Effects of Gonadotropin-Releasing Hormone Receptor Antagonists on Ectopic Human Trophoblast Cell Invasion

by

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Abstract

Ectopic pregnancy (EP) is a life-threatening condition responsible for 6.5% of Canadian mothers' death between 1993-2004. EP occurs when an embryo implants outside the uterine endometrium, commonly in the Fallopian tube (98%). Tubal EP often requires surgical intervention as a life-saving measure. Early and precise diagnosis allow many women to choose methotrexate, the only available medical option, for treatment. Methotrexate is effective in 70% of patients; however, they may require multiple treatments, suffer moderate-to-severe side effects, or experience sub-optimal responses. The remaining 30% fail to respond to the treatment.

Gonadotropin-releasing hormone (GnRH) has an inducing effect on intrauterine placental cell invasion and human chorionic gonadotropin hormone (hCG) production. GnRH receptor (GnRH-R) antagonists have been used safely to treat endometriosis, chronic pelvic pain, and in the artificial fertilization techniques. For the first time, we separated primary trophoblast cells from tubal EP placentas. We validated their trophoblastic origin using trophoblast-validating markers. The preliminary findings showed that primary EP trophoblast cells express GnRH and GnRH receptor. Treating these cells with clinical GnRH-R antagonist, cetrorelix, suppressed their viability. We used primary trophoblast cells from tubal pregnancies, immortalized HTR-8/SVneo cell line, and placental villous explants established from the first-trimester human intrauterine placenta in this study. We investigated the effect of clinical GnRH-R antagonists, cetrorelix and ganirelix, on the invasion of different trophoblast models. Finding a safe, effective, and more tolerant medical treatment for tubal pregnancy will significantly improve women's health and reduce healthcare-related costs.

Lay Summary

Ectopic pregnancy (EP) occurs when embryo implants outside the womb, commonly in Fallopian tube. Tubal rupture and catastrophic bleeding may occur that necessities life-saving tubal removal surgery. Methotrexate (MTX) is the only medical treatment available for EP and is effective in 70% of patients who may require multiple treatments or experience moderate-to-severe side effects. The remaining 30% do not respond to MTX and require surgery. Gonadotropin-releasing hormone (GnRH) receptor blockers (antagonists) used clinically to treat several gynecological conditions. Their potential effect as a novel medical treatment for EP was studied in this thesis. EP supporting cells express GnRH and GnRH receptors. Treating them with GnRH antagonists (GnRH-ants) in culture interferes with their growth and invasion. Antagonists influence on EP cells and placental explants will support future clinical trial for GnRH-ants as a medical treatment. Providing a safer and effective EP medical treatment will significantly improve women health and reduce costs.

Preface

The research project of this thesis was developed in Dr. Bedaiwy laboratory at BC Children's Hospital Research Institute (BCHRI). The research was supported by Nelly Auersperg Award from the Women's Health Research Institute and BC Women's Hospital & Health Centre.

I participated in the experimental design under the supervision of Dr. Bedaiwy and Dr. Bo Peng (a previous post-doctoral fellow) and conducted all listed experiments, data collection, and analysis in chapters 6 and 7.

Chapter 6 is based on the isolation of primary trophoblast cells from tubal pregnancy placentas. The tissue collection was approved by the University of British Columbia (UBC) Ethics Board, under the approval number: H15-02234. I was responsible for transferring tissues from Vancouver General Hospital (VGH) to our laboratory located at BCHRI. The isolation of trophoblast cells from these samples was also among my responsibilities. The validation step of these cells was led by Dr. Peng. Finally, I have conducted the trans-well invasion assays and data analysis.

Chapter 7 is based on placental explant establishment that was conducted at Dr. Alexander Beristain laboratory, (UBC, Vancouver, BC). The experiment was performed with the assistance of Jenna Treissman**,** a graduate student at Dr. Beristain laboratory, who helped in culturing the explants, picking the images, and fixing the tissue.

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List of Symbols

$	American dollar
<	Less than
>	More than
β	Beta
α	Alpha
^{0} C	Degrees Celsius
%	Percentage

List of Abbreviations

AA	Antimicrobial antibiotic
ADAM	A disintegrin and metalloprotease domain
ACOG	American College of Obstetricians and Gynecologists
ANOVA	Analysis of variance
BCHRI	BC Children's Hospital Research Institute
BrdU	Bromodeoxyuridine
CAM	Cellular adhesion molecule
CB	Endocannabinoid receptor
CBF	Ciliary beat frequency
CH2FH4	Methylenetetrahydrofolate
CK	Cytokeratin
CO_2	Carbon dioxide
COS	Controlled ovarian stimulation
COX	Cyclooxygenase
CSEP	Caesarean scar ectopic pregnancy
CT	Cytotrophoblast cells
Ctx	Cetrorelix
DELFIA	Dissociation-enhanced lanthanide fluorescence immunoassay
DMEM	Dulbeco's modified eagle's medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline

dUMP	Deoxyuridine monophosphate
ECM	Extracellular matrix
eEVT	Endovascular extravillous trophoblast
EP	Ectopic pregnancy
ERB	Ethics Research Board
EVCT	Extravillous cytotrophoblast
EVTs	Extravillous trophoblast cells
FBS	Fetal bovine serum
FDA	Food and drug administration
FSH	Follicle-stimulating hormone
Gx	Ganirelix
h	Hour
hCG	Human chorionic gonadotropin hormone
HOXA	Homeobox genes
iEVT	Interstitial extravillous trophoblast
ICC	Interstitial cells of Cajal
ICSI	Intracytoplasmic sperm injection
IL	Interleukin
IU	International unit
GnRH	Gonadotropin-releasing hormone
GnRH-ants	GnRH antagonists
GnRH-R	Gonadotropin-releasing hormone receptor

L	Liter
LH	Luteinizing hormone
LIF	Leukemia inhibitor factor
mg	Milligram
mIU	Milli-international unit
mL	Milliliter
mRNA	Messenger RNA
MTX	Methotrexate
nM	Nanomolar
NMR	Nuclear magnetic resonance
NO	Nitric oxide
nmol/L	Nanomoles per liter
NOS	Nitric oxide synthases
OR	Odds ratio
OEP	Ovarian ectopic pregnancy
PAF	Platelet-activating factor
PGE2	Prostaglandin E2
PUL	Pregnancy of unknown location
PROKS	Prokineticins
PROKR	Prokineticin receptor
SEM	Standard error of the mean
TA	Glucocorticoid triamcinolone acetonide

TGCs	Trophoblast giant cells
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
UBC	University of British Columbia
USA	United States of America
VGH	Vancouver general hospital

Acknowledgements

I am sincerely thankful for my supervisor Dr. Mohamed Bedaiwy who gave me the opportunity to pursue my graduate studies in his laboratory that enabled me to continue my future career in this field, and for his endless support during the hard times and years of studies.

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My sincere appreciation to my lab members, Dr. Bo Peng, Dr. Amr Osman, and Fahad Alotaibi, for providing coherent answers to my endless questions.

Special thanks owed to my wonderful parents, Mohamed Yassin and Magda Ewis, who have supported me throughout my years of education, both morally and financially, and without their prayers, I would not have been able to achieve my goals.

Lastly, I am so grateful to my wonderful family who encouraged me during my research studies and participated with their endless time and prayers. Thanks to Safwat; my husband, Muhammad, Omar, and Leena; my lovely kids.

Dedicated to all women suffered from ectopic pregnancy and its complications

Chapter 1: Ectopic Pregnancy

Ectopic pregnancy (EP) is a life-threatening condition (1) that occurs when an embryo implants outside the uterine endometrium. It represents 1.4%-2.4% of all pregnancies (2). Emergency units report that the incidence of EP among women in the first trimester of pregnancy complaining of vaginal bleeding with or without abdominal pain is as high as 18% (3). The most common site for ectopic implantation is the Fallopian tube (tubal pregnancy). Other types include an ovarian, abdominal, cervical, interstitial, cesarean scar, and heterotopic EP (4).

1.1 Types of Ectopic Pregnancy

1.1.1 Tubal EP

More than 90%-95% of ectopic pregnancies occur in the Fallopian tube (5). Of these, 70 % occur in the ampulla of the tube, 12% in the isthmus, and 11% in the fimbria (**Figure 1.1**). Interstitial EP, in which implantation occurs in the intramural segment of the Fallopian tube, is represented in 3% of cases and is classified as a distinct type of EP.

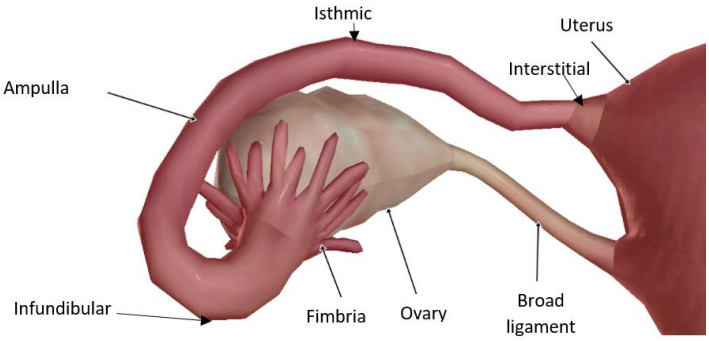

Figure 1. 1: Segments of human Fallopian tube

(https://commons.wikimedia.org/wiki/File:Female_reproductive_system-ar2.png), Labeling by Lobna Abdellatif, https://creativecommons.org/licenses/by-sa/4.0/legalcode

Fallopian tubes are the physiological site for fertilization, providing a conduit for the oocyte from the ovary and for the fertilized egg to the uterus (**Figure 1.2**). Secretions of the

Fallopian tube provides nourishment for the ovum and spermatozoa (**Figure 1.2**). Also, tubal secretions enhance the embryo and maternal cross talk that maintains embryo propelling towards the uterus where it implants. Abnormal signals or disturbed cross talks are believed to be the cause of tubal EP, and this is discussed later under the mechanism of tubal EP.

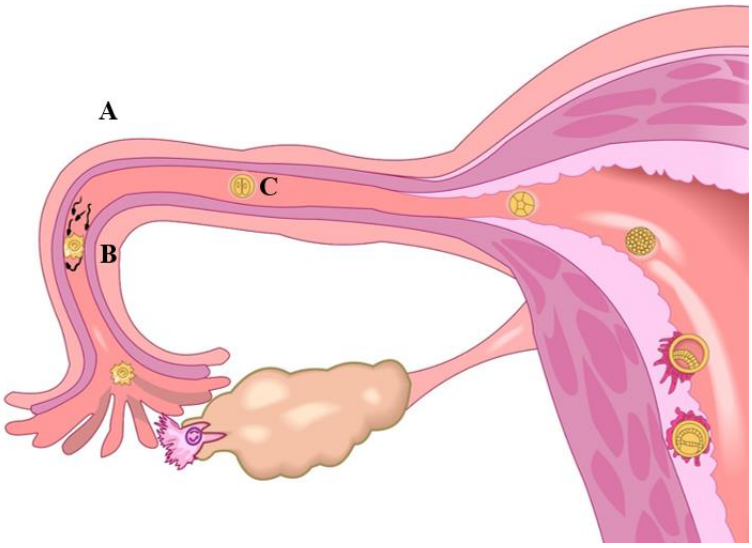

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Figure 1. 2: Fallopian tube importance for human reproduction. It transports the oocyte to the uterus (**A**) meanwhile might be fertilized (**B**) and start the early development (**C**).

1.1.2 Interstitial EP

Interstitial pregnancy, often referred to as cornual pregnancy in the medical literature (2, 5), represents 3% of all EPs (5). It occurs when an embryo passes into the myometrium at the junction between the interstitial portion of the Fallopian tube and the cornual portion of the uterus (5).

1.1.3 Ovarian EP (OEP)

Ovarian EP also accounts for 3% of EPs (6, 7). The actual incidence is believed to be higher because of the undetermined percentage of pregnancies of unknown origin could include ovarian pregnancies (8). The early diagnosis is crucial for the prevention of severe complications. However, preoperative diagnosis is challenging (9). Although ovarian pregnancy was first reported in 1682 (10), the first laparoscopy to treat ovarian pregnancy was in 1988 (7, 11). Of all ovarian pregnancies, 75% terminate during the first trimester, 12.5% in the second trimester, and 12.5% may reach term gestation (12). Spiegelberg (13) proposed four criteria for the diagnosis of OEP including:

- The Fallopian tube of the affected side must be intact
- The EP is attached to the uterus by the ovarian ligament
- The gestational sac is located in the region of the ovary
- The ovarian tissue in the gestational sac is seen histologically

1.1.4 Abdominal EP

Abdominal EP accounts for 1.3% of EPs (2). In primary abdominal EP, implantation occurs in the abdominal cavity while secondary abdominal EP occurs after ruptured tubal pregnancy or abortion through tubal fimbriae (2). Several sites for abdominal implantation are reported, as shown in **Figure 1.3** (14, 15).

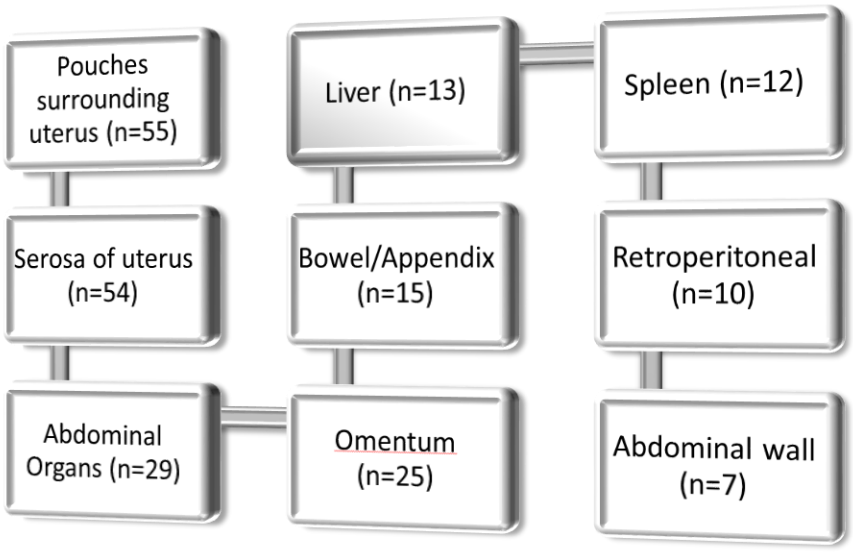


Figure 1. 3: Categories of early abdominal pregnancy, in order from most to least frequently reported (15).

1.1.5 Cervical EP

Cervical EP accounts for 0.15% of EPs (2). Before 1979, cervical pregnancy was treated mostly by hysterectomy because of the uncontrolled vaginal bleeding. Treatment focused on saving the lives of patients rather than on preserving their future fertility. Today, advances in the ultrasonography have enabled early diagnosis that allows better management options.

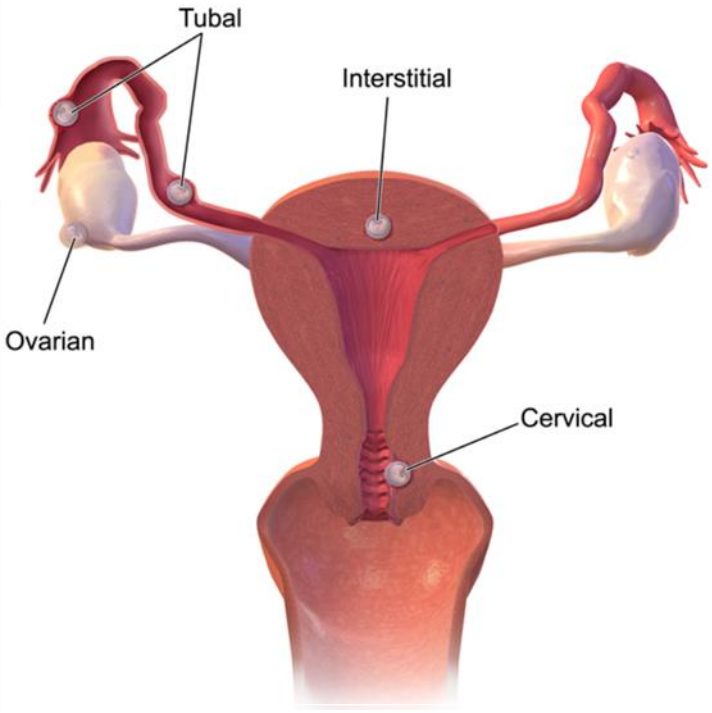
1.1.6 Caesarean scar EP (CSEP)

In this type, the embryo implants in the uterine myometrium at the site of a previous cesarean section scar (2). It represents 6% of all EPs (5). Although the number of cesarean deliveries is increasing, CSEP is not related to the number of cesarean deliveries (5). The

outcomes rely on early diagnosis to prevent the uterine rupture and massive bleeding that will impair future fertility or increase the likelihood of maternal mortality (5).

1.1.7 Heterotopic pregnancy

It occurs when one of two fertilized oocytes implants in the uterus and the second implants ectopically. The incidence of heterotopic pregnancy is < 1:30,000 with natural conception, and 1:100 in pregnancies achieved using assisted reproduction techniques (2, 16).



BruceBlaus (https://commons.wikimedia.org/wiki/File:Ectopic_Pregnancy.png), https://creativecommons.org/licenses/by-sa/4.0/legalcode

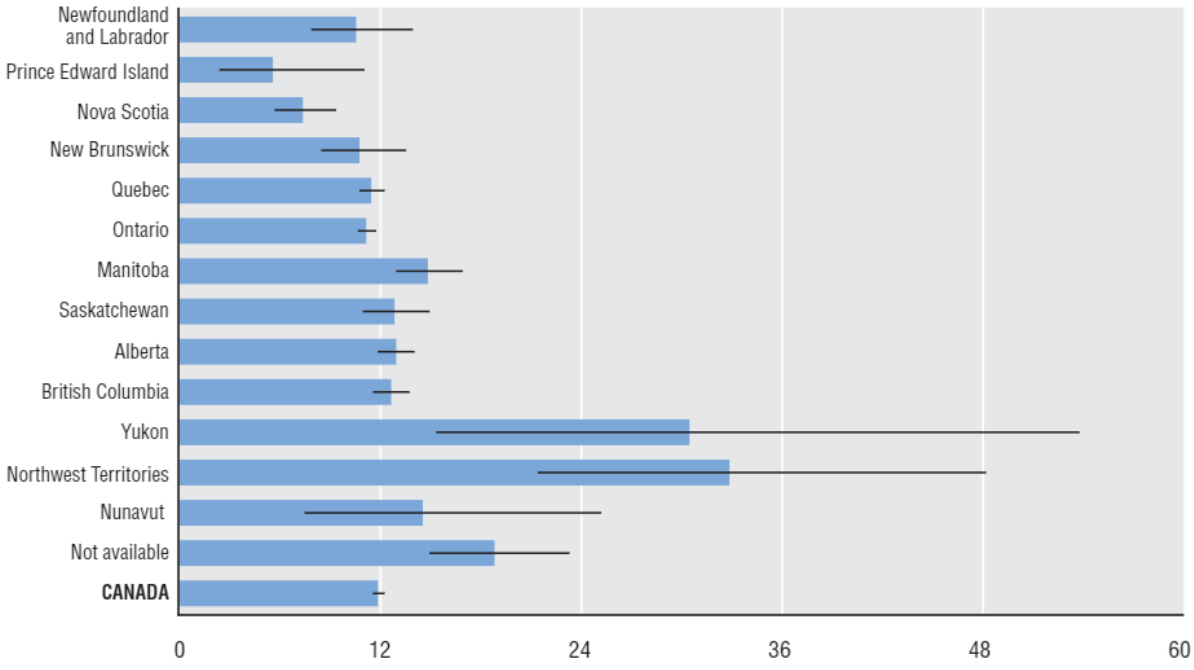
Figure 1. 4: Possible sites of EP

1.2 Maternal Mortality and Morbidity in EP

Hemorrhage from tubal rupture is one of the leading causes of maternal mortality in the first trimester (17). In western countries, EP accounts for 9%-13% of pregnancy-related deaths (2, 18). According to the Public Health Agency of Canada, 6.5% of maternal deaths between 1993 and 2004 were due to EP (19). In the 1980s, 75% of early pregnancy maternal deaths were due to EP (2, 20).

Furthermore, several co-morbidities are associated with EP that can be fertility-threatening (1) or can increase the risk of EP recurrence (20). Among women with one previous EP, the incidence of EP recurrence is 10%, while in women with two or more previous EPs, this increases up to 25% (21, 22).

Figure 1. 5: Rate of EP by Canadian province/territory (19)



1.3 Economic Burden of EP

Despite recent advances in the diagnostic techniques that allow early detection of EP cases, the economic burden of treatment increases; amounting to millions of dollars each year in western countries. For example, the annual cost to treat EPs in the United Kingdom is about $15 million dollars (17, 23), and in the USA, it was estimated to be $1.1 billion dollars in 1992 (24). Costs include diagnostic and excluding investigations, hospitalization costs, and treatment. The most cost-effective treatment is methotrexate (MTX), which costs approximately $1000 per patient (25). However, the treatment of adverse effects and complications associated with MTX add to that cost. A rescue surgery may also be needed, adding around $2500 per patient (25). In a Canadian study of the costs of unintended pregnancy outcomes (26), the expenses in live birth cases were $3500 per case, while in EPs were $2600 per case. This study agreed with the Canadian Institute for Health Information report, 2006 edition, about the cost of antepartum hospitalization of EP cases, which represented in **Figure 1.6** (27).

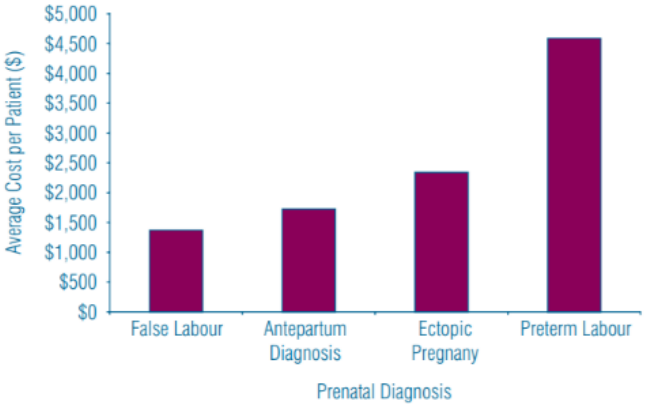


Figure 1. 6: Cost of antepartum hospitalization in Canada (27)**.**
In 2002-2003, the total average cost to care for patients with EP was $2600/patient. Note, data do not include Quebec or rural Manitoba.

1.4 Risk Factors of EP

Several risk factors contribute to the tubal pregnancy occurrence including, cigarette smoking, tubal infection, history of pelvic inflammatory disease, history of tubal surgery, and tubal pregnancy occurring during intrauterine device usage or after assisted reproduction procedure (28, 29). These risk factors are classified as high risk, moderate risk, or mild risk factors as shown in **Table 1.1**.

Table 1. 1: Risk factors of a tubal pregnancy (30)

High risk	Moderate risk	Mild risk
Prior tubal surgery	Sterility	Age above 40 years
Prior tubal pregnancy	Current or prior ascending infection	
Sterilization	Cigarette smoking	
Use of intrauterine device	More than one sexual partner	
	Tubal pathology	

Although the maternal age is considered a mild risk factor, it was responsible for a 5-fold increase in severe morbidity associated with hospitalized women diagnosed with EP (31). The highest increase was among women over 35 years old (**Figure 1.7**) (31).

Figure 1. 7: Age-specific rates of death/severe morbidity per 100 women hospitalized for EP 1987-2014 (32).

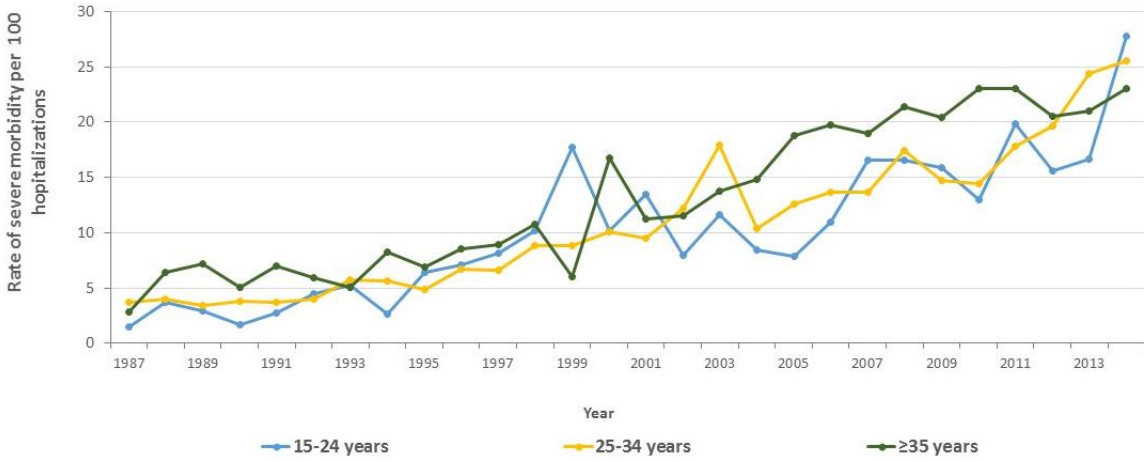
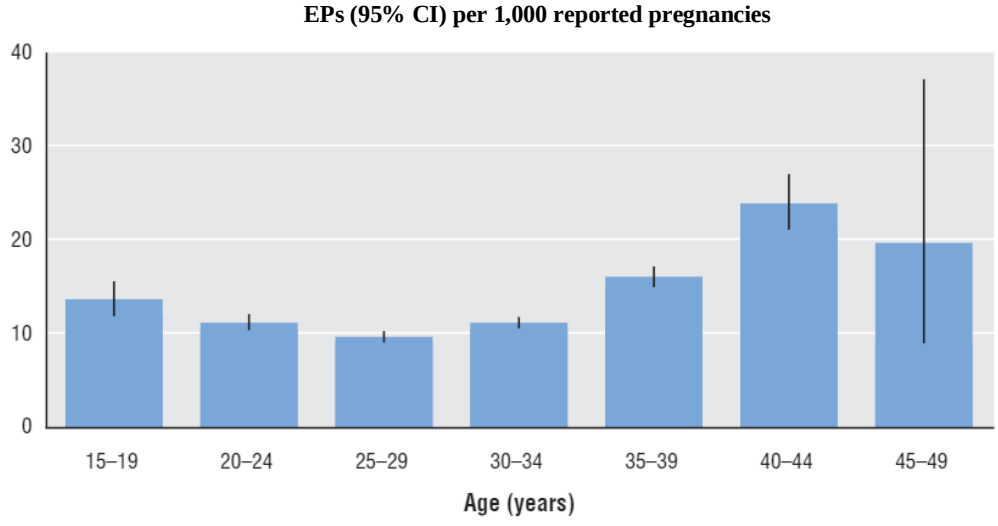


Figure 1. 8: Rate of EP by maternal age (19)



Source: Canadian Institute for Health Information. Hospital Morbidity Database, 2004−2005.
CI—confidence interval

1.5 Mechanism of Tubal EP

Several studies investigating the underlying mechanisms of tubal EP have supported the view that the cause is either impairment of embryo-tubal transport or alteration in the tubal environment, which enhances embryo implantation in the tube (**Figure 1.9**) (17, 33). In humans, tubal functions are mediated by paracrine signals between tubal cells (34-36). Coordination between tubal epithelial cells, tubal smooth muscles, and the embryo enhances the early embryo development and its propulsion in the Fallopian tube during its journey towards the uterus (37).

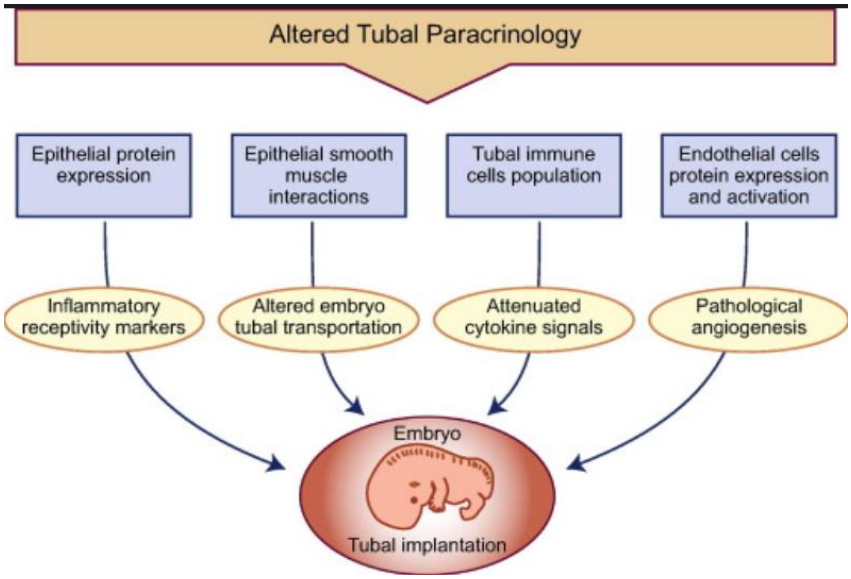


Figure 1. 9: Mechanisms of tubal EP (38)

1.5.1 Tubal epithelial–smooth muscle cross talk:

There are two epithelial cell types lining the wall of the human Fallopian tube (**Figure 1.10**): (1) ciliated epithelial cells that push the fertilized egg during its passage through the tube,

and (2) secretory cells, which are fewer in number, and produce the tubal fluid that mediate the tubal environment important for maternal-embryonic signals.

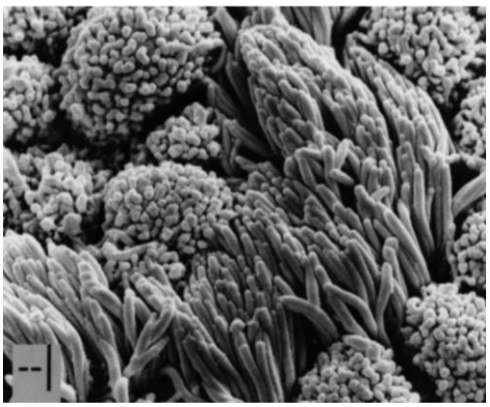


Figure 1. 10: A scanning electron micrograph for a human Fallopian tube showing the hair-like ciliated cells that help oocyte propelling to the uterus and secretory cells that nourish it (39).

Progesterone regulates the ciliary beats and the secretory cell function (34). The absence of ciliated epithelial cells or the presence in low numbers has been shown to contribute to tubal pregnancy (40). Risk factors, such as cigarette smoking, have been shown to decrease the ciliary beat frequency of animal oviducts (41). Tubal gonorrheal infection upregulates tumor necrosis factor (TNF-α), which sloughs the ciliated epithelial lining (42) and suppresses the propulsive activity that causes embryo retention.

The tubal muscle contractility is controlled by interstitial cells of Cajal (ICC) (43), which are regulated by progesterone. ICC was first discovered in the gastrointestinal tract and known for its regulatory role in smooth muscle contractility (38). Lower ICC numbers are correlated with high levels of nitric oxide synthases (NOS) enzyme (44) that increases ICC apoptosis or suppress cell proliferation (44). The upregulated NOS was reported after *Chlamydia muridarum* infection in animals (44) and *Chlamydia trachomatis* infection in

humans (45). The increased NOS increases nitric oxide (NO) levels that have a relaxing effect on tubal smooth muscles (46) and impair embryo-tubal transport leading to tubal implantation.

1.5.2 Embryo–smooth muscle cross talk:

Many trophoblast-derived factors are expressed in the human Fallopian tube (38). Human chorionic gonadotropin, hCG, is produced by the embryo and its receptor has been detected in the Fallopian tube (47). Treating the Fallopian tube mucosa with hCG upregulates prostaglandin E2 levels which have a relaxing effect on tubal smooth muscle (48).

Platelet-activating factor (PAF), produced by human embryos, has a pivotal role during placentation (49), acting as a direct autocrine growth factor during the preimplantation stage (50). PAF receptor expression was detected in the tubal epithelial cells that may contribute to the embryo adhesion and tubal implantation (17, 38). Results in an animal study have shown that a PAF inhibitor significantly delayed the embryo transport in hamster oviduct, but did not affect the oocyte transport (51) suggesting that disruption of signals from the embryo could contribute to tubal pregnancy.

1.5.3 Tubal epithelial–embryo cross talk:

The implantation process is a complex maternal-embryo dialogue mediated by several factors and proteins that induce receptivity, embryo adhesion, and invasion. Tubal damage due to the presence of risk factors provides a pro-inflammatory status that is believed to upregulate cytokines, which are the main inducers for implantation. Smoking and *Chlamydia trachomatis* infection affect prokineticins (PROKS) expression, a protein family that is responsible for cytokine upregulation. A significant high expression of PROKS receptors was observed in the

Fallopian tubes of women who smoke or post-*Chlamydia trachomatis* infection (52, 53). PROKS were also reported to upregulate the endometrial leukemia inhibitor factor (LIF) (54) that is essential for implantation in mice (55). Increasing PROKS expression in the Fallopian tube could upregulate factors like LIF that increase the tubal receptivity and enhance embryo tubal implantation (38).

Activin A is another protein that is upregulated in the tubal epithelium after *Chlamydia trachomatis* infection (45) and induces LIF expression (38). *In vitro* studies showed that activin A induces villous trophoblast transition to the invasion phenotype (56).

Interleukin 1 (IL-1) is also crucial for normal embryo implantation (57) by upregulating IL-8 levels. IL-8 has a role in neutrophils recruitment and tissue damage promotion (58, 59). Tubal *Chlamydia trachomatis* infection induces IL-1 production from tubal epithelial cells (58). Inhibiting tubal IL-1 receptors has shown to suppress cytokine production and tubal epithelium deciliation after *Chlamydia trachomatis* infection (58). Results show that IL-1 acts on maternal-embryonic interactions that might induce tubal receptivity.

Other regulators for the apposition, adhesion, and invasion process in normal pregnancies are reported to be expressed in the Fallopian tube during tubal pregnancy, such as integrins and mucin 1 (17, 33, 38), suggesting that tubal receptivity contributes to tubal pregnancy occurrence.

Several genes, such as estrogen- and progesterone-regulating genes, have been shown to alter in the Fallopian tubes of women with tubal EP (17, 38). GnRH is another hormone that is expressed in intrauterine placentas (60, 61), as well as in the Fallopian tube epithelium (62). However, its expression in the tubal implantation sites was not determined until 2016 (63), the details of GnRH expression in the tubal pregnancies will be discussed in Chapter 3.

1.5.4 Tubal immune cell-embryo cross talk:

Maternal immune cells have a crucial role in the regulation of trophoblast invasion via the induction of trophoblast cell apoptosis. In tubal pregnancy, the absence of $CD56^{bright}$ natural killer cells in the Fallopian tubes is thought to be the cause of the uncontrolled invasion associated with tubal EP (38). Also, $CD56^{dim}$ cells in the Fallopian tube of women with EP have a direct role in the uncontrolled invasion. $CD56^{dim}$ cells found in tubal implantation sites are non-cytotoxic (64), unlike the same cells in the peripheral blood (65); however, they do not produce factors that induce trophoblasts apoptosis. Consequently, this leads to unrestrained invasion of the tubal wall layers and possible tubal rupture (38).

The alteration of tubal immune cells was reported to induce the production of cytokines which enhance tubal receptivity with subsequent signals to the embryo to be implanted in the tube (38).

1.6 Diagnosis of Tubal EP

Diagnosis of tubal pregnancy occurs through blood analysis and imaging procedures. The diagnostic clues for tubal pregnancy are the serial increase in β-hCG levels in the maternal blood and ultrasound findings for an empty uterus with a gestational sac that presents at one side of the uterus, anatomical site of the Fallopian tube (66-68).

1.6.1 Trends of serial serum hCG levels:

A single value of the hCG level is insufficient to assess the location and viability of gestation (69). Serial measurements are needed to differentiate between normal and abnormal pregnancies (69, 70). The serum hCG level increases gradually until 10 weeks of gestation when they plateau (71). The rate of increase depends on the initial hCG value; with high initial values, the rate of increase is low, and with low initial values, the rate is high (71). According to the American College of Obstetricians and Gynecologists (ACOG) bulletin, there is 49% increase with an initial hCG level of < 1500 mIU/mL, a 40% increase with an initial value of 1500-3000 mIU/mL, and a 33% increase with an initial level of > 3000 mIU/mL (71). During early pregnancy, when the expected 2-day increase in serum hCG level does not occur, suspicion of EP or pregnancy of unknown location (PUL) is increased, but this alone should not be considered diagnostic (71). However, a normal pattern of hormonal increase does not exclude the possibility of EP or PUL (72). Decreasing levels of serum hCG indicate failing pregnancy and spontaneous resolution (71); however, they should be monitored until they reach the non-pregnancy level because ruptured tubal pregnancy can occur while hCG levels are decreasing or even when they are at very low levels (71).

1.6.2 Transvaginal ultrasonography:

Definitely, ultrasound screening can diagnose EP when a gestational sac with a yolk sac or embryo is visualized at the adnexal region (73, 74). However, most EPs do not progress until this stage (73). Early diagnosis depends on the high index of suspicion. In an intrauterine pregnancy, the gestational sac is likely to be visible on transvaginal ultrasound by 5.5 to 6 weeks of gestation, calculated from the first date of the last menstrual period or estimated from the

serum β-hCG level (75, 76). The absence of an intrauterine gestational sac with increasing β-hCG levels indicates an abnormal gestation and raises the possibility of EP or PUL.

The presence of a mass with a hypoechoic area separated from the ovary increases the suspicion for EP with 80% positive predictive value (73).

Visualizing a gestational sac with a yolk sac or embryo in the uterine endometrium eliminates the possibility of EP, except in heterotopic cases (71). Also, finding a uterine hypoechoic structure 'sac like' can be seen in EP due to the collection of fluid or blood, which represented as pseudo-gestational sac (77, 78).

1.7 Treatment of Tubal EP

EP can be managed expectantly in patients with no significant symptoms (2, 17, 79). In other patients, the choice of medical versus surgical treatment relies on several factors, including the clinical, laboratory, and radiological data, as well as patient's preference once she informed of the advantages and risks of each approach (71).

1.7.1 Expectant management:

According to the most recent guidelines by ACOG, expectant management may have a role in 98% of EP cases in which the initial hCG value is < 200 IU/L and in a declining phase (71). This role decreases when the initial value increases (2). For example, watchful waiting is successful in 73% of cases with an initial hCG level < 500 IU/L and in 25% with an initial hCG < 2000 IU/L (71). Also, the size of the EP and the viability of the fetus should be considered.

1.7.2 Surgical management:

Surgical managment involves either salpingectomy (**Figure 1.11**), excision of the Fallopian tube, or salpingostomy, which involves removal of the ectopic mass but not the Fallopian tube (**Figure 1.11**). The surgical intervention, performed by laparotomy or laparoscopy, is most often used to save the patient's life after tubal rupture or for patients who cannot take the medical treatment.

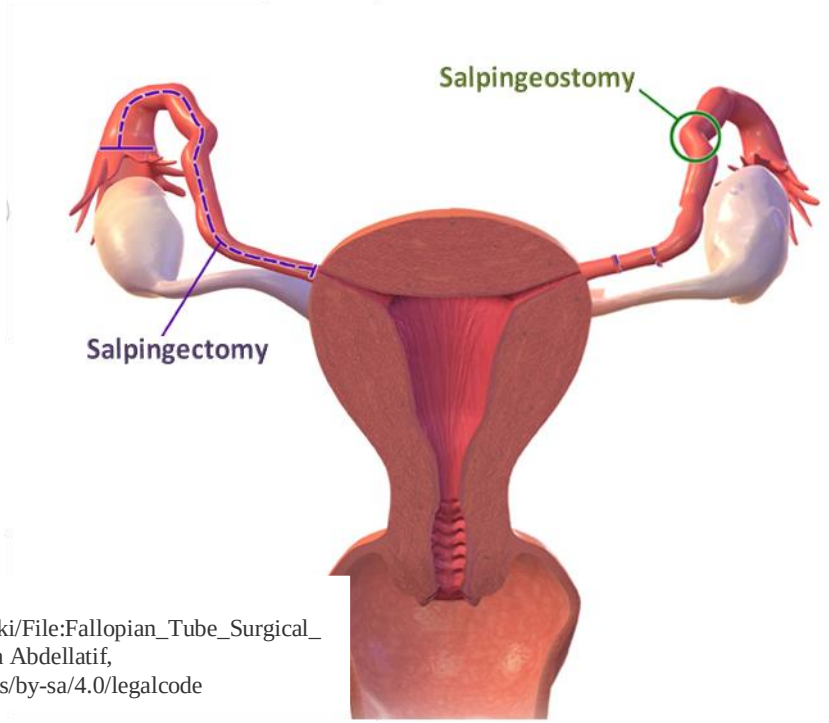
1.7.2.1 Salpingostomy:

It involves a linear incision or transampullary expression to remove the trophoblastic tissue from the Fallopian tube (30). The gold standard technique for this procedure is a laparoscopy. The advantages of laparoscopy over laparotomy are rapid abdominal access, short surgical time, fewer surgical complications, faster healing, and lower hospitalization and recovery costs (80). Monitoring the serum hCG levels is mandatory to ensure total removal of the ectopic trophoblastic tissue (71). The presence of remaining trophoblastic tissue (4%-15%) (20, 30) and increasing the risk of tubal pregnancy recurrence are the most common disadvantages of this procedure.

1.7.2.2 Salpingectomy:

That is preferred in EP patients who do not want to preserve their future fertility or who have severe Fallopian tube damage with massive bleeding (71, 81). However, it is not recommended in patients with desired future fertility in case of having a healthy contralateral Fallopian tube (71). Impaired fertility and anesthesia complications are the most common adverse effects.

Figure 1. 11: Surgical management of tubal EP



BruceBlaus (https://commons.wikimedia.org/wiki/File:Fallopian_Tube_Surgical_Procedures.png), Labeling by Lobna Abdellatif, https://creativecommons.org/licenses/by-sa/4.0/legalcode

1.7.3 Medical management:

Despite advances in the health system, there is only one medical treatment available for EP, which is methotrexate (MTX). MTX is a chemotherapy agent that is used in the treatment of lung and breast cancer, as well as in immunodeficiency disorders such as rheumatoid arthritis and psoriasis.

1.7.3.1 Mechanism of action:

MTX interferes with the metabolism of folic acid (21) which is crucial for cell proliferation particularly during the S-phase of the cell cycle (82). MTX suppresses DNA synthesis by inhibiting the dihydrofolate reductase enzyme, which is involved in purine and pyrimidine synthesis (**Figure 1.12**) (21). Therefore, it affects the rapidly proliferating cells like

cancer and trophoblastic cells. MTX has many side effects and contraindications that limit its clinical usage or lead to MTX withdrawal and change of the treatment (21).

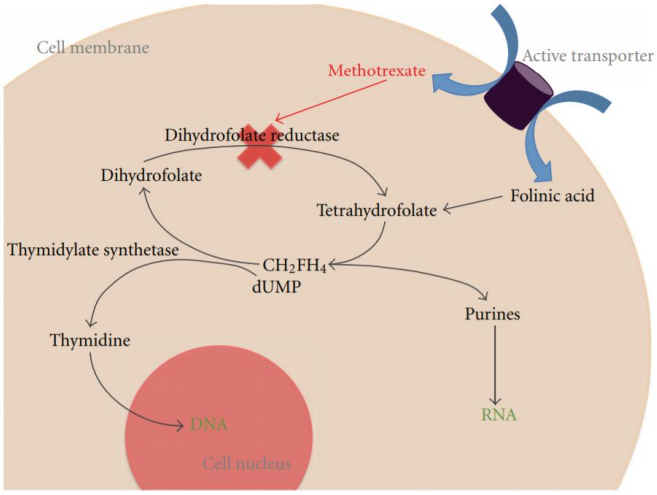


Figure 1. 12: Mechanism of action of methotrexate (83)
By which it inhibits cellular proliferation. Active transporter includes the reduced folate carrier and an endocytic pathway activated by a folate receptor; dUMP: deoxyuridine monophosphate; CH2FH4: methylenetetrahydrofolate.

1.7.3.2 Adverse effects:

MTX-associated side effects include elevation of liver enzymes, kidney dysfunction, bone marrow suppression, dermatitis, and reversible hair loss (84, 85).

1.7.3.3 Contraindications:

MTX is contraindicated in patients who have liver or kidney diseases, or who have pulmonary, stomach, or immunosuppressive disorders.

1.7.3.4 Criteria:

Patients with tubal EP must be hemodynamically stable and preferably have a β-hCG level < 5000 IU/L, and not to be shown on ultrasound to have a viable fetus with cardiac activity in the Fallopian tube, to be eligible for MTX (85-87). However, MTX could be used in these conditions with particular precautions.

1.7.3.5 Challenges of MTX:

In addition to the side effects and contraindications associated with MTX therapy, it is reported to have 30% failure to treat EP patients (88),. Thus, it is clear that safer and better-tolerated medical treatment is necessary.

Chapter 2: Human Placenta

2.1 Formation and Development

The placenta develops during each pregnancy and is expelled after delivery of the fetus. It connects the fetal blood circulation with the maternal blood (89) to provide the fetus with nutrition and to enable filtration of gases and waste (90, 91). The human placenta also has an endocrine function. It produces hormones that are responsible for pregnancy maintenance such as hCG, human placental lactogen, and placental growth hormone (92).

The human placenta originates from the trophectoderm cell layer of a blastocyst (93). After blastocyst implantation in the endometrium, placental finger-like projections develop, forming trophoblastic primary villi (94). Primary villi infiltrated with mesenchymal cells that form secondary villi (94) (**Figure 2.1**). The infiltrating mesenchymal cells differentiate into blood vessel components of the fetal circulatory system forming tertiary villi (94) (**Figure 2.1**).

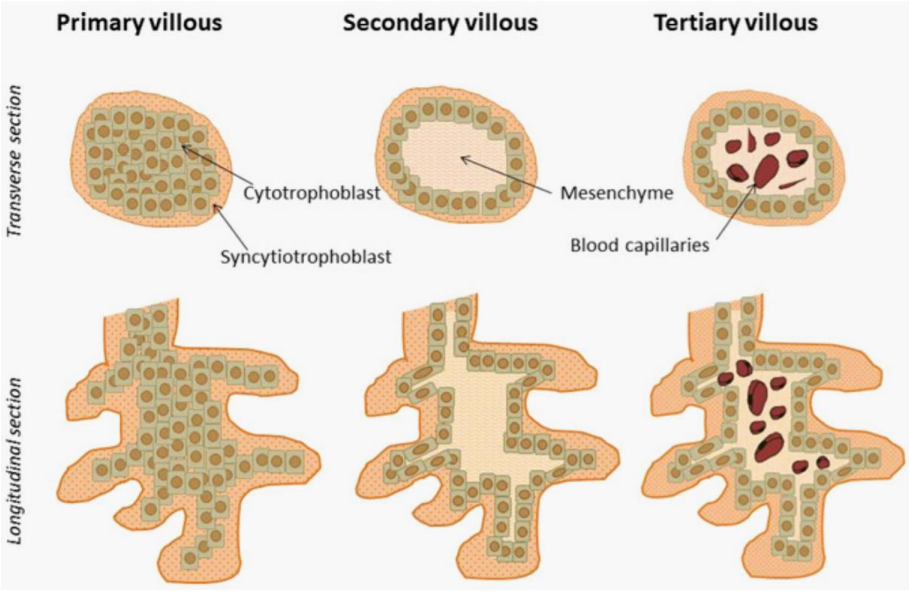


Figure 2. 1: Stages of human placental villi formation (95)**.**

Villous cytotrophoblasts differentiate into extravillous trophoblasts (EVTs) and multinucleated syncytiotrophoblasts (91) (**Figure 2.2**). Each trophoblast subpopulation has a different phenotype and function that collectively provides the supportive and endocrine functions of the placenta. Cytotrophoblasts are progenitor cells that proliferate and differentiate into other trophoblastic cell types (91). Differentiated EVTs invade the maternal tissues (89, 91), while syncytiotrophoblasts are responsible for hCG hormone production (89, 91) (**Figure 2.2**).

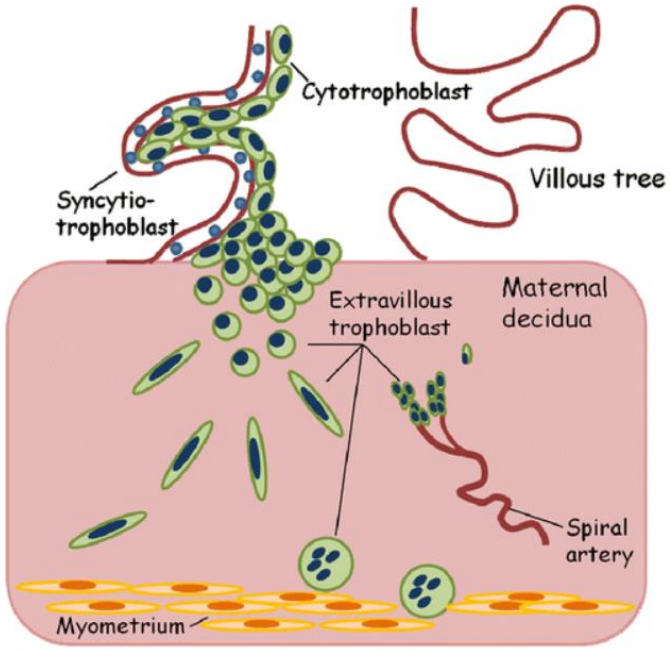


Figure 2. 2: Villous and extravillous trophoblast in human placentation (93)

2.2 Placentation in Uterine Pregnancy and Ectopic Pregnancy

There are differences and similarities between intrauterine placentation and placentation in tubal pregnancy. The invasion behavior of EVTs and the receptivity markers that enhance placentation in normal and tubal pregnancy will be discussed in this section.

2.2.1 EVT invasion

In intrauterine placentation, the interstitial EVTs (iEVT) invade the maternal decidua (91), and the endovascular EVTs (eEVT) invade the maternal spiral arteries (91). EVTs invade the maternal tissue until they reach the inner third of the myometrium (89, 91), where EVTs fuse together forming multinucleated trophoblast giant cells (TGCs). TGCs act as a limiting zone that prevents further invasion into the myometrium (91) (**Figure 2.3**). Also, EVTs invasion is controlled by the induction of their apoptosis. The presence of apoptotic cells that is mediated by maternal immune cells enhances the EVT cell death (96).

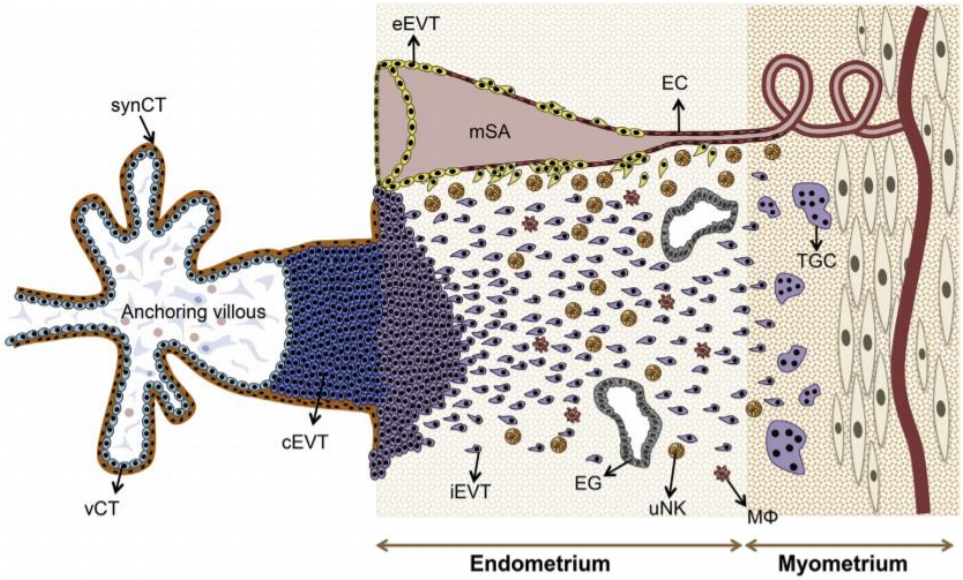
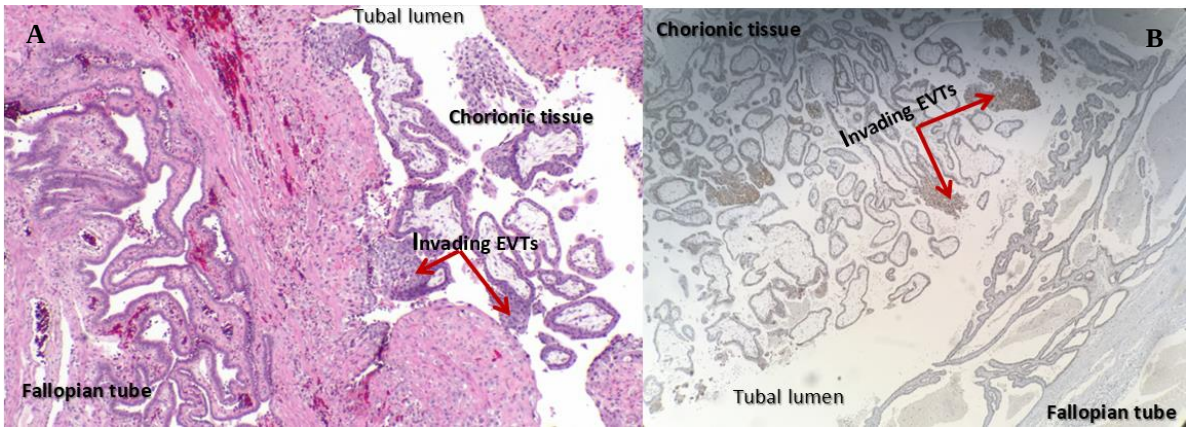


Figure 2. 3: Schematic diagram of trophoblast differentiation (89). Mononuclear villous cytotrophoblasts differentiate into multinucleated syncytiotrophoblast or invasive EVTs. vCT, villous cytotrophoblasts; synCT, syncytiotrophoblast; cEVT, columnar extravillous cytotrophoblast; eEVT, endovascular extravillous cytotrophoblast; iEVT, interstitial extravillous cytotrophoblast; mSA, maternal spiral artery; EC, endothelial cells; EG, endometrial gland; uNK, uterine Natural Killer cell; Mϕ, macrophages; TGC, trophoblast giant cell.

During tubal placentation (**Figure 2.4**), EVTs invade different layers of the tubal wall without reaching a stopping zone and cause a tubal rupture. Although tubal thickness is not supportive of any pregnancy and can be the direct cause for tubal rupture, it was reported that tubal perforation is due to the uncontrolled invasion to the tubal wall (96). That was deemed because of the lack of apoptotic cells at the tubal implantation sites (96). Alteration of the tubal environment affects tubal immune cells which mediate the apoptotic cells (96). Therefore, it was suggested that the failure to induce EVTs apoptosis in tubal pregnancy leads to uncontrolled invasion and tubal rupture (96).



(A) Nephron (https://commons.wikimedia.org/wiki/File:Ectopic_pregnancy_-- low mag.jpg), „Ectopic pregnancy -- low mag“, labeling by Lobna Abdellatif, https://creativecommons.org/licenses/by-sa/3.0/legalcode. **(B)** A slide preserved at Bedaiwy laboratory.

Figure 2. 4: Microscopic views for tubal pregnancy. **(A)** Trophoblast cells invade the wall of Fallopian tube, (**B)** Tubal pregnancy implantation site expressing GnRH-l obtained from a previous study in our laboratory.

2.2.2 Receptivity markers

Several factors that enhance endometrial receptivity in normal pregnancies are detected in the Fallopian tubes of women with tubal pregnancy (63). The implantation process relies on three crucial steps; apposition, adhesion, and invasion. Cellular adhesion molecule (CAM) family groups including integrins, selectins, cadherins, and immunoglobulins are essential for blastocyst apposition and adhesion. They are expressed in the endometrium during the window of implantation in the intrauterine pregnancy (97). Their absence is reported to cause implantation failure, and their abnormal expression causes placentation complications. CAM family members have been detected in tubal implantation sites (17, 33, 98) **(Figure 2.5)**, and have been suggested to promote tubal receptivity and enhance the tubal embryo implantation.

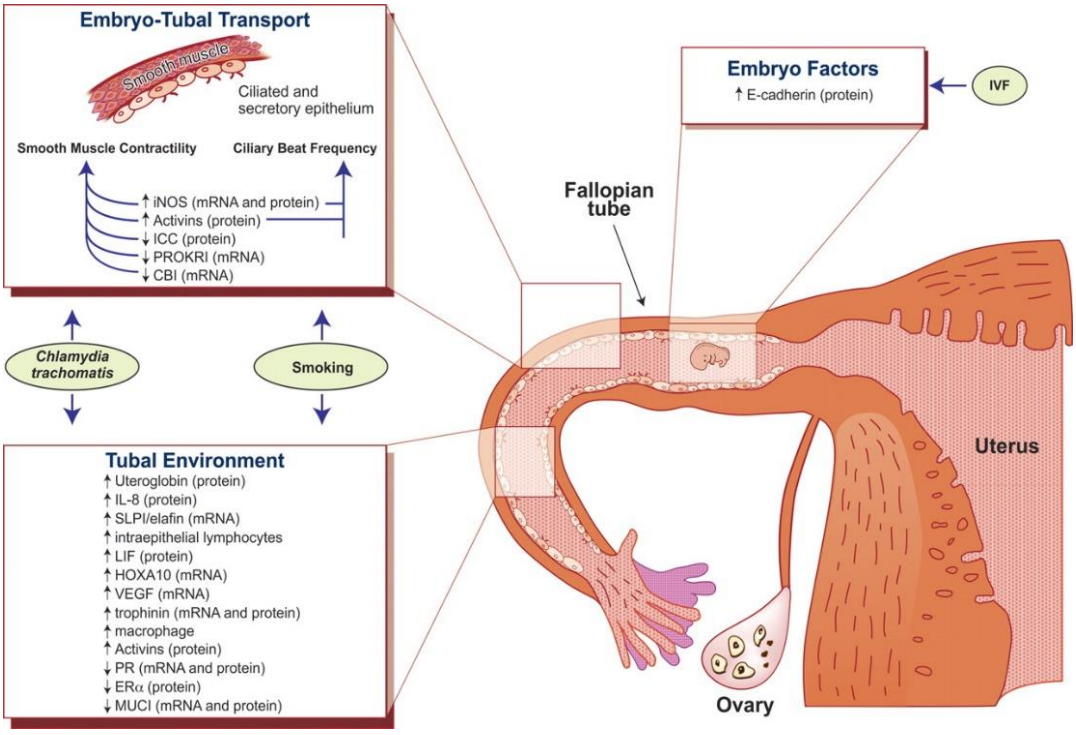


Figure 2. 5: Schematic diagram summarizing the potential factors contributing to the development of tubal EP (33)

2.3 Biological Tools to Study Trophoblast Cell Functions

Several biological models have been used to examine different trophoblastic functions such as cytotrophoblast cell proliferation, EVT cell invasion, and hCG hormone production from syncytiotrophoblasts cells. Models used include primary trophoblasts isolated from first trimester intrauterine placentas, multiple immortalized trophoblastic cell lines, and human placental explants. The trophoblastic immortalized HTR-8/SVneo cells, primary trophoblast cells from tubal pregnancies, and first-trimester placenta explants are examined in this study.

2.3.1 Trophoblastic cell lines

Immortalized cell lines from trophoblastic or choriocarcinoma origin have been used widely to study the different functions of primary trophoblast cells (99). Trophoblastic cell lines that are used extensively in the placenta research are HTR-8/SVneo, BeWo, JAR, and JEG-3 immortalized cell lines (99, 100).

The choriocarcinoma BeWo cell line shows function and phenotypic characteristics similar to those of villous syncytiotrophoblasts, such as the fusion (101) and hCG production (102). However, the upregulation of two criteria at the same time is not mandatory to indicate the syncytiotrophoblast cells activity.

On the other hand, HTR-8/SVneo cell line is widely used to investigate the primary EVTs invasion phenotype (93). HTR-8/SVneo cells were also reported to be a valid model to examine primary cytotrophoblasts function. They show similar characteristics to primary cytotrophoblasts such as the absence of transformed phenotype and their response to TGF-β (103-105). Kilburn B.A. et al showed the effect of hypoxia and Matrigel contact on HTR-8/SVneo different cell functions, which are summarized in **Table 2.1** (106).

The low O_2 levels in culture induced cell proliferation and suppressed the invasion even in the presence of Matrigel (106). As well, cells in hypoxic status seemed to preserve the epithelial phenotype by increasing α6β4 expression and decreasing expression of α1β1 that is expressed in mesenchymal cells (106). Thus, HTR-8/SVneo can be a useful model to study cytotrophoblast cells if cultured in a low O_2 level. However, to study EVT cell functions; cells should be kept in non-hypoxic conditions. Data in **Table 2.1** should be considered during the usage of HTR-8/SVneo cells because any changes in O_2 levels in the incubator due to low gas levels in the cylinder or gas leakage could alter the epithelial-mesenchymal cell phenotype and affect cell response to treatment.

Table 2. 1. Summary of HTR-8/SVneo cells response to culture conditions (106)

	Effect of hypoxia	Effect of Matrigel contact
Proliferation	Increased	Decreased
Invasion	Decreased*	Increased
HLA-G expression	Decreased*	Increased
α6β4 expression	Increased*	Decreased
α1β1 expression	Decreased*	Increased
α5β1 expression	Neutral*	Neutral

* Determined in the presence of Matrigel; therefore, not evaluated independently

Another consideration is that HTR-8/SVneo cells have only two criteria out of 4 that were proposed by Lee et al (107) to identify primary first-trimester trophoblast cells. The 4 criteria include: 1- Expression of trophoblast protein markers such as cytokeratin 7 and *GATA*–binding protein 3 (GATA3), 2- Hypomethylation of ELF 5 promoter, 3- Overexpression of chromosome 19 miRNA cluster, and 4- Expression of HLA class 1 molecules including HLA-G, -C, -E, and -F with no expression for HLA-A or -B.

The immortalized HTR-8/SVneo cells express cytokeratin 7, GATA3, and HLA-G as same as in primary trophoblasts. However, they differ in the following: ELF 5 promoter is hypermethylated (107), they are not expressing chromosome 19 miRNAs (108, 109) and not having the same HLA profile that was reported in villous cytotrophoblast or EVT cells (107).

For these reasons, HTR-8/SVneo cell line is considered as an acceptable model to study EVT cell functions but not the ideal tool.

Moreover, HTR-8/SVneo cells are used to examine hCG production (110). Although the fact that hCG *in vivo* secretion is confined to the villous syncytiotrophoblast cells (99), several studies have reported hCG hormone production *in vitro* as a common feature of tumor cell lines (**Table 2.2)** (99, 111, 112). HTR-8/SVneo cell line is derived from normal trophoblast cells, not tumor cells; however, hCG production has been studied in this cell line and results showed profound secretion in the response to some treatments such as GnRH in a time- and dose-dependent manner (110).

Table 2. 2. Types of immortalized cell lines used to study trophoblast functions and the corresponding primary trophoblastic features of each cell line (99)

Table 2.2a. Human trophoblast cell lines

Cell line	hCG	hPL	CK	CK-18	CK-8	CK-7	Vimentin
TCL-1	+	n	+	n	n	n	−
IST-1	−	+	+	+	n	+	n
HT	+	−	+	n	n	n	−
TL	−	−	+	n	n	n	−
NPC	+	n	+	+	n	n	−
NHT	+	−	+	n	n	n	−
SGHPL-4	+	+	n	−	n	n	+
SGHPL-5	+	+	+	+	n	n	+
SGHPL-6	n	n	n	n	n	n	n
SGHPL-7	n	n	n	n	n	n	n
HTR-8	−	+	+	+	+	n	n
HTR-8/Svneo	+	n	+	+	+	n	n
RSVT-2	n	+	+	n	n	n	n
RSVT2/C	n	+	+	n	n	n	n
HT116	n	n	+	n	n	n	n
ED77	+	+	+	+	+	n	−
ED27	+	+	+	+	+	n	−
ED31	+	+	+	+	+	n	−
SPA-26+7 lines	+	n	n	n	n	n	n
HP-W1	+	n	n	n	n	n	n
HP-A1	+	n	n	n	n	n	n
HP-A2	+	n	n	n	n	n	n

Table 2.2b. Choriocarcinoma cell lines

Cell line	hCG
BeWo	+
BeWo MC-1	−
BeWo MC-2	−
JAR	+
JEG	+
AC1	n
AC1-5	n
AC1-9	n
*AC1-1	n
*AC-1M59	n
*AC-1M32	n
*AC-1M46	n
*AC-1M81	n
*AC-1M88	n

n: not available

2.3.2 Primary trophoblast cells

The isolation of cytotrophoblast cells has been reported using enzymatic digestion protocols (93). Cytotrophoblasts differentiate *in vitro* into multinucleated syncytiotrophoblast cells (93) and EVTs. Cytotrophoblasts lose their proliferation capability in the *in vitro* environment and cannot be cultured for a long time (113, 114). Therefore, trophoblastic immortalized cell lines have been established to study particular aspects and functions of human placental cells *in vitro* (100) (**Figure 2.6**).

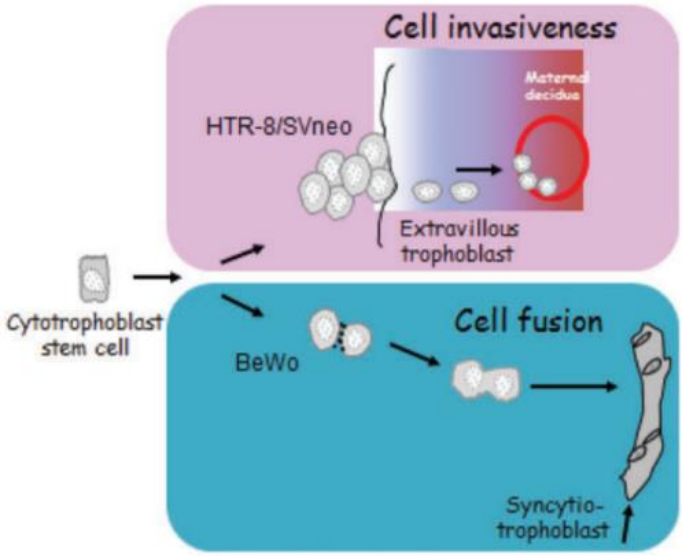


Figure 2. 6: Different pathways differentiation of the HTR-8/SVneo and BeWo trophoblast cells (93)

Although experiments using cell lines are highly reproducible, results obtained might be far away from the *in vivo* environment and do not represent the responses to treatments *in vivo* (93). Also, cell lines culture identify mono cell function, while placental function relies on multicellular interaction (93). Thus, several *ex vivo* studies investigated the different trophoblast cell populations in the same culture, called the co-culture system (115, 116). The

primary placental explant is another model that has been established to closely resemble the *in vivo* trophoblast differentiation and the outgrowth pattern (93, 115).

2.3.3 Placental explants

Explants from human placenta have been widely used to study various placental functions and organ behavior. They were first established to measure oxygen consumption (117, 118), then placental explant transport studies were widely investigated during the 1960s to 1970s (115). Fox examined the effect of various oxygen concentrations on cytotrophoblast proliferation using first-trimester placenta explant in 1970 (119) and was the first to report their stimulatory effect on cytotrophoblast proliferation after low oxygen exposure (119).

In the early 1990s, studies on trophoblast proliferation and differentiation in explants from early gestation increased, using the anchoring and floating villi (120, 121). In addition, the effect of the extracellular matrix in the explant columns reconstitution and EVT differentiation was recognized. First-trimester anchoring villi explants have provided *in vitro* models to understand the post-implantation events such as early placentation stages, cytotrophoblast cell differentiation, and placental invasion (115).

2.3.3.1 Advantages:

a. It preserves the intact villi structure with the presence of all cell types including cytotrophoblast cells, syncytiotrophoblasts cells, endothelial cells, and villous stroma that maintain the cell-cell interaction and the paracrine regulation (93).
b. It mimics the different biological functions of trophoblast cells including proliferation, differentiation, and hormones production (93) (**Figure 2.7**).

2.3.3.2 Disadvantages:

a. The inability to identify which cell is responsible for a particular function (93).

b. Short-term experiment

c. The irreproducibility of the explant

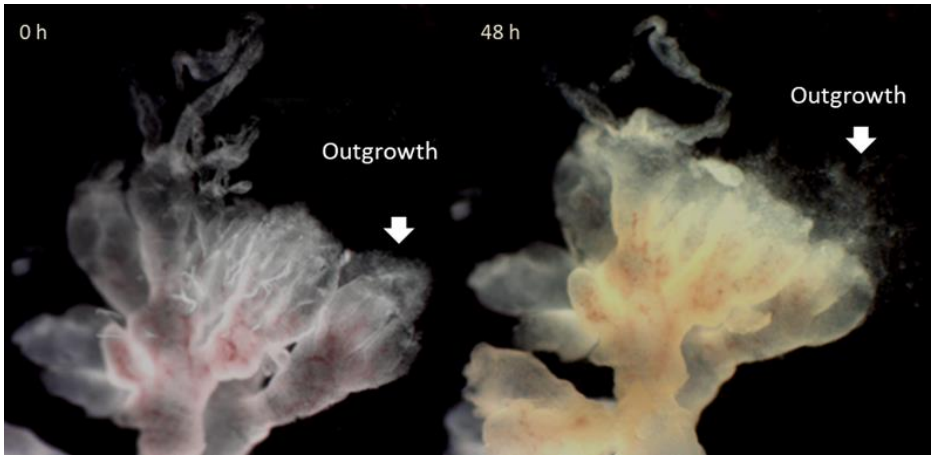


Figure 2. 7: Placental explant *in vitro* culture. The placental column outgrowth imaged at 0 hours (0 h) and after 48 hours (48h). This villous was cultured in untreated medium and used in the control group of the experiment performed to address aim 2 in this study.

2.4 Laboratory Models to Study Tubal EP

2.4.1 Descriptive studies

The use of Fallopian tube tissue from women with tubal pregnancy has been approved in most EP research projects. However, the ethical concerns involved in obtaining healthy Fallopian tube tissue from women with intrauterine pregnancies has made it difficult to create an ideal control arm (122). Thus, collecting Fallopian tubes in the mid-luteal phase of the menstrual cycle from non-pregnant women undergoing hysterectomy for any gynecological condition (123, 124) or from women in a pseudo-pregnant state treated with hCG hormone days before the surgery (125) are the only options. Both have limitations and could be largely irrelevant, particularly if the studies aim to identify diagnostic markers for EP (122). However, these options can be useful in the tubal functional studies that are critical to identifying the role of Fallopian tube dysfunction and tubal pregnancy occurrence (122).

Also, they were used to recognize some risk factors that cause tubal dysfunction and consequently affect embryo-tubal transport (122). For example, the role of PROKs receptors in response to smoking and past *Chlamydia trachomatis* infection on tubal cells function determined in human. The expression of PROK receptors −1 (PROKR1) and −2 (PROKR2) increases in response to smoking or after *Chlamydia trachomatis* infection, respectively. In addition to the descriptive studies, functional studies confirmed that cotinine, a smoking metabolite, and *Chlamydia trachomatis*, upregulate PROKR1 and PROKR2 expression *in vitro* (53, 126) (**Figure 2.8**).

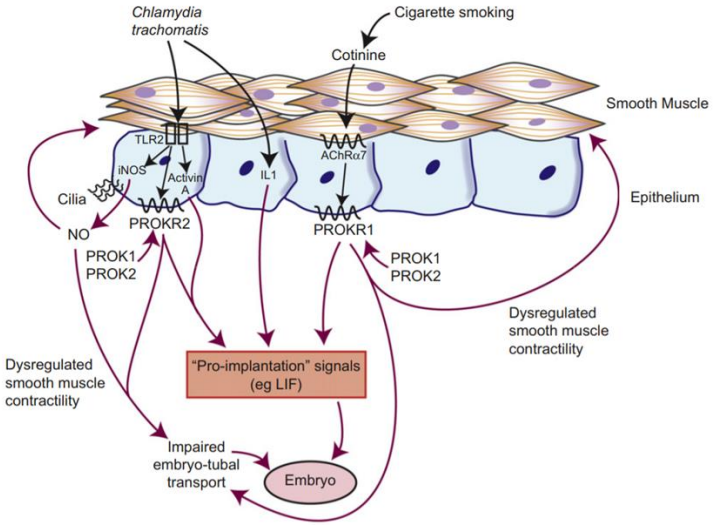


Figure 2. 8: Representative scheme for altered paracrine signals in the Fallopian tube in response to cigarette smoking and *Chlamydia trachomatis* (38).

Investigations on the endometrium from women with tubal pregnancies have shown similarities and differences in decidualization between intrauterine and EP. The similarities were in cellular composition (127, 128), while the differences were in morphology and expression. Several decidualization markers such as prolactin, insulin-like growth factor binding protein 1, and inhibins/activins were expressed differently in the EP endometrium than in the endometrium of intrauterine pregnancies of a similar gestational age (129, 130). These markers have been proposed as serum biomarkers for EP implantation.

Gestation-matched comparisons between intrauterine and tubal pregnancies have also made by obtaining trophoblast tissues from women undergoing elective pregnancy termination or surgical management of miscarriage and from tubal pregnancy (122). Although isolation of trophoblast cells from normal pregnancy placentas is well established, purification of the same

cells from tubal EP implantation sites was not reported until a successful establishment performed by our research team (131).

Biological fluids are another tool used to differentiate ectopic from intrauterine pregnancies. They are more accessible for laboratory studies and enable identifying a biomarker profile for EP (122). Despite recent advances in protein separation and mass spectrometry, these techniques did not help in determining novel biological markers for EP (122). Using new technologies such as one-dimensional gel electrophoresis/multidimensional protein identification technology (1-DE MudPIT) enabled recognizing 70 proteins that appear to be differentially expressed in women with EP (132). A disintegrin and metalloprotease-12 (ADAM-12) is one of the proteins effective in differentiating EP from viable intrauterine pregnancy (133). It was tested in 199 EP patients using the commercial DELFIA assay (134). Results showed lower levels in the serum of the EP group compared to the intrauterine pregnancy group (133).

2.4.2 Functional studies

Tubal embryo transport is regulated by tubal ciliary beat activity and tubal smooth muscle contractions (17, 33, 38). Although this dynamic process has not been examined *in vivo*, several *ex vivo* studies were developed to study ciliary beat frequency (CBF) in animals such as guinea pigs, (135) and mice (136, 137), and in human Fallopian tube biopsy samples (138, 139). The methodology used to measure CBF is well identified (122, 140) and adjusted for the agonist/antagonist studies (122), which helped in determining the effect of various factors including the beta-adrenergic stimuli (141), prostaglandins (142), and angiotensin-II (143), which increase CBF, while progesterone in high doses decreases CBF (122). Unfortunately, the

presence of endogenous agonists/ antagonists and the complex nature of the tissue biopsies has presented technical problems (122). Also, the difficulties in tissue imaging because of the size, mobility, and transparency have required sophisticated tissue preparation (122, 136). In addition, the ciliary activity showed cyclic changes (136, 144) that increase in the luteal phase of the menstrual cycle in humans (144) and decrease in the estrous phase of the mouse menstrual cycle (136).

Similarly, Fallopian tube contractility models have been established by isolating smooth muscle tissue strips or circles from Fallopian tube biopsy samples under a stereomicroscope (122). The muscle strips were mounted in chambers, then contractions were recorded by a force-displacement transducer under tension (122). Data were registered on a polygraph or digital data acquisition system (122).

The effect of adrenergic receptors on oviductal smooth muscle was reported after the use of this approach, and results revealed that stimulation of alpha receptors promotes smooth muscle contraction, while the stimulation of beta receptors suppresses it (145). Also, sex steroid hormones and other factors such as NO (46, 146), prostacyclin (147), and prostaglandins (147-149) have a regulating effect on tubal smooth muscles that control the embryo transport. Studies on murine oviducts have shown that chlamydial infection recruits macrophages expressing nitric oxide synthases 2, which produce NO (44, 150) that damages the oviductal muscle activity (44, 150).

2.4.3 Cell culture

Numerous studies have examined co-culture methods using human embryos and endometrial cells to identify endometrial biology (151). There have been few similar studies

using Fallopian tube cells to determine the causes of tubal pregnancy because of the unavailability of correlated cell lines and the problems associated with isolated tubal cells (122). Only two epithelial cell lines from the Fallopian tubes were established (152, 153). That was deemed due to the technical difficulty to isolate cells from tubal tissues because of the number of cells isolated in each case is variable (122). As well, tubal epithelial cells grown in monolayer culture lose their morphological features such as cilia (122). Developing a novel Fallopian tube epithelial culture system (154) that overcomes the co-culture obstacles could open the door for researchers to use it with trophoblast tissue or *in vitro* fertilized embryos and could be a useful tool to analyze gene expression changes during tubal ectopic implantation (122). The presence of ciliated cells in this model will enable understanding of the mechanisms regulating the functions of cilia (122). Results from these studies coupled with microarray and proteomic technologies may provide clues for the similarities and differences between ectopic and intrauterine placentation (122).

2.4.4 Animal models

Abdominal EP is the most frequent EP that occurs in animals with only 3 cases of tubal pregnancy reported to date (122, 155). However, studies on rodent models have yielded crucial information on the etiology of tubal EP (156). For example, the importance of endocannabinoid receptor 1 (CB1) in the tubal transport process shown in mice lacking CB1 or CB1/2, or treated with the synthetic CB1 antagonist, in which embryos retained in the oviducts (157). Also, the importance of Dicer1, a ribonuclease III enzyme required for micro-RNA processing, have been published after studies on female mice carrying a floxed allele of Dicer1 (122, 158). In these female mice, there was tubal hypotrophy with the formation of tubal cysts that disturb tubal

embryo transport (159-161). Future animal studies should aim to induce tubal implantation in mice by knocking down specific genes that contribute to tubal pregnancy, such as PROKs, LIF, or HOXA10 (122). Disturbing tubal ciliary beat frequency and tubal smooth muscle contractility by treating mice with agents that antagonize several genes as PROK, CB1, and NO may also provide useful tubal pregnancy animal models (122).

Chapter 3: Gonadotropin-Releasing Hormone (GnRH) System

3.1 GnRH Types

The peptide hormone GnRH and its G-protein-coupled receptor (GnRH-R) play a crucial role in human reproductive functions (63, 162, 163). Three GnRH isoforms have been identified in humans, named GnRH-I, -II, and -III (110, 164, 165). The mammalian GnRH, GnRH-I or hypothalamic GnRH, is primarily expressed in the central nervous system and regulates the gonadal functions via the hypothalamic-pituitary-gonadal axis. It stimulates the anterior pituitary gland to secrete the gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which control the gonadogenesis and hormone production (166).

The second GnRH isoform is GnRH-II or midbrain GnRH that was first discovered in the chicken and has a 70% similarity to GnRH-I (110, 167, 168). Both GnRH-I and GnRH-II are expressed in the central nervous system and produced by the peripheral tissues including the reproductive organs and the placenta in normal and neoplastic conditions (167, 169).

The third isoform is GnRH-III or telencephalic GnRH that was first isolated from the sea lamprey (170) and has 60% homology to GnRH-I. In the lamprey, studies have shown the essential role of GnRH-III in gametogenesis and steroidogenesis (171). In humans, GnRH-III is expressed in the hypothalamus and midbrain (165, 172, 173), but there is no evidence for expression in peripheral organs.

3.1.1 Structure:

The sequence of GnRH as a decapeptide was identified in 1971 by Andrew A. Schally (174); however, the function of each residue was not studied until some time later. **Figure 3.1**

shows the sequence of GnRH isoforms in humans, a 3D structure of GnRH-I, and the functions of residues.

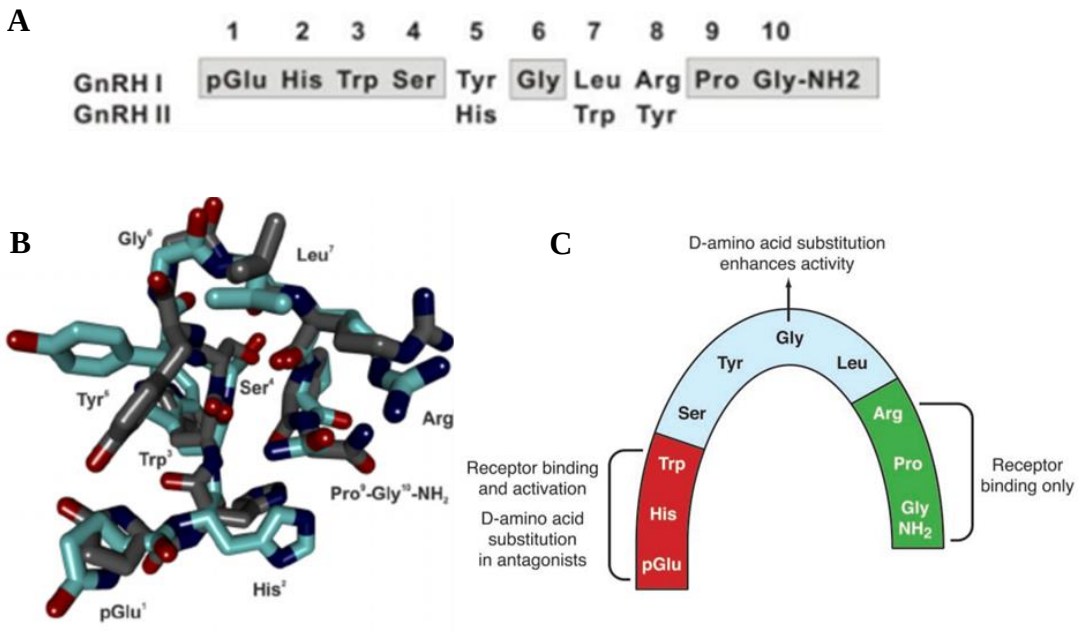


Figure 3. 1: A. the sequence of GnRH isoforms in humans. **B.** The NMR structure of GnRH showing the location of the residues (175). **C.** Representative image showing the function of residues (163)

3.2 GnRH Receptor (GnRH-R)

To date, two GnRH-R isoforms, GnRH-IR and GnRH-IIR, have been identified in humans (165, 169). GnRH receptors are expressed in the pituitary and extra-pituitary tissues, including placenta (164, 169). Studies on the structure of GnRH-R have shown the presence of 2 different binding domains for the GnRH agonist and antagonist ligands (176, 177). Both GnRH agonist and antagonist can bind to the same GnRH-R, but the cellular biological response relies on several factors including the receptor affinity for the ligand, the volume of the ligand, and the ligand structure.

The structure of the GnRH analog can alter the cell response (177-179). For instance, peptide GnRH antagonists such as cetrorelix and ganirelix cannot stabilize the receptor configuration after the initiation of receptor activation by the agonist. In contrast, non-peptide GnRH antagonist such as elagolix can induce allosteric changes in the receptor and induce receptor stabilization even after the agonist stimulation.

The receptor affinity for GnRH analog affects the subsequent cellular function. Synthetic GnRH agonists and antagonists are designed to have a higher GnRH-R affinity than GnRH native form. For example, ganirelix that used as a clinical GnRH antagonist has a 9-fold higher affinity for GnRH-R than GnRH-I (165, 180), while cetrorelix has 20-fold. Also, cetrorelix has a 2-fold higher affinity to the receptor than buserelin, a synthetic GnRH agonist, or antide, a different antagonist (181).

3.3 Synthetic GnRH Agonists Vs Antagonists in the Clinical Practice

GnRH synthetic analogs, agonists and antagonists, are widely used to treat many gynecological disorders including infertility, central precocious puberty, endometriosis, and uterine fibroid, as well as endometrial carcinoma, and breast cancer (182-184). GnRH agonists are used clinically because of their reversible blocking effect on the pituitary gonadotropin hormones secretion (**Figure 3.2**). However, agonists first induce an initial LH increase, which results in increased symptoms (165, 185). GnRH antagonists have the advantage of avoiding this initial hormonal increase before the central suppression (**Figure 3.2**) (186), but their effect is shorter than the agonist suppressive effect and ceases shortly after the discontinuation of treatment (186).

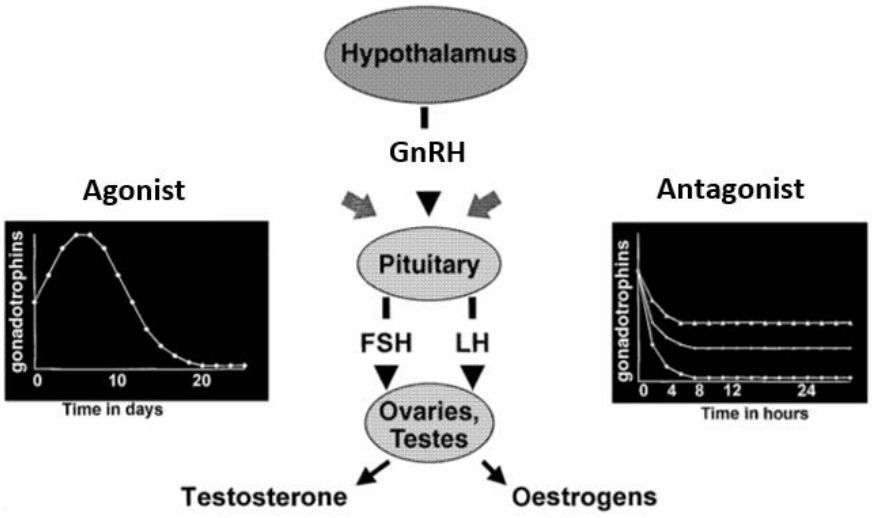


Figure 3. 2: GnRH analogs mode of action

Various clinical GnRH antagonists have been used in reproductive medicine to treat hormone-dependent diseases (187). First generation GnRH antagonists were associated with anaphylactic reactions because of significant histamine release (188-190) (**Figure 3.3**). Subsequent generations have overcome this adverse effect by adding D-Ala in position 10 and Ac-D-Nal-D-Cpa-D-Pal in the N-terminal with several amino acid substitutions in positions 5, 6, and 8 (185, 191) (**Table 3.1**).

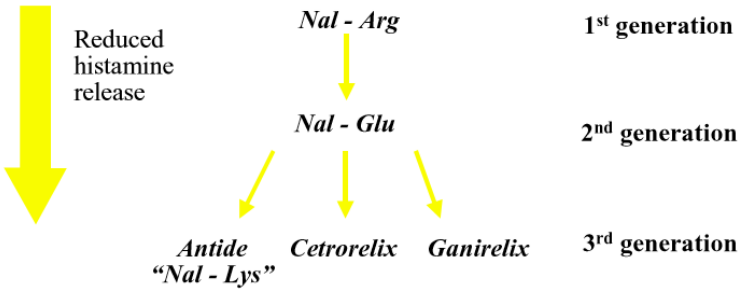


Figure 3. 3: GnRH antagonist generations (190)

Cetrorelix, ganirelix, abarelix, and degarelix are the third generation GnRH-R antagonists with the highest receptor binding affinity and inhibitory effect (185). Cetrorelix was the first on the market for controlled ovarian stimulation (COS) (192). Cetrorelix and ganirelix are administrated as subcutaneous injections and mainly employed to treat endometriosis and uterine fibroid, as well as being used in for *in vitro* fertilization protocols (185) (**Table 3.2**). Degarelix was approved by the Food and Drug Administration (FDA) to treat prostate cancer (**Table 3.2**). Abarelix has immediate systemic allergic reactions, which caused its withdrawal from the USA market (185).

Elagolix is a new GnRH-R antagonist that was approved by the FDA in 2018. It is a non-peptide antagonist that can be administrated orally and overcome the complications of the injection route (193). In addition, as a non-peptide molecule, it can stop GnRH receptor subsequent response after being activated by GnRH. Several studies investigated the effect of elagolix on endometriosis-associated pain and heavy menstrual bleeding associated with uterine fibroids (194-197). Results showed that elagolix had a suppressive effect on symptoms in patients during the phases of clinical trials (194, 197). On 23 July 2018, US-FDA approved elagolix 150 mg and 200 mg tablets as an oral GnRH antagonist to treat moderate-to-severe pain associated with endometriosis (198). It is still under phase 2 clinical trial for heavy menstrual bleeding associated with uterine fibroids (198).

Antide, also known as Iturelix, is another GnRH-R antagonist that is used extensively in the agonist/antagonist *in vitro* studies. It has a poor solubility that causes complications such as erythema and nodule formation at the injection site (199). Its suppressive effect on testosterone production in rats continued for 30 days after a single 2 mg antide injection (200).

Table 3. 1: Amino acids sequence of GnRH analogs (190)

	1	2	3	4	5	6	7	8	9	10
Agonists										
GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Buserelin	1	2	3	4	5	D-Ser	7	8	9ethylamide	
Goserelin	1	2	3	4	5	D-Ser	7	8	9	AzGly
Leuprolin	1	2	3	4	5	D-Leu	7	8	9ethylamide	
Triptorelin	1	2	3	4	5	D-Trp	7	8	9	Gly-NH_2
Nafarelin	1	2	3	4	5	D-Nal	7	8	9	Gly-NH_2
Antagonists										
Cetrorelix	D-Nal	D-Phe	D-Pal	4	5	D-Cit	7	8	9	D-Ala
Nal-Glu	D-Nal	D-Phe	D-Pal	4	Arg	D-Glu	7	8	9	D-Ala
Antide	D-Nal	D-Phe	D-Pal	4	NicLys	D-Niclys	7	Lys(iPr)	9	D-Ala
Ganirelix	D-Nal	D-Phe	D-Pal	4	5	D-hArg	7	hArg	9	D-Ala
AzalineB	D-Nal	D-Phe	D-Pal	4	Phe	D-Phe	7	Lys(iPr)	9	D-Ala
Degarelix	D-Nal	D-Cpa	D-Pal	4	Aph	D-Aph	7	Lys(iPr)	9	D-Ala

Table 3. 2: Decapeptides commercially available or in human trials (200)

Decapeptides commercially available or in human trials.

Name	Manufacturer/ patent holder	Route	Trial phase	Approved indication	Clinically tested application	Preclinical applications
Decapeptides						
Abarelix (Plenaxis®)	Praecis	SC	Phase III	NA	Prostate cancer, endometriosis	NA
Acyline	NICHD/Salk Institute	SC	Phase II	NA	NA	Hormonal male contraception
Teverelix (Antarelix®)	Asta Medica AG/Europeptides	SC	Phase I	NA	NA	NA
Cetrorelix (Cetrotide®)	Serono, ASTA Medica AG	SC	Commercially available	IVF	BPH, uterine fibroids, prostate cancer	Precocious puberty, gynecological cancer
Degarelix (FE200486)	Ferring Pharmaceuticals	SC	Phase II	NA	Prostate cancer	IVF, endometriosis
Ganirelix (Antagon®, Orgalutran®)	Organon Inc	SC	Commercially available	IVF	Ovarian hyperstimulation syndrome, PCOS	NA
Iturelix® (Antide)	Serono	SC	Preclinical	NA	NA	leiomyoma
Orntide (Ornirelix®)	Oakwood Laboratories/ Tulane	SC	Phase I/II	NA	NA	NA
Non-peptides						
CMPD1	Pfizer, Agouron	Oral	Preclinical	NA	NA	NA
NBI-42902	Neurocrine Inc	Oral	Phase I	NA	NA	NA
NOX 1255	NOXXON Pharma, Schering	SC	Preclinical	NA	NA	NA
TAK-013	Takeda	Oral	Phase II	NA	NA	NA

NA, not available; NICHD, National Institute of Child Health and Human Development; PCOS, polycystic ovarian syndrome; SC, subcutaneous.

3.4 GnRH System in Normal Placentation

The expression of GnRH and GnRH-R has been studied in human placenta. Their detection was reported in the maternal and fetal compartments in the first trimester (201, 202) and in term placentas (61). GnRH expression is differential within trophoblast cell subpopulations and during the period of gestation. GnRH-I is expressed in all trophoblast subpopulations throughout the gestational period (61, 203), while GnRH-II is expressed in the villous cytotrophoblast and EVT cells during early pregnancy (61, 169).

GnRH-IR expression has been detected in the cytotrophoblast and syncytiotrophoblast cells (169, 204), while the presence of full-length functional GnRH–IIR RNA transcript was not detected in the human peripheral tissues (205). The effect of GnRH-I and GnRH-II on the reproductive organs has been shown to be mediated by GnRH-IR activation (206).

3.4.1 GnRH analogs and uterine receptivity

Studies using GnRH agonists/ antagonists in women undergoing *in vitro* fertilization showed a significant higher endometrial thickness and pregnancy rate in women using the GnRH agonist protocol (207). Results before suggested this was attributable to the higher endometrial receptivity after GnRH agonist administration (207). On the other hand, the use of GnRH antagonists in the assisted reproductive technology resulted in significantly lower pregnancy rates compared with the long agonist protocol users, when used in high doses without adequate luteal support, as shown in a Cochrane review of clinical trials (208). However, recent data showed the pregnancy rate with agonist and antagonist protocols are the same.

3.4.2 GnRH analogs and embryo implantation

The administration of GnRH agonist alone (209) or in combination with other hormones (210) for ovarian stimulation and intracytoplasmic sperm injection (ICSI) was reported to improve embryo implantation and live birth rates compared with placebo (185).

Meanwhile, ethical concerns have limited the study of GnRH antagonists on embryo implantation. However, a study showed lower implantation rates in the group treated with ganirelix in doses more than 0.25mg per day (211). Another study showed that treating rats with cetrorelix during the implantation period had a teratogenic effect, poor pregnancy outcomes, and decreased placental development (212).

3.5 GnRH System in Trophoblastic Cell Lines

GnRH and its receptor are also expressed in several trophoblastic cell lines. Previous studies showed GnRH system expression in the HTR-8/SVneo (213), BeWo (214, 215), and JEG-3 cell lines (216). Also, the influence of GnRH on hCG hormone production in HTR-8/SVneo (110) and BeWo cell lines was examined. GnRH-I and GnRH-II induce hCG secretion from HTR-8/SVneo immortalized cells in a dose-dependent manner (**Figure 3.4**) (110).

The effects of GnRH analogs on the cell viability of HTR-8/SVneo and BeWo immortalized cells were studied in our laboratory (63). GnRH-I increased BeWo cell viability and this effect was suppressed by treatment with the GnRH antagonist antide (**Figure 3.5**) (63). HTR-8/SVneo cells did not have different responses in the agonist and antagonist groups (**Figure 3.5**) (63). The effect of clinical antagonists such as cetrorelix and ganirelix on hCG production was also investigated in our laboratory (data not yet published). Results revealed a

significant upregulation of hCG mRNA levels after treating BeWo cells with GnRH-I, which was suppressed in the groups treated with cetrorelix or ganirelix (**Figure 3.6**).

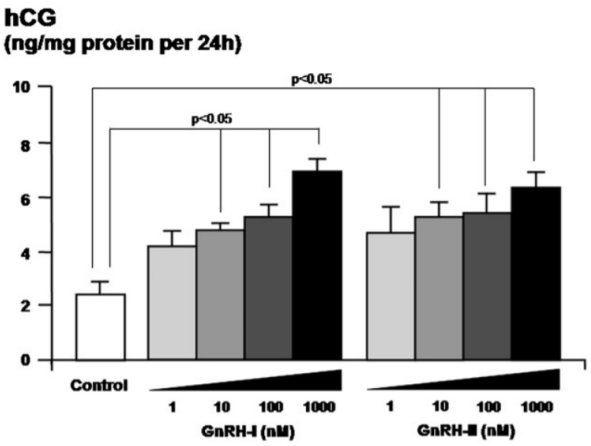


Figure 3. 4: GnRH effect on hCG production in HTR-8/SVneo immortalized cells (110). HCG secretion significantly induced after GnRH-I and -II treatment in a dose-dependent manner.

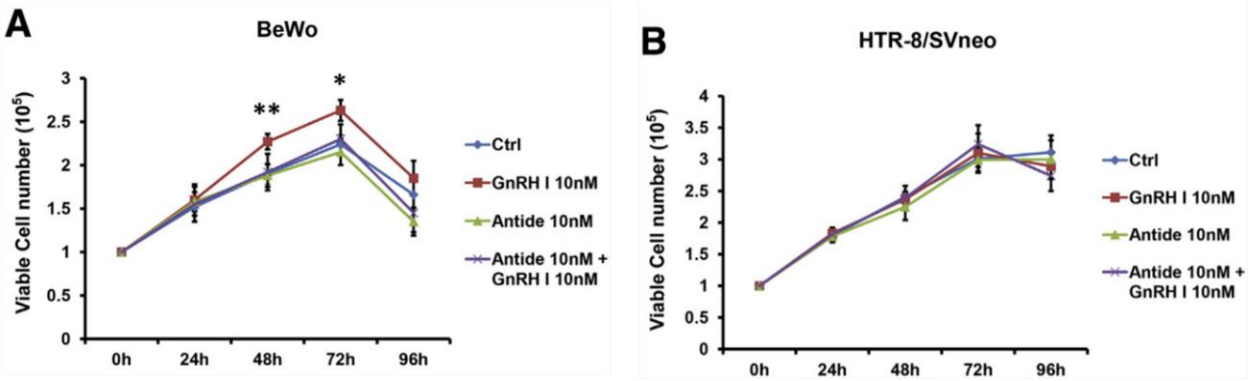


Figure 3. 5: Effect of GnRH analogs on immortalized cell viability (63)**.** GnRH antagonist (Antide) abolished the induced effect of GnRH in the number of viable BeWo cells, but there was no effect of GnRH or GnRH antagonist in HTR-8/SVneo cells (62). BeWo cells and HTR-8/SVneo cells treated with 10 nmol/L GnRH I for 24, 48, 72 or 96 hours. (A) BeWo and (B) HTR-8/SVneo viable (non-stained) cell numbers measured at each time point by means of trypan blue cell counting. Results are presented at the mean SEM of three independent experiments, and significant differences are indicated by asterisks (n = 3; * P-value < .05; ** P-value < .01).

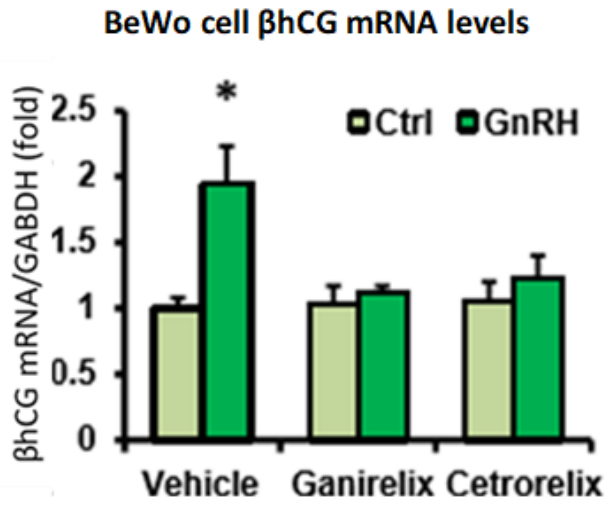


Figure 3. 6: Effect of clinical GnRH antagonists, cetrorelix and ganirelix, on βhCG mRNA levels in BeWo choriocarcinoma cells. GnRH (100nM)-induced βhCG mRNA production (9 hours) was abolished by pre-treating BeWo cells with GnRH antagonists (Ganirelix and cetrorelix, 100nM). P-value <0.05. (Dr. Peng did this experiment. Not published)

3.6 GnRH System in Tubal Pregnancy

Although Casañ et al. identified GnRH expression in the human Fallopian tube epithelium in 2000 (217), expression of the GnRH system in tubal pregnancy implantation sites had not been investigated until a recent study performed in our laboratory (63). The results showed a differential GnRH/GnRH-R expression among trophoblast subpopulations, Fallopian tube epithelial cells, and Fallopian tube stromal cells (63) (**Figure 3.7**).

Syncytiotrophoblasts showed the highest GnRH expression, then EVTs, which had a higher GnRH immunoreactivity than cytotrophoblasts. GnRH expression in the tubal epithelium was similar to that observed in the cytotrophoblasts but significantly lower than syncytiotrophoblasts and EVTs expression (63).

The expression of GnRH-R was the highest in cytotrophoblast and EVT cells when compared with the whole cell populations. However, there was no significant difference

between them. The tubal stromal cells showed the lowest GnRH-R expression (**Figure 3.7**) (63).

Though so far the only study exploring GnRH/GnRH-R expression in tubal EP implantation sites, the effect of GnRH analogs on primary EP trophoblast cells has yet to be determined due to the unavailability of primary EP trophoblasts and the difficulty to isolate them from EP placenta. Peng et al. successfully established viable primary trophoblast cultures from tubal implantation sites in 2017 (131).

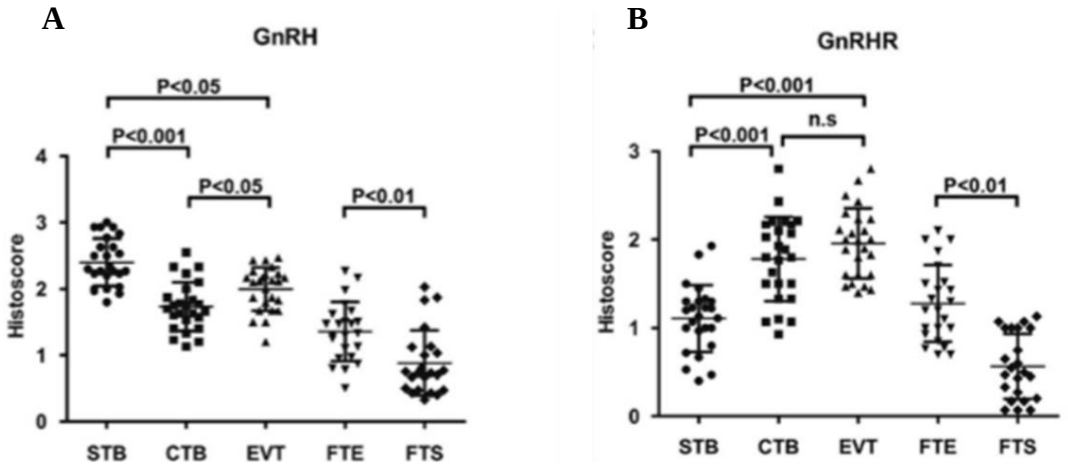


Figure 3. 7: Comparison of GnRH and GnRH-R histoscore between trophoblasts and Fallopian tube cells from women with tubal EP(63)**.**

Dot plots representing the histoscores of GnRH (**A**) and GnRH-R (**B**) between trophoblast cell subpopulations (cytotrophoblast [CTB], syncytiotrophoblast [STB], and extravillous trophoblast [EVT]) and Fallopian tube cells (epithelium [FTE] and stroma [FTS]). CTB, STB, and EVT: n = 25; FTE and FTS: n = 23.

Chapter 4: Study Hypothesis and Aims

4.1 Hypothesis

GnRH-I is essential for the establishment and early maintenance of pregnancy. It enhances placental cell invasion (216) and hCG production (218). GnRH and its receptor expression were identified in the Fallopian tube epithelium (217) and in EP trophoblastic cells (63). We hypothesize that clinical GnRH-R antagonists could suppress the different biological functions of EP trophoblast cells including the cell invasion, that could be used clinically to treat abnormal placentation such as tubal EP.

4.2 Specific Aims

Aim 1: To determine the effect of clinical GnRH-R antagonists on placental cell invasion using:

- HTR-8/SVneo immortalized trophoblast cells
- Primary trophoblast cells isolated from tubal implantation sites.

Aim 2: To investigate the effect of clinical GnRH-R antagonists on placental explants established from first-trimester human placentas.

Chapter 5: Materials and Methods

5.1 Tissues

5.1.1 EP human placentas

First-trimester human placentas were obtained from women with tubal pregnancy. Tissue collection was approved by the UBC Ethics Board (Approval number: H15-02234), and study participants provided written consents (**Appendix A**).

5.1.1.1 Inclusion criteria

Patients were included if

- They were 18 years-of-age or older.
- They had presented to the general gynecology department at Vancouver General Hospital (VGH) with tubal EP.
- They were eligible for tubal surgery, (salpingectomy or salpingostomy).

5.1.1.2 Exclusion criteria

Patients were excluded if

- They were eligible for medical treatment, MTX, for tubal EP.
- They presented with non-tubal EP.
- They did not read and understand the English language or had difficulty in understanding the consent form.

5.1.1.3 Time to consent

Participants were informed about the consent within 24 hours ahead of the procedure. They were presented with a consent form (**Appendix A**) and were given the opportunity to accept or decline participation in the study.

5.1.1.4 Recruitment

When physicians counseled women about the proposed procedure, they also provided information about this study and asked women whether they were willing to participate in the study by providing placental tissue derived from the tubal EP. The consent emphasized that their decision to participate in the study or not would not affect their treatment or the procedure they were offered. Because of the emergency nature of the procedure, it was not feasible for a member of our research team to be involved in consenting the participants. Most completed the consent with staff gynecologists or a resident. For those participants who presented to the hospital in an acute situation with a narrow window of time between presentation and necessary surgery, every effort was made to ensure that the participants would make an informed choice within the rules and the regulations of this process.

5.1.1.5 Collection

Specimens were retrieved from the Research Biobank, at the VGH Department of Anatomical Pathology, and transferred by one of the research team members, in a saline solution, to our laboratory at the BC Children's Hospital Research Institute.

5.1.2 Normal human placenta

Human placenta at 6-week gestation was obtained from a woman who underwent elective termination of pregnancy in the CARE program at BC women's hospital (ERB #: H13-00640).

5.2 Cells

5.2.1 Primary cells

We isolated primary villous cytotrophoblast cells from tubal EP implantation sites. The isolation steps started with dissecting the tree-like structure of the placental villi. This particular portion was chosen to prevent trophoblastic cells from becoming contaminated with the Fallopian tube epithelial cells (**Figure 5.1**). The dissected tissue was minced to fine particles using a sharp blade, washed with sterile Dulbecco's phosphate-buffered saline (DPBS) (Thermo Scientific, Waltham, MA), and centrifuged. Collagenase enzyme type 1 was added to the minced tissue for an hour in a warm water bath to allow the enzymatic digestion. The primary EP trophoblast cells were cultured in Dulbecco's minimum essential medium (DMEM) (Life Technology), supplemented with 10% fetal bovine serum (FBS) and 1% antimicrobial antibiotic treatment (AA), and then incubated at 37^0 C with 5% CO_2. Fresh enriched DMEM medium was replaced every 48 hours (**Figure 5.2**).

Our research team established primary trophoblast in vitro cultures from 5 tubal placental specimens out of 14, and I isolated 3 of them. These cells were validated for their trophoblastic origin by labeling them with trophoblast markers using the immunofluorescence staining (**Appendix B**).

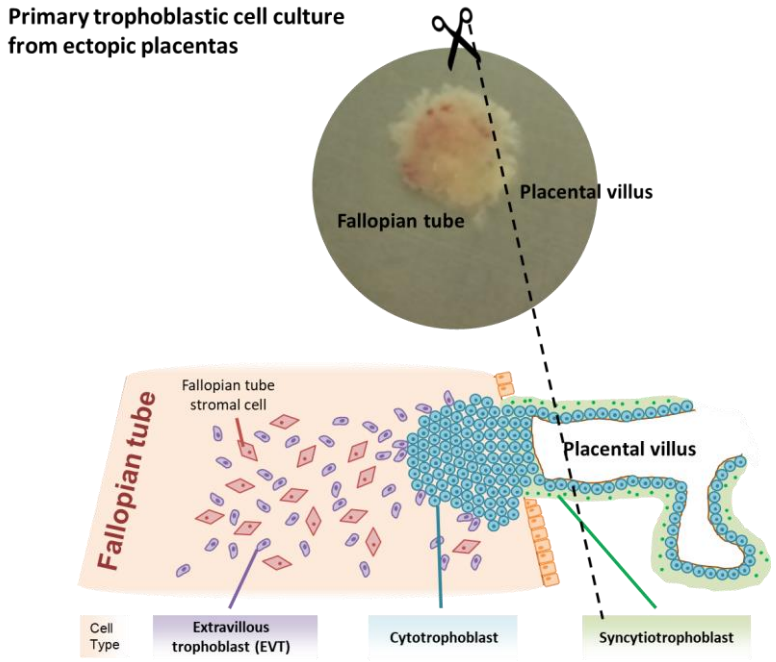


Figure 5. 1: A Schematic diagram for the particular part of the placental villi dissected during primary EP trophoblasts *in vitro* culturing. This portion represents the villous cytotrophoblast cells that differentiate in culture into syncytiotrophoblasts and EVTs.

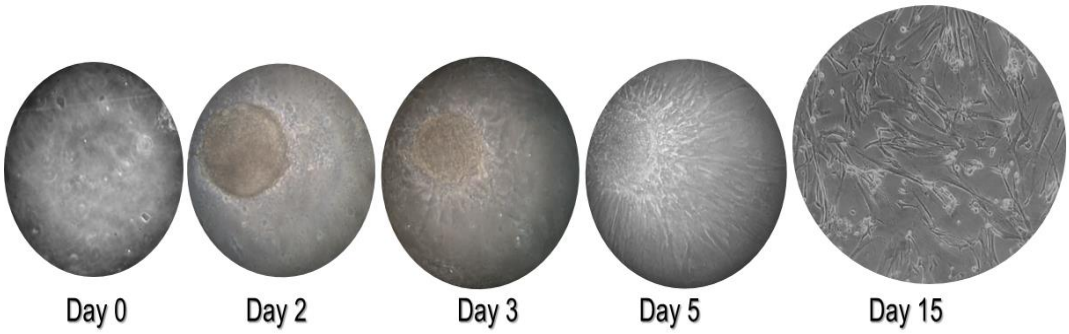


Figure 5. 2: A Panel Diagram shows a microscopic view for primary trophoblasts establishment from tubal EP placenta during the first passage of *in vitro* culturing.

5.2.2 Cell lines

The immortalized human HTR-8/SVneo cell line was generously provided by Dr. Peter C.K. Leung, (UBC, Vancouver, BC). HTR-8/SVneo cells were cultured in DMEM supplemented with 10% FBS and 1% AA treatments. Cells were maintained in a 37°C-humidified incubator with 5% CO_2. The culture medium was replaced with fresh medium every 48 hours.

5.3 Reagents and Treatments

Native human GnRH-1 and clinical GnRH-R antagonists (ganirelix acetate and cetrorelix acetate) were purchased from Sigma-Aldrich (St. Louis, MO). Antide, another antagonist, was obtained from Bachem (Belmont, CA). Growth factor reduced Matrigel was purchased from Corning (Bedford, MA).

5.4 Immunofluorescence

Trophoblast labeling markers were used to validate the primary EP trophoblastic cells. Cytokeratin-7 was used as a marker for cytotrophoblast cells, HLA-G for EVTs, hCG for syncytiotrophoblasts, and vimentin as a mesenchymal marker (**Appendix B**). The expression of GnRH and GnRH-R in these cells was also detected (**Appendix B**). Dr. Bo Peng led the cell immunofluorescence staining to which I made a significant contribution.

5.5 Trans-well Invasion Assay

Immortalized and primary cells were seeded in enriched DMEM culture medium with 10% FBS and 1% AA for 24 hours. DMEM medium containing 0.1% FBS was replaced for

another 24 hours. At day 3, the starved cells were treated with 100 nM distilled water (group 1), 100 nM GnRH-I (group 2), 100 nM Ganirelix (group 3), and 100 nM GnRH-I after 100 nM Ganirelix pre-treatment (group 4). At day 4, growth factor-reduced Matrigel was added to the culture inserts for at least 4 hours before the cells were seeded. The cells were suspended in 0.1% FBS supplemented DMEM and treated for a second time before seeding them into cell culture inserts, 8.0 µm pore size, in a concentration of $5x10^4$ cells per insert (**Figure 5.3**). The lower chambers were provided with DMEM containing 10% FBS to allow the chemotactic gradients that force the cells to migrate. The cells were incubated in 37^0 C with 5% CO_2 for 24 or 48 hours. Invading cells were fixed with cold methanol and stained with the hemacolor solution, (EMD Chemicals Inc., Gibbstown, NJ) (**Figure 5.3**). The non-invading cells were removed using cotton swabs, and the membranes were cut and mounted on glass slides. These slides were observed under the microscope, and the invading cells were counted.

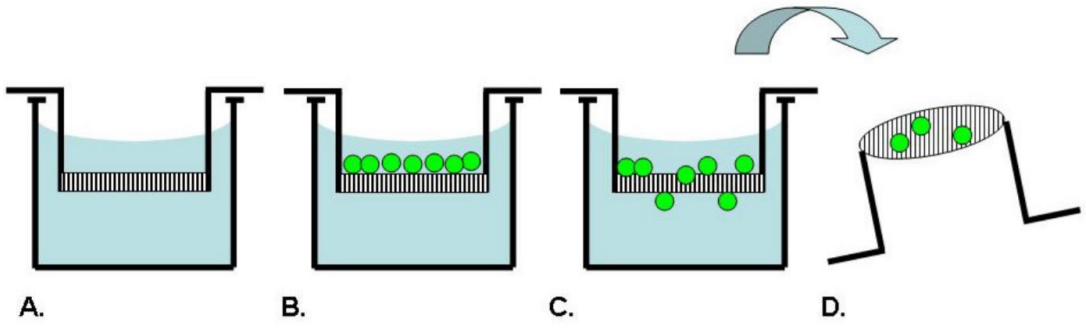


Figure 5. 3: Trans-well invasion assay (219)

5.6 Placental Explant *In vitro* Culture

The Milli cell culture inserts were coated with growth factor-reduced Matrigel (200µL/insert). Coated inserts were maintained in 37^0 C incubator with 5% CO_2 for 30 minutes to allow the Matrigel to solidify. During that time, good anchoring tips of the placental villi were selected carefully and dissected under a microscope. Each villous was dissected to include 3-4 columns and placed gently on top of the coated inserts (**Figure 5.4, a**). The lower chambers were provided with 400 µL of phenol red-free DMEM/F-12K (1:1) media, supplemented with 10% FBS and 1% AA. The explants were incubated in 37^0 C with 5% CO_2 for 24 hours.

At day 2, the medium in the lower chambers was replaced with 400 µL of fresh treated medium (100nM), and 200 µL of the treated medium (100nM) was added carefully on top of the explants (**Figure 5.4, b, c**). Images were taken, before incubation, by a digital camera connected to Nikon SMZ 7454T trinocular dissecting microscope as 0 hours (0h) time point for all explants in the control and GnRH-R antagonist groups. Images were taken again after 48 hours (48h) of treatment; then explants were fixed with paraformaldehyde overnight and embedded in paraffin for immunostaining.

The changes in the columns outgrowth, the length and the area of growth, were quantified using the ImageJ software (NIH) by subtracting the column length (10 measurements/column) or the total growth area at 0h from 48h.

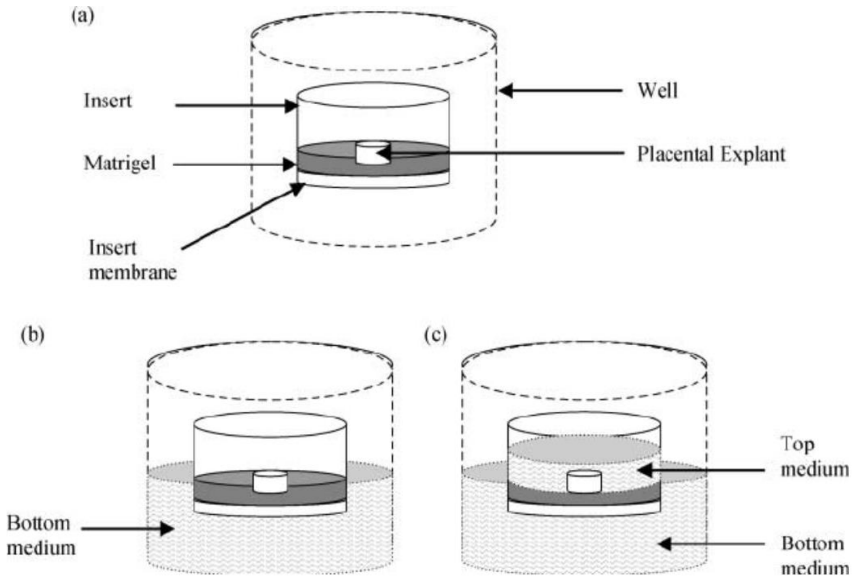


Figure 5. 4: Diagram of the explant establishment (220)**.**
(**a**) Diagram of the well, the insert, the Matrigel and the placental explant. (**b**) The explant cultures pre-incubated overnight in the presence of the medium in the lower chambers only. (**c**) The treated medium added gently above (top medium) and replaced below the placental explants (bottom medium).

5.7 Statistical Analysis

Three independent experiments were performed to address the effect of GnRH-R antagonists on the primary and immortalized cells invasion. One pilot experiment was done to determine the effect of cetrorelix on the placental explant growth in 3 replicates.

One-Way ANOVA test, followed by post-hoc Tukey's test, used to compare the 4-6 treated groups in the invasion experiment, represented in chapter 6.

Student t-test was used to analyze the results of 2 treated groups in the placental explants experiment, represented in chapter 7.

Chapter 6: Effect of Clinical GnRH-R Antagonist on Placental Cell Invasion

6.1 Introduction

Trophoblast cell invasion is a crucial step for embryo implantation and normal placentation. During early placentation, villous cytotrophoblast cells proliferate and differentiate into other trophoblast cell types. They either fuse and form multinucleated syncytiotrophoblast cells or undergo a different pathway to become EVTs. The invasive EVTs are non-proliferative cells that invade the maternal tissue and spiral arteries.

GnRH is expressed in the placental cells and in the Fallopian tube epithelium. Also, expression of GnRH and GnRH-R have been demonstrated at tubal pregnancy implantation sites. Several studies showed that GnRH-I has a stimulatory effect on primary EVT and HTR-8/SVneo cell invasion through distinct signaling pathways, which is abolished by antide, the experimental GnRH-R antagonist.

Experiments in this chapter determined the effect of GnRH-I and ganirelix, a clinical GnRH-R antagonist, on placental cell invasion. We also showed the effect of ganirelix compared to the antide effect in the same experiment. Antide has a known suppressive effect on HTR-8/SVneo cell invasion. We used the HTR-8/SVneo immortalized cells and primary EVT cells isolated from tubal EP placentas.

6.2 Results

6.2.1 Ganirelix attenuates GnRH-induced cell invasion in HTR-8/SVneo immortalized trophoblast cells

HTR-8/SVneo cell invasion induced after treating cells with 100nM GnRH-I, the dose used in the experiments based on previous studies performed in our laboratory. The induced cell invasion significantly diminished in cells pre-treated with 100 nM ganirelix for 24 hours ($P = 0.02$) (**Figure 6.1**). Ganirelix effect compared with the antide effect on same experiments in our study. Both antagonists significantly suppressed the cell invasion, with a higher significance shown in groups treated with the antide ($P = 0.01$) (**Figure 6.1**).

The inhibitory effect observed in groups treated with the antagonists, either alone or combined with GnRH-I (**Figure 6.1**).

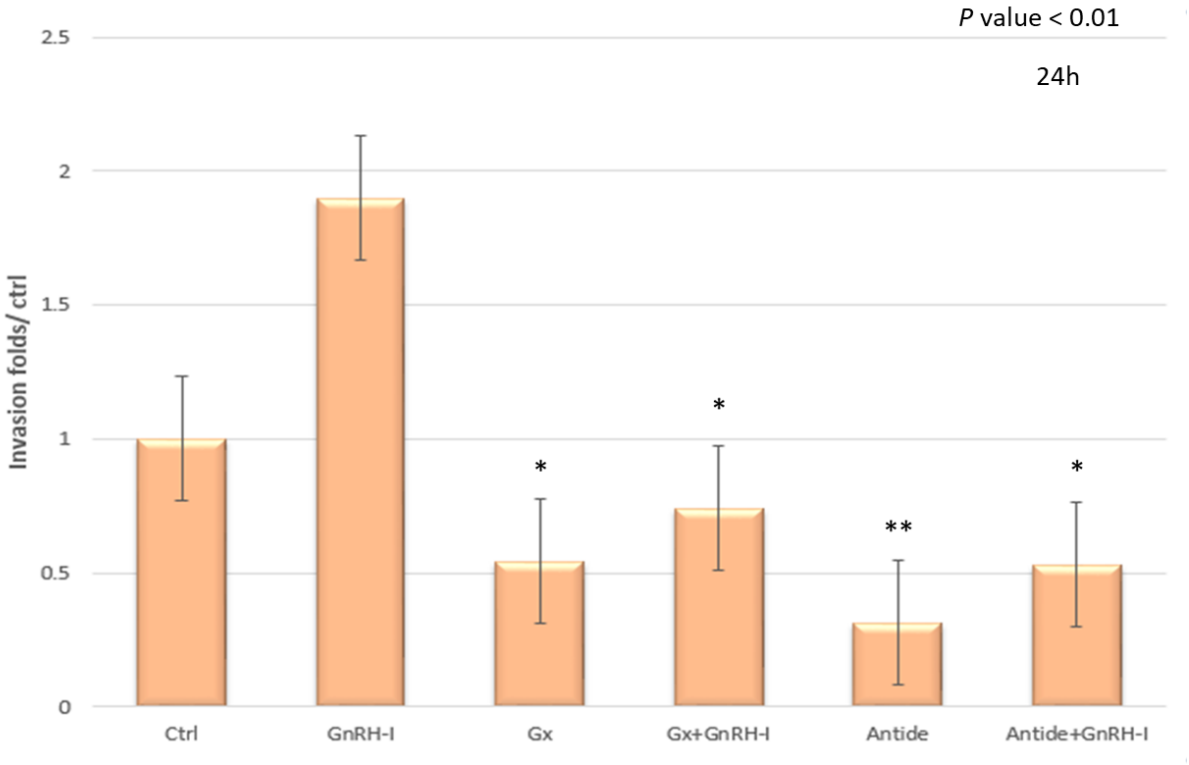


Figure 6. 1: Ganirelix, as well as antide, attenuate HTR-8/SVneo cell invasion

GnRH-R antagonists (100nM) suppress GnRH-I (100nM)-induced effect on HTR-8/SVneo cell invasion after 24 hours. Asterisks indicate the significant differences (*P-value < 0.05, ** P-value < 0.01).

6.2.2 Ganirelix effect on the invasion of primary trophoblasts isolated from tubal EP placentas

Results of 3 independent experiments using cells isolated from 2 patients showed no difference in the numbers of invading primary cells treated with GnRH analogs after 48 hours. Despite the well-known inducing effect of GnRH-I on the invasion of intrauterine placental cells, our results did not show the same response in trophoblast cells isolated from EP. On the other hand, EP cells treated with ganirelix showed a slight decrease in the invasion when compared with the control, but this difference was not statistically significant (**Figure 6.2**).

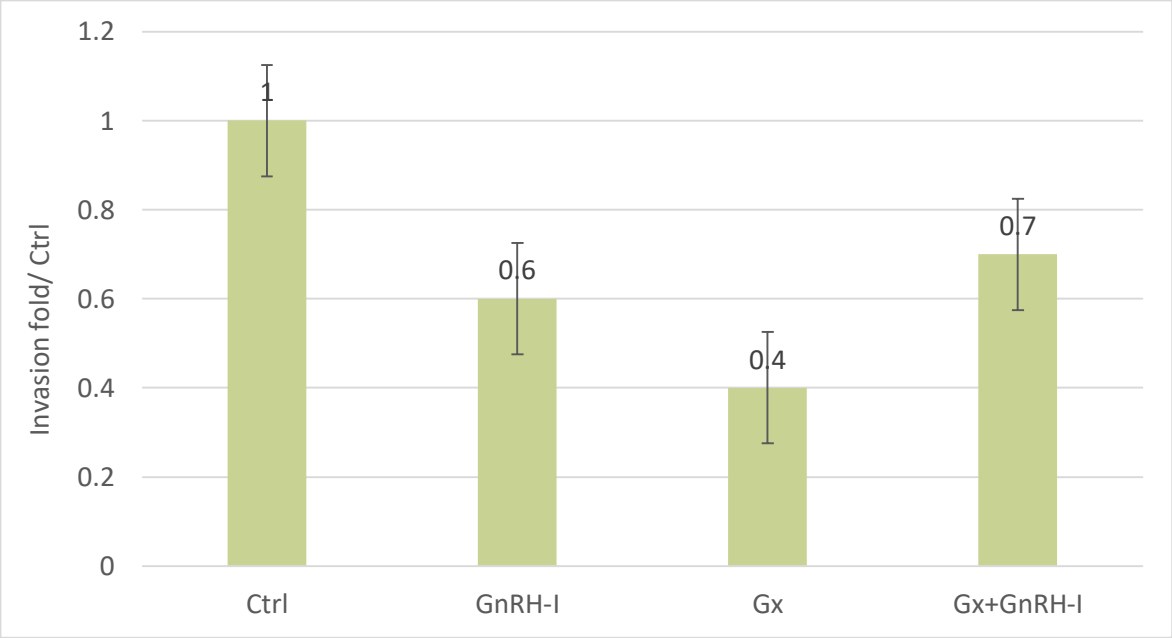


Figure 6. 2: Effect of ganirelix on primary EP trophoblast cells invasion

Primary trophoblast cells isolated from tubal implantation sites did not show differences between groups treated with 100 nM GnRH analogs after 48 hours.

6.3 Discussion

Although GnRH-I induced-effect on the placental cell invasion has been extensively studied in several trophoblast immortalized cells and primary EVTs isolated from intrauterine placentas, their effect on trophoblast cells isolated from EPs is not known. In this study, we examined the effect of GnRH analogs on EP trophoblast cell invasion as well as on HTR-8/SVneo cell line.

Our team isolated 5 primary cultures from 14 tubal implantation sites. We propose that the low success rate of isolation could be due to the nature of tissue collected, the small size of ectopic placentas with fewer numbers of villi, or the tissue damage after MTX therapy.

In 2 specimens, there were no tree-like projections that resemble the placental villi, and the tissues collected were white elongated structure that we assume to be the Fallopian tube.

Another specimen was collected from a patient who was treated with MTX after adding amendments to the inclusion criteria. There was total damage in the MTX-treated placenta (**Figure 6.3 A**), which did not enable us to recognize the villi or even identify different tissue layers within the specimen. Another challenge we had during isolation was the small size of tubal placentas (**Figure 6.3 B**). Most of the collected specimens had few numbers of villi due to the early gestational age of EP or the undeveloped tubal placenta because of the pathological location of the EP placenta. Finally, the bloody placentas we got in more than one specimen, could have affected the trophoblast isolation success. The presence of lots of blood cells in culture could have affected the adherence of isolated trophoblast cells to the plastic plates. Also, blood cells could affect the trophoblast quality by consuming the nutrition provided in the medium, particularly we changed the medium every 48-72 hours. The presence of the blood

cells is not related to the skill of isolation, but it is a part of the nature of ectopic placentation (**Figure 6.3 C**).

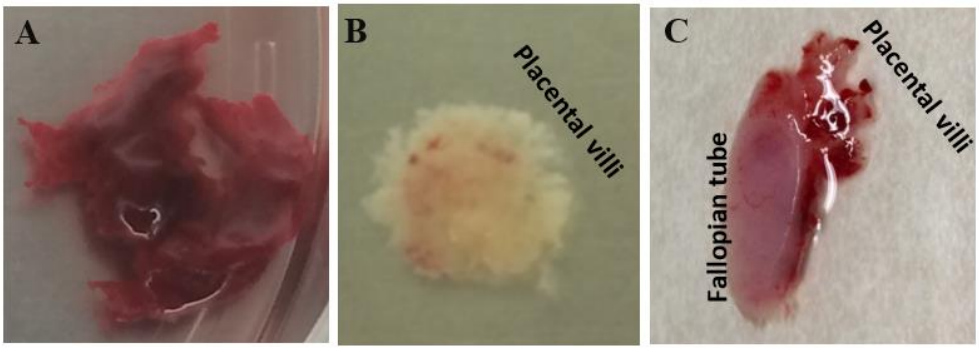


Figure 6. 3. Different ectopic placentas collected from tubal pregnancies in our study. Image (**A**) is a tubal placenta from a patient who was treated with MTX. Image (**B**) is the best ectopic placenta I collected that has clear villi; however, its size is small due to the early gestational age. Image (**C**) is a tubal placenta obtained by Dr. Peng that shows the bloody tubal pregnancy implantation site.

Results of 3 independent experiments showed that GnRH analogs have a distinct effect on the invasion of primary EP and immortalized cells; we suggest this could be due to the following:

The *in vivo* invasion of EVTs in EP was reported to be different from intrauterine pregnancy (96), which could be the direct cause of the different response or may require different experimental conditions. In this experiment, we used the same invasion assay used for intrauterine EVTs. It is possible that the culture conditions for EP-EVTs are different and a modified culture approach may be necessary.

Another reason could be the cell response to treatment differs among different cells when the same function is studied. For instance, GnRH-I (10 nM) significantly increased the cell viability in BeWo cells that were suppressed after antide (10 nM) pre-treatment (63). Meanwhile, using the same experimental conditions in HTR-8/SVneo cells, results did not show

different response between GnRH analogs (63). In another study, the effect of glycodelin A, a glycoprotein secreted from the endometrial glands and decidual glandular epithelium (221), was investigated on the hCG secretion in different cell lines. Results showed that hCG production increased in JEG3 cell line in a dose- and time-dependent manner (222). However, using the same conditions in BeWo cells, there was no effect (222). It was suggested that hCG production is not regulated by glycodelin in BeWo cells (222).

Furthermore, changes in the experimental conditions might show a different response to treatment in the same cell population. For example, BeWo cell proliferation showed a good response to synthetic glucocorticoid triamcinolone acetonide (TA), but hCG production did not alter under the same conditions (205). However, hCG secretion increased when BeWo cells treated in the presence of serum (205). It was suggested that the absence of serum suppresses hCG production (205). Consequently, for future experiments using primary EP trophoblasts, we propose changing the cell culture protocol and treating cells in an enriched medium could show a different response.

Another possibility could be the type of GnRH-R antagonist used in the experiments might be responsible for the inadequate cell response. Ganirelix could have a non-sufficient effect on EP trophoblast cells. Therefore, more validation with other commercially available antagonists is required. This suggestion is based on knowing that several clinical studies have shown that each antagonist has an optimal effect on different cells from different organs. When the FDA approves a drug, it stipulates the exact clinical use for which the drug is approved and the related clinical conditions. For example, abarelix was approved for advanced prostate cancer treatment, while ganirelix and cetrorelix were approved for the alleviation of moderate-to-

severe endometriosis pain and for the use in infertility treatment protocols. Consequently, we suggest that other clinical GnRH-R antagonists should be examined.

Finally, the failure to detect a different response between experimental groups does not indicate there is no treatment effect. The treatment may not affect the cell function but affect the quality of functioning cells. For instance, Brüssow et al. showed that antarelix, an antagonist, did not affect the LH pulse frequency when compared with controls in a pig study. However, the overall LH secretion was diminished (223). In addition, researchers showed that the follicular growth and the number of follicles in pigs treated with antarelix were similar to those in the control group. However, those animals failed to ovulate (223); indicating that despite the absence to detect an effect on cells after antagonist treatment, the function was not preserved.

For these reasons, more experiments with further amendments in the experimental conditions and using different clinical antagonists are needed to clarify the effect of GnRH-R antagonists on EP trophoblast cell invasion.

Chapter 7: Effect of Cetrorelix, a Clinical GnRH-R Antagonist, on Placenta Explant Outgrowth

7.1 Introduction

Among different *in vitro* models used to understand and study placental morphology and functions, placental explants showed their close similarity to the *in vivo* environment (93). As mentioned in chapter 2, preservation of the tissue structure and cell-cell interactions are the main advantages of the explant model (93), which provide a near approximation of the *in vivo* tissue response to treatments. Early placental explants were used to detect the effect of several factors or treatments on cytotrophoblast proliferation, EVT cells differentiation, and invasion (115). In this study, we investigated the effect of cetrorelix (100nM), a clinical antagonist, on the growth of placental explants (**Figure 7.1**). We chose cetrorelix in this experiment because it is the most common commercially and clinically available GnRH-R antagonist in gynecology.

7.2 Results

7.2.1 Effect of cetrorelix on placental explant outgrowth

Cetrorelix decreased the explant outgrowth area ($P = 0.02$) (**Figure 7.2**) and the length of the outgrowth ($P = 0.03$) (**Figure 7.3**) after 48h when compared with the control. This effect was quantified on 3 replicates of explants treated with GnRH-R antagonist or control. In each

replicate, there were 3-4 villous columns. A total of 9 or 10 columns in the control and cetrorelix groups were measured and analyzed.

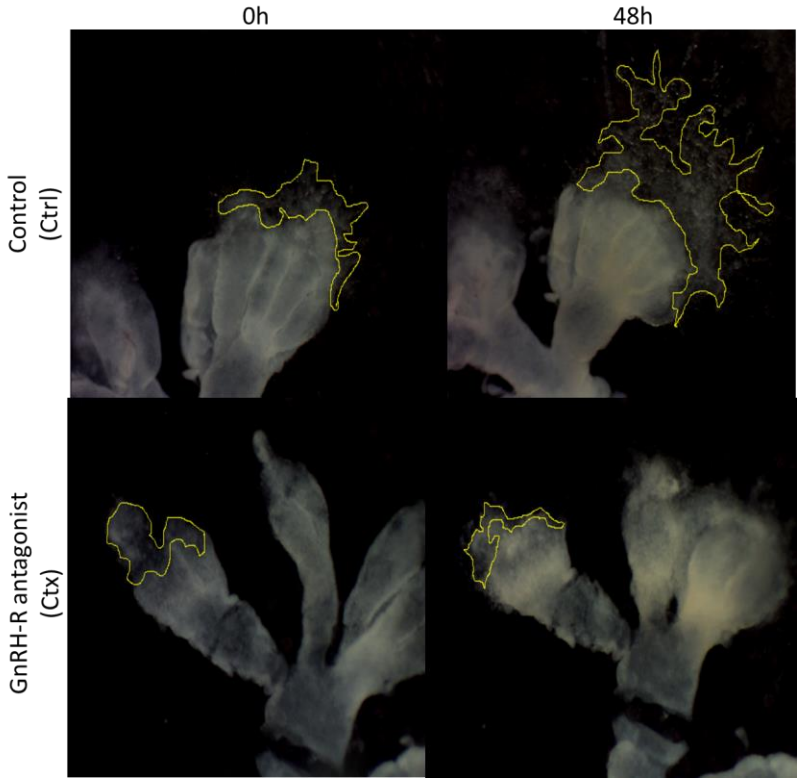


Figure 7. 1: Effect of cetrorelix on placenta explant outgrowth

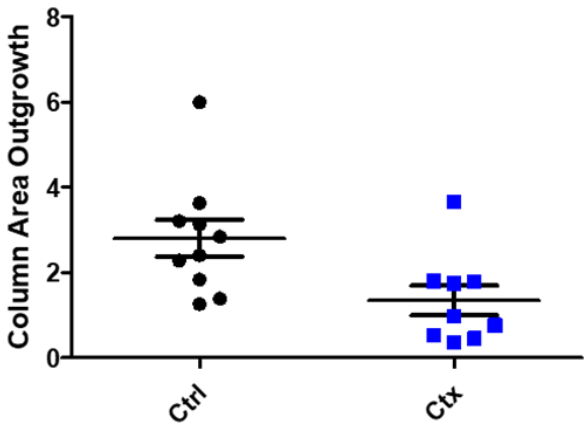


Figure 7. 2: Effect of cetrorelix on placental explant outgrowth area

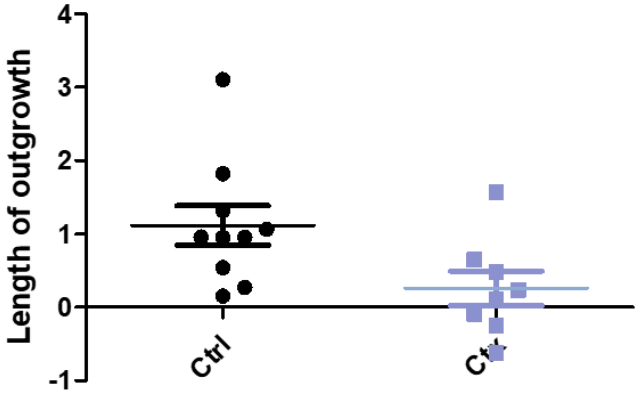


Figure 7. 3: Effect of cetrorelix on placental explant outgrowth length

7.3 Discussion

The results in chapter 7 are based on the quantification of two different measurements for the tissue outgrowth: the area of growth (**Figure 7.2**) and the length of outgrowth (**Figure 7.3**). The inhibitory effect of cetrorelix on of the explant growth provides a promising indicator for the same possibility *in vivo*.

Placental explant outgrowth arises in two ways (115): by the attachment and cell migration from cytotrophoblast villous columns tips at 0 time, or from cytotrophoblast cell proliferation at the attachment site, particularly with the removal of the overlying syncytium (115). Based on the experimental period and conditions, pulse-chase studies with BrdU (224) showed the inadequate cytotrophoblast division after the first 2 days of explant culture (115); so tissue outgrowth came mostly from cell columns formed in the first hours of culture and what is observed is a reorganization process for these cells to aggregate and be directed to migrate (115).

In this placental model, we examined the cytotrophoblast cell outgrowth on extracellular matrix (ECM), Matrigel, which is not frequently contaminated with fibroblasts until much later, if at all (225). Also, ECM substrates suppress cell death even in the presence of TNF-α (226). Therefore, culturing placental explants on ECM substrates is preferred than culturing them on plastic plates, which is associated with a high rate of apoptosis (115) and frequent contamination with fibroblasts (227). Additionally, Matrigel in this model can address the first steps of tissue invasion *in vitro* but without a full evaluation of the subsequent pathways (115).

For these reasons, we showed in this chapter the effect of cetrorelix on cytotrophoblast cell proliferation. Also, we determined the preliminary effect of the antagonist on the invasion capability of differentiated cells. Results supported our previous preliminary results

(unpublished data) showing the significant inhibitory effect of cetrorelix on EP trophoblast cell proliferation (**Figure 7.4**).

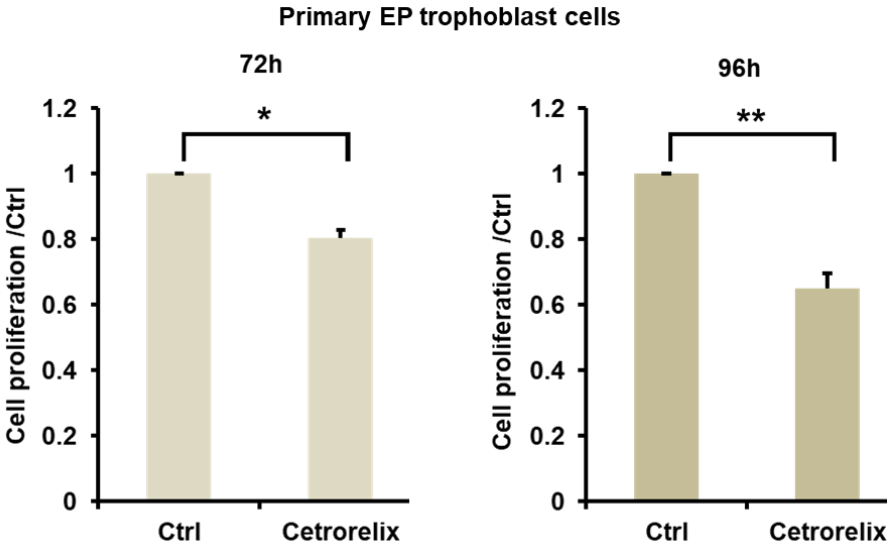


Figure 7. 4: Clinical GnRH antagonist effect on primary EP trophoblast cell proliferation. Cetrorelix significantly abolished the primary EP trophoblastic cell proliferation, as compared with the control. Asterisks indicate the significant differences (**P*-value < 0.05, ** *P*-value < 0.01).

Further experiments are needed to confirm the effect of cetrorelix and ganirelix on placental explants established from intrauterine and EP placentas. Also, investigating the effect of other available GnRH-R antagonists is required.

Finally, the same model can determine the effect of GnRH-R antagonists on other functions such as placental transport, and the hormone and protein production. Also, this model can be used to study the effect of GnRH-R antagonists on ECM, cytokines, and growth factors and their role on cytotrophoblast proliferation, the formation of new cell columns, and differentiation into EVTs.

Chapter 8: Conclusion

During the course of this project, our research investigated the effect of clinical GnRH-R antagonists on trophoblast cell invasion. There are several antagonists available in the market, but we studied the effect of the most common drugs used in the gynecology clinics (Cetrorelix and ganirelix). Both drugs are successfully used to treat endometriosis, uterine fibroids, and in *in vitro* reproduction protocols. However, there are no reports for their usage in pregnancy-related disorders. The only guideline for their effect on pregnancy is described in clinical studies that investigated the advantages of GnRH-R agonists versus antagonists in *in vitro* fertilization protocols. These studies showed that women who used high doses of GnRH-R antagonists without adequate luteal phase support had lower pregnancy outcomes.

Building on the fact that GnRH upregulates the placental cell invasion via various signaling pathways, which are suppressed by antide pre-treatment. Antide is an experimental GnRH-R antagonist that is extensively used in the agonist-antagonist *in vitro* studies. We examined the effect of ganirelix on HTR-8/Svneo immortalized cell line and on primary trophoblast cells, isolated from a tubal pregnancy. In addition, we determined the cetrorelix effect on early pregnancy placental explants. We hypothesized that clinical antagonists would suppress the placental cell invasion and placental explants outgrowth.

One clinical antagonist was used in each experiment to evaluate the effect on one trophoblast model. In chapter 6, ganirelix was used to evaluate its effect on trophoblast cell invasion. Results showed a significant suppressive effect on GnRH induced HTR-8/SVneo cell invasion (P value < 0.001). Ganirelix effect was compared with antide, which has a known suppressive effect on HTR-8/SVneo cell invasion. However, these results were not replicated

in primary EP trophoblast cells. We used primary cells isolated from 2 tubal placentas in different passages and results showed response variability between cells.

In chapter 7, cetrorelix was used to determine the effect of GnRH-R antagonist on first-trimester intrauterine placental explants. Cetrorelix suppressed outgrowth of the explants compared with the control after 48h. Two outcomes were measured: the outgrowth area (*P*-value = 0.02) and the length of growth (*P*-value = 0.03). Only one experiment was performed for this model. However, the experiment included 3 replicates in each group. Each replicate had 3 to 4 columns to evaluate their outgrowth. There were in total 9-to-10 columns in each group that was quantified and analyzed.

This study has several strengths including the following: 1- To our knowledge, it is the first reported study investigating the effect of clinical GnRH-R antagonists on 3 placental models. 2- We cultured primary trophoblasts isolated from tubal implantation sites and used them in functional studies as a novel step in the *in vitro* EP studies. 3- We compared the effect of a known suppressive treatment, antide, on HTR-8/SVneo cell invasion with the proposed drug in the same experimental conditions.

However, this study has faced an obstacle due to collecting few numbers of EP tissue specimens during the period of the research, which did not enable us to do further experiments on primary cells or establishing EP placental explant model.

We propose, with better availability of EP tissues, evaluating the effect of different antagonists on various functions including the effect on hCG production, cell proliferation, and the induction of apoptosis. Also, we envision examining different models established from EP placentas such as placental explants or by using primary EP cells in a co-culture system with

Fallopian tube epithelial cells or endometrial cells to show the role of the maternal and fetal cells in the tubal pregnancy occurrence.

When good EP models are established, we suggest determining the subsequent signaling pathways of GnRH and its antagonists involved in tubal pregnancy that control EP trophoblasts invasion.

Finally, we suggest investigating the effect of GnRH-R antagonists combined with MTX on various trophoblast functions. This suggestion could help to reduce MTX side effects or eliminate the failure to respond to MTX.

In conclusion, we have shown that clinical GnRH-R antagonists, cetrorelix and ganirelix, generally suppress placental cell invasion and explant growth. After further validation, the clinical antagonists could be investigated for possible use to treat EP or abnormal placentation.

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Appendices

Appendix A

a place of mind
THE UNIVERSITY OF BRITISH COLUMBIA

BC WOMEN'S HOSPITAL+ HEALTH CENTRE
An agency of the Provincial Health Services Authority

Vancouver CoastalHealth
Promoting wellness. Ensuring care.

Department of Obstetrics and Gynaecology
Faculty of Medicine
The University of British Columbia
D415A - 4500 Oak Street
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Informed Consent

Study Title:	Gonadotropin-releasing Hormone Antagonist for Ectopic Pregnancy Treatment
Study Number:	H15-02234
Funded by:	Nelly Auersperg Award in Women's Health Research, Women's Health Research Institute (WHRI)
Version:	November 05, 2015

Principal Investigator:	Dr. Mohamed A. Bedaiwy
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(For urgent matters, the Principal Investigator can be reached through BC Women's Locating at 604-875-2161)

INVITATION

Our researchers are conducting a study to look into the causes of and new medical treatment for tubal pregnancy and as you currently are experiencing tubal pregnancy, we are inviting you to participate in our study.

This is a research study. Before agreeing that you will participate in this study, you should read this informed consent form to understand the purpose of the study, potential risks, and procedures.

This informed consent form provides information about the research study and describes the purpose, procedures, benefits, risks, and precautions of the study related to you.

Please ask the study staff to explain any information that you do not understand. When all of your questions have been answered and you feel that you understand this study, you will be invited to participate in the study and to sign this informed consent form if you agree for you to participate in the study. You will be given a signed and dated copy of this informed consent form to keep.

YOUR PARTICIPATION IS VOLUNTARY

Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. Before you decide, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what will happen to you during the study and the possible benefits, risks and discomforts.

If you wish to participate, you will be asked to sign this form. If you decide to take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision.

If you do not wish to participate, you do not have to provide any reason for your decision not to participate nor will you lose the benefit of any medical care to which you are entitled or are presently receiving.

Please take time to read the following information carefully and discuss it with your support person and the medical team before you decide.

WHO IS CONDUCTING THIS STUDY?

The study is being conducted by Dr Mohamed Bedaiwy and his research team. Funding for this study came from Nelly Auersperg Award in Women's Health Research administered by the Women's Health Research Institute (WHRI).

BACKGROUND

Normal placenta growth is important during pregnancy as it can provide oxygen and nutrition to the growing baby. Placenta related diseases can be harmful to the health of mother and baby, and can result in unhealthy pregnancy. In particular, placenta growing outside the womb, for example, in the tubal area, can result in rupturing, bleeding and other very serious conditions.

What causes tubal pregnancy is still not clear. Understanding the conditions of placenta growth related to tubal pregnancy would help us discover the early signs of tubal pregnancy and how it forms. It would enable new tools to be made and used to detect tubal pregnancy, and to design effective treatment for tubal pregnancy. Therefore, with the assistance of the samples from women with tubal pregnancy, we may be able to further understand the cause of tubal pregnancy and develop potential treatment of tubal pregnancy.

WHAT IS THE PURPOSE OF THIS STUDY

This study will look at the behavior of one specific hormone, called gonadotropin-releasing hormone (GnRH), and its related molecule, called gonadotropin-releasing hormone receptor (GnRHR) in tubal pregnancy. We will examine the function of GnRH and GnRHR in increasing the placenta cell growth and attachment in tubal pregnancy. Additionally, we will test to see if changing the levels of GnRH and GnRHR via a pharmaceutical drug can lower the growth and attachment of placenta cells from tubal pregnancy in a cell culture system.

WHO CAN PARTICIPATE

All women with diagnosis of tubal ectopic pregnancy who will undergo surgical treatment are eligible to participate.

WHO SHOULD NOT PARTICIPATE

- Women who have opted for medical treatment for tubal ectopic pregnancy
- Women with non-tubal ectopic pregnancy
- Women who do not read and understand English or have difficulty to understand the written consent form

WHAT DOES THE STUDY INVOLVE?

All research will be conducted at the Women Health Research Institute and Child & Family Research Institute which is adjacent to the BC Women's Hospital and Health Care Centre. All participants will be recruited from BC women's hospital and Vancouver General hospital (Vancouver Coastal Health).

A piece of the uterine tube containing placenta from participants with tubal pregnancy will be collected. The tissues will be transported to the research laboratory. Placental cells from these tissues will be separated and maintained in the laboratory. The levels of GnRH and GnRHR will be measured. Drug altering the levels of GnRH and GnRHR will be added to the cells. The ability of the cells to grow and attach will be evaluated. After obtaining the results, the cells will be safely destroyed.

STUDY PROCEDURES

Screening:

After you have talked about the study with the research staff and have signed the informed consent form, you will be screened for possible inclusion in the study. Screening will consist of the following:

- Medical and obstetric history: the study physician or their staff will ask you questions about your medical history including details about your current pregnancy and any prior pregnancies and their outcomes
- Medical records review: your study physician or their staff will review your medical records to see if you meet the criteria to be included in the study.
- Current medications: you will be asked about any medications you are currently taking or have recently taken
- Physical exam: the study physician will perform a physical exam.
- Your weight and height will be measured.
- Vital signs: your blood pressure, heart rate, respiratory rate (the rate of your breaths) and temperature will be measured.

- Obtain laboratory tests: Blood tests were already done at your hospital within the day before you sign this consent form. No other blood and urine samples will be sent to another laboratory especially for the study.

The study screening will be analyzed and the investigator will decide if you are eligible to participate in the study.

The tissue will be collected as indicated in above section.

WHAT ARE MY RESPONSIBILITIES

You are responsible for the following:

- Reading this informed consent form and asking any questions before you agree for you to participate in the study and you sign this informed consent form.
- Contact the study doctor if you desire to end your participation

WHAT ARE THE HARMS AND DISCOMFORTS

There will be no increased risk or personal inconvenience to you. These studies will not modify or interfere with your surgical procedure in any way. Participation in the study will not require any extra time over that of normal care.

WHAT ARE THE POTENTIAL BENEFITS OF PARTICIPATING?

There are no direct benefits to you for participating in these studies. The results of the study will not be made known to you.

It is however hoped that these studies will improve the diagnosis and treatment of women with ectopic pregnancy, infertility or recurrent pregnancy loss who are trying to establish a family and/or women suffering from disorders of pregnancy.

WHAT ARE THE ALTERNATIVES TO THE STUDY TREATMENT?

This study does not involve giving you any treatments or modify any of your current treatment plans.

WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT TO PARTICIPATE?

You may withdraw from this study at any time without giving reasons. If you choose to enter the study and then decide to withdraw at a later time, all information about you collected up to the point of your withdrawal, including information obtained from your biological samples, will be retained for analysis in order to protect the integrity of the research, which may benefit future research participants and patients. However, no further information will be collected.

If samples have been collected before you withdraw, they will be destroyed or returned to the facility from which they were obtained. There may be exceptions where the samples will not be able to be withdrawn for example where the sample is no longer identifiable (meaning it cannot be linked in any way back to your identity). If your participation in this study includes enrolling in any optional studies or long term follow-up, you will be asked whether you wish to withdraw from these as well.

CAN I BE ASKED TO LEAVE THE STUDY?

Not applicable

HOW WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

Your confidentiality will be respected. However, research records and health or other source records identifying you may be inspected in the presence of the Investigator or his or her, by representatives of the Women Health Research Institute, and the UBC Research Ethics Board for the purpose of monitoring the research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

You will be assigned a unique study number as a participant in this study. This number will not include any personal information that could identify you (e.g., it will not include your Personal Health Number, SIN, or your initials, etc.). Only this number will be used on any research-related information collected about you during the course of this study, so that your identity will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique study number that is used on your research-related information will not be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to ensure that your privacy is respected. You also have the legal right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study doctor.

WHAT HAPPENS IF SOMETHING GOES WRONG?

By signing this form, you do not give up any of your legal rights and you do not release the study doctor, participating institutions, or anyone else from their legal and professional duties.

If you have any questions or desire further information about this study before or during participation, you can contact Dr. Mohamed Bedaiwy at 604-202-7635.

WHAT WILL THE STUDY COST ME?

Routine medical care for your condition (care you would have received whether or not you were in this study) and is provided to you by your provincial health insurance as a resident of Canada.

REMUNERATION

There are no direct benefits to you for participating in these studies. The results of the study will not be made known to you.

You will not be delayed or inconvenienced as a direct result of this study and will therefore not be remunerated (paid) for your participation.

WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY DURING MY PARTICIPATION?

If you have any questions or desire further information about this study before or during participation, you can contact principle investigator Dr. Mohamed Beidawy at 604-202-7635.

WHO DO I CONTACT IF I HAVE ANY QUESTIONS OR CONCERNS ABOUT MY RIGHTS AS A PARTICIPANT?

If you have any concerns or complaints about your rights as a research participant and/or your experiences while participating in this study, contact the Research Participant Complaint Line in the University of British Columbia Office of Research Ethics by e-mail at RSIL@ors.ubc.ca or by phone at 604-822-8598 (Toll Free: 1-877-822-8598).

AFTER THE STUDY IS FINISHED

The data collected from your sample will be kept in a secured server for five years after publication of this work. The samples and cells collected in this study will be safely destroyed by autoclaving to eliminate the bioacitivity of these samples.

INFORMED CONSENT SIGNATURE PAGE

My signature on this consent form means:

- I have read and understood the information in this consent form.
- I have had enough time to think about the information provided.
- I have been able to ask for advice if needed.
- I have been able to ask questions and have had satisfactory responses to my questions.
- I understand that all of the information collected will be kept confidential and that the results will only be used for scientific purposes.
- I understand that my participation in this study is voluntary.
- I understand that I am completely free at any time to refuse to participate or to withdraw myself from this study at any time, and that this will not change the quality of care that I receive.
- I authorize access to my health records and samples as described in this consent form.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I understand that there is no guarantee that this study will provide any benefits to me.
- I agree to take part in this study and to follow the instructions provided to me. I will contact the study doctor immediately if I experience any unexpected or unusual symptoms.
- I have told the study doctor about my previous and present illnesses and medications, and any recent visits with my doctor.

I will receive a signed copy of this consent form for my own records.

Participants' Signature:_____ Date and Time:_____

Printed Name:_____

Investigator or person who conducted the Informed Consent discussion

I confirm that I have personally explained the nature and extent of the planned research, study procedures, potential risks and benefits, alternative therapeutic options, and confidentiality of personal information to the participant named above.

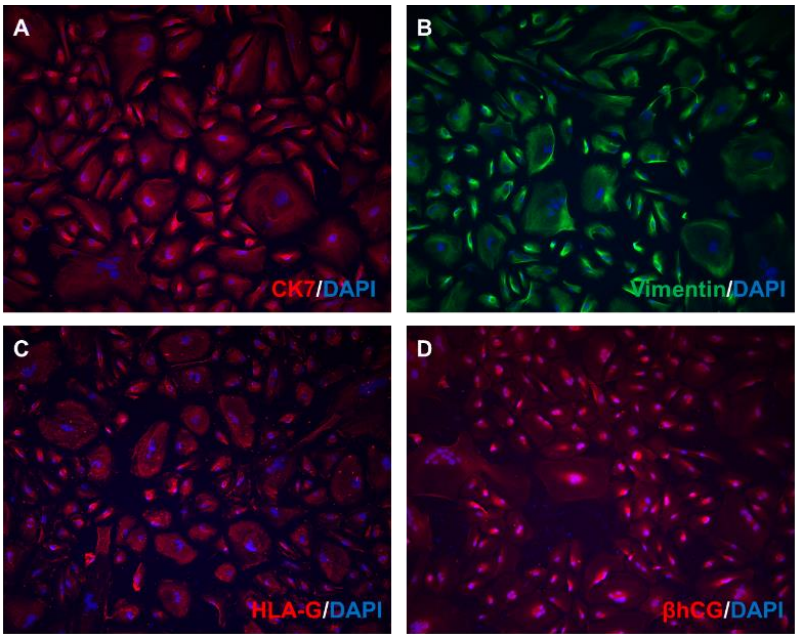
Signature:_____ Date:_____

Printed Name:_____

Appendix B

B1. Validation

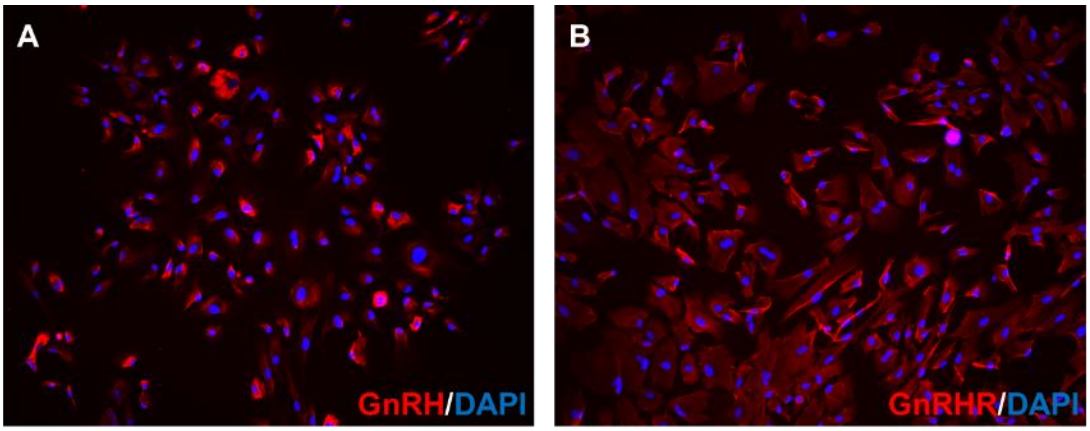
The primary EP trophoblastic cells validated by trophoblast labeling markers showed positive immunoreactivity to cytokeratin-7, HLA-G, hCG, and vimentin. The validation revealed culturing different trophoblast cell populations including cytotrophoblasts that were positive to cytokeratin-7, syncytiotrophoblasts that were positive to hCG, and EVTs that were positive to HLA-G immune marker. Positive immunoreactivity to vimentin was due to the epithelial-mesenchymal transition phenotype (EMT) that characterize the trophoblastic cells.



Immunolocalization of primary trophoblasts isolated from the placenta of tubal pregnancy. Representative images show the immunocytochemistry of (A, red) cytokeratin-7, (B, green) Vimentin, (C, red) HLA-G, and (D, red) hCG in primary EP trophoblastic cells. Cytokeratin-7 and Vimentin are epithelial and mesenchymal cell markers, respectively. HLA-G is a cell marker for extravillous trophoblast cells, and hCG is abundantly expressed in syncytiotrophoblasts. DAPI used to label the cell nucleus.

B2. GnRH system expression in Primary EP Trophoblasts

The expression of GnRH and GnRH-R was examined in the isolated primary cells. Positive immunoreactivity to GnRH and GnRH-R antibodies observed in the primary EP trophoblast cells.



Immunofluorescence staining shows (A, red) GnRH and (B, red) GnRH-R expression in primary EP trophoblasts isolated from tubal pregnancies.

Appendix C

Conference Presentations

C1. Establishment of primary trophoblast cell culture from ectopic pregnancy placenta

This abstract was presented in the 63rd Annual Meeting of the Canadian Fertility and Andrology Society (CFAS) on September 16, 2017, in Vancouver, BC.

It was awarded CFAS Travel Grant.

C2. The role of GnRH antagonists in a novel ectopic pregnancy cell model

This abstract was presented in the 73rd annual meeting of the American Society for Reproductive Medicine (ASRM) in October 2017 in San Antonio, Texas.

The poster was awarded the ASRM In-Training Awards for Research.

Appendix D

Publications

1. Lisonkova S, Wen Q, **Abdellatif L**, Alfaraj S, Yong P, Bedaiwy M. Temporal trends in severe morbidity associated with ectopic pregnancy requiring hospitalization. Fertility and Sterility. 2017;108(3):e105.

2. Peng B, **Abdellatif L**, Klausen C, Leung P, Bedaiwy M. The role of GnRH antagonists in a novel primary ectopic pregnancy cell model. Fertility and Sterility. 2017;108(3):e103-e4.