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Regulation of angiogenic factors by pituitary tumor transforming gene (PTTG) in tumorigenesis.

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REGULATION OF ANGIOGENIC FACTORS BY PITUITARY TUMOR TRANSFORMING GENE (PTTG) IN TUMORIGENESIS

By

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A Dissertation Approved on

May 25th 2006

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DEDICATION

To my wife Saira and children Naureen and Danial Malik for their support and encouragements

They began my education.
They motivated me to continue it.
They always contributed to it.

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I would like to express my deep and sincere gratitude to my mentor Dr. Sham S. Kakar, his wide knowledge, and his logical way of thinking have been great value for me. His understanding and encouragement and personnel guidance provided me the basis of my thesis.

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Finally, I would like to thanks all who directly and indirectly supported and helped me completing my thesis on time.

ABSTRACT

UPREGULATION OF ANGIOGENIC FACTORS BY PITUITARY TUMOR TRANSFORMING GENE (PTTG) IN TUMORIGENESIS

MOHAMMAD TARIQ MALIK

May, 25TH 2006

Pituitary tumor transforming gene (*PTTG*), also known as securing, is a novel oncogene that is expressed at high levels in most of the tumors analyzed to date. Overexpression of *PTTG* in mouse fibroblast (NIH 3T3) cells increases cell proliferation, induces cellular transformation and promotes tumor formation in nude mice. PTTG is a multi-domain and multifunction protein. Some functions of PTTG include inhibition of pre-mature sister chromatid separation, activation of transcription of c-myc oncogene, cell cycle regulation, and DNA repair. In some cancers such as thyroid, pituitary, esophageal, and colorectal tumors, high *PTTG* expression correlates with metastasis and poor prognosis. The PTTG gene encodes a protein of 203 amino acids and is consists of six exons and four introns. The *PTTG* promoter is specifically activated in tumors and is highly regulated by Sp1 and NF-Y nuclear factors. However, the precise mechanism by which *PTTG* mediates its tumorigenic function remains unclear. To this end, we determined the effect of *PTTG* on tumor metastasis and angiogenesis by determining its effect on secretion and expression of various

angiogenic and metastatic factors including bFGF, VEGF, IL-8 and MMP-2. Using RT/PCR, ELISA and zymography we showed a significant increase in expression and secretion of bFGF, VEGF, IL-8 and MMP-2 in HEK293 cells on transfection of PTTG cDNA. Down regulation of PTTG expression by siRNA and a genetically deleted HCT116 (PTTG -/-) cell line showed significantly decrease in expression of MMP-2, which led to decreased invasion and migration of HCT116 (PTTG-/-) cells as compared to the HCT116 wild type. Taken together, our results confirm that PTTG is a potent tumorigenic gene that exerts its tumorigenic functions by increasing secretion and expression of VEGF, bFGF, IL-8 and MMP-2 and MT1-MMP. In addition expression of other angiogenic and adhesion molecules including beta-1, beta-3, alpha-5 and alpha-V integrins was also modulated by PTTG in HEK293 cells.

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CHAPTER I

INTRODUCTION

Cancer is the second leading cause of death in the United States. An estimated 1.4 million new cases and 570 thousand deaths are expected in the year 2006 (1). Overall worldwide, every year more than 11 million people are diagnosed with cancer, leading to an estimated seven million deaths (2). Despite substantial advances made in understanding tumorigenesis, complete knowledge of the underlying mechanisms that lead to cancer initiation and progression remain unclear. Some of the factors that have been reported to influence cancer development include, but are not limited to environmental factors, genetic predisposition, and aging. Cancer development is a complex and multi-step process that involves activation of proto-oncogenes, growth factors, and their receptors, or deregulation of tumor suppressor genes leading to abnormal cellular processes. Most importantly, the cancer cells exploit every opportunity to deviate from the standard functions and restraints of normal cells. Genetic aspect of cancer can be traced back more than 100 years when von-Hansenmann in 1890 and Boveri et al. in 1914 suggested aneuploidy, due to chromosomal missegregation, as the fundamental basis of cancer (3-5). Since the Boveri prediction it now seems that all solid tumor cells are not only aneuploid (6) but they have acquired a number of mutations in oncogenes and tumor suppressor

genes. Some of these genes include KRAS, TP53, APC, BRCA1, and RB1 among others. In addition cancer cell lines show a high level of chromosomal instability (CIN) as a result of the loss or gain of whole chromosome or partial loss of chromosomes during cell division (7).

The cause of chromosomal instability (CIN) is unknown, but one idea is that it occurs due to a defect in the processes that control chromtid separation during mitosis (8). Whether aneuploidy is a contributor, or just a reason for tumor development, remains unanswered. It is known that a tumor originates from a single altered cell, followed by sequential somatic, genetic or epigenetic changes over a generation with increased aggressive subpopulation within the tumor mass (9,10). Somatic evolution phenomena have been confirmed by numerous molecular studies; it has been estimated that cells have to acquire five to seven successive mutations to allow tumor growth, invasion and metastasis (11-13).

To identify the cause of tumorigenesis many cancer-causing genes have been identified. Although they differ from tumor to tumor, loss or change in their functions allows most cancers to acquire phenotypic differences. Hanahan and Weinberg (14) described in detail phenotypic differences between healthy and cancerous cells. The basic six phenotypic changes reported to occur at the cellular level as essential hallmarks of cancer are: (a) unlimited mitosis; (b) evasion of apoptosis; (c) resistance to growth-inhibition signals; (d) tissue invasion and metastasis; (e) sustained angiogenesis; and (f) escape from the dependence on external growth stimulation (14). Genetic instability is an additional factor which accounts for the high incidence of mutations in cancer cells. Cancer susceptible

genes can be generally categorized into three classes: gatekeepers, caretakers and landscapers (15,16).

Broadly speaking, gatekeeper genes are divided into two categories: (1) oncogenes and (2) tumor suppressor genes (17,18). Mutations in any or both of these types of genes operate similarly at the physiological level, and can exert positive or negative regulatory properties on cell growth and proliferation. Oncogenes are frequently activated by gain-of-function mutations, fusion with other genes, amplification, increased expression as a result of increased promoter activity, or protein stabilization (19,20). Hence these oncogenes genes play an important role in diverse signaling pathways involved in various stages of human cancer initiation, progression, angiogenesis and metastasis (17). Caretakers, or stability genes, function to maintain the genomic integrity of the cell and regulate DNA repair mechanisms, chromosome segregation and cell cycle check points (18). Defects in caretaker genes lead to genetic instabilities that contribute to the mutations in other genes, including oncogenes and suppressor genes that directly affect cell proliferation and survival, thus promoting tumorigenesis (21-23). Defects in landscaper genes do not directly affect cellular growth, but instead generate an abnormal stromal environment that contributes to neoplastic transformation of cells (17,23). Some examples of landscaper genes are metalloproteinases (*MMPs*), uroplasminogen activator (*uPA*), tissue plasminogen activator (*tPA*), fibroblast growth factor-2 (*FGF-2*), and platelet-derived growth factor (*PDGF*).

In summary, cancer is a complex and multistep genetic disease that involves accumulation of genetic changes in somatic cells. These genetic changes often involve activation of oncogenes and inactivation or loss of tumor suppressor genes. However the mechanisms that initiate and promote tumorigenesis remain unclear.

1.1 PITUITARY TUMOR TRANSFORMING GENE (PTTG)

To help understand the mechanisms of human tumorigenesis and to identify the possibility for the existence of a common gene or signaling mechanism, our laboratory cloned and characterized a novel oncogene: the pituitary tumor transforming gene (PTTG), also known as mammalian securin, from human testis (24) and ovarian tumors (25). Our initial studies were based on the work of Pei et al (26), who used an mRNA differential display technique to clone PTTG from a rat pituitary tumor. We and others showed that induction of PTTG in mouse NIH3T3 fibroblast cells induces cell transformation *in vitro* and *in vivo* (26-28). PTTG was found to be expressed in almost all tumors analyzed to date, including tumors derived from the pituitary, adrenal, thyroid, liver, kidney, endometrium, uterus, breast, testis, ovary, colon, as well as melanoma, leukemia and various cell lines derived from these tumors (24,26,29-35). Expression of PTTG is either very low or undetectable in normal tissues, except in the testis. PTTG is reported to be a novel oncogene shown as a molecular signature gene, whose expression in solids tumors is associated with metastasis and poor

prognosis (36). Inactivation of PTTG results in chromosomal loss with high levels of chromosomal instability (37,38) and a defect in cell division (39). Overexpression of PTTG causes tumor formation in nude mice (26) and tumor cells are aneuploid (40,41). A strong correlation between aneuploidy, genomic instability and overexpression of PTTG was shown by Kim et al (42) in follicular thyroid cancer cells. Zhang et al (35) showed a positive correlation between PTTG levels and invasiveness of pituitary tumors. Similarly, Genkai et al (43) showed a higher level of expression of PTTG in high grade tumor tissues from patients with poor prognosis. PTTG has also been shown to be involved in angiogenesis by inducing the expression and secretion of FGF (27) and VEGF (44). In summary, PTTG is an important proto-oncogene, involved in many cellular processes, and importantly, in tumorigenesis. Therefore, understanding the mechanism by which PTTG induces tumorigenesis will help in identifying new signaling pathways and gene products that may help to understand complex diseases and allow for the development of novel and more potent therapeutics opportunities.

1.2 PRIMARY STRUCTURE OF PTTG

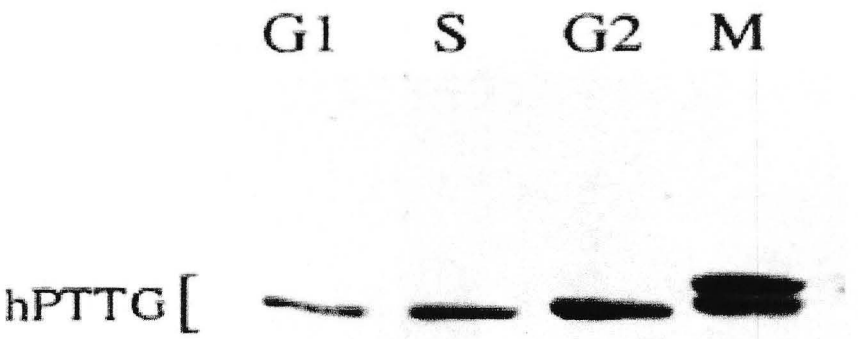
PTTG cDNA cloned from testis (24) and ovarian tumors (25) is composed of 656 nucleotides, and encodes a protein of 202 amino acids, with an approximate molecular weight of 24 kDa (25). However, PTTG migrates at 28 – 30 kDa on SDS-PAGE, suggesting post-translational modifications (27,34,45,46).

PTTG also migrates as a doublet, which is suggestive of phosphorylation status as shown in (Figure 1) (26,47).

Sequence analysis of the PTTG protein showed no homology to other proteins in the human genome (24,26). It is a multi-domain, natively unfolded protein (48), and can be divided into two parts: the N-terminal regulatory domain and C-terminal functional domain. The N-terminus contains a KEN box and D-box surrounded by lysine residue, and serves as a target for anaphase promoting complex (APC) for degradation (Figure 2) (49). A mutation in the KEN box does not prevent PTTG degradation, while a mutation in the D-box region partially prevents degradation (45). However, a mutation in both KEN and D-boxes completely abolishes PTTG degradation (50). The C-terminal functional domain is more acidic and contains proline-rich motifs (PXXP), a casein kinase II (CKII) phosphorylation site, and cyclic AMP and cyclic GMP protein kinase phosphorylation sites (27,29,30,47). The proline-rich domain (PXXP) is known to bind to various Src homology 3 (SH3) domain-containing proteins (51-53). The SH3 binding domains mediate protein-protein interactions in signal transduction, suggesting that PTTG may interact with other proteins that control its function, regulation or trans-localization. However, no protein that interacts with these sites has been identified.

Figure 1: PTTG is expressed in cell cycle dependent manner. A doublet for phosphorylated PTTG protein is observed at M phase of cell cycle suggesting potential for PTTG phosphorylation.

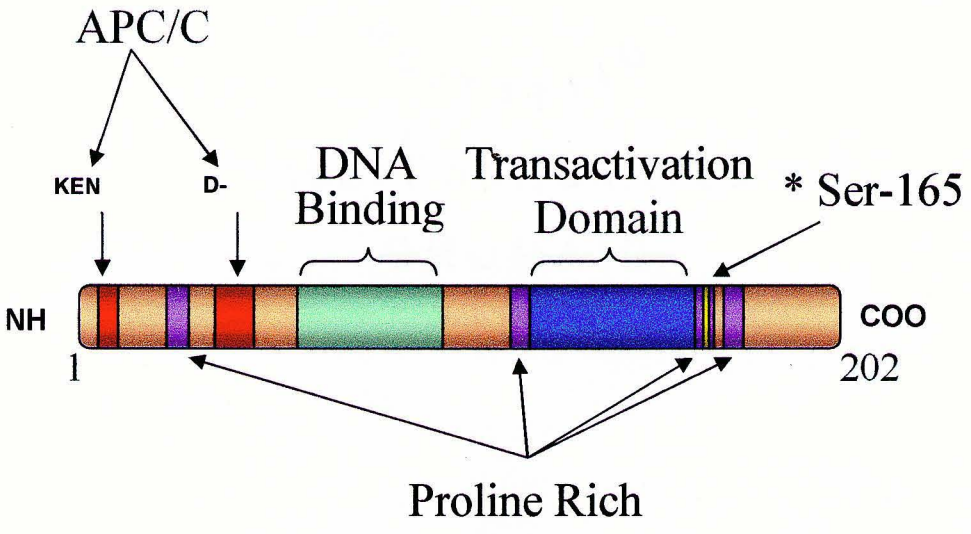
Figure 1



Adapted from Morales et al. (47)

Figure 2: Major functional domains of PTTG, including DNA binding domain, transactivation domain, and the critical proline rich (SH3) domains. Taken from Bradshaw et al (54).

Figure 2


APC/C
KEN
D-
DNA Binding
Transactivation Domain
* Ser-165
NH
COO
1
202
Proline Rich

However, the PXXP domains of PTTG have been reported to be involved in bFGF and VEGF transactivation (55), as well as being involved in cell transformation (27). It has been reported that serine 165 within the proline-rich domain is the sole phosphorylation site for cyclin dependent kinase-2 (cdc2), (47) and MAPK (56) is necessary during cell division to facilitate the function of PTTG (47,55). Deletion or mutation of the proline-rich motifs abolishes the transformation and tumorigenesis function of PTTG (27,55). Mutation of the analogous murine serine at 162 position reduces PTTG mediated transactivation by 75% (57). PTTG is mainly present in the cytoplasm with a partial nuclear localization. Although PTTG lacks a nuclear localization sequence, the protein is directed to the nucleus by interaction with PTTG binding factor (PBF) (47,56) or phosphorylation by mitogen-activated protein kinase (MAPK) (56). It has also been speculated that the small size of PTTG may help in the translocation into the nucleus. In addition, PTTG has been shown to interact with p53 (58), Ku-heterodimer (59), ribosomal protein S10, and a novel human homologue of bacterial heat shock protein DnaJ; HSJ2 (60).

1.3 PTTG FAMILY OF GENES

The PTTG genes cloned from three species (rat, mouse and human) are highly homologous. The human homologue of PTTG shares approximately ~80 % homology at the amino acid and nucleotide level, respectively, with the rat and mouse genes. The PTTG gene was mapped to human chromosome 5q35.1, a locus frequently associated with the reoccurrence of lung cancer and leukemia

(Figure 3) (61,62). In adult human tissues, PTTG is most abundantly expressed in testis, as well as in thymus, colon, small intestine, brain, placenta and pancreas (24). Two additional members of the PTTG family have been identified (30). After cloning of these members, PTTG was renamed as PTTG1 and the other two members as PTTG2 and PTTG3. All members of the family are transcribed from different genes located on different chromosomes. PTTG1 mapped to chromosome 5q35.1 (61), PTTG2 to chromosome 4p12, and PTTG3 to chromosome 8q22. PTTG1 and PTTG3 encode the proteins of 202 amino acids. Structurally they are same, but the function PTTG3 is not known (30,63). PTTG2 contains a deletion at nucleotide 541 which results in a frame shift and an early stop codon, resulting in a truncated protein of 191 amino acids (Figure 4). PTTG1, PTTG2 and PTTG3 are differentially expressed in normal and tumor tissues (30). PTTG1 has been most extensively studied. For simplicity, we will refer to PTTG1 as PTTG for the remainder of this dissertation.

Figure 3: Genomic organization of the human PTTG. PTTG gene is located on Chromosome 5 at 5q35.1. PTTG has six exon and five introns and spans at least 10 kb. The cDNA is composed of 656 nucleotides and encodes a protein of 202 amino acids (30).

Figure 3

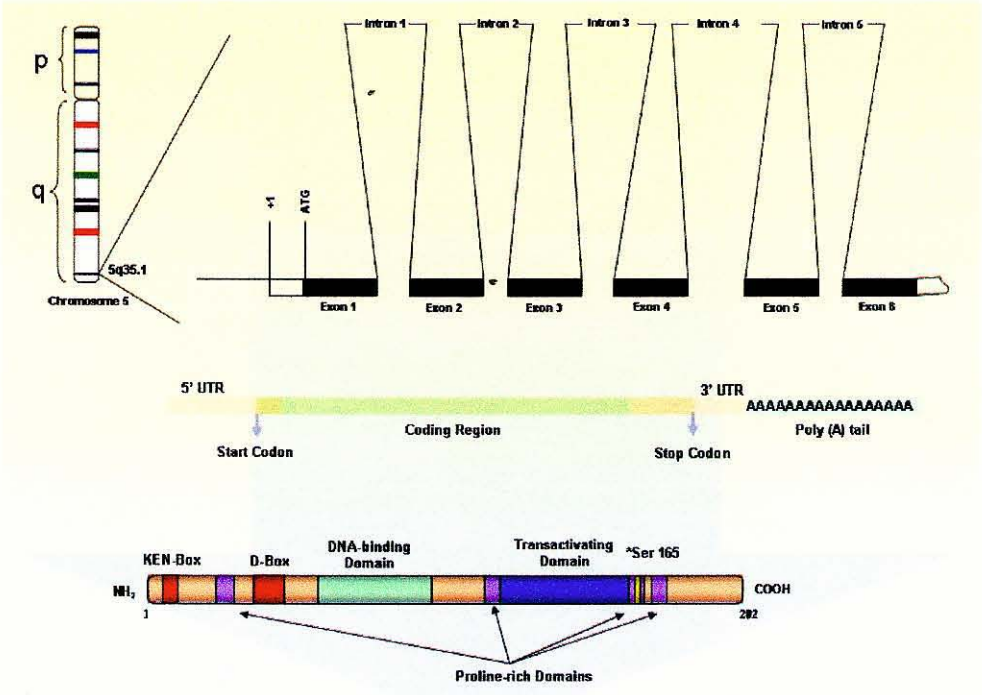
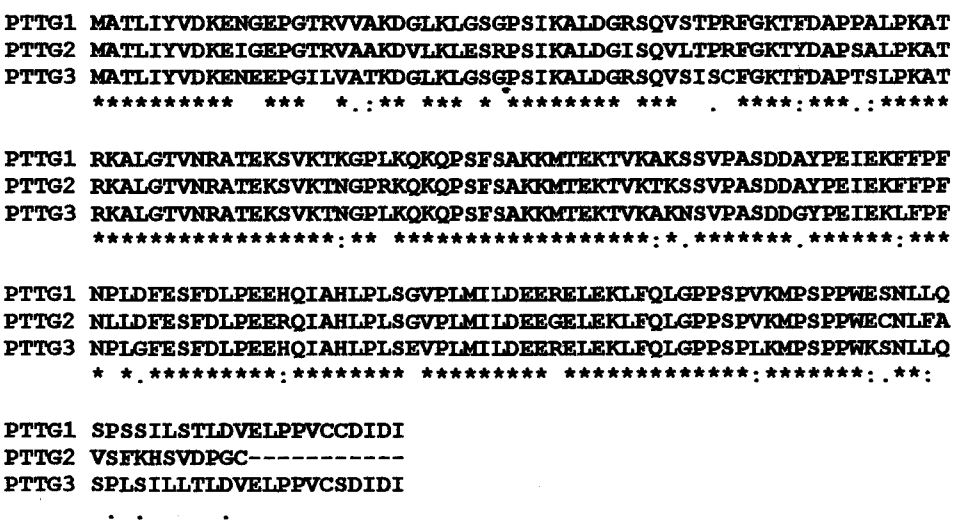

p
q
5q35.1
Chromosome 5
Intron 1
Intron 2
Intron 3
Intron 4
Intron 5
+1
ATG
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
5' UTR
3' UTR
AAAAAAAAAAAAAAAA
Poly (A) tail
Coding Region
Start Codon
Stop Codon
KEN-Box
D-Box
DNA-binding Domain
Transactivating Domain
^Ser 165
NH2
COOH
1
282
Proline-rich Domains

Figure 4: Amino acid sequence alignment of PTTG1, PTTG2 and PTTG3 gene products. Clustal View (European Bioinformatics Institute).

Figure 4


PTTG1 MATLIYVDKENGEPGTRVVAKDGLKLGSGPSIKALDGRSQVSTPRFGKTFDAPPALPKAT
PTTG2 MATLIYVDKEIGEPGTRVAAKDVLKLESRPSIKALDGISQVLTPRFGKTYDAPSALPKAT
PTTG3 MATLIYVDKENEEPGILVATKDGLKLGSGPSIKALDGRSQVSISCFGKTFDAPTSLPKAT
PTTG1 RKALGTVNRATEKSVKTKGPLKQKQPSFSAKKMTEKTVKAKSSVPASDDAYPEIEKFFPF
PTTG2 RKALGTVNRATEKSVKTNGPRKQKQPSFSAKKMTEKTVKTKSSVPASDDAYPEIEKFFPF
PTTG3 RKALGTVNRATEKSVKTNGPLKQKQPSFSAKKMTEKTVKAKNSVPASDDGYPEIEKLFPF
PTTG1 NPLDFESFDLPEEHQIAHLPLSGVPLMILDEERELEKLFQLGPPSPVKMPSPPWESNLLQ
PTTG2 NLLDFESFDLPEERQIAHLPLSGVPLMILDEEGELEKLFQLGPPSPVKMPSPPWECNLFA
PTTG3 NPLGFESFDLPEEHQIAHLPLSEVPLMILDEERELEKLFQLGPPSPLKMPSPPWKSNLLQ
PTTG1 SPSSILSTLDVELPPVCCDIDI
PTTG2 VSFKHSVDPGC----------
PTTG3 SPLSILLTLDVELPPVCSDIDI

1.4 BIOLOGICAL ROLES OF PTTG IN NORMAL CELLULAR FUNCTIONS.

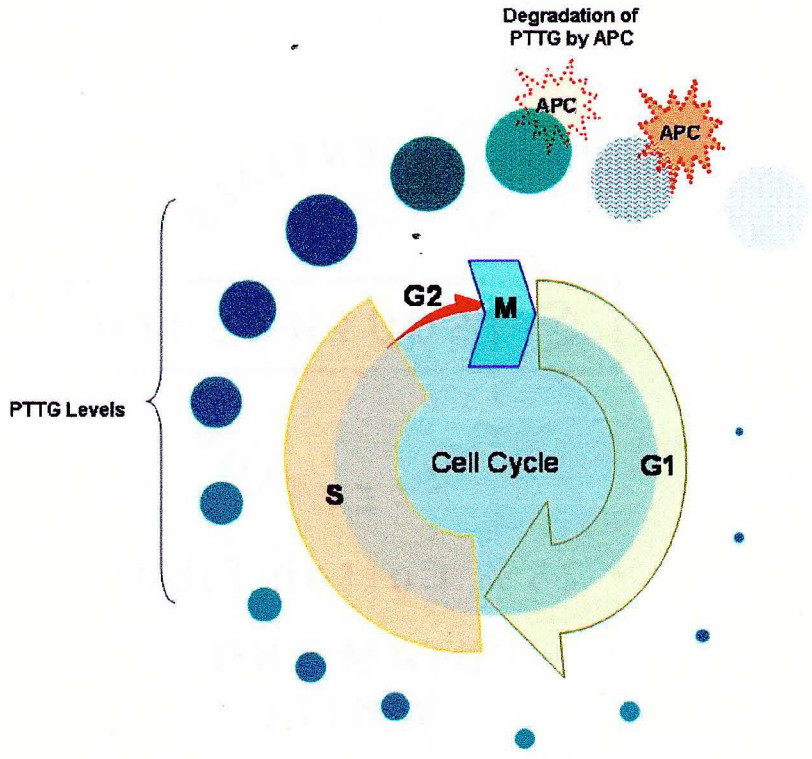
PTTG is a multi-functional protein that plays critical roles in cell cycle progression, chromosomal stability and cell division (64,65). PTTG functions as human securin to ensure that there is no premature separation of sister chromatids by inhibiting separase, which is liberated from securin to degrade cohesin bound to sister chromatids at anaphase (Figure 5) The level of PTTG expression is increased rapidly in proliferating cells and is regulated in a cell cycle-dependent manner (47,66). PTTG mRNA and protein levels are low at the G1/S inter-phase, and gradually increases during S phase with maximum expression at M phase. PTTG undergoes complete degradation at anaphase by ubiquitinization and by the recognization of the destruction box (D-box) consensus sequence of RxxLxxxxN (67). The daughter cells after cell division express low levels of PTTG protein (45). Overexpression of PTTG inhibits separation of sister chromatids, resulting in gene translocations, truncations and unequal number of chromosomes in daughter cells (aneuploidy) (41,66).

1.5 EXPRESSION AND BIOLOGICAL ROLE OF PTTG IN TUMORIGENESIS.

PTTG is abundantly expressed only in testis where it is believed to be necessary for normal spermatogenesis (24). Moderate expression is found in the

Figure 5: PTTG expression is cell cycle dependent. The mRNA and protein expression of PTTG are low at the G1 / S interphase, gradually increases during S phase and peaking at G2 / M phase. PTTG is degraded as the cells enter the anaphase and daughter cells express low levels of PTTG protein.

Figure 5


Degradation of
PTTG by APC
APC
APC
G2
M
PTTG Levels
Cell Cycle
G1
S

thymus, colon, and small intestine, brain, and pancreas (27), and very low or undetectable levels are found in the kidney, ovary, breast, liver and adrenal (49,60). In contrast, high levels of PTTG mRNA and protein are detected in most tumors including tumors of the pituitary, adrenal, ovary, endometrium, liver, colon, lung, stomach, breast, thyroid and testicles (24,25,33,35,68-70). In colon cancers, expression of PTTG strongly correlates with the degree of angiogenesis and metastasis (33). The pituitary tumor transforming gene (PTTG) has been shown as a signature gene among eight other genes associated with highly metastatic solid tumors, and its expression was associated with metastasis and poor prognosis (36). A relationship between the survival rate and level of expression of PTTG in esophageal cancer has also been reported (71). Overexpression of human PTTG in mouse NIH3T3 fibroblasts and human embryonic kidney (HEK293) cells resulted in increased cell proliferation, induction of cells to form foci in monolayer culture and promotion of tumor formation in nude mice (24,26,27,72). These results clearly suggest that PTTG is a human oncogene.

The most important candidates for downstream targets of PTTG function are *c-myc* oncogene and basic fibroblast growth factor (bFGF). Induction of PTTG results in increased cell proliferation through activation of *c-myc* (73), suggesting direct transactivation of c-myc by PTTG. In recent studies, Bernal et al. (58) reported interaction of the PTTG protein with the p53 protein thereby

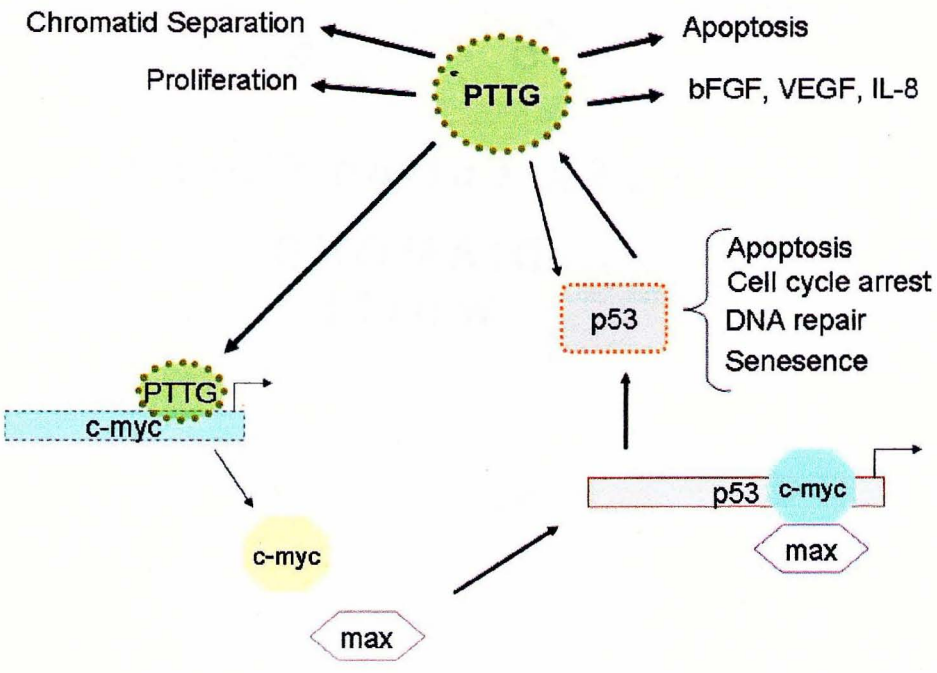
blocking the specific binding of p53 to DNA resulting in inhibition of its transcriptional activity (Figure 6).

As mentioned earlier, PTTG possesses transactivation activity (29,56). Transient transfection of a fusion construct containing the GAL4-DNA-binding domain linked to the C-terminal region of PTTG which contains the multiple glutamic acid and proline residues characteristic of transcription activation domains transactivates a luciferase reporter gene (29). PTTG mutants defective in transactivating activity are unable to transform mouse fibroblast NIH3T3 cells, suggesting that transactivating activity is important for the oncogenic functions of PTTG. Importantly, PTTG interacts with the *c-myc* oncogene product. Induction of PTTG results in increased cell proliferation through the activation of *c-myc* (73) by binding to the *c-myc* promoter near the transcriptional initiation site. Furthermore, inactivation of PTTG results in chromosomal loss with high levels of chromosomal instability (37,38). PTTG knockout mice show high levels of chromosomal instability and abnormal cell division (39). On the other hand, over-expression of PTTG promotes tumors in nude mice (26), and these tumors are aneuploid (40,41). Zhang et al (35) have shown a positive correlation between PTTG levels and the invasiveness of pituitary tumors.

Interesting effects of PTTG on cellular proliferation have been reported, Pei et al have shown that overexpression of PTTG in NIH3T3 cells slows down the rate of cell proliferation, which is in contrast to the function of an oncogene

Figure 6: PTTG up-regulates p53 transcription and its own expression.

Figure 6


Chromatid Separation
Proliferation
PTTG
Apoptosis
bFGF, VEGF, IL-8
Apoptosis
Cell cycle arrest
DNA repair
Senesence
p53
PTTG
c-myc
c-myc
max
p53
c-myc
max

(26). Data from our laboratory and others have since shown that PTTG overexpression results in cell cycle arrest and apoptosis (40,66,74), and these effects are mediated through both *p53* dependent and independent pathways.

PTTG overexpression also induces the expression of the Bax protein a known target of *p53* (74). However, in another study where inducible PTTG constructs were used, there was an increase in cell proliferation due to increases in *c-myc* and MEK1 expression (73). Altogether it seems that both cell proliferation and inhibition of cell proliferation may be a function of cellular PTTG levels. When PTTG levels are modestly increased, cell proliferation is stimulated, while the converse is seen when PTTG levels are high.

Data from our laboratory (Malik et al; unpublished data) and others (75) have shown that media from PTTG over-expressing cells promotes angiogenesis. Many growth factors including bFGF, EGF, TGF-α and IGF-1 in turn increase PTTG expression in human cultured cell lines (33,76,77). It is not clear how these growth factors increase PTTG expression, but increases in either PTTG or growth factors result in enhanced expression of both. There is a positive feedback of PTTG and growth factors, suggesting that PTTG and growth factors play important complimentary roles in tumorigenesis. Besides these growth and pro-angiogenic factors, other angiogenic genes such as an inhibitor of DNA binding 3 (ID_3), insulin-like growth factor-1 (IGF-1) (76), matrix metalloproteinase-2 (MMP-2) (Malik et al; unpublished data), interleukin-8 (IL-8) (72), interleukin 10

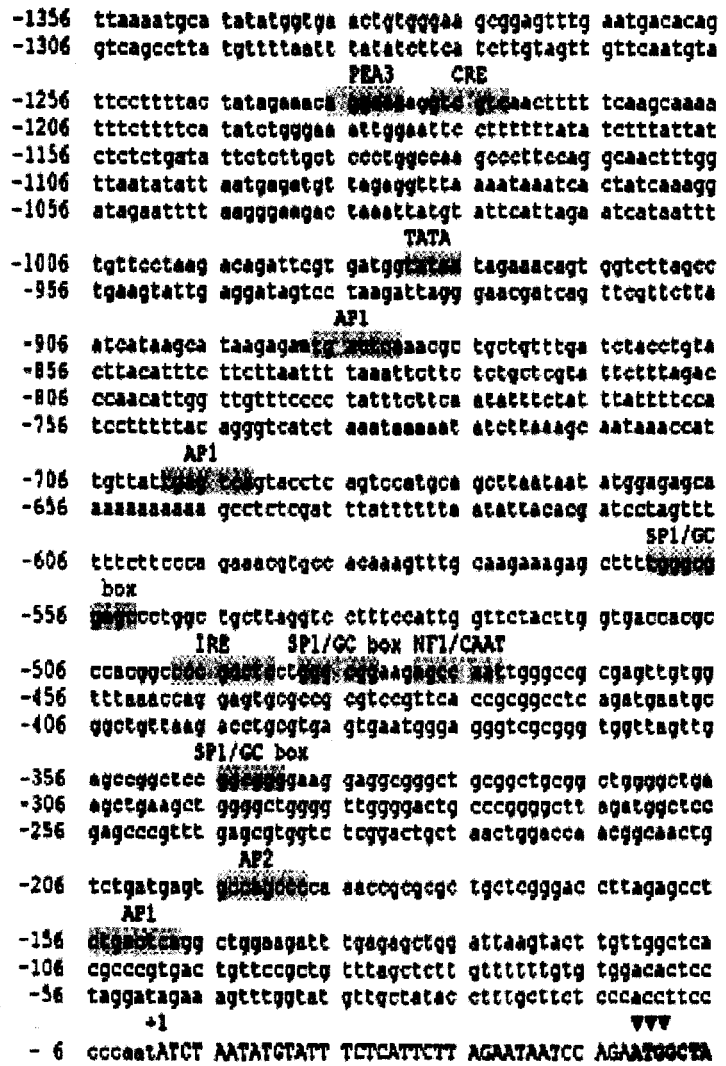
(IL-10) and mothers against decapentaplegic, dorsophila, homolog of 1 (SMAD-1) are expressed after overexpression of PTTG in thyroid cells (76,78,79). Decreases in expression of anti-angiogenic genes including thrombospondin-1 (TSP1), endostatin (42), and tissue inhibitor of metalloproteinases 2 (TIMP2) have been observed (Malik et al unpublished data)

1.6 PTTG GENE REGULATION

To aid in understanding the mechanisms that regulate the expression of the the in tumors, the PTTG gene was cloned from a human genomic library (61,62). The PTTG gene is composed of six exons and five introns and spans at least 10kb. Analysis of the 5' flanking region of the PTTG gene revealed that there is no TATA box sequence within 25-35 nucleotides upstream of the putative transcriptional start site. However, a CAAT box sequence at -474 bp from the transcriptional start site was identified (80). In addition, a number of transcriptional binding sites were identified including AP1, AP2, PEA3, SP1, IRE, CHR (cell cycle homology region), CDE (cell cycle-dependent element), CRE, ERE and NF-Y (34,46,80) (Figure 7). Recently our laboratory identified Sp1 and NF-Y DNA binding sites in the PTTG promoter sequence. Sp1 increases the PTTG promoter activity using luciferase reporter assays (81), whereas in the presence of DNA damaging agents, p53 suppresses the expression of PTTG in an NF-Y dependent manner (82).

Figure 7: PTTG gene 5' flanking sequence. Various transcriptional regulatory sequences are highlighted. Inverted triangles represent translational start codon ATG.

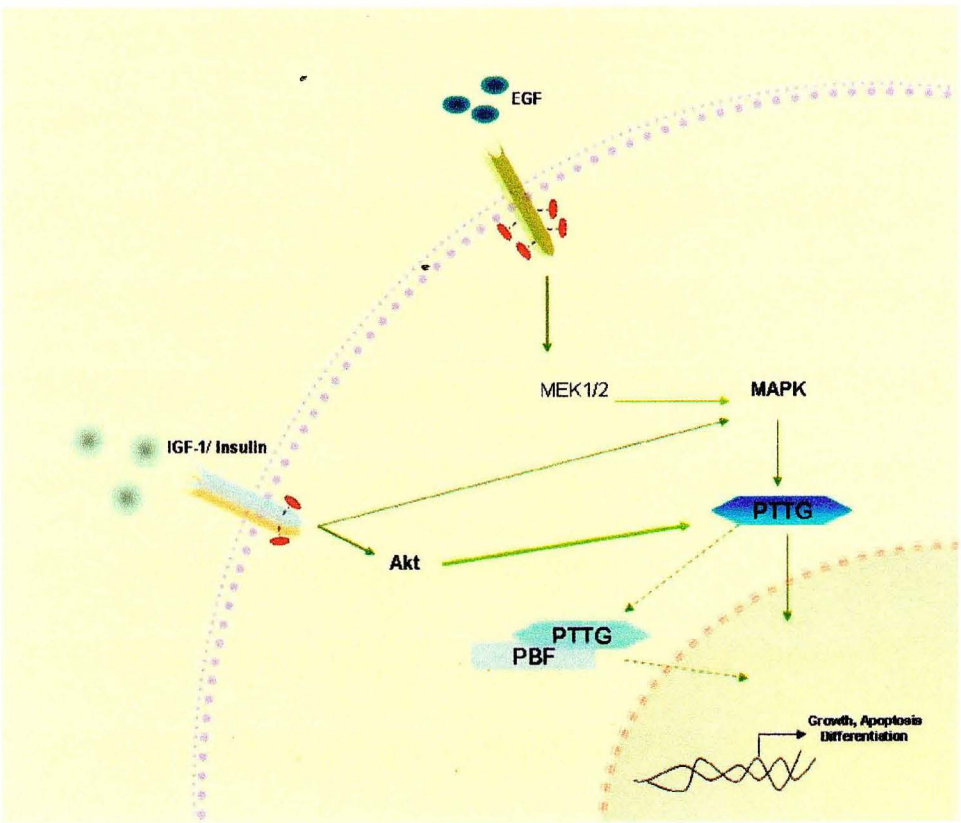
Figure 7


PEA3
CRE
TATA
AP1
AP1
IRE
SP1/GC box NF1/CAAT
SP1/GC box
AP2
AP1
+1
SP1/GC
box

It has been established that PTTG expression correlates with tumorigenesis; however, the signaling pathways that regulate expression and tumorigenesis have not been defined. Tfelt-Hansan et al (77) reported regulation of PTTG expression in human astrocytes cells by two promaglinant mitogens, epithelial growth factor (EGF) and TGF-α. Data from our laboratory and others have demonstrated that both insulin and IGF-1 regulate the expression of PTTG in breast tumor cell lines (76,79). The effects of insulin and IGF-1 were found to be mediated through activation of the Akt pathway, a downstream signaling pathway common for insulin and IGF-1 (Figure 8). Therefore, it is possible that insulin and IGF-1 regulated pathways contribute to the growth and proliferation of tumors by activating oncogenic molecular targets such as PTTG. Taken together thses data strongly suggests an important role of PTTG in cell proliferation and tumorigenesis.

Figure 8: IGF / Insulin and EGF regulate the expression of PTTG through insulin / IGF-1 and EGF signaling pathway.

Figure 8


EGF
MEK1/2
MAPK
IGF-1/ Insulin
PTTG
Akt
PTTG
PBF
Growth, Apoptosis
Differentiation

1.7 ANIMAL AND CELL CULTURE MODELS

To understand the importance of PTTG *in vivo,* Melmed et al (83) generated mice lacking PTTG (PTTG -/-) and showed that such animals exhibited aberrant cell cycle progression, premature centromere division chromosomal instability, as well as tissue specific phenotypes, such as testicular and splenic hypoplasia and thymic hyperplasia (83). In their extended studies these investigators showed that male mice lacking PTTG (PTTG-/-) in late adulthood showed impaired proliferation of β-cells and developed type-I diabetes at their later adult hood, suggesting the importance of PTTG in β-cells proliferation and insulin production (64). The function of PTTG in tumorigenesis was demonstrated by the PTTG(-/-)/Rb(+/-) animal that showed reduced pituitary cell proliferation and pituitary tumors (84). On the other hand, Abbud et al (85); generated transgenic animals driving the PTTG expression under the control of α-GSU-promoter and showed that transgenic male mice develop pituitary adenomas and prostate hyperplasia due to increase in luteinizing hormone (LH) secretion (85). We developed tissue specific transgene mice that expressed PTTG under the control of murine inhibitory substance 2 receptor (MISIIR) gene promoter to target ovarian surface epithelium. This transgenic animal developed cystic glandular hyperplasia of endometrium, and generalized hypertrophy of the myometrium. There were no visible ovarian tumors in these transgenic female mice (Shahenda et al, unpublished data). These studies clearly demonstrate that PTTG play a critical role in cell proliferation and tumorigenesis.

1.8 EFFECT OF DOWNREGULATION OF PTTG

As described earlier, overexpression of PTTG in mouse fibroblast cells increases cell proliferation, induces cellular transformation, and promotes tumor formation in nude mice (24,27). These results suggest that there may exist a relationship between the level of PTTG and tumorigenesis. Based on these results we and others hypothesized that down regulation of PTTG mRNA levels should inhibits tumor cell proliferation and reverse the cancer phenotype. Use of small interfering RNA (siRNA) technology (86), and adenovirus mediated siRNA (87) or antisense oligodeoxynucleotides (anti-ONDs) (86,88) are well established techniques to reduce the expression of a specific gene. To demonstrate the possibility that down regulation of PTTG may reverse the cancer phenotype and PTTG may serve as a molecular target for cancer treatment, Kakar and Malik (54) showed that transfection of lung tumor cells with PTTG specific siRNA resulted in a significant reduction in PTTG mRNA, and protein. Furthermore, these transfected cells also demonstrated reduction of both colony formation on soft agar and tumor formation in nude mice. siRNA effects are short–lived. Therefore, to study the function of PTTG and MMP-2 expression and secretion, we used a human colorectal cancer cell line (HCT116), in which both the copies of PTTG gene are inactivated genetically by homologous recombination (PTTG -/- HCT116) cell (89). More details of this study are explained in chapter IV.

In summary, cancer is the leading cause of death in the United States. It has been established that tumorigenesis is a multistep process, involving activation of proto-oncogenes, growth factors and their receptors and inactivation of tumor suppressor genes. Abnormal expression of a gene in tumor cells can be associated with several of characteristics that distinguish these cells from normal cells. These characteristics includes altered cell differentiation, DNA repair, cell-cell communication, cell matrix interaction, tumor invasion, migration, metastasis, angiogenesis and apoptosis. Our lab cloned a potent oncogene pituitary tumor transforming gene (PTTG) from human testis. The PTTG cDNA is composed of 656 nucleotide and encodes a protein of 202 amino acid with no homology to other known proteins. Over-expression of PTTG in mouse fibroblast NIH3T3 cells resulted in increased cell proliferation and induced cellular transformation. Furthermore, injection of PTTG stably transfected NIH3T3 cells into nude mice resulted in tumor formation, suggesting a role for PTTG in tumorigenesis. PTTG may induce its tumorigeneic function through increased bFGF, VEGF, and IL-8 expression and secretion. With the exception of testis and fetal liver, the levels of PTTG are either very low or undetectable in normal tissue. The PTTG is expressed at high levels in almost all tumors including ovary, kidney, pituitary, breast, uterus, liver and testis. There are two more members of PTTG family, PTTG2 and PTTG3. All three members of PTTG family are differentially expressed in normal and tumor tissue. PTTG1 has been most extensively studied. Down regulation of PTTG by siRNA results in loss of cell's tumorigeneic function.

1.9 HYPOTHESIS AND SPECIFIC AIMS

There is compelling evidence in the literature to suggest that PTTG is a proto-oncogene overexpressed in most tumors and tumor-derived cell lines. Overexpression of PTTG inhibits chromatid separation leading to genetic instability and ultimately tumorigenesis (45). Studies have shown that transient expression of PTTG in mouse fibroblast NIH3T3 cells increases the expression and secretion of VEGF and bFGF, (27,90) potent mitogens in angiogenesis (91,92). PTTG also possesses transactivation activity, which is related to its transformation ability, suggesting a role in regulating the transcription of target genes that can regulate its function (47,57). Taken together these results suggest that PTTG is a multifunctional protein and mediates its functions through multiple signaling pathways. All functional studies of the oncogenic properties of PTTG have been performed using mouse fibroblast cells (NIH3T3) (29,35), an ideal cell line for studying the behavior of oncogenes and tumor suppressor genes because of its indefinite growth in culture, retention of contact inhibition, and ease of transformation (93). Due to biological differences between human and rodent cells, however, care must be taken in extrapolating results obtained using rodent cells to human cells. There are many examples in which overexpression of an oncogene can induce transformation of primary rodent cells, but fails to induce transformation in human counterpart. Therefore, it is important to test the oncogenic abilities of PTTG in human cell line to establish its role in human tumorigenesis.

1.10 SPECIFIC AIMS

SPECIFIC AIM 1:

To determine the role of PTTG in human tumorigenesis using human immortalized cell line.

RATIONALE

Overexpression of PTTG in mouse primary cells induces cellular transformation and promotes tumor formation without the co-operation of other genes. Data from our lab and others (24,27) have shown that over-expressing PTTG in mouse NIH3T3 cells increased cell proliferation and cellular transformation *in vitro,* and promoted tumor formation *in vivo.* PTTG over-expression is also known to induce expression of bFGF and VEGF (90). Increased expression of bFGF and VEGF has been reported in several human tumors and is considered to be stimulating for angiogenesis (94). However, whether PTTG transforms human cells has not been demonstrated. Major biological differences between human cells and rodent cells make it difficult to extrapolate the results from rodents to humans (95). There are a number of instances where over-expression of oncogene in rodent cells fails to transform human cells (96,97).

SPECIFIC AIM: 2

To determine the role of PTTG in up-regulation and secretion of metalloproteinases in tumor angiogenesis and metastasis.

RATIONALE

PTTG has been reported to increase levels of angiogenic and metastatic factors including bFGF and VEGF (27,90). In limited studies of colorectal and pituitary adenocarcinomas, a relationship between PTTG expression and metastasis has been reported (33,68). However, the mechanism by which PTTG regulates metastasis remains unclear. For a tumor to metastasize, many degrading proteinases must be activated to facilitate movement of tumor cells from the primary site to invade distance sites and metastasize. Matrix metalloproteinases (MMPs) are known to play a key role in such processes by degrading the extracellular matrix and controlling activities of growth factors, chemokines and cytokines that favor tumor metastasis (98). We hypothesized that regulation of tumor angiogenesis and metastasis is controled by PTTG by regulating the expression and secretion of matrix metalloproteinases.

SPECIFIC AIM: 3.

To determine if decreased PTTG expression in cancer cells reverses the cancer phenotype and reduces angiogenesis and metastasis through down regulation of metalloproteinases.

RATIONALE

PTTG is a potent oncogene that promotes tumor formation in nude mice. PTTG has also been shown to regulate the expression of many growth factors and cytokines including bFGF, VEGF, and IL-8 (72). These factors are key regulators of tumor angiogenesis and metastasis. PTTG increases the expression and secretion of MMP-2. MMP-2 is known to be implicated in the aggressive and metastatic tumor phenotypes with poor survival outcomes. If PTTG is essential in regulating angiogenesis and metastasis, we perdict deletion of PTTG will reverse the cancer phenotype remain unknown. In our study, we will deplete the expression of PTTG by using specific siRNA or will use HCT116 (PTTG -/-) null cell line where the PTTG gene is inactivated by homologous recombination, and its counterpart wild type HCT116 (PTTG +/+) as a control to study the effect of PTTG on MMP-2 expression and secretion.

CHAPTER II

SECTION I

GROWTH FACTORS AND CYTOKINES

INTRODUCTION

Growth factors and cytokines are small secreted molecules. That activates signaling cascades in target cells by binding to specific cell specific surface receptors. Two types of receptor are important: receptor tyrosine kinases and G-protein-coupled receptors. The action of growth factors is mainly mediated through receptor tyrosine kinases (RTK) while cytokines act through G-protein-coupled receptors (GPCR). These mediators influence cell proliferation in a positive or negative manner by inducing a series of intracellular signaling cascades that regulate diverse biological responses. Cytoplasmic molecules that mediate these responses are called second messengers. Among the intracellular signaling proteins are kinases, phosphates, GTP-binding proteins. The ultimate transmission of these signals to the nucleus effects the expression of many genes involved in mitogenic and differentiation response. Pathogenic expression of critical genes in response to constitutive expression of growth factors and cytokines can contribute to altered cell growth associated with malignancy (99). Almost all tumors can activate the angiogenic switch by changing the balance of

angiogenic inducers such as growth factors and cytokines required to sustain neo-vascularization. These growth factors include bFGF, VEGF, IL-8, IL-10 and countervailing inhibitors such as thrombospondin-1 (98,100,101).

Cancer development is a complex and multi-step process that involves activation of oncogenes, growth factors, and their receptors, or deregulation of tumor suppressor genes, leading to abnormal cellular processes. The growth and proliferation of cells requires heterotypic mitogenic growth signaling, i.e. growth factors are synthesized by one cell type in order to stimulate the proliferation of another cell type (14). Instead, cancer cells acquire the ability to synthesize growth factors to which they are responsive to, thus creating a positive feedback signaling loop. This autocrine signaling obviates the dependence of cancer cell on growth factors from other cells and provides growth signal autonomy. In this manner cancer cells modulate external and internal signals that regulate their uncontrolled proliferation and evade apoptosis (14,100). Furthermore, all mammalian cells are programmed for a limited number of cell divisions cycles, which must be disrupted in cancer cells in order to expand to a macroscopic size beyond 1 – 2 mm. Cancer cells, may continue to proliferate until their oxygen and nutrient supply is limited by access to the vasculature. Thus, angiogenesis is a crucial factor in the progression of tumor growth. During angiogenesis, tumors activate an angiogenic switch by shifting the balance of gene transcription towards angiogenesis inducers, such as growth factors and cytokines, and countervailing angiogenesis inhibitors (98). Many tumors increase the expression of vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and acidic and basic

fibroblast growth factors (FGF 1 and 2), while expression of endogenous inhibitors thrombospodin-1 or β-interferon is decreased (78). Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (102,103) are strong mitogens for endothelial cells and have been reported to play an essential roles in angiogenesis, and stimulation of cell migration and metastasis (104-108). Interleukin-8 (IL-8) belongs to a super-family of CXC chemokines, and is a multifunctional cytokine that exhibits potent angiogenic and pro-inflammatory activities both *in vitro* and *in vivo* (109).

Data from our laboratory (24,72) and others (27) have shown that over-expression of PTTG cDNA in mouse NIH3T3 fibroblast cells increases the expression of bFGF and induces cellular transformation and increased cell proliferation. Further, stably transfected mouse NIH3T3 fibroblast cells constitutively expressing PTTG injected into nude mice produce tumors. However, a role for PTTG in transformation of human cells has not been demonstrated.

There are discrepancies between mouse and human carcinogenesis (95,110). Despite similarities between mouse and humans, murine models of human carcinogenesis are questionable due to differences in environmental conditions, metabolic stability, and the robustness of the cellular regulatory network (111). Major biological differences between human and rodent cells make it difficult to directly extrapolate the results from mice to human (95). There are a number of instances in

which over-expression of oncogenes transformed rodent cells but failed to transform the same cells from humans (96,97).

To determine the role of PTTG in human tumorigenesis, we used the human embryonic kidney cell line (HEK293) in our studies as a model. The cell line was transformed by human adenovirus type 5 to prevent senescence (112). This cell has a moderate tumorigenic potential and have been extensively used to study the oncogenic potential of a number of genes (113-115). Mice xenografted with these cells do not develop tumors, even three months after injection (115).

SECTION II

Ectopic expression of PTTG/Securin promotes tumorigenesis in human embryonic kidney cells.

2.2 SUMMARY

Pituitary tumor transforming gene (PTTG) encodes a protein that is primarily involved in the regulation of sister chromatid separation during cell division. The oncogenic potential of PTTG has been well characterized in the mouse, particularly mouse fibroblast (NIH3T3) cells, where it induces cell proliferation, promotes tumor formation and angiogenesis. To determine if PTTG functions as an oncogene in humans, we have characterized its effects on human embryonic kidney (HEK293) cells. We found that introduction of human PTTG into HEK293 cells through transfection with PTTG cDNA resulted in increased cell proliferation, anchorage-independent growth in soft agar, and formation of tumors after subcutaneous injection of nu/nu mice. Pathologic analysis revealed that these tumors were poorly differentiated. Analyses of HEK293 cells transiently transfected with PTTG cDNA and tumors developed after injection of HEK293 cells stably transfected with PTTG cDNA indicated significantly higher levels of secretion and expression of bFGF, VEGF and IL-8 compared to HEK293 cells transfected with pcDNA3.1 vector or uninvolved tissues collected from the mice. Mutation of the proline-rich motifs at the C-terminal region of PTTG abolished its oncogenic properties. Mice injected with this

mutated PTTG either did not form tumors or formed very small tumors. These results suggest that PTTG is a human oncogene that possesses the ability to transform human without cooperation of other oncogenes in part through regulation of expression or secretion of bFGF, VEGF and IL-8.

2.2.1 INTRODUCTION

Pituitary tumor transforming gene (PTTG), a recently characterized oncogene, was initially identified on analysis of a rat pituitary tumor (26); subsequently, a human homologue of PTTG was cloned by us and others (24,27,29). Three members (PTTG, PTTG2 and PTTG3) of the PTTG family, which exhibit differential expression in normal and tumor cells have been reported (30), although only PTTG has been studied in detail. PTTG is located on chromosome 5q33 (61), a locus associated with recurrent lung cancer and myelogenous leukemias (62). Moreover, it has been shown to be expressed highly in various tumors, and cell lines derived from such tumors, including tumors of the pituitary, thyroid, colon, ovary, testicles and breast (25,33,68-70). In normal tissues, its expression is low or undetectable except in testis (24,26). Recent studies have indicated that elevated expression of PTTG in some tumors may serve as a prognostic marker for tumor invasiveness and metastasis (36). A clue to its function was gained from its structural similarity with the yeast securin, which led to its identification as a human securin (45), and suggested that it may play a role in regulation of sister chromatid separation. It appears, to have multiple effects in cells including enhanced expression associated with an increase in the expression of the c-

myc oncogene (73), an increase in the expression of p53 (40,74), an increase in the secretion and expression of basic growth factor (bFGF) (75), and an increase in the secretion and expression of vascular endothelial growth factor (VEGF) (75,90).

To date, evidence for the oncogenic function of PTTG has been obtained by overexpression of PTTG in mouse fibroblast cells (NIH3T3) followed by assessment of its ability to induce cellular transformation in vitro (colony formation in soft agar) and tumor formation in nude mice (24,27). Due to differences between human and rodent cells, care must be taken in extrapolating results obtained using rodent cells to human cells. There are now several examples in which overexpression of an oncogene induces transformation of primary rodent cells(96), the same strategy failed to induce transformation of the same cell type derived from humans. Usually this failure is attributable to the requirement for co-expression of another gene or oncogenic cooperation of other genes (116-120).

Similarly, much of the evidence concerning the mechanisms by which PTTG may affect the phenotype of the cell has been obtained using transfected NIH3T3 cells. It is known that the secretion of growth factors and cytokines by tumor cells, and the cells that infiltrate and surround the tumor mass play an essential role in the regulation of tumor growth and metastasis (121). Both bFGF and VEGF have been implicated in tumorigenesis and the expression and secretion of these molecules has been demonstrated on transfection of NIH3T3 cells with PTTG cDNA (75,90), but this has not been confirmed with transfection of human cells. The effect of PTTG

expression on another cytokine that is known to play a key role in tumorigenesis, interleukin-8, (IL-8), have not yet been analyzed.

The purposes of this study were three-fold. First, to determine whether PTTG can induce cellular transformation of normal human cells; second, to determine if PTTG is sufficient to induce transformation; and, third, to characterize changes in secretion and expression of key metastatic, angiogenic and chemokine factors (bFGF, VEGF and IL-8). For these studies, we selected the human embryonic kidney (HEK293) cell line as our model. The expression of the SV40 large T antigen by these cells prevents their senescence, but they do not exhibit tumorigenic characteristics nor do they develop into tumors when xenografted into nude mice (120,122).

2.2.3 MATERIAL AND METHODS

Generation of cell lines constitutively expressing PTTG:

The human embryonic kidney cell line (HEK293), which had been transfected previously with SV40 large-T antigen, was purchased from ATCC (American Type Culture Collection; Rockville, MD) and cultured according to the instructions provided. The cells were transfected with pcDNA3.1 vector, pcDNA3.1-PTTG or pcDNA3.1-mPTTG to generate stable clones that constitutively express human wild-type PTTG or mutated PTTG (mPTTG) protein as described previously (24). The mPTTG, which carries a double amino acid change within the SH3 binding domain of

PTTG (P^{163} to A^{163}, P^{170} to A^{170} and P^{172} to A^{172}, and P^{173} L^{173}), was generated by site-directed mutagenesis using the Quick-change mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Mutation of these amino acids has been reported to abrogate the tumorigenic function of PTTG and to block the secretion and expression of bFGF in mouse NIH3T3 cells (27). The primers used for this site-directed mutagenesis were 5'-GATGCTCTCCGCACTCTGGGAATCCAATCTG-3' and 5'-TTCACAAGTTGAGGGGCGCCCAGCTGAAACAG-3'. The transfected cells were then selected in neomycin G418 (500 μg/ml) and the clones that expressed high levels of PTTG protein or mPTTG protein were selected. One clone from pcDNA3.1 transfected cells (HEKpcDNA3.1) two clones from pcDNA3.1-PTTG transfected cells (HEKPTTG-1 and HEKPTTG-3) and two clones from pcDNA3.1-mPTTG transfected cells (HEKmPTTG-2 and HEKmPTTG-4) were propagated into cell lines.

Cell proliferation assay:

Cell proliferation was assayed using the CellTiter 96 non-radioactive cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's instructions and as described previously (24). Briefly, cells growing in log phase were trypsinized and seeded in 96-well plates (5,000 cells/well in a final volume of 100 μl) in replicates of 4 and incubated at 37° C in 5% CO_2 and 95% air. After incubation for

24 h, 48 h, 72 h or 96 h, 20 μl of dye solution from the kit was added to each well and incubated at 37° C for an additional 2 h. The quantity of formazan product was measured by its absorbance at 490 nm using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). Each experiment was repeated at least three times.

Soft agar colony formation (anchorage-independent cell growth) assay:

Anchorage-independent cell growth was determined by analyzing the formation of colonies in soft agar. Cells (10^4) from each cell line were suspended in 0.3% agar in DMEM containing 10% fetal bovine serum and plated on solidified agar (0.7%) in 35 mm dishes. After 14 days of culture, colonies formed were counted and photographed as described previously (24).

In vivo tumor growth assay:

Cells growing in log phase were harvested by trypsinization and washed twice with PBS. The cells were resuspended in PBS to a final concentration of $5x10^6$/ml. The cells ($1x10^6$ cells in 200 μl PBS/site) were injected subcutaneously (s.c.) into both flanks of 5- to 6-week old female nu/nu mice (4 mice/group) (Charles River Laboratory, Wilmington, MA). All procedures were carried out following the protocol approved by The University of Louisville Institutional Animal Care and Use

Committee. Four weeks after injection, the mice were sacrificed, and the tumors and other tissues harvested. The skin and connective tissues were dissected from the tumors, and the tumor volume was calculated from measurements of length x width x height. The tissues were divided into two parts, one part being fixed in 10% buffered formalin and the other stored in liquid nitrogen. For histopathologic analysis, 5μm sections were cut from paraffin-embedded tissues, and mounted on slides. Sections were stained with H&E (123), and processed for histopathologic evaluations.

Western blot analysis:

Cells growing in log phase were lysed in chilled lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF] supplemented with Complete Mini Protease Inhibitor tablets (Roche Molecular Biochemicals, Indianapolis, IN). Equal amounts of protein extract (40μg) were resolved on 12% SDS-PAGE gel, and transferred onto a nitrocellulose membrane (Amersham, Piscataway, NJ). Blots were probed with PTTG antiserum at a dilution of 1:1,500 as described previously (124). Immunoreactive proteins were visualized using the Enhanced Chemiluminescent Detection System (Amersham) according to the instructions provided.

ELISA analysis of bFGF, VEGF and IL-8

The levels of bFGF, VEGF and IL-8 in tissue culture supernatants and tissue homogenates were measured using commercially available ELISA kits from BD

Biosciences (Minneapolis, MN). To measure bFGF, VEGF and IL-8 in the culture supernatants, HEK293 cells were transiently transfected with pcDNA3.1 or pcDNA3.1-PTTG cDNA using Fugene6 as the transfectant reagent as described previously (81). After 24 h of transfection, the medium was replaced with serum free DMEM medium. Twenty-four hr later, the medium was collected and concentrated 5-fold (1.0 ml to 200 μl) using a speedVac system (Savant, Holbrook, NY). To measure bFGF, VEGF and IL-8 in tumor and other tissues, tissues were homogenized in 50 mM Tris (pH 7.4), 0.25% Triton X-100, 5 mM EDTA and 0.1% NP40 supplemented with Complete Mini Protease Inhibitor tablets (Roche Molecular Biochemicals, Indianapolis, IN) using a polytron homogenizer. Homogenates were centrifuged to remove particulate matter and then diluted with the diluent provided in the ELISA kit. The concentration of bFGF, VEGF and IL-8 in a sample was determined by interpolation from a standard curve. All measurements were normalized to protein concentration and performed in triplicate.

Semi-quantitative reverse transcriptase/polymerase chain reaction (RT/PCR):

Total RNA from tumors and other tissues was purified using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The RNA pellets were resuspended in RNase-free water and the contaminating DNA was removed from the preparations with DNaseI. The yield of total RNA was measured using a spectrophotometer and the quality was assessed by electrophoresis through a 1% agarose gel. First strand cDNA was synthesized using the iScript™ cDNA

synthesis kit (BioRad, Hercules, CA). PCR primers (Table 1) were designed based on the human PTTG, bFGF, VEGF and IL-8 cDNA sequences. The PCR conditions for each gene are listed in Table 1.GAPDH amplification was used as an internal control. Ten μl from a total of 50μl PCR reaction mix was applied to a 2% agarose gel and after electrophoresis; the gel was stained with ethidium bromide to visualize PCR products. The densitometric values for the PCR-amplified products were quantified using BioRad software and normalized against the GAPDH values.

Table 1: Primer sequences and PCR conditions for the amplification of PTTG, bFGF, VEGF, IL-8 and GAPDH.

Table 1

	Sense Primer Sequence	Antisense Primer Sequence	PCR Conditions
PTTG	ATGGCTACTCTGAT CTAT	AAAATCTATGTCAC AGCAAAC	95°C 5 min, 95°C 30 s, 54°C 30 s, 72°C 30s. 28 cycles.
bFGF	TTCTTCCTGCGCAT CCACCC	CTCTTAGCAGACAT TGGAAG	95°C 5 min, 95°C 30 s, 56°C 30 s, 72°C 30s. 26 cycles.
VEGF	GAATCATCACGAA GTGGTGA	AACGCGAGTCTGTG TTTTTG	95°C 5 min, 95°C 30 s, 56°C 30 s, 72°C 30s. 28 cycles.
IL-8	ACCACCGGAAGGA ACCATCT	GAATTCTCAGCCCT CTTCAA	95°C 5 min, 95°C 30 s, 58°C 30 s, 72°C 30s. 28 cycles.
GAPDH	TGATGACATCAAG AAGGTGGT	TCCTTGGAGGCCAT GTGGGCC	95°C 5 min, 95°C 30 s, 54°C 30 s, 72°C 30s. 26 cycles.
Mutated-PTTG	GATGCTCTCCGCAC TCTGGGAATCCAAT CTG	TTCACAAGTTGAGG GGCGCCCAGCTGA AACAG	95°C 5 min, 95°C 30 s, 54°C 30 s, 72°C 30s. 35 cycles.

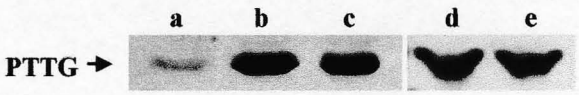
2.2.4 RESULTS

Generation of HEK293 cells stably expressing PTTG and mPTTG:

HEK293 cells were transfected with pcDNA3.1-PTTG, pcDNA3.1-mPTTG or pcDNA3.1 vector. After G418 selection, 10 clones from each of pcDNA3.1, pcDNA3.1-PTTG or pcDNA3.1-mPTTG transfected cells were picked, cultured, and expanded. The PTTG protein expression of these transfectants was detected by western blot analysis using PTTG antiserum. Two representative clones from PTTG transfected (named HEKPTTG-1 and HEKPTTG-3) and mPTTG transfected (named HEKmPTTG-2 and HEKmPTTG-4), and one clone from pcDNA3.1vector (named HEKpcDNA3.1) was selected for further studies. Selection of clones was based on the level of expression of PTTG protein. (Figure 9) shows the protein expression of these clones. Transfection of cells with the pcDNA3.1 vector resulted in expression of a very low level of PTTG protein. The clones of the pcDNA3.1-PTTG- and pcDNA3.1-mPTTG-transfected cells that exhibited approximately equivalent levels of expression of PTTG and mPTTG proteins were processed to establish stable cell lines.

Figure 9: Western blot analysis of HEK293 cells transfected with pcDNA3.1, pcDNA3.1-PTTG or pcDNA3.1-mPTTG. a: HEK pcDNA3.1, b: HEK PTTG clone 1, c: HEK PTTG clone 3, d: HEK m-PTTG clone 2, and e: HEK m-PTTG clone 4.

Figure 9

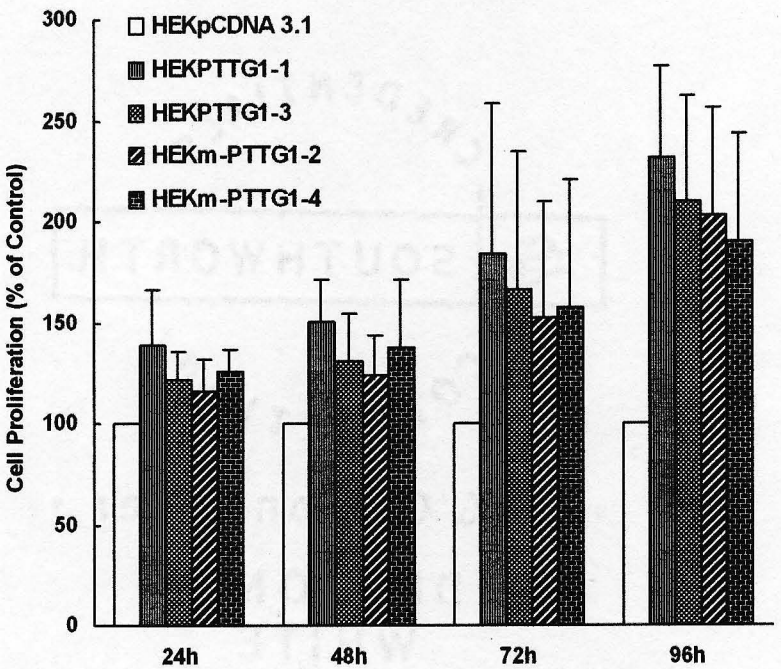


Stable transfection of PTTG induces cell proliferation and transformation of HEK293 cells overexpressing PTTG.

Previously we have shown that over-expression of PTTG in mouse fibroblast NIH3T3 cells results in an increase in cell proliferation (24). To determine if over expression of PTTG in HEK293 cells produces similar effects, we estimated the proliferation at 24, 48, 72 and 96 hours after plating of stably transfected HEK293 cells expressing high levels of PTTG or mPTTG protein. Both clones of PTTG-transfected cells (HEKPTTG-1 and HEKPTTG-3) exhibited significantly greater proliferation than the cells transfected with vector only at all time points tested, and the time course of proliferation was very similar in both clones, increasing by 30-40% after 24 hours, 40-50% after 48 hours, 60-80% after 72 hours and 110-130% after 96 hours (Figure 10). Surprisingly, the proliferation of the cells expressing the mutated PTTG was equivalent to that of the cells expressing the wild-type PTTG and was significantly higher than that of the cells transfected with vector only. These experiments indicate that over expression of PTTG induces a significant proliferative effect in HEK293 cells; however, at least under the conditions used, mutation of the proline-rich motifs of PTTG did not affect this response.

Figure 10: Cell proliferation of HEK293 cells stably transfected with pcDNA3.1, PTTG1 or mPTTG1. 5 x 103 cells were plated / well. The results are expressed as percent of control (HEK293 cells stably transfected with pcDNA3.1 control vector). Error bars ± SEM (n = 4) of three independent experiments. $p<0.05$

Figure 10

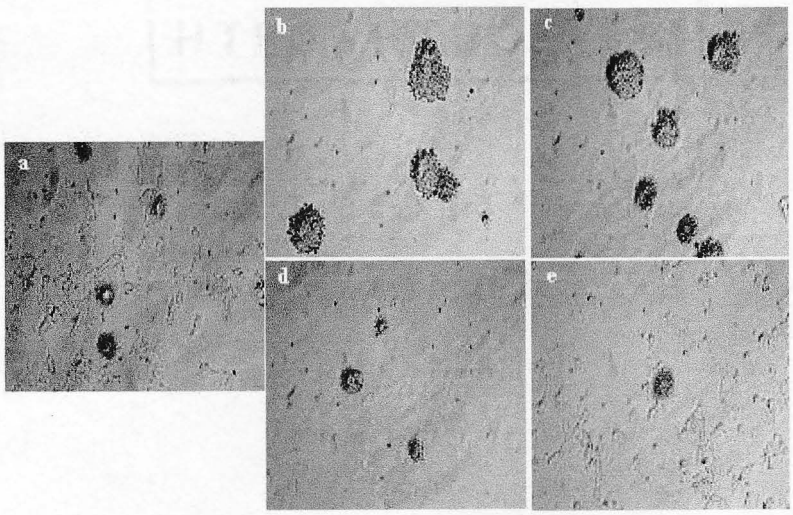

HEKpCDNA 3.1
HEKPTTG1-1
HEKPTTG1-3
HEKm-PTTG1-2
HEKm-PTTG1-4
Cell Proliferation (% of Control)
300
250
200
150
100
50
0
24h
48h
72h
96h

Overexpression of PTTG induces cellular transformation.

Next, we assayed the effects of transfection with PTTG on the ability of the HEK293 cells to form colonies in soft agar (anchorage-independent growth). As shown in (Figure 11), over-expression of PTTG in HEK293 cells resulted in a higher incidence of colony formation than that observed on transfection with the vector only. The cells transfected with vector only formed few colonies and these were of small size during 14 days of culture, whereas both the cell lines expressing wild type PTTG formed a significantly higher number of colonies, which were of a large size. The incidence of colony formation was 2% for HEKpcDNA3.1 cell line but was 19% for the HEKPTTG-1 cell line and 30% for the HEKPTTG-3 cell line. In this case, mutation of the proline-rich motifs of PTTG resulted in a significant reduction in the number of colonies formed with the incidence of colony formation for the HEKmPTTG-2 and HEKmPTTG-4 cell lines being similar to the vector-only transfected cells (HEKpcDNA3.1). These results suggest that over expression of PTTG in HEK293 cells induces cellular transformation, and mutation of proline-rich motifs does not effect the cell proliferation, but abrogates the cellular transformation ability of PTTG.

Figure 11: Colony formation of HEK293 cells stably transfected with pcDNA3.1, pcDNA3.1-PTTG1 or pcDNA3.1-m-PTTG1. a: HEK pcDNA3.1, b: HEK PTTG1, c: HEK-PTTG1-3, d: HEK m-PTTG1-2 and e: HEK m-PTTG1-4.

Figure 11



PTTG induces tumor formation in nude mice injected with HEK293 cells stably expressing PTTG protein.

To determine whether PTTG promotes tumor formation in nude mice, we subcutaneously injected nude mice with HEK293 cells expressing PTTG or mPTTG. Three out of four mice injected with the HEKPTTG-1 or HEKPTTG-3 cell lines developed large tumors within four weeks of injection (Figure 12). Pathologic analysis of the tumors revealed that they were poorly differentiated (Figure 13). Mice injected with the HEKmPTTG-2 cell line also developed tumors, but the tumors were of a small size. None of the mice injected with the other cell line-expressing mutant PTTG (HEKmPTTG-4) or the vector-only cell line (HEKpcDNA3.1) developed tumors within the time frame of this experiment. The tumor volumes, measured at the end of experiment (six weeks after injection of cells), were 150-1320 mm^3 for HEKPTTG-1, 72-1404 mm^3 for HEKPTTG-3 and 8.8-12.6 mm^3 for HEKmPTTG-2 (Table 2).

These results clearly demonstrate that PTTG gene is a potent oncogene. Moreover, they demonstrate that PTTG possesses the ability to act alone to induce transformation of human cells, and does not require the oncogenic cooperation of other gene(s) to achieve its tumorigenic function.

Figure 12: Tumor development in nu/nu mice on injection of HEK293 cells stably transfected with pcDNA3.1, pcDNA3.1 PTTG1 or pcDNA3.1 m-PTTG1 plasmids. Each mouse was injected with 1 x 10^6 cells. Six weeks after injection, the mice were photographed and sacrificed, tumor and other tissues were collected and tumor volumes were measured. a: Mouse injected with HEK pcDNA3.1 cells, b: mouse injected with HEK pcDNA3.1 PTTG1 cells, c: mouse injected with HEK pcDNA3.1 PTTG1-3 cells, and d: mouse injected with HEK pcDNA3.1 m-PTTG1-2. Arrows indicate the tumors.

Figure 12

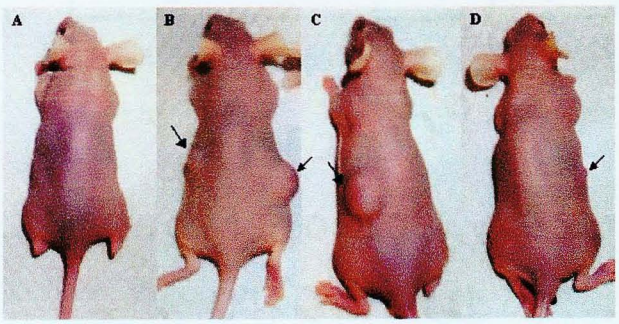


Figure 13: Histopathological analysis of the tumors excised from animals injected with HEK293 expressing PTTG1 or m-PTTG1. a: Normal HEK293 cells stained with hematoxylin, b: tumor from animal injected with HEK pcDNA3.1 PTTG1, c: tumor from animal injected with HEK pcDNA3.1 PTTG1-3.

Figure 13

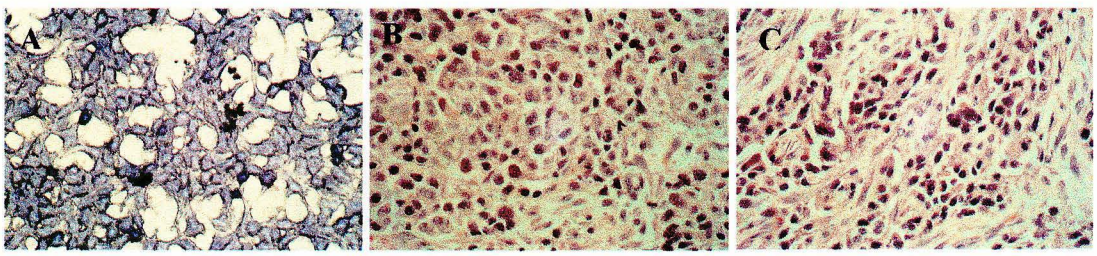


Table 2: Tumor formation induced by PTTG1 expressing HEK293 cells in nude mice.

Table 2

Clone	Animals with tumor	Tumor Volume Range
HEK pcDNA3.1	0/4	NA
HEK PTTG1-1	3/4	150-1320 mm^3
HEK PTTG1-3	3/4	72-1404 mm^3
HEK m-PTTG1-4	2/4	8.8-12.6 mm^3
HEK m-PTTG1-2	0/4	NA

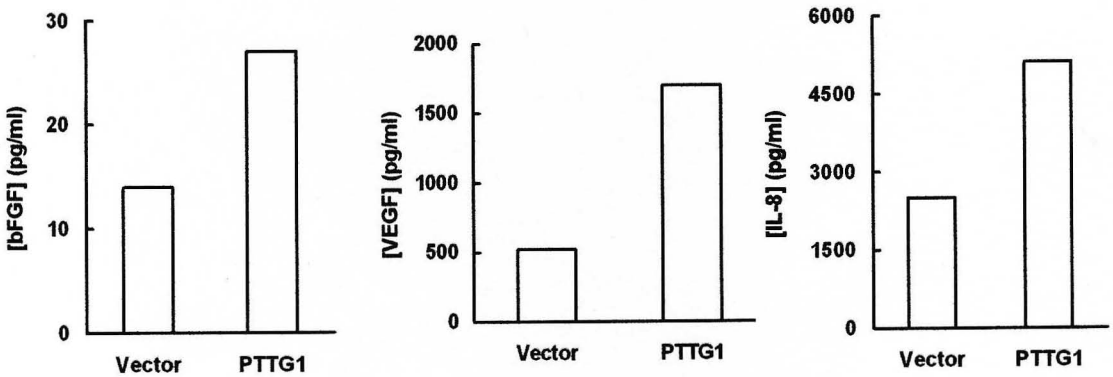
PTTG stimulates expression and secretion of bFGF, VEGF and IL-8.

Local invasive growth is a key feature of primary malignant tumors. A correlation between the levels of expression of PTTG with increased tumor invasiveness and with the degree of malignancy has been demonstrated in pituitary and colorectal tumors (33,125). The specific mechanisms by which PTTG facilitates the invasive behaviors of tumor cells remain obscure. Recently Melmed et al, (75,90) have shown that transfection of NIH3T3 cells with PTTG cDNA results in an increase in secretion and expression of both bFGF and VEGF. A direct correlation between high IL-8 expression and tumor metastases has been shown in a number of cancers (109,126,127), and IL-8 also has been reported to possess mitogenic (128) and angiogenic effects (129). We therefore measured the levels of bFGF, VEGF and IL-8 in HEK293 cells transiently transfected with pcDNA3.1 or pcDNA3.1-PTTG cDNA and in tumors developed on injection of nude mice with HEK293 cells that constitutively express PTTG. As shown in (Figure 14A), the levels of bFGF, VEGF and IL-8 were comparatively higher in conditioned medium of cells transfected with pcDNA3.1-PTTG cDNA than from cells transfected with pcDNA3.1 vector only. Cells transfected with pcDNA3.1-PTTG showed a 2-fold increase in bFGF, a 3.5-fold increase in VEGF and a 2-fold increase in IL-8 levels compared to cells transfected with pcDNA3.1 vector only. Measurement of the mRNA levels of these proteins by RT/PCR showed significantly higher levels in cells transfected with pcDNA3.1-PTTG cDNA

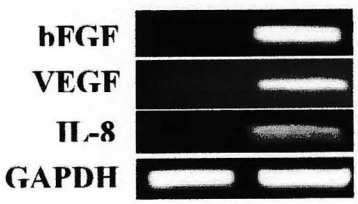
Figure14: Overexpressing of PTTG in HEK293 cells induces secretion and expression of bFGF, VEGF, IL-8. HEK293 cells were transiently transfected with pcDNA3.1 or pcDNA3.1-PTTG1 cDNA. The culture media was removed, lyophilized and bFGF, VEGF, and IL-8 secreted in culture medium were measured by ELISA. a: amount of bFGF, VEGF and IL-8 in culture medium. Vector: cells transfected with pcDNA3.1 vector DNA; PTTG1: cells transfected with pcDNA3.1-PTTG1 cDNA. B: Expression of bFGF, VEGF and IL-8 mRNA in cells. Lane 1: pcDNA3.1 transfected cells and Lane 2: pcDNA3.1-PTTG1 transfected cells. GAPDH was used as a loading control. The data is representative of two independent experiments.

Figure 14

A


[bFGF] (pg/ml)
30
20
10
0
Vector
PTTG1
[VEGF] (pg/ml)
2000
1500
1000
500
0
Vector
PTTG1
[IL-8] (pg/ml)
6000
4500
3000
1500
0
Vector
PTTG1

B


bFGF
VEGF
IL-8
GAPDH

compared to cells transfected with pcDNA3.1 vector (Figure 14B). To determine if over expression of PTTG results in increase in levels of bFGF, VEGF and IL-8 in vivo, we measured the levels of bFGF, VEGF and IL-8 proteins in lysates from tumors developed on injection of nude mice with HEK293 cells stably transfected with PTTG. As shown in (Figure 15), the levels of bFGF, VEGF and IL-8 were significantly higher in three out of four tumors compared to normal tissues (kidney, liver, lung and heart) collected from the same animals. Since the size of the tumors that developed on injection of cells expressing mutated PTTG (HEKmPTTG-2) were small, we were unable to analyze the bFGF, VEGF and IL-8 levels in these tumors. Measurement of mRNA for bFGF, VEGF and IL-8 revealed significantly higher levels of expression in tumors compared to normal tissues and tumors developed on injection of HEKmPTTG-2 cells (Figure 16). bFGF levels were found to be comparatively higher in heart which is consistent with other investigators (130) Our results show that over-expression of PTTG in HEK293 cells resulted in an increased secretion and expression of bFGF, VEGF and IL-8 in vitro and in vivo, suggesting that increase in secretion and expression of bFGF, VEGF and IL-8 by PTTG may be one mechanism by which PTTG achieves its oncogenic function and increases tumor angiogenesis.

Figure 15: Analysis of bFGF, VEGF and IL-8 expression by ELISA. Tumors and other tissue were excised from the animals injected with HEK293 cells stably transfected with PTTG1 (clone 1 and 3) and homogenized. bFGF, VEGF and IL-8 in the homogenates were analyzed by ELISA. Each analysis was performed in triplicate tissue and was normalized to mg of protein. Error bars represent ± SEM of three independent experiments.

Figure 15

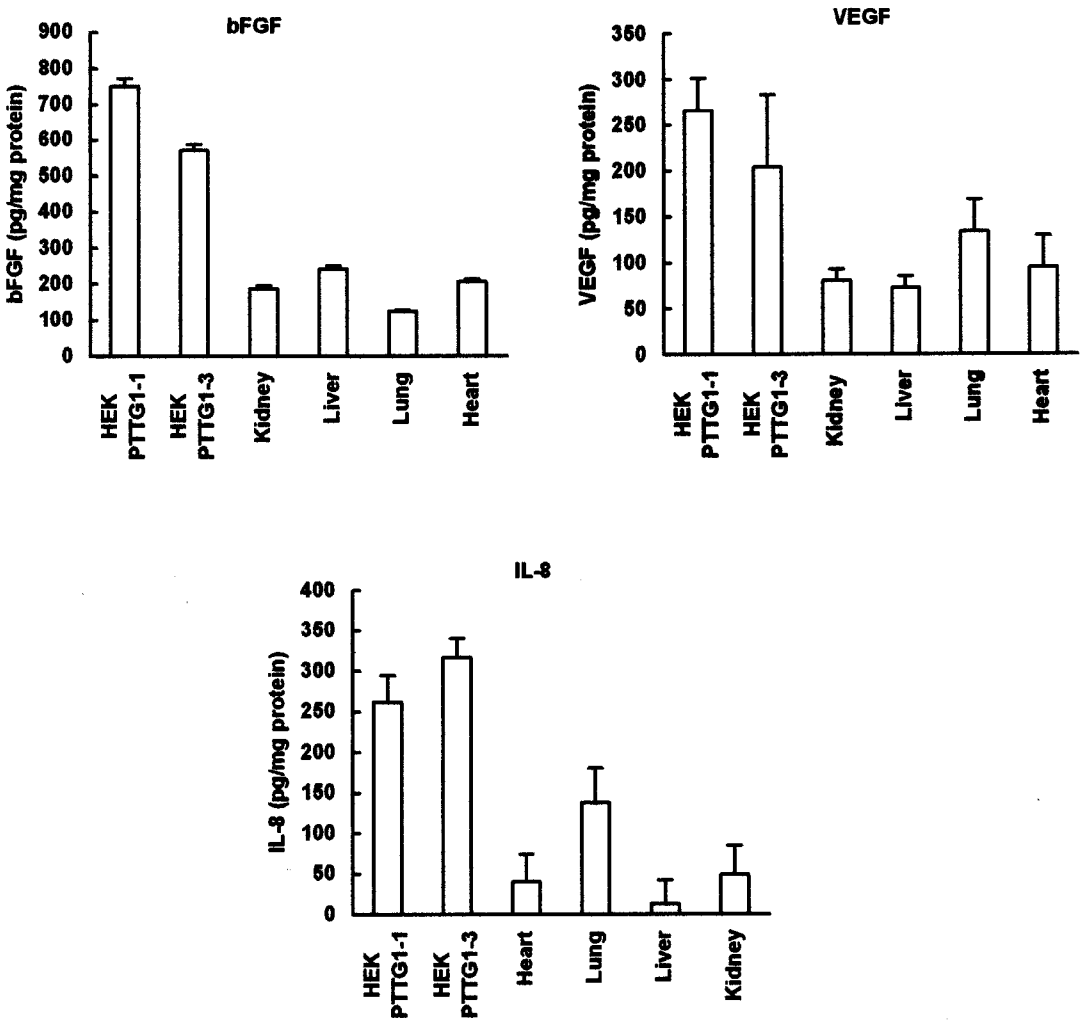
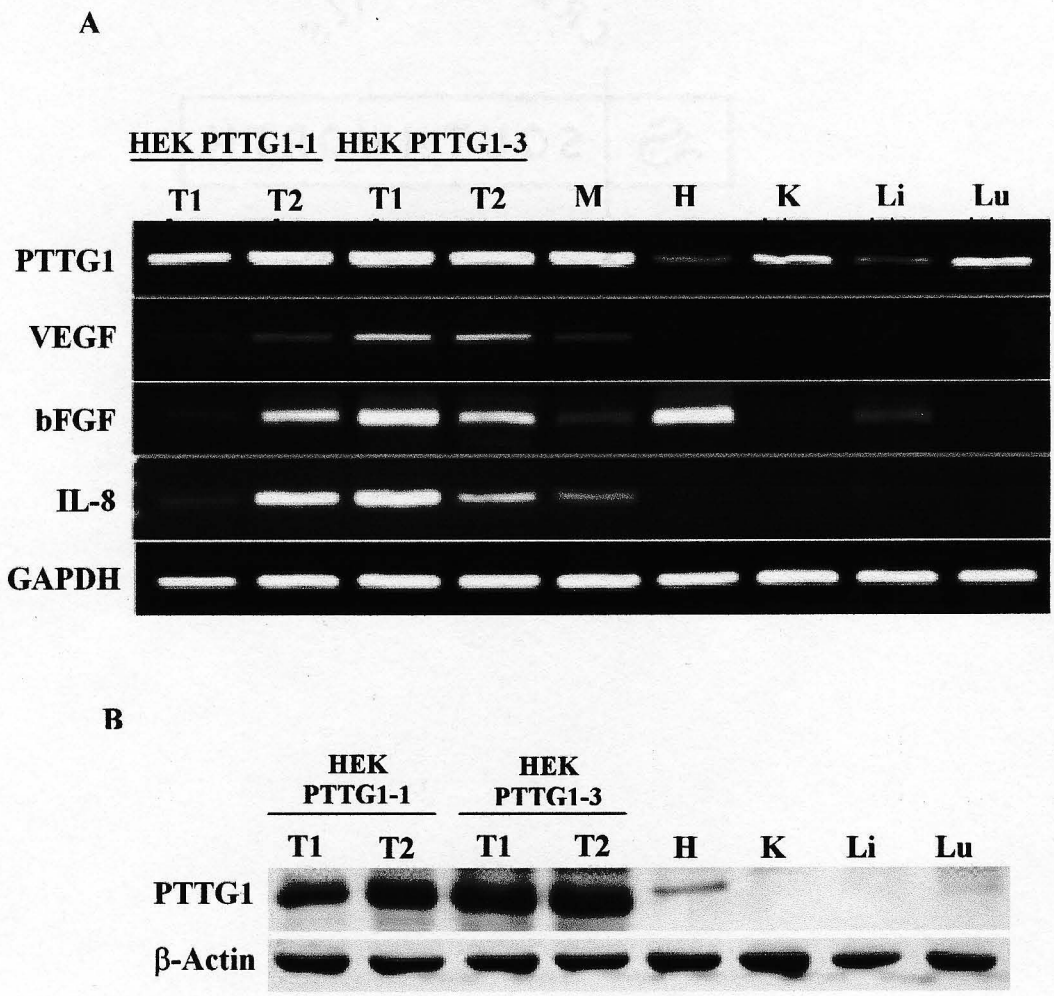

bFGF
bFGF (pg/mg protein)
900
800
700
600
500
400
300
200
100
0
HEK PTTG1-1
HEK PTTG1-3
Kidney
Liver
Lung
Heart
VEGF
VEGF (pg/mg protein)
350
300
250
200
150
100
50
0
HEK PTTG1-1
HEK PTTG1-3
Kidney
Liver
Lung
Heart
IL-8
IL-8 (pg/mg protein)
400
350
300
250
200
150
100
50
0
HEK PTTG1-1
HEK PTTG1-3
Heart
Lung
Liver
Kidney

Figure16: Analysis of expression of PTTG, bFGF, VEGF and IL-8 from tumors and other tissues collected from mice injected subcutaneously with HEK293 cells stably expressing PTTG1 protein. a: RT-PCR analysis and , b: western blot analysis. T1: tumor 1, T2: tumor 2, M: HEK m-PTTG1-2, H: heart, K: kidney, Li: liver, Lu: Lung. GAPDH and β-actin were used as control to examine equal loading. The gels are representative of two independent experiments.

Figure 16


A
HEK PTTG1-1
HEK PTTG1-3
T1
T2
T1
T2
M
H
K
Li
Lu
PTTG1
VEGF
bFGF
IL-8
GAPDH
B
HEK PTTG1-1
HEK PTTG1-3
T1
T2
T1
T2
H
K
Li
Lu
PTTG1
β-Actin

2.2.4 DISCUSSION

The oncogenic function of PTTG was established by its overexpression in a mouse fibroblast cell line (NIH 3T3) followed by assessment of its ability to induce cellular transformation and tumor formation in nude mice (24,27). However, the differences in biology between the rodent cells and human cells have brought the validity of this model into question. There are a number of instances in which an oncogene has been shown to induce transformation in rodent cells but failed to induce transformation of the same types of cells obtained from humans. To test the ability of PTTG to induce transformation in human cells, we selected the human embryonic kidney- 293 (HEK293) cell line as our model. HEK293 cells have been transfected with the large-T antigen and the expression of this molecule results in the cells bypassing senescence and being immortalized. It has been demonstrated that expression of large-T antigen does not convert these cells to the tumorigenic phenotype nor do these cells develop tumors when injected into nude mice (120). Our data clearly demonstrate that over-expression of PTTG in HEK293 results in an increase in cell proliferation, induces cellular transformation *invitro* (increase in anchorage-independent growth), and promotes tumor formation in nude mice. Cells transfected with pcDNA3.1 vector did not form colonies in soft agar or develop into tumors on implantation in nude mice, confirming that HEK293 cells do not possess a tumorigenic phenotype. Thus, our results suggest that PTTG is a potent oncogene and is capable of inducing tumorigenesis in human cells.

A second issue that we were able to address using the HEK293 cell model is the question of the ability of PTTG to induce transformation of normal human cells, i.e., whether it acts alone or in cooperation with another oncogene to achieve its tumorigenic function. It has been reported that a single oncogene may not be sufficient for induction of transformation, but requires co-expression, or oncogenic cooperation, of another oncogenes(s) to induce tumorigenesis in normal primary human cells (96,116,118,119,131,132). Our data clearly show that PTTG is sufficient to achieve its tumorigenic function without the cooperation of another gene.

PTTG contains several-proline rich motifs (PXXP); two of these that are located in the C-terminal domain have been reported to be potential binding sites for SH3-domians (133). In our study we confirm that mutation of these C-terminal proline-rich motifs abrogates the tumorigenicity of PTTG in human cells. Such loss of tumorigenicity on mutation of PTTG could be due to a loss of expression. Our western blot analysis of the stable cell lines (HEKmPTT1-2 and HEKmPTTG-4) that constitutively express mutated PTTG protein showed high levels of expression of mPTTG protein, suggesting that the loss of tumorigenic function of mPTTG protein is not due to loss of expression but due to the loss of its ability to induce cellular transformation. These results are consistent with other investigators for rodent cells (27) and confirm the importance of C-terminal proline-rich motifs to mediate the oncogenic function of PTTG.

The molecular mechanisms by which PTTG achieves its tumorigenic function remain unclear. PTTG has been reported to induce expression of the c-myc oncogene

(56), bFGF (75) and VEGF (90). bFGF is a broad spectrum and pleiotropic mitogen for growth and differentiation affecting various mammalian cells and organ systems and a large number of cells lines (134). Besides stimulating wound healing, tissue repair and hematopoiesis (135), bFGF induces cell migration and proliferation (91) and acts as an agiogenic factor that induces migration, proliferation and differentiation of endothelial cells (136). In addition, it has been reported to modulate the invasion of tumor cells through surrounding tissue to form new capillary cord structures by regulating the activities of extracellular molecules including collagenase, proteinases and integrins (136). Regulation of secretion and expression of bFGF by PTTG in NIH 3T3 cells has been shown (27). Consistent with these reports, our results demonstrate a significant increase in secretion and expression of bFGF in HEK293 cells on transient transfection with PTTG cDNA as well as in tumors developed by injection of stable cell lines that constitutively express PTTG.

VEGF is a potent stimulant of the vascularization of tumors and is one of the most specific markers of tumor vasculature observed to date (105,106). VEGF is a multifunctional cytokine acting as a potent permeability agent, an endothelial cell chemotactic agent, an endothelial cell survival factor and an endothelial cell proliferation factor (137). The expression and secretion of VEGF has been shown to be a crucial rate-limiting step during tumor progression (108). Our results demonstrate a significant increase in secretion and expression of VEGF in HEK293 cells on transfection with PTTG and also from tumors excised from animals injected with HEK293 cells that stably express PTTG.

A direct correlation between high IL-8 expression and metastases in melanoma (109), ovarian cancer (126), prostrate cancer (127) and pancreatic cancer (138) has been reported. To determine if overexpression of PTTG induces change in secretion and expression of IL-8, we measured its levels in HEK293 cells on transfection with PTTG cDNA and in tumors developed on injection of HEK293 cells transfected with PTTG. Our results demonstrate for the first time that overexpression of PTTG induces IL-8 expression in vitro and also in tumors in vivo.

In summary, our results demonstrate that PTTG is a potent human oncogene and has the ability to induce cellular transformation of human cells. Overexpression of PTTG in HEK293 cells leads to an increase in the secretion and expression of bFGF, VEGF and IL-8. Mutation of C-terminal proline-rich motifs abrogates the oncogenic function of PTTG. To our knowledge, this is the first study demonstrating the importance of PTTG in human tumorigenesis.

CHAPTER III

SECTION I

MATRIX METALLOPROTEINASES

INTRODUCTION

Metalloproteinases are a multigene family of metal-containing proteases that share common structural and functional characteristics. All of the family members sequenced to date have at least three common domains: (i) a prodomain which contains a conserved cysteine residue that is lost on activation; (ii) a catalytic domain which contains a conserved metal-binding site and (iii) a highly conserved zinc-binding active site (Figure 17) (139). There are at least 24 members of the MMP family, and that can be subdivided according to their substrate specificity. Regulation of MMPs occurs at three different levels: alteration of gene expression, activation of latent zymogens, and inhibition by tissue inhibitors of metalloproteinases (TIMP). Among the members of the MMP family, gelatinases (MMP-2 and MMP-9) are the most common metalloproteinases associated with tumor metastasis and angiogenesis. Overexpression of MMP-2 has been correlated to an invasive phenotype in several cancer types and is often predictive of poor survival (140). MMP-2 knockout mice display lowered lung colonization following intravenous administration of cancer cells, and reduced tumor angiogenesis resulting in

Figure 17: Major functional domains of Matrix Metalloproteinases-2 (MMP-2).

Figure 17


MMP-2
Propeptid
Signal
Catalytic
Zn^2
Hemopexin-like
Fibronectin type II
Signal
Linker

reduced tumor growth (141). Moreover, MMP-2 has been shown to be a prognostic marker in ovarian cancer, lung cancer, and gastric cancer (142-144). MMP-2 is also known to be involved in tumor angiogenesis through degradation of extracellular matrix (ECM), which can result in tumor cells, and endothelial cell migration due to loss of cell-matrix and cell-cell contacts (145). MMP-2 is also capable of releasing growth factors that are from ECM, cleaving certain growth factor receptors and activating growth factors, excreted as pre-pro-enzymes, such as transforming growth factors (TGFα, TGFβ), macrophage-colony stimulating factor (M-CSF), insulin like growth factor (IGF), and fibroblast growth factor receptor (FGFR) (146-148). However, as the MMP-2 activity may not always lead to angiogenesis and metastasis, the mechanisms by which metalloproteinases are regulated remain unclear.

SECTION II

Regulation of Angiogenesis and Invasion by *human Pituitary tumor transforming gene (hPTTG)* through increased the expression and secretion of Matrix Metalloproteinase-2.

INTRODUCTION:

Primary tumor growth is restricted due to a limited supply of oxygen, nutrients, and growth factors. Tumor progression and invasion to distant organs depends on tumor angiogenesis. To achieve angiogenesis various factors including transforming genes and growth factors turn on to enable tumor progression (44). Once angiogenesis is initiated, the tumors expand exponentially and invade to local and distance tissues. From a patient survival perspective, understanding the mechanisms of angiogenesis and development of new therapeutics to inhibit angiogenesis is a critical step leading to inhibition of tumor growth and metastasis (98,99).

Many oncogenes have been reported to play important role in tumor angiogenesis. Recently, a novel oncogene, pituitary tumor transforming gene (PTTG), also known as securin, has been reported to play a vital function in tumor angiogenesis (90). Using an mRNA differential technique, PTTG was originally cloned from rat a pituitary tumor (26), followed by cloning homologue from humans (4-6). The predominant cellular location of the PTTG protein is the cytoplasm, although it is partially localized in the nucleus (66). Nuclear translocation of PTTG can be facilitated by either interaction with PTTG binding factor (PBF) (149), or by

interaction with the mitogen-activating protein (MAP) kinase cascade (56). The level of PTTG expression is increased in rapidly proliferating cells and is regulated in a cell cycle-dependent manner (45). PTTG mRNA and protein expression are low at the G1/S interphase, gradually increase during the S phase, and peak at the G2/M phase (45). As the cells enter anaphase, PTTG is degraded and daughter cells express very low amounts of PTTG. The degradation of PTTG most likely occurs via ubiquitination since PTTG contains a D box which is required for such proteolysis.

Numerous studies have demonstrated that human PTTG displays a distinct pattern of expression. In normal tissues, PTTG expression is restricted, with high levels in testis, and low levels in the thymus, colon, and small intestine (24,27). In contrast, PTTG is highly expressed in a variety of human primary tumors as well as tumor cell lines including carcinomas of the ovary, lung, testis, kidney, colon, thyroid, pituitary, liver, adrenal, breast, prostate, melanoma, leukemia, and lymphoma (24,25,29,33,36,47,66,80,150-153), suggesting that PTTG may be involved in tumorigenesis. Furthermore, the expression level of PTTG correlate with increased tumor invasiveness in human pituitary tumors with hormone overproduction (151), and with the degree of malignancy, pathogenesis and/or progression of colorectal and thyroid tumors (33,151,154). PTTG has been identified as one of eight signature genes associated with tumor metastasis and up-regulated in human primary solid tumors (36). A relationship between the survival rate and level of expression of PTTG in esophageal cancer has been reported (71).

We and others have shown that overexpression of human PTTG in mouse fibroblasts (NIH3T3) and human embryonic kidney (HEK293) cells results in increased cell proliferation, induction of cellular transformation in vitro and formation of tumors in nude mice (24,27). Currently, the precise mechanism by which PTTG causes cell transformation remains unclear. Data from our laboratory and others suggest that PTTG may act through basic growth factor (bFGF) (24,155), vascular endothelial growth factor (VEGF) (72,90), and/or interleukin-8 (IL-8) (72). Additional mechanisms by which PTTG may induce its oncogenic function are indicated by findings that implicate it in sister chromatid separation during cell division (45). PTTG, by virtue of its function as human securin, ensures that there is no premature separation of sister chromatids. Mice that lack PTTG show aberrant cell cycle progression, premature centromere division, and problems with chromosomal stability, as well as tissue specific phenotypes, such as testicular and splenic hypoplasia and thymic hyperplasia (83). In addition, PTTG null mice exhibit impaired proliferation of pancreatic beta cells and developed type I diabetes during late adulthood (64,83). Furthermore, animal deficient in PTTG (-/-) demonstrat inhibition of tumor development compared to Rb (+/-) induced tumors (84). On the other hand, transgenic animals that express PTTG under the control of the αGSU promoter developed an enlarged pituitary and hyperplasia of prostate (85). Taken together, these data strongly suggest an important role of PTTG in cell proliferation and tumorigenesis and suggest mechanisms that may contribute to these effects.

Local invasive growth is a key feature of primary malignant tumors. A correlation between the level of expression of PTTG with increased tumor invasiveness and degree of malignancy in pituitary and colorectoral tumors has been reported (33). However, the specific mechanisms facilitating the invasive behavior of cancers remain obscure. Interactions between cancer cells and surrounding normal cells and the extra-cellular matrix (ECM) are thought to be key event in tumor cell invasion (140,146). To invade and spread through the surrounding normal tissue, tumor cells must degrade multiple elements of the ECM, including fibronectin, laminin, and type IV collagen (139,156). Several metalloproteinases (MMPs) are required for the degradation of the ECM, and these are classified according to their substrate specificity. MMP-2 and MMP-9 are the most common MMPs in tumors, and the elevated levels have been reported in various cancers (139,157,158). In the present study, we show that overexpression of PTTG in HEK293 cells results in up-regulation of the secretion and expression of MMP-2, but not MMP-9, leading to increased cell migration and invasion. These data suggest an important role for PTTG in tumor cell migration and invasion.

3.2.2 MATERIAL AND METHODS

Material

Human embryonic kidney cells (HEK293) and Human umbilical vein endothelial cells (HUVEC) were obtained form American Type Culture Collection (ATCC, Manassa, VA) and maintained at 37°C in a humidified atmosphere of 5% CO_2. HEK293 cells were cultured in DMEM (GIBCO) supplemented with

penicillin/streptomycin (100 IU/ml and 100μg/ml) (GIBCO) and 10% FCS (Hyclone, Atlanta GA.). HUVEC cells were cultured in ECM medium (Clonetics, US) supplemented with Bullet Kit (EGM-2, Clonetics, US). Cells passages were performed routinely. The MMP-2 blocking antibody (MMP2BA) was obtained from Chemicon USA. Porcine gelatin and clostridium collagenases were from Sigma Chemical Co. (St. Louis, MO). DQ Collagen fluroescein conjugate was purchased from Molecular Probes (Carlsbad, CA).

GE Array Analysis of human extracellular matrix and adhesion molecules

The expression profile of extracellular matrix & adhesion molecules genes were analyzed using the non-radioactive SuperArray GEArray Q series human gene array (HS-010 SuperArray Bioscience Corp., Frederick, MD). This array membrane is composed of 96 extracellular matrix & adhesion molecules genes, a plasmid pUC18 negative control, and four housekeeping genes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin A, ribosomal protein L13a, and β-actin. Radioactive [α-^{32}P]dCTP labeled cDNA probes were prepared from 1 μg of RNA from pcDNA3.1 and pcDNA-PTTG transfected HEK293 cells each, denatured and hybridized to extracellular matrix & adhesion molecules gene-specific cDNA fragments spotted on the membranes. After pre-hybridization with GEAhyb Hybridization Solution (SuperArray) of denatured salmon sperm DNA (Invitrogen). The array membrane was hybridized with denatured cDNA (labeled with α-^{32}P-dCTP) probes overnight at 60°C. After washing of the membrane twice with 2 × SSC, 1%

SDS and twice with 0.1 × SSC, 0.5% SDS for 15 min at 55°C, the membranes were exposed to x-ray film at -80°C for 24-48 h. The results were analyzed with ScanAlyzer and GEArray Analyzer program. The relative expression levels of different genes were estimated by comparing its signal intensity with that of internal control β-actin.

ELISA for MMP-2 and MMP-9:

The levels of MMP-2 and MMP-9 in tissue culture supernatants were measured using commercially available ELISA kits from BD Biosciences (Minneapolis, MN). To measure MMP-2 and MMP-9 in the culture supernatants, HEK293 cells were transiently transfected with pcDNA3.1 or pcDNA3.1-PTTG cDNA using Fugene6 as the transfectant reagent as described previously (81). After 24 h of transfection, the medium was replaced with serum free DMEM medium. Twenty-four hr later, the medium was collected and concentrated 5-fold (1.0 ml to 200 μl) using a speedVac system (Savant, Holbrook, NY). The concentration of MMP-2 and MMP-9 in a sample was determined by interpolation from a standard curve. All measurements were normalized to protein concentration and performed in triplicate.

Transfection and collection of conditioned media:

For transient transfection, HEK293 cells were seeded into six well plates approximately 24 h prior to transfection,. Cells were transfected in serum-free medium using 1μg of plasmid DNA and 3μl of transfectin (BioRad) according to the manufacture's instructions. After 18 hr of transfection, medium was changed to

serum-free medium (DMEM GIBCO) ± MMP-2 blocking antibody 2 μg/ml. After 48 hr, conditioned medium (CM) were collected, centrifuged and stored at -80°C for future use. Tissue lysates and stable clones of HEK293 cells transfected with pcDNA3.1 or pcDNA-PTTG were generated as describe previously (72).

Matrix Metalloproteinases-2 (MMP-2) expression and secretion: Zymography

Secretion and activity of MMP-2 in conditioned medium collected from cells transfected with pcDNA3.1 or pcDNA-PTTG cDNA was used to perform gelatin zymography analysis as described by Heussen et.al (159,160). Briefly conditioned medium with or without MMP-2 blocking antibody (1:1000) was collected and one ml of medium was lyophilized and reconstituted with 100 μl of water. Protein was determined by the Bradford method (161) and 20 μg of protein was loaded on polyacrlyamide gels containing 0.1% gelatin Sigma Co. (St. Louis, MO). Electrophoresis was performed under non-reducing conditions at 20 mA for 3 hr at room temperature. The gel was washed twice for 30 min each in 2.5% Triton X-100 to remove SDS, incubated in substrate buffer (50 mM Tris-HCl, 5mM $CaCl_2$, 0.01% NaN_3, pH 7.6) for 24 h at 37°C. Gels was stained with 0.5% Coomassie brilliant blue G-250 (Pierce Rockford, IL) for 30 min at room temperature, and destained in the destaining buffer (30% ethanol, 10% acetic acid, deionized water). The presence of metalloproteinases (MMP-2) was indicated by an unstained proteolytic zone of

substrate. The gel was scanned using NIH image software, and data are presented as fold change relative to control i.e, pcDNA transfected conditioned medium.

Detection of collagenase activity in conditioned medium and tissue sections.

Collagenase activity in conditioned medium (CM)

Twenty µl of collagen fluorescent substrate reconstituted in PBS was mixed with 80 µl of reaction buffer (0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM NaN_3, pH 7.6) and added to the fluorescent reader plate. Twenty µl of conditioned medium treated with or without MMP-2 blocking antibody was added to each well. Clostridium collagenase, serially diluted was used as a positive control and 100 µl of reaction buffer was used as a negative control. The plate was incubated at room temperature for 1 h. After incubation, the fluorescent intensity of the digested product from DQ collagen was measured at excitation 495 nm and emission at 515 nm in the fluorescent microplate reader. The fold change was calculated by dividing the mean of fluorescence values of conditioned media collected from pcDNA transfected by the mean value of fluorescence of conditioned media collected from pcDNA-PTTG transfected with or without MMP-2 blocking antibody.

Collagenase activity in tumor tissue.

Collagenase activity was determined in tumor tissues extracted from *nu/nu* mice as describe previously (72). Briefly, DQ collagen substrate (1mg/ml) was mixed with reaction buffer (0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM NaN_3, pH

7.6) containing 0.8% low melting agarose (Invitrogen) at a ratio of 1:1 as described by Peter et al (162). After melting the agarose mixture at 60°C, the mixture was applied on the frozen tissue section on glass slides and allowed to solidify at room temperature. Slides were incubated in a humidified chamber with a few drops of reaction buffer on each slide for 5-7 days. Assessment of degradation of substrate was examined with an Olympus IX50 fluorescent microscope, and photographed with a KODAK DC290 digital camera.

Western blot analysis

Cells were lysed with lysis buffer (20mM Tris HCl pH7.5, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF, and 1μg/ml each of pepstatin, leupeptin and aprotinin) and subjected to SDS-PAGE. The proteins were blotted onto nitrocellulose membranes as described previously (124). PTTG protein was detected by using polyclonal antiserum (1:1500) to protein and HRP-conjugated secondary anti-rabbit antibody (1:5000), by the enhanced chemiluminescent substrate (ECL, Amersham).

Immunohistochemistry analysis

HEK293 cells were transiently transfected with pcDNA3.1 or pcDNA3.1+PTTG vector in chamber slides (Fisher Scientific, Springfield, NJ). After 24 h of transfection, cells were fixed using 4% freshly prepared paraformaledehyde for 8 min and then permeabilized by treating with 0.1% Nonidet P-40 for 5 min. Cells were pretreated with 5% normal goat serum for 60 min to block nonspecific binding

sites. The tissue was washed with PBS, incubated with anti-PTTG antiserum as described (124), and MMP-2 monoclonal antibody(R&D) diluted at (1:1500). Control samples were incubated with preimune serum. After several rinses with PBS buffer, the sections were incubated for 45 min with Texas Red conjugated anti-rabbit secondary antibody and Texas green conjugated anti-mouse secondary antibody (1:100) obtained from Jackson Immuno Research Laboratories (West Grove, PA). Cells were analyzed using a fluorescent microscope (Olympus X50).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from transfected HEK293 cells in log phase and tumor tissues using Trizol reagent (Gibco-BRL) as described previously (25). In brief, samples were homogenized in 5 ml of Trizol reagent, after vortexing for 2 min, 1 ml of homogenate was distributed in five 1.5 ml Eppendroff tubes and kept at room temperature for 5 min, 1/5 volume of chloroform was added to each sample followed by vigorous mixing and incubation for 2-3 min. The aqueous phase was removed after centrifugation at 15,000 rpm and precipitated with isopropanol. The RNA was washed once with 70% ethanol. After air-drying, the RNA was resuspended in *RNase* free water. Total RNA concentration was determined spectrophotometrically at 260 nm. Using iScript RT-PCR kit (Bio-Rad), first strand cDNA was synthesized and subsequently used for PCR amplification using the specific primers for MMP-2, MT1-MMP, PTTG, and GAPDH (72,163) (Table 3) using Taq polymerase (Takra Co.). PCR conditions were 95°C for 5 min, 95°C for 1 min, 54°C for 30 sec and 72°C for 30

sec for 30 cycles with a final extension for 5 min at 72°C. Ten μl PCR product from each sample was subjected to electrophoresis on a 1.5% agarose gel.

In-vitro invasion assay

PTTG stably transfected HEK293 cells were used for invasion and migration assays. Boyden chamber matrigel coated wells (BD BioCoat Invasion System) were used according to the manufactuer's instructions. HEK293 cells were dislodged non–enzymatically (TryplE, Invitrogen, IL) and resuspended in DMEM serum-free culture medium, with or without MMP-2 blocking antibody at $2x10^5$ cells/ml and 0.25 ml of cell suspension was transferred to the top chamber. Medium with 2% FBS was added to the lower chamber. The plate was incubated at 37°C, 5% CO_2 atmosphere for 22 h. Non-migrated cells in the upper chamber were removed with clean cotton swab, and migrated cells on the bottom of the membrane were stained with Eosin and Gimsa stain. The migrated cells were counted and invasion index was calculated by dividing the percent of cells that migrated through the matrigel by the percent of cells that moved through the pores of uncoated wells. The effect of secreted MMP-2 on HUVEC cells invasion and migration was also performed using conditioned media in the lower chambers of Boyden chamber wells.

Table 3: Primer Sequences for MMPs and Integrins.

Table 3

	Forward Primer	Reverse Primer
MMP-2	5'-tggcgatggatacccctttt-3'	5'-ttctcccaaggtccatagctcat-3'
MT1-MMP	5'-ccccgaagcctggctaca-3'	5-'gcatcaggctttgcctgttact-3'
TIMP-2	5'-gtagtgatcagggccaaagc-3'	5'-caggccctttgaacatcttt-3'
Integrin Beta-1	5'-catctgcgagtgtggtgtct-3'	5'-ggggtaatttgtcccgactt-3'
Integrin Beta-3	5'-gacaagggctctggagacag-3'	5'-actggtgagctttcgcatct-3'
Integrin Alpha-5	5'-gtgggccaacaaagaacact-3'	5'-tggagcaggcccaaatatag-3'
Integrin Aplha-V	5'-aactcaagcaaaagggagca-3'	5'-gggttgcaagcctgttgtat-3'
GAPDA	5'-atcttcttttgcgtcgccag-3'	5'-ttccccatggtgtctgagc-3'

Wound migration assay.

The wound migration assay was performed as described by Cheresh et al (164) with slight modifications. HUVEC cells were grown in 35mm culture dishes to 80% confluence. A wound was formed using a 200 µl pipette tip to clear the cell monolayer, and the boundary of the wound was marked. Cells were then washed three times with PBS. The conditioned medium (CM) was applied in different combinations with and without the MMP-2 blocking antibody. Mouse IgG (Sigma) was used as a control. Cells were incubated for 18-20 h at 37°C, under a 5% CO_2 atmosphere. After incubation, cells were fixed with absolute methanol, stained with diluted (1:10) Giemsa stain (Sigma Chemical Co., St. Louis MO), and photographed. Cell migration was measured by counting the number of cells that migrated into the clear space using an Olympus X-50 microscope at 100 X fitted with an ocular grid. The values represented are the mean of four different random fields. The fold change was determined by dividing the mean number of cells that moved from the wound edge in the experimental cultures by those that moved from the wound edge in the control cultures. HEK293 cells constitutively expressing PTTG were grown in 35 mm tissue culture dishes to 80% confluence. A wound was formed with a 200 ul pipette tip. Cells were washed thrice with 1X PBS. Five ml of DMEM serum-free medium was added to the dishes with or with MMP-2 blocking antibody (MMP2BA). After 24 hr of incubation, cells were fixed with methanol, stained with 1:10 diluted Giemsa stain, and photographed as described earlier.

Tubule formation assay in 3D matrigel matrix:

Forty-eight well plates were coated with 20 μg/ml of matrigel (GF-reduce BD Bedford MA). HUVEC cells ($2x10^3$ cell/ml) were plated on the matrigel matrix with conditioned medium (CM) with or with out MMP-2 blocking antibody for 24 h. After 4 h 100 μl of matrigel-CM (matrigel + condition medium mixture 1:2 ratio) was overlaid on cells. Cells were allowed to differentiate into tubule according to Lewis et al (165). After 3 days, tubule formations were assessed by fixing the cells with methanol for 15 min, followed by rinsing with PBS. Three random fields of view in three replicate wells for each test condition was visualized under high power Olympus IX50 (X400). Color images were captured using Kodak DC290 digital camera linked to a computer with Adobe Photoshop 7 software. Quantification of tubule formation was carried out by counting the number of tubule branches and the total area covered by tubules in each field of view using image analysis software Photoshop version 7. A numerical value was assigned to each pattern according to the network of tubule formation. A numerical value of 0-1 is given when the cells are well separated, 2-3 when cells begin to migrate and align to form tubes, and 4-5 when capillary tubes or closed polygons begin to form.

Luciferase Assay:

A human MMP-2 promoter (1959 bp of the sequence upstream from the transcription start site) cloned into luciferase reporter construct (pGL-MMP2) was obtained from Dr. Etty Beneveniste, University of Alabama at Birmingham, AL (Figure 18) (166). Five hundred ng of the MMP-2 promoter construct or pGL2

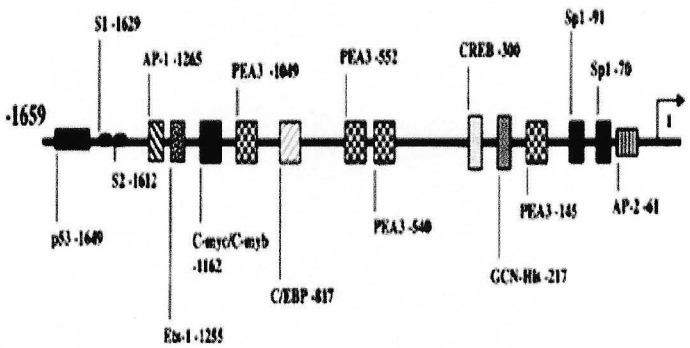
construct was co-transfected with 500 ng pcDNA-PTTG and 100 ng of the renilla construct into 3 x 10^6 HEK293 cells using Transfectin (Bio-Rad). After transfection, cells were allowed to recover for 18 h and then cultured in 10% FCS/DMEM for 24 h. Cells were washed in PBS and lysed with 500 µl of 1X lysis buffer (Promega); luciferase activity was then determined in triplicate as described by the manufacturer. The luciferase activity of each sample was normalized to renilla activity. The fold change in luciferase activity was calculated by dividing the relative luciferase activity of PTTG-pGL-MMP2 promoter activity by PTTG-pGL luciferase relative activity.

Statistical Analysis:

Statistical comparison of data sets was carried out by the Student's t test (single comparison) or by one-way ANOVA (multiple comparison) when data sets complied with the condition of normality and equal variance. Under other conditions, comparisons were carried out by nonparametric analysis using the Mann-Whitney rank-sum test (single comparisons) or the Kruskal-Wallis one-way ANOVA on ranks (multiple comparisons). The Bonferroni methods (parametric test) or the Dunett methods (nonparametric test) were used to identify data sets that differed from the control data in multiple comparisons. Probability of $p < 0.05$ determined from the two-sided test were considered significant. The statistical analysis was carried out by using SPSS 10.0 software.

Figure 18: Key regulatory elements in 1959 bp upstream of transcription start site of human MMP-2 promoter sequence.

Figure 18


S1 -1629
AP-1 -1265
PEA3 -1049
PEA3 -552
CREB -300
Sp1 -91
Sp1 -70
-1659
S2 -1612
p53 -1649
C-myc/C-myb -1162
Ets-1 -1255
C/EBP -817
PEA3 -540
PEA3 -145
AP-2 -61
GCN-His -217

RESULTS

PTTG increases the expression and activity of MMP-2 *in-vitro*

Gelatinase A (MMP-2) was reported to correlate with invasive and metastatic behavior of malignant tumors. Induction of MMP-2 protein had been implicated in various diseases including tumor progression and metastases. We therefore measured the expression and secretion of MMP-2 in HEK293 cells transiently transfected with pcDNA3.1 and pcDNA3.1-PTTG cDNA using The GE array for human extracellular matrix and adhesion molecules and ELISA. As shown in (Figure 19 A and B), MMP-2 expression was ~8 fold increased in the cells transfected with pcDNA-PTTG as compared to cell transfected with pcDNA vector alone. Also, there was ~5 fold increases in the MMP-2 secretion in pcDNA-PTTG transfected cells as compared to pcDNA transfected cells. As shown in (Figure 19 C and D), MMP-2 mRNA levels of MMP-2 protein in pcDNA3.1-PTTG transfected cells were increased by 22 fold ($p<0.05$) compared to HEK293 cells transfected with vector-alone. Co-transfection of HEK293 cells with pGL-MMP-2-Luc reporter gene and with pcDNA-PTTG resulted in luciferase activity increased by 13 fold ($p<0.05$) relative to control pGL2 vector (Figure 20). Measurement of the functional activity of MMP-2 by zymography (Figure 21) and fluorescent collagenase substrate degradation revealed more than two-fold increased in MMP-2 activation in cells transfected with PTTG cDNA compared to cells transfected with vector alone (Figure 22). These data suggest that PTTG increases MMP-2 expression as well as its activity, both of which may be required for the tumor angiogenesis and invasive phenotype to degrade ECM to pave the way for metastases.

Figure 19: A: Selected data from GE Array for human extracellular matrix and adhesion molecules are increased by more than two fold in HEK293 cells transfected with pcDNA-PTTG vs pcDNA3.1. B: Measurement of MMP-2 and MMP-9 in conditioned medium using ELISA, MMP-2 was approximately increased by 5-fold in HEK293 cells transfected with pcDNA-PTTG compared to pcDNA transfected cells. C: Semi quantitative RT-PCR for MMP-2 and GAPDH. D: Graphical representation of the data shown in MMP-2 expression was increased by ~22 fold in HEK293 cells transfected with pcDNA-PTTG compared to pcDNA3.1 transfected cells. (** $p<0.05$).

Figure 19 A and B

A

Microarray analysis

Cell Adhesion Molecules	Fold Change	Extracellular Matrix Proteins	Fold Change	Proteases	Fold Change
Integrin alpha 5	4.6 ↑	Thrombospodin-2	2.8 ↑	MMP-2	8.0 ↑
Integrin alpha V	3.6 ↑	Thrombospodin-3	2.0 ↑	MMP-9	No Change
Integrin beta 1	3.8 ↑	COL1A1	3.8 ↑	MMP-10	3.0 ↑
Integrin beta 3	4.2 ↑	Vitronectin	3.4 ↑	MMP-16	2.0 ↑
Integrin alpha 6	2.6 ↑	Fibronectin 1	4.2 ↑	MMP-14	6.5 ↑
				TIMP-1	2.0 ↓
				TIMP-2	3.2 ↓

B

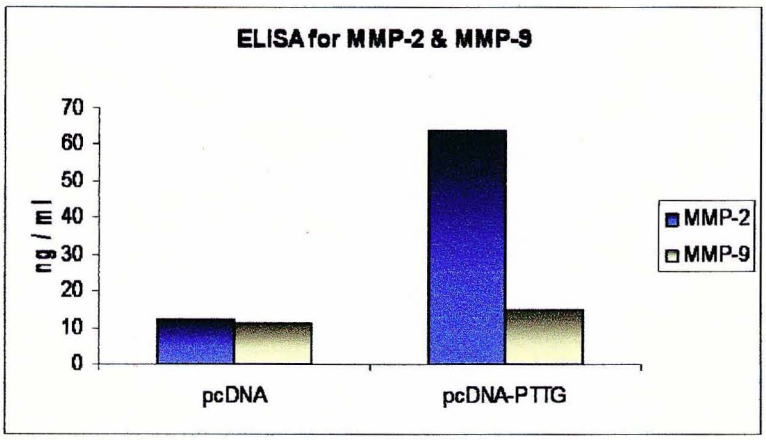


Figure 19 C and D

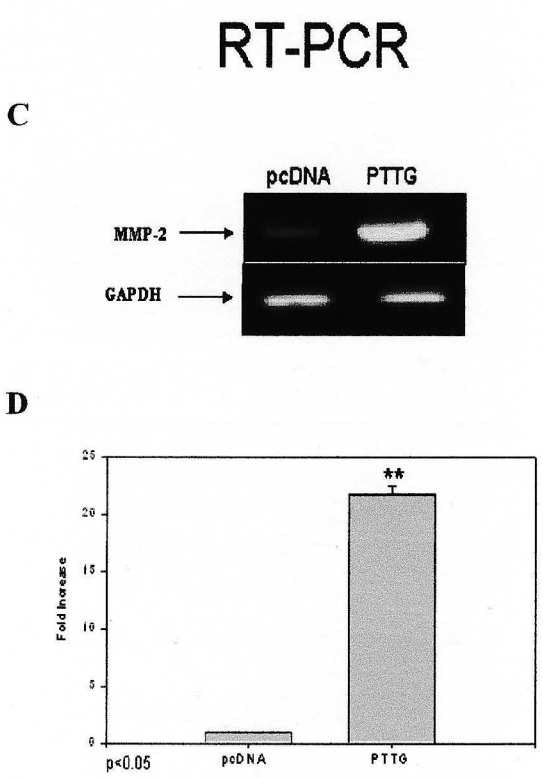


Figure 20: MMP-2 promoter activation. MMP-2 promoter activity was increased by ~13 fold in cells transfected with pcDNA-PTTG as compared to pcDNA transfected cells. Error bars represent mean ± SEM (n = 3). (** $p<0.05$)

Figure 20

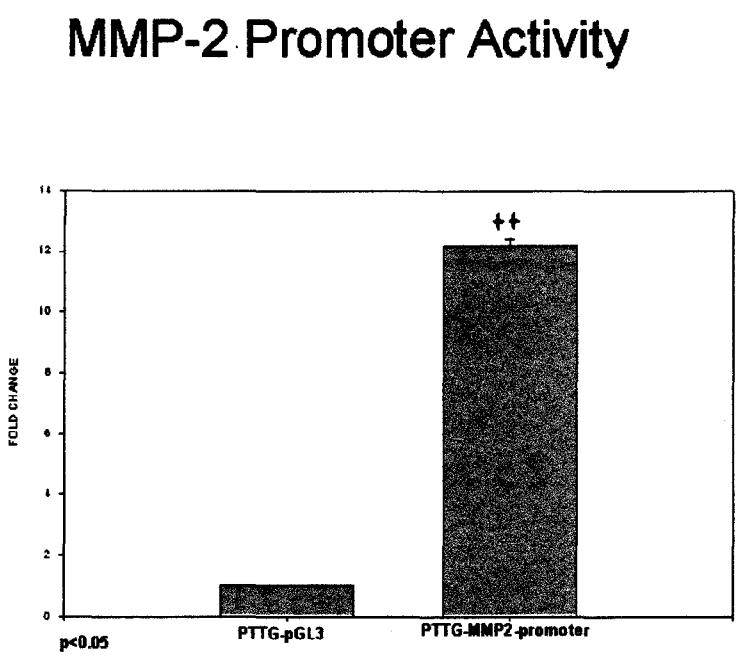

MMP-2 Promoter Activity
FOLD CHANGE
12
10
2
0
++
p<0.05
PTTG-pGL3
PTTG-MMP2-promoter

Figure 21: A: Gelatin zymography. Activity of MMP-2 was increased in conditioned medium from HEK293 cells transfected with pcDNA-PTTG compared to cells transfected with pcDNA plasmid. B: Densometric analysis of zymography showing increase in inactive and active forms of MMP-2 in conditioned medium from HEK293 transfected with pcDNA-PTTG and pcDNA transfected cells.

Figure 21

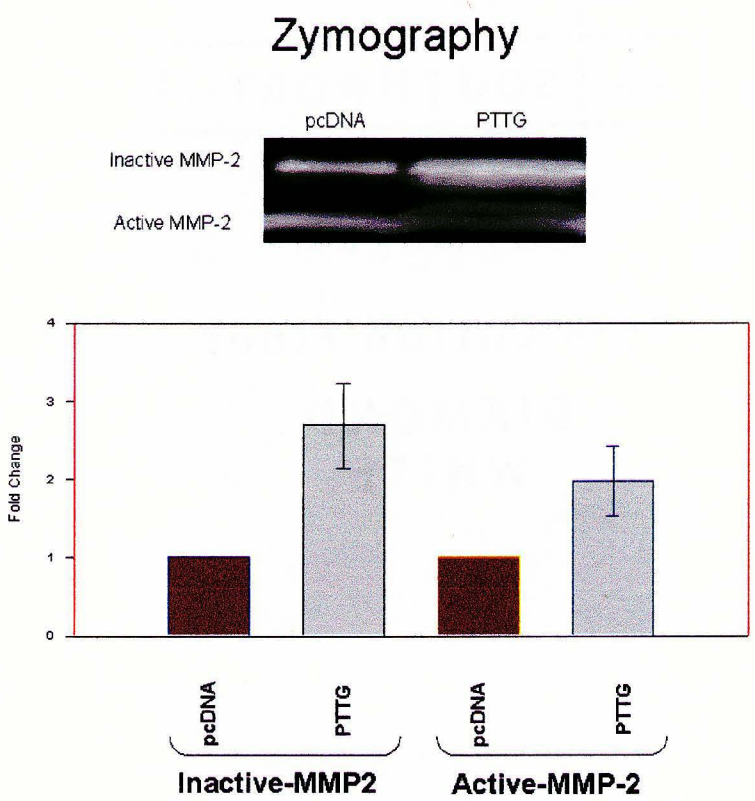
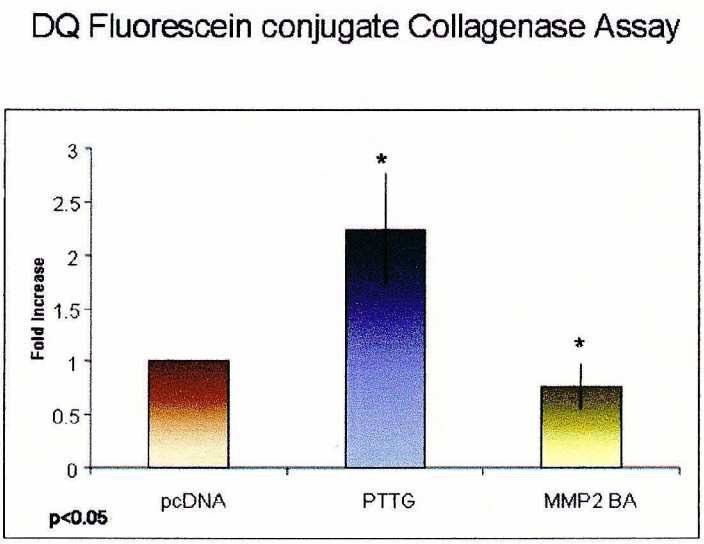

Zymography
pcDNA
PTTG
Inactive MMP-2
Active MMP-2
Fold Change
0
1
2
3
4
pcDNA
PTTG
pcDNA
PTTG
Inactive-MMP2
Active-MMP-2

Figure 22: Direct Quenching (DQ) Fluorescein conjugate collagenease analysis. Activity of collagenase was approximately 2.5 fold higher in conditioned medium from HEK293 cells transfected with pcDNA-PTTG as compared to cells transfected with pcDNA plasmid. Addition of MMP-2 blocking antibody (MMP2BA) completely blocks the collagenase activity. Data represent the mean ± SEM of experiments performed in triplicate. (* p0.05)

Figure 22


DQ Fluorescein conjugate Collagenase Assay
Fold Increase
3
2.5
2
1.5
1
0.5
0
*
*
pcDNA
PTTG
MMP2 BA
p<0.05

PTTG increases invasion and migration *in-vitro*.

To determine the role of PTTG on invasion and migration through MMP-2, we used stably transfected HEK293 cells seeded on Boyden chamber wells. HEK293 stably transfected with PTTG cDNA cells showed higher invasive index compared to HEK293 stably transfected with vector alone (Figure 23). We also observed a complete inhibition of invasion and migration of cells in medium containing MMP-2 (2μg/ml) blocking antibody (MMP2-Ab) as compare to conditioned medium from pcDNA-PTTG transfected cells, and no effect on invasion with the addition of mouse IgG (2μg/ml) as control.

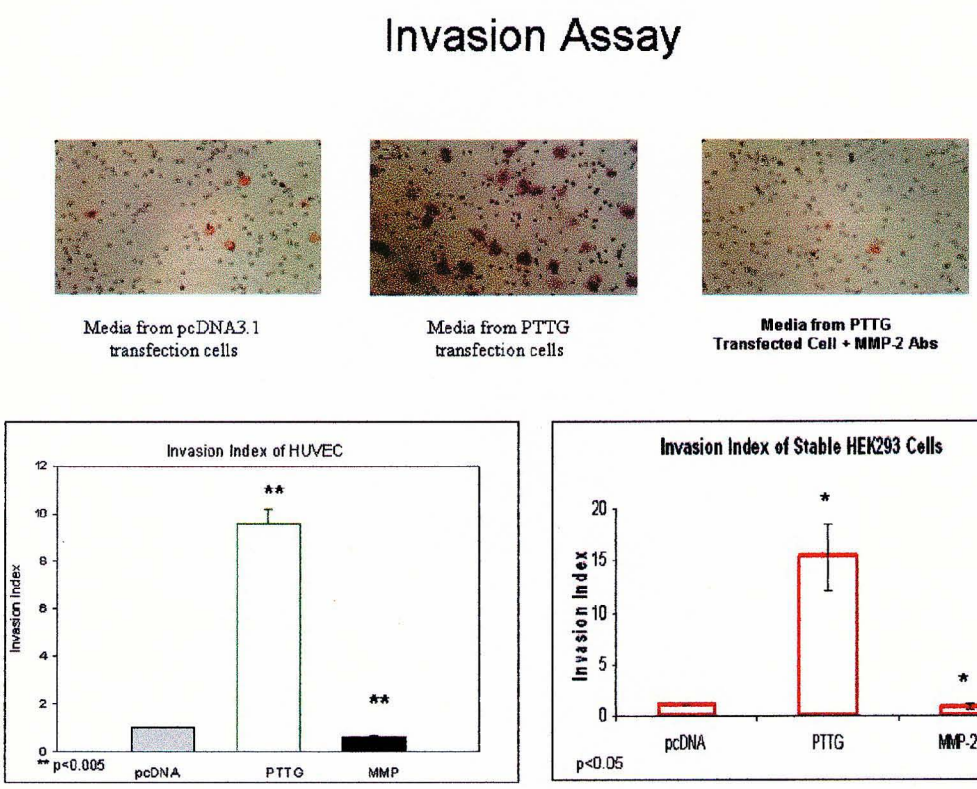
Similar results were obtained using HUVEC cells. Increased invasion and migration was observed in the conditioned medium from HEK293 transfected with PTTG cDNA as compared to conditioned medium from vector transfected cells. Complete inhibition of migration and invasion was observed when MMP-2 (2μg/ml) blocking antibody was used as compared to control or without antibody.

PTTG increases the migration of HUVEC Cells *in-vitro*.

A wound migration assay was performed in 35mm culture plates seeded with HUVEC, and migration was quantified by counting the number of cells that migrated into the non-wounded area using a microscope eyepiece marked

Figure 23: PTTG increased the invasion of HUVEC and HEK293 stably transfected cells. A Matrigel coated Boyden invasion chamber assay was performed to evaluated the invasion of HUVEC and HEK293 stably transfected cells.

Figure 23


Invasion Assay
Media from pcDNA3.1 transfection cells
Media from PTTG transfection cells
Media from PTTG Transfected Cell + MMP-2 Abs
Invasion Index of HUVEC
Invasion Index
12
10
8
6
4
2
0
**
**
** p<0.005
pcDNA
PTTG
MMP
Invasion Index of Stable HEK293 Cells
Invasion Index
20
15
10
5
0
*
*
p<0.05
pcDNA
PTTG
MMP-2 BA

grid (1 mm^2). HUVEC cultures were incubated for 48 h in conditioned media from HEK293 cells transfected with pcDNA3.1 or pcDNA-PTTG. There was a 95% increase in cell migration into non-wound area when conditioned medium from pcDNA3.1-PTTG compared to medium from cells transfected with vector alone (Figure 24). Specific MMP-2 blocking antibody suppressed migration by 87% into the wounded area as compared to conditioned medium from pcDNA-PTTG, but there was no effect on migration with rabbit/mouse IgG (data not shown).

PTTG increases tubule formation and growth in 3D matrigel matrix.

MMP-2 plays an important role in the proteolysis of ECM that allows endothelial cells to migrate toward the angiogenic stimuli and form the blood vessels that nurture tumor development. A HUVEC cell in three dimensional matrigel matrix was mixed with conditioned medium from HEK293 transfected cells with pcDNA or pcDNA-PTTG with or without MMP-2 blocking antibody. As shown in (Figure 25) the tubule formation was enhanced in CM from pcDNA3.1-PTTG as compared to vector alone. Treatment with MMP-2 blocking antibody completely abolished tubule formation, suggesting an important role of MMP-2 in angiogenesis. On the other hand, when stable transfected HEK293 cells were grown on a matrigel matrix, cells which were transfected with vector alone did not grow and remained spherical after 48 hrs (Figure 26), whereas cells transfected with pcDNA-PTTG spread out and started

Figure 24: Conditioned medium from PTTG transfected HEK293 cells increased the migration of HUVE cells as compared to pcDNA transfected HEK293 cells.

Figure 24

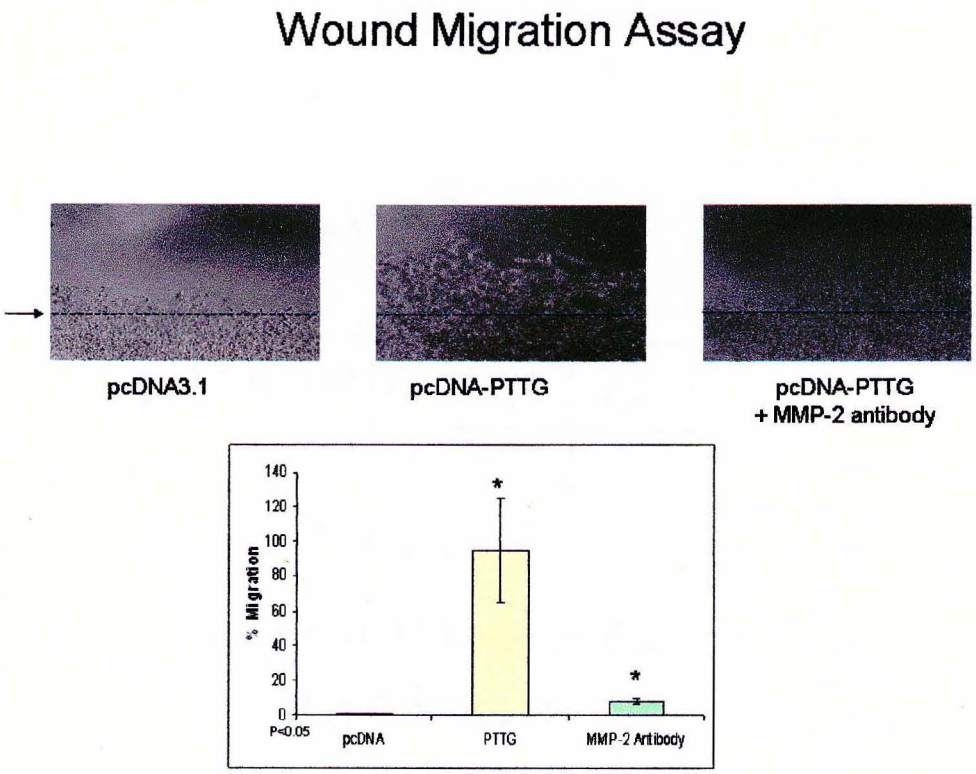
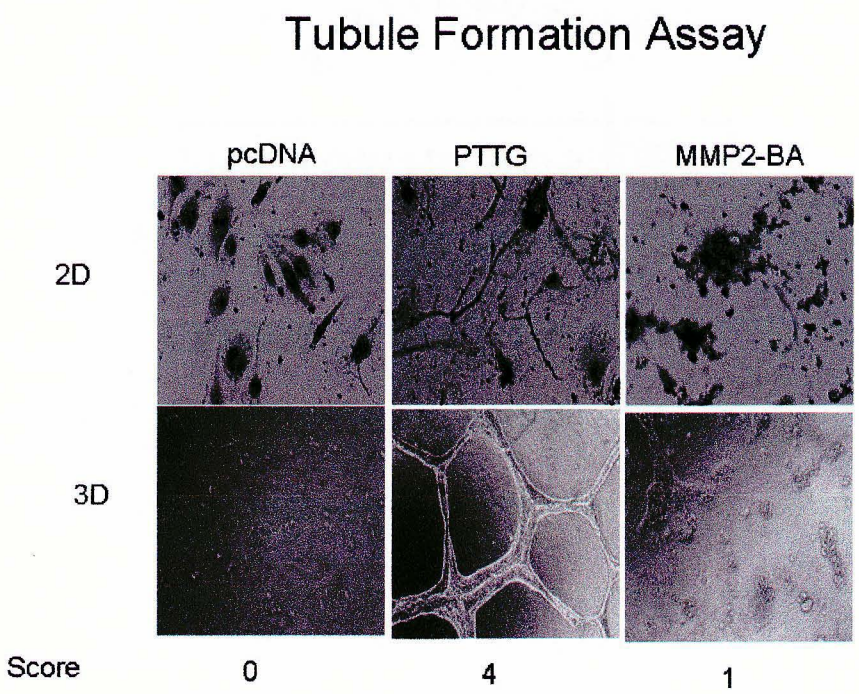

Wound Migration Assay
pcDNA3.1
pcDNA-PTTG
pcDNA-PTTG
+ MMP-2 antibody
% Migration
140
120
100
80
60
40
20
0
P<0.05
pcDNA
PTTG
MMP-2 Antibody
*
*

Figure 25: Tubule formation assay: HUVEC cells were grown in matrigel matrix mixed with conditioned medium collected from pcDNA-PTTG or pcDNA3.1 transfected HEK293 cells, and pcDNA-PTTG + MMP2 blocking antibody (MMP2BA).

Figure 25



growing within 24 h. On the other hand, inclusion of MMP-2 blocking antibodies, completely blocked the growth of these cells. These data suggest a role for MMP-2 in proliferation and growth of cells.

PTTG increases the expression of alpha-5, alpha-V, beta-1 and beta-3 integrins.

Integrins are a diverse family of glycoproteins that form hetrodimer receptors for ECM molecules. The family can form at least 25 distinct pairs of 18 α-subunit and 8 β-subunits, with each other specific for a unique set of ligands. Integrin $\alpha_V\beta_3$ binds a wide range of ECM molecules including fibronectin, fibrogen, vitronectin and MMP, and proteolyse collagen and laminin, whereas $\alpha_5\beta_1$ selectively binds to fibronectin (167). Numerous studies have documented marked differences in surface expression and disregulation of integrins in malignant tumors compared with pre-neoplastic tumor of the same types (168). Integrin $\alpha_5\beta_3$ is strongly expressed at the invasive front of malignant melanoma cells and angiogenic blood vessels (169), and expression of α_V and β_3 integrin increases the metastatic potential of various tumor cell lines (170). As shown in (Figure 27) the mRNA levels of alpha-5, alpha-V, beta-1 and beta-3 are significantly increased in HEK293 cells transfected with pcDNA-PTTG as compared to pcDNA3.1

PTTG increases the expression and secretion of MMP-2 *in-vivo*.

Analysis of MMP-2 expression, secretion, and activity in tumors excised from nude mice overexpressing PTTG confirmed the higher expression of MMP-2 mRNA

(Figure 28) measured by RT/PCR, activity measured by zymography, and DQ fluorescent substrate activity.

3.2.5 DISSCUSSION:

Pituitary tumor transforming gene (PTTG) is a potent oncogene expressed at high levels in almost all malignant tumor analyzed to date. Its oncogeneic potential was demonstrated by its over-expression in mouse fibroblast NIH3T3 and HEK293 cells, which showed increased proliferation, colony formation on soft agar and tumor formation in nude mice (24,27,72). Expression of PTTG has been reported to correlate with highly aggressive and metastastic tumors (33,71), and to regulate the expression of many growth and angiogenic factors such as bFGF, VEGF and IL-8 (72). However, the mechanism by which this aggressive behavior is regulated remains unclear. Metalloproteinases are central to the ability of cancer cells by regulating the extracellular matrix (ECM) around them. They degrade the matrix, and make way for the tumor cells to migrate and metastasize to distance sites. It is well recognized that MMPs are also involved in the expression and activation of various chemokines, growth factors and their receptors (171). Multiple MMPs are involved in the degradation of the surrounding matrix, but MMP-2 has been at the forefront in tumor invasion, angiogenesis, and formation of metastases. Tumor and stromal cells express high levels of MMP-2, which allows the cells to invade and metastasize. However cellular mechanism by which MMP-2 is regulated is not fully understanded. To

Figure 26: Three dimensional growth assay. HEK293 cells were grown in matrigel with or without MMP-2 blocking antibody mixed with conditioned media from pcDNA-PTTG or pcDNA3.1 transfected HEK293 cells.

Figure 26

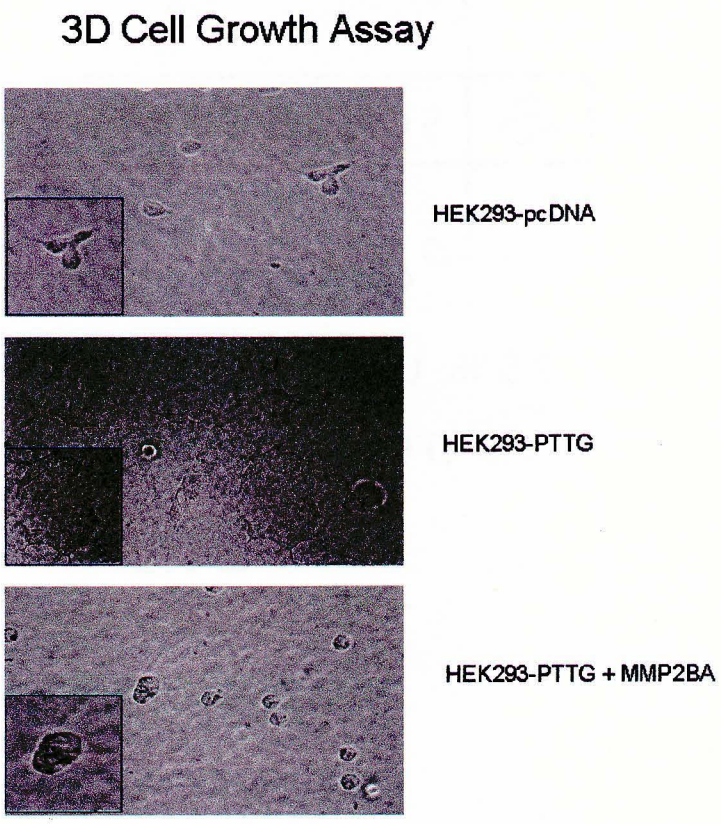
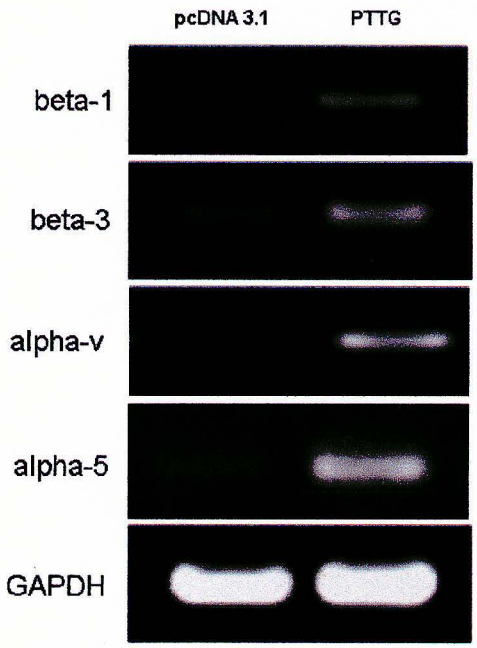

3D Cell Growth Assay
HEK293-pcDNA
HEK293-PTTG
HEK293-PTTG + MMP2BA

Figure 27: Semi quantitative RT-PCR for Integrins and GAPDH. Expression of beta-1, beta-3, alpha-5, and alpha-V was increased in HEK293 transfected with pcDNA-PTTG cells as compared to pcDNA3.1 transfected cells. Densometric analysis of gel showing the expression of integrins in HEK293 cells transfected with pcDNA-PTTG or pcDNA3.1.

Figure 27


pcDNA 3.1
PTTG
beta-1
beta-3
alpha-v
alpha-5
GAPDH

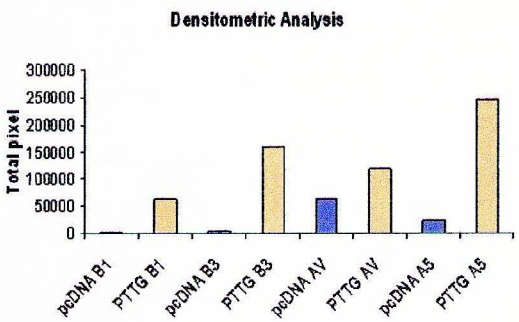
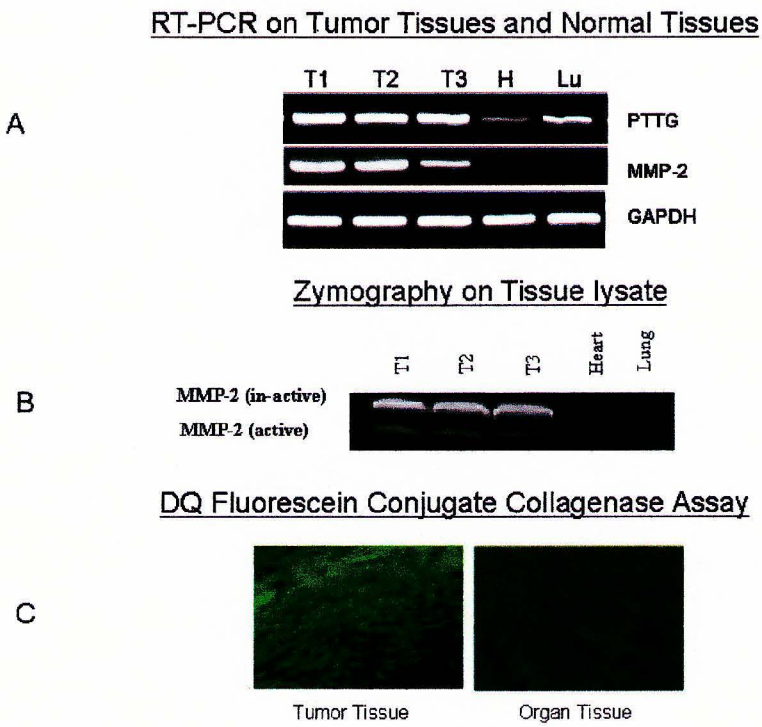

Densitometric Analysis
Total pixel
300000
250000
200000
150000
100000
50000
0
pcDNA B1
PTTG B1
pcDNA B3
PTTG B3
pcDNA AV
PTTG AV
pcDNA A5
PTTG A5

Figure 28: Expression of PTTG and MMP-2 in tumor excised from nude mice. A: Semi-quantitative RT-PCR. B: Zymography. C. *In-situ* fluorescence collagenase activity.

Figure 28


RT-PCR on Tumor Tissues and Normal Tissues
A
T1
T2
T3
H
Lu
PTTG
MMP-2
GAPDH
Zymography on Tissue lysate
B
T1
T2
T3
Heart
Lung
MMP-2 (in-active)
MMP-2 (active)
DQ Fluorescein Conjugate Collagenase Assay
C
Tumor Tissue
Organ Tissue

investigate the interrelation between PTTG and MMP-2 in angiogenic and invasiveness of tumor cells, we selected HEK293 cells. Stable overexpression of PTTG in HEK239 increased the expression and secretion of MMP-2, which in turn increased invasion, migration, and tubule formation of HUVEC cells treated with conditioned medium. Treatment with specific MMP-2 blocking antibody inhibited migration, invasion, and tubule formation of HUVEC cells, suggesting that MMP-2 is crucial in migration, invasion, and angiogenesis. Many studies have implicated MMP-2 in tumor angiogenesis and metastases (172-174). Itoh et al reported a significant reduction in angiogenic activity and subsequent tumor progression in MMP-2 deficient mice (141). Others have shown that MMP-2 is required for tumor angiogenesis and metastases (158,175). As cancer cells become metastatic and endothelial become angiogenic, they develop altered affinity for their extracellular matrix. Some of these changes are mediated by alteration in the expression of cell-surface molecules known as integrins. Integrins are involved in regulating the activities of proteolytic enzymes that degrade the basement membrane, the initial barrier to surrounding tissue, by directly mediating adhesion to ECM, and also by regulating intracellular signaling pathways that control cytoskeleton organization. As PTTG is involved in regulation of cell cytoskeleton (45) these changes could occur through the expression of different integrins on the cell surface, as we have showed that integrin α_V, α_5, β_1 and β_3 expression is measured by overexpression of PTTG in HEK293 cells. Integrin α_V and β_1 have been impicated in the regulation of metalloproteinases MMP-2 and MT1-MMP with whom it forms a complex in highly invasive phenotypes of metastatic tumors (176,177). In previous studies, we (72)

showed that PTTG stimulates the expression of bFGF, VEGF and IL-8, which may induce the expression of MMP-2 (178). Overexpression of PTTG in HEK293 cells may have autocrine effects on the expression of MMP-2 initiated by growth factors. However, the studies done by Hyuga et al (179) showed autocrine factors were not responsible for the upregulation of MMP-2 in highly metastastic cell line. Tumors excised from nude mice also displayed higher expression and collagenase activity when compared to normal organs from the same animals, except in the liver and kidney which express MMP-2 for their normal function (180,181).

In summary, our results provide evidence that PTTG contributes to cell migration, invasion and angiogenesis by induced of MMP-2 expression and secretion. Further, we showed that tumors from nude mice have high expression and secretion of MMP-2 which contributed to high gelatinolytic activity. However, due to the expression of other contributing growth factors we are unable to show autocrine effect on MMP-2 expression and secretion. Further studies are needed to separate the autocrine effect of growth factors on MMP-2 expression.

Chapter IV

Regulation of Matrix Metalloproteinase-2 (MMP-2) in human tumor cells lacking Pituitary Tumor Transforming Gene (PTTG -/-).

INTRODUCTION

Tumorigenesis is a multistep process. Abnormal gene expression is associated with several characteristics that differentiate tumor cells from normal cells, including alterations in cell differentiation, invasion, migration, metastasis, angiogenesis and apoptosis. Over-expression of human pituitary tumor transforming gene (PTTG) in mouse fibroblast NIH3T3 cells and human embryonic kidney (HEK239) cells results in increased cell proliferation, induction of the cell foci formation and promotion of tumor formation in nude mice (24,26,27,72). These finding suggest that PTTG is a potent human oncogene. Data from our laboratory and others suggested that PTTG may act through bFGF, VEGF, IL-8 and MMP-2 to achieve cellular transformation leading to tumorigenesis.

Local invasive growth is a key event of primary malignant tumors. A correlation between the expression levels of PTTG and invasiveness and degree of

malignancy in pituitary, colorectal and thyroid tumor has been demonstrated (33,35,36). However, specific mechanisms facilitating the invasive behavior of cells expressing PTTG remains unclear. Interaction between cancer cells and surrounding normal cells, involving extra-cellular matrix (ECM) are thought to be the key to tumor cell invasion (182,183). To invade and metastasize into the surrounding normal tissues, tumor cells must degrade multiple elements of the ECM, including fibronectin, laminin, and type IV collagen (183). Several metalloproteinases (MMPs) are required for the degradation of the ECM and these are classified according to their substrate specificity. Gelatinases (MMP-2 and MMP-9) are the most common MMPs found in tumors, and elevated levels of these enzyme have been reported in various cancers (183). MMP-2 is also known to be involved in tumor angiogenesis through degradation of extracellular matrix which can result in tumor cell as well as endothelial cell migration due to loss of cell-matrix and cell-cell contacts (145). MMP-2 is also capable of releasing growth factors from ECM, cleaving growth factor receptors and activating growth factors excreted as pre-pro-enzymes, such as transforming growth factors (TGFα, TGFβ), macrophage-colony stimulating factor (M-CSF), insulin like growth factor (IGF) and fibroblast growth factor receptor (FGFR) (146-148).

As discussed in the chapter III, PTTG regulates the expression and secretion of MMP-2 in HEK293 cells. Recent data from our laboratory Kakar et al; (184) and others (70,86-88) showed that attenuating PTTG expression using siRNA, antisense oligos or adenovirus mediated transfer of siRNA against PTTG, inhibit tumor growth

of ovarian, lung and hepatoma cancer cell lines *in vitro* and *in vivo*. None of these studies examined the metastastic or invasive properties of these tumors. The aim of this study is to utilize cancer cells genetically lacking PTTG gene to determine its effect on tumor invasiveness and determine the role of MMP-2 in such regulation. We hypothesized that deletion of PTTG will reverses the invasive phenotype by decreasing MMP-2 expression and secretion. To support our hypothesis we used human colorectal tumor cell line HCT116 lacking PTTG (PTTG -/-), and wild type HCT116 (PTTG +/+) cells as control. These cell lines were a generous gift from Dr. Vogelstein (John Hopkins Oncology Center and Howard Hughes Medical Institute. Baltimore. Maryland) (89).

4.2 MATERIAL AND METHODS:

Cell cultures

Human colorectal cancer cells lines were obtained from Dr. Bret Vogelstein (The John Hopkins Oncology Center and Howard Hughes Medical Institute. Baltimore Maryland) (89) and propagated as described elsewhere (185,186).

Western Blot:

Cells growing in log phase were lysed in chilled lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF] supplemented

with Complete Mini Protease Inhibitor tablets (Roche Molecular Biochemicals, Indianapolis, IN). Equal amounts of protein extracts (40 ug) were resolved on 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ). Blots were probed with PTTG antiserum at a dilution of 1:1,500 as described previously (124). Immunoreactive proteins were visualized using the Enhanced Chemiluminescent Detection System (Amersham) according to the instructions provided by the supplier.

Reverse Transcription Polymerase Chain Reaction (RT-PCR):

Total RNA from cells was purified using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The RNA pellets were resuspended in RNase-free water, and contaminating DNA was removed from the preparations with DNaseI. The yield of total RNA was measured using a spectrophotometer, and the quality was assessed by electrophoresis through a 1% agarose gel. First strand cDNA was synthesized using the iScript™ cDNA synthesis kit (BioRad, Hercules, CA). PCR primers were designed, based on the human PTTG, MMP-2 and GAPDH cDNA sequences. The PCR conditions for each gene are listed in Table 1 & 2. GAPDH amplification was used as an internal control. Ten μl from a total of 50μl PCR reaction mix was applied to a 2% agarose gel. After electrophoresis the gel was stained with ethidium bromide to visualize PCR products. The densitometric values for the PCR-amplified products were quantified using BioRad software and normalized against the GAPDH values.

Zymography:

Secretion and activity of MMP-2 in conditioned medium collected from cells was used to perform gelatin zymography analysis as described by Heussen et al (159,160). Briefly conditioned medium was collected and one ml of medium was lyophilized and reconstituted to 100 µl with water. Protein was determined by Bradford method (161) and 20 µg of protein from each sample was loaded on polyacrlyamide gel containing 0.1% gelatin Sigma Co. (St. Louis, MO). Electrophoresis was performed under non-reducing conditions at 20 mA for 3 hr at room temperature. The gel was washed twice for 30 min each in 2.5% Triton X-100 to remove SDS, incubated in substrate buffer (50 mM Tris-HCl, 5mM $CaCl_2$, 0.01% NaN_3, pH 7.6) for 24 h at 37°C. The gel was stained with 0.5% Coomassie brilliant blue G-250 (Pierce Rockford, IL) for 30 min at room temperature, and destained in the destaining buffer (30% ethanol, 10% acetic acid, deionized water). The presence of metalloproteinases (MMP-2) was indicated by an unstained proteolytic zone of substrate. The gel was scanned using NIH image software, and data are presented as fold change relative to control i.e, HCT116 WT-conditioned medium.

Detection of collagenase activity in conditioned medium and tissue sections.

Collagenase activity in conditioned medium (CM)

Twenty microliters of collagen fluorescent substrate reconstituted in PBS was mixed with 80 µl of reaction buffer (0.5 M Tris-HCL, 1.5 M NaCl, 50 mM $CaCl_2$, 2

mM NaN_3, pH 7.6) and added to the fluorescent reader plate. Twenty µl of conditioned medium was added to each well. Clostridium collagenase, serially ten fold serially diluted used as a positive control and 100 µl of reaction buffer was a negative control. The plate was incubated at room temperature for 1 h. After incubation, the fluorescent intensity of the digested product from DQ collagen was measured by excitation at 495 nm and emission at 515 nm in the fluorescent microplate reader. The fold change in activity was calculated by dividing the mean of fluorescence values of conditioned media collected from HCT116 WT by the mean value of fluorescence of conditioned media collected from HCT116 Null (PTTG-/-) cells.

Luciferase Assay:

A human MMP-2 promoter (1959 bp upstream from transcription start site) luciferase reporter construct (pGL-MMP2) was obtained from by Dr. Etty Beneveniste, University of Alabama at Birmingham, AL (166). Five hundred ng of the MMP-2 promoter construct or pGL2 was co-transfected with 100 ng of a renilla construct into 3 x 10^6 HCT116 WT and HCT116 Null (PTTG-/-) cells using Transfectin (Bio-Rad). After transfection, cells were allowed to recover for 18 h and then cultured in 10% FCS/DMEM for 24 h. Cells were washed in PBS and lysed with 500 µl of 1X lysis buffer (Promega). Luciferase activity was determined in triplicate as described by manufacturer. The luciferase activity of each sample was normalized to renilla activity to determine the relative luciferase activity (RLA). The fold change

in RLA was calculated by dividing the relative luciferase activity of HCT116 WT-pGL-MMP2 promoter activity by HCT116 Null-pGL-MMP-2 luciferase activity.

Migration Assay:

HCT116 WT and HCT116 Null (PTTG-/-) cells were cultured in McCoy's medium containing 100 ml/L FBS and 10 g/L penicillin-streptomycin in a 50 ml/L CO_2 incubator at 37°C. Assessment of HCT116 migration was performed as recently described (187) with minor modifications. HCT116 cells were dispersed into homogeneous single cell suspensions after trypsinization. These cells were extensively washed with McCoy medium containing 1 g/L fatty acid-free BSA and resuspended in the same medium. HCT116 cells (10^5) were dispersed onto the upper chamber of transwell compartment with an 8 μm pore size filter (QCM Cell Migration Assay, Chemicon. USA). The cells were allowed to adhere for 1 h at 37°C. The medium in the lower chamber was removed and replaced by growth medium alone. Migration was allowed to proceed for 24 h at 37°C. The remaining cells attached to the upper surface of the filters were carefully removed with cotton swabs. Migrated cells were stained with crystal violet and examined by light microscopy and photographed. The number of migrated cells was quntitated by dissolving stained cells in 10% acetic acid and transfering a 100 μl of dye/solute mixture to a 96 well plate for colorimetric reading at at 560 nm. Data were expressed as mean ± SD of the three independent experiments.

Invasion Assay:

HCT116 WT and HCT116 Null (PTTG-/-) cells were cultured in McCoy medium containing 100 ml/L FBS and 10 g/L penicillin-streptomycin in a 50 ml/L CO_2 incubator at 37°C. Assessment of HCT116 migration was performed as recently described above (187-189).

Percent Invasion:

Percent invasion was calculated by dividing mean of cell invaded through matrigel insert membrane by mean of cells migrating through control insert membrane and multiplied by 100.

Statistical Analysis:

Statistical comparison of data sets was carried out by the Student's t test, and by one-way ANOVA for multiple sets using SPSS v 10.

4.3 RESULTS:

Loss of PTTG expression in HCT116 (PTTG -/-) correlates with the expression of MMP-2.

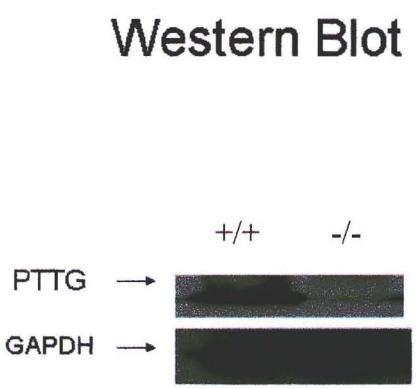
Over-expression of MMP-2 correlates to an invasive phenotype in several cancer types and is often predictive of poor survival (140). MMP-2 null mice display

lower lung colonization following intravenous administration of cancer cells and reduced tumor angiogenesis resulting in reduced tumor growth (141). PTTG regulates the MMP-2 expression in the HEK293 cell line, which increases the invasive behavior of these cells. To determine the functional effect of PTTG on MMP-2 regulation, colorectal cancer cell lines lacking PTTG gene HCT116 (PTTG -/-) and its control HCT116 wild type (PTTG +/+) were used.

To test the validity of HCT116 (PTTG +/+ and -/-) cell lines as a model system, western blot analysis for PTTG was carried out. As shown in (Figure 29), expression of PTTG protein in HCT1116 (PTTG -/-) was undetectable, whereas HCT116 (PTTG +/+) cells showed high levels of PTTG protein. Further, to demonstrate an effect of PTTG on MMP-2 gene transcription, MMP-2 promoter activity was measured in HCT116 (PTTG +/+) and HCT116 (PTTG -/-) cell lines.

Figure 29: Western blot analysis of HCT116 wild type and HCT116 (PTTG -/-) cells showing PTTG and GAPDH protein expression.

Figure 29



A 1659 bp DNA fragment containing human MMP-2 gene promoter cloned into the pGL3 (Promega) vector was co-transfected with renilla vector. After 24 h growth medium was changed to serum-free medium. 24 h after the change in medium, the cells were lysed and assayed for luciferase activity as described previously (80). As shown in (Figure 30) MMP-2 promoter activation was increased by ~8 fold higher ($p<0.05$) in HCT116 (PTTG +/+) compared to the HCT116 (PTTG -/-) cell line. Similar results were obtained by the semi-quantitative reverse transcription (RT-PCR) analysis of messenger RNA of PTTG and MMP-2. As shown in (Figure 31), the level of PTTG mRNA was higher in wild type HCT116 cells but undetectable in HCT116 (PTTG -/-) cell line. Similarly, the mRNA level of MMP-2 was higher in HCT116 (PTTG +/+) compared to HCT116 (PTTG -/-) cells. As described in chapter III, over-expression of PTTG increases the expression of MMP-2 in HEK293 cells. To confirm our results of MMP-2 we measured MMP-2 secretion and activity in HCT116 cells. DQ-collagenase and zymography assays. As shown in (Figure 32), there was substantially less secretion of MMP-2 by the HCT116 (PTTG -/-) cell lines as compared to HCT116 (PTTG +/+) cell line. Furthermore, the collagenolytic activity of MMP-2 was reduced by 30% in medium from HCT116 (PTTG -/-) as compared to HCT116 (PTTG +/+) cells.

Figure 30: Luciferase assay of MMP-2 promoter transfected in HCT116 wild type and HCT116 (PTTG -/-) cells. ** $p<0.005$

Figure 30

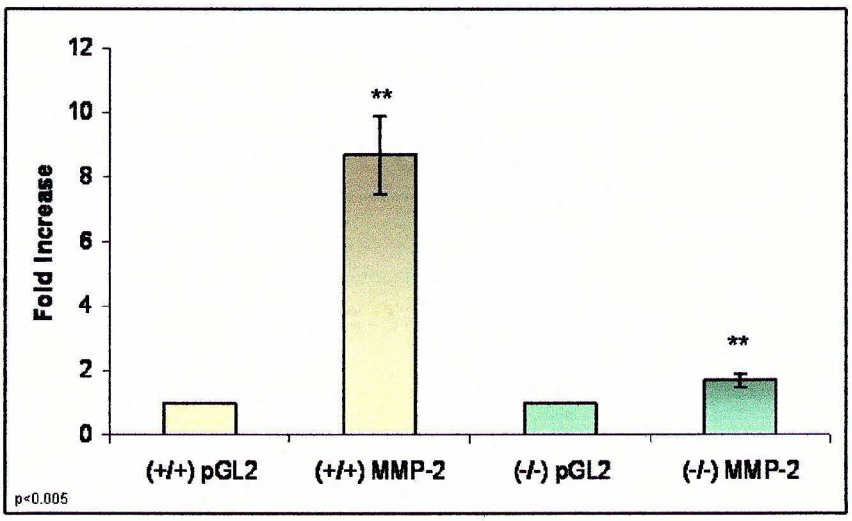


Figure 31: Semi-quantitative RT-PCR for PTTG, MMP-2 and GAPDH in HCT116 (PTTG +/+) and HCT116 (PTTG -/-) cells.

Figure 31

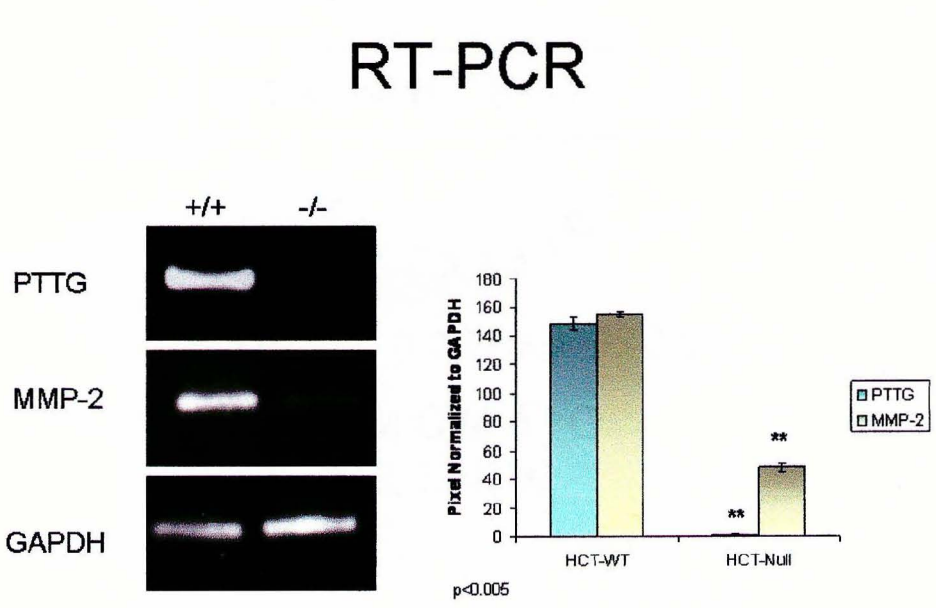
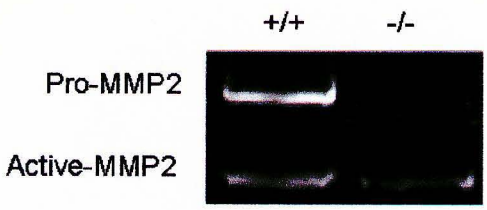

RT-PCR
+/+
-/-
PTTG
MMP-2
GAPDH
Pixel Normalized to GAPDH
180
160
140
120
100
80
60
40
20
0
**
**
HCT-WT
HCT-Null
PTTG
MMP-2
p<0.005

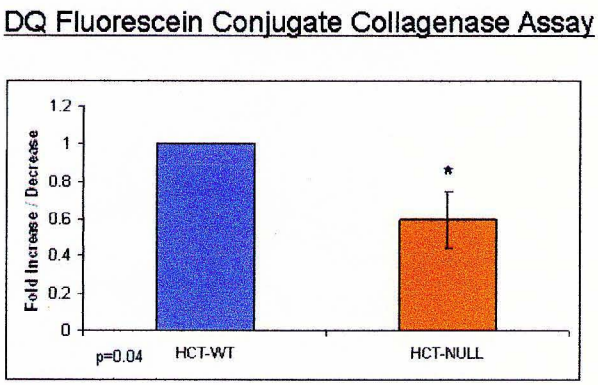
Figure 32: Zymography and Collagenase assay of conditioned media collected from HCT116 wild type (PTTG +/+) and HCT116 (PTTG -/-) cells

Figure 32

A


+/+
-/-
Pro-MMP2
Active-MMP2

B


DQ Fluorescein Conjugate Collagenase Assay
Fold Increase / Decrease
1.2
1
0.8
0.6
0.4
0.2
0
*
p=0.04
HCT-WT
HCT-NULL

Decrease in MMP-2 expression reduces the invasive phenotype of HCT116 (PTTG -/-) cell line.

A relationship between PTTG expression and metastasis has been reported (33,68) in limited studies in colorectal and pituitary adenocarcinomas. As mentioned earlier, MMP-2 is thought to have a prominent role in tumor cell invasion and is increased by PTTG. We next tested if PTTG stimulated the expression of MMP-2, the invasive behavior of HCT116 (PTTG +/+) and HCT116 (PTTG -/-) cells. The Boyden chamber matrigel invasion assay were used to measure the invasive index of both cell lines. As shown in (Figure 33 A) there was a significant decrease in both invasion and migration of HCT116 (PTTG -/-) cells as compared to HCT116 wild type. There was also a ~39 % reduction in invasion of HCT116 (PTTG -/-) compared to HCT116 (PTTG +/+) (Figure 33 B). These results suggest that PTTG modulates the expression of MMP-2 in mediating its tumorigenic function.

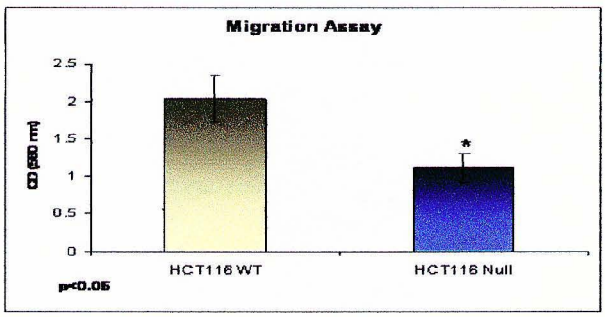
4.4 DISCUSSION

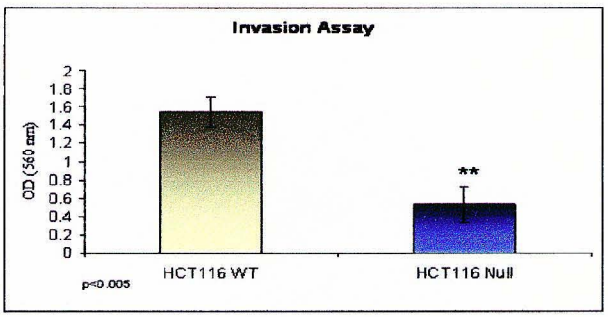
In this chapter it is shown that PTTG plays an important role in regulating the expression of MMP-2. Deletion of the PTTG gene from the HCT116 cell line is associated with a reduction of MMP-2 expression and decreased invasive capacity of this cancer cell line. These finding are consistent

Figure 33: Invasion and migration assay using HCT116 wild type and HCT116 (PTTG -/-) cells. Percent invasion was calculated as mentioned in material and methods.

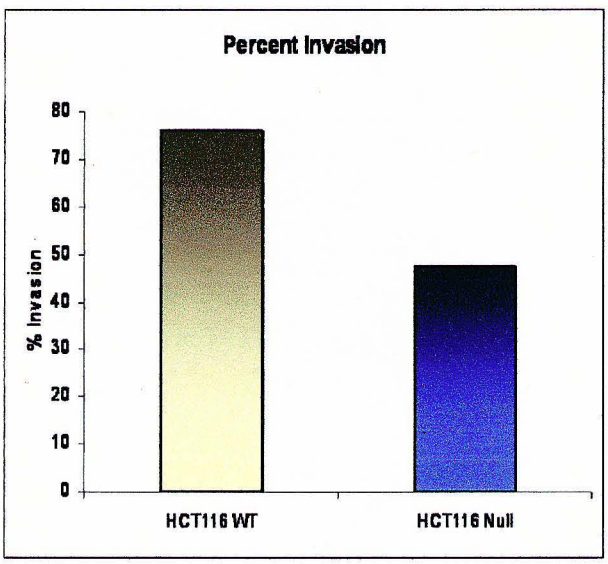
Figure 33

A


Migration Assay
2.5
2
1.5
1
0.5
0
*
HCT116 WT
HCT116 Null
p<0.05


Invasion Assay
OD (560 nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
HCT116 WT
HCT116 Null
p<0.005

B


Percent Invasion
% Invasion
80
70
60
50
40
30
20
10
0
HCT116 WT
HCT116 Null

with the previous results from our group using siRNA against PTTG (184) and others using PTTG full length anti-sense mRNA (88), or oligonucleotides against PTTG mRNA (190), reduced colony formation on soft agar, and suppression of tumor development in nude mice. PTTG is over-expressed in most types of cancer and plays an important role in tumor angiogenesis, metastasis and growth (24,25,33,71,72,124,151). Over-expression of PTTG has been shown to induce cellular transformation and to promote tumor development in nude mice (72). MMP-2 plays an important role in local invasion, angiogenesis and growth. A correlation between the levels of expression of PTTG with increased tumor invasiveness and degree of malignancy in pituitary and colorectal tumors have been demonstrated (33,68). Melmed et al (85) showed that when the PTTG gene is ablated animals exhibited impaired proliferation of pancreatic β-cells and developed type I diabetes during late adulthood, suggesting the importance of PTTG in β-cell proliferation. These results are in agreement with Perez et al (191) showing that MMP-2 is required for pancreatic islet cell formation (191), and other studies of diabetes and β-cell dysfunction where MMP-2 expression is impaired (192). These results suggest that PTTG null animals have impaired pancreatic β-cell proliferation and development of diabetes may be due to impaired MMP-2 expression.

MMP-2 activity was still observed in PTTG -/- cells line, suggesting that PTTG is not the only factor which can lead to the regulation of MMP-2 (193-196). On the other hand, PTTG ablation in HCT116 may change the phenotypic characteristic

of the cells during positive selection, and other genetic or biochemical alterations which might have occurred that lead to cell survival cells without PTTG.

In conclusion, PTTG expression correlates with the expression of MMP-2 in HCT116 cell lines. Depletion of PTTG from HCT116 (PTTG -/-) decreased the transcription of MMP-2, which in-turn decreased the expression, secretion, and activity of MMP-2. These together effect cell migration and invasion characteristics of HCT116 cells. The data suggest that PTTG is a potent oncogene that may act through bFGF, VEGF, IL-8 and MMP-2 to induce transformation, cell proliferation, angiogenesis, invasion, and metastasis. MMP-2 is crucial in all steps of tumorigenesis; blocking MMP-2 activity can inhibit the multistep process of tumorigenesis.

Chapter V

CONCLUSIONS AND DISCUSSION

Pituitary tumor transforming gene (PTTG) is a relatively recently isolated proto-oncogene that has been identified as a mammalian securin protein implicated in chromatid separation during cell division (45). Expression of PTTG is cell cycle-dependent both at the protein and mRNA levels (45,47,66). By virtue of mammalian securing activities, PTTG over-expression inhibits of chromatid separation which may lead to genetic instability, aneuploidy and thereby tumorigenesis (45) The oncogeneic function of PTTG was established by over–expressing the protein in mouse fibroblast cell line (NIH3T3) and assessing its ability to induce cellular transformation and tumor formation in the nude mice (24,27). Expression of PTTG in NIH3T3 cells increaseds bFGF and VEGF expression thereby leading to tumorigenesis and angiogenesis *in vitro* and *in vivo* (35,90). bFGF and VEGF are major activating factors in mitogenesis and angiogenesis (90,91). Thus, PTTG may play a role in tumor progression and angiogenesis.

There are numerous instances in which overexpression of oncogenes induce transformation rodent cell lines, but have failed to transform the same cells type from humans (96). It is now widely accepted that human tumorigenesis is a multistep process and requires activation of more than one oncogene (197).

The goal of this study was three fold. First, to determine whether PTTG can induce the transformation of normal human cells; second, to determine if PTTG is sufficient to induce transformation, and third, to characterize the changes in secretion and expression of the key angiogenic and metastatic factors bFGF, VEGF, IL-8, and MMP-2. To address our hypothesis and to investigate the role of PTTG in tumorigenesis, I used the human embryonic kidney cell line (HEK293). This cell line has been transformed by human adenovirus type 5 to prevent senescence (112) and HEK293 cells displays moderate tumorigenic potential (113).

Our data demonstrate that overexpression of PTTG in HEK293 cells increases cell proliferation and induces cellular transformation in vitro as demonstrated by colony formation on soft agar and tumor growth in nude mice. By contrast, cells transfected with pcDNA 3.1 vector as control did not cause colonies of tumor cells to grow in soft agar nor did they promote the development of tumors when implanted in nude mice. These results are in agreement with the previous studies (24,27) that demonstrated an effect of PTTG to transform NIH3T3 mouse fibroblast cells. Second, demonstrated that PTTG alone is sufficient to induce transformation of primary human cells without the cooperation of other oncogenes to achieve its tumorigenic function.

Often, a single oncogene is not sufficient for the induction of transformation, but requires co-operation of other oncogene(s) to induce tumorigenesis in primary human cells (96,116,118,119,134,198). However, HEK293 cells are transformed by adenovirus type 5, have achieved the limitless growth advantage over their normal counterpart. Our data suggest that PTTG is sufficient by itself to initiate the transformation of HEK293 cell line, and we have demonstrated that PTTG over-expression in HEK293 cells accelerates their tumorigenic capacity compared to non-transfected or vector transfected HEK293 cells. PTTG contains two PXXP motif in the proline rich region near C-terminal identified as SH3-binding site (27); these sites are critical as mutation of these regions causes the loss of transforming and transactivating activity of PTTG. Our data clearly demonstrate that the mutation of these sites abrogates the tumorigenic function of PTTG in human cells. The question arises that the loss of tumorigenic function of PTTG may be due to loss of expression due to mutation, but western blot analysis of a stable clone demonstrated constitutive expression of mutated PTTG protein in HEK293 cells. This suggests that the loss of tumorigenic function of mutated PTTG clones was not due to loss of expression but loss of its ability to induce cellular transformation. Consistent with the results of other investigators using rodent cells (27,55), we confirmed the importance of the C-terminal proline-rich motif in mediating the oncogenic function of PTTG.

The mechanism of tumorigenesis by which PTTG induces cell transformation remains unclear. Apart from the role its cell division, PTTG regulates bFGF expression (27). bFGF has previously been implicated in the growth and development

of other tumors (75) and the growth of tumors depends mainly on adequate vascularization (91). VEGF and IL-8 have also been shown to increase tumor vascularization and migration of endothelial cells (108,199,200). Consistent with the hypothesis that PTTG may promote tumorigenesis by upregulation of angiogenic factors; our data clearly show significant increases in the expression and secretion of bFGF, VEGF and IL-8 when PTTG is over-express in HEK293 cells, suggesting, a role of PTTG in angiogenesis and tumor promotion. This notion recently raised the question that PTTG may form collaborate with other genes to activate the angiogenic switch in tumor initiation and progression. McCabe et al (78) investigated several angiogenic genes regulated by PTTG in primary thyroid cells. Result from cDNA microarray analysis of angiogenic factors showed upregulation of several angiogenic promoter genes, including inhibitor of DNA binding-3 (ID_3), and down-regulation of inhibitors of the angiogenesis gene thrombospondin-1 (TSP-1), suggesting that PTTG may play an important role in the angiogenesis.

For a tumor to grow successfully and metastasize, it requires a continuous supply of nutrient and oxygen. Without angiogenesis tumors cannot grow larger than few mm^3 (91). One of the early steps in tumor angiogenesis is degradation of ECM catalyzed by MMPs that enable endothelial cells to migrate and proliferate to form new vessels (201,202) and to metastasize, by facilitating the movement of tumor cells from primary site, to distant organs. Matrix metalloproteinases (MMPs) play a key role in this processes by degrading the extracellular matrix and controlling the biological activities of growth factors, chemokines and cytokines to favor tumor

angiogenesis and metastasis (98). Our data clearly indicate that over-expression of PTTG in HEK293 cells increases the expression and secretion of MMP-2. This increase of MMP-2 activity is due to an increase in at both mRNA and protein levels as demonstrated by MMP-2 promoter activity and zymography analysis. MMP-2 involved in tumor angiogenesis, mainly through it degradative capacity, and can result in tumor cell and endothelial cell migration due to loss of cell-matrix contacts and cell-cell contact (145,156,174). Our data indicates that the increase in expression and secretion of MMP-2 by PTTG over-expression enhances the invasion and migration of both HEK293 and endothelial cells. Increases in MMP-2 secretion also promote the tubule formation of HUVEC cells in 3D collagen matrix that is analogous to angiogenesis. By using a specific inhibitor that inhibits MMP-2 activity, we showed a decrease in MMP-2 activity and loss of its ability for invasion, migration and tubule formation of HUVEC, suggesting a specific role of MMP-2 in angiogenesis and cell invasion.

Tumors developed in nude mice show increased expression of MMP-2 mRNA and MMP-2 activity, compared to other tissue from same animals. MMP-2 is secreted as a latent pro-MMP-2 form, for the activation of MMP-2 it requires a active interaction of another class of membrane bound metalloproteinase know as MT-1-MMP and tissue inhibitor of metalloproteinases-2 (TIMP2) (139). A balance between the concentration of MMP-2 and TIMP-2 is needed for the activation of MMP-2 at cell surface (203). Our data clearly demonstrate an increase in the expression of MT1-MMP and decreased expression of TIMP-2 in the cells over-expressing PTTG

compared to cells transfected with vector. These data indicate that PTTG not only increases the expression of MMP-2 but also regulates the activity of MMP-2 by altering the expression of MT-1-MMP and TIMP-2.

PTTG over-expression has been reported in various tumors, including pituitary (35), thyroid (69,154), colon (33), ovary and breast (25). In thyroid, pituitary, oesophageal and colorectal tumors, high PTTG expression correlates with tumor invasiveness (27,33,71,154). Furthermore, PTTG has recently been identified as a key metastatic "signature gene" with high expression in multiple tumors (36). It remains unknown whether PTTG is essential for regulating angiogenesis and metastasis by regulating MMP-2 and whether deletion of PTTG will reverse the cancer phenotype by decreasing the expression and secretion of MMP-2. Data from our studies show that deletion of PTTG from a human colorectal cancer line HCT116 (PTTG -/-) decreased expression and secretion of MMP-2. This decrease was associated with decreased potential of HCT116 (PTTG -/-) cells to invade through matrigel compared to wild type HCT116 (PTTG +/+) cells. Consistent with the results of Kakar and Malik (184) and Hassanain et al (unpublished data) and others (70,86,88) using siRNA or oligonucleotides method to decrease the expression of PTTG in tumor cells resulted in decrease cell proliferation, increase in apoptotic figures, decrease in number of colonies formed on soft agar, and inhibition of tumor formation in nude mice.

In conclusion I demonstrated that PTTG is a potent oncogene, its over-expression in human cells can induce cellular transformation by increasing the cell

proliferation, enhancing colony formation in soft agar *in-vitro* and, promote tumor formation on injection human cells transfected with PTTG cDNA in nude mice. The possible mechanism by which PTTG induces angiogenesis, invasion and tumorigenesis is by increasing the expression of growth factors such as bFGF, VEGF, IL-8 and MMP-2. Depletion of PTTG from the HCT116 cells appears to abrogate its possible role in tumorigenesis by suppression of MMP-2 expression.

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ABSTRACT AND POSTER PRESENTATION:

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