REGULATION OF MATRIX METALLOPROTEINASE EXPRESSION AND ACTIVITY BY THE ARYL HYDROCARBON RECEPTOR IN A2058 HUMAN MELANOMA CELLS

by

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ABSTRACT OF THE DISSERTATION

REGULATION OF MATRIX METALLOPROTEINASE EXPRESSION AND ACTIVITY BY THE ARYL HYDROCARBON RECEPTOR IN A2058 HUMAN MELANOMA CELLS

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Malignant melanoma has a high incidence of mortality due to its resistance to chemotherapy and tendency to metastasize, and in the past 60 years has seen an increase in industrialized nations. HYPOTHESIS: We propose that activation of the aryl hydrocarbon receptor (AhR) by environmental chemicals contributes to melanoma invasion through enhancing expression and activity of the matrix metalloproteinases (MMPs). Further, we hypothesize that AhR interactions with other signaling pathways are critical for AhR-induced MMP expression in these cells. The AhR, originally identified as the receptor for the polycyclic aromatic hydrocarbon (PAH) family of environmental contaminants, is activated by endogenous and exogenous compounds, including: flavonoids, UV photoproducts of tryptophan, as well as some synthetic retinoids. We have previously shown that TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)-activation of the AhR results in increased expression and activity of MMPs -1, -2 and -9.

MMPs are a family of zinc- and calcium-dependent proteinases that degrade extracellular matrix (ECM) substrates that are involved in melanoma progression and metastasis. In this thesis we show that maximal TCDD-induced MMP-1 activation in A2058 melanoma cells requires 3 cis-acting response elements in the distal portion of the MMP-1 promoter, the NFκB, CCAAT and MITF sites. These elements are known to be downstream targets of the Ras/Raf signaling pathway, and our data also show that Ras/Raf activation is critical for AhR-induced MMP-1, -2 and -9 expression. Our data further suggest that AhR-Ras/Raf interactions result in deregulation of ECM metabolism, through alterations in expression of MMPs and their endogenous inhibitors, TIMP-1/-2 (tissue inhibitor of metalloproteinases). Lastly we demonstrate crosstalk between Ras/Raf signaling and the AhR pathway, and show that loss of AhR results in a reduction in Ras/Raf-mediated phosphorylation of ERK. Crosstalk is also demonstrated by a reduction of AhR expression and activity observed following Ras/Raf inhibition. Interestingly, 60% of all melanomas contain an activating mutation, ^{V600E}BRAF, in the Ras/Raf pathway, suggesting that melanomas may be more sensitive to AhR-activation. These data demonstrate that AhR-activated expression of MMPs in A2058 melanoma cells requires Ras/Raf signaling and that these pathways are directly involved in the regulation of enzymes vital to melanoma progression.

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1.0. GENERAL INTRODUCTION

1.1. Melanoma

1.1.1. Skin Structure and Function

The integumentary organ system, which consists of the nails, sweat glands, hairs and skin, serves primarily as a physical barrier between an organism and its environment (Champion, 1970). The most expansive portion of the integumentary organ system is the skin, which facilitates various functions such as protection, temperature control, adaptive immune response, sensation, absorption, respiration and secretion. Human skin consists of two distinct layers, the epidermis and dermis. These layers serve structural and functional purposes and consist of a characterized compliment of cellular and extracellular components.

During development mesenchymal cells within the mesoderm of an embryo give rise to most of the cells of the dermis, such as the mast cells, fibroblasts and also the adipose cells of the hypodermis (Champion, 1970). The most abundant dermal fibrillar collagen, type I collagen accounts for over 80% of all the collagenous proteins in the dermis (van Kempen et al., 2008). The developed dermal layer is divided into two sections, the papillary region adjacent to the epidermis and the deeper reticular region (Figure 1.1.A.). The two regions differ in their connective tissue composition in that the papillary region contains a finely woven meshwork of collagen I fibers while the reticular region contains a distinctive pattern of thick collagen bundles (van Kempen et al., 2007).

A sheet of extracellular matrices called the basement membrane exists between the basal layer of the epidermis and the papillary region of the dermis (Amano et al., 2001). The basement membrane serves as structural support, linking the epidermal to the

dermal layer but also facilitates keratinocytes polarity for growth and differentiation (De Arcangelis et al., 1996). The basement membrane between the dermis and the epidermis contains mainly collagens type IV and VII, laminins, nidogen and perlecan and provides a physical barrier against epidermal cell invasion and hence metastasis (Marinkovich et al., 1993).

The epidermal layer resides above the basement membrane and consists predominantly of keratinocytes in contact with melanocytes, Langerhans' and Merkel cells. This layer of the skin consists of multiple strata defined by various stages of differentiated keratinocytes and is under constant renewal. The melanocytes are dendritic cells derived from the neural-crest and are located at the basal layer of the epidermis (Sulaimon and Kitchell, 2003). One melanocyte will contact approximately 36 proximal keratinocytes and this grouping is referred to as an epidermal unit (Fitzpatrick, 1971). The melanocyte within the epidermal unit interacts with surrounding keratinocytes in a biochemical and physical fashion through dendritic processes. The primary role of melanocytes is to deliver a photoprotective, pigment producing molecule called melanin to the surrounding keratinocytes. Melanin is synthesized and transported to the keratinocytes in a membrane bound organelle called the melanosome (Schallreuter, 2007).

1.1.2 Melanoma Incidence and Stages

Melanoma is a malignant tumor arising from normal human melanocytes. Melanoma related mortalities in the United States have risen 34% from 1973 to 1992 and are still on the rise in countries world wide (Greenlee et al., 2000; Hoffmann et al., 1998; Parkin et al., 2005; Rigel et al., 1996). Melanomas can be ocular, mucosal,

genitourinary, gastrointestinal, leptomeninges, as well as metastasize to lymph nodes however, 95% of melanomas originate in the skin. The incidence of cutaneous melanoma is rising in several European countries (Karim-Kos et al., 2008; Pellacani et al., 2008) and the age-adjusted incidence is 68 and 29.3 of every 100,000 persons in Australia and the United States respectively (Parkin et al., 2005). Due to both increasing trends in incidence world-wide, and the lack of many successful treatment options, the characterization of malignant melanoma pathology needs further research and understanding (Rigel et al., 1996).

Melanocyte growth control and proliferation is normally suppressed by neighboring epithelial cells through homodimer interactions involving the cell surface receptor E-cadherin. The change in expression from E-cadherin to N-cadherin by melanoma cells results in release from growth suppression and formation of small clusters of melanocytes called benign nevi (Li et al., 2001; Sanders et al., 1999; Silye et al., 1998). The formation of benign melanocytic nevi is a preliminary step in the development of melanoma and is characterized by local proliferation without dermal invasion (Figure 1.1.B.). The transition from benign nevi to malignant melanoma requires invasion and is categorized into distinct phases based on invasion Figure 1.1.B.). Cutaneous melanomas initiate in the epidermal layer of the skin as a pigmented cluster of melanocytes that grow in a radial fashion called the radial growth phase (RGP). This phase of growth does not require extravasion from the epidermis into the dermis (Elder, 2006). The vertical growth phase of melanoma is defined by invasion into the papillary and reticular dermis and requires deposition of the basement

membrane consisting of mostly collagen type IV as well as macromolecules such as laminins, proteoglycans, entactin and other collagens (Elder, 2006; Pasco et al., 2004).

1.1.3. Causes of Melanoma

The main etiological risk factor for acquiring cutaneous melanoma is related to sporadic, intense sun exposure, especially in adolescence (Breitbart et al., 1997; Gandini et al., 2005; Walter et al., 1999). However, pyrimidine dimer formation, a typical signature of UV-mediated DNA damage, is lacking in melanomas. This observation is consistent with the resistance to UV-induced cell death of melanocytes compared to keratinocytes and suggests that pathways other than UV radiation may contribute to melanoma initiation and progression (de Leeuw et al., 1994). Epidemiological and environmental evidence demonstrate that environmental carcinogens contribute to melanoma initiation and progression (Beane Freeman et al., 2004; Broome Powell et al., 1999; Kennedy et al., 2005; Wolfe et al., 1990). Previous finding from the White laboratory at Rutgers University show that exposure of melanoma cells to an environmental carcinogen, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and resultant activation of the aryl hydrocarbon (AhR) pathway resulted in increased expression of matrix metalloproteinases (MMPs) which are essential to matrix catabolism and metastasis (Villano et al., 2005). These data are consistent with the possibility that environmental carcinogens are not only able to affect melanoma incidence, but can also facilitate enhanced progression through the AhR pathway.

Mutations in members of the Ras/Raf/MEK/ERK signaling pathway are commonly found in melanomas and the most frequent mutation occurs in an isoform of

Raf, BRaf (Davies et al., 2002). BRaf is a serine/threonine protein kinase that resides down stream of Ras in the Ras/Raf kinase signaling pathway. Somatic mutations of BRaf occur in approximately 7% of all cancers indicating that BRaf may have an important role in cancer maintenance (as reviewed (Dhomen and Marais, 2007). The most common activating mutation, accounting for approximately 90% of all BRaf mutations in melanomas, involves the substitution of valine for a glutamic acid residue at position 600 (BRaf ^{V600E}), which was previously thought to be position 599 (Davies et al., 2002). BRaf ^{V600E} is considered an oncogene due to the ability of melan-a cells expressing BRaf ^{V600E} to form tumors capable of growth in nude mice verses no growth for the same cells expressing wild-type BRaf (Wellbrock et al., 2004). In addition, the suppression of BRaf ^{V600E} resulted in slower growth of melanoma tumors in mice (Hoeflich et al., 2006). Interestingly, BRaf ^{V600E} is not sufficient for cancer progression due to 80% of benign skin lesion containing the mutation as well as reports of transgenic zebrafish expressing BRaf ^{V600E} resulting in only nevi formation (Patton et al., 2005; Pollock et al., 2003; Yazdi et al., 2003). Together these data suggest that BRaf ^{V600E} may play an important role in melanoma initiation however, other signals are also necessary for tumor progression. The A2058 melanoma cells utilized previously by the White laboratory and throughout this thesis harbor the BRaf ^{V600E} mutation making the Ras/Raf pathway of particular interest in studying the regulation of TCDD-induced MMP expression.

1.2.1. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent proteinases that together are able to degrade all the components of the ECM. Family members contain a cysteine switch motif in the prodomain, a zinc binding motif and have sequence homology to MMP-1, with the exception of MMP-23 which lacks a cysteine switch motif. There are currently 23 MMPs characterized in humans that are classified based upon their substrate specificity, sequence similarity and domain organization (Visse and Nagase, 2003) (Table 1.1.). MMP classifications are as follows: collagenases, which degrade connective tissue collagens; gelatinases that degrade denatured collagens and gelatins, stromelysins, which have a broad substrate specificity for proteoglycans, laminins, fibronectins and gelatins and also contain MMP-3, a known activator of pro-MMP-1 (Suzuki et al., 1990); matrilysins that lack a hemopexin domain; membrane-type MMPs (MT-MMP) of which there are two types, the type I transmembrane proteins and the glycosylphosphatidylinositol anchored proteins with varying substrate specificities; and a group of "other" MMPs consisting of seven MMPs that do not fit specific classifications of the other five groups.

MMPs are mainly regulated at the level of transcription or activity and are essential for tissue remodeling events such as wound healing and embryogenesis and loss of control of MMPs can result in cancer, arthritis, atherosclerosis, aneurysms, nephritis ulceration and fibrosis (Chakraborti et al., 2003; Visse and Nagase, 2003). MMP transcription is induced by various growth factors and cytokines. Inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are two well studied stimulators of MMP production and IL-1-induced MMP-1 expression was recently seen

to require both ERK and NFkB signaling (Chakraborti et al., 2003; Fan et al., 2006). Other factors such as retinoic acid and glucocorticoids are known to inhibit MMP expression (Chakraborti et al., 2003). The effect of a particular cytokine or growth factor is sometimes cell type specific as is seen with transforming growth factor-β (TGF-β) and retinoic acid. In keratinocytes, TGF-β activates MMP-1 expression, however it represses MMP-1 expression in fibroblasts (Pilcher et al., 1997b), while retinoic acid, which has been demonstrated to suppress MMP-1 in breast cancer cells (Benbow et al., 1999c), results in MMP-1 induction in keratinocytes (Murphy et al., 2004).

MMP gene expression has been shown to be mediated through many cis- and trans-acting factors. Fos and Jun transcription factor family members have been shown to bind to two activator protein-1 (AP-1) sites located in the proximal promoter of MMP-1 and activate expression (Benbow and Brinckerhoff, 1997). These AP-1 sites are also required for TCDD-induced MMP-1 expression in normal human keratinocytes (Murphy et al., 2004). There are also PAE-3 elements located in the MMP-1 promoter that are known to mediate binding of the ETS family of transcription factors. A PEA-3 site in the MMP-1 promoter located at -80bp of the MMP-1 promoter regulates TPA (PMA) induction (White and Brinckerhoff, 1995). Another site at -3108 has been implicated in the suppression of MMP-1 due to retinoic acid treatment (Benbow et al., 1999a). Recent data suggest that MEKK1 overexpression of MMP-1 is mediated by NFκB-p65 and C/EBPβ heterodimer binding to -2008 and -1972bp, respectively.

MMPs are produced as pro-enzymes and require cleavage of the propeptide for activation. proMMP activation occurs in a two-step fashion generating cleavage intermediates (Nagase, 1997). The first step generally involves a proteolytic attack upon

an exposed loop region between the first and second helix of the pro-peptide. After this cleavage, it is thought that destabilization of the pro-peptide occurs, which allows the remaining pro-peptides removal by other MMPs resulting in an active MMP molecule. Plasmin is also a known activator of MMPs. The precursor of plasmin, plasminogen is cleaved by urokinase plasminogen activator to generate plasmin. Plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10 and proMMP-13 (Lijnen, 2001).

Aside from degrading extracellular matrix components, MMPs are also known to cleave non-matrix substrates including signaling molecules, receptors, and adhesion molecules. Precise processing of these non-matrix molecules expand the biological role of MMPs beyond that of matrix turnover (Overall, 2004). Apolioprotein E (apoE) suppresses growth factor-induced cell migration and proliferation in smooth muscle cells and is a substrate for MT1-MMP. Cleavage of apoE by MT1-MMP abrogates suppression of cell proliferation (Aoki et al., 2005). Further, extracellular matrix metalloproteinase inducer (EMMPRIN) is cleaved in an MMP-dependent manner to produce soluble EMMPRIN molecules (Tang et al., 2004). Other molecules cleaved by MMPs are syndecan-1, ADAMTS4 and cyclophilin B (De Ceuninck et al., 2003; Endo et al., 2003; Gao et al., 2004). The recent findings that have revealed MMPs increased substrate repertoire, termed the substrate degradome, have spurred researchers to alter their experimental methods to encompass the complete change of cleavage products produced within a cell or tissue to understand changes in the cellular microenvironment resulting from MMP activity (Lopez-Otin and Overall, 2002).

1.2.2. Regulation of Matrix Metalloproteinase Activity

The primary inhibitor of MMP activity in plasma is the α2-macroglobulin, while in tissue the primary inhibitors are the tissue inhibitors of metalloproteinases (TIMPs). TIMPs are specific endogenous inhibitors of MMPs that regulate them through 1:1 stoichiometric interactions (Nagase and Woessner, 1999). There are four known TIMPs (1-4) and they function by binding the catalytic domain of MMPs rendering them unable to metabolize ECM substrates (Gomez et al., 1997). The inhibition of TIMPs can be facilitated by the N-terminal region of the TIMPs (Murphy et al., 1991). TIMPs can inhibit all MMPs excluding MT1-MMP, which TIMP-1 fails to inhibit (Will et al., 1996). Some examples of specific TIMP/MMP interactions are known, such as TIMP-1 inhibiting proMMP-9 (Airola et al., 1999; Goldberg et al., 1992). ProMMP-2 is not easily activated by general proteases and TIMP-2 for its activation. Studies show that TIMP-2 and proMMP-2 form a tight complex through their C-terminal domains and that TIMP-2's N-terminal domain binds to MT1-MMP (Butler et al., 1998; Strongin et al., 1995). proMMP-2 is activated by MT1-MMP and is freed from TIMP-2 (Visse and Nagase, 2003). The ratio between TIMP:MMP expression is most likely crucial for controlled matrix degradation by MMPs.

1.2.3. Matrix Metalloproteinase in Melanoma

MMP expression and activity during vertical invasion is essential to the melanoma cells' ability to traverse the basement membrane and extracellular matrix. Therefore, MMP expression correlates with the invasive potential of melanoma and are markers of an unfavorable clinical prognosis (Parsons et al., 1997). The most common

MMPs associated with melanoma progression are the gelatinase, MMP-2 and the collagenase, MMP-1. MMP-2 is involved in degradation of fibrillar collagens after partial degradation by collagenases, indicating that MMP-2 aids in invasion through the dermal layer and upregulation of MMP-2 increases both tumor growth and metastasis (Luca et al., 1997). Collagenases are essential to remodeling, because they are the only enzymes capable of initiating degradation of interstitial collagens at neutral pH and it has been shown that MMP-1 reduction by antisense RNA and also by RNA interference that MMP-1 is crucial to both basement membrane type IV collagen degradation as well as reducing angiogenesis, respectively (Blackburn et al., 2007; Durko et al., 1997). Immunolocalization studies of human melanoma tissue show the presence of MMP-1, MMP-2, and MMP-3 both within melanoma and in host tissue cells, but not in distant normal tissue (Walker and Woolley, 1999). The role of MMP-3 in melanoma progression may be in the activation of proMMP-1. The expression of MMP-1 and MMP-3 have also been seen to correlate with invasive stage melanomas (Airola et al., 1999) indicating that MMPs are a good indicator of invasiveness.

Degradation of the ECM by MMPs can lead to the production of cleavage products or the release of previously sequestered macromolecules and are called matrikines. Some released products are defined as biologically active protein domains derived from ECM macromolecules (Pasco et al., 2004). Studies have shown that basement membrane molecules released during degradation influence melanoma progression. Laminin-5 enhances melanoma cell motility through $\alpha 3\beta 1$ or $\alpha 6\beta 4$ integrin signaling by up-regulating MMP-9 secretion (Tsuji et al., 2002). Other matrikines are implicated in the suppression of melanoma such as endostatin.

Endostatin is released from collagen XVIII degradation and implicated as a possible anti-angiogenic factor. The mode of action of endostatin seems multifaceted in that it has been seen to compete FGF-1 or VEGF for heparin sulfate chain binding resulting in decreased angiogenesis, while it also binds to the catalytic domain of MMP-2 and suppresses its activation as well as actication of MT1-MMP (Egeblad and Werb, 2002; Kim et al., 2000; Lee et al., 2002; Marneros and Olsen, 2001). This indicates that the role of MMPs in melanoma progression isn't limited solely to disruption of physical barriers, but also results in the creation of many biologically active molecules with various activities [as reviewed (Egeblad and Werb, 2002; Pasco et al., 2004)].

1.3.1. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a polycyclic aromatic hydrocarbon (PAH) (Figure 1.2.) and is one of many unintentional by-products of chlorinated waste incineration and industrial combustion (Travis and Hattemer-Frey, 1991). Due to its lipophilicity and resistance to biological degradation, TCDD accumulates in adipose tissue and body burdens generally increase over time. In 1997, the International Agency for Research on Cancer (IARC) changed TCDDs status from a group 2 (possible) human carcinogen to a group 1 carcinogen (Steenland et al., 2004). TCDD's effectiveness as tumor promoter is seen in the rodent model where it is one of the strongest tumor promoters, promoting at 1/100 the dose of the tumor promoter 12-*o*-tetradecanoylphorbol-13-acetate (PMA) (Pitot et al., 1980). These findings are supported in human cohort studies showing that factory workers exposed to elevated levels of TCDD displayed a higher incidence of cancer related mortalities (Hooiveld et al., 1998; Steenland et al., 1999). Further, data from individuals exposed to TCDD in

an industrial accident in Seveso, Italy in 1976 reports that there are increases in mortality due to lymphatic and hematopoietic cancers in the area of highest contamination while mortality from rectum and lung cancer existed in lower contaminated areas as compared to a proximal reference site in Seregno, Italy (Cope et al., 2003). Support for TCDDs classification as a carcinogen regarding melanoma comes from a Ranch Hand cohort study of Air Force personnel who sprayed TCDD contaminated Agent Orange in Vietnam. This study has recently shown a significant increase in melanoma incidence in TCDD-exposed Air Force personnel as compared to the general population or other Air Force personnel in Southeast Asia who did not spray Agent Orange (Akhtar et al., 2004). These data indicate that TCDD exposure correlates to an increase in a variety of cancers including melanoma.

1.3.2. Aryl Hydrocarbon Receptor (AhR) Pathway

AhR, and its dimerization partner, the AhR Nuclear Translocator (Arnt), are members of the basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) domain family of transcription factors. This is a family of proteins with diverse biological roles ranging from regulation of development, to hypoxia signaling and circadian rhythms (Crews and Fan, 1999). The AhR is a ligand activated transcription factor that resides inactive in the cytoplasm while bound to accessory proteins including two heat shock protein 90 molecules, a HSP-90-interacting co-chaperone p23, and an immunophilin-like protein, ARA9/XAP2/AIP (Carver et al., 1998; Denis et al., 1988; Kazlauskas et al., 2001; Nair et al., 1996; Schmidt and Bradfield, 1996) (Figure 1.3.). Binding to the ligand binding domain of AhR results in exposure of the nuclear localization sequence through alterations of XAP2 binding (Kashuba et al., 2006; Ramadoss and Perdew, 2005). After

nuclear localization and dissociation from HSP-90 and ARA9, the AhR forms a heterodimer with heterodimeric binding partner, Arnt (Heid et al., 2000). The AhR/Arnt heterodimer functions as a transcriptional activator by binding to specific DNA enhancer element sequences in the 5' regions of AhR-responsive genes termed xenobiotic response elements (XRE: 5V-GCGTG-3V) (Matsushita et al., 1993; Reyes et al., 1992; Sogawa et al., 1995; Watson and Hankinson, 1992).

A common theme of the PAS family of proteins is the production of repressor molecules to terminate signal transduction (Haarmann-Stemmann and Abel, 2006). The Aryl Hydrocarbon Repressor (AhRR) is similar to both AhR and Arnt in that they all have a bHLH region that facilitates DNA binding and a PAS domain. Like Arnt however, the AhRR does not bind ligand and the AhR has a carboxy-terminal that differs greatly from AhRR (Mimura et al., 1999). The AhRR is involved in negative feedback inhibition of activated AhR/Arnt signal transduction through its increased expression mediated by XREs in its promoter (Evans et al., 2005) (Mimura et al., 1999). Possible mechanisms of AhRRs repression consist of AhRR sequestering of Anrt or direct competition for XRE binding which both would affect the AhR pathway's ability to activate XRE-mediated transcription. The current theory is that AhRR is involved in transrepression through protein-protein interactions at the site of promoter activation (Evans et al., 2008). A role for AhRR in human cancer has recently been realized through a report demonstrating that AhRR expression is suppressed in a variety of human malignant tissues from various regions including the colon, breast, lung, stomach, cervix and ovary (Zudaire et al., 2008). This report suggests that the AhRR may be a tumor suppressor and also implies that AhR signaling is critical to such malignancies.

1.3.3. Endogenous Role of the AhR Pathway

The role of the AhR in carcinogenic progression has focused on chemical-induced activation; however recent studies have shown that the AhR signaling pathway has functions other than response to exogenous chemicals. AhR knockout mice and cell culture experiments demonstrate that without ligand binding, the AhR promotes cell proliferation [see (Marlowe and Puga, 2005)]. A role for the AhR in controlling cell proliferation is further supported by results showing that TCDD exposure causes a decrease in cell cycle progression and proliferation (Huang and Elferink, 2005; Jin et al., 2004; Ray and Swanson, 2004). These data suggest that the AhR may play the role of an environmental/cellular stress sensor and inhibit cell cycle progression when the cell is stressed and that activation of the AhR by chemicals such as TCDD result in dysregulation of AhR's normal role.

The AhR has also been implicated in malignant progression involving direct interactions with other pathways and potential regulation of AhR and AhRR expression. AhR has been shown to physically interact with the known cell cycle control protein, retinoblastoma (RB) and this interaction represses cell cycle progression and E2F-dependent gene expression (Puga et al., 2000). Recent data also demonstrate an enhanced expression of AhR in both DMBA-induced pre-malignant mammary tissue and malignant mammary cells (Yang et al., 2007). These data combined with previous data showing higher expression of AhR in breast carcinoma cell lines as compared to immortalized, non-tumor-derived cell lines suggest a role for the AhR pathway in mammary tumorigenesis (Spink et al., 1998). Up-regulation of AhR in DMBA-induced mammary tissue results in increased expression of xenobiotic

metabolizing enzyme CYP1B1, which could facilitate the production of carcinogenic metabolites ultimately enhancing progression (Buters et al., 1999). Furthermore, the AhRR, has been implicated as an tumor suppressor due to its consistent down-regulation in malignant colon, breast, lung, stomach, cervix and ovary tissue (Zudaire et al., 2008). The presence of AhRR in normal tissue may be required to control AhR-dependent repression of cell cycle progression through interactions with the RB protein while the inverse expression of AhR and AhRR in breast cancer also suggests that AhR pathway modulation is a critical step in tumorigenesis. These current findings involve endogenous roles of the AhR pathway suggest that the AhR and the AhRR may be involved in mediating non-chemical as well as chemical induced tumorigenesis.

1.3.4. TCDD and the AhR Pathway

Studies involving the activation of the AhR pathway by TCDD have focused on the heterodimer's ability to activate transcription of phase I xenobiotic metabolizing genes including members of the cytochrome p450 (CYP450) family of monooxygenase enzymes (Fujii-Kuriyama et al., 1992; Swanson, 2004; Watson and Hankinson, 1992) and phase II xenobiotic metabolizing enzymes, including UGT1A1, GST-Ya subunit and NADPH-quinone-oxido-reductase [reviewed in (Mimura and Fujii-Kuriyama, 2003)]. TCDD also activates genes unrelated to xenobiotic metabolism. These include genes encoding proteins involved in growth control, such as transforming growth factor-α (Hankinson, 1995), transforming growth factor-β2 (Hankinson, 1995), Bax (Matikainen et al., 2001) and p27kip1 (Kolluri et al., 1999); cytokines interleukin-1β and interleukin-2 (Jeon and Esser, 2000; Sutter et al., 1991; Yin et al., 1994), and nuclear transcription

factors such as c-fos, Jun-B, c-Jun and Jun-D (Hoffer et al., 1996; Marlowe and Puga, 2005; Puga et al., 1992).

Exposure to TCDD results in a number of pathological lesions involving matrix remodeling. For example, in utero exposure to TCDD in rats results in reduced postnatal seminal vesicle branching and differentiation (Hamm et al., 2000). In addition to rats, C57BL/6 mice exposed to TCDD either in utero or through lactation exposure to TCDD, as well as a mice containing a null mutation of the AhR develop altered seminal vesicles and prostrates (Lin et al., 2002). Furthermore, studies of mammary gland development reveal that weanling rats exposed to TCDD display aberrant mammary gland development characterized by decreased mammary tubule branching (Brown and Lamartiniere, 1995). Malformations of the palatal shelves resulting in cleft palate are also seen in mice exposed to TCDD in utero (Abbott and Birnbaum, 1989). The effects of TCDD and the AhR pathway upon ECM remodeling are also seen in the teleost, Danio rerio (zebrafish). The common cardinal vein delivers blood to the heart of the zebrafish and undergoes remodeling as it regresses dorsally during development. This regression is decreased by TCDD treatment in an AhR-dependent fashion (Bello et al., 2004). Zebrafish caudal fin regeneration coincides with MT1-MMP, MMP-1 and TIMP-2 expression and TCDD treatment results in an AhR-dependent inhibition of regeneration (Bai et al., 2005; Zodrow and Tanguay, 2003). These data show that TCDD activation of the AhR can result in aberrant ECM remodeling events in a wide variety of physiological processes.

Data from the White laboratory have demonstrated that activation of the AhR by TCDD results in alterations in expression and activity of matrix metalloproteinases

(MMP) in melanoma and normal human keratinocytes cells (Murphy et al., 2004; Villano et al., 2005). Over-expression and increased activity of MMPs in cancerous or proximal stromal cells can result in aberrant ECM deposition and cell motility (Benbow et al., 1999b; Blackburn et al., 2007; Hofmann et al., 1999). The White laboratory has specifically shown that TCDD can induce MMP-1, MMP-2 and MMP-9 expression and activity in A2058 melanoma cells and that this is mediated by the AhR signaling pathway and requires de novo protein synthesis (Villano et al., 2005). Furthermore, it was demonstrated that TCDD-induced activation of MMP-1 is not mediated through any known AhR-responsive elements in the MMP-1 promoter. Further studies are needed to elucidate the mechanisms and/or signaling pathways required for TCDD-induced MMP-1 expression in melanoma cells.

1.4. Objective and Hypothesis

The objectives of this thesis are to elucidate the pathways and molecular mechanisms involved in mediating AhR-dependent TCDD induction of MMP-1, MMP-2 and MMP-9 in A2058 melanoma cells. It is expected that this knowledge will help develop a better understanding of how AhR signaling mediates MMP production as it relates to malignant melanoma. It is hoped that such an understanding will lead to the elucidation of a novel participating pathway or molecule that could be targeted for inhibition and utilized in treatment. It is hypothesized here that: (1) TCDD-induction of MMP-1 expression is mediated, in part, by novel transcription factor binding site(s) in the distal MMP-1 promoter and that these binding sites require AhR-mediated signaling; (2) additional second messenger signaling is required for TCDD-mediated induction of

MMP-1, MMP-2 and MMP-9 expression; and (3) the AhR signaling pathway will modulate the second messenger signal required for TCDD-mediated induction of MMPs. A2058 melanoma cells containing an activating BRaf mutation (BRaf ^{V600E})

Data presented here demonstrate that TCDD induction of MMP-1 promoter activity in A2058 melanoma cells is mediated by AhR and that maximal activation involves binding of NFκB, CCAAT and MITF regulatory elements to 5' proximal regions of the MMP-1 promoter, as well as by histone acetylation. Inhibition of either the Ras/Raf MAP kinase pathway or the p38 MAP kinase pathway results in reduction of MMP-1, MMP-2 and MMP-9 TCDD-induced MMP expression demonstrating a need for second messenger signaling for TCDD induction of MMPs. Either p38 inhibition or AhR inhibition resulted in decreased phosphorylation of the Ras/Raf signaling pathway component, ERK. This decrease in phosphorylation together with effects of Ras/Raf signaling inhibition on MMPs demonstrate the necessity of ERK phosphorylation for maximal TCDD-induced MMP expression. Ras/Raf signaling inhibition also results in a decrease of AhR expression and a concomitant increase in AhRR expression, suggesting that the Ras/Raf inhibitor is altering AhR responsiveness by changing AhR:AhRR balance. Less expression of AhR and more of AhRR would result in an overall repression of the AhR signaling pathway and data here show that normal human melanocytes express a much greater amount of AhRR compared to AhR. In comparison, the BRaf ^{V600E} containing A2058 melanoma cells express a high amount of AhR compared to AhRR, however after Ras/Raf inhibition A2058 cells express less AhR and more AhRR. This change in expression due to Ras/Raf inhibition causes A2058 cells expression levels of AhR and AhRR to resemble that of normal human melanocytes.

These data demonstrate that Ras/Raf signaling pathway has a direct effect on the expression of AhR and AhRR, indicating that melanoma progression may involve regulation of the AhR pathway through AhR and AhRR intracellular concentrations.

A.

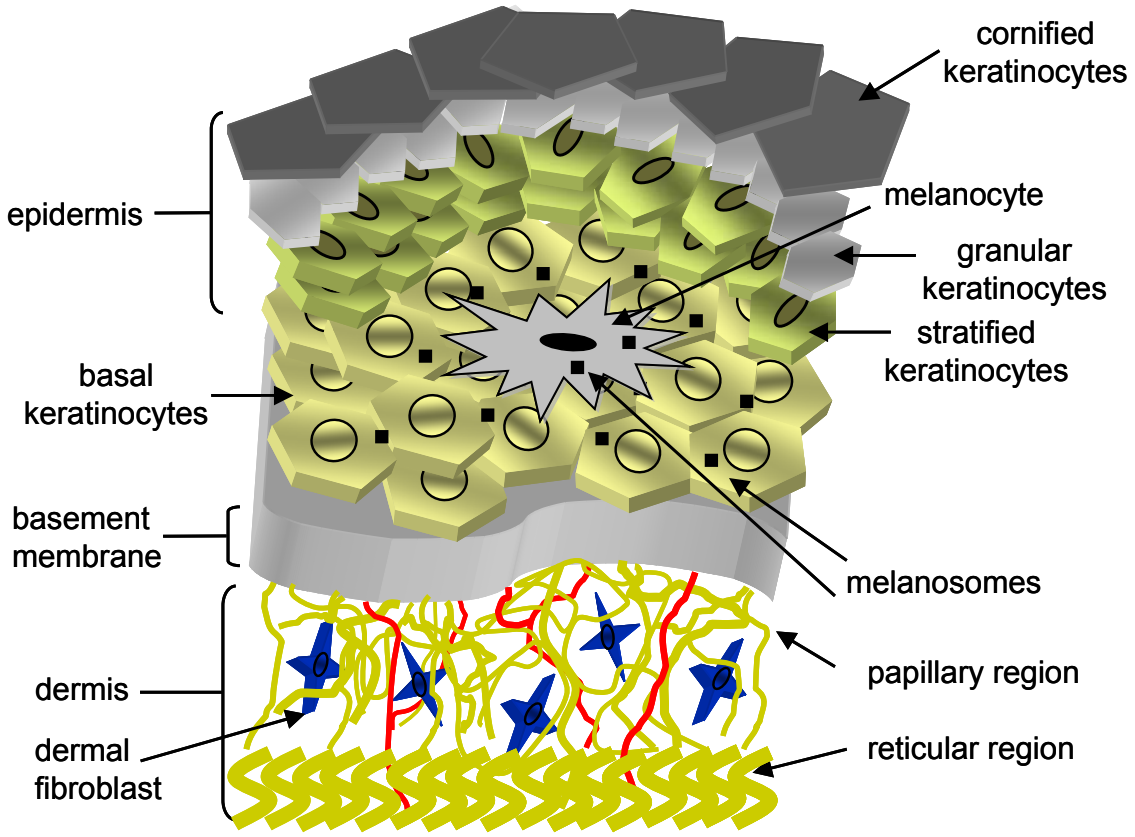


Figure 1.1. Continued on next page

B.

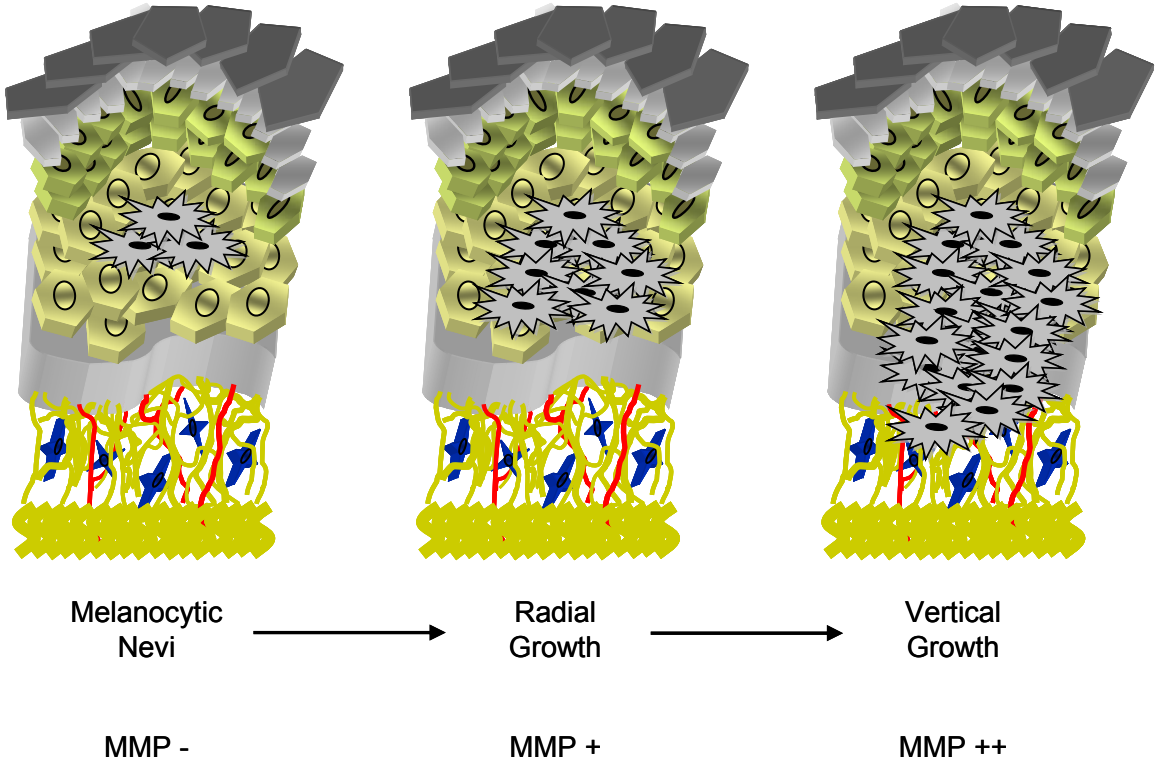


Figure 1.1. Depiction of human skin and melanoma progression

(A) Human skin consists of two distinct layers, the epidermis and the dermis. The layers are separated by a basement membrane consists mainly of collagens type IV and VII and provides a physical barrier to epidermal cell invasion to the dermis. Melanocytes are pigment producing cell of neural crest origin and reside in the basal layer of the epidermis in contact with approximately 36 keratinocytes and together these cells form what's termed the epidermal unit. (B) Benign nevi transition to malignant melanoma initially growing within the epidermis in circular fashion (radial growth phase) where increased expression of MMPs are observed. Further progression involves degradation of the basement membrane by MMPs and results in dermal invasion (vertical growth phase).

Subfamily	Name	ECM substrates
Collagenases	Collagenase-1/MMP-1	Collagen I, II, III,VII,VIII,X,XI; gelatin, entactin/nidogen, fibronectin, laminin, vitronectin, aggrecan
	Collagenase-2/MMP-8	Collagen I, II, III; gelatin, entactin, aggrecan, tenescin
	Collegenase-3/MMP-13	Collagen I, II, III,VI,VIII,IX,X, XIV; gelatin, fibronectin, vitronectin, aggrecan, osteonectin
Gelatinases	Gelatinase-A/MMP-2	Collagen I, III, IV, V,VII,X,XI; gelatin, fibronectin, fibrillin, elastin, laminin-5, vitronectin, aggrecan, osteonectin, tenescin
	Gelatinase-B/MMP-9	Collagen IV, V, XI, XIV; gelatin, decorin, fibrillin, elastin, laminin, vitronectin, aggrecan
Stromelysins	Stromelysin-1/MMP-3	Collagen III, IV, V,VII,IX, X, XI; gelatin, decorin, elastin, entactin/nidogen, fibronectin, fibrillin, laminin, vitronectin, aggrecan, osteonectin
	Stromelysin-2/MMP-10	Collagen III, IV, V; gelatin, elastin, fibronectin, aggrecan
Membrane-Type	MT-MMP-1/MMP-14	Collagen I, II, III; gelatin, fibronectin, vitronectin, aggrecan
	MT-MMP-2/MMP-15	Proteoglycan
	MT-MMP-3/MMP-16	Collagen III; fibronectin
	MT-MMP-4/MMP-17	Gelatin, fibronectin/fibrin
	MT-MMP-5/MMP-24	Fibronectin, proteoglycans, gelatin
	MT-MMP-6/MMP-25	Collagen IV, gelatin, laminin-1, fibronectin, proteoglycans, fibrin/fibrinogen
Matrilysin	Matrilysin-1/MMP-7	Collagen I, IV; decorin, elastin, fibrillin, fibronectin, laminin, vitronectin, aggrecan, osteonectin
	Matrilysin-2/ MMP-26	Collagen IV, gelatin, fibronectin, fibrin/fibrinogen
Other MMPs	Stromelysin-3/MMP-11	Fibronectin, laminin, aggrecan
	Elastase/MMP-12	Fibronectin, elastin, laminin, proteoglycans, fibrin/fibrinogens
	RASI-1/MMP-19	Collagen IV; gelatin, laminin, fibronectin, fibrin/fibrinogen
	Enamelysin/MMP-20	Ameologenin, aggrecan
	CA-MMP/MMP-23 & MMP-24	Gelatin
	Epilysin/MMP-28	

Table 1.1. Matrix metalloproteinases and substrate specificity
Table of matrix metalloproteinases with their corresponding substrate specificities reproduced from (Hillegass et al., 2006).

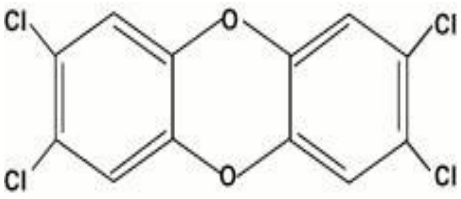


Figure 1.2. Chemical structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

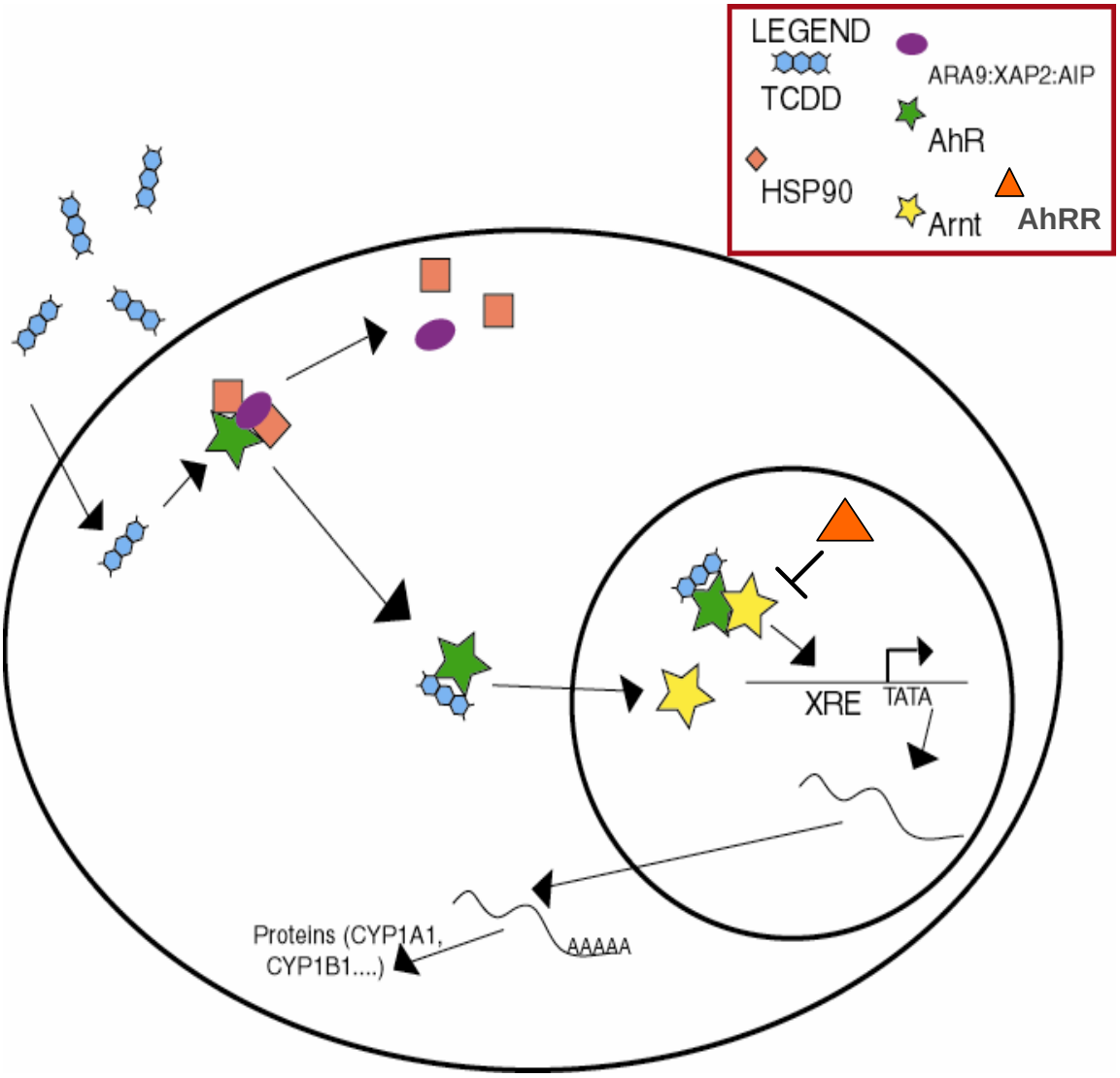


Figure 1.3. The aryl hydrocarbon signaling pathway
The aryl hydrocarbon receptor (AhR) is a receptor for many polycyclic aromatic hydrocarbons, such as TCDD. The AhR resides inactive in the cytoplasm associated with accessory proteins HSP90, p23 and XAP2. TCDD binding to AhR results in nuclear translocation where AhR forms a heterodimer with the Aryl Hydrocarbon Nuclear Translocator (Arnt). The AhR/Arnt heterodimer functions as a transcription factor able to bind to xenobiotic response elements of target genes and induce transcription. The AhR Repressor (AhRR) is an endogenous repressor of the AhR/Arnt heterodimer and can inhibit AhR/Arnt-mediated transcription.

2.0. MATERIALS AND METHODS

2.1. Cell Culture

A2058 melanoma cells were purchased from ATCC, and cultured in Dulbecco's Modification of Eagle's Medium (DMEM), 1X with 4.5g/L, L-glutamine, & sodium pyruvate (Mediatech) with Pen/Strep (Mediatech) and Fetal Clone II (HyClone). Normal Human Melanocyte (NHM) lightly pigmented primary cultures were purchased from Cascade Biologics and cultured in Media 154 (Cascade Biologics) supplemented with Human Melanocyte Growth Supplement (Cascade Biologics). Cultures were incubated at 37^{o}C, in a humidified, 5% CO_2 incubator.

2.2. Treatments

Confluent A2058 and NHM cultures were washed three times in Ca^{2+}/Mg^{2+} free PBS and serum starved for 24h in serum and additive-free media. Pretreatments containing chemical inhibitors alone were applied for 30min. prior to co-treatments. Treatments were performed at the following concentrations: TCDD (10nM; UltraScientific), α-naphthoflavone (αnap) (1μM; Sigma), U0126 (1.25-10μM; Calbiochem), SB203580 (4-15μM; Biosource), LY294002 (10μM; Cell Signaling Tech) and PP2 (10μM; Biomol), Scriptaid (5uM; Calbiochem), Nullscript (5uM; Biomol), Trichostatin A (Calbiochem; 12.5-25nM).

2.3. Quantitative Real Time- PCR (qRT-PCR)

For Real Time-PCR analysis, total RNA was isolated using Trizol Reagent (Invitrogen) per manufacturer's instructions. DNA contamination was removed by DNA-free treatment (Ambion) and cDNA was generated using the iScript cDNA Synthesis kit (BioRad). Primer sets for quantitative PCR and standard curves are described previously (Villano *et al.*, 2005) except for BRaf primers obtained from Kim et al (Kim *et al.*, 2006). Fluorescent PCR products were detected using a Bio-Rad iCyclerQ real-time PCR detection system using iQ SYBR green supermix (BioRad) and all results were normalized to GAPDH levels. Melt curves were generated for each sample after amplification for determination of primer dimers.

2.4. Transient Transfections and Luciferase Assays

MMP-1 promoter constructs 4400bp and 1500bp cloned into the pGL3-basic luciferase vector (Promega) was the kind gift of Dr. C. E. Brinckerhoff (Rutter *et al.*, 1997). A 2011, 1989, 1949, 1909, 1787, 1610 and 1500bp MMP-1 luciferase constructs were PCR amplified, cloned into and sequenced from TOPO pCRII (Invitrogen). The proximal promoter region was either cloned into the Hind III or XhoI site of pGL3-basic luciferase vector and the distal region at Xho I or SacI, depending on its orientation in TOPO pCRII. The CYP1A1 luciferase plasmid (pRNH3/235) was the kind gift of Dr. M. Denison and Dr. Ron Hines and is described previously (Rushing and Denison, 2002).

A2058 melanoma cells were transfected with the pGL3 constructs using Lipofectamine 2000 (Invitrogen) in serum and additive-free medium (0.55μg DNA : 10μl Lipofectamine). Cultures were co-transfected with the renilla luciferase vector

phRL-TK (Promega) at a ratio of 1:100 phRL-TK: pGL3 MMP-1 constructs to control for variations in transfection efficiency. A2058s were plated onto 35mm plates at 90 percent confluence. One day post-plating, cells were transfected as stated above, and at two days post-plating the cells were pretreated with inhibitors following co-treatment with vehicle (DMSO) or TCDD and co-treated with inhibitors U0126, SB203580, LY294002 or PP2 at indicated concentrations. Cellular lysates were harvested 24h following treatment using the Dual Luciferase Kit (Promega) and luciferase activities determined using a Dynex Multiplate Luminometer. Results were normalized to total protein concentrations using a modified Lowry assay (BioRad). Luciferase activity was normalized for transfection efficiency using phRL-TK (Promega). Treatments and transfections were performed in triplicate and statistical analysis was performed using SigmaStat version 1.0 for Windows statistical analysis program (Jandel Scientific).

2.5. siRNA Transient Transfections

A2058s were plated to 90 percent confluence on a 6 well plate one day prior to transient transfections of siRNA molecules. A2058 cells were washed three times in Ca^{2+}/Mg^{2+} free PBS and cells were transiently transfected with TriFECTa Kit Dicer Substrate RNAi duplexes (IDT) specific to BRaf (siBraf) or AhR (siAhR) at 10nM concentration per well using Lipofectamine 2000 (Invitrogen) at 15μL per well. In experiments cotransfected with the 2011bp luciferase construct and either siAhR or siBRaf, 0.55μg luciferase + 10nM siRNA : 15:l Lipofectamine ratio was used for a 24hr transfection. After transfection, the cells were washed three times in Ca^{2+}/Mg^{2+} free PBS and treatments were applied for indicated times. Treated cells were harvested

24h following treatment using the Dual Luciferase Kit (Promega) and luciferase activities determined using a Dynex Multiplate Luminometer. Results were normalized to total protein concentrations using a modified Lowry assay (BioRad). Luciferase activity was normalized for transfection efficiency using phRL-TK (Promega). Treatments and transfections were performed in triplicate and statistical analysis was performed using SigmaStat version 1.0 for Windows statistical analysis program (Jandel Scientific).

siBraf Duplex:

5'GGAACAUAUAGAGGCCCUAUUGGAC 3'

5'GUCCAAUAGGGCCUCUAUAUGUUCCUG 3'

siAhR Duplex:

5'UCUGUAUUAAGUCGGUCUCUAUGCCGC 3'

5'GGCAUAGAGACCGACUUAAUACAGA 3'

2.6. Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays

Oligonucleotides for mobility shifts were prepared by Integrated DNA Technologies and sequences can be seen in figure 3.3. The following sequences represent -2012 through -1945bp of the human MMP-1 promoter as wild-type (2012 wt) or containing bold, italic and underlined base-pair mutation of specified binding sites.

(2012 wt) agggtgacgtcttag**gcaat**ttcctgt**ccaat**cacagatggtc**a*catg***ctgctttcctgagttaacctat

(2012 NFkB) agggtgacgtcttagg***gt***a***cc***tcctgtccaatcacagatggtcacatgctgctttcctgagttaacctat

(2012 CCAAT)agggtgacgtcttaggcaatttcctgt**ggt**a**c**cacagatggtcacatgctgctttcctgagttaacctat

(2012 Mitf) agggtgacgtcttaggcaatttcctgtggtaccacagatggtc**t**c**ga**gctgctttcctgagttaacctat

2012 (N/C) agggtgacgtcttagg**gt**a**cc**tcctgt**ggt**a**c**cacagatggtcacatgctgctttcctgagttaacctat

2012 (N/C/M) agggtgacgtcttagg**gt**a**cc**tcctgt**ggt**a**c**cacagatggtc**t**c**ga**gctgctttcctgagttaacctat

Nuclear extracts were prepared from A2058 monolayers cultures grown to 90% confluence, serum-starved for 24h and treated with TCDD or DMSO or cotreated with U0126 inhibitor. After treatment for indicated times, cells were washed two times with ice cold Ca^{2+}/Mg^{2+} free PBS and transferred in PBS to microcentrifuge tubes where they were centrifuged in a refrigerated centrifuge (4^oC) at 1,850*g for 5mins. Cells were resuspended in buffer A: 10mM HEPES,pH 7.9; 1.5mM $MgCl_2$; 10 mM KCl; 0.5mMDTT; 0.5mM PMSF and lysed using a 25 gauge needle. The samples were centrifuged in a refrigerated centrifuge (4^oC) at 3,300*g for 5mins and resuspended in buffer C: 20mM HEPES, pH 7.9; 25 % glycerol; 0.42M KCl; 1.5mM $MgCl_2$; 0.2mM EDTA; 0.5mM DTT; 0.5mM PMSF. Cells were centrifuged in a refrigerated centrifuge (4^oC) at 14,000*g for 30mins and supernatants were stored in 20μL volumes at -80^oC. Protein content of the extracts was determined using the Modified Lowry assay with a bovine serum albumin standard curve (Pierce). For electrophoretic mobility shift assays, 10μg of nuclear protein combined with buffer C and 1μg poly dIdC totaling 10μL were incubated with 30,000cpm of [α-^{32}P]dCTP (GE Healthcare, PERKin-Elmer) double-stranded probes for 30mins at room temperature. After incubation, binding reactions were electrophoresed on a 7% acrylamide gel in 0.5 X

Tris borate/EDTA at 125 V. Gels were dried, exposed on a phosphorimager screen, and scanned using a PhosphorImager.

2.7. *In vitro* Zymography

Media from A2058 cultures were concentrated at 4^{o}C using YM-30 Microcon Centrifugal Concentrator size exclusion spin columns (Millipore). Concentrated sample protein concentrations were determined using a modified Lowry assay (BioRad) and 15 μg of sample was analyzed for collagenase activity using EnzChek Gelatinase/Collagenase Assay Kit (Molecular Probes) with collagenase type I substrate. After incubation, samples were read in an HT-Soft fluorescence plate reader at 492nm excitation / 535nm emission. Samples were plated in quadruplicate and statistical analysis was performed using SigmaStat version 1.0 for Windows statistical analysis program (Jandel Scientific).

2.8. Western Analysis

Whole cell protein was isolated from A2058s using RIPA lysis buffer and 50 μg of sample were separated on a NuPAGE 4-12% Bis-Tris acrylamide gel (Invitrogen) under reducing conditions. Separated proteins were transferred to a Hybond membrane (Amersham) and transfer was confirmed by Ponceau-S staining. Western blots were blocked in TBST containing 5% milk and probed in TBST containing 3% milk with the following antibodies; p44/42 , phospho-p44/42, p38, phospho-p38 (Cell Signaling), β-actin (Sigma). Secondary HRP conjugated antibodies specific to rabbit and mouse were diluted in TBST containing 5% milk and were detected using an ECL Plus Kit (Amersham) and exposed to Kodak X-ray film or read on a Storm 860 Molecular Imager

(GMI). Blots of whole cell protein were stripped and re-probed using an antibody specific to human β-actin as a loading control.

Table 2.1. Primers for quantitative real-time PCR

Name	Sequence	Size	Reference
GAPDH	F: 5'- CGCCAGCCGAGCCACAT-3' R: 5'- TCGCCCCACTTGATTTTG-3'	295 bp	(Murphy et al., 2004)
MMP-1	F: 5'-CCACAAACCCCAAAAGCGTG-3' R: 5'-CGTGTAGCACATTCTGTTGAA-3'	262 bp	(Nagata et al., 2003)
MMP-2	F: 5'-GTGCTGGGCTGCTGCTTTCGT-3' R:5'-GTCGCCCCTCAAAGGTTTGGAAAT-3'	487 bp	(Villano et al., 2005)
MMP-9	F: 5'-CCACGTGACAAGCCCATGGGGCCCC-3' R: 5'-GCAGCCTAGCCCAGTCGGATTTGATG-3'	303 bp	(Villano et al., 2005)
TIMP-1	F: 5'-AATTCCGACCTCGTCATCA-3' R: 5'-TGCAGTTTTCCAGCAATGA-3'	230 bp	(Paek et al., 2006)
TIMP-2	F: 5'-AAAGCGGTCAGTGAGAAGG-3' R: 5'-CTTCTTTCCTCCAACGTCC-3'	183 bp	(Paek et al., 2006)
CYP1A1	F: 5'-TAACACTGATCTGGCTGCAG-3' R: 5'-GGGAAGGCTCCATCAGCATC-3'	420 bp	(Abbott et al., 1999)
AhR	F: 5'-GGACTTGGGTCCAGTCTAATGCAC-3' R: 5'-AGCCAGGAGGGAACTAGGATTGAG-3'	296 bp	(Abbott et al., 1999)
AhRR	F: 5'-ACCGCGGATGCAAAAGTAAAA-3' R: 5'-GCTCCTTCCTGGTGAGTAATTGG-3'	71 bp	
BRAF	F: 5'-CCTCAGAGTAAAAATAGGTG-3' R: 5'-ATAGCCTCAATTCTTACCA-3'	315 bp	(Kim et al., 2006)

3.0. AHR-DEPENDENT MATRIX METALLOPROTEINASE-1 (MMP-1) EXPRESSION IS MEDIATED BY MULTIPLE BINDING SITES IN THE DISTAL PROMOTER IN A2058 MELANOMA CELLS

3.1. Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix transcription factor involved in regulation of circadian rhythm, drug metabolism and immunosupression. Originally identified as the receptor for environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the AhR is now known to be activated by a variety of exogenous and endogenous compounds. Previous data from the White laboratory demonstrate that TCDD-induced AhR activation alters expression and activity of MMPs in human melanoma cells and in normal human keratinocytes. MMP expression and activity are essential for tissue remodeling and cellular migration, and are inappropriately expressed in a variety of pathologies that involve extracellular matrix (ECM) remodeling, such as rheumatoid arthritis and tumor metastasis. In this study we use siRNA specific to AhR to confirm previous findings indicating that the AhR is necessary for TCDD-induced MMP expression. Although these data indicate TCDD-activation of MMP-1 is AhR-dependent, it also showed that AhR-activation of MMP-1 expression is not mediated through typical AhR-binding sites. Using electrophoretic mobility shift and MMP-1 promoter assay analysis, we identified the binding sites required for TCDD-induced MMP-1 expression, including an NFkB-like and a CCAAT binding site.

3.2. Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a polycyclic aromatic hydrocarbon (PAH) and is one of many unintentional by-products of chlorinated waste incineration and industrial combustion (Travis and Hattemer-Frey, 1991). Due to its resistance to biological and chemical degradation, TCDD accumulates in adipose tissue and body burdens increase over time. Although TCDD appears non-mutagenic, it is believed to act by promoting neoplastic transformation in cells that have been initiated (Safe, 2001). Health risks have been demonstrated in human cohort studies showing that factory workers exposed to elevated levels of TCDD displayed a higher incidence of cancer related mortalities (Hooiveld et al., 1998; Steenland et al., 1999). The molecular mechanism of tumor promotion by TCDD is not clearly understood; however, it is accepted that TCDD exerts its effects through activation of the AhR pathway (Poellinger, 2000).

The AhR resides inactive in the cytoplasm while bound to accessory proteins including two heat shock protein 90 molecules, a HSP-90-interacting co-chaperone p23, and an immunophilin-like protein, ARA9/XAP2/AIP (Carver et al., 1998; Denis et al., 1988; Kazlauskas et al., 2001; Nair et al., 1996). Ligand binding results in activation of AhR and nuclear localization (Reyes et al., 1992). In the nucleus, the AhR forms an active transcription factor through heterodimerization with the aryl hydrocarbon nuclear translocator (Arnt) and this heterodimer can bind to specific DNA sequences in the 5' regions of AhR responsive genes termed xenobiotic response elements (XRE: 5'-GCGTG-3') (Matsushita et al., 1993; Reyes et al., 1992; Sogawa et al., 1995; Watson and Hankinson, 1992). Studies of AhR/Arnt and TCDD have focused on the heterodimer's

ability to activate transcription of xenobiotic metabolizing genes including members of the cytochrome p450 (CYP450) family of monooxygenase enzymes (Fujii-Kuriyama et al., 1992; Swanson, 2004; Watson and Hankinson, 1992), as well as genes unrelated to xenobiotic metabolism. These include genes encoding proteins involved in growth control, cytokines, nuclear transcription factors and regulators of extracellular matrix (ECM) proteolysis (Marlowe and Puga, 2005; Murphy et al., 2004; Sutter et al., 1991; Villano et al., 2005; Yin et al., 1994).

Matrix metalloproteinases (MMPs) are a family of calcium- and zinc-dependent endopeptidases which are categorized into six groups based on substrate specificity, sequence and domain similarities; collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs (Visse and Nagase, 2003). Tumor invasion and metastasis relies on the expression of MMPs to degrade ECM and the basement membrane resulting in cell invasion (Brinckerhoff et al., 2000). Malignant melanoma is a disease of normal human melanocytes and relies on ECM remodeling to invade through the basement membrane and interstitial ECM. Once in the dermal layer degradation of the most abundant protein found in the human dermis, fibrillar type I collagen, is required and the most effective MMP known to accomplish this is MMP-1 (Kerkela and Saarialho-Kere, 2003; Pilcher et al., 1997a; Weber et al., 1984). Immunohistochemical and in situ hybridization experiments have revealed that MMP-1 expression correlates with invasiveness of melanomas (Airola et al., 1999; Walker and Woolley, 1999) and recently RNA interference inhibition of MMP-1 prevent angiogenesis in a mouse model (Blackburn et al., 2007).

Acetylation and deacetylation of particular lysine residues of histones alters nucleosome structure allowing or denying transcription factors to access the DNA (as reviewed (Wolffe and Guschin, 2000)). Recent data have implicated acetylation in control over MMP expression. In one report a retinoid X ligand, LG268, was shown to increase MMP-1 transcript levels through histone H4 acetylation (Burrage et al., 2007). Another report demonstrated that histone deacetylase inhibition resulted in reduced MMP-1 expression (Young et al., 2005). Furthermore, histone deacetylases show anti-cancer effects and are already in use in clinical trials (davRasheed et al., 2007). In addition to control of histones, acetylation has been shown to alter the activation or availability of transcription factors (Musri et al., 2007; Quivy and Van Lint, 2004).

Previous data from the White laboratory demonstrates that TCDD induces MMP-1 expression in A2058 melanoma cells and that this induction requires de novo protein synthesis (Villano et al., 2005). Further these data indicate, using a chemical antagonist of AhR (α-naphthoflavone), that this induction is most likely dependant on TCDD-AhR activation. The data presented here confirm that MMP-1 expression is specifically regulated through the AhR pathway through use of short inhibitory RNA (siRNA) specific to AhR. Furthermore, these data show that TCDD/AhR activation of MMP-1 is mediated through multiple cis-acting elements in the distal portion of the MMP-1 promoter, including a NFκB-like, CCAAT and a MITF site. These findings also indicate that histone acetylation is modified upon TCDD-activation of AHR and this also contributes to MMP-1 activation.

3.3. Results

3.3.1. Effect of TCDD on distal deletions of MMP-1 promoter

It has been previously demonstrated that a luciferase construct containing 4400bp of proximal MMP-1 promoter sequence (4400-pGL3) was induced in response to TCDD and that this response is lost in luciferase constructs containing 1500bp or less of MMP-1 proximal promoter sequence, indicating that proximal AP-1 sites that were TCDD-responsive in normal human keratinocytes were not responsive in A2058 melanoma cells (Villano et al., 2005). To refine the TCDD/AhR responsive region of the MMP-1 promoter, I generated six new luciferase deletional constructs containing 2011, 1989, 1949, 1909, 1787 or 1610bp of proximal MMP-1 promoter. Additionally, the MMP-1 promoter was analyzed using a transcription factor binding site database (MatInspector,Genomatix) and five elements of interest were identified in this region; a microphthalmia-associated transcription factor (MITF) element, a CCAAT/enhancer binding protein elements (CCAAT), a NFkB-like element (NFkB), a polyoma enhancing activity-3/E26 virus (ETS) element, and an activator protein-1 (AP-1) site. The 2011bp construct contains all five potential responsive elements, while the 1989bp construct lacks the NFkB-like element, and the remaining constructs only contain AP-1 and ETS elements (Figure 3.1.A). A2058 melanoma cells were transiently transfected with the luciferase constructs containing the luciferase gene (pGL3: Promega) under the transcriptional control of 4400, 2011, 1989, 1949, 1909, 1787, 1610 and 1500bp of the MMP-1, 5' regulatory region. Cells were exposed to TPA (12-*O*-tetracanoyl phorbol-13-acetate) as a positive control for activation of the MMP-1 promoter

sequences. All sequences tested maintained TPA responsiveness as seen previously (Murphy et al., 2004; White and Brinckerhoff, 1995). The 2011bp construct was induced 4 fold over control levels while loss of the 22bps between -2011 and -1989 resulted in loss of all significant TCDD-responsiveness (Figure 3.1.B). These data demonstrate that TCDD-induced MMP-1 promoter activity requires sequences located between -2011bp and -1989bp for maximal activation.

3.3.2. Nuclear protein binding to the minimal MMP-1 promoter region

To assess the potential binding of transacting factors to the MMP-1 promoter, overlapping oligonucleotides approximately 70bp in length were generated spanning from 2011bp through 1592bp of the MMP-1 promoter for electrophoretic mobility shift assays (EMSA). Seven oligonucleotides designed for gel shift analysis divide potential MMP-1 promoter transacting binding elements such that a 2012-1942bp oligonucleotide contains the NFκB-like, CCAAT and MITF putative binding elements and the 1792-1722bp oligonucleotide containing an AP-1 site (Figure 3.2.). Nuclear proteins from DMSO (vehicle) or TCDD treated A2058s were incubated with ^{32}P-labeled oligonucleotides in the presence and absence of specific or non-specific unlabeled competitor and DNA•Protein complexes are electrophoresed on a polyacrylamide TBE gel. Nuclear protein binding was observed in five of the seven oligonucleotides, -2011 to -1941bp, -1951 to -1882bp, -1892 to -1822bp, -1838 to -1769bp and -1792 to -1722bp (Figure 3.3.A., B., C.) while -1733 to -1658bp (Figure 3.3.C) and -1668 to -1593bp (Figure 3.3.D.) did not demonstrate binding of nuclear proteins. Binding to the -1951 to -1882bp (Figure 3.3.A.) and the -1792 to -1722bp

(Figure 3.3.C). oligonucleotides was unable to be competed by addition of 100-fold excess of unlabelled specific (S) oligonucleotide hence binding is considered not specific. Oligonucleotides spanning -1892 to -1822bp and -1838 to -1769bp (Figure 3.3.B.) displayed weak binding of both DMSO and TCDD nuclear extracts and this binding was lost when competition applied by either unlabeled specific (S) or non-specific (NS) oligonucleotides demonstrating that these bands are non-specific. Specific binding was only observed in the oligonucleotide spanning -2012 to -1942bp (Figure 3.3.A.). This region of the MMP-1 promoter contains three putative TCDD-responsive elements consisting of a NFkB-like, CCAAT and MITF sites (Figure 3.2.). Nuclear protein binding results observed here demonstrate that specific binding only occurs in the -2012 to -1942bp oligonucleotide which contains distal segments of the minimally TCDD-responsive 2011bp MMP-1 luciferase promoter and this binding does not increase due to TCDD treatment.

3.3.3. MMP-1 promoter binding involves multiple response elements

To elucidate elements responsible for specific binding of nuclear proteins in the proximal region of the MMP-1 promoter spanning 2012-1942bp, oligonucleotides of the same length were synthesized containing specific mutations in the cis-elements of interest and used in EMSA. Although all statistically significant responsiveness in luciferase assays is lost at 1989bp, two mutations involving potential TCDD-responsive elements CCAAT and MITF, residing 5 and 20bp respectively from the end of the 1989bp luciferase construct, were made to determine whether they also may contribute to TCDD-binding. The wild-type oligonucleotide (2012 wt) showed strong binding with both the DMSO control (C) and TCDD (TD) treatment (Figure 3.4.). Single site

mutation of the NFkB, CCAAT or MITF elements each resulted in a decrease in binding of the 2012-1942bp oligonucleotide. In a double NFkB and CCAAT mutant oligonucleotide binding was also reduced, however both specific and non-specific oligonucleotides were both able to successfully compete binding, suggesting that the affinity for the nuclear proteins for this oligonucleotide was diminished by the mutation of these elements. When the NFkB, CCAAT and MITF sites were all mutated in the same oligonucleotide, all binding was lost. These results demonstrate that multiple binding sites in the region of the MMP-1 promoter between -2012 to -1942bp are capable of binding nuclear proteins and that mutations of either NFkB, CCAAT or MITF elements result in a decrease of nuclear protein binding.

3.3.4. NFkB and CCAAT sites are required for minimal MMP-1 reporter transactivation by TCDD

In order to determine whether differences in promoter binding result in alterations of transactivation, 2011bp mutant MMP-1 luciferase constructs were synthesized containing the same mutations in the NFkB, CCAAT, MITF, NFkB/CCAAT and NFkB/CCAAT/MITF that were used in EMSA (Fig. 3.4.A.). A2058 cells were transiently transfected with one of the following 2011bp length MMP-1 pGL3 constructs; wild-type promoter (2011wt), NFkB-like mutant (NFkB), CCAAT/Enhancer Binding Protein site mutant (CCAAT), MITF site mutant (MITF), NFkB-like-CCAAT/Enhancer Binding Protein double mutant (N/C), and NFkB-like-CCAAT/Enhancer Binding Protein-MITF triple mutant (N/C/M). Mutation of NFkB, CCAAT and MITF binding sites alone all retained significant TCDD-responsiveness, albeit reduced in comparison to the wild-type construct (Figure 3.5.A). The data also suggest a role for the NFkB and

MITF binding sites, at -1997 and -1970bp respectively, to be involved in basal level production of MMP-1 as is seen in the significant reduction of basal level as compared to wild-type. Mutation of the NFkB site results in a 52% fold reduction of TCDD-induction as compared to the wild-type promoter (Figure 3.5.B.). Similarly, mutation of the CCAAT site at -1985bp yielded a 60% fold reduction. MITF mutation also alters MMP-1 induction by TCDD, but to a lesser extent, 21% fold reduction. In addition, both N/C and N/C/M mutations resulted in a loss of induction of promoter activity (Fig. 3.5.A.). These data indicate that both the NFkB-like and CCAAT binding sites are involved in TCDD-induced transactivation of the MMP-1 promoter and that MITF may also contribute to maximal TCDD-induction.

3.3.5 siAhr reduces TCDD-induced MMP-1 and CYP1A1 promoter activation

It has been previously shown that TCDD-induced MMP-1 expression in A2058 cells relies upon the AhR signaling pathway by using the chemical AhR antagonist, α-naphthoflavone (Villano et al., 2005). To confirm these findings and determine whether A2058 cells were transiently transfected with the minimal responsive 2011bp MMP-1 reporter construct alone or cotransfected with siRNA specific to AhR (siAhR) or a non-specific siRNA (Scrambled). Cells were treated with DMSO or TCDD for 24h and luciferase activity was analyzed. Cotransfection with siAhR resulted in an average reduction in TCDD-induced MMP-1 promoter activity of 3 fold. Scrambled control siRNA had no effect on TCDD-induction of the MMP-1 promoter (Figure 3.6.A.). Quantitative real-time PCR (qRTPCR) was used to confirm that siAhR knocked down transcript levels of AhR. AhR transcript levels were reduced by 64% in siAhR treated

cells as compared to non-treated cells confirming that siAhR is effective at lowering AhR transcript levels (Figure 3.6.B.).

To confirm that knock down of AhR transcript levels inhibits TCDD-dependent induction of the MMP-1 promoter, A2058 cells were cotransfected with siAhR and a known AhR-dependent, TCDD-inducible reporter construct, cytochrome P4501A1 (CYP1A1) promoter. CYP1A1 luciferase levels are induced approximately 9.2 fold by TCDD as compared to only 3 fold when co-transfected with siAhR and treated with TCDD (Figure 3.6.C). This 3 fold repression by siAhR matches the 3 fold repression of MMP-1 induction by TCDD supporting the argument that the reduction is result of inhibiting TCDD's effects upon the AhR signaling pathway. Taken together, these data show that TCDD-induced MMP-1 expression is dependent on the AhR signaling pathway in A2058 melanoma cells.

3.3.6 TCDD-induced MMP-1 expression requires changes in acetylation

Recent findings suggest that MMP gene expression can be mediated, at least in part, to changes in histone acetylation (Young et al., 2005). To investigate the role of chromatin remodeling on TCDD-induced MMP-1 gene expression A2058 cells were cotreated with histone deacetylase (HDAC) inhibitors, Trichostatin A (TSA) and Scriptaid for 24h. Total RNA was harvested and qRTPCR was performed using primers specific to MMP-1 and CYP1A1 (Table 2.1.). Cotreatment with Scriptaid resulted in a loss of TCDD-induced MMP-1 expression while a structurally similar, inactive negative control (Nullscript) retained significant induction (Figure 3.7.A.). TSA did not result in complete loss of MMP-1 induction, however it was significantly reduced (Figure 3.7.B.). TCDD induction of CYP1A1 was also inhibited by both Scriptaid (Figure 3.7.C.) and

TSA (Figure 3.7.D.) Both MMP-1 and CYP1A1 TCDD-induced expressions are affected by inhibition of histone deacetylation suggesting that transcription of theses genes relies not only upon transactivation of their promoters by direct binding of transcription factors, but may also require either recruitment of HDACs or histone acetyltransferases (HAT) resulting in changes in chromatin structure or acetylation status of transcription factors.

3.4. Discussion

Data presented here demonstrate that TCDD induction of MMP-1 is dependent on the AhR signaling pathway and multiple response elements in the MMP-1 promoter including; CCAAT, MITF and NFκB are involved in mediating TCDD induction. The data also show that acetylation events are required for both TCDD-induced MMP-1 and CYP1A1 expression suggesting that either epigenetic or transcription factor modification is required for AhR-dependent TCDD induction.

Previous data from the White laboratory demonstrated that MMP-1 expression could be inhibited through the use of a chemical antagonist of the AhR, α-naphthoflavone (Villano et al., 2005). Current opinions of the use of chemical inhibitors for specific targeting of singular pathway components lead to the utilization of siRNA technology to validate the above findings with α-naphthoflavone. Cotransfection of siRNA targeted to the AhR (siAhR) and the 2011bp MMP-1 luciferase promoter revealed that siAhR could suppress TCDD induction of MMP-1. Data presented here using siAhR agree with the previous published data from the White lab and further show that AhR signaling is required for TCDD-induced MMP-1 expression.

Nuclear protein binding and TCDD-induced MMP-1 promoter activity were both hindered by mutation of the CCAAT/enhancer binding protein (CCAAT) site located at -1985bp. CCAAT/enhancer binding proteins and peroxisome proliferator-activated receptors (PPAR) are important transcription factors involved in adipocyte differentiation which TCDD and the AhR pathway has been shown to affect. The conversion of fibroblasts to adipocytes can be stimulated in the absence of PPAR inducers when CEBPα was co-transfected indicating that CEBPα is sufficient for differentiation (Tontonoz et al., 1994). More recently, ERK signaling was found to promote adipogenesis by enhancing expression of both PPARγ and CEBPα in preadipocyte cells (Prusty et al., 2002). It has also been demonstrated that TCDD exposure mediates suppression of adipocyte differentiation through inhibition of the CCAAT/ enhancer binding protein-α (CEBPα) isoform (Liu et al., 1996) as well as through suppression of PPAR-γ1 (Hanlon et al., 2003). Hanlon et al. (2003) also showed that the suppression of PPAR-γ1 required synergistic activation of AhR as well as ERK kinase. Together, these data provide evidence that AhR activation by TCDD can modulate CEBP transcription factor protein binding resulting in profound biological effects. These studies lead us to speculate that TCDD activation of AhR in A2058 melanoma cells may result in alteration of CEBP binding ability to the MMP-1 promoter since mutation of the putative CCAAT binding site in the MMP-1 promoter did result in both a reduction of nuclear protein binding as well as a reduction of TCDD-induced MMP-1 promoter activity.

NFκB has been shown to be involved in many disease states that involve inappropriate activity of MMPs including; arthritis, neurodegenerative diseases and cancers (Tian et al., 2002). NFκB regulates cytokine, growth factor and oxidative stress

signals resulting in MMP expression and can be seen when cytokine, interleukin-1 (IL-1) induces MMP-1 through the use of NFκB and AP-1 elements (Barchowsky et al., 2000; Vincenti et al., 1998). Furthermore, Interleukin-1β induces MMP-1 expression in a NFkB- and ERK kinase-dependent fashion in human articular chondrocytes (Fan et al., 2006). In addition to regulating chondrocytes, responsiveness to MEKK1 overexpression in human synovial fibroblasts relies upon a NFkB and a CCAAT element located at -1997 and -1985bp of the MMP-1 promoter respectively where p65 and C/EBPβ are found to bind (Faour et al., 2006). There have also been reports of p65 and AhR physical interactions demonstrated by co-immunoprecipitation of a p65 homodimer with activated AhR (Tian et al., 1999) indicating that NFκB signaling may be involved in TCDD-mediated induction of MMP-1 in A2058 melanoma cells.

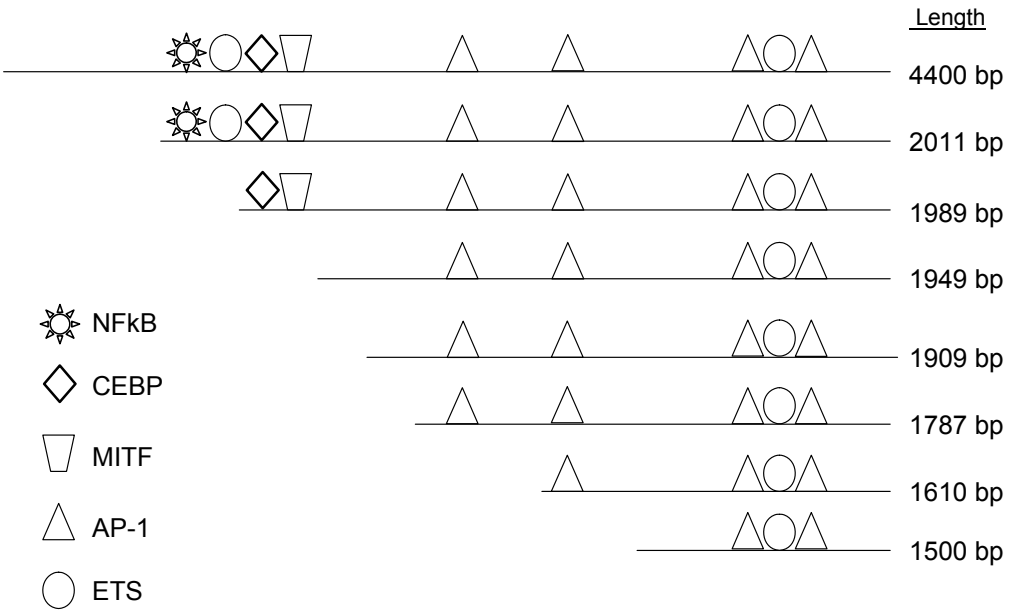
A MITF binding site located at -1970bp is also of interest due to its role as a master regulator of melanocyte differentiation (Levy et al., 2006). It's also known that reduced expression of MITF occurs during melanoma progression (Zhuang et al., 2007) suggesting that MITF may play a role in melanoma progression through the control of ECM degradation. Mutations in the MITF binding site resulted in reduced nuclear protein binding in EMSA; while in luciferase assays the MITF binding site contributes more to basal level production, retaining most of its TCDD-responsiveness when mutated. These data indicate that although MITF does affect nuclear protein binding, it's minimally involved in TCDD-induction of MMP-1. Although the MITF mutation does not drastically alter TCDD-induction of MMP-1 expression, it does significantly reduce the basal level of expression suggesting that MITF is involved in the basal expression of MMP-1 in malignant melanoma.

Epigenetic regulation of gene transcription involving alterations in chromatin structure via histone deactylation has been recently realized as a potential target for anti-tumor therapy (as reviewed (Boyle et al., 2005; Glozak and Seto, 2007). In regards to ECM deposition, histone deacetylase (HDAC) inhibitors have been shown to alter *in-vitro* invasion through a Matrigel substrate in A2058 cells (Kim et al., 2004b) and alter MMP-2 activity in CL-1 lung cancer cells (Liu et al., 2003). Another study has shown that increased MMP-1 expression and activity due to interleukin-1 α and Oncostatin M cotreatement in human chondrosarcoma cells and bovine nasal cartilage explants are affected by HDAC inhibitors. Furthermore, evidence of direct acetylation involving the transactivation potential of NFκB family members p65 and p50 (Quivy and Van Lint, 2004) and C/EBPβ (Musri et al., 2007) suggest that acetylation events may also be necessary for transcriptional activation at the NFκB and CCAAT sites of the MMP-1 promoter. Recent data revealed that CYP1A1 activation by the AhR/Arnt transcription factor complex requires the release of a histone deacetylase (HDAC1) (Schnekenburger et al., 2007). Acetylation events are also seen to affect AhR pathway members AhRR and Arnt through control of expression and trophpblast differentiation, respectively (Maltepe et al., 2005; Oshima et al., 2007). Data presented here indicate that HDAC inhibitors reduce TCDD-induced MMP-1 and the AhR-dependent CYP1A1 gene expression indicating that there is epigenetic or modification-dependent regulation of AhR-dependent MMP transcription.

In summary, data presented here demonstrate that a minimal TCDD-responsive promoter consisting of -2011bp contains an NFκB-like, a CCAAT and a MITF site that are all involved in maximal induction. Furthermore, nuclear protein binding is also

reduced when any of the three sites above are mutated in an oligonucleotide spanning -2012 to -1942bp of MMP-1 promoter. Inhibition of the minimal responsive MMP-1 promoter by small inhibitory RNA confirmed previous reports of AhR pathway inhibition upon MMP expression utilizing α–naphthoflavone. Lastly, AhR-dependent TCDD induction of MMP-1 and CYP1A1 expression were reduced after co-treatment with histone deacetylase inhibitors, TSA and Scriptaid, indicating involvement of acetylation for AhR-dependent MMP-1 expression. Taken together these data suggest a role for both NFκB and C/EBP transcription factors in mediating AhR-dependent MMP-1 expression and that acetylation is crucial either to MMP-1 availability or transcription factor activation.

A.



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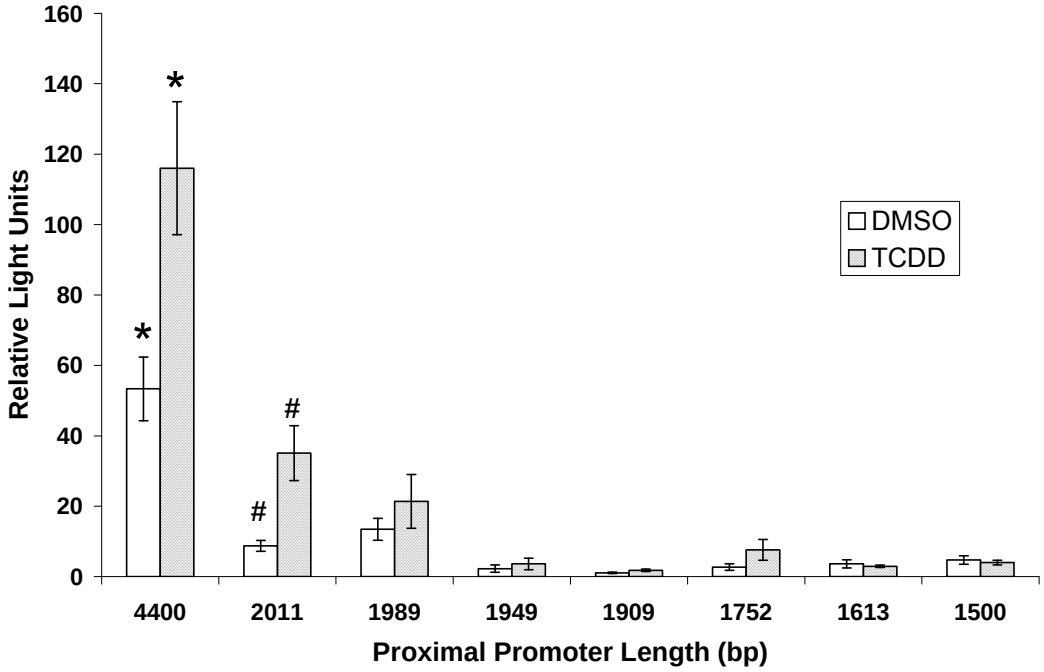
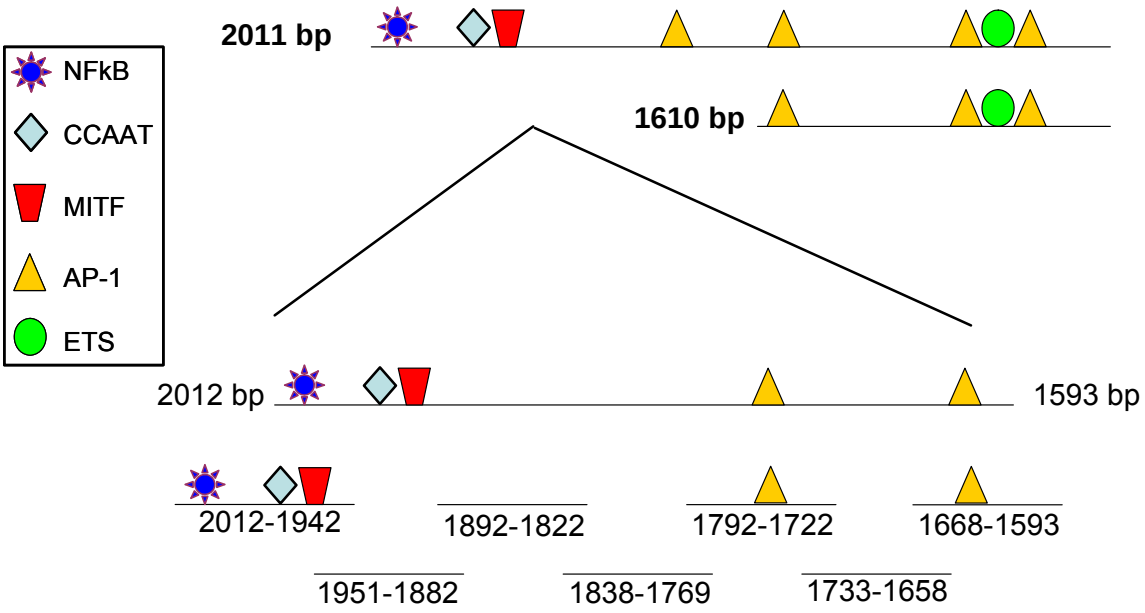


Figure 3.1. Schematic representation of the human MMP-1 promoter
The human MMP-1 promoter contains multiple putative TCDD-responsive elements in portions ranging from -4400 to -1500bp in length. Some known TCDD-responsive elements and their locations in MMP-1 luciferase reporter constructs are depicted above. (A) Nuclear Factor kappa B (NFkB), CCAAT Enhancer Binding Protein (CEBP), Microphthalmia-associated Transcription Factor (Mitf), Activator Protein-1 (AP-1) and polyoma enhancing activity-3/E26 virus (ETS) have been highlighted. B) A2058 cells were transiently cotransfected with proximal MMP-1 promoter containing luciferase constructs and phRL-TK renilla luciferase for 24hrs. Cells were treated with DMSO or TCDD (10^{-8}M) for 24h. Cell lysates were harvested 24 h after treatment and luciferase assays performed using the Promega Dual Luciferase Kit and detected on a Dynex luminometer plate reader. Data are presented as RLU (relative light units). All treatments and transfections were performed in sextuplet, and normalized to total protein as well as an internal transfection control (phRL-TK: Promega). Data shown are representative of three separate transfections. (* and # indicate significance using t-test ($p < 0.05$)

A.



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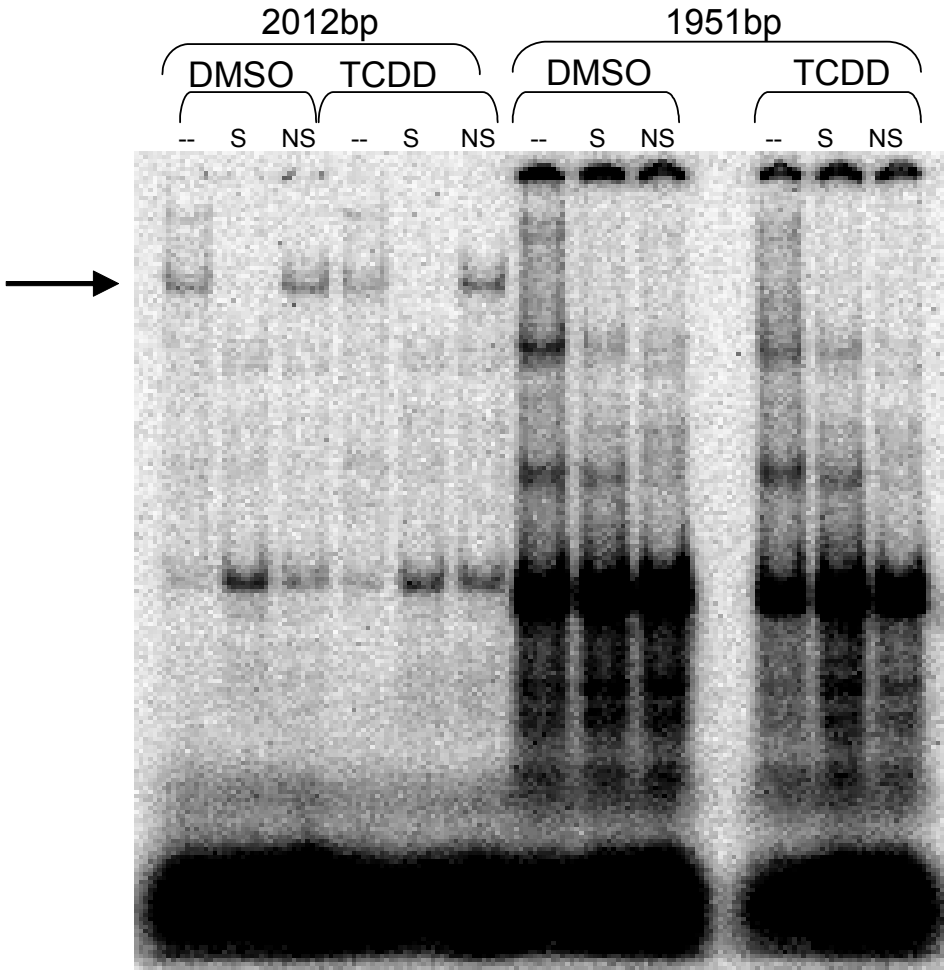
B.

MMP-1 Promoter Location	Oligonucleotide Sequence/Response Element Location	Length (base pairs)
-2012 to -1942bp	agggtgacgtcttag***gcaatt****tcc*tgt***ccaat***cacagatggt*ca**catg***ctgctttcctgagttaacctat *NFkB c-ETS CCAAT Mitf*	70
-1951 to -1882bp	Ttaacctattaactcacccttgtttcccaggcctcagtggagctaggcttgtcacgtcttcacagtgac	69
-1892 to -1822bp	Tcacagtgactagattccctcacagtcgagtatatctgccactccttgacttttaaaacatagtctatgt	70
-1838 to -1769bp	Aaaacatagtctatgttcaccctctaatatgaagagccccttttcactattttctttgtctgtgctggag	69
-1792 to -1722bp	tattttctttgtctgtgct***ggagtca***cttcagtggcaagtgttctttggtctctgccgcaccctccctct *AP-1*	70
-1733 to -1658bp	accctccctctgatgcctctgagaagaggatttccttttcgtgagaatgtcttcccattcttcttaccctcttga	75
-1668 to -1593bp	accctcttgaactcacatgttatgccacttagatgaggaaattgtagttaaataattagaaagata***tgactta***tc *AP-1*	75

Figure 3.2. Diagram of EMSA probes (A) Overlapping diagram of EMSA probes used to span 2012-1593bp of the MMP-1 promoter (B) Oligonucleotides sequences for EMSA and with putative binding sites underlined and bold.

A.



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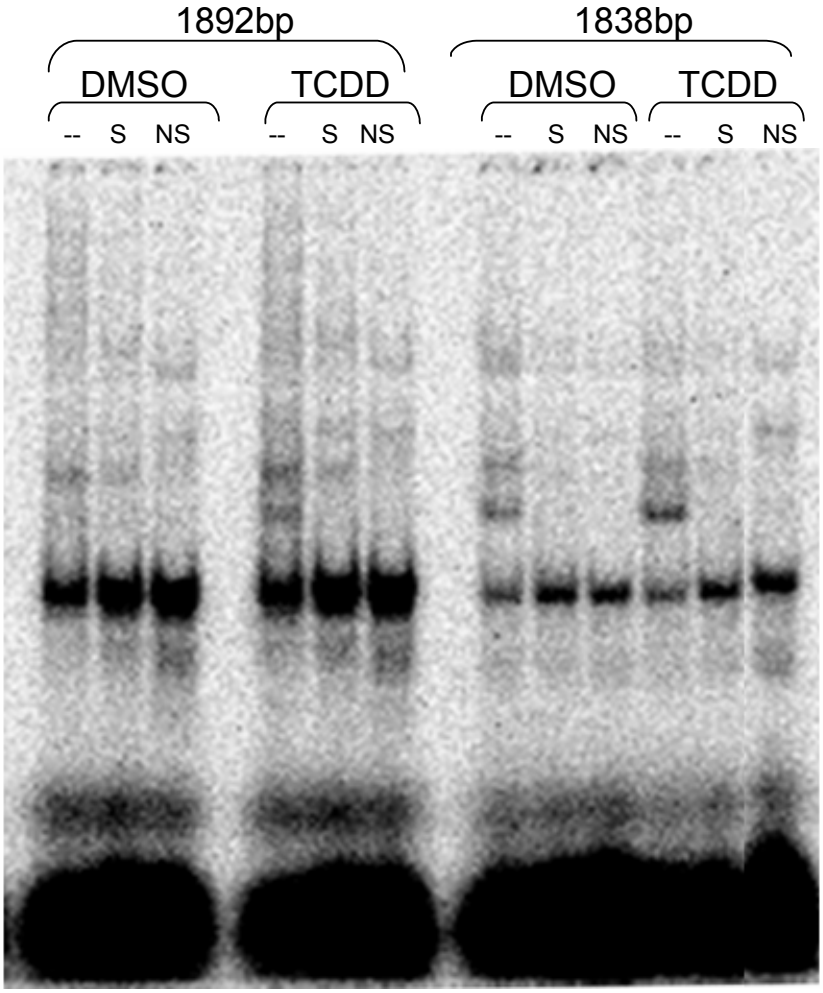

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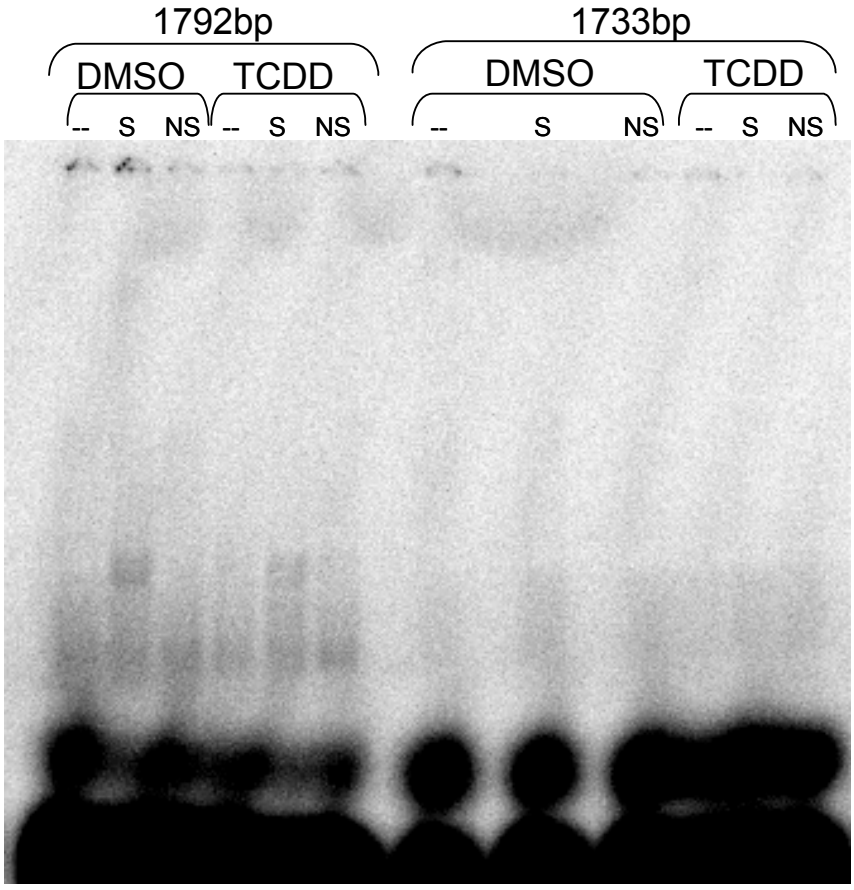

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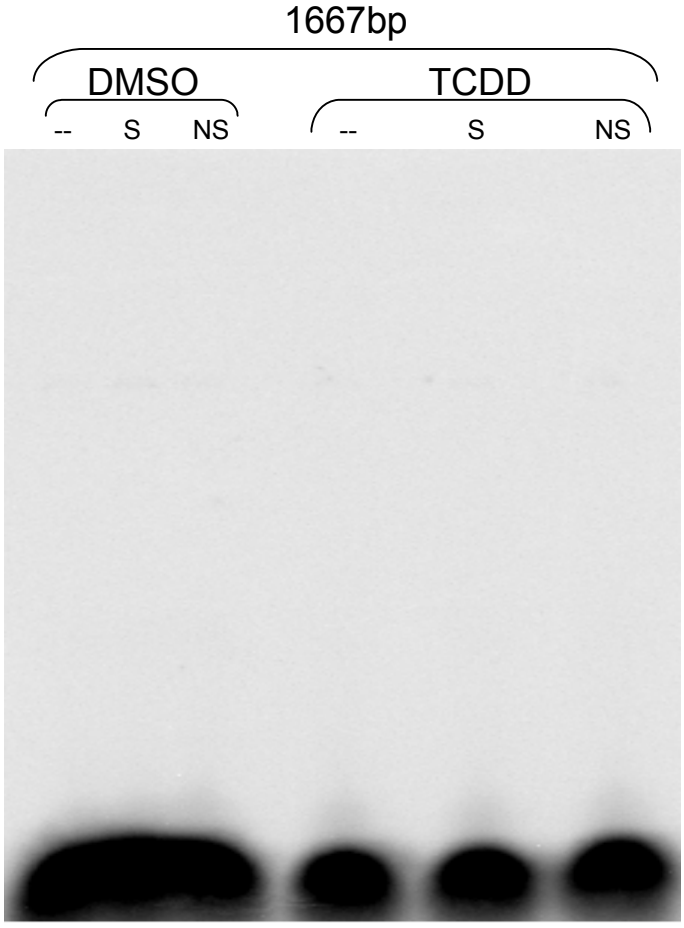

Figure 3.3. Nuclear proteins specifically bind to 2012-1942bp of the MMP-1 promoter Random labeled α-^{32}P dCTP oligonucleotides representing regions of the human MMP-1 promoter (figure 3.2.) were incubated in binding reactions with 10µg of nuclear extracts prepared from A2058 cells treated with DMSO or TCDD (10^{-8}M). Extracts were electrophoresed on a non-denaturing polyacrylamide TBE gel and exposed to a phosphorimager screen, and scanned using a PhosphorImager. Specific binding occurred only in the 2012bp oligonucleotide, indicated by an arrow. (--) α-^{32}P dCTP oligonucleotide; (S) α-^{32}P dCTP oligonucleotide + 100-fold excess of a non-radiolabeled "self" oligonucleotide; (NS) α-^{32}P dCTP oligonucleotide + 100-fold excess of a non-radiolableled "non-self" oligonucleotide.

A.

	NFkB	CCAAT	Mitf
(2012 wt)	agggtgacgtcttag**gcaat**ttcctgt**ccaat**cacagatggtca**catg**ctgctttcctgagttaacctat		
(2012 NFkB)	agggtgacgtcttagg***gt***a***cc***tcctgtccaatcacagatggtcacatgctgctttcctgagttaacctat		
(2012 CCAAT)	agggtgacgtcttaggcaatttcctgt***ggt***a***c***cacagatggtcacatgctgctttcctgagttaacctat		
(2012 Mitf)	agggtgacgtcttaggcaatttcctgtggtaccacagatggtc***t***c***ga***gctgctttcctgagttaacctat		
2012 (N/C)	agggtgacgtcttagg***gt***a***cc***tcctgt***ggt***a***c***cacagatggtcacatgctgctttcctgagttaacctat		
2012 (N/C/M)	agggtgacgtcttagg***gt***a***cc***tcctgt***ggt***a***c***cacagatggtc***t***c***ga***gctgctttcctgagttaacctat		

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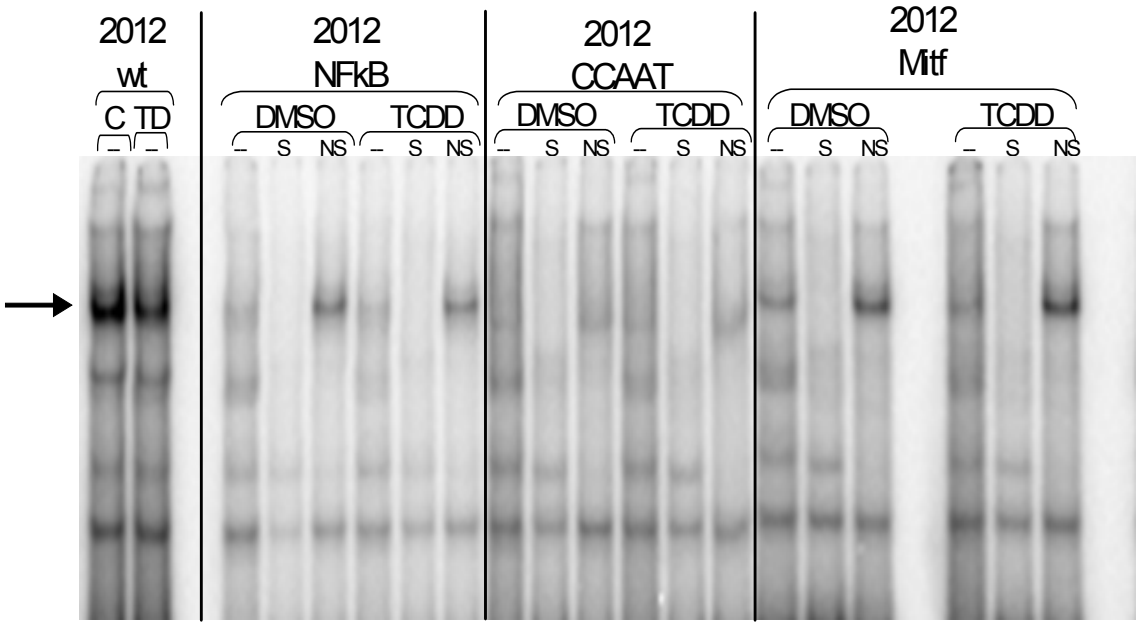
B.



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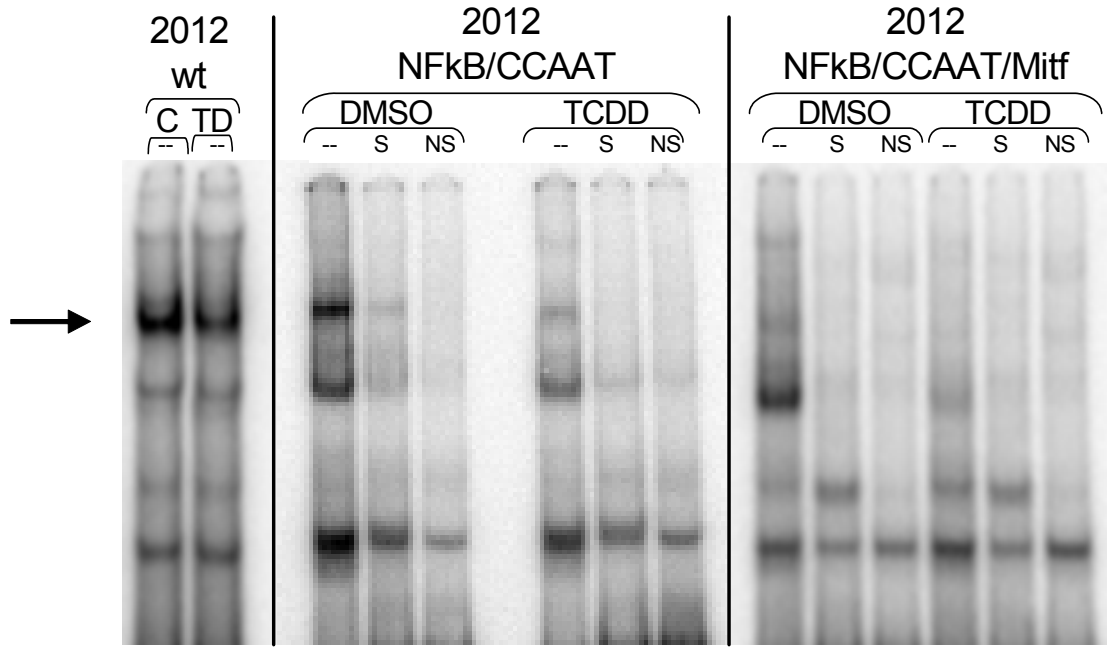
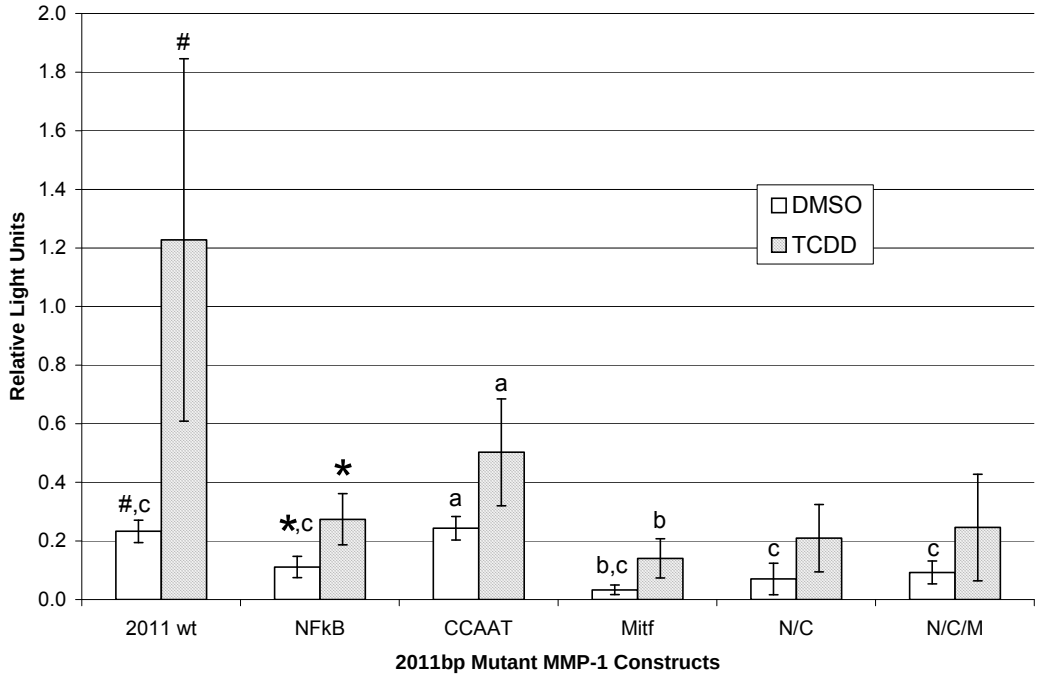


Figure 3.4. Mutational analysis of nuclear binding on 2012-1942bp promoter region
(A) Sequence alterations in the single, double and triple mutants of 2012-1942bp of the MMP-1 promoter consisting of single mutated NFkB-like, CCAAT, and Mitf binding sites, and (C) double mutant N/C (NFkB/CCAAT) and triple N/C/M (NFkB/CCAAT/Mitf). Binding sites are underlined and in bold print in the wild-type while altered base pairs in these sites are underlined and in bold and italicized print in the mutants. Wild-type and mutated oligonucleotides involving a random labeled α-^{32}P dCTP oligonucleotide were prepared and analyzed as in figure 3.3. (B) Single mutant oligonucleotides display reduced binding compared to wild-type. (C) The N/C double mutant binding was reduced and was no longer specific while the N/C/M triple mutant binding is reduced even more. Specific binding is indicated by an arrow. (--) α-^{32}P dCTP oligonucleotide; (S) α-^{32}P dCTP oligonucleotide + 100-fold excess of a non-radiolabeled "self" oligonucleotide; (NS) α-^{32}P dCTP oligonucleotide + 100-fold excess of a non-radiolableled "non-self" oligonucleotide.

A.



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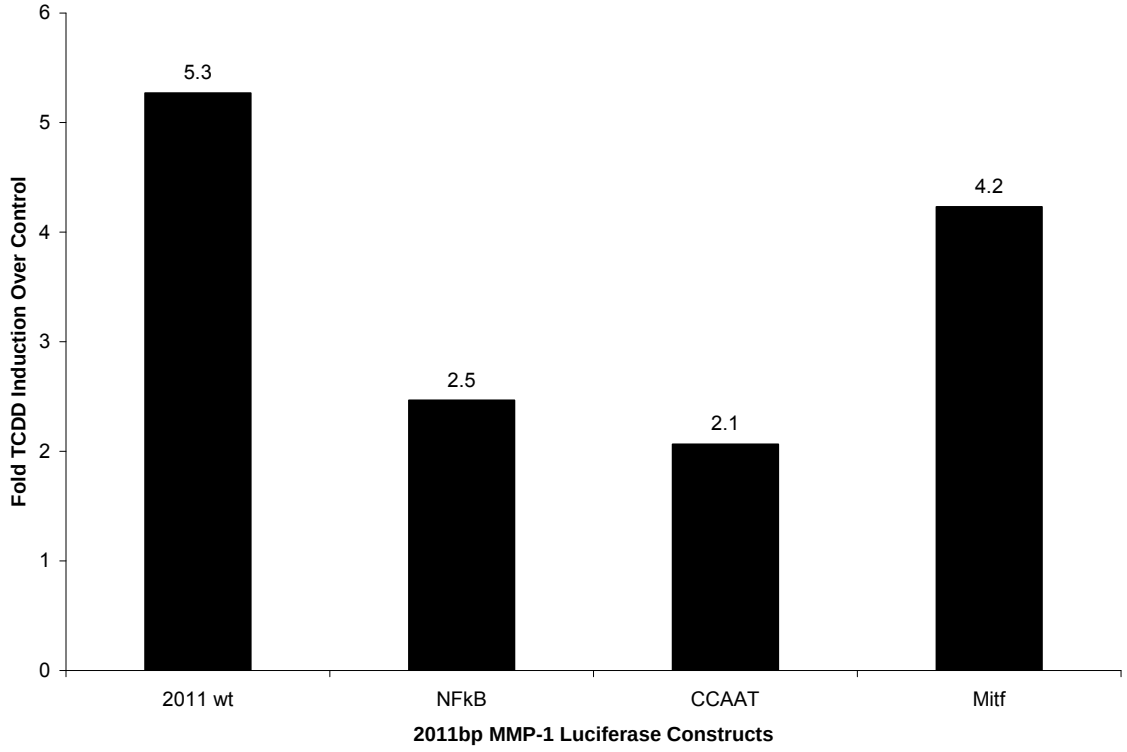
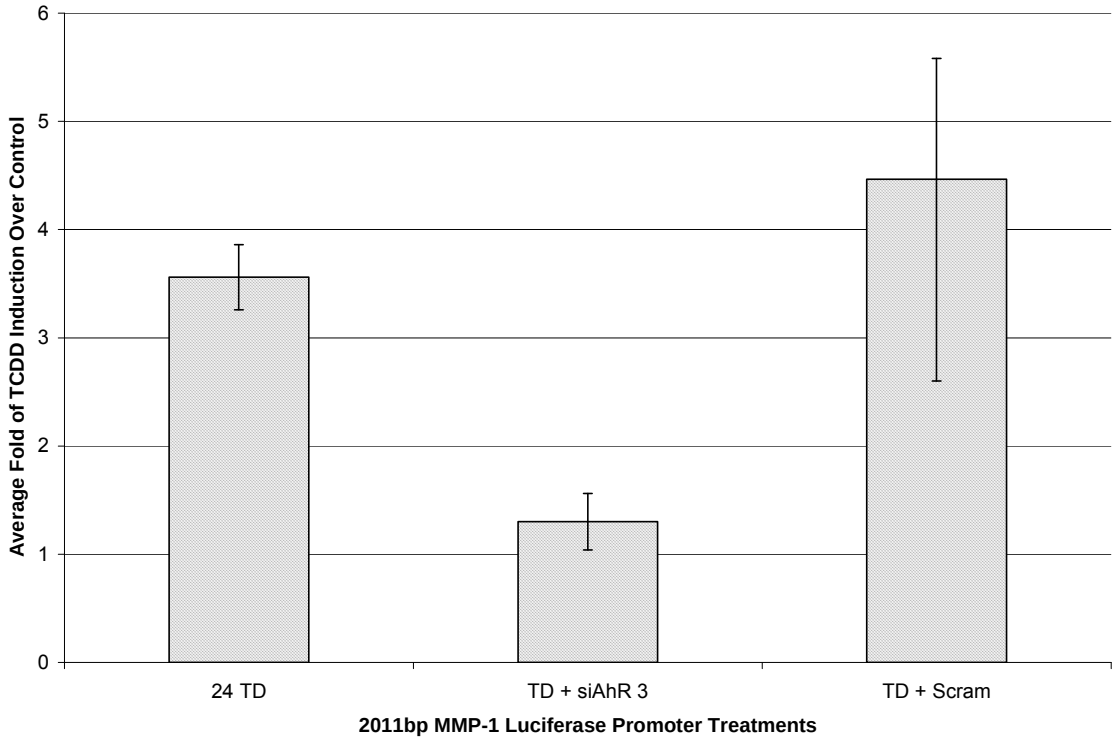


Figure 3.5. Mutational analysis of the MMP-1 minimal promoter Mutations in the 2011bp human MMP-1 promoter luciferase construct were synthesized identically to mutations used in electrophoretic mobility shift assays (figure 3.4.A.). A2058 cells were transiently cotransfected with proximal MMP-1 promoter containing luciferase constructs and phRL-TK renilla luciferase for 24hrs. Cells were treated with DMSO or TCDD (10^{-8}M) for 24h. Cell lysates were harvested 24 h after treatment and luciferase assays performed using the Promega Dual Luciferase Kit and detected on a Dynex luminometer plate reader. Data are presented as RLU (relative light units). All treatments and transfections were performed in sextuplet, and normalized to total protein as well as an internal transfection control (phRL-TK: Promega). (A) #, *, a, and b were marked significance between DMSO and TCDD treated samples while c designates significance between basal levels of various construct lengths using t-test ($p < 0.05$). (B) 2011bp wilt-type and mutant luciferase constructs from figure 3.5.A. are expressed in fold of TCDD-induction over DMSO levels.

A.



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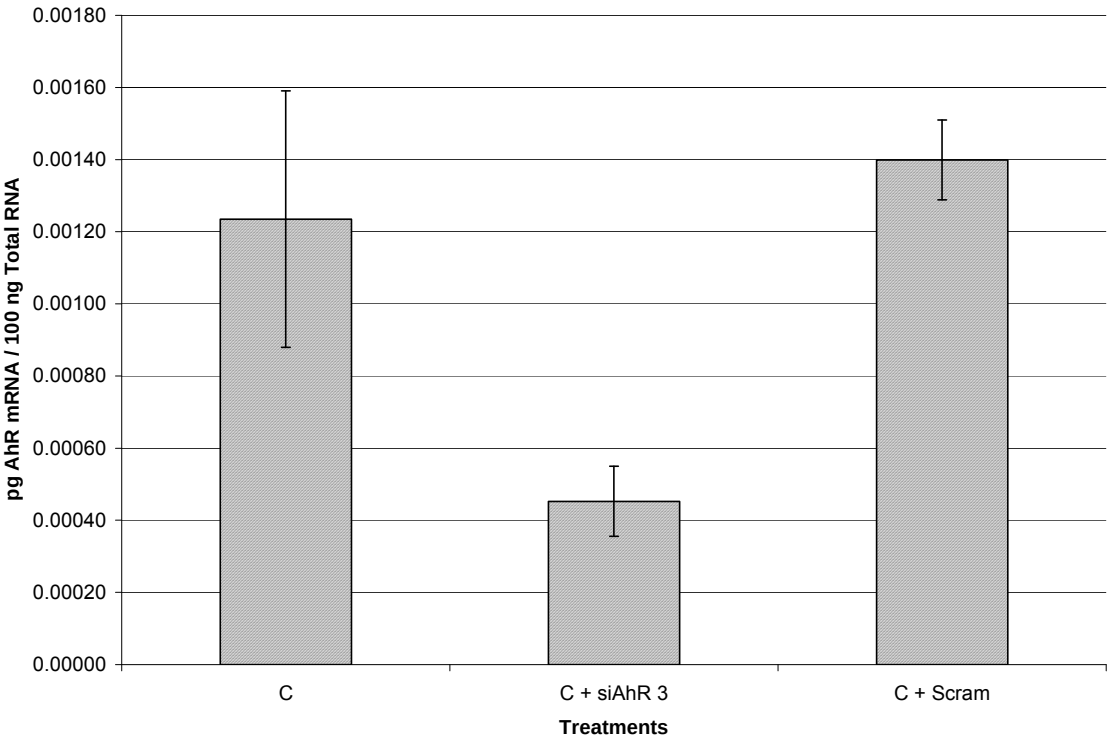

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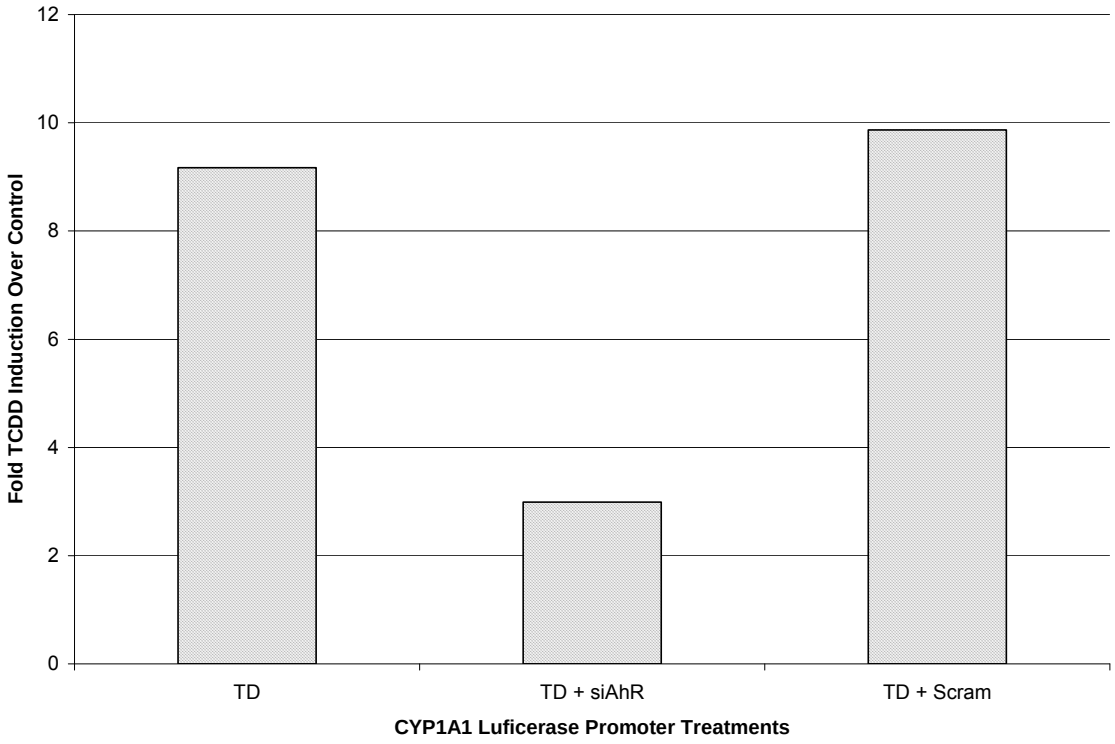
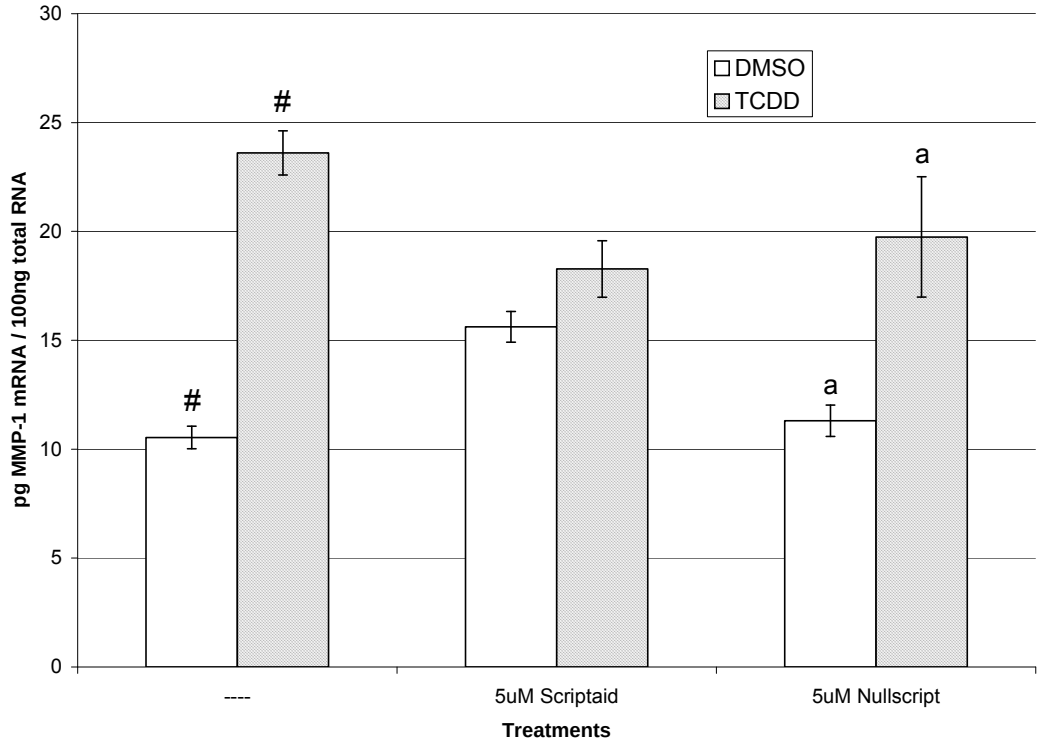


Figure 3.6. siAhR inhibits TCDD-induced promoter activation A2058 cells were transiently cotransfected for 24h with luciferase reporter constructs, alone and with siRNA specific to AhR (siAhR), or with a non-specific siRNA (Scram). Cells were treated with DMSO or TCDD (10^{-8}M) for 24h. Cell lysates were harvested 24 h after treatment and luciferase assays performed using the Promega Dual Luciferase Kit and detected on a Dynex luminometer plate reader. (A) 2011bp MMP-1 luciferase promoter data is presented as the average fold of TCDD over control of three separate experiments. (B) A2058 cells were transiently transfected with siAhR for 24h and treated with DMSO or TCDD (10^{-8}M) for 24h. Total RNA was harvested and expression levels of AhR were analyzed using q-RT-PCR. (C) CYP1A1 luciferase promoter was cotransfected with siAhR as in (A) and data is presented as fold of TCDD over control.

A.



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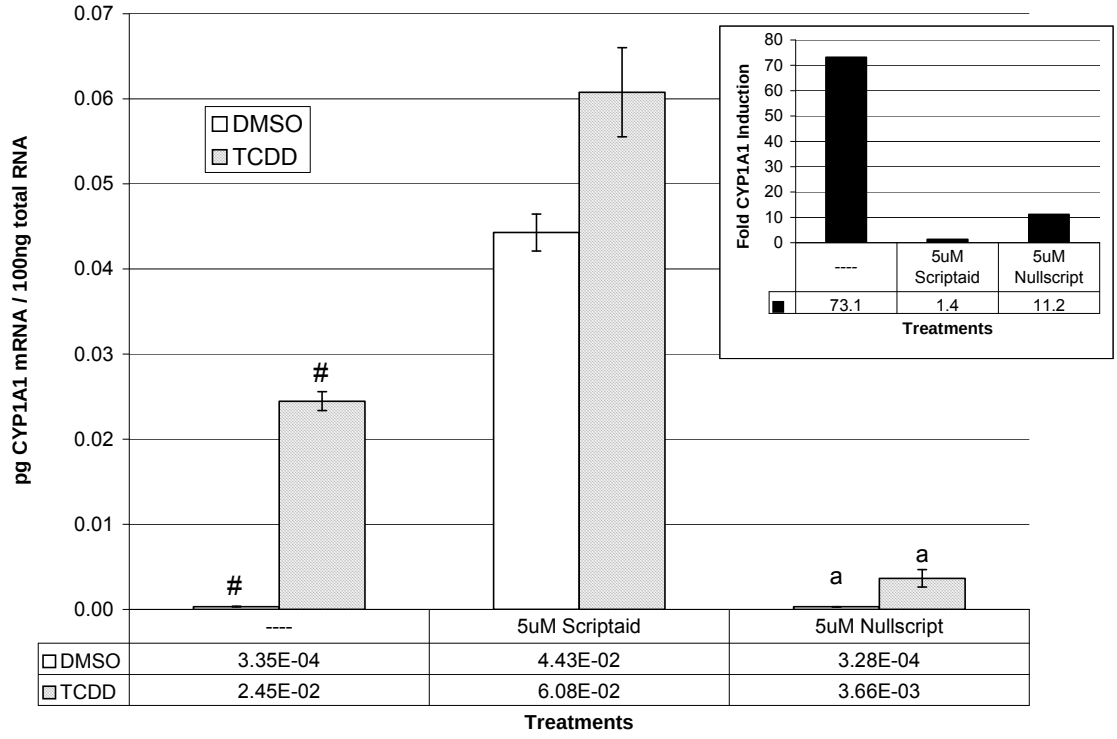

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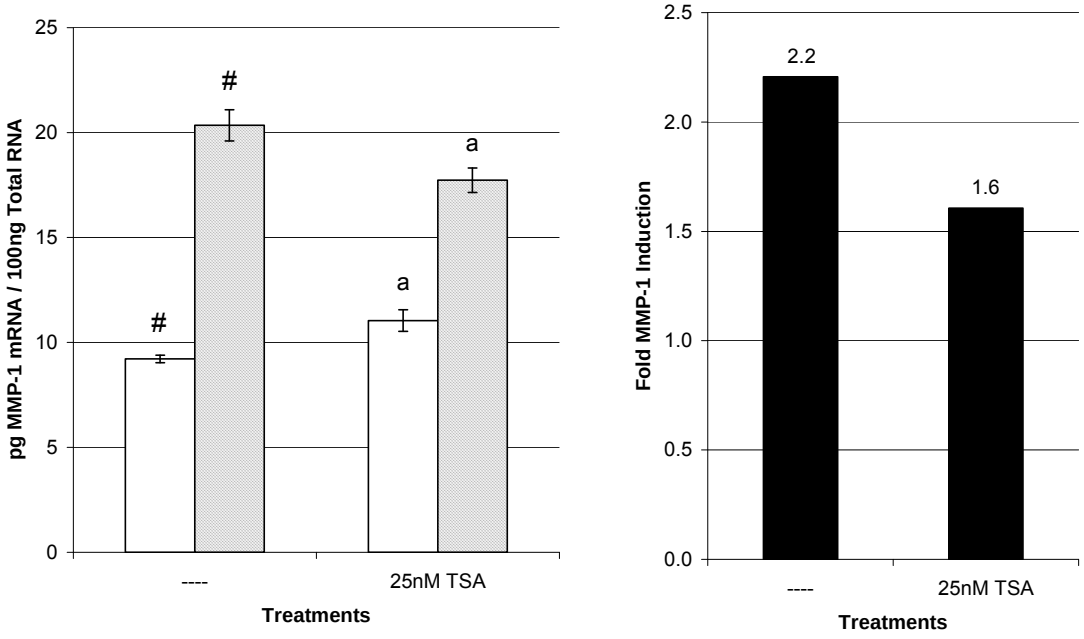

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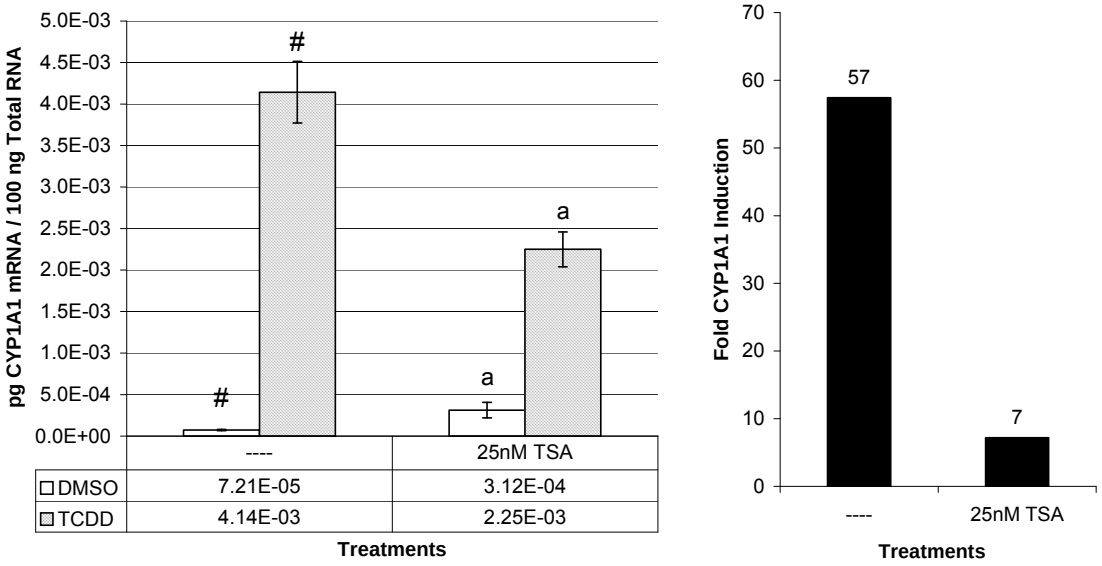


Figure 3.7. Histone deacetylase inhibitors alter TCDD-induced expression A2058 cells were cotreated with histone deacetylase inhibitors Scriptaid (5μM) and negative control Nullscript (5μM) for 24h, total RNA was harvested and (A) MMP-1 and (B) CYP1A1 transcript levels were analyzed via q-RT-PCR. A2058 cells were also cotreated with another histone deactylase inhibitor, trichostatin A (TSA, 25nM) for 24 and transcript levels of (C) MMP-1 and (D) CYP1A1 were analyzed as above. (∗, # ,a denote statistical significance between like-marked treatments ($p<0.05$)).

4.0 THE RAS/RAF SIGNALING PATHWAY IS REQUIRED FOR AHR-INDUCED EXPRESSION OF MATRIX METALLOPROTEINASES (MMP) IN MELANOMA CELLS

4.1. Abstract

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent ligand of the aryl hydrocarbon receptor (AhR) in humans. TCDD exposure results in many pathological lesions including wasting syndrome, inflammation chloracne and cancer. In addition to its effect on AhR, TCDD exposure is known to result in the activation of various other signaling pathways such as PI3 kinase, Src kinase, as well as p38 and ERK MAP kinase pathways. Data from the White laboratory demonstrates that activation of the AhR pathway by TCDD results in increased expression and activity of several matrix metalloproteinases (MMPs). Matrix metalloproteinase (MMP) expression and activity are essential for tissue remodeling and cellular migration, and are inappropriately expressed in diseases such as tumor metastasis. The goal of these studies is to determine whether other signaling pathways are involved in modulating AhR-induced MMP expression. To investigate contributions of intracellular signaling pathways on TCDD/AhR-induced MMP-1 expression, we utilized chemical inhibitors specific to PI3, Src, p38 and ERK kinase pathways. The data presented here demonstrate that Ras/Raf signaling and potentially the p38 signaling pathways are involved in TCDD-induced mRNA expression of MMP-1, -2, and -9. These data also examine the effect of Ras/Raf signaling inhibition on the expression of the endogenous MMP inhibitors, tissue inhibitors of metalloproteinase TIMP -1 and -2.

4.1. Introduction

The aryl hydrocarbon receptor (AhR) is member of the basic Helix-Loop-Helix Per-Arnt-Sim (bHLH PAS) domain family of transcription factors, and was originally identified as the receptor for polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Legraverend et al., 1982). Recently data has shown that the AhR can also be activated by alternative ligands (Bohonowych et al., 2008; Denison et al., 2002; Helferich and Denison, 1991; Peters et al., 2006). AhR activation by TCDD can activate the transcription of many types of down-stream target genes such as: members of phase I xenobiotic metabolism (CYP450), cytokines, growth factors, transcription factors, as well as matrix metalloproteinases (Fujii-Kuriyama et al., 1992; Murphy et al., 2004; Sutter et al., 1991; Villano et al., 2005; Watson and Hankinson, 1992; Yin et al., 1994).

In addition to activation of AhR, TCDD exposure also alters the activation of a variety of other second messenger pathways in mammalian cells. The src tyrosine kinase family member c-src has been shown to associate with the cytosolic AhR complex and is activated upon TCDD binding to the AhR in both guinea pig and mouse models and (Enan and Matsumura, 1996; Kohle et al., 1999). Src knockout mice were also recently used to prove that TCDD-induced wasting syndrome is Src-dependent (Vogel et al., 2003). In humans, increased Src kinase activity is also seen in mammary cells following TCDD treatment (Park et al., 2007; Park et al., 2004). TCDD is also known to affect the PI3 kinase pathway. One example is seen when the suppression of PI3 kinase by LY294002 results in inhibition of TCDD-induced CYP1A1 levels as well as preventing TCDD-induced growth of human mammary cells (Guo et al., 2000; Tannheimer et al.,

1998). Indications of requirements of TCDD-induced CYP1A1 expression appeared to be blocked by use of a p38 inhibitor and results in cytoplasmic localization of the AhR (Shibazaki et al., 2004a; Shibazaki et al., 2004b). Lastly, the ERK MAP kinase pathway has also been shown to be involved in modulation of AhR nuclear localization and inhibition of ERK kinase results in ligand-independent AhR nuclear translocation and stabilization while not resulting in CYP1A1 transactivation (Chen et al., 2005).

Intracellular signaling resulting in ERK phosphorylation has an effect on AhR function in many cell types (Hanlon et al., 2003; Tan et al., 2004), but is of great interest in melanoma where NRas and BRaf mutations are prevalent and result in constitutive signaling via the Ras/Raf MAP kinase signaling pathway (Davies et al., 2002; Pollock et al., 2003; Satyamoorthy et al., 2003; Yazdi et al., 2003). The most common activating mutation involves the substitution of a glutamic acid for a valine at position 600 in the kinase domain of BRaf (BRaf ^{V600E}) (Davies *et al.*, 2002). Although this mutation alone is not sufficient to induce invasion, invasive melanoma cell lines containing this mutation do exhibit elevated expression levels of MMP-1 under the control of the ERK pathway (Brauchle et al., 2000; Huntington et al., 2004; Yazdi et al., 2003).

Most of the mortalities caused by melanoma are a result of distant metastases which require extracellular matrix remodeling (Hofmann et al., 2005) and previous studies indicate that polycyclic and halogenated hydrocarbons (PAH/HAH), may stimulate melanoma progression by altering matrix remodeling enzyme expression levels and activities (Villano et al., 2005). In order for melanoma cells to gain motility and traverse the basement membrane, they must degrade extracellular membrane

(ECM) components. Matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent endopeptidases classified by ECM substrate specificity. MMPs are expressed as latent and inactive proenzymes whos cleavage and activation enable normal tissue remodeling events such as wound healing and embryogenesis; however, inappropriate expression is involved in disease states such as arthritis and cancer progression (Chakraborti *et al.*, 2003; Visse and Nagase, 2003; Wernicke *et al.*, 2006). Degradation of ECM components also can lead to release or activation of latent extracellular signaling molecules contained within the intact matrix (as reviewed, (McCawley and Matrisian, 2000)). MMP expression has been shown to correlate with the invasive potential of melanoma and is a marker for unfavorable clinical prognosis (MacDougall *et al.*, 1995; Vaisanen *et al.*, 1996). The collagenase, MMP-1 and gelatinases, MMP-2 and MMP-9 are essential proteases that enable migration *in vitro* through specified substrates (Durko et al., 1997) (Villano et al., 2005) and are also expressed in the tumor microenvironment (Hofmann et al., 1999; Itoh et al., 1999; Nikkola et al., 2002). MMP activity can be regulated by stoichiometric interactions with the physiological protein inhibitors called tissue inhibitors of matrix metalloproteinases (TIMPs). There are four known TIMPs (1-4) and they function by binding the catalytic domain of MMPs rending them unable to metabolize ECM substrates (Gomez et al., 1997). TIMP regulation of MMP activity has been demonstrated by TIMP-1 inhibition of the activation of latent pro form of MMP-9 (pro-MMP-9) as well as MMP-1 (Airola et al., 1999; Goldberg et al., 1992). Further, studies involving TIMP-2 and MMP-2 have revealed that TIMP-2 along with MT1-MMP play a pivotal role in pro-MMP-2 activation (Butler et al., 1998; Strongin et al., 1995). The

balance between TIMP and MMP expression is therefore critical in controlling ECM degradation.

In this report it's demonstrated that Ras/Raf signaling is required for TCDD-mediated expression of MMPs and TIMPs. Ras/Raf signaling is essential for TCDD-induced mRNA expression of MMP-1, -2 and -9 as well as activity of MMP-1. TIMP-2 was also affected by Ras/Raf inhibition resulting in an overall increased expression. Furthermore, a decrease of ERK phosphorylation indicates that reduction of AhR-induced MMP expression by inhibition of the p38 pathway is also dependent upon Ras/Raf signaling.

4.3. Results

4.3.1. Role of various signaling pathways on MMP-1 promoter induction

Previous data show that TCDD-induced MMP-1 expression is AhR-dependent, however recent data suggest that other signaling pathway may be involved in TCDD/AhR activation. To investigate the impact of kinase signaling pathways on TCDD/AhR activation of MMP-1 expression we cotreated BRaf ^{V600E} mutated A2058 melanoma cells with specific pathway inhibitors (Figure 4.1.A.) and examined their effects on TCDD/AhR activation of MMP-1 promoter activity. Inhibition of the Src signaling pathway by chemical inhibitor PP2 did not reduce TCDD-induced MMP-1 promoter activity, while inhibition of both PI3 (LY294002) and p38 (SB203580) kinase pathways resulted in a 2.3 and 2.7 fold decrease in TCDD-induced MMP-1 promoter activity (Figure 4.1.B.). The only complete inhibition of TCDD-induced MMP-1 promoter activity occurred when A2058 cells were co-treated with the MEK 1/2

inhibitor, U0126. These data indicate that TCDD-induced MMP-1 expression is dependent on Ras/Raf/MEK/ERK signaling inhibitor. Further, as SB203580 and LY294002 inhibitors both partially inhibited MMP-1 promoter activation, these data suggest that maximal TCDD/AhR-activation of MMP-1 promoter activity also involves the PI3 and p38 MAP kinase signaling pathways.

4.3.2. Inhibition of TCDD-induced MMP-1 mRNA expression by inhibition of Ras/Raf or p38 MAP kinase pathways

To demonstrate that the effects of the inhibitors on MMP-1 promoter activation lead to similar effects on mRNA production, we examined TCDD-induced MMP-1 mRNA expression in the presence of the Ras/Raf and the p38 MAPK pathway inhibitors. I focused on these two pathways and not the PI3 kinase pathway because data in chapter 5 showed that PI3 inhibition is not AhR-dependent (Figure 5.2.A.). Inhibition of Ras/Raf signaling with U0126 resulted in a marked reduction of MMP-1 expression at all concentrations tested, as well as a reduction of TCDD-responsiveness (Figure 4.2.A.). Co-treatment with TCDD and the p38 MAP kinase inhibitor SB203580 did reduce TCDD/AhR induced MMP-1 expression, although there is still a statistically significant increase (Figure 4.2.B.). These data indicate that Ras/Raf signaling is essential for TCDD-induced expression of MMP-1 and suggest that p38 MAP kinase may play a less significant role.

4.3.3. Collagenase activity is repressed by both Ras/Raf and p38 inhibition

The above data indicate that TCDD-induced MMP-1 mRNA expression and promoter induction are modulated by Ras/Raf signaling and that to a certain extent, promoter induction is also influenced by the p38 MAPK pathway (Figure 4.1., 4.2.A, 4.2.B). To determine whether the inhibition seen at the level of gene expression corresponds to changes in MMP enzymatic activity, we performed an *in vitro* zymography assay for collagenase activity. Conditioned media was collected from samples 24h post-treatment with TCDD, control or co-treated with U0126 or SB203580. These data show a 2.5 fold increase in collagen type I degradation due to TCDD treatment (Figure 4.2.C.). Co-treatment of cells with TCDD plus U0126 or SB203580 abrogated TCDD-induced collagenase activity in A2058s. Therefore, inhibition of the Ras/Raf and p38 MAP kinase pathways also results in reduction of TCDD/AhR-induced collagenase activity.

4.3.4 siRNA inhibition of BRaf

Approximately seventy percent of all human melanoma tumors have a mutation in BRaf (V600E) that results in a constitutively active Ras/Raf signaling pathway (Davies *et al.*, 2002). The A2058 melanoma cells contain this mutation, and other laboratories have demonstrated that basal expression of MMP-1 is a target of the increased Ras/Raf pathway activity (Huntington et al., 2004; Tower et al., 2002). To specifically investigate the role of BRaf in TCDD-induced signaling we used siRNA to selectively knock-down BRaf levels in A2058 cells. Transient transfection of A2058 cells with siRNA targeted to Braf significantly reduced levels of Braf mRNA while a negative control consisting of

random siRNA not homologous to any known human sequence (scrambled) had no significant effect (Figure 5A). A2058 cells were transiently co-transfected with 2011bp of MMP-1 promoter and siRNA to BRaf (siBRaf) or a negative control (scrambled). TCDD-induced MMP-1 promoter induction was reduced 2-fold (5 vs 2.5 fold) when siRNA targeted to BRaf was co-transfected into the cells along with the MMP-1 luciferase construct (Fig 5B**).** These data confirm the U0126 inhibitor data indicating that inhibition of Ras/Raf kinase activation, through down-regulation of the BRaf (V600E) kinase, reduces TCDD-induced MMP-1 expression and activity.

4.3.5 Alterations in TCDD-induced MMP-2 and -9 expression due to Ras/Raf or p38 pathway inhibition

In order to examine whether TCDD-induction of other MMPs is also dependent on Ras/Raf signaling, we utilized the MEK1/2 inhibitor (U0126) and the p38 inhibitor (SB203580) and examined expression of MMP-2 and MMP-9. Previous data from the White lab indicate that MMP-2 and -9 are known to be induced by TCDD in A2058 cells (Villano et al., 2005). The multifunctional gelatinases are often over-expressed in melanomas(Hofmann et al., 2000a). Cells were pre-treated with MAPK inhibitors 30min prior to co-treatment with vehicle control or TCDD and total RNA was used as template for qRTPCR using primers specific for MMP-2 and MMP-9 (Table 2.1.).

Inhibition of both Ras/Raf (U0126) and p38 signaling (SB203580) lead to a statistically significant increase in MMP-2 basal expression, indicating an inhibitory role for these pathways in MMP-2 expression (Figure 4.4.A.). However, there is no significant alteration in TCDD-induced MMP-2 expression between co-treated samples

with inhibitor and TCDD, or samples treated with TCDD alone. Therefore, neither Ras/Raf nor p38 are required TCDD induced MMP-2 expression.

The modulation of MMP-9 expression by Ras/Raf and p38 signaling is dissimilar to that observed for MMP-2 expression. TCDD treatment results in a three-fold induction of MMP-9 expression compared to solvent control (Figure 4.4B.) When either the Ras/Raf or p38 kinase pathways are inhibited, there is no significant TCDD-induced expression of MMP-9, similar to our findings with MMP-1. MMP-9 basal levels are not altered by inhibition of either pathway indicating that kinase signaling effects TCDD-induced MMP-2 and -9 expression differently in A2058 cells.

4.3.6. Tissue inhibitors of matrix metalloproteinases (TIMPs) are affected by inhibition of Ras/Raf signaling

Tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenous inhibitors of MMP enzymatic function and are essential for controlled matrix remodeling (as reviewed (Hofmann et al., 2000a)). Dysregulation of MMP/TIMP ratio can lead to enhanced matrix degradation by uninhibited MMPs or conversely lead to its inhibition through high levels of TIMPs as compared to MMPs. I therefore analyzed TIMP expression in response to TCDD treatment in the presence and absence of Ras/Raf inhibitor, U0126.

Data show for the first time that expression of TIMP-1 and TIMP-2 are induced by TCDD treatment in A2058 melanoma cells (Figures 4.4.C. and 4.4.D.). TIMP-1 is significantly induced 2.3 fold by TCDD and is still remains induced upon co-treatment with the MEK1/2 inhibitor, U0126 (Figure 4.4.C.). There isn't a statistically significant difference between basal levels or TCDD treated levels with or without U0126,

indicating that Ras/Raf inhibition has no effect on TCDD-induced TIMP-1 expression. TIMP-2 was significantly induced by TCDD treatment (Figure 4.4.D.) Although inhibition of MEK1/2 did not alter TCDD-mediated induction of TIMP-2, it did have an effect on overall expression. The basal level of TIMP-2 significantly increased upon treatment with U0126 as did the level of TCDD-induced expression. These data suggests that Ras/Raf signaling may be involved in repression of TIMP-2 expression and that upon treatment with U0126 that repression is released. Interestingly a similar increase in basal expression is observed with MMP-2 after U0126 treatment of cells suggests that repression of expression may be similarly regulated (Figure 4.4.A.) Taken together TCDD-induction of two gelatinases, MMP-2 and MMP-9 depends on both Ras/Raf and p38 signaling. Conversely, TIMP-1 and TIMP-2 TCDD-induced expression are both unaffected by Ras/Raf inhibition.

4.3.7. ERK and p38 levels of phosphorylation in response to TCDD treatment

To determine the effect of TCDD on Ras/Raf and p38 MAPK signaling in A2058s, the phosphorylation status of two terminal proteins in these signaling pathways, ERK and p38, respectively, was investigated. TCDD treatment did not result in any change in ERK phosphorylation (pERK) (Figure. 4.5.A.). To ensure that ERK phosphorylation was the result of Ras/Raf signaling in the A2058 melanoma cells, pERK levels were determined following co-treatment with U0126, which resulted in complete loss of pERK. Interestingly, inhibition of p38 MAPK pathway by SB203580 also partially inhibited pERK levels, suggesting that there is cross talk between these MAP kinase pathways as previously seen in human umbilical vein endothelial cells at the level

of MEK activation (Houliston *et al.*, 2001). Phosphorylated p38 levels were undetectable in control or TCDD-treated samples at all time point tested (Figure 4.5.B.), This is supported by prior reports of low levels of p38 phosphorylation in A2058 cells (Benbow *et al.*, 2002). Lack of discernable phosphorylation suggests that activation of the p38 MAPK is not the primary mode of action affected when p38 inhibitor, SB203580, modulates TCDD-induced MMP production.

4.4. Discussion

These data demonstrate that the AhR- and Ras/Raf signaling pathways cooperate to mediate TCDD-induced expression of MMPs and their physiological inhibitors, TIMPs, in melanoma cells. Furthermore, these data show that although AhR activation by TCDD does not alter ERK phosphorylation, ERK inhibition through the use of either a MEK1/2 or p38 inhibitor results in a reduction in phosphorylated ERK. This suggests that p38 signaling influences the Ras/Raf signaling pathway. However, data presented here show no evidence of p38 phosphorylation (pp38) and therefore no visible evidence supporting a role for p38 kinase repression. Due to a lack of pp38 in A2058 melanoma cells, the resulting inhibition of MMP-1 expression by the p38 inhibitor, SB203580, is proposed to result from SB203580 acting upon a Ras/Raf signaling pathway constituent directly.

Over 70 percent of all human melanomas contain an activating mutation in the BRaf molecule (BRaf ^{V600E}) resulting in elevated kinase activity (Davies *et al.*, 2002), and this mutation affects the basal levels of MMP-1 in other melanoma cell lines (Benbow et al., 2002; Huntington et al., 2004; Tower et al., 2002). Interestingly, this mutation is not

limited to skin cancers, and is also found in ovarian (30%), thyroid (30%), and colorectal cancers (15%) (Frasca et al., 2008; Preto et al., 2008; Ueda et al., 2007). Data generated here extend these findings to not only include increased basal levels of MMP-1 in BRaf ^{V600E} containing melanoma cells, but also shows that TCDD-mediated expression and activity of MMP-1 as well as induction of other ECM remodeling proteins require Ras/Raf signaling. The Ras/Raf signaling pathways therefore may influence melanoma progression in the presence of environmental contaminants.

Previous findings from the White laboratory demonstrate that TCDD-induced MMP-1 expression in melanoma cells is restricted to the most invasive cell line used in the study, the A2058 line (Villano *et al.*, 2005). Although, normal human melanocytes and less invasive melanoma cell lines were responsive to TCDD/AhR, as demonstrated by activation of CYP1A1 and CYP1B1, no induction of MMPs was observed. Interestingly, a similar situation has been observed in breast cancer cell lines, where AhR expression correlates with invasive characteristics (Schlezinger *et al.*, 2006). One difference between the A2058 melanoma cells and the normal melanocytes, is that the A2058 cells contain an activating mutation in the BRaf gene that changes a valine residue to a glutamate (BRaf ^{V600E}) and this mutation has been shown to cause elevated Ras/Raf signaling as well as increased expression of MMPs (Benbow *et al.*, 2002; Huntington *et al.*, 2004). The current data show that activated Ras/Raf signaling is critical for AhR-mediated changes and occur at the level of transactivation as seen using the 2011bp minimal-responsive MMP-1-luciferase promoter (Figure 4.1.). Inhibition of the Ras/Raf pathway by MEK 1/2 inhibitor, U0126, results in a complete loss of TCDD induction of the 2011bp MMP-1 construct while both p38 inhibitor and PI3 kinase inhibitor partially

reduced TCDD-induced 2011bp promoter activity. The inhibition of the MMP-1 promoter induction observed with PI3 inhibitor LY294002 (Figure 4.1.) may not involve the AhR pathway due to a lack of similar inhibition of the CYP1A1 promoter (See Chapter 5, Figure 5.2.A.). Furthermore, PI3 pathway inhibition of CYP1A1 promoter (Figure 5.2.A.) with LY294002 resulted in the same fold response as Src inhibition (PP2), which showed no affect on MMP-1 responsiveness (Figure 4.1.). This indicates that only two pathways are likely to play a role in AhR-mediated, TCDD-induction of MMP-1, the p38 and Ras/Raf kinase signaling pathways.

The data presented here further demonstrate that Ras/Raf pathway inhibition not only results in a reduction of TCDD-induced MMP-1 promoter activity, but also results in reduced TCDD/AhR-mediated mRNA induction (Figure 4.2.A.). The specificity of the MEK 1/2 chemical inhibitor's mode of repression is supported by siRNA knockdown of BRaf transcript levels, which also leads to a reduction in MMP-1 promoter inductions (Figures 4.3.B.). This was not the case when the p38 inhibitor was used to analyze TCDD-induced MMP-1 transcript levels induction. p38 inhibition did not alter the ability of TCDD to induce MMP-1 transcripts, however the basal levels of transcripts were significantly reduced (Figure 4.2.B.).

ECM degradation and basement membrane infiltration are facilitated by the expression and activity of MMPs (as reviewed (McCawley and Matrisian, 2000; Pasco et al., 2004) . In particular elevated levels of MMP-1 and MMP-2 expression are found in invasive melanoma samples (Vaisanen et al., 1996; Walker and Woolley, 1999) while the importance of MMP-9 and MMP-2 is displayed by lack of invasiveness in MMP-9-deficient mice (Itoh et al., 1999; Itoh et al., 1998). Previous data from the White

laboratory indicate that MMP-1, MMP-2 and MMP-9 expression are all induced by TCDD in an AhR-dependent fashion (Villano et al., 2005). Current data demonstrate that intracellular mitogen activated kinase pathways, Ras/Raf/ERK and p38, are also required for TCDD induced expression of other MMPs, but they differ in regulation. For example, MMP-1 basal levels are significantly reduced following both Ras/Raf and p38 kinase inhibition, while MMP-2 basal expression is increased due to Ras/Raf and p38 kinase inhibition. This suggests that serine/threonine phosphorylation not only plays a role in TCDD induction, but also in control of basal expression. These data further suggests that melanomas containing the BRaf ^{V600E} mutation may contain elevated basal expression of both MMP-1 and MMP-9 due to constitutive activation of the Ras/Raf signaling pathway and that inhibition of this pathway will result in reduction of both MMPs. However, MMP-2 expression is repressed by constitutive Ras/Raf signaling and released by MEK 1/2 inhibition which could potentially offset MMP-9 basal inhibition by MEK 1/2 inhibition. Furthermore, TCDD induction of MMP-1 and MMP-9 is dependent on Ras/Raf signaling while MMP-2 induction in not affected by Ras/Raf inhibition, indicating that AhR-dependent TCDD induction of MMP-2 involves different regulatory pathways than MMP-1 and MMP-9.

MMPs are secreted as inactive, pro-enzymes and their ability to degrade ECM substrates depends upon their activation as well as expression. An *in vitro* collagenase type I assay was used to determine whether Ras/Raf or p38 inhibition had any effect on MMP-1 activity. Both Ras/Raf and p38 inhibition greatly reduced TCDD-induced collagenase activity further implicating both pathways requirement in TCDD-induction (Figure 4.2.C.). Since there are no reports of MMP-13 or MMP-8 activity in human skin,

it is assumed that the prevalent protease, MMP-1 mediates the degradation of type I collagen.

Inhibition of MMP activity can be facilitated by a family of reversible endogenous inhibitors called tissue inhibitors of matrix metalloproteinases (TIMPs) Typically inhibition of specific MMPs are performed by one of four TIMPs resulting in 1:1 stoichiometric inhibition by direct binding. TIMP-1 binds and inhibits pro-MMP-9 (Airola et al., 1999; Goldberg et al., 1992) and TIMP-2 along with MT1-MMP are involved in pro-MMP-2 sequestering (Butler et al., 1998; Strongin et al., 1995) Expression of TIMP-1 correlates with more invasive melanoma while and TIMP-2 expression can be seen in less invasive melanomas (Airola et al., 1999; Hofmann et al., 2000b). These data show that both TIMP-1 and TIMP-2 are induced by TCDD in A2058 melanoma cells and that induction is not affected by Ras/Raf inhibition, while MMP-1 and -9 expression do require Ras/Raf signaling. Both basal and TCDD-induced TIMP-2 expression is increased upon U0126 treatment similarly to that of MMP-2 expression. Further studies involving gelatinase activity are required to determine if the effect of overall increases in both TIMP-2 and MMP-2 expression due to Ras/Raf inhibition will result in enhanced activation of MMP-2 or simply offset one another.

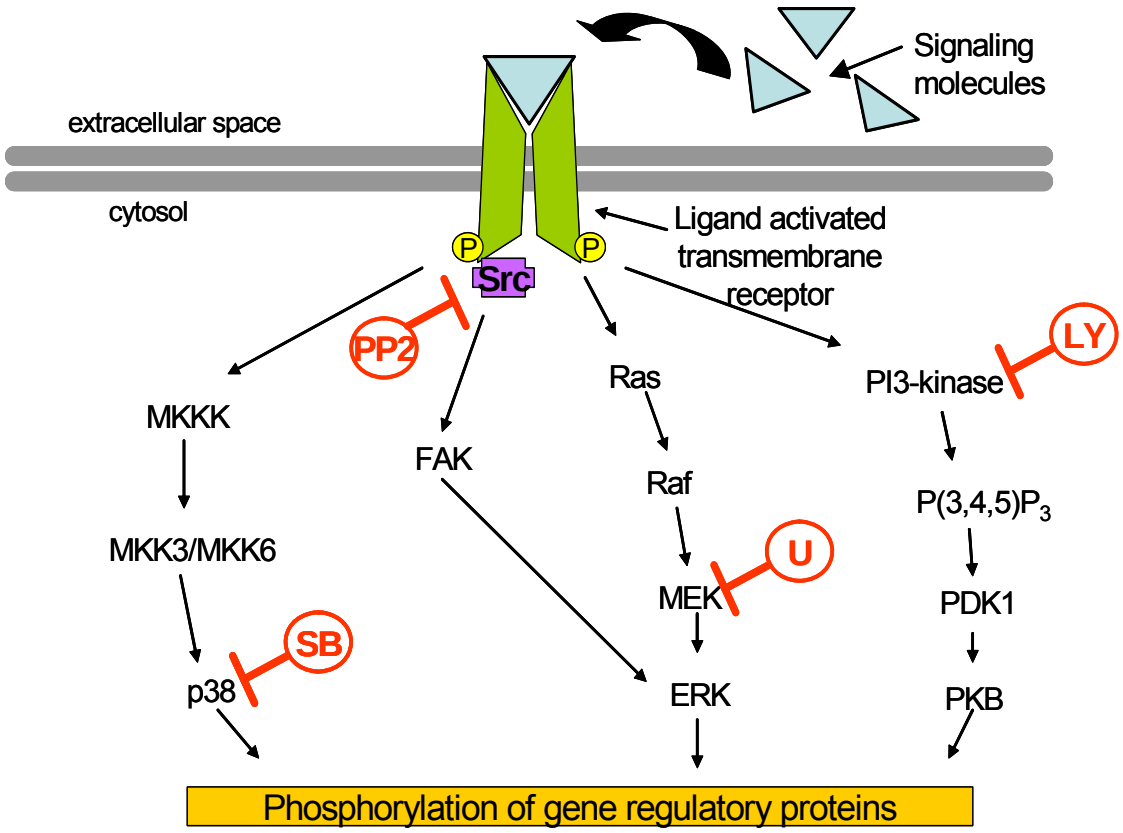
The decrease of MMP-1 promoter responsiveness and activity, as well as MMP-2 and MMP-9 expression, due to p38 inhibition may have resulted from cross talk between p38 and Ras/Raf pathways. Data here demonstrate that p38 inhibition down-regulated ERK phosphorylation (Fig 4.5.A.). Recent evidence supporting agonist-specific cross talk involving p38 and Ras/Raf signaling has been shown in control of prostacyclin production in human umbilical vein endothelial cells where p38 activation positively

regulates the Ras/Raf pathway (Houliston *et al.*, 2001). p38 phosphorylation was not evident in data produced in this report (Figure 4.5.B.), however low levels of p38 phosphorylation have been previously reported in A2058 cells indicating that undetectable p38 kinase signaling may exist in this current study as well (Benbow et al., 2002). It therefore remains possible that p38 inhibition may still affect ERK phosphorylation via a similar unidirectional cross talk, however it is also possible that p38 kinase signaling is directly required for maximal AhR-mediated TCDD induction. Yet a third and most likely possibility involves non-specific inhibition of the p38 chemical inhibitor, SB203580, resulting in decreased ERK phosphorylation. Which ever the case is, the data show that the p38 signaling pathway is required for maximal TCDD-induction of the known AhR responsive CYP1A1 gene (Figure 5.2.C.) as indicated by a significant decrease from 18 fold responsiveness to less than 3 fold. This however is unlike the strict requirement for Ras/Raf signaling (Figure 4.3.A.) which when inhibited results in total loss of TCDD-induced MMP-1 expression.

In summary, data presented here demonstrate the involvement of the Ras/Raf pathway in TCDD-enhanced expression of MMP-1, MMP-2 and MMP-9 as well as TIMP-2 in A2058 melanoma cells. Therefore, activation of the AhR pathway in melanoma cells with a constitutively active BRaf mutation may enhance the metastatic potential of those cells by altering the MMP:TIMP ratio and could have a negative impact on disease progression. It is interesting to note that ERK signaling is required for MMP-1 induction by cigarette smoke (Kim et al., 2004a) and that a known AhR ligand is a constituent of cigarette smoke (Alam et al., 2008). Given the recent findings that the AhR pathway can be activated by a variety of endogenous and exogenous ligands,

including UV-photoproducts (Fritsche *et al.*, 2007), the data presented here suggest that the combination of BRaf activation and the AhR pathway may contribute to the progression of melanoma and perhaps to other tumor types as well.

A.



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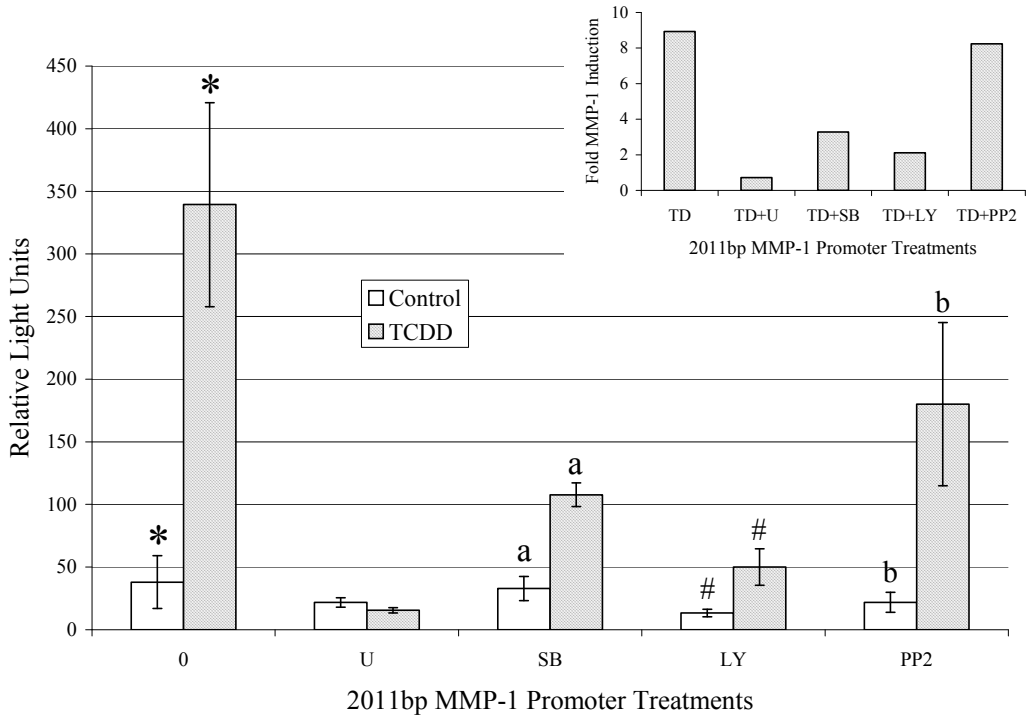
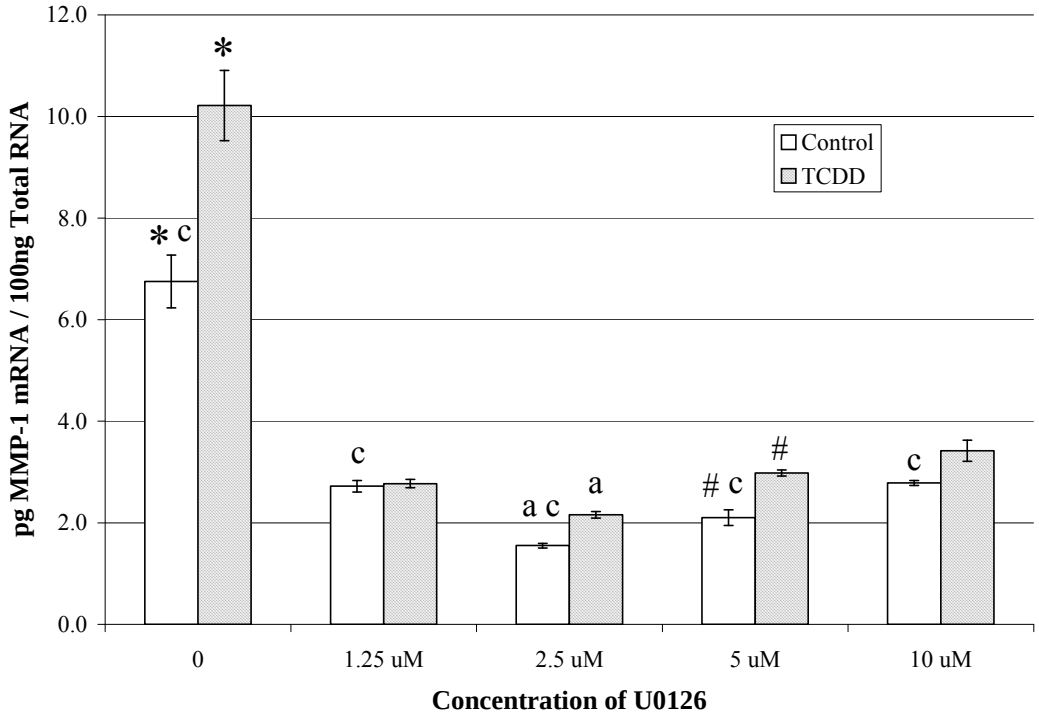


Figure 4.1. Effects of various MAPK inhibitors on 2011bp MMP-1 (A) Depiction of kinase signaling pathways and inhibitor used. (B)TCDD-induced 2011bp minimal MMP-1 promoter activity was analyzed in the presence of U=U0126 (MEK1/2 inhibitor), SB=SB203580 (p38 MAPK inhibitor), LY=LY294002 (PI3 kinase inhibitor), PP2 (Scr tyrosine kinase inhibitor). Cells were pretreated in serum and additive-free media with each inhibitor for 30min. Cells were co-treated with inhibitors and DMSO (vehicle) or 10nM TCDD for 24h. Data are representative of three separate experiments and error bars represent standard deviations. The inset represents fold TCDD responsiveness over vehicle control. (∗,#,a, b, $p<0.05$ using t-test for Control vs TCDD). Data are representative of three separate experiments.

A.



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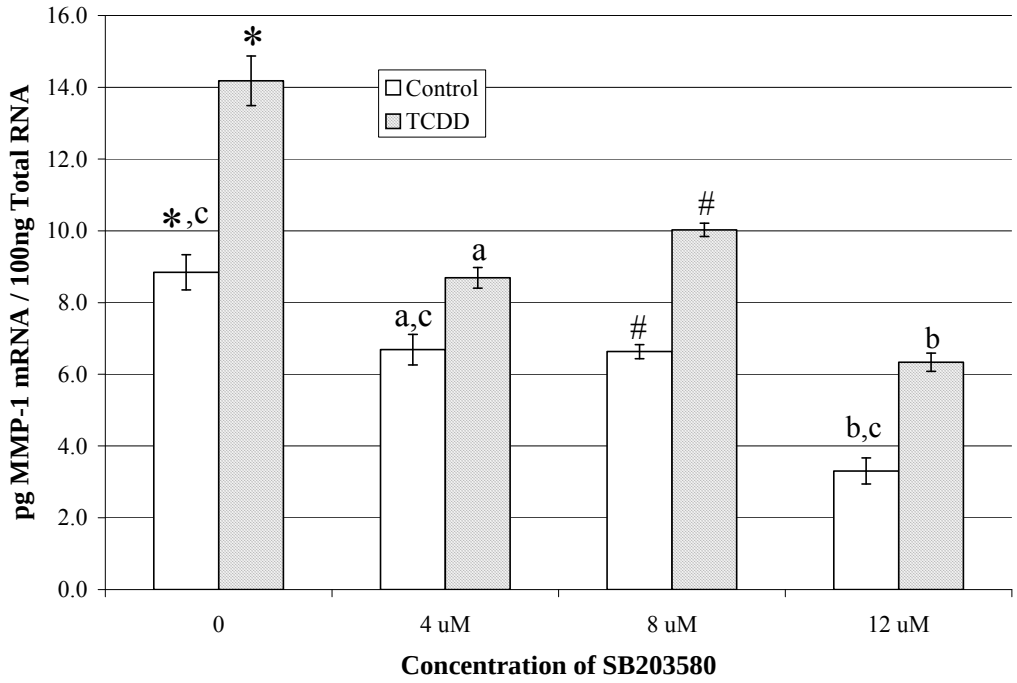

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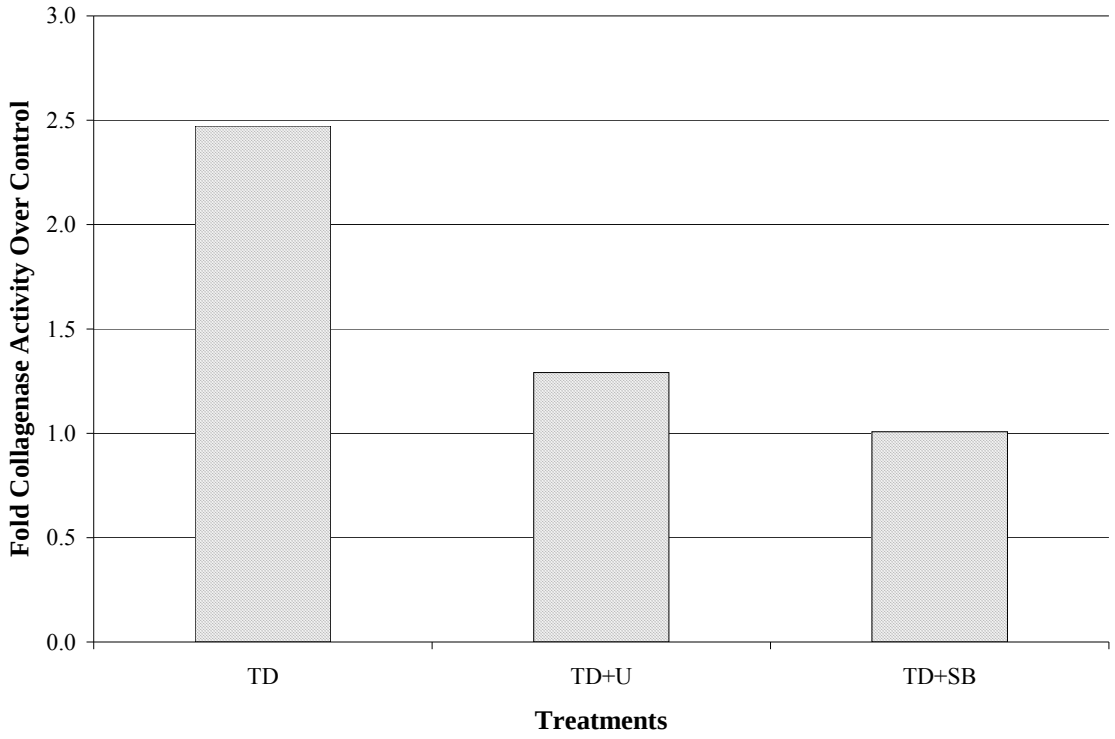

Figure 4.2. Inhibition of TCDD-mediated expression and activity of MMP-1 The effect of Ras/Raf and p38 pathway inhibition on TCDD-induced MMP-1 gene expression levels were analyzed by qRT-PCR. Confluent A2058 cultures were washed three times in PBS and cultured in serum and additive-free media. Cells were pretreated in serum and additive-free media with each inhibitor for 30min followed by 24h with DMSO (vehicle) or 10nM TCDD alone and also co-treated for 24h with serial dilutions of U0126 ranging from 10-1.25μM (A) or serial dilutions of SB203580 ranging from 12μM-4μM (B). Treated cultures were harvested for total RNA and qRT-PCR was performed. Results were normalized to GAPDH expression and MMP-1 expression levels are expressed as pg of MMP-1/100ng of total RNA. Data are representative of three separate experiments and error bars represent standard deviations. C=DMSO, TD=TCDD (* $p<0.05$ using t-test for Control vs TCDD). (C) Conditioned media was collected and concentrated by nominal molecular weight filtration and total protein concentrations were determined using a modified Lowry assay. 15μg of total protein was used in a fluorescent labeled collagen type I assay. Wells were loaded in quadruplicate and data is expressed as fold TCDD induction over control (DMSO). Data are representative of three separate experiments. (∗,#,a, b, c denote significance $p<0.05$ using t-test).

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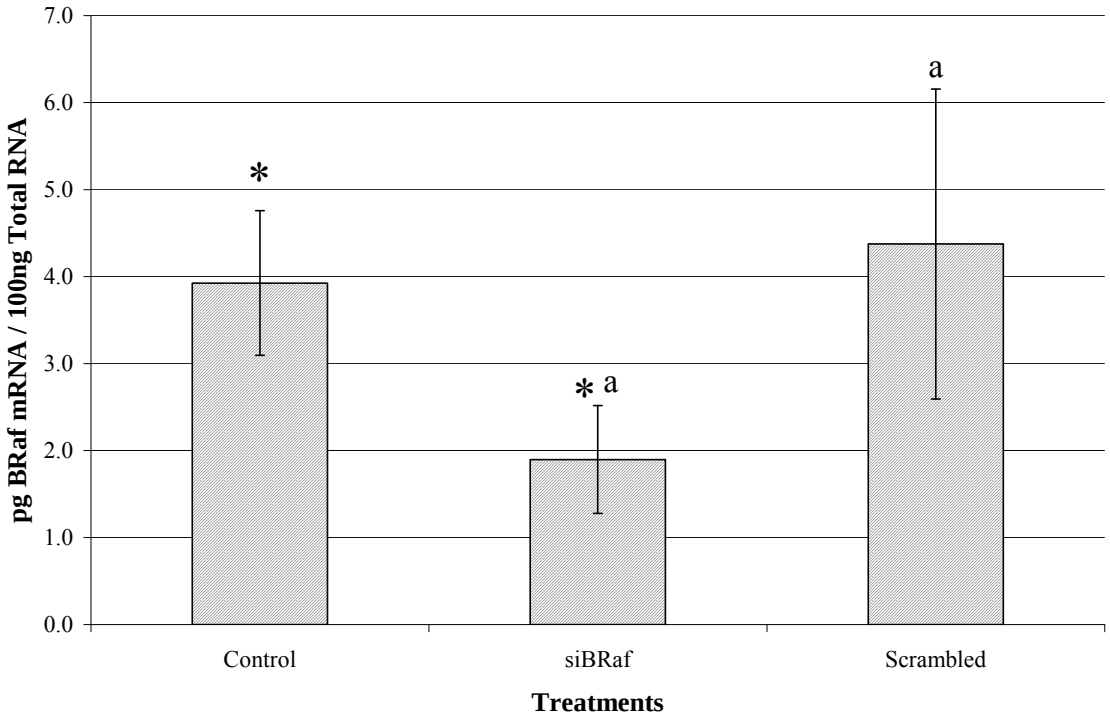

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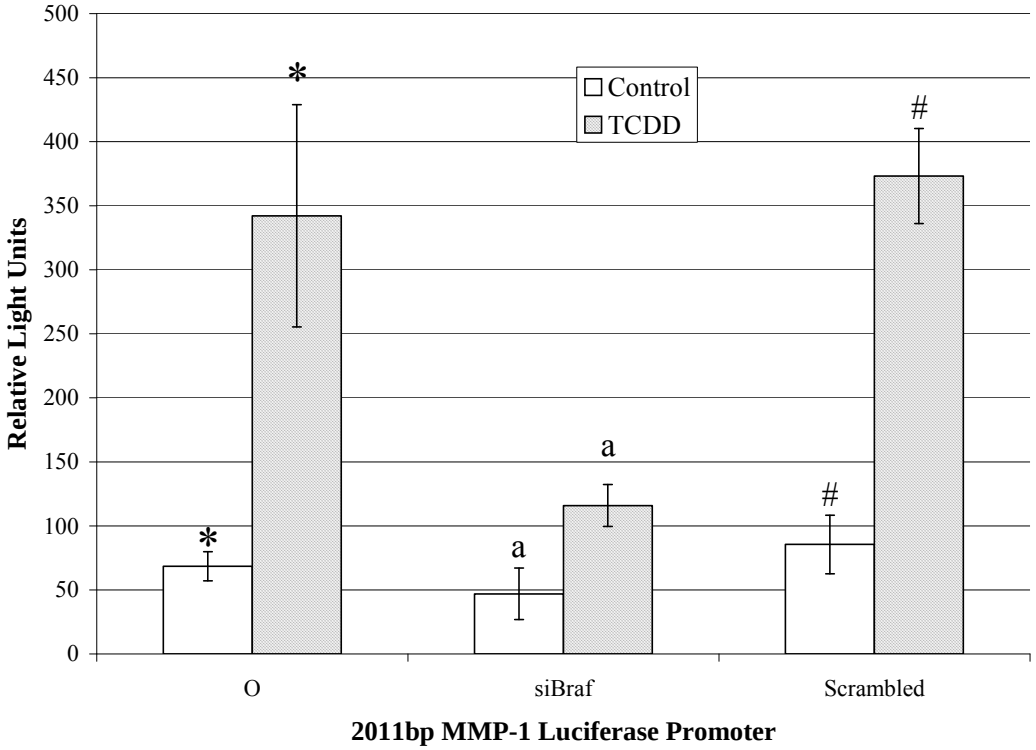
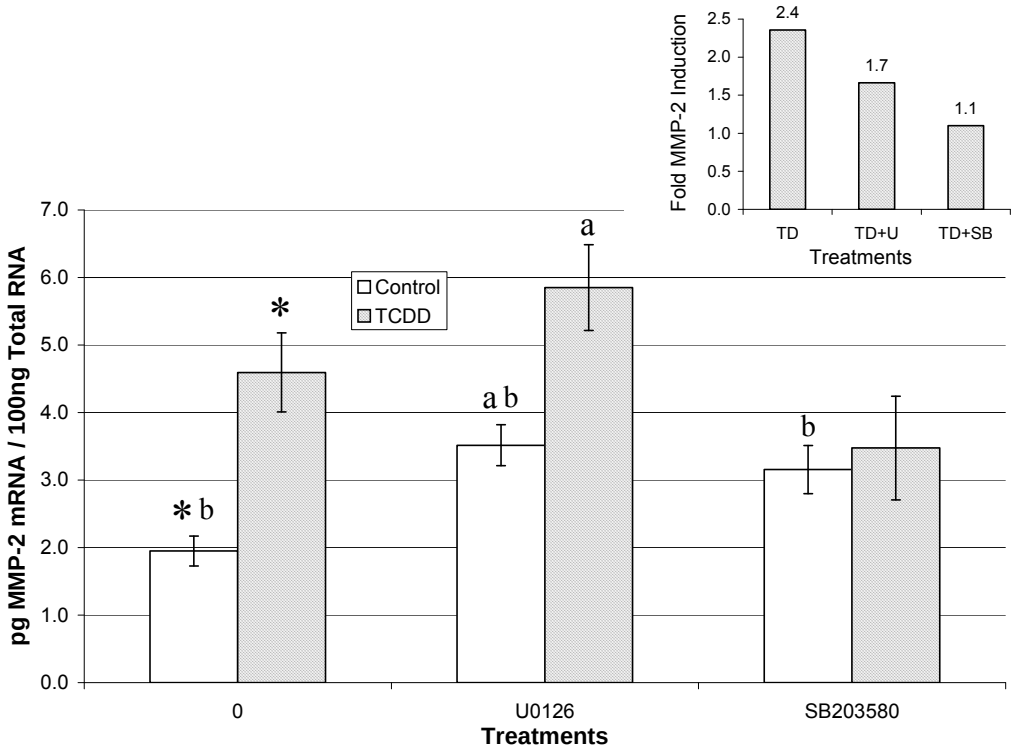


Figure 4.3. Inhibition with siRNA reduces TCDD-induced MMP-1 transactivation (A) A2058 cells were transiently transfected with siRNA targeted to the Braf molecule (siBraf) and a negative control consisting of random siRNA not homologous to any known human sequence (scrambled) to show specificity. Total RNA was harvested from A2058 cells (control), siBRaf transfected cells and scrambled transfected cells. Relative Braf RNA levels were analyzed by real-time PCR. A2058 cells were transiently co-transfected with the 2011bp minimal MMP-1 luciferase-promoter construct (B) promoter along with siRNA to BRaf. Cells were treated with DMSO (vehicle) or 10nM TCDD for 24h, harvested and luciferase assays were performed as described. Treatments were performed in quadruplicate. Data are representative of three separate experiments and error bars represent standard deviation (* $p<0.05$ for t-test using Control vs TCDD).

A.



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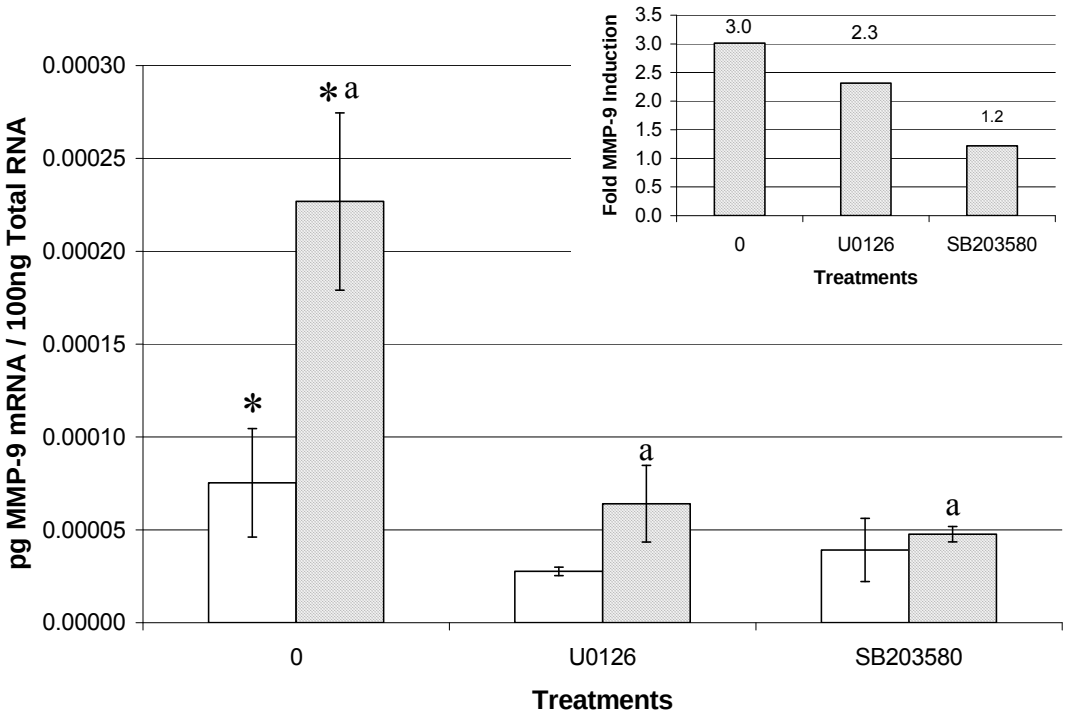

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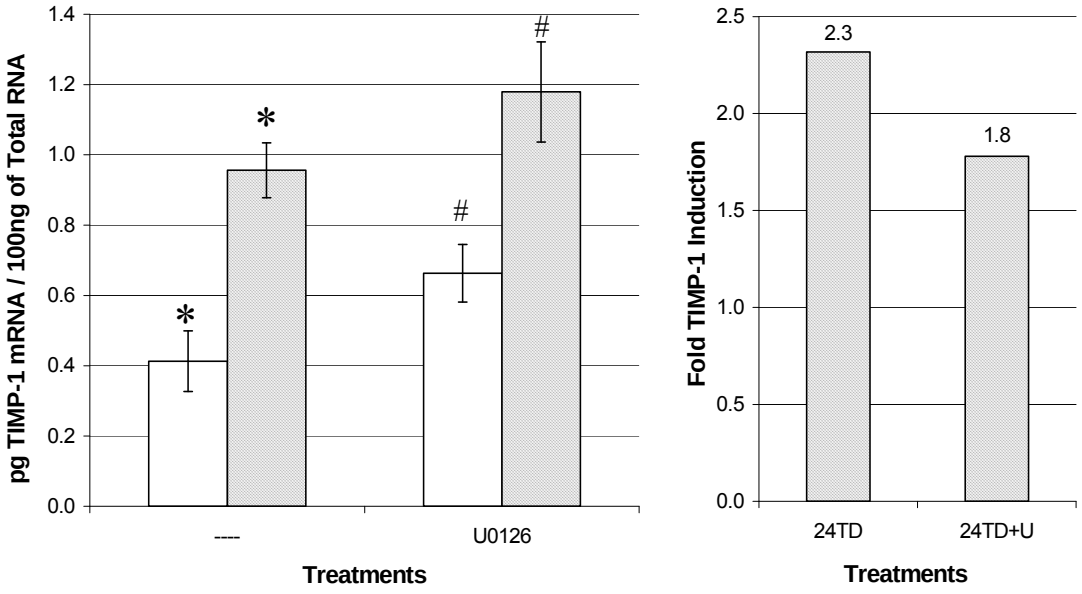

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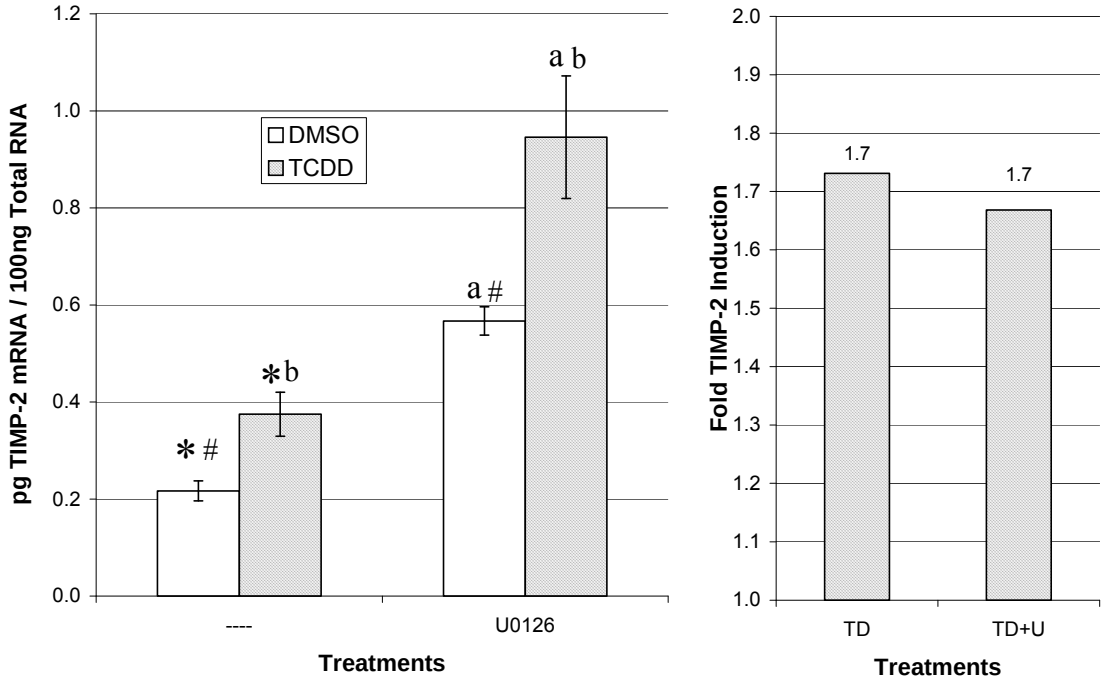
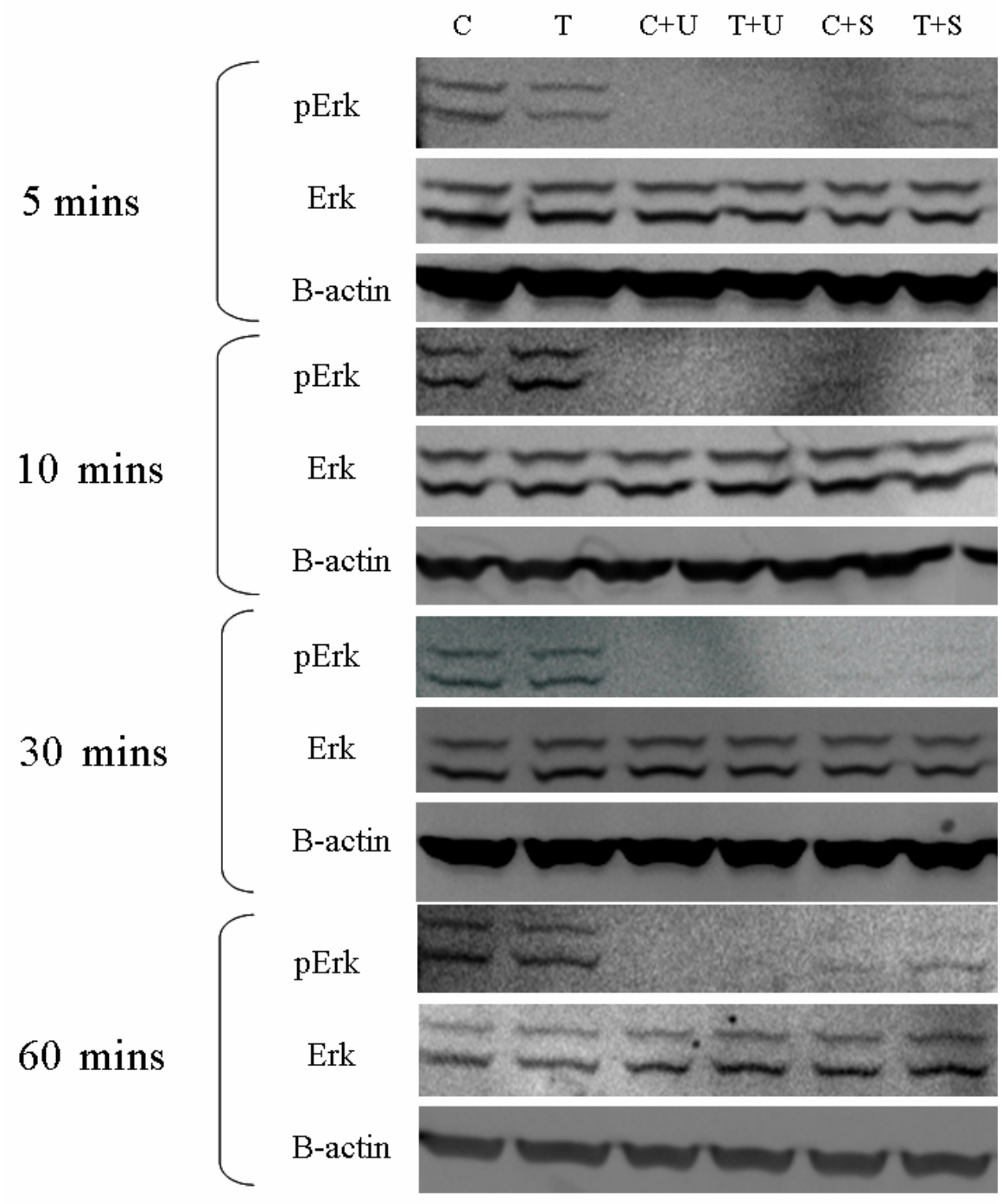


Figure 4.4. Involvement of Ras/Raf signaling upon TCDD-induced expression of MMP-2, MMP-9, TIMP-1 and TIMP-2

The level of TCDD-induced (A) MMP-2, (B) MMP-9, (C) TIMP-1, and (D) TIMP-2 mRNA expression were analyzed by qPCR in the presence of U=U0126 (MEK1/2 inhibitor) and SB=SB203580 (p38 MAPK inhibitor). The inset represents fold TCDD responsiveness over vehicle control. Confluent A2058 cells were washed three times in PBS, pretreated in serum and additive-free media with each inhibitor for 30mins, then treated for 24h with DMSO (vehicle) or 10nM TCDD alone and also co-treated above indicated inhibitors. Treated cultures were harvested for total RNA and quantitative PCR was performed. Results were normalized to GAPDH expression and MMP-2 and -9 expression levels are expressed as pg expression mRNA /100ng of total RNA. Data are representative of three separate experiments and error bars represent standard deviations. C=DMSO, TD=TCDD (*, a, b, c, #, $p<0.05$ using t-test for marked comparisons).

A.



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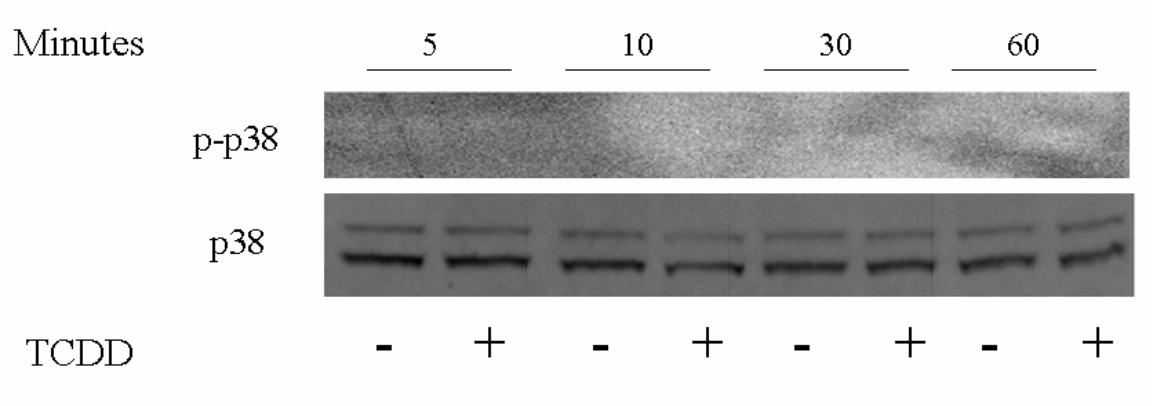

Figure 4.5. ERK phosphorylation levels change with inhibition of MEK and p38 inhibition Confluent A2058 cells were washed three times in Ca^{2+}/Mg^{2+} free PBS and cultured in serum and additive-free media for 24h. Cells were pretreated in serum and additive-free media with either SB203589 (S) or U0126 (U) inhibitors for 30min. Following pretreatment, cultures were co-treated with inhibitors and DMSO (vehicle) or 10nM TCDD for 5, 10, 30 and 60min. Treated cells were washed with ice cold PBS and harvested in fresh RIPA buffer. Protein concentration was determined using a modified Lowry assay. Samples were electrophoresis on a 4-12% Bis-Tris gel and transferred to nitrocellulose for western blot analysis. Blots were probed with primary antibodies to detect (A) total ERK levels and phosphorylated ERK levels as well as (B) total p38 and phosphorylated p38 levels. β-actin was used as a loading control. Blots were scanned using a Storm 860 Molecular Imager. Blots are representative of three independent experiments.

5.0. CROSS-TALK BETWEEN THE RAS/RAF/MEK/ERK AND ARYL HYDROCARBON RECEPTOR (AHR) PATHWAYS IN A2058 MELANOMA CELLS

5.1. Abstract

The aryl hydrocarbon receptor (AhR) is a member of the basic Helix-loop-Helix Per-Arnt-Sim (bHLH-PAS) domain family of transcription factors and was identified as the receptor for polycyclic aromatic hydrocarbons and related compounds. Recently, studies indicate that the AhR binds to a variety of endogenous and exogenous compounds including UV photoproducts of tryptophan. The AhR pathway and the Ras/Raf signaling pathway have been shown to interact resulting in AhR stabilization and reduced AhR DNA binding ability and transactivation. ERK kinase signaling has been shown to be involved with AhR nuclear localization and activation. It has been shown that 70% of melanomas contain a constitutively activating mutation in the BRaf molecule (BRafV600E) of the Ras/Raf/ERK signaling pathway resulting in hyperphosphorylation of ERK. Furthermore, acute sun expose correlates to melanomas possessing the BRaf activating mutation and UV radiation has been shown to cause AhR activation suggesting that there may be a tie between AhR and Ras/Raf signaling in melanomas. We have shown that AhR-dependent MMP induction requires Ras/Raf signaling resulting in ERK phosphorylation in A2058 melanoma cells, however the exact role of ERK phosphorylation is not known. It is demonstrated here that Ras/Raf signaling alters the expression of AhR and aryl hydrocarbon receptor repressor (AHRR) and that cotreatment with an AhR antagonist α-napthoflavone results in a decrease in

ERK phosphorylation. We also show that Ras/Raf but not p38 signaling can abrogate AhR-mediated expression of CYP1A1.

5.2. Introduction

The AhR and Arnt are members of the basic Helix-loop-Helix Per-Arnt-Sim (bHLH-PAS) domain family of transcription factors and were originally identified as receptors for 2,3,7,8-tetrachloldibenzo-*p*-dioxin (TCDD) and other related polycyclic aromatic hydrocarbons (PAH) (Hoffman et al., 1991; Poland et al., 1976). AhR resides inactive the cytoplasm complexed to two heat shock protein 90 (HSP90) molecules, co-chaperone p23, and an immunophilin-like protein XAP2 (Carver et al., 1998; Kazlauskas et al., 2001). Upon ligand binding, the AhR dissociates from XAP2 and the HSP90-ligand bound AhR complex translocates to the nucleus where it bind Arnt (Reyes et al., 1992; Sogawa et al., 1995). Together AhR and Arnt form an active transcription factor that can bind to and facilitate transcription from xenobiotic response elements (Matsushita et al., 1993; Watson and Hankinson, 1992). Recent data indicate that the AhR binds and is activated by a variety of endogenous and exogenous compounds including flavonoids, UV photoproducts of tryptophan as well as synthetic retinoids (Carver and Bradfield, 1997; Denison et al., 2002; Oberg et al., 2005; Song et al., 2002; Soprano et al., 2001; Soprano and Soprano, 2003). The aryl hydrocarbon receptor repressor (AhRR) is also a member of the bHLH-PAS family and is an endogenous inhibitor of AhR signaling (Haarmann-Stemmann and Abel, 2006). The AhRR functions as a negative regulator of the AhR pathway by inhibiting

transactivation events initiated by the AhR/Arnt transcription factor (Evans et al., 2008).

Recent studies indicate that there is an interaction of Ras/Raf and AhR signaling pathways, and demonstrate that activation of the Ras/Raf pathway stabilizes the AhR, reduces AhR DNA binding ability and is essential for AhR complex activity (Chen et al., 2005; Tan et al., 2002; Tan et al., 2004; Yim et al., 2004). Data also show that transiently transfected, constitutively active MEK1, a down-stream target of Raf, can elevate the level of AhR-dependent luciferase activity (Tan et al., 2004). This indicates the need for Ras/Raf signaling for AhR-dependent activation and downstream effects demonstrating that Ras/Raf signaling can mediate an AhR-dependent response.

Malignant melanoma is an aggressive disease that develops from the transformation of normal human melanocytes and 70% of all melanomas contain a constitutively activating mutation of the serine/threonine kinase BRaf molecule (BRaf ^{V600E}) of the Ras/Raf/ERK signaling pathway resulting in hyperphosphorylation of ERK (Davies et al., 2002; Pollock et al., 2003; Satyamoorthy et al., 2003). The main etiological risk factor for acquiring cutaneous melanoma is related to sporadic, intense sun exposure, especially in adolescence (Breitbart et al., 1997; Gandini et al., 2005; Walter et al., 1999) and Braf activating mutations have been found to correlate with melanocytic lesions resulting from acute sun exposure rather than chronic exposure (Dhomen and Marais, 2007). In addition, UV radiation has been shown to induce AhR-mediated gene transcription of CYP1A1 though oxidation of tryptophan (Sindhu and Kikkawa, 1999). Recent studies also indicate that UVB radiation results in the formation of and AhR ligand, 6-formylindolo[3,2-b]carbazole, and results in epidermal

growth factor receptor internalization and activation of ERK (Fritsche et al., 2007). These studies suggest that there is constant relation between ERK kinase pathway and AhR activation and that the main risk factors associated with melanoma initiation also results in AhR activation. Data from the White laboratory have shown that activated AhR is necessary for increased *in vitro* invasion of BRaf ^{V600E} containing A2058 melanoma cells through increased expression and activity of matrix metalloproteinase. A2058 melanoma cells were also shown to express the most AhR when compared to less aggressive melanoma cell lines as well as normal human melanocytes, suggesting that increased AhR expression and constitutive Ras/Raf signaling result in increased TCDD-induced MMP expression (Villano et al., 2005).

Data produced here demonstrate that Ras/Raf inhibition results in alteration in basal levels of two AhR pathway constituents, the AhR and the AhRR. Additionally, data presented here show that the AhR antagonist, α-naphthoflavone, affects ERK phosphorylation in A2058 cells. AhR-mediated induction of CYP1A1, a known AhR-responsive gene, was abrogated by Ras/Raf inhibition while only partial inhibition was seen by p38 kinase inhibition. Taken together these data suggest that constitutive activation of BRaf is required for AhR-mediated MMP response. Furthermore, BRaf constitutive activation results in alterations of the AhR pathway expression and therefore may enable TCDD-mediated responses.

5.3. Results

5.3.1. A comparison between normal human melanocytes and A2058 AhR and AhRR expression

Recent data indicate that AhRR is a tumor suppressor in a variety of cancers demonstrating the importance of AhR mediated signaling in cancer progression (Zudaire et al., 2008). Interestingly, AhR is expressed at high levels in immortalized and malignant mammary gland cell lines but not in normal, finite life-span cells (Yang et al., 2007). The above data correspond to what was previously seen in the White lab in that normal human melanocytes (NHMs) do not produce a large amount of AhR mRNA as compared to A2058 melanoma cells and correlated increased TCDD-responsiveness (Villano et al., 2005). AhRR expression was analyzed to determine if there is any correlation between AhRR expression and the previously seen increases in AhR expression in A2058 melanoma cells. Results from qRTPCR revealed that NHMs produce three orders of magnitude more AhRR mRNA than A2058 cells do (Figure 5.1.). This is the exact opposite expression profile for AhR in A2058s and NHMs and indicates an inverse relationship for the expression of AhR and AhRR that depends on the status of the cells, "normal" verses malignant.

5.3.2. AhR and AhRR expression levels in response to inhibition of the Ras/Raf pathway

One difference between the A2058s and NHMs is that the A2058s contain the BRaf ^{V600E} constitutively active mutation that results in hyperactive BRaf signaling. To determine whether the BRaf ^{V600E} mutation contributes to AhR and AhRR expression in A2058s, we examined AhR and AhRR expression following treatment with the down-stream target of Ras/Raf signaling, MEK1/2 inhibitor, U0126. Exposure of A2058 melanoma cells to U0126 resulted in a significant decrease in overall AhR expression

(Figure 5.1.B.). These data demonstrate that Ras/Raf signaling directly effects AhR expression levels in A2058 cells indicating that Ras/Raf signaling is necessary to maintain levels of AhR and therefore may affect AhR signaling in A2058 cells.

To examine if AhRR expression levels are affected by Ras/Raf signaling inhibition, A2058 cells were co-treated with the MEK1/2 inhibitor, U0126 and DMSO or TCDD. qRTPCR analysis revealed that AhRR expression does not show induction by TCDD treatment, but significantly increases in the presence of U0126 co-treatment approximately 3 fold compared to uninhibited (Figure 5.1.C.). Taken together these data show that the expression of AhR and AhRR in A2058 melanoma cells is modulated by Ras/Raf signaling such that when Ras/Raf signaling is elevated, AhR expression will be increased and AhRR expression will be decreased.

5.3.3. Inhibition of Ras/Raf and p38 kinase pathways inhibit CYP1A1 promoter activity and mRNA expression

The Ras/Raf signaling pathway is only one pathway that TCDD/AhR is known to interact with. To assess if other signaling pathways can also modulate AhR-dependent expression the well characterized AhR-dependent, monooxygenase gene, Cytochrome P4501A1 (CYP1A1) was used as a reporter of AhR activation. A human CYP1A1 luciferase construct (pRNH3/235, described previously) (Fujisawa-Sehara et al., 1986) containing four dioxin response elements (XREs) through the 5' region (-1140 to +59bp) was transiently transfected into A2058 cells. TCDD-induced CYP1A1 promoter activity was reduced 2.4 fold following co-treatment with the chemical inhibitors for PI3 kinase and Src (Figure 5.2.A.). Ras/Raf inhibition resulted in a complete loss of TCDD-

mediated induction of CYP1A1 while p38 kinase inhibition resulted in over 5 fold reduction in TCDD-mediated CYP1A1 promoter activity (Figure 5.2.A.) Cotreatment with PI3, Src and P38 pathway inhibitors showed induction due to TCDD treatment, while Ras/Raf inhibition resulted in complete inhibition of CYP1A1 promoter activity. These data indicate that only the Ras/Raf pathway is essential for AhR-mediated TCDD-responses.

Due to previous data suggesting that p38 signaling could affect AhR-dependent MMP expression, CYP1A1 mRNA expression was analyzed following cotreatment with both the Ras/Raf inhibitor (U0126) as well as the p38 inhibitor (SB203580) and TCDD. Ras/Raf pathway inhibition resulted in a complete loss of TCDD-mediated CYP1A1 expression at all concentrations used (Figure 5.2.B.). p38 inhibition inhibited TCDD-induced CYP1A1 expression, lowering induction 6.8 fold, similarly to CYP1A1 reporter levels above; however, there was still significant TCDD-induced increase in CYP1A1 mRNA expression even at the highest concentration, 12μM (Figure 5.2.C.). The effects of Ras/Raf pathway inhibition on CYP1A1 expression using the chemical inhibitor, U0126 were verified through the use of small inhibitory RNA specific to BRaf (siBRaf). A2058s were transiently cotransfected with pRNH3/235 and siBRaf and were harvested after 24h of TCDD treatment. There was a significant reduction in CYP1A1 promoter activity when cells were co-transfected with siBRaf that was recovered in cells co-transfected with a negative control siRNA (scrambled) (Figure 5.2.D.). Taken together, CYP1A1 promoter responsiveness and expression levels suggest that Ras/Raf signaling is required for AhR-mediated TCDD induction and also suggest p38 signaling is necessary for a maximal response.

5.3.4. Inhibition of AhR signaling, inhibits ERK phosphorylation

The constitutive BRaf mutation, Braf ^{V600E}, in A2058 melanoma cells results in phosphorylation of down stream MAPK target, ERK (Mercer and Pritchard, 2003). Data from Chapter 4 indicate that the Ras/Raf pathway alters AhR signaling however, little is known about the effects of AhR upon Ras/Raf signaling. In order to examine potential effects of AhR signaling upon the Ras/Raf pathway, whole cell protein lysates prepared from A2058 cells co-treated with α-naphthoflavone and either TCDD or DMSO were electrophoresised on a 4-12% TBE gel. After separation the proteins were transferred to a nitrocellulose membrane and probed for ERK, phosphorylated ERK and β- actin. Phosphorylated ERK (pERK) levels were unchanged at 5min of TCDD and α-naphthoflavone cotreatment (Figure 5.3.). Levels of pERK decreased when co-treated with α-naphthoflavone at 10 and 30min followed by a partial recovery at 60min. These data provide evidence that regulation between the Ras/Raf and AhR signaling pathways is not unidirectional and suggests that cross-talk may occur at the level of ERK phosphorylation.

5.4. Discussion

Findings presented in this chapter demonstrate that the AhR and Ras/Raf signaling pathways interact and this interaction can modulate AhR and AhRR expression as well as ERK phosphorylation. Previous data indicate that normal human melanonocyte (NHM) cells express low levels of AhR and melanoma cell lines express AhR in direct correlation to invasiveness with A2058 cells being the most invasive and highest AhR expressing cell line (Villano et al., 2005). Data from experiments conducted

with “normal”, immortalized and malignant mammary cell lines show a similar expression pattern for AhR in that higher levels of AhR are found in malignant human mammary cells lines as compared to immortalized or limited life-span “normal” cells suggesting that increased AhR expression/signaling may be a common step in the progression of both melanoma and breast cancer (Yang et al., 2007). These data also show the resultant activation of CYP1B1 by over-expressed AhR in immortalized MCF-10F mammary cells is able to be reversed by expression of AhRR suggesting that these cells are overexpressing an active form of AhR and that AhR-mediated events in immortalized cells can be reversed by AhRR expression. Increased expression of AhR and reduced expression of AhRR in A2058 melanoma cells may also alter AhR-mediated activation of downstream target expression as it did in mammary cells. Further support of a role for increased AhR expression in cancer, AhRR was just proposed as a tumor supressor due to its reduced expression in malignant tissue from human colon, breast, lung, stomach, cervix and ovary (Zudaire et al., 2008). Furthermore, the down-regulation of AhRR expression in a human lung cancer cell line conferred resistance to apoptosis and enhanced invasion indicating that AhRR modulation in melanoma cells may have a similar effect through the regulation of MMPs (Zudaire et al., 2008).

A2058 melanoma cells contain an activating mutation in the Ras/Raf signaling pathway molecule, BRaf (BRaf ^{V600E}) which results in phosphorylation of MEK 1/2 and ERK(Mercer and Pritchard, 2003). Data presented here suggest that elevated Ras signaling, a common alteration in a variety of cancers, confers increased sensitivity to AhR ligands, and reduced expression of AhRR, a putative tumor suppressor. These data are supported by findings in other cell types demonstrating that the Ras/Raf MAP kinase

pathway has an impact on the AhR signaling pathway. In Hepa-1 cells, ERK is activated by TCDD in an AhR-independent manner and pERK is critical to AhR-dependent gene transcription (Tan *et al.*, 2002). Further, Ras/Raf signaling is required for AhR-dependent inhibition of adipocyte differentiation (Hanlon *et al.*, 2003). Ras/Raf signaling has also been shown to regulate the AhR pathway in mouse Hepa-1 cells (Tan *et al.*, 2004). Data presented here show that inhibition of constitutively active Ras/Raf signaling in A2058 melanoma cells results in a decrease in AhR expression (Figure 5.1.B.) and an increase in AhRR expression (Figure 5.1.C). This change in AhR pathway mediators causes A2058 expression profile of AhR and AhRR to completely reverse making their expression profile appear more like NHM cells and this change in A2058 expression profile could have profound effects on the AhR signaling.

The data presented here indicate that the interaction between AhR and Ras has affected the level of promoter activation. In order to analyze the effects that pathway signaling inhibition has on AhR-dependent gene transcription we utilized a CYP1A1 luciferase reporter. TCDD-induced, AhR-dependent CYP1A1 promoter activation shows that Ras/Raf signaling is essential in mediating AhR transactivation and that this also extends to increased CYP1A1 mRNA expression. These data are supported by finding that wild-type mouse Hepa-1c1c7 cells over-expressing a dominant negative form of ERK resulted in suppression of TCDD-dependent transcription of a XRE-driven reporter (Yim et al., 2004). Similar requirements of Ras/Raf signaling for AhR mediated CYP1A1 production are seen in mouse hepatoma (Hepa-1) cells, however the inhibition of ERK phosphorylation leads to decreases in Arnt and not AhR (Tan et al., 2004). On the other hand, some data show that inhibition of the Ras/Raf pathway can lead to AhR nuclear

localization and is able to induce CYP1A1 expression (Chen et al., 2005). Such results may be explained if Ras/Raf signaling affected ligand activated AhR differently from unliganded AhR. However, our data do not demonstrated an increase in CYP1A1 basal level expression with increasing amount of U0126 inhibitor in A2058 melanoma cells , as observed in hepa1c1c7 cells (Chen et al., 2005). To confirm that the effect of the U0126 on CYP1A1 was primarily through down regulation of BRAF, BRAF expression was reduced using siBRaf and showed a similar reduction in TCDD- induced CYP1A1 promoter activity (Figure 5.2.D.).

Preliminary data from the White lab suggests that the p38 MAPK pathway may also have an impact on AhR signaling in the A2058 melanoma cells (Figure 4.1.B.). The data presented in this chapter demonstrate that inhibition of the p38 MAPK pathway reduces CYP1A1 mRNA expression and promoter activity**,** but does not completely abrogate it. These data indicate, p38 signaling may also be required for a maximal AhR-mediated response. This is similar to a situation where both ERK and JNK MAP kinase pathways are required for TCDD-induction of CYP1A1, however, cell type specificity may account for the use of JNK kinase signaling in mouse hepatoma cells over p38 in human melanoma cells (Tan et al., 2004). However, it is unclear as to the importance of p38 MAPK in A2058 melanoma cells. Data presented in Chapter 4 indicate that the p38 MAPK inhibitor also reduces ERK phosphorylation, suggesting that the down regulation observed may be via ERK 1/2 phosphorylation. Furthermore, in chapter 4, results have also shown that A2058 melanoma cells have no observable activity of p38MAPK, which would not support this pathway as being critical for AhR-mediated gene expression n this cell type.

In addition to demonstrating that the Ras/Raf pathway has an impact on AhR signaling in A2058 melanoma cells, the data presented here also supports the hypothesis that the AhR pathway modulates Ras/Raf phosphorylation in this cell type. The data show that inhibition of the AhR signaling pathway using the AhR antagonist α-naphthoflavone causes a reduction in ERK phosphorylation after ten minutes of treatment with DMSO or TCDD and lasted up to 60 min indicating that AhR signaling is necessary for ERK phosphorylation. This is the first evidence seen that indicates that the AhR may regulate the ability of the MAP kinase pathway to phosphorylate down stream effector molecules such as ERK. This indicates that AhR and the Ras/Raf pathway are co-regulated through cross-talk involving both the activation of AhR and the ultimate phosphorylation of ERK.

In summary, data presented here demonstrate that the AhR and Ras/Raf signaling pathways modulate each others effecter molecules in A2058 melanoma cells. This is seen in changes of AhR and AhRR expression due to Ras/Raf inhibition as well as the resulting decrease in activation of a known AhR-responsive gene, CYP1A1. On the other hand, AhR inhibition is able to decrease ERK phosphorylation indicating that the two pathways cross-talk and potentially interact with one another. This suggests that the elevation of these two pathways in invasive cell type may results from synergistic positive regulation.

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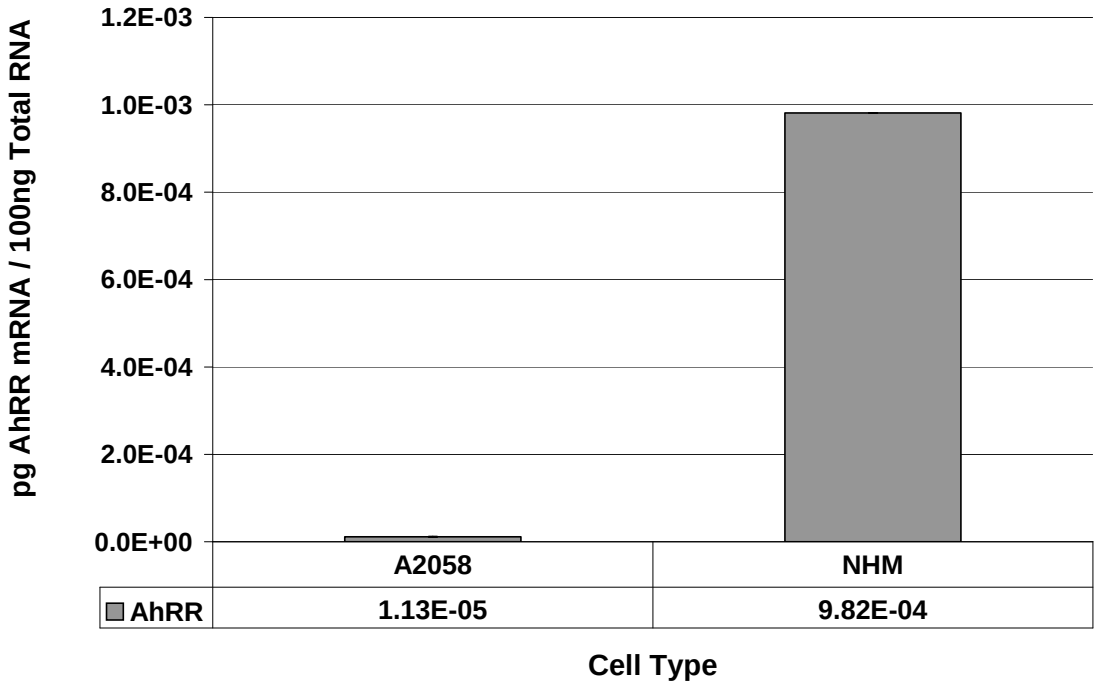

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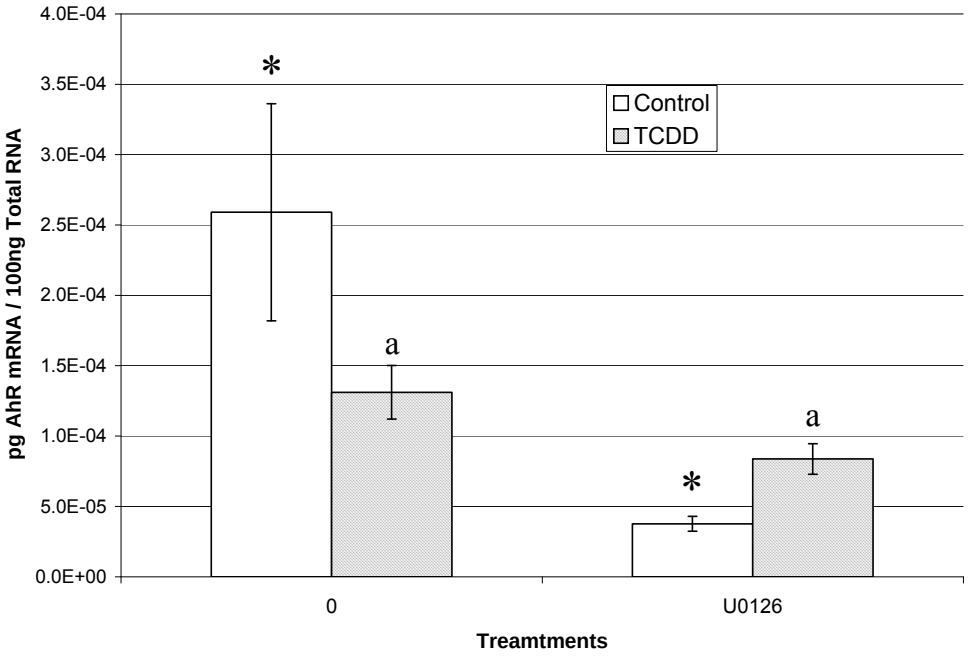

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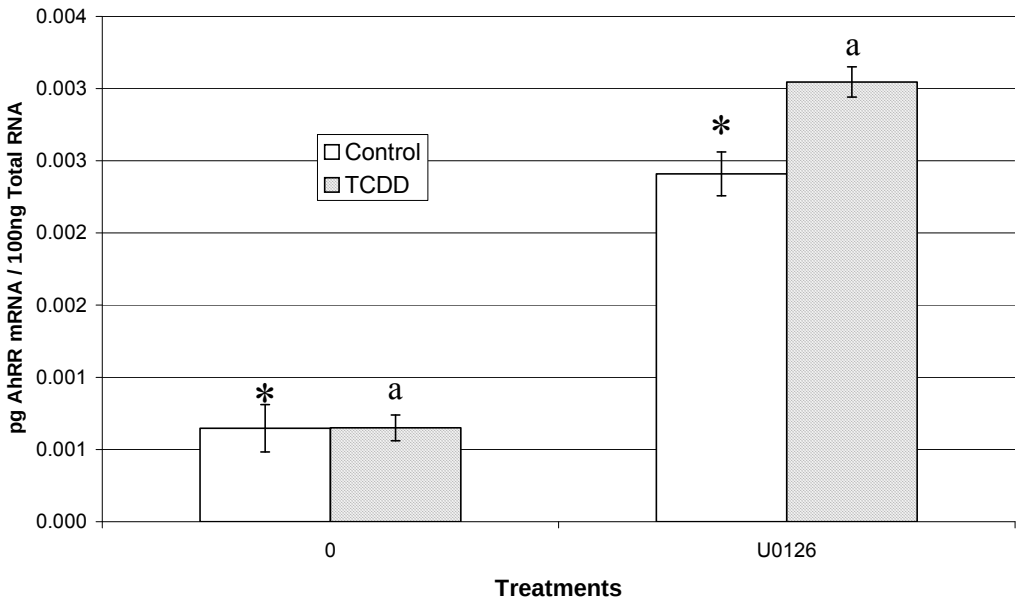
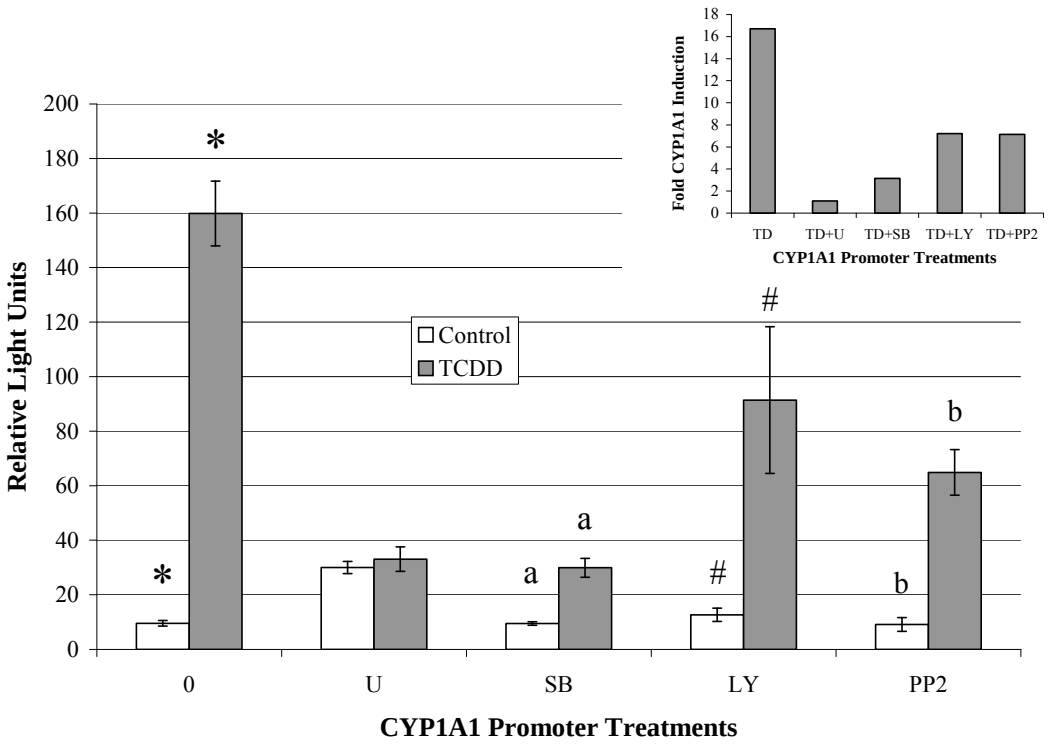


Figure 5.1. Differential expression of aryl hydrocarbon receptor and aryl hydrocarbon receptor repressor levels between normal human melanocytes and A2058 melanoma cells

Gene expression levels of AhR and AhRR were analyzed by qRT-PCR. Confluent A2058 cultures were washed three times in PBS and cultured in serum and additive-free media. A2058 cells and normal human melanocytes expression levels of AhRR (A) were treated with DMSO for 24h and analyzed via qRT-PCR. A2058 cells were pretreated in serum and additive-free media with each inhibitor for 30min followed by 24h with DMSO (vehicle) or 10nM TCDD alone and also co-treated for 24h with 10μM U0126 inhibitor. Treated cultures were harvested for total RNA and qRT-PCR was performed for AhR (B) and AhRR (C) expression levels. Results were normalized to GAPDH expression and MMP-1 expression levels are expressed as pg of MMP-1/100ng of total RNA. Data are representative of three separate experiments and error bars represent standard deviations. C=DMSO, TD=TCDD (∗, a, denote significance $p<0.05$ using t-test).

A.



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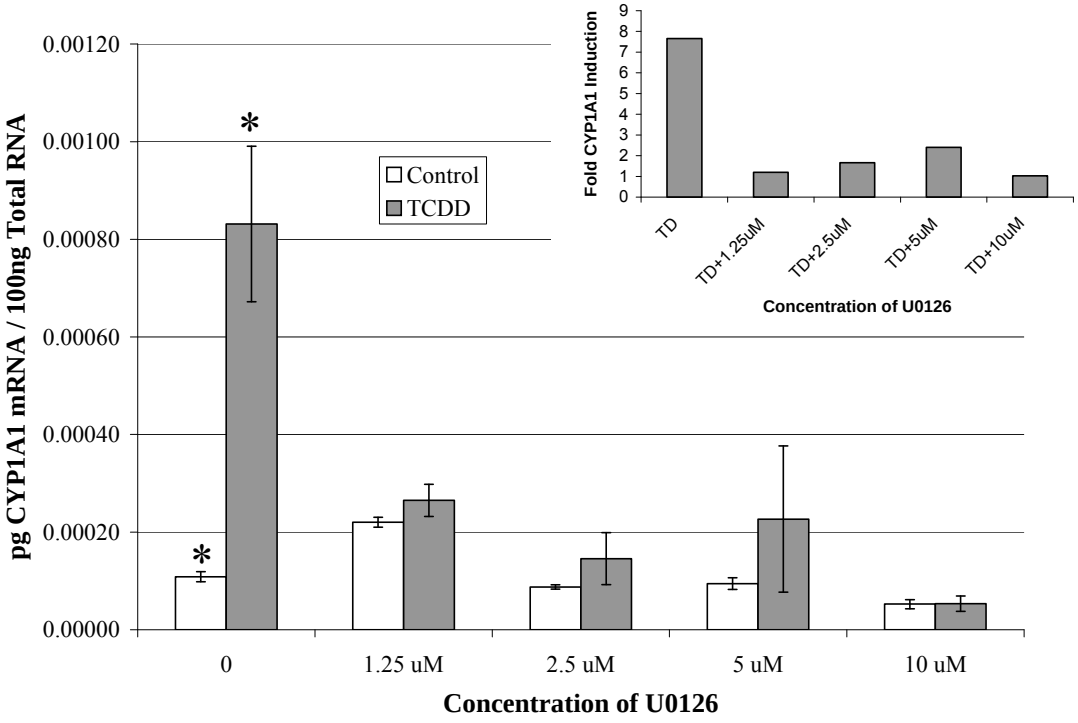

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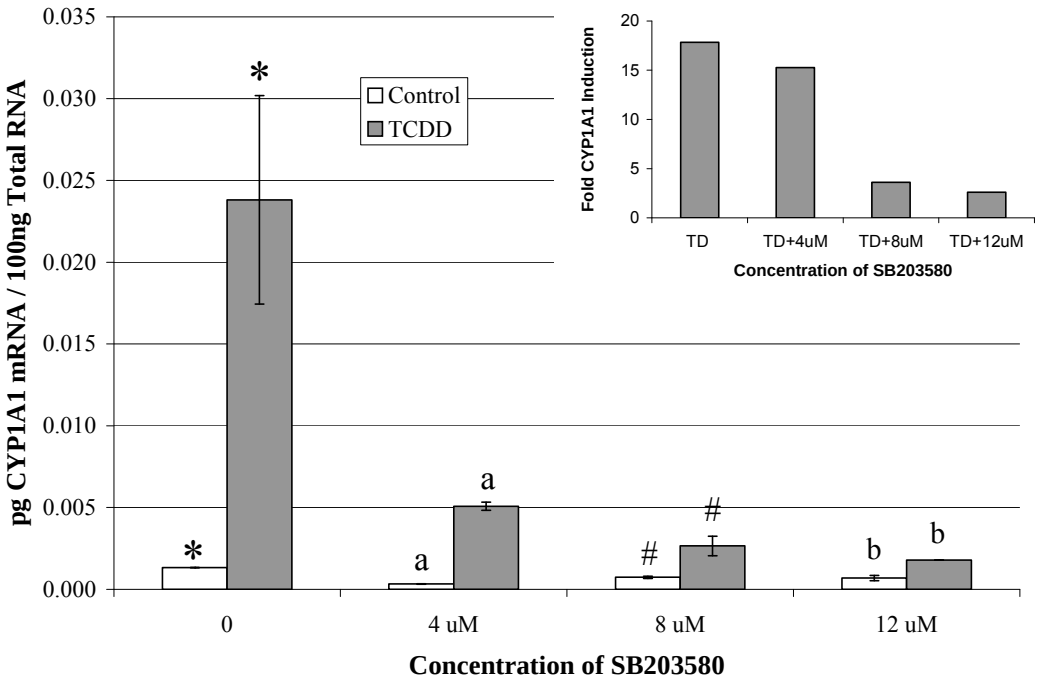

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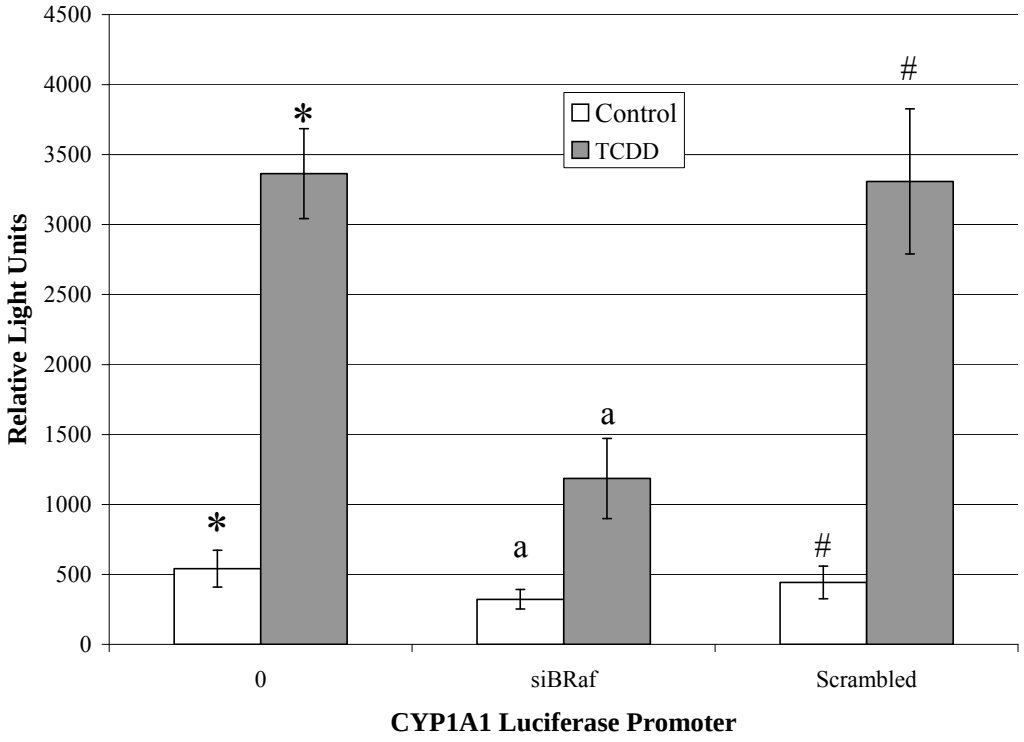

Figure 5.2. Inhibition of TCDD-mediated CYP1A1 promoter induction and gene expression

(A) The level of TCDD-induced CYP1A1 promoter driving luciferase expression was analyzed in the presence of U=U0126 (MEK1/2 inhibitor), SB=SB203580 (p38 MAPK inhibitor), LY=LY294002 (PI3 kinase inhibitor), PP2 (Scr tyrosine kinase inhibitor). Cells were pretreated in serum and additive-free media with each inhibitor for 30min and co-treated with inhibitors and DMSO (vehicle) or 10nM TCDD for 24h. The inset represents fold TCDD responsiveness over vehicle control. TCDD-induced expression levels of CYP1A1 were examined after co-treatment with (B) U0126 or (C) SB203580. Confluent A2058 cells were washed three times in PBS, pretreated in serum and additive-free media with each inhibitor for 30mins, then treated for 24h with DMSO (vehicle) or 10nM TCDD alone and also co-treated for 24h with serial dilutions of U0126 ranging from 10-1.25μM (B) or serial dilutions of SB203580 ranging from 12μM-4μM (C). Treated cultures were harvested for total RNA and quantitative PCR was performed. Results were normalized to GAPDH expression and CYP1A1 expression levels are expressed as pg of CYP1A1/100ng of total RNA. Data are representative of three separate experiments and error bars represent standard deviations. C=DMSO, TD=TCDD (∗, a, #, b denote significance $p<0.05$ using t-test).

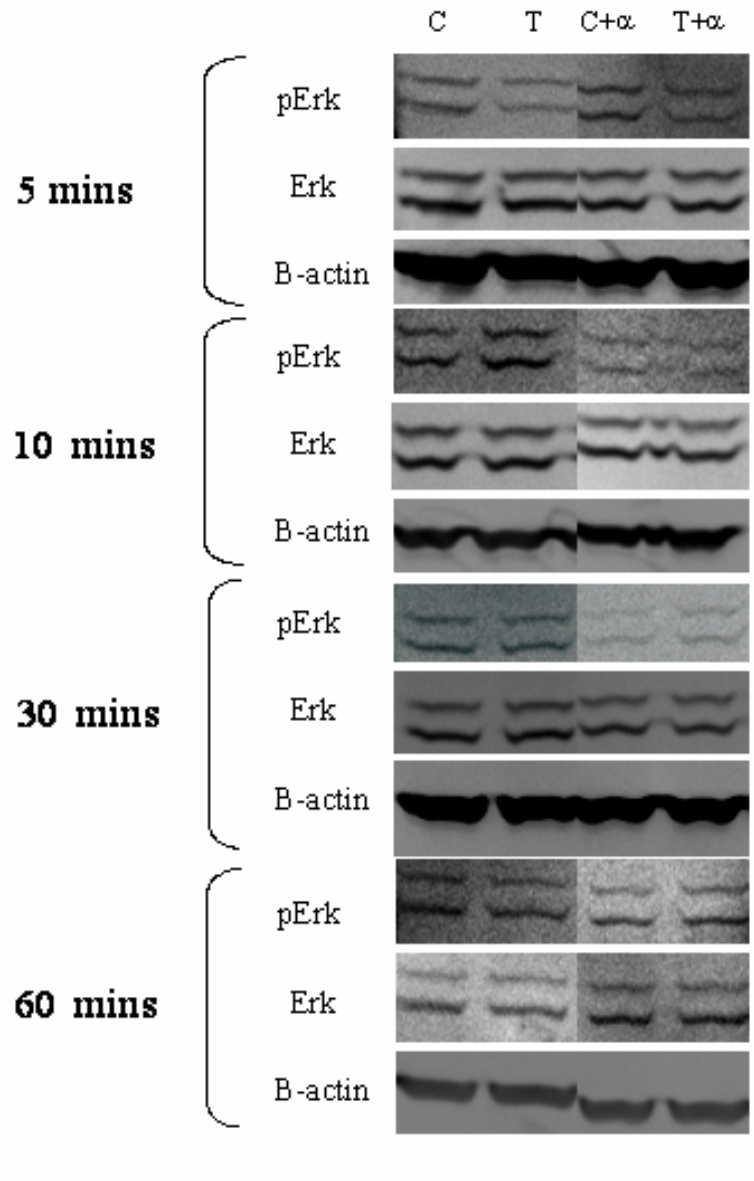

Figure 5.3. Treatment with AhR antagonist alters ERK phosphorylation status
Confluent A2058 cells were washed three times in Ca^{2+}/Mg^{2+} free PBS and cultured in serum and additive-free media for 24h. Cells were pretreated in serum and additive-free media with AhR antagonist, α-naphthoflavone (α) for 30min. Following pretreatment, cultures were co-treated with inhibitors and DMSO (vehicle) or 10nM TCDD for 5, 10, 30 and 60min. Treated cells were washed with ice cold PBS and harvested in fresh RIPA buffer. Protein concentration was determined using a modified Lowry assay. Samples were electrophoresis on a 4-12% Bis-Tris gel and transferred to nitrocellulose for western blot analysis. Blots were probed with primary antibodies to detect (A) total ERK levels and phosphorylated ERK levels as well as (B) total p38 and phosphorylated p38 levels. β-actin was used as a loading control. Blots were scanned using a Storm 860 Molecular Imager. Blots are representative of three independent experiments.

6.0. General Discussion

The data presented in this thesis contribute to our understanding of the interactions between the Ras/Raf and AhR signaling pathways. Furthermore, these data also suggest that the interaction between these pathways may be important for melanoma progression through dysregulation of matrix metabolism in invasion and metastasis. Given that a large proportion of melanocytic tumors contain mutations resulting in an overactive Ras/Raf signaling pathway, these data may also add to our knowledge of pathway interactions involved in Ras/Raf mediated events in melanoma, and provide potential targets for chemotherapy or diagnosis.

Previous findings from the White laboratory demonstrated that MMP expression and activity is enhanced by exposure to TCDD and activation of the AhR pathway (Murphy et al., 2004; Villano et al., 2005). The data presented in this thesis build upon these findings by identifying novel responsive elements in the distal portion of the MMP-1 promoter that are involved in AhR mediated TCDD induction, including NFκB, CCAAT and MITF cis-acting elements. Interactions between the NFκB and AhR pathways have been demonstrated in a variety of cell types (Camacho et al., 2005; Ruby et al., 2002; Singh et al., 2007). The CCAAT site has also been shown to have roles in AhR signaling as seen in adipocyte differentiation (Hanlon et al., 2003; Liu et al., 1996). Although the final element, the MITF site, may have less of an effect than the NFκB or CCAAT elements in TCDD induction, its gene amplification has been found in 10% of primary and 20% of metastatic melanomas suggesting it active involvement in melanoma progression (Fecher et al., 2007).

Interestingly, the identified cis-acting elements in the MMP-1 promoter support other data indicating that the interactions between the Ras/Raf signaling pathway and the AhR signaling pathway modulate matrix metabolism. The cell line used in these studies, the A2058 melanoma cells, contain a mutation in the BRaf gene that is common in human melanomas (Davies et al., 2002). This mutation results in constitutive activation of the Ras/Raf signaling and the data presented here suggest that Ras/Raf activation modulates AhR signaling. There is direct evidence linking transcription factors of the binding sites found to facilitate MMP-1 responsiveness to Ras/Raf signaling. Ras/Raf/Mek/ERK signaling, has been shown to induce NκB signaling (Liu et al., 2007) suggesting a possible reasoning for elevated MMP-1 production in A2058s compared to normal human melanocytes. A possible option for TCDD-increased MMP-1 signaling due to the NκB site located in its promoter comes from the formation of a heterodimer of NκB family member, RelB and AhR that is able mediate interleukin-8 transcription (Vogel et al., 2007). The CCAAT element transcription factor, C/EBPβ, is also effected by oncogenic Ras signaling. Oncogenic Ras stimulated C/EBPβ activation of a C/EBP-responsive promoter and this activation required ERK phosphorylation (Zhu et al., 2002). Lastly it was seen that both of these sites are involved in MEKK1-induced MMP-1 expression in synovial fibroblasts through NFkB p65 and C/EBPβ binding (Faour et al., 2006). Taking all the above data into consideration it is probable that both an NFκB and C/EBP family member are binding to these sites and regulating TCDD-induced MMP-1 expression. The role of AhR in mediating this activation requires further investigation.

Data resulting from this thesis also show that interactions between the Ras/Raf and AhR signaling pathways alter expression of other MMPs besides MMP-1 and that

differential effects of MMPs and their endogenous inhibitors may result in dysregulated matrix metabolism. TCDD-induced expression of both MMP-2 and MMP-9 are reduced by Ras/Raf inhibition. MMP-2 and MMP-9 are members of the gelatinase family of MMPs and have been implicated in tumor invasion through mediating degradation of basement membrane and extracellular matrix components (Hofmann et al., 1999; Itoh et al., 1999; Walker and Woolley, 1999). I further show that TCDD-induced MMP-9 expression levels are completely lost upon Ras/Raf inhibition, similar to that seen with MMP-1. TCDD-induced MMP-2 expression is reduced by Ras/Raf signaling inhibition, but this may be mechanistically different from that observed with MMP-1 and MMP-9, as there was a significant increase in MMP-2 basal expression following Ras/Raf inhibition. This indicates that that Ras/Raf signaling may be responsible for repression of MMP-2 and when this pathway is inhibited the repression is released. Interestingly, the MMP-2 inhibitor, TIMP-2, also shows a similar increase in basal level expression upon Ras/Raf inhibition. Since TIMP-2 expression also increases in coordination with MMP-2 it is likely that this potential increase in MMP-2 activity would be offset by TIMP-2 inhibition.

Data presented in this thesis also suggest that the effect of Ras/Raf signaling may, in part, be due to alterations in the major constituents of the AhR pathway, the AhR and the AhRR. Previous data from the White laboratory, along with data presented in this thesis, demonstrate that the A2058 cells express relative high levels of AhR compared to AhRR and this expression profile favors TCDD-induced AhR signaling. Inhibition of Ras/Raf signaling changes this expression profile such that AhR levels decrease in the A2058 cells while AhRR levels increase and this alteration in expression may cause a

decrease in AhR signaling due to increased AhRR repression. These data are the first indication that Ras/Raf signaling can affect AhR signaling pathway constituents and further studies on protein levels are necessary to see if these expression changes correlate to changes in receptor and repressor levels. It is interesting to note that a very recent publication implicates AhRR as a putative tumor suppressor due to reduced expression in malignant tissues of the lung, colon, breast, stomach cervix and ovary (Zudaire et al., 2008). Data presented in this thesis show that compared to normal human melanocytes, A2058 melanoma cells also have reduced AhRR expression. Therefore, Ras/Raf signaling inhibition is able to lower levels of AhR and increase levels of AhRR, indicating that Ras/Raf may facilitate inhibition of TCDD through modulation of the AhR signaling pathway mediators. This inhibition of AhR may ultimately result in decreased MMP-1 and MMP-9 AhR-dependent production.

For the first time, it has been demonstrated that inhibition of AhR signaling can result in alterations of Ras/Raf signaling. Treatment of A2058 cells with an AhR antagonist, α-naphthoflavone, resulted in decreased ERK phosphorylation, indicating that the AhR signaling pathway is also able to mediate Ras/Raf-dependent signaling. Physical interactions between the AhR and ERK have been noted in hepa1c1c7 cells and it is possible that if there were such a complex in A2058 cells, inhibition of the AhR could result in decreases of ERK phosphorylation (Chen et al., 2005).

In conclusion the results presented here demonstrate that Ras/Raf signaling is essential for AhR-mediated TCDD induction of MMPs (Figure 6.1.). Furthermore, cross-talk between the Ras/Raf and the AhR signaling pathways has been demonstrated through effects of inhibition of each pathway on the alternate endpoints, such as AhR/AhRR

expression and ERK phosphorylation. Taken to together these data suggest that tumor cells containing alterations in the Ras/Raf signaling pathway may be more susceptible to AhR-ligands, and exposure to such ligands may favor a more invasive phenotype. Furthermore, these data also suggest that the AhR pathway is in some way modulating Ras/Raf signaling, by contributing to its overactivity. This suggests that the AhR pathway may be an ideal target for chemotherapies, as it would have a low impact on normal cells, but may disrupt the overactive Ras/Raf signaling that contributes to tumor growth and progression (DeLuca et al., 2008).

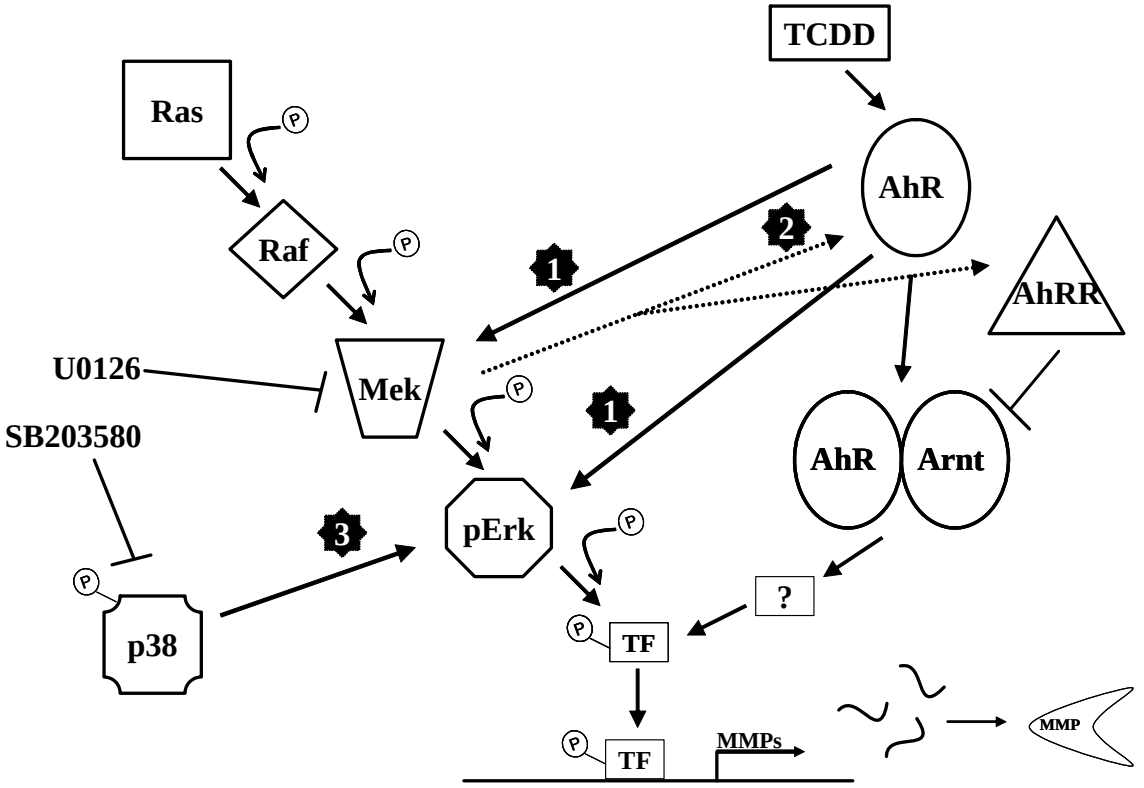

Figure 6.1. Schematic of potential AhR-Ras/Raf signaling pathway interactions
The data in this report indicate that the AhR pathway and the Ras/Raf signaling pathway intersect in A2058 melanoma cells: 1) The AhR is required for maximal Erk phosphorylation and is therefore either inhibiting up-stream activation of the Ras/Raf pathway or directly inhibiting Erk phosphorylation (Fig 6A). 2) Inhibition of Mek1/2 of the Ras/Raf signaling pathway by U0126 results in decreased AhR and increased AhRR expression indicating a role for Ras/Raf signaling in the regulation of AhR signaling pathway (Fig 7). 3) Inhibition of p38 by SB203580 results in a reduction of Erk phosphorylation suggesting that p38 signaling may be required for maximal Ras/Raf signaling or that SB203580 has an effect on Erk phosphorylation (Fig 6A). Data presented here reveal a requirement for Ras/Raf and AhR signaling for TCDD-induced MMP and TIMP expression.

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Cirriculum Vitae

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RESEARCH EXPERIENCE

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PUBLICATIONS

Murphy KA, White LA. The aryl hydrocarbon receptor (AhR) and the Ras/Raf signaling pathways are required for AhR- induced expression of matrix metalloproteinase-1(MMP-1) in melanoma cells. J Cell Physiol (submitted)

Hillegass JM, **Murphy KA**, Villano CM, White LA. 2006. The impact of aryl hydrocarbon receptor signaling on matrix metabolism: implications for development and disease. Biol Chem 387(9):1159-1173.

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Murphy KA, Villano CM, Dorn R, White LA. 2004. Interaction between the aryl hydrocarbon receptor and retinoic acid pathways increases matrix metalloproteinase-1 expression in keratinocytes. J Biol Chem 279(24):25284-25293.

PRESENTATIONS

SEMINARS

2007 Department of Biochemistry and Microbiology Seminar Series
The Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on matrix metalloproteinases.

POSTERS

Murphy, K.A. and White, L. A. (2008) Interaction between the aryl hydrocarbon receptor (AhR) and the Ras/Raf signaling pathways is required for AhR- induced expression of matrix metalloproteinases. Society of Toxicology 47th Annual Meeting

Murphy, K.A. and White, L. A. (2007) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced matrix metalloproteinase-1 expression in A2058 melanoma cells requires the AhR and Erk pathways. Society of Toxicology 46th Annual Meeting

Murphy, K.A. and White, L.A. (Nov 30th 2006) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced matrix metalloproteinase expression in A2058 melanoma cells. Frontiers of Biopharmaceutical Sciences Symposium, Rutgers University, Piscataway, NJ

Murphy, K.A. and White, L.A. (2006) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced matrix metalloproteinase expression in A2058 melanoma cells. Society of Toxicology 45th Annual Meeting

Murphy, K.A. and White, L.A. (2005) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin alters expression of retinoic acid receptors in normal human keratinocytes. Society of Toxicology 44th Annual Meeting