OSTEOPONTIN AS A REGULATOR OF LEUKEMIA INHIBITORY FACTOR mRNA LEVELS IN THE AtT-20 MOUSE PITUITARY CELL LINE

By

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ABSTRACT OF THE THESIS

Osteopontin as a Regulator of Leukemia Inhibitory Factor mRNA Levels

in the AtT-20 Mouse Pituitary Cell Line

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The highly phosphorylated glycoprotein Osteopontin (OPN) is a multifaceted protein with a diversity of roles in many immunological processes, and has recently been found to have a significant role in the Hypothalamus-Pituitary-Adrenal (HPA) axis. Its role was discovered when unstressed OPN-knockout mice were found to have abnormally high basal corticosterone levels, which is the hormone typically elevated following stress induction of the HPA axis. Another protein rigorously studied and repeatedly identified in the successful functioning of the HPA axis is Leukemia Inhibitory Factor (LIF). I propose that OPN may possess a regulatory role in the expression of LIF, with the absence of OPN leading to a greater abundance of LIF mRNA, and consequently, over-production of corticosterone in non-stressful situations.

Using the mouse anterior pituitary cell-line AtT-20, a common and highly useful model in HPA axis research, I have found evidence that treatment of these cells with OPN partially inhibits the expression of LIF mRNA. The dose-dependency of this inhibition appears to behave as either positive or negative depending on the cellular density of the culture treated with OPN.

Should OPN turn out to be a regulator of LIF mRNA expression, then absence of OPN may lead to an over-abundance of LIF, therefore affecting the expression of several proteins downstream of LIF that potently stimulate corticosterone production, such as the cholesterol transport protein StAR. It may turn out that OPN has an especially significant and indispensable role in the HPA axis via regulation of LIF mRNA levels.

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ABBREVIATIONS

ACTH……..……..Adrenocorticotropic Hormone

AVP……...……Arginine Vasopressin

CRH…….Corticotropin-Releasing Hormone

CRS…….....Chronic Restraint Stress

GC…….……Glucocorticoid

HPA……Hypothalamic-Pituitary-Adrenal

JAK…….……..Janus Kinase

LIF…….Leukemia Inhibitory Factor

LPS…….Lipopolysaccharide

OPN……....Osteopontin

POMC……..…Pro-opiomelanocortin

StAR……..Steroidogenic Acute Regulatory Protein

STAT……..Signal Transducer and Activator of Transcription

1. INTRODUCTION

1.1 Purpose of this review

Osteopontin (OPN) is a pleiotropic phosphoglycoprotein expressed in a variety of tissues that can act as a cell-to-cell messenger, bind to integrins, and induce NF-κB activation during inflammation and other stress conditions (Wang et al., 2008). It plays a particularly important role in immune function as a chemoattractant for T cells and macrophages, as well as a modulator of T helper cell cytokine polarization through enhancement of IL-12 and inhibition of IL-10 cytokine production by macrophages, augmenting a greater TH1-to-TH2 response ratio (O' Reagan et al., 2000; Denhardt et al., 2001). OPN even assists in stress-induced lymphocyte apoptosis within the spleen and thymus (Wang et al., 2007). The OPN protein has been observed in a secreted (sOPN) and intracellular (iOPN) form, with each isoform expressed differently depending on immune cell type and situational response (Wang et al., 2008). Both sOPN and iOPN are a product of the same full-length OPN mRNA, differing as a result of alternative translation (Shinohara et al., 2008). The two forms are believed to be strongly implicated with proper immunological response. And so it is evident from a multitude of preceding studies that OPN is a highly multifaceted protein substantially involved in immune function.

The immune system is one of the major communicators in the activation and regulation of the primary stress response system known as the hypothalamic-pituitary-adrenal (HPA) axis. In fact, proper HPA axis operation is highly dependent on the immune system. As previously stated, OPN expression is involved in an array of immunological reactions, which includes being up-regulated at sites of tissue

inflammation and repair (Denhardt et al., 2001). Based upon these facts, it may be hypothesized that OPN plays some role in the vital bidirectional communication of the immune system and HPA axis.

In 2009 it was reported that OPN is indeed somehow involved in maintaining proper HPA axis activity, before and after chronic restraint stress (CRS) (Wang et al., 2009). OPN's significance in the stress pathway was identified when researchers observed that mice deficient in OPN were found to display much higher than normal basal corticosterone levels in comparison to wild type mice (Wang et al., 2009). This unusually high corticosterone phenotype was displayed in these mice both before and after stress.

Typically in wild-type mice plasma levels of both corticosterone and adrenocorticotropic hormone (ACTH, the primary stimulator of corticosterone) are low prior to stress. Once subjected to CRS, there is an increase in ACTH levels subsequently followed by an increase in corticosterone levels (Wang et al., 2009). The rising corticosterone levels lead to an inhibitory feedback on ACTH release, followed by plasma reduction of corticosterone levels due to a lack of stimulation by ACTH (Wang et al., 2009). Unexpectedly, OPN knockout mice showed substantially elevated corticosterone basal levels compared with WT mice, and following CRS had only a marginal increase in ACTH in addition to just a marginal increase of the already elevated corticosterone levels (Wang et al., 2009). Despite the low ACTH levels, corticosterone levels remained high in knockout mice whether subjected to CRS or no stress at all (control) (Wang et al., 2009). It makes sense that ACTH levels in OPN knockout mice would always be relatively low due to the inhibitory effect created by elevated

corticosterone, but the mechanisms by which corticosterone levels remain high in the absence of stress (i.e. low ACTH) is not yet understood.

The following essay is a review of the HPA axis and how it relies on its close partnership with the immune system in regulating homeostasis due to inflammatory and pathogenic stresses. Throughout the following pages, I provide an argument as to why we believe a specific component of the immune stress pathway called leukemia inhibitory factor (LIF) may be responsible for the abnormal corticosterone production observed in mice deficient in OPN, in large part by identifying OPN as a regulator of LIF mRNA amount. Evidence reported in the published literature, along with our preliminary data, provide evidence that LIF may be a key component in explaining the abnormally high corticosterone levels displayed by OPN-deficient mice in the absence of stress.

1.2 Introduction to the HPA axis

The sustainability of a living organism is dependent on its capacity to maintain a physiological steady state or homeostasis, and therefore, must possess the ability to react and adapt to constant assault by physical or psychological stressors in order to survive (Bornstein et al., 1999). An adaptive and highly complex response system has evolved in order to combat internal and external stressors through a comprehensively coordinated response involving the endocrine, nervous, and immune systems (Haddad et al., 2002). Despite being classified as separate networks, these three systems must operate in a synergistic fashion in order for an organism to fully benefit in dealing with stress. The hypothalamus, pituitary, and the adrenal glands are the central organs involved in one of the two major peripheral limbs of the stress response system (the other being the

Sympathetic Nervous System), and their coordinated function between one another forms a hormonal axis (Bornstein et al., 1999).

The term “axis” (see **Fig. 1** below) refers to the ability of the hypothalamus, pituitary, and adrenal glands to interact with one another through sequential signaling, leading to alterations in hormone secretion in both a stimulating and negative feedback manner (Blaney and Millon, 2009). This hypothalamic-pituitary-adrenal (HPA) axis covers the functional use of the endocrine, central nervous, and circulatory systems, along with an increasingly profound bidirectional interaction with the immune system, which together sum up the most important body adapter defense to both endogenous and exogenous stress (Kudielka et al., 2005). This axis has been the subject of active investigation because of its role in adaptive stress, regulation of homeostasis, effect on diurnal systems, involvement in an assortment of physiological and psychological illnesses, and its immune-related therapeutic (Boumpas et al., 1993).

1.3 HPA Axis: The stress response system

The primary purpose of the HPA axis is to initiate an appropriate biochemical response to stressful stimuli whether it is non-cognitive such as physical trauma or immunological insult, or cognitive such as emotional distress (Buckingham et al., 1996). With its activation comes the modulation of an individual's body chemistry in order to cope with the given stressful situation, and an attempt to restore homeostasis (Buckingham et al., 1996).

Basically anything that challenges homeostatic systems that must be maintained in narrow ranges such as blood pH or body temperature will stimulate HPA axis activity

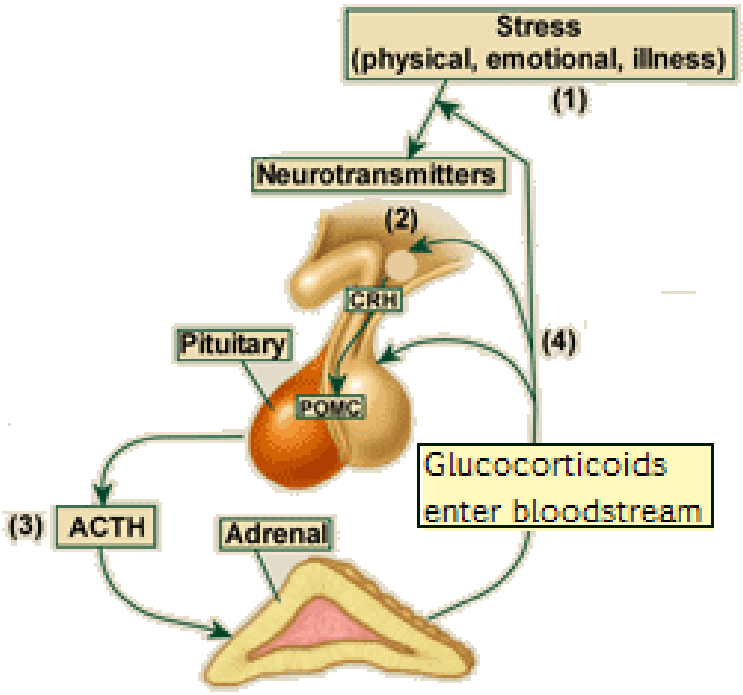
(McEwen et al., 1998). Allostasis must also be maintained, although these adaptive systems have much broader boundaries (McEwen et al., 1998). Allostatic systems enable us to cope and adapt to our changing physical states such as noise, crowding, physical threat, or infectious agents (McEwen et al., 1998). Other specific examples of stressors that activate the HPA axis are hunger, temperature extremes, physical restraint, viral or bacterial infection, taking an exam, or public speaking to name just a few (Turnbull et al., 1999; McEwen et al., 1998; Adam et al., 2007). Disruption of either homeostatic or allostatic systems (referred to as allostatic load) initiates a biochemical response that ultimately leads to the secretion of glucocorticoids from the adrenal cortex, leading to restoration of a physiological steady state (Newton, 2000; McEwen et al., 1998).

Glucocorticoids (GC) are the principal effecter hormones of the HPA axis that initiate the necessary alterations within the body in response to stress which include acceleration of motor reflexes, improvement of attention and cognitive function, an increase in pain threshold, and/or alterations in cardiovascular function (Chrousos, 1995). These responses help the body to quickly adapt and cope with the present stressful situation with the overall intention of restoring the body to homeostasis (Chrousos, 1995). The secretion of GCs into the bloodstream is not usually a direct endeavor, but the end result of a cascade of hormones and cell-signaling molecules from the hypothalamus, pituitary, and the adrenal gland (Bornstein et al., 2004).

The main players involved in GC release are corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) secreted from the hypothalamus, and the secreted anterior pituitary hormone ACTH, which is the primary stimulator of GCs from the

adrenal gland (Charmandari et al., 2005). This cascade of hormones all begins with a select group of neurosecretory neurons cells located in a region of the hypothalamus known as the paraventricular nucleus (PVN) (Haddad et al., 2002). A stressful stimulus leads to the release of CRH and AVP from the PVN into the hypophyseal portal system, where it migrates to the anterior pituitary and binds to a subpopulation of cells known as corticotrophs (Herman et al., 1997). CRH binding to corticotrophs leads to the up-regulation of pro-opiomelanocortin (POMC), which is subsequently cleaved via proconvertase 1 (PC1) into four separate peptides: β-lipotropin, β-melanocyte stimulating hormone, β-endorphin, and ACTH (Ware et al., 2005). The most pertinent of these molecules to the HPA axis is ACTH, which is the principal stimulator of GC production (Haddad et al., 2002). Although AVP binding does not stimulate the release of ACTH significantly per se, its contribution lies in its ability to act as a potent synergistic factor with CRH, amplifying the CRH-induction of ACTH (Charmandari et al., 2005).

Upon release, ACTH migrates through the bloodstream until it reaches its main target, the adrenal cortex (Charmandari et al., 2005). Binding of ACTH to the zona fascidulata of the adrenal cortex initiates steroidogenesis, leading to the production of GCs (Bornstein et al., 2004). Once in the bloodstream GCs will initiate a physiological response in a variety of tissues to help restore homeostasis. In the end, GCs will make their way to the anterior pituitary and hypothalamus and bind to type II glucocorticoid receptors, exerting negative feedback on CRH and ACTH secretion (Charmandari et al., 2005). The drop-off in ACTH plasma concentrations in turn leads to a reduction in GC plasma levels due to lack of ACTH-stimulated steroidogenesis of the adrenal cortex (Buckingham et al., 2006). **Fig. 1** provides a visual overview of the HPA axis.



[Thomas, 2010]

Fig. 1: The HPA Axis. **(1)** Stressful stimuli are perceived by the body, triggering chemical messengers that signal the release of CRH from the hypothalamus into the pituitary gland **(2)** CRH binds to corticotrophs within the anterior pituitary, simulating the expression of POMC, which is subsequently cleaved into ACTH. **(3)** ACTH migrates through the bloodstream where it ultimately acts on cells within the adrenal cortex, stimulating glucocorticoid production and release into the bloodstream. **(4)** Glucocorticoids such as cortisol (humans) and corticosterone (mice) initiate the physiological changes in order to cope with the particular stress, and at the same time exert negative feedback on the hypothalamus and anterior pituitary suppressing CRH and ACTH, respectively.

1.4 Glucocorticoids: The primary effectors of the HPA axis

GCs are steroid hormones produced by the adrenal gland that control carbohydrate, fat and protein metabolism, changes in cardiovascular function, such as increased heart rate, and inhibition of immune-mediated inflammation (Chrousos, 1995). GCs are the hormones predominantly expressed in response to stimulation of the HPA axis, and thus are the molecules predominantly responsible for mediating physiological changes necessary to cope with this stress. Although there are a variety of different GCs released from the adrenal cortex during stress, it is cortisol and corticosterone that are

most abundantly expressed during HPA activation in humans and rodents, respectively (Spackman and Riley, 1978; Kudielka et al., 2005). These two hormones carry out the majority of the metabolic changes that result from GC expression (Spackman and Riley, 1978; Kudielka et al., 2005).

Following secretion into the bloodstream, about 10% of GCs circulating in the body are found in their active freeform, while the rest are bound to corticosteroid binding globulin (CBG) and albumin (Chrousos, 1995; Kudielka et al., 2005). Free form GCs can diffuse through the plasma membrane readily and bind with cytoplasmic glucocorticoid receptors (Chrousos, 1995). In its non-activated state, the glucocorticoid receptor (GR) resides in the cytosol, and forms a complex together with two hsp90 heat shock proteins and immunophilin (Ku Tai et al., 1992; Charmandari et al., 2005). Once GC binds with GR the attached immunophilin and heat shock proteins dissociate and the new receptor-ligand complex then translocates from the cytoplasm into the nucleus through the nuclear pores (Chrousos, 1995). Once the hormone/receptor complex is inside the nucleus it then binds to glucocorticoid-responsive elements within the DNA, upon which it modulates the transcription rates of targeted genes (Drouon et al., 1992). GCs also display post-transcriptional effects in addition to transcription modulation, such as altering protein secretion rates and stability of specific messenger RNA (Chrousos, 1995; Dayanithi et al., 1989). Examples include the ability of the GC dexamethasone to reduce the half-life of Surfactant protein-A mRNA in human fetal lung in vitro, and as mentioned, the ability of GCs to suppress ACTH secretion from corticotrophs (Charmandari et al., 2005; Boggaram et al., 1991).

In addition to its metabolic effects, GCs have the ability to greatly alter immunological reactions as well. They are impressively potent at suppressing immune activity, primarily due to their ability to physically interact with the transcription factor nuclear factor kappa B (NF-κB) (Charmandari et al., 2005; Lee et al., 1998). NF-κB is a rapid response transcription factor that is highly involved in immune and inflammatory events, and is responsible for affecting the expression of pro-inflammatory genes such as cytokines, chemokines, and immunoreceptors (Chesnokova et al., 2002; Lee et al., 1998). GCs also repress inflammation by increasing the expression of inhibitory NF-κB (iNF-κB) (Chesnokova et al., 2002). Conversely, NF-kB abolishes transcriptional activation of glucocorticoid responsive genes, making GCs and NF-kB mutually antagonistic of each other (Chesnokova et al., 2002). This allows them to balance one another's activity, helping to avoid a runaway inflammatory response or a prolonged suppression of the immune system (Chesnokova et al., 2002). Needless to say, the coordination between GC secretion and immune function clearly demonstrates how imperative this relationship is to both HPA axis activity and immunity as a whole.

2. HPA AXIS AND THE IMMUNE SYSTEM

2.1 Cytokines as mediators of the HPA axis

Viral and bacterial infections, along with many non-infectious inflammatory reactions are among the most frequent stressors the body encounters, making the immune system a highly involved participant in the HPA axis (Haddad et al., 2002). The neuroendocrine and immune systems communicate in a bidirectional manner, and without this reciprocal communication the full function of both the HPA axis and the immune system would be incomplete (Turnbull et al., 1999). This is because GCs and immunologic processes have a profound regulatory affect on one another via negative feedback control (Turnbull et al., 1999). This elegant regulation is crucial because overstimulation of the immune system can lead to an assortment of autoimmune diseases such as multiple sclerosis, fibromyalgia, and rheumatoid arthritis; while conversely, overproduction of GCs can also lead to severe health consequences such as cardiovascular problems and enhanced susceptibility to infectious diseases due to prolonged immune suppression (Kudielka et al., 2005). The prominent components of the immune system that play a pivotal role in HPA axis stimulation and assist in GC regulation are the immunoregulators known as cytokines.

In general, cytokines are pleiotropic polypeptide mediators that regulate the function of a variety of different cell types and contribute to cell growth and differentiation (Turnbull et al., 1999). In addition to being anatomically intertwined, the immune, endocrine, and central nervous systems all share cytokine signaling as a common chemical language (Patterson, 1994). These peptides contribute to regulating tissue repair, haemopoiesis, inflammation, and the specific and non-specific immune

responses, as well as acting as endocrine factors regulating pituitary development, hormone secretion, and feedback control of the HPA axis (Haddad et al., 2002). Cytokines are rapidly induced in response to tissue injury, infection, or inflammation, and affect HPA axis activity mainly by stimulating the secretion of CRH and ACTH (Chesnokova et al., 2002; Bornstein et al., 2004).

In healthy tissue cytokine expression is typically low, however, physiological stresses to tissue homeostasis such as disease, infection or trauma will induce rapid production (Turnbull et al., 1999). There is a vast collection of different individual cytokines, and the particular cytokine expression profile in response to stress is dependent on the nature of the threat. For example, the bacterial cell membrane component and endotoxin lipopolysaccharide (LPS) will lead to slightly different cytokine expression than infection by cytomegalovirus (CMV) in mice (Silverman et al., 2005; Beishuizen et al., 2003). These two responses appear to be exactly the same except that bacterial LPS also induce the cytokine IL-1β (Turnbull et al., 1999). This subtle difference provides evidence of how cytokine expression contributes to the complexity and specificity of the immune systems participation in the HPA axis.

Although numerous cytokines have been linked with the stress response, the vast majority of HPA axis stimulating activity found in plasma can be attributed to the cytokines IL-6, IL-1, and TNF alpha (Chrousos, 1995). All three share similar biological activities and are expressed sequentially during immunological and inflammatory challenge, with TNF alpha secretion occurring first, quickly followed by IL-1 secretion, and finally IL-6 (Chrousos, 1995). These three as well as with most pro-inflammatory cytokines stimulate the HPA axis at the hypothalamic level through CRH and AVP

induction, and can also directly increase POMC expression and circulating ACTH by acting on corticotrophs of the anterior pituitary (Chrousos, 1995; Chesnokova et al., 2002; Ware et al., 2005).

While the resulting plasma levels of immune-derived cytokines during stress are believed to be too low to have a direct impact on adrenal production of GCs, the adrenal cortex is extensively infiltrated by macrophages that readily secrete different cytokines locally, including IL-1, IL-6, and TNF alpha, providing perhaps great enough concentrations for adrenal GC stimulation (Bornstein et al., 2004). It is already known that IL-6 can exert a direct effect on adrenal production of GCs if in great enough supply by acting on adrenocortical cells (Bamberger et al., 2000). In fact, one study has shown that transgenic mice constitutively expressing IL-6 have a greater increase in corticosterone levels than wild-type mice when subjected to acute immobilization stress (Raber et al., 1997). Fascinatingly, although these IL-6 transgenic mice had enhanced corticosterone production when compared with their control counterparts, they did not exhibit any significant elevation in circulating ACTH. This suggests that not only does IL-6 stimulate the HPA axis at the hypothalamic and pituitary level, but can also act directly on the adrenal gland to stimulate steroidogenesis without the need for elevated ACTH release (Raber et al., 1997).

The publications surrounding the ability of IL-6 to enhance corticosterone production independent of ACTH have led me to propose that aberrant cytokine expression is a possible explanation for the elevated corticosterone levels observed in unstressed OPN-knockout mice mentioned in the introduction. LIF is another cytokine within the same family as IL-6 that has also been shown to be an integral component to

the HPA axis. I suggest that the studies involving IL-6 are supportive to the idea that LIF can also enhance GC production in the absence of ACTH due to the fact IL-6 and LIF are closely related cytokines that share the similar receptor subunit gp130 and both activate JAK/STAT signaling pathway (Bamberger et al., 2000; Heinrich et al., 1998). I have chosen to investigate LIF as the culprit instead of IL-6 due to the fact that LIF plays a much more active role in ACTH and corticosterone levels before and after stress, whereas IL-6 is involved primarily following stress.

2.2 LIF: A key cytokine in proper HPA axis maintenance

Although many of the published findings surrounding the activation of the HPA axis via cytokines involve specifically IL-1, IL-6, and TNF-α, there are a plethora of additional cytokines that participate in this stress pathway as well. Other interleukins such as IL-8, IL-4, IL-9, IL-10, IL-13, IL-12, IFN-γ, and CSF, all contribute as either inhibitory or augmenting factors upstream of CRH and ACTH expression (Turnbull et al., 1999; Buckingham et al., 1996). Of particular interest is that of LIF, a pleiotropic polypeptide originally given its name from its ability to induce the terminal differentiation of myeloid leukemic cells. LIF has since been discovered to be a highly multifaceted cell signaling molecule involved in bone metabolism, cachexia, neural development, embryogenesis and inflammation (Chesnokova et al., 1998**)**.

LIF is expressed in many tissues including human fetal, adult, and murine pituitary cells; its expression within anterior pituitary corticotrophs has been discovered to be imperative in maintaining basal level POMC transcription and ACTH release (Akita et al., 1995; Patterson, 1994; Ray et al., 1996). LIF knockout mice show an extremely blunted increase in plasma ACTH in response to stress, although corticosterone plasma

levels appear no different than wildtype mice (Chesnokova et al., 1998). When in higher than normal supply, LIF can actually further enhance POMC gene expression and subsequent ACTH release (Chesnokova et al., 1998). For instance, studies involving incubation of murine corticotroph AtT-20 cells or human fetal pituitary cells with added LIF (1nM) documented a 2 to 4-fold increase and 29% increase of ACTH, respectively (Auernhammer and Melmed, 2000). Not only can LIF induce ACTH secretion alone but has a profound synergistic affect with the actions of CRH, a property also seen with AVP. AtT-20 cells co-incubated with CRH and LIF exerts a 2 to 3-fold greater increase of ACTH secretion in comparison to CRH alone (Auernhammer and Melmed, 2000). LIF's ability to enhance ACTH secretion appears to be a result of its affect on POMC expression as well as its ability to increase the activity of PC-1, the enzyme responsible for cleaving POMC into ACTH (Ware et al., 2005). Altogether, LIF is highly involved in the proper maintenance *and* activation of the HPA axis, making this protein relevant to almost any research on the stress pathway.

The mechanism by which LIF exerts its affects on POMC transcription is through binding to the LIF receptor (LIFR) (Auernhammer and Melmed, 2000). LIF receptors, along with IL-6 receptors, possess a key receptor subunit called gp130, an important feature that places them within the same cytokine family (Chesnokova et al., 2002). Both LIF and its receptor are expressed centrally in the hypothalamus, within the anterior pituitary cells, and have been detected throughout the normal human adrenal cortex (Chesnokova et al., 2002; Mikhaylova et al., 2008). LIF and its relatives such as IL-6 affect transcription by first binding to their respective receptors, which stimulates JAK kinases, leading to subsequent phosphorylation of STAT1 and STAT3 (Ray et al., 1996).

The phosphorylated STAT proteins translocate to the nucleus and participate in the assembly of transcription factor complexes, which in turn alter POMC gene expression (Ray et al., 1996). The expression of LIF in pituitary cells predominantly occurs within corticotrophs, making it readily available in assisting POMC expression (Ray et al., 1997).

The importance of LIF expression to the HPA axis is of particular interest to our current study involving the phenomenon of elevated corticosterone levels in OPN-knockout mice for several reasons. First, it is an essential component in maintaining proper HPA axis activity. Second, LIF has been found to be able to stimulate steroidogenesis independent of ACTH in a similar fashion to its close relative, IL-6 (Mikhaylova et al., 2008). And finally, LIF and OPN are both expressed at sites of inflammation, which leads me to the prediction that these two immune-induced proteins have a regulatory affect on one another (Patterson et al, 1994; Wang et al., 2008). Our research involves the treatment of the AtT-20 mouse anterior pituitary cell line with OPN followed by analysis of LIF mRNA expression. The AtT-20 cell line is of murine corticotrophs derived from pituitary tumors developed in mice following exposure to ionizing radiation of atomic blasts (Schiller, 2000). These tumors constitutively expressed POMC and ACTH, along with regular expression of LIF, and because of these characteristics the AtT-20 cell line is used extensively in HPA axis research. Dr. Richard E. Mains, who has spent several decades investigating the murine cell line, has kindly supplied AtT-20 cells for the work of this thesis (Mains et al., 1999). AtT-20 cells serve as a good model in observing changes in LIF mRNA levels from OPN-treatment in a way that is relevant to the HPA axis. Through careful experimentation we have discovered

that OPN may have a role in regulating the abundance of LIF mRNA in murine corticotrophs, which has led us to later hypothesize why aberrant LIF expression may explain the elevated basal corticosterone levels exhibited by OPN-deficient mice.

3. MATERIALS & METHODS

Cell Culture. AtT-20 cells, kindly provided by Dr. Richard Mains (University of Connecticut), were grown in DMEM supplemented with L-glutamine, streptomycin, and penicillin (all supplied by Invitrogen). Mouse OPN was purified from medium conditioned by a ras-transformed murine embryonic fibroblasts line (275-3-2) (Wu et al, 2000).

Osteopontin Purification. Murine osteopontin used was from the same source as that used in Wang et al., 2009, in which the purification protocol was described by the authors exactly as the following, "Mouse OPN was purified from serum-free medium conditioned by a rastransformed murine embryonic fibroblast line (275-3-2) (17). The medium was incubated with 1 ml of protein G beads (Pierce), to which the 2A1 anti-OPN mAb had been cross-linked. The beads were washed and packed into a 2-ml disposable column. OPN was eluted from the 2A1-protein G beads with 100 mM glycine and 500 mM NaCl (pH 2.5) and collected into tubes containing a neutralizing pH 8 Tris-Cl buffer. Fractions were analyzed by SDS-PAGE and proteins were visualized by non-ammoniacal silver staining and Western blotting. Positive fractions were pooled, desalted on PD-10 columns (GE Healthcare Bio-Sciences), quantified by ELISA, and lyophilized (Wang et al., 2009)."

In Vitro Assays. AtT-20 cell cultures were grown to ~60-75% confluency, unless stated otherwise. Cell confluency was determined by visual estimation of cell cultures under a light microscope. AtT-20 cells were cultured in serum-free medium for 18 hr prior to experimentation. Cells were then treated with a variety of OPN concentrations (1.0 μg/ml

0.7 μg/ml, 7 ng/ml, 0.07 ng/ml) and incubated for 6 hr. RNA extraction was initiated immediately upon completion of the 6-hr incubation.

RNA Extraction. RNA isolation was accomplished immediately following completion of each treatment. Cell culture medium was aspirated, and TRIZOL Reagent (Invitrogen; Carlsbad, CA) and was added (500 μl of Trizol was added per 2 cm^2 well of a six-well plate). Subsequent purification was performed as described by Invitrogen. The final preparation of RNA was then tested for purity by spectophotometry and accepted if 260/280 > 1.8.

RT-PCR and PCR DNA Analysis. Complementary DNA (cDNA) was synthesized from total RNA recovered from each treatment of AtT-20 cells. Through O.D. measurements, the concentration of RNA could be acquired for each sample and allowed for the ability to calculate what volume of each sample contained 4 μg of RNA for the equal creation of cDNA. Also, it should be noted that cDNA levels are assumed to accurately represent mRNA levels, although there is no way to confirm this and may not ultimately be the case. The RT reaction volume was 27 μl and included 4 μg RNA of interest, 4 μl of 2'deoxynucleoside 5'-triphosphate mix (2.5 mM dNTP; Invitrogen; Carlsbad, CA), 5 μM N6 random hexamer (Integrated DNA Technologies; Coralville, IA), and sterile H_2O, into a 200 μl centrifuge tube. The contents were first heated at 65° C for 5 min, followed by adding 4 μl of 5X first strand buffer and 2 μl of 1.0 M DTT (Invitrogen; Carlsbad, CA), with the contents incubated at 25° C for 10 min. Finally, 1 μl (1 Unit) of Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen;

Carlsbad, CA) was added and incubated at 37° C for 50 min. The reaction was inactivated by heating at 70° C for 15 min.

PCR was carried out using 4 µl of cDNA combined with a reaction mixture that included 5 µl of 10X PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 4 µl of 2.5 mM dNTP mix, 1 µl of *Taq* polymerase, and 30.5 µl of H_2O. PCR was performed for 35 cycles (95° C for 30 sec, 53.5° C for 60 sec, 72° C for 30 sec) with a 3-min extension at 72° C. RT-PCR and PCR were carried out using the GENEAMP PCR system 9600. Primer sequences were as follows: Murine LIF primers were kindly provided by Dr. Yi Ren, primer sequence is unknown, amplicon size-720 bp; LIF2 forward, 5'-AAACGGCCTGCATCTAAGG-3', LIF2 reverse, 5'-AGCAGCAGTAAGGGCACAAT-3', amplicon size 93-bp.

Quantification of PCR Products. PCR amplicons were quantified using the Java imaging processing program ImageJ. Following electrophoresis DNA gels were photographed and saved as a JPEG file. JPEG images were opened using ImageJ and each cDNA band was individually highlighted using the square tool. Band intensity was then measured using the analyze and measure features. The results displayed a series of peaks that corresponded to the highlighted bands, and the area of these peaks were measured and given a numerical value based on area. The area of the peak increases with brightness.

4. RESULT

4.1 Osteopontin affects LIF mRNA levels in AtT-20 cells

Our initial experiment involving the addition of OPN to AtT-20 cell cultures resulted in an inhibition of LIF mRNA levels in a dose-dependent manner. The highest concentration of OPN used (700 ng/ml) resulted in the greatest reduction of LIF mRNA (**Fig. 2**). Using a lower concentration of OPN (7.0 ng/ml) resulted in a reduction of LIF mRNA, although to a lesser extent (**Fig. 2)**. Finally, the lowest concentration of OPN (0.07 ng/ml) incubated with AtT-20 cells showed a slight reduction in LIF mRNA when compared with control (**Fig. 2**). Unfortunately, the primer set that was used became inactive following this initial experiment for reasons unknown. A new set of primers were synthesized (LIF2) and were used for all follow-up experiments.

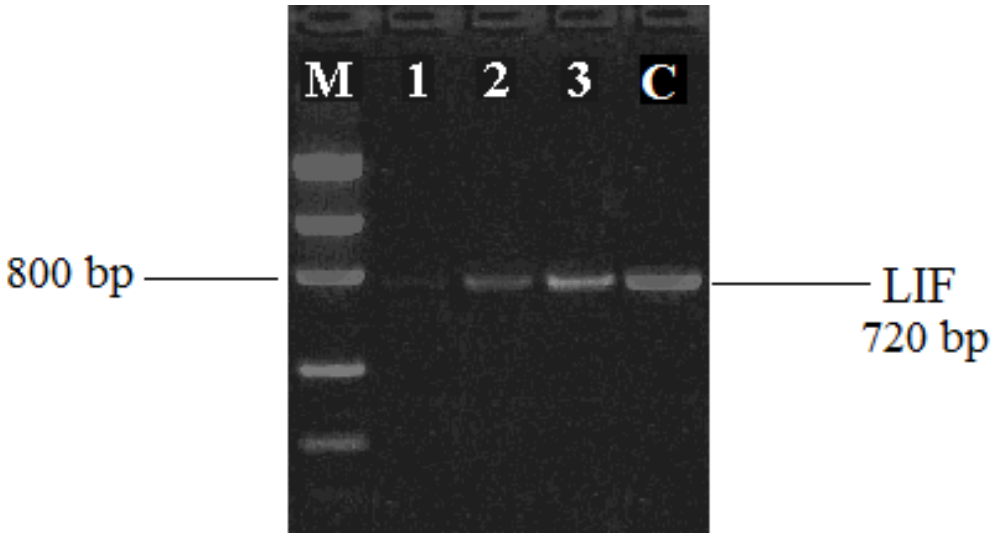


Fig. 2: LIF mRNA Levels in OPN-treated AtT-20 Cells. Agarose gel electrophoresis of amplified cDNA products of LIF isolated from AtT-20 cells either untreated (lane C), or treated with OPN at concentrations of 700 ng/ml (lane 1), 7.0 ng/ml (lane 2), or 0.07 ng/ml (lane 3). Inhibition of LIF by OPN appears to be dose-dependent. Lane M represents Low-MW RNA markers.

4.2 OPN affects LIF mRNA levels in AtT-20 cells of 60% confluency

A significant omission of the original experiment was the failure to record the confluency of the AtT-20 cell cultures treated with OPN. This led to two follow-up experiments in order to determine if the observed changes of LIF mRNA via OPN were dependent on the confluency of the cell culture. The first follow-up experiment performed involved the culturing of AtT-20 cells to ~60% confluency, while keeping the treatment concentrations of OPN from the previous experiment the same. Amplification of LIF was accomplished using the newly synthesized primer set LIF2. Additionally, the housekeeping gene GAPDH was also amplified to ensure equal loading. Unfortunately, the concentration of GAPDH primer used was too high leading to saturation upon amplification, and thus negating its intended purpose. Similar results were observed in this experiment as in the previous one, in which the highest concentration of OPN resulted in the greatest reduction of LIF mRNA, with this effect becoming less pronounced as the concentration of OPN decreased (**Fig. 3)**. This experiment confirmed the initial observation that OPN appears to affect the abundance of LIF mRNA in a negative manner. Also worth noting is that the product size using LIF2 primers is a rather small 100 base pairs, which is very close to location of bands formed from primer dimer (50 bp). While these two bands are similar in size, we strongly believe that the PCR products depicted in all figures are in fact LIF. This deduction was made following multiple experiments loading primer alone and observing the relative band intensity of primer dimer compared to putative LIF amplicons (data not shown). However, the band found in lane 3 appears too suspiciously low to be LIF and should be discredited.

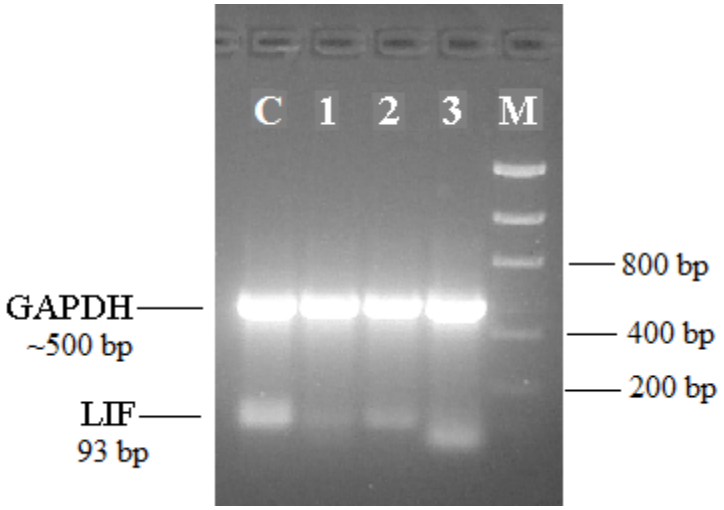


Fig. 3: LIF + GAPDH mRNA Levels in 60% Confluent OPN-treated AtT-20 Cells. Agarose gel electrophoresis of amplified cDNA products of LIF isolated from AtT-20 cells either untreated (lane C), or treated with OPN at concentrations of 700 ng/ml (lane 1), 7.0 ng/ml (lane 2), or 0.07 ng/ml (lane 3). Gel electrophoresis of GAPDH from the same RT-PCR samples is depicted as a semi-quantitative reference for equivalent amplification between samples. Lane M represents Low-MW RNA markers.

4.3 OPN inhibition of LIF mRNA in AtT-20 cells of 100% confluency

The first two experiments have provided evidence of OPN's ability to negatively affect LIF mRNA levels. To ultimately rule out confluency as a contributing factor to the previous observations, the next experiment performed was using fully confluent AtT-20 cell cultures. Once AtT-20 cells were grown to 100% confluency they were then incubated with the same OPN concentrations as in the prior two experiments, plus an additional treatment of OPN at 1000 ng/ml. The treatment of OPN at 1000 ng/ml was added to see if a higher concentration of OPN could exert an even greater reduction in LIF mRNA, and thus help define a limit to its inhibitory affect.

Unexpectedly, the affect of OPN on inhibiting LIF mRNA in fully confluent AtT-20 cells appears not to follow the trend previously seen in our experiments. The control still possessed the highest amount of LIF mRNA when compared with OPN-treated

groups, indicating that the ability of OPN to inhibit LIF mRNA was not lost in fully confluent AtT-20 cells. However, the pattern to this inhibition appeared to follow a trend that was dose-dependent in an inverse manner to previous experiments. In fact, the two highest OPN concentrations displayed the lowest reduction of LIF mRNA. Conversely, the lower OPN concentrations displayed the highest inhibition of LIF mRNA (**Fig. 4)**. The intensity of each band found in **Fig. 4** was measured using the program Image J, and the quantified data was placed in **Fig. 5**. As you can see in **Fig. 5,** lower concentrations of OPN are more effective at inhibiting LIF mRNA in fully confluent AtT-20 cells. **Fig. 5** provides a visual of the how the inhibition of LIF mRNA by OPN still follows a dose-dependent trend, albeit opposite of the trend observed in 60% confluent cells. Nonetheless, the inhibitory aspect of OPN against LIF mRNA appears present regardless of cell confluency.

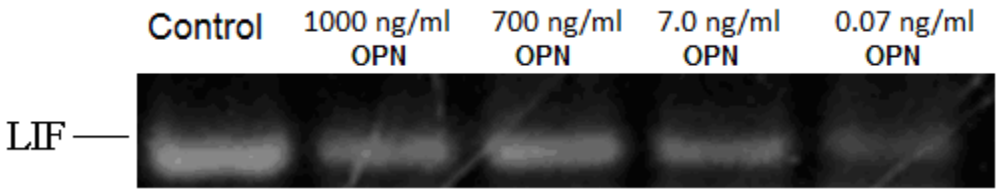


Fig. 4: LIF mRNA Levels in Confluent OPN-treated AtT-20 Cells. Agarose gel electrophoresis of amplified cDNA products of LIF isolated from AtT-20 cells either untreated (control), or treated with OPN at concentrations of 1000 ng/ml, 700 ng/ml, 7.0 ng/ml, or 0.07 ng/ml. The inhibitory effect in fully confluent cells appears to follow a dose-dependent trend that is opposite to that observed in non-confluent cells.

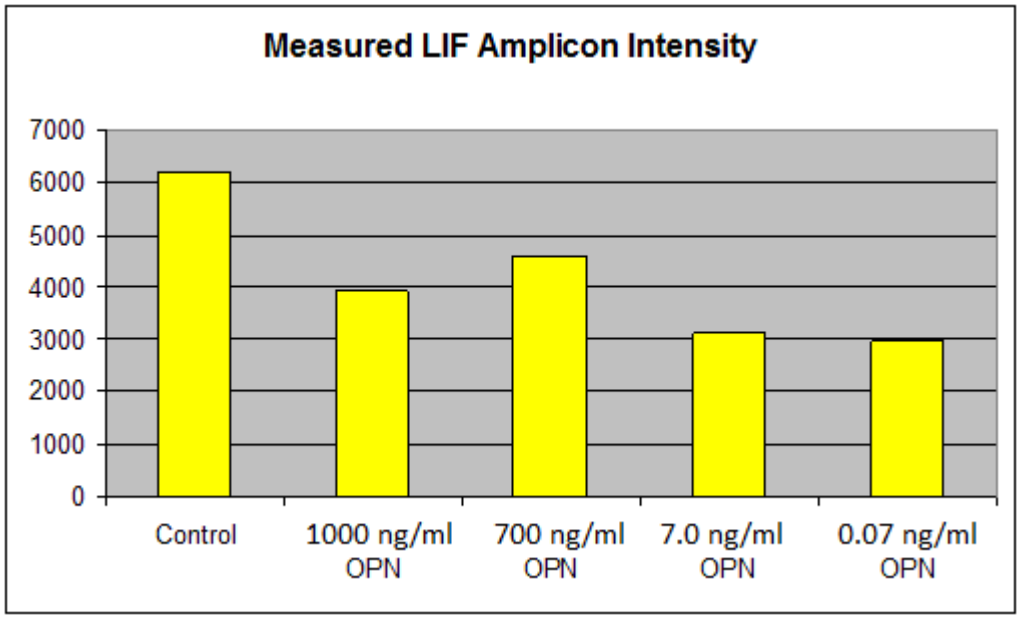


Fig. 5: Quantization of Fig. 4 LIF Amplicons using ImageJ Analysis. Quantitative data confirms LIF mRNA reduction by OPN in confluent AtT-20 cells is dose-dependent and that lower OPN concentrations show a greater inhibitory effect. Note the downward trend. Units are arbitrary.

4.4 Inhibition trend of LIF in confluent AtT-20 cells is not due to protocol error

To ensure that the previously obtained data were not a result of improper loading of mRNA used for the creation of cDNA, the housekeeping gene GAPDH was amplified in addition to the amplification of LIF. The intensity exhibited by each GAPDH amplicon appeared to be similar for all treatments, although they may have reached a saturation point (**Fig. 6)**. Since it is unclear whether or not GAPDH amplicons in **Fig. 6** are indeed saturated, the analysis continued as if they were not.

Given that each PCR reaction was set up using equal amounts of cDNA, it appears that this apparent reversed trend in the inhibition properties of OPN against LIF mRNA is a result of AtT-20 cell confluency. LIF amplicons from **Fig. 6** were quantified using the computer program ImageJ, and the resulting data show that the higher concentrations of OPN are less effective at inhibiting LIF when compared with the two

lowest concentrations (**Fig. 7)**. Reproducibly, the two lowest concentrations of OPN were the most effective at inhibiting LIF in both experiments involving fully confluent AtT-20 cells. Also worth mentioning is that the highest concentrations of OPN at 1000 ng/ml and 700 ng/ml varied considerably in the extent to which they inhibited LIF between the two experiments involving fully confluent cells (**Fig. 5, Fig. 7)**.

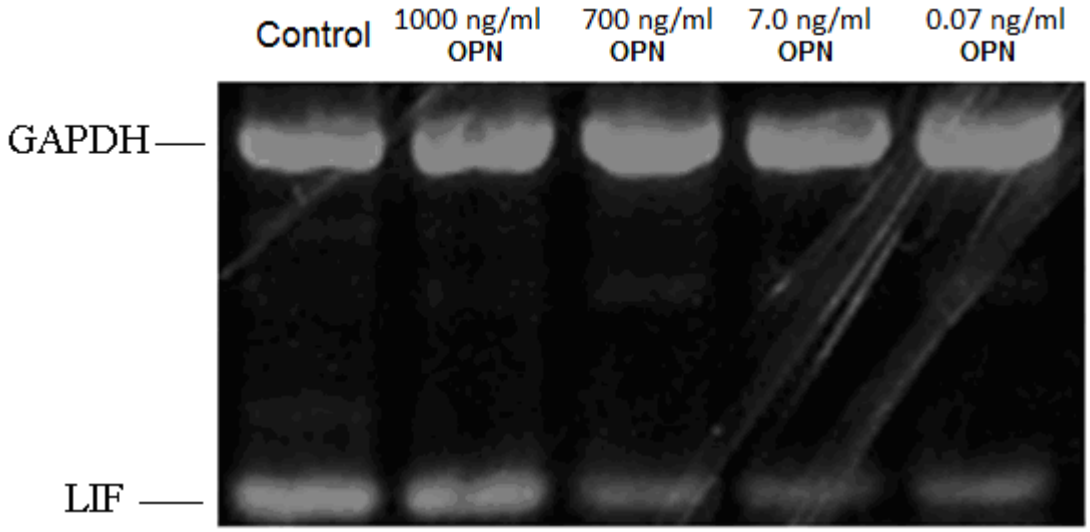


Fig. 6: LIF + GAPDH mRNA Levels in Confluent OPN-treated AtT-20 Cells. Agarose gel electrophoresis of amplified cDNA products of LIF isolated from AtT-20 cells either untreated (control), or treated with OPN at concentrations of 1000 ng/ml, 700 ng/ml, 7.0 ng/ml, or 0.07 ng/ml. Gel electrophoresis of GAPDH from the same RT-PCR samples is depicted as a semi-quantitative reference for equivalent amplification between samples. Note the clear downward trend with OPN concentration.

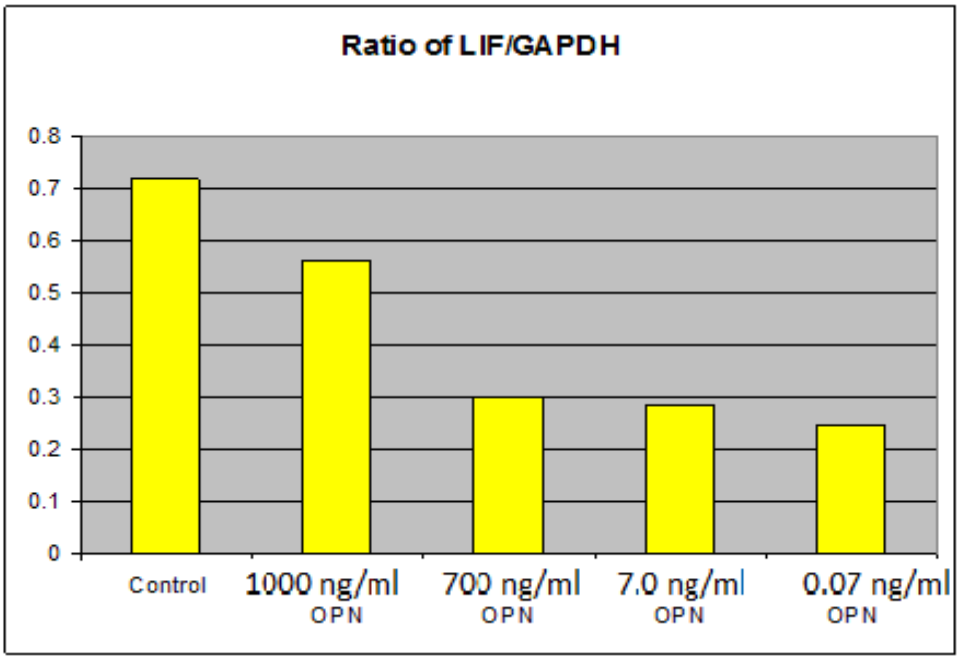


Fig. 7: LIF/GAPDH Ratio of Fig. 6 Amplicons using ImageJ Analysis. LIF/GAPDH ratios indicate that smaller rather than larger concentrations of OPN may have a greater reducing affect on LIF mRNA levels in 100% confluent AtT-20 pituitary cells. Units are arbitrary.

4.5 Osteopontin reduces LIF mRNA abundance in AtT-20 cells

Our data clearly suggests that OPN has the ability to affect LIF mRNA levels in AtT-20 cells. When cells are at ~60% confluency, OPN reduces LIF mRNA abundance in a dose-dependent manner, with the higher the concentration of OPN leading to a greater reduction in LIF mRNA. However, when AtT-20 cells are fully confluent the inhibitory affect on LIF mRNA by OPN is appears to follow a reverse trend with the lower concentrations of OPN being greater inhibitors of LIF mRNA abundance.

It is unfortunate that the original LIF primer set became mysteriously inactive after just one use since it provided the clearest and most dramatic distinction of LIF reduction by various OPN treatments. The problem may be that the original primer set produced amplicons of a more suitable size for LIF mRNA analysis (~723 bps) while the new LIF2 primers produced smaller and possibly less reliable amplicons of only around

100 bps. The reason for choosing primers that would generate a smaller product size was to double their use as primers for future qPCR reactions.

Even though there are discrepancies in the manner in which OPN inhibits LIF mRNA in AtT-20 cells, it appears to have an inhibitory affect on LIF mRNA levels nonetheless. Taking this one step further, we suspect that OPN may ultimately serve as a regulator of LIF transcription. And as we will now explain, perhaps the lack of regulatory control of LIF by OPN is in part an explanation for the elevated corticosterone levels observed in OPN knockout mice discussed in the introduction.

DISCUSSION

The aforementioned experiments provide evidence that OPN possesses the ability to reduce LIF mRNA amount in AtT-20 cells. This inhibitory effect appears to be dose-dependent, although the nature of this dependency may be determined by cell density. When AtT-20 cells are at 60-70% confluency the dose-dependent trend appears positive, with the inhibition of LIF becoming greater as OPN concentration increases. Fully confluent AtT-20 cultures seem to follow a dose-dependent trend in a negative manner, with LIF inhibition being greater as OPN concentration decreases.

While there appears to be some promising evidence of OPN as an inhibitor of LIF, there are a few caveats that need to be addressed. First, the method by which cell confluency was determined using visual estimation is admittedly subjective. What I estimate as confluent (>98% of the plate surface covered with cells) may not be considered so by another individual. There needs to be a way of cultivating a consistent cell density from one experiment to the next in order for the results to be considered truly reproducible. Also, it is well documented that as a cell culture nears confluency a variety of physiological changes may occur including cell differentiation and alterations in genetic expression, all of which could have a profound impact on experimental outcome (Pieper et al., 1999). Keeping the cell culture density consistent will be imperative for all future experiments.

A second word of caution comes when reporting that OPN inhibits LIF mRNA since this is not directly confirmed in my experiments. It was presumed in my research that the activity of reverse transcriptase successfully converted all mRNA into cDNA

equally, but such an assertion was not proved. As a result, it is more accurate to declare that OPN inhibits LIF cDNA amount in AtT-20 cells instead. For now, I maintain that these results are an indication of decreased LIF mRNA levels, albeit indirectly.

By correcting a few errors in the methodology, such as maintaining a consistent culture confluency between experiments and adjusting the concentration of GAPDH primer to prevent saturation levels, better attempts at providing more convincing evidence of OPN as an inhibitor of LIF mRNA levels can be made. It will be interesting to see if these protocol modifications can lead to a more consistent dose-dependent pattern by which LIF inhibition occurs. If so, future research will then consist of identifying the exact mechanism of this inhibition. A few possibilities are OPN binding with surface receptors (signal transduction) and affecting LIF transcription or by OPN directly interacting with the LIF RNA transcript.

Though the research presented in this thesis involves pituitary corticotrophs, it is the potential action of OPN as an inhibitor of LIF that I believe may possibly answer the larger question of how OPN regulates corticosterone in mice, which I will now attempt to explain.

Publications leading up to our current research have reported LIF and OPN as critical components in maintaining proper basal ACTH and corticosterone levels, respectively. Loss of OPN function leads to elevated basal corticosterone levels as seen in OPN-deficient mice. Identifying exactly how this phenotype arises, i.e. how does OPN contribute to the regulation of corticosterone in the absence of stress, is the focus of the presented thesis. With our preliminary studies providing evidence that OPN is a regulator of LIF mRNA levels in mouse corticotrophs, we hypothesize that the disruption of this

putative relationship is the cause for the elevated basal corticosterone levels observed in OPN-deficient mice.

Most studies involving LIF participation in the stress pathway center on its ability to enhance POMC transcription and ACTH release in pituitary corticotrophs. However, what we know from previous studies involving mice lacking OPN is that these mice display abnormally high corticosterone levels *without* the prerequisite increase of circulating ACTH. In defense of our proposed hypothesis, the question becomes how might a lack of regulatory control of OPN over LIF lead to aberrant steroidogenesis in mice in ways other than LIF enhancement of POMC transcription and ACTH release?

In order to begin answering this question we must narrow our search by locating the specific dysfunctional area(s) suspected in the stress pathway. This is a truly necessary endeavor when you consider that the HPA axis spans three main organs, in addition to a vastly intertwined communication between the nervous and immune systems via cytokines and catecholamines. A good starting place is in the identification of all known and suspected actions LIF possesses within this pathway. As previously mentioned, LIF has thus far been found to be most significant in its ability to stimulate POMC expression and ACTH release in anterior pituitary corticotrophs. However, the lack of elevated basal ACTH levels in OPN-deficient mice would lead one to believe that the augmented corticosterone production occurs downstream of the hypothalamus/pituitary. This examination has led us to deduce that the irregular corticosterone production in these mice is most likely some type of dysregulation at/within the adrenal glands. In order to support this prediction, it is imperative to identify the characteristic expression and function of LIF within the adrenal gland, and more

importantly, does it possess the capability to directly affect steroidogenesis in the absence of ACTH.

Unfortunately, there has been no preceding research surrounding LIF expression in mouse adrenal tissue at all (Woods et al., 2008). In fact, the recent discovery of LIF in bovine adrenal tissue is the first such identification within a species other than humans to be published (Woods et al., 2008). So in order to begin unraveling this mystery we must look to the studies involving LIF within the human adrenal gland, which are extensive enough to provide useful clues (Bamberger et al., 2000; Mikhaylova et al., 2008; Woods et al., 2008). A word of caution is that numerous drug candidates developed by pharmaceutical and biotech companies have proven very successful in laboratory mice, only to go on to be complete failures in humans (Reagan-Shaw et al., 2008; Pisano, 2006). In general, the disagreement in results is due to subtle differences between mouse and human biology (Reagan-Shaw et al., 2008). For all intents and purposes of this study and many others, the back and forth conversion between human and murine cellular biology is currently the best method we have in obtaining insight to one another's physiology.

What has been known for at least the last decade is that LIF/LIF-R mRNA and protein are expressed throughout all three zones of the human adrenal cortex, but not within the adrenal medulla (Bornstein and Rutkowski, 2002; Bamberger et al., 2000). This revelation has led some researchers to imply the possibility of a LIF/LIF-R paracrine/autocrine action in regulating steroidogenesis directly since this process takes place within adrenocortical cells of the adrenal cortex (Bamberger et al., 2000). As predicted, studies involving the addition of LIF to the human adrenocortical carcinoma

cell-line NCI-H295 resulted in an increase in cortisol release a minimum of 122.6%, and as high as 190% of the control in some studies (Bamberger et al., 2000; Mikhaylova et al., 2008). These results, while they may have been conducted on human cell-lines, clearly showcase LIF's capacity to directly elevate glucocorticoid output without the assistance of ACTH, and help provide the necessary foundation for our hypothesis.

Additional studies involving NCI-H295R (derived from the parent cell-line NCI-H295 and produces more androgens) have further uncovered that LIF's ability to stimulate cortisol production is through the activation of key genes involved in steroidogenesis, in particular Steroidogenic Acute Regulatory protein (StAR) (Mikhaylova et al., 2008; Samandari et al., 2007). StAR is a 37-kDa phosphorylated protein involved in the transport of cholesterol, the substrate that all steroid hormones are synthesized from, into the inner mitochondrial membrane (IMM) where steroidogenesis occurs (Hu et al., 2010). The implications of LIF's ability to induce the StAR protein cannot be overstated, as StAR is essential to the synthesis of glucocorticoids and all other steroid classes (Lin et al., 1995; Hu et al., 2010). The translocation of cholesterol from the outer mitochondrial membrane (OMM) to the IMM is mandatory in adrenal steroidogenesis and is generally accepted as the rate-limiting step, which is why the function of StAR is indispensable (Lin et al., 1995; Hu et al., 2010).

Typically following stress, ACTH is released into the bloodstream where it then acts as a tropic hormone on adrenocortical cells inducing the expression of StAR (Hu et al., 2010). The availability of cholesterol is obviously of critical importance too, but there are multiple sources that contribute to its cellular supply, including ACTH stimulated cholesterol recruitment as well as cholesterol accumulation as an ester derivative in lipid

droplets of steroidogenic cells (Hu et al., 2010; Roostaee et al., 2008). While the necessity of cholesterol could be considered rate-limiting, there are many ways for its acquisition by cells, leaving its accessibility rarely in doubt. But it is the StAR protein, and its induction by ACTH, that leads to the all important step of cholesterol transport to the IMM, where steroid synthesis is immediately initiated by the enzyme P450scc (Hu et al., 2010). Cleavage of the cholesterol side chain by P450scc results in pregnenolone, the starting substrate within the IMM for every class of steroids (Rhéaume et al., 1992).

Simply put, if cholesterol does not cross the mitochondrial membrane then pregnenolone cannot be synthesized and steroidogenesis will cease (Hu et al., 2010). Studies involving COS-1 monkey kidney cells possessing inactive StAR proteins resulted in a dramatic drop in the formation of pregnenolone, whereas an approximately 8-fold increase in pregnenolone production was seen when these COS-1 cells coexpressed wildtype StAR, providing further evidence to the essential nature of this protein in initiating steroid synthesis (Arakane et al., 1996). StAR induction by LIF becomes crucial to the support of our hypothesis because the formation of pregnenolone is not limited by the activity of P450scc, but by the availability of cholesterol substrate in the IMM where the enzyme resides (Hu et al., 2010). Taken together, the aforementioned findings indicate that StAR upregulation by overexpressed LIF may be enough for enhanced steroidgenesis to occur without the assistance of ACTH (Hu et al., 2010).

Explained so far is that ACTH stimulates the production of cortisol/corticosterone in adrenocortical fasciculata-reticularis cells in large part by exerting positive effects on the transcription of StAR, and that LIF mimics this effect. But there are other key enzymes that participate in the conversion of pregnenolone to glucocorticoids within the

IMM (Rhéaume et al., 1992). It turns out that the study showing LIF induction of StAR also provided data on LIF's ability to induce one of the key enzymes involved in cortisol synthesis known as 17α-hydroxylase/17,20-lyase (CYP17) (Mikhaylova et al., 2008). In all, NCI-H295R adrenocortical cells treated with LIF resulted in an increase in StAR and CYP17 mRNA by up to 138% and 178% of control, respectively (Mikhaylova et al., 2008). Further analysis revealed that LIF increased the protein amounts of StAR and CYP17 by up to 140% and 130% of control, respectively (Mikhaylova et al., 2008). Maybe most relevant to our hypothesis is that both StAR and CYP17 genes and/or their upstream sequences contain several possible binding sites for STAT proteins, indicating the potential for transcriptional regulation by way of LIF/LIFR signaling (Mikhaylova et al., 2008). LIF's ability to directly influence steroidogenesis in adrenal tissue appears to be more robust than was probably first envisioned.

The enzyme CYP17 is essential in the successful conversion of pregnenolone to cortisol, which is the predominant stress hormone in humans but not mice. However, it can't be discounted that LIF might also enhance the expression of the enzyme CYP11B1, which is responsible for the final step in the synthesis of the most abundant murine stress hormone, corticosterone (Hu et al., 2010). The reason CYP11B1 expression was not examined in studies involving NCI-H295R adrenocortical cells is most likely due to the nature of this particular cell-line, which is of human origin and therefore CYP11B1 was probably deemed not significantly important to the study. It would be interesting to identify whether or not LIF does indeed exert a positive effect on the expression of CYP11B1, as well as to confirm that induction of StAR and other additional steroid synthesizing enzymes by LIF occurs in mice as it does in humans. If such is the case, this

would imply that LIF, if expressed in great enough abundance, could to a degree mimic the effects of ACTH, and may begin to explain how and why corticosterone levels in OPN-deficient mice remain elevated in the absence of ACTH or stressful input.

Here is where the findings revealed by our research become so interesting. If OPN does help to regulate LIF through an inhibitory effect, as our preliminary data would suggest, the idea that LIF could supplant ACTH in increasing corticosterone levels in OPN-deficient mice begins to make sense. If our initial findings hold true, a possible scenario would be the following; in *healthy* mice immunological and inflammatory stress results in the expression of LIF, which in response enhances corticosterone levels, perhaps in part, by acting on adrenocortical cells directly. OPN expression then inhibits LIF mRNA, in turn blocking any downstream actions LIF may contribute to steroid production, allowing for restoration of baseline corticosterone levels. However, when the OPN gene is nonfunctional LIF expression is left unchecked, consequently leading to persistent LIF-stimulated overproduction of corticosterone in adrenocortical cells.

There is already circumstantial evidence of a possible regulatory relationship between OPN and LIF, most significant of which is their characteristic co-expression in response to inflammatory stress. In mice, OPN and LIF levels are increased in response to LPS injection, and both are chronically overexpressed in the synovial fluid of arthritic patients (Patterson et al, 1994; Wang et al., 2008). Not to mention the ability of OPN to influence the activation of NF-κB, a potent regulator of inflammatory cytokine expression, as well as OPN's ability to act as an anti-inflammatory in later stages of certain pathological events (Wang et al., 2008). The frequent co-expression of LIF and OPN at inflamed tissue sites is certainly enough to suspect them of having some type of

interaction with one another, whether it is regulatory or adjuvant. Presuming the aforementioned ideas are true, we believe the findings surrounding another precursor to all glucocorticoids known as progesterone and its effects on OPN mRNA levels help to further connect our theory that LIF is responsible for elevated basal corticosterone levels in OPN-deficient mice.

What makes progesterone so intriguing to our hypothesis is that it has been discovered to increase OPN mRNA levels in human trophoblasts and to a degree on the skin of female mice (Omigbodun et al., 1997; Craig et al., 1991). The murine OPN gene has been well characterized, and like the human OPN gene, appears to have putative response elements for progesterone, but also for glucocorticoids (Omigbodun et al., 1997). The ability of progesterone to enhance OPN mRNA transcription, plus the strong suspicion that GCs are capable of doing the same is fascinating to our research. Connecting the evidence regarding LIF, GC synthesis, and OPN leads us to believe they all participate together in forming a regulatory loop. I propose such a regulatory loop displayed in **Fig. 8**, which shows a schematic of how LIF, StAR, progesterone, corticosterone, and OPN might sequentially regulate one another.

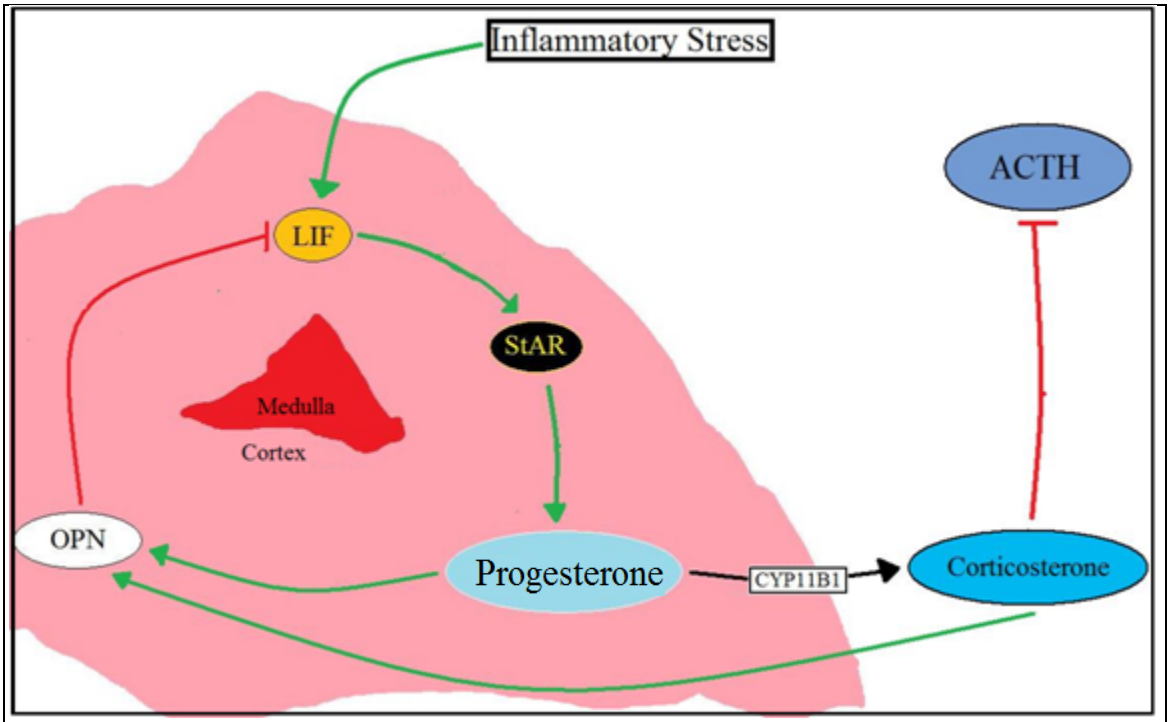


Fig. 8: Proposed OPN/LIF Regulatory Loop. LIF stimulated expression via inflammatory stress leads to increased progesterone formation and corticosterone release through induction of StAR and CYP11B1. Corticosterone and progesterone up-regulate OPN which acts as an inhibitor to LIF mRNA expression. Corticosterone and progesterone levels decrease due to the lack of available cholesterol in absence of LIF-stimulated StAR expression. Lowered corticosterone levels allow ACTH to elevate in response to future stress. **Green** arrow denotes stimulation; **Red** blunted line Denotes inhibition.

As **Fig. 8** depicts, LIF enhances steroidogenesis through induction of StAR and possibly other steroidogenic enzymes such as CYP11B1. The formation of progesterone and corticosterone stimulate OPN mRNA transcription, with the mature OPN protein then exerting a negative feedback on LIF mRNA expression and all its downstream targets. Corticosterone production would then diminish due to the lack of StAR activity and available cholesterol within the IMM. **Fig. 9** illustrates how disruption of this loop would occur if OPN is rendered inactive, effectively removing the "shutoff valve" to LIF transcription. The result would be over-expression of basal LIF triggering a constant stimulation of GCs, namely corticosterone, and chronic suppression of ACTH. In fact,

ACTH would be suppressed in such a way that it could not be elevated even when the organism is stressed, which is an observed characteristic of OPN-deficient mice.

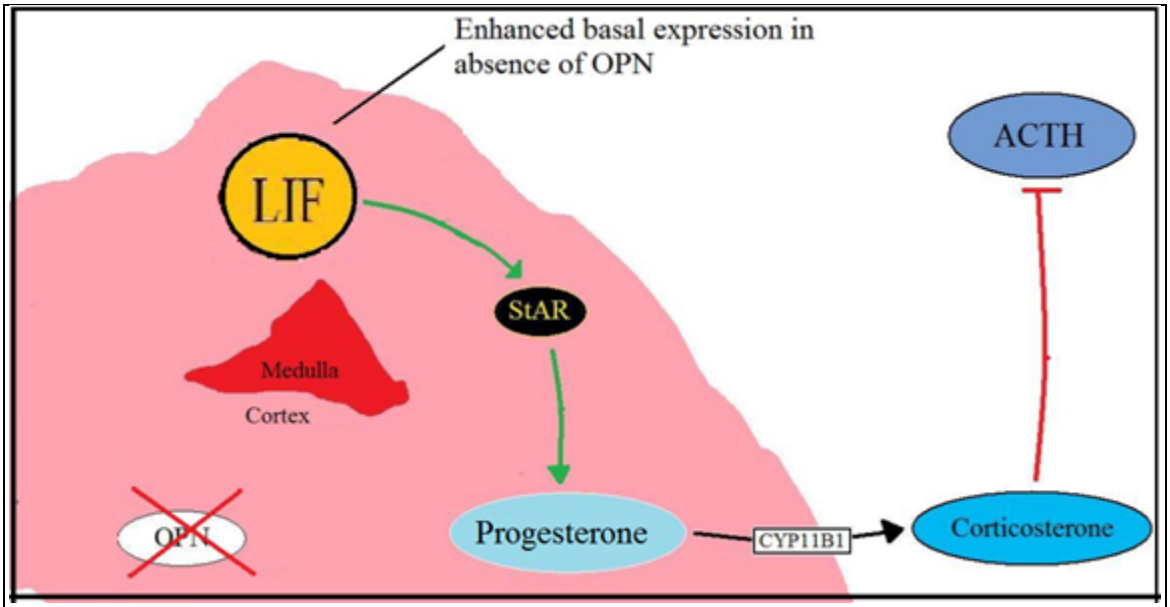


Fig. 9: Disruption of Proposed OPN/LIF Regulatory Loop. Without OPN present, basal LIF would be elevated in the absence of stress causing continued induction of StAR and possibly other steroidogenic enzymes leading to chronic elevation in corticosterone levels. ACTH suppression would persist permanently. **Green** arrow denotes stimulation; **Red** blunted line denotes inhibition.

Since OPN appears to inhibit basal transcription of LIF in our studies involving AtT-20 cells, mice lacking OPN may experience elevated basal LIF levels within adrenocortical cells, which might prompt continuous production of StAR-induced corticosterone in these mice in the absence of stress. If such is the case, then the steroid inducing effects of LIF are in essence, always ON.

While identifying OPN as a regulator of LIF mRNA levels will ultimately be a useful discovery, in the end, it may not explain the characteristic expression of corticosterone in OPN-deficient mice. I suggest several hypotheses in explaining the unusual corticosterone production in OPN-knockout mice in the event that LIF regulation is ruled out as the cause. One is that OPN may play a role in the proper growth and

development of the adrenal cortex, which controls steroidgenesis. Perhaps dysfunctional growth of the adrenal cortex could lead to an unnecessarily high constitutive expression of corticosterone. Such an event is seen in a certain form of Cushing's syndrome where the adrenal glands become hyperplastic, resulting in hypersecretion of glucocorticoids (Findlay et al., 1993). A quick way to study this may be to compare the mass of adrenal glands between OPN-knockout mice and wildtype mice. Another explanation is that OPN may decrease sensitivity of the adrenal cortex to ACTH. This may explain why mice lacking OPN have high basal corticosterone levels in the presence of relatively low ACTH. As mentioned in the introduction, AVP enhances the sensitivity of CRH induction of ACTH, which helps provide a visible example of this concept, and perhaps OPN contributes an opposing affect (Charmandari et al., 2005).

As it stands now, with the combination of published findings and our preliminary studies showcasing OPN as an inhibitor of LIF mRNA, our proposed hypothesis that up-regulation of LIF is responsible for elevated corticosterone in unstressed OPN-deficent mice is starting to take a reliable form and warrants future investigation. However, there is still plenty of forthcoming work to be done in order to fully validate our convictions and include:

- Does OPN act as an inhibitor of LIF mRNA in murine adrenocortical cells?

  The basis of our hypothesis is that the inhibitory effects of OPN against LIF observed in pituitary cells must also occur similarly in adrenocortical cells, since we suspect that it is a disruption of the putative OPN/LIF relationship within the adrenal gland that is leading to aberrant corticosterone production in OPN-deficient mice.

- Does LIF possess the ability to enhance corticosterone, as well as steroidogenic proteins such as StAR in murine adrenocortical cells?

  There is plenty of evidence that LIF enhances cortisol and StAR in human adrenocortical cells, but again, we need to see if this is a phenomenon that occurs in murine biology as well.

- Is there a greater abundance of LIF, LIFR, and/or StAR mRNA or protein present within the adrenal tissue of OPN-deficient mice compared with wildtype mice?

  Analysis of homogenized adrenal tissue of OPN-deficient mice could reveal that there is indeed a higher abundance of these critical proteins predicted to be up-regulated in our hypothesis. This would be a relatively simple *in vivo* experiment that would help provide insight into whether the *in vitro* findings will ultimately translate to a live mouse.

As of now we suspect that over--expression of LIF is the cause of enhanced basal corticosterone output in OPN-deficeint mice stemming from a lack of regulatory control of basal LIF mRNA levels by OPN. Confirming this hypothesis would reveal LIF as a much more prominent participant in the regulation of the HPA axis, and would provide another vital component, and possibly therapeutic target, **w**hen researching this essential pathway.

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