Annexin 2–CXCL12 Interactions Regulate Metastatic Cell Targeting and Growth in the Bone Marrow
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Abstract
Annexin 2 (ANXA2) plays a critical role in hematopoietic stem cell (HSC) localization to the marrow niche. In part, ANXA2 supports HSCs by serving as an anchor for stromal-derived factor-1 (CXCL12/SDF-1). Recently, it was demonstrated that prostate cancer cells, like HSCs, use ANXA2 to establish metastases in marrow. The present study determined the capacity of ANXA2–CXCL12 interactions. Significantly more CXCL12 was expressed by BMSCAnxa2+/− than by BMSCAnxa2−/−, resulting in more prostate cancer cells migrating and binding to BMSCAnxa2+/− than BMSCAnxa2−/−, and these activities were reduced when CXCL12 interactions were blocked. To further confirm that BMSC signaling through ANXA2–CXCL12 plays a critical role in tumor growth, immunocompromised SCID mice were subcutaneously implanted with human prostate cancer cells mixed with BMSCAnxa2+/− or BMSCAnxa2−/−. Significantly larger tumors grew in the mice when the tumors were established with BMSCAnxa2+/− compared with the tumors established with BMSCAnxa2−/−. In addition, fewer prostate cancer cells underwent apoptosis when cocultured with BMSCAnxa2+/− compared with BMSCAnxa2−/−, and similar results were obtained in tumors grown in vivo. Finally, significantly more vascular structures were observed in the tumors established with the BMSCAnxa2+/− compared with the tumors established with BMSCAnxa2−/−. Thus, ANXA2–CXCL12 interactions play a crucial role in the recruitment, growth, and survival of prostate cancer cells in the marrow.

Implications: The tumor microenvironment interaction between ANXA2–CXCL12 is critical for metastatic phenotypes and may impact chemotherapeutic potential. Mol Cancer Res; 13(1); 197–207. ©2014 AACR.
similar roles in recruiting and localizing tumor cells into the HSC niche (27–30).

In the present study, we explored whether ANXA2 facilitates metastasis and growth of prostate cancer through its interactions with CXCL12. We demonstrate that ANXA2-expressing BMSCs express more CXCL12 than do ANXA2-deficient BMSCs, and the production of these 2 proteins increases the recruitment of prostate cancer cells into the marrow niche. In addition, CXCL12 produced by ANXA2-expressing BMSCs promotes prostate cancer proliferation, protects prostate cancer from chemotherapy-induced apoptosis, and increases the development of vascular structures. Our results suggest that ANXA2 and CXCL12 interactions facilitate the recruitment, growth, and survival of prostate cancer cells in marrow.

Materials and Methods

Cell culture

The human prostate cancer cell line PC3 was obtained from the ATCC. C4-2B, the metastatic subline of the prostate cancer cell line LNCaP, was originally isolated from a lymph node of a patient with disseminated bony and lymph node involvement. Luciferase-expressing prostate cancer cell lines (PC3Luc and C4-2BLuc cells) and GFP-expressing prostate cancer cell lines (PC3GFP and C4-2B GFP cells) were established by lentiviral transduction. Human bone marrow endothelial cells (HBMEC) were isolated from a normal Caucasian male and immortalized with SV40 large T-antigen (31). Prostate cancer cells and HBMEC were grown in RPMI-1640 (Invitrogen) supplemented with 10% FBS and 1% T-antigen (31). Prostate cancer cells in marrow.

Adhesion assay

BMSCs from Anxa2fl/fl or Anxa2+/− mice (5 to 7-week-old) were used for this study. Dr. K.A. Hajjar (Weill Medical College of Cornell University, New York, NY) graciously provided our laboratory with a pair of the homozygous Anxa2−/− mice for breeding (32). After sacrifice, marrow was flushed from femurs and tibias of both Anxa2−/− and Anxa2+/− mice with α-MEM (Invitrogen) containing 2% FBS and cultured in α-MEM containing 15% heat-inactivated FBS and 1% P/S to generate primary BMSCs. Once confluent, the cells were passaged 2 to 3 times with trypsin to minimize macrophage contamination. Subsequently, repopulated BMSCs were obtained, and the cells were cultured in α-MEM containing 10% heat-inactivated FBS and 1% P/S. These cells were termed as BMSCsAnxa2+/− and BMSCsAnxa2−/−. For differentiation assays, mouse primary BMSCs were cultured in adipogenic, osteogenic, or chondrogenic conditions for 2 weeks, fixed and stained for identification with Alizarin Red S, Oil Red O, and Alcian Blue, respectively.

Proliferation assay

BMSCsAnxa2+/− or BMSCsAnxa2−/− (1 × 10^5 cells per well) were seeded onto 12-well culture plates for 24 hours and PC3Luc cells (1 × 10^5 cells per well) were directly cocultured with BMSCsAnxa2+/− or BMSCsAnxa2−/− for 48 hours. The amount of luciferase in these cultures was measured by Dual-Luciferase Reporter Assay kit (cat. E1910; Promega) and detected using a luminometer (BD Monolight 2010; BD Biosciences).

Adhesion assay

BMSCsAnxa2+/− or BMSCsAnxa2−/− were plated onto 96-well plates at a concentration of 1 × 10^4 cells/100 µL/well in growth medium, and the cultures were incubated for 2 days. Prostate cancer cells were labeled with 2.5 µg/mL of the lipophilic dye carboxyfluorescein diacetate (CFDA, cat. V12883; Molecular Probes) in RPMI for 30 minutes at 37°C and washed in PBS. Thereafter, prostate cancer cells (1 × 10^5 cells per well) were added to each well and were left for 30 minutes at room temperature to allow binding to BMSCs. In some cases, adhesion assays of prostate cancer cells to BMSCs were done in the presence or absence of function blocking antibody to ANXA2 (cat. 610069; BD Pharmingen), with matched IgG as an antibody control. Total fluorescence per well was determined initially, followed by washing wells with PBS to quantify fluorescence for prostate cancer cells adherent to BMSCs.

Transwell chemotaxis assay

BMSCsAnxa2+/− or BMSCsAnxa2−/− (1 × 10^5 cells per well) were seeded onto 24-well culture plates. Prostate cancer cells or HBMECs were labeled with 2.5 µg/mL of CFDA as described above. Prostate cancer cells or HBMECs were resuspended in serum-free RPMI-1640 and equilibrated for 10 minutes at 37°C. CFDA-labeled prostate cancer cells or HBMECs were loaded into the top chambers of 8-mm Transwell microporous membrane 24-well plates (cat. 3422; Costar Corp.). A total of 650 µL of conditioned medium (CM) from 3-day cultures of BMSCsAnxa2+/− or BMSCsAnxa2−/− (1 × 10^5 cells per well) was added into the bottom well. The plates were incubated at 37°C for 3 hours. At the termination of the experiments, the intensity of fluorescence in the lower chamber, indicating the number of cells that had migrated, was determined by plate reader (Molecular Probes). In some cases, migration of prostate cancer cells to CM isolated from BMSCs was analyzed in the presence of neutralizing anti-CXCL12 monoclonal antibodies (120 µg/mL, cat. MAB310; R&D Systems) or AMD3100, a selective CXCR4 antagonist (4 ng/mL, cat. A5602; Sigma).

Cell death assay

BMSCsAnxa2+/− or BMSCsAnxa2−/− (1 × 10^5 cells per well) were seeded onto 12-well culture plates for 24 hours. GFP expressing prostate cancer cells (1 × 10^5 cells per well) were added to the wells and cultured together with the BMSCs for 48 hours before the addition of the anticancer drug, taxotere (1 µg/mL, cat. NDC0409-0201-10; Hospira) for an additional 48 hours. In addition, HBMECs (1 × 10^5 cells per well) were seeded onto 12-well culture plates for 24 hours and the cells were treated with ANXA2 (1 µg/mL, cat. 11-511-248-344; Genway Biotech) or CXCL12 (200 ng/mL, cat. 350-NS; R&D Systems) for 48 hours. Apoptosis in these cultures was measured by flow cytometry (FACSAria dual laser flow-cytometer, Becton Dickinson) using PE Annexin V Apoptosis Detection Kit I (cat. 559763; BD Biosciences). Assays were performed in triplicate and the results are representative of 3 independent experiments. In tissue sections from mice inoculated with human prostate cancer, apoptosis of prostate cancer cells and endothelial cells in the tumors was measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (cat. 11684817910; Roche).

Quantitative RT-PCR

Total RNA was extracted using the RNeasy mini or micro kit (Qiagen). cDNA was established using a First-Strand synthesis kit (Invitrogen). Quantitative PCR was performed on an ABI 7700 sequence detector (Applied Biosystems) using TaqMan Universal...
PCR Master Mix Kit (Applied Biosystems) according to the directions of manufacturer. TaqMan MGB probes (Applied Biosystems) were as follows: human Anxa2r (Hs01588662_s1) and human Ccr4 (Hs00223705_m1). β-Arrestin (Hs9999903_m1) was used as an internal control for the normalization of target gene expression.

**ELISAs**

BMSCs*Anxa2r+/+* or BMSCs*Anxa2r−/−* (1 x 10^5 cells per well) were cultured in 12-well culture plates for 48 hours. An antibody sandwich ELISA was used to evaluate CXCL12 levels in the CM by the modification of directions of manufacturer (R&D Systems). CXCL12 secretion levels were normalized to total cell numbers.

**Immunostaining**

Cells and tumor sections were used for immunostaining. Cells were fixed with 10% neutral-buffered formalin (cat. HTS01320; Sigma) and permeabilized with PBT (1:500 dilution of Triton-X100 in PBS). Tumor sections were deparaffinized, hydrated, and then blocked with Image-iT FX signal enhancer (cat. I36933; Life Technology) for 30 minutes and incubated for 2 hours at room temperature with primary antibodies combined with reagents of Zenon Alexa Fluor 488 (green) or 555 (red) labeling kit (Life Technology). Polyclonal anti-ANXA2 (cat. 610069; 1:25 dilution; BD Pharmingen), polyclonal anti-CXCL12 (cat. ab25117; 1:100 dilution; Abcam), monoclonal anti-HLA (cat. 311402; 1:50 dilution; BioLegend), CD31 (cat. ab32457; 1:100 dilution; Abcam), monoclonal p-AKT (cat. ab81283; 1:100 dilution; Abcam), and monoclonal anti-AKT (cat. ab32505; 1:100 dilution; Abcam) were used as primary antibodies. After washing with PBS, tumor sections were mounted with ProLong Gold antifade reagent with DAPI (cat. P36931; Invitrogen). Images were taken with Olympus FV-500 confocal microscope. In some cases, the tumor sections were stained by TUNEL staining (cat. 11684817910; Roche).

**Western blotting**

PC3 cells (89%–90% confluent) were cultured in 6-well plates in RPMI (1 mL) without FBS for 18 hours. After serum starvation, the cells were treated with Anxa2 (1 µg/mL), CXCL12 (200 ng/mL), or a combination of Anxa2 (1 µg/mL) and CXCL12 (200 ng/mL) for 30 minutes at 37°C. Whole-cell lysates were prepared from cells, separated on 10% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were first incubated with 5% milk for 1 hour before the addition of antibodies to p-AKT (cat. 9271; 1:1,000 dilution; Cell Signaling) and AKT (cat. 9272; 1:1,000 dilution; Cell Signaling) followed by overnight at 4°C. Subsequently the blots were incubated with peroxidase-coupled anti-rabbit IgG secondary antibody (cat. 7074; 1:2,000 dilution; Cell Signaling) for 1 hour, and protein expression was detected with SuperSignal West Dura Chemiluminescent Substrate (cat. Prod. 34075; Thermo Scientific).

**Subcutaneous tumor growth**

To evaluate tumor growth, subcutaneous tumors were established. The PC3 cells (2 x 10^5 cells) mixed with BMSCs*Anxa2r+/+* or BMSCs*Anxa2r−/−* (2 x 10^5 cells) in Collagen I (cat. 354236; BD Bioscience) were injected subcutaneously into 5- to 7-week-old male SCID mice. Tumors were imaged weekly by bioluminescence imaging (BLI). After 5 weeks, the animals were sacrificed and tumors were prepared for histology.

**Intratibial tumor growth**

To evaluate tumor growth in bone marrow, PC3Luc* (1 x 10^5 cells) or C4-2BLuc* (3 x 10^5 cells) were inoculated intratibially into 5- to 7-week-old male SCID mice. For intratibial injection, mice were anesthetized with 2.5% isoflurane/air, and both legs were cleaned with betadine and 70% ethanol. The knee was flexed and a 27-G/8-inch needle was inserted into the proximal end of right tibia followed by injection of 20 µL single-cell suspensions of prostate cancer cells. Tumors were allowed to become established for 5 weeks.

All animals were sacrificed at week 5 and tibiae with tumors were collected for histology.

**Statistical analyses**

Results are presented as mean ± SD. Significance of the difference between 2 measurements was determined by unpaired Student t test, and multiple comparisons were evaluated by the Newman–Keuls multiple comparison test. Values of P < 0.05 were considered significant.

**Results**

**ANXA2 and CXCL12 expression are correlated in the bone marrow environment**

We examined the extent to which Anxa2 expression is correlated with CXCL12 expression in the long bones of wild-type and Anxa2-deficient mice (Fig. 1A). In vitro, we tested CXCL12-expressing cells that were identified in BMSCs derived from wild-type mice (BMSCs*Anxa2r+/+* or BMSCs derived from Anxa2 knockout mice (BMSCs*Anxa2r−/−*; Fig. 1B). CXCL12 protein levels in the CM of BMSCs*Anxa2r−/−* were significantly greater than those generated by BMSCs*Anxa2r+/+* cultures (Fig. 1C). The findings suggest that ANXA2 expression by BMSCs is correlated with CXCL12 expression in bone marrow microenvironment.

**Interactions between ANXA2 and CXCL12 by BMSCs contribute to prostate cancer cell adhesion and migration**

To test whether expression of the ANXA2 receptor (Anxa2r) and the receptor for CXCL12, Cxcr4, are correlated, mRNA levels were quantified using qRT-PCR. Significantly higher levels of Anxa2r or Cxcr4 expression was observed in the bone-homing prostate cancer cell lines PC3 and C4-2B, compared with human BMSCs (Fig. 2A and B). Studies were next performed to determine whether ANXA2 serves as an adhesion molecule for prostate cancer cell binding to BMSCs. As shown in Fig. 2C, the binding of prostate cancer cells to BMSCs was significantly greater than the binding of prostate cancer cells to BMSCs. When the experiments were repeated in the presence of a function blocking antibody targeting ANXA2, significant reduction of prostate cancer binding to BMSCs was observed (Fig. 2D).

CXCL12 is a key regulator of hematopoietic stem cell migration and tumor metastasis into the marrow. As ANXA2 expression is linked to expression of CXCL12, we next performed migration assays of prostate cancer cells in response to BMSCs*Anxa2r+/+* or BMSCs*Anxa2r−/−*. For these studies, prostate cancer cells were added to the upper chamber of a migration assay dish and CM derived from BMSCs*Anxa2r+/+* or BMSCs*Anxa2r−/−* was added to the
Expression of ANXA2 and CXCL12 by BMSCs contributes to prostate cancer cell growth

Next, we evaluated whether or not the interaction of ANXA2 and CXCL12 plays a role in prostate cancer tumor growth in vitro. Here, prostate cancer cells (PC3<sub>Luc</sub>, 1 × 10<sup>5</sup>) were cocultured with BMSC<sub>ANXA2<sup>−/−</sup> or BMSC<sub>ANXA2<sup>+/−</sup> or BMSC<sub>ANXA2<sup>+/+</sup></sub> for 48 hours in 12-well culture plates. Thereafter, luciferase activity was measured by Dual-Luciferase Reporter Assay kit to monitor prostate cancer growth. More luciferase activity was observed when the tumor cells were cocultured with BMSC<sub>ANXA2<sup>−/−</sup></sub> than with BMSC<sub>ANXA2<sup>+/−</sup></sub> (Fig. 3A).

On the basis of the in vitro studies, we examined whether there are differences in the ability of BMSCs to support prostate cancer growth in vivo linked to expression of ANXA2 and CXCL12. For these studies, tumors were established by subcutaneously injecting PC3<sub>Luc</sub> cells (2 × 10<sup>5</sup>) mixed with BMSC<sub>ANXA2<sup>+/+</sup></sub> or BMSC<sub>ANXA2<sup>−/−</sup></sub> (2 × 10<sup>5</sup>) in type I collagen into 5- to 7-week-old male SCID mice. Bioluminescent imaging at 5 weeks was used to monitor tumor growth. Significantly larger tumor growth occurred when the tumors were mixed with BMSC<sub>ANXA2<sup>−/−</sup></sub> compared with tumors established with BMSC<sub>ANXA2<sup>+/−</sup></sub> (Fig. 3B and C). To further test the growth of prostate cancer cells in the bone marrow, tumors were established by injecting PC3<sub>Luc</sub> (1 × 10<sup>5</sup>) or C4-2B<sub>Luc</sub> (3 × 10<sup>5</sup>) intratibially into 5- to 7-week-old male SCID mice. We found that prostate cancer cells are able to grow in bone marrow and these tumor cells interact with ANXA2 or CXCL12-expressing osteoblast cells on the bone surface in marrow (Fig. 3D).

To explore the molecular mechanisms whereby ANXA2 and CXCL12 regulate tumor growth, we examined whether ANXA2 alone, CXCL12 alone, or the combination of ANXA2 and CXCL12 activates AKT in prostate cancer cells. For these investigations, prostate cancer cells were treated with ANXA2 and/or CXCL12 and phosphorylation of AKT was evaluated by Western blotting. The data demonstrate that ANXA2 or CXCL12 alone highly induces AKT phosphorylation in PC3 cells, and the combination of ANXA2 and CXCL12 significantly enhances activation of AKT phosphorylation (Fig. 3E). To validate the in vitro results, we examined induction of AKT phosphorylation on the tumors generated in Fig. 3B. The immunofluorescence data demonstrate that the expression levels of p-AKT was significantly enhanced in the tumor cells mixed with BMSC<sub>ANXA2<sup>−/−</sup></sub> compared with tumors established with BMSC<sub>ANXA2<sup>+/−</sup></sub> or tumors alone (Fig. 3F). These data suggested that ANXA2 and CXCL12 facilitate prostate cancer growth via the AKT pathway.

Expression of ANXA2 and CXCL12 by BMSCs contributes to protection from chemotherapy-induced apoptosis

Prostate cancer cells are known to develop resistance to chemotherapies, particularly in marrow. To explore the role that ANXA2 and CXCL12 play in this process, cocultures of BMSCs and prostate cancer cells were evaluated by FACS for Annexin V fluorescent staining. Blue, DAPI nuclear stain. Bar, 20 μm. C, secretion of CXCL12 by BMSC<sub>ANXA2<sup>−/−</sup></sub> or BMSC<sub>ANXA2<sup>+/−</sup></sub> as determined by ELISA. Data are representative of mean with SD of 3 independent experiments (Student t test).

Expression of ANXA2 and CXCL12 by BMSCs contributes to protection from chemotherapy-induced apoptosis

Prostate cancer cells are known to develop resistance to chemotherapies, particularly in marrow. To explore the role that ANXA2 and CXCL12 play in this process, cocultures of BMSCs and prostate cancer cells were evaluated by FACS for Annexin V staining (Fig. 4). Apoptotic levels for prostate cancer cells cocultured with BMSC<sub>ANXA2<sup>−/−</sup></sub> were lower than when prostate cancer cells were cultured alone or with BMSC<sub>ANXA2<sup>+/−</sup></sub>. Further protection from apoptosis was noted when the microtubule-inhibiting drug taxotere was included in the cultures (Fig. 4A and B). To evaluate whether these in vitro studies are also relevant to our in vivo studies, TUNEL staining was next performed on the tumors generated in Fig. 3B. Fewer apoptotic tumor cells were found in the tumors established with PC3<sub>Luc</sub> and BMSC<sub>ANXA2<sup>−/−</sup></sub> compared
with tumors generated from PC3\textsuperscript{Luc} mixed with BMSC\textsuperscript{Anxa2\(+/+\)} (Fig. 4C and D). Together the data suggest that ANXA2 and CXCL12 expression by BMSCs are likely to play a role in the resistance of prostate cancer cells to chemotherapy.

Expression of ANXA2 and CXCL12 by BMSCs contributes to the generation of vascular structures in tumors

To determine whether ANXA2 and CXCL12 contribute to endothelial cell recruitment into tumor beds, migration by endothelial cells in response to CM isolated from BMSCs was evaluated. When HBMEC were added to the upper chamber of the culture dish with BMSCs CM present in the bottom, fewer HBMECs migrated toward BMSC\textsuperscript{Anxa2\(+/+\)} CM compared with BMSC\textsuperscript{Anxa2\(+/+\)} CM. When the experiments were performed in the presence of neutralizing anti-CXCL12 antibody or AMD3100, a selective CXCR4 antagonist. Data are representative of mean with SD of 3 independent experiments (Student t test).

Discussion

Recent studies suggest that niche cells in the bone marrow participate in the cellular and molecular events for the tumor progression and metastasis (5–9). In this study, we show ANXA2 and CXCL12 interactions produced by BMSCs facilitate prostate cancer recruitment and proliferation, aid in protection
Figure 3.
ANXA2 and CXCL12 interact to regulate prostate cancer cell growth. A, PC3 cells were placed in coculture with BMSC\textsuperscript{Anxa2\textsuperscript{+}/+} or BMSC\textsuperscript{Anxa2\textsuperscript{-}/-}, and proliferation was evaluated at 48 hours by determining the change in luciferase levels using a Dual-Luciferase Reporter Assay kit. Data are representative of mean with SD of 3 independent experiments (Student t test). B and C, SCID mice were implanted subcutaneously PC3 cells alone or mixed with BMSC\textsuperscript{Anxa2\textsuperscript{+}/+} or BMSC\textsuperscript{Anxa2\textsuperscript{-}/-}. Tumor growth was evaluated by BLI at 5 weeks (n = 5 animals per group, mean ± SD, Student t test). D, SCID mice were implanted intratibially with PC3 or C4-2B\textsuperscript{LUC} to directly test tumor growth in bone marrow. H&E staining demonstrates tumor growth in marrow (left). Bar, = 50 μm. (Continued on the following page.)
of prostate cancer cells from chemotherapy-induced apoptosis, and facilitate the generation of vascular structures in tumors. Together these data suggest that ANXA2 and CXCL12 interactions may be integral to tumor development in the marrow microenvironment.

The engagement of DTCs to the bone marrow niche and the maintenance of these cells in the niche are essential steps to establish metastases. In this context, ANXA2, an osteoblast-expressed adhesion molecule, may be a crucial docking signal (30). CXCL12 is a chemokine, which in addition to functioning as a secreted molecule is known to bind to extracellular matrix proteins (27–29). Previously, we and other groups have identified several factors that may be involved in establishing a tumor niche. We have shown that CXCL12 produced by the niche participates in the regulation of prostate cancer metastasis (27–29), and we have also demonstrated that ANXA2 and its receptor ANXA2R regulate the localization and maintenance of prostate cancer cells in the niche (30). More importantly, tumor cell metastases share molecular mechanisms that regulate HSC homing and localization to the bone marrow (27–30). Subsequently, we reported that ANXA2 and CXCL12 bind directly to each other, and these interactions facilitate hematopoietic progenitor cell migration in response to CXCL12 (26). We therefore hypothesized that ANXA2-expressing bone marrow stromal cells facilitate prostate cancer metastasis and growth by inducing CXCL12. We found that ANXA2-expressing BMSCs express elevated levels of CXCL12, which increases the binding and recruitment of prostate cancer cells into the marrow niche and promotes prostate cancer proliferation. Thus, a model can be envisioned in which CXCL12 facilitates the recruitment of DTCs to the niche, ANXA2 facilitates prostate cancer engagement into the niche, and CXCL12 produced by the niche cells stimulates prostate cancer growth. CXCL12 is known to play a central role in promoting the growth of tumor cells including ovarian carcinoma (33), small cell lung cancer (34), head and neck squamous cell carcinoma (35), pancreatic cancer (36), and prostate cancer (37). It has also been demonstrated that secreted CXCL12 induces prostate

Figure 4.
ANXA2 and CXCL12 in the marrow environment enhance prostate cancer survival and drug resistance. PC3(GFP) or C4-2B(GFP) cells were directly cultured with BMSC(ANXA2+/-) or BMSC(ANXA2-/->) for 48 hours and taxotere was introduced into the culture for an additional 48 hours. Annexin V staining was performed on the recovered (A) PC3 or (B) C4-2B cells and quantified by FACS. Data in A and B are representative of mean with SD of 3 independent experiments (Student t test). C, TUNEL staining of apoptotic prostate cancer cells (black arrows) of tumors grown from PC3 cells alone or mixed with BMSC(ANXA2+/-) or BMSC(ANXA2-/->) implanted into SCID mice. Bar, = 50 μm. D, quantification of percentage of TUNEL-positive cells in C (mean ± SD, Student t test).

(Continued.) Colocalization of ANXA2 and CXCL12 on osteoblasts (yellow arrows) associated with the bone surface was detected by immunofluorescent staining (top). Blue, DAPI nuclear stain. Bar = 50 μm. HLA-positive tumor cells (red arrows) are associated with ANXA2 (green arrows)-expressing osteoblasts (middle) or CXCL12 (green arrows)-expressing osteoblasts (bottom) as detected by immunofluorescent staining. Bar, = 50 μm. E, ANXA2, CXCL12, or a combination of ANXA2 and CXCL12 were treated to PC3 cells for 30 minutes. Western blotting was performed to detect p-AKT or AKT levels. F, immunofluorescent staining of p-AKT or AKT on the tumors generated in B.
Figure 5.
ANXA2 and CXCL12 expression by BMSCs increases vascular formation. A, migration of HBMECs toward CM isolated from BMSC<sup>Anxa2<sup>+</sup>/+</sup> or BMSC<sup>Anxa2<sup>/</sup>/</sup> with and without AMD3100, a selective CXCR4 antagonist. Data are representative of mean with SD of 3 independent experiments (Student t test). B, CD31 staining (red) for blood vessel cells in tumors grown from PC3<sup>luc</sup> cells alone or mixed with BMSC<sup>Anxa2<sup>+</sup>/+</sup> or BMSC<sup>/</sup>/ implanted into SCID mice. Blue, DAPI nuclear stain. Bar, 50 μm. C, quantification of percentage of CD31-positive cells in B (mean ± SD, Student t test). D, apoptotic HBMECs in the presence of ANXA2, CXCL12, or combination of ANXA2 and CXCL12 were quantified by FACS analysis using Annexin V staining. Data are representative of mean with SD of 3 independent experiments (Student t test). E, TUNEL staining of apoptotic endothelial cells (black arrows) in tumors grown from PC3<sup>luc</sup> cells mixed with BMSC<sup>Anxa2<sup>+/</sup>/</sup> or BMSC<sup>/</sup>/ implanted into SCID mice. Bar, 50 μm. F, quantification of percentage of TUNEL-positive cells in E (mean ± SD, Student t test).
cancer proliferation in in vitro (37–39), following activation of ERK1/2 and AKT pathways (34, 36, 37). We also found that ANXA2 and CXCL12 interactions effectively activate through AKT signaling pathways that, in many respects, have been linked to tumor growth.

In marrow, osteoblasts play important roles in the production of a mineralized matrix that serves as the basis of bone remodeling. Osteoblasts are also known to express many cell adhesion molecules, including ANXA2, which may facilitate bone activities. Recently, it was demonstrated that ANXA2 regulates osteogenic differentiation, yet the molecular mechanisms remain unclear (40–42). Hypoxia and VEGF both induce ANXA2 expression by osteoblasts and endothelial cells through SRC and MEK kinase–dependent pathway (40). ANXA2 increases the mineralization of osteoblastic cells and alkaline phosphatase activity (41). ANXA2 also supports osteogenic differentiation of mesenchymal stem cells (MSC) and further regulates both intramembrane and endochondral ossification in adolescent idiopathic scoliosis (AIS; ref. 42). We have also reported that fewer osteoblasts are found in the long bone of Anxa2 knockout (Anxa2−/−) mice compared with wild-type (Anxa2+/+) mice and, likewise, a decrease of bone phenotype markers (Runx2, osteocalcin, Collagen 1a) is seen in bones from these knock-out mice (data not shown). Critical to this report, lower CXCL12-expressing cells in BMSCs derived from Anxa2 knockout-mice (BMSCAnxa2−/−) were identifies compared with cells isolated from wild-type mice (BMSCAnxa2+/+). As ANXA2 mediates osteogenic activities in marrow, the release of tumor-supporting activities from either osteoblasts or bone matrix may provide some clues to how ANXA2 regulates metastasis and CXCL12 signaling simply by providing lower levels of substrates or adhesive surfaces for DTCs to lodge. Alternatively, while the molecular mechanisms need to be further investigated, our data also suggest that an ANXA2-deficient environment may lead to less osteoblastic regulation of bone remodeling, which ultimately results in a decrease of prostate cancer tumor growth and survival in bone marrow. Both of these possibilities are worthy of further study.

CXCL12 is known to regulate tumor cell apoptosis. CXCL12 may protect tumor cells from drug-induced apoptosis directly through the activation of antiapoptotic pathways but also indirectly by modulating the adherence of cancer cells. CXCL12 mediates adhesion of small cell lung cancer cells (SCLC) to marrow stromal cells and prevents SCLC against drug-induced apoptosis (34). CXCL12 activates NF-κB, which inhibits radiation-induced TNFα production and tumor apoptosis (43, 44). Moreover, NF-κB signaling is involved in the expression of CXCR4, thus promoting tumor cell migration and metastasis (43–45). In this study, we found that more prostate cancer cells survived when cocultured with BMSCAnxa2−/− vs. coculture with BMSCAnxa2+/+ in in vitro. Similar results were obtained in in vivo studies, as fewer apoptotic cells were found in tumors established with BMSCAnxa2−/− versus those with BMSCAnxa2+/+ cells. In part, these results may be due to regulation of CXCL12 expression by ANXA2 or enhanced ability to localize CXCL12 to cell surfaces, which may be more efficient in activating cell functions than in a soluble form. Whether either of these 2 possibilities is responsible for how ANXA2 interacts with CXCL12 remains unclear and will require additional investigation.

Finally, migration, expansion, and survival of vascular endothelial cells are essential for establishment of a functional network of angiogenesis. Ling and colleagues demonstrated that Anxa2-deficient mice have markedly impaired neoangiogenesis. This contributes to a failure to localize plasmatic activity to the endothelial cell surface and a failure to activate selected matrix metalloproteinase (32). It has also been demonstrated that ANXA2-dependent plasmatic generation in breast tumor is necessary to trigger the switch to neoangiogenesis, thereby stimulating tumor growth (46, 47). We found that PC3Anxa2−/− cells implanted with BMSCAnxa2−/− showed significantly more and larger CD31-expressing blood vessels than PC3Anxa2+/− mixed BMSCAnxa2−/+. It has also been demonstrated that CXCL12 stimulates the formation of capillary-like structures with human vascular endothelial cells (48–51). Recently, we demonstrated that prostate cancer cells use CXCL12 and its receptors (CXCR4 and CXCR7) as key elements in metastasis and growth in bone, where CXCR4 signaling leads to an angiogenic switch (48–50). We have further demonstrated that CXCR4 signaling results in the activation of MAP and AKT pathways, which ultimately increases the secretion of VEGF and IL8 (48–50). More importantly, CXCR4 directly regulates blood vessel formation (48), due to inhibiting the secretion of the vascular inhibitor phosphoglycerate kinase 1 (PGK1; ref. 50). Thus, there are both direct and indirect mechanisms whereby ANXA2 and CXCL12 interactions may regulate tumor growth. Clearly, more studies are warranted.

In summary, this work provides further evidence for the roles that ANXA2 and CXCL12 play in the tumor microenvironment and specifically in prostate cancer migration, growth, and survival in bone marrow. Our data also provide evidence that ANXA2 and CXCL12 regulate vascular formation in tumors. Together these studies suggest that ANXA2 and CXCL12 interactions facilitate a number of activities critical for tumor growth.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Y. Jung, R.S. Taichman

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