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Original Articles

The impact of bone morphogenetic protein 4 (BMP4) on breast cancer metastasis in a mouse xenograft model

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ABSTRACT

Bone morphogenetic protein 4 (BMP4) is a key regulator of cell proliferation and differentiation. In breast cancer cells, BMP4 has been shown to reduce proliferation *in vitro* and interestingly, in some cases, also to induce migration and invasion. Here we investigated whether BMP4 influences breast cancer metastasis formation by using a xenograft mouse model. MDA-MB-231 breast cancer cells were injected intracardially into mice and metastasis formation was monitored using bioluminescence imaging. Mice treated with BMP4 developed metastases slightly earlier as compared to control animals but the overall number of metastases was similar in both groups (13 in the BMP4 group vs. 12 in controls). In BMP4-treated mice, bone metastases were more common (10 vs. 7) but adrenal gland metastases were less frequent (1 vs. 5) than in controls. Immunostaining revealed no differences in signaling activation, proliferation rate, blood vessel formation, EMT markers or the number of cancer-associated fibroblasts between the treatment groups. In conclusion, BMP4 caused a trend towards accelerated metastasis formation, especially in bone. More work is needed to uncover the long-term effects of BMP4 and the clinical relevance of these findings.

Introduction

Bone morphogenetic protein 4 (BMP4) is a member of the transforming growth factor β (TGFβ) superfamily of extracellular signaling molecules. BMP4 is one of 20 BMPs that were first identified based on their ability to form bone at extraskeletal sites but are now known to have multiple roles both during development and in adult tissues [1,2]. In the cellular context, BMPs regulate fundamental processes such as cell proliferation, differentiation, migration and survival, i.e. characteristics that are of great relevance also in cancer pathogenesis [3]. The versatile functions of BMPs are conveyed through the canonical SMAD pathway where the extracellular ligands first bind to specific cell surface serine-threonine kinase receptor dimers [4]. Intracellular SMAD proteins, which include receptor-regulated SMADs (SMAD1/5/9) and SMAD4, transmit the BMP signal by forming a complex that translocates to the nucleus in order to control the expression of BMP target genes [2,5]. The signals generated by BMPs may also be transferred via ERK, JNK and p38 mitogen-activated protein kinases (MAPKs) [6]. In addition, there is evident crosstalk between BMP and other signaling pathways, such as Wnt, JAK/STAT and Notch [5].

In breast cancer, the expression of several BMPs is deregulated [7,8]. In the case of BMP4, overexpression as compared to normal mammary gland has been described both in cancer cell lines [9,10] and in primary tumors [11,12]. Functional assays in multiple breast cancer cell lines implicated BMP4 as a strong inhibitor of cell proliferation through the induction of G1 cell cycle arrest [9,10]. Interestingly, BMP4 also influenced the migratory properties of breast cancer cells. BMP4 treatment increased migration and invasion of a subset of breast cancer cell lines either directly or via the functions of cancer-associated fibroblasts [9,10,12,13]. The MDA-MB-231 cells demonstrated an especially prominent increase in migration and invasion upon BMP4 stimulation. However, in a study by Shon and colleagues [14], MDA-MB-231 was reported to respond to BMP4 stimulation with reduced migration and invasion. Yet, data from 3D breast cancer cell models, which better mimic the *in vivo* environment, further sustained that BMP4 indeed enhances the migratory capacity of MDA-MB-231 cells [15]. The functional role of BMP4 as an inhibitor of cell growth and promoter of cell migration and invasion is further supported by breast cancer patient data. Strong BMP4 protein expression, which was detected in 25% of breast tumors, associated with low proliferation index and increased frequency of tumor recurrence [11].

The impact of BMP4 on breast cancer formation *in vivo* has been studied surprisingly little, but results from other tissue types mainly point to its role in tumor suppression [16]. For example, direct

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manipulation of BMP4, either through overexpression or administration of recombinant protein, led to reduced tumor growth in xenograft models of brain, colorectal and lung cancers [17–21], whereas enhanced proliferation was seen in hepatocellular carcinoma [22]. Using mouse mammary cancer cells and an orthotopic xenograft model, Cao and colleagues [23] showed that overexpression of BMP4 had no effect on either *in vitro* cell proliferation or primary tumor growth. Nevertheless, BMP4 inhibited the metastatic ability of mouse mammary cancer cells in this model [23]. Other *in vivo* experiments in breast cancer have not focused on direct effects of BMP4 but have instead used manipulation of the BMP pathway and thus these data might not exclusively reflect BMP4 activity. For example, administration of DMH1, a BMP antagonist, and deletion of BMP receptor BMPR1A resulted in reduced mammary tumor growth in MMTV.PyVmT mouse model [24,25]. Furthermore, expression of dominant negative BMPR1A in a mouse model of breast cancer bone metastasis resulted in smaller osteolytic lesions and improved survival [26]. Manipulation of upstream regulators implicated BMP4 as a metastasis promoter in two breast cancer xenograft models [12,27] and a metastasis suppressor in one study [14]. Taken together, *in vivo* data on the functional effects of BMP4 and especially its possible role in breast cancer metastasis formation are very limited and contradictory. Here we sought to address this issue using intracardiac injection of MDA-MB-231 cells into nude mice together with direct treatment of the animals with BMP4. The MDA-MB-231 cells were specifically selected for this study since they exhibit distinct increase in migration and invasion in response to BMP4 treatment *in vitro* [9,10,15]. Metastasis formation was followed with bioluminescence imaging (BLI) and the possible contribution of BMP4 to basic characteristic of the metastasis samples as well as the surrounding tumor stroma were evaluated using immunohistochemistry and immunofluorescence.

Materials and methods

Cell lines

Breast cancer cell line (MDA-MB-231) and embryonic kidney cells (293T) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained under the recommended culture conditions.

Plasmids, virus production and transduction

Lentiviral plasmid vector pHIV-Luciferase (pHIV-Luc) that contains the firefly Luciferase as a reporter gene was obtained from Addgene (plasmid no. 21375 provided by Bryan Welms, Addgene, Cambridge, MA, USA). Plasmid identity was verified by sequencing with ABI3130xl Genetic Analyzers using vector specific primers. GenElute Endotoxin-free Plasmid Maxiprep kit (Sigma-Aldrich, St. Louis, MO, USA) was used for plasmid purification. Altogether, 7 μg of lentivector was used to produce concentrated lentiviruses in 293T cells according to instructions in Lenti-X Tet-On Advanced Inducible Expression System (Clontech, Mountain View, CA, USA). Concentrated lentivirus was used to transduce 8.0×10^4 of MDA-MB-231 cells (6-well plates) in the presence of 8 μg/ml polybrene and normal culture medium for 24 h. Transduction medium was discarded the next day and cells were passaged five times in a ratio of 1:4 to ensure that they were free of viral particles before performing any experiments. Luciferase expression was confirmed using Luciferase Assay System (Promega, Madison, WI, USA) and luminescence was measured with Luminoskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA).

3D Matrigel assay

Recombinant human BMP4 (rhBMP4) was obtained from R&D Systems (Minneapolis, MN, USA). Cells were cultured on growth factor-reduced Matrigel (Corning, Corning, NY, USA) using the overlay method as described previously [15]. Briefly, 24-well plates were coated with Matrigel. Cells (1.0×10^4 cells/ml) suspended in 2.5% Matrigel solution containing 100 ng/ml BMP4 or vehicle control (4 mM HCl with 0.1% BSA) were added on coated wells. Medium with BMP4 or vehicle control was replenished every two to three days and the cells were allowed to grow up to 14 days.

BMP4 treatment for the in vivo *experiment*

Before intracardiac inoculation, MDA-MB-231/Luc cells were pretreated with rhBMP4 (100 ng/ml) or equivalent volume of vehicle control for seven days and fresh medium was replenished every third day. For the dosage of mice, rhBMP4 was diluted to a concentration of 20 μg/ml PBS with pH ca. 3.8. The vehicle control stock solution was similarly diluted before dosage.

Mice

All experiments were performed by Pharmatest Services Ltd (Turku, Finland) that holds the ethical approval of the National Committee for Animal Experiments. Female athymic nude mice (athymic nude Foxn1nu, Harlan, The Netherlands) were used for this study. BMP4- or vehicle control-treated MDA-MB-231/Luc cells (2×10^5 cells in 0.1 ml of PBS) were inoculated into the left cardiac ventricle of the mice under anesthesia and analgesia at day 0. Mice were given 100 μg/kg rhBMP4 or vehicle control through tail vein injection starting at day 0, three times a week for seven weeks. Animal welfare was monitored daily. The animals were weighed before each dosing and appearances of any clinical signs were recorded. Four mice died or were euthanized due to complications related to the cell inoculation and one mouse due to a dosing-related complication. These animals were excluded from the analyses, thus leaving 10 mice in the BMP4 group and 11 in control group. There was no statistical difference in the weight of the animals between the groups either during or at the end of the study.

Bioluminescence imaging (BLI) and sample collection

Whole body tumor burden and the number of metastases were quantified by imaging the bioluminescence emitted by the MDA-MB-231/Luc cells using IVIS Lumina imaging system (PerkinElmer, Waltham, MA, USA). 100 mg/kg of D-luciferin (Gold Biotechnology, St Louis, MO, USA) was administered intraperitoneally and the animals were anesthetized and imaged within 10–30 minutes after the luciferin administration. Imaging was performed weekly from week 3 until sacrifice at 7 weeks after inoculation.

Gross necropsy was performed on all animals at the end of the study, and all macroscopic signs were recorded. Samples from all tissues with metastases as well as corresponding control tissues with no signs of metastases were harvested, and collected. The tissues were fixed in 10% formalin, bone tissues were decalcified with EDTA, and all were embedded on paraffin (BiositeHisto, Tampere, Finland). 5 μm slides were cut and the tissue sections were deparaffinized and rehydrated for subsequent analyses. Hematoxylin and eosin (H&E) staining was performed using routine procedures.

Immunostainings

Antigen retrieval using citrate buffer was performed. In immunohistochemistry (IHC) with mouse antibodies, M.O.M. kit was used (Vector laboratories, Burlingame, CA, USA). The following primary antibodies were used in IHC: Phospho-Smad1/5/9 (1:200, cat 9511, Cell Signaling Technology, Danvers, MA, USA), Ki67 (1:200, cat KI67-MM1-L-CE-S, Leica Biosystems, Nussloch, Germany) and MECA32 (1:100, cat 550563, BD Biosciences, Franklin Lakes, NJ, USA). The following primary antibodies were used in immunofluorescence (IF): vimentin (1:500, cat 919101, BioLegend, San Diego, CA, USA), keratin 5 (1:500, cat 905501, BioLegend), keratin 14 (1:500, cat 905301, BioLegend) and α-SMA (1:500, cat A2547, Sigma-Aldrich, St. Louis, MO, USA). Antibodies in IHC were diluted in goat or rabbit serum or Normal antibody Diluent (ImmunoLogic, Duiven, the Netherlands) and for IF the dilution was done in 12% BSA. Secondary antibodies for IHC stainings were biotinylated goat anti-rabbit IgG and biotinylated rabbit anti-rat IgG (both at a dilution of 1:100, from Vector laboratories) or Simple Stain MAX PO (MULTI) Universal Immunoperoxidase polymer (Nichirei biosciences, Tokyo, Japan). DAB based detection was used to visualize target proteins. The three secondary antibodies used in IF were goat anti-chicken, anti-rabbit or anti-mouse Alexa Fluor 488 antibody (at a dilution of 1:200, all from Thermo Fisher Scientific). The IHC slides were counterstained with hematoxylin. IF slides were mounted in SlowFade +DAPI (Molecular Probes/Invitrogen) and IHC slides in AquaPolyMount (Polysciences, Inc., Warrington, PA, USA) or dehydrated and mounted in DPX Mountant (VWR, Radnor, PA, USA). Stainings were performed as described [24].

Bone stainings

The tissues were stained with Toluidine blue for bone and cartilage visualization. TRAP (tartrate-resistant phosphatase) staining for osteoclasts was performed by incubation in naphthol AS-BI phosphate solution (cat N-2125, Sigma Aldrich) followed by color reaction in sodium nitrate and pararosaniline dye (cat P-3750, Sigma Aldrich).

Image analysis

IHC and H&E images were taken with an Olympus microscope (Olympus, Tokyo, Japan) connected to Surveyor software (Objective Imaging, Cambridge, UK) and IF images with Zeiss Axio Imager M2 microscope (Carl Zeiss, Oberkochen, Germany) connected to an ApoTome slider module (Carl Zeiss). Quantification of Ki67 data were

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