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Parathyroid Hormone Related Protein: A Marker of Breast Tumor Progression and Outcome

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1. Introduction

1.1 Breast cancer

Breast cancer is the second leading cause of death among women. According to the American Medical Association and American Cancer Society, it is the most common disease of women, although it usually does not affect them until they reach their '20s. Every year millions of women are diagnosed with this disease and screening for breast cancer is now widespread, particularly among older women. Breast cancer is the most common form of cancer in women accounting for 1 in 4 of female cancers and is the leading cause of death among gynecological cancers in developed countries. Almost 10% of women in France will develop breast cancer and 75% of newly detected cases are women over 50 years. This number is constantly increasing. (The Lancet, Volume 374, Issue 9701, Page 1567, 7 November 2009 (Breast cancer in developing countries).

1.2 Background

Breast tissue is an exocrine gland of ectodermal origin. Each breast is composed of 10-20 glandular lobes, each of which is divided into lobules and acini. The acini are grouped densely around an alveolar duct (lactiferous duct 3rd order). Several alveolar ducts come together to form a lobular channel (channel 2nd order) which drains a lobule. Several lobular channels unite to form a milk duct that drains all the lobules within the glandular lobe. Each lobe acts as an independent gland with its own excretory duct (lactiferous duct). These lactiferous ducts converge toward the nipple. The mammary gland can be affected by various lesions. The more benign, include pseudo-tumoral lesions, benign epithelial tumors, fibro-epithelial and cystic fibrosis. These are also called benign breast disease. Malignant epithelial lesions may be non-invasive (lobular carcinoma *in situ* and ductal carcinoma) or infiltrating (invasive lobular carcinoma and infiltrating ductal carcinomas). Infiltrating ductal cancers are the most common, accounting for 84% of breast cancers, while infiltrating lobular cancers account for only 4% of breast tumors and non-invasive forms for 1-3%.

Breast cancer cells often metastasize to bone where they produce large amounts of parathyroid hormone-related protein (PTHrP). We have investigated the possible roles of PTHrP in breast cancer bone metastases.

1.3 Parathyroid hormone-related protein (PTHrP) gene

The sequence of parathyroid hormone-related peptide (PTHrP) is very similar to that of the N-terminal portion of parathyroid hormone (PTH). The PTHrP gene is complex and can generate at least three mature peptides by alternative splicing. PTHrP acts via a receptor that it shares with PTH and also via specific receptors. Physiologically, PTHrP is produced locally in many normal tissues where it has autocrine/paracrine functions, particularly during embryonic development, growth regulation and the differentiation of many cell types. PTHrP has an endocrine action on bone and kidney.

1.3.1 Gene structure

The PTHrP gene is located on the short arm of chromosome 12 in a position homologous to the PTH gene on chromosome 11 in humans (Naylor et al. 1983; Mangin et al. 1988; Suva et al., 1989) *(Figure 1*). Both chromosomes carry more genes belonging to the same family such as the A and B isoforms of lactate dehydrogenase (LDH), Sox 5 and 6 and forms H and K of the ras gene (Yang and Stewart, 1996). The chromosomal location and structural homology of the two genes suggest that they were produced by duplication of a common ancestral chromosome. But the PTHrP gene is much more complex than the PTH gene (Philbrick et al., 1996) *(Figure 1).*

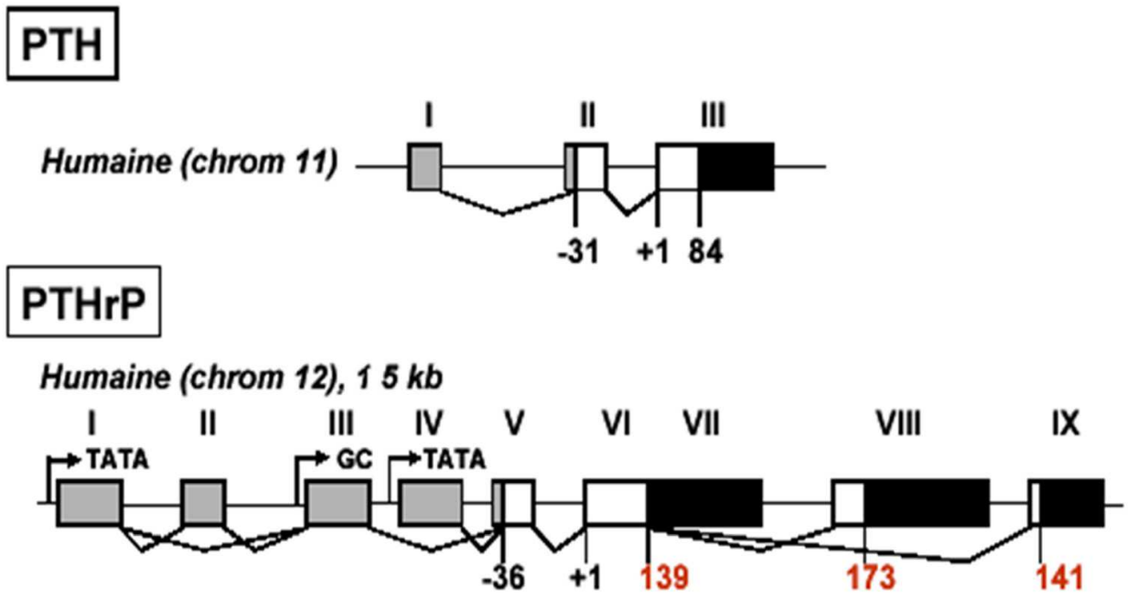


Fig. 1. *Structure and organization of the Human PTH and PTHr P genes*: Organization of the PTH (chromosome 11 in humans) and PTHrP (chromosome 12 in humans) genes and phylogenetic variations. Exons are in Roman numerals. The coding regions are shown as white rectangles, untranslated 5′ regions are in gray, untranslated 3' regions are in black, and introns are shown by solid lines. The promoter sequences are indicated by black arrows. The alternative splicings that generate the isoforms of PTHrP in each species are represented by the broken lines in each sequence.

The 15 kb-long human PTHrP gene is a complex transcriptional unit composed of nine exons and 15 different transcripts can be generated by alternative splicing of the 5′ and 3′ exons (Broadus and Stewart, 1994; Gillespie and Martin, 1994). Transcription may be initiated at any of the three promoters, P1, P2 and P3. P1 and P3 are canonical TATA promoters (Mangin et al., 1990; Campos et al., 1992; Suva et al., 1989) and initiate transcription at exons 1 and 4 respectively, while P2 is a GC-rich promoter that initiates transcription upstream of exon IC (Vasavada et al., 1993). Evaluation of PTHrP alternative promoter usage by qualitative (Southby et al., 1995 and 1996) and quantitative reverse transcription RT-PCR (Bouizar et al., 1999; Richard et al., 2003) revealed high concentrations of P3-initiated transcripts in most tumors, including breast cancers (Bouizar et al., 1999) and many tumor cell lines (Cataisson et al., 2003). The amino acid sequences encoded by only two of the 9 exons in the human gene are present in all transcripts. One is exon 5, which encodes the prepro-sequence and the other is exon 6, which encodes the common sequence of 139 amino acids. Polymerase II will transcribe exon 7 if the splice donor site located at the end of exon 6 is skipped; this exon encodes the stop codon and the 3' untranslated region of the 139 residue isoform. The splicing of exon 6 to exon 8 provides a transcript encoding the 173-residue isoform, while splicing exon 6 to exon 9 produces a transcript encoding the 141-residue isoform. *(Figue 1)*

2. PTHrP and mammary gland physiopathology

Recent studies using targeted overexpression or disruption of the PTHrP gene have demonstrated that the peptide regulates the development of the cartilaginous growth plate, the skin, teeth and mammary glands (Broadus et al 1994, Karaplis et al 1994, .Philbrick et al 1998,Wysolmerski et al 1998)

2.1 PTHrP and mammary development

Overexpression and underexpression of PTHrP disrupt the process of branching morphogenesis by which the mammary gland develops (Broadus et al 1994, Wysolmerski,et al 1994,). Wysolmerski and colleagues demonstrated that amino-terminal PTHrP is required for the development of the mammary epithelial duct system in mice. PTHrP gene expression is limited to the mammary epithelium during embryonic mammary development, while expression of the PTH/PTHrP receptor gene is restricted to the mesenchyme. In addition, mammary stromal cells in culture bind aminoterminal PTHrP and respond with an increase in intracellular cAMP (Dunbar et al 1998). This suggests that PTHrP acts as an epithelial message that must be received by mammary mesenchyme before it can support branching growth. Collagen II-PTHrP (rescued) /PTHrP knockout mice that have no PTHrP in any tissue except the cartilage lack even a vestige of mammary epithelial ducts. PTHrP-knockout mouse embryos exhibit a primary failure of branching morphogenesis during embryonic mammary gland development. Deletion of the PTH/PTHrP receptor recapitulates the failure of mammary development seen in the PTHrP knockout embryos. Transgene-mediated reintroduction of PTHrP into the mammary epithelial cells of PTHrP-knockout animals reversed the failure of embryonic mammary development and allowed the development of a complete mammary duct system within the mammary fat pad. Embryonic mammary glands are formed in two steps; the mammary bud is formed first, followed by branching morphogenesis to form the immature ductal tree (Sakakura et al 1987). The mammary buds are formed appropriately in PTHrP-knockout embryos but they fail to

undergo the initial round of branching growth that leads to a typical immature ductal tree. The mammary epithelial structure fails to elongate and/or penetrate into the developing fat pad in the absence of PTHrP. Instead, the mammary bud remains in the upper dermis surrounded by dense fibro-connective tissue. The mammary epithelial cells have degenerated in newborn PTHrP-knockout mice, the nipple sheath has not formed, and all traces of the mammary epithelial duct system has disappeared, explaining the lack of mammary structures in the mature rescued PTHrP-knockout mice (Dunbar et al 1998).

2.2 PTHrP gene expression, puberty and early pregnancy

The mammary glands of newborn mice undergo little development until the onset of puberty at 3-4 weeks of age (Sakakura et al 1991). The hormonal changes that occur at this time influence the distal ends of the mammary ducts to form specialized structures called terminal end buds, where there is active cell proliferation and differentiation during ductal growth and morphogenesis (Daniel et al 1988). By the time mice are 8-10 weeks old, the epithelial duct system has grown to the borders of the mammary fat pad and the terminal buds disappear, leaving the typical branched duct system found in the adult virgin gland. Pregnancy triggers another round of epithelial proliferation that leads to the production of terminal ducts and lobular-alveolar structures.

PTHrP may also play a role in the epithelial/mesenchymal interactions that govern these later rounds of ductal morphogenesis. PTHrP was first found in the mature mammary gland during late pregnancy and lactation (Budayr et al 1987, Thiede et al 1988). More recently, Dunbar and colleagues found mRNAs encoding PTHrP in the mammary epithelium and PTH/PTHrP receptor mRNAs in the stroma during puberty and early-to-mid pregnancy. Furthermore, both genes are most actively expressed in those regions of the mammary gland that are proliferating and undergoing ductal morphogenesis, the terminal end buds. Overproduction of PTHrP in the mammary myoepithelial cells of transgenic mice results in abnormal mammary duct development during puberty and early pregnancy (Wysolmerski,et al 1994). PTHrP overproduction during puberty severely impairs both the overall rate of ductal proliferation and the pattern of side branching. In addition, PTHrP overproduction during early pregnancy impairs terminal duct development. These effects appear to be mediated by amino-terminal PTHrP acting through the PTH/PTHrP receptor.

2.3 PTHrP and lactation

Parathyroid hormone-related protein does not only influence the development of breast tissue. Human and bovine milk contain high concentrations of PTHrP (Thiede et al 1988,): a thousand times higher than the concentration in the blood plasma. The glandular epithelial and myoepithelial cells of the lactating breast produce large amounts of parathyroid hormone-related protein. Most of it is secreted into the milk although small quantities are released into the blood (Khosla et 1990). It has therefore been proposed that PTHrP may enhance blood flow to the mammary gland during lactation (Davicco et al 1993). Suckling stimulates PTHrP gene expression in the mammary gland and prolactin has been reported to increase synthesis of the peptide. This led to the idea that the protein may be the long-postulated signal that adapts maternal calcium metabolism to the stress of lactation.

While an infusion of PTHrP can increase mammary blood flow in goats (Posser et al 1994) and sheep (Rodda et al 1988), this does not appear to alter milk production or enhance the calcium content of the milk. Moreover, there is no evidence that the plasma concentration of

PTHrP induced by lactation can mobilize calcium from bone or enhance its renal reabsorption. However, there is evidence that PTHrP is involved in calcium transfer. Barlet et al showed that PTHrP increases the $P0_4$, $Ca_{2+,}$ and Mg_{2+} content of breast milk (Barlet et al 1990). Parathyroid hormone-related protein has been detected in the serum of nursing mothers (Grill et al 1991, Sowers et al 1996). But there is no conclusive evidence that it is the long-sought lactation signal. Yet mammary parathyroid hormone-related protein probably has systemic effects, because its concentrations are high in the serum of women with rare syndromes of hypercalcemia associated with lactation and massive mammary hypertrophy (Lepre et al 1993). PTHrP increases the cAMP in myoepithelial cells and decreases the intracellular calcium induced by oxytocin in these cells (Ferrari,et al 1993, Seitz et al 1993).

3. PTHrP and breast cancer

Several studies have reported finding PTHrP in primary tumors. Ikeda and coworkers identified PTHrP transcripts in two breast tumors by Northern blotting in patients with hypercalcemia (Ikeda et al.,1988). Many subsequent immunohistochemical studies with polyclonal antibodies have revealed a significant percentage of cells staining positive for PTHrP: 60% of 102 primary tumors (Southby et al. 1990), 69% of 81 breast carcinomas (Liapis et al., 1993) and up to 72% of 367 primary tumors (Henderson et al., 2001, Henderson et al., 2006). Other more recent studies using monoclonal antibodies detected positive signals in 100% of invasive carcinomas tested (Iezzoni et al. 1998; Surowiak et al., 2003) .Similarly, both PTHrP and its receptor PTH1-R have been detected in breast cancers (Carron et al., 1997, Downey et al., 1997, Iezzoni et al. 1998; Linforth et al. 2002). There is now considerable evidence that PTHrP influences the growth and differentiation of mammary tissue, both *in vitro* and *in vivo* (Wysolmerski,et al 1995, Ferrari, et al 1992, Birch,et al 1995, Lupareilo et al 1997, Cataisson et al 2000). PTHrP is secreted by epithelial cells in the breast (Sebag et al 1994) and that both normal myoepithelial and tumoral epithelial mammary cells (Seitz et al 1993) secrete PTHrP in vitro (Birch,et al 1995 Luparello et al 1997,Cataisson et al 2000). Analyses of human breast cancer cell lines and immunological and in situ hybridization studies of human tumors indicate that neoplasic epithelial cells express both PTHrP and the PTH/PTHrP receptor (Birch,et al 1995,Iezzoni et al 1998, Cataison et al, 2000). In contrast, antibody labeling of human breast cancer tumors suggests that stromal fibroblasts surrounding the tumor produce significant amounts of immunoreactive PTHrP (Iezzoni et al 1998, Cros et al 2002).

3.1 Parathyroid hormone in invasive breast cancer

3.1.1 Role of PTHrP in the development of bone metastases

Parathyroid hormone-related protein (PTHrP) is a PTH-like calcitropic hormone, a growth factor and regulator of development (Murray et al., 2005). Tumor-derived PTHrP appears to be a crucial component of a chain of signalling events among cells of the bone microenvironment that facilitates both the destruction of bone and the growth of tumor cells (Guise et al., 1996; Thomas et al., 1999, Kozlow& Guise 2005). Many clinical studies have examined the impact of PTHrP on metastatic primary tumors. PTHrP has been detected by immunocytochemistry and *in situ* hybridization in 92% of bone metastases, but in only 17% of non-bone (soft tissue) metastases (Vargas et al., 1992). Others have found that 83% of patients who developed bone metastases had PTHrP in the primary tumor (Kohno et al.,

1994). A retrospective RT-PCR study showed increased transcripts of PTHrP in the primary tumors of patients who subsequently developed bone metastases (Bouizar et al., 1993). The results of an extension of this study to a larger cohort suggested that transcripts encoding the 139-residue isoform were overexpressed in tumors that had metastasized to bone (Bouizar et al. 1999; Guise et al. 2002).

PTHrP produced by breast cancer cells may facilitate the development of skeletal metastases by enhancing the survival of tumor cells in bone, or by promoting the invasion of bone by tumor cells (Bundred et al., 1992). Tumor cells tha have become established in bone produce factors that change normal bone remodeling, such as parathyroid hormone-related protein (PTHrP). Bone metastases of breast cancers are predominantly osteolytic and cause skeletal lesions leading to fractures, intractable bone pain, nerve compression, and hypercalcemia (Arguello et al 1988, Bouizar et al, 1993, 1999).

In vitro studies identified PTHrP in primary cultures of mammary tumors and in breast cancer cell lines, although the amount varied from one line to another (Birch et al. 1995; Guise et al. 1996; Cataisson et al., 2000, Saito et al., 2005). Breast cancer cells frequently spread to bone, where they form osteolytic metastases. Coleman and Rubens found that 69% of patients who died from breast cancer had bone metastases (Coleman and Rubens, 1987). And PTHrP has a major role in the development of such metastases. It is not only linked to the development of bone metastases; it concentration is also increased when breast tumor cells metastasize to bone (Bundred et al. 1992) Metastasis is a complex process involving a cascade of relatively well known cellular events . Many factors (Guise et al., 1996 and 2002), including PTHrP, can facilitate metastasis to bone (Vargas et al. 1992; , Guise et al., 2002, Bouizar et al., 1993 and 1999, Cataisson et al , 2000) *(Figure 4)* .These factors promote the differentiation and activation of osteoclasts, and hence bone resorption. There is considerable evidence that PTHrP promotes metastases to bone (Guise et al 1996, 2002). Studies on Animal models that mimic metastatic bone disease have helped identify the mechanisms responsible for osteolytic metastasis. A popular one is athymic mice injected with human breast carcinoma cells (Arguello et al. 1988; Guise and Mundy, 1998; Peyruchaud et al., 2001). Another uses the injection of MCF-7 mammary carcinoma cells transfected to overexpress PTHrP into the hearts of immunodeficient mice (Thomas et al., 1999). Clinical and experimental evidence indicates that it is important in malignancy; it mediates bone destruction during osteolytic metastasis. Neutralizing antibodies against PTHrP inhibit both the development and the progression of bone metastasis by cells of the human breast cancer cell line MDA-MB-231 in mice (Burtis et al 1990, 1992). The PTHrP produced by breast cancer cells may enable them to grow and invade bone by stimulating osteoclastic activity and bone resorption (Kozlow& Guise 2005).

3.1.2 Malignant humoral hypercalcemia and PTHrP

Parathyroid hormone-related protein (PTHrP) was initially identified because of its involvement in hypercalcemia associated with malignancy Bundred et al 1992. PTHrP is produced and secreted by tumors whose targets are primarily bone and kidney. It has thus been considered to be primarily a deleterious protein responsible for humoral hypercalcemia of malignancy (HHM). This metabolic phenomenon is mediated by the paraneoplastic secretion of PTHrP.The amino-terminal peptide of PTHrP induces hypercalcemia of magliancy, osteoclast-mediated resorption, elevated nephrogenous cAMP, and phosphate excretion. The plasma concentration of PTHrP may be useful for predicting

the occurrence of bone metastasis in breast cancer patients. Immunochemical studies have detected the peptide in 60% of normocalcemic women with a primary breast cancer (Southby et al., 1990). Brunded et al (1992) used this methodology to show that patients who went on to develop bone metastases were more likely to have marked staining. Most (65%) of the hypercalcemic patients with breast cancers metastasizing to bone had elevated plasma PTHrP concentrations (Pvve1i et al 1991). PTHrP mRNA was detected by *in situ* hybridization more frequently in bone metastases than in non-bone metastases (Vargas et al 1992). Our semi-quantitative RT-PCR studies, which are considerably more accurate than immunochemistry, showed that PTHrP mRNA was present in 82% of primary breast cancers (Bouizar et al 1993). We also found PTHrP mRNA in significantly more (97%) patients with primary breast cancer who subsequently developed bone metastases than those who remained free of metastases or developed metastases in soft tissues (Bouizar et al 1993, 1999). Patients with cancer often have severe hypercalcemia (Strewler, 2000). This is and synonymous with a poor prognosis since their median survival time is estimated to be about six weeks (Strewler, 2000; Solimando, 2001). Moreover, the PTHrP concentration is elevated in the serum of 80% of the patients who develop cancer, particularly those with solid tumors (Rankin et al. 1997).

4. PTHrP in different breast cell models

Cultured 8701-BC cells express the genes encoding PTHrP and its receptor and release immunoreactive PTHrP (iPTHrP) fragments into the extracellular medium (Luparello et al 1999) PTHrP 1–34, 67–86 and, to a minor extent, 107–139 have also been described as anti-mitogenic but invasion-promoting for the same cell line (Luparello et al 1999).

Studies with transfected MCF-7 cells have shown that the overproduction of PTHrP is associated with increased mitogenesis via the intracrine pathway. Adding PTHrP 1-34 to cultures of MCF-7 cells stimulated increases in cAMP and cell proliferation , (Falzon et al 2000), but its effects on the PLC signaling arm were not evaluated. Walker carcinoma cells are derived from mammary glands, and are used in animal models of HHM. N-terminal PTHrP and PTH act exclusively through the PLC signal pathway in these cells, and these peptides stimulate these cells to proliferate *in vitro* (Benitez-Verguizas et al 1994).

Endogenous PTHrP appears to stimulate a variety of carcinoma cell lines to proliferate (Martin et al 1997). This may be due to tumor-specific responses to the dysregulated production of PTHrP by these cancerous cells, or a reflection of the tissue-specific growth effects of the peptide. PTHrP gene expression, PTH/PTHrP receptor signaling and PTHrP-induced mitogenesis was investigated in three SV-40 large T immortalized human mammary epithelial cell lines with different degrees of tumorigenicity (Cataissionet al 2000). The S1T3 and S2T2 cell lines were established by immortalizing primary cultures of normal human breast epithelial cells from two individuals with SV40-T Ag. *(Table 1)*Like breast epithelial cells in primary culture, the S1T3 cells did not grow in soft agar and did not form tumors in nude mice. In contrast, S2T2 cells, which also did not grow in soft agar, produced slow growing tumors (<8 mm in diameter 8 to 10 weeks after inoculation) in nude mice. The NS2T2A1 cell line was derived from an S2T2 tumor that had been grown in nude mice, re-established in vitro and then repassaged in nude mice(Berthon et al 1992 , Cataisson et al 2000). The NS2T2A1 line produced colonies in soft agar and tumors that grew rapidly in nude mice. All the cell lines stained for *pan-cytokeratin and cytokeratin 18*, confirming their epithelial nature. S2T2 and NS2T2A1 cells exhibit specific chromosomal markers resembling

those of human breast cancer (Berthon et al., 1992). Cells were cultured in Dulbecco's modified Eagle's and Ham's F12 medium (DMEM/F12, 1/1, v/v) with reduced calcium, 10 mM HEPES, 2 mM glutamine, 10 mg/ml insulin, 5mM cortisol, 2 ng/ml epidermal growth factor, 50 IU/ml penicillin, 50 mg/ ml streptomycin and 5% Chelex-treated horse serum. MDA-MB-231 breast cells lines derived from pleural effusions (Cailleau et al., 1974) were used as positive control of PTHrP gene expression, were cultured in RPMI medium supplemented with 10% fetal calf serum.

	NS2T2A1 Invasive Cell line	S2T2 Tumoregenic Cell line	S1T3 Nontumorogenic Cell Line
Soft agar colonies formation	+++	-	-
Nude mouse Tumors	100%, 3 Weeks	30% , 3 Months	0%
Estrogen Receptor Status	-	-	-
Bone Mestastasis Production	+++	-	-

Table 1. Tumorigenicity, phenotype and caryotype of human mammary epithelial cell line immortalised with SV40 T antigen. (+++) strongly stained by anti–cytoketin 18 and anti-Pan-cytokeratin antibodies , 100 % of positively staining epithelial breast cancer cells (from, Berthon et al1992; Cataisson et al 2000).

The most tumorigenic NS2T2A1 cells contained the most PTHrP mRNA, produced aggressive tumors (Cataisson et al., 2000) and gave rise to bone metastases in mice. PTH/PTHrP receptor1 (PTHrP-R1) mRNA and proteins were detected in all three cell lines. Treatment with hPTHrP (1-34) and hPTH (1-34) increased intracellular cAMP, but not free Ca2+ in the non-tumorigenic S1T3 cells. They increased both cAMP and free Ca2+ levels in the moderately tumorigenic S2T2 cells, but only increased free Ca2 in the highly tumorigenic NS2T2A1 cells. Cataisson et al., 2000, studied the mitogenic effect of PTHrP (1-34) and the signaling pathways coupled to this effect in these three immortalized mammary epithelial cell lines. The tumorigenic NS2T2A1 cells had the highest concentration of PTHrP mRNA and protein. PTHrP 1-34 stimulated NS2T2A1 cells to proliferate, acting via its receptor and the R-PTH1 activation pathway of PLC. The PTHrP-R1 receptor antagonist (Asn^{10} , Leu^{11} , D Trp^{12}) PTHrP (7-34) or anti-PTHrP antibodies dose-dependently reduced [^{3}H] thymidine incorporation into the highly tumorigenic cells but did not affect the other lines. This finding that a PTHrP-R1 receptor antagonist reduced cell proliferation suggests

that PTHrP signaling mediated by the phospholipase C pathway stimulated the proliferation of these highly tumorigenic NS2T2A1 cells. PTHrP also causes 8701-BC breast tumor cells to be more invasive while inhibiting tumor growth (Luparello, et al., 1995). The heterogeneity of clones within a tumor may condition the effect of PTHrP on proliferation by giving responding differently to exogenous PTHrP (Luparello, et al., 1997). Another study by Luparello et al. found that PTHrP (38-94) only inhibited the proliferation of several breast cancer cell lines in vitro and tumor growth in vivo (Luparello et al., 2001). By evaluating PTHrP protein and mRNA expression in the SV40 immortalized human mammary epithelial cell lines, we found that PTHrP protein and mRNA expression levels of the various lines are associated with tumorigenicity. The highly tumorigenic NS2T2A1 and tumorigenic S2T2 cells expressed significantly greater amounts of PTHrP protein and mRNA than the nontumorigenic S1T3 cells. (Cataisson et al 2000).

5. Regulation of PTHrP gene expression in breast cancer cell lines

PTHrP seems to influence the malignant progression of breast cancer. About 60% of primary breast neoplasms contain PTHrP immunoreactivity, as do more than 90% of metastatic foci in bone (Southby et al 1990, Downey et al 1997,). And patients with primary breast cancer who later develop bone metastases have elevated plasma PTHrP mRNA concentrations (Bouizar et al 1993, 1999). The presence of PTHrP immunoreactivity in many breast cancers has led to the suggestion that the peptide has an autocrine effect on tumor growth (Iezzoni et al 1997). And Downey et al found that 100% of the invasive breast carcinomas surveyed had PTHrP immunoreactivity and 96% were labeled with anti-PTH/PTHrP receptor antibodies (Downey et al 1997). Significantly more cells in breast cancer tumors and metastases that are rich in PTH/PTHrP receptor mRNA and protein are labeled with antibodies to the proliferation marker Ki-67, suggesting that this receptor is associated with the increased proliferation of these tumors (Downey et al 1997). We found high concentrations of PTHrP mRNA in primary breast cancers of 110 patients who later develop bone metastases (Bouizar et al 1993, 1999). It is therefore very likely that the peptide influences aspects of the disease that precede metastasis to bone.

5.1 Splicing patterns of PTHrP mRNAs

The complexity of the PTHrP gene suggests that regulation is transcriptional and post-transcriptional, which provides considerable opportunity for dysregulation in neoplastic diseases. We investigated the mechanisms underlying the synthesis of PTHrP in the three SV-40 large T immortalized human mammary epithelial cell lines (S1T3, S2T2 and NS2T2A1) with differing tumorigenicities in order to identify the splicing patterns of their PTHrP mRNAs. The PTHrP gene has three distinct promoters, P1, P2 and P3. The TATA-containing promoter, P3, lies in an intron 35 bp upstream to exon 4 [–4 94 to –486 bp, and the transcription start site is –464 relative to the ATG translation start site in exon 5]. The second TATA promoter, P1, lies 25 bp 5′ to exon 1 and ~2.7 kb upstream of P1[–3250 to –3242 and the transcription start site is –3221]. P2 is a GC-rich regulatory region located upstream of exon 3 and the transcription initiation site is 11 bp upstream of the exon 3 splice acceptor site [–726]. Initial studies of the PTHrP promoters suggested tissue-specific promoter usage. P1 was used infrequently, generally in cell lines and tumors of squamous cell origin.

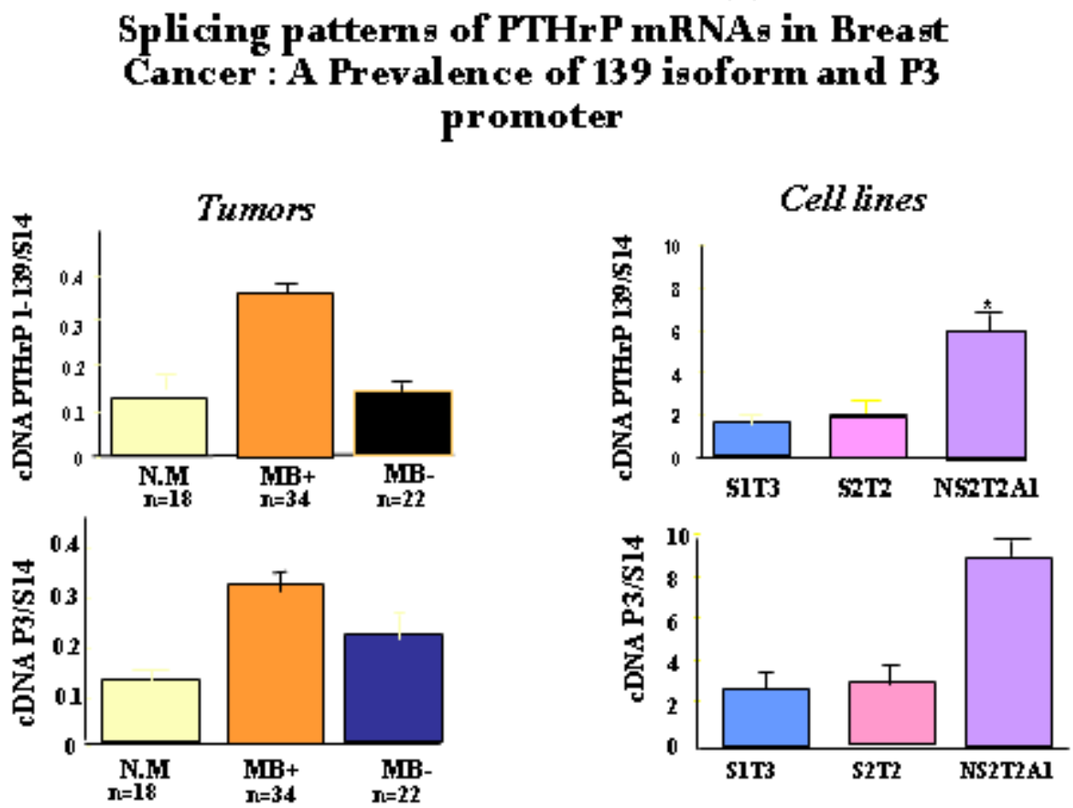
5.2 Splicing patterns of PTHrP mRNAs in breast tumors

We studied 74 primary breast cancer samples by semiquantitative RT-PCR. P2- and P3-derived transcripts were much more abundant than those derived from P1. And P3-derived transcripts were more abundant in samples from patients who later developed bone metastases. The amounts of P2-derived transcripts are significantly higher in samples from patients with mixed metastasis (bone and extra-bone metastasis) than in samples of non-metastatic tumors (Bouizar et al 1999). However, almost all breast tumors that contained P2-initiated transcripts also lacked estrogen receptors and P3 transcripts were the most abundant in breast tumors that metastasized to bone (Bouizar et al., 1999). The concentration of the 1-139 isoform was significantly elevated in primary tumors from breast cancer patients who later developed bone metastases (Bouizar et al 1999), transcripts of the 1-139 isoform derived from the P2 and P3 promoters were more abundant in tumors that ultimately metastasized, and P3 transcripts were the most abundant in tumors that metastasized to bone (Bouizar et al 1999). We found a positive correlation between PTHrP-amplified cDNA and histological node involvement (Bouizar et al 1993). Levels of PTHrP 139 cDNA were increased in the breast tumors of patients with many invaded lymph nodes (Bouizar et al 1999). This suggests that the production of PTHrP 139 is directly or indirectly linked to the ability of malignant breast cancer cells to spread through the lymph system.

Various studies have reported that the incidence of bone metastases is correlated with the histologic grade in breast cancers, and 65% of breast cancers with bone metastases were SBR GRII, while only 30% were GRIII (Coleman et al 1987, Diel et al 1992). We found more 139 PTHrP cDNA in the tumors with SBR GRIl than in those with SBR GR I or III. Thus, the PTHrP 139 isoform is the most abundant in breast cancers (Bouizar et al 1993, Bouizar et al 1999) and its presence is linked to a poor prognosis and the development of bone metastases. This isoform could be a useful prognostic marker in breast cancers. The same trend was observed for P2-initiated transcripts, but the values between groups were not statistically different. The activities of the P2 and P3 promoters were significantly correlated. The upstream TATA box promoter (P1) was rarely used. Therefore, the PTHrP gene seems to use mainly its downstream TATA (P3) and mid-region GC-rich (P2) promoters in breast cancers, especially in those that developed metastases (Bouizar et al 1999) *(Figure2,3).* The tumorigenic NS2T2A1 cells contained increased levels of the transcripts that encode for the 1–139 isoform and showed that the P3 promoter was most active in lines produced high levels of PTHrP mRNA (Cataisson et al, 2003) *(Figure 2,3).*

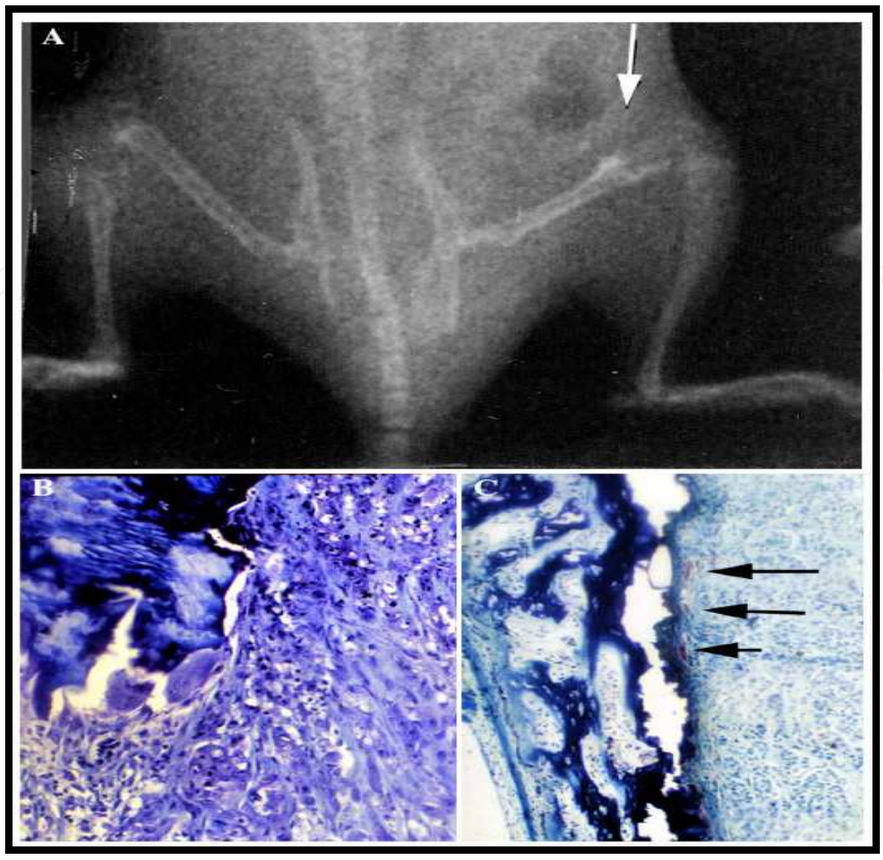
GC-rich *cis* regulatory regions do not appear to be limited to a particular class of genes. They have been located in housekeeping genes and those encoding viruses, receptors, ion-channels, cytokines, structural proteins, and DNA-binding proteins. They are frequently located in the 5´ region of genes lacking typical TATA- or CCAAT-consensus elements. The 5′ untranslated region of the PTHrP gene contains a ‘CpG island' upstream of exon 1C (Vasavada et al, 1993). P2-initiated transcripts only in the S2T2 and NS2T2A1 neoplasic cells and not in the non-tumoral S1T3 cells. The isolated promoter 1 construct (P1) produced very little reporter gene activity in any of the cell lines, and it is not clear whether this result reflected the low endogenous activity of this promoter or whether it lacks appropriate activating sequences. Both the full length (FL) and the promoter 2 (P2) constructs, which has a minimal (30 bases) sequence upstream of the transcription initiation site, produced relatively high reporter gene activities in all three lines. Even the S1T3 line, which contained very few endogenous P2-derived transcripts, produced substantial P2-driven luciferase activity. This finding limits the utility of larger PTHrP P2 containing reporter constructs

with these specific lines. In contrast, reporter gene activity from an isolated promoter 3 construct that contained 140 bp of upstream sequence (P3-BH) reflected endogenous P3 activity because luciferase activity gradually increased in the S2T2 and NS2T2A1 cells. While P2 appears to be active in a wide variety of cell lines and tumors quantitative studies will be needed to determine the contribution of this promoter to PTHrP gene expression in breast cancer cells *(Figure 4)*



RT-PCR of the PTHrP isoforms 1–139 and 1–141/ S14 according to the development of metastases.
S14: internal control gene S14 (human small ribosomal protein 14)
n : number of patients
Tumors from patients without metastases (No Metastasis) (NM)
Tumors from patients that formed Bone Metastases (BM+)
Tumors from patients developing soft tissue metastases (BM-)

Fig. 2. Prevalence of PTHrP 139 and the P3 (TATA) promoter in invasive breast cancer and invasive breast cancer cell line (NS2T2A1)(Bouizar et al, 1999, Cataisson et al 2003).



(A) Radiograph of bone metastases (white arrows) induced by injecting NS2T2A1 cells intracardially into athymic mice. (B) Histological image showing invasion of the bone marrow by the tumor mass. (C) osteoclasts stained for alkaline phosphatase (black arrows). (Bouizar et al., unpublished data).

Fig. 3. The invasive nature of NS2T2A1 cells.

6. The P3 (TATA) promoter in PTHrP transcription

The way PTHrP gene expression differs among tumors appears to result from altered transcription (Gillespie and Martin, 1994).The P3 promoter contains a series of transcription factor binding sites located ~ 50 bp upstream and downstream of the TATA box. The Ets, SMAD, and Sp1 binding sites and a CRE-like site may be involved in the control of PTHrP gene expression in some cancer cell lines (Cataisson et al., 2003; Lindemann et al., 2001; Karperien et al., 1997; Chilco et al., 1998). Also, bone osteoclasts may mediate the release of TGF-β from the bone matrix in order to stimulate PTHrP gene expression in breast cancer cells (Yin et al., 1999, Guise et al., 2002). A recent study of PTHrP promoter activity in MDA MB-231 breast cancer cells indicated that the P3 promoter is activated by TGF-β, and requires the synergistic interaction of SMADs, components of the TGF-β signaling pathway, and Ets factors (Lindemann et al., 2001).

6.1 Transcription factors that regulate the P3 promoter

We have established immortalized human mammary epithelial cell lines whose differing tumorigenicities are correlated with their PTHrP gene expression (Cataisson et al., 2000).

The Ets binding site of the PTHrP P3 promoter and the amount of Ets1 were crucial for PTHrP production by the most tumorigenic NS2T2A1 cells (Cataisson et al., 2003). However the precise mechanisms by which Ets1 activates PTHrP gene expression during tumor progression in breast cancer are unknown. We have also shown that the transcription factor Ets1 and its DNA binding site (EBS) are major determinants in the activation of PTHrP transcription in tumorigenic NS2T2A1 cells. These cells also contain higher concentrations of Ets1 protein (and mRNA) than S2T2 and S1T3 cells (Cataisson et al., 2003), which again suggests that this factor is responsible for the increased activity of the EBS-dependent PTHrP reporter gene. However, since not enough Ets1 was produced to activate PTHrP reporter gene expression in S2T2 and S1T3 cells, there are probably other transcription factors and/or coactivators contributing to the regulation of PTHrP gene expression in the fully tumorigenic cells (Cataisson et al., 2003).

Ets factors typically have a conserved winged helix-turn-helix DNA-binding domain (Wasylyk et al., 1993) that recognizes the core motif 5′GGA(A/T)3′ whose flanking nucleotides determine specificity (Wasylyk et al., 1993 and (Sementchenko and Watson, 2000). Ets1 is overproduced in a variety of malignant tumors, including mammary carcinomas and tumorigenic cell lines (Delannoy-Courdent et al., 1996 and Cataisson et al., 2003). Like other regulators, the Ets proteins regulates transcription by interacting with other nuclear factors such as NFκB (Bassuk et al., 1997), AP-1 (Bassuk and Leiden, 1995), Pax (Fitzsimmons et al., 1996), (Dittmer et al., 1997 and Gitlin et al., 1993), Sp1 (Dittmer et al., 1997 and Block et al., 1996), CREB (cyclic AMP response element binding protein) (Wardle et al., 2002), CBP (Foulds et al., 2004 and Wang et al., 2004), and STAT factors (signal transducers and activators of transcription) (Aittomaki et al., 2000 and Rameil et al., 2000). CREB and Ets may cooperate to recruit CBP. We know that the P3 promoter in lung squamous carcinoma cell lines is sensitive to cAMP stimulation (Chilco et al., 1998) and there is a CRE-like site in the non-coding portion of the exon 4, 56bp downstream the TATA box. We investigated the relationship between the binding of Ets1 to the EBS site and the concentration of CBP and P3 promoter function in our three tumorigenic cell lines and whether the transcription factor CREB helps control of the PTHrP P3 promoter. The transcription factor CREB and its coactivator, CBP, were first identified as mediators of cAMP/PKA signaling (Gonzalez et al., 1989; Arias et al., 1994 and Chrivia et al., 1993). It is now clear that the coactivator CBP and the related protein p300 activate specific genes and recruit other coactivators to transcription initiation complexes. They interact with several transcription factors to form multimolecular complexes that regulate the transcription of eukaryotic genes (Janknecht and Hunter, 1996 and Goodman and Smolik, 2000). Ets1 recruits CBP and p300 during the activation of the human stromelysin promoter, while the histone acetyl transferase (HAT) activity of the proteins is required for effective transactivation of this gene (Jayaraman et al., 1999). Thus, the recruitment of CBP/p300 by DNA-bound transcription factors like Ets1 could facilitate the formation of pre-initiation complexes at promoters like P3. We used gel retardation assays with competition studies and supershift assays with specific anti-Ets1 antibodies to show that Etsl interacts with its DNA binding site (EBS) on the PTHrP P3 promoter. We found that extracts of tumorigenic cell lines contained more Ets1-DNA-binding complexes than pre-tumorigenic and non-tumorigenic cells, which correlated with their Ets1 contents (Cataisson et al., 2003). Western blot analyses showed that there were higher basal concentrations of CBP/P300 in invasive tumorigenic NS2T2A1cells than in the pre-tumoral S2T2and non-tumoral and S1T3 cells. The transfection of Ets1 and CBP/p300 expression vectors enhanced Ets1-activated P3

reporter gene activity. This cooperation was specific to tumorigenic NS2T2A1 cells. These cells not only have high concentrations of CBP/p300 and Ets1 cooperate in PTHrP P3 reporter gene transactivation, but this action is strictly dependent on the integrity of the EBS, indicating that CBP is directly recruited by Ets1. The involvement of CBP in basal and Ets1-induced P3 promoter. Our results are in agreement with previous findings showing that 12S-E1A represses PTHrP gene expression in murine keratinocytes and in human squamous carcinoma cell lines. Foley et al., 1999, Jayaraman et al., (1999) found that Ets1 recruited CBP and p300 during the transcriptional activation of the human stromelysin promoter and their HAT activity was required for effective transactivation. It is generally believed that the interaction of transcription factors with CBP/p300 localizes the coactivators to specific DNA regions, resulting in site-specific histone acetylation, chromatin remodeling and the activation of specific genes. Thus, the recruitment of CBP/p300 by DNA-bound transcription factors could facilitate the formation of pre-initiation complexes at relevant promoters (Oikawa and Yamada, 2003).

6.2 Crosstalk between transcription factors

Crosstalk between transcription factors is a commonly recognized mode of gene regulation. A STAT DNA binding site called GAS in the PTHrP P3 promoter (Richard et al., 2005) overlaps the EBS site. STAT proteins are latent cytoplasmic factors that can be activated by tyrosine phosphorylation by members of the Janus tyrosine kinase (JAK) family in response to a variety of cytokines and growth factors (Wang et al., 2005). STAT3 and STAT5 are constitutively activated in several cancers, including breast cancer, and during cell growth and transformation (Garcia et al., 2001). We found only phosphorylated STAT3 in our three cell lines and no evidence that it interacted with the EBS/GAS or was involved in the expression of the PTHrP gene. However, it is always possible that bone-derived cytokines disrupt the JAK-STAT pathway during bone invasion and influence PTHrP gene transcription.

NS2T2A1 cells should also possess additional factors required for the Ets1-mediated activation of PTHrP reporter genes (Cataisson et al., 2003). There are other regulatory sites on the P3 promoter in addition to EBS/GAS; they are SMAD, AP1, AP2 and SP1. We found binding activities for the Ap1, Ap2 and Sp1 consensus sequences in nuclear extracts of NS2T2A1 cells (data not shown), suggesting that the cognate transcription factors may participate in PTHrP-P3 promoter activity. Further studies with P3 promoter sequences, specific mutations used for EMSA and transfection experiments are required to understand how other transcription factors participate in PTHrP P3 promoter activity in tumorigenic cells.

6.3 CREB transcription factor

We also found that the transcription factor CREB was present in the cells of all three lines and that the p-CREB/CREB ratio increased with their tumorigenicity. Electrophoretic mobility shift assay (EMSA) or gel shift assay is one of the most powerful methods for studying protein–DNA interactions. Typically, ^{32}P-labeled DNA probes containing the sequence bound by the protein of interest are used in EMSA (Lance et al, 2007). However, using an electrophoresis mobility shift assay indicated that CREB was not bound to CRE-like sequences (Hamzaoui et al, 2007). This suggests no binding or a low affinity interaction, possibly due to the presence of T instead of G in the CRE-like sequence (data not shown). Nevertheless mutations in the CRE-like site abrogated activation by Ets1 as well as cooperativity between Ets1 and CBP, indicating that sequences downstream of the EBS

recruit factors and coactivators that help stabilize the EBS-Ets1 complex. The CRE-like region is followed by an AP2 site. So, member of the b-ZIP family of transcription factor may form heterodimers at this composite site, recruit CBP and take part in EBS functioning. The enhancement of P3 promoter activity by CREB or constitutively active CREB overproduction, independently of the integrity of EBS and/or CRE-like sequences, may also indicate that CREB promotes DNA-binding-independent transcriptional activation, as it does in other models (Giebler et al., 2000). CREB may thus tether CBP to an as-yet-unidentified transcription factor/regulatory site on the P3 promoter. We are now working to determine how CREB acts on the P3 promoter and its relationship with the tumorigenic potential of breast cancer cell lines.

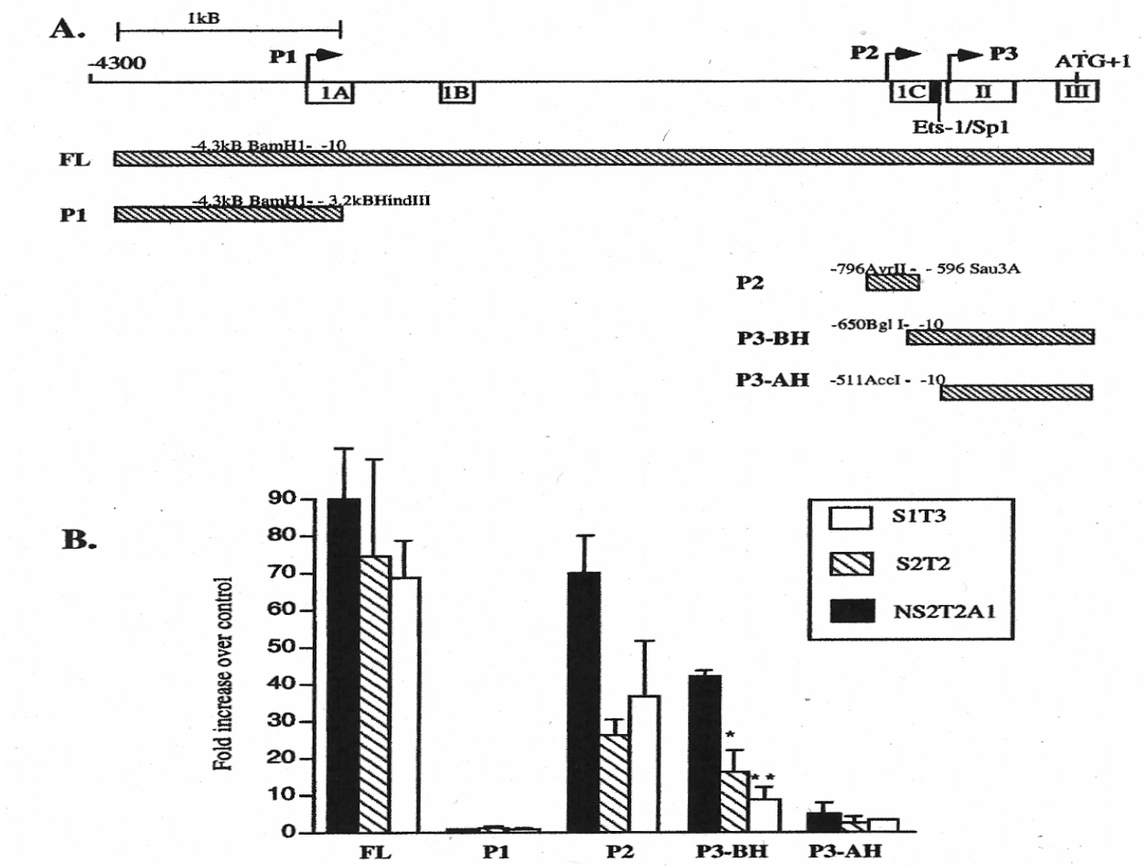


Fig. 4. Expression of reporter genes driven by 5'PTHrP sequences in S1T3, S2T2 and NS2T2A1 cells. Panel A: a schematic representation of the 5'- flanking region of the human PTHrP gene and the 5'PTHrP-Luc constructs used in the analysis: exons are indicated by boxes, promoters by arrows and the shaded region represents the region of the EBS-1 and Sp1trascription factors sites. The construct represented as FL (full length) contains 4.3 kb of upstream sequence and all three promoters. P1 contains the P1 promoter in isolation and P2 contains the P2 promoter and minimal (30 bases) sequence upstream of the transcription initiation site. P3-BH contains 140 bp upstream of the P3 TATA including the Ets-1/Sp1 site and the downstream non-translated exon II. P3-AH contains only 15 bp of upstream sequence, thus eliminating the Ets-1/Sp1 site, but contains downstream sequences. Panel B: Substantial P2-driven luciferase activity and relatively high level of the reporter gene

activity in the NS2T2A1 line *V/S* S1T2 and S1T3 lines .The bar graph depicts reporter gene activity normalized to the activity of a promoter less luciferase vector pGL-2. Bars represent the average of three samples and error bars indicate SEM. If an error bar is not present it indicates the error was not of sufficient magnitude to be illustrated on the graph. Every construct was transfected into each of the three lines in three separate experiments and similar results were obtained. * represents PB/0.05 NS2T2A1 vs S2T2; ** represents PB/0.01NS2T2A1 vs S1T3.(Cataisson et al 2003).

In conclusion, we have clearly established the importance of cooperation between Ets1 and CBP at the EBS regulatory sequence of the P3 promoter. We have proposed that there is cross-talk between Ets1 and transcription factors (like CREB) that have affinity to and share a linkage with coactivators such as CBP. (Son et al 2010), reported that metastatic MDA-MB-231 breast cancer cells contained more CREB than did non-metastatic MCF-7 cells. They used wild-type CREB and a dominant-negative form (K-CREB) to show that CREB signaling positively regulated the proliferation, migration, and invasion of MDA-MB-231 cells. K-CREB also prevented MDA-MB-231 cell-induced osteolytic lesions in a mouse model of cancer metastasis. Lastly, CREB signaling regulated the expressions of the PTHrP, MMPs, and OPG genes, which are all closely involved in cancer metastasis and bone destruction. Thus CREB is overproduced in developing breast cancer cells and this CREB upregulation is important for many steps in the metastasis of breast cancers to bone, along with the stimulation of PTHrP gene expression. These findings open up new horizons in the study of the modulation of the PTHrP promoter activity and its impact on the devastating results of PTHrP overexpression (invasion, metastasis, HHM, etc.). They may even reveal new targets forbreast c ancer therapy.

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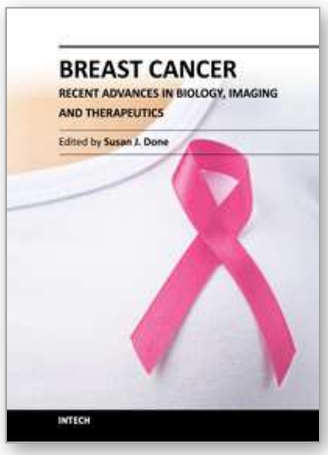

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In recent years it has become clear that breast cancer is not a single disease but rather that the term encompasses a number of molecularly distinct tumors arising from the epithelial cells of the breast. There is an urgent need to better understand these distinct subtypes and develop tailored diagnostic approaches and treatments appropriate to each. This book considers breast cancer from many novel and exciting perspectives. New insights into the basic biology of breast cancer are discussed together with high throughput approaches to molecular profiling. Innovative strategies for diagnosis and imaging are presented as well as emerging perspectives on breast cancer treatment. Each of the topics in this volume is addressed by respected experts in their fields and it is hoped that readers will be stimulated and challenged by the contents.

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