UTERINE *Gpr83* EXPRESSION AND GPR83 SIGNALING DURING EARLY PREGNANCY IN THE MOUSE

By

NATALIYA PAROBCHAK

A thesis submitted to

The School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Toxicology

Written under the direction of

Dr. Andy Babwah

And approved by

_____

_____

_____

New Brunswick, New Jersey

OCTOBER, 2020

ABSTRACT OF THE THESIS

Uterine *GPR83* expression and GPR83 signaling during early pregnancy in the mouse

By NATALIYA PAROBCHAK

Thesis Director: Dr. Andy Babwah

The goal of this study was to characterize the expression and signaling of uterine GPR83 *in vivo* in the nonpregnant and pregnant mouse and *in vitro* in human endometrial and non-endometrial cells. Expression of uterine *Gpr83* was determined by quantitative polymerase chain reaction throughout the estrous cycle and during early pregnancy in ovarian-stimulated and non−ovarian-stimulated mice and pregnant and pseudopregnant mice. Expression was also determined in ovariectomized mice after the administration of oil, E2, P4, or E2 + P4 and in stromal cells following 6 days of *in vitro* decidualization. GPR83 signaling was studied in human endometrial and embryonic kidney cell lines. Cells were treated with PEN, a GPR83 ligand, and PEN-induced extracellular signal-regulated kinase **(**ERK) phosphorylation was assayed under conditions that blocked $G\alpha_{q/11}$ and/or β-arrestin signaling. Results show that uterine *Gpr83* is expressed throughout the estrous cycle and during early pregnancy; expression increases dramatically at the time of uterine receptivity, embryo implantation, and stromal cell decidualization. In the ovariectomized mouse, hormone add-back reveals that *Gpr83* expression is highly responsive to the

combined treatment of E2 and P4, and studies in the ovarian-stimulated mouse show that expression is also very sensitive to changes in E2 and P4 and is therefore tightly regulated by E2 and P4. At the implantation site, expression is elevated up to D6 of pregnancy and then declines rapidly on D7 and D8, suggesting that if there is any involvement in decidualization, it is likely associated with primary but not secondary stromal cell decidualization. This premise was supported by the observation that stromal cell decidualization *in vitro* progresses with a decline in *Gpr83* expression. In ERα/PR-expressing endometrial Ishikawa cells, GPR83 mediates PEN signals in a $G\alpha_{q/11}$-dependent manner, and studies conducted in HEK 293 cells lacking β-arrestin revealed that GPR83 also signals via a β-arrestin−dependent manner. When signaling by either one or both pathways is downregulated, cells exhibit a major reduction in responsiveness to PEN treatment, demonstrating that signaling by both pathways is significant. Based on our findings, we hypothesize that PEN/GPR83 signaling regulates uterine receptivity, embryo implantation, and primary stromal cell decidualization by coupling to $G\alpha_{q/11}$- and β-arrestin−dependent pathways.

ACKNOWLEDGEMENTS

My deepest gratitude goes first and foremost to Dr. Andy Babwah, who expertly guided me through my graduate education. I am thankful for his continuous support of my master's study and research; for his patience, enthusiasm, motivation and immense knowledge. Without his guidance and persistent help, this research project would not have been possible. I could not have imagined having a better advisor and mentor for my master's study.

Additionally, I would like to thank my committee members, Dr. Lauren Aleksunes and Dr. Kenneth Reuhl. I am greatly indebted to your time, support and valuable guidance that allowed me to fulfill one of my greatest achievements. I have learned a lot throughout these years given the opportunity to be part of such a respected program. I am thankful to Dr. Aleksunes for accepting me into the program and the constant guidance throughout the years. To the most intelligent man I had the pleasure of meeting, Dr. Reuhl, I am forever grateful for the knowledge that he has bestowed upon me, for his wisdom and most importantly for his friendship; he is the reason I am here today.

Next, I would like to thank my laboratory colleagues and fellow students that were involved in helping me with my thesis project. I am extremely grateful with the assistance and suggestions throughout my project.

Lastly, I must express my very profound gratitude to my parents and my sister for providing me with unfailing support and continuous encouragement throughout my years of study and through the final steps. This accomplishment would not have been possible

without them. Thank you to Victoria for being my everything, this accomplishment I dedicate to you. Finally, a special thank you to my mom, a strong and gentle soul who taught me to believe in hard work and that so much could be done with very little.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

hCG	human chorionic gonadotropin
CDC	centers for disease control and prevention
ESC	endometrial stromal cells
E2	17 β-Estradiol
P4	progesterone
GPCR	G protein-coupled receptor
MSX	muscle segment homeobox
ER	estrogen receptor
PR	progesterone receptor
LH	luteal hormone
CL	corpora luteum
D	day
FGF	fibroblast growth factor
IGF	insulin-like growth factors
HAND2	heart- and neural crest derivatives-expressed protein 2
LIF	leukemia inhibitory factor
LIF-R	leukemia inhibitory factor receptor
IL-6	interleukin 6
JAK-STAT3	Janus tyrosine kinase-Signal transducer and activator of transcription 3
PDZ	primary decidual zone
SDZ	secondary decidual zone
uNK	uterine natural killer cells
BMP2	bone morphogenetic protein 2
HOXA10	homeobox gene
7TMR	seven-pass-transmembrane domain receptors
ECL	extracellular
ICL	intracellular
GDP	guanosine diphosphate
GTP	guanosine triphosphate
MAPK	mitogen-activated protein kinase
ERK	extracellular-signal-regulated kinase
AC	adenylyl cyclase
AMP	adenosine monophosphate
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
PKA	protein kinase A
PKC	protein kinase C
DAG	diacylglycerol
PLC	phospholipase C
PIP_2	phosphatidylinositol bisphosphate
IP_3	inositol 1,4,5-triphosphate
ER	endoplasmic reticulum

CREB	cAMP-response element binding protein
LPAR3	lysophosphatidic acid 3
LGR4	leucine-rich repeat-containing G protein-coupled receptor 4
KISS1R	kisspeptin receptor
HPG	hypothalamic pituitary gonadal axis
PG	prostaglandin or prostanoid receptor
COX	cyclooxygenase
CT	calcitonin receptor
OXTR	oxytocin receptor
IVF	*in vitro* fertilization
GPR83	G protein-coupled receptor 83
NC-IUPHAR	International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification
IS	implantation site
IIS	interimplantation site
WT	wild type
IU	international unit
PMSG	pregnant mare serum gonadotropin
IP	intraperitoneal
HB-EGF	Heparin-binding epidermal growth factor-like growth factor

INTRODUCTION

Human Pregnancy

Pregnancy, which is also known as gestation, is the term used to describe the events in which a fetus develops inside a woman's uterus following sexual intercourse or assisted reproduction. A healthy pregnancy lasts about 40 weeks based on gestational age (Abman, 2010, p. 46-47). Gestational age is defined to as the age of an embryo or fetus from the first day of the woman's last menstrual period (Abman, 2011). The term embryo refers to the developing offspring during the first 10 weeks of gestation while the term fetus applies to the rest of gestation. Clinically, pregnancy is confirmed following the detection of the β subunit of the human chorionic gonadotropin (hCG) hormone in the blood or urine (Cole, 2010). hCG is produced by the syncytiotrophoblast of the implanted embryo and typically urine tests will show a positive result around the fourth week of gestation or just before when the next menstrual period is expected (Cole, 2010). Early pregnancy can also be detected by obstetric ultrasonography. This technique detects the early gestational sac and/or yolk sac which can be visualized by the fifth week of gestation. A viable pregnancy is confirmed via the ultrasound detection of the heartbeat which can occur by the sixth week of gestation.

My research is focused on better understanding molecular uterine events that occur early in pregnancy and which are critical for the establishment of a healthy and successful pregnancy. Specifically, these molecular events are required for the acquisition of uterine receptivity, embryo implantation and decidualization. In women, the acquisition of uterine receptivity, embryo implantation and the early stages of

decidualization are accomplished by the time the woman recognizes she is pregnant based on a positive hCG test. Disruption of any of these three early pregnancy events leads to infertility and early pregnancy loss.

Infertility and Early Pregnancy Loss

Infertility is characterized as the inability of a woman to become pregnant (as determined by the inability to detect blood or urine β-hCG or a gestational sac and/or yolk sac) after one year of unprotected sex (Cole, 2010). Infertility is a poorly understood condition and according to the Centers for Disease Control and Prevention (CDC), in the United States, about 10% of women (6.1 million) of reproductive age (15-44) have difficulty getting pregnant or staying pregnant. Infertility can arise due to unhealthy sperm, eggs and/or embryos (CDC, 2019). An unhealthy embryo accounts for one-third of failed implantation events, while an unreceptive uterus accounts for the remaining two-thirds failed embryo implantation events (Simon et al., 1998; Ledee-Bataille et al., 2002). An unreceptive uterus can arise because of anatomical and/or molecular defects. Anatomical defects include congenital (septate uterus) and acquired (myomas and synechiae) diseases which are easily detected and often effectively treated surgically (Okada et al., 2018). Uterine molecular defects are wide-ranging and are more difficult to identify and treat. Even with successful implantation, early pregnancy loss can still occur, which can be due to an unhealthy embryo and/or defect in decidualization of endometrial stromal cells (ESCs) (Okada et al., 2018). Decidualized ESCs provide an early source of nourishment for the developing embryo, regulate the depth of penetration of the embryo into the endometrium and contribute directly to placenta development. Abnormalities in

decidualization have been linked to recurrent miscarriages, utero-placental disorders and early pregnancy loss. It is therefore very important to understand and keep all these factors in mind when dealing with infertility issues.

The Uterus

In humans and most other mammals, the uterus is a major female hormone-responsive secondary sex organ of the reproductive system. In a woman, the uterus is divided anatomically into four regions known as the fundus, which is the uppermost rounded part of the uterus and is connected to the fallopian tubes, the corpus (the body), the cervix, which is the lower portion of the uterus and the cervical canal (D'Amico and Barbarito, 2015, p.645). Histologically, the uterus is made up of three layers and together form what is known as the uterine wall. The innermost to outermost layers of the uterus are as follows: the endometrium, myometrium and perimetrium (Tortora and Derrickson, 2011, p.1105). The endometrium consists of a single layer of columnar epithelial cells and the underlying stroma. The stroma is made up of connective tissue that varies in thickness due to hormonal influences. In the uterus, simple tubular glands, arising from invaginations of the uterine epithelium, span from the endometrial surface through to the base of the stoma. In a woman of reproductive age, functionally, the endometrium is comprised of two layers, the basal layer and a functional layer (Franasiak and Scott, 2015). The functional layer is the layer that builds up from cellular proliferation in response to increasing estrogen levels during the proliferative phase (first part) of the menstrual cycle. At the end of the menstrual cycle, in response to declining progesterone levels, the functional layer is shed. In contrast, the basal layer, which lies next to the

myometrium and below the functional layer, does not shed during the menstrual cycle. The myometrium is the middle layer of the uterine wall and is made up of smooth muscle cells. The myometrium stretches during pregnancy to allow the uterus to expand and one of its important functions is to induce uterine contractions (Aguilar et al., 2010). Finally, the perimetrium is the outer serous layer of the uterus. The main function of the perimetrium is to protect the uterus from friction from muscle movements by forming a smooth layer of simple squamous epithelium along its surface and by secreting a lubricating fluid (Aguilar et al., 2010).

In mice, unlike humans, the uterine fundus splits into the right and the left uterine horn that converge at the cervix. Thus, the mouse uterus is referred to as bicornuate whereas in human, the uterus is pyriform (Ratajczak, et al., 2010). The uterine horn is referred to having a mesometrial side (or pole) and an anti-mesometrial side. The mesometrial side is the side where the horn is attached to the mysentery, a membrane that secures the horn to the abdominal wall and through which arteries and veins connect the uterus to the rest of the peripheral circulation (Ratajczak, et al., 2010). Histologically, the mouse uterus is very similar to the human uterus, which the exception that mice do not undergo menstruation (Supp. Fig. 6). It too is made up of the endometrium which consists of the epithelium and the stroma layers and these are surrounded by the myometrium (Das, 2010). During early pregnancy in the mouse, stromal cell decidualization is first initiated at the antimesometrial pole where the blastocyst implants and then spreads to the mesometrial pole where the placenta develops (Das, 2010). Because of their physiological similarities to humans, the ease of breeding and maintaining them in research laboratories, the pregnant mouse has long served as a major animal model of human reproduction.

Estradiol and Progesterone Signaling The acquisition of uterine receptivity, embryo implantation and stromal cell decidualization are complex early pregnancy events regulated by numerous factors. Two major factors are ovarian estradiol (E2)- and progesterone (P4) which signal via their uterine receptors (Pawar et al., 2014). The correct balance of hormones is essential for a successful pregnancy. Together, E2 and P4 signaling in the uterus regulate the expression of a multitude of uterine factors that control early pregnancy events. These factors include transcription factors and a wide array of secreted signaling molecules (ligands) and their cognate receptors. Among these receptors are the G protein-coupled receptors (GPCRs), the largest protein superfamily in mammalian genomes. While not very common, early pregnancy is also regulated by molecules whose expression is independent of E2 and P4; this includes the well-studied muscle segment homeobox (MSX) 1 and MSX2 (Pawar et al., 2014). These molecules regulate epithelial proliferation and differentiation by controlling epithelial-stromal cross talk (Pawar et al., 2014). Studies have shown that conditional ablation of *Msx1* and *Msx2* in the uterus resulted in female infertility because of failure in implantation (Pawar et al., 2014). Although there are many aspects of infertility and the underlying issues associated with it, my research is specifically focused on better understanding how GPCRs regulate early pregnancy (to be discussed later). Very little is known about GPCRs as regulators of early pregnancy and their regulation by the steroid hormones, E2 and P4. Thus, an important goal of my studies is to identify novel uterine GPCR regulators of early pregnancy and to determine how their expression and signaling are regulated.

E2 and P4 signal via both membrane and intracellular receptors but studies using knockout mouse models have shown that it is signaling via specific intracellular receptors that regulates the acquisition of uterine receptivity, embryo implantation and decidualization (Bagchi et al., 2001). The uterus expresses two intracellular estrogen receptors, ERα and ERβ, and two intracellular progesterone receptors, PR-A and PR-B (Cha et al., 2012). Studies have shown that mice lacking ERα, but not ERβ, are infertile concluding that ERα is essential for uterine receptivity and implantation (Lubahn et al., 1993). Another study determined that while loss of PR-A and PR-B resulted in infertility (Lydon et al., 1995), loss of PR-B only was not sufficient to trigger infertility revealing that it is PR-A which is important for uterine receptivity and implantation (Mulac-Jericevic et al., 2000). From here on, PR-A is simply referred to as PR.

In preparation for embryo implantation, when P4 levels are still low, the uterus is under the control of E2 and E2 acts to prepare the luminal epithelium for embryo implantation. Since the blastocyst attaches to the epithelium, the major role of E2 is to create an intact epithelial lining. E2 signals via stromal and epithelial ERα to trigger the proliferation of the luminal epithelial cells which are tightly packed and connected by tight junctions (Bhurke et al., 2016). At the same time E2 induces the expression of PR allowing P4, which is increasing in levels, to start signaling. The major action of P4 signaling is to inhibit the E2-dependent proliferation of the uterine epithelium while undergoing differentiation (Bhurke et al., 2016). Upon differentiation, the luminal epithelium undergoes an epithelial–mesenchymal transition, loses its tight junctions between the cells and this facilitates embryo attachment.

Uterine Receptivity Uterine or endometrial receptivity refers to a brief ovarian steroid-dependent period when the endometrium attains the functional capacity to permit blastocyst implantation (Zhang et al., 2013). The acquisition of uterine receptivity is E2- and P4-dependent and in both humans and rodents, depending on E2 and P4 levels, the uterus cycles between a pre-receptive, receptive and refractory phase.

In women, ovarian E2 levels rise during the first 14 days of the menstrual cycle and upon peaking mid-cycle it triggers the (luteal hormone) LH surge and ovulation (Georgadaki et al., 2016). Upon ovulation, the empty ovarian follicle is transformed into the corpora luteum (CL) which then produces P4. In women, the pre-receptive uterine phase corresponds to the first five days after ovulation during the rise in P4 levels. Uterine receptivity is achieved around day (D) 20 of the menstrual cycle and remains maximally receptive to embryo implantation for about five days; this period corresponds with peak P4 levels and is referred to as the window of implantation (Georgadaki et al., 2016). If embryo implantation does not occur (for example, due to the absence of an embryo from the egg not being fertilized), the CL regresses, P4 levels decline and the uterus transitions into a non-receptive or refractory phase and this leads into menstruation (Dey et al., 2004). In the mouse, the preovulatory E2 is high during the first two days after ovulation and during this period P4 levels rise rapidly. On the fourth day after ovulation, the uterus becomes receptive to embryo implantation for a brief period and on the fifth day it enters the refractory phase.

Although female sex steroid hormones are the primary regulators of the cellular and molecular mediators of implantation, numerous other molecules play an important role.

Most of these molecules are directly or indirectly regulated by E2 and/or P4. Among the major regulators of uterine receptivity are the E2-induced fibroblast and insulin-like growth factors, FGF1, FGF2, FGF9, FGF18 and IGF-1, the P4-induced heart- and neural crest derivatives-expressed protein 2 (HAND2) (Pawar et al., 2014). It has been shown that the fibroblast and insulin-like growth factors are produced by stromal cells and act in a paracrine manner to mediate E2-driven uterine epithelial growth via FGF and IGF-I receptors located on the luminal and glandular epithelia (Pawar et al., 2014). It was also shown that in the mouse endometrium, these growth factors are negatively regulated by P4-induced stromal HAND2 (Pawar et al., 2014). This was determined in mice bearing a conditional deletion of *Hand2* in the uterus. These knockout mice showed persistent FGF expression and luminal epithelial cell proliferation on D4 of pregnancy coupled to embryo implantation failure. These findings revealed that in the pregnant uterus HAND2 is a major mediator of the antiproliferative effects of P4 in the latter part of the pre-receptive phase and is required for the uterus to achieve a receptive state.

Another major regulator of uterine receptivity is the E2-induced leukemia inhibitory factor (LIF), an IL-6 family member, which signals via the LIF receptor (LIF-R β) (Kimber, 2005). It is expressed in various embryonic and adult tissues with particularly high levels in the uterus. In LIF knockout mice, embryos fail to attach to the luminal epithelium and studies indicate that LIF mediates the final step in the acquisition of uterine receptivity (Stewart et al., 1992, Cheng et al., 2002). Just before implantation begins on the afternoon of D4, in response to a surge in E2 secretion on the morning of D4, LIF is secreted by the endometrial glands. LIF then acts on the LIF receptors in the luminal epithelium and activates the Janus tyrosine kinase-Signal transducer and activator of

transcription 3 (JAK-STAT3) pathway (Yoo et al., 2019). This pathway suppresses the expression of tight and adherens junction complexes in the luminal epithelium and disrupts cell-cell linkages triggering a loss of epithelial polarization. These are all essential steps in luminal epithelial cells adopting a final phenotype to accommodate embryo implantation, establishing LIF as a critical regulator of uterine receptivity. LIF most likely plays a role in endometrial function in humans as well. In humans, LIF mRNA and protein are highly expressed in the endometrial glands during the luteal phase of the menstrual cycle, the time when implantation would occur (Yoo et al., 2019). Defects in LIF expression in some studies have been associated with recurrent miscarriages and unexplained infertility thus possibly implicating LIF as one of the important factors involved in achieving a successful pregnancy in humans.

Embryo Implantation Embryo implantation is a critical aspect of viviparous birth and it is the process where the blastocyst attaches to the uterine epithelium and invades the underlying stroma cells to an extent that is species specific (Lee and DeMayo, 2004). Successful implantation requires a receptive uterus, a healthy embryo and synchronized communication between the embryonic and maternal tissue (Simon et al., 2000). Implantation is divided into three stages: apposition, adhesion and invasion (Enders, 1967).

In mice, upon blastocyst formation in the luminal horn, the zona pellucida yields to physical stress that is coupled to localized lysis and it is through the ruptured zona pellucida the blastocyst hatches allowing the embryonic trophectoderm to make physical contact with the luminal epithelium (Ruane et al, 2017). Within the luminal horn, the blastocyst

undergoes apposition. During apposition, the blastocyst adopts a specific orientation relative to the luminal epithelium. In humans following successful apposition, the inner cell mass of the blastocyst faces the epithelial cells. In mice, the blastocyst localizes to special evaginations of the luminal epithelium called implantation chambers (Ruane et al, 2017). Implantation chambers form along the mesometrial pole of the luminal epithelium and embryos undergo apposition with their inner cell mass directed towards the mesometrial pole (Ruane et al, 2017). In the process of apposition, the blastocyst interacts with the epithelium using adhesion molecules, including cadherins, integrins, selectins and immunoglobulins. These molecules are expressed on the surface of the trophectoderm and interact with ligands expressed on the epithelial cells.

It is reported that the expression of integrins, which are transmembrane glycoproteins, are up-regulated during the window of implantation, implicating them as important markers of implantation. E-cadherins present in both the trophoblast and endometrial epithelium may also play a crucial role in the initiation of attachment and implantation (Rowlands et al., 2000). In addition, selectins are a group of carbohydrate-binding proteins. Specifically, L-selectin expressed by trophoblast cells was shown to be important during implantation process. Blocking L-selectin using specific antibodies leads to impaired adhesion of the trophoblasts to the endometrial epithelium, further implicating their importance during embryo implantation (Genbacev et al., 2003).

This interaction between the adhesion molecules and their ligands ushers in the adhesion stage of implantation, during which the polarized interaction between the blastocyst and endometrium is established and becomes stronger, securing the embryo to

the uterine lining. Once adhesion to the epithelium is initiated, the trophectoderm differentiates into the cellular cytotrophoblast which then gives rise to the overlying and invasive syncytiotrophoblast (Cohen et al., 2006). In the mouse, luminal epithelial cells interacting with the embryo within the implantation chamber undergo entosis, allowing the syncytiotrophoblast to interact with the stromal cells. Adhesion molecules expressed on the surface of the invasive syncytiotrophoblast interact with ligands expressed by the stromal extracellular matrix (Cohen et al., 2006). Once this is achieved, the invasion stage of implantation begins. Syncytiotrophoblast invades the stroma thus completing the implantation process. In mice this occurs on the fifth day after ovulation and is a critical step that precedes stromal cell decidualization and placentation.

Decidualization During the process of murine embryo implantation, stromal cells undergo E2-dependent proliferation and following implantation, in the mouse, stromal cells surrounding the implanting embryo undergo stromal–epithelial transition in a process referred to as decidualization. In women, localized decidualization begins in the uterine secretory phase even before an implantation has occurred. However, upon implantation, the decidualization reaction spreads beyond the implantation site to adjacent stromal regions. Decidualization involves biochemical, vascular and morphological changes that are driven by E2 and P4 (Ramathal and Bagchi, 2010). These changes lead to the formation of a differentiated maternal tissue called the decidua which supports embryonic growth and the maintenance of early pregnancy (Ramathal and Bagchi, 2010).

In the mouse, stromal cell decidualization begins on the afternoon of D5 in cells surrounding the implantation chamber in the antimesometrial region of the uterus; this

forms the primary decidual zone (PDZ), a transient tissue that is well developed by D6 but has undergone demise by D8 (Parobchak et al., 2020). The PDZ is avascular and is enriched in intercellular tight junctions, gap junctions, and adherens junctions. It creates a permeability barrier around the implantation chamber, isolating the embryo from immune cells and harmful molecules present in the maternal circulation (Parobchak et al., 2020). Decidualization progresses from around the PDZ towards the myometrium in the antimesometrial and mesometrial regions, where it gives rise to the secondary decidual zone (SDZ). In the antimesometrial region, the SDZ is comprised of the antimesometrial decidua while in the mesometrial region it forms the mesometrial decidua that eventually gives rise to the decidual basalis during placentation. Development of the secondary decidua peaks on D8 with its decline corresponding to the development of the placenta (Parobchak et al., 2020).

More specifically, the process of decidualization is characterized by morphological changes in the endometrial stromal cells (ESCs). ESCs are elongated, fibroblast-like mesenchymal cells that differentiate into rounded, epithelioid-like cells (Coulam, 2016). Decidualization is also characterized by an influx of resident and peripheral uterine natural killer cells (uNK) and vascular remodeling in order to supply blood to the developing embryo (Schatz et al., 2016). Decidualized ESCs are the main cell types in the decidua and they provide nutrients for the implanting blastocyst. In women, decidualization begins around six days after ovulation in the mid-secretory phase of the menstrual cycle, or approximately at the onset of the window of implantation (Gellersen and Brosens, 2014). This process continues with the stromal cells surrounding the spiral arteries in the upper

endometrium regardless of the presence or absence of an embryo. In mice, decidualization is stimulated by the blastocyst (Cha et al., 2012).

Decidualization is regulated by many E2 and P4-regulated factors. Some important factors include the bone morphogenetic protein 2 (*Bmp2*). *Bmp2* is a P4-regulated gene and deletion of *Bmp2* in the uterus is associated with infertility in mice primarily due to decidualization failure (Lee et al., 2007; Lim et al., 1997). Since *Bmp2* is expressed in the underlying stroma, uterine deletion of the gene in mice was consistent with a failure in decidualization (Lee et al., 2007). Another important regulator of decidualization is the Abdominal B-like homeobox gene (*Hoxa10*), another P4-regulated gene expressed in stromal cells during decidualization (Bhurke et al., 2016). An important role for *Hoxa10* in stromal cell decidualization was established through studies showing that *Hoxa10* null mutations in mice are associated with defects in decidualization and infertility (Benson et al., 1996; Das et al., 2010).

G Protein-Coupled Receptors (GPCRs)

As stated earlier, there are many factors that regulate early pregnancy and most of these factors are regulated by E2 and/or P4. These include transcription factors and a wide array of secreted signaling molecules (ligands) and their cognate receptors. Among these receptors are the G protein-coupled receptors (GPCRs), the largest protein superfamily in mammalian genomes. Very little is known about GPCRs as regulators of early pregnancy and their regulation by E2 and P4. Thus, an important goal of my studies is to identify novel uterine GPCR regulators of early pregnancy and to determine how their expression and signaling are regulated.

Introduction to GPCRs GPCRs, also referred to as seven-pass-transmembrane domain receptors (7TMRs), form the largest protein superfamily and are involved in a variety of pathophysiological processes. In humans, there are about 800 GPCRs representing about 3% of the encoded genome (Babwah, 2020). Of the approximately 800 human GPCRs, about 440 are sensory receptors, of which the majority (about 390) are olfactory receptors. The primary function of a GPCR is to transduce extracellular stimuli into intracellular signals. G proteins play important roles in determining the specificity and temporal characteristics of the cellular responses to signals being transmitted (Hamm, 1998). GPCRs are found in eukaryotes, including flagellate eukaryotes, bacteria, yeast, plants, nematodes, amphibians, reptiles and vertebrates including mammals (King et al., 2003).

GPCRs are made up of extracellular (ECL) and intracellular (ICL) loops within the cells' plasma membrane. The extracellular environment contains the NH_2 terminus and the intracellular part contains the COOH terminus. The intracellular loops and domains regulate the interaction between the receptor and G proteins (Gether, 2000). GPCRs are activated by many extracellular ligands which include light-sensitive compounds, pheromones, hormones, neurotransmitters, ions, amino acids, peptides and lipids. GPCRs are ubiquitously expressed in the body and therefore regulate almost every physiological and cellular process (Babwah, 2020). Since they are intimately associated with human pathophysiology, pharmacologically complaint and readily targeted at the outer cell surface, they represent major drug targets where to date about 475 drugs or approximately 35% of the drugs approved by the US Food and Drug Administration target over 100 GPCRs (Babwah, 2020).

GPCRs interact with G proteins at the plasma membrane. When an exogenous ligand binds the receptor, the binding changes the conformation of the intracellular domains of the receptor. Such changes support the association of the receptor with a variety of heterotrimeric G proteins, leading to the activation of a G protein. When the GPCR is in its inactive state, the receptor is bound to a heterotrimeric G protein complex comprised of the Gα, Gβ and Gγ subunits (Hamm, 1998). In the inactive state, the Gα subunit is bound to GDP. When a ligand binds the receptor, this triggers the exchange of GDP for GTP on the Gα subunit and the disassociation of the Gα subunit from the Gβγ dimer.

The disassociated Gα and Gβγ subunits will go on to interact with other intracellular proteins to continue the signal transduction cascade. Signal transduction is terminated with the hydrolysis of GTP and the re-association of Gα and Gβγ subunits. It is understood that the Gβ and Gγ subunits function as a dimer and activate numerous signaling molecules such as lipid kinases, phospholipases and ion channels (Neumann et al., 2014). In addition to the regulation of these secondary-messenger systems mentioned above, the Gα and Gβγ subunits have also been reported to regulate the activation of major key intracellular signal-transducing molecules such as GTP-binding proteins of Ras and Rho families as well as members of the mitogen-activated protein kinase (MAPK) family of serine-threonine kinases which includes the extracellular signal-regulated kinase (ERK) (Neumann et al., 2014; Tuteja, 2009).

While the heterotrimeric G-protein is made up of three subunits, the Gα, Gβ and Gγ subunit, it is the α subunits that defines the basic functional property of the protein. At least 23 α-subunits derived from 17 different genes have been identified and are classified

into four main classes which include $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{12/13}$, and $G\alpha_{q/11}$ (Gautam et al., 1998). Once disassociated from the heterotrimeric complex, the Gα subunits interact with various effector enzymes in a very specific manner.

For example, $G\alpha_s$ stimulates the cAMP-dependent pathway by activating adenylyl cyclase (AC) and thus increases cyclic AMP (cAMP) levels, which in turn activates the cAMP-dependent protein kinase (PKA) pathway. The $G\alpha_{i/o}$ subunit primarily inhibits the cAMP dependent pathway by inhibiting AC, thereby decreasing the production of cAMP from ATP which in turn results in a decreased activity of cAMP-dependent protein kinase (Wettschureck and Offermanns, 2005).

The $G\alpha_{12/13}$ subunit activates Rho and further downstream signals. In addition, studies have shown that $G\alpha_{12/13}$ can induce a variety of signaling pathways which lead to the activation of numerous downstream effectors including Na^+/H^+ exchanger, phospholipase A_2 and/or c-*jun* NH_2-terminal kinase, as well as the formation of diacylglycerol (DAG) (Wettschureck and Offermanns, 2005).

The $G\alpha_q$ and $G\alpha_{11}$ subunits are ubiquitously expressed and those receptors that bind to $G\alpha_q$ and $G\alpha_{11}$ do not seem to discriminate between $G\alpha_q$ and $G\alpha_{11}$ (Wettschureck and Offermanns, 2005). The major function of $G\alpha_q$ and $G\alpha_{11}$ is to couple receptors to the β-isoform of phospholipase C (PLC). PLC cleaves phosphatidylinositol bisphosphate (PIP_2), a plasma membrane phospholipid, into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Wettschureck and Offermanns, 2005). While DAG remains bound to the plasma membrane, IP_3 is released into the cytosol. In the cytosol, IP_3 binds to the IP_3

calcium channel receptors on the endoplasmic reticulum (ER) leading to the release of calcium. Cells actively sequester calcium in the ER in order to maintain low cytoplasmic levels. When released, increased cytosolic calcium concentrations cause a cascade of intracellular changes in activity via the calcium binding proteins and calcium-dependent processes. In addition, DAG works together with the released calcium to activate the function of PKC, inducing the phosphorylation of other molecules and further altering cell activity (Skiba et al., 1996; Hamm, 1998).

Besides signaling via G proteins, GPCRs can also activate G protein-independent signaling pathways. This is mainly accomplished through the multi-functional adaptor proteins referred to as arrestins (Bologna et al., 2017). The arrestins are a small family of proteins that were discovered in the visual system. They include arrestin-1 and arrestin-4 expressed in retinal cones and rods, and arrestin-2 (β-arrestin1) and arrestin-3 (β-arrestin2) which are expressed in almost all cells in the body (Shukla et al., 2011; Lefkowitz, 2013). In an unstimulated cell, arrestins are localized in the cytoplasm in an "inactive" conformational state, however, upon GPCR activation, arrestins translocate to the plasma membrane where they block GPCR coupling to G protein in two ways. First, arrestins physically disrupt GPCR-G protein coupling and then link the receptor to the internalization machinery. This promotes receptor internalization via clathrin-coated pits, following transport to internal compartments known as endosomes (Bologna et al., 2017). In this way, the receptor can either be directed for degradation or recycled back to the plasma membrane where it can once again signal.

In addition to its roles in GPCR desensitization, β-arrestins have been found to play an important role in signaling, trafficking and ubiquitination of receptors (Shenoy et al., 2001). Studies have shown that internalized receptor-β-arrestin complexes can form a framework for MAPKs including ERK1/2 and many others to generate signalosome (a group of proteins which are involved in protein degradation), which may facilitate long lasting cell signaling in the cytosol (Luttrell et al., 1999; McDonald et al., 2000; Gong et al., 2008; Song et al., 2009). The role of β-arrestins in the regulation of MAPK cascades is the most studied mechanism. The first signaling function of β-arrestin was described in 1999, when Luttrell and colleagues reported that β2-adrenergic receptor-bound β-arrestin triggered Src-dependent activation of MAP kinases ERK1 and ERK2 (Luttrell et al., 1999). This finding demonstrated that in addition to activating MAPKs via the G protein-coupled pathway, GPCRs can also activate MAPKs via the β-arrestins (Ma and Pei, 2007). Recent work revealed that β-arrestins can also translocate from the cytoplasm to the nucleus and associate with many different cofactors such as p300 and cAMP-response element-binding protein (CREB) at the promoters of target genes to regulate transcription (Ma and Pei, 2007).

GPCR Regulators of Early Pregnancy

Based on a molecular screen conducted in the Babwah laboratory, it was determined that in the mouse on the day of embryo implantation, the uterus moderately or strongly expresses about 15 GPCRs, of which about 10 have known ligands while the rest are classified as orphan receptors (receptors for which there are no well-established endogenous ligands). Among the GPCRs with known ligands, only a small number has

been shown, through direct and detailed investigations, to be major regulators of uterine receptivity, embryo implantation and/or stromal cell decidualization. Some of these GPCRs include lysophosphatidic acid receptor 3 (LPAR3), leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4) and the kisspeptin receptor (KISS1R). Other GPCRs that might also be important regulator of early pregnancy include the oxytocin, prostanoid and calcitonin receptors.

Lysophosphatidic acid receptor 3 The Lysophosphatidic acid receptor 3 (*Lpar3*) couples to both $G\alpha_{i/o}$ and $G\alpha_{q/11}$ downstream signaling pathways that regulate cell proliferation, differentiation and survival. Studies in female mice in which the LPA receptor was deleted identified important roles for the receptor-mediated LPA signaling in fertility. These include ovarian function, fertilization, decidualization, embryo implantation and development, pregnancy maintenance and parturition (Ye and Chun, 2010). Based on studies conducted on the *Lpar3* knockout mouse during early pregnancy, *Lpar3* was shown to be important in the attainment of uterine receptivity and embryo implantation. (Pandy-Szekeres et al., 2018). *Lpar3* knockout female mice displayed delayed embryo implantation with reduced cell proliferation in the stroma as well as uneven embryo spacing and reduced litter size (Ye et al., 2005). In addition, deletion of *Lpar3* in the uterus disrupts the balance between E2 and P4 and causes persistent progesterone signaling, resulting in the inability to exit the pre-receptive phase during early pregnancy (Diao et al., 2015). This is critical because prolonged PR expression in the uterine epithelium beyond the expected implantation window is detrimental to embryo implantation. In addition to data from non-human species, recent data show that LPAR3 might also play an important function in human reproduction. One study has shown that

LPAR3 levels decrease in the middle-and later-secretory endometrium (note implantation occurs in the secretory phase) of patients with endometriosis (Wei et al., 2009).

Leucine-rich repeat-containing G protein-coupled receptor 4 The leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4) is a $G\alpha_s$-coupled receptor (Pandy-Szekeres et al., 2018). Studies showed that LGR4 plays a critical functional role in embryonic development (Mohri et al., 2010; Sone et al., 2013). More importantly, mice lacking *Lgr4* expression in the epithelial tissues exhibited reduced fertility and the embryos had impaired development (Mohri et al., 2010). Additionally, uterine *Lgr4* knockout mice displayed altered epithelial differentiation, evident by the reduction in the number of uterine glands. Furthermore, the uteri of mice that lacked *Lgr4* expression lost the ability to undergo induced decidualization (Sone et al., 2013). Therefore, *Lgr4* expression in the uterine epithelium is required for healthy gland development and decidualization in mice. In mice that lacked *Lgr4* expression, P4 failed to inhibit luminal epithelium proliferation and P4 signaling was significantly down-regulated, again suggesting that the LGR4 is an important uterine GPCR that is required for the acquisition of endometrial receptivity (Kida et al., 2014).

Kisspeptin receptor The kisspeptin receptor (KISS1R) is a $G\alpha_{q/11}$-coupled receptor (Pandy-Szekeres et al., 2018). The kisspeptin/KISS1R signaling system is an important regulator of reproduction through its direct actions along the hypothalamic-pituitary-gonadal (HPG) axis (reviewed in Radovick and Babwah, 2019). Accumulating evidence has shown that kisspeptin signaling is involved in the regulation of trophoblast invasion, placentation and most importantly, the process of implantation (Radovick and

Babwah, 2019). In the mouse uterine luminal epithelium, kisspeptin was shown to trigger phosphorylation of p38 and ERK1/2 on day 4 of pregnancy thus implicating epithelial KISS1R in embryo implantation (Fayazi et al., 2015). Studies in female *Kiss1r* knockout mice showed uterine defects and infertility (Calder et al., 2014; Leon et al., 2016). Uterine kisspeptin and KISS1R regulate embryo implantation by controlling the availability of endometrial glandular secretions needed for embryo adhesion to the uterine epithelium (Calder et al., 2014; Leon et al., 2016; Radovick and Babwah, 2019). It has also been shown that decidual kisspeptin and KISS1R signaling may be involved in regulating decidual function in humans (Radovick and Babwah, 2019).

Prostanoid receptors The prostanoid receptors are G protein-coupled receptors activated by the endogenous ligands prostaglandins PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, PGH_2, prostacyclin [PGI_2] and thromboxane A_2 (Niringiyumukiza et al., 2018). The most common type of PG found in animal species is PGE_2. Studies with PGE_2, specifically subtype-2 or EP_2, have shown that mice that are EP_2 deficient displayed problems with ovulation, fertilization, implantation and embryo development (Chakraborty et al., 1996; Matsumoto et al., 2001). PGs play a crucial role in embryo implantation. For instance, PGE_2S and $PGF_2\alpha S$ localize in the human endometrial epithelia with a significantly noticeable increase in both concentrations in the uterine fluid during the implantation window period (Vilella et al., 2013). The expression of cyclooxygenase 1 and 2 (COX-1 and COX-2) is also important during early pregnancy. COX is an important enzyme in the PG biosynthesis. In humans, COX-1 is expressed in the luminal and glandular epithelia while COX-2 is mostly expressed in the luminal epithelia and perivascular cells during the implantation window. Female mice lacking COX-2 were infertile with decidualization and

implantation defects (Lim et al., 1997). Most importantly, however is that, mice deficient in the PG receptor specifically EP_2 have been shown to exhibit impaired reproductive functions (Tilley et al., 1999).

Calcitonin receptor Another GPCR implicated in regulating early pregnancy is the calcitonin receptor (CT) that binds the peptide hormone calcitonin. This receptor couples to $G\alpha_s$ and $G\alpha_{q/11}$, thus, the CT receptor couples to and activates both adenylyl cyclase and phospholipase C and is involved in the maintenance of calcium homeostasis (Purdue et al., 2002). However, direct studies have not been conducted on the receptor itself but instead most of the studies have been on its ligand. Therefore, the importance of this receptor has only been implied through a detailed analysis of its ligand, calcitonin. Calcitonin has been shown to play an important role in calcium homeostasis in many tissues and its expression levels are dramatically increased in the glandular epithelium of rat uterus between days 3-5 of pregnancy (peri-implantation period). It appears that calcitonin functions as a regulatory signal in the uterus during the pre-implantation period and is critical for successful pregnancy. (Zhu et al., 1998).

Oxytocin receptor Another GPCR implicated in regulating early pregnancy is the $G\alpha_{q/11}$-coupled oxytocin receptor (OXTR) (Gimpl and Fahrenholz., 2001). To date, only limited evidence suggests that OXTR plays an important role in achieving successful pregnancy. For instance, the differential expression of the *Oxtr* in a mouse uterus during embryonic apposition and invasion period suggests a potentially important role of oxytocin in embryo implantation process (Beretsos et al., 2007). It has also been demonstrated that

oxytocin antagonists improve uterine receptivity during *in vitro* fertilization (IVF) treatments which leads to improved pregnancy rates (Pierzynski et al., 2007).

G protein-coupled receptor 83 As stated earlier, a molecular screen conducted in the Babwah laboratory determined that in the mouse on the day of embryo implantation, the uterus moderately or strongly expresses about 15 GPCRs, of which about 10 have known ligands. Among the GPCRs with known ligands is G protein-coupled receptor 83 (GPR83). Just recently, GPR83 was classified as an orphan receptor, but in 2016 research from the Devi laboratory identified GPR83 as a major receptor for the proSAAS-derived peptide called PEN (Gomes et al., 2016). It is worth noting that according to a primary recommendation of the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) [Babwah, 2020], an orphan GPCR is only "deorphanized" when two or more peer-reviewed papers from independent research groups demonstrate activity of the ligand at the receptor, with a potency that is consistent with a physiologic function. Based on this criterion, our findings (Parobchak et al., 2020), the basis of this thesis, that provides the evidence to officially deorphanize GPR83.

GPR83 is novel GPCR widely expressed in the brain and immune system in humans and mice. In-depth *in situ* hybridization analysis revealed high expression levels in hypothalamus, amygdala and hippocampus regions on the brain. These discrete areas in the brain are known to be involved in anxiety, depression, stress modulation and memory. *Gpr83* mRNA was first identified as a glucocorticoid/cAMP-induced gene in a mouse T-lymphocyte cell line (Harrigan et al., 1989; Harrigan et al., 1999) and later was shown to

be highly up-regulated in regulatory T (Treg) cells (Fontenot et al., 2005; Sugimoto et al., 2006). In the uterus, both glucocorticoid signaling and T cells are reported to play an important role in pregnancy, such as stimulating immune tolerance at the maternal-fetal interface. Although the underlying mechanism of this tolerance is not fully understood, it can potentially involve GPR83. Previous studies have reported that GPR83 is regulated by the stress hormone corticosterone and that GPR83 knockout mice are resistant to stress-induced anxiety (Lueptow et al., 2017). Newly emerging evidence reveals roles for the centrally located GPR83 in regulating dopamine release and morphine reward-learning in the brain (Fakira et al., 2019). This is important because it appears that GPR83 is a functionally versatile molecule that might have a significant role in regulating early pregnancy events.

Preliminary data from the Babwah laboratory show that *GPR83* mRNA is expressed in the endometrial scrapings from healthy women throughout the menstrual cycle (Parobchak et al., 2020). Thus, it appears that uterine GPR83 is a potentially promising regulator of human pregnancy. Prior to my research, there were no reports on the expression of uterine GPR83 and for that reason my research is focused on characterizing uterine *Gpr83* expression during early pregnancy and to identify the mechanisms by which GPR83 signals in the endometrium using mouse models of human pregnancy and human endometrial and non-endometrial cell lines.

PEN is Derived from ProSAAS

PEN is one of the most abundant hypothalamic neuropeptides and is derived from the processing of the precursor protein called proSAAS (named because it contains a Ser-

Ala-Ala-Ser motif) (Fricker et al., 2000). ProSAAS is a 26-kDa (~260 amino acid) protein that is encoded by the *PCSK1N* gene (chromosomal localization Xp11.3 in humans) and is widely expressed in a number of species including humans and mice. The human form shares 84% homology with mouse and rat while the mouse and rat form share 97% sequence homology (Mzhavia et al., 2001; Sayah et al., 2001; Feng et al., 2001).

Some of the most abundant intermediary peptides were discovered to be derived from proSAAS. For instance, the N-terminal region of proSAAS is cleaved to yield GAV, littleSAAS, and KEP peptides. Meanwhile, the C-terminal region yields PEN and bigLEN (Mzhavia et al., 2002; Mzhavia et al., 2001; Wardman et al., 2014). In order to better understand some of these peptides, researchers were able to gain more information by conducting either overexpression or knockout studies of proSAAS. Additional information about the physiological role of proSAAS derived peptides has come from genetically modified mice. For instance, mice overexpressing proSAAS exhibited obesity when compared to wild-type, while those that lacked proSAAS yielded slightly underweight mice (Morgan et al., 2010 and Wei et al., 2004). Mice that had a proSAAS-knockout, displayed an anxiety phenotype (Morgan et al., 2010).

To date, not much is known about the specific roles of individual proSAAS derived peptides except for littleSAAS, BigLEN and more recently, PEN. Evidence for a role for PEN has been linked to metabolism regulation and food intake.

Location of PEN Expression In humans, the precursor proSAAS is highly expressed in the brain, pituitary, adrenal glands and pancreas (Fricker et al., 2000). Since proSAAS mRNA is widely expressed in brain regions such as the hypothalamus,

hippocampus and amygdala these peptides, including PEN, have been proposed in the regulation of the neuroendocrine system (Fricker et al., 2000).

While it remains to be determined whether PEN is also available in the uterine environment, it was clearly shown that *Pcsk1n*, the gene encoding ProSAAS (the PEN precursor), is expressed in the mouse uterus during the peri-implantation period (Decaillot et al., 2006). Therefore, it is likely that PEN is produced in the uterus and exerts uterine functions at the time of embryo implantation.

Location of GPR83 Expression PEN neuropeptide has been identified as an endogenous ligand for GPR83 (Gomes et al., 2016). In mice, *Gpr83* mRNA is found to be expressed in limbic structures in the forebrain, in the striatum, in some regions of the hypothalamus and low levels in the periphery (Pesini et al., 1998). In contrast, in humans, GPR83 mRNA is highly expressed in the brain and spinal cord and low levels in the thymus and other tissues (Brezillon et al., 2001). Specifically, within the brain, GPR83 is found to be highly expressed in the hypothalamus, striatum, hippocampus, amygdala and prefrontal cortex (Lueptow et al., 2018). Human GPR83 shares 89.5% sequence homology with mice and 88% homology with rats (Harrigan et al., 1991 and De Moerlooze et al., 2000).

MATERIAS AND METHODS

Animal studies Animal studies were approved by Rutgers University according to guidelines established by the Institutional Animal Care and Use Committee. Studies were conducted on C57 Bl/6 WT mice. All data presented in this study are based on data generated with the C57 Bl/6 mice.

Non-pregnant cycling females Estrous staging (proestrus, estrus, or metestrus and diestrus) of adult females (10 weeks old) was done as previously described (Caligioni, 2009). Mice, at each estrous stage were euthanized between 1:00 and 3:00 PM and uteri collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada).

Unstimulated pregnancy Females (8-10 weeks old) in proestrus were mated to either stud or vasectomized males and the day of mating is defined as day 0. Only females that showed a copulatory plug (evidence of successful mating) on the morning of D1 were studied further. Mice were euthanized between 8:00-9:00 AM on D1, D4-8 of pregnancy and uteri collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada). Based on the visibility of implantation swellings, D6-8 uteri were cut and separated into implantation sites (IS) and inter-implantation sites (IIS).

Ovarian-stimulated pregnancy Females (8 weeks old) were administered 7.5 IU pregnant mare serum gonadotropin (PMSG; Folligon; Intervet) i.p. followed 48 hours later by 7.5 IU human chorionic gonadotropin (hCG; Chorulon; Intervet) i.p. Immediately after the hCG injection, mice were mated to stud males and subsequently analyzed as described for the unstimulated females (see above).

E2, P4 or E2 + P4 regulation of gene expression Two weeks following ovariectomy, mice (8 weeks old) were administered either peanut oil (100 µl), E2 (100 ng in a final volume of 100 µl), P4 (1 mg in a final volume of 100 µl), or E2 (100 ng) + P4 (1 mg) (in a final volume of 200 µl) by s.c. injection (Jeong et al., 2010). Twenty-four and forty-eight hours after hormone administration, mice were euthanized and uteri were collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada). E2 and P4 were purchased from Tocris Bioscience (Minneapolis, MN).

Uterine cellular fractionation D4 pregnant uteri from unstimulated pregnancies were enzymatically processed to yield epithelial, stromal only and stromal + myometrial fractions (Li et al., 2011). Day 4 unstimulated pregnant uteri were suspended in Trypsin which broke down the epithelial layer into single cells. The remaining fraction, stromal and myometrial was collected. To separate the stromal fraction, collagenases were added to the stromal and myometrial fraction. This was filtered in a 40 micron filter which allowed the stromal cells to flow through thus yielding the stromal fraction. Tissue fractions were rapidly frozen until used for RNA isolation.

***In vitro* decidualization** Mouse uterine stromal cells were collected on day 4 of pseudopregnancy and grown on glass cover slips in DMEM/F-12 (1∶1), plus 10% charcoal stripped serum and antibiotic. Following 1 hr of attachment, cells were washed thoroughly in HBSS and re-incubated in fresh medium for 24 hr. The medium then was replaced with the decidualization medium composed of DMEM/F-12, 1% charcoal-stripped FBS, 1 µM P4, 10 nM E2, 0.5% HB-EGF and antibiotic, and cells were grown for an additional six days before being analyzed for *Gpr83* expression.

qPCR analysis of gene expression RNA was prepared from uterine horns and uterine tissue fractions using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Gene expression studies were conducted using mouse *Gpr83*, human *GPR83*, mouse *Rpl13a* and human *RPL13A* PrimePCR Assays (Bio-Rad Laboratories). Gene expression was determined on total RNA. Gene expression was calculated as relative expression to the housekeeping gene (*Rpl13a*), using the $2^{-\Delta\Delta Ct}$ method (de Oliveira et al., 2019).

Cell line studies The Ishikawa cell line (human Asian endometrial adenocarcinoma) was purchased from Sigma-Aldrich (ECACC 99040201) (St. Louis, MO, USA) and is part of the European Collection of Authenticated Cell Cultures (ECACC). Ishikawa cells were cultured according to vendor's instructions. Human Embryonic Kidney (HEK) 293 cells (parental WT and *Arrb1*$^{-/-}$/*Arrb2*$^{-/-}$) were generous gifts from Professor Graeme Milligan (University of Glasgow, Glasgow, UK). The generation and characterization of the HEK 293 *Arrb1*$^{-/-}$/*Arrb2*$^{-/-}$ line is described in (Alvarez-Curto et al., 2016; Milligan and Inoue, 2018). The HEK 293 cell lines were cultured as described in (Alvarez-Curto et al., 2016). The human endometrial stromal cell line (SHT290) was purchased from Kerafast (Boston, MA, USA) and cultured as described in (Barbier et al., 2005).

$CaPO_4$ transfection of HEK 293 cells The HEK 293 cell lines were transfected with C-terminal FLAG epitope (DDK)-tagged human GPR83 cDNA (Origene, Rockville, MD, USA) using the $CaPO_4$ transfection kit (ThermoFisher Scientific, Branchburg, NJ, USA) according to the manufacturer's recommendations. Transfected cells were analyzed 48 hours later. GPR83-FLAG expression was quantified by SDS-PAGE and western blotting using the rabbit polyclonal anti-FLAG antibody at a 1:1000 dilution (Sigma-Aldrich, St. Louis, MO, USA).

PEN-triggered ERK1/2 phosphorylation in Ishikawa and HEK 293 cell lines Ishikawa and GPR83-FLAG expressing HEK 293 cells were either treated with PEN (100 nM) or left untreated. Cells were then lysed, and pERK1/2 levels were quantified by SDS-PAGE and western blotting. In some studies, cells were treated with the cyclic depsipeptide and selective $G\alpha_{q/11}$ inhibitor YM-254890 (Takasaki et al., 2004). (FUJIFILM Wako Pure Chemical Corporation, Richmond, VA, USA). YM-254890 was reconstituted in DMSO and used at a final concentration of 1 μM (Ando et al., 2010). Cells were preincubated in YM-254890 for 30 mins at 37^{o}C before adding PEN (100 nM).

siRNA-mediated downregulation of *GPR83* in Ishikawa cells Silence Select siRNAs against human *Gpr83* and negative control siRNA were purchased from ThermoFisher Scientific (Branchburg, NJ, USA). Ishikawa cells were transfected with either the *Gpr83* specific siRNA or control siRNA using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific, Branchburg, NJ, USA). Forty-eight hours later, cells were treated with PEN (100 nM) for 10mins. Non-treated WT cells served as an additional control to establish basal pERK1/2 levels. Quantification of pERK levels in control and experimental samples is described in this article.

SDS-PAGE and western blotting pERK1/2 levels in the Ishikawa and HEK 293 cell lines were assayed by SDS-PAGE and western blotting. Briefly, cellular lysates were prepared, and protein were fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were then probed for either GPR83-FLAG, pERK1/2 and total ERK. The following day, blots were washed extensively and incubated with the appropriate species-specific horseradish peroxidase-conjugated secondary antibody. Blots were developed using an ECL detection system (ThermoFisher Scientific).

cAMP assay cAMP levels were determined in Ishikawa cells treated with (i) PEN only (serial dilutions: 1000-15.6 nM, shown as log values 3-1.19 in Figure 3C) for either 3 or 15 mins, or (ii) forskolin (40 μM), either in the absence of PEN or in the presence of varying concentrations of PEN (serial dilutions: 1000-15.6 nM) for either 3 or 15 mins. cAMP levels were measured using the Cyclic AMP XP Assay Kit (Cell Signaling Technologies, Danvers, MA, USA) according to the manufacturer's instructions. Adenylyl cyclase activity was calculated according to the manufacturer's instructions.

Statistics The differences between groups were determined using unpaired Student's t-test or one-way ANOVA followed by post-hoc Bonferroni test (GraphPad Prism Software, Inc, La Jolla, CA). All values are expressed as mean ± SEM and a value of $p<0.05$ was considered statistically significant. Each assay was performed 3 to 4 times.

RESULTS

Uterine *Gpr83* mRNA is expressed in the non-pregnant and pregnant mouse

Due to a lack of reliable antibodies to study GPR83 protein expression, the data presented is based on *Gpr83* mRNA quantification only. The expression of uterine *Gpr83* mRNA transcripts was quantified by qPCR and based on unprocessed cycle threshold (Ct) values of approximately 24 (compared to the *Rpl13a* Ct value of about 16) we concluded that *Gpr83* is expressed throughout the estrous cycle (Figure 1A). Expression was also determined on D1 and D4-8 of pregnancy in the WT mouse. D1 is when the uterus is unreceptive to embryo implantation, D4 is the day of uterine receptivity, D5 is when the embryo implantation occurs followed by decidualization. On D6 to D8, implantation swellings are visibly distinct, and this permitted the separation of the uterus into implantation and inter-implantation sites. Based on the analysis it was observed that *Gpr83* mRNA expression increased about 25-fold from D1 to D4 (Ct values of about 24) and remained unchanged on D5 (Figure 1B), strongly suggesting roles in early pregnancy. On D6, expression increased further, and this was observed at both the implantation and inter-implantation sites (Figure 1B). Interestingly, expression dropped significantly at the implantation sites on D7 and D8 but remained elevated at the inter-implantation sites suggesting that *Gpr83* is involved in decidualization but particularly in the early parts of decidualization because as decidualization continues at the implantation site, on D7 and D8 the expression decreases in the inter-implantation sites (Figure 1B).

Uterine *Gpr83* mRNA is an E2/P4-responsive gene

Observations that uterine *Gpr83* mRNA expression increased dramatically on D4 suggested strongly that it could be both E2- and P4-responsive and the further increase in expression on D6 during a heightened period of stromal decidualization strengthened this possibility. To test whether this was the case, *Gpr83* expression was assessed in females that received either vehicle (oil), E2, P4 or E2 + P4 for 24 or 48 hrs, two weeks after ovariectomy (OVX). The results revealed that *Gpr83* expression was responsive to E2 but unresponsive to P4 treatment when compared to vehicle (oil) (Figure 1C). However, when E2 and P4 were co-administered, expression increased dramatically (about 65-fold) at 24 hours and while diminished at 48 hours it was still significantly greater compared to vehicle. (Figure 1C). The dramatic E2 + P4 combined effect leads us to hypothesize that *Gpr83* may play an important role in the regulation of stromal cell decidualization.

Uterine *Gpr83* mRNA expression is downregulated in the ovarian-stimulated mouse during early pregnancy

Ovarian stimulation in IVF protocols results in supraphysiological levels of E2 and P4 and a change in the E2:P4 ratio. These changes can have significant impact on uterine gene expression and the acquisition of uterine receptivity, embryo implantation and decidualization during early pregnancy and present a potential obstacle to increasing higher pregnancy rates during assisted reproduction (Zhang et al., 2015). To determine whether uterine *Gpr83* expression was sensitive to changes in E2 and P4 following ovarian stimulation, expression was assessed on D1 and D4-7 in ovarian-stimulated females (Figure 1D). The results revealed that following stimulation, expression increased only about 2.5-fold on D4 relative to D1, and the massive rise in expression observed in non-stimulated females (Figure 1B) was strikingly absent. Furthermore, a significant change in

expression, relative to D1, was not seen at any of the other time points analyzed (Figure 1D).

The preimplantation embryo does not modulate uterine *Gpr83* mRNA expression on D4 of pregnancy

For some genes, uterine expression is modulated by the preimplantation embryo (Das et al., 1994). In the studies described thus far, *Gpr83* mRNA expression was assessed in the pregnant mouse. Therefore, to determine whether its expression is influenced by unimplanted embryos in the uterine horn on the morning of D4, gene expression was compared between the pregnant female (mated to a stud male) and the pseudopregnant female (mated to a vasectomized male). The results revealed that the presence of unimplanted embryos had no effect on uterine *Gpr83* expression (Figure 1E).

Uterine *Gpr83* mRNA is highly expressed in the uterine stroma on D4 of pregnancy

The spatial distribution of *Gpr83* mRNA in the D4 pregnant uterus was determined by cellular fractionation. The results revealed that on D4, expression was localized to the stroma (Figure 1F) but not the epithelium (Figure 1F). Due to the technical difficulty in isolating the myometrium as a separate fraction, a combined stromal/myometrial fraction was prepared, and this was compared to the stromal fraction. This comparison revealed a reduction in *Gpr83* expression (resulting from a myometrial-diluting effect) indicating that *Gpr83* is not expressed in the myometrium (Figure 1F). Similar investigations were also conducted on fractionated D6 and 7 uteri. However, due to the difficulty in fractionating the epithelium and stroma as pregnancy progresses, only a preliminary assessment of

Gpr83 mRNA expression was made at these time points and the findings were consistent with the D4 data that *Gpr83* is only expressed in the uterine stroma.

Stromal cells express *Gpr83* and *Gpr83* mRNA level declines following decidualization *in vitro*

Data presented in Figure 1B showed that the expression of *Gpr83* diminished significantly at the implantation sites on D7 and D8 but remained elevated at the inter-implantation sites. This suggests that if *Gpr83* regulates decidualization, it likely only regulates the formation of the primary decidual zone. To examine this further, stromal cells from D4 pregnant females were isolated, attached to glass coverslips and then washed thoroughly with HBSS; yielding a purified population of stromal cells. The stromal cells were then subjected to an *in vitro* decidualization protocol as previously described (Ma et al., 2011; Deng et al., 2016). As predicted based on the *in vivo* findings, after six days of decidualization, there was a decline in *Gpr83* expression. However, while the difference was not significant (n=3 for CTRL and experimental groups), a clear trend towards reduced expression was apparent (Figure 1G).

The data presented in Figure 1G also strengthens the conclusion that *Gpr83* is expressed by uterine stromal cells. Since *Gpr83* is expressed in regulatory T (Treg) cells (Fontenot et al., 2005; Sugimoto et al., 2006) and Treg cells are abundant in the uterus during early pregnancy (Sharma, 2014), it was important to demonstrate that *Gpr83* expression was not only due to T-reg cells but also due to uterine cells. In the experiment described above, following the removal of all non-adherent cells like T-reg cells by thorough washing in HBSS, the purified D4 stromal cells were analyzed for *Gpr83* expression and were found to express *Gpr83* (Figure 1G). Furthermore, T-reg and other

immune cells like the uterine natural killer cells accumulate in the decidua at the maternal-fetal interface and myometrium at the implantation site (Sharma, 2014). In this study, it was observed that on D7 and D8, while *Gpr83* expression declined at the implantation site, it remained elevated at the inter-implantation site further strengthening the evidence that *Gpr83* is highly expressed by uterine cells.

PEN triggers ERK phosphorylation in Ishikawa endometrial cells

Through the use of the Neuro2A mouse neuroblastoma cell line and the heterologous Chinese Hamster Ovary (CHO) cell line, it was determined that GPR83 is a major receptor for PEN and that PEN signals via GPR83 in a $G\alpha_{q/11}$-dependent manner. To build on these important findings in an endometrium-specific context, *GPR83* mRNA expression first was determined in two cell models of the human endometrium. Here it was observed that *GPR83* is highly expressed in the human Ishikawa endometrial adenocarcinoma cell line but weakly in the human SHT290 endometrial stromal cell line (Figure 2A). This potentially implies that *GPR83* regulates early pregnancy by regulating the epithelium in an autocrine manner and/or the stroma in a paracrine manner.

Next, it was determined whether Ishikawa and SHT290 cells are responsive to PEN. Cells were treated with 100 nM human PEN and lysates were assayed by SDS-PAGE and western blotting for pERK1/2. Phosphorylation of ERK1/2 was selected as the readout since numerous cell surface receptors, including GPCRs, rapidly trigger ERK1/2 phosphorylation. Results showed that in the continuous presence of PEN, PEN treatment of Ishikawa cells induced a rapid rise in pERK2 levels with maximum phosphorylation detected after 10 mins of treatment (Figure 2B and C). It was observed that, pERK1 levels were undetected or barely so. Following the maximum response, pERK2 levels declined

gradually (Figure 2B and C). In contrast, PEN failed to trigger a visible change in pERK levels in the SHT290 cells (Supplementary Figure 1). These findings suggest that while both cell lines express *GPR83*, Ishikawa cells produce higher levels of functional GPR83 than the SHT290 cells and could serve as an ERα/PR-expressing endometrial cell model to study PEN/GPR83 signaling.

PEN triggers ERK phosphorylation in a GPR83-dependent manner in Ishikawa endometrial cells

To determine whether the PEN-triggered ERK phosphorylation in Ishikawa cells occurred via GPR83, *GPR83* mRNA levels were downregulated using *GPR83*-specific siRNAs (Figure 2D) and cells were subsequently treated with human PEN. Results showed that in *GPR83* downregulated cells, PEN-dependent ERK phosphorylation was greatly diminished relative to PEN-treated WT cells and cells transfected with a negative control siRNA (Figure 2E and F). These findings establish that in Ishikawa cells, GPR83 is a major receptor for PEN (Figure 2G).

PEN triggers ERK phosphorylation in a $G\alpha_{q/11}$-dependent manner in Ishikawa endometrial cells

To determine whether GPR83 mediates PEN signals in a $G\alpha_{q/11}$-dependent manner Ishikawa cells were treated with PEN following pre-treatment with the cyclic depsipeptide and selective $G\alpha_{q/11}$ inhibitor YM-254890 (dissolved in DMSO). A control group of cells was pre-treated with DMSO only. Results showed in vehicle treated cells, as expected, PEN triggered a maximum response in ERK phosphorylation after 10 mins and this response subsequently desensitized (Figure 3A and B). However, in the presence of YM-

254890, at most time points, PEN-induced ERK phosphorylation was significantly diminished (Figure 3A and B). These findings confirm that in Ishikawa cells, GPR83 mediates PEN signals in a $G\alpha_{q/11}$-dependent manner.

PEN treatment of Ishikawa endometrial cells does not activate $G\alpha_i$ or $G\alpha_s$

It was reported that PEN also activates $G\alpha_i$-dependent signaling. Since $G\alpha_i$ and $G\alpha_s$ have opposite effects on adenylyl cyclase activity, it was determined whether PEN activates $G\alpha_i$ or $G\alpha_s$ in Ishikawa cells. Cells were treated with PEN for either 3 or 15 mins and the results showed that PEN failed to trigger an increase in cAMP levels suggesting that GPR83 is not coupled to $G\alpha_s$ (Figure 3C, 15 mins data shown). PEN treatment, for either 3 or 15 mins, also failed to inhibit forskolin-induced cAMP formation (Figure 3C, 15 mins data shown), suggesting that GPR83 is not coupled to $G\alpha_i$ and therefore unable to block the direct stimulation of adenylyl cyclase by forskolin. Overall, the data presented in Figure 3A and B, reveal that in Ishikawa endometrial cells, GPR83 mediates PEN signals independently of $G\alpha_i$ or $G\alpha_s$ (Figure 3D).

PEN triggers ERK phosphorylation via GPR83 in a $G\alpha_{q/11}$- and β-arrestin-dependent manner in HEK 293

Since some GPCRs trigger ERK phosphorylation in a $G\alpha_{q/11}$-independent manner via the major regulatory molecules, β-arrestin-1 and -2 (11, 36), it was determined whether PEN also couples GPR83 to β-arrestin. To determine this, human GPR83-overexpressing HEK 293 cells lacking β-arrestin-1 and -2 (KO) as a result of CRISPR/Cas9 genome editing were used (generously donated from Professor Graeme Milligan, University of Glasgow). Like CHO cells, HEK 293 cells have proven to be an excellent cell model for studying

GPCR signaling. Additionally, like CHO cells, we found responsiveness to PEN was only achieved following overexpression of a human GPR83 cDNA expression construct (FLAG-epitope tagged on the C-terminal end).

Results showed that in WT HEK 293 cells, PEN triggered pERK formation and this peaked after 10 mins of treatment (Figure 4A and B). When KO cells were treated with PEN, a similar response was observed, but importantly, the response was significantly diminished relative to the WT cells (Figure 4A and B). When KO cells were pre-treated with YM-254890 and then administered PEN, ERK phosphorylation was also abolished except at 10 mins (Figure 4A and B). The increased response at 10 mins suggests that non-$G\alpha_{q/11}$- and non-β-arrestin-dependent pathways have been activated that may nor may not be PEN-triggered. The changes in pERK levels were independent of GPR83 expression levels, as determined by western blotting using the anti-FLAG antibody (Figure 4B). These results suggest that $G\alpha_{q/11}$- and β-arrestin-dependent pathways are major conduits of PEN/GPR83-dependent ERK phosphorylation in HEK 293 cells (Figure 4G).

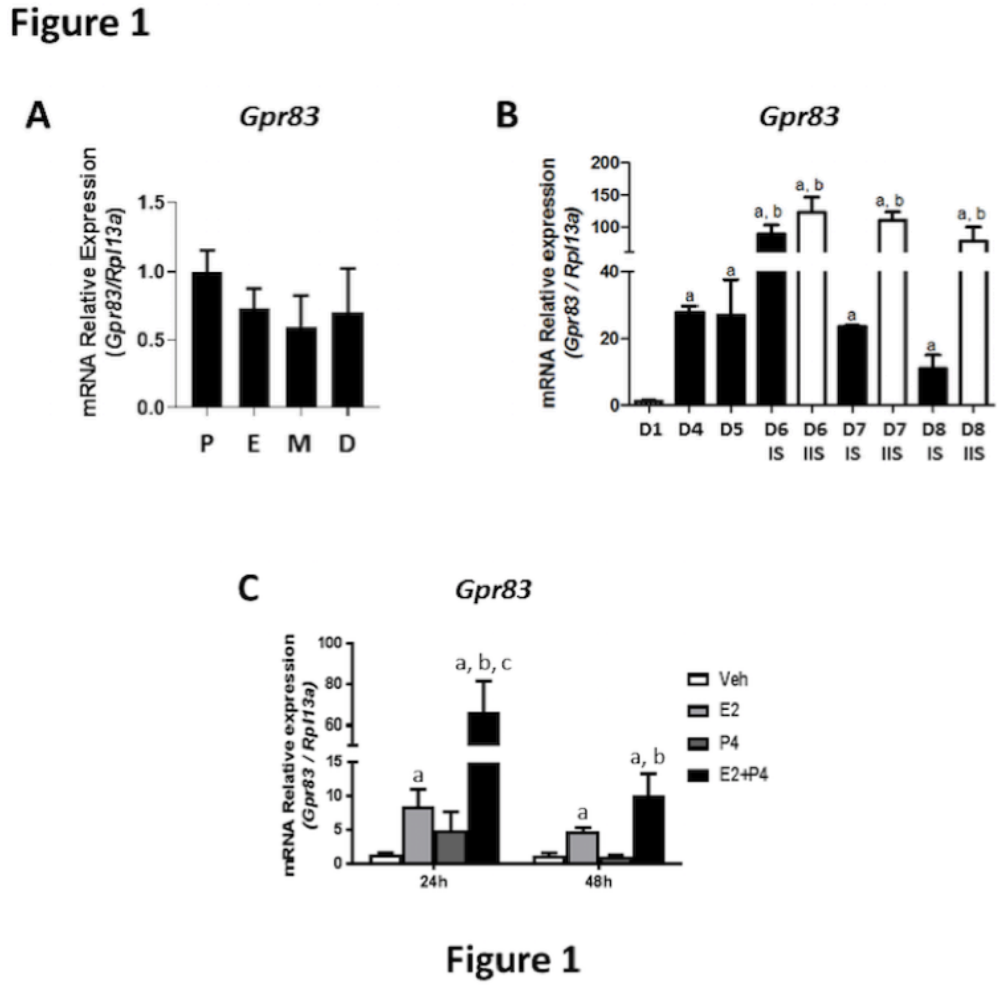


Figure 1. Uterine *Gpr83* mRNA is an E2+P4 responsive gene that is highly expressed in the peri-and postimplantation period and diminishes in expression with progressive decidualization at the implantation site. *Gpr83* expression was determined by quantitative polymerase chain reaction in the (**A**) nonpregnant mouse uterus during proestrus (P), estrus (E), metestrus (M), and diestrus (D); (**B**) pregnant mouse uterus during early pregnancy (D1-D8); a: $P<0.05$ vs. D1; b: $P<0.05$ vs. D4, D5, D7 IS, D8 IS; (**C**) OVX mouse + oil, E2, P4, or E2 + P4 following 24 and 48 hour of treatment; a: $P<0.05$ vs oil; b: $P<0.05$ vs. E2, c: $P<0.05$ vs. P4. (IS = implantation site; IIS = interimplantation site).

Figure 1

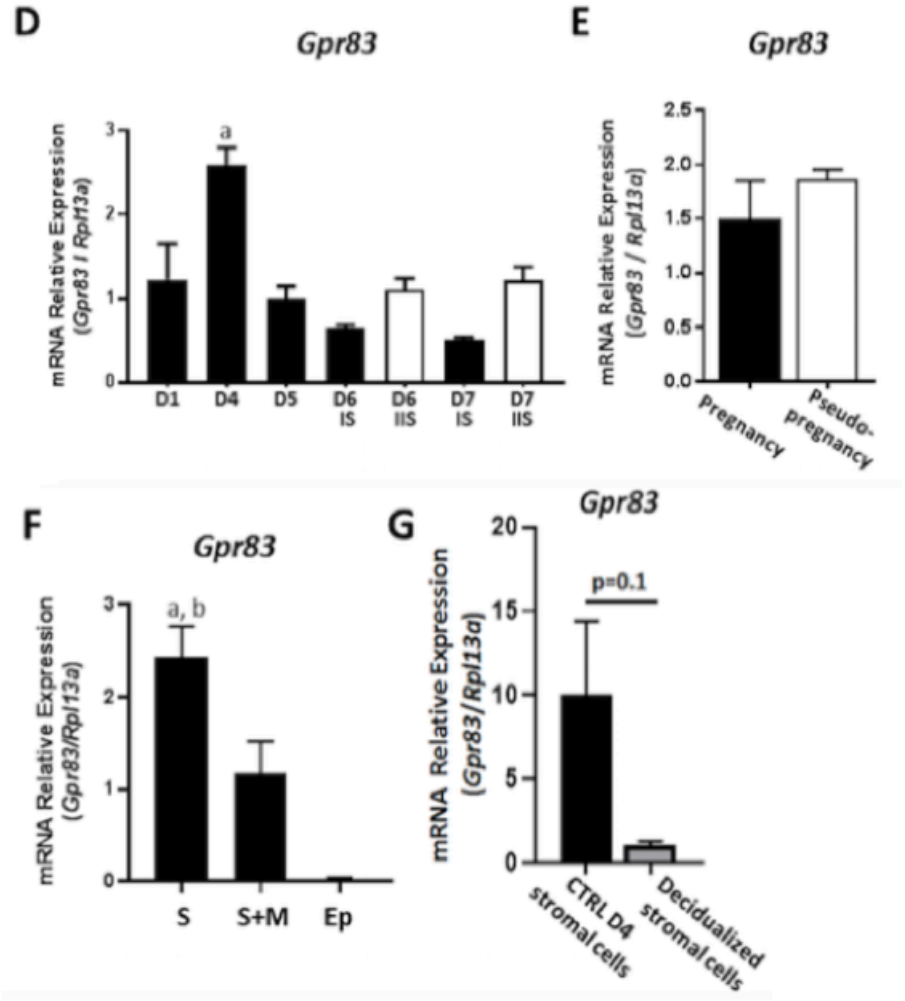


Figure 1. *Gpr83* expression is highly expressed on D4 of mouse pregnancy. (**D**) pregnant ovarian-stimulated mouse uterus during early pregnancy; a:$P<0.05$ vs. D1, D5, D6-8 IS, and IIS (D1-D7); (**E**) pregnant and pseudopregnant mouse on D4 of pregnancy; (**F**) fractionated D4 pregnant uterus; a:$P<0.01$ vs. S+M, b:$P<0.0001$ vs. Ep. and (**G**) in purified stromal cells isolated on D4 of pregnancy (CTRL) and following 6 days of in vitro decidualization. Except for data presented in (**G**), uteri were collected from five to 10 mice; in (**G**) data was collected from three mice in each group. Ep = epithelial fraction; IS = implantation site; IIS = interimplantation site; S = stromal fraction; S+M = combined stromal + myometrial fractions.

Figure 2

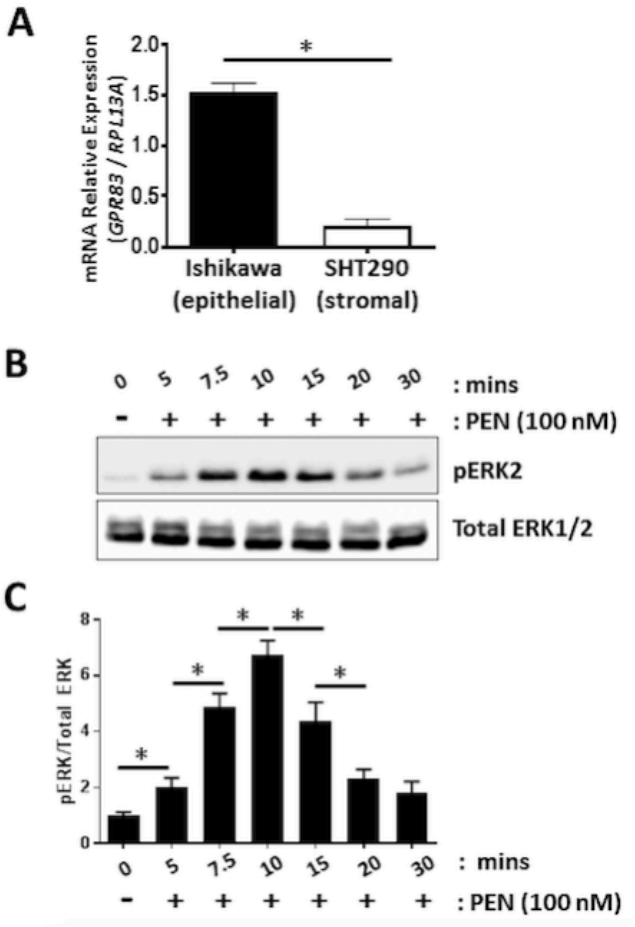


Figure 2. PEN triggers extracellular signal-regulated kinase (ERK) phosphorylation in a GPR83-dependent manner in Ishikawa endometrial epithelial cells. (**A**) *GPR83* expression was determined by qPCR in Ishikawa and SHT290 cells. (**B** and **C**) Time course assay of PEN-triggered ERK phosphorylation in Ishikawa cells as analyzed by western blotting and densitometric analysis of the blots.

Figure 2

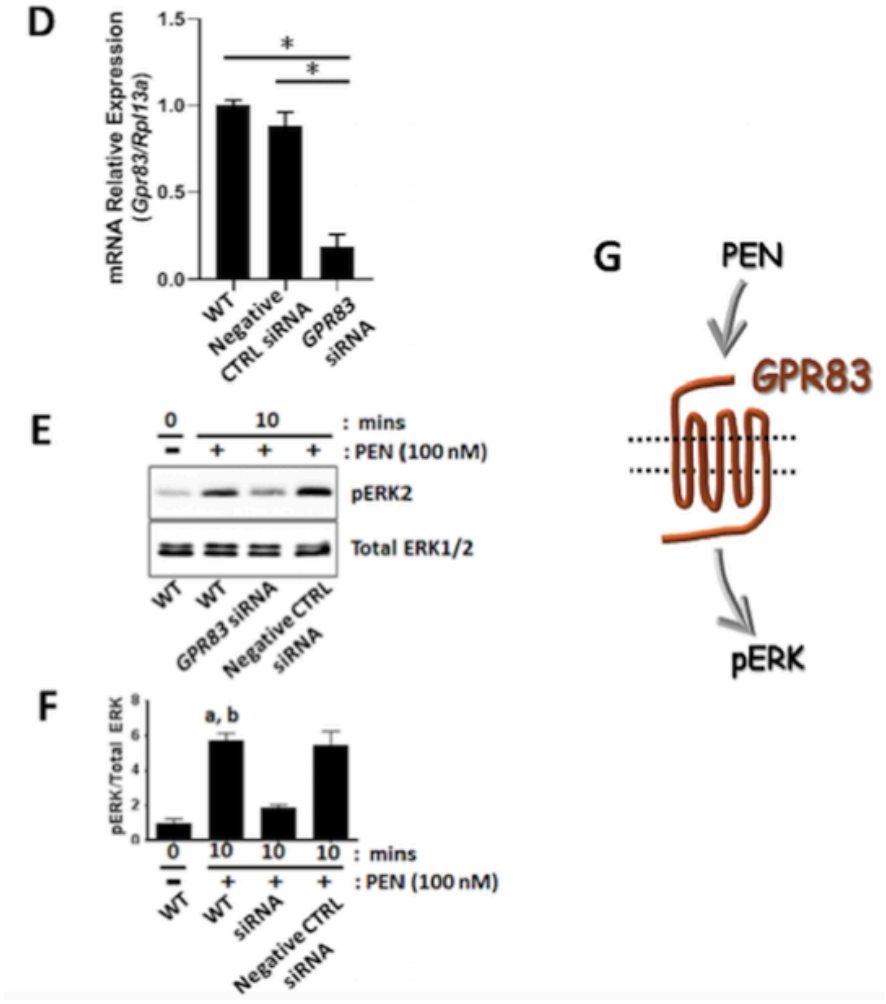


Figure 2. siRNA-mediated downregulation of *GPR83* diminishes PEN-triggered ERK phosphorylation in Ishikawa cells. (**D**) Effect of *Gpr83* siRNA transfection on *Gpr83* expression. (**E** and **F**) Effect of siRNA-mediated downregulation of *GPR83* on PEN-triggered ERK phosphorylation in Ishikawa cells. a: $P<0.01$ vs. 0 min WT cells, b: $P<0.01$ vs. *GPR83* siRNA downregulated cells. (**G**) An illustration showing PEN triggers ERK phosphorylation in a GPR83-dependent manner. All experiments were repeated a minimum of three to four times with $P<0.05$.

Figure 3

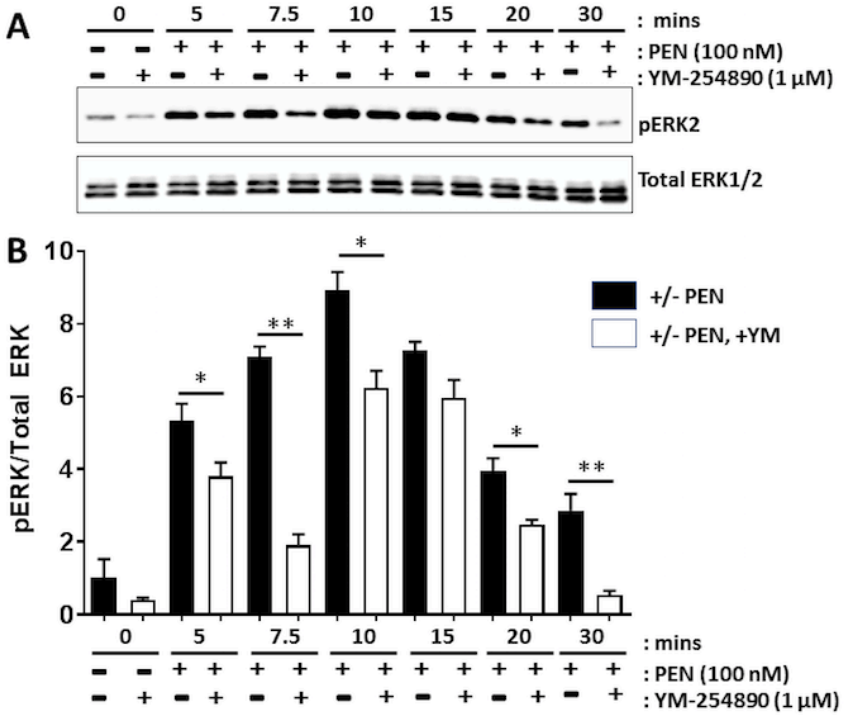
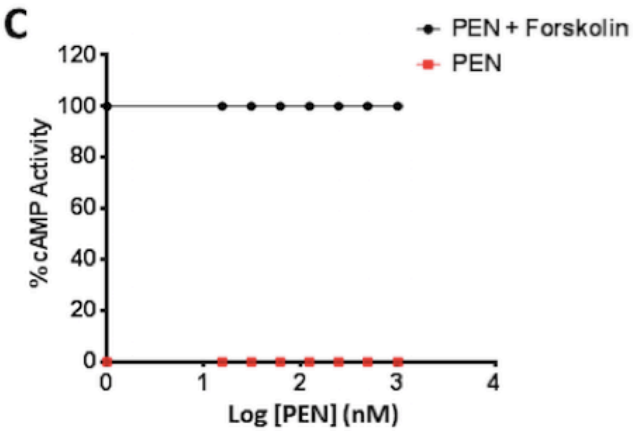


Figure 3. PEN triggers extracellular signal-regulated kinase (ERK) phosphorylation via GPR83 in a $G\alpha_{q/11}$ -dependent manner in Ishikawa endometrial epithelial cells. (**A** and **B**). Effect of YM-254890 on PEN-triggered ERK phosphorylation in Ishikawa cells as analyzed by western blotting and densitometric analysis of the blots. All experiments were repeated a minimum of three to four times. (**P*<0.05; ***P*<0.01).

Figure 3



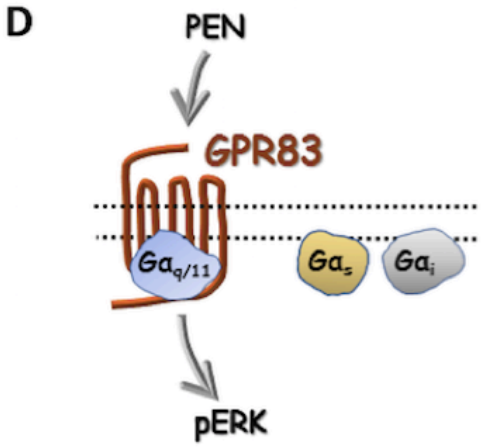


Figure 3. PEN treatment of Ishikawa endometrial cells does not activate $G\alpha_i$ or $G\alpha_s$. (**C**) cAMP levels were measured in Ishikawa cells treated with PEN (filled red squares) or in forskolin-treated cells in the presence or absence of PEN (filled black circles) to assess GPR83 coupling to $G\alpha_s$ and $G\alpha_i$ following PEN treatment. (**D**) Cartoon illustrating PEN triggers ERK phosphorylation via GPR83 in a $G\alpha_{q/11}$ -dependent manner and independently of $G\alpha_s$ and $G\alpha_i$. All experiments were repeated a minimum three to four times. (*P<0.05; **P<0.01).

Figure 4

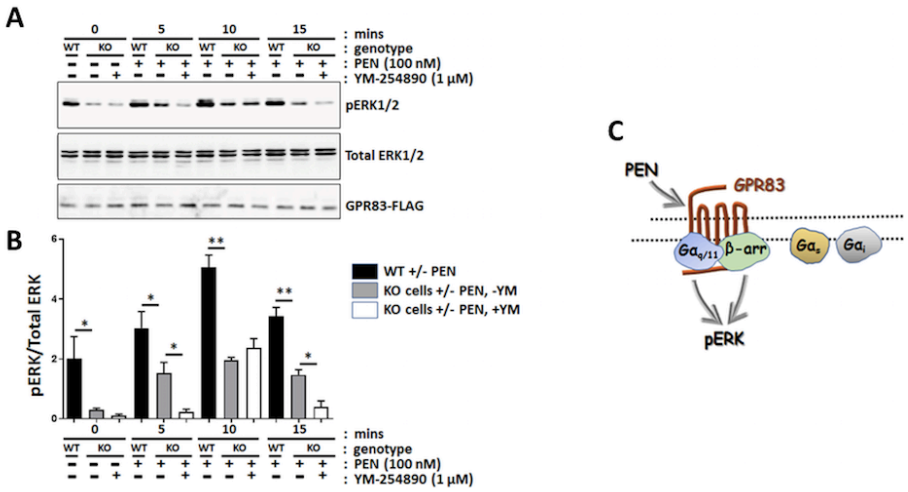
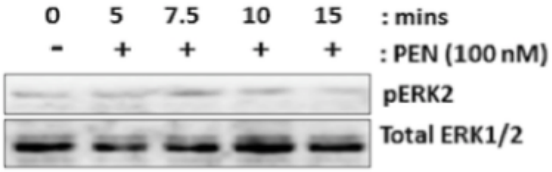


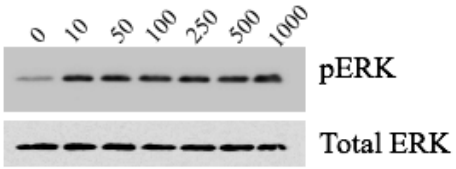
Figure 4. PEN triggers extracellular signal-regulated kinase (ERK) via GPR83 in a $G\alpha_{q/11}$ - and β-arrestin-dependent manner in HEK 293 cells. (**A** and **B**) Effect of the loss of β-arrestin-1/2 (KO) and YM-254890 on PEN-triggered ERK phosphorylation in GPR83-FLAG overexpressing HEK 293 cells as analyzed by western blotting and densitometric analysis of the blots. (**C**) An illustration showing that PEN triggers ERK phosphorylation via GPR83 in a $G\alpha_{q/11}$ - and β-arrestin dependent manner and independently of $G\alpha_s$ and $G\alpha_i$. All experiments were repeated a minimum of three to four times. (*P<0.05; **P<0.01).

Supplementary Figure 1



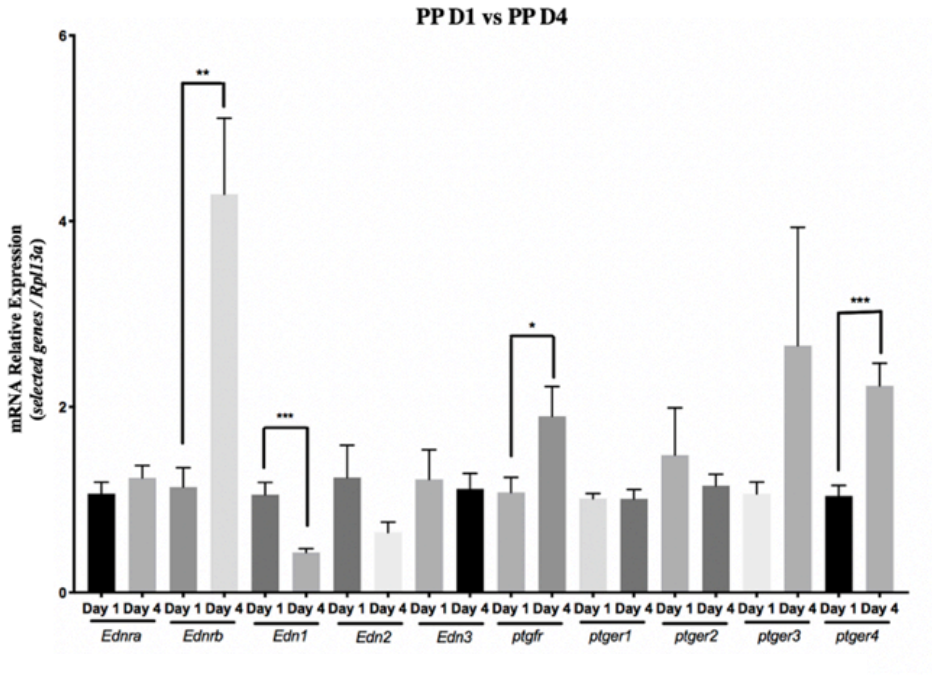
Supplementary Figure 1. PEN time course assay on ERK phosphorylation in SHT90 cell line. PEN failed to trigger a visible change in pERK levels in the SHT290 cells when compared to Ishikawa cell line (Figure 2B). These findings suggest that while both cell lines express *GPR83*, Ishikawa cells produce higher levels of functional GPR83 than the SHT290.

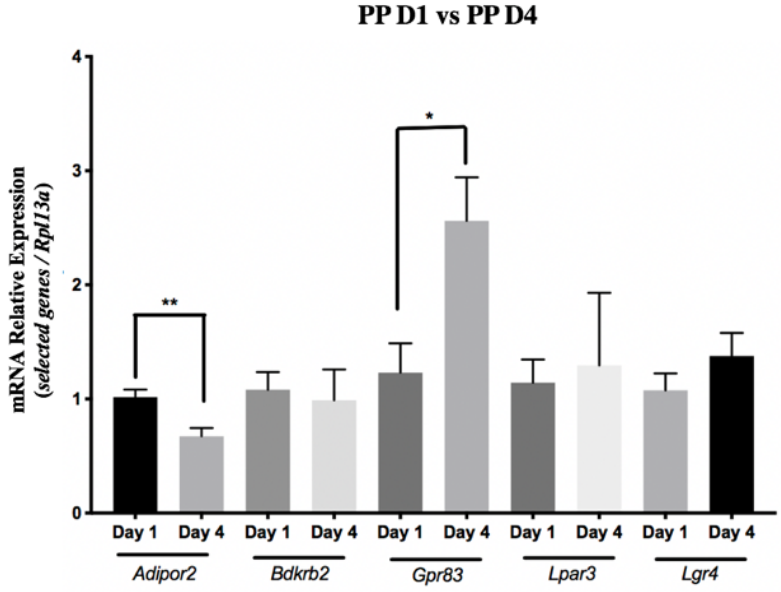
Supplementary Figure 2



Supplementary Figure 2. PEN dose response assay. Ishikawa cells were treated with various concentrations of PEN (10-1000 nM) and 10 mins later, cell lysates were prepared, and pERK was measured by western blotting. A representative western blot of pERK levels is shown. It was found that among independent assays, the response in pERK formation was variable at 10 and 50 nM PEN but consistent at 100 nM PEN and higher. For that reason, we selected 100 nM PEN as the lowest PEN concentration that gave a consistent and maximal response in pERK formation. Therefore, 100 nM PEN is used in all studies presented in this thesis.

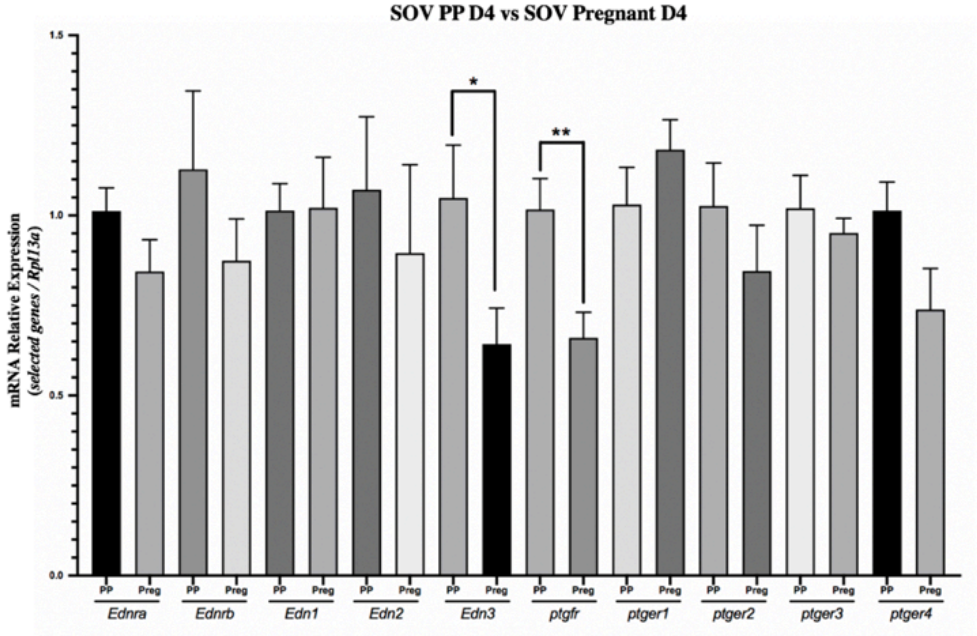
Supplementary Figure 3

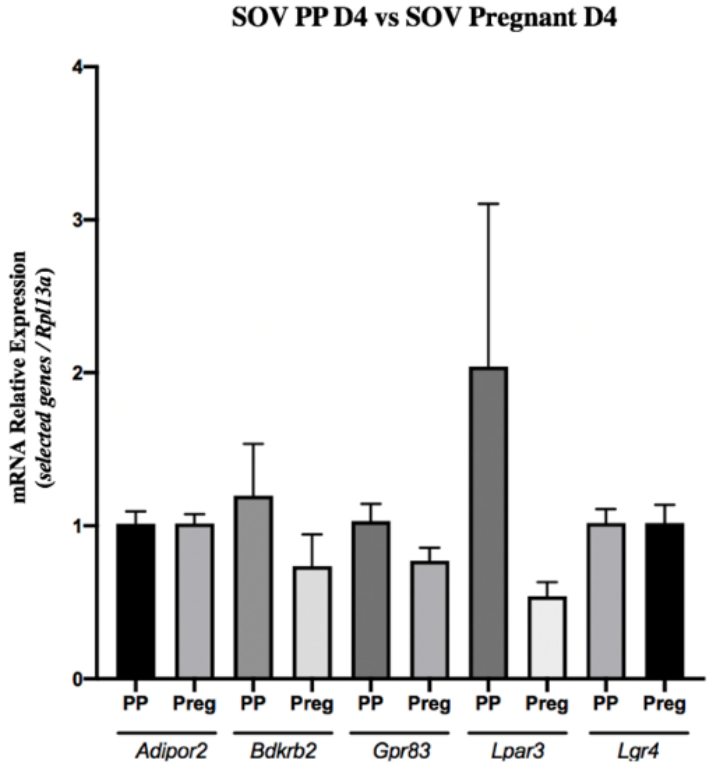




Supplementary Figure 3. Pseudopregnant D1 vs Pseudopregnant D4 gene expression profile: A molecular screen was done looking at various GPCR gene expression in pseudopregnant WT female mice on D1 vs D4 of pregnancy. D1 is characterized as the day that the uterus is "unreceptive" to embryo implantation and D4 is characterized as the day the uterus is "receptive" to embryo implantation should there be one present. Therefore, on D4 the progesterone regulated genes that are crucial for the attainment of uterine receptivity should be elevated when compared to D1. The results revealed that several GPCRs screened were elevated on D4 of pregnancy with *Gpr83* being one of them. This study was conducted by mating WT pseudopregnant female with vasectomized male mice. Female mice that showed a copulatory plug were euthanized on D1 and D4 of pregnancy. Uteri from each group were collected and stored in RNAlater. The tissues were subjected to RNA Extraction. A screen for various GPCRs was performed via qPCR analysis. It was found that the following progesterone regulated genes were observed to be statistically significant between D1 and D4 of pregnancy: *Ednrb*, *Edn1*, *Ptgfr*, *Ptger4*, *Adipor2* and *Gpr83*. ($P<0.05$)

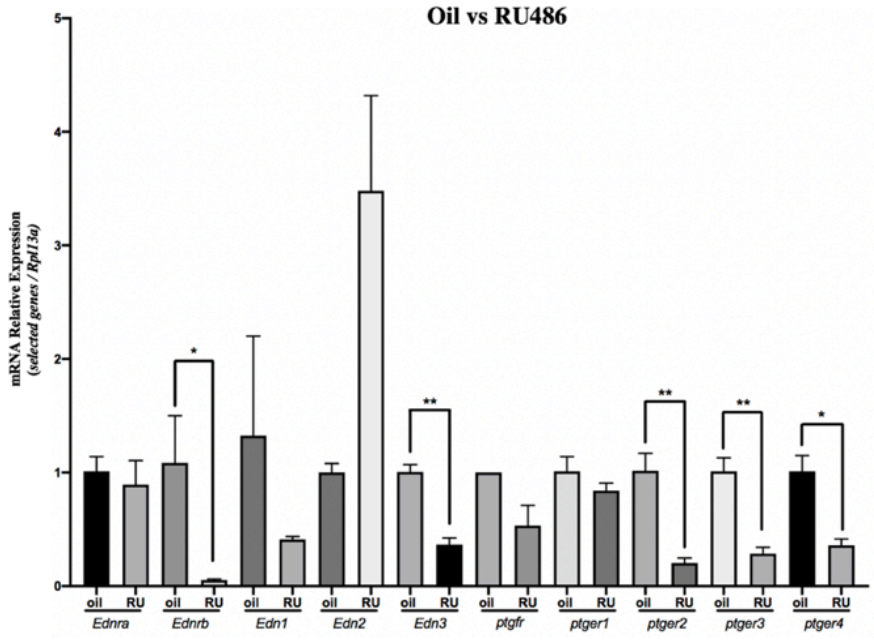
Supplementary Figure 4

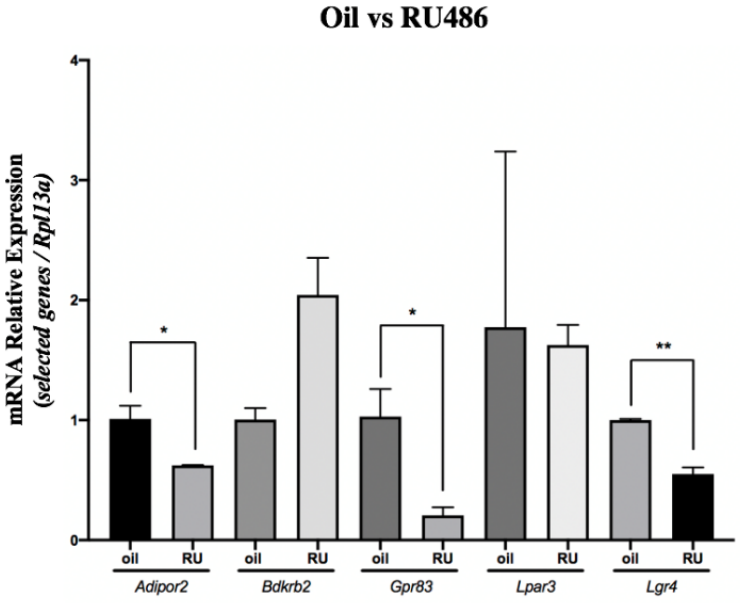




Supplementary Figure 4. Superovulated Pseudopregnant D4 vs Superovulated Pregnant D4: In this study, WT pseudopregnant female mice were mated with vasectomized male mice that resulted in a pseudopregnancy. This was compared to WT females mated to stud males that resulted in a pregnancy. Female mice that showed a copulatory plug were euthanized D4 of pregnancy from both groups. Uteri from each group were collected and stored in RNAlater. The tissues were subjected to RNA Extraction. A qPCR analysis was performed to determine if the embryos in the reproductive tract influence various GPCRs' gene expression on D4 of pregnancy. The data revealed that for majority of GPCRs, the embryo does not influence gene expression and no statistical significance difference was seen in the pregnant vs pseudopregnant group. ($P<0.05$)

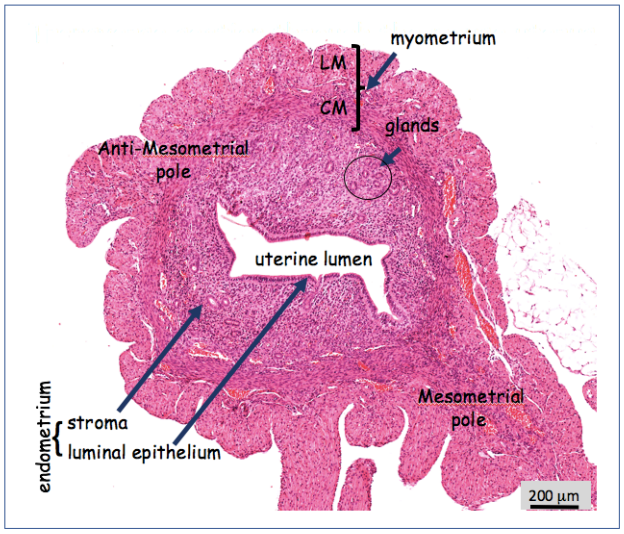
Supplementary Figure 5





Supplementary Figure 5. Oil vs RU486 (pseudopregnant): WT female mice were mated with vasectomized male mice to create a pseudopregnancy. Female mice were injected in the morning of D3 of pregnancy with either oil (control) or RU486. Then 24 hours later, on the morning of D4 the animals were euthanized and uteri were collected for RNA Extraction. The qPCR analysis revealed that there were a number of GPCRs that had a statistically significant change between the two groups analyzed. These genes were as follows: *Ednrb*, *Edn3*, *ptger2*, *ptger3*, *ptger4*, *Adipor2*, *Gpr83* and *Lgr4*. (P<0.05)

Supplementary Figure 6



Supplementary Figure 6 shows a transverse section of the mouse uterine horn stained with hematoxylin and eosin

DISCUSSION

Ovulation on the evening of proestrus (D0 of pregnancy) triggers changes in the uterus that prepares it for embryo implantation on D4 of pregnancy in the event the eggs are fertilized. These changes begin with E2-induced proliferation of the luminal epithelium as seen on D1 and 2 of pregnancy (part of the pre-receptive phase). With the formation of the corpora lutea, P4 signaling on D3 and 4 of pregnancy results in the inhibition of E2-mediated luminal epithelial cell proliferation; this is required for the luminal epithelial cells to differentiate and express a phenotype that can support embryo implantation from the evening of D4 into the morning of D5 during a brief period called the receptive phase. On D4, in the P4-primed uterus, E2 regulates new functions essential for embryo implantation; these are the expression of glandular *Lif* which is viewed as the final step in the acquisition of uterine receptivity and together with P4, E2 induces stromal cell proliferation and decidualization (Calder et al., 2014; Dessauer et al., 2002; Kelleher et al., 2018).

Stromal cell decidualization begins on the afternoon of D5 in cells surrounding the implantation chamber in the antimesometrial region of the uterus and this forms the primary decidual zone (PDZ), a transient tissue that is well developed by D6 but has undergone regression by D8 (Rosario and Stewart, 2016; Abrahamsohn and Zorn, 1993; Welsh and Enders, 1985; Tan eta l., 2002). This PDZ is avascular and is enriched in intercellular tight junctions, gap junctions, and adherens junctions and creates a permeability barrier around the implantation chamber isolating the embryo from immune cells and harmful molecules present in the maternal circulation (Yuan et al., 2019; Tung et al., 1986). Decidualization progresses from around the PDZ towards the myometrium in

the antimesometrial and mesometrial regions where it gives rise to the secondary decidual zone (SDZ). In the antimesometrial region, the SDZ is comprised of the antimesometrial decidua while in the mesometrial region it forms the mesometrial decidua that eventually gives rise to the decidual basalis during placentation. Development of the secondary decidua peaks on D8 with its decline corresponding to the development of the placenta (Parr and Parr, 1986).

While *Gpr83* is expressed throughout the estrous cycle, its expression increases dramatically on D4-6 of pregnancy suggesting roles in embryo implantation and decidualization. While it remains to be determined whether PEN is also available in the uterine environment at the time of GPR83 expression, it was clearly demonstrated that *Pcsk1n*, the gene encoding ProSAAS (the PEN precursor), is expressed in the mouse uterus during the peri-implantation period (Babwah, 2015). Therefore, during this period, it is likely that GPR83 is actively signaling and transmitting PEN signals intracellularly. In the ovariectomized mouse, *Gpr83* expression is unresponsive to P4, suggesting it is not involved in the P4-dependent inhibition of proliferation of the luminal epithelium. However, since *Gpr83* expression is highly induced following the combined treatment of E2 and P4 in the ovariectomized mouse, it is more likely that *Gpr83* regulates stromal cell decidualization and perhaps even decidual function (Wu et al., 2016). This idea is further strengthened by the observation that *Gpr83* is highly expressed in the stroma. Interestingly, maximal *Gpr83* expression at the implantation site was only observed on D6, suggesting that any roles in decidualization might be more pronounced during primary decidualization.

While *Gpr83* mRNA expression declined rapidly at the implantation site on D7 and D8, it remained elevated at the inter-implantation site. *Gpr83* expression also declined in stromal cells as they underwent decidualization *in vitro*. Thus, *Gpr83* appears to be involved in early but not late decidualization. In a transcriptomic analysis of the implantation and inter-implantation sites of the mouse uterus on D5 of pregnancy, pathway analysis found that genes expressed at the inter-implantation site mainly regulated metabolic pathways that provide energy for embryo implantation (Pawar et al., 2015). In contrast, genes expressed at the implantation site encoded proteins that support embryo development and trophoblast invasion (Pawar et al., 2015). Based on the dynamic expression pattern of *Gpr83* during early pregnancy, it is possible that on D4-6 it plays a role in regulating implantation and PDZ development and function while on D7 and D8 it regulates metabolism, a role it also plays in the hypothalamus through its interaction with the ghrelin receptor (Muller et al., 1968). These exciting possibilities await investigation.

It is now well established that in addition to signaling via G proteins, some GPCRs signal via other molecules (Szereszewski et al., 2010). A major class of these molecules is made up of the β-arrestins (Babwah et al., 2015). While β-arrestins terminate G protein-dependent GPCR signaling and mediate receptor internalization, they can also act as molecular scaffolds, coupling receptors to other signaling pathways. In this study, it was demonstrated that the GPR83-$G\alpha_{q/11}$-and-β-arrestin-coupled pathways are major mediators of PEN-induced ERK phosphorylation. These pathways might not necessarily represent redundant signaling as it was previously demonstrated that downstream of the protease-activated receptor-2 (PAR2), G proteins and β-arrestins regulate different pools of cytoplasmic ERK, resulting in different downstream functions (Defea, 2008).

As seen in Figure 3A and B, in Ishikawa cells at 15 mins YM-254890 did not reduce ERK phosphorylation via the $G\alpha_{q/11}$-coupled pathway. But in Figure 4A and B, it is observed that in HEK 293 KO cells at 15 mins, in the absence of β-arrestin signaling, the residual ERK phosphorylation that persisted and would be accounted for by $G\alpha_{q/11}$ signaling was completely blocked by YM-254890. Taken together, the data suggest that $G\alpha_{q/11}$ signaling turns on first and begins to decline by 7.5 mins (as indicated by declining ERK phosphorylation) but by 10 mins β-arrestin signaling has turned on resulting in an increase in ERK phosphorylation before it begins to go back down again. These findings are consistent with what is reported for many other GPCRs that signal via G proteins and β-arrestin, that is, G protein signaling occurs first but is then desensitized by β-arrestin; following desensitization, β-arrestin couples the receptor to ERK resulting in another wave of ERK phosphorylation (Szereszewski et al., 2010).

We recently reported that uterine $G\alpha_{q/11}$ signaling is a major mechanism underlying the acquisition of uterine receptivity and embryo implantation (de Oliveira et al., 2019). It was that finding which led to this study and the identification of uterine GPR83 as a novel $G\alpha_{q/11}$-coupled receptor. This study provides the first report on *Gpr83* expression in the mouse uterus and GPR83 signaling in human endometrial cells. Future investigations will be aimed at studying the function of uterine GPR83 and such studies would be greatly aided through the development of robust antibodies that can be used to study GPR83 protein expression. Based on the current data regarding its expression profile, uterine GPR83 shows promise in emerging as an important regulator of early pregnancy.

REFERENCES

Abman, SH. (2011). Fetal and neonatal physiology (4th ed.). *Philadelphia: Elsevier/Saunders. pp. 46–47.*

Abrahamsohn, P.A., and Zorn, T.M. (1993). Implantation and decidualization in rodents. *J Exp Zool* **266**, 603-28.

Aguilar, H.N., Mitchell, S., Knoll, A.H., and Yuan, X. (2010). Physiological pathways and molecular mechanisms regulating uterine contractility. *Human Reproduction Updates* **16**(6), 725-744.

Alvarez-Curto, E., Inoue, A., Jenkins, L., Raihan, S.Z., Prihandoko, R., Tobin, A.B., et al. (2016). Targeted elimination of G proteins and arrestins defines their specific contributions to both intensity and duration of G protein-coupled receptor signaling. *J Biol Chem* **291**, 27147-59.

Ando, K., Obara, Y., Sugama, J., Kotani, A., Koike, N., Ohkubo, S., et al. (2010). P2Y2receptor Gq/11 signaling at lipid rafts is required for UTP-induced cell migration in NG 108-15 cells. *J Pharmacol Exp Ther* **334**, 809-19.

Babwah, A.V. (2015). Uterine and placental KISS1 regulate pregnancy: what we know and the challenges that lie ahead. *Reproduction* **150**, R121-8.

Babwah, A.V. (2020). The wonderful and masterful G protein-coupled receptor (GPCR): a focus on signaling mechanisms and the neuroendocrine control of fertility. *Molecular and Cellular Endocrinology* **515**, 110886.

Babwah, A.V., Navarro, V.M., Ahow, M., Pampillo, M., Nash, C., Fayazi, M. *et al.* (2015). GnRH neuron-specific ablation of Galphaq/11 results in only partial inactivation of the neuroendocrine-reproductive axis in both male and female mice: in vivo evidence for Kiss1r-coupled Galphaq/11-independent GnRH secretion. *J Neurosci* **35**, 12903-16.

Bagchi, I.C., Li, Q., and Cheon, Y.P. (2001). Role of steroid hormone-regulated genes in implantation. *Ann N Y Acad Sci* **943**, 68-76.

Barbier, C.S., Becker, K.A., Troester, M.A., and Kaufman, D.G. (2005). Expression of exogenous human telomerase in cultures of endometrial stromal cells does not alter their hormone responsiveness. *Biol Reprod* **73**,106-14.

Benson, G.V., Lim, H., Paria, B.C., Satokata, I., Dey, S.K., and Maas, R.L. (1996). Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10 expression. *Development* **122**, 2687–2696.

Beretsos, P., Loutradis, D., Koussoulakos, S., Margaritis, L. H., Kiapekou, E., Mastorakos, G., Papaspirou, I., Makris, N., Makrigiannakis, A., and Antsaklis, A. (2006). Oxytocin receptor is deferentially expressed in mouse endometrium and embryo during blastocyst implantation. *Ann. N. Y. Acad. Sci* **1092**, 466–479.

Bhurke, A.S., Bagchi, I.C., and Bagchi, M.K. (2016). Progesterone-regulated endometrial factors controlling implantation. *Am J Reprod Immunol* **75**(3), 237-245.

Bhurke, A.S., Bagchi, I.C., and Bagchi, M.K. (2016). Progesterone-regulated endometrial factors controlling implantation. *Am J Reprod Immunol* **75**, 237-245.

Brezillon, S., Detheux, M., Parmentier, M., Hokfelt, T., and Hurd, Y.L. (2001). Distribution of an orphan G-protein coupled receptor (JP05) mRNA in the human brain. *Brain Res* **921** (1–2), 21–30.

Bologna, Z., Teoh, J., Bayoumi, AS., Tang, Y., and Kim, I. (2017). Biased G protein-coupled receptor signaling: new player in modulating physiology and pathology. *Biomolecules & Therapeutics* **25**, 12-25.

Calder, M., Chan, Y. M., Raj, R., Pampillo, M., Elbert, A., Noonan, M., Gillio-Meina, C., Caligioni, C., Be´rube´, N. G., Bhattacharya, M., Watson, A. J., Seminara, S. B., and Babwah, A. V. (2014) Implantation failure in female Kiss1-/- mice is independent of their hypogonadic state and can be partially rescued by leukemia inhibitory factor. *Endocrinology* **155**, 3065–3078.

Caligioni, C.S. (2009). Assessing reproductive status/stages in mice. Curr Protoc Neu-rosci, Appendix4: Appendix 4I.

Cha, J., Sun, X., and Dey, S.K. (2012). Mechanisms of implantation: strategies for successful pregnancy. *Nat Med* **18**, 1754–1767.

Cha, J., Sun, X., and Dey, SK. (2012). Mechanisms of implantation: strategies for successful pregnancy. *Nat Med* **18**, 1754-1767.

Chakraborty, I., Das, S., Wang, J., and Dey, S. (1996). Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids. *Journal of Molecular Endocrinology **16***(2), 107-122.

Chen, J.R., Cheng, J.G., Shatzer, T., Sewell, L., Hernandez, L., and Stewart, C.L. (2000). Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinol* **141**, 4365-4372.

Chen, K., Chen, X., He, J., Ding, Y., Geng, Y., Liu, S. *et al.* (2015). Mouse endometrium temporal and spatial expression mRNA and microRNA associated with embryo implantation. *Reprod Sci* **22**, 1399 408.

Cohen, M., Meisser, A., and Bischof, P. (2006). Metalloproteinases and human placental invasiveness. *Placenta* **27**, 783-793.

Cole, LA. (2010). Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology: RB&E* (8**)**, 102.

Conneely, O.M., and O'Malley B.W. (1995). Mice lacking progesterone receptors exhibit pleiotropic reproductive abnormalities. *Genes and Dev* **9**, 2266–2278.

Coulam, C. (2016). What about superfertility, decidualization, and natural selection? *J Assist Reprod Genet* **33**, 577-580.

D'Amico, D., and Barbarito, C. (2015). Health & Physical Assessment in Nursing. *Boston: Pearson*. p.645.

Das, S.K. (2010). Regional development of uterine decidualization: molecular signaling by Hoxa-10. *Molecular Reproduction & Development* **77**, 387-396.

Das, S.K., Wang, X.N., Paria, B.C., Damm, D., Abraham, J.A., Klagsbrun, M., et al. (1994). Heparin binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development* **120**, 1071-83.

Das, SK. (2010). Regional development of uterine decidualization: molecular signaling by Hoxa-10. *Molecular Reproduction and Development* **77**(5), 387–396.

De Moerlooze, L., Williamson, J., Liners, F., Perret, J., and Parmentier, M. (2000). Cloning and chromosomal mapping of the mouse and human genes encoding the orphan glucocorticoid-induced receptor (GPR83). *Cytogenet Cell Genet* **90**, 146–150.

De Oliveira, V., Schaefer, J., Calder, M., Lydon, J.P., DeMayo, F.J., Bhattacharya M. *et al.* (2019). Uterine Galphaq/11 signaling, in a progesterone-dependent manner, critically regulates the acquisition of uterine receptivity in the female mouse. *FASEB J* **33**, 9374-87.

Decaillot, F.M., Che, F.Y., Fricker, L.D., and Devi, L.A. (2006). Peptidomics of Cpefat/fat mouse hypothalamus and striatum: effect of chronic morphine administration. *J. Mol. Neurosci* **28** (3), 277–84.

Defea, K. (2008). Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. *Br J Pharmacol* **153** Suppl 1, S298-309.

Deng, W., Cha, J., Yuan, J., Haraguchi, H., Bartos, A., Leishman, E., et al. (2016). p53 coordinates decidual sestrin 2/AMPK/mTORC1 signaling to govern parturition timing. *J Clin Invest* **26**, 2941-54.

Dey, S.K., Lim, H., Das, S.K., Reese, J., Paria, B.C., Daikoku, T., and Wang, H. (2004). Molecular cues to implantation. *Endocr Rev* **25**, 341–373.

Diao, H., Li, R., El Zowalaty, A.E., Xiao, S., Zhao, F., Dudley, E.A., and Ye, X. (2015). Deletion of lysophosphatidic acid receptor 3 (Lpar3) disrupts fine local balance of progesterone and estrogen signaling in mouse uterus during implantation. *Biol. Reprod* **93**, 123.

Enders, A. (1967). A morphological analysis of the early implantation stages in the rat. *Am J Anat* **125**, 1-29.

Fakira, A.K., Peck, E.G., Liu, Y., Lueptow, L.M., Trimbake, N.A., Han, M.H., et al. (2019). The role of the neuropeptide PEN receptor, GPR83, in the reward pathway: relationship to sex-differences. *Neuropharmacology* **157**, 107666.

Fayazi, M., Calder, M., Bhattacharya, M., Vilos, G. A., Power, S., and Babwah, A. V. (2015). The pregnant mouse uterus exhibits a functional kisspeptin/KISS1R signaling system on the day of embryo implantation. *Reprod. Biol. Endocrinol* **13**, 105.

Feng, Y., Reznik, S.E., and Fricker, L.D. (2001). Distribution of proSAAS-derived peptides in rat neuroendocrine tissues. *Neuroscience* **105**, 469–478.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A, and Rudensky, A.Y. (2005). A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* **6**, 1142-51.

Franasiak, J.M., and Scott, R.T. (2015). Reproductive tract microbiome in assisted reproductive technologies. *Fertility and Sterility* **104**(6), 1364-1371.

Fricker, L.D., McKinzie, A.A., Sun, J., Curran, E., Qian, Y., Yan, L., Patterson, S.D., Courchesne, P.L., Richards, B., Levin, N., Mzhavia, N., Devi, L.A., and Douglass, J. (2000). Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. *J. Neurosci* **20** (2), 639–48.

Gautam, N., Downes, G. B., Yan, K. and Kisselev, O. (1998). The G- protein β gamma complex. *Cell. Signal* **10**, 447-455.

Gellersen, B., and Brosens, J.J. (2014). Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev* **35**, 851-905.

Genbacev, O.D., Prakobphol, A., Foulk, R.A., Krtolica, A.R., Ilic, D., Singer, M.S., Yang, Z.Q., Kiessling, L.L., Rosen, S.D., and Fisher, S.J. (2003). Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. *Science* **299**, 405-408.

Georgadaki, K., Khoury, N., Spandidos, D. A., and Zoumpourlis, V. (2016). The molecular basis of fertilization (Review). *International Journal of Molecular Medicine* **38**(4), 979–986.

Gether, U. (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrine Reviews* **21**(1), 90-113.

Gimpl, G., and Fahrenholz F. (2001). The oxytocin receptor system: structure, function, and regulation. *Physiological reviews* **81**(2), 629-83.

Gomes, I., Bobeck, E.N., Margolis, E.B., Gupta, A., Sierra, S., Fakira, A.K., et al. (2016). Identification of GPR83 as the receptor for the neuroendocrine peptide PEN. *Sci Signal* **9**, ra43.

Gong, K.Z., Li, Z.J., Xu, M., Du, J.H., Lv, Z.Z., and Zhang, Y.Y. (2008). A novel protein kinase A independent, β-arrestin-1-dependent signaling pathway for p38 mitogen-activated protein kinase activation by β2-adrenergic receptors. *J. Biol. Chem* **283**, 29028-29036.

Hamm, H. E. (1998). The many faces of G protein signaling. *J. Biol. Chem* **273**, 669-672.

Harrigan, M.T., Baughman, G., Campbell, N.F., and Bourgeois, S. (1989). Isolation and characterization of glucocorticoid-and cyclic AMP-induced genes in T lymphocytes. *Mol Cell Bio* **9**, 3438-46.

Harrigan, M.T., Campbell, N.F., and Bourgeois, S. (1991). Identification of a gene induced by glucocorticoids in murine T-cells: a potential G protein-coupled receptor. *Mol Endo* **5**, 1331-8.

Jeong, J.W., Kwak, I., Lee, K.Y., Kim, T.H., Large, M.J., Stewart, C.L., et al. (2010). Foxa2 is essential for mouse endometrial gland development and fertility. *Biol Repro* **83**, 396-403.

Kelleher, A.M., Milano-Foster, J., Behura, S.K., and Spencer, T.E. (2018). Uterine glands coordinate on time embryo implantation and impact endometrial decidualization for pregnancy success. *Nat Commun* **9**, 2435.

Kida, T., Oyama, K., Sone, M., Koizumi, M., Hidema, S., and Nishimori, K. (2014), Lgr4 is required for endometrial receptivity acquired through ovarian hormone signaling. *Biosci. Biotechnol. Biochem* **78**, 1813–1816.

Kimber, S. (2005). Leukaemia inhibitory factor in implantation and uterine biology. *Reproduction* **130**(2), 131-145.

King, N., Hittinger, C.T., and Carroll, S.B. (2003). Evolution of key cell signaling and adhesion protein families predates animal origins. *Science* **301**(5631), 361–3.

Ledee-Bataille, N., Lapree-Delage, G., Taupin, J.L., Dubanchet, S., Frydman, R., and Chaouat G. (2002). Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. *Hum Reprod* **17**, 213–218.

Lee, K.Y., and DeMayo, F.J. (2004). Animal models of implantation. *Reproduction* **128**(6), 679-95.

Lee, K.Y., Jeong, J-W., Tsai, S.Y., Lydon, J.P., and DeMayo, F.J. (2007). Mouse models of implantation. *Trends Endocrinol Metab* **18**, 234– 239.

Lefkowitz, R. J. (2013). Arrestins come of age: a personal historical perspective. *Prog. Mol. Biol. Transl. Sci* **118**, 3-18.

Lefkowitz, R.J. (2000). β-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**, 1574-1577.

Leon, S., Barroso, A., Vazquez, M.J., Garcia-Galiano, D., Manfredi-Lozano, M., Ruiz-Pino, F., et al. (2016). Direct actions of kisspeptins on GnRH neurons permit attainment of fertility but are insufficient to fully preserve gonadotropic axis activity. *Sci Rep* **6**, 19206.

Li, Q., Kannan, A., DeMayo, F.J., Lydon, J.P., Cooke, P.S., Yamagishi, H., et al. (2011). The anti proliferative action of progesterone in uterine epithelium is mediated by Hand2. *Science* **331**, 912-6.

Lim, H., Paria, B.C., Das, S.K., Dinchuk, J.E., Langenbach, R., Trzaskos, J.M., and Dey, S.K. (1997). Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91**, 197-208.

Lim, H., Paria, B.C., Das, S.K., et al. (1997). Multiple female reproductive failures in cyclooxygenase 2 deficient mice. *Cell* **91**(2), 197–208.

Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S., and Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences* **90**(23), 11162-11166.

Lueptow, L.M., Devi, L.A., and Fakira, A.K. (2018). Targeting the recently deorphanized receptor GPR83 for the treatment of immunological, neuroendocrine and neuropsychiatric disorders. *Prog Mol Biol Transl Sci* **159**, 1-25.

Luttrell, L.M., Ferguson, S.S., Daaka, Y., Miller, W.E., Maudsley, S., Della Rocca, G.J., Lin, F.T., Kawakatsu, H., Owada, K., Luttrell, D.K., Caron, M.G., and Lefkowitz, R.J. (1999). β-arrestin-dependent formation of β(2) adrenergic receptor-Src protein kinase complexes. *Science* **283**, 655-661.

Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, C.A Jr., Shyamala, G., Ma, L., and Pei, G. (2007). Beta-arrestin signaling and regulation of transcription. *J Cell Sci* **120**(2), 213-8.

Ma, X., Gao, F., Rusie, A., Hemingway, J., Ostmann, A.B., Sroga, J.M., et al. (2011). Decidual cell polyploidization necessitates mitochondrial activity. *PLoS One* **6**, e26774.47.

Matsumoto, H., Ma, W., Smalley, W., Trzaskos, J., Breyer, R.M., and Dey, S.K. Diversification of Cyclooxygenase-2-Derived Prostaglandins in Ovulation and Implantation. *Biology of Reproduction* **64**(5), 1557–1565.

McDonald, P.H., Chow, C.W., Miller, W.E., Laporte, S.A., Field, M.E., Lin, F.T., Davis, R.J., and Milligan, G., and Inoue, A. (2018). Genome editing provides new insights into receptor controlled signaling pathways. *Trends Pharmacol Sci* **39**, 481-93.

Mohri, Y., Umezu, T., Hidema, S., Tomisawa, H., Akamatsu, A., Kato, S., Nawa, A., and Nishimori, K. (2010). Reduced fertility with impairment of early-stage embryos observed in mice lacking Lgr4 in epithelial tissues. *Fertil. Steril* **94**, 2878–2881.

Morgan, D.J., Wei, S., Gomes, I., Czyzyk, T., Mzhavia, N., Pan, H., Devi, L.A., Fricker, L.D., and Pintar, J.E. (2010). The propeptide precursor proSAAS is involved in fetal neuropeptide processing and body weight regulation. *J. Neurochem* **113** (5), 1275–84.

Mulac-Jericevic, B., Mullinax, R.A., DeMayo, F.J., Lydon, J.P., and Conneely, O.M. (2000). Subgroup of

reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* **289**, 1751–1754.

Muller, T.D., Muller, A., Yi, C.X., Habegger, K.M., Meyer, C.W., Gaylinn, B.D. *et al.* (2013). The orphan receptor Gpr83 regulates systemic energy metabolism via ghrelin-dependent and ghrelin-independent mechanisms. *Nat Commun* **4**, 1968.

Mzhavia, N., Berman, Y., Che, F.Y., Fricker, L.D., and Devi, L.A. (2001). ProSAAS processing in mouse brain and pituitary. *J Biol Chem* **276**, 6207–6213.

Mzhavia, N., Qian, Y., Feng, Y., Che, F.Y., Devi, L.A., and Fricker, L.D. (2002). Processing of proSAAS in neuroendocrine cell lines. *Biochem. J* **361** (Pt 1), 67–76.

Neumann, E., Khawaja, K., and Müller-Ladner U. (2014). G protein-coupled receptors in rheumatology. *Nature Reviews Rheumatology* **10**(**7**), 429.

Niringiyumukiza, J.D., Cai, H. and Xiang, W. (2018). Prostaglandin E2 involvement in mammalian female fertility:ovulation, fertilization, embryo development and early implantation. *Reprod Biol Endocrinol* **16**, 43.

Okada, H., Tsuzuki, T., and Murata, H. (2018). Decidualization of the human endometrium. *Reproductive Medicine and Biology* **17**(3), 220–227.

Pandy-Szekeres, G., Munk, C., Tsonkov, T.M., Mordalski, S., Harpsøe, K., Hauser, A.S., Bojarski, A.J., and Gloriam, D.E. (2018). GPCR in 2018: adding GPCR structure models and ligands. *Nucleic Acids Res* **46**(D1), D440–D446.

Parobchak, N., Rao, S., Negron, A., Schaefer, J., Bhattacharya, M., Radovick, S., and Babwah, A.V. (2020). Uterine Gpr83 mRNA is highly expressed during early pregnancy and GPR83 mediate the actions of PEN in endometrial and non-endometrial cells. *F&S Science*. (in press).

Parr, M.B., and Parr, E.L. (1986). Permeability of the primary decidual zone in the rat uterus: studies using fluorescein-labeled proteins and dextrans. *Biol Reprod* **34**, 393-403.

Pawar, S., Hantak, A.M., Bagchi, I.C., and Bagchi, M.K. (2014). Minireview: steroid regulated paracrine mechanisms controlling implantation. *Molecular Endocrinology* **28**(9,1), 1408-1422.

Pawar, S., Laws, M.J., Bagchi, I.C., and Bagchi, M.K. (2015). Uterine epithelial estrogen receptor-alpha controls decidualization via a paracrine mechanism. *Mol Endocrinol* **29**, 1362-74.

Pesini, P., Detheux, M., Parmentier, M., and Hokfelt, T. (1998). Distribution of a glucocorticoid- induced orphan receptor (JP05) mRNA in the central nervous system of the mouse. *Mol. Brain Res* **57** (2), 281–300.

Pierzynski, P., Reinheimer, T.M., and Kuczynski, W. (2007). Oxytocin antagonist may improve infertility treatment. *Fertility and Sterility* **88**(1), 213.e19-213.e22.

Purdue, B.W., Tilakaratne, N., and Sexton, P.M. (2002). Molecular pharmacology of the calcitonin receptor. *Receptors & Channels* **8**(3-4), 243–55.

Radovick, S., and Babwah, A.V. (2019). Regulation of pregnancy: evidence for major roles by the uterine and placental Kisspeptin/KISS1R signaling systems. *Semin Reprod Med* **37**, 182-190.

Ramathal, C.Y., Bagchi, I.C., Taylor, R.N., and Bagchi, M.K. (2010). Endometrial decidualization: of mice and men. *Seminars in Reproductive Medicine* **28**(1), 17–26.

Ratajczak, C., Fay, J.C., and Muglia, L. (2010). Preventing preterm birth: The past limitations and new potential of animal models. *Disease Models and Mechanisms* **3**(7-8), 407-14.

Rosario, G.X., and Stewart, C.L. (2016). The Multifaceted Actions of Leukaemia Inhibitory Factor in Mediating Uterine Receptivity and Embryo Implantation. *Am J Reprod Immunol* **75**, 246-55.

Rowlands, T.M., Symonds, J.M., Farookhi, R., and Blaschuk, O.W. (2000). Cadherins: Crucial regulators of structure and function in reproductive tissues. *Rev Reprod* **5**, 53-61.

Ruane, P.T., Berneau, S.C., Koeck, R., Watts, J., Kimber, S.J., Brison, D.R., Westwood, M., and Aplin, J.D. (2017). Apposition to endometrial epithelial cells activates mouse blastocysts for implantation. *Molecular Human Reproduction* **23**(9), 617–627.

Sayah, M., Fortenberry, Y., Cameron, A., and Lindberg, I. (2001). Tissue distribution and processing of proSAAS by proprotein convertases. *J Neurochem* **76**, 1833–1841.

Schatz, F., Guzeloglu-Kayisli, O., Arlier, S., Kayisli, U.A., and Lockwood, C.J. (2016). The role of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy outcomes and abnormal uterine bleeding. *Hum Reprod Update* **22**, 497-515.

Sharma, S. (2014). Natural killer cells and regulatory T cells in early pregnancy loss. *Int J Dev Biol* **58**, 219-29.

Shenoy, S.K., McDonald, P.H., Kohout, T.A., and Lefkowitz, R.J. (2001). Regulation of receptor fate by ubiquitination of activated β2- adrenergic receptor and β-arrestin. *Science* **294**, 1307-1313.

Shukla, A.K., Xiao, K.H., and Lefkowitz, R.J. (2011). Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem. Sci* **36**, 457-469.

Simon, C., Martin, J.C., and Pellicer A. (2000). Paracrine regulators of implantation. *Baillieres Best Pract Res Clin Obstet Gynaecol* **14**, 815–826.

Simon, C., Moreno, C., Remohi, J., and Pellicer, A. (1998). Cytokines and embryo implantation. *J Reprod Immuno* **39**, 117–131.

Skiba, N.P., Bae, H., and Hamm, H.E. (1996). Mapping of effector binding sites of transducin α-subunit using Gαt/Gαi1 chimeras. *J. Biol. Chem* **271**, 413-424.

Sone, M., Oyama, K., Mohri, Y., Hayashi, R., Clevers, H., and Nishimori, K. (2013). LGR4 expressed in uterine epithelium is necessary for uterine gland development and contributes to decidualization in mice. *FASEB J* **27**, 4917–4928.

Song, X.F., Coffa, S., Fu, H.A., and Gurevich, V.V. (2009). How does arrestin assemble MAPKs into a signaling complex? *J. Biol. Chem* **284**, 685-695.

Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S.J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **35**, 976–79.

Sugimoto, N., Oida, T., Hirota, K., Nakamura, K., Nomura, T., Uchiyama, T., et al. (2006). Foxp3 dependent and independent molecules specific for CD25+CD4+natural regulatory T cells revealed by DNA microarray analysis. *Int Immunol* **18**, 1197-209.

Szereszewski, J.M., Pampillo, M., Ahow, M.R., Offermanns, S., Bhattacharya, M., and Babwah, A.V. (2010). GPR54 regulates ERK1/2 activity and hypothalamic gene expression in a Galpha(q/11) and beta-arrestin-dependent manner. *PLoS One* **5**, e12964.

Takasaki, J., Saito, T., Taniguchi, M., Kawasaki, T., Moritani, Y., Hayashi, K., et al. (2004). A novel Galphaq/11-selective inhibitor. *J Biol Chem* **279**, 47438-45.

Tan, J., Raja, S., Davis, M.K., Tawfik, O., Dey, S.K., and Das, S.K. (2002). Evidence for coordinated interaction of cyclin D3 with p21 and cdk6 in directing the development of uterine stromal cell decidualization and polyploidy during implantation. *Mech Dev* **111**, 99-113.

Tilley, S.L., Audoly, L.P., Hicks, E.H., Kim, H.S., Flannery, P.J., Coffman, T.H., and Koller, B.H. (1999). Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E2 receptor. *J Clin Invest* **103**(11), 1539-45.

Tortora, G., and Derrickson, B. (2011). Principles of Anatomy & Physiology (13th ed.). *Wiley. P.1105*.

Tung, H.N., Parr, M.B., and Parr, E.L. (1986). The permeability of the primary decidual zone in the rat uterus: an ultrastructural tracer and freeze-fracture study. *Biol Reprod* **35**, 1045-58.

Tuteja, N. (2009). Signaling through G protein coupled receptors. *Plant Signaling & Behavior* **4**(10), 942 947.

U.S. Department of Health and Human Services Office on Women's Health. Infertility. Retrieved April 2019, from https://www.women- shealth.gov/a-z-topics/infertility.

Vilella, F., Ramirez, L., Berlanga, O., et al. (2013). PGE2 and PGF2*α* concentrations in human endometrial fluid as biomarkers for embryonic implantation. *Journal of Clinical Endocrinology & Metabolism* **98**(10), 4123–4132.

Wardman, J.H., and Fricker, L.D. (2014). ProSAAS-derived peptides are differentially processed and sorted in mouse brain and AtT-20 cells. *PLoS One* **9** (8), No. e104232.

Wei, Q., St Clair, J.B., Fu, T., Stratton, P., and Nieman, L.K. (2009). Reduced expression of biomarkers associated with the implantation window in women with endometriosis. *Fertil Steril* **91**, 1686–1691.

Wei, S., Feng, Y., Che, F.Y., Pan, H., Mzhavia, N., Devi, L.A., McKinzie, A.A., Levin, N., Richards, W.G., and Fricker, L.D. (2004). Obesity and diabetes in transgenic mice expressing proSAAS. *J. Endocrinol* **180** (3), 357–68.

Welsh, A.O., and Enders, A.C. (1985). Light and electron microscopic examination of the mature decidual cells of the rat with emphasis on the antimesometrial decidua and its d degeneration. *Am J Anat* **172**, 1-29.

Wettschureck, N., and Offermanns, S. (2005). Mammalian G proteins and their cell type specific functions. *Physiol. Rev* **85**, 1159–1204.

Wu, W., Kong, S., Wang, B., Chen, Y., and Wang, H. (2016). Spatiotemporal expression of endogenous opioid processing enzymes in mouse uterus at peri-implantation. *Cell Tissue Res* **363**, 555-65.

Ye, X., and Chun, J. (2010). Lysophosphatidic acid (LPA) signaling in vertebrates reproduction. *Trends in Endocrinology and Metabolism: TEM* **21**(1), 17-24.

Ye, X., Hama, K., Contos, J.J., Anliker, B., Inoue, A., Skinner, M.K., Suzuki, H., Amano, T., Kennedy, G. Arai, H., Aoki, J., and Chun, J. (2005). LPA3-mediated lysophosphatidic acid signaling in embryo implantation and spacing. *Nature* **435**(7038), 104-108.

Yoo, I., Chae, S., Han, J., Lee, S., Kim, H. J., & Ka, H. (2019). Leukemia inhibitory factor and its receptor: expression and regulation in the porcine endometrium throughout the estrous cycle and pregnancy. *Asian Australasian Journal of Animal Sciences **32***(2), 192–200.

Yuan, J., Aikawa, S., Deng, W., Bartos, A., Walz, G., Grahammer, F., *et al.* (2019). Primary decidual zone formation requires Scribble for pregnancy success in mice. *Nat Commun* **10**, 5425.

Zhang, S., Kong, S., Lu, J., Wang, Q., Chen, Y., Wang, W., Wang, B., and Wang, H. (2013). Deciphering the molecular basis of uterine receptivity. *Mol. Reprod. Dev* **80**, 8-21.

Zhang, Y., Chen, Q., Zhang, H., Wang, Q., Li, R., Jin, Y., et al. (2015). Aquaporin-dependent excessive intrauterine fluid accumulation is a major contributor in hyper-estrogen induced aberrant embryo implantation. *Cell Res* **5**, 139-42.

Zhu, L.J., Bagchi, M.K., and Bagchi, I.C. (1998). Attenuation of calcitonin gene expression in pregnant rat uterus leads to a block in embryonic implantation. *Endocrinology* **139**(1), 330–339.