Expression and function of the novel proto-oncogene PBF in thyroid cancer: a new target for augmenting radioiodine uptake

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Abstract

Pituitary tumor-transforming gene (PTTG)-binding factor (PBF; PTTG1IP) was initially identified through its interaction with the human securin, PTTG. Like PTTG, PBF is upregulated in multiple endocrine tumours including thyroid cancer. PBF is believed to induce the translocation of PTTG into the cell nucleus where it can drive tumourigenesis via a number of different mechanisms. However, an independent transforming ability has been demonstrated both in vitro and in vivo, suggesting that PBF is itself a proto-oncogene. Studied in only a limited number of publications to date, PBF is emerging as a protein with a growing repertoire of roles. Recent data suggest that PBF possesses a complex multifunctionality in an increasing number of tumour settings. For example, PBF is upregulated by oestrogen and mediates oestrogen-stimulated cell invasion in breast cancer cells. In addition to a possible role in the induction of thyroid tumourigenesis, PBF over-expression in thyroid cancers inhibits iodide uptake. PBF has been shown to repress sodium iodide symporter (NIS) activity by transcriptional regulation of NIS expression through the human NIS upstream enhancer and further inhibits iodide uptake via a post-translational mechanism of NIS governing subcellular localisation. This review discusses the current data describing PBF expression and function in thyroid cancer and highlights PBF as a novel target for improving radioiodine uptake and thus prognosis in thyroid cancer.

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Introduction

Pituitary tumor-transforming gene (PTTG)-binding factor (PBF) is a proto-oncogene with particular importance to endocrine neoplasia. Although lack of significant homology with other human proteins limits prediction of a specific protein role, it also suggests that PBF has unique functionality, and ongoing studies of PBF reveal increasing number of modes of action. Further, PBF is ubiquitously expressed and highly conserved across a wide diversity of animal species suggesting significant evolutionary importance.

PBF was discovered through a yeast two-hybrid screen to identify proteins that interact with the human securin PTTG (Chien & Pei 2000). Specific binding between PTTG and PBF was demonstrated both in vitro and in vivo through pull-down and co-immunoprecipitation assays in COS-7 cells. Through deletion analysis, the PTTG binding domain in PBF was identified within the C-terminal 30 amino acids and the corresponding domain in PTTG was found to be between amino acids 123 and 154 (Chien & Pei 2000).

PBF, also known as PTTG1-interacting protein (PTTG1IP), encodes a protein of 180 amino acids with a predicted molecular mass of 22 kDa (Chien & Pei 2000). Located on chromosome 21q22.3, PBF had previously been cloned and termed C21orf3 (also known as C21orf1). Given the low incidence of solid tumours in trisomy 21, it is intriguing that PBF is found in the distal portion of chromosome 21 to which the majority of phenotypic features of Down’s syndrome map (Lyle et al. 2009). There are currently no data available pertaining to the loss of PBF in either animal knock-out models or in human disease. However, a large heterozygous deletion spanning the integrin β2 (ITGB2) and PBF genes that resulted in the loss of PBF was detected in a patient with leukocyte adhesion deficiency type-1 (LAD-1). This condition is caused by genetic alterations in the ITGB2 gene, and no apparent novel or exacerbated phenotype that could be attributed to the loss of one PBF allele was reported in this patient. Further, the mutation was inherited from the patient’s father who was seemingly phenotypically normal. Hence, a single functional PBF allele appears to be sufficient in humans (Bernard Cher et al. 2011).

Initial protein prediction studies suggested that C21orf3 was a cell surface glycoprotein due to a potential N-terminal signal peptide, transmembrane domain, endocytosis motif and
two putative N-glycosylation sites (Yaspo et al. 1998). PBF also possesses an extracellular N-terminal cysteine-rich region, similar to that found in the membrane-associated plexins, semaphorins and integrins, and hence referred to as a PSI domain (Bork et al. 1999). In contrast to evidence supporting the characterisation of PBF as a membrane protein, the presence of a bipartite nuclear localisation signal (NLS) near the C-terminus suggested that PBF may also be a nuclear protein (Chien & Pei 2000).

The presence of the putative NLS at the C-terminal end of PBF, therefore, prompted subcellular localisation studies. In COS-7 cells, haemagglutinin-tagged PBF (HA-PBF) was located mainly in the nucleus, with significant staining also in the cytoplasm. Mutation of the NLS predominantly shifted PBF expression to a perinuclear and cytoplasmic location, confirming its requirement for the nuclear localisation of PBF. PTTG, tagged with green fluorescent protein (GFP-PTTG), was predominantly observed in the cytoplasm, with partial nuclear localisation, whereas co-transfection of HA-PBF resulted in increased nuclear translocation of GFP-PTTG. This effect was abrogated when the mutated NLS was used, indicating that PBF with an intact NLS is required for PTTG translocation into the nucleus (Chien & Pei 2000).

PTTG is a complex, multifunctional protein involved in a wide range of cellular processes such as cell cycle regulation, genetic instability and gene transactivation (Vlotides et al. 2007). Overexpression of PTTG has been observed in a number of cancers, including thyroid cancer (Heaney et al. 2001, Boelaert et al. 2003, Kim et al. 2006). PTTG induces cellular transformation in vitro and tumourigenesis in vivo, and a number of mechanisms by which PTTG contributes to tumourigenesis have been investigated. These are discussed in detail in a recently published review (Smith et al. 2010). PBF and its interaction with PTTG have been utilised in an innovative potential therapeutic tool to knock-down PTTG. A fusion protein comprising PBF and part of the F box protein β-TrCP, a subunit of a ubiquitin protein ligase complex, specifically targets PTTG for degradation. Overexpression of the chimeric protein in HeLa cells resulted in a significant reduction in PTTG protein levels, accompanied by encouraging antitumour effects such as reduced fibroblast growth factor (FGF-2) expression, cell growth and ability to form colonies in soft agar (Mo et al. 2009).

All known functions of PTTG require its presence in the nucleus; therefore, PBF may contribute to the oncogenic effects of PTTG through increasing its nuclear entry. However, similar to PTTG, PBF demonstrates transforming ability and is overexpressed in a growing number of tumour types (McCabe et al. 2003, Stratford et al. 2005, Watkins et al. 2010). Following its discovery, many of the subsequent studies of PBF have also assessed PTTG and attempted to delineate the contribution of each protein (Smith et al. 2010). However, the individual functions of PBF have been most thoroughly examined in the thyroid, and hence this review focuses on PBF function in the thyroid and the implications of its overexpression on the progression and treatment of thyroid cancer.

**PBF expression**

As determined by northern blot analysis, PBF is widely expressed in normal human tissues, including normal thyroid (Yaspo et al. 1998, Chien & Pei 2000). Initially, PBF expression was found to be upregulated in pituitary tumours compared with normal pituitary tissue suggesting a potential involvement of PBF in tumourigenesis (McCabe et al. 2003). Further, a significant positive correlation between PTTG and PBF expression was observed in pituitary adenomas but not in normal pituitary tissue, suggesting that an association between PTTG and PBF mRNA expression may be important in tumourigenesis. However, expression levels per se were not associated with clinical parameters (McCabe et al. 2003).

Although expression was low or absent in normal breast tissue, immunohistochemical analysis of tissue microarray (TMA) tumour samples demonstrated that PBF was strongly expressed in epithelial cells of all types and grades of breast tumour assessed (Watkins et al. 2010). The region of the PBF promoter −399 to −291 relative to the translational start site contains between 1 and 6 repeats of an 18 bp sequence housing a putative oestrogen response element (ERE) half-site. This region was bound by estrogen receptor-α (ER-α) in ChIP studies and conferred most of the responsiveness of PBF to oestrogen. Subjects with greater numbers of ERE repeats showed higher PBF mRNA expression, and PBF protein expression positively correlated with ER-α status. PBF knockdown significantly abrogated oestrogen-mediated MCF-7 cell invasion through Matrigel (BD Biosciences, Oxford, UK). Further, secretion of PBF was critical in the induction of cell invasion (Watkins et al. 2010).

**PBF in thyroid cancer**

PBF expression was found to be significantly increased in thyroid cancer and independently associated with early tumour recurrence (Stratford et al. 2005). When stratified by tumour type, there was no significant difference in PBF expression between papillary (n = 17) and follicular (n = 7) carcinomas. No mutations were observed when the coding region of PBF was sequenced in these 24 thyroid tumours (Stratford et al. 2005). PBF expression was significantly and positively correlated with PTTG expression, and a potential contributing factor to this relationship was identified through subsequent in vitro studies, which found that over expression of PTTG in primary human thyroid cultures and PTTG-null HCT116 colorectal carcinoma cells significantly increased PBF expression. However, abrogation of its proline-rich region containing potential binding sites for Src-homology-3 (SH3) domains, mediators of intracellular signal transduction pathways, rendered PTTG unable to stimulate PBF expression. NIH3T3 cells stably transfected with wild-type PTTG also demonstrated increased PBF expression. Conversely, PTTG expression was not stimulated in PBF stable cell lines (Stratford et al. 2005).
Given the high levels of PBF and PTTG expression in multiple tumours, transforming potential was assessed using soft agar assays (Stratford et al. 2005). Stably transfected NIH3T3 clones with high expression of either PBF or PTTG led to significant colony formation (Fig. 1A).

Interestingly, mutated forms of PTTG that could neither stimulate PBF mRNA expression (SH3 domain binding site) nor interact with PBF (amino acids 123–154) did not induce colony formation. Hence, PBF is a transforming gene in vitro and may mediate the transforming ability of PTTG. Further, the tumourigenic ability of PBF in vivo was observed when injection of NIH3T3 cells stably expressing PBF into nude mice induced tumour formation (Stratford et al. 2005; Fig. 1B).

PTTG increases the expression and secretion of basic FGF-2 in a manner that is dependent on its SH3 domain binding motif (Zhang et al. 1999, Ishikawa et al. 2001). Conditioned medium from NIH3T3 cells overexpressing PTTG induced the proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) in vitro, although a neutralising FGF-2 antibody abrogated these effects. This suggests that PTTG transactivates FGF-2 via its SH3 domain binding site, which in turn mediates the induction of angiogenesis (Ishikawa et al. 2001).

In differentiated thyroid cancer, FGF-2 expression is increased, suggesting overall that either one or two copies of PBF allows normal angiogenic responses, whereas three copies are inhibitory (Reynolds et al. 2010). These studies appear to conflict with the existing data that strongly suggest that PBF is pro-tumourigenic. The mechanism behind the anti-angiogenic action of PBF in Down’s syndrome remains to be elucidated but represents an intriguing area of research.

PBF and iodide uptake

Radioiodine is central in the treatment of thyroid tumours and their metastases and has been used effectively over 60 years. This treatment is dependent on the sodium iodide symporter (NIS), which under normal conditions mediates the uptake of iodide from the bloodstream across the basolateral plasma membrane of thyroid follicular cells for thyroid hormone biosynthesis. Prognosis is good for the majority of patients with differentiated thyroid cancer, as radioiodine ablation of the thyroid bed following surgery is highly effective in removing the primary tumour. However, up to 35% of these tumours recur (Mazzaferri & Kloos 2001). Crucially, NIS activity is diminished in thyroid cancers due to down-regulated expression levels and impaired targeting to, or retention at, the plasma membrane where NIS is functional. Even after TSH stimulation to increase iodide uptake, 10–20% of differentiated tumours do not accumulate sufficient radioiodine for destruction. Thyroid tumours and metastases that are radioiodine refractory are associated with a poor prognosis. Understanding and overcoming the mechanisms by which iodide uptake is reduced in thyroid
PTTG overexpression was found to decrease iodide uptake in both rat thyroid FRTL-5 cells and human primary thyroid cells, and NIS expression was reduced in the human thyrocytes (Heaney et al. 2001). More recently, high PTTG expression has significantly been associated in vivo with decreased radioiodine uptake during patient follow-up (Saez et al. 2006). The initial observation that PTTG may be involved in the regulation of iodide uptake led us to investigate the possibility of a similar role for PBF. We subsequently found that in our series of thyroid cancers, which demonstrated increased PTTG and PBF expression, NIS expression was significantly reduced compared with normal thyroid (Boelaert et al. 2007), showing a significant negative correlation with both PTTG and PBF expression (K Boelaert, VE Smith, JA Franklyn, CJ McCabe unpublished data). Reduced NIS expression was also associated with early tumour recurrence and nodal involvement, further emphasizing the importance of NIS activity in patient outcome (Boelaert et al. 2007).

A significant reduction in NIS mRNA expression and $^{125}$I uptake following PTTG overexpression was replicated in vitro in primary human thyroid cultures (Boelaert et al. 2007; Fig. 2A). PTTG with a disrupted SH3 domain binding site also repressed NIS mRNA, suggesting that the transactivation capability of PTTG is not required for the inhibition of NIS mRNA expression, and PTTG may act independently of PBF upregulation. However, the SH3 mutant PTTG exhibited a reduced ability to repress iodide uptake compared with wild-type PTTG, indicating that this repression is at least partially dependent on SH3 domain binding. Interestingly, PBF overexpression also resulted in significantly reduced NIS expression and iodide uptake. Further, the greatest reduction in iodide uptake seen was a synergistic inhibition following the co-transfection of both PBF and PTTG cDNA (Boelaert et al. 2007).

Within this model, PTTG-mediated repression of iodide uptake was significantly abrogated by FGF-2 antibody treatment, suggesting that it is influenced by FGF-2 secretion, whereas repression by PBF remained unchanged, suggesting that this is independent of FGF-2 (Boelaert et al. 2007).

Promoter studies identified the human NIS upstream enhancer (hNUE) as the element responsible for mediating the repression of NIS by PTTG and PBF using both FRTL-5 cells and human primary thyroid cultures (Boelaert et al. 2007; Fig. 2B). The hNUE found 7–9 kb upstream of NIS is thyroid-specific and TSH-responsive. A PAX8 binding site, located between bases −9286 and −9298, and a CAMP-responsive element (CRE)-like sequence, are both essential for full hNUE activity (Taki et al. 2002). Interestingly, a putative binding site for upstream stimulating factor 1 (USF1) lies within the PAX8 site and this overlapping PAX8/USF1 sequence is highly conserved between species (Lin et al. 2004). Because PTTG binds to the c-myc promoter in a complex with USF1 (Pei 2001) and putative USF1 binding sites are found upstream of genes regulated by PTTG, such as FGF-2 and VEGF (unpublished observations), the possibility that this region might confer the regulation of NIS by PTTG and PBF was examined (Boelaert et al. 2007). Discrete mutation of either the USF1 or the PAX8 site resulted in a significant disruption of promoter activity, confirming the importance of the PAX8 binding site and suggesting that USF1 binding is also required for full hNUE activity (Boelaert et al. 2007). Interestingly, the substitution of just two bases within the USF1 consensus significantly abrogated the ability of both PTTG and PBF to inhibit NIS activity. Disruption of the PAX8 binding site failed to significantly diminish the ability of PTTG to repress the hNUE but prevented PBF repression of the hNUE. Overall, this suggests that the PAX8/USF1 site plays a critical role in mediating the repression of PTTG and PBF on the human NIS promoter (Boelaert et al. 2007).

We have also recently demonstrated a post-translational mechanism of NIS repression by PBF (Smith et al. 2009). Knowledge regarding the regulation of NIS trafficking in the thyroid is still limited. TSH is required for the post-translational regulation of NIS, in addition to stimulating cAMP-mediated NIS expression (Riedel et al. 2001). There is
evidence that TSH is required for either the targeting of NIS to the plasma membrane or its retention there, as NIS is located in the plasma membrane of thyroid cells in the presence of TSH but is redistributed into intracellular vesicles upon TSH withdrawal (Kaminsky et al. 1994, Kogai et al. 1997, Riedel et al. 2001). Differing patterns of NIS phosphorylation have also been observed in FRTL-5 cells maintained in the presence or absence of TSH (Riedel et al. 2001). However, although a number of phosphorylated residues have been confirmed, none have been shown to affect NIS trafficking (Vadysirisack et al. 2007).

Through immunofluorescent studies in COS-7 cells, exogenous NIS localisation was found to be predominant within the plasma membrane with some staining within intracellular vesicles (Smith et al. 2009), the latter reminiscent of those previously described in TSH studies (Kaminsky et al. 1994, Riedel et al. 2001). PBF was expressed at relatively low levels in the nucleus, whereas the majority of PBF protein was concentrated within intracellular vesicles in the cytoplasm. When NIS and PBF were co-expressed in both COS-7 cells and FRTL-5 cells, strong colocalisation was observed, particularly within intracellular vesicles. Glutathione S-transferase (GST) pull-down assays and co-immunoprecipitation assays revealed that NIS and PBF not only colocalised but also interacted in vitro. Critically, when PBF was co-transfected with NIS, there was increased NIS staining within intracellular vesicles and a concomitant reduction in membrane staining (Fig. 3A). Cell surface biotinylation assays quantitatively confirmed that PBF overexpression resulted in a reduction in plasma membrane expression of NIS (Smith et al. 2009; Fig. 3B).

PBF demonstrated strong colocalisation with endogenous expression of the late endosomal marker CD63, a member of the tetraspanin family that is commonly associated with clathrin-dependent endocytosis (Smith et al. 2009). NIS also colocalised with CD63. Analysis of caveolin-1, the main constituent of caveolae that acts as a regulator of caveolae-dependent lipid trafficking and endocytosis, revealed no colocalisation between caveolin-1 and either PBF or NIS. These data suggest that clathrin-dependent rather than caveolae-dependent endocytosis is responsible for the internalisation of these proteins (Smith et al. 2009).

To further investigate the relationship between PBF and NIS, three deletion mutants of PBF (M1–M3) were created (Smith et al. 2009). WT PBF was again predominantly localised within intracellular vesicles. By contrast, M1 (Δ149–180) was located almost exclusively in the plasma membrane, potentially through the deletion of a putative tyrosine-based sorting signal at the C-terminus. M2 (Δ29–93) and M3 (Δ94–149) appeared to be predominantly expressed in the endoplasmic reticulum. Markedly, none of the mutants colocalised with CD63, strongly suggesting that they were not expressed within late endosomes (Smith et al. 2009). In FRTL-5 cells, endogenous NIS activity was measured in response to WT PBF and mutant PBF overexpression. Although WT PBF significantly repressed iodide uptake, none of the three mutants, which did not localise within intracellular vesicles with either CD63 or NIS, were able to inhibit NIS activity.

Overall, these studies suggest a mechanism by which PBF binds NIS and redistributes NIS away from the plasma membrane into late endosomes, resulting in significantly repressed cellular iodide uptake. To this end, we are currently challenging these observations in vivo, with the effect of PBF on NIS expression and function being analysed in a murine model in which PBF overexpression is targeted specifically to the thyroid gland.

Figure 3 PBF alters the subcellular localisation of NIS. (A) Compared with vector only (VO) control (i), immunofluorescent detection of NIS-HA in COS-7 cells shows increased NIS staining within intracellular vesicles with PBF overexpression (ii). Scale bars: 20 μM. (B) Cell surface biotinylation assay in COS-7 cells transfected with a VO control or PBF. Representative immunoblot analysis of membrane-bound NIS (i) and total cellular NIS, (ii) Flotillin-1 was used as a marker of membrane protein expression to determine loading between samples. Graphs indicate mean differences in expression as determined by scanning densitometry (n = 3). *P < 0.05; ns, not significant. Taken from Smith et al. (2009).
Conclusions

PBF is a proto-oncogene that is upregulated in thyroid cancer (Stratford et al. 2005). In addition to a potential role in driving thyroid tumorigenesis, PBF also hinders one of its key treatments, radioiodine. PBF potently represses iodide uptake in the thyroid both through the transcriptional inhibition of NIS expression and via a post-translational mechanism that regulates NIS localisation (Boelaert et al. 2007, Smith et al. 2009).

PBF may contribute to thyroid tumorigenesis through increased translocation of PTTG into the cell nucleus and, in support of this, nuclear PBF has been observed (Chien & Pei 2000, Smith et al. 2009). However, there is increasing evidence to suggest that PBF can act independently of PTTG (Stratford et al. 2005, Smith et al. 2009, Watkins et al. 2010). Although the earlier study did not describe vesicular staining (Chien & Pei 2000), more recent studies have identified PBF predominantly within intracellular vesicles and also at the plasma membrane (Stratford et al. 2005, Smith et al. 2009). This suggests that PBF is indeed a membrane-associated protein as originally predicted (Yaspo et al. 1998), and further studies may elucidate the functional roles of this protein in the cell membrane and within the cytoplasmic compartments, both under normal conditions and in tumourigenesis.

Owing to the long half-life of NIS, estimated at 3–5 days (Riedel et al. 2001), post-translational regulation would allow cells to regulate iodide uptake over a much shorter timeframe. In support of this, significant internalisation of NIS has been demonstrated within 48 h of PBF transfection (Smith et al. 2009). Membrane vesicles containing NIS have been found to be enriched for plasma membrane content, suggesting that NIS is regulated through an endocytosis-mediated pathway (Kaminsky et al. 1994). PBF appears, therefore, to significantly modulate this process, although it is yet to be determined whether PBF is involved in NIS regulation in the normal thyroid (Smith et al. 2009).

Although TSH is the only known regulator of NIS plasma membrane targeting in normal thyroid, within the thyroid tumour setting NIS localisation may also be modified by the activating mutation BRAF<sup>V600E</sup>, the most common genetic alteration in thyroid cancers. In vitro studies have demonstrated that, in addition to repressing NIS expression, BRAF<sup>V600E</sup> impairs NIS trafficking via increased transforming growth factor-β secretion (Riesco-Eizaguirre et al. 2006, 2009). Given the ability of PBF to similarly influence NIS expression and localisation, we might hypothesise that an association exists between PBF, BRAF<sup>V600E</sup> and TSH. However, this has not been investigated to date.

Overall, PBF is emerging as a fascinating protein with multiple roles in numerous tumour types including thyroid cancer. These promising studies demonstrate that PBF clearly warrants further investigation as a prospective prognostic or therapeutic tool in thyroid cancer. Further, the inhibitory effect on NIS activity identifies PBF as a potentially important therapeutic target for improving radioiodine uptake and hence prognosis of thyroid cancer.

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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