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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF A NEWLY IDENTIFIED HUMAN MT5-MMP TRANSCRIPT VARIANT ISOLATED FROM MULTIPOTENT NT2 CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

ABP	AMPA Binding Protein
AD	Alzheimer's Disease
ADAM	A disintegrin and matrix metalloprotease
ADAMTS	ADAM with thrombospondin-like repeats
AP-1	Activating Protein-1
AP-2	Adaptor Protein-2
APP	Amyloid Precursor Protein
β-DG	Beta-Dystroglycan
BDNF	Brain Derived Neurotrophic Factor
CD44	Cluster Designation 44, hyaluronan receptor
CNS	Central Nervous System
CSPG	Chondroitan Sulfate Proteoglycan
CREB	cAMP Response Element-Binding
CTAP-III	Chemokine connective tissue activating peptide III
DRG	Dorsal Root Ganglion
DSPG	Dermatin Sulfated Proteoglycan
ECM	Extracellular Matrix
EGL	External Granular Layer
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
GBM	Glioblastoma Multiforme
GPI	Glycosyl-phosphatidylinositol
GRIP	Glutamate Receptor Interaction Protein
HB-EGF	Heparin-Binding Epidermal Growth Factor
HSPG	Heparin Sulfate Proteoglycan
IGL	Internal Granular Layer
IS	Insertion Sequence
LTP	Long Term Potentiation
MINT-3	Munc-18 Interacting Protein
MMP	Matrix Metalloproteinase
MS	Multiple Sclerosis
MT-MMP	Membrane Type Matrix Metalloproteinase
NFkB	Nuclear Regulatory Factor kappa Beta

NGF	Neural Growth Factor
NO	Nitric Oxide
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PDGF-BB	Platelet Derived Growth Factor-BB
PNS	Peripheral Nervous System
RA	Retinoic Acid
RECK	Reversion-inducing cysteine-rich protein with Kazal Motifs
RT	Reverse Transcription
SDF-1	Stromal cell-derived factor 1
TGN	Trans-golgi Network
TIMP	Tissue Inhibitor of Metalloproteases
TNF-alpha	Tumor necrosis factor-alpha
VEGF	Vascular Endothelial Growth Factor

Abstract

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF A NEWLY IDENTIFIED HUMAN MT5-MMP TRANSCRIPT VARIANT ISOLATED FROM MULTIPOTENT NT2 CELLS

By Heather Hamilton Ross, MPT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University School of Medicine.

Virginia Commonwealth University School of Medicine, 2006

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Membrane-type 5 matrix metalloproteinase (MT5-MMP) is unique among MMP family members as it is predominately expressed in the CNS. Its expression is ubiquitous during brain development and restricted to regions of neurogenesis and neuroplasticity in the adult. MT5-MMP is a mediator of pericellular proteolysis and is thought to have a functional impact on neurite outgrowth. The studies presented in this work were designed to examine MT5-MMP expression in cultured NT2 cells, a model of neurogenesis and neuronal differentiation, and in adult neurogenic brain regions. We

further sought to overexpress MT5-MMP and test the hypothesis that it plays a role in substrate-specific cell motility.

MT5-MMP mRNA was expressed in NT2 cells and was significantly higher in differentiated neuronal hNT cells. MT5-MMP cDNA cloned from NT2 cells unexpectedly revealed a novel sequence (MT5-MMPvar) which was 92% homologous with the published MT5-MMP and was characterized by a 162 bp deletion. Both transcripts could be identified in NT2, hNT and adult human hippocampus. The newly cloned MT5-MMPvar cDNA translated into an approximately 52 kDa protein as seen in *in vitro* expression studies. Using an MT5-MMPvar specific antibody designed to span the 162 bp deletion, MT5-MMPvar protein could be detected in NT2 cells and these protein levels increased in their neuronal counterparts, hNT cells. MT5-MMPvar protein was also expressed in adult human hippocampal tissue. MT5-MMPvar protein was shown to be expressed in a murine region of neurogenesis and plasticity, suggesting the existence of a murine homolog of this variant.

Based on bioinformatic analysis, the MT5-MMPvar transcript was predicted to lack a sufficient signal peptide and to remain a Type-I membrane protein. This computer assisted modeling suggests that the most significant functional implication of MT5-MMPvar sequence variations is to affect its direction into the ER for processing.

Functional studies using COS-7 cells genetically modified to overexpress MT5-MMPvar demonstrated no difference in cellular motility compared to parental or vector control cells. Preliminary studies show MT5-MMPvar expression in COS-7 cells associated with perinuclear structures and the cell membrane. Adult human neural

progenitor cells stimulated to differentiate into immature neurons demonstrated MT5-MMPvar expression associated with the cell membrane and process outgrowths.

This work has identified a novel transcript variant of the human MT5-MMP gene and shown that the protein product of this gene is significantly higher in differentiated NT2 cells. This, combined with preliminary results suggesting MT5-MMPvar cellular redistribution in more mature cell types, indicates a role for MT5-MMPvar in neural differentiation and function.

CHAPTER 1

GENERAL INTRODUCTION

Overview

The matrix metalloproteinase family is associated with a variety of physiological and pathological processes. The defining characteristics and protein domains inherent to these family members as well as their regulation and general function are well understood. Several members of the MMP family have been identified in the normal CNS and changes in their expression levels have been characterized in association with a number of CNS insults and pathologies. MT5-MMP is unique among MMP family members in that its expression is largely limited to the CNS and accumulating evidence suggests that MT5-MMP may play a role in neurogenesis and axonal outgrowth.

Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) are a well-characterized family of endopeptidases. The first member was described in 1962 (Gross and Lapiere, 1962) and, to date, 23 family members have been identified. The MMPs are notable for their proteolytic capabilities. The MMPs, along with members of the plasmin system, ADAM

family and ADAMTS family, constitute those proteases with the ability to cleave peptide bonds within insoluble ECM proteins (Nagase, 1997; Somerville et al., 2003). The MMPs, also known as the matrixin subfamily of the larger metalloprotease family, are categorized as metalloproteases due to their dependence on interaction with zinc for enzyme activity. The conserved binding domain, HEXGHXXGXXHS/T found within the catalytic domain of all MMPs, mediates the chelation of a zinc molecule. This conserved zinc binding domain along with a conserved regulatory sequence, PRCGXPD, within the propeptide domain comprises what is called the "cysteine" switch (Van Wart and Birkedal-Hansen, 1990). This proposed model refers to the association of the conserved cysteine residue with the zinc molecule. When the enzyme is in the latent state, the switch is "off" and the cysteine residue is bound to the zinc molecule. Chemical agents such as organomercurials or proteolytic enzymes lead to the disassociation of the cysteine residue from the zinc thereby allowing a water molecule to interact with the zinc resulting in activation (reviewed in Chakraborti et al., 2003).

Protein Domains Found within the Matrix Metalloproteinases

MMP family members are composed of a conserved set of protein domains present in a uniform orientation (Figure 1.1.). All MMP family members contain a minimal domain comprised of the signal, propeptide, and catalytic domains. The signal peptide functions to direct co-translational insertion into the endoplasmic reticular plasma membrane, which ultimately directs the soluble protein to the extracellular space. The propeptide domain contains the aforementioned cysteine switch regulatory

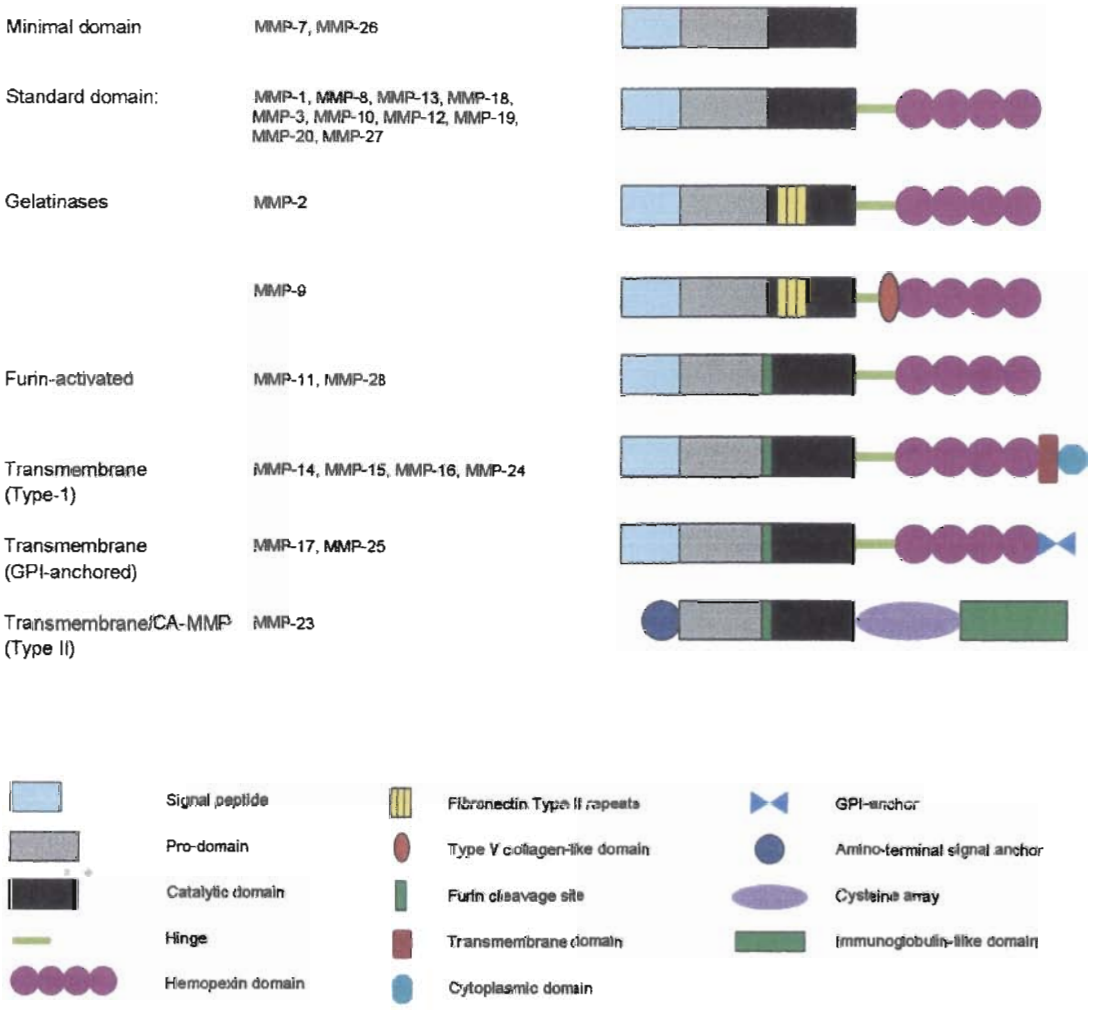


Figure 1.1. Schematic representation of MMP functional domains. Adapted from Somerville et al., 2003.

motif while the catalytic domain houses the previously described zinc-binding region. MMP-7 and MMP-26 contain just the signal, propeptide, and catalytic domains (minimal domain). Other MMP family members contain additional functional domains. All other MMPs (except CA-MMP/MMP-23) contain a flexible hinge domain and a hemopexin domain for substrate recognition. The gelatinases, MMP-2 and MMP-9 contain fibronectin type II repeats and MMP-9 contains a type V collagen binding domain between the hinge and propeptide domains. The furin-activated secreted MMPs contain a furin cleavage site within the propeptide.

Membrane-Type Matrix Metalloproteinases

Most members of the MMP family are processed through the secretory pathway as secreted proteins, destined for the extracellular environment. Members of the Membrane-Type MMP (MT-MMP) subfamily are structurally similar to the secreted MMPs except for the addition of three unique conserved insertion sequences (Reviewed in Fillmore et al., 2001). The first insertion sequence, IS-1, confers a furin or proprotein convertase recognition site between the propeptide and catalytic domain and represents a novel site of activation (Knauper and Murphy, 1998). The second insertion sequence, IS-2, is found 43 residues downstream of the start of the catalytic domain and differs among the MT-MMP group. There is some evidence to suggest that the IS-2 motif plays a role in MT-MMP mediated activation of MMP-2 (reviewed in Fillmore et al., 2001). The third insertion sequence, IS-3, is located in the transmembrane domain of the MT-MMPs. The MT-MMPs associate with the membrane as either a Type-I

Table 1: Structural Components of the Membrane-Type Matrix Metalloproteinases

MMP	Alternate Name(s)	IS1	IS2	IS3	Documented Alternative Transcripts	Type of Membrane Protein
MMP 14	MT1-MMP	Present	Present	Present	Yes	Type I
MMP 15	MT2-MMP	Present	Present	Present	No	Type I
MMP 16	MT3-MMP	Present	Present	Present	Yes	Type I
MMP 17	MT4-MMP	Present	Absent	Absent	No	GPI-anchored
MMP 24	MT5-MMP	Present	Present	Present	Yes	Type I
MMP 25	MT6-MMP/leukolysin	Present	Absent	Absent	No	GPI-anchored
MMP 23	CA-MMP	Present	Absent	Absent	No	Type II

transmembrane protein or as a GPI-anchored membrane protein (Table 1.1.). Type I transmembrane MT-MMPs contain a hydrophobic single-pass transmembrane domain followed by a cytoplasmic tail. The transmembrane domain is variable in size while the cytoplasmic tail contains conserved cysteine residues flanked by tyrosine and serine residues (Knauper and Murphy, 1998). There are reports that demonstrate that the cytoplasmic tail is required for cellular localization to the membrane. The GPI-anchored MT-MMPs do not possess the transmembrane and cytoplasmic domains, but instead encode a GPI anchor domain, the functions of which are speculated to possibly include increased lateral mobility within the plasma membrane, lipase-mediated protein release/secretion or involvement in signal transduction (Cole et al., 1997; Anderson, 1993; Kinoshita et al., 1993; Sargiacomo et al., 1993). A final unique membrane-bound MMP is cysteine array-MMP/CA-MMP/MMP23, a Type II transmembrane protein characterized by an amino-terminal signal anchor in place of the signal peptide. Other sequence deviations of MMP-23 include a cysteine rich motif downstream of the catalytic domain, termed the cysteine array, and an immunoglobulin-like domain attached to the core protease domain in place of the hinge, hemopexin, transmembrane and cytoplasmic domains (Pei, 1999).

Regulation of the Matrix Metalloproteinases

The MMPs have the potential to be destructive in nature due to their powerful proteolytic capabilities. Their expression and activation is therefore tightly regulated at the transcriptional, mRNA and protein levels.

Certain MMPs are constitutively transcribed and secreted while the expression of others must be stimulated. The expression profile is influenced by the cis-regulatory factors present within the proximal promoter. These contrasting patterns of constitutively secreted versus stimulated expression are exemplified by the two gelatinases, MMP-2 and MMP-9. In the case of MMP-9, basal levels tend to be low, with documented induction of higher levels of gene expression occurring in response to trans-activating factors such as AP-1 and NFkB (Van Den Steen et al., 2002). Alternatively, basal levels of MMP2 expression are high purportedly due to the high GC-content within the proximal promoter region (Haas, 2005). However, transcriptional activation above such high basal levels does occur due to the presence of several DNA binding elements activated by transcription factors such as ETS family members, NFkB and CREB (Van del Steen et al., 2002; Haas, 2005; Zhang et al., 2003; Melnikova et al., 2005; Liu et al., 2005).

MMPs are regulated at the mRNA level by mechanisms such as RNA editing, alteration of stability and spatial redistribution. Alternative splicing of pre-mRNA has been reported for certain MMP family members such as MMP-8 (Hu et al., 1999). It has been reported that nitric oxide (NO) mediates a decrease in binding of the mRNA stabilizing molecule HuR to MMP-9, resulting in increased instability of MMP-9 mRNA (Akool et al., 2003). Additionally, neuronal MMP-9 mRNA has been shown to translocate to the dendritic layer of the dentate gyrus during Kainate-induced excitotoxicity (Szklarczyk et al., 2002).

MMPs are regulated at the protein level through multiple mechanisms. Enzyme latency is maintained through the previously mentioned cysteine switch (Van Wart and Birkedal-Hansen, 1990). Most are regulated by other MMP molecules, or by other proteases such as plasmin (reviewed by Chakraborti et al., 2003). MMP zymogen activation is mediated by members of the proprotein convertase/furin family within the trans-golgi network (Sato et al., 1996; Wang and Pei, 2001; Kang et al., 2002; Pei and Weiss, 1995). The activation of secreted MMPs by members of the MT-MMP subfamily is well described. A tertiary complex between MT1-MMP, TIMP-2 and MMP-2 can occur on the cell membrane, allowing a free MT1-MMP molecule to activate MMP-2 (Sato et al., 1994). While this MT1-MMP-mediated activation of MMP-2 requires TIMP-2, the activation of MMP-13 by MT1-MMP does not (Knauper et al., 1996).

MMPs are inhibited by several different types of molecules. The tissue inhibitors of metalloproteinases (TIMP) consist of four family members of 20-30 kDa that each interact with different MMP family members to directly inhibit zymogen activation (Reviewed by Baker et al., 2002). Interactions between the MMPs and the TIMPs can result in both inhibition and activation of MMP activity and the resultant effect is dependent on the levels of these opposing family members. (Lehti et al., 2000; Lehti et al., 2002; Rozanov et al., 2001). Procollagen C-proteinase, which has structural similarity to the TIMP family, also acts post-translationally to inhibit MMP family members (Mott et al., 2000). Reversion-inducing cysteine-rich protein with Kazal motifs (RECK), has been shown in recent reports to inhibit MMP-2, MMP-9 and MT1-

MMP through unknown mechanisms (Oh et al., 2001). Finally, ∝-macroglobulin is a known MMP inhibitor which mediates its function in the circulatory system through a complex "bait and trap" mechanism (Takahashi et al., 1998). In this case, cleaved ∝-macroglobulin physically binds with the MMP molecule, isolating it from potential substrates.

Regulation of the Membrane-Type Matrix Metalloproteinases

A growing body of literature has focused on additional levels of MT-MMP regulation including their cellular trafficking and membrane localization (reviewed by Hernandez-Barrantes et al., 2002). As mentioned above, the MT-MMPs are subject to intracellular furin activation (Sato et al., 1994; Pei, 1999; Pei and Weiss, 1996). MT-MMPs are also recruited to the cell membrane for dimerization and oligomerization, where they participate in pericellular proteolysis, activation of secreted MMPs, interaction with other bioactive molecules, and possibly cell signaling (Lehti et al., 2000; Lehti et al., 2002). The localization of MT-MMPs to cell membrane specializations such as the invadopodia of invasive cells and caveolae invaginations occurs as a result of interactions of the cytoplasmic domain with intracellular signaling molecules (Nakahara et al., 1997; Annabi et al., 2001; Remacle et al., 2003). Cell compartment cycling via clatharin-coated vesicles or lysosomal compartments within the secretory pathway has recently been described (Jiang et al., 2001; Uekita et al., 2001; Conner and Schmid, 2003). The cycle between cell membrane location and endocytosis can be assisted by cellular chaperones such as AP-2 and Mint family

members and serve to regulate exposure of the active form of MT-MMPs to potential peri- and extracellular targets (Uekita et al., 2001; Wang et al., 2004). MT-MMP subfamily members also undergo shedding of the extracellular portion of the protein (ectodomain shedding). Shedding of the ectodomain of the MT-MMPs occurs via autocatalytic as well as non-autocatalytic means (reviewed by Osenkowski et al., 2004). In sum, these studies show that the MT-MMPs are subject to additional levels of regulation and cellular targeting.

General Function of the Matrix Metalloproteinases

In general, the MMPs are known as secreted enzymes that degrade extracellular matrix (ECM) proteins. Most MMPs are named based on the particular ECM component they cleave. In addition to these proteolytic interactions, MMPs play an important role in growth factor release from growth factor binding proteins or structural proteins in the extracellular environment. This serves to regulate interactions of growth factors with their receptors resulting in the initiation of signaling cascades (Imai et al., 1997; Whitelock et al., 1996; Manes et al., 1997; Fowlkes et al., 1995; Fowlkes et al., 1994). MMP-3 is responsible for the shedding of membrane-docked heparin-binding-EGF (HB-EGF) and MT1-MMP can act as a sheddase by mediating the release of the hyaluronan receptor, CD44 (Suzuki et al., 1997; Kajita et al., 2001). Conversely, MMPs can indirectly inhibit growth factor signaling by the shedding of growth factor receptors thereby resulting in a decrease in receptors on the cell surface (Levi et al., 1996). Direct inactivation occurs through proteolytic cleavage of the growth factor,

such as in the cases of chemokine connective tissue activating peptide III (CTAP-III) and stromal cell-derived factor 1 (SDF-1) (Fujiwara et al., 2002; McQuibban et al., 2001). Due to their cellular location and the known mechanisms of their regulation, the MT-MMPs have been targeted for a more diverse set of functions involving pericellular cell signaling, migration and invasion and activation/inactivation of bioactive molecules.

Matrix Metalloproteinases in the Central Nervous System

It is widely recognized that while dysregulation of MMP expression contributes to CNS pathology, their regulated expression is essential for normal tissue morphogenesis and for neuroplasticity (Gasche et al., 2006). A growing body of literature has focused on regions and cell types that express different MMP family members, how those levels change during pathology and how MMP family members function in the CNS. Of particular interest to the work proposed in this thesis is a review of the known role of MMP family members in nervous system repair and plasticity. Also included are insights relating to the recently identified pool of adult neural progenitor cells found in the postmitotic neural environment.

The Gelatinases in CNS Physiology and Pathology

Of the MMP subfamilies, the most widely studied in the CNS have been the gelatinases (MMP-2/gelatinase A and MMP-9/Gelatinase B) (Dzwonek et al., 2004). MMP-2, the constitutively active of the two, has been detected in normal brain

astrocytes at the message and protein levels using expression and activity based assays (Szklarczyk A et al., 2002; Rivera et al., 2002; Phillips and Reeves, 2001; Wright et al., 2003; Zhang et al., 2000; Zhang et al., 1998). Neuronal expression of MMP-2 and its activity have been reported in the normal neocortex (Planas et al., 2001), cerebellar Purkinje neurons (Vaillant et al., 1999; Wright et al., 2003; Zhang et al., 2000; Zhang et al., 1998) and fetal neuronal cultures (Vecil et al., 2000). MMP-2 has also been detected in oligodendrocytes (Oh et al., 1999; Uhm et al., 1998) and microglia (Rosenberg et al., 2001). Induction of hippocampal MMP-2 above basal levels occurs in response to a variety of traumatic CNS insults such as ischemia, kainate acid treatment, trauma and viral infection (Rivera et al., 2002; Planas et al., 2001; Khuth et al., 2001; Phillips and Reeves, 2001; Zhang et al., 1998; Patrick et al., 2002; Zhang et al., 2003; Johnston et al., 2002). Additionally, increased MMP-2 levels are seen in the pons associated with spinocerebellar ataxia (Evert et al., 2001). Interestingly, astrocytic and microglial MMP-2 levels are decreased in the parkinsonian substantia nigra while neuronal levels are unchanged (Lorenzl et al., 2002). MMP-2 is expressed by invading brain cancer cells and contributes to the ensuing pathology via increased angiogenesis, invasion and cell proliferation (Bello et al., 2001; Guo et al., 2005). Other proposed mechanisms of MMP-2 function in neural damage include its potential involvement in cell death and mediation of the neuroinflammatory response (Zhang et al., 2003).

MMP-9 expression is observed in astrocytes and microglia (Vecil et al., 2000; Vaillant et al., 1999; Rosenberg et al., 2001; Oh et al., 1999; Uhm et al., 1998) as well as in oligodendrocytes (Uhm et al., 1998; Oh et al., 1999). Studies indicate that

predominate expression levels reside, however, in CNS neuronal populations from regions such as the neocortex, cerebellum (granule and Purkinje cells) and hippocampus (Szklarczyk et al., 2002; Rivera et al., 2002; Wright et al., 2003; Zhang et al., 2000; Zhang et al., 1998; Taishi et al., 2001; Vaillant et al., 2003). Similar to MMP-2, MMP-9 neuronal expression is increased in CNS pathologies (Gu et al., 2002; Rivera et al., 2002; Lorenzl et al., 2002; Khuth et al., 2001; Rosenberg et al., 2001; Lim et al., 1996; Backstrom et al., 1996; Asahina et al., 2001; Rosell et al., 2005; Dewil et al., 2005). Whether MMP-9 in neurodegenerative pathologies is expressed by recovering neurons or by dying ones is currently unclear (reviewed in Dzwonek et al., 2004). It has been shown that nitric oxide (NO)-induced activation as well as S-nitrosylation of MMP-9 promotes neuronal cell death (Manabe et al., 2005; Gu et al., 2002). MMP-9 is expressed by activated microglia and, when activated by TNF-alpha, mediates neurotoxicity associated with neurodegenerative pathology, as neurons in MMP-9 knock out animals experience significantly lower levels of cell death in response to activated microglia (Kauppinen and Swanson, 2005). It has also been shown that MMP-9 participates in caspase-3-mediated neurotoxicity of brain endothelial cells through the disruption of cell-matrix interactions and integrin-mediated signaling (Lee and Lo, 2004). Increased MMP-9 is associated with an invasive phenotype of brain tumor cells (Tanimura et al., 1999; Sahin et al., 2005) and in the mediation of extracellular proteolysis accompanying inflammatory demyelination in MS lesions (Cuzner and Opdenakker, 1999).

The Collagenases in CNS Physiology and Pathology

The collagenases are so named for their ability to degrade the fibrillar collagens found in the ECM. Although these ECM targets are seemingly absent from the brain, (Sugita et al., 2001) two of the collagenases, neutrophil collagenase/MMP-8 and collagenase-3/MMP-13, have been detected in the normal CNS at the mRNA level (Anthony et al., 1998: Sekine-Aizawa et al., 2001). Increased mRNA and protein levels of MMP-8 are found in infiltrating granulocytes associated with rat and murine models of MS (Anthony et al., 1998; Nygardas et al., 2002; Toft-Hansen et al., 2004). Elevated neuronal MMP-13 is expressed in NeuN positive cells associated with remodeling following focal cerebral ischemia (Nagel et al., 2005) and is found in human brain following stroke (Rosell et al., 2005). MMP-13 may also participate in activation cascades involving MMP-2, MMP-9 and MT1-MMP in cancer (Johansson et al., 1999). It is not known if this has been examined in CNS cancer cells.

Collagenase-1/MMP-1 is not detectable in normal CNS but is highly expressed in glioblastoma multiforme (Nakagawa et al., 1994, McCready et al., 2005). Overexpression of MMP-1 in GBM is theorized to affect invasion as higher levels have a positive correlation with aggressiveness of tumors (Rao et al., 2003; Nabeshima et al., 2002; Binder et al., 2002). Increased MMP-1 levels have also been reported in patients suffering from MS (Kurzepa et al., 2005).

The Stromelysins in CNS Physiology and Pathology

All four stromelysin subfamily members have been detected in the normal CNS (Ulrich et al., 2005). Increased MMP-7 and MMP-10 expression is documented in the rat EAE model (Cuzner and Opdenakker, 1999) and in human patients suffering from MS (Toft-Hansen et al., 2004; Kurzepa et al., 2005). MMP-3 has been the subject of more extensive study in the CNS. Stromelysin-1/MMP-3 has been shown to be expressed in cerebellar neurons (interneurons, Purkinje cells and IGL granular cells), cortical neurons and hippocampal neurons (Vaillant et al., 1999; Wetzel et al., 2003; Kim et al., 2005). Additionally, basal levels of MMP-3 are reported in Bergman glia and in cultured astrocytes (Vaillant et al., 1999; Muir et al., 2003). Pathologically, increases in MMP-3 levels are seen in cortical AD plaques and in neurons and microglia associated with neuroinflammation (Yoshiyama et al., 2000; Rosenberg et al., 2001). MMP-3 is also reported to be increased following CNS viral infection (Giraudon et al., 1997; Zhou et al., 2005), brain ischemia (Wetzel et al., 2003) and is reported to be associated with CNS brain tumor invasion (Tanimura et al., 2003). During brain ischemia, MMP-3 subcellular immunoreactivity changed from scattered and punctate to diffuse staining of the neuronal cell body (Wetzel et al., 2003) and was induced in infiltrating microglia (Rosenberg et al., 2001). The proposed mechanisms for MMP-3 induction in brain pathology include shedding of membrane-anchored death receptors from damaged neurons, thus exerting an anti-apoptotic effect (Wetzel et al., 2003).

The Membrane-Type Matrix Metalloproteinases in CNS Physiology and Pathology

MT1-MMP mRNA has been detected in normal murine and rat total brain extracts (Vecil et al., 2000; Sekine-Aizawa et al., 2001; Ulrich et al., 2005) and more specifically in adult cortex and hypothalamus (Khuth et al., 2001). MT2-MMP and MT3-MMP are expressed at low basal levels in total brain extract (Sekine-Aikawa et al., 2001; Anthony et al., 1998). MT1-MMP overexpression is associated with brain tumor progression (Tanimura et al., 2003; Guo et al., 2005), viral infection (Khuth et al., 2001), hypoxic injury in retinal glial cells (Noda et al., 2005) and has been implicated in neurotoxic cascades associated with neuroinflammation (Rosenberg, 2002). MT1-MMP and potentially other MT-MMPs exert their invasive effect through structural pericellular interactions with secreted MMPs and integrins (Reviewed by Van Meter et al., 2001; Nuttall et al., 2003). It has been proposed that MT-MMPs can additionally affect cell functions including tumor growth and angiogenesis through interaction with bioactive molecules such as the growth factor VEGF (Deryugina et al., 2002). MT3-MMP has been identified as one of many proteins in the nucleus accumbens regulated by CREB in reward systems associated with drug addiction (McClung et al., 2003). The evidence for MT5-MMP in CNS physiology and pathology will be discussed in detail in a subsequent section.

Matrix Metalloproteinases in CNS Repair and Plasticity

A review of MMP function in neuroplasticity and neurogenesis is essential to the central theme of the proposed research. The fact that few of the known classical ECM substrates are present in the brain has previously limited the study of MMP

expression and their potential role in normal brain physiology. However, the regulated expression of MMP family members following CNS insult is now well-documented; and there is an emerging position that the MMPs are involved in CNS plasticity. Two examples of neuroplasticity that have been studied are the plasticity associated with cerebellar morphogenesis and reactive synaptogenesis in the hippocampus. In addition, the MMPs have been shown to participate in physiological neuronal cell motility and process outgrowth. Further, the discovery of a maintained neural stem cell niche in adult brain and the potential for MMP function in the maintenance of that niche, as has been shown in other stem cell niches (Heissig et al., 2002), call for new investigations in these areas.

Cerebellar Histogenesis

The histogenesis of the cerebellar cortex occurs during the perinatal period of brain development. Several of the MMPs are expressed in the cerebellum during this period (Reviewed by Luo, 2005). Cerebellar granule neuronal precursors from the EGL, a secondary germinal zone, proliferate, migrate, and mature during postnatal development. Additionally, extensive dendritogenesis of Purkinje cells occurs during this period. MMP-2 and MMP-9 are both expressed during cerebellar morphogenesis and their mRNA and protein expression patterns in the EGL during this period suggest that both the gelatinases participate in the migration of cerebellar granule neuronal precursors as well as neuronal process outgrowth, dendritogenesis and synaptogenesis (Ayoub et al., 2005; Vaillant et al., 1999; Vaillant et al., 2003). MMP-3, a known activator of MMP-9, has also been detected in the developing cerebellum and this

expression pattern mimicked that of MMP-9 (Vaillant et al., 1999; Hahn-Dantona et al., 1999). MMP-9 activation is regulated by MMP-3 and members of the plasmin system (Hahn-Dantona et al., 1999). When the tissue plasminogen activator gene is knocked out, radial migration of cerebellar granule neurons is strongly inhibited, supporting the notion that an MMP-3/MMP-9/plasmin cascade plays an essential role in cerebellar histogenesis (Seeds et al., 1999). MT5-MMP is also expressed in the developing cerebellum and expression levels are localized to mature granule neurons rather than immature precursor cells (Jaworski, 1999). The temporal expression of MT5-MMP was shown to drastically increase during the period of cerebellar dendritogenesis (Hayashita-Kinoh et al., 2001; Sekine-Aizawa et al., 2001).

Hippocampal Synaptogenesis

MMP activation has also been associated with hippocampal stimulation and reactive synaptogenesis. Neuronal MMP-9 protein, mRNA, enzyme activity levels and membrane localization are increased following hippocampal seizure activity (Zagulska-Szymczak et al., 2001; Zhang et al., 1998; Szklarczyk et al., 2002). Interestingly, these increased seizure-related MMP-9 mRNA levels were seen in both cell body and dendritic layer, suggesting activity-driven translocation of MMP-9 mRNA, presumably to control protein synthesis of MMP-9 in a synapse-specific manner. Synapse-specific expression of proteins such as MMP-9 has been proposed to occur during events of learning and memory such as LTP (Frey and Morris, 1998; Martin et al., 2000). Both gelatinases are found pre- and post-synaptically during LTP (Moore et al., 2002) and MMP-9 in the course of spatial learning (Wright et al., 2002). Interestingly, increases

in MMP-9 were also seen in cortical regions during different forms of learning, suggesting more widespread MMP expression associated with neural plasticity (Wright et al., 2004). Reactive synaptogenesis and resultant capacity for LTP in the hippocampus associated with functional recovery following deafferentation is reduced following administration of an MMP inhibitor (Reeves et al., 2003). The same study reported a decrease in deafferentation-related clearance of cellular debris and synaptic proliferation in the presence of an MMP inhibitor (Reeves et al., 2003). Additionally, TBI-induced reactive sysnaptogenesis was correlated with increased MMP-3 levels (Kim et al., 2005). MMP-7 has also been reported to dramatically alter dendritic morphology in cultured hippocampal neurons, suggesting a role in maturation of dendritic spines and synaptic stability (Bilousova et al., 2006).

Neural Cell Process Outgrowth

Process outgrowth of neural cell types is also affected by MMP expression. The growth cone is part of the axon where the leading edge of neurite outgrowth occurs. NGF induction of MMP-3 expression has been shown in differentiated PC12 cells (Fillmore et al., 1992) and has been shown to be spatially localized to the growth cone of cultured PC12 cells (Nordstrom et al., 1995). RA-induced expression of MT5-MMP is found in the active growth cone of cultured murine DRG cells (Hayashita-Kinoh et al., 2002). This expression was shown to be required for growth cone motility through brain-specific ECM molecules using exogenously added MT5-MMP peptide and non-specific MMP inhibition using BB94 (Hayashita-Kinoh et al., 2001). Neuronal MMP-2 can also be induced by administration of NGF, and has been shown to affect neuronal

process outgrowth in a three dimensional matrix (Muir, 1994). Pharmacological inhibition of MMPs results in aberrant axon guidance of *Xenopus laevis* retinal ganglion cells while in transit during brain development (Hehr et al., 2005). Similarly, high levels of MMP-9 and MMP-12 expression are associated with oligodendrocyte process outgrowth (Luke et al., 1999; Larsen and Yong, 2004). In addition to process outgrowth, MMP-12 has been shown to be involved in oligodendrocyte differentiation (Larsen and Yong, 2004).

MMP activation has been proposed to participate in reparative remyelination through degradation of the inhibitory proteoglycan, NG2 (Larsen et al., 2003). MMP-2 and MMP-9 expression patterns following nerve injury and the ability of MMP-2 to degrade the inhibitory proteoglycan, CSPG, suggest their involvement in axonal degeneration and regeneration (Kherif et al., 1998; Heine et al., 2004). Regulated expression of MMP-2, MT1-MMP and MT5-MMP are seen in another model of axonal degeneration and regeneration, the murine nasal epithelium following neural ablation (Tsukatani et al., 2003; Hamilton Ross et al., unpublished observation).

Precursor Cell Biology

Very little information is available concerning MMP expression and function in neural stem/progenitor cells. It has been shown that oligodendrocyte precursor cells utilize MMPs for motility (Amberger et al., 1997) and for process elongation in the case of MMP-9 (Uhm et al., 1998). In addition, MMP-2 is expressed by human embryonic CNS stem cells at higher levels than in mature CNS cells tested (Frölichsthal-Schoeller et al., 1999).

Matrix Metalloproteinase Substrates/Targets in the Brain

The inter-neuronal ECM space does not contain a classical basal lamina, which would contain several of the identified MMP target ECM proteins (Sugita et al., 2001). However, several brain ECM proteins and other molecules have been identified as physiological and pathological target proteins for MMP activity. MT5-MMP cleaves sulfated proteoglycans such as CSPG and HSPG (Hayashita-Kinoh et al., 2001). Aggrecan is one CSPG targeted by MMP-3 (Kim et al., 2005) and possibly other MMPs such as MT5-MMP. Another identified physiological ECM target of MMP family members is dystroglycan (β-DG) (Yamada et al., 2001; Kacmarek et al., 2002). β-DG is a cell surface protein found on the postsynaptic membrane that binds to presynaptic neurexins, membrane-bound proteins associated with neurotransmitter release (Sugita et al., 2001; Missler et al., 2003). LTP is substantially reduced in animals null for β-DG (Moore et al., 2002), and this has been proposed to be mediated by membrane localized processing of β-DG by MMP family members (Yamada et al, 2001). Amyloid precursor protein (APP) is another CNS target protein and has been reported as a substrate for MT1-MMP, MT3-MMP and MT5-MMP (Ahmad et al., 2006). MMP-9 and MT5-MMP are associated with senile plaques and neurofibrillary tangles found with AD (Backstrom et al., 1996; Sekine-Aizawa et al., 2001). Another structural protein, alpha-synuclein, is the main component of Lewy bodies associated with PD and is processed via the activation of MMP-3 (Sung et al., 2005).

The brain ECM, while providing structural support, also anchors many growth factors. MMP family members can cleave these proteins resulting in the release from the ECM. The precursor proteins for neurotrophins NGF and BDNF contain consensus sites for cleavage by MMP-3 and MMP-

fact, the members of the plasmin system as well as MMPs have been shown to extracellularly cleave secreted pro-neurotrophins (Lee et al., 2001).

MT5-MMP

Identification of MT5-MMP Gene Products

The fifth member of the MT-MMP subfamily was independently cloned by two groups of investigators in 1999. It was identified as a candidate gene from a human cerebellar EST clone and was then cloned and characterized in the mouse (Pei, 1999). Human MT5-MMP was subsequently cloned, sharing 95% homology to the murine gene, and mapped to chromosome 20q11.2-12 (Llano et al., 1999). MT5-MMP shares significant sequence homology with other MT-MMPs, with areas of sequence divergence occurring in the signal, propeptide, stem and hinge domains (Pei, 1999; Llano et al., 1999). Northern blot analysis recognizes a transcript of ~4.5 kb in adult murine, rat and human brain tissue and faintly in adult rat liver (Pei, 1999; Jaworski, 1999; Llano et al., 1999; Sekine-Aizawa et al., 2001). A smaller transcript is reported in adult human kidney, pancreas and lung (Llano et al., 1999) and adult rat brain (Sekine-Aizawa et al., 2001). Human, murine and rat MT5-MMP respectively encode proteins of 645, 614 and 618 amino acids prior to signal peptide cleavage or other

posttranslational modifications (Llano et al., 1999; Pei, 1999; Sekine-Aizawa et al., 2001). Independent transfection studies confirm that murine and human MT5-MMP associate mainly with the membrane fraction of cell protein preparations and through immunohistochemistry have been localized on the cell plasma membrane (Pei, 1999; Llano et al., 1999). However, further work reveals that MT5-MMP can also be seen co-localized with furin in the TGN, where furin acts to activate the proenzyme at a conserved furin cleavage site (Wang et al., 1999). In the TGN, furin also cleaves MT5-MMP at a second site, resulting in a protein form described as a "shed" species found in the cytoplasm (29, 40-46 kDa) as well as secreted from the cell (44-46 kDa) (Pei, 1999).

MT5-MMP Expression in the CNS

Independent studies have investigated MT5-MMP expression patterns through development in both the mouse and rat. An initial study using northern blot analysis on adult murine tissue panels found MT5-MMP to be primarily expressed in brain tissue with low expression in the testis (Pei, 1999). In whole brain tissue developmental onset of mRNA expression was embryonic day 11 (E11) with high levels persisting until at least E17, when levels began to drop to adult basal levels (Pei, 1999). Interestingly, when murine cerebral tissue was differentiated from cerebellar tissues different expression patterns were revealed (Hayashita-Kinoh et al., 2001). Northern blot analysis showed peak levels of cerebral MT5-MMP expression to occur embryonically, dropping around P0. Conversely, cerebellar expression, expressed at low levels embryonically, peaked at P30. Notably, these temporal expression patterns indicate that

peak cerebral MT5-MMP expression corresponds to neocortical morphogenesis while peak cerebellar expression corresponds to granular cell differentiation in the EGL and their migration across the molecular layer into the IGL (Hayashita et al., 2001). Developmental expression was further analyzed in murine brain structures at E15.5, P14 and P60. In situ hybridization and immunohistochemistry revealed that MT5-MMP expression was diffusely found at E15.5 but was temporally and spatially regulated to the granular cell layer of the cerebellum, olfactory bulb, dentate gyrus, subventricular zone of the lateral ventricle, rostral migratory stream and at low levels in the cortex at P14. By P60, these levels were further restricted to the granular cell layer of the cerebellum and to the dentate gyrus (Hayashita et al., 2001).

Similar work has been conducted on rat tissues. Initial experiments using northern blot analysis showed MT5-MMP mRNA expression appears on E16, with levels peaking at P0 and dropping off to adult basal levels by P60 (Jaworski, 1999). In situ hybridization localized this expression abundantly throughout the nervous system beginning at E14. Other regions having enriched MT5-MMP expression on E18 included the thymus and aorta (Jaworski, 1999). By E20, expression was clearly enriched in the brain parenchyma. This robust expression in neural tissue was temporally and spatially restricted through postnatal development to the hippocampus, dentate gyrus, cerebellar cortex and olfactory bulb by P60. Notably, these are all regions with documented postnatal plasticity (Jaworski, 1999). When adult rat tissue was examined by Northern blot analysis, only the cerebellum retained high levels of expression, compared to modest levels found in cerebral and brain stem extracts.

Similarly, in situ hybridization on adult rat brain slices showed expression predominately in the granular layer of the cerebellum, with lower levels seen in the cortex. However, immunohistochemistry revealed the presence of rat MT5-MMP protein in the cerebellum, hippocampus, dentate gyrus, cortex and thalamus in the adult (Sekine-Aizawa et al., 2001).

Results from these studies demonstrate that MT5-MMP expression is robust during development and is temporally and spatially regulated to regions of plasticity into adulthood. In rat cells, MT5-MMP mRNA expression was predominately found in the postmitotic parenchyma surrounding the mitotically active ventricular zone during development although some staining was seen within it (Jaworski, 1999). Closer examination of expression patterns in these tissues revealed that MT5-MMP is predominately expressed in neuronal cell types. In all, MT5-MMP has been specifically localized to granule cells of the cerebellum and hippocampus, cortical pyramidal cells, thalamic neurons and Purkinje cells of the cerebellum (Sekine-Aizawa et al., 2001; Hayashita-Kinoh et al., 2001). Subcellular distribution has been found predominately in the somata and dendrites of several cell types (Sekine-Aizawa et al., 2001; Hayashita-Kinoh et al., 2001).

Pathologically, increased MT5-MMP expression is seen in brain tumors of glial cell origin (astrocytoma, anaplastic astrocytoma and glioblastoma) and in pancreatic and lung malignancies (Llano et al., 1999). Conversely, expression is decreased in human prostatic tumor tissue (Jung et al., 2003). MT5-MMP is also found to co-localize with Aβ deposits near the surface of senile plaques and neurofibrillary tangles

associated with AD (Sekine-Aizawa et al., 2001). In murine tissue, MT5-MMP mRNA expression was detected by RT-PCR at low levels in normal cornea and much higher levels in epithelial cells of the substantia propria seven days following bacterial infection (Dong et al., 2001). The temporal expression pattern in this model was proposed to coincide with macrophage infiltration (Dong et al., 2001). In addition, increased MT5-MMP protein levels have been reported in human diabetic kidney and were found to be localized to epithelial cells associated with atrophy of the proximal and distal tubules, collecting duct and loop of Henle (Romanic et al., 2001).

MT5-MMP Functional Studies

Like other Type-I transmembrane MT-MMP subfamily members, MT5-MMP activates MMP-2 (Llano et al., 1999; Pei, 1999) and degrades ECM proteins (Wang et al., 1999). Substrate profiling revealed that MT5-MMP's proteolytic targets include the sulfated proteoglycans (CSPG, HSPG and DSPG), gelatin and to a limited extent fibronectin, however did not include laminin or Type 1 collagen (Wang et al., 1999; Hayashita-Kinoh et al., 2001). Of note, MT5-MMP is the first MT-MMP reported to degrade the sulfated proteoglycans. These ECM proteins are rich in the brain ECM and their MMP-mediated degradation neutralizes the inhibitory effect the sulfated proteoglycans exert on neurite outgrowth (Ashley et al., 1995).

Members of the MT-MMP subfamily are reportedly associated with cellular invasion and cancer cell progression (reviewed by Fillmore et al., 2001). MT5-MMP is upregulated in brain tumors and also in invading cytotrophoblast cells (Llano et al.,

1999; LaMarca et al., 2005). KiSS-1, a metastasis suppressor gene, has been identified as a target of MT5-MMP. KiSS-1 is a tumor suppressor gene that forms a complex with pro-MMP-2 and MMP-9 to achieve a metastasis suppressor effect linked functionally to tumor progression (Takino et al., 2003). Cleavage of this protein into an alternate form termed metastin results in attenuation of cellular motility and invasion through increased formation of focal adhesions.

Neuron-specific MT5-MMP message and protein were found in cultured P19 cells when treated with RA in the presence of Ara-C to induce neuronal differentiation (Hayashita-Kinoh et al., 2001). In situ zymography combined with immunohistochemistry revealed that during perinatal cerebellar morphogenesis (P9), MT5-MMP-positive cells migrating to the granule cell layer exhibited gelatinolytic activity. MT5-MMP-positive Purkinje cells and granule cells in the adult murine cerebellum were further shown to maintain this gelatinolytic phenotype (Hayashita-Kinoh et al., 2001). This same group of investigators reported that MT5-MMP was localized to the growth cones of cultured murine dorsal root ganglion cells by immunohistochemistry (Hayashita-Kinoh et al., 2001). *In vitro* assays conducted on cultured DRG neurons revealed that MT5-MMP expression was sufficient to promote neurite outgrowth through degradation of laminin, CSPG and HSPG substrata (Hayashita-Kinoh et al., 2001).

Recently, the creation of an MT5-MMP knock out mouse was reported (Komori et al., 2004). Histological analysis revealed no obvious abnormalities in brain, spinal cord or DRG anatomy, however MT5-MMP deficiency was characterized in a

neuropathic pain model (mechanical allodynia) caused by nervous system lesion (Komori et al., 2004). A lesion was induced through ligation or transection to the sciatic nerve, whereupon pathological sprouting in the dorsal horn of the spinal cord resulted in a marked reduction in threshold to a painful stimulus. This response was notably absent in MT5-MMP knock out animals, reflecting an absence of pathological neurite sprouting in the dorsal horn. Subsequent immunohistochemistry showed loss of myelinated Aβ fibers in the dorsal horn. These results confirmed that MT5-MMP loss is sufficient to reduce the pathological sprouting by the nerve fibers in the spinal cord (Komori et al., 2004).

Recent work has shown that MT5-MMP binds to the AMPA receptor binding protein (ABP) and co-localizes with synaptic markers and the Glutamate Receptor Interaction Protein (GRIP). ABP was shown to form complexes with MT5-MMP that were recruited to the growth cone of developing neurons and to the synapse of mature neurons. Further, this work identified neuronal cadherin cell adhesion molecules as potential MT5-MMP substrates (Monea et al., 2006). This work adds to accumulating data suggesting that MT5-MMP may play a significant role in synaptic morphology and remodeling.

Proposed Research

Rationale and Significance

Converging lines of evidence outline the potential importance of MT5-MMP to CNS function. MT5-MMP is expressed ubiquitously during nervous system

development and is spatially regulated in adult CNS regions known to be neurogenic and areas where synaptic plasticity occurs. ECM proteins known to be proteolytic targets for MT5-MMP are present in the brain and MT5-MMP misexpression is associated with certain CNS pathologies. MT5-MMP is localized to and functions in axonal outgrowth and has been proposed to function in synaptic remodeling. Taken together, these data suggest MT5-MMP may play a significant role in neuronal function. Although the spatiotemporal regulation of its expression hints that MT5-MMP could be important to neural stem cell biology, no studies to date have studied its function in regard to residual pools of neural stem/progenitor cells.

The significance of the proposed work is multifaceted. Work aimed at extending published reports of MT5-MMP neuronal expression will provide additional insight into the potential functions of MT5-MMP in the CNS. Studies seeking to clone MT5-MMP from cells of human origin for *in vitro* overexpression experiments will provide a novel experimental tool in which to address the nature of MT5-MMP and its effect on cell migration and invasion on ECM substrates.

Hypothesis

MT5-MMP is expressed in regions of physiological and pathological plasticity and neurogenesis. It is also a known mediator of pericellular proteolysis, specifically of those substrates found in the brain ECM and known to have a functional impact on cell migration, neurite outgrowth and tissue morphogenesis but its function is unknown in relation to neural stem/progenitor cell deployment and motility. Therefore, we posit

that MT5-MMP levels will be differentially expressed in an *in vitro* model of neurogenesis and neural differentiation. We predict that MT5-MMP expression will increase as a function of neural differentiation. In addition the introduction of MT5-MMP into MT5-MMP null cells will result in an enhanced migratory phenotype.

Specific Aims

Specific Aim 1: To profile MT5-MMP expression in adult CNS tissues and in the NT2-hNT model of neurogenesis and differentiation.

Specific Aim 2: To clone MT5-MMP cDNA from NT2 cells.

a. Perform sequence and bioinformatic analysis of MT5-MMP cDNA obtained from NT2 cells.
b. Create MT5-MMP overexpressing cell lines for functional studies.

Specific Aim 3: To investigate the effects of MT5-MMP overexpression in ECM substrate-specific cell migration and invasion.

Chapter 2

IDENTIFICATION OF A NOVEL HUMAN MT5-MMP TRANSCRIPT VARIANT IN MULTIPOTENT NT2 CELLS

Introduction

The MMP family of zinc-dependent endopeptidases is comprised of 23 members, several of which are known to be present in the physiological and pathological CNS (Yong et al., 1998; Lukes et al., 1999). In addition to their established role in ECM degradation, the MMP family has a wide range of influences on biologic processes including the generation of bioactive proteins (Egeblad and Werb, 2001; Somerville et al., 2003; McCawley et al., 2001). The most recently identified subfamily of the MMP family is the membrane-type MMPs or MT-MMPs (reviewed in Fillmore et al., 2001). MT5-MMP, the fifth cloned MT-MMP, is expressed in few tissues including testes, liver, lung and kidney; however is predominately found in the nervous system (Jaworski, 1999; Pei, 1999; Sekine-Aizawa et al., 2001). MT5-MMP expression is ubiquitously expressed during neural development while in the adult CNS

it is restricted to specific regions, some of which are known to be neurogenic, such as the hippocampus (Jaworski, 1999). Previous work indicates that neuronal MT5-MMP participates in plasticity associated with neurite outgrowth (Hayashita-Kinoh et al., 2001).

While existing studies have investigated the presence and function of MT5-MMP in murine and rodent models, little data is available on MT5-MMP expression in the human CNS and neurogenic regions. To begin investigations on the role of MT5-MMP in human neurogenesis and neural differentiation, we initiated studies using a well-characterized neurogenic *in vitro* model, the NT2 cell line. Stimulation of NT2 cells with RA leads to the differentiation of NT2 cells into a 99% purely post-mitotic neuronal population (hNT cells) (Pleasure et al., 1992).

In this report, we show that MT5-MMP mRNA is significantly increased in differentiated hNT cells. MT5-MMP cDNA cloned from NT2 cells resulted in the identification of a novel transcript (herein referred to as MT5-MMPvar) differing from that of the previously reported MT5-MMP human gene (Llano et al., 1999). While 92% homologous to the reported sequence this transcript is characterized by a 162 bp deletion within the signal and propeptide domains. Both the previously reported and MT5-MMPvar transcripts were identified in NT2 and hNT cells and in adult human brain tissue. *In vitro* transcription/translation studies demonstrated the translation of an approximately 52 kDa protein from MT5-MMPvar. Western blot analysis using a deletion spanning antibody revealed the presence of the corresponding MT5-MMPvar

protein in NT2 and hNT cells and in adult human hippocampus. These results suggest that more than one human MT5-MMP transcript may exist in the CNS.

Materials and Methods

NT2 cell culture and human tissue acquisition

Undifferentiated NT2/D1 cells were obtained from ATCC (#CRL-1973) and were maintained in high-glucose DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were differentiated according to an established protocol (Pleasure et al., 1992). Cells were treated with 10 μmol *all-trans* retinoic acid (RA) (Sigma-Aldrich, St Louis, MO) for four weeks. Cells were manually tapped off the flasks and replated onto Matrigel (BD Bioscience, Bedford, MA) in the presence of mitotic inhibitors (cytosine arabinoside [1 μM], fluorodeoxyuridine [10 μM] and uridine [10 μM]) for one week. Treatment with cytosine arabinoside was discontinued and cultures maintained an additional four weeks. Cells were replated onto poly-L-lysine coated six well plates and constituted a 99% pure neuronal culture (hNT). Human adult brain tissue was obtained from temporal lobe resections to treat epilepsy in accordance with an approved IRB protocol from our institution. Tissue was acutely dissected at the time of harvest into samples corresponding to parahippocampal gyrus or hippocampal formation and stored at -80°C in freezing media (DMEM supplemented with 10% DMSO and 20% FBS) until use. At the time of use, frozen samples were subjected to either RNA extraction or protein extraction as described below.

Quantitation of MT5-MMP mRNA levels (real time PCR)

Total RNA was isolated from NT2 cells, hNT cells and adult human brain tissue using TRIzol reagent, the manufacturer's protocol (Invitrogen, CA) and standard extraction procedure (Sambrook and Russell, 2001). To prepare tissue extracts for RNA isolation, flash-frozen pieces were ground to a fine powder with a frozen mortar and pestle prior to being added to TRIzol reagent. RNA was quantitated using spectrophotometry and treated with RQ1 DNase (Promega, Madison, WI) using the manufacturer's protocol. A primer set complimentary to human MT5-MMP (Table 2.1.) was designed for automated real time PCR (VCU Nucleic Acid Research Facility-NARF). Primers for human Cyclophilin A were also designed through the VCU NARF and served as an internal control. Results (n=3) were analyzed by Student's t-Test.

Western blot analysis, antibodies and peptide competition assay

Cultured adherent cells (T-75) were washed with PBS and incubated with 1 ml of ice-cold RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% SDS, 1% sodium deoxycholate, 1% Nonidet P-40) for 5 minutes on ice and with gentle agitation. Flasks were scraped, sheared with a 26½ gauge needle and centrifuged at 16,000 rpm for 30 minutes at 4°C. Supernatant was collected and stored at -80°C until use. Alternatively, frozen tissue samples from adult human hippocampal samples were thawed and suspended in 800 µl ice-cold RIPA buffer containing protease inhibitor cocktail (Calbiochem, San Diego, CA) and mechanically homogenized. The homogenized tissue was incubated at 4°C for 15 minutes and centrifuged at 16,000 rpm for 30

Table 1: Primer Design, location and expected sizes for all PCR experiments

MT5-MMP Primers	Sequence	Location	Expected Amplicon Size (bp)
Real Time PCR	5′ tgagccatggacgctagga 3′ 5′ tgcacagccaccaggaaga 3′	Spanning Intron 4	62
Full cDNA	5′ acacatgccgaggagccggggcg 3′ 5′ gcacccattcctggactggccgc 3′ **	Start ATG Terminating Codon	1938 (MT5-MMP) 1779 (MT5-MMPvar)
5′ End	5′ acacatgccgaggagccggggcg 3′ 5′ gttgggtttggtgcctggtgt 3′	Start ATG Exon 2	1140 (MT5-MMP) 978 (MT5-MMPvar)

**Llano et al, 1999

minutes at 4°C. Protein supernatants were aliquoted and stored at -80°C until use. Proteins were quantified using the Lowry method and Biorad DC reagent (Biorad, Hercules, CA) using the manufacturer's protocol. A BSA (Pierce, Rockford, IL) standard curve was used (25 μg/ml to 2000 μg/ml range). Equal amounts of protein were loaded onto Bis-Tris 4-12% density gradient gels (Invitrogen, Carlsbad, CA) for SDS-PAGE at 120 V using the manufacturer's power source and protocol (Novex). Proteins were transferred (1 hour at 30 V) onto nitrocellulose (Invitrogen) and incubated with 15 ml blocking buffer [5% bovine milk in Tris-buffered saline (50 mM Tris, pH 7.6, 150 mM NaCl) plus 0.05% Tween-20] for 1 hour at room temperature with gentle agitation. Primary antibody [anti-synaptophysin (1:400, Sigma); anti-cyclophilin A (1:2000, Upstate Biotech) or anti-MT5-MMPvar (1:2000, Primm Biotech)] was added to 10 ml blocking buffer and incubated overnight at 4°C with gentle agitation. Membranes were washed with rinse buffer [Tris-buffered saline (50 mM Tris, pH 7.6, 150 mM NaCl) plus 0.05% Tween-20] for 6 x 5 minutes with vigorous agitation. HRP-conjugated secondary antibody was added to 10 ml blocking buffer (1:5000, Rockland) and incubated for 1 hour at room temperature with gentle agitation. Membranes were washed as before and developed using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ) and the manufacturer's protocol. Immunoreactivity was visualized by exposure onto autoradiography film (Marsh) and was developed using an X-OMAT developer (Kodak, Rochester, NY). For quantitative analysis, optical densities of protein bands were measured using ImageQuant™ software (Amersham Biosciences, Piscataway, NJ). For peptide competition, anti-

MT5-MMPvar antibody (1:2000) was pre-incubated with or without the control peptide supplied by the manufacturer (Primm Biotech) used to generate MT5-MMPvar antibody (1:200) in blocking buffer at 4°C for one hour prior to primary antibody incubation.

Cloning of a novel MT5-MMP transcript

Total RNA was isolated from NT2 cells (TRIzol reagent) and the manufacturer's protocol. 1μg was used as template for reverse transcription (RT). RT was performed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), Oligo dT primer (Invitrogen, Carlsbad, CA) and the manufacturer's protocol. RT product was checked for degradation by agarose gel electrophoresis (1%) and 4 μl used for PCR template. PCR amplification was performed using primers (1.5 μl) specific for the human MT5-MMP cDNA (Table 2.1.), Advantage GCII DNA polymerase system (Clontech, Mountain View, CA) and manufacturer's protocol with the following cycling conditions: 1 minute at 92°C followed by 35 cycles of 1 minute at 92°C, 1 minute at 70°C, 3 minutes at 72°C with a final extension for 10 minutes at 72°C. Amplicons were visualized by agarose gel electrophoresis (1.2%). Bands were cut out of agarose gels and purified (Qiagen, Valencia, CA) using the manufacturer's protocol. PCR product (2 μl) was cloned into pTARGET (Appendix) eukaryotic expression vector (Promega, Madison, Wisconsin) using the manufacturer's protocol and transformed into TOP10F bacteria (Invitrogen, Carlsbad, CA). Bacteria were heat shocked at 42°C for 30 seconds and incubated on ice for 2 minutes. Then, 450 μl of SOC media was added to the transformation reaction and tubes incubated in an orbital shaker set at 250 rpm at 37°C

for 2 hours. Fifty or 100 µl of the transformation reaction was streaked onto LB plates including ampicillin resistance antibiotic (100 µg/ml) and plates incubated overnight at 37°C. Three ml of LB media containing ampicillin (100 µg/ml) was inoculated with selected colonies and incubated in an orbital shaker set at 250 rpm at 37°C for 6 hours. One ml of inoculate was subjected to miniprep analysis (Qiagen, Valencia, CA) using the manufacturer's protocol. Inclusion of cDNA insert in the sense orientation was verified by restriction enzyme digest with DraIII and incubated overnight at 37°C (Appendix). Digested DNA was visualized by agarose gel electrophoresis (1.2%). Clones confirmed to either contain no insert (vector control) or the MT5-MMP cDNA in the sense orientation were purified using a maxiprep (Qiagen, Valencia, CA) and sent for DNA sequencing (VCU Nucleic Acid Research Facility and University of Iowa Sequencing Facility). Sequence analysis and alignment was performed online using available software and databases (Chromas, NCBI, Clustal). The resultant sequence was submitted to the NCBI database (GenBank accession number **DQ300264**).

RT- PCR of the 5′ end of MT5-MMP

Total RNA was obtained as described above. RT product was PCR amplified using primers specific for the 5′ end of the MT5-MMP gene (Table 2.1.) and Advantage GCII DNA polymerase system as described above but with the following cycling conditions: 1 minute at 92°C followed by 30 seconds at 92°C, 30 seconds at 69°C, 1 minute at 72°C for 35 cycles and final extension for 3 minutes at 72°C. Amplified DNA was visualized using agarose gel electrophoresis (1.2%).

In vitro transcription/translation

Sequence-confirmed MT5-MMPvar (2 µl) was subcloned into the pCRT7/CT bacterial expression vector (Invitrogen, Carlsbad, CA) designed to yield a fusion protein containing a 3-5 kDa C-terminal tag (Fig 2.4.A) (Appendix). To this end, the sequence-confirmed 1779 bp MT5-MMPvar DNA insert was digested out of the multicloning site of the pTARGET/MT5-MMPvar construct using EcoRI. Resultant insert and plasmid fragments were isolated using agarose gel electrophoresis (1.2%) and the DNA insert purified (Qiagen, Valencia, CA) using the manufacturer's protocol. MT5-MMPvar (2 µl) was then used as input into the cloning reaction with the pCRT7/NT vector using the manufacturer's protocol. Then, 2 µl of the cloning reaction was added to TOP10F′ propagation bacteria. Transformation, colony selection and miniprep analysis were performed as described above. Inclusion and orientation of the subcloned MT5-MMPvar cDNA insert was confirmed with restriction enzyme digest with AgeI. 2 µl of pCRT7/NT-MT5-MMPvar was added to BL21Dea3pLysS expression bacteria (Invitrogen) and incubated on ice for 30 minutes. Bacteria were then heat shocked at 42°C for 30 seconds and incubated on ice for 2 minutes. Then, 250 µl of SOC media was added to the cloning reaction and tubes incubated in an orbital shaker set at 250 rpm at 37°C for 2 hours. The entire transformation reaction was used to inoculate 5 ml of LB media containing ampicillin (100 µg/ml) and chloramphenicol (37 ug/ml) and incubated in an orbital shaker set at 250 rpm at 37°C for 1 hour. The cultures were then divided for -/+ IPTG conditions. Expression of fusion protein was induced using IPTG

(1uM) for 0-6 hours. Then, 500 µl of inoculate was collected at 0, 1, 2, 3, 4, 5 or 6 hours and bacteria pelleted by centrifugation (10,000 rpm for 10 minutes at 4°C) and stored at -80°C until use. Pellets were resuspended (recipe volumes here) and heated at 70°C for 10 minutes. Heated product was sheared with a 26½ gauge needle and proteins loaded onto 4-12% density gradient gels and subjected to western blot analysis as described above. Antibody against the V5 epitope on the C-terminal tag was used for detection (1:2000, Invitrogen).

Results

MT5-MMP mRNA levels are significantly increased in NT2 cells following neuronal differentiation

The temporal expression pattern of MT5-MMP in rodent neural development and its expression in adult rodent hippocampal neurons have been reported (Jaworski, 1999; Sekine-Aizawa et al., 2001). The purpose of our studies was to begin investigations into the potential role of MT5-MMP in neurogenesis and to expand on previous findings in adult human brain tissue (Llano et al., 1999). We first examined MT5-MMP mRNA expression in an *in vitro* model of adult neurogenesis and neuronal differentiation. Using an established protocol, multipotent NT2 cells were differentiated into neuronal hNT cells (Figure 2.1.A, B) as verified by immunoblotting for synaptophysin, a mature neuronal marker (Figure 2.1.C). Total RNA isolated from NT2 and hNT cells was subjected to qPCR analysis using primers specific for MT5-MMP (Table 2.1.). Message levels were modest in NT2 cells and significantly

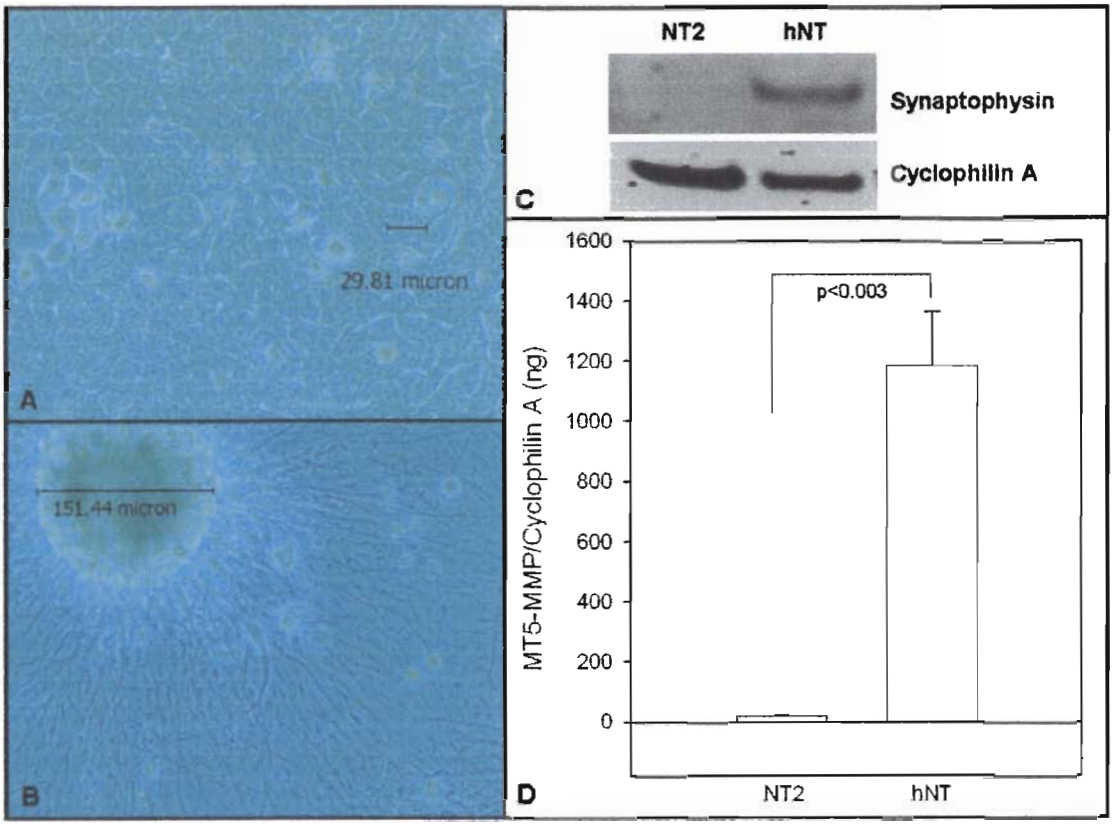


Figure 2.1. Expression of MT5-MMP mRNA in NT2 and hNT cells. A: Phase contrast image of NT2 cells prior to differentiation. B: Phase contrast image of differentiated hNT neurons. C: Verification of differentiation by synaptophysin western blot analysis. Cyclophilin A was used as an internal control. D: MT5-MMP mRNA levels in NT2 and hNT cells. Results expressed as the ratio of MT5-MMP expression over cyclophilin A (mean ± SEM), n = 3.

($p<0.003$) higher in differentiated hNT cells (Figure 2.1.D). Relative values of NT2 (18.6) and hNT (1183.7) RNA were normalized to those of the housekeeping gene, cyclophilin A in NT2 and hNT cells (7.0 and 11.4 respectively).

An MT5-MMP transcript cloned from NT2 cells does not share full sequence homology with the previously reported sequence

For both functional and expression studies, the full length cDNA of MT5-MMP was cloned from NT2 cells using primers directed at the full coding sequence for the human MT5-MMP gene (Table 2.1.). Once subcloned into pTARGET expression vector, the cDNA was sequenced and alignment analysis was performed. Results from sequence analysis showed that the obtained sequence (MT5-MMPvar) aligned with the published sequence of human MT5-MMP, however with only 92% identity (Figure 2.2. A). To determine the location and nature of the divergent DNA sequence, differences in sequence identity were plotted to the corresponding domain feature of the protein (Figure 2.2.B). Analysis of MT5-MMPvar revealed a 162 base pair deletion corresponding to an area within the signal and propeptide domains. A significant portion of the signal peptide domain (39 amino acids of 52) was deleted while no significant feature to the propeptide domain appeared to be deleted. Additional analysis using the PredictProtein program (ExPASy Proteomic tools) indicated that although the signal domain is missing amino acids, this protein is predicted to insert into the cell membrane in the correct orientation. Finally, a single amino acid substitution in the catalytic domain (Y to H) is observed.

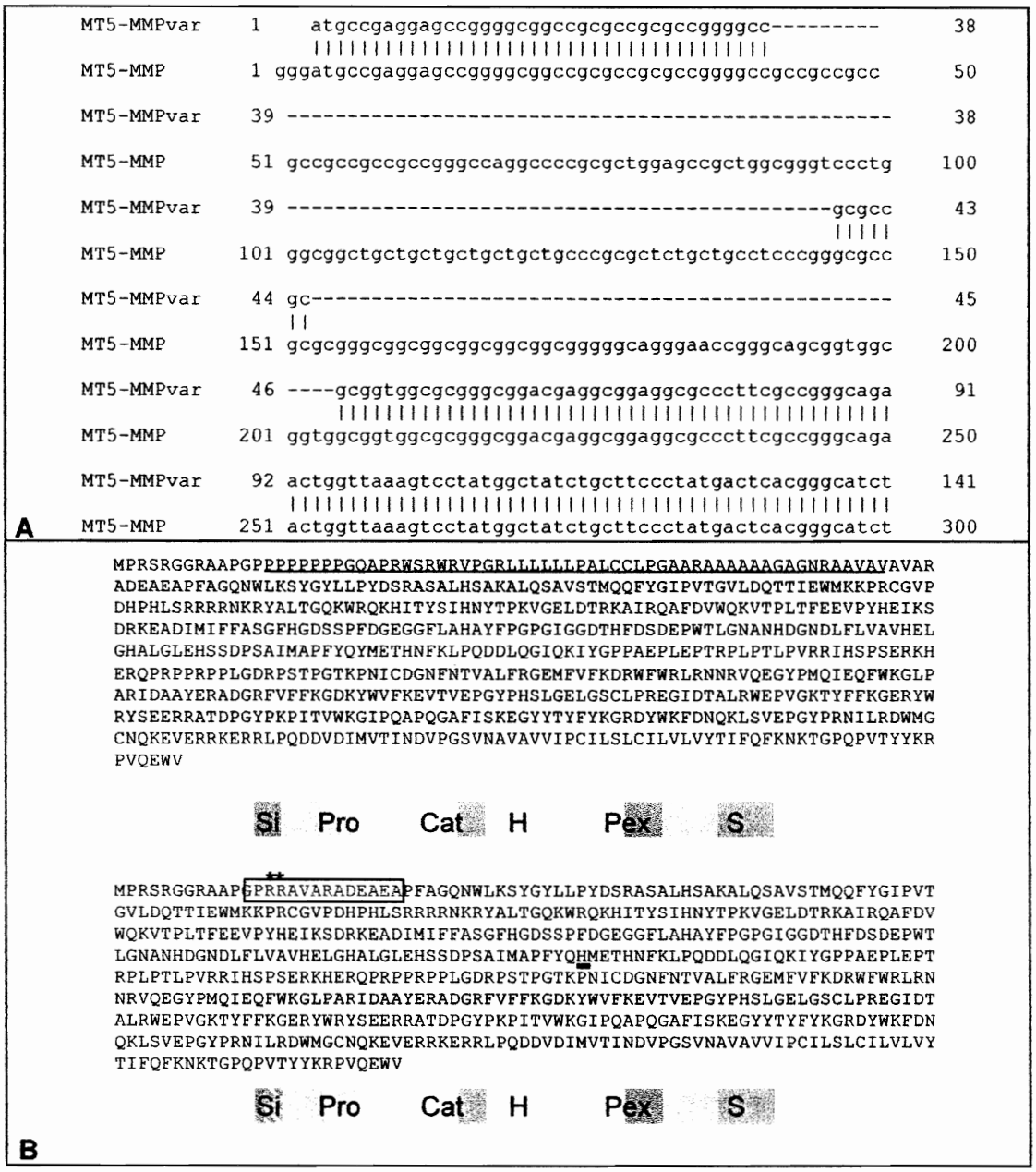

MT5-MMPvar 1 atgccgaggagccggggcggccgcgccgcgccggggcc--------- 38
MT5-MMP 1 gggatgccgaggagccggggcggccgcgccgcgccggggccgccgccgcc 50
MT5-MMPvar 39 ---------- 38
MT5-MMP 51 gccgccgccgccgggccaggccccgcgctggagccgctggcgggtccctg 100
MT5-MMPvar 39 ----------gcgcc 43
MT5-MMP 101 ggcggctgctgctgctgctgctgcccgcgctctgctgcctcccgggcgcc 150
MT5-MMPvar 44 gc---------- 45
MT5-MMP 151 gcgcgggcggcggcggcggcggcgggggcagggaaccgggcagcggtggc 200
MT5-MMPvar 46 ----gcggtggcgcgggcggacgaggcggaggcgcccttcgccgggcaga 91
MT5-MMP 201 ggtggcggtggcgcgggcggacgaggcggaggcgcccttcgccgggcaga 250
MT5-MMPvar 92 actggttaaagtcctatggctatctgcttccctatgactcacgggcatct 141
MT5-MMP 251 actggttaaagtcctatggctatctgcttccctatgactcacgggcatct 300
A
MPRSRGGRAAPGPPPPPPPPGQAPRWSRWRVPGRLLLLLLPALCCLPGAARAAAAAAGAGNRAAVAVAVAR
ADEAEAPFAGQNWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGIPVTGVLDQTTIEWMKKPRCGVP
DHPHLSRRRRNKRYALTGQKWRQKHITYSIHNYTPKVGELDTRKAIRQAFDVWQKVTPLTFEEVPYHEIKS
DRKEADIMIFFASGFHGDSSPFDGEGGFLAHAYFPGPGIGGDTHFDSDEPWTLGNANHDGNDLFLVAVHEL
GHALGLEHSSDPSAIMAPFYQYMETHNFKLPQDDLQGIQKIYGPPAEPLEPTRPLPTLPVRRIHSPSERKH
ERQPRPPRPPLGDRPSTPGTKPNICDGNFNTVALFRGEMFVFKDRWFWRLRNNRVQEGYPMQIEQFWKGLP
ARIDAAYERADGRFVFFKGDKYWVFKEVTVEPGYPHSLGELGSCLPREGIDTALRWEPVGKTYFFKGERYW
RYSEERRATDPGYPKPITVWKGIPQAPQGAFISKEGYYTYFYKGRDYWKFDNQKLSVEPGYPRNILRDWMG
CNQKEVERRKERRLPQDDVDIMVTINDVPGSVNAVAVVIPCILSLCILVLVYTIFQFKNKTGPQPVTYYKR
PVQEWV
Si
Pro
Cat
H
Pex
S
MPRSRGGRAAPGPRRAVARADEAEAPFAGQNWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGIPVT
GVLDQTTIEWMKKPRCGVPDHPHLSRRRRNKRYALTGQKWRQKHITYSIHNYTPKVGELDTRKAIRQAFDV
WQKVTPLTFEEVPYHEIKSDRKEADIMIFFASGFHGDSSPFDGEGGFLAHAYFPGPGIGGDTHFDSDEPWT
LGNANHDGNDLFLVAVHELGHALGLEHSSDPSAIMAPFYQHMETHNFKLPQDDLQGIQKIYGPPAEPLEPT
RPLPTLPVRRIHSPSERKHERQPRPPRPPLGDRPSTPGTKPNICDGNFNTVALFRGEMFVFKDRWFWRLRN
NRVQEGYPMQIEQFWKGLPARIDAAYERADGRFVFFKGDKYWVFKEVTVEPGYPHSLGELGSCLPREGIDT
ALRWEPVGKTYFFKGERYWRYSEERRATDPGYPKPITVWKGIPQAPQGAFISKEGYYTYFYKGRDYWKFDN
QKLSVEPGYPRNILRDWMGCNQKEVERRKERRLPQDDVDIMVTINDVPGSVNAVAVVIPCILSLCILVLVY
TIFQFKNKTGPQPVTYYKRPVQEWV
Si
Pro
Cat
H
Pex
S
B

Figure 2.2. Isolation of a novel human MT5-MMP transcript from NT2 cells. A: 5' end DNA alignment of MT5-MMP previously reported and MT5-MMPvar. B: Amino acid code with corresponding schematic for published MT5-MMP (top panel) and MT5-MMPvar (bottom panel). Underline, amino acids that are deleted from MT5-MMPvar. *, arginine residues found at deletion site; Box, peptide sequence used for antibody production; Bold underline, Y to H amino acid change. *Protein domains: Si, signal; Pro, Propeptide; Cat, Catalytic; H, Hinge; Pex, Hemopexin; S, Stem; T, Transmembrane; C, Cytoplasmic tail.*

Two MT5-MMP transcripts can be identified in NT2 cells, hNT cells and adult human brain tissue

To analyze further the 5′ region of MT5-MMP, primers were designed to amplify the divergent region (Table 2.1.). Expected bands at 978 and 1140 bp represented MT5-MMPvar and MT5-MMP transcripts, respectively. Results from RT-PCR analysis demonstrated the presence of both transcripts in NT2 cells, hNT cells and in adult human brain samples (Figure 2.3.B). When PCR amplification was again performed and analyzed to determine if two full transcripts could be detected in NT2 cells, only one amplicon corresponding to the MT5-MMPvar transcript could be identified (Figure 2.3.A).

MT5-MMPvar cDNA subcloned into a bacterial expression system translates into a ~52 kDa protein

In order to determine if the cDNA obtained was capable of translating into a protein, MT5-MMPvar cDNA was subcloned into an *in vitro* transcription/translation system to generate a fusion protein containing a C-terminus tag (Figure 2.4.A). Induction of MT5-MMP protein expression in bacterial cells with IPTG resulted in the translation of a ~52 kDa protein, detected using an antibody directed against the V5 epitope (Figure 2.4.B).

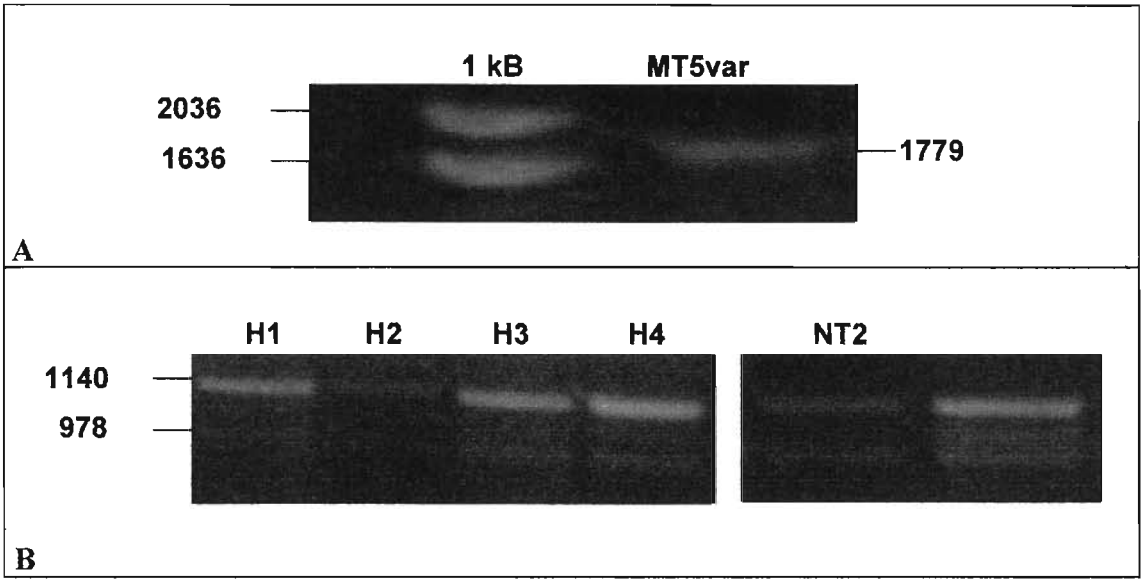


Figure 2.3. Analysis of the 5′ region of MT5-MMP cDNA in NT2, hNT and adult human tissues. A: RT-PCR results amplifying full MT5-MMP cDNA. B: RT-PCR results amplifying the divergent region of the MT5-MMP gene. Primer sequences and expected amplicon sizes listed in Table 1. H1-H4, independent hippocampal surgical samples.

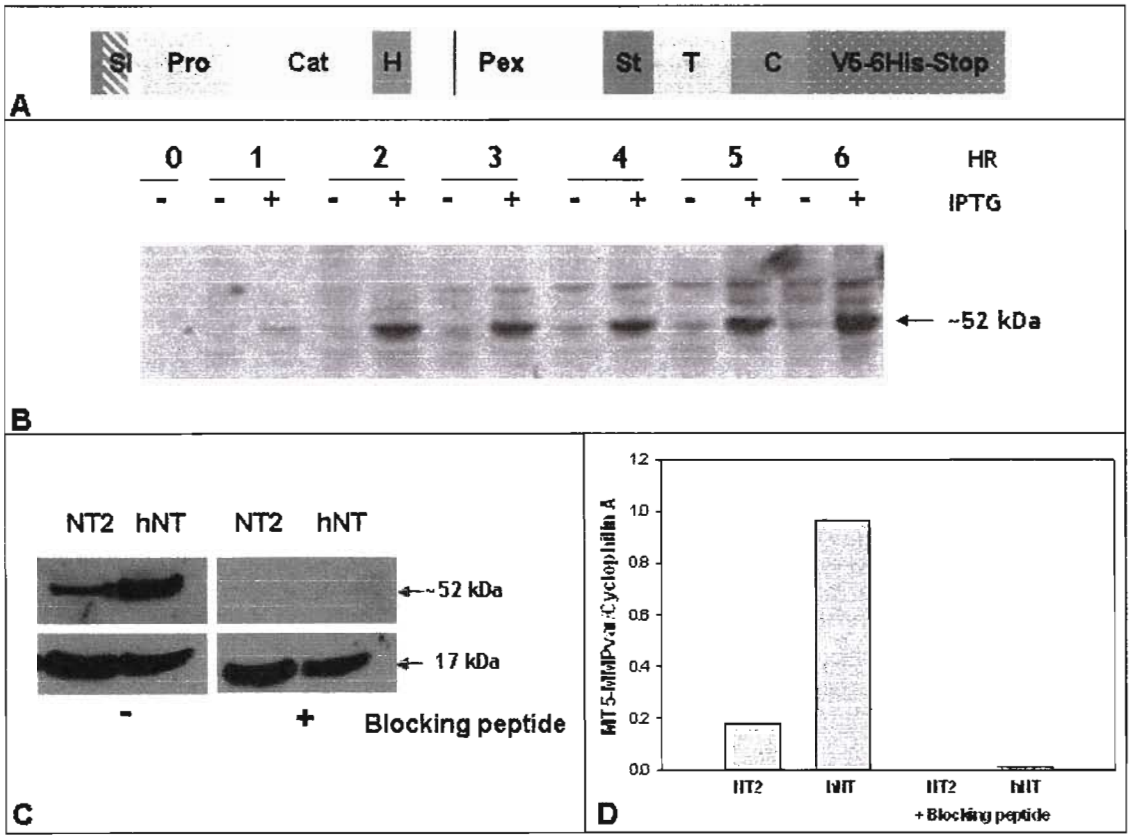


Figure 2.4. Translation of MT5-MMPvar cDNA. A: Schematic representation of MT5-MMPvar cDNA fused with a C-terminal tag containing a V5 epitope. B: IPTG (1mM) induction of protein synthesis in bacteria containing MT5-MMPvar/V5 fusion construct resulted in a ~52 kDa protein species recognized by an antibody specific to the V5 epitope. C: Western blot analysis of MT5-MMP in NT2 and hNT cells using an antibody developed from a peptide specific to the region which spans the deletion site. Antibody specificity was examined by peptide competition experiments (C, right panel). Cyclophilin A (17 kD) was used as an internal control. D: Densitometric analysis was performed to quantitate MT5-MMPvar protein levels in NT2 and hNT samples normalized to cyclophilin A.

To confirm that the variant protein product could be detected in the cells of origin, an antibody made using a peptide sequence spanning the deleted region of MT5-MMPvar transcript was utilized (Figure 2.2.B, shaded box). This sequence (PGPRRAVARADEAEA) was designed to correspond to the two amino acids before the deleted region, the intervening two arginine residues present in the MT5-MMPvar sequence and the eleven amino acids following the region deleted from published MT5-MMP. The MT5-MMPvar antibody was designed to this region as it would only be antigenic to the MT5-MMPvar sequence. Because the first 2 amino acids of the peptide are 162 bp away from the last 11 amino acids on the published MT5-MMP, and because the intervening two arginine residues are not present on the published MT5-MMP sequence, this antibody should not interact with the published MT5-MMP protein. Western blot analysis of NT2 and hNT protein using this antibody confirmed the presence of the MT5-MMPvar protein product (Figure 2.4.C, left panel). Antibody specificity was demonstrated by peptide competition using the purified peptide corresponding to the sequence of the MT5-MMPvar-specific antibody (Figure 2.4.C, right panel). Densitometric quantitation revealed substantially higher levels of MT5-MMPvar protein in hNT cells over NT2 levels (Figure 2.4.D). In all experiments, cyclophilin A was used as an internal control.

MT5-MMPvar protein is expressed in adult human neurogenic regions

We next sought to determine the presence of MT5-MMPvar protein in neurogenic adult human brain tissue. Western blot analysis using the above mentioned deletion spanning antibody revealed the presence of MT5-MMPvar protein in hippocampi obtained from temporal lobe resection (Figure 2.5.A, lanes 1-6, top panel). Peptide competition confirmed antibody specificity by blocking the ~52 kDa protein species (Figure 2.5.B). NT2 cell lysate was used as positive control (Figure 5A, B lane 7) and equal protein loading confirmed using cyclophilin A (Figure 2.5.A, B 17 kDa).

Discussion

Due to published reports on the temporal expression pattern of MT5-MMP in the rodent nervous system (Jaworski, 1999) and the potential role of this gene in adult neuroplasticity (Hayashita-Kinoh et al., 2001), we sought to clone this gene for functional as well as expression studies relating to adult human neurogenesis. Moderate levels of total MT5-MMP were found in NT2 cells with levels significantly increasing in differentiated hNT cells (Figure 2.1.D). cDNA subsequently cloned from NT2 cells shared a 92% homology to the reported MT5-MMP sequence (Figure 2.2.A). Compared to reported MT5-MMP, this novel transcript contained a deletion containing part of the signal peptide region spanning into the propeptide domain. The amino terminus of both the previously reported MT5-MMP transcript and this novel MT5-MMP transcript were identified in NT2 cells, hNT cells and in adult human brain tissue (Figure 2.3.B), however in this cell line we were unable to clone the full cDNA of the published MT5-MMP sequence (Figure 2.3.A). It should be noted that quantitative

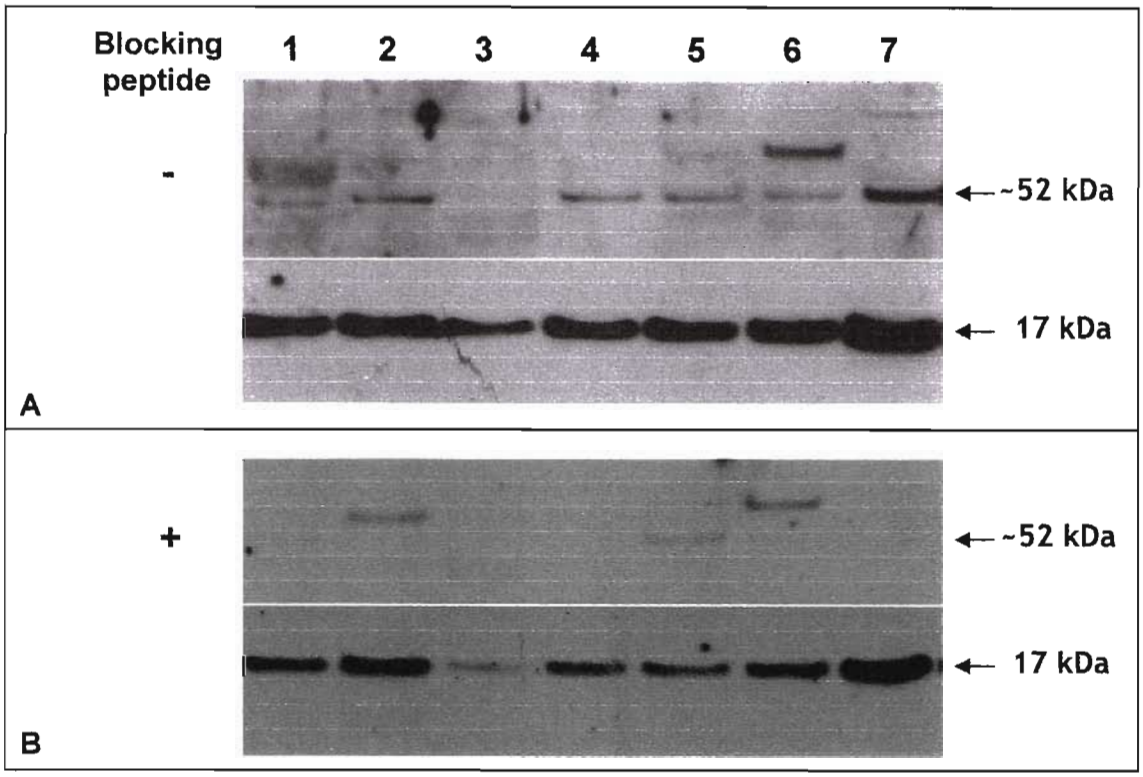


Figure 2.5. MT5-MMP protein expression in six independent adult human surgical tissues. A: Western blot analysis of proteins isolated from adult hippocampus using the deletion-spanning MT5-MMPvar antibody revealed expression of a ~52 kDa protein in 5 of 6 samples. NT2 served as positive control for MT5-MMP expression. B: Antibody specificity was examined by peptide competition. Cyclophilin A (17 kD) was used as internal control. Lane assignments: 1-6, independent surgical samples; 7, NT2 cells.

results in Figure 2.1. corresponded to total MT5-MMP levels as the gene is probed in a region common to both transcripts. Due to high GC content in the deleted region quantitative analysis of the relative expression levels of the two transcripts was unable to be performed by the VCU Nucleic Acid Research Facility. However, MT5-MMPvar cDNA was shown to translate into a full length protein and was further shown to be present in both NT2 and hNT cells (Figure 2.4.B, C). MT5-MMP protein levels were significantly higher in hNT cells (Figure 2.4.D). Current work in our laboratory includes the cloning of the previously reported open reading frame for comparative expression and functional studies of both transcripts.

In this report our sequencing and RT-PCR results indicate that more than one gene product may exist for the human MT5-MMP gene. Kajita et al. have reported similar findings for variable MT4-MMP transcripts, in which a second transcript was found to encode a more highly transcribed sequence and the only translated protein product (Kajita et al., 1999). The results presented here indicate that multiple gene products from the human MT5-MMP gene may exist and that this newly described transcript is expressed in human CNS tissue. Indeed, MT5-MMPvar protein was found to be expressed in six independent human hippocampal samples (Figure 2.5.). Our results demonstrate the overexpression of MT5-MMPvar in differentiated human neurons and are in agreement with previous reports in which the authors report the localization of MT5-MMP to neurons in the rodent CNS (Sekine-Aizawa et al., 2001). The significance of the 162 bp deletion is not known, however it is interesting that this deletion corresponds to a region of trinucleotide repeats containing eight Pro, six Leu,

and six Ala in the reported sequence. It has been proposed that this area could give rise to protein variants due to the instability known to be associated with trinucleotide repeats (Llano et al., 1999). Taken together these studies support the further characterization of this gene and its protein products.

CHAPTER 3

BIOINFORMATIC CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF MT5-MMPVAR TRANSCRIPT VARIANT

Introduction

MT5-MMPvar, a newly identified transcript variant of the human MT5-MMP gene, has been identified in both multipotent NT2 cells and in their neuronal counterparts, hNT cells. This novel transcript directs the translation of a 593 amino acid protein, and can be identified in the adult human hippocampus at both the RNA and protein levels. MT5-MMPvar shares 92% homology with the published sequence and is divergent in the signal and propeptide domains.

Genetic variance has been described for other members of the MMP family in this region. For example, genomic analysis of MMP-1 and MMP-13 supports potential alternative splicing of the first intron (Collier et al., 1988; Anglard et al., 1995; Pendas et al., 1997). Human neutrophil collagenase (MMP-8) can undergo alternative splicing of the first intron, yielding a second transcript (MMP-8alt) (Hu et al., 1999). This variant directs translation of an additional MMP-8 protein product with an insufficient signal peptide. In an *in vitro* model of protein secretion, designed to detect co-translational processing and glycosylation, MMP-8alt protein could not undergo such

modifications; thus MMP-8alt was predicted to be an intracellular protein (Hu et al., 1999). However, MMP-8alt translated *in vitro* was able to maintain its autocatalytic activation ability, suggesting it to be a functional protein with potentially altered cellular distribution or processing cascades (Hu et al., 1999).

The human MT4-MMP gene yields two gene products with a heterogeneous 5′ end. In this case, the original transcript (MT4-MMP Puente-type) does not contain a signal peptide domain and was unable to direct translation of a functional protein (Puente et al., 1996). In contrast, the variant transcript contained a signal peptide and directed translation of two protein products (Kajita et al., 1999). The proposed mechanism for the generation of the two MT4-MMP variants was alternative splicing of the first intron. In the case of MT5-MMP itself, the existence of two transcripts has been described in the ovary of the medaka fish, neither of which encoding a signal peptide domain (Kimura et al., 2001). Both transcripts could be detected in ovarian tissue using in situ hybridization with probes specific to each 5′ end (Kimura et al., 2001). Taken together, these data reveal a trend of sequence variation within the 5′ sequence of some MMP family members.

While it is known that the signal peptide domain functions to direct newly synthesized proteins to the endoplasmic reticular plasma membrane, the variation within this region in MMP family members is poorly understood. The functional impact of potentially altered cellular distribution or membrane orientation among the MT-MMP family members having such variation has not been defined. The goal of the present study was to conduct a comprehensive bioinformatic database analysis of MT5-

MMP and MT5-MMPvar DNA and corresponding protein sequences. We first aimed to compile information regarding the potential structural and functional implications of the loss or inclusion of the variable sequences. We then tested the ability of MT5-MMPvar to affect cellular chemotaxis and invasion in the presence or absence of ECM substrates. A preliminary immunocytochemical study was performed to examine MT5-MMPvar localization in MT5-MMPvar stably transfected COS-7 cells and in adult human neural progenitor cells. Lastly, a pilot study in which we examined protein expression in a murine region of postnatal neurogenesis was conducted.

Based on computer analysis using several programs, it is predicted that the MT5-MMPvar will not retain an adequate signal peptide for direction to the endoplasmic reticular membrane; however it is predicted to be a type-1 membrane protein. Studies using COS-7 cells genetically modified to overexpress MT5-MMPvar demonstrate no difference in cellular motility compared to parental or vector control cells. Immunocytochemistry results indicate that MT5-MMPvar appears to be localized primarily to the perinuclear region and to the cell membrane to a lesser extent in stably transfected COS-7 cells. Further, MT5-MMPvar is expressed in cultured adult human neural progenitor cells and appears to be expressed throughout the cytoplasm with intense immunoreactivity seen in the perinuclear region. Following stimulation of differentiation with serum withdrawal and administration of PDGF-BB, MT5-MMPvar expression appears to be associated with the membrane. Lastly, results from pilot studies investigating MT5-MMPvar expression in an adult murine neurogenic region suggest a murine homolog of MT5-MMPvar may exist. Taken together, these results

support further investigation of MT5-MMPvar expression patterns and function in neuronal cells.

Materials and Methods

Database Analyses

DNA Sequence Alignments

The NCBI database (www.ncbi.nlm.nih.gov) was utilized to acquire DNA sequences for MT5-MMP (Accession Number NM 006690) and MT5-MMPvar (Accession Number DQ300264). NCBI's Basic Local Alignment Search Tool (BLAST) was utilized to align these sequences. The EBI alignment tool, ClustalW, was utilized to depict sequence alignments (www.ebi.ac.uk/clustalw/). The genomic sequence for chromosome 20q.12 (Accession Number AL121753) was aligned to both MT5-MMP and MT5-MMPvar using the EBI alignment tool, Wise2, (www.ebi.ac.uk/Wise2/) (Appendix).

Protein domain and motif analysis

The analytical tools Prosite and MotifScan found on the protein analysis site ExPASy (http://us.expasy.org/) were utilized to analyze the protein sequences for the presence of protein domains and functional motifs.

Hydrophobicity plots, signal peptide analysis and transmembrane prediction

The Kyte-Doolittle method was used for hydrophobicity analysis (http://occawlonline.pearsoned.com, http://bioinformatics.weizmann.ac.il) (Kyte and Doolittle, 1982). SignalP version 3.0 (Bendtsen et al., 2004) from the Center for

Biological Sequence Analysis (CBS) was used for analysis of signal peptide sequences (www.cbs.dtu.dk/services/SignalP). TMPred (www.ch.embnet.org/software/TMPRED/) and DAS (www.sbc.su.se) databases were used for prediction of membrane insertion and orientation. The Phobius server (Käll et al., 2004; Käll et al., 2005) was used for combined analysis of signal peptide sequence and transmembrane domains and orientations (http://phobius.cgb.ki.se/). MT1-MMP was analyzed as a positive control.

Establishment of Stable Cell lines Overexpressing MT5-MMPvar

MT5-MMPvar cDNA previously cloned into the eukaryotic expression vector pTARGET (pTARGET/MT5-MMPvar) or an empty vector (pTARGET) was stably transfected into COS-7 cells (a kind gift from Dr. Dorne R Yager) using the Lipofectamine 2000 reagent and manufacturer's protocol. Following transfection, cells were maintained in serum-free DMEM supplemented with L-glutamine (1%) for twenty four hours. Serial dilutions of trypsinized cells (1:20, 1:50, 1:100) were then replated for twenty four hours. Fetal calf serum (10%) and G418 selection antibiotic (300 mg/ml) were added to media and cells maintained for 14 days. Sixteen colonies containing pTARGET/MT5-MMPvar and eight colonies containing pTARGET vector control were cloned using 5 mm disks into individual wells of twenty four well plates and maintained until confluent, at which time they were expanded in first T25 then T75 cell culture flasks. MT5-MMPvar expression status was confirmed by qPCR as described in Chapter 2 and using the following primers: 5′ tgagccatggacgctagga 3′; 5′ tgcacagccaccaggaaga 3′.

Chemotaxis and Invasion Assays

All COS-7 cell lines were maintained in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin-streptomycin and 300 μg/ml G418. Parental COS-7 cells received no G418 selection antibiotic. Membranes of 5.7 mm diameter, 8 μm pore Neuroprobe ChemoTx 96 well plates (Product number 116-8; Neuroprobe, Gaithersburg, MD) were left uncoated or coated with matrigel or CSPG as follows. Matrigel was prepared by diluting the product obtained from the manufacturer (Product number 354234; BD Bioscience, Bedford, MA) 1:12 in serum free DMEM and aliquoted into 1 ml units. Aliquots were thawed on ice prior to use. Lyophilized CSPG (Product number C9819-5G; Sigma, St Louis, MO) was resuspended in PBS and aliquoted in 1 ml units. Each aliquot was further stored as 50 μl units for individual use and adjusted to working dilutions (200 μg/ml or 450 μg/ml) in serum free DMEM. To coat the Neuroprobe membranes, 20 μl of prepared matrix was coated onto each membrane well and incubated at 37°C for 1 hour. Excess matrix was aspirated just prior to cell plating. 315 μl of either 0% or 10% media was added to the wells of Neuroprobe plates. Once the membrane was positioned over the media-filled wells, 20,000 cells per condition were plated in serum free media onto each uncoated membrane or membrane coated in matrix in a 50 μl volume. For blockade of cell chemotaxis and invasion, O-phenanthroline (50 μg/ml) (Product number 516705; Calbiochem, San Diego, CA) or DMSO vehicle control were added to cells 1 hour prior to plating. Similarly, MT5-MMPvar blocking peptide (1:200) or control IgG (1:200)

was added to cells 1 hour prior to plating. Neuoprobe plates were then incubated for 24 hours at 37°C. Membranes were removed and the top sides of the membranes, containing non-motile cells, were vigorously washed sequentially with PBS, a cell scrapper and cotton swabs with a final PBS wash. Membranes were incubated in 100% methanol for 30 minutes at room temperature to fix cells on the underside of the membrane. Cells were stained with Giemsa (diluted 1:50 in deionized water) for 30 minutes. To quantify the intensity of staining, plates were read on a ThermoMax microplate reader using SoftMax software (Molecular Devices, Sunnyvale, CA) at 570 nm. Raw numbers minus background were entered into Sigma plot for data analysis. On individual plates, each condition (cells alone, cells plus inhibitor, cells plus control IgG, etc) was prepared in triplicate or quadruplicate. Results are representative of three or more independent plates. For Figure 3.7., fold increase in cell motility in the presence of serum was calculated over baseline in the absence of serum and included results from all experiments conducted (no matrix, n=6; matrigel, n=4; CSPG, n=7). Comparison between conditions was performed using Student's t-Test.

Immunocytochemistry

Adult human neural progenitor cell lines were obtained from the VA Parkinson's Disease Research Education and Clinical Center's (PADRECC) adult human neural stem/progenitor laboratory. COS-7 cells or undifferentiated progenitor cells were maintained in DMEM containing 10% fetal serum, 1% penicillin-streptomycin and 1% L-glutamine. To fix cells, media was removed and 4%

paraformaldehyde added for 30 minutes at room temperature. Fixative was removed and cells washed with PBS three times. All washes were with PBS at room temperature with gentle agitation. Fixed cells were blocked with 0.5 ml/well of blocking solution (5% normal donkey serum, 0.25% TritonX-100 in PBS) for 30 minutes at room temperature, and then washed. Primary antibody (MT5-MMPvar 1:500, Primm Biotech) was adjusted to a 0.5 ml volume and applied to cells and allowed to incubate at 4°C overnight with gentle agitation. The following day, cells were washed three times with PBS and then incubated with 0.5 ml blocking solution for 30 minutes at room temperature. Secondary antibody (Rabbit Cy2 or Cy3, 1:250, Jackson Immunoresearch) was added in a 0.5 ml volume and incubated for 3 hours at room temperature with gentle agitation. Plates were stored with the wells covered with PBS until viewed under the fluorescent microscope.

Western blot analysis of murine cortex, SVZ, RMS and OB Tissue

Adult mice were sacrificed by rapid decapitation and the skulls hemisected. Tissue from the anterior subventricular zone, rostral migratory stream, olfactory bulb and frontal cortex were dissected at the time of sacrifice. Tissue was suspended in 800 µl ice-cold RIPA buffer containing protease inhibitor cocktail (Calbiochem, San Diego, CA) and mechanically homogenized with a Kontes motorized mortar and pestle. The homogenized tissue was incubated at 4°C for 15 minutes and centrifuged at 16,000 rpm for 30 minutes at 4°C. Protein supernatant was aliquoted and stored at -80°C until use. Protein was quantified and subjected to western blot analysis as described in Chapter 2

Material and Methods. Membranes were probed with the following primary antibodies: MT5-MMPvar (Primm Biotech, 1:2000) or cyclophilin A (Upstate Biotech, 1:2000). Immunoreactivity was quantified using a densitometer and analyzed using ImageQuant™ software (Amersham Biosciences, Piscataway, NJ).

Results

The divergent region of human MT5-MMP gene transcripts contains known functional motifs

For database analysis, a comparative DNA sequence alignment was first conducted. DNA sequence alignments were depicted in full and any deviation noted (Figure 3.1.). As reported in Chapter 2, MT5-MMPvar DNA sequence aligned to the MT5-MMP reported sequence with 92% homology (Figure 3.1., vertical line between aligned base pairs). The 162 bp deletion identified at the 5` end of the cDNA represented the majority of this deviation (Figure 3.1., alignment lines 1-5). Within this deletion, there is a seven base pair mis-alignment that is out of frame with the published MT5-MMP coding sequence. The MT5-MMPvar remains in frame and results in translation of two arginine residues in the MT5-MMPvar sequence (Figure 3.1., alignment lines 3 and 4). In addition to the 162 bp deletion, three single base pair changes were found downstream of this deletion, at base pairs 156, 648 and 760 of MT5-MMPvar (Figure 3.1.). Only the third single base pair change resulted in an amino acid conversion, a tyrosine to a histidine (Figure 3.1., underlined codons TAC and CAC respectively). The location of this amino acid change is located in the

```
MT5-MMPVar        1    atgccgaggagccggggcggccgcgccgcgccggggcc---------    38
                       ||||||||||||||||||||||||||||||||||||
MT5-MMP           1 gggatgccgaggagccggggcggccgcgccgcgccggggccgccgccgcc    50

MT5-MMPVar       39 --------------------------------------------------    38

MT5-MMP          51 gccgccgccgccgggccaggccccgcgctggagccgctggcgggtccctg    100

MT5-MMPVar       39 ----------------------------------------------gcgcc    43
                                                                  |||||
MT5-MMP         101 ggcggctgctgctgctgctgctgcccgcgctctgctgcctcccgggcgcc    150

MT5-MMPVar       44 gc------------------------------------------------    45
                    ||
MT5-MMP         151 gcgcgggcggcggcggcggcgggggcagggaaccgggcagcggtggc    200

MT5-MMPVar       46 ----gcggtggcgcgggcggacgaggcggaggcgcccttcgccgggcaga    91
                        ||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         201 ggtggcggtggcgcgggcggacgaggcggaggcgcccttcgccgggcaga    250

MT5-MMPVar       92 actggttaaagtcctatggctatctgcttccctatgactcacgggcatct    141
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         251 actggttaaagtcctatggctatctgcttccctatgactcacgggcatct    300

MT5-MMPVar      142 gcgctgcactcagcaaaggccttgcagtcggcagtctccactatgcagca    191
                    |||||||||||||||.||||||||||||||||||||||||||||||||||
MT5-MMP         301 gcgctgcactcagcgaaggccttgcagtcggcagtctccactatgcagca    350

MT5-MMPVar      192 gttttacgggatcccggtcaccggtgtgttggatcagacaacgatcgagt    241
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         351 gttttacgggatcccggtcaccggtgtgttggatcagacaacgatcgagt    400

MT5-MMPVar      242 ggatgaagaaaccccgatgtggtgtccctgatcacccccacttaagccgt    291
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         401 ggatgaagaaaccccgatgtggtgtccctgatcacccccacttaagccgt    450

MT5-MMPVar      292 aggcggagaaacaagcgctatgccctgactggacagaagtggaggcaaaa    341
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         451 aggcggagaaacaagcgctatgccctgactggacagaagtggaggcaaaa    500

MT5-MMPVar      342 acacatcacctacagcattcacaactataccccaaaagtgggtgagctag    391
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         501 acacatcacctacagcattcacaactataccccaaaagtgggtgagctag    550

MT5-MMPVar      392 acacgcggaaagctattcgccaggctttcgatgtgtggcagaaggtgacc    441
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         551 acacgcggaaagctattcgccaggctttcgatgtgtggcagaaggtgacc    600

MT5-MMPVar      442 ccactgacctttgaagaggtgccataccatgagatcaaaagtgaccggaa    491
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         601 ccactgacctttgaagaggtgccataccatgagatcaaaagtgaccggaa    650

MT5-MMPVar      492 ggaggcagacatcatgatctttttgcttctggtttccatggcgacagct    541
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         651 ggaggcagacatcatgatctttttgcttctggtttccatggcgacagct    700

MT5-MMPVar      542 ccccatttgatggagaaggggattcctggcccatgcctacttccctggc    591
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         701 ccccatttgatggagaaggggattcctggcccatgcctacttccctggc    750
```

```
MT5-MMPVar      592 ccagggattggaggagacacccactttgactccgatgagccatggacgct    641
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         751 ccagggattggaggagacacccactttgactccgatgagccatggacgct    800

MT5-MMPVar      642 aggaaatgccaaccatgacgggaacgacctcttcctggtggctgtgcatg     691
                    |||||||.||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         801 aggaaacgccaaccatgacgggaacgacctcttcctggtggctgtgcatg     850

MT5-MMPVar      692 agctgggccacgcgctgggactggagcactccagcgaccccagcgccatc     741
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         851 agctgggccacgcgctgggactggagcactccagcgaccccagcgccatc     900

MT5-MMPVar      742 atggcgcccttctaccagcacatggagacgcacaacttcaagctgcccca     791
                    ||||||||||||||||||.||||||||||||||||||||||||||||||||||
MT5-MMP         901 atggcgcccttctaccagtacatggagacgcacaacttcaagctgcccca     950

MT5-MMPVar      792 ggacgatctccagggcatccagaagatctatggacccccagccgagcctc     841
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         951 ggacgatctccagggcatccagaagatctatggacccccagccgagcctc    1000

MT5-MMPVar      842 tggagcccacaaggccactccctacactccccgtccgcaggatccactca     891
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1001 tggagcccacaaggccactccctacactccccgtccgcaggatccactca    1050

MT5-MMPVar      892 ccatcggagaggaaacacgagcgccagcccaggccccctcggccgccccct     941
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1051 ccatcggagaggaaacacgagcgccagcccaggccccctcggccgccccct    1100

MT5-MMPVar      942 cggggaccggccatccacaccaggcaccaaacccaacatctgtgacggca     991
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1101 cggggaccggccatccacaccaggcaccaaacccaacatctgtgacggca    1150

MT5-MMPVar      992 acttcaacacagtggccctcttccggggcgagatgtttgtctttaaggat    1041
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1151 acttcaacacagtggccctcttccggggcgagatgtttgtctttaaggat    1200

MT5-MMPVar     1042 cgctggttctggcgtctgcgcaataaccgagtgcaggagggctaccccat    1091
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1201 cgctggttctggcgtctgcgcaataaccgagtgcaggagggctaccccat    1250

MT5-MMPVar     1092 gcagatcgagcagttctggaagggcctgcctgcccgcatcgacgcagcct    1141
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1251 gcagatcgagcagttctggaagggcctgcctgcccgcatcgacgcagcct    1300

MT5-MMPVar     1142 atgaaagggccgatgggagatttgtcttcttcaaaggtgacaagtattgg    1191
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1301 atgaaagggccgatgggagatttgtcttcttcaaaggtgacaagtattgg    1350

MT5-MMPVar     1192 gtgtttaaggaggtgacggtggagcctgggtacccccacagcctggggga    1241
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1351 gtgtttaaggaggtgacggtggagcctgggtacccccacagcctggggga    1400

MT5-MMPVar     1242 gctgggcagctgtttgccccgtgaaggcattgacacagctctgcgctggg    1291
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP        1401 gctgggcagctgtttgccccgtgaaggcattgacacagctctgcgctggg    1450
```

```
MT5-MMPVar      1292 aacctgtgggcaagacctacttttttcaaaggcgagcggtactggcgctac   1341
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1451 aacctgtgggcaagacctacttttttcaaaggcgagcggtactggcgctac   1500

MT5-MMPVar      1342 agcgaggagcggcgggccacggaccctggctaccctaagcccatcaccgt   1391
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1501 agcgaggagcggcgggccacggaccctggctaccctaagcccatcaccgt   1550

MT5-MMPVar      1392 gtggaagggcatcccacaggctccccaaggagccttcatcagcaaggaag   1441
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1551 gtggaagggcatcccacaggctccccaaggagccttcatcagcaaggaag   1600

MT5-MMPVar      1442 gatattacacctatttctacaagggccgggactactggaagtttgacaac   1491
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1601 gatattacacctatttctacaagggccgggactactggaagtttgacaac   1650

MT5-MMPVar      1492 cagaaactgagcgtggagccaggctacccgcgcaacatcctgcgtgactg   1541
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1651 cagaaactgagcgtggagccaggctacccgcgcaacatcctgcgtgactg   1700

MT5-MMPVar      1542 gatgggctgcaaccagaaggaggtggagcggcggaaggagcggcggctgc   1591
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1701 gatgggctgcaaccagaaggaggtggagcggcggaaggagcggcggctgc   1750

MT5-MMPVar      1592 cccaggacgacgtggacatcatggtgaccatcaacgatgtgccgggctcc   1641
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1751 cccaggacgacgtggacatcatggtgaccatcaacgatgtgccgggctcc   1800

MT5-MMPVar      1642 gtgaacgccgtggccgtggtcatcccctgcatcctgtccctctgcatcct   1691
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1801 gtgaacgccgtggccgtggtcatcccctgcatcctgtccctctgcatcct   1850

MT5-MMPVar      1692 ggtgctggtctacaccatcttccagttcaagaacaagacaggccctcagc   1741
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
MT5-MMP         1851 ggtgctggtctacaccatcttccagttcaagaacaagacaggccctcagc   1900

MT5-MMPVar      1742 ctgtcacctactataagcggccagtccaggaatgggtg      1779
                     ||||||||||||||||||||||||||||||||||||
MT5-MMP         1901 ctgtcacctactataagcggccagtccaggaatgggtgtg   1940
```

Figure 3.1. Full cDNA sequence alignment of MT5-MMP and MT5-MMPvar. NCBI BLAST analysis reflected 92% sequence homology between the two transcript variants. The start ATG is boldfaced. (---) denotes base pairs deleted in MT5-MMPvar. (.) denotes single base pair differences. Only the underlined codon at base pairs 759-761 of MT5-MMPvar (TAC to CAC) results in an amino acid change within the translated protein product. A seven residue alignment of the two sequences (GCGCCGC) occurs within the deletion and corresponds to two arginine (R) residues encoded by the MT5-MMPvariant sequence.

carboxy end of the catalytic domain and did not correspond to any functional motif present in the domain. Next, the amino acid sequences of both transcripts were aligned and examined to determine if all functional motifs inherent to MMP family members were intact (Figure 3.2.). While the first thirteen of 52 amino acids that comprise the signal peptide were present in MT5-MMPvar, the remaining 45 residues were absent including the signal peptide cleavage site (Figure 3.2., black arrowhead). Also absent from MT5-MMPvar were the nine initial amino acids of the propeptide domain. However, the cysteine switch and conserved furin cleavage site, both important for MMP activation status, were present (Figure 3.2., bold blue and black, respectively). Two sites of N-myristoylation, one at the signal peptide cleavage site and one slightly downstream, were notably absent in MT5-MMPvar (Figure 3.2., underlined in red).

MT5-MMPvar is predicted not to contain an adequate signal peptide

The signal peptide, composed of a conserved stretch of hydrophobic amino acid residues, serves to direct newly synthesized proteins to the membrane of the endoplasmic reticulum for secretion from the cell. The signal peptide is then cleaved, and in the case of MMP proteins, the propeptide domain undergoes regulatory events for activation (described in Chapter 1). While the functional moieties within the propeptide domain appeared intact in the MT5-MMPvar protein, it was not known if there was a sufficient signal peptide. A strong predictive tool for determining the presence of hydrophobic regions of a protein is the hydropathic plot (Kyte and Doolittle, 1982). This analysis was performed to determine if the remaining signal

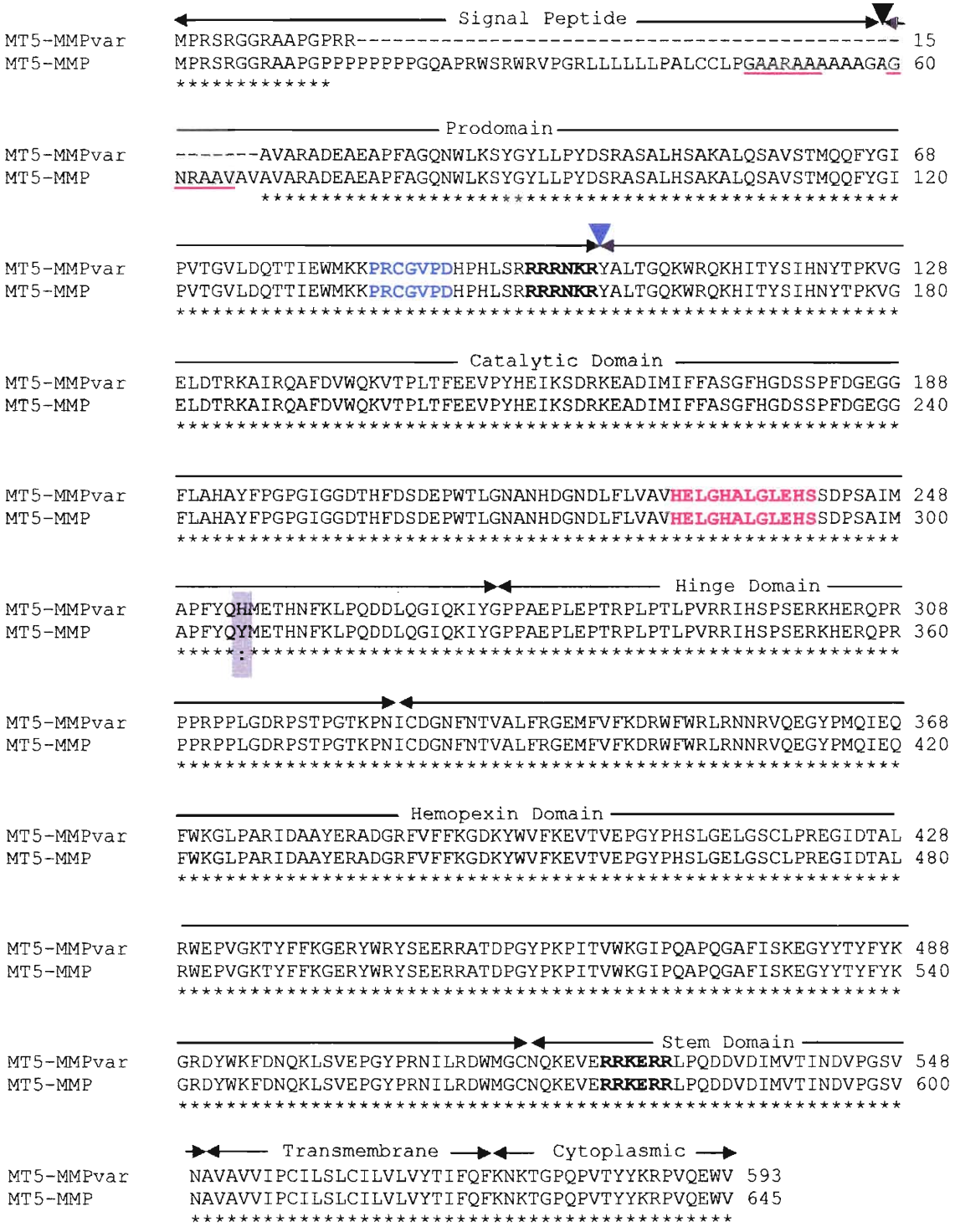
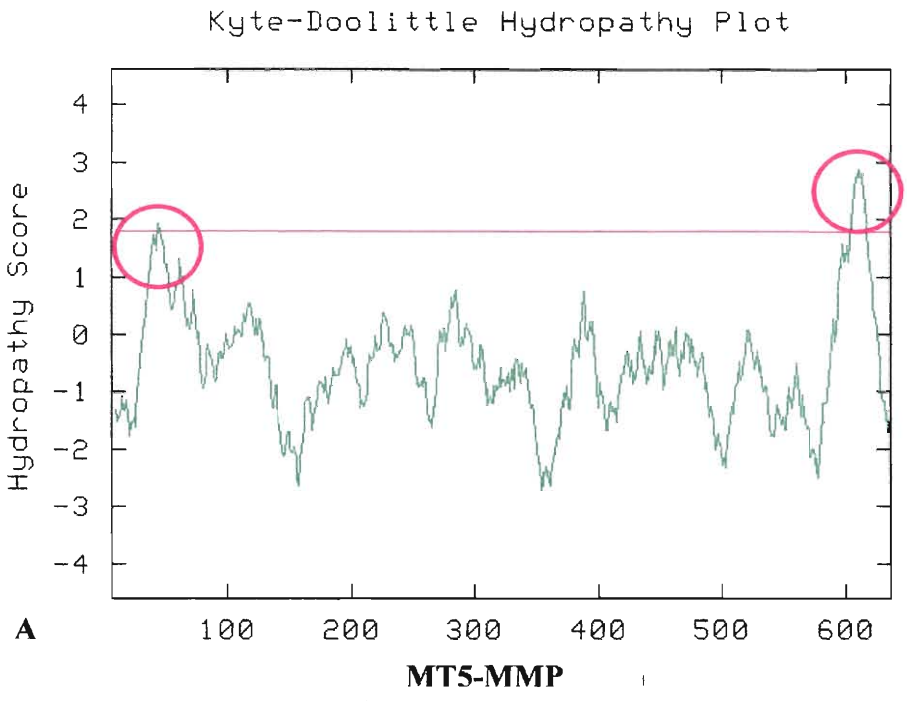

Signal Peptide
MT5-MMPvar MPRSRGGRAAPGPRR---------- 15
MT5-MMP MPRSRGGRAAPGPPPPPPPPGQAPRWSRWRVPGRLLLLLLPALCCLPGAARAAAAAAGAG 60
Prodomain
MT5-MMPvar -------AVARADEAEAPFAGQNWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGI 68
MT5-MMP NRAAVAVAVARADEAEAPFAGQNWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGI 120
MT5-MMPvar PVTGVLDQTTIEWMKKPRCGVPDHPHLSRRRRNKRYALTGQKWRQKHITYSIHNYTPKVG 128
MT5-MMP PVTGVLDQTTIEWMKKPRCGVPDHPHLSRRRRNKRYALTGQKWRQKHITYSIHNYTPKVG 180
Catalytic Domain
MT5-MMPvar ELDTRKAIRQAFDVWQKVTPLTFEEVPYHEIKSDRKEADIMIFFASGFHGDSSPFDGEGG 188
MT5-MMP ELDTRKAIRQAFDVWQKVTPLTFEEVPYHEIKSDRKEADIMIFFASGFHGDSSPFDGEGG 240
MT5-MMPvar FLAHAYFPGPGIGGDTHFDSDEPWTLGNANHDGNDLFLVAVHELGHALGLEHSSDPSAIM 248
MT5-MMP FLAHAYFPGPGIGGDTHFDSDEPWTLGNANHDGNDLFLVAVHELGHALGLEHSSDPSAIM 300
Hinge Domain
MT5-MMPvar APFYQHMETHNFKLPQDDLQGIQKIYGPPAEPLEPTRPLPTLPVRRIHSPSERKHERQPR 308
MT5-MMP APFYQYMETHNFKLPQDDLQGIQKIYGPPAEPLEPTRPLPTLPVRRIHSPSERKHERQPR 360
MT5-MMPvar PPRPPLGDRPSTPGTKPNICDGNFNTVALFRGEMFVFKDRWFWRLRNNRVQEGYPMQIEQ 368
MT5-MMP PPRPPLGDRPSTPGTKPNICDGNFNTVALFRGEMFVFKDRWFWRLRNNRVQEGYPMQIEQ 420
Hemopexin Domain
MT5-MMPvar FWKGLPARIDAAYERADGRFVFFKGDKYWVFKEVTVEPGYPHSLGELGSCLPREGIDTAL 428
MT5-MMP FWKGLPARIDAAYERADGRFVFFKGDKYWVFKEVTVEPGYPHSLGELGSCLPREGIDTAL 480
MT5-MMPvar RWEPVGKTYFFKGERYWRYSEERRATDPGYPKPITVWKGIPQAPQGAFISKEGYYTYFYK 488
MT5-MMP RWEPVGKTYFFKGERYWRYSEERRATDPGYPKPITVWKGIPQAPQGAFISKEGYYTYFYK 540
Stem Domain
MT5-MMPvar GRDYWKFDNQKLSVEPGYPRNILRDWMGCNQKEVERRKERRLPQDDVDIMVTINDVPGSV 548
MT5-MMP GRDYWKFDNQKLSVEPGYPRNILRDWMGCNQKEVERRKERRLPQDDVDIMVTINDVPGSV 600
Transmembrane
Cytoplasmic
MT5-MMPvar NAVAVVIPCILSLCILVLVYTIFQFKNKTGPQPVTYYKRPVQEWV 593
MT5-MMP NAVAVVIPCILSLCILVLVYTIFQFKNKTGPQPVTYYKRPVQEWV 645

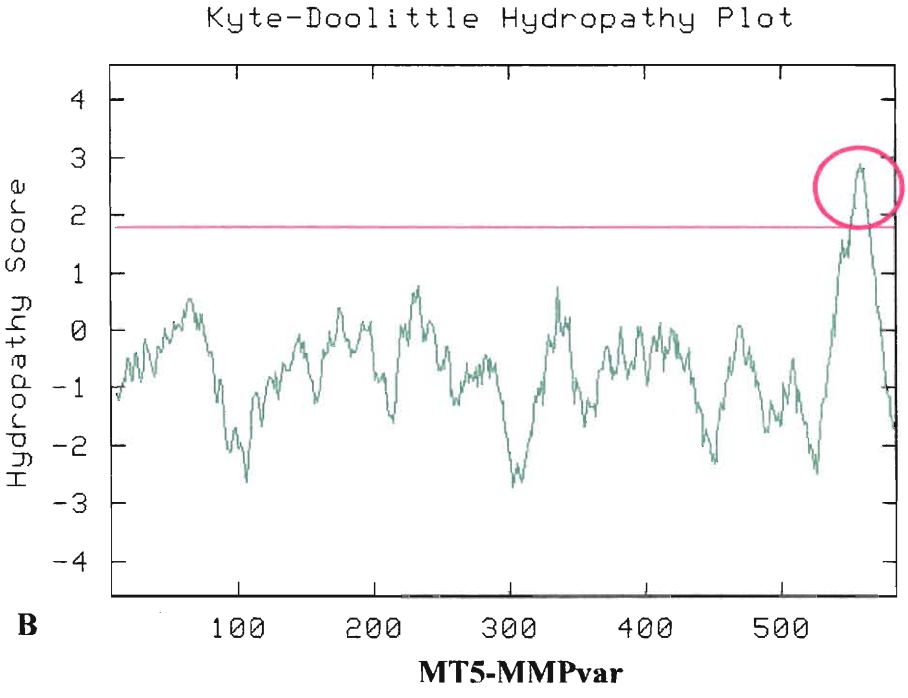
Figure 3.2. MT5-MMP and MT5-MMPvar amino acid alignment, domain identification and location of structural components. Each protein domain is listed above the amino acid sequence. The cysteine switch (PRCGVPD) is listed in bold blue; the conserved furin recognition motif (RXRXKR) in bold black and the zinc binding sequence (HELGHALGLEHS) in bold red. The signal peptide cleavage site is indicated with a black arrowhead. The furin cleavage site is indicated with a blue arrowhead. N-myristoylation sites of interest are underlined in red. A single amino acid change (Y→H) is shaded in grey.

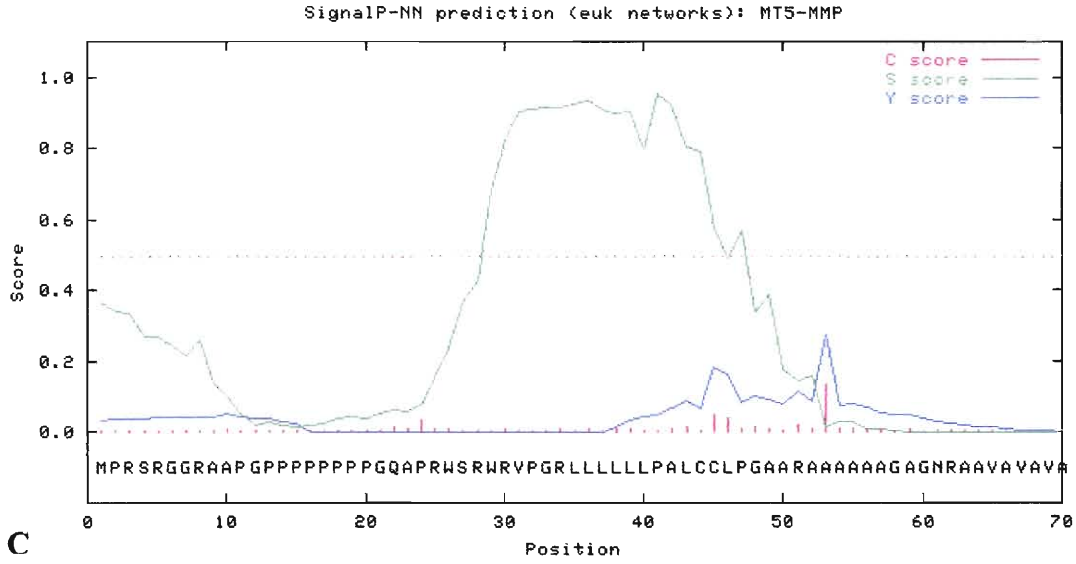
peptide contained adequate hydrophobicity to direct membrane insertion. MT1-MMP was used as a positive control (Appendix). Kyte-Doolittle plots revealed that while both MT5-MMP and MT5-MMPvar possessed the known carboxy terminal hydrophobic amino acids corresponding to the transmembrane domain preceding the cytoplasmic tail, only MT5-MMP was predicted to possess adequate hydrophobicity for membrane insertion at the signal peptide sequence (Figure 3.3. A, B). Analysis with the SignalP database, used to predict the presence of a signal peptide based on the amino acid content, predicted that MT5-MMPvar does not possess adequate amino acid sequence to be identified as a classical signal peptide sequence (Figure 3.3. C, D).

MT5-MMPvar is predicted to be a Type-1 transmembrane protein

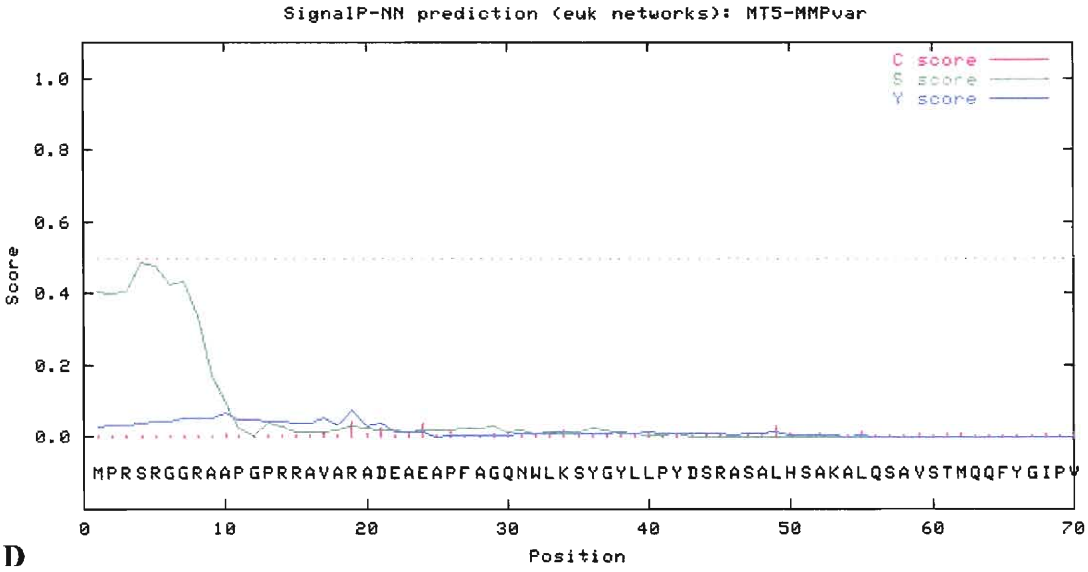
The predicted loss of the signal peptide in the MT5-MMPvar amino acid sequence led us to question if this would alter its insertion and orientation as a Type-I transmembrane protein. A combined predictive analytical tool, Phobius, was employed to analyze the protein sequence both for presence of signal peptide and effect on membrane orientation. This analysis confirmed the prediction that MT5-MMPvar did not possess a functional signal peptide, however predicted that the protein would insert into the plasma membrane in the same orientation as MT5-MMP, as a type-1 membrane protein with the amino terminus outside the cell (Figure 3.4. A, B). This analysis was confirmed using multiple analytical tools, and all results confirmed only one region of MT5-MMPvar, that of the transmembrane domain, was predicted to insert into plasma membrane structures. Taken together, these structural analyses predict that while this


Kyte-Doolittle Hydropathy Plot
Hydropathy Score
4
3
2
1
0
-1
-2
-3
-4
100
200
300
400
500
600
A
MT5-MMP


Kyte-Doolittle Hydropathy Plot
Hydropathy Score
4
3
2
1
0
-1
-2
-3
-4
100
200
300
400
500
B
MT5-MMPvar

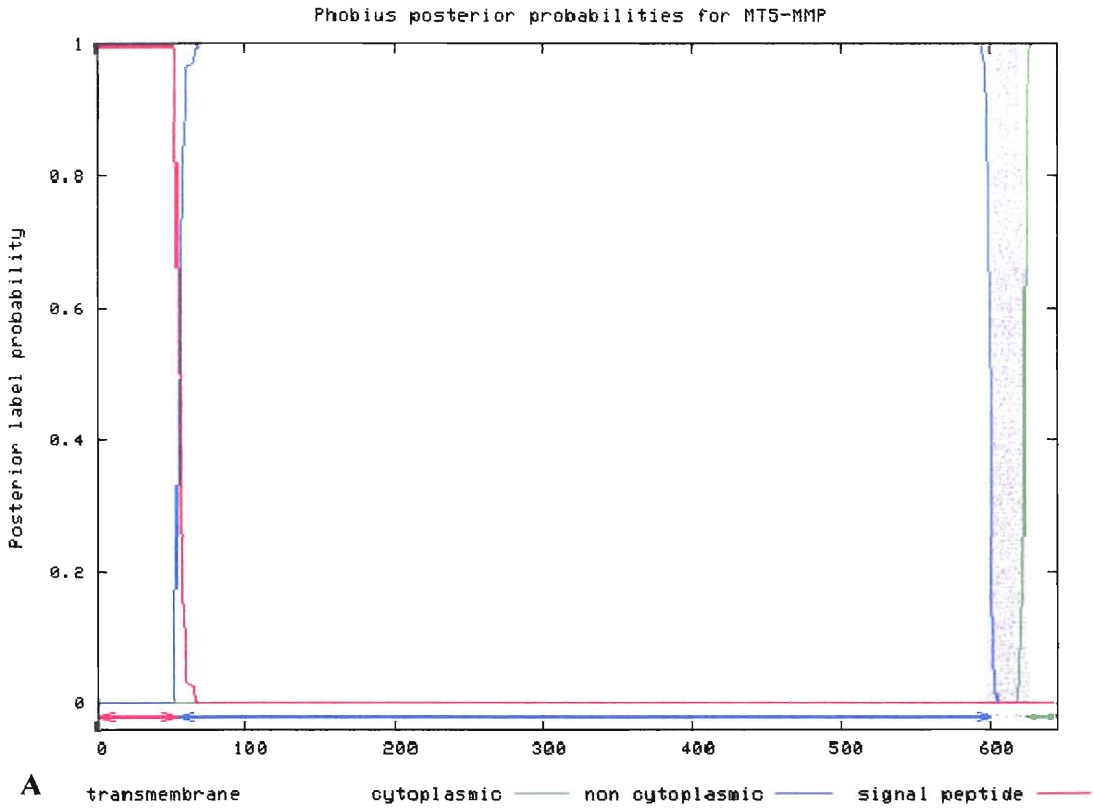

SignalP-NN prediction (euk networks): MT5-MMP
C score
S score
Y score
Score
1.0
0.8
0.6
0.4
0.2
0.0
MPRSRGGRAAPGPPPPPPPPPGQAPRWSRWRVPGRLLLLLLPALCCLPGAARAAAAAAGAGNRAAVAVAVA
0
10
20
30
40
50
60
70
Position

C


SignalP-NN prediction (euk networks): MT5-MMPvar
C score
S score
Y score
Score
1.0
0.8
0.6
0.4
0.2
0.0
MPRSRGGRAAPGPRRAVARADEAEAPFAGQNWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGIPV
0
10
20
30
40
50
60
70
Position

D

Figure 3.3. Hydropathic profile and SignalP analysis of MT5-MMP and MT5-MMPvar protein sequences. Kyle-Doolittle hydropathic profiles with a window of 19 were plotted for both MT5-MMP (A) and MT5-MMPvar (B) amino acid sequences. Peaks with a score greater than 1.8 represent regions strongly predicted to be transmembrane regions. In MT5-MMP two such regions are found while only one is found in MT5-MMPvar, all circled in red. The SignalP database was used to analyze MT5-MMP (C) and MT5-MMPvar (D) for the presence of sufficient signal peptide for direction to the endoplasmic reticulum plasma membrane and for the presence of signal peptide cleavage sites. C-score, S-score and Y-score predict signal peptide cleavage site, overall homology to signal peptide identity and combined cleavage prediction, respectively.


Phobius posterior probabilities for MT5-MMP
Posterior label probability
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
transmembrane
cytoplasmic
non cytoplasmic
signal peptide

A

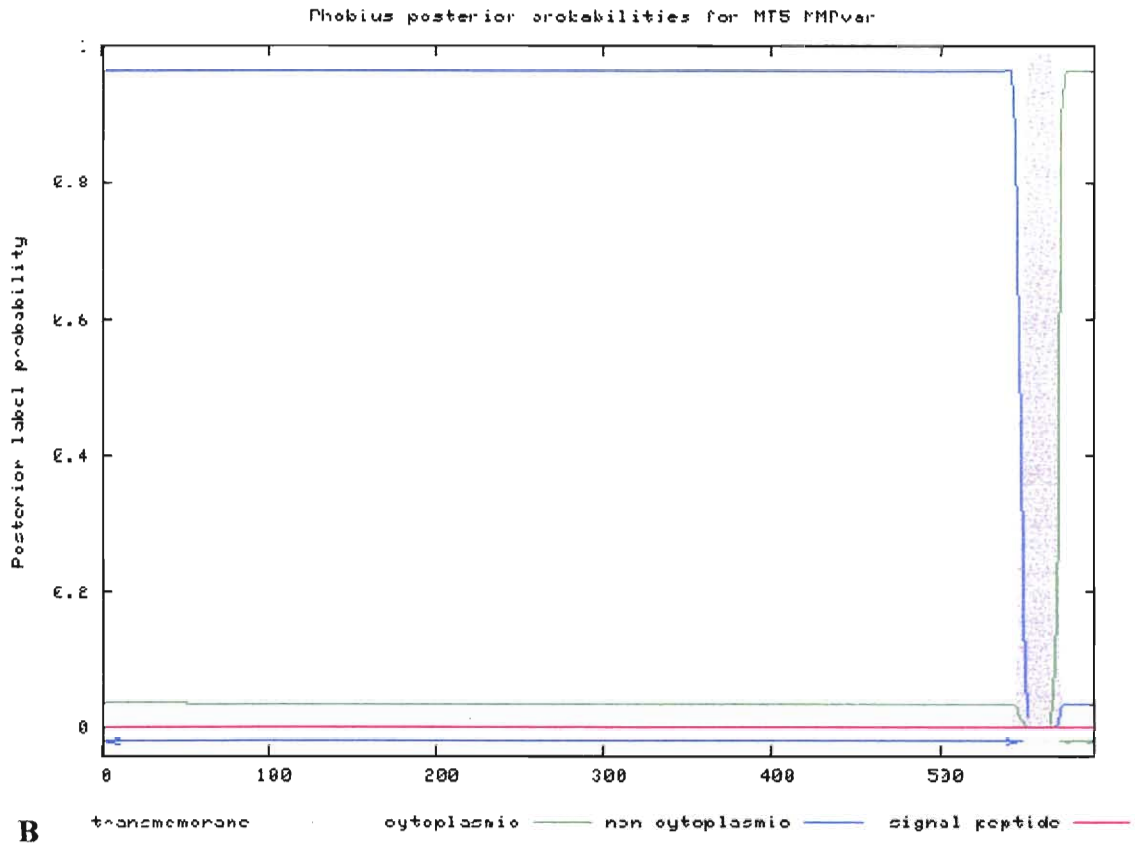


Figure 3.4. Combined prediction of signal peptide status and membrane orientation. Phobius analysis tool was utilized to analyze the sequences of both MT5-MMP (A) and MT5-MMPvar (B) and is designed to predict membrane orientation in combination with presence of signal peptide status. Signal peptide is depicted in red, non-cytoplasmic region in blue, insertion into the plasma membrane in grey and cytoplasmic region in green. Peaks represent strength of predictive signal and the arrows along the bottom confirm the model's prediction of peptide motifs and orientation. MT5-MMP has a distinguishing signal peptide prediction at the amino terminus (indicated by the red arrow) while in MT5-MMPvar this event is notably absent.

transmembrane protein is predicted to correctly insert into the plasma membrane, mechanisms for direction to the secretory pathway other than the classical signal peptide, thus differentiating MT5-MMPvar from MT5-MMP.

MMPvar Overexpression in COS-7 Cells

In order to directly test how MT5-MMPvar expression affects cell behavior, MT5-MMP-null COS-7 cells were stably transfected with MT5-MMPvar cDNA. MT5-MMPvar plasmid construct (pTARGET/MT5-MMPvar) or an empty pTARGET vector was transfected into COS-7 cells and selected using the antibiotic, G418. Following initial selection, individual colonies were isolated and expanded. Sixteen colonies containing pTARGET/MT5-MMPvar and nine colonies containing pTARGET were expanded for analysis. MT5-MMPvar expression status was confirmed by qPCR. All sixteen pTARGET/MT5-MMPvar clones expressed MT5-MMPvar to varying degrees while neither parental COS-7 cells nor pTARGET vector controls expressed any appreciable MT5-MMPvar (Figure 3.5.). Two sense constructs and one vector control construct were chosen for further analysis along with COS-7 parental cells.

Transfection of MT5-MMPvar does not exert a significant effect on COS-7 chemotaxis or invasion.

Previous work has shown that members of the MT-MMP subfamily act as mediators of cell motility through various migration and invasion assays. In order to determine if MT5-MMPvar could exert a similar effect on cell movement, parental

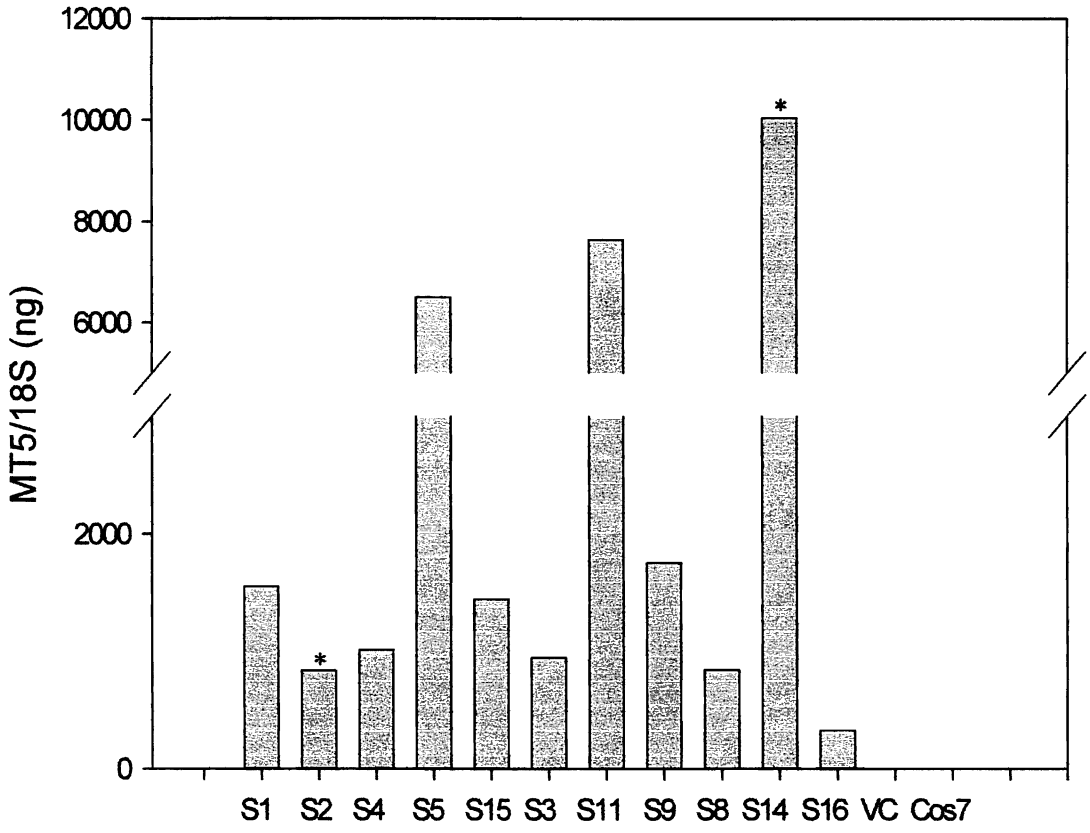


Figure 3.5. qPCR analysis of COS-7 cells stably transfected with MT5-MMPvar. Individual clones were maintained until confluence in G418 selection media (300 ug/ml). Total RNA was isolated and subjected to qPCR using primers against MT5-MMP as described in Chapter 2. Sixteen clones transfected with pTARGET/MT5-MMPvar (S1-S16) were found to express variable levels of MT5-MMPvar. One vector control cell line (VC) and COS-7 parental cells (Cos7) were null for MT5-MMP expression. An asterisk (*) denotes MT5-MMPvar- overexpressing cell lines selected for further analysis.

COS-7 cells, vector control cells and COS-7 cells over-expressing MT5-MMPvar were subjected to Neuroprobe chemotaxis and invasion assays. Cells (20,000/well) were plated on Neuroprobe membranes and positioned over a 96-well plate containing either 0% or 10% media. Initial experiments were conducted to assess the efficacy of cell migration through Neuroprobe membranes (Figure 3.6.). In the presence of serum, the migration of parental COS-7 cells (COS), vector control cells (VC) and sense MT5-MMPvar overexpressing cells (S2) was significantly higher than in the absence of serum at 24 hours. There was no difference in migration between the parental COS-7 cells (COS), vector control cells (VC) and sense MT5-MMPvar overexpressing cells in the presence or absence of serum.

We next wanted to determine if a difference in cell migration/invasion could be discerned using specific ECM molecules coated on the surface of the Neuroprobe membrane. Migration/invasion assays were conducted using Neuroprobe membranes coated with matrigel, a mixed-ECM protein provisional matrix, or CSPG, a novel substrate of MT5-MMP present in the nervous system. We hypothesized that there would be a significant increase in migration in the presence of CSPG in cells over-expressing MT5-MMPvar and that this effect could be blocked with both a non-specific MMP inhibitor and an MT5-MMPvar blocking peptide. Although not significant, there was a trend suggesting an increase in cell motility with the MT5-MMPvar overexpressing cells compared to vector controls in the presence of CSPG (Figure 3.7.). In the presence of the zinc-chelating metalloprotease inhibitor, σ-phenanthroline, migration of all cell lines on all matrix conditions was partially inhibited (Figure 3.8.-

3.10., second histogram bar in each series). This blockade of cell chemotaxis could therefore not be isolated to MT5-MMPvar expression alone. Inclusion of an MT5-MMPvar blocking antibody partially blocked cell motility in all cell lines and on all matrix conditions (Figure 3.8.-3.10., third histogram bar in each series) while inclusion of control IgG had no blocking effect (Figure 3.8.-3.10., fourth histogram bar in each series). Blockade with inclusion of MT5-MMP blocking antibody was similar to the extent of blockade with σ-phenanthroline with the exception of sense constructs in the presence of CSPG. Therefore, overexpression of MT5-MMPvar alone in COS-7 cells was not sufficient to result in a more motile or invasive phenotype. Inclusion of transiently transfected MMP-2 in preliminary experiments did not alter these results (n=1; data not shown).

Immunocytochemical analysis of MT5-MMPvar in COS-7 and cultured adult human progenitor cells.

Based on the computer analyses presented, MT5-MMPvar is predicted to lack a sufficient signal peptide and to remain a Type-I membrane protein. This protein variant is thus likely directed to subcellular locations via different pathways than published MT5-MMP. To begin to examine the localization of MT5-MMPvar, preliminary experiments were conducted using the MT5-MMPvar antibody made to a synthetic peptide to the region that is unique to the MT5-MMPvar protein (see Chapter Two for description of antibody). Immunoreactivity was seen in vector control COS-7 at low levels (Figure 3.11.A). More intense immunoreactivity was seen in MT5-MMPvar

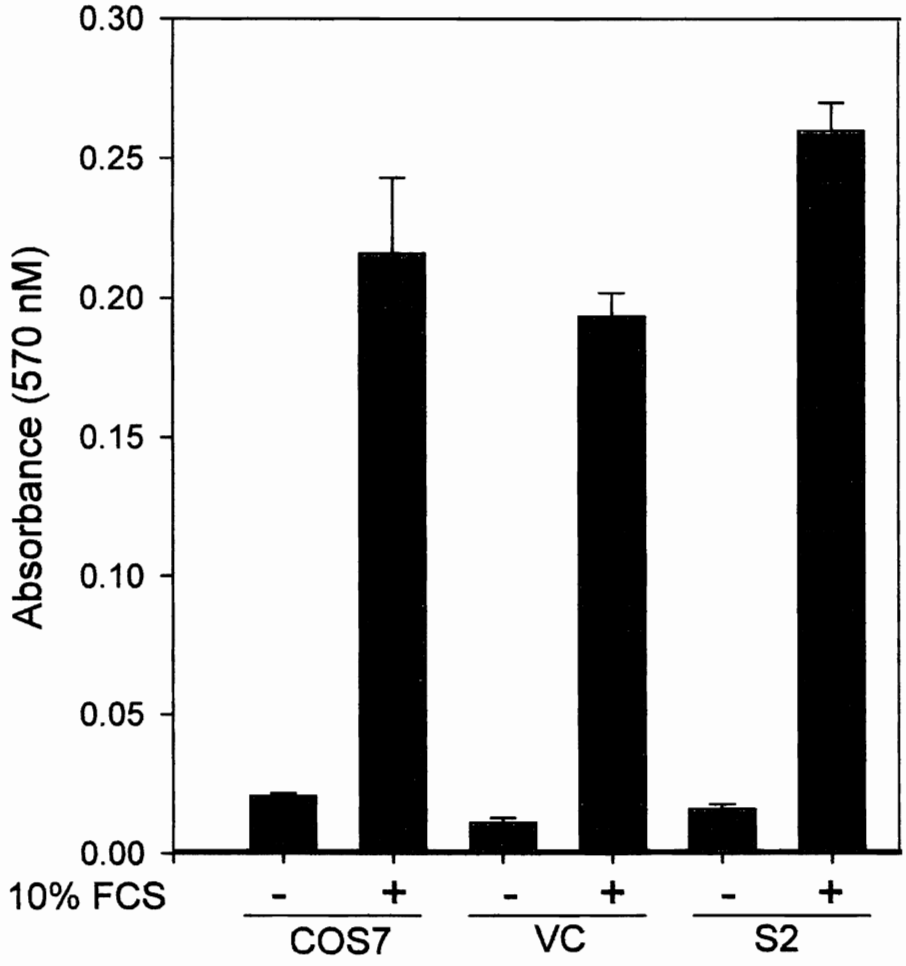


Figure 3.6. Chemotaxis of COS-7 cell lines as assessed by the Neuroprobe Assay. Parental COS-7 cells, cells overexpressing MT5-MMPvar (S2) or vector control (VC) cells were plated on Neuroprobe membranes in the presence or absence of 10% serum in lower chamber. After 24 hours, membranes were processed as described in the material and methods. Plates were read at 570 nm and background subtracted from raw data. Histogram bars represent column means ± standard deviation.

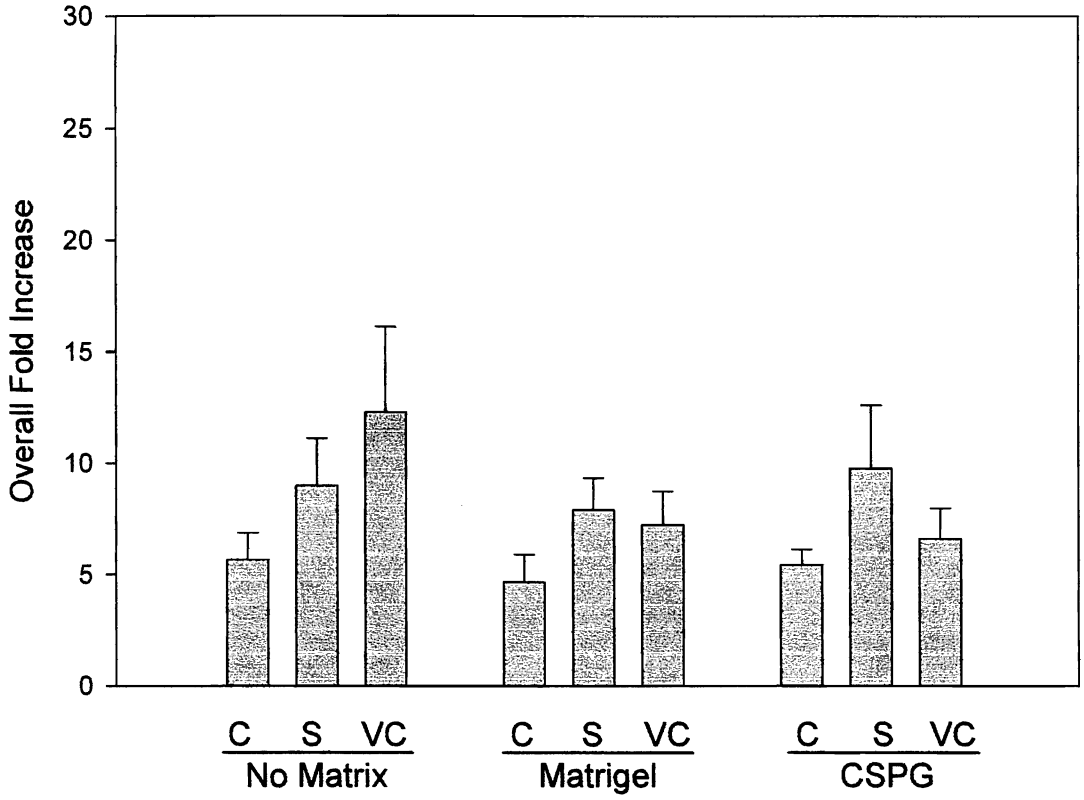


Figure 3.7. Serum induced COS-7 cell motility in the presence or absence of ECM proteins. Parental COS-7 cells (C), cells overexpressing MT5-MMPvar (S), or vector control (VC) cells were plated on Neuroprobe membranes containing no matrix, matrigel or CSPG. After 24 hours, membranes were processed as described in the material and methods. Fold increase of cell motility in the presence of 10% serum was calculated over baseline cell motility (no matrix, n=6; matrigel, n=4; CSPG, n=7). Histogram bars represent fold increase over all experiments conducted (column means ± standard error). Comparison between conditions was performed using Student's t-Test

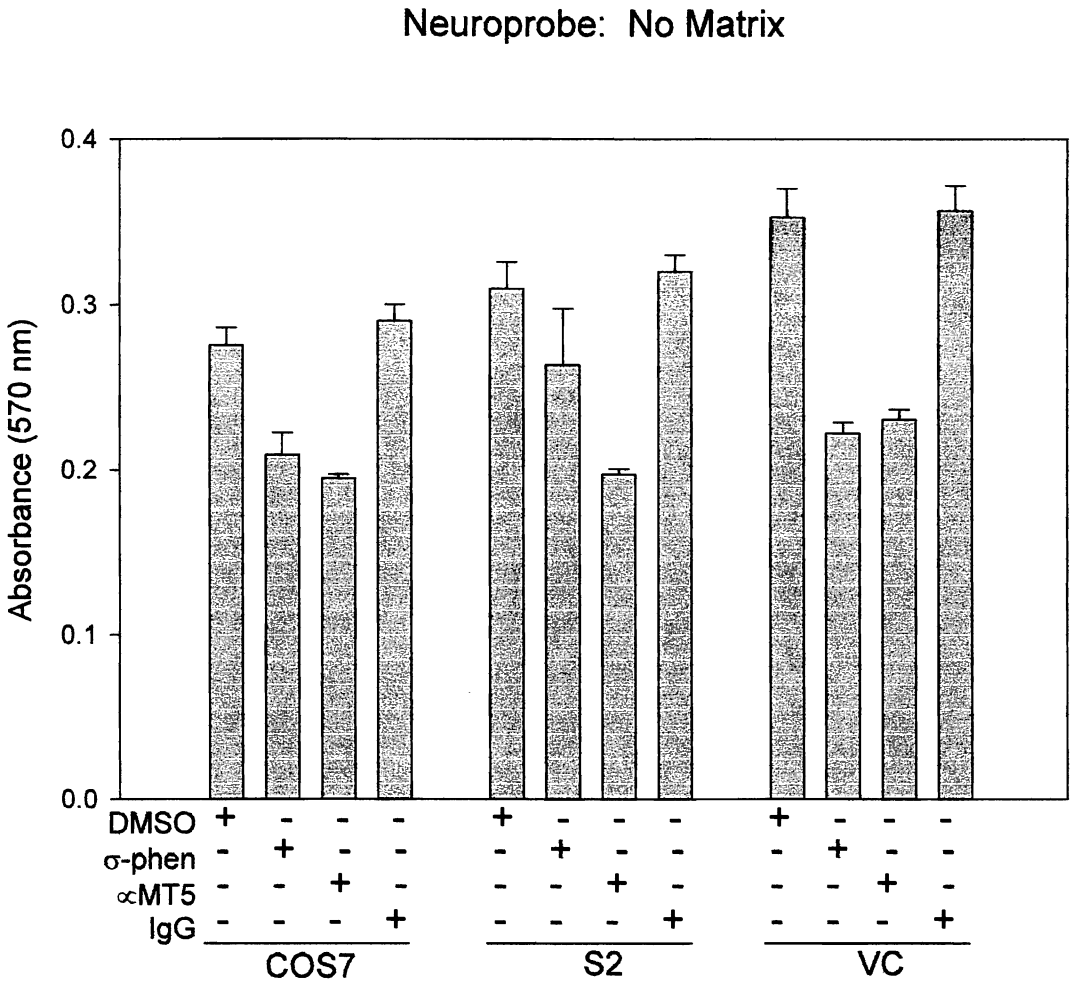


Figure 3.8. Effect of inhibitors on cell chemotaxis through neuroprobe membranes. Parental COS-7 cells (COS7), cells overexpressing MT5-MMPvar (S2) or vector control cells (VC) were plated on Neuroprobe membranes in the presence of 10% serum plus one of the following conditions: DMSO vector control, phenanthroline (50 ug/ml), MT5-MMPvar blocking peptide (1:200), control IgG (1:200). After 24 hours, membranes were processed as described in the material and methods. Plates were read at 570 nm and background subtracted from raw data. Histogram bars represent column means ± standard deviation.

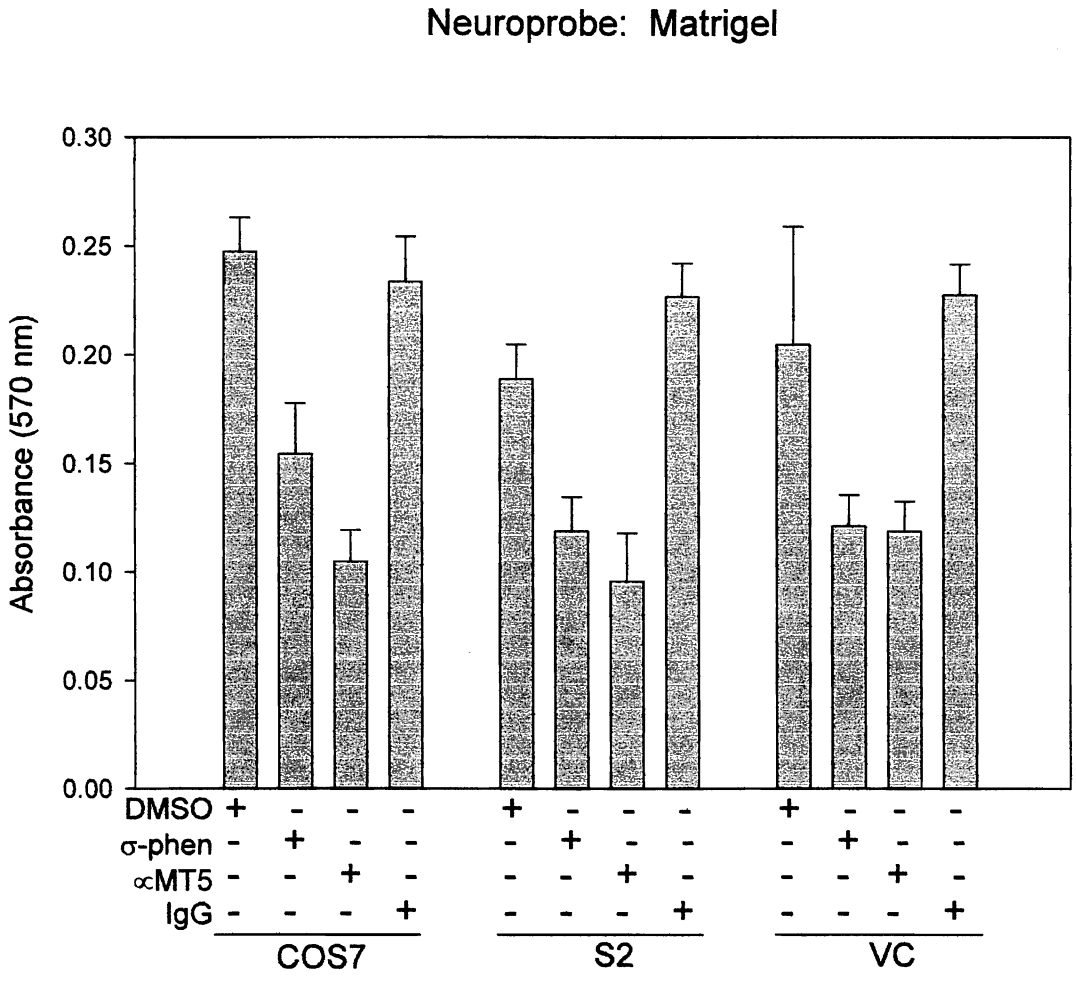


Figure 3.9. Effect of Inhibitors on cell invasion through matrigel. Parental COS-7 cells (COS7), cells overexpressing MT5-MMPvar (S2) or vector control cells (VC) were plated on Neuroprobe membranes coated with matrigel (diluted 1:12) in the presence of 10% serum plus one of the following conditions: DMSO vector control, phenanthroline (50 ug/ml), MT5-MMPvar blocking peptide (1:200), control IgG (1:200). After 24 hours, membranes were processed as described in the material and methods. Plates were read at 570 nm and background subtracted from raw data. Histogram bars represent column means ± standard deviation.

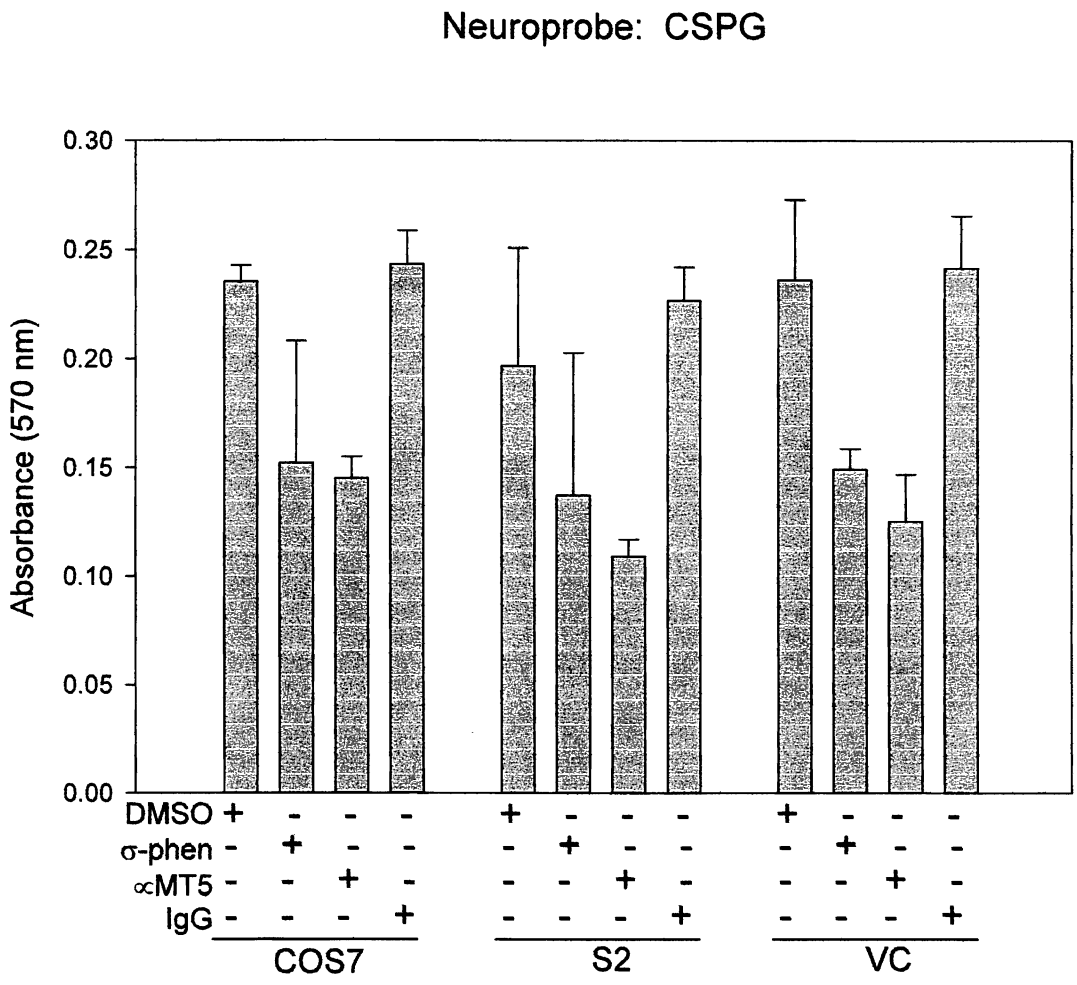


Figure 3.10. Effect of inhibitors on cell invasion through CSPG. Parental COS-7 cells (COS7), cells overexpressing MT5-MMPvar (S2) or vector control cells (VC) were plated on Neuroprobe membranes coated with CSPG (200 ug/ml) in the presence of 10% serum plus one of the following conditions: DMSO vector control, phenanthroline (50 ug/ml), MT5-MMPvar blocking peptide (1:200), control IgG (1:200). After 24 hours, membranes were processed as described in the material and methods. Plates were read at 570 nm and background subtracted from raw data. Histogram bars represent column means ± standard deviation.

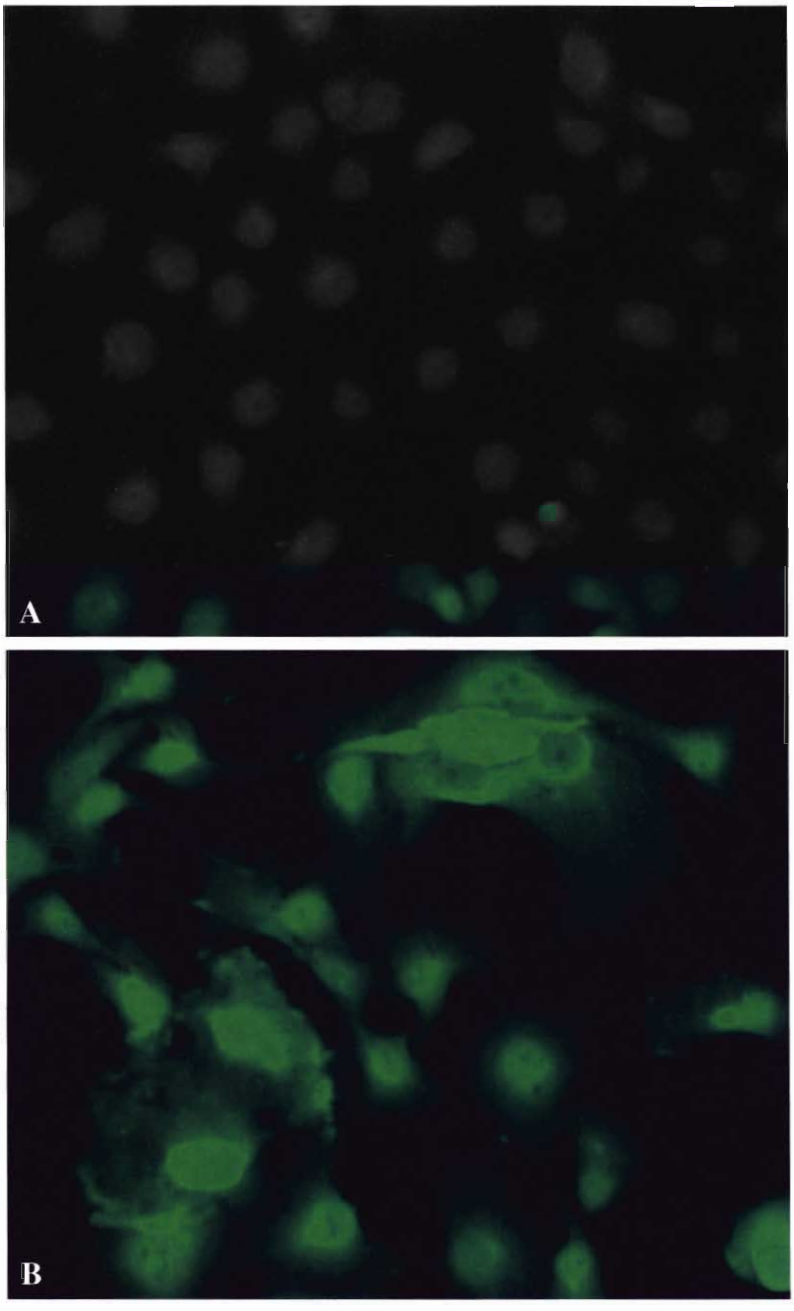


Figure 3.11. Immunocytochemical analysis of MT5-MMPvar in COS-7 cells. COS-7 cells stably transfected with vector control (A) or with pTARGET/MT5-MMPvar (B) were plated on 48 well plates and maintained in DMEM containing 10%FBS. Cells were fixed and stained as described in Material and Methods. MT5-MMPvar immunoreactivity was visualized using a FITC filter. N=1

overexpressing cells and appeared to accumulate in the perinuclear region and to a lesser extent on the plasma membrane (Figure 3.11.B).

Our previous work showed that MT5-MMPvar mRNA and protein was expressed in adult human cortical tissue (Chapter 2). We next wanted to extend these findings in cultured adult human neural progenitor cells cultured from these tissues and examine MT5-MMP cellular distribution by immunocytochemical methods. MT5-MMPvar was expressed in undifferentiated cells cultured in the presence of serum (Figure 3.12.B). No signal was seen with pre-immune serum (Figure 3.12.D) or with secondary antibody alone (Figure 3.12.F) controls. Neural differentiation was induced based on an established protocol (Vescovi et al., 2002) involving serum withdrawal and/or the addition of specific growth factors. Cells cultured in the presence of serum expressed MT5-MMPvar in the perinuclear region (Figure 3.13.B) however in cells subjected to serum withdrawal, MT5-MMPvar expression was seen throughout the cell and in process outgrowths (Figure 3.13.A). We have shown that administration of PDGF-BB facilitates the differentiation of cultured progenitor cells into immature neurons (McClain, Gigliotti, and Fillmore, 2006 abstract). MT5-MMPvar expression in cells treated with PDGF-BB in the presence of serum appeared to be localized to plasma membrane structures and in the small number of process outgrowths (Figure 3.14.B). PDGF-BB treatment of cells subjected to serum withdrawal resulted in an increased number of differentiating progenitor cells based on morphology and MT5-MMPvar expression appeared to be present throughout the cell and on process outgrowths (Figure 3.14.A). Staining with the neuronal marker, β-III-tubulin and the astrocytic

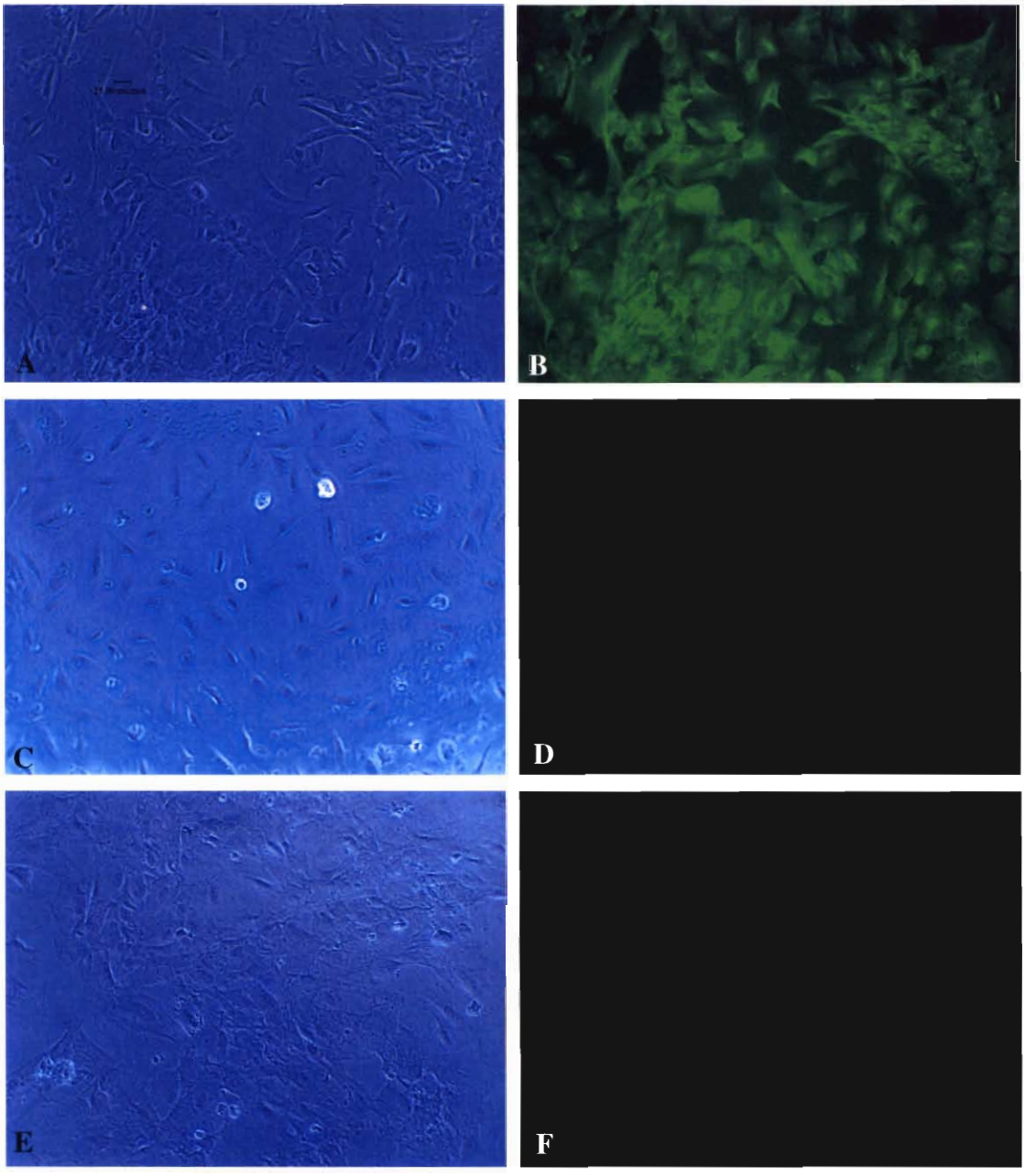


Figure 3.12. Immunocytochemical of MT5-MMPvar in cultured adult human cortical progenitor cells. Cultured cells were plated on 48 well plates and maintained in 10% DMEM. Cells were fixed and stained as described in Material and Methods. MT5-MMPvar immunoreactivity was visualized using a FITC filter. A., C., E., phase contrast images of cultured cells. B., MT5-MMPvar. D., Pre-immune serum. F., No primary antibody.

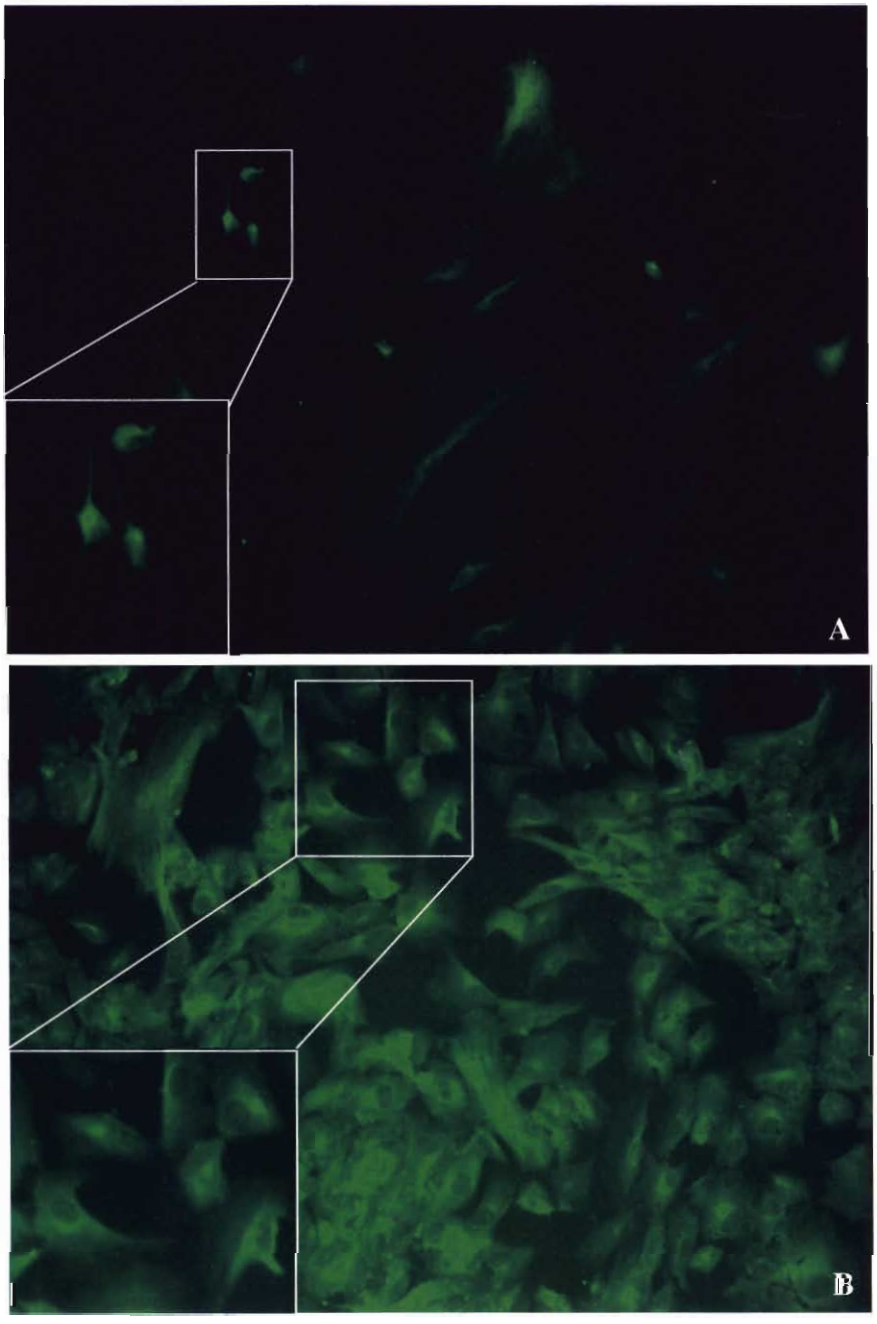

A
B

Figure 3.13. Immunocytochemical subcellular localization of MT5-MMPvar in adult human cortical precursor cells cultured with or without serum. Cultured cells were plated on 48 well plates and maintained in 0% or 10% DMEM. Cells were fixed and stained as described in Material and Methods. MT5-MMPvar immunoreactivity was visualized using a FITC filter. Following four days of serum withdrawal to induce differentiation (A), precursor cells undergo morphological changes and resemble a more mature cell type as compared to cells cultured in the presence of serum (B). MT5-MMPvar is localized to primarily intracellular membrane structures in non differentiated cells (B). MT5-MMPvar immunoreactivity is more strongly localized to process outgrowths following induction of differentiation (A).

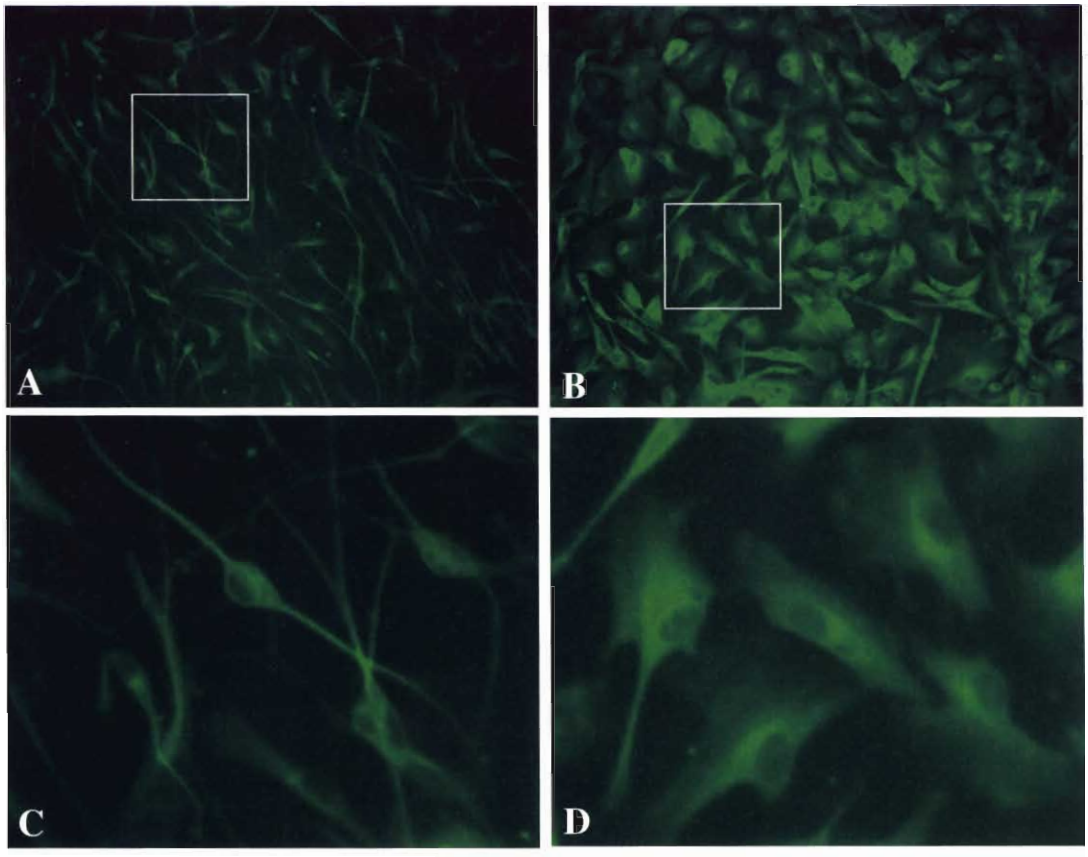


Figure 3.14. Immunocytochemical subcellular localization of MT5-MMPvar in adult human cortical precursor cells cultured with or without serum following treatment with PDGF. Cultured cells were plated on 48 well plates and maintained in 0% or 10% DMEM. All cells were treated with PDGF (30 ng/ml). Cells were fixed and stained as described in Material and Methods. MT5-MMPvar immunoreactivity was visualized using a FITC filter. PDGF treated cells cultured in the presence of serum (B) results in a more undifferentiated morphology compared to PDGF treated cells subjected to serum withdrawal (A). PDGF treatment results in a much more robust population of differentiated cells. MT5-MMPvar is localized to both intracellular membrane structures and cell processes in the presence of serum (B) and is localized strongly in process outgrowths in differentiating cells. (C). Closer view of box indicated in (A). (D). Closer view of box indicated in (B).

marker, GFAP, showed that cultures of differentiating MT5-MMPvar positive cells (Figure 3.15.A) were positive for both β-III-tubulin and GFAP (Figure 3.15.B), consistent with the phenotype of a maturing neural progenitor cell described by (Laywell et al., 2005). These cells were negative for fibroblast cell markers (data not shown). Taken together, this preliminary data suggests that MT5-MMPvar may be expressed in pre-differentiated and maturing neural progenitor cells and that upon differentiation cellular distribution changes from primarily perinuclear membrane structures to a more wide distribution pattern.

A possible murine homolog to human MT5-MMPvar can be detected in a neural stem cell niche.

Previous work has identified MMP-9 to be essential in the deployment of hematopoetic stem cells (Heissig et al, 2002). Because of the temporal expression of MT5-MMP in regions known to harbor a neurogenic niche in the adult, we hypothesized that MT5-MMP could play a role in neurogenesis. To begin this line of investigation, we were interested to further characterize MT5-MMPvar expression in the murine subventricular zone and rostral migratory stream. In this model, adult progenitors reside in the anterior subventricular zone and constitutively migrate along the rostral migratory stream to repopulate interneurons in the olfactory bulb (Gritti et al., 2002). Using our MT5-MMPvar specific antibody, MT5-MMP expression was observed in the subventricular zone, rostral migratory stream and olfactory bulb as well as in adjacent cortex (Figure 3.16.B). Densitometric quantification of protein levels

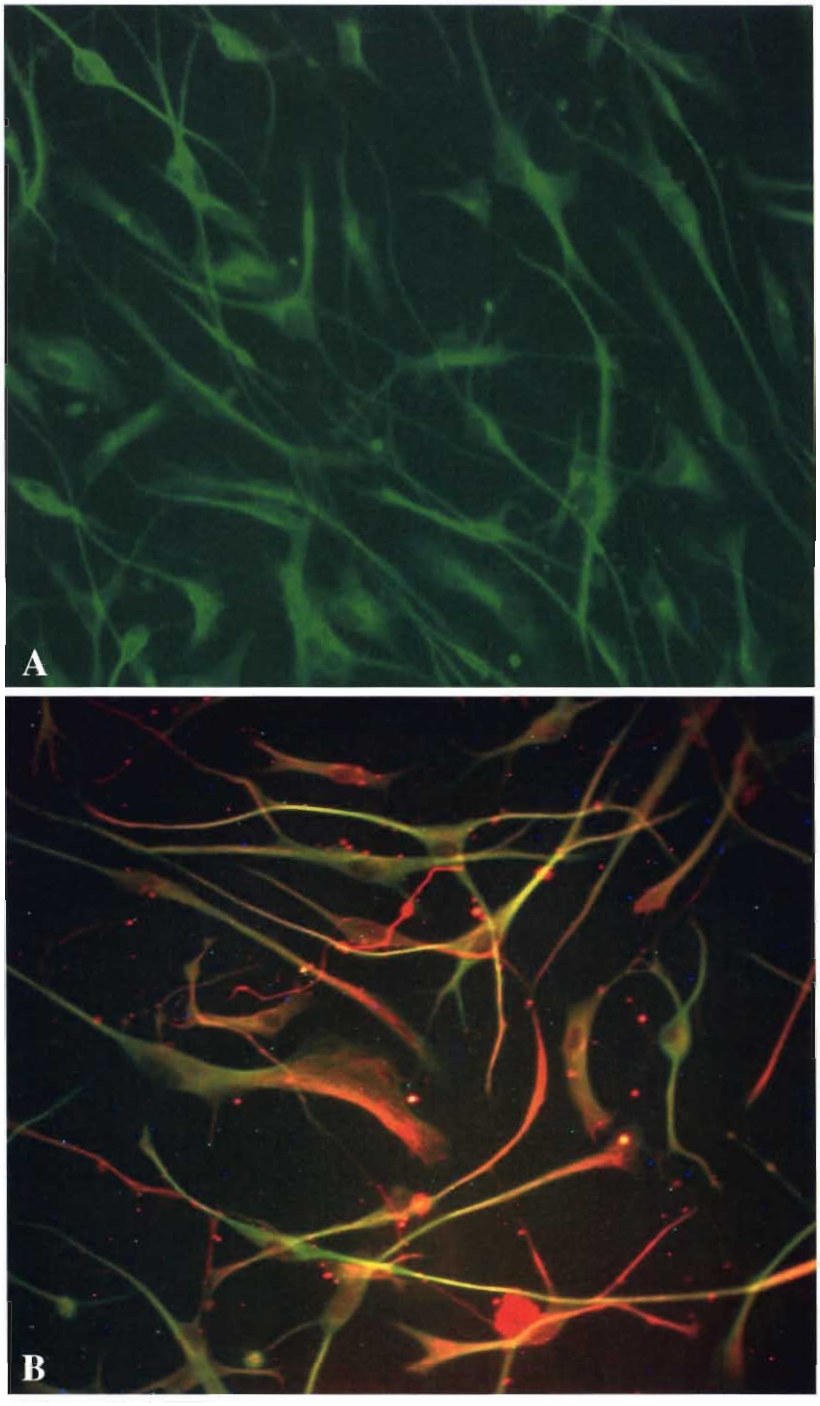

A
B

Figure 3.15. Expression of MT5-MMPvar and CNS cell specific markers in differentiating adult human cortical progenitor cells following treatment with PDGF-BB. Cultured cells were plated on 48 well plates and maintained in 0% DMEM. All cells were treated with PDGF-BB (30 ng/ml). Cells were fixed and stained as described in Material and Methods. MT5-MMPvar and GFAP immunoreactivity were visualized using a FITC filter. β-III-tubulin immunoreactivity was visualized with a Rhodamine filter. Images were merged using SPOT Software Version 3.2.4. for Windows. MT5-MMPvar expression is associated with the plasma membrane and on process extensions shown on a representative field of differentiating cells treated with PDGF-BB in the absence of serum (A). These MT5-MMPvar-expressing cells are both β-III-tubulin and GFAP positive (B), consistent with the maturing progenitor cell phenotype described by Laywell et al., 2003.

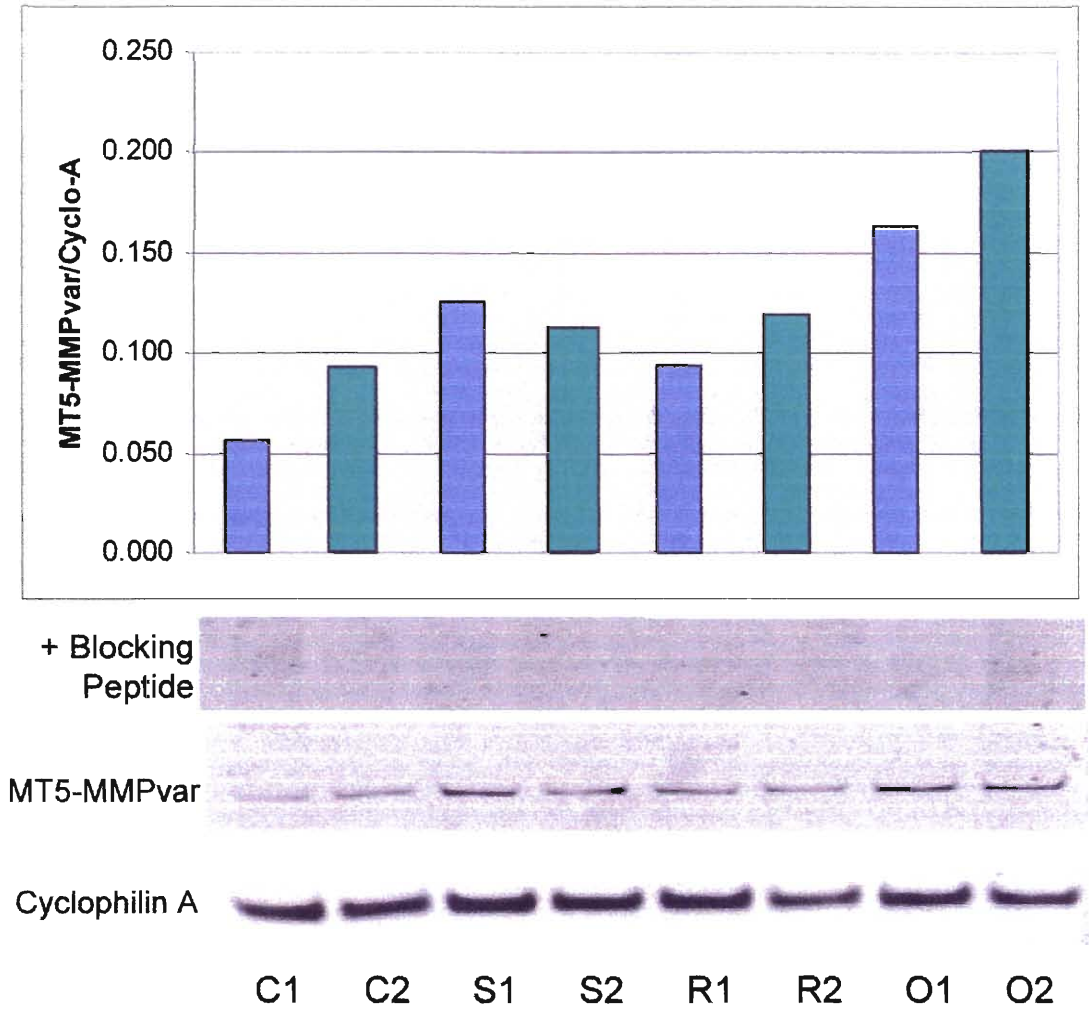


Figure 3.16. MT5-MMPvar expression in a murine model of neuronal progenitor cell mobilization. Tissue was obtained from the frontal lobe of the cortex (C), anterior subventricular zone of the ventricle (S), rostral migratory stream (R), and olfactory bulb (O) from two animals (1 and 2). Protein was isolated from tissue samples and subjected to western blot analysis as described in Material and Methods. A ~52 kDa protein was immunoreactive to the MT5-MMPvar antibody (B, top panel). Expression levels were compared to cyclophilin A loading control (B, bottom panel) and relative levels calculated by densitometry (A). Histogram bars represent the ratio of MT5-MMPvar expression to that of cyclophilin A.

indicated that while present in all regions tested, levels appear lowest in the cortex and highest in the olfactory bulb, the site of differentiation and integration of the migrated progenitor cells (Figure 3.16.A). This pilot study is the first to report that an alternative murine MT5-MMP transcript may exist and indicates its *in vivo* constitutive expression in a region of ongoing neural progenitor cell mobilization.

Discussion

In this work, a comprehensive database analysis was undertaken in order to outline important information regarding potential functional differences between MT5-MMP and MT5-MMPvar at the DNA and protein levels. We have previously reported that the 162 bp deletion at the 5′ end of the gene corresponds to portions of the signal peptide and propeptide domains. Interestingly, the DNA sequence in this deleted region found only in the published MT5-MMP sequence contains sets of trinucleotide repeats (CCG, CTG and GCG) that generate stretches of proline, leucine and alanine repeats (Llano et al, 1999). Of the MT-MMPs cloned to date, the published MT5-MMP transcript is the only MT-MMP reported to contain this region. These sequences, through the potential of being polymorphic in nature, allow for genetic diversity and multiple gene products (reviewed by Ashley and Warren, 1995). Whether the observed variance between the 5′ region of MT5-MMP and MT5-MMPvar transcript variants is due to the presence of these trinucleotide repeats is unknown. Further studies are required to determine if other mechanisms such as alternative splicing cause the generation of multiple MT5-MMP gene products.

The presented bioinformatic analysis confirmed that the functional motifs inherent to MMP family members are present in the MT5-MMPvar protein code (propeptide domain), specifically the cysteine switch and furin cleavage site, important for latency and activation, respectively. The most significant structural feature lost in MT5-MMPvar is a sufficient signal domain and the signal peptide cleavage site. This loss of the signal peptide could result in the loss of co-translational direction to the ER for processing; however this has not been examined. The ultimate consequence of this loss and on the loss the two N-myristolyation sites on cellular trafficking, distribution, and function is unknown.

qPCR analysis verified that MT5-MMPvar was overexpressed in COS-7 cells but was not expressed in either parental or vector control cells. All COS-7 cell lines, when assessed for chemotaxis and invasion, were able to adequately respond to serum, confirming the presence of cellular machinery in COS-7 cells to mediate this phenotype. No robust increase in cell chemotaxis or invasion was observed in the presence of ECM proteins and/or serum in MT5-MMPvar overexpressing cells above vector control or parental cells. Inhibition with σ-phenanthroline was effective in all cell lines, suggesting the presence of other metalloproteases or zinc-dependent proteins sensitive to treatment that participate in cell motility. Unexpectedly, inhibition with MT5-MMP blocking antibody also occurred in all cell lines, presumably due to either a nonspecific response to the immune peptide or specific interaction with a similar protein inherent to COS-7 cells. These results suggest that MT5-MMPvar overexpression alone was not sufficient to create a more motile phenotype in the system utilized, raising the

question of whether MT5-MMP was localized to the membrane to mediate this response.

It is possible that MT5-MMPvar alone does not mediate motility and invasion directly, but acts through the activation of MMP-2. A preliminary western blot and zymogram analysis modeled after previous work (Pei, 1999) suggests that MT5-MMPvar in the COS-7 experimental system may not activate MMP-2 (data not shown). It is possible that MT5-MMPvar is not localized to the membranes of COS-7 cells which would be needed for substrate remodeling of the ECM or activation of MMP-2. However, the immunocytochemical results suggest that in some of the stably transfected cells the MT5-MMPvar may be associated with the cell membrane. Experiments designed to examine MT5-MMPvar expression in isolated subcellular preparations in addition to more sensitive immunohistochemical examination using confocal imaging are needed to address these issues.

Immunocytochemistry performed on undifferentiated neural progenitor cells isolated from adult human cortex demonstrated that endogenous MT5-MMPvar was similarly found diffusely within the cell and a high amount was found in the perinuclear region. Two stimuli for differentiation of cultured progenitor-like cells, serum withdrawal and treatment with PDGF-BB, resulted in the redistribution of MT5-MMPvar protein to process outgrowths and a reduced concentration in the perinuclear membrane structures. It is therefore likely that signals such as this would be required in COS-7 cells to facilitate the recruitment of MT5-MMPvar to the cell membrane to mediate cell migration/invasion and activation of MMP-2.

We previously demonstrated that MT5-MMPvar is expressed in an *in vitro* model of neurogenesis and neuronal differentiation. We examined MT5-MMPvar expression in a region known to harbor a constitutively active pool of adult progenitor cells in the murine brain in order to extend these findings. Western blot analysis revealed that MT5-MMPvar was present in the regions tested, suggesting the existence of a murine homolog to this gene transcript. These results also indicated that MT5-MMPvar levels were highest in the olfactory bulb, the site of neuronal differentiation and functional integration of the progenitor cell pool. These results support previous findings of increased expression of MT5-MMPvar in maturing neuronal cell types. An important avenue of future investigation is to confirm the existence of such a homolog and begin investigations of its potential function in this model.

Taken together, these results raise important questions regarding the cellular processing and localization of MT5-MMPvar. Published work has shown that MT5-MMP is subject to multiple cellular trafficking pathways (discussed below), and our findings provide insight into future lines of investigation. The known cellular trafficking pathways for MT5-MMP are schematically depicted in Figure 3.17. The published sequence, having a conventional signal peptide sequence, is co-translationally directed to the ER plasma membrane (Figure 3.17., Pathway 1). Intracellular activation in the Golgi network occurs at a furin activation site present in the propeptide (Green diamond, Pathway 1), and MT5-MMP is then directed to the cell membrane as an activated enzyme (Pathway 1). MT5-MMP is also cleaved at a second site of furin activation (Green diamond, Pathway 2) (Wang and Pei, 2001). Pei's work identified a

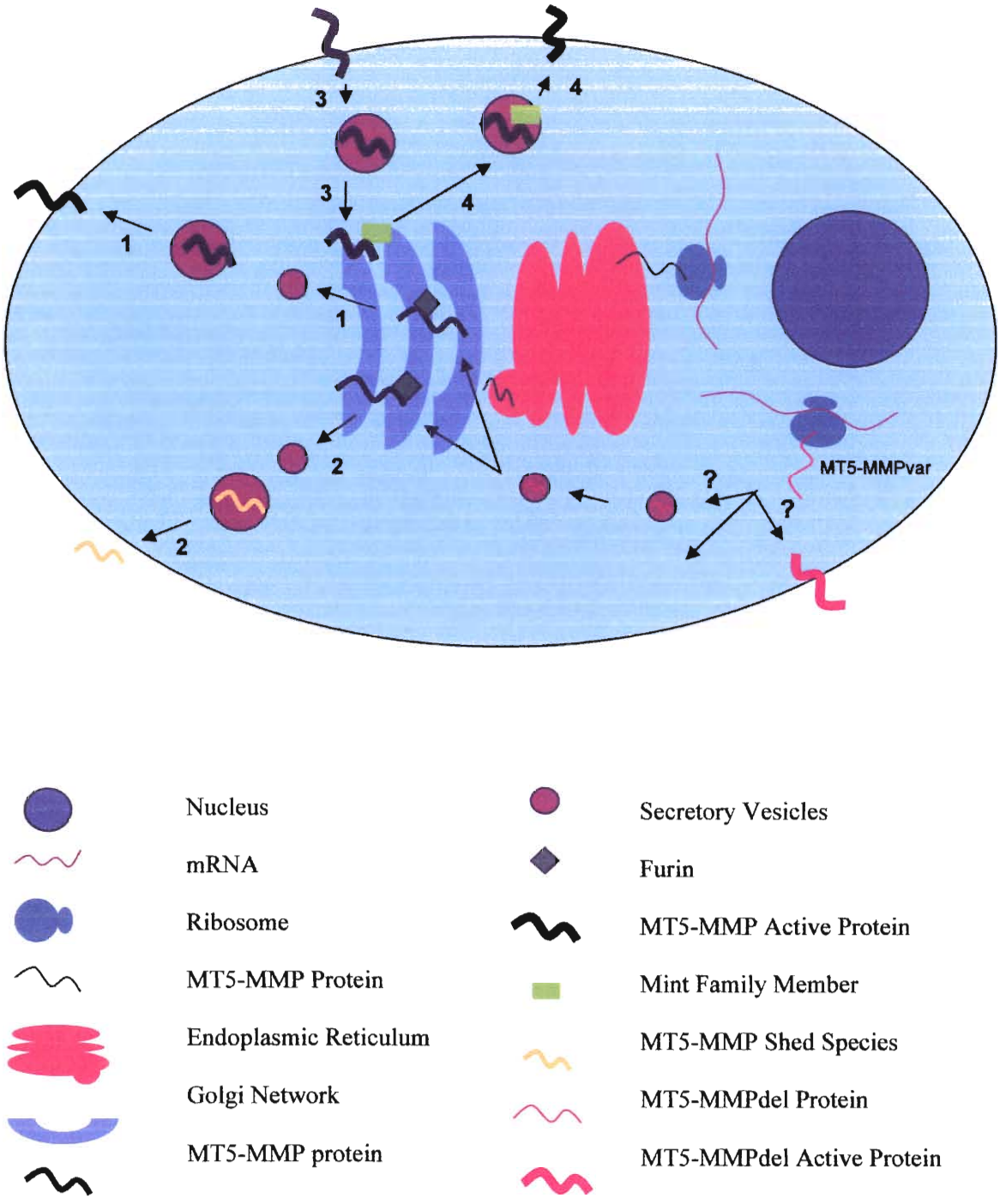


Figure 3.17. Schematic representation of MT5-MMP cellular trafficking

novel furin activation site in the stem region that is cleaved also in the Golgi network. Cleavage of MT5-MMP at this site results in a form of MT5-MMP that is shed from the cell as a secreted protein (Figure 3.17., Pathway 2). This serves to downregulate its presentation on the cell membrane and its activation of MMP-2 (Wang and Pei, 2001). MT5-MMP, once presented on the membrane, is also known to be constitutively endocytosed through a clatharin-dependent pathway and is chaperoned back to the cell membrane by Mint family proteins, presumably as another pathway to limit constitutive membrane localization (Figure 3.17., Pathway 3) (Wang et al, 2002).

The loss of the signal peptide in MT5-MMPvar suggests that MT5-MMPvar is not initially directed to the secretory pathway by the conventional manner. Other pathways for protein secretion independent of the classical signal peptide have been reported. FGF-9, a glycosylated neurotrophic protein highly expressed in the brain, has no classical signal peptide and is co-translationally directed to the endoplasmic reticular plasma membrane through a novel hydrophobic domain (Miyakawa et al., 1999). However, analysis of MT5-MMPvar did not identify such a hydrophobic domain. An alternate means of direction to the secretory pathway has been proposed for MMP family members lacking the classical signal peptide (Kimura et al., 2001), but specific mechanisms have not been identified.

MT5-MMPvar is still predicted to orient to the cell membrane as a type one membrane protein due to the retention of the carboxy terminus transmembrane domain and cytoplasmic tail. Immunocytochemical results suggest that under certain conditions MT5-MMPvar is recruited to the cell membrane. Future work should focus on the

elucidation of how direction of MT5-MMPvar to the cell membrane is achieved (Figure 3.17., pathway 4). Our preliminary work suggests that PDGF-BB is one candidate growth factor resulting in this membrane localization, and future study should focus on the elucidation of the role of MT5-MMPvar in neuronal maturation and in progenitor cell biology.

Chapter 4

GENERAL DISCUSSION

MT5-MMP is expressed ubiquitously in the developing CNS and is temporally restricted to regions of plasticity in the postnatal and adult CNS (Jaworski, 1999). MT5-MMP has been shown be induced during neuronal differentiation and to facilitate neurite outgrowth (Hayashita-Kinoh et al., 2001). Recent reports indicate that MT5-MMP is associated with synaptic proteins and plays a role in reactive synaptogenesis and perhaps in physiological synaptic function (Komori et al, 2004; Monea et al., 2006). This emerging literature indicates that MT5-MMP may play a significant role in neuronal development and function. The objective of these studies was to examine MT5-MMP expression in adult human brain tissue and in NT2-hNT cells, a model of neurogenesis and neural differentiation as well as to begin investigations of MT5-MMP function by *in vitro* overexpression experiments. Unexpectedly, while trying to clone MT5-MMP cDNA for functional experiments, we discovered a novel cDNA transcript (MT5-MMPvar).

MT5-MMP cDNA was cloned from multipotent NT2 cells and these cloning experiments yielded a transcript (MT5-MMPvar) sharing 92% homology to the published MT5-MMP cDNA. *In vitro* translation studies showed that MT5-MMPvar

cDNA translated into an approximately 52 kDa protein, different from the previously reported 64 or 58 kDa species corresponding to the propeptide and active enzyme, respectively (Llano et al., 1999; Romanic et al., 2001). Both MT5-MMPvar mRNA and protein were expressed in NT2 cells; however MT5-MMPvar protein was significantly higher in their neuronal counterparts, hNT cells. In addition, MT5-MMPvar RNA and protein were expressed in adult human hippocampal tissue.

Functional studies showed that cellular motility and invasion was not significantly altered in COS-7 cells engineered to overexpress MT5-MMPvar. Subsequent experiments examining MT5-MMPvar cellular distribution showed that MT5-MMPvar was primarily localized to intracellular membrane structures rather than being heavily recruited to the cell membrane. Immunocytochemistry experiments further revealed that endogenous MT5-MMPvar was expressed in adult human neural progenitor cells, and that when these cells were induced to differentiate into immature neurons, MT5-MMPvar cellular distribution became more localized to process outgrowths. Finally, experiments probing murine brain tissue from adult neurogenic regions revealed an immunoreactive protein of approximately 52 kDa using an antibody specific for human MT5-MMPvar, suggesting that a murine homolog could exist in a region of constitutive adult progenitor cell mobilization. Taken together, these data identify a novel human MT5-MMP transcript, MT5-MMPvar, and show that its expression and cellular distribution is regulated in neuronal cells (hNT) and cultured adult human neural progenitor cells. In addition, these data demonstrate the presence of MT5-MMPvar protein in a murine region of adult neurogenesis.

Increases of the MT5-MMPvar protein product in differentiated hNT cells were shown using western blot analysis and a MT5-MMPvar transcript-specific antibody designed in our laboratory. RT-PCR analysis utilizing primers that amplified the 5′ end of the MT5-MMP gene showed that both transcripts were present at the RNA level. When RNA levels were quantitated, total MT5-MMP levels (probed in a common region of the two transcripts) increased in hNT cells, however quantitation of each of the two transcripts could not be conducted due to the nucleic acid composition within the area of divergence. This region is a high-GC content region and a suitable Taqman Probe could not be designed using current resources. Future studies should aim to quantitate MT5-MMP and MT5-MMPvar transcripts through alternate qPCR strategies or techniques such as RNA Protection Assay (RPA) which can identify and quantitate multiple RNA products.

The mechanism responsible for the generation of this transcript variant is currently not known. Alternative splicing is one possibility, as this editing mechanism has been described for MMP family members such as MMP-8, MMP-1 and MMP-13 (Hu et al., 1999). Efforts to complete the sequence of the 5′ end of the human MT5-MMP gene to conduct splicing analysis and further studies has been limited due to conflicting genomic data (Appendix Figure 2). Methodological limitations associated with mapping this region support similar difficulties reported by others (Llano et al., 1999). Future studies should continue efforts at genomic mapping of the first intron and exon of the human MT5-MMP gene. The RPA experiments proposed above could also aid in the characterization of MT5-MMP transcripts present in the cell.

As mentioned above the protein product of the transcript identified in this work translates into an approximately 52 kDa protein, and this protein product was detected in NT2 cells, higher in hNT cells and was also detected in adult human hippocampal tissue and in murine subventricular zone, rostral migratory stream and olfactory bulb. The expression of the MT5-MMPvar protein using a cDNA *in vitro* translation system was not compared to the published MT5-MMP open reading frame, the cDNA of which was never obtainable by the standard cloning methods employed. Future studies will seek to clone the published MT5-MMP reading frame using alternate cloning strategies and conduct pilot protein expression studies to determine if this entire open reading frame can translate into protein, as this has not been shown. Comparative functional analyses would follow.

The described MT5-MMPvar transcript lacks a sufficient signal peptide. The newly joined sequence resulting from the deletion did not encode a de novo signal peptide sequence at the amino terminus for direction to endoplasmic reticular membrane insertion. Based on computer assisted modeling it is predicted that the most significant functional implication of the MT5-MMPvar sequence variations is to effect its direction into the ER for processing. How the MT5-MMPvar transcript is intracellularly processed is currently unknown. Future lines of investigation should comparatively assay MT5-MMP and MT5-MMPvar transcripts in a eukaryotic expression system and in this system test for co-translational processing and post-translational modifications (Ju et al., 1999). Further, confocal imaging studies would yield more conclusive information on MT5-MMPvar subcellular localization. Future

work directed at identifying the cellular trafficking of the MT5-MMPvar peptide would aid in the understanding of where this protein resides in the cell and the nature of its intracellular processing

When comparing the two cDNA sequences, the major difference is the deletion of 45 residues of the 58 amino acid signal peptide. This region in the published MT5-MMP does not share significant sequence homology with other MT1-MMP, MT2-MMP or MT3-MMP (Type-I transmembrane MT-MMPs) and shares little homology to MT4-MMP and MT6-MMP (GPI-anchored MT-MMPs). Within this region of the published MT5-MMP are two sites of N-myristoylation. It is reported that interaction of N-myristoylated side chains stabilize protein aggregates on the cell membrane (Sankaram, 1994), however, an understanding of how this affects MT-MMP aggregation has not been studied. In addition to the N-myristoylation sites, this region also contains a region of trinucleotide repeats which are deleted from the MT5-MMPvar sequence. The observed trinucleotide repeats present on the published MT5-MMP sequence contribute to genetic diversity (reviewed by Ashley and Warren, 1995), but it is not clear if this region of repeats contributes to the generation of the variant form of MT5-MMPvar. Another interesting observation within this region of the published MT5-MMP sequence is that it contains four potential binding sites for signaling molecules belonging to the SH3 and Erk kinase family, however the functional implications of these sites are unknown.

Results from the functional and immunocytochemical studies presented suggest that in COS-7 cells MT5-MMPvar can successfully be overexpressed, MT5-MMPvar

appears to localize mainly to perinuclear membrane structures at baseline and MT5-MMPvar does not confer a more migratory or invasive phenotype using the Neuroprobe migration and invasion assay. We predicted that the overexpression of MT5-MMPvar would result in an increase in migration. With the addition of known MT5-MMP ECM substrates we predicted that the mechanism of increased migration would be due to its own proteolytic activity. Another predicted mechanism is the activation of MMP-2 via interactions with TIMP-2 and MT5-MMP. There are several potential reasons for the negative migratory results. One is the cellular localization of MT5-MMPvar. For example, if not localized to the cell membrane, it would not be available to associate with membrane-localized tertiary complexes involving MMP-2 and TIMP-2. This could be due to cellular localization of MT5-MMPvar in the conditions tested or the lack of TIMP-2 in this system. While the experimental paradigm utilized recapitulated those previously reported (Pei, 1999), these studies have been conducted in an alternate cell type (MDCK) and other cellular proteins involved in this cascade such at TIMP-2 or Claudin-5 (Miyamori et al., 2001) may not be expressed in COS-7 cells. Future experiments should investigate the activation of MMP-2 by MT5-MMPvar by ensuring membrane localization of MT5-MMPvar in experimental conditions and by determining if the cells utilized express TIMP-2.

Future work should include comparative functional studies between different cell types overexpressing MT5-MMP and MT5-MMPvar cDNA constructs in order to determine if these transcripts behave similarly in the same cell types. Readily available for overexpression studies in our laboratory are the NT2 neuronal differentiation model,

multipotent cells harvested from adult human hippocampal samples and COS-7 cells. These studies would extend the current work by helping to understand if this protein is, under basal levels, processed in the cell for post-translational modifications and/or presented on the cell membrane to participate in known MT5-MMP cell functions, such as MMP-2 activation. It could also be determined if MT5-MMPvar functions in a cell-type specific manner. Based on preliminary work in which MT5-MMPvar appears to undergo cellular redistribution under signals directing differentiation, additional studies could be planned to more directly examine MT5-MMPvar processing in response to growth factor stimulation.

Essential to the understanding of signals altering MT5-MMP transcriptional regulation is the cloning and identification of the proximal promoter. For example, administration of RA has been reported by us and others to result in increased MT5-MMP RNA levels, possibly through transcriptional activation (Hayashita-Kinoh et al., 2001; Ross and Fillmore, unpublished observation). These studies, following the physical mapping of the 5′ end of the gene, and the identification of cis-responsive elements would greatly assist in the characterization of transcriptional control of the MT5-MMP gene.

In sum, these studies raise interesting questions regarding the nature of the MT5-MMP gene and its ultimate function in the developing and mature CNS. This work has identified a novel transcript variant of the human MT5-MMP gene, MT5-MMPvar, which is likely subject to alternate regulatory and/or trafficking pathways compared to the published MT5-MMP. The pathways resulting in the processing,

activation and membrane presentation of MT5-MMPvar are currently unknown. Further, the functional role of MT5-MMPvar in cell migration, invasion, neurogenesis and neuroplasticity is not understood. The significance of the presence of two MT5-MMP transcripts in the developing and mature CNS is not clear and should be the focus of future work. It should be considered that the activity of a membrane-bound degradative enzyme in a post-mitotic neuron is likely subject to additional methods of regulation and cellular distribution. Such CNS-specific levels of regulation are undocumented in MMP literature but should be considered in work seeking to further our understanding of MMP function in CNS development and plasticity.

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APPENDIX

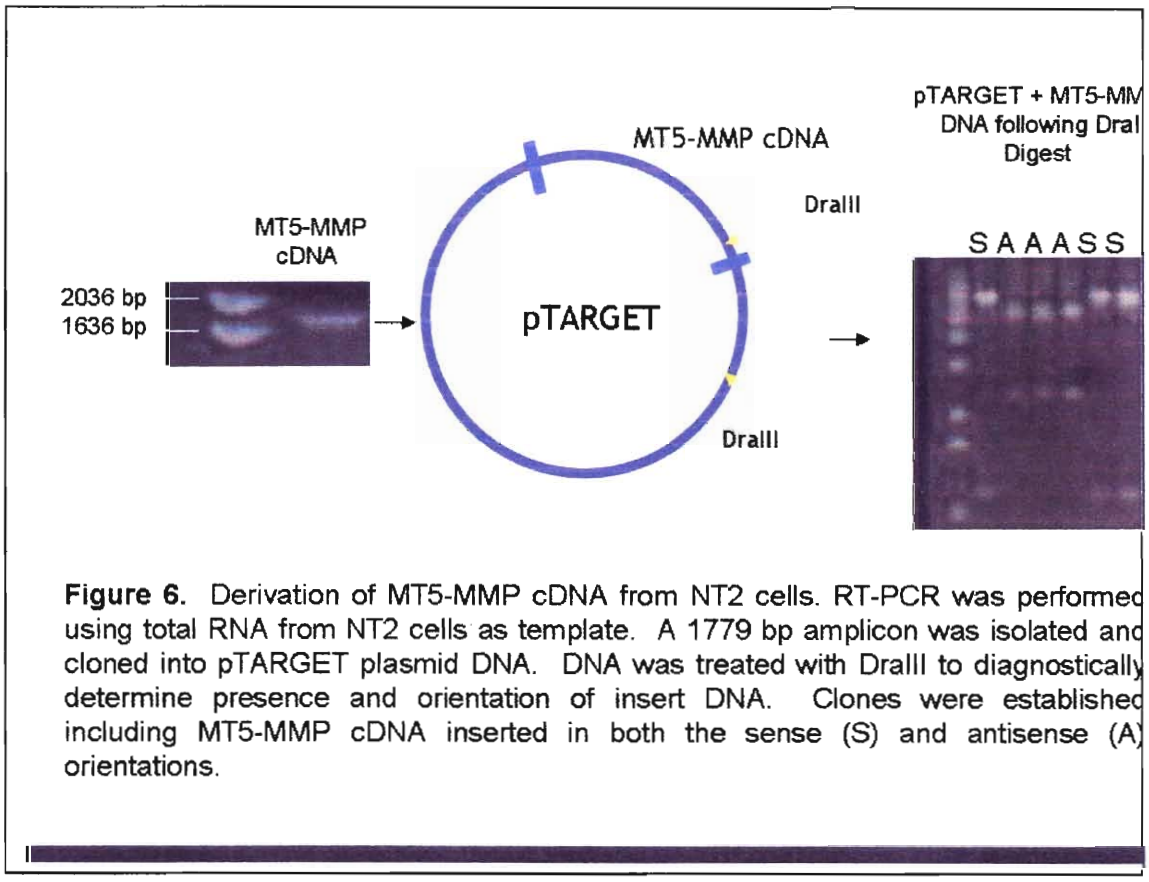
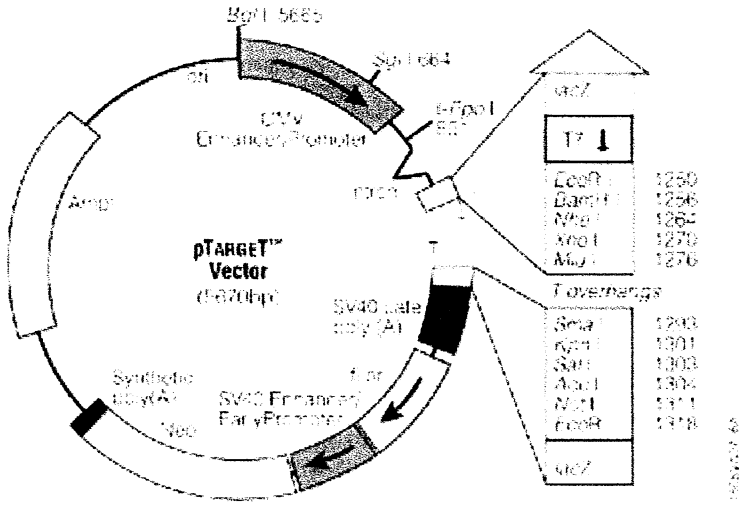
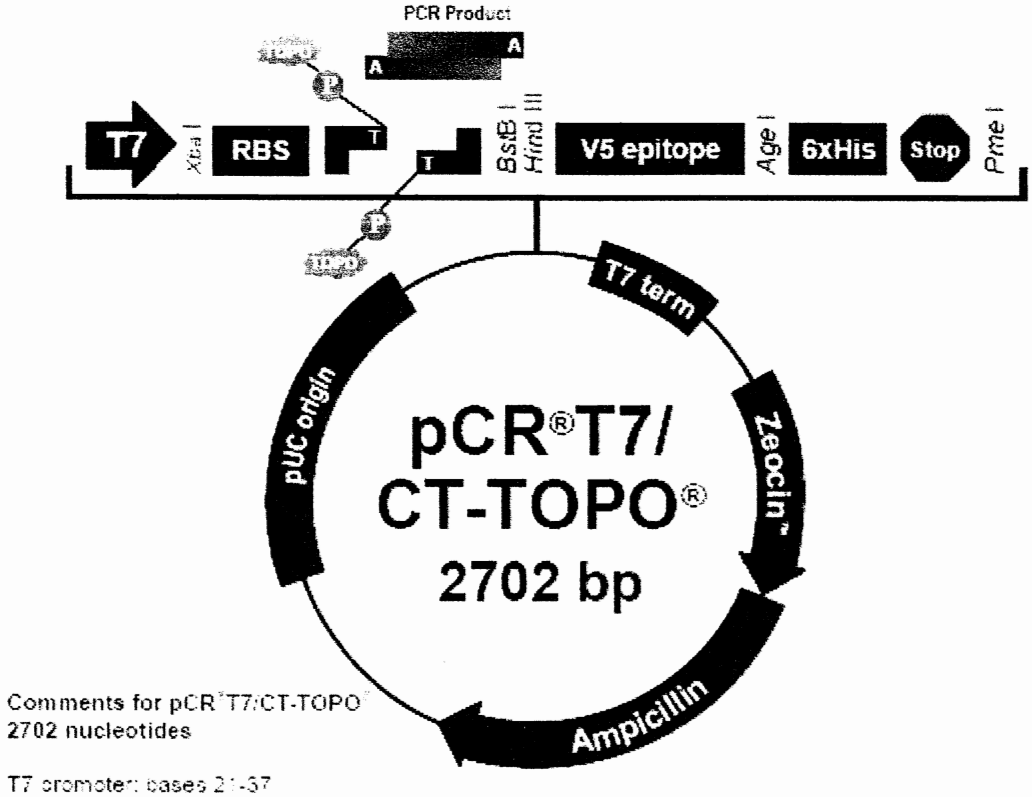


Figure 6. Derivation of MT5-MMP cDNA from NT2 cells. RT-PCR was performed using total RNA from NT2 cells as template. A 1779 bp amplicon was isolated and cloned into pTARGET plasmid DNA. DNA was treated with DraIII to diagnostically determine presence and orientation of insert DNA. Clones were established including MT5-MMP cDNA inserted in both the sense (S) and antisense (A) orientations.

Appendix Figure 1. Cloning Procedure and Restriction Enzyme Digest of pTARGET Expression Vector and MT5-MMP cDNA. For full description of materials and methods please see Chapter 2.



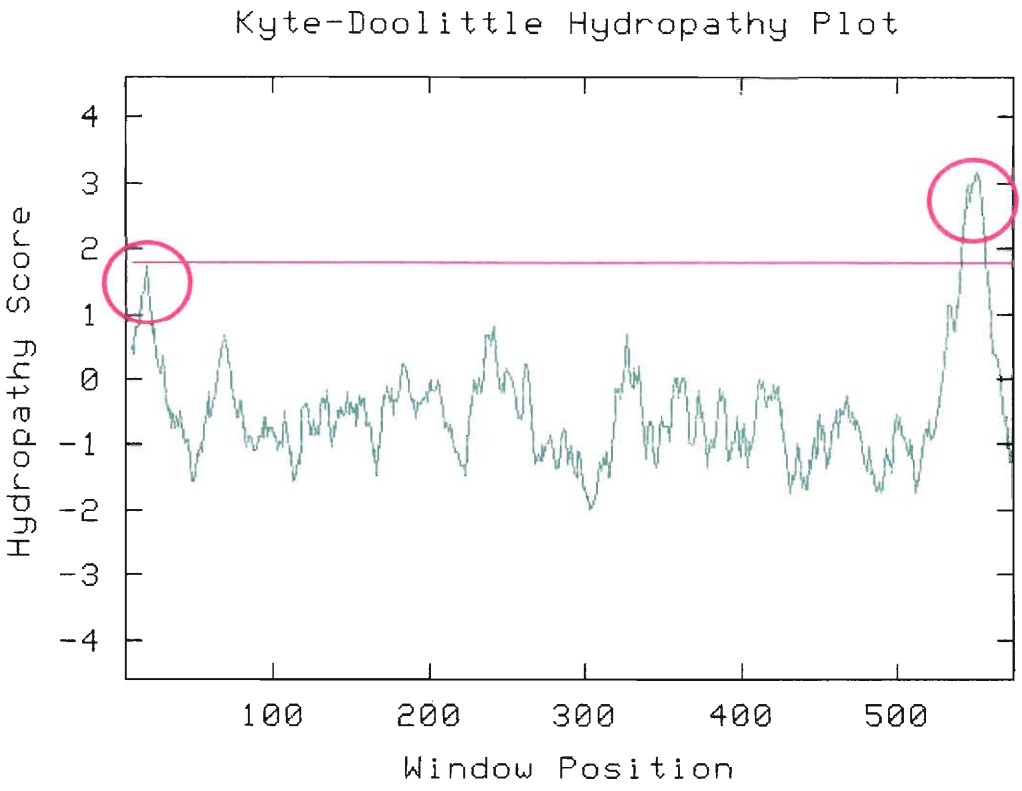
Appendix Figure 2. Vector Map of pTARGET Mammalian Expression Vector.



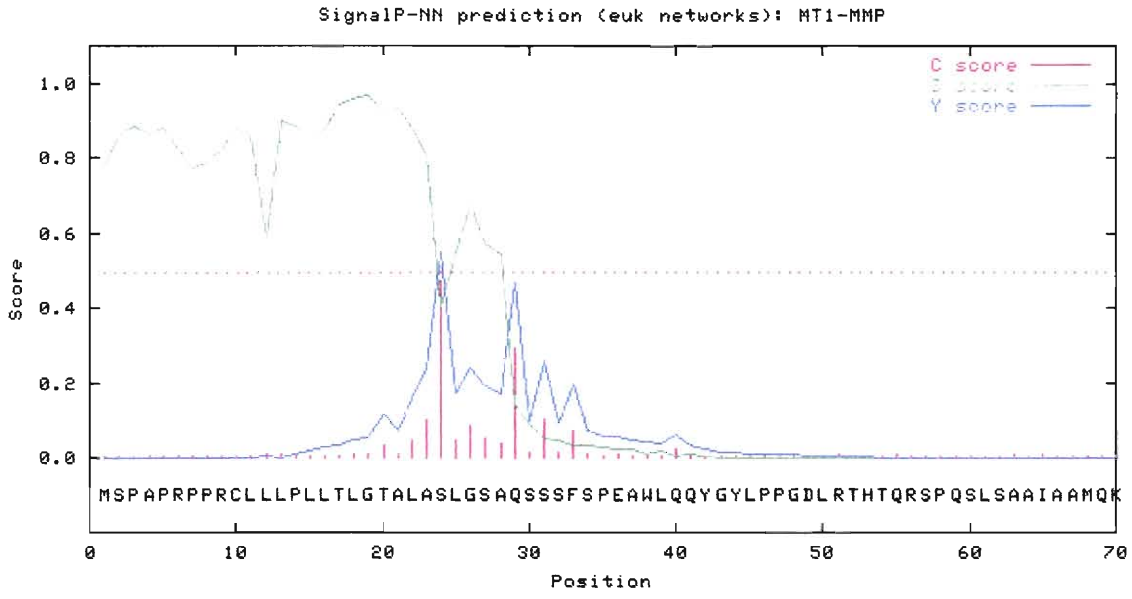
Comments for pCR®T7/CT-TOPO®
2702 nucleotides

T7 promoter: bases 21-37
T7 promoter priming site: bases 21-40
Ribosome binding site: bases 65-81
TOPO® Cloning site: bases 100-101
V5 epitope: bases 122-163
V5 (C-term) Reverse priming site: bases 131-151
Polyhistidine (6xHis) region: bases 173-190
T7 transcription terminator: bases 240-287
Zeocin™ resistance gene: bases 367-830
ORF: bases 466-830
Ampicillin resistance gene: bases 934-1711
ORF: bases 951-1711
pUC origin: bases 1956-2629

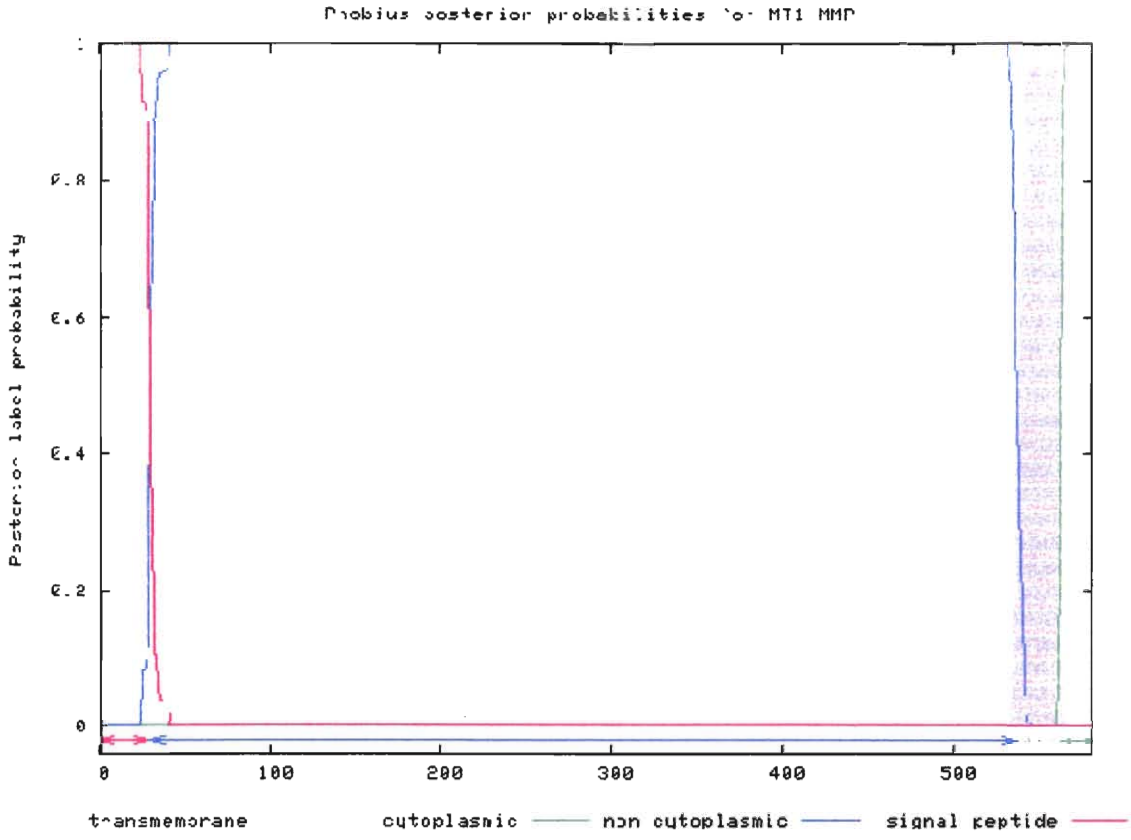
Appendix Figure 3. Vector Map of pCRT7/CT-TOPO Bacterial Expression Vector.



Appendix Figure 4. Hydropathic profile of MT1-MMP protein sequence. Kyle-Doolittle hydropathic profiles with a window of 19 was plotted for the MT1-MMP amino acid sequence as a positive control for MT5-MMP transcript plots. Peaks with a score greater than 1.8 represent regions strongly predicted to be transmembrane regions. In MT1-MMP two such regions are found, both circled in red.



Appendix Figure 5: SignalP Analysis of MT1-MMP



Appendix Figure 6: Phobius Plot for MT1-MMP

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MT5-MMP            54 AAAAGAGNRAAVAVAVARADEAEAPFAGQ
MT5-MMPvar         16                AVARADEAEAPFAGQ
                     AAAAGAGNRAAVAVAVARADEAEAPFAGQ
Chromosome 20     373 gggggggacgggggggggcgggggggctggcGTGGGGC  Intron 1
                      ccccccctgccccccccgccccccgaccg

MT5-MMP            83    NWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGIPVTGVLDQ
MT5-MMPvar               NWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGIPVTGVLDQ
                         NWLKSYGYLLPYDSRASALHSAKALQSAVSTMQQFYGIPVTGVLDQ
Chromosome 20   20312 CAGattattgtccctgtcgtgcctgagtctggtaaccttgacgaggtgc
                      -0>agtacagattcaacgccctaccactacctcctaatagtctcgttaa

MT5-MMP           129 TTI                              WMKKPRCGVPDHPHLSRRRR
MT5-MMPvar              TTI                          WMKKPRCGVPDHPHLSRRRR
                        TTI           E:E[gag]       WMKKPRCGVPDHPHLSRRRR
Chromosome 20   20453 aaaGAGTAAGAT  Intron 2   CAGGtaaacctggcgccctacaca
                      cct  <2-----[20464:25379]-2> gtaacgggtcaacatggggg

MT5-MMP           153 NKRYALTGQKWRQKHITY                           IHNYT
MT5-MMPvar              NKRYALTGQKWRQKHITY                       IHNYT
                        NKRYALTGQKWRQKHITY           S:S[agc]    IHNYT
Chromosome 20   25441 aactgcagcatacacaatAGGTGCTTC  Intron 3   CAGCacata
                      aagactcgaaggaaatca  <2-----[25497:27924]-2> taaac

MT5-MMP           177 PKVGELDTRKAIRQAFDVWQKVTPLTFEEVPYHEIKSDRKEADIMIFFA
MT5-MMPvar              PKVGELDTRKAIRQAFDVWQKVTPLTFEEVPYHEIKSDRKEADIMIFFA
                        PKVGELDTRKAIRQAFDVWQKVTPLTFEEVPYHEIKSDRKEADIMIFFA
Chromosome 20   27941 cagggcgacagaccgtggtcagaccatgggctcgaaagcagggaaattg
                      catgatacgactgactatgaatcctctaatcaaatagagaacatttttc

MT5-MMP           226 SGFHGDSSPFDGEGGFLAHAYFPGPGIGGDTHFDSDEPWTLGNANHD
MT5-MMPvar              SGFHGDSSPFDGEGGFLAHAYFPGPGIGGDTHFDSDEPWTLGNANHD
                        SGFHGDSSPFDGEGGFLAHAYFPGPGIGGDTHFDSDEPWTLGNANHD
Chromosome 20   28088 tgtcggatctgggggtcgcgttcgcgagggactgtggctacgagacg
                      cgtagagcctagaggttcacatcgcgtggacatacaacgctgacaaa

MT5-MMP           273                              NDLFLVAVHELGHALGLEHSSDP
MT5-MMPvar                                         NDLFLVAVHELGHALGLEHSSDP
                                 G:G[ggg]          NDLFLVAVHELGHALGLEHSSDP
Chromosome 20   28229 GGTAAGGC  Intron 4   CAGGGagctcgggcgcgcgcgctagc
                       <1-----[28230:37265]-1>  aattttctaatgactgtaacgac

MT5-MMP           297 SAIMAPFYQYMETHNFKLPQDDLQGIQKIY
MT5-MMPvar              SAIMAPFYQYMETHNFKLPQDDLQGIQKIY
                        SAIMAPFYQYMETHNFKLPQDDLQGIQKIY
Chromosome 20   37337 agaagcttctagacatacccggccgacaat
                      gcttcctaaatacaatatcaaatagtaata
```

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MT5-MMP          327                                PPAEPLEPTRPLPTLPVRRIHSP
MT5-MMPvar                                          PPAEPLEPTRPLPTLPVRRIHSP
                                 G:G[gga]           PPAEPLEPTRPLPTLPVRRIHSP
Chromosome 20  37427 GGTGTGTG  Intron 5   CAGGAccggccgcaacccaccgcaactc
                      <1-----[37428:40677]-1>  cccactaccgctcctctggtacc


MT5-MMP          351 SERKHERQPRPPRPPLGDRPSTPGTKPNICDGNFNTVALFRGEMFVFK
MT5-MMPvar           SERKHERQPRPPRPPLGDRPSTPGTKPNICDGNFNTVALFRGEMFVFK
                     SERKHERQPRPPRPPLGDRPSTPGTKPNICDGNFNTVALFRGEMFVFK
Chromosome 20  40749 tgaacgcccacccccggcctacgaacaatggataaggctcggatgta
                     cagaaagacgccgcctgagccccgcacatgagatactcttggatttta


MT5-MMP          399                              DRWFWRLRNNRVQEGYPMQIEQFWKG
MT5-MMPvar                                        DRWFWRLRNNRVQEGYPMQIEQFWKG
                                                  DRWFWRLRNNRVQEGYPMQIEQFWKG
Chromosome 20  40893 GTAGGGC  Intron 6   CAGgctttcccaacgcggtcacagcttag
                     <0-----[40893:43232]-0>aggtggtgaagtaagactataatgag


MT5-MMP          425 LPARIDAAYERADGRFVFFK                               DKY
MT5-MMPvar           LPARIDAAYERADGRFVFFK                               DKY
                     LPARIDAAYERADGRFVFFK          G:G[ggt]             DKY
Chromosome 20  43311 ccgcagggtgagggatgttaGGTAATGT  Intron 7   CAGGTgat
                     tccgtaccaagcaggtttta <1-----[43372:45041]-1>  aaa


MT5-MMP          449 WVFKEVTVEPGYPHSLGELGSCLPREGIDTALRWEPVGKTYFFKGERYW
MT5-MMPvar           WVFKEVTVEPGYPHSLGELGSCLPREGIDTALRWEPVGKTYFFKGERYW
                     WVFKEVTVEPGYPHSLGELGSCLPREGIDTALRWEPVGKTYFFKGERYW
Chromosome 20  45053 tgtaggaggcgtccacggcgattccggagagcctgcggaatttaggctt
                     gttaatctacgacagtgatgggtcgagtacctggactgacattagagag


MT5-MMP          498 RYSEERRATDPGYPKPITVWKGIPQAPQGAFISKEG
MT5-MMPvar           RYSEERRATDPGYPKPITVWKGIPQAPQGAFISKEG
                     RYSEERRATDPGYPKPITVWKGIPQAPQGAFISKEG
Chromosome 20  45200 ctaggccgagcgtcacaagtagaccgccggtaaagg
                     gagaaggccacgacactctgagtcaccagcttgaag


MT5-MMP          534                                YTYFYKGRDYWKFDNQKLSVEPG
MT5-MMPvar                                          YTYFYKGRDYWKFDNQKLSVEPG
                                 Y:Y[tat]           YTYFYKGRDYWKFDNQKLSVEPG
Chromosome 20  45308 TGTACGTA  Intron 8   CAGATtatttagcgttatgacacaggcg
                      <1-----[45309:47744]-1>  acataaggaagataaaatgtacg


MT5-MMP          558 YPRNILRDWMGCNQKEVERRKERRLPQDDVDIMVTINDVPGSVNAVAVV
MT5-MMPvar           YPRNILRDWMGCNQKEVERRKERRLPQDDVDIMVTINDVPGSVNAVAVV
                     YPRNILRDWMGCNQKEVERRKERRLPQDDVDIMVTINDVPGSVNAVAVV
Chromosome 20  47816 tccaaccgtagtacagggccagcccccggggaagaaaggcgtgaggggg
                     acgattgagtggaaaataggaaggtcaaatatttctaatcgctactctt


MT5-MMP          607 IPCILSLCILVLVYTIFQFKNKTGPQPVTYYKRPVQEWV
MT5-MMPvar           IPCILSLCILVLVYTIFQFKNKTGPQPVTYYKRPVQEWV
                     IPCILSLCILVLVYTIFQFKNKTGPQPVTYYKRPVQEWV
```

```
Chromosome 20  47963 actactctacgcgtaatctaaaagcccgattaccgcgtg
                     tcgttctgtttttacttataaacgcactcaaagctaagt
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Appendix Figure 7: MT5-MMP and MT5-MMPvar alignment with human Chromosome 20. MT5-MMP (top amino acid sequence) represents the published MT5-MMP sequence. MT5-MMPvar (middle amino acid sequence) represents the variant transcript described. Chromosome 20 (lower amino acid sequence and the double-stranded DNA complement) represents the available sequence for human chromosome 20q11.2. All intron and exon boundaries are labeled accordingly. Numbers corresponding to base pairs found on the genomic sequence are arbitrary.

VITA

Heather Hamilton Ross was born in Atlanta, Georgia on March 9, 1975 and is an American citizen. She graduated from Southeast Guilford High School in Greensboro, North Carolina in 1993. She earned an Associate of Science from Louisburg College in 1995, a Bachelor of Arts from the University of North Carolina at Chapel Hill in 1997 and a Master of Physical Therapy from East Carolina University in 2000. She then joined the Department of Anatomy and Neurobiology at Virginia Commonwealth University in 2000 and earned a Doctor of Philosophy in 2006. Mrs. Ross has authored one manuscript pertaining to her work currently in review for peer-reviewed publication as well as multiple abstracts. She has co-authored one book chapter and two peer reviewed papers. She has presented her work at several national and international conferences and has served as a teaching assistant and lecturer in multiple graduate level courses. Her academic career has been distinguished by awards such as being named a Distinguished Academic All American student athlete by the NCJAA, earning academic and athletic scholarships as well as the Brantley Award in recognition of the highest GPA from Louisburg College and earning the School of Medicine Award of Excellence and the Graduate School C.C. Clayton Award in recognition of academic achievement from Virginia Commonwealth University.