ALTERATIONS IN THE EXPRESSION OF CANCER ASSOCIATED MEDIATORS IN H-RAS TRANSFORMED MURINE FIBROBLAST CELLS

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

In the Department of Biology

Faculty of Science

University of Prince Edward Island

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Charlottetown, Prince Edward Island

February, 2011

Your file Votre référence
ISBN: 978-0-494-82249-4
Our file Notre référence
ISBN: 978-0-494-82249-4

SIGNATURE PAGE(S)

Not numbered in thesis

REMOVED

CONDITIONS FOR THE USE OF THE THESIS

ABSTRACT

ALTERATIONS IN THE EXPRESSION OF CANCER ASSOCIATED MEDIATORS IN H-*RAS* TRANSFORMED MURINE FIBROBLAST CELLS

The progression of cancer is characterized by the alteration in key cancer associated activities. This study investigates the expression of proliferation and proinflammatory linked proteins in H-*ras* transformed murine fibroblast cells. The proteins examined include spermidine/spermine N^1-acetyltransferase (SSAT), ornithine decarboxylase (ODC), inducible nitric oxide (iNOS), cyclooxygenase-2 (COX-2), matrix metalloproteinases (MMPs), and associated mediators of MMP activity which include extracellular matrix metalloproteinase inducer (EMMPRIN), reversion-inducing-cysteine-rich protein with Kazal motifs (RECK), and TIMPs (tissue inhibitors of MMPs). Treatment of NR3 cells (H-*ras* transformed, benign tumor forming) with phorbol-12-myristate acetate (PMA) resulted in an induction of SSAT protein expression, whereas SSAT expression was unaltered in 10T ½ cells (non-transformed). Studies investigating the possible signal transduction pathways involved in the alteration of SSAT protein expression suggests a possible role for protein kinase-C (PKC), mitogen activated protein (MAP) kinase, and phosphatidylinositol-3-kinase (P-I-3 kinase) pathways. The effects of oncostatin M (OSM) on MMPs and associated mediators were also examined in NR3 cells. OSM resulted in an induction of MMP-9 and MMP-2 activity in the transformed cells, with a possible involvement of the P-I-3

kinase pathway and PKC pathways, respectively. OSM treatment on NR3 cells also resulted in an induction of EMMPRIN, TIMP-1 and TIMP-2, with possible involvement of specific proteins of the JAK/STAT pathway. Various other proteins associated with proliferation and proinflammatory was also investigated in transformed and non-transformed cells. Tumor necrosis factor-α (TNF-α), iNOS, COX-2, and ODC protein expression were all elevated in NR3 cells in response to OSM, whereas only COX-2 expression was elevated in the non-transformed cells. The altered expression and regulation of the cancer associated proteins represents an aspect of the altered cellular growth program inherent to H-*ras* transformed cells.

ACKNOWLEDGEMENTS

First of all, I would like to sincerely thank my supervisor Dr. Robert Hurta. It has been a long journey and without your patience, understanding, and guidance I would not have finished this degree. You are a great friend Rob and I have truly learned a great deal from you in the past years, so Thank You. I would also like to thank my committee members, Dr. Marva Sweeney-Nixon and Dr. Kathy Gottschall-Pass. I appreciate all the patience and help the two of you have showed over the past years and thank you for the support. To all the post and current members of the Hurta lab, we have learned a great deal from each other and thank you for the help and laughs along the way. I would also like to thank all the members of the Biology department for all your help along the way and for all motivation in the last couple years. Most importantly I would like to thank my fiancé. You are an amazing person and without your help, understanding and constant pressure I would not be where I am today. I would also like to thank the Prince Edward Island Division of the Canadian Cancer Society for financially supporting my research.

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LIST OF ABBREVIATIONS

α-MEM	alpha minimal essential medium
APAO	acetylated polyamine oxidase
BCIP	5-bromo-4-chloro-3-indolylphosphate
BSA	bovine serum albumin
BENSPM	N^1-N^{11}-bisethylnorspermine
COS-7	**C**V-1 **O**rigin **S**V40
COX-2	cyclooxygenase-2
dcSAM	decarboxylated S-adenosylmethionine
DENSPM	N^1-N^{11}-diethylnorspermine
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EMMPRIN	extracellular matrix metalloproteinase inducer
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
HRP	horse radish peroxide
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	janus kinase
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
NBT	nitro blue tetrazolium
NO	nitric oxide
ODC	ornithine decarboxylase
OSM	oncostatin m
pAL8A	plasmid AL8A
PAO	polyamine oxidase
PDGF	platlet-derived growth factor
PI3K	phosphoinositide 3-kinase
PIAS	protein inhibitor of activated STAT
PKC	protein kinase C
PMA	phorbol-12-myristate acetate
PMF	polyamine modulated factor
PMSF	phenylmethylsulfonylfluride
RAF	rapidly accelerated fibrosarcoma
RAS	**ra**t sarcoma
RECK	reversion-inducing cysteine-rich protein with KAZAL motifs
SAMDC	S-adenosylmethionine decarboxylase
SDS	sodium dodecyl sulfate

SOCS	suppressor of cytokine signalling
SSAT	spermidine/spermine N^1-acetyltransfersase
STAT	signal transducer and activator of transcription
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween-20
TIMP	tissue inhibitor of matrix metalloproteinase
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor - α
TPA	tissue plasminogen activator
TYK2	tyrosine protein kinase 2

1. CHAPTER ONE

Literature Review

1.1 Cancer

Cancer is a very complex disease, being both incredibly ordered and dysfunctional at the same time. Normal cellular development follows an ordered sequence consisting of growth, differentiation and death. In cancer this ordered process is manipulated so that cancer cells are able to utilize the normal processes and mechanisms of the body to grow uncontrollably, invade neighbouring tissue and metastasize to various tissues in the body (Liotta et al., 1991, Duffy et al., 2008). The disease is grouped into broad categories: carcinoma includes cancers that begin in the skin or in tissue that line or cover internal organs, sarcoma include cancers that begin in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue, leukemia include cancers that originate in blood-forming tissue such as bone marrow, and lymphoma include cancers that begin in the immune system (Zuckerniann, 1942, Berman, 2004).

1.2 Cancer: associated properties and pathways

1.2.1 H-*ras* – metastatic potential and properties

Cellular function is a highly regulated process; however, alterations in genetic material can produce mutations in the cell that affect normal cellular growth and

division. A variety of environmental factors including alcohol, radiation, stress, and infections can cause abnormalities and mutations in the genetic material of cells resulting in normal genes (proto-oncogenes) becoming oncogenes (Fontham et al., 2009). The oncogene then has the ability to turn a normal cell into a tumour cell which has a significant growth advantage that includes uncontrollable cellular proliferation and growth, the ability to inhibit programmed cell death, and the ability to invade neighbouring tissue and metastasize.

One of the most studied oncogenes is the *ras* family of genes. The *ras* family of proteins are important signalling molecules that regulate various cellular processes including growth, differentiation, and survival (Lin et al., 1998, Downward, 1998, Kauffman-Zeh et al., 1997). In response to stimuli, *ras* activates various downstream effectors that initiate signalling cascades via activation of protein kinases (White et al., 1995). Oncogenic mutations in the *ras* gene have been linked to many types of human cancers such as colorectal and lung cancer (Zlobec et al. 2010, Smith et al., 2010, Brose et al., 2002). *Ras* proteins function as internal switches that control intracellular signalling networks. The *ras*-regulated signalling pathways control proliferation, differentiation, and apoptosis (Park et al., 2003). The *ras* proteins are often dysregulated in cancer cells which can lead to permanent activation and constant stimulation of downstream signalling pathways and, ultimately, activation of various transcription factors that have the ability to influence cell proliferation (Coleman et al., 2004, Pozzi et al., 2006). The main signalling pathways that have been shown to be affected by *ras* proteins include mitogen-activated protein Kinase (MAPK), phosphatidylinositol-3-

kinase (P-I-3 kinase), AKT, and the protein kinase C (PKC) (Liu and Ringer, 2003, Yoon et al., 2008, Seales et al., 2005).

1.2.2 Signalling transduction pathways- regulation and roles in cancer cell growth

MAP kinase pathway

The MAP kinase pathway is a signal transduction pathway that relays signals from cell surface receptors to the cell nucleus, resulting in the regulation of specific genes that are involved in apoptosis, cell growth and cell differentiation. The cascade of the pathway is complex but initially relies on the activation of extracellular receptors by specific ligands (Fig.1.1). The binding of the ligand activates a cascade of kinase activity that leads to potential end targets of MAPK activity (Cobb, 1999). Various extracellular stimuli, including growth factors, stress, radiation, and inflammatory cytokines, induce a sequential activation of specific kinases used during the MAPK cascade (Kortenjann and Shaw, 1995, Pearson et al., 2001). The specific kinase depends directly on the specific extracellular ligand/receptor complex.

Generally, the kinases can be grouped into three main categories: MAP3K, MAP2K, and MAPK. One main example of the MAPK signalling pathway is the ras/ERK MAPK pathway that regulates a wide array of cellular responses (Lewis et al., 1998, Hunter, 2000, Chang and Karin, 2001). Activation of the MAPK pathway has been shown to be essential for *ras*-induced cellular events including proliferation and

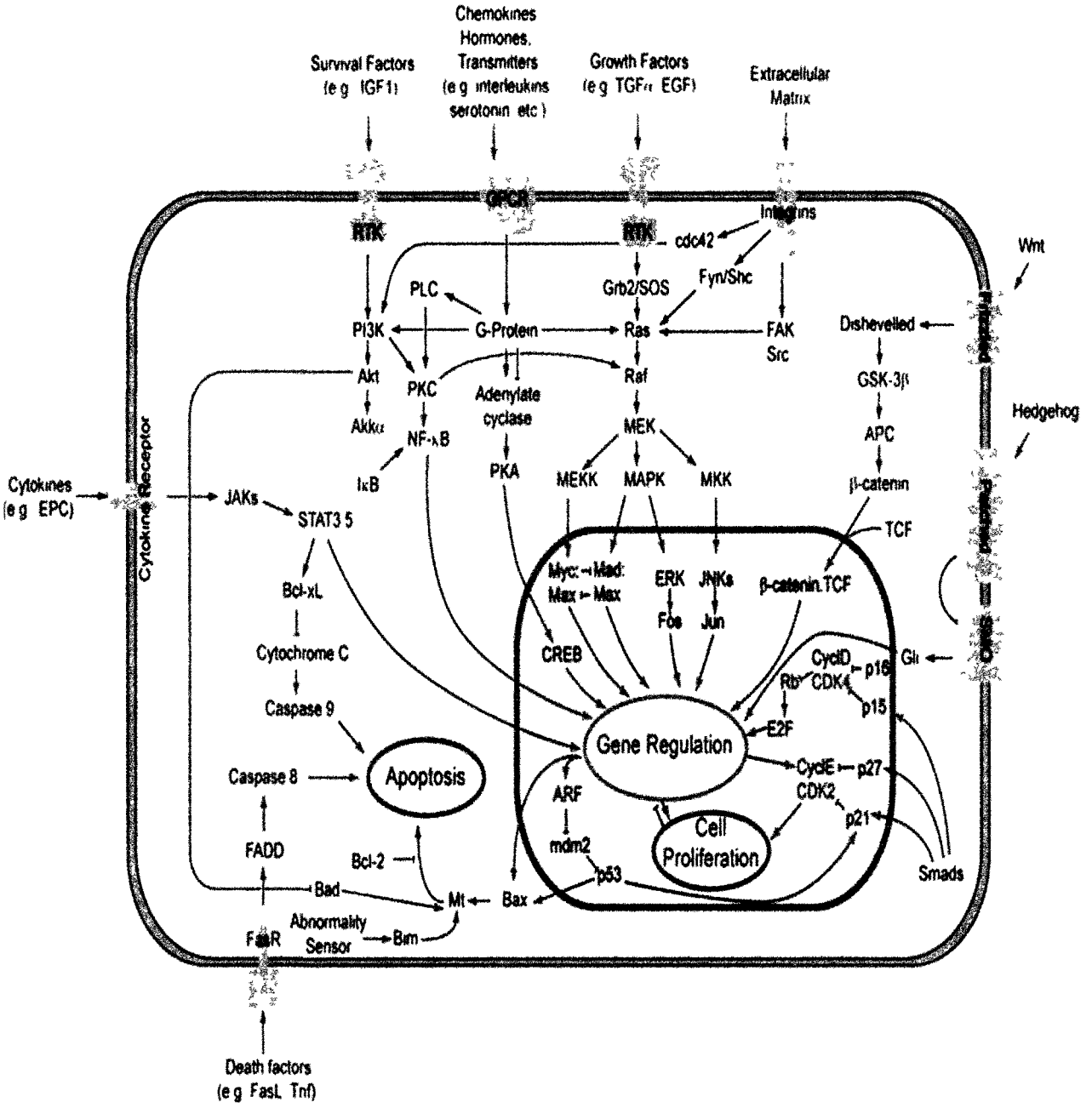


Figure 1.1. Signalling transduction pathway. Signalling transduction pathway illustrating the MAP kinase, P-I-3 kinase, PKC, AKT, and the JAK/STAT pathway. (Hanahan and Weinberg, 2000).

differentiation (Cowley et al., 1994). The ras/ERK MAPK pathway induces the activation of three protein kinases, Raf, MEK, and ERK. After extracellular stimulation, *ras* becomes active and activates the protein kinase activity of RAF (MAPKKK). MEK (MAPKK) localizes to the cytoplasm and is phosphorylated and activated by RAF. In response to stimulation, MEK phosphorylation of ERK induces the activation of ERK and promotes the dissociation of ERK from the MEK-ERK complex (Adachi et al, 1999). The activated ERK translocates from the cytoplasm to the nucleus where ERK activates several nuclear targets (Khokhlatchev et al., 1998). The MAP kinase pathway is downstream of the P-I-3 kinase pathway and has been shown to be activated by the P-I-3 pathway. In COS-7 cells, LPA activation of MAP kinase was blocked by inhibitors of P-I-3K, such as wortmannin and LY294002, suggesting that P-I-3K is required for MAP kinase activation at a point upstream of *ras* activation (Hawes et al, 1996, Takeda et al., 2009).

P-I-3 kinase pathway

The P-I-3 kinase pathway is composed of a family of enzymes that regulate the basic cellular functions such as transcription, proliferation, growth, and survival (Fig.1.1). Alterations in the activation of the pathway have been suggested to result in the development of major diseases such as diabetes, autoimmune disorders, and cancer. The kinases involved in the pathway are intracellular signal transducer enzymes that activate various downstream effectors by phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (Vimalanathan et al., 2009). The P-I-3 kinase

family is divided into three different classes: Class I, Class II, and Class III (Foster et al., 2003). The classifications are based upon primary structure and regulation. During cancer, kinases of the P-I-3 kinase pathway become mutated, resulting in the uncontrolled activation of the pathway causing the kinase to be more active. Normally, the antagonist kinase is also absent in tumours which contributes significantly to the development of cancer. Recent studies have concluded that the P-I-3 kinase pathway is one of the most commonly activated pathways in cancer, and the high frequency of P-I-3 kinase pathway alterations in cancer has led to an increase in the development of P-I-3 kinase inhibitors that are currently in trial stages in humans (Yuan and Cantley, 2008, Van der Heijden and Bernards, 2010 , Courtney et al., 2010).

JAK/STAT pathway

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is a major pathway in altering cellular growth, proliferation, differentiation, and apoptosis. The pathway consists of various extracellular ligands and their respective receptors that stimulate the JAK/STAT pathway and the pathway is principle pathway for many cytokines and growth factors (Dumler et al, 1999, Liu and Ringer, 2002, Simon et al., 1998). For the JAK/STAT signal to commence, the cytoplasmic domain of two subunits must be associated with JAK tyrosine kinases (Fig.1.1). Once the ligand-receptor complex is formed, the JAKs are able to phosphorylate additional receptors and STAT proteins. STAT proteins are latent transcription factors that are located in the cytoplasm until they are phosphorylated (Rawlings et al., 2005, Schindler, 2002).

Activated STATs are able to enter the nucleus and activate and/or inactivate the transcription of target genes (Leonard, 2001). Thus, the pathway enables direct relay from an external signal. The JAK/STAT pathway consists of two subgroups; the JAK subgroup which consists of JAK-1, JAK-2, JAK-3 and Tyk-2, and the STAT subgroup which consists of STAT1-7. Both groups are tightly regulated by protein tyrosine phosphatases, suppressors of cytokine signalling (SOCS), and protein inhibitors of activated STATs (PIAS) (Rawlings et al., 2004). There is increasing evidence that the dysregulation of the JAK/STAT pathway is responsible for renal disease, myocardial injury, and tumourigenesis (Chuang and He, 2010, Ananthakrishnan et al., 2005, Barry et al., 2007, Espert et al., 2005)

AKT pathway

The serine/threonine protein kinase AKT, also known as protein kinase B, plays a key role in mediating signals for cell growth, cell survival, transcription and migration (Chang et al., 2003). AKT is activated by a diverse array of growth factors, cytokines, and other physiological stimuli in a P-I-3 kinase-dependant manner (through a cascade effect involving both membrane translocation and phosphorylation). AKT is a key downstream effector of P-I-3 kinase and becomes activated upon binding of the p85 subunit of P-I-3 kinase to phosphorylation sites in the cytoplasmic domain of specific tyrosine kinases (such as FAK and PYK2). Upon activation, AKT recruits inactive AKT and phosphorylates a wide range of protein substrates that regulates and phosphorylate the activity of kinases, transcription factors, and other regulatory molecules (Fig.1.1).

(Paez and Sellers, 2002). The dysregulation of the pathway has been shown in a wide range of lung tumours and hematologic malignancies (Massion et al., 2004).

PKC pathway

Protein Kinase C (PKC) family consists of a number of serine-threonine kinases that are divided into three groups based on their activation factors (Koivunena et al., 2006). The basic pathway of PKC involves allosteric activation by the intracellular messengers, diacylglycerol and Ca^{2+}, however, activation of PKC has also been shown from several kinases, phosphatases and intracellular binding proteins (Fig.1.1). PKC plays an important role in several signal transduction cascades and has been shown to regulate cell growth, transcription, and cellular differentiation in various cell types (Dekker, 2004). Upon activation by phorbol ester tumour promoters, PKC can phosphorylate potent activators of transcription and increase expression of certain oncogenes, thereby promoting cancer progression. PKC activators are also known to act as a tumour promoter, which provides another link between carcinogenesis and PKC (Koivunen et al., 2006).

1.2.3 Tumour promoters

Cancer is initiated when normal functions of a cell become altered resulting in the abnormal growth of the cell. The resulting cell becomes a distinct, well defined benign tumour. With exposure and stimulation from tumour promoters, the benign tumour is able to convert to a malignant tumour and gain the ability to invade

surrounding tissue. The malignant cells are further able to metastasize to distant sites in the body and the allow the disease to spread. This two-stage model of carcinogenesis commences with a carcinogen that is capable of irreversibly altering the function of a cell and a promoter that is capable of speeding the progression of the disease (Tennant, 1999). A wide range of chemical compounds have been shown to promote tumour activity but the most studied class of tumour promoters are phorbol ester tumour promoters (Todaro, 1980). Phorbol-12-myristate-13-acetate (PMA) is the most studied phorbol ester and is used as a major research tool in carcinogenesis. PMA is also a known activator of PKC and has been shown to promote cancer in various cell lines. For example in melanoma cells, PMA was shown to activate the PKC pathway, and cells experience increased cell proliferation and protection from apoptosis. In human prostate cancer cells, PMA was shown to increase migratory and invasive capacities through the PKC pathway (Jorgensen, 2005 and He, 2010).

1.3 Polyamine pathway- biosynthesis and catabolism

One focus of past and recent cancer research is the possible involvement of the polyamine pathway in understanding the progression of the disease. The polyamine pathway is important in the regulation of cell proliferation, cell differentiation and cell death (Wallace et al., 2003). All cell growth requires some degree of involvement of the polyamine pathway as polyamines have been discovered to be essential for the proper maintenance of the high metabolic activity of a normal healthy body (Criss, 2003). The importance of the polyamine pathway is reflected by the strict regulatory control of the intracellular polyamine levels and the strict regulation between the biosynthesis and

catabolism of the intracellular polyamines (as levels are set low enough to allow for absolute minimal cell growth and set at higher limits to prevent toxicity within the cell) (Pegg, 1986, Thomas and Thomas, 2003).

The polyamine pathway is composed of two distinct components: the anabolic and catabolic portion. The anabolic portion of the pathway consists of two highly regulated enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC), and two constitutively expressed synthase enzymes: spermidine synthase and spermine synthase (as shown in Fig. 1.2) (Wallace et al., 2003). ODC is responsible for converting ornithine to putrescine while SAMDC and the synthase enzymes convert putrescine to spermidine and spermine. ODC acts as the rate-limiting enzyme and ultimately controls the anabolic pathway (Criss et al., 2003, Auvinen et at., 1992). For this reason ODC has generated the most attention in anti-cancer and cancer prevention studies. Research has found that ODC mRNA and ODC activity levels are upregulated in a variety of human cancers, including esophagus, stomach, colon, and liver tumours (Yoshida et al., 1992, Elitsur et al., 1992, Weiss et al., 2002). Further, ODC overexpression has been is related to the early stages of prostate carcinogenesis; unfortunately, ODC overexpression in prostate cancer has been shown to decrease as the cancer progresses (Young, 2006). In another study researchers transfected murine 10T ½ fibroblasts with an ODC transfect that overexpressed ODC and discovered that the process lead to increased invasiveness and malignant transformation in other cell types (Kubota et al., 1997). Also, NR3 cells treated with PMA showed an increase in ODC and SAMDC gene expression and NR3 cells treated

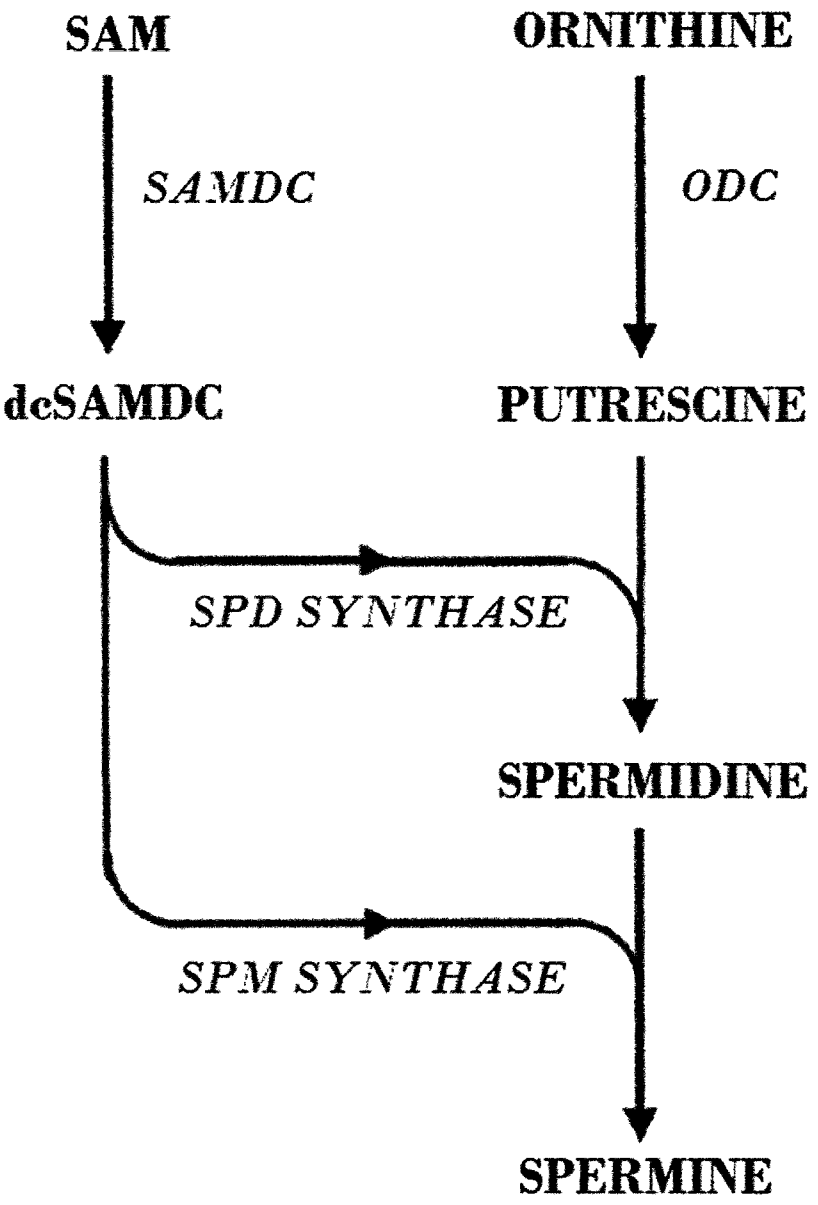


Figure 1.2. Polyamine biosynthesis pathway. Enzymes are shown in italics (SAMDC- S-adenosylmethionine, ODC- ornithine decarboxylase, Spd synthase- spermidine synthase; Spm synthase- spermine synthase)

with TGF-β1 also showed an increase in ODC, which illustrates the importance of the anabolic polyamine pathway and cancer progression (Hurta et al., 2001, Voskas et al., 2001).

The catabolic portion of the pathway consists of a strictly regulated enzyme spermidine/spermine N^1-acetyl transferase (SSAT), and a constituvely expressed acetylpolyamine oxidase (APAO). SSAT converts spermine and spermidine to their acetylated version while APAO converts N-acetyl-spermine to spermidine and N-acetyl-spermidine to putrescine (as shown in Fig. 1.3). Although most anti-cancer studies have focused on inhibiting the biosynthesis portion of the polyamine pathway, trial studies using inhibitors of biosynthetic enzymes have not proven useful for cancer therapy. Recent cancer studies involving the polyamine pathway are now focusing on the catabolic portion of the pathway and more importantly the SSAT enzyme.

1.3.1 SSAT- functions and regulation

Polyamines are vital molecules in regulating normal cellular and neoplastic growth (Wallace et al., 2003). The importance of polyamines is reflected in the strict regulatory control of intracellular levels (Chopra and Wallace, 1998). One major regulatory component of the pathway is SSAT. SSAT is a tightly regulated enzyme that has been implicated in many processes including obesity/glucose tolerance, stress response, and oxygen homeostasis, and has also shown importance in pathophysiology events such as liver regeneration, ischemic damage, pancreatitis, and cancer (Pegg, 2008).

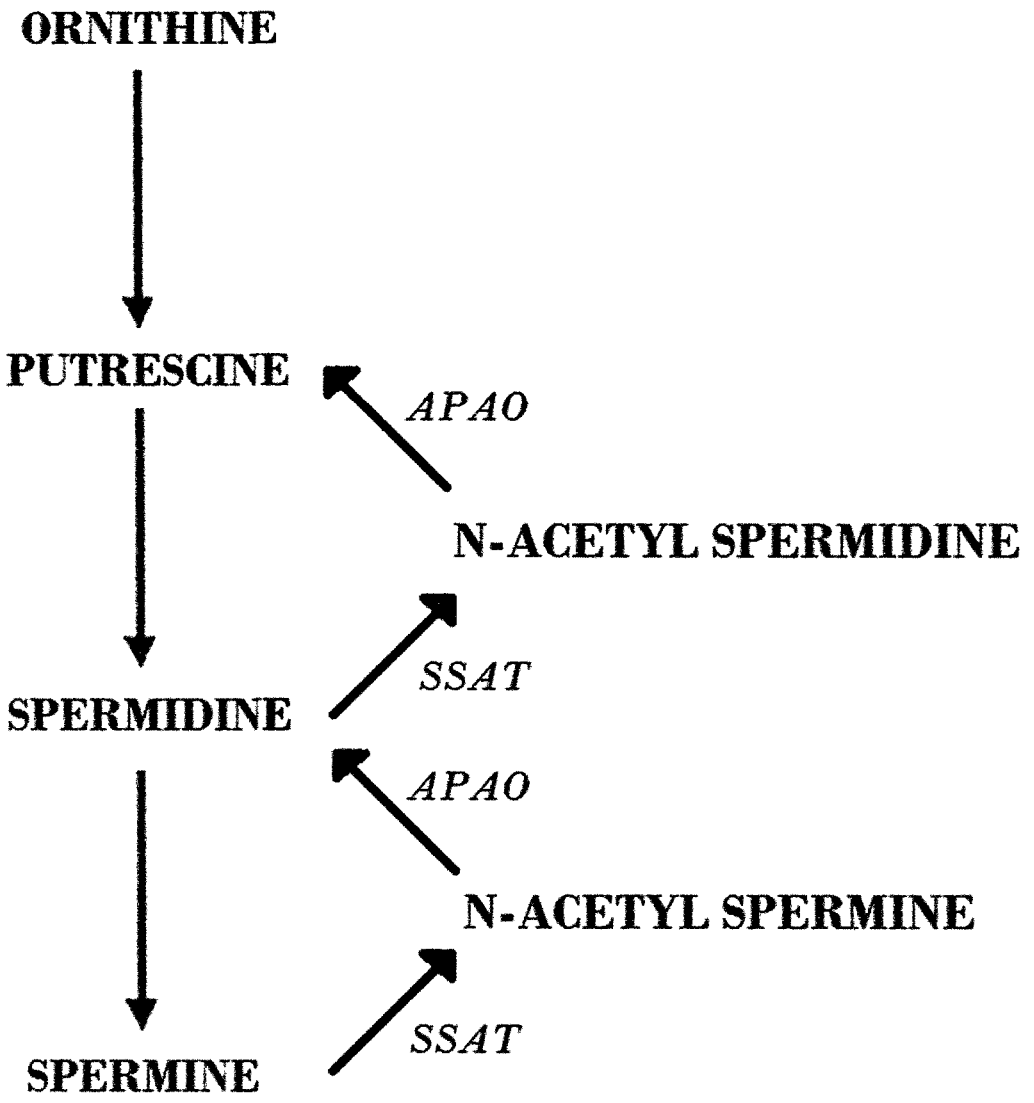


Figure 1.3. Polyamine catabolishm pathway. Enzymes are shown in italics. (APAO-acetylpolyamine oxidase; SSAT- spermidine/spermine N^1-acetyl transferase).

Under normal conditions, cellular levels of SSAT are very low but can be induced and/or inhibited by a variety of metabolites and polyamine analogues (Criss, 2003). Researchers have reported that SSAT overexpression suppresses tumour outgrowth in a mouse prostate cancer model (Tucker et al., 2005); however, a study using a polyamine analogue DENSPM, also known as BENSPM, is able to deplete polyamine pools in colorectal cancer cells and potentially induce SSAT expression (Allen et al., 2007). Other studies using polyamine analogues that overexpress SSAT have showed apoptotic results in human melanoma and lung cancer cell lines (Casero et al., 1989, Kramer et al., 1997). Other researchers have also concluded that SSAT overexpression by polyamine analogues suppress cell growth in various *in vitro* and *in vivo* models (Marverti et al., 2005, Pledgie-Tracy et al., 2010, Wang et al., 2004). Although SSAT can be regulated by many factors, including toxins, hormones, and cytokines, the enzyme is largely regulated by polyamine levels. Increases in polyamine levels regulate SSAT during transcription, mRNA processing, mRNA translation, and protein stabilization. For example, transcriptional regulation of SSAT occurs at the polyamine-responsive element (PRE), along with the polyamine modulated factor-1 (PMF-1), only when polyamine levels are high (Pegg, 2008). Also, during translation of SSAT mRNA high intracellular polyamine levels allow for the removal of repressor proteins which increases translation of the enzyme.

1.3.2 SSAT and ODC relationship

SSAT and ODC are two important and highly regulated enzymes in the polyamine pathway. Although both enzymes differ in structure and function, they play key roles in maintaining intracellular polyamine homeostasis. The enzymes participate in a futile cycle that consists of biosynthetic enzymes (ODC and SAMDC) becoming upregulated in response to a decrease in intracellular polyamine levels, while the activity of the catabolic enzymes (SSAT and APAO) becoming upregulated in response to an increase in polyamine levels thereby decreasing polyamine levels overall. The regulation and the coordination of the polyamine pathway by specific biosynthetic and catabolic enzymes is evident; however there is also a direct correlation between ODC and SSAT levels in many cancers including human prostate (Bettuzzi, et al., 2000). This suggests the possibility that the two enzymes may "cross talk" to affect the activity of each other.

1.4 Matrix metalloproteinases – functions and regulation

Matrix metalloproteinases (MMPs) are members of a family of proteins that are involved in tissue remodelling and degradation of extracellular matrix. They also play a role in a variety of normal physiological processes such as embryonic development, ovarian function, and diseases such as cancer and arthritis (Verma and Hansch, 2007, Egebald and Werb, 2002). MMPs belong to the family of zinc endopeptidases collectively known as metzincins. The metzincins superfamily is subdivided into four families: seralyzsins, astacins, adamalysins, and MMPs (Nagase and Woessner, 1999). The MMP family is comprised of more than 20 related zinc-dependent enzymes that

share a common functional domain (Chang and Werb, 2001, Birkedal-Hansen et al., 1993). The enzymes have both a descriptive name based on a preferred substrate and a MMP numbering system based on the order the enzymes were discovered. The main characteristic of MMPs is their ability to effectively degrade extracellular matrix proteins (collagens, proteoglycans, laminin, fibronectin, vitronectin, aggrecan, enactin, tenascin and elastin); however, the enzymes also have the ability to cleave specific peptides and proteins (Stetler-Stevenson and Yu, 2001). MMPs are secreted by stromal cells including fibroblasts, osteoblasts, and endothelial cells, and are expressed as zymogens. Zymogens are processed by various other proteolytic enzymes to generate the active MMP (Verma and Hansch, 2007). The basic structure of MMPs contains the following domains: (1) signal peptide, (2) a prodomain that occupies the active zinc site so the enzyme is inaccessible to substrates, (3) zinc containing catalytic domain (4) a hemopexin domain which controls interactions with substrates and (5) a hinge region. Among MMPs, different subgroups exist due to additional structural domains and complexity of the enzyme (Verma and Hansch, 2007, Lijen, 2001).

1.4.1 Gelatin-binding MMPS

MMP-2

The gelatin-binding MMPs or type IV collagenases consist of MMP-2 and MMP-9, and are named according to their ability to cleave type IV collagen. MMP-2 (gelatinase-1) is a 72 kDa enzyme which has been found to be expressed in several normal and malignant human and murine tissues. MMP-2 exists in the extracellular

matrix bound to gelatin and laminin molecules, and is one of few latent MMPs that localize to the cell membrane for proteolytic activation (Sato et al., 1994). Since MMPs are initially secreted as inactive zymogens, they must be activated by proteases such as plasmin, elastase, and trypsin. Among MMPs, proMMP-2 is unique in that it is unable to be activated by these compounds; however, it has been shown to be regulated by various compounds such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and urokinase (Lim et al., 1996, Kazes et al., 1998), and the majority of the MMP-2 activation is regulated by membrane type 1-matrix metalloproteinases (MT1-MMPs). MT1-MMP is secreted by macrophages and expressed in tumour cells, and the activation of proMMP-2 by MT-MMP is regulated in a complex manner by TIMP2. TIMP2 binds to MT1-MMP and this complex acts as a receptor for proMMP2. The tricomplex of MT1-MMP / TIMP2 / proMMP-2 is cleaved by an adjacent free MT1-MMP and induces the activation of proMMP-2 (Kinoh et al., 1996).

MMP-9

MMP-9 is a 92 kDa enzyme that plays an important role in tissue invasion/metastasis and angiogenesis. The enzyme is secreted as an inactive zymogen and can be activated *in vitro* by several proteolytic enzymes and growth factors (Yao, 2001). In normal cellular processes, MMP-9 is activated when there is an imbalance in intracellular levels of proMMP-9 to TIMP. The progression works through a protease cascade including plasmin and stromelysin 1 (MMP-3). Although plasmin can process proMMP-9 it yields an inactive MMP-9, therefore plasmin recruits endogenous MMP-3 to activate MMP-9 (Ramos-DeSimone et al., 1999). Many studies have shown a direct

correlation between MMP-9 expression and breast, ovarian, and prostate cancer (Pellikainen et al., 2004, Sillanpaa et al., 2007, Xu et al., 2008)

1.4.2 MMP Regulation

The activity of MMPs is a tightly regulated process and is controlled at three levels: transcription, pro-enzyme activation and post transcriptional regulation.

Transcriptional Regulation - The enzymes are generally expressed in low amounts and their transcription is regulated either positively or negatively by a number of factors including cytokines (IL-6), growth factors [(platelet derived growth factor (PDGF)], hormones (corticosteroids), tumour promoters (TNF-α), and aging (Birkedal-Hansen, 1993 et al., Hornebeck and Lafume, 1991).

Pro-enzyme Activation - All MMPs are synthesised as latent pro-enzymes (zymogens) and require activation by the disruption of the cysteine-zinc interaction of the cysteine switch, and removal of the pro-peptide. Some examples include intracellular activation of MMPs by furin, extracellular activation by other MMPs, especially MMT-MMPs, and extracellular activation by non-proteolytic agents such as plasmin, which directly degrades the ECM components and catalyzes the activation of many proMMPs.

Post transcription Regulation - Inhibition of proteolytic activity of MMPs can be regulated by other active MMPs, by endogenous inhibitors, autodegradation, and selective endocytosis (Xue et al., 2003). Tissue inhibitors of matrix metalloproteinases (TIMPs) are an example of endogenous inhibitors.

1.4.3 Associated mediators of MMPs

TIMPs bind both the active and/or latent forms of MMPs, and the net proteolytic activity depends upon the TIMP/MMP concentration balance. An imbalance in MMPs and TIMPs has been linked to liver and cardiovascular disorders as well as many types of cancer (Hemmann et al., 2007, Messerli, 2004, Kousidou et al., 2004, Daja et al., 2003, Wang et al., 2006). TIMPs consists of a family of MMP inhibitors (TIMP1, 2, 3, and 4) that have a far higher concentration in tissue than MMPs. The transcription of TIMPs is regulated by similar cytokines and growth factors that control MMP expression, and studies have shown that increased expression levels of MMPs correlates to increased expression of the inhibitors, TIMPs, and the reversion inducing cysteine rich protein with Kazal motifs (RECK) protein (Figueira et al., 2009).

Another endogenous inhibitor of MMPs is the membrane bound protein RECK. RECK is widely expressed in normal cells but is expressed at lower levels in transformed cells, tumour derived cell lines, and cells that undergo malignant transformation (Takeuchi et al., 2004, Kang et al., 2007, Meng et al., 2008). When RECK is restored in these cancer cell lines it has been shown to regulate MMP-2, MMP-

9, and MT1-MMP, and results in strong suppression of invasion, metastasis, and tumour angiogenesis suggesting the importance of the RECK protein in cancer progression (Masui et al., 2003, Noda et al., 2003, Oh et al., 2001). RECK is characterized as having two major functions: RECK is important in extracellular matrix remodelling, and the downregulation of RECK can lead to excessive MMP activation and cancer progression (Noda et al., 2003).

1.5 Oncostatin M

Oncostatin M (OSM) is a member of the interleukin-6 (IL-6) related cytokine subfamily that includes IL-6, IL-11, and leukemia inhibitory factor (LIF). The protein is produced mostly by monocytes, T-cells, and macrophages and functions as a growth regulator that inhibits the proliferation of a number of tumour cells lines by regulating cytokine production. OSM is a 28 kDa glycoprotein with a length of 196 amino acids that is capable of stimulating cells through two different receptor subunits: LIF and gp130 (Mosley et al., 1992). Both receptor subunits are found in most mammalian species, however, only humans are capable of utilizing both the OSM/gp130 and OSM/LIF receptor complexes (Wang et al. 2000).

The OSM pathway is a complex pathway that relies on the JAK/STAT signalling cascade to relay extracellular messages (Auernhammer, 2004). In brief, the OSM/LIF and OSM/gp130 receptor complexes use JAK-1, JAK-2, and Tyk-2 to relay signals to the nucleus via STAT-1, STAT-3, and STAT-5 protein. The transcription factors are then able to control the remodelling of the ECM, control growth modulation, and

regulate inflammatory responses through various proteins, growth factors and cytokines (Halfter et al., 2000). Results of research also suggest that most OSM mediated effects are via endothelial cells due to the extreme abundance of OSM receptors in these cells (Modur et al., 1997). Data from other studies suggest that OSM also uses the MAP kinase pathway to relay extracellular signals in murine adrenocortical Y-1 tumour cells and dermal fibroblasts; however, not through the PKC pathway (Auernhammer et al., 2004, Ihn and Tamaki, 2000). Nagata et al. (2003) suggested that OSM is able to use the MAP kinase pathway as growth factors were able to upregulate MMP-9 in smooth muscle cells through MAP kinase but not through the JAK/STAT pathway (Nagata et al., 2003).

1.6 Pro-inflammatory molecules- regulation of iNOS and COX-2

Nitric Oxide (NO) is an important signalling molecule that has been found to have major roles in many human systems. NO is synthesized, only on demand, for short periods of time following enzyme activation of constitutively expressed endothelial NO synthase (eNOS) or neuronal NO synthase (nNOS). In contrast, inducible NO synthase (iNOS) is expressed only after cell activation and then produces NO for long periods of time. iNOS, at the transcriptional level, is under the control of a variety of inflammatory molecules such as IL-6, cytokines, and lipopolysaccharide, and is linked to the progression of vascular disease (Wong et al. 1996, Syapin et al., 2001). Studies also show that altered iNOS protein concentrations are significantly correlated to gastric carcinogenesis and human melanomas (Wei Feng et al., 2002, Sikora et al., 2010, Watanabe et al., 2000). Another pro-inflammatory molecule, cyclooxygenase (COX) - 2

has also been directly linked with iNOS, and the production of NO is correlated with altered COX-2 synthesis. COX-2 is an enzyme that is responsible for the formation of substances that cause inflammation and pain. Formally called prostaglandin H2 synthase-2 (PGHS-2), COX-2 has been shown to be negatively regulated by NO in response to lipopolysaccharide (Lamon et al., 2010), and the overexpression of the enzyme has been linked to various cancers including colon cancer (Watanabe et al., 2000, Bahkle, 2001).

General hypothesis and research objectives

The following is the general hypothesis of this thesis:

H-ras mediated cellular transformation results in the overexpression of cancer associated activities including cellular proliferation and inflammation.

To test this hypothesis the following specific objectives were addressed:

1. Examination of the effects of phorbol-12-myristate acetate (PMA), a tumor promoter, on spermidine/spermine N1-acetyltransferase (SSAT) protein expression in H-ras transformed (NR3 cells) murine fibroblasts and the elucidation of the pathways involved.

   Previous studies in the laboratory have examined the effects of PMA on ODC and SAMDC (anabolic enzymes of the polyamine pathway) and have discovered

that PMA upregulated ODC and SAMDC levels in H-ras transformed cell but no studies have examined the effects of PMA on SSAT (catabolic enzyme of polyamine pathway). The polyamine pathway is a tightly regulated pathway so the possibility of tumor promoters upregulating both the anabolic and catabolic enzymes of the pathway would signify the possibility of ODC and SSAT directly impacting each other.

2. Examination of the effects of oncostatin M (OSM) on the expression and regulation of matrix metalloproteinases (MMP) and associated mediators in H-ras transformed fibroblasts and the elucidation of the signalling pathways involved.

   No previous work has examined the effects of OSM on NR3 cells and this novel study will elucidate the effects of OSM, a known growth regulator, on H-*ras* transformed NR3 cells, capable of benign tumor formation, compared to untransformed cells (10T ½ murine fibroblasts).

3. Examination of the effects of OSM on proinflammatory and proliferation linked activities in H-*ras* transformed fibroblasts (NR3 cells). Inflammation and proliferation of cancer cell are essential components of the progression of the disease. This study will examine the possible link between the expression of proinflammatory markers, iNOS and COX-2, proliferation linked genes, ODC and SSAT, and H-*ras* cellular transformation.

2. CHAPTER TWO

Tumor Promoter Mediated Regulation of Spermidine/Spermine N^1-Acetyltransferase in NR3 Murine Fibroblast Cells by Phorbol-12-Myristate Acetate

2.1. Abstract

Altered cellular growth and regulation are important in the progression of tumors. Cell growth regulation by tumor promoters can be very complex. This present study demonstrates a novel link between phorbol ester tumor promoter mediated alterations in the expression of spermidine/spermine N^1-acetyltransferase (SSAT), a key rate-limiting enzyme in the polyamine degradation pathway, and H-*ras* mediated cellular transformation. Treatment of NR3 cells (H-*ras* transformed, benign tumor forming cells) with PMA resulted in an induction of SSAT expression. SSAT expression increased dramatically in NR3 cells exposed to phorbol-12-myristate acetate (PMA) for 4 hours, whereas SSAT expression was apparently unaffected in parental non-transformed, normal 10T 1/2 cells. The effective PMA concentration in NR3 cells was determined to be 0.1uM. Studies investigating the possible mechanism(s) whereby PMA mediated alterations in SSAT expression occurs in NR3 cells suggest a role for protein kinase-C, mitogen activated protein kinase, and P-I-3 kinase mediated events playing a regulatory role. Altered expression and regulation of SSAT by tumour promoters represents an aspect of the altered cellular growth program inherent to H-*ras* transformed cells.

2.1. Introduction

Polyamines are essential for the proper growth, proliferation, and function of all normal cells (Wallace et al., 2003). Normal cell growth is intricately controlled by alterations in polyamine expression by anabolic and catabolic enzymes (Thomas and Thomas, 2003). In cancerous tissue, polyamine levels and specific enzymes involved in polyamine catabolism are altered compared to the surrounding normal tissue, making polyamines an excellent marker for cancer studies (Criss, 2003). The polyamine pathway in all mammalian cells is divided into a biosynthetic and catabolic component. Spermidine/spermine N^1-acetyltransferase (SSAT) is the rate-limiting enzyme of the catabolic component of the pathway that converts spermidine and spermine to N-acetyl spermidine and N-acetyl spermine, respectively (Wallace et al., 2003, Criss, 2003, Pegg, 1986, Pegg, 2008). The activity of SSAT is low under basal cellular conditions but can be inhibited, activated and/or induced rapidly in response to a number of metabolites and polyamine derivatives (Matsui and Pegg, 1998). An increase in SSAT activity can decrease polyamine levels and prevent cellular proliferation in a variety of cell types (Casero and Pegg, 1993, Chen et al., 2003). The rate of tumor progression can also be accelerated when cells are stimulated by non-mutagenic factors such as tumor promoters. Tumor promoters are capable of affecting gene expression by stimulating cellular proliferation (Zoumpourlis, 2003). Alone, tumor promoters can only increase the sensitivity of cells to tumor formation; but, when they act in concert with mutagenic agents (tumor initiators) they are capable of forming tumors (Henning et al., 1983). Phorbol ester tumor promoters, such as phorbol-12-myristate acetate (PMA), are commonly used in cancer studies due to their ability to bind to and affect the protein

kinase C (PKC) pathway and mitogen-activated protein kinase (MAPK) pathway causing a wide array of effects in cells (Amaral et al., 1993, Tiberio, 2001).
In this specific study the effects of PMA on SSAT protein expression in H-ras transformed murine fibroblast cells was investigated.

2.3. Materials and Methods

2.3.1 Cell lines

Mouse 10 T ½ cells were transfected with the plasmid pAL8A which contains T-24-H-*ras* and the neomycin resistance gene. Post-transfection, cell lines were established which included H-ras transformed NR3 cells which are morphologically non-transformed (Egan et al, 1987, Hurta et al, 1996). It is important to note that the 10 T ½ cell line is not tumorigenic, whereas the NR3 cell line is capable of forming benign tumors in syngeneic hosts (Egan et al, 1987, Hurta et al, 1996).

2.3.2. Cell culture and treatment conditions

10 T ½ cells and NR3 cells were cultured on 100-mm plastic culture dishes (Falcon) (BD Biosciences, Mississauga, ON) in α-minimal essential medium (MEM) (Invitrogen, Burlington, ON) supplemented with antibiotic/antimycotic (Invitrogen, Burlington, ON) and 10% (v/v) fetal bovine serum (Hyclone Laboratories, Mississauga, ON) and were cultured at 37°C in 5% CO_2. Cells were cultured to confluence prior to exposure to PMA (Sigma, Oakville, ON). In experiments using PMA, a serum-free

medium (defined medium) was used that contained 4 μg/ml transferrin (Sigma) and 2 μg/ml insulin (Sigma). Cells were cultured to confluence and then placed in defined medium for 24 hr prior to PMA treatment. Post PMA treatment cells were removed from the tissue culture plates using a 0.3% buffered trypsin solution (Sigma).

To evaluate possible involvement of signal transduction pathways, cells were pre-treated for 1 hour with specific pathway inhibitors prior to 4 hour exposure of PMA. The inhibitors used were protein kinase C (PKC) inhibitor calphostin C (Sigma), mitogen activated protein (MAP) kinase inhibitor PD98059 (Sigma), and phosphatidylinositol-3 (P-I-3) kinase inhibitor LY294002 (Sigma) at concentrations of 500nM, 50μM and 10μM, respectively.

2.3.3. SDS-PAGE and western blot analysis-

Cells were harvested and lysed in cell lysate buffer containing 10mM Tris and 1mM phenylmethylsulphonyl fluoride (PMSF) (Sigma). Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, ON). Equal amounts of protein were electrophoresed on 15% SDS-polyacrylamide gels and transferred for 1.5 hr onto 0.2μm nitrocellulose membranes (Bio-Rad Laboratories). Blots were blocked in Tris-buffered saline (TBS) (50mM Tris-HCl and 150mM NaCl) containing 1% bovine serum albumin (BSA) (Sigma), followed by incubation in TBS supplemented with 0.05% Tween-20 (TBS-T) (Sigma) and 1:200 diluted SSAT primary antibody (a gift from Robert Anthony Casero, John Hopkins University, Baltimore, MD). Blots were washed with TBS-T and incubated in 1:2000 goat anti-rabbit

secondary antibody for 2 hr at room temperature. Blots were washed with TBS-T and developed using BCIP-NBT tablets. The housekeeping protein β-actin was used as a loading control. Complementary membranes were incubated with β-actin antibody at 1:100 dilution followed by incubation in 1:500 β-actin secondary antibody and processed as described above with the exception that actin protein was electrophoresed on 7.5% SDS-polyacrylamide gels.

All western blot analysis was performed in triplicate and quantified using Bio-rad Quantity One Software. Quantity One Software calculates band density by detecting the pixel intensity and the volume of the band.

2.4. Results

2.4.1. SSAT basal protein expression levels are elevated in NR3 cell lines compared to 10T ½ cell lines.

SSAT protein expression was evaluated in non-transformed 10T ½ cells compared to NR3 cells by western blot analysis (Fig. 2.1A). Results revealed a 2.0 fold increase compared to basal levels in SSAT protein expression in NR3 cells compared to 10T ½ cells (Fig. 2.1B). This response demonstrates a direct relationship between H-*ras* transformation and SSAT protein expression.

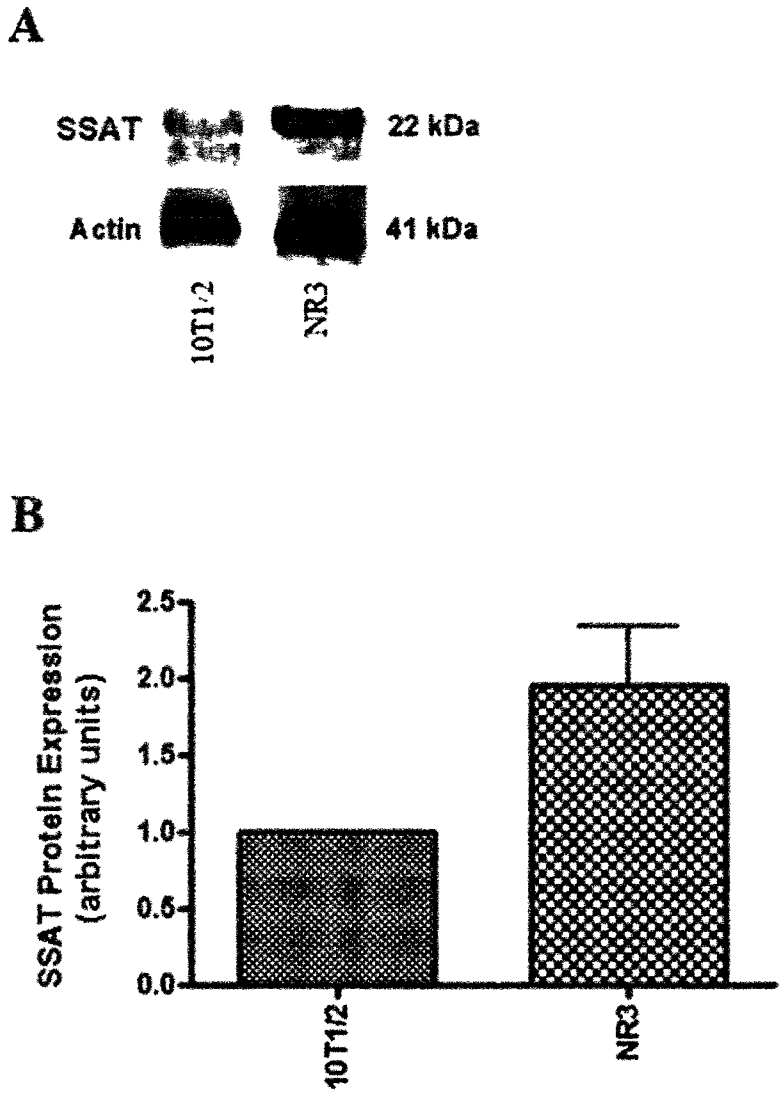


Figure 2.1. Basal expression levels of SSAT protein was elevated in NR3 cells (H-*ras* transformed) compared to 10T ½ cells (non-transformed phenotype). Protein expression of SSAT and actin were analyzed by western blot analysis (Fig.2.1A) and protein expression was quantified and represented with a histogram (Fig.2.1B). (1) 10T ½ cells with the vehicle DMSO (2) NR3 cells with DMSO.

2.4.2. Phorbol-12-Myristate Acetate upregulates SSAT protein expression in H-*ras* transformed murine fibroblast cells.

Protein expression of SSAT was examined in response to PMA by western blot analysis. In the non-transformed phenotype (10T ½ cells) SSAT protein expression was unaltered in response to 0.1μM PMA for 4 hours (Fig.2.2A). A 2.6-fold increase was observed in SSAT protein expression in NR3 cells in response to 0.1μM PMA (Fig. 2.2B).

2.4.3. Optimal effects of Phorbol-12-Myristate Acetate mediated alteration in SSAT protein expression.

The optimal effects of PMA was investigated on SSAT protein expression in 10T ½ cells and NR3 cells and included a range of PMA concentrations (0.001, 0.01, 0.1, 1.0 μM). SSAT protein expression was apparently unaffected in 10T ½ cells in response to PMA treatment at all PMA concentrations examined (Figure 2.3A). As shown in Figure 2.3B, NR3 cells showed an increase in SSAT protein expression commencing at 0.01μM and increasing dramatically in a dose dependent manner at 0.1μM. A 3.5 fold increase was observed in SSAT protein expression at 0.01 μM PMA, a 7.1 fold increase at 0.1 μM PMA, and a 2.1 fold increase at 1.0 μM in NR3 cells. There was no apparent alteration in SSAT protein expression at 0.001μM.

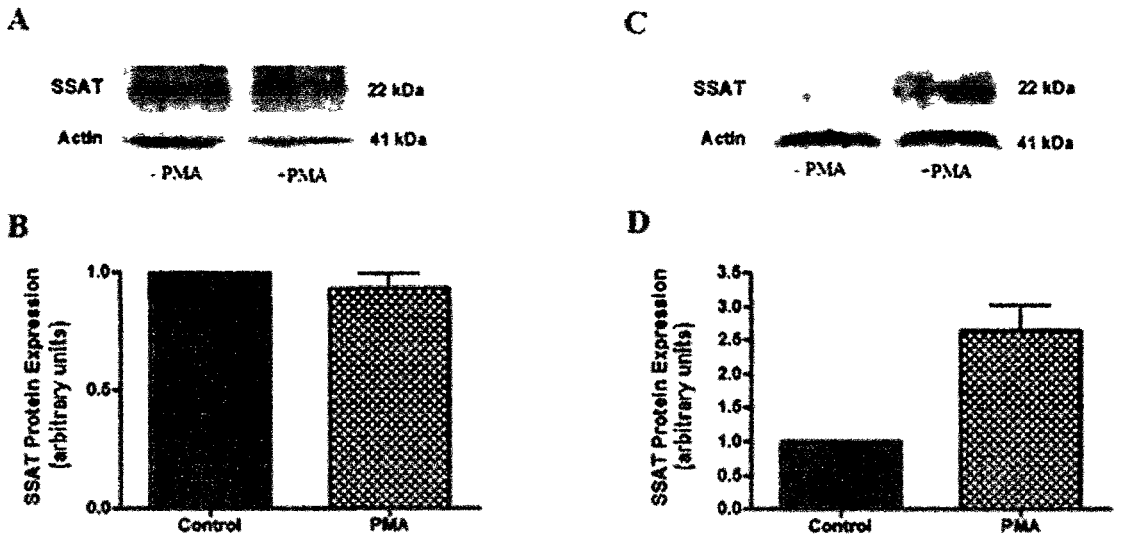


Figure 2.2. 10T ½ and NR3 cells were treated for 4 hours with 0.1μM PMA and protein expression of SSAT and actin were analyzed by Western blot analysis and represented with histograms. Fig. 2.2A- Western blot analysis of 10T ½ cells treated with PMA, Fig. 2.2B- Quantification of SSAT protein expression in 10T ½ cells treated with PMA, Fig. 2.2C- Western blot analysis of NR3 cells treated with PMA, and Fig. 2.2D- Quantification of SSAT protein expression in NR3 cells treated with PMA. Cells were treated as follows: 1. control (DMSO) 2. 0.1μM PMA

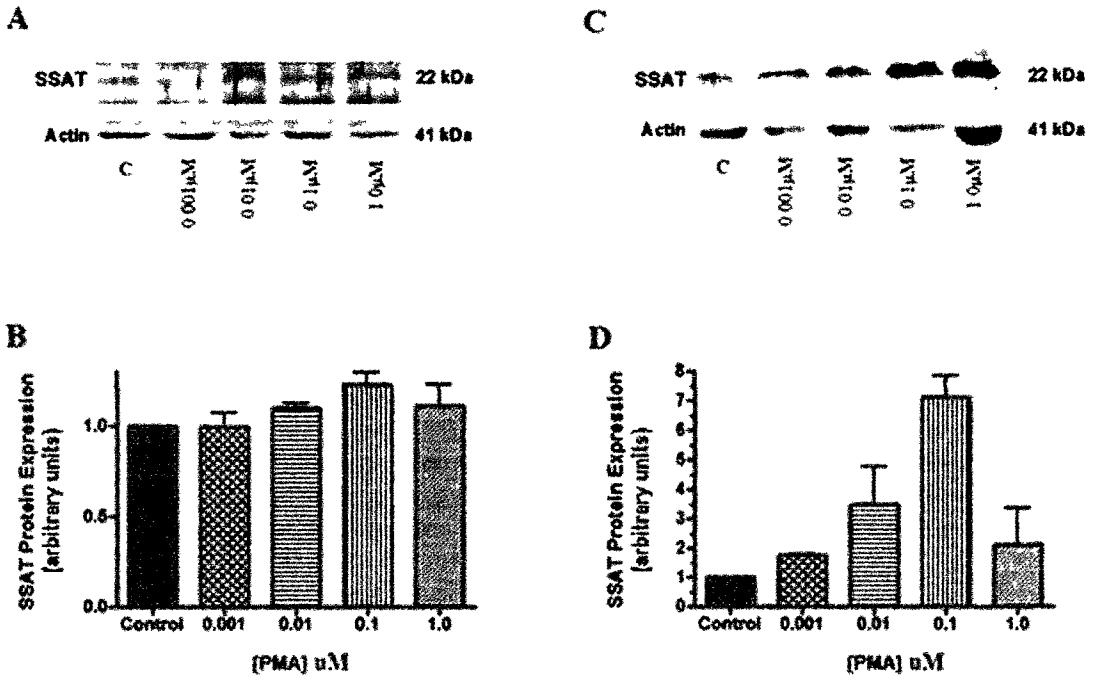


Figure 3. PMA progressively upregulates SSAT protein expression in transformed murine fibroblasts (NR3). Protein expression was determined by western blot analysis and protein expression was quantified and represented with histograms. Fig. 2.3A- Western blot analysis of 10T ½ cells treated with PMA, Fig. 2.3B- Quantification of SSAT protein expression in 10T ½ cells treated with PMA, Fig. 2.3C- Western blot analysis of NR3 cells treated with PMA, and Fig. 2.3D- Quantification of SSAT protein expression in NR3 cells treated with PMA. Cells were treated under the following conditions: 1. control (DMSO) 2. 0.001μM PMA 3. 0.01μM PMA 4. 0.1μM PMA 5. 1.0μM PMA 6. 10.0μM PMA.

2.4.4. Phorbol-12-Myristate Acetate affects SSAT protein expression in NR3 cells in a time dependent manner.

Various time intervals were investigated to determine the earliest time point that PMA (0.1μM) altered SSAT protein expression in NR3 cells. Time points examined included 0, 2, 4 and 8 hours. Results showed (Figure 2.4A) that PMA altered SSAT protein expression as early as 2 hours (1.8 fold increase); however, optimal SSAT protein expression was observed at 4 hours (5.6 fold increase). At 8 hours PMA treatment there was also a 3.4 fold increase in SSAT protein expression.

2.4.5. PMA mediated alterations in SSAT protein expression is regulated by specific signal transduction pathways in NR3 cells.

Using western blot analysis, mitogen activated protein kinase (MAPK) pathway, protein kinase C (PKC) pathway, and the P-I-3 kinase pathways were investigated to determine any possible involvement in the PMA mediated alterations in SSAT protein expression in NR3 cells (Figure 2.5A/2.5C). The PMA concentration used was 0.1μM. Concentrations (v/v) of the PKC, MAPK and P-I-3 pathway inhibitors used were calphostin C (500 nM), PD 098059 (50μM), and LY 294002 (10 μM), respectively.

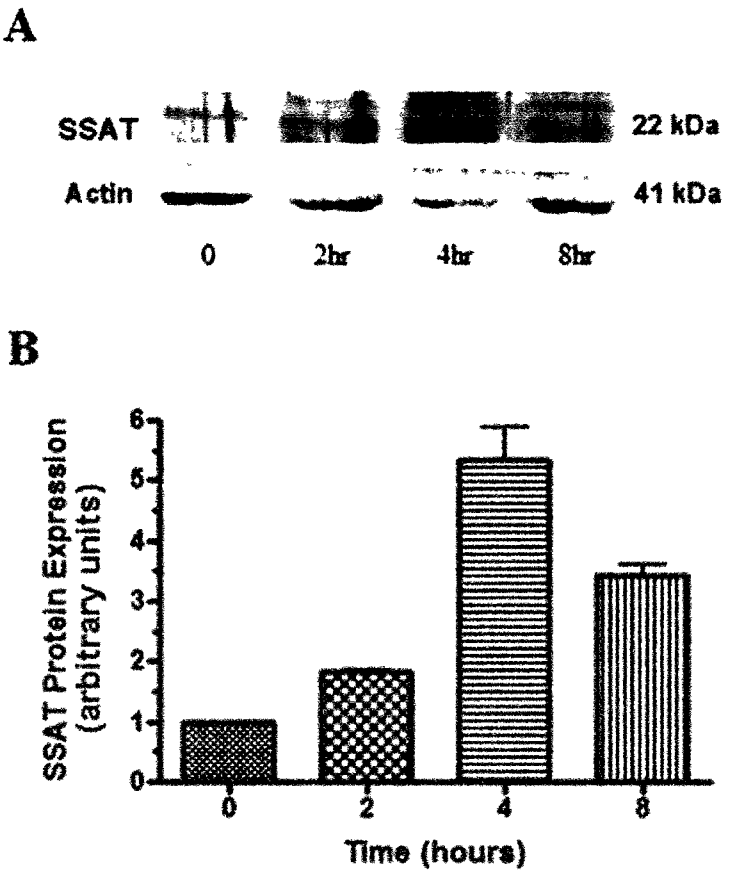


Figure 4. PMA mediated alterations in SSAT protein expression in NR3 cells was apparent as early as 4 hours at 0.1μM PMA. SSAT protein expression was determined by western blot analysis (Fig. 4A) and represented with a histogram (Fig. 4B). Cells were treated as follows: 1. control (DMSO) 2. 2hrs 3. 4hrs 4. 8hrs.

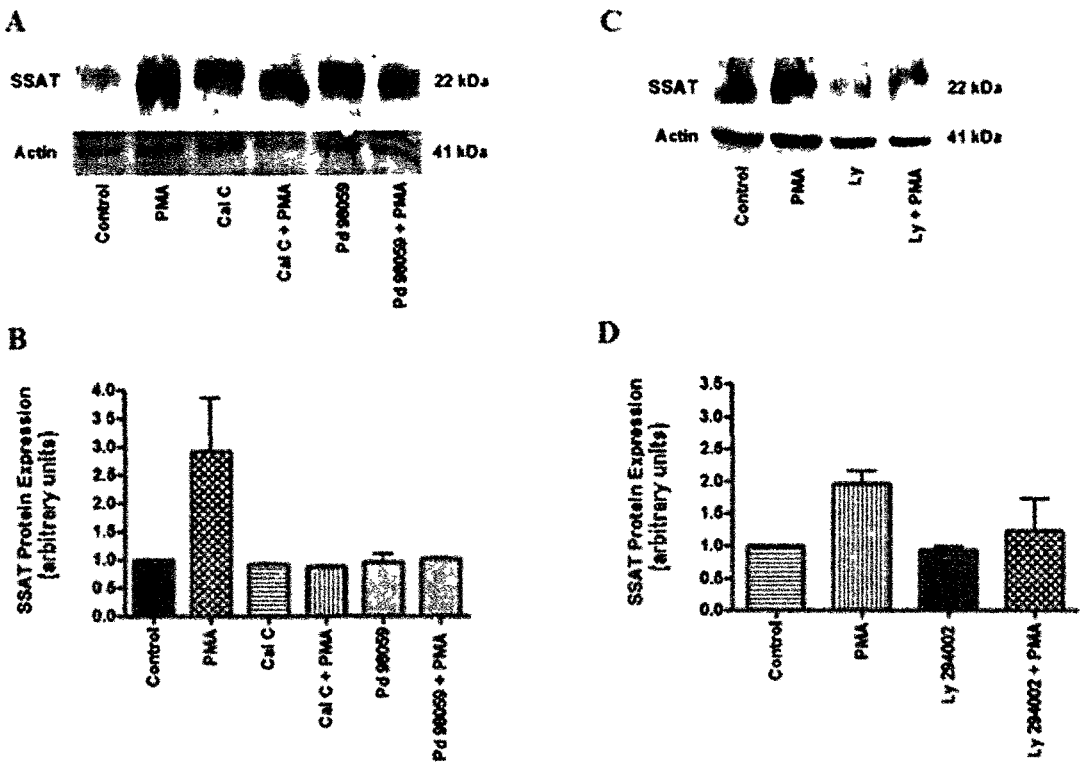


Figure 5. NR3 cells were treated for 4 hours with 0.1μM PMA and/or inhibitor, and the protein expression of SSAT and actin were analyzed by western blot analysis. Fig. 2.5A/2.5B shows the involvement of the PKC pathway and the MAP kinase pathway in the PMA mediated events in SSAT alteration. NR3 cells were treated under the following conditions: 1. control (DMSO) 2. 0.1μM PMA 3. 0.65M calphostin C 4. calphostin C + 0.1μM PMA 5. 0.65M PD 098059 6. PD 098059 + 0.1μM PMA. Fig. 2.5C/2.5D -The P-I-3 kinase pathway is also involved in the regulation of SSAT protein expression in NR3 cells post PMA treatment. NR3 cells were treated as follows: 1. control (DMSO) 2. 0.1μM PMA 3. LY 294002 4. LY 294002 + 0.1μM PMA.

2.5. Discussion

The regulation of SSAT has been shown to be vitally important for cellular proliferation, cellular differentiation and cell death in a variety of cell types (Zahedi et al., 2006, Rodriguez et al., 2001, Hong Min, 2002, Babbar et al., 2006). SSAT is the rate limiting catabolic enzyme that is responsible for converting spermidine and spermine to N-acetyl spermidine and N-acetyl spermine, respectively (Tabor and Tabor, 1985). Previous studies have discovered high intracellular polyamine levels in cancerous cells compared to non-cancerous cells (Devens et al., 2000, Saverio et al., 2000). More specifically, studies have discovered that cancerous cells show an increase in the catabolic intermediates (N-acetyl spermidine and N-acetyl spermine) of the polyamine pathway (Hurta et al., 2001), which possibly suggests that SSAT activity is increasing in cancerous cells by acetylating excess spermidine and spermine molecules. Phorbol-12-myrsitate acetate was shown to induce spermidine/spermine N^1-acetyltransferase (SSAT) in NR3 cells (H-*ras* transformed, benign tumor forming). SSAT induction in protein expression was apparently unaffected in the non-transformed 10T ½ cells which suggests a direct relationship between H-*ras* cellular transformation and PMA mediated upregulation of SSAT protein expression. Previous studies investigating PMA effects on NR3 cells have showed an increase in ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) gene expression, both short-lived enzymes (Hurta et al., 2001). This evidence correlates with SSAT induction with PMA in H-*ras* transformed cells suggesting that there is a direct relationship between alterations in anabolic enzymes (ODC) and catabolic enzymes (SSAT) in the polyamine pathway allowing for intracellular polyamine homeostasis.

The ODC and SSAT relationship also suggests a possible mechanism by which PMA stimulation of H-*ras* transformed cells may allow cancer cells to continue to proliferate.

Using specific inhibitors of signal transduction pathways, PMA mediated induction in SSAT protein expression in NR3 cells was found to be regulated through specific pathways; the protein kinase C (PKC) pathway, the mitogen activated protein (MAP) kinase pathway, and the P-I-3 kinase pathway. The PKC pathway has been reported to be elevated in breast tumors, leukemic cells, lung cancer cells, and mouse and human colon tumors (Nishizuka, 1984). SSAT expression was also shown to be up-regulated after PMA-evoked activation of PKC in human myeloid leukemia cells and PKC activation was also apparent in murine papilloma cells in the regulation of ODC post hydrogen peroxide treatment (Gavin et al., 2004, Otieno and Kensler, 2000). Activation of the MAP kinase pathway is essential for signal transduction in response to numerous stimuli. In various human melanoma cell lines and porcine uterine endometrial cells, MAP kinase activation was found to be linked to the induction of SSAT activity (Chen et al., 2003).

When studying the progression of cancer in any cell, SSAT is a logical enzyme to examine; SSAT is present in very low quantities in normal cells but becomes extremely elevated in cancerous tissue, SSAT is the rate limiting enzyme in the degradation pathway of polyamines, and SSAT has a very short half-life but is stabilized by other polyamines in the pathway which shows the importance of SSAT to polyamine metabolism. Further investigating how SSAT is able to regulate and interact with other

polyamines will yield a better understanding of how the polyamine pathway might be a key pathway in regulating the uncontrollable cell growth and proliferation inherent to cancer progression.

3. CHAPTER THREE

Oncostatin M Mediated Expression and Regulation of Matrix Metalloproteinases in an H-*ras* Transformed Fibroblast Capable of Benign Tumour Formation.

3.1. Abstract

Oncostatin M (OSM) is a growth and differentiation factor that has been shown to exert both stimulatory and inhibitory effects on tissue invasion in various cell lines. Tissue invasion can be monitored by examining markers of extracellular matrix degradation such as matrix metalloproteinase (MMP) activity. This present study elucidates a link between OSM, MMPs and various mediators associated with MMP activity. Treatment of NR3 cells (H-*ras* transformed, benign tumour forming cells) with OSM resulted in an induction of MMP-9 and MMP-2 activity. MMP-9 and MMP-2 activity increased in response to 50 ng/ml OSM at 24 hr. MMP-9 activity also increased as early as 2 hours when exposed to OSM at a concentration of 200ng/mL, whereas MMP-2 activity decreased. The expression of associated mediators which affect MMP expression was determined in response to OSM treatment. EMMPRIN (extracellular matrix metalloproteinase inducer), RECK (reversion-inducing-cysteine-rich protein with Kazal motifs), and TIMP-1 and TIMP-2 (tissue inhibitors of MMP) expression was determined. NR3 cells were exposed to OSM at 200ng/mL for 2 hours and to OSM at 50ng/mL for 24 hours, respectively. Western blot analysis revealed that EMMPRIN protein expression increased dramatically at50ng/ml for 24 hours and increased markedly at 200ng/ml for 2 hours. RECK protein expression was unaltered in response

to 200ng/ml OSM but was decreased at 50ng/ml for 24 hours. TIMP-1 and TIMP-2 protein expression increased in response to 200ng/ml but was slightly decreased at 50ng/ml for 24 hours. The upregulation of MMP-9 activity in NR3 cells in response to OSM treatment apparently involves the phosphatidylinositol-3 (P-I-3) kinase pathway and the up-regulation of MMP-2 activity apparently involves protein kinase C (PKC) and MAP kinase (MAPK) pathways. The involvement of the JAK/STAT pathway in this OSM-mediated response in NR3 cells was also demonstrated.

3.2. Introduction

Oncostatin M (OSM) is a pleiotropic cytokine that belongs to the interleukin-6 superfamily. It is synthesized by monocytes, macrophages and activated T lymphocytes and acts through specific receptors in a wide variety of cells including lung and endothelial cells and many tumour cell lines (Nagata et al., 2003, Weiss et al., 2006). The receptors for OSM lack an intrinsic tyrosine kinase domain and, therefore, rely on the JAK/STAT cytoplasmic pathway as a mediator of signal transduction. This pathway utilizes Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) to relay signals from cytokines (and growth factors) to the cell nucleus allowing STAT proteins to modify gene expression (Chen and Benveniste, 2004, Chen et al., 2005). The pathway plays a central role in cell fate decisions such as proliferation, differentiation and apoptosis (Gomez-Lechon, 1999). Recent studies also suggest a role for the JAK/STAT pathway in extracellular matrix (ECM) metabolism in human astrocytes (Nagase and Woessner, 1999). The ECM has been shown to be tightly

regulated by matrix metalloproteinases (MMPs). MMPs are enzymes that are essential for the proteolytic degradation of collagens and proteoglycans of the ECM. There are numerous members of the MMP family and each are classified according to the substrate the enzyme is capable of degrading (Deryugina and Quigley, 2006). One important subfamily of MMPs includes the gelatinases which consists of matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2). Both MMP-9 and MMP-2 are considered to be important in the metastasis of cancer cells due to their ability to break down the ECM of the cells (Li and Zafarullah, 1998, Stetler-Stevenson and Yu, 2001).

MMPs play vital roles in normal cellular dynamics and therefore their expression is a highly regulated process. Tissue inhibitors of metalloproteinase (TIMPs) are one method of MMP regulation; TIMP-1 and TIMP-2 have been shown to regulate MMP-9 and MMP-2, respectively. TIMPs bind to and inhibit the active form of MMPs and have been shown to inhibit cell invasion *in vitro* and tumorigenesis/metastasis *in vivo* (Chirco et al., 2006, Lambert et al., 2004). MMPs are also highly regulated by EMMPRIN (extracellular matrix metalloproteinase inducer) and RECK (reversion-inducing-cysteine-rich protein with kazal motifs). EMMPRIN is very important in tissue remodelling and has been reported to be induced by several MMPs, including MMP-9 and MMP-2 (Gabison et al., 2005, Nabeshima, 2006). Down-regulation of RECK in cancer cell lines has been shown to result in excessive activation of MMPs thereby promoting invasion and metastasis (Noda et al., 2003). This study will examine the

effects of OSM on the expression and regulation of MMP and associated mediators in H-*ras* transformed murine fibroblasts.

3.3 Materials and Methods

3.3.1. Cell lines and growth conditions

Mouse 10 T ½ cells were transfected with the plasmid pAL8A which contains T-24-H-*ras* and the neomycin resistance gene. Post-transfection, cell lines were established which included NR3 cells which are morphologically non-transformed (Noda et al., 2003, Egan et al., 1987). It is important to note that the 10 T ½ cell line is not tumorigenic whereas the NR3 cell line is capable of forming benign tumours in syngeneic hosts (Noda et al., 2003, Egan et al., 1987).

10 T ½ cells and NR3 cells were cultured on 100-mm plastic culture dishes (Falcon) (BD Biosciences, Mississauga, ON) in α-minimal essential medium (MEM) (Invitrogen, Burlington, ON) supplemented with antibiotic/antimycotic (Invitrogen) and 10% (v/v) fetal bovine serum (Hyclone Laboratories, Mississauga, ON) and were cultured at 37°C in 5% CO_2, as previously described (Hurta and Voskas, 2001). Cells were cultured to confluence prior to exposure to Oncostatin M (OSM) (R&D Systems, Burlington, ON). In experiments using OSM, a serum-free medium (defined medium) was used that contained 4μg/ml transferrin (Sigma, Oakville, ON) and 2μg/ml insulin (Sigma). Cells were placed on the defined medium for 24 hr prior to OSM treatment.

Cells were removed from the tissue culture plates using a 0.3% buffered trypsin solution (Sigma).

3.3.2. SDS-PAGE and western blot analysis

Cells were harvested and lysed in cell lysate buffer containing 10mM Tris and 1mM phenylmethylsulphonyl fluoride (PMSF) (Sigma). Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, ON). Equal amounts of protein were electrophoresed on SDS-polyacrylamide gels and transferred for 1.5 hr onto 0.2μm nitrocellulose membranes (Bio-Rad Laboratories). The Western blots were blocked in Tris-buffered saline (TBS) (50mM Tris-HCl and 150mM NaCl) containing 1% bovine serum albumin (BSA) (Sigma), followed by incubation in TBS supplemented with 0.05% Tween-20 (TBS-T) (Sigma) and specific primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for JAK-1(A-9), JAK-2(C-20), JAK-3(L-20), EMMPRIN(G-19), RECK(S-18), TIMP-1(H-150), TIMP-2(C-20), STAT-1 p84/p91 (C-136), STAT-2(H-190), STAT-3(F-2), and STAT-4(L-18). Blots were washed with TBS-T and incubated in secondary antibody for 2 hr at room temperature. Specific secondary antibodies were used for different proteins. Secondary antibodies used were as follows: actin, JAK-1, and JAK-2 used goat-anti mouse IgG; EMMPRIN, RECK, and JAK-3 used donkey-anti goat IgG; TIMP-1, TIMP-2, and STAT 1-4 used goat-anti rabbit IgG. Blots were developed using Sigma Fast BCIP/NBT developing tablets (Sigma). As a loading control, the housekeeping protein β-actin was used.

All western blot analysis was performed in triplicate and quantified using Bio-rad Quantity One Software. Quantity One Software calculates band density by detecting the pixel intensity and the volume of the band.

3.3.3. Gelatin zymography

Gelatinolytic activity was analyzed by gelatin gel electrophoresis essentially as described previously (Leber and Balkwill, 1997, Matchett, 2005). Briefly, an aliquot of conditioned medium was mixed (4:1) with sample buffer which consisted of 10% SDS, 0.1% bromophenol blue in 0.312 M Tris-HCl, pH 6.8, and incubated at 37°C for 10 min. Aliquots of each sample were loaded into wells of a 5% stacking gel and resolved by electrophoresis at a constant current (30mA). The 10% resolving gel contained Type A gelatin (Sigma) at a final concentration of 1mg/ml. Following electrophoresis, gels were washed in 0.05 M Tris–HCl, pH 7.4, containing 2% Triton X-100 for 1 hr at room temperature followed by a 30 min wash in 0.05 M Tris–HCl, pH 7.4, at room temperature. Gels were then incubated at 37°C for 24 hr in a substrate buffer containing 0.05 M Tris–HCl, pH 7.4, 1% Triton X-100 and 0.005 M $CaCl_2$. Following this incubation period, gels were stained with 0.1% Coomassie Blue R-250 in a solution of acetic acid, methanol and water (5:50:45 v/v/v), and destained in a solution of acetic acid, methanol and water (5:10:85 v/v/v). Gelatinolytic activity appears as zones of clearing (due to the degradation of gelatin) against a blue background.

All gelatin zymography was performed in triplicate and quantified using Bio-rad Quantity One Software. Quantity One Software calculates band density by detecting the pixel intensity and the volume of the band.

3.4. Results

3.4.1. Effects of OSM on MMP activity

OSM treatment of NR3 cells up-regulates the activity of MMP-9 and MMP-2. Gelatinolytic activity of conditioned medium from NR3 cells was determined following 24 hr treatment of NR3 cells with 1, 10, 25, and 50 ng/ml of OSM, respectively. As shown in Figure 3.1, MMP-9 activity and MMP-2 (72 kDa, 68 kDa, and 66 kDa isoforms) activity increased at 50ng/ml treatment of OSM for 24 hr. MMP-9 and MMP-2 activity were unaffected in 10 T ½ cells with similar OSM treatments (data not shown). Figure 3.2 shows a histogram of the correlating gelatinolytic activity of MMP-9 (Fig. 3.2A) and MMP-2 (Fig. 3.2B) in NR3 cells in response to OSM.

The OSM-induced up-regulation of MMP-9 and MMP-2 (72 kDa and 68 kDa isoforms) activity was time dependent when NR3 cells were stimulated with 50ng/ml OSM. Gelatinolytic activity of conditioned medium from NR3 cells was determined following 12, 24, 36, and 48 hr exposure to 50ng/ml OSM. As shown in Figure 3.3, MMP-9 activity increased slightly at 12 hr and continued to increase markedly at 24, 36, and 48 hr. MMP-2 (72 kDa and 68 kDa isoforms) activity increased at 12 and 24 hr but

increased substantially at 36 and 48 hr. Figure 3.4 shows a histogram of the correlating gelatinolyic activity of MMP-9 (Fig. 3.4A) and MMP-2 (Fig. 3.4B). The effect of a higher dose of OSM for a short exposure time was also examined to determine whether MMP-9 and MMP-2 activity was stimulated in NR3 cells under these conditions. Cells were treated with 200 ng/ml OSM for 2 hr. As shown in Figure 3.5A and 3.5B, MMP-9 activity increased markedly in response to 200 ng/ml for 2 hr, however MMP-2 activity decreased.

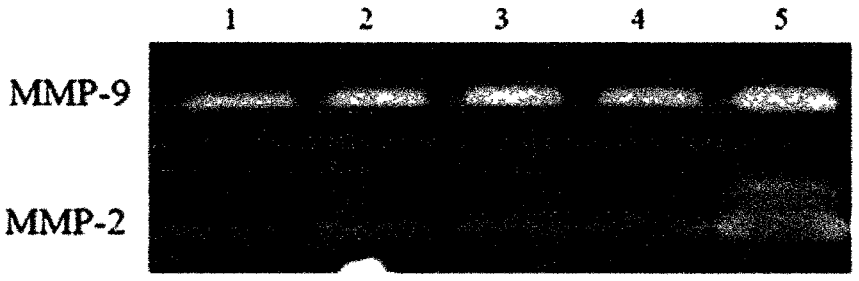
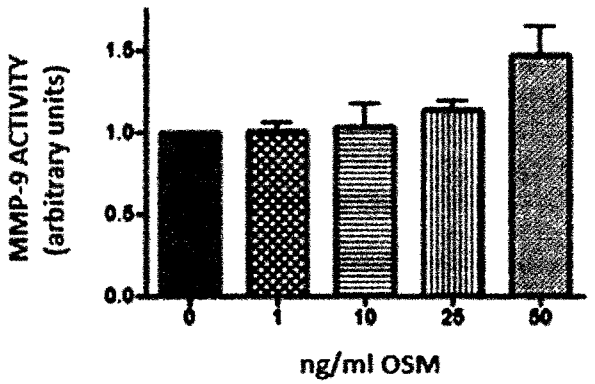


Figure 3.1: Effects of OSM treatment on the activity of MMP-9 and MMP-2 in NR3 cells. Gelatin zymography was performed on aliquots of conditioned medium from NR3 cells treated with various concentrations of OSM for 24 hr exposure. (1) Control, (2) 1ng/ml OSM, (3) 10ng/ml, (4) 25ng/ml, and (5) 50ng/ml.

A



B

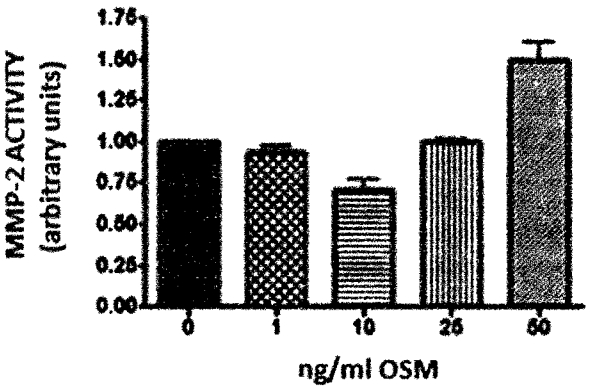


Figure 3.2: Effects of OSM treatment on NR3 cells up-regulates the activity of MMP-9 and MMP-2. Gelatin zymography was performed on aliquots of conditioned medium from NR3 cells treated with various concentrations of OSM for 24 hr exposure. Results were quantified for MMP-9 protein expression (Fig. 3.2A) and MMP-2 protein expression (Fig. 3.2B).

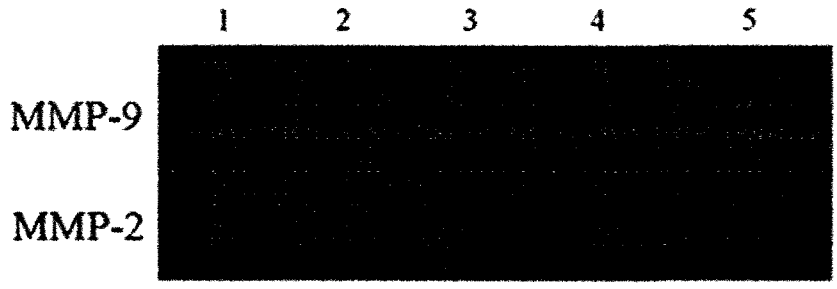
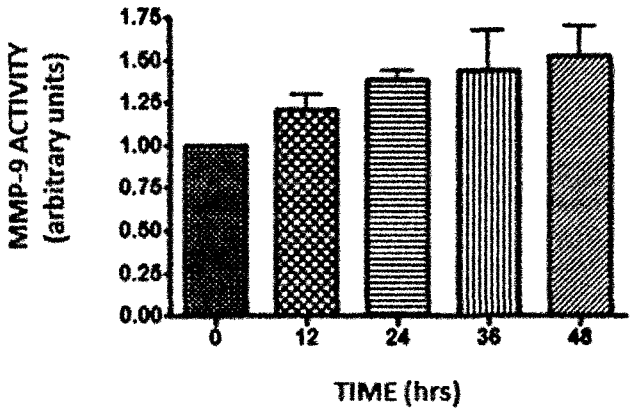


Figure 3.3: Effects of OSM treatment on NR3 cells up-regulates the activity of MMP-9 and MMP-2 in a time dependent response. Gelatin zymography was performed on aliquots of conditioned medium from NR3 cells treated with 50ng/ml OSM at various time intervals. (1) Control, (2) 12 hr, (3) 24 hr, (4) 36 hr and (5) 48 hr exposure.

A



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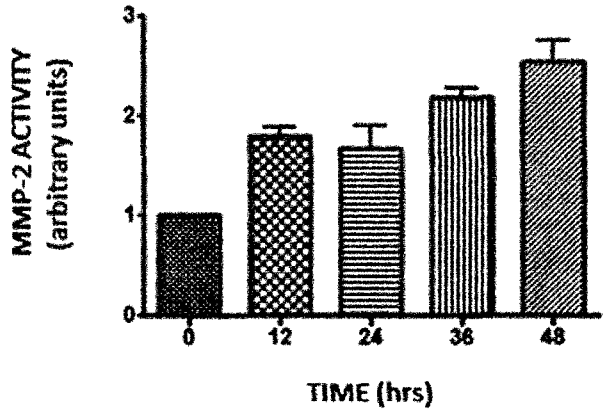


Figure 3.4: Effects of OSM treatment on NR3 cells up-regulates the activity of MMP-9 and MMP-2 in a time dependent response. Gelatin zymography was performed on aliquots of conditioned medium from NR3 cells treated with 50ng/ml OSM at various time intervals. Results were quantified for MMP-9 protein expression (Fig. 3.4A) and MMP-2 protein expression (Fig. 3.4B).

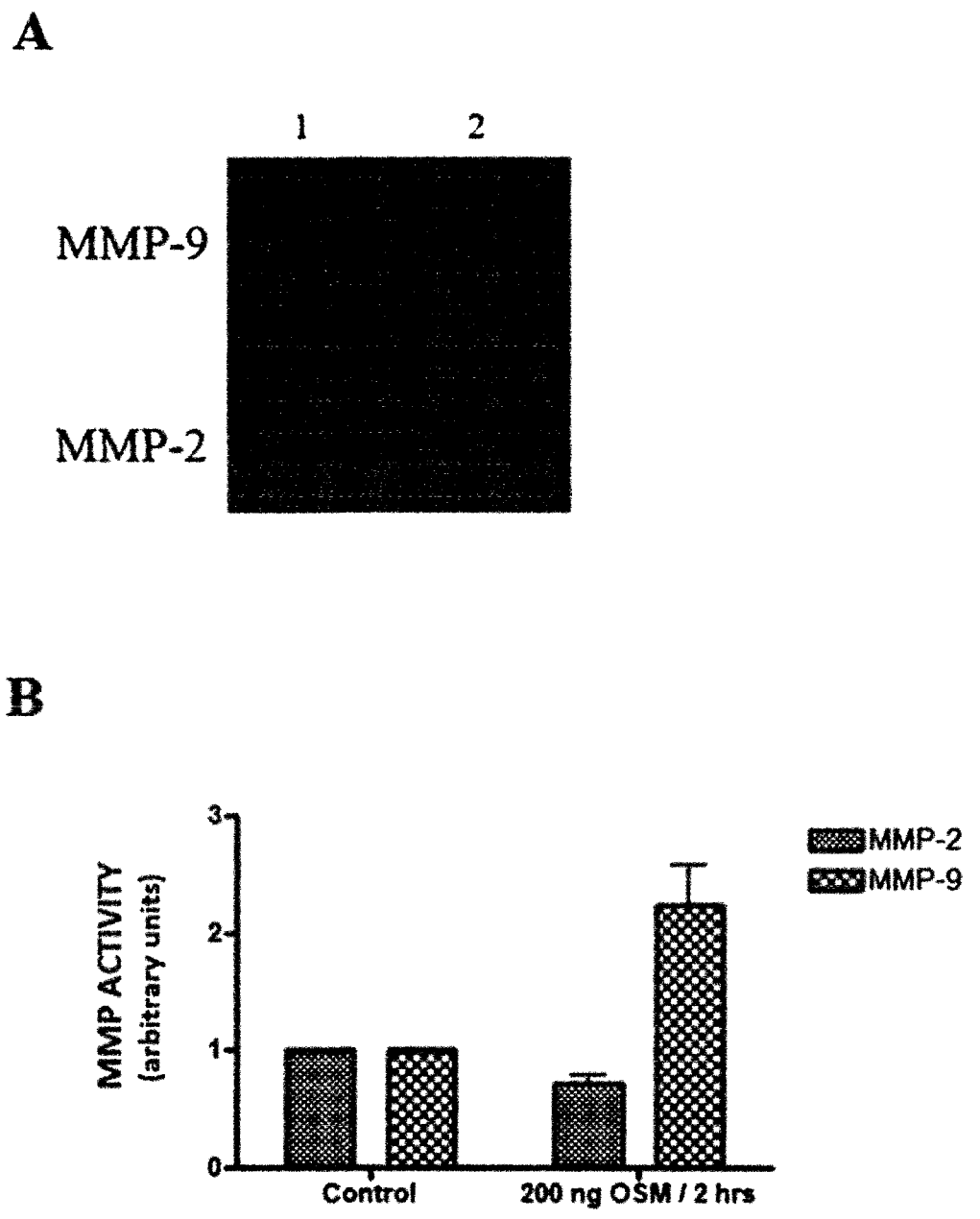


Figure 3.5: Effects of elevated OSM concentration on NR3 cells up-regulates MMP-9 activity but apparently down-regulates MMP-2 activity. Gelatin zymography was performed on aliquots of conditioned medium from NR3 cells treated with 50ng/ml OSM for 24 hr and 200ng/ml OSM for 2 hr (Fig. 3.5A). Results were quantified for MMP-9 and MMP-2 protein expression (Fig. 3.5B).

3.4.2. Effects of cellular signalling inhibitors on OSM mediated alterations in MMP-9 and MMP-2 activity in NR3 cells

Gelatin gel electrophoresis was performed using conditioned medium from NR3 cells pre-treated for 1 hr with known inhibitors of signalling pathways, prior to a 24 hr exposure of cells to 50ng/ml OSM. As shown in Figure 3.6, phosphatidylinositol-3 (P-I-3) kinase inhibitor LY 294002 (10μM) apparently alleviated the elevated MMP-9 activity in NR3 cells in response to OSM but had no effect on the induction of MMP-2 suggesting a possible involvement of the P-I-3 kinase pathway in the OSM stimulation of MMP-9 activity in NR3 cells. The protein kinase C inhibitor calphostin C (500 nM) had no effect on the up-regulation of MMP-9 activity in NR3 cells in response to OSM; but, was able to inhibit the up-regulation of MMP-2 activity in response in OSM, showing that the protein kinase C pathway is involved in the OSM-mediated stimulation of MMP-2 activity in NR3 cells. Cells were also treated with the MAP kinase inhibitor PD98095 (50μM). The inhibitor had no effect on the OSM-mediated up-regulation of MMP-9 activity but inhibited the effects of OSM on MMP-2 activity in NR3 cells suggesting that the MAP kinase pathway is involved in the OSM-mediated stimulation of MMP-2 activity but apparently not involved in the stimulation of MMP-9 activity. AKT IV inhibitor (10μM), a known inhibitor of the AKT pathway, apparently had no effect on inhibiting the up-regulation of MMP-9 and MMP-2 activity in response to OSM. Figure 3.7 shows a histogram of gelatin zymography results from Fig. 3.6.

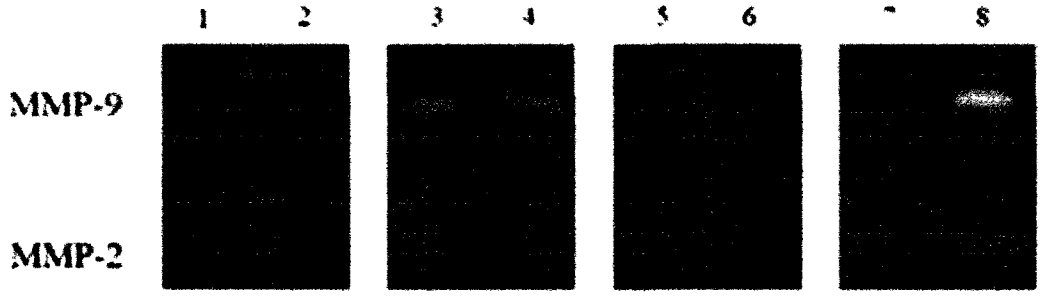
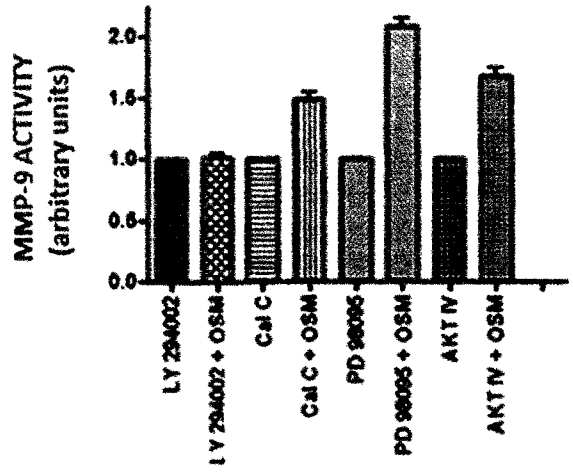


Figure 3.6: OSM regulates MMP-9 and MMP-2 activity in NR3 cells through specific signal transduction pathways. Gelatin zymography was performed on aliquots of conditioned medium from NR3 cells treated for 24 hr with 50ng/ml OSM and/or 1 hr pre-treatment with an inhibitor. MMP-9 and MMP-2 activity was analyzed by Gelatin zymography. Cells were treated under the following conditions: (1) 10μM LY 294002, (2) 10μM LY 294002 + 50ng/ml OSM, (3) 500nM Calphostin C (4) 500nM Calphostin C + 50ng/ml OSM (5) 50μM PD 098059, (6) PD 098059 + 50ng/ml OSM, (7) 10μM AKT Inhibitor IV (8) 10μM AKT Inhibitor IV + 50ng/ml OSM.

A



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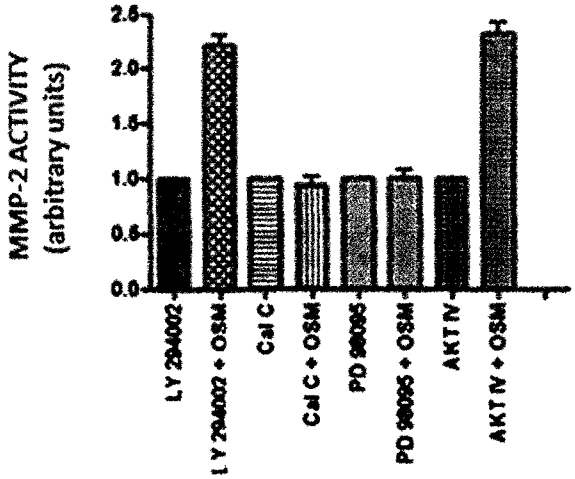


Figure 3.7: OSM regulates MMP-9 and MMP-2 activity in NR3 cells through specific signal transduction pathways. Gelatin zymography was performed on aliquots of conditioned medium from NR3 cells treated for 24 hr with 50ng/ml OSM and/or 1 hr pre-treatment with an inhibitor. MMP-9 and MMP-2 activity was analyzed by Gelatin ymography and results were quantified for MMP-9 protein expression (Fig.3.7A) and MMP-2 protein expression (Fig. 3.7B).

3.4.3. Effects of OSM on TIMPs, EMMPRIN, AND RECK protein expression

MMP activity can also be regulated through specific mediators that are able to inhibit and/or stimulate MMP activity. Protein expression levels of some mediators of MMP activity were investigated under the following conditions: (1) NR3 cells were treated with 50ng/ml OSM for 24 hr and (2) 200ng/ml OSM for 2 hr. Protein expression levels of TIMP-1 and TIMP-2 in NR3 cells were determined following treatment of NR3 cells with 50ng/ml OSM treatment for 24hr and 200ng/ml OSM treatment for 2hr. In Figures 3.8 and 3.9, TIMP-1 and TIMP-2 protein expression levels were apparently unaffected at 50ng/ml OSM for 24 hr but TIMP-1 and TIMP-2 protein expression levels were increased when cells were treated with 200ng/ml for 2hr. OSM treatment of NR3 cells also alters the protein expression of EMMPRIN and RECK in a dose and time dependent response. Protein expression levels of EMMPRIN and RECK in NR3 cells were determined by Western blot analysis. In Figure 3.10, EMMPRIN protein expression dramatically increased at 50ng/ml OSM treatment for 24 hr and at 200ng/ml OSM for 2 hr. As shown in Figure 3.11, RECK protein expression levels decreased at 50ng/ml OSM treatment for 24 hr. There was no apparent change in RECK protein expression levels at 200ng/ml OSM treatment for 2 hr.

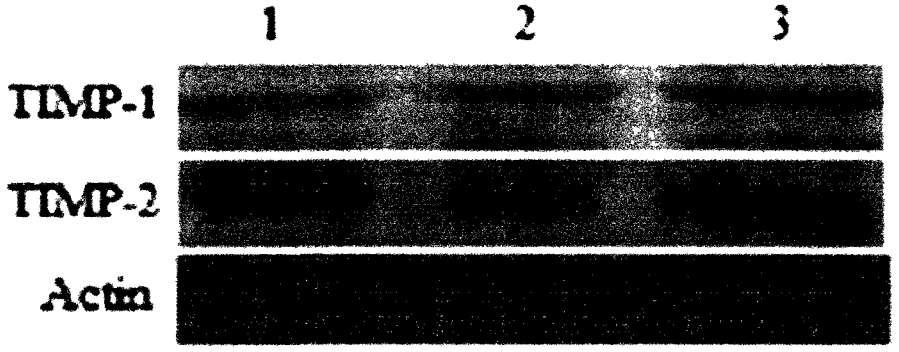
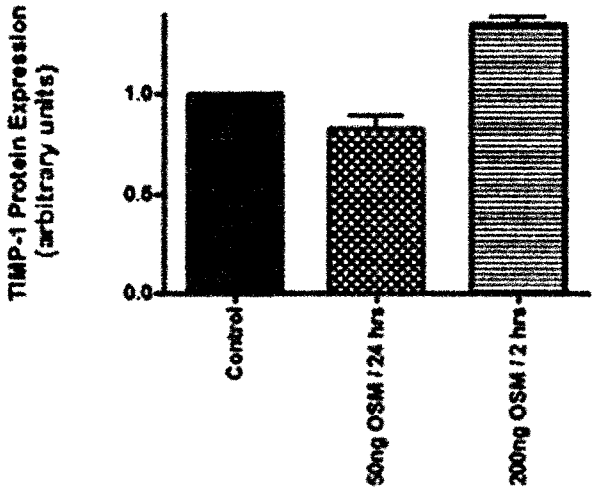


Figure 3.8: Effects of OSM treatment on NR3 cells alters the protein expression of TIMP-1 and TIMP-2. Western blot analysis was performed on NR3 cells treated with 50ng/ml OSM for 24 hr and 200ng/ml OSM for 2 hr. (1) Control, (2) 50ng/ml OSM for 24 hr, and (3) 200ng/ml OSM for 2 hr.

A



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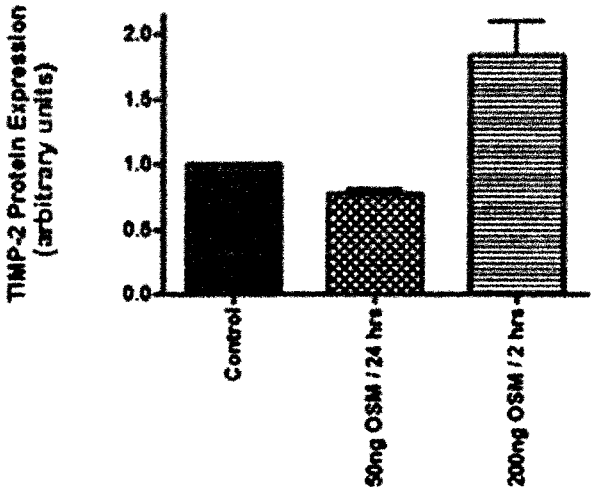
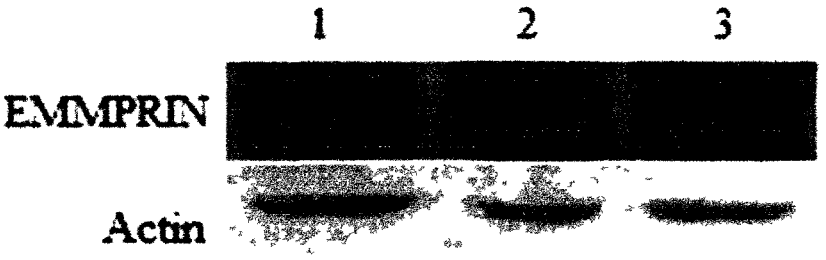


Figure 3.9: Effects of OSM treatment on NR3 cells alters the protein expression of TIMP-1 and TIMP-2. Western blot analysis was performed on NR3 cells treated with 50ng/ml OSM for 24 hr and 200ng/ml OSM for 2 hr. Results were quantified for TIMP-1 protein expression (Fig. 3.9B) and TIMP-2 protein expression (Fig. 3.9B).

A



B

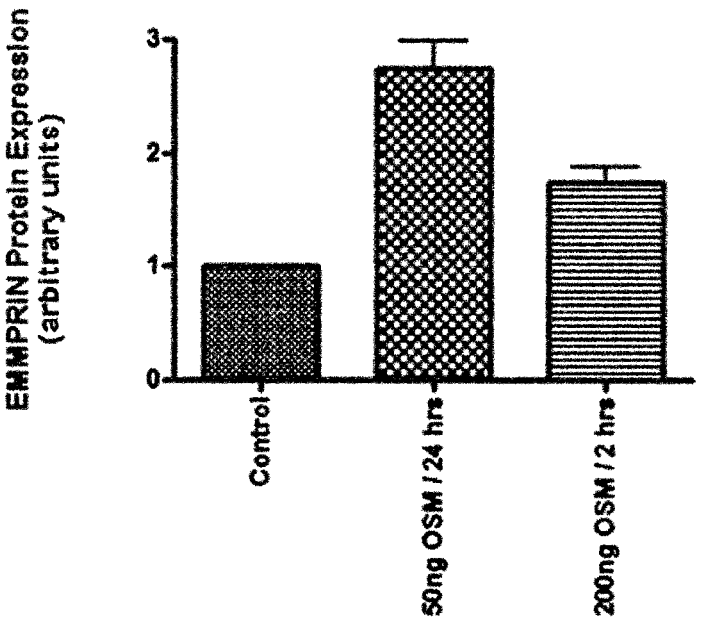


Figure 3.10: Effects of OSM treatment on NR3 cells alters the protein expression of EMMPRIN. Western blot analysis was performed on NR3 cells treated with 50ng/ml OSM for 24 hr and 200ng/ml OSM for 2 hr. (1) Control, (2) 50ng/ml OSM for 24 hr, and (3) 200ng/ml OSM for 2 hr (Fig.6A). Results were quantified for EMMPRIN protein expression (Fig. 6B).

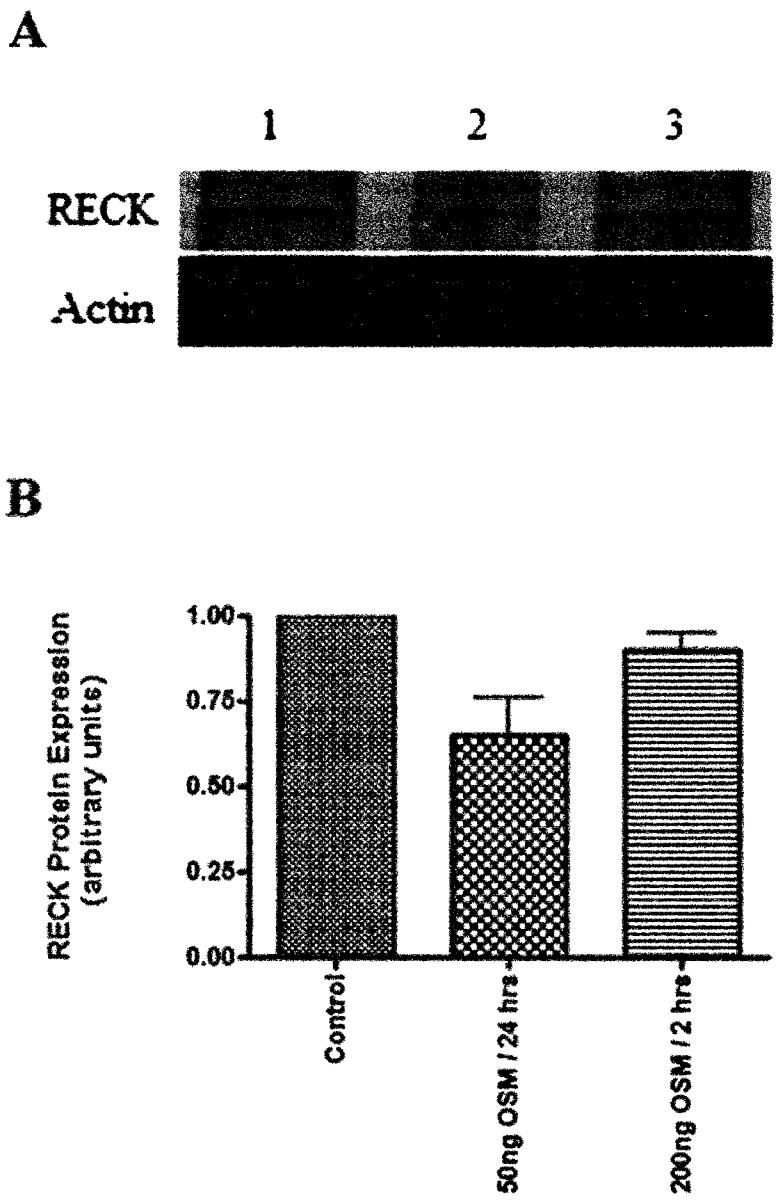


Figure 3.11: Effects of OSM treatment on NR3 cells alters the protein expression of RECK. Western blot analysis was performed on NR3 cells treated with 50ng/ml OSM for 24 hr and 200ng/ml OSM for 2 hr. (1) Control, (2) 50ng/ml OSM for 24 hr, and (3) 200ng/ml OSM for 2 hr (Fig.7A). Results were quantified for RECK protein expression (Fig. 7B).

3.4.4. OSM alters the protein expression of specific members of the JAK-STAT signalling pathway in NR3 cells

OSM receptors recruit the JAK/STAT signalling pathway to modify gene expression (Grant et al., 2002, Korzus et al., 1997). OSM binds to specific receptors and activates the JAK family of kinases that selectively phosphorylate STAT proteins to bind to specific gene promoters. Protein expression levels of the Janus family of kinases (JAK-1, JAK-2, and JAK-3) and the STAT family (STAT-1, STAT-2, STAT-3, and STAT-4) was determined by Western blot analysis following 50ng/ml OSM for 24 hr. As shown in figure 3.12 and figure 3.13, respectively, only specific JAKs and STATs were recruited (up-regulated) in the OSM signalling pathway. Protein expression levels of JAK-1, JAK-2, and JAK-3 were up-regulated in response to OSM. STAT-2 and STAT-3 were the only members of the STAT family to be up-regulated whereas STAT-1 and STAT-4 protein expression was unaltered in the OSM treatment of NR3 cells.

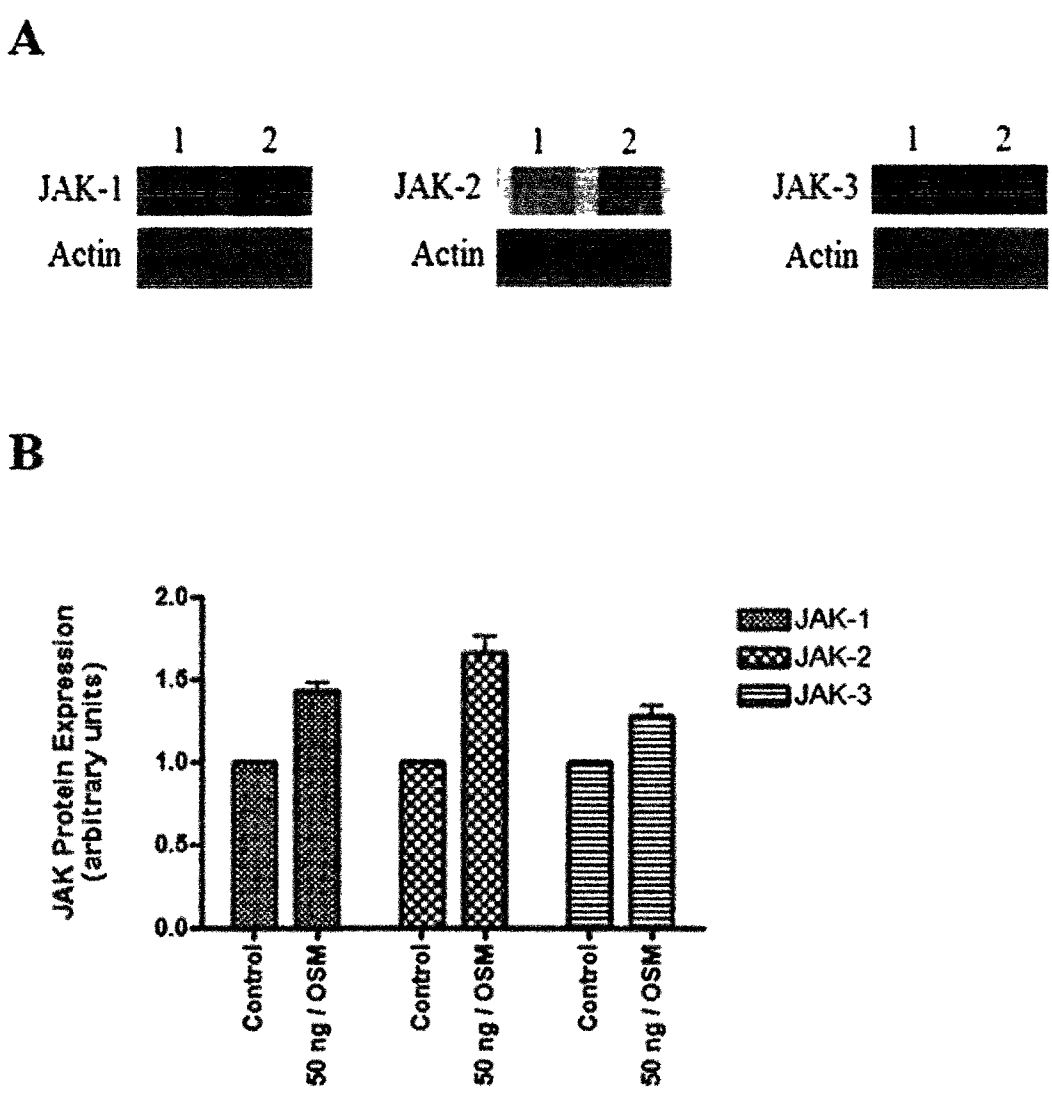


Figure 3.12: OSM alters specific JAK proteins of the JAK-STAT signalling pathway in NR3 cells. Protein expression levels of JAK-1, JAK-2, and JAK-3 were determined by western blot analysis (Fib. 3.12A). NR3 cells were treated with 50 ng/ml OSM for 24 hr. (1) Control and (2) 50 ng/ml OSM for 24 hr (Fig. 8A). Results were quantified for JAK-1, JAK-2, and JAK-3 protein expression (Fig. 3.12B).

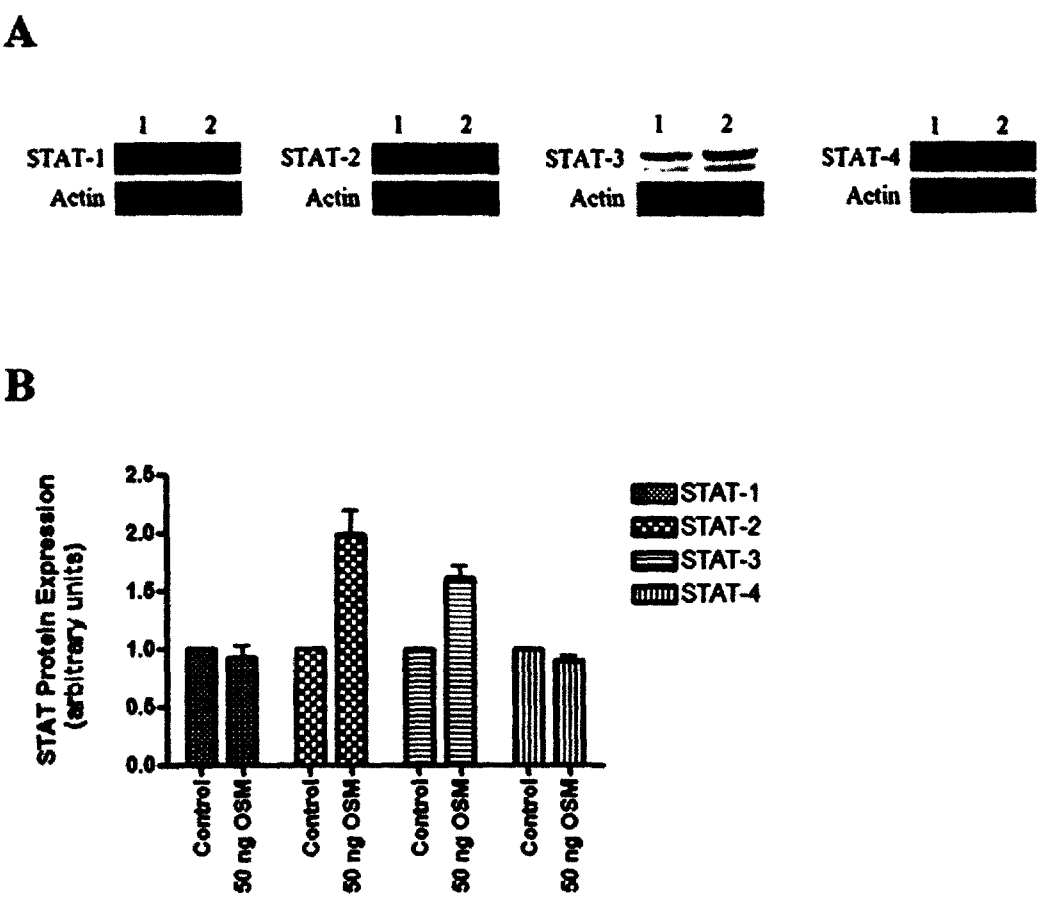


Figure 3.13: OSM alters specific STAT proteins of the JAK-STAT signalling pathway in NR3 cells. Protein expression levels of STAT-1, STAT-2, STAT-3, and STAT-4 were determined by western blot analysis (Fig. 3.13A). NR3 cells were treated with 50 ng/ml OSM for 24 hr. (1) Control and (2) 50 ng/ml OSM for 24 hr (Fig. 9A). Results were quantified for STAT-1, STAT-2, STAT-3, and STAT-4 protein expression (Fig. 3.13B).

3.5. Discussion

The progression of cancer relies upon a number of critical events. Continuous growth of the tumour, angiogenesis, the ability to evade programmed cell death, tissue invasion, and the ability to metastasize are some examples. MMPs are a family of enzymes that have the ability to degrade the extracellular matrix of cells and critical enzymes that enable metastasis and tissue invasion (Chang and Werb, 2001). There are numerous members of the MMP family and they are characterized according to their ability to degrade various components of the extracellular matrix of cells (Chang and Werb, 2001). Two important members of the MMP family are MMP-9 and MMP-2, enzymes which are capable of degrading gelatin (Nagase and Woessner, 1999). Increased MMP-9 and MMP-2 activity has been demonstrated in variety of human cancers (Stetler-Stevenson and Yu., 2001).

The current study has demonstrated that MMP activity in NR3 cells increases following exposure to the growth factor Oncostatin M (OSM). NR3 cells (H-*ras* expressing, benign tumour forming cells) produced elevated amounts of MMP-9 and MMP-2 activity in response to OSM whereas 10 T ½ (untransformed, normal) cells showed no alteration in MMP-9 and MMP-2 activity (data not shown). Other studies have also shown that OSM stimulates MMP-9 and MMP-2 activity in smooth muscle cells (Nagata et al., 2003, Korzus et al., 1997, Qing et al., 2001). Our studies found a direct relationship between the OSM mediated alteration in MMP activity and H-*ras*

cellular transformation and revealed that only H-*ras* transformed cells were capable of altering MMP expression with OSM treatment.

The findings in this study suggest that MMP-9 and MMP-2 activity in response to OSM is differentially regulated. MMP-9 activity was found to be regulated through P-I-3 kinase pathway and apparently not regulated through the protein kinase C, the MAP kinase and the AKT pathways. MMP-2 was regulated through protein kinase C and MAP kinase pathways, and not via the P-I-3 kinase and the AKT pathways in response to OSM. Numerous studies have shown that that OSM utilizes the MAP kinase pathway on a variety of different cell types, including human astroglioma and astrocytes, rat aortic smooth muscle cells, breast cancer cells, and murine fibroblasts (Halfter et al., 2000, Song et al., 2005, Stancato et al., 1997, Van Wagoner et al., 2000). Other studies have showed that in human airway smooth muscle, OSM has no effect on the activation of the MAP kinase pathway (Faffe et al., 2005). The PKC pathway has been reported to be elevated in breast tumours, leukemic cells, lung cancer cells, and mouse and human colon tumours (Blobe et al., 1994, Zhang et al., 2004). This pathway has also been shown to be activated by OSM in murine fibroblasts and human endothelial cells activity (Smyth et al., 2006, Saijonmaa et al., 1998).

The P-I-3 kinase/Akt pathway plays a critical role in tumorigenesis and has been shown to promote breast cancer cell survival (Albert et al., 2006). Results from Godoy-Tundidor et al (2005) showed that the P-I-3 kinase pathway and the AKT pathway both

play an important role in the OSM mediated proliferation of prostate cancer 22Rv1 cells. Other studies have also confirmed the involvement of the P-I-3 kinase pathway in the OSM mediated inhibition of cell proliferation in breast carcinoma cells (Albert et al, 2006). Interestingly, Smyth et al. (2006) showed that OSM induced interleukin-6 expression in murine fibroblasts and the activation of the P-I-3 kinase pathway was involved but the AKT inhibitor pathway showed no apparent involvement.

MMPs are typically increased during tissue degradation and are important factors in cancer invasion and metastasis. MMP expression is a tightly regulated process and can be regulated at the transcriptional and post translational level, and via TIMPs and various mediators of MMP expression, such as EMMPRIN and RECK. In this present study TIMP-1 and TIMP-2 were shown to be up-regulated at elevated OSM concentration (200ng/ml for 2 hr); however, both were slightly decreased at the lower OSM concentration (50ng/ml for 24 hr).TIMP-1 and TIMP-2 are inhibitors of MMP activity and the different doses of OSM on H-*ras* transformed cells should affect TIMP protein expression in a similar manner; however, the findings suggest that lower doses of OSM has the ability to inhibit the effects of TIMP-1 and TIMP-2 and allow MMP expression to be unaffected by TIMPs which is a possible cancer advantage in H-*ras* transformed NR3 cells.

EMMPRIN was dramatically up-regulated in response to 50ng/ml for 24 hr OSM and RECK was down-regulated. Both proteins were also significantly affected at

200ng/ml for 2hr. EMMPRIN is a protein that has been shown in numerous studies to induce MMP activity and reduced expression of the RECK protein has also been shown to lead to excessive activation of MMP-2, MMP-9, and MT1-MMP (Noda et al., 2003, Chang et al., 2006, Gabison et al., 2005). Our findings suggests that MMP activity in NR3 cells in response to OSM (50ng/ml for 24 hr) are regulated through EMMPRIN and RECK and suggests that H-*ras* transformation in NR3 cells mediates the alteration in both EMMPRIN and RECK. Our studies also suggest that TIMP-1 and TIMP-2 works in concert with EMMPRIN but not RECK to regulate the OSM mediated response in MMP-9 and MMP-2 activity at higher OSM concentrations. TIMP-1 and TIMP-2 complex with collagenases/gelatinases and normally result in the irreversible inactivation of MMP-9 and MMP-2, respectively (Chirco et al., 2006, Lambert et al., 2004), and in various cell types OSM has been shown to alter TIMPs mRNA level (Nagata et al, 2003). EMMPRIN is known to be involved in the regulation of matrix remodelling and can stimulate the production of various MMPs in fibroblasts and endothelial cells and RECK has been shown to be involved in MMP regulation because of its ability to bind to and inhibit the proteolytic activity of MMP-9, which prevents the digestion of the ECM. Researchers have also found that RECK is widely expressed in normal tissue, whereas it is strongly inhibited in several tumour-derived cells and in fibroblasts transformed with oncogenes (Sasahara et al., 1999, Takahashi et al., 1998).

This study also suggests that the JAK-STAT activation pathway is utilized in H-*ras* transformed NR3 cells in response to OSM treatment. Only specific members of the JAK-STAT pathway were shown to be activated in the OSM response which suggests

that the JAK/STAT pathway is capable of mediating a response in NR3 cells independent of all members of the pathway. In this study JAK-1, JAK-2, JAK-3 and STAT-2, STAT-3 showed an involvement in the NR3 response to OSM whereas STAT-1 and STAT-4 where apparently not involved in this response. It is well known that a variety of cytokines, including OSM, utilizes the JAK/STAT pathway in both human and murine cells. Recent studies have found that JAK-1, JAK-2, and JAK-3 all become activated upon stimulation by OSM and both JAK-1 and JAK-2 compete for receptor binding sites (Gomez-Lechon, 1999, Song et al, 2005). However, JAK-1 seems to be the critical JAK required for OSM signalling (Parganas et al., 1998). Studies have also found that OSM is a potent activator of STAT-3 in a variety of cell types and to a lesser extent STAT-1 (Faffe et al, 2005, Grant et al, 2002, Chen and Benveniste, 2004). The JAK/STAT pathway can also act in concert with the MAP kinase pathway upon OSM stimulation [Qing et al, 2001, Halfter et al, 2000, Stancato et al., 1997), while another study has showed that the JAK-STAT pathway and the MAP kinase pathway are independently involved in the OSM stimulated proliferation of human adipose-derived mesenchymal stem cells (Song et al., 2005). The PKC pathway has also been shown to be linked with the JAK-STAT pathways and is a regulator of STAT-3 in keratinocyte proliferation (Gartsbein, 2006).

In summary this study has demonstrated an interesting relationship between Oncostatin M mediated signalling/regulation and MMP expression in H-*ras* transformed cells capable of benign tumour formation. Furthermore, this study has elucidated

another aspect of the H-*ras* mediated altered growth regulatory program associated with cellular transformation.

4. CHAPTER FOUR

Oncostatin M Mediated Expression of Proinflammatory and Proliferation Linked Activities in H-*ras* Transformed Fibroblast Cells Capable of Benign Tumor Formation.

4.1. Abstract

Oncostatin M (OSM) is a glycoprotein that is produced by monocytes and T-cells. The cytokine is a known inducer of inflammation and has been shown to promote proliferation in cancerous cells. This present study examines a link between OSM, TNF-α, proinflammatory linked enzymes [inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)] and proliferation linked enzymes [ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyl transferase (SSAT). Treatment of 10T ½ cells (non-transformed cells) and NR3 cells (H-*ras* transformed, benign tumour forming cells) with OSM resulted in an induction of TNF-α activity in a dose and time dependent manner in only NR3 cells. NR3 cells were treated with various concentrations of OSM and TNF-α activity increased at 10ng/ml for 24 hr and also increased at 25 and 50 ng/ml OSM. Effects of OSM were time dependent with TNF-α activity induction seen as early as 24 hr. iNOS and COX-2 protein levels were also examined after 50ng/ml OSM treatment for 24 hr. In 10T ½ cells there was no apparent change in iNOS protein expression but COX-2 protein expression increased. In NR3 cells both iNOS and COX-2 protein expression were increased. The protein expression of proliferation linked enzymes were also examined. ODC protein expression levels were increased in

transformed, NR3 cells but not in 10T ½ cells, and there was no apparent change in SSAT protein expression in both 10T ½ and NR3 cells. In conclusion, OSM induces both proinflammatory and proliferation linked proteins suggesting that OSM is capable of providing H-*ras* transformed cells with a distinct growth advantage.

4.2. Introduction

OSM is a member of the interleukin-6 (IL-6) cytokine family and is expressed in low levels in normal cells; but, is elevated in various pathologies such as cancer (Chen and Benveniste, 2004). In cancer, OSM has been found to play a major role in regulating various mediators of inflammation and proliferation, which are two key components of the progression of cancer (Tanaka and Miyajima, 2003, Zarling, 1986). Various proinflammatory mediators have been found to be associated with gastric, colon, and breast cancers (Zhao et al., 2010, Desai et al., 2010, Glynn et al., 2010). Tumor necrosis factor- α (TNF-α) is a proinflammatory cytokine that regulates cellular homeostasis by inhibiting proliferation and differentiation through apoptosis (Petersen et al., 2007). TNF-α mRNA levels were found to be elevated in two types of gastric cancer cells, HGC27 and MKN45 cells (Zhao et al. 2010). iNOS and COX-2 are two key proinflammatory enzymes that have been linked to cancer (Sappayatosok et al., 2009). iNOS is an enzyme that is capable of producing nitric oxide (NO) which is a major signalling molecule that is elevated in colon cancers and recent studies are targeting the molecule for a potential treatment for cancer progression (Desai et al., 2010). COX-2 is an enzyme that synthesizes prostaglandins which are responsible for inflammation and

vasodilation and prostaglandin levels have been shown to be elevated in many breast cancer cells (Glynn et al., 2010).

Another key component of cancer progression is the ability of cancer cells to grow uncontrollably and the polyamine pathway is a key pathway that has been shown to influence cell growth in cancer cells. (Higuchi and Wang, 1995, Hurta et al.,1996, Hurta et al., 2001). Two key enzymes in the polyamine pathway, ODC and SSAT, have been shown to be important markers in cellular proliferation in numerous cancer studies and the overexpression of ODC and SSAT have been linked to colon, esophagus, and prostate cancers (Yoshida et al, 2002, Tucker et al., 2005). This study will examine the effects of OSM on proinflammatory linked proteins (iNOS and COX-2) and proliferation linked proteins (ODC and SSAT) in H-*ras* transformed murine fibroblasts.

4.3. Materials and Methods

4.3.1. Cell lines and growth conditions

Mouse 10 T ½ cells were transfected with the plasmid pAL8A which contains T-24-H-*ras* and the neomycin resistance gene. Post-transfection, cell lines were established which included NR3 cells which are morphologically non-transformed (Noda et al., 2003, Egan et al., 1987). It is important to note that the 10 T ½ cell line is not tumorigenic whereas the NR3 cell line is capable of forming benign tumours in syngeneic hosts (Noda et al., 2003, Egan et al., 1987).

10 T ½ cells and NR3 cells were cultured on 100-mm plastic culture dishes (Falcon) (BD Biosciences, Mississauga, ON) in α-minimal essential medium (MEM) (Invitrogen, Burlington, ON) supplemented with antibiotic/antimycotic (Invitrogen) and 10% (v/v) fetal bovine serum (Hyclone Laboratories, Mississauga, ON) and were cultured at 37°C in 5% CO_2, as previously described (Hurta and Voskas, 2001). Cells were cultured to confluence prior to exposure to Oncostatin M (OSM) (R&D Systems, Burlington, ON). In experiments using OSM, a serum-free medium (defined medium) was used that contained 4μg/ml transferrin (Sigma, Oakville, ON) and 2μg/ml insulin (Sigma). Cells were placed on the defined medium for 24 hr prior to OSM treatment. Cells were removed from the tissue culture plates using a 0.3% buffered trypsin solution (Sigma).

4.3.2. SDS-PAGE and western blot analysis

Cells were harvested and lysed in cell lysate buffer containing 10mM Tris and 1mM phenylmethylsulphonyl fluoride (PMSF) (Sigma). Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Mississauga, ON). Equal amounts of protein were electrophoresed on SDS-polyacrylamide gels and transferred for 1.5 hr onto 0.2μm nitrocellulose membranes (Bio-Rad Laboratories). The Western blots were blocked in Tris-buffered saline (TBS) (50mM Tris-HCl and 150mM NaCl) containing 1% bovine serum albumin (BSA) (Sigma), followed by incubation in TBS supplemented with 0.05% Tween-20 (TBS-T) (Sigma) and specific primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies used were

as follows: ODC (N-15), iNOS (K-20), COX-2 (C-20) purchased from Santa Cruz Biotech. and SSAT (a gift from A.E. Pegg, University of Pennsylvania). Blots were washed with TBS-T and incubated in secondary antibody for 2 hr at room temperature. Specific secondary antibodies were used for different proteins. Secondary antibodies used were as follows: actin, COX-2, and ODC used donkey-anti goat IgG; iNOS and SSAT used goat anti-rabbit IgG. All secondary antibodies were allowed to incubate for 2 hr at room temperature before blots were developed using Sigma Fast BCIP/NBT developing tablets (Sigma). As a loading control, the housekeeping protein β-actin was used. Complementary membranes were incubated with β-actin antibody at 1:100 dilution followed by incubation in 1:500 β-actin secondary antibody and processed as described above with the exception that actin protein was electrophoresed on 7.5% SDS-polyacrylamide gels.

All western blot analysis was performed in triplicate and quantified using Bio-rad Quantity One Software. Quantity One Software calculates band density by detecting the pixel intensity and the volume of the band.

4.3.3. TNF- α ELISA

A tumor necrosis factor- α ELISA kit (RPN2744) was used to quantify TNF-α (Amersham Biosciences, New Jersey). All cells and reagents used during the analysis

were stored at room temperature before use in the ELISA. 50 ul of pre-treatment buffer was added to each well, followed by 50ul of both standards and samples. All standards and samples were assayed in triplicate. The 96 well plate was covered and allowed to incubate at room temperature for 1 hr. After incubation the plate was washed three times followed by the addition of 50ul of biotinylated antibody reagent to each well. The plate was then incubated at room temperature for 1 hr before it was washed again. 100ul of streptavidin-HRP reagent was added to each well and the plate was allowed to incubate at room temperature for 30 minutes followed by three washes and the addition of 100ul of TMB substrate to each well. The plate was allowed to develop at room temperature in the dark for 10 mins. The reaction was stopped by adding 100ul of Stop Solution to each well. The absorbance was measured on a plate reader at 450nm minus 550nm.

4.4. Results

4.4.1. OSM treatment alters TNF-α activity in H-*ras* transformed murine fibroblasts.

OSM treatment on 10T ½ and NR3 cells up-regulates the activity of TNF-α in a dose and time dependent manner. In the dose dependent study, OSM was used at various concentrations including 0, 10, 25, 50, and 100ng/ml for 24 hr on 10T ½ and NR3 cells (Figure 4.1). As shown in Fig. 4.1, there was no apparent change in TNF-α activity in 10T ½ cells, however in NR3 cells TNF-α activity increased at 10ng/ml and activity continued to increase at 25 and 50 ng/ml but decreased slightly at 100ng/ml.

TNF-α activity was measured in NR3 cells at 0, 24, 36, and 48 hr at 50ng/ml OSM. As shown in Fig. 4.2 TNF-α activity increased dramatically at 24 hr in NR3 cells and increased slightly at 36 and 48 hr.

4.4.2. OSM regulates pro-inflammatory enzymes in 10T ½ and NR3 cells

iNOS and COX-2 protein expression was evaluated in non-transformed 10T ½ cells compared to transformed NR3 cells, by western blot analysis. Western blot analysis revealed no apparent change in iNOS protein expression in 10T ½ cells when treated with 50ng/ml OSM (Fig. 4.3A) and an increase in iNOS protein expression when H-*ras* transformed NR3 cells were treated with OSM (Fig. 4.3B). Fig. 4.3C represents a quantification of the western blot analysis for iNOS protein expression in 10T ½ and NR3 cells treated with 50ng/ml OSM for 24 hr.

Western blot analysis revealed an increase in COX-2 protein expression in 10T ½ cells and also an increase in NR3 cells when treated with 50ng/ml OSM (Fig. 4.4A and Fig. 4.4B, respectively). Fig. 4.4C represents a quantification of the western blot analysis for COX-2 protein expression in 10T ½ and NR3 cells treated by 50ng/ml OSM for 24 hr.

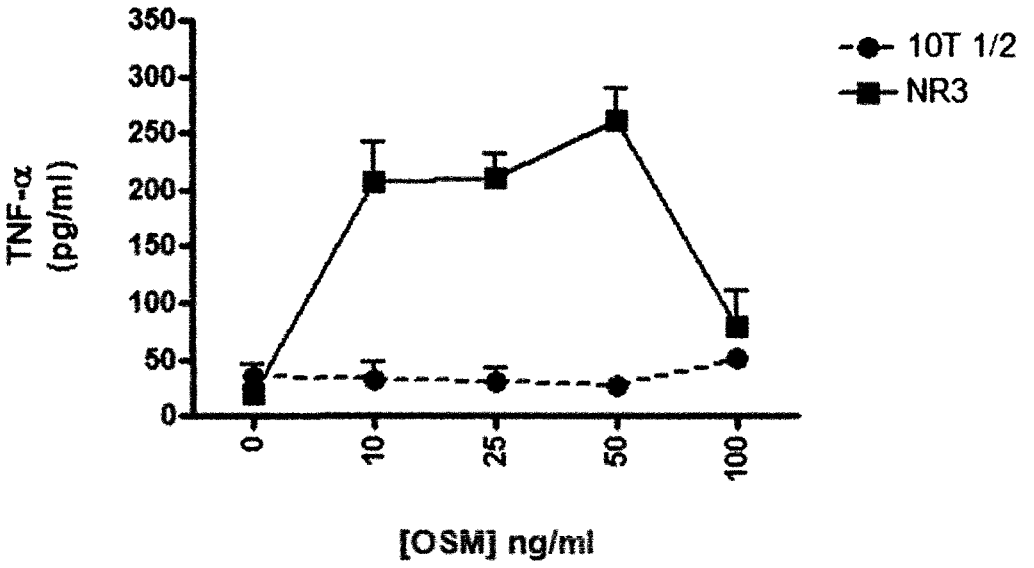


Figure 4.1: OSM alters TNF-α levels in transformed murine fibroblast cells. TNF-α ELISA was performed on OSM treatment on 10T ½ and NR3 cell. OSM concentrations used include 10, 25, 50, and 100 ng/ml. Cells were treated with the various OSM concentrations for 24 hours.

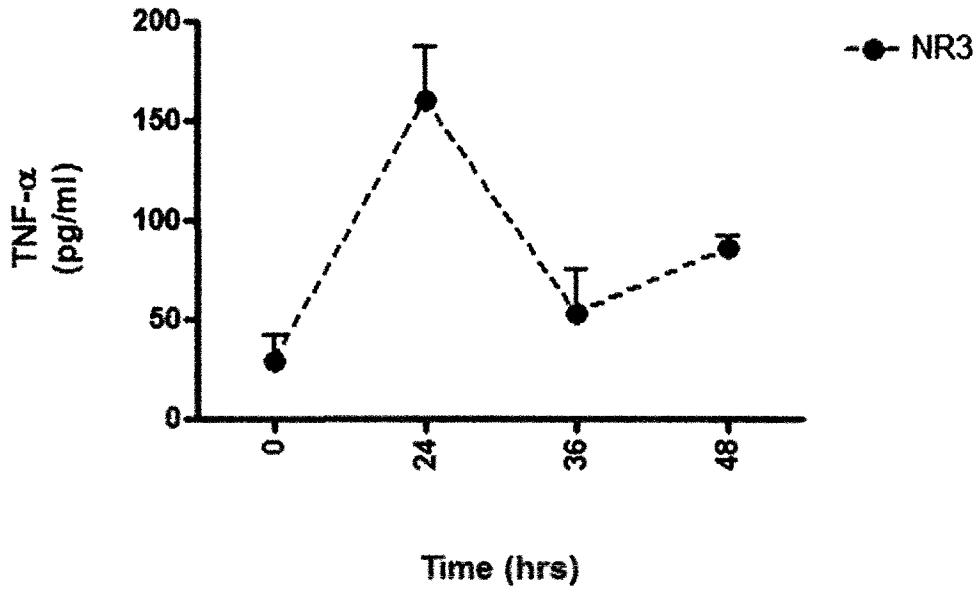


Figure 4.2: OSM elevates TNF-α levels in a time dependent manner. TNF- α ELISA was performed on OSM treatment on NR3 cells. Cells were treated with 50ng/ml OSM for 0, 24, 36, and 48 hr.

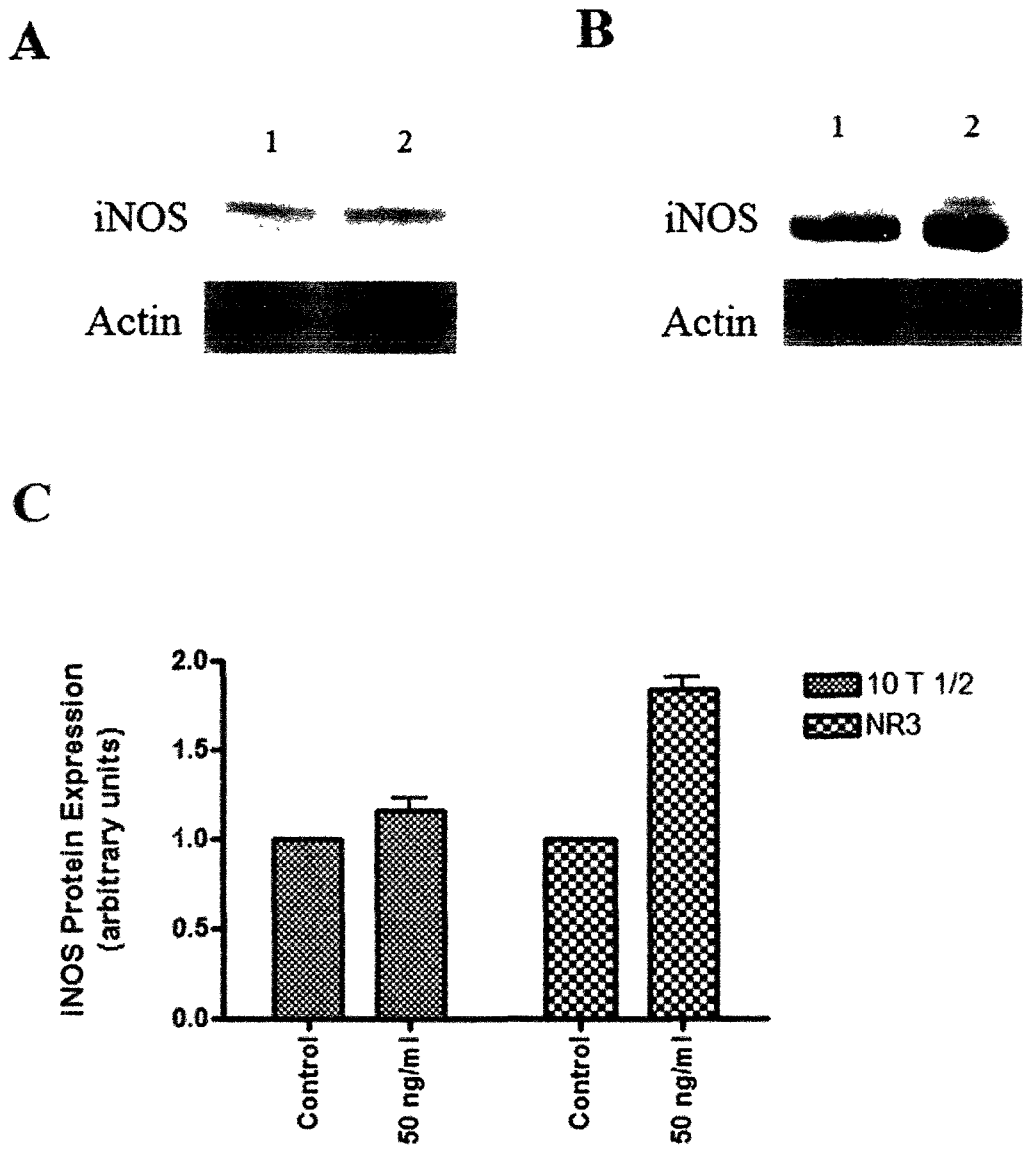


Figure 4.3: OSM alters the protein expression of specific proinflammatory enzymes in 10T ½ and NR3 cells. Protein expression of iNOS was determined by western blot analysis. 10T ½ cells (Fig. 4.3A) and NR3 cells (Fig. 4.3B) were treated with 50 ng/ml OSM for 24 hr. (1) Control and (2) 50 ng/ml OSM for 24 hr. Results were quantified for iNOS protein expression (Fig. 4.3C).

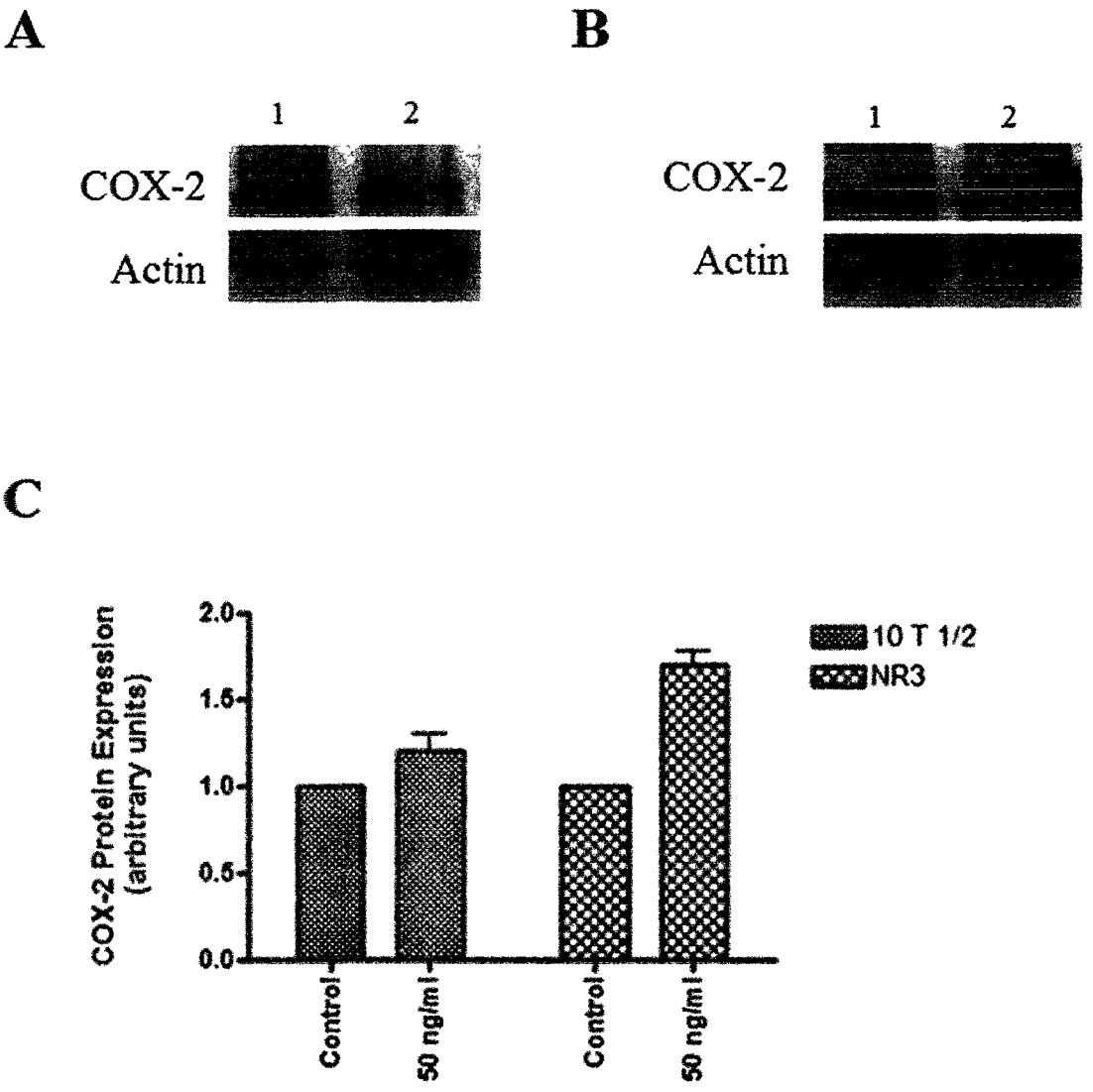


Figure 4.4: OSM alters the protein expression of specific proinflammatory enzymes in 10T ½ and NR3 cells. Protein expression of COX-2 was determined by western blot analysis. 10T ½ cells (Fig. 4.4A) and NR3 cells (Fig. 4.4B) were treated with 50 ng/ml OSM for 24 hr. (1) Control and (2) 50 ng/ml OSM for 24 hr. Results were quantified for COX-2 protein expression (Fig. 4.4.C).

4.4.3. OSM alters protein expression of specific polyamine pathway enzymes in H-ras transformed fibroblasts.

ODC and SSAT protein expression in response to OSM treatment was evaluated in non-transformed 10T ½ cells compared to NR3 cells by western blot analysis. Western blot analysis revealed no change in ODC protein expression in 10T ½ cells when treated with 50ng/ml (Fig. 4.5A). In transformed NR3 cells, ODC protein expression increased in response to OSM treatment (Fig.4.5B). Fig. 4.5C represents a histogram of the western blot analysis for ODC protein expression in 10T ½ and NR3 cells treated by 50ng/ml OSM.

Western blot analysis of SSAT protein expression in response to OSM treatment was examined in 10T ½ and NR3 cells. No apparent alteration in SSAT protein expression in response to OSM treatment was noted in either 10T ½ cells (Fig. 4.6A) or NR3 cells (Fig. 4.6B). Fig. 4.6C represents a histogram of the OSM treatment on SSAT protein expression in 10T ½ and NR3 cells.

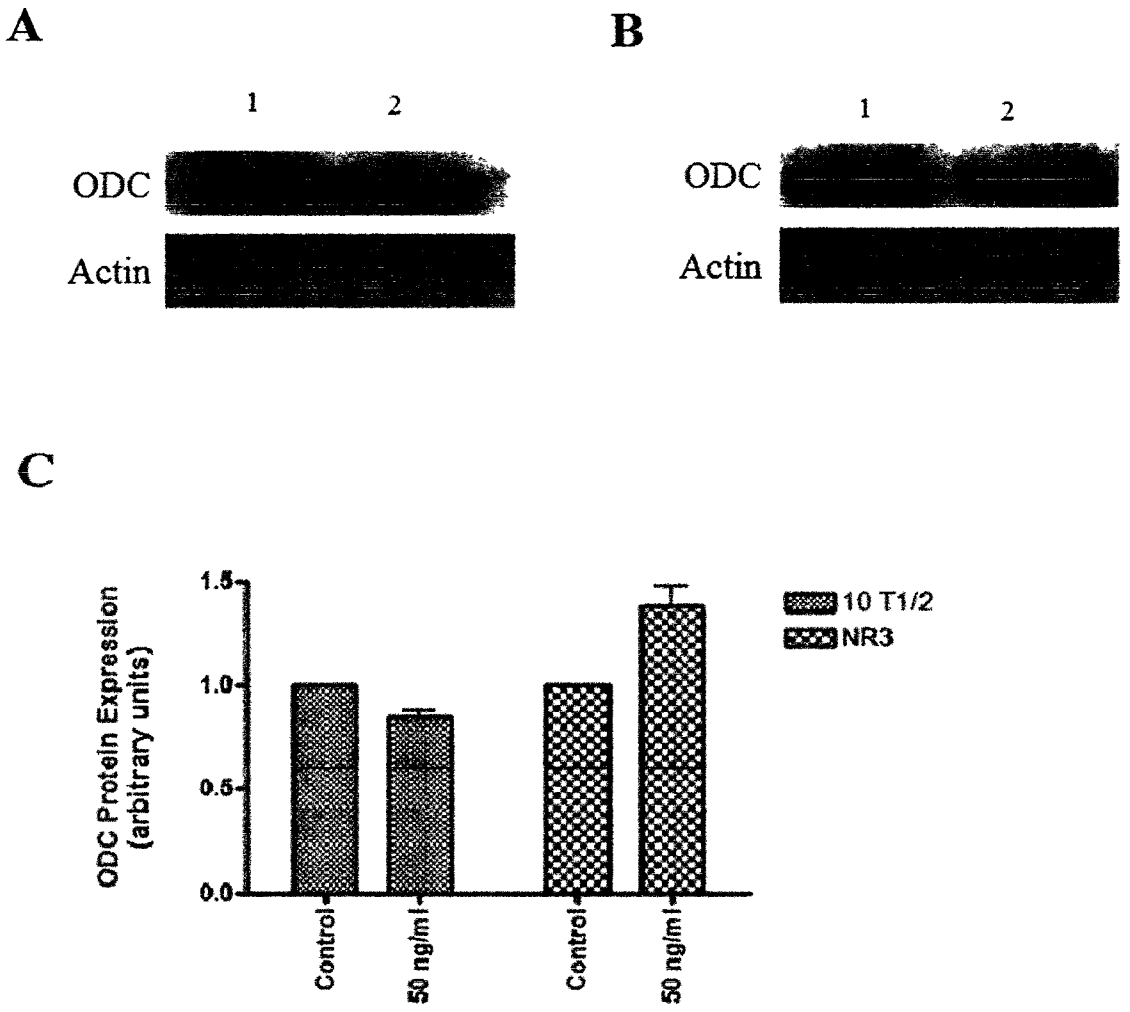


Figure 4.5: The effect of OSM was examined on specific proliferation linked enzymes (ODC) in 10T ½ and NR3 cells. Protein expression of ODC was determined by western blot analysis. 10T ½ cells (Fig. 4.5A) and NR3 cells (Fig. 4.5B) were treated with 50 ng/ml OSM for 24 hr. (1) Control and (2) 50 ng/ml OSM for 24 hr. Results were quantified for ODC protein expression (Fig. 4.5C).

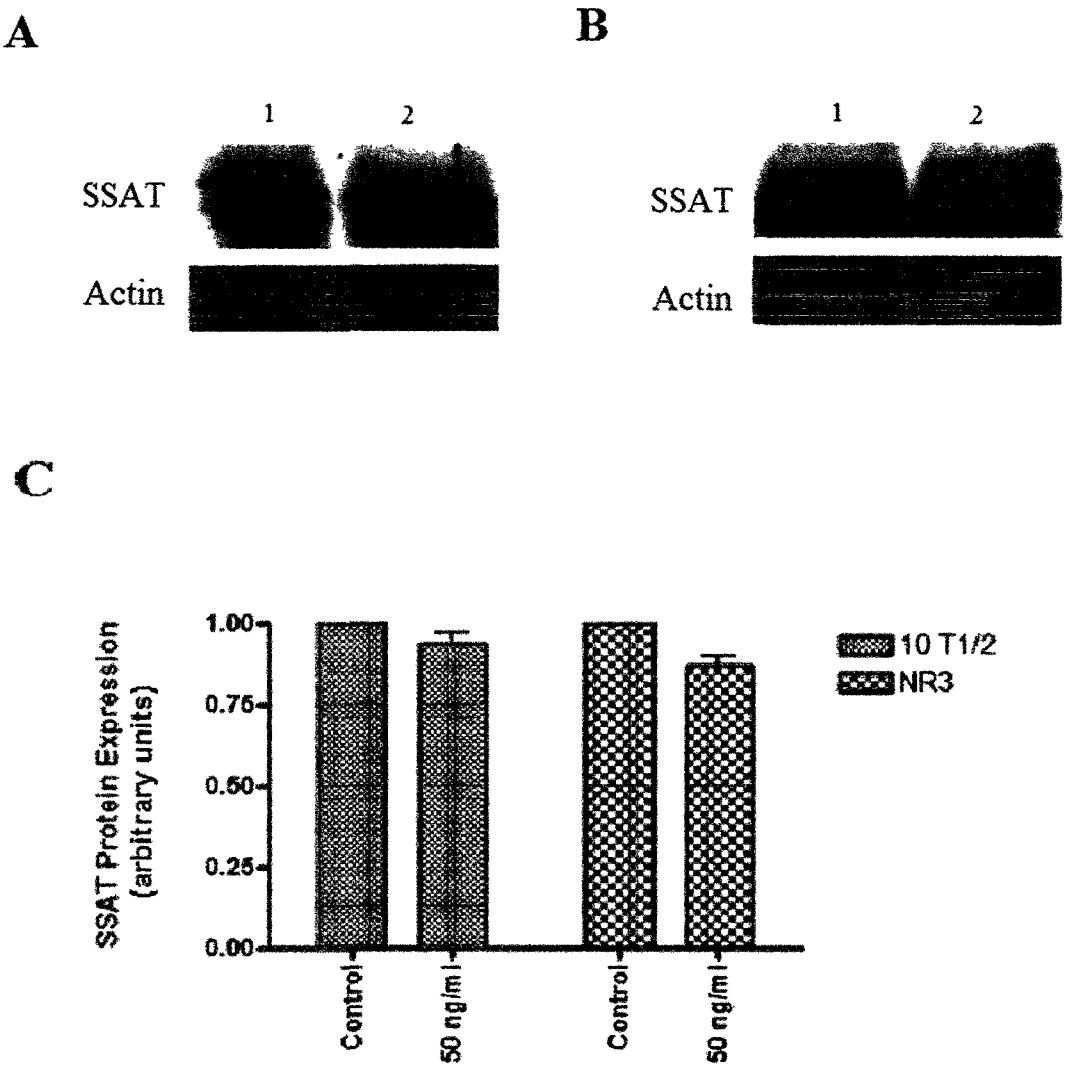


Figure 4.6: The effect of OSM was examined on specific proliferation linked enzymes (SSAT) in 10T ½ and NR3 cells. Protein expression of SSAT was determined by western blot analysis. 10T ½ cells (Fig. 4.6A) and NR3 cells (Fig. 4.6B) were treated with 50 ng/ml OSM for 24 hr. (1) Control and (2) 50 ng/ml OSM for 24 hr. Results for SSAT protein expression were quantified (Fig. 4.6C).

4.5. Discussion

Inflammation and proliferation are crucial processes for the progression of cancer, and this study demonstrates a direct relationship between H-*ras* mediated cellular transformation and various mediators of inflammation and proliferation. In 10T ½ cells OSM was unable to alter TNF-α activity, iNOS protein expression, and ODC protein expression; however, OSM did increase COX-2 protein expression in 10T ½ cells. In NR3 cells, TNF-α activity increased dramatically in response to OSM and iNOS, COX-2, and ODC protein expression were also increased markedly. Interestingly, SSAT protein expression was unaltered in both the transformed and non-transformed cells, which suggests that OSM may only affect the anabolic and not the catabolic portion of the polyamine pathway but further investigation is required to verify this observation.

TNF-α, which is normally important in the necrosis of tumor cells, has also been shown to promote the proliferation of cancer cells and provide cells with an uncontrollable growth advantage. In ovarian cancer cells, MDAH 2772, TNF-α induced proliferation by suppressing interleukin expression and in breast cancer cells, T47D, TNF-α enhanced proliferation by acting through TNFR1 (Kim et al., 2007, Rivas, et al., 2008).

Inflammation has been found to be induced by various stimuli and has been linked with most types of human cancer including pulmonary, prostate, bladder and

renal cancers. The findings in this study correlate with previous research and demonstrates that iNOS and COX-2 protein expression are increased in *ras* transformed cells and not normal cells (Park et al., 2008, Proctor et al., 2010). iNOS and COX-2 levels are normally increased during tumorigenesis and recent studies have also shown that the suppression of iNOS and COX-2 can decrease colorectal cancer (Aoi et al., 2010).

Proliferation linked enzymes also play a key role in the progression of cancer. One major pathway that controls proliferation includes the polyamine pathway that is partly regulated by two rate limiting enzymes, ODC and SSAT. In this study, ODC and SSAT protein levels were examined in the presence of OSM. The findings suggest that OSM only affects the protein expression of the ODC and not SSAT. ODC and SSAT usually work in concert to regulate the polyamine pathway and studies have found that that both enzymes are usually elevated in cancerous tissue as compared to normal tissue (Linsalata et al., 2006). Some studies have concluded that the two enzymes can be altered differentially as in prostate cancer where only ODC is significant overexpressed (Schipper et al., 2006). Also, breast cancer progression studies concluded that only the anabolic enzymes in the polyamine pathway (ODC) are overexpressed which allows cells uncontrollable growth which is shown during cancer progression (Manni et al., 1995).

These novel studies all suggest that OSM is a very important cytokine in mediating some aspects of cancer progression in H-*ras* transformed cells and further research should be concluded to investigate the effects of OSM on various other mediators of cancer progression.

5. CHAPTER FIVE

5.1. General Discussion

The major research findings described in this thesis presents information regarding the novel observation that PMA, a tumor promoter, was able to increase the expression of SSAT, a key enzyme in polyamine catabolism, in H-*ras* transformed cells. The signal transduction mechanisms involved were also determined. In transformed NR3 cells, SSAT protein expression was elevated in response to OSM treatment compared to non-transformed 10T ½ cells that exhibited no alteration in SSAT protein expression in response to OSM (Chapter 2). In previous research, ODC and SAMDC (two key enzymes in the anabolic portion of the polyamine pathway) were altered in response to PMA in NR3 cells (Voskas et al., 2001). These findings suggest that ODC, SAMDC, and SSAT are coordinately regulated in NR3 cells in response to PMA which suggests that the transformed cells may possibly work in concert to enhance polyamine synthesis and provide polyamine homeostasis in NR3 cells. Voskas et al. (2001) also found that the PKC pathway was involved in the PMA mediated alteration in ODC and SAMDC gene expression, whereas SSAT protein alteration in NR3 cells was also regulated through the PKC pathway but involvement from the P-I-3 kinase and MAP kinase pathways was also discovered in the PMA mediated alteration in SSAT protein expression. The alteration in SSAT protein levels in response to PMA which occurs in H-ras transformed NR3 cells may provide these cells with an added growth advantage and may also represent the cells increased ability to preserve homeostasis.

The research in this thesis also examines the effects of OSM on MMP activity in transformed cells. Elevated expression of MMP-2 and MMP-9 activity was shown in NR3 cells in response to OSM; however, no apparent alteration in MMP-2 and MMP-9 activity in non-transformed, 10T ½ cells was observed. Mediators of MMP expression (EMMPRIN, RECK, TIMP-1 and TIMP-2) were also found to be altered in NR3 cells in response to OSM treatment (Chapter 3). EMMPRIN is induced by MMPs and plays a major role in tissue remodeling and is typically increased in cancerous cells (Gabison et al. 2005, Zheng et al., 2010), and RECK, TIMP-1, and TIMP-2 all play key roles in inhibiting MMP expression and typically inhibit tissue invasion and metastasis (Chang et al., 2008, Fan et al., 2003). EMMPRIN protein expression was elevated in NR3 cells in response OSM treatment and TIMP-1 and TIMP-2 protein expression were also elevated. Interestingly, RECK protein expression decreased which suggests that the increase in MMP-2 and MMP-9 protein expression is possibly only being regulated by TIMPs and not by RECK protein levels. Downregulation of RECK, which is a known tumor suppressor, has been shown to promote tissue invasion and metastasis (Chang et al., 2007), and this study suggests that OSM promotes an H-*ras* mediated downregulation of RECK which may possibly promote tumor progression in NR3 cells.

The P-I-3 kinase, PKC, and MAP kinase pathways are all important pathways in regulating many cellular behaviors including cellular alterations found in cancer. As was hypothesized, OSM utilizes these pathways in regulating MMP-2 and MMP-9 protein expression, however, MMP-2 and MMP-9 utilize different signaling pathways in

response to OSM (Chapter 3). In response to OSM, MMP-2 expression was shown to be elevated by acting through the PKC and the MAP kinase pathway, meanwhile, MMP-9 expression was shown to be elevated possibly through only the P-I-3 kinase pathway. The JAK/STAT pathway usually takes part in regulating cellular responses to cytokines such as OSM. In this study, specific members of the JAK/STAT pathway were elevated in response to OSM. Of the JAK family of proteins, JAK-1, JAK-2, and JAK-3 were upregulated in response to OSM and of the STAT family only STAT-2 and STAT-3 were involved in the OSM mediated alteration in NR3 cells.

Inflammation has also been shown to be a hallmark of cancer progression. In this thesis, expression of specific protein mediators of inflammation was examined in response to OSM (Chapter 4) . As hypothesized, OSM altered the regulation of TNF-α, iNOS and COX-2 protein expression in transformed cells (NR3 cells) but not in non-transformed cells (10T ½ cells). TNF-α, which is known for inducing proliferation and invasiveness in cancer cells, was elevated in response to OSM and the levels of iNOS and COX-2, which are commonly overexpressed in cancers (Watanabe et al., 2000), were also elevated in response to OSM. The research findings described in this thesis suggest the H-*ras* mediated cellular transformation may increase the transformed cells responsiveness to growth signals including OSM. This increased responsiveness results in altered expression of some cancer associated activities. This may provide the transformed cells with a selective growth advantage over non-transformed cells.

Overall, the findings in this project investigate key aspects of cancer progression. The purpose of the research was to determine the mediators and pathways involved in cellular proliferation and inflammation and allow for future studies to further investigate these findings for a possible solution for the inhibition of cancer growth. Studying the possible reasons for the initiation of tumors is a very complex and almost impossible chore, but the research in this study will allow for future investigators to study the deadly aspects of cancer, cellular proliferation and inflammation, and possibly eliminate these threats.

5.2 Future Research

Research in this thesis provides further support for the hypothesis that H-ras mediated cellular transformation is associated with alterations in the expression of mediators of cancer progression. All findings in this study suggest that there is a direct link between H-*ras* transformation and altered cellular processes that promote either tissue remodeling, invasion, and / or proliferation of cells which are all activities which support cellular growth.

The polyamine pathway is a major pathway involved in proliferation regulation in cancerous cells and is an area of cancer research that should be examined further. By studying both protein and message level of other enzymes involved in the catabolic polyamine pathway, such as polyamine oxidase (PAO), we could increase our

understanding of the direct interaction between all enzymes involved in polyamine biosynthesis and catabolism and their association with cancer proliferation. Future studies could also be conducted to determine if PMA is able to stabilize the protein level of SSAT in transformed cells. SSAT is the rate-limiting enzyme in the catabolic portion of polyamine metabolism and although it exists in low amounts in normal cells it also has a high turnover rate. Investigations to determine if SSAT protein is being stabilized by tumor promoters, such as PMA, would provide a better understanding of how SSAT is able to regulate catabolism in NR3 cells. Similar studies examining PMA effects of SSAT message expression would also be helpful.

Future research on the effects of OSM on polyamine metabolism and cancer progression should also be investigated. OSM is known as a growth stimulator in fibroblast cells and has also been shown to enhance angiogenesis and metastasis (Queen et al., 2005). In certain cancers OSM production has also been shown to increase (Queen et al., 2005), and this study suggests that OSM overexpression promotes activities associated with invasion and tissue remodelling. Future research should investigate the mechanisms through which OSM affects these processes in H-ras transformed cells and studies should be conducted to determine if OSM inhibition in transformed cells has any effect on tissue remodelling and proliferation. The research of MMP expression in H-*ras* transformed cells should also be continued. MMP expression was evaluated by gelatin zymography but further studies on mRNA levels of MMPs in response to OSM and mRNA levels of P-I-3 kinase, PKC, and MAP kinase pathways would further dissect the

specific pathway signalling that occurs in MMP expression in H-*ras* transformed cells.

Finally, the findings described in this thesis have contributed to our understanding of how H-ras overexpression results in altered responses which are supportive of an enhanced transformed cell phenotype which then provides such H-ras transformed cells with a selective growth advantage over "normal" non-transformed cells.

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