NOVEL PATHWAYS PROMOTING THYROID TUMOURIGENESIS AND GROWTH

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SUMMARY

Thyroid cancers are the most common endocrine malignancies and their incidence continues to rise. Over-expression of the human pituitary tumor transforming gene (hPTTG) in thyroid carcinomas is a prognostic indicator of tumour recurrence. hPTTG is multifunctional with roles in mitotic control, DNA repair, genetic instability, cell transformation and apoptosis. Importantly, hPTTG transactivates expression of growth factors implicated in proliferation and angiogenesis, and represses the sodium iodide symporter (NIS), which is essential to radioiodine treatments in thyroid cancer. hPTTG interacts with a binding factor (PBF) which is an independent transforming gene and also represses iodine uptake.

Work described in this thesis provides evidence for the existence of thyroidal autocrine regulatory pathways involving hPTTG and growth factors *in vitro*. We directly investigated the role of hPTTG in thyroid tumourigenesis through the generation and characterisation of a murine transgenic model with thyroid-targeted hPTTG over-expression (hPTTG-Tg mice). Unexpectedly, hPTTG over-expression was not sufficient for thyroid tumourigenesis. Investigations performed in hPTTG-Tg and $Pttg^{-/-}$ knockout mice indicated a particularly important relationship between hPTTG and the epidermal growth factor (EGF) *in vivo*. hPTTG and PBF were confirmed as repressors of NIS *in vivo* following studies in hPTTG-Tg and PBF-Tg mice respectively. The studies described in this thesis highlight the therapeutic potential of targeting hPTTG and PBF in thyroid cancer. To this effect, specific tyrosine kinase inhibitors prevented autocrine induction of hPTTG by growth factors, and siRNA depletion of PBF restored NIS function to normal levels in hPBF-Tg thyrocytes.

Based on these data, hPTTG appears to play a dual role in endocrine tumourigenesis, being involved in both tumour initiation and subsequent progression towards more aggressive phenotypes.

DEDICATION

I would like to dedicate this Doctoral dissertation to my parents, Oliver and Sandy, and to my sister, Natalie.

Thank you for your continual love and support.

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ABBREVIATIONS

ABT1	Activator of basal transcription 1
AFP	Alpha-fetoprotein
AIT	Apical iodide transporter
AITD	Auto-immune thyroid disease
AKT1	V-akt murine thymoma viral oncogene homolog 1
AP1	Activator protein 1
AP2	Activator protein 2
APC	Anaphase promoting complex
BRAF	Serine/threonine-protein kinase
CAM	Chorio-allantoic membrane
CAT	Chloroamphenicol acetyltransferase
cAMP	Cyclic adenosine monophosphate
CCNB1	Cyclin B
CDC-2	Cyclin-dependent kinase 2
CDC-20	Cyclin-dependent kinase 20
CDE	Cell cycle dependent element
CDH1	Cadherin-1
CDK4	Cyclin-dependent kinase 4
CHR	Cell cycle homology region
CIN	Chromosomal instability
CRK	V-crk sarcoma virus CT10 oncogene homolog
CTNNB1	Catenin (cadherin-associated protein), beta 1
DB	Destruction box
DES	Diethylstilbestrol
DNA-PK	DNA-dependent protein kinase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERα	Oestrogen receptor alpha
EREs	Oestrogen receptor elements
ESCC	Esophageal squamous cell carcinoma
EST	17β-estradiol
FGF-2	Fibroblast growth factor 2
FISSR-PCR	Fluorescent inter-simple sequence repeat PCR
FLT-1	Vascular endothelial growth factor receptor 1
FNA	Fine needle aspiration
FNAC	Fine needle aspiration cytology
FGFR1-4	Fibroblast growth factor receptor (1-4)
GH	Growth hormone
HAT	Histone acetyltransferase

HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HEK293	Human embryonic kidney 293
HGF	Hepatocyte growth factor
HLGAGs	Heparin-like glycosaminoglycans
hNUE	Human upstream enhancer element
hPTTG	Human pituitary tumor transforming gene
HRAS	Harvey rat sarcoma viral oncogene
HSP70	Heat shock protein 70
HUVEC	Human umbilical vein endothelial cells
ID3	Inhibitor of DNA-binding 3
IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
KDR	Vascular endothelial growth factor receptor 2
KRAS	Kirsten rat sarcoma viral oncogene
LB	Lysogeny broth
LH	Luteneising hormone
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
MEFs	Mouse embryonic fibroblasts
MEK1	Mitogen-activated protein kinase kinase 1
MEK3	Mitogen-activated protein kinase kinase 3
M-FISH	Multiplex fluorescent in situ hybridisation
MMP-2	Matrix metalloproteinase 2
MNG	Multinodular goitre
MRI	Magnetic resonance imaging
NF-Y	Nuclear factor Y
NGF	Nerve growth factor
NHEJ	Non-homologous DNA end-joining
NLS	Nuclear localisation signal
NIS	Sodium iodide symporter
NRAS	Neuroblastoma rat sarcoma viral oncogene
NTRK1	Neurotrophic tyrosine receptor kinase type 1
OCT-1	Octamer-binding transcription factor 1
PAX8	Paired box gene 8
PBF	Pituitary tumor transforming gene binding factor
PCNA	Proliferating cell nuclear antigen
PET	Positron emission topography

PI3K	Phosphoinositide-3-kinase
PIK3CA	Phosphoinositide-3-kinase catalytic alpha polypeptide
PKC	Protein kinase C
PKCβ1	Protein kinase C beta
PLCγ	Phospholipase C gamma
PLK1	Polo-like kinase 1
PPARγ1	Peroxisome proliferator-activator receptor gamma 1
PRL	Prolactin
PTCs	Papillary thyroid carcinomas
PTEN	Phosphatase and tensin homologue
PTTG	Pituitary tumor transforming gene
PTTG1IP	Pituitary tumor transforming gene 1 interacting protein (PBF)
RAR-β	Retinoic acid receptor beta
RET	Rearranged in transformation
RET/PTC	Rearranged in transformation/Papillary thyroid carcinomas
shRNA	Short hairpin ribonucleic acid
SH-3	Src-homology-3
SP1	Specificity protein 1
T_3	Triiodothyronine
T_4	Thyroxine
TBPL1	TATA box-binding protein-like protein 1
TCF-4	Transcription factor 4
TG	Thyroglobulin
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
TNM	Tumour size, node metastases and distant metastases
TP53	Tumour protein p53
TPO	Thyroid peroxidase
TRα	Thyroid hormone receptor alpha
TRβ	Thyroid hormone receptor beta
TSHR	Thyroid stimulating hormone receptor
TSP-1	Thrombo-spondin 1
TSH	Thyroid stimulating hormone
TTF1	Thyroid transcription factor 1
USF1	Upstream stimulatory factor 1
VEGF	Vascular endothelial growth factor
WT	Wild-type

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1 CHAPTER ONE

General Introduction

1.1 Pathogenesis of thyroid cancer

1.1.1 Epidemiology and classification

Thyroid cancers are the most common endocrine malignancies and have the fastest rising incidence of all cancers (Howlader et al. 2011). In the United Kingdom, thyroid carcinomas have an annual incidence of 2.6 per 100,000, affecting 1 in 1200 adults (British Thyroid Association and Royal College of Physicians 2007), while in the USA, they account for 0.85 % and 2.5 % of new cancer cases in men and women respectively (Jemal et al. 2003). Most patients have a favourable prognosis following surgery and administration of radioiodine, resulting in low mortality rates of 0.21 % and 0.3 % for men and women respectively (Schneider and Ron 2005). However, thyroid cancer incidence has been steadily rising over the past 30 years and has approximately tripled in the USA and other developed regions during this time (Albores-Saavedra et al. 2007; Davies and Welch 2006; Burgess and Tucker 2006; Colonna et al. 2007). The increase in incidence is generally attributed to advances in diagnostic approaches including high-resolution imaging, as well as the increased use of fine-needle aspiration (FNA) biopsies and susbsequent cytologic examination. However, exposure to ionizing radiation due to wider use of medical radiation or nuclear power accidents such as Chernobyl are well known risk factors in thyroid cancer that may also contribute to increased incidence (Rabes et al. 2000; Nikiforov et al. 1997; Bounacer et al. 1997; Smida et al. 1999; Hamatani et al. 2008; Takahashi et al. 2007).

Three to five percent of thyroid cancers arise from parafollicular or C cells and give rise to medullary thyroid carcinomas. All other thyroid cancers originate in thyroid follicular epithelial cells and are further classified as well-differentiated papillary (80-85 %) and follicular carcinomas (10-15 %), poorly differentiated or insular carcinoma (< 2 %) and anaplastic or dedifferentiated carcinoma (1-2 %). Follicular adenomas are benign entities that

may develop into follicular carcinomas. Disease progression and subsequent dedifferentiation of follicular carcinomas can result in development of insular or anaplastic carcinomas, although these subtypes can also develop *de novo* (Nikiforov and Nikiforova 2011; Nikiforov 2009; Delellis et al. 2004).

In contrast to thyroid cancer, benign thyroid disease in the form of diffuse or nodular thyroid enlargement, termed goitre, is highly prevalent. Goitres are characterised by hyperplasia, which is typically driven by elevated levels of thyroid stimulating hormone (TSH), growth factors and circulating stimulatory antibodies. While erroneous thyroidal metabolism, dietary goitrogens and goitrogenic chemicals can cause goitres, nutritional iodine deficiency represents the major cause of TSH-induced goitrogenesis worldwide. Consequently, incidence rates of goitre are highly influenced by the iodine status of a population, but goitre occurrence remains prevalent even in iodine-sufficient regions. For example, the Whickham survey described a comprehensive population survey of 2,749 people in Northern England and identified that 15.5 % of the participants had a palpable goitre (8.6 % had a small goitre) with a female to male ratio of 4.5:1 (Tunbridge et al. 1977).

1.1.2 Molecular genetics of thyroid cancer

External irradiation is a well-defined pathogenetic factor associated with thyroid carcinoma, but others such as hormonal (oestrogen and TSH), dietary, environmental and genetic factors have all been implicated (Schneider and Ron, 2005). Most malignant thyroid cancers are monoclonal in origin, meaning that transforming events are caused by genetic alterations in a single cell, giving rise to clonal cell populations conferred with a growth advantage (Moniz et al. 2002; McCarthy et al. 2006; Fagin 2005b). Subsequent propagation of tumour growth is driven by extracellular growth factors regulating critical growth and

survival pathways. Molecular components of these highly complex and intricate pathways may act as oncogenes or tumour suppressor genes. In recent years, we have enhanced our knowledge of genetic aberrations that cause activation of oncogenes or inactivation of tumour suppressor genes, which appear to have important roles in thyroid tumourigenesis (Fagin 2005b; Nikiforov and Nikiforova 2011).

1.1.3 Oncogenes in thyroid cancer

Initiation and progression of thyroid cancer occurs through gradual accumulation of genetic alterations. Various critical genes, mutated by single point mutations or chromosomal rearrangements, are associated with specific etiologic factors in thyroid carcinogenesis. The prevalence of these genetic alterations are summarised in Table 1-1 and discussed in detail in subsequent sections.

Characteristics	Papillary Carcinoma	Follicular Carcinoma	Poorly Differentiated Carcinoma	Anaplastic Carcinoma	Medullary Carcinoma
Cell type origin	Follicular	Follicular	Follicular	Follicular	C cell
Prevalence (%)	80-85	10-15	< 2	1-2	3-5
Route of spread	Local lymph-node metastasis	Haematogenous metastasis (bones and lungs)	Invasive local growth, lymph-node and haematogeneous metastases	Invasive local growth, lymph-node and haematogeneous metastases	Lymph-node and haematogeneous metastases
10-year survival (%)	95-98	90-95	~50	< 10	60-80
Prevalence of common mutations (%)	*BRAF* 40-45 *RAS* 10-20 *RET/PTC* 10-20 *TRK* <5	*RAS* 40-50 *PAX8/PPARγ* 30-35 *PIK3CA* <10 *PTEN* <10	*RAS* 20-40 *TP53* 20-30 *BRAF* 10-20 *CTNNB1* 10-20 *PIK3CA* 5-10 *AKT1* 5-10	*TP53* 50-80 *CTNNB1* 5-60 *RAS* 20-40 *BRAF* 20-40 *PIK3CA* 10-20 *PTEN* 5-15 *AKT1* 5-10	Familial: *RET* > 95 Sporadic: *RET* 40-50 *RAS* 25

Table 1-1: Summary of thyroid cancer classifications and associated genetic mutations. Adapted from (Nikiforov and Nikiforova 2011).

1.1.3.1 Tyrosine receptor kinase rearrangements

The Rearranged in Transformation (*RET)* gene encodes a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor family of extracellular signalling molecules and is usually only expressed in neuronal, neuro-endocrine and kidney cells (Knowles et al. 2006). Rearranged in transformation/papillary thyroid carcinomas (*RET/PTC)* is a clonal chromosomal rearrangement observed in 10-20 % of papillary thyroid cancers (PTCs) (Zhu et al. 2006; Guerra et al. 2011; Santoro et al. 1992), where its presence is associated with a high risk of development of metastases (Jhiang et al. 1992). Detection of *RET/PTC* rearrangements are very common in small PTCs, leading to speculation that it represents an early event in thyroid cell transformation (Kim et al. 2003). In these rearrangements, a fragment of the *RET* gene coding for the C-terminal tyrosine kinase domain of the RET protein forms chimeric fusion genes with active promoters of other genes. Transgenic mouse models have demonstrated that this results in the expression of a constituitively active RET/PTC protein that persistently stimulates mitogen-activated protein kinase (MAPK), independent of ligand-binding, thereby promoting thyroid tumourigenesis (Jhiang et al. 1996; Santoro et al. 1996; Powell et al. 1998).

Neurotrophic tyrosine receptor kinase type 1 *(NTRK1)* codes the receptor for nerve growth factor (NGF). *NTRK1* forms fusion genes with at least 3 different partner genes in thyroid papillary carcinomas, but is significantly less prevalent than *RET/PTC* rearrangements (Radice et al. 1991; Greco et al. 1992; Miranda et al. 1994; Bongarzone et al. 1989).

1.1.3.2 Nuclear receptor mutants

The *PAX8/PPARγ* rearrangement results in a fusion gene between part of the thyroid transcription factor gene, paired box gene 8 (*PAX8)*, and the peroxisome proliferator-activator

receptor γ1 (*PPARγ)* gene, which encodes a DNA-binding nuclear receptor that is involved in regulating adipocyte differentiation and lipid metabolism (Kroll et al. 2000; Powell et al. 2004; Evans et al. 2004). Although the transforming mechanisms of PAX8/PPARγ are poorly understood, this genetic translocation occurs in 30-35 % of follicular thyroid carcinomas (French et al. 2003; Nikiforova et al. 2003b; Dwight et al. 2003).

In addition, mutations in the thyroid hormone receptor (TR) α1 and β1 transcripts have been reported in papillary thyroid cancers (Puzianowska-Kuznicka et al. 2002). Aged transgenic mice with thyroid targeted expression of a mutant TRβ that is inactive ($TR\beta^{PV/PV}$ mice), develop invasive follicular thyroid cancers, suggesting a role for TRβ in thyroid cancer progression (Suzuki et al. 2002).

1.1.3.3 *RAS* mutations

Human Harvey rat sarcoma viral oncogene (*HRAS)*, Kirsten rat sarcoma viral oncogene (*KRAS)* and neuroblastoma rat sarcoma viral oncogene (*NRAS)* encode G-proteins which are involved in the transduction of intracellular signalling from cell surface receptor tyrosine kinases and G-protein coupled receptors, to the nucleus (Marshall 1996). RAS proteins hydrolyse GTP to GDP and activate MAPK, phosphoinositide 3-kinase (PI3K) and other pathways to stimulate cell proliferation and suppress differentiation (Mendoza et al. 2011; Marshall 1996). Activating point mutations in *RAS* genes have been identified in various thyroid tumours, including 10-20 % of papillary carcinomas, 40-50 % of follicular carcinomas and 20-40 % of insular and anaplastic carcinomas (Suarez et al. 1990; Esapa et al. 1999; Motoi et al. 2000; Manenti et al. 1994; Namba et al. 1990; Karga et al. 1991; Ezzat et al. 1996). The presence of *RAS* mutations in benign follicular adenomas (Esapa et al. 1999; Motoi et al. 2000; Namba et al. 1990) and in thyroid malignancy as above, suggests that

aberrant control of RAS may drive thyroid tumour progression from benign to well-differentiated to anaplastic thyroid tumour subtypes.

1.1.3.4 *BRAF* mutations

Serine/threonine-protein kinase (BRAF) is one of the three isoforms of the serine-threonine kinase RAF, which is activated by RAS, resulting in its translocation to the cell membrane where it phosphorylates and activates the MAPK pathway. In thyroid cancer, BRAF can become constituitively active following point mutations or chromosomal rearrangements. The most common mechanism of BRAF activation is caused by a point mutation resulting in a valine-to-glutamate replacement at residue 600 ($BRAF^{V600E}$) (Kimura et al. 2003; Cohen et al. 2003). This particular mutation represents 99 % of all BRAF mutations found in thyroid cancer, where it occurs in 40-45 % of papillary carcinomas (Xing 2005; Fukushima et al. 2003; Nikiforova et al. 2003a; Fagin 2005a) and in 20-40 % of insular and anaplastic carcinomas (Namba et al. 2003; Nikiforova et al. 2003a; Begum et al. 2004; Ricarte-Filho et al. 2009). The results of the various studies above imply that $BRAF^{V600E}$ mutations are an early event, predisposing tumours to progressive dedifferentiation and development of an anaplastic disease state. In support of this, transgenic mice with thyroid targeted $BRAF^{V600E}$ expression develop goitre and papillary thyroid cancers, which progressively transitions to poorly differentiated carcinomas (Knauf et al. 2005).

1.1.3.5 Accumulation of other oncogene mutations

In addition to *BRAF* and *RAS* mutations, a number of additional oncogenic mutations have been identified in poorly differentiated and anaplastic carcinomas, but not in well-differentiated tumour types, suggesting these mutations are late events in thyroid

tumourigenesis. The catenin (cadherin-associated protein) beta 1 (*CTNNB1)* gene encodes a β-catenin involved in cell-adhesion and Wnt signalling, and a point mutation in this gene has been found in up to 60 % of anaplastic carcinomas (Garcia-Rostan et al. 1999; Garcia-Rostan et al. 2001; Kurihara et al. 2004). In addition, studies have demonstrated that poorly differentiated and anaplastic carcinomas harbour mutations of PI3K pathways components including phosphoinositide-3-kinase catalytic alpha polypeptide (*PIK3CA)* itself (10-20%), phosphatise and tensin homologue (*PTEN)* (5-15%) and v-akt murine thymoma viral oncogene homolog 1 (*AKT1)* (5-10%) (Ricarte-Filho et al. 2009; Garcia-Rostan et al. 2005; Santarpia et al. 2008; Hou et al. 2007; Dahia et al. 1997).

1.1.4 Tumour suppressor genes in thyroid cancer

Tumour suppressor genes serve to arrest cell division and/or induce apoptosis in response to genetic mutations. Mutations of the important cell cycle regulator tumour protein p53 (TP53) exist in over 50 % of all cancers (Hollstein et al. 1994). p53 is stabilised in response to DNA damage and elicits protective effects by activating downstream target genes that prevent cell cycle progression, such as p21 (Dulic et al. 1994). Mutations of p53 have been found in 50-80 % of anaplastic carcinomas where its inactivity results in unrestrained tumour cell growth (Fagin et al. 1993; Donghi et al. 1993; Dobashi et al. 1994; Ito et al. 1992). The rare occurrence of this mutation in preceding tumour stages suggests that p53 mutations are a late event in thyroid tumourigenesis.

Another tumour suppressor gene is p16, a direct inhibitor of cyclin-dependent kinase 4 (CDK4), inactivation of which is associated with the development of spontaneous neoplasms and increased sensitivity to carcinogenic stimuli (Serrano et al. 1996). The expression of p16 has been shown to be reduced in papillary thyroid cancer (Liang et al. 2009).

1.1.5 Prognosis of thyroid cancer

Retrospective studies have enabled us to recognise certain factors that adversely affect prognosis of thyroid cancer and risk is assessed in patients with DTC using a prognostic scoring system. **T**umour size, **N**ode metastases and distant **M**etastases (TNM) is the most frequently used system and is summarised in Table 1-2. While most patients have a good prognosis following surgery and administration of radioiodine, BTA guidelines suggest 10 year disease specific mortality for stage I as 1.7 %, rising to 60.9 % for stage IV disease (British Thyroid Association and Royal College of Physicians 2007; Loh et al. 1997). 5-30% of patients will develop locoregional recurrence (British Thyroid Association and Royal College of Physicians 2007; Jonklaas et al. 2006; Mazzaferri and Jhiang 1994), with subsequent five year survival of 87 %. If distant metastases are present, five year survival falls to 72 % (Jonklaas et al. 2006). The prognostic accuracy of the staging and grading systems used in differentiated thyroid cancer has increased (D'Avanzo et al. 2004). Factors associated with a worse prognosis are male gender, age greater than 40 years and tumours of the follicular subtype or greater than 1 cm in size (British Thyroid Association and Royal College of Physicians 2007; Mazzaferri and Jhiang 1994).

Primary tumour

pT1 Intrathyroidal tumour, ≤ 1 cm in greatest dimension

pT2 Intrathyroidal tumour, >1-4 cm in greatest dimension

pT3 Intrathyroidal tumour, > 4 cm in greatest dimension

pT4 Tumour of any size, extending beyond thyroid capsule

pTX Primary tumour cannot be assessed

Regional lymph nodes (cervical or upper mediastinal)

N0 No nodes involved

N1 Regional nodes involved
If possible, subdivide
N1a Ipsilateral cervical nodes
N1b Bilateral, midline or contralateral cervical nodes or mediastinal nodes

NX Nodes cannot be assessed

Distant metastases

M0 No distant metastases

M1 Distant metastases

MX Distant metastases cannot be assessed

Disease Stage	< 45 years of age	> 45 years of age	10 year cancer-specific mortality (%)
STAGE I	- Any T, any N, M0	- pT1, N0, M0	1.7
STAGE II	-Any T, any N, M1	-pT2, N0, M0 - pT3, N0, M0	15.8
STAGE III		-pT4, N0, M0 - Any pT, N1, M0	30.0
STAGE IV		- Any pT, any N, M1	60.9

* Undifferentiated or anaplastic carcinomas are all STAGE IV.

*Table 1-2: Table summarising **T**umour size, **N**ode metastases and distant **M**etastases (TNM) prognostic scoring system and associated mortality rates. Adapted from (British Thyroid Association and Royal College of Physicians 2007; D'Avanzo et al. 2004; Loh et al. 1997)*

The gold standard for diagnosing thyroid malignancy remains FNA cytology (FNAC). Recent advances in molecular analysis of FNA present novel approaches to providing individualised treatment strategies to patients. Currently, indeterminate cytology indicating an inability to conclusively discern benign from malignant nodules is found in 10-20 % of patients with thyroid nodules and malignancy is diagnosed in only 20 % of these. Consequently, unnecessary surgery is performed in 60-90 % of these patients (Baloch et al. 2002; Baloch et al. 2008; Mazzaferri 1993). Advances in the molecular analysis of the

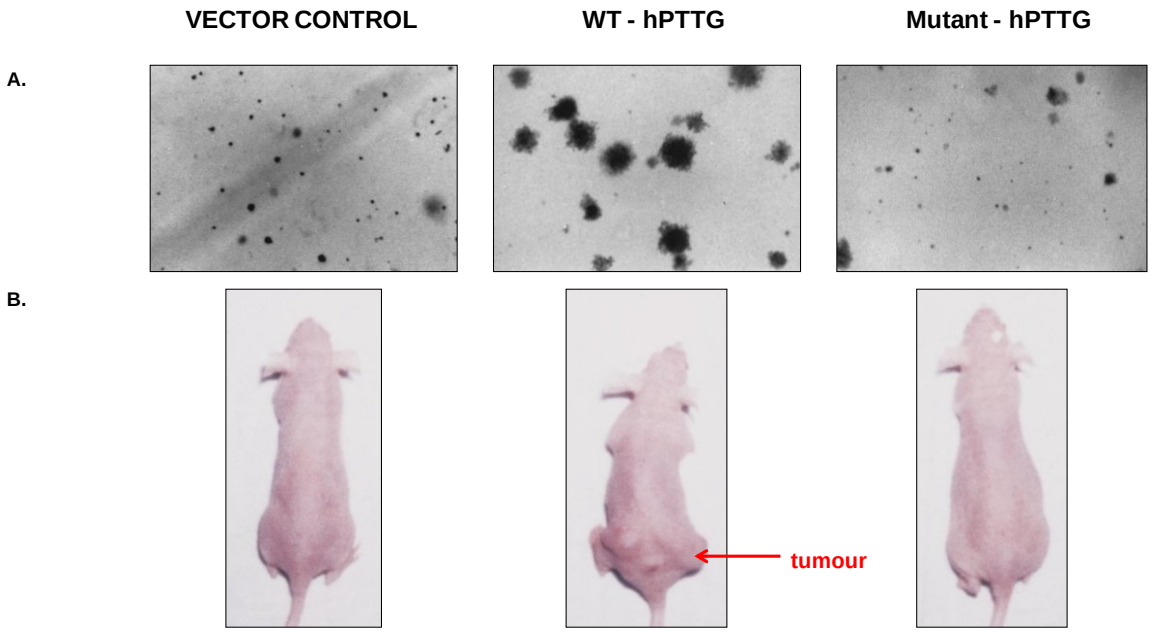
mutational markers described above (see section 1.1.3), represent opportunities to identify specific cancer subtypes and to provide highly improved diagnostic accuracy and to avoid unnecessary surgical procedures. For example, the collective results of 22 studies of thyroidal FNA samples revealed that in 1,117 nodules deemed positive for the $BRAF^{V600E}$ mutation, 99.3 % were subsequently histologically classified as papillary carcinomas, thereby demonstrating the irrefutable accuracy of $BRAF^{V600E}$ mutations as an indicator of cancer (Nikiforova and Nikiforov 2009; Kim et al. 2010; Kim et al. 2011; Nam et al. 2010).

1.2 Identification of the Pituitary Tumor Transforming Gene (PTTG)

Rat Pttg was first cloned using mRNA differential display polymerase chain reaction, where mRNA was exclusively expressed at high levels in rat pituitary tumour cells (GH4) but not in normal pituitary tissue (Pei and Melmed 1997). A human homologue of *PTTG* was cloned from a foetal liver cDNA library by using rat *Pttg* cDNA as a screening probe, and subsequently 89 % sequence homology was shown between rat and human *PTTG* (*hPTTG*) (Zhang et al. 1999b). Contemporaneously, a human homologue was cloned from thymus (Dominguez et al. 1998) and testis (Kakar and Jennes 1999). A further two *hPTTG* homologues have now been identified, including *hPTTG2* on chromosome 4p12 (Kakar and Jennes 1999; Prezant et al. 1999) and *hPTTG3* on 8q22 (Prezant et al. 1999), which are 91 % and 89 % homologous to *hPTTG1* respectively. A further variant of *hPTTG1* with altered functional capabilities was also detected (Wang and Melmed 2000b). However, *hPTTG1* on chromosome 5q33 (Zhang et al. 1999b) is the most abundant variant that is highly associated with oncogenesis and hence the most extensively studied form of *hPTTG1*. All of the studies described in subsequent chapters are focused on hPTTG1 and is denoted as hPTTG in accordance with convention.

1.2.1 Characterisation of hPTTG

Following observation of hPTTG over-expression in tumour cells, several studies have demonstrated the potent transforming capabilities of both rat and hPTTG *in vitro* and *in vivo*. Stable transfection of *hPTTG* in NIH3T3 mouse fibroblast cells caused an increase in cellular proliferation, augmented anchorage-independent growth in soft agar and induced tumour formation after subcutaneous injection into athymic nude mice (Pei and Melmed 1997; Zhang et al. 1999b; Kakar and Jennes 1999). Thus, the identification of *hPTTG* as a novel transforming gene has prompted many studies investigating its potential involvement in carcinogenesis.



C.

	TUMOUR WEIGHT (mg)		
N1	0	1000	100
N2	0	560	0
N3	0	670	0
N4	0	770	0
N5	0	940	0

*Figure 1-1: Characterisation of hPTTG as a potent transforming gene in vitro and in vivo. **A** Colony formation of NIH3T3 cells transfected with vector only (pCI-neo), wild type or mutant hPTTG expression vector on soft agar. The transforming ability of hPTTG, abrogated by mutation of its*

proline rich domain is observed by the formation of large and abundant colonies. ***B.*** *Tumour formation induced following subcutaneous injection of NIH3T3 stable transfectants into nude mice. Each mouse was injected with 3 x 10^5 control (n = 5), wild type (n = 5) or mutant hPTTG-over-expressing (n = 5) cells. After 2 weeks mice were photographed, sacrificed and their tumours excised and weighed (weight data in* ***C****) (Adapted from (Zhang et al. 1999b)).*

1.2.1.1 Structure of hPTTG

hPTTG has an open reading frame of 609 bp that encodes a 202 amino-acid protein (Zhang et al. 1999b). hPTTG is functionally divided into an N-terminal basic domain and a C-terminal acidic domain that harbour regulatory and functional domains respectively (Dominguez et al. 1998; Zhang et al. 1999b; Kakar and Jennes 1999).

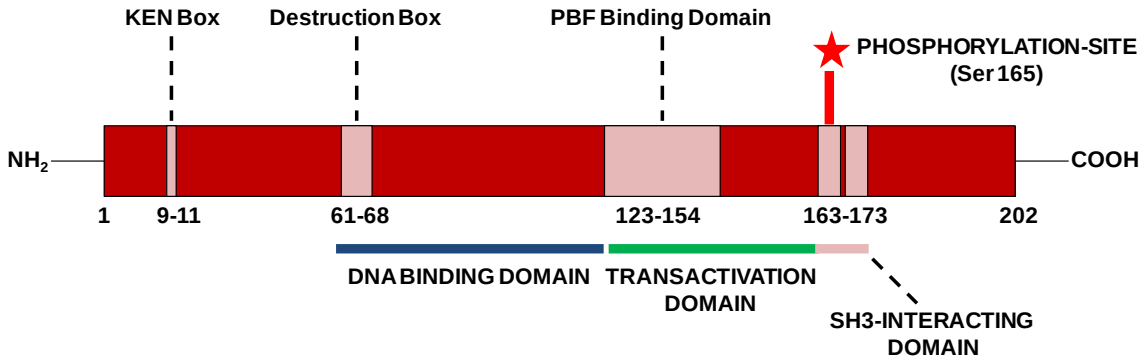


Figure 1-2: Schematic representation of the hPTTG protein. The following functional domains are displayed in pink (amino acids are given in brackets): KEN box (9 - 11), Destruction box (61 - 68), PBF binding domain (123 - 154) and SH3-interacting domain (163 - 173). The DNA binding domain is found between amino acids 61 and 118 and a transactivation domain lies between 119 and 164. The red star indicates the phosphorylation site of human PTTG.

In the regulatory N-terminal domain, a KEN box (amino acids 9-11) and a destruction box (DB) (amino acids 61-68) are substrates for the anaphase promoting complex (APC), and together are important for ubquitination of hPTTG by the APC to allow progression from metaphase to anaphase during mitosis (Zou et al. 1999; Zur and Brandeis 2001). Ubiquitination of hPTTG was unaffected or only partially inhibited by sole mutations in the KEN box and DB respectively. In contrast, double mutations of both motifs resulted in

completely abolished hPTTG degradation, suggesting they act in unison to ensure hPTTG levels are regulated correctly (Zou et al. 1999; Zur and Brandeis 2001).

Incorporating the DB box, a DNA binding domain is present from amino acids 61-118 with a transactivational region immediately downstream of this (Zhang et al. 1999b; Pei 2000; Wang and Melmed 2000b), allowing hPTTG to act as a transcription factor. Interestingly, prospective transcriptional targets of hPTTG include growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) (Kim et al. 2006a; Zhang et al. 1999b) the sodium iodide symporter (NIS) (Boelaert et al. 2007) and the proto-oncogene c-myc (Pei 2001). Although the transactivational properties of hPTTG require its presence in the nucleus, hPTTG is predominantly a cytoplasmic protein (Chien and Pei 2000) without a consensus nuclear localisation sequence. Studies have implicated a role for both MAPK phosphorylation cascades (Pei 2000) and an interaction with a specific hPTTG binding factor (PBF) (Chien and Pei 2000) for the nuclear translocation of hPTTG (see section 1.5.1). The PBF interacting domain lies within the transactivating domain (amino acids 123-154) (Chien and Pei 2000).

hPTTG contains a proline-rich region (amino acids 163-173) in the C-terminal that is critical to its transactivational and transforming capabilities (see sections 1.3.6 and 1.4). Two PXXP motifs confer a highly conserved and putative Src-homology-3 (SH-3) interacting domain (Zhang et al. 1999b) that when mutated result in abrogation of hPTTG-mediated transformation *in vitro*, tumourigenesis *in vivo* and growth factor induction (Zhang et al. 1999b; Pei 2000; Wang and Melmed 2000b; Ishikawa et al. 2001; McCabe et al. 2002; Boelaert et al. 2003a). The serine at residue 165 is within the SH-3 interacting domain and is the only reported phosphorylation site of hPTTG, which is thought to be important for the actions of hPTTG during mitosis (Pei 2000; Ramos-Morales et al. 2000; Boelaert et al. 2004).

1.2.1.2 hPTTG expression in human tissues

hPTTG is expressed at low levels in most normal adult tissues including colon, small intestine, pancreas, brain and thymus (Zhang et al. 1999b; Pei 1999). It is abundantly expressed in the testis, with evidence for a role during spermatogenesis (Pei 1999). Following the discovery of high hPTTG expression in pituitary adenomas (Pei and Melmed 1997), increased hPTTG expression has been found in various neoplasms including those of the thyroid (Heaney et al. 2001; Boelaert et al. 2003a), breast (Puri et al. 2001), colon (Heaney et al. 2000), ovary (Puri et al. 2001), oesophagus (Shibata et al. 2002), lung (Kakar and Malik 2006) and liver (Jung et al. 2006). Other studies report hPTTG over-expression in haematopoietic neoplasms (Dominguez et al. 1998; Saez et al. 2002), astrocytomas (Tfelt-Hansen et al. 2004) and in metastatic gastric carcinomas (Wen et al. 2004). Furthermore, high hPTTG expression is present in all transformed cell lines (Zhang et al. 1999b; Yu and Melmed 2001; McCabe and Heaney 2003; Yu and Melmed 2004). With over-expression so frequently observed in tumour tissues, hPTTG presents itself as a potentially crucial proto-oncogene.

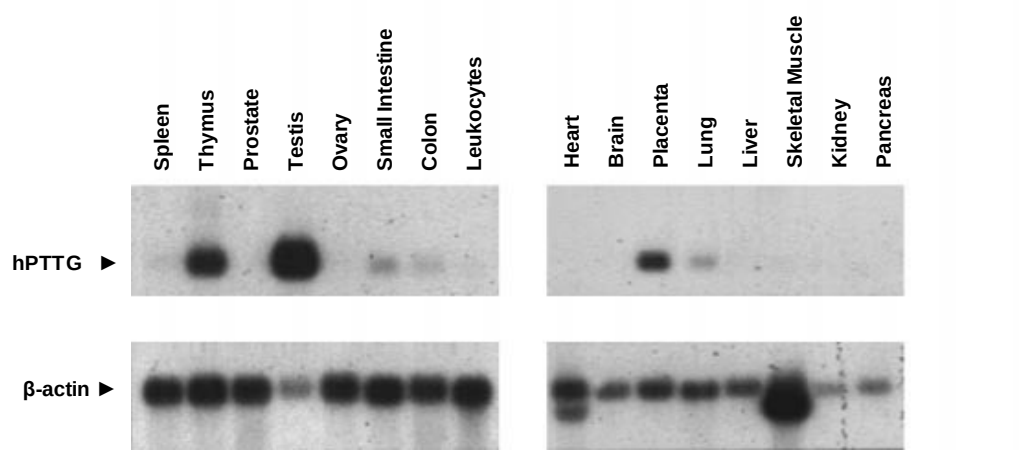


Figure 1-3: hPTTG expression in normal tissues. Northern blot analysis of hPTTG (top panel) and human β-actin (bottom panel showing relative amounts of RNA on each lane) mRNA expression in the indicated human adult tissues. Figure adapted from (Dominguez et al. 1998).

1.2.1.3 hPTTG expression in thyroid cancer

Several studies have independently demonstrated the over-expression of hPTTG in thyroid cancer. hPTTG mRNA and protein was found to be significantly over-expressed in thyroid tumours compared to normal tissue controls with particularly high levels in hyperplastic thyroid lesions and follicular tumours (adenomas and carcinomas) (Heaney et al. 2001). In this study, over-expression of hPTTG was less marked in papillary carcinomas than in hyperplastic or follicular lesions, a result that contrasts with our own findings of hPTTG over-expression in 19 papillary as well as 8 follicular carcinomas, but not in 38 hyperplastic thyroid lesions compared to normal tissue controls (Boelaert et al. 2003a). In this study, hPTTG was identified as an independent prognostic indicator of early tumour recurrence (Boelaert et al. 2003a). An additional study using immunohistochemistry showed high expression of hPTTG in 65 % of differentiated thyroid carcinoma tissue samples that were associated with nodal and distant metastases, as well as disease persistence (Saez et al. 2006).

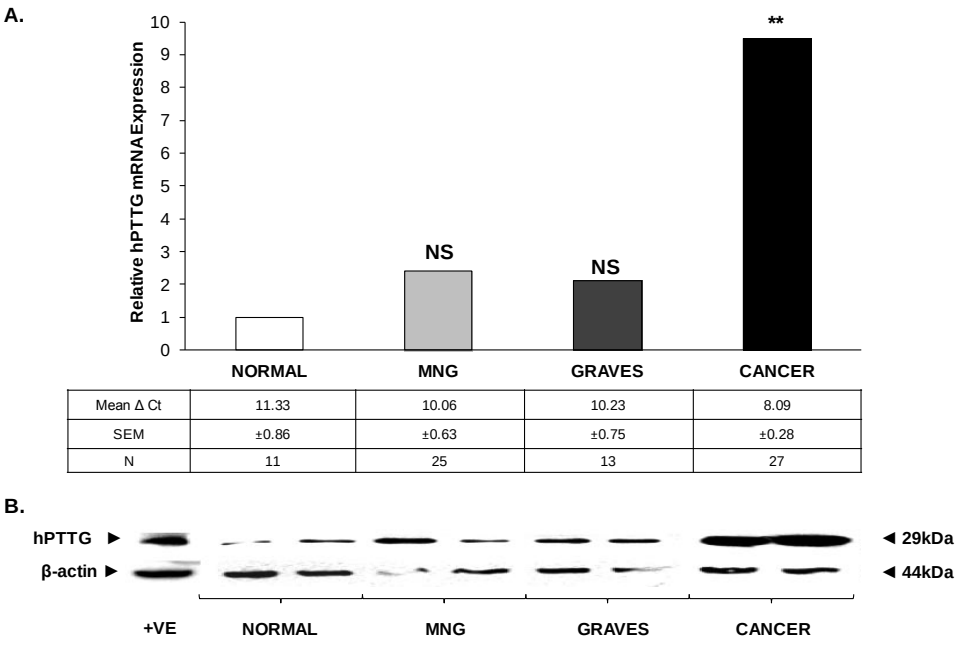


*Figure 1-4: hPTTG is over-expressed in differentiated thyroid cancer. **A** Qualitative RT-PCR data displaying relative fold changes in hPTTG mRNA expression in 11 normal, 25 multinodular*

goiter (MNG), 13 Graves' disease, and 27 cancer specimens. ***B*** *Western blot analysis of hPTTG protein expression in two representative samples of normal thyroid, two MNGs, two Graves' disease specimens, and two thyroid cancers showing markedly increased expression of PTTG in thyroid cancer. JEG-3 choriocarcinoma cells served as a positive control (+VE).* ** *$p < 0.01$. NS = non-significant. (Adapted from (Boelaert et al. 2003a)).*

1.2.1.4 Regulation of hPTTG expression

Thus far, no mutations have been discovered in either the coding or non-coding regions of *hPTTG* (Zhang et al. 1999b; Kanakis et al. 2003), suggesting hPTTG upregulation rather than mutational events underly its pathological effects described in section 1.2.1.2. The following sections describe studies that have investigated various mechanisms of hPTTG regulation.

1.2.1.4.1 Regulation of hPTTG by hormones

Oestrogen was the first hormonal regulator of Pttg to be identified when it was shown to induce pituitary Pttg expression *in vitro* and *in vivo* (Heaney et al. 1999). *In* vitro, *Pttg* mRNA expression was induced in rat pituitary GH3 cells at 24 hours following treatment with the synthetic oestrogen, diethylstilboestrol (0.1-10 nM). This effect was abolished by addition of the oestrogen-receptor antagonist, anti-E4-hydroxytamoxifen (Heaney et al. 1999). GH3 cells transiently transfected with full length mouse *Pttg* and treated with oestrogen, demonstrated an approximate 220 % increase in *Pttg* promoter activity, as determined by luciferase reporter assays. This induction was shown to be via the action of oestrogen upon oestrogen response elements (EREs) (Heaney et al. 1999). Subsequent studies have confirmed the presence of EREs in the *hPTTG* promoter (Kakar and Jennes 1999).

In vivo, ovariectomised Fischer 344 rats develop pituitary lactotroph tumours 4 weeks after treatment with oestrogen coincident with increased levels of circulating prolactin (PRL).

Pttg mRNA and protein expression was optimally induced by a 1000 ng dose of oestrogen after 48 hours of administration via an osmotic pump (Heaney et al. 1999). Notably, the highest Pttg expression was observed at an early stage of pituitary transformation, consistent with a role for hPTTG in the initiating events of tumourigenesis (see section 1.3). Furthermore, the increased pituitary Pttg expression at this stage coincided with induction of pituitary Vegf and Fgf-2 expression, as well as the development of pituitary arterial networks, supporting the hypothesis that elevated hPTTG expression contributes to tumour progression (Heaney et al. 1999). A subsequent study by the same group provided further support for these findings, demonstrating cyclical expression of Pttg, Vegf and Fgf-2, concordant with oestrogen levels during the oestrus cycle of Fischer 344 rats (Heaney et al. 2002). There was an approximately 3-fold induction of pituitary *Pttg*, *Vegf* and *Fgf-2* mRNA during pre-oestrus and oestrus, concomitant with increased proliferating cell nuclear antigen (PCNA) expression during maximal pituitary growth proliferation.

Having observed that treatment of primary human pituitary tumour cells with anti-oestrogen suppressed hPTTG expression *in vitro*, reduced Pttg expression was demonstrated *in* vivo following co-administration of oestrogen and anti-oestrogen via osmotic mini-pump in rats. Furthermore, serum levels of PRL were reduced by 88 % and pituitary tumour growth was suppressed by 41 %, suggesting anti-oestrogens could be an important therapeutic tool in treatment of pituitary tumours through repression of hPTTG expression (Heaney et al. 2002).

TSH has been directly implicated in the regulation of thyroidal hPTTG, where treatment of rat thyroid FRTL-5 and human primary follicular thyroid cells with TSH resulted in a significant induction of rat *Pttg* and *hPTTG* mRNA respectively (Heaney et al. 2001). Adding further complexity to our understanding of the regulation of hPTTG by hormones is the finding that oestrogen can stimulate increased TSH secretion in women (Clark et al. 1985;

McTiernan et al. 1984; Prestonmartin et al. 1987; Prestonmartin et al. 1993). It is therefore possible that oestrogen's regulation of hPTTG in thyroid cells is through increased serum TSH levels. Thyroid cancer is approximately 3 times more common in women (Fagin 2005b) and 25 % of thyroid cancers express oestrogen receptors (Clark et al. 1985), in keeping with a potential role for oestrogen in thyroid neoplasia. It is clear that further investigation is required to elucidate the exact interactions between hPTTG and hormones and to determine whether such hormones are in fact primary tumour promoters.

Other studies have demonstrated a role for insulin in the regulation of hPTTG. There was an approximate 2.5-fold increase in *hPTTG* mRNA expression following treatment with insulin in human breast cancer, MCF-7 cells. Luciferase reporter assays revealed a dose-dependent increase in the *hPTTG* promoter activity following insulin treatments and pre-treatment with the transcription inhibitor actinomycin-D completely abrogated induction of *hPTTG* mRNA expression, suggesting that hPTTG regulation by insulin is transcriptionally driven (Thompson and Kakar 2005). Another study reported upregulation of hPTTG mRNA and protein expression by insulin in U87MG and U138MG malignant astrocytes. This effect was completely abrogated by treatment with the PI3K inhibitors LY294002 and Wortmannin, but only partially blocked by the MAPK inhibitor PD98059. However, Pttg was not induced by insulin in non-tumourous primary rat embryonal astrocytes suggesting that insulin regulation of hPTTG may differ between malignant and non-malignant cell types (Chamaon et al. 2005).

1.2.1.4.2 Regulation of hPTTG by growth factors

There is increasing evidence that hPTTG has an important relationship with growth factors, where its expression is induced by various growth factors and hPTTG itself is capable

of transactivating expression of some of these mitogenic factors (see sections 1.4.3 and 1.4.4).

One study described over-expression of hPTTG in astrocytomas and subsequently demonstrated hPTTG induction by the epidermal growth factor receptor (EGFR) ligands, epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), in xenotransplantable U87 human glioma cells. This effect was abrogated by treatment with the specific EGFR inhibitor, AG1478. The c-met ligand, hepatocyte growth factor (HGF), also induced hPTTG expression but to a lesser extent (Tfelt-Hansen et al. 2004). A subsequent study demonstrated similar regulation of hPTTG by EGF and TGF-α in pituitary folliculostellate TtT-GF cells. Once again, these effects were blocked following inhibition of EGFR, by treatment with either AG1478 or gefitinib. Rapid phosphorylation of EGFR and subsequent activation of the MAPK and PI3K pathways was demonstrated, suggesting that these pathways are important in hPTTG regulation. EGF induction of hPTTG was cell-cycle dependent where its expression peaked at the S-G2 transition and the effect was absent following early S-phase blockade (Vlotides et al. 2006).

Insulin-like growth factor 1 (IGF-1) has been shown to regulate hPTTG expression in human breast cancer MCF-7 cells (Thompson and Kakar 2005) and in malignant and non-malignant astrocytes (Chamaon et al. 2005). In both studies, hPTTG induction by IGF-1 was partially or completely blocked by treatment with either of the specific PI3K inhibitors, LY294002 and Wortmannin, or the specific MAPK inhibitor PD98059. Interestingly, the latter study demonstrated interactions between both kinases and endogenous hPTTG in both malignant astrocytes and non-tumourous neuronal cells. In addition to enhancement of hPTTG transcription following treatment with IGF-1, it was speculated that a second and more direct route of hPTTG regulation may be mediated via direct binding to MAPK or PI3K (Chamaon et al. 2005).

The relationship between hPTTG and FGF-2 is well established (see sections 1.4.1 and 1.4.3) with studies demonstrating induction of hPTTG by FGF-2 treatment in NIH3T3 cells (Heaney et al. 1999) and in primary cultures of uterine leiomyomas, benign tumours of myometrial smooth muscle tissue (Tsai et al. 2005).

1.2.1.4.3 Transcription factors involved in regulation of hPTTG

Promoter studies of the mouse (Wang and Melmed 2000a), rat (Pei 1998) and human (Kakar and Jennes 1999) *PTTG* homologues have revealed the presence of one or more conserved specificity protein 1 (SP1) box motifs, implying that this transcription factor may play a role in regulating hPTTG expression. Mutation of the SP1 binding site -520 bp upstream of the *hPTTG* translational start site resulted in 70 % reduced overall promoter activity, whereas over-expression of SP1 in human PC-3 prostate cancer cell and HS27 fibroblast cells significantly increased *hPTTG* promoter activity (Clem et al. 2003). However, site-directed mutagenesis targeting the SP1 binding site in the rat *Pttg* promoter did not significantly affect promoter activity (Pei 1998).

hPTTG mRNA and protein expression were repressed by triiodothyronine (T_3) in hepatocellular carcinoma (HCC) cell lines and this was dependent on expression of thyroid hormone receptors (TRs) and mediated through repression of SP1 (Chen et al. 2008). Further, stable short hairpin RNA (shRNA) knockdown of SP1 resulted in reduced hPTTG expression and reduced proliferation. Stable shRNA knockdown of *hPTTG* directly, had a similar effect on proliferation. *In vivo*, Pttg and Sp1 expression were repressed in the livers of thyroidectomised rats treated with T_3. Furthermore, in human HCCs, hPTTG and SP1 were over-expressed while TR expression was reduced in comparison to matched normal liver, providing a potential mechanism for hPTTG over-expression in HCC, through modulation by thyroid hormone. (Chen et al. 2008).

A recent study treated various thyroid cancer cell lines with drugs synthetically derived from the triterpenoid glycyrrhetinic acid, CDODA-Me and CF3DODA-Me, and reported inhibition of their growth (Chintharlapalli et al. 2011). This was at least in part due to reduced expression of specificity proteins (SPs), including SP1. Reduced SP1 expression was associated with subsequent repression of hPTTG, FGF-2, VEGF and c-myc, as well as induction of apoptosis (Chintharlapalli et al. 2011). As well as providing further evidence for hPTTG regulation by SP1, these findings provide additional support for the relationship between hPTTG, growth factors and other proto-oncogenes (see sections 1.3.6 and 1.4).

Further studies of the hPTTG promoter identified four CAAT boxes, which are binding sites for the nuclear factor Y (NF-Y) transcription factor (Clem et al. 2003). Interestingly, there is evidence to suggest that NF-Y and SP1 act in unison to control transcriptional activation on various gene promoters (Inoue et al. 1999; Chang et al. 1999; Hu et al. 2000; Xiong et al. 2000; Kim et al. 2001). Two studies have described direct interaction between NF-Y and SP1 (Roder et al. 1999; Yamada et al. 2000). The interaction between NF-Y and SP1 appears to be significant in hPTTG regulation where mutation of the NF-Y binding site in the *hPTTG* promoter caused a 25 % repression of promoter activity, whereas a double mutation of both NF-Y and SP1 binding sites caused 90 % reduced promoter activation (Clem et al. 2003). A further study proposed a potential mechanism for hPTTG downregulation by p53 (see section 1.3.5), whereby p53 was shown to interact with NF-Y thereby preventing initiation of transcription on the *hPTTG* promoter (Zhou et al. 2003).

Several studies have reported a role for transcription factor 4 (TCF-4), a downstream effector of β-catenin signalling pathways, in the regulation of hPTTG. A TCF-4 binding element was identified in the *hPTTG* promoter and binding of TCF-4 to this region was demonstrated (Zhou et al. 2005; Hlubek et al. 2006; Pan et al. 2007). Subsequently, stable

transfectants of β-catenin in human embryonic kidney 293 (HEK293) cells demonstrated increased hPTTG mRNA and protein expression (Zhou et al. 2005) and siRNA transfections against β-catenin in colorectal carcinoma cells resulted in reduced *hPTTG* mRNA expression (Hlubek et al. 2006). In both of these studies, an accumulation of β-catenin correlated with over-expression of hPTTG in human esophageal squamous cell carcinomas [ESCCs] (Zhou et al. 2005) and in human colorectal adenomas and carcinomas (Hlubek et al. 2006), respectively. Regulation of hPTTG and the proto-oncogene c-myc by γ-catenin was observed in colon cancer cell lines, following both over-expression and siRNA knockdown studies (Pan et al. 2007).

Another study provides strong evidence for the involvement of a further transcription factor, Octamer-binding transcription factor-1 (OCT-1), in the regulation of hPTTG expression. Two OCT-1-binding motifs were identified in the *hPTTG* promoter and chromatin immunoprecipitation assays using HEK293 cells demonstrated direct binding of OCT-1 to these regions. Over-expression of OCT-1 resulted in increased *hPTTG* promoter activity and a subsequent 4-fold increase in hPTTG mRNA and protein expression. Conversely, siRNA knockdown of OCT-1 resulted in reduced hPTTG mRNA and protein expression. Furthermore, confocal immunofluorescent imaging showed a strong correlation between OCT-1 and hPTTG expression in 75 % of pituitary tumours (n=79), 61 % of breast tumours (n=77) and 65 % of colorectal tumours (n=71) (Zhou et al. 2008).

Other transcription factors may have a role in hPTTG expression. Additional motifs present in the *hPTTG* promoter include activator protein 1 (AP1) and 2 (AP2) binding sequences, a cyclic-AMP (cAMP) response element sequence, a cell cycle dependent element (CDE) motif as well as a cell cycle homology region (CHR) motif (Kakar 1999). Further studies are required to determine the functional significance of these promoter elements.

1.2.1.4.4 Epigenetic mechanisms and hPTTG expression

No mutations in the coding nor in the non-coding regions of *hPTTG* were identified by RT-PCR in pituitary tumours (Zhang et al. 1999b), and following studies of the *hPTTG* promoter in 25 pituitary adenomas (Kanakis et al. 2003) respectively. A small number of studies have investigated epigenetic mechanisms that may be involved in the control of hPTTG expression.

One investigation treated prostate cancer cells with 5-Azacytidine to cause genome wide demethylation, but reported no effect on hPTTG protein expression (Hidalgo et al. 2008). A CpG island comprised within a 650 bp region was identified as having high probability of being subject to epigenetic control. However, subsequent methylation-specific PCR analysis revealed unmethylated CpG island in both differentiated thyroid carcinomas and matched normal samples. Furthermore, loss of heterozygosity (LOH) studies using Affymetrix microarray technology and FRET analysis did not identify any allelic imbalances encompassing the *hPTTG* locus (Hidalgo et al. 2008).

However, histone modifications have recently been implicated in hPTTG regulation, where over-expression of histone acetyltransferase (HAT) p300 resulted in increased *hPTTG* promoter activity, as well as mRNA and protein expression in 293T cells. The HAT activity of p300 was crucial for its regulation of hPTTG and chromatin immunoprecipitation assays revealed increased histone H3 acetylation on the *hPTTG* promoter following over-expression of p300. Further, treatment of 293T cells with histone deacetylase (HDAC) 3 resulted in reduced hPTTG expression and conversely, treatment with an HDAC inhibitor caused upregulation of hPTTG. Interestingly, co-expression of NF-YA and NF-YB with p300 synergistically increased *hPTTG* promoter activity, providing further insights into the

involvement of NF-Y transcription factors in hPTTG regulation, whereby they possibly bind and recruit p300 to the *hPTTG* promoter (Tian et al. 2009).

1.2.1.4.5 Cell-cycle dependent subcellular localisation of hPTTG

Various studies have demonstrated that hPTTG expression is cell-cycle dependent. An early study identified hPTTG as the vertebrate securin, where levels of hPTTG protein were shown to increase during S-phase and peak in G2- and M-phase in HeLa S3 cells (Zou et al. 1999). Consistent with this, extracts from HeLa cells arrested in each stage of the cell cycle revealed low hPTTG expression in S, increasing expression in G2 and highest expression in M-phase, before returning to low levels once more in G1 (Ramos-Morales et al. 2000). Furthermore, in both HeLa and COS-7 cells, hPTTG expression was low following serum starvation, elevated during rapid proliferation following addition of serum and downregulated once again as cells became confluent (Ramos-Morales et al. 2000). Another study confirmed increased hPTTG mRNA and protein expression progressively through S-, G2- and M-phase as above, and subsequently these observations were elegantly supported following live cell imaging in synchronised human choriocarcinoma JEG-3 cells (Yu et al. 2000b). Following over-expression of hPTTG or hPTTG-EGFP in JEG-3 cells, immunofluorescent staining during live cell imaging revealed that hPTTG was largely localised to the nucleus during interphase with only a small amount of cytosolic hPTTG detected. Predominantly nuclear hPTTG expression in JEG-3 cells was confirmed by Western blots of nuclear and cytoplasmic cell fractions following transfection with *hPTTG-EGFP* (Yu et al. 2000b).

Although the same study replicated these findings in NIH-3T3, GH3 and AtT20 rat pituitary tumour, SKOV-3 human ovarian cancer, MCF-7 and COS-7 cell lines, there are

contrasting data to suggest otherwise. Early studies using subcellular fractionation in Jurkat T lymphoma cells (Dominguez et al. 1998) and immunohistochemistry in pituitary adenomas, and in lung and breast adenocarcinomas (Saez et al. 1999) indicated that hPTTG was predominantly localised to the cytoplasm, with only partial nuclear expression (see Figure 1-5). A further study used fluorescence microscopy to demonstrate that transfection of *pCMX-GFP-hPTTG* resulted in predominantly nuclear hPTTG in HeLa, COS-7 and DU145 cells, but that hPTTG expression was diffuse throughout the cytoplasm and nucleus in A549, DLD-1 and NIH3T3 cells (Mu et al. 2003). It therefore seems possible that the subcellular localisation of hPTTG is cell-specific and dependent on experimental techniques employed. Thus far, no functional role for cytoplasmic hPTTG has been identified and its presence here is considered to be a form of negative regulation of hPTTG presence in the nucleus where it has functional outputs. Indeed, hPTTG's presence in the nucleus is essential for all its functions described in subsequent sections. hPTTG itself does not have a nuclear localisation signal (NLS) (see section 1.2.1.1), but is thought to be able to diffuse freely across the nuclear membrane given its small molecular weight. However, an hPTTG binding factor (PBF) has been identified and shown to specifically interact with hPTTG and facilitate its nuclear entry via its own NLS (Chien and Pei 2000).

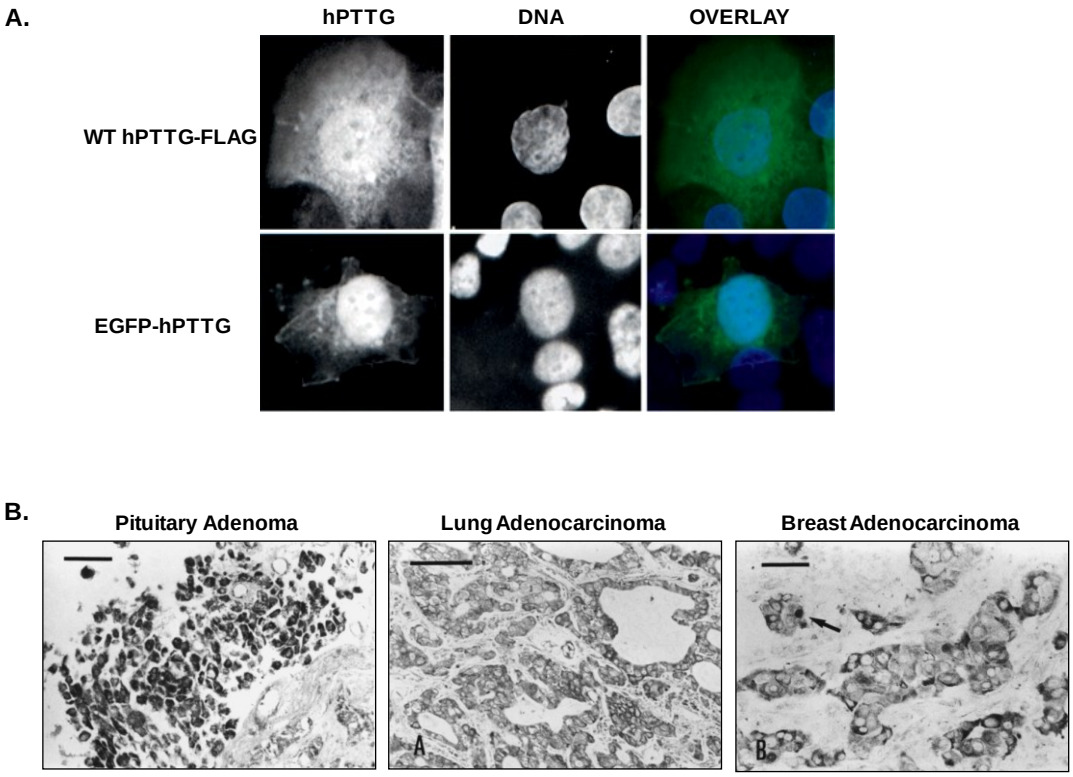


Figure 1-5: Contrasting data of hPTTG localisation during interphase. ***A*** *Interphase localisation of hPTTG in JEG-3 cells. Cells were transfected with plasmids encoding WT-hPTTG-FLAG or EGF-hPTTG. hPTTG protein was visualised using immunofluorescent staining and EGFP-hPTTG protein was visualised directly (left) and was localised mainly in the nucleus. Cells were also stained with Hoechst 33258 to highlight the nuclei and chromosomes and to determine the cell cycle (middle). The images of cells expressing hPTTG or hPTTG-EGFP were overlaid with images of the cells stained with Hoechst (right), again illustrating hPTTG mainly to be localised to the nucleus (figure adapted from (Yu et al, 2000b)).* ***B*** *Immunohistochemical detection of hPTTG in pituitary adenomas (left), lung adenocarcinomas (middle) and breast adenocarcinomas (right), demonstrating predominantly cytoplasmic localisation of hPTTG, with rare staining of nuclear PTTG indicated by the black arrow (right) (figure adapted from (Saez et al. 1999)).*

Interestingly, observations from one study imply that hPTTG is a secreted protein. hPTTG expression was observed in the Golgi apparatus and vesicles in both human pituitary adenomas and mouse pituitary AtT-20 cells, consistent with a potential role in membrane trafficking mechanisms. Further, secreted hPTTG was detected in the cell culture supernates taken from pituitary tumour cell lines (Minematsu et al. 2007). The authors speculated that hPTTG itself may be a signalling molecule involved in pituitary autocrine and paracrine

pathways. However, additional studies are required to validate this finding and to identify specific functions for secreted hPTTG.

The live cell imaging studies in JEG-3 cells described previously, made the critical observation that hPTTG co-localised with the mitotic spindles while it was upregulated during mitosis (Yu et al. 2000b). Similar studies by the same group reported that hPTTG localised to mitotic chromosomes in H1299 cells (Yu et al. 2003). These observations are consistent with hPTTG's primary function as the human securin involved in mitotic regulation (see section 1.3.1) and further support for this was provided by a study that reports the phosphorylation of hPTTG during this phase of the cell cycle (Ramos-Morales et al. 2000).

1.2.1.5 Phosphorylation of hPTTG

In the study described above, where hPTTG protein expression in HeLa cell extracts was cell cycle dependent, it was also demonstrated in Western blot analyses that hPTTG migrated as a doublet in M-phase extracts compared to a single band observed in other phases, indicating that hPTTG is phosphorylated during mitosis (see Figure 1-6). These findings were confirmed in studies incubating M-phase HeLa cell extracts with an alkaline phosphatase in the presence or absence of a phosphatase inhibitor. In the absence of phosphatase inhibitor, expression of the heavier band representing phospho-hPTTG was reduced and there was a concomitant upregulation of the lighter band representing non-phosphorylated hPTTG. Addition of the inhibitor prevented this, suggesting the effect was specific to phosphorylation status of hPTTG. Further support for mitotic phosphorylation of hPTTG was provided by demonstrating enhanced phosphorylation of purified recombinant hPTTG *in vitro* following incubation with mitotic HeLa cell lysates compared to interphase lysates (Ramos-Morales et al. 2000). hPTTG contains putative phosphorylation sites for

cAMP- and cGMP-dependent protein kinases, casein kinase II, AKT and protein kinase C (PKC) (Dominguez et al. 1998) but thus far, the Ser165 residue on hPTTG is the only confirmed phosphorylation site of hPTTG. This residue is located within the first PXXP motif (see Figure 1-2) and is a consensus site (T/SPXK/R) for cyclin-dependent kinase 2 (CDC2) (Moreno and Nurse 1990; Nigg 1991; Zhou et al. 1994; Ramos-Morales et al. 2000), which is important for mitotic progression (Nurse 1994; Yu et al. 2000a). Immunodepletion of the kinase CDC2 in mitotic cell lysates resulted in reduced hPTTG phosphorylation and, conversely, immunopurified CDC2 from mitotic cell lysates increased hPTTG phosphorylation in phosphorylation assays *in vitro*. Treatment with the specific CDC2 inhibitor, butyrolactone I, significantly reduced hPTTG phosphorylation *in vitro*. Furthermore, mutation of the Ser165 residue abolished CDC2-mediated phosphorylation of hPTTG (Ramos-Morales et al. 2000). CDC2 is a regulator of many downstream targets involved in crucial mitotic events and so the finding that CDC2 directly phosphorylates hPTTG could be indicative of a requirement for hPTTG to be phosphorylated to perform its role as a securin (see section 1.3.1).

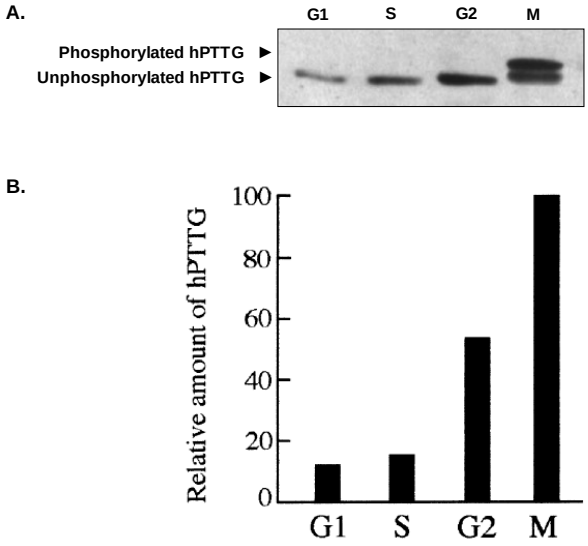


*Figure 1-6: Cell cycle-dependent expression and phosphorylation of hPTTG protein. **A** Western blot of HeLa cell extracts arrested in the different phases of the cell cycle demonstrating the double*

band during mitosis. ***B*** *Densitometry of the immunoblot indicating highest levels of hPTTG expression during mitosis (figure adapted from(Ramos-Morales et al. 2000)).*

Phosphorylation of hPTTG by the DNA-dependent protein kinase (DNA-PK) was reported as part of a mechanism that involves hPTTG in DNA damage responses (see section 1.3.5). However, further studies are required to identify the site of hPTTG phosphorylation by this kinase, as well as the exact functional output following DNA-PK induced phosphorylation (Romero et al. 2001).

The presence of a consensus MAPK phosphorylation site (Pro-X-Ser/Thr-Pro) (Alvarez et al. 1991) in the transactivation domain of Pttg, prompted one group to investigate the regulation of Pttg by Mapk activation. It was found that rat Pttg is phosphorylated by Mapk at Ser162, which is the analogous site to human Ser165, and this was mediated by a direct interaction between an N-terminus Pttg Sh3-interacting domain and mitogen-activated protein kinase kinase 1 (Mek1). Phosphorylation of Ser162 by Mapk facilitated nuclear translocation and subsequent transactivational function of Pttg, as determined by increased Fgf-2 transcription (see section 1.3.6). Point mutations in either Mek1 or Mapk interacting regions of Pttg resulted in cytoplasmic retention of Pttg and loss of its transactivational functions (Pei 2000).

Together, these results suggest that phosphorylation of hPTTG is important for its nuclear translocation where it has multiple roles including those as a mitotic regulator and gene transactivator. Given the presence of other putative phosphorylation sites on hPTTG, it is clear that further studies are required to identify alternative mechanisms of hPTTG phosphorylation and to resolve the various functional outputs of phosphorylated hPTTG.

1.3 hPTTG initiates tumourigenesis

1.3.1 hPTTG as a securin

The findings of the various studies described above, that hPTTG is periodically expressed and phosphorylated in the nucleus throughout the cell cycle, peaking at the metaphase-anaphase boundary when it colocalises with mitotic spindles, are indicative of a crucial role for hPTTG in mitosis.

Securins are proteins that are involved in the prevention of premature separation of sister chromatids during the metaphase to anaphase transition in mitosis. In normal cell division, chromosomes are replicated before sister chromatids are segregated to opposite poles of the cell, ensuring production of diploid daughter cells that are genetically identical to the parent cell (Nasmyth et al. 2001; Nasmyth 2001; Nasmyth 2002). Failure of the mechanisms that control the timely separation of sister chromatids results in inappropriate cell division causing aneuploidy and genetic instability.

Eukaryotic chromosomal replication occurs during S-phase and sister chromatids are bound together by cohesin complexes that oppose the splitting forces exerted by microtubules (Michaelis et al. 1997; Zur and Brandeis 2001). The cohesion complex is made up of multiple subunits of which mutation has been reported to cause improper separation of sister chromatids (Nasmyth 2001; Peters 2002). The cysteine protease, separase, is involved with proteolytic degradation of cohesins during mitosis (Nasmyth et al. 2001; Nasmyth 2001; Nasmyth 2002). Separase is a large 2121 amino-acid protein that is critically phosphorylated at Ser1126 and mediates its proteolytic activity through the key catalytic residue cys2029, which is conserved in all proteases (Uhlmann et al. 2000; Chestukhin et al. 2003). In the first instance, cohesins are largely removed from the long arms of chromosomes during prophase and prometaphase by the actions of the kinases Aurora B and polo-like kinase 1 (PLK1)

(Losada et al. 1998; Losada et al. 2002; Waizenegger et al. 2000; Hauf et al. 2001; Peters 2002; Sumara et al. 2002). Subsequently, separase is activated at the start of anaphase and is required for the degradation of remaining cohesin complexes that are preferentially located proximally to centromeres, to allow the segregation of sister chromatids (Waizenegger et al. 2000; Hauf et al. 2001; Peters 2002).

Throughout most of the cell cycle, the proteolytic activity of separase is inhibited by a direct interaction with securin/hPTTG (Jallepalli et al. 2001; Jallepalli and Lengauer 2001). As described previously in human cells (see sections 1.2.1.4.5 and 1.2.1.5), hPTTG accumulates during G1 and G2 phases, with maximal expression in M-phase before an abrupt decline of hPTTG expression at the end of metaphase immediately prior to the onset of anaphase. hPTTG expression diminishes rapidly at this stage as it is a substrate of the APC, which is a large cell cycle regulated ubiquitin-protein ligase made up of at least 11 subunits. The APC ubiquitinates a number of proteins involved in cell cycle regulation, resulting in their degradation at the 26S proteasome (Zachariae and Nasmyth 1999; Nasmyth 2001). Regulation of hPTTG/securin in this way allows the release of functional separase to breakdown remaining cohesins and ultimately, the progression to anaphase (Zou et al. 1999; Jallepalli et al. 2001). APC substrates possess D-box and KEN box consensus sequences essential for their recruitment to the APC and mutations of both of these domains in hPTTG abolished hPTTG degradation during mitosis (Zou et al. 1999; Zur and Brandeis 2001; Glotzer et al. 1991; Pfleger and Kirschner 2000; Peters 2002). Co-activators, such as CDC20 and CDH1 bind to the the D-box and KEN box of APC substrates before assembling with the APC as part of a degradation recruitment mechanism. In early mitosis, MAD2 (mitotic arrest deficient 2) inhibits Cdc20-APC assemblies as part of a complex spindle checkpoint system that prevents premature separase activity while chromosomes align correctly on mitotic

spindles (Peters 2006; Nasmyth 2001; Michel et al. 2001). Once this checkpoint is passed, Cdc20-APC becomes active until the end of anaphase when Cdh1-APC activity is reported to be more prominent and continues until the next G1-phase is reached (Peters 2002).

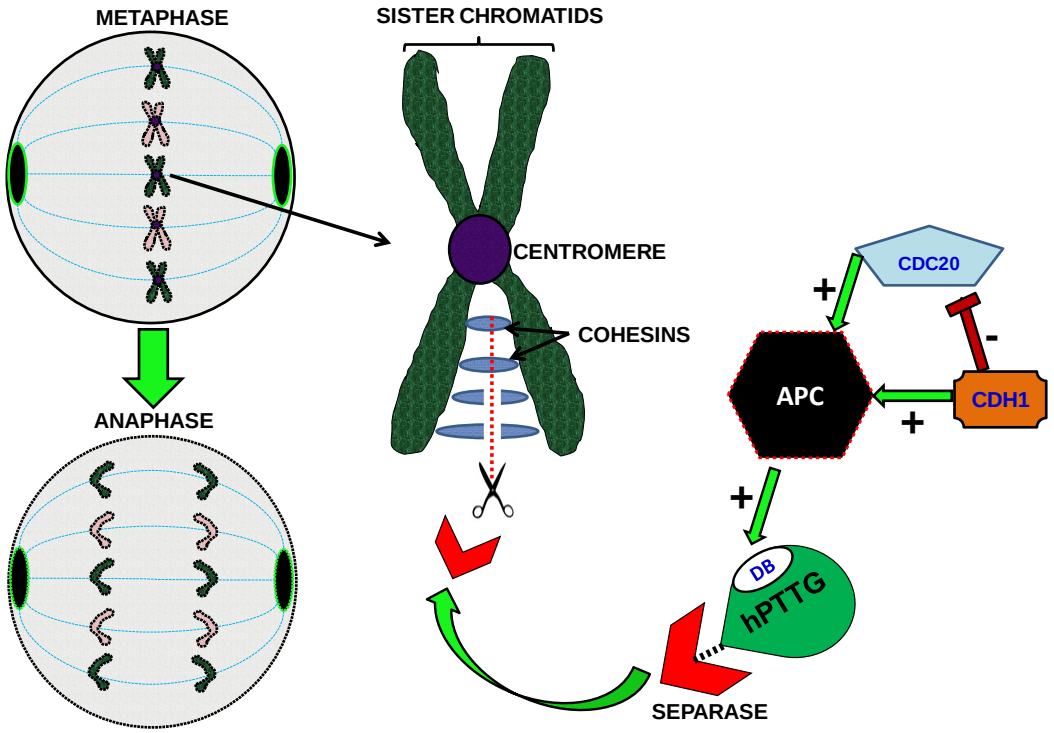


Figure 1-7: Schematic diagram and simplified representation of metaphase to anaphase transition during mitosis. hPTTG is ubquitinylated at the destruction box (DB) by the APC, which in turn is controlled by CDC20 and CDH1. Following the degradation of hPTTG, separase cleaves the cohesins and both sister chromatids migrate to one side of the mitotic spindle.

The APC complex mediates proteolysis of various other mitotic regulators including cyclins A and B (Zachariae and Nasmyth 1999; Nasmyth 2001; Peters 2002). Cyclin B (CCNB1) is the regulatory subunit of CDC2 and together they inactivate separase by direct phosphorylation at Ser1126. The CDC2/CCNB1 complex cannot phosphorylate separase while it is associated with hPTTG, suggesting that the APC complex releases active separase by proteolysis of hPTTG and cyclin B in two independent regulatory pathways (Gorr et al. 2005; Stemmann et al. 2001; Peters 2002; Holland and Taylor 2006). The existence of

multiple regulatory mechanisms provides a possible explanation for cell viability in the absence of hPTTG *in vitro* and *in vivo* (Jallepalli et al. 2001; Wang et al. 2001).

1.3.2 hPTTG over-expression and genetic instability

Thyroid cancers originate from a single transformed cell that acquires enhanced activity of proliferation and survival pathways. Genomic instability in a cell can initiate further genetic aberrations including chromosomal imbalances, amplifications, deletions and translocations (Cooper 1995). A number of studies have highlighted the presence of genetic instability in thyroid cancer, including the identification of specific chromosomal regions that are susceptible to allelic deletions in thyroid tumours (Kubo et al. 1991; Zedenius et al. 1995; Grebe et al. 1997; Marsh et al. 1997; Tung et al. 1997; Ward et al. 1998). Aneuploidy is a common form of chromosomal instability (CIN) whereby various chromosomal rearrangements occur, including the complete loss or gain of whole chromosomes. As a result of such aberrations, daughter cells may over-express oncogenes or under-express tumour suppressor genes. Indeed, aneuploidy is characteristic of differentiated thyroid tumours and transformed thyroid cancer cell lines (Joensuu et al. 1986; Joensuu and Klemi 1988) and is associated with high mortality rates in papillary thyroid carcinomas (Sturgis et al. 1999). Mitosis is a complex process that is stringently regulated in order to ensure diploid cell division and genomic stability (Nasmyth et al. 2001; Nasmyth 2001; Nasmyth 2002). The complexity of the processes required for the segregation of sister chromatids during metaphase and anaphase depends on multiple proteins that if aberrantly expressed may cause aneuploidy. Identification of hPTTG as the human securin directly involved in sister chromatid segregation (see section 1.3.1) meant hPTTG was presented as a strong candidate for inducing aneuploidy in thyroid cancer when aberrantly expressed (see Figure 1-8).

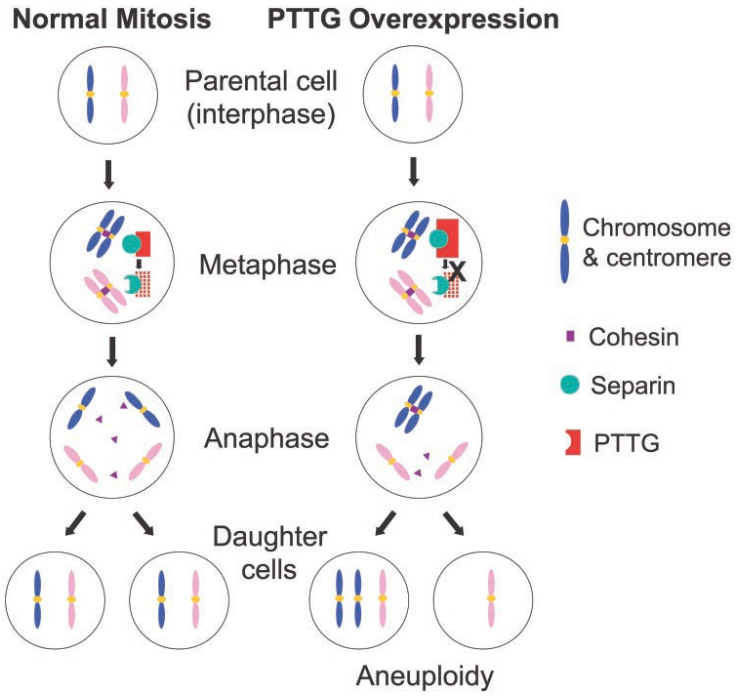


Figure 1-8: Proposed model of hPTTG-induced aneuploidy. ***Left****: normal mitosis, where the degradation of securin by the APC at the end of metaphase releases tonic inhibition of separin (now known as separase) thereby allowing cohesin degradation and equal distribution of chromatids between 2 daughter cells.* ***Right****: Abnormal mitosis in cells over-expressing hPTTG, when aneuploidy results from dysregulated sister chromatid separation (figure from(Yu et al. 2003)).*

Studies have shown induction of aneuploidy following transient or stable over-expression of EGFP-tagged hPTTG in MG-63 osteosarcoma cells, as determined by increased frequency and severity of micronuclei, macronuclei and chromosomal bridges. Untransfected or EGFP control transfected MG-63 cells had very low levels of basal aneuploidy (Yu et al. 2000a).

One important study employed an approach of single cell live imaging to study the effects of hPTTG over-expression on mitosis, where they were able to use a CCD digital camera to capture phase-contrast or fluorescence images every minute (during metaphase and anaphase) or every few hours (post-telophase) over a 48-hour period (Yu et al. 2003) (see Figure 1-9). Individual human lung cancer H1299 cells with low endogenous hPTTG expression were utilised for the study, where untransfected or EGFP control transfected cells

displayed normal cell division resulting in diploid daughter cell production (see Figure 1-9 A). Cells transfected with *hPTTG-EGFP* demonstrated effects dependent on expression levels. In cells where hPTTG-EGFP levels were low, hPTTG was typically degraded before anaphase and normal mitosis ensued. However, high levels of hPTTG-EGFP over-expression resulted in a prolonged prophase and metaphase, asymmetric cytokinesis, ultimately causing catastrophic cell division (see Figure 1-9 B). Most frequently, total failure to segregate metaphase chromosomes led to all chromosomes moving towards one pole in conjunction with cell elongation and development of a midline furrow. Consequentially, one daughter cell contained all the parental DNA content forming a macronucleus and the other was a non-viable cell without a nucleus. In other cells, hPTTG-EGFP over-expression caused partial segregation of sister chromatids. This resulted in development of 'anaphase bridges' where chromatin breakages between partially fused chromatids occur under the opposing forces exerted by microtubules and ultimately leading to the appearance of micronuclei. Furthermore, over-expression of a mutated hPTTG lacking the KEN box and D-box domains that allow it to be targeted for degradation by the APC (see section 1.3.1), also resulted in asymmetrical cytokinesis (Yu et al. 2003). The results discussed above strongly indicate that that over-expression of hPTTG or lack of hPTTG degradation results in inappropriate cell division and a state of aneuploidy.

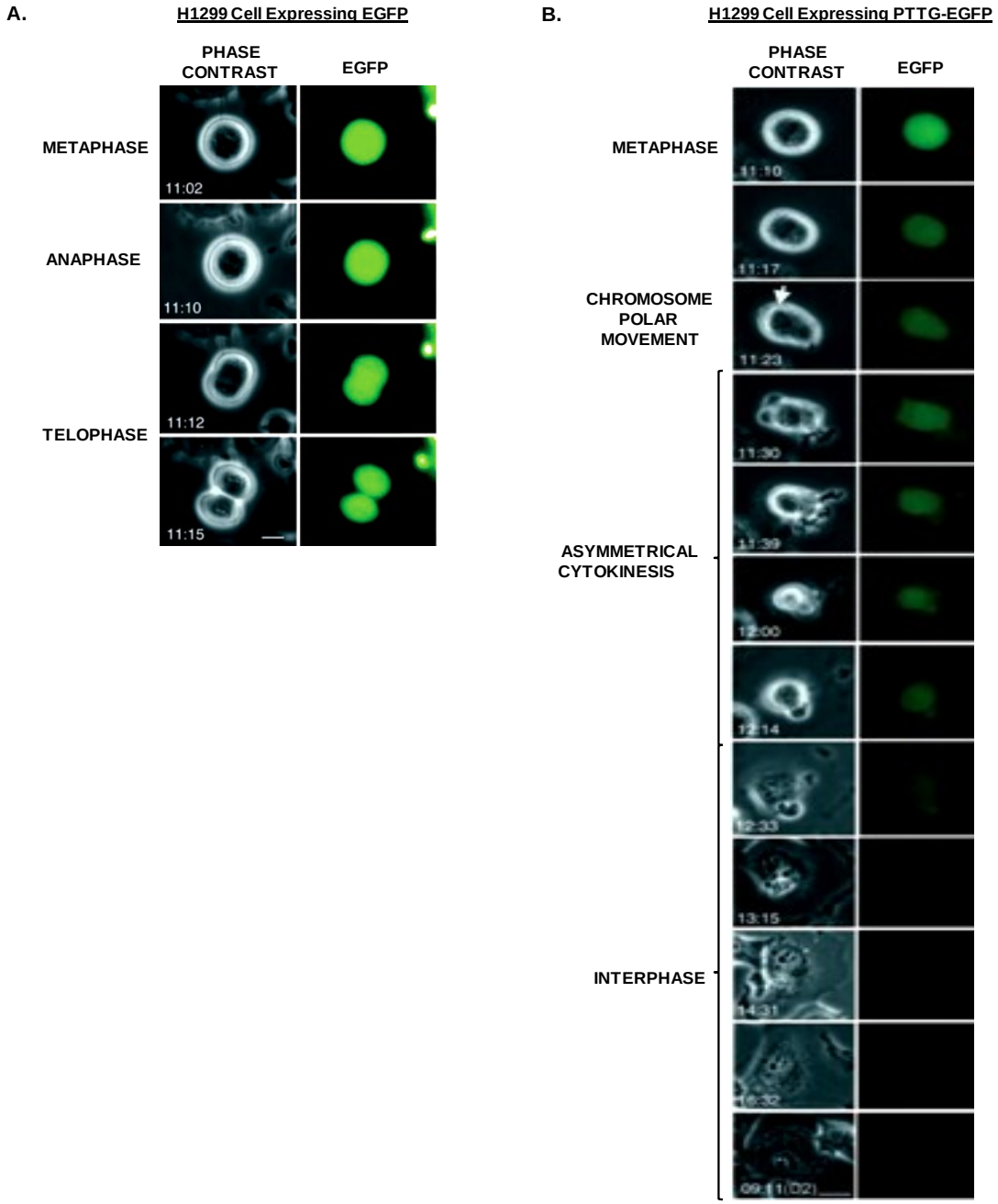


Figure 1-9: Live-imaging of single H1299 cells with phase-contrast microscopy and EGFP fluorescent-microscopy. ***A*** *Visualisation of normal cell division of a single H1299 cell expressing EGFP alone into 2 daughter cells during mitosis. Time when images were taken is shown for each frame; Bar: 10 μm.* ***B*** *Chromosome non-segregation and aneuploidy resulting from failure of hPTTG-EGFP degradation. Single live H1299 cells expressing hPTTG-EGFP were continuously observed. The complete absence of cytokinesis results in one daughter cell with a macronucleus and one non-viable daughter cell. Arrow: non-segregated chromosomes. (Figure adapted from (Yu et al. 2003)).*

To assess the importance of hPTTG in inducing genetic instability in thyroid cancer, our group employed a fluorescent intersimple sequence repeat PCR assay (FISSR-PCR) which was first used to measure intra-chromosomal instability in sporadic colorectal cancers (Basik et al. 1997). We demonstrated that genomic instability was variable, but overall higher in thyroid cancer tissue samples compared to normal tissue controls (increased genomic instability index of 6.7-72.7 %). Importantly we found a very strong correlation ($R^2 = 0.8$, $p = 0.007$) between hPTTG expression and genomic instability in thyroid cancers (Kim et al. 2005). Furthermore, over-expression of hPTTG in FTC133 human thyroid follicular carcinoma cells induced genetic instability in a dose-dependent manner (Kim et al. 2005). These findings strengthen evidence for a relationship between hPTTG and genetic instability *in vitro* and *in vivo*, indicating that hPTTG at least in part, may be a causal factor in the chromosomal aberrations observed in differentiated thyroid cancers.

1.3.3 hPTTG over-expression in mouse models

Two studies have directly investigated the effects hPTTG over-expression *in vivo* through studies in transgenic mice. hPTTG was targeted to the pituitary glands of mice using the α-subunit of glycoprotein hormone (α-GSU) promoter, which is the earliest expressing pituitary hormone gene product. Female αGSU.PTTG mice had significantly enlarged pituitary glands and elevated serum IGF-1 levels compared with WT mice. However, the study focused on a more aggressive phenotype in male αGSU.PTTG mice, which demonstrated plurihormonal focal pituitary transgene expression with LH-, TSH- and, unexpectedly, also GH-cell focal hyperplasia and adenoma, associated with increased serum LH, GH, testosterone, and IGF-I levels. MRI scans demonstrated large and irregularly shaped pituitary glands in αGSU.PTTG mice (see Figure 1-10). Some of these mice died prematurely

from urinary tract obstruction, caused by prostate and seminal vesicle hyperplasia due to elevated serum hormone levels. Critically, this study provided evidence of a role for hPTTG over-expression in enhanced pituitary cell growth and specifically in the promotion of differentiated polyhormonal cell focal expansion (Abbud et al. 2005).

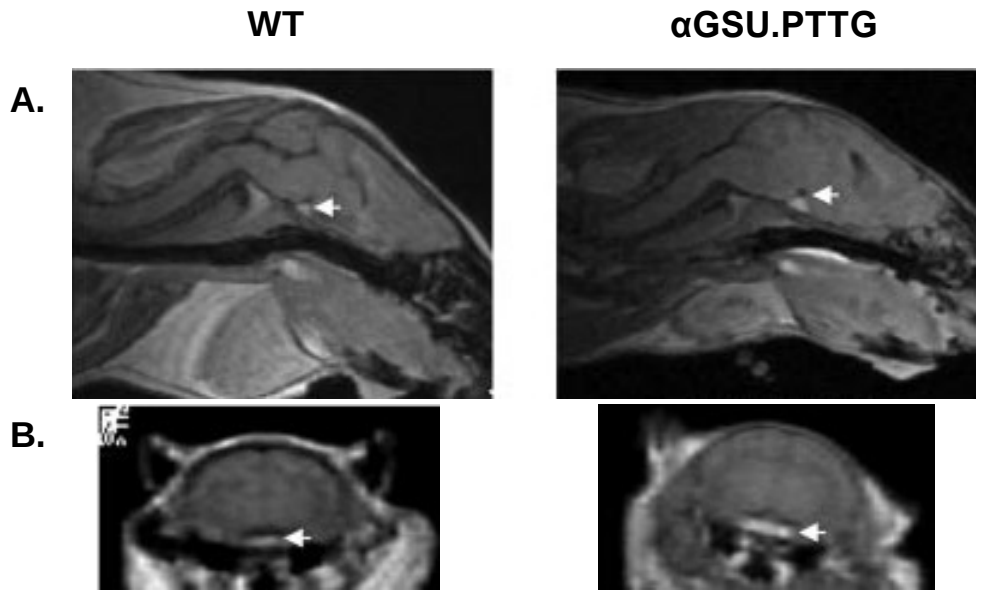


*Figure 1-10: Enlarged pituitary glands in αGSU.PTTG mice. Sagittal (**A**) and coronal (**B**) MRI images of representative WT and αGSU.PTTG mice. White arrows indicate the pituitary gland. Adapted from (Abbud et al. 2005).*

Following observation of hPTTG over-expression in ovarian cancers (Puri et al. 2001), a transgenic mouse model was developed to directly investigate the effect of hPTTG over-expression in the ovary (El-Naggar et al. 2007). Increased hPTTG expression was evident in the ovarian surface epithelium and granulosa cells of transgenic mice, driven by the Mullerian inhibitory substance type II receptor gene promoter (MISIIR). Although female MISIIR-PTTG transgenics did not develop ovarian tumours, the study reported an overall increased mass of the corpus luteum, generalised hypertrophy of the myometrium of uteri, with cystic glandular and hyperplasia of the endometrium. The study concluded that hPTTG is involved with the initial transformation of ovarian epithelial cells and may be important in

creating pre-cancerous conditions, but its over-expression alone is not sufficient for ovarian tumourigenesis (El-Naggar et al. 2007).

1.3.4 hPTTG under-expression and genetic instability

Following the identification of hPTTG as the human securin with a crucial role in regulating sister chromatid segregation thereby ensuring the maintenance of euploidy, it was predicted that cells lacking hPTTG would not be viable. It was therefore surprising that both hPTTG-null cells and Pttg knockout mice are viable (Jallepalli et al. 2001; Wang et al. 2001).

Human colorectal HCT116 cells, which have a stable karyotype and intact DNA damage and mitotic spindle checkpoints, were used to establish an hPTTG-null cell line ($hSecurin^{-/-}$) by means of homologous recombination. Although $hSecurin^{-/-}$ cells generally grew more slowly, the percentage of cells in apoptosis, mitosis or interphase was almost identical to that of $hSecurin^{+/+}$ cells, thereby indicating their viability. Multiplex fluorescent in situ hybridisation (M-FISH) was used to analyse chromosome number and structure in metaphase spreads and loss of chromosomes was revealed in greater than 80 % of $hSecurin^{-/-}$ cells. Time-lapse microscopy studies were used to analyse key mitotic events and over one third of $hSecurin^{-/-}$ cells failed to separate metaphase chromosomes. Despite non-completion of anaphase, $hSecurin^{-/-}$ cells eventually exited mitosis after an extensive delay at the metaphase to anaphase transition and daughter cells were associated with budded nuclei, chromosomal instability and gross aneuploidy (Jallepalli et al. 2001).

Subsequently, the development of a Pttg knockout ($Pttg^{-/-}$) mouse has provided insight to the effects of the absence of Pttg *in vivo* (Wang et al. 2001; Wang et al. 2003). $Pttg^{-/-}$ mice are viable and fertile, despite females being deemed subfertile following observation of reduced average litter sizes. Major characteristics of $Pttg^{-/-}$ mice are testicular and splenic

hypoplasia, thymic hyperplasia and thrombocytopenia. Although the reason for these phenotypes is unclear, Pttg levels are abundant in normal testes and thymus, so the organ-specific varying phenotypes described in this study indicate cell type specific regulation of growth by Pttg. Mouse embryonic fibroblasts (MEFs) derived from $Pttg^{-/-}$ mice had a shortened G1-phase and a prolonged G2- to M-phase transition. Interestingly, the phenotype of a high percentage of $Pttg^{-/-}$ MEFs being in G2- and M-phase, was reversed by retroviral introduction of Pttg. Cytogenetic analyses revealed that $Pttg^{-/-}$ MEFs had frequently damaged nuclei, chromosomal abnormalities particularly in centromeric regions and up to 15 % of nuclei were bi- or multinucleated. Metaphase chromosomal spreads revealed premature centromere division, up to 6 % chromosomal abnormality including quadriradial and triradial chromosome formations and overall 10-15 % aneuploidy in $Pttg^{-/-}$ MEFs compared to $Pttg^{+/+}$ MEFs (Wang et al. 2001).

The results of the studies described above clearly lend further support to the function of hPTTG as a securin, though the viability of $hSecurin^{-/-}$ HCT116 cells and $Pttg^{-/-}$ mice is indicative of the existence of alternative mechanisms driving cell cycle progression and survival when hPTTG expression is lacking. As discussed in section 1.3.1, one potential mechanism is the inhibitory phosphorylation of separase at Ser1126 by the CCNB1/CDC2 complex, where separase is only activated once cyclin B (CCNB1) is ubiquitinylated by the APC (Stemmann et al. 2001; Gorr et al. 2005; Holland and Taylor 2006). Supporting this, a further study in $hSecurin^{-/-}$ HCT116 cells demonstrated that progression to anaphase and specifically the event of centromere separation, was dependent on an active proteasome (Gimenez-Abian et al. 2005).

Further work using $hSecurin^{-/-}$ HCT116 cells revealed that the characteristic loss of chromosomes in these cells, as discussed above (Jallepalli et al. 2001), is in fact overcome

following continued passaging of these cells. Despite persistently low separase activity, cells regained chromosomal stability and normal cell division with complete segregation of sister chromatids (Pfleghaar et al. 2005). Another study highlighted the redundancy of Pttg and separase in mitotic regulation by mutating the Ser1121 phosphorylation residue of separase in $Pttg^{-/-}$ MEFs, thereby generating double mutant *securin* $^{-/-}$ *separase* $^{-/S1121A}$ MEFs. Following arrest of asynchronously growing cells in G2-/M-phase through nocodazole treatments, it was apparent that sister chromatids were separated prematurely in double mutant cells, though cells remained viable. However, only a small degree of activation of separase was observed suggesting other mechanisms of separase inhibition may exist, at least in MEFs (Huang et al. 2005). Although the studies described above indicate Pttg function is subject to redundancy, a complex phenotype observed in 6 month old male $Pttg^{-/-}$ mice suggests that this is not true for all cell types. These mice exhibit hyperglycaemia and insulinopaenia typical of diabetes, though collectively the phenotype did not conform to either Type I or Type II diabetes. The observed features were due to impaired proliferation in $Pttg^{-/-}$ pancreatic β-cells, where the presence of macronuclei were also observed, consistent with aberrant cell division due to lack of Pttg (Wang et al. 2003). Further investigations of this phenotype revealed that p21 was activated in young mice and genes associated with DNA damage were progressively upregulated in adult $Pttg^{-/-}$ mice. It was concluded that reduced pancreatic β-cell mass was caused by a combination of apoptosis and senescence, secondary to DNA damage (Chesnokova et al. 2009).

Despite some evidence for hPTTG redundancy, the studies described above clearly demonstrate that lack of hPTTG causes genetic instability. Taken together with the abundance of studies demonstrating genetic instability as a result of hPTTG over-expression (see section 1.3.2), it is possible that *hPTTG* represents an important gene at an early stage of

tumourigenesis, where genetic instability is widely recognised as a critical factor of tumour development (Melmed 2003).

1.3.5 hPTTG, P53, apoptosis and DNA damage responses

It is possible that over-expression of hPTTG can further contribute to initiating events in thyroid cancer, as well as to subsequent phases of disease progression, through interactions with vital cell cycle and DNA repair genes. *p53* is mutated in over 50 % of malignancies and is therefore of particular importance in many cancers (Hollstein et al. 1994). It is a tumour suppressor gene that is activated in response to DNA damage and elicits protective effects through induction of cell cycle arrest, DNA repair and apoptotic pathways (Levine 1997; Vousden 2000). The protective activities of p53 are mediated through transcriptional activation of downstream target genes (El-Deiry 1998; Lakin and Jackson 1999; Wahl and Carr 2001) and loss of p53 activity or the ability to induce a p53 response can cause malignant progression (Vousden 2006). Over-expression of oncogenes causes cellular stress through DNA damage, as is true for over-expression of hPTTG in cells (see section 1.3.2) and thus elicits protective cellular responses. Interestingly, several studies have described interactions between hPTTG and p53 in association with apoptosis.

hPTTG induction of apoptosis was initially observed following over-expression of EGFP-hPTTG in JEG-3 cells (Yu et al. 2000b). In a subsequent study by the same group, over-expression of hPTTG in MCF-7 cells with WT levels of p53 caused induction of apoptosis following upregulation and nuclear translocation of p53. This effect was enhanced by contransfections with *p53* and abrogated by expression of the p53 inactivator, human papillomavirus E6 protein. However, hPTTG over-expression in p53 null MG-63 cells also resulted in apoptosis suggesting that hPTTG over-expression activates apoptotic pathways via

p53-dependent and p53-independent mechanisms. Moreover, hPTTG induced aneuploidy in p53 null MG-63 cells, but not in MCF-7 or JEG-3 cells with WT p53 expression, suggesting p53 is capable of preventing hPTTG-induced aneuploidy (Yu et al. 2000a). Another study provided further support for p53-mediated hPTTG induction of apoptosis, where over-expression of either hPTTG or p53 in HEK293 cells caused apoptosis. hPTTG was shown to indirectly mediate p53 transcription via activation of c-myc expression, which subsequently bound directly to the *p53* promoter. Enhanced expression of the well established pro-apoptotic p53 effector, BAX, was representative of increased p53 function following hPTTG over-expression (Hamid and Kakar 2004; Nagashima et al. 2003). Together, these results suggest that cells can respond to potentially harmful levels of hPTTG over-expression through induction of apoptotic pathways. However, failure of these mechanisms could support survival of aneuploid cells and sustained tumour growth.

The relationship between hPTTG and p53 is ambiguous due to contrasting data between studies. Pull-down and coimmunoprecipitation assays demonstrated a direct and specific interaction between hPTTG and p53 *in vitro* and *in vivo* (Bernal et al. 2002). This interaction blocked p53 binding to DNA, p53 transcriptional capabilities and in contrast to the findings above, p53-induced apoptosis was prevented by over-expression of hPTTG. In addition, a potentiation of the apoptotic and transactivating functions of p53 was demonstrated in hPTTG deficient cells ($hPTTG^{-/-}$ human colorectal HCT116 cells), highlighting the physiological relevance of this interaction (Bernal et al. 2002). Adenoviral vector delivery of siRNA against hPTTG in SH-J1 hepatoma cells resulted in activation of p53 leading to increased p21 levels and induction of apoptosis. Further, *in vivo* study of SH-J1 tumor xenografts established in nude mice observed reduced tumour growth following intra-tumour delivery of adenoviral hPTTG siRNA (Jung et al. 2006). Apoptosis was induced

by UV irradiation in HeLa cells, and apoptosis was enhanced in cells with depleted hPTTG levels following siRNA knockdown and reduced in cells over-expressing hPTTG (Lai et al. 2007). In the context of hPTTG inhibition of p53-mediated apoptosis, reduced apoptosis in tumour cells with high hPTTG expression could explain the prolonged survival of these cells, thereby promoting tumourigenesis.

The relationship between hPTTG and p53 is further complicated by the finding that hPTTG is itself a transcriptional target of p53. Doxorubicin and bleomycin are drugs known to cause double stranded DNA breaks (Povirk 1996; Gewirtz 1999). Treatment of cells with these drugs induced DNA damage and repression of hPTTG expression was reported, an effect that was entirely dependent on the presence of p53. Further analysis of the *hPTTG* promoter indicated that following DNA damage, functionally activated p53 prevents NF-Y binding to the *hPTTG* promoter and subsequent regulation of its transcription (Zhou et al. 2003). An additional study has since demonstrated direct binding of p53 to the *hPTTG* promoter resulting in hPTTG suppression (Kho et al. 2004). Repression of hPTTG expression following DNA damage induced by UV irradiation was commonly observed in multiple cell lines and occurred independently of p53. Instead, reduced hPTTG expression was attributed to two independent mechanisms involving decreased hPTTG protein synthesis and increased proteasomal degradation of hPTTG. hPTTG was however required for cell cycle arrest following UV irradiation and lack of hPTTG resulted in premature mitosis and increased apoptosis (Romero et al. 2004). hPTTG degradation was thought to be APC-mediated due to the stability of a double D-box and KEN box mutant (Romero et al. 2004), though more recently, SKP1-CUL1-βTrCP E3 was identified as the ubiquitin ligase that mediates hPTTG degradation following UV irradiation (Limon-Mortes et al. 2008). In contrast, hPTTG was induced in both RKO (wild-type p53) and SW480 (mutant p53)

colorectal carcinoma cells following X-ray radiation. Further, cytotoxicity and apoptosis was increased in hPTTG-null HCT116 cells compared with wild-type HCT116 cells (Chiu et al. 2007). Together these results suggest that increased levels of hPTTG in colorectal carcinoma cells following X-ray radiation may be associated with protective induction of apoptosis.

Further evidence for the critical role of hPTTG in DNA damage response pathways is the finding that hPTTG can form complexes with Ku heterodimers (Romero et al. 2001). The Ku heterodimer containing Ku70 and Ku80 binds to DNA and facilitates non-homologous DNA end-joining (NHEJ) repair of double-strand DNA breaks through recruitment of the catalytic component of DNA-PK (Kharbanda et al. 1998; Smith and Jackson 1999). hPTTG binds to Ku70 at a region located at its N-terminal forming an inhibitory complex; in the presence of DNA damage, Ku70 dissociates to allow DNA-PK-dependent DNA repair. The ability of DNA-PK to phosphorylate hPTTG may facilitate its dissociation from Ku70 (Romero et al. 2001). Concordant with this, hPTTG over-expression inhibits Ku70 DNA binding and represses repair of double-stranded DNA breaks (Kim et al. 2007b). Together, these data suggest that hPTTG over-expression can cause aberrant sequestration of Ku proteins and thus inhibit appropriate DNA repair mechanisms, leading to further genetic instability. Consistent with this, studies observed reduced cellular proliferation rates accompanied by altered patterns of end resection in NHEJ assays following DNA damage in hPTTG-null HCT116 cells compared with normal HCT116 cells (Bernal et al. 2008).

It is clear that we only have a partial understanding of the complex and intricate relationship between hPTTG, p53 and other DNA repair mechanisms and these remain to be elucidated in the context of the thyroid. This is a fascinating and important area of research since the progression of many cancers requires cells to escape normal regulation of apoptosis by p53 and the activation of DNA repair pathways, which could indeed be a fundamental

consequence of hPTTG over-expression observed in differentiated thyroid cancer, causing prolonged cell survival and increased genetic instability.

1.3.6 hPTTG as a gene transactivator

The early observations that hPTTG has a similar peptide sequence to DNA binding proteins, and shows partial nuclear localisation, suggested that hPTTG may transactivate other genes (Dominguez et al. 1998). A fusion protein of hPTTG and the DNA binding protein GAL4 was able to activate GAL4 responsive elements to drive expression of the *his3* and *lacZ* genes in yeast, as well as the luciferase gene in mammalian cells. Deletion of amino acids 124-202 abolished the transactivational ability of hPTTG, whereas mutation of the N-terminus (amino acids 1-122) did not, confirming that the C-terminal part of hPTTG is responsible for this function (Dominguez et al. 1998).

Indeed, the C-terminus of hPTTG is an acidic region containing multiple glutamic acid and proline residues, which are characteristic of transactivation domains (Dominguez et al. 1998; Wang and Melmed 2000b; Pei 2000). Within the proline-rich region, there are two PXXP motifs that are putative SH3-interacting sites; highly conserved regions that govern transduction of intracellular signalling pathways. Crucially, point mutations in the PXXP motifs resulted in abolished transcriptional activation, as well as hPTTG's *in vitro* transforming and *in vivo* tumour-inducing activity (Zhang et al. 1999b). Similarly, point mutation of a key proline residue in murine Pttg abrogated transcriptional activity and transforming capability in NIH3T3 cells (Wang and Melmed 2000b), suggesting that the transactivation and transforming abilites of hPTTG are intrinsically linked. Interestingly, *in vitro* studies demonstrated that Egf stimulation of the Mapk cascade resulted in nuclear translocation of rat Pttg and subsequent transcriptional activity. These effects were dependent

on Mapk phosphorylation of Ser162 within the transactivation domain, in addition to a specific interaction between Mek1 and an SH3-interacting domain located between amino acids 51 and 54 (Pei 2000). The corresponding residue in hPTTG, Ser165, has thus far only been described as a phosphorylation target for CDC2 (Ramos-Morales et al. 2000) and the association with MAPK remains to be verified (see section 1.2.1.5).

1.3.6.1 hPTTG and c-Myc

Several transcriptional targets of hPTTG have been identified, including genes involved in cellular growth and angiogenesis, as will subsequently be discussed (see section 1.4). Initially, cDNA PCR array analysis was performed in order to broadly identify transcription targets of hPTTG. A number of genes were upregulated in cell lines following induction of rat Pttg including Mek1, mitogen-activated protein kinase kinase 3 (Mek3), protein-kinase C beta (Pkcβ-1) and heat shock protein 70 (Hsp70). However, the c-Myc oncogene was identified as a major Pttg transcription target and was subject to further investigation (Pei 2001). c-Myc is a transcription factor that is deregulated in various human tumour types (Patel et al. 2004), possibly through its critical role in control of cellular proliferation, with over-expression of c-Myc resulting in cell-cycle progression, cell transformation and block of differentiation (Henriksson and Luscher 1996). Induction of Pttg in HeLa cells resulted in increased proliferation and transformation following activation of c-Myc expression by direct binding to the c-Myc promoter in a complex with the ubiquitously expressed upstream stimulatory factor (USF1). Interestingly, Pttg-mediated c-Myc activation was dependent on phosphorylation of Pttg, suggesting Pttg phosphorylation may be of importance in Pttg gene transactivation (Pei 2001). Induction of p53 by hPTTG (see section 1.3.5) was shown to be an indirect effect dependent on prior activation of c-Myc, which

formed heterodimers with its binding partner MAX to drive the *p53* promoter (Hamid and Kakar 2004).

Given the similar effects of hPTTG and c-Myc over-expression, in addition to the dependency of some hPTTG effects on c-Myc activation, it is possible that hPTTG's oncogenic actions are mediated via c-Myc. However, high levels of hPTTG expression in pituitary tumours was not associated with increased c-Myc expression (Boggild et al. 1994; Woloschak et al. 1994; Saez et al. 1999; Zhang et al. 1999a). Furthermore, hPTTG is predominantly expressed during G2/M-phase (see section 1.3.1), whereas c-Myc is an S-phase protein (Thompson 1998) suggesting they are independently expressed throughout the cell-cycle. Although these observations suggest hPTTG has tumourigenic effects independent of c-Myc, it remains plausible that hPTTG induction of c-Myc plays a role in tumour initiation.

1.3.6.2 hPTTG and other interacting factors

Chromatin immunoprecipitation (ChIP)-on-Chip experiments were used to simultaneously assess 20,000 gene promoters in JEG-3 cells, and demonstrated a global transcriptional effect of hPTTG (Tong et al. 2007). Binding of hPTTG was observed in a total of 746 promoters with functions relating to cell cycle, metabolic control and signal transduction. SP1 binding sites were frequently identified in hPTTG-binding promoters and subsequent co-immunoprecipitation and pull-down assays confirmed a specific interaction between hPTTG and SP1 both *in vivo* and *in vitro* via hPTTG's N-terminal (amino acids 1-120). The results of this study show that hPTTG exhibits properties of a global transcription factor and may act through formation of transcription factor complexes by binding other proteins (Tong et al. 2007).

Yeast two-hybrid systems were designed to identify proteins physically associating with hPTTG in human testicular cells. *In vitro* binding assays and co-immunoprecipitation assays identified a specific interaction, conferred by the C-terminal of Pttg, between rat Pttg and the molecular chaperone HSJ2 and ribosomal protein S10. The functional significance of these interactions remain unknown, but Pttg was expressed at specific stages of spermatogenesis suggesting it has an important role in this process (Pei 1999).

Using protein microarray screening, hPTTG was found to have protein-protein interactions with approximately 80 proteins. Interaction with S10 was confirmed and associations with transcription factors such as TATA box-binding protein-like protein 1 (TBPL1) and activator of basal transcription 1 (ABT1) were identified, providing further evidence that hPTTG is important in transcriptional regulation (Tong et al. 2008; Tong and Eigler 2009). A specific interaction with Aurora-A kinase was identified and validated using His-tag pull-down assays and co-immunoprecipitation assays in HCT116 cells. hPTTG over-expression resulted in inhibition of Aurora-A kinase activity and abnormally condensed chromatin. hPTTG-null cell proliferation was more sensitive to Aurora-A knock down and to Aurora kinase Inhibitor III treatment compared to WT cells, indicating that hPTTG knockdown could be a potential approach to improve the efficacy of Aurora kinase inhibitors (Tong et al. 2008).

Overall, hPTTG seems to exert its transcriptional effects either by direct DNA binding or by interacting with proteins at both the N- and C-terminal regions to facilitate or inhibit transcriptional processes. However, the effects observed following mutations in the C-terminal transactivation domain suggest that this region is particularly important in hPTTG's transactivational and transforming capabilities.

1.4 hPTTG promotes tumour progression

Aside from the role of hPTTG in initiating thyroid cancer through the pathways described above, hPTTG has been shown to interact with growth factors and cytokines that are implicated in further dysregulation thereby driving disease progression. The relationships between hPTTG, FGF-2 and VEGF are the most clearly established and appear to be of critical importance in tumour progression through promotion of growth and angiogenesis. Angiogenesis is the normally quiescent process of blood vessel development that is thought to become highly activated during tumourigenesis and is necessary for tumour growth and invasion (Folkman 1992; Folkman and Shing 1992; Risau 1997; Hanahan and Folkman 1996; Folkman 1972; Folkman 1990). Endocrine organs are typically highly vascular, but thyroid tumours acquire an even greater blood supply (Goldenberg et al. 1998; Akslen and LiVolsi 2000; Turner et al. 2003), driven by altered levels of pro-angiogenic growth factors such as FGF-2 (Bikfalvi et al. 1997; Powers et al. 2000; Turner et al. 2003) and VEGF (Ferrara and DavisSmyth 1997) and inhibitors, as well as extracellular matrix changes that allow endothelial migration (Folkman 1992; Folkman and Shing 1992; Hanahan and Folkman 1996; Turner et al. 2003). The critical roles of FGF-2 and VEGF are well established where both molecules act synergistically to modulate tumour angiogenesis and invasion (Gospodarowicz et al. 1987; Bikfalvi et al. 1997; Ferrara and DavisSmyth 1997).

1.4.1 FGF-2

FGF-2 belongs to a family of small poly-peptide growth factors, comprising 23 members, which share common certain structural characteristics, are expressed by almost all tissues and are mitogenic to many cell types. FGFs are secreted into the extracellular environment where they form a reservoir by binding the heparin-like glycosaminoglycans

(HLGAGs) or the extracellular matrix (ECM). Subsequently, their activity is mediated through digestion of the ECM or by secreted FGF binding proteins (Powers et al. 2000). Though four FGF-receptors (FGFR 1-4) sharing common structures including an intracellular tyrosine kinase domain have been identified, FGF-2 preferentially interacts with FGFR1 (Powers et al. 2000; Maher 1996). FGFR1 is expressed in the nuclear fraction of various cell types (Maher 1996), including thyroid follicular cells (Patel et al. 1996), and nuclear FGFR1 is associated with proliferation (Maher 1996; Stachowiak et al. 1997). The binding of FGF-2 to FGFR1 results in receptor dimerisation, autophosphorylation of tyrosine kinase residues and the subsequent activation of various signal transduction pathways (Lemmon and Schlessinger 1994; Bikfalvi et al. 1997; Pawson 1995). The major pathways with reported involvement in FGF signalling are the Phospholipase C-γ (PLC-γ), v-crk sarcoma virus CT10 oncogene homolog (CRK) and RAS/MAPK signalling pathways (Bikfalvi et al. 1997; Kouhara et al. 1997; LaVallee et al. 1998; Powers et al. 2000). FGF-2 has important physiological roles in embryonic development where it is involved in the regulation of growth, differentiation and function of various systems (Bikfalvi et al. 1997; Powers et al. 2000; Logan et al. 1991; McAvoy et al. 1991; Unsicker et al. 1992; Baird 1994). In addition, FGF-2 is a potent angiogenic molecule which stimulates smooth muscle cell growth, wound healing and tissue repair *in vivo and in vitro* (Folkman and Shing 1992; Bikfalvi et al. 1997; Powers et al. 2000) and through these mitogenic and angiogenic capabilities can contribute to tumour development (Murai et al. 1996).

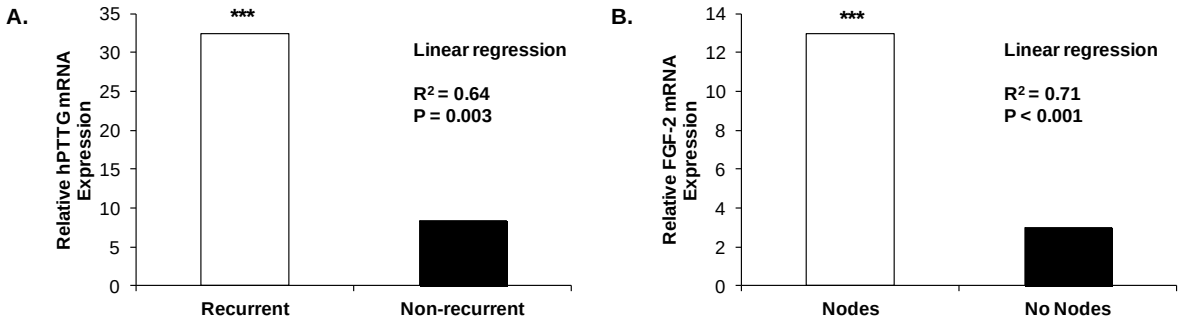
1.4.2 VEGF

Similarly to FGF-2, VEGF is a potent mitogen but acts in a highly specific manner on micro- and macrovascular endothelial cells (Leung et al. 1989; Plouet et al. 1989) and

activates serine proteases urokinase-type and tissue-type plasminogen activators known to be involved in cellular invasion and tissue remodelling (Pepper and Montesano 1990). Furthermore it affects vascular permeability, promotes the expression of adhesion molecules on endothelial cells, induces vasodilatation and influences monocyte chemotaxis (Senger et al. 1983; Roberts and Palade 1995; Ferrara and DavisSmyth 1997). Alternative splicing of a single *VEGF* gene results in the synthesis of four different isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) that have different tissue expression. However, $VEGF_{165}$ is the predominant isoform and thus commonly referred to as VEGF (Ferrara and DavisSmyth 1997; Ramsden 2000). The actions of VEGF are facilitated by binding to either VEGFR-1 (FLT-1) or VEGFR-2 (KDR), which both have consensus tyrosine kinase sequences that are autophosphorylated following formation of multimeric VEGF receptor complexes (Vaisman et al. 1990; Ferrara and DavisSmyth 1997; Ramsden 2000; Guo et al. 1995). Subsequently, SH2- containing proteins are recruited to the complex and activate intracellular transduction pathways involving PLC-γ and the ras GTP-ase activating protein (Ferrara and Davis-Smyth, 1997). The importance of VEGF in tumour development is evidenced by a correlation between *VEGF* mRNA expression and tumour vascularity (Brown et al. 1995; Viglietto et al. 1997; Kerbel et al. 1998) and increased levels of circulating VEGF in cancer patients (Kondo et al. 1994). Furthermore, upregulation of FLT-1 and KDR mRNA has been found in the endothelial cells associated with tumours (Plate et al. 1994; Brown et al. 1995). Specifically in thyroid malignancies VEGF represents an important bio-marker as there is a strong correlation between its expression and tumour aggressiveness and metastasis (Soh et al. 1997; Bunone et al. 1999; Klein et al. 2001).

1.4.3 hPTTG, FGF-2, VEGF and angiogenesis

Various studies have described the induction of FGF-2 by hPTTG. Stable over-expression of hPTTG in NIH3T3 cells resulted in increased FGF-2 mRNA expression and secretion as determined by ELISA. Mutation within the SH3-interacting domain of hPTTG resulted in abrogation of these effects and also prevented *in vitro* transformation and *in vivo* tumourigenesis, indicating that enhanced FGF-2 signalling is an important event in these processes (Zhang et al. 1999b). Subsequently, it was demonstrated that FGF-2 in turn induces hPTTG mRNA expression in NIH3T3 cells, suggesting the existence of autocrine pathways of regulation between hPTTG and FGF-2. This effect was blocked by antiserum against FGF-2, which also reduced basal hPTTG expression providing further evidence for an auto-regulatory feedback mechanism (Heaney et al. 1999). Our group's own studies demonstrated that expression of FGF-2 was induced following hPTTG over-expression in human primary follicular thyroid cells. This effect was unaltered by mutation of the hPTTG phosphorylation site (S165A) but was significantly reduced by mutation of the SH3-interacting domain (Boelaert et al. 2003a). Furthermore, a positive correlation between high levels of hPTTG and FGF-2 expression was observed in differentiated thyroid cancers. Both can be considered important prognostic indicators, as hPTTG over-expression was associated with tumour recurrence and the expression of *FGF-2* mRNA was independently associated with lymph node invasion and distant metastases (Boelaert et al. 2003a). Further, use of luciferase reporter assays in COS-7 cells demonstrated that hPTTG regulation of FGF-2 was a direct and promoter-specific effect (Chien and Pei 2000).



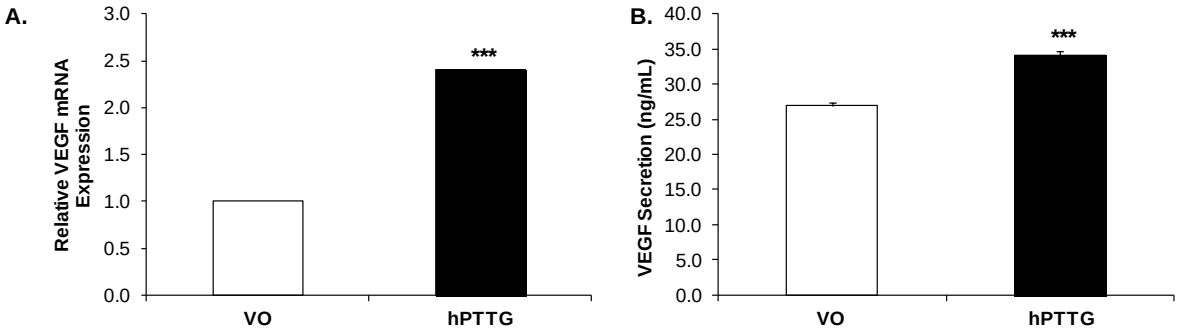
Mean Δ Ct	6.30	8.27
SEM	±0.14	±0.28
N	6	18

Mean Δ Ct	13.90	15.99
SEM	±0.24	±0.17
N	9	15

*Figure 1-11: hPTTG and FGF-2 as potential prognostic indicators. **A** Fold changes in hPTTG mRNA in recurrent and non-recurrent thyroid cancers. **B** Fold changes in FGF-2 mRNA in thyroid cancers with and without nodal involvement. Gene expression is relative to a value of 1.0 in normal thyroid tissue. ***, $p < 0.001$. Also displayed are the results of multiple linear regression analysis relating hPTTG mRNA to recurrence (**A**) and relating FGF-2 mRNA to nodal metastases (**B**). Results shown are mean ΔCt ± SEM. Adapted from (Boelaert et al. 2003a).*

Our group has also performed studies that have elucidated an additional auto-regulatory mechanism involving hPTTG and VEGF. Having reported increased hPTTG levels in 111 pituitary adenomas examined (McCabe *et al*, 2003), we also examined the expression of VEGF and KDR in these tumours (n = 103) (McCabe *et al*, 2002). Non-functioning pituitary tumours (n = 81) demonstrated markedly raised VEGF mRNA (3.2-fold, $p < 0.05$) and protein concentrations compared with samples of normal pituitary (n = 10). Moreover a significant positive correlation between *VEGF* and *hPTTG* mRNA was noted ($R^2 = 0.22$, $p < 0.001$). The expression of *KDR* mRNA was also highly induced in non-functioning tumours (14-fold, $p < 0.001$) as well as in the whole cohort of pituitary tumours (14-fold, $p < 0.0001$) and again a positive correlation with *hPTTG* mRNA was demonstrated ($R^2 = 0.14$, $p < 0.001$). Western blotting confirmed higher KDR expression at protein level (McCabe *et al*, 2002). hPTTG over-expression resulted in upregulation of both FGF-2 and VEGF expression in NT-2, MCF-7 and JEG-3 cells. Once again the phosphorylation status did not alter hPTTG upregulation of FGF-2 and VEGF, whereas mutation of the SH3 domain reduced this effect (McCabe et al. 2002). FGF-2 itself has previously been shown to

modulate VEGF expression in endothelial cells (Seghezzi et al. 1998; Saadeh et al. 2000). However, in this study, depletion of secreted FGF-2 in the cell media failed to influence the effect of hPTTG on VEGF expression suggesting that hPTTG affects VEGF expression via a mechanism that is independent of hPTTG-mediated transactivation of FGF-2 (McCabe et al. 2002). In a further study by our group, cDNA PCR array analyses revealed that mRNA expression of *VEGF* and the pro-angiogenic gene *DNA-binding 3* (*ID3*) were induced following over-expression of hPTTG in human primary thyroid follicular cells. Induction of ID3 was subsequently demonstrated to be via the SH3-interacting domain, further evidencing the importance of this region in the transactivational capabilities of hPTTG (Kim et al. 2006b). Subsequent studies in follicular thyroid cancer FTC-133 cells demonstrated that hPTTG induces expression of ID3 following upregulation of VEGF and its receptor KDR revealing another potentially important autocrine feedback mechanism involving VEGF in thyroid cancer. Thyroid epithelial cells were shown to express a functional KDR receptor through which VEGF could promote proliferation via the KDR-dependant MAPK pathway. Crucially, in FTC133 thyroid cells, over-expression of hPTTG resulted in an increase in VEGF mRNA expression and secretion (see Figure 1-12). There was also an increase in KDR expression, which is consistent with the finding that KDR expression is elevated in thyroid cancer (Kim et al. 2006a). Together, these data suggest that autocrine pathways of interaction exist between growth factors and hPTTG in thyroid cells, and that dysregulation of these pathways in thyroid cancer can contribute to tumour proliferation and angiogenesis.



Mean Δ Ct	7.2	6.0
SEM	±0.2	±0.3
N	4	4

Figure 1-12: hPTTG regulates expression and secretion of VEGF in thyroid follicular carcinoma FTC-133 cells. ***A*** *Fold change in VEGF mRNA expression in FTC133 cells transfected with hPTTG compared with and normalized to VO-transfected controls.* ***B*** *VEGF secretion from hPTTG-transfected cells compared with VO-transfected control cells measured using ELISA. Results shown are mean ΔCt ± SEM. *** $p < 0.001$. Adapted from (Kim et al. 2006a).*

The evidence that hPTTG regulates expression of growth factors such as FGF-2 and VEGF provides possible mechanisms by which hPTTG mediates angiogenesis and overall disease progression. One study directly investigated the effects of hPTTG on angiogenesis. Consistent with previous findings (Zhang et al. 1999b), hPTTG over-expression induced FGF-2 secretion in NIH3T3 cells (Ishikawa et al. 2001). Subsequently, the conditioned medium from NIH3T3 cells stably over-expressing WT-hPTTG induced proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC cells) *in vitro*. *In vivo*, concentrated media from *WT-hPTTG* transfected NIH3T3 cells induced chick chorio-allantoic membrane (CAM) spoke-wheel-like appearances (see Figure 1-13). Moreover, conditioned medium derived from hPTTG transfectants harbouring the same C-terminal mutation which abrogated FGF-2 induction (Zhang et al. 1999b), induced weaker angiogenic activity than that of *WT-hPTTG* transfected cells (Ishikawa et al. 2001).

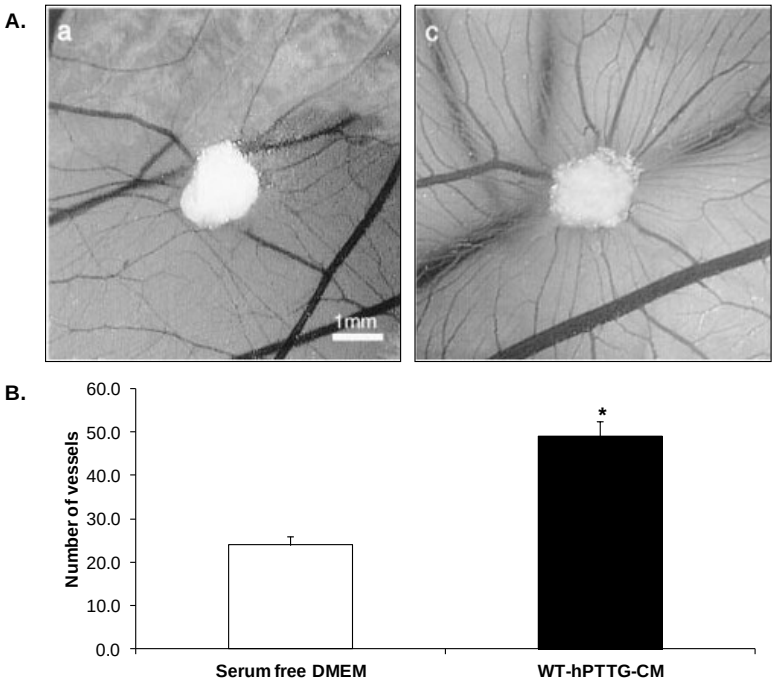


*Figure 1-13: hPTTG induces angiogenesis in vivo. **A** Chrorio-allantoic membrane (CAM) assays demonstrating enhanced vascularisation following incubation with concentrated medium from WT-hPTTG transfected (WT-hPTTG-CM) NIH3T3 cells (right) compared with those treated with serum free DMEM (left). **B** Quantification of induced vessels counted under stereomicroscopy. * $p < 0.01$. Adapted from (Ishikawa et al. 2001).*

A number of investigations in animal models have provided further *in vivo* evidence of the role of Pttg and growth factors in tumour progression. Notably, in the rat model of oestrogen-induced pituitary lactotroph tumour formation, increased expression of Pttg is an early event, thereby supporting a role for Pttg in the earlier stages of tumourigenesis (Heaney et al. 1999). However, the same study makes observations that support the hypothesis that Pttg's relationship with growth factors could similarly promote tumour progression by driving proliferation and angiogenesis. In this model, an increase in Fgf-2 and Vegf expression correlated with the peak in Pttg expression at the hypertrophic to hyperplastic transition of pituitary lactotroph tumour formation (Heaney et al. 1999). A subsequent study in rats showed that expression of Pttg, Fgf-2 and Vegf is increased in a cyclic manner in line with the

rat oestrus cycle, further highlighting the interplay between Pttg, hormones and growth factors (Heaney et al. 2002).

TRβ$^{PV/PV}$ mice spontaneously develop follicular thyroid cancer, and show increased vascularisation, progression to metastasis and high levels of Pttg in the primary thyroid tumours, as well as in the secondary and distant lung metastases (Ying et al. 2006). A double mutant transgenic line, TRβ$^{PV/PV}$*/Pttg*$^{-/-}$, with deletion of Pttg, provided important insights into the effects of Pttg on tumour development (Kim et al. 2007a). In the absence of Pttg, mice developed thyroid tumours, but vascular invasion and metastases were reduced, in line with a reduction in expression of Fgf-2, Fgfr1 and Vegf (Kim et al. 2007a), providing strong support for a role of Pttg in thyroid tumour progression through interactions with growth factors. Similarly, a recent study indicated reduced FGF-2 and VEGF mRNA and protein expression in thyroid cancer cell lines treated with drugs that reduce hPTTG expression (Chintharlapalli et al. 2011).

Additionally, there is evidence in various types of human tumours that links hPTTG with angiogenesis adding to our own study described above (McCabe et al. 2002). Higher *hPTTG* mRNA expression was observed in highly vascular colorectal cancers compared with less vascular specimens (Heaney et al. 2000). High hPTTG expression was associated with poor prognosis in hepatocellular carcinoma (HCC). hPTTG, FGF-2 and VEGF expression were all positively correlated and associated with intratumoural microvessel density, indicating transactivation of FGF-2 and VEGF by hPTTG as a key event in HCC progression (Fujii et al. 2006). Similar results were obtained in studies of hPTTG and FGF-2 in endometrial carcinoma (Wang et al. 2004) and hPTTG and VEGF in pituitary adenomas (Minematsu et al. 2006).

1.4.4 hPTTG and other growth factors and cytokines involved in tumour progression

Despite a significant body of evidence demonstrating the relationship between hPTTG, FGF-2 and VEGF, there is evidence for regulation of other factors by hPTTG. This was highlighted by our own studies identifying *ID3* as a pro-angiogenic gene regulated by hPTTG in human primary thyroid follicular cells. In the same array, *IGF-1*, which stimulates proliferation, survival and anti-apoptotic pathways, was induced 3.1-fold. Further, anti-angiogenic genes such as *thrombo-spondin 1* (*TSP-1*) were downregulated (Kim et al. 2006b). In addition to *FGF-2*, *IGF-1* mRNA expression was positively correlated with *hPTTG* in pituitary adenomas, providing further evidence that hPTTG-mediated IGF-1 transcription may be involved in tumourigenesis (Chamaon et al. 2010). Further, in thyroids from mutant $TR\beta^{PV/PV}$ mice, which spontaneously develop follicular thyroid cancers, *Pttg* and *Tgf-α* expression was elevated at the time of metastatic spread (Ying et al. 2003). Together these results indicate that interactions between hPTTG and multiple growth factors is of importance in tumour development.

hPTTG over-expression in HEK293 cells upregulated interleukin-8 (IL-8) expression *in vitro* and *in vivo,* in tumours developed in nude mice following injection of these cells (Hamid et al. 2005). The expression and secretion of matrix metalloproteinase 2 (MMP-2) was also elevated in these models and subsequent treatments of HUVECs with the conditioned medium taken from hPTTG-transfected HEK293 cells induced proliferation, migration and tube formation *in vitro*. The addition of an MMP-2 antibody significantly decreased these effects (Malik and Kakar 2006).

Together, these findings suggest that hPTTG regulates multiple downstream targets that are involved in proliferative and angiogenic pathways. The described autocrine mechanisms of interaction between hPTTG, FGF-2 and VEGF are well established and

clearly important but further investigation is required in thyroid models to determine whether hPTTG is a key mediator of other autocrine mechanisms involving growth factors that drive tumour progression when aberrantly controlled.

1.5 PTTG Binding Factor

1.5.1 PBF characterisation and structure

The PTTG-Binding Factor (PBF) was isolated and identified as a novel protein that interacts with hPTTG using a two yeast hybrid screening system. PBF, also known as PTTG1-interacting protein (PTTG1IP), is located on chromosome 21q22.3 and contains an open reading frame of 179 amino-acids with a predicted molecular mass of 22 kDa (Chien and Pei 2000). It has since become clear that *PBF* had previously been cloned, when it was termed *C21orf3* (also known as *C21orf1*). C21orf3 was thought to have a role in cell trafficking mechanisms as a Type 1a integral membrane cell surface glycoprotein, following initial protein prediction studies identifying a potential N-terminal signal peptide, transmembrane domain, endocytosis motif and two putative N-glycosylation sites (Yaspo et al. 1998). Further analysis identified potential sites for post-translational modifications such as phosphorylation sites for cAMP- and cGMP-dependent kinase, PKC and casein kinase II and five glycosylation sites for *N*-linked and *O*-linked oligosaccharides (Chien and Pei 2000). Additionally, an extracellular N-terminal cysteine-rich putative protein-binding domain, common to plexins, semaphorins and integrins and thereby referred to as a PSI domain, is also found in PBF (Bork et al. 1999). However, PBF was demonstrated to contain a C-terminal bipartite NLS suggesting that it may also have nuclear functions (Chien and Pei 2000). The direct and specific interaction between hPTTG and PBF was confirmed *in vitro* and *in vivo*, using glutathione S-transferase pull-down and co-immunoprecipitation assays in

COS-7 cells. Furthermore, deletion analysis identified the hPTTG binding domain in PBF to be located within the C-terminal 30 amino acids and the corresponding domain in hPTTG to be between amino acids 123 and 154 within the transactivating domain (Chien and Pei 2000).

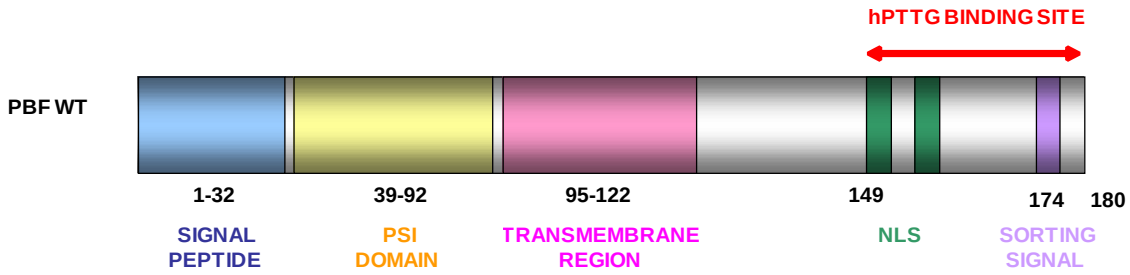
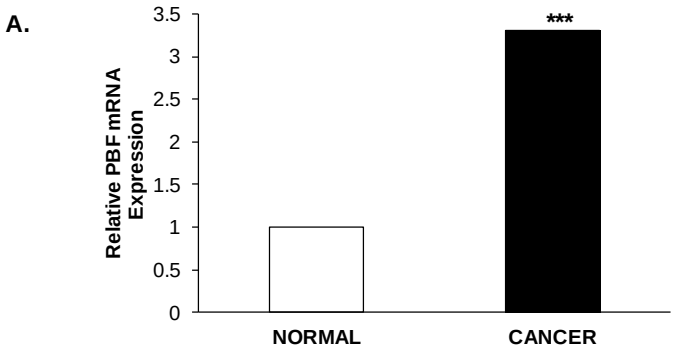


Figure 1-14: Schematic representation of the putative domains of the PBF protein. A cleavable signal peptide is located at the N-terminus between amino acids 1 - 32. The PSI domain forms the remaining extracellular region between amino acids 39 - 92, next to the transmembrane domain located between amino acids 95 - 122. An NLS and endocytosis motif are found at the C-terminus from amino acids 149 and 174 respectively. The site of interaction with hPTTG, within the C-terminal 30 amino acids, is highlighted in red (Figure adapted from (Smith et al. 2010)).

1.5.2 PBF expression and regulation

Northen blot analysis in a variety of human tissues demonstrated that PBF is ubiquitously expressed in normal tissue (Chien and Pei 2000; Yaspo et al. 1998). Subsequently, studies within our own group demonstrated a 5.7-fold over-expression of *PBF* mRNA in 111 pituitary tumours (described in section 1.4.3) compared with normal pituitary tissue. Interestingly, a significant positive correlation between the expression of *hPTTG* and that of *PBF* mRNA was observed in pituitary adenomas but not in normal pituitaries (McCabe et al. 2003). Furthermore, we found significant over-expression of PBF mRNA and protein in the same thyroid cancer specimens in which hPTTG was over-expressed (see section 1.2.1.3), indicating an important relationship between hPTTG and PBF in thyroid cancer (Stratford et al. 2005). Additionally, PBF itself was identified as an independent predictor of early tumour

recurrence. The coding region of *PBF* was sequenced in 24 thyroid carcinomas but no mutations were detected (Stratford et al. 2005).



Mean Δ Ct	12.51	10.78
SEM	±0.43	±0.21
N	11	27

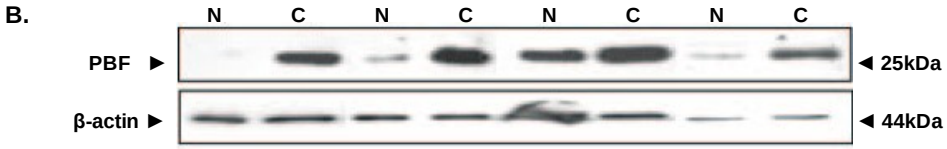


*Figure 1-15: PBF is over-expressed in thyroid cancer. **A** TaqMan RT-PCR data demonstrating over-expression of PBF mRNA in thyroid cancer. **B** Representative Western blot analysis of four normal and matched thyroid tumour specimens, demonstrating over-expression of PBF protein in thyroid cancer (Figure adapted from (Stratford et al. 2005)).*

Interestingly, over-expression of hPTTG in human primary thyroid cells or hPTTG-null HCT116 cells induced *PBF* mRNA expression, an effect that was dependent on the SH3-interacting domain. Similarly, stable over-expression of hPTTG in NIH3T3 cells resulted in elevated *PBF* mRNA expression and conversely, stable PBF over-expression had no effect on *hPTTG* transcription (Stratford et al. 2005).

Studies comparing C3H10T1/2 mouse embryonic fibroblasts constitutively over-expressing Runx2 with WT cells, identified *Pbf* as a transcriptional target gene for the Runx2 transcription factor. Runx2 bound to consensus sequences upstream of *Pbf* in gel shift assays

and Runx2 over-expression increased activity of a 1.8 kb *Pbf* promoter fragment in reporter assays (Stock et al. 2004).

Treatment with the growh factors, EGF and TGF-α, had no effect on *PBF* mRNA expression in folliculostellate TtT-GF cells, indicating alternative mechanisms of PBF regulation compared with that for hPTTG (Vlotides et al. 2006). However, treatment of ER-α-positive MCF-7 cells with diethylstilbestrol (DES) and 17β-estradiol (EST) induced significant PBF mRNA and protein expression, following direct binding of the estrogen receptor α (ERα) to the *PBF* promoter (Watkins et al. 2010).

1.5.3 PBF localisation and function

PBF demonstrates no sequence homology with other human proteins, indicating that it is functionally unique. It is however, highly conserved across other species (77 % homology to rat, 75 % mouse, 60 % chicken), suggesting that it is functionally important, though its primary function remains to be determined.

Identification of the interaction between PBF and hPTTG prompted further characterisation studies to investigate the functional significance of this relationship (Chien and Pei 2000). Immunofluorescence and subcellular fractionation techniques were used to investigate the subcellular localisation of hPTTG and PBF following their transfection into COS-7 cells (see Figure 1-16). GFP-tagged hPTTG demonstrated mainly cytoplasmic localisation, but its expression in the nucleus was greatly enhanced following co-transfection with PBF-HA, suggesting PBF facilitates translocation of hPTTG to the nucleus. When the NLS site was deleted in PBF, GFP-hPTTG expression was confined to the cytoplasm, demonstrating a specific dependency on PBF for nuclear translocation. Functionally, PBF translocation of hPTTG to the nucleus resulted in a more than 3-fold induction in FGF-2

promoter activity, whereas in the absence of PBF little *FGF-2* transcription occurred. It therefore appears that PBF may be required for some of hPTTG's transactivational capabilities (Chien and Pei 2000).

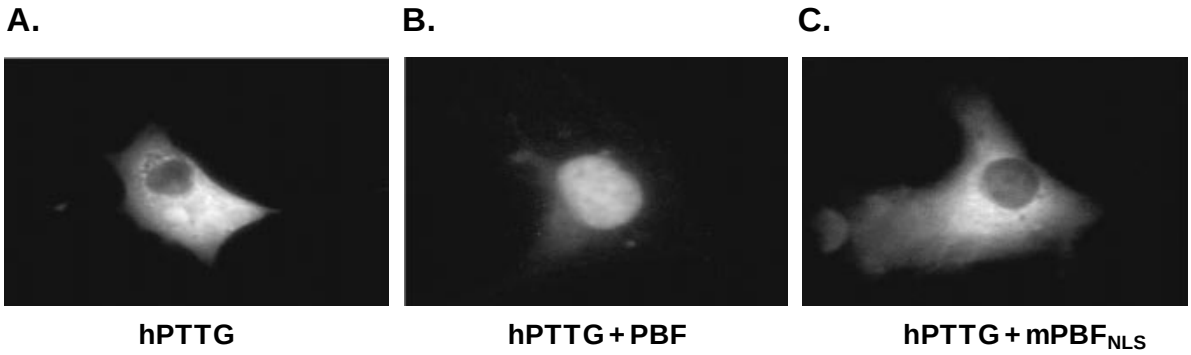


Figure 1-16: Nuclear translocation of hPTTG by PBF. COS-7 cells were transfected with ***A.*** *hPTTG-GFP;* ***B.*** *hPTTG-GFP + PBF-HA or* ***C.*** *hPTTG-GFP and HA-tagged mutant PBF with a deleted NLS (mPBF$_{NLS}$). 24 hours after transfection, cells were fixed and stained. GFP-hPTTG was detected by green fluorescence (figure adapted from(Chien and Pei 2000)).*

The original prediction that PBF is a cell-surface glycoprotein (Yaspo et al. 1998) was further supported by immunofluorescent studies within our own group, demonstrating a strong presence of PBF in intracellular vesicles where it colocalised with the late endosomal marker CD63, following internalisation (Smith et al. 2009). Deletion of the C-terminal 30 amino acids results in PBF accumulation at the plasma membrane suggesting the tyrosine-based sorting signal within this region is functional (Smith et al. 2009).

1.5.4 PBF as a proto-oncogene

Crucially, clones selected from NIH3T3 cells with stable over-expression of either hPTTG (see section 1.2.1) or PBF led to significant colony formation in soft agar assays, thus demonstrating the transforming capabilities of both *in vitro*. hPTTG mutants lacking a functional SH3-interacting domain, as described previously, cannot induce *PBF* mRNA expression or interact with the PBF protein. In this study, the hPTTG SH3-mutant did not

induce transformation, indicating that PBF could be an effector of hPTTG-induced transformation and tumourigenesis. To this effect, PBF was identified as an independent proto-oncogene following injection of NIH3T3 cells stably expressing PBF into nude mice. This induced tumour formation, thereby demonstrating the tumourigenic ability of PBF *in vivo* (Stratford et al. 2005).

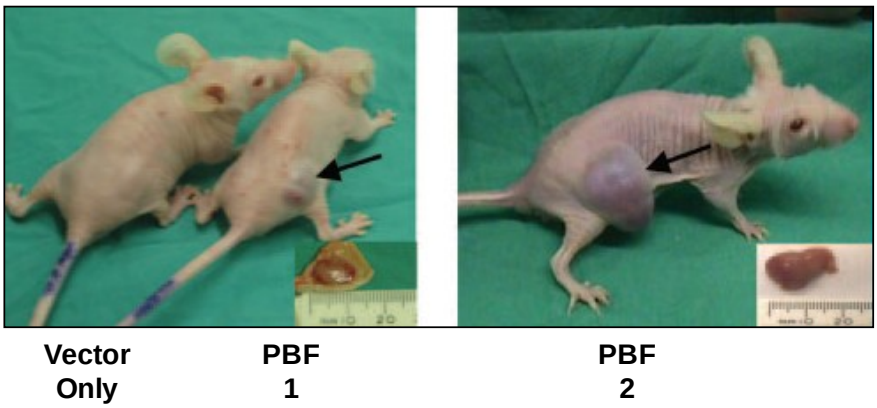


Figure 1-17: PBF has transforming capabilities independent of hPTTG. A total of 5 x10^6 cells from vector-only or PBF-expressing stable NIH3T3 lines were injected into athymic nude mice. Examples of tumour formation in PBF injected mice are indicated by the black arrows, where the PBF mouse on the right developed a tumour weighing 1.4g (inset) (Figure adapted from (Stratford et al. 2005)).

To further investigate the oncogenic potential of PBF *in vivo,* our group recently generated a murine transgenic model with thyroid targeted over-expression of PBF. This model was the subject of some of the investigations reported within this thesis, which led to publication of these data. hPBF-Tg transgenic mice exhibited a striking enlargement of the thyroid gland associated with hyperplastic and macrofollicular lesions, mediated by activation of Akt and Tshr, both known regulators of thyrocyte proliferation (Read et al. 2011).

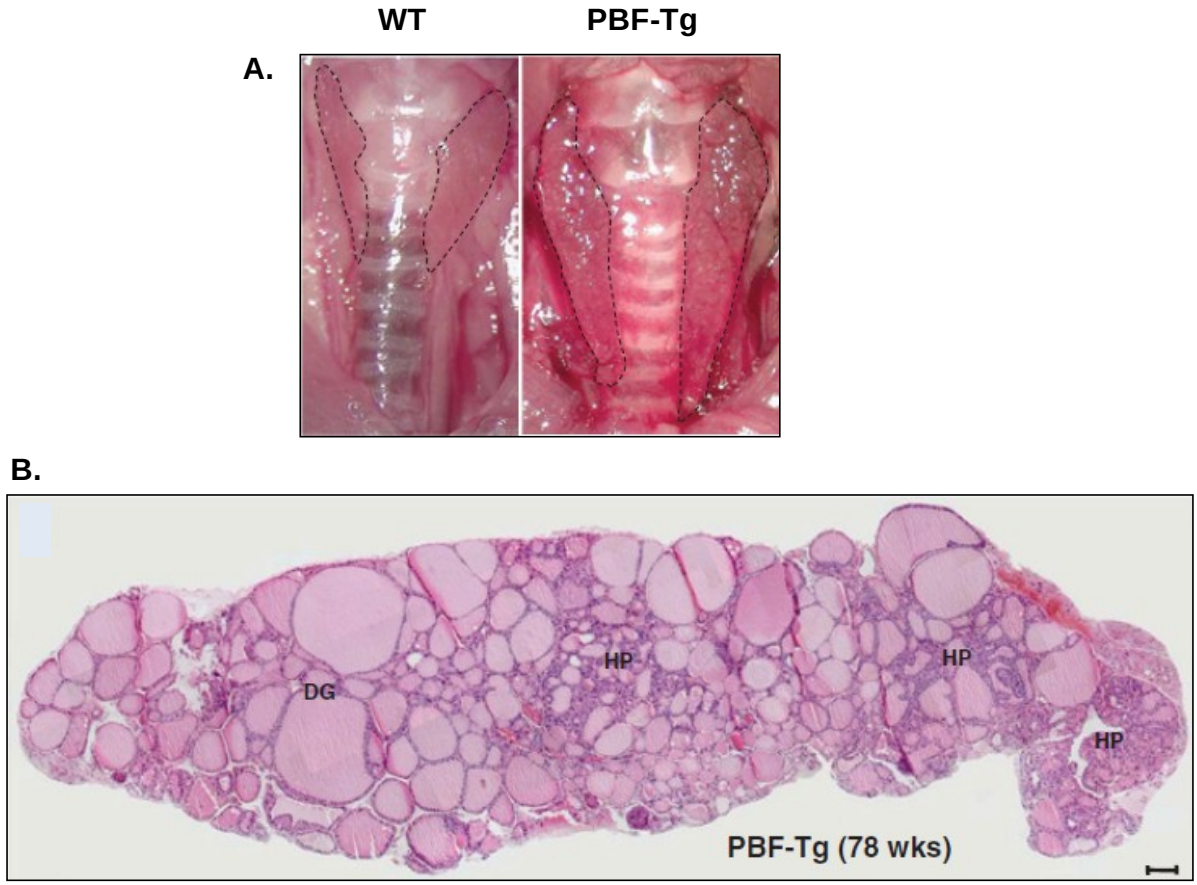


*Figure 1-18: Mice with thyroid targeted over-expression of PBF develop enlarged thyroid glands. **A** Representative images of thyroid glands from 52-week-old PBF-Tg and WT mice. Dotted line indicates margin of thyroid lobes. **B** Composite image of an entire thyroid lobe from a 78-week-old PBF-Tg mouse with diffuse goitre (DG) and hyperplastic regions (HP). Scale bars, 100 mm.*

1.6 Sodium Iodide Symporter (NIS)

1.6.1 Characterisation of NIS

The ability of thyroid cells to accumulate iodide has been known for a long time, being reported as early as 1915 (Marine and Feiss 1915). Though the existence of an iodide transporter was inferred from studies elucidating some of the properties of this phenomenon throughout the 20th century, *NIS* was only cloned in 1996 and marked a major advance in the field of thyroid biology (Dai et al. 1996; Smanik et al. 1996; Dohan et al. 2003). NIS mediates the active transport of I^- into thyroid follicular cells, which is the critical and rate-limiting step of thyroid hormone biosynthesis, where I^- is a fundamental constituent of

triiodothyronine (T_3) and thyroxine (T_4). NIS is an integral plasma membrane glycoprotein located at the basolateral membrane of thyroid follicular cells and co-transports 2 Na^+ and 1 I^- ion into the cytoplasm of these cells. This occurs against the I^- electrochemical gradient, but is driven by the Na^+ electrochemical gradient maintained by the Na^+/K^+ ATPase (Spitzweg et al. 2001; Boelaert and Franklyn 2003; Dohan et al. 2003; Riesco-Eizaguirre and Santisteban 2006). Cell polarisation is important in I^- transport and cytoplasmic I^- is driven across the apical membrane of thyroid follicular cells by the Cl^-/I^- transporter, pendrin (Scott et al. 1999), or the apical I^- transporter (AIT) (Rodriguez et al. 2002) in a process called I^- efflux. At this stage, free I^- is organified at the cell-colloid interface. In a complex reaction catalysed by thyroid peroxidase (TPO), I^- is oxidised and incorporated onto tyrosyl residues of thyroglobulin (Tg), leading to the coupling of iodotyrosine residues to form T_3 and T_4. Iodinated Tg is stored in the colloid until it is endocytosed back into thyroid follicular cells and T_3 and T_4 are secreted into the blood stream in response to a biological demand for thyroid hormones. This entire process is primarily regulated by activation of the adenylate cyclase-cAMP pathway following binding of TSH to the TSH-receptor (TSHR) located in the basolateral membrane of thyroid follicular cells (Spitzweg et al. 2001; Dohan et al. 2003; Riesco-Eizaguirre and Santisteban 2006; Weiss et al. 1984; Nissim et al. 1987).

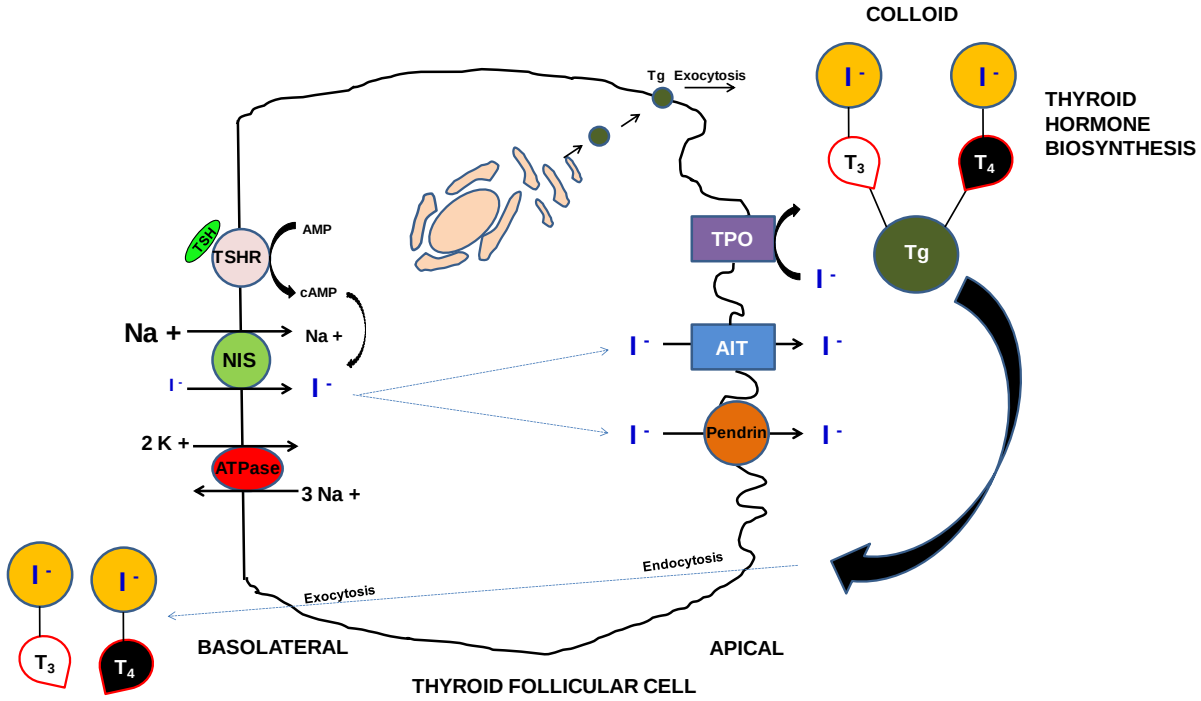


Figure 1-19: Schematic illustration of the key aspects of iodine transport and organification in the thyroid gland. TSHR, TSH receptor, TPO thyroid peroxidase, Tg, thyroglobulin, AIT, apical iodide transporter (Figure adapted from (Spitzweg et al. 2001; Dohan et al. 2003).

Importantly, our knowledge of the thyroid's ability to take up iodide has allowed us to exploit this mechanism to provide diagnostic and therapeutic tools in benign and malignant thyroid disease. Scintigraphic imaging is used diganostically to visualise the thyroid and thyroid function, as well as to detect thyroid cancer metastases. In addition, therapeutic doses of ^{131}I are administered to patients with Graves' disease, multinodular goitre, functional differentiated thyroid cancers and their metastases in order to ablate pathological tissue in a highly targeted manner (Spitzweg et al. 2001; Boelaert and Franklyn 2003; Dohan et al. 2003; Riesco-Eizaguirre and Santisteban 2006).

1.6.2 NIS in thyroid disease

Immunohistochemical studies of normal thyroid tissue have demonstrated that NIS protein is heterogeneously expressed at the basolateral membrane of a minority of thyroid follicular cells (Caillou et al. 1998; Castro et al. 1999a). However, thyroidal NIS expression is often altered in diseased states. Immunohistochemical analysis of thyroid specimens from Graves' disease patients revealed strong over-expression of NIS protein in the basolateral membrane of most thyroid follicular cell, consistent with the clinically observed increase in radioiodine uptake in acute Graves' thyrotoxicosis (Saito et al. 1997; Caillou et al. 1998; Joba et al. 1999; Castro et al. 1999a). Further, there is evidence that NIS is a novel thyroid autoantigen, where NIS autoantibodies have been detected in the sera of patients with auto-immune thyroid disease (AITD). The results of these studies are detailed in two reviews, which both conclude that the biological function of anti-NIS autoantibodies remains undetermined (Spitzweg and Morris 2002; Dohan et al. 2003). Expression of NIS protein in AITD is similar to normal thyroid tissue with strongest expression near to lymphocytic infiltrates (Caillou et al. 1998). Toxic multinodular goitres demonstrate stronger heterogeneous expression of NIS protein than normal thyroid (Caillou et al. 1998), whereas levels are low in non-toxic MNG and diffuse iodine deficiency goitres (Joba et al. 1999). Similarly, NIS levels are increased in autonomously functioning thyroid nodules (hot nodules) with increased radio-iodine uptake and are decreased in cold nodules with low or absent uptake of radio-iodine (Joba et al. 1999).

Critically, I^- uptake is reduced in many thyroid cancers and their metastases compared with normal thyroid, thereby limiting the efficacy of radio-iodine therapies (Maxon and Smith 1990). Even after TSH stimulation, 10-20 % of tumours with reduced I^- uptake remain incapable of sufficient I^- concentration for effective radio-ablation therapy (Kogai et al. 2006;

Robbins et al. 1991; Schmutzler and Koehrle 2000). A correlation between NIS expression and I^- uptake in thyroid tumours has been confirmed (Caillou et al. 1998; Castro et al. 1999a) and tumours with reduced NIS activity are associated with a poor prognosis (Ward et al. 2003).

Various mRNA (Smanik et al. 1997; Lazar et al. 1999; Ringel et al. 2001; Arturi et al. 2003; Park et al. 2000; Ryu et al. 1999; Ward et al. 2003) and protein studies using specific poly- and monoclonal antibodies (Jhiang et al. 1998b; Caillou et al. 1998; Castro et al. 1999a; Castro et al. 1999b; Faggiano et al. 2007; Gerard et al. 2003; Trouttet-Masson et al. 2004) have demonstrated reduced NIS expression in thyroid cancer tissue, suggesting repression of I^- uptake is caused by a transcriptional effect. However, contrasting studies observed normal or even increased levels of NIS expression in as many as 70 % of thyroid cancers at the level of mRNA (Arturi et al. 1998; Luciani et al. 2003; Saito et al. 1998; Tanaka et al. 2000) or protein (Dohan et al. 2001; Saito et al. 1998; Wapnir et al. 2003). Immunohistochemical analysis of NIS over-expression in these thyroid cancers, revealed that NIS was primarily localised to the cytoplasm and not at the basolateral membrane where it is required to be in order to be functional (Castro et al. 1999b; Dohan et al. 2001; Wapnir et al. 2003). This indicates that reduced I^- uptake is at least in part attributable to mislocalisation of NIS following interference with intracellular trafficking or plasma membrane retention mechanisms. Together these results suggest that an understanding of the mechanisms of both NIS induction and NIS localisation are required to develop therapeutic strategies to improve radioiodide treatment in thyroid cancer (Spitzweg and Morris 2002; Dohan et al. 2003).

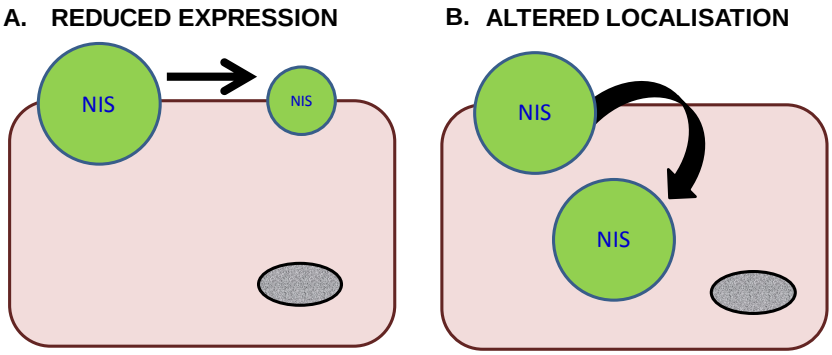


*Figure 1-20: Functional levels of NIS are regulated by factors affecting expression (**A**) and subcellular localisation of NIS (**B**).*

1.6.3 Regulation of NIS expression and function

Regulation of NIS expression and function is characteristically intricate, with multiple factors effecting NIS transcription, translation, post-translational modification and cellular trafficking mechanisms controlling its localisation. As mentioned previously, TSH is the major regulator of thyroid function and has been shown to induce NIS transcription and translation, resulting in enhanced I^- uptake by rat thyroid FRTL-5 cells (Kogai et al. 1997; Ohno et al. 1999; Riedel et al. 2001) and human primary thyroid follicular cells (Saito et al. 1997; Kogai et al. 2000). Further, TSH regulation of NIS has been demonstrated *in* vivo, including studies in rat (Levy et al. 1997) and human (Martino et al. 2000) systems. In a secondary mechanism of NIS regulation, TSH levels have also been shown to effect the localisation or retention of NIS to the basolateral membrane where it is functional (Riedel et al. 2001; Kogai et al. 1997). NIS is a phospho-protein with phosphorylation patterns governed by TSH in FRTL-5 cells (Riedel et al. 2001). Targeting of NIS to the basolateral membrane was proposed to be mediated by phosphorylation of the C-terminal, but despite the presence of various phosphorylation residues, none have been confirmed to play a role in NIS trafficking (Vadysirisack et al. 2007). However, activation of the PI3K pathway in MCF-7 cells interfered with cell-surface trafficking and induction of endogenous and exogenous NIS

function (Knostman et al. 2007b). Furthermore, two recent studies have described regulation of NIS expression by the PI3K pathway (Zaballos et al. 2008; Kogai et al. 2008).

Aside from TSH, high levels of I^- itself have long been known to reduce thyroid function. The Wolff-Chaikoff effect describes a highly specialised intrinsic autoregulatory system, whereby I^- organification by TPO is acutely blocked upon receipt of high doses of I^-, resulting in reduced NIS activity. However, an adaptation of the system occurs as early as 2 days after, restoring I^- uptake to allow normal biosynthesis of thyroid hormones (Wolff and Chaikoff 1948; Wolff et al. 1949). Despite extensive investigation, the precise mechanisms underlying the inhibition of iodide organification by high levels of I^- remain poorly understood, though studies in FRTL-5 cells have reported transcriptional (Spitzweg et al. 1999) and post-translational (Eng et al. 2001) regulation of NIS following treatment with I^-.

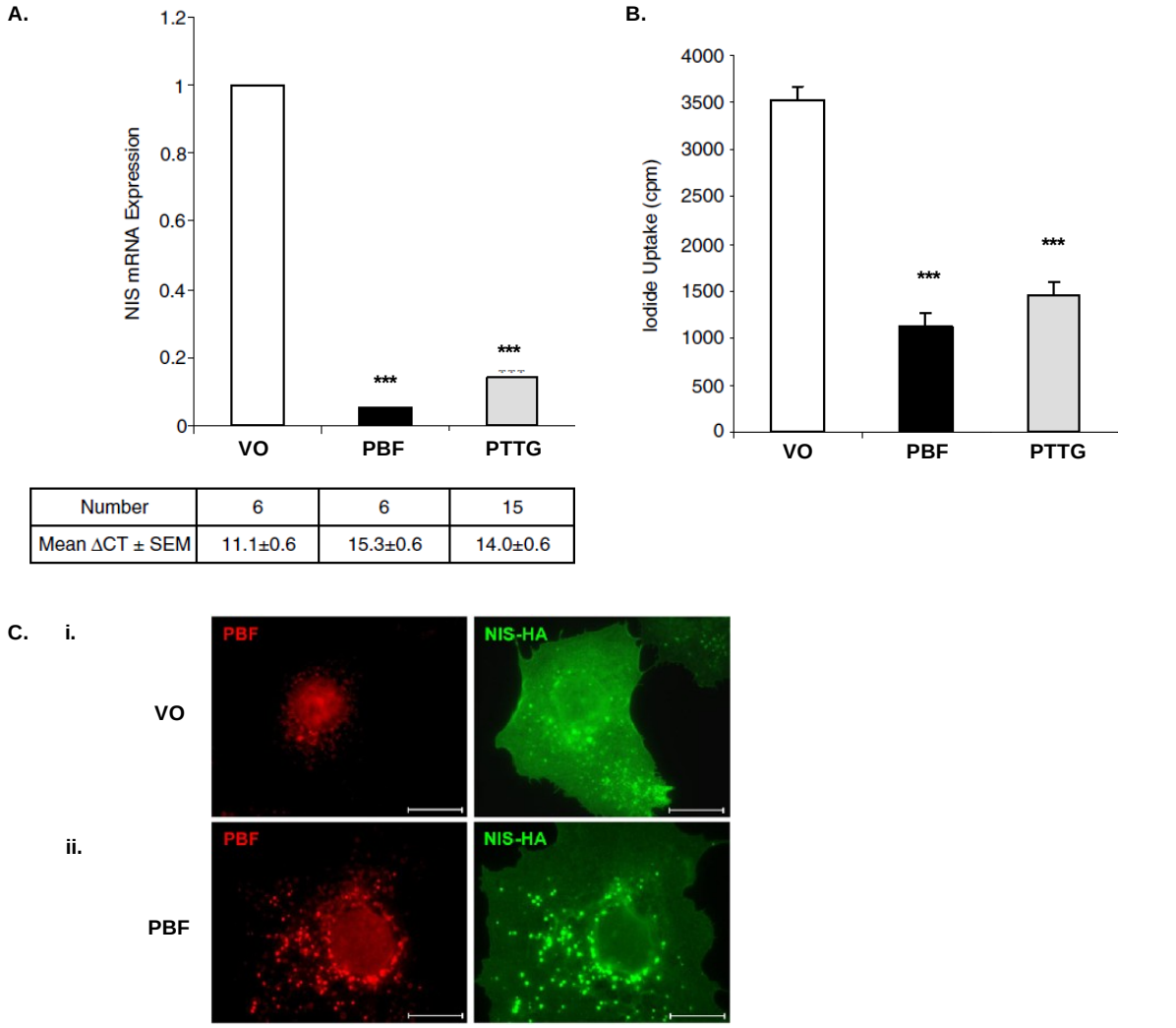
Other regulators of NIS include various cytokines and growth factors that repress NIS expression and function in a time- and dose-dependent manner. Transforming growth factor beta (TGF-β), IGF-1, tumor necrosis factor alpha (TNF-α) and beta (TNF-β), interferon gamma (IFN-γ), interleukin alpha (IL-1α), beta (IL-1β) and 6 (IL-6) have been proven to inhibit *NIS* mRNA expression and iodide uptake activity in FRTL-5 and human thyroid cells (Ajjan et al. 1998; Pekary et al. 1997; Pekary et al. 1998; Garcia and Santisteban 2002). IFN-γ repression of NIS has also been demonstrated *in vivo* (Caturegli et al. 2000). Thyroglobulin also acts as a potent suppressor of *NIS* transcription and subsequently I^- uptake (Suzuki et al. 1999; Ulianich et al. 1999; Kohn et al. 2001). Though the mechanism is poorly understood, it is thought that this may represent a negative feedback autoregulatory mechanism that counterbalances TSH stimulation of follicular function. Furthermore, oestradiol promotes proliferation and inhibits NIS expression and I^- uptake in FRTL-5 cells, providing a

mechanism that potentially contributes to higher incidence of thyroid disease in women (Furlanetto et al. 1999; Furlanetto et al. 2001).

1.6.4 hPTTG, PBF and NIS

Of particular importance to our own research interests, both hPTTG and PBF have been implicated in the regulation of NIS and I^- uptake. hPTTG regulation of NIS was originally observed in rat FRTL-5 cells over-expressing hPTTG (Heaney et al. 2001). I^- uptake was strongly repressed in cells over-expressing hPTTG and subsequent Northern blot analysis demonstrated that this was due to reduced *NIS* mRNA expression. Further, hPTTG over-expression induced transcription and secretion of FGF-2. Treatment of FRTL-5 cells with FGF-2 also caused reduced *NIS* mRNA expression and I^- uptake, at similar levels to those observed following hPTTG over-expression (Heaney et al. 2001). FGF-2 mediated repression of I^- uptake has also been demonstrated in human primary thyrocytes (Cocks et al. 2003). Together these results suggest that hPTTG effects on NIS are mediated at least in part through induction of FGF-2 as part of autocrine feedback mechanisms involving hPTTG and FGF-2 (Heaney et al. 2001). Subsequently, in a study confirming hPTTG expression as a prognostic indicator for persistent disease, immunohistochemical analysis of 16 human differentiated thyroid tumours demonstrated strong hPTTG over-expression that was associated with reduced I^- uptake in patients (Saez et al. 2006). Studies within our own group demonstrated that both hPTTG and its binding factor PBF repress expression of *NIS* mRNA and inhibit I^- uptake in FRTL-5 cells and human primary thyroid follicular cells (Boelaert et al. 2007). Promoter studies in FRTL-5 cell and human primary thyroid cells demonstrated that repression of NIS by hPTTG and PBF was via specific binding to a PAX8-upstream stimulating factor 1 (USF1) response element within a 1 kb element in the *NIS* promoter known as the human upstream enhancer element (hNUE). Depletion of secreted

FGF-2 partially inhibited NIS repression by hPTTG, providing further support for the existence and importance of autocrine pathways involving hPTTG and growth factors. FGF-2 depletion had no effect on NIS repression by PBF in human primary thyroid cells, implying that PBF acts independently of FGF-2. Increased hPTTG, PBF and FGF-2 expression were significantly correlated with reduced NIS expression in human thyroid cancers demonstrating the importance of these interactions *in vivo* (Boelaert et al. 2007). Furthermore, our group have demonstrated a further mechanism of NIS regulation by PBF, whereby PBF modulates the internalisation of NIS from the basolateral membrane of thyroid follicular cells (Smith et al. 2009). Subcellular localisation studies in COS-7 cells, demonstrated the redistribution of NIS from the plasma membrane into intracellular vesicles following over-expression of PBF. Reduced expression of NIS in the plasma membrane was confirmed in cell-surface biotinylation assays and coimmunoprecipitation and GST-pull-down experiments demonstrated a direct interaction between NIS and PBF. I^- uptake studies were performed in FRTL-5 cells to demonstrate the functional consequence of this interaction. Over-expression of WT PBF repressed iodide uptake by FRTL-5 cells by 39.4 ± 5 % (P<0.001) compared with VO control. In contrast, over-expression of three deletion mutants of PBF, which do not localise within intracellular vesicles in COS-7 or FRTL-5 cells, lost the ability to inhibit NIS activity (Smith et al. 2009).



Number	6	6	15
Mean ΔCT ± SEM	11.1±0.6	15.3±0.6	14.0±0.6

Figure 1-21: PBF and hPTTG repress expression and activity of the sodium iodide symporter in human primary thyroid cultures. ***A*** *TaqMan RT-PCR data demonstrating repression of NIS mRNA following over-expression of either PBF or hPTTG.* ***B*** *Iodide uptake assays were performed in 24-well plates, 48 h after transient transfection and demonstrate reduced uptake following PBF or hPTTG over-expression. Data were adjusted for transfection efficiency, as assessed by β-gal staining (Figures A and B adapted from (Boelaert et al. 2007)).* ***C*** *In an alternative mechanism, PBF alters the subcellular localisation of NIS into intracellular vesicles.* ***(i)*** *Immunofluorescent detection of NIS-HA and endogenous PBF in cells transfected with NIS-HA and VO control.* ***(ii)*** *PBF over-expression is associated with an increase in NIS staining within intracellular vesicles. Scale bars: 20 μM. (Figure adapted from (Smith et al. 2009)).*

Together, these findings suggest that over-expression of hPTTG and PBF in thyroid cancer (see sections 1.2.1.3 and 1.5.2) has serious implications for expression and activity of NIS, resulting in a significant negative impact upon the efficacy of radioiodine ablation and

therapy. We have conducted further studies of these relationships reported in CHAPTER and some of these results were recently published (Read et al. 2011).

1.7 Hypothesis and aims

The hypothesis that is paramount to the investigations described in this thesis is that hPTTG has a dual role in thyroid tumourigenesis. In the first instance, hPTTG initiates tumourigenesis by causing aneuploidy and genetic instability through its role as a securin. Secondly, hPTTG promotes mitogenic and angiogenic mechanisms of tumour expansion through auto- and paracrine interactions with growth factors. In addition, hPTTG negatively impacts upon efficacy of radiodine treatment through repression of NIS expression and function (see Figure 1-22).

Following on from previous studies demonstrating that hPTTG is a transforming gene over-expressed in thyroid tumours and is involved in the regulation of growth factors and NIS, we further explored this hypothesis under the following broad aims:

1. Work described in this thesis has sought to establish whether hPTTG is involved in autocrine mechanisms of interaction with the growth factors EGF, TGF-α and IGF-1, in thyroid cells *in vitro*. Transient transfection studies and growth factor treatments were performed in thyroid cancer cell lines and human primary thyroid follicular cells.

2. In order to directly test our dual hypothesis, we set out to generate a murine transgenic model with thyroid-targeted hPTTG over-expression (hPTTG-Tg mice). Transgenic colonies were obtained following pronuclear injection of a DNA construct containing hPTTG under control of the bovine thyroglobulin promoter.

3. Having obtained a transgenic colony of hPTTG-Tg mice, we sought to determine the effects of thyroidal hPTTG over-expression on thyroid cell growth and function through extensive characterisation studies including histological and thyroid function analysis, as well as primary murine thyroid cell cultures.

4. In parallel studies, we aimed to determine gene expression alterations in hPTTG-Tg mice, including effects on growth factor induction by hPTTG *in vivo*, using gene-specific TaqMan RT-PCR assays and ELISAs. Angiogenesis-specific PCR cDNA arrays were performed to assess gene expression in hPTTG-Tg thyroid glands.

5. Finally, we sought to challenge our group's *in vitro* observations of NIS repression by hPTTG and PBF, through conducting *in vivo* studies in hPTTG-Tg and PBF-Tg mice. Importantly, we aimed to determine whether hPTTG and PBF represent novel therapeutic targets for enhancing treatment with radioiodine in thyroid disease.

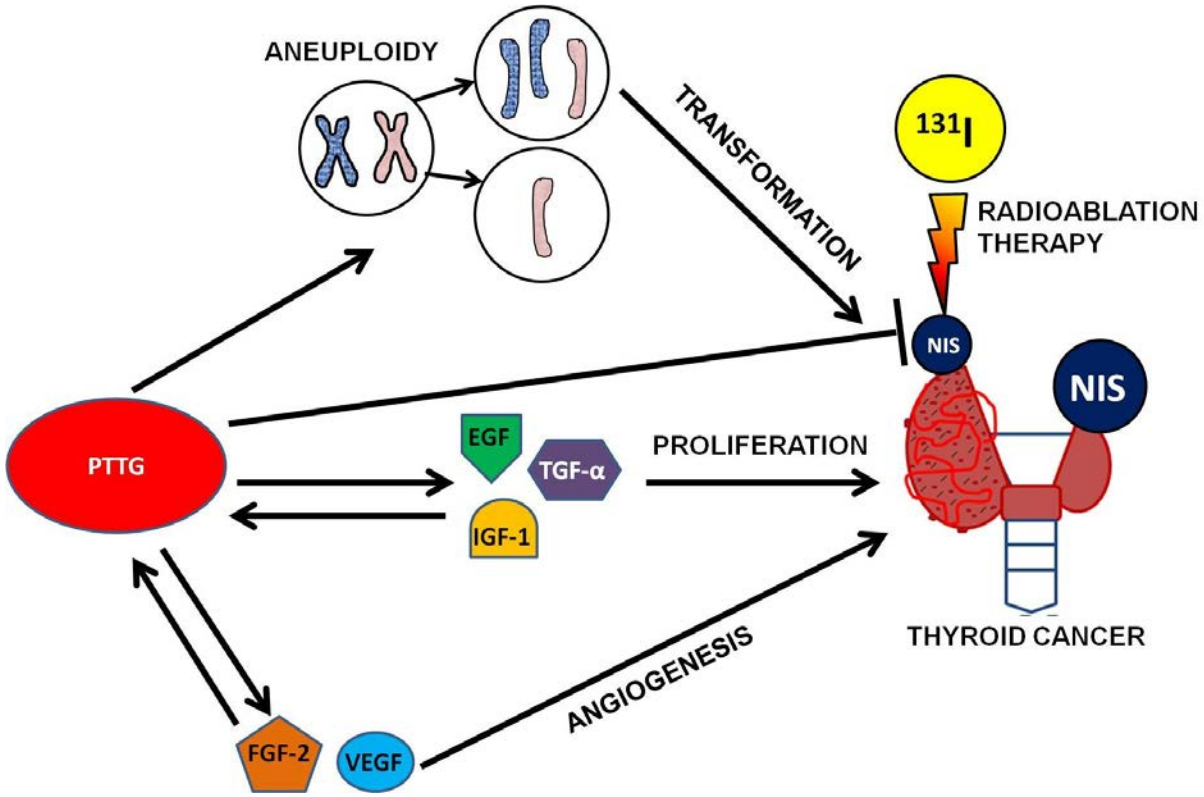


Figure 1-22: Schematic diagram illustrating the proposed model of thyroid tumourigenesis, including the major hypotheses investigated within this thesis. hPTTG initiates tumourigenesis through induction of aneuploidy and genetic instability. hPTTG contributes to tumour development through autocrine interactions with growth factors that promote proliferation and angiogenesis. hPTTG impairs ^{131}I radioiodine ablation therapy through downregulation of NIS expression and function.

2 CHAPTER TWO

Materials and Methods

2.1 Cell lines

Thyroid papillary carcinoma TPC-1 and anaplastic thyroid carcinoma SW1736 cells were kindly provided by Dr Rebecca Schweppe (Division of Endocrinology, Metabolism, & Diabetes, University of Colorado Denver, Aurora, Colorado), and thyroid papillary carcinoma K1 cells were obtained from the Health Protection Agency Culture Collections, UK. All thyroid carcinoma cell lines were routinely cultured in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Invitrogen; EU approved, South American origin), penicillin (10^5 U/l) and streptomycin (100 mg/l) [Invitrogen]. Cells were passaged twice weekly.

2.2 Thyroid samples and human primary thyroid culture

Collection of thyroid specimens was with approval of the Local Research Ethics committee and following informed written consent from patients. All tissues were surplus to pathological requirement. Human thyroid follicular cells were prepared from surgical specimens, and consisted mainly of multinodular goitres and normal thyroid tissue from the contralateral lobe, as previously described (Eggo et al. 1996; Eggo 1998; Ramsden et al. 2001). Thyroid tissue was digested by 0.2 % type II collagenase (Worthington Biochemicals). Follicles were plated in 12-well plates in medium described previously by Ambesi-Impiombato *et al*. (Ambesiimpiombato et al. 1980), supplemented with thyrotrophin (300 mU/l) [Sigma], insulin (100 μg/l) [Sigma], penicillin (105 U/l), streptomycin (100 mg/l) and 5 % fetal calf serum. Serum was omitted after 72 hours of culture, and experiments were performed between days 7-11 of the culture. Generally, these cultures survive and maintain function for more than 4 weeks and fibroblast as well as endothelial cell contamination is negligible under these conditions (Eggo 1998).

2.3 Murine primary thyrocyte culture

A method for mouse primary thyrocyte culture was adapted from a previously described approach (Jeker et al. 1999). Thyroids from mice of different genotypes were aseptically dissected before being disrupted mechanically in PBS and digested in 0.2 % collagenase for 45 minutes at 37 ºC on a rotator. Collagenase was inactivated by addition of culture medium and cells were centrifuged for 10 minutes at 700 g to obtain a pellet containing mainly single thyroid follicles. After discarding the supernatant, the pellet was resuspended in 1 ml of culture medium and pipetted up and down until no fragments were macroscopically visible. Cells from each thyroid (2 wells for each hPTTG-Tg or WT thyroid; 4 wells for each hPBF-Tg thyroid) were seeded into 12-well plates in medium described by Ambesi-Impiombato *et al*. (Ambesiimpiombato et al. 1980), supplemented with thyrotrophin (300 mU/l), insulin (10 mg/ml), transferrin (5 mg/ml) [Sigma], hydrocortisone (3.5 ng/ml) [Sigma], somatostatin (10 ng/ml) [Sigma], glycyl-L-histidyl-L-lysine acetate (2 ng/ml) [Sigma], penicillin (105 U/l), streptomycin (100 mg/l) and 5 % fetal calf serum. After 72 hours, serum was omitted and experiments were performed between days 7-14 of the culture.

2.4 RNA extraction and reverse transcription

Total RNA was extracted from cells, utilising a single step acid guanidinium phenol-chloroform extraction method [Tri-reagent (250 µl per well of a 12-well plate), Ambion, Austin, TX, USA] (Chomczynski and Sacchi 1987). Following manufacturer's guidelines, chloroform (99+ %, 50 µl; Sigma-Aldrich, St. Louis, MO, USA) was added to each reaction, mixed thoroughly and left to stand at room temperature for 15 minutes before centrifugation at 12,000 g for 15 minutes at 4 ºC, which separates the mixture into 3 phases. The uppermost colourless aqueous phase containing RNA was transferred into a fresh tube and isopropanol (99+ %, 125 µl; Sigma-Aldrich, St. Louis, MO, USA) was added. After mixing, the sample

was left to stand at room temperature for 5-10 minutes before centrifugation at 12,000 g for 10 minutes at 4 ºC, which results in the formation of an RNA pellet at the bottom of the tube. The supernatant was discarded and the pellet washed in 75 % ethanol (250 µl) then centrifuged at 7500 g for 5 minutes at 4 ºC. The ethanol was discarded and the pellet dried at room temperature before elution in 30 µl of nuclease-free water and storage at -80 ºC.

Tissue samples from mouse organs excised for RNA studies were stored at -20 ºC in RNAlater RNA stabilisation reagent (Qiagen, UK). Total RNA was extracted from up to 5 mg of tissue using the RNeasy Microkit (Qiagen, UK), as per the manufacturer's instructions.

500 nanograms of RNA (determined by spectroscopy at λ260 nm using a NanoDrop spectrometer (NanoDrop Products, Wilmington, DE, USA)) was reverse transcribed (RT) with avian myeloblastosis virus (AMV) reverse transcriptase in a total reaction volume of 10 µl, with 5 pmol random hexamer primers, 1 µl 10 AMV reverse transcriptase buffer, 1 µl deoxynucleotide triphosphate (dNTP) mix (200 µM each), 10 units ribonuclease inhibitor (RNasin™) and 7.5 units AMV reverse transcriptase (All reagents from Promega, Madison, WI, USA).

2.5 Quantitative polymerase chain reaction PCR (QT-PCR)

Relative fold changes in expression of mRNAs encoding various proteins were calculated with control samples as a reference (given an arbitrary value of 1). These experiments were performed using the ABI 7500 Sequence Detection System, which employs TaqMan™ chemistry for highly accurate quantitation of mRNA levels (Wang and Brown 1999).

A fluorogenic TaqMan probe consists of an oligonucleotide with a 5' reporter dye (FAM 6-carboxy-fluorescein or VIC) and a 3' quencher dye (TAMRA 6-carboxy-tetramethyl-rhodamine). Fluorescent quenching of the reporter depends on the spatial

proximity of its quencher. PCR amplification releases the reporter into solution, away from its quencher, yielding a signal that can be read by a LASER and CCD camera. One such event occurs for each PCR product generated, enabling real time detection of cDNA amplification. Quantitative primers and probe were designed (Primer Express™ software, PE Biosystems, USA) for a unique sequence of each gene ensuring that the PCR product spanned at least one exon boundary, and hence avoiding genomic DNA amplification. Alternatively, pre-optimised specific gene-expression assays for QT-PCR were purchased (Applied Biosystems, Warrington, UK).

PCR was carried out in 25 µl volumes on 96 well plates, in a reaction buffer containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK) [3 mM $Mg(OAc)_2$, 200 µM dNTPs, 1.25 units Ampli-Taq Gold polymerase, 1.25 units AmpErase UNG], 175 nM TaqMan probe and 900 nM primers with 1 µl of the RT reaction. All target gene probes were labelled with FAM, and the housekeeping genes, 18S and DS-CAM, with VIC. All reactions were multiplexed where possible. For specific gene-expression assays that were not validated for multiplexing, reactions were singleplexed. The 18S primers and probe are provided as a pre-optimised control system (Applied Biosystems, Warrington, UK), enabling data to be expressed in relation to an internal reference, to allow for differences in RT efficiency. Reactions were as follows: 50 °C for 2 minutes, 95 °C for 10 minutes; then 44 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

As per the manufacturer's guidelines, data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values (ΔCt = Ct of the target gene minus Ct of the housekeeping gene 18S). Measurements were carried out in duplicate for each sample. The fold change of mRNA in an experimental group compared to the control group is calculated using the equation fold change = $2^{-(\Delta Ct \text{ of experimental group} - \Delta CT \text{ of control group})}$ or $2^{-\Delta\Delta Ct}$.

2.6 Western blot analysis

Proteins were prepared from thyroid tissues in lysis buffer (100 mmol/L sodium chloride, 0.1 % Triton X-100, and 50 mmol/L Tris, pH 8.3) containing enzyme inhibitors (1 mmol/L phenylmethylsulphonylfluoride, 0·3 μmol/L aprotinin, and 0·4 mmol/L leupeptin). Protein concentration was determined using the BCA Protein Assay kit (Thermo-Fisher Scientific, Rockford, IL, USA) with bovine serum albumin standards. 10-60 μg of protein was denatured in 4 x laemlli buffer (Bio-Rad, Hertfordshire, UK) for 5 minutes at 95 ºC.

Western blot analyses were performed as we have described previously (Boelaert et al. 2003a; Kim et al. 2005; Smith et al. 2009). Soluble proteins (10-60 μg) were separated by electrophoresis in 12.5 % sodium dodedecyl sulphate polyacrylamide gels, transferred to polyvinylidene fluoride membranes, incubated in 5 % non-fat milk in tris-buffered saline with 0.1 % Tween, followed by incubation with appropriate primary antibodies for 16 hours at 4 °C. After washing in tris-buffered saline plus 0·1 % Tween, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature. After further washes, antigen-antibody complexes were visualised by the ECL plus and ECl advance chemiluminescence detection systems (GE Healthcare, UK) on x-ray film (Kodak, UK). Actin expression was determined in all Western blot analyses (monoclonal anti-β-Actin Clone AC-15 (Sigma-Aldrich), used at 1:10 000) to assess potential differences in protein loading. Scanning densitometry studies were performed to quantify differences in protein expression (Image J Software).

2.7 Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of wild-type and PTTG-Tg mouse thyroid specimens were immunostained using an avidin-biotin peroxidise technique (Vectastain Elite;

Vector Laboratories, Peterborough,UK). All reagents were prepared according to the kit instructions.

Briefly, the slides were dewaxed in Histoclear and rehydrated in an ethanol concentration gradient. After washing in 20 mm Tris/0.15 m sodium chloride with 0.3 % Tween 20 (pH 7.4) (TBS-T), slides were incubated in 1 mg/ml hyaluronidase in 0.1 m sodium acetate (pH 5.5) at 37 ºC for 30 minutes, washed, and then incubated in 0.03 % hydrogen peroxide in 20 mM Tris/0.15 M sodium chloride (pH 7.4) to block endogenous peroxidase activity. Slides were then blocked in 5 % normal goat serum (NGS) in TBS-T for 30 minutes in a humidity chamber before being incubated with primary antibody in blocking buffer for 16 hours at 4 ºC in a humidity chamber. For negative controls, the primary antibody was replaced by 10 % normal goat serum. After three 5-minute washes in TBS-T, the sections were incubated in biotinylated anti-rabbit antibody for 30 minutes at room temperature, followed, after additional TBS-T washes, by addition of the avidin-biotin-peroxidase complex. The reaction was developed using the DAB peroxidise substrate kit for 5-10 minutes and then counterstained in Mayer's hematoxylin. Slides were dehydrated, cleared, and mounted. Immunostained thyroid tissue sections were viewed under a light-microscope (Zeiss) and images captured using Axiovision software (Version 4).

2.8 Statistical analysis

Data were analysed using Sigma Stat and SPSS version 11.5 software (SPSS Science Software UK Ltd, Birmingham UK). Distributions of data were determined using the Kolmogorov-Smirnov test. Student's t-test and the Mann Whitney U test were used for comparison between two groups of parametric and non-parametric data respectively. Analysis of Variance (ANOVA) and Kruskal-Wallis tests were used for between-group comparisons of multiple groups of parametric and non-parametric data respectively. The

combined effects of mouse genotypes and time on body weight changes were analyzed using a two-way ANOVA with repeated measurements over time. Correlations between pairs of mRNA results were examined using Spearman rank correlation. Scanning densitometry data with fewer than five data points were assumed to be Gaussian following use of the Anderson-Darling test, and subsequently subject to analysis by student's t-test or ANOVA for comparison between two groups or multiple groups of data respectively. Kaplan-Meier mouse survival analysis was performed using the XLSTAT add-on to Microsoft Excel. The log-rank (Mantel-Cox) test was used to compare the survival distribution of two sample sets (non-parametric data, non-informative censorship). Significance was taken as $p < 0.05$.

3 CHAPTER THREE

Regulation of hPTTG Expression by Growth Factors *In Vitro*

3.1 Introduction

hPTTG is expressed at low levels in most normal adult tissues but over-expression of hPTTG has been described in numerous primary tumours and neoplasms (see section 1.2.1.2).

hPTTG over-expression in thyroid disease was first described in a relatively small study, where Northern blot analysis revealed an abundance of *hPTTG* in a subset of specimens of thyroid hyperplasia, follicular adenomas and follicular carcinomas, with no increased expression evident in papillary cancers (Heaney et al. 2001). Furthermore, TSH treatment of rat FRTL5 thyroid cells and human thyroid cells in primary culture, was reported to induce hPTTG expression *in vitro*, consistent with a role for hPTTG in controlling thyroid cell growth (Heaney et al. 2001). A further study conducted by our own group used highly accurate quantitative RT-PCR to demonstrate an approximately 2-fold increase in *hPTTG* mRNA expression in hyperplastic thyroid conditions (n = 38) and a striking 9.5-fold induction in pretranslational *hPTTG* expression in differentiated thyroid cancers (n = 27). In contrast to the study by Heaney et al., no difference in hPTTG expression was detected when comparing follicular and papillary carcinomas (Boelaert et al. 2003a).

hPTTG over-expression is associated with inappropriate cell divison, cell transformation and transactivation of other genes that have critical roles in tumourigenesis (see sections 1.3 and 1.4). Furthermore, hPTTG represses *NIS* mRNA expression in thyroid cells leading to reduced iodide uptake (Boelaert et al. 2007), an effect which has significant consequences for the treatment of thyroid cancer with radioiodine. Based on the current evidence, hPTTG over-expression may have significant implications in the progression and treatment of thyroid cancer. Thus far, no mutations have been discovered in either the coding or non-coding regions of *hPTTG* (Zhang et al. 1999b; Kanakis et al. 2003) and there is insufficient explanation for hPTTG over-expression in tumour tissues. Whilst the regulation

of hPTTG has not been clarified completely, hormones such as oestrogen (Heaney et al. 1999; Kakar and Jennes 1999; Heaney et al. 2002), TSH (Heaney et al. 2001) and insulin (Chamaon et al. 2005; Thompson and Kakar 2005) have been implicated (see section 1.2.1.4). Furthermore, there is increasing evidence that hPTTG expression is regulated by a number of growth factors.

One study described over-expression of hPTTG in astrocytomas and subsequently demonstrated hPTTG induction by the EGFR ligands, EGF and TGF-α, in xenotransplantable U87 human glioma cells. This effect was abrogated by treatment with the specific EGFR inhibitor, AG1478. The c-met ligand, HGF, also induced hPTTG expression but to a lesser extent (Tfelt-Hansen et al. 2004).

A subsequent study demonstrated similar regulation of hPTTG by EGF and TGF-α in pituitary folliculostellate TtT-GF cells. Once again, these effects were blocked following inhibition of EGFR, by treatment with either AG1478 or gefitinib. Rapid phosphorylation of EGFR and subsequent activation of the MAP-Kinase and PI3-Kinase pathways were demonstrated following treatment with EGF and TGF-α, suggesting that these pathways are important in hPTTG regulation. EGF induction of hPTTG was cell-cycle dependent where its expression peaked at the S-G2 transition and the effect was absent following early S-phase blockade (Vlotides et al. 2006).

IGF-1 has been shown to regulate hPTTG expression in human breast cancer MCF-7 cells (Thompson and Kakar 2005) and in malignant and non-malignant astrocytes (Chamaon et al. 2005). In both studies, hPTTG induction by IGF-1 was partially or completely blocked by treatment with either of the specific PI3-Kinase inhibitors, LY294002 and Wortmannin, or the specific MAP-Kinase inhibitor PD98059. Interestingly, the latter study demonstrated a direct interaction between both kinases and endogenous hPTTG in both malignant astrocytes and non-tumourous cells. In addition to enhancement of hPTTG transcription following

treatment with IGF-1, it was suggested that a second and more direct route of hPTTG regulation may be mediated via direct binding of either kinase (Chamaon et al. 2005).

The induction of hPTTG expression by FGF-2 (see section 1.2.1.4.2) has been demonstrated in NIH3T3 cells (Heaney et al. 1999) and in primary cultures of uterine leiomyomas, benign tumours of myometrial smooth muscle tissue (Tsai et al. 2005).

The transcription factor SP1 is well established as a critical regulator of hPTTG expression (Pei 1998; Kakar and Jennes 1999; Wang and Melmed 2000b; Clem et al. 2003; Zhou et al. 2003), where mutation of the SP1 binding site in the hPTTG promoter resulted in 70 % reduced overall promoter activity (Clem et al. 2003). A recent study described reduced hPTTG, FGF-2 and VEGF expression in various thyroid cancer cell lines treated with drugs inhibiting SP1 (Chintharlapalli et al. 2011) and VEGF expression was upregulated following phosphorylation of SP1 by MAP-Kinase and PI3-Kinase (Milanini-Mongiat et al. 2002; Pore et al. 2004).

hPTTG expression is cell-cycle dependent and it is most abundant during G2/M-phase where it is reportedly phosphorylated by the kinase CDC2 at the Ser-165 residue (Zou et al. 1999; Yu et al. 2000b; Ramos-Morales et al. 2000). While hPTTG expression is upregulated during mitosis, the observation that over-expression of hPTTG in differentiated thyroid cancer did not correlate with expression of the proliferation marker, PCNA, suggests that hPTTG over-expression is at least partly independent of increased mitotic rates (Boelaert et al. 2003a).

Collectively, these studies suggest that hPTTG is induced by a number of mitogenic factors via complex pathways involving activation of multiple kinases, transcription factors and cell-cycle regulators. The aim of this study was to assess whether hPTTG expression is regulated by growth factors in thyroid cells as part of the investigation of our hypothesis that hPTTG and growth factors are involved in autocrine and paracrine mechanisms of regulation

that may be aberrantly controlled in thyroid tumours. Malignant thyroid cell lines and human primary thyrocytes were treated with various growth factors and potential mechanisms of hPTTG regulation were investigated through use of specific inhibitors and siRNA knockdown studies.

3.2 Materials and Methods

3.2.1 Cell lines and treatment with growth factors and inhibitors

Human thyroid papillary carcinoma TPC-1 and K1 cells, and human anaplastic thyroid carcinoma SW1736 cell lines, were routinely cultured as described in section 2.1. For treatment experiments with growth factors, TPC-1, K1 and SW1736 cells were seeded in six-well plates at a density of 9 x 10^4, 1.3 x 10^5 and 1.2 x 10^5 cells/well respectively. After 24 hours, media was replaced with serum free media for a further 24 hours to obtain synchronised cells. In the first instance, preliminary dose-response and timecourse experiments were performed in synchronised K1 cells as described in section 3.3.3.1. Subsequently, synchronised TPC-1, K1 and SW1736 cells were incubated with EGF (5 nM), TGF-α (5 nM) [Sigma-Aldrich, UK], IGF-1 (10 ng/ml) or FGF-2 (5 nM) [Peprotech, UK] with or without PD98059 (30 μM), Wortmannin (50 nM) or LY294002 (50 μM) [Calbiochem, La Jolla, CA]. Concentrations of inhibitors were determined from the literature (Thompson and Kakar 2005; Chamaon et al. 2005). Cells were harvested in protein lysis buffer at the times indicated.

3.2.2 siRNA transfection studies

TPC-1 cells were transfected with siRNAs targeting *SP1* (60 nM # S13318) or *CDC2* (30 nM # S464) transcripts and controlled for by equivalent scrambled (# AM4635) siRNA transfections (Life Technologies, Grand Island, NY, USA). All siRNA transfections were

performed using the siPORT™ *NeoFX*™ Transfection Agent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. 24 hours post-transfection, cells were synchronised and treated with growth factors as described above (see section 3.2.1), or with anisomycin (100 μM) for 8 hours (Sigma-Aldrich, UK). Cells were harvested in protein lysis buffer or 0.5 ml Tri Reagent (Life Technologies, Grand Island, NY, USA).

3.2.3 Thyroid samples, human primary thyroid culture and transfections

Collection of thyroid samples was with approval of the Local Research Ethics committee and thyroid cells were cultured as described in section 2.2. All serum, insulin and TSH was removed from human primary thyrocytes for at least 24 hours before treatment with growth factors or inhibitors, which were performed as described above in section 3.2.1.

Human primary thyrocytes were transfected with 1 μg DNA/well using FuGENE-6 Transfection Reagent (Roche, Indianapolis, IN, USA), with an optimised ratio of 6 μl per 1 μg plasmid DNA using either pCI-neo-hPTTG or equal amounts of blank plasmid (vector-only, VO, control). In siRNA studies, human primary thyrocytes were transfected with a specific CDC2 siRNA (60 nM) and controlled for by equivalent scrambled siRNA transfections (see section 3.2.2) using the lipofectamine-2000 transfection agent (Invitrogen, UK) according to the manufacturer's instructions.

3.2.4 RNA extraction, reverse transcription, QT-PCR

Total RNA was extracted from TPC-1, K1, SW1736 cells and human primary thyrocytes as described in section 2.4. Reverse transcription and QT-PCR techniques were as described above (see sections 2.4 and 2.5). Primers and probes for *hPTTG* mRNA detection were designed as described above (see section 2.5) and the sequences are given in Table 3-1.

Gene-specific expression assays for *EGFR* (Hs01076078_m1), *IGF1R* (Hs00609566_m1) and *FGFR1* (Hs00915142_m1) were purchased from Applied Biosystems (Warrington, UK).

Sequence Name	Sequence
hPTTG Probe	CGTCTTGCCACCGGCTTCCCT
hPTTG Forward Primer	GAGAGAGCTTGAAAAGCTGTTTCAG
hPTTG Reverse Primer	TCCAGGGTCGACAGAATGCT

Table 3-1: Oligonuleotide sequences of PCR primers and TaqMan probe used to detect hPTTG mRNA expression. All TaqMan primers run at 59°C and yield amplicons of 70-150bp.

3.2.5 Western blot analysis

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see section 2.6). Blocked membranes were subsequently incubated with primary antibodies against hPTTG (2 µg/ml) [Invitrogen, UK], CDC-2 (1:1000), SP1 (1:500), phospho-P44/42 ERK1/2 (1:1000) and phospho-AKT (1:1000) [Cell Signalling Technology, Boston, MA, USA]. After washing in TBS-T, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidise (Dakocytomation, UK) for 1 hour at room temperature before being visualised by techniques described in section 2.6.

3.2.6 MTT cell viability assays

The rate of cellular proliferation of synchronised TPC-1 cells treated with growth factors was assessed by internalisation and reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were seeded in 96-well plates, synchronised and treated with growth factors as described above (see sections 3.2.1 and 3.2.2). Cells were incubated with 0.8 mg/ml MTT (Sigma-Aldrich, UK) for the last 3 hours of culture incubation. Cells were then washed with cold PBS before formazan crystals were solubilised

by addition of 100 µl dimethyl sulfoxide for 15 minutes. The absorbance at 540 nm was measured using a Wallac 1420 Victor plate-reader (Perkin-Elmer, Massachusetts, USA).

3.2.7 Statistical analysis

Data were analysed as described in section 2.8.

3.3 Results

3.3.1 Endogenous hPTTG expression in thyroid cells

To predict whether hPTTG is regulated by growth factors in thyroid cells, we first analysed the endogenous expression levels of hPTTG and growth factor receptors (see section 3.3.2) in human primary thyrocytes, TPC-1 and K1 thyroid papillary carcinoma cells and in SW-1736 anaplastic thyroid carcinoma cells. Real-Time PCR analysis revealed a 8.1-, 11.9- and 25.5-fold increased expression of *hPTTG* mRNA in TPC-1 ($n = 4$, $p < 0.001$), K1 ($n = 4$, $p < 0.001$) and SW1736 ($n = 4$, $p < 0.001$) cells respectively, compared with normal human primary thyrocytes ($n = 12$). SW1736 cells had significantly higher expression of *hPTTG* mRNA compared with TPC-1 ($p < 0.01$) but not K1 cells. Western blot analysis confirmed hPTTG protein over-expression in transformed cell lines compared with normal human primary thyrocytes, where expression was heterogeneous but at generally low levels. Further Western blot analysis using a lower length of exposure was performed to determine the relative levels of hPTTG protein in thyroid cancer cell lines. TPC-1 cells had the lowest levels of over-expression of hPTTG protein; K1 cells had moderate over-expression of hPTTG protein; and SW1736 cells had very strong over-expression of hPTTG protein. These results are indicative of higher levels of hPTTG expression in cells derived from a more aggressive tumour type (see Figure 3-1).

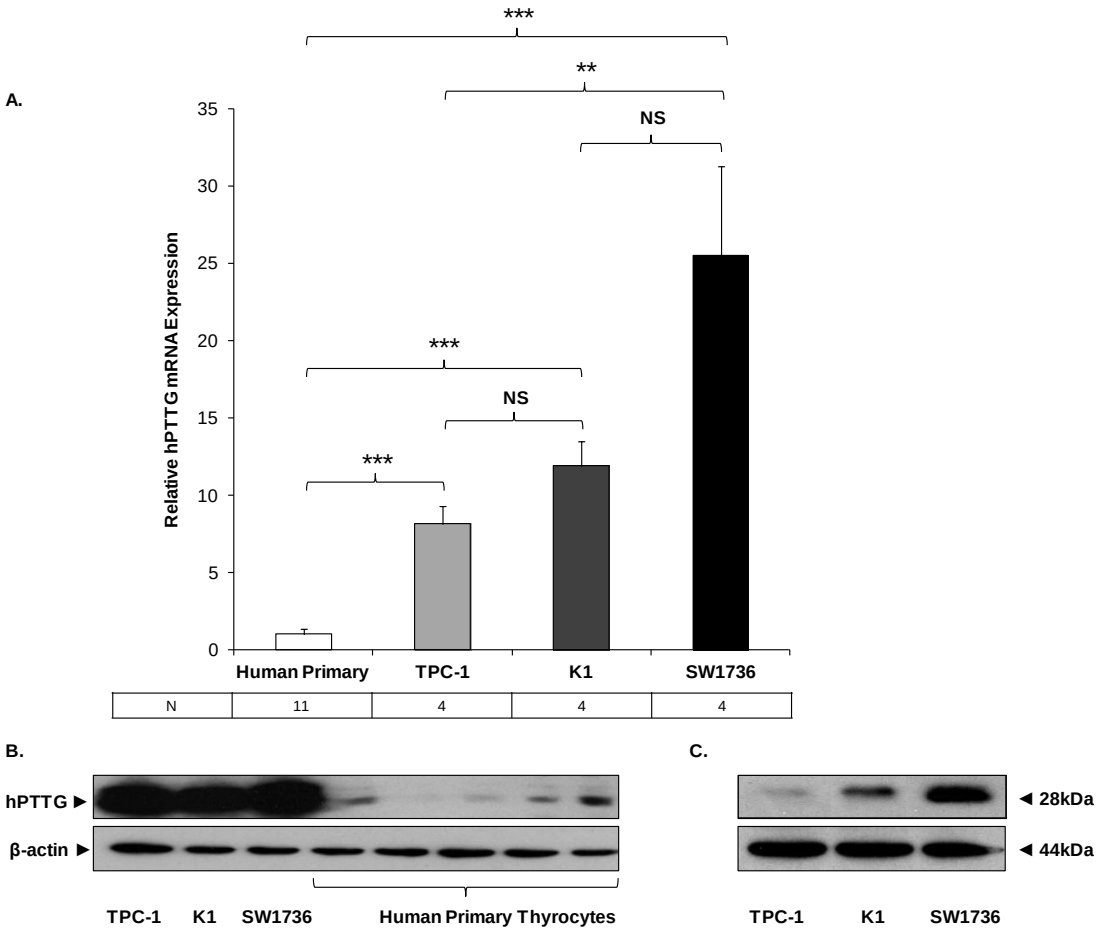


Figure 3-1: *Endogenous expression of hPTTG in human primary thyrocytes, TPC-1, K1 and SW1736 cell lines.* ***A*** *Endogenous hPTTG mRNA expression levels in thyroid cells, determined by TaqMan RT-PCR using hPTTG-specific primers and probes. In this and following histograms, gene expression is displayed relative to a value of 1.0 for human primary thyrocytes (normal thyroid cells). The number of samples used for each group are given in the corresponding columns in the table underneath the graph. Error bars represent the standard error of the mean (SEM) (** $p < 0.01$, *** $p < 0.001$, NS = non-significant).* ***B*** ***E****ndogenous hPTTG protein expression levels in thyroid cells, determined by Western blot analysis, demonstrating strong over-expression of hPTTG in thyroid cancer cell lines compared to low heterogeneous expression in normal human primary thyrocytes.* ***C*** *Lower exposure Western blot analysis demonstrating relative hPTTG expression levels in thyroid cancer cell lines.*

3.3.2 Growth factor receptor expression in thyroid cells

EGFR, IGF1R and FGFR1 are growth factor receptors that have been implicated in thyroid malignancies and in facilitating signalling by growth factors that regulate hPTTG (Chamaon et al. 2005; Tfelt-Hansen et al. 2004; Thompson and Kakar 2005; Heaney et al. 1999; Tsai et al. 2005; Lee et al. 2007; Landriscina et al. 2011; Lam et al. 2011; Belfiore et

al. 1999; St Bernard et al. 2005; Cocks et al. 2003). The mRNA expression of these growth factor receptors was analysed in thyroid cell lines and compared to expression levels in normal human primary thyrocytes (n = 12) to determine responsiveness of these cell types to treatment with growth factors. *EGFR* mRNA was over-expressed 1.75-, 2.38- and 3.08-fold in TPC-1 (n = 4, p = NS), K1 (n = 4, p = 0.05) and SW1736 (n = 4, p < 0.01) cells respectively. *IGF1R* was expressed in transformed thyroid cell lines but showed a 0.45-, 0.23- and 0.45-fold reduction of mRNA expression in TPC-1 (n = 4, p < 0.05), K1 (n = 4, p < 0.001) and SW1736 (n = 4, p < 0.05) cells respectively, when compared with primary cells. *FGFR1* mRNA was over-expressed 5.86-, 6.53- and 5.24-fold in TPC-1 (n = 4, p < 0.05), K1 (n = 4, p < 0.05) and SW1736 (n = 4, p < 0.05) cells respectively, compared to normal human primary thyrocytes (n = 12) (see Figure 3-2).

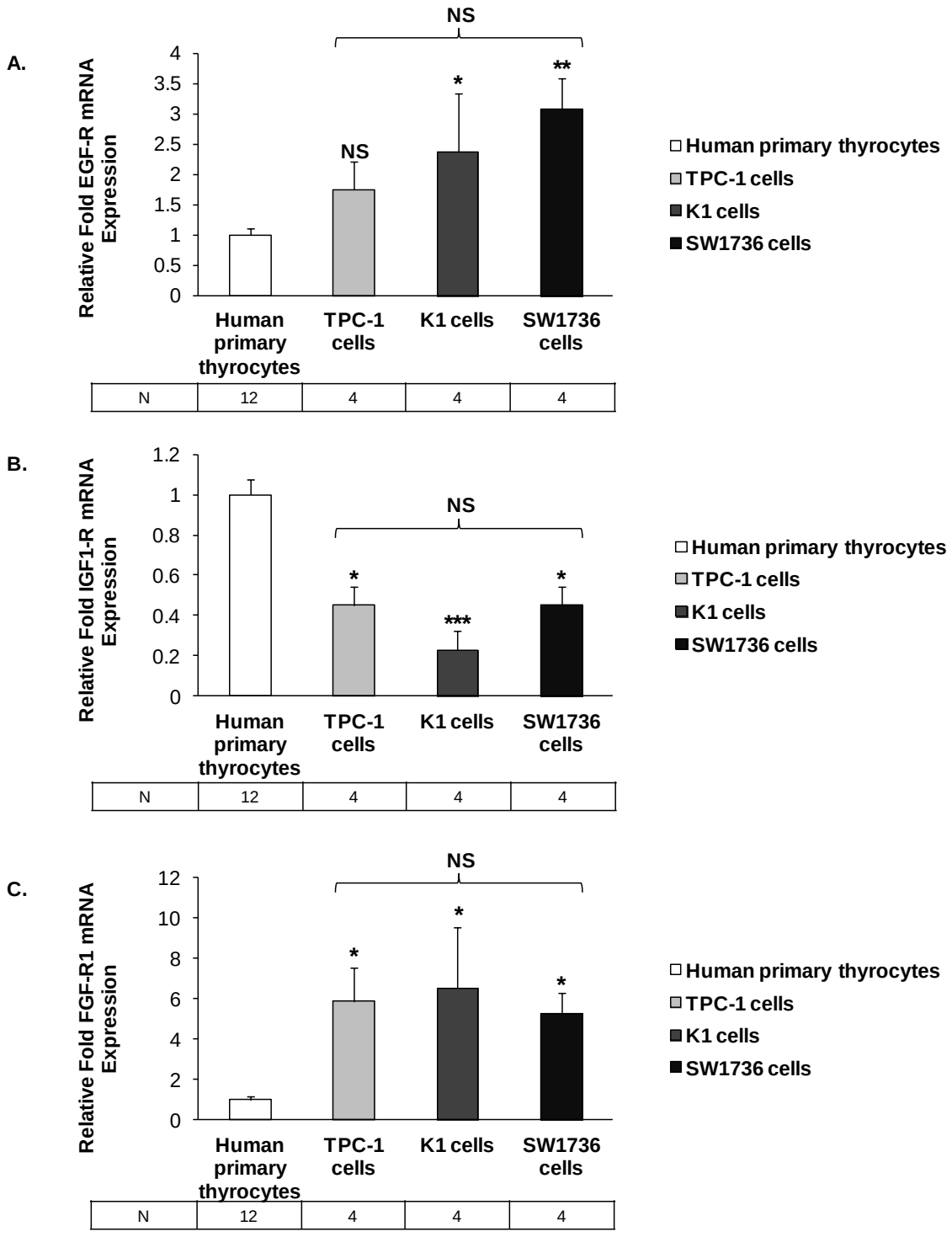


*Figure 3-2: Histograms displaying endogenous mRNA expression of growth factor receptors in human primary thyrocytes, TPC-1, K1 and SW1736 cell lines, determined by TaqMan RT-PCR. **A** EGF-R mRNA expression in thyroid cells, showing significant over-expression in K1 and SW1736 thyroid cancer cell lines. **B** IGF1R mRNA expression in thyroid cells, showing significantly reduced expression in thyroid cancer lines. **C** FGF-R1 mRNA expression in thyroid cells, showing significant over-expression in thyroid cancer cell lines. Error bars represent the SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = non-significant).*

3.3.3 hPTTG regulation by growth factors in thyroid cells

Having determined expression levels of hPTTG and growth factor receptors in those thyroid cells being tested, we investigated the regulation of hPTTG protein expression following treatment with either EGF, TGF-α (EGFR ligands), IGF-1 (IGF1R ligand) or FGF-2 (FGFR1 ligand).

3.3.3.1 Preliminary time-course and dose-response experiments

In the first instance, time-course and dose-response experiments were performed in K1 cells to determine the optimal duration of treatment time and growth factor concentration. For dose-response experiments, synchronised K1 cells were incubated with varying growth factor concentrations for 24 hours. hPTTG protein was induced in a dose-dependent manner by EGF and TGF-α (0.5, 5 and 50 nM) peaking with a dose of 5 nM, and no further induction was observed following treatment with 50 nM doses. Similarly, IGF-1 (1, 10 and 100 ng/mL) induced hPTTG protein in a dose-dependent manner but hPTTG protein expression was only marginally increased with the higher 100 ng/mL dose compared to treatment with 10ng/mL. All concentrations of FGF-2 (0.5, 5 and 50 nM) caused a mild induction of hPTTG protein. Subsequently, the most relevant doses for each growth factor were decided to be 5 nM EGF, 5 nM TGF-α, 10 ng/mL IGF-1 and 5 nM FGF-2, based on the responses observed (see Figure 3-3 A).

In timecourse experiments, treatment of K1 cells with the doses of growth factor decided above caused no response at 0-12 hours, but there was evidence of hPTTG protein induction at 24-36 hours, with maximal growth factor induced hPTTG expression observed at 24 hours (see Figure 3-3 B).

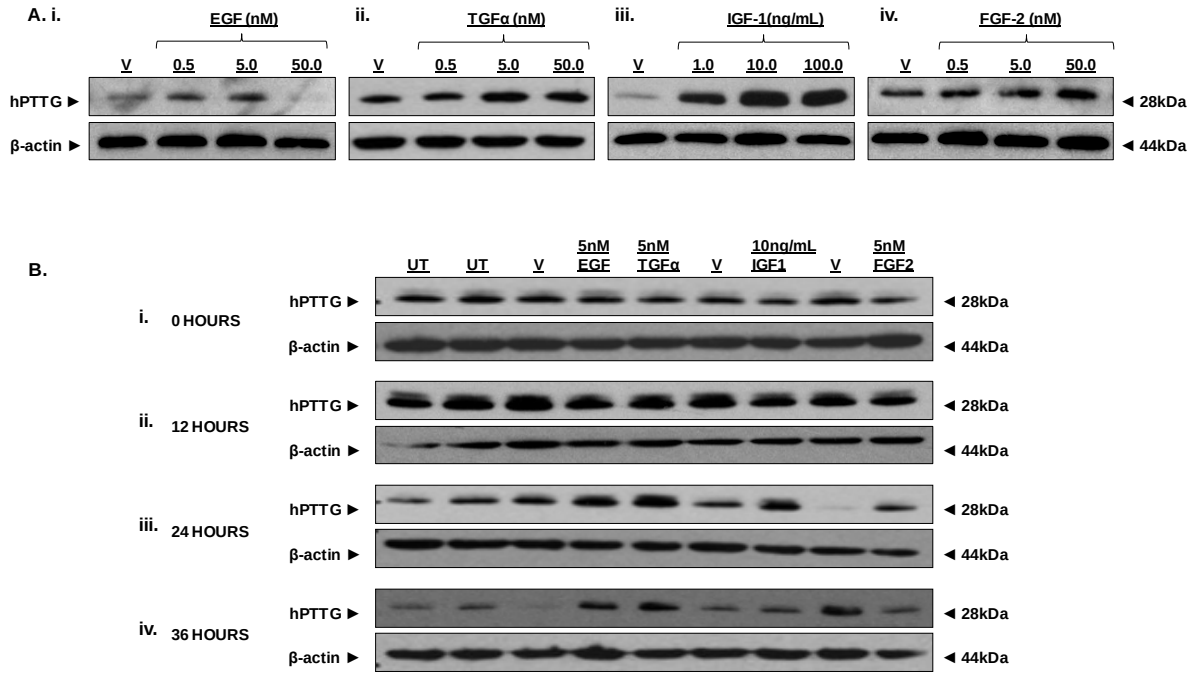


*Figure 3-3: Preliminary dose-response and timecourse treatments of K1 cells with growth factors. **A** Western blot analyses demonstrating hPTTG regulation by different doses of EGF [0.5, 5.0, 50 nM] **(i)**, TGF- α [0.5, 5.0, 50.0 nM] **(ii)**, IGF-1 [1.0, 10.0, 100.0 ng/mL] **(iii)** and FGF-2 [0.5, 5.0, 50.0 nM]**(iv)**. **B** Western blot analyses demonstrating regulation of hPTTG by 5 nM EGF, 5 nM TGF-α, 10 ng/mL IGF-1 and 5 nM FGF-2 at 0 **(i)**, 12 **(ii)**, 24 **(iii)** and 36 hours **(iv)**. UT = untreated, V = vehicle treated.*

3.3.3.2 hPTTG regulation by growth factors in presence and absence of inhibitors

Having determined appropriate treatment times and doses, synchronised thyroid cells (TPC-1, K1, SW1736, human primary thyrocytes) were incubated with either 5 nM EGF, 5 nM TGF-α, 10 ng/ml IGF-1 or 5 nM FGF-2 for the duration of 24 hours. Western blot analysis and scanning densitometry studies were performed to determine hPTTG protein expression in GF-treated cells compared to vehicle-only treated cells. Treatment with 5 nM EGF induced hPTTG protein expression in human primary thyrocytes (3.2-fold, $n = 3$, $p < 0.05$), TPC-1 cells (6.4-fold, $n = 5$, $p < 0.001$), K1 cells (3.2-fold, $n = 5$, $p < 0.001$) and in SW1736 cells (1.7-fold, $n = 3$, p = NS). Similarly, 5 nM TGF-α treatments induced 3.5-, 6.9-, 3.8- and 1.5-fold elevation of hPTTG protein expression in human primary thyrocytes ($n = 3$, $p < 0.01$), TPC-1 cells ($n = 5$, $p < 0.001$), K1 cells ($n = 5$, $p < 0.001$) and SW1736 cells (n

= 3, p = NS), respectively. For all cell types except SW1736 cells (where hPTTG was not significantly induced by EGFR ligands) these effects were abrogated upon addition of the MAP-Kinase-specific inhibitor PD98059 (30 μM) (see Figure 3-4).

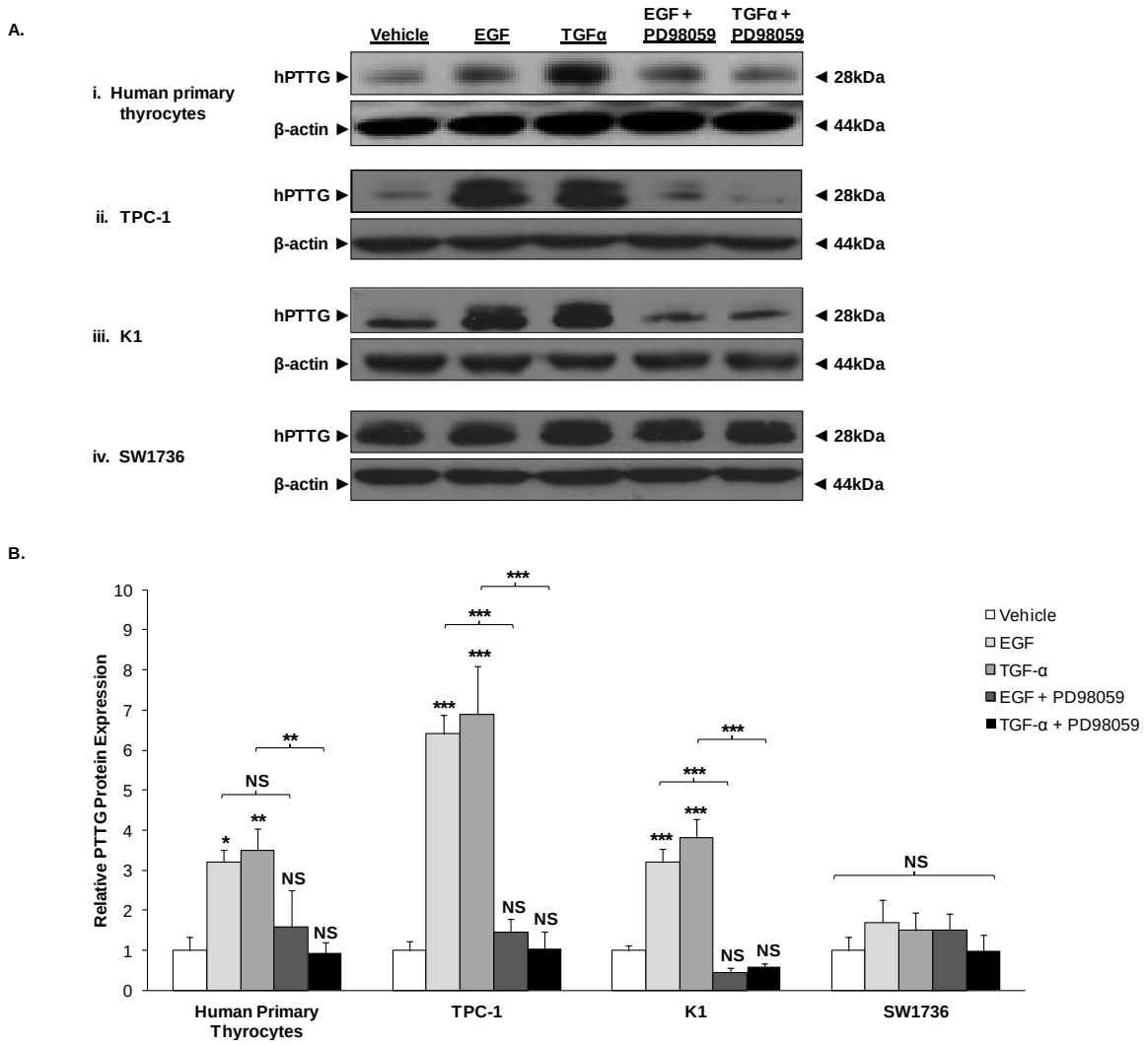


Figure 3-4: Induction of hPTTG protein expression following treatment of thyroid cells with growth factors ± inhibitors. ***A*** *Representative Western blot analyses demonstrating hPTTG protein expression following treatment with EGF (5 nM) and TGF-α (5 nM) ± PD98059 (30 μM), for 24 hours, in synchronised human primary thyrocytes* ***(i)****, TPC-1* ***(ii)****, K1* ***(iii)*** *and SW1736 cell lines* ***(iv)****.* ***B*** *Scanning densitometry studies demonstrate hPTTG protein expression levels given as a value relative to 1.0 for vehicle only control treatments, ± SEM. Results are based on ≥ 3 separate experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = non-significant).*

Treatment with 10 ng/ml IGF-1 caused a 1.9-, 4.2-, 2.2- and 1.7-fold upregulation of hPTTG protein expression in human primary thyrocytes (n = 3, $p < 0.05$), TPC-1 cells (n = 4, $p < 0.01$), K1 cells (n = 4, $p < 0.01$) and SW1736 cells (n = 3, p = NS) respectively. In all

cell types except SW1736 cells, hPTTG induction by IGF-1 was abrogated by treatment with either of two PI3-Kinase specific inhibitors, Wortmannin (50 nM) or LY294002 (50 μM). Treatment with 5 nM FGF-2 had no effect on hPTTG protein expression in human primary thyrocytes (1.2-fold, $n = 3$, p = NS), K1 cells (1.4-fold, $n = 3$, p = NS) or SW1736 cells (1.5-fold, $n = 3$, p = NS), but resulted in a striking 8.8-fold induction in TPC-1 cells ($n = 3$, $p < 0.001$). This effect was not investigated further due to the inconsistent responses demonstrated between different thyroid cell types (see Figure 3-5).

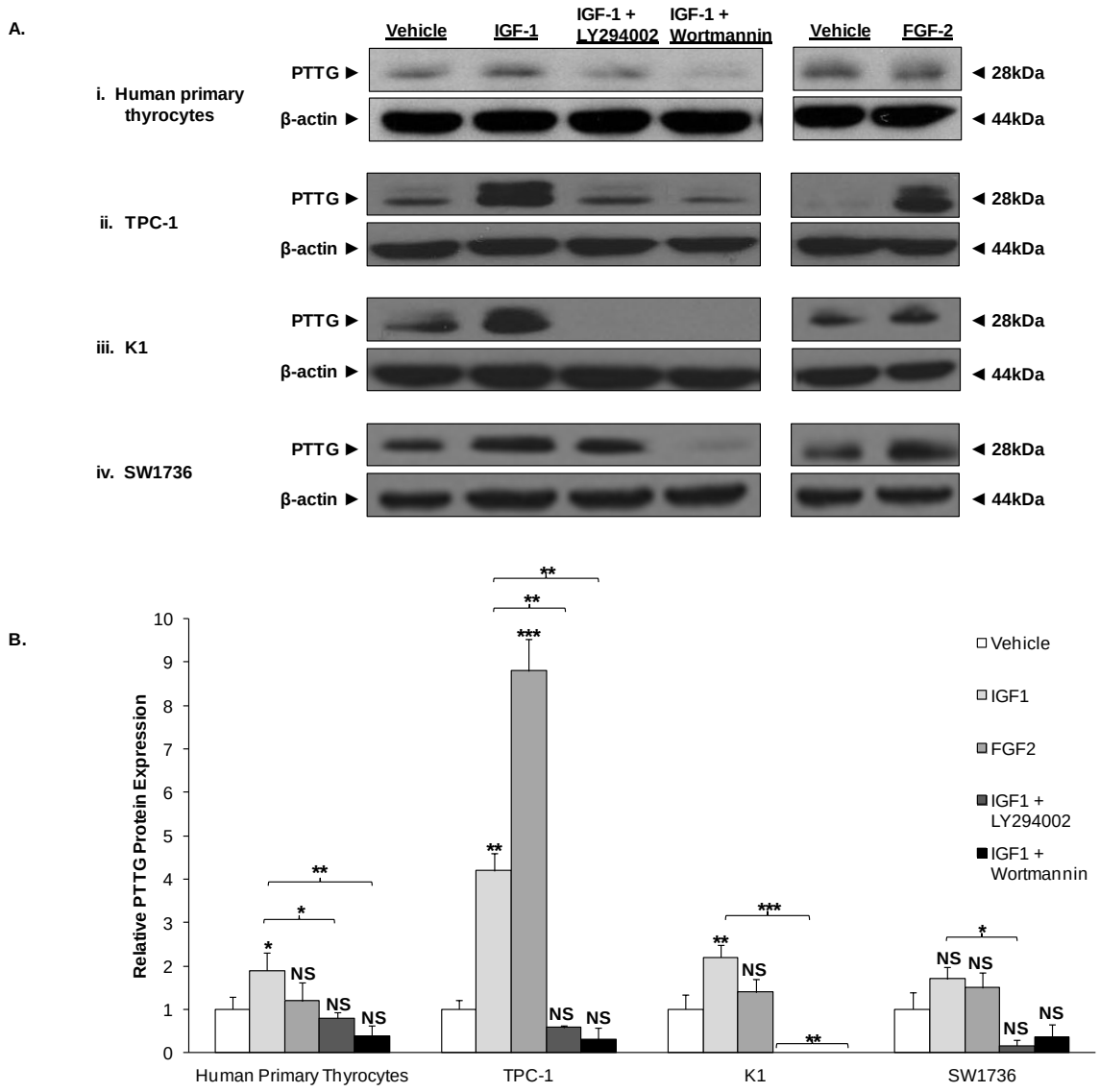


Figure 3-5: Induction of hPTTG protein expression following treatment of thyroid cells with growth factors ± inhibitors. ***A*** *Representative Western blot analyses demonstrating hPTTG protein expression following treatment with IGF-1 (10 ng/mL) ± LY294002 (50 μM) or Wortmannin (50 nM) and FGF-2 (5 nM), for 24 hours, in synchronised human primary thyrocytes* ***(i)****, TPC-1* ***(ii)****, K1* ***(iii)*** *and SW1736 cells* ***(iv)****.* ***B*** *Scanning densitometry studies demonstrating hPTTG protein expression*

levels given as a value relative to 1.0 for vehicle only control treatments, ± SEM. Results are based on ≥ 3 separate experiments. ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = non-significant).*

Notably, treatment with growth factors resulted in increased expression of both unphosphorylated and phosphorylated hPTTG, where the heavier upper band of the doublet represents phosphorylated hPTTG (Ramos-Morales et al. 2000). Together these results provide evidence that hPTTG protein expression and phosphorylation is upregulated in thyroid cells via the MAP-Kinase and PI3-Kinase pathways, following activation of the EGF-Receptor and the IGF1-Receptor respectively.

3.3.4 Confirmation of MAP-kinase and PI3-kinase activation by treatment with growth factors

To confirm activation of the MAP-kinase and PI3-kinase pathways in response to growth factor treatment, we analysed phospho-P44/42 ERK1/2 and phospho-AKT expression 10, 30 and 90 minutes following treatment in TPC-1, K1 and SW1736 cells (Vlotides et al. 2006). Treatment with EGF (5 nM) or TGF-α (5 nM) stimulated ERK-phosphorylation from 10 minutes post-treatment and was sustained throughout the timecourse. Furthermore, there was significant activation of the PI3-kinase pathway as evidenced by increased p-AKT expression. These effects were partially or completely abrogated following pre-incubation with PD98059 (30 μM). Similarly, treatment with IGF-1 (10 ng/mL) resulted in the AKT-phosphorylation after 10 minutes that was sustained throughout the experiment. Once again, increased p-ERK expression was indicative of MAP-kinase pathway activation following IGF-1 treatment. These effects were partially or completely abrogated following pre-incubation with LY294002 (50 μM) (see Figure 3-6).

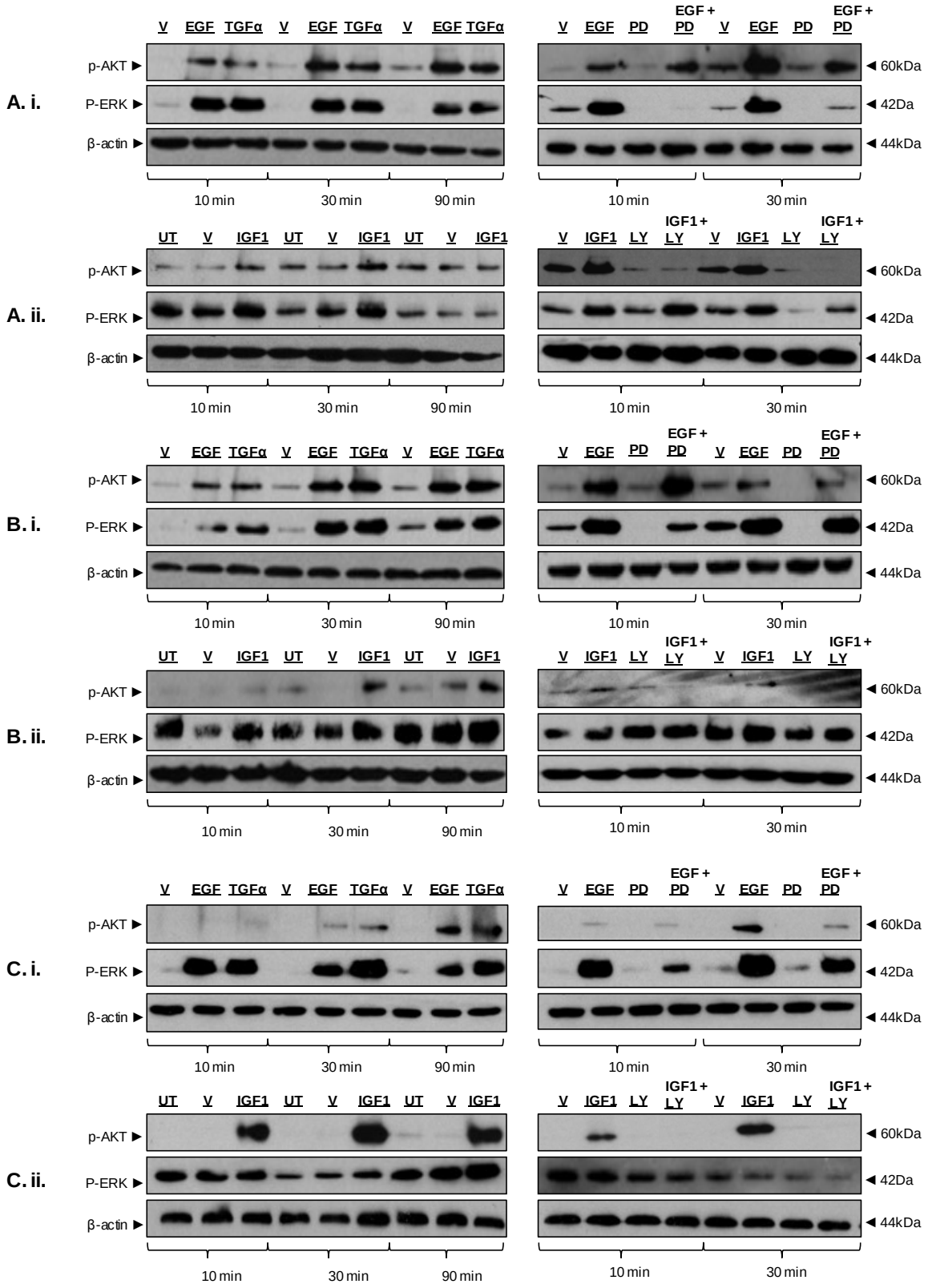


Figure 3-6: Western blot analyses confirming MAP-kinase and PI3-kinase activation in thyroid cell lines following treatment with EGF, TGF-α and IGF-1. ***A-C****, Western blot analyses of timecourse experiment showing detection of phospho-P44/42 ERK1/2 and phospho-AKT in TPC-1 (****A****), K1 (****B****) and SW1736(****C****) cells following treatment with EGF (5 nM) and TGF-α (5 nM) ± PD98059 (30 μM) (****i****) or IGF-1 (10 ng/mL) ± LY249002 (50 μM) (****ii****) at early time-points of 10, 30 and 90 minutes. V = vehicle, PD = PD98059, LY = LY294002.*

3.3.5 Regulation of hPTTG by growth factors is independent of SP1 and CDC2

Given the reported roles of the transcription factor SP1 and the kinase CDC2 (CDK1) in the regulation of hPTTG expression and phosphorylation respectively (Clem et al. 2003; Ramos-Morales et al. 2000), we set out to investigate the potential involvement of these proteins in the mechanism of hPTTG regulation by growth factors in thyroid cells. TPC-1 cells were chosen for these studies as they were the most responsive to treatment with growth factors and were consistently amenable to transfection. Growth factor treatments of TPC-1 cells were repeated following knockdown of *SP1* and *CDC2* transcripts. hPTTG protein expression was determined following successful SP1 and CDC2 knockdown through Western blotting and scanning densitometry. As expected, we observed a strong induction of hPTTG protein expression by either EGF (2.7-fold, $n = 4$, $p < 0.05$) or TGF-α (3.1-fold, $n = 4$, $p < 0.01$) following transfection with scrambled siRNA. Basal levels of hPTTG protein in vehicle treated cells showed a marked 0.27-fold reduction in expression following SP1 knockdown ($n = 4$, $p < 0.05$) compared to scrambled controls ($n = 4$), consistent with a role for SP1 in regulating basal hPTTG expression. Nonetheless, even with depleted SP1 levels, hPTTG protein expression was induced following treatment with EGF (4.6-fold, $n = 4$, $p < 0.01$) and TGF-α (5.2-fold, $n = 4$, $p < 0.01$) compared with vehicle treated controls ($n = 4$) (see Figure 3-7).

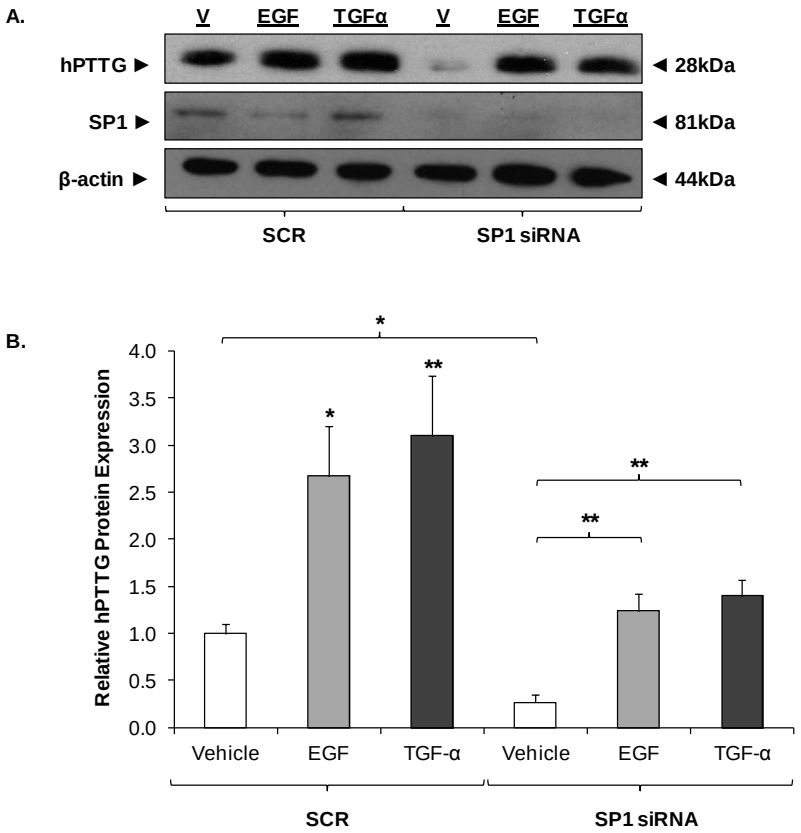


*Figure 3-7: hPTTG regulation by growth factors is independent of SP1. **A** Western blot analysis demonstrating persistent regulation of hPTTG by growth factors when SP1 levels are depleted in TPC-1 cells. **B** Scanning densitometry studies demonstrating hPTTG protein expression levels relative to a value of 1.0 for vehicle treated scrambled only controls, ± SEM. Results are based on n = 4 repeats of the described experiment. (* $p < 0.05$, ** $p < 0.01$). V = vehicle.*

CDC2 siRNA transfections resulted in successful knockdown of CDC2 expression, determined by Western blot analysis at the time of treatment and the time of termination. As expected, there was a strong induction of both unphosphorylated and phosphorylated hPTTG by growth factors in cells transfected with a scrambled siRNA control and treated with 5nM EGF (3.7-fold, $n = 3$, $p < 0.05$). Interestingly, when CDC2 expression was repressed, basal levels of hPTTG expression in vehicle treated cells were strikingly induced in comparison to scrambled controls (9.9-fold, $n = 4$, $p < 0.05$). However, treatment with EGF resulted in further induction of hPTTG expression (13.8-fold, $n = 3$, p = NS), compared with vehicle treated controls (see Figure 3-8).

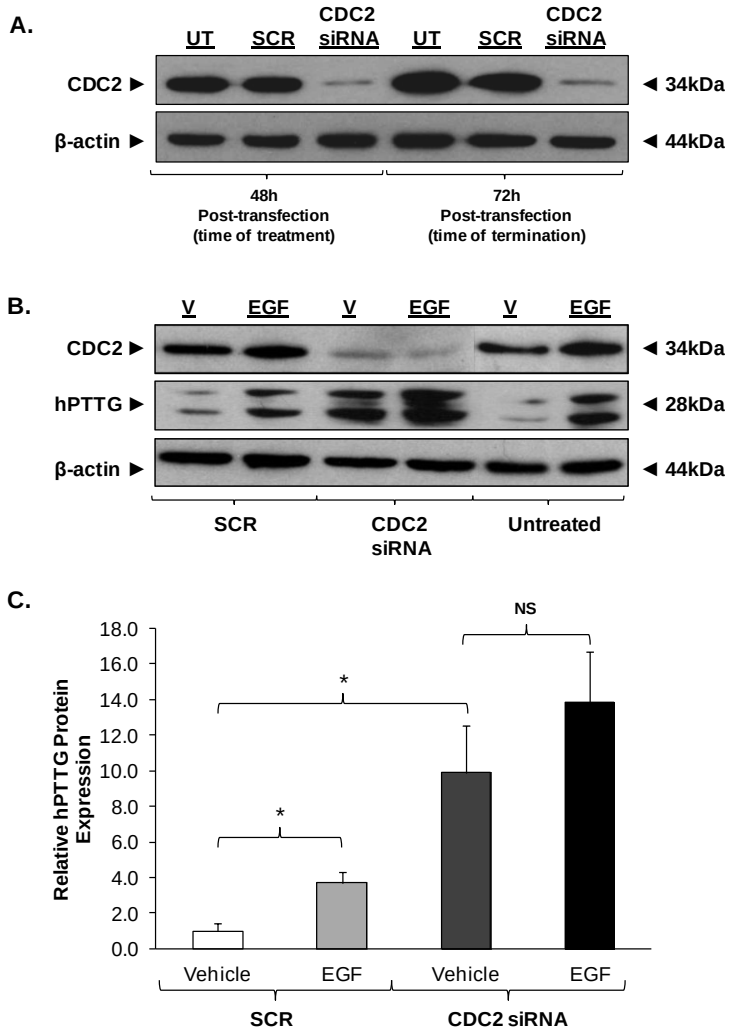


Figure 3-8: hPTTG regulation by growth factors is independent of CDC2. ***A*** *Western blot analysis demonstrating successful knockdown of CDC2 protein throughout the duration of the experiment.* ***B*** *Representative Western blot analysis demonstrating successful knockdown of CDC2, causing a generic increase in hPTTG expression and phosphorylation that is further enhanced upon addition of EGF in TPC-1 cells.* ***C*** *Scanning densitometry studies demonstrating hPTTG protein expression levels relative to a value of 1.0 for vehicle treated scrambled only controls, ± SEM. Results are based on n = 3 repeats of the described experiment. (* $p < 0.05$, NS = non-significant). V = vehicle.*

3.3.6 CDC2 regulates expression and phosphorylation of hPTTG

The observation that repression of CDC2 results in increased levels of hPTTG implies that CDC2 is a key regulator of hPTTG expression and we investigated this further. Firstly, we confirmed that knockdown of CDC2 results in increased hPTTG expression in both TPC-1 cells (4.1-fold, n = 6, $p < 0.01$) and human primary thyrocytes (1.9-fold, n = 4, $p < 0.05$)

(see Figure 3-9). To clearly demonstrate that CDC2 depletion results in upregulation of both phosphorylated and unphosphorylated hPTTG we used primary thyrocytes transfected with WT or Phos- hPTTG (which cannot be phosphorylated) as controls. The lack of the heavier band following transfection with Phos- hPTTG clearly illustrates that this band represents a phosphorylated form of hPTTG. This band migrates more slowly due to the increased molecular mass caused by an additional phosphate group (PSO43-), where the additional negative charge is negated by use of SDS (see Figure 3-9 A.i.).

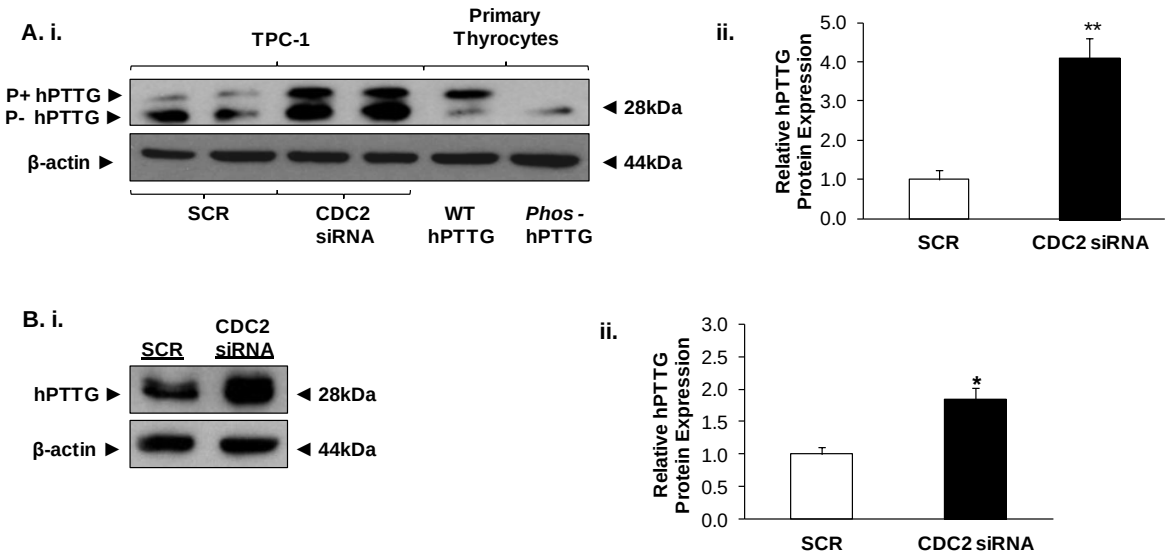


Figure 3-9: CDC2 regulates expression and phosphorylation of hPTTG. ***A+B*** *Representative Western blot analyses and scanning densitometry confirming an increase in hPTTG expression and phosphorylation following repression of CDC2 in TPC-1 cells (**A i-ii**) and human primary thyrocytes (**B i-ii**). Scanning densitometry studies demonstrate hPTTG protein expression levels relative to a value of 1.0 for scrambled only transfection controls, ± SEM. Results are based on n ≥ 3 repeats of the described experiment. (* p < 0.05, ** p < 0.01). P+ hPTTG = Phosphorylated hPTTG, P- hPTTG = Unphosphorylated hPTTG.*

We employed two approaches to investigate whether the regulation of hPTTG by CDC2 is due to transcriptional or post-translational events. Firstly, we performed CDC2 knockdown experiments in TPC-1 cells followed by treatment with anisomycin (100 μM) to inhibit translation 36 hours post-transfection. Western blot analysis demonstrated that the upregulation of hPTTG caused by repression of CDC2 is abrogated upon the addition of anisomycin, suggesting that this effect is transcriptionally driven. Concordant with this,

Real-Time PCR analysis revealed that *hPTTG* mRNA expression was significantly upregulated (1.7-fold, $n = 8$, $p < 0.01$) when CDC2 was depleted, providing further evidence that CDC2 is a criticial mediator of *hPTTG* transcription (see Figure 3-10).

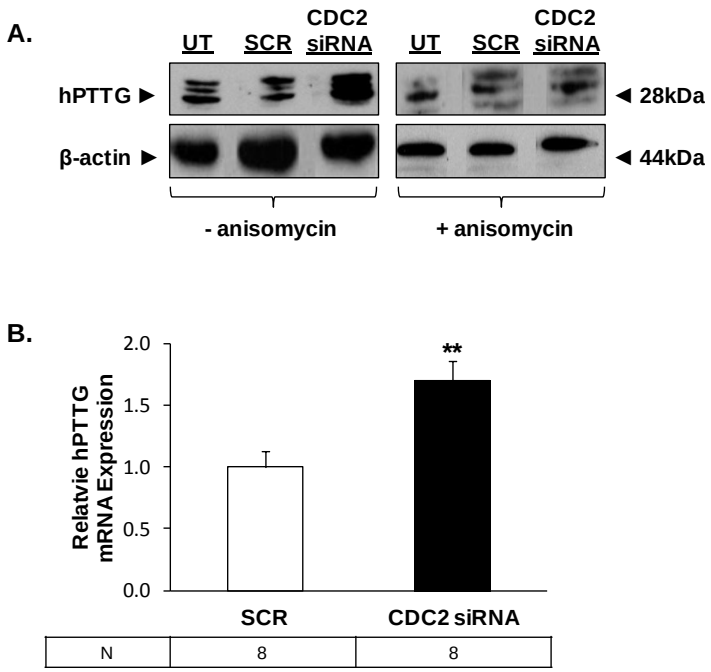


*Figure 3-10: CDC2 regulates transcription of hPTTG. **A** Representative Western blot analysis demonstrating that hPTTG upregulation following CDC2 repression is inhibited by treatment with anisomycin (100 μM), implicating this as a transcriptional effect. **B** Consistent with this, TaqMan RT-PCR analysis confirmed that hPTTG mRNA expression was induced following CDC2 repression. hPTTG mRNA expression is given as value relative to 1.0 for scrambled only transfection controls. N = 3 experiments. Error bars represent the SEM (** $p < 0.01$).*

3.3.7 Upregulation of hPTTG by growth factors is not associated with increased proliferation; CDC2 repression causes reduced cell proliferation

Given hPTTG's crucial role in mitosis as the human securin, we investigated if the increased hPTTG expression in response to growth factors was due to increased cellular proliferation. To test this we performed MTT cell viability assays and simultaneously investigated proliferative effects following CDC2 depletion in the absence or presence of growth factors. There was no significant difference in the cell viability of TPC-1 cells following treatment with growth factors ($n = 3$) compared to vehicle treated controls. Interestingly, when CDC2 was repressed, there was a 24 % reduction in cell viability in

vehicle treated cells (n = 3, p < 0.001) compared to scrambled siRNA controls, suggesting that the observed increase in hPTTG expression following CDC2 knockdown results in reduced cell proliferation. When CDC2 expression was repressed, there was no significant difference in cell viability following treatment with growth factors (n = 3) compared to vehicle treated controls (see Figure 3-11).

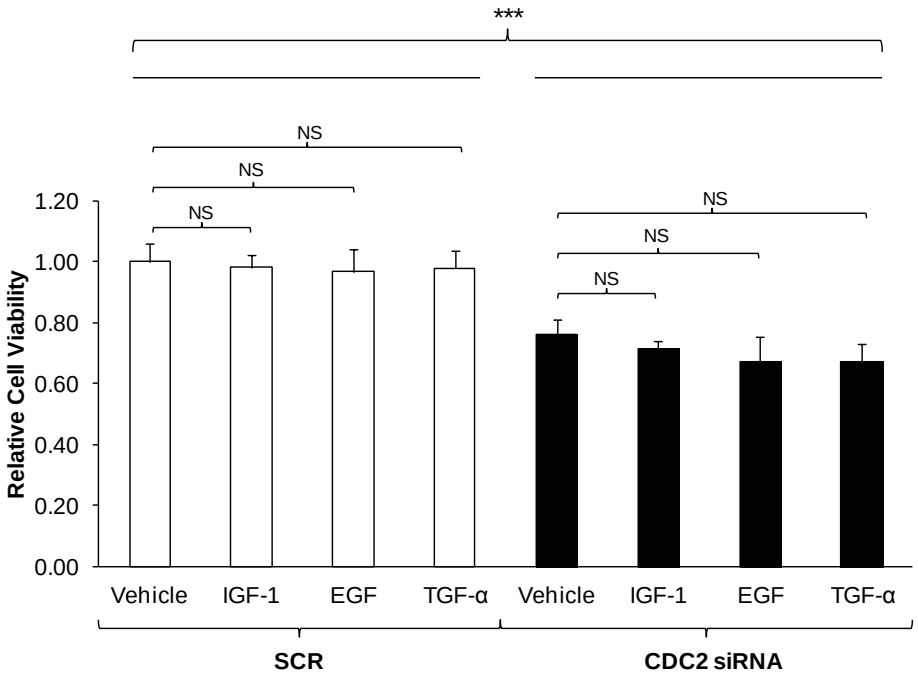


*Figure 3-11: MTT cell viability assays demonstrating that hPTTG induction by growth factors is not as a result of increased proliferation, but that CDC2 repression results in a significantly reduced proliferation rate in TPC-1 cells. N = 3 experiments. Error bars represent the SEM (*** p < 0.001, NS = non-significant).*

3.4 Discussion

The precise mechanisms of hPTTG over-expression in thyroid cancer have not been fully elucidated. Since hPTTG is induced by EGF, TGF-α, IGF-1 and FGF-2 in non-thyroid human cell types (Tfelt-Hansen et al. 2004; Vlotides et al. 2006; Chamaon et al. 2005; Thompson and Kakar 2005; Heaney et al. 1999; Tsai et al. 2005), we investigated if similar effects were observed in human thyroid cells. This study demonstrated that hPTTG expression and phosphorylation is regulated by EGF, TGF-α and IGF-1 in both malignant and non-malignant thyroid cells, though hPTTG was not induced by FGF-2 in all cell types

investigated. Interestingly, these effects were independent of the known regulators of hPTTG expression (SP1) and phosphorylation (CDC2). Furthermore, CDC2 was identified as a novel regulator of hPTTG expression in thyroid cells.

3.4.1 Expression of hPTTG and growth factor receptors in thyroid cells

Endogenous hPTTG mRNA and protein were over-expressed in transformed thyroid papillary carcinoma cell lines compared to normal human primary thyrocytes, consistent with hPTTG over-expression in thyroid malignancies, where its expression correlates with aggressiveness of tumour subtypes (Boelaert et al. 2003a; Heaney et al. 1999). Indeed, hPTTG over-expression was greater in SW1736 cells derived from an anaplastic thyroid carcinoma, compared with TPC-1 and K1 cells derived from papillary thyroid cancers.

EGFR, IGF1R and FGFR1 are tyrosine kinase receptors that facilitate the signalling capabilities of EGF and TGF-α, IGF-1 and FGF-2, respectively. The MAPK and PI3K signalling cascades are the major pro-survival, anti-apoptotic pathways activated by EGFR, IGF1R and FGFR1 following ligand binding, receptor dimerisation and tyrosine auto-phosphorylation (Oda et al. 2005; Riedemann and Macaulay 2006). High expression of *EGFR* in TPC-1 (albeit statistically non-significant), K1 and SW1736 cell lines was consistent with previous studies demonstrating its over-expression in six thyroid cancer cell lines (Yeh et al. 2006) and in thyroid tumours where elevated EGFR expression was associated with disease progression (Landriscina et al. 2011; Lee et al. 2007; Lam et al. 2011). Taken together with observations that the thyroid is an EGF-rich environment (Kajikawa et al. 1991) and that TGF-α is over-expressed in thyroid tumours (Lam et al. 2011), enhanced EGFR signalling appears to be an important process in thyroid cancer progression.

Similarly, our findings of elevated levels of *FGFR1* in TPC-1, K1 and SW1736 cells compared to normal human primary thyrocytes is consistent with other observations of

enhanced FGFR1 expression in *ex vivo* human specimens of thyroid hyperplasia (Thompson et al. 1998) and cancer (St Bernard et al. 2005). In addition, expression of the FGFR1 ligands FGF-1 and FGF-2 is increased in thyroid hyperplasia and cancer (Thompson et al. 1998; Eggo et al. 1995), suggesting FGF signalling is involved in thyroid disease progression. Moreover, FGF-2 expression positively correlated with elevated hPTTG expression in thyroid cancer specimens, where it was identified as an independent prognostic indicator of metastasis (Boelaert et al. 2003a). Subsequently, FGF-2 induction by hPTTG was demonstrated in thyroid cells (Boelaert et al. 2004). Together, these observations suggest that hPTTG could have an important role in mediating increased autocrine FGF-2 signalling to drive thyroid tumour progression.

Under-expression of *IGF1R* mRNA in TPC-1, K1 and SW1736 cells compared with human primary thyrocytes is contradictory to reports of increased IGF1R protein expression in TPC-1 and three other thyroid carcinoma cell lines (Belfiore et al. 1999). Further, both IGF-1 (mRNA and protein) and IGF1R (protein) upregulation has been reported in *ex vivo* human thyroid adenomas and carcinomas, supporting a role for enhanced IGF1R signalling in disease progression where their expression positively correlated with tumour aggression (Maiorano et al. 2000). However, our results were consistent, statistically significant and obtained using a robust technique in TaqMan RT-PCR.

3.4.2 Regulation of hPTTG expression and phosphorylation by growth factors

Despite varying levels of growth factor receptor expression, all thyroid cells tested were responsive to treatments with EGF, TGF-α and IGF-1, resulting in induction of hPTTG protein expression and phosphorylation. However, induction of hPTTG by growth factors in SW1736 cells was less clear than in TPC-1, K1 and human primary thyroid cells and scanning densitometry studies revealed non-significant hPTTG induction. It is plausible that

since endogenous hPTTG expression levels are high in SW1736 cells, the effects of growth factor treatment on further hPTTG induction are attenuated.

hPTTG expression and phosphorylation were strikingly enhanced following treatment with FGF-2 in TPC-1 cells, whereas no significant effects were observed in other cell types. It is possible that normal human primary thyrocytes were not responsive due to low FGFR1 expression compared with increased expression in thyroid cancer cells as observed in this and previous studies (St Bernard et al. 2005). Effects of growth factors on hPTTG expression were generally less striking in SW1736 cells and further dose-response and time-course studies may clarify hPTTG regulation by FGF-2 in K1 cells, since preliminary dose-response and timecourse experiments indicated FGF-2 induction of hPTTG expression. FGF-2 effects were not investigated further due to the inconsistent responses demonstrated between different thyroid cell types.

Given the role of hPTTG as the human securin (Zou et al. 1999; Zur and Brandeis 2001; Yu et al. 2000b), it may be difficult to discern direct effects on hPTTG and those secondary to stimulation of cellular proliferation. MTT assays in TPC-1 cells revealed no change in proliferation following addition of growth factors, implying direct effects on hPTTG regulation, consistent with the observation that hPTTG over-expression did not correlate with PCNA expression in human thyroid tumours (Boelaert et al. 2003a).

The effects of EGFR ligands and IGF-1 were abrogated following additional treatments with specific MAPK (PD98059) or PI3K (LY294002 and Wortmannin) inhibitors respectively, consistent with observations in non-thyroid cells (Chamaon et al. 2005; Tfelt-Hansen et al. 2004). Analysis of phospho-P44/42 ERK1/2 and phospho-AKT expression confirmed rapid activation of the MAPK and PI3K cascades respectively, by both EGFR ligands and IGF-1. Notably, these pathways were also activated in SW1736 cells suggesting high levels of endogenous hPTTG in this cell type is driven by alternative pathways. Our

findings add to increasing evidence of significant levels of crosstalk between these signalling pathways (Castellano and Downward 2011; Vlotides et al. 2006), and therapeutic targeting of both pathways has been proposed in the management of thyroid cancer (Miller et al. 2009).

3.4.3 Mechanisms of hPTTG regulation by growth factors

Subsequent investigations were performed in TPC-1 cells since these were the most responsive to growth factors and highly amenable to siRNA transfection. Given the established role of SP1 as a regulator of hPTTG transcription (Chintharlapalli et al. 2011; Clem et al. 2003) and its reported activation by MAPK and PI3K (Milanini-Mongiat et al. 2002; Pore et al. 2004), we investigated whether SP1 mediates hPTTG induction by growth factors. Observation of reduced hPTTG in vehicle treated cells following SP1 depletion confirmed its role in *hPTTG* transcription, consistent with siRNA studies in hepatocellular carcinoma cells (Chen et al. 2008). Following SP1 knockdown, hPTTG was nonetheless inducible by growth factors suggesting the effect is independent of SP1, concurrent with studies of hPTTG regulation by EGF in pituitary folliculostellate TtT/GF cells (Vlotides et al. 2006).

CDC2 is the sole-confirmed phosphorylator of hPTTG (Ramos-Morales et al. 2000) and we assessed its importance in growth factor mediated phosphorylation of hPTTG. Unexpectedly, CDC2 depletion caused a striking upregulation of unphosphorylated and phosphorylated hPTTG protein in vehicle-only treated cells. This finding is in contrast to a study demonstrating direct phosphorylation at Ser165 of hPTTG by CDC2 during mitosis in HeLa cells (Ramos-Morales et al. 2000). Induction of hPTTG was further enhanced, albeit non-significantly, upon addition of EGF, and so it is conceivable that growth factor effects on hPTTG expression and phosphorylation are independent of CDC2 and that alternative mechanisms of hPTTG phosphorylation exist. MAPK directly phosphorylates Ser162 on rat Pttg, which is the analogous site to Ser165 in hPTTG (Pei 2000). Moreover, a direct

interaction between hPTTG and both MAPK and PI3K was observed in malignant and non-neoplastic astrocytes, as well as human testis (Chamaon et al. 2005). It is tempting to speculate that activation of these kinases by growth factors leads to the direct phosphorylation of hPTTG, though this requires further investigation.

3.4.4 Regulation of hPTTG by CDC2

Induction of hPTTG protein expression and phosphorylation following CDC2 depletion was confirmed in TPC-1 cells and human primary thyrocytes. This effect was abrogated by treatment with the translation inhibitor, anisomycin, suggesting the effect is not post-translational. Subsequently, TaqMan RT-PCR studies revealed upregulation of *hPTTG* mRNA expression following knockdown of CDC2, suggesting a transcriptionally driven effect. Thus we have identified CDC2 as a novel regulator of hPTTG expression. Proliferation of TPC-1 cells was significantly reduced following CDC2 depletion, potentially as a result of TPC-1 induced hPTTG expression with high levels of hPTTG resulting in mitotic inhibition through its role as a securin (Boelaert et al. 2003b).

3.4.5 Conclusion

This study confirmed that EGF, TGF-α and IGF-1 activate MAPK and PI3K pathways via the EGFR and IGF1R respectively to induce hPTTG expression and phosphorylation in thyroid cells. Enhanced expression of growth factors and their receptors in thyroid cancer could provide an explanation for hPTTG over-expression in thyroid malignancies. SP1 and CDC2 are not involved in regulating growth factor mediated hPTTG expression and phosphorylation, though CDC2 was identified as an independent regulator of hPTTG expression. Further investigations of the exact mechanisms by which growth factors regulate hPTTG expression and phosphorylation may elucidate novel potential targets to reduce hPTTG expression in thyroid cancer.

4 CHAPTER FOUR

Regulation of Growth Factor Expression by hPTTG

4.1 Introduction

As demonstrated and discussed in Chapter 3, hPTTG expression and phosphorylation is induced by growth factors. In addition, several studies have demonstrated that hPTTG in turn transactivates growth factors.

hPTTG induction of the angiogenic growth factors, FGF-2 and VEGF, has been described in various studies. Initially, an increase in FGF-2 expression was observed following hPTTG over-expression in NIH3T3 fibroblasts. Further, increased FGF-2 secretion was detected in the conditioned media of cells, and both of these effects were dependent on the SH3-interacting domain of hPTTG (Zhang et al. 1999b). A subsequent study made similar observations through investigating the effect of hPTTG over-expression in angiogenesis. Increased proliferation, migration and tube formation were observed in human umbilical vein endothelial cells (HUVECs), following treatment with the conditioned media from hPTTG-transfected NIH3T3 cells. The pro-angiogenic effects of hPTTG over-expression were significantly reduced by mutation of the SH3-interacting domain or by addition of a neutralising FGF-2 antibody (Ishikawa et al. 2001). Luciferase reporter assays performed in COS-7 cells indicated that hPTTG regulation of FGF-2 expression is as a result of direct effects on the *FGF-2* promoter (Chien and Pei 2000). Two studies from our own group have provided strong evidence for FGF-2 upregulation by hPTTG in thyroid cancer. In the first instance, over-expression of both hPTTG and FGF-2 was demonstrated in differentiated thyroid cancer, where there was a strong positive correlation of the expression of *hPTTG* and *FGF-2* transcripts. Importantly, this study identified hPTTG and FGF-2 as potential prognostic indicators of tumour recurrence and metastasis, respectively (Boelaert et al. 2003a). Subsequently, hPTTG induction of FGF-2 in thyroid cells was demonstrated more directly following over-expression of hPTTG in primary human thyrocytes, where the

SH3-interacting domain of hPTTG was once again critical to regulation of FGF-2 (Boelaert et al. 2004).

A further study from our laboratory indicated there was increased expression of VEGF and its receptor, KDR, in pituitary tumours with high hPTTG expression. In addition, hPTTG over-expression induced both FGF-2 and VEGF expression in NT-2, MCF-7 and JEG-3 cells via its SH3-interacting domain. hPTTG regulation of VEGF was shown to be independent of FGF-2 induction through depletion of secreted FGF-2 in the cell media (McCabe et al. 2002).

Our group used an angiogenesis-specific cDNA PCR array to investigate the regulation of other angiogenic genes by hPTTG in thyroid cells. Notably, cells over-expressing hPTTG induced the pro-angiogenic genes *VEGF*, *ID3* and *IGF-1*, and downregulated the anti-angiogenic gene *thrombospondin-1* (*TSP-1*), suggesting hPTTG may be a critical regulator of multiple genes that are important in angiogenesis and tumour progression in thyroid cancer (Kim et al. 2006b). The SH3-interacting domain was necessary for regulation of *ID3*, which was later shown to be mediated by the stimulation of VEGF and KDR (Kim et al. 2006a).

A recent study revealed positive correlations between expression of *hPTTG*, *FGF-2* and *IGF-1* transcripts in pituitary adenomas, providing further evidence of hPTTG's interaction with these growth factors in tumours (Chamaon et al. 2010). It is possible that other growth factors are also regulated by hPTTG and thyroids from mutant $TR\beta^{PV/PV}$ mice which spontaneously develop follicular thyroid cancers demonstrated elevated *Pttg* and *TGF-α* expression at the time of metastatic spread (Ying et al. 2003).

The growing body of research that describes a correlation between increased hPTTG and growth factor expression in a variety of tumours strongly suggests that their relationship is important in angiogenesis and disease progression. We have demonstrated that hPTTG is regulated by growth factors in thyroid cells (Chapter 3) and in turn, hPTTG has been shown

in previous studies to induce expression of some growth factors in both thyroid (Boelaert et al. 2003a; Kim et al. 2006a; Kim et al. 2006b) and non-thyroid cell types (Zhang et al. 1999b; Heaney et al. 1999; Chien and Pei 2000; Ishikawa et al. 2001; McCabe et al. 2002). In addition, human thyroid follicular and endothelial cells express growth factors as well as their receptors, mediating cross talk between these cell types and thus facilitating mitogenesis and angiogenesis (Fagin 2005b; Patel et al. 2003). Based on these observations we performed studies to test the hypothesis that hPTTG and growth factors may regulate each other through auto/paracrine feedback mechanisms that are aberrantly controlled in thyroid cancer.

Following on from studies in Chapter 3, which confirmed that hPTTG expression is induced by EGF, TGF-α and IGF-1, the aim of this study was to investigate whether hPTTG itself transactivates these growth factors. Thyroid cells were transiently transfected with hPTTG and growth factor expression was determined through quantitative TaqMan RT-PCR. Thyroid cells were treated with conditioned media taken from vector only control or WT/mutant hPTTG-transfected cells in order to investigate autocrine and paracrine induction of hPTTG and to infer a mechanism of growth factor regulation. Specific neutralising antibodies and growth factor receptor inhibitors were used to determine the significance of each signalling pathway implicated in the auto-regulation of hPTTG and growth factors in thyroid cells.

4.2 Materials and methods

4.2.1 Cell culture and transfections

Human thyroid papillary carcinoma TPC-1 and K1 cell lines were routinely cultured as described in section 2.1. For transfection experiments, TPC-1 and K1 cells were seeded in 12-well plates at a density of 4.5×10^4 and 6.5×10^4 cells, respectively. Cells were left 24 hours before transfection.

Collection of thyroid samples was with approval of the Local Research Ethics committee and samples were cultured as described in section 2.2. All serum, insulin and TSH was removed from human primary thyrocytes for at least 24 hours before transfection.

For hPTTG over-expression studies, we utilised a collection of constructs consisting of the pCI-neo vector housing either *wild-type hPTTG*, hPTTG with a Ser165 mutation (termed *Phos*-), or hPTTG with PXXP motif mutations (termed *SH3*-), all as previously described (Boelaert et al. 2004). Cells were transfected with 1 µg DNA/well using FuGENE-6 Transfection Reagent (Roche, Indianapolis, IN, USA), with an optimised ratio of 6 µl per 1 µg plasmid DNA. Control transfections utilised equal amounts of blank plasmid (vector-only, VO, control). Transfection efficiency of human primary thyrocytes was predicted by Western blotting, parallel transfections of β-galactosidase (β-gal staining) or GFP (fluorescence microscopy). Cells were harvested 48 hours post-transfection in protein lysis buffer or 250 µl Tri Reagent. Growth factor expression was determined in experiments where a minimum of 30 % transfection efficiency had been achieved.

4.2.2 Conditioned media treatments

Conditioned media from transfected human primary thyrocytes were collected and centrifuged at 600 g for 10 minutes at room temperature to remove particulates. Cell culture supernatant was then used to treat primary thyrocytes derived from the same specimen, with or without the addition of neutralising antibodies against EGF (20 µg/ml), IGF-1 (20 µg/ml) [R&D SYSTEMS, UK] and TGF-α (1 : 40) [Biovision Inc., California, USA], or gefitinib (Iressa, 100 µM) and picropodophyllin (PPP, 20 µM) [Tocris Bisoscience, Missouri, USA]. Gefitinib and picropodophyllin are highly specific inhibitors of phosphorylation of the tyrosine kinase domains of EGFR and IGFR respectively. Inhibitors were added to cells 30 minutes prior to treatment with conditioned media in order to block growth factor receptor activity. Further inhibitor was added at the same time as conditioned media in order to

sustain inhibition of growth factor receptor activity. Cells were harvested 24 hours later in protein lysis buffer.

4.2.3 RNA extraction, reverse transcription, QT-PCR

Total RNA was extracted from TPC-1, K1 cells and from human primary thyrocytes as described in section 2.4. Reverse transcription and QT-PCR techniques were as described above (see sections 2.4 and 2.5). hPTTG mRNA was detected using the primers and probe described above (see Table 3-1). Gene-specific expression assays for *EGF* (Hs01099999_m1), *TGF-α* (Hs_00608187_m1) and *IGF-1* (Hs01547656_m1) were purchased from Applied Biosystems (Warrington, UK).

4.2.4 Western blot analysis

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see section 2.6). Blocked membranes were subsequently incubated with primary antibodies against hPTTG (2 µg/ml) [Invitrogen, UK]. After washing in TBS-T, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidise (Dakocytomation, UK) for 1 hour at room temperature before being visualised by techniques described in section 2.6.

4.2.5 Enzyme-linked immunosorbent assays (ELISAs)

Conditioned medium from primary thyrocytes was collected 48 hours following transfection with *VO* and *WT-hPTTG* plasmids. The medium was then centrifuged at 12000 g for 15 minutes at 4 ºC to remove particulates. EGF and IGF-1 concentration were assayed in 200 µl and 50 µl of the supernatant respectively, using the Quantikine HS human EGF and human IGF-1 enzyme-linked immunosorbent assays (R&D Systems, Inc.) as per the manufacturer's instructions. Following the preparation of reagents and standards, cell culture or standard (200 µl EGF; 50 µl IGF-1) was added to assay diluent (50 µl EGF; 150 µl IGF-1)

in each well of a 96 well plate. After 3 hours incubation at room temperature (18-23 °C), plates were washed and 200 µl of either EGF or IGF-1 basic conjugate was added to each well. After a further 2 hour incubation period at room temperature, plates were washed and 200 µl of substrate solution was added to each well. Following a further 30 minute incubation period, the reaction was terminated by the addition of 50 µl stop solution to the wells. The optical density of each well was determined within 30 minutes using a microplate reader set to 450 nm. Readings were also taken at 570 nm wavelength and these were subtracted from the readings at 450 nm to correct for optical imperfections in the plate.

4.2.6 Statistical analysis

Data were analysed as described in section 2.8.

4.3 Results

4.3.1 Effects of hPTTG expression on growth factor expression in thyroid cells

hPTTG has been shown to induce expression of proangiogenic growth factors including VEGF and FGF-2 (Kim et al. 2006a; Boelaert et al. 2004). We set out to investigate whether hPTTG is capable of upregulating expression of mitogenic growth factors implicated in thyroid tumour progression, including EGF, TGF-α and IGF-1 (Hoelting et al. 1994; Holting et al. 1995; Lam et al. 2011; Maiorano et al. 2000). We explored this through transient transfection studies in thyroid cells where either vector only controls or wild-type hPTTG were over-expressed in thyroid cells.

4.3.1.1 Growth factor expression following hPTTG over-expression in transformed thyroid cell lines

In the first instance, we performed transient transfection experiments in TPC-1 and K1 papillary thyroid carcinoma cells since they were most consistently amenable to transfection. Successful hPTTG over-expression was confirmed by Western blot analysis.

Forty eight hours post-transfection, TaqMan RT-PCR revealed that there was no significant induction of *EGF* or *TGF-α* mRNA expression by hPTTG in either TPC-1 or K1 cells. *IGF-1* mRNA expression was completely undetectable in both cell lines (see Figure 4-1).

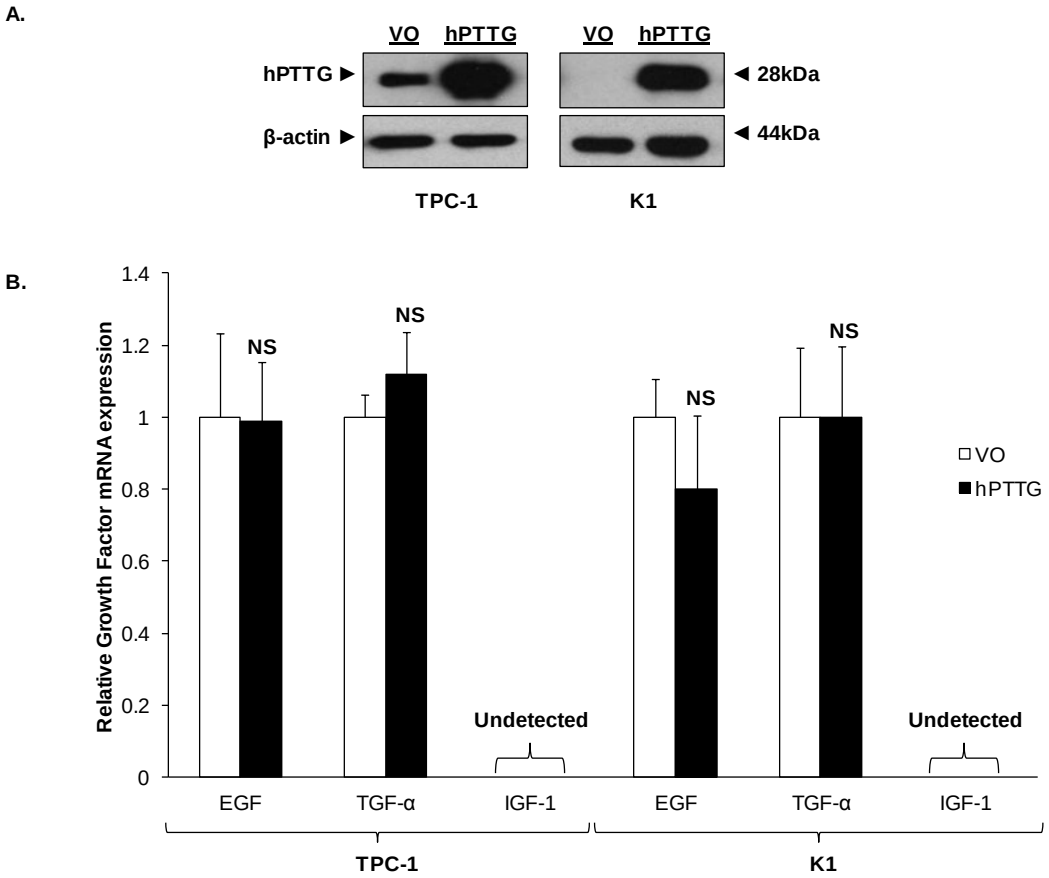


Figure 4-1: hPTTG does not induce growth factor expression in TPC-1 and K1 transformed cell lines. ***A*** *Representative Western blot anlalysis demonstrating successful transfection of TPC-1 and K1 cells with wild-type hPTTG.* ***B*** *TaqMan RT-PCR data showing relative mRNA expression levels of EGF, TGF-α and IGF-1 following transient hPTTG over-expression compared to vector only transfection controls. N = 3 experiments. Error bars represent the SEM (NS = non-significant).*

4.3.1.2 hPTTG over-expression induces growth factor expression in human primary thyrocytes

Given the above findings, we performed further transient transfection experiments in human primary thyrocytes in which endogenous hPTTG expression is low (see Figure 3-1) and which express all three growth factors being investigated. Transfection efficiency was determined by Western blot analysis or by detection of beta-galactosidase and GFP control transfections. In cell preparations where transfection efficiency was demonstrated to be >30

%, growth factor expression was analysed. At 48 hours post-transfection TaqMan RT-PCR revealed that hPTTG over-expression induced *EGF* (1.7-fold, $n = 6$, $p < 0.001$), *TGF-α* (1.5-fold, n = 5, p < 0.001) and *IGF-1* (1.6-fold, $n = 7$, $p < 0.001$) mRNA expression compared to vector only transfected cells (see Figure 4-2).

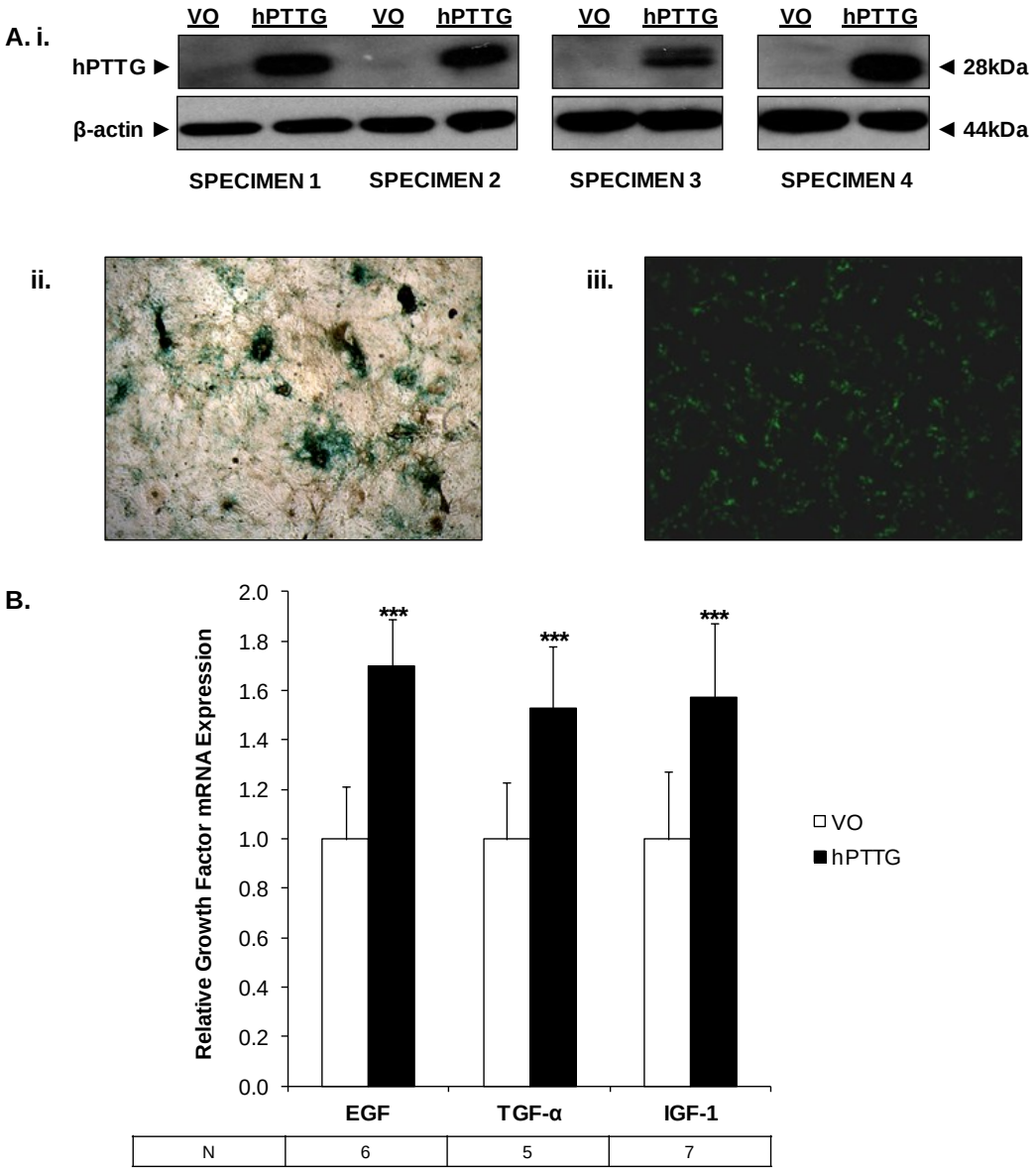


Figure 4-2: Over-expression of hPTTG in human primary thyrocytes induces growth factor mRNA expression. ***A*** *Representative Western blot analyses* ***(i)*** *demonstrating successful transient over-expression of hPTTG in human primary thyrocytes derived from different specimens. β-galactosidase staining* ***(ii)*** *and GFP fluorescence microscopy* ***(iii)*** *demonstrating 30-50 % transfection efficiency of human primary thyrocytes.* ***B*** *TaqMan RT-PCR data showing relative mRNA expression levels of EGF, TGF-α and IGF-1 following transient hPTTG over-expression compared to vector only transfection controls. N indicates number of repeat experiments. Error bars represent the SEM (*** $p < 0.001$).*

4.3.2 hPTTG and growth factors are involved in paracrine and autocrine feedback mechanisms

To investigate whether the increase in growth factor mRNA expression results in enhanced translation and secretion of these growth factors and subsequent paracrine upregulation of hPTTG by these growth factors, we performed treatments of human primary thyrocytes with the conditioned media of transfected cells from the same thyroid specimen. We also transfected hPTTG mutants to infer the mechanism involved in growth factor regulation by hPTTG. Western blot analysis demonstrated that cells treated with the conditioned media from *wild-type hPTTG* transfectants showed a 7-fold induction of endogenous hPTTG ($n = 4$, $p < 0.001$) compared to vector only controls ($n = 4$). This effect was dependent upon the SH3-interacting domain of hPTTG, since cells treated with conditioned media following transfection with *SH3- hPTTG* showed a non-significant upregulation of endogenous hPTTG (1.9-fold, $n = 4$, p = NS). However, transfection with *Phos- hPTTG* resulted in a 7.3-fold induction of hPTTG protein ($n = 4$, $p < 0.001$), similar to effects following *wild-type hPTTG* transfection, indicating that these effects are independent of hPTTG phosphorylation (see Figure 4-3).

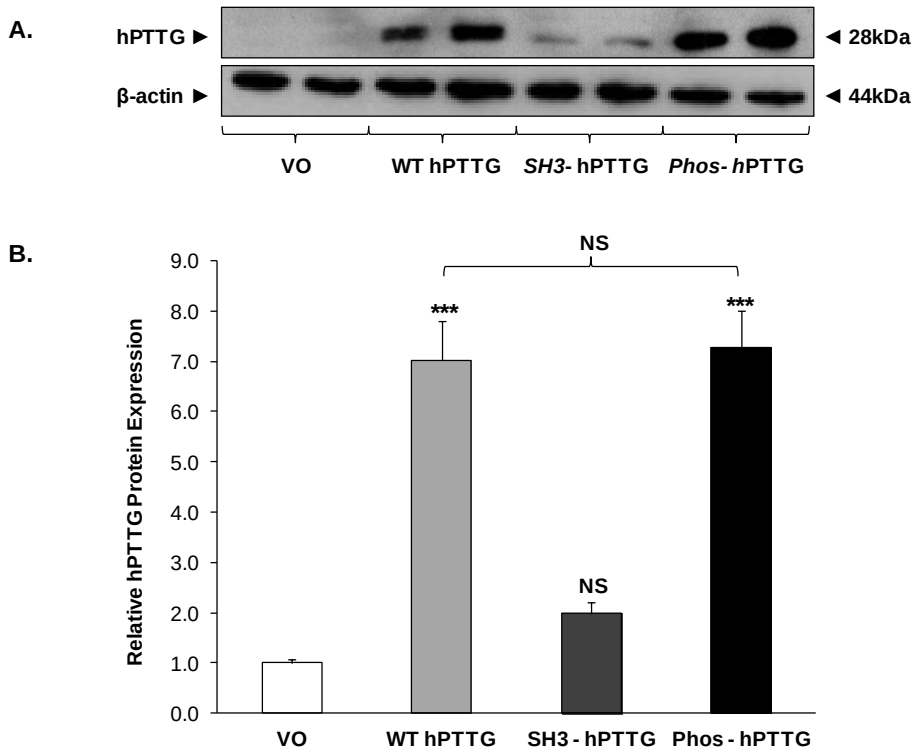
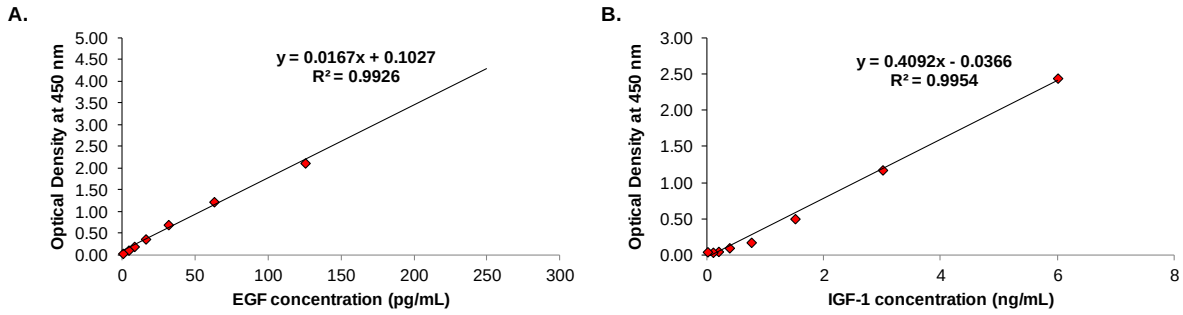


Figure 4-3: Over-expression of hPTTG in vitro induces further autocrine and paracrine upregulation of hPTTG. ***A*** *Representative Western blot analysis and scanning densitometry data (****B****) demonstrating hPTTG expression following treatment of human primary thyrocytes with conditioned media from wild-type or mutant hPTTG-transfected cells from the same specimen. Results are based on n = 4 repeats of the described experiment (*** $p < 0.001$, NS = non-significant).*

4.3.3 Detection of growth factor secretion by ELISA

In order to identify the specific growth factors involved in autoregulation of hPTTG expression, and to determine whether increased transcription of growth factors (see section 4.3.1.2) results in increased translation and secretion, we used ELISAs to analyse EGF and IGF-1 concentrations in cell culture supernatants from human primary thyrocytes following transient transfection with *hPTTG*. Although technical success of the assays was demonstrated, both EGF and IGF-1 were undetectable, suggesting that the levels of EGF and IGF-1 in the cell culture medium of human primary thyrocytes are beyond the limits of detection of these assays (see Figure 4-4).



Sample	Abs @ 450nm	Abs @ 570nm	Corrected Abs [450-570]	EGF concentration (pg/mL) [(Corrected Abs – c)/ x-coefficient]
Specimen 1 VO A	0.111	0.030	0.081	-1.30
Specimen 1 VO B	0.104	0.028	0.076	-1.60
Specimen 1 hPTTG A	0.116	0.031	0.084	-1.10
Specimen 1 hPTTG B	0.114	0.030	0.084	-1.11
Specimen 2 VO A	0.111	0.031	0.080	-1.35
Specimen 2 VO B	0.112	0.032	0.080	-1.36
Specimen 2 hPTTG A	0.122	0.030	0.090	-0.73
Specimen 2 hPTTG B	0.120	0.034	0.085	-1.04

Sample	Abs @ 450nm	Abs @ 570nm	Corrected Abs [450-570]	IGF-1 concentration (ng/mL) [(Corrected Abs – c)/ x-coefficient]
Specimen 1 VO A	0.054	0.026	0.028	-4.49
Specimen 1 VO B	0.056	0.030	0.027	-4.52
Specimen 1 hPTTG A	0.057	0.028	0.028	-4.48
Specimen 1 hPTTG B	0.054	0.028	0.025	-4.63
Specimen 2 VO A	0.058	0.026	0.027	-4.56
Specimen 2 VO B	0.053	0.029	0.029	-4.41
Specimen 2 hPTTG A	0.066	0.034	0.031	-4.26
Specimen 2 hPTTG B	0.057	0.030	0.028	-4.49

*Figure 4-4: EGF and IGF-1 levels in human primary thyrocyte cell culture supernatants were undetectable by ELISA. **A-B** Concentration standard curves and exemplary calculations of growth factor concentrations for EGF (**A**) and IGF-1 (**B**).*

4.3.4 Inhibition of hPTTG auto-regulation

Having failed to confirm increased growth factor secretion by ELISA, we performed further investigations into hPTTG auto-regulation to establish the importance of specific growth factors. Treatments of human primary thyrocytes with conditioned media were repeated (see section 4.3.2) and neutralising antibodies against growth factors or specific inhibitors of growth factor receptor tyrosine kinase activity were introduced. Western blot analysis revealed that addition of neutralising antibodies, against either TGF-α or EGF, to conditioned media treatments abolished the upregulation of hPTTG observed in untreated controls. Addition of an antibody that neutralises IGF-1 only partially abrogated the hPTTG response. Gefitinib and picropodophyllin (PPP) are currently used therapeutically as highly

specific inhibitors of phosphorylation of the tyrosine kinase domains of EGFR and IGF1R respectively. Western blot analysis demonstrated that additional treatment of cells with 100 μM gefitinib completely prevented upregulation of hPTTG following treatment with conditioned media. Additional treatments with 20 μM PPP only partially abrogated the hPTTG response. Thus, autocrine upregulation of hPTTG expression could be attenuated by preventing EGF, TGF-α and IGF-1 signalling via their respective receptors (see Figure 4-5).

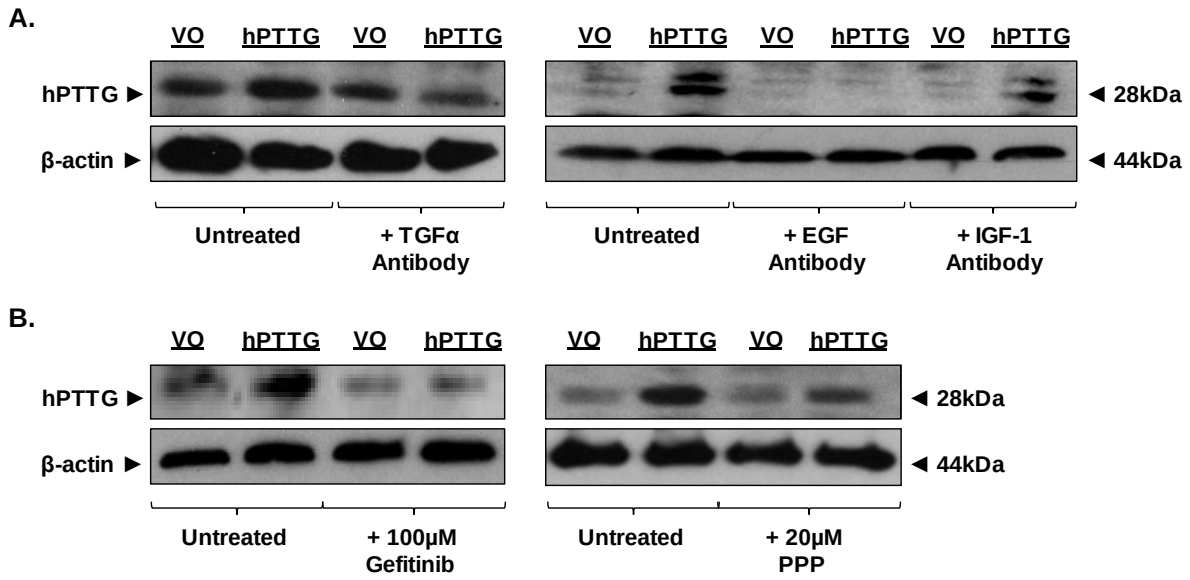


Figure 4-5: Intervention of hPTTG auto-regulation with neutralising antibodies against growth factors and selective inhibitors of growth factor receptor tyrosine kinase activity. ***A+B*** *Representative Western blot analyses showing effects on hPTTG induction following treatment with conditioned media, by addition of neutralising antibodies against EGF, TGF-α, IGF-1(**A**), the EGFR inhibitor gefitinib (100 μM) or the IGFR inhibitor picropodophyllin (20 μM) (**B**). Western blots are representative of n ≥ 2 separate experiments.*

4.3.5 Positive correlation between hPTTG and growth factor expression in human thyroid specimens

To further characterise the relationship between hPTTG and growth factors in the thyroid, we investigated the correlation between expression of hPTTG and growth factor transcripts in 34 human thyroid specimens from either normal thyroid tissue or multinodular goitres. TaqMan RT-PCR revealed a positive correlation between levels of mRNA encoding

hPTTG and that of both *EGF* (R_s = 0.2177, $p < 0.01$) and *IGF-1* (R_s = 0.2104, $p < 0.01$). There was a weak correlation between *hPTTG* and *TGF-α* mRNA expression (R_s = 0.0793, p = 0.101) that was not statistically significant (see Figure 4-6).

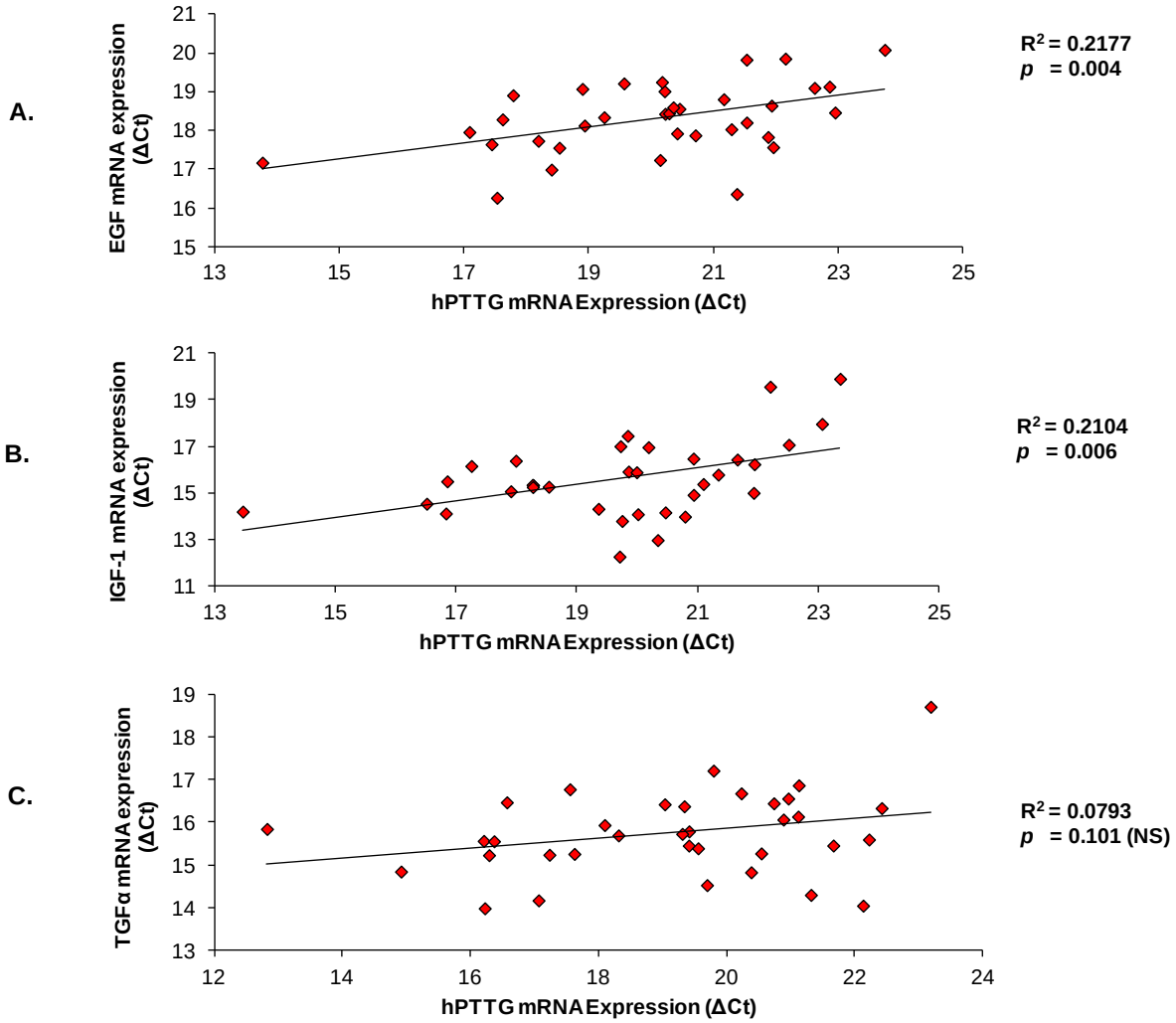


*Figure 4-6: Correlation between mRNA expression for hPTTG and growth factors in normal thyroids and multinodular goitres. Significant associations are illustrated between hPTTG and EGF (**A**) and IGF-1 (**B**), but not with TGF-α (**C**). Data are given as ΔCT values and the R^2 and p values are also displayed. N = 34 normal or multinodular goitre thyroid specimens.*

4.4 Discussion

Increased hPTTG expression causes transactivation of growth factors such as VEGF and FGF-2 that contribute to sustained tumour growth and angiogenesis (Ishikawa et al. 2001; Boelaert et al. 2004; McCabe et al. 2002; Kim et al. 2006a). Having established that

hPTTG is regulated by EGF, TGF-α and IGF-1 in thyroid cells, we investigated whether hPTTG in turn regulates these growth factors. To this effect, we demonstrated that hPTTG regulated expression and secretion of EGF, TGF-α and IGF-1 as part of autocrine feedback mechanisms *in vitro*, which led to further upregulation of hPTTG. Treatments with specific neutralising antibodies or tyrosine kinase inhibitors resulted in abrogation of autocrine hPTTG induction. Thus, intervention of these autocrine pathways represents a potential therapeutic strategy to reduce hPTTG over-expression in thyroid cancer.

4.4.1 Induction of growth factors by hPTTG in thyroid cells

In the first instance, studies were performed in TPC-1 and K1 papillary thyroid carcinoma cells due to our ability to consistently over-express hPTTG in these cell types. However, TaqMan RT-PCR revealed no significant alterations in *EGF* and *TGFα* mRNA expression following hPTTG over-expression. *IGF-1* mRNA was not expressed in either cell type, consistent with studies demonstrating lack of *IGF-1* expression in thyroid cancer cell lines (Belfiore et al. 1999). We considered the possibility that pathways driving expression of EGF and TGFα may already be strongly activated in these cells where endogenous hPTTG expression is already very high (see section 3.3.1). To test this, we performed further transient transfection studies in human primary thyrocytes, which have low levels of endogenous hPTTG (see section 3.3.1) and which express all three growth factors being investigated. Achieving a consistent level of transfection was challenging, but we were able to verify or refute successful transfection through Western blot analysis, β-gal staining and GFP microscopy. All cells examined had 30-50 % transfection efficiency and TaqMan RT-PCR analysis revealed that *EGF*, *TGF-α* and *IGF-1* mRNA expression was induced following hPTTG over-expression. EGFR (Landriscina et al. 2011; Lee et al. 2007; Lam et al. 2011) and IGF1R (Belfiore et al. 1999; Maiorano et al. 2000) upregulation in thyroid cancer is associated with disease progression. Moreover, the thyroid is an EGF-rich

environment (Kajikawa et al. 1991) and TGF-α (Lam et al. 2011) and IGF-1 (Maiorano et al. 2000) are over-expressed in thyroid tumours. Together these findings suggest that enhanced EGFR and IGF1R signalling appears to be critical in thyroid cancer progression. Our findings suggest that over-expression of growth factors in thyroid cancer may be partly mediated by hPTTG over-expression and that pathways of growth factor regulation by hPTTG could provide novel therapeutic targets to reduce growth factor expression and prevent disease progression.

4.4.2 Secretion of growth factors and autocrine regulation of hPTTG

Given the induction of hPTTG by growth factors (see section 3.3.3) and having determined upregulation of *EGF*, *TGF-α* and *IGF-1* mRNAs by hPTTG in thyroid cells, we sought to establish whether growth factor secretion is enhanced and leads to autocrine upregulation of hPTTG. Treatments with the conditioned media of transfected cells induced hPTTG protein expression in cell cultures derived from the same specimen, suggesting increased secretion of growth factors. Transfections with *hPTTG* mutants revealed that this effect was dependent on the SH3-interacting domain but independent of hPTTG phosphorylation, concurrent with various studies demonstrating transactivational capabilities of hPTTG owing to its SH3-interacting domain. Induction of FGF-2 by hPTTG is well established and this effect was dependent on an intact SH3-interacting domain in NIH3T3 mouse fibroblasts (Zhang et al. 1999b) and human primary thyrocytes (Boelaert et al. 2004). Furthermore, FGF-2 mediated angiogenesis following hPTTG over-expression in HUVEC cells was diminished following introduction of a point mutation in the region coding for the SH3-interacting domain (Ishikawa et al. 2001). Similarly, hPTTG induction of VEGF in NT2, MCF-7 and JEG-3 cells (McCabe et al. 2002) and of ID3 in human primary thyrocytes (Kim et al. 2006b) was mediated via the SH3-interacting domain. These findings, together

with the observations of the current study, emphasise the importance of hPTTG's C-terminal SH3-interacting domain in the transactivation of growth factors involved in tumour growth and vascularisation.

We used ELISAs to try and confirm the specific growth factors involved and their relative contribution to autocrine regulation of hPTTG. However, failure to detect EGF or IGF-1 in this approach suggested that concentration levels were beyond the limits of detection of the assays used. Alternatively, there could have been a technical issue with growth factor degradation as a result of long term storage of samples.

As a result, an alternative strategy was adopted that allowed us to confirm the specific pathways involved in hPTTG autoregulation and provide insight into possible therapeutic approaches. In repeats of conditioned media treatments, additional treatments with neutralising antibodies against EGFR ligands or the specific EGFR tyrosine kinase inhibitor gefitinib prevented autocrine regulation of hPTTG, strongly supporting a relationship between hPTTG, EGFR ligands and EGFR in thyroid cells. Addition of an IGF-1 neutralising antibody or the specific IGF1R inhibitor PPP only partially abrogated the effect, suggesting this pathway may be less important. Alternatively, hPTTG may be involved in interactions with IGF-2, which also binds IGF1R and is implicated in neoplasia (Riedemann and Macaulay 2006; Pollak et al. 2004). In addition, other receptor types may be involved since IGF signalling is complicated by the ability of the IGF1R to dimerise with the insulin receptor [IR] (Riedemann and Macaulay 2006). Some IGF1R inhibitors including PPP only inactivate IGF1R and not IR (Vasilcanu et al. 2008) and thus IGF1R inhibition may not completely prevent IGF-1/IGF-2 activity. Indeed, IGF1R/IR dimers are over-expressed in thyroid cancer (Belfiore et al. 1999) and this may be important in terms of specificity and toxicity when employing treatments targeting the IGF system.

This section of work provokes discussion regarding the benefits of targeting EGFR and IGF1R in thyroid cancer. Gefitinib (Iressa, AstraZeneca) selectively binds the ATP site of the EGFR tyrosine kinase enzyme and has been shown to slow or block growth of thyroid cancer cell lines (Schiff et al. 2004; Nobuhara et al. 2005). It is currently used in the treatment of non-small cell lung cancer where the clinical response correlates with mutational activation of EGFR (Brown et al. 2010). An early phase II clinical trial using gefitinib to treat 27 patients with advanced differentiated thyroid cancer reported mild tumour volume reduction and prolonged stable disease in some patients, although this did not meet 'partial response criteria' (Pennell et al. 2008). Erlotinib (Tarceva, OSI/Genentech) and Lapatanib (Tykerb, GlaxoSmithKline) are alternative EGFR tyrosine kinase inhibitors that are FDA approved for lung/pancreatic and breast cancer respectively, while cetuximab (Erbitux, ImClone/Bristol-Myers Squib) and panitumumab (Vectibix, Amgen) are FDA approved monoclonal antibodies that block the EGFR ligand binding site.

PPP is a cyclolignan that inhibits tyrosine phosphorylation of Y1136 in the activation loop of IGF1R (Girnita et al. 2004; Vasilcanu et al. 2004), and treatment with this agent has been shown to induce significant tumour regression and to inhibit metastasis in various cell systems and xenografted mice (Girnita et al. 2006; Vasilcanu et al. 2006; Stromberg et al. 2006). In order to assess its suitability as a drug for targeting IGF1R, ongoing research continues to investigate mechanisms of tumour cell resistance to PPP following long-term exposure (Hashemi et al. 2011) and PPP remains a highly promising drug for future cancer treatments. Many other drugs targeting IGF1R are in phase I clinical trials (Lopez-Calderero et al. 2010), including monoclonal antibodies that bind IGF1R and induce internalisation of the receptor by endocytosis. αIR3 was the first monoclonal antibody targeting IGF1R to demonstrate anti-cancer effects *in vivo* (Arteaga et al. 1989), and more recently developed antibodies such as CP-751,871 [figitumumab, Pfizer] (Cohen et al. 2005) and IMC-A12

[cixutumumab, Imclone] (Wu et al. 2005) are showing promise in trials in a wide range of malignancies including multiple myeloma, Ewing's sarcoma, neuroendocrine tumours, colorectal, prostate, breast, pancreatic, liver, and head and neck cancers (Lopez-Calderero et al. 2010). Moreover, trials of newly developed tyrosine kinase inhibitors targeting IGF1R such as INSM-18 [Insmed] (Ryan et al. 2008) and OSI-906 [OSI Pharmaceuticals] (Carden et al. 2009; Lindsay et al. 2009) have demonstrated significant potential for future use in clinical practice.

Further trials in patients with thyroid cancer are required to determine the overall benefits of treatments targeting EGFR and IGF1R signalling but the studies described in this chapter demonstrate that one advantage of this approach could be the desirable intervention in the autocrine feedback mechanisms involving hPTTG and growth factors.

4.4.3 hPTTG and growth factor correlation studies in human thyroid samples

Several studies have investigated the correlation between hPTTG and growth factors in endocrine malignancies. Our group's own study demonstrated a positive correlation of *hPTTG* and *FGF-2* expression in 27 differentiated thyroid cancers compared with 11 normal thyroids, where hPTTG and FGF-2 were identified as independent markers of tumour recurrence and metastasis, respectively (Boelaert et al. 2003a). There was also strong positive correlation between *hPTTG* and *FGF-2*, as well as *IGF-1* mRNA expression in 103 pituitary adenomas (Chamaon et al. 2010). Another study in pituitary adenomas, conducted by our own group, observed positive correlation between *hPTTG* and *VEGF*, as well as the VEGF receptor, *KDR* (McCabe et al. 2002). Further, in mutant $TR\beta^{PV/PV}$ mice which spontaneously develop follicular thyroid cancers, *Pttg* and *Tgf*-α were both strongly upregulated at the metastatic stage (Ying et al. 2003). Given these findings and the observations of the current study that hPTTG induces expression of *EGF*, *TGF-α* and *IGF-1*, we performed studies to correlate mRNA expression in 37 human thyroid specimens taken

from normal thyroid tissue or multinodular goitre. Moderate positive correlations were observed between *hPTTG* and all three growth factor transcripts investigated, though the relationship between *hPTTG* and *TGF-α* was not significant. These observations further support the existence of important relationships between hPTTG and growth factors in normal and hyperplastic thyroid cells. In this setting, only a weak to moderate correlation was evident, potentially since growth factor expression is influenced by activation of multiple pathways. It would be of great interest to investigate whether this relationship is maintained or enhanced in a malignant setting, by performing further correlation studies in thyroid cancer samples.

4.4.4 Conclusion

This study confirmed that hPTTG over-expression causes upregulation of EGF, TGF-α and IGF-1 expression and secretion in human thyroid cells via its SH3-interacting domain. Moreover, treatments with conditioned media demonstrated that hPTTG induced growth factor secretion is capable of autocrine upregulation of hPTTG. The work in this and the previous chapter collectively establish the existence of autocrine pathways of interaction between hPTTG and growth factors, which may be aberrantly controlled in thyroid tumours. Specific depletion of growth factors with neutralising antibodies or treatments with growth factor receptor tyrosine kinase inhibitors revealed that EGFR signalling may be of particular importance in hPTTG autoregulation, while IGF1R signalling may also be significant. Treatments with gefitinib and PPP, already used in some clinical settings, may be used to disrupt autocrine growth factor signalling at the site of the EGFR and IGF1R respectively, thereby reducing hPTTG expression. Further investigations of the exact mechanisms by which hPTTG regulates growth factors and vice versa, may elucidate novel potential targets to reduce hPTTG and growth factor expression in thyroid cancer.

5 CHAPTER FIVE

Generation of a Murine Model with Thyroid Targeted hPTTG Expression

5.1 Introduction

Following the discovery of rat Pttg (Pei and Melmed 1997) and the subsequent cloning of *hPTTG* in several contemporaneous studies (Zhang et al. 1999b; Dominguez et al. 1998; Kakar and Jennes 1999), it was demonstrated that both rat and *hPTTG* are potent transforming genes *in vitro* and *in vivo*. Stable transfection of *hPTTG* in NIH3T3 mouse fibroblast cells caused an increase in cellular proliferation, augmented anchorage-independent growth in soft agar and induced tumour formation after subcutaneous injection into athymic nude mice (Pei and Melmed 1997; Zhang et al. 1999b; Kakar and Jennes 1999).

Subsequently, hPTTG was found to be over-expressed in various human tumours including those of the pituitary (Zhang et al. 1999a; McCabe et al. 2003), breast (Puri et al. 2001), colon (Heaney et al. 2000), ovary (Puri et al. 2001), oesophagus (Shibata et al. 2002), lung (Kakar and Jennes 1999) and liver (Jung et al. 2006). Other studies reported hPTTG over-expression in haematopoietic neoplasms (Dominguez et al. 1998; Saez et al. 2002), astrocytomas (Tfelt-Hansen et al. 2004) and in metastatic gastric carcinomas (Wen et al. 2004). Furthermore, studies have elucidated critical tumour-promoting mechanisms of hPTTG, including induction of aneuploidy and genetic instability through its role as a securin (see sections 1.3.1 and 1.3.2) and stimulation of growth factors and angiogenesis (see section 1.4 and Chapter 5).

Together, these findings suggest that hPTTG over-expression may have crucial roles in both the initiation and progression of many tumour types. However, the precise function of hPTTG *in vivo*, and the oncogenic potential of hPTTG in a specific organ has not been widely tested. Further insight has been provided by two studies that investigated the effects of targeted hPTTG over-expression in the pituitary glands (Abbud et al. 2005) and ovaries (El-Naggar et al. 2007) of transgenic mice. These studies reported hPTTG-induced pituitary hyperplasia and cystic glandular hyperplasia of the endometrium respectively (see section

1.3.3 and Chapter 6). Studies by our own group and those of others have demonstrated that hPTTG is over-expressed in thyroid tumours (Heaney et al. 2001; Boelaert et al. 2003a). Furthermore, our group's own *in vitro* studies have demonstrated specific interactions between hPTTG and growth factors in thyroid cells (Boelaert et al. 2003a; Kim et al. 2006a), as well as an inhibitory effect on NIS expression and function, with implications for radioiodide therapies in thyroid disease (Boelaert et al. 2007). At this stage, it was therefore of significant interest to generate a transgenic mouse model with thyroid targeted hPTTG expression in order to perform *in vivo* investigations of the precise role of hPTTG in thyroid tumourigenesis.

A major approach for the generation of transgenic mice was first described in 1980, where exogenous DNA becomes randomly integrated into the mouse genome following microinjection into the male pronuclei of fertilised oocytes, which are subsequently implanted into pseudopregnant foster mothers (Gordon et al. 1980). Typically, thyroid-targeted transgene expression has been achieved using this technique to introduce DNA constructs with transgene expression under control of the thyroid-specific bovine thyroglobulin promoter. For example, this technique has been employed successfully to demonstrate that transgenic mice with thyroid targeted $BRAF^{V600E}$ expression develop goitre and papillary thyroid cancers (Knauf et al. 2005). More recently, out group has also successfully generated a murine transgenic model with thyroid-specific over-expression of PBF, using a DNA construct containing the bovine thyroglobulin promoter and demonstrated significant goitrogenesis and hyperplasia (Read et al. 2011). Given the success of these and numerous other studies, the same methodology was chosen to investigate the *in vivo* effects of thyroid-specific over-expression of hPTTG.

The aims of the work reported in this chapter were therefore to generate a murine transgenic model with thyroid-targeted hPTTG over-expression, to investigate the hypothesis

that increased hPTTG expression in thyroid follicular epithelial cells promotes thyroid growth and neoplasia. Moreover, we sought to perform studies to determine whether several important *in vitro* observations, including increased growth factor expression and repression of NIS in response to hPTTG over-expression, could be validated in an *in vivo* model.

5.2 Materials and methods

5.2.1 Generation of transgene construct: *TG-hPTTG-FLAG*

5.2.1.1 PCR amplification of 3'-FLAG tagged *hPTTG* cDNA

PCR primers were designed to amplify *hPTTG* cDNA with the addition of a sequence encoding a FLAG epitope (N-DYKDDDDK-C) at the 3' end, from the vector *pCI-neo-hPTTG* (Zhang et al. 1999b; Boelaert et al. 2004). The forward primer contained an EcoRI restriction enzyme site and the reverse primer contained a BamH1 restriction enzyme site following the stop codon (see Figure 5-1).

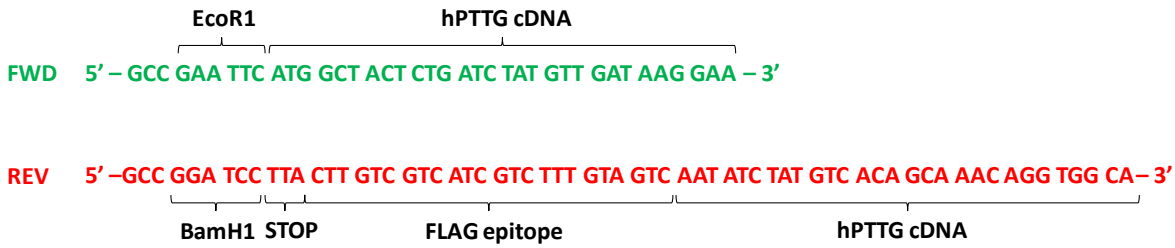


Figure 5-1: Sequence and features of the PCR primers designed to amplify and tag the hPTTG cDNA on the 3' end with the FLAG epitope.

The PCR reaction consisted of 500 ng *pCI-neo-hPTTG* plasmid DNA template, 600 nM each of forward and reverse primers (Alta Bioscience), 250 µM dNTPs, 1.5 mM $MgCl_2$ (Bioline, London, UK), 0.5x NH_2 reaction buffer (Bioline), 2.5 U Biotaq™ DNA polymerase (Bioline), which was made up to a total volume of 50 µl with nuclease-free water.

The PCR cycling conditions used were 95 ºC for 5 min for the initial denaturation of DNA template before holding at 78 ºC at which stage the DNA polymerase in NH_2 reaction

buffer was added. Subsequently, there were 40 cycles of 94 ºC for 30 sec (denaturation), 65 ºC for 30 sec (primer annealing) and 72 ºC for 1 min 30 sec (extension), followed by a final extension step of 72 ºC for 7 min. This was performed on a Mastercycler gradient (Eppendorf, Hamburg, Germany). The PCR product was subsequently electrophoresed on a 1.5 % agarose (Bioline) gel in 1x TAE (Tris-Acetate-EDTA) buffer (Eppendorf) (see Figure 5-2).

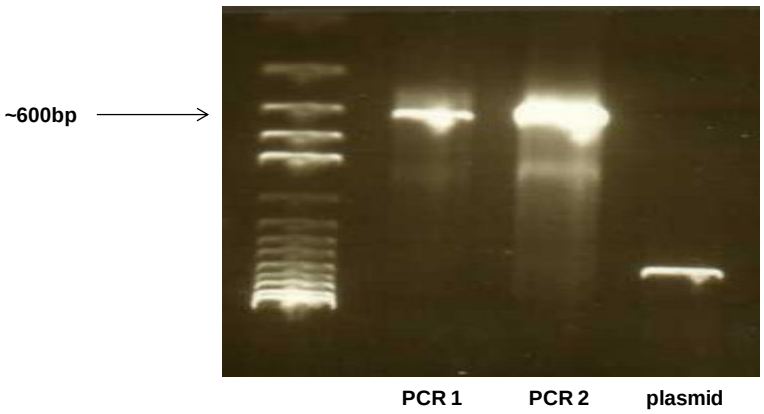


Figure 5-2: Agarose gel illustrating the hPTTG-FLAG PCR product (~600 bp). PCR1 and 2 show PCR products for two separate PCR reactions. Plasmid = undigested/unamplified pCI-neo-hPTTG plasmid. DNA Hyperladder IV was used to indicate band sizes (Bioline).

5.2.1.2 Gel extraction of PCR product

The PCR product (~600 bp in size) was excised from the gel using a scalpel and extracted from the agarose using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Briefly, the gel fragment was dissolved in 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 ml) at 50 ºC. One gel volume of isopropanol was added and the solution centrifuged through a QIAquick column. After discarding the flow-through, the column was washed with Buffer QG and then twice with Buffer PE, which contained ethanol. A final centrifugation step minus wash solution ensured maximal removal of ethanol before elution in 30 µl nuclease-free water.

5.2.1.3 Restriction digestion and ligation of *pSG5* and PCR product

The next step was to subclone the FLAG-tagged *hPTTG* gene into the *pSG5* plasmid downstream of the beta-globin intron II and a T7 promoter, and upstream of an SV40 polyadenylation (poly-A) tail, between the EcoR1 and BamH1 restriction enzyme sites (see Figure 5-4).

In a total volume of 25 µl, 1 µg *pSG5* and 14 µl gel extracted PCR product were digested using 15 U EcoRI (Promega), 15 U BamH1 (Promega), 10x acetylated BSA and 10x Buffer H (Promega) for 2 hours at 37 ºC. Digested vector and PCR product were electrophoresed on a 1.5 % agarose gel and gel extracted using the QIAquick gel extraction kit as described in section 5.2.1.2. The ligation reaction was carried out at 6 ºC overnight and consisted of digested vector and PCR product in a 1:3 Molar ratio, 10x T4 DNA ligase buffer (Promega) and 3 U T4 DNA ligase (Promega) in a total volume of 20 µl.

5.2.1.4 Transformation into DH5α cells

According to the manufacturer's instructions, 2 µl of the ligation reaction were transformed into Subcloning Efficiency™ DH5™ Competent Cells (Invitrogen). The ligation reaction was added to 50 µl bacterial cells and incubated on ice for 30 minutes. The cells were heat-shocked for 20 seconds at 42 ºC and returned to ice for a further 2 minutes. Lysogeny broth (LB) was added to a total volume of 1 ml and the cells incubated at 37 ºC for 1 hour with shaking at 200 rpm. The cells were subsequently pelleted by centrifugation at 13,000 rpm for 3 minutes and, after the supernatant was decanted off, resuspended in the ~50-100 µl remaining LB. The cell suspension was then spread on an LB-agar (1 % agar) plate with 100 µg/ml carbenicillin (Sigma-Aldrich) and incubated at 37 ºC for 16 hours.

Colonies were screened for plasmid containing hPTTG-FLAG cDNA by PCR using the primers described in Figure 5-1. Each bacterial colony was boiled for 10 minutes at 95 ºC in 10 µl water to release the plasmid DNA. The PCR reaction comprised of 10 µl plasmid

DNA, 600 nM forward and reverse primers (Alta Bioscience), 500 µM dNTPs, 1.5 mM $MgCl_2$ (Bioline), 1x NH_2 reaction buffer (Bioline) and 2.5 Units Biotaq™ DNA polymerase (Bioline), which was made up to a total volume of 50 µl with nuclease-free water. The PCR cycling conditions used were 95 ºC for 5 minutes, 40 cycles of 94 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 45 seconds, followed by 72 ºC for 7 minutes. The 600 bp PCR products were subsequently electrophoresed on a 1.5 % agarose gel.

5.2.1.5 Plasmid DNA purification

Bacterial colonies in which the *hPTTG-FLAG* cDNA was successfully ligated into the plasmid vector were used to inoculate 5 ml LB containing carbenicillin (100 µg/ml) and incubated at 37 ºC for 1 hour with shaking at 200 rpm. Plasmid DNA was purified from these cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Briefly, the cells were pelleted by centrifugation and supernatant discarded. The pellet was resuspended and cells lysed. The addition of alkaline protease solution was followed by incubation at room temperature for 5 minutes to inactivate endonucleases. Cell lysis was terminated with neutralisation solution and centrifugation produced a cleared lysate, which was transferred to a spin column. The DNA bound to the column was washed twice with an ethanol-based wash solution and then centrifuged without wash solution present to ensure maximal removal of ethanol. Finally, plasmid DNA was eluted from the column with 50 µl nuclease-free water.

5.2.1.6 DNA sequencing of *pSG5-hPTTG-FLAG* minipreps

10 µl DNA sequencing reactions containing 500 ng miniprep DNA, 2 µl sequencing primer (5 pmol/µl) and water were analysed by the Genomics Laboratory (University of Birmingham) using an ABI 3730 DNA analyser. DNA sequence analyses were viewed using

Chromas Lite Version 2.0 (Technelysium Pty Ltd, Australia) and compared with template sequences using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequencing primers used at this step were a T7 primer (5' TAA TAC GAC TCA CTA TAG GG '3) and primers E, F, G and K (see Figure 5-6 B) [All provided by Alta Biosciences, UK].

5.2.1.7 Maxiprep of *pSG5-hPTTG-FLAG*

Once the cDNA sequences had been confirmed, larger quantities of DNA were purified by maxiprep. Originating from the same colonies that were selected initially for plasmid DNA purification and sequencing, 150 ml bacterial cultures were incubated at 37 ºC for 16 hours. Plasmid DNA was subsequently isolated using the GenElute™ HP Plasmid Maxi-Prep Kit (Sigma) following the manufacturer's instructions. To pellet the bacterial cells, 100 ml culture was centrifuged at 7500 g for 10 minutes at 4 ºC. Following resuspension, the cells were lysed with an alkaline-SDS solution, in which chromosomal DNA was denatured. When the lysate was neutralised by acidic sodium acetate, chromosomal DNA renatured and formed aggregates, which were co-precipitated along with protein-SDS complexes and high molecular weight RNA. This material was removed by passing through a filter syringe. The filtered lysate was centrifuged at 3000 g for 2 minutes to bind the plasmid DNA to a column and again to wash the DNA with an ethanol-based solution. Another 5 minute wash step was performed before the DNA was eluted in 3 ml nuclease-free water by centrifugation at 3000 g for 7 minutes. The quantity of plasmid DNA obtained was determined by measuring absorbance at a wavelength of 260 nm using a NanoDrop spectrometer (NanoDrop Products, Wilmington, DE, USA).

5.2.1.8 Introduction of XhoI restriction enzyme site to *pSG5-hPTTG-FLAG*

PCR primers were designed to introduce a XhoI restriction enzyme site to *pSG5-hPTTG-FLAG* at the 3' end downstream of the poly-A tail (see Figure 5-3).

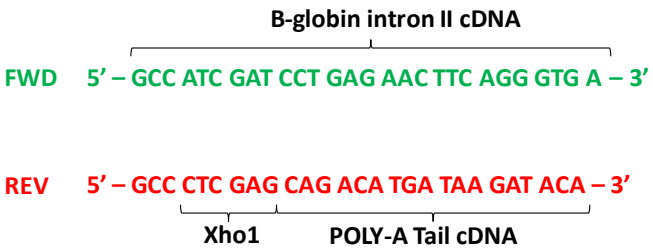


Figure 5-3: Sequence and features of the PCR primers designed to introduce a Xho1 restriction enzyme site to pSG5-hPTTG-FLAG at the 3' end downstream of the poly-A tail.

PCR amplification of *pSG5-hPTTG-FLAG* was carried out as per reactions and thermo-cycling protocols described in section 5.2.1.1. The PCR product (~1500 bp in size) was excised from the gel using a scalpel and extracted from the agarose using the QIAquick gel extraction kit (Qiagen) as described in section 5.2.1.2.

5.2.1.9 Restriction digestion and ligation of *pBSK* plasmid and PCR product

The *pBSK* plasmid containing the bovine thyroglobulin promoter was kindly provided by Dr. Jeffrey Knauf and Prof. James Fagin (Memorial Sloan-Kettering Cancer Centre, New York, USA). This *pBSK* plasmid and the PCR product with a region of cDNA containing the beta-globin intron II, *hPTTG-FLAG* and the poly-A tail, were digested using Xho1 and Cla1 restriction enzymes. Reactions were identical to those described in section 5.2.1.3. Digested vector and PCR product were electrophoresed on a 1.5 % agarose gel and gel extracted using the QIAquick gel extraction kit as described in section 5.2.1.2. The ligation reaction was carried out as described in section 5.2.1.3. Transformation of the ligation product into DH5α cells and subsequent PCR screening of colonies was performed as decribed in section 5.2.1.4. The PCR primers used were those described in Figure 5-3.

5.2.1.10 Plasmid DNA purification and sequence verification

Bacterial colonies in which the cDNA was successfully ligated into the plasmid vector were cultured overnight as described in section 5.2.1.5. Plasmid DNA was purified from

these cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega) as described in section 5.2.1.5.

The correct nucleotide sequence was confirmed using the primers described in the table in Figure 5-6 B and by DNA sequencing analysis as described in 5.2.1.6. Once cDNA sequences had been confirmed, larger quantities of DNA were purified by maxiprep using the Qiagen EndoFree Plasmid Maxi Kit (Qiagen). The protocol is similar to that described in section 5.2.1.7, except for the inclusion of an extra step that removes all endotoxins released during the lysis step of plasmid purification. This extra step was essential to obtain the high levels of DNA purity for transgenic animal work.

5.2.1.11 Isolation and microinjection of the transgene

The final linearised transgene construct was isolated using restriction enzyme digest reactions and diagnostic agarose gels. Restriction enzyme digest of *pBSK-TG-hPTTG-FLAG* was performed in reactions similar to those described in 5.2.1.3 using Xho 1, Spe 1 and Pvu 1. The reaction product following digestion with Xho 1, Spe 1 and Pvu 1 was electrophoresed on a 1.5 % agarose gel and the presence of a DNA fragment for the predicted size of ~3500 bp was gel extracted and purified as described in 5.2.1.2. Following further purification steps carried out by Andrea Bacon (Biomedical Services Unit, University of Birmingham), the generation of the hPTTG-Tg transgenic line was performed by microinjection of fertilized mouse oocytes with the *TG-hPTTG-FLAG* transgene and subsequent transfer into pseudopregnant females, as per standard protocols (Bacon et al. 2007; Hogan et al. 1994; Read et al. 2011). WT and transgenic PTTG-Tg mice were bred at the University of Birmingham and all experiments performed in accordance with U.K. Home Office regulations.

5.2.2 Cell culture

Normal rat thyroid FRTL5 cells (purchased from the Health Protection Agency Culture Collections, UK) were maintained in identical media to that described for human primary thyrocytes (see section 2.2). Human non-small cell lung carcinoma H1299 cells were kindly provided by Dr. Andy Turnell (School of Cancer Sciences, University of Birmingham), were grown in RPMI 1640 medium, supplemented with 10 % fetal bovine serum, 1 % L-glutamine and 1 % pen/strep (Life Technologies, Inc., Grand Island, NY, USA). Human colorectal cancer HCT116 cells (kindly provided by Prof. Vogelstein, MD, USA) were grown in McCoy's 5A modified medium, supplemented with 10 % fetal bovine serum, 1 % L-glutamine and 1 % pen/strep (Life Technologies, Inc., Grand Island, NY, USA).

5.2.3 Transfections

FRTL5, H1299 and HCT116 cells were seeded at 5.0 x 10^4 and 1.0 x 10^5 cells/well in 12 and 6-well plates respectively. Cells were transfected with 1 µg (12-well plate) and 2 µg (6-well plate) of plasmid DNA/well using FuGENE-6 Transfection Reagent (Roche, Indianapolis, IN, USA), with an optimised ratio of 3 µl per 1 µg plasmid DNA. Control transfections utilised equal amounts of blank plasmid (vector-only, VO, control). Cells were harvested 48 hours post-transfection in protein or RNA lysis buffer.

5.2.4 Tissue DNA extraction

Genomic DNA of potential founder mice was extracted from mouse ear clippings using the DNeasy Blood and Tissue kit (Qiagen), as per the manufacturer's instructions. In brief, tissue samples were first lysed using proteinase K for 3 hours at 56 ºC. RNase A was added for 2 minutes to ensure RNA free genomic DNA was yielded. Subsequently, buffering conditions were adjusted to provide optimal DNA binding conditions and the lysate was

loaded onto the DNeasy Mini spin column. During centrifugation, DNA was selectively bound to the DNeasy membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors are removed in two efficient wash steps and DNA is then eluted in water. DNA concentration and purity was determined by spectroscopy at λ260 nm using a NanoDrop spectrometer (NanoDrop Products, Wilmington, DE, USA) where DNA routinely demonstrated high purity with A260/A280 ratios of 1.8–2.0.

5.2.5 Screening by conventional PCR

Potential transgenic mice were screened by PCR using up to four different primer sets (Figure 5-9 B) in order to demonstrate reproducibility of genotyping results and to improve the reliability of this assay. The PCR reaction consisted of 100 ng mouse genomic DNA template, 600 nM each of forward and reverse primers (Alta Bioscience), 200 µM dNTPs, 2.5 mM $MgCl_2$ (Bioline, London, UK), 1x TaqMaster PCR Enhancer (5Prime, Hamburg, Germany), 0.5x NH_2 reaction buffer (Bioline), 2.5 U Biotaq™ DNA polymerase (Bioline), which was made up to a total volume of 50 µl with nuclease-free water.

The PCR cycling conditions used were 95 ºC for 5 minutes for the initial denaturation of DNA template before holding at 78 ºC at which stage the DNA polymerase in NH_2 reaction buffer was added. Subsequently, there were 40 cycles of 94 ºC for 30 seconds (denaturation), 58/62 ºC for 30 seconds (primer annealing) and 72 ºC for 1 minute 30 seconds (extension), followed by a final extension step of 72 ºC for 7 minutes. This was performed on a Mastercycler gradient (Eppendorf, Hamburg, Germany). PCR products were subsequently electrophoresed on a 1.5 % agarose (Bioline) gel in 1x TAE (Tris-Acetate-EDTA) buffer (Eppendorf) before gels were visualised to identify ~ 600 bp bands representative of transgenic mice.

5.2.6 Screening and genotyping through QT-PCR zygosity assay

The genotype of transgenic mice was determined by Real-Time RT-PCR analysis essentially as described previously (Ballester et al. 2004; Read et al. 2011). Primers used to specifically detect the human *PTTG* gene were as described in Table 3-1. Real-Time PCR reactions and data analysis were carried out as described in section 2.5, except that PCR reactions were also performed using primers and probe directed to an 89 bp sequence in the *Dscam* gene to normalise DNA levels, which is a conserved sequence in both mouse and human genomes (Table 5-1). Low amounts of mouse genomic DNA (5 ng) were amplified and ΔΔCT values calculated from Ct values for hPTTG in human placenta and liver DNA controls.

Sequence Name	Sequence
DSCAM Probe	5′ VIC-TTCAAGTGCATTATCCCCTCCTCGGTG-3′
DSCAM Forward Primer	5′-CAGAAAACCATGAGAGGCAATG-3′
DSCAM Reverse Primer	5′-TTCTCCCATGAGACGACAGTGA-3′

Table 5-1: Oligonuleotide sequences of PCR primers and TaqManTM probe used to detect an 89 bp sequence in the DSCAM gene expression as a genomic DNA control for zygosity assays. VIC = 5' reporter dye.

5.2.7 RNA extraction, reverse transcription, QT-PCR

Total RNA was extracted from FRTL-5, HCT116 and H1299 cells using the Stratagene Absolutely RNA miniprep kit [Agilent Technologies UK Ltd.]. Total RNA was extracted from mouse thyroid glands and control tissue organs using the RNeasy microkit (Qiagen, UK). In both instances, adherent cells or mouse tissue was homogenised in lysis buffer containing β-Mercaptoethanol before isolating RNA according the manufacturer's protocols. In both kits, RNA was isolated using an RNA-binding spin column and treated with DNase I to ensure an RNA yield with a high level of purity. Extracting RNA of high

purity is critical because contaminating DNA present in the RNA sample can give rise to amplification products that mimic the amplification product expected from the RNA target.

Subsequent reverse transcription and QT-PCR techniques were as described (see sections 2.4 and 2.5). *hPTTG* mRNA was detected using the primers and probe described previously (see Table 3-1).

5.2.8 Western blot analysis

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see section 2.6). Blocked membranes were subsequently incubated with either of two primary antibodies against hPTTG; Pds1 (mouse anti-hPTTG; 1:1000) [Labvision, Thermofisher, UK] or hPTTG1 (rabbit anti-hPTTG; 2 μg/ml) [Invitrogen, UK]. After washing in TBS-T, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidise (Dakocytomation, UK) for 1 hour at room temperature before being visualised by techniques described in section 2.6.

5.2.9 Immunohistochemistry

Immunohistochemical analysis of hPTTG expression in formalin-fixed, paraffin-embedded sections of wild-type and PTTG-Tg mouse thyroid specimens was carried out as described in section 2.7. A primary antibody against hPTTG was used (2 μg/ml) [Invitrogen, UK].

5.2.10 Statistical analysis

Data were analysed as described in section 2.8

5.3 Results

5.3.1 Generation of an *hPTTG-FLAG* transgene construct

A multi-step cloning strategy was adopted in order to generate a transgene construct consisting of a human PTTG-FLAG-tagged cDNA downstream of the bovine thyroglobulin promoter. In the first instance, a FLAG-tagged *hPTTG* cDNA was cloned into the *pSG5* vector downstream of the rabbit beta-globin intron II and upstream of an SV40 polyadenylation (poly-A) tail. Subsequently, a DNA fragment containing the rabbit beta-globin intron II, FLAG-tagged *hPTTG* and the poly-A tail was cloned into a *pBSK* plasmid containing the bovine thyroglobulin promoter, kindly provided by Professor James Fagin (Memorial Sloane-Kettering Cancer Centre, New York) (see Figure 5-4).

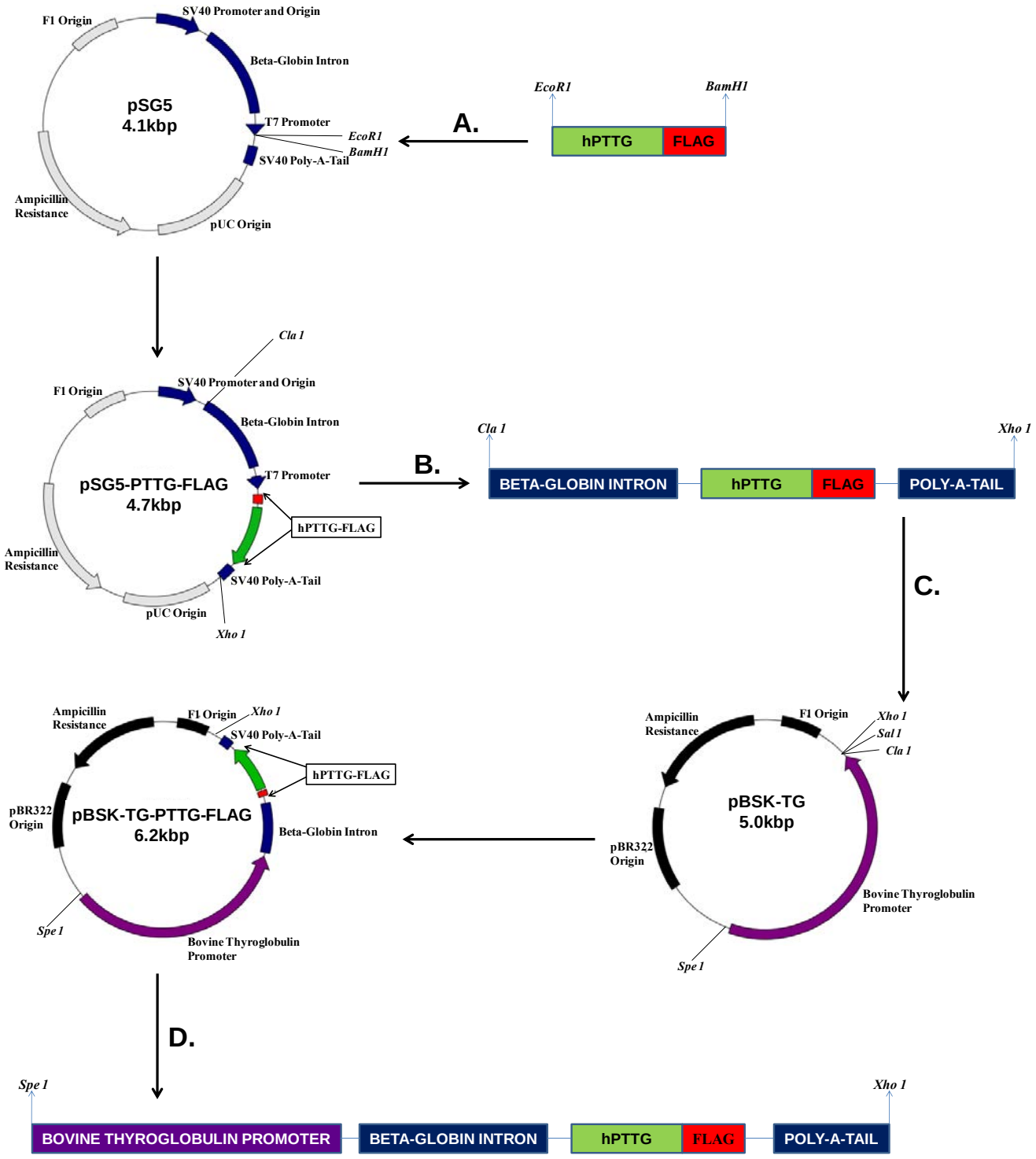


Figure 5-4: Schematic overview of the multi-step cloning strategy for the development of the Tg-hPTTG-FLAG transgene construct. ***A*** *hPTTG-FLAG cDNA was ligated into the pSG5 construct following digestion with the EcoR1 and BamH1 restriction enzymes.* ***B*** *A DNA fragment consisting of the beta-globin intron II, hPTTG-FLAG and a poly-A-tail was isolated following digestion with Cla 1 and Xho 1 restriction enzymes.* ***C*** *The fragment obtained in* ***B*** *was ligated into the pBSK plasmid containing the bovine thyroglobulin promoter (TG), following digestion with Xho 1 and Cla 1 restriction enzymes.* ***D*** *The final transgene construct was isolated following restriction digests with Xho 1 and Spe 1.*

5.3.2 Verification of Transgene Activity and Specificity

To assess the activity and specificity of the transgene construct, transient transfections of *pBSK-VO/hPTTG* or a control plasmid *pSG5-VO/hPTTG*, were performed in normal rat thyroid FRTL5, human colorectal cancer HCT116 and human lung cancer H1299 cells. *hPTTG* mRNA expression was analysed using TaqMan RT-PCR to assess transgene activity in each cell type. As expected, *hPTTG* mRNA expression was increased in FRTL5 (5692-fold, $n = 3$, $p < 0.001$), HCT116 (8-fold, $n = 3$, $p < 0.001$) and H1299 (128-fold, $n = 3$, $p < 0.001$), following transfection with *pSG5-hPTTG* compared with *VO* controls. Importantly, there was a 421-fold increase of *hPTTG* mRNA expression in FRTL-5 cells transfected with *pBSK-hPTTG* ($n = 3$, $p < 0.001$) with no expression observed in either HCT116 or H1299 cells (see Figure 5-5).

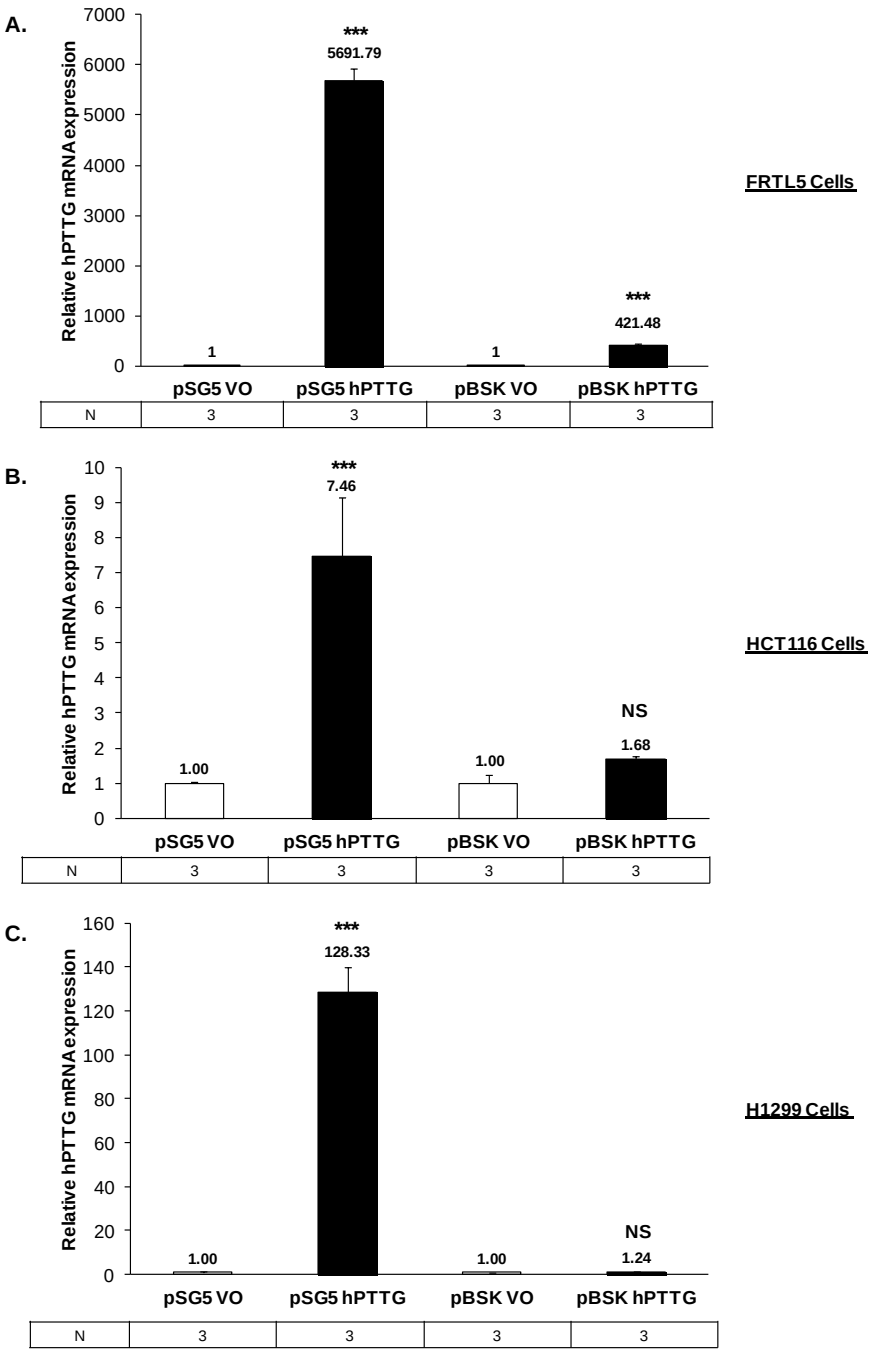


*Figure 5-5: TaqMan RT-PCR demonstrating relative hPTTG mRNA expression following transient transfection with either pSG5-PTTG or pBSK-PTTG in FRTL5 cells (**A**), HCT116 cells (**B**) and H1299 cells (**C**). Data are given as a value relative to 1.0 for appropriate transfection controls. Error bars represent the SEM (*** $p < 0.001$, NS = non-significant).*

5.3.2.1 Verification of construct by DNA sequencing

Having confirmed thyroid specific expression of *hPTTG* mRNA following transfection with *pBSK-hPTTG*, the transgene construct was further validated by direct

sequencing that verified the correct nucleotide sequence and orientation of all construct components (see Figure 5-6).

A.

BOVINE THYROGLOBULIN PROMOTER
BETA GLOBIN INTRONI
T7 PROMOTER
HUMAN PTTG
FLAG-TAG
POLY-A TAIL
STOP SITE TAA

Spe I site ACTAGT
BamH1 site GGATCC
Cla I site ATCGAT
EcoR1 site GAATTC
Xho 1 site CTCGAG
Sal 1 site GTCGAC

ATTAACCCTCACTAAAGGGA.....ACTAGTGGATCCAGGAGGGAGAGGTGAGAGGGCCAGAGGTGAGGAGCCTGCAGAGAGGATGGAGACCCAAGCCAGGAGCGG
GCTCACGGAGGCCAGACCAAGGAGTTGGTGCTGAGTTTCCGTAGAGCATCCTCCTGGCTCACGAGGCATCAGATGATGGTAGGAGCTGAGTCATCGTGAGAAAGAGGTTTTC
ACGGTGTGGAGGCAGCGCCTGGCATGGCTCTCTCCAAGCTCATCGCTTCATTGCTGGAGTGGAGAGAAGCACCTCTCCTTCCAGGTACACTGTTTTGACTCAGAAGGAAGCC
CGGGCG*GCTCTCACCCTCACTGA*CTCTGCTGTGCCTCTTCCCAGGGGCCAGGCCAGGGAGAATGACTCAGCACACAAGCTGGCCTGTGCAAGACCCTGCCTGCATGCATGG
GTGGGGGAAGCATGAGGAGTGGGCTTTCTGGTAGACCTCAGTCATGGTCTCAGCATTATTTTTTAAAAGGATTTTATTTATTTGTTTTGATGTGGACCAGTTTTAAAATCTTTATT
GAATTTGTTACCATGTTGCTTCTGTTTTATGTTTTGGTTTTTTGGCCACGAGGCATGGGGGATGTTAGCTCCCCGACCAGGGACTGAACCCTTGCCCCCTGCATGGAAAGGTCA
AGTCTTAACTTCTTGACTGCCTAGGAAGTCCCGGTTTCAACATTTTAGAAAATTCATCTGCTTTAGAACACCTGGACCTGGAGAGCTGGCAAGGGCTGCACATGGCCAGGCTC
ACGGTAATATTACAGCGTCTCCATAGCCTGGGCAGAACTTGGCTTGTGTGATTGGGGCTGTGGGCTCCCCAGGTGGTGC*TAGTGGTAAAGAACCCTTCT*GCCAGTGCAGGAG
ACATAAGAGATGCGGGTTCTATCCCTGGATCAGGAAGATCCC*CTGGAGAAGGAAATGGC*AACCTGCTCCAGGATTCTTGCCTGGAGAATCCCAAGGACAGAAGAGCCTGGC
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GCATGTCTTCTCAAATCATCTTTACCTAAAACCTAAGGCTCATTGTGATCTCCCTCACTCCCAAGT*GCTCTCCCACACCTGCC*CACCTACCTTCTGAACACCCATGGAGCCTGT
CTCCATCCCCCACCCCTTTAATGGGAGAAGCCACTGGAGAAAGCTCACTGATGTACAGGCTTTATGTGGAAGACAAGCTTCCTGCTGCCTTTTGGTTGTCTGACGTCCTGGGA
CAGAGGGGAAAGGGGGATGACTACGAGTATGACTGTGCGTGTGTTTGGCTTATCTCATCAAAATCTCTACATTCTGTGTTAATGGATCTGCCTGTTTTGTTCCCTGCCATATCC
TCATGGCCTAGAATAGTGTCTGCTTCTCTATCAGACTCTAAAGAAACATTGCTAGGAGGGAAGGAAGGAGCATGGATGAGGAGGGAGGGAGCATTGTGTTTCTCTCACGGTG
GGCCTGAACGTGTGGCCCACCAAGTTGTTAACTTTGGCCTTTACCCCTGAAGATGAATTATGAAGCCACACCCCCAGTTCTTCCTTGGTGGCTCAGATGGTCAAGAATCCACC
TGCAATGCGGGAGACCTGGGTTTGATCCCTGGGTTGGGAAGATCC*CCTGGAGAAGGGAATGGCTAC*CCCACTCCAGTATTCTGGCCTGGAGAATCCCATGGACAGAGGAGCC
TGGCGGGATGCAGTCCATGGGGTCTCAGAGAGTCAGATGTGACTGAGCGACTTTCACACACATTCGTCCCTGGTTCTGCTCCCCTACAGCCTCCACAAGATTTTCACCCCACA
CTGGCCACATGAGTGTCCTCCAGGGGAACAGACGCAGGTGGAGGACCTCCTTGTGACCAGCAGAGAAAACAGGGTGGGCACTGCTTCCCTGAGTGCCTGTGGGTGGGGGC
TAAGTACCCACAG*CAGTGCTATAAAGGCTC*CTTGGCCAGAGCCCTAAGGTGGGCAGCAGGGGATCCCCCGGGCTGCAGGAATTCGATAtCAAGCTTATCGATCCTGAGAACT
TCAGGGTGAGTTTGGGGACCCTTGATTGTTCTTTCTTTTTCGCTATTGTAAAATTCATGTTATATGGAGGGGGCAAAGTTTTCAGGGTGTTGTTTAGAATGGGAAGATGTCCCTT
GTATCACCATGGACCCTCATGATAATTTTGTTTCTTTCACTTTCTACTCTGTTGACAACCATTGTCTCCTCTTATTTTCTTTTCATTTTCTGTAACTTTTTCGTTAAACTTTAGCTTG
CATTTGTAACGAATTTTTAAATTCACTTTTGTTTATTTGTCAGATTGTAAGTACTTTCTCTAATCACTTTTTTTTCAAGGCAATCAGGGTATATTATATTGTACTTCAGCACAGTTTT
AGAGAACAATTGTTATAATTAAATGATAAGGTAGAATATTTCTGCATATAAATTCTGGCTGGCGTGGAAATATTCTTATTGGT*AGAAACAACTACATCCTGGTC*ATCATCCTGCC
TTTCTCTTTATGGTTACAATGATATACACTGTTTGAGATGAGGATAAAATACTCTGAGTCCAAACCGGGCCCCTCTGCTAACCATGTTCATGCCTTCTTCTTTTTCCTACAGCTC
CTGGGCAACGTGCTGGTTATTGTGCTGTCTCATCATTTTGGCAAAGAATTGTAATACGACTCACTATAGGGCGAATTCATGGCTACTCTGATCTATGTTGATAAGGAAAATGGA
GAACCAGGCACCCGTGTGGTTGCTAAGGATGGGCTGAAGCTGGGGTCTGGACCTTCA*ATCAAAGCCTTAGATGG*GAGATCTCAAGTTTCAACACCACGTTTTGGCAAAACGT
TCGATGCCCCACCAGCCTTACCTAAAGCTACTAGAAAGGCTTTGGGAACTGTCAACAGAGCTACAGAAAAGTCTGTAAAGACCAAGGGACCCCTCAAACAAAAACAGCCAA
GCTTTTCTGCCAAAAAGATGACTGAGAAGACTGTTAAAGCAAAAAGCTCTGTTCCTGCCTCAGATGATGCCTATCCAGAAATAGAAAAATTCTTTCCCTTCAATCCTCTAGACT
TTGAGAGTTTTGACCTGCC*TGAAGAGCACCAGATTGC*GCACCTCCC*CTTGAGTGGAGTGCCTC*TCATGATCCTTGACGAGGAGAGAGAGCTTGAAAAGCTGTTTCAGCTGGG
CCCCCCTTCACCTGTGAAGATGCCCTCTCCACCATGGGAATCCAATCTGTTGCAGTCTCCTTCAAGCATTCTGTCGACCCTGGATGTTGAATTGCCACCTGTTTGCTGTGACAT
AGATATTGACTACAAAGACGATGACGACAAGTAAGGATCCAGATCTTATTAAAGCAGAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTC
ACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGCTCGACGTCGAC.....*AATGGGTTGAATTAGCGGAA*

B.

PRIMER NAME	SEQUENCE	DIRECTION	START REGION
Primer A	*5'-ATTAACCCTCACTAAAGGGA-3'*	FORWARD	T3 promoter (plasmid)
Primer B	*5'-CTGGAGAAGGAAATGGC-3'*	FORWARD	Bov. Tg. promoter
Primer C	*5'-TGAAGAGCACCAGATTGC-3'*	FORWARD	Bov. Tg. promoter
Primer D	*5'-CCTGGAGAAGGGAATGGCTA-3'*	FORWARD	Bov. Tg. Promoter
Primer E	*5'-AGAAACAACTACATCCTGGTC-3'*	FORWARD	Beta-globin intron II
Primer F	*5'-TGAAGAGCACCAGATTGC-3'*	FORWARD	hPTTG
Primer G	*5'-CTTGAGTGGAGTGCCTC-3'*	FORWARD	hPTTG
Primer H	*5'-TCAGTGAGGGTGAGAGC-3'*	REVERSE	Bov. Tg. Promoter
Primer I	*5'-GGCAGGTGTGGGAGAGC-3'*	REVERSE	Bov. Tg. Promoter
Primer J	*5'-GAGCCTTTATAGCACTG-3'*	REVERSE	Bov. Tg. Promoter
Primer K	*5'-CCATCTAAGGCTTTGAT-3'*	REVERSE	hPTTG
Primer L	*5'-TTACCCAACTTAATCGCCTT-3'*	REVERSE	pBSK plasmid

*Figure 5-6: Direct sequencing of the pBSK-TG-hPTTG-FLAG construct confirmed the correct nucleotide sequence and orientation of the construct. **A** The nucleotide sequence for the transgene construct from the Spe 1 to Xho I restriction sites (3563 bp). **B** Table of sequencing primers used (marked as underlined and in italics in **A**).*

5.3.2.2 Preparation and microinjection of transgene construct

A linearised transgene construct was isolated using restriction enzyme digest reactions and diagnostic agarose gels. Digestion with only Xho 1 and Spe 1 resulted in two bands of

DNA migrating very closely together, representing the ~3500 bp transgene and a similarly sized fragment from the remainder of the plasmid. Additional digestion with Pvu 1 caused cuts at two further sites within the *pBSK* plasmid so that the transgene fragment was more easily isolated on an agarose gel. The reaction product obtained following digestion with Xho 1, Spe 1 and Pvu 1 was migrated on an agarose gel and the presence of a DNA fragment for the predicted size of ~3500 bp was confirmed. Following further restriction digests of *pBSK-TG-hPTTG-FLAG* and migration of the products on an agarose gel, the transgene fragment was extracted from the gel and purified (see Figure 5-7).

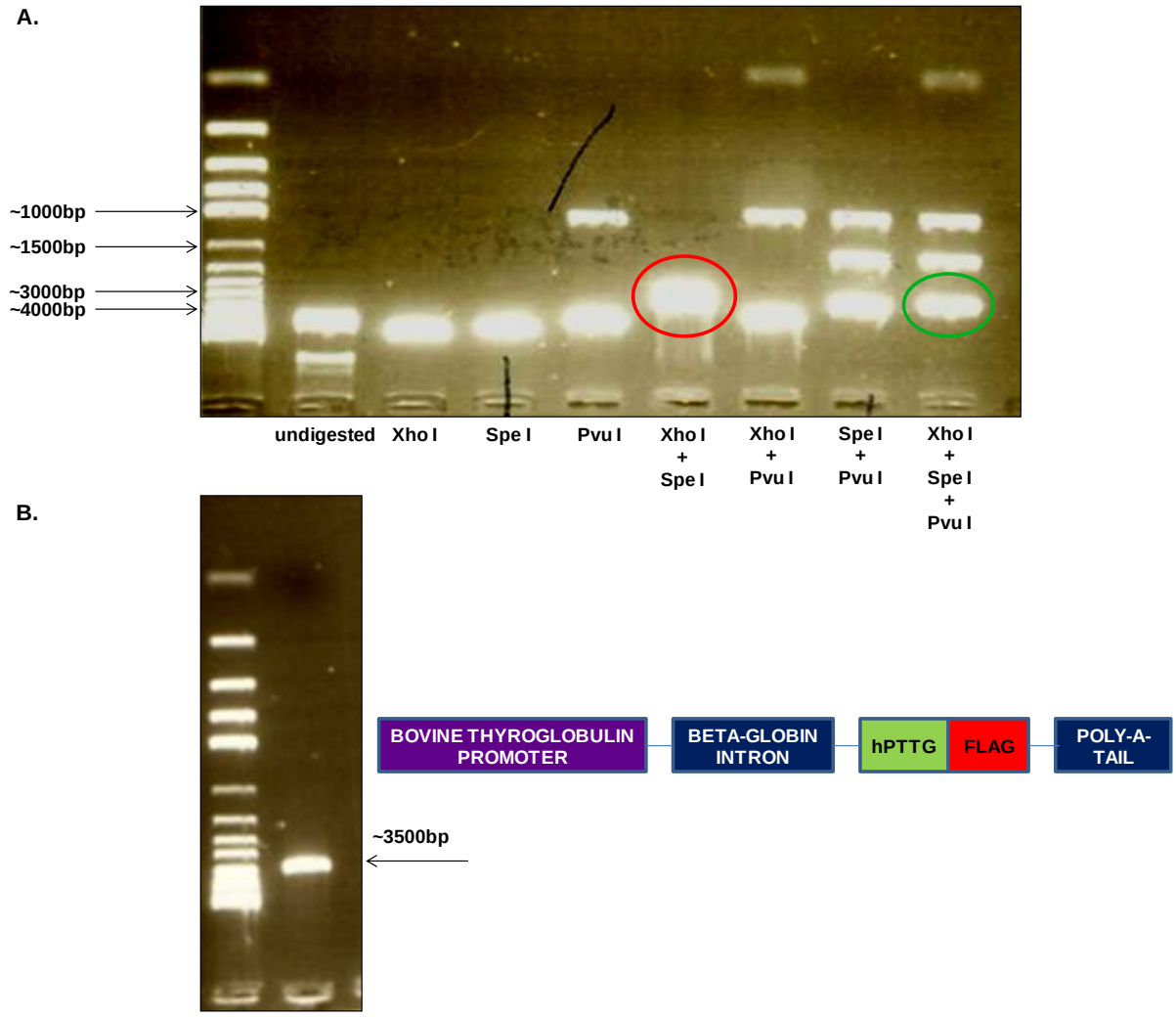


*Figure 5-7: Isolation of the linearised TG-hPTTG-FLAG transgene construct. **A** Diagnostic agarose gel illustrating the products of various restriction digest reactions. Red circle demonstrates the difficulty in resolving between the two fragments when just Xho 1 and Spe 1 are used. Green*

circle highlights the identification of the transgene fragment (~3500 bp) following digestion with Xho 1, Spe 1 and Pvu 1. ***B*** *Agarose gel showing migration of a sample of the linearised transgene following excision from original agarose gels and a purification step. The clean, sharp appearance of the band indicates a pure and non-denatured DNA sample.*

The purified transgene construct was given to Andrea Bacon at the transgenic facility of the Biomedical Services Unit, University of Birmingham, who performed further purification steps before injecting into the male pronuclei of fertilised oocytes from the FVB/N mouse strain. Oocytes were then implanted into the oviducts of pseudopregnant foster mothers for development to term (see Figure 5-8).

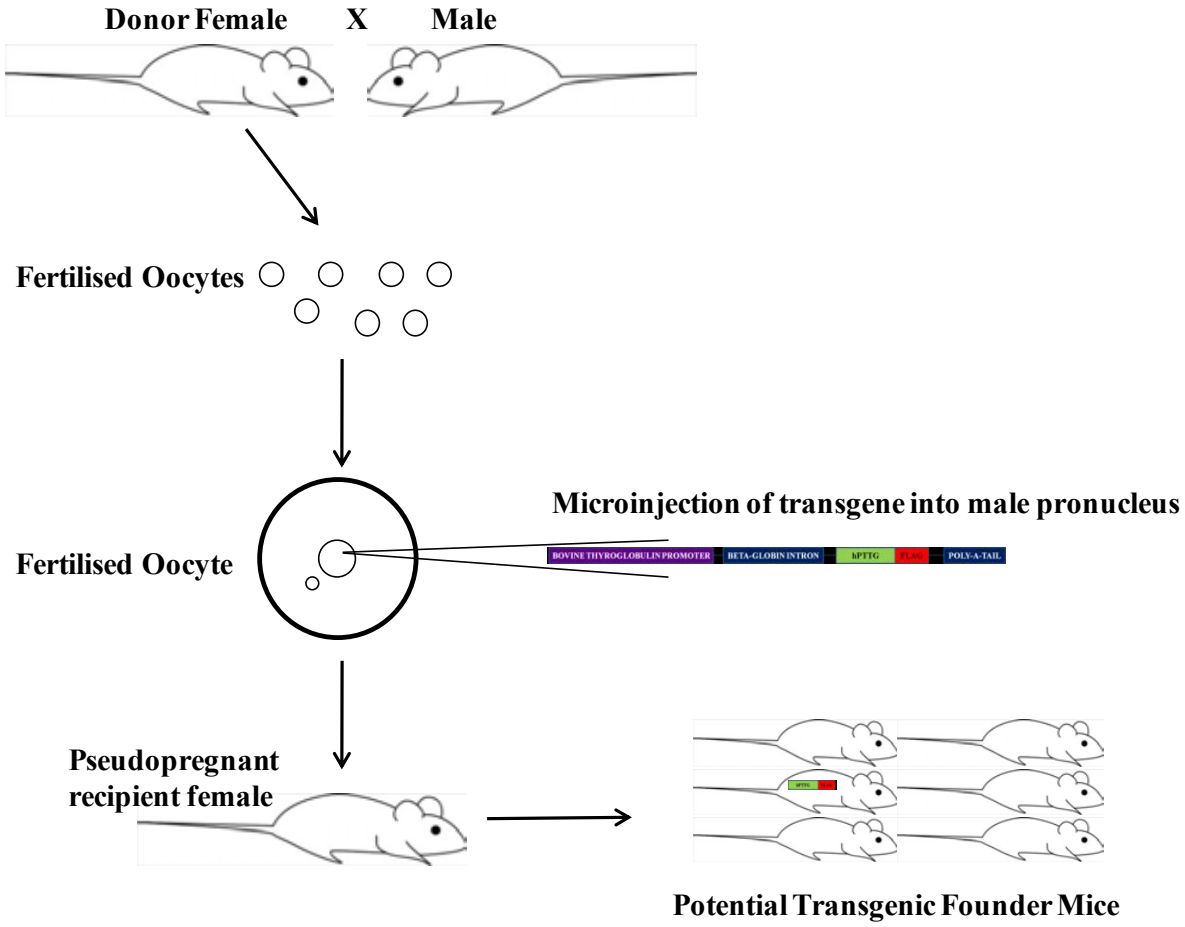


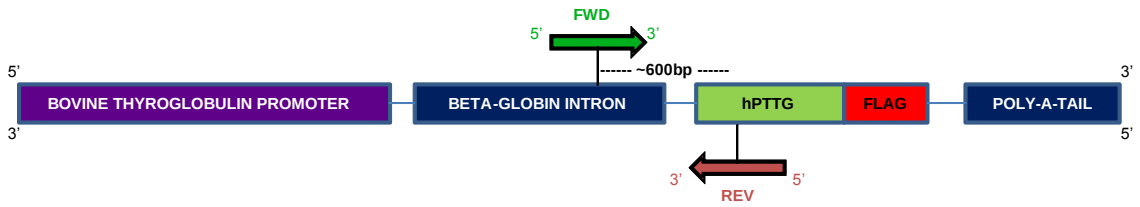
Figure 5-8: Schematic diagram illustrating pronuclear injection of the transgene construct before introduction to a pseudopregnant foster mother, resulting in offspring litters containing potential founder mice.

5.3.3 Identification and propogation of transgenic lines

5.3.3.1 Conventional PCR analysis to identify potential founders

PCR primer sets were designed in order to amplify an approximately 600 bp fragment spanning the region between the beta-globin intron and *hPTTG*. This design strategy was adopted to ensure specific detection of the transgene and avoid amplification of the endogenous murine *Pttg* gene. PCR primer sets and reaction conditions were validated and optimised by performing PCR amplifications directly from the *pBSK-Tg-hPTTG* plasmid and visualising agarose gels (see Figure 5-9).

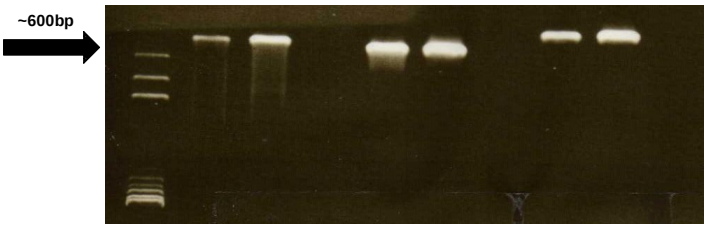
A.



B.

PRIMER PAIR	PRIMER NAME	PRIMER SEQUENCE	Optimal Melting Temp. (°C)
PAIR 1	Primer 1 FWD	CTGGTCATCATCCTGCCTTTCTC	62
	Primer 3 REV	GCTTGGCTGTTTTTGTTTGAGG	
PAIR 2	Primer 4 FWD	CGTGCTGTTATTGTGCTGT	62
	Primer 5 REV	GAGAGGCACTCCACTCAAGG	
PAIR 3	Primer 4 FWD	CGTGCTGTTATTGTGCTGT	62
	Primer 6 REV	CATCATCTGGAGGCAGGAACA	
PAIR 4	Primer 8 FWD	GCCTTCTTCTTTTTCCTACAGC	58
	Primer 9 REV	CAGGCAGGTCAAAACTCTCA	

C.



Amount of DNA (µg)	0.05	0.10	0.00	0.05	0.10	0.00	0.05	0.10	0.00
Primer Pair	1			2			3		

*Figure 5-9: Design and validation of PCR primers for screening of transgene in potential founders. **A** Schematic diagram illustrating PCR primer design strategy for specific detection of the transgene. **B** Table of primer pairs and sequences with validated optimal melting temperatures. **C** Migration of PCR products on an agarose gel following transgene amplification directly from the pBSK-TG-hPTTG-FLAG plasmid using primer pairs 1, 2 and 3. (Primer set 4 was similarly validated).*

Subsequently, DNA was extracted from ear tissue clippings from mice in potential transgenic founder litters and subject to rigorous screening by conventional PCR, using all four different PCR primer sets to validate results. Positive control PCR reactions were routinely set up to detect mouse Notch1 in order to confirm the presence of genomic mouse DNA in each sample and to verify efficient PCR amplifications. These were important controls to minimise the risk of false negatives whereby transgene-positive founder mice may have been misidentified as transgene-negative WT mice (see Figure 5-10).

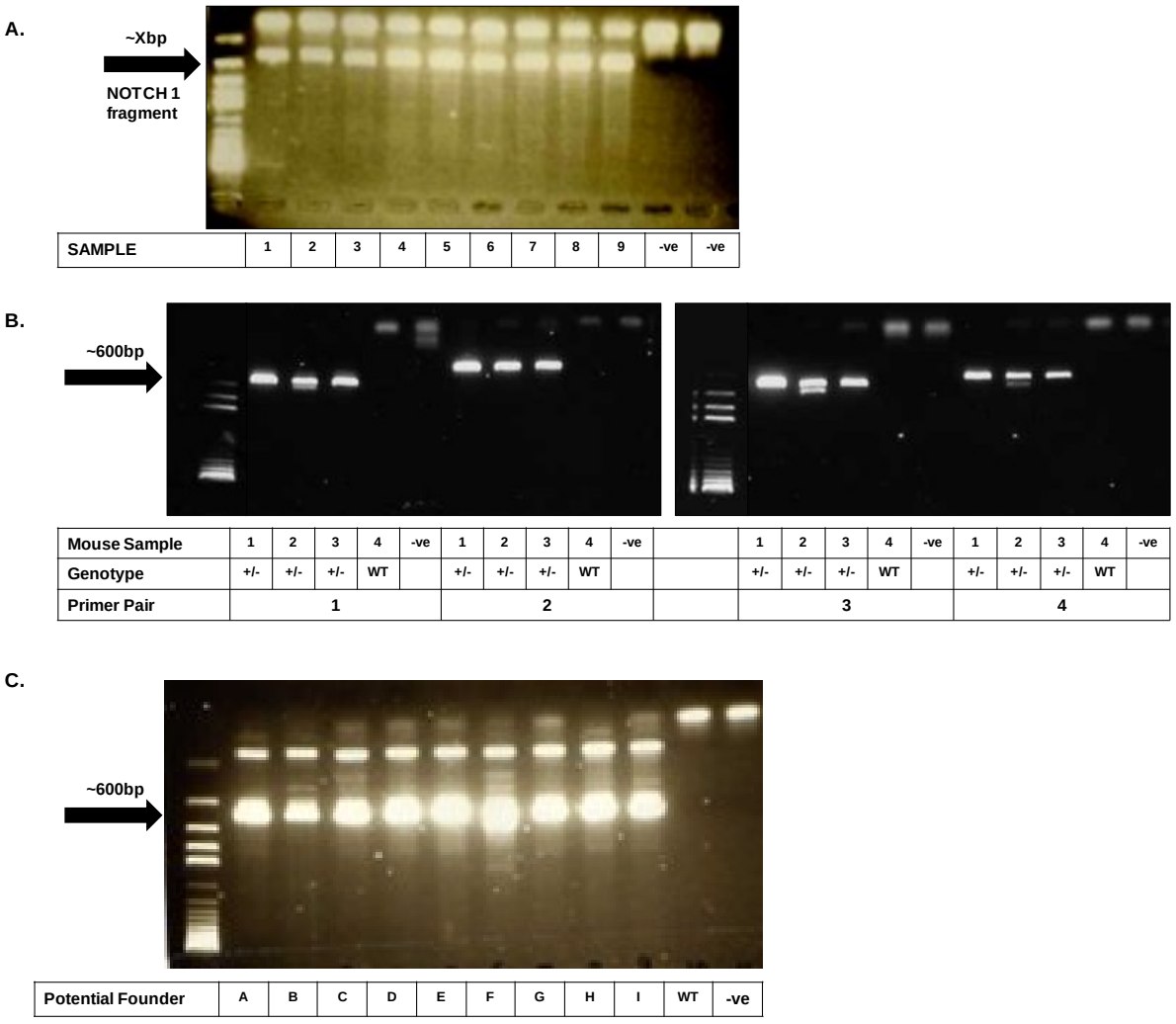


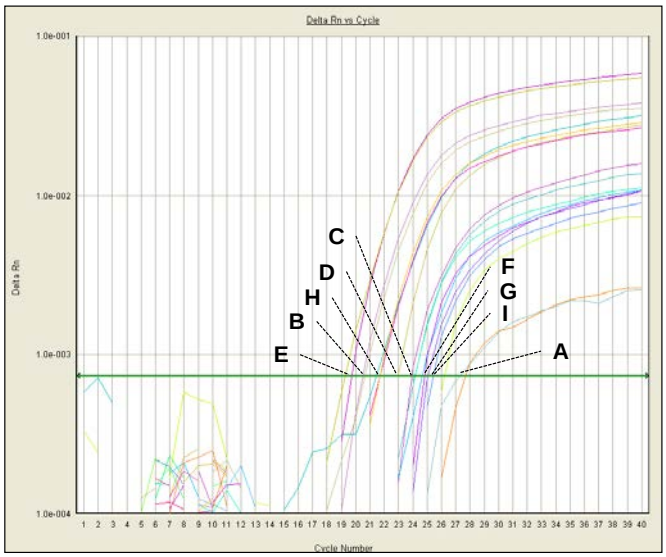
Figure 5-10: Screening of potential founder mice by conventional PCR. ***A*** *Demonstration of Notch-1 amplification in 9 potential founder DNA samples, confirming the presence of mouse genomic DNA and successful PCR amplification.* ***B*** *Agarose gels demonstrating identification and confirmation of 3 TG-hPTTG-FLAG transgenic founder mice (# 1-3) and 1 WT mouse (# 4) through use of all four primer pairs.* ***C*** *Agarose gel demonstrating the identification of 9 founder mice (A-I) out of a total of 95 screened. +/- = hemizygous hPTTG founder mouse.*

Further confirmation of screening results was achieved by TaqMan RealTime-PCR using a primer and probe set that specifically amplified *hPTTG* and not murine *Pttg*. Using this approach, 9 founder mice identified above were confirmed as transgene-positive (see Figure 5-11).

A.

Sequence Name	Sequence
hPTTG Probe	5'-CGTCTTGCCACCGGCTTCCCT-3'
hPTTG Forward Primer	5'-GAGAGAGCTTGAAAAGCTGTTTCAG-3'
hPTTG Reverse Primer	5'-TCCAGGGTCGACAGAATGCT-3'

B.



C.

SAMPLE	hPttg Ct	Average Ct
FOUNDER A	27.7	27.5
	27.3	
FOUNDER B	20.4	20.5
	20.5	
FOUNDER C	24.1	24.0
	23.9	
FOUNDER D	23.0	22.2
	21.4	
FOUNDER E	19.6	19.4
	19.2	
FOUNDER F	24.6	24.6
	24.7	
FOUNDER G	26.2	25.1
	24.1	
FOUNDER H	21.6	21.6
	21.6	
FOUNDER I	25.3	25.2
	25.0	
PF 10	Undet.	Undet.
	Undet.	
PF 11	Undet.	Undet.
	Undet.	
PF 12	Undet.	Undet.
	Undet.	
PF 13	Undet.	Undet.
	Undet.	
PF 14	Undet.	Undet.
	Undet.	

Figure 5-11: Confirmation of conventional PCR screening results through quantitative TaqMan RealTime-PCR. ***A*** *Table showing sequences of primers and probe that specifically amplify hPTTG and not murine Pttg.* ***B*** *Real-Time PCR reaction curves for the 9 founder mice (in duplicate) demonstrating amplification of the hPTTG transgene.* ***C*** *Table of Ct values highlighting the presence of the hPTTG transgene in the 9 founder mice (A-I) and complete lack of detection in potential founder mice considered transgene-negative.*

Nine mice were identified as transgenic founders from 95 mice in a total of 20 potential founder litters. Mice identified as positive for the transgene were set up with WT breeding partners to propagate transgenic lines in order to confirm transmission of the transgene to subsequent generations, and to obtain mouse tissue for the assessment of hPTTG expression in the thyroid glands and control organs of transgenic mice.

5.3.3.2 Evaluation of *hPTTG* mRNA expression in transgenic thyroids

From the original 9 founder mice, 1 was infertile while the 8 others successfully transmitted the transgene. Total RNA was extracted from the thyroid glands of transgenic mice in each of the remaining 8 lines and *hPTTG* mRNA expression was evaluated by TaqMan RT-PCR. Five of these lines failed to express *hPTTG* mRNA in the thyroid and were terminated (data not shown). Hemizygous mice in the remaining 3 lines (termed lines A, B and C) demonstrated expression of *hPTTG* mRNA in excised thyroid glands. Hemizygous mice in line A showed a 13,076-fold increase in thyroid *hPTTG* mRNA expression ($n = 3$, $p < 0.001$), whilst lines B and C demonstrated a significantly lower 22- ($n = 3$, $p < 0.05$) and 13-fold ($n = 3$, $p < 0.05$) over-expression of *hPTTG* mRNA respectively (see Figure 5-12).

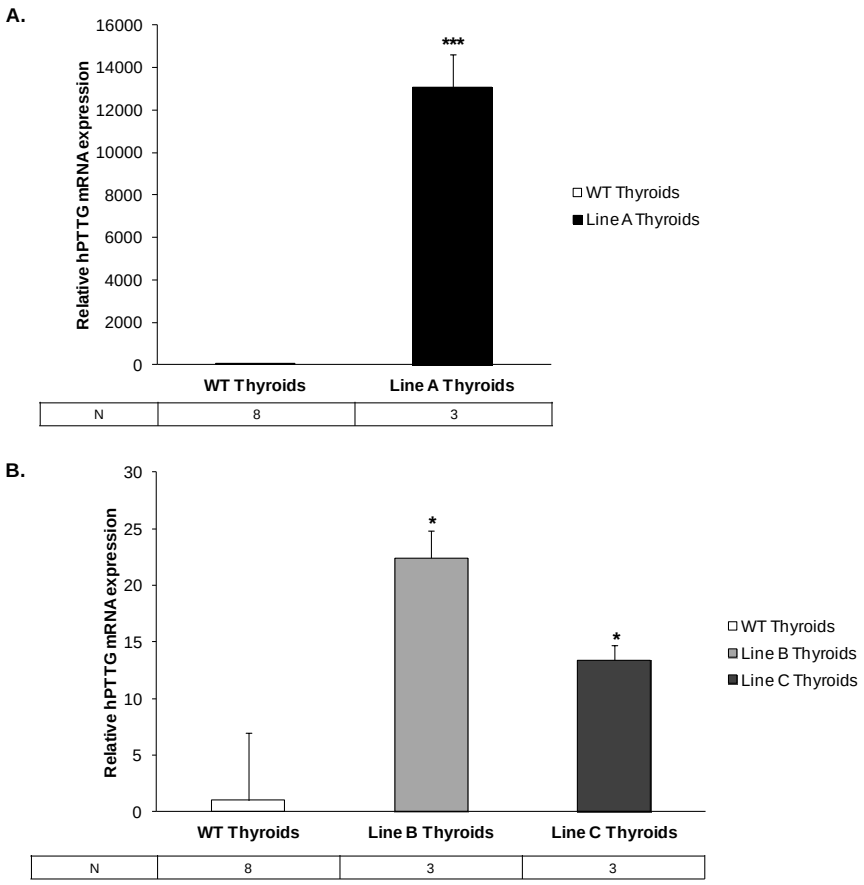


*Figure 5-12: TaqMan RT-PCR data demonstrating strong thyroid hPTTG mRNA expression in Line A (**A**) and significantly weaker over-expression in Lines B and C (**B**). Error bars represent the SEM (* $p < 0.05$, *** $p < 0.001$).*

5.3.3.3 Evaluation of hPTTG protein expression in transgenic thyroids

Concomitantly to analysis of thyroid *hPTTG* mRNA over-expression in lines A, B and C, we determined hPTTG protein expression levels in excised thyroid glands of 6-week-old transgenic mice by Western Blot analysis. Surprisingly, FLAG-tagged hPTTG expression was undetectable using any of three commercial FLAG antibodies (data not shown). However, using a hPTTG antibody (Cambridge Biosciences), it was confirmed that transgenic mice in line A had over-expression of hPTTG protein (n=4) in the thyroid gland but mice from lines B (n=5) and C (n=6) did not (see Figure 5-13).

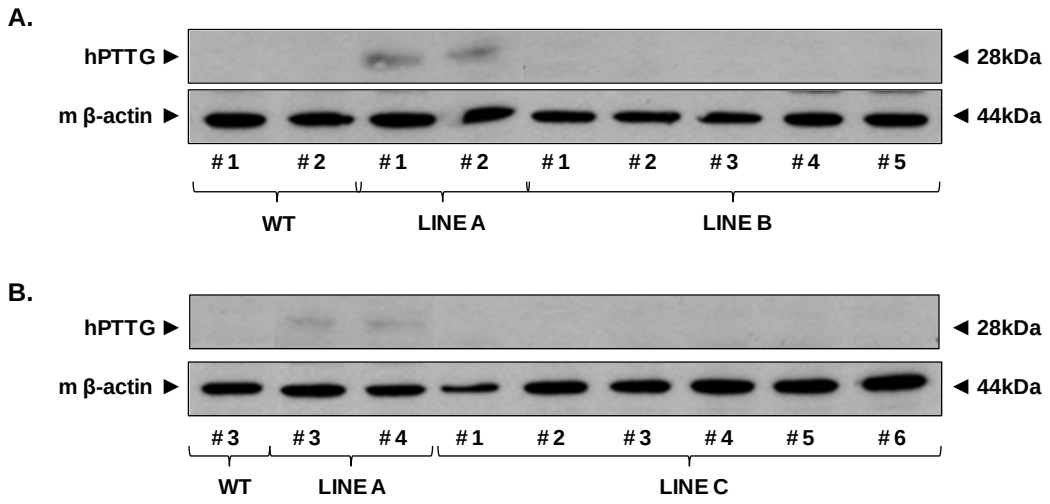


*Figure 5-13: Western blot analyses confirming hPTTG protein expression in thyroids from transgenic mice in line A (**A+B**) but not in lines B (**A**) and C (**B**).*

Consequently, lines B and C were terminated, while line A was investigated further to determine whether hPTTG expression was successfully confined to the thyroid gland.

5.3.3.4 Determination of thyroid specific *hPTTG* mRNA expression in Line A

Having identified line A as a transgenic line with significant over-expression of *hPTTG* mRNA and protein in the the thyroid gland, we investigated the specificity of transgene expression. Total RNA was extracted from the thyroid glands, heart, lungs, kidneys, spleen and liver of transgenic mice from line A and *hPTTG* mRNA expression was

evaluated by TaqMan RT-PCR. As before, stong over-expression of *hPTTG* mRNA was demonstrated in the thyroid gland (28,143-fold, $n = 4$, $p < 0.001$). *hPTTG* mRNA was completely undetected in liver, heart, kidney and spleen ($n = 4$) but was mildly over-expressed in lung tissue (280.5-fold, $n = 4$, $p < 0.001$). As murine *Pttg* is not amplified by our hPTTG TaqMan RT-PCR primers, fold changes were calculated by comparing to the average WT background signal and are therefore not representative of absolute expression differences (see Figure 5-14).

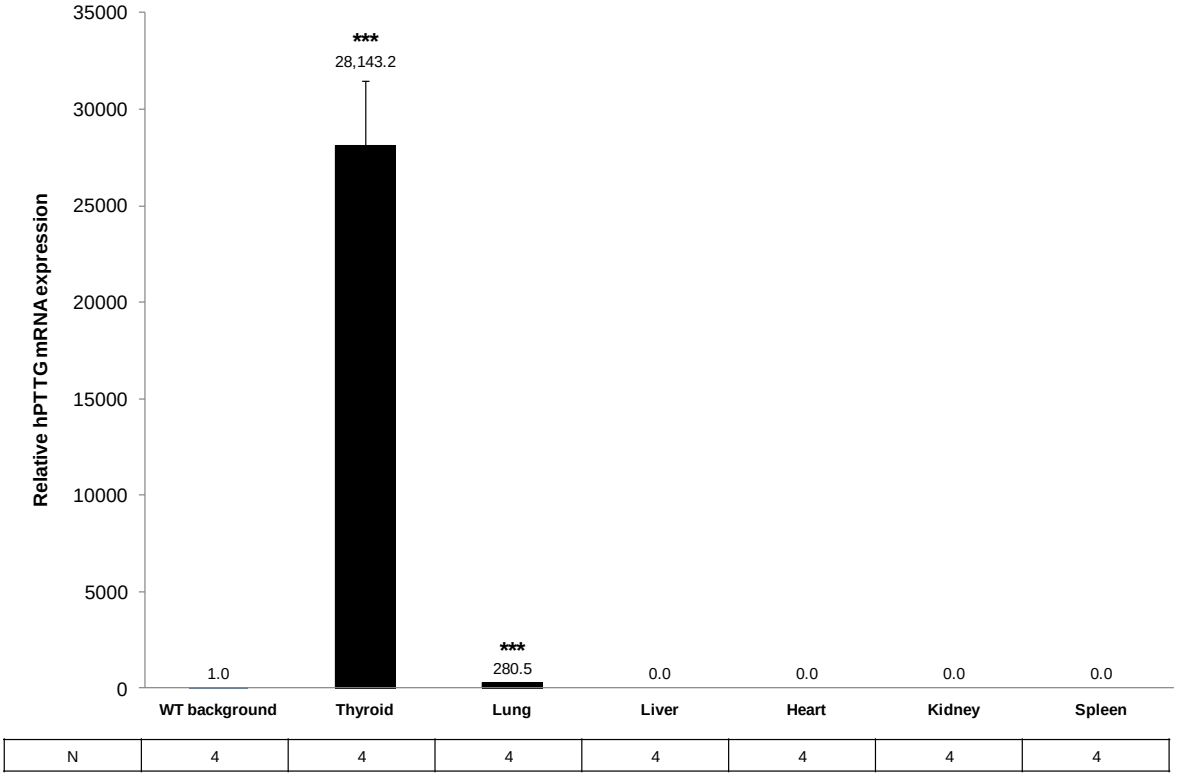


*Figure 5-14: TaqMan RT-PCR demonstrating significant over-expression of hPTTG mRNA in the thyroid glands compared to control organs of transgenic mice in line A. hPTTG mRNA was completely undetected in liver, heart, kidney and spleen control organs but relatively mild over-expression of hPTTG mRNA in lung tissue suggests partial activation of the transgene in the lung. Error bars represent the SEM (*** $p < 0.001$).*

5.3.3.5 Propagation and maintenance of a murine transgenic colony

Following confirmation of line A as an appropriate transgenic line, this line was propagated by strategically inbreeding hemizygous transgenic mice in order to fully establish

a murine colony with thyroid-specific over-expression of hPTTG for subsequent investigations (see Figure 5-15).

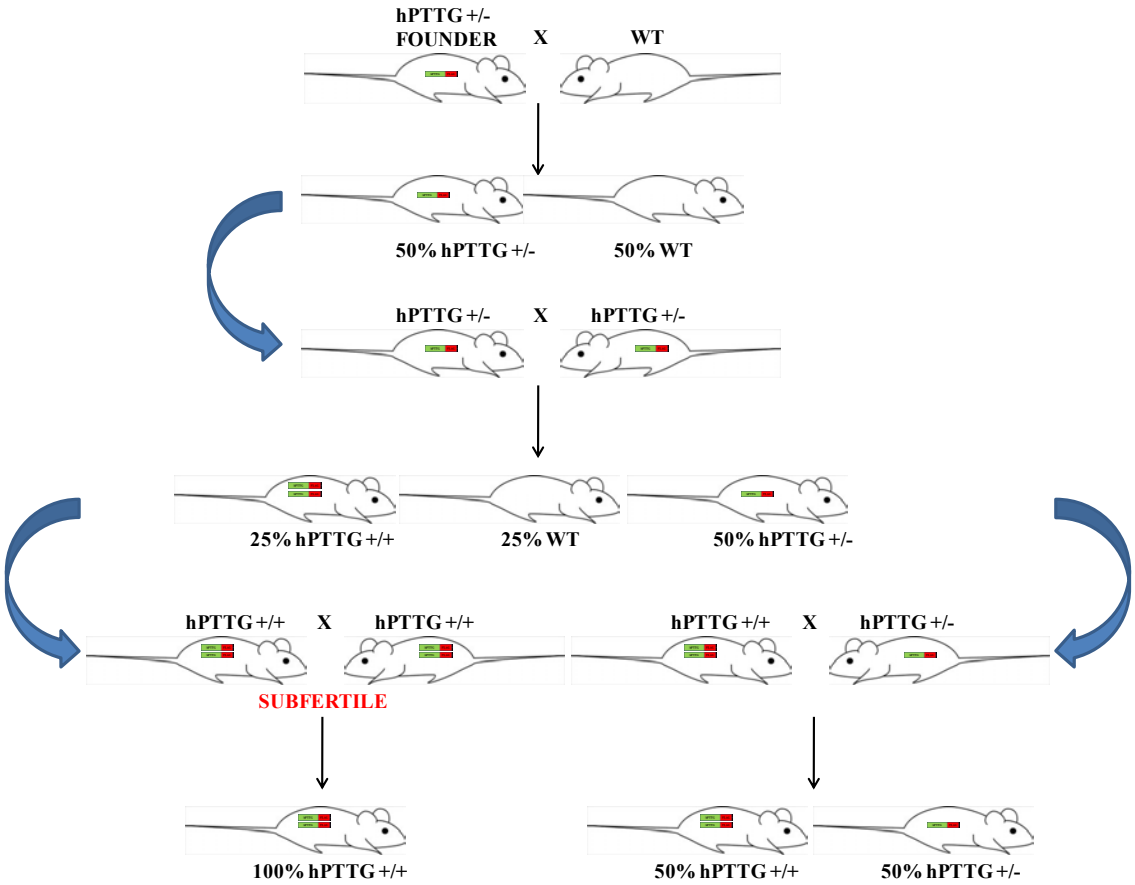
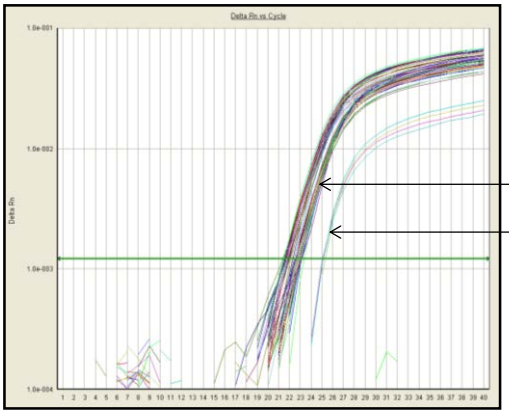


Figure 5-15: Schematic diagram of the inbreeding strategy employed to establish a homozygous murine transgenic colony with thyroid hPTTG over-expression.

DNA was extracted from tissue clippings taken from the offspring of hemizygote-hemizygote breeding pairs to determine the genotype of mice using a RealTime-PCR-based zygosity assay. *hPTTG* DNA was detected using the primer and probe set specifically designed for *hPTTG* amplification and results were normalised to expression of the *Dscam* genomic DNA control. From the zygosity assay it was predicted by comparison to human DNA controls that hemizygous mice have approximately 9 randomly inserted copies of the transgene, resulting in homozygous mice possessing approximately 18 (see Figure 5-16).

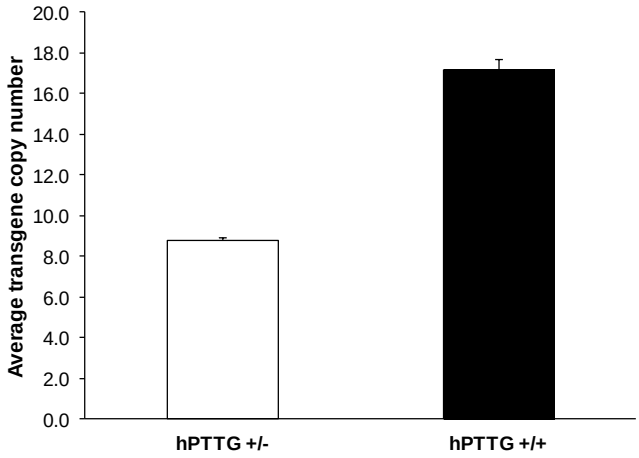
A.



B.

SAMPLE	DSCam	Average	Pttg Ct	Average	ΔCt	Ave ΔCt	ΔΔCt	Fold Change	Fold change*2	Zygosity
1	25.318	25.427	23.394	23.289	-1.924	-2.138	-2.138	4.402	8.803	hemizygote
	25.536		23.184		-2.352					
2	25.071	25.189	23.094	22.982	-1.977	-2.206	-2.206	4.614	9.228	hemizygote
	25.306		22.871		-2.435					
3	25.549	25.514	22.61	22.565	-2.939	-2.949	-2.949	7.722	15.444	homozygote
	25.478		22.519		-2.959					
4	25.221	25.319	22.109	22.228	-3.112	-3.091	-3.091	8.518	17.036	homozygote
	25.417		22.348		-3.069					
5	25.298	25.342	Undet.		Undet.	Undet.				WT
	25.386	25.342	Undet.		Undet.					
6	25.758	25.751	Undet.		Undet.	Undet.				WT
	25.744	25.751	Undet.		Undet.					
7	25.033	25.135	23.079	22.933	-1.954	-2.202	-2.202	4.600	9.199	hemizygote
	25.236		22.787		-2.449					
8	25.193	25.212	23.239	23.086	-1.954	-2.127	-2.127	4.368	8.736	hemizygote
	25.232		22.932		-2.3					
9	25.077	25.094	21.832	21.927	-3.245	-3.167	-3.167	8.979	17.957	homozygote
	25.111		22.023		-3.088					
10	25.105	25.105	22.048	22.035	-3.057	-3.071	-3.071	8.401	16.801	homozygote
	25.106		22.022		-3.084					
11	25.291	25.268	21.845	21.941	-3.446	-3.326	-3.326	10.028	20.057	homozygote
	25.244		22.038		-3.206					
Human Liver	26.132	26.078	26.774	26.738	0.642	0.661	Average ΔCt Human Controls 0.570			
	26.023		26.703		0.68					
Human Placenta	25.734	25.938	26.306	26.418	0.572	0.48				
	26.143		26.531		0.388					
DNA -ve control	Undet.		Undet.		Undet.					
	Undet.		Undet.		Undet.					

C.



	hPTTG +/-	hPTTG +/+
Mean copy number	8.80	17.14
N	16	9

*Figure 5-16: TaqMan Real-Time PCR zygosity assay. **A** Representative image of hPTTG amplification reaction curves for mouse DNA samples and human DNA controls. **B** Table demonstrating an example of mouse genotyping calculations. hPTTG is completely undetectable in WT mice, while hPTTG-Tg$^{+/+}$ mice have approximately double the amount compared to hPTTG-Tg$^{+/-}$ mice. **C** Histogram presenting the predicted copy number of the randomly inserted hPTTG transgene in hPTTG-Tg$^{+/-}$ and hPTTG-Tg$^{+/+}$ mice, determined by comparison to human DNA controls.*

In the first instance, mice identified as homozygotes were set up into homozygote-homozygote breeding pairs in order to generate a colony of homozygous mice for investigation of our hypotheses, removing the need to perform any further genotyping. However, it was identified early on that homozygous mice were subfertile with only 2 out of 8 homozygote-homozygote pairings able to reproduce and the finding of small litter sizes (≤ 6 pups). Homozygote-hemizygote breeding pairs were set up as an alternative approach to maintaining a transgenic colony and were significantly more productive, giving rise to both hPTTG hemi- and homozygous colonies for subsequent studies.

5.3.3.6 Further validation of hPTTG protein expression in transgenic mice

Following establishment of a transgenic line, hPTTG protein expression was fully validated in both hemi- and homozygous hPTTG transgenic mice. Having identified an optimal hPTTG antibody (Zymed laboratories), hPTTG protein expression in 6-week-old excised transgenic thyroid glands was demonstrated by Western Blot analyses, where there was an approximately 2-fold increase in homozygous mice compared to that of hemizygous mice, consistent with transgene copy number. This observation was reproduced in a further Western Blot that simultaneously demonstrated the thyroid specificity of hPTTG protein over-expression, where hPTTG protein was completely undetectable in protein extracts from lung, kidney, spleen and liver controls (see Figure 5-17).

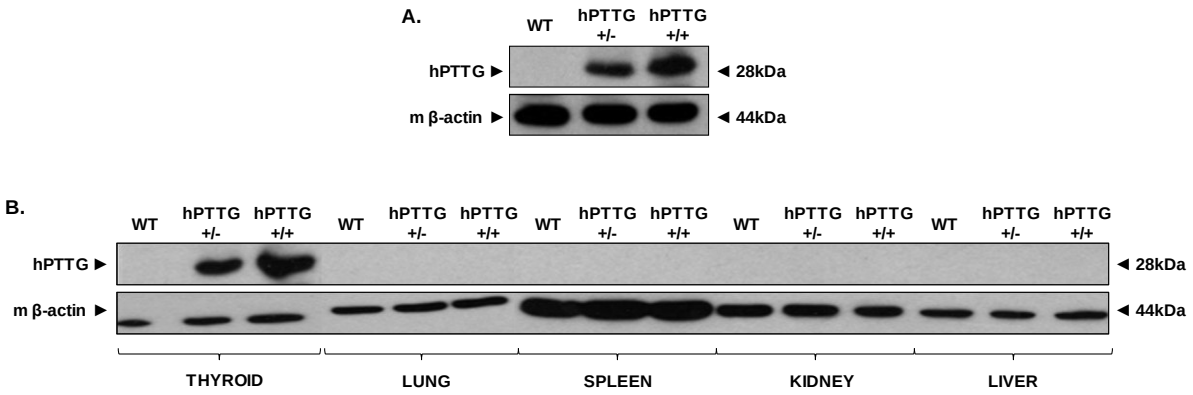


Figure 5-17: Western blot analyses confirming thyroid-specific hPTTG protein expression in transgenic mice from line A. ***A*** *Western blot analysis demonstrating an approximately 2-fold increase in hPTTG protein expression in hPTTG-Tg$^{+/+}$ mice compared to hPTTG-Tg$^{+/-}$ mice with none being detected in WT mice.* ***B*** *Further Western blot analysis demonstrating hPTTG protein expression in the thyroid glands but not in any of the other organs of transgenic mice.*

Analysis of hPTTG protein expression in the thyroids of transgenic mice was further studied by immunohistochemistry techniques using paraffin embedded thyroid tissue from WT, hemi- and homozygous hPTTG mice. As expected, hPTTG was undetectable in WT thyroid sections thereby confirming the specificity of the antibody to hPTTG. There was significant staining of thyroid follicular cells in thyroid sections from hPTTG hemizygotes that was further intensified in those from hPTTG homozygotes. At 100x magnification, it was clearly apparent that hPTTG was localised to both the cytoplasm and the nuclei of thyroid follicular epithelial cells, consistent with a role for hPTTG in both cellular compartments (see Figure 5-18).

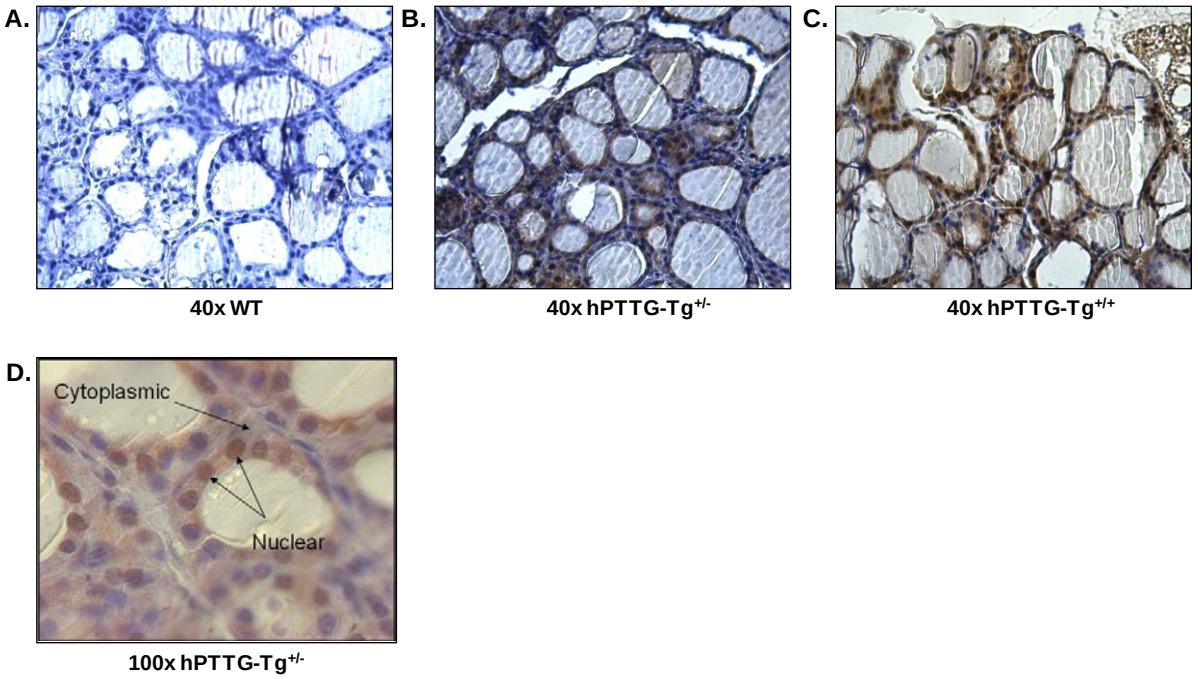


Figure 5-18: Immunohistochemistry demonstrating hPTTG expression and localisation in the thyroid glands of hPTTG transgenic mice. ***A*** *Lack of brown staining indicative of absence of hPTTG expression in WT mice.* ***B-C*** *Significant brown staining in hPTTG-Tg$^{+/-}$ sections (**B**) with increased intensity of staining in hPTTG-Tg$^{+/+}$ sections (**C**) consistent with thyroidal hPTTG protein expression levels dependent on transgene copy number.* ***D*** *Magnification at 100X clearly shows detection of hPTTG protein in both the cytoplasm and the nucleus of thyroid follicular cells in hPTTG transgenic mice.*

5.4 Discussion

The work reported in this chapter describes the successful generation of a murine model with thyroid targeted hPTTG over-expression. Key stages of the development of this transgenic model raise important discussion points and considerations regarding the complexity and limitations of such a model. Nevertheless, completion of this chapter of work represents an important milestone in the overall project, providing an *in vivo* model of hPTTG over-expression in the thyroid glands of mice for subsequent investigation of its role in thyroid pathogenesis and physiology.

5.4.1 Considerations of human PTTG over-expression in murine thyroids

As we set about our investigation, we had to decide whether to create a murine transgenic line with thyroidal over-expression of hPTTG or murine Pttg. Since *hPTTG* was cloned (Zhang et al. 1999b; Kakar and Jennes 1999; Dominguez et al. 1998), most studies have focused on characterisation of the functions and interactions of hPTTG. We therefore believed that the best approach to build on these studies was to over-express hPTTG, with the aim of validating some of the important findings of these previous studies. hPTTG protein shares 66 % sequence homology with murine Pttg (Wang and Melmed 2000a) and so we could expect that hPTTG functions similarly to murine Pttg. However, it was important for us to consider that the full functional properties of hPTTG may be compromised when over-expressed in a murine organ. Providing further confidence in our decision, two other groups observed phenotypes associated with over-expression of hPTTG in the pituitary glands (Abbud et al. 2005) and ovarian epithelium (El-Naggar et al. 2007) of transgenic mice. In addition, we believe that by over-expressing hPTTG, any phenotypes we observe will provide more compelling insight in relation to human thyroid disease.

5.4.2 Validation of transgene construct

Following a series of restriction enzyme digests and ligation steps, we successfully cloned *hPTTG-FLAG* into the *pBSK* plasmid downstream of the bovine thyroglobulin promoter. At this stage, we sought to validate the activity and specificity of the transgene by analysing hPTTG expression following transient transfections in human cell lines. Analysis by TaqMan RT-PCR revealed that *hPTTG* mRNA expression was specifically induced in FRTL5 cells, but not in non-thyroid cell lines. Direct sequencing of the *pBSK-hPTTG* plasmid demonstrated the correct nucleotide sequence and orientation of the construct, providing us with further confidence. Moreover, a parallel study by our own group successfully demonstrated transgenic PBF expression in the thyroids of transgenic mice,

having used an identical cloning strategy to that described in this study (Read et al. 2011). Together, these results provided enough evidence for the validity of our transgenic construct for us to proceed with microinjections.

5.4.3 Random integration of the transgene

Microinjection of exogenous DNA into the male pronuclei of fertilised oocytes was first described by Gordon et al. in 1980 where recombinant plasmid DNA derived from the bacterial plasmid *pBR322* was injected into the fertilised oocytes of CD-1 mice. DNA is injected at an early stage into the single-cell zygote to avoid mosaicism and becomes randomly integrated into the genome, if at all (Gordon et al. 1980). We observed around a 10 % success rate of random integrations (9/95 potential founders identified as positive for our transgene), which was considered highly successful. Interestingly, 8/9 of the lines derived from these transgenic founder mice failed to produce mice that over-expressed thyroid-specific hPTTG protein, with 1 founder considered infertile and 7 lines failing to demonstrate either enhanced thyroid hPTTG transcription or translation despite successful transmission of the transgene. This low success rate highlights the inefficiency and challenges faced in this approach to *in vivo* genetic manipulation. It is apparent that successful random integration of the transgene does not necessarily confer its desired activity and expression. Though the mechanisms are not fully understood, randomly inserted transgenes are highly susceptible to silencing and expression levels are thought to be influenced by the specific insertion site and the variable copy number inserted at this site (Clark et al. 1994; Dorer 1997; Garrick et al. 1998). However, we have successfully identified and established a transgenic line that overcomes this issue and expresses high levels of hPTTG in the thyroid glands of mice.

Another important issue concerning random integration is that the transgene may localise to part of the coding or regulatory regions of endogenous genes or is in fact completely deleterious, causing disruption of their normal function and inducing secondary

phenotypes. It is possible that the subfertility observed in homozygous mice is due to transgene insertion at an undesirable locus, where male subfertility and infertility are frequently reported in other transgenic models (Jhappan et al. 1994; Magram and Bishop 1991; Meng et al. 2002; Woychik and Alagramam 1998; Walters et al. 2009; Zhang et al. 2011). Alternatively, the effects of high thyroidal hPTTG expression may be embryonically lethal, resulting in reduced litter sizes. The latter seems more likely as the subfertility was not gender specific. Further investigations are required to provide a full explanation for this. However, it was important to consider the possibility of insertional mutagenesis during our characterisation studies (Chapter 6) as we tried to discern between specific and non-specific phenotypes.

Elegant studies have combined the use of a thyroglobulin promoter with the Cre-lox expression system to specifically alter thyroid gene expression *in vitro* and *in vivo* (Lin et al. 2004; Muller et al. 2011). Indeed, recent studies have generated highly targeted and inducible transgenic models using homologous recombination of Cre-lox and FLP-FRT systems in embryonic stem cells (Zwaka and Thomson 2003; Urbach et al. 2004; Suzuki et al. 2008; Di Domenico et al. 2008; Irion et al. 2007; Davis et al. 2008; Ruby and Zheng 2009; Zou et al. 2009; Sakurai et al. 2010). This approach enables site-specific insertion of a single copy of a transgene, thus overcoming mistargeting and multiple copy number issues, and we accept some of the limitations of the approach employed in this study.

5.4.4 Transgene copy number and expression levels

As mentioned above, a limitation of *in vivo* genetic modification by random integration of a transgene is that the total number of transgene copies inserted cannot be controlled. In a TaqMan Real-Time PCR based zygosity assay, it was predicted by comparing to human DNA controls that in our transgenic model hemi- and homozygous mice possessed approximately 9 and 18 copies respectively. The zygosity assay has proven to be

highly reliable and we observed consistent predictions of transgene copy number across multiple generations resulting in consistent genotyping throughout the study. Our results suggest that the founder mouse had ~9 transgene copy insertions on the same chromosome at the same integration site (probably in the form of head-tail concatemers, as is typically described in such models (Bishop and Smith 1989)) as opposed to 9 separate genome integration events in different genomic regions, which would have made for a highly complex transgenic model to manage.

In some transgenic models, severity of phenotype is directly proportional to transgene copy number. For example, high-copy RET/PTC1 transgenic mice have an earlier onset of papillary thyroid tumour formation, increased thyrocyte proliferation, aberrant follicle formation and reduced iodide uptake in comparison to low-copy number transgenics (Jhiang et al. 1998a). Having decided to maintain a colony of both hemi- and homozygous hPTTG mice, it has been of interest in downstream investigations to compare phenotypes for both genotypes in order to determine whether they are copy number dependent. Certainly, a clear increase in thyroid hPTTG protein expression was observed in homozygotes compared to hemizygotes, as determined by Western Blot and immunohistochemical analyses, and subsequent studies have sought to compare the functional outputs of this expression difference. However, even hemizygotes in our transgenic line have high thyroid expression of hPTTG (up to 28,000-fold *hPTTG* mRNA expression) and it would have been interesting to study an alternative line with much lower expression levels. Indeed, we might have expected to observe dose-dependent phenotypes of thyroid hPTTG over-expression given the *in vitro* findings in fetal brain NT-2 cells that low hPTTG over-expression causes enhanced proliferation while high levels of hPTTG over-expression has the opposite effect (Boelaert et al. 2003b).

Given the discussed disadvantages of this model, an alternative transgenic line would have been useful for parallel investigations in order to validate key phenotypes as being specific to thyroid hPTTG over-expression, and Chapter 8 describes the generation of an alternative line for this purpose. Unfortunately, with the failings of 8/9 transgenic founder lines, only one transgenic line was available for investigation at this stage.

5.4.5 Thyroid-specificity of hPTTG expression

In order to investigate the effects of increased thyroid hPTTG expression, it was of great importance to successfully target expression of the transgene specifically to the thyroid gland. As described, we aimed to achieve this through the creation of a construct where hPTTG expression is driven by the upstream activation of the bovine thyroglobulin promoter. Hypothetically, the bovine thyroglobulin promoter should only be activated in thyroid cells by thyroid-specific transcription factors such as TTF1, TTF2 and PAX8 (Civitareale et al. 1989; Mizuno et al. 1991; Lazzaro et al. 1991; Mascia et al. 1997; Plachov et al. 1990; di Magliano et al. 2000; Di Palma et al. 2003), but this promoter has not been fully characterised and it is possible that it has 'leaky' activity in non-thyroid cells, resulting in partial mistargeting of hPTTG expression. Strong induction of *hPTTG* mRNA expression was demonstrated in the thyroid glands of our transgenic mouse line compared to background. However, a relatively mild induction of *hPTTG* mRNA expression was detected in lung tissue from transgenic mice, where there was none in other control tissues. The group that first characterised the tissue-specificity of the bovine thyroglobulin gene promoter did so by generating transgenic mice that express the bacterial enzyme chloramphenicol acetyltransferase (CAT), and they reported entirely thyroid-specific CAT activity (Ledent et al. 1990). Subsequently other thyroid-specific transgenic models were generated using this promoter sequence (Ledent et al. 1991; Ledent et al. 1992). Another study used the same promoter to generate a murine transgenic line with thyroid over-expression of mutant guanine

nucleotide stimulatory factor (G_s) α-subunit, and specifically reported no expression in the lung tissue of transgenic mice following analysis of reverse transcripts by conventional PCR (Michiels et al. 1994). Two more recent studies on thyroid cancer used the same promoter sequence to drive thyroid expression of the $BRAF^{V600E}$ mutation and the RET/PTC1 gene rearrangement (Knauf et al. 2005; Jhiang et al. 1998a; Cho et al. 1999), and thyroid-specificity was declared, though data were not shown.

Taking these studies into account, it is tempting to speculate that our transgene has randomly inserted at a locus in close proximity to a promoter or enhancer that is specific to lung cells, thus causing some expression in the lung tissue of transgenic mice. However, in our group's murine model of thyroid targeted expression of PBF using the same promoter sequence, *PBF* mRNA expression was also detected in lung tissue from transgenic mice. Given that this model is likely to have originated from a random insertion at an altogether different locus, it seems that we are consistently detecting a specific activity of the bovine thyroglobulin promoter in the lung cells of mice. Thyroid transcription factor 1 (TTF1) is expressed in the lung where it regulates the expression of lung-specific genes (Guazzi et al. 1990; Boggaram 2009). It is therefore a possible explanation that TTF1 is capable of limited activation of the bovine thyroglobulin promoter in lung cells, but that this promoter is more strongly activated in thyroid cells where other thyroid-specific transcription factors are present. However, hPTTG protein expression was not demonstrated in lung tissue from transgenic mice, which was consistent with validation results in the PBF transgenic mouse study. It is possible that the increase in lung *hPTTG* transcription does not result in hPTTG translation, and that the mRNA may be targeted for degradation. Alternatively, the expression of lung hPTTG protein may be so low that it was undetectable by Western Blot analyses. Irrespective of these possibilities, it was important to consider if mistargeted

hPTTG expression was present, especially when characterising the hPTTG-Tg mouse phenotypes (Chapter 6).

Furthermore, hPTTG protein expression was observed by immunohistochemistry in both the cytoplasm and the nucleus. While hPTTG is predominantly a cytoplasmic protein (Chien and Pei 2000), its translocation to the nucleus facilitates its transforming and transactivational properties (see section 1.2.1.1). The presence of hPTTG in the nuclei of transgenic mouse thyroid follicular cells may be indicative of fully functional hPTTG protein. Thus, we would expect the phenotype corresponding to hPTTG over-expression to manifest in subsequent characterisation work.

5.4.6 Conclusion

It is clear from the work completed in this chapter that *in vivo* transgenesis can provide a variety of challenges. Through rigorous expression studies, development of a robust genotyping assay and strategic inbreeding, we were able to overcome some of these difficulties and generated a murine transgenic colony with thyroid-targeted hPTTG protein expression. This model provided us with a platform to directly study the effects of thyroidal hPTTG over-expression on thyroidal cell growth and tumourigenesis *in vivo* for the first time.

6 CHAPTER SIX

Characterisation of a Murine Model with Thyroid-Targeted hPTTG Expression

6.1 Introduction

Many studies to date have described that hPTTG acts as a critical transforming gene with significant oncogenic potential conferred by its induction of genetic instability and transactivation of other tumour promoting genes. However, direct *in vivo* investigations of hPTTG's tumourigenic capabilities are currently limited. The successful generation of a transgenic mouse model with thyroid-specific over-expression of hPTTG described in Chapter 5 has allowed us to directly investigate the hypothesis that thyroidal hPTTG over-expression induces thyroid growth and neoplasia.

Currently, only two other studies have investigated the effects of organ specific hPTTG over-expression. hPTTG was targeted to the pituitary glands of mice using the α-subunit of glycoprotein hormone (α-GSU) promoter, which is the earliest expressing pituitary hormone gene product. Female αGSU.PTTG mice had significantly enlarged pituitary glands and elevated serum IGF-1 levels compared with WT mice. However, the study focused on a more aggressive phenotype in male αGSU.PTTG mice, which demonstrated plurihormonal focal pituitary transgene expression with LH-, TSH- and GH-cell focal hyperplasia and adenoma, associated with increased serum LH, GH, testosterone, and IGF-I levels. Some mice died prematurely due to obstruction of the urinary tract, caused by prostate and seminal vesicle hyperplasia due to elevated serum hormone levels. Critically, this study provided evidence of a role for hPTTG over-expression in enhanced pituitary cell growth and specifically in the promotion of differentiated polyhormonal cell focal expansion (Abbud et al. 2005).

hPTTG is also over-expressed in ovarian cancers (Puri et al. 2001) and a transgenic model was generated to directly investigate the effect of hPTTG over-expression in the ovary (El-Naggar et al. 2007). Increased hPTTG expression was evident in the ovarian surface epithelium and granulosa cells of transgenic mice, driven by the Mullerian inhibitory

substance type II receptor gene promoter (MISIIR). Although female MISIIR-PTTG transgenics did not develop ovarian tumours, the study reported an overall increased mass of the corpus luteum, generalised hypertrophy of the myometrium of uteri, with cystic glandular and hyperplasia of the endometrium. The study concluded that hPTTG is involved with the initial transformation of ovarian epithelial cells and may be important in creating pre-cancerous conditions, but its over-expression alone is not sufficient for ovarian tumourigenesis (El-Naggar et al. 2007).

In addition to a potential role in cell transformation, hPTTG has transactivational properties that can promote tumour progression as discussed in detail previously (see sections 1.3.6 and 1.4). Our group's own studies have demonstrated hPTTG-mediated induction of FGF-2 and VEGF in thyroid cells (Boelaert et al. 2003a; Kim et al. 2006a) and we have elucidated further relationships between hPTTG, EGF, TGF-α and IGF-1 in thyroid cells, in studies described in Chapters 3 and 4. In addition, our group previously performed an angiogenesis cDNA PCR array following over-expression of hPTTG in human primary thyroid follicular cells. A number of pro-angiogenic genes such as *ID3* (3.5-fold induction), and anti-angiogenic genes such as *TSP-1* (2.5-fold reduction), demonstrated altered expression, indicating that hPTTG may represent a critical regulator of the angiogenic switch in thyroid tumourigenesis (Kim et al. 2006b).

Following on from the successful generation of a murine transgenic model (see Chapter 5), the aim of this study was to fully characterise this model to investigate the hypothesis that thyroid-targeted hPTTG over-expression will induce thyroid tumourigenesis. hPTTG-Tg mice were carefully monitored throughout the study and were evaluated macroscopically and histologically to determine phenotypes. Qualitative RT-PCR, murine primary thyrocyte culture and ELISA assays were used in order to verify our *in vitro* observations including the induction of growth factors. Further investigations were

performed in order to contrast some of these observations in the thyroid glands of $Pttg^{-/-}$ knockout mice. Angiogenesis cDNA PCR arrays were performed in order to broadly evaluate the effect of enhanced thyroidal hPTTG expression on genes involved in angiogenesis *in vivo*.

6.2 Materials and methods

6.2.1 Transgenic mice

hPTTG-Tg mice were generated and validated as described in Chapter 5. $Pttg^{-/-}$ knockout mice were kindly provided by Professor Melmed [UCLA, USA] (Wang et al. 2001). Transgenic hPTTG-Tg, $Pttg^{-/-}$ knockout and WT mice were bred at the University of Birmingham and all experiments performed in accordance with U.K. Home Office regulations.

6.2.2 Murine primary thyrocyte culture

Murine thyroid follicular cells were isolated and cultured from WT and hPTTG-Tg thyroid glands as described previously in section 2.3. This culture system was extensively validated in Chapter 7 (see section 7.3.2.2).

6.2.3 Post-mortem and macroscopic evaluation of transgenic mice

hPTTG-Tg mice that became ill were culled by cardiac puncture under terminal anaesthesia. Subsequently, mice were dissected and thorough post-mortem analysis performed with the guidance of expert mouse pathologists at the Biomedical Services Unit, University of Birmingham.

6.2.4 Mouse thyroid dissection

A microdissection technique was developed to routinely excise mouse thyroid glands for downstream analyses. In summary, the outer layer of fur and skin was removed to reveal the salivary glands, which are relatively large compared to their human counterparts. The salivary glands were pulled laterally to reveal the infrahyoid muscles running across the trachea. Using a dissecting microscope, these "strap muscles" were cut away to clearly expose the trachea, to which the thyroid gland is attached. Thyroid glands were then carefully excised, weighed and stored in formalin (histology), RNA later (RNA analysis), liquid nitrogen (protein analysis) or PBS (primary cell culture) (see Figure 6-1).

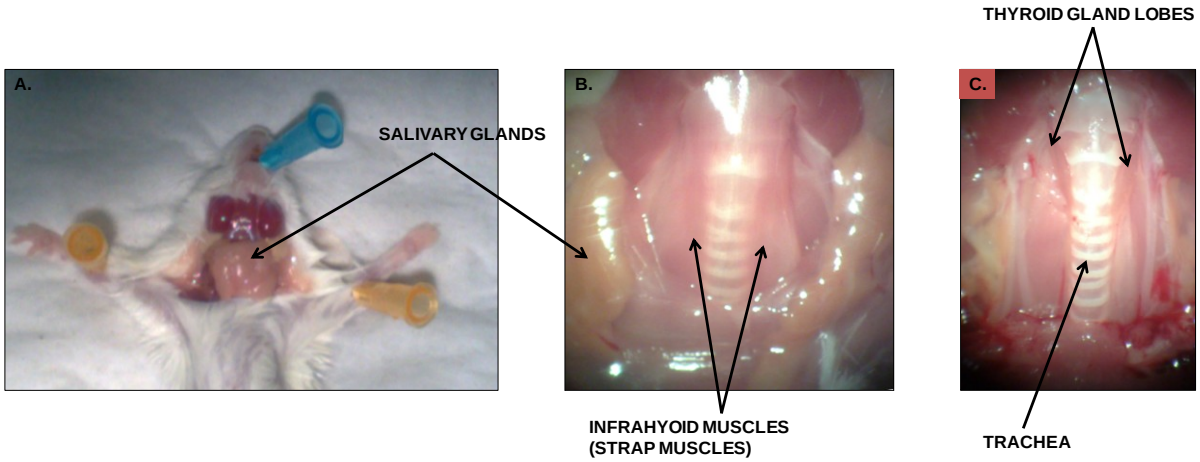


*Figure 6-1: Mouse thyroid dissection. **A** Fur and skin are cut away to expose neck region. **B** Salivary glands are pulled laterally to reveal infrahyoid muscles. **C** Infrahyoid muscles are cut away to expose thyroid lobes attached to the trachea.*

6.2.5 Histological evaluation of mouse thyroids

Thyroid glands were removed from mice aged between 1.5 and 18 months using a dissecting microscope and stored in formalin. Mouse thyroids were sectioned and subject to hematoxylin and eosin (H&E) staining performed by the Cellular Pathology Department, University Hospital Birmingham NHS Foundation Trust. Stained thyroid tissue sections were viewed under a light-microscope (Zeiss) and images captured using Axiovision software (Version 4). Histological analysis was performed with the guidance of expert

human thyroid histopathologist, Dr. Adrian Warfield (University Hospital Birmingham NHS Foundation Trust).

6.2.6 Serum thyroid hormone concentration

Mouse blood samples were extracted from mice by cardiac puncture and left to clot overnight at 4 ºC. Subsequently, bloods were centrifuged at full speed for 30 minutes at 4 ºC and the supernatant (serum) was stored at -80 ºC. Mouse serum TSH concentrations were determined by the laboratory of Professor Samuel Refetoff (University of Chicago). Details of this assay have been published (Pohlenz et al. 1999).

Total T_4 and total T_3 in serum of WT and hPTTG-Tg mice were measured using radioimmunoassay kits (MP Biomedicals). In brief, standards and mouse serum samples were added to tubes coated with either T_4 or T_3 monoclonal antibodies. Subsequently, a tracer solution containing radiolabelled T_4 or T_3 was added to the tube. Unlabeled and labeled analyte compete for a limited number of available binding sites on an antibody which has equal affinity for the standard and the analyte in the sample. Increasing quantities of unlabeled analyte reduce the amount of labeled analyte bound to antibody. The level of radioactivity bound is, therefore, inversely related to the concentration of analyte. At the end of a 1 hour incubation period, tubes were decanted and rinsed with distilled water before the bound radioactivity on coated tubes was counted using a gamma counter. The concentration of T_4 or T_3 in serum samples was determined by interpolation from a standard curve of % of Trace Level versus μg/dL T_4 or T_3.

6.2.7 RNA extraction, reverse transcription and QT-PCR

Total RNA was extracted from murine primary thyrocytes as described previously in section 2.4. Mouse thyroid glands were stored in RNAlater RNA stabilisation reagent at

-20 ºC before total RNA was extracted using the RNeasy microkit (Qiagen, UK), as per the manufacturer's instructions.

Subsequent reverse transcription and QT-PCR techniques were as described above (see sections 2.4 and 2.5). Murine gene-specific expression assays for *Pttg* (Mm00479224_m1), *Pbf* (Mm00521473_m1), *Vegf* (Mm01281449_m1), *Fgf-2* (Mm00433287_m1), *Tshr* (Mm00442027_m1), *Tg* (Mm01200340_m1), *Nis* (Mm01351811_m1), *Egf* (Mm00438696_m1), *Igf-1* (Mm00439560_m1), *Tgf-α* (Mm00446232_m1), *Egfr* (Mm00433023_m1) and *Igf1r* (Mm00802831_m1) were purchased from Applied Biosystems (Warrington, UK). Primers used to amplify *hPTTG* were those described in Table 3-1.

6.2.8 Enzyme-linked immunosorbent assays (ELISAs)

Conditioned medium was collected from WT and hPTTG-Tg murine primary thyrocytes 5 days following serum removal. Cell culture supernatants were prepared as previously described (see section 4.2.5). Egf and Igf-1 concentrations were assayed in 50 µl of the supernatant, using the Quantikine mouse EGF and mouse/rat IGF-1 enzyme-linked immunosorbent assays (R&D Systems, Inc.) as per the manufacturer's instructions described in brief previously (see section 4.2.5).

6.2.9 Angiogenesis-specific cDNA PCR arrays

RNA from mouse thyroid glands was obtained as described in section 6.2.7. RNA was converted to cDNA using the RT^2 First Strand Kit (SA Biosciences) as per the manufacturer's guidelines. Briefly, 25 ng of RNA was mixed with 2 µl of Genomic DNA Elimination Mixture and water in 10 µl reactions, and incubated at 42 ºC for 5 minutes before being chilled on ice. 10 µl of RT cocktail containing RT buffer (BC3; 4 µl), Primer and external control mix (P2; 1 µl), RT enzyme mix (RE3; 2 µl) and water (3 µl) was added to each 10 µl RNA mixture. Reactions were incubated at 42 ºC for 15 minutes before

termination by heating at 95 ºC for 5 minutes. Each 20 µl cDNA sample was diluted by addition of 91 µl of water. cDNA quality and specific expression of hPTTG and Nis was determined in samples by TaqMan RT-PCR, as described in section 6.2.7. Subsequently, 102 µl of cDNA was added to 1350 µl of 2X RT^2 SYBR Green qPCR Master Mix (SA Biosciences) and 1248 µl of water. 25 µl of experimental cocktails were loaded into each well of mouse angiogenesis-specific cDNA PCR array plates (96-well format) (# PAMM-024, SA Biosciences). PCR plates were sealed and centrifuged for 1 minute at 1000 g to remove bubbles. PCR reactions were performed using an ABI 7900HT Fast Real-Time PCR System. Reactions were as follows: 95 °C for 10 minutes; then 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Inspection of in-built controls determined that PCR array results were not affected by genomic DNA contamination or RNA impurities. Data were compared using the ΔΔCt method described in section 2.5, but by comparing genes of interest to an average of multiple house-keeping genes.

6.2.10 Statistical analysis

Data were analysed as described in section 2.8.

6.3 Results

6.3.1 Survival of hPTTG-Tg mice

Overall, survival of hPTTG-$Tg^{+/-}$ mice (75.7 ± 9.6 %, n = 43) was non-significantly decreased compared with WT mice (96.5 ± 2.4 %, n = 68) by 18 months of age ($p = 0.147$). In contrast, survival of hPTTG-$Tg^{+/+}$ mice (28.5 ± 8.1 %, n = 57) was strikingly reduced compared with either WT ($p < 0.0001$) or hPTTG-$Tg^{+/-}$ mice ($p < 0.0001$) by 18 months of age. Notably, hPTTG-$Tg^{+/+}$ mice became ill from ~3 months of age onwards, with 38.1 ± 6.8 % dying by 6 months of age. Comparison of survival trends between male (29.9 ± 10.5 %, n = 35) and female (24.2 ± 13.1 %, n = 31) hPTTG-$Tg^{+/+}$ mice demonstrated that both genders

of this genotype have decreased survival, with no significant difference observed by 18 months of age ($p = 0.702$) (see Figure 6-2).

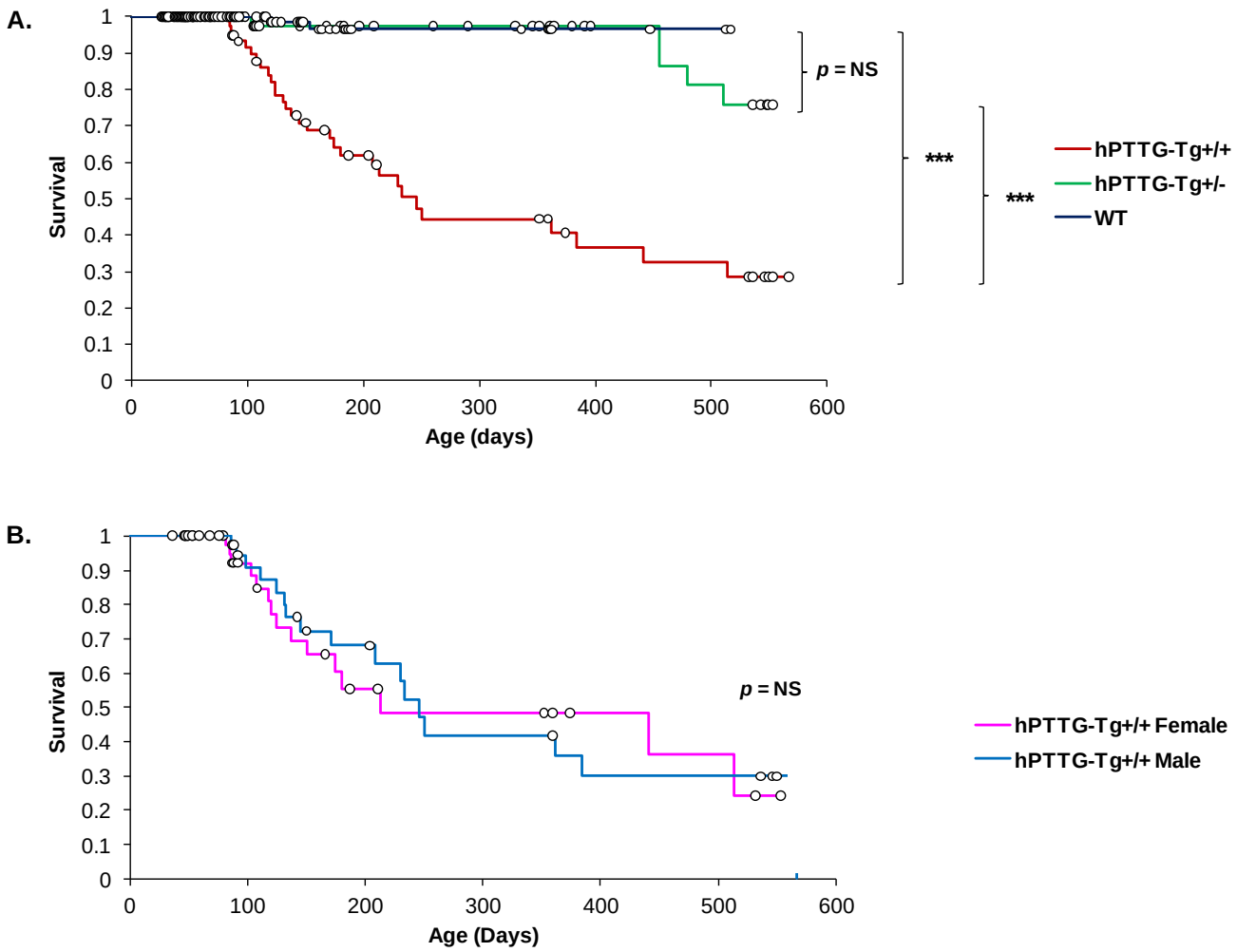


*Figure 6-2: Kaplan-Meier survival analyses. **A** Survival curves for WT (blue, n =68), hPTTG-Tg$^{+/-}$(green, n = 43), hPTTG-Tg$^{+/+}$(red, n = 57) mice demonstrating significantly reduced survival rates of hPTTG-Tg$^{+/+}$mice. **B** Survival curves for male (n = 35) and female (n =31) hPTTG-Tg$^{+/+}$mice demonstrating gender-independent phenotype. Circles represent censored event (mouse deliberately harvested for experiments), intersection represents spontaneous death of mouse (*** p < 0.001, NS = non-significant).*

Wherever possible, ill mice were culled by cardiac puncture under terminal anaesthesia prior to post-mortem evaluation. Subsequently, serum and histological analyses were performed to investigate cause of illness/death, as described in subsequent sections.

6.3.2 Total body weight of transgenic mice

Total body weight data were collected by weighing mice at the time of harvest at 1.5, 3, 6, 12 or 18 months of age. Overall, there were no significant differences in the total body weight of female hPTTG-Tg mice, except at 3 months of age, where hPTTG-$Tg^{+/+}$ females (n = 11) were significantly under-weight compared with WT females (9.5 % reduced weight, n = 23, $p < 0.01$). In contrast, a number of significant differences in total body weight of hPTTG-Tg males were identified. At 1.5 months of age, hPTTG-$Tg^{+/-}$ males (n = 4) were significantly over-weight compared with WT controls (12.5 % excess weight, n = 39, $p < 0.05$) and hPTTG-$Tg^{+/+}$ mice (14.2 % excess weight, n = 14, $p < 0.01$). At 3 months, hPTTG-$Tg^{+/+}$ males (n = 10) were significantly under-weight compared with WT controls (8.3 % reduced weight, n = 25, $p < 0.05$) and hPTTG-$Tg^{+/-}$ mice (9.4 % reduced weight, n = 20, $p < 0.01$). At 6 and 12 months there were no significant differences, but at 18 months of age, hPTTG-$Tg^{+/+}$ males (n = 5) were significantly under-weight compared with hPTTG-$Tg^{+/-}$ males (17.7 % reduced weight, n = 13, $p < 0.05$) (see Figure 6-3 A). However, throughout the study we observed great variation in weight data due to factors including parentage and litter size. In setting up hPTTG-$Tg^{+/+}$ x hPTTG-$Tg^{+/-}$ breeding pairs, we had an in-built control in that we could compare the weights of the resulting hPTTG-$Tg^{+/+}$ and hPTTG-$Tg^{+/-}$ littermates. We consistently observed that between ~ 3 and 7 weeks of age that both male and female hPTTG-$Tg^{+/+}$ mice were significantly under-weight compared to hPTTG-$Tg^{+/-}$ littermates. Representative results are presented in Figure 6-3 B and C.

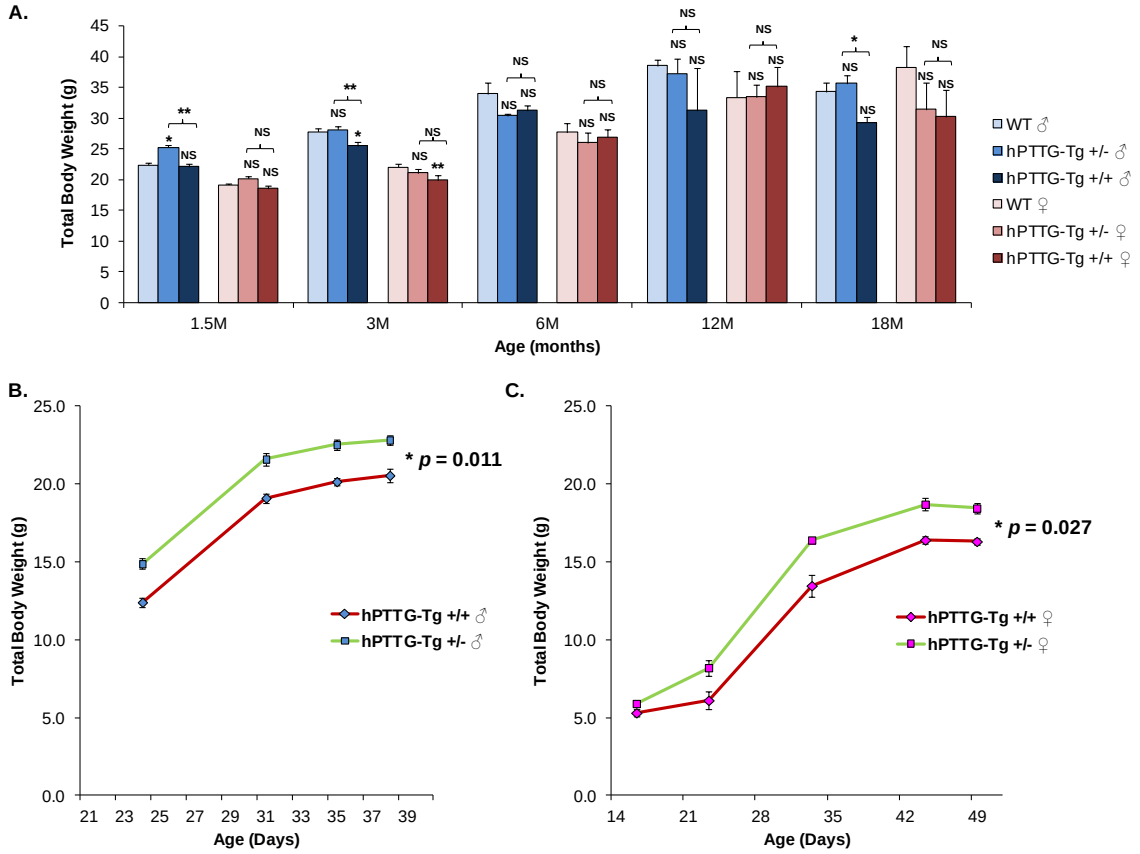
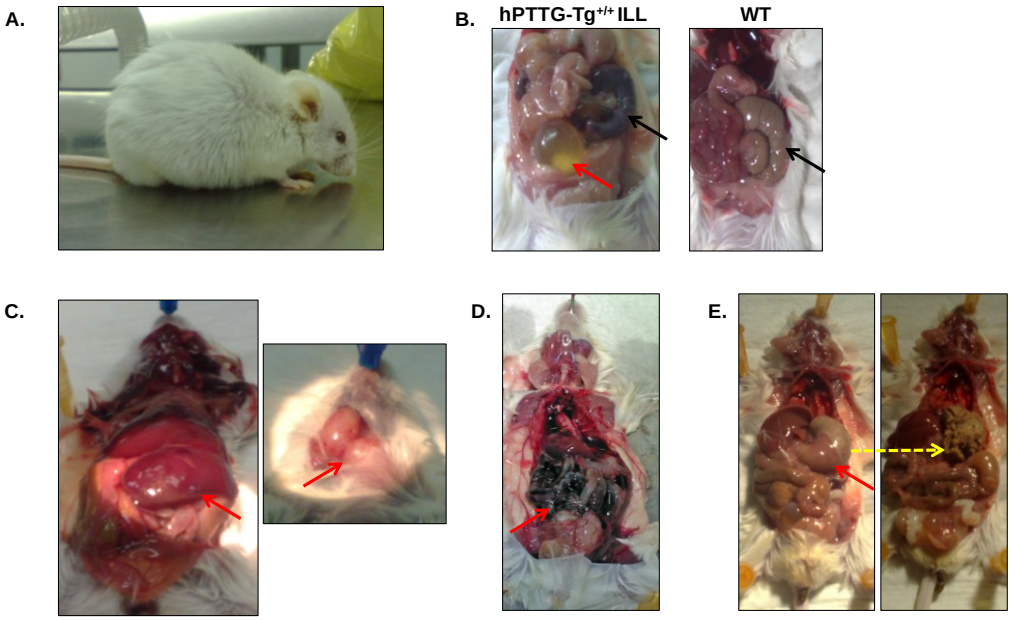


*Figure 6-3: Analysis of mouse total body weight data. **A** Bar chart displaying total body weight of WT, hPTTG-Tg$^{+/-}$ and hPTTG- Tg$^{+/+}$ mice (Males in shades of blue, females in shades of pink) at 1.5, 3, 6, 12 and 18 months of age. **B+ C** Representative total body weight analyses demonstrating reduced body weight of hPTTG- Tg$^{+/+}$ males (**B**) and females (**C**) compared with hPTTG-Tg$^{+/-}$ littermates. Error bars represent the SEM (* $p < 0.05$, ** $p < 0.01$, NS = non-significant).*

6.3.3 Evaluation of ill hPTTG-Tg mice

hPTTG-Tg mice that became ill were culled by cardiac puncture under terminal anaesthesia. Post-mortem examination of 3-8 month old hPTTG-Tg$^{+/+}$ mice that became ill rarely revealed any abnormalities that were macroscopically evident. Many of these mice were lean, had a distended bladder and an abnormal caecum that was shrivelled and compact. However, this was later described by a mouse pathologist as typical features found in non-specifically ill mice. One 2.5 month old female hPTTG-Tg$^{+/+}$ mouse that became ill had obvious enlargement of the salivary glands, kidneys, spleen and lymph nodes, and histopathologoical evaluation confirmed the diagnosis of B-cell lymphoma. An ill 3.5 month

old male hPTTG-Tg$^{+/+}$ mouse presented with an abnormal intestine, the cause of which remains unknown. Similarly, an 8 month old male hPTTG-Tg$^{+/+}$ mouse had a distended stomach containing a large amount of undigested food, and again the cause remains unknown. Crucially, thyroid glands in all ill hPTTG-Tg mice at this age (3-8 months) were of a normal size, with no macroscopic evidence of goitre or tumour formation (see Figure 6-4).



GENOTYPE	hPTTG-Tg$^{+/+}$	hPTTG-Tg$^{+/+}$	hPTTG-Tg$^{+/+}$
GENDER	♀	♂	♂
AGE	2.5 MONTHS	3.5 MONTHS	8 MONTHS
BODY WEIGHT	NA	18.5g	25.5g
THYROID WEIGHT	1.6mg	1.8mg	1.6mg
OBSERVATION	B-CELL LYMPHOMA (enlarged salivary glands, kidneys, spleen, lymph nodes)	ABNORMAL INTESTINE	DISTENDED STOMACH

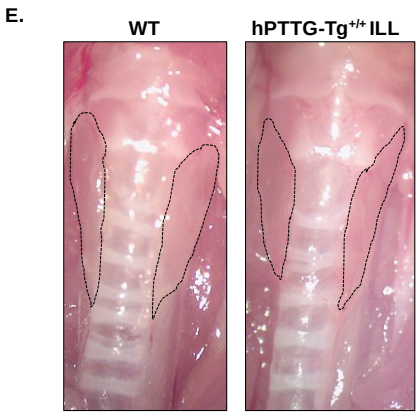
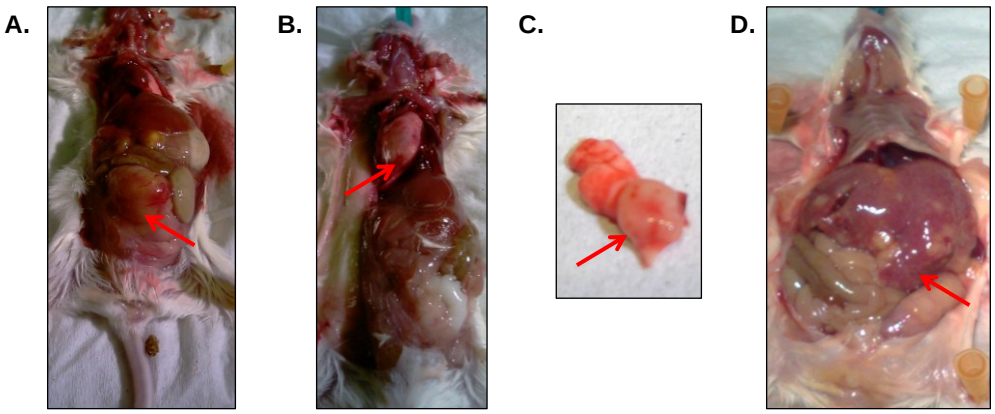


Figure 6-4: Macroscopic evaluation of ill hPTTG-Tg mice. ***A*** *Dishevelled and hunched appearance of an ill mouse.* ***B*** *Representative photographs of a frequent observation in both male and female hPTTG-Tg$^{+/+}$ mice that become ill between 3-8 months of age. A distended bladder is indicated by the red arrow. The caecum is indicated by the black arrow and is noticeably dark and compact in hPTTG-Tg$^{+/+}$ mice (left).* ***C*** *Photographs of a hPTTG-Tg$^{+/+}$ female with a B-cell lymphoma. Red arrows indicate enlarged spleen (left) and salivary glands (right).* ***D*** *Photograph of hPTTG-Tg$^{+/+}$ male with an abnormal intestine indicated by the red arrow.* ***E*** *Photograph of a hPTTG-Tg$^{+/+}$ male with a distended stomach indicated by the red arrow and its undigested contents by a yellow arrow.* ***F*** *Representative photographs demonstrating no macroscopically evaluated differences between the thyroid glands of WT (left) and ill hPTTG-Tg$^{+/+}$ (right) mice at ~ 3.5 months of age.*

hPTTG-Tg$^{+/-}$ and hPTTG-Tg$^{+/+}$ mice were aged to 12 and 18 months in order to study the long term effects of thyroidal over-expression of hPTTG. As hPTTG-Tg mice approached the 18 month time point, there were a number of mice that presented with pathologies. An 18 month old hPTTG-Tg$^{+/-}$ female had a large uterine tumour (2.3 g); two 18 month old hPTTG-Tg$^{+/-}$ males had lung tumours (0.98 g and 0.32 g); and one hPTTG-Tg$^{+/+}$ female had a cancerous liver (7.1 g) (see Figure 6-5).



	A.	B.	C.	D.
GENOTYPE	hPTTG-Tg$^{+/-}$	hPTTG-Tg$^{+/-}$	hPTTG-Tg$^{+/-}$	hPTTG-Tg$^{+/+}$
GENDER	♀	♂	♂	♀
AGE	18 MONTHS	18 MONTHS	18 MONTHS	17 MONTHS
BODY WEIGHT	26.6g	26.8g	30.0g	40.3g
THYROID WEIGHT	3.3mg	2.8mg	2.6mg	2.3mg
TUMOUR TYPE (Weight [g])	UTERINE - 2.3g	LUNG- 0.98g	LUNG- 0.320g	LIVER- 7.1g

*Figure 6-5: Pathologies identified in aged hPTTG-Tg mice (indicated by red arrows). **A** 18 month old hPTTG-Tg$^{+/-}$ female with a uterine tumour (2.3 g). **B** 18 month old hPTTG-Tg$^{+/-}$ male with a lung tumour (0.98 g). **C** Lung tumour from an 18 month old hPTTG-Tg$^{+/-}$ male. **D** 17 month old hPTTG-Tg$^{+/+}$ female with a cancerous liver (7.1 g).*

6.3.4 Evaluation of thyroid weight in hPTTG mice

Thyroid glands in aged hPTTG-Tg$^{+/-}$ and hPTTG-Tg$^{+/+}$ mice appeared to be of normal size compared to age-matched WT mice, with no macroscopic evidence of goitre or tumour formation (see Figure 6-6).

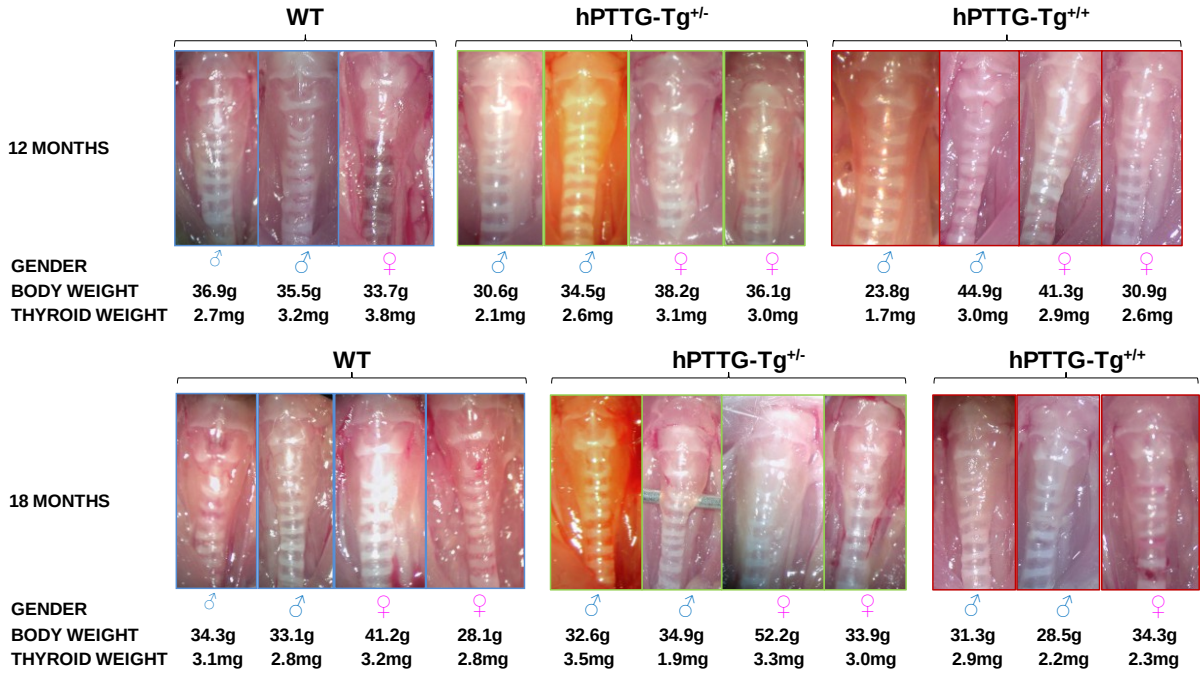


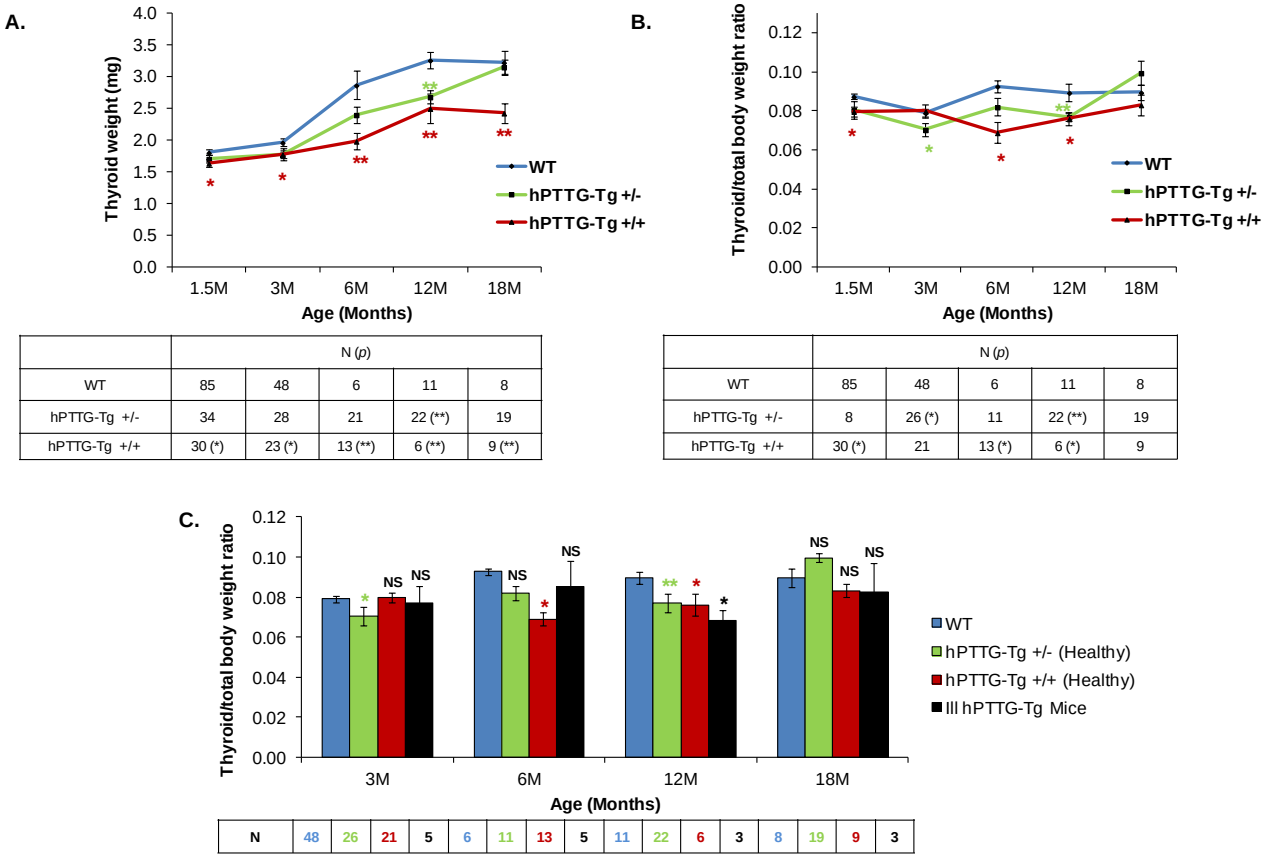
Figure 6-6: Macroscopic evaluation of thyroid glands in aged hPTTG-Tg mice. Representative photographs of thyroid glands in situ of WT (blue), hPTTG-Tg$^{+/-}$ (green) and hPTTG-Tg$^{+/+}$ (red) thyroids at 12 months (top row) and 18 months (bottom row). The gender, body weight and thyroid weight of each animal is also indicated beneath each photograph.

Thyroid glands were excised from mice and the weights were determined. Interestingly, hPTTG-Tg thyroids were reduced in size compared with WT, with a stronger phenotype evident in hPTTG-Tg$^{+/+}$ mice. hPTTG-Tg$^{+/-}$ thyroids demonstrated a consistent trend of reduced size compared with WT controls at all ages, though this difference was only statistically significant at 12 months ($p < 0.01$), where hPTTG-Tg$^{+/-}$ thyroids had a mean size of 2.68 ± 0.1 mg (n = 22) compared with 3.26 ± 0.13 for WT mice (n = 11). hPTTG-Tg$^{+/+}$ mice had significantly smaller thyroids at 1.5 months (1.64 ± 0.06 mg, n = 30, $p < 0.05$), 3 months (1.78 ± 0.05 mg, n = 23, $p < 0.05$), 6 months (1.98 ± 0.14 mg, n = 13, $p < 0.01$), 12 months (2.5 ± 0.23, n = 6, $p < 0.01$) and 18 months (2.43 ± 0.15, n = 9, $p < 0.01$) compared with age-matched WT thyroids, which had mean weights of 1.80 ± 0.04 mg (n = 85), 1.96 mg ± 0.06 (n = 48), 2.87 mg ± 0.22 (n = 6), 3.26 mg ± 0.13 (n = 10) and 3.23 mg ± 0.18 (n = 8) respectively (see Figure 6-7 A**).**

Given that hPTTG-Tg mice variously have reduced body weight (see section 6.3.2), it is possible that the reduced thyroid size observed is a consequence of the lower total body weight. We therefore also analysed weight data by calculating the thyroid:total body weight ratio of mice. Between 1.5 and 12 months of age hPTTG-Tg$^{+/-}$ mice still demonstrated a generally lower mean thyroid:total body weight ratio compared with WT controls. However, this was only statistically significant at 3 months (0.070 ± 0.004, n = 8, $p < 0.05$) and 12 months (0.077 ± 0.002, n = 22, $p < 0.01$), compared with age-matched WT controls, with a mean of 0.079 ± 0.002 (n = 48) and 0.090 ± 0.005 (n = 11) respectively. By 18 months of age, hPTTG-Tg$^{+/-}$ mice (0.099 ± 0.006, n = 19, p = NS) had a thyroid:total body weight ratio comparable to WT controls (0.089 ± 0.004, n = 8).

hPTTG-Tg$^{+/+}$ mice had significantly lower thyroid:total body weight ratios at 1.5 months (0.080 ± 0.002, n = 30, $p < 0.05$), 6 months (0.069 ± 0.005, n = 13, $p < 0.05$) and 12 months (0.076 ± 0.003, n = 6, $p < 0.05$) compared with age-matched WT controls, with mean ratios of 0.087 ± 0.001 (n = 85), 0.093 ± 0.003 (n = 6) and 0.077 ± 0.002 (n = 22), respectively. At 3 months (0.080 ± 0.004, n = 21, p = NS) and 18 months (0.083 ± 0.005, n = 9, p = NS) of age, there was no significant difference compared with WT control ratios of 0.079 ± 0.002 (n = 48) and 0.089 ± 0.004 (n = 8), respectively. See Figure 6-7 B.

Thyroid:total body weight ratios were calculated for ill hPTTG-Tg mice to confirm that these mice did not have enlarged thyroids. Data from ill mice was compared with WT data from the most relevant time point. At 3 (0.077 ± 0.009, n = 5, p = NS), 6 (0.085 ± 0.013, n = 5, p = NS) and 18 (0.083 ± 0.014, n = 3, p = NS) months of age, ill hPTTG-Tg mice showed no significant difference compared with WT ratios (detailed above). At 12 months of age, ill hPTTG-Tg mice demonstrated a significantly reduced mean ratio of 0.069 ± 0.005 (n = 3, $p < 0.05$) compared with 12 month WT mice (as above) (see Figure 6-7 C).



	N (*p*)				
WT	85	48	6	11	8
hPTTG-Tg +/-	34	28	21	22 (**)	19
hPTTG-Tg +/+	30 (*)	23 (*)	13 (**)	6 (**)	9 (**)

	N (*p*)				
WT	85	48	6	11	8
hPTTG-Tg +/-	8	26 (*)	11	22 (**)	19
hPTTG-Tg +/+	30 (*)	21	13 (*)	6 (*)	9

N	48	26	21	5	6	11	13	5	11	22	6	3	8	19	9	3

*Figure 6-7: Analysis of thyroid gland weights in hPTTG-Tg mice. **A** Comparison of raw thyroid weights in WT and hPTTG-Tg mice. **B** Comparison of thyroid:total body weight ratios in WT and hPTTG-Tg mice. **C** Bar chart form of data presented in **B** (3-18 months only), with additional comparative analysis of thyroid:total body weight ratio in ill hPTTG-Tg mice. WT (blue), hPTTG-Tg$^{+/-}$ (green), hPTTG-Tg$^{+/+}$ (red), ill hPTTG-Tg (black). Error bars represent the SEM (* $p < 0.05$, ** $p < 0.01$, NS = non-significant).*

6.3.5 Histological evaluation of hPTTG-Tg mouse thyroids

To examine mouse thyroid glands microscopically, we performed H&E staining on sections from hPTTG-Tg and WT mice. Composite images of complete thyroid lobes demonstrated normal thyroid histology in ill hPTTG-Tg$^{+/+}$ mice compared with WT mice (see Figure 6-8).

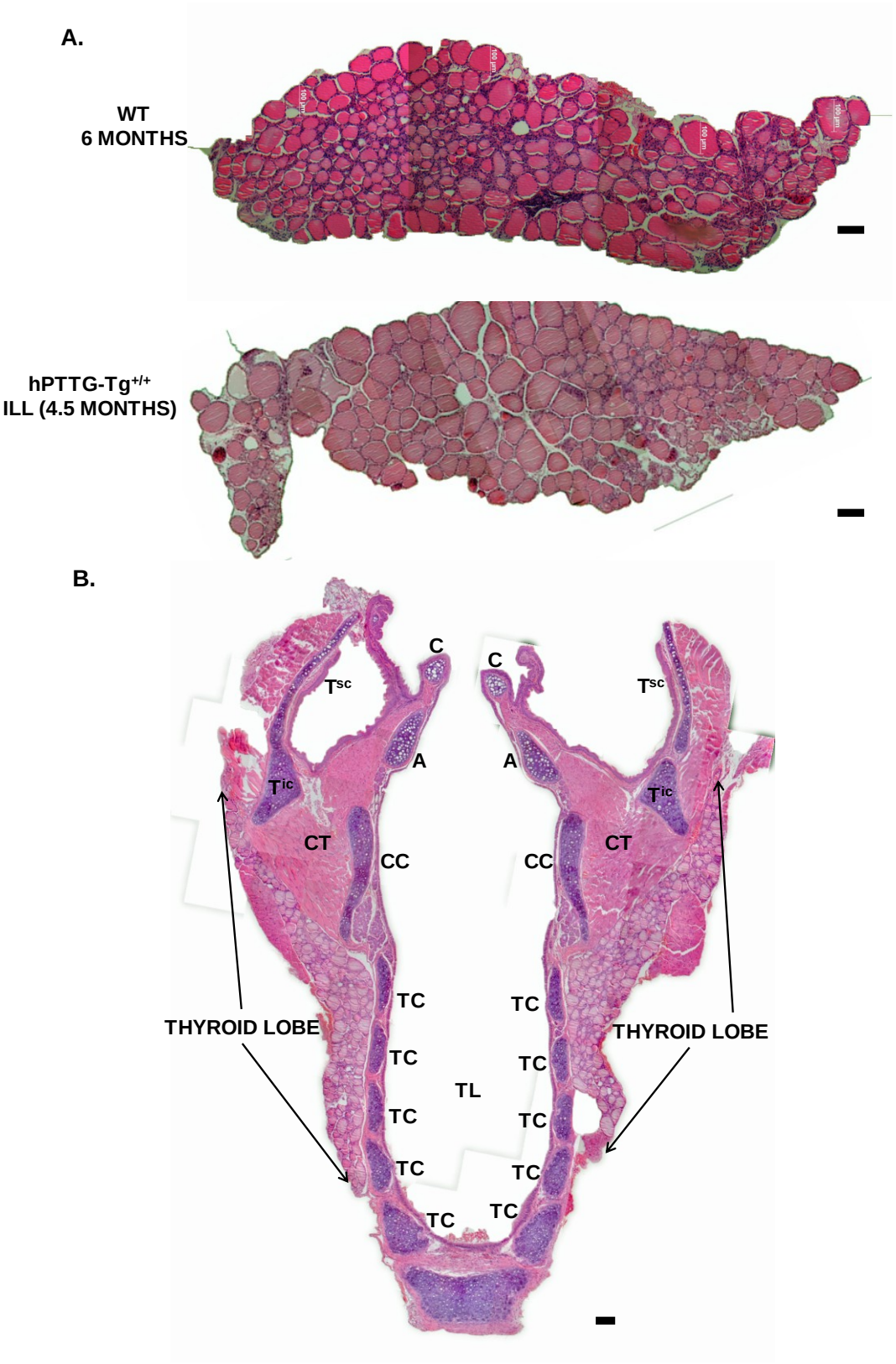


Figure 6-8: Histological analysis of thyroids from ill hPTTG-Tg$^{+/+}$ mice. ***A*** *Representative images of H&E stained thyroid sections from ill hPTTG-Tg$^{+/+}$ mouse and WT mouse (4.5-6 months of age).* ***B*** *Coronal section of neck region from ill hPTTG-Tg$^{+/+}$ mouse (age 3 months). Scale bars: 100 µm. TC = Tracheal cartilage, TL = Tracheal lumen, CC = Cricoid cartilage, CT = Cricothyroid muscle, T = Thyroid cartilage (ic = inferior cornu, sc = superior cornu), A = Arytenoid cartilage, C = Corniculate cartilage.*

Composite images of thyroid lobes from 12 and 18 month old hPTTG-Tg$^{+/-}$ and hPTTG-Tg$^{+/+}$ mice revealed no histological abnormalities and were comparable to age-matched WT controls (see Figure 6-9).

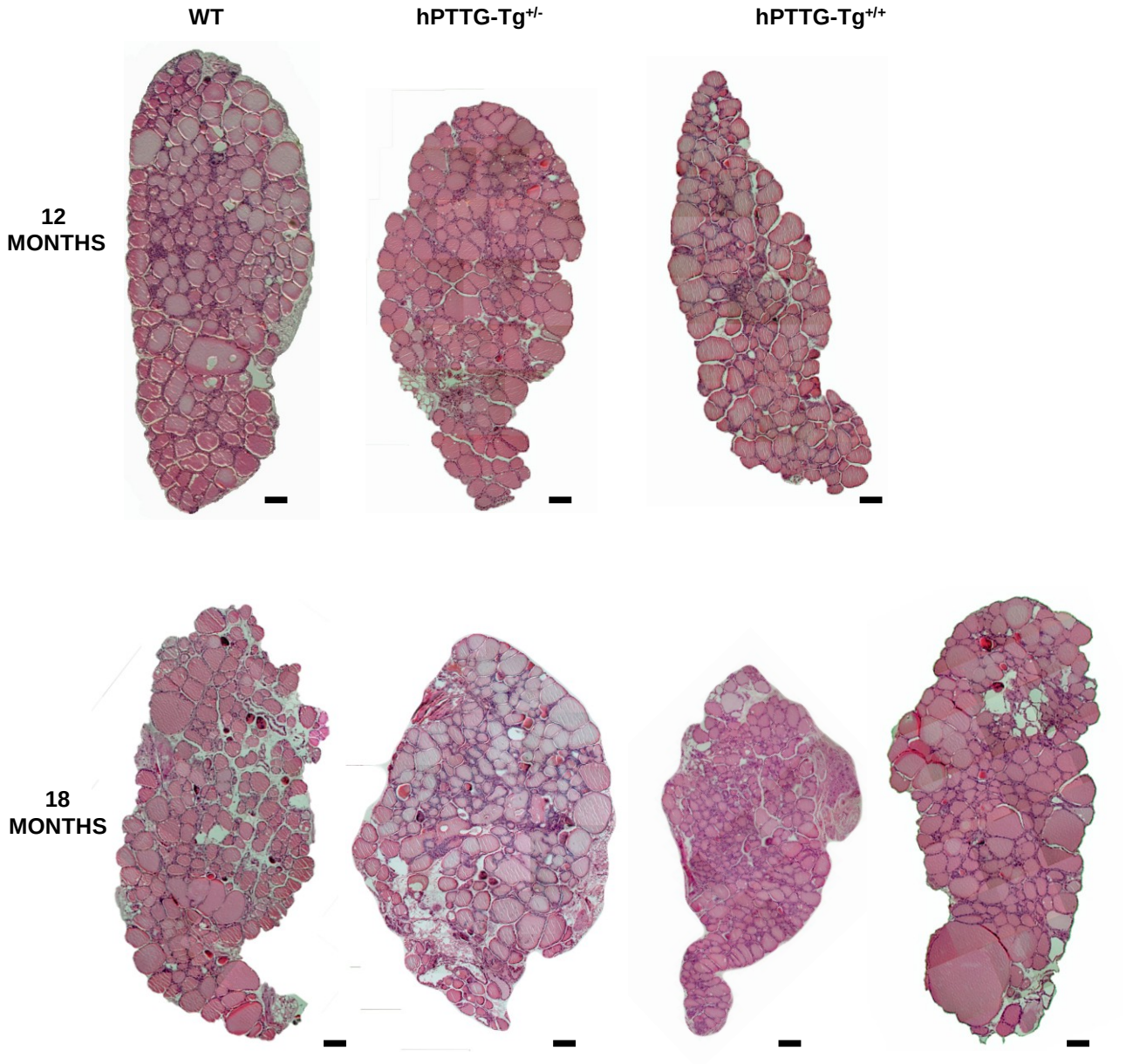


Figure 6-9: Histological analysis of thyroids from aged hPTTG-Tg mice. Representative images of H&E stained thyroid sections from 12 and 18 month old WT (left), hPTTG-Tg$^{+/-}$ (middle) and hPTTG-Tg$^{+/-}$ (right). Scale bars: 100 µm.

6.3.6 Thyroid hormone concentrations

Serum thyroid hormone concentrations were determined in order to assess the effect of transgene expression on thyroid function. Measurement of serum TSH concentration was restricted by sample availability (no samples available from ill hPTTG-Tg$^{+/+}$ male mice or 18 month old WT mice) and assay limitations. We therefore focused on characterisation of thyroid function in hPTTG-Tg$^{+/-}$ mice due to their increased rate of survival, but were able to include some samples from ill and aged hPTTG-Tg$^{+/+}$ mice to give an indication of their thyroid status. There was no significant difference in serum TSH concentrations in male hPTTG-Tg$^{+/-}$ mice at either 6 weeks (57.4 ± 12.9 ng/mL, n = 6, p = NS) or 12 months (89.8 ± 16.7 ng/mL, n = 3, p = NS) of age, compared with age-matched WT controls with average concentrations of 48.2 ± 13.9 ng/mL (n = 6) and 118.3 ± 7.2 ng/mL (n = 3), respectively. In contrast, serum TSH concentrations were below the limits of detection in hPTTG-Tg$^{+/-}$ females at 6 weeks of age. In accord with the lowest detectable value in the assay, hPTTG-Tg$^{+/-}$ females were assigned a nominal mean of 10 ng/mL which was significantly lower (n = 6, $p < 0.05$) compared with age-matched WT controls (24.1 ± 3.7 ng/mL, n = 6). There was no significant difference in serum TSH concentration in 12 month old hPTTG-Tg$^{+/-}$ females (25.4 ± 8.1 ng/mL, n = 3, p = NS) compared with age-matched WT females (28.2 ± 16.5 ng/mL, n = 3). Serum TSH concentrations in hPTTG-Tg$^{+/+}$females that became ill at ~ 3.5 months of age (29.7 ± 6.0 mg/mL, n = 5, p = NS) were comparable to those in 6 week old WT females (as above). 18 month old hPTTG-Tg$^{+/+}$ males (114.6 ± 44.6 mg/mL, n = 3, p = NS) and females (48.5 ± 1.6 mg/mL, n = 3, p = NS) had serum TSH concentrations that were comparable to 12 month old gender-matched WT controls (as described above) (see Figure 6-10).

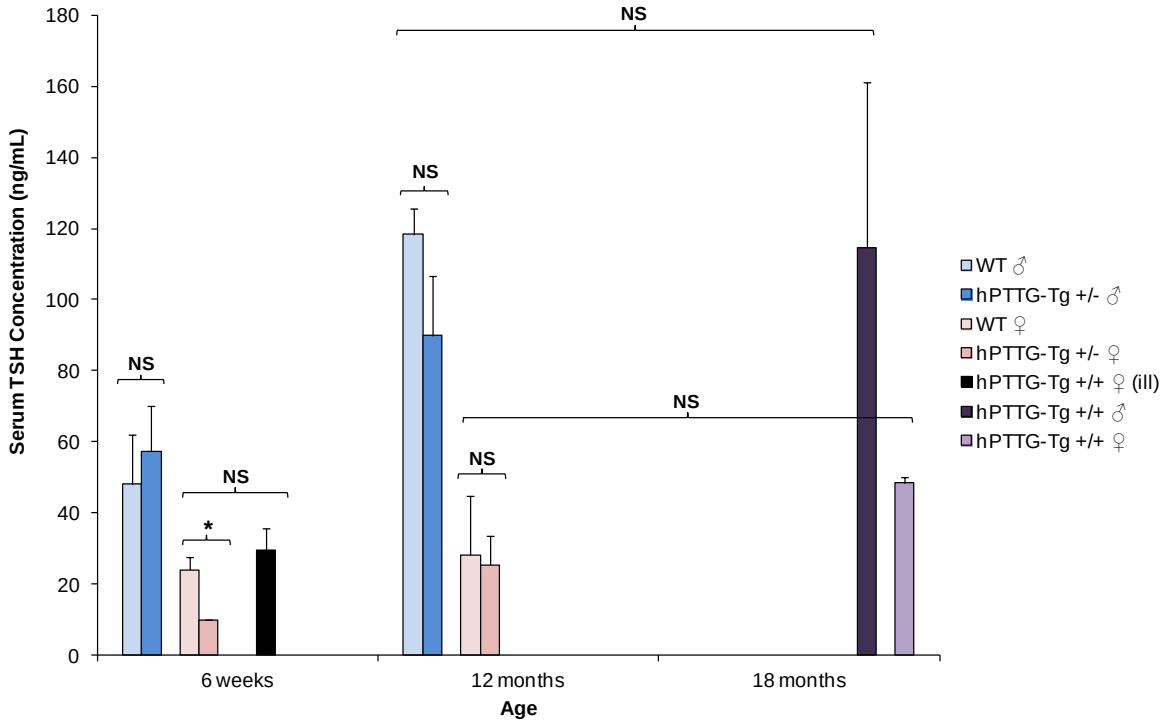


Figure 6-10: Serum TSH concentrations in 6 week and 12 month old WT (males – light blue, females – light pink) and hPTTG-Tg$^{+/-}$ mice (males – dark blue, females – dark pink). TSH concentrations in 3.5 month old ill hPTTG-Tg$^{+/+}$ females (black) were compared against 6 week old WT females. TSH concentrations in 18 month old hPTTG-Tg$^{+/+}$ males (dark purple) and females (light purple) were compared against gender-matched 12 month old WT mice. Error bars represent SEM ($p < 0.05$, NS = non-significant).*

Further analyses were performed to determine serum concentrations of total triiodothyronine (T_3) and thyroxine (T_4). Once again, 3.5 month old ill hPTTG-Tg$^{+/+}$ females were compared to 6 week old WT females, and since no 18 month old WT samples were available, 18 month old hPTTG-Tg mice were compared to gender matched 12 month old WT mice. There were no significant alterations of serum T_3 concentrations in hPTTG-Tg male or female mice at any time-point, compared with WT controls. Similarly, there were no significant alterations of serum T_4 concentrations in hPTTG-Tg female mice at any time-points and there were no marked alterations of total T_4 concentrations in aged hPTTG-Tg males. However, 6 week old hPTTG-Tg$^{+/+}$ (15.8 ± 3.8 ng/dL, n = 5, $p < 0.001$) males had significantly higher total T_4 concentrations compared with WT controls (2.0 ± 0.2 ng/dL, n = 6) (see Figure 6-11).

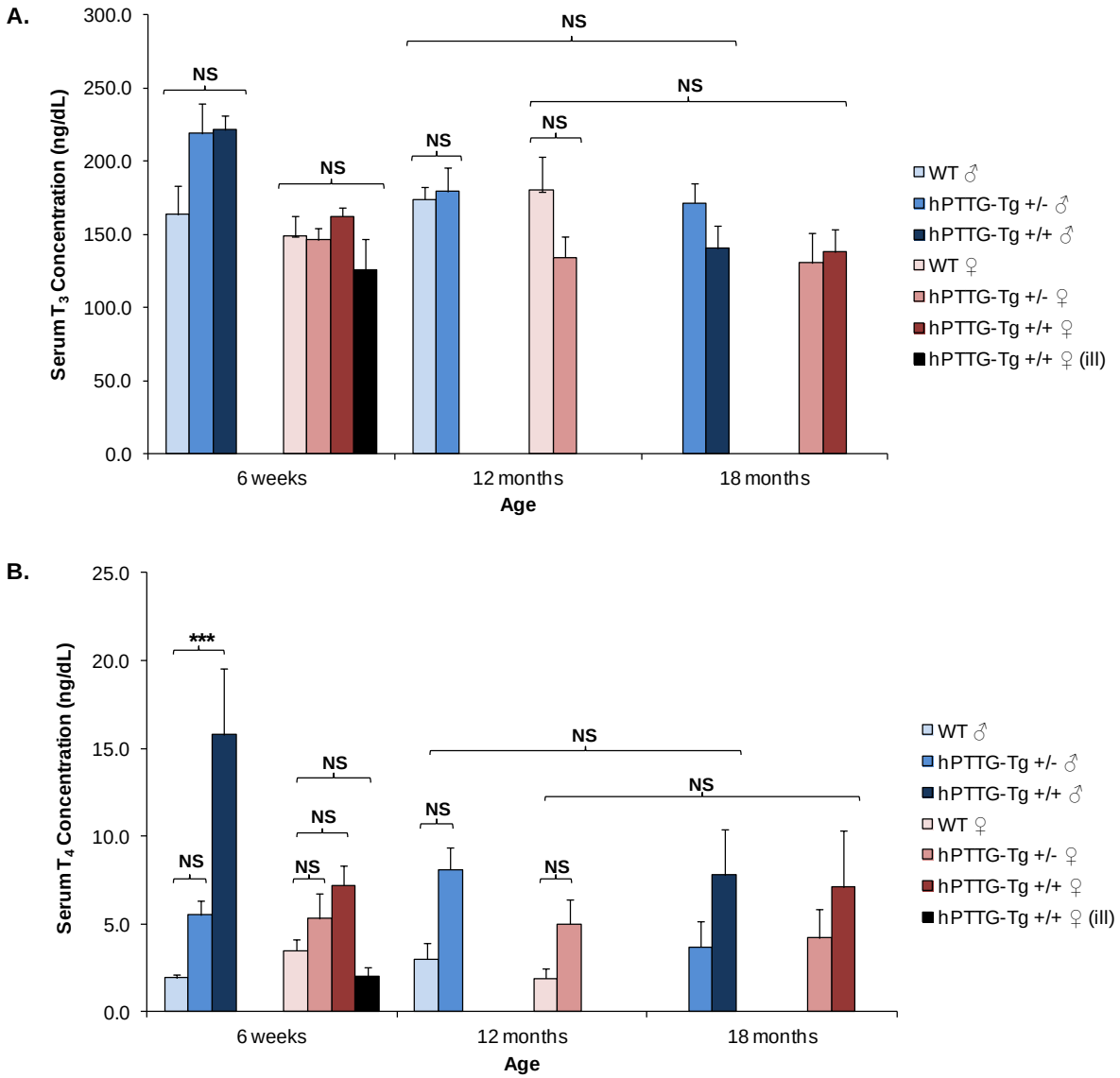


*Figure 6-11: Analysis of serum concentration of total triiodothyronine (T_3) [**A**] and thyroxine (T_4) [**B**]. WT (males – light blue, females – light pink), hPTTG-Tg$^{+/-}$ (males- middle blue, females – middle pink), hPTTG-Tg$^{+/+}$ (males – dark blue, females – dark pink). T_3 and T_4 concentration in 3.5 month old ill hPTTG-Tg$^{+/+}$ females (black) were compared against 6 week old WT females. T_3 and T_4 levels in 18 month old hPTTG-Tg mice were compared against gender-matched 12 month old WT mice. Error bars represent SEM (*** $p < 0.001$, NS = non-significant).*

6.3.7 Effects of thyroid-targeted hPTTG expression on gene expression

To further characterise hPTTG-Tg mice, we analysed thyroidal expression of mRNAs encoding endogenous *Pttg*, markers of thyroid differentiation (*Tshr, Tg and Nis*) and of those genes that have been shown previously to be induced by hPTTG, including *Pbf* (Stratford et al. 2005), *Vegf* (Kim et al. 2006b; Kim et al. 2006a) and *Fgf-2* (Boelaert et al. 2003a). Expression levels of *Nis* were part of an extensive study described in Chapter 7. *Pttg* levels

were unaltered in hPTTG-Tg$^{+/-}$ mice (0.51-fold, n = 11, *p* = NS) and hPTTG-Tg$^{+/+}$ mice (1.44-fold, n = 3, *p* = NS) compared with WT controls (n = 9). Similarly, *Pbf* levels were unaltered in hPTTG-Tg$^{+/-}$ mice (0.74-fold, n = 11, *p* = NS) and hPTTG-Tg$^{+/+}$ mice (0.86-fold, n = 3, *p* = NS) compared with WT controls (n = 9). Surprisingly, hPTTG-Tg$^{+/-}$ mice (1.22-fold, n = 12, *p* = NS) and hPTTG-Tg$^{+/+}$ mice (1.03-fold, n = 3, p = NS) had similar levels of *Vegf* compared with WT controls (n = 14). While hPTTG-Tg$^{+/-}$ mice had significantly elevated levels of *Fgf-2* (1.88-fold, n = 12, $p < 0.001$), hPTTG-Tg$^{+/+}$ mice unexpectedly demonstrated contrasting data with unaltered levels of *Fgf-2* (1.36-fold, n = 3, p = NS) compared with WT controls (n = 9). hPTTG-Tg$^{+/+}$ mice showed unaltered levels of *Tshr* (1.41-fold, n = 3, *p* = NS) and *Tg* (1.31-fold, n = 3, *p* = NS) compared with WT controls (n = 10) (see Figure 6-12).

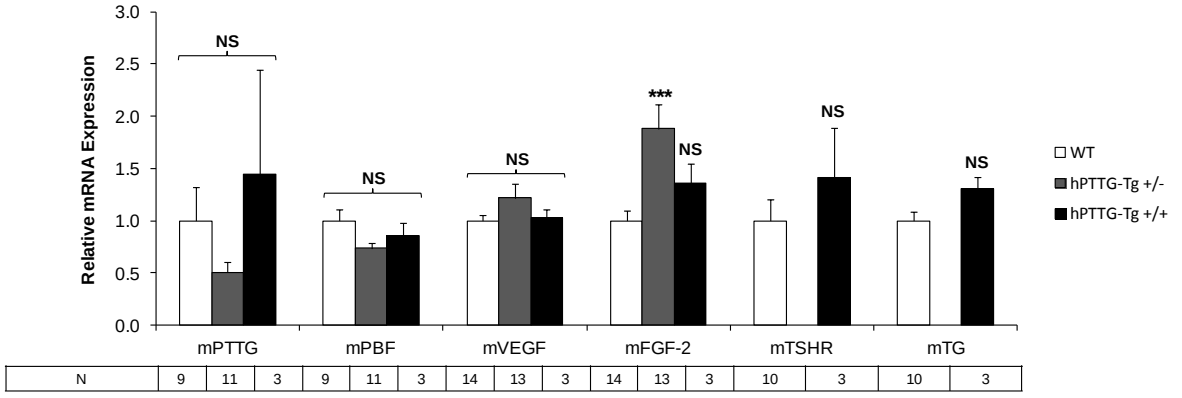


*Figure 6-12: TaqMan RT-PCR data displaying expression levels of mRNAs encoding Pttg, Pbf, Vegf, Fgf-2, Tshr and Tg in 6 week old WT and hPTTG-Tg mice. Error bars represent SEM (*** $p < 0.001$, NS = non-significant).*

6.3.8 hPTTG regulation of Egf, Igf-1 and Tgf-α *in vivo*

In Chapter 5, induction of EGF, IGF-1 and TGF-α mRNA by hPTTG was observed in human primary thyrocytes. To test this *in vivo*, thyroidal expression of *Egf, Igf-1* and *Tgf-α* were evaluated in 6 week old hPTTG-Tg mice. Thyroids excised from hPTTG-Tg$^{+/-}$ mice had similar levels of *Egf* (0.89-fold, *n* = 7, *p* = NS) and *Igf-1* (0.84-fold, *n* = 7, *p* = NS) mRNA expression compared with age-matched WT controls (*n* = 29). In contrast, *Egf* (2.02-

fold, n = 13, $p < 0.001$) and *Igf-1* (1.46-fold, n = 12, $p < 0.05$) levels were significantly elevated in hPTTG-Tg$^{+/+}$ mice compared with age-matched WT controls (n = 29). *Tgf-α* expression levels were only evaluated in hPTTG-Tg$^{+/+}$ mice (1.26-fold, n = 10, p = NS) where levels were not significantly altered compared with WT controls (n =11) (see Figure 6-13).

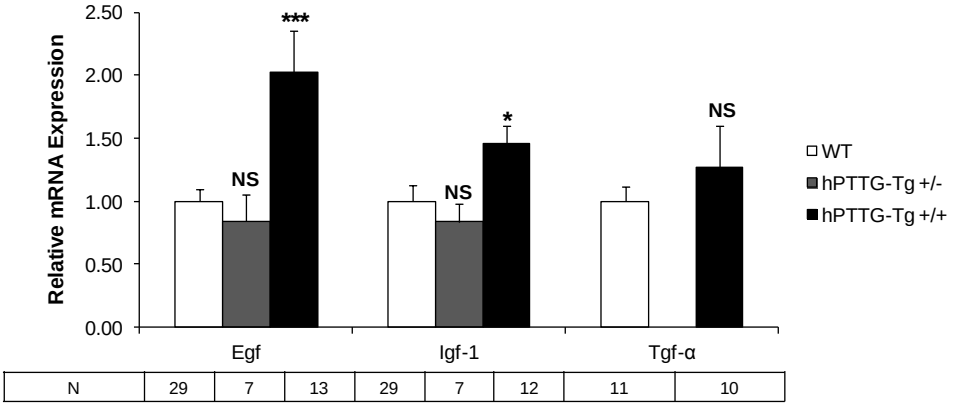


Figure 6-13: TaqMan RT-PCR analysis demonstrating mRNA expression levels of Egf, Igf-1 and Tgf-α in 6 week old WT and hPTTG-Tg mice. Error bars represent SEM (p < 0.05, *** p < 0.001, NS = non-significant).*

Subsequently, mouse primary thyrocytes were isolated and cultured from 6 week old WT and hPTTG-Tg mice (see Chapter 7 for in-depth details on mouse thyroid cell culture), and the conditioned media collected 5 days after serum removal were analysed by ELISAs specific to Egf and Igf-1. Consistent with the mRNA expression data, there was no significant increase in Egf secretion by hPTTG-Tg$^{+/-}$ transgenic thyrocytes (13.1 ± 2.1 pg/mL, n = 7, p = NS), but Egf secretion was significantly elevated in hPTTG-Tg$^{+/+}$ transgenic thyrocytes (20.9 ± 3.4 pg/mL, n = 12, $p < 0.01$) compared with WT controls (9.5 ± 2.0 pg/mL, n = 17). In contrast, thyroid cultures from either hPTTG-Tg$^{+/-}$ (217.5 ± 53.4 pg/mL, n = 13, p = NS) or hPTTG-Tg$^{+/+}$ (170.4 ± 20.0 pg/mL, n = 26, p = NS) transgenic mice showed no significant difference in Igf-1 secretion compared to WT controls (160.9 ± 23.5 pg/mL, n = 28) (see Figure 6-14).

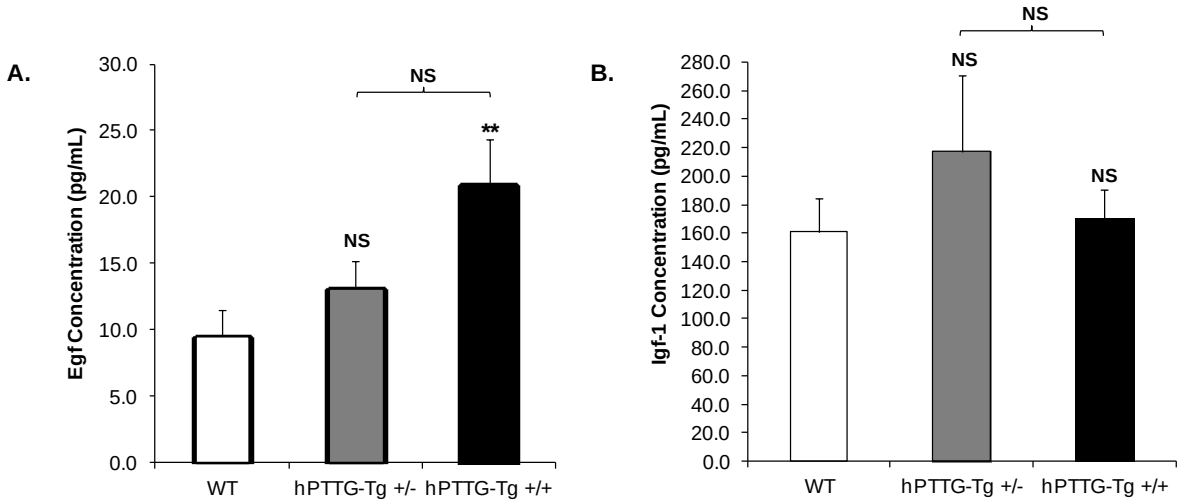


*Figure 6-14: Histograms showing secretion levels of Egf (**A**) and Igf-1 (**B**) respectively, from primary mouse thyrocytes derived from WT and hPTTG-Tg mice, determined by ELISA. Data are presented as the mean growth factor concentration (pg/mL) ± SEM. (** $p < 0.01$, NS = non-significant).*

Further qualitative RT-PCR confirmed that mouse primary thyrocytes expressed both Egfr and Igf1r, and thus have all components required to facilitate autocrine mechanisms involving Pttg and growth factors. There was no significant difference in *Egfr* or *Igf1r* expression between WT and hPTTG-Tg transgenic mice (see Figure 6-15).

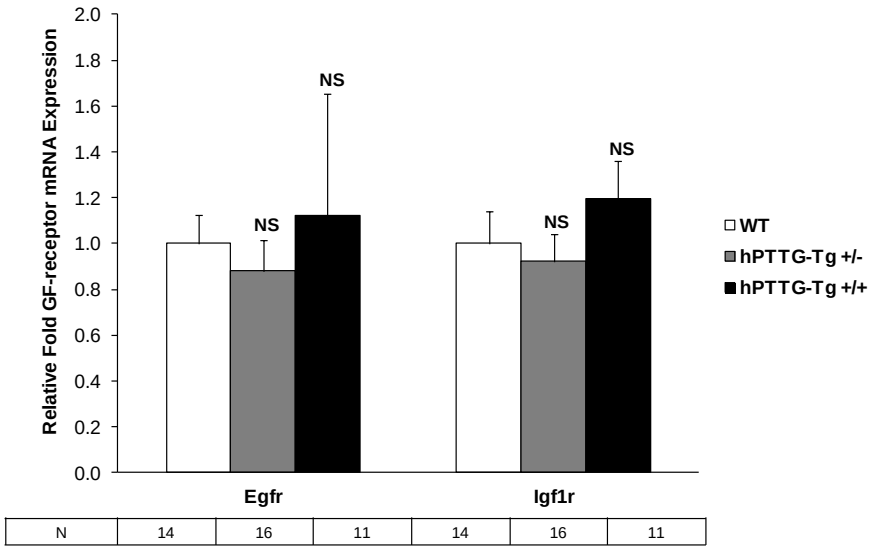


Figure 6-15: TaqMan RT-PCR analysis demonstrating mRNA expression levels of Egfr and Igf1r in WT and hPTTG-Tg mice. Error bars represent the SEM (NS = non-significant).

6.3.9 Expression of Egf, Igf-1 and Tgf-α in Pttg$^{-/-}$ knockout mice

To explore the effects of reduced endogenous PTTG expression *in vivo*, we next examined *Egf*, *Tgf-α* and *Igf-1* mRNA expression in thyroid glands of Pttg$^{-/-}$ knockout mice (Wang et al. 2001). Qualtitative RT-PCR analysis revealed a significant reduction in *Egf* mRNA expression in thyroid glands of Pttg$^{-/-}$ mice (0.4-fold, $n = 5$, $p < 0.01$), whereas *Tgf-α* (1.0-fold, $n = 5$, p =NS) and *Igf-1* (1.6-fold, $n = 5$, p =NS) mRNA expression levels were unaltered compared to WT controls ($n = 5$) (see Figure 6-16).

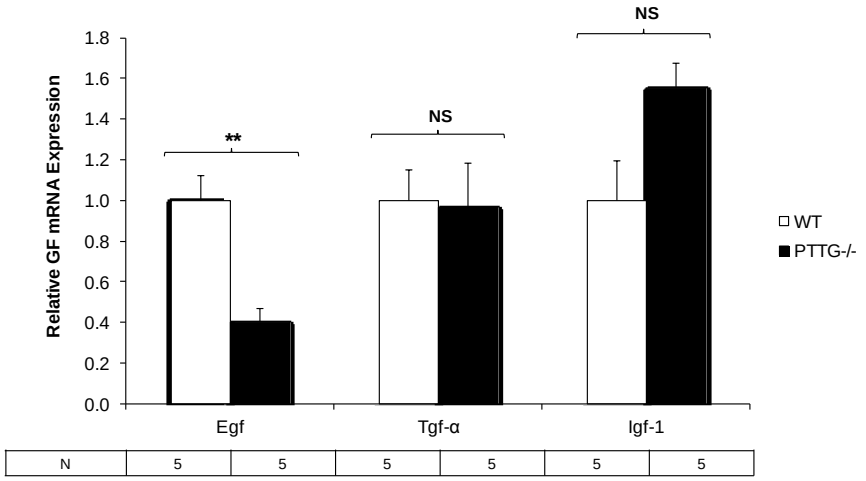


*Figure 6-16: TaqMan RT-PCR demonstrating reduced Egf and unaltered Tgf-α and Igf-1 mRNA expression in the thyroid glands of Pttg$^{-/-}$ knockout mice. Error bars represent the SEM (** $p < 0.01$, NS = non-significant).*

6.3.10 Angiogenesis PCR array analysis in hPTTG-Tg$^{+/+}$ thyroids

In order to analyse the effects of thyroidal hPTTG over-expression on the expression of genes involved in proliferation and angiogenesis, we performed angiogenesis cDNA PCR array analyses on mouse thyroid glands. Four separate PCR arrays were performed comparing hPTTG-Tg$^{+/+}$ mice ($n = 2$) with WT controls ($n = 2$). Given the variability of mRNA results observed thus far, specific hPTTG-Tg$^{+/+}$ mouse thyroid cDNA samples were chosen based on TaqMan RT-PCR validation of high *hPTTG* expression and low *Nis*

expression in accordance with the phenotype described in detail in Chapter 7 (see Figure 6-17).

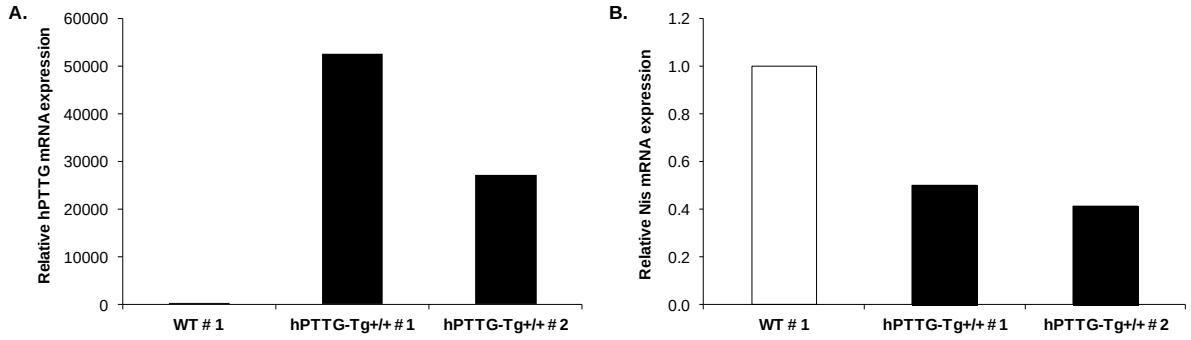


*Figure 6-17: Validation of individual hPTTG-Tg$^{+/+}$ thyroid cDNA samples for cDNA PCR array analyses. TaqMan RT-PCR data demonstrating high expression levels of hPTTG mRNA (**A**) and reduced Nis mRNA expression (**B**) in individual hPTTG-Tg$^{+/+}$ mouse thyroid glands.*

Subsequent PCR array analysis revealed various alterations in gene expression in hPTTG-Tg$^{+/+}$ thyroids compared with WT. These included changes in expression of Cytokine (C-X-C motif) ligand 1 (*Cxcl1*; 3.24-fold increase), Coagulation Factor II (*F2*; 2.06-fold increase), Fibroblast growth factor 6 (*Fgf-6*; 11.1-fold reduction), Midkine (*Mdk*; 2.34-fold increase), Matrix metallopeptidase 9 (*Mmp9*; 2.0-fold reduction), T-box 1 (*Tbx1*; 3.8-fold reduction), T-box 4 (*Tbx4*; 14.3-fold reduction), Tumour necrosis factor (*Tnf-α*; 2.0-fold reduction) and Tumor necrosis factor alpha-induced protein 2 (*Tnfaip2*; 2.16-fold increase). We also looked closely at other genes of specific interest including *Egf* (1.33-fold reduction), *Tgf-α* (1.43-fold increase), *Igf-1* (1.44-fold increase), *Vegf* (1.47-fold increase), *Fgf-2* (1.82-fold increase), *Tsp-1* (1.67-fold increase) and *Kdr* (1.46-fold increase) (see Figure 6-18).

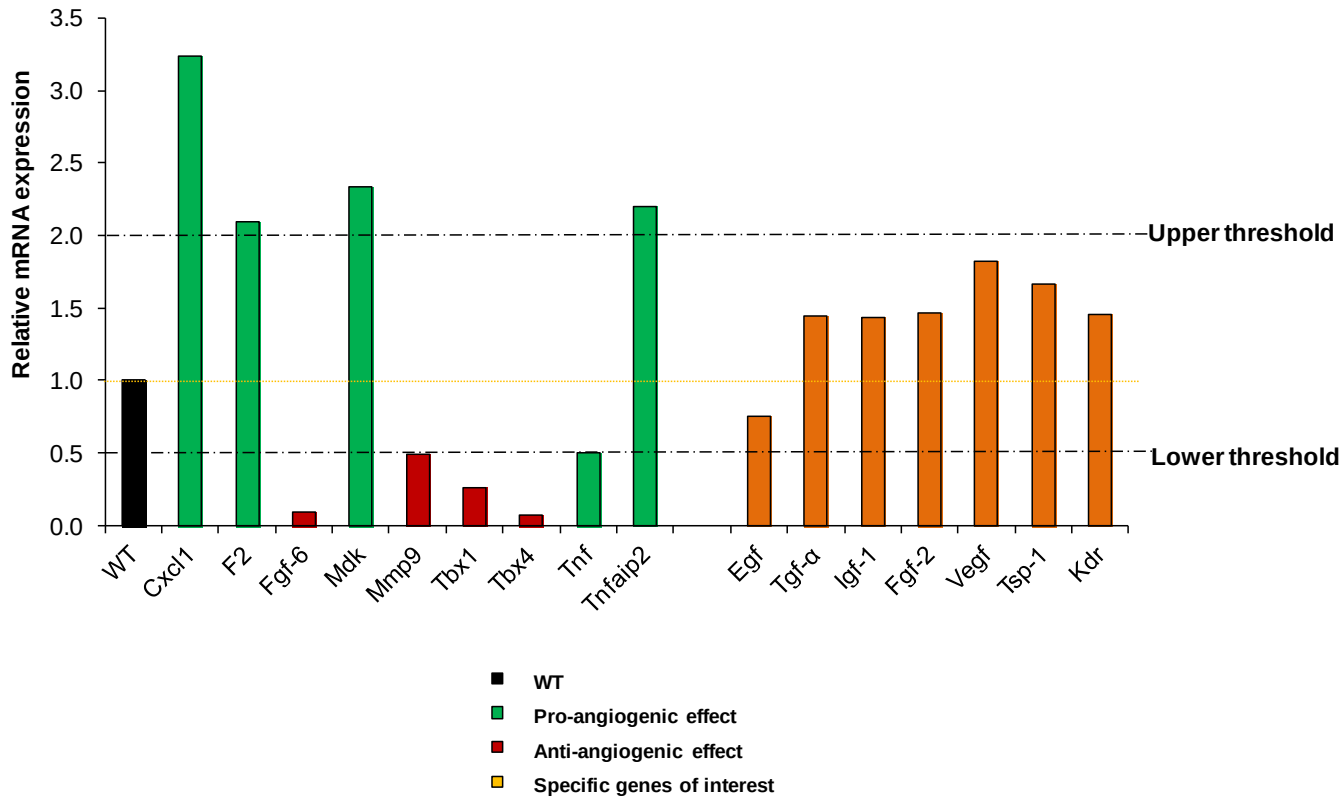


Figure 6-18: Angiogenesis cDNA PCR array analysis in hPTTG-Tg$^{+/+}$ thyroids compared with WT. Genes that demonstrated a 2.0-fold differential expression (either increased or reduced) in hPTTG-Tg$^{+/+}$ thyroids compared with WT controls were identified as significant and these thresholds are indicated by black dashed lines. Pro-angiogenic and anti-angiogenic gene expression alterations are shown in green and red respectively. The average fold change of other genes of specific interested are shown in orange.

6.4 Discussion

The mechanisms governing thyroid cell growth in hyperplasia and neoplasia remain to be fully defined, despite recent progress in our understanding of the role of other oncogenes such as BRAF (Knauf et al. 2005; Franco et al. 2011). The aim of the present chapter was to investigate the hypothesis that thyroidal over-expression of hPTTG would induce thyroid growth and neoplasia. Following the successful generation of a murine transgenic model with thyroid-targeted hPTTG expression (see Chapter 5), the current chapter of work describes the characterisation of this transgenic model.

6.4.1 Survival of hPTTG-Tg mice

Given that various murine models of thyroid cancer developed cancers from 6 months onwards (Knostman et al. 2007a; Kim and Zhu 2009), hPTTG-Tg mice were aged up to 18 months in order to study the potential development of thyroid cancers. In our concurrent investigation of transgenic mice with thyroidal PBF over-expression, PBF-Tg mice demonstrated a higher incidence of macrofollicular lesions and hyperplasia at 18 months of age compared with earlier time-points (Read et al. 2011). Strikingly, only 28.5 % of hPTTG-$Tg^{+/+}$ survived to 18 months of age and many became ill from ~ 3 months onwards. In contrast, hPTTG-$Tg^{+/-}$ mice generally survived to 18 months of age, with a survival trend comparable to WT mice. There was no significant difference in survival trends between male and female hPTTG-$Tg^{+/+}$ mice suggesting that this phenotype was independent of gender. Ill hPTTG-$Tg^{+/+}$ mice were subject to extensive investigations to determine a cause of death. Crucially, thyroids from ill hPTTG-$Tg^{+/+}$ mice were microscopically and histologically normal. Further post-mortem evaluation with the support of expert mouse pathologists revealed no consistent cause of death in these mice, where frequent observation of a distended bladder or abnormal gut were dismissed as typical effects seen in any ill mouse.

There were no consistent differences observed between genotypes of mice when evaluating mouse total body weight data across all time points, though general trends suggested both male and female hPTTG-$Tg^{+/+}$ mice were under-weight compared with WT and hPTTG-$Tg^{+/-}$ mice. Total body weight varied considerably and was dependent on factors such as litter size and parentage, and this was investigated more closely. Following our strategy of having hPTTG-$Tg^{+/-}$ X hPTTG-$Tg^{+/+}$ breeding pairs (see section 5.3.3.5), total body weights of the resulting hPTTG-$Tg^{+/-}$ and hPTTG-$Tg^{+/+}$ offspring were compared, which confirmed that male and female hPTTG-$Tg^{+/+}$ mice were consistently under-weight compared with hPTTG-$Tg^{+/-}$ littermates between 3 and 7 weeks of age.

In a mouse model of inherited hypothyroidism, hyt/hyt autosomal recessive mutants demonstrate similarly retarded growth from weaning age into adulthood, in association with low serum T_4 and elevated serum TSH (Beamer et al. 1981). However, analysis of serum thyroid hormone levels in ill hPTTG-$Tg^{+/+}$ females revealed that there was no significant difference in TSH, T_3 or T_4 concentrations compared with WT controls. The reasons for illness and death in hPTTG-$Tg^{+/+}$ mice were therefore not identified after post-mortem analysis, histological evaluation and thyroid function analyses. In the absence of any thyroid phenotype, it appears increasingly likely that this may have been a non-specific effect, possibly caused by the disruption of a critical gene at the insertion site of the transgene construct. Supporting this logic, hPTTG-$Tg^{+/-}$ mice demonstrated a similar survival pattern to WT mice, possibly explained by the fact that they would presumably possess one normal copy of the potentially disrupted gene that is sufficient for normal function and survival. In order to verify this, it may be possible to determine the transgene insertion site by performing inverse-PCR analyses in order to amplify and subsequently sequence the flanking regions of DNA (Ochman et al. 1988). However, following careful consideration work to fully characterise surviving hPTTG-$Tg^{+/-}$ mice and hPTTG-$Tg^{+/+}$ mice was continued.

The survival of 28.5 % of hPTTG-$Tg^{+/+}$ mice to 18 months raises further questions as to why some mice of this genotype become ill but others do not. Aged hPTTG-$Tg^{+/+}$ mice continued to express hPTTG protein (data not shown) and therefore survival was not due to "switching off" of the transgene. It is possible that disruption of a gene at the site of transgene insertion does not effect hPTTG-$Tg^{+/+}$ mice with 100 % penetrance. Alternatively, reduced hPTTG-$Tg^{+/+}$ survival could be the fatal effect of toxicity caused by excessive over-expression of the transgene, which is feasible given the multiple transgene copy numbers and high levels of hPTTG expression observed in this transgenic line (see Figure 5-14, 5-17, 5-18

and 5-19). On this basis, varying levels of total hPTTG expression in hPTTG-Tg$^{+/+}$ mice may determine death or survival.

6.4.2 Characterisation of surviving hPTTG-Tg mice

Despite many hPTTG-Tg$^{+/+}$ mice becoming ill before reaching an age at which enhanced thyroid growth would be expected, we were able to investigate phenotypes in those that survived, and in hPTTG-Tg$^{+/-}$ mice, which also demonstrated significant over-expression of hPTTG in the thyroid (see Figure 5-14, 5-17, 5-18, 5-19). A number of aged hPTTG-Tg mice presented with different tumour types at around 18 months of age. Given that a low level of mistargeted hPTTG mRNA expression was observed in the lungs of hPTTG-Tg mice (see Figure 5-14), it was interesting that two hPTTG-Tg$^{+/-}$ mice developed lung tumours.

However, the occurrence of lung tumours in 18 month old WT mice (data not shown) indicated that this was not a specific effect of hPTTG over-expression in the lung. Indeed, spontaneous development of a variety of lesions, and of lung tumours in particular, has been reported in non-transgenic ageing FVB/N mice (Mahler et al. 1996).

Microscopic analysis at the point of dissection revealed no visible evidence of goitre formation or tumour development in either hPTTG-Tg$^{+/-}$ or hPTTG-Tg$^{+/+}$ mice between 1.5 and 18 months of age. Furthermore, hPTTG-Tg thyroid glands were deemed histologically normal with no evidence of benign or malignant lesions. Unexpectedly, thyroid weight analysis indicated that hPTTG-Tg$^{+/-}$ thyroids were generally smaller than those of WT mice, though this was only statistically significant at 12 months of age. hPTTG-Tg$^{+/+}$ thyroids were even smaller compared with WT and demonstrated statistical significance at every age investigated, indicating a copy number dependent phenotype of reduced thyrocyte proliferation. However, following the observation that hPTTG-Tg$^{+/+}$ mice were under-weight, thyroid weight data were also analysed in relation to total body weight. Thyroid:total body weight ratios of hPTTG-Tg mice were still generally lower than that of WT mice,

though this was not consistently observed or statistically significant at every time-point. The inconsistency of results potentially reflects a subtle phenotype that has been difficult to elucidate given high levels of variation and difficulties in accurately determining thyroid weights. Future studies should aim to verify or refute reduced cellular proliferation in hPTTG-Tg thyroids by analysis of mitotic markers such as PCNA and cyclin D1.

Nonetheless, the lack of enhanced thyroid growth was inconsistent with previous data showing that subcutaneous expression of murine fibroblasts over-expressing hPTTG induced high-grade malignant tumour formation in athymic nude mice (Zhang et al. 1999b; Kakar and Jennes 1999). Furthermore, our observations contrast with other transgenic models of organ-specific hPTTG over-expression describing hPTTG-induced hyperplasia in the pituitary glands (Abbud et al. 2005) and ovaries (El-Naggar et al. 2007) of transgenic mice. One study from our laboratory identified hPTTG as a critical regulator of proliferation in fetal neuronal NT-2 cells, showing that low transient expression of hPTTG resulted in a significant proliferative effect, whereas high levels of hPTTG expression inhibited cell turnover through its role as a securin (Boelaert et al. 2003b). It is possible that a dose-dependent response to hPTTG exists *in vivo*, where both hPTTG-$Tg^{+/-}$ and hPTTG-$Tg^{+/+}$ mice demonstrate high levels of hPTTG expression and reduced thyroid size. Consistent with this theory, MISIIR-PTTG transgenic mice that develop hypertrophy of the corpus luteum have relatively low expression of transgenic hPTTG in the ovarian epithelium (El-Naggar et al. 2007). Alternatively, hPTTG-induced apoptosis (Yu et al. 2000a) might explain reduced thyroid size in hPTTG-Tg mice and this could be investigated through the use of apoptosis assays.

The lack of thyrocyte transformation in hPTTG-Tg mice indicates that over-expression of hPTTG alone is not sufficient for thyroid tumourigenesis. Major pathogenetic factors associated with thyroid cancer include dietary iodine deficiency and exposure to

external radiation (Schneider and Ron 2005; Nikiforov and Nikiforova 2011). It would therefore be interesting to test whether hPTTG over-expression facilitates the transition to malignancy following induction of TSH-driven goitre formation in hPTTG-Tg mice on an iodine deficient diet. Similarly, given the potential role of hPTTG in DNA damage response pathways (see section 1.3.5), and in particular the observation that over-expression of hPTTG can repress DNA repair (Kim et al. 2007b), it would be of significant interest to test whether exposure to ionizing radiation causes a greater incidence of thyroid tumour development in hPTTG-Tg mice compared with WT mice. Alternatively, it is possible that another oncogene is required to facilitate hPTTG mediated thyroid cell transformation. It has already been described that follicular thyroid tumours in $TR\beta^{PV/PV}$ mice demonstrated reduced proliferation and vascular invasion when Pttg was absent ($TR\beta^{PV/PV}$/*Pttg*$^{-/-}$ mice) (Kim et al. 2007a). It is therefore possible that hPTTG over-expression may lead to more aggressive phenotypes acting in concert with other oncogenes in other mouse models of thyroid cancer.

It is often the case that the introduction of two oncogenes is required for tumourigenesis in transgenic models of disease. For example, crossing of the $Pten^{L/L}$;TPO-Cre mouse with mice harboring a Kras oncogenic mutation led to invasive and metastatic follicular carcinomas, whereas neither the $Pten^{L/L}$;TPO-Cre or $Kras^{G12D}$ mouse model alone developed thyroid carcinomas (Miller et al. 2009). The most obvious oncogenic partner for hPTTG is its binding factor PBF and to this effect, we have established a hPTTG.PBF-Tg bitransgenic colony which is currently being investigated in our laboratory. While hPTTG and PBF have important functions that are independent of each other, PBF facilitates nuclear localisation and transactivational capabilities of hPTTG (Chien and Pei 2000) and their interaction at oncogenic expression levels may yield highly interesting phenotypes. PBF-Tg mice develop goitres with 100 % penetrance (Read et al. 2011) and it will be of great interest

to see whether concerted over-expression of hPTTG results in the development of thyroid malignancies.

6.4.3 Serum thyroid hormone concentrations

Serum concentrations of TSH were determined by the laboratory of Professor Samuel Refetoff (University of Chicago) in a method previously described (Pohlenz et al. 1999). Generally, there were no significant differences in serum TSH concentrations in either young or aged hPTTG-Tg mice. However, TSH concentrations were undetectable in female hPTTG-$Tg^{+/-}$ serum samples and thus significantly lower than WT controls. This may have been a result of sample degradation and Professor Refetoff advised that assay measurements at the lower limit of detection are unreliable, and it is therefore unlikely that this is a true observation. In addition, the phenotype of reduced thyroid size was not female specific, suggesting that low TSH concentration does not underly this phenotype in hPTTG-Tg mice. Serum levels of T_3 were also similar in hPTTG-Tg and WT mice and although hPTTG-Tg mice demonstrated a general trend of increased serum T_4 concentration, this was only significant at 6 weeks of age in hPTTG-$Tg^{+/+}$ males. Elevated concentrations of serum T_3 and T_4 might reflect underlying hyperthyroidism, which could represent an explanation for reduced total body weight in 3-7 week old hPTTG-$Tg^{+/+}$ mice. However, this was not accompanied by reduced serum TSH concentration, and relative T_3 and T_4 levels did not always correlate as would be expected. Given the discrepancies and high variability of the data, it appears that hPTTG-Tg mice are euthyroid, though further measurements should seek to confirm this.

6.4.4 Gene expression analyses

hPTTG-$Tg^{+/+}$ mice showed no changes in mRNA expression of *Tshr* and *Tg* compared with WT, indicating that hPTTG-Tg mice maintain thyroid differentiation.

hPTTG-Tg mice demonstrated similar levels of endogenous *Pttg* and *Pbf* compared with WT mice. The lack of induction of *Pbf* expression by exogenous hPTTG in hPTTG-Tg mice was in contrast with previous *in vitro* observations (Stratford et al. 2005). Although hPTTG protein shares 66 % homology with murine Pttg (Wang and Melmed 2000a), it is possible that the full transactivational capabilities of hPTTG are compromised in murine thyroid cells. Failure of hPTTG to transactivate other protooncogenes such as Pbf and c-Myc (not investigated in the current study) may contribute to the failure of thyroid cell transformation in hPTTG-Tg mice. Given that PBF causes transformation independently of hPTTG *in vivo* (Stratford et al. 2005), it is conceivable that hPTTG-induced transformation is mediated via transactivation of PBF. Indeed, a mutant form of hPTTG that does not induce PBF expression failed to transform NIH3T3 cells *in vitro* (Stratford et al. 2005). The points raised here emphasise the importance of our ongoing investigation of phenotypes in hPTTG.PBF-Tg bitransgenic mice.

The finding that hPTTG-Tg mice had levels of *Vegf* and *Fgf-2* thyroidal mRNA expression comparable to WT was inconsistent with studies from our own group demonstrating hPTTG induction of *VEGF* and *FGF-2* mRNA expression in human thyroid cells *in vitro* (Boelaert et al. 2003a; Kim et al. 2006a). Again, this may be attributed to the inability of hPTTG to induce these factors in murine thyroid cells. In addition, we generally observed high variability in mRNA expression in mouse thyroids and *Vegf* and *Fgf-2* expression should be studied in greater numbers of hPTTG-Tg mice to obtain more meaningful data. Indeed, having decided to focus on validation of EGF, IGF-1 and TGF-α induction by hPTTG as described in Chapter 4, investigation of a large number of mice was required to overcome the high variability observed and elucidate important relationships. We successfully demonstrated that WT and hPTTG-Tg thyroid tissues express Egf, Tgf-α and Igf-1, as well as their receptors, Egfr and Igf1r respectively. While *Egf* and *Igf-1* mRNA

expression were unaltered in hPTTG-Tg$^{+/-}$ mice, their expression was significantly increased in hPTTG-Tg$^{+/+}$ mice, indicating a copy number dependent phenotype. However, *Tgf-α* mRNA expression was not significantly induced in hPTTG-Tg$^{+/+}$ mice. Subsequent ELISAs demonstrated that Egf secretion was enhanced in cultures derived from hPTTG-Tg$^{+/+}$ mice compared with WT, whereas in contrast to mRNA expression data Igf-1 secretion was unaltered (there is currently no mouse Tgf-α assay available). The failure of hPTTG to induce Tgf-α emphasises that the full transactivational capabilities of hPTTG may be compromised in this model. In addition, increased transcription may not necessarily lead to increased translation and secretion of growth factors as was the case for Igf-1. However, these observations confirm important pathways of interaction between hPTTG and EGF, and validate the *in vitro* findings in human primary thyrocytes described in Chapter 4. Supporting this further, *Egf* mRNA was under-expressed in Pttg$^{-/-}$ thyroids, whereas *Tgf-α* and *Igf-1* mRNA expression were not significantly altered.

Following the observations in this chapter and in Chapter 4, that hPTTG induces the expression of various growth factors, angiogenesis-specific cDNA PCR array analyses were performed in mouse thyroid glands to broadly assess the effect of thyroidal hPTTG over-expression on other genes involved in proliferation and angiogenesis. *Cxcl1*, *F2*, *Mdk* and *Tnfaip2* are all pro-angiogenic genes that demonstrated a greater than 2.0-fold upregulation in hPTTG-Tg$^{+/+}$ thyroids. Interestingly, human homologues of *Cxcl1* and *Mdk* were also induced in an angiogenesis-specific microarray following transient expression of hPTTG in human primary thyroid follicular cells (Kim et al. 2006b), and MDK is over-expressed in papillary thyroid carcinomas (Kato et al. 2000). *Tnf-α*, an anti-angiogenic gene that promotes cell death and tumour regression (Carswell et al. 1975; Old 1985), was repressed in hPTTG-Tg$^{+/+}$ thyroids. Notably, hPTTG also downregulated several pro-angiogenic genes including *Fgf-6*, *Mmp-9*, *Tbx-1* and *Tbx-4*. Repression of *Fgf-6* was in contrast to induction of *FGF-6*

by hPTTG in the microarray analysis described above (Kim et al. 2006b). Despite not reaching the 2.0-fold threshold, *Fgf-2* and *Igf-1* were induced by hPTTG, consistent with our detailed mRNA expression studies (see Figure 6-12 and 6-13). In contrast with previous observations, *Egf* expression was repressed while *Vegf* and *Tgf-α* were induced in hPTTG-$Tg^{+/+}$ thyroids (see Figure 6-12 and 6-13), illustrating the variability of mRNA expression results we have observed throughout the study. Repression of the anti-angiogenic gene, *TSP-1*, has been implicated in thyroid tumour progression (Tanaka et al. 2002). The observation of hPTTG-induced *Tsp-1* (below the threshold) was inconsistent with our group's previous report of *TSP-1* repression by hPTTG (Kim et al. 2006b). Expression of the pro-angiogenic gene, *Kdr*, was induced by hPTTG albeit to levels below the 2.0-fold threshold, consistent with previous observations of *KDR* induction by hPTTG in follicular thyroid carcinoma FTC-133 cells (Kim et al. 2006a). Although extensive validation of these observations are required, it is clear that hPTTG may promote tumour progression by regulating the expression of multiple genes with both pro- and anti-angiogenic properties, and may thus be a key gene in triggering the angiogenic switch in thyroid tumorigenesis.

6.4.5 Conclusions

This *in vivo* investigation of transgenic mice with thyroid-targeted hPTTG expression has presented a variety of challenges. Nonetheless, the data shows that transgenic mice with high levels of thyroidal hPTTG expression do not develop thyroid hyperplasia or neoplasia, but in fact have smaller thyroid glands. The exact mechanisms underlying this phenotype remain to be determined. It will be important to create an alternative transgenic line with low levels of thyroidal hPTTG over-expression to define the *in vivo* dose-dependent effects of hPTTG on thyroid cell proliferation and transformation. Work towards this aim is described in Chapter 8. Several important considerations have been discussed within this chapter, but the results suggest that ongoing study of hPTTG.PBF-Tg bitransgenic mice represents an

important avenue of research whereby concerted over-expression of hPTTG and PBF may induce tumour formation.

Importantly, several of our *in vitro* observations described in Chapter 4 were validated in the transgenic mouse model. Chiefly, an important relationship between hPTTG and Egf *in vivo* was confirmed through studies in both hPTTG-Tg and $Pttg^{-/-}$ knockout mice. Angiogenesis-specific cDNA PCR array analysis highlighted the potential relationship between hPTTG and a number of genes critically involved in tumour proliferation and angiogenesis and has opened up opportunities for further research.

7 CHAPTER SEVEN

Regulation of Expression and Function of the Sodium Iodide Symporter (NIS) by hPTTG and PBF

7.1 Introduction

The sodium iodide symporter (NIS) is an integral membrane glycoprotein which mediates active transport iodide (I^-) into thyroid follicular cells, as the critical and rate-limiting first step in thyroid hormone biosynthesis (Kogai et al. 2006; Dohan et al. 2003; Boelaert and Franklyn 2003). Importantly, this physiological mechanism has been exploited using ^{131}I or ^{99m}Tc scintigraphic imaging as a diagnostic tool or by administering therapeutic doses of ^{131}I for the treatment of patients with benign and malignant thyroid diseases, as well as their metastases, through ablation of pathological tissue in a highly targeted manner (Spitzweg et al. 2001; Dohan et al. 2003; Boelaert and Franklyn 2003; Riesco-Eizaguirre and Santisteban 2006; Kogai et al. 2006).

However, I^- uptake is reduced in most thyroid cancers and their metastases compared with normal thyroid, thereby limiting the efficacy of radio-iodine therapies (Maxon and Smith 1990). Treatment with recombinant human TSH may be used to stimulate I^- uptake by the thyroid, but even then, 10-20 % of tumours remain incapable of sufficient I^- concentration for effective radio-ablation therapy (Kogai et al. 2006; Robbins et al. 1991; Schmutzler and Koehrle 2000). It is therefore of critical importance to understand the mechanisms involved in repression of I^- uptake in thyroid cancer, in order to develop strategies to improve treatment with radioiodine.

Various mRNA (Smanik et al. 1997; Lazar et al. 1999; Ringel et al. 2001; Arturi et al. 2003; Park et al. 2000; Ryu et al. 1999; Ward et al. 2003) and protein studies using specific poly- and monoclonal antibodies (Jhiang et al. 1998b; Caillou et al. 1998; Castro et al. 1999a; Castro et al. 1999b; Faggiano et al. 2007; Gerard et al. 2003; Trouttet-Masson et al. 2004) have demonstrated reduced NIS expression in thyroid cancer tissue, suggesting repression of I^- uptake is caused by a reduction in the amount of NIS expression. However, in several contrasting studies NIS expression was normal or increased (Arturi et al. 1998; Luciani et al.

2003; Saito et al. 1998; Tanaka et al. 2000; Dohan et al. 2001; Wapnir et al. 2003), but immunohistochemical analysis of NIS cellular localisation showed that NIS was predominantly present in the cytoplasm, indicating that intracellular trafficking of NIS away from the basolateral membrane may be a further cause of reduced I^- uptake in malignancy (Castro et al. 1999b; Dohan et al. 2001; Wapnir et al. 2003).

While TSH and I^- itself are well established major regulators of NIS expression and function, modulation of NIS activity is complicated and is effected by various factors (see section 1.6.3). Both hPTTG and PBF have been implicated in regulation of NIS *in vitro*. Over-expression of hPTTG in rat thyroid FRTL-5 cells resulted in reduced *NIS* mRNA expression and I^- uptake. This effect was at least partly dependent on hPTTG-induced FGF-2 expression, providing further evidence for important interactions between hPTTG and growth factors (Heaney et al. 2001). Subsequently, in a study confirming hPTTG expression as a prognostic indicator for persistent disease, immunohistochemical analysis of 16 human differentiated thyroid tumours demonstrated strong hPTTG over-expression that was associated with reduced I^- uptake in patients (Saez et al. 2006). Studies within our group demonstrated reduced *NIS* mRNA expression and I^- uptake in FRTL-5 cells and human primary thyrocytes following over-expression of hPTTG or PBF (Boelaert et al. 2007). Detailed promoter studies confirmed that this was a transcriptional effect mediated by specific binding of PBF or hPTTG to a PAX8-upstream stimulating factor 1 (USF1) response element within a 1 kb element in the *NIS* promoter known as the human upstream enhancer element (hNUE). Once again, hPTTG repression of NIS was mediated partly through FGF-2 induction, while PBF acted independently of FGF-2 to suppress NIS. Increased *hPTTG*, *PBF* and *FGF-2* expression was significantly correlated with reduced *NIS* expression in human thyroid cancers demonstrating the importance of these interactions *in vivo* (Boelaert et al. 2007). In an additional study, our group have demonstrated a further mechanism of NIS

regulation by PBF, whereby PBF directly binds to and internalises NIS into intracellular vesicles. The result of this mechanism was reduced NIS protein expression in the basolateral membrane and subsequent reduction of I^- uptake by FRTL-5 cells (Smith et al. 2009). Together, these findings suggest that over-expression of hPTTG and PBF in thyroid cancer (see sections 1.2.1.3 and 1.5.2) has significant implications for expression and function of NIS, thereby reducing the efficacy of ^{131}I in the ablation and treatment of thyroid tumours.

The aim of this study was to assess whether hPTTG and PBF over-expression affect the expression and function of NIS *in vivo*. Thyroidal expression of NIS mRNA and protein was evaluated in our recently generated transgenic mouse models with thyroid-targeted expression of hPTTG (see Chapter 5) and PBF (see section 1.5.4). Further, ^{125}I radioiodine uptake assays were performed in murine primary thyroid cultures derived from transgenic mice. siRNA knockdown studies were performed in transgenic murine primary thyrocytes and normal human primary thyrocytes to evaluate the potential of hPTTG and PBF as targets for enhancing NIS activity and therefore improving radioiodide therapies.

7.2 Materials and methods

7.2.1 Murine primary thyrocyte culture

Murine thyroid follicular cells were isolated and cultured from WT, hPTTG-Tg and PBF-Tg thyroid glands as described previously in section 2.3.

7.2.2 Human thyroid samples and primary culture

Collection of thyroid samples was with approval of the Local Research Ethics committee and samples were cultured as described in section 2.2.

7.2.3 siRNA transfection studies

Between days 7-10 of culture, murine and human primary thyrocytes cells were transfected with siRNAs targeting *hPTTG* (100 nM # 46028) or human *PBF* (50 nM #

147350, 50 nM # 14399) transcripts and controlled for by equivalent scrambled (100 nM # AM4635) siRNA transfections (Life Technologies, Grand Island, NY, USA). All siRNA transfections were performed using the lipofectamine-2000 transfection agent (Invitrogen, UK) according to the manufacturer's instructions. Cell culture medium was replaced between 4-6 hours to minimise the toxic effects of lipofectamine-2000. After 72 hours, iodide uptake assays were performed and cell lysates were harvested in protein lysis buffer or 0.25 ml Tri Reagent (Life Technologies, Grand Island, NY, USA).

7.2.4 RNA extraction, reverse transcription, QT-PCR

Total RNA was extracted from murine and human primary thyrocytes as described previously in section 2.4. Mouse thyroid glands and human thyroid tissue samples were stored in RNAlater RNA stabilisation reagent at – 20 ºC before total RNA was extracted using the RNeasy microkit (Qiagen, UK), as per the manufacturer's instructions. Subsequent reverse transcription and QT-PCR techniques were as described above (2.4 and 2.5). *hPTTG* mRNA was detected using the primers and probe described above (Table 3-1). Gene-specific expression assays for *Pbf* (Mm00521473_m1), *Tshr* (Mm00442027_m1) and *Nis* (Mm01351811_m1) were purchased from Applied Biosystems (Warrington, UK).

7.2.5 Immunohistochemistry

Immunohistochemical analysis of NIS protein expression in formalin-fixed, paraffin-embedded sections of wild-type, hPTTG-Tg and hPBF-Tg mouse thyroid specimens was carried out as previously described in section 2.7. A rabbit anti-rat NIS antibody was used (20 µg/mL) [# TIT11-A, Alpha Diagnostic International, SA, Texas, USA] (Josefsson et al. 2002).

7.2.6 Immunoperoxidase cell staining

Murine primary thyrocytes were cultured on 8-well chamber slides [BD Biosciences Ltd.]. On day 7 of cultures, cells were fixed in ethanol for 10 minutes at room temperature. Cells were briefly washed in PBS before being incubated in 5 % normal goat serum (NGS) in PBS for 20 minutes at room temperature, followed by a specific primary anti-HA antibody (1:500) [# sc-805, Santa Cruz Biotechnology] for 1 hour at room temperature. After two 5-minute washes in PBS, cells were incubated in biotinylated anti-rabbit secondary Ab for 30 minutes at room temperature. Cells were again washed with PBS, before incubation with 3 % hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. After further PBS washes, the avidin-biotin-peroxidase complex was added to cells for at least 30 minutes, followed by further PBS washes before the reaction was developed using the DAB peroxidase substrate kit for 5-10 minutes and then counterstained in Mayer's hematoxylin. Slides were dehydrated, cleared, and mounted.

7.2.7 Western blot analysis

Protein extraction, quantification and subsequent Western blotting methods were as described previously (see section 2.6). For PBF detection, membranes were incubated with rabbit primary antibodies against PBF (1:1000) (Smith et al. 2009; Watkins et al. 2010) and HA (1:500) [# sc-805, Santa Cruz Biotechnology].

7.2.8 Iodide uptake assays

Murine and human primary thyrocytes were grown in 12 well plates in 0.5 ml medium. The incorporation of ^{125}I was used as a standard marker of thyroid cell function and was assayed by the addition of NaI (final concentration of 10^{-7} and 10^{-9} moles/l in human and murine cells, respectively) and 0.05 μCi ^{125}I (Hartmann Analytic, Germany) per well for 2 hours as previously described (Eggo et al. 1996; Boelaert et al. 2007). The cell layer was

rapidly washed with HBSS to remove unincorporated iodide and the cells lysed in 2 % SDS protein lysis buffer or 0.25 mL Tri Reagent. Incorporated radioactivity was counted in a gamma counter. Relative iodide uptake was corrected for protein concentration as measured by the Bradford assay.

7.2.9 Statistical analysis

Data were analysed as described in section 2.8

7.3 Results

7.3.1 hPTTG and PBF repress NIS expression *in vivo*

To investigate the effects of thyroidal hPTTG and PBF over-expression on NIS *in vivo*, we performed investigations using hPTTG-Tg and hPBF-Tg mice. Analysis of whole thyroid glands using Real-Time PCR revealed reduced *NIS* mRNA expression in 6-week-old age and gender matched hPTTG-$Tg^{+/-}$ (0.62-fold, $n = 5$, $p < 0.05$), hPTTG-$Tg^{+/+}$ (0.64-fold, $n = 3$, $p < 0.05$) and hPBF-Tg (0.52-fold, $n = 10$, $p < 0.001$) mice compared with WT controls (see Figure 7-1).

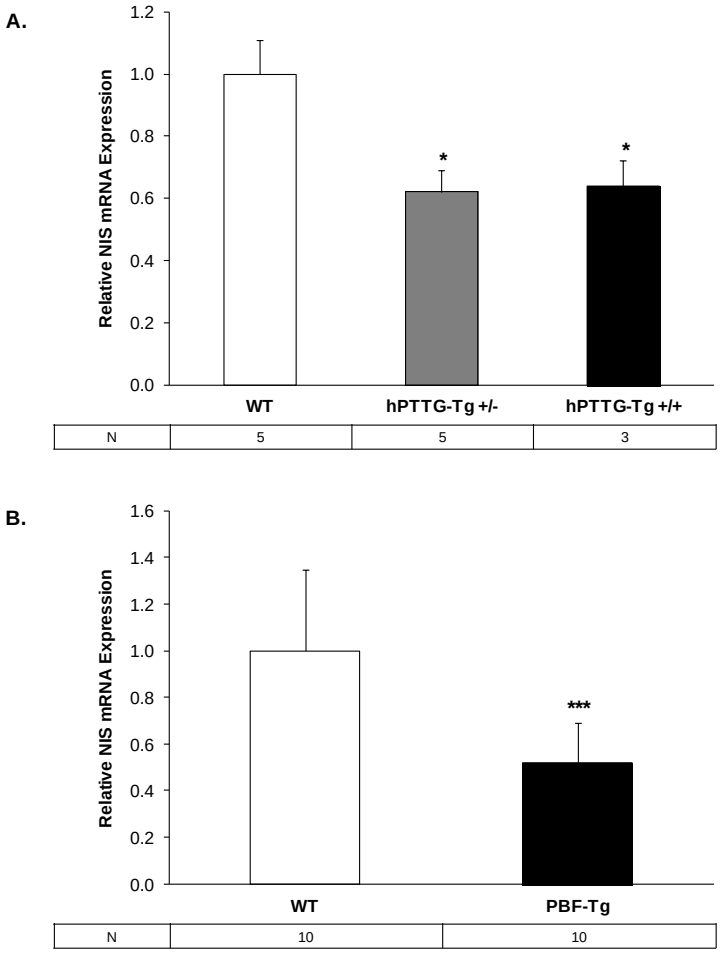


*Figure 7-1: hPTTG and PBF over-expression reduce NIS mRNA expression in vivo. TaqMan RT-PCR data showing relative thyroidal NIS mRNA expression in hPTTG-Tg$^{+/-}$ and hPTTG-Tg$^{+/+}$ (**A**), and hPBF-Tg (**B**)mice compared with wild-type controls. Error bars represent the SEM (* $p < 0.05$, *** $p < 0.001$).*

Assessment of NIS immunostaining in thyroid sections from 6-week-old age and gender matched mice, revealed a heterogeneity of NIS expression across whole thyroid lobes, but with an overall reduction in NIS protein expression in hPTTG-Tg and hPBF-Tg mice compared to WT (see Figure 7-2).

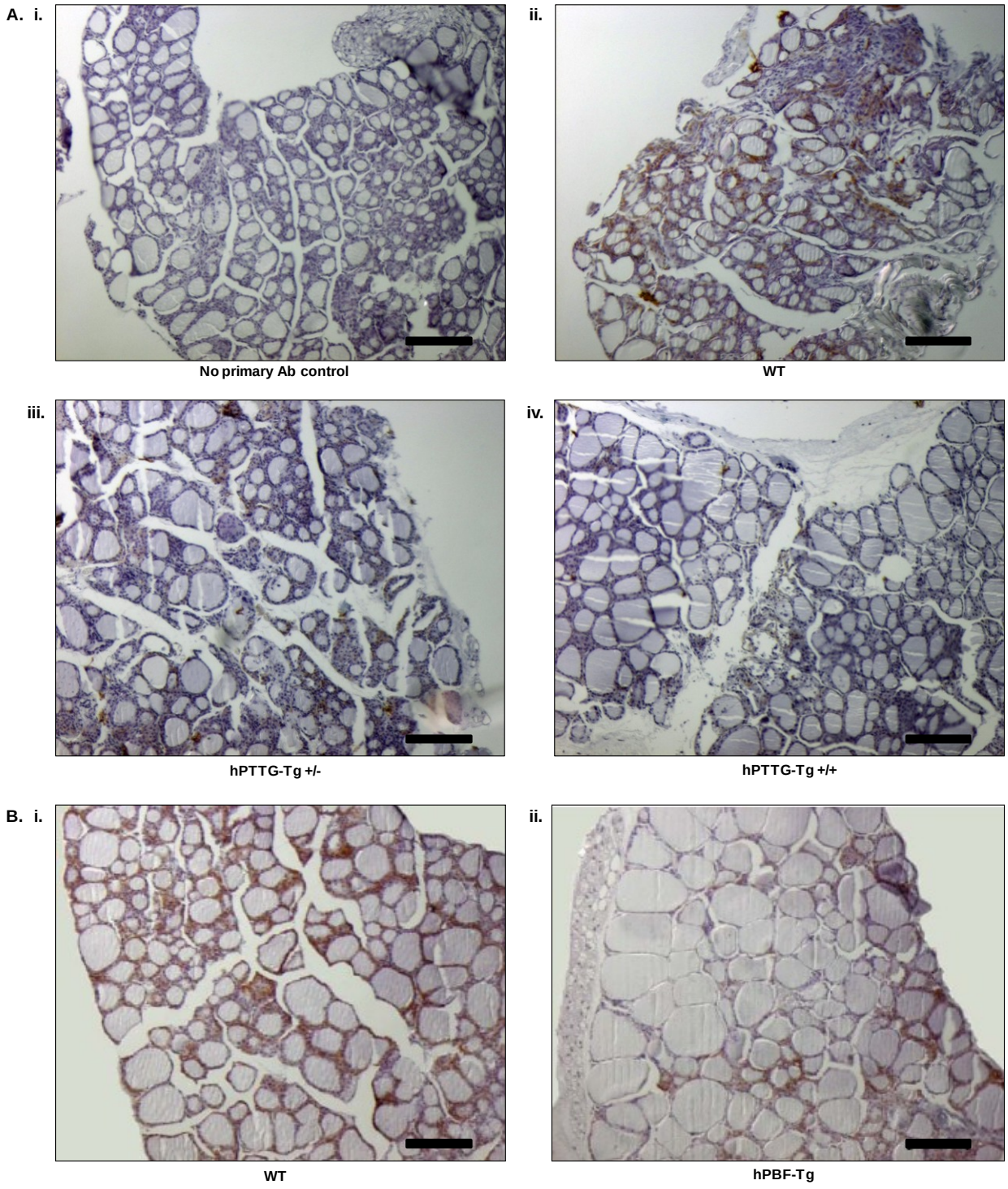


Figure 7-2: hPTTG and PBF over-expression reduce NIS protein expression in vivo. ***A*** *Detection of NIS by immunohistochemistry of WT* ***(ii)****, hPTTG-Tg$^{+/-}$* ***(iii)*** *and hPTTG-Tg$^{+/+}$* ***(iv)*** *thyroids. A no primary antibody control demonstrates the specificity of the HRP-conjugated secondary antibody* ***(i)****.* ***B*** *Detection of NIS by immunohistochemistry of WT* ***(i)*** *and hPBF-Tg* ***(ii)*** *thyroids. Scale bars: 100 µm. Images are representative of n ≥ 3 experiments.*

7.3.2 Analysis of NIS function *in vivo*

Given that hPTTG-Tg and hPBF-Tg mice showed repressed NIS mRNA and protein expression, we set out to investigate NIS function in these transgenic mouse models.

7.3.2.1 Establishment of murine thyrocyte cell culture

We adopted an approach involving the routine culture of murine primary thyrocytes, using a technique adapted from previously described methods for murine and human primary thyroid culture. The morphology of cell cultures was microscopically examined daily to help determine the optimal culture conditions, culminating in the final protocol described in the materials and methods (see section 2.3). Isolated thyroid follicles (primary follicles) adhered to culture plates on day one and progressively flattened to form multiple layers of cells. By day 5, most follicles had lost their 3-dimensional structure and a monolayer of thyrocytes began filling the inter-follicular spaces. Cells continued to spread, becoming more confluent by day 10, at which point numerous secondary follicles appeared to form (see Figure 7-3).

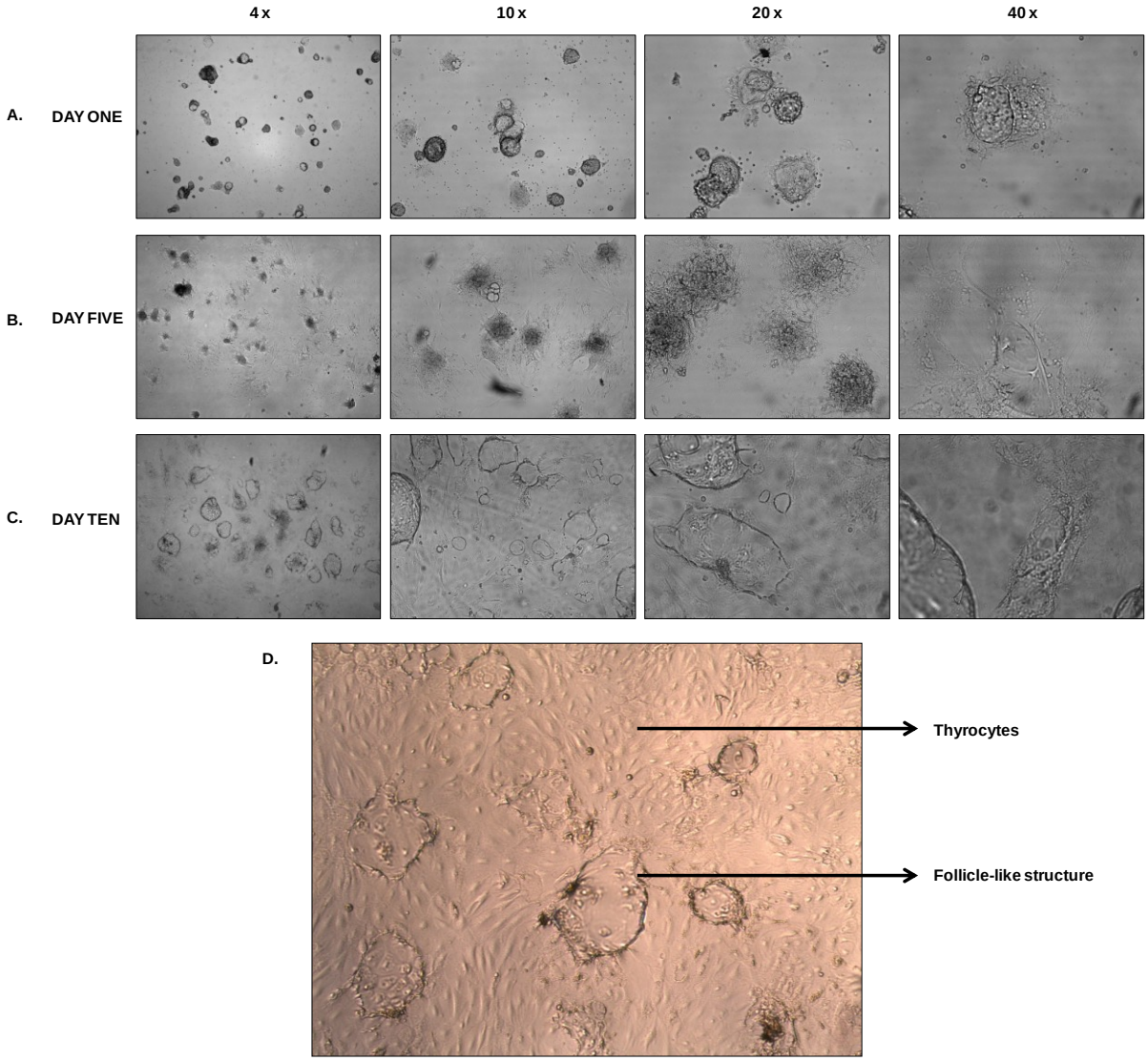


Figure 7-3: Murine primary thyroid cell culture. ***A*** *Day one – isolated primary follicles adhere to cell culture plate.* ***B*** *Day 5 – Primary follicles have flattened and a monolayer of thyrocytes begins to fill inter-follicular spaces.* ***C/D*** *Day 10 – Cells have continued to spread and form secondary follicular structures.*

7.3.2.2 Validation of murine thyrocyte cell culture system

Prior to carrying out full scale investigations, we performed several critical validation experiments to determine the suitability of murine primary thyrocyte cultures as a model for our studies. We adopted two approaches to determine whether transgene expression is maintained by primary thyrocytes in culture, following digestion of whole thyroids. Firstly, analysis of PBF-HA expression in murine primary thyrocytes by immunocytochemistry demonstrated strong staining in PBF-Tg cultures compared with minimal non-specific

staining in WT cultures. Secondly, Real-Time PCR analysis revealed strong *hPTTG* mRNA expression in hPTTG$^{+/-}$ cultures, which was approximately double in hPTTG$^{+/+}$ cultures, compared with completely undetectable levels in WT cultures. Importantly, these results demonstrate that our cultures were thyroid-specific and maintain transgene expression (see Figure 7-4).

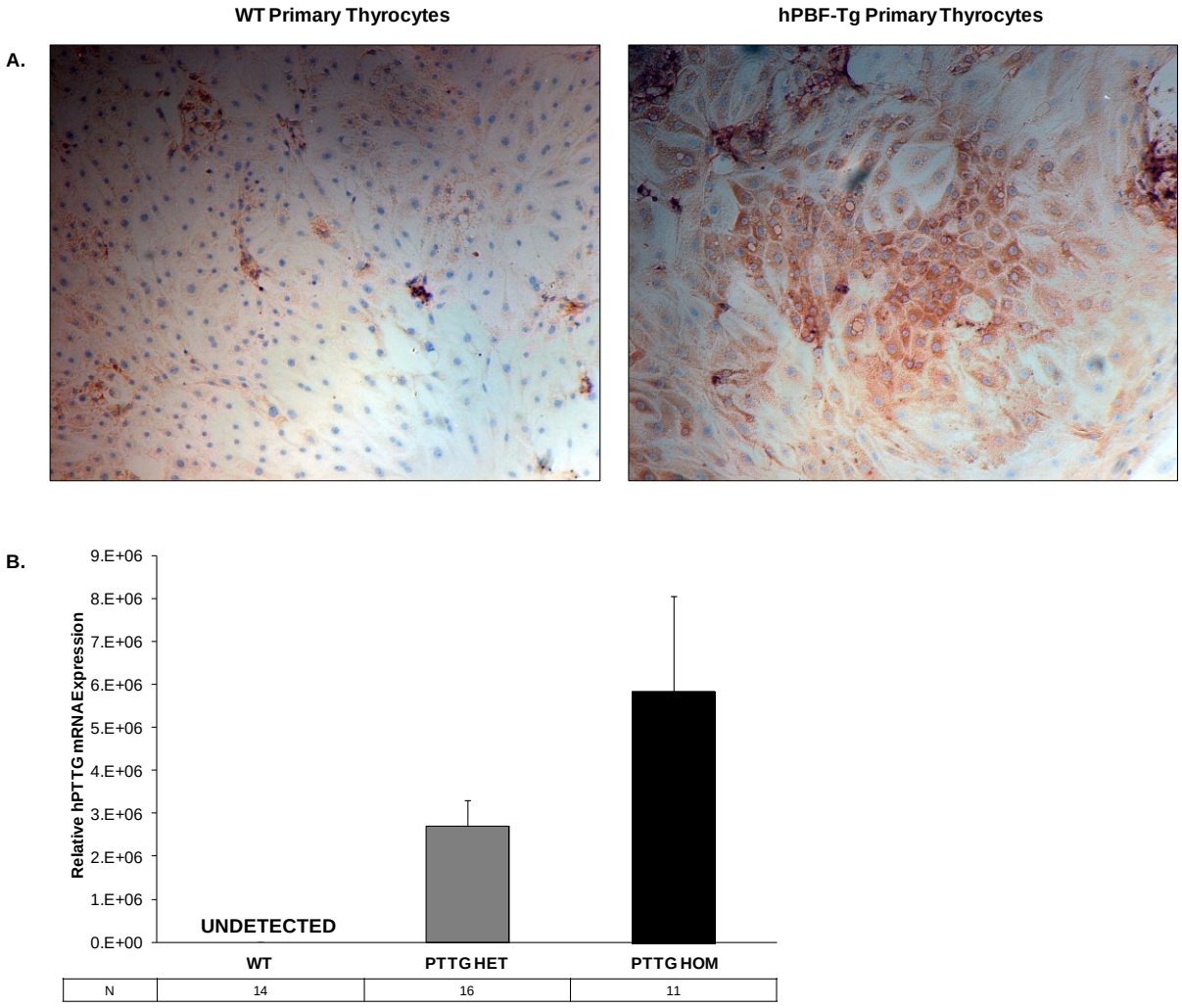


Figure 7-4: Murine primary thyrocytes derived from transgenic thyroid glands maintain transgene expression in culture. ***A*** *Immunohistochemistry demonstrating detection of hPBF-HA protein in hPBF-Tg but not WT primary thyrocytes.* ***B*** *TaqMan RT-PCR data showing detection of high hPTTG expression in hPTTG$^{+/-}$ and hPTTG$^{+/+}$ primary thyrocytes. Error bars represent the SEM.*

Subsequently, Real-Time PCR analysis determined that the TSH-R is expressed by murine primary thyrocytes (ΔCt = 13.56, n = 6) where WT cultures were used for this purpose. Further Real-Time PCR analysis revealed that WT primary thyrocytes (0.9-fold, n = 14, p = NS) have a similar level of *NIS* mRNA expression to whole WT thyroid glands (n =

3). hPTTG-Tg$^{+/-}$ (0.77-fold, n = 16, p = NS) and hPTTG-Tg$^{+/+}$ (0.6-fold, n = 11, p = NS) mouse primary thyrocytes (n = 14) demonstrated a trend of reduced *NIS* mRNA expression compared with WT cultures, similar to that described in whole thyroid glands in section 7.3.1. In this instance, the trend was non-significant due to highly variable results observed in the cultures. Importantly, these results indicated that murine primary thyrocytes maintained NIS expression in culture and were likely to be responsive to TSH in the culture medium – a critical regulator of NIS expression and function (see Figure 7-5). Preliminary iodide (^{125} I) uptake assays in WT cultures verified that our murine primary thyrocyte cultures were functional, demonstrating an average uptake of 4222.1 cpm/µg protein (n = 20).

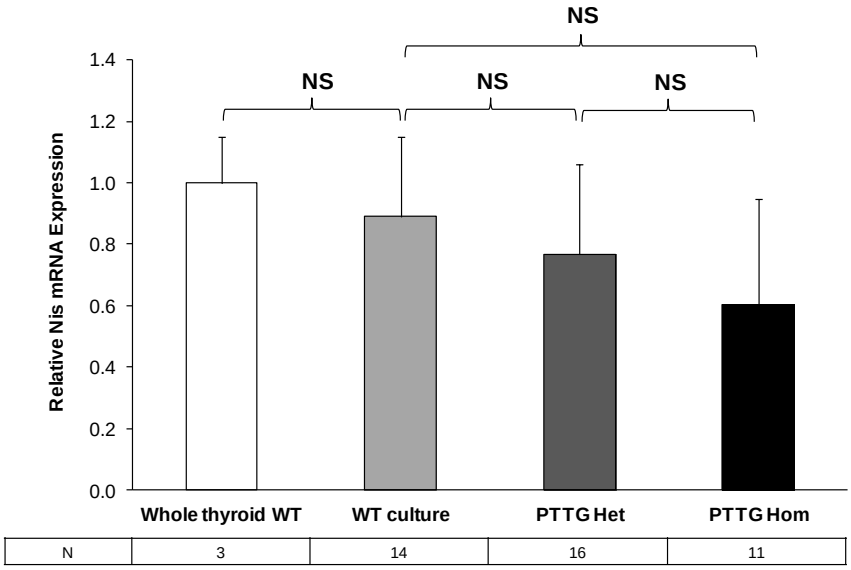


Figure 7-5: TaqMan RT-PCR data demonstrating maintained NIS mRNA expression in WT and hPTTG-Tg murine primary thyrocytes. Error bars represent the SEM (NS = non-signifcant).

7.3.2.3 hPTTG and PBF repress NIS function *in vivo*

Having demonstrated our capability to culture functional murine primary thyrocytes that maintain transgene expression, we examined NIS function in our transgenic mouse models. We performed primary cultures of thyroid cells from 4-6 week-old age and sex-matched WT, hPTTG-Tg$^{+/-}$, hPTTG-Tg$^{+/+}$ and hPBF-Tg mice, and performed ^{125}I uptake

assays after 7 days of culture. Results were corrected for protein concentration and revealed potent and significant repression of ^{125}I uptake in hPTTG-Tg$^{+/-}$ (0.57-fold, n = 15, $p < 0.05$), hPTTG-Tg$^{+/+}$ (0.35-fold, n = 14, $p < 0.001$) and hPBF-Tg mice (0.27-fold, n = 14, $p < 0.001$) compared with WT (n = 14) (see Figure 7-6).

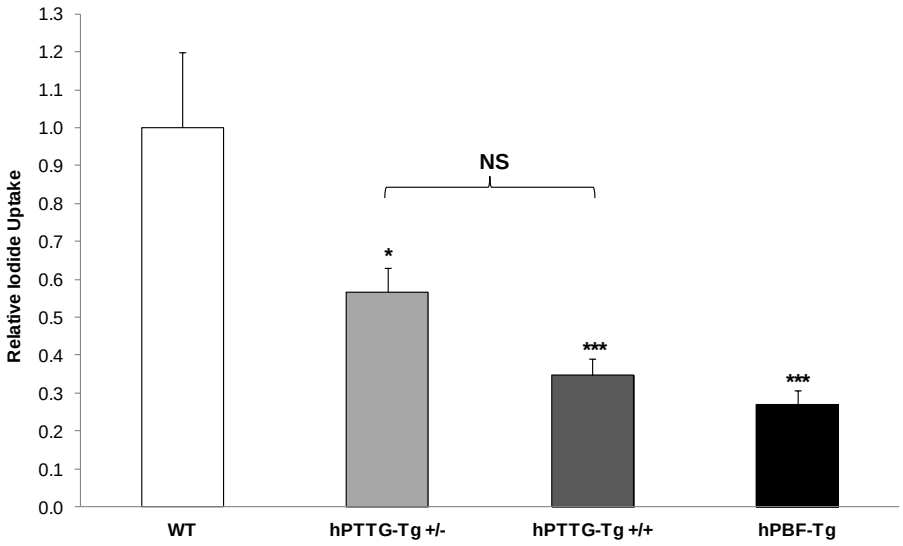


Figure 7-6: Relative ^{125}I uptake in primary thyrocyte cultures from WT (n = 14), hPTTG-Tg$^{+/-}$ (n = 15), hPTTG-Tg$^{+/+}$ (n = 14) and hPBF-Tg mice (n =14). Error bars represent the SEM (p < 0.05, *** p < 0.001, NS = non-significant).*

7.3.3 PBF knockdown rescues NIS phenotype

Adequate radioiodide uptake is a critical determinant of successful treatment of benign and malignant thyroid disease. We set out to investigate whether repressed ^{125}I uptake in hPTTG-Tg and hPBF-Tg thyroid cultures could be ameliorated. Primary thyrocyte cultures from hPTTG-Tg or hPBF-Tg mice were transfected with siRNA targeted to *hPTTG* and human *PBF* respectively. Unfortunately, we were unable to consistently knockdown transgenic hPTTG expression in hPTTG-Tg thyrocytes (data not shown). However, following successful PBF knockdown in hPBF-Tg thyrocytes, ^{125}I uptake increased 2.4 ± 0.64-fold (n = 20, $p < 0.05$) to levels indistinguishable from WT (n = 8, p = NS), whereas transfection using a scrambled siRNA did not alter ^{125}I uptake (n = 20, p = NS) (see Figure 7-7).

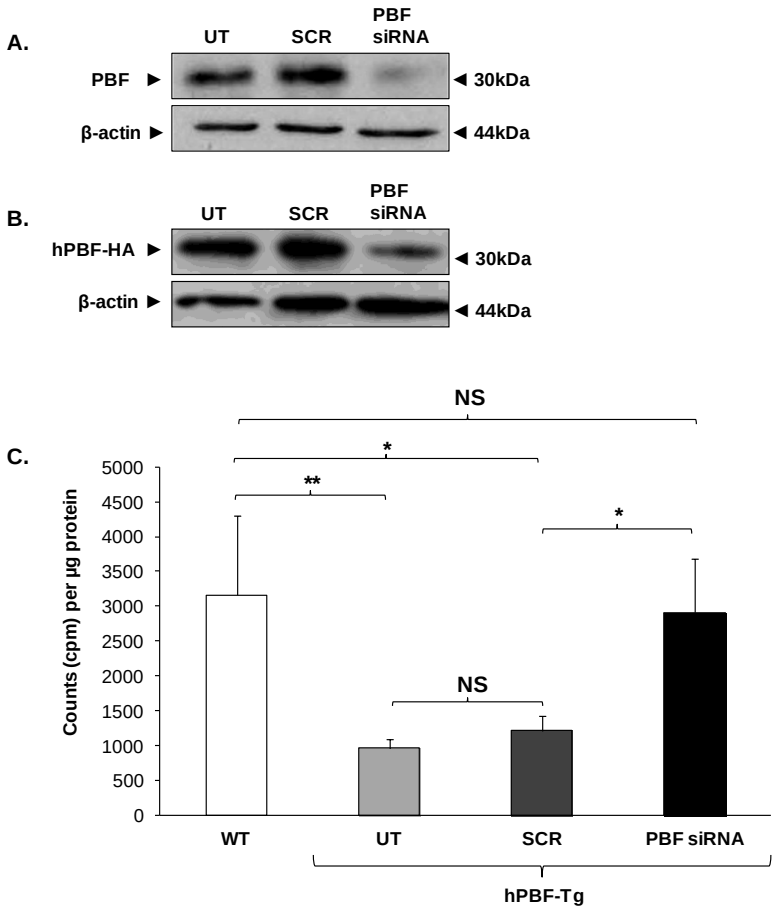


Figure 7-7: NIS function is restored following knockdown of PBF in hPBF-Tg primary thyrocytes. ***A + B*** *Western blot analyses demonstrating successful knockdown of transgenic PBF expression in hPBF-Tg primary thyrocytes, using either a PBF (**A**) or an HA (**B**) antibody.* ***C*** *Histogram displaying restoration of ^{125}I uptake in hPBF-Tg primary thyrocytes following PBF knockdown, to levels similar to those in WT cultures. Error bars represent the SEM (* $p < 0.05$, ** $p < 0.05$, NS = non-significant).*

7.3.4 PBF and hPTTG as therapeutic targets

To investigate the potential of PBF and hPTTG as therapeutic targets in human thyroid hyperplasia and neoplasia, we performed radioiodide uptake studies in primary cultures of normal human thyrocytes following transient knockdown of PBF or hPTTG. As in murine cultures, Western blot analysis confirmed successful repression of PBF protein expression in human thyrocytes. Reduced PBF expression was associated with a significant increase in *NIS* mRNA expression (2.1-fold, $n = 9$, $p < 0.05$) at 72 hours post-transfection. Crucially, targeted repression of endogenous PBF expression in human thyrocytes resulted in

a significant increase in radioiodide uptake (2.0-fold, n = 11, p < 0.001), compared with scrambled controls (n = 11) (see Figure 7-8).

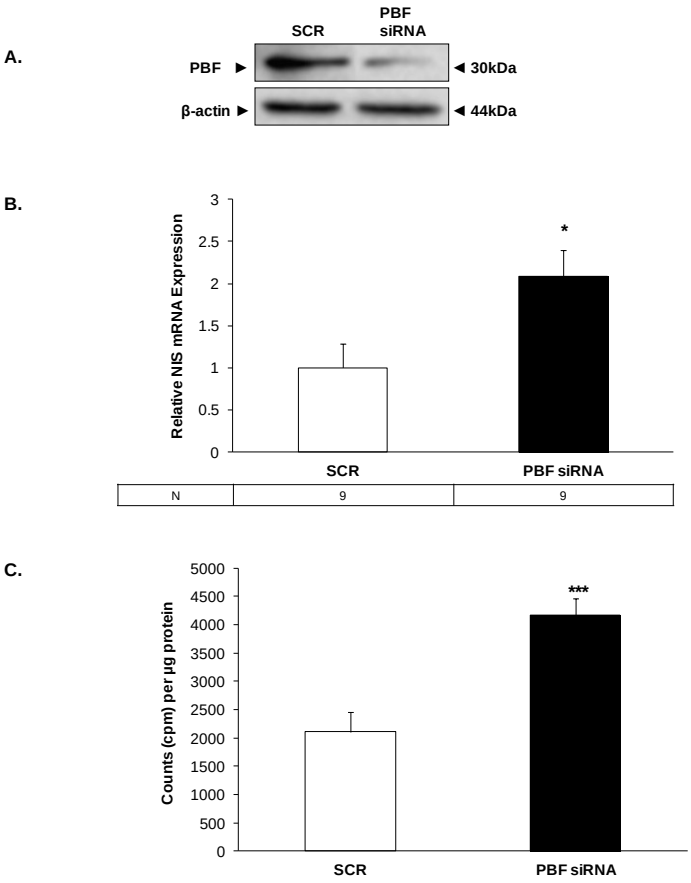


Figure 7-8: PBF as a therapeutic target in human thyroid disease. ***A*** *Western blot analysis demonstrating successful knockdown of endogenous PBF in normal human primary thyrocytes.* ***B*** *TaqMan PCR data demonstrating increased NIS mRNA expression in normal human primary thyrocytes following PBF knockdown.* ***C*** *Histogram displaying enhanced ^{125}I uptake by normal human primary thyrocytes following repression of PBF. N = 4 experiments. Error bars represent the SEM (* $p < 0.05$, ** $p < 0.001$).*

Transfection with a specific hPTTG siRNA resulted in approximately 50 % knockdown of hPTTG protein expression in human primary thyrocytes, as determined by Western blot analysis. Although radioiodide uptake was elevated following hPTTG protein depletion, there was no significant difference in comparison to scrambled transfection controls (1.4-fold, n = 12, p = NS) (see Figure 7-9).

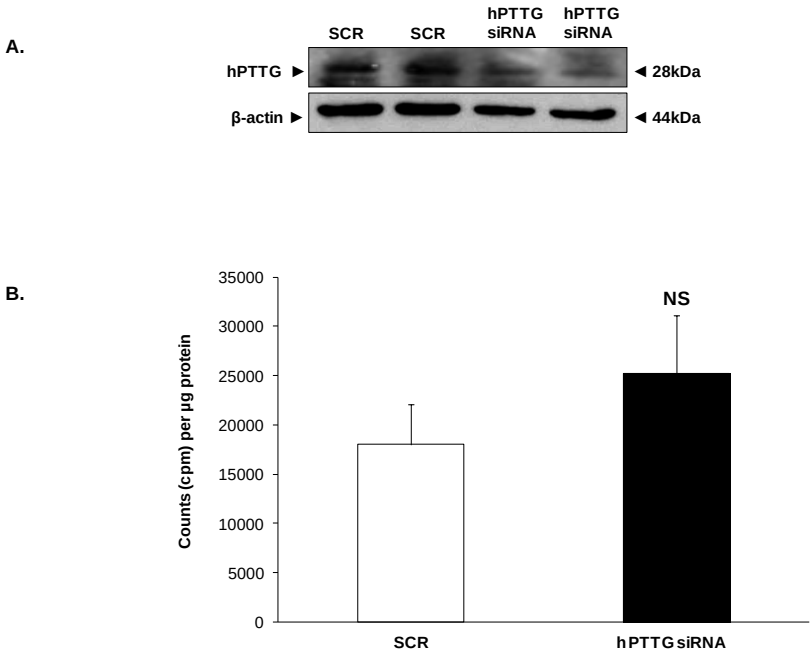


*Figure 7-9: hPTTG as a therapeutic target in human thyroid disease. **A** Western blot analysis demonstrating partial knockdown of endogenous hPTTG in normal human primary thyrocytes. **B** Histogram displaying ^{125}I uptake levels by normal human primary thyrocytes following repression of hPTTG. N = 3 experiments. Error bars represent the SEM (NS = non-significant).*

7.3.5 PBF-Tg murine primary thyrocyte control study

In the PBF siRNA studies described in section 7.3.3, a commercial siRNA against human *PBF* was used, where we had no knowledge of its activity against endogenous mouse *PBF* transcripts. Following the above observations that repression of endogenous PBF results in enhanced NIS expression and function in normal human primary thyrocytes, we performed a control experiment in WT murine primary thyrocytes to attempt to discern the effects caused by repression of either endogenous or transgenic PBF expression. Real-Time PCR analysis following transfection of WT murine primary thyrocytes with human PBF siRNA revealed that neither endogenous mouse *Pbf* (1.09-fold, $n = 6$, p = NS) or *Nis* (1.69-fold, $n = 6$, p = NS) mRNA expression was significantly altered. Crucially, there was no significant difference observed in ^{125}I uptake between PBF (1.03-fold, $n = 20$, p =NS) and scrambled ($n = 20$) siRNA transfected WT murine primary thyrocytes. Together these results suggest that the

reversal of hPBF-Tg NIS phenotypes described in section 7.3.3 was due to specific repression of transgenic human PBF expression. See Figure 7-10.

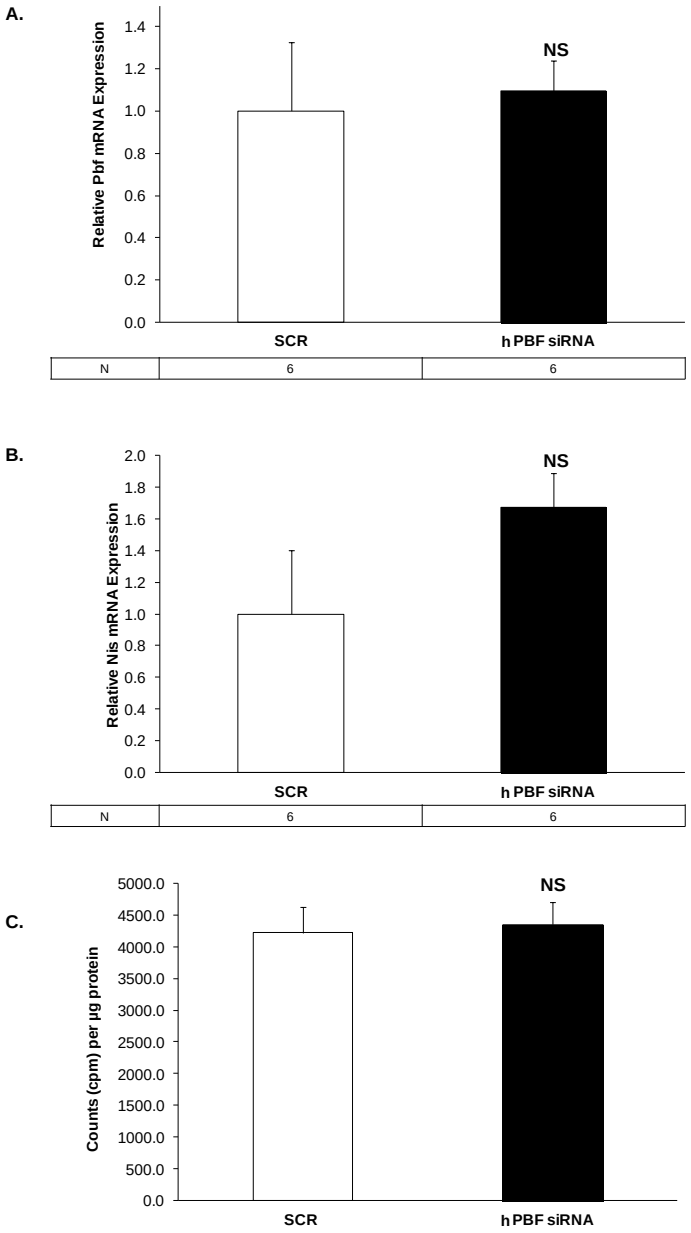


Figure 7-10: Effect of PBF siRNA transfections on endogenous mouse PBF and NIS expression and function in WT murine primary thyrocytes. ***A + B*** *TaqMan RT-PCR demonstrating that endogenous mouse PBF (**A**) and NIS (**B**) mRNA expression are unaltered in WT murine primary thyrocytes following transfection with hPBF siRNA.* ***C*** *Histogram displaying no alteration in ^{125}I uptake in WT cultures following transfection with hPBF siRNA. Error bars represent the SEM (NS = non-significant).*

7.4 Discussion

Radioiodine ablation therapies are central to the treatment of thyroid cancer and toxic nodular hyperthyroidism, and are also increasingly used as first-line therapy in Graves' disease (Weetman 2007) and to induce size reduction of benign goiters (Hegedus et al. 2003). The sodium iodide symporter (NIS) facilitates delivery of radioiodine but expression and activity of NIS are often repressed in thyroid cancers (Dohan et al. 2003; Boelaert and Franklyn 2003; Kogai et al. 2006). *In vitro* studies have demonstrated that hPTTG and PBF repress *NIS* transcription (Heaney et al. 2001; Boelaert et al. 2007) and that PBF also regulates intracellular localisation of NIS (Smith et al. 2009), both resulting in reduced I^- uptake by thyroid cells. In this chapter of work, we have conducted studies in transgenic mice with thyroid-targeted hPTTG and PBF expression to investigate whether these interactions exist *in vivo*. This, together with our knowledge that hPTTG (Heaney et al. 2001; Boelaert et al. 2003a) and PBF (Stratford et al. 2005) are over-expressed in thyroid cancer, suggests they represent potential targets for enhancing active NIS expression and radioiodide therapies in thyroid disease. To explore this, we performed siRNA knockdown studies in murine and human primary thyroid cells to determine if targeting PBF and hPTTG expression enhances NIS expression and activity.

7.4.1 NIS expression and function in hPTTG-Tg and PBF-Tg mice

Given that hPTTG and PBF regulate NIS expression and activity *in vitro*, we analysed expression levels of NIS in our transgenic mouse models with thyroid-targeted over-expression of hPTTG and hPBF (Heaney et al. 2001; Boelaert et al. 2007; Smith et al. 2009). TaqMan RT-PCR analysis revealed that *NIS* mRNA expression was reduced by approximately 40 % in both hPTTG-$Tg^{+/-}$ and hPTTG-$Tg^{+/+}$ thyroids, and by approximately 50 % in hPBF-Tg thyroids. Reduced NIS protein expression in hPTTG-$Tg^{+/-}$, hPTTG-$Tg^{+/+}$ and hPBF-Tg thyroids was confirmed by immunohistochemical analysis.

To investigate the functional consequence of reduced NIS expression in our transgenic mouse models, we established a protocol for routine culture of murine primary thyroid follicular cells and performed extensive validation studies to ascertain its suitability as an experimental model. Microscopic analysis of murine thyroid cultures demonstrated our ability to isolate thyroid follicular cells that adhere, spread and form secondary follicular structures, as previously described (Jeker et al. 1999). Critically, transgene expression was maintained in murine primary cultures derived from transgenic thyroid glands, as determined by immuncytochemistry and TaqMan RT-PCR, suggesting that we could expect any *in vivo* transgene effects to be preserved in *ex vivo* cultures. In addition, given that transgene expression is theoretically confined to thyrocytes, this confirmed the purity of our cultures, specifically containing mouse thyroid follicular cells with minimal contamination by fibroblasts.

TSH is the major hormonal regulator of thyroid cell function (Dohan et al. 2003; Boelaert and Franklyn 2003; Kogai et al. 2006) and TaqMan RT-PCR analysis demonstrated expression of TSH-R in WT cultures, indicating that our murine primary thyroid cells would be responsive to TSH in the culture medium. Further TaqMan RT-PCR analysis demonstrated that expression of *NIS* mRNA in WT cultures was comparable to that of WT whole thyroid glands, indicating our *ex vivo* cell culture system was representative of *in vivo* biology and possessed the major components required for I^- uptake. In addition, hPTTG-$Tg^{+/-}$ and hPTTG-$Tg^{+/+}$ cultures demonstrated a trend of reduced *NIS* mRNA expression compared with WT cultures, similar to that described in whole thyroid glands in section 7.3.1. In this instance, the trend was non-significant due to the relatively variable results observed in the cultures, but suggested that transgenic phenotypes were preserved in culture. Finally, preliminary ^{125}I uptake assays demonstrated significant uptake by WT murine primary thyrocytes with an average of 4222.1 cpm/μg protein, thereby confirming functionality.

Crucially, ^{125}I uptake was reduced in hPTTG-Tg$^{+/-}$ (0.57-fold), hPTTG-Tg$^{+/+}$ (0.35-fold) and PBF-Tg (0.27-fold) thyroid cultures compared with WT, confirming that reduced NIS mRNA and protein expression in transgenic mice caused reduced I^- uptake. PBF-Tg cultures showed a 73 % repression of I^- uptake, which in combination with our observation of ~50 % inhibition of *NIS* mRNA expression, suggests that repressed I^- uptake in PBF-Tg cultures may additionally encompass altered subcellular localisation of NIS in accord with our group's *in vitro* observations (Smith et al. 2009). Thyroid hyperplasia in PBF-Tg mice is driven by activation of the TSH-R and AKT (Read et al. 2011); thus, repression of NIS expression and function in PBF-Tg thyroids may be partly mediated by activation of PI3-Kinase, which has been implicated in downregulating NIS expression (Zaballos et al. 2008; Kogai et al. 2008) and trafficking to the basolateral membrane (Knostman et al. 2007b).

7.4.2 PBF and hPTTG as targets to improve I^- uptake in thyroid disease

hPTTG (Heaney et al. 2001; Boelaert et al. 2003a) and PBF (Stratford et al. 2005) are over-expressed in thyroid cancer, and while hPTTG expression is unaltered in multinodular goitre (MNG) (Boelaert et al. 2003a), PBF expression is increased in MNG in association with reduced NIS expression (Read et al. 2011). PBF-Tg mice have normal thyroid function and exhibit a striking enlargement of the thyroid gland associated with hyperplastic and macrofollicular lesions, bearing similarities to human MNG (Read et al. 2011), while no thyroid growth was observed in hPTTG-Tg mice (see Chapter 6).

Following confirmation of reduced I^- uptake in transgenic thyroid cultures, we set out to investigate hPTTG and PBF expression as potential therapeutic targets by performing siRNA knockdown studies. Unfortunately, we were unable to consistently knockdown hPTTG protein expression in hPTTG-Tg thyroid cultures, even with a high dose of 100 nM hPTTG siRNA. Future repeats of this study should focus on optimisation of hPTTG knockdown in hPTTG-Tg primary thyrocytes. However, PBF protein expression was

strongly and reproducibly repressed following transfection with PBF siRNA. Critically, PBF depletion resulted in full rescue of the PBF-Tg NIS phenotype, where $^{125}I^-$ uptake by PBF-Tg primary thyrocytes was restored to levels comparable to those of WT cultures. This exciting result verifies the specificity of the PBF-induced NIS phenotype and highlights PBF as a potential therapeutic target.

In order to investigate whether PBF represents a potential therapeutic target in human thyroid disease, we used primary cultures of normal human thyrocytes, and investigated the effects of altered PBF expression on radioiodide uptake. PBF depletion resulted in a ~2.0-fold enhancement of $^{125}I^-$ uptake by human primary thyrocytes in association with increased expression of *NIS* mRNA, providing further evidence for the functional interaction between PBF and NIS. In a parallel study, we investigated the therapeutic potential of hPTTG. In contrast to studies performed in hPTTG-Tg murine primary thyrocytes, moderate knockdown of hPTTG protein was successful but did not result in altered $^{125}I^-$ uptake. It is possible that knockdown of hPTTG was not strong enough to elicit an alteration in NIS function. Alternatively, hPTTG regulation of NIS transcription could be an acquired function that is only active when hPTTG is over-expressed as it is in thyroid malignancies, but does not regulate NIS when expressed at physiological levels as it is in normal thyroid tissue or MNG (Boelaert et al. 2003a). In contrast, PBF appears to regulate NIS expression and activity in normal thyroid tissue and this function is enhanced when PBF is over-expressed in thyroid disease (Read et al. 2011; Stratford et al. 2005).

7.4.3 Conclusion

Overall, these results provide critical *in vivo* evidence that both hPTTG and PBF downregulate thyroidal NIS expression and reduce I^- uptake, which has important implications for the efficacy of radiodine therapies in thyroid disease where hPTTG and PBF are over-expressed. The resemblance between PBF-Tg mouse phenotypes and human MNG indicates

that PBF may be important in the aetiology and treatment of hyperplastic thyroid diseases, in addition to its more established role in thyroid cancer. While further investigations are required to determine the benefits to radioiodide uptake by targeting hPTTG expression, we have crucially demonstrated that the binding partner of hPTTG, PBF, represents a novel therapeutic target to overcome radioiodine resistance in thyroid tumors and their metastases, as well as more generally in other thyroid diseases.

8 CHAPTER EIGHT

Creation of a Transgene Construct for the Generation of an Alternative hPTTG-Tg Transgenic Colony

8.1 Introduction

In Chapters 5 and 6 we described the generation and characterisation of a murine transgenic model with thyroid-targeted over-expression of hPTTG (hPTTG-Tg mice). The major limitation of this study was that only one transgenic line was available for investigation. Our observation that hPTTG-Tg mice had smaller thyroids compared with WT mice was in contrast to studies reporting hPTTG-induced tumour formation in athymic nude mice (Zhang et al. 1999b; Kakar and Jennes 1999), and hPTTG-induced hyperplasia in the pituitary glands (Abbud et al. 2005) and ovaries (El-Naggar et al. 2007) of transgenic mice. In a study from our own group, low levels of hPTTG over-expression caused increased proliferation in fetal neuronal NT-2 cells, while high levels of hPTTG resulted in inhibited cell growth (Boelaert et al. 2003b). Accordingly, hPTTG-Tg mice demonstrated high levels of thyroidal hPTTG over-expression associated with reduced thyroid size. This observation prompted the hypothesis that hPTTG effects on thyroid cell proliferation *in vivo* may be dependent on expression levels. In order to test this hypothesis, we set out to create alternative transgenic lines and aimed to investigate thyroid phenotypes in a model with low levels of thyroidal hPTTG over-expression.

Having previously failed to detect hPTTG-FLAG using FLAG antibodies (Chapter 5), the aim of the work described in this chapter was to adapt the *Tg-hPTTG-FLAG* transgene construct to replace the FLAG epitope with an HA epitope (Tg-hPTTG-HA). This work provides another transgene construct for pronuclear injection in order to generate alternative transgenic lines with varying levels of thyroidal hPTTG expression. In turn, this will allow us to define if phenotypes are dependent upon the level of thyroidal hPTTG over-expression *in vivo*.

8.2 Materials and methods

8.2.1 Site-directed mutagenesis of *pBSK-Tg-hPTTG-FLAG*

pBSK-Tg-hPTTG-FLAG was as described in Chapter 5 (see Figure 5-4 and 5-7). The C-terminal FLAG sequence was converted to an HA sequence through site-directed mutagenesis (see Figure 8-1), using the QuickChange site-directed mutagenesis kit (Stratagene, UK), as per the manufacturer's instructions. Briefly, 50 µl mutagenesis reactions contained 40 ng of plasmid dsDNA, 5 µl 10 x reaction buffer, 1 µl dNTP mix, 125 ng of forward and reverse mutagenic primers, water and 1 µl *PfuTurbo* DNA polymerase (2.5 U/µl). Mutagenic primers were as described in Figure 8-1 C and D. Mutagenic PCR reactions were performed using a Mastercycler gradient (Eppendorf, Hamburg, Germany). Reactions were as follows: 95 °C for 30 seconds; then 17 cycles of 95 °C for 30 seconds, 55 °C for 1 minute, and 68 °C for 6 minutes (1 minute/1 kb plasmid DNA). Subsequently, 1 µl of DpnI restriction enzyme (10 U/µl) was added to reaction products and incubated at 37 °C for 1 hour in order to digest digest the parental DNA template and to select for mutation-containing synthesised DNA.

8.2.2 Transformation into DH5α cells

Transformation of 1 µl of mutation-containing synthesised DNA into 50 µl DH5α cells was performed as decribed in section 5.2.1.4.

8.2.3 Plasmid DNA purification and sequence verification

Bacterial colonies were cultured overnight as described in section 5.2.1.5. Plasmid DNA was purified from these cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega) as described in section 5.2.1.5.

8.2.4 DNA sequencing of miniprep DNA

DNA sequencing was performed as described in section 5.2.1.6 in order to confirm incorporation of the desired mutations. Sequencing primers F and L (see Figure 5-6 B) were used for this purpose.

8.2.5 Maxiprep of *pBSK-Tg-hPTTG-HA*

Following confirmation of successful mutation, bacterial cultures originating from the same colonies were set up and larger quantities of DNA were purified by maxiprep as described in section 5.2.1.7.

8.2.6 DNA sequencing of *pBSK-Tg-hPTTG-HA*

The correct nucleotide sequence and orientation was confirmed using the primers described in the table in Figure 5-6 B and by DNA sequencing analysis as described in section 5.2.1.6.

8.2.6.1 Isolation of transgene for pronuclear injection

The final linearised transgene construct was isolated using restriction enzyme digest reactions and diagnostic agarose gels. Restriction enzyme digest of *pBSK-TG-hPTTG-FLAG* was performed in reactions similar to those described in section 5.2.1.3 using Xho 1, Spe 1 and Pvu 1. The reaction product following digestion with Xho 1, Spe 1 and Pvu 1 was electrophoresed on a 1.5 % agarose gel and the presence of a DNA fragment for the predicted size of ~3500 bp was gel extracted and purified as described in section 5.2.1.2.

8.2.6.2 Isolation of transgene for pronuclear injection

The final linearised transgene construct was isolated using restriction enzyme digest reactions and diagnostic agarose gels as previously described (see section 5.2.1.11).

8.3 Results

8.3.1 Strategy for generation of *hPTTG-HA* transgene construct

Following failure to detect hPTTG-FLAG in hPTTG-Tg mice using FLAG antibodies (Chapter 5), we decided to create alternative transgenic lines using a construct consisting of *hPTTG* cDNA with a C-terminus HA-tag. Other components of the transgene construct remained identical. The *pBSK-Tg-hPTTG-FLAG* created in Chapter 5 (see Figure 5-4 and 5-6), was mutated directly to avoid other cloning steps. A two-step site-directed mutagenesis strategy was adopted to mutate 16 base pairs within the FLAG sequence, converting this to a sequence encoding the HA-epitope. See Figure 8-1.

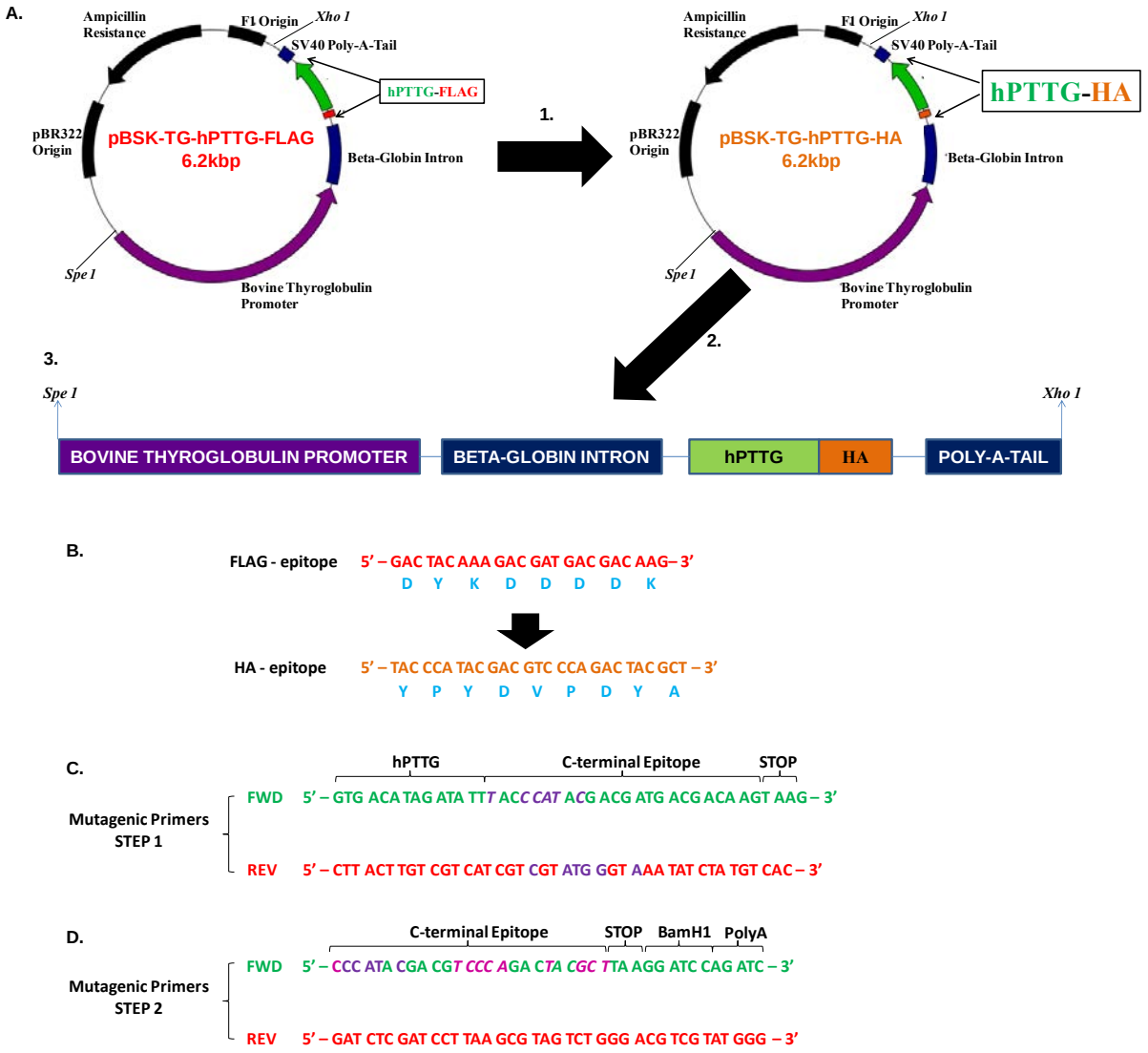


*Figure 8-1: Generation of the Tg-hPTTG-HA transgene construct. **A** Schematic diagram illustrating the strategy for generation and isolation of the Tg-hPTTG-HA construct (**1.** Site-directed mutagenesis, **2.** Restriction enzyme digest, **3.** Isolated Tg-hPTTG-HA construct). **B** Nucleotide sequences of FLAG (red) and HA (orange) epitopes, with amino-acid sequences underneath (blue). **C + D** Mutagenic primers for step 1 (**C**) and step 2 (**D**). Mutated base pairs following step 1 are indicated in purple, while those from step 2 are indicated in pink.*

8.3.2 Verification of construct by DNA sequencing

Following each mutagenesis step, sequencing of DNA minipreps using Primers F and L (see Figure 5-6 B) confirmed that reactions had successfully incorporated the desired mutations. Having extensively validated activity of the *pBSK-Tg-hPTTG-FLAG* construct *in vitro* and *in vivo* in Chapter 5, we did not perform any further *in vitro* validation studies at this stage with the *pBSK-Tg-hPTTG-HA* contstruct. However, as undesired spontaneous

mutations can occur during site-directed mutagenesis and DNA maxi-preps, we used the same primers as detailed previously (see Figure 5-6 B) to verify the correct nucleotide sequence and orientation of all construct components (see Figure 8-2).

Figure 8-2: Direct sequencing of the pBSK-Tg-hPTTG-HA construct confirmed the correct nucleotide sequence and orientation of the construct. The nucleotide sequence for the transgene construct from the Spe 1 to Xho I restriction sites (3566 bp) is shown. Sequencing primer details are given in Figure 5-6 B.

8.3.3 Isolation and preparation of the transgene construct

A linearised transgene construct was isolated using restriction enzyme digest reactions and diagnostic agarose gels. As described previously (Chapter 5), digestion with only Xho 1 and Spe 1 resulted in two bands of DNA migrating very closely together, representing the ~3500 bp transgene and a similarly sized fragment from the remainder of the plasmid. Additional digestion with Pvu 1 caused cuts at two further sites within the *pBSK* plasmid so

that the transgene fragment was more easily isolated on an agarose gel. The reaction products obtained following multiple digestions using Xho 1, Spe 1 and Pvu 1 were migrated on an agarose gel and the presence of DNA fragments for the predicted size of ~3500 bp were confirmed. The transgene fragments were excised from the gel and subject to gel extraction and purification before being passed on to the Biomedical Services Unit for further purification and microinjection as described in section 5.3.2.2 (see Figure 8-3).

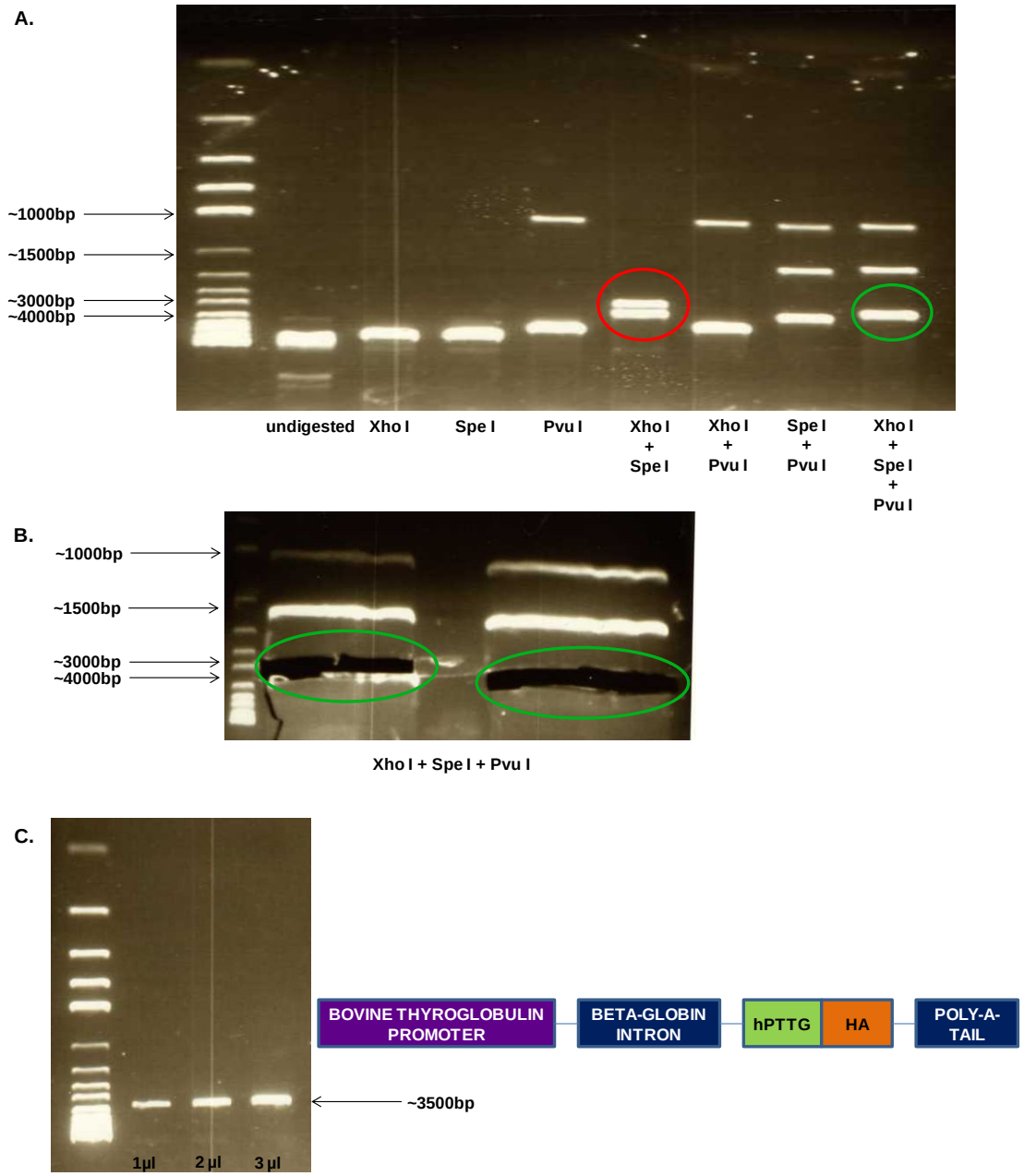


*Figure 8-3: Isolation of the linearised Tg-hPTTG-HA transgene construct. **A** Diagnostic agarose gel illustrating the products of various restriction digest reactions. Red circle demonstrates the difficulty in resolving between the two fragments when just Xho 1 and Spe 1 are used. Green circle*

highlights the identification of the transgene fragment (~3500 bp) following digestion with Xho 1, Spe 1 and Pvu 1. ***B*** *Agarose gel demonstrating migration and excision of transgene fragments (Green circles) following digestion with Xho 1, Spe 1 and Pvu 1.* ***C*** *Agarose gel showing migration of 1, 2 and 3 µl samples of the linearised transgene following excision from original agarose gels and a purification step. The clean, sharp appearance of the band indicates a pure and non-denatured DNA sample.*

8.4 Discussion

The work reported in this chapter describes the successful creation of a transgene construct containing *hPTTG* cDNA with a C-terminal HA-epitope downstream of the bovine thyroglobulin promoter. The linearised transgene construct was isolated and purified in preparation for pronuclear microinjection. Subsequent generation of alternative transgenic lines with thyroid-targeted hPTTG over-expression will facilitate the investigation of relative hPTTG over-expression levels on thyroid phenotypes *in vivo*. From this point onwards, investigations were carried out by other members of the group.

9 CHAPTER NINE

Final Conclusions and Future Studies

The work in this thesis has investigated some of the roles of the proto-oncogene hPTTG in the growth and function of thyroid cells. Based on the hypothesis that hPTTG affects thyroid neoplasia by the initiation as well as the promotion of tumourigenesis, the complex interplay between hPTTG, growth factors and other key genes was examined in thyroid cells *in vitro* and *in vivo*. *In vivo* investigations were facilitated by the successful generation of a murine transgenic line with thyroid-targeted hPTTG over-expression. Furthermore, due to the key roles of hPTTG and PBF in the regulation of NIS expression and function, the expression of these oncogenes as potential therapeutic targets for enhancing radioiodine treatments was investigated.

9.1 Autocrine interactions between hPTTG and growth factors

Expression and phosphorylation of hPTTG was induced by EGF, TGF-α and IGF-1 following activation of pathways involving MAPK and PI3K in both malignant and non-malignant human thyroid cells. These effects were independent of known regulators of hPTTG expression and phosphorylation, SP1 and CDC2 respectively. Interestingly, CDC2 was identified as an independent regulator of hPTTG expression. Subsequently, we confirmed that in turn, hPTTG over-expression causes upregulation of EGF, TGF-α and IGF-1 expression and secretion in human follicular thyroid cells via its SH3-interacting domain, and enhanced levels of growth factors caused autocrine induction of hPTTG. Collectively, these studies define the existence of autocrine pathways of interaction between hPTTG and growth factors, which may be aberrantly controlled in thyroid tumours. Subsequent investigations in hPTTG-Tg and $Pttg^{-/-}$ knockout mice confirmed a particularly important interaction between hPTTG and EGF *in vivo*.

Continued investigations into the exact mechanisms by which growth factors regulate hPTTG expression and phosphorylation may elucidate novel potential targets to reduce

hPTTG expression in thyroid cancer. To this effect, further siRNA knockdown studies of other transcription factors implicated in the regulation of hPTTG including OCT-1 (Zhou et al. 2008), AP1 and AP2 (Kakar 1999), may provide further insights and identify novel strategies in this respect.

CDC2 is the only reported regulator of hPTTG phosphorylation (Ramos-Morales et al. 2000), which is in contrast with the work described in this thesis. Future studies should aim to identify alternative mechanisms of hPTTG phosphorylation. Given reports of a direct interaction between MAPK and PI3K with hPTTG (Chamaon et al. 2005), and the fact that Mapk directly phosphorylates rat Pttg (Pei 2000), it would be of specific interest to investigate whether MAPK and PI3K directly phosphorylate hPTTG in thyroid cells in response to growth factors.

Whilst a moderate correlation between transcripts of hPTTG and growth factors was observed in normal human thyroid tissue, it would be interesting to perform correlation studies in thyroid cancer specimens. We would propose to assess expression of hPTTG, EGF, TGF-α and IGF-1 in our group's recently collected cohort of "matched" thyroid cancer specimens. Thus, we would be able to verify the relationship between hPTTG and these growth factors in human thyroid cancer and determine whether the expression of these growth factors is, in addition to FGF-2 (Boelaert et al. 2003a), useful in predicting aggressive thyroid tumour behaviour.

9.2 Further investigations of thyroid-targeted hPTTG over-expression

We generated a murine transgenic model with thyroid-targeted over-expression of hPTTG (hPTTG-Tg mice) in order to directly investigate the tumourigenic effects of thyroidal hPTTG over-expression *in vivo*. Unexpectedly, transgenic mice with high levels of thyroidal hPTTG expression do not develop thyroid hyperplasia or neoplasia, but in fact have smaller thyroid glands.

Future studies should aim to verify reduced proliferation in thyroids from hPTTG-Tg mice through expression analyses of proliferation markers such as PCNA and cyclin-D1. In addition, following on from work described in Chapter 8, we are currently generating and characterising alternative murine transgenic lines with thyroid-targeted hPTTG expression. These studies will enable us to define the *in vivo* dose-dependent effects of hPTTG on thyroid cell proliferation and transformation, and to test the hypothesis that low levels of thyroidal hPTTG over-expression results in thyroid tumourigenesis. Furthermore, additional transgenic lines with higher levels of thyroidal hPTTG over-expression will provide the means to validate and give credence to the conclusions derived from our studies in current hPTTG transgenic mice.

We recently sourced a low-iodine mouse chow (Teklad Laboratories) for ongoing studies using our standard model of goitre induction (Ramsden et al. 2005) in WT, hPTTG-Tg and $Pttg^{-/-}$ knockout mice to investigate the effects of thyroidal hPTTG over- and under-expression on TSH-driven goitrogenesis. In addition, we are performing preliminary experiments to investigate whether we can successfully target mouse thyroid glands *in situ*, with doses of ionising radiation using the irradiator facility at the Biomedical Services Unit (University of Birmingham). These studies will provide important insight into the oncogenic potential of thyroidal hPTTG over-expression in the context of elevated serum TSH levels or DNA damage following exposure to external radiation.

Ongoing characterisation of hPTTG.PBF-Tg bitransgenic mice remains an investigation of priority. Given the striking hyperplasia observed in PBF-Tg mice (Read et al. 2011) and that PBF facilitates the nuclear translocation and transactivational capability of hPTTG (Chien and Pei 2000), it will be interesting to see if tumourigenesis is facilitated by their concerted over-expression in the thyroid. In addition, given the reported roles of hPTTG and PBF in causing genetic instability, investigations using fluorescent inter-simple sequence

repeat PCR (FISSR-PCR) are being conducted to determine the relative levels of genetic instability in hPTTG-Tg, PBF-Tg and hPTTG.PBF-Tg mice compared with WT controls. This study may provide important insight concerning genetic aberrations that underlie key phenotypes.

9.3 hPTTG and regulation of other angiogenic genes

Angiogenesis-specific cDNA PCR array analysis highlighted the potential relationship between hPTTG and a number of genes critically involved in tumour proliferation and angiogenesis, and has opened up opportunities for further research.

hPTTG upregulation of the pro-angiogenic genes *Cxcl1* and *Mdk*, and repression of the anti-angiogenic gene *Tnf-α*, was of particular interest. We propose that thyroidal expression changes of these genes should be validated in greater numbers of hPTTG-Tg mice using gene-specific TaqMan RT-PCR assays (Applied Biosystems) and immunohistochemistry on paraffin embedded thyroid sections. Upon validation of these alterations in hPTTG-Tg mice, expression analyses should also be performed in our cohort of 'matched' human thyroid specimens.

9.4 hPTTG and PBF as therapeutic targets for enhancing treatment with radioiodine

Work described in this thesis confirmed that both hPTTG and PBF downregulate thyroidal NIS expression and reduce I^- uptake *in vivo*, which has important implications for the efficacy of radiodine therapies in thyroid disease where hPTTG and PBF are over-expressed. Crucially, we demonstrated that PBF represents a novel therapeutic target to overcome radioiodine resistance in thyroid tumors and their metastases, as well as more generally in other thyroid diseases.

Our inability to consistently achieve strong repression of hPTTG expression in hPTTG-Tg thyrocytes or human primary thyrocytes hampered our investigations of hPTTG as a

potential therapeutic target for enhancing ^{131}I uptake. Successful knockdown of PBF was achieved by using a combination of siRNAs targeting *PBF* transcripts. We propose that a similar strategy should be adopted to optimise knockdown of hPTTG expression in order to fully determine the potential of hPTTG as a therapeutic target to alleviate NIS repression in thyroid cancer.

Following demonstration of these critical phenotypes in an *ex vivo* primary culture system, it would be interesting to confirm these findings through *in vivo* visualisation of ^{124}I accumulation using positron emission topography (PET) imaging, as has been described previously (Roepke et al. 2009). Further, studies of non-thyroid cancers have used novel approaches using hPTTG siRNA to reduce hPTTG expression *in vivo*, resulting in inhibition of tumour development (Kakar and Malik 2006; Jung et al. 2006). Therefore, an exciting prospective study would be to combine *in vivo* siRNA treatments with PET-imaging in hPTTG-Tg and PBF-Tg mice to demonstrate the therapeutic potential of targeting hPTTG and PBF over-expression in live mice. In the future, this approach may evolve into a therapeutic approach, with progress continuing in the development of siRNA delivery techniques that allow over-expressed genes to be specifically targeted in cancer cells without affecting normal cells.

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