ROBYN D. PEREIRA – M.SC. THESIS
THE EFFECTS OF OLANZAPINE ON TROPHOBLAST INVASION

THE EFFECTS OF OLANZAPINE ON TROPHOBLAST INVASION

By

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LAY ABSTRACT

Pregnant women are highly vulnerable to mental illness, with prevalence rates estimated to be as high as 30%. Atypical antipsychotic medications such as olanzapine (Zyprexa®) are increasingly being prescribed for many mental illnesses, and this increased use is also evident during pregnancy. Women are advised to remain on these medications throughout pregnancy; however, the metabolic side effects on the developing baby are poorly understood. The objective of this project was to determine how taking olanzapine during pregnancy can affect the placenta, an organ that substantially influences the development of the baby. We found that olanzapine can directly increase the invasion of cells, which is required for placental development. These findings allow for a better understanding of how olanzapine can impact the offspring of women taking this antipsychotic. Such knowledge can also better inform and encourage healthcare providers to improve strategies for managing pregnancies where women need to take atypical antipsychotics.

ABSTRACT

Olanzapine (OLN) is one of the most commonly taken antipsychotics during pregnancy. Exposure of the fetus to OLN during gestation leads to altered birth weight. This is of concern because altered fetal growth increases the risk of metabolic syndrome later in life, which negatively impacts an individual's quality of life, lifespan, and increases the burden on the healthcare system. However, the mechanisms of how antipsychotics, such as OLN, contribute to fetal growth effects have yet to be determined. The placenta plays a critical role in modulating the *in utero* environment to promote optimal fetal growth and is dysfunctional in pregnancies complicated by fetal growth restriction. However, no previous work has examined the direct effect of OLN on the placenta.

We assessed whether OLN can impact the placenta by exposing a human trophoblast cell line (HTR-8/SVneo cells) to OLN and determining its effects on trophoblast invasion. Altered invasion is heavily implicated in adverse pregnancy outcomes such as preeclampsia, and intrauterine growth restriction. This thesis demonstrates that OLN increases trophoblast cell invasion, as well as integrin gene expression and the amount of active extracellular MMPs, which are important for invasion. Although the cellular signaling pathway(s) by which OLN mediates these effects remains unclear, it may involve OLN's ability to decrease the maximal activity of the mitochondrial electron transport chain complexes. These findings provide the first evidence that OLN has the potential to affect placentation. This thesis contributes to the current understanding of how antipsychotic drugs can result in adverse fetal outcomes and allows healthcare

providers to better predict and manage fetal outcomes of women taking OLN during pregnancy.

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LIST OF ABBREVIATIONS

ΔCt	Comparative cycle times
α_{1A}	α adrenergic receptor subtype 1A
α_{1B}	α adrenergic receptor subtype 1B
α_{2A}	α adrenergic receptor subtype 2A
α_{2B}	α adrenergic receptor subtype 2B
α_{2C}	α adrenergic receptor subtype 2C
5-HT	Serotonin
5-HT_{1A}	Serotonin receptor subtype 1A
5-HT_{1B}	Serotonin receptor subtype 1B
5-HT_{1D}	Serotonin receptor subtype 1D
5-HT_{1E}	Serotonin receptor subtype 1E
5-HT_{2A}	Serotonin receptor subtype 2A
5-HT_{2B}	Serotonin receptor subtype 2B
5-HT_{2C}	Serotonin receptor subtype 2C
5-HT_{5A}	Serotonin receptor subtype 5A
5-HT_{6}	Serotonin receptor subtype 6
5-HT_{7}	Serotonin receptor subtype 7
5-MU	5-methylurapidil
AKT	Protein kinase B
ANOVA	Analysis of variance
ANT	Antimycin A

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAT	Catalase
cDNA	Complementary DNA
CT	Cytotrophoblast
CuZnSOD	Copper/Zinc superoxide dismutase
CYP450	Cytochrome P450
D_1	Dopamine receptor subtype 1
D_2	Dopamine receptor subtype 2
D_3	Dopamine receptor subtype 3
D_4	Dopamine receptor subtype 4
D_5	Dopamine receptor subtype 5
DCFDA	2',7'-dichlorofluorescin diacetate
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DRP1	Dynamin-related protein 1
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
EVT	Extravillous trophoblast
FAK	Focal adhesion kinase
FBS	Fetal bovine serum

FIS1	Mitochondrial fission 1
FOSL1	FOS-Like antigen
GCM1	Glial cell missing 1
GD	Gestational day
GPCR	G-protein coupled receptor
GPX1	Glutathione peroxidase 1
H_1	Histamine receptor subtype 1
HIF	Hypoxia-inducible factor
HLX	H2.0-like homeobox
HNE	4-Hydroxynonenal
HSP	Heat-shock protein
ID2	Inhibitor of DNA binding 2
JAK	Janus kinase-signal transducers
kDa	Kilodalton
LDHA	Lactate dehydrogenase A
M_1	Muscarinic receptor subtype 1
M_2	Muscarinic receptor subtype 2
M_3	Muscarinic receptor subtype 3
M_4	Muscarinic receptor subtype 4
M_5	Muscarinic receptor subtype 5
MAPK	Mitogen activated protein kinase
MFN	Mitofusion

MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
$O_2^{\cdot -}$	Superoxide
O/N	Overnight
OLN	Olanzapine
OPA1	Optic atrophy 1
PAI	Plasminogen activator inhibitor
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
RFU	Relative fluorescence units
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SNAP23	Synaptosomal-associated protein 23
ST	Syncytiotrophoblast
STAT3	Signal transducer and activator of transcription
TBST	Tris-buffered saline with Tween 20
TGF-β	Transforming growth factor β

TIMP	Tissue inhibitor of metalloproteinase
TNF-β	Tumor necrosis factor β
uNK	Uterine natural killer cells
uPA	Urokinase plasminogen activator
VAMP3	Vesicle associated membrane protein 3

DECLARATION OF ACADEMIC ACHIEVEMENT

Robyn D. Pereira (RDP) and Dr. Sandeep Raha (SR) designed this thesis, with support and guidance from Dr. Alison Holloway (AH) and Dr. Valerie Taylor (VT). All laboratory experiments were performed by RDP with the assistance of Anson Cheung, Gurrattan Chandhoke, and Jasmine Liew. Data was analyzed by RDP and SR. RDP also contributed greatly to the survey of literature and prepared a review paper (*1*). RDP wrote this thesis dissertation with the help of SR, AH, and VT.

1. INTRODUCTION

1.1 Prevalence and Impact of Atypical Antipsychotic Use During Pregnancy

1.1.1 Mental health disorders during pregnancy

Mental disorders are the leading cause of years lived with disability worldwide (*2*). In Canada, it is estimated that one in five people will personally experience a mental illness in their lifetime, of which mood disorders such as depression, bipolar disorder, and psychosis such as schizophrenia are prevalent (*3*). Many disorders manifest in young adulthood (*4, 5*), and women of reproductive age are especially susceptible to developing a new or the worsening of an existing mental illness (*6*). Indeed, most women with psychoses are mothers (*7, 8*) and it is estimated that between 14-30.5% of women who are pregnant suffer from mental illnesses such as schizophrenia, bipolar disorder, depression, and anxiety (*9-12*). Left untreated, mental illnesses are associated with an increased risk of pregnancy complications such as premature delivery, low birth weight, and stillbirth as well as poor maternal-infant bonding (*13-15*); all of which can result in lifelong impairment and increase the risk of disease for the developing child (*16-19*). Moreover, individuals who discontinue their treatments when pregnant experience higher rates of relapse (can be an increase of more than 50%) than if they remain on their medications (*20-23*). Therefore, guidelines and physicians typically recommend that mentally ill patients, especially those who suffer from bipolar disorder and schizophrenia, remain on their prescribed medications throughout pregnancy (*24, 25*).

1.1.2 Prevalence of atypical antipsychotic use

Atypical antipsychotic use for the treatment of mental illnesses has increased dramatically (one group reports an increase of more than 200%) over the past 20 years in various countries (*26-30*). Canada is no exception. Despite a steady-state population in Manitoba between 1996 and 2006, there was an increase of 227% in the dispensed antipsychotic medications across all age groups and annual expenditures increased from $1.7 million to $22.0 million (*31*). Moreover, there was also an increase of 37.2% in antipsychotic prescription rates from 2005 to 2012 across Canada (*32*). This increase in antipsychotic use has been largely attributed to atypical antipsychotics being prescribed for the treatment of a range of mental illnesses including schizophrenia, bipolar disorder, depression, and anxiety (*26, 28, 33-35*).

Given that women are at high risk of mental illness during their childbearing years, it is not surprising that there has also been a considerable increase in the use of atypical antipsychotics by pregnant women (*36, 37*); use in this patient population increased by approximately 15% between 1985 and 2005 (*36*). More recently, the use of antipsychotics during pregnancy has increased 2.5-fold (between 2001 to 2007) in the United States (*37*). This elevated prevalence of use during pregnancy results in higher incidences of fetal exposure to atypical antipsychotics. Despite this, the impact of antipsychotic use during pregnancy remains poorly researched and under-resourced (*38, 39*), and the effects of antipsychotics on fetal development, especially with respect to their metabolic impact, are unclear (*40-43*).

1.1.3 Fetal outcomes of atypical antipsychotic use during pregnancy

Recent meta-analyses have demonstrated that exposure to atypical antipsychotics during pregnancy is associated with a significant increase in risk for babies born preterm and with major malformations (*44, 45*) as well as being small for gestational age and having a low birth weight (*44*). This is supported by some studies, which were not included in either meta-analysis, that found a significant increase in the risk of malformations (*46, 47*), prematurity at birth (*46*) and altered birth weight or size for gestational age (*46, 48*). Additionally, exposure to atypical antipsychotics during pregnancy has been shown to increase neonatal intensive care or hospitalization visits (*46, 47, 49, 50*). However, other reports found no effect of antipsychotics on malformations (*51, 52*), incidence of preterm delivery (*52-54*), or birth weight/size (*53-55*). Therefore, the impact of exposure to atypical antipsychotics during pregnancy remains unclear.

Moreover, little is known about the long-term development of these infants. One study found that babies exposed to atypical antipsychotics had poor neurodevelopmental outcomes at six months of age compared to infants born to women with a mental illness but not exposed to drugs during fetal life (*56*). This observation is supported by a study demonstrating that infants exposed to atypical antipsychotics had lower scores of cognitive, motor, social-emotional and adaptive behaviours at two months of age (*48*). However, there were no differences in functioning of infants who were exposed to atypical antipsychotics during gestation compared to those who were not at 12 months of age (*48*). This indicates that atypical antipsychotic exposure during pregnancy may cause

infants to suffer a short-term delay in development within the first six months of birth, however they are able to catch-up to the functioning of other infants unexposed to atypical antipsychotics during gestation.

Current study designs have faced difficulties in determining whether adverse fetal outcomes observed are due to the antipsychotic exposure, independent of other factors such as: the maternal mental illness itself, maternal comorbidities (such as obesity or diabetes), or unidentified maternal behaviours (such as smoking, or illicit drug use) (*38*). Other challenges include small sample sizes, the comparison group available and used (mental illness with no drug use, drug use not thought to have effects on pregnancy or population norms) as well as the timing, type, and duration of medication use (*38*). Therefore, *in vivo* animal models along with *in vitro* studies are warranted to separate the effect of specific atypical antipsychotics from other influencing systemic factors to overcome shortcomings of current data and better elucidate the impact of atypical antipsychotics on fetal outcomes.

Olanzapine (OLN; Zyprexa®; 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5]benzodiazepine), is one of the most commonly prescribed atypical antipsychotics in the United Kingdom (*57*), Australia (*27*), as well as in Canada (*32*), and is the 10th biggest selling pharmaceutical globally (*28*). Moreover, OLN is consistently within the top two commonly reported atypical antipsychotics used in pregnancy (*37, 43, 46, 58*). However, only two studies have attempted to specifically assess the effects of OLN use

during pregnancy on fetal growth. These studies have found conflicting results, with one study reporting an increased incidence of high birth weight (*59*), and the other indicating a trend towards babies with low birth weight (*47*). Altered fetal growth (either being small or large compared to population norms) is associated with an increased risk of adult onset of disease including type 2 diabetes, cardiovascular disease, and obesity (*60-64*). This is problematic because these chronic diseases contribute to a burden on the individual's quality of life and the healthcare system (*65*). Thus, the paucity of data on OLN's impact, coupled with the observed effect of atypical antipsychotics as a class on fetal growth, suggests that further attention regarding the effects of OLN on fetal growth is warranted.

1.1.4 Pharmacology of OLN

OLN is a thienobenzodiazepine (Figure 1) that mainly exerts its beneficial neurological effects through a combination of dopamine 2 (D_2) receptor and serotonin receptor subtype 2A (5-HT_{2A}) antagonism (*66*). OLN is also capable of binding other dopamine (D_1, D_3, D_4 and D_5) and serotonin receptor subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6 and 5-HT_7) as well as α adrenoreceptors (α_{1A}, α_{1B}, α_{2A}, α_{2B} and α_{2C}), muscarinic receptors (M_1-M_5), and histamine 1 (H_1) receptors (*67*), and can act as an inverse agonist at some of these receptors (*68-71*).

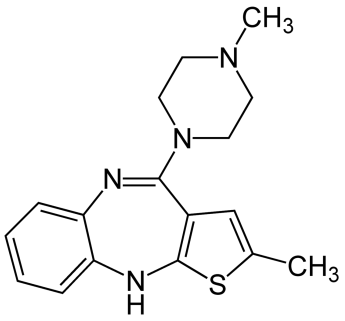

Figure 1. The structure of OLN (http://www.drugbank.ca/drugs/DB00334).

Following oral administration, 83% of OLN is readily absorbed, however due to a high first pass effect, only 60% of OLN is bioavailable (*72*), with 93% of OLN bound to plasma protein (highly to albumin, moderately to α1-acid-glycoprotein and minimally to γ globulins) (*73, 74*). OLN's half-life is approximately 33 hours, and has an apparent volume of distribution of 1150L (*73*). OLN is primarily metabolized in the liver by direct glucuronidation to inactive metabolites (*73, 75*), 10-*N*-glucuronide and 4'-*N*-glucuronide (*74*). Other minor pathways include metabolism by flavin-containing monooxygenase 3 to *N*-oxide, or by cytochrome P450 (CYP450) oxidation via CYP1A2 to N-desmethyl-olanzapine (*74*). OLN can also be converted to 2-hydroxymethylolanzapine and 2-carboxylic acid via CYP1A2, or minorly through CY2D6 (*76*). 10-*N*-glucuronide is considered the primary metabolite since it contributes significantly to drug-related circulating species in plasma (44% of the parent drug concentration), and forms the largest metabolic fraction that is excreted (*74*). OLN and its metabolites are cleared mainly through renal excretion (50% parent drug, 7% metabolites) and feces (2% parent drug, 28% metabolites) (*74*).

Once OLN is administered orally (typically 10mg-40mg daily), a steady state is reached in about a week, which is approximately 2-fold higher than the serum concentration following a single initial dose. The therapeutic range of the average patient receiving OLN treatment is 20-80ng/mL (0.064μM-0.256μM) in serum (*72, 77*). Currently, there is limited clinical data on the pharmacokinetics of OLN in pregnancy. However, the pharmacokinetics (and thus the plasma concentration), of atypical antipsychotics such as OLN can be impacted by physiological changes during pregnancy (*42*). Therefore, an increased dose during pregnancy may be required to achieve the same serum concentrations of OLN when a patent is not pregnant, however, close monitoring and more evidence-based studies are required (*76, 78-80*).

OLN is lipophilic, has relatively small molecular size (*81*), and has been postulated to be capable of diffusing through the placenta via passive transport or tissue transporters (*75*). Within 4 hours, up to 14% of OLN can traverse a human placenta *ex vivo*, with 83% of that passed being the parent drug and the other 17% as a glucuronide metabolite (*75*). However, *in vivo* measurements of placental transfer have found that 72.1% of OLN can pass through the human placenta, with high variability between patients attributable to variations in pregnancy and patient specific factors including: drug distribution, metabolism, pharmacokinetic interactions with other substances ingested, and genetic polymorphisms for transporters in the placenta (*47*). Not only can OLN cross the placental barrier, but large amounts of OLN is also sequestered within the placenta itself (*75*). Therefore, OLN may be impacting fetal growth by directly affecting the placenta.

Indeed, placental dysfunction is heavily implicated in pregnancy complications, and altered fetal growth (*1, 82-84*).

Importantly, OLN also has one of the highest propensities to induce metabolic syndrome (a clustering of medical conditions predisposing individuals to chronic diseases) compared to other antipsychotics (*85, 86*). OLN can substantially increase weight gain and the risk of acquiring both type 2 and gestational diabetes (*87-89*), which are all known to influence the birth size of babies (*90-92*). Therefore, part of the mechanism underpinning the effect of OLN on birth size may be secondary to its effects on the maternal metabolic state (*93*). Thus, OLN has the potential to affect fetal growth by altering the *in utero* environment: 1) indirectly via alterations in maternal metabolic homeostasis, 2) by crossing the placenta and directly interacting with developing fetal tissues, or 3) by influencing placenta development and function. My thesis will focus on OLN's direct effects on human placenta development and function.

1.2 The Placenta and Its Important Role in Fetal Growth

1.2.1 Structure and functions of the human placenta

The placenta is a specialized organ situated at the maternal-fetal interface and is vital in modulating the *in utero* environment to promote normal fetal development. The placenta maintains this supportive environment by secreting important hormones and growth factors, delivering nutrients, exchanging gases between fetal and maternal circulations and by providing protection against the maternal immune system and environmental

substances, such as drugs (*82, 94*).

The mature human placenta is a highly vascularized organ composed of both fetal and maternal tissue (*94*). The fetal portion of the placenta is called the chorionic plate, which is composed of fetal vessels protruding from the umbilical cord (*94*). The maternal portion of the placenta is known as the basal plate, and between these two areas is the intervillous space. This space is composed of tree-like protrusions called villi, which are made up of branching fetal blood vessels (*94*). Maternal blood bathes the villi in the intervillous space via the maternal spiral arteries, enabling maternal-fetal exchange between the fetal and maternal circulations (*94*). However, the fetal blood vessels of the villi never come into direct contact with the maternal circulation in the intervillous space due to the multinucleated syncytiotrophoblasts that layer the villi and facilitate the exchange between the fetal and maternal circulations (*95*). This form of placenta barrier observed in humans is referred to as hemochorial (from Greek heme=blood and chorion=trophoblast membrane) (*95*). The villi within the intervillous space are anchored to the maternal decidua via cytotrophoblast cells (CTs), which provide structural support and give rise to invasive trophoblast cells that are responsible for remodeling the maternal vasculature to facilitate this blood flow into the intervillous space (*94*).

1.2.2 Implantation and human placenta development

For the formation of a functional human placenta to begin, the human blastocyst must first implant into the receptive maternal decidua (*96*). The maternal decidua is established

during the normal menstrual cycle where the uterine wall endometrium is transformed into the decidua to better support pregnancy upon conception (*96*). The maternal decidua contains a different cell milieu compared to endometrium with more secretory stromal cells, decidual-specific leukocytes, and altered blood vessels (*96*). Implantation occurs approximately 7-9 days after fertilization, at which point the conceptus is called a blastocyst, consisting of the inner cell mass, which eventually gives rise to the fetus, and the trophectoderm, composed of cells that facilitate the blastocyst attachment to the maternal decidua and give rise to trophoblasts (*97*).

1.2.3 Trophoblast differentiation

Following implantation, the cells of the trophectoderm begin to proliferate and differentiate into an inner CT cell layer and a non-proliferative outer multinucleated syncytium (*98*), which is responsible for initial invasion into the decidua (*94, 97*). The outer multinucleated syncytium then loses its invasive potential (*97*), and the inner CTs undergo extensive proliferation and follow either the villous or extravillous differentiation path to establish mature, specialized trophoblast subtypes (*94, 97*). The villous pathway involves CTs fusing to form multinucleated syncytiotrophoblasts (ST), which form the villi of the placenta and are primarily responsible for placental transport, and endocrine functions (*94, 99, 100*). By 14 days of pregnancy, inner CTs following the extravillous pathway penetrate the layer of STs and form CT columns (*94*). CTs within these columns can differentiate into extravillous trophoblasts (EVTs), which are responsible for invading both interstitially (interstitial trophoblasts), to provide space for the villous region to

expand, and invading to the maternal spiral arteries (endovascular trophoblasts), to remodel them in order to initiate and sustain adequate blood flow into the intervillous space (*94, 99*) (Figure 2).

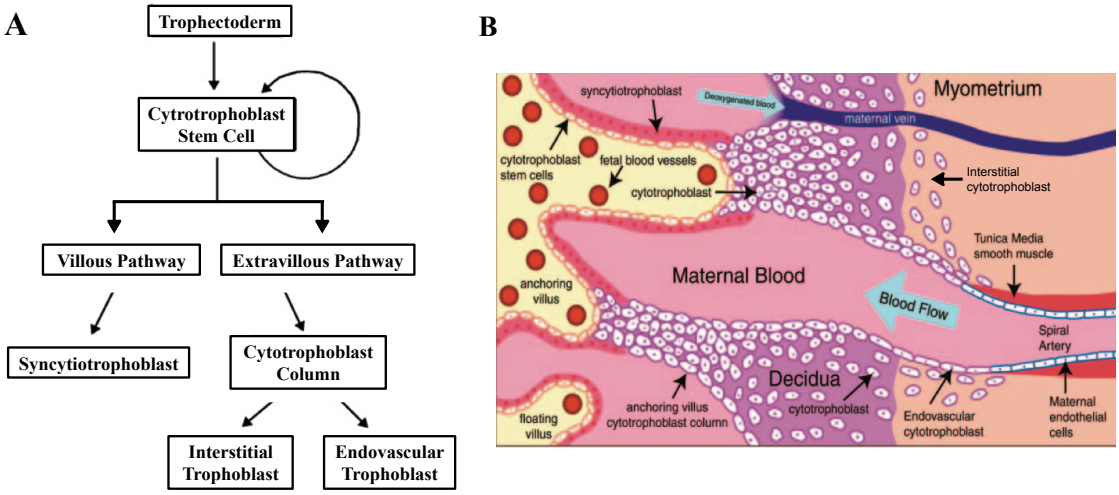

Figure 2. The role of trophoblast cell lineages in normal human placentation. A) All trophoblasts originate from blastocyst trophectoderm. Cytotrophoblast stem cells from the trophectoderm continue to proliferate and differentiate into syncytiotrophoblasts (villous pathway) or aggregate together to form the cytotrophoblast column (extravillous pathway). Cytotrophoblasts in the cell columns then differentiate into either interstitial or endovascular trophoblasts (adapted from (*97*)). **B)** Cytotrophoblast stem cells and syncytiotrophoblasts constitute the villi. Some cytotrophoblasts form cytotrophoblast cell columns at the tips of the villi and will differentiate into interstitial and endovascular trophoblasts that invade the maternal decidua. Interstitial trophoblasts invade laterally to expand the villous space while endovascular trophoblasts invade longitudinally to remodel the maternal spiral arteries and facilitate optimal blood flow to the placenta (adapted from (*101*)).

Trophoblast differentiation and invasion is regulated by a variety of factors present in the local decidual environment, such as immune cells (e.g., uterine natural killer cells; uNK), oxygen levels, hormones, growth factors, cytokines, and cell adhesion molecules expressed by interacting cells (extensively reviewed in (*102-105*)). Additionally, EVTs

also exhibit an intrinsic differentiation program supporting cell invasion, independent of the decidual environment through the secretion of autocrine factors (*102*). However, little is known about the signaling pathways, transcription factors, and their putative targets controlling the differentiation of invasive trophoblasts (*102*). However, the differentiation can be tracked because CT populations are known to express different adhesion proteins compared to EVTs (*106, 107*). For instance, CTs express the adherent junction protein E-cadherin, which is down-regulated in EVTs, while VE-cadherin and platelet-endothelial cell adhesion molecule-1 (PECAM-1) are up-regulated in EVTs (*106-108*). Moreover, in preeclampsia placentae (where there is altered trophoblast invasion), there is a failure in the up-regulation of PECAM-1 and VE-cadherin, but a strong expression of E-cadherin (reviewed in (*107*)). Additionally, CTs express α6β4 integrin (predominant laminin-5 receptor), but there is an integrin switch to α1β1 (collagen/laminin receptors) and α5β1 (fibronectin receptor) as EVTs become more invasive (*109*). Moreover, EVTs that have invaded deep into the decidua also highly express integrin αvβ3 (*106*). Additionally, in an *in vitro* study where the function of α1 or β1 was inhibited, a shift from cell-matrix to cell-cell interactions was observed along with a decrease of invasive properties (*106*). Therefore, this integrin switch appears to be essential for the invasive phenotype of EVTs (*110, 111*).

Understanding the transcriptional regulation of genes involved in placenta development and function has also given insights into the proper control of placentation to support successful pregnancies (*112*). However, little is known about the activation of

transcription factors that distinguish between various CT lineages. The transcription factors, inhibitor of DNA binding 2 (ID2) and H2.0-like homeobox (HLX) appear to promote trophoblast proliferation and negatively regulate invasion by targeting genes important in regulating the cell cycle. Whereas the transcription factors glial cell missing 1 (GCM1) and signal transducer and activator of transcription (STAT3) activate invasive trophoblast differentiation (*102*) and regulate proteins that both inhibit (tissue inhibitor of metalloproteinase; TIMP), and stimulate invasion (matrix metalloproteinase; MMP) (*113, 114*). Moreover, FOS-Like antigen (FOSL1) is more highly expressed in invasive EVTs and modulates the expression of MMPs important to trophoblast invasion (*115*). There is also evidence that the expression of these transcription factors are altered in the placentae of pregnancies complicated by preeclampsia and intrauterine growth restriction (*116-118*).

Therefore, if exposure to drugs during pregnancy can alter the expression or activation of transcription factors or adhesion proteins important to trophoblast invasion (summarized in Figure 3), there could be negative impacts on the placenta, and thus fetal growth. Therefore, appropriate trophoblast differentiation is necessary to ensure proper placenta development and optimal fetal growth.

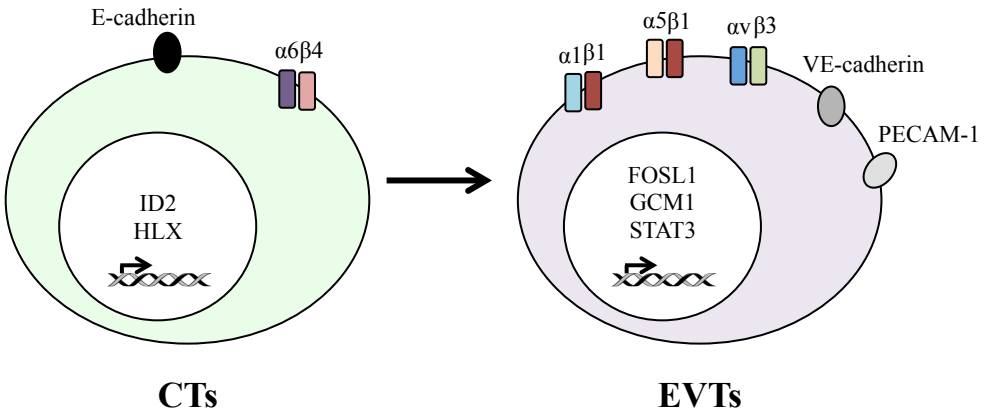

Figure 3. **Adhesion proteins and transcription factors that differ between the CT and EVT lineages.** As CTs differentiate (represented by →) to EVTs they up-regulate different transcription factors (depicted in the inner circle, which represents the nucleus), integrins, and adhesion proteins (located on the outer circle, representing the plasma membrane of the cells, where they facilitate cell adhesion and movement).

1.2.4 Trophoblast invasion

Trophoblast invasion is accomplished by EVT expression of integrins, that facilitate movement (migration), and by secretion of proteases, such as MMPs and urokinase plasminogen activator (uPA), that degrade the extracellular matrix (ECM) of the decidua (*119, 120*). Additionally, EVTs also produce inhibitors of these enzymes, such as TIMPs and plasminogen activator inhibitors (PAIs) (*121*), to fine-tune and limit the amount of trophoblast invasion (*120*). The regulation of MMP activity seems to be particularly crucial for successful trophoblast invasion, with MMP2 and MMP9 being the most important and well-characterized. The ratio of these proteases alters throughout the beginning of pregnancy, and invasion is inhibited in the absence of MMP9 activity (*20, 83*). Additionally, MMP2 and MMP9 expression are most intensive in EVTs and the surrounding ECM area (*120, 122-124*). Moreover, the expression of both proteases is also reduced in clinical conditions where typical invasion patterns are disrupted, such as

preeclampsia and intrauterine growth restriction (*118, 125, 126*). Moreover, in cases where there is excessive trophoblast invasion, such as placenta accrete, there is an increase in MMP expression (*127*).

EVT invasion is tightly controlled both spatially and temporally (*119*). Invasion is restricted spatially to the inner third of the myometrium by interstitial trophoblasts, and to the maternal spiral arteries by endovascular trophoblasts (*119*). At 8 weeks of human pregnancy, the interstitial trophoblasts have reached the decidual-myometrium border, and the endovascular trophoblasts have formed a temporary plug at the spiral arteries to prevent maternal blood flow into the placenta (*120, 128*). Trophoblast invasion tends to peak around 10-12 weeks of pregnancy, at which point the EVT spiral artery plug dissipates to allow maternal blood to enter the intervillous space of the placenta (*105*). In addition to establishing blood flow to the placenta, proper EVT invasion is also important for the successful remodeling of maternal spiral arteries (*83*). EVTs are recruited to the arteries by factors secreted by uNK cells and macrophages that surround the maternal spiral arteries. These leukocytes begin the remodeling process by disrupting the vascular smooth muscle cells in the absence of EVTs, and then the recruited EVTs completely remove the remaining muscle layer and replace the endothelial cells (*104, 105*). Remodeling the maternal spiral arteries is necessary to create low-resistance, high-capacity vessels, which improves the blood flow from the maternal arteries to the villi within the placenta (*128*). This remodeling occurs at about 8-12 weeks of human pregnancy, and by this time there are extensive fetal blood vessels within the placenta

villi to facilitate efficient exchange of nutrients and gases between the fetus and mother (*1*).

Altered trophoblast invasion and spiral artery remodeling can result in compromised growth, development, or function of the placenta leading to placental insufficiency and an inability of the placenta to meet the growth and developmental needs of the fetus. Abnormal trophoblast invasion has been associated with a number of pregnancy complications including gestational hypertension, preeclampsia, and placenta accrete, which have negative effects on fetal growth and survival (*1, 129-135*). Consequently, the modulation of trophoblast invasion is a crucial component regulating fetal growth (*83, 135*). Therefore, it is possible that if OLN impacts trophoblast invasion, this may be a mechanism by which OLN use during pregnancy results in altered fetal growth (*84*).

1.3 Regulation of Trophoblast Invasion

1.3.1 Signaling transduction pathways regulating invasion

Trophoblast invasion is regulated by the coordinated actions of various extrinsic factors such as cytokines, growth factors and hormones that are released from decidual stromal cells, and leukocytes such as uNK cells, decidual macrophages and T cells within the decidua (*102, 105*). Trophoblast cells also exhibit autocrine signaling mechanisms that can regulate their invasion, independent of the local decidual environment (*102, 105*). Both these extrinsic and intrinsic pathways influence a number of signaling cascades known to be important in trophoblast invasion, including the focal adhesion kinases

(FAKs), Rho/ROCK, mitogen-activated protein kinases (MAPKs) (such as extracellular signal-regulated kinases (ERKs)), phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), and janus kinase-STAT (JAK-STAT) (*99, 102, 105, 136*).

The activation of ERK1/2, AKT1, and STAT3 have been extensively linked to controlling trophoblast invasion and migration via regulating integrin expression and gene transcription of FOSL1, MMPs, and TIMPs (reviewed in (*99, 136*)). Moreover, the activation (via phosphorylation) of ERK1/2 is significantly higher in invasive trophoblasts within placentae of normal pregnancies compared to those from pregnancies complicated by preeclampsia (*137*). Furthermore, a loss of activated AKT1 (*138*) or STAT3 (*118*) is evident in placentae of patients with preeclampsia.

These signaling pathways are affected by extracellular stimuli of G protein-coupled receptors (GPCRs), many of which are also targeted receptors of OLN. For instance, in mouse models of α adrenergic receptor deletion, there is a reduction of phosphorylated ERK and placental vascular development, indicating that the MAPK pathway is altered with improper adrenergic signaling (*139*). MAPK and PI3K/AKT pathways have also been implicated in muscarinic and serotonin receptor signaling in various cancer and trophoblast cells (*140-143*). Moreover, OLN can affect ERK1/2 and AKT phosphorylation *in vitro* as well as in various regions of the rat brain (*144-146*). Previous work has also demonstrated that OLN can bind and internalize the 5- HT_{2A} receptor, which then activates the JAK2-STAT3 signaling pathway in neuronal cells (*147*).

Therefore, OLN may influence these signaling pathways, and affect trophoblast invasion. Elucidating which receptors and signaling pathways are important for OLN mediated effects on trophoblast invasion will give insights into the mechanism of OLN on trophoblast function as well as the role of various signaling molecules on pregnancy outcomes.

1.3.2 Mitochondrial signaling pathways

The mitochondrial electron transport chain (ETC) is one of the primary sources of reactive oxygen species (ROS), which are important signaling molecules for proper placental development and function (*1, 148, 149*). Altered placental mitochondrial ETC function and excessive ROS can lead to pregnancies complicated by preeclampsia (*150, 151*), premature birth (*152, 153*), and altered fetal growth (*152, 154, 155*); ailments that may be caused by altered trophoblast invasion.

Moreover, EVT invasion into the maternal decidua occurs under hypoxic conditions (*148, 156-158*), which can cause an excess of ROS and oxidative stress (a state of cellular imbalance between the generation of ROS and cellular antioxidant defenses) (*159, 160*). Maternal hypoxia (and resultant oxidative stress) has been shown to activate CT differentiation along the invasive pathway, and hypoxic conditions can increase the expression of proteins important in facilitating the degradation of the ECM (*161-164*). ROS has also been shown to effect EVT survival, differentiation, and motility (*165*), while increasing the expression of an antioxidant enzyme (thereby decreasing ROS) can

directly impair the ability of CTs to differentiate (*166*). Therefore, an increase in ROS may affect trophoblast differentiation and potentially, invasion.

In support of this, previous work in our laboratory has demonstrated that reducing mitochondrial ETC activity and increasing the production of mitochondrial ROS, increases HTR-8/SVneo cell invasion (unpublished data). Moreover, mitochondrial ETC dysfunction and ROS production can activate transcription factors, such as members of the hypoxia-inducible factor (HIF) family (*167*), which can regulate cell adhesion, ECM degradation, and invasion (*168-172*). The tight regulation of HIF-1α activation during pregnancy has also been associated with proper trophoblast differentiation and invasion (*173, 174*). Therefore, mitochondrial ETC dysfunction and the generation of mitochondrial ROS can impact trophoblast invasion, potentially through HIF-1α.

Importantly, there is some evidence to suggest that OLN can reduce mitochondrial respiration (*175*), inhibit complex I (*176, 177*), complex II (*178, 179*), and complex IV (*176*). Additionally, OLN can induce ROS production (*180, 181*), increase oxidative stress (*182*), and increase plasma lipid peroxidation (*183*). Therefore, given the importance of mitochondrial ETC and ROS for trophoblast invasion and normal placenta development, it is plausible that OLN may disrupt the mitochondria and affect trophoblast invasion, which in turn could be the mechanism by which OLN impacts fetal growth.

2. STUDY OBJECTIVES

2.1 Rationale and Hypothesis

Therefore, the rationale for this Master's thesis was founded upon the following:

(1) Maternal OLN exposure in pregnancy can lead to altered fetal growth. However the mechanism underpinning this is unclear,

(2) Altered fetal growth is strongly associated with placental insufficiency and improper trophoblast invasion, and;

(3) Improper trophoblast invasion can occur as a consequence of alterations in various pathways, including those associated with OLN putative receptor targets and the MAPK, PI3K/AKT, STAT3 and mitochondrial signaling.

Therefore, I hypothesize that OLN exposure directly leads to altered signaling pathways that contribute to changes in trophoblast invasion.

2.2 Objectives

The long-term objective of this study is to better characterize the direct effects OLN can have on trophoblast invasion and therefore, elucidate how maternal exposure to this atypical antipsychotic impacts placenta development. By identifying the cellular mechanisms underlying the adverse outcomes of OLN exposure, we will contribute to the current knowledge of atypical antipsychotic side effects and potentially elucidate predictive measures to better manage fetal outcomes.

The short-term objectives of this study are:

(1) To determine whether OLN can effect trophoblast invasion,

(2) To assess the mechanistic signaling pathway(s) responsible for linking the effects of OLN to changes in trophoblast invasion, and;

(3) To evaluate the expression and contribution of receptors by which OLN may mediate its effects on trophoblast invasion.

3. MATERIALS AND METHODS

3.1 Cell Culture Maintenance and Treatments

We used a human trophoblast cell line (HTR-8/SVneo cells) to study the effects of OLN on trophoblast invasion because we did not have access to human placenta samples at early gestational time points (when trophoblast invasion occurs). HTR-8/SVneo cells were donated by Dr. P.K. Lala (Western University, ON, Canada). These cells were derived from human first trimester placenta explant cultures and immortalized with a SV40 large T antigen (*184*). HTR-8/SVneo cells have more similar expression profiles and properties to invasive EVTs (*184*) relative to other trophoblast cell lines (*185, 186*), and thus are well established for investigating functions typical to that of human trophoblasts such as invasion (*121, 186*).

HTR-8/SVneo cells were cultured at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 in RPMI-1640 (Lonza, 12-115F, Walkersville, MD, USA) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Seradigm, 1500-500, Radnor, PA, USA), 1mM L-glutamine (Life Technologies, 25030-081, Burlington, ON, Canada), 1U/mL penicillin, and 1µg/mL streptomycin (Life Technologies, 15140-122) unless otherwise noted. Cells were maintained at sub-confluent conditions and sub-divided when they became 80%-90% confluent. For experimental purposes 150 000 HTR-8/SVneo cells were seeded on uncoated 100mm x 20mm polystyrene tissue culture plates (Corning, 353003, Corning, NY, USA) and allowed to adhere overnight (O/N). Cells were then

exposed to OLN (Toronto Research Chemicals Inc., Toronto, ON, Canada) or vehicle control, dimethyl sulfoxide (DMSO) (Caledon Laboratory Chemicals, 4100-1, Georgetown, ON, Canada) for 48 hours. There are several challenges when comparing *in vivo* and *in vitro* OLN concentrations. Firstly, plasma levels of antipsychotics are highly variable among individual patients (with a 4-fold variability between individuals (*72, 73*)), and secondly, the complex microenvironment *in vivo* that influences drug availability is not present *in vitro* (*187*). Moreover, OLN concentrations in serum during pregnancy is not well established (*42*). Therefore, HTR-8/SVneo cells were treated with 100nM OLN; within the mid-range of reported OLN serum concentrations in non-pregnant individuals (*72, 188*) as well as 1μM OLN to account for the variability between individuals, and to represent a high, yet physiologically feasible concentration of OLN (*189-192*). Currently, there is no reliable data on the stability of OLN in cell culture media (*193*); however, the estimated half-life in patients is 33 hours (*72, 194*). Therefore, treatments were replenished every 24 hours unless otherwise specified to ensure OLN concentrations remained consistent throughout the exposure time.

3.2 Rat Mating and Placenta Sampling

A rat model was used to explore the expression of receptors in the placenta at a time when trophoblast invasion has been initiated compared to when invasion is complete. The rat was a suitable model to use because the process by which trophoblasts extensively invade the decidua and remodel maternal spiral arteries is remarkably similar to humans (*195, 196*).

Animal procedures for this study were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocol 140209) in accordance with the guidelines of the Canadian Council of Animal Care. Female Wistar rats from Harlan (Indianapolis, IN, USA) were maintained under controlled lighting (12:12 hour light-dark cycle) and temperature (22°C) with *ad libitum* access to water and standard rat chow. Mating was carried out by pairing one male with one virgin female per cage O/N. Successful mating was confirmed, and gestational day (GD) 0 was determined by the presence of sperm in vaginal flushes the following morning (as described previously (*197*)). Sperm positive females were subsequently weighed weekly to confirm pregnancy. At GD15 and GD20, placentae were extracted via laparotomy from dams under 2% isoflurane anesthetic (Baxter Corporation, 071970212, Mississauga, ON, Canada). Both sides of the uterine horn were clamped to minimize blood loss and three whole placentas (one from the terminal, one from the middle and one near the cervix of the right uterine horn) were harvested from each dam, snap-frozen in liquid nitrogen, and stored at -80°C.

3.3 MTS Viability Assay

A [3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) assay was performed to determine a compound's effect on cell viability. HTR-8/SVneo cells were seeded in 96-well polystyrene tissue culture plates (Corning, 3595) at a density of 7 000 cells/well. Cells were allowed to attach O/N, and then treated with vehicle control or concentrations of the compound ranging from $1x10^{-9}$ M to 1×10^{-4} M. After the desired exposure time, cell viability was measured using the

MTS assay (Promega, G5421, Madison, WI, USA) according to the manufacturer's instructions. The viability of the cells was quantified by the amount of formazan present, which was determined by a 490nm absorbance measurement taken using the Multiskan Spectrum microplate spectrophotometer (Thermo Scientific, Rockford, IL, USA).

3.4 Invasion Assay

BioCoat Growth Factor Reduced Matrigel Invasion Chambers (Corning, 40483) were used to assess HTR-8/SVneo cell invasion. These chambers include Matrigel, which mimics a solubilized tissue basement membrane and contains common ECM components found in the maternal decidua (*198-200*). HTR-8/SVneo cells were serum-deprived (0.5% FBS) for 16 hours to allow for FBS to be used as a chemoattractant in this experiment. The Matrigel within the 24-well plate was rehydrated using serum free RPMI-1640 media for 2 hours. 50 000 HTR8/SVneo cells in serum-free RPMI-1640 supplemented with vehicle or drug treatments were seeded into the transwell inserts, on Matrigel (6.5-mm diameter polycarbonate membrane, 8-μm pore). RPMI-1640 with 5% FBS was added to the bottom of the insert, and the cells were allowed to invade through the Matrigel for 48 hours in a humidified cell culture incubator at 37°C, based on parameters defined previously (*201*). Non-invading cells were then gently removed from the top surface of the Matrigel using a cotton swab. Invaded cells on the undersurface were fixed with ice-cold 100% methanol for 2 minutes and stained with 1% Toluidine Blue O in 1% Borax (Sigma-Aldrich, 198161, St. Louis, MO, USA) for 2 minutes. The undersurface of the Matrigel was then removed and mounted bottom-up on glass slides. Five representative

brightfield images (10X objective) of each slide were obtained with an Eclipse Ni-U upright microscope (Nikon Instruments Inc., Mississauga, ON, Canada). The number cells that traversed the Matrigel were counted by an investigator blinded to the treatment group using ImageJ freeware (National Institutes of Health, Bethesda, MD, USA). Only cells that were clearly stained and had visible outer boundaries were included in the analysis. The average cell number per slide was determined, and the invasion index was expressed as the mean number of invaded cells treated with the drug relative to invaded cells treated with vehicle control.

3.5 Migration Assay

To determine whether OLN's effects on invasion may be due to its effect on HTR-8/SVneo cell mobility, trophoblast migration rate was evaluated using Ibidi μ-Dish 35mm, high culture inserts (Ibidi GmbH, 81176, Martinsried, Germany) according to the manufacturer's instructions. Briefly, 14 000 HTR-8/SVneo cells were seeded into each side of the culture inserts and allowed to attach in a humidified cell culture incubator O/N at 37°C. Inserts were then removed, leaving a 500μm ± 50μm space between two cell patches. HTR-8/SVneo cells were treated with 0.01% DMSO, 100nM or 1μM OLN and the center of the gap was imaged at 4.5X using Motic AE2000 inverted microscope (Motic, Richmond, BC, Canada) and VWR® V10 Microscope Camera (VWR International, Mississauga, ON, Canada). Images were taken every six hours until 30 hours when 100% of the gap was covered in all three treatment groups. Wimasis Image Analysis software (Wimasis GmbH, Munich, Germany) was used to determine the

percentage of cell-covered area and space area in each image. Percent of cell-covered area over time was plotted, and the linear migration phase was determined as per Wimasis manual edge detection analysis guidelines. The slope within this time period (6 and 24 hours) was then determined to establish the migration rate of HTR-8/SVneo cells treated with OLN relative to DMSO. The migration rate of each treatment was determined in order to account for any differences observed in the initial gap created at the outset of the experiment.

3.6 Trophoblast Gene Expression Analyses

3.6.1 Total RNA extraction and quantification

After the treatment period, cells were collected in Trizol Reagent (Life Technologies, 15596018, Carlsbad, CA, USA) and lysed using a 20-guage needle. Samples were then incubated for 5 minutes at room temperature to dissociate nucleoprotein complexes. Chloroform was added, and the samples were centrifuged at 4200 x g for 15 minutes at 4°C to separate the RNA-rich aqueous phase. RNA was then extracted using the RNeasy Mini Kit (Qiagen, 74104, Hilden, Germany) with the RNase-Free DNase set (Qiagen, 79254) to reduce DNA contamination according to the manufacturer's instructions.

To extract RNA from HTR-8/SVneo cells in Matrigel (i.e. 24 hours into the invasion assay) Cell Recovery Solution (Corning, 354253) was used as per the manufacturer's instructions. Briefly, cells in Matrigel were scraped in Cell Recovery Solution and incubated for 40 minutes at 4°C with gentle agitation to dissolve the Matrigel. Cells were

subsequently washed and centrifuged at 2500 rpm for 5 minutes at 4°C. RNA from these cells was then isolated using the Trizol-RNeasy Mini Kit RNA extraction method described above.

RNA from 50mg of rat placenta was isolated by first homogenizing the tissue in Trizol using a Polytron homogenizer (Kinematica GmbH, Kreins-Luzern, Switzerland). Placenta homogenates were then subjected to the RNA extraction protocol described above. RNA quantification and purity was determined using the NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific). The absorbance ratio of 260nm to 280nm (A_{260}/A_{280}) and 260nm to 230nm (A_{260}/A_{230}) for each sample used in this study was above cut-off thresholds of 1.7 and 1.8-2.2, respectively. All RNA was stored at -80°C until used.

3.6.2 Complementary DNA (cDNA) synthesis

A maximum of 4µg of total RNA was transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814, Foster City, CA, USA) according to manufacturer's instructions with the iCycler thermocycler (BioRad Laboratories, Hercules, CA, USA). The cycling conditions were 25°C for 10 minutes, followed by 37°C for 120 minutes and 85°C for 5 minutes. All cDNA was stored at -20°C.

3.6.3 Primer design and validation

Primers targeting genes of interest (Supplemental Table 1) were designed using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD, USA) with the following changes from default settings:

a) PCR product size: Minimum 50 base pairs and maximum 150 base pairs
b) Primer melting temperature: Minimum 58°C, optimal and maximum 60°C
c) Exon-exon junction span when possible to ensure no amplification of genomic DNA contaminants or introns

Each primer set was then assessed for homo- and hetero-dimerization using the OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA, USA). Primers with ΔG (kcal/mol) values less than -9.0 for both dimers were excluded as potential primers for validation. Primer sets were then synthesized by the MOBIX Lab (DNA Sequencing and Oligo Synthesis Facility, Hamilton, ON, Canada) with the following specifications: 25nM synthesis scale and de-salted purification. Stock primers were diluted to 100μM with pure water, and primers were validated through dissociation analyses of melt curves using a 5-fold serial dilution of a pooled cDNA samples. Only primer sets producing a single melting peak in the dissociation curves were used to quantify gene expression in samples.

3.6.4 Real-time quantitative polymerase chain reaction (RT-qPCR) assay

The relative abundance of each target transcript was determined by RT-qPCR using a LightCycler 480 384-multiwelll plate (Roche, 04729749001) and LightCycler 480 II

machine (Roche, Mannheim, Germany). For each experiment, cDNA samples (or water as a negative control) were mixed with 66nM of target primers and PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, 95072-05K, Gaithersburg, MD, USA) in triplicate. The cycling conditions were polymerase activation (95°C for 10 minutes), followed by 50 cycles of denaturing (95°C for 15 seconds) and annealing/elongation (60°C for 1 minute).

3.6.5 Analysis of RT-qPCR results

The relative fold change in target gene expression between OLN and DMSO treatments, or between GD15 and GD20 rat placenta, was determined via the 2^{nd} Derivative Maximum Method in the LightCycler 480 Software Release 1.5.1.62 (Roche). The crossing point was set at the exponential increase in amplification, and the relative fold changes were calculated using the comparative cycle times (ΔCt) method (*202, 203*). The expression of β-actin and 18S were used as reference genes for HTR-8/SVneo cell samples as done previously (*204, 205*). RNA polymerase II and 18S were used as reference genes for rat placenta samples, since the absolute expression of other tested housekeepers that had been previously recommended (*206-209*) altered significantly with gestational age (Supplemental Figure 1). ΔCt values were then normalized to the average of the vehicle control, or GD15, (ΔΔCt) and underwent a linear transformation by the formula, $2^{-\Delta\Delta Ct}$.

3.7 Protein Preparations

3.7.1 Total cell protein extraction

After treatment periods, dead cells were aspirated, and remaining adherent live, cells were scraped and pelleted by centrifugation at 2500 rpm for 5 minutes. Cells were then resuspended in either radioimmunoprecipitation assay (RIPA) lysis buffer (25mM Tris-HCL pH 7.6, 1% (v/v) NP-40, 0.1% (w/v) SDS, 150nM NaCl, 1% (w/v) sodium deoxycholate) or HEPES buffer (5mM HEPES pH 7.5, 100mM KCl, 70mM sucrose, 220mM mannitol, 1mM EGTA), supplemented with cOmplete Mini EDTA-free protease inhibitor (Roche, 11836170001) and phosphatase inhibitor cocktail tablets (Roche, 04693159001). Samples were then sonicated using the Microson Ultrasonic Cell Disrupter XL 2000 (Misonix, Farmingdale, NY, USA) at 7Hz for 15 seconds. All isolates were stored at -80°C until used.

3.7.2 Mitochondrial enriched protein preparation

Cells were washed in Dulbecco's Phosphate-Buffered Saline (DPBS), harvested in HEPES buffer and mitochondria were isolated by differential centrifugation. Briefly, cells were centrifuged at 2000 rpm for 5 minutes, and the resulting pellet was resuspended in 1mL HEPES buffer with cOmplete Mini EDTA-free protease inhibitor cocktail tablets and 2mg/mL fatty acid free bovine serum albumin (BSA; Roche, 10775835001). The cell suspension was sonicated on ice using the Microson Ultrasonic Cell Disrupter XL 2000 (Misonix, Farmingdale, NY, USA) for three 10-second pulses at 7Hz, then centrifuged at 1000 x g for 1 minute at 4°C. The supernatant was removed and centrifuged at 16000 x g

for 5 minutes at 4°C and the mitochondrial fraction, in the pellet, was then resuspended in a minimal volume of HEPES buffer supplemented with protease inhibitors, without BSA, and flash frozen in liquid nitrogen. Samples were stored at -80°C until use.

3.7.3 Nuclear and cytoplasmic cell protein extraction

To isolate nuclear and cytoplasmic protein fractions from HTR-8/SVneo cells, the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, 78833) were used as per manufacturer's instructions. Following treatment period, cells were washed with DPBS, harvested with trypsin-EDTA and centrifuged at 500 x g for 5 minutes to remove cell debris. Cells were resuspended in ice-cold Cytoplasmic Extraction Reagents I and II and incubated on ice to disrupt the cell membranes and cause the release of cytoplasmic contents. Suspensions were subsequently centrifuged for 5 minutes at 16 000 x g in 4°C to pellet intact nuclei. The cytoplasmic extract (supernatant) was then removed and stored at -80°C until required. The pellet, containing the nuclei, was suspended in ice-cold Nuclear Extract Reagent and incubated on ice for 40 minutes. While incubating, samples were vortexed (for 15 seconds) every 10 minutes. Following incubation, samples were centrifuged for 10 minutes at 16 000 x g in 4°C to remove debris, and the supernatant was stored at -80°C until needed.

3.7.4 Bicinchoninic acid protein assay

Protein concentration in each sample was determined using a bicinchoninic acid protein assay kit (Thermo Scientific, 23227) according to manufacturer's instructions. Protein

samples were diluted 1:10 in RIPA or HEPES buffer and all samples and BSA standards were assayed in duplicate. Absorbance was monitored at 562nm using the Multiskan Spectrum microplate spectrophotometer (Thermo Scientific).

3.8 Immunoblotting

3.8.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins by molecular weight prior to Western blotting. 10μg or 20μg of protein loading samples were prepared with 2X sample loading buffer (0.5M Tris-HCl, pH 6.0, 1% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol) and double distilled water then heated at 90°C for 5 minutes to denature proteins. Proteins were subsequently separated on 7.5%, 10% or 12.5% bis-polyacrylamide gels by being subjected to SDS-PAGE at 125V (constant current) for 1.5hours. Separated proteins were then wet transferred onto a 0.45μm nitrocellulose membrane (BioRad Laboratories) for 1.25 hours at 110V on ice. Both the SDS-PAGE and transfer was completed using the Mini-PROTEAN® Tetra Cell Electrophoresis System (BioRad Laboratories).

3.8.2 Western blotting

Membranes were blocked for 2 hours in 5% (w/v) fat free skim milk (Carnation, Markham, ON, Canada) or BSA (Roche, 10735078001) diluted in Tris-buffered saline with Tween 20 (TBST; 150mM NaCl, 20nM Tris Base, pH 7.6, 0.1% (v/v) Tween 20) at room temperature. Membranes were then incubated with primary antibodies (Supplemental Table 2) O/N at 4°C. Following incubation, membranes were washed

seven times in TBST for 3 minutes each time. Membranes were then incubated with horseradish peroxidase-conjugated secondary anti-rabbit IgG or anti-mouse IgG antibody (1:5000; GE Healthcare, Buckinghamshire, UK) in the same conditions as the primary antibody for 1-2 hours at room temperature. Membranes were again washed in TBST and developed using Immobilon Western Chemiluminescent HRP substrate (Millipore, WBKLS0500, Billerica, MA, USA). Images were captured using Image Reader LAS-3000 (FujiFilm, Mississauga, ON, Canada). If necessary for normalization purposes, membranes were stripped of primary antibodies using Restore Western Blot Stripping Buffer (Thermo Scientific, 21059) and re-blocked for 1 hour. Membranes were then probed O/N for the appropriate normalizing protein of β-actin (total cell protein), lactate dehydrogenase A (LDHA; cytosolic protein fraction), porin (mitochondrial protein fraction), or histone H2A (nuclear protein fraction) to control for protein loading. Membranes were then prepared for image detection as described above. Protein expression was quantified using ImageJ software (National Institutes of Health) to measure the densitometry of the target bands.

3.9 MMP Activity Assay

7 000 HTR-8/SVneo cells were seeded in 96-well tissue culture treated plates (Corning, 3595) and allowed to attach O/N. Cells were then treated with 0.01% DMSO, 100nM or 1μM OLN in RPMI-1640 supplemented with 0.5% FBS, and without phenol red (Life Technologies, 11835-030). Following 48 hours, media was removed, and total MMP activity in media samples was then assessed using the Fluorometric-Green MMP Activity

Assay kit (Abcam, ab112146, Cambridge, MA, USA) as per manufacturer's instructions. Briefly, 15ng of a positive control, human MMP9 full-length protein (Abcam, ab168863) was mixed with 2mM 4-Aminophenylmercuric acetate buffer and added to a white opaque 96-well microplate (Corning, 353296). Media samples were also added to the microplate, and incubated in a humidified cell culture incubator at 37°C for 2 hours to activate the MMP9 positive control. A fluorescence resonance energy transfer MMP substrate was then added and incubated for 1 hour to allow active MMPs in samples to cleave the substrate into two separate fragments, resulting in fluorescence recovery. The fluorescent intensity was measured at 490nm/525nm (Ex/Em) using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA).

3.10 Gelatin Zymography

Gelatin zymography was performed to detect whether changes in total MMP activity may be attributable to the activity of gelatinases MMP2 or MMP9. HTR-8/SVneo cells were treated with 0.01% DMSO, 100nM OLN or 1μM OLN in RPMI-1640 supplemented with 0.5% FBS for 48 hours. Media was then collected, and proteins with a molecular weight greater than 10kDa were concentrated using Nanostop centrifugal devices (Pall Life Sciences, OD010C33, Ann Arbor, MI, USA). Samples for electrophoresis were prepared by diluting protein samples with zymogram sample buffer (BioRad Laboratories 161-0764). Proteins were separated on a 10% Zymogram Ready Gel precast gel with gelatin (BioRad Laboratories, 161-1167) via SDS-PAGE at 125V for 30 minutes then 110V for 1.5 hours. The gel was then imaged using Image Reader LAS-3000 (FujiFilm) to view the

protein standard. During electrophoresis, SDS causes MMPs to denature and become inactive (*211, 212*); therefore, MMPs were renatured by washing and incubating in renaturation buffer (BioRad Laboratories, 1610765) for 30 minutes at room temperature with gentle agitation. MMPs in the gel were then initially activated by incubating the gel in development solution (BioRad Laboratories, 1610766) for 30 minutes at room temperature, and continually activated/allowed to degrade the gelatin (*212*) by incubating in zymogram development buffer O/N at 37°C. The gel was then stained with Coomassie Blue (0.5% Coomassie Blue R-250 in a solution of 40% methanol, 10% acetic acid) for 1 hour at room temperature with gentle agitation and destained (40% methanol, 10% acetic acid in water) for 1 hour under the same conditions. The zymogram was then imaged, and bands were analyzed using the same technique as for immunblots.

3.11 Assessment of Mitochondrial ETC Activity

ETC activity assays were performed on protein samples isolated from the mitochondria of HTR-8/SVneo cells exposed to OLN (as outlined in section 3.7.2). This allows for minimal cross contamination with cytosolic proteins (Supplemental Figure 2), and thus, changes in measured activities are due to mitochondrial ETC complexes as opposed to cytosolic proteins, such as NADPH oxidase.

3.11.1 Citrate synthase assay

Citrate synthase activity, an indicator of total mitochondrial mass (*213*), was measured and used to normalize mitochondrial ETC complex activity. Mitochondrial fractions were

added to a mixture of 100mM Tris buffer (200mM Tris-HCL with 0.2% Triton X-100, pH 8.0), 100µM 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich, 69783), and 300µM acetyl co-A (Roche, 1010189300). A baseline reading was taken at 1 minute then the reaction was initiated by adding 510µM oxaloacetic acid (Sigma-Aldrich, O4126). Readings were taken in duplicate at 412nm and 37^{o}C using the Cary 300 Bio UV-Visible Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The specific activity of citrate synthase was determined using the methods described previously (*213*).

3.11.2 NADH:cytochrome c oxidoreductase assay

NADH:cytochrome *c* oxidoreductase (Complex I+III) activity was assayed by mixing mitochondrial fractions with 50mM potassium phosphate buffer (pH 7.5), 1 mg/mL of fatty acid free BSA (Roche, 10775835001), 300µM potassium cyanide (Sigma-Aldrich, 151508) and 50µM oxidized cytochrome *c* (Sigma-Aldrich, 9007436). In the reference cuvette, 10µM rotenone (Sigma-Aldrich, 83794) was added at which point baseline readings were taken for 1 minute. The reaction was started with the addition of 40µM NADH (Roche, 10107735001) and the change in absorbance at 550nm and 37^{o}C was assessed using the Cary 300 Bio UV-Visible Spectrophotometer (Agilent Technologies). The specific activity of Complex I+III was calculated as described previously (*213*).

3.11.3 Succinate:cytochrome *c* oxidoreductase *assay*

Succinate:cytochrome *c* oxidoreductase (Complex II+III) activity was measured by mixing mitochondrial fractions with 50mM potassium phosphate (pH 7.5), 300µM of

potassium cyanide (Sigma-Aldrich, 151508) and 10mM of succinate (Sigma-Aldrich, 150903). In the reference cuvette 10mM malonate (Sigma-Aldrich, 141957) was added and incubated for 10 minutes at 37^{o}C. The reaction was started by adding 50μM of oxidized cytochrome *c* (Sigma-Aldrich, 9007436) and the change in absorbance was assessed at 550nm and 37^{o}C using the Cary 300 Bio UV-Visible Spectrophotometer (Agilent Technologies). The specific activity of Complex II+III was determined using methods described previously (*213*).

3.11.4 Cytochrome c oxidase assay

Cytochrome *c* (Complex IV) activity was assessed by determining the ability of this complex to reduce exogenously added reduced cytochrome *c*. Mitochondrial samples were mixed with 50mM of potassium phosphate buffer (pH 7.5), and 60μM reduced cytochrome *c* (made using ascorbic acid (*213*) and purified via dialysis). The oxidation of cytochrome *c* was then determined in duplicate by measuring the decline of reduced cytochrome *c* absorbance at 550nm over time using the Cary 300 Bio UV-Visible Spectrophotometer (Agilent Technologies) at 37^{o}C. The specific activity of Complex IV was calculated using the methods described previously (*213*).

3.12 Total Cellular ROS Detection

ROS can act as important signaling molecules, and includes free radicals such as superoxide ($O_2^{\cdot -}$), the hydroxyl radical, and other reactive molecules such as hydrogen peroxide (*148*). Therefore, the total amount of ROS produced in HTR-8/SVneo cells

treated with DMSO and OLN was quantified using the 2',7'-dichlorofluorescin diacetate (DCFDA) Cellular Reactive Oxygen Species Detection Assay kit (Abcam, ab113851). HTR-8/SVneo cells were also exposed to Antimycin A (ANT; Sigma-Aldrich, A8674-25MG) as a positive control, since ANT induces ROS production in HTR-8/SVneo cells (unpublished data). After exposure to drugs, DCFDA dye was added and deacetylated by cellular esterases to a non-fluorescent compound, which can be oxidized by ROS into the highly fluorescent 2',7'-dichlorofluorescin. The resultant fluorescence was detected at wavelengths 495nm/529nm (Ex/Em) using the Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek).

3.13 ADP/ATP Assay Kit

To determine whether potential alterations in mitochondrial ETC function by OLN resulted in a decrease in cellular energy (adenosine triphosphate; ATP), the ADP/ATP ratio within HTR-8/SVneo cells was determined using the luciferin-luciferase bioluminescent ADP/ATP Ratio Assay kit (Abcam, ab65313). A background luminescent measurement was taken using the Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Biotek) after the ATP reaction mixture (made of Nucleotide Releasing Buffer and ATP Monitoring Enzyme) was incubated in a black 96-well tissue culture plate (Corning, 353219). After exposure to OLN, HTR-8/SVneo cells were washed with DPBS and incubated in Nucleotide Releasing Buffer for 5 minutes at room temperature to permeabilize the membrane and cause the release of ADP and ATP into solution. This solution was then added to the ATP reaction mixture, incubated for 2 minutes at room

temperature and a luminescent reading was taken again. The ADP was then converted to ATP by adding ADP Converting enzyme during a 2 minute incubation, and another luminescent read was taken. The ADP/ATP ratio was then calculated as per the manufacturer's guidelines.

3.14 Statistical Analysis

All analyses were performed using GraphPad Prism 6.0g (GraphPad Software Inc., San Diego, CA, USA). Data was tested for outliers, normality, and equal variance. Values classified as statistical outliers by the Grubb's test using GraphPad QuickCalcs (GraphPad Software Inc., San Diego, CA, USA) were removed from analyses. In cases where data was not normally distributed, the data was log transformed in order to achieve normality. Data was analyzed using a two-tailed Student's T-test or a one-way analysis of variance (ANOVA) where appropriate and when significance ($p \leq 0.05$) was found, the Tukey *post hoc* test was used for multiple comparisons. For RT-qPCR analysis of rat placenta receptor gene expression, at least three placentae were examined from each dam, and each group of placentas from one dam was considered a statistical unit (*215*). All results are expressed as the mean ± standard error of the mean (SEM).

4. RESULTS

4.1 OLN Does Not Affect HTR-8/SVneo Cell Viability

Exposing HTR-8/SVneo cells to increasing concentrations ($1x10^{-9}$M to $1x10^{-4}$M) of OLN did not significantly alter cell viability when compared to untreated cells following 48 hours (Figure 4), 24 or 72 hours (Supplemental Figure 3). Moreover, physiologically relevant OLN concentrations (i.e., 100nM and 1μM) did not significantly affect HTR-8/SVneo cell growth over seven days (Supplemental Figure 4).

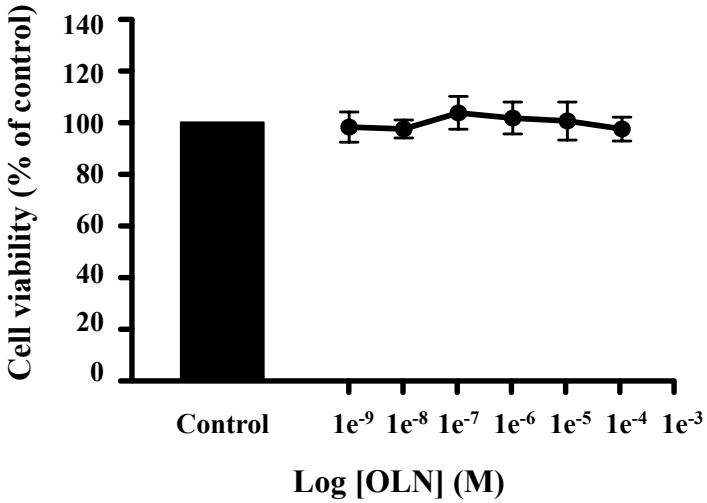

Figure 4. HTR-8/SVneo cell viability is not altered following OLN exposure. Cells were plated at a density of 70 000 cells/mL in a microplate and allowed to attach O/N. Cells were then treated for 48 hours with ranging OLN concentrations of $1x10^{-9}$M to $1x10^{-4}$ M (n=5). Viability following OLN exposure was assessed using a MTS assay. Values represent the mean ± SEM relative to untreated, control cells. Significance ($p \leq 0.05$) was determined using a one-way ANOVA.

4.2 OLN Affects HTR-8/SVneo Cell Invasion

Significantly more HTR-8/SVneo cells invaded Matrigel (Figure 5; 558±150 cells per field) when exposed to 1μM OLN compared to cells treated with 0.01% DMSO (Figure 5;

398±131 cells per field). However, no significant difference in invasion was found between HTR-8/SVneo cells exposed to 100nM OLN (Figure 5; 526±148 cells per field) compared to 0.01% DMSO.

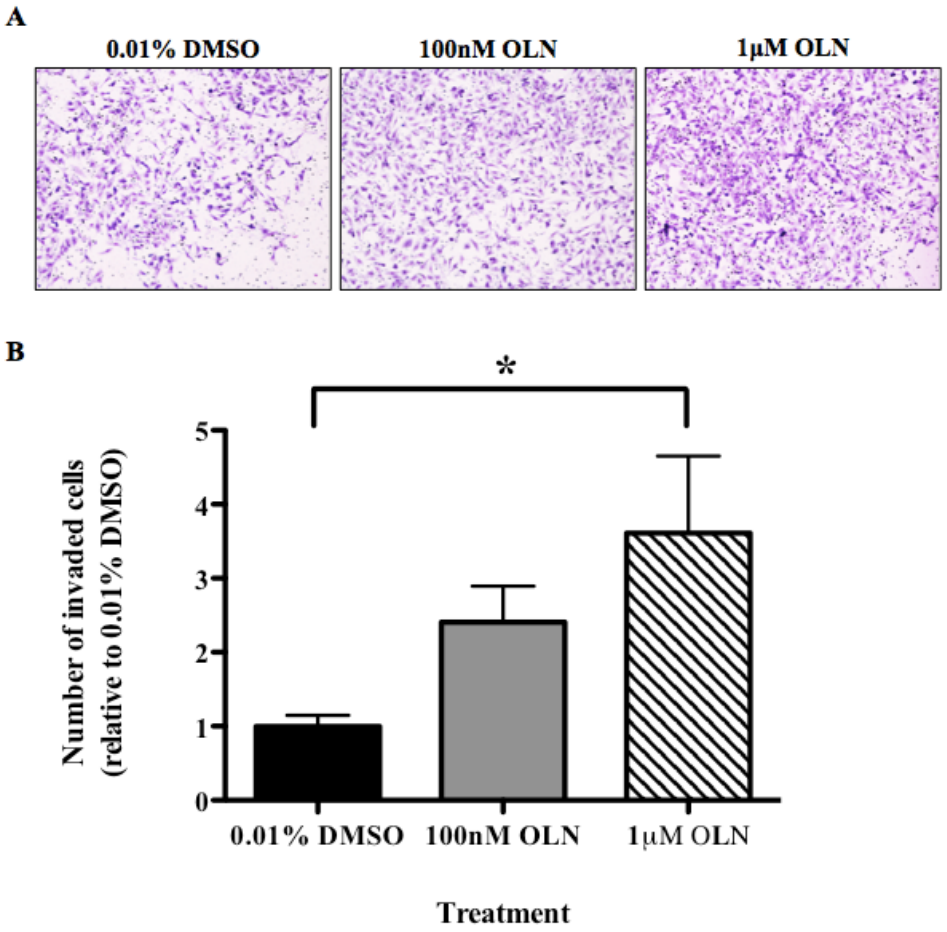

Figure 5. 1µM OLN increases the number of HTR-8/SVneo cells that invade Matrigel relative to 0.01% DMSO after 48 hours. Representative brightfield images of invaded cells treated with 0.01% DMSO, 100nM OLN and 1µM OLN are shown in **(A)**. HTR-8/SVneo cell invasion following 48 hours of exposure to 0.01% DMSO, 100nM OLN and 1µM OLN relative to 0.01% DMSO, vehicle control, are represented in (**B**). All values are presented as the mean ± SEM (n=6). The data was log transformed to achieve normality and significant differences (*$p<0.05$) between treatment groups was determined by one-way ANOVA and Tukey's *post hoc* analysis. Images were captured at 10X magnification using an upright Nikon microscope.

Trophoblast invasion is dependent on CTs differentiating into invasive EVTs, and their ability to migrate through the maternal decidua by degrading the ECM (*99, 102*). Therefore, we assessed how OLN may be increasing the invasion of HTR-8/SVneo cells by determining which of these processes are affected by OLN treatment. The gene expression of transcription factors known to have distinct profiles in trophoblast lineages (including: ID2, HLX, GCM1, STAT3 or FOSL1) (*99, 115*), proteases (MMPs, uPA), their inhibitors (TIMPs, PAI-1) (*99, 120*) as well as trafficking proteins (vesicle-associated membrane protein 3 (VAMP3) and synaptosomal-associated protein 23 (SNAP23) (*216*)) were not significantly different between cells exposed to 0.01% DMSO and 1μM OLN for 48 hours (Table 1). However, OLN treatment did result in a dose-dependent increase in the expression of MMP2 (Table 1). Additionally, the uPA inhibitor PAI-1 was significantly increased in OLN relative to DMSO-treated cells (Table 1).

Despite MMPs being subject to post-transcriptional regulation (*217*), OLN treatment did not significantly affect the protein expression of MMPs or their TIMP inhibitors (Figures 6 and 7). For trophoblast cells to invade the maternal decidua, intracellular MMPs are secreted and activated by cleavage. Only upon activation are MMPs able to degrade ECM proteins, such as those in the Matrigel of the invasion assay (*120*). Indeed, HTR-8/SVneo exposure to 1μM OLN for 48 hours significantly increased the total extracellular MMP activity compared to 0.01% DMSO and 100nM OLN (Figure 8), corresponding to the increase in invasion observed. Both the activity of MMP2 (Figure 8C) and MMP9 (Figure 8D) were not significantly different in media samples from cells treated with 0.01%

DMSO compared to 1μM OLN. Trophoblast invasion also involves the migration/movement of trophoblasts to the decidua and through the ECM once degraded (*218*). This involves the ability of EVTs to modulate the expression of certain adhesion molecules, which provide appropriate traction to, and mobility through, components of ECM (*110, 219*). However, OLN exposure did not significantly alter trophoblast migration (Figure 9) or gene expression of integrins and cell adhesion molecules (Table 2) that are differentially expressed in invasive compared to non-invasive trophoblasts (*106, 107, 110, 111*).

Table 1

Relative gene expression of transcription factors and invasion markers following 48 hour treatments

	Group		
Gene	0.01% DMSO	100nM OLN	1μM OLN
HLX	1.08±0.02	1.32±0.36	0.78±0.18
ID2	1.02±0.11	1.71±0.31	**0.77±0.09**[c]
FOSL1	1±0.01	0.92±0.01	0.84±0.09
GCM1	0.86±0.15	0.77±0.16	0.85±0.10
STAT3	1.18±0.18	1.56±0.12	1.20±0.14
MMP1	1.06±0.20	1.16±0.20	1.55±0.36
MMP2	1.12±0.32	**1.30±0.26**[a]	**2.97±0.42**[bc]
MMP3	0.66±0.09	0.71±0.30	0.86±0.31
MMP9	1.05±0.19	1.31±0.30	1.56±0.05
MMP14	1.06±0.18	1.17±0.22	0.88±0.13
TIMP1	1.00±0.04	1.05±0.03	1.02±0.06
TIMP2	1.00±0.04	0.95±0.02	1.01±0.04
uPA	1.08±0.23	1.24±0.29	1.51±0.04
PAI-1	1.05±0.18	1.04±0.17	**1.64±0.06**[b]
VAMP3	1.01±0.07	1.19±0.10	1.00±0.01
SNAP23	1.02±0.12	1.23±0.21	0.86±0.13

Data is presented as the mean fold change ± SEM relative to HTR-8/SVneo cells treated with 0.01% DMSO. Statistical significance ($p \leq 0.05$) was determined using a one-way ANOVA and Tukey *post hoc* test. [a] and [b] indicates statistical significance between 0.01% DMSO and 100nM or 1μM OLN, respectively while [c] represents a significant difference between 100nM and 1μM OLN; $n \geq 4$ per group.

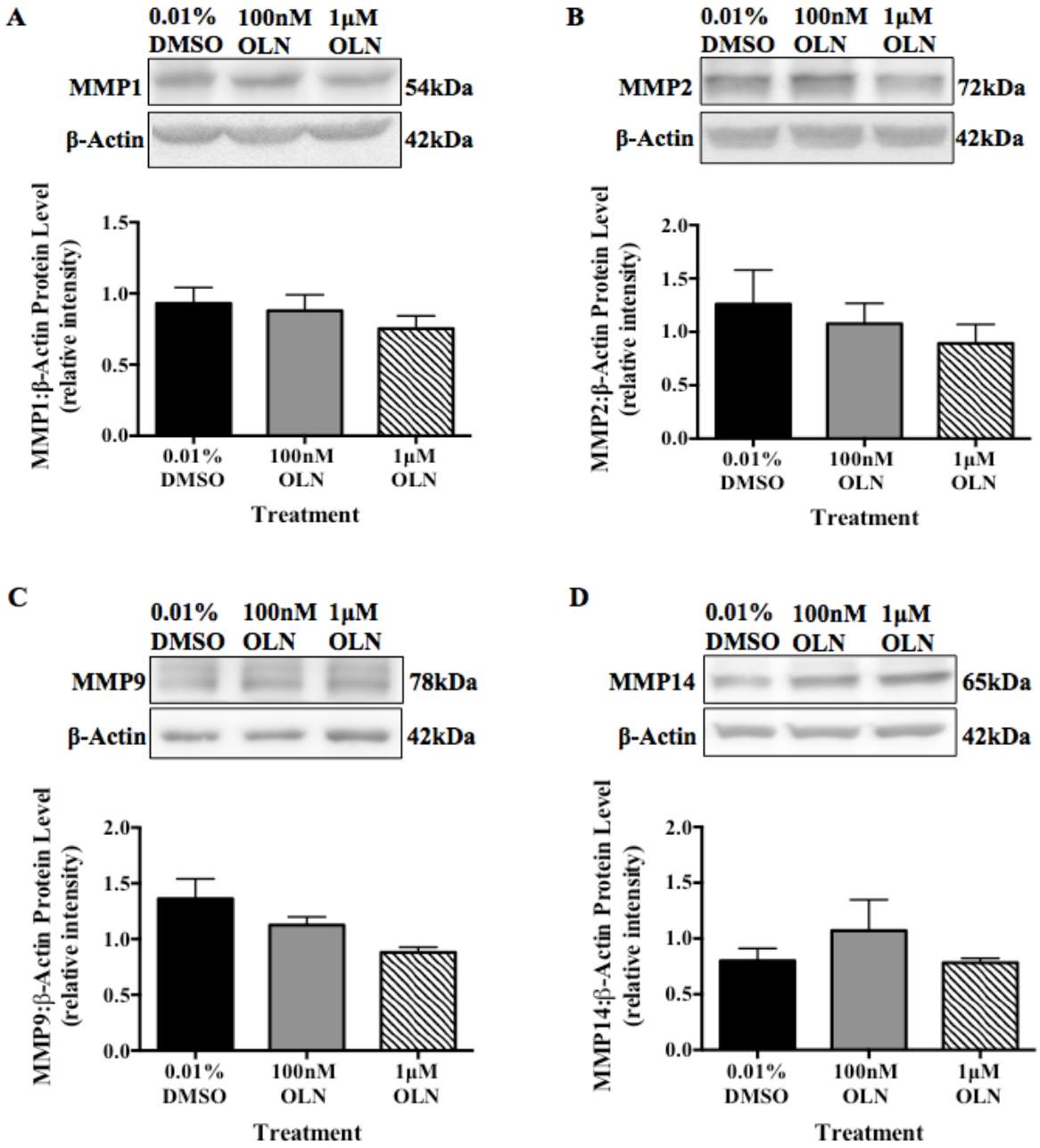

Figure 6. MMP protein levels in HTR-8/SVneo cells are not significantly different following 48-hour exposure to OLN. HTR-8/SVneo cells were cultured in the presence of 100nM, 1µM OLN or 0.01% DMSO for 48 hours on uncoated polystyrene. Proteins were extracted and separated on 10% polyacrylamide gels, transferred to nitrocellulose then incubated with antibodies targeted for proteins of interest: MMP1 (n=3) (**A**), MMP2 (n=3) (**B**), MMP9 (n=4) (**C**) and MMP14 (n=4) (**C**). All protein levels were normalized to β-Actin. The data is presented as the mean intensity of the target bands ± SEM. Significance ($p \leq 0.05$) was determined using a one-way ANOVA.

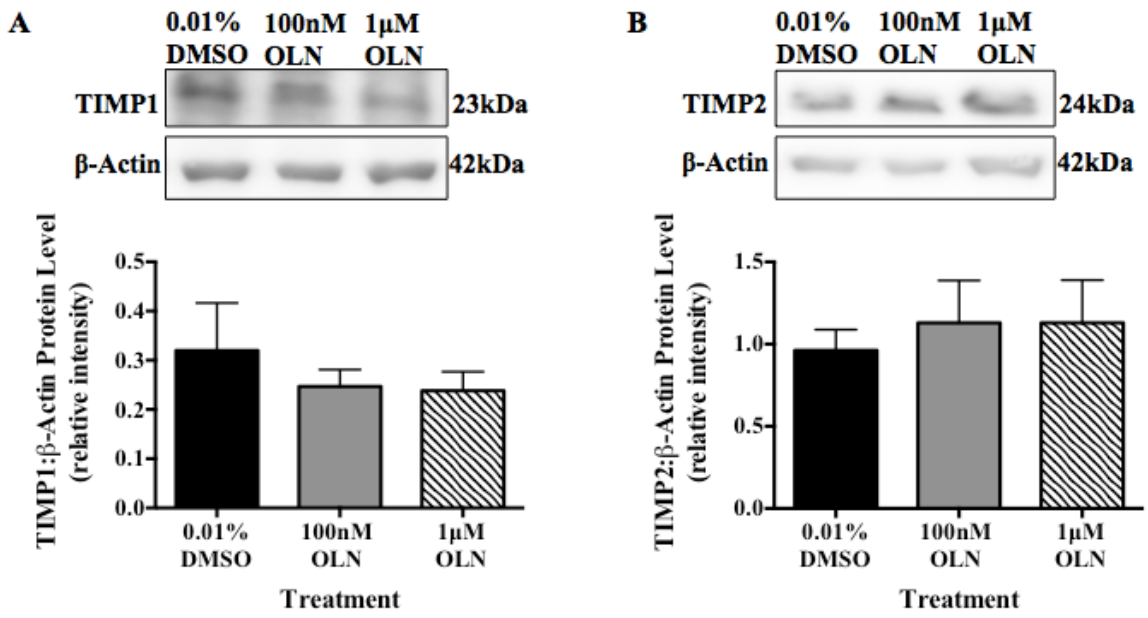

Figure 7. TIMP protein levels in HTR-8/SVneo cells are not affected following 48-hour exposure to OLN. HTR-8/SVneo cells were cultured in the presence of 100nM, 1μM OLN or 0.01% DMSO for 48 hours on uncoated polystyrene. Proteins were extracted and separated on 12.5% polyacrylamide gels, transferred to nitrocellulose then incubated with antibodies targeted for proteins of interest: TIMP1 (**A**) and TIMP2 (**B**). All protein levels were normalized to β-Actin. The data is presented as the mean intensity of the target bands ± SEM (n=4 per group). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

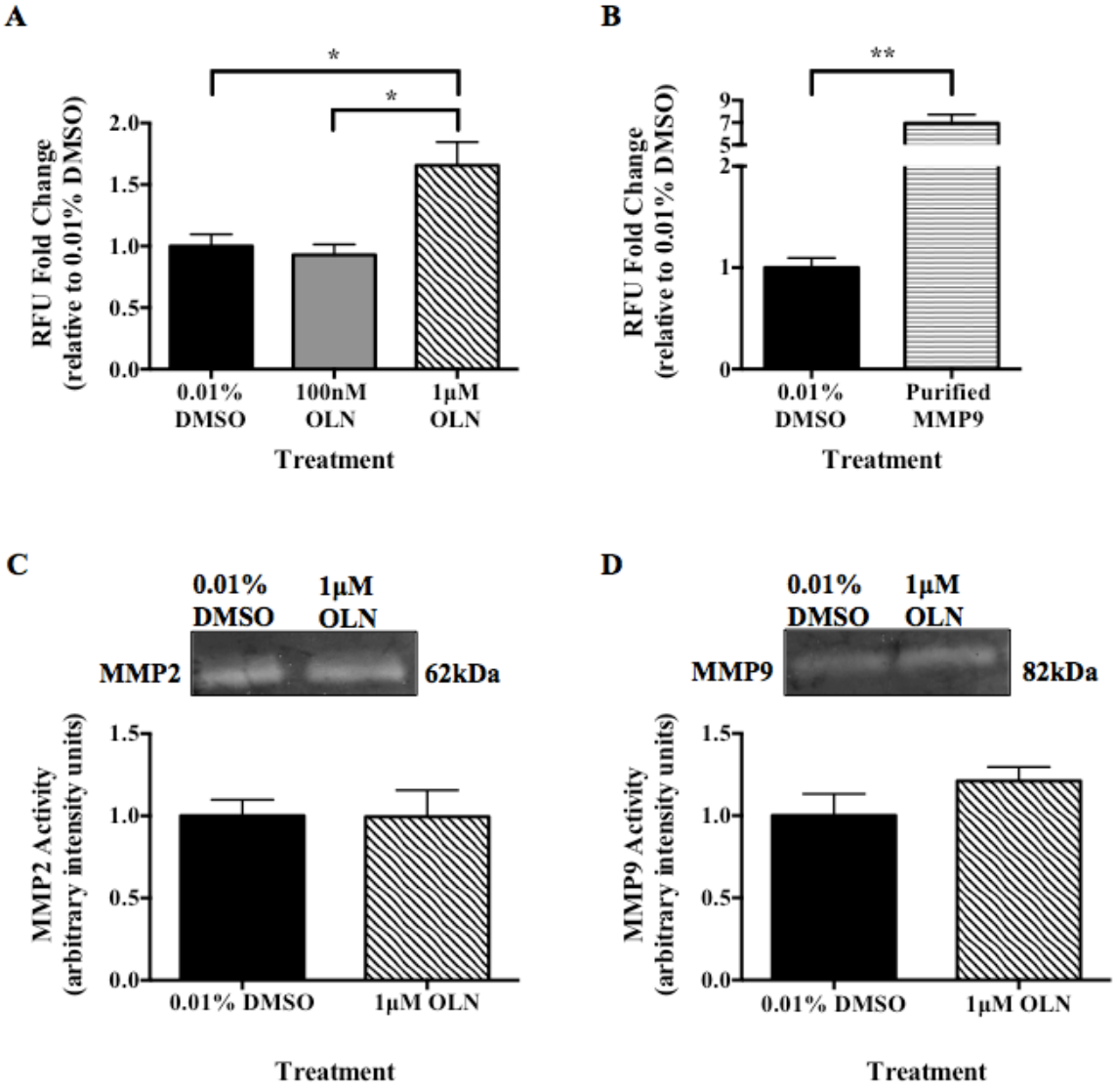

Figure 8. The effect of OLN on HTR-8/SVneo MMP activity. Media was collected from HTR-8/SVneo cells treated with 0.01% DMSO, 100nM or 1μM OLN and the total MMP activity in media samples was measured using a commercially available MMP activity assay kit (**A**). 15ng of activated MMP9 protein was used as a positive control **(B)**. Media from HTR-8/SVneo cells treated with 0.01% DMSO and 1μM OLN was also collected to assess the specific activities of MMP2 **(C)** and MMP9 **(D)** using gelatin zymography. Concentrated media samples were separated on a 10% polyacrylamide gel with gelatin, incubated to allow for degradation of gelatin, developed, and stained with Coomassie Blue. All values represent the mean intensity of the bands ± SEM (n=3 per group). Significance was assessed using a one-way ANOVA with Tukey *post hoc* analysis for multiple comparisons for the MMP activity assay kit (n=3; *$p<0.05$). The significance between the MMP9 positive control and DMSO as well as the zymogram experiment was determined using a two-tailed Student's T-test.

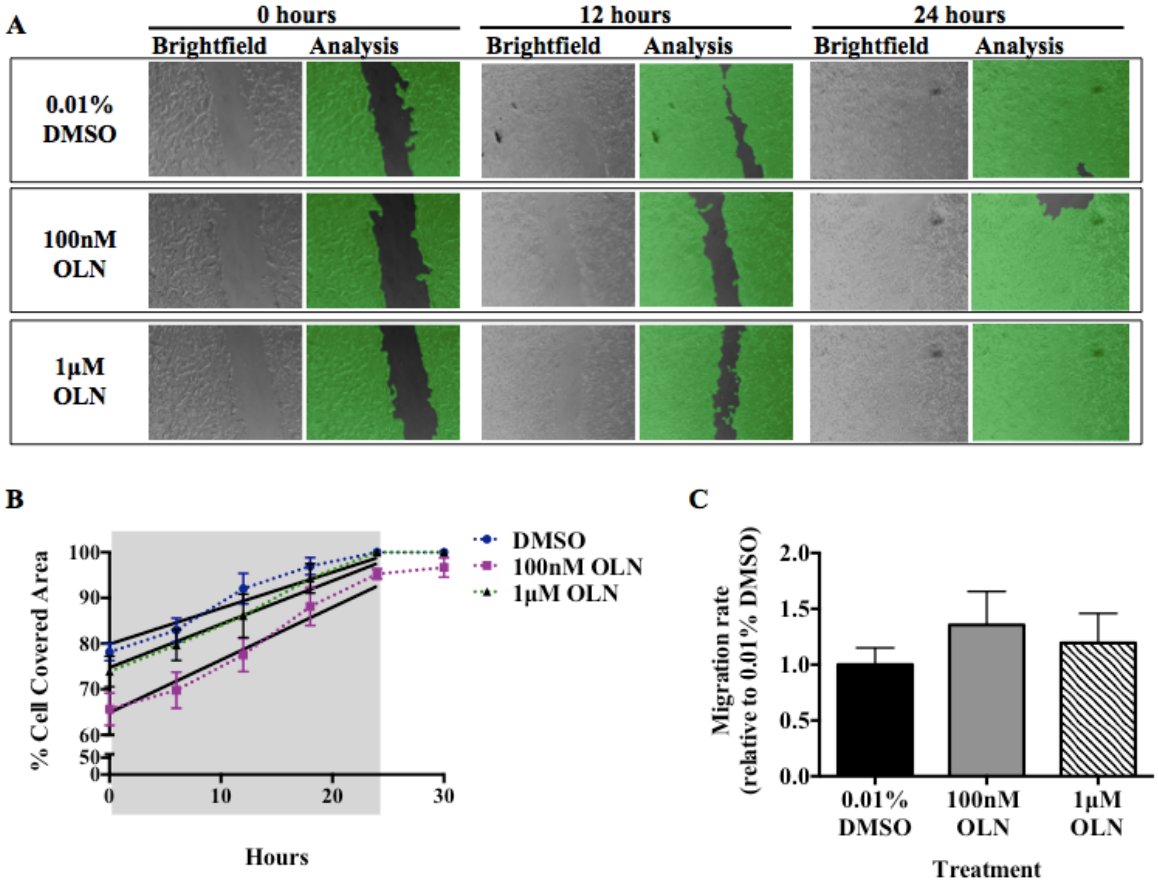

Figure 9. OLN does not significantly alter HTR-8/SVneo cell migration. HTR-8/SVneo cells were treated with 0.01% DMSO, 100nM or 1µM OLN and allowed to migrate to close a gap created on an uncoated polystyrene plate. Images were taken every six hours post-treatment initiation until 30 hours; representative images shown in (**A**). The linear migration phase (gray area) was identified by plotting % of cell-covered area over time and fitting the data with a linear regression (black lines overlaying plots) (**B**). The slope between time points giving the line of best fit (as determined by R^2>0.80) was determined to elucidate the migration rate (**C**). All data points represent the mean ± SEM (n=3). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

Table 2

Relative gene expression of adhesion molecules following 48 hour treatments			
	Group		
Gene	0.01% DMSO	100nM OLN	1μM OLN
ITGAV	1.01±0.08	1.07±0.10	1.41±0.28
ITGA1	1.51±0.47	1.73±0.47	1.63±0.26
ITGA5	1.05±0.18	1.06±0.22	1.60±0.15
ITGA6	1.03±0.16	1.03±0.07	1.12±0.13
ITGB1	1.00±0.04	0.81±0.12	0.87±0.13
ITGB4	1.04±0.18	0.94±0.16	1.03±0.12
CDH1	1.11±0.24	1.53±0.39	1.14±0.17
CDH5	1.11±0.22	0.71±0.17	0.87±0.21
PECAM-1	1.03±0.15	1.07±0.13	0.91±0.15

Data is presented as the mean fold change ± SEM relative to HTR-8/SVneo cells treated with 0.01% DMSO. Statistical significance ($p \leq 0.05$) was determined using a one-way ANOVA; n=4 per group.

4.3 A Survey Of Possible Receptor Targets for OLN in Trophoblasts

OLN can bind a variety of receptors with moderate to high affinity including: dopamine (D_1-D_5), serotonin (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{2A}, 5-HT_{2B}, 5- HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6 and 5-HT_7) as well as α-adrenoreceptors (α_{1A}, α_{1B}, α_{2A}, α_{2B} and α_{2C}), muscarinic (M_1-M_5), and histamine 1 (H_1) receptors (*67*). HTR-8/SVneo cells express all putative OLN target receptors, with the exception of dopamine D_3 (Table 3). A comparison of the receptor expression in the rat placenta between GD15 (a time of prolific invasion) and GD20 (term pregnancy) was done to elucidate which receptors known to be targeted by OLN may be responsible for the increase in HTR-8/SVneo invasion observed. The gene expression of the following receptors did not change between GD15 and GD20 placenta: serotonin 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{5A}, 5-HT_6, 5-HT_7, dopamine D_1, D_2, D_3, D_4, muscarinic M_2, M_3, M_5, as well as adrenergic α_{1B}, α_{2A},

and α_{2B} (Figure 10). While, adrenergic α_{2C}, muscarinic M_1, and M_4, histamine 1, as well as serotonin 5-HT_{1D} and 5-HT_{2B} receptors were up-regulated in GD20 placenta relative to GD15 during rat pregnancy. The adrenergic α_{1A} receptor was the only receptor whose gene expression was significantly decreased in GD20 rat placenta relative to GD15 (Figure 10). Therefore, the effect of the α_{1A} adrenergic receptor antagonist 5-methylurapidil (5-MU) on HTR-8/SVneo cell viability and invasion was determined. Because 5-MU can also act as an agonist at the serotonin 5-HT_{1A} receptor subtype, the effect of serotonin on HTR-8/SVneo invasion was also assessed. There was no significant difference between vehicle control and any concentration of 5-MU or 5-HT on HTR-8/SVneo cell viability or invasion through Matrigel (Figure 11).

Table 3

Gene expression of putative OLN receptor targets in HTR-8/SVneo cells

Class	Subtype										
Serotonin	1A	1B	1D	1E	2A	2B	2C	3	5A	6	7
	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
Dopamine	1	2	3	4	5						
	✔	✔	**ND**	✔	✔						
Muscarinic	1	2	3	4	5						
	✔	✔	✔	✔	✔						
Adrenergic	1A	1B	2A	2B	2C						
	✔	✔	✔	✔	✔						
Histamine	1										
	✔										

A checkmark (✔) represents the mRNA presence of that respective receptor subtype in HTR-8/SVneo cells. ND represents a receptor whose expression was not detected in cells ($n \geq 6$).

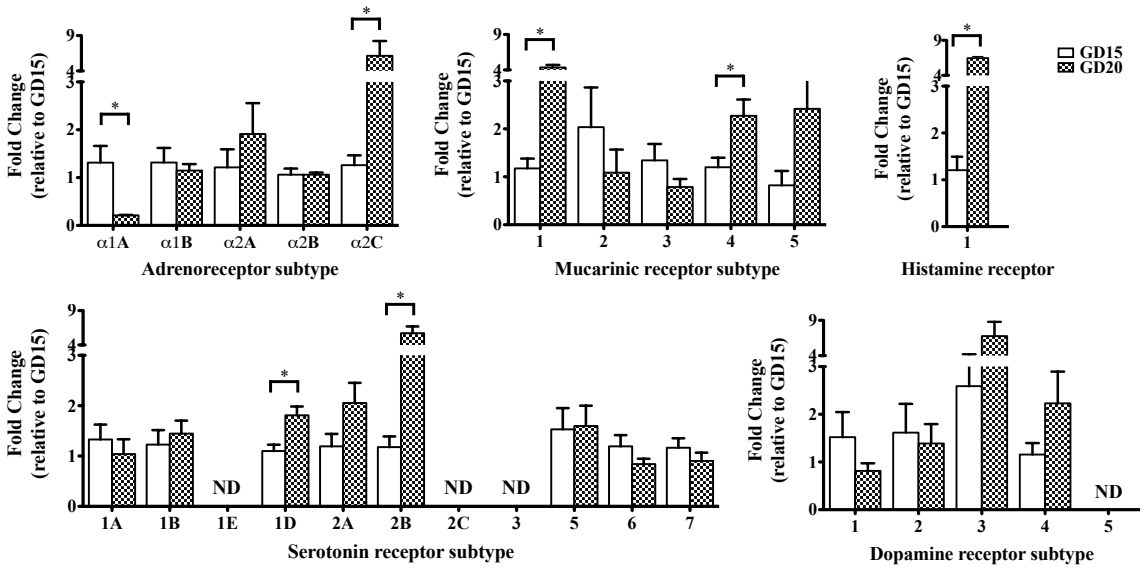

Figure 10. Gene expression of putative OLN target receptors are temporally regulated during rat gestation. The gene expression of receptors in the rat placenta was determined at GD20 relative to GD15 (n=5 dams). ND indicates a receptor whose expression was not detected. All receptor expression data was normalized to 18S and RNA polymerase II housekeeper genes. Significance was assessed using a two-tailed Student T-test, *p<0.05.

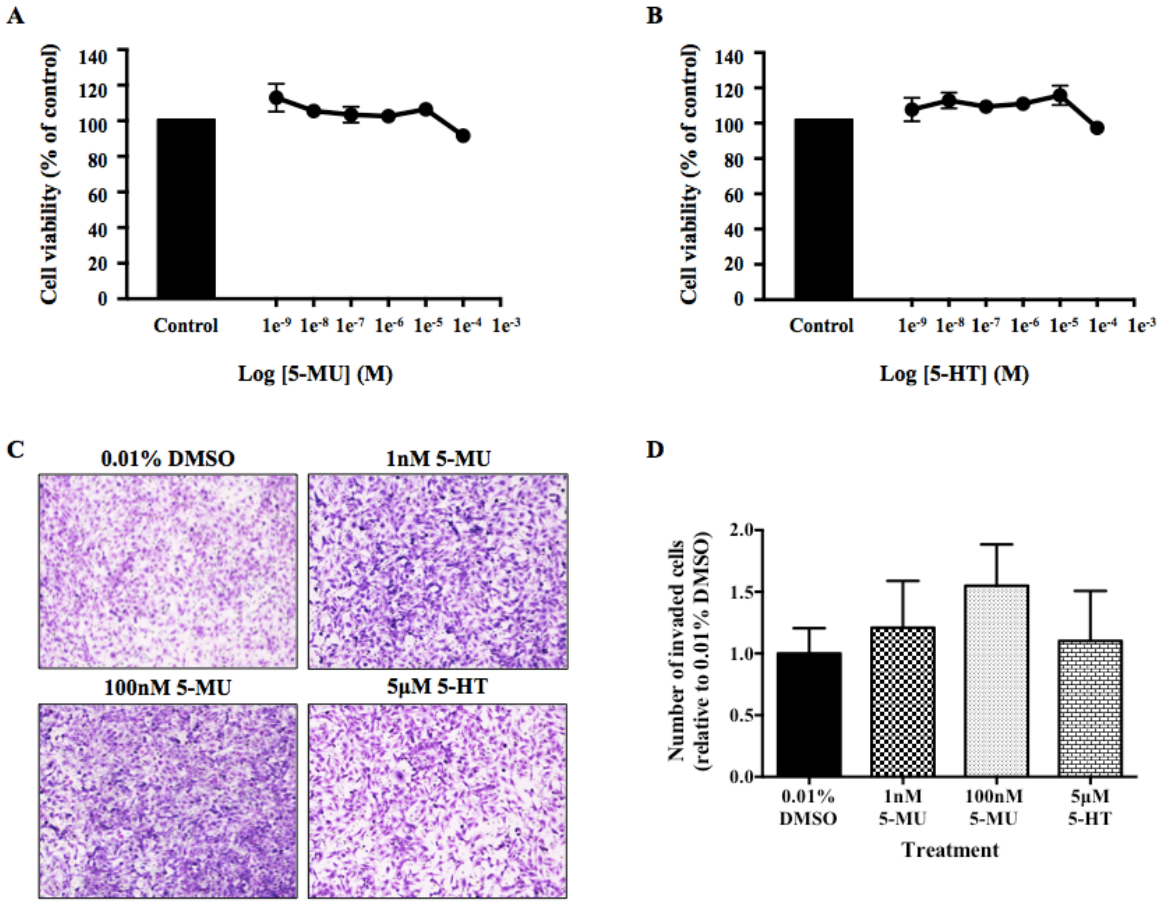

Figure 11. 5-MU and 5-HT do not affect HTR-8/SVneo cell viability or invasion. Treatment of HTR-8/SVneo cells for 48 hours with $1x10^{-9}$ to $1x10^{-4}$ M 5-MU (n=4) **(A)** or with $1x10^{-9}$ to $1x10^{-4}$ M 5-HT (n=3) **(B)** did not alter cell viability compared to untreated control cells. Representative brightfield images of invaded cells treated with 0.01% DMSO, 1nM 5-MU, 100nM 5-MU and 5µM 5-HT **(C)** show that HTR-8/SVneo cells treated with 10nM 5-MU, 100nM 5-MU or 5µM 5-HT did not alter cell invasion relative to 0.01% DMSO (n=3) **(D)**. Results are presented as the mean ± SEM relative to control. Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

4.4 Signaling Pathways Linking OLN to Increased HTR-8/SVneo Invasion

There are many signaling pathways that regulate trophoblast invasion, some of which are known to be stimulated by GPCRs, such as the MAPK, PI3K/AKT and STAT3 pathways (*99, 102*). Since many of the OLN putative receptor targets present in HTR-8/SVneo cells are GPCRs, the phosphorylation levels of two key kinases, AKT, and ERK1/2, and transcription factor STAT3 in the PI3K-AKT, MAPK, and JAK-STAT pathways, respectively was assessed following HTR-8/SVneo OLN exposure. Activation of these pathways by extracellular stimuli can occur over a broad temporal range (*220-222*). Therefore, the effect of 1μM OLN exposure on the activation of these pathways in HTR-8/SVneo cells was evaluated over a time span of 5 minutes to 24 hours (Supplemental Figure 5). There was no significant difference in the amount of phosphorylated relative to total protein expression of ERK1/2 (Figure 12), AKT1 (Figure 13) or STAT3 (Figure 14) with OLN exposure at any of the time points assessed; including 48 hours (Figure 15), the length of OLN exposure in invasion experiments.

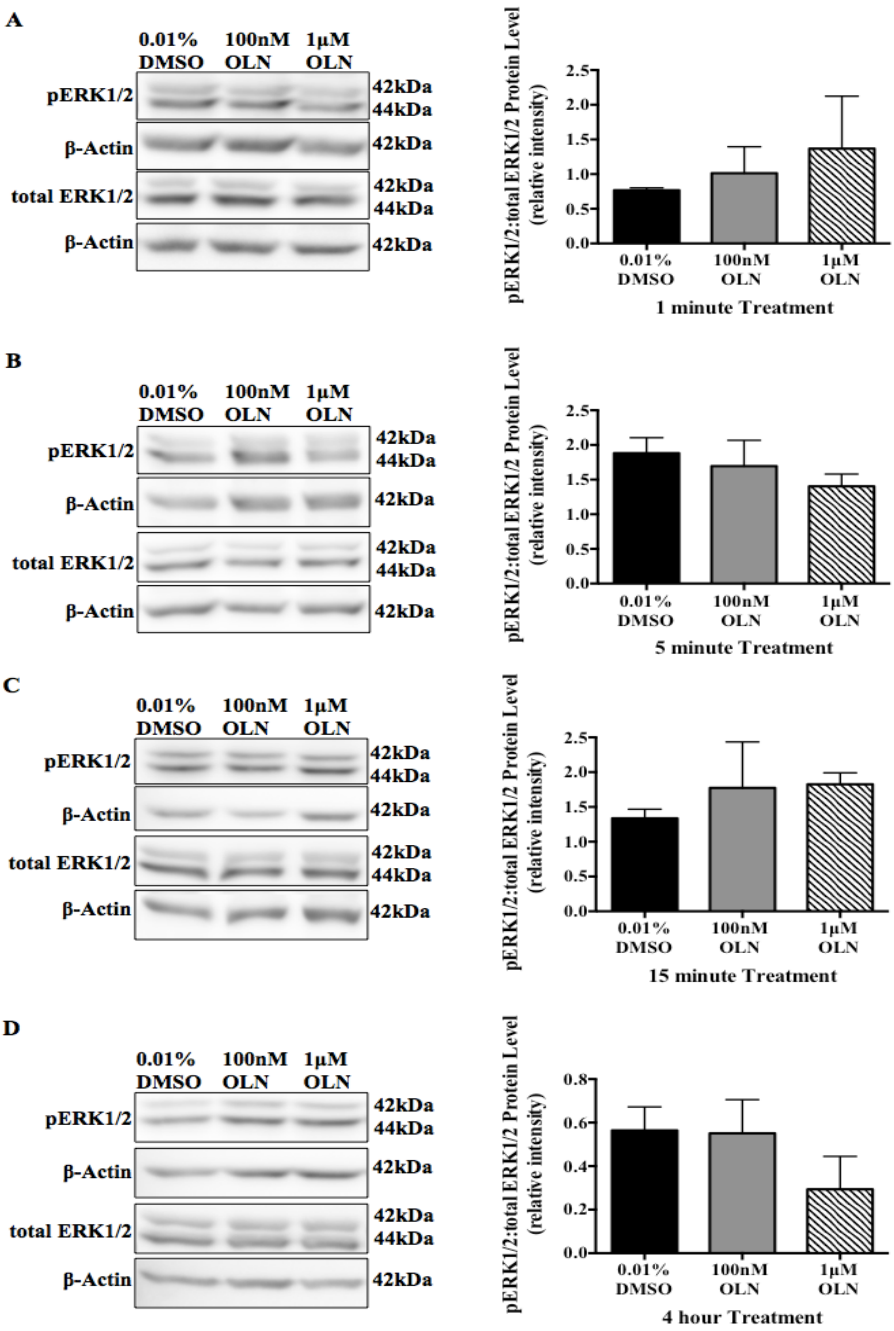

Figure 12. The effect of various OLN exposure times on the phosphorylation of ERK1/2 in HTR-8/SVneo cells. The protein levels of pERK1/2:ERK1/2 in HTR-8/SVneo cells grown in serum-deprived conditions was measured following exposure to DMSO or OLN for 1 minute **(A)**, 5 minutes **(B)**, 15 minutes **(C)** and 4 hours **(D)** by Western blot. All protein levels were normalized to β-Actin. The data is presented as a ratio of pERK1/2 to total ERK1/2 mean intensities of the target bands ± SEM (n=4). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

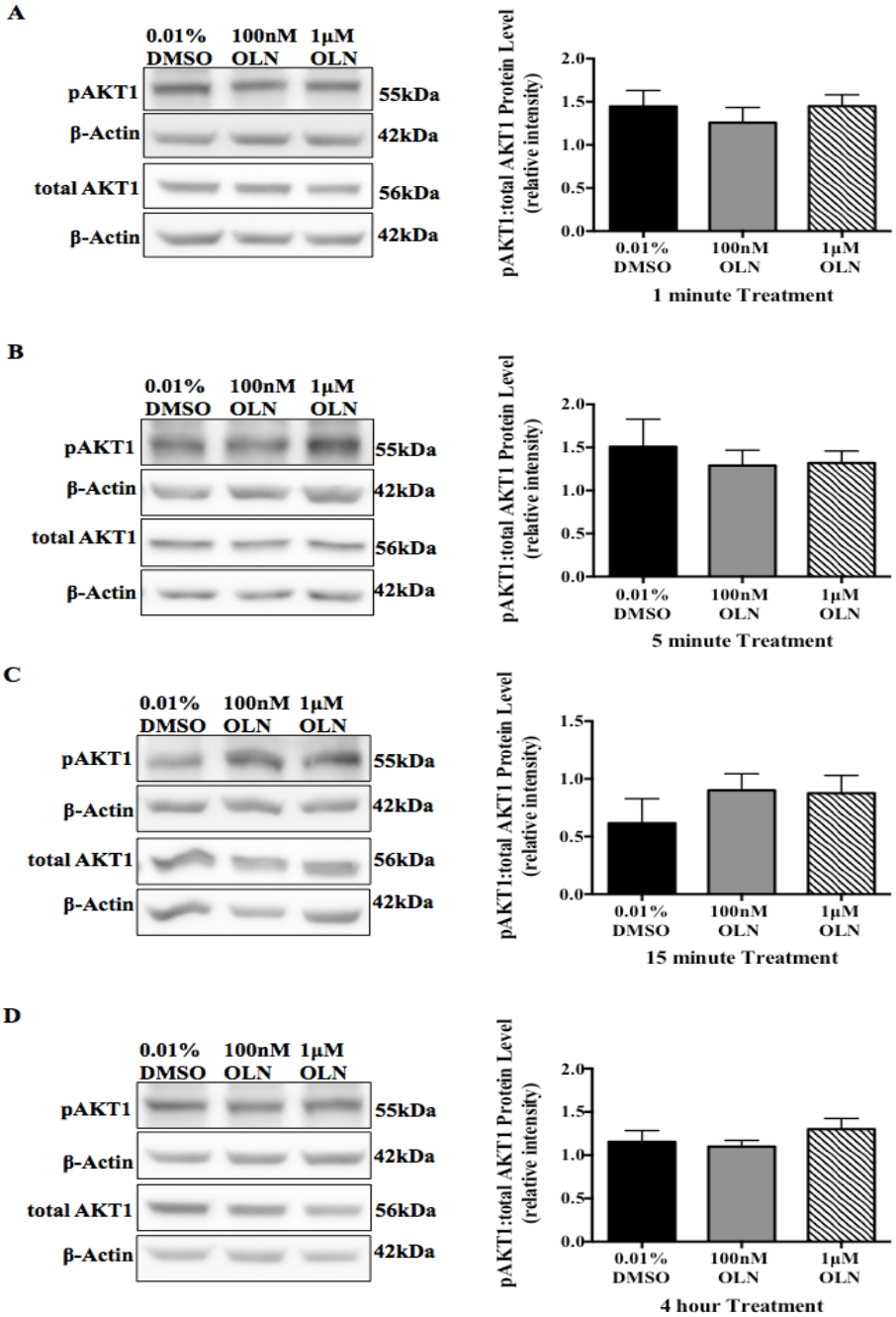

Figure 13. The effect of various OLN exposure times on the phosphorylation of AKT1 in HTR-8/SVneo cells. The pAKT1:AKT1 protein level in HTR-8/SVneo cells grown in serum-deprived conditions was measured following exposure to DMSO or OLN for 1 minute **(A)**, 5 minutes **(B)**, 15 minutes **(C)** and 4 hours **(D)** by Western blot. All protein levels were normalized to β-Actin. The data is presented as a ratio of pAKT1 to total AKT1 mean intensities of the target bands ± SEM (n=4). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

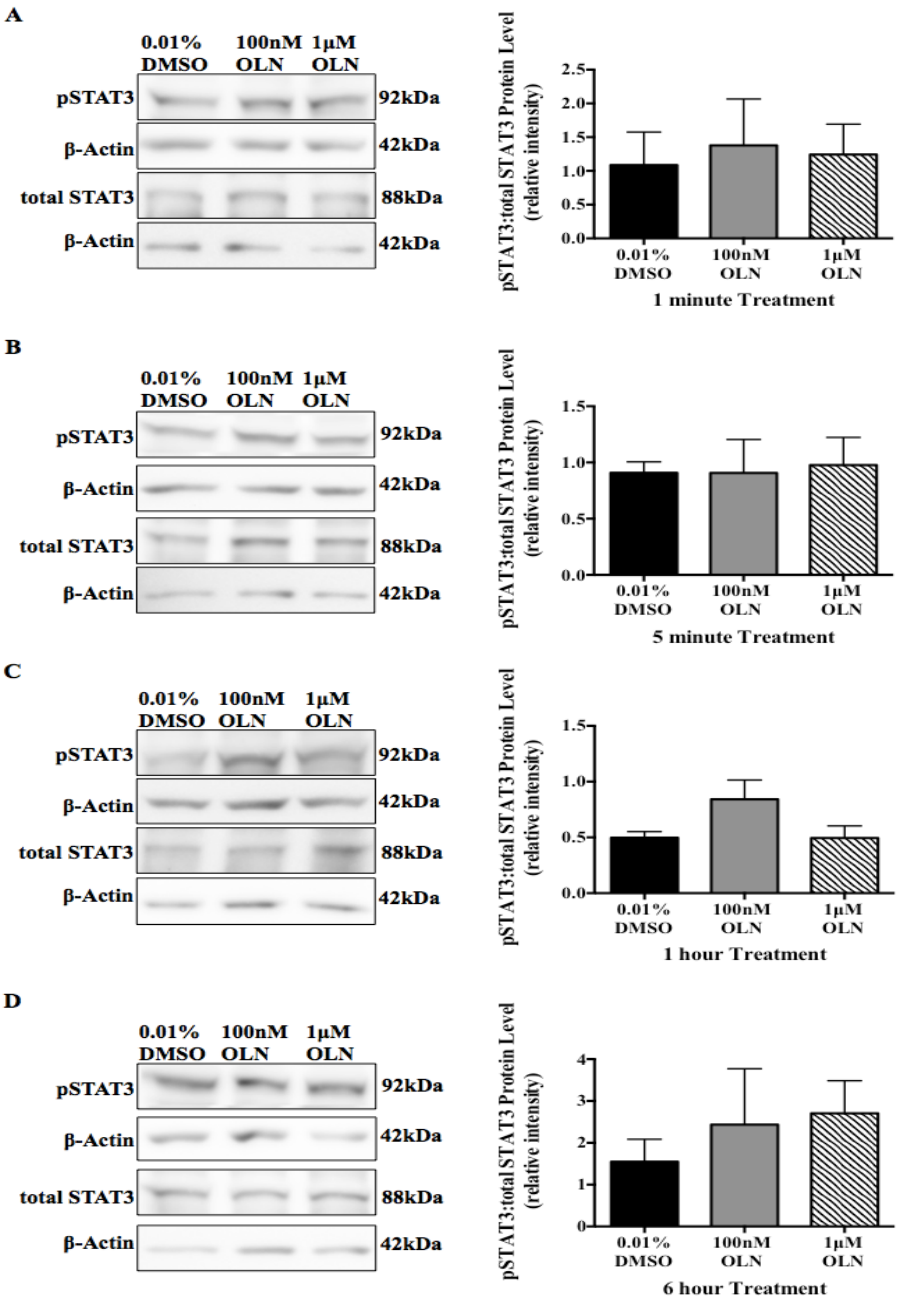

Figure 14. The effect of various OLN exposure times on the phosphorylation of STAT3 in HTR-8/SVneo cells. The pSTAT3:STAT3 protein level in HTR-8/SVneo cells grown in serum-deprived conditions was measured after 1 minute **(A)**, 5 minutes **(B)**, 1 hour **(C)** and 6 hours **(D)** of exposure to DMSO or OLN by Western blot. All protein levels were normalized to β-Actin. The data is presented as a ratio of pSTAT3 to total STAT3 mean intensities of the target bands ± SEM (n=4). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

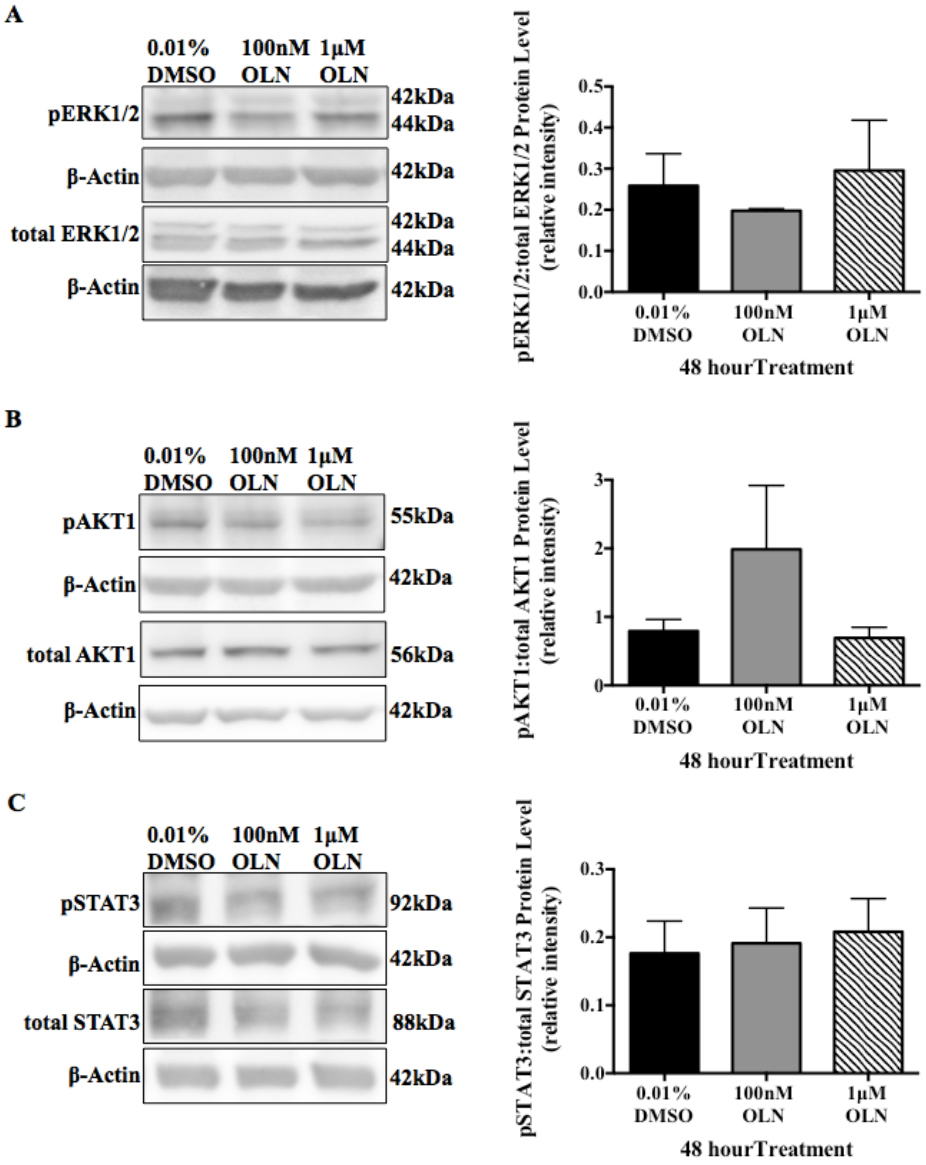

Figure 15. The effect of OLN on signaling pathways in HTR-8/SVneo cells following 48 hours of exposure. The protein levels of pERK1/2:ERK1/2 **(A)** pAKT1:AKT1 **(B)** and pSTAT3:STAT3 **(C)** following 48-hour exposure to DMSO or OLN in serum-deprived conditions was detected by Western blot. All protein levels were normalized to β-Actin. The data is presented as the mean intensities of the phosphorylated to total protein ratio ± SEM (n=4). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

Mitochondrial ETC function, ROS production, and a state of oxidative stress have all been associated with cell invasion and are affected by OLN exposure. Therefore, it is possible that OLN may be impacting HTR-8/SVneo invasion by altering mitochondrial ETC function, or causing a state of oxidative stress. 100nM and 1μM OLN exposure caused a significant reduction in complex I+II and complex I+III ETC activity, but not in complex IV ETC activity (Figure 16) within the mitochondria of exposed HTR-8/SVneo cells. These observed changes in mitochondrial ETC complex activities were not related to a decrease in protein expression (Figure 17). Moreover, although decreased mitochondrial ETC activity is often associated with a decrease in ATP production and increase in oxidative stress, OLN did not affect the ADP/ATP ratio, expression of ATP synthase (Figure 18), amount of ROS, or oxidative damage (i.e., 4-hydroxynonenal (HNE); Figure 19). Additionally, there was no evidence of OLN-induced changes in the gene (Table 4) or protein expression of factors involved in regulating ROS, including the antioxidants: CuZnSOD, MnSOD, CAT, and GPX1 (Figure 20), stress chaperones HSP60 and HSP70 (Figure 21) or fission-fusion markers dynamin-related protein 1 (DRP1), optic atrophy 1 (OPA1) and mitofusion 2 (MFN2) (Figure 22). HIF-1α translocation to the nucleus is also known to be influenced by mitochondrial ETC function and play a signaling role in trophoblast invasion, however following 48 hours of OLN exposure there was no significant change in the ratio of nuclear HIF-1α compared to cytosolic HIF-1α (Figure 23).

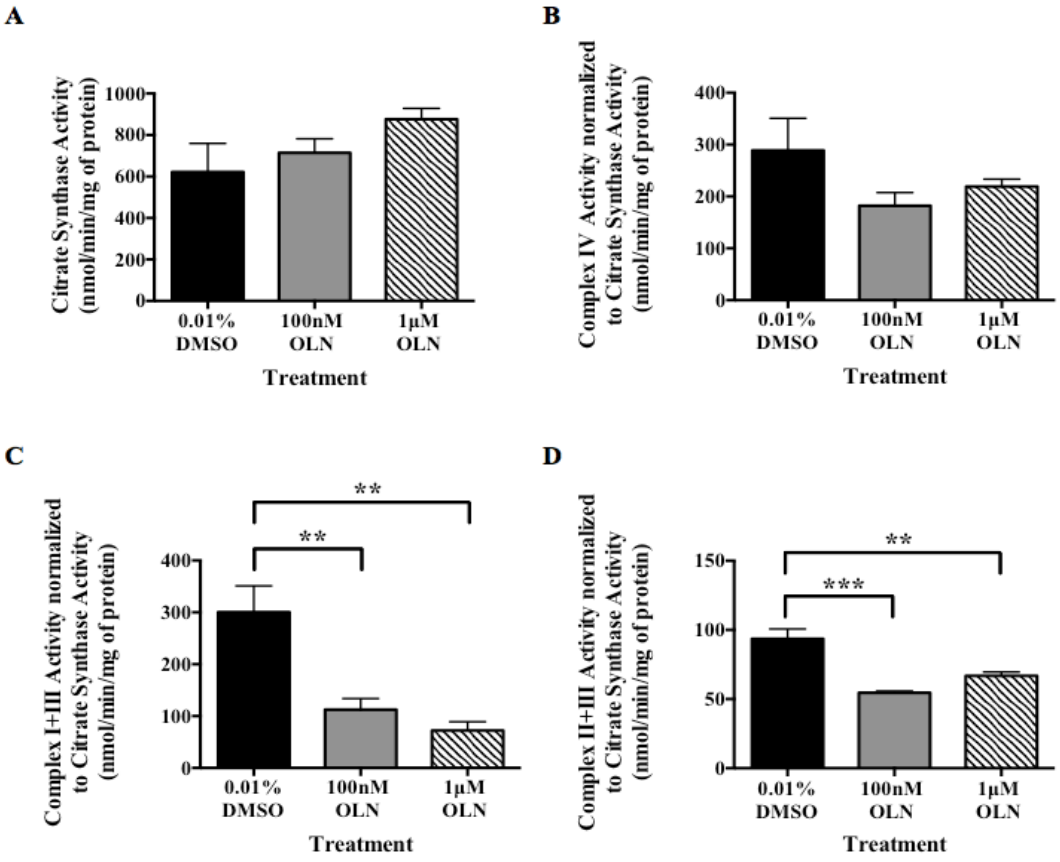

Figure 16. Effect of OLN on HTR-8/SVneo mitochondrial ETC complex function. Isolated mitochondria from HTR-8/SVneo cells treated with 0.01% DMSO, 100nM or 1µM OLN for 48 hours was used to measure mitochondrial ETC complex activity. 100nM OLN and 1µM OLN exposure did not affect the activity of citrate synthase (**A**) or complex IV activity **(B)**. However, both 100nM and 1µM OLN treatments decreased the activity of complex I+III **(C)** and complex II+III **(D)**. All values represent the mean activity normalized to citrate synthase activity ± SEM (n=4 per group). Significance was assessed using a one-way ANOVA and Tukey *post hoc* analysis for multiple comparisons with ** indicating $p<0.01$ and *** indicating $p<0.001$.

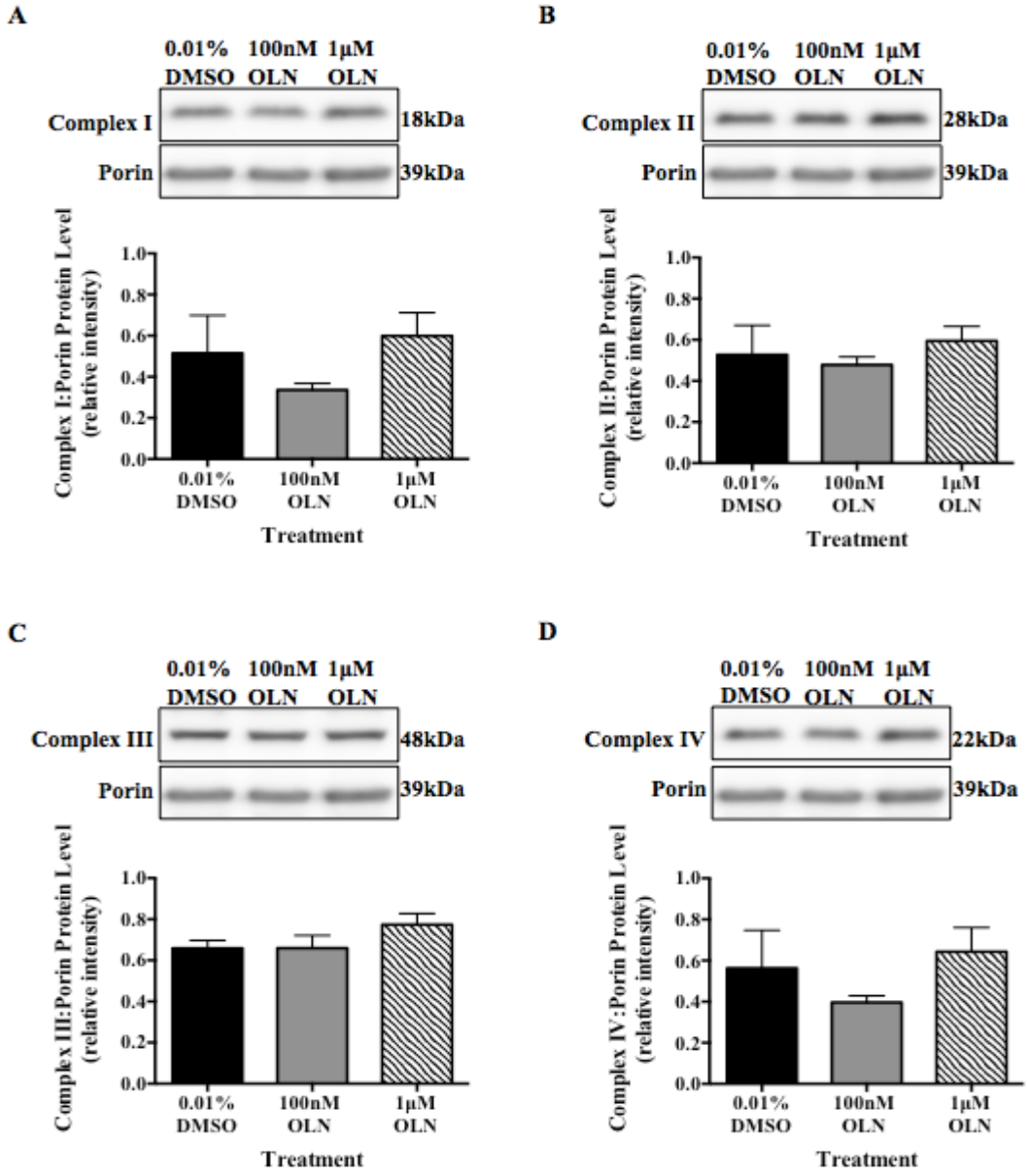

Figure 17. Mitochondrial ETC complex protein expression in mitochondria isolates of HTR-8/SVneo cells treated with OLN for 48 hours. HTR-8/SVneo cells were cultured in the presence of 100nM, 1µM OLN or 0.01% DMSO for 48 hours. Mitochondria was isolated, and proteins were extracted and separated on polyacrylamide gels, transferred to nitrocellulose then incubated with antibodies targeted for the proteins of interest. Neither 100nM nor 1µM OLN significantly altered the protein levels of complex I **(A)**, complex II **(B)**, complex III **(C)**, or complex IV **(D)**. All protein levels were normalized to porin. The data is presented as the mean intensity of the target bands ± SEM (n=4 per group). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

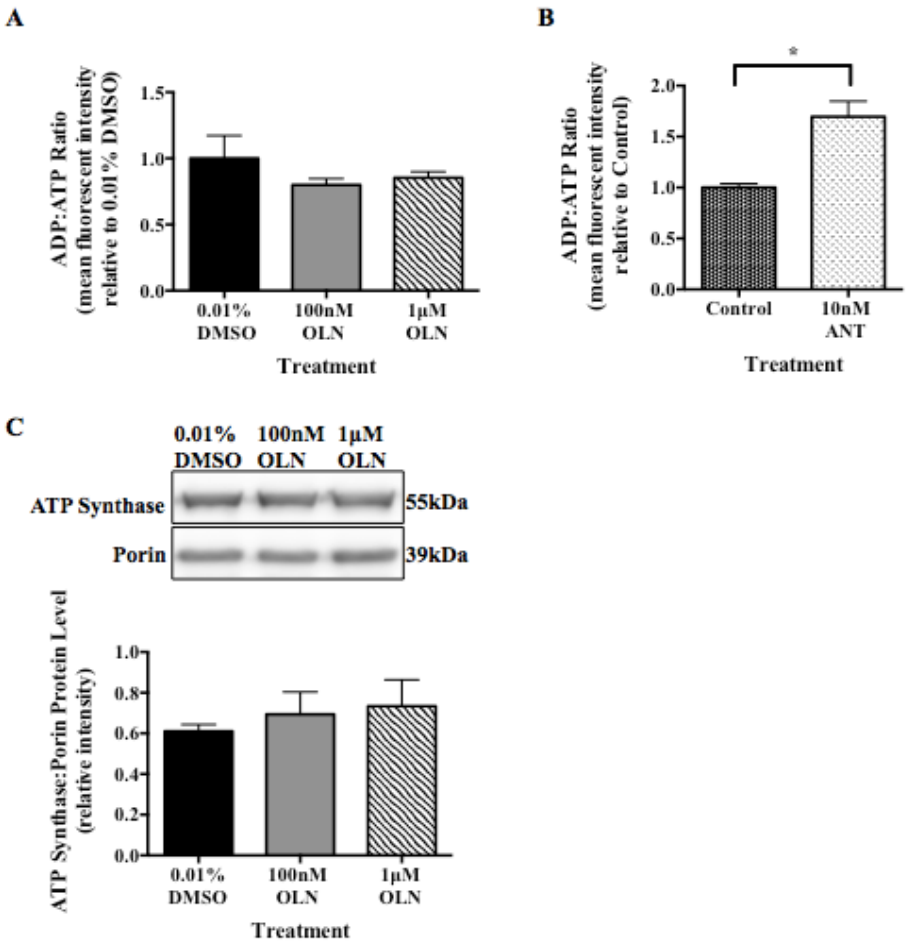

Figure 18. ATP production abilities of HTR-8/SVneo cells treated with OLN. HTR-8/SVneo cells were cultured in the presence of 0.01% DMSO, 100nM OLN, or 1μM OLN for 48 hours. The ADP/ATP ratio in the cells was assessed using a commercially available kit **(A)**, with 10nM of ANT as a positive control **(B)**. The ADP/ATP ratio is presented as the mean fluorescent intensity relative to the 0.01% DMSO treatment ± SEM (n=4 per group). The protein level of ATP synthase was measured via Western blot in isolated mitochondria of HTR-8/SVneo cells exposed to 0.01% DMSO, 100nM OLN, or 1μM OLN for 48 hours **(C)**. Protein levels were normalized to porin and data is presented as the mean intensity of the target bands ± SEM (n=4 per group). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA for the ADP/ATP activity assay and Western blots, while a two-tailed Student's T-test was performed for the ANT positive control comparison (*$p < 0.05$).

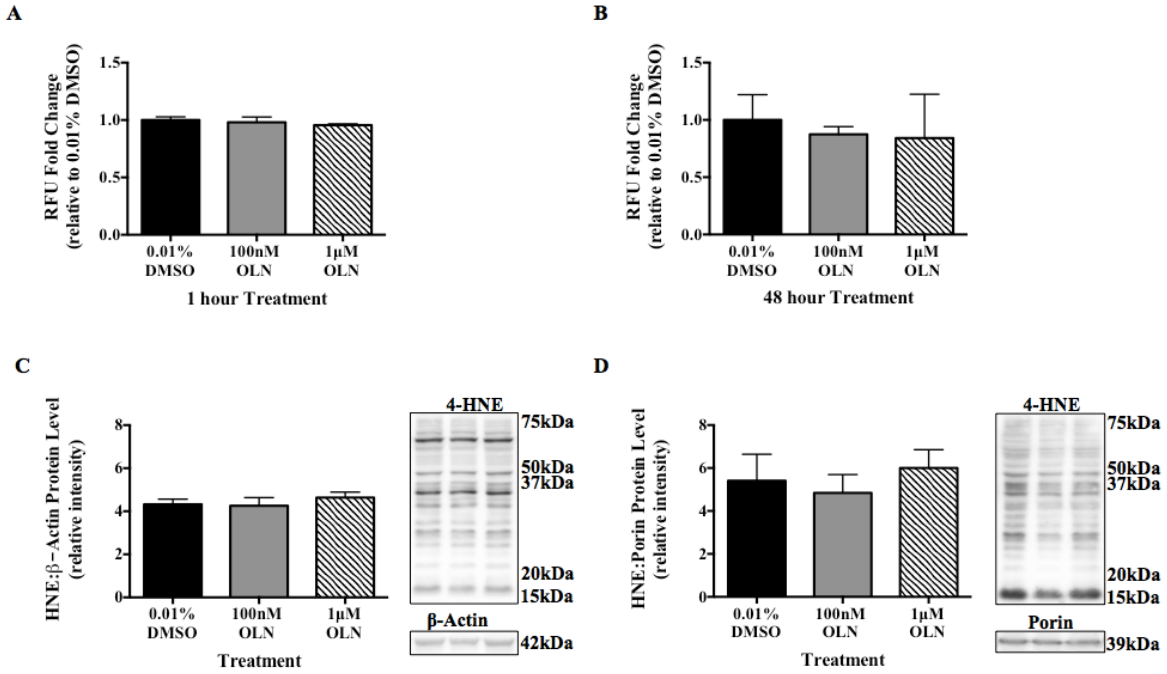

Figure 19. ROS production and oxidative damage in HTR-8/SVneo cells exposed to OLN. HTR-8/SVneo cells were cultured in the presence of 0.01% DMSO, 100nM OLN, or 1μM OLN for 1 hour **(A)** or 48 hours **(B)** and then the amount of intracellular ROS was measured using a commercially available kit. Data is presented as the fluorescent unit fold change relative to the 0.01% DMSO treatment ± SEM (n≥3 per group). HNE levels in cell lysates **(C)** and mitochondria-enriched fractions **(D)** of HTR-8/SVneo cells exposed to 0.01% DMSO, 100nM OLN, or 1μM OLN for 48 hours was assessed via Western blot. Protein levels were normalized to β-Actin for cell lysates and porin for mitochondria isolates. Data is presented as the mean intensity of the target bands ± SEM (n=4 per group). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

Table 4

Relative gene expression of oxidative stress related genes after 48 hour treatments

	Group		
Gene	0.01% DMSO	100nM OLN	1μM OLN
MnSOD	1.07±0.22	1.05±0.16	1.41±0.07
CuZnSOD	1.03±0.14	0.95±0.17	0.83±0.05
GPX	1.01±0.09	0.95±0.10	0.78±0.17
CAT	0.87±0.03	1.08±0.24	0.92±0.04
HSP60	1.11±0.26	0.87±0.08	1.56±0.31
HSP70	1.07±0.22	1.11±0.18	1.45±0.08
NRF2	1.03±0.15	1.02±0.24	0.94±0.04
DRP1	1±0.06	0.9±0.16	1.21±0.11
OPA1	1.01±0.10	0.97±0.13	1.06±0.07
MFN1	1±0.05	0.93±0.08	1.05±0.07
MFN2	1.01±0.07	0.85±0.11	1.19±0.11
FIS1	1.01±0.07	1.03±0.08	1.09±0.09

Data is presented as the mean ± SEM fold change of gene expression relative to HTR-8/SVneo cells treated with 0.01% DMSO. Statistical significance ($p \leq 0.05$) of OLN treatments compared to DMSO was assessed using a one-way ANOVA; n=3 per group.

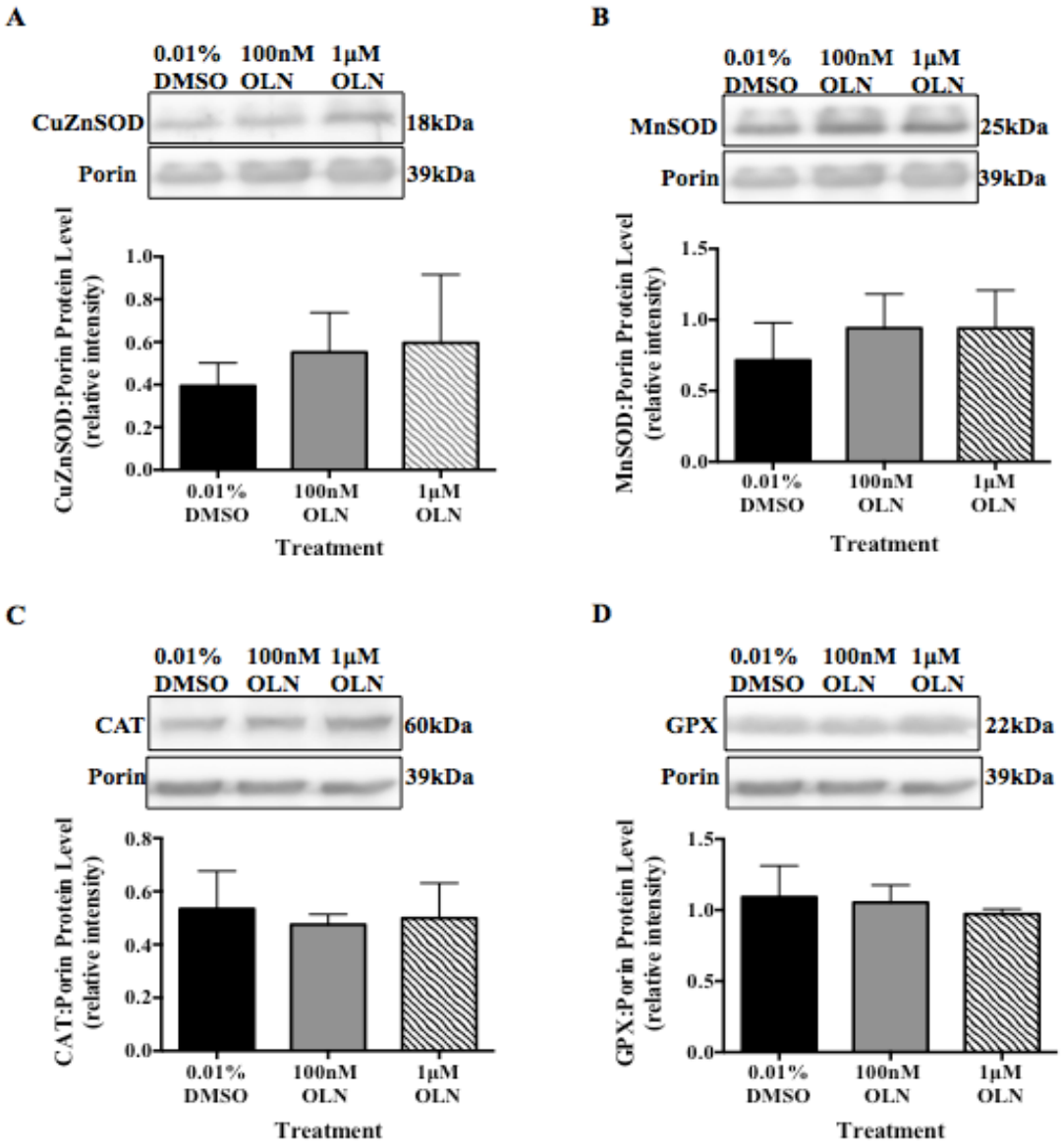

Figure 20. No significant change in protein expression of antioxidants in HTR-8/SVneo cells following 48-hour exposure to OLN. HTR-8/SVneo cells were cultured in the presence of 100nM, 1µM OLN or 0.01% DMSO for 48 hours. Protein was isolated from cells and separated on polyacrylamide gels, transferred to nitrocellulose then incubated with antibodies targeted for proteins of interest. Neither 100nM nor 1µM OLN significantly altered CuZnSOD **(A)**, MnSOD **(B)**, CAT **(C)** or GPX1 **(D)** protein expression (n=4 per group). Protein levels were normalized to porin. The data is presented as the mean intensity of the target bands ± SEM. Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

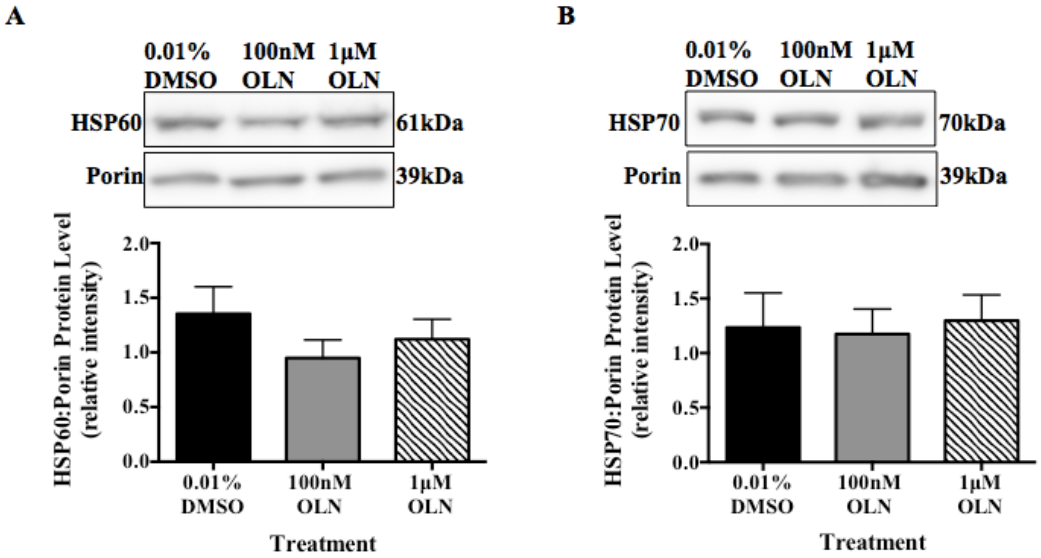

Figure 21. No significant change in protein expression of stress chaperones in HTR-8/SVneo cells following 48-hour exposure to OLN. HTR-8/SVneo cells were cultured in the presence of 100nM, 1µM OLN or 0.01% DMSO for 48 hours. Protein was isolated from cells and separated on polyacrylamide gels, transferred to nitrocellulose then incubated with antibodies targeted for proteins of interest. Neither 100nM nor 1µM OLN significantly altered HSP60 **(A)** or HSP70 **(B)** protein expression (n=4 per group). Protein levels were normalized to porin. The data is presented as the mean intensity of the target bands ± SEM. Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

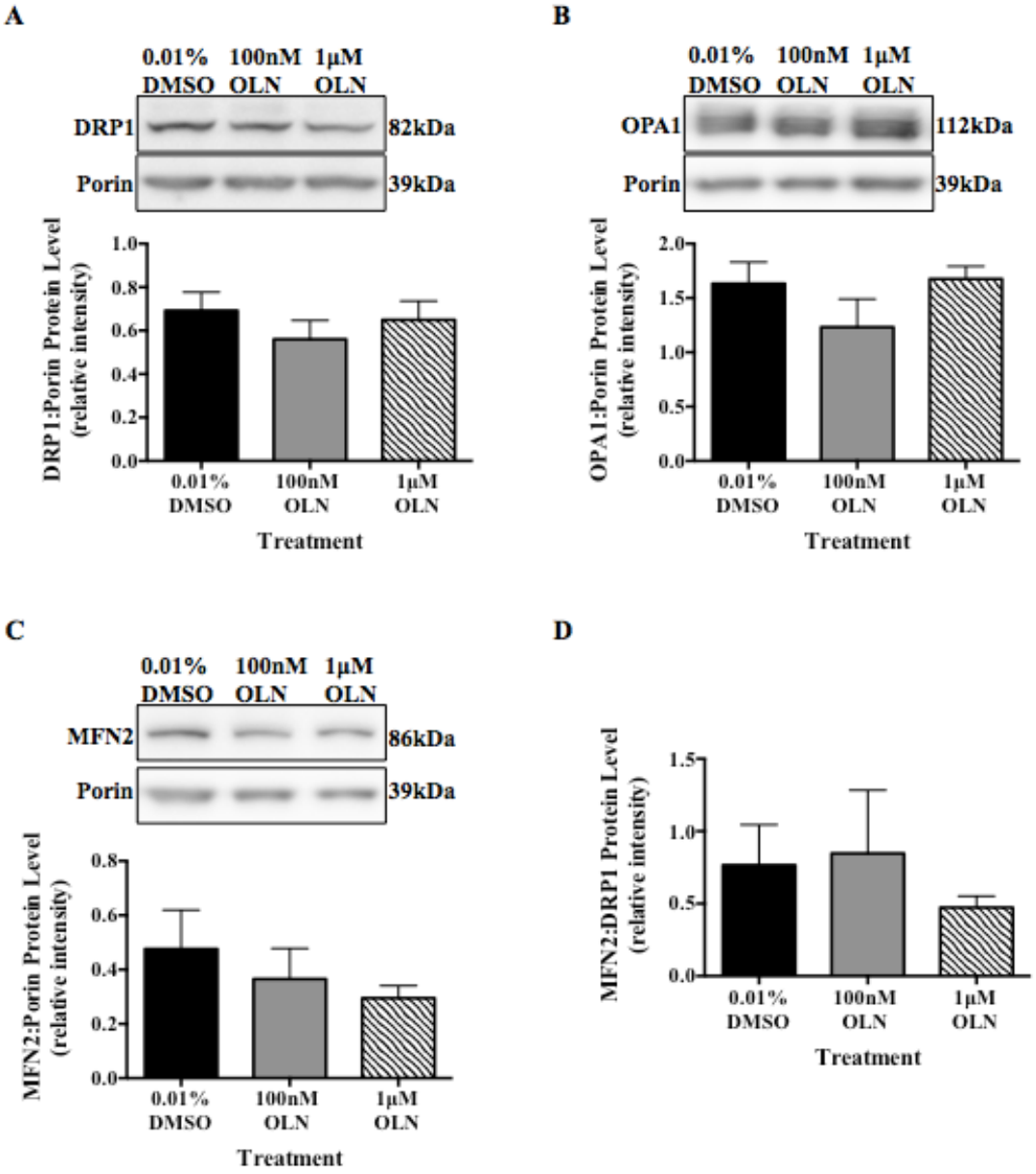

Figure 22. No change in the fission or fusion protein levels between HTR-8/SVneo cells treated with DMSO and OLN for 48 hours. HTR-8/SVneo cells were cultured in the presence of 100nM, 1µM OLN or 0.01% DMSO for 48 hours. Mitochondria was isolated, and proteins were extracted and separated on polyacrylamide gels, transferred to nitrocellulose then incubated with antibodies targeted for proteins of interest. Neither 100nM nor 1µM OLN significantly altered the protein levels of DRP1 **(A)**, OPA1 **(B)**, MFN2 **(C)**, or the ratio between MFN2:DRP1 **(D)**. All protein levels were normalized to porin. Data is presented as the mean intensity of the target bands ± SEM (n=4 per group). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

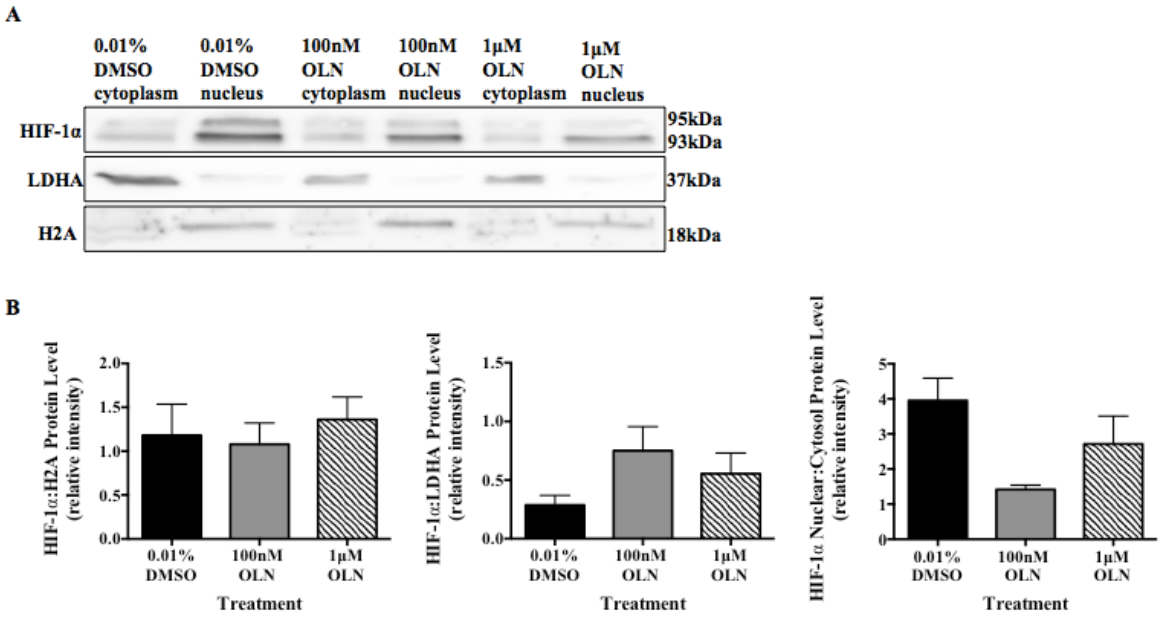

Figure 23. Nuclear translocation of HIF-1α in HTR-8/SVneo cells exposed to OLN for 48 hours. HTR-8/SVneo cells were cultured in the presence of 0.01% DMSO, 100nM OLN, or 1μM OLN for 48 hours. Cytosolic and nuclear proteins were extracted using a commercially available kit and specific targeted protein bands were detected via Western blot **(A)**. Neither 100nM nor 1μM OLN significantly altered the protein level of HIF-1α in the nucleus, cytosol, or the ratio between nuclear:cytosolic HIF-1α **(B)**. Cytosolic protein levels were normalized to LDHA while nuclear protein levels were normalized to histone H2A. Data is presented as the mean intensity of the target bands ± SEM (n=3 per group). Significance ($p \leq 0.05$) was assessed using a one-way ANOVA.

4.5 OLN Affects Gene Expression of Invasive Trophoblast Markers and Antioxidants During HTR-8/SVneo Invasion

To evaluate whether OLN mediates significant changes within HTR-8/SVneo cells during the invasion process, a preliminary experiment assessing gene expression was performed. Following 24 hours of HTR-8/SVneo cell invasion through Matrigel, the gene expression of MMP9 (Table 5) was significantly increased in cells treated with 1μM OLN and both 100nM and 1μM OLN treatments increased in the expression of PAI-1 (Table 5). Moreover, there was a dose-dependent increase in the expression of integrin ITGAV, and

an increase in both ITGA1, and ITGA5 when cells were exposed to 1μM OLN (Table 6). Additionally, cells exposed to 1μM OLN expressed significantly more ITGA5 than when exposed to 100nM OLN (Table 6). Interestingly, there was also a significant decrease of CuZnSOD and a significant increase in GPX1 expression with OLN treatment during cell invasion (Table 7).

Table 5

Relative gene expression of invasion markers following 24 hours of invasion

	Group		
Gene	0.01% DMSO	100nM OLN	1μM OLN
MMP1	1.13±0.34	1.47±0.20	1.79±0.52
MMP9	1.01±0.09	1.55±0.19	**1.85±0.25***
MMP14	1.02±0.15	1.21±0.14	1.02±0.42
TIMP1	1.00±0.04	1.25±0.19	1.48±0.02
TIMP2	1.03±0.18	1.18±0.07	1.08±0.17
uPA	1.00±0.70	1.33±0.07	1.30±0.12
PAI-1	1.02±0.12	**1.78±0.07****	**2.19±0.11*****

Data is presented as the mean fold change ± SEM relative to HTR-8/SVneo cells treated with 0.01% DMSO. Statistical significance ($p \leq 0.05$) between OLN and DMSO was assessed by a one-way ANOVA and Tukey *post hoc* as indicated by *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq .001$; n=3 per group.

Table 6

Relative gene expression of adhesion molecules following 24 hours of invasion			
	Group		
Gene	0.01% DMSO	100nM OLN	1μM OLN
ITGAV	1.00±0.03	**1.22±0.01**[a]	**1.43±0.06**[de]
ITGA1	1.00±0.05	1.17±0.08	**1.36±0.10**[b]
ITGA5	1.00±0	1.57±0.13	**2.15±0.13**[ce]
ITGA6	1.01±0.08	1.09±0.12	1.26±0.08
ITGB1	1.01±0.07	1.06±0.06	1.13±0.05
ITGB4	1.06±0.26	1.34±0.32	1.53±0.03

Data is presented as the mean fold change ± SEM relative to HTR-8/SVneo cells treated with 0.01% DMSO. Statistical significance ($p \leq 0.05$) was determined with a one-way ANOVA and Tukey *post hoc* test. Significant differences between 0.01% DMSO and 100nM OLN is indicated by [a]$p \leq 0.05$. [b]$p \leq 0.05$ denotes a significant difference between 0.01% DMSO and 1μM OLN, while [c]($p \leq 0.01$) and [d]($p \leq 0.001$) indicates a significant difference between 0.01% DMSO and 1μM OLN and [e] represents statistical significance ($p \leq 0.05$) between 100nM and 1μM OLN treatments; n=3 per group.

Table 7

Relative gene expression of antioxidant genes after 24 hours of invasion			
	Group		
Gene	0.01% DMSO	100nM OLN	1μM OLN
MnSOD	1±0.06	1.1±0.08	1.03±0.03
CuZnSOD	1±0.02	1.02±0.02	**0.92±0.01**[bc]
GPX1	1±0.06	**1.52±0.08**[a]	**1.53±0.16**[b]
CAT	1±0.05	1.15±0.05	1.13±0.01

Data is presented as the mean fold change ± SEM relative to HTR-8/SVneo cells treated with 0.01% DMSO. Statistical significance ($p \leq 0.05$) was determined using a one-way ANOVA and Tukey *post hoc* test with [a] and [b] indicating statistical significance between 0.01% DMSO and 100nM or 1μM OLN, respectively and [c] representing a significant difference between 100nM and 1μM OLN; $n \geq 4$ per group.

5. DISCUSSION

5.1 Implications of OLN Increasing HTR-8/SVneo Cell Invasion

In the current study, we demonstrated that exposure to OLN can increase the number of HTR-8/SVneo cells that invade Matrigel. Increased trophoblast invasion can cause aberrant spiral artery remodeling within the placenta and lead to reduced fetal growth in cases of maternal obesity (*223, 224*), fetal growth restriction (*134, 225*) and preeclampsia (*226*). OLN use can also lead to altered fetal growth (*44, 45, 47, 59*). Thus, the increase in trophoblast invasion observed with OLN treatment may result in altered spiral artery remodeling within the placenta, and may be a mechanism by which *in utero* OLN exposure leads to altered fetal growth.

It is understood that preeclampsia, a disease where altered birth weight in infants is common, is caused by reduced spiral artery remodeling due to altered trophoblast invasion. Although the precise pathogenesis of preeclampsia remains elusive (*128, 227, 228*), a study using a rat model of preeclampsia found that trophoblasts invaded deeper in the maternal decidua, and the placenta had less maternal blood vessels that had been remodeled compared to controls (*226*). Moreover, there were higher resorption rates, lower litter sizes and lower fetal weights relative to controls (*226*). Therefore, the authors suggest there is an early increase in trophoblast invasion in pregnancies complicated by preeclampsia, but this is rarely seen in humans because placentas are typically sampled during late second and early third trimesters (*226*). By this time, trophoblasts have since been eliminated (*226*) potentially through apoptosis (*225, 229*) due to a lack of decidual

cell support. Moreover, a study comparing the placenta from healthy women and women with pregnancies complicated by severe fetal growth restriction found incomplete spiral artery remodeling despite an increase in extravillous trophoblast densities by maternal blood vessels (*134*). This suggests that failed artery remodeling and reduced fetal growth is due to altered trophoblast remodeling functions, perhaps secondary to local decidual factors being ineffective at initiating the remodeling of maternal vessels, inadequate at modulating invasion, or be a consequence of reduced support of these invasive trophoblasts by other cells (*134, 225*). Therefore, an increase in trophoblast invasion (such as that observed with OLN in this thesis) may result in inadequate support by, or coordination with, other cells of the decidua required for proper spiral artery remodeling and fetal growth.

Moreover, a rat model of maternal obesity (*223*) found more invasive trophoblasts in the placenta of high-fat fed dams at mid-gestation compared to control, but a similar number of invasive trophoblasts later in gestation when reduced spiral artery remodeling was evident (*224*). This was associated with a poor placental vasculature network, reduced litter sizes and lower fetal weights compared to controls, which suggests that impacting the progression of trophoblast invasion results in inadequate vascular remodeling and poor fetal outcomes (*224*). This may occur because altered timing of trophoblast invasion may alter the normal coordination and timing of interactions between trophoblasts and other cell types in the decidua that are required for proper placental vascular remodeling. For instance, uNK cells play a central role in the initiation of maternal vasculature

remodeling by disrupting maternal spiral arteries before trophoblasts reach these arteries (*104, 230*). The premature presence of trophoblasts in the maternal decidua may result in inadequate vessel disruptions by uNK cells, and in turn, incomplete vessel remodeling by trophoblasts and insufficient blood vessel maturity (*231, 232*).

Therefore, the increase in HTR-8/SVneo invasion caused by OLN may alter the typical coordination of cells or cause the cells to be inadequately supported during *in vivo* placental development. Thus, OLN's direct impact on HTR-8/SVneo cell invasion could indeed affect placental development and function, contributing to altered fetal growth.

5.2 OLN Does Not Alter HTR-8/SVneo State of Differentiation

OLN could be directly influencing HTR-8/SVneo cell invasion through a number of pathways, such as affecting the differentiation status of the cells, their subsequent migration through the ECM and/or their ability to degrade the ECM (*94, 100*). OLN has been shown to increase the differentiation of precursor cells to oligodendrocytes (*187, 233, 234*), and neurons (*235*) and has also been proposed to enhance adipocyte differentiation (*236, 237*). However, there was no effect of OLN in altering the gene expression of transcription factors known to have distinct profiles in the different trophoblast lineages. This indicates that the increase in HTR-8/SVneo cell invasion with OLN exposure was independent of OLN influencing trophoblast differentiation. This discrepancy might be related to the concentrations of OLN used in our study. Many of the studies that have assessed OLN's affects on differentiation have used concentrations

equal to or greater than 10μM (*233-237*). Other studies have shown there is no change in the differentiation of precursor cells *in vitro* to mature adipocytes (*238*), oligodendrocytes, astrocytes or neurons with OLN over a range of 10nM to 1μM (*193*), which is consistent with the results of this thesis.

5.3 OLN Does Not Alter HTR-8/SVneo Migration

OLN may be facilitating the increase in HTR-8/SVneo invasion by mediating the expression of integrins that are important for proper trophoblast mobility through the decidua (*106, 110, 111*). However, an increase in integrins and other adhesion molecules typically up-regulated in invasive trophoblasts was not observed when HTR-8/SVneo cells were treated with OLN, in the absence of ECM or invasion assay. Moreover, although there was an increase in invasion with OLN exposure, there was no change in HTR-8/SVneo migration. Therefore, OLN may increase invasion without affecting the ability of the cells to migrate. This has previously been observed in a human breast cancer cell line, where exposure to TNF-α induced invasion, but not migration (*239*). However, the interaction of trophoblasts with ECM has been considered an important factor in numerous decisions directing cell fate and behaviour (*199*) as it provides cells with a substrate for attachment, and migration (*240*). Additionally, *in vivo* trophoblast invasion and migration is mediated by an oxygen gradient and signaling factors secreted by decidual cells (i.e., chemoattractants) (*102-105, 164*). Therefore, I conclude that cell migration and expression of integrins in the presence of matrix proteins (as found in Matrigel), and chemoattractants may result in a more biologically relevant assessment of

migration (*241*). Thus, significant changes in integrin expression and migration with OLN exposure may have been missed in this experiment due to the lack of ECM or chemoattractant.

5.4 OLN Facilitates the Increase in HTR-8/SVneo Invasion by Increasing Total Extracellular MMP Activity

OLN's increase in trophoblast invasion occurs in concert with an increase in extracellular MMP activity, which is independent of significant changes in intracellular protease or protease inhibitor expression.

Proteases, such as uPA and MMPs, are particularly important in facilitating trophoblast invasion, as they degrade ECM proteins and allow space for trophoblast cells to move through the decidua. Dysregulation of MMPs, uPA, and their inhibitors (TIMPs and PAI-1, respectively) have been linked to altered trophoblast invasion (*83, 242*) and deficiencies in placentation (*120, 125, 126*). In this study, we found that 1μM OLN increased trophoblast invasion as well as increased extracellular MMP activity. This increase in MMP activity cannot be attributed to a specific increase in MMP2 or MMP9 (MMPs extensively studied and implicated in trophoblast invasion (*120, 122-124*)) since there was no significant difference between DMSO and 1μM OLN treatments in the gelatin zymogram experiment performed. Therefore, the total MMP activity increase with 1μM OLN may not be due to an increase in MMP2 or MMP9 activity, but instead, an increase in the activity of other MMPs. However, an ELISA would need to be performed

to further confirm if there were changes in specific MMP activities (*212, 243*) with OLN treatment.

OLN's increase in extracellular MMP activity is independent of significant changes in the protein expression of intracellular proteases (MMP1, MMP2, MMP9 or MMP14) or protease inhibitors (TIMP1 and TIMP2) important in regulating trophoblast invasion (*120, 122, 244*). However, the gene expression of MMP2 was significantly up-regulated. This may be explained by the ability of MMPs to be post-transcriptionally regulated by growth factors, hormones as well as pharmacological reagents (*217*). Therefore, it is possible that OLN may regulate MMP expression by affecting mRNA stability, increasing the cellular reserve of MMP mRNA. This type of protein regulation has been observed in neurons, especially those that mediate learning and memory, where mRNA is transported to specific cellular regions and often remain translationally dormant until certain signaling pathways are activated (*245, 246*). Therefore, OLN may be up-regulating the gene expression of MMP2, however only under conditions that signal the translational activation of the mRNA (perhaps, invasion through Matrigel or serum-deprived conditions) would we observe a corresponding change in protein expression. Alternatively, it is possible that OLN may be increasing the transcription of MMPs, and an increase in the intracellular protein level of MMPs is not observed because there are more MMPs secreted by the cells to facilitate invasion. However, there was no significant difference in gene expression of secretion chaperones (VAMP3 and SNAP23) that associate with cellular vesicles containing MMPs (*216*). However, the mechanism of

MMP secretion remains elusive, so other implicated MMP vesicle trafficking proteins, such as myosin and kinesin isoforms, need to be evaluated (*216, 247, 248*). Therefore, from the experiments completed in this study, I conclude that the increase in MMP extracellular activity with 1μM OLN exposure in the absence of intracellular protein expression changes is because OLN may increase the mRNA reserve for proteins important for invasion, and only under conditions where cells are invading or in conditions that may more closely simulate *in vivo* conditions during invasion would we observe changes in protein expression.

5.5 OLN Affects HTR-8/SVneo Invasion by Modulating PAI-1 Expression

PAI-1 gene expression was significantly elevated with OLN treatment when HTR-8/SVneo cells were not invading Matrigel. PAI-1 plays an important role in trophoblast invasion through uPA regulation (*249-252*); it is produced and secreted by trophoblasts and inhibits uPA, an important modulator of cell invasion by facilitating MMP activation and ECM degradation (*120, 252*). The increase in PAI-1 may reflect an attempt of the HTR-8/SVneo cells to regulate the increased invasion as a result of OLN treatment. A significant increase in PAI-1 expression may have been observed in the absence of HTR-8/SVneo cells invading Matrigel because it is among the most highly induced transcriptional outputs that characterize a migratory or invasive phenotype in cancer (*253*).

Taken together, I conclude that OLN can increase HTR-8/SVneo cell invasion through Matrigel, by increasing the cellular reserve of MMP2, the amount of active extracellular MMPs, and the PAI-1 expression, which may be a downstream compensatory effect of the HTR-8/SVneo cells regulating the OLN-mediated increase in invasion. Moreover, I believe that the absence of significance in OLN-induced migration, adhesion proteins and other markers of the invasive trophoblast phenotype may be due to the fact that these targets were measured in cells that were not actively invading through ECM.

5.6 OLN Does Not Increase HTR-8/SVneo Invasion Solely Through Its Action at the α_{1A} Adrenergic Receptor

HTR-8/SVneo cells express the majority of receptors OLN is known to bind, with the exception of dopamine D_3. Using a rat model to characterize the temporal changes in placental receptors that could be targeted by OLN, we found that the gene expression of the following receptors increased significantly in GD20 placenta relative to GD15: histamine H_1, muscarinic M_1 and M_4, serotonin 5-HT_{1D}, 5-HT_{2B}, and the α_{2C} adrenergic receptors. It is not surprising to find a general increase in the expression of these receptors between GD15 and GD20 since many of OLN's putative receptor targets are associated with placental functions that are required to support successful fetal growth (such as the production and secretion of growth factors and hormones (*141, 254-259*)), and there is substantial increase in placental weight and fetal growth between GD15 and GD20 in rat pregnancy to support the rapid rate of fetal growth during this period (*260*).

The α_{1A} adrenergic receptor was the only receptor more highly expressed in the placenta at GD15 compared to GD20, suggesting that it may be important to processes early in pregnancy, such as trophoblast invasion. Consistent with this hypothesis is that there is a shift from α adrenergic receptor subtypes to β adrenergic receptor subtypes in trophoblasts as pregnancy progresses (*261*). The α adrenergic receptor has also been linked to trophoblast invasion. Norepinephrine, a catecholamine agonist of adrenergic receptors, is a negative regulator of trophoblast invasion by modulating progesterone production via the α_{1A} adrenergic receptor (*262*). Moreover, blocking norepinephrine's actions has resulted in increased MMP activity (*263*). Thus, there was rationale to support that OLN's antagonistic actions at the α_{1A} adrenergic receptor may affect HTR-8/SVneo invasion. However, the 5-MU antagonist of the α_{1A} adrenergic receptor did not significantly impact HTR-8/SVneo cell invasion through Matrigel. Therefore, OLN antagonism exclusively at this receptor may not be responsible for the increase in invasion observed.

Despite the lack of data regarding most of these receptors and their effects on trophoblast function, some of these OLN-targeted receptors have been shown to affect cancer cell invasion. For instance, dopamine can decrease ovarian cancer cell invasion (*264*), and dopamine receptor antagonism has been linked to breast cancer risk (*265*). Therefore, OLN's antagonistic actions at dopamine receptors may be responsible for the increase in HTR-8/SVneo invasion observed. Moreover, OLN can act as an inverse agonist at some receptors (*68-71*). Therefore, OLN's actions as an inverse agonist at receptors that reduce

trophoblast invasion may be responsible for the increase in HTR-8/SVneo cell invasion observed with 1μM OLN. Discovering which receptors OLN may be eliciting its affects on HTR-8/SVneo cells will provide mechanistic insight into OLN's actions on the placenta during pregnancy and also may aid clinicians in determining what other drugs concomitantly prescribed with OLN should be closely monitored.

In conclusion, HTR-8/SVneo cells express many of OLN's receptor targets and to date, the receptor(s) responsible for the OLN-mediated increase in HTR-8/SVneo cell invasion remains elusive.

5.7 OLN Does Not Activate ERK1/2, AKT or STAT3 Signaling Pathways

OLN did not appear to affect PI3K/AKT, MAPK, and JAK/STAT3 signal transduction pathways, which are heavily implicated in regulating trophoblast invasion (*99, 102, 136*). Previous studies that have found OLN alters the phosphorylation of AKT and ERK1/2 in PC12 cells (differentiated into neurons) used high concentrations (40μM and 120μM) of OLN (*145, 146*). Additionally, other groups have found that OLN is capable of activating ERK1/2 (*237*) and STAT3 (*147*) in the frontal cortex only after long-term treatment *in vivo*. Therefore in this study, OLN is likely increasing HTR-8/SVneo invasion through pathways that have yet to be investigated, such as the FAK and Rho-GTPase signaling pathways, which can be activated via GPCRs and affect trophoblast invasion (*102, 136*). Since the receptors that OLN is known to bind to are mainly GPCRs, OLN may be mediating its effects on invasion through these other pathways. For instance, signaling

through GPCRs in focal adhesions (contact sites between invasive cells and surrounding ECM) can influence FAK activation through phosphorylation and inducing FAK phosphorylation in trophoblasts up-regulates MMP2 and MMP9 activity in primary trophoblast cells and JEG-3's (*266*). Moreover, down-regulating FAK phosphorylation results in decreased cell migration and invasion as well as reduced MMP2 activity in placenta explants (*267*) and isolated CTs (*268*). Since we found that 1μM OLN increased the activity of total extracellular MMPs and HTR-8/SVneo invasion, and similar results are found with increases in FAK activation, OLN-mediated effects observed may be due to activation of the FAK pathway. Moreover, OLN may also be mediating its effects on HTR-8/SVneo invasion by affecting the activation of the Rho-GTPase signaling pathway. OLN can induce changes in the expression of genes involved in the Rho-GTPase (*269, 270*) and Wnt (*233, 270, 271*) signaling pathways; and the inhibition of a major effector in the Wnt pathway reduces the ability of trophoblasts to migrate and invade (*272*). Therefore, OLN may be inducing HTR-8/SVneo invasion by affecting signaling pathways that have not yet been assessed and require further analysis.

5.8 OLN Decreases the Activity of Mitochondrial ETC Complex(s) Without Inducing Oxidative Stress

HTR-8/SVneo exposure to OLN did not alter the activity of complex IV but did decrease the activity of complex I, II, and/or III of the mitochondrial ETC, without altering protein expression. Discrepancies between mitochondrial ETC function and protein expression is not without precedent in the literature (*210, 273, 274*), and may be attributed to post-

translational modifications, such as acetylation and phosphorylation (*275-278*). It is possible that only complex III is affected with OLN treatment since the activity of complex I and complex II were assessed alongside complex III activity. However, no previous study has reported a change in complex III activity with OLN exposure. Therefore, it is more likely that OLN is affecting both complex I and complex II activity, which is consistent with previous literature where OLN treatment resulted in a decrease in complex I (*176, 177*), and complex II (*178, 179*) in rat brain, or when isolated neuronal mitochondria were treated directly with high concentrations (>100μM) of OLN. Therefore, this work demonstrates for the first time that OLN can impair mitochondrial ETC function following treatment with physiologically relevant concentrations of OLN in intact trophoblast cells.

Mitochondrial ETC function is associated with ROS production (*279, 280*), which is a modulator of trophoblast differentiation and motility (*165, 166*), as well as placenta development (*1, 149, 281*). Previous work in our lab has also demonstrated that a reduction in mitochondrial ETC activity and increased mitochondrial ROS production can increase HTR-8/SVneo cell invasion and activity of proteases important in trophoblast invasion (unpublished data). Additionally, there is evidence that mitochondrial ETC activity is related to cancer cell invasion and metastasis through the modulation of HIF-1α (*170, 171, 173, 174*). Moreover, alterations in mitochondrial ETC functioning and a state of oxidative stress has been observed in pregnancy complications that result in fetal growth changes (*148*), an observed effect of atypical antipsychotic and OLN exposure

during pregnancy (*40, 44, 45, 59*). Therefore, the OLN-mediated reduction in mitochondrial ETC function in HTR-8/SVneo cells may be a mechanism by which OLN mediates an increase in trophoblast invasion and leads to adverse pregnancy outcomes.

However, despite causing a significant reduction in the activity of mitochondrial ETC complexes, OLN did not alter the amount of ADP/ATP, the expression of ATP synthase, HIF-1α, antioxidants, mitochondria fission or fusion markers, stress chaperones, ROS production, or ROS-mediated damage. The lack of evidence linking HTR-8/SVneo mitochondrial ETC dysfunction with ATP production or induction oxidative stress following OLN treatment may indicate that the effect of OLN on mitochondrial ETC activity is independent of its effect on invasion. However, I believe that the lack of significance in ATP production or induction of oxidative stress may be attributsble to the different conditions of the mitochondrial ETC function assay compared to the conditions of experiments assessing the ADP/ATP, mitochondrial protein expression and state of oxidative stress. The mitochondrial ETC complex activity assays performed in this study represent how these complexes are functioning when provided with saturating amounts of substrates, and thus, working at their maximal capacity (*213*). Perhaps under conditions of high energy demands, such as trophoblast invasion, the mitochondrial ETC complexes I, II and/or III would be forced to work at or near their maximal capacity, and during this time, we would have observed changes in the ADP/ATP ratio, ROS production or indicators of oxidative stress following OLN treatment. This is supported by preliminary

results (outlined below in section 5.9) on the gene expression of antioxidants using a revised model to address the response of HTR-8/SVneo cells to OLN during invasion.

5.9 OLN Induces Changes in Antioxidants and Invasive Markers in HTR-8/SVneo Cells During Invasion

OLN evoked significant HTR-8/SVneo responses only in certain experimental conditions; an important differential response to OLN that was uncovered due to standard assay procedures differing from standard cell culture conditions. There were no (or only minimal changes) with OLN treatment in HTR-8/SVneo cell migration, and the expression of genes and proteins when evaluated in cells that were cultured on uncoated polystyrene tissue culture surfaces, in 5% serum. However, for the invasion assay, HTR-8/SVneo cells were serum-deprived O/N (to allow for serum to be used as a chemoattractant), plated on Matrigel in serum-free media and incubated to allow for invasion through Matrigel. OLN also significantly increased the extracellular MMP activity in HTR-8/SVneo cells in serum-deprived conditions (done to avoid interference from endogenous MMPs in serum). Additionally, OLN induced significant impairment in mitochondrial ETC function when forced to work at maximal capacity, which may represent a more similar condition to when HTR-8/SVneo cells are invading as opposed to basal activity under standard cell culture conditions. Therefore, I have performed preliminary experiments where gene expression of a select few invasion and oxidative stress targets were assessed when cells were exposed to OLN in serum-deprived media

and invading through Matrigel to justify the importance of these culture conditions in elucidating the impact of OLN on HTR-8/SVneo cells.

OLN was found to significantly impact the expression of antioxidants (CuZnSOD and GPX1) when cells were invading Matrigel in the invasion assay. The increase in GPX1 expression may indicate that HTR-8/SVneo cells exposed to OLN are attempting to manage an increase in hydrogen peroxide. The decrease in CuZnSOD may indicate that OLN can impact this gene and in turn, the ability of cells to manage $O_2^{\cdot-}$. An increase in $O_2^{\cdot-}$ production could be due to OLN's ability to reduce the mitochondrial ETC function (observed in isolated mitochondria from HTR-8/SVneo cells) during invasion, as mitochondrial ETC dysfunction has been associated with ROS production (*279, 280*). Moreover, $O_2^{\cdot-}$ is known to activate factors such as HIF-1α that can affect invasion (*103, 167, 169-174*). Therefore, when HTR-8/SVneo cells are exposed to OLN during invasion, it is possible that more $O_2^{\cdot-}$ is present compared to vehicle control, which may be a mechanism by which OLN increases invasion. Changes in $O_2^{\cdot-}$ (and potentially HIF-1α) may not have been observed in previous experiments because ROS and HIF-1α expression and translocation was measured when cells were grown on polystyrene without ECM or in the presence of standard serum conditions. Therefore, further experiments are required to elucidate whether OLN may be increasing HTR-8/SVneo cell invasion by impacting mitochondrial ETC function, ROS production, oxidative stress induction, and HIF-1α.

Additionally, integrin subunits that are up-regulated when CTs have differentiated and are established as invasive EVTs, such as αV, α1, and α5 (*106, 109*), as well as MMP9 (necessary for trophoblast invasion) (*120, 122-124*) were significantly increased in invading HTR-8/SVneo cells exposed to 1μM OLN. PAI-1 expression also increased when HTR-8/SVneo cells were treated with OLN during invasion. PAI-1 can inhibit uPA activity, which is important in activating MMPs and degrading ECM components (*120, 252*). Moreover, PAI-1 can also influence uPA in Matrigel by binding ECM components similar to those which the uPA activating receptor typically binds, allowing there to be more unbound uPA activating receptor available to cleave and activate uPA (*253*). Therefore, an increase in PAI-1 may suggest an increase in uPA activity. Although there was no significant change in uPA gene expression, uPA can be post-transcriptionally modified and has a long mRNA stability (*282, 283*). Thus, the increase in PAI-1 may reflect an attempt to reduce the OLN-mediated increase in extracellular MMP activity or ECM degradation via uPA inhibition or, alternatively, contribute to the increase in invasion observed with OLN by increasing the amount of available uPA activating receptors. Moreover, PAI-1 is also involved in the regulation of cell surface integrin localization, particularly integrin αv, which is important for appropriate traction to and through ECM allowing for efficient cell migrate and invade (*110, 219*). PAI-1 can interact with cell receptors to trigger the detachment of cell surface integrins from their ECM ligands and redistribute to the leading edge of migration, to facilitate cell locomotion (*253*). In support of this, there was a dose-dependent increase in the integrin αV and PAI-1 gene expression, with OLN when cells were invading through Matrigel.

Therefore, it is possible that the increase in PAI-1 gene expression in HTR-8/SVneo cells treated with OLN may be: 1) attempting to regulate invasion by reducing uPA and MMP activity or 2) be contributing to the increase in invasion by either increasing uPA and MMP activity or altering integrin expression and function, but this remains to be determined.

Taken together, I conclude that 1μM OLN can increase HTR-8/SVneo cell invasion, by increasing the amount of active extracellular MMPs, and regulating integrin expression. Furthermore, an increase in PAI-1 expression may be a compensatory mechanism of the HTR-8/SVneo cells regulating the OLN-mediated increase in invasion or contribute to the increase in invasion through its interaction with ECM proteins or involvement in integrin expression at the cell surface. The increase in invasion also may be mediated by OLN's influence on mitochondrial ETC function, as supported by OLN's ability to impair of mitochondrial ETC complex activity when forced to work at maximal capacity coupled with the changes observed in antioxidants when HTR-8/SVneo cells were invading. This preliminary study also highlights the importance of the extracellular environment on the trophoblasts response to drugs; invading HTR-8/SVneo cells treated with OLN may not respond similarly to those exposed to OLN when grown in the absence of ECM, or in typical serum conditions.

5.10 Importance of *in vitro* Conditions on HTR-8/SVneo Cell Response to OLN

Serum concentration and basement membrane substrates may have more profound affects on trophoblast sensitization to drugs or extracellular stimuli than what has been reported in studies assessing trophoblast invasion (*284-287*). The changes associated with HTR-8/SVneo cells plated on Matrigel may more closely represent the environment and phenotype of trophoblasts *in vivo*. Matrigel is an assortment of ECM proteins, as well as some growth factors and cytokines extracted from Enflebreth-Holm-Swarm tumors in mice, which is similar to the matrix present around invasive trophoblasts in the maternal decidua (*198-200*). Previous studies have demonstrated that *in vitro* experimentation on Matrigel causes cells to display a phenotype that more comparable to that seen *in vivo* for a variety of genes in primary hepatocytes (*288*), and trophoblast cells are no exception. The interaction of trophoblasts with ECM has been considered an important factor in trophoblast invasion as it provides cells with a protein substrate for attachment, and migration (*240*) and has an important role in numerous decisions directing cell fate and behaviour (*199*). Studies have demonstrated differences in trophoblast characteristics when cultured on Matrigel compared to standard cell culture dishes (*289, 290*). HTR-8/SVneo and primary isolated trophoblast cells have a phenotype more similar to EVT lineage when cultured in dilute Matrigel (*198, 289, 291*), as opposed to on uncoated polystyrene cell culture surfaces, where they may more closely resemble CTs within the proximal region of the cell column (*289*).

Therefore, Matrigel promotes a more *in vivo* invasive trophoblast phenotype in culture, and my data clearly demonstrates that when cells are invading Matrigel, OLN significantly impacts integrin expression, consistent with the increased invasion observed. This underscores the importance of utilizing functionally relevant conditions when evaluating the effects of drugs on trophoblasts.

Serum concentration is another potential contributing factor to the differential OLN effects observed. Serum deprivation *in vitro* has been shown to elicit cellular responses (in cancer cells) that are also induced during *in vivo* invasion. For instance, HIF-1 is a key cellular regulator of trophoblast differentiation (*174, 292*) and invasion (*290, 292-294*) and can be regulated by both oxygen levels (hypoxia) and extracellular stimuli, such as growth factors and hormones, present in serum (*295, 296*). Serum starvation is known to increase the HIF-1α expression in cancer cells independent of low oxygen level, with a corresponding increase in invasion (*98, 297-299*). HIF-1α is also known to regulate genes important for invasion (including PAI-1, integrins, and MMPs) (*174, 300, 301*), which were altered with HTR-8/SVneo exposure to OLN in serum-deprived media in this current study. Interestingly, there is support for OLN being able to regulate the expression of HIF-1 in the hippocampus (*302*). Therefore, in *in vitro* conditions with serum-deprivation (as those used in Matrigel invasion experiments and MMP activity assay), there may be an increase in HIF-1, priming cells for invasion. OLN may then impact these activated pathways to significantly up-regulate factors important for invasion, as

opposed to OLN being able to affect these pathways independent of serum-deprivation-induced pathway priming.

Therefore, I have demonstrated for the first time that OLN does impact HTR-8/SVneo invasion, and thus may impact placentation. Though the specific receptor(s) and signaling pathway responsible remains unclear, OLN can impact integrin, MMP, and PAI-1 gene expression as well as increase extracellular MMP activity; all mechanistically consistent with the observed increase in invasion. Moreover, OLN can also affect the mitochondrial ETC function in HTR-8/SVneo cells, which may contribute to the affects observed on invasion. Additionally, my work highlights the important contribution that serum concentration and ECM can have on cellular responses to pharmaceuticals like OLN.

6. FUTURE DIRECTIONS

6.1 Compare OLN's Influence on HTR-8/SVneo Cells in Various *in vitro* Conditions

In light of the findings underscoring the need to clearly evaluate the impact of cell culture substratum and media on trophoblast cell signaling, it would be beneficial to systematically characterize OLN's effects on HTR-8/SVneo cells in the context of different Matrigel and serum conditions *in vitro*. For instance, since HIF-1α expression is heavily implicated in trophoblast invasion and is up-regulated with serum-deprivation in cancer cells, the effects of Matrigel and various serum concentrations on HIF-1α translocation to the nucleus and expression and activity of proteins important in regulating invasion such as, integrin dimerization, MMPs, TIMPs, uPA, and PAI-1 could be assessed with and without OLN treatment. This would 1) clarify the effects of ECM substrate and serum concentrations on HTR-8/SVneo cells in relation to *in vivo* invasion, and 2) elucidate whether OLN's effects on invasion may only be observed if the HTR-8/SVneo cells are sensitized to invasion.

Additionally, it would be worthwhile to improve some limitations in experiments under these various conditions. For instance, OLN's effect on HTR-8/SVneo cell migration could be evaluated when cells are stimulated to migrate using serum as a chemoattractant. This would be more relevant to *in vivo* situation of trophoblast migration where cell movement is regulated by factors secreted by cells within the decidua. Moreover, since there was a significant impairment in the maximal functioning capacity of some mitochondrial ETC complexes with OLN treatment as well as changes in antioxidant

gene expression during cell invasion, further evaluation into ETC function of intact mitochondria within live HTR-8/SVneo cells following OLN exposure is warranted. This should be done by taking polarographical measurements of oxygen consumption and measuring ATP production in intact serum-deprived HTR-8/SVneo cells treated with OLN in order to determine the live-cell functioning, and end product of ETC function, respectively, of the intact and coupled mitochondrial ETC (*213*). Additionally, mitochondrial ROS production and protein expression changes in markers of oxidative stress as a consequence of OLN exposure should be evaluated when cells are grown on ECM in various serum concentrations.

Moreover, these current studies were carried out under normoxic conditions. However, trophoblast invasion and the initiation of spiral artery remodeling occur in a hypoxic environment *in vivo* (*156, 158*). Moreover, there is evidence (outlined in section 5.10) that HIF-1α expression is affected by both hypoxia, and serum-deprivation. Therefore, it would be worthwhile to perform future studies on the effects of OLN on HTR-8/SVneo signaling pathways in a hypoxic environment alongside serum-deprivation conditions. This would further elucidate how OLN may impact trophoblast invasion *in vivo* and which *in vitro* conditions can simulate the *in vivo* environment during trophoblast invasion.

6.2 OLN's Effects on Spiral Artery Remodeling in the Placenta

OLN can directly increase HTR-8/SVneo cell invasion and previous literature has shown that increased invasion, is associated with poor maternal spiral artery remodeling and the establishment of an immature placental vascular network (*131, 134, 223, 224, 226*). Inadequate vascularization of the placenta leads to placental insufficiency, resulting in a decrease in the exchange of nutrients and wastes between maternal and fetal circulations and adverse pregnancy outcomes such as preterm delivery and altered fetal growth (*1, 303, 304*); which are observed in cases of antipsychotic exposures (*44-46, 49*). Therefore, it would valuable to extend the relevance of our current findings by examining whether OLN can affect trophoblast invasion *in vivo*, and whether this can subsequently impact artery remodeling, since this may be a mechanism by which OLN causes altered fetal growth.

Previous investigations on preeclampsia pathology have suggested that increased trophoblast invasion may not be adequately supported in the maternal decidua, potentially resulting in subsequent apoptosis later in gestation (*134, 225, 226*). Moreover, increased early trophoblast invasion has led to immature placental blood vessel development possibly due to the change in coordination between trophoblasts and other cells in the decidua (*223, 224, 231, 232*). Therefore, it would be beneficial to examine the effect of OLN on the depth of trophoblast invasion at different time points of invasion, such as GD15 (initiation), GD18 (peak) and GD20 (term, completion). It would also be important to jointly assess the degree of trophoblast apoptosis and support provided by the other

decidual cells (such as uNK cells) at these gestational time points by examining apoptosis markers in invasive trophoblasts as well as factors secreted by trophoblasts and decidual cells that influence trophoblast survival, invasion and remodeling functions. Moreover, the extent of spiral artery disruption by uNK when trophoblasts arrive to these sites and the subsequent remodeling and functioning of the placental vasculature following trophoblast invasion would allow us to determine whether the effect of OLN on invasion translates to altered placental vasculature.

This proposed work would be the first to evaluate OLN's impact on placental development and function *in vivo* and would greatly benefit the current knowledge of how OLN can cause pregnancy complications. Furthermore, to extend the relevance findings back to the clinical setting, it would be informative to pair this examination of trophoblast invasion and placental vasculature assessment to human placentae exposed to OLN during pregnancy.

7. CONCLUSION

Though there are still many facets to be investigated regarding OLN's effects during pregnancy, our findings provide novel contributions to characterizing OLN's direct influence on trophoblast function. OLN can increase trophoblast invasion by altering extracellular MMP activity and integrin expression, which may potentially be mediated through OLN's actions on mitochondrial ETC activity. This work also brings to light the importance of various substrates and medium conditions when assessing *in vitro* responses to drugs such as OLN. By beginning to elucidate these underlying molecular mechanisms, we may better understand the clinical pathologies associated with OLN exposure during pregnancy. Our *in vitro* experiments provide further rationale for future studies on the effects of OLN during placental development and on placental function *in vivo*. If we can identify the mechanisms(s) by which OLN affects placental development and function we can then begin to explore how these changes impact fetal growth.

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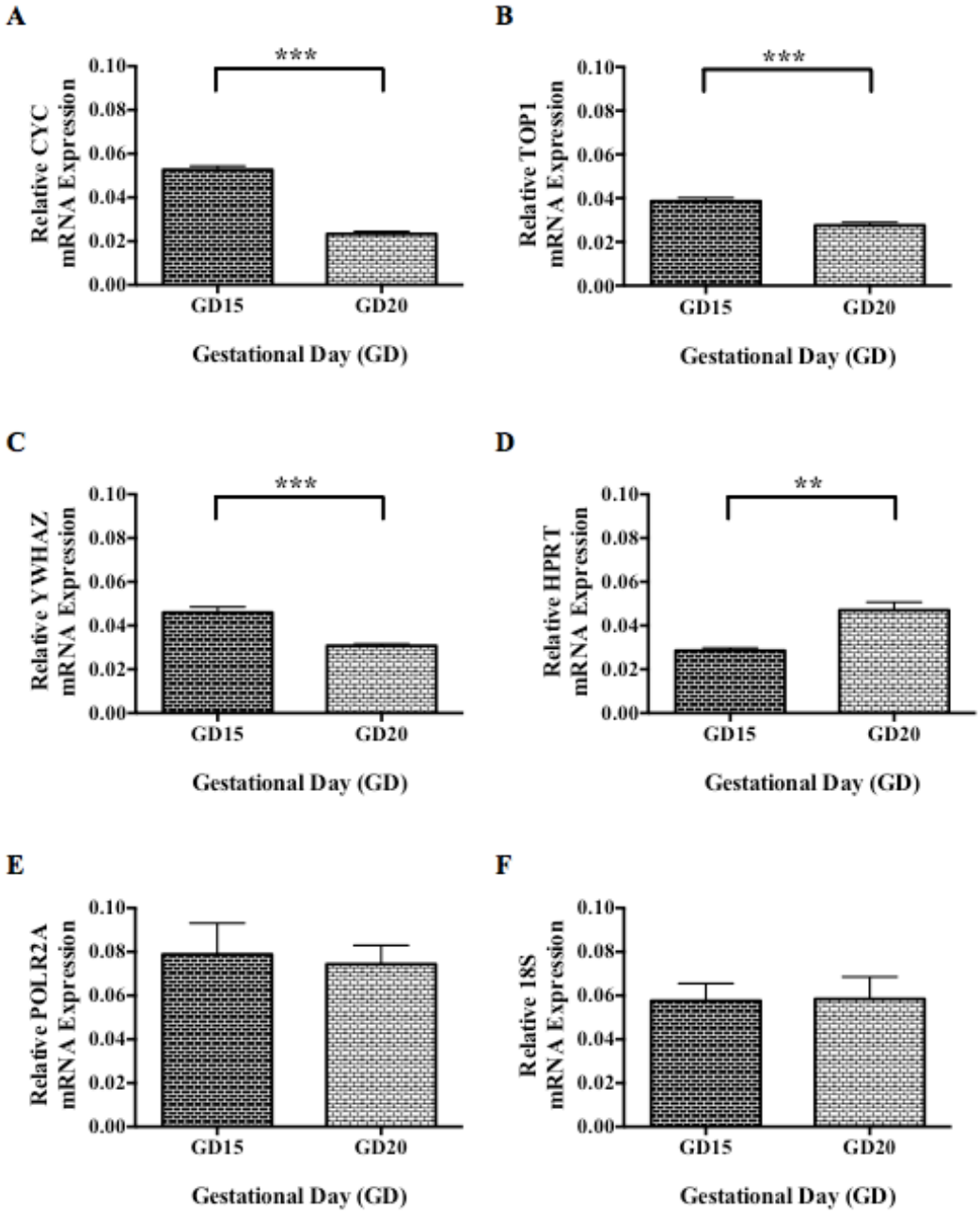
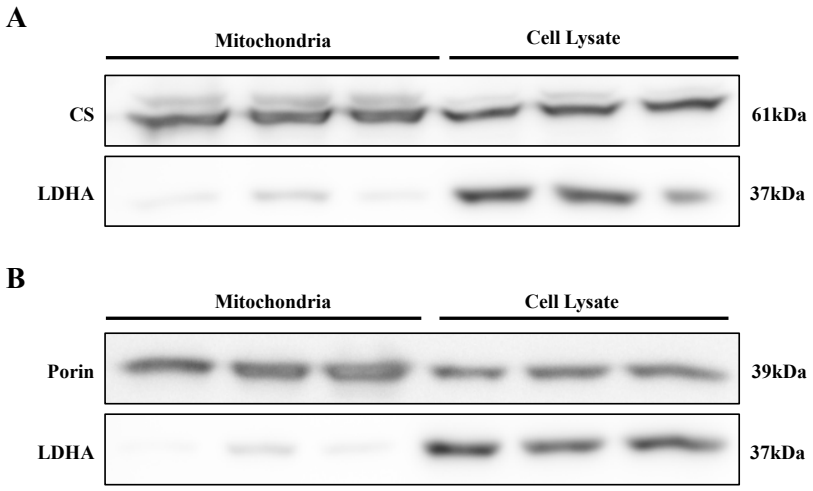
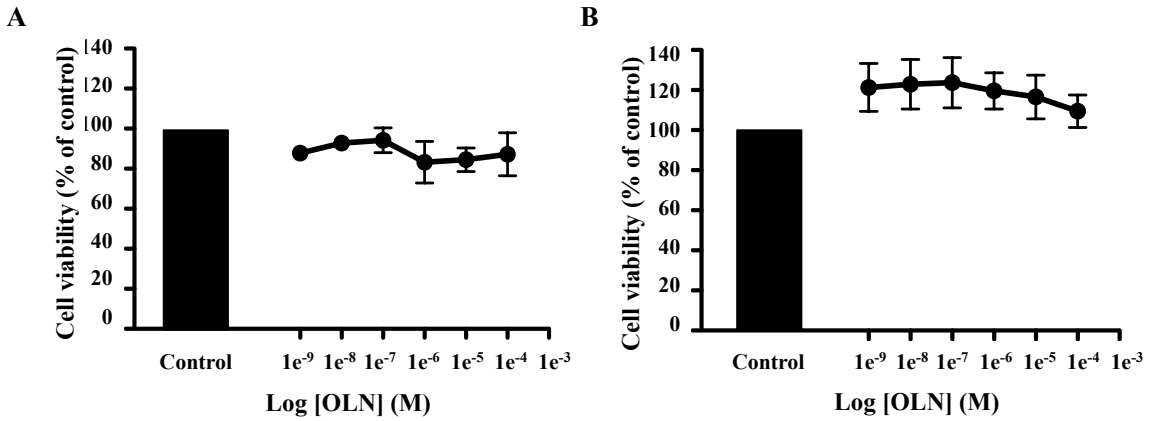
APPENDIX



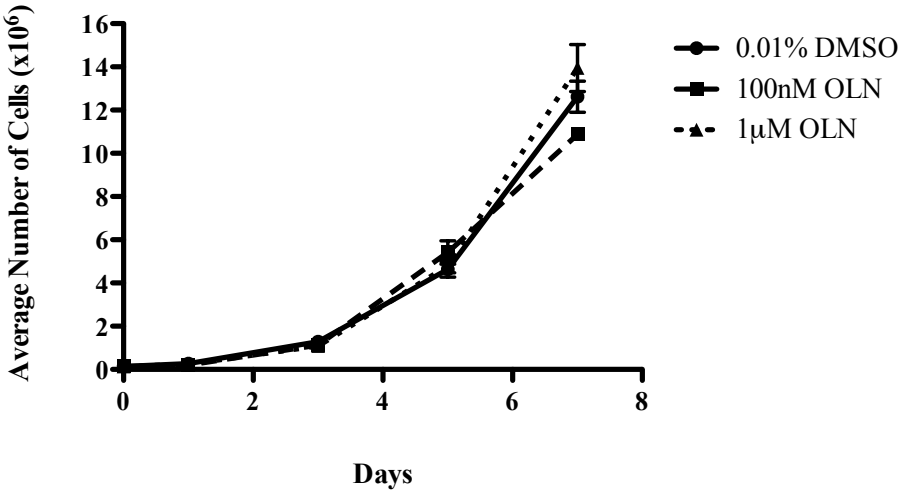
Supplemental Figure 1. The gene expression of recommended placental housekeepers at two different gestational ages. The relative expression of recommended placenta housekeepers: CYC **(A)** TOP1 **(B)** YWHAZ **(C)** HPRT **(D)** POLR2A **(E)** and 18S **(F)** from mRNA extracted from rat placenta at GD15 and GD20. Values represent the mean ± SEM. Significance was determined using a two-tailed Student's T-test; **p<0.01 and ***p<0.0001.



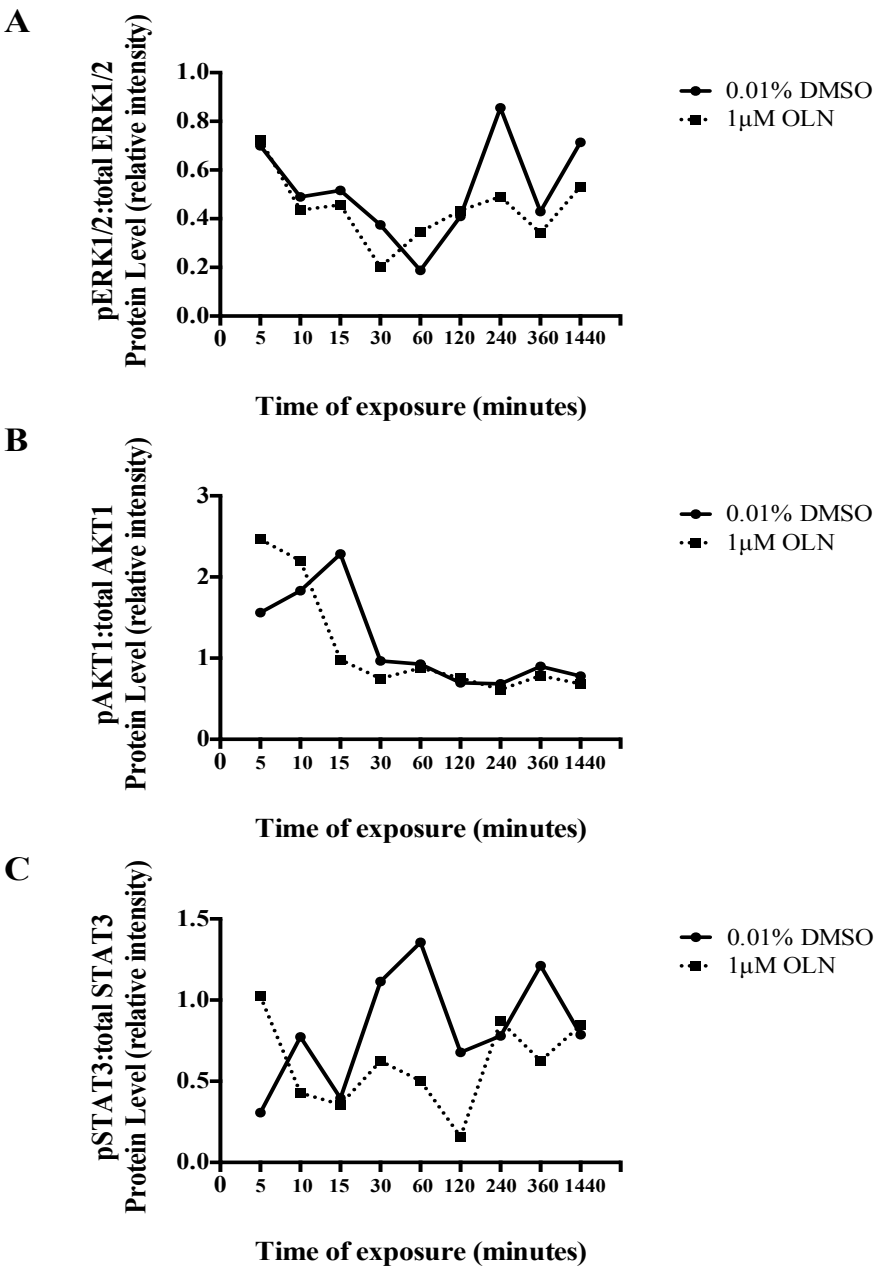
Supplemental Figure 2. The quality of the mitochondria enrichment method. Representative Western blot images of targeted proteins isolated from HTR-8/SVneo cells using the mitochondria enrichment or total cell lysate extraction methods. Target proteins for the mitochondria were citrate synthase **(A)** and porin **(B)**, which are juxtaposed against LDHA, a cytosolic protein.



Supplemental Figure 3. HTR-8/SVneo cell viability following OLN exposure. Cells were plated in a microplate and allowed to attach O/N then treated for 24 hours (**A**) and 72 hours (**B**) with ranging OLN concentrations of $1x10^{-9}$M to $1x10^{-4}$ M (n=5). Viability following OLN exposure was assessed using the MTS assay. Values represent the mean ± SEM. Significance was determined using a one-way ANOVA with Tukey's correction for multiple comparisons.



Supplemental Figure 4. OLN exposure did not alter HTR-8/SVneo cell growth. 80 000 cells were seeded and after 24 hours cells were treated with 100nM or 1μM OLN (Day 0). Cell growth over a period of seven days was determined by manual counting every 48 hours following Day 1 using the trypan-exclusion method. All values represent the mean ± SEM (n=4). Significance was determined using a one-way ANOVA with Tukey's correction for multiple comparisons.



Supplemental Figure 5. Phosphorylated proteins of interest following various lengths of exposures to 0.01% DMSO and 1μM OLN. Proteins extracted from HTR-/SVneo cells that were treated with 0.01% DMSO and 1μM OLN for indicated times between 5 minutes and 24 hours were probed for both phosphorylated and total ERK1/2 **(A)**, AKT **(B)**, and STAT3 **(C)** via Western blot.

Supplemental Table 1

Primer sequences for the genes of interest

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Housekeepers		
18S	CACGCCAGTACAAGATCCCA	AAGTGACGCAGCCCTCTA
18s	GCGATGCGGCGGCGTTAT	AGACTTTGGTTTCCCGGAAGC
β-actin	GCACTCTTCCAGCCTTCCTT	AATGCCAGGGTACATGGTGG
Cyc	CCGCTGTCTCTTTTCGCC	GCTGTCTTTGGAACTTTGTCTGC
Hprt1	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT
Polr2a	CCTGGAGCGAGAACTGTACC	ACAGAAGAGCCAAATGCCGA
Top1	ACTGTGGGTTGCTGTTCACT	ACTCCACCACGTACTCCTGA
Ywhaz	GTGGTACAAAGACAGCACGC	GCAGACAAAGGTTGGAAGGC
Transcription Factors		
FOSL1	GCCCACTGTTTCTCTTGAGC	GATGGAGAGTGTGGCAGTGA
GCM1	CATCCAAAACCAGAAACCAAG	TCCCCTGACTTTGTGTTTC
HLX	ATGTTGGCGACTTGGTAGGG	AGGACACTGAGACTCCCCTC
ID2	CATCTTCCCAGGGTGTTCTCT	TCTGGTATTCACGCTCCACC
STAT3	CATATGCGGCCAGCAAAGAA	ATACCTGCTCTGAAGAAACT
Receptors		
ADRA1A	TTTTTGCAGGTCTGCTGCTG	AGACTTCCTCCCCGTTCTCA
ADRA1B	ACGGGGGAAGCAAAGTTTCAG	TGTCCAGGTCGGGATTCATCT
ADRA2A	CACTCCAGCGCCTTCTTCC	TGAACGATCAGCTCTCCAGG
ADRA2B	CGCATCTACCTGATCGCCAA	ACTTCGAGTGTCCGTTGACC
ADRA2C	GGGCTTCCTCATCGTCTTCAC	GCCCGAAGTACCAGTAGGC
Adra1a	TGCATCATCTCCATCGACCG	CAAAGAAAGCACCCAGACGC
Adra1b	GGCGGGAGTCATGAAGGAAA	CCTTGGTACTGCTGAGGGTG
Adra2a	ACAGCGATGGACCAAGACAG	ACCGCAGCGTCAGTTCTAAA
Adra2b	CTCCTGCGTACCGTGAGTTT	TGAGAAAGGTGATGGCCGAC
Adra2c	TGTGGTGTGGTGTGTACCTG	GTACTCTACCGCTTGCGTCA
CHRM1	AATCACTGGCTGTGCCTCTC	AAGAGGGGTCTGTAGGGTCC
CHRM2	AAGCGGACCACAAAAATGGC	AGCCGTACCAAAGGTGACAG
CHRM3	AGCACTTGTGTTCTGATTAGTGG	CACGCCACAGCAAAACCTTA
CHRM4	CTGGCAGTTTGTGGTGGGTA	TGTGGATGTACAGCACCGTC
CHRM5	GAGAAGGCATTTTGGGTCTCG	CTAGGTTCCTGGCAGTGGTT
Chrm1	AGCTACAGTGACAGGCAACC	TCTTGAGCTCGGTGTTGACC
Chrm2	GCTCTTGGTGGTGAACCAGA	AGGCGTGAAACGATCATCCA
Chrm3	GGAGCTGACGGAGGAAAGTA	TTCAGAAGGTGCCAGGTAGC
Chrm4	TAATCGGTGCTTTCCGCCTT	AAGGTCTTGGTTTCCCACCC
Chrm5	CTTTGGCTGGCACTCGACTA	CGGTACGTCAGTGGTCTTGT
DRD1	TGACACTGACGTCTCTCTGGA	GTTGGGTGCTGACCGTTTTG
DRD2	CAGTCGAGCTTTCAGAGCCA	CCATTCTCCGCCTGTTCACT
DRD4	TACGTGGTCTACTCGTCCGT	CCAGTAGAGCAGCAGCATGA
DRD5	CGGCTTGCTCTCTTCTCTCG	GCTGTTCTCGGGAGTCTGTAG
Drd1	GCCATAGAGACGGTGAGCAT	GCTCATGGTGGCTGGAAAAC
Drd2	CTCTACTCTCCAATCCACTC	CCCAAGTTACCACATCCAG
Drd3	CTACATAGTCCTGAGGCAAAG	CCCTTATTGAAAACTGCCG
Drd4	CAAGATCACTGGAAGGGAG	AAACAAGCCGGACACAG

Supplemental Table 1 (continued)

Primer sequences for the genes of interest

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Receptors		
HRH1	GCAGGGACTATGTAGCCGTC	CCGTTCGAGAGAAGGACTGG
Hrh1	CTGTCTTCAGCCAAGAGGGG	ATGTCGAGACGGAAACAGGG
HTR1A	GGCTCTTGTTCTGCCCTTCT	GTTGGAGTAGCCCAGCCAAT
HTR1B	GATTGCCACAGTGTACCGGA	ATCAGGTAGTTAGCCGGGGT
HTR1D	CATCTGCATCTCCATCCCCC	GTGTTCACCAGACAGTCCGA
HTR1E	GTGGCTGAGTGTGGACATGA	GTCCAGGGCAATGACACAGA
HTR2A	GGCCCTGCTCAATGTGTTTG	CTTGTAGGCCAAAGCCGGTA
HTR2B	CTCACTGGCTGCCTTCTTCA	GCGTTGAGGTGGCTTGTTTT
HTR2C	CCGAAGGGCAATCTCCAACT	CTCCCTCCCAGACAAAGCAG
HTR3A	TGTCCTCCATCCGGCAATTC	CGCCAGCAGGTAAATGTGGA
HTR5A	CTCCTTTTCCCTCTCCACCC	AAAGCCCAGCAAGGTGAGAA
HTR6	CTGCCTTTTGTCCTTGTGGC	AGGTGAAGCATATGGCACCC
HTR7	GTTGTGATCGGCTCCATCCT	TTGACGAAGCACACGGAGAT
Htr1a	CCGTGAAAGGAAGACGGTGA	AATGAAAAACGGCAGCCAGC
Htr1b	CTTTCTATTTACCCACCCTGCTC	GTCTGAGACTCGCACTTTGACTT
Htr1d	CCCGGAGTCGAATCCTGAA	TGATAAGCTGTGCTGTGGTGAA
Htr2a	AGCTCTGTGCGATCTGGATT	CCCCTCCTTAAAGACCTTCG
Htr2b	TGGCAGTTTCATGCTCTTTG	TTCCCTTTGGAGAACTGTGG
Htr5a	AGTGAACTCGGTGACTGCAT	GCTGCGGTTAGGTTCCAAAG
Htr6	ACATCAGTACCCCTCCCCAA	AGGTCATCGAGCAGCGATTT
Htr7	TGCTGGCTGCCGTTTTTC	CTACAGGAGGTGCCACAGATAAAG
Invasion Markers		
MMP1	CCCATCGGCCCACAAACCCC	AGCAGCTTCAAGCCCATTTGGCA
MMP2	ATACCATCGAGACCATGCGG	CTGGGTCCAGATCAGGTGTG
MMP3	TGCCGCATATGAAGTTACTAG	TCGATTTTCCTCACGGTTG
MMP9	CCGGCATTCAGGGAGACGCC	TTGAACCACGACGCCCTTGC
MMP14	CCGATGTGGTGTTCCAGACA	TATGTGGCATACTCGCCCAC
SERPINE1	TCTCAGGAAGTCCAGCCACT	CACAAGGTGGCAGTGTGGG
SNAP23	TGAAATGGAAGAGAACCTGAC	AATACGATCTCTGTTGGTGTC
TIMP1	GGGCTTCACCAAGACCTACA	TGCAGGGGATGGATAAACAG
TIMP2	GAAGAGCCTGAACCACAGGT	GGGGGAGGAGATGTAGCA
TIMP3	CTCTGGACCGACATGCTCTC	AGGCGTAGTGTTTGGACTGG
TIMP4	CCTTGGTGCAGAGGGAAAGT	TTGGCAGCCACAGTTCAGAT
PLAU	ACTGGAATTGTGAGCTGGGG	GCCAGGCCATTGTCTTCCTT
VAMP3	CAAAATGTCTACAGGTCCAAC	CTCTTTCCAGAACCTTGTCC

Supplemental Table 1 (continued)

Primer sequences for the genes of interest

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Adhesion Factors & Integrins		
CDH1	AAGTGACTGATGCTGATGCCC	CCAGGGGACAAGGGTATGAA
CDH5	TCACCCAGACCAAGTACACAT	TGGTGAAAGCGTCCTGGTAG
ITGA1	CAGTCTATCCACGGAGAAATG	GGCTCAAAATTCATGGTCAC
ITGA5	CCAAAAGAAGCCCCCAGCTA	TCCTTGTGTGGCATCTGTCC
ITGA6	CTCCCTGAGCACATATTCG	CACCTCCAACTTCTCCATC
ITGAV	TCACTAAGCGGGATCTTGCC	AGCACTGAGCAACTCCACAA
ITGB1	AAGCGAAGGCATCCCTGAAA	GTCTACCAACACGCCCTTCA
ITGB4	TGAGATCACAGCCTACGA	CTCCCGAAGGTTCTCAATAAG
PECAM1	ATTGTTCCCGGTTTCCAG	AATTGCTCTGGTCACTTCTC
Mitochondrial & Oxidative Stress Markers		
CAT	TGAAGATGCGGCGAGACTTT	GAGGGGTACTTTCCTGTGGC
DNM1L	AAACTTCGGAGCTATGCGGT	AGGTTCGCCCAAAAGTCTCA
GPX	GGCAGATGTGACAAGCAAGG	TGAATGCACTAAGGGCTGGC
FIS1	CCAAGAGCACGCAGTTTGAG	AGACGTAATCCCGCTGTTCC
HSPA1A	GGAGTTCAAGAGAAAACACAAG	AAGTCGATGCCCTCAAAC
HSPD1	GTGTGATGTTAGCTGTTGATG	AATTTCTTTGTCTCCGTTTGC
MFN1	TTGGAGCGGAGACTTAGCAT	GCCTTCTTAGCCAGCACAAAG
MFN2	CACAAGGTGAGTGAGCGTCT	ACCAGGAAGCTGGTACCACG
OPA1	GCTCTGCATACATCTGAAGAACA	AGAGGCTGGACAAAAGACGTT
SOD1	AAAGATGGTGTGGCCGATGT	CAAGCCAAACGACTTCCAGC
SOD2	CACTGCAAGGAACAACAGGC	TAGTAAGCGTGCTCCCACAC

Supplemental Table 2

Antibody conditions for the proteins of interest

Antibody name	Source	Block conditions	Antibody dilution	Antibody conditions
β-actin	Millipore (612657)		1 in 100 000	3% BSA
4-HNE	Abcam (ab48506)	5% BSA	1 in 2000	3% BSA
AKT1	Abcam (ab82216)	5% milk	1 in 200	3% milk
AKT1 (phosphorylated S473)	Abcam (ab66138)	5% milk	1 in 1000	3% BSA
ATP synthase	MitoSciences (MS601)	5% milk	1 in 500	1.5% milk
CAT	Abcam (ab16731)	5% milk	1 in 1000	3% milk
Complex I	MitoSciences (MS601)	5% milk	1 in 500	1.5% milk
Complex II	MitoSciences (MS601)	5% milk	1 in 500	1.5% milk
Complex III	MitoSciences (MS601)	5% milk	1 in 500	1.5% milk
Complex IV	MitoSciences (MS601)	5% milk	1 in 500	1.5% milk
CS	Prepared in house (Dr. S Raha)	5% milk	1 in 3000	3% milk
CuZnSOD	Abcam (ab16831)	5% milk	1 in 5000	3% milk
DRP1	Abcam (ab56788)	5% milk	1 in 500	3% milk
ERK1/2	Cell Signaling (4348)	5% BSA	1 in 1000	5% BSA
ERK1/2 (phosphorylated Thr202/Tyr204)	Cell Signaling (8544)	5% BSA	1 in 1000	5% BSA
GPX	Abcam (ab22604)	5% milk	1 in 1000	3% BSA
H2A	GeneTex (GTX129418)	5% BSA	1 in 1000	3% BSA
HIF-1α	GeneTex (GTX127309)	5% milk	1 in 1000	3% milk
HSP60	GeneTex (GTX110089)	5% milk	1 in 5000	3% milk
HSP70	GeneTex (GTX104126)	5% milk	1 in 1000	3% milk
LDHA	Cell signalling (20125)	5% milk	1 in 2000	3% milk
MFN2	Millipore (ABC42)	5% milk	1 in 1000	3% milk
MMP1	Abcam (ab137332)	5% milk	1 in 2000	3% BSA
MMP2	Abcam (ab51125)	5% milk	1 in 2000	3% BSA
MMP9	GeneTex (GTX61537)	5% milk	1 in 1000	3% BSA
MMP14	GeneTex (GTX61603)	5% milk	1 in 1000	3% BSA
MnSOD	Abcam (ab13534)	5% milk	1 in 1000	3% milk
STAT3	GeneTex (GTX110587)	5% BSA	1 in 1000	3% BSA
STAT3 (phosphorylated Tyr705)	GeneTex (GTX61820)	5% milk	1 in 500	3% BSA
TIMP1	GeneTex (GTX112096)	5% milk	1 in 800	3% BSA
TIMP2	GeneTex (GTX16392)	5% milk	1 in 500	3% milk
OPA1	Abcam (ab119685)	5% milk	1 in 1000	3% milk
Porin	MitoSciences (MSA03)	5% milk	1 in 1000	3% milk