THE ROLES OF GNA11 AND GNA14 IN MEDIATING FGF2- AND VEGFA-INDUCED ENDOTHELIAL FUNCTION UNDER PHYSIOLOGICAL CHRONIC NORMOXIA

by

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Overall Abstract

Background: During pregnancy, remarkable fetoplacental vascular growth and development occur to support the growing fetus. Interruption of such processes may cause adverse pregnancy outcomes such as intrauterine growth restriction and preeclampsia (PE). Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) are two key regulators of fetoplacental endothelial function. G protein α subunit (Gα) mediates many cellular signaling networks. G protein α subunit 11 (GNA11) and 14 (GNA14) are a members of Gαq/11 subfamily. GNA11 is actively involved in mediating vascular growth and basal blood pressure. GNA14 is identified as a human hypertension-susceptibility gene. However, expression pattern in human placentas and roles of GNA11 and GNA14 in mediating FGF2- and VEGFA-induced fetal endothelial function remains unclear. Here we hypothesize that GNA11 and/or 14 is expressed in human placentas and have significant roles in mediating FGF2- and/or VEGFA-induced human fetoplacental endothelial cell function under physiological chronic normoxia. **Methods:** Human umbilical cord vein endothelial cells (HUVECs) were isolated from healthy pregnant women after term, and were constantly cultured under 3% O_2 (37^0C, 5% CO2, ~92% N2, which is Physiological Chronic Normoxia) till passages 4-5 for experiments. Specific-targeted siRNAs and adenoviruses were used for decrease and increase protein levels of GNA11 and/or GNA14. Subsequently, cell migration, proliferation, monolayer integrity, and/or $[Ca^{++}]_i$ were performed. Western blotting was used to determine protein levels and phosphorylation. **Results:** Knockdown of GNA11 decreases FGF2- and VEGFA-induced cell migration, but not proliferation and monolayer integrity. Such decrease is associated with enlarged phosphorylation of PLCβ3 at S537.

GNA14 overexpression impairs FGF2-induced cell migration, but enhances FGF2-mediated monolayer integrity. However, GNA14 overexpression does not affect neither FGF2-mediated $[Ca^{++}]_i$ nor VEGFA-induced cell function. Such altered FGF2-mediated cell function is associated with elevated phosphorylation of PLCβ3 at S1105. **Conclusion:** these two members of Gαq/11 subfamily may play important roles in mediating peptide growth factors-induced fetoplacental endothelial function. GNA11 and GNA14 may serve as therapeutic targets for correcting endothelial dysfunction occurring in many endothelial cell related cardiovascular diseases.

Abbreviation

AKT1	v-akt murine thymoma viral oncogene homolog 1
CDC42	cell division control protein 42 homolog
ERK1/2	mitogen-activated protein kinase 3/1
FGF2	fibroblast growth factor-2
FGFR	FGF receptor
Flt	VEGFR1
FRS2α	FGFR substrate 2α
G protein	heterotrimeric GTP-binding protein
GDP	guanosine diphosphate
GNA	G protein subunit α
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
HSPG	heparin sulfate proteoglycan
HUAEC	human umbilical cord vein endothelial cell
HUVEC	human umbilical cord vein endothelial cell
IUGR	intrauterine growth restriction
KDR	VEGFR2
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
p.c.	post conception
PDGF	platelet-derived growth factor
PE	preeclampsia

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLC	phospholipase C
PlGF	placental growth factor
RAC1	ras-related C3 botulinum toxin substrate 1
RGS	regulators of G protein signaling
RhoA	ras homolog gene family A
SCN	standard culture normoxia
STAT3	signal transducer and activator of transcription 3
VEGFA	vascular endothelial growth factor-A
VEGFR	VEGF receptor

Chapter I. Literature Review

I.1. Significance

During pregnancy, fetal and placental vasculatures undergo dramatic growth, remodeling, and dilation to accommodate the remarkable increases in fetal and placental blood flows which are required for supporting the developing fetus (Barcroft & Barron, 1946; Magness & Zheng, 1996; Raio *et al.*, 2003; Benirschke *et al.*, 2006*a*; Reynolds *et al.*, 2010). Impaired endothelial function is associated with several pregnancy complications, such as preeclampsia (PE) and intrauterine growth restriction (IUGR) (Zygmunt *et al.*, 2003). These complications not only jeopardize maternal health during and after pregnancy (Ghulmiyyah & Sibai, 2012), but also often cause low fetal birth weight which may adversely affect future health of offspring (Barker, 1998; Neiger, 2017).

Fibroblast Growth Factor-2 (FGF2) and Vascular Endothelial Growth Factor-A (VEGFA) are two potent regulators of endothelial functions (e.g., proliferation, migration, and permeability) (Folkman & Shing, 1992; Klein *et al.*, 1997; Ferrara *et al.*, 2003; Hicklin & Ellis, 2004; Koch & Claesson-Welsh, 2012). Actions of VEGFA and FGF2 are mediated via a serial of protein kinases (Wang & Zheng, 2012) and also via heterotrimeric GTP-binding proteins (G proteins) (Heyworth *et al.*, 1986; Poppleton *et al.*, 1996; Conway *et al.*, 1999; Zeng *et al.*, 2003; Shan *et al.*, 2006; Pyne & Pyne, 2011). G protein subunit α-11 (GNA11) and 14 (GNA14) as transducers participate in various transmembrane and intracellular signaling pathways. The molecules in these signaling pathways include phospholipase C (PLC), mitogen-activated protein kinase 3/1 (ERK1/2), v-akt murine thymoma viral oncogene homolog 1 (AKT1), and Ca^{++}, all of which are critical to endothelial function (Hubbard & Hepler, 2006). A combined action of GNAq and GNA11 has been shown to be required for VEGFA-stimulated proliferation and migration of

human umbilical cord vein endothelial cells (HUVECs) via activation of a small GTPase Ras homolog gene family A (RhoA) (Zeng *et al.*, 2003). Mutation and/or abnormal expression of GNA14 is associated with human hypertension (Kohara *et al.*, 2008), pulmonary artery hypertension (Abdul-Salam *et al.*, 2010), and childhood vascular tumor (Lim *et al.*, 2016). Nothing, however, is known about actions of GNA11 and 14 in FGF2- and VEGFA-regulated endothelial function.

Nowadays, function of G protein α subunits is widely studied and well defined. However, roles of $G\alpha_{q/11}$ family members, especially GNA11 and 14, in mediating FGF2- and VEGFA-induced signaling pathways in human fetoplacental endothelial cells are rarely understood. In addition, throughout angiogenesis of human pregnancy, fetoplacental endothelial cells reside under low oxygen environments (~2-8% O_2 or pO_2 ~17-60 mmHg) (Granger *et al.*, 2001; Danilov & Fiskum, 2008; Halliwell & Gutteridge, 2015). This physiological chronic normoxia is critical to cellular homeostasis. Nonetheless, most of *in vitro* studies regarding endothelial function are performed under a hyperoxic condition (~21% O_2) as compared with physiological environments. Here, we hypothesize that GNA11 and/or 14 have significant roles in mediating FGF2- and/or VEGFA-induced human fetoplacental endothelial cell function under physiological chronic normoxia. Data acquired from these studies will shed light on understanding the roles of G protein α subunits in signaling pathways of peptide growth factors in regulating human fetoplacental endothelial function.

I.2. Placental Anatomy and Physiology

A normally developed mature placenta is composed by two main parts: fetal tissue derived from the chorionic sac and maternal tissue derived from the endometrium (Jauniaux *et al.*, 2000). In term pregnancies, the fetal placental tissue is referred to the chorionic plate which contains vast amount of fetal chorionic vessels with a radial branching pattern from umbilical cord vessels. The maternal placental tissue is named as the basal plate (Jauniaux *et al.*, 2000). As the space between these two parts, intervillous space functions as a maternal-fetal exchange site for oxygen, nutrients, hormones, and wastes, etc. At the maternal and fetal interface, oxygen-rich maternal blood enters intervillous space through endometrial spiral arteries. While after exchange, fetal blood enriched with oxygen and nutrient flow into the fetus via a single umbilical cord vein. Oxygen- and nutrients-deficient fetal blood returns to placental interface via two umbilical cord arteries. On the maternal side, oxygen- and nutrient-deficient maternal blood is drained into the maternal circulation through endometrial veins.

At villi, maternal and fetal bloods are separated by the placental membrane (also termed as placental barrier). During early stage of pregnancy, the placental membrane is composed by four layers of cells: syncytiotrophoblasts which have direct contact with maternal blood; cytotrophoblasts; connective tissue; and endothelium (Jauniaux *et al.*, 2000). With the progress of pregnancy, the cytotrophoblast layer differentiate into syncytiotrophoblasts. By term, the placental membrane is primarily composed by three layers, syncytiotrophoblast, connective tissue of villus, and endothelium of fetal capillaries, from outside to inside, respectively. In some areas, the placental membrane, however, is

composed by only syncytiotrophoblasts with direct contact with endothelium (Jauniaux *et al.*, 2000).

In addition to serving as a maternal and fetal exchange site, the placenta also exhibits abilities of metabolism, protection and endocrine. Most research regarding placental transportation has been based on term placentas. However, evidence has showed that placental transportation during early stage of gestation may differ from that at term (Jauniaux *et al.*, 2000; Glazier & Jansson, 2004). For example, since placental circulation is not established before 10-12 weeks of gestation, early nutrient transportation is through trophoblast phagocytosis from endometrial gland secretion (Burton *et al.*, 2002). After placental circulation is established, maternal blood direct contacts with terminal villi, and subsequently exchanges respiratory gases, nutrients and wastes through placental membrane.

Lack of the nervous system in human placenta makes fetal-maternal communication highly rely on blood borne substances; hence placental endocrine, paracrine, and/or autocrine plays important roles at both fetal and maternal sides of placentas. The placenta is able to produce steroid hormones, peptide growth factors, cytokines, chemokines and many others. Some of them exert major roles in placental and fetal development. For example, after 9th week of gestation, the placenta becomes the major source of circulating progesterone and estrogen (Page, 1993; Jauniaux *et al.*, 2000).

During pregnancy, the fetus needs to be delicately protected from xenobiotics, such as bacteria, viruses, and chemicals. Although limited, protection is still one major aspect of placental function. As one of many other physiological barriers, the placenta can block many bacteria transmitting from mother to fetus, although, some bacteria and

many viruses still can penetrate through the placental barrier and cause poor pregnancy outcomes (Jauniaux *et al.*, 2000; Arechavaleta-Velasco *et al.*, 2002). Mechanisms of such protective function include expression of export pumps at maternal-facing side of syncytiotrophoblasts (Jauniaux *et al.*, 2000; Marin *et al.*, 2003). Expression of a set of cytochrome *P450* enzymes may contribute to metabolize drugs and other xenobiotics as well (Pasanen, 1999). Despite of the imperfect of placental barrier function, some of maternal antibodies (including immunoglobulin G class) can be transported through placentas by pinocytosis, which participates greatly neonatal passive immunity (Jauniaux *et al.*, 2000).

I.3. Endothelial Cells

Endothelial cells are a layer of cells lining the interior surface of blood vessels and lymphatic vessels. Endothelial cells in an adult human are approximately ~1 x 10^{13}, weigh approximately 1 kg, and cover a surface area of approximately 1 - 7 m^2 (Augustin *et al.*, 1994). Based on this estimation, in an adult human weighting 100 kg, endothelial cells account for ~33% of total cell numbers (Sender *et al.*, 2016).

Significant heterogeneity of endothelial cells is well recognized (Cines *et al.*, 1998; Aird, 2012), and has been described at the level of cell morphology, function, gene expression, and antigen composition (Aird, 2007*a*, 2007*b*, 2012). Endothelial phenotypes may vary between different organs, between different segments of the vascular loop within the same organ, and between neighboring endothelial cells of the same organ and blood vessel type (Aird, 2007*a*). For example, our recent study reveals that the human umbilical cord vein and artery endothelial cells (HUVECs and HUAECs, respectively)

have distinct gene expression profiles, which may lead to unique phenotypes of different origins of endothelial cells (Jiang *et al.*, 2013*a*, 2013*b*).

Other than serving as a cell barrier separating blood from its surrounding tissues, endothelial cells regulate many vascular function including vascular tone, coagulation, inflammatory status, fibrinolysis, and oxidization, primarily via secreting agonists and antagonists (Esper *et al.*, 2006). More importantly, it is well established that endothelial dysfunction could lead to vascular diseases including pathogenesis of atherosclerosis (Cines *et al.*, 1998; Hansson, 2005), heart disease (Hadi *et al.*, 2005), hypertension (Hamasaki *et al.*, 2000), and the pregnancy-complications such as PE (Roberts *et al.*, 1989) and IUGR (Kingdom *et al.*, 2000). Thus, maintaining normal endothelial function is particularly important for vascular function, which has a great impact on human health and wellbeing (Cines *et al.*, 1998; Michiels, 2003).

I.4. Placental Vascular Formation and Angiogenesis

During normal pregnancy, dramatic increases of fetal and maternal blood flows are required in order to support fetal growth and survival. These increases in early pregnancy are mainly achieved by two distinct processes, vasculogenesis and angiogenesis (Wang & Zheng, 2012).

Vasculogenesis is referred as *de novo* formation of blood vessels from non-vascular cells, and it takes place approximately from day 18 to 35 post conception (*p.c.*) in human. In order to form the primitive capillaries, early stage of vasculogenesis is featured with a series of *in situ* differentiation of hemagiogenic stem cells to angioblast cells (progenitors of endothelial cells), and these processes are regulated by multiple

growth factors, such as fibroblast growth factor (FGF) 2 (FGF2) and vascular endothelial growth factor (VEGF) A (VEGFA) (Wang & Zhao, 2010). At day 21 to 32 *p.c.*, primitive vascular tubes that are composed of a single layer of endothelial cells can be observed (Wang & Zhao, 2010).

Around day 32 *p.c.* is a milestone of separating vasculogenesis and angiogenesis. From this day to delivery, once primitive fetoplacental circulation is established, vasculogenesis will be replaced by angiogenesis (a process of new blood vessels growth from existing blood vessels) (Benirschke *et al.*, 2006*b*; Wang & Zhao, 2010). At this time, endothelial tubes in villous start to connect with each other and fetoplacental circulation is initially established. From day 32 to week 25 *p.c.*, capillary network in immature intermediate villous is established by branching angiogenesis. On the other hand, from weeks of 15 to 32 *p.c.*, blood vessels in stem villous and terminal villous are formed by capillary regression and non-branching angiogenesis, respectively (Benirschke *et al.*, 2006*b*; Wang & Zhao, 2010). Angiogenesis is tightly controlled by angiogenic factors (i.e., FGF2 and VEGFA) that can be produced by placental cells (trophoblasts, pericytes and endothelial cells) (Wang & Zhao, 2010).

I.5. Oxygen Homeostasis and Physiological Normoxia in Human Placentas

Maintenance of oxygen homeostasis in humans is critical. Oxygen is essential for driving oxidative phosphorylation for generating ATP energy in mitochondria. During pregnancy, in order to meet a dramatically increased level of metabolism for both maternal adaptation and fetal development, the demand of oxygen increases accordingly.

Such demand subsequently causes significant vascular remodeling, vasodilation and angiogenesis (Pasanen, 1999; Arechavaleta-Velasco *et al.*, 2002; Marin *et al.*, 2003).

Contrary to the canonical concept of placentas for supplying fetal development-required oxygen and nutrient, during pregnancy, differentiation and development of placentas are undergoing in a relatively low oxygen (referred as physiological normoxia) environment (~2-3% O2, ~15.2-22.8 mmHg at ≤ 8-10 weeks of gestation, ~8% O_2, ~61 mmHg between 8-10 weeks of gestation; ~6% O2, ~45.7 mmHg at the end of third trimester) as compared with the ambient O2 level at sea level (21% O2, 160 mmHg) (Granger *et al.*, 2001; Danilov & Fiskum, 2008; Halliwell & Gutteridge, 2015). While in intracardiac fetal blood, the O2 is ~3% during relative early stage of pregnancy (13-16 weeks of gestation) (Jauniaux *et al.*, 2001). The relatively low oxygen level in placentas is believed to be caused by the blockage of spiral arteries by extracellular trophoblasts during early stage of pregnancy. Such a low oxygen status is considered a defensive mechanism for protecting vulnerable early embryo from contacting excessive reactive oxygen species (Intaglietta *et al.*, 1996). After 10 weeks of gestation, the blockage is released and maternal blood is allowed to flow into intervillous space through dilated and remodeled spiral arterioles (Rodesch *et al.*, 1992; Jaffe *et al.*, 1997).

Growing evidence shows that such physiological normoxia occurring in placentas and fetuses during pregnancy is beneficial for embryonic and placental growth and development (Rodesch *et al.*, 1992; Genbacev *et al.*, 1997; Caniggia & Winter, 2002; Red-Horse *et al.*, 2004; Danilov & Fiskum, 2008). For example, as compared with 21% O_2, 2-3% O_2 promote extravillous trophoblasts outgrowth and proliferation in placental explants obtained from first trimester human placentas (Genbacev *et al.*, 1997). In

contrast, oxygen lower than physiological range (~1.5%) stalls outgrowth and proliferation of extravillous trophoblasts (James *et al.*, 2006). This piece of evidence indicates important roles of physiological oxygen levels (3-8%) in mediating normal development of placenta during early stage of pregnancy.

Recent reports have showed that physiological chronic normoxia (~20- 25 days, 3% O_2, 5% CO_2, ~92% N_2) enhances FGF2- and VEGFA-induced cell proliferation and migration in HUVECs and HUAECs in comparison to standard culture normoxia (~21% O_2) (Jiang *et al.*, 2013*a*, 2013*b*). Such low oxygen-promoted growth factors-induced cell responses are achieved at least partially via enlarged ERK1/2 and AKT1 activation (Jiang *et al.*, 2013*a*, 2013*b*).

I.6. FGF Family and FGF Receptors

So far, 18 members of FGF family have been discovered (FGF1-10 and FGF16-23). Among them, 6 subfamilies are described based on sequence similarity and phylogeny, including 5 paracrine subfamilies and 1 endocrine subfamily (Beenken & Mohammadi, 2009; Yang *et al.*, 2015). FGFs function by binding to high affinity receptors FGF receptors (FGFRs). Paracrine FGFs bind with FGFRs in association with heparin sulfate proteoglycans (HSPGs) (Spivak-Kroizman *et al.*, 1994; Beenken & Mohammadi, 2009; Ornitz & Itoh, 2015). Such binding complex can prevent FGFs from being released from the cells and being degraded (Rosengart *et al.*, 1988; Friesel & Maciag, 1995; Murakami *et al.*, 2008*a*; Beenken & Mohammadi, 2009). On the other hand, endocrine FGFs only bind HSPGs weakly, hence they are able to be transported distantly and function as endocrinal factors. Other than HSPGs, endocrine FGFs require another co-

receptor, Klotho, for binding with FGFRs (Kurosu *et al.*, 2006, 2007; Urakawa *et al.*, 2006; Goetz *et al.*, 2007). However, unlike VEGFA, bioactivity of FGF is predominantly mediated by FGFR expression and activity at target tissues, but not dependent on FGF expression (Cao *et al.*, 2003; Simons, 2004, 2005).

FGF2 is one of the most extensively studied members of FGF family and is known for its potent roles in inducing angiogenesis (Folkman & Shing, 1992; Klein *et al.*, 1997; Ornitz & Itoh, 2015). Despite of different isoforms of FGF2 with molecular weight ranging from 18 to 24 kDa that are encoded by a single copy of human *fgf2* gene, only 18 kDa isoform is found in human placentas (Wolff *et al.*, 1996; Hamai *et al.*, 1998). FGF2 is expressed in both trophoblasts and endothelial cells and acts as pro-angiogenic factor (Zheng *et al.*, 1997; Hamai *et al.*, 1998).

By far, four members of FGFRs have been described in human, including FGFR1-4 (Johnson & Williams, 1992; Ornitz & Itoh, 2015). FGFR1 is considered the major receptor for FGF2 in endothelial cells (Presta *et al.*, 2005; Turner & Grose, 2010). Upon binding to FGF, FGFR dimerizes and trans-/auto-phosphorylates its intracellular tyrosine kinase, subsequently initiating downstream signaling pathways (Ornitz & Itoh, 2015). For example, phosphorylation of FGFR2 at Y653 and 654 lead to promotion of tyrosine activity up to ~ 1000 folds, and phosphorylation of Y677 and 766 creates binding sites for signal transducer and activator of transcription 3 (STAT3) and PLCγ (Peters *et al.*, 1992; Dudka *et al.*, 2010; Ornitz & Itoh, 2015). Initiation of some of FGFR1-induced signaling pathways, including Ras-mitogen-activated protein (MAP) kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-AKT, is mediated by phosphorylation of FGFR substrate 2α (FRS2α) (Ornitz & Itoh, 2015). Although FGFR

canonically activates several signaling pathways, such as Ras-MAPK, PI3K-AKT, PLCγ, and STAT; however, numerous isoforms of FGFR generated due to splice variants render FGFR distinct roles in regulating downstream signaling molecules. By truncating extra-/intracellular domains, lacking of Ig-like domains or utilizing different coding regions for the same Ig-like domain, FGFR1 has at least 12 different isoforms in vertebrates (Johnson & Williams, 1992). Among them, there are 3 soluble isoforms lacking kinase domains and 9 transmembrane isoforms (Groth & Lardelli, 2002). One of these isoforms with defected Ig-like domains is identified in human placentas; instead of activating canonical downstream signaling molecules, such as FRS2 and PLCγ, this defected FGFR1 isoform mediates rat myoblasts cell proliferation through activation of ERK1/2 and increase in c-fos mRNA expression (Lopez & Korc, 2000).

As mice lacking *Fgf2* develop normally, FGF2 has less important roles in regulating embryonic development (Ortega *et al.*, 1998). However, knockout of *Fgf2* gene in mice may cause many impairments including reduced vascular tone (Zhou *et al.*, 1998), decreased endothelial cell proliferation and vascular density (Virag *et al.*, 2007), impaired cardiomyocyte hypertrophy (Virag *et al.*, 2007), as well as retarded cutaneous (Ortega *et al.*, 1998) and pulmonary wound healing (Guzy *et al.*, 2015). In contrast, FGFR1 is required for embryonic development since lacking of *Fgfr1* at both alleles causes embryonic death at embryonic day 8.5. (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994). In these *Fgfr1* knockout mice embryos, mesoderm formation and initiation of somitogenesis undergo normally; however, mesoderm migration and embryonic growth are largely delayed (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994).

I.7. VEGF Family and VEGF Receptors

To date, five members have been found of the VEGF family in mammal, including VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (PlGF) (Hicklin & Ellis, 2004; Koch & Claesson-Welsh, 2012). Although heterodimers are found as alternative forms in some members, normally, VEGF family members are homodimeric polypeptides (DiSalvo *et al.*, 1995). For example, in rat GS-9L glioma cell line not only produce VEGFA and PlGF homodimer, but also produce VEGFA-PlGF heterodimer with a endothelial mitogenic potency similar to VEGFA homodimer (DiSalvo *et al.*, 1995).

Being discovered and cloned in 1980's, VEGFA (also referred as VEGF) has been intensely studied as a prototype member of VEGF family, as well as a pivot regulator of complex angiogenic process in both physiological and pathological conditions (Ferrara *et al.*, 2003; Hicklin & Ellis, 2004; Koch & Claesson-Welsh, 2012). VEGFA is required for vasculogenesis as loss of even a single allele of VEGFA causes embryonic death of mice due to abnormal blood vessel formation (Ferrara *et al.*, 1996). Well-documented evidence has showed potent ability of VEGFA for anti-apoptosis, promoting cells growth and permeability of endothelial cells derived from veins, arteries and lymphatics in both *in vivo* and *in vitro* models (Plouet *et al.*, 1989; Leung *et al.*, 1989; Ferrara & Davis-Smyth, 1997; Ferrara *et al.*, 2003). Immunohistochemistry studies have showed expression of VEGFA in trophoblasts and stromal cells, but not in villous endothelium in human placenta (Jackson *et al.*, 1994; Shiraishi *et al.*, 1996).

Albeit there are three members in VEGF receptor (VEGFR; VEGFR1, 2 and 3) family, VEGFR 1 (VEGFR1 or Flt1) and VEGFR 2 (VEGFR2 or KDR) are main receptors that mediate VEGFA actions (Ferrara *et al.*, 2003). Both VEGFR1 and 2 are essential for

embryo development. For instance, *vegfr1*$^{-/-}$ mice embryos die at embryonic day 9, primarily caused by disoriented development of vasculature (Fong *et al.*, 1995) and excessive production of haematopoietic progenitors (Shalaby *et al.*, 1995); VEGFR2-knockout mice embryo died at embryonic day 8.5 from damaged development of hematopoietic and endothelial cells (Ferrara *et al.*, 2003; Koch & Claesson-Welsh, 2012). VEGFR1 and 2 are expressed in both placental villous endothelium and cytotrophoblasts (Cooper *et al.*, 1992; Clark *et al.*, 1996). However, their biological features and bioactivity in response to VEGFA are distinct from each other. VEGFR1 may exhibit anti-angiogenic effects through intracellular signaling pathways as well. Zeng *et al*. have reported that VEGFR1 inhibits VEGFR2-mediated cell proliferation, but not migration, through activation of cell division control protein 42 homolog (CDC42) and ras-related C3 botulinum toxin substrate 1 (Rac1) in HUVECs and such activation is mediated by PI3K (Zeng *et al.*, 2002*a*). Although VEGFR2 has a 10 fold lower affinity for binding with VEGFA as compared to VEGFR1, VEGFR2 is the major transducer of pro-angiogenic signaling of VEGFA (Fuh *et al.*, 1998; Shinkai *et al.*, 1998; Ferrara *et al.*, 2003; Koch & Claesson-Welsh, 2012).

Other than its full length version, VEGFR1 has an alternative splice isoform which is soluble (soluble VEGFR1; sVEGFR1 or sFlt1). Both of these two isoforms have higher VEGFA-binding affinity than VEGFR2. However, since sFlt1 lacks cytoplasmic kinase domains and cannot induce cellular responses, the binding of sVEGFR1 to VEGFA decreases the bioavailability of VEGFA; hence this possibly prevents and/or limits activation of VEGFR1 and VEGFR2 by VEGFA (Koch & Claesson-Welsh, 2012). More importantly, the imbalance of sVEGFR1 and VEGFA, as well as other angiogenesis-

related factors, is considered one of possible mechanisms of PE (Karumanchi & Bdolah, 2004). Besides, accumulation of sVEGFR1 variant sFlt-14 is found in placentas of preeclamptic women (Sela *et al.*, 2008).

It is well established that VEGFR2 is the dominant receptor for mediating VEGFA-induced endothelial responses such as cell proliferation, migration and permeability (Neufeld *et al.*, 1999; Ferrara *et al.*, 2003; Koch & Claesson-Welsh, 2012). Binding of VEGFA to VEGFR2 promotes and stabilizes receptor dimerization, which leading to auto- or tans-phosphorylation of tyrosine residues of receptor kinases at cytoplasmic domain. Subsequently, activated VEGFR2 induces a complex of signaling network in endothelial cells, such as MEK/MAPK and PI3K/AKT1 (Jiang *et al.*, 2013*a*), Rho (Zeng *et al.*, 2002*b*), and PLC (Mukhopadhyay & Zeng, 2002; Bhattacharya *et al.*, 2009).

I.8. Heterotrimeric Guanine Nucleotide-binding Proteins (G Proteins)

G proteins mediate a vast array of cell function. G protein function by interacting with G protein-coupled receptors (GPCRs), which are the largest family of cell-surface molecules by far (Wettschureck & Offermanns, 2005). In mammalian genome, at least 1000 GPCRs are encoded. Among them, most of them mediate sensory signaling transduction, such as taste; while ~400-500 recognize non-sensory stimuli, such as hormones and neurotransmitters. Physiological binding partners of ~200 GPCR are still unknown (Wettschureck & Offermanns, 2005).

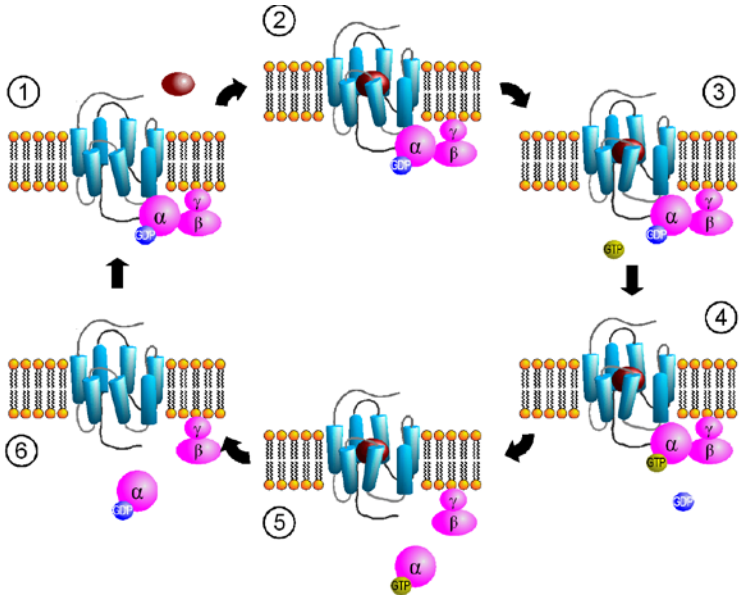

Figure I.1 (https://en.wikipedia.org/wiki/G_protein)

Upon binding to their ligands (Figure I.1), G proteins transmit GPCR-mediated signals, which are constituted by three subunits, α (Gα), β and γ (Gβ and Gγ). α subunits remain inactivated by binding with guanosine diphosphate (GDP); naturally, β and γ subunits are structurally intertwined to form an undissociable complex (Wettschureck & Offermanns, 2005). When at inactivated status, GDP-bound Gα is associated with Gβγ. Binding to GPCR promotes GDP exchange to guanosine triphosphate (GTP). GTP-bound Gα subsequently disassociates from Gβγ. Both of Gα and Gβγ have biological roles in regulating downstream signaling pathways. Termination of Gα signaling is accomplished by hydrolysis of GTP to GDP via RGS (regulators of G protein signaling)-regulated GTPase, GDP-bound α subunit eventually reunites with βγ complex to complete the cycle (Wettschureck & Offermanns, 2005).

Crystal structure analysis reveals a conserved protein fold among Gα subunits, composed by a GTPase domain and a helical domain (Sánchez-Fernández *et al.*, 2014). In GTPase domain, five highly conserved loops with consensus sequences are

responsible for guanine nucleotide binding, while other three flexible loops undergo conformational changes during GTP/GDP binding of Gα subunit (Sánchez-Fernández *et al.*, 2014). The helical domain contains six α-helices and serves as lid over the nucleotide-binding sites. Such structure facilitates the binding of Gα and guanine nucleotides and increases the activity of intrinsic GTP hydrolysis activity (Echeverría *et al.*, 2000). Though more information is needed, both N- and C-terminus are believed to be essential for receptor specificity, interaction with Gβγ subunits, and relocation of Gα on membrane (Oldham & Hamm, 2006).

Other than canonical modulation of GDP-GTP exchange rate, activity and function of G protein subunits can be modulated by post-translational modifications as well, such as lipid modification and phosphorylation (Jennings & Linder, 2010). N-myristoylation and/or palmitoylation of Gα, as well as prenylation of Gγ, may promote membrane attachment and interaction with Gα, respectively (Jones *et al.*, 1990; Mumby *et al.*, 1990; Jennings & Linder, 2010). Bacterial exotoxins also have great impacts on mediating G protein activity. Both $G\alpha_s$ and $G\alpha_i$ can be ADP-ribosylated by cholera toxin and pertussis toxin (Jennings & Linder, 2010). Such modification prolongs $G\alpha_s$ activation, but disrupts interaction of $G\alpha_i$ with activated receptors (Jennings & Linder, 2010).

Based on the similarity of their sequences, Gα subunits can be classified into different subfamilies. For example, α subunit is classified into 4 subfamilies ($G\alpha_s$, $G\alpha_i$/$G\alpha_o$, $G\alpha_q$/$G\alpha_{11}$, and $G\alpha_{12}$/$G\alpha_{13}$), whereas Gβ and Gγ have 5 and 12 members, respectively (Wettschureck & Offermanns, 2005). Together they can make up hundreds of combinations of G proteins. Each subfamily has several family members with distinct tissue distributions and function. Upon activation, these G proteins can induce a diverse

array of downstream signal molecules (e.g., PLC, RhoA, ERK1/2, PI3K, p38 MAPK, and Ca^{2+}), thereby mediating cell processes (Neves, 2002; Wettschureck & Offermanns, 2005).

Several studies suggest the importance of Gα subunits in mediating placental and endothelial function during pregnancy. Expression of many members of $G\alpha_s$ and $G\alpha_{i/o}$ subfamilies have been confirmed in human placentas (Kenton & Johnson, 1994; Bourgeois *et al.*, 1996). Although, Petit *et al.*, 1997 reported that protein levels of $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{11}$ are not changed in placental tissues from preeclamptic pregnancy as compared with those from normal pregnancy (Petit *et al.*, 1997). Recently, significant elevated levels of protein and mRNA of $G\alpha_{12}$ have been reported in human placentas obtained from preeclamptic pregnancy (Cox *et al.*, 2011; Ye *et al.*, 2016).

I.9. Introduction of $G\alpha_{q/11}$ Subfamily

$G\alpha_{q/11}$ subfamily is very important in mediating cellular signaling transduction, as nearly 40% of all GPCR rely on them (Hubbard & Hepler, 2006). This subfamily is constituted of 4 members, $G\alpha_q$ (GNAq), $G\alpha_{11}$ (GNA11), $G\alpha_{14}$ (GNA14), and $G\alpha_{15/16}$ (GNA15/16; different gene names in mouse or human, respectively) (Hubbard & Hepler, 2006). Within this family, GNAq and 11 are most extensively studied, while GNA14 and GNA15/16 are much less known (Hubbard & Hepler, 2006). In addition, it is possible that GNA14 and 15/16 may have similar function as GNAq and/or 11 since they can activate same downstream signaling molecules (Hubbard & Hepler, 2006). Further evidence has shown that $G\alpha_{q/11}$ family members have both redundant and unique function (Hubbard & Hepler, 2006).

$G\alpha_{q/11}$ family members were discovered in the early 1990's by affinity purification and molecular cloning (Pang & Sternweiss, 1990; Taylor *et al.*, 1990; Strathmann & Simon, 1990). Both GNAq and 11 are ubiquitously expressed in mammalian tissues (Nakamura *et al.*, 1991; Wilkie *et al.*, 1991; Chen *et al.*, 1996). Tissue distributions of GNA14 and 15/16 are believed to be more restricted. Expression of GNA14 has been identified in the spleen, lung, kidney, pancreas, liver, testis and bone marrow adherent stromal cells (Nakamura *et al.*, 1991; Wilkie *et al.*, 1991; Zigman *et al.*, 1994). GNA15/16 may have the most limited expression location as they are only detected in either tissues containing large amount of hematopoietic cells or cells that are hematopoietic origin (Amatruda *et al.*, 1991; Wilkie *et al.*, 1991).

In amino acid sequences, GNA11, GNA14, and GNA15/16 have 90%, 80%, and 57% similarity to GNAq. However, N-termini (1-40 amino acids) of $G\alpha_{q/11}$ family members are highly variable as within this area, GNA11, GNA14, and GNA16 are only 83%, 65% and 35% identical to GNAq (Hubbard & Hepler, 2006). Studies on crystal structures of Gα reveal importance of an α-helix in N-terminus (Wall *et al.*, 1995; Lambright *et al.*, 1996; Sprang, 1997), of which it is critical in regulating post-translational modifications, Gβγ interactions, as well as protein-protein/protein-lipid interactions (Hepler *et al.*, 1996; Nakamura *et al.*, 1996). The high diversity and principle roles of N-terminus imply function differences between different Gα subunits. For example, GNA11 and 14 display opposite actions in mediating PLC activity in oocytes of xenopus (Nakamura *et al.*, 1996).

After coupling with receptors and being activated, one of the important downstream binding partners of $G\alpha_{q/11}$ family members is PLC-β. Among all four isoforms of PLC-β (PLC-β1-4), GNAq, GNA11, and GNA15/16 activate PLC-β in an order of potency (PLC-

β3 ≥ PLC-β1 >> PLC-β2), in which activation of PLC-β3 and -β1 has a 10-fold greater potency than -β2 (Hepler *et al.*, 1993; Jhon *et al.*, 1993; Smrcka & Sternweis, 1993; Runnels & Scarlata, 1999). Certainly, $G\alpha_{q/11}$ family members also activate PLC-β4, however, such activation is limited by the sensitivity of PLC-β4 to ribonucleotide inhibition (Lee *et al.*, 1994; Jiang *et al.*, 1994). Different $G\alpha_{q/11}$ family members have been proving to have variant ability of activating PLC-β1-4. For example, although all members of $G\alpha_{q/11}$ subfamily are expressed in hematopoietic cells, GNA16 activates PLC-β2 more effectively than GNAq, 11 and 14 (Lee *et al.*, 1992; Jiang *et al.*, 1994). Other than PLC-β, the $G\alpha_{q/11}$ family can also mediate activity of other signaling molecules, such as RhoA (Ras homolog gene family, member A) (Hubbard & Hepler, 2006), PKC (protein kinase C) (Hawes *et al.*, 1995), STAT (Chan & Wong, 2000; Wu *et al.*, 2003; Lo *et al.*, 2003; Lo & Wong, 2004), and various MAPKs (Hubbard & Hepler, 2006).

I.10. Roles of GNA11 and 14 *in vitro* and *in vivo*

Both GNA11 and 14 are involved in mediating different cell function (Hubbard & Hepler, 2006). By using rat portal vein myocytes, knockdown of GNAq decreases α1-adrenoreceptor-mediated $[Ca^{++}]_i$ elevation in Ca++-free culture solution; while in Ca++-containing solution, knockdown of GNA11 suppresses α1-adrenoreceptor-induced $[Ca^{++}]_i$ (Macrez-Leprêtre *et al.*, 1997). These data suggest differential roles of GNAq and 11 in mediating cell function. In addition, double knockdown of GNAq and 11 blocks VEGFA-stimulated endothelial migration via activation of RhoA (Zeng *et al.*, 2002*b*), whereas it only inhibits VEGFA-, but not FGF2-induced endothelial proliferation via phosphorylation of MAPK (Zeng *et al.*, 2003). The constitutively active form of GNAq or

14, but not GNA15, induces cell apoptosis in vascular smooth muscle cells via activation of caspase-3 (Peavy *et al.*, 2005). These findings further reveal the complexity of $G\alpha_{q/11}$ family-mediated cell signaling pathways, which may render both functional redundancy and distinction among $G\alpha_{q/11}$ family members.

In animal models, double knockout of GNAq and 11 is lethal for embryonic development of mice (embryo died at embryonic day 10.5). Heterozygous offspring with one intact allele of either of two genes have heart malformation and die shortly after birth (Offermanns *et al.*, 1998). Nowadays, inducible and tissue-specific knockout mice model allow mice to survive till adulthood and hence provide more broad understanding of roles of $G\alpha_{q/11}$ family members *in vivo*. Instead of GNA12 and 13, double knockout of GNAq and 11 decreases basal blood pressure in mice vascular smooth muscle tissues (Wirth *et al.*, 2008). The integrity of these two genes is also necessary for salt-induced hypertension and its development in mice (Wirth *et al.*, 2008). These data indicate possible important roles of GNA11 in mediating cardiovascular function.

Abnormal expression of GNA11 or 14 is related to many human diseases. Somatic mutation-induced constitutive activation of GNAq and/or 11 is associated with different phenotypes of melanoma (eye cancer) (Shoushtari & Carvajal, 2014). In addition, mutation of GNA11 is correlated with familial hypocalciuric hypercalcemia (a hereditary endocrinal disease featured with high blood calcium levels and low uric calcium levels) (Nesbit *et al.*, 2013; Arnold & Marx, 2013; Li *et al.*, 2014). More importantly, GNA14 is considered as the hypertension-susceptibility gene in human (Kohara *et al.*, 2008). Protein expression of GNA14 is significantly increased in lung tissues from pulmonary artery hypertensive patients (Abdul-Salam *et al.*, 2010). Constitutively activated mutation

of GNA14 is linked with childhood vascular tumor and possibly via upregulated MAPK pathway (Lim *et al.*, 2016). These findings imply important roles of GNA11 and 14 in pathogenesis of many human diseases.

Interestingly, other than canonical G protein-binding GPCRs, receptor tyrosine kinases (RTKs) are also capable of interacting with G proteins (Pyne & Pyne, 2011). Pertussis toxin (an inhibitor of $G\alpha_i$ family) can eliminate cell responses induced by insulin- (Heyworth *et al.*, 1986) and platelet-derived growth factor (PDGF) (Conway *et al.*, 1999). $G\alpha_s$ (Poppleton *et al.*, 1996) and $G\alpha_{13}$ (Shan *et al.*, 2006) have also be reported to mediate epidermal growth factor- and PDGF-induced signaling transduction and cell function, respectively. Although the exact mechanisms of these interactions still remain unknown, a study showed that GNAq and 11 may function upstream of VEGFR2 activation (Zeng *et al.*, 2003).

Chapter II. Expression of G Protein Subunit α-14 is Increased in Human Placentas from Preeclamptic Pregnancies

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I am the co-first author of this paper. I participated in designing and performing experiments, analyzing data. Specifically, I involved in generating and analyzing data presented in all figures. The current version has been formatted for this dissertation.

Abstract

G proteins mediate cellular function upon interaction with G-protein coupled receptors. Of the 16 mammalian G protein α subunits identified, G protein subunit α 11 (GNA11) and 14 (GNA14) have been implicated in modulating hypertension and endothelial function. However, little is known about their expression and roles in human placentas. Here, we examined GNA11 and GNA14 protein expression in first trimester (FT), normal term (NT), and severe preeclamptic (sPE) human placentas as well as in NT human umbilical cords. We found that GNA11 and GNA14 were immunolocalized primarily in trophoblasts, villous stromal cells, and endothelial cells in placentas as well as in endothelial and/or smooth muscle cells of the umbilical cord artery and vein. Western blotting revealed that the GNA14, but not GNA11, protein levels were increased (2.5-2.9 fold; $p < 0.01$) in sPE vs. NT placentas. GNA11 protein was detected only in NT, but not FT, placentas, whereas GNA14 protein levels were increased (7.7-10.6 fold; $p < 0.01$) in NT vs. FT placentas. Thus, GNA11 and GNA14 may mediate the function of several cell types in placentas. Moreover, the high expression of GNA14 in sPE placentas may also imply its importance in sPE pregnancies as in the other hypertension-related disorders.

II.1. Introduction

During pregnancy, normal placental growth and development, in association with remarkable vascular formation and growth, are required to provide effective materno-fetal exchange in support of the rapid growing fetus (Reynolds & Redmer, 2001). Thus, impaired placental development, such as that seen with inadequate trophoblast invasion or dysfunction of the placental vasculature, could contribute to pregnancy complications, such as preeclampsia and intrauterine growth retardation (Cerdeira & Karumanchi, 2012).

Preeclampsia, a leading cause of neonatal and maternal morbidity and mortality, occurs in approximately 5-10% of all pregnancies worldwide and is characterized by the onset of high blood pressure and proteinuria (Solomon & Seely, 2004, 2006). To date, the exact etiology of preeclampsia remains elusive; however, defective placentation, placental ischemia, and endothelial dysfunction are thought to be three major insults associated with the increased risk of preeclampsia (Solomon & Seely, 2004, 2006).

G proteins mediate a diverse array of cellular functions upon interaction with G protein coupled receptors (GPCRs), which represent by far the largest family of cell-surface molecules (Neves, 2002; Dorsam & Gutkind, 2007). The heterotrimeric G protein comprises α, β and γ subunits, the latter two forming an indissociable complex (Wettschureck & Offermanns, 2005; Hubbard & Hepler, 2006). The mammalian Gα subunit, which binds to and hydrolyzes guanosine-5'-triphosphate (GTP) and thus defines the basic properties of a G protein, can be grouped into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (Wettschureck & Offermanns, 2005; Hubbard & Hepler, 2006). Each family further contains various members. For example, the Gαq family consists of $G\alpha_q$, $G\alpha_{11}$ (GNA11), $G\alpha_{14}$ (GNA14), and $G\alpha_{15/16}$. Upon activation, these G proteins induce a number of

downstream signal molecules (e.g., PLC, ERK1/2, and Ca++), thereby initiating cellular processes (Wettschureck & Offermanns, 2005; Hubbard & Hepler, 2006). The importance of the Gα subunit in the fetus has been recognized since the knockdown of $G\alpha_{12/13}$, $G\alpha_q$, and GNA11 in mice results in intrauterine fetal death, largely due to severe defects in fetal vasculature (Offermanns *et al.*, 1997, 1998; Ruppel *et al.*, 2005). Recent evidence has also shown that GNA11 is an essential mediator in the activation of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) and is required for the VEGF-stimulated cell migration and proliferation as well as VEGF-induced ERK1/2 activation in human umbilical vein endothelial cells (HUVECs) (Zeng *et al.*, 2002*b*, 2003). Moreover, although GNA14 has received much less attention, it has been identified as a hypertension-susceptibility gene in humans (Kohara *et al.*, 2008). Along the same lines, proteomic analysis also revealed that GNA14 protein expression was significantly increased in lung tissues from patients with pulmonary artery hypertension (Abdul-Salam *et al.*, 2010), suggesting importance of GNA14 in hypertension-related diseases.

The expression of GNA11 and GNA14 has been detected in various mammalian tissues including brain, heart, lung, liver, kidney, thyroid, testis, and skeletal muscle tissues (Nakamura *et al.*, 1991; Wilkie *et al.*, 1991; Laugwitz *et al.*, 1996). However, information on the cellular distribution and expression of GNA11 and GNA14 in human placentas is lacking. Thus, in this study, we examined GNA11 and GNA14 protein expression in human placentas obtained from the first trimester (FT), normal term (NT), and severe preeclamptic (sPE) pregnancies, as well as in human umbilical cord vessels from NT pregnancies using immunohistochemistry and/or Western blot analysis.

II.2. Materials & Methods

Collection of Placental Tissues

Two sets of placental tissue samples were collected from two different sites. The first set of samples were collected from NT (n=10; nine were from vaginal delivery and one from cesarean section delivery) and sPE (n=10; all were from cesarean section delivery) pregnancies in the Meriter Hospital, Madison, WI, as previously described (Chung *et al.*, 2004; Jiang *et al.*, 2010). Umbilical cords from NT pregnancies were also collected. The tissue collection protocol was approved by the Institutional Review Board of Meriter Hospital, and the Health Sciences Institutional Review Board of the University of Wisconsin-Madison, and followed the recommended guidelines for using human subjects.

The second set of samples were obtained from FT (n=10) and NT (n=10) pregnancies in Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, China. These FT placentas were collected from patients with induced abortion at the gestational age of 6-8 weeks, as described elsewhere (Hao *et al.*, 2012). Collection of the placentas was approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital. Written informed consent to participate in the study was obtained from each patient.

Preeclampsia was defined according to the standard criteria (Roccella, 2000). Preeclampsia was considered severe if one or more of the following criteria were present: maternal blood pressure higher than or equal to 160/110 mmHg on two separate readings; proteinuria more than 2+ by dipstick or more than 2 g/24 hr. None of the study subjects had signs of infection. Smokers were excluded. Patient ages were similar (23 ± 0.9 years)

between NT and sPE pregnancies. Gestational ages for NT pregnancies (39 ± 0.2 weeks) were significantly ($p < 0.05$) higher than those for sPE pregnancies (34 ± 0.7 weeks). Fetal weights for NT pregnancies (3404 ± 90.9 g) were higher ($p < 0.05$) than in sPE pregnancies (2198 ± 236.4 g). Each of these parameters was analyzed using the Student's t-test.

Placental villi from beneath the chorionic and basal plates were quickly dissected (~10 g each), snap-frozen, and stored in liquid nitrogen for Western blot analysis. Additional placental tissues and umbilical cords were fixed overnight at 4^0C in 4% paraformaldehyde in 10 mM PBS and embedded in paraffin for immunohistochemistry.

HUVECs were also isolated from NT pregnancies and cultured as described previously (Wang *et al.*, 2009; Dai *et al.*, 2011). At passage 2, five cell preparations were pooled and expanded. These HUVECs express GNA11 and GNA14 (Zeng *et al.*, 2002*b*, 2003; Jiang *et al.*, 2013*a*), and were used as a standard in the Western blot analysis.

Validation of GNA11 and GNA14 Antibodies

A rabbit antibody against human GNA11 antibody (cat # AP19441) was purchased from Abgent (San Diego, CA). The specificity of the GNA11 antibody was confirmed in cell lysates from GNA11-overexpressing 293T cells (10 µg; sc-120367, Santa Cruz Biotech, Santa Cruz, CA; a positive control), GNA14-overexpressing 293T cells (10 µg; sc-174410, Santa Cruz Biotech, a negative control), and HUVECs (20 µg; a positive control) using Western blotting (see below). As shown in Fig. II.1A, this GNA11 antibody detected a single band at ~42 kDa in GNA11-overexpressing 293T cells and a

predominant ~42 kDa band in HUVECs; however, no band was observed between 24-76 KDa in GNA14-overexpressing 293T cells.

GNA14 antibody was generated by immunizing 2 rabbits with synthesized peptides based on the predicted sequence of human GNA14 (GenBank access # NM_004297) by GeneTel, Madison, WI. The antisera were pooled, affinity purified, and used for immunohistochemistry and Western blot analysis. The specificity of the GNA14 antibody was first verified in cell lysates from GNA11-overexpressing 293T cells (5 µg; a negative control), GNA14-overexpressing 293T cells (5 µg; a positive control), and HUVECs (20 µg; a positive control) using Western blotting. As shown in Fig. II.1A, GNA14 antibody detected one predominant band at ~42 kDa in GNA14-overexpressing 293T cells and HUVECs, but not in GNA11-overexpressing 293T cells. The specificity of the GNA14 antibody was further confirmed in human placental tissue sections using immunohistochemistry (Fig. II.1B). Placental tissue sections were probed with the GNA14 antibody (4 µg/ml) or with the GNA14 antibody which was preimmunoneutralized at 4^0C overnight with an excess amount (100-fold) of the synthesized GNA14 peptide immunogen. We observed that the synthesized GNA14 peptide immunogen greatly decreased the intensity of the GNA14 staining (Fig. II.1B). All of these data attest to the specificity of the GNA14 antibody.

Immunohistochemistry

Immunolocalization of GNA11 and GNA14 was visualized by indirect detection via the avidin: biotinylated-peroxidase complex method (Vector Laboratories, Burlingame, CA), as previously described (Chung *et al.*, 2004; Jiang *et al.*, 2010). Paraffin-embedded

tissue sections (n=4–5 for each experimental group) were cut at 5-μm thickness. Antigen retrieval was performed in sodium citrate buffer solution (10 mM, pH 6.5) before staining. After endogenous peroxidase quenching with 3% H_2O_2, tissue sections were counterstained lightly with hematoxylin. Nonspecific binding was blocked with 1% horse serum albumin. Tissue sections were then probed with a rabbit antibody against human GNA11 (4 μg/ml; Abgent) or GNA14 (4 μg/ml; GeneTel).

The controls consisted of replacing the primary antibody with preimmune rabbit IgG at the same concentration as the primary antibody. The secondary antibody used was a biotinylated universal antibody. The specific immunoreactivity was visualized by 3-amino-9-ethylcarbazole (Vector Laboratories).

Western Blot Analysis

Western blot analysis was conducted as described (Chung *et al.*, 2004; Jiang *et al.*, 2010). Placental tissues were homogenized and lysed by sonication in buffer (50 mM HEPES, 0.1 M NaCl, 10 mM EDTA, 4 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate [pH 7.5], 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin). After centrifugation, protein concentrations of the supernatant were determined with BSA (fraction V; Sigma, St. Louis, MO) as a standard. Protein samples of placental tissue supernatants (50 or 100 μg/sample) were separated on 10% SDS-PAGE gels, and electrically transferred to polyvinylidene difluoride membranes. In parallel, supernatants of HUVECs (20 μg protein/sample) were included in each gel as standards.

The membranes were first probed with GNA11 (1:1000) or GNA14 antibody (1:500), followed by reprobing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; Novus, Littleton, CO) and β-actin (1:10,000; Life Technologies, Grand Island, NY) as loading controls. Proteins were visualized using enhanced chemiluminescence (ECL) reagents from Amersham Biosciences (Piscataway, NJ), followed by exposure to chemiluminescence films. The immunoreactive signals were analyzed by densitometry using NIH Image-J imaging analysis software (Bethesda, MD).

Statistical Procedures

Data were analyzed using t-test (SigmaStat, Jandel Co., San Rafael, CA). Differences were considered significant at $p < 0.05$.

II.3. Results

Immunolocalization

To determine the cellular distribution of GNA11 and GNA14 in human placentas and umbilical cord vessels, immunohistochemistry was conducted. We observed that GNA11 and GNA14 were localized in the FT, NT and sPE placentas as well as in umbilical cord vessels (Fig. II.1 and 2). In placental villi, GNA11 and GNA14 were present primarily in syncytiotrophoblasts and cytotrophoblasts in FT, and syncytiotrophoblasts in NT and sPE placentas (Fig. II.2A-2C and 2F-2H). GNA11 and GNA14 were also present in villous stromal cells and vascular endothelial cells (Fig. II.2A-2C and 2F-2H). In addition, GNA14 was also detected in erythrocytes in NT and sPE placentas (Fig. II.2H). In umbilical cords, GNA11 and GNA14 was immunolocalized in endothelial cells of the umbilical cord artery

and vein (Fig. II.2D, 2E, 2I, and 2J), whereas only GNA11 was present in the smooth muscle cells of the umbilical artery (Fig. II.2E). No positive GNA11 and GNA14 staining was observed in the IgG control in all tissues studied. These data indicate that GNA11 and GNA14 are expressed in multiple cell types in human placentas and umbilical cords.

Western Blot Analysis

To quantify changes in GNA11 and GNA14 proteins in human placentas, Western blot analysis was performed. Both GNA11 and GNA14 were detected at ~42 kDa in human placental tissues and in HUVECs (positive control) (Fig. II.1 and 3), corresponding to the reported molecular masses of GNA11 (UniProtKB # P29992; Nakamura *et al.*, 1991; Wilkie *et al.*, 1991; Laugwitz *et al.*, 1996) and GNA14 (UniProtKB # O95837; Wilkie *et al.*, 1991). When normalized to GAPDH and β-actin, the levels of GNA14, but not GNA11 protein were increased ($p < 0.01$) by ~2.5- and 2.9-fold, respectively, in sPE vs. NT placentas (Fig. II.3A). GNA11 was undetectable in FT but was abundantly present in NT placentas (Fig. II.3B). When normalized to GAPDH and β-actin, the protein levels of GNA14 were increased ($p < 0.01$) by ~10.7- and 7.7-fold, respectively, in NT vs. FT placentas (Fig. II.3B). These data demonstrate that, whereas the protein levels of both GNA11 and GNA14 in placentas are increased from FT to NT, only the GNA14, and not GNA11, protein levels are elevated in sPE vs. NT placentas.

II. 4. Discussion

In the current study, we have demonstrated the cellular distribution and expression of GNA11 and GNA14 proteins in human placentas from FT, NT and sPE pregnancies.

We found that GNA11 and GNA14 were present in several cell types in placentas and umbilical cord vessels (e.g., trophoblast cells, endothelial cells, vascular smooth muscle cells, and villous stromal cell). More importantly, we observed that, as the protein levels of both GNA11 and GNA14 in placentas exhibited similar increases from in FT to NT, only GNA14 was further elevated in sPE as compared to NT. Thus, together with the evidence resulting from the knockout mice (Offermanns *et al.*, 1997, 1998; Wang *et al.*, 2015), a systemic multiple gene approach (Kohara *et al.*, 2008), and in vitro studies (Zeng *et al.*, 2002*b*, 2003; Wang *et al.*, 2015; Sivaraj *et al.*, 2015), our current data suggest that although both GNA11 and GNA14 may affect the function of multiple human placental cells, GNA14 may have a unique role in sPE placentas as in other hypertension-related diseases, such as hypertension and pulmonary artery hypertension (Kohara *et al.*, 2008; Abdul-Salam *et al.*, 2010; Lei *et al.*, 2014).

It is not surprising that GNA11 and GNA14 are expressed in syncytiotrophoblasts, trophoblasts, stromal cells and endothelial cells in all placentas from FT, NT and sPE pregnancies because GNA11 and GNA14, especially GNA11, are known to be expressed in many cell types of various mammalian tissues (Nakamura *et al.*, 1991; Wilkie *et al.*, 1991; Laugwitz *et al.*, 1996). Thus, given that GNA11 and GNA14 are critically involved in mediating fetal vascular development (Offermanns *et al.*, 1998), vascular functions (Wang *et al.*, 2015; Sivaraj *et al.*, 2015), hypertension (Kohara *et al.*, 2008; Abdul-Salam *et al.*, 2010; Lei *et al.*, 2014) and endothelial function (Zeng *et al.*, 2002*b*, 2003; Wang *et al.*, 2015; Sivaraj *et al.*, 2015), these data suggest that both GNA11 and GNA14 may also mediate functions of these placental cells.

The current finding that only the GNA14, and not GNA11, protein levels were elevated in sPE over NT placentas implies that GNA14 may be a key mediator in placentas from sPE pregnancies, in which hypertension is one of the hallmarks (Solomon & Seely, 2004, 2006). This observation is extremely interesting, as other investigators have reported that GNA14 expression is also high in lung tissues from patients with pulmonary artery hypertension (Abdul-Salam *et al.*, 2010; Lei *et al.*, 2014). Thus, our current data support the notion that GNA14 is a hypertension-susceptibility gene in humans (Kohara *et al.*, 2008) and suggest that GNA14 overexpression might be used as an index for predicting hypertension-related diseases, especially when in conjunction with other clinical diagnoses. To date, it is unclear what are the underlying mechanisms elevating GNA14 expression or the consequences of GNA14 overexpression in meditating endothelial functions. However, we have recently shown that chronic low oxygen significantly increases expression of GNA14 mRNA in HUAECs (Jiang *et al.*, 2013*b*). Thus, chronic low oxygen and/or hypoxia within the tissues may upregulate GNA14 expression in the placenta tissues. Moreover, the exact role of GNA14 in hypertension also remains elusive. Nonetheless, because many hypertension-related diseases are associated with endothelial dysfunction (Ross, 1999; Bradford C. Berk, Judith Haendeler, 2000; Granger *et al.*, 2001) and endothelium of placenta and umbilical cord vessels is one of major cell types expressing GNA14 (Fig. II.2), it is possible that GNA14 overexpression in endothelial cells may cause endothelial dysfunction (e.g., decreased cell proliferation, migration and/or viability), leading to hypertension-related diseases.

One may consider that the different expression of GNA14 between NT and sPE placentas is due to the different gestational ages of sPE and NT pregnancies, as observed in the current study. However, the protein levels of both GNA11 and GNA14 were increased in placentas from FT to NT pregnancies (Fig. II.3B), suggesting an increasing trend in the expression of placental GNA11 and GNA14 proteins from early pregnancy to full term. Thus, together with the observation that only GNA14 protein levels were elevated in sPE placentas (Fig. II.3A), it is unlikely that the shorter gestational age in PE pregnancies would be a major factor contributing to high GNA14 expression in sPE placentas, unless GNA14 expression uniquely (relative to GNA11) varies in a biphasic fashion (e.g., low in FT, high in ~33 weeks, and low again in NT).

In conclusion, the current data suggest that GNA11 and GNA14 may play important roles in mediating normal cellular function in human placentas; however, GNA14 overexpression in placentas may contribute to placental cellular dysfunction during sPE pregnancies, a hypertension-related disease. Further studies are warranted and are currently underway to explore the actions and signaling mechanisms of GNA11 and GNA14 in placental endothelial cells.

II.5. Figures

Figure II.1.

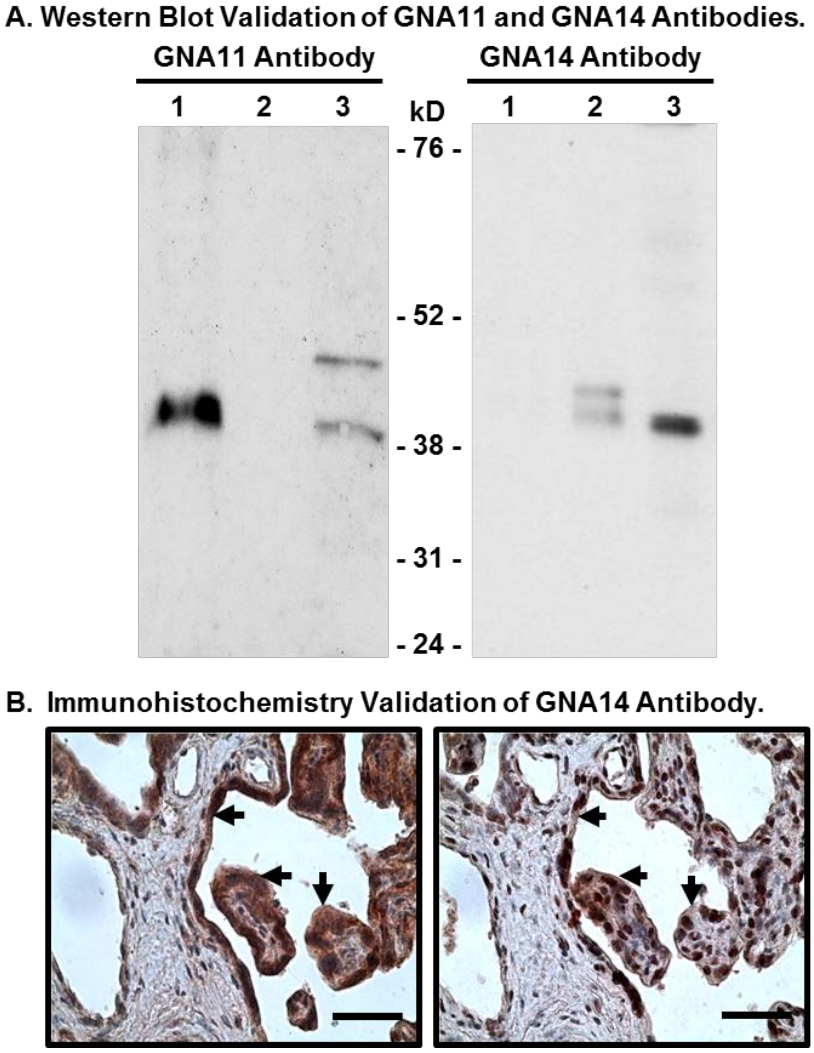

Figure II.1. Validation of GNA11 and GNA14 antibodies. (A) Western blot validation of GNA11 and GNA14 antibodies. Lane 1: GNA11-overexpressing 293T cells (10 µg protein); Lane 2: GNA14-overexpressing 293T cells (10 µg protein); Lane 3: HUVECs (20 µg protein). (B) Immunohistochemistry validation of GNA14 antibody in human placentas (n=4) from severe preeclamptic (sPE) pregnancies. After counterstaining with hematoxylin, the adjacent tissue sections were probed with the GNA14 antibody alone (4 µg/ml, left panel) or the GNA14 antibody pre-immunoneutralized with its peptide immunogen (right panel). Representative images are shown. Arrows: syncytiotrophoblasts. Bar, 50 µm.

Figure II.2. A-C, F-H

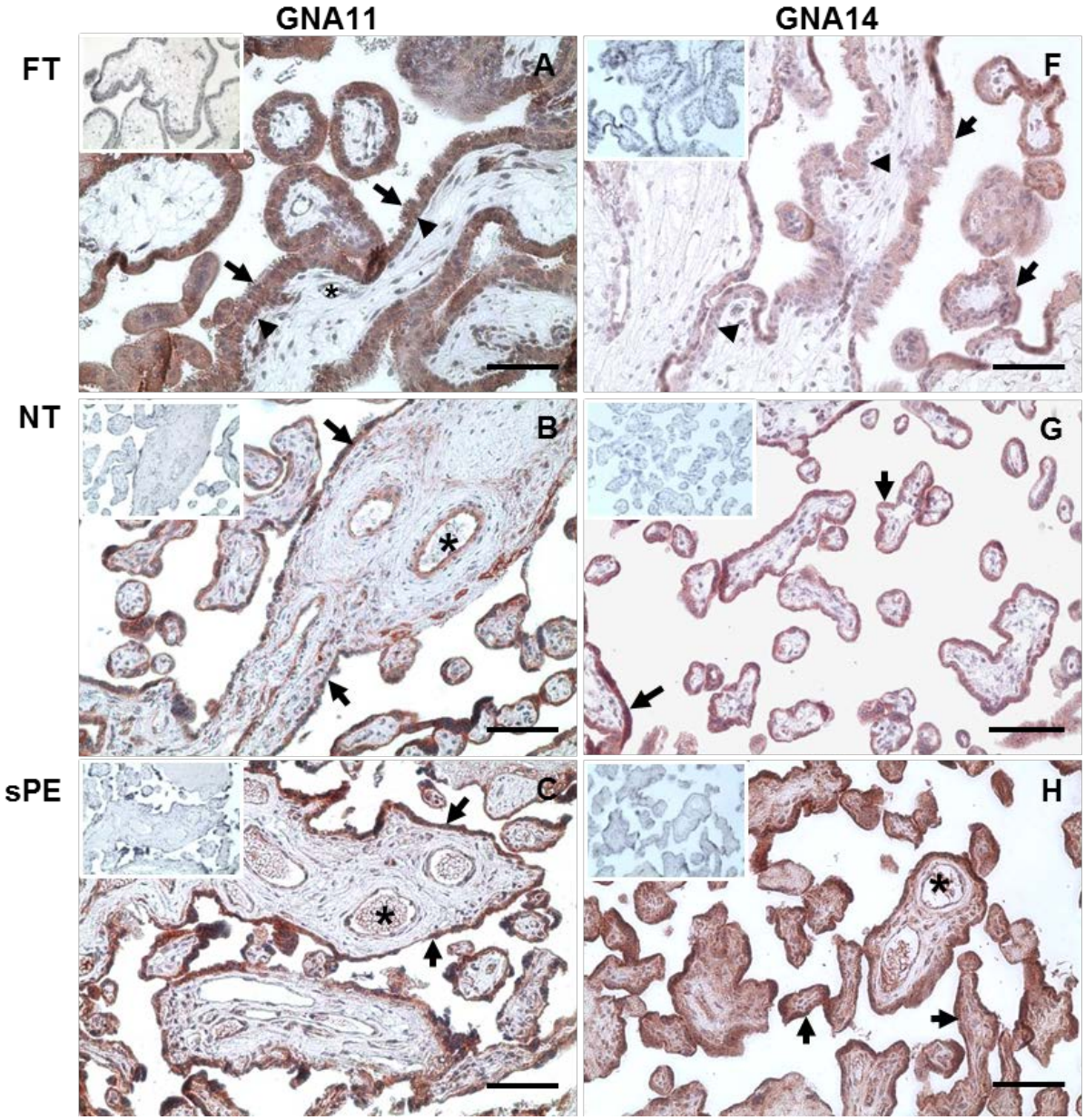

Figure II.2. D, E, I, J

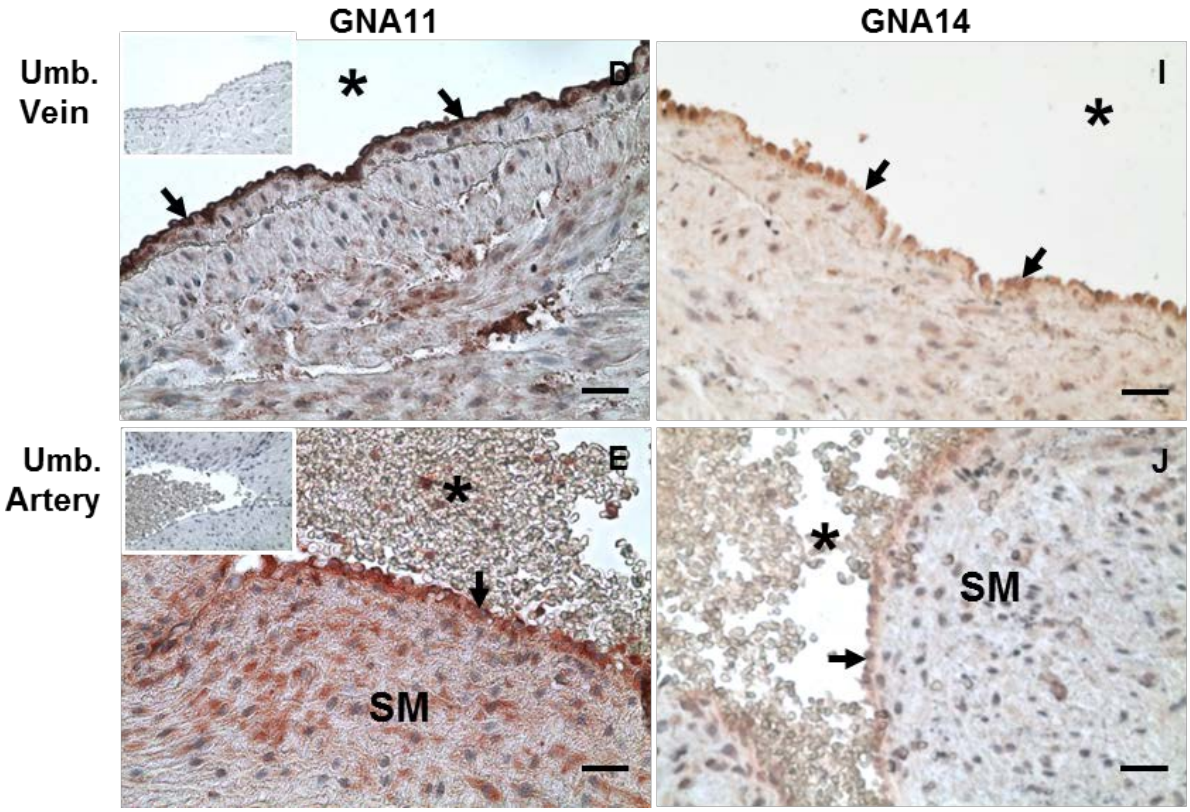

Figure II.2. Immunolocalization of GNA11 and GNA14 in human placentas from first trimester (FT), normal term (NT), and severe preeclamptic (sPE) pregnancies as well as in the umbilical (Umb.) vein and artery from NT pregnancies. The tissue sections (n=4–5 placentas for each group) were probed with GNA11 or GNA14 antibodies (4 μg/ml) after light counterstaining with hematoxylin. The preimmune IgG controls (4 μg/ml) are shown in the inset images. SM: smooth muscle layer. *: lumen of blood vessels; arrows: syncytiotrophoblasts in placental tissues and endothelial cells in umbilical vessels; arrow heads: trophoblasts. Bar, 50 μm.

Figure II.3.

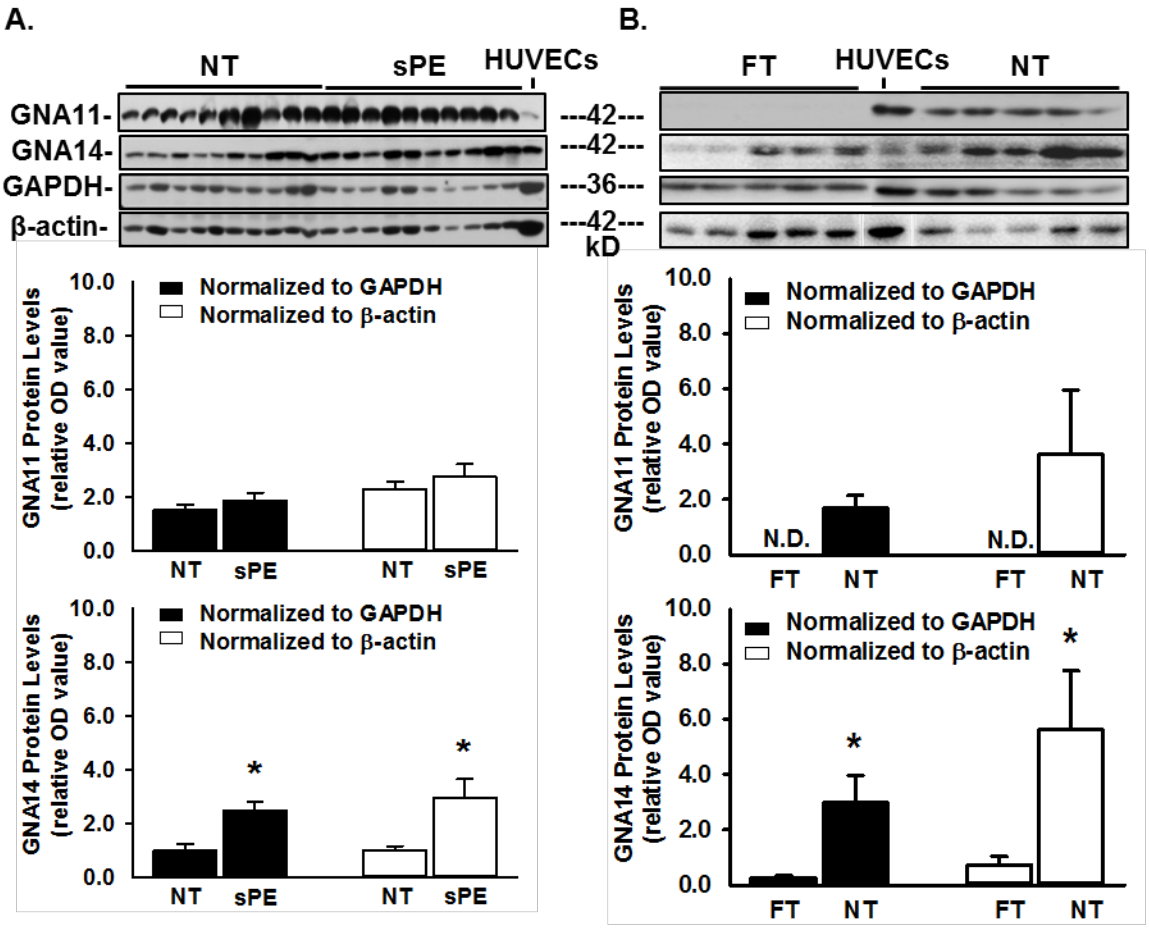

Figure II.3. Western blot analysis for GNA11 and GNA14 in human placentas and in HUVECs. (A) Placentas from normal term (NT) and severe preeclamptic (sPE) pregnancies (n=10/group) and HUVECs from NT pregnancies. (B) Placentas from FT and NT pregnancies (n=10/group). A representative image is shown for Panel B. Proteins (100 µg in A and 50 µg in B for each placental sample, as well as 20 µg for HUVECs) were subjected to Western blotting. Data are expressed as mean ± SEM of OD values normalized to GAPDH or β-actin. N.D.: non-detectable. *$p < 0.01$.

Chapter III. GNA11 Differentially Mediates FGF2- and VEGFA-Induced Cellular Responses in Human Fetoplacental Endothelial Cells

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I am the first author of this paper. I participated in conception and design of experiments; collection and assembly of data; data analysis and interpretation; drafting and critical revision of manuscript. The current version has been formatted for this dissertation.

Key Point Summary

- Fetoplacental vascular growth is critical to fetal growth. Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) are two major regulators of fetoplacental vascular growth. G protein α subunit 11 (GNA11) transmits signals from many external stimuli to the cellular interior and may mediate endothelial function.
- It is unknown if GNA11 mediates FGF2- and VEGFA-induced endothelial cell responses under physiological chronic low O_2.
- We show that knockdown of GNA11 significantly decreases FGF2- and VEGFA-induced fetoplacental endothelial cell migration, but not proliferation and permeability.
- Such decreases in endothelial migration are associated with increased phosphorylation of phospholipase C-β3 (PLCβ3).
- Our results suggest differential roles of GNA11 in mediating FGF2- and VEGFA-induced fetoplacental endothelial function.

Abstract

During pregnancy, fetoplacental angiogenesis is dramatically increased in association with rapidly elevated blood flows. Any disruption of fetoplacental angiogenesis may lead to pregnancy complications such as intrauterine growth restriction. Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) are crucial regulators of fetoplacental angiogenesis. G protein α subunits q (GNAq) and 11 (GNA11), two members of $G\alpha_{q/11}$ subfamily are involved in mediating vascular growth and basal blood pressure. However, little is known about roles of GNA11 alone in mediating the FGF2- and VEGFA-induced fetoplacental endothelial function. Using a cell model of human umbilical cord vein endothelial cells (HUVECs) cultured under physiological chronic low O_2 (3% O_2), we showed that GNA11 siRNA dramatically inhibited ($p < 0.05$) FGF2- and VEGFA-stimulated fetoplacental endothelial migration (~36% and ~50%, respectively), but not proliferation and permeability. GNA11 siRNA also elevated ($p < 0.05$) FGF2- and VEGFA-induced phosphorylation of phospholipase C-β3 (PLCβ3) at S537 in a time-dependent fashion, but not ERK1/2, and AKT1. These data suggest that GNA11 mediates FGF2- and VEGFA-stimulated fetoplacental endothelial cell migration partially via altering activation of PLCβ3.

III. 1. Introduction

During pregnancy, the fetoplacental vasculature undergoes tremendous growth and remodeling to support normal fetal growth (Magness & Zheng, 1996). In addition, impaired fetoplacental vascular growth and function are associated with several pregnancy complications including preeclampsia and intrauterine growth restriction (Zygmunt *et al.*, 2003). These complications not only jeopardize maternal and fetal health, but also increase risk of cardiovascular disease onset in adult offspring (Wang *et al.*, 2002; Powe *et al.*, 2011; Boeldt *et al.*, 2014; Brodowski *et al.*, 2017; Zhou *et al.*, 2017). Thus, a better understanding of the regulation of fetoplacental endothelial growth and function is a prerequisite for the design of therapeutic controls for fetoplacental vascular function.

Two major regulators of fetoplacental angiogenesis, fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA), activate high-affinity receptor tyrosine kinases (RTKs) (Klein *et al.*, 1997; Ferrara *et al.*, 2003; Wang & Zheng, 2012). Subsequently, they induce phosphorylation of a cascade of protein kinases, such as mitogen-activated protein kinase 3/1 (ERK1/2) and v-akt murine thymoma viral oncogene homolog 1 (AKT1), thus leading to various cell responses (Ferrara *et al.*, 2003; Podar & Anderson, 2008; Turner & Grose, 2010). These two protein kinases are actively involved in mediating fetal endothelial proliferation, migration, and survival (Wang & Zheng, 2012; Jiang *et al.*, 2013*a*, 2013*b*).

Besides RTKs, peptide growth factors may also interact with G protein and mediate a vast array of cell function (Pyne & Pyne, 2011). G protein is composed of α, β, and γ subunits, among which α subunit is a major functional component. G protein α subunits are classified into four subfamilies, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. $G\alpha_{q/11}$ consists of four

members, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$ (GNAq, 11, 14, and 15/16, respectively) (Wettschureck & Offermanns, 2005; Hubbard & Hepler, 2006). Among these four members, GNAq and 11 are key mediators of cardiovascular function. For instance, double knockout of GNAq and 11 in mice results in embryonic death, due to malformation of the heart (Offermanns *et al.*, 1998). Mice carrying only one intact allele of GNAq or GNA11 also die shortly after birth (Offermanns *et al.*, 1998). In addition, both GNAq and 11 are necessary for maintaining basal blood pressure and developing salt-induced hypertension (Wirth *et al.*, 2008). We have reported expression of GNA11 in human placentas and in human umbilical cord vein endothelial cells (HUVECs) (Zhao *et al.*, 2014). In HUVECs, double knockdown of GNAq and 11 impairs VEGFA-induced cell migration (Zeng *et al.*, 2002), and inhibits VEGFA-, but not FGF2-induced cell proliferation (Zeng *et al.*, 2003). Our knowledge on roles of GNA11 in mediating endothelial function is limited. Sivaraj et al. (2015) have demonstrated that knockout of GNA11 in endothelial cells does not affect postnatal angiogenesis in mice retina, while knockout of GNAq or both of GNAq and 11 in endothelial cells significantly decreases postnatal angiogenesis in mice. More recently, Couto et al. (2017) have reported that a somatic mutation of GNA11 is associated with capillary malformation in human. These data suggest the importance of GNA11 in mediating endothelial function, possibly depending on the origins of endothelial cells.

Members of the $G\alpha_{q/11}$ subfamily can activate phospholipase C (PLC), predominantly PLCβ3 (Offermanns, 1999; Rhee, 2001). In HUVECs, knockdown of PLCβ3 inhibits VEGFA-stimulated cell migration, whereas it enhances VEGFA-stimulated

cell proliferation in association with increased phosphorylation of PLCβ3 at serine 537 and 1105 (S537 and S1105) (Bhattacharya *et al.*, 2009).

Fetoplacental endothelial cells *in vivo* reside under low oxygen environments (3-8% O_2) relative to ambient oxygen level (~ 21%) at sea level throughout pregnancy (Jauniaux *et al.*, 2001; Meschia, 2013; Rodesch *et al.*, 1992). The oxygen level is 3.7% (range 2.3–5.1%) in the umbilical vein at the end of gestation (Meschia, 2013). These low oxygen levels are believed to be important for normal endothelial function (Burton *et al.*, 1996; Mayhew, 2003; Meschia, 2013; Zamudio, 2007), including gene expression and FGF2- and VEGFA-induced fetoplacental endothelial responses (Jiang *et al.*, 2013*a*, 2013*b*). As such, HUVECs cultured in a physiological low oxygen condition were used in this study, as physiological low oxygen may more closely mimic *in vivo* condition.

To date, little is known regarding roles of GNA11 in mediating FGF2- and VEGFA-induced fetal endothelial function under physiological low oxygen levels during pregnancy. Here, we tested the hypothesis that GNA11 alone mediates FGF2- and VEGFA-induced fetal endothelial cell proliferation, migration and permeability via altering phosphorylation of ERK1/2, AKT1, and PLCβ3 using HUVECs constantly cultured under physiological low oxygen as a cell model.

III.2. Materials and Methods

Ethical Approval

The umbilical cord collection protocol was approved by the Institutional Review Board of UnityPoint Meriter Hospital (Madison, WI), and the Health Sciences Institutional Review Boards of the University of Wisconsin–Madison (Protocol # 2004-006). The

subjects gave written, informed consent before participating. All procedures were conducted in accordance to the Declaration of Helsinki, except for registration in a database.

Primary HUVECs and Culture Conditions

HUVECs were isolated from umbilical cord veins of normal pregnancy using the previously described collagenase enzyme protocol (Jiang *et al.*, 2013*a*, 2013*b*, 2014; Li *et al.*, 2015; Zhou *et al.*, 2017). After isolation, cells were cultured in endothelial culture medium (ECM; #1001 Sciencell, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (p/s), 1% amphotericin B (AB; #15290018, Thermo Fisher Scientific, Waltham, MA), and 1% endothelial cell growth supplement (ECGS) under a physiological chronic normoxia (PCN; 37^0C, 5% CO_2, 3% O_2, ~92% N_2) condition.

Cells were purified and verified for their endothelial phenotypes as described (Jiang *et al.*, 2013*a*, 2013*b*, 2014). After verification, cells from five individual cell preparations were pooled and cells at passages 4-5 were used in this study.

All media used for cell culture and experiments were pre-purged with N_2 and equilibrated in a hypoxia incubator adjusted to the PCN condition before addition to cells. Dissolved O_2 in media was monitored using a dissolved oxygen meter. All experiments were conducted either in a hypoxia incubator adjusted to the PCN condition or a 3% O_2 heated oxygen-controlled glovebox (Coy Laboratory Products, Grass Lake, MI) as described (Jiang *et al.*, 2013*a*, 2013*b*, 2014).

GNA11 siRNA Transfection

To study roles of GNA11 in FGF2- and VEGFA-induced cell responses in HUVECs, siRNA transfection was performed as described (Wang *et al.*, 2008; Li *et al.*, 2015; Jiang *et al.*, 2014). A pool of 4 siRNAs specifically targeting human GNA11 (Catalog # L-010860-00-0005; GenBank # NM_002067.4) and a pool of four scrambled siRNAs (ssiRNA; #D-001810-10-05) were purchased from Dharmacon (Lafayette, CO). The siRNA and ssiRNA were pre-mixed with Lipofectamine RNAiMAX transfection reagent (vehicle; #13778030, Invitrogen, Carlsbad, CA) at room temperature. Subconfluent cells were cultured in antibiotic- and serum-free media (ECMb; #1001, Sciencell) containing GNA11 siRNA (20 nM) or ssiRNA (20 nM). After 4 hr culture (transfection), an equal amount of ECM supplied with 10% FBS, 2% p/s, 2% AB and 2% ECGS was added. Cells were harvested and subjected to Western blotting 2, 3, and 4 days post transfection.

Cell Migration

For cell migration assays, after 2 days of transfection with the vehicle, ssiRNA and GNA11 siRNA, cells were serum-starved and placed (30,000/insert) into each insert using transwell system (Corning, Corning, NY) as described (Jiang *et al.*, 2013*a*, 2013*b*, 2014; Li *et al.*, 2015, 2017). FGF2 or VEGFA was added into the bottom wells (final concentration 100ng/ml; #10014-HNAE and #80006-RNAB, respectively, Sino Biologic Inc, China). After 16 hr of culture, calcein AM (#C3100MP, Invitrogen) was added into bottom wells (final concentration 4 μg/ml). Five pictures were taken at random sites using a Nikon TE2000U inverted microscope. Cell number was quantified by Metamorph imaging analysis program (Molecular Devices, Sunnyvale, CA). ECMb supplemented

with 2% heated inactivated FBS, 1% p/s, and 1% AB was used for preparing control media and final growth factor solutions.

Cell Proliferation

Cell proliferation was evaluated using the crystal violet method as previously described (Wang *et al.*, 2008; Song *et al.*, 2009; Li *et al.*, 2015, 2017). Briefly, after 2 days of transfection, cells were inoculated into 96-well plates (5 - 8,000 cells/well) and cultured overnight. After serum starvation for 16-24 hr, cells were treated with control media, FGF2, or VEGFA (100 ng/ml) for 48 hr. Cells were rinsed with PBS, fixed in a methanol, and stained with 0.1% crystal violet solution (Sigma, St. Louis, MO). After solubilization, the OD value of each well was measured by a microplate reader at 570 nm (Bio-Tek, Winooski, VT). ECMb supplemented with 0.2% heated inactivated FBS, 1% p/s, and 1% AB was used for preparing control media, and final growth factor solutions.

Cell Permeability

Cell permeability was examined using an electric cell-substrate impedance sensing system (ECIS Zθ; Applied Biophysics, Troy, NY) as described (Zhou *et al.*, 2017). Briefly, HUVECs were treated with vehicle, ssiRNA, or GNA11 siRNA as described above. After 2 days of transfection, cells (50,000 cells/well) were inoculated into 96W10E+ ECIS array plates (Applied Biophysics) pre-coated with 10nM cysteine and 0.1% gelatin. Resistance of each electrode was monitored at 4000 Hz. After culturing for 16-20 hr, resistance reaches a plateau, indicating 100% confluence. Cells were serum-starved for

3-4 hr in ECMb containing 1% p/s and 1% AB. FGF2 or VEGFA were added (final concentration 100 ng/ml). Changes in resistance were monitored for up to 24 hr.

Adenoviral Transduction

To further explore roles of GNA11 in FGF2- and VEGFA-induced cell responses in HUVECs, GNA11 was overexpressed using adenoviruses carrying *GNA11* with a green fluorescent protein (GFP) reporter (Ad-*GNA11;* Vector Biolabs, Malvern, PA) as previously described (Jiang *et al.*, 2014). Adenoviruses carrying GFP (Ad-*GFP*) were used as a control vector (Ren *et al.*, 2014). Amplification and transduction of adenovirus were performed as described (Liao *et al.*, 2009; Jiang *et al.*, 2014). We first verified the specificity of Ad-*GNA11* using polymerase chain reaction (PCR) with TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA). TaqMan primers for GNA11 (assay #: Hs00976153_m1, amplicon length 103 bp) and GNA14 (assay #: Hs01030246_m1, amplicon length 98 bp) were purchased (Applied Biosystems). PCR products were separated in a 2% agarose gel using the E-Gel system according to manufacturer's instructions (Thermo Fisher Scientific).

After confirmation of the specificity of Ad-*GNA11*, HUVECs at 50-60% confluency were transfected with Ad-*GNA11* or Ad-*GFP* at different multiplicity of infection (MOI). Cells were harvested and subjected to Western blotting after 3 days of transfection.

Western Blotting

Western blot analysis was conducted as described (Dai *et al.*, 2011; Jiang *et al.*, 2013*a*, 2013*b*; Li *et al.*, 2015*a*, 2017). Cells were lysed in the lysis buffer (50 mM HEPES,

0.1 M NaCl, 10 mM EDTA, 4 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate [pH 7.5], 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 5 µg/ml leupeptin, 5 µg/ml aprotinin). Cell lysates were collected and centrifuged. Protein samples (20-30 µg/sample) were separated on 10% SDS-PAGE gels, and electrically transferred to polyvinylidene difluoride membranes. Membranes were probed by primary antibodies (Table 1). Proteins were visualized by enhanced chemiluminescence (ECL) or ECL2 (Thermo Fisher Scientific). Signals were recorded by Epson Perfection 4990 Photo Scanner (Long Beach, CA). Data were analyzed using NIH Image J software. Polyclonal rabbit GNA14 antibody (GeneTel Laboratory, Madison, WI) was used for detecting GNA14 protein in GNA11 siRNA knockdown verification (Fig. III.1). We have previously verified and used this GNA14 antibody in human placental tissues and HUVECs (Zhao *et al*., 2014). Monoclonal mouse GNA14 antibody (Abnova, Walnut, CA) was used for examine GNA14 protein in Ad-*GNA11* overexpression assay (Fig. III.5).

Statistics

Data were analyzed using one-way ANOVA (SigmaStat software, Jandel Co., San Rafael, CA). When an F-test was significant, data were compared using the Bonferroni's Method (all pairwise or versus control multiple comparison procedures) or Student's t-test. $p < 0.05$ was considered statistically significant.

III.3. Results

GNA11 siRNA Suppresses GNA11 Protein Levels in PCN-HUVECs

To study the role of GNA11 in mediating FGF2- and VEGFA-induced cell responses, human-specific GNA11 siRNA was used to knock down GNA11 expression (Fig. III.1). Western blotting revealed that GNA11 siRNA, but not ssiRNA, significantly ($p < 0.05$) suppressed protein levels of GNA11 by ~70% compared with vehicle after 3 and 4 days of transfection (Fig. III.1). As a demonstration of the specificity of GNA11 siRNA, GNA11 siRNA transfection did not significantly alter protein levels of GNA14 for up to 4 days. GNA14 is another member of $G\alpha_{q/11}$ subfamily and shares ~70% of identity with GNA11 in amino acid sequences in human (Hubbard & Hepler, 2006). We also observed that GNA11 protein levels in the vehicle group were slightly decreased after 3 and 4 days (0.7 ± 0.17 fold of Day 2 after data were normalized to β-actin) of culture as compared with Day 2 (1.0 ± 0.15 fold of Day 2 mean), but this decrease did not reach statistical significance.

GNA11 siRNA Differentially Mediates Endothelial Migration, Proliferation, and Monolayer Integrity in Response to FGF2 and VEGFA

Compared with the control (serum-free media), FGF2 and VEGFA stimulated ($p < 0.05$) cell migration of HUVECs by ~2.4 and ~3.5 fold, respectively (Fig. III.2A). Compared with vehicle, GNA11 siRNA, significantly ($p < 0.05$) attenuated FGF2- and VEGFA-stimulated cell migration by ~36% and ~50%, respectively (Fig. III.2A) while ssiRNA had no effect.

Both FGF2 and VEGFA promoted ($p < 0.05$) proliferation of HUVECs (~2.7 and ~2.5 folds of control, respectively; Fig. III.2B). However, GNA11 siRNA did not significantly change FGF2- and VEGFA-stimulated cell proliferation (Fig. III.2B).

Compared with the control, VEGFA, but not FGF2, decreased ($p < 0.05$) the resistance of the cell monolayer in a time-dependent fashion (Fig. III.2D and C), suggesting that VEGFA increases cell permeability. After 2.5 hr of treatment, VEGFA maximally decreased ($p < 0.05$) resistance of monolayer by ~28%, which was maintained up to 24 hr. However, GNA11 siRNA had no significant effects on cell monolayer integrity in response to FGF2 and VEGFA.

GNA11 siRNA does not Alter FGF2- and VEGFA-Induced Phosphorylation of ERK1/2 and AKT1

To explore the signaling mechanisms by which GNA11 mediates FGF2- and VEGFA-induced cell migration, phosphorylation of ERK1/2 (T202/Y204) and AKT1 (S473) was examined (Fig. III.3). FGF2 and VEGFA time-dependently elevated ($p < 0.05$) phosphorylation of ERK1/2 (~3.22 and ~3.05 fold at 5 min vs. time 0, respectively). However, GNA11 siRNA did not significantly alter phosphorylation patterns of ERK1/2 compared with ssiRNA control (Fig. III.3).

Both FGF2 and VEGFA slightly induced phosphorylation of AKT1, but neither induction reached statistical significance compared with time 0, nor did GNA11 siRNA significantly change phosphorylation patterns of AKT1 (Fig. III.3). GNA11 siRNA did not significantly change protein levels of total ERK1/2 and AKT1 (Fig. III.3, quantitative data not shown).

GNA11 siRNA Increases Phosphorylation of PLCβ3 at S537 in Response to FGF2 and VEGFA.

In the ssiRNA group, FGF2 treatment did not induce phosphorylation of PLCβ3 S537 for up to 60 min (Fig. III.4). VEGFA rapidly increased ($p < 0.05$) phosphorylation of S537, starting at 5 min; this stimulatory effect was maintained through the final 30 min time point (Fig. III.4). Interestingly, GNA11 siRNA time-dependently increased ($p < 0.05$) phosphorylation of S537 when cells were treated with FGF2 and VEGFA (Fig. III.4). Specifically, compared with the ssiRNA control at the corresponding time point, GNA11 siRNA elevated ($p < 0.05$) FGF2-inducd phosphorylation of S537 by ~ 1.5 fold at 10, 20, and 30 min, while it increased VEGFA-induced phosphorylation of S537 by ~15.2 and ~12.0 fold, at 10 and 20 min, respectively. Nonetheless, in both ssiRNA and siRNA groups, neither FGF2 nor VEGFA induced significant phosphorylation of S1105 for up to 60 min (Fig. III.4). GNA11 siRNA did not significantly change protein levels of total PLCβ3 (Fig. III.4, quantitative data not shown).

Elevation of GNA11 and 14 Protein Levels by Ad-*GNA11*

We also attempted to further dissect roles of GNA11 by overexpressing GNA11 using Ad-*GNA11* in HUVECs. We verified the specificity of Ad-*GNA11* by PCR assay (Fig. III.5A). GNA11, but not GNA14 primers, generated a single band at ~ 100bp from the primary Ad-*GNA11* stock. Likewise, in the primary Ad-*GNA14* stock, GNA14, but not GNA11 primers produced a single band at ~98 bp (Fig. III.5A). These two bands correspond to the predicted amplicon lengths of GNA11 and 14 indicating the specificity of Ad-*GNA11*.

After confirmation of Ad-*GNA11* specificity, HUVECs were transfected with Ad-*GNA11* (Fig. III.5B). Surprisingly, after 3 days of transfection, Ad-*GNA11* increased ($p <$

0.05) GNA11 and GNA14 protein levels comparably, while Ad-*GFP* had no significant effect on either GNA11 or GNA14 protein levels in HUVECs (Fig. III.5B-D). This Ad-*GNA11*-induced overexpression started at 5 MOI of Ad-*GNA11* (~1.8 vs. ~1.5 fold for GNA11 and 14 protein, respectively), was maintained at 10 MOI (~5.2 vs. ~2.7 fold, respectively), and further elevated at 20 MOI (~18.2 vs. ~8.6 fold, respectively). Since we were unable to exclusively overexpress GNA11 in HUVECs using Ad-*GNA11*, no further gain of function assay was performed.

III.4. Discussion

In this study, we demonstrated that knockdown of GNA11 alone inhibits FGF2- and VEGFA-stimulated fetoplacental endothelial cell migration, but not proliferation and permeability under physiological chronic low oxygen. Such inhibition is associated with elevated phosphorylation of PLCβ3 S537, but without any change in phosphorylation of PLCβ3 S1105, ERK1/2 and AKT1 in response to FGF2 and VEGFA. These data indicate that GNA11 differentially mediates different FGF2- and VEGFA-induced fetoplacental endothelial responses. These data also suggest that GNA11 plays an important role in mediating FGF2- and VEGFA-induced fetoplacental cell migration, one major step of angiogenesis, possibly in part via enhanced phosphorylation of PLCβ3 S537.

To date, only a few studies have explored the roles of the $GNA_{q/11}$ subfamily in mediating endothelial angiogenic responses. Specifically, recent evidence has implicated the potential importance of GNAq (Sivaraj *et al.*, 2015) and GNA11 (Couto *et al.*, 2017) in mediating angiogenesis. In addition, double knockdown of GNAq and 11 in HUVECs blocks VEGFA-induced cell migration (Zeng *et al.*, 2002; Sivaraj *et al.*, 2015), and inhibits

VEGFA-, but not FGF2-induced, cell proliferation (Zeng *et al.*, 2003). Our current study provides novel evidence that GNA11 alone critically mediates not only VEGFA-, but also FGF2-induced migration in HUVECs under physiological chronic low oxygen. However, in contrast to the inhibitory effects of double knockdown of GNAq and GNA11 on VEGFA-stimulated proliferation and permeability (Zeng *et al.*, 2003; Sivaraj *et al.*, 2015), knockdown of GNA11 alone did not significantly affect either FGF2- or VEGFA-induced cell proliferation and permeability. Collectively, these data suggest that while GNAq is a major mediator for endothelial proliferation and permeability, GNA11 critically mediates endothelial cell migration in response to FGF2 and VEGFA during placental angiogenesis.

Alternatively, different oxygen levels (3% vs presumably 21% O_2) used to culture cells in the current study and the studies by Zeng *et al*. (2003) and Sivaraj *et al*. (2015) might explain such discrepancies. Particularly, we have recently shown that physiological chronic low oxygen (3% O_2) robustly alters gene expression profiles, and promotes cell responses (migration, proliferation, and signaling pathway activation) to FGF2 and VEGFA in HUVECs (Jiang *et al.*, 2013*a*, 2013*b*). In addition, we developed the primary cell line used in the current study by pooling five individual cell preparations, which might more closely represent the HUVEC phenotype as each individual cell preparation could vary highly in their response to stimuli. Given that the GNA11 siRNA only partially suppressed the GNA11 protein expression, it is possible that the residual GNA11 protein is sufficient to mediate cellular responses such as cell proliferation and permeability in HUVECs.

Both ERK1/2 and AKT1 critically mediate fetal endothelial function including proliferation, migration, and survival (Wang & Zheng, 2012; Jiang *et al.*, 2013*a*, 2013*b*).

However, our current data showed that GNA11 had no effect on activation of these two protein kinases, in agreement with the previous study when both GNAq and GNA11 are knocked down in HUVECs (Sivaraj *et al.*, 2015). These data indicate that GNAq and GNA11 do not play an important role in mediating FGF2- and VEGFA-mediated ERK1/2 and AKT1 activation in HUVECs.

Upon activation, PLC can trigger the release of intracellular calcium and further enhances protein kinase C activity, leading to modulation of various cell function (Offermanns, 1999; Hubbard & Hepler, 2006). In the PLCβ subfamily, PLCβ3 has the highest affinity for the $G\alpha_{q/11}$ subfamily (PLCβ4 and β3 ≥ PLC-β1 >> PLC-β2; Offermanns, 1999), and is a key mediator of VEGFA action in endothelial cells (Mukhopadhyay & Zeng, 2002; Bhattacharya *et al.*, 2009). For example, knockdown of PLCβ3 inhibits VEGFA-stimulated cell migration, yet promotes VEGFA-stimulated cell proliferation in HUVECs (Bhattacharya *et al.*, 2009). The phosphorylation of PLCβ3 at different sites is considered to be one of major mechanisms by which the activity of PLCβ3 is regulated (Yue *et al.*, 1998; Xia *et al.*, 2001; Yue & Sanborn, 2001; Bhattacharya *et al.*, 2009). In this regard, phosphorylation of PLCβ3 S1105 is believed to inhibit PLCβ3 activity (Yue *et al.*, 1998; Xia *et al.*, 2001), while phosphorylation of S537 appears to be active (Yue & Sanborn, 2001). However, VEGFA can increase phosphorylation of PLCβ3 S537 and S1105 in HUVECs in association with increased cell migration and proliferation (Bhattacharya *et al.*, 2009), suggesting that both sites could be active for VEGFA-induced endothelial function in HUVECs. In our current study, we also observed that in the control (ssiRNA) group, VEGFA, but not FGF2, elevated phosphorylation of PLCβ3 S537; however, neither VEGFA nor FGF2 altered phosphorylation of PLCβ3 S1105. Thus, increasing

phosphorylation of PLCβ3 S537 to certain levels might be important for VEGFA-, but not FGF2-stimulated cell function in HUVECs. Nonetheless, the current finding that GNA11 knockdown robustly elevated FGF2- and VEGFA-mediated phosphorylation of PLCβ3 S537 implies that GNA11 is a negative regulator of PLCβ3 S537 phosphorylation in response to FGF2 and VEGFA in HUVECs. More importantly, as GNA11 knockdown-increased PLCβ3 S537 phosphorylation is associated with decreases in FGF2- and VEGFA-stimulated cell migration, the excessive phosphorylation of PLCβ3 S537 could adversely affect cell migration in HUVECs under physiological chronic low oxygen as we proposed in Figure III.6. Further studies are needed to define the exact interaction of GNA11 and PLCβ3, as well as the role of PLCβ3 S537 phosphorylation in regulating activity of PLCβ3 as GNA11 and phosphorylation of PLCβ3 could be potential targets for therapeutic intervention for aberrant angiogenesis, which is associated with many human diseases such as fetal growth restriction.

Surprisingly, we observed that overexpression of GNA11 also elevated protein levels of GNA14, even at low MOI of Ad-*GNA11,* although the specificity of the Ad-*GNA11* expression vector was confirmed. To date, the mechanisms underlying such crossover overexpression are yet to be determined.

In conclusion, our current data clearly indicate differential roles of GNA11 in mediating FGF2- and VEGFA-induced fetoplacental endothelial function in association with different regulation of phosphorylation of PLCβ3 under a physiological low oxygen condition, suggesting the importance of GNA11 in fetoplacental angiogenesis.

III.5. Figures

Figure III.1.

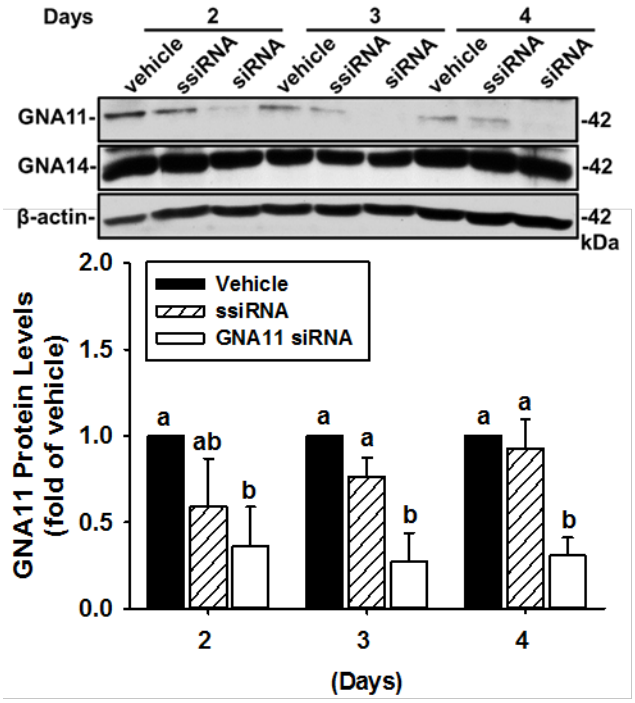

Figure III.1. Effects of GNA11 siRNA on GNA11 and 14 protein levels in HUVECs. Subconfluent cells were treated with transfection reagent (vehicle), scrambled siRNA (ssiRNA, 20 nM), or GNA11 siRNA (siRNA, 20 nM) for up to 4 days. Cellular proteins (20-30 µg) were subjected to Western blotting to detect GNA11, GNA14, and β-actin. Data are expressed as means ± SEM of the fold change relative to vehicle control. [a,b] Means with different letters differ (Bonferroni's all pairwise multiple comparison procedures; $p < 0.05$; n = 3 independent experiments).

Figure III.**2**.

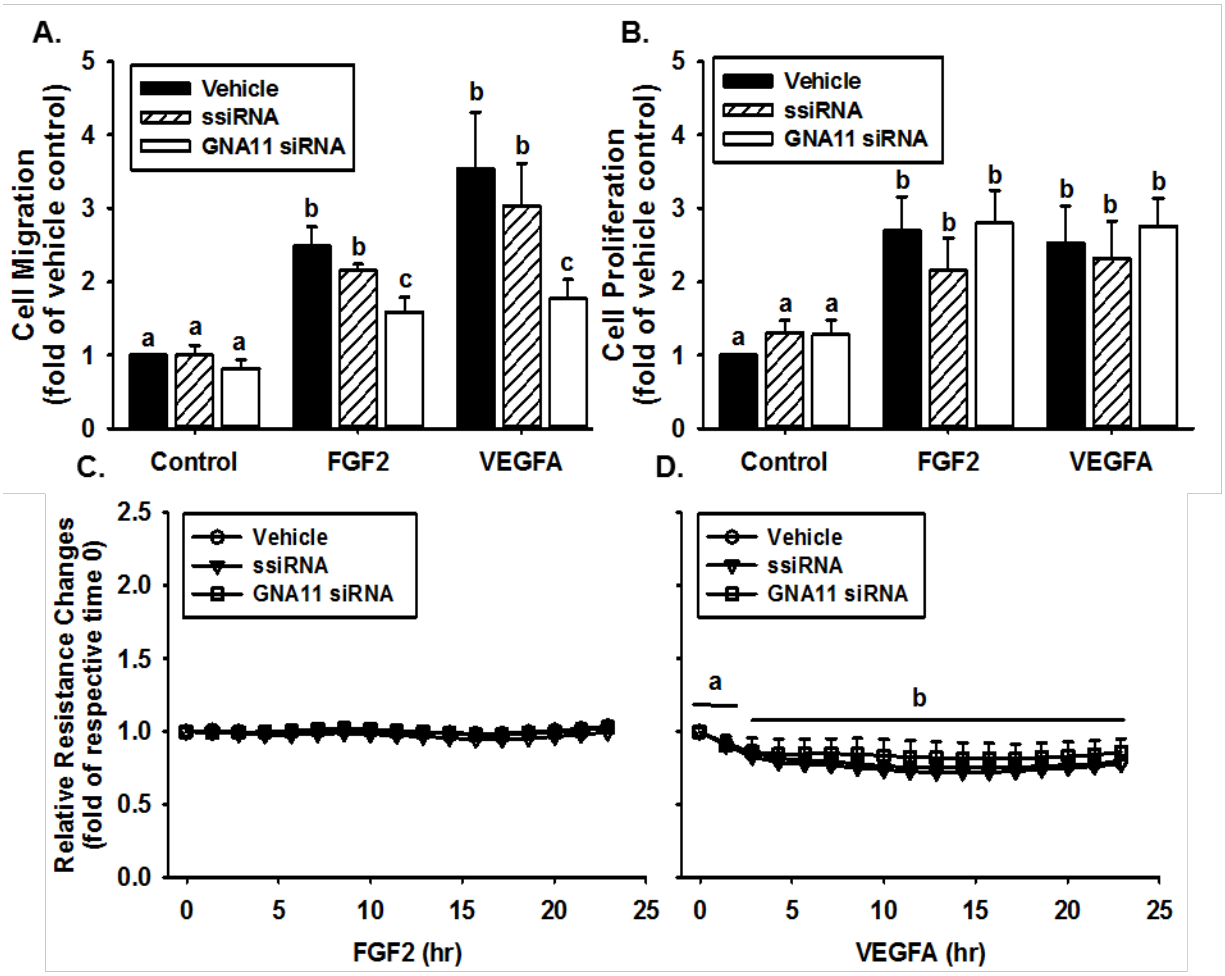

Figure III.2. Effects of GNA11 siRNA on FGF2- and VEGFA-mediated cell migration, proliferation, and permeability in HUVECs. Cells were transfected with vehicle, ssiRNA, or GNA11 siRNA for 2 days. After serum-starvation for 24 (migration and proliferation) or 3 hr (permeability), cells were treated with FGF2 and VEGFA (100 ng/ml) for 16 hr (cell migration, n = 6, A), 48 hr (cell proliferation, n = 4 independent experiments, B), or 24 hr (cell permeability, n = 3 independent experiments, C and D). Data are expressed as means ± SEM of the fold change relative to vehicle control or relative to time 0 for the control group. [a,b,c] Means with different letters differ (Bonferroni's Method all pairwise multiple comparison procedures; $p < 0.05$).

Figure III.**3**.

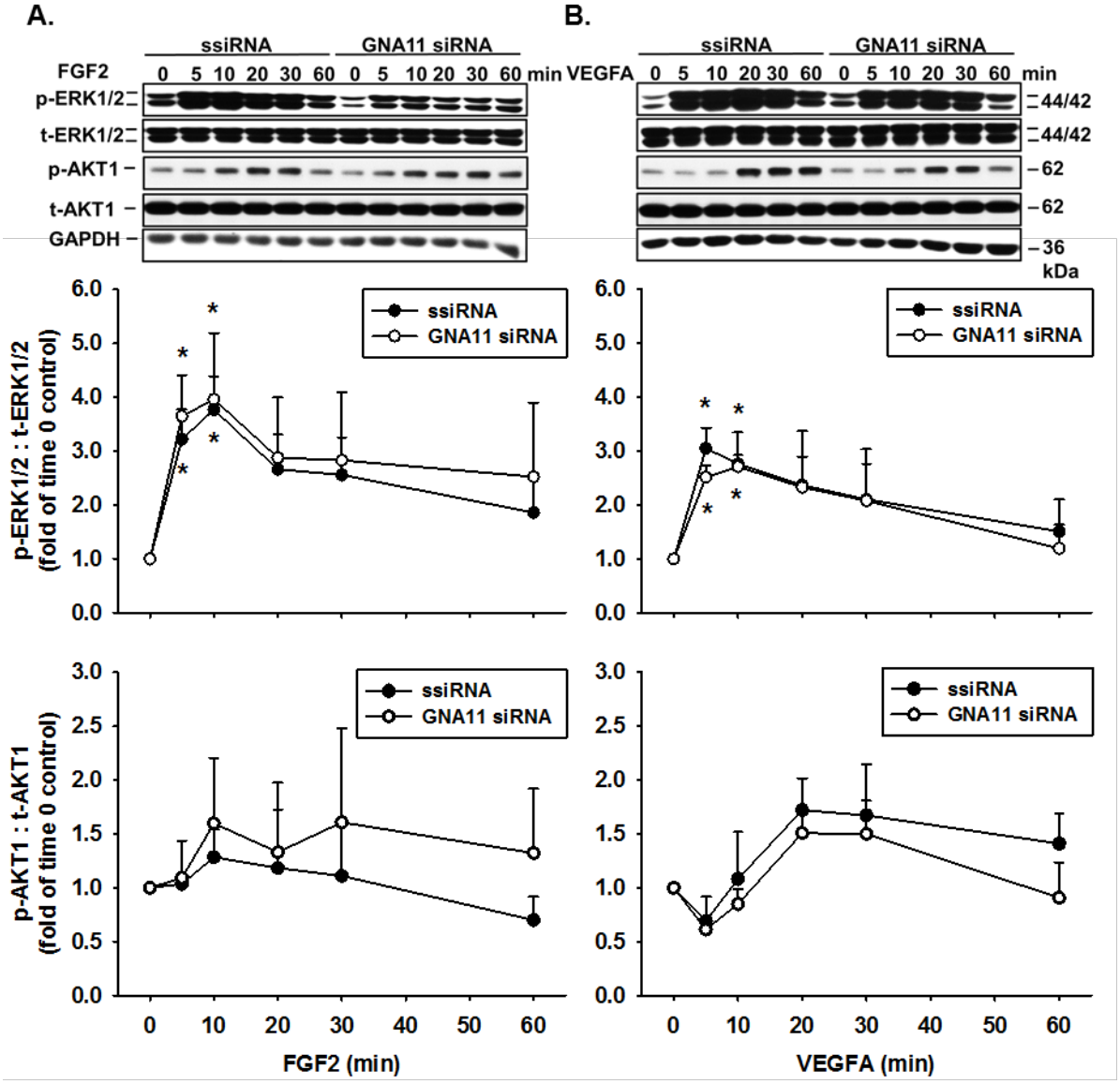

Figure III.3. Effects of GNA11 siRNA on phosphorylation of ERK1/2 and AKT1 in response to FGF2 and VEGFA in HUVECs. Cells were transfected with ssiRNA or GNA11 siRNA for 2 days. After 24 hr serum-starvation, cells were treated with 100 ng/ml of FGF2 (A) or VEGFA (B) for up to 60 min. Cellular proteins (20-30 µg) were subjected to Western Blotting. Data are expressed as means ± SEM of the fold change relative to the time 0 control. * Differ from the respective time 0 control (Bonferroni's multiple comparison procedures versus time 0 control; $p < 0.05$; n = 3 independent experiments).

Figure III.4.

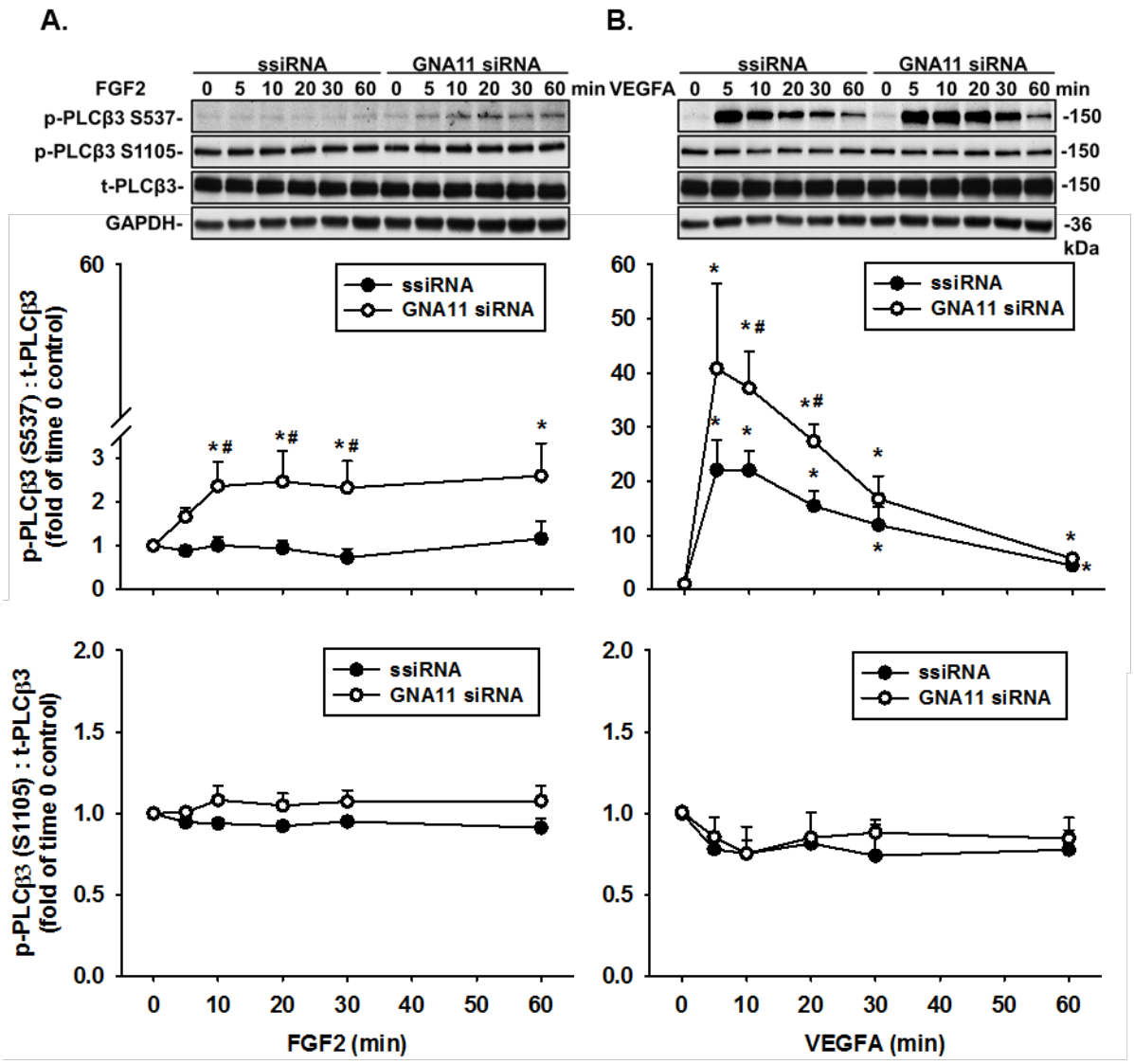

Figure III.4. Effects of GNA11 siRNA on mediating phosphorylation of PLCβ3 S537 and S1105 in response to FGF2 and VEGFA in HUVECs. Cells were transfected with ssiRNA or GNA11 siRNA. After 24 hr serum starvation, cells were treated by 100 ng/ml of FGF2 (A) and VEGFA (B) for up to 60 min. Proteins were subjected to Western Blotting. Data are expressed as means ± SEM of the fold change relative to the time 0 control. * Differ from the respective time 0 control (Bonferroni's multiple comparison procedures versus time 0 control; $p < 0.05$); # Differ from the corresponding time point (Student's t-test; $p < 0.05$; n = 4 independent experiments).

Figure III.5.

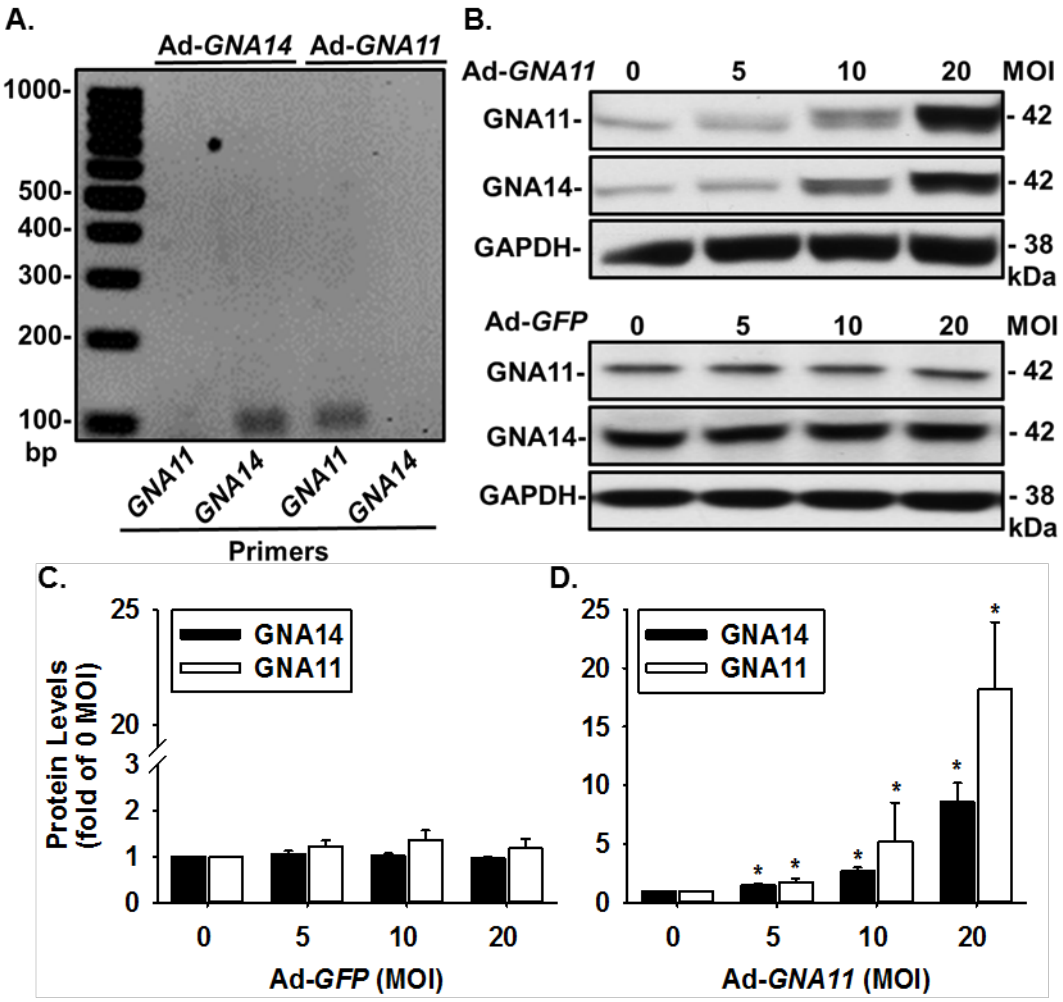

Figure III.5. Verification of primary stock of Ad-*GNA11* and effects of Ad-*GNA11* on GNA11 and GNA14 protein levels. A: PCR was used to verify primary stock of Ad-*GNA11* and *GNA14* as a control. Products of GNA11 and 14 primers are shown (~103 and ~98 bp, respectively). B: cells were transfected with Ad-*GNA11* or Ad-*GFP* for 3 days. Proteins (20-30 µg) were subjected to Western blotting to detect the indicated proteins. C and D: quantitative data for Ad-*GFP*- (C) and Ad-*GNA11*- (D) induced changes in GNA11 and 14 protein levels. Data are expressed as means ± SEM of the fold change relative to 0 MOI. * Differ from 0 MOI (Bonferroni's multiple comparison procedures versus 0 MOI; $p < 0.05$; n = 4 independent experiments).

Figure III.**6**.

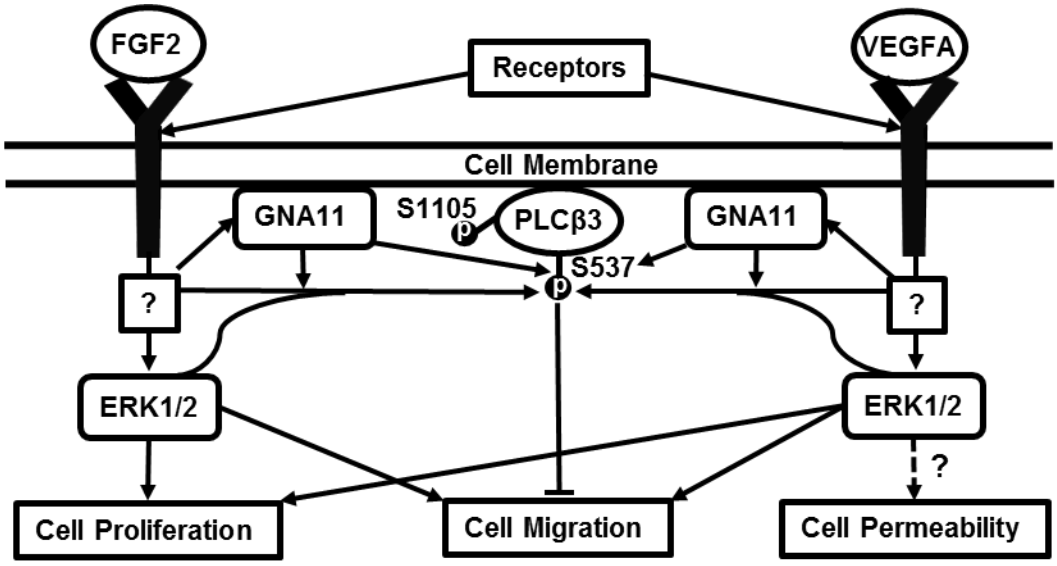

Figure III.6. A hypothesized signaling model for FGF2- and VEGFA-regulated fetal endothelial function via GNA11, PLCβ3, and ERK1/2 under physiological chronic low oxygen. In this model, phosphorylation of PLCβ3 S537 is mediated via two pathways. First, FGF2- and VEGFA-activated RTK and/or downstream signals including ERK1/2 phosphorylates GNA11, which blocks/does not affect (FGF2) or only partially increases (VEGFA) phosphorylation of PLCβ3 S537, depending on the degree and/or sites of GNA11 phosphorylation. Secondary, activated downstream signal can also directly phosphorylates PLCβ3 S537; this phosphorylation is either blocked (FGF2) or only partially increased (VEGFA) by phospho-GNA11. However, downregulation of GNA11 causes overphosphorylation of PLCβ3 S537, possibly via increasing and decreasing its sensitivity to FGF2- and VEGFA-activated protein kinases and phosphatase, respectively. This overphosporylation, along with alternations in phosphorylation on other active and inhibitory sites of PLCβ3 partially, inhibits FGF2- and VEGFA-induced cell migration. In this model, the effect of ERK1/2 on endothelial cell proliferation and migration are well established, while its effect on cell permeability remains elusive.

Chapter IV. GNA14 Mediates FGF2-, but not VEGFA- Induced Cellular Responses in Human Fetoplacental Endothelial Cells

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Key Point Summary

- Fetoplacental growth and function are critical to maternal and fetal health. Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) are two major regulators of fetoplacental vascular growth and function. G protein α subunit 14 (GNA14) is a signal transmitter inside cells.
- GNA14 overexpression is associated with hypertensive diseases. It is unknown if GNA14 mediates FGF2- and VEGFA-induced fetoplacental endothelial function.
- In this study, we show that GNA14 overexpression decreases FGF2-stimulated fetoplacental endothelial cell migration, while enhancing cell monolayer integrity in response to FGF2. GNA14 overexpression does not alter VEGFA-stimulated cell migration and proliferation, nor does it affect VEGFA-weakened cell monolayer integrity. GNA14 overexpression also fails to change intracellular Ca^{++} mobilization in response to FGF2 and VEGFA.
- GNA14 overexpression is associated with increased phosphorylation of phospholipase C-β3.
- These data indicate differential roles of GNA14 in mediating FGF2- and VEGFA-induced fetoplacental endothelial function.

Abstract

During pregnancy, a tremendous increase in fetoplacental angiogenesis is associated with elevated blood flow. Aberrant fetoplacental angiogenesis and vascular function may lead to pregnancy complications including fetal growth restriction and preeclampsia. Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) are crucial regulators of fetoplacental endothelial function. G protein *α* subunit 14 (GNA14), a member of Gαq/11 subfamily is involved in mediating hypertensive diseases and tumor vascularization. However, little is known about roles of GNA14 in mediating the FGF2- and VEGFA-induced fetoplacental endothelial function. Using a cell model of human umbilical vein endothelial cells (HUVECs) cultured under physiological chronic low oxygen (3% O_2), we show that transfecting cells with adenovirus carrying GNA14 cDNA (Ad-*GNA14*) dose-dependently increases ($p < 0.05$) protein expression of GNA14. GNA14 overexpression blocks ($p < 0.05$) FGF2-stimulated endothelial migration, whereas it enhances ($p < 0.05$) endothelial monolayer integrity (maximum increase of ~35% over the control at 24 hours) in response to FGF2. In contrast, GNA14 overexpression does not significantly alter VEGFA-stimulated cell migration, VEGFA-weakened cell monolayer integrity, and intracellular Ca^{++} mobilization in response to ATP, FGF2, and VEGFA. GNA14 overexpression does not alter either FGF2- or VEGFA-induced phosphorylation of ERK1/2. However, GNA14 overexpression time-dependently elevates ($p < 0.05$) phosphorylation of phospholipase C-β3 (PLCβ3) at S1105 in response to FGF2, but not VEGFA. These data suggest that GNA14 distinctively mediates fetoplacental endothelial

cell migration and permeability in response to FGF2 and VEGFA, possibly in part by altering activation of PLCβ3 under physiological chronic low oxygen.

IV.1. Introduction

During normal pregnancy, increased fetoplacental angiogenesis and vasodilation are associated with dramatically elevated fetoplacental blood flow, to support the increasing need for maternal and fetal exchange (Magness & Zheng, 1996). Dysregulation of fetoplacental angiogenesis may lead to adverse pregnant outcomes (Zygmunt *et al.*, 2003) such as fetal growth restriction and preeclampsia (PE) (Zygmunt *et al.*, 2003; Cerdeira & Karumanchi, 2012; Boeldt & Bird, 2017). Thus, a better understanding of mechanism underlying placental angiogenesis will help us develop strategies to control abnormal angiogenesis.

G protein mediates many cell function, which in turn regulate diverse biological processes such as cardiovascular growth and function (Pyne & Pyne, 2011). The G protein α subunit is a major signaling transducer of heterotrimeric G protein, and is currently classified into four subfamilies: Gα_s, G$\alpha_{i/o}$, G$\alpha_{q/11}$ and G$\alpha_{12/13}$ with a total of 18 members in human (Wettschureck & Offermanns, 2005; Syrovatkina *et al.*, 2016). Among these members, G protein *α* subunit 14 (GNA14), a member of G$\alpha_{q/11}$ subfamily, is expressed in endothelial cells of human placentas and umbilical cord vessels and its expression is elevated during pregnancy (Zhao *et al.*, 2014). More importantly, while GNA14 has been identified as a human hypertension-susceptibility gene (Kohara *et al.*, 2008), its protein levels are elevated in lung tissues from pulmonary artery hypertension (PAH) patients (Abdul-Salam *et al.*, 2010; Lei *et al.*, 2014) and in human placental tissues from patients with PE (Zhao *et al.*, 2014). To date, it is unknown if changes in endothelial GNA14 protein expression contribute to these increases in PAH lung and in PE placental

tissues (Zhao *et al.*, 2014; Zou *et al*., 2018), and collectively, these data suggest importance of GNA14 in mediating endothelial function both under physiological and pathological (e.g., human hypertensive diseases) states.

Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) are key regulators of fetoplacental angiogenesis and other endothelial function (Wang & Zheng, 2012). FGF2- and VEGFA-induced cellular functions are mediated primarily *via* activation of their high affinity receptor tyrosine kinases (RTKs; Klein *et al.*, 1997; Ferrara *et al.*, 2003; Wang & Zheng, 2012). These RTKs, in turn induce a vast array of signaling pathways, such as mitogen-activated protein kinase 3/1 (ERK1/2) (Wang & Zheng, 2012), and subsequent cell responses (Ferrara *et al.*, 2003; Podar & Anderson, 2008; Turner & Grose, 2010; Wang & Zheng, 2012).

Co-expression of GNAq and 11, two members of $G\alpha_{q/11}$ subfamily, has been reported to mediate FGF2 and/or VEGFA-induced endothelial responses including endothelial proliferation, migration, and intracellular Ca^{++} mobilization (Mukhopadhyay & Zeng, 2002; Zeng *et al.*, 2002, 2003; Sivaraj *et al.*, 2015). Recently, we have also demonstrated that GNA11 alone mediates FGF2- and VEGFA-induced cell responses in human umbilical cord endothelial cells (HUVECs) (Zou *et al*., 2018). Nothing is known about the roles of GNA14, which has ~70% amino acid sequence identity with GNA11 in humans (Hubbard & Hepler, 2006) in mediating fetoplacental endothelial function. However, as a somatic constitutively active mutation of GNA14 promotes excessive vascular growth in vascular tumors, and expressing this mutation in primary HUVECs maintains cell viability in association with increased activation of ERK1/2 under a serum-

free state (Lim *et al.*, 2016), GNA14 dysregulation may also lead to fetoplacental endothelial dysfunction during PE.

Phospholipase C β subfamily (PLCβ) is the major downstream signaling molecule of the $G\alpha_{q/11}$ subfamily (Offermanns, 1999; Rhee, 2001). Among all four members of PLCβ subfamily (PLCβ1 to 4), PLCβ3 has the highest affinity to $G\alpha_{q/11}$ subfamily members (PLCβ4 and β3 $\geqslant$ PLCβ1 >> PLCβ2; Offermanns, 1999). PLCβ3 exhibits distinct roles in mediating VEGFA-induced different endothelial responses, as evidenced by the observation that knockdown of PLCβ3 inhibits VEGFA-stimulated cell migration, whereas it enhances VEGFA-stimulated endothelial cell proliferation (Bhattacharya *et al.*, 2009). Currently, it is unknown exactly how PLCβ3 is activated; however, phosphorylation of PLCβ3 at different sites such as serine 537 (S537) or 1105 (S1105) is thought to be one of the major mechanisms in regulating PLCβ3 activity (Yue *et al.*, 1998; Xia *et al.*, 2001; Yue & Sanborn, 2001; Bhattacharya *et al.*, 2009). Specifically, phosphorylation of PLCβ3 S1105 has been reported to inhibit PLCβ3 activity in COSM6 and COS-7 cells (Yue *et al.*, 1998; Xia *et al.*, 2001), but the function of S537 phosphorylation remains ambiguous (Yue & Sanborn, 2001). Our recent study has also shown that elevated phosphorylation of PLCβ3 S537 is associated with VEGFA-stimulated cellular responses; however, over-phosphorylation of PLCβ3 S537 is accompanied with reduced FGF2- and VEGFA-stimulated cell migration induced by GNA11 knockdown in HUVECs (Zou *et al.*, 2018). These data suggest that phosphorylation of PLC*β*3 might differentially mediate PLCβ3 S537 activity, leading to distinct cell responses.

In the present study, we tested the hypothesis that GNA14 overexpression disrupts FGF2- and VEGFA-induced fetoplacental endothelial function *via* altering phosphorylation of ERK1/2 and PLCβ3 using HUVECs as a cell model, which is constantly cultured under physiological low oxygen (3% O_2).

IV. 2. Materials and Methods

Ethical approval

The protocol for collecting umbilical cord was approved by the Institutional Review Board of UnityPoint Meriter Hospital (Madison, WI), and the Health Sciences Institutional Review Boards of the University of Wisconsin–Madison (Protocol number 2004-006). The patients gave written, informed consent before participating. All procedures were conducted in accordance to the Declaration of Helsinki, except for registration in a database.

Primary HUVECs isolation and culture

HUVECs were isolated from umbilical cord vein of normal pregnancy using a standard collagenase enzyme-based protocol and cultured as previously described (Jiang *et al.*, 2013*a*, 2013*b*, 2014; Zhou *et al.*, 2017; Zou *et al.*, 2018). After isolation, cells were cultured in endothelial culture medium (ECM; catalog number 1001 Sciencell, Carlsbad, CA). ECM consisted of ECM basal media (ECM-b; catalog number 1001-b, Sciencell) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (p/s), 1% amphotericin B (AB; catalog number 15290018, Thermo Fisher Scientific,

Waltham, MA), and 1% endothelial cell growth supplement (ECGS) under a physiological low oxygen conditions (37^0C, 5% CO_2, 3% O_2, ~92% N_2) (Jiang *et al.*, 2013*a*, 2013*b*, 2014; Zou *et al.*, 2018).

Cell preparations were screened for endothelial characteristics as previously described (Jiang *et al.*, 2013*a*, 2013*b*, 2014). After verification, cells were pooled from five individual cell preparations, and cells at passage 4 were used in this study (Zou *et al.*, 2018).

All media used for cell culture and experiments were pre-purged with N_2 and equilibrated in a hypoxia incubator adjusted to physiological low oxygen condition before use. Dissolved O_2 in medium was monitored using a meter measuring dissolved oxygen. All experiments were conducted either in a hypoxia incubator adjusted to physiological low oxygen condition or a 3% O_2 heated oxygen-controlled glovebox (Coy Laboratory Products, Grass Lake, MI).

Adenoviral transfection

To study the roles of GNA14 in FGF2- and VEGFA-induced cell responses in HUVECs, and to mimic its placental expression in PE, GNA14 was overexpressed using commercially available adenoviruses carrying *GNA14* and green fluorescent protein (GFP, as reporter) (Ad-*GNA14;* Vector Biolabs, Malvern, PA). Adenoviruses carrying GFP reporter alone (Ad-*GFP*) were used as a vector control (Ren *et al.*, 2014). Amplification and transduction of adenovirus were performed as described (Liao *et al.*, 2009; Jiang *et*

al., 2014; Zou *et al.*, 2018). The specificity of Ad-*GNA14* has been verified as described (Zou *et al.*, 2018).

After verification of specificity of Ad-*GNA14*, HUVECs at 50-60% confluence were transfected by Ad-*GNA14* or Ad-*GFP* for 3 days, and cell lysates were subjected to Western blotting to confirm GNA14 overexpression. Additional cells were transfected for 2 days and serum-starved, followed by function assays as described below.

GNA14 siRNA transfection

In order to further dissect roles of GNA14 in FGF2- and VEGFA-induced cell responses in HUVECs, GNA14 siRNA transfection was performed as described (Wang *et al.*, 2008; Jiang *et al.*, 2014; Li *et al.*, 2015*a*; Zou *et al.*, 2018). A pool of four siRNAs specifically targeting human GNA14 (Catalog number L-008561-00-0005; GenBank number NM_004297.3; Dharmacon, Lafayette, CO) and a pool of 4 scrambled siRNAs (ssiRNA; catalog number D-001810-10-05) were purchased (Dharmacon). The GNA14 siRNA and ssiRNA were pre-mixed with Lipofectamine RNAiMAX transfection reagent (vehicle; catalog number 13778030, Invitrogen, Carlsbad, CA) at room temperature. Sub-confluent cells were cultured in antibiotic- and serum-free media (ECMb) containing GNA14 siRNA or ssiRNA (20 nM). After 4 hours of transfection, an equal amount of 2x ECM containing 10% FBS, 2% p/s, 2% AB 2% and ECGS was added. Cells were cultured for 2, 3, and 4 days and lysed. Lysate proteins (20-30 μg) were subjected to Western blot. Additional cells were transfected with a second dose of GNA14 siRNA at 4 days after the

first transfection, and cultured for an additional 2, 3, or 4 days before cells were harvested for Western blot.

Cell migration

Cell migration was assayed using a transwell system (Corning, Corning, NY) as described (Jiang *et al.*, 2014; Li *et al.*, 2015*a*; Zou *et al.*, 2018). After two days of transfection with Ad-*GFP* or Ad-*GNA14*, cells were serum-starved for 24 hours and seeded into inserts at 30,000 cells/insert. FGF2 or VEGFA was added to bottom chambers (final concentration: 100ng/ml; catalog number 10014-HNAE and 80006-RNAB, respectively, Sino Biologic Inc, China). After 16 hours of culture, calcein AM (catalog number C3100MP, Invitrogen) was added to bottom chambers (0.2 μg/ml). Five pictures were taken randomly using a Nikon TE2000U inverted microscope. Cell numbers were quantified using the Metamorph imaging analysis program (Molecular Devices, Sunnyvale, CA). ECMb supplemented with 2% heated inactivated FBS, 1% p/s, and 1% AB was used for preparing control medium and growth factor solutions.

Cell proliferation

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8; catalog number CK04-05, Dojindo Molecular Technologies, Rockville, MD) according to manufacturer's instructions. Briefly, after 2 days of transfection with Ad-*GFP* or -*GNA14*, cells were inoculated into 96-well plates (5,000-8,000 cells/well) and cultured overnight. After serum starvation for 16 hours, cells were treated with control medium, FGF2 or

VEGFA (100ng/ml) for 48 hours. CCK-8 solution was added and incubated for additional 4 hours. The OD value of each well was measured by a microplate reader at 450 nm (Bio-Tek, Winooski, VT). ECMb supplemented with 0.2% heated inactivated FBS, 1% p/s, and 1% AB was used for preparing control medium, and growth factor solutions.

Cell monolayer integrity

Cell monolayer integrity was examined using an electric cell-substrate impedance sensing system (ECIS Zθ; Applied Biophysics, Troy, NY) as described before (Zhou *et al.*, 2017; Zou *et al.*, 2018). After two days of transfection with the Ad-*GFP* or Ad-*GNA14*, cells (50,000 cells/well) were inoculated into 96W10idf ECIS array plates (Applied Biophysics) pre-coated with 10 nM cysteine and 0.1% gelatin. Resistance of each well was monitored at 4000 Hz. After resistance reached a plateau (indicating 100% confluence), cells were serum starved for 3-4 hours in ECMb containing 1% p/s and 1% AB. FGF2 or VEGFA was added (100 ng/ml) and the resistance was monitored for up to 24 hours as an indicator of cell monolayer integrity.

$[Ca^{++}]_i$ imaging

Intracellular calcium concentration ($[Ca^{++}]_i$) was monitored as described (Krupp *et al.*, 2013; Boeldt *et al.*, 2017). HUVECs at passage 4 (300,000 cells) were plated into 35 mm glass-bottom dishes and cultured overnight. After transfection with Ad-*GNA14* or Ad-*GFP* for 3 days, HUVECs were incubated in 10 μM Fura-2 AM with 0.05% pluronic acid F127 (Life Technologies, Carlsbad, CA) dissolved in 1 ml ECM for an hour in a hypoxic

incubator. Cells were incubated in Krebs buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 6 mM glucose, 2 mM $CaCl_2$, 25 mM HEPES, pH 7.4) for 30 minutes for ester hydrolysis. Fura-2 loading was verified by viewing 380-nm UV excitation on a Nikon inverted microscope and 80-90 cells were selected for imaging. An initial 5 minutes basal level recording was performed before subsequent addition of 100 μM ATP, a positive control for endothelial $[Ca^{++}]_i$ response mediated via G-protein coupled heptahelical receptors, FGF2 (100 ng/ml) or VEGFA (100 ng/ml; catalog number 293-VE-010, R & D Systems, Minneapolis, MN). In preliminary studies, similar $[Ca^{++}]_i$ responses were also observed in cells treated with VEGFA from Abgent (catalog number 80006-RNAB; data not shown). After treatment with ATP, FGF2, and VEGFA, $[Ca^{++}]_i$ was monitored for up to 30 minutes. $[Ca^{++}]_i$ for each cell was calculated in real time against an established ratiometric standard curve using InCyt Im2 software (Intracellular Imaging, Cincinnati, OH). Changes in $[Ca^{++}]_i$ were expressed as fold of the mean value of the last minute of the basal level. The area under curve for VEGFA-induced changes in $[Ca^{++}]_i$ was calculated using SigmaPlot software (Jandel Co., San Rafael, CA).

Western blotting

Western blot analysis was conducted as described (Jiang *et al.*, 2013*a*, 2013*b*; Li *et al.*, 2015*a*, 2017; Zou *et al.*, 2018). Cells were disrupted in lysis buffer (50 mM HEPES, 0.1 M NaCl, 10 mM EDTA, 4 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate [pH 7.5], 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin) and centrifuged. Protein samples (20-30 μg/sample)

were separated on 10% SDS-PAGE gels, and electrically transferred to polyvinylidene difluoride membranes. Membranes were probed by primary antibodies (Table 1). Proteins were visualized by enhanced chemiluminescence (ECL) or ECL2 (Thermo Fisher Scientific). Signals were recorded by Epson Perfection 4990 Photo Scanner (Long Beach, CA). Data were analyzed using the NIH Image J software.

Statistics

Data were analyzed using one-way ANOVA or Student's t-test (SigmaPlot software). When an F-test was significant, data were analyzed using the Student-Newman-Keuls (SNK) Method for pairwise multiple comparisons. $p < 0.05$ was considered statistically significant.

IV.3. Results

Ad-*GNA14* increases protein levels of GNA14.

After 3 days of transfection, Ad-*GNA14* ($p < 0.05$) elevated protein levels of GNA14 and GNA11 in a dose-dependent fashion (Fig. IV.1A). Specifically, Ad-*GNA14* at 5 MOI significantly increased ($p < 0.05$) GNA14 (~5 fold of control), but not GNA11, while Ad-*GNA14* at 10 and 20 MOI elevated ($p < 0.05$) both GNA14 (~7.8 and ~18.5 fold, respectively) and GNA11 (~2.5 and ~7.4 fold, respectively). In addition, GNA14 increases induced by Ad-*GNA14* at 5 and 10 MOI were significantly higher ($p < 0.05$) than those of GNA11 (Fig. IV.1A). Ad-*GFP* had no significant effects on either GNA14 or GNA11 protein levels in HUVECs as previously reported (Zou *et al.*, 2018).

Since Ad-*GNA14* at 5 MOI significantly ($p < 0.05$) elevated only GNA14, but not GNA11 protein (Fig. IV.1A), and this elevation was comparable to the ~2.5 - 2.9 fold increase in GNA14 protein observed in human placentas from preeclamptic vs. normotensive pregnancy (Zhao *et al.*, 2014), Ad-*GNA14* at 5 MOI was used in all of the following experiments.

Either a single or double dosing of GNA14 siRNAs at 20 nM did not significantly alter GNA14 protein levels (Fig. IV.1B; quantitative data not shown). We had also transfected cells with 40 and 100 nM of GNA14 siRNA for up to 4 days. However, neither dose of GNA14 siRNA significantly altered GNA14 protein levels (data not shown). As we were unable to suppress GNA14 protein expression in HUVECs, no further loss of function assays were performed.

Ad-*GNA14* alters cell migration and monolayer integrity in response to FGF2, but not VEGFA.

Compared to the control (ECM-b), FGF2 and VEGFA significantly ($p < 0.05$) stimulated cell migration by ~3.1 and ~4.8 fold, respectively (Fig. IV.2A). However, Ad-*GNA14*, but not Ad-*GFP*, blocked ($p < 0.05$) FGF2-induced cell migration, whereas it did not significantly affect VEGFA-stimulated cell migration.

Compared to the control in the 0 MOI group, FGF2 and VEGFA dramatically ($p < 0.05$) stimulated cell proliferation in the 0 MOI group (~2.2 and ~1.8 fold, respectively) and in the Ad-*GFP* group (~1.9 and ~1.8 folds, respectively) (Fig. IV.2B). However, Ad-*GFP* alone also significantly ($p < 0.05$) stimulated cell proliferation (~1.6 fold) in the 0 MOI

group (Fig. IV.2B), suggesting an off-target stimulatory effect of Ad-*GFP*. Thus, as Ad-*GNA14* contained *GFP*, we cannot exclude the possibility that *GFP* would partially contribute to FGF2- and VEGFA-stimulated cell proliferation in Ad-*GNA14* group. Nevertheless, Ad-*GNA14* completely inhibited ($p < 0.05$) FGF2/GFP- and VEGFA/GFP-stimulated cell proliferation (Fig. IV.2B).

Compared to the control, FGF2 did not significantly affect cell monolayer integrity, whereas VEGFA significantly ($p < 0.05$) decreased the cell resistance or decreased cell monolayer integrity by ~40%, starting at ~4 hours (Fig. IV.2C and D) in the 0 MOI and Ad-*GFP* groups. Interestingly, Ad-*GNA14* time-dependently elevated ($p < 0.05$) the cell resistance or elevated monolayer integrity in response to FGF2, but not VEGFA (Fig. IV.2C and D), indicating decreases in cell permeability. This elevation in cell resistance began at ~8 hours of FGF2 treatment (~17%) and was maintained for up to 24 hours (~35% at 24 hours).

Ad-*GNA14* does not change $[Ca^{++}]_i$ in response to ATP, FGF2, and VEGFA.

We also examined roles of Ad-*GNA14* in mediating $[Ca^{++}]_i$ mobilization in response to ATP, FGF2, or VEGFA. We observed that ATP (Fig. IV.3A and B) and VEGFA (Fig. IV.3E and F), but not FGF2 (Fig. IV.3C and D), robustly ($p < 0.05$) increased $[Ca^{++}]_i$. In the 0 MOI group, the VEGFA-induced $[Ca^{++}]_i$ peak was delayed for 2-3 minutes compared with ATP treatments, and the magnitude of this peak appeared to be slightly lower than that induced by ATP (~2 vs. ~3 fold, respectively). However, compared to the 0 MOI group, Ad-*GFP* and -*GNA14* slightly increased VEGFA-induced $[Ca^{++}]_i$ peak, but this increase

did not reach significant(Fig. IV.3). The area under curve between 0 and 10 minutes of VEGFA treatment was also calculated. Again, no significant differences *(p* = 0.205) were found between 0 MOI (1.0 ± 0.09 of fold of 0 MOI), Ad-*GFP* (1.3 ± 0.14), and Ad-*GNA14* (1.3 ± 0.09) groups.

The proportions of cells responding to ATP (89.7 ± 3.05%, 85.9 ± 4.01%, and 89.9 ± 3.32%, respectively) or VEGFA (65.7 ± 10.54%, 72.9 ± 7.09%, and 73.6 ± 9.13%, respectively) were also similar among the 0 MOI, Ad-*GFP*, and Ad-*GNA14* groups. No cell responded to FGF2.

Ad-*GNA14* does not affect FGF2-induced phosphorylation of ERK1/2.

Both FGF2 and VEGFA robustly increased ($p < 0.05$) phosphorylation of ERK1/2 at T202/Y204 in a time-dependent fashion (~16.6 and ~ 6.1 fold increases at 10 min vs. time 0 for FGF2 and VEGFA, respectively; Fig. IV.4). Ad-*GNA14* did not alter FGF2-induced phosphorylation of ERK1/2 over a 60 min window (Fig. IV.4A). Ad-*GNA14* slightly increased VEGFA-induced phosphorylation of ERK1/2 at 10, 20, and 30 min; however, only at 30 min did this increase (~3.3 folds) reach statistical significance (Fig. IV.4B).

Ad-*GNA14* increases phosphorylation of PLCβ3 S1105 in response to FGF2, but not VEGFA.

In the 0 MOI group, compared to the time 0 control, FGF2 did not induce phosphorylation of PLCβ3 S537 and S1105 for up to 60 min (Fig. IV.5A), whereas VEGFA rapidly (≤ 5 min) increased ($p < 0.05$) phosphorylation of PLCβ3 S537 (~14.8 fold at 5

min), but not S1105 (Fig. IV.5B). This VEGFA-increased phosphorylation of PLCβ3 S537 was maintained at relatively high levels for up to 60 min (Fig. IV.5B).

Ad-*GNA14* rapidly (≤ 5 min) elevated phosphorylation of PLCβ3 S537 (~ 3.4 and 6.2 folds over Ad-*GFP* at 5 and 20 min, respectively; $p < 0.05$) in response to FGF2 in a time-dependent fashion (Fig. IV.4B). However, Ad-*GNA14* did not alter the phosphorylation patterns of PLCβ3 either at S1105 in response to FGF2, or at S537 and S1105 in response to VEGFA.

IV.4. Discussion

In the present study, we demonstrate that under physiological low oxygen, GNA14 overexpression significantly inhibits endothelial migration and permeability, but not proliferation in response to FGF2, but not in response to VEGFA. This inhibition is associated with elevated phosphorylation of PLCβ3 S1105, but not PLCβ3 S537 and ERK1/2. These results indicate that GNA14 differentially regulates FGF2- and VEGFA-induced fetoplacental endothelial responses. These findings also suggest that GNA14 overexpression might impair FGF2-induced endothelial function, perhaps contributing to fetoplacental endothelial dysfunction in PE.

Previous studies have revealed important roles of GNAq and/or 11 in mediating FGF2 and/or VEGFA-induced endothelial function (Zeng *et al.*, 2002*b*, 2003; Sivaraj *et al.*, 2015; Zou *et al.*, 2018). In addition to GNAq and/or 11 in G$\alpha_{q/11}$ subfamily, GNA14 has been identified as one of the human hypertension-susceptible genes (Kohara *et al.*, 2008). Moreover, protein expression of GNA14 is increased in lung from PAH patients

(Abdul-Salam *et al.*, 2010) and in placentas from PE patients (Zhao *et al.*, 2014), which is a disorder of pregnancy characterized by hypertension. Together, these data suggest that GNA14 overexpression is involved in human hypertensive and other vascular diseases (e.g. congenital and sporadic vascular tumor, Lim *et al.*, 2016), possibly partially via impaired endothelial function.

Here we show that overexpression of wild type GNA14 in primary HUVECs significantly alters cell migration, monolayer integrity, and PLCβ3 phosphorylation in response to FGF2, but not VEGFA. In addition, GNA14 overexpression abolishes both FGF2/GFP- and VEGFA/GFP-stimulated cell proliferation, but does not alter $[Ca^{++}]_i$ in response ATP, FGF2 or VEGFA. To our knowledge, this is the first report of differential roles of GNA14 in mediating FGF2- and VEGFA-induced fetoplacental endothelial function. Interestingly, while GNA11 mediates both FGF2- and VEGFA-stimulated cell migration, but not proliferation and permeability in HUVECs (Zou *et al.*, 2018), GNA14 exclusively acts on FGF2-, but not VEGFA-regulated several cell responses examined in the present study. Together, these data support the concept that GNA11 and GNA14 have common and differential roles in mediating FGF2- and VEGFA-induced cellular response as previously suggested (Nakamura *et al.*, 1996; Hubbard & Hepler, 2006).

We attempted to further define the role of GNA14 in HUVECs using siRNA to knockdown GNA14 function. However, no dosage schedule of GNA14 siRNA examined over 8 days suppressed GNA14 protein expression (Fig. IV.1B). This is despite the evidence of real-time qPCR showing that after 2 days of transfection, GNA14 siRNA at 20 nM decreased GNA14 mRNA levels by ~50% (data not shown). We also tested

GNA14 siRNA from two additional vendors (Integrated DNA Technologies, Skokie, IL and Santa Cruz Biotechnology, Dallas, Texas) and similarly failed to detect any significant knockdown in GNA14 protein (data not shown). These data suggest that GNA14 siRNAs used in the current study are insufficient to suppress GNA14 protein expression in HUVECs. Alternatively, another possible explanation could be that GNA14 protein expression is uniquely regulated at the post-translational level in HUVECs such that suppression of GNA14 mRNA expression might decrease degradation of GNA14 protein in HUVECs.

One major finding in the present study is that GNA14 overexpression inhibits FGF2-induced cell migration is accompanied by enhanced cell monolayer integrity (or decreased cell permeability) in HUVECs. The exact causal relationship between cell migration and cell monolayer integrity is unclear. Nonetheless, for a cell to migrate it must first disconnect from its neighbors. Thus, GNA14 overexpression might decrease FGF2-stimulated cell migration via strengthening endothelial monolayer integrity.

Interestingly, we observed an off-target, stimulatory effect of Ad-*GFP*, specifically on cell proliferation. This finding raises a caution against using Ad-*GFP* as a control. However, this observation is unique since Ad-*GFP* alone stimulates cell proliferation, migration, and tube formation in human lung microvascular endothelial cells under a serum-free condition (Kornberg & Grant, 2007). It has been suggested that activation of focal adhesion kinase may contribute to such effects in human lung microvascular endothelial cells (Kornberg & Grant, 2007). In addition, compared to Ad-*GFP*, neither FGF2 nor VEGFA further promotes cell proliferation, indicating no synergistic effects on

cell proliferation between these two peptide growth factors and Ad-*GFP*. Thus, we cannot draw a conclusion about the specific role of GNA14 overexpression in FGF2- and VEGFA-stimulated cell proliferation. Nonetheless, it is noteworthy that even though Ad-*GFP* exhibits the off-target effect on cell proliferation, GNA14 overexpression blocks cell proliferation stimulated by FGF2/Ad-GFP and VEGFA/Ad-*GFP*, supporting its anti-proliferative activity.

Not surprisingly, in Ad-*GFP*-transfected cells, both FGF2 and VEGFA stimulate ERK1/2 phosphorylation in HUVECs, comparable to our previous reports (Wang & Zheng, 2012; Jiang *et al.*, 2013*a*). However, GNA14 overexpression does not change the overall phosphorylation pattern of ERK1/2 at T202/Y204 induced by FGF2 and VEGFA. Thus, similar to GNA11 (Zou *et al.*, 2018), GNA14 may not play a major role in mediating FGF2- and VEGFA-induced activation of ERK1/2 under physiological chronic low O_2. This prompted us to ask what other signaling molecules might be involved in GNA14-mediated FGF2's action in HUVECs. One potential molecules is PLCβ3, since it is a major downstream target of members of the $G\alpha_{q/11}$ subfamily and a key mediator of many VEGFA's actions in endothelial cells (e.g., migration, proliferation) beyond acute $[Ca^{++}]_i$ signaling (Offermanns, 1999; Mukhopadhyay & Zeng, 2002; Zeng *et al.*, 2002*b*, 2003; Hubbard & Hepler, 2006; Sivaraj *et al.*, 2015; Zou *et al.*, 2018).

In the present study, we observe that VEGFA induces phosphorylation of PLCβ3 S537, though FGF2 does not induce phosphorylation of PLCβ3 S537 and 1105 in Ad-*GFP* group. These observations are consistent with our recent report (Zou *et al.*, 2018), further confirming our earlier suggestion that phosphorylation levels of PLCβ3 537 and

1105 is involved in VEGFA-, but not FGF2-induced cell function under basal physiological conditions (Zou *et al.*, 2018). More importantly, GNA14 overexpression greatly elevates phosphorylation of PLCβ3 S1105 in response to FGF2 in parallel to decreased cell migration, increased monolayer integrity, and unaltered ERK1/2 activation. Thus, it is reasonable to propose that GNA14 serves as a positive mediator of phosphorylation of PLCβ3 S1105 in response to FGF2. In addition, it also indicates that similar to GNA11, GNA14 also does not mediate ERK1/2 activation in HUVECs. Together with the earlier reports that phosphorylation of PLCβ3 S1105 inhibits PLCβ3 activity (Yue *et al.*, 1998, 2000; Xia *et al.*, 2001), our observations from the present study suggest that GNA14 overexpression-elevated phosphorylation of PLCβ3 S1105 may attenuate activity of PLCβ3 and partially contribute to the abolition of FGF2-stimulated cell migration and FGF2-enhanced monolayer integrity.

In contrast to the previous report demonstrating that VEGFA induces phosphorylation of both PLCβ3 S537 and 1105 (Bhattacharya *et al.*, 2009) in HUVECs, we only see phosphorylation of PLCβ3 S537, but not S1105, in response to VEGFA. It is unclear if this discrepancy can be attributed to the different oxygen levels used to culture cells (3% vs. presumably ~21% O_2). Nonetheless, Ad-*GNA14* fails to affect phosphorylation of PLCβ3 S537 and S1105 in response to VEGFA. These data indicate that although VEGFA-induced phosphorylation of PLCβ3 S537 may critically mediate VEGFA-induced cell function; but GNA14 does not mediate such phosphorylation. Instead, GNA11, another member of $G\alpha_{q/11}$ subfamily may be a key mediator for such phosphorylation in response to VEGFA as we recently suggested (Zou *et al.*, 2018).

Unlike GNAq and GNA11 which regulate VEGFA-stimulated $[Ca^{++}]_i$ mobilization in HUVECs (Mukhopadhyay & Zeng, 2002; Zeng *et al.*, 2003), GNA14 overexpression does not alter VEGFA-induced $[Ca^{++}]_i$ responses, suggesting that GNA14 may not participate in the VEGFA-PLCβ3-IP3-Ca^{++} signaling pathway in HUVECs.

To date, the mechanism controlling phosphorylation of PLCβ3 S537 and S1105 is undefined in HUVECs. However, the constitutively active form of calcium/calmodulin-dependent protein kinase II induces phosphorylation of PLCβ3 S537 in COSM6 cells (Yue & Sanborn, 2001). In addition, protein kinase A, C, and G can all phosphorylate PLCβ3 S1105 in COSM6 and COS-7 cells, and hence inhibit PLC activity (Yue *et al.*, 1998, 2000; Xia *et al.*, 2001). Thus, it is possible these protein kinases may also be involved in phosphorylation of PLCβ3 S537 and S1105 in response to FGF2 and VEGFA in HUVECs. In addition, collectively, this, combined with our finding that the $[Ca^{++}]_i$ response to VEGFA is delayed by a few minutes while that to ATP (a stimulant of heptahelical receptors coupled to PLCβ3) is immediate suggests that PLCβ3 is not the central player in mediating changes in $[Ca^{++}]$i and that role is fulfilled by PLCγ. It is more likely PLCβ3 is a player in further downstream cell signaling such as the small G protein CDC42 as described by Bhattacharya et al (2009).

To date, the exact interactions between the RTK cascades and GNA14 are unclear. Based on the data from the present and previous studies (Wang & Zheng, 2012; Jiang *et al.*, 2013*a*), we would like to hypothesize that GNA14 differentially mediates phosphorylation of PLCβ3 S537 and S1105 via distinctive activation of RTK downstream signals in response to FGF2 and VEGFA under physiological chronic low oxygen as

described in Fig. IV.6. Further studies are needed to define the RTK/GNA14/PLCβ3 pathway as well as the role of PLCβ3 S1105 and/or S537 phosphorylation in regulating the activity of PLCβ3 in response to FGF2 and VEGFA. This is important as GNA14 and phosphorylation of PLCβ3 at different sites could be potential targets for therapeutic intervention for endothelial dysfunction, which is closely associated with many human cardiovascular diseases.

In conclusion, our results indicate differential roles of GNA14 in mediating FGF2- and VEGFA-induced fetoplacental endothelial function in association with distinguish phosphorylation of PLCβ3. Such findings suggest GNA14 overexpression in endothelial cells may impair endothelial function, leading to hypertension-related diseases (e.g., hypertension, PAH, and PE). Thus, our data demonstrate a unique crosstalk between GNA14 and PLCβ3, and reveal a novel signaling mechanism of FGF2 in endothelial cells.

IV.5. Figures

Figure IV.1.

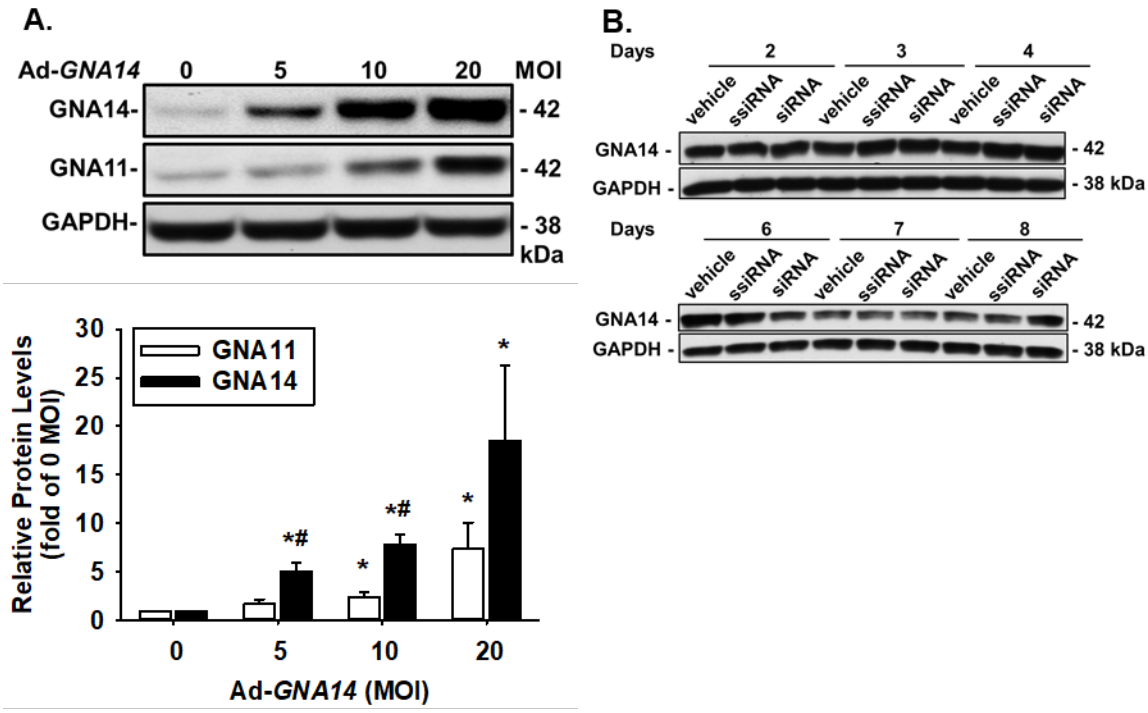

Figure IV.1. Effects of Ad-*GNA14* and GNA14 siRNA on protein levels of GNA14 in HUVECs. A: Ad-*GNA14* transfection: After 3 days of transfection, cellular proteins (20-30 μg) were subjected to Western blotting for GNA14, GNA11, and GAPDH. Data normalized to GAPDH were expressed as means ± SEM. *Different from 0 MOI control (SNK method for pairwise multiple comparisons); #Different from GNA11 at the corresponding dose of Ad-*GNA14* (Student's t-test). $p < 0.05$; n = 4 individual experiments. B: Western blotting images for GNA14 siRNA transfection: after transfecting with a single dosing of 20 nM of GNA14 siRNA for up to 4 days (upper panel) or with double dosing (Days 1 and 6) of 20 nM GNA14 siRNA for up to 8 days (lower panel), cells were harvested and cellular proteins (20-30 μg) were subjected to Western blotting. n = 4 individual experiments.

Figure IV.2.

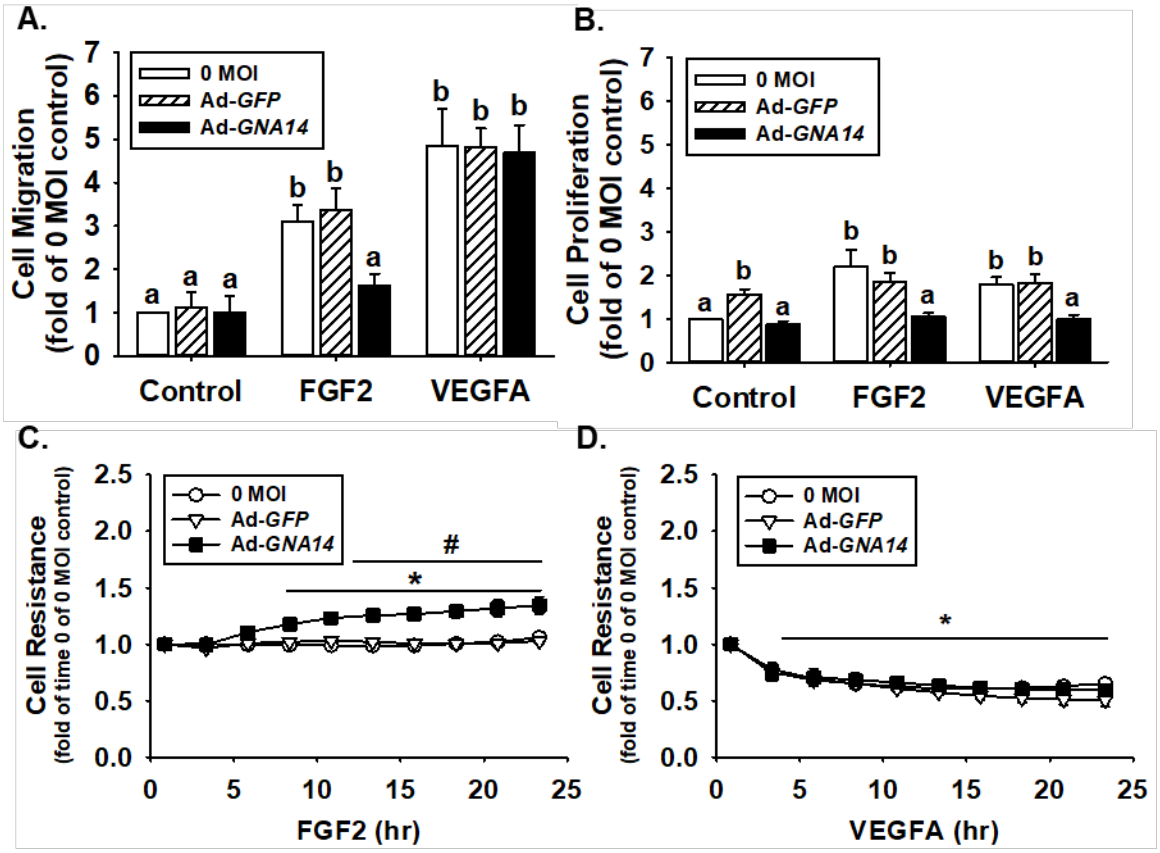

Figure IV.2. Effects of Ad-*GNA14* on FGF2- and VEGFA-induced cell migration, proliferation, and monolayer integrity in HUVECs. Cell migration (A), proliferation (B), and permeability (C) were determined using Transwell system, CCK-8, and ECIS, respectively. Cells were transfected with Ad-*GFP* or Ad-*GNA14* at 5 MOI for 2 days. After serum starvation for additional 24 hr (cell migration and cell proliferation) or 3 hr (cell permeability), cells were treated with FGF2 and VEGFA (100 ng/ml) for 16 hr (cell migration; n = 3-5 individual experiments), 48 hr (cell proliferation; n = 4 individual experiments) or 24 hr (cell permeability; n = 3 individual experiments). Data were expressed as means ± SEM. [a,b]Means with different letters differ (SNK method for pairwise multiple comparisons). *Different from the time 0 control of 0 MOI (SNK method for pairwise multiple comparisons); #Different from the corresponding dose of Ad-*GFP* (Student's t-test). $p < 0.05$.

Figure IV.3.

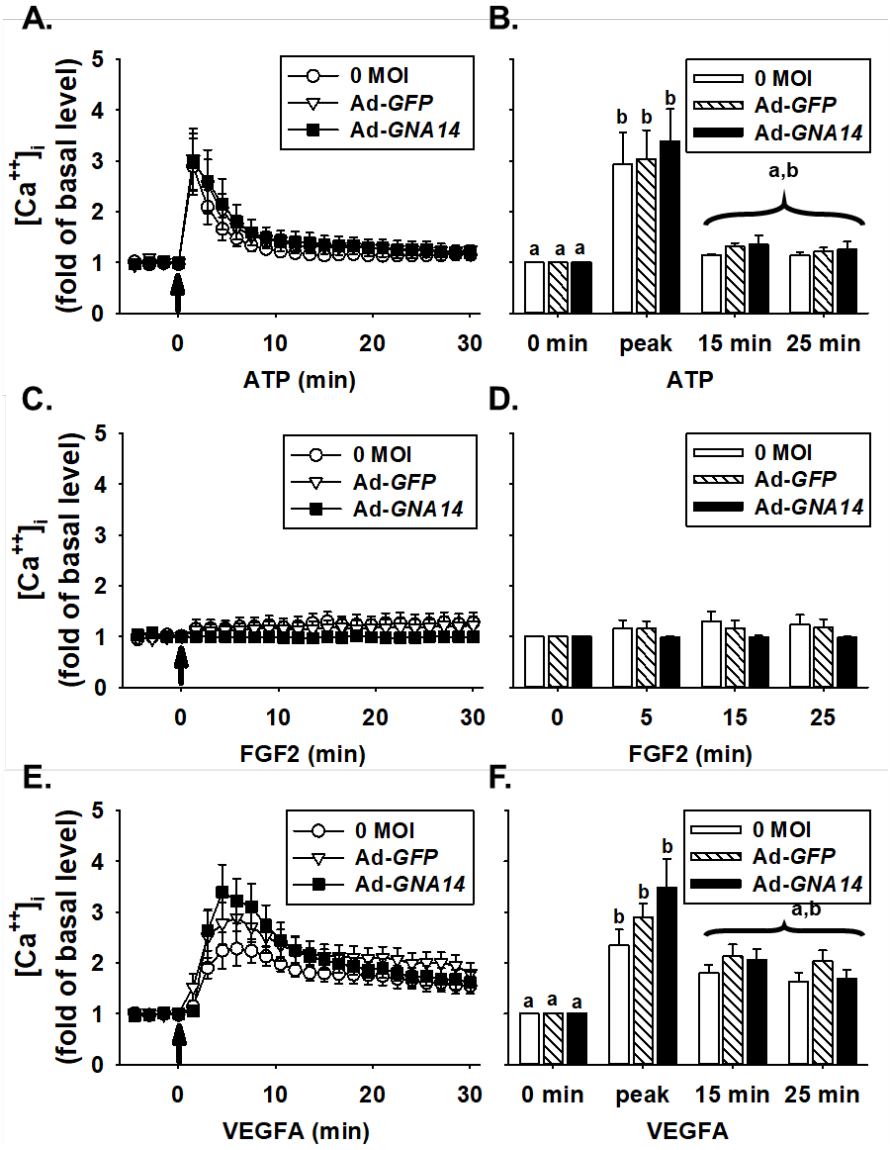

Figure IV.3. Effects of Ad-*GNA14* on $[Ca^{++}]_i$ in response to ATP, FGF2 or VEGFA. Cells were transfected by Ad-*GFP* or Ad-*GNA14* at 5 MOI for 3 days. Cells were loaded with Fura-2 AM for 1 hr, and incubated in Krebs buffer for ester hydrolysis for 0.5 hr. 80-90 cells were randomly selected for real-time $[Ca^{++}]_i$ imaging under microscope. After basal levels of $[Ca^{++}]_i$ were recorded for 5 min, ATP, FGF2 or VEGFA was added, followed by sequentially recording $[Ca^{++}]_i$ for up to 24 hr. A, C, and E: $[Ca^{++}]_i$ of responding cells (ATP and VEGFA) or of all cells selected (FGF2). B, D, and F: $[Ca^{++}]_i$ at different time points. Data were expressed as means ± SEM fold of mean of the last min of basal levels. Arrow: addition of agonists. ^{a,b}Means with different letters differ (SNK method for pairwise multiple comparisons). $p < 0.05$. n = 3-5 individual experiments.

Figure IV.4.

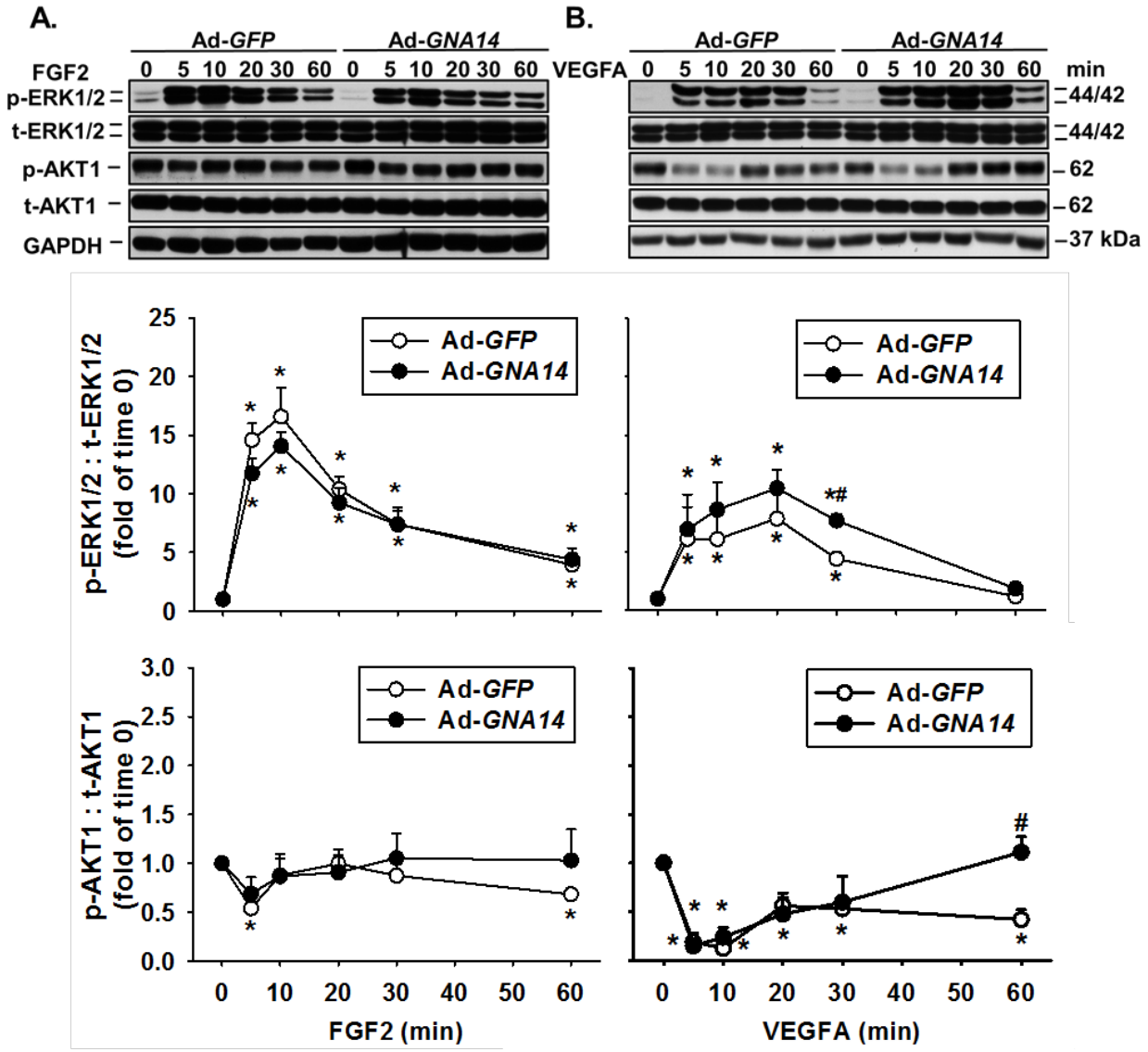

Figure IV.4. Effects of Ad-*GNA14* on FGF2- and VEGFA-induced phosphorylation of ERK1/2. Cells were transfected with Ad-*GFP* or Ad-*GNA14* for 2 days. After serum starvation for additional 24 hr, cells were treated with FGF2 and VEGFA (100 ng/ml). Cellular proteins (20-30 μg) were subjected to Western blotting. Data were expressed as means ± SEM. *Different from the corresponding time 0 (SNK method for pairwise multiple comparisons); #Different from corresponding time point of Ad-*GFP* group (Student's t-test). $p < 0.05$; n = 4 individual experiments.

Figure IV.5.

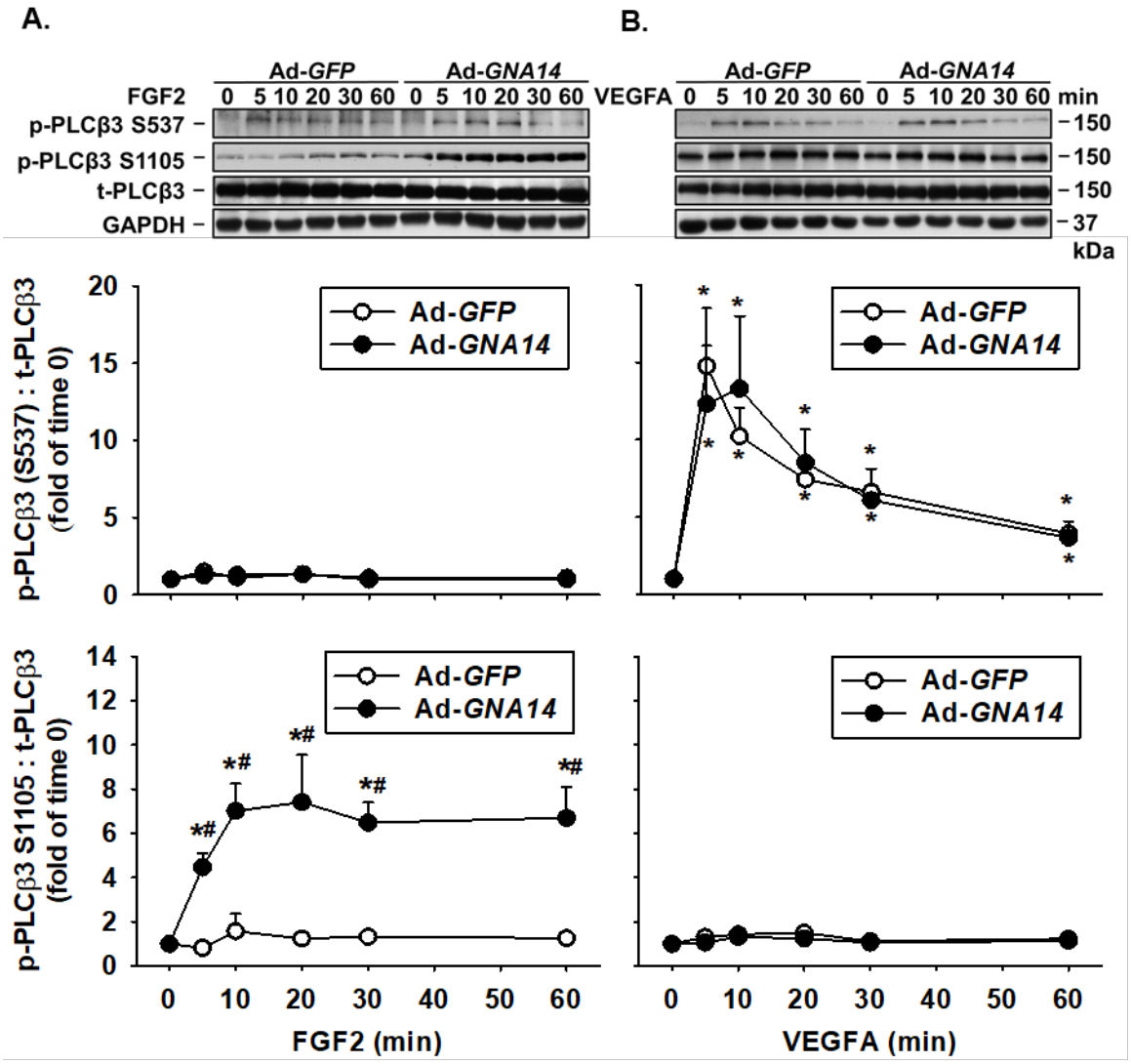

Figure IV.5. Effects of Ad-*GNA14* on phosphorylation of PLCβ3 S537 and S1105. After 2 days of transfection, HUVECs were serum starved for additional 24 hr. Cells were treated with FGF2 and VEGFA (100 ng/ml). Cellular proteins (20-30 μg) were subjected for Western blotting. Data were expressed as means ± SEM. *Different from the corresponding time 0 (SNK method for pairwise multiple comparisons); #Different from the corresponding time point of Ad-*GFP* (Student's t-test); $p < 0.05$; n = 3 individual experiments.

Figure IV.6.

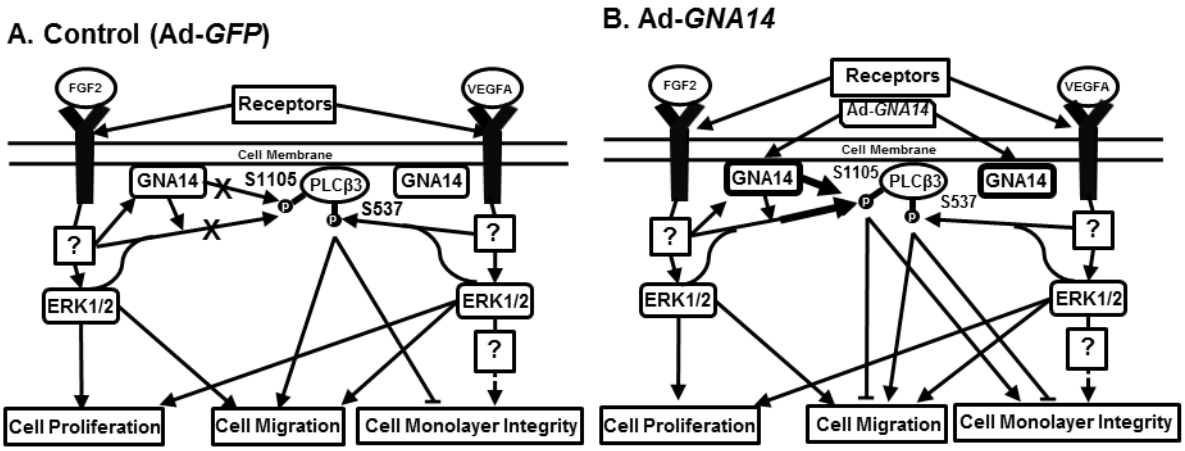

Figure IV.6. A hypothesized signaling model for the role of GNA14 in FGF2- and VEGFA-regulated fetal endothelial function via PLCβ3 and ERK1/2 under physiological chronic low oxygen. A. Ad-GFP (a control): under this condition, FGF2- and VEGFA-stimulate cell proliferation and migration at least via activation of ERK1/2 (Jiang et al., 2013a). GNA14 at the basal level does not affect RTK downstream signals-mediated phosphorylation of PLCβ3 S537 and S1105 in response to FGF2. However, while VEGFA increases phosphorylation of PLCβ3 S537, but not S1105, in association with VEGFA-increased cell proliferation, migration, and permeability, GNA14 does not appear to mediate all these changes as GNA14 overexpression does not dysregulate all these cellular responses as shown in B. B. GNA14 overexpression by Ad-GNA14: Upon FGF2 stimulation, RTK downstream signals activate GNA14 (possibly via phosphorylation). This activated form of overexpressed GNA14 facilitates PLCβ3 S1105 phosphorylation in response to FGF2 by directly acting and/or indirectly via enhancing activity of RTK downstream signals on PLCβ3 S1105. Such excessive phosphorylation may inhibit FGF2-stimulated cell migration and enhance FGF2-maintained cell monolayer integrity. In contrast, GNA14 overexpression does not affect PLCβ3 S537 phosphorylation and cell function in response to VEGFA. Crosses: blocked or no stimulation; Bold-lined squares: increased GNA14 protein; Bold arrows: enhanced stimulation; Question marks: unknown signals.

Chapter V. Final Discussion

V.1. Summary of Chapters

By far, seventeen members of G protein α subunits have been identified in mammals are classified into four subfamilies (Wettschureck & Offermanns, 2005).

Recently, our studies revealed elevated levels of GNA14 protein in HUAECs cultured under physiological low oxygen (3%) (Jiang *et al.*, 2013*b*). This finding prompts us to focus on members of $G\alpha_{q/11}$. GNA11 has been considered functionally redundant to GNAq (Offermanns *et al.*, 1998; Offermanns, 1999; Sivaraj *et al.*, 2015). However, evidence also shows that GNA11 and GNAq may also have distinct roles in regulating norepinephrine-induced intracellular calcium mobilization in rat portal vein myocyte (Macrez-Leprêtre *et al.*, 1997). In addition, as we discussed in Chapter IV, previous studies have reported the increased protein levels of GNA14 in several hypertension related diseases (Kohara *et al.*, 2008; Abdul-Salam *et al.*, 2010; Lei *et al.*, 2014; Zhao *et al.*, 2014). Despite that these pieces of evidence have suggested the importance of GNA11 and 14 in multiple types of cells including placental cells, their roles, especifically in mediating peptide growth factors-induced fetoplacental endothelial function are largely unknown.

In the present study, I focused on dissecting the roles of GNA11 and GNA14 in fetoplacental endothelial cells. I am specifically interested in clinical significance of GNA14 as its overexpression is related to human hypertensive diseases (Kohara *et al.*, 2008; Abdul-Salam *et al.*, 2010; Zhao *et al.*, 2014). We have determined protein expression of GNA11 and 14 in human placentas and umbilical cord vessels (Chapter II). Further, we individually studied roles of GNA11 and GNA14 in mediating FGF2- and VEGFA-induced fetoplacental endothelial function using HUVECs as a cell model under physiological low oxygen (Chapters III and IV).

In Chapter II, we determined protein expression of GNA11 and 14 in human placental tissues from FT, NT and sPE, as well as umbilical cords from NT pregnancy. We found that both GNA11 and 14 proteins were immunolocalized in villous vascular endothelial cells and stromal cells in FT, NT and sPE placentas, as well as in endothelial cells of umbilical cord vein and artery. Both proteins were also present in cyto- and syncytiotrophoblasts of FT and in syncytiotrophoblasts of NT and sPE placentas.

We also quantified protein levels of GNA11 and 14 in placental tissues from FT, NT and sPE. Compared with NT placentas, GNA14, but not GNA11, was significantly increased in sPE placentas. While GNA11 was undetected in FT placentas, both GNA11 and 14 were significantly elevated in NT compared with FT placentas. Together, these data indicate that GNA11 and GNA14 are widely expressed in human placental cells, implicating their importance in mediating function of multiple types of human placental cells including endothelial and trophoblast cells.

In Chapter III (summarized in Table 2), we dissected roles of GNA11 in mediating FGF2- and VEGFA-induced fetoplacental endothelial function and underlying potential signaling mechanisms in HUVECs. We found that GNA11 siRNA specifically suppressed protein expression of GNA11, but not GNA14. This GNA11 downregulation significantly decreased FGF2- and VEGFA-induced endothelial migration, but not cell proliferation and permeability. We further discovered that VEGFA, but not FGF2, significantly and time-dependently increased phosphorylation of PLCβ3 S537. However, neither FGF2 nor VEGFA induced notable phosphorylation of PLCβ3 S1105. GNA11 siRNA time-dependently elevated phosphorylation of PLCβ3 S537 in response to FGF2 and VEGFA.

However, phosphorylation of PLCβ3 S1105, ERK1/2 T202/Y204 and AKT1 S473 was not affected.

We also provided evidence that FGF2 and VEGFA did not induce activation of RhoA (suppl. Fig. 3). Moreover, surprisingly, we found that Ad-*GNA11* dose-dependently overexpressed not only GNA11 but also GNA14 proteins. These data imply that GNA11 alone indeed critically mediates FGF2- and VEGFA-induced function; but this mediation is limited to certain endothelial function.

In Chapter IV (summarized in Table 3), we determined the roles of GNA14 in mediating FGF2- and VEGFA-induced endothelial function and underlying possible signaling mechanisms in HUVECs. Ad-*GNA14* at a relatively low dose (5 MOI) specifically up-regulated protein expression of GNA14. GNA14 overexpression significantly suppressed FGF2-, but not VEGFA-, induced cell migration, whereas it dramatically enhanced cell monolayer integrity in response to FGF2, but not VEGFA. We further observed that only VEGFA, but not FGF2 induced phosphorylation of PLCβ3 S537, while neither FGF2 nor VEGFA induced phosphorylation of PLCβ3 S1105. Moreover, GNA14 overexpression significantly increased phosphorylation of PLCβ3 S1105 in response to FGF2, but not VEGFA. GNA14 overexpression, did not alter phosphorylation of PLCβ3 S537 in response to FGF2 and VEGFA. Additionally, ATP- (a positive control for endothelial $[Ca^{++}]_i$ response mediated via G-protein coupled heptahelical receptors), and VEGFA-induced $[Ca^{++}]_i$ were not affected by GNA14 overexpression. These results suggest the crucial roles of GNA14 in mediating FGF2-, but not VEGFA-, induced endothelial function, specifically in cell migration and monolayer integrity.

Interestingly, we found that similar to FGF2 and VEGFA, Ad-*GFP* alone also significantly increased cell proliferation under a serum-free medium condition. However, GNA14 overexpression abolished the combined stimulatory effects of FGF2/Ad-*GFP* and VEGFA/Ad-*GFP* on cell proliferation, suggesting GNA14 overexpression is anti-proliferative in HUVECs.

In summary, results generated from this thesis elucidate unique and complicated roles of GNA11 and 14 in mediating FGF2- and VEGFA-induced different endothelial response. These findings extend our knowledge regarding interactions between G protein α subunits and peptide growth factors in endothelial function, and provide new information for developing clinical intervention for human hypertensive diseases in the future.

V.2. Implication of Key Findings

V.2.1 Distinct Roles of FGF2 and VEGFA in Mediating Endothelial Function.

In the present study, we found that FGF2 and VEGFA display similar but distinct roles in regulating endothelial function. For example, FGF2 and VEGFA exhibit relatively similar potency of inducing placental endothelial migration and proliferation and activation of ERK1/2 and AKT1 as shown in the present study and previously (Jiang *et al.*, 2013*a,b*). On the other hand, FGF2 maintains endothelial monolayer integrity, whereas VEGFA decreases it. This is not surprising since FGF2 is the key factor of maintaining vascular monolayer integrity (Murakami & Simons, 2009) and disruption of FGF2 signaling impairs endothelial monolayer integrity, leading to disintegration of vasculature *in vivo* (Murakami *et al.*, 2008*b*). In contrast, VEGFA is originally identified as a potent vascular permeability

factor and can induce endothelial permeability (Lal *et al.*, 2001; Bates, 2010). In addition, FGF2 does not induce phosphorylation of PLCβ3 at neither S537 nor S1105, whereas VEGFA significantly elevates PLCβ3 S537 phosphorylation.

Under a physiological condition, as disruption of endothelial cell monolayer integrity is required for initiating angiogenesis, FGF2 and VEGFA may act on different steps of angiogenesis: VEGFA, but not FGF2, promotes breakdown of endothelial cell integrity, and then VEGFA and FGF2 work synergistically to stimulate endothelial cell proliferation and migration. However, under a pathological condition such as PE, elevated FGF2 and/or VEGFA expression in placentas (Chung *et al.*, 2004; Özkan *et al.*, 2008), along with other abnormal expression of endothelial function related factors (e.g., sFlt-1, endothelin-1, TNF-α, IL6, and microRNAs ;Granger *et al.*, 2001; Karumanchi & Bdolah, 2004; Zhou *et al.*, 2017) could cause defective normal endothelial cell responses, leading to impaired fetoplacental vascular function (e.g., increased permeability and decreased blood flow to fetus).

V.2.2 Physiological Roles of GNA11 and 14 in Fetal Endothelial Cells

As shown in Chapter II, both GNA11 and 14 protein are significantly elevated in NT vs. FT placentas. Levels of GNA11 protein in FT are undetectable but are relative high in NT placentas. While GNA14 protein is dramatically increased ~7.7 – 10.7 folds in NT vs. FT. These increases are associated with drastically increased placental angiogenesis, which is required for normal placental and fetal growth throughout pregnancy (Magness

& Zheng, 1996), suggesting critical roles of $G\alpha_{q/11}$ subfamily, including GNA11 and 14 in fetoplacental growth during normal pregnancy.

Indeed, co-expression of GNAq and 11 has been shown to be required for VEGFA-induced fetoplacental angiogenic responses via directly interacting with and activating VEGFR2. The questions followed are: 1) can GNA11 or GNA14 alone mediate FGF2- and VEGFA-induced fetoplacental endothelial function? 2) do GNA11 and GNA14 have differential actions on endothelial cell function? The data from the present study indicate either GNA11 or GNA14 alone can differentially mediate FGF2- and VEGFA-induced fetoplacental function as GNA11 knockdown inhibits FGF2- and VEGFA-stimulated cell migration, but not cell proliferation, while GNA14 overexpression attenuates FGF2-, but not VEGFA-induced migration and monolayer integrity. These data also clearly indicate that any dysregulation (either suppression or overexpression) of GNA11 and GNA14 expression in fetal endothelial cells can lead to fetoplacental endothelial dysfunction (Table 2 and 3).

The next question l asked is what are signaling pathways involved in GNA11- and GNA14-mediated cell responses. Obviously, ERK1/2 and AKT1, two of key signaling pathways in fetoplacental endothelial function (Wang and Zheng, 2012) are unlikely to be targets of GNA11 and/or GNA14 as we have shown in Chapters III and IV. Thus, other signaling pathways must be involved. These signaling pathways may include RhoA and PLCβ3 as both of them have been implicated in actions of GNAq and GNA11 in fetoplacental cells (Zeng *et al.*, 2002*b*; Wettschureck & Offermanns, 2005; Hubbard & Hepler, 2006). However, RhoA appears not to be a major player in such mediation under

physiological low oxygen as neither FGF2 nor VEGFA robustly induces activation of RhoA (Suppl. Fig. 3). Instead, PLCβ3 emerges as an important candidate signaling given PLCβ3 has been shown to be a major downstream mediator in GNAq/11-mediated cell response (Wettschureck & Offermanns, 2005; Hubbard & Hepler, 2006).

Like many proteins (e.g., enzymes, protein kinases, and protein phosphatases), the activity of PLCβ3 could be regulated by its phosphorylation status (Yue *et al.*, 1998, 2000; Xia *et al.*, 2001; Yue & Sanborn, 2001). To date, it is estimated that there are a total of 113 potential phospho–sites in PLCβ3 (consisting of 1234 amino acids in human) based on prediction of PLCβ3 amino acid sequences from NetPhos (Technical University of Denmark; http://www.cbs.dtu.dk/services/NetPhos/), although overall effects of such phosphorylation on PLCβ3 activity are largely undefined. In the present study, I choose to focus on PLCβ3 S537 and S1105 since both are implicated in regulation of PLCβ3 and are only two sites with commercially available antibodies against them. We have found that excess phosphorylation of PLCβ3 S537 (FGF2 and VEGFA) and/or S1105 (FGF2) is associated with impaired cell migration in response to FGF2 and VEGFA (Fig. V.1 and 2). Canonically, PLC catalyzes conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG), and subsequently induces activity of protein kinase C (PKC). PKC activity is associated with nitric oxide (NO) production (Matsubara *et al.*, 2003) and vascular endothelial cadherin (VE-Cad) activity (Vandenbroucke St Amant *et al.*, 2012), both of which may regulate endothelial migration (Murohara *et al.*, 1999; Gavard, 2014). Moreover, though GNA11 and 14 are members from the same GNAq/11 subfamily and share highly similar amino acid sequences,

GNA11 mediates not only FGF2-, but also VEGFA-induced cell migration. However, GNA11 is not involved in FGF2- and VEGFA-mediated other cell functions, such as monolayer integrity and cell proliferation. In contrast, GNA14 only modulates FGF2-, but not VEGFA-induced cell function. Thus, different members of $G\alpha_{q/11}$ differentially mediate FGF2- and VEGFA-induced fetoplacental endothelial function via different activation of PLCβ3 and its downstream signaling.

V.2.3. Potential Mechanisms of GNA14 Overexpression in Endothelial Cells during Pathological Conditions

To date, there is no report to link altered GNA11 expression to any human disease. In contrast, GNA14 protein overexpression has been identified in several tissues from hypertensive diseases (e.g. PAH and PE; Abdul-Salam *et al.*, 2010; Zhao *et al.*, 2014). The first question one may ask in this regard is if there is a potential cause and effect relationship between GNA14 overexpression and these hypertensive diseases. Although there is no direct evidence to support this relationship, such a relationship is possible if one considers that GNA14 overexpression either induced by gene mutation or epigenetic modifications cause endothelial dysfunction, leading to impaired vascular function in these tissues. Alternatively, GNA14 overexpression is induced by hypertensive diseases-associated cell microenvironments including dysregulated humoral factors (e.g, growth factor, cytokines and their receptors) and/or relatively prolonged hypoxia (Granger *et al.*, 2001; Bird *et al.*, 2013; Roberts, 2014; Hod *et al.*, 2015; Karumanchi & Granger, 2016; Charolidi & Carroll, 2017)

Indeed, one of the theories regarding the pathogenesis of PE is that defective placentation and/or reduced uterine blood flow lead to placental hypoxia and hence induce increased cytokines, such as FGF2, VEGFA, TNFα, IL6, and/or IL8 (Granger *et al.*, 2001; Bird *et al.*, 2013; Roberts, 2014; Hod *et al.*, 2015; Karumanchi & Granger, 2016). These elevated cytokines may also contribute to hyperpermeability, which is one of the main characteristics of PE symptoms (Uzan *et al.*, 2011). At the level of cells, HUVECs from PE patients exhibit hyperpermeability (Wang *et al.*, 2002) and impaired $[Ca^{++}]_i$ (Krupp *et al.*, 2013). These abnormalities are associated with impaired and disorganized junction proteins, such as connexin 43 (Bird *et al.*, 2013), as well as VE-cadherin and occludin (Wang *et al.*, 2002).

The prolonged hypoxia-elevated GNA14 is directly supported by our recent report that chronically lower oxygen increases expression of GNA14 mRNA by ~ 2.5 fold in HUAECs (Jiang *et al.*, 2013*b*). Thus together with results from the present thesis (Chapter IV), GNA14 overexpression is more likely to a second reaction, rather than a major causing factor, to PE. This overexpression is likely to be induced by dysregulation of placental expressing humoral factors and/or hypoxia (Fig. V.1).Future gain of function in animal models may be designed to test these hypotheses.

However, by far, no direct evidence shows that GNA14 is overexpressed in endothelial cells in tissues from these hypertensive diseases. The recent RNA-seq data from our laboratory reveal no differences in mRNA levels of GNA11 and GNA14 between PE vs. NT HUVECs (unpublished data from Dr. Jing Zheng's lab), suggesting no dysregulation of GNA11 and 14 in endothelial cells from placental macrovasculature.

However, we cannot exclude the possibility that such overexpression occurs in microvascular endothelial cells within placental villi. In addition, if GNA14 is overexpressed primarily in trophoblasts, the question if such overexpression will interfere with expression and release of angiogenic factors in trophoblasts is still needed to be answered in the future studies.

V.2.4. Further Implication of Roles of GNA14 Overexpression in Cardiovascular Diseases.

Nowadays, whether PE impairs fetoplacental angiogenesis is still debatable as both increased capillary volume density in severe PE (Boyd & Scott, 1985), and decreased placental microvessel counts in PE placentas have been reported (Uras *et al.*, 2012). Moreover, others, including a study from Dr. Jing Zheng's lab indicate that PE does not affect placental angiogenesis (as indexed by capillary number density Teasdale, 1987; Li *et al.*, 2015). However, considering that placentas from PE are generally small in size as compared with those from NT (Roberts & Post, 2008; Dahlstrøm *et al.*, 2008; Kim *et al.*, 2014), the overall fetoplacental angiogenesis could be decreased in parallel with GNA14 overexpression.

In addition to angiogenesis, I speculate that GNA14 overexpression may adversely affect endothelial vasodilation via attenuating endothelial production of vasodilators, such as NO and prostacyclin (PGI2) (Magness & Zheng, 1996) and/or increasing production of endothelial vasoconstrictors (ie., endothelin), which warrant further future studies.

Furthermore, if GNA14 overexpression in HUVECs observed in the present study reflects its change in endothelial cells in the entire fetal vascular bed besides fetoplacental endothelial cells, GNA14 overexpression might inhibit fetal angiogenesis or other fetal endothelial function partially via induction of over-phosphorylation of PLCβ3 S537 and S1105, which ultimately contributes to increased risk of cardiovascular diseases in PE offspring during adulthood (Kajantie *et al.*, 2009; Koleganova *et al.*, 2012). Moreover, endothelial-derived FGF2 contributes to the progression of PAH via inducing proliferation of pulmonary artery smooth muscle cell (Izikki *et al.*, 2009). Thus, results presented in this thesis, could shed light on generating therapeutic interventions for endothelial-associated cardiovascular diseases.

V.2.5 Human Disease-Related Mutations of GNA11 and 14.

Beside changes in expression levels, mutations of GNA11 and GNA14, are also identified to be related with several human diseases. These mutations of GNA11 and 14 studied are all cause constitutive activation of G protein α subunits.

A somatic mutation of GNA11 is found in human melanoma which is a skin cancer (Shoushtari & Carvajal, 2014). Such form of mutation disables the ability of hydrolysis GTP to GDP, hence “locks” GNA11 into an active status (Shoushtari & Carvajal, 2014). This mutated GNA11 activates downstream signaling through enhanced activation of MEK/ERK and PI3K/ATK pathways, facilitating tumor progress (Shoushtari & Carvajal, 2014). Recently, a mutation of GNA11at sites different from the melanoma also exhibit a similar constitutive activation and is associated with extremity capillary malformation and

overgrowth in a congenital vascular lesion (Couto *et al.*, 2017). However, the mechanisms of this somatic mutation of GNA11 in mediating such abnormal vascular growth remain unknown.

GNA14 mutation is also linked to congenital and sporadic vascular tumors which are featured with vascular overgrowth (Lim *et al.*, 2016). Transfecting this form of mutation into HUVECs significantly prolongs cell survival under a serum-free condition and causes morphological changes via enhance phosphorylation of ERK1/2 (Lim *et al.*, 2016).

Thus, together with the data from the present study, it appears that expression dysregulation and mutations of GNA11 and GNA14 can both impair endothelial function, possibly via different signaling pathways as no changes in ERK1/2 and Akt1 activation are detected in the present study.

V.3. Proposed Signal Mechanisms underlying Altered GNA11 and GNA14 Expression

Before further discussion, I would like to emphasize the extremely complex singling pathways (e.g. protein kinases, protein phosphatases, and enzymes) that are involved in FGF2- and VEGFA-induced endothelial responses. Most recently, using a commercially available antibody-based protein kinase array, we found that 10 min of VEGFA treatment elevated (≥ 200%) phosphorylation of 48 proteins including ERK1/2 and AKT1 as shown in Chapters III and/or IV, as well as EGFR, FGFR1, and FAK; whereas it suppressed (≥ 60% decreases) phosphorylation of 59 proteins including MEK1, VEGFR1 and VEGFR2 in HUVECs. Thus, singling molecules studied in the present study only represent a very

limited spectrum of the whole signaling networks involved in FGF2- and VEGFA-regulated cell function.

V.3.1. Activation of ERK1/2 and AKT1

Our earlier studies have showed that activation of ERK1/2 and AKT1 play important roles in mediating FGF2- and VEGFA-induced cell migration and proliferation in HUVECs (Wang & Zheng, 2012; Jiang *et al.*, 2013*a*, 2013*b*). In contrast to constitutively activated GNA11 and 14 mutations which enhance activation of ERK1/2 and/or AKT (Zeng *et al.*, 2003; Murray *et al.*, 2010; Shoushtari & Carvajal, 2014 Lim *et al.*, 2016), altered GNA11 and 14 expression does not change FGF2- and VEGFA-induced activation of ERK1/2 and AKT1. These results are consistent with the previous report that knockdown of GNAq and 11 in HUVECs does not change VEGF-induced phosphorylation of ERK1/2 and AKT1 (Sivaraj *et al.*, 2015).

V.3.2. Phosphorylation of PLCβ3 at S537 and 1105

Although PLCβ3 is one of the most potent responder to $G\alpha_{q/11}$ subfamily among other members of PLCβ subfamily, knowledge regarding regulation of PLCβ3 activity through phosphorylation within a intact cell system is extremely limited. Ionomycin can induce PLCβ3 S537 phosphorylation in COSM6 cells (Yue & Sanborn, 2001). In the present study, VEGFA, but not FGF2 robustly elevates phosphorylation of PLCβ3 S537, whereas GNA11 suppression further increases phosphorylation of PLCβ3 S537 in response to FGF2 and VEGFA. Thus, under a normal status, GNA11 appears to function

as an inhibitor of phosphorylation of PLCβ3 S537; whereas once GNA11 is suppressed, this inhibition is released, leading to overphosphorylation of PLCβ3 S537 and ultimately decreasing cell migration stimulated by FGF2 and VEGFA. Similarly, GNA14 overexpression induces phosphorylation of PLCβ3 S1105 in response to FGF2 but not VEGFA, indicating that GNA14 only at relatively high levels can facilitate FGF2-mediated phosphorylation of PLCβ3 S1105 in HUVECs. Such overphosphorylation of PLCβ3 S1105 is also associated with decreased cell migration and strengthened cell monolayer integrity in response to FGF2. Thus, overexpression of either PLCβ3 S537 or PLCβ3 S1105 has negative impacts on fetoplacental angiogenesis.

Phosphorylation of PLCβ3 S537 and S1105 can be induced by multiple protein kinases including calcium/calmodulin-dependent protein kinase II (CaMK II; Yue & Sanborn, 2001), PKC (Yue *et al.*, 2000), PKA (Yue *et al.*, 1998), or PKG (Xia *et al.*, 2001). As one of the downstream targets of PLC, it is not surprising that PKC regulates activity of PLC in a negative feedback fashion (Nishizuka, 1992). Shizukuda *et al.*, 1999 have showed that VEGFA-induced cell migration and proliferation were mediated via decreased PKCδ activity in HUVECs. Thus, it is possible that the increased level of GNA14 potentiates the activity of PKC and/or facilitates PKC-depend phosphorylation of PLCβ3 at S1105, therefore inhibits PLCβ3 activity and further down-regulates FGF2-induced endothelial migration.

V.3.3. Interaction between GPCRs and RTKs though G Protein α Subunits

To date, the exact mechanism governing interactions between GPCRs and RTKs is not clear yet. Here we hypothesize a signaling network model between GPCRs and RTKs based on the present study and previous reports from other investigators (Fig. V.2.; Sivaraj *et al.*, 2015).

One possible mechanism we purpose is that the binding of growth factors to RTKs induces the release of pre-existing unknown GPCR agonists. After release, these GPCR agonists bind with corresponding GPCRs on cells produced them in an autocrine fashion, and hence activate Gα and/or Gβγ signaling pathways. Activated Gα and/or Gβγ further incorporate with RTKs facilitating the signaling transduction (Fig. V.2A). However, Sivaraj *et al*., 2015 have showed that VEGFR2-blocking antibody completely abolishes $[Ca^{++}]_i$ elevation in response to conditioned media from VEGFA-treated HUVECs. In addition, Zeng *et al*., 2003 have also demonstrated that $G\alpha_{q/11}$ are essential for phosphorylation of VEGFR2, which occurs within seconds after VEGFA stimulation. Hence, it is less likely that RTKs communicate with GPCRs in an autocrine fashion.

Another possible mechanism is that, as there exist constant activated GPCRs on cell membrane, a small portion of Gα may be retained in an activated form located in the same lipid domain as RTKs (Fig. V.2B; Bond & Ijzerman, 2006). Hence, upon the binding of growth factors with RTKs, these Gα subunits can immediately bind with RTKs and facilitate signaling transduction of growth factor. In this scenario, if levels of Gα are impaired, it may inhibit basal activity of RTK signalings. Indeed, knockdown of $G\alpha_{q/11}$ impairs basal level of inositol monophosphate accumulation (a stable downstream metabolite of inositol triphosphate), suggesting reduced PLC activity at basal status

(Sivaraj *et al*., 2015). However, more studies are needed to elucidate interactions between RTKs and GPCRs.

V.4. Limitations and Future Directions

V.4.1. Limitations of This Thesis

The first major limitation of this thesis is using HUVECs from term pregnancy as a model for fetoplacental endothelial cells. This cell model may differ from those microvascular endothelial cells residing in placental villi although they may respond similarly to peptide growth factors (Lang *et al.*, 2003; Aird, 2012). However, considering it is unethical to obtain human placentas at early stage of pregnancy and technical difficulty of isolating villous endothelial cells, HUVECs are by far the most widely used cell models to study fetoplacental endothelial function with plenty of information for comparisons.

Secondly, although we constantly culture HUVECs under an O_2 level comparable to that in umbilical cord vessels at term and in placental villi in the first trimester of human pregnancy, this is a static culture condition without any flow stress applied to the cells, which obviously does not reflect *in vivo* state for placental endothelial cells.

In addition, although we make our best effort to keep culturing HUVECs and performing experiments in a low oxygen condition, cells are inevitably to be exposed to atmosphere O_2 levels for a short period of time during cell isolation and experimental preparations in the cell culturehood. Thus, this acute exposure to high O_2 may impact cell phenotypes examined in the present study.

Another major limitation is that all of the present studies are conducted *in vitro*. However, as many signaling mechanisms can only be determined in detail in *in vitro* cell models, this is a stepping stone for us to perform *in vivo* study to confirm these signaling mechanisms in the future studies.

In the present study, we have established an association between PLCβ3 and GNA11/14 in cellular responses to FGF2 and VEGFA in HUVECs. However, no cause and effect interaction is defined. One option to address this issue to use specific and efficient activators for PLCβ3. I have tried the only commercial available PLC activator *m*-3m3FBS (Bae *et al.*, 2003), which is supposed to activate multiple members of PLC family; however, our preliminary data show that *m*-3m3FBS induces PLC activity at the level of, or even lower, than VEGFA (data not shown). Thus, more specific and potent activators are needed before we can attack this challenge.

Further, as discussed earlier in this chapter (Section V.3.), signaling molecules that are involved in actin of GNA11 and 14 are covered in a very limited fraction in the current thesis. Recent preliminary data from our lab using antibody-based protein kinase array reveals hundreds of activated/inhibited kinases induced by VEGFA. Thus, if time and finance permit, it would be interesting to screen FGF2 and VEGFA signaling pathways in endothelial cells after altering GNA11 and GNA14 expression.

Technically, we have also the limited choices of primary antibodies which can be used to target GNA11 and phosphorylation of PLCβ3. The primary antibody of GNA11 used in this thesis is from one of very few products that work efficiently in Western blotting. Soon after a bulk purchase of this antibody, the vendor no longer maintains this product

line. In addition, primary antibodies for phosphorylated PLCβ3 S537 and 1105 are the only two commercially available antibodies.

V.4.2. Future Directions

In the present study, we have only established an association between GNA11/14 with PLCβ3 in cellular responses to FGF2 and VEGFA in HUVECs. However, no cause and effect interaction is defined. Thus, one direct future study can be performed is to specifically alter GNA11 and GNA14 expression using CRISPR/Gas 9 genome editing technology, followed by examining cell responses as described in the present study. If successful, it may provide us with an answer for this question.

In the present study, I have only explored a very limited signaling molecules which may be involved in GNA11 and GNA14 mediation of cell function. It would be interesting to assess the global changes in signaling networks using antibody-based protein kinase array as discussed above, since this method may allow us to find potential target signaling cascades in GNA11- and GNA14-mediated FGF2 and VEGFA cell responses. After screening, specific attention can be focus on Src, CaMK II, PKC, PKA, and PKG as all of these have been shown to be important in regulating PLCβ3 activity.

An earlier publication has showed that, knockout of GNA11 does not interrupt pregnancy and birth of mice (Offermanns *et al.*, 1998). And most of these GNA11 knockout offspring can survive to adulthood and are fertile. However, many key pregnancy related events (e.g. birth weight, placental angiogenesis and fetal cardiovascular functions) in GNA11 knockout mice and fetuses have not been discussed

yet. So far, roles of GNA14 in mediating cardiovascular function in animal models are not yet defined. Thus, if desired resources are given, conditional knockdown of GNA11 and GNA14 in endothelial cells in animal models could enlighten our understanding of roles of GNA11 and 14 in mediating endothelial function.

V.5. Conclusions

Firstly, this thesis further widens our sight of the complexity of G-protein regulation of fetoplacental endothelial function and its underlying signaling mechanisms. Secondly, this thesis proves that GNA11 may not only be functionally redundant, or even replaceable, to GNAq, but also has its unique roles in mediating peptide growth factors-induced fetoplacental endothelial function. Thirdly, for the first time, roles of GNA11 and GNA14 in mediating peptide growth factors-induced endothelial function are determined. Together, these two members of $G\alpha_{q/11}$ subfamily may play important roles in mediating peptide growth factors-induced fetoplacental endothelial function. GNA11 and GNA14 may serve as therapeutic targets for correcting endothelial dysfunction occurring in many endothelial cell related cardiovascular diseases.

V.6. Figures

Figure V.1.

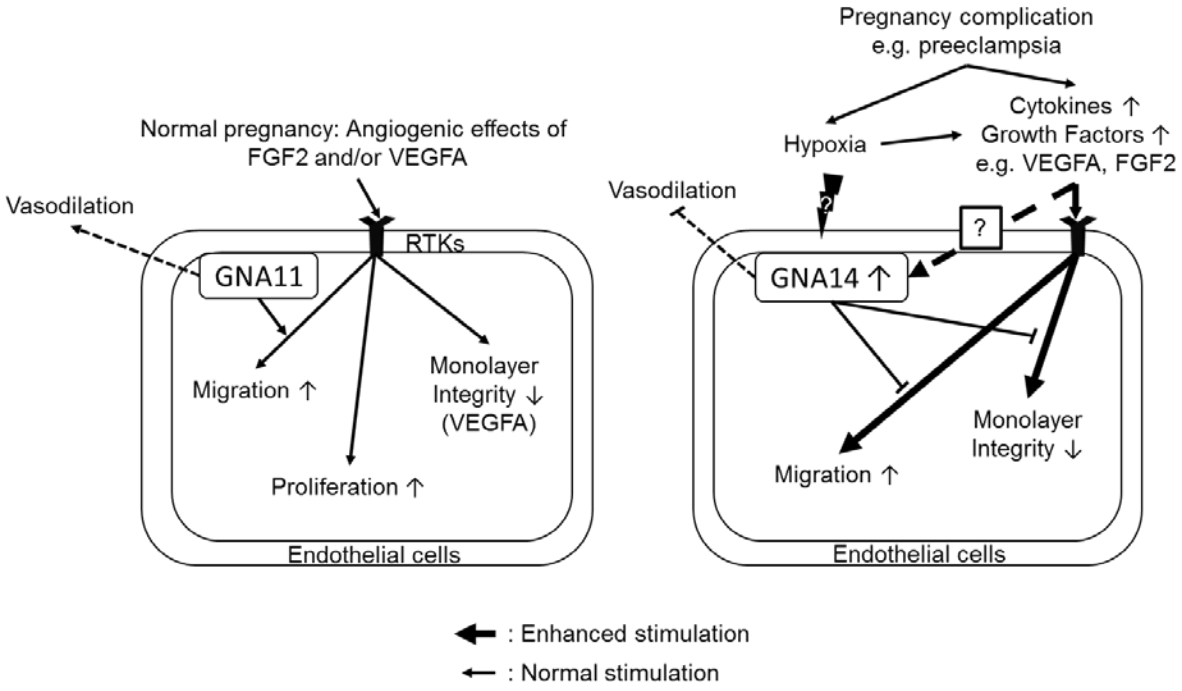

Figure V.1. A proposed model for the roles of GNA11 and 14 in mediating fetoplacental angiogenesis in normal (left) and PE (right) pregnancy. Left penal: During normal pregnancy, GNA11 serves as a mediator of FGF2- and VEGFA-induced endothelial migration, but not proliferation and monolayer integrity. Loss function of GNA11 may impairs normal fetoplacental angiogenesis. Right panel: During PE, improper placentation induces hypoxia and placental ischemia and hence elevates production of cytokines and/or growth factors (e.g. VEGFA, sFlt-1, FGF2, TNFα, IL6, and IL8). These abnormal conditions induce GNA14 overexpression, leading to endothelial dysfunction. Question marks: unknown relationships; Bold lines and arrows: enhanced stimulation.

Figure V.2.

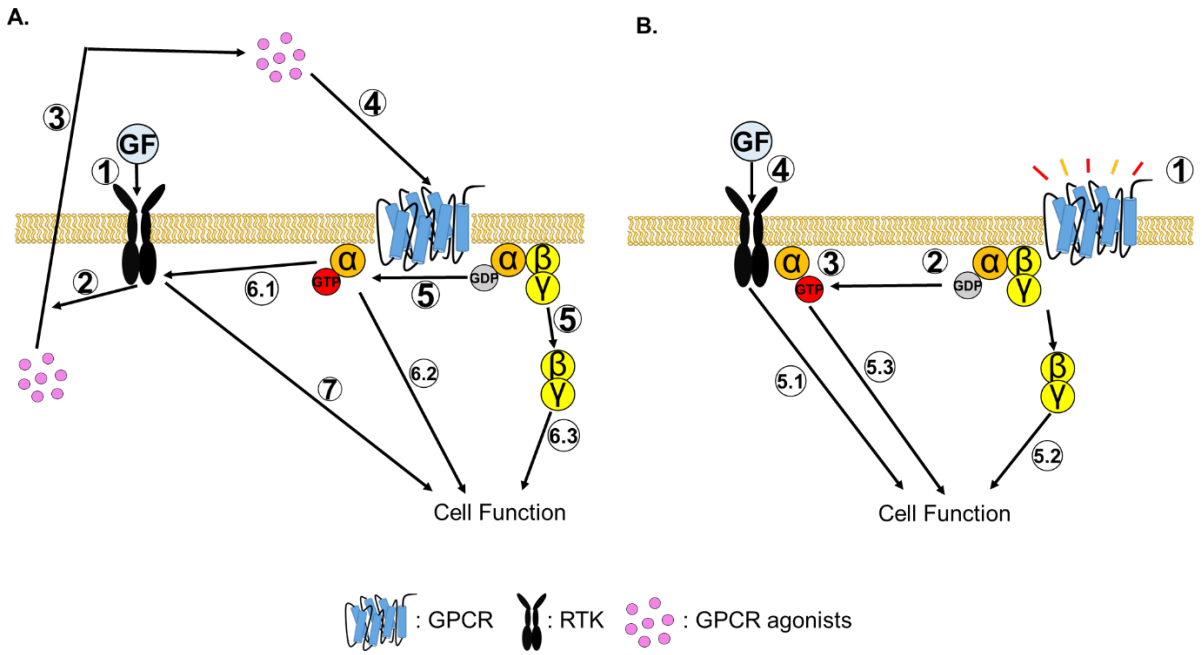

Figure V.2. Proposed mechanisms of interactions between Gα and RTK. A: The binding of peptide growth factors to RTKs (A1) induces secretion (A2-A3) of yet-to-be identified GPCR agonists, which in turn activate GPCR (A3 and 4), ultimately activating Gα and βγ (A5) to mediate cell function via coupling of Gα with RTK (A6.1), Gα (A6.2) and/or by βγ complex (A6.3). B: The basal activity of GPCRs (B1) maintains a tonic activity of Gα (B2), which are temporally closely associated with RTK (B3). Hence, upon binding of growth factors to RTKs (B4), these activated Gα rapidly facilitate RTK dimerization and/or autophosphorylation, activating RTKs- (B5.1), βγ complex- (B5.2), and/or Gα- (B5.3) induced cell function. GF: peptide growth factors. GPCR: G protein-coupled receptor. RTK: receptor tyrosine kinase.

V.7. Reference

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Table 1.

Antibody	Vendor	Catalog #	Dilution
β-actin	Cell Signaling Technology	4967	1 : 1000
GAPDH	Novus Biologicals	H00002597-M01	1 : 10000
GNA11	Abgent	AP19441a	1 : 1000
GNA14	Abnova	H00009630-M06A	1 : 500
GNA14	GeneTel Laboratories	Rb139-Rb140	1 : 500
phospho-p44/42 MAPK T202/Y204 (p-ERK1/2)	Cell Signaling Technology	9101	1 : 2000
p44/42 MAPK (t-ERK1/2)	Cell Signaling Technology	9102	1 : 2000
phospho-AKT1 S473 (p-AKT1)	Santa Cruz Biotech	sc-7985-R	1 : 2000
AKT1 (t-AKT1)	Cell Signaling Technology	9272	1 : 2000
phospho-PLCβ3 S537 (p-PLCβ3 S537)	Cell Signaling Technology	2481	1 : 500
phospho-PLCβ3 S1105 (p-PLCβ3 S1105)	Thermo Fisher Scientific	PA5-38089	1 : 1000
PLCβ3 (t-PLCβ3)	Millipore	ABS-512	1 : 1000
RhoA	Cytoskeleton	ARH04	1 : 500
RhoA, B, C	Millipore	05-778	1 : 500

Table 2.

			FGF2	VEGFA
Cell Function	Cell migration	control (ssiRNA)	↑↑	↑↑
		GNA11 siRNA	↑	↑
	Cell proliferation	control (ssiRNA)	↑↑	↑↑
		GNA11 siRNA	↑↑	↑↑
	Monolayer integrity	control (ssiRNA)	--	↓
		GNA11 siRNA	--	↓
Phosphorylation	ERK1/2	control (ssiRNA)	↑↑	↑↑
		GNA11 siRNA	↑↑	↑↑
	AKT1	control (ssiRNA)	--	--
		GNA11 siRNA	--	--
	PLCβ3 S537	control (ssiRNA)	--	↑↑
		GNA11 siRNA	↑↑	↑↑↑↑
	PLCβ3 S1105	control (ssiRNA)	--	--
		GNA11 siRNA	--	--

Stimulation | No change | Inhibition

Color intensity indicates degree of change

Table 3.

	Serum-free media (or ATP)	FGF2	VEGFA

Cell Function	Cell migration	control (Ad-*GFP*)	--	↑↑	↑↑
		Ad-*GNA14*	--	--	↑↑
	Cell proliferation	control (Ad-*GFP*)	↑↑	↑↑	↑↑
		Ad-*GNA14*	--	-- (?)	-- (?)
	Monolayer integrity	control (Ad-*GFP*)	--	--	↓
		Ad-*GNA14*	--	↑	↓
	$[Ca^{++}]_i$	control (Ad-*GFP*)	↑↑	--	↑↑
		Ad-*GNA14*	↑↑	--	↑↑
Phosphorylation	ERK1/2	control (Ad-*GFP*)	x	↑↑	↑↑
		Ad-*GNA14*	x	↑↑	↑↑
	AKT1	control (Ad-*GFP*)	x	--	↓
		Ad-*GNA14*	x	--	↓
	PLCβ3 S537	control (Ad-*GFP*)	x	--	↑↑
		Ad-*GNA14*	x	--	↑↑
	PLCβ3 S1105	control (Ad-*GFP*)	x	--	--
		Ad-*GNA14*	x	↑↑	--

Stimulation | No change | Inhibition

Color intensity indicates degree of change

X: experiments were not performed

?: unknown relationship

Supplemental methods

mRNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA were extracted using AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) and quantified by using NanoDrop 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). The quality and integrity of total RNA were confirmed using the Agilent RNA6000 NanoChip in the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with RNA integrity number scores larger than 9.0 were used. Samples of total RNA (1 μg) were reverse transcribed to cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen) in a 20 μl volume.

RT-qPCR was performed as described in Chapter III in a StepOne^{Plus} qPCR system (Life Technologies). Each RT-qPCR reaction was performed in triplicate. In parallel, XenoRNA control (a synthetic RNA transcript) and ACTB from TaqMan Cells-to-CT Control Kit (Invitrogen) was used as endogenous and exogenous control. The normalized RT-qPCR data were then further analyzed using the $2^{-\Delta\Delta CT}$ method to determine the relative abundance.

Rho activation assay

Rho activity was examined by Rho Activation Assay Kit (#17294, Merck Millipore, Burlington, MA) as manufacturer's instruction. Briefly, HUVECs were inoculated into three 60 mm petri dishes and serum-starved for 24 hr before addition of FGF2/VEGFA (100 ng/ml) for 0, 1, 5, 15, and 30 min. Cells were rinsed twice by ice cold PBS and lysed by 0.2 ml Mg2+ lysis buffer (MLB, #20168, Merck Millipore). Cells were detached by rubber

policeman and cellular lysates were incubated for 15 min at 4 ºC with agitation. Cellular lysates were then centrifuged (14000g) for 5 min at 4 ºC. Cellular debris were discarded and protein concentrations were measured by BCA Protein Assay Kit (#23225, ThermoFisher). For the following procedures, 400 µg protein per treatment were used. For GTPγS and GDP loading, 500 µl of cellular extract were used. GTPγS (100 µM; a form of GTP that is hardly hydrolyzed by GTPase serving as positive control) and GDP (1 mM; a negative control) with EDTA (10 mM) were added and incubated for 30 min at 30 ºC with agitation.

Active Rho pull-down assay was performed as manufacturer's instruction. Briefly, 15-23 µl (20 - 30 µg) of Rho Assay Reagent slurry were added to cellular lysates (with or without GTPγS or GDP) and incubate for 45 min at 4 ºC with gentle agitation. Agarose beads were briefly centrifuged and washed by MLB for three times. Finally, 2 µl of dithiothreitol (1 M) was added and samples were boiled in 2x Laemmli reducing sample buffer for 5 min and subsequently subjected for Western blotting.

Supplemental results

Ad-*GNA14* dramatically increases GNA14 mRNA, while decreases GNA11 mRNA.

Ad-*GNA14* was transfected at 0, 5, 10, and 20 MOI for 3 days in HUVECs. Ad-*GNA14* drastically elevated GNA14 mRNA for ~1100, ~3500, and ~7900 folds comparing 0 MOI at 5, 10, and 20 MOI, respectively. However, Ad-*GNA14* significantly attenuated GNA11 mRNA ~33%, ~28%, and ~30% at 5, 10, and 20 MOI, respectively.

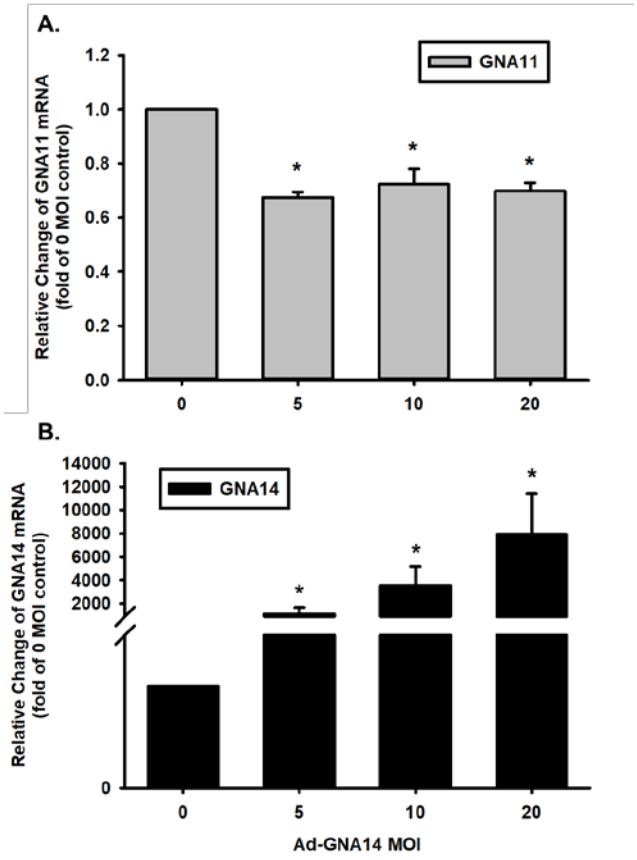
GNA14 siRNA significantly decreases GNA14 mRNA.

HUVECs were transfected with GNA14 siRNA for 2, 3, and 4 days. GNA14 siRNA significantly decreases GNA14 mRNA for ~53%, ~46%, and ~31% at 2, 3, and 4 days of transfection, respectively. However, mRNA levels of GNA11 were not affected by GNA14 siRNA (data not shown).

FGF2 and VEGFA does not induce activation of Rho Subfamily.

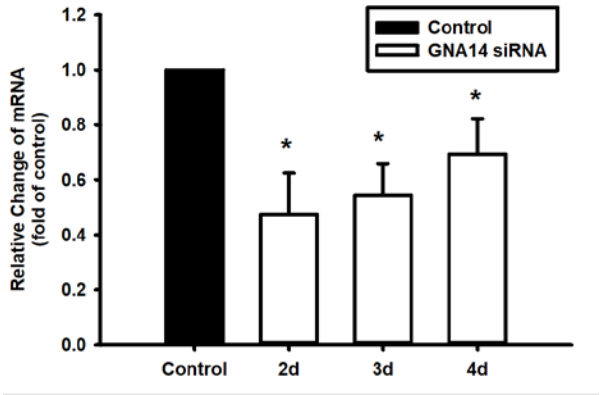
FGF2 and VEGFA-induced activation of Rho subfamily were also examined by immunoprecipitation. In GTPγS and GDP pretreat groups, immunoprecipitation showed clear band, indicating activated RhoA. However, cellular lysates treated by FGF2 or VEGFA for 5, 15, and 30 min did not show significant bands as compared with 0 min control, suggesting in PCN-HUVECs, FGF2 and VEGFA do not significantly induce activation of RhoA. Shorter time points of treatments (1 and 2 min) did not show significant band of active RhoA. Another primary antibody detecting RhoA, B, and C was used, however, similar results were observed (data not shown).

Supplimental Figure 1.



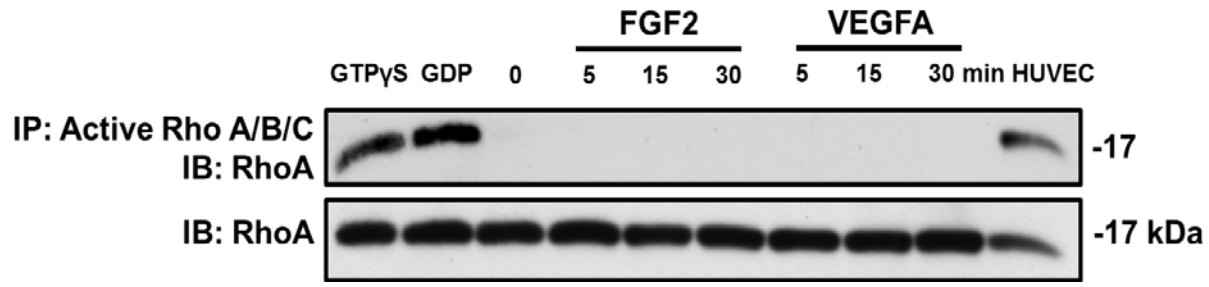
Supplemental Figure 1. Effects of Ad-*GNA14* on relative gene expression of GNA11 and GNA14 in HUVECs. HUVECs were transfected by Ad-*GNA14* at 5, 10, and 20 MOI for 3 days. Cells were then subjected to RT-qPCR for expression levels of (A) GNA11 and (B) GNA14. Data were displayed as mean ± SEM. * Different from respective 0 MOI control ($p < 0.05$; Dunnett's multiple comparison vs 0 MOI control), n = 3 individual experiments.

Supplemental Figure 2.



Supplemental Figure 2. Effects of GNA14 siRNA on relative mRNA levels of GNA14. HUVECs were transfected with GNA14 siRNA (20 nM) for 2, 3, and 4 days. Cells were then subjected to RT-qPCR. Data were expressed as means ± SEM. *Different from control ($p < 0.05$; student's t-test). n = 3 individual experiments.

Supplemental Figure 3.



Supplemental Figure 3. Effects of FGF2 and VEGFA on activation of RhoA. HUVECs were treated with FGF2 or VGEFA for up to 30 min. Control cellular lysates were firstly incubated with GTPγS or GDP for 45 min, then activated Rho were pull-downed by agarose-conjugated antibody. FGF2- and VEGFA-treated cellular lysates were directly pull-downed using agarose-conjugated antibody of active RhoA. Upper panel: Cellular lysates were immunoprecipitated against active Rho, then immunoblotted using monoclonal primary antibody targeted on RhoA. Lower panel: same amount of proteins were immunoblotted by monoclonal primary antibody targeted RhoA.