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Applications of Transgenics in Studies of Bone Sialoprotein

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Abstract

Bone sialoprotein (BSP) is a major non-collagenous protein in mineralizing connective tissues such as dentin, cementum and calcified cartilage tissues. As a member of the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) gene family of glycoproteins, BSP is involved in regulating hydroxyapatite crystal formation in bones and teeth, and has long been used as a marker gene for osteogenic differentiation. In the most recent decade, new discoveries in BSP gene expression and regulation, bone remodeling, bone metastasis, and bone tissue engineering have been achieved with the help of transgenic mice. In this review, we discuss these new discoveries obtained from the literatures and from our own laboratory, which were derived from the use of transgenic mouse mutants related to BSP gene or its promoter activity.

Keywords

bone sialoprotein; transgenic mice; bone metastasis; bone remodeling; osteogenic differentiation

Introduction

Bone sialoprotein (BSP) is a major non-collagenous protein in mineralizing connective tissues such as dentin, cementum and calcified cartilage tissues. It is a 34 kDa protein that contains predominantly aspartic acid, glutamic acid and glycine (Ganss et al., 1999). BSP has a high carbohydrate content (Fisher et al., 1987; Fisher et al., 1983) and is post-translationally modified by glycosylation (Fisher et al., 1987; Young M, 1993), phosphorylation (Franzen and Heinegard, 1985), and sulphation of tyrosines (Ecarot-Charrier et al., 1989). The Arg-Gly-Asp (RGD) sequence near the carboxy-terminus of BSP protein is a cell attachment site recognized by $\alpha_v\beta_3$ integrin receptor (Oldberg et al., 1988a; Oldberg et al., 1988b), while polyglutamic acid regions in the amino terminus mediate the binding of BSP to hydroxyapatite (Oldberg et al., 1988a). As a member of the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) gene family of glycoproteins, BSP is involved in regulating hydroxyapatite crystal formation in bones and teeth (Fisher et al., 2001).

Since the first successful gene transfer by microinjection into mouse embryos in 1980 (Gordon et al., 1980), transgenic mice have become an important animal model which allows researchers to observe gene function, tissue specificity of gene expression, and the mechanisms by which specific gene expressions are regulated. Furthermore, foreign gene expression-related phenotypic changes can also be observed. The system also allows for the creation of animal models for human diseases and their treatments (Gordon and Ruddle, 1985). In the most recent decade, new discoveries in BSP gene expression and regulation, bone remodeling, bone metastasis, and bone tissue engineering have been achieved with the help of this powerful system. In this review, we discuss these new discoveries obtained from the literatures and from

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our own laboratory, which were derived from the use of transgenic mouse mutants related to BSP gene or its promoter activity.

BSP gene expression and regulation

In the last several decades, many studies created specific transgenic animals carrying a reporter gene to investigate gene regulation mechanisms. A reporter protein is a protein or an enzyme whose expression or the expression of its catalytic product can easily be detected by routine fluorometric, colorimetric, or luminometric techniques (Spergel et al., 2001). The luciferase from the firefly *Photinus pyralis* is one of the most commonly used reporter proteins for bioluminescence (Herschman, 2003). In contrast to the traditional approaches to monitoring gene expression such as immunohistochemistry and *in situ* hybridization, the main advantage of luciferase is that it enables detection of very low levels of gene expression (Spergel et al., 2001) and non-invasive monitoring of gene expression in living animals (Contag et al., 2000; Wu et al., 2001). Using transgenic mice harboring promoter region of a certain gene linked to a luciferase reporter gene, it can be assessed whether the investigated gene can be regulated by transcription factors and growth factors or whether *cis*-acting DNA elements involved in temporospatial specificity of gene expression are present (Palmiter and Brinster, 1985). Furthermore, by producing transgenic mice with different lengths of the promoter region, these *cis*-acting DNA elements required for tissue-specific expression can be precisely located (Paz et al., 2005). Moreover, although valuable information about tissue-specific regulatory elements can be achieved by transfection of genes into appropriate cell lines, *in vitro* approaches cannot duplicate the complexity of the molecular and cellular changes occurring in a live animal (Dente et al., 1988). The most stringent demonstration that a particular promoter sequence is sufficient for tissue specific gene expression requires testing the function of that sequence in every possible cell type throughout development, which is a goal easily achieved by creating transgenic animals (Palmiter and Brinster, 1985).

BSP promotes the initial formation of mineral crystals and has long been considered as an early marker of osteogenic differentiation (Chen et al., 1994; Chen et al., 1992; Kasugai et al., 1992; Sodek et al., 1992). Therefore it is of particular interest to investigate the effects of transcription factors, growth factors, and hormones on BSP expression. Among all the available approaches towards that end, the integration of the BSP promoter-reporter construct into the normal murine genome allows more meaningful *in vivo* analysis of gene expression at different developmental stages and more significant *in vivo* analysis of gene regulation by various hormones and cytokines.

As early as in 1996 four transgenic mouse lines (rBSP2.7Luc) were generated in our laboratory, in which a luciferase reporter gene driven by a ~2.7 kb rat BSP promoter was stably integrated into the mouse genome to monitor the selective expression of the BSP gene (Chen et al., 1996b). In this study it was reported that high luciferase expression levels were detected consistently in bone tissues while the luciferase activities in kidney, liver, stomach, intestine, and spleen were negligible (Chen et al., 1996b). In addition to the spatial BSP expression patterns, temporal analyses also revealed different luciferase expression patterns at different developmental stages (Chen et al., 1996b). To investigate the regulatory mechanisms underlying the specific temporospatial expression pattern of BSP, dexamethasone was injected into the rBSP2.7Luc transgenic mice and was found to significantly stimulated the luciferase activity in bone tissues (Chen et al., 1996a). A putative glucocorticoid response element (GRE) within the 2.7 kb rat BSP promoter sequence (Li and Sodek, 1993; Ogata et al., 1995; Sodek et al., 1995) was postulated to mediate the dexamethasone-induced increase in BSP gene expression (Chen et al., 1996a). In another study using this transgenic mouse line (rBSP2.7Luc), vitamin D₃ deficiency was induced to establish a rachitic animal model (Chen et al., 1999). In contrast to the stimulative effects of glucocorticoids on BSP expression *in*

vivo, vitamin D₃ was shown to inhibit BSP expression while stimulating OPN expression (Chen et al., 1999).

In 2000, it was reported that a homeodomain binding element (named as OSHE1; osteoblast-specific homeodomain element 1) in a 2.5 kb fragment of the murine BSP promoter is required for osteoblast-specific expression in cell culture (Benson et al., 2000). The researchers then generated transgenic mouse lines in which both luciferase and lacZ reporter gene expressions were driven by the wild-type 2.5 kb BSP promoter or the same promoter containing a 2 bp mutation in OSHE1 (Gopalakrishnan et al., 2003). The results demonstrated a much higher specificity of exogenous gene expressions driven by the wild type BSP promoter. However, some soft tissue expression of the luciferase gene driven by the wild type 2.5 kb BSP promoter was also detected although luciferase activity was much higher in mineralized tissues (Gopalakrishnan et al., 2003). These studies demonstrated that additional regulatory elements within the extended regions of the BSP promoter are required to obtain absolute tissue-specificity.

In 1999 four consensus sequences for Cbfa1/Runx2 binding were identified within a large DNA segment of the murine BSP gene extending 16 kb upstream from the BSP transcription start site. Two of these sequences bind Cbfa1/Runx2 and show transactivating activity in transient transfection assays (Yamauchi et al., 1999). To further investigate the mechanisms by which tissue- and time-specific expression of BSP is regulated, transgenic mice harboring luciferase reporter gene driven by longer BSP promoter sequence were established. We reported the generation of two transgenic mouse lines in which 4.8 kb (mBSP4.8Luc) or 9.0 kb (mBSP9.0Luc) of the BSP promoter was ligated to the luciferase reporter gene. Luciferase assays revealed a higher specificity of luciferase expression in mBSP9.0Luc mice, which indicated that the 9.0 kb mouse BSP promoter including all the four putative Cbfa1/Runx2 binding sites is necessary for tissue-specific expression of the BSP gene (Paz et al., 2005).

However, research results regarding the effect of *Runx2* on BSP expression is controversial. An early *in vitro* study showed that *Runx2* enhanced BSP expression (Ducy et al., 1997). In another study it was reported that within the proximal region of the mouse, rat and human BSP genes, there are two putative *Runx2*-binding sites (R1 and R2) which act as osteoblast-specific transcriptional enhancers (Roca et al., 2005). In contrast to these studies, there are also some studies reporting that *Runx2* regulates BSP expression in an inhibitory way, and it has been suggested that the possible mechanisms could be a corepressor partner interacting with *Runx2* such as histone deacetylase 3 (HDAC3) (Lamour et al., 2007), or promoter architecture changes induced by *Runx2* binding which allows binding by other potential suppressive transcription factors (Javed et al., 2001). To further investigate the transcriptional regulation of BSP by *Runx2* *in vivo*, we crossbred the mBSP9.0Luc or mBSP4.8Luc (lacking two upstream *Runx2* elements) transgenic mice (Paz et al., 2005) with the *Runx2*^{+/-} mouse line. Our recent results demonstrated that the direct effect of *Runx2* on mouse BSP promoter is to enhance its activity, and *Runx2* overexpression induced a higher increase in the activity of luciferase driven by a longer promoter. In contrast, the suppression effect of *Runx2* on BSP promoter is in an indirect way, and during early developmental stages a corepressor may interact with *Runx2* and restrict excessive BSP expression (Tu et al., 2008). These results clearly indicated that *in vivo* expressions of bone matrix proteins are regulated in a subtle and complicated way, and the complexity of *in vivo* environment could not be completely replicated by *in vitro* experiments.

Bone tissue engineering

Emergence of new technologies based on rapidly expanding knowledge of the molecular mechanisms regulating cellular differentiation and matrix formation has stimulated the

introduction of novel clinical approaches for the repair of damaged tissues and reconstruction of oral and maxillofacial defects. Nevertheless, there is still much to be learned about cellular histogenesis before these approaches for regeneration of the complex tissues that comprise the skeletal structures in orofacial regions become routine clinical procedures. As a major protein of the bone matrix, BSP has long been used as a marker for osteoblastic differentiation, especially in the studies aimed at estimating osteogenic effects of the new therapies for bone tissue engineering. Furthermore, transgenic mouse models carrying various exogenous genes driven by BSP promoter have also been demonstrated to be a powerful tool in bone tissue engineering research field, particularly in *ex vivo* studies.

Recently in our lab a new transgenic mouse model (BSP-Luc/ACTB-EGFP) was created by cross-breeding the male ACTB-EGFP mouse (Jackson Lab, Bar Harbor, ME) with a homozygote mBSP9.0Luc(Paz et al., 2005) female mouse. These transgenic mice have two genetic markers which provided much convenience in bone tissue engineering studies. One of the two genetic markers is a luciferase reporter gene driven by a 9.0kb mouse BSP promoter, which indicates that luciferase expression in this mouse model is restricted to mineralized tissues as BSP expression (Chen et al., 1992; Chen et al., 1996b; Fisher et al., 1990; Oldberg et al., 1988b; Paz et al., 2005). Therefore the chimeric gene of BSP-Luc can serve as a faithful and sensitive marker for osteogenic differentiation of transplanted BMSCs. Detection of BSP promoter expression reflects the functional stage of the osteogenic cells derived from the embryonic or adult stem cells. The other genetic marker is the enhanced green fluorescent protein (EGFP) driven by a β -actin promoter and cytomegalovirus enhancer. After the transplantation of stem cells isolated from BSP-Luc/ACTB-EGFP transgenic mice into a wild type mouse, the integration of these two reporters, luciferase and GFP, enables the identification of cells derived from transplanted stem cells from host cells present in the wound sites. In short, GFP staining can be used to track the fate and migration of all transplanted stem cells, whereas luciferase staining serves as a marker for osteogenic differentiation of the transplanted stem cells because the luciferase expression is switched on as these cells differentiated into osteoblasts. This is a critical method as it permits the determination of where these cells originate and how they undergo osteogenic differentiation.

A recent study using this transgenic mouse model was aimed at determining the extent to which BMSCs from distant sites are involved in tissue regeneration (Li et al., 2008). In this study BMSCs isolated from transgenic mice BSP-Luc/ACTB-EGFP were transplanted into irradiated wild type mice and artificial bone wounds were created in the recipient mice. In addition to traditional immunohistochemical staining and RT-PCR analysis, the temporospatial expressions of luciferase and GFP were also monitored using Xenogen IVIS Imaging System, which allows for detection of reporter gene expression in living animals. Our results demonstrated that transplanted BMSCs can be detected in wound sites as early as two weeks after surgery and lasted the whole experimental period. Luciferase expression peaked at 2 weeks after surgery and decreased thereafter, similar to the expression pattern of BSP, while GFP expression was relatively stable during the experimental period (Li et al., 2008). These results, achieved by using this powerful and invaluable transgenic mouse model, not only provided direct evidences on functions of BMSCs in bone regeneration, but also indicated the manner in which they can be delivered to the regeneration sites.

Another transgenic mouse model developed in our laboratory was applied in bone tissue engineering research. BSP-TVA transgenic mice demonstrated a skeletal tissue-specific expression of an avian retroviral receptor (TVA) gene driven by the 4.9kb BSP promoter (Li et al., 2005). In this transgenic mouse model BSP expressing cells also produce the receptor (TVA) for the ALV-A to allow selective infection by replication-competent avian leucosis virus (ALV) splice acceptor (RCAS) viral vectors, which is derived from the avian retrovirus, ALV subgroup A (Holland and Varmus, 1998). After systemic infection with a construct

expressing a mutated form of Cbfa1/Runx2 (Cbfa1mu), which acts as a dominant-negative regulator of Cbfa1/Runx2 because Cbfa1mu has a higher affinity for DNA than Cbfa1/Runx2 but has no transcriptional activity, the BSP-TVA mice showed successful Cbfa1mu expression and suppressed BSP expression in the bone tissues. In addition, the bone and tooth formation were delayed in these Cbfa1mu injected BSP-TVA mice (Li et al., 2005). These studies, showing that BSP-TVA transgenic mice can be used to target genes to sites of osteogenesis, proved that these transgenic mice is a unique system for studying molecular events associated with bone formation in vivo. Using this versatile transgenic mouse model various gene expressions and their regulatory mechanisms can be studied in vivo.

In future studies the BMSCs isolated from BSP-Luc/ACTB-EGFP mice can be use to determine the effect of osteogenic transcription factors on cell osteogenic differentiation in the context of tissue regeneration. To achieve the same purpose, bone defects can also be generated in TVA mice followed by viral infection using a gene therapy method.

Bone remodeling

The integrity and strength of the adult skeletal structure is maintained by bone remodeling, a process that involves a well-organized balance of old bone resorption by osteoclasts (Teitelbaum, 2000) and new bone formation by osteoblasts (Ducy et al., 2000). Although both bone formation and bone resorption continue throughout adult life and about 10% of the bone tissue is replaced each year with complete renewal every 10 years (Cohen, 2006), their net effects on bone mass are zero in healthy individuals.

Bone remodeling is normally regulated by a variety of cytokines, extracellular matrix proteins, and hormones, among which the RANKL/RANK and the decoy receptor, osteoprotegerin (OPG), have been identified as the key regulators of bone remodeling (Akatsu et al., 1998; Kong et al., 1999; Lacey et al., 1998; Yasuda et al., 1998). Bone sialoprotein (BSP), which is a major non-collagenous extracellular matrix protein in bone, is produced by osteoclasts, osteoblasts, osteocytes and hypertrophic chondrocytes (Chen et al., 1994; Chen et al., 1992; Kasugai et al., 1992; Sodek et al., 1992). Although BSP expression has been shown to be coincident with de novo bone formation (Chen et al., 1994; Chen et al., 1992; Kasugai et al., 1992; Sodek et al., 1992), substantial evidences suggested that BSP plays a role not only in bone formation but also in bone resorption. As early as 1995, it was found that human osteoclasts (FLG 29.1) synthesize BSP with the induction of phorbol 12-myristate 13-acetate (TPA) (Masi et al., 1995). Using immunohistochemistry and *in situ* hybridization methods, it was shown that osteoclasts express BSP gene at both protein and mRNA levels (Bianco et al., 1991). Raynal and colleagues found that BSP significantly stimulates bone resorption in a dose-dependent manner at concentrations as low as 50 nM. They suggested that this stimulation could be partially due to an increase in osteoclast adhesion to bone via its RGD sequence or its acidic sequences (Raynal et al., 1996). Using a cell culture system, another group found that BSP significantly increased attachment of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts and suggested that post-translational modification of BSP phosphorylation is necessary for *in vitro* osteoclastic bone resorption (Razzouk et al., 2002). We also reported that BSP acts synergistically with the receptor activator of nuclear factor κ B ligand (RANKL)/RANK signaling to induce osteoclastogenesis and bone resorption in RAW264.7 cells. Furthermore, BSP was shown to decrease RANKL-induced apoptosis (Valverde et al., 2005). The effects of BSP found in RAW264.7 cells also were shown in primary cultures of bone marrow-derived monocytes/macrophages (BMM) (unpublished data). However, in vivo data about the overall effects of systemically BSP overexpression on bone metabolism were not available until recently a novel transgenic mouse model was established (Valverde et al., 2008).

The transgenic mouse model, name as CMV-BSP transgenic mice, was created in which constitutively over-expressed mouse BSP cDNA is driven by the cytomegalovirus (CMV) promoter. The mineralized tissues isolated from these transgenic mice demonstrated higher BSP levels than those from wild-type mice, and BSP expression could be detected in all soft tissues derived from the transgenic mice. As a result of constitutional BSP overexpression in the whole body, the CMV-BSP transgenic mice exhibited serum BSP levels which were within the range of individuals with excessive bone resorption (50-100 ng/ml) in postmenopausal osteoporosis, multiple myeloma, metastatic breast cancer or Paget's disease (Bellahcene et al., 1996; Fedarko et al., 2001; Seibel et al., 1996; Shaarawy and Hasan, 2001; Stork et al., 2000). Further experimental analyses revealed that CMV-BSP transgenic mice exhibit phenotypes including mild dwarfism, thinner calvarial and mandibular bones, increased multinucleated osteoclast numbers, lower values of bone mineral density (BMD), and an upregulation in markers of osteoclastic activity, all of which indicated an increase in bone resorption. *Ex vivo* experiments indicated that the mechanisms underlying the increased bone resorption observed in CMV-BSP mice may be the enhanced differentiation of osteoclast precursor cells and elevated osteoclastic activity (Valverde et al., 2008).

The phenotypic analysis of CMV-BSP transgenic mouse points out a cause and effect relationship between BSP overexpression and increased bone resorption. It also provides a molecular mechanism for the excessive bone resorption of a variety of bone pathologies characterized by their high serum levels of BSP and high RANKL-to-OPG ratio. In future studies, this transgenic mouse model could be used to evaluate the therapeutic effects of antibodies against BSP on bone diseases characterized by increased bone resorption, especially on those with high serum BSP levels.

Bone metastasis

Metastasis of primary malignancy is a major factor in the morbidity and mortality associated with cancer. Breast cancer is among the most common cancers that disseminate to bone (Yoneda, 1998) with bone metastases found in virtually all advanced breast cancer patients. Severe bone pain, hypercalcemia, pathological fractures and neural compression syndrome often accompany osteolytic bone metastasis of breast cancer (Reddi et al., 2003). In breast cancer, bone metastases are predominantly osteolytic and destructive. The literature review presented below summarizes the role of transgenic mice in studying BSP-associated neoplastic osteolysis and tumor cell activities that occur in bone metastasis of breast cancer.

In addition to the studies indicating the presence of BSP in human breast cancers (Barnes et al., 2003; Bellahcene et al., 1994) and higher serum BSP levels in patients with primary breast cancer which is closely related to the prognosis of subsequent bone metastasis (Diel et al., 1999), it was shown that BSP was higher in bone metastases than in visceral metastases in breast and prostate cancer patients (Waltregny et al., 2000). It was also reported that BSP overexpressing human breast cancer cells transplanted through ventricle injection caused extensive bone metastatic lesions in a tumor metastasis mouse model (Zhang et al., 2004).

To further investigate the effects of host tissue-derived BSP on breast cancer metastasis, we transplanted luciferase-labeled 4T1 murine breast cancer cells into two distinct transgenic mouse lines and evaluated the resulted bone metastasis (Tu et al., 2009). One of the transgenic mouse lines, CMV-BSP mice, is characterized by high serum BSP level and ectopic BSP expression in soft tissues as mentioned above (Valverde et al., 2008). Using a combination of real-time IVIS Imaging, histological analysis and radiographic analysis, we found that CMV-BSP mice receiving intracardiac cancer cell inoculation developed more severe metastases in multiple vital organs including liver, lung, and kidney, and more CMV-BSP mice died of vital organ metastases before the scheduled sacrifice time point (Tu et al., 2009). These results are

interesting because originally 4T1 murine breast cancer cells show a primary characteristic of bone-seeking metastases. Our previous *in vitro* studies showed that BSP overexpression in MDA-231 human breast cancer cells promotes tumor cell migration through endothelial cells as well as through the chicken embryonic vasculature (Chen et al., 2003). Thus a high serum BSP level observed in CMV-BSP mice might facilitate the migration of breast cancer cells through the blood vessels. In addition, BSP was reported to promote tumor cell proliferation, adhesion and migration (Byzova et al., 2000; Sung et al., 1998), which may also contribute to the high metastatic rate observed in CMV-BSP mice.

Tumor metastasis is a multi-step process which begins with tumor cells departing from the primary site, followed by their entering circulation, and then ended with their seeding and growing in a remote site. To focus on the effects of host BSP on tumor cell seeding and growing in remote bone sites, we developed a novel transgenic mice line in which targeted BSP overexpression in osteoclasts was driven by a Cathepsin K (CtpsK) promoter (Li and Chen, 1999; Tu et al., 2009). This transgenic mouse line, named as CtpsK/BSP mice, showed increased osteoclastogenesis, reduced trabecular bone volume and decreased bone mineral density (BMD) (Tu et al., 2009). After intracardiac and intratibial inoculation of 4T1 murine breast cancer cells, more CtpsK/BSP mice developed bone metastatic lesions when compared with wild type mice, and the osteolytic lesion area was significantly larger in CtpsK/BSP mice than in the wild type mice (Tu et al., 2009). Ross et al. reported the direct binding of osteoclasts to intact bone matrix via $\alpha_v\beta_3$, using osteopontin and BSP for attachment (Ross et al., 1993). Consistent with these previous findings, our results clearly indicated that endogenous BSP synthesized by osteoclasts not only induces tumor cell seeding into the bones via $\alpha_v\beta_3$, but also promotes osteoclast differentiation in metastatic osteolysis of breast cancer in an autocrine manner (Tu et al., 2004), thus further promotes bone metastasis of breast cancer.

Conclusion

Bone sialoprotein (BSP), which is a major non-collagenous extracellular matrix protein in bone, is produced by osteoclasts, osteoblasts, osteocytes and hypertrophic chondrocytes and has long been identified as early marker of osteogenic differentiation. With the help of various transgenic mouse lines harboring reporter genes driven by different lengths of BSP promoter, the temporospatial expression patterns of BSP was thoroughly determined *in vivo*. Furthermore, regulation of BSP gene expression by various hormones and cytokines was also investigated. Using these powerful tools, interesting results of detecting stem cell origin, differentiation, and stem cell fate in living animals were also generated. In addition, *in vitro* studies have suggested that BSP may intervene at several steps of bone remodeling by its ability to nucleate hydroxyapatite crystal formation under steady-state conditions, promote mineralization of some osteoblastic cell lines, induce osteoblast and osteoclast adhesion and increase osteoclastogenesis and bone resorption. Using transgenic mouse lines in which BSP gene over-expression was achieved at different levels, the roles of BSP in osteoclastogenesis, bone resorption and osteolytic bone metastasis were revealed *in vivo*. These transgenic mouse lines, serving as invaluable tools in bone and mineralized tissue studies, could be further utilized in future studies aimed at developing new strategies in tissue engineering, developing new therapies to osteolytic bone diseases, and investigating bone development and bone remodeling.

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