EXPRESSION AND SECRETION OF BASIGIN BY HUMAN PLACENTAL TROPHOBLAST CELLS OCCURS VIA MICROVESICLE SHEDDING AND IS A REGULATED PROCESS

BY

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THESIS
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ABSTRACT

Successful pregnancy depends on the ability of trophoblast cells to invade into maternal uterine tissues in a tightly controlled manner. Subsequently the trophoblasts must remodel the uterine spiral arteries in order to establish an adequate blood supply for normal fetal and placental development. Failure of sufficient trophoblast invasion and defective remodeling of the maternal uterine spiral arteries is linked to the obstetrical disease preeclampsia. Trophoblast invasion and spiral artery remodeling are influenced by many factors such as matrix metalloproteinases (MMPs), as well as cytokines, growth factors and oxygen tension. Previous studies have demonstrated that basigin (BSG)/extracellular matrix metalloproteinase inducer (EMMPRIN) is able to regulate MMP production and is critical for successful implantation, angiogenesis and parturition. However, expression and specific functions of BSG during the first trimester of pregnancy in human placenta have not been investigated. This study focused on determining whether BSG is present in the first trimester human placenta, how secretion of BSG protein is regulated, and what the specific functions of BSG during early placentation may be. The main findings of our work are: 1) BSG protein is present in the human trophoblast throughout the first trimester of pregnancy; 2) human trophoblast-like cell lines in culture express BSG transcript variants -2, -3 and -4 and secrete BSG protein into surrounding medium; 3) release of BSG from the surface of trophoblast-like cells occurs through microvesicle shedding; 4) the signaling cascade responsible for microvesicle release is the protein kinase C (PKC) pathway; 5) BSG-containing microvesicle release by trophoblast-like cells is altered in response to hypoxia/reoxygenation injury; 6) the inflammatory cytokine interleukin-1β (IL-1β) and transforming growth factor-β1 (TGF-β1) regulate BSG protein abundance in microvesicles at the post-transcriptional level; 7) recombinant BSG protein (rBSG) stimulates release of MMP-
1 and MMP-3 by cultured human umbilical vein endothelial cells (HUVEC). These findings show that BSG secretion occurs through a controlled process of microvesicle shedding in trophoblasts, and supports our hypothesis that BSG regulates interactions of trophoblast cells with other uterine cells during early placental development.
DEDICATION

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LIST OF ABBREVIATIONS

ATP: adenosine triphosphate
Bis: bisindolylmaleimide I HCl
BM: basement membrane
BSG: basigin
cAMP: cyclic adenosine monophosphate
COX-2: cyclooxygenase-2
DAG: diacylglycerol
E2: estradiol
EC: endothelial cell
ECM: extracellular matrix
EMMPRIN: extracellular matrix metalloproteinase inducer
END1: endothelin-1
ER: endoplasmic reticulum
ESCRT: Endosomal Sorting Complex Required for Transport
EVT: extravillous trophoblast
FAK: focal adhesion kinase
FasL: Fas ligand
GPCR: G-protein-coupled receptor
hCG: human chorionic gonadotropin
HIF: hypoxia inducible factor
HIV: human immunodeficiency virus
HPL: human placental lactogen
HUVEC: human umbilical vein endothelial cell

IL-1: interleukin-1

IL-1β: interleukin-1β

IL-6: interleukin-6

IL-8: interleukin-8

IL-10: interleukin-10

IL-11: interleukin-11

LIF: leukemia inhibitory factor

LSB: laemmli sample buffer

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

MCP1: monocyte chemotactic protein1

MCT: monocarboxylate transporter

MEK 1 / 2: mitogen-activated protein kinase kinase

MMP: matrix metalloproteinase

MT-MMP: membrane-type matrix metalloproteinase

MVB: multivesicular body

NF-kB: nuclear factor kappa B

P4: progesterone

PAI: plasminogen activator inhibitor

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PMA: phorbol myristate acetate
POP₄: processing of precursor 4, ribonuclease P/MRP subunit

PTP: permeability transition pore

rBSG: recombinant BSG protein

ROS: reactive oxygen species

RTK: receptor tyrosine kinase

SDS: sodium dodecyl sulfate

TBS: tris-buffered saline

TBST: tris-buffered saline with tween 20

TBS-TT: tris-buffered saline with triton-x and tween 20

TCSF: tumor cell-derived collagenase stimulatory factor

TF: tissue factor

TGF-β: transforming growth factor-β

TGF-β1: transforming growth factor-β1

TIMP: tissue inhibitor of matrix metalloproteinase

TNF-α: tumor necrosis factor- α

TPA: 12-O-tetradecanoylphorbol-13-acetate

TRAIL: TNF-related apoptosis inducing ligand

TUN: trophouteronectin

uNK: uterine natural killer cell

uPA: urokinase type plasminogen activator

VEGF: vascular endothelial growth factor

VSMC: vascular smooth muscle cell

XDH/XO: xanthine dehydrogenase/xanthine oxidase
CHAPTER 1
INTRODUCTION

Successful pregnancy outcome depends on the ability of trophoblast cells to invade into maternal uterine tissues in a highly controlled (both in space and time) manner and also on proper remodeling of the uterine spiral arteries to ensure establishment of a sufficient blood supply between the mother and the fetus [1-4]. Insufficient placental invasion and defective remodeling of spiral arteries during the first trimester of pregnancy can result in severe outcomes such as preeclampsia. This obstetrical disease is a leading cause of morbidity and mortality to both the fetus and mother [5-9].

During the initial stages of implantation, apposition and attachment of the blastocyst trophectoderm to the uterine endometrial luminal epithelium occurs. This is followed by the invasion of trophoblast cells through the basement membrane into the underlying stromal compartment. Trophoblast cells must invade into the maternal uterine tissues in order to be able to anchor the fetus and the placenta within the uterine endometrium and to reach the maternal circulation [1, 4, 10].

In the initial weeks of pregnancy the placental villi are covered by an inner mononuclear layer of cytotrophoblasts. These cytotrophoblasts can take on two differentiation paths. They can fuse and form a multinucleated syncytiotrophoblast layer that overlies cytotrophoblasts or they can take on an invasive phenotype differentiating into extravillous trophoblast (EVT). During the invasion process the EVT grows out of the villi migrating from the basement membrane (BM) of anchoring villi into the decidua migrating as far as the inner layer of the myometrium and into the walls of uterine spiral arteries [11, 12]. These migration and differentiation processes are
regulated by multiple factors including proteases (MMPs, urokinase type plasminogen activator (uPA), cathepsins), oxygen tension, cytokines, chemokines, growth factors, and others [3, 13-15].

Our laboratory studies the role of the protein BSG in reproduction. BSG, also known as CD147 or EMMPRIN, is a transmembrane glycoprotein that is a member of the immunoglobulin superfamily [16-18]. BSG was originally identified as tumor cell-derived collagenase stimulatory factor (TCSF) that was isolated from tumor cell conditioned medium and was able to stimulate production of MMPs by fibroblasts [17]. Since then, BSG protein has been found to be robustly expressed by cancer cells and has been recognized to play a key role as a local regulator of MMP production [19-22]. Furthermore, it has been shown that BSG is released from both tumor and normal cells through plasma membrane microvesicle shedding [23, 24]. In addition to induction of MMPs, BSG has multiple other functions including involvement in neurological processes [25, 26], retinal function [27], lymphocyte migration and activation [28, 29], and shuttling of monocarboxylate transporters (MCTs) to the cell surface membrane [30].

BSG exerts important functions in both male and female reproductive processes including spermatogenesis, fertilization and embryonic implantation [31, 32]. Bsg null mutant mouse embryos fail to implant into the uterus despite developing normally during the pre-implantation stages [31]. It was shown later that BSG protein is expressed in mouse pre- and peri-implantation embryos and is located in both inner cell mass and trophectoderm layers of the blastocyst [33]. In addition, BSG is expressed in human term placenta by trophoblast cells and amniotic epithelial cells. Within the placenta, it is believed that BSG may induce fetal membrane rupture and lead to placental detachment from the uterus at the time of parturition through induction of MMPs [34]. However, despite previous studies done on the role of this protein
during implantation and parturition, the presence and secretion of BSG in the first trimester of pregnancy have not been examined and the specific functions of BSG during placentation are not clear.

In the first portion of this project, we wanted to confirm that BSG is present in first trimester human placenta and determine how BSG protein is secreted by trophoblast cells. We hypothesized that BSG protein is present in the first trimester trophoblast cells and that trophoblast cells release BSG protein into surrounding medium through microvesicle shedding allowing for paracrine signaling to other uterine cells. The specific aims for this study were to 1) determine whether BSG protein is present in human placental tissues throughout the first trimester of pregnancy; 2) determine whether BSG is secreted by trophoblast cells in culture using human immortalized first trimester trophoblast cells and choriocarcinoma cell lines; 3) determine whether BSG is released from trophoblast-like cells through microvesicle shedding; and, 4) determine whether rBSG protein may act on endothelial cells (ECs) to regulate MMP production.

The second portion of this project focused on determining how microvesicle release by trophoblast cells may be regulated. Our specific aims were to: 1) determine whether microvesicle shedding by trophoblast-like cells is regulated through the PKC pathway; 2) determine how microvesicle shedding by trophoblast-like cells may be altered by hypoxia/reoxygenation; and 3) determine the effect of the cytokine IL-1β and growth factor TGF-β1 on microvesicle shedding and abundance of BSG protein in microvesicles in trophoblast-like cells.

Data from these experiments will help to unravel the role of the BSG protein in early placental development and clarify the mechanism of BSG release from placental trophoblast cells into surrounding environment. We also hope to elucidate the signaling pathway that
regulates BSG secretion via microvesicle shedding and identify other external stimuli that regulate microvesicle release from placental trophoblast cells.
CHAPTER 2
LITERATURE REVIEW

implantation

Conception begins with fertilization of the oocyte in the ampulla of the fallopian tube. The newly formed zygote remains in the fallopian tube for approximately three days during which it undergoes cell cleavage leading to development of the morula, a mass of totipotent cells still enclosed within the zona pellucida. A series of differentiation events occur after compaction of the morula leading to development of a blastocyst, a ball-like structure with a fluid-filled inner cavity. Cells that lie on the outside of the morula become trophectoderm while undifferentiated cells within form the inner cell mass. Formation of the blastocyst occurs after the morula has moved from the fallopian tube into the uterine cavity. During this time the maternal endometrium is prepared for implantation. All of the processes described above are referred to as preimplantation development [1, 10].

Implantation begins when a dialogue is initiated between the blastocyst which floats freely in the lumen of the uterine cavity and the receptive endometrium. The timely arrival of the embryo to the receptive endometrium is very important and is called the ‘window of implantation’, the time when the uterine endometrium is receptive to blastocyst implantation [5, 35]. The ‘window of implantation’ does not only include changes in the endometrial epithelium. In rodents and humans uterine stromal cells undergo the process of decidualization. Stromal cells alter their morphology and secretions and become terminally differentiated, rounded decidual cells [36].
Once blastocyst apposition and adherence to the endometrial epithelium occurs, an invasive process begins whereby the blastocyst finally breaks through the BM into the underlying stroma [10]. This process of invasion is for the most part controlled by trophoblast cells. However, the decidua controls the extent of invasion. It creates a physical barrier that prevents excessive penetration by trophoblast cells. In addition, the decidua regulates cytokine production and secretes protease inhibitors to impede invasion [35-37].

ANATOMY OF PLACENTATION DURING THE FIRST TRIMESTER OF PREGNANCY

Development and maturation of the placenta requires highly controlled processes of trophoblast proliferation, differentiation and invasion into maternal uterine tissues as well as remodeling of spiral arteries to establish an adequate blood supply between the mother and fetus [2, 38]. During the first trimester of human pregnancy, the placental villi are covered by the inner mononuclear layer of cytotrophoblasts. Cytotrophoblast progenitors in the placental villi follow one of two different paths; some fuse and form multinucleated syncytiotrophoblast that overlies the cytotrophoblast layer, others follow an invasive path differentiating into the EVT. During this invasion process, the EVT grows out from the villous forming cell columns and begins to migrate from the BM of the anchoring villi into the decidua reaching as far as the inner layer of myometrium as well as into the walls of uterine spiral arteries. The syncytiotrophoblast layer has a barrier function in human placenta and also massively increases the surface area available for nutrient exchange between the mother and the fetus whereas the invasive EVT plays an important role in remodeling of the uterine spiral arteries. EVT that invades into the decidua can be classified into either endovascular trophoblast cells that migrate down the spiral arteries and
replace ECs or interstitial trophoblast cells that invade into the decidua (Fig.1). Interstitial trophoblast cells also destroy the arterial media which is replaced by fibrinoid material and then fuse in the myometrium to form placental-bed giant cells [5, 11]. Both interstitial and endovascular trophoblast cells play important roles in spiral artery remodeling by participating in remodeling of extracellular matrix (ECM) and disruption of ECs/ECM/vascular smooth muscle cells (VSMCs) interactions. Late in the first trimester of pregnancy such active EVT invasion results in conversion of low-flow, high-resistance vessels into high-flow, low resistance spiral arteries with increased circumference and vascular compliance [38, 39]. Such vessel restructuring is necessary for delivery of large amounts of blood that are required in the second and third trimesters of pregnancy to ensure there is sufficient blood supply for adequate fetal development independent of maternal vasoconstriction [40].

FACTORS REGULATING FORMATION OF DIFFERENT TROPHOBLAST CELL TYPES

Cytotrophoblast progenitor cells take on diverse paths of differentiation in response to specific factors that direct them to one or another pathway. For example, it is known that conversion of cytotrophoblast progenitor cells to human chorionic gonadotropin (hCG) producing, multinucleated syncytiotrophoblasts requires factors including charged phospholipids such as phosphatidylserine, divalent cations such as calcium and magnesium, and proteins of the syncytin family [41]. Moreover, cyclic adenosine monophosphate (cAMP) and its analogues are able to induce trophoblast fusion \textit{in vitro} [42]. Human cytотrophoblasts can also be induced to differentiate into syncytiotrophoblasts in response to hCG [43]. Trophoblast cells within cell columns, also called junctional trophoblast, produce a special type of fibronectin called
trophouteronectin (TUN). Junctional trophoblasts make contact with external ECM and TUN facilitates attachment of the placenta to the uterus. Transforming growth factor-β (TGF-β) and leukemia inhibitory factor (LIF) have been shown to up regulate TUN and down regulate hCG production [37, 44]. Invasive EVTs, also called intermediate trophoblasts because of their intermediate size between cyto- and syncytiotrophoblast, acquire an invasive phenotype and actually leave the placenta. EVT cells express various proteases and switch to a different type of integrin expression in order to be able to invade. These cells produce human placental lactogen (HPL) [12, 37].

REMODELING OF SPIRAL ARTERIES

Prior to undergoing remodeling, the uterine spiral arteries consist of three distinct layers. The first layer is intima. It is the most internal layer of ECs that lies on top of a BM consisting mainly of collagen type-IV and laminins. Elastin, collagen type-IV and fibronectin form the internal elastic lamina that surrounds the intima and separates the ECs from the next layer. The internal elastic lamina is reduced or absent in the decidual segments of the uterine spiral arteries. The second layer is formed by VSMCs surrounded by elastic fibers forming the external elastic lamina. ECs and VSMCs have direct cell to cell contact within the matrix through the myoendothelial junctions. There may be several layers of VSMCs at the more proximal end of the spiral arteries, while the distal end has a single layer of VSMCs. The third layer is the adventitial layer which surrounds the external elastic lamina and consists mainly of collagen fibers and fibroblasts. Thus, the internal elastic lamina functions as a barrier to invading endovascular trophoblast cells while the external elastic lamina and the adventitial layer serve as a barrier for interstitial trophoblast cells [39].
The first step in remodeling of the spiral arteries is decidua-associated vascular remodeling characterized by EC vacuolation and VSMC swelling prior to EVT invasion [39]. An abundance of macrophages and uterine natural killer (uNK) cells have been observed in close proximity of the spiral arteries at the time of decidua-associated vascular remodeling [45, 46]. These changes in the decidua are thought to be under the control of steroid hormones [47].

The next step in spiral artery remodeling involves both interstitial and endovascular trophoblast cell invasion. Invasion occurs retrograde to the blood flow [39]. Endovascular trophoblasts invade through the distal openings of the spiral arteries. By 8 weeks of gestation almost all the spiral arteries contain endovascular trophoblast cells at their distal segments. After 10 weeks endovascular trophoblast cells are found more proximally, deeper in the decidua [48]. Interstitial trophoblast cells reach the myometrium by 8 weeks of gestation [49]. Extensive loss of VSMCs and ECs occurs during this stage as well as disappearance of decidual leukocytes [46].

The final step in spiral artery remodeling is regeneration of the arterial wall when the vascular cells are completely replaced by EVTs that secrete and become embedded in fibrinoid material [46]. This fibrinoid material consists of fibronectin, collagen type IV and laminin. The result is formation of high-flow, low-resistance remodeled blood vessels [39, 50]. The endothelium that is replaced by trophoblast layer is restored later during pregnancy [39].

Overall, several mechanisms are proposed to be involved in spiral artery remodeling. These include ECM remodeling, vascular cell de-differentiation, cell migration, changes in the adhesive properties of cells, and apoptosis and may be interdependent [39].
OXYGEN ENVIRONMENT WITHIN THE FETOPLACENTAL UNIT

During EVT invasion into the spiral arteries during first trimester of pregnancy, EVT cells form plugs that occlude the uterine spiral arteries. This results in restricted flow of maternal blood into the intervillous space, creating a low oxygen environment [38, 40]. Direct ultrasonography measurements of oxygen tension in endometrium and placenta with oxygen sensitive probes showed that, at 8 to 10 weeks of gestation, oxygen concentrations within the placenta are less than 20 mm Hg as compared to endometrium where the oxygen concentration is around 40 mm Hg. The oxygen tension in the placenta is therefore lower than in endometrium [51]. Establishment of such a low oxygen environment of 3-5% O2 within the intervillous space is referred to as a physiologic hypoxic environment, and is essential for early placental invasion and development [52].

At 11-12 weeks of gestation, the spiral arteries become unobstructed allowing an increase in maternal blood flow that leads to an increase in placental oxygen levels to 8-10% O2 [11, 38] (Fig.2). By 12-13 weeks gestation, when placental invasion is almost complete, the oxygen tension in the placenta increases to 60 mm Hg [51].

Both the normal process of placentation as well as a pathological process such as tumor growth occur in a low oxygen environment. Cells subjected to hypoxia alter their expression of genes involved in angiogenesis such as vascular endothelial growth factor (VEGF) [39-42] and endothelin-1 (END1) [53, 54] as well as genes involved in invasion processes such as those necessary for invasion and degradation of the ECM [55, 56]. Thus, oxygen levels play an important role in regulation of cellular invasion [57] and hypoxia inducible factors (HIFs) have been shown to be key mediators of the response to low oxygen concentrations [58].
PREECLAMPSIA - A DISEASE OF IMPAIRED PLACENTAL DEVELOPMENT

Preeclampsia is an obstetrical disease that is the leading cause of morbidity and mortality to both fetus and the mother. It is diagnosed by gestational proteinuria and hypertension and the only intervention for this disease is delivery of the fetus. Thus, the greatest contributing factor to perinatal morbidity is premature birth. It is estimated that preeclampsia is responsible for 15% of all preterm births [6-9].

The cause of preeclampsia is still unknown. The most widely recognized predisposing factor for this disease is poor placentation characterized by insufficient cytotrophoblast invasion and defective remodeling of the maternal uterine spiral arteries [59]. In contrast to vascular transformation of spiral arteries during the normal course of pregnancy, spiral artery changes in preeclampsia are insufficient. Invasion of the spiral arteries by endovascular trophoblast occurs only at the superficial aspect of the endometrium or is absent. Insufficient invasion results in reduction of spiral artery diameter in preeclamptic pregnancies. The spiral arteries are less than half the diameter of spiral arteries in normal pregnancies [6, 59] (Fig.3).

Previously it was hypothesized that the abnormalities observed in preeclampsia result from a state of chronic hypoxia. However, this view has changed recently. Because of the incomplete remodeling of spiral arteries, high pressure flow of blood results in hydrostatic damage to the placental villi and perfusion by intermittent pulses of fully oxygenated blood. Fluctuations in oxygen concentrations resulting from this intermittent perfusion, also referred to as hypoxia/reoxygenation damage, lead to oxidative stress. Placental oxidative stress further leads to over-activation of the maternal systemic inflammatory network. Thus, the changes seen in preeclampsia are the result of oxidative stress and physical disruption of the placental villi [9, 59]. More recent studies have proposed that inadequate placentation is actually the result of poor
immunoresponsiveness to paternal antigens and inadequate maternal tolerance of feto-paternal antigens [9, 60-61].

HYPOXIA/REOXYGENATION

Hypoxia/reoxygenation injury results from fluctuations in oxygen concentrations. The pathological changes observed in preeclampsia have been proposed to result from intermittent perfusion of the placenta by fluctuating oxygen levels [59]. Hypoxia-reperfusion type injury can result in oxidative stress that leads to the generation of reactive oxygen species (ROS) through mitochondrial pathways and the xanthine dehydrogenase/xanthine oxidase (XDH/XO) system [62]. ROS can cause cellular dysfunction, growth arrest and even cell death [59]. Levels of malondialdehyde, a product of lipid peroxidation, are increased in women with preeclampsia while levels of antioxidants such as peroxide dismutase, glutathione peroxidase, glutathione reductase and catalase are decreased compared to normal pregnant women and non-pregnant controls [63]. Moreover, expression of XDH/XO holoenzyme and the activity of xanthine oxidase, that is known to generate ROS, are increased in cytotrophoblasts of women with preeclampsia. Peroxide dismutase levels are reduced in the same cells [64].

Calcium homeostasis is also perturbed as a consequence of hypoxia/reoxygenation injury and leads to formation of mitochondrial permeability transition pores (PTP). ROS produced in response to oxidative stress damage the endoplasmic reticulum (ER) Ca^{2+}-uptake system and interfere with the efflux of Ca^{2+} through the plasma membrane, thus, causing increases in intracellular free Ca^{2+} and PTP formation. The PTPs open, the mitochondrial membrane collapses, leading to loss of adenosine triphosphate (ATP) and the cell undergoes apoptosis or necrosis [59].
Hypoxia/reoxygenation injury of placental explants in vitro leads to up-regulation of tumor necrosis factor-α (TNF-α) mRNA in placental tissues as well as increased levels of secreted TNF-α protein in conditioned medium [65, 66]. Hypoxia-reoxygenation of placental explants in vitro activates the p38, mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-kB) signaling pathways. This leads to increases in TNF-α, IL-1β, and other pro-inflammatory cytokines and also increases expression of cyclooxygenase-2 (COX-2) and apoptosis [67].

FACTORS REGULATING TROPHOBLAST INVASION

Trophoblast invasion is a highly controlled process. In contrast to tumor invasion, trophoblast invasion is controlled both spatially (invasion does not go beyond the proximal third of the myometrium) and temporarily (occurs first 12 weeks of pregnancy) [3]. Trophoblast migration and invasion are controlled by a complex network of cell types, multiple autocrine and paracrine factors, hormones, signaling pathways and regulatory transcription factors. These include critical proteases and adhesion molecules; multiple cell types such as trophoblasts, uterine epithelial and stromal cells, uNK cells, and macrophages; and cytokines and angiogenic factors [13-15].

Invasive trophoblast cells secrete specific proteases that degrade ECM allowing trophoblasts to migrate and invade. The proteases involved are MMPs, serine proteases such as uPA [68] and cysteine proteases such as cathepsins [69]. Trophoblast and decidual cells also produce tissue inhibitors of matrix metalloproteinases (TIMPs) and plasminogen activator inhibitor (PAI) that act to limit excessive trophoblast invasion.
Acquisition of an invasive phenotype by trophoblast cells also requires a switch in expression of adhesion molecules. In the first trimester of pregnancy $\alpha_6$ integrins are expressed in cytotrophoblast stem cells but are downregulated in invasive cytotrophoblasts. Differentiating and invasive cytotrophoblasts, on the contrary, express $\alpha_5\beta_1$ and $\alpha_1\beta_1$ integrins. Later in gestation cytotrophoblast cells with reduced invasive capacity lose expression of $\alpha_1\beta_1$ integrins [70]. The $\alpha_1\beta_1$ integrins are thought to promote invasiveness through interaction with collagens and laminins which are abundantly expressed in the decidua [13].

Many cytokines, chemokines and growth factors produced by both decidual and trophoblast cells such as IL-1$\beta$ [71], TNF-$\alpha$ [72], interleukin-6 (IL-6) [73], interleukin-11 (IL-11) [74], TGF-$\beta$ [75], chemokines CX3CL1, CCL14 and CCL4 [76], have been shown to be involved in the regulation of trophoblast invasion. Hormones such as hCG, progesterone (P4) and estradiol (E2) are also thought to influence trophoblast invasion [77].

The actions of cytokines, growth factors, chemokines, hormones and cell-matrix contacts initiate signal transduction pathways through receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs), integrins and others. This leads to activation of signaling pathways such as MAPKs, focal adhesion kinases (FAKs), phosphoinositide 3-kinase (PI3K)-Akt, Wnt and others that result in effects on cell proliferation, differentiation, migration and apoptosis [13, 14].

In summary, the process of trophoblast migration and invasion, which is absolutely essential for normal placental development and successful pregnancy outcome, is a highly complex process and involves multiple regulatory mechanisms.
BASIGIN/EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER

BSG is a transmembrane glycoprotein that is a member of the immunoglobulin superfamily [18]. Originally identified in LX-1 lung carcinoma cells as a secreted factor capable of stimulating the collagenase activity of human fibroblasts, BSG has been identified independently in several different model systems resulting in a long list of acronyms for this molecule including TCSF [78, 16], EMMPRIN [17], neurothelin [79], OX-47 and CE9 [80], gp42 [81,82], 5A11 and HT7 [83], M6 [84], OK blood antigen [85], and most recently CD147 [19]. BSG protein is encoded by a single gene designated BSG. The BSG gene is located on chromosome 19p13.3 and encodes a 29 kDa protein whose molecular mass varies depending on the degree of N-linked glycosylation. This glycosylation serves as a regulatory mechanism of basigin function. BSG expressed on tumor cells contains complex β1, 6-branched polylactosamine sugars that may contribute to invasive potential of tumor cells as compared to low glycosylated species which contain high-mannose-type carbohydrates [86]. BSG glycosylation allows for greater cellular association with the ECM [87].

BSG contains two extracellular immunoglobulin-like domains, a 24 amino acid residue transmembrane domain, and a 40 amino acid cytoplasmic domain [87]. The extracellular domain of BSG contains four cysteines that form two disulfide bonds which in turn organize the extracellular region of BSG into two immunoglobulin-like domains [18, 84] (Fig.4). Another BSG transcript that encodes three immunoglobulin-like domains was identified in the mouse retina [88, 89]. More recently, four BSG transcript variants -1, -2, -3 and -4, that encode four different BSG isoforms (BSG isoforms -1, -2, -3 and -4), were deposited in the NCBI Entrez Gene database. The larger BSG isoform-1, with three Ig-like domains, is a retina-specific isoform. BSG isoform-2 is the most predominant isoform and is also the only isoform that is
secreted. BSG isoforms -3 and -4 are less abundant and were first identified in human endometrial stromal cells and cervical carcinoma cell lines [90, 91].

BSG has multiple functions including involvement in neurological processes such as learning, memory and sensory functions and plaque formation in Alzheimer’s disease [25, 26], retinal function [27], shuttling of monocarboxylate transporters (MCTs) to the cell surface membrane [30], lymphocyte migration and activation [28], and involvement in tissue repair and remodeling. The most well-known function of BSG is induction of MMPs. BSG was originally identified as Tumor Cell-Derived Collagenase Stimulatory Factor that was isolated from tumor cell conditioned medium and was able to stimulate production of MMPs in fibroblasts [17]. Studies using recombinant or native EMMPRIN protein have confirmed the stimulatory effect on MMP production [92, 93]. BSG is robustly expressed by a variety of cancer cells and is a critical molecule for tumor progression [20, 21]. Furthermore, studies have shown that BSG is released via plasma membrane microvesicle shedding both from tumor and normal cells [23, 24]. In addition, BSG released in microvesicles by human ovarian carcinoma cells has been shown to stimulate the angiogenic capability of ECs [94]. Thus, BSG has multiple functions including the regulation of glycolytic activity through its role as a chaperone protein for MCTs, promotion of endothelial capillary structure formation, and an important role in tumor progression by regulating tissue remodeling through induction of MMPs.

**BASIGIN AND TUMOR INVASION**

BSG is known to be one of the key regulators of tumor growth and metastasis. Successful tumor invasion requires changes in the architecture of the cell cytoskeleton, expression of surface adhesion molecules, induction of angiogenesis and penetration of the BM.
All of these events have been associated with the function of BSG [87]. BSG, secreted by tumor cells, stimulates MMP-1, MMP-2 and MMP-3 production by surrounding stromal cells [95]. MMPs in turn mediate ECM degradation that allows cancer cells to move. BSG is expressed in various malignant tumors including breast, colon, lung, skin, bladder and head/neck tumors [96-100]. Moreover, BSG mRNA is overexpressed in many carcinomas as compared to normal or benign tissues. Antibodies to BSG are being tested as potential anti-cancer treatments as they have been shown to inhibit MMP expression and consequently decrease tumor invasion [87, 101].

In addition to its effects on MMP production, BSG can also stimulate its own expression via a positive feedback mechanism and has also been shown to induce VEGF production by stromal cells [87]. Moreover, tumor-derived BSG is able to stimulate MMP-1, MMP-2 and MMP-3 production by vascular ECs [101]. BSG is released from the plasma membrane of tumor cells through microvesicle shedding, microvesicles are broken down and soluble BSG is released and may signal to cells in a paracrine manner that does not require cell to cell contact [23]. Microvesicle-associated BSG from epithelial ovarian cancer cells has been shown to modulate MMP production by ECs implicating its role in angiogenesis [94].

BSG is also associated with the α3β1 and α6β1 integrins which interact with laminins. Integrins are the major receptors of cell attachment to ECM and laminin is major component of the BM. Levels of both integrin types are elevated in metastatic cancers. Since BSG and α3β1 and α6β1 integrins are associated together, it suggests that they act jointly to facilitate ECM breakdown and therefore tumor invasion [87, 102]. BSG acts as both a ligand and receptor as soluble BSG can bind to the transmembrane form of BSG to induce Erk activation and MMP production in uterine fibroblast cells [91, 23].
BASIGIN FUNCTION IN REPRODUCTION

BSG exerts important functions in both male and female reproductive processes including spermatogenesis, fertilization and embryonic implantation [31, 32]. BSG plays an essential role in implantation because most Bsg null mutant embryos are unable to implant into the uterus despite developing normally during the early stages of preimplantation. Most Bsg null mutant embryos die before induction of the decidual reaction and those few that survive to birth are sterile [31]. Increased expression of BSG in the uterine endometrial epithelium is observed at the sites of mouse embryo apposition in the endometrium [32]. These observations indicate that BSG expression is required both by the embryo and the maternal uterus for successful implantation. Later studies showed that BSG protein is expressed in mouse pre- and peri-implantation embryos both in the inner cell mass and the trophectoderm layer of blastocysts [33]. BSG is also expressed in human uterine endometrium [103]. The distribution of BSG differs between the proliferative, secretory and menstrual phases of the menstrual cycle [103].

Additionally, BSG is expressed in term human placenta in trophoblast cells and amniotic epithelial cells. It is believed that BSG in placenta and fetal membranes acts to stimulate MMP production, thereby inducing fetal membrane rupture and leading to detachment of the placenta from the uterus at the time of parturition [34].

In the mouse testis BSG is expressed in the spermatocytes and spermatids. Male Bsg null mice are sterile due to arrest of spermatocytes at the metaphase of the first meiotic division during spermatogenesis [31]. BSG is expressed in the human testis with the onset of spermatocyte differentiation [104]. BSG is also present on mature sperm. Antibodies directed against BSG inhibit sperm binding to oocytes with an intact zona pellucidae which supports the importance of BSG during fertilization [105].
MONOCARBOXYLATE TRANSPORTER PROTEINS (MCTs)

Monocarboxylate transporter proteins (MCTs) function to facilitate proton-linked transport of monocarboxylates, such as lactate and pyruvate, across the plasma membrane in diverse organs. MCTs play a fundamental role in metabolic homeostasis. MCT proteins have twelve transmembrane spanning regions with both the C- and N- termini being in the cytosol and contain a large loop region between the sixth and seventh transmembrane domains. To date, 14 isoforms of the MCT family have been described (MCT1-14), but only isoforms MCT1 through MCT4 are known to be involved in proton-linked transport [87, 106-107].

MCT1 and MCT4 are known to require BSG protein for its full activity. BSG is tightly bound to MCT1 and MCT4 proteins in the cell plasma membrane [30]. Several studies have demonstrated the colocalization of BSG and MCTs in the same region within cells [30, 108]. Studies suggest that BSG is associated with two MCT1 molecules at the plasma membrane [109]. Blocking BSG expression with an antibody led to disruption of MCT expression as well [30]. Moreover, overexpression of BSG in highly invasive tumors has been correlated with upregulation of MCT1. MCT expression is most evident in cells with a high glycolytic rate associated with hypoxic energy production. Tumor growth is characterized by highly glycolytic metabolism and excessive lactic acid production. This process is under the control of MCTs and is necessary for tumor survival. For this reason MCT inhibitors are being used in cancer treatment [87].

MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes that are involved in degradation and remodeling of ECM [110]. MMPs are produced and
secreted as proenzymes (inactive proforms) into the ECM where they must be proteolytically cleaved to become active. Both latent and activated MMPs can be found in extracellular matrices. These enzymes all consist of 3 common domains. The ‘pre’ domain is located at the amino terminus and its function is to initiate cellular export of the enzyme. The next is the ‘pro’ domain. Latent MMPs, or proMMPs become active after cleavage and removal of this domain. MMPs also have a catalytic domain where the active site of these enzymes is located and which holds a zinc ion by bonding to a molecule of water and three histidines. Almost all MMPs, with some exceptions, also have a hemopexin-like domain. Its function is to mediate associations with ECM components and MMP inhibitors. The activity of MMPs is regulated by specific TIMPs [111, 112].

The MMP family of enzymes can be divided into 4 groups. The first group are the collagenases. They are responsible for degradation of fibrillar collagens I, II, and III. The second group are the gelatinases. This group degrades mostly denatured collagens (gelatins) and native collagen IV. The third group are the stromelysins. These have the most extensive range of substrates including fibronectins, laminin, elastin, proteoglycan core proteins and collagens III, IV, V. The last group is a new group of membrane-type matrix metalloproteinases (MT-MMPs). They are localized at the surface of the cell membrane and their substrate specificity is not yet clear [110, 112].

MMP activity is known to be regulated at the levels of transcription, enzyme activation and inhibition, complex formation and compartmentalization [113]. MMPs have manifold functions such as involvement in cell invasion, migration, cell proliferation and apoptosis. MMPs are extremely important for processes such as embryo implantation, placentation,
angiogenesis, mammary gland development, wound healing, tissue morphogenesis, bone
development and many others [114].

**MATRIX METALLOPROTEINASES PRODUCED BY TROPHOBLAST AND INVOLVED IN TROPHOBLAST INVASION**

MMPs have been found to be highly involved in the processes of embryo implantation [115], proper placental development [110] and parturition [116]. In order to be able to invade maternal uterine tissue, trophoblast cells must degrade the BM and ECM of the endometrium [112]. Trophoblast invasion and migration are tightly controlled occurring only during first trimester of pregnancy and invasion is limited to the proximal third of the myometrium. There is a fine balance between secretion of MMPs from trophoblast cells and their inhibition by TIMPs in contrast to the more uncontrolled tumor invasion [115, 3].

MMP-2 and MMP-9 are the most studied MMPs produced by trophoblast cells [117]. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) degrade mainly collagen IV and other ECM proteins such as collagens I, V, VII, IX, elastin, fibronectin, laminin and vitronectin [110, 115]. MMP-2 and MMP-9 are differentially expressed during the first trimester of pregnancy: MMP-2 is known to be the primary gelatinase during the early first trimester (6-8 weeks of pregnancy) after which its expression decreases. In contrast, no MMP-9 secretion by cytotrophoblastic cells is observed by week 6, but between 7 to 11 weeks of gestation its secretion increases gradually [110, 115, 117]. During the first trimester, MMP-2 has been shown to be expressed in EVT while MMP-9 is expressed mainly in villous cytotrophoblast [118]. Thus, MMP-2 is the predominant gelatinase in the early first trimester while MMP-9 is dominant in the late first trimester of pregnancy. MMP-14 and MMP-15 have also been found to be expressed in the first trimester of
pregnancy. Their function is to activate other MMPs, especially MMP-2, but they also act as proteolytic enzymes. Moreover, other MMPs associated with placentation have been found in trophoblast cells such as MMP-1, -3, -7, -18, -19 and -26 [110, 115].

Invasiveness of trophoblast is controlled by the balance in expression of MMPs and TIMPs. TIMPs prevent the enzymatic activity of MMPs by binding to their catalytic domain. TIMPs include four proteins: TIMPs -1-4. All four of these TIMPs are known to be present in trophoblast tissues [115, 119]. TIMP-1 preferentially binds MMP-9 in both its active and latent forms. TIMP-2 preferentially binds to active or latent MMP-2 [115].

**MATRIX METALLOPROTEINASES INVOLVED IN SPIRAL ARTERY REMODELING**

Remodeling of the uterine spiral arteries is a key event in early pregnancy necessary for establishment of adequate blood supply between mother and the fetus. The remodeling of spiral arteries can be roughly divided into three stages. The first stage involves changes in ECs and VSMCs such as vacuolization, dilation, muscular hypertrophy and disorganization prior to trophoblast arrival. Second stage is removal of VSMCs and ECs by invasive EVT and other decidual cells. Third stage is when ECs are replaced by endovascular trophoblast cells and fibrinoid material is deposited [5]. Several mechanisms are proposed to be involved in these stages including ECM restructuring, vascular cell de-differentiation, cell migration, changes in the adhesive properties of cells, and sensitivity to death-inducing stimuli and may be interdependent [39]. Some of these processes may be mediated by the actions of invading trophoblasts. Roles played by interstitial trophoblasts that invade decidua and endovascular trophoblasts that invade blood vessels may be different. Vascular and maternal immune cells also
play an important role in this process [39]. The MMP degrading proteases released by both the vascular and trophoblast cells are extremely important for remodeling of the spiral arteries. ECs produce MMPs-1, -2, -9, -10 and -14. VSMCs produce MMPs-2, -9, -10, -12 and -14, interstitial EVT MMPs-1, -2, -3 and endovascular trophoblast cells MMPs-7 and -9. In addition, decidual uNK cells contribute to the remodeling process by producing MMPs-2, -7, and -9 while macrophages produce MMPs-7 and -9 [39]. Excessive remodeling is controlled concomitantly by TIMPs. ECs are known to produce TIMP-2; VSMCs and interstitial EVT TIMPs-1 and -2; uNK cells TIMPs -1,-2 and -3 [39].

Trophoblast cells can influence the synthesis of MMPs by VSMCs [39]. In vitro studies showed that conditioned medium from BeWo cells, an invasive trophoblast choriocarcinoma cell line, was able to induce MMP-12 expression in VSMCs to facilitate ECM breakdown. Moreover, when BeWo cells were perfused into segments of uterine spiral arteries, they induced apoptosis of VSMCs [120]. Trophoblast cells are known to express/produce apoptotic factors such as TNF-α, TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) that can induce VSMC death [121-123]. This may contribute to the second stage of spiral arteries remodeling. Therefore, trophoblast cells normally produce MMPs themselves but can also control production of MMPs by VSMCs. In general, MMPs action during spiral arteries remodeling is necessary for processes such as ECM restructuring, destruction of interactions between vascular cells (ECs and VSMCs) and between vascular cells and ECM, and cell migration.

MICROVESICLES

Microvesicles are small cell membrane-bound vesicles that are shed into body fluids such as blood, urine and ascites [124-126]. They are generated by outward budding of the plasma
membrane from the cell surface. Microvesicles are secreted by both normal and malignant cells and their membranes contain cell surface elements. Microvesicles contain cell surface receptors, proteins, mRNAs, microRNAs and organelles and their function is to facilitate transfer of these components between cells [127]. They also deliver infectious agents into cells such as human immunodeficiency virus (HIV) and prions [128, 129]. Microvesicles may not only circulate in body fluids but also act in a paracrine manner. The function of microvesicles appears to be dependent on the components of their membranes and, therefore, dependent on the cell type from which they originate. For example, microvesicles that are shed from the cellular membrane of endothelial cells carry in their membranes components that facilitate angiogenesis. Microvesicles shed from tumor cells carry in their membranes metalloproteinases and other proteolytic enzymes and, thus, facilitate invasion into the ECM. Tumor vesicles also contain components in their membranes that help tumor cells to escape immune surveillance. Microvesicles were originally thought to be heterogeneous shaped, large vesicles of up to 1 µm in diameter. More recently, however, they have been characterized as having a spherical shape with a diameter less than 200 nm [130, 131].

Microvesicles are often characterized as or grouped together with exosomes. However, microvesicles are unique vesicles that are distinct from exosomes. Microvesicles are formed by the outward budding of plasma cell membranes while exosomes arise from exocytic multivesicular bodies (MVBs) that are released upon fusion of the exocytic MVB with the plasma membrane. Exosomes are also smaller and range from 40 to 100 nm in diameter. It is important to note that separation of the exosome fraction from the microvesicle fraction of vesicles is a big challenge for researchers. Ultracentrifugation techniques commonly used to
isolate microvesicle fractions at a speed of 100,000g are likely to sediment a mixture of microvesicles and exosomes [130, 131].

**FORMATION AND MODE OF RELEASE OF MICROVESICLES**

Release of microvesicles requires an increase in intracellular calcium concentration that may come from either intracellular or extracellular stores, this leads to activation of cytosolic enzymes important for formation of microvesicles such as calpain and cysteine protease. Activation of these cytosolic enzymes leads to fragmentation of the cytoskeleton that is in close proximity to the plasmalemma by proteolysis. The cell membrane loosens and everts as submembranous cytoskeletal spectrin (or fordin, depending on the cell type) loosens. A membrane bleb grows, forming a protrusion, and finally this protrusion, now called a microvesicle, detaches. Afterwards, the cell membrane reestablishes its continuity [132] (Fig.5).

Resting cells do release some amount of microvesicles, however, it is possible to increase the rate of release. Ca²⁺ is known to induce microvesicle shedding [130]. For example, the calcium ionophore A23187 has been shown to activate shedding of microvesicles from platelets [133]. Phorbol esters that activate PKC are also able to induce microvesicle release [130]. Exposure of lung carcinoma cells to phorbol myristate acetate (PMA), a well-known activator of PKC, stimulated shedding of microvesicles into conditioned medium [23].

Release of exosomes requires a completely different mechanism from that required for release of microvesicles. Formation of exosomes begins with the generation of endosomes at the plasma membrane which fuse and form endocytic cisterna. Endocytic cisterna, in turn, may be targeted to recycling endosomes, degradative MVBs that evolve into lysosomes or exocytic MVBs. Exosomes are generated within the MVB lumen. Generation of exosomes targeted for
lysosomes requires a multiprotein complex called the Endosomal Sorting Complexes Required for Transport (ESCRTs). The accumulation of exosomes within exocytic MVBs has been shown to involve metabolism of sphingomyelin to ceramide. It was shown that ceramide triggers budding of exosome vesicles into exocytic MVBs and that release of exosome vesicles is reduced after the inhibition of sphingomyelinases. Sphingomyelinases remove phosphocholine moiety from sphingomyelin that results in formation of ceramide [134]. Exocytic MVBs then fuse their external membrane with the plasma membrane of the cell after which the exosomes are released into the extracellular space [130] (Fig.6).

MICROVESICLE BREAKDOWN AND INTERACTION WITH TARGET CELLS

Once microvesicles are released from their cell of origin, they do not remain in the extracellular space in intact form for long, but are broken down over a period of time [127]. Microvesicles isolated from lung carcinoma cells had a relatively short life span as they appeared to break down within a few hours and released full-length EMMPRIN protein into conditioned medium [23]. When microvesicles are broken down, they release their components into extracellular space [130]. Release of their cargo has corresponding consequences. For example, microvesicles released by human breast carcinoma cells are enriched in MMP-9 protein [135]. The release of microvesicles enriched in MMP-9 plays an important role in the directional proteolysis of the ECM during cancer cell migration [135]. Microvesicles shed by the lung carcinoma cells are rich in EMMPRIN protein [23]. EMMPRIN released in microvesicles by tumor cells is able to induce MMP expression in fibroblasts thereby controlling ECM degradation by fibroblasts [23].
Upon release from their cell of origin, microvesicles do not interact with just any cell they come into contact with but only with cells they recognize specifically [130]. For example, microvesicles released by platelet cells interact with monocyte cells but not with neutrophils [136]. Interaction of microvesicles with cell of interest can occur in several ways. Microvesicles released into extracellular space can interact with target cell by binding to specific cell surface receptors on target cell. It leads to initiation of cell signaling events. Another way microvesicles can interact with cell of interest is through direct fusion with plasma membrane of their target cell. It leads to discharge of their cargo into the cytosol. It can also happen through the endocytic uptake of the microvesicle. Endocytic uptake can be followed by transcytosis, fusion of the microvesicle membrane with membrane of endosome which is followed by release of microvesicle cargo into cytosol or endocytosed microvesicle can be targeted to the lysosome by entering the endosomal pathway [130].

**MICROVESICLE FUNCTION**

Microvesicles play important roles in many biological processes mediating cell to cell communication. They are important for both physiological and pathological processes such as coagulation, thrombosis, inflammation, angiogenesis, immunity and cancer progression [127, 130-131]. The function of microvesicles was first recognized as an important part of blood coagulation and thrombosis. Microvesicles derived from platelets are very important because they contain a specific protein involved in the initiation of coagulation and thrombosis called tissue factor (TF) [137]. In response to endothelium damage, after platelets make contact with collagen, platelets release microvesicles containing TF that bind to macrophages, neutrophils and
other platelets via P-selectin 1, their surface ligand [130]. This action of microvesicles aids in the process of thrombus formation.

Microvesicles have an important role in different inflammatory diseases, because they have been found in high concentrations at the sites of inflammation. The role of microvesicles in inflammation depends on the stage of the inflammatory process. At early stages, microvesicles shed by neutrophils can stimulate macrophages to release anti-inflammatory molecules such as TGF-β1 and interleukin-10 (IL-10) while reducing levels of TNF-α and interleukin-8 (IL-8). At later stages, microvesicles become pro-inflammatory by stimulating release of IL-6 and monocyte chemotactic protein 1 (MCP1) [130].

Microvesicles are highly important for tumor progression. Microvesicles released spontaneously from the surface of a highly metastatic B16 mouse melanoma cell subline (F10), when fused with a poorly metastatic B16 mouse melanoma cell subline (F1), increased the ability of F1 cells to stop in the lung and establish metastases in this organ [138]. Several proteins contained in tumor-derived microvesicle play critical roles in cancer progression. CD147/EMMPRIN released in tumor-microvesicles is involved in proangiogenic activity and ECM degradation [23, 94], VEGF promotes angiogenesis [139], the proteases MMP2, MMP9 and uPA are involved in ECM degradation [140, 141], and β1 integrin released by tumor microvesicles is involved in ECM attachment [135]. Moreover, FasL has been shown to be involved in immune evasion and promotion of T-cell apoptosis, while LMP-1 participates in immune evasion as well inhibiting leukocyte proliferation [131].
RELEASE OF BASIGIN-CONTAINING MICROVESICLES FROM TUMOR CELL

BSG/EMMPRIN protein is shed from the surface of tumor cells through microvesicle shedding. A recent study showed that BSG is released from the surface of NCI-H460 lung carcinoma cells as a full-length protein containing both N- and C- termini, not in its truncated form due to proteolytic cleavage of the N-terminal extracellular domain. Full-length BSG protein was shown to bud from the surface of tumor cells in microvesicles and after some time these microvesicles lysed releasing BSG into the surrounding conditioned medium. Investigators suggested that it is BSG within microvesicles that is able to bind to its receptor on target fibroblast cells initiating MMP induction and thereby facilitating tumor invasion and metastasis [23] (Fig.7).

Another recent study demonstrated that BSG-containing microvesicles shed from the surface of human epithelial ovarian carcinoma cells were able to promote an angiogenic phenotype in HUVECs in vitro. Microvesicles shed by human ovarian carcinoma cell lines OVCAR3, SKOV3, and A2780 expressed different levels of BSG (OVCAR3>SKOV3>A2780) and stimulated expression of MMPs by HUVECs as well as formation of capillary-like structures by ECs in a BSG-dependent manner. These findings support the importance of BSG-containing microvesicles in tumor-induced angiogenesis [94].

INTERLEUKIN-1β

Interleukin-1β (IL-1β) is an inflammatory cytokine belonging to the interleukin-1 (IL-1) cytokine family [142, 143]. Cytokines are soluble glycoproteins released by cells that act nonenzymatically to regulate cellular function [144]. During early pregnancy cytokines are involved in embryo implantation, regulation of trophoblast invasion, spiral artery remodeling and
Cytokines also play a role in the initiation of parturition [145].

IL-1β is the predominant form of IL-1 in the placenta and is produced by both trophoblasts and placental macrophages [144]. Immunohistochemical studies showed that IL-1β is localized to villous syncytiotrophoblast and to EVT [146]. During the first trimester, EVT production of IL-1β does not change between 8-10 and 12-14 weeks gestation remaining at around 230 pg/ml while secretion of this cytokine secreted by villous cytotrophoblast increases from 132 to 360 pg/ml by 12-14 weeks of gestational age [147]. Additionally, first trimester human cytotrophoblasts express the type I IL-1 receptor suggesting that IL-1β may act in both a paracrine and autocrine manner [71].

IL-1β plays an important role in trophoblast invasion. In vitro studies showed that IL-1β stimulated MMP-9 production and activity in cytotrophoblasts [71]. Moreover, IL-1β also up regulates MMP-2, MMP-9, MT-MMP1, MT-MMP2, and uPA expression in first trimester trophoblastic cells and the JEG-3 choriocarcinoma cell line [148]. IL-1β has other effects on trophoblast function, specifically on hormone production by trophoblast cells. It has been shown to stimulate hCG production by first trimester human trophoblast [149]. Trophoblast-derived IL-1β stimulates the release of hCG in first trimester human trophoblasts possibly by activating IL-6 and the IL-6 receptor system [150].

TRANSFORMING GROWTH FACTOR-β1

The transforming growth factor (TGF-β) superfamily consists of three related homodimeric proteins: TGF-β1, -2, -3 and also includes activins, inhibins and bone morphogenetic proteins. TGF-β1, -2, -3 signal through the SMAD family of proteins by binding
to cell surface receptors I, II and III [75]. TGF-βs are involved in processes such as cell proliferation, ECM production, angiogenesis, and immunosuppressive effects [151]. TGF-βs are also important molecules in the regulation of reproductive processes such as oocyte development, ovarian function, mammary gland morphogenesis, regulation of hypothalamic-pituitary-gonadal axis function, regulation of endometrial function, decidualization, early embryo development, and placental development [152, 153].

TGF-β1 has been recognized as an important regulator of invasion of the EVT into uterine endometrium and spiral arteries. TGF-β1 mRNA and protein are present in both decidua and villous placenta with greatest expression in villous tissues [154]. TGF-β1 is expressed in first trimester placental membranes at fairly constant levels [144]. Studies showed that TGF-β1 inhibited trophoblast invasion in an EVT explant culture system and also reduced secretion of both MMP-9 and uPA [75]. Proliferation and apoptosis were not altered in response to growth factor [75]. TGF-β1 was also shown to inhibit trophoblast invasion in vitro using a first trimester, immortalized, extravillous trophoblast cell line (HTR-8/SVneo) [155]. Another study confirmed that TGF-β1 inhibits MMP-9 and uPA production and induces expression of protease inhibitors such as TIMP-1, TIMP-2 and PAI-1 in first trimester trophoblast cells and JEG-3 choriocarcinoma cells [148]. Thus, TGF-β1 is one of the important factors that control trophoblast growth and invasion during early pregnancy.
Figure 1. Anatomy of the trophoblast populations that are present at fetal-maternal boundary in the first trimester of pregnancy. CT - cell columns of growing cytotrophoblast; E – endovascular trophoblast cells; T – interstitial trophoblast cells; A – uterine spiral arteries; F – fibrinoid material; GC – placental-bed giant cells. Adapted by permission from Macmillan Publishers Ltd: Nature [11], copyright (2002). http://www.nature.com/nri/journal/v2/n9/full/nri886.html
Figure 2. Oxygen environment in the first trimester of pregnancy. A – Narrow spiral arteries prior to pregnancy. B – Occlusion of apical portions of spiral arteries by invading EVT which creates low oxygen environment within the intervillous space (3-5 % O2). C – Late in the first trimester of pregnancy spiral arteries become unobstructed from trophoblast cells which results in increased oxygen levels (8-10% O2) within the intervillous space. “Reprinted from Placenta, Vol 31, Patel J, Landers K, Mortimer RH, Richard K, Regulation of hypoxia inducible factors (HIF) in hypoxia and normoxia during placental development, Pages 951-957, Copyright (2010), with permission from Elsevier.”
Figure 3. Comparison of non-pregnant (A), pathological conditions of pregnancy (preeclamptic) (B) and normal pregnancy (C) spiral arteries. Deficient trophoblast invasion in preeclampsia leads to inadequate transformation of spiral arteries (B). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology [11], copyright (2002).
http://www.nature.com/nri/journal/v2/n9/full/nri886.html
Figure 4. The structure of BSG protein. The immunoglobulin-like domains are represented by circles. S-S are disulfide bridges formed by cysteins. “Reprinted from Neuroscience Letters, Vol 140, Seulberger H, Unger CM, Risau W, HT7, Neurothelin, Basigin, gp42 and OX-47 - many names for one developmentally regulated immuno-globulin-like surface glycoprotein on blood-brain barrier endothelium, epithelial tissue barriers and neurons, Pages 93-97, Copyright (1992), with permission from Elsevier.”

Figure 7. Microvesicle release of BSG. Cartoon shows that BSG/EMMPRIN is released in microvesicles (MVs) from tumor cells (A). It acts as both a ligand and a receptor as soluble full-length BSG shed in MVs upon release into surrounding medium (B) can bind to transmembrane form of BSG (C) and can induce MMP production in target fibroblast cells (D). Adapted by permission from Macmillan Publishers Ltd: Oncogene [23], copyright (2004).
http://www.nature.com/onc/journal/v23/n4/full/1207070a.html
CHAPTER 3
HUMAN PLACENTAL TROPHOBLAST CELLS EXPRESS BASIGIN AND SECRETE THIS PROTEIN VIA MICROVESICLE SHEDDING

ABSTRACT

Controlled trophoblast invasion into maternal uterine tissues and remodeling of uterine spiral arteries are necessary processes for the establishment of successful pregnancy. One of the factors regulating these processes are MMPs, a family of zinc-dependent proteases responsible for degradation and remodeling of ECM and BM. BSG/EMMPRIN protein plays a critical role in induction of MMP production in a number of tissues. BSG is shed from the surface of tumor and normal cells through the process of microvesicle shedding. BSG shed in microvesicles from tumor cells acts as both a ligand and a receptor as soluble BSG shed in microvesicles can bind to the transmembrane form of BSG and induce MMP production in fibroblast cells. BSG exerts important functions in fertilization, implantation and parturition. Bsg null mouse embryos fail to implant into the uterus, and transfer of wild-type mouse embryos into pseudopregnant Bsg null females results in significant reduction of live births. BSG is expressed in term human placenta in trophoblast and amniotic epithelial cells and its expression may be increased at the time of labor. However, the presence of BSG in the first trimester human placenta and its specific functions during early placental development are currently unknown nor has the mode of release of BSG protein from trophoblast cells been investigated. Our objectives were: a) to examine presence of BSG in human placenta throughout the first trimester of pregnancy; b) determine whether BSG is secreted from first trimester placental cells; and c) determine whether release of this protein occurs via plasma membrane microvesicle shedding. Human placental tissues from
clinically normal pregnancies were analyzed for BSG presence throughout the first trimester of pregnancy. Immortalized first trimester, human trophoblast cells and choriocarcinoma cell lines were used for in vitro experiments. Our results showed that BSG immunolocalized to both cytotrophoblast and syncytiotrophoblast layers in first trimester human placental tissues. BSG was expressed and BSG protein was present in cell lysates and secreted into surrounding medium by all the human trophoblast-like cell lines. Moreover, the mRNAs for BSG transcript variants 2 through 4 were expressed in trophoblast-like cells. Further, we identified that the mode of release of BSG from the surface of trophoblast-like cells occurs through microvesicle shedding. We also found that rBSG protein stimulated release of MMP-1 and MMP-3 by cultured HUVECs. Taken together, these data suggest that BSG, shed in microvesicles, may be important for paracrine interactions between trophoblast cells and other uterine cells during the early stages of placental development.
INTRODUCTION

Successful pregnancy is dependent on the tightly regulated processes of trophoblast invasion into the maternal endometrium and remodeling of maternal uterine spiral arteries to establish an adequate blood supply between mother and fetus. Trophoblast cells are extremely invasive; they penetrate the uterine epithelium and invade into the decidua and maternal spiral arteries. However, trophoblast invasion is tightly controlled both spatially and temporally as it normally occurs only during the first trimester of pregnancy and normally does not proceed beyond the proximal third of the myometrium [1-4, 38].

One of the important factors that regulate trophoblast invasion and spiral artery remodeling are proteolytic enzymes (MMPs) that are secreted by both trophoblast and vascular (ECs and VSMCs) cells. MMPs degrade the ECM and BM and disrupt interactions between the ECM and vascular cells as well as between ECs and VSMCs during implantation and placentation [39, 110-112]. BSG/EMMPRIN, a transmembrane glycoprotein that is a member of the immunoglobulin superfamily [16-18], plays a key role as a local regulator of MMP production in a number of tissues and cancers [16, 19, 22].

BSG is a critical molecule in reproductive processes including fertilization [32], successful implantation [31], angiogenesis [101,156] and parturition [34]. Studies demonstrated that BSG expression by both the embryo and the maternal uterus is required for successful implantation. Increased expression of BSG in the uterine endometrial epithelium is observed at the sites of mouse embryo apposition [32]. Embryos lacking Bsg fail to implant into the uterus despite developing normally during the preimplantation period [31]. BSG is expressed in mouse pre- and peri-implantation embryos in both the inner cell mass and trophectoderm layer of blastocyst [33]. Moreover, BSG is expressed in trophoblast cells and amniotic epithelial cells of
human placenta at term where it is thought to stimulate MMP production and facilitate rupture of fetal membranes and placenta detachment at the time of labor [34].

Bioactive BSG is released from the surface of tumor cells into conditioned medium through microvesicle shedding [23]. These microvesicles degrade over time releasing bioactive BSG and BSG, in turn, binds to its receptor on fibroblasts to up regulate MMP production by these cells [23]. In addition, another group reported that BSG is contained in microvesicles shed by epithelial ovarian cancer cells and that these microvesicles induce angiogenesis and promote MMP gene expression in endothelial HUVEC cells in a BSG-dependent fashion [94]. Moreover, proteomic characterization of cell membrane blebs/microvesicles released by human retinal pigment epithelium showed presence of BSG inside these blebs. This BSG protein may be involved in ECM remodeling processes at sites distant from the site of microvesicle release by the retinal pigment epithelium and may contribute to the progression of age-related macular degeneration of the retina [24].

Despite studies that have investigated expression of BSG and its function during implantation and labor, little is known about the function of BSG during the first trimester of pregnancy. Additionally, the mode of release of BSG from trophoblast cells has not been studied so far. Human placental tissues from clinically normal pregnancies were used to investigate presence of BSG throughout the first trimester of pregnancy. Human immortalized HTR-8/SVneo trophoblast cells and JAR and JEG-3 choriocarcinoma cell lines were used as in vitro model systems for our experiments.

The HTR-8/SVneo cell line is a first trimester, human trophoblast cell line with an expanded life span. It was established by introducing the gene encoding simian virus 40 large T antigen into first trimester human trophoblast cells. The transfected HTR-8/SVneo cells exhibit
prolonged growth in culture and retain phenotypic properties and functional characteristics of parental trophoblast cells [157, 158]. JAR and JEG-3 cell lines are malignant choriocarcinoma cell lines. They may have characteristics related to their malignant origins [159, 160].

The goals of this study were to 1) analyze BSG protein presence in human placental tissues throughout the first trimester of pregnancy; 2) determine which BSG transcript variants are expressed in trophoblast-like cell lines and determine whether BSG protein is present in cell lysates and secreted by trophoblast cells into surrounding medium in these trophoblast-like cell lines; 3) determine whether BSG protein is released through microvesicle shedding; and, 4) determine whether rBSG protein can act on endothelial cells to regulate MMP production. Our results showed, for the first time, that BSG is present in human placenta throughout the first trimester of pregnancy and that BSG is released from the surface of trophoblast cells through plasma membrane microvesicle shedding. Moreover, rBSG stimulates secretion of MMP-1 and MMP-3 by human endothelial cells. Our findings show that BSG can act in a paracrine manner to regulate the interaction of trophoblast cells with other uterine cells, such as ECs, during early placental development.
MATERIALS AND METHODS

Collection of Placental Tissues and Acquisition of HUVECs

Human placental tissues were obtained from clinically normal pregnancies at 7 weeks 3 days (n=1), 9 weeks (n=3), 12 weeks 3 days (n=1), and 17 weeks of gestation (n=1), or term labor (n=2). Early gestation samples were obtained from pregnancy terminations. HUVECs were obtained from umbilical cords of term placentas and isolated by collagenase digestion, as previously described [161]. Both placental tissues and umbilical cords were collected after written informed consent and the study was approved by the Cambridge University Research Ethics Committee.

Immunohistochemistry for Basigin

Human placental tissues were collected and fixed in neutral buffered formalin. Fixed tissues were then embedded into paraffin. For immunohistochemical staining, 5-μm tissue sections were cut from paraffin embedded tissue and mounted onto microscope slides. Tissue sections were deparaffinized in xylene, rehydrated in serial gradient alcohol solutions, and washed in dH2O. Endogenous peroxide activity was blocked with 3% hydrogen peroxide in dH2O for 15 min at room temperature. Sections were then rinsed in tap water and then dH2O. Antigen retrieval was performed in a pressure cooker for 25 min in 0.01M Citric buffer (pH6.0). Sections were then incubated in 5% Goat serum (GS) + 2% BSA in Tris-Buffered Saline (TBS) to block nonspecific binding for 1 hour at room temperature. Tissue sections were then incubated overnight at 4°C with a goat anti-human BSG antibody (hEMMPRIN Affinity Purified Goat IgG; R&D Systems, Minneapolis, MN) at 2.5 μg/ml in TBS containing 5%GS. Non-specific goat IgG was used as a negative control. Sections were washed three times in Tris-Buffered Saline.
with Triton-X and Tween 20 (TBS-TT) and then incubated with biotinylated rabbit anti-goat IgG antibody (Vector Laboratories, Peterborough, UK) diluted 1:200 in 5%GS in TBS at room temperature for 1 h followed by three washes in TBS-TT. This was followed by incubation with ABC reagent (avidin-biotin-peroxidase complex) for 45 min at room temperature. Sections were further washed in TBS-TT and an additional wash in Tris-maleate buffer was performed. Staining was developed using SIGMAFAST™ 3, 3’- Diaminobenzidine tablets (D4168; Sigma, St. Louis, MO). The slides were counter-stained with hematoxylin for 10 sec, dehydrated, cleared in xylene, and mounted in DPX.

**Cell Culture**

The immortalized human first trimester trophoblast cell line (HTR-8/SVneo) and human choriocarcinoma cell lines (JAR and JEG-3) were a gift from Dr. Stephen Charnock-Jones (Cambridge University, UK). All cell culture experiments were conducted at 37°C and 5% CO₂. JAR, JEG-3 and HTR-8/SVneo cell lines were cultured in RPMI 1640 medium (Cellgro, Manassas, VA) and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) in all cell culture experiments. Additionally, HTR-8/SVneo cells were supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) that was heat inactivated. Fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) was inactivated by placing thawed bottles in a water bath at 56°C for 30 min. During incubation the contents were swirled every 5 min to ensure they were evenly heated. After 30 min, serum was transferred on ice to cool. Aliquots were made and stored at -20°C until use. JAR cells were supplemented with 10% and JEG-3 cells with 5% fetal bovine serum. EGM-2 BulletKit (CC-3162; Lonza, Walkersville, MD) was used for culture of HUVECs. EGM-2 BulletKit included 500 ml of EBM-2 basal medium and
EGM-2 Quot Kit Suppl. & Growth Factors containing 10 ml fetal bovine serum, 0.5 ml heparin, 0.2 ml hydrocortisone, 0.5 ml vascular endothelial growth factor, 0.2 ml human recombinant fibroblast growth factor-B, 0.5 ml recombinant long R insulin-like growth factor-1, 0.5 ml ascorbic acid, 0.5 ml gentamicin sulfate/amphotericin B and 0.5 ml human recombinant epidermal growth factor. HUVEC cells were cultured until passage 4.

**RT-PCR for Identification of BSG Splice Variants**

**Extraction of Total RNA**

JAR, JEG-3 and HTR-8/SVneo trophoblast-like cells were grown in six-well plates until 80% confluency. RNA was extracted using RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. The RNA concentration and purity of each sample were determined by measuring the absorbency at 260 nm and evaluating the ratio of absorbencies at 260/280 (optical density > 1.8) using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware). Samples were then stored at -80°C until cDNA synthesis.

**cDNA Synthesis and Reverse-Transcriptase PCR**

Two micrograms of total RNA per sample were reverse transcribed into cDNA in a 20-μl volume of RT reactions using Super Script III First-Strand Synthesis System for RT-PCR (Cat. No.: 180080-051, Invitrogen, Carlsbad, CA) as described below. Briefly, RNA/primer mixture including 2 μg of total RNA, 10 mM dNTP mix, random primers and DEPC-treated water were incubated at 65°C for 5 min, then placed on ice for at least 1 min. cDNA synthesis mix including 5xFS Buffer, 0.1 M DTT, RNaseOUT and SuperScript III RT were added to the RNA/primer mix and incubated at 25°C for 5 min, then at 50°C for 60 min and finally inactivated at 70°C for
15 min. Synthesized cDNAs were used for real-time PCR analysis. Real-time PCR analysis was performed in a 50-μl volume containing Takara Prime STAR™ HS DNA Polymerase Reaction Mix (Code No. R010A, Takara Bio Inc, Otsu, Shiga, Japan) that includes Prime STAR™ HS DNA polymerase, 5x Prime STAR™ PCR Buffer (Mg2+) and dNTP mixture. cDNA, primer F, primer R and sterilized dH2O were added to the reaction as well. Reverse transcription-PCR oligonucleotide primers were ordered from Integrated DNA Technologies: BSG-2, basigin isoform 2 (F: 5’-GCGAGGAATAGGAATCATGG-3’, R: 5’-TACTCTCCCCACTGGTGTCGTC-3’); BSG-3, basigin isoform 3 (F: 5’-TTAGTCTGGTCTCTTGC-3’, R: 5’-TACTCTCCCCACTGGTGTCGTC-3’); BSG-4, basigin isoform 4 (F: 5’-TTAGTCTGGTCTCTTGC-3’, R: 5’-TACTCTCCCCACTGGTGTCGTC-3’). Amplification conditions included an initial denaturation step at 94°C for 1 min followed by 36 thermal cycles of denaturing for 30 sec at 94°C, reannealing for 30 sec at 55°C, and elongation for 30 sec at 72°C. PCR amplified products were resolved on 2% agarose gels (Fisher Scientific, Pittsburgh, PA).

**Cell Lysates Collection and Concentration of Conditioned Cell Medium**

Cell lysates were collected in hot 1X Laemmli Sample Buffer (LSB) preheated at 95°C for 5 min. Cells were manually scraped and lysates sonicated and spun at 14,000g for 10 minutes at 25°C to remove cell fragments. Samples were collected and stored at -20°C until further analysis. Conditioned cell medium was collected directly from the treated cell plates, spun at 1000g for 10 min to remove cell debris and applied to Amicon Ultra-15 10-kDa centrifugal filter units (Millipore Corp., Billerca, MA). Tubes were spun and concentrated either 10, 30 or 50 fold. Samples were collected and stored at -20°C until further analysis. For immunoblotting,
15µl of cell lysate samples (containing either 5 or 10 ug total protein) or 30 µl of conditioned medium (unconcentrated or concentrated 10, 30 or 50 fold) were loaded onto gels.

Microvesicle Isolation

Human trophoblast cell lines were grown until 80% confluence. Once cells reached this confluency, they were transferred to serum-free medium containing only L-glutamine and antibiotics for 24 h. The conditioned cell medium was collected from the cell plates and centrifuged at 1500g for 10 min, transferred to a new tube and centrifuged again at 1500g for 15 min to pellet cellular debris. Some of the conditioned cell medium was saved at -20°C for further concentration and immunoblot analysis. The remaining supernatant was then ultracentrifuged at 40,000 rpm for 1 hr at 4°C. The supernatant from this spin was stored at -20°C for further immunoblot analysis. The microvesicle pellet was resuspended in 180 µl 1x LSB preheated at 95C for 5 min. 1x LSB contained 10% glycerol (Fisher Scientific, Pittsburgh, PA), 62.5 mM Tris Base pH=6.8 (Fisher Scientific, Pittsburgh, PA) and 2% sodium dodecyl sulfate (SDS) (Fisher Scientific, Pittsburgh, PA). Volume was adjusted with water to 50 ml and 1x LSB was stored at room temperature. Samples were frozen at -20°C until further immunoblot analysis. A schematic for microvesicle collection is shown in Fig.8. For immunoblotting, equal volumes (18 µl) of unconcentrated conditioned medium collected before ultracentrifugation, 30 fold concentrated conditioned medium collected before ultracentrifugation, supernatant from ultracentrifugation or resuspended microvesicle pellet were loaded onto gels.
**Immunoblotting for Basigin and MCT1**

Samples were denatured in 4X LSB dye at 95°C for 5 min. 4X LSB dye contained 10% glycerol (Fisher Scientific, Pittsburgh, PA), 50 mM Tris Base (Fisher Scientific, Pittsburgh, PA), 2% SDS (Fisher Scientific, Pittsburgh, PA), 100 mM dithiothreitol (DTT) (Fisher Scientific, Pittsburgh, PA) and 0.025% Bromphenol Blue (Sigma, St. Louis, MO). The volume was adjusted to 10 ml with water. Aliquots of 4xLSB dye were made and stored at -20°C until use. Samples were applied to 4-20% Precise Protein Gradient Gels (Thermo Scientific, Rockford, IL) and transferred onto 0.45-μm pore size nitrocellulose membranes (Thermo Scientific, Rockford, IL) overnight. Nonspecific binding sites were blocked by incubating membranes in Tris-Buffered Saline containing 0.1% Tween 20 (TBST), pH 8 with 5% instant, nonfat dry milk for 1 hr at 25°C with shaking. Membranes were incubated with primary specific antibody in TBST pH 8 containing 2% BSA overnight at 4°C and then washed three times 10 min each in TBST pH 8 to remove unbound antibody. Membranes were incubated with HRP-conjugated secondary antibody in TBST, pH 8 with 2.5% nonfat dry milk for 1 hr at 25°C. Purified mouse anti-human CD147 (BD Biosciences, San Jose, CA) and rabbit polyclonal anti-human MCT1 (H-70) (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies were used at 1:1000 dilution. HRP-linked anti-mouse IgG (Cell Signaling, Danvers, MA) and anti-rabbit IgG (Cell Signaling, Danvers, MA) secondary antibodies were used at 1:10000 dilution. Membranes were washed again 6 times 5 min each in TBST pH 8 before visualization of immunocomplexes using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). Precision Plus Protein all blue molecular weight marker was used as standards (Bio-Rad Laboratories, Hercules, CA).
Effect of Basigin on HUVECs

To evaluate the effect of rBSG protein on MMP-1 and MMP-3 production by HUVECs, cells were cultured in EBM-2 medium with Single Quots from the EGM-2 Bullet-Kit (CC-3162; Lonza, Walkersville, MD) at 37°C in a 5% CO₂ atmosphere until 70-75% confluent. Once cells reached confluence, they were treated in serum-free EGM-2 medium with 0 µg/ml, 1 µg/ml or 5 µg/ml rBSG or 3 pg/ml lipopolysaccharide (LPS) for 48 hrs. This concentration of LPS corresponded to the amount of LPS present in the highest dose of rBSG tested. The rBSG protein was produced in our lab as previously described [33, 91]. Conditioned medium samples were collected, spun at 1600 rpm for 8 min to pellet cell fragments and medium was then concentrated 4-fold using the Amicon Ultra-15 10-kDa centrifugal filter units (Millipore Corp., Billerica, MA). Samples were then saved at -20°C for immunoblotting analysis. Cells were harvested and counted to determine any effects of rBSG on cell proliferation.

Immunoblotting was performed as described above for BSG and MCT1 with the following modifications. Samples were denatured in 4X LSB at 100°C for 5 min. The PageRuler PreStained Protein Ladder was used as standard (Fermentas, Glen Burnie, MD). 5% milk powder in TBST was used as blocking solution as well as for preparation of primary and secondary antibodies. Goat anti-human MMP-1 antibody (R&D Systems, Minneapolis, MN) was used at 1:250 dilution and goat anti-human MMP-3 antibody (R&D Systems, Minneapolis, MN) was used at 1:1000 dilution. Polyclonal rabbit anti-goat HRP-conjugated secondary antibody was used at 1:10000 dilution (Dakocytomation, Carpinteria, CA). ECL detection solution (Amersham, Pittsburgh, PA) was used to visualize immunocomplexes.
RESULTS

**Immunolocalization of Basigin Protein in First Trimester Human Placenta**

Representative sections demonstrating immunolocalization of BSG in human placental tissues throughout the first trimester of pregnancy are shown in Fig.9 A-D. Placental tissues were obtained from first trimester pregnancies terminated at 7 weeks 3 days (Fig.9 A), 9 weeks (Fig.9 B), 12 weeks 3 days (Fig.9 C) and 17 weeks (Fig.9 D). Immunohistochemical evaluation of BSG protein presence showed that basigin protein was present in both cytotrophoblast and syncytiotrophoblast layers with no immunoreactive staining in the villous core. Strong immunoreactivity was observed predominantly in the cell membranes for all the first trimester samples. A representative positive control section from term placenta is shown in Fig.9 E.

**Human Trophoblast-Like Cells JAR, JEG-3 and HTR-8/SVneo Express BSG transcript variants 2-4 and Secrete Basigin Protein into Medium**

Based on our finding that BSG protein is present in human placental tissues throughout the first trimester, we next investigated the expression of BSG mRNA transcript variants and BSG protein in JAR and JEG-3 choriocarcinoma cell lines and first trimester immortalized HTR-8/SVneo cells (Fig. 10). The expression of BSG transcript variants was determined using RT-PCR with transcript variant-specific primer pairs. We identified three distinct bands in all three human trophoblast-like cell lines tested which corresponded to BSG transcript variants 2, 3 and 4 (Fig.11A). BSG transcript variant 2 was the most predominant transcript variant in all three cell lines since 6 times more PCR product was loaded onto gels in order to detect transcript variants 3 and 4 (10 µl of PCR product was loaded for BSG transcript variant 2 and 60 µl for isoforms 3,4). We also verified presence of BSG protein in cell lysates of all three trophoblast-like cell lines.
(Fig.11B). BSG protein ran as a smear confirming previous reports that BSG has a wide range of molecular weights since it is a highly glycosylated protein. Our immunoblots recognized several bands ranging between 37 and 75 kD due to varying degrees of glycosylation. Moreover, the degree of glycosylation varied amongst the different cell lines with more highly glycosylated forms of BSG found in the choriocarcinoma cells. BSG was also detected in trophoblast cell-conditioned medium. Immunoblotting results indicated that BSG protein is released into conditioned medium from trophoblast-like cell lines (Fig.11C).

**Human Trophoblast-Like Cells Release Basigin Protein via Microvesicle Shedding**

Since we were able to detect BSG protein in conditioned medium from trophoblast-like cells, we next investigated the mode of release of this transmembrane protein. We wanted to determine whether release occurs through plasma membrane microvesicle shedding as has been shown to occur in other cells. Using a series of centrifugation steps, we isolated the microvesicle fraction from conditioned medium of the three cell lines as shown in Fig.8. Fractions of conditioned medium before and after ultracentrifugation as well as the microvesicle pellets were probed for BSG protein using immunoblotting (Fig.12). BSG protein was not detected in conditioned medium before ultracentrifugation or in supernatant after ultracentrifugation. However, the microvesicle fractions shed by the trophoblast-like cell lines were highly positive for BSG. Concentration of the conditioned medium collected before ultracentrifugation by 30 fold allowed us to detect a small amount of BSG protein in the medium. These results led us to conclude that BSG is released through microvesicle shedding from the plasma membrane of trophoblast-like cells, and that the majority of BSG protein is in the microvesicle fraction.
Immunoblotting for MCT1, another cell membrane protein, was performed to confirm isolation of the microvesicle fraction.

**Basigin Treatment Stimulates MMP-1 and MMP-3 Secretion by Endothelial Cells**

We next investigated whether BSG protein is involved in trophoblast-endothelial cell interactions. HUVECs were treated with purified rBSG protein (0, 1 or 5 µg/ml) for 48 hours, conditioned medium collected and concentrated 4 fold, and levels of MMP-1 and MMP-3 secretion were assessed by immunoblotting. In order to control for the effect of LPS contamination in our recombinant protein, HUVECs were also treated with 3 pg/ml LPS for 48 h. This amount of LPS corresponded to the levels present in the highest dose of rBSG used. Immunoblotting analysis showed that 1 or 5 µg/ml rBSG treatment increased endothelial (HUVEC) cell secretion of MMP-1 and MMP-3 proteins into conditioned medium (Fig.13). We also performed cell counts to determine whether treatment with rBSG protein affected proliferation of HUVECs but saw no effects on cell proliferation (data not shown).
DISCUSSION

Our study has shown that BSG protein is present in human placental tissues throughout the first trimester of pregnancy. Furthermore, although BSG is a transmembrane protein, it is secreted into surrounding medium by trophoblast cells. We determined that the mode of release of BSG from the surface of trophoblast cells is via microvesicle shedding. Moreover, rBSG protein stimulates secretion of MMP-1 and MMP-3 by endothelial cells. These results suggest that BSG, shed in microvesicles, may play an important role in paracrine interactions between trophoblast cells and other uterine cells during implantation and placental development.

Our study shows the presence of BSG in human placenta throughout the first trimester of pregnancy. BSG protein was detected by immunohistochemistry in both cytotrophoblast and syncytiotrophoblast layers. These results complement previous findings reporting presence of BSG in mouse pre- and peri- implantation embryos in the trophectoderm layes of blastocyst and inner cell mass [33], as well as the presence of BSG in trophoblast cells of human term placentas [34]. An additional study published in a Chinese journal reported that, during the first trimester, BSG was immunolocalized mainly to cytotrophoblast, syncytiotrophoblast and cytotrophoblast columns of the chorionic villi [162]. The syncytiotrophoblast layer serves an important barrier function in the human placenta and massively increases the surface area available for nutrient exchange between the mother and the fetus [163]. Thus, the localization of BSG in the syncytiotrophoblast layer may be linked to the function of BSG as an important chaperone protein that facilitates the trafficking of MCT1 and MCT4 to the plasma membrane [30, 109].

Monocarboxylate transporters are necessary for the transport of monocarboxylates such as lactate across the plasma membrane [106, 107]. One study reported that MCT1 predominantly immunolocalizes to basal plasma membrane (fetal-facing) and MCT4 to microvillous plasma
membrane (maternal-facing) and that BSG was expressed equally by both membranes in the syncytiotrophoblast of term human placenta [164]. Therefore, one potential role for BSG in syncytiotrophoblast is the regulation of MCT trafficking and activity. Cytotrophoblast cells can follow one of two different paths, either conversion to syncytiotrophoblasts or acquisition of a more invasive phenotype [37, 12]. BSG localization in the cytotrophoblasts may also regulate MCTs but may be possibly linked to regulation of MMPs [95, 22] that would allow invasion and migration or integrin-mediated cell adhesion [165].

Immunoblotting, utilizing the JAR, JEG-3 and HTR-8/SVneo trophoblast-like cell lines, demonstrated abundant expression of BSG protein in all three cell lines. BSG protein in cell lysates ran as a smear confirming previous reports that BSG is a highly glycosylated protein [18, 34]. The antibody used in our studies recognized several bands between 37 and 75 kD. Myauchi et al. reported that BSG from embryonal carcinoma cells had a molecular weight ranging between 43 and 66 kD. The core protein with the putative signal sequence is approximately 30 kD [18]. Later, Kanekura et al. showed that treatment of BSG protein from COS cells with endoglycosidase F (N-glycosidase) shifted the molecular mass of BSG from 54 to 28 kD [166]. Li et al. detected 30 kD and 40-55 kD sizes of BSG in human term placentas of laboring or nonlaboring patients [34]. Thus, the molecular mass of BSG varies depending on the degree of glycosylation. In addition the degree of glycosylation differs depending on the cell type or tissue. Our results showed that the molecular weight range of BSG protein secreted by HTR-8/SVneo cells was smaller than that of the choriocarcinoma cell lines. These differences in glycosylation may affect specific biological functions of BSG and merit further investigations.

We also investigated which of the four BSG transcript variants are expressed by trophoblast cells. Recently, four basigin BSG transcript variants -1, -2, -3 and -4 that encode
different BSG isoforms (BSG isoforms -1, -2, -3 and -4) were identified [90, 91]. Belton et al. showed that BSG-2 is the most predominant splice variant encoding the original transmembrane protein with two immunoglobulin-like domains and the only one that is secreted [91]. They also identified two other BSG transcript variants, -3 and -4, that were much less abundant in human endometrial stromal cells and cervical carcinoma cell lines [91]. Another BSG transcript variant, BSG-1, encoding a protein with three immunoglobulin-like domains has been found to be specifically expressed in the retina [88, 89]. Most recently, Liao et al. identified the presence of BSG-2, -3 and -4 in various normal tissues and reported up regulation of these transcript variants in hepatocellular carcinoma [90]. The results we obtained in the present study are consistent with those discussed above. We identified expression of the transmembrane form (BSG transcript variant-2) and the two intracellular forms (BSG transcript variants -3 and -4) in all trophoblast-like cell lines tested. BSG transcript variant-1 is retina-specific and was not assessed in this study. Consistent with the studies of Belton et al. and Liao et al., transcript variant 2 was the most predominant. BSG isoform-2 on the cell surface has been shown to be able to interact homophilically with soluble basigin [91]. BSG isoform-3 can interact with BSG isoform-2 via hetero-oligomerization possibly regulating the functional activity of BSG isoform-2. The effects of these different BSG isoforms need to be studied further to increase our understanding of BSG gene function.

Our results demonstrated that BSG protein is released from the surface of trophoblast cells through shedding of microvesicles, small membrane blebs generated by the outward budding of plasma membrane [130, 131]. Microvesicle shedding has been observed in various cell types including platelets [136], dendritic cells [167] and cancer cells [23, 138]. Microvesicles have been recently recognized as important messengers involved in cell-to-cell
communication through the transfer of cell surface receptors, proteins, mRNAs, and even infectious agents between cells [127, 130-131]. Sidhu et al. showed that BSG is released from the surface of tumor cells via microvesicle shedding [23]. Moreover, tumor microvesicles underwent spontaneous breakdown releasing soluble BSG into the surrounding medium [23]. A subsequent study in 2007 showed that BSG-containing microvesicles shed from the surface of human epithelial ovarian carcinoma cells were able to promote an angiogenic phenotype and up regulate MMPs in cultured endothelial HUVEC cells [94]. Alcazar et al. used proteomic analysis to show the presence of 42 kD BSG in microvesicles derived from human retinal pigment epithelium [24].

Recently, proteomic studies reported the presence of BSG isoform 2 in exosomes derived from Swan71 human first trimester trophoblast cells [168] and showed that these trophoblast-derived exosomes are able to mediate monocyte recruitment and differentiation [169]. The authors of these papers distinguished trophoblast-derived microvesicles as exosomes based on the presence of exosome marker proteins and on their morphology and density [168]. However, our results indicate that microvesicles from trophoblast cells are released though plasma membrane blebbing since high amounts of the transmembrane protein BSG were found in the microvesicle fraction. The presence of MCT1 protein, another cell membrane protein, in our microvesicle fraction, further supports that these are cell-membrane-rich fractions. More likely, the microvesicle population isolated by ultracentrifugation sediments a mixture of microvesicles and exosomes. Atay et al. identified 147 proteins amongst 282 proteins present in microvesicles that had not previously been described in exosomes including integrin β1 [168] which has been shown to be concentrated in the membranes of microvesicles [130, 135].
Overall, based on the studies described above and our novel finding of release of BSG protein from surface of trophoblast cells through plasma membrane microvesicle shedding, we hypothesize that BSG protein is released through microvesicle shedding from trophoblast cells into surrounding medium, the microvesicles are broken down over a period of time, and soluble BSG protein is released and can presumably bind to target cells. Thus, BSG protein may be involved in cross-talk between trophoblast cells and other uterine cells during the process of early placental development.

In support of our hypothesis we have also shown that BSG can regulate MMP production by ECs. Treatment of HUVEC cells with rBSG increased secretion of MMP-1 and MMP-3 into the surrounding medium. The physiological significance of MMP-1 and MMP-3 up regulation in ECs by BSG has not been fully investigated. A previous study also demonstrated that rBSG increased the secretion of MMP-1, -2 and -3 by ECs and proposed that these MMPs are involved in ECM remodeling around tumor cells [101]. It is also known that trophoblast cells, ECs, and VSMCs produce various MMPs that contribute to remodeling of the spiral arteries [39]. Moreover, it was shown that conditioned medium from trophoblast cells was able to induce MMP-12 expression in VSMCs to facilitate ECM breakdown [120]. Based on these previous findings and our own results, we hypothesize that during extravillous trophoblast invasion in the first trimester of pregnancy, BSG released from trophoblast cells acts on ECs to promote production of specific MMPs. MMP action may lead to the loss of adhesive interactions between vascular cells and to remodeling of the ECM as part of the process of remodeling of the uterine spiral arteries.

In summary, our findings confirm that BSG is present in placental trophoblasts throughout the first trimester of pregnancy. BSG is shed in microvesicles from the plasma
membrane of trophoblast cells and is released into surrounding medium where it can signal to other uterine cells, including ECs, to up regulate MMPs. BSG therefore may act as a paracrine factor that may contribute to the process of spiral artery remodeling during early placental development.
Figure 8. Schematic for microvesicle collection. Adapted from www.ars.usda.gov website.
Figure 9. Immunolocalization of BSG protein in the first trimester human placenta. The brown staining shows the presence of immunoreactive BSG. (A-D) are representative sections of human placental tissues from first trimester of pregnancy. (A) 7 weeks 3 days, (B) 9 weeks, (C) 12 weeks 3 days and (D) 17 weeks human placental tissues. (E) is representative positive control section of normal term placental tissues. (F) is representative negative control section of placenta. CTB, cytotrophoblast layer; STB, syncytiotrophoblast layer; VC, villous core. Scale bars, 100 µm; insert scale bar, 20 µm.
Figure 1. Trophoblast-like cell lines used. (A) JAR, choriocarcinoma cell line, (B) JEG-3, choriocarcinoma cell line, (C) HTR-8/SVneo, human first trimester immortalized cell line.
Figure 11. Human trophoblast-like cells JAR, JEG-3 and HTR-8/SVneo express BSG transcript variants 2-4 and secrete BSG protein into surrounding medium. (A) Reverse transcriptase PCR for BSG transcript variants 2-4 in cultured trophoblast-like cell lines JAR, JEG-3 and HTR-8/SVneo. M, base pair marker; Ctr, no template negative control; +, detected BSG transcript variants; -, no RT negative control; EEC, human uterine epithelial cell line used as positive control; BSG-2, transcript variant 2 (270 bp); BSG-3, transcript variant 3 (435 bp), BSG-4, transcript variant 4 (279 bp). 10 µl of the PCR reaction was loaded for BSG transcript variant 2 and 60 µl for transcript variants 3, 4. (B) Immunoblot showing BSG in JAR, JEG-3 and HTR-8/SVneo cell lysates (5 or 10 µg total protein was loaded). C, human uterine epithelial cell lysate (HES) used as positive control. (C) Immunoblot showing BSG in JAR, JEG-3 and HTR-8/SVneo 1x (unconcentrated) or 10, 30, 50 x concentrated conditioned medium collected after 24 h incubation.
Figure 12. BSG protein is released from JAR, JEG-3 and HTR-8/SVneo human trophoblast-like cells via microvesicle shedding. Lane 1, 1x unconcentrated trophoblast-cell conditioned medium before ultracentrifugation; lane 2, 30x concentrated conditioned medium before ultracentrifugation; lane 3, supernatant after ultracentrifugation; lane 4, microvesicle pellet. Equal volumes of 1x or 30x conditioned medium, supernatant or resuspended microvesicle pellet were loaded and analyzed by immunoblotting for BSG and MCT1 proteins. C, human uterine epithelial cell line (HES) used as positive control.
Figure 13. BSG stimulates MMP-1 and MMP-3 secretion by ECs. Immunoblot analysis of 4x concentrated conditioned medium from HUVECs after 48 h treatment with rBSG (0, 1 or 5 µg/ml) showed increased levels of MMP-1 and MMP-3 secretion. L, 3 pg/ml LPS control.
CHAPTER 4
SECRETION OF BASIGIN-CONTAINING MICROVESICLES BY HUMAN PLACENTAL TROPHOBLAST CELLS IS A REGULATED PROCESS

ABSTRACT

BSG is a cell surface glycoprotein that is a member of the immunoglobulin superfamily. BSG is crucial for successful implantation and may also be involved in parturition. BSG is expressed in first trimester placental trophoblast cells and is secreted from the surface of trophoblast cells through microvesicle shedding. Microvesicles are small membrane blebs generated by the outward budding of the plasma membrane. They act as important messengers mediating cell-to-cell communication. However, whether microvesicle release by trophoblast cells is a regulated process is unknown. Our goal was to determine what some of the signaling cascades responsible for microvesicle release by placental trophoblast cells are; specifically, the potential involvement of the PKC pathway. Further, our aim was to investigate whether shedding of BSG-containing microvesicles is regulated by external stimuli such as the pro-inflammatory cytokine IL-1β or the growth factor TGF-β1. We also wanted to determine whether microvesicle release by trophoblast cells is altered by hypoxia/reoxygenation. We utilized the human first trimester immortalized HTR-8/SVneo trophoblast cell line as our experimental model. Our results showed that treatment of trophoblast-like cells with an activator of protein kinase C, PMA, stimulated an increase in release of BSG-containing microvesicles in both a time- and dose-dependent manner as shown by immunoblotting. Treatment with the PKC inhibitor bisindolylmaleimide I HCl (Bis) suppressed this induction of release of microvesicles. Release of BSG in microvesicles was increased in response to oxidative stress such as
hypoxia/reoxygenation as determined by BCA protein assays and immunoblotting. The inflammatory cytokine IL-1β increased, while the growth factor TGF-β1 decreased, incorporation of BSG protein into microvesicles. This occurred at the post-transcriptional level as determined from messenger RNA and immunoblotting analysis. However, these treatments did not affect the total amount of microvesicles shed by the HTR-8/SVneo cells. Our findings confirm that one of the signaling cascades responsible for release of BSG-containing microvesicles by trophoblast cells is the PKC pathway. Moreover, trophoblast cells release microvesicles by mechanisms that differ, depending on the nature of the stimulus.
INTRODUCTION

The important prerequisites for successful pregnancy are trophoblast invasion into the maternal uterine tissues and remodeling of the uterine spiral arteries during the first trimester of pregnancy [1-2, 4-5]. These processes result in the remodeling of ECM and restructuring of spiral arteries from low-flow, high-resistance into high-flow, low resistance vessels with increased circumference and vascular compliance [38, 39]. This is critical for the establishment of adequate blood flow for normal fetal and placental development. Insufficient trophoblast invasion and incomplete spiral artery remodeling are linked to the obstetrical disease preeclampsia. This disease is a leading cause of morbidity and mortality to both the fetus and the mother [6-9]. Recently, it has become widely accepted that the pathological changes observed in preeclampsia are a result of intermittent perfusion of the placenta by fluctuating oxygen levels (hypoxia/reoxygenation) secondary to deficient trophoblast invasion of the endometrial arteries [59].

Trophoblast invasion and spiral artery remodeling are controlled by several factors including MMPs [110, 112, 117], cytokines [71, 73-74], growth factors [75] and changes in oxygen tension [40, 57]. BSG, also known as EMMPRIN, is a glycosylated transmembrane protein expressed on the surface of normal cells and tumor cells [18, 20, 33]. BSG plays a critical role in the induction of MMPs and regulates remodeling of ECM [16-17, 19, 22]. BSG is crucial for successful implantation [31] and may also be involved in parturition [34]. In chapter 3 we showed that BSG is present in first trimester human trophoblast cells and also demonstrated that BSG protein is released from the surface of trophoblast cells via microvesicle shedding. BSG is also been shown to be shed in microvesicles from the surface of tumor and other normal cells [23, 24]. Microvesicles are small cell membrane vesicles that are shed into the extracellular
environment and contain cell surface elements. Microvesicles have different compositions and biological effects depending on the cell of origin. The proteins and other factors in microvesicles can change substantially to allow enrichment of particular proteins needed to perform specific functions. Microvesicles are now recognized as important mediators of cell-to-cell communication [127, 130-131, 170].

BSG release from the surface of tumor cells occurs through microvesicle shedding in a PKC-dependent manner [23]. However, the signaling cascade responsible for release of microvesicles by placental trophoblast cells is unknown. Therefore, the goal of this study was to determine whether release of BSG-containing microvesicles by first trimester trophoblast cells is regulated through the PKC pathway. We also wanted to determine whether the cytokine IL-1β and growth factor TGF-β1, factors known to control trophoblast invasion, could affect the release of BSG in microvesicles by trophoblast cells. Furthermore, the amount of circulating microvesicles is increased in patients with preeclampsia suggesting that placental microvesicles may play a role in the pathophysiology of this disease [171]. Hypoxia/reoxygenation has been proposed as a possible mechanism for placental oxidative stress in preeclampsia [59]. Therefore, we also wanted to determine whether release of BSG-containing microvesicles by first trimester trophoblast cells is altered by hypoxia/reoxygenation.

Immortalized human first trimester HTR-8/SVneo trophoblast cells were used as the model system for this study. We chose this cell line over others used in chapter 3 because, based on our results, JEG-3 cells are not very suitable for studying microvesicle shedding since they release very low levels of microvesicles. Moreover, the JEG-3 and JAR choriocarcinoma cell lines have characteristics related to their malignant origin. For example, while TGF-β inhibits invasion of normal trophoblast cells [172], JEG-3 and JAR choriocarcinoma cells are resistant to
the anti-invasive properties of TGF-β [159]. HTR-8/SVneo cells retain phenotypic properties and functional characteristics of the parental trophoblast cells [157, 158].

Our results showed that microvesicle shedding by immortalized first trimester trophoblast cells is regulated by the PKC signaling pathway. Microvesicle shedding and release of BSG were increased in response to hypoxia/reoxygenation. The inflammatory cytokine IL-1β increased and the growth factor TGF-β1 decreased incorporation of BSG into microvesicles at the post-transcriptional level but did not affect the amount of trophoblast microvesicles shed. These data show that microvesicle shedding and release of BSG protein in microvesicles by first trimester trophoblast cells is not random, but is a well-regulated process.
MATERIALS AND METHODS

Cell Culture

The immortalized human first trimester trophoblast cell line HTR-8/SVneo was a gift from Dr. Stephen Charnock-Jones (Cambridge University, UK). HTR-8/SVneo cells were cultured in RPMI 1640 medium (Cellgro, Manassas, VA) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) that was heat-inactivated, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO₂ atmosphere. Fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) was inactivated by placing thawed bottles in a water bath at 56°C for 30 min. During incubation the contents were swirled every 5 min to ensure they were evenly heated. After 30 min, serum was transferred on ice to cool. Aliquots were made and stored at -20°C until use. Cell culture conditions described above were used for PMA, Bis, IL-1β and TGF-β1 experiments. For hypoxia/reoxygenation experiments HTR-8/SVneo cells were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin (Invitrogen, Carlsbad, CA).

Experimental Design for PMA and Bis Treatment Studies

HTR-8/SVneo cells were grown to 80% confluence. Once cells reached this confluency, they were transferred to serum-free medium containing only L-glutamine and antibiotics for 24 hours. After this serum starvation period, the cells received treatments in serum-free medium. After the treatment period, conditioned medium was collected and used for isolation of microvesicles and supernatant. Some conditioned medium was collected at the 0 hr time point as well for microvesicle isolation. Microvesicle pellets and supernantants were stored at -20°C for further immunoblotting analysis. Microvesicle pellets were also analyzed for total protein content by BCA protein assay. PMA was obtained from Sigma-Aldrich (St. Louis, MO),
dissolved in 100% ethanol and a 2 mM stock stored in the dark at -20°C until use. Cells were treated with 0, 100 or 200 ng/ml PMA (catalog no. P1585-1MG; Sigma-Aldrich, St. Louis, MO) for 2 or 4 hours in serum-free medium. Bis was obtained from Calbiochem (#203291), dissolved in water to a 0.1mM stock and kept at 4°C until use. Cells were treated with serum free medium alone, vehicle control for PMA (ethanol), 100 ng/ml PMA, 4.5 ng/ml Bis alone, both 100 ng/ml PMA and 4.5 ng/ml Bis for 2 hours and 4.5 ng/ml Bis for 1 hour followed by treatment with 100 ng/ml PMA for 2 more hours.

**Hypoxia/Reoxygenation Experiment**

Cells were grown until ~60-70% confluence. Once cells reached this confluency, they were transferred to serum-free medium containing only L-glutamine and antibiotics for 24 hours. After this serum starvation period the cells continued to be cultured under either 1) 2% \( \text{O}_2 \), 2) 8% \( \text{O}_2 \), or 3) 20% \( \text{O}_2 \) for 4 hours 30 min, or 4) were subjected to hypoxia/reoxygenation (4 hours at 2% oxygen, followed by 30 min at 8% oxygen). Culture at low \( \text{O}_2 \) was achieved by placing cells into a humidified incubator that consists of chambers where each chamber can be individually regulated (Hypoxia control system, Plas Labs, Inc., Lansing, MI). Incubator Forma, Thermo Scientific Corp., Rockford, IL was used for culture of cells under normal incubator conditions. The oxygen level in the medium was checked using an oxygen probe (Ocean Optics, Dunedin, FL). Following the incubation period the conditioned cell medium was collected for isolation of microvesicles and supernatant. Microvesicle pellets and supernatants were stored at -20°C for immunoblotting analysis. Microvesicle pellets were also analyzed for total protein content by BCA protein assay.
IL-1β and TGF-β1 Experimental Design and Treatments

Cells were grown until they reached 80% confluence. They were then transferred to serum-free medium containing only L-glutamine and antibiotics for 24 hours. After this serum starvation period, the cells were treated with serum free medium alone; 1, 2.5 or 5 ng/ml IL-1β (catalog no. 201-LB, R&D Systems, Inc., Minneapolis, MN); or 1, 2.5 or 5 ng/ml TGF-β1 (catalog no. 240-B, R&D Systems, Inc., Minneapolis, MN) for 0, 8 or 24 hours. Following the treatment periods, conditioned medium was collected and microvesicles were isolated; RNA samples were harvested and analyzed using quantitative real-time PCR. Microvesicle pellets were stored at -20°C for immunoblotting analysis and were also analyzed for total protein by BCA protein assay.

Microvesicle Isolation

Conditioned medium was collected from the culture plates, centrifuged at 1500g for 10 min, transferred to a new tube and centrifuged again at 1500g for 15 min to pellet cellular debris. Some of the conditioned cell medium was saved at -20°C for further immunoblot analysis. The supernatant was then ultracentrifuged at 40,000 rpm for 1 hr at 4°C. The supernatant from this ultracentrifuge spin was stored at -20°C for immunoblot analysis. Microvesicle pellets for each treatment were resuspended in 1X LSB preheated at 95°C for 5 min. 1xLSB contained 10% glycerol (Fisher Scientific, Pittsburgh, PA), 62.5 mM Tris Base pH=6.8 (Fisher Scientific, Pittsburgh, PA) and 2% SDS (Fisher Scientific, Pittsburgh, PA). Volume was adjusted with water to 50 ml and 1xLSB was stored at room temperature. Samples were frozen at -20°C until further analysis. Schematic for microvesicle collection is shown in Fig.8. For immunoblotting,
equal volumes of supernatant collected after ultracentrifugation and of the resuspended microvesicle pellets were loaded on gels.

**Immunoblotting**

Samples were denatured in 4X LSB dye at 95°C for 5 min. 4X LSB dye contained 10% glycerol (Fisher Scientific, Pittsburgh, PA), 50 mM Tris Base (Fisher Scientific, Pittsburgh, PA), 2% SDS (Fisher Scientific, Pittsburgh, PA), 100 mM DTT (Fisher Scientific, Pittsburgh, PA) and 0.025% Bromphenol Blue (Sigma, St. Louis, MO). Volume was adjusted to 10 ml with water. Aliquots of 4XLSB dye were made and stored at -20°C until use. The samples were then loaded onto 4-20% Precise Protein Gradient Gels (Thermo Scientific, Rockford, IL) and transferred to 0.45-μm pore size nitrocellulose membranes (Thermo Scientific, Rockford, IL) overnight. Nonspecific binding sites were blocked by incubating membranes in Tris-buffered saline containing 0.1% Tween-20 (TBST) pH 8 with 5% instant, nonfat dry milk for 1 hr at 25°C on a shaker. Membranes were incubated with specific primary antibodies in TBST pH 8 containing 2% BSA overnight at 4°C and then washed three times 10 min each in TBST pH 8 to remove unbound antibody. Membranes were incubated with HRP-conjugated secondary antibody in TBST pH 8 with 2.5% nonfat dry milk for 1 hr at 25°C. Purified mouse anti-human CD147 (BD Biosciences) and rabbit polyclonal anti-human MCT1 (H-70) (Santa Cruz) primary antibodies were used at 1:1000 dilution. HRP-linked anti-mouse IgG (Cell Signaling, Danvers, MA) and HRP-linked anti-rabbit IgG (Cell Signaling, Danvers, MA) secondary antibodies were used at 1:10000 dilution. Membranes were washed again 6 times for 5 min each in TBST pH 8 before visualization of immunocomplexes using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). Precision Plus Protein all blue molecular weight marker
was used as standards (Bio-Rad Laboratories, Hercules, CA). Densitometry was performed using the ImageJ Program from the National Institutes of Health (available at http://rsbweb.nih.gov/ij/download.html).

**BCA Protein Assay**

The concentration of protein in microvesicle pellets was determined by the BCA protein assay. The BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) was used according to the manufacturer’s instructions using the microplate procedure. The absorbance measurements were made using a μQuant microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT).

**RNA Isolation and cDNA Synthesis**

Total RNA was harvested and extracted from cells using the RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. Total RNA (0.5 µg) was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems; Atlanta, GA) according to the manufacturer’s instructions.

**Quantitative Reverse-Transcriptase PCR**

Synthesized cDNA was used for real-time PCR analysis. Real-time PCR analyses were performed in triplicate in 10 µl reactions containing TaqMan® Universal PCR Master Mix (4304437, Applied Biosystems, Atlanta, GA), diluted cDNA, nuclease-free water, and 20X Assays-on-Demand™ Gene Expression Assay (Applied Biosystems, Atlanta, GA) reagents. 20X Assays-on-Demand™ Gene Expression Assays were purchased for the following genes: basigin (HS00174305-m1) and processing of precursor 4, ribonuclease P/MRP subunit (POP4)
Real-time PCR amplification and detection were performed in MicroAmp® Optical 384-Well Reaction Plates with Barcode (4309849, Applied Biosystems, Atlanta, GA) using the TaqMan ABI7900 sequence detection system. Amplification conditions included: 10 min hold at 95°C, 40 thermal cycles of denaturing for 15 sec at 95°C, and an annealing/extension for 1 min at 60°C. Relative fold changes were calculated using the comparative C_T method. Fold induction was expressed relative to POP₄ endogenous control gene.

**Statistical analysis**

Outcome measures for each experiment were analyzed by an ANOVA model using SAS (Copyright, SAS Institute Inc.). To evaluate experimental statistical differences between treatments, orthogonal contrast statements were designed for planned comparisons. Outcome measures are described as follows: integrated density values for densitometry analysis, total microvesicle protein determined by BCA assay and the difference between the threshold cycle of target gene and POP₄ (DCt) for gene expression studies. Outcome measures were averaged and variation of the mean was expressed as ± S.E. The criterion for significance was p < 0.05. For gene expression studies, the threshold cycle was defined as the cycle number at which all transcripts are in the linear phase of amplification. The difference between target and POP₄ was then normalized for control treatment expression and expressed as the relative fold difference.
RESULTS

*Secretion of Basigin-Containing Microvesicles by Trophoblast-Like Cells is Regulated by the PKC Activator PMA and the PKC Inhibitor Bis*

To explore the mechanism of BSG release in microvesicles from trophoblast cells, we exposed HTR-8/SVneo trophoblast-like cell line to the well-known tumor promoter, PMA. PMA is a diacylglycerol (DAG) analogue known to specifically stimulate the PKC signaling pathway. Treatment of HTR-8/SVneo cells with PMA (0, 100 or 200 ng/ml) for 2 or 4 hours dramatically increased BSG release in microvesicles (P) in both a time- and dose-dependent manner (Fig.14A). Supernatants (S) were included to confirm that microvesicles did not show appreciable breakdown in this time frame. We also saw no measurable release of BSG in microvesicles at 0 hours of treatment. To further prove that microvesicle shedding by trophoblast cells is controlled by PKC, we treated HTR-8/SVneo cells with either serum free medium (SF), 0.1% ethanol (EtOH), 100 ng/ml PMA, 4.5 ng/ml Bis, or Bis and PMA combined for 2 h (Bis+PMA). Cells were also pretreated with Bis for 1 h followed by PMA treatment (Bis/PMA) for 2 h (Fig.14B). Our results showed that induction of BSG-containing microvesicles was inhibited by the PKC inhibitor Bis (4.5 ng/ml). This result confirms that BSG release in membrane vesicles from trophoblast cells is regulated, at least partially, by PKC. Immunoblotting for MCT1, another cell membrane protein, was performed as a control to confirm isolation of the cell-membrane fraction (Fig.14A and B).
Microvesicle Shedding and Basigin Release by Trophoblast-Like Cells are Increased in Response to Hypoxia/Reoxygenation

We next wanted to determine whether BSG release in microvesicles and microvesicle shedding may be altered in response to hypoxia/reoxygenation. Immunoblotting was performed to determine whether BSG release in microvesicles was altered in response to varying oxygen conditions while protein analysis assays were used to quantitate the total amount of microvesicles shed. Immunoblotting analysis showed that BSG release in microvesicles was increased in response to hypoxia/reoxygenation (2%/8%) as compared to culture of cells in 2%, 8% or 20% O_2 (Fig. 15A). This increase in BSG release was due to an increase in overall microvesicle shedding as indicated by the increase in total protein in the microvesicle fraction (Fig.15B).

TGF-β1 Inhibits Basigin Release in Microvesicles but Does Not Affect Overall Microvesicle Shedding or Basigin mRNA Levels in Human Trophoblast-Like Cells

The growth factor TGF-β1 is known to inhibit trophoblast invasion. To elucidate the effect of this molecule on release of BSG protein, HTR-8/SVneo cells were treated with 0, 1, 2.5 or 5 ng/ml TGF-β1 for 8 or 24 hours, microvesicle pellets were isolated and analyzed by immunoblotting for BSG protein. Immunoblot analysis showed that BSG protein levels in microvesicle fractions were decreased in response to TGF-β1 treatments for 8 hours (Fig.16A) as well as for 24 hours (Fig.17A) in a dose-dependent manner. Total protein released in microvesicles was measured by BCA protein assay and showed that the amount of vesicles shed did not differ between treatments at 8 (Fig.16B) or 24 hours (Fig.17B). To determine whether BSG transcript levels were affected by TGF-β1 treatment, quantitative RT-PCR was performed.
We observed no change in the mRNA levels when compared to 0 ng/ml control treatment after 8 hours (Fig.16C) or 24 hours (Fig.17C) of treatment with 1, 2.5 or 5 ng/ml TGF-β1. These results indicate that the decrease in microvesicle-associated BSG in response to TGF-β1 treatment was not due to a reduction in microvesicle shedding but likely due to selective inhibition of BSG incorporation into microvesicles at the post-transcriptional level.

**IL-1β Stimulates Basigin Release in Microvesicles but Does Not Affect Overall Microvesicle Shedding or Basigin mRNA Levels in Human Trophoblast-Like Cells**

The inflammatory cytokine IL-1β is known to stimulate trophoblast invasion. We therefore wished to determine the effect of this cytokine on release of BSG protein in microvesicles. HTR-8/SVneo cells were treated with 0, 1, 2.5 or 5 ng/ml IL-1β for 8 or 24 hours, microvesicle pellets were isolated and analyzed by immunoblotting for BSG protein. Immunoblot analysis showed that BSG levels in the microvesicle fractions were increased in response to IL-1β treatment for 8 hours, however, treatments were not dose-dependent (Fig.18A). At 24 hours of treatments, we did not detect a significant increase in BSG protein levels in microvesicles as compared to the 0 ng/ml control (Fig.19A). Total protein released in microvesicles was measured using a BCA protein assay and showed that the amount of vesicles shed did not differ among treatments at either 8 (Fig.18B) or 24 hours (Fig.19B). To identify whether BSG transcript levels were affected by IL-1β treatment, quantitative RT-PCR was performed. There was no change in the mRNA levels when compared to 0 ng/ml control after either 8 (Fig.18C) or 24 hours (Fig.19C) of treatment with 1, 2.5 or 5 ng/ml IL-1β. These results indicate that the inflammatory cytokine IL-1β, a very potent stimulator, causes a rapid increase in BSG protein accumulation in microvesicles at 8 hours of treatment. Our data also showed that
this increase in BSG protein release in microvesicles does not occur through an increase in \textit{BSG} gene transcription but likely occurs due to selective BSG protein incorporation into microvesicles at a post-transcriptional level.
DISCUSSION

The goal of our study was to determine whether BSG secretion via microvesicle shedding by human placental trophoblast-like cells is a regulated process. The main findings presented here are that one of the signaling cascades responsible for BSG release in microvesicles by trophoblast-like cells is the PKC pathway. We also found that exposure of trophoblast-like cells to hypoxia/reoxygenation led to a burst of BSG-containing microvesicle shedding. Treatment of trophoblast-like cells with the inflammatory cytokine IL-1β increased while TGF-β1 decreased incorporation of BSG protein into microvesicles at the post-transcriptional level but did not affect the overall amount of microvesicle shedding. These data show that BSG release via microvesicle shedding by first trimester trophoblast-like cells is not a random process but is regulated at multiple levels.

Microvesicles are small cell membrane bound vesicles generated by the outward budding of the plasma membrane. Recently microvesicles have been recognized as important messengers that mediate cell-to-cell communication. Interestingly, microvesicles are normally shed from the surface of healthy cells [173]. However, the number of microvesicles shed from the cells increases upon cell activation, hypoxia or irradiation, oxidative injury, exposure to the proteins from an activated complement cascade and exposure to shearing stress [127, 130-131]. Microvesicles are released from viable cells and are usually smaller in size compared to the apoptotic bodies derived from damaged cells and they do not contain fragmented DNA [127].

Our studies in chapter 3 showed that the first trimester trophoblast-like cells release microvesicles containing abundant levels of BSG protein. These BSG-containing microvesicles may be paracrine mediators of trophoblast and EC cross-talk. BSG is an important molecule for invasion processes. BSG released in microvesicles from trophoblast-like cells may contribute to
spiral artery remodeling during early placental development. The mechanisms responsible for release of BSG in microvesicles are not completely understood. Sidhu et al. showed that BSG release via microvesicle shedding from tumor cells is amplified upon exposure of cells to PMA, a PKC activator, and thapsigargin which evokes the release of Ca\(^{2+}\) from the ER. This group also showed that treatment with inhibitors of PKC such as Bis, Ca\(^{2+}\) chelator BAPTA/AM, and inhibitors of mitogen-activated protein kinase kinase 1/2 (MEK 1/2) inhibited the ability of PMA to stimulate BSG release suggesting that full length BSG release via budding of microvesicles is regulated in a physiologically controlled manner by a PKC, Ca\(^{2+}\) and MEK 1/2-dependent signaling pathway [23]. Our results showed that under normal conditions trophoblast cells release only a limited amount of microvesicles. Results from our study are consistent with those discussed above. BSG release in membrane microvesicles from first trimester trophoblast-like cells was increased in both a time- and dose-dependent manner upon cell exposure to PMA, a well-defined DAG analogue known to stimulate PKC consistent with previous results. BSG release in microvesicles was decreased after exposure of cells to the PKC inhibitor Bis, a synthetic derivative of the microbial product staurosporine [174, 175]. PMA, also known as 12-O-tetradecanoylphorbol-13-acetate (TPA) is a phorbol ester that contains a DAG-like structure in its molecule and is a powerful tumor promoter and activator of PKC [176, 177]. DAG is the earliest product of signal-induced inositol phospholipid breakdown and is able to increase the affinity of PKC for Ca\(^{2+}\), thereby activating it [176]. When PMA/TPA is added to resting cells, PKC is converted from a soluble inactive form to a form tightly associated with the cell membrane [176]. Activation of PKC has been demonstrated to affect central signaling pathways such as MAPK/ERK and the PI3K pathways [178]. It remains to be determined whether microvesicle shedding from trophoblast-like cells is regulated by these two signaling pathways.
During early pregnancy, the EVT cells invade the uterine spiral arteries and transform the spiral arteries into high-flow, low resistance blood vessels with a large diameter. In preeclampsia, this transformation of the spiral arteries is not complete and is restricted to the superficial endometrial layer or may even be absent. Oxidative stress is considered to be a key intermediate step responsible for the changes observed in preeclampsia. Recently, it has been proposed that increased placental oxidative stress is a result of intermittent placental perfusion within the intervillous space by pulses of fully oxygenated blood, also referred to as hypoxia/reoxygenation [59]. Several studies have examined the effects of hypoxia/reoxygenation on placental oxidative stress. For example, it was shown in vitro that ROS are actively generated in the villous endothelium, syncytiotrophoblast and stromal cells subjected to hypoxia/reoxygenation [179]. Other investigators also showed that invasive cytotrophoblasts generate ROS as evidence of oxidative stress in preeclamptic as compared to control normal pregnancies [64]. However, the effects of hypoxia/reoxygenation (an ischemia-reperfusion) leading to oxidative stress have been much better described in organ systems such as the heart and brain [180, 181]. Interestingly, elevated levels of circulating microvesicles are associated with diseases such as acute ischemic stroke [182], ischemic heart disease and cerebrovascular syndromes [132, 183].

We therefore wanted to assess the effect of hypoxia/reoxygenation on microvesicle release by first trimester trophoblast cells. Our results showed that HTR-8/SVneo cells exposed to hypoxia/reoxygenation shed increased amounts of microvesicles. BSG secretion by these cells was also elevated due to the overall increase of vesicles shedding. We believe that hypoxia/reoxygenation leads to an increased release of trophoblast cell-derived plasma membrane microvesicles as a stress response. To date there have been no published studies
examining the mechanisms for microvesicle shedding by EVT. However, it has been shown that syncytiotrophoblast microvesicles are shed into the maternal circulation during normal pregnancy [184] and that amounts are higher in women with preeclampsia [185].

During the first trimester of pregnancy trophoblast invasion is highly aggressive. However, it is still tightly controlled both in space and time [3, 13-15]. Trophoblast cells are constantly exposed to a uterine microenvironment rich in cytokines and growth factors. IL-1β and TGF-β1 are secreted by the trophoblast cells, decidual cells, or both [147-148, 153]. Karmakar et al. showed that IL-1β and TGF-β1 are critical regulators of trophoblast invasion. They reported that during the first trimester, trophoblast proteases including MMPs and uPA are up regulated by IL-1β, while TIMPs and PAIs are up regulated by TGF-β1 [148]. TGF-β1 also suppresses secretion of MMP-9 and uPA [75] and inhibits trophoblast invasion in the HTR-8/SVneo cell line [155]. BSG is a critical molecule involved in stimulation of MMP production [95, 101]. We showed in chapter 3 that first trimester trophoblast cells release microvesicles containing BSG protein. In this study we examined the effect of IL-1β and TGF-β1 on BSG release in microvesicles and overall levels of microvesicle shedding by trophoblast cells. We also examined effects of IL-1β and TGF-β1 on BSG gene expression at the transcriptional level. Our results show that TGF-β1 inhibits and IL-1β stimulates BSG release in microvesicles. However, these factors did not affect the overall level of microvesicles shed. The increase in BSG protein release in microvesicles upon treatment with IL-1β or decrease upon treatment with TGF-β1 was not a result of changes in BSG transcription. The decrease in microvesicle-associated BSG upon TGF-β1 treatment and increase upon IL-1β treatment were also not due to decreased/increased numbers of microvesicles shed but likely due to selective incorporation of BSG protein into microvesicles.
Our results show that IL-1β and TGF-β1 can regulate incorporation of BSG protein into microvesicles. IL-1β increased BSG release in microvesicles and it is likely that this may be a mechanism by which this cytokine stimulates the invasive potential of trophoblast cells during the first trimester of pregnancy. We hypothesize that these BSG-containing microvesicles may signal to surrounding trophoblast cells or other uterine cells to stimulate MMPs and promote the invasion processes. TGF-β1, on the other hand, suppressed BSG protein incorporation into microvesicles. We hypothesize that TGF-β1 can inhibit trophoblast cell invasive potential by inhibiting BSG release in microvesicles. This would result in a modified response of target cells in terms of MMP production and invasion.

In summary, BSG release in microvesicles by placental trophoblast cells is regulated through the PKC pathway. First trimester trophoblast cells release elevated numbers of microvesicles in response to hypoxia/reoxygenation as a result of a stress response. Moreover, treatment of trophoblast cells with the cytokine IL-1β or the growth factor TGF-β1 did not change the amount of microvesicles shed. However, IL-1β induced and TGF-β1 inhibited incorporation of BSG protein into microvesicles. This happens at a post-transcriptional level, and the mechanisms responsible merit further investigation.
FIGURES

Figure 14. BSG secretion via microvesicle shedding by trophoblast-like cells is stimulated by the PKC activator PMA and inhibited by the PKC inhibitor Bis. (A) Immunoblot showing that PMA (100 ng/ml, 200 ng/ml) stimulates an increase in BSG and MCT1 levels in the microvesicle fraction isolated from HTR-8/SVneo cell ultracentrifuged conditioned medium collected after 2 or 4 h incubation in a time- and dose-dependent manner. S, supernatant after ultracentrifugation; P, resuspended microvesicle pellet; C, human uterine epithelial cell line (HES) used as positive control. (B) Immunoblot of HTR-8/SVneo microvesicle fractions collected after 2 h treatment showing that induction of BSG and MCT1 release in microvesicle fractions by PMA (100 ng/ml) is inhibited by the PKC inhibitor Bis (4.5 ng/ml). Immunoblot shows resuspended microvesicle pellets isolated from ultracentrifuged conditioned medium after treatment with serum free medium (SF), 0.1% ethanol (EtOH), 100 ng/ml PMA, 4.5 ng/ml Bis, 100 ng/ml PMA and 4.5 ng/ml Bis combined for 2 h (Bis+PMA), or a 1 h 4.5 ng/ml Bis pretreatment followed by 2 h 100 ng/ml treatment with PMA (Bis/PMA).
Figure 15. BSG release via microvesicle shedding by trophoblast-like cells and overall amounts of microvesicles shed are increased in response to hypoxia/reoxygenation. (A) Immunoblot showing BSG levels in microvesicle fractions isolated from HTR-8/SVneo cell ultracentrifuged conditioned medium after exposure of trophoblast cells to 2%, 8% or 20% oxygen levels for 4 h 30 min, or hypoxia/reoxygenation conditions (4 h at 2% followed by 30 min at 8% oxygen). P, resuspended microvesicle pellet; S, supernatant after ultracentrifugation; C, human uterine epithelial cell line (HES) used as positive control. Histogram shows densitometric analysis of immunoblots. * indicates significance between treatment groups (n=3; *p<0.05); (B) BCA assay analysis of total protein content within microvesicle fractions isolated from HTR-8/SVneo cell ultracentrifuged conditioned medium showing significant increase in total protein amounts in microvesicles after exposure to hypoxia/reoxygenation. * indicates significance between treatment groups (n=3; *p<0.05).
Figure 16. Incorporation of BSG protein into microvesicles is decreased while overall amounts of microvesicles shed and BSG mRNA levels did not change after treatment with 0, 1, 2.5 and 5 ng/ml TGF-β1 for 8 hours. (A) Immunoblots showing decrease in BSG and MCT1 levels in microvesicle fractions isolated from ultracentrifuged conditioned medium after TGF-β1 treatments for 8 hours. 0h showing no release of BSG in microvesicles occurred at 0 hours of treatment. C, human uterine epithelial cell line (HES) used as positive control. Histogram shows densitometric analysis of immunoblots for BSG. The * indicates a significance between untreated samples at 0 hours compared to 8 hours. Bars with different letters are significantly different from each other within the 8 hours time period (n=3; p<0.05). (B) BCA assay analysis of total protein content in microvesicle fractions indicating no change in overall amounts of microvesicles shed. The * indicates a significance between untreated samples at 0 hours compared to 8 hours (n=3; p<0.05) (C) No change in BSG mRNA occurred in response to treatments (n=3).
Figure 17. Incorporation of BSG protein into microvesicles is decreased while overall amounts of microvesicles shed and BSG mRNA levels did not change after treatment with 0, 1, 2.5 and 5 ng/ml TGF-β1 for 24 hours. (A) Immunoblots showing decrease in BSG and MCT1 levels in microvesicle fractions isolated from ultracentrifuged conditioned medium after TGF-β1 treatments for 24 hours. 0h showing no release of BSG in microvesicles occurred at 0 hours of treatment. C, human uterine epithelial cell line (HES) used as positive control. Histogram shows densitometric analysis of immunoblots for BSG. The * indicates a significance between untreated samples at 0 hours compared to 24 hours. Bars with different letters are significantly different from each other within the 24 hours time period (n=3; p<0.05). (B) BCA assay analysis of total protein content in microvesicle fractions indicating no change in overall amounts of microvesicles shed. The * indicates a significance between untreated samples at 0 hours compared to 24 hours. (C) No change in BSG mRNA occurred in response to treatments (n=3).
Figure 18. Incorporation of BSG into microvesicles is increased while overall amounts of microvesicles shed and BSG mRNA levels did not change after treatment with 0, 1, 2.5 and 5 ng/ml IL-1β for 8 hours. (A) Immunoblots showing increase in BSG and MCT1 levels in microvesicle fractions isolated from ultracentrifuged conditioned medium after IL-1β treatments for 8 hours. 0h showing no release of BSG in microvesicles occurred at 0 hours of treatment. C, human uterine epithelial cell line (HES) used as positive control. Histogram shows densitometric analysis of immunoblots for BSG. The * indicates a significance between untreated samples at 0 hours compared to 8 hours. Bars with different letters are significantly different from each other within the 8 hours time period (n=3; p<0.05). (B) BCA assay analysis of total protein content in microvesicle fractions indicating no change in overall amounts of microvesicles shed. The * indicates a significance between untreated samples at 0 hours compared to 8 hours (n=3; p<0.05) (C) No change in BSG mRNA occurred in response to treatments (n=3).
Figure 19. Incorporation of BSG into microvesicles, overall amounts of microvesicles shed and BSG mRNA levels did not change after treatment with 0, 1, 2.5 and 5 ng/ml IL-1β for 24 hours. (A) Immunoblots showing no change in BSG and MCT1 levels in microvesicle fractions isolated from ultracentrifuged conditioned medium after IL-1β treatments for 24 hours. 0h showing no release of BSG in microvesicles occurred at 0 hours of treatment. C, human uterine epithelial cell line (HES) used as positive control. Histogram shows densitometric analysis of immunoblots for BSG. The * indicates a significance between untreated samples at 0 hours compared to 24 hours (n=3; p<0.05). (B) BCA assay analysis of total protein content in microvesicle fractions indicating no change in overall amounts of microvesicles shed. The * indicates a significance between untreated samples at 0 hours compared to 24 hours (n=3; p<0.05). (C) No change in BSG mRNA occurred in response to treatments (n=3).
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

The placenta is a complex organ that has manifold functions necessary for establishment and support of successful pregnancy. Controlled trophoblast invasion into maternal uterine tissues and remodeling of the uterine spiral arteries are key events in early pregnancy. These key events are influenced by many factors such as MMPs, cytokines, growth factors and oxygen tension. Another regulatory factor is the protein BSG that induces MMP secretion and is critical for embryonic implantation, angiogenesis and parturition.

Several significant studies have shown that BSG is essential for multiple reproductive processes including those listed above. However, the specific functions of BSG in the early placenta have not been studied. As a result, our goals for this project were twofold. First, we wanted to determine whether BSG is present in human placenta throughout the first trimester of pregnancy, whether BSG is secreted by trophoblast cells and what the mode of release of this transmembrane protein is. Additionally, we wanted to determine whether BSG can act on ECs to regulate MMP production. Second, we wanted to assess how BSG secretion in microvesicles by trophoblast cells might be regulated. We wanted to determine what signaling mechanisms might be responsible for microvesicle shedding by first trimester trophoblast cells and what the effects of oxidative stress, cytokines and growth factors on microvesicle shedding and BSG release are.

Our first hypothesis was that BSG is present in human placenta throughout the first trimester of pregnancy and that during the first trimester of pregnancy, when EVT invasion occurs, trophoblast cells express BSG and release BSG into surrounding medium through plasma membrane microvesicle shedding. Furthermore, BSG acts on ECs and promotes production of
specific MMPs by ECs. Our results showed that BSG protein is present in the human placenta throughout the first trimester of pregnancy. *BSG* transcript variants 2, 3, and 4 are expressed in first trimester EVT cells and these cells secrete BSG protein into the medium. In addition, we determined that BSG was released from the surface of trophoblast cells via plasma membrane microvesicle shedding. Finally, our data show that rBSG protein is able to stimulate release of MMP-1 and MMP-3 by ECs into culture medium. Our results suggest that BSG, shed in microvesicles, may be important for paracrine interactions between trophoblast cells and other uterine cells including ECs, during the process of early placental development.

Our data support the role of BSG as an inducer of MMP production and add to the increasingly growing body of literature regarding microvesicle shedding which occurs in a broad spectrum of cell types. These trophoblast cell-derived microvesicles may deliver signals from trophoblast cells to other uterine cells and serve as mediators of cell-to-cell communication. Earlier studies showed that BSG is shed from the surface of tumor cells in microvesicles [23]. Moreover, microvesicles shed by different carcinoma cell lines express different levels of BSG and stimulate expression of MMPs by ECs in a BSG-dependent manner [94]. Consistent with these studies, our data also show that BSG is secreted from the surface of normal trophoblast cells via microvesicles and that rBSG is able to stimulate MMP production by ECs.

In order to answer the question of whether BSG from microvesicles can directly stimulate MMPs, one of the future directions for this project would involve carrying out two-chamber co-culture experiments. Trophoblast cells would be cultured in the upper chamber and ECs in the bottom chamber. A permeable membrane would separate the two chambers and will allow microvesicles to move from the top chamber to the bottom. This would allow cells to receive soluble factors in a cell-cell contact independent manner. This experiment would also involve
treating cells with a stimulator of microvesicle release to determine whether increased microvesicle shedding by trophoblast cells would result in further increases in MMP production by ECs. An alternative approach would be to use siRNAs to knock down BSG expression in trophoblast cells and assess the effect of BSG-depleted microvesicles on MMP secretion by ECs. BSG-depleted microvesicles should not have the same ability to stimulate MMP secretion. Another approach would be to immunoprecipitate BSG from microvesicles and treat ECs with this protein. However, this approach would involve collection of large quantities of microvesicle which may be an obstacle. In addition, no studies have been performed to assess the effects of BSG protein or BSG-containing microvesicles on uPA production by ECs. This could be another future direction for our studies.

We also wished to determine which signaling pathway is responsible for BSG release in microvesicles by first trimester trophoblast cells. Additional aims were to investigate the effect of hypoxia/reoxygenation and factors present in the uterine environment at the time of trophoblast invasion on BSG release in microvesicles.

Our data show that trophoblast cells do release some microvesicles in their resting state. However, treatment of trophoblast cells with PMA, a well-defined analogue of DAG known to stimulate PKC, increased BSG release in microvesicles substantially in time- and dose-dependent manner. Moreover, treatment of cells with the PKC inhibitor Bis, inhibited the ability of PMA to stimulate BSG release. These results confirm that BSG-containing microvesicle release is regulated at least partially through the PKC signaling mechanism. Studies from other groups using various cell types have shown that microvesicle shedding increases dramatically upon stimulation with Ca\(^{2+}\). One of our future directions could be to assess whether microvesicle shedding by trophoblast cells can be induced after exposure to Ca\(^{2+}\) ionophores or stimuli that
evoke the release of Ca\(^{2+}\) from the ER, and whether it is possible to inhibit microvesicle shedding upon treatment with Ca\(^{2+}\) chelators. Moreover, PKC has been demonstrated to affect central signaling pathways such as MAPK/ERK and the PI3K pathways. Another future direction for our studies would be determining what downstream signaling molecules are involved in regulation of microvesicle shedding. This would be possible to accomplish by using inhibitors of signaling molecules of ERK or MEK 1/2 and see whether these would inhibit the ability of PMA to stimulate BSG-containing microvesicle release. Since chemical inhibitors are not always very specific, another experimental approach would be to use RNAi. Most of the current research studies examining the function of microvesicles are conducted on cells *in vitro* and utilize ultracentrifugation techniques to isolate the microvesicle fractions. These are likely to sediment a mixture of both microvesicles and exosomes, and it may be important to determine which stimuli act specifically on microvesicle release without affecting secretion of other types of vesicles.

**Oxygen is a powerful factor regulating placental trophoblast cell behavior.** Oxidative stress of placental tissues plays a key role in pathological diseases of pregnancy such as preeclampsia. Oxidative stress has been proposed to result from intermittent perfusion of the placenta by fluctuating oxygen levels (hypoxia/reoxygenation injury). Therefore, we were interested in determining the effects of hypoxia/reoxygenation on microvesicle release by first trimester extravillous trophoblast cells. Our results showed that cells exposed to hypoxia/reoxygenation shed increased amounts of microvesicles as a result of a stress response. BSG secretion within the microvesicles was also elevated due to an overall increase of microvesicles shed. These data raise various questions such as what the effects of hypoxia-
reoxygenation and oxidative stress on BSG expression in trophoblast cells are and whether BSG expression is altered in placentas of patients with preeclampsia.

Lastly, our data showed that the inflammatory cytokine IL-1β and the growth factor TGF-β1 appear to regulate incorporation of BSG protein into microvesicles. IL-1β and TGF-β1 have been recognized as important regulators of invasion of the EVT into uterine endometrium and spiral arteries. Our results showed that treatments of trophoblast cells with IL-1β or TGF-β1 did not appear to change the overall amount of microvesicles shed. However, IL-1β induced and TGF-β1 inhibited incorporation of BSG protein into microvesicles. We did not observe any changes in BSG mRNA levels upon treatments. This suggests that IL-1β and TGF-β1 control BSG protein incorporation into microvesicles at a post-transcriptional level. However, it still remains to be determined how BSG incorporation into microvesicles occurs. We believe it happens through selective insertion of BSG protein already present in the cytoplasm into trophoblast cell plasma membranes. Another further direction would be to perform immunofluorescence for BSG in trophoblast cells to determine whether treatments cause alterations in BSG localization within the cytoplasm or cell membrane. For example, upon IL-1β treatment we would expect to see a shift in localization of BSG from cellular stores to trophoblast plasma membrane and we would expect intensity for BSG protein in the membrane to be higher upon treatment as compared to untreated control.

The data presented in this thesis suggest a number of additional further studies for this project. It would be important to assess the effects of BSG and BSG-containing microvesicles derived from trophoblast cells on trophoblast cell proliferation, MMPs, uPA, cytokine production and trophoblast cell migration. Cell count experiments can be performed to assess the effect on proliferation; immunoblotting, qRT-PCR and gelatin zymography can be performed to determine
effects on MMPs and uPA production; enzyme-linked immunosorbent assay (ELISA) can be
done to measure the amounts of cytokines released into surrounding medium after treatment and
a trans-filter invasion assay could be used to assess effect of treatments on trophoblast migration.
It would further be important to carry out time course microarray analysis to identify the genes in
trophoblast and ECs that show altered expression in response to treatments with BSG or BSG-
containing microvesicles.

There has been a rapid increase in the number of scientific reports pointing out the
importance of microvesicles as mediators of communication between cells. Microvesicles carry
specific proteins and other factors to target cells of interest. These target cells may have specific
receptors for a particular protein that may elicit specific responses in these cells depending on
their physiologic state. In our studies we demonstrated that BSG protein is highly expressed in
trophoblast cells and that it is released through microvesicle shedding. BSG-containing
microvesicles may act as mediators of trophoblast/EC or trophoblast/other uterine cell cross-talk.
The various glycosylated forms of BSG present in trophoblast microvesicles may be involved in
trophoblast invasion and spiral artery remodeling. Moreover, incorporation of BSG protein into
microvesicles is controlled by cytokines and growth factors present in the microenvironment
surrounding trophoblast cells and may contribute to the regulation of trophoblast invasion. BSG
protein, identified in microvesicles, is of special relevance to the process of trophoblast invasion
into the decidua and into spiral arteries as it promotes MMP activation. Many interesting
questions remain to be answered in future studies.
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