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Notch regulates the growth-promoting nitric oxide cGMP-dependent pathway in epithelial ovarian cancer and ovarian surface epithelium cells

by

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Abstract

Ovarian cancer is the leading cause of mortality among gynecologic cancers. There are several subtypes of ovarian cancer, amongst which epithelial ovarian cancer (EOC) makes up to 90% of all ovarian cancers. The current treatment for EOC consists of debulking surgery followed by a chemotherapeutic regimen composed of platinum-derivative/paclitaxel agents. This regimen is inefficient due to the high rate of chemoresistance development. Thus, targeted therapeutic strategies are in demand in order to control this disease.

To this end, a better understanding of the molecular pathways and mechanisms underlying the initiation and development of EOC is required. Here, we report the interaction between the oncogenic Notch and nitric oxide (NO) pathways and provide novel insights into the molecular nature of this interaction. Moreover, we show that NO signaling promotes the growth of EOC cells *in vitro* and propose blocking this pathway as a potential targeted therapeutic strategy in EOC.

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List of Abbreviations

Abbreviation	Full name
°C	degree Celsius
µg	microgram
µM	micromolar
8-Br-cGMP	8 Bromoguanosine 3', 5' –cyclic monophosphate
AP-1	activating protein-1
Ca	calcium/calmodulin
CAP	cyclophosphamide
Caspase	cysteine-aspartic proteases
CDDP	cisplatin
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
DAPT	γ-Secretase Inhibitor IX
DLL-1, 3, 4	Delta-like-1, 3, 4
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E_2	estradiol
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial to mesenchymal transition
eNOS	endothelial nitric oxide synthase
EOC	epithelial ovarian cancer
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGF-2	fibroblast growth factor-2
g	gravity
G148	neomycin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescence protein
GOG	gynecologic oncology groups
GSNO	s-Nitrosoglutathione
GTP	guanosine triphosphate
GUCY1A1	guanylate cyclase soluble subunit alpha-1
GUCY1B3	gunaylate cyclase soluble subunit beta-3

h	hour
HCL	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES	human hairy and enhancer of split
HEY	hairy/enhancer- of- split related with YRPW motif
i.p	intraperitoneal
ICAM-1	intercellular adhesion molecule 1
IGF1-R	Insulin-Like Growth Factor I Receptor
IgG	Immunoglobulin G
iNOS	inducible nitric oxide synthase
IOSE	Immortalized ovarian surface epithelial cells
JAG 1-2	Jagged 1-2
KOH	potassium hydroxide
Mg^{+2}	magnesium ion
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
MMP	matrix metalloproteinase
mm	millimeter
MSCV	murine stem cell virus
MYC	Myelocytomatosis Viral Oncogene Homolog
NaCl	sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	sodium fluoride
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
nm	nanometer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOD SCID	non-obese diabetic severe combined immunodeficiency
NOS	nitric oxide synthase
ODQ	1H-[1,2, 4]oxadiazolo[4,3-a]quinoxalin-1-one
OSE	ovarian surface epithelium
P_4	progesterone
PBS	phosphate buffered saline
PDEs	phosphodiesterases

PDGF	platelet derived growth factor
PKG	cGMP-dependent protein kinase
pmol	Picomol
Rev	HIV-responsive expression vector
RIPA	radioimmune precipitation
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute medium
RRE	rev responsive element
RT-PCR	real time- polymerase chain reaction
SDS	Sodium dodecyl sulfate
SE	standard error
sGC	soluble guanylate cyclase
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SLUG	Snail Homolog 2
Src	Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene Homolog
TIMP	tissue inhibitor of matrix metalloproteinases
VASP	vasodilator-stimulated phosphoprotein
VEGF	vascular endothelial growth factor
VSVG	vesicular stomatitis virus GP
WT1	Wilms tumor gene product 1

Chapter 1

Introduction

1.1 Ovarian cancer and epithelial ovarian cancer (EOC)

1.1.1 Introduction to ovarian cancer

Ovarian cancer is the fifth leading cause of cancer-related mortality in women and the first among gynecologic cancers (Jemal *et al.*, 2011). Approximately 225,000 women all over the world were diagnosed with ovarian cancer in 2011 (Jemal *et al.*, 2011). In Canada, approximately 2,600 women are diagnosed with this disease annually (Canadian Cancer statistics 2013). Almost 95% of ovarian cancer incidents are sporadic, with a significantly increased risk after the age of 50 (Burkman *et al.*, 2002; Merino *et al.*, 1993). About one third of all ovarian malignancies that occur in women younger than 40 years old are considered borderline ovarian tumor; a type of tumor which occurs in earlier stages of disease and has a better prognosis (Skirnisdottir et al., 2008), indicating a correlation between age and prognosis.

In spite of being able to provoke symptoms, ovarian cancer is termed ‘the silent killer’ because its symptoms are generalized, nonspecific and can be mixed with many normal processes in the female physiology (Goff *et al.*, 2000). Unlike many other cancers, ovarian cancer is characterized by the absence of anatomical barriers to the peritoneal cavity, thus making metastasis and implanting new nodules of tumor throughout the peritoneal surface much easier (Bast *et al.*, 2009).

The 5-year ovarian cancer survival rate when diagnosed at the first stage is very high (approximately 90%); however, only 20% of ovarian cancers are diagnosed at stage 1 (Bast *et al.*, 2009). When the disease develops to stage 2 (metastasis into the pelvis), stage 3 (metastasis to the abdomen) or stage 4 (metastasis beyond the peritoneal cavity), the survival rates dramatically go down (Lutz *et al.*, 2011). In fact, survival rates are strongly correlated with the stage of the disease, ranging from 90% 5-year survival rates at early stages, to a maximum of 25% at stages 3 and 4 (Grewal *et al.*, 2013).

There are several types of ovarian cancer, with epithelial ovarian cancer (EOC) being the most predominant subtype (comprising 90% of ovarian cancers) (Bell *et al.*, 2005). Other ovarian cancer types include germ cell, sarcoma, stromal and mixed-type tumors (Chan *et al.*, 2006).

Recently, immunohistochemistry powered by molecular pathogenesis helped the differential and accurate diagnosis of ovarian carcinoma subtypes. For example, using antibodies against Wilms Tumor Gene Product 1 (WT1) helped clinicians specifically diagnose serous EOC which is the only subtype of ovarian cancer that is positive in this test (McCluggage *et al.*, 2011).

1.1.2 Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) is the most common type of ovarian carcinomas, comprising approximately 90% of all ovarian cancers (Chan *et al.*, 2006). EOC is believed to arise from ovarian surface epithelium (OSE) or fallopian tube fimbria (Auersperg *et al.*, 2001; Lax *et al.*, 2009; Levanon *et al.*, 2008). Recent studies provide evidence that serous EOC originates from fallopian tube but doesn't become clinically detectable until it has spread to the ovarian surface (Crum *et al.*, 2007; Kindelberger *et al.*, 2007; Kurman *et al.*, 2010). EOC is a very aggressive type of cancer and approximately 85% of patients who achieve full remission following first-line therapy rapidly develop recurrent disease (Foley *et al.*, 2013).

There are several distinct histological subtypes among EOC, including serous, mucinous, endometrioid, clear cell types, and transitional cell tumors; the latter including Brenner tumors. The serous EOC is the most common subtype, comprising approximately 70% of all EOC incidences (Jarboe *et al.*, 2008; kaku *et al.*, 2003). According to the biological behavior, EOC can be classified into benign, borderline and malignant subtypes (Foley *et al.*, 2013). Moreover, serous EOC itself is now classified into two groups: low grade and high grade serous EOC (McCluggage *et al.*, 2011).

1.2 Treatment of ovarian cancer

1.2.1 The standard treatment regimen for ovarian cancer

Due to the severity and low survival rates of ovarian cancer, the treatment strategies are complicated and employ combinations of multiple methods and agents. The standard treatment for ovarian cancer, and specifically EOC, is a combined surgery and chemotherapy regimen (Dinh *et al.*, 2008; du Bois *et al.*, 2003). The first-line and most effective chemotherapeutic regimen is a combination of platinum-based (e.g. cisplatin) and taxane-based (e.g. paclitaxel) agents (Piccart *et al.*, 2000).

Significant advances in chemotherapeutic regimens and surgical techniques through rational clinical trials in the past decades have resulted in significant improvements in treatment outcomes (*Yap et al.*, 2009). In spite of these advances in chemotherapy of ovarian cancer, debulking surgery remains a key and the most effective part in the treatment. Debulking surgery is performed by removing the biggest visible bulk of the tumor, even if complete resection is impossible (Bast *et al.*, 2009). Patients with residual tumor size of greater than 2 cm have a survival rate of 12-14 months while patients with a tumor of less than 2 cm size have a survival rate of 40-45 months indicating the substantial effect of residual tumor size on survival rates (Mutch *et al.*, 2002).

Following surgery, combined platinum/paclitaxel regimen gives initial impressive response rates of as high as 80% complete remission (Agarwal *et al.*, 2003). Unfortunately, most of the patients relapse within an average of 18 months (Greenlee *et al.*, 2001). This is primarily due to the stress-induced selection of a small group of drug resistant cells which survive the chemotherapy regimen. After killing the sensitive cells that usually represent most of the tumor bulk, the tumor becomes undetectable by the currently available pathological and imaging techniques. Hence, the drug-resistant population of cells starts to propagate and gives rise to a more severe tumor which is drug resistant and unresponsive to the first-line chemotherapeutic regimen. Obviously, the same chemotherapeutic regimen is ineffective against recurrent disease, resulting in poor outcomes (Gore *et al.*, 1990). Currently, several newly-developed agents are being used for treating relapsed disease including doxorubicin, gemcitabine, topotecan, etoposide and hormonal therapies (Agarwal *et al.*, 2003). However, response rates remain quite low, and go down with every subsequent relapse. In addition, none of these suggested agents for treatment of relapsed disease is well established or with no clear consensus in the field (Yap *et al.*, 2009).

As previously mentioned, the first-line chemotherapeutic strategy for ovarian cancer is a combination of platinum-based and taxane-based agents. The first combination therapy for ovarian cancer was proposed in the mid 1980s, when two studies showed that using doxorubicin and

cyclophosphamide (CAP) in addition to cisplatin (CDDP) significantly enhanced both patients' response and survival rates (Neijt *et al.*, 1984; Omura *et al.*, 1986). A great advance in treatment outcome was achieved by replacing doxorubicin with paclitaxel as first reported by the Gynecologic Oncology Group (GOG) (McGuire *et al.*, 1996) and then confirmed by European–Canadian intergroup study (Piccart *et al.*, 2000). As a result, the new combination of cisplatin/paclitaxel became the standard first-line chemotherapeutic regimen for ovarian cancer. Due to the high toxicity of cisplatin which lowers its tolerability, several clinical studies have been conducted to test the feasibility of replacing cisplatin with its less toxic and more tolerable relative, carboplatin, a less potent platinum-derivative (du Bois *et al.*, 2003). Nowadays, carboplatin is in clinical use in place of cisplatin (Katsumata *et al.*, 2013).

1.2.2 The need to develop new targeted therapies

As stated above, the use of platinum/paclitaxel combination is the first-line chemotherapeutic regimen in ovarian cancer; however, there are many problems facing this regimen. Despite an initial good response, cancer cells eventually become resistant to chemotherapeutic agents. A better understanding of the molecular mechanisms and driver pathways that control the growth and development of ovarian cancer is needed in order to develop targeted therapies that help overcome the problem of the rapid development of drug resistance against traditional regimens. Here,

we shed more light on one of the important pathways in EOC biology, which is the NO/sGC signaling pathway. We decipher molecular details on how this important pathway is regulated in EOC and whether it can be used for targeted and efficient therapy.

1.3 Nitric oxide signaling in mammalian cells

1.3.1 The history of the discovery of nitric oxide signaling

Nitric oxide (NO) is one of the important signaling molecules in mammalian systems. Since its discovery, NO has been extensively studied over decades with more than 86,000 published papers to date (Bryan *et al.*, 2009). NO was found to be the main regulator for smooth muscle relaxation, an important signaling molecule in endothelial and nerve cells and a killer molecule produced by the immune system (Bryan *et al.*, 2009). Moreover, decreased bioavailability and prolonged over-production of NO were linked with many diseases (Moncada 1993; Moncada and Higgs 1993). The discovered physiological roles of NO captured the interest of researchers and resulted in a lot of effort to uncover the molecular pathways underlying NO signaling in mammalian cells.

In 1970s, two milestone studies reported that NO stimulates soluble guanylyl cyclase which in turn elevates cGMP levels leading to vascular relaxation (Arnold *et al.*, 1977; Katsuki *et al.*, 1977). At this time, it was

very surprising that a gaseous molecule could lead to smooth muscle relaxation. Later studies by the same group proposed the role of NO as a messenger in mammalian cells (Murad *et al.*, 1978; Murad *et al.*, 1979). Subsequent studies were conducted in order to uncover further details about this proposed pathway (Bryan *et al.*, 2009). Collectively, all of these studies over time resulted in the identification of the molecular events mediating NO signaling in mammalian cells. In 1998, Ferid Murad, Robert Furchgott and Louis Ignarro were awarded the Nobel Prize in Physiology or Medicine for their discoveries concerning NO as a signaling molecule in the cardiovascular system.

1.3.2 Biosynthesis and release of NO in mammalian cells

In mammals, nitric oxide synthase (NOS) is activated by binding calcium/calmodulin (Ca). Activated NOS synthesizes NO from L-Arginine by oxidation of guanidine nitrogen, releasing NO and L-citrulline as a byproduct (Figure 1) (Liu and Gross 1996). Released NO then diffuses into target cells and binds its main target enzyme, soluble guanylyl cyclase (sGC) (Derbyshire and Marletta, 2012). There are two types of NOS isoforms: Ca- dependent and –independent. Ca-dependent NOS isoforms include endothelial NOS (eNOS), neuronal NOS (nNOS), while inducible NOS (iNOS) is Ca-independent since calmodulin is already bound to the enzyme. eNOS and nNOS synthesize NO that acts as a signaling molecule mediating physiological responses (Bredt, 1999; Marletta, 2001)

while iNOS is induced after activation of macrophages, endothelial cells and a number of other cell types by cytokines. iNOS is responsible for the production of NO for prolonged periods of time (Bryan *et al.*, 2009; Moncada *et al.*, 1991).

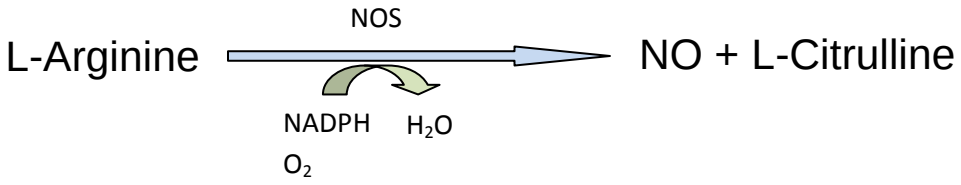


Figure 1. The production of nitric oxide from L-Arginine in mammalian cells. In mammalian cells, nitric oxide synthase (NOS) synthesizes NO from L-Arginine in the presence of NADPH, releasing L-Citrulline as a byproduct.

Traditionally, it was thought that eNOS is only produced in endothelial cells, nNOS is only produced in neuronal cells and iNOS is only produced when induced by the immune system in response to stimuli. Nowadays, it is widely appreciated that all of the isoforms are produced constitutively in several cell types (Bryan *et al.*, 2009). All three isoforms of eNOS, nNOS and iNOS are catalytically self-sufficient if all substrates and cofactors required for continuous low production of NO are available (Abu-Soud *et al.*, 1994).

NO signaling in mammalian cells can take place through two different routes: cGMP-independent or NO/sGC (cGMP-dependent) (Figure 2).

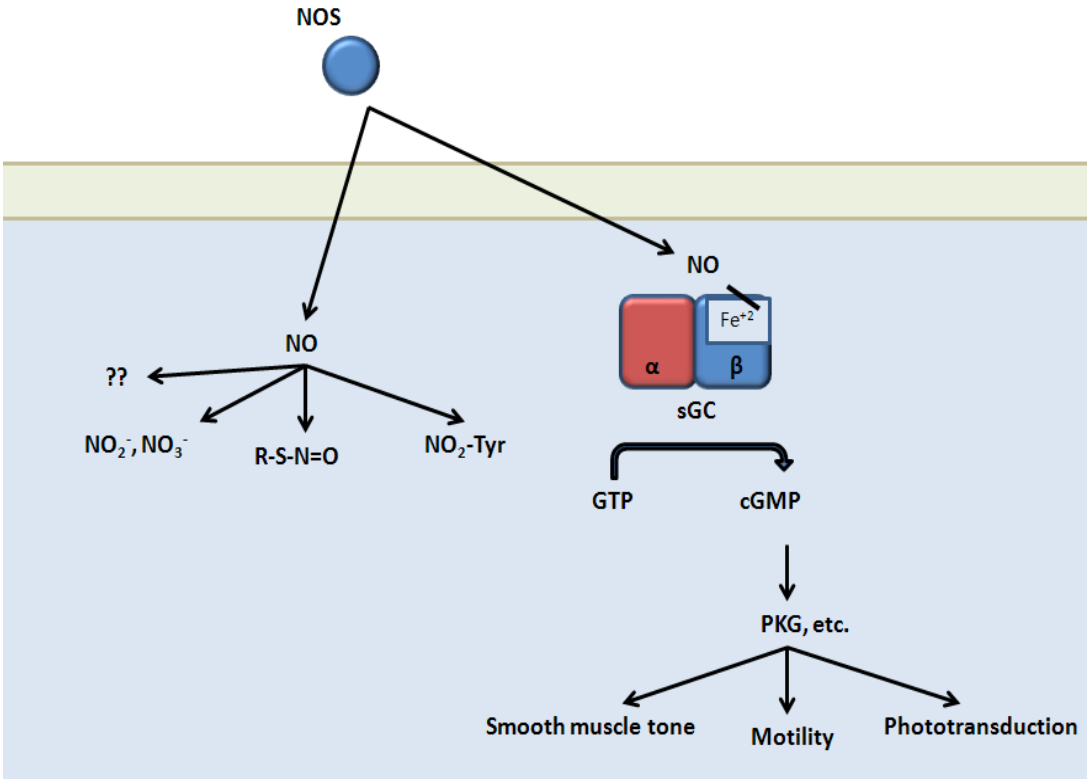


Figure 2. NO signaling pathways in mammalian cells. Upon activation, nitric oxide synthase (NOS) synthesizes NO from the amino acid L-Arginine (L-Arg) and produces L-Citrulline (L-Cit) as a byproduct. NO is then diffused into target cells where it provokes signaling cascade through either cGMP-dependent or –independent pathways. NOS: Nitric Oxide Synthases; (??): Unknown pathways to be discovered; NO_2^-: Nitrite; NO_3^-: Nitrate; R-S-N=O: the general structure of S-Nitrothiols; NO_2-Tyr: Nitrotyrosine; sGC: Soluble Guanylyl Cyclase (with α and β subunits); Fe^{+2}: Heme group; GTP: Guanosine Triphosphate; cGMP: Cyclic Guanosine Monophosphate; PKG: Protein Kinase G.

1.3.3 cGMP-independent NO signaling

There are several aspects of NO signaling inside mammalian cells that are independent of cGMP. These cGMP-independent signaling pathways of NO signaling are quite complicated and not very well understood. To date, there are three major discovered NO cGMP-independent signaling pathways which are mediated by inorganic nitrite and nitrate, S-nitrothiols or nitrotyrosine (Bryan *et al.*, 2009).

Nitrite and nitrate work as a store of NO in the human body, which can be recycled under specific conditions to release NO (Benjamine and Vallance, 1994; Gladwin *et al.*, 2005; Lundberg *et al.*, 2008). Currently, both nitrite and nitrate signaling have a wide range of applications in therapeutics and health research (Bryan *et al.*, 2007; Duranski *et al*., 2005; Pluta *et al.*, 2005, Webb *et al.*, 2004). In fact, nitrate needs to be reduced to nitrite inside the body to become bioactive (Lundberg *et al.*, 2004; Spiegelhalder *et al.*, 1076).

The second major cGMP-independent NO signaling pathway is mediated by S-nitrothiols. S-nitrothiols are thio-esters of nitrite conjugated to amino acids, peptides or proteins. Common examples of S-nitrothiols include S-nitrocysteine and S-nitroglutathione. Today, it is widely accepted that S-nitrothiols are intermediates in cGMP-independent NO signaling with protein thiols acting as major targets for NO signaling in the cell (Foster *et al.*, 2003). The S-nitrosylation signaling is massively involved in controlling many biological processes including apoptosis, G-protein

coupled receptor signaling and the inflammation response (Hara *et al.*, 2005; Hess *et al.*, 2005; Kelleher *et al.*, 2007; Whalen *et al.*, 2007).

The third major route of cGMP-independent NO signaling is the nitrotyrosine pathway. Nitrotyrosine is of specific importance because it is closely related to formation and production of peroxidases. Moreover, it is considered as a marker of reactive nitrogen species and nitrative stress (Sampson *et al.*, 1998; Wu *et al.*, 1999). There are other proposed routes of cGMP-independent NO signaling in mammalian cells; however, none of them are confirmed and cGMP-independent NO signaling is still poorly understood.

1.3.4 NO/sGC (cGMP-dependent) signaling

In this study we focused on the NO/sGC pathway which involves sGC stimulation by NO binding to its heme domain, leading to a more than 200 fold increase in its enzymatic activity (Humbert *et al.*, 1990; Lee *et al.*, 2000). Once activated, sGC starts to dramatically increase the level of cGMP production. cGMP in turn activates downstream targets including phosphodiesterases (PDEs), ion-gated channels and cGMP-dependent protein kinases (PKGs) (Munzel *et al.*, 2003; Sanders *et al.*, 1992; Warner *et al.*, 1994). This pathway controls many physiological functions including smooth muscle tone, motility, photo-transduction and maintaining electrolyte homeostasis (Biel *et al.*, 1999; Boolell *et al.*, 1996; Lucas *et al.*,

2000). cGMP-dependent and –independent NO signaling routes are summarized in Figure 2.

1.3.4 The structure and importance of sGC enzyme

sGC acts as the main receptor of NO and the key enzyme in cGMP-dependent NO signaling in mammalian cells. sGC is a heterodimeric protein consisting of two subunits referred to as α and β with a bound heme in the active site of the β subunit (Grezer *et al.*, 1991). The heme group is crucial for the activation of the enzyme and removal of this group is sufficient to abolish NO-induced activation that can be restored by re-addition of the same group (Crave and DeRubertis, 1978; Foerster *et al.*, 1996; Ignarro, 1990; Ignarro *et al.*, 1986). Similar to other nucleotide converting enzymes, sGC requires the cofactor Mg^{+2} for catalysis (Friebe and Koesling, 2003).

There are several isoforms of sGC subunits and the termed α3 and β3 isoforms represent the human versions (Giuili *et al.*, 1992). The β subunit is the critical subunit for the enzymatic activity (Koesling *et al.*, 2004). In fact, both of the subunits were shown to be important for proper activity of the enzyme; however, the β subunit (the heme binding subunit) plays a more important role in the enzymatic activity and substrate binding (Koglin and Behrends, 2003). Despite the fact that sGC is only active as a heterodimer (Herteneck *et al.*, 1990; Kamisaki *et al.*, 1986), recombinant homodimers of the N-terminal parts of β subunit expressed in bacteria

were able to bind the heme group in a manner similar to that of the wild type sGC (Zhao and Marletta, 1997). In the β1 isoform, the heme containing residue was identified as His-105 (Wedel *et al.*, 1994; Zhao *et al.*, 1998). Moreover, mutations in two conserved cysteines surrounding His-105 lead to loss of enzyme-bound heme and response to NO, which can be regained after heme rebinding (Friebe *et al.*, 1997). Taken together, these studies show that the β subunit (also known as GUCY1B3 in human) plays a more important role in the enzymatic activity, mainly through heme binding.

sGC is critical for mediating most of the functions of NO in mammalian cells. To confirm the critical role of sGC in NO signaling, sGC knockdown mice were generated (Friebe *et al.*, 2007; Mergia *et al.*, 2006; Nimmegeers *et al.*, 2007). Deletion of sGC resulted in severe symptoms which led to the premature death of mice at the age of 4 weeks. Collectively, these studies show that sGC is the key enzyme in mediating physiological functions of NO and transducing NO signaling cascade in mammalian cells.

1.3.6 The role of NO signaling in cancer

Despite the fact that NO signaling was first studied in the context of cardiovascular disorders, this pathway is now believed to be highly involved in many aspects of cancer initiation and progression. Many cancer-related processes including angiogenesis, cell cycle control,

invasion, metastasis and apoptosis are affected and modulated at least in part by NO signaling (Ying and Hofseth, 2007). On the other hand, NO has been reported to have anti-tumor effects as well (Choudhari *et al.*, 2013). Comparing the several studies which report multifaceted and sometimes opposing roles of NO in tumor biology, it appears that the biological effect of NO on cancer is greatly dependent on factors such as treatment dose and cellular context.

In fact, NO is involved in tumor initiation and formation of neoplastic lesions by promoting mutagenesis and toxicity in human cells (deRojas-Walker *et al.*, 1995; Gal and Wogan, 1996). Moreover, NO was shown to inhibit DNA repair mechanisms in human cells (Wink *et al.*, 1998). Notably, it was shown that NO may directly induce GC to AT mutation in p53 gene leading to loss of its normal activity (Wink *et al.*, 1998). In addition, NO inhibits caspases and cytochrome c and increases the expression of BCL-2 leading to efficient blockade of apoptosis (Choi *et al.*, 2002). Other studies show that NO effects on apoptosis are mediated by activation of cyclooxygenase-2 (COX-2) (von-Knethen and Brune, 1997).

The role of NO in angiogenesis has been extensively studied due to the clear link between NO and blood vessels relaxation. As expected, it was reported that NO promotes blood flow around solid tumors by inhibiting adhesive interactions between endothelial cells and increasing vascular permeability (Ziche and Morbidelli, 2000). The activity of NO/sGC pathway is essential for vascular endothelial growth factor (VEGF) to

promote neovascular growth (Ziche and Morbidelli, 2009). Also, NO activates COX-2 that in turn promotes the production of pro-angiogenic factors and prostaglandins (Morbidelli *et al.*, 2003; Morbidelli *et al.*, 2004). There are other reported mechanisms by which NO modulates angiogenesis including S-nitrosylation of redox-sensitive transcription factors like AP-1 and NF-kB, leading to transcriptional activation of several angiogenic factor genes as well as stimulation of FGF-2 (Donnini and Ziche, 2002; Gallo *et al.*, 1998).

NO upregulates several matrix metalloproteinases including MMP-2 and MMP-9 which in turn enhance tumor invasiveness (Gallo *et al.*, 1998; Lala and Orucevic, 1998). Moreover, NO inhibits several TIMPs (tissue inhibitors of MMPs) including TIMP-2 and TIMP-3 in order to further enhance the activity of MMPs and promote invasion of the tumor (Lala and Orucevic, 1998). Other studies indicate several roles of NO in promoting tumor growth, including for instance the inhibition of anti-tumor immune response by inhibiting the proliferation of leukocytes (Wink *et al.*, 1991).

On the other hand, an anti-tumor role of NO was reported as well (Choudhari et al., 2013). The effect of NO can be pro- or anti-tumorigenic based on several factors including concentration, type of synthase, target cell type, and surrounding conditions (Fukumura et al., 2006). Further studies are needed in order to understand the detailed mechanism and molecular interactions related to NO signaling in tumor biology.

1.4 The role of NO signaling in ovarian cancer biology

The role of NO signaling in ovarian cancer has become the focus of several groups proposing new therapeutic initiatives based on the manipulation and modification of this signaling pathway.

1.4.1 Role of NO in growth

NO plays a significant yet complicated role in ovarian cancer growth. The effect of NO on ovarian cancer growth can be promoting or inhibiting depending on many factors including the dose, the isoform of biosynthesis enzyme, the mechanism of release and production and the microenvironment. For instance, it was reported that downregulation of iNOS expression and NO production is a possible mechanism for mediating the inhibitory effects of high doses of estradiol (E_2), progesterone (P_4) or E_2+P_4 on ovarian cancer cell growth (Keith Bechtel and Bonavida, 2001). This finding indicates that basal levels of NO promote ovarian cancer cell growth. Moreover, it was reported that targeting iNOS results in less S-nitrosylation of caspase-3. S-nitrosylation of caspase-3 is a common mechanism by which many cells become resistant to apoptosis. Thus, siRNA knockdown of iNOS enhances apoptosis in EOC cells, confirming the role of iNOS in promoting growth of EOC by evading apoptosis (Saed *et al.*, 2010). More specifically, another study reported that protein kinase G type Iα (PKG-Iα), a downstream kinase of cGMP-dependent NO signaling, contributes to enhanced Src

activation, DNA synthesis and cell proliferation in ovarian cancer cells. Subsequently, the authors proposed targeting NO/sGC signaling as a potential therapeutic strategy to inhibit the growth of ovarian cancer (Leung *et al.*, 2010). In addition, the basal levels of NO seem to suppress apoptosis primarily through the NO/sGC pathway. It was reported that the basal activity of NO/sGC/cGMP signaling regulates p53 and caspase-3 protein levels and function, possibly by regulating the phophorylation state of p53, to suppress apoptosis and promote cell survival and growth of EOC cells (Fraser *et al.*, 2006). Collectively, these studies indicate that low and basal levels of NO signaling promote the growth and suppress apoptosis of ovarian cancer cells.

1.4.2 Role of NO in tumorigenesis

In an interesting study, Ozel and colleagues investigated the expression of COX-2 and iNOS in specimens from 100 ovarian carcinomas and correlated the results with other prognostic parameters. They concluded that expression of iNOS may be critical in the initial steps of carcinogenesis of EOC (Ozel *et al.*, 2006). Moreover, inhibition of NO synthesis resulted in inhibition of tumorigenesis and reduction in the size of ovarian cysts in mice models (Nemade *et al.*, 2002), confirming the role of NO in early tumorigenesis. Similarly, Tavares Murta and colleagues reported higher levels of NO in cystic fluids collected from women with malignant ovarian cystic tumors than women with benign tumors,

suggesting a tumorigenic role for NO in the microenvironment of ovarian cystic tumors (Tavares Murta *et al.*, 2004). Recently, it was shown that targeting eNOS reduced the expression of the intercellular adhesion molecule 1 (ICAM-1) and prevented stroma-mediated epithelial invasion in ovarian cancer (Trachootham *et al.*, 2013). Taken together, these results say that that NO plays a role in stimulating tumorigenicity at the early stages of ovarian cancer development.

1.4.3 Significance of deciphering the basics of NO signaling in ovarian cancer

As shown earlier, there is strong evidence supporting the important role of NO signaling in ovarian cancer biology; however, the underlying mechanisms remain poorly understood. The effect of NO on several aspects of ovarian cancer biology is multifaceted, depending on the dose, the isoform of synthesis enzyme, the type of signaling (cGMP-dependent or –independent) and the surrounding conditions in the cellular context as well as microenvironment. As stated above, there is evidence indicating the involvement of NO low and basal levels in promoting growth, tumorigenicity and suppressing apoptosis in ovarian cancer. Specifically, the NO/sGC signaling pathway was reported to promote growth and inhibit apoptosis of ovarian cancer cells (Fraser *et al.*, 2006; Leung *et al.*, 2010). To better understand this complicated role and design targeted therapies manipulating NO in ovarian cancer, in-depth mechanistic studies are

required in order to understand the molecular pathways and interactions utilized by this gaseous molecule in ovarian cancer cells. Here, we report the ability of Notch signaling to upregulate NO/sGC signaling in OSE and EOC cells.

1.5 Notch signaling

In mammalian cells, the highly conserved Notch signaling pathway is responsible for determination of cell fate and regulation of cell-cell interaction (Artavanis-Tsakonas *et al.*, 1999). Generally, Notch maintains the undifferentiated state of the cell; however, in some rare cases it induces differentiation (Capaccione and Pine, 2013; Nickoloff *et al.*, 2002).

1.5.1 Overview of Notch signaling

The Notch gene was first characterized in flies almost 95 years ago, and was named “Notch” after the phenotype resulting from partial inhibition of this gene: several notches at the wing margins (Artavanis-Tsakonas *et al.*, 1999).

The Notch genes encode an approximately 300 kDa transmembrane receptor proteins (Hopfer *et al.*, 2005). After translation, Notch proteins are cleaved by a furin-like protease and heterodimerize to form mature receptors (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998). The resulting Notch heterodimers are autoinhibited unless activated by binding Notch ligands attached to the surface of a neighbouring cell (Gordon *et al.*,

2007). Thus, it was traditionally believed that Notch signaling has an effect on neighboring cells only through cell-cell contact; however, a recent study showed that one of the Notch ligands (Jagged-1) can be cleaved and secreted to activate Notch in nearby cells in a paracine manner (Lu *et al.*, 2013).

Upon activation by binding the ligand, Notch receptors undergo a set of complex cleavage processes, resulting in the release of the intracellular domain of the protein (Capaccione and Pine, 2013). The activated intracellular domain (ICD) translocates to the nucleus where it interacts with the DNA bound CSL protein complex (Known as CBF1/RBP-Jκ in human) (Borggrefe *et al.*, 2009). The CBF1/RBP-Jκ complex acts as a transcriptional repressor unless it binds to Notch ICD, a process that converts it into a transcriptional activator to regulate the Notch target genes (Kao *et al.*, 1998).

1.5.2 Notch receptors, ligands and target genes

In mammalian cells, the Notch family is composed of four receptors (Notch 1-4) (Fortini, 2009). These Notch receptors are activated by 12 ligands organized into four families: DSL/DOS, DSL only, DOS coligands and the noncanonical ligands (Kopan and Ilagan, 2009). The canonical pathway activating ligands, Jagged 1-2 (JAG1 and JAG2) and Delta-like-1, 3, 4 (DLL1, 3, 4), are expressed on the surface of the signal sending cell,

to activate Notch signaling in a neighboring target cell by binding to its transmembrane Notch receptors (Milner and Bigas, 1999).

Notch target genes are highly dependent on cell type and context; however, there are a few genes which are constitutively activated by Notch regardless of the cell type. The best studied examples of such genes include the human hairy and enhancer of split (HES) and the hairy/enhancer-of-split related with YRPW motif (HEY) families (Bailey *et al.*, 1995; Jarriault *et al.*, 1998). Both HES and HEY proteins act as transcriptional repressors of lineage commitment genes by binding target DNA through their helix-loop-helix and WPRW domains (Fisher *et al.*, 1996).

Several identified Notch targets are known to play important roles in cancer including MYC (Weng *et al.*, 2006), IGF1-R (Eliasz *et al.*, 2010), NF-kB (Vilimas *et al.,* 2007), Survivin (Chen *et al.*, 2011) and SLUG (Niessen *et al.*, 2008).

1.5.3 Notch signaling in ovarian cancer

Notch signaling can be oncogenic or tumor suppressing depending on cancer types. Several studies have reported the involvement of Notch signaling in ovarian cancer, especially in the processes of proliferation, survival, tumorigenicity, angiogenesis, epithelial to mesenchymal transition (EMT) and chemoresistance (Cappaccione and Pine, 2013; Espinoza and Miele, 2013; Gupta *et al.*, 2013, Hopfer *et al.*, 2005; Hu *et al.*, 2011;

McAuliffe *et al.*, 2012). Notch signaling was previously reported to interact with NO/sGC signaling in endothelial and glioma cells (Chang *et al.*, 2011; Charles *et al.*, 2010). These reports led us to investigate whether Notch is involved in the regulation of NO/sGC signaling in ovarian cancer as well. We posited that Notch activation of NO/sGC signaling is one of the ways that mediate the tumorigenicity of Notch in ovarian cancer. Furthermore, this regulation by Notch may reveal new insight into NO/sGC signaling in epithelial ovarian cancer and whether it has a role in the transformation of normal OSE into EOC.

1.6 Hypothesis

We hypothesize that Notch activates NO/sGC signaling in EOC as well as OSE cells by regulating the expression of some key signaling components of the pathway. We also propose that inhibiting sGC activity inhibits the growth of EOC cells *in vitro* and may provide the basis for a targeted therapeutic strategy against advanced EOC.

Chapter 2

Materials and Methods

2.1 Cell culture

Immortalized ovarian surface epithelial cells (IOSE 364 and IOSE 386) were provided by the Canadian Ovarian Tissue Bank at the BC Cancer Agency. Cells were cultured in M199/MCDB105 medium containing 5% FBS and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Primary EOC cells isolated from patients' ascites were cultured in M199/MCDB105 medium containing 10% FBS and supplemented with 100U/ml penicillin and 100 µg/ml streptomycin. OVCAR3 and OVCA429 EOC cells from our lab stock were cultured in RPMI 1640 medium containing 10% FBS and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. SKOV3 cells from our lab stock were cultured in DMEM/F12 medium containing 10% FBS and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Retroviral and lentiviral packaging cell lines (Phoenix-Ampho and 293T cells) from our lab stock were cultured in DMEM high glucose medium containing 10% FBS and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in 5% CO_2. Protocol for primary ovarian cancer sample collection was approved by the Alberta Cancer Research Ethics Committee and Research Ethics Office of the University of Alberta.

2.2 Generation of stable overexpression and knockdown cell lines

IOSE 364 cells were stably transduced using retroviral vectors containing Notch1 intracellular domain (NICD1), Notch3 intracellular domain (NICD3) or hGUCY1B3 as previously described (Niessen *et al.*, 2008) to generate cells overexpressing the corresponding proteins. Cells stably transduced with the respective empty vectors (MSCVneo, pCEG, and MSCVpac respectively) were used as controls. Phoenix-Ampho cells were transfected using the calcium phosphate method. Media from Phoenix-Ampho cells transfected with the previously mentioned vectors were collected and used to infect IOSE 364 cells after being passed through filters to prevent cell line cross-contamination. IOSE/NICD3 cells and their corresponding control cells were purified by FACS sorting for GFP positive cells. IOSE/NICD1 cells and their corresponding control cells were purified by G418 selection. IOSE/hGUCY1B3 cells and their corresponding control cells were purified by puromycin selection. OVCA429 cells were infected with pLentiLox-GFP lentiviral vector expressing short hairpin RNA (shRNA) with scrambled sequence (referred to as shRandom) or shRNA against GUCY1B3 (referred to as shGUCY1B3) to generate GUCY1B3 knockdown cells and their corresponding control. The sequences of shRandom and shGUCY1B3 constructs were GTT GCT TGC CAC GTC CTA GAT and GGA CTG AGA TCA GCT GCT TAC, respectively. Briefly, 293T cells were transfected using pLentiLox-GFP-shRandom or pLentiLox-GFP-shGUCY1B3 along

with packaging and assembly vectors (RRE, REV, VSVG) using the calcium phosphate method. Conditioned media were collected and passed through filters to infect OVCA429 cells. Generally, pLentiLox-GFP infected cells are purified using FACS sorting for GFP positive cells, but in our case the infection efficiency was quite high, so we used the cells directly to avoid the side effects of FACS sorting on cells. The infection efficiencies of all prepared cell lines were confirmed by Western blotting. Notch target genes HES1 and HEY1 were used as readouts of Notch activation.

We had difficulty in detecting the expression of NICD1 in IOSE cells stably transduced with MSCVneo-NICD1 construct by Western blotting. This is likely due to the short half-life of NICD1 in these cells. However, we confirmed the overexpression of NICD1 by real-time PCR using primers that amplify the intracellular domain of Notch1 and the induction of classic Notch target genes HES1 and HEY1 in NICD1-overexpressing IOSE cells.

2.3 siRNA knockdown

IOSE cells with NICD1 or NICD3 overexpression were seeded in 6-well plates along with their corresponding control cells. Cells were cultured in antibiotic-free media for 24h to reach the approximate confluency of 80%. Two ON-TARGET PLUS small interefering RNA (siRNA) oligonucleotides against GUCY1B3 were obtained from Thermo Scientific

Dharmacon. The sequence of siGUCY1B3A siRNA was UCA UGA ACC UGG ACG AUU U whereas the sequence of siGUCY1B3B siRNA was GGU AGU UAC AGG UGU CAU A. One ON-TARGET PLUS non-targeting pool obtained from the same source and referred to as siControl was used as a control. siRNAs at final concentrations of 25 nM were transiently transfected into target cells using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. Cells were incubated in the transfection mixture for 48h before treatment. After treatment, cell lysates were collected for Western blotting analysis.

2.4 Chemical reagents and treatments

S-Nitrosoglutathione (GSNO, a NO donor), 1H-[1,2, 4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, the sGC specific inhibitor) and 8 Bromoguanosine 3', 5' –cyclic monophosphate (8-Br-cGMP, a cGMP donor) were purchased from Sigma. γ-Secretase Inhibitor IX (DAPT, a Notch inhibitor) was purchased from EMD Millipore. cGMP enzyme immunoassay kit was obtained from the Cayman Chemical Company. siRNAs were obtained from Thermo Scientific Dharmacon. Lipofectamine RNAiMAX transfection reagent was obtained from Invitrogen. Throughout the study, cells were treated with 50 µM GSNO for 2h to activate NO/sGC signaling or with 10 µM 8-Br-cGMP for 2h to supply the cells with exogenous cGMP. To block Notch, cells were treated with 10 µM DAPT

for 48h. For sGC inhibition, we treated cells with 50 µM ODQ for 2h. In cases of DAPT and ODQ, equal volumes of the vector (DMSO) were used as controls.

2.5 Quantification of intracellular cGMP levels

Prior to cGMP quantification using the Cayman Chemical Company immunoassay kit, cells were treated with GSNO or left untreated for 2h. Cell lysates were then collected in 0.1 M HCl according to the manufacturer's instructions. Protein concentrations of different samples were assessed using the DC protein assay (Bio-Rad). Samples and standards were acetylated using 4 M KOH and Acetic Anhydride to allow detection of cGMP less than 1 pmol/ml. cGMP concentrations in the cell lysates were normalized to the protein amount and represented as pmol/mg protein.

2.6 RNA isolation and reverse transcription

RNA isolation was performed using TRIzol reagent from Invitrogen following the manufacturer's instructions. RNA concentrations were assessed using a DU 730® spectrophotometer from Beckman Coulter. cDNAs were synthesized using SuperScript II reverse transcriptase reagents from Invitrogen in the presence of RNase inhibitor.

2.7 Real-time PCR

Real time RT-PCR was performed using Mastercycler® ep realplex real-time PCR machine from Eppendorf and SYBR green dye from QIAGEN. Fold changes were calculated based on $\Delta\Delta C_T$ method. Experimental samples were first normalized to GAPDH and then to the control samples. Primer sequences used for real time RT-PCR were: Notch1, forward 5' -CGC ACA AGG TGT CTT CCA G- 3', reverse 5' -AGG ATC AGT GGC GTC GTG- 3'; Notch3, forward 5' -CAA TGC TGT GGA TGA GCT TG- 3', reverse 5' -AAG TGG CTT CCA CGT TGT TC- 3'; HEY1, forward 5' -AGA GTG CGG ACG AGA ATG GAA ACT- 3', reverse 5' -CGT CGG CGC TTC TCA ATT ATT CCT- 3'; HES1, forward 5' -AGG CGG ACA TTC TGG AAA TG- 3', reverse 5' -CGG TAC TTC CCC AGC ACA CTT- 3'; GUCY1B3, forward 5' -GGA AAT TGC TGG CCA GGT TCA AGT- 3', reverse 5' -TTC TCC TGT GGT TTC TGT TCG GCT- 3'; GAPDH, forward 5' -GGA CCT GAC CTG CCG TCT AGA A- 3', reverse 5' -GGT GTC GCT GTT GAA GTC AGA G- 3'.

2.8 Extraction of whole cell lysates and Western blotting analysis

Whole cell lysates were extracted using modified radioimmune precipitation (RIPA) buffer. After being washed once with phosphate buffered saline (PBS), cells were incubated in RIPA buffer composed of 50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM

EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and proteinase inhibitor cocktail. Then, lysates were collected, sonicated, and centrifuged at 14,000 X g for 15 min at 4 °C. The supernatants were labeled as whole cell lysates. Protein concentrations were assessed using the DC protein assay (Bio-Rad) and an equal amount of proteins was used for Western blotting (50 µg/well). Western blotting was performed as previously described (Fu *et al.*, 2001). Membranes were scanned and analyzed using Odyssey® IR scanner and Odyssey® imaging software 3.0. Primary antibodies against cleaved Notch1 (C-Notch1), Notch1, Notch3, VASP, phosphorylated VASP (p-VASP) (Ser 239) and PKG-1 were purchased from Cell Signaling Technology. Primary antibodies against GUCY1B3 and GUCY1A1 were purchased from Sigma. Tubulin primary antibody was obtained from Abcam. Secondary antibodies IR Dye 800CW conjugates of donkey anti-rabbit-IgG, anti-rat IgG and anti-mouse IgG were purchased from LI-COR Biosciences. Data shown in this study are representatives of three independent experiments to confirm significance. Tubulin was used as a loading and transfer control.

2.9 Neutral red uptake assay

Neutral red uptake assay was used either to determine the effect of ODQ on the growth of different EOC cell lines or to assess the effect of

GUCY1B3 knockdown on the growth of EOC cells *in vitro* as previously described (Gupta *et al.*, 2013). Briefly, OVCA429, OVCAR3 and SKOV3 cells were seeded in 96 well plates at the densities of 5000, 7500, 5000 cells/well respectively. After 24h when cells settled down, they were either treated with increasing doses of ODQ or an equal volume of DMSO for 72h. Then, treatment containing media were replaced with fresh media containing 33 µg/ml neutral red dye. After 3 h of incubation, cells were washed once with PBS before being lysed in 100 µl lysis buffer (50% ethanol and 1% acetic acid) to develop the color that represents the cell number. Absorbance was read at 540 nm using an Omega microplate reader. For developing growth curves of OVCA429 cells with or without GUCY1B3 knockdown, cells were seeded at a density of 500 cells/well. Cultured cells were incubated for one, three or five days. After each time point, neutral red uptake assay was performed on one plate as described earlier to assess cell number. Cell numbers were then represented as relative percentages compared day 1 reading that represents 100% growth. Experiments were repeated at least three times to confirm statistical significance.

2.10 Soft agar colony formation assay

To assess the ability of EOC cells to form visible colonies with or without GUCY1B3 knockdown we used the soft agar colony formation

assay as described earlier (Liu *et al.*, 2013). After cells were trypsinized to form a single cell suspension, we seeded 1 X 10^4 cells in 60 mm diameter plastic culture dishes in triplicate. Plates contained two layers of RPMI 1640-agar medium. The base layer consisted of 0.5% agarose while the top consisted of 0.3% agarose. Cells were included in the top layer. Plates were supplied with approximately 500 µl of fresh medium every 3 to 4 days and incubated at 37°C in 5% CO_2 for 23 days. Visible colonies (approximately 50 to 100 cells) were counted after the incubation using a binocular microscope. Data shown are generated from one experiment; however, the assay was repeated more than three times in our lab and the results were confirmed to be consistent and statistically significant.

2.11 Statistical analysis

Data are shown as means ± SE of three to five independent experiments. Statistical significances between groups were determined by the paired student's *t* test and significance was defined as $p<0.05$. ANOVA analysis was performed for ODQ response experiments to confirm significance.

Chapter 3

Results

3.1 GUCY1B3 is overexpressed in EOC cell lines

NO/sGC pathway is involved in the survival (Leung *et al.*, 2008), protection against apoptosis (Fraser *et al.,* 2006) and promoting DNA synthesis/proliferation of ovarian carcinoma cells (Leung *et al.*, 2010). sGC, the main receptor of NO in the cGMP-dependent NO signaling, comprises α (GUCY1A1) and β (GUCY1B3) subunits (Brian *et al.*, 2009; Nakane *et al.*, 1994; Koesling *et al.*, 1990). In order to elucidate how NO/sGC pathway is regulated in ovarian cancer, we examined the expression of the subunits of sGC in three different established EOC cell lines (OVCAR3, SKOV3 and OVCA429) in comparison to non-cancerous controls of two IOSE cell lines. Interestingly, we found that the expression of GUCY1B3 subunit is elevated in all examined EOC cell lines compared to the IOSE controls (Figure 3). On the other hand, the expression level of GUCY1A1 was comparable among cancerous and non-cancerous cell lines (Figure 3). Notably, GUCY1B3, which contains the heme group that represents the active site of the enzyme, was reported to be the more important subunit for sGC enzymatic activity (Koglin and Behrends, 2003). In addition, we tested the expression of Notch1 and Notch3. The expression of Notch3 was exclusively upregulated in OVCAR3 cells, which showed the highest level of GUCY1B3 expression as well. The expression of Notch1 was comparable among cancerous and non-cancerous cell lines (Figure 3).

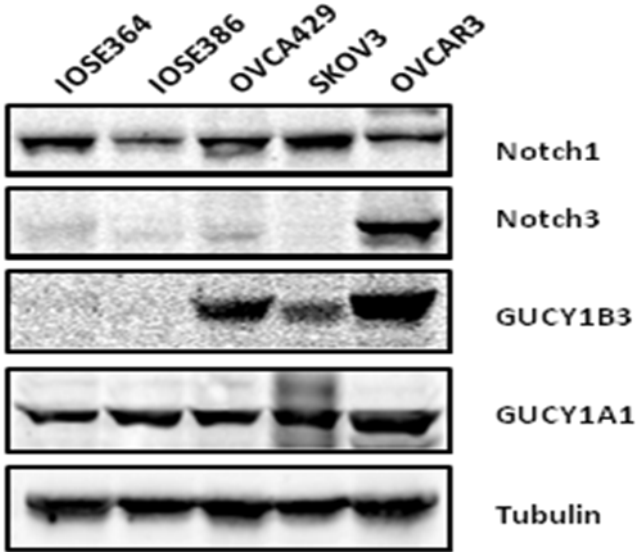


Figure 3. The expression of GUCY1B3 is elevated in EOC cells compared to IOSE controls. The expression levels of GUCY1B3, GUCY1A1, NOTCH1 and NOTCH3 were compared among three established EOC cell lines (OVCAR3, SKOV3 and OVCA429) and two non-cancerous IOSE cell lines as controls. Tubulin was used as a control to confirm equal protein loading and transfer.

3.2 GUCY1B3 is a downstream target of Notch signaling in IOSE and EOC cells

3.2.1 Notch overexpression elevates the expression of GUCY1B3 in IOSE cells

A published study reported that Notch elevates the expression of sGC and activates NO/sGC signaling in endothelial cells (Chang *et al.*, 2011). Here, we examined whether Notch is a regulator of GUCY1B3 expression and NO/sGC activity in IOSE and EOC cells as well. First, we examined the basal levels of Notch1 and Notch3 in three EOC and two IOSE cell lines (Figure 3). There was no significant difference in the expression levels of Notch1 among cancerous and non-cancerous cell lines. However, there was an impressive elevation of Notch3 expression in OVCAR3 cell line (Figure 3).

To determine the effect of Notch signaling on GUCY1B3 expression, we stably transduced IOSE364 cell line with retroviral vectors expressing Notch1 intracellular domain (NICD1) or Notch3 intracellular domain (NICD3) and the respective empty vectors. We selected IOSE cell line for this experiement because it has no basal expression of Notch3. In addition, OSE is believed to be one of the potential origins of ovarian cancer (Auersperg *et al.*, 2001). We specifically focused on Notch1 and Notch3 because they are the two members of Notch family of receptors that are important for ovarian cancer prognosis and known to be involved

in ovarian cancer biology and progression (Hopfer *et al.*, 2005; Choi *et al.* 2008). Activation of Notch1 or Notch3 in IOSE cells resulted in significant elevation of the expression of GUCY1B3 (Figure 4, Figure 5). Interestingly, we report that activation of Notch signaling also increased the expression of another important enzyme in the cGMP-dependent NO signaling; cGMP-dependent protein kinase 1 (PKG-1). Notch activation failed to increase the expression of GUCY1A1 (Figure 4, Figure 5). Collectively, these results show that Notch signaling elevates the expression of at least two key proteins in the NO/sGC pathway, GUCY1B3 and PKG-1.

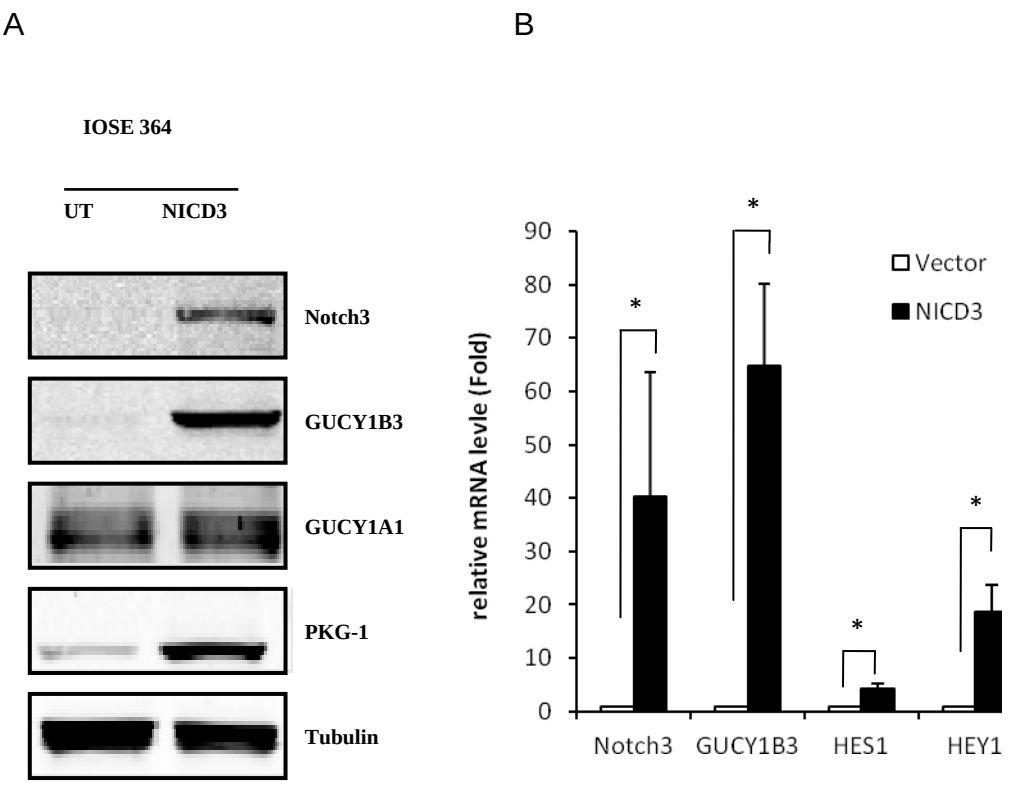


Figure 4. Notch3 activation increases the expression of GUCY1B3 in IOSE cells. IOSE 364 cells were infected with an empty retroviral vector of PCEG or vector of pCEG-NICD3 and were FACS sorted for GFP-positive cells. **(A)** Expression of Notch3, GUCY1B3, GUCY1A1, and PKG-1 in cell lysates was examined by Western blotting. Tubulin was used as a loading control. **(B)** mRNA levels of Notch3, GUCY1B3, HES1 and HEY1 were assessed using real-time PCR and normalized to GAPDH level as a housekeeping control. mRNA levels were represented as the fold change relative to corresponding controls and shown as means of three independent experiments. *Statistically significant ($p<0.05$).

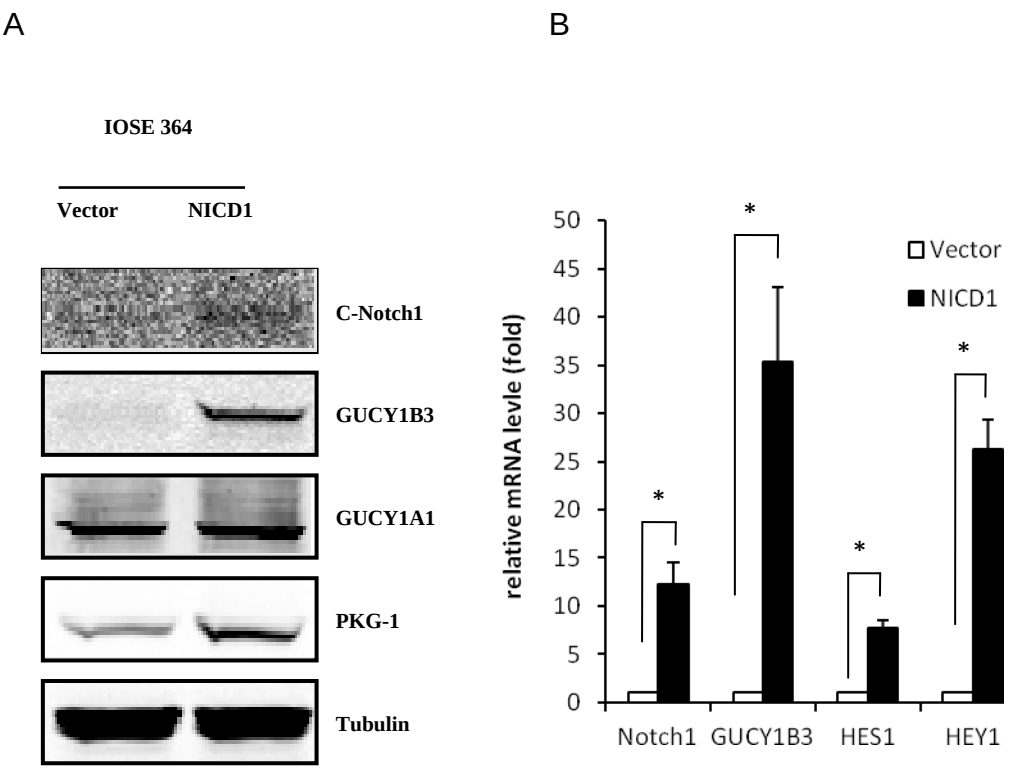


Figure 5. Notch1 activation increases the expression of GUCY1B3 in IOSE cells. IOSE 364 cells were infected with an empty retroviral vector of MSCVneo or vector of MSCVneo-NICD1 and were selected using G418. **(A)** Expression of cleaved Notch1, GUCY1B3, GUCY1A1, and PKG-1 in cell lysates was examined by Western blotting. Tubulin was used as a loading control. **(B)** mRNA levels of Notch1, GUCY1B3, HES1 and HEY1 were assessed using real-time PCR and normalized to GAPDH level as a housekeeping control. mRNA levels were represented as the fold change relative to corresponding controls and shown as means of three independent experiments. *Statistically significant ($p<0.05$).

3.2.2 Notch inhibition by DAPT decreases the expression of GUCY1B3 in OVCAR3 cells

As previously shown in Figure 3, OVCAR3 cells exhibit significantly higher expression levels of Notch3 and GUCY1B3 than all other tested cell lines. To determine whether the overexpression of Notch3 in these cells is related to the higher expression level of GUCY1B3 protein, we used γ-secretase inhibitor IX (DAPT), a known inhibitor of Notch activation (Sastre et al.,2001; Geling et al.,2002), and observed the resulting effect on GUCY1B3 expression. Western blotting and RT-PCR results showed that inhibiting Notch by DAPT in OVCAR3 cells significantly decreased the expression of GUCY1B3 (Figure 6), confirming our hypothesis regarding the role of Notch signaling in regulating the expression of GUCY1B3. Moreover, we found that inhibiting Notch signaling was able to slightly decrease the expression level of PKG-1 (Figure 6), confirming that PKG-1 is also a downstream target of Notch signaling. Taken together, we show that Notch signaling upregulates GUCY1B3 and PKG-1 expression levels in IOSE and EOC cells.

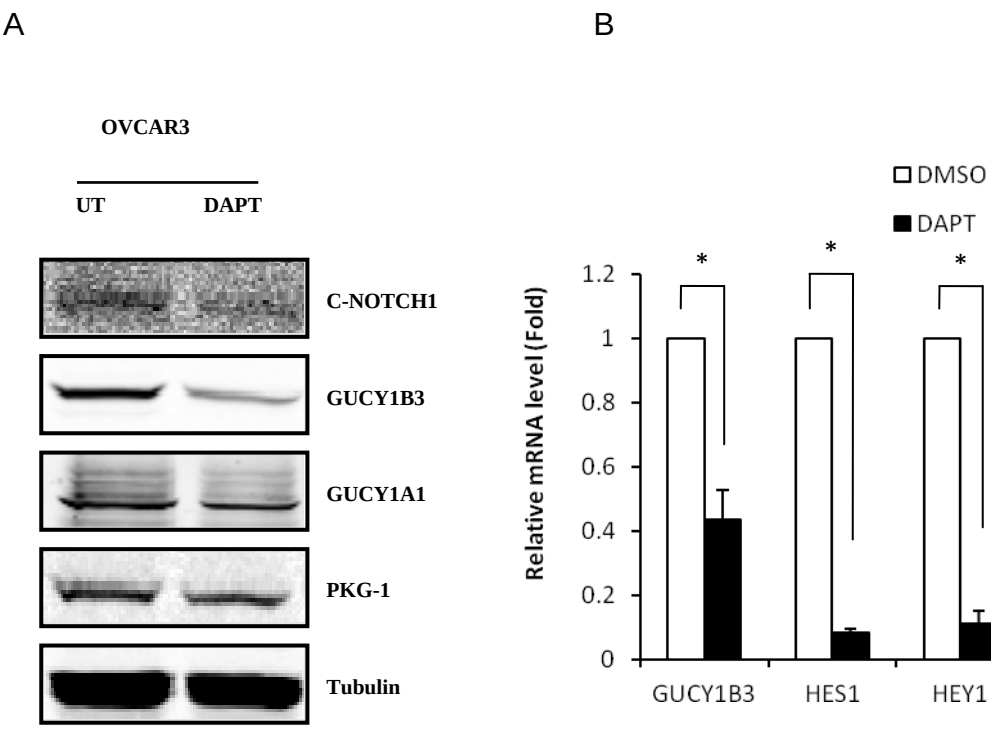


Figure 6. Blocking Notch signaling by DAPT inhibits GUCY1B3 expression in OVCAR3 cells. (A) OVCAR3 cells were treated with 10 µM DAPT or left untreated for 48h. Expression of GUCY1B3, GUCY1A1, and PKG-1 in cell lysates was examined by Western blotting. Inhibition of cleavage of Notch1 was used as readout to confirm the effectiveness of the treatment, and tubulin was used as a loading control. **(B)** mRNA levels of GUCY1B3, HES1 and HEY1 were assessed by real-time PCR and normalized to GAPDH levels. mRNA levels were represented as the fold change relative to corresponding controls and shown as means of three independent experiments. *Statistically significant ($p<0.05$).

3.3 Notch signaling regulates NO/sGC signaling activity in IOSE and EOC cells

3.3.1 Notch activation enhances NO/sGC signaling in IOSE cells

As mentioned in the introduction section, NO activates sGC to convert GTP to cGMP which in turn activates several downstream kinases (e.g., PKGs) to phosphorylate vasodilator-stimulated phosphoprotein (VASP). As shown in Figures 4 and 5, Notch activation increases the expression of GUCY1B3, the main enzymatically important subunit of sGC. Thus, we wanted to test whether Notch-induced upregulation of GUCY1B3 augments NO/sGC signaling activity in IOSE cells. Hence, we treated control cells, NICD1 overexpression cells or NICD3 overexpression cells with 50 µM GSNO (a NO donor) or left them untreated for 2h. After treatment, cell lysates were collected and used to determine the cGMP production and phosphorylation of VASP levels. We used phosphorylation of VASP as readout to determine the level of NO/sGC activity at the protein level. Interestingly, only Notch overexpressing cells treated with GSNO showed detectable levels of cGMP production (Figures 7 and 8). Consistent with this observation, clear phosphorylation of VASP was only detected in Notch overexpressing cells treated with GSNO by Western blotting (Figures 7 and 8). Collectively, these results indicate the crucial role of Notch in regulating and enhancing NO/sGC signaling in IOSE cells.

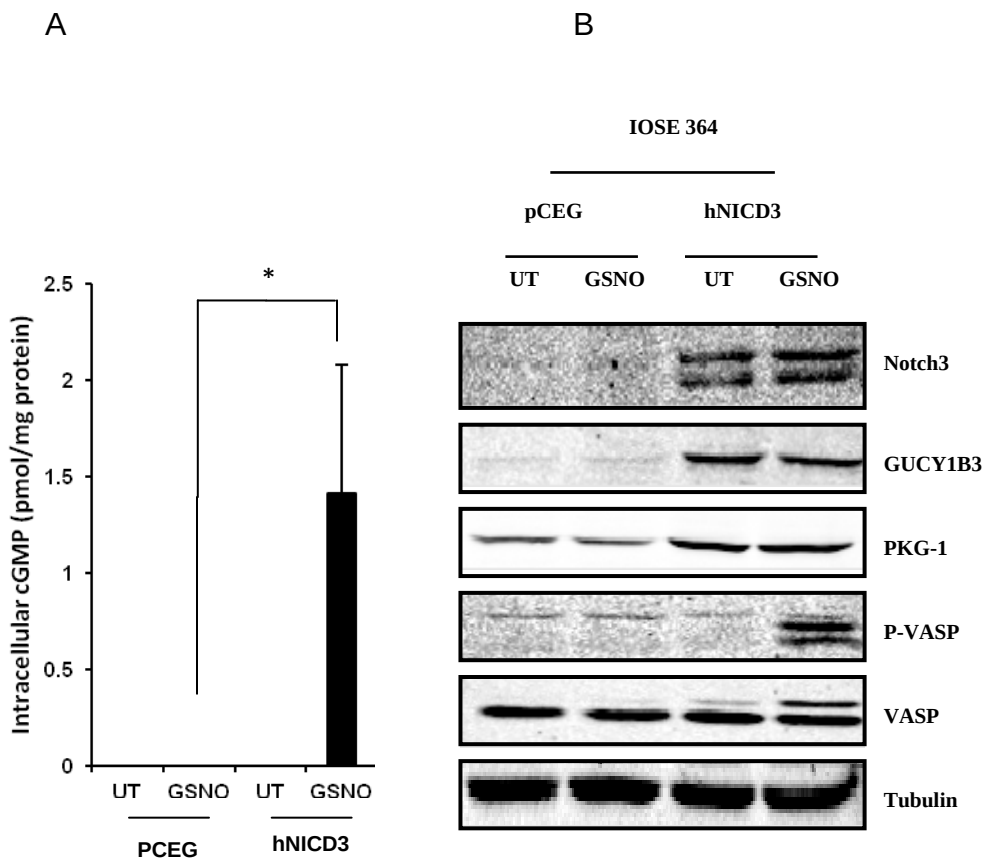


Figure 7. Notch3 overexpression enhances GSNO-induced NO/sGC signaling in IOSE cells. IOSE364 cells were infected with an empty retroviral vector pCEG or pCEG-NICD3 vector and were FACS sorted for GFP-positive cells. Then, cells were treated with 50 µM GSNO (a NO donor) or left untreated for 2h. **(A)** Intracellular cGMP levels were assessed using EIA kit. Data shown are means of three independent experiments. *Statistically significant ($p<0.05$). **(B)** After treatment, expression of Notch3, GUCY1B3, PKG-1, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

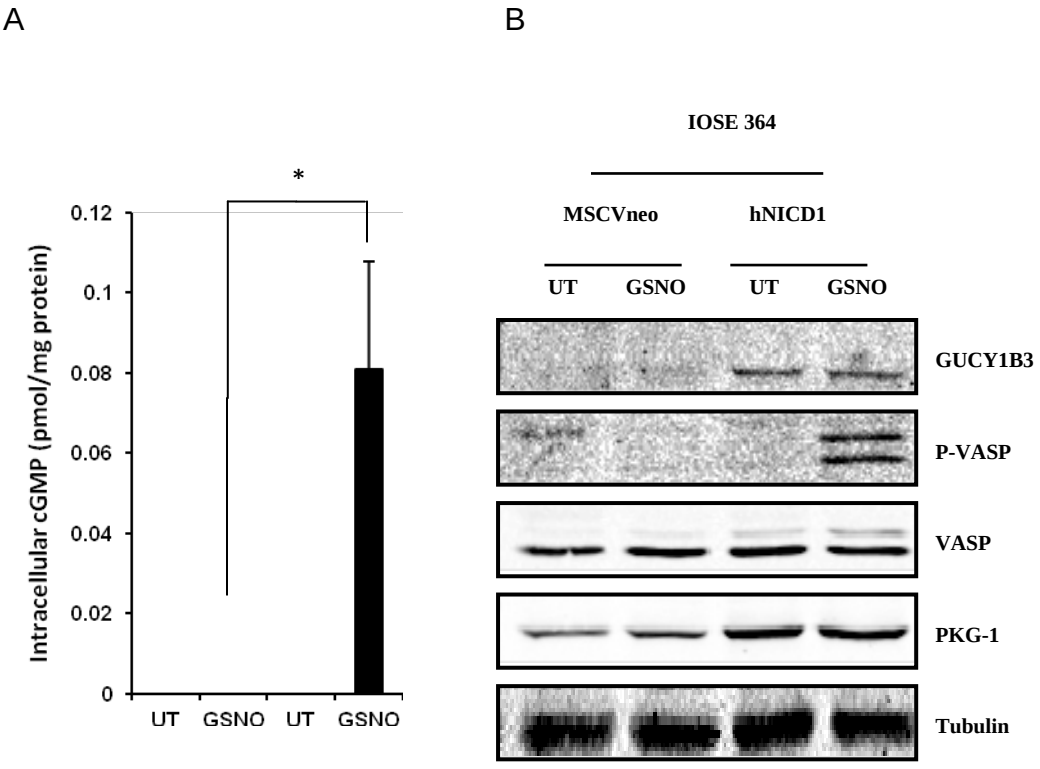
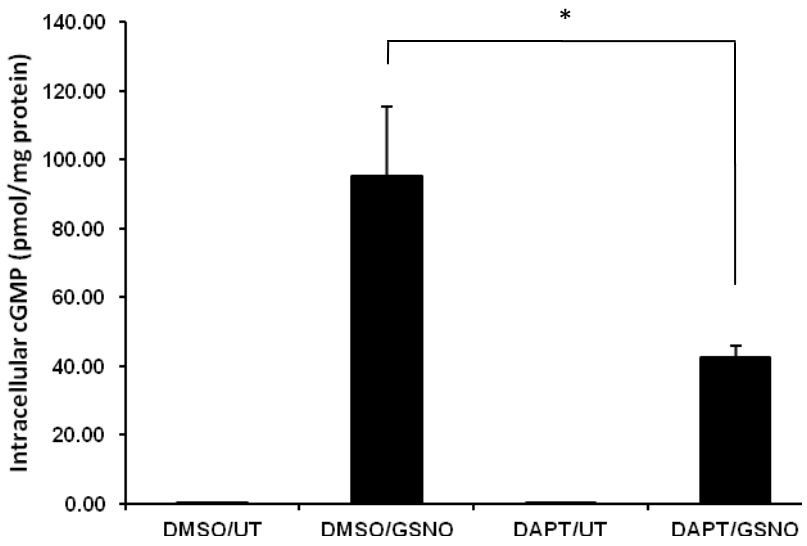


Figure 8. Notch1 overexpression enhances GSNO-induced NO/sGC signaling in IOSE cells. IOSE364 cells were infected with an empty retroviral MSCVneo vector or MSCVneo-NICD1 vector and were selected using G418. Then, cells were treated with 50 µM GSNO (a NO donor) or left untreated for 2h. **(A)** Intracellular cGMP levels were assessed using EIA kit. Data shown are means of three independent experiments. *Statistically significant ($p<0.05$). **(B)** After treatment, expression of GUCY1B3, PKG-1, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

3.3.2 Blocking Notch signaling by DAPT inhibits GSNO-induced NO/sGC activity in OVCAR3 cells

The OVCAR3 cell line exhibits the highest level of Notch3 and GUCY1B3 expression among all tested cell lines (Figure 3). Moreover, blocking Notch signaling using DAPT was able to significantly inhibit the expression of GUCY1B3 (Figure 6). Subsequently, we posited that Notch signaling is important in regulating the activity of NO/sGC signaling in OVCAR3 cells. To test this hypothesis, we first pre-treated OVCAR3 cells with 10 μM DAPT to block Notch signaling or left them untreated for 48h. Then, we treated cells with 50 μM GSNO under continuous DAPT treatment to test the level of GSNO-induced NO/sGC activity after blocking Notch. As expected, DAPT inhibition of Notch was able to diminish the level of GSNO-induced activation of NO/sGC represented by the level of cGMP production and VASP phosphorylation (Figure 9). This result confirms the proposed role of Notch signaling in regulating NO/sGC activity as shown in the previous part. Taken together, the results of these two experiments indicate that Notch signaling is an important activator of NO/sGC signaling in IOSE and EOC cells.

A



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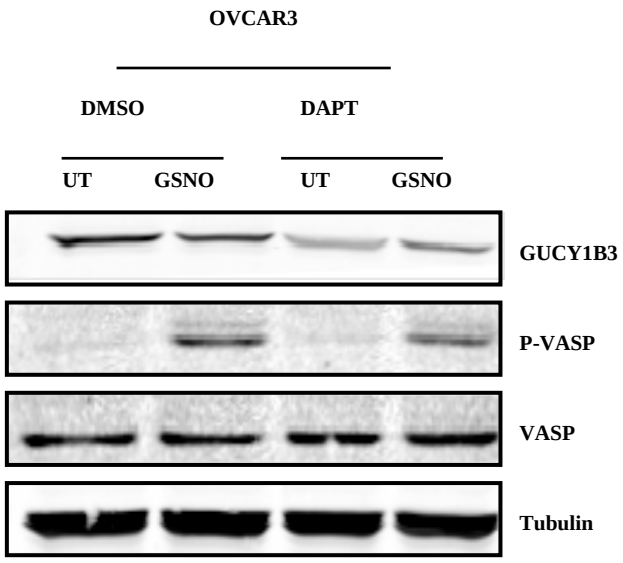


Figure 9. Blocking Notch by DAPT diminishes GSNO-induced NO/sGC signaling in OVCAR3 cells. OVCAR3 cells were pre-treated

with 10 μM DAPT or left untreated for 48h then co-treated with 50 μM GSNO under the continuous DAPT treatment. **(A)** Intracellular cGMP levels were assessed using EIA kit. Data shown are means of three independent experiments. *Statistically significant ($p<0.05$). **(B)** After treatment, expression of GUCY1B3, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting.. Tubulin was used as a loading control.

3.4 Notch regulates NO/sGC activity by increasing the expression of GUCY1B3

3.4.1Inhibiting sGC activity by ODQ blocks Notch-induced phosphorylation of VASP

After confirming that Notch signaling is a limiting factor in regulating NO/sGC activity in IOSE and EOC cells, we wanted to investigate the molecular basis underlying this interaction. As shown previously, Notch is able to significantly increase the expression of GUCY1B3. Thus, we posited that Notch augmentation of NO/sGC activity is mediated, at least in part, through enhancing sGC enzymatic activity. To test this hypothesis, we pre-treated IOSE vector, NICD1 overexpression or NICD3 overexpression cells with 15 μM 1H-[1,2, 4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a specific inhibitor of sGC (Garthwaite *et al.*, 1995) for 2h. Then, we treated the cells with 50 μM GSNO under continuous ODQ treatment to test whether blocking sGC activity will abolish Notch-induced phosphorylation of VASP. Indeed, ODQ treatment was able to almost completely compensate for the effect caused by Notch overexpression (Figures 10 and 11). These results show that the effect of Notch on NO/sGC signaling is primarily mediated through regulation of sGC activity in IOSE cells.

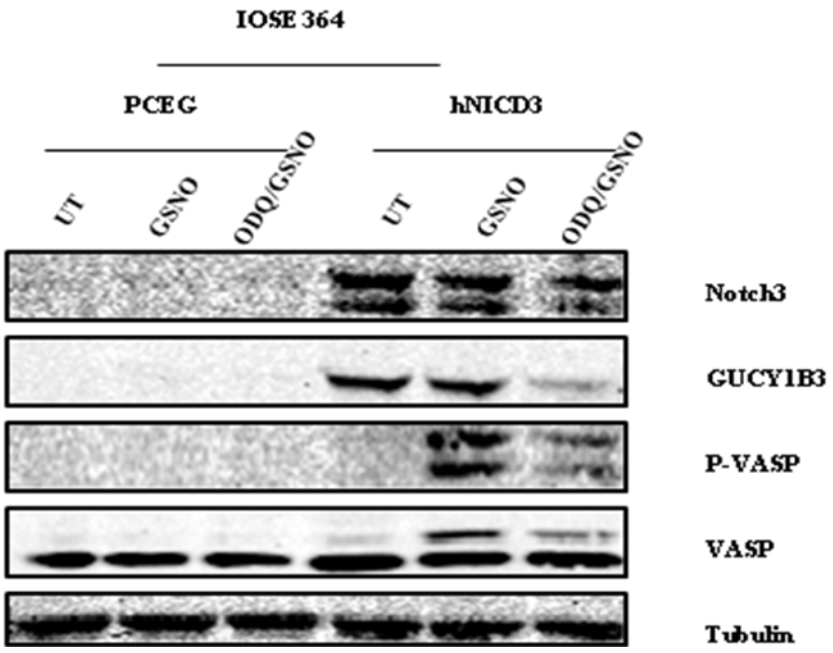


Figure 10. ODQ blocks Notch3-induced activation of NO/sGC signaling. IOSE 364 cells were infected with an empty pCEG retroviral vector or pCEG-NICD3 vector and were FACS sorted for GFP-positive cells. Cells were pre-treated with 15 µM ODQ or left untreated for 2h then treated with 50 µM GSNO under continuous ODQ treatment for additional 2h. Expression of Notch3, GUCY1B3, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

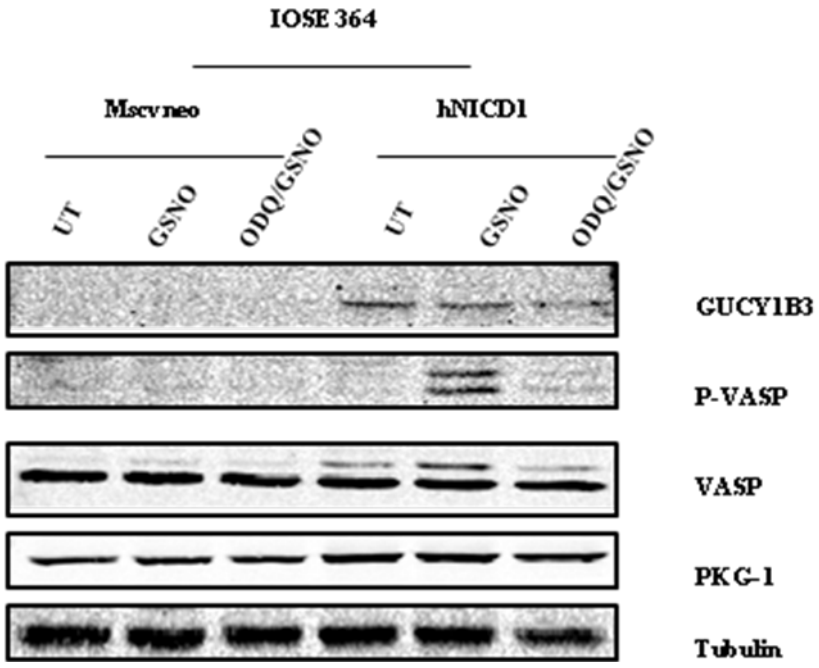


Figure 11. ODQ blocks Notch1-induced activation of NO/sGC signaling. IOSE 364 cells were infected with an empty MSCVneo retroviral vector or MSCVneo-NICD1 vector and were selected using G148. Cells were pre-treated with 15 μM ODQ or left untreated for 2h then treated with 50 μM GSNO under continuous ODQ treatment for additional 2h. Expression of GUCY1B3, PKG-1, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

3.4.2 Knocking down GUCY1B3 diminishes the effect of Notch on NO/sGC signaling

In the previous experiment we showed that Notch stimulation of NO/sGC signaling is mediated through regulation of the activity of the sGC enzyme. Here, we try to assess more specifically whether the effect of Notch on NO/sGC signaling is due to the elevated expression of GUCY1B3. To this end, we used two siRNA constructs (sequences are indicated in chapter 2) to knock down GUCY1B3 in NICD1 overexpressing or NICD3 overexpressing IOSE cells. A non-targeting pool of siRNA sequences was used as a control. Cells were transfected with the siRNAs and incubated for 48h before treating them with 50 μM GSNO to test the resulting activity of NO/sGC signaling. In NICD1 overexpression cells, knocking down GUCY1B3 almost completely blocked Notch-induced activation of NO/sGC (Figure 12). Similar results were obtained in NICD3 overexpression cells; however, the effect was not as dramatic (Figure 13). The weaker effect in NICD3 overexpression cells is probably due to the inefficient knockdown, as well as the higher expression levels of GUCY1B3 in NICD3 overexpression cells. Nevertheless, these results show that GUCY1B3 is the main mediator through which Notch regulates the activity of NO/sGC signaling.

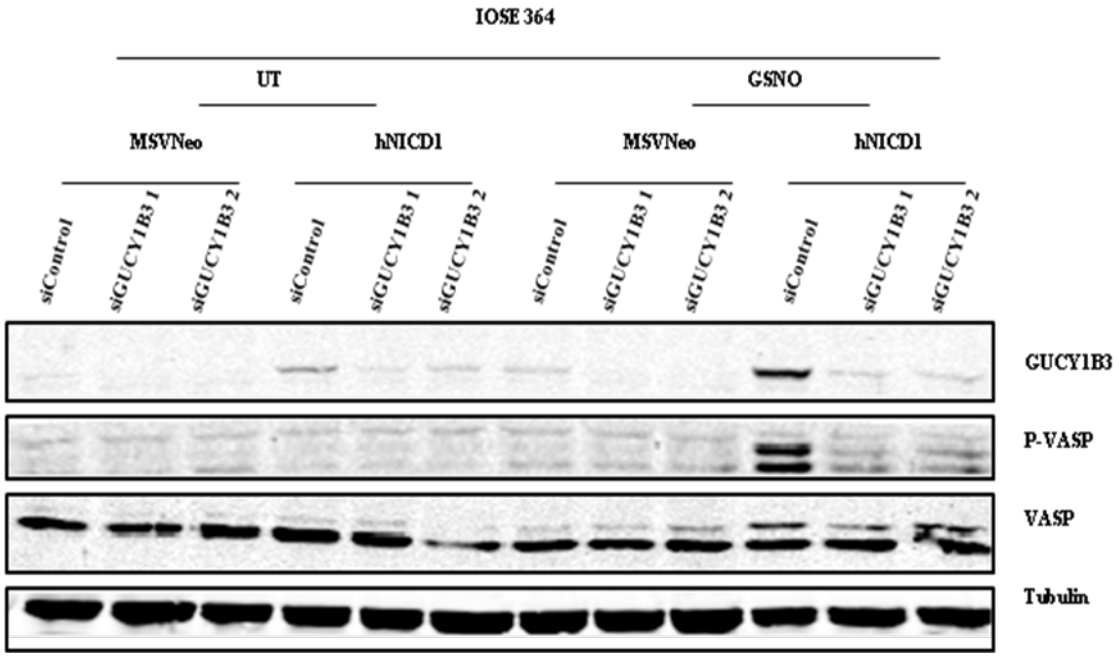


Figure 12. Knocking down GUCY1B3 abolishes the effect of Notch1 overexpression in IOSE cells. IOSE 364 cells were infected with an empty MSCVneo retroviral or MSCVneo-NICD1 vector and were selected using G148. Cells were transfected with 25 nM corresponding siRNA (siControl, siGUCY1B3 1or siGUCY1B3 2) then treated with 50 µM GSNO for 2h. Expression of GUCY1B3, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

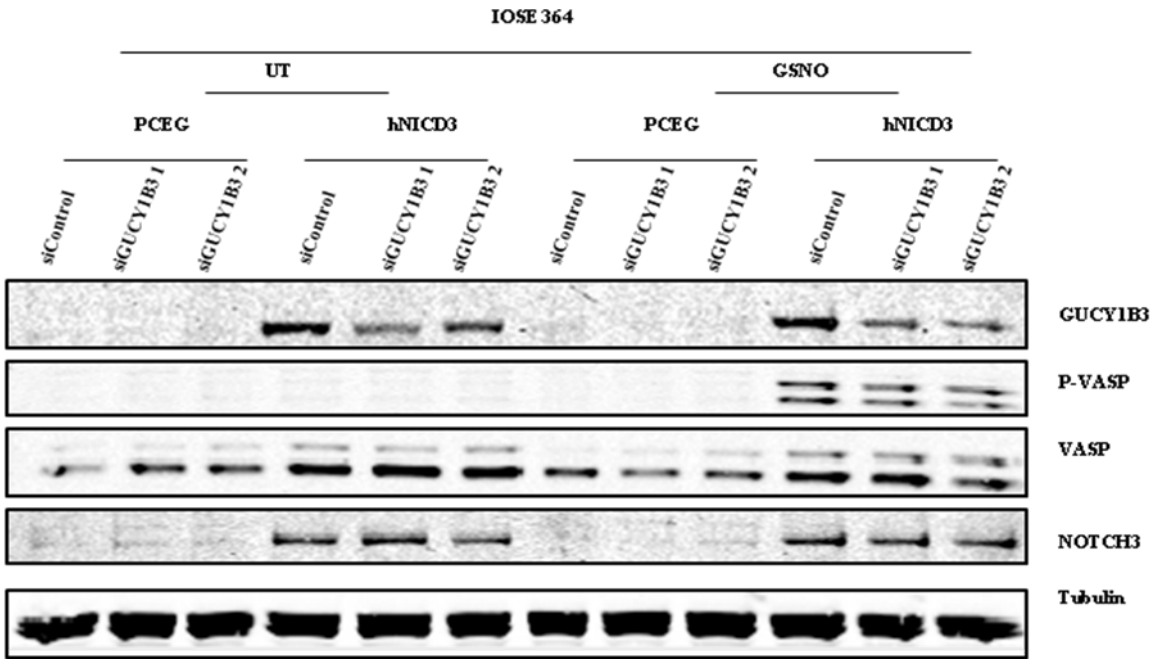


Figure 13. Knocking down GUCY1B3 weakens the effect of Notch3 overexpression in IOSE cells. IOSE 364 cells were infected with an empty pCEG retroviral vector or pCEG-NICD3 vector and were FACS sorted for GFP-positive cells. Cells were transfected with 25 nM corresponding siRNA (siControl, siGUCY1B3 1or siGUCY1B3 2) then treated with 50 µM GSNO for 2h. Expression of Notch3, GUCY1B3, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

3.4.3 GUCY1B3 overexpression in IOSE is sufficient for activation of NO/sGC signaling

After reporting that Notch augments NO/sGC signaling primarily through increased expression of GUCY1B3, we wanted to further confirm the proposed role of GUCY1B3 overexpression in this context. Therefore, we stably transduced IOSE 364 cells with MSCVpac-GUCY1B3 to overexpress GUCY1B3 or the empty MSCVpac vector as a control. Then, we treated these cells with 50 μM GSNO for 2h to test the effect on phosphorylation of VASP as readout of NO/sGC activity. Overexpression of GUCY1B3 alone was sufficient to activate NO/sGC signaling upon treatment with the NO donor GSNO (Figure 14). Notably, there was only one detectably induced band of phosphorylated VASP (Ser239) upon GUCY1B3 overexpression (Figure 14) in comparison to two clearly induced bands in the case of Notch overexpression (Figures 7 and 8). Moreover, the effect of GUCY1B3 overexpression on activating NO/sGC seems to be weaker than that of Notch overexpression. Also, the level of PKG-1 expression was not affected by GUCY1B3 overexpression. Collectively, these results show that GUCY1B3 is the determinant factor in regulating Notch effect on NO/sGC signaling, but they also give an indication that Notch regulates other molecules in the pathway such as PKG-1.

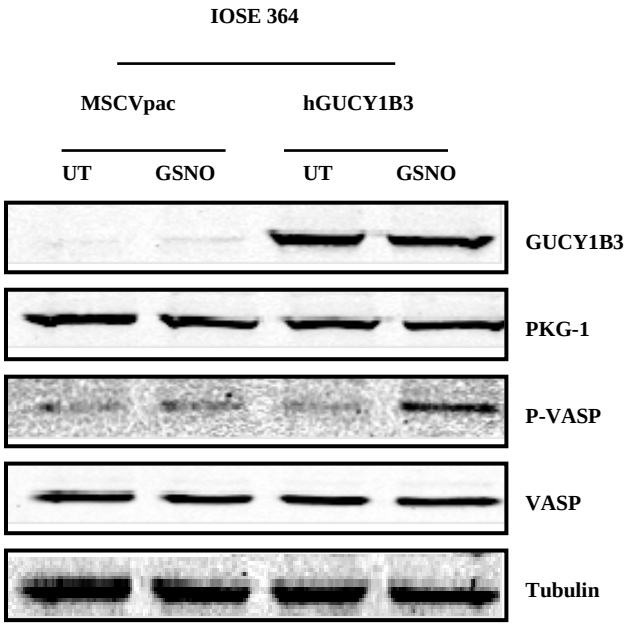


Figure 14. GUCY1B3 overexpression enhances GSNO-induced NO/sGC activity in IOSE cells. IOSE 364 cells were infected with empty MASCVpac retroviral vector or MSCVpac-GUCY1B3 vector and were selected using puromycin. Then, cells were treated with 50 µM GSNO or left untreated for 2h. Expression of GUCY1B3, PKG-1, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

3.5 GUCY1B3 is the primary but not the only regulator of Notch effect on NO/sGC signaling

Notch overexpression in IOSE cells increased the expression of GUCY1B3 as well as at least one more downstream enzyme in the pathway, PKG-1 (Figures 4 and 5). Moreover, overexpression of GUCY1B3 was unable to induce the expression of PKG-1, indicating that Notch upregulation of PKG-1 is independent of GUCY1B3 (Figure 14). This led us to posit that, in spite of the primary role of GUCY1B3 in Notch regulation of NO/sGC signaling as shown in the previous experiment, there may be roles for other downstream factors such as PKG-1 in this process. To test this hypothesis, we treated IOSE control cells, NICD1 overexpression cells or NICD3 overexpression cells with 8-Br-cGMP (a cGMP donor) for 2h. Interestingly, exogenous cGMP (which is a downstream of sGC in the pathway) enhanced the phophorylation level of VASP (Figures 15 and 16). This finding indicates that there are other factors in addition to GUCY1B3 that take part in mediating the effect of Notch on NO/sGC signaling, including PKG-1. In summary, we provide evidence that Notch augmentation of NO/sGC/cGMP signaling is mainly, but not solely through elevated levels of GUCY1B3 protein.

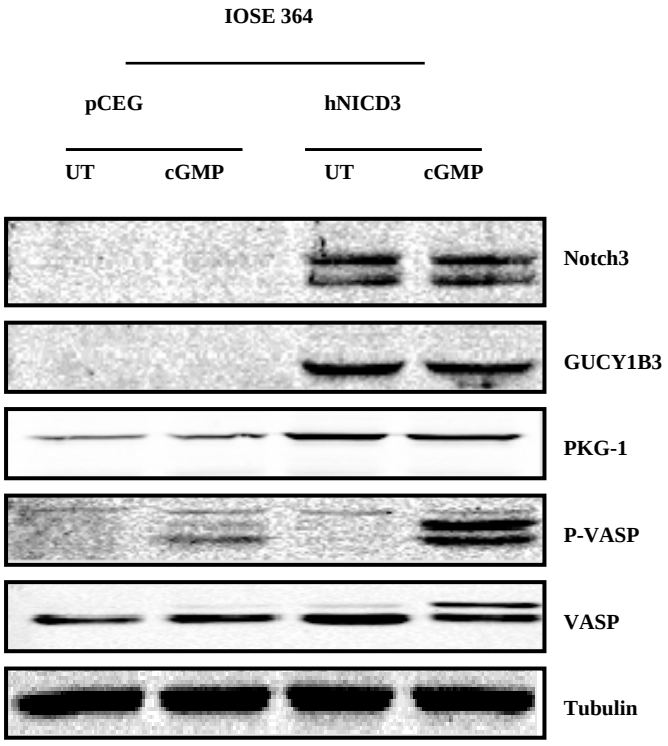


Figure 15. Exogenous cGMP enhances NO/sGC signaling in Notch3 overexpression IOSE cells. IOSE 364 cells were infected with an empty pCEG retroviral vector or pCEG-NICD3 vector and were FACS sorted for GFP-positive cells. Then, cells were treated with 10 µM 8-Br-cGMP or left untreated for 2h. Expression of Notch3, GUCY1B3, PKG-1, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

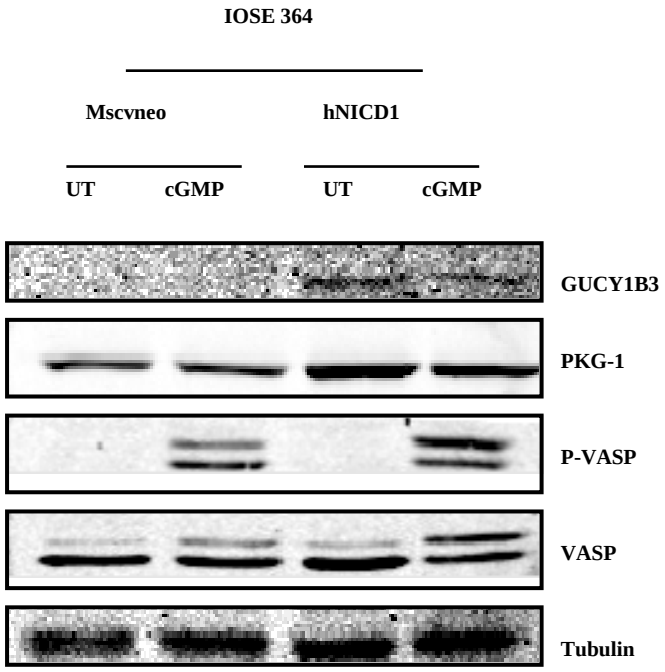


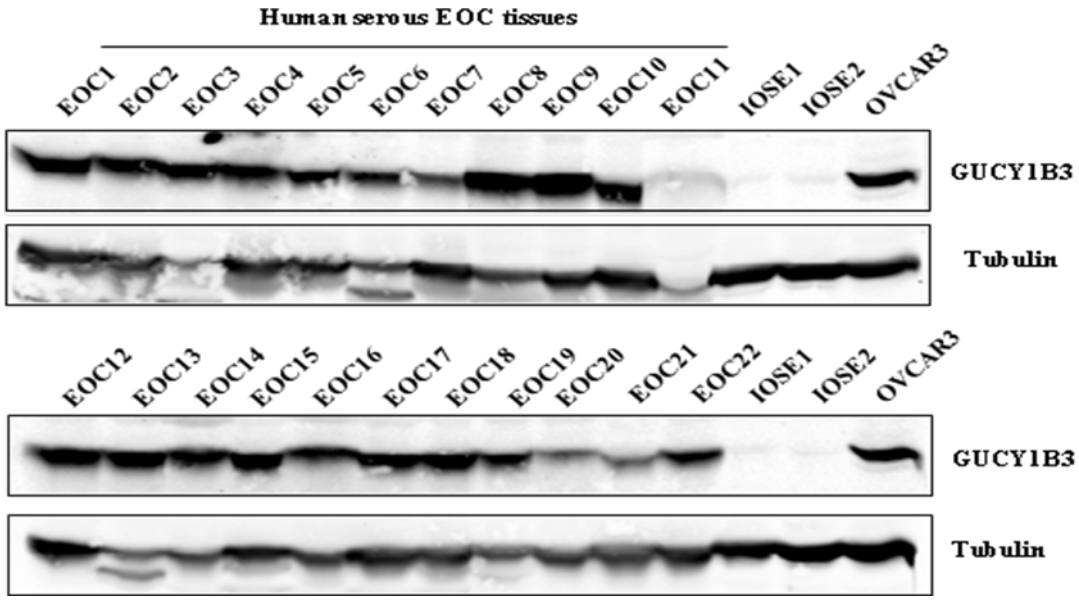
Figure 16. Exogenous cGMP enhances NO/sGC signaling in Notch1 overexpression IOSE cells. IOSE364 cells were infected with an empty MSCVneo retroviral vector or MSCVneo-NICD1 vector and were selected using G418. Then, cells were treated with 10 µM 8-Br-cGMP or left untreated for 2h. Expression of GUCY1B3, PKG-1, the phosphorylated form of VASP, and total VASP in cell lysates was examined by Western blotting. Tubulin was used as a loading control.

3.6 NO/sGC signaling promotes the growth of EOC cells *in vitro*

3.6.1 ODQ inhibits the growth of EOC cell lines *in vitro*

After confirming that Notch augments NO/sGC signaling, we wanted to assess the functional role of this augmented activity in EOC. Interestingly, we observed that GUCY1B3 (the critical functional subunit of sGC) expression in primary EOC tissues and cells was impressively upregulated in comparison to IOSE non-cancerous controls (Figure 17). Published work of two groups reported that NO/sGC activity plays an important role in promoting the growth of ovarian cancer cells and protecting them against apoptosis (Fraser *et al.*, 2006; Leung *et al.*, 2010). To further confirm this conclusion, we cultured three different EOC cell lines (OVCA429, OVCAR3 and SKOV3) in the presence of ODQ treatment or a control of DMSO. Cells were cultured at proper densities to allow signaling cascades and cell-cell contact, and were treated with ODQ for 72h. Then, cell numbers were measured using the neutral red uptake assay. Confirming the reported role of NO/sGC activity in promoting EOC growth, all of the three cell lines showed significant inhibition of growth when treated with ODQ in comparison to the controls (Figure 18). Taken together, these results show the role of augmenting NO/sGC signaling in promoting ovarian cancer cell growth, and indicate that augmentation of NO/sGC is likely one of the mechanisms that mediate the tumorigenic role of Notch in EOC.

A



B

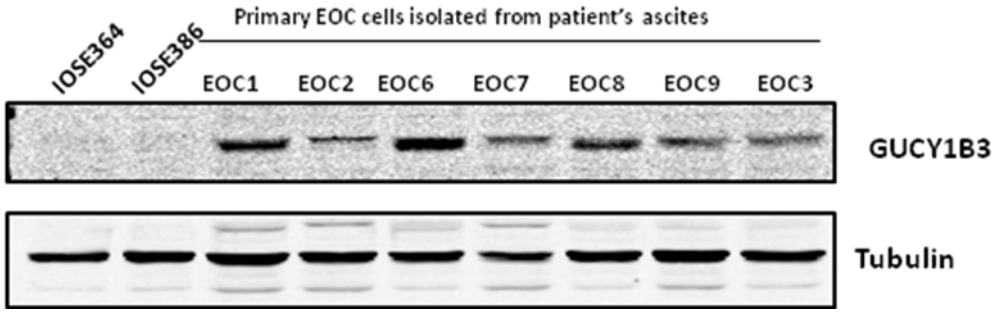
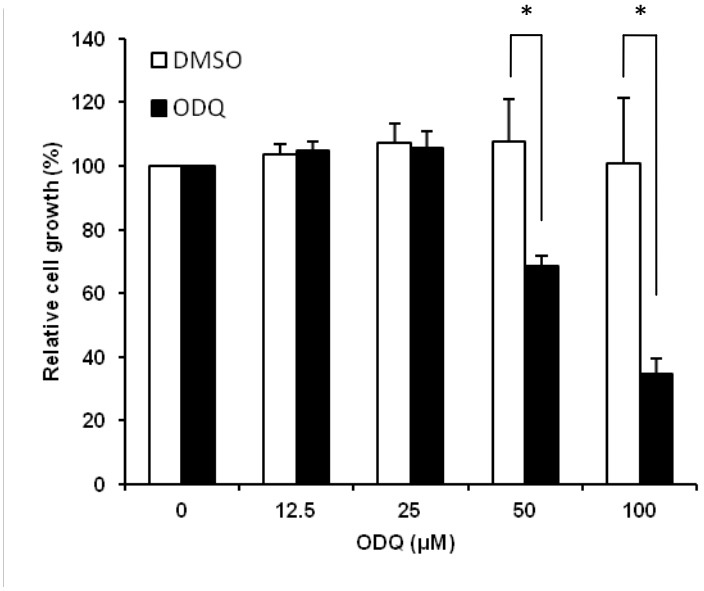


Figure 17. Expression of GUCY1B3 in EOC primary tissues and cells. (A) Twenty two human serous EOC tissues isolated from different patients were cultured and cell lysates were collected to examine the expression of GUCY1B3 in comparison to the non-cancerous IOSE controls. OVCAR3 cells were used as a positive control for GUCY1B3

expression. **(B)** EOC cells from the ascites of 7 different patients were isolated and cultured in optimum medium. Expression of GUCY1B3 in cell lysates was examined by Western blotting and compared to expression levels in IOSE controls. Tubulin was used as a loading control.

A


DMSO
ODQ
Relative cell growth (%)
140
120
100
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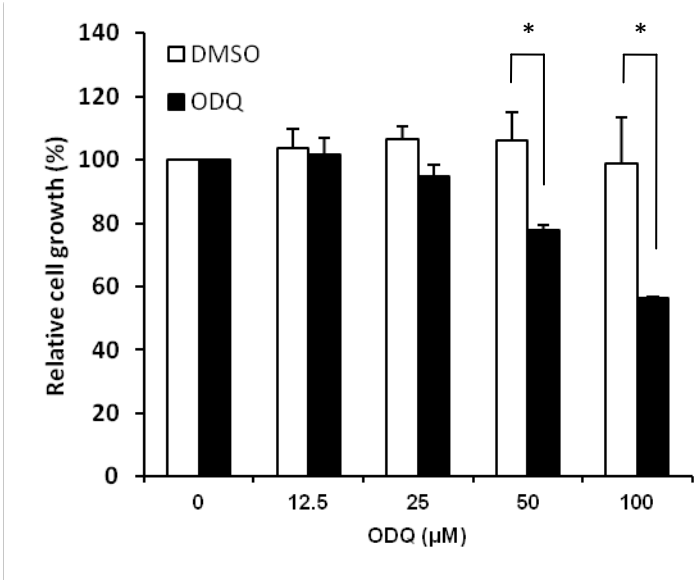
DMSO
ODQ
Relative cell growth (%)
140
120
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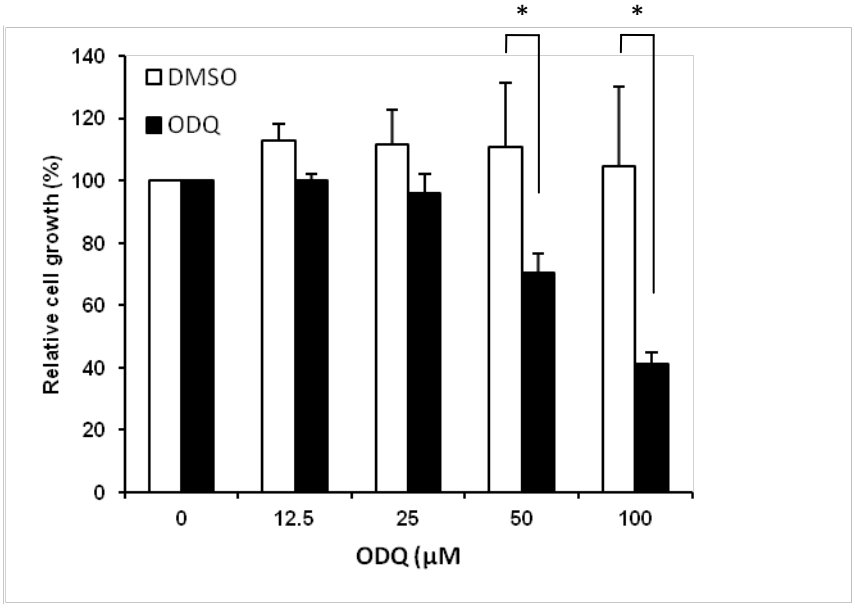

Figure 18. Blockade of sGC activity by ODQ significantly inhibits the growth of EOC cell lines *in vitro*. OVCA429 **(A)**, SKOV3 **(B)** and OVCAR3 **(C)** cells were cultured for 72h in increasing doses of ODQ or equal amounts of DMSO as control. After the incubation, cell number was assessed using neutral red uptake assay and expressed as relative percentages to the corresponding controls. Data shown are means of three independent experiments. *Statistically significant ($p<0.05$).

3.6.2 Knockdown of GUCY1B3 using shRNA inhibits the growth and colony formation ability of EOC cells *in vitro*

As previously stated, we found that the expression of GUCY1B3 is upregulated in all tested primary EOC cells, tissues and cell lines in comparison to IOSE controls (Figures 3 and 17). After confirming the contribution of NO/sGC signaling to the growth of EOC *in vitro*, we speculated whether knocking down GUCY1B3 can efficiently inhibit the growth of EOC. To this end, we infected OVCA429 cells with a lentiviral vector of shGUCY1B3 or shRandom as a control. Then, we cultured OVCA429/shGUCY1B3 and OVCA429/shRandom cells for different time points (1, 3 or 5 days) under optimum conditions for growth. After each time point, cell numbers were measured using the neutral red uptake assay. Numbers of GUCY1B3 knockdown cells were shown as percentages relative to their corresponding controls of shRandom cells. Interestingly, there was a significant inhibition of OVCA429 cell growth upon GUCY1B3 knockdown by almost 50% (Figure 19). Moreover, when we tested the colony forming ability of GUCY1B3 knockdown cells in comparison to shRandom cells using soft agar assay technique, we found a significant inhibition in the colony forming ability of the knockdown cells, supporting the proposed role for GUCY1B3 in EOC tumorigenesis (Figure 20). To confirm the observations shown in Figures 19 and 20, we generated two more shGUCY1B3 constructs. Unfortunately, we failed to reproduce these results using the two new constructs.

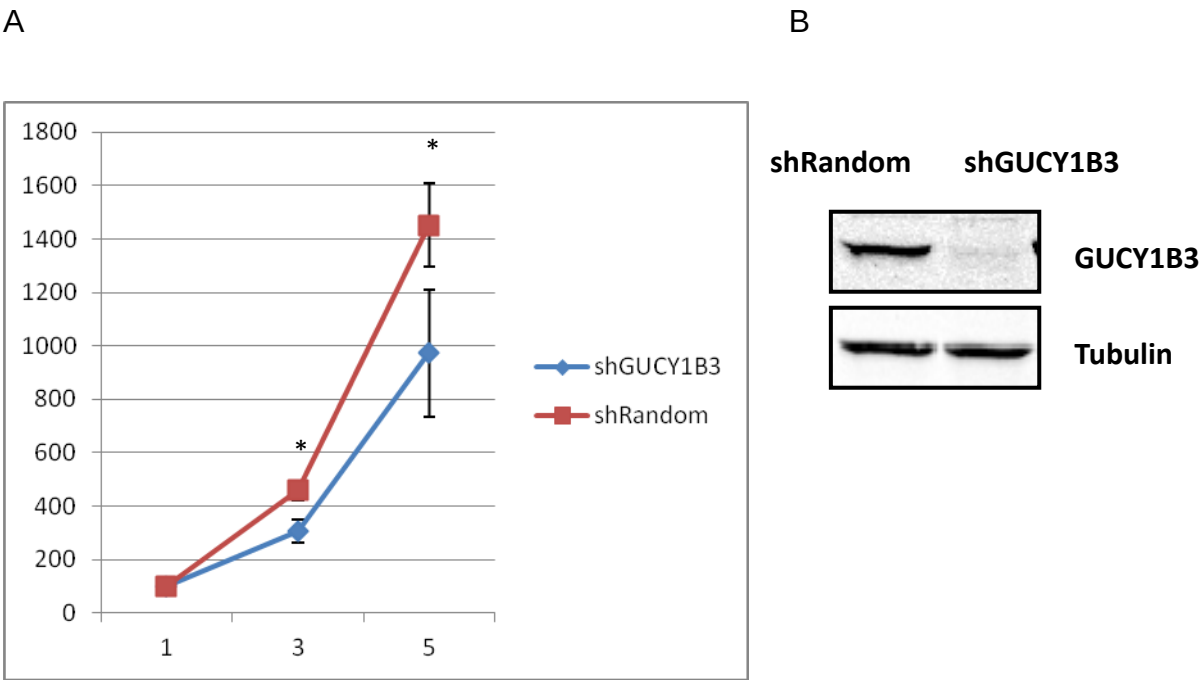

Figure 19. Knockdown of GUCY1B3 inhibits the growth of OVCA429 cells *in vitro*. OVCA429 cells were lentivirally infected with shRNA against GUCY1B3 (shGUCY1B3) or shRNA of a scrambled sequence as a control (shRandom). **(A)** Cells were cultured under optimum conditions for 1, 3 or 5 days to develop the growth curves. Cell numbers were measured using the neutral red uptake assay and expressed as relative percentages to the corresponding controls. Data shown are means of three independent experiments. *Statistically significant ($p<0.05$). **(B)** Cell lysates of shGUCY1B3 and shRandom cells were collected to test the expression of GUCY1B3 by Western blotting to confirm the knockdown efficiency. Tubulin was used as a loading control.

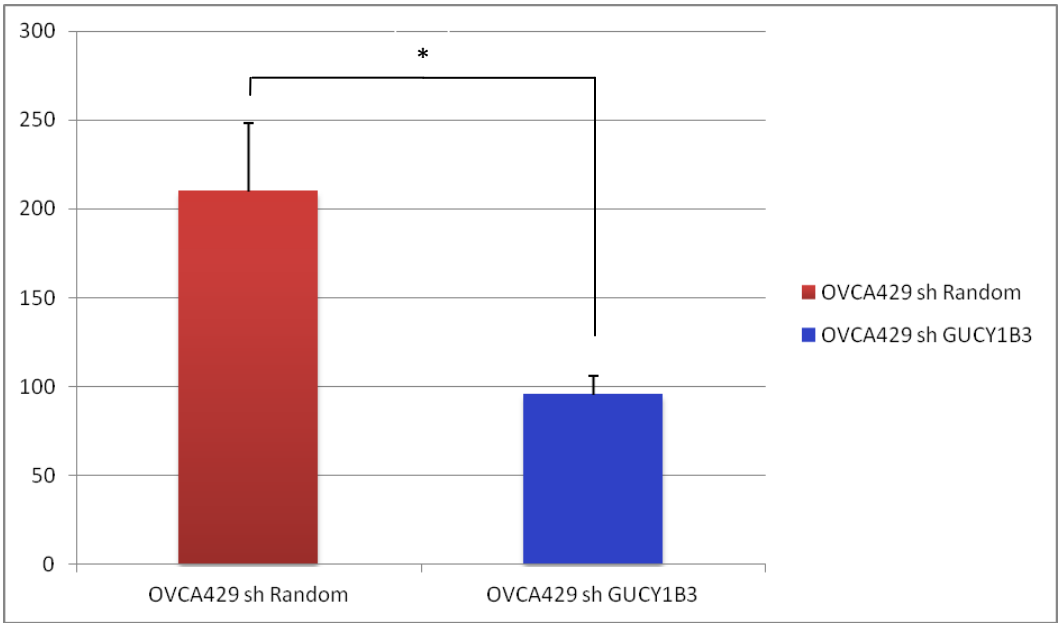

Figure 20. Knockdown of GUCY1B3 inhibits colony forming of ability of OVCA429 cells. OVCA429 cells were lentivirally infected with shRNA against GUCY1B3 (shGUCY1B3) or shRNA of a scrambled sequence as a control (shRandom). Then, cells were cultured using soft agar assay for 23 days to test their colony formation ability in the semi solid media. Colonies were quantified using a binocular microscope, by counting 50-100 cells-size colonies. Data shown are means of three independent experiments. *Statistically significant ($p<0.05$) (Nidhi Gupta, Fu lab).

Chapter 4

Discussion

4.1 Epithelial ovarian cancer and NO/sGC signaling: A driving pathway

Ovarian cancer is composed of several subtypes, amongst which epithelial ovarian cancer (EOC) remains the most predominant, the most severe (Bell *et al.*, 2005; du Bois *et al.*, 2003; Yap *et al.*, 2009) and the leading cause of mortality among gynecological cancers (Canadian Cancer Statistics 2013). There is a serious need to better understand the molecular basis of EOC in order to develop more efficient treatments and control the high mortality rates. In this study, we shed more light on the regulation of one important pathway in the biology and progression of ovarian cancers in general, the NO/sGC pathway (Fukumura *et al.*, 2006).

In ovarian cancer, NO/sGC is known to be implicated in many important biological processes including survival of ovarian carcinoma cells (Leung *et al.*, 2008), protection against spontaneous apoptosis (Fraser *et al.*, 2006) and promoting DNA synthesis/proliferation (Leung *et al.*, 2010). Moreover, it has been reported that NO plays an important role in the chemoresistance of ovarian carcinoma cells; however, the nature of this role is quite controversial and possibly dependent on the dose and conditions of NO treatment (Engels *et al.*, 2008; Leung *et al.*, 2008; Turchi, 2006). In fact, NO signaling inside tumor cells is quite complicated and can interact with many other molecules and counterparts constituting a large connected signaling network (Turchi *et al.*, 2006).

Given the importance of NO/sGC signaling in ovarian cancer along with its complicated network of interactions, there is a real need for more in-depth and specific molecular studies to better understand its regulation and effects on ovarian cancer progression. These specific studies may lead to better targeted therapies against chemoresistant ovarian cancer by manipulating NO/sGC signaling.

4.2 Notch regulates NO/sGC signaling in OSE and EOC cells at several levels

In this study, we uncover new details about the regulation of NO/sGC signaling activity in OSE and EOC cells. We have shown that there is a significant upregulation of GUCY1B3 (the main functional subunit of sGC) in all tested primary EOC tissues and cells in comparison to non-cancerous controls (Figure 17). Moreover, we show new insights into how this upregulation happens, and identify a potential functional role of this upregulation in EOC development and progression.

Notch has been reported to be essential for ovarian cancer proliferation, survival, tumorigenicity and chemoresistance (Choi et *al.*, 2008; Gupta *et al.*, 2013; Hu *et al.*, 2011; Ivan *et al*., 2013; Park *et al.*, 2010; Rose *et al.*, 2010; Steg *et al.*, 2011). In addition, targeting Notch has been proposed as an efficient therapeutic approach in ovarian cancer (Egloff and Grandis, 2012; McAuliffe *et al.*, 2012; Morgan *et al.*, 2013).

Indeed, Morgan and colleagues suggested that Notch targeting should be used as a first line therapy against ovarian cancer in combination with platinum agents (Morgan *et al.*, 2013). Also, the importance of Notch signaling components in prognosis of ovarian cancer has been pointed out (Mitsuhashi *et al.*, 2012).

Notch was reported to interact with NO signaling in endothelial and glioma cells (Chang *et al.*, 2011; Charles *et al.*, 2010); however, these interactions have quite different natures. Charles and colleagues reported that in PDGF-induced glioma cells, GSNO (a nitric oxide donor) activates Notch signaling through NO/sGC/cGMP/PKG, and this activation is both necessary and sufficient for NO-induced elevation of side population phenotypes (Chares *et al*., 2010). On the other hand, Chang and colleagues demonstrate that Notch induces sGC expression and NO production in endothelial cells (Chang et al., 2011). Moreover, this latter study concluded that NO/sGC signaling is required for early endothelial-mesenchymal transition (EMT) in the developing artiventicular canal (Chang *et al.*, 2011). Here, we test whether this type of interaction takes place in OSE and EOC. In addition, we provide insight into the molecular mechanism of this interaction.

Despite the important role of Notch signaling in ovarian cancer biology and progression, the molecular basis and functional role of this pathway in ovarian cancer is not very well characterized (Chen *et al.*, 2012). In this study, we show that Notch augments NO/sGC signaling in

non-cancerous OSE as well as EOC cells. We used the level of cGMP production and/or VASP phosphorylation (a known downstream target of NO/sGC pathway) as readouts of NO/sGC signaling activity. Our findings suggest that Notch regulates NO/sGC signaling at several levels, including GUCY1B3 and PKG-1 as direct targets of Notch signaling. In addition, we suggest that probably there are other molecules in the NO/sGC pathway that are regulated by Notch as well.

OSE is believed to be one of the potential origins of ovarian cancer (Auersperg *et al.*, 2001). Our results in IOSE cells reflect that one of the mechanisms by which Notch promotes the initiation and progression of ovarian cancer is through stimulating NO/sGC signaling and its subsequent biological effects. It would be interesting to address this issue in more specific subsequent studies. In this study, we show that NO/sGC signaling is almost totally inactive in parental IOSE cells (Figures 7 and 8). Upon activation of Notch signaling through overexpression of Notch1 or Notch3 intracellular domains we observe a significant and dramatic augmentation of NO/sGC signaling in these cells (Figures 7 and 8). This augmentation is mainly through upregulation of GUCY1B3 and PKG-1 (Figure 4 and 5). Moreover, we show that GUCY1B3 is upregulated in all tested EOC primary tissues, cells and established cell lines in comparison to IOSE cells that show almost no expression of this protein (Figures 3 and 17). Taking into consideration the suggested role of NO in the tumorigenesis of ovarian cancer at early stages (Nemade *et al.*, 2002;

Ozel *et al.*, 2006; Traves Murta *et al.*, 2004), it would be interesting to develop specific experiments to test whether the augmentation of NO/sGC signaling by Notch plays a role in initiation and/or progression of EOC.

Our results demonstrate that the effect of Notch on NO/sGC activity is mediated through upregulation of at least two different molecules in the pathway, GUCY1B3 and PKG-1. More in-depth and broad studies are needed to further assess the complete and detailed molecular interaction at all levels. In this study, we show results which uncover parts of this mechanism. The augmentation of NO/sGC by Notch activation was dramatically diminished when we blocked sGC activity using ODQ (Figure 10 and 11). This indicates that the stimulation of sGC enzymatic activity is the primary route in mediating the effect of Notch on the NO pathway. To further characterize the molecular mechanism we wanted to identify the specific subunit of sGC which is responsible for mediating this interaction. Our results show that Notch significantly upregulates GUCY1B3 (Figure 4 and 5) without affecting the expression level of GUCY1A1. Subsequently, we posited that the effect of Notch on NO/sGC is specifically and primarily through upregulation of GUCY1B3. To test this hypothesis we developed a set of knockdown and overexpression experiments. At first, we knocked down GUCY1B3 expression through siRNA approach. Knockdown of GUCY1B3 abolished the effect of NICD1 (Figure 12) and reduced the effect of NICD3 (Figure 13) on NO/sGC signaling. The weaker effect of GUCY1B3 knockdown on NICD3 overexpression cells is likely due to the

incomplete inhibition of GUCY1B3 expression by siRNA in comparison to the complete inhibition in case of Notch1 (Figure 13). Interestingly, overexpression of GUCY1B3 alone was adequate to mediate NO-induced downstream molecular events, showing that GUCY1B3/sGC is the major determinant of NO/sGC signaling in IOSE cells (Figure 14). Our results show that Notch not only upregulated GUCY1B3, but also PKG-1, another key enzyme in the NO/sGC pathway (Figure 4 and 5). After we reported that GUCY1B3 is the primary mediator of the effect of Notch on NO/sGC signaling, we questioned whether the upregulation of PKG-1 plays an additive role in augmenting the effect of Notch. To answer this question we supplied Notch overexpression cells with cGMP donor because cGMP is a downstream of sGC in the pathway. Interestingly, the exogenous cGMP was able to significantly enhance the phosphorylation of VASP (Figure 16 and 17) indicating a role of PKG-1 (the downstream target of cGMP) in this process.

Notably, we were able to detect the elevation of two bands of phosphorylated VASP by polyclonal anti-p-VASP (Ser239) antibody upon Notch activation and GSNO treatment. However, when we overexpressed GUCY1B3 solely, we detected the elevation of a single band (the lower one). On the other hand, the supplement of exogenous cGMP mainly elevates the expression of the other band (the upper one). Considering that there are currently three confirmed phosphorylation sites on VASP (Ser157, Ser239, Thr278) (Samolenski *et. al.*, 1998), these results give an

indication that probably GUCY1B3 and PKG-1 affect different phosphorylation sites on VASP. Taken together, these results show that Notch augments NO/sGC signaling primarily through upregulation of GUCY1B3 and that PKG-1 plays an additive role in the process.

After reporting the previous results in OSE cells (the potential origin of EOC), we wanted to expand our model to EOC cells. Hence, we used OVCAR3 cells because these cells express the highest level of the endogenous Notch3 and GUCY1B3 (Figure 3). We used DAPT to block Notch signaling and observed the effect on the expression level of GUCY1B3, PKG-1 and NO/sGC signaling activity. Parallel to our results in IOSE cells, Notch blockade by DAPT significantly inhibited the expression of GUCY1B3 and slightly inhibited the expression of PKG-1 in OVCAR3 cells (Figure 6). Moreover, the levels of cGMP production and phosphorylation of VASP, readouts of NO/sGC activity, were significantly inhibited by DAPT treatment (Figure 9). Collectively, these results indicate that, similar to IOSE cells, the interaction between Notch and NO/sGC pathways takes place in EOC cells as well.

4.3 NO/sGC signaling contributes to the growth of EOC *in vitro*

After reporting the interaction between Notch and NO/sGC, we wanted to address the functional implications of upregulating NO/sGC in EOC. First, we found that GUCY1B3 (the main functional subunit of sGC)

is significantly upregulated in all primary EOC cells and tissue we tested in comparison to IOSE controls (Figure 17), indicating a possible role for this upregulation in EOC initiation and/or progression.

In fact, some studies reported a role for NO/sGC activity in ovarian cancer cell proliferation and survival (Fraser et al., 2006; Leung *et al.*, 2010). To further confirm this reported role we used three different EOC cell lines which are commonly used as models for *in vitro* studies (OVCAR3, OVCA429 and SKOV3) to test the contribution of NO/sGC to the growth of EOC. We used ODQ to specifically inhibit sGC and block NO/sGC activity in these cell lines and compared the growth of these cells to that of the control cells treated with DMSO. Confirming the results of the published studies, ODQ treatment caused significant inhibition of growth of the three cell lines *in vitro* (Figure 18). These results show that NO/sGC activity is required for efficient growth of EOC cells and suggest that augmentation of NO/sGC signaling is a mechanism by which Notch activation promotes growth of EOC cells.

Based on our observation of the elevated GUCY1B3 expression in primary EOC samples, it is important to conduct specific studies to test whether GUCY1B3 expression can be a faithful and reliable prognostic marker in EOC. Clinical studies will be required to test if the expression level of GUCY1B3 and the activity of NO/sGC are correlated to other prognostic factors and/or stages in disease development.

Our results confirm the previously reported observation that NO/sGC generally contributes to ovarian cancer growth *in vitro*. However, more specific studies are needed to link this progression-promoting role to the Notch-stimulated NO/sGC signaling in particular. In other words, it is important to inhibit NO/sGC signaling in Notch overexpression cells to assess the contribution of NO/sGC to mediating Notch carcinogenesis.

From another prospective, the results of the current study suggest that blocking or inhibiting NO/sGC signaling can be a potential efficient therapeutic strategy in EOC. Subsequently, *in vivo* studies will be required to test the effect of ODQ on EOC progression in mice models. Current therapeutic regimes are ineffective against advanced EOC (Yap *et al.*, 2009). Thus, it is important to develop new effective and targeted therapies against advanced and recurrent stages of this disease. Our results provide a promising initiative for a pre-clinical study and suggest a new targeted therapy against EOC.

To propose a more specific and targeted treatment initiative, we considered the molecular targeting of GUCY1B3 in specific. To test this proposal, we targeted GUCY1B3 by short hairpin RNA (shRNA) to specifically knock down its expression in OVCA429 cells. Interestingly, our initial experiments showed that knockdown of GUCY1B3 was able to significantly inhibit the growth of OVCA429 cells to almost 50% (Figure 19) and inhibit their colony formation ability by almost 65% (Figure 20). To confirm this observation, we generated two more shRNA constructs

against GUCY1B3. However, we found that, unlike the first shGUCY1B3 construct, the two new constructs had no significant effect on growth or colony formation ability of OVCA429 cells. The efficiencies of all three shRNA constructs were confirmed by Western blotting which showed that all of them were able to completely knockdown the expression of GUCY1B3. Our results thus suggest that the phenotype generated by the first construct was likely an off-target effect. Interestingly however, we found that OVCA429 cells with a stable knockdown of GUCY1B3 using the two new shGUCY1B3 constructs formed smaller tumors compared with shRandom cells in xenografted mice (Fu lab, unpublished data). Therefore, these results suggest that knockdown of GUCY1B3 may not affect the behavior of EOC cells *in vitro*, but may affect the growth of EOC tumors *in vivo* due to the effect of the microenviroment. Nevertheless, more investigation will be required to obtain reliable results and come to a conclusion. In summary, blocking NO/sGC signaling may serve as a potential efficient therapeutic strategy against EOC; however pre-clinical experiments are needed before proposing this drug initiative for clinical trials. Although our first construct to target GUCY1B3 gave very promising results *in vitro*, we were unable to reproduce these results using the other constructs. On the other hand, the two new constructs were efficient in inhibiting EOC growth *in vivo*. More specific experiments are needed to determine the genuine effect of GUCY1B3 knockdown *in vitro* and to

elucidate the mechanism underlying the discrepancy between the results observed *in vitro and in vivo*.

4.4 Future directions

In the current study we report that Notch upregulates NO/sGC mainly by increasing GUCY1B3 and PKG-1 expression. Further experiments are required to determine the effect of Notch on other molecules in the different NO signaling pathways. Both Notch and NO/sGC are important signaling pathways in ovarian cancer cell survival and proliferation (Choi et *al.*, 2008; Mitsuhashi *et al.*, 2012; Leung *et al.*, 2010), so the interaction between them is of specific importance. Here, we provide insight into the molecular interaction between Notch and NO/sGC through several knockdown/overexpression experiments. We defined GUCY1B3 to be the primary mediator of the effect of Notch on NO/sGC signaling. We also defined PKG-1 to play an additive role in the process. There are probably other mediators to be shown in subsequent studies. To this end, mechanistic studies testing the effect of Notch on the entire signaling network of NO are in demand.

As shown in the results section, experiments specific for GUCY1B3 affect a VASP phosphorylation site different from the VASP phosphorylation site of PKG-1 (Figures 14 and 15). It would be interesting to further study this observation to identify the different phosphorylation

sites of VASP targeted by Notch through GUCY1B3 and PKG-1 and observe whether different phosphorylation sites result in different biological effects.

Studies on other types of cancers are required to test whether the interaction between Notch and NO/sGC is a global phenomenon in a broad spectrum of cancers or specific to a few types, which will give further indication of its functional and biological roles.

It is also important to develop functional and mechanistic set of studies to understand the role of GUCY1B3 which is impressively and impressively overexpressed in EOC primary cells, tissue and cell lines compared to the non-cancerous controls (Figure 17). Notably, the expression levels of the other subunit of sGC (GUCY1A1) are comparable among cancerous and non-cancerous cells. Specific studies are required to decipher the implications of this upregulation; whether the elevated expression of GUCY1B3 is involved in cell transformation, tumor initiation and/or tumor progression. Another important direction is to accurately define the role of NO/sGC signaling in mediating the tumorigenic effect of Notch.

We also suggest developing clinical studies to test if GUCY1B3 expression level has a prognostic value for EOC patients. It is important to test if there is a correlation between the level of GUCY1B3 expression and

the chance of developing EOC and/or the severity of the developed tumors (i.e. the stage and average survival rates).

It is important to assess the molecular and functional consequences of this reported interaction. In other words, to identify the downstream targets and biological effects of Notch mediated by NO/sGC signaling. Genetic approaches, proteomics platforms and high throughput screening techniques can be used to obtain a global view on all Notch targets that are mediated through NO/sGC signaling.

NO/sGC is reported to be involved in proliferation and survival of ovarian cancer cells (Fraser et al., 2006; Leung *et al.*, 2010). This conclusion was supported by our results using three different EOC cell lines (Figure 18). However, studies involving both Notch and NO/sGC are required to confidently conclude that Notch promotes ovarian cancer progression through activating NO/sGC.

Based on our *in vitro* experiments using ODQ, NO/sGC seems to be a potential efficient druggable pathway in EOC. However, *in vivo* experiments using mice models are needed to propose this therapeutic strategy for human trials and advanced drug development stages. The behavior of cancer cells is dramatically affected by the tumor microenvironment (Fang *et al.*, 2013; Yokota *et al.*, 2013), so it is essential to test the effect of blocking NO/sGC on the progression of EOC developed in models which are close to human. We plan to conduct

intraperitoneal (i.p.) inoculation of EOC cells into NOD SCID (non-obese diabetic severe combined immunodeficiency) mice, because these mice are easy to handle and monitor tumor progression. Also, i.p. injection more accurately models advanced EOC processes in human (e.g., dissemination and metastasis throughout the peritoneum) (Garson *et al.*, 2005; Shaw *et al.*, 2004). Moreover, it would be valuable to use the developed tumors in mechanistic studies to address how NO/sGC affects ovarian cancer growth and proliferation at the molecular level. These molecular and mechanistic studies will be important in the drug pre-development stage to propose an initiative for a targeted therapeutic strategy against advanced EOC.

In this study, we tried to develop a more specific therapeutic strategy against EOC by targeting GUCY1B3 *in vitro* expecting it will exert the same effect of blocking NO/sGC using ODQ. Despite generating promising results using the first shRNA construct to knockdown GUCY1B3 *in vitro*, the data were not reproducible using two other shRNA constructs against GUCY1B3. Further detailed studies are needed to address this issue. First, we need to define and confirm the most faithful construct for *in vitro* studies. To this end, other knockdown approaches including the use of siRNA constructs can be applied. In addition, overexpression approaches can be used as well. We conducted a pilot study to test the effect of these constructs *in vivo*. Despite generating conflicting results *in vitro*, the two new shRNA constructs against GUCY1B3 were able to

significantly inhibit the growth of EOC *in vivo* (Fu lab, unpublished data). On the basis of these promising results, we are following up this experiment to confirm the effect of GUCY1B3 knockdown *in vivo*. On the other hand, it is important to address why blocking NO/sGC using ODQ (a specific inhibitor of sGC) has inhibitory effect on ovarian cancer growth while specific targeting of the main functional subunit of sGC does not have the same effect *in vitro*. In all cases, in-depth mechanistic and molecular studies will be needed to characterize the effect of GUCY1B3 knockdown in relation to the effect of blocking NO/sGC activity using the chemical inhibitor ODQ.

4.5 Conclusions

The severity of EOC and ineffectiveness of current therapeutic regimes make it essential to introduce new and more effective targeted therapies. To do that, better understanding of the molecular mechanisms and pathways that control the initiation, development and progression of EOC are required. As described in the introduction section, the NO/sGC pathway is essential to the survival and proliferation of EOC cells; however, the regulation of this pathway is not very well characterized. Here, we report for the first time the interaction between the NO/sGC pathway and Notch in EOC. We show that Notch, which is an important pathway in cancer biology, upregulates NO/sGC signaling in EOC as well

as normal OSE cells. Moreover, we describe some of the molecular events that mediate this interaction. We also show that GUCY1B3, the primary mediator of the effect of Notch on NO/sGC, is significantly upregulated in all tested EOC samples in comparison to non-cancerous controls, suggesting that GUCY1B3 might play a role in tumor initiation and/or progression. Supporting the previously reported observation, we show that NO/sGC activity contributes to EOC growth *in vitro* suggesting that activating NO/sGC signaling might be one of the routes that mediate Notch tumorigenic effect on EOC. Subsequently, we propose using ODQ, the specific inhibitor of sGC, as a potential effective therapeutic agent against advanced EOC. Thus, we plan to test the effect of ODQ on EOC progression and growth *in vivo*.

Besides the novel description of the interaction between the two pathways of Notch and NO/sGC in EOC, this study provides an initiative for a pre-clinical study using ODQ as an effective therapeutic agent against advanced EOC.

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