Bone morphogenetic proteins in breast cancer: dual role in tumourigenesis?

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Abstract

The human bone morphogenetic protein (BMP) family consists of over 20 growth factor proteins that are involved in bone formation and developmental processes. BMPs are extracellular signalling molecules that are able to regulate various cellular functions, proliferation, differentiation, apoptosis and migration. For the last 10 years, these powerful cytokines have increasingly been studied in several cancers, and aberrant expression patterns of BMPs have been reported. Functional studies have suggested that BMPs are involved in both cancer promotion and inhibition. The role these signalling molecules play in breast cancer is only starting to emerge: thus far, studies have been even contradictory. Different BMP ligands have been shown to decrease as well as increase cancer cell growth and migration. Furthermore, they are involved in bone metastases, which are a common feature in breast cancer. In this sense, BMPs resemble a closely related protein transforming growth factor β , which possesses a bidirectional role in cancer cell regulation. In this review, we focus on the current knowledge of BMP expression, functional roles and involvement in bone metastasis in breast cancer.

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Introduction

Bone morphogenetic proteins (BMPs) are extracellular signalling molecules that constitute the largest branch of the transforming growth factor β (TGF β) superfamily (Kawabata *et al.* 1998, Chang *et al.* 2002). By regulating target gene transcription, these growth factors control various cellular processes, such as proliferation, differentiation, apoptosis and migration. BMPs were originally identified based on their ability to form bone at extraskeletal sites (Wozney *et al.* 1988, Reddi 1997, Wozney 2002); currently, they are well known to play critical roles in diverse developmental phases (Hogan 1996a, Zhao 2003). These kinds of powerful developmental pathways are frequently disrupted in cancer (Kelleher *et al.* 2006), and for the last decade, BMPs have been increasingly focused upon in cancer research.

Cancer is in essence a genetic disease that is caused by accumulation of genetic alterations in oncogenes and tumour suppressor genes. Studies of inheritable cancer predisposition syndromes indicate that components of the BMP signalling pathway could act as tumour suppressors. In Juvenile polyposis syndrome,

germline mutations of the *BMPRIA* receptor are detected in 20–25% of cases, and mutations of the cytosolic signalling transducer *SMAD4* are detected in 15–20% of cases (Waite & Eng 2003a). *BMPRIA* mutations are also associated with some cases of Cowden syndrome (Harradine & Akhurst 2006). In addition, *SMAD4* is mutated in half of all sporadic pancreatic cancers and in one-third of sporadic metastatic colon cancers (Massague *et al.* 2000). *SMAD8*, another cytosolic signalling transducer, was shown to be silenced by an epigenetic mechanism in one third of breast cancers (Cheng *et al.* 2004). Thus far, in breast cancer, either heritable or somatic genetic alterations have not been detected in the BMP ligands themselves. In somatic cancers, several *in vitro* and *in vivo* studies have examined BMPs in various cancer types originating from a plethora of different tissues such as breast, prostate, bone, skin, lung, pancreas, colon, intestine, brain and ovaries (Kleeff *et al.* 1999, Yoshikawa *et al.* 2004, Hsu *et al.* 2005, Kim & Kim 2006, Langenfeld *et al.* 2006, Piccirillo *et al.* 2006, Bleuming *et al.* 2007, Deng *et al.* 2007, Theriault *et al.* 2007, Ye *et al.* 2007).

There has been a gradual increase in the understanding of the role that BMPs play in cancer. However, the number of studies is still limited even within breast and prostate cancer, which are the most studied tumour types. Furthermore, results are often contradictory. The same ligand can act differently depending on the cancer type, and it is likely that multiple members in the BMP family should not be examined as simply equals. Even the same BMP ligand within the same cancer type seems to act differently depending on the study. The current consensus is that BMPs are involved in both promotion and inhibition of cancer progression. Such a phenomenon has been reported for the superfamily member TGF β , which inhibits epithelial cell proliferation through a well-characterised cytostatic programme; however, during cancer progression, this growth inhibition is often lost (Derynck *et al.* 2001, Siegel & Massague 2003, Massague & Gomis 2006). Moreover, TGF β induces epithelial–mesenchymal transition (EMT), has pro-angiogenic and immunosuppressive effects, and is evidently also involved in the metastatic process. Whether similar bidirectional action applies for BMPs is still unknown.

The purpose of this review is to elucidate the current knowledge of BMPs in breast cancer pathogenesis.

Bone morphogenetic proteins

Structure

Members of the TGF β superfamily (BMPs, TGF β s, activins, inhibins, NODAL and anti-Müllerian hormone) share a similar structure and a signalling pathway (Schmierer & Hill 2007). BMPs, some of which are called growth and differentiation factors (GDF), are dimeric molecules composed of two identical monomers linked with a disulfide bond (Kingsley 1994, Reddi 1998, Ducky & Karsenty 2000, Sebald *et al.* 2004). The core monomer contains a common structure called a ‘cysteine knot’ with seven highly conserved cysteines (Griffith *et al.* 1996). Currently, there are 21 known members in the human BMP family (BMP2–BMP7, BMP8A/B, BMP10, BMP15, GDF1–3, GDF5–7, myostatin, GDF9–11 and GDF15; Schmierer & Hill 2007). These growth factors can be further divided into subgroups according to their amino acid sequences (Kawabata *et al.* 1998, Newfeld *et al.* 1999, Botchkarev 2003, Ye *et al.* 2007).

BMPs are translated as a large pre-proprotein containing a signal peptide sequence, a pro-domain and a mature growth factor domain (Kingsley 1994, Sebald *et al.* 2004). Prodomains are required in the

dimerisation process and are cleaved at the consensus site RXXR (Ducky & Karsenty 2000). Mature dimeric BMP is secreted out of the cell, but the prodomain may remain attached non-covalently; thus, the prodomain is thought to regulate BMP activity and availability (Constam & Robertson 1999, Gregory *et al.* 2005, Sopory *et al.* 2006). In contrast to the well-known regulation of TGF β isoforms by their prodomains (Keski-Oja *et al.* 2004), functions of different BMP prodomains are not yet thoroughly studied. Homodimers seem to be the major form of BMPs, but they exist also as heterodimers that can be even more biologically active than homodimers (Aono *et al.* 1995, Israel *et al.* 1996, Zhu *et al.* 2006).

Function

BMPs are unique in their ability to initiate bone formation (Wozney & Rosen 1998). They stimulate the differentiation of mesenchymal cells into chondroblasts and osteoblasts and the subsequent new bone construction during embryogenesis as well as during bone repair processes in adult tissues (Reddi 1997, Wozney & Rosen 1998, Wozney 2002). BMP subfamily members differ greatly in their osteogenic activities, and not all ligands are osteoinductive by nature (Luu *et al.* 2007). BMP2 and BMP7 have been studied the most in clinical applications and are currently used as treatment options in orthopaedics (Luo *et al.* 2005). Perhaps even more important than osteoinduction is the role BMPs play in different developmental phases. They regulate the primal stages of embryogenesis, formation of left–right asymmetry, neural and skeletal patterning, limb formation, and organogenesis (Hogan 1996a, Zhao 2003). Certain BMPs are vital for development because it has been previously shown that Bmp2 and Bmp4 null mutant mice die during embryogenesis, and Bmp7 null mutant mice shortly after their birth (Hogan 1996b). Not much is known about the function of BMPs during breast development. Because BMP-specific receptors are expressed in the developing mammary gland, this suggests that there is also active BMP signalling (Wakefield *et al.* 2001). Thus far, only Bmp2 and Bmp4 have been proposed to regulate the development of mouse mammary glands (Phippard *et al.* 1996, Cho *et al.* 2006).

Signalling pathway

In addition to their common structure, BMPs and other members of the TGF β family share a common signalling pathway. There are several excellent reviews that illustrate well the rough backbone of the

TGF β superfamily signalling pathway (Heldin *et al.* 1997, Kawabata *et al.* 1998, Shi & Massague 2003, ten Dijke & Hill 2004, Miyazono *et al.* 2005, Massague & Gomis 2006, Schmierer & Hill 2007). Briefly, the ligand binds to two separate transmembrane serine–threonine kinase receptors, type I and type II, forming a heterotetrameric complex. In the complex, type II receptor phosphorylates and activates the type I receptor that in turn is able to phosphorylate and activate cytosolic SMAD proteins. Receptor-regulated SMADs (R-SMADs) form complexes with common SMAD (Co-SMAD), and these active SMAD complexes regulate transcription of target genes in the nucleus. A schematic illustration of BMP signalling is presented in Fig. 1. Because there are numerous ligands in the superfamily and only limited number of receptors and SMADs, extensive signalling regulation is necessary to achieve a specific response (Feng & Derynck 2005). The BMP-specific features of the signalling pathway and its regulation are discussed below.

BMP ligands can bind three different type I and three different type II receptors (Kawabata *et al.* 1998, ten Dijke *et al.* 2003, Nohe *et al.* 2004, Miyazono *et al.* 2005). Type I receptors include BMP receptor type IA (BMPRIA or ALK-3), BMP receptor type IB (BMPRIB or ALK-6) and activin A receptor type I

(ACVR1 or ALK-2). Type II receptors include BMP receptor type II (BMPRII), activin A receptor type IIA (ACVR2A or ActR-II) and activin A receptor type IIB (ACVR2B or ActR-IIB). Both receptor types consist of an N-terminal extracellular ligand-binding domain, a single transmembrane domain and a C-terminal intracellular kinase domain (de Caestecker 2004). In addition, towards the N-terminus from the kinase domain, type I receptors contain a conserved GS domain (a sequence rich of glycine and serine residues) that is needed for phosphorylation (Shi & Massague 2003, de Caestecker 2004). Both receptors are required for signalling, but the ligand specificity is mainly dictated by the type I receptor because it binds to the ligand with higher affinity than the type II receptor (Rosenzweig *et al.* 1995, ten Dijke *et al.* 2003). During ligand binding, a stable receptor complex is formed, which contains a receptor dimer of each type (Feng & Derynck 2005). Constitutively, active type II receptors phosphorylate the GS domain of type I receptors, which in turn phosphorylate and thus activate downstream SMAD proteins (Feng & Derynck 2005).

Different BMPs bind to the three type I receptors with different affinities (de Caestecker 2004). BMP2 tends to bind BMPRIIA more than BMPRIIB, BMP4 binds both with similar affinity, whereas BMP7 prefers ACVR1 and BMPRIIB (Kawabata *et al.* 1998, Macias-Silva *et al.* 1998, ten Dijke *et al.* 2003, Sebald *et al.* 2004). More variation in receptor activation is provided in the complex formation. For example, BMP receptors BMPRIIA, BMPRIIB and BMPRII can form oligomeric complexes with each other in any combination (Gilboa *et al.* 2000, Sebald *et al.* 2004). Furthermore, depending on the ligand, the receptor complex is formed in two alternative ways. BMP7 and BMP6 interact first with the type II receptor and then recruit type I receptors, whereas BMP2 and BMP4 bind first to the type I receptor and then recruit type II receptors (de Caestecker 2004). Finally, there is evidence that receptor complex formation can even affect the downstream signalling alternatives. If BMP binds to a preformed receptor complex, the Smad pathway is activated; in contrast, if complex formation occurs after BMP binding, the p38 mitogen-activated protein kinase (MAPK) pathway is activated (Nohe *et al.* 2002, 2004).

The major pathway for BMP signals is composed of intracellular SMAD proteins. BMP-specific receptors activate three R-SMADs (SMAD1, SMAD5 and SMAD8; Attisano & Wrana 2000, Itoh *et al.* 2000, Derynck & Zhang 2003). There is only one common SMAD (SMAD4), which is shared by all superfamily ligands (Derynck & Zhang 2003). R-SMADs and

Figure 1 Schematic presentation of BMP signalling pathway.

SMAD4 are closely related; their structures are composed of two conserved, globular domains (Mad homology domain, MH) that are joined together by a non-conserved linker domain (Massague *et al.* 2005). Upon activation, type I receptor phosphorylates a SXS motif of R-SMADs; thus, activated R-SMADs can interact with SMAD4, thereby forming a heteromeric complex that is translocated into the nucleus (ten Dijke *et al.* 2003, Feng & Derynck 2005). It has been reported that BMP4 and GDF5 activate all three R-SMADs, whereas BMP6 and BMP7 activate only SMAD1 and SMAD5 (Aoki *et al.* 2001).

The SMAD complex interacts with specific DNA sequences called Smad-binding element or BMP response elements in the promoters or enhancers of target genes (Feng & Derynck 2005). In fact, SMADs themselves have quite low affinity for DNA, and to obtain high affinity and target gene selectivity, numerous other factors co-operate with SMADs (ten Dijke *et al.* 2002, Feng & Derynck 2005, Massague *et al.* 2005). Transcription factors that bind to adjacent promoters can either function in transcriptional activation or repression. For example, SMAD co-operating transcription factors include the Runx family of transcription factors, Menin, Hoxc-8, zinc finger proteins OAZ and YY1, and oestrogen receptor (ER; Hata *et al.* 2000, Zwijsen *et al.* 2003, Feng & Derynck 2005, Miyazono *et al.* 2005). In addition, several nuclear co-activators (such as p300/CBP and GCN5) and co-repressors (such as c-Ski, Sno, Tob, SIP1 and Evi-1) amplify and specify the signal response (von Bubnoff & Cho 2001, Zwijsen *et al.* 2003, Feng & Derynck 2005, Miyazono *et al.* 2005). The expression pattern and binding site preferences of these co-factors as well as their function in integration of cues from other signalling pathways all confer the specificity of target gene transcription (Schmierer & Hill 2007).

In addition to the SMAD pathway, other intracellular pathways are activated in response to BMP ligands. For example, BMPs induce MAPK, but the exact mechanism of activation is not clear (Derynck & Zhang 2003, Nohe *et al.* 2004, Javelaud & Mauviel 2005). Studies in *Xenopus laevis* have indicated that BMP receptor activation leads to MAPK activation via X-linked inhibitor of apoptosis, TGF β -activated kinase and Tak-binding protein (Shibuya *et al.* 1998, Yamaguchi *et al.* 1999, Herpin & Cunningham 2007). BMP7 has been shown to induce p38 MAPK through integrin-linked kinases (Piscione *et al.* 2001, Hu *et al.* 2004, Leung-Hagesteijn *et al.* 2005). Furthermore, the ligands BMP2 and BMP4 can activate p38 and extracellular signal-related kinase (ERK) MAPK, but not JNK (Kimura *et al.* 2000, Nohe *et al.* 2002, 2004,

Jin *et al.* 2006, Otani *et al.* 2007, Yang *et al.* 2007b). MAPK can also modulate SMAD activation. Phosphorylation of the SMAD1 linker region in response to MAPK signalling can lead to attenuation of the BMP signal (Kretschmar *et al.* 1997, Derynck & Zhang 2003, Massague 2003, Sapkota *et al.* 2007). Besides MAPK, it has been reported that BMP2 can activate the protein kinase C and phosphatidylinositol 3-kinase pathways (Hay *et al.* 2001, Ghosh-Choudhury *et al.* 2002). BMPs are also known to crosstalk with other major signalling pathways, such as Wnt (Attisano & Labbe 2004), JAK/STAT (von Bubnoff & Cho 2001, Nohe *et al.* 2004, Miyazono *et al.* 2005) and Notch (Miyazono *et al.* 2005, Herpin & Cunningham 2007).

At first glance, BMP signalling seems to function in a rather linear fashion. However, the considerable variation in the use of receptors, abundance of transcriptional co-regulators and links to other major pathways create more of a signalling network than a straight line.

Regulation of the signalling pathway

The BMP signalling pathway is heavily regulated at multiple levels. As discussed previously, diversity is achieved by heteromeric ligands, receptors, SMADs and their co-regulators, as well as other pathways. Furthermore, BMP signalling is adjusted by extracellular antagonists, accessory receptors on the membrane and intracellular control of SMAD activity.

Extracellularly, BMP ligands are inhibited by secreted peptides, called BMP antagonists (Gazzerro & Canalis 2006). Antagonists can either bind the BMP ligand or BMP receptor to prevent ligand–receptor interaction (Balemans & Van Hul 2002, Groppe *et al.* 2002, Canalis *et al.* 2003). Several different antagonists regulate different BMP activities, which include follistatin, noggin, the chordin family members, twisted gastrulation and DAN family members (such as gremlin, cerberus, dan and sclerostin; Reddi 2001, Balemans & Van Hul 2002, Ebara & Nakayama 2002, Canalis *et al.* 2003, Gazzerro & Canalis 2006). Appropriate doses of extracellular BMP are ensured by feedback loops, because many of the antagonists themselves are target genes of BMPs (Miyazono 2000, Gazzerro & Canalis 2006). Another level of regulation is provided by membrane-bound proteins. The antagonist-like function of the transmembrane protein CRIM1 prevents the actions of BMP7 and BMP4 already in the Golgi compartment by affecting ligand processing (Wilkinson *et al.* 2003). The pseudoreceptor BAMBI in turn interferes with the receptor complex, thus inhibiting BMP signalling

(Onichtchouk *et al.* 1999, Canalis *et al.* 2003). There are also few membrane-bound peptides that can enhance BMP signalling, which include membrane-anchored proteins DRAGON and RGMaA (repulsive guidance molecule) (Samad *et al.* 2005, Gazzoer & Canalis 2006) and accessory receptors endoglin (Barbara *et al.* 1999, Schermer *et al.* 2007) and betaglycan (Kirkbride *et al.* 2008).

Intracellularly, BMP signalling is restrained by inhibitory SMADs (I-SMADs) and SMURF ubiquitin ligases (ten Dijke *et al.* 2002, Canalis *et al.* 2003, Massague *et al.* 2005). Two known I-SMADs, SMAD6 and SMAD7, are structurally similar to the R-SMADs. I-SMAD competes with R-SMAD in the interaction of activated type I receptors and with SMAD4 for the complex formation with SMAD1 (Massague *et al.* 2005). As with extracellular antagonists, I-SMADs are also BMP target genes, thus creating a feedback mechanism for BMP signals. SMURF1 and SMURF2 in turn target R-SMADs to degradation via the proteasome machinery resulting in BMP signalling inhibition (von Bubnoff & Cho 2001, ten Dijke *et al.* 2002, Massague *et al.* 2005). In addition, SMURF1 directs BMP-specific receptors for degradation through interaction with I-SMADs (Murakami *et al.* 2003).

Some of the BMP target genes involved in bone induction and development are already known, and they include transcription factors (inhibitor of differentiation, Id), Runx2, MFH-1, Vent2, Msx2 and Dlx5, as well as antagonists and I-SMADs, which have already been mentioned (Canalis *et al.* 2003). Recent microarray-based studies have shown that expression levels of hundreds of genes are altered during osteoblastic differentiation (Balint *et al.* 2003, Korchynskiy *et al.* 2003, Peng *et al.* 2003, Gu *et al.* 2004, de Jong *et al.* 2004). Taking into account that BMPs function in diverse tissues at various phases of development, numerous different target genes can be expected. Only a part of these target genes are currently known, and several are still waiting to be discovered.

In normal tissues, there are several options to ensure proper spatiotemporal BMP signal that results in the desired phenotypic response. At the same time, this network of BMP signalling is highly sensitive to cancer-specific disturbances. Next, the role of BMP family members in breast cancer is discussed.

Bone morphogenetic proteins in breast cancer

Aberrant expression of the different BMP ligands has been detected in breast cancer (Table 1). We will first discuss the expression and possible functional

significance of the different BMP ligands, for which BMP2, BMP6 and BMP7 are currently known best in breast cancer. There are only a few studies that have investigated the connection between BMP signalling and patient outcome, and most of the current knowledge of BMPs in breast cancer is based on *in vitro* and *in vivo* studies. In the last chapter, the connection between BMP signalling and the process of bone metastasis in breast cancer will be assessed.

Expression and function

BMP2

BMP2 expression has been demonstrated in breast cancer, most often at low or similar levels as compared to normal cells. Transcripts of *BMP2* have been reported in a few breast cancer cell lines (Arnold *et al.* 1999, Clement *et al.* 2000, Schwaninger *et al.* 2007); however, based on a panel of 22 breast cancer cell lines, *BMP2* is expressed mainly at low levels in less than half of the cases (Alarmon *et al.* 2007). Similarly, significantly lower levels of *BMP2* were observed in both non-invasive and invasive breast tumours as well as in liver metastatic tumour tissues than in normal mammary gland samples, demonstrating that *BMP2* expression is downregulated in breast cancer (Reinholz *et al.* 2002). However, Clement *et al.* (2000) detected *BMP2* expression in breast tumour specimens at similar levels than in the normal appearing cells in the tumour-free resection margin in the majority of patient samples. Here, it must be noted that such normal appearing adjacent tissues might contain cancer-specific alterations; and thus, they do not necessarily reflect the expression status of a truly normal tissue. Likewise, *BMP2* expression was demonstrated in the majority of 39 primary tumour samples at very low and similar levels compared to normal mammary gland cDNA (Alarmon *et al.* 2007). Examination of *BMP2* protein expression in breast cancer patient samples did not reveal any association with survival or clinicopathological parameters (Raida *et al.* 2005a). However, the antibody used in this study recognises both *BMP2* and *BMP4*; and therefore, the individual ligand expression profiles could not be discerned.

Functionally, *BMP2* is one of the most frequently studied ligands in breast cancer. *BMP2* inhibits breast cancer cell proliferation (Ghosh-Choudhury *et al.* 2000a,b, Pouliot & Labrie 2002). In these three functional studies, *BMP2* was demonstrated to induce the level of p21 (a cyclin-dependent kinase inhibitor) and to cause the hypophosphorylation of the

Table 1 Expression of bone morphogenetic protein (BMP) family members in breast cancer cell lines, primary tumours and recurrent tumours

Ligand	References	RNA/protein	Cell line		Primary tumour		Recurrence		Expression in tumour samples
			F	n	F	n	F	n	
BMP2	Arnold <i>et al.</i> (1999)	RNA	100%	2					Similar expression compared to tumour-free resection margin Primaries and recurrences downregulated compared to 18 normal samples
	Clement <i>et al.</i> (2000)	RNA	75%	8	81%	36			
	Reinholz <i>et al.</i> (2002)	RNA			100%	29	100%	5 ^a	
BMP3	Alarmo <i>et al.</i> (2007)	RNA	41%	22	85%	39			
	Schwaninger <i>et al.</i> (2007)	RNA	33%	3					
	Arnold <i>et al.</i> (1999)	RNA	100%	2					
BMP4	Alarmo <i>et al.</i> (2007)	RNA	36%	22	41%	39			
	Schwaninger <i>et al.</i> (2007)	RNA	0%	3					
	Arnold <i>et al.</i> (1999)	RNA	0%	2					
BMP5	Alarmo <i>et al.</i> (2007)	RNA	95%	22	100%	39			
	Schwaninger <i>et al.</i> (2007)	RNA	33%	3					
	Arnold <i>et al.</i> (1999)	RNA	100%	2					
BMP6	Alarmo <i>et al.</i> (2007)	RNA	45%	22	56%	39			
	Autzen <i>et al.</i> (1998)	RNA					60%	5 ^a	
	Arnold <i>et al.</i> (1999)	RNA	100%	2					
	Clement <i>et al.</i> (1999)	RNA	100%	5	100%	44			Variable expression compared to tumour-free resection margin
	Alarmo <i>et al.</i> (2007)	RNA	50%	22	97%	39			
	Schwaninger <i>et al.</i> (2007)	RNA	0%	3					
BMP7	Zhang <i>et al.</i> (2007)	RNA	100%	3					
	Arnold <i>et al.</i> (1999)	RNA	0%	2					
	Schwalbe <i>et al.</i> (2003)	Protein	100%	3	100%	170			
	Alarmo <i>et al.</i> (2006)	Protein	100%	11	100%	91			Upregulated compared to ten normal samples
	Alarmo <i>et al.</i> (2007)	RNA	82%	22	100%	39			
	Buijs <i>et al.</i> (2007a,b)	RNA			100%	67			
BMP8	Alarmo <i>et al.</i> (2008)	Protein			47%	409	13%	38 ^b	
	Alarmo <i>et al.</i> (2007)	RNA	95%	22	77%	39			
	GDF9a	RNA	71%	7	100%	109			
BMP15	Hanavadi <i>et al.</i> (2007)	RNA			100%	109			Upregulated compared to non-neoplastic back-ground tissue (n=33) Upregulated compared to non-neoplastic back-ground tissue (n=33)

F, frequency.
^aDistant recurrence.
^bLocal recurrence.

retinoblastoma protein, resulting in G₁ arrest of breast cancer cells. This growth arrest required both cytoplasmic signal transducers SMAD1 and SMAD4 (Pouliot & Labrie 2002). Another study showed that the PTEN (a tumour suppressor) levels were increased in breast cancer cells after BMP2 treatment (Waite & Eng 2003b). BMP2 can be influenced by other factors that affect breast cancer cell growth. Vitamin D is

known to inhibit growth of breast cancer cells (Welsh 2007). BMP2 is upregulated in response to vitamin D analogue treatment, which resulted in growth reduction of breast cancer cells (Lee *et al.* 2006a,b). Oestrogen is a well-known factor that regulates breast cancer cell proliferation, and it was shown to suppress BMP2 activity in one breast cancer cell line (Yamamoto *et al.* 2002).

BMP2 reduces cancer cell growth, but it also has a beneficial impact on cancer cells. Breast cancer cell resistance to apoptosis is increased by BMP2, and based on microarray studies, BMP2 influences apoptosis-related genes (Raida *et al.* 2005a, Steinert *et al.* 2008). Furthermore, migration and invasion of breast cancer cells are enhanced by BMP2 (Clement *et al.* 2005). BMP2 also increases the endothelial cell tube formation *in vitro*, and BMP2 overexpressing MCF-7 breast cancer cells form tumours with pronounced vascularisation in a mouse xenograft model (Clement *et al.* 2005, Raida *et al.* 2005b). BMP2-induced endothelial cell activation was accompanied by increased p38 phosphorylation (Raida *et al.* 2005b). BMP2 can also influence tumour propagation in a more complex manner. BMP2 was shown to induce expression of tenascin-W, an extracellular matrix regulator that is found in the tumour stroma (Scherberich *et al.* 2005). In another study, BMP2 did not affect growth of tumours, but was able to initiate microcalcification in a rat breast tumour model (Liu *et al.* 2008). Although the functional significance of microcalcification is not yet clear, it is an important diagnostic marker because it is visible on mammograms of 30–50% of breast cancer patients.

To summarise, BMP2 expression is either down-regulated or unaltered in breast cancer cells. Because BMP2 reduced cancer cell proliferation, it is reasonable to expect that it is downregulated in breast cancer. However, studies indicate that in certain contexts, BMP2 can also promote oncogenic behaviour by affecting apoptosis, migration, invasion and angiogenesis.

BMP6

BMP6 mRNA has also been detected in a few breast cancer cell lines (Arnold *et al.* 1999, Clement *et al.* 1999, Schwaninger *et al.* 2007, Yang *et al.* 2007a). A wide range of BMP6 expression levels have been reported in primary tumour samples with a minority of samples having elevated levels compared to normal appearing cells in the tumour resection margin (Clement *et al.* 1999). In a set of 22 breast cancer cell lines and 39 primary tumours, BMP6 was expressed mainly at rather low levels (Alarmo *et al.* 2007). BMP6 expression is associated with E-cadherin expression, which links it to non-invasive breast cancer phenotypes (Yang *et al.* 2007a). Interestingly, BMP6 transcripts have been detected in breast cancer skeletal metastases (Autzen *et al.* 1998). An epigenetic mechanism might be involved in the regulation of expression because BMP6 hypermethylation and

inactivation were detected in breast cancer patient samples (Zhang *et al.* 2007). Hypermethylation was seen only in ER-negative breast tumours, but not in ER-positive tumours. Oestrogen has been shown to induce as well as inhibit BMP6 expression in breast cancer cells (Ong *et al.* 2004, Zhang *et al.* 2005, Takahashi *et al.* 2008). Vitamin D and epidermal growth factor (EGF) have also been shown to stimulate BMP6 expression in a few breast cancer cell lines (Clement *et al.* 1999, Lee *et al.* 2006a,b).

BMP6 function in breast cancer has been studied mainly in a few breast cancer cell lines where it inhibits growth and migration of cancer cells. Combined treatment with BMP6 and oestrogen inhibits growth of MCF-7 breast cancer cells and reduces p38 MAPK activity; however, BMP6 alone did not affect growth of these cells (Takahashi *et al.* 2008). BMP6 attenuates MDA-MB-231 breast cancer cell migration by regulating expression of the transcriptional repressor zinc finger E-box binding homeobox 1 (ZEB1), also known as δ EF1; Yang *et al.* 2009). BMP6 inhibits ZEB1 expression by repressing the promoter as well as by increasing miR-192 expression, which subsequently downregulates ZEB1 (Yang *et al.* 2009). Reduced ZEB1 expression in turn results in decreased miR-21 expression, an miRNA implicated in invasion and metastasis (Du *et al.* 2009). BMP6 treatment also reduced MDA-MB-231 cell proliferation, but still protected these cells from apoptosis, demonstrating a dual function for BMP6 in breast cancer (Du *et al.* 2007).

Taken together, BMP6 expression in breast cancer does not dramatically differ from the patterns seen in normal cells, but interestingly, BMP6 inactivation by hypermethylation has been detected in ER-negative breast cancer patients. Functional studies have mostly revealed the cancer-inhibiting properties of BMP6. These mechanisms are often rather complex in nature, and they also involve miRNAs.

BMP7

BMP7 transcripts have been detected with highly variable expression levels in tumour samples (Alarmo *et al.* 2006, Buijs *et al.* 2007a). The expression of BMP family members has rarely been studied at the protein level in breast cancer, and BMP7 is the only ligand that has been reasonably well studied in patient samples. BMP7 protein expression was first reported in 3 breast cancer cell lines, 170 primary breast tumours and 1 normal mammary gland sample (Schwalbe *et al.* 2003). In this set of primary tumours, BMP7 protein expression levels were highly variable but were

associated with hormone receptor status. Similarly, frequent BMP7 expression was detected in 11 breast cancer cell lines and in primary breast tumour samples ($n=91$; Alarmo *et al.* 2006). The majority of the patient samples demonstrated BMP7 overexpression compared to ten normal mammary gland samples (Alarmo *et al.* 2006). We also found that *BMP7* is not an amplification target gene even though it resides in the chromosomal area 20q13, which is frequently amplified in breast cancer (Kallioniemi *et al.* 1994, Hodgson *et al.* 2003). Amplification is a common mechanism of oncogene activation in solid tumours (Albertson *et al.* 2003, Albertson 2006). A follow-up study using a substantially larger set ($n=409$) of patient samples also identified BMP7 overexpression in half of the samples (Alarmo *et al.* 2008).

We have shown that BMP7 protein expression in primary tumours is associated with accelerated bone metastasis formation, and BMP7 expression was further shown to be an independent prognostic factor for early bone metastasis (Alarmo *et al.* 2008). Interestingly, BMP7 expression is often lost in the corresponding local recurrent tumour (Alarmo *et al.* 2008). In another patient series, the median *BMP7* transcript level in primary tumours was higher in patients with visceral rather than bone metastases, but not significantly different in patients with or without metastasis (Buijs *et al.* 2007a).

The functional significance of BMP7 has been studied in breast cancer cell line models where different phenotypes were observed after BMP7 manipulation by siRNA-based silencing or BMP7 treatment (Alarmo *et al.* 2009). BMP7 was shown to stimulate growth of two breast cancer cell lines and inhibit growth of four cell lines. In addition, exogenous BMP7 significantly increased the migration and invasion of MDA-MB-231 cells *in vitro* (Alarmo *et al.* 2009). On the contrary, in a MDA-MB-231 xenograft mouse model, exogenous BMP7 was shown to decrease tumour growth (Buijs *et al.* 2007a). Buijs *et al.* (2007a) also showed that endogenous and exogenous BMP7 treatment results in diminished formation and growth of bone metastases.

Different phenotypic responses to BMP7 could be due to other factors that have an impact on BMP7 expression or activity. BMP7 was identified as a novel target gene for Lim only protein 4, which is frequently overexpressed in breast cancer (especially in ER-negative tumours) and is associated with poor outcome (Wang *et al.* 2007). A recent study also revealed that BMP7 is a direct target gene for the p53 family of proteins (Yan & Chen 2007). This study showed that in a p53 mutant breast cancer cell line,

BMP7 reduction leads to decreased growth, whereas in a p53 wild-type breast cancer cell line, no growth reduction was detected. Hence, BMP7 could function to maintain cell survival in p53-deficient breast cancer cells (Yan & Chen 2007). *BMP7* expression has been shown to diminish in response to oestrogen (Kusumegi *et al.* 2004, Takahashi *et al.* 2008). Similarly to BMP6, BMP7 and oestrogen together inhibit MCF-7 proliferation, but BMP7 alone does not alter cell growth (Takahashi *et al.* 2008).

To conclude, BMP7 is widely expressed in breast cancer and is associated with early bone metastasis. Several factors seem to influence the expression or activity of BMP7. This might explain that phenotypes supporting both cancer progression and regression have been described after manipulation of BMP7 signalling.

Other BMP ligands

The role of the other BMP family ligands in breast cancer has been studied less intensively. The expression levels of GDF9a and BMP15 are down-regulated in breast tumours compared to normal samples, and diminished transcript expression of these genes is associated with poor prognosis (Hanavadi *et al.* 2007). Transcripts of *BMP3*, *BMP4*, *BMP5* and *BMP8* have been reported only in a few breast cancer cell lines (Arnold *et al.* 1999, Clement *et al.* 2000, Schwaninger *et al.* 2007). An expression survey using 22 breast cancer cell lines and 39 primary breast tumours revealed that among these ligands, *BMP4* had a very prominent profile, and it was overexpressed in the majority of cell lines and tumour samples (Alarmo *et al.* 2007, Ketolainen *et al.* 2010). *BMP3* and *BMP5* were detected, but at more modest expression frequencies and levels, and *BMP8*, although it was frequently detected, clearly at lower levels (Alarmo *et al.* 2007).

There are only a few functional studies that have concentrated on ligands other than BMP2, BMP6 or BMP7. BMP4 has been suggested to promote invasive behaviour of mammary epithelial cells, and thus also cancerous cells because it disturbs the formation of the lumen of mammary epithelial cells (Montesano 2007). In another study, it reduced migration and invasion of breast cancer cells *in vitro* and suppressed matrix metalloproteinase (MMP-9) expression, thereby inhibiting invasive behaviour (Shon *et al.* 2009). BMP4 expression was shown to be dependent on *N-myc* downstream-regulated gene 2 (NDRG2) overexpression (Shon *et al.* 2009). The same dependency on other factors was detected when BMP4 stimulated immortalised murine mammary epithelial cell proliferation in

combination with different other cytokines (fibroblast growth factor (FGF)-2, FGF-7, FGF-10, EGF and hepacyte growth factor (HGF)) but not alone (Montesano *et al.* 2008). In a recent work done by Ketolainen *et al.* (2010), BMP4 treatment decreased growth of nine breast cancer cell lines studied by inducing a G₁ arrest. Interestingly, at the same time, BMP4 increases migration and invasion of a subset of these cell lines, demonstrating a truly dualistic function for BMP4 in breast cancer cells (Ketolainen *et al.* 2010).

BMP receptors and BMP signalling components

The expression profiles of BMP-specific receptors have been rather infrequently studied. We showed that type I (BMPRI1A, BMPRI1B and ACVR1) and type II (BMPRI2, ACVR2A and ACVR2B) BMP-specific receptor transcripts were detected in a comparably more uniform manner than the ligands in all breast cancer cell lines and primary tumour samples studied (Alarmo *et al.* 2007). Helms *et al.* (2005) demonstrated that BMPRI1B is overexpressed in breast cancer, and that it is associated with poor prognosis of breast cancer patients. BMPRI1B expression in ER-positive breast cancer specimens correlates with high tumour grade, high tumour proliferation index and cytogenetic instability (Helms *et al.* 2005). In addition, BMPRI1B expression is accompanied by SMAD1/5/8 activation as well as anti-apoptotic activity, linking active BMP signalling to tumour progression (Helms *et al.* 2005). Similarly, phosphorylated SMAD1/5/8 protein was detected in primary breast tumours, lymph node and bone metastases (Katsuno *et al.* 2008). In their study, expression of a dominant negative BMPRI1A in a mouse xenograft model resulted in decreased invasiveness and bone metastasis, as well as prolonged survival of the mice (Katsuno *et al.* 2008). Blocking active BMP signalling *in vitro* using a dominant negative form of BMPRI2 receptor instead of BMPRI1A was shown to lead to growth reduction of breast cancer cells (Pouliot *et al.* 2003). Thus, depending on the receptor, BMP signalling is able to modulate cell growth or invasion.

As discussed above, several factors can modulate the activity of both BMP ligands and other pathway components. Oestrogen was shown to reduce the mRNA levels of BMP-specific receptors (Takahashi *et al.* 2008). In addition to upregulation of BMP2 and BMP6, vitamin D reduced SMAD6 expression and resulted in SMAD1/5 phosphorylation, implicating that vitamin D can mediate growth inhibitory effects through active BMP signalling in breast cancer (Lee *et al.* 2006a,b). Variable BMP functions could

also be a result of alternative pathway activation, which has been detected at least with the ligands BMP2, BMP4 and BMP6. MAPK activation was seen in BMP4-induced disruption of the mammary epithelial lumen as well as BMP2-induced endothelial cell activation in breast cancer (Raida *et al.* 2005b, Montesano 2007). Both SMAD and p38 MAPK pathways were shown to be activated in breast cancer cells in response to BMP6-induced anti-apoptotic effect (Du *et al.* 2007). Interpretation of BMP function in breast cancer can be further complicated by the involvement of stromal cells in mammary tumourigenesis. Sneddon *et al.* (2006) detected elevated levels of GREMLIN 1 in breast tumour stroma, and suggested a model where BMP antagonists produced by the tumour stroma maintain tumour cell expansion, analogous to the stem cell expansion in the normal tissues.

Compared to the amount of studies concentrating on BMP ligands, there are only few studies that have focused on BMP-specific receptors or other BMP signalling pathway components in breast cancer. Whereas BMP ligands produce alternating phenotypes in breast cancer cells, thus far, BMP-specific receptors seem more likely to stimulate cancer progression.

BMPs and bone metastasis

A natural focus for BMP research in cancer is their possible involvement in the process of bone metastases. Cancers originating in the epithelia of breast and prostate tissues are particularly known to frequently metastasise to the bone. Studies in prostate cancer suggest that BMP signalling plays an active role in this process (Keller *et al.* 2001, Vessella & Corey 2006, Ye *et al.* 2007). In breast cancer, fewer studies have examined the contribution of BMPs to bone metastases.

BMP-regulated factors have been suggested to be involved in bone metastasis. Breast cancer cell line-derived BMPs were shown to upregulate bone sialoprotein (BSP) expression in preosteoblast cells (Bunyaratavej *et al.* 2000). BSP is involved in new bone formation and could therefore provide a link between the metastatic process to bone and breast cancer. A known BMP target gene and a co-factor for BMP signalling, RUNX2, might be another such link. Intact RUNX2 is required for the formation of breast cancer osteolytic metastases in bones (Barnes *et al.* 2004, Javed *et al.* 2005). Schwaninger *et al.* (2007) showed that forced expression of the BMP antagonist noggin in osteoinductive (osteoblastic bone metastases forming) prostate cancer cells *in vivo* diminishes the

osteoblastic response in bone (i.e. no excess bone formation and reduced number of osteoclasts). However, noggin overexpression does not affect the tumour growth in this intraosseous xenograft model. Because noggin was not expressed in osteoinductive prostate or breast cancer cell lines, this implies that even though active BMP signalling does not affect tumour growth, it might contribute to the formation of osteoblastic bone metastases by changing the bone architecture (Schwaninger *et al.* 2007).

Recent work of Buijs *et al.* (2007a) has shown that overexpression of BMP7 as well as exogenous BMP7 treatment significantly reduces the formation of bone metastases and growth in a mouse xenograft model of breast cancer. They detected BMP7 expression in breast cancer cell lines with a more epithelial than invasive phenotype (high E-cadherin expression and low vimentin expression; Buijs *et al.* 2007a). BMP7 has been shown to reverse TGF β -induced EMT by decreasing vimentin expression and increasing E-cadherin expression in breast cancer cells and in normal mouse mammary epithelial cells (Zeisberg *et al.* 2003, Valcourt *et al.* 2005, Buijs *et al.* 2007a). Kowanzetz *et al.* (2004) showed that increased E-cadherin expression occurs through BMP7-induced upregulation of Id2 and Id3. Interestingly, when Id2–3 is knocked out, BMP7 actually induces the smooth muscle actin (SMA), a mesenchymal phenotype marker) expression and stimulates EMT in mouse mammary epithelial cells (Kowanzetz *et al.* 2004, Valcourt *et al.* 2005). These studies illustrate well the complex network of proteins that are involved in BMP-regulated cellular processes.

In contrast to the results of Buijs *et al.* (2007a), a recent study using a similar mouse xenograft model of breast cancer has showed that active BMP signalling actually increases invasion and bone metastases (Katsuno *et al.* 2008). They detected activated SMAD1/5/8 in primary and metastatic tumours, and utilised functional bioluminescence imaging to demonstrate that BMP signalling results in transcriptional activity in bone metastases *in vivo*. Inhibition of BMP signalling through the dominant negative BMPRI1A reduces interleukin-11 expression and invasiveness *in vitro* and bone metastasis *in vivo*, and it resulted in prolonged survival of the mice (Katsuno *et al.* 2008). As discussed earlier, BMP ligands bind their receptors with different affinities (Sebald *et al.* 2004). For example, BMPRI1A is preferred by BMP2 and BMP4 instead of BMP7; thus, the discrepancy in the work done by Buijs *et al.* might be partly explained by the receptor. However, we observed that BMP7

expression is associated with accelerated bone metastasis formation and acted as an independent prognostic factor for early bone metastases (Alarmo *et al.* 2008).

Conclusions

BMPs have diverse cancer-specific expression patterns that differ from the expression patterns observed in normal mammary tissues, indicating aberrant behaviour in breast cancer. Common receptor expression in breast cancer also indicates that BMP signalling is not restrained, which is sometimes seen in some inheritable cancer syndromes. These expression patterns vary when different cancer types are compared. Contrary to breast cancer, BMP2 is overexpressed in virtually every lung cancer (Langenfeld *et al.* 2005), and BMP7 is downregulated in prostate cancer (Masuda *et al.* 2004, Buijs *et al.* 2007b). Similarly, BMPRI1A, BMPRI1B and BMPRI2 expression has been shown to be lost in advanced prostate cancer (Kim *et al.* 2000, 2004), but such a phenomenon has not been observed in breast cancer (Alarmo *et al.* 2007). One can reasonably expect that diverse expression profiles also lead to diverse phenotypic effects depending on the cancer type in question.

The main theme in the functional studies of BMPs in breast cancer is that the same ligand can both promote and inhibit cancer progression depending on the study. Many of the *in vitro* or *in vivo* studies rely on a single cell line; thus, it is difficult to ascertain how well the results can be generalised. We showed that among eight breast cancer cell lines, BMP7 stimulated growth in two, inhibited growth in four, and two cell lines did not respond to BMP7 at all (Alarmo *et al.* 2009). Similarly, in prostate cancer, BMP7 elicits diverse functional responses depending on the cell line (Yang *et al.* 2005). Therefore, conclusions based simply on one cell line might be too straightforward. It is thus highly important to use large, unbiased patient materials to explore the possible impact of particular BMPs in a comprehensive manner.

BMP-inducible phenotypes in breast cancer clearly seem to be context dependent as can be expected based on the heavily regulated signalling pathway discussed in the beginning of this review. Some studies have detected that other growth factors and hormones alter the response to BMP. Oestrogen, an important hormonal player in breast cancer (Anderson 2002), modulates BMP ligands themselves as well as the responses elicited by BMPs. ER is also a known transcription factor operating with SMADs.

More detailed analysis on BMP signalling and its regulation in tumorigenesis is obviously needed to clarify the impact of BMPs in breast cancer. Whether the seeming discrepancy of results between studies is actually a reflection of bidirectionality remains to be seen. BMP2, BMP4 and BMP7 have been shown to both inhibit and promote breast cancer progression; thus, they possess bidirectional functions similar to the dual role established for TGF β in breast cancer.

As a whole, despite the fact that a great number of the studies on BMPs in cancer have concentrated on breast carcinomas, a systematic view on the actions of BMPs in breast cancer is still developing. These studies illustrate that the pleiotropy BMPs exert in normal tissues is also possible in cancer. The naturally occurring extra- and intracellular inhibitors of BMP signalling will eventually offer a plethora of therapeutic intervention points and might allow fine tuning of therapeutic options in breast cancer (Tsuchida *et al.* 2006, Gazzoer & Minetti 2007). Even though at this time solid conclusions are hard to draw due to conflicting results, BMPs are no doubt significant players in the breast cancer development, and the future challenge will be to find out what brings the worst out of BMPs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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