 affected breast cancer cell proliferation and migration and enhanced tumor progression by upregulating MMP-9, MMP-12, cathepsin K, and cathepsin G. Therefore, IL-20 produced by breast cancer cells promotes tumor progression not only by its direct autocrine effect but also by nurturing a microenvironment for tumor growth and metastasis. We previously showed (22, 28, 30, 39) that IL-20 was induced in response to hypoxia; promoted angiogenesis in endothelial cells; induced IL-6, IL-8, and MCP-1 expression in several types of cell; and was a chemoattractant for neutrophils. In the current study, we revealed that IL-20 enhanced tumor proliferation and migration, and upregulated the production of MMPs, cathepsin K, and cathepsin G. These properties of IL-20 further provide evidence that IL-20 is involved in many phases of tumor progression. That 7E enhanced tumor cell apoptosis, reduced tumor growth, and suppressed bone colonization in vivo confirmed the findings.

To investigate whether exogenous IL-20 enhanced 4T1 growth in vivo, 4T1-bearing mice were treated with IL-20. No significant difference was observed between PBS- and IL-20–treated groups (data not shown). This finding may be attributed to the existence of large quantities of IL-20 endogenously produced by 4T1 cells. We also found that 7E prolonged survival and reduced metastasis to vital organs other than bone in the intraventricular metastatic model. Additional investigations of the molecular mechanism by which IL-20 is involved in tumor metastasis to other vital organs are necessary.

A previous study (40) indicated that IL-20 was upregulated in tumor tissues during the progression of tumor growth in mice that had been s.c. challenged with breast adenocarcinoma SB5b cells. In our tumor model of 4T1 cells, we also found that IL-20 was highly expressed in the tumor mass, which is consistent with our clinical findings. IL-20 is a hypoxia-inducible gene, and the hypoxia response element has been identified on the IL-20 promoter (28). Therefore, one possible mechanism upregulating IL-20 in the tumor may be the response of cancer cells to hypoxia in the solid tumor mass. 7E suppressed 4T1 tumor growth and tumor weight in vivo. The reduction of tumor growth and tumor size in 7E-treated mice was correlated with the reduced protein levels of IL-20 and the number of IL-20–positive cells in the tumor mass, which supported the idea that IL-20 was directly involved in the...
tumor progression of breast cancer by regulating tumor cell proliferation and migration in vivo.

Breast cancer that metastasizes to bone leads to pathological bone destruction and erosion, which causes fractures and loss of mobility. Continuation of the vicious circle depends on a continuous supply of osteoclast precursors and mature osteoclasts that can be activated by RANKL (41). IL-20 was strongly expressed in breast tumors from bone-metastasis tissue. Our recent studies (23) demonstrated that IL-20 markedly induced RANKL production in synovial fibroblasts, and Th17 cells. IL-20 promoted osteoclast differentiation by upregulating RANK expression on osteoclast precursor cells and upregulating RANKL expression on osteoblasts (29). Therefore, IL-20 is an upstream regulator for expression of RANK and RANKL, which are very critical molecules for osteoclastogenesis. These findings also provide evidence that IL-20 is involved in breast cancer-mediated bone osteolysis by modulating osteoclastogenesis.

In the progression of tumor-mediated osteolysis, cathepsin G is pivotal in amplifying the vicious circle by cleaving the extracellular domain of RANKL, which increases RANKL-mediated osteoclast differentiation and activation (42). Cathepsin G is also a chemoattractant for osteoclast precursors by proteolytically activating PAR-1 (43). Our recent study (29) demonstrated that IL-20 upregulated the expression of cathepsin G in osteoclasts, which dose-dependently induced the cleavage of RANKL from the surface membrane of osteoclasts. In the current study, IL-20 also upregulated the expression of cathepsin G in breast cancer cells. Therefore, we hypothesize that IL-20 facilitates the entrance of breast tumor cells into the bone microenvironment and induces osteolysis through four possible mechanisms: (1) IL-20 promotes tumor progression by enhancing tumor cell proliferation and migration; (2) IL-20 nurtures a microenvironment for tumor colonization in bone by upregulated MMP-9, MMP-12, cathepsin K, and cathepsin G; (3) IL-20 acts on osteoclasts and tumor cells to produce in the bone microenvironment a large amount of cathepsin G, which leads to the generation and shedding of soluble RANKL and increased osteoclastogenic activity; and (4) IL-20 also modulates osteoclastogenesis by upregulating RANK on osteoclast precursor cells and RANKL on osteoblasts, which provides a link between inflammation and tumor-induced osteolysis.

IL-20 acted on osteoclast and enhanced osteoclast differentiation (29), suggesting that IL-20’s neutralization of the Ab 7E might affect normal bone turnover. We treated the healthy mice with 7E and analyzed the BMD. Although micro-CT scans showed that 7E treatment, compared with PBS treatment, slightly increased BMD in the tibia, we did not observe a significant difference between these two data analyzed by ANOVA. Thus, we further analyzed the serum concentrations of the following two bone turnover markers: osteocalcin secreted from osteoblasts and the C-terminal telopeptide of collagen secreted from osteoclasts in 7E-treated healthy mice. Healthy mice receiving 7E treatment showed lower serum osteocalcin and C-terminal telopeptide of collagen than did PBS-treated control mice (Y.H. Hsu, unpublished observations). This finding suggested that 7E could modulate bone turnover and create an unfavorable microenvironment for tumor cells in which to colonize and grow. 7E potently reduced bone colonization and prevented bone loss in our 4T1-induced metastatic model, which supports our hypothesis.

In contrast to the proangiogenic functions of IL-20 in murine tumor models, IL-24, another member of the IL-10 family, inhibited tumor formation by suppressing tumor vascularization (44). Both cytokines seem to influence tumor angiogenesis differently, despite signaling through the same receptor complexes.

The detailed mechanism used by these two cytokines to regulate breast cancer awaits further investigation.

In summary, our findings show that IL-20 is an important mediator in breast cancer progression. IL-20 expression in breast cancer tissue is associated with higher mitotic rate, advanced tumor stage, and bone metastasis. IL-20 not only enhanced the proliferation and migration of cancer cells but also nurtured a microenvironment that facilitated tumor progression by upregulating MMPs and cathepsins. 7E suppressed bone colonization, decreased osteolytic bone lesions, and prevented bone loss in our intratibial osteolytic model. We conclude that 7E is a potential therapeutic agent for reducing tumor growth and bone colonization, and for protecting against osteolytic bone loss.

Disclosures
The authors have no financial conflicts of interest.

References


