Equine Trophectoderm Cells and Their Role in Fetal-Maternal Recognition.

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ACADEMIC ABSTRACT

Establishment and maintenance of a successful pregnancy requires signaling from the embryo to the mare, a process known as maternal recognition. Six days after fertilization, the trophectoderm (TE), a placenta precursor is formed. Signals emanating from the TE to the uterine environment are critical to maternal recognition of pregnancy. The identity of factors necessary for this process remain unknown. A novel equine induced trophoblast cell line (iTr) that closely mimics the genotype and phenotype of native equine TE was created. Transcriptome analysis of iTr revealed increased expression of growth factor (GF) receptors for Epidermal GF (EGF), Hepatocyte GF (HGF), Fibroblast GF-2 (FGF-2) and Insulin GF (IGF-1), suggesting these GF may be important targets during TE development in the early embryo. We hypothesized that treatment of iTr cells with these GF would induce changes in cell proliferation and expression of genes likely involved in maternal recognition. The objectives of this experiment were to evaluate the effect of these GFs on iTr mitotic response and regulation of genes involved in steroidogenesis.

Equine iTr cells (n = 3) were cultured with 10 ng/mL EGF, HGF, FGF-2 or IGF-1 for 24 hr, with 5-ethynyl-2'-deoxyuridine (EdU) supplementation during the final 2 hr. Subsequently, cells were fixed and EdU positive and total nuclei were enumerated. A parallel plate of iTr cells was treated in a similar manner and lysed for total RNA isolation. Quantitative PCR using gene-specific primers for CYP11A1, PTGS2, PTGES2, and PTGES3 was performed. Data were analyzed by ANOVA with Tukey's post hoc adjustment using the GLM procedure of SAS. Treatment with EGF, FGF-2, HGF, and IGF-1 increased ($P < 0.05$) iTr proliferation from control levels of 25.33

± 1.03% to 38.58 ± 1.61%, 45.50 ± 2.94%, and 38.23 ± 2.01% respectively. The 2-ΔΔCT

method was used to calculate the fold change (FC) using GAPDH as the reference gene for

normalization. Expression of CYP11A2, PTGES2, and PTGES3 was not affected by GF, as

measured by qPCR. By contrast, PTGS2 transcript abundance increased ($P < 0.05$) following

FGF-2 (FC = 3.327 ± 0.8291) and HGF (FC = 11.88 ± 4.572) treatment. These results indicate

that FGF-2 and HGF may simultaneously induce proliferation and prostaglandin production by

TE cells. The combined results of these experiments will improve our understanding of TE

morphogenesis and its response to uterine-derived growth factors.

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GENERAL AUDIENCE ABSTRACT

Establishment and maintenance of a pregnancy requires that the mare uterus recognize the presence of the embryo, a process known as maternal recognition of pregnancy. The trophectoderm (TE) are cells on the outer layer of the embryo formed six days after fertilization, which later give origin to the placenta. The TE sends signals from the embryo to the uterus, that are very important for the mare's recognition of the embryo's presence. The specific nature of these signals are still unknown in the horse. A cell line (iTr) very similar in aspect and genes to the horse's native TE has been created in our laboratory. A set of comparative assays have showed that, during the developmental stage of maternal recognition, both the horse TE and the iTr cells share significant identity, and have receptors for the same set of growth factors (GF), suggesting these GF are important for early embryo development and potentially involved in the signaling process of maternal recognition.

We proposed that treatment with these GF would induce iTr cells to proliferate and express signals likely involved in maternal recognition in horses. The objectives of this experiments were to evaluate the effect of EGF, HGF, FGF-2 and IGF-1 on iTr cells by measuring proliferation and cellular mechanisms of maternal recognition already established in in other species.

Equine iTr cells were cultured with different GF and right before analysis a fluorescent dye that stain dividing cells was added in order to measure the proliferation. Equivalent cell cultures were used to evaluate if the treatment affected the production of hormones involved in signaling maternal recognition. Treatment with all GF induced higher cell proliferation, but HGF also increased the production of one enzyme that participates in producing a very important

hormone (prostaglandin E_2). The combined results of these experiments add to our understanding of maternal recognition in horses.

KEY WORDS: equine, maternal recognition of pregnancy, trophectoderm.

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AP, Alkaline phosphatase

CDX2, caudal type homeo box transcription factor 2

CL, corpus luteum

c-MET, hepatocyte growth factor receptor

CYP11A1, cholesterol side-chain cleavage enzyme

eCG, equine chorionic gonadotropin

EGF, epidermal growth factor

EOMES, eomesodermin

ESC, embryonic stem cells

ESR, estrogen receptor

FBS, fetal bovine serum

FGF, fibroblast growth factor

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GATA, GATA-binding protein

GALNT1, polypeptide N-acetylgalactosaminyltransferase

HAND1, heart and neural crest derivatives-expressed protein 1

HSD3, 3Beta Hydroxysteroid dehydrogenase

ICM, inner cell mass

IGF, insulin-like growth factor

iPSC, Induced pluripotent stem

ISG15, interferon-stimulated gene 15

iTr, induced trophoblast

LIF, leukemia inhibitory factor

MAPK, mitogen activated protein kinase

MRP, maternal recognition of pregnancy

mTOR, mammalian target of rapamycin

MUC, mucin-coding

NEU, Neuraminidase

NANOG, Nanog homeobox

OCT4, octamer-binding transcription factor 4

PGE_2, prostaglandin E_2

PGF2α, prostaglandin F 2 alpha

PGFM, PGF2α metabolite

PI3K, phosphatidylinositol 3-kinase

PO, post ovulation

PR, progesterone receptors

PTGS2, prostaglandin-endoperoxide synthase 2

RTK, receptors tyrosine kinase

SOX2, sex determining region Y-box 2

SPLA2, secretory Type II Phospholipase A2

STAR, Steroidogenic acute regulatory protein

TE, trophectoderm

TEAD4, transcriptional enhancer activator domain family member 4

TFAP2A, transcription factor activating protein 2-alpha

TGM3, transglutaminase 3

VEGF, vascular endothelial growth factor

ZP, zona pellucida

CHAPTER I

INTRODUCTION

The Thoroughbred horse industry is driven by the production of foals with racing aspirations as two-years old. Every foal on the ground brings with it substantial financial investments as well as a concerted effort to rebreed the mare for the subsequent year. The average pregnancy rates with natural breeding is around 50-60% per cycle (Scherzer, 2010). The cost of maintaining a mare every year, oscillates between US$15,000 and US$18,000 (personal communication) and these values are generally buffered with the incomes generated from the sale of a foal. Thus, a mare that does not get pregnant at the end of a breeding season reflects in a full year of costs without any financial income. As an example, the average selling price in 2016 of a Thoroughbred weanling in the US is U$64,848 and in 2018 for yearling is U$129,335. Since there is no guarantee of a pregnancy in the following season, barren mares create a significant burden to the equine industry.

To establish and successfully maintain a pregnancy, the mare's uterine environment undergoes a series of events in preparation to receive the embryo. The equine embryo migrates from the oviduct to the uterus around 6.5 days after fertilization, initiating a continuous process of interaction with the mare's uterus. The early embryo signals its presence in the uterus through diverse biochemical signaling processes that allow for the maintenance of a corpus luteum and interruption of the oestrous cycle (Klein, 2015). As a consequence, the secretion of prostaglandin F2-α (PGF2α) is delayed preventing the process of luteolysis, and thus prolonging the supply of progesterone required to maintain pregnancy (Klohonatz et al., 2015). This process is known as "maternal recognition of pregnancy" (MRP) (Short, 1969). The cells of the outer layer of the equine blastocyst drive the primary signaling events from the embryo to the mare's

endometrium, resulting in embryo fixation, implantation and the development of the placenta. Despite a firm understanding of the global processes of pregnancy establishment and maintenance in the mare, there are important gaps in knowledge concerning the signaling molecules and mechanisms involved with MRP in horses.

Equine induced trophoblasts (iTr) cells display similar *in vitro* behavior as embryo-derived trophoblasts, in addition to a highly overlapping transcriptome (Reinholt et al., 2017). Thus, iTr cells represent an invaluable tool to study trophectoderm mediated events involved with MRP. A better understanding of the factors underlying MRP can lead to advances in equine subfertility, and provide a basis for therapeutic approaches to pregnancy failure in horses.

The thesis tested the hypothesis that equine iTr cells proliferate in response to growth factors commonly found in the uterine milieu and these growth factors alter steroidogenic gene transcription.

LITERATURE REVIEW

Reproductive physiology in the mare

The mare is a seasonal breeder with estrus occurring during the long days of spring and summer. The mare is mainly monovulatory with an estrous cycle that lasts on average 21 to 22 days with a period of 5-7 days of estrus (Fig 1-1) (Aurich, 2011). Before each ovulation, high concentrations of estrogens and luteinizing hormone cause the mare to show behavioral signs of estrus. After the ovulation occurs, progesterone levels increase reaching a maximum concentration at day 8 post-ovulation (PO). If the mare is not pregnant at day 14 to 15 PO, the endometrium will produce PGF2α leading to luteolysis and a decrease in progesterone levels. Within a few days after luteolysis, the mare will start a new estrous cycle (Brinsko et al., 2010) . If the mare is pregnant, the ovulatory CL will be the primary source of progesterone with

secondary CLs providing additional progesterone. The placenta becomes the primary source of progesterone secretion at day 150-200 of gestation (Brinsko et al., 2010).

Hormonal profile in pregnant and non-pregnant mares

Pregnant and non-pregnant mares have a similar hormonal profile until d 14 PO (Klohonatz et al., 2015). In pregnant mares, progesterone and estrogen receptors (PR and ESR, respectively) are present in the uterine endometrium at day 10 PO and decline in number between days 15 – 20 PO. In non-pregnant mares, *PR* and *ESR* expression are greater during estrus and late diestrus, preparing for the next cycle (McDowell et al., 1999).

Oxytocin (OXT) in the mare is produced by the endometrium in contrast to other species, in which the endometrium secretes prostaglandin, but not OXT. The mare also has OXT receptors in the endometrium thus, creating an autocrine circuit (Aurich, 2011). Similar to other species, the mare uterine endometrium releases PGF2α in response to OXT, producing a positive feedback loop that is important to luteolysis (Burns et al., 1997; Sharp et al., 1997). OXT and PGF2α are released in pulses, but PGF2α's pulses are more discrete (Douglas and Ginther, 1976). During estrus and late diestrus, *OXT* expression is greater than during other phases of the cycle (Behrendt-Adam et al., 1999). In agreement, mares injected with OXT during late diestrus were reported to exhibit a plasmatic increase PGF2α metabolite (PGFM), the main metabolite of PGF2α (Goff et al., 1987)**.** Similarly, in vitro observations of endometrial explants treated with OXT, resulted in a rise in PGF2α secretion (Ealy et al., 2010). On the other hand, OXT administration at or before day 8 PO does not induce luteolysis, suggesting that the OXT-dependent luteolytic mechanisms are not established until late diestrus (Neely et al., 1979), being the endometrium only responsive to OXT secretion at or after day 11 PO (Goff et al., 1987).

The effects of OXT and PGF2α are different between pregnant and non-pregnant mares. In early pregnancy, mares show a limited ability to secrete PGF2α in response to OXT. However, in non-pregnant mares, OXT is produced by the endometrium and pituitary gland, resulting in a greater production of OXT and PGF2α. Consequently, luteolysis of the CL will occur inducing the mare to cycle again (Sharp et al., 1997). Compared to pregnant mares, the non-pregnant mare's endometrium expresses greater amounts of prostaglandin-endoperoxide synthase 2 (PTGS2) and secretory Type II Phospholipase A2 (SPLA2 also known as PLA2G2A) mRNA, both enzymes involved in prostaglandin synthesis (Klohonatz et al., 2015).

A study evaluating uterine flush content, found that at day 14-16 PO pregnant mares contain less PGF2α than cycling mares (Berglund et al., 1982). Uterine flushes done after day 18 of pregnancy contain concentrations of PGF2α similar to cyclic mares undergoing luteolysis, suggesting that the equine embryo doesn't prevent PGF2α secretion during pregnancy, but delays it (Stout and Allen, 2002). *In vitro* exposure of endometrial explants to embryo secretions decreased PTGS2 expression while those exposed to OXT had increased PTGS2 expression. This indicates that during early pregnancy the equine embryo could be targeting the enzyme as an antiluteolytic signal (Ealy et al., 2010).

At day 25 of pregnancy, part of the trophoblast goes through cellular changes and form the chorionic girdle. Around day 38 of pregnancy, these trophoblastic cells of the chorionic girdle invade the uterine epithelium forming the endometrial cups. The endometrial cups secrete equine chorionic gonadotropin (eCG). This hormone is first detectable in the maternal blood around day 35-42 and around day 100-150 is undetectable. The role of this hormone is to assist the supplementary CL formation and stimulates the maintenance of the primary CL. In addition, it plays a key role in immunoregulation during the pregnancy (Brinsko et al., 2010)

Maternal recognition of pregnancy

The window during which maternal-fetal recognition occurs is unclear. However, the embryo-derived signal should be present before 14 PO in order to prevent PGF2α secretion from the endometrium, resulting in the lysis of the corresponding CL (Boerboom et al., 2004). Dialysis of conditioned media from equine embryos at day 14 PO co-cultured with endometrial explants from mares at the same length of gestation, identified a protein of molecular weight within the 1 to 6 kDa range (Sharp et al., 1989). Another study, using equine embryos at day 13.5 PO, treated with either proteinase K or dextran-coated charcoal, identified a proposed MRP signal (s) of molecular weight within the 3-10 kDa range (Ababneh et al., 2000). These studies indicate that the trophectoderm of the embryo synthesizes and releases a protein molecule during the MRP timeframe that can suppress PGF2a secretion.

The phenomenon of MRP has been studied in several different species for the past 50 years. Several pathways involved in MRP have been discovered providing a detailed understanding of this process in cattle, sheep and pigs (Klein, 2015). The elongation of the trophoblast through the uterus in ruminants and pigs allows the materno-fetal interaction that is key for the implantation and placentation (Geisert et al., 1990). In the pig between day 10 and 14 PO, the spherical embryo elongates and the trophoblast cells start to produce and release estrogens that interfere with the normal secretion of endometrial PGF2α. The embryo-derived estrogens are able to redirect endometrial PGF2α secretion away from the uterine vein (endocrine secretion) to the uterine lumen (exocrine secretion). By extrapolation, several studies evaluated the effects of estradiol administration on luteolysis in the mare. Uterine infusions with estradiol were performed in non-pregnant mares from day 10 to day 16 PO. Nonetheless, the lifespan of the CL was not different from sham-treated mares suggesting that the hormone does

not delay luteolysis (Vanderwall et al., 1994). Systemic injections of different concentrations of exogenous estradiol over the course of an estrous cycle (or up to 32 days maximum) also failed to prevent CL regression (Woodley et al., 1979). Experiments introducing estradiol impregnated spheres to mare's uteruses were performed (Goff et al., 1993), though none of the treatments managed to delay luteolysis, with mares returning to cyclicity (Woodley et al., 1979). Daily supplementation of diethylstilbestrol from day 7 though day 18 PO (Berg and Ginther, 1978), prolonged the CL lifespan and suppressed a new wave of follicular growth. Diethylstilbestrol is a synthetic estrogen and thus this response does not mimic the physiological context. More recent studies, performed uterine infusions with different types of oils and estradiol at different days PO. The only mixture that prolonged the lifespan of the CL in mares was a combination of coconut oil and estradiol administered at day 6 PO. Based on these results, the authors concluded that this vegetable oil could be involved in prostaglandins synthesis in the mare's endometrium leading to delayed luteolysis (Wilsher and Allen, 2011). Since all these experiments were performed in non-pregnant mares, it can't be ruled out that the embryo-derived estrogen could be involved in MRP.

Interferon tau is the MRP factor in ruminants. The establishment of a pregnancy involves the suppression of the endometrial secretions of PGF2α in order to maintain the CL secretion of progesterone. The trophoblast produces interferon tau around day 10-16 PO, inhibiting the endometrial receptors for OXT thereby disrupting the uterine secretion of PGF2α and maintaining the pregnancy (Bazer et al., 1997).

Equine embryos express two interferon delta genes, EqIFN-delta 1 and EqIFN-delta, during early pregnancy at day 16 and day 22 (Cochet et al., 2009). Different types of interferons were measured in equine embryos at day 13, 15,20 and 25 of pregnancy. The results indicated

that equine embryos were unable to produce interferon-like protein molecules that can avoid luteolysis (Baker et al., 1991). In horse has been observed that interferon-stimulated gene 15 (ISG15) is present in the mare's endometrium during early pregnancy and at the time of embryonic implantation. However, the expression of ISG15 was not different between non-pregnant and pregnant mares at day 14 and 50 PO leading the authors to conclude that ISG15 is probably involved in cellular functions unrelated to MRP. In summary, there is no evidence that interferon molecules are modulators of MRP in the mare (Klein et al., 2011).

Prostaglandin E_2 (PGE_2) is part of the prostaglandin hormone family. It is associated with the process of luteolysis in pigs and ruminants. During pregnancy, gilts and ewes, have an increase in intrauterine PGE_2 concentrations. Uterine infusions with PGE_2 delays the process of luteolysis in both swine and sheep (Pratt et al., 1977; Christenson et al., 1994). In a similar manner, equine embryos produce PGE_2 as early as 5 days PO until at least day 15 PO, doubling the amount between day 13 and 15 of pregnancy (Weber et al., 1991). PGE_2 receptors are present in the mare's endometrium, making it a perfect target for the PGE_2 secreted by the embryo (Weber et al., 1992). Additionally, several cases with unexplained fertility problems have resolved with a PGE_2 injection into the serosa of the oviduct (Arnold and Love, 2013). Uterine infusion of PGE_2 in non-pregnant mares showed an increase in CL lifespan compared with control and sham treatment suggesting that PGE_2 secreted from the embryo is related with prolonging CL lifespan in pregnant mares (Vanderwall et al., 1994).

Early equine embryonic development

Embryonic development in mammals is characterized by the early differentiation of trophoblast lineage, which is in charge of providing means by which the embryo, and further the fetus, survive in the uterus (Pfeffer and Pearton, 2012). After the morula is formed it divides in

two very different and special structures reaching the stage of blastocyst. The trophectoderm (TE) that further gives origin to the placental tissues, and the inner cell mass (ICM) that contributes to form the fetus itself. The TE, is the first extra-embryonic tissue that forms the outer cell layer of the blastocyst (Iqbal et al., 2014). The TE originates all the trophoblast cells lineage that later will form part of the placenta and the periphery of the embryo (Gardner and Beddington, 1988; Rossant, 2007), and plays a key role in embryonic implantation (Pfeffer and Pearton, 2012). The ICM, also differentiates into two structures, the hypoblast and epiblast. Hypoblast or primitive endoderm, like the TE, gives rise to extra-embryonic tissues such as the visceral yolk sac and parietal endoderm. On the other hand, the epiblast or primitive ectoderm, is a structure that lies above the hypoblast, and generates the allantois, amnion, extra-embryonic mesoderm of the visceral yolk sac and the three primary layers (endoderm, mesoderm and ectoderm) (Gardner and Beddington, 1988).

The mare's oviduct is very selective with regards to cell transit through its lumen. Around 6.5 days PO, fertilized ova are able to pass the through oviduct into the uterus due in part to PGE_2 directed oviduct dilatation (Weber et al., 1991). When the embryo reaches the uterus, it is still surrounded by the zona pellucida (ZP) with a thick acellular glycoprotein membrane immediately beneath it and atop the trophectoderm (Betteridge et al., 1982; Battut et al., 1997; Betteridge, 2007). As the ZP starts to get thinner, the embryo is able to expand in size and the ZP is shed. The capsule is then the only surrounding protection for the embryo. The capsule is a unique structure amongst domestic animals, and provides mechanical resistance to uterine contractions that propels the embryo during the mobility phase (Oriol et al., 1993). Artificial removal of the capsule from embryos at day 6-7 PO demonstrated their inability to survive after transfer into recipient mares reinforcing its role in promoting early embryo protection and

survival (Stout et al., 2005). The capsule is composed by mucin-like glycoproteins produced by the trophoblast (Albihn et al., 2003). The embryo expresses sialyltransferases and sialic acid transporters, which builds the carbohydrate content of the capsule glycoproteins. Sialic acid content is greatest during day 8-14 PO and this seems to be related to an antiadhesive function (Klein and Troedsson, 2011a). The sialic acid content within the capsule decreases around day 16-17 PO, coinciding with embryonic fixation (Arar et al., 2007). Neuraminidase 2 (NEU2), is an enzyme expressed by the embryo, is responsible for removing the sialic acid from glycoproteins suggesting that the embryo regulates the content of sialic acid of its own capsule (Klein and Troedsson, 2012). The capsule is absent at day 21-23 PO (Betteridge, 2007). Mucin-coding genes (MUC1 and 2), genes involved in proteoglycan synthesis (GALNT1,4 and 12) are also transcribed by the TE and important for capsule formation (Iqbal et al., 2014). Although it has been demonstrated that the trophoblast cells produce capsule material, researchers are unable to reproduce this phenomenon suggesting that the equine embryo needs the endometrial microenvironment to accomplish the task. Permeation of capsule material through membrane channels, probably due to poor binding of the glycoprotein to the trophectodermal surface, also contributes to the failed capsule formation in vitro (Tremoleda et al., 2003). The embryo doesn't need the ZP in order to form the capsule because bisected blastocysts form a capsule after transfer into recipient mares (McKinnon et al., 1989).

Unlike ruminants or pigs that elongated their trophoblast in order to interact with the endometrium, equine embryo mobility throughout the uterus is a remarkable feature among domestic animals, and was documented by ultrasound beginning at day 9 PO with peak movement during 11-15 PO (Ginther, 1983). At day 16 PO, the embryo's size limits its movements through the uterus, as well as an increase in uterine tone (Gastal et al., 1996). The

embryo is propelled through the uterus by myometrial contractions stimulated by the embryo

secretion of prostaglandin E2 and F2a (Watson and Sertich, 1989; Weber et al., 1991). Embryo

mobility is reduced in pregnant mares treated with flunixin meglumine (a prostaglandin

inhibitor) from day 10 to 18 of pregnancy. Restricting embryo mobility to only one horn during

10-18 days PO increases endometrial secretion of PGF2α and terminates the pregnancy. The

embryo needs to be in contact with at least 2/3 of the endometrium to avoid luteolysis and

maintain the pregnancy, demonstrating that movement is vital for equine MRP (McDowell et al.,

1988).

Trophectoderm and induced trophectoderm (iTr)

Trophectoderm cells are the precursors of the primitive placenta in eutherian mammalian.

These cells are crucial mediators in the survival and growth of embryos (Rielland et al., 2008).

Any type of disruption in the trophoblast can cause problems embryonic or fetal development

(Godfrey, 2002). Even though placentas from different species can have some functional and

structural similarities, there is a big molecular diversity among species, generating a wide set of

targets of research interest (Douglas et al., 2009).

Transcription factors including TEAD4, GATA3, OCT4, CDX2 (Iqbal et al., 2014) and

EOMES are present in the equine TE (Iqbal et al., 2014; Reinholt et al., 2017). CDX2 and

EOMES are the main transcription factors during the early TE development in mice. Bovine TE

does not express EOMES indicating specie differences (Russ et al., 2000). CDX2 is one of the

first trophoblast-specific genes to be expressed and it is indispensable for the trophoblast lineage

formation and the TE function. Mice embryos genetically ablated of CDX2 are unable to

implant (Strumpf et al., 2005). TEAD4 is required in mice for CDX2 expression (Ralston et al.,

2010). By contrast, EOMES is important for differentiation and proliferation of the TE after the

blastocyst expands (Strumpf et al., 2005). EOMES null mice demonstrate arrested blastocyst development prior to implantation. Suggesting that EOMES is required for the differentiation of the trophectoderm into the trophoblast (Russ et al., 2000).

The GATA transcription factor family is involved in the regulation of mammalian embryo development. Two of the family members, GATA2 and GATA3, are expressed in both the human and mouse trophoblast lineage. A study showed that GATA2/GATA3 knockout mouse embryos were unable to mature to the blastocyst stage due to an abnormal TE growth (Paul and Home, 2015). They also demonstrated that GATA2/GATA3 are crucial for the trophoblast lineage and to regulate and maintain balance the maternal-fetal interface. In mice, GATA3 ablation reduces CDX2 expression (Home et al., 2009) and in cattle, CDX2 expression is increased when GATA2 is overexpressed (Bai et al., 2012).

A hallmark of trophoblast differentiation is the formation of giant cells, also called binucleated cells. These cells are involved in the maternal-fetal union and in the synthesis of hormones and proteins required to maintain a pregnancy (Hu and Cross, 2010). GCM1 and HAND1 play a critical role stimulating giant cells differentiation (Scott et al., 2000), and in the horse they are expressed between 21-25 days PO, when CDX2 expression is decreasing (de Mestre et al., 2009).

A novel equine TE cell line (iTr) that closely mimics the genotype and phenotype of native equine TE, was recently created (Reinholt et al., 2017). This cell line has an epithelial-like morphology with a flat cobblestone appearance that are capable of forming dome-shaped structures (Saito et al., 2002). When cultured in suspension, iTr form hollow spheres that are very similar to an equine day 8 embryo. The transcriptome of equine iTr displays identical core TE lineage markers when compared to that of native TE cells from d8 embryos as revealed by

RNA-sequencing. Both express EOMES, TFAP2A, TEAD4, CDX2, GATA2 and POU5F1 (OCT4) (Reinholt et al., 2017) A similar cell line was created through incomplete reprogramming of porcine mesenchymal stem cells. Porcine iTr cells spontaneously form floating spheres when confluent, and can form non-hemorrhagic teratomas. The iTr cells express several TE markers with few pluripotency genes (Ezashi et al., 2011).

Embryonic stem cells and related variations

Cells derived from the ICM, known as embryonic stem cells (ESC), are pluripotent and capable of self-renewal, differentiating into cells of all germ layer (Desmarais et al., 2011). They express the pluripotency markers (OCT4, SOX2 and NANOG) (Han et al., 2011). Studies done in equine embryos from days 8 to 14 PO showed that NANOG is present in the ICM but not in the TE (Iqbal et al., 2014). Immunosurgical dissection of ICM cells of day 8 blastocysts could be passaged up to 28 times and expressed OCT4, FUT4, AP, TRA-1-81, TRA-1-60, a defined panel of ESC markers. However, once these cells were injected in mouse, teratoma(s) failed to develop (Li et al., 2006)

The fibroblast growth factor (FGF) family is involved in signaling pathways also present in the ICM. In human embryonic stem cells, FGF2 signaling favors their pluripotency and self-renewal (Diecke et al., 2008). Expression of FGF2 and its receptors (FGFR1, FGFR4 and GAB1) is higher in the equine ICM than TE cells (Iqbal et al., 2014). During blastocyst development, FGF signaling is crucial for the differentiation process from ICM to epiblast (Yamanaka et al., 2010). The generation of embryonic stem cells from the ICM in the horse have had several attempts, but all have failed because ICM derived cells transform into TE. Therefore, it is suggested that the unsuccessful transformation to horse ES cells could be because of the high FGF pathway activity in the ICM of the horse (Iqbal et al., 2014).

Growth factor regulation of TE

To gain more information about gene expression in equine iTr cells, Reinholt et al (2017) conducted a bioinformatics study of the iTr transcriptome. The analysis revealed many ligand and receptors tyrosine kinase (RTK). Several growth factors' receptors signal to different biological responses through mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). The receptors for platelet derived growth factor family was highly expressed in the transcripts, including the hepatocyte growth factor receptor (c-MET), EGF and FGF receptors 1, 3 and 4. Also, IGF-1 and -2 ligand and receptors were present, indicating an autocrine signal (Reinholt et al., 2017).

In the mouse, c-MET is the mediator of the allantoic mesenchyme and trophoblast interaction, inducing proliferation and differentiation (Stewart, 1996). Homozygous c-MET mutant (-/-) mouse embryos that died before day 16.5 of pregnancy, showed developmental defects in the placenta, damage of liver parenchyma, a smaller liver and poor skeletal muscle development, bringing evidence that, at least in mice, c-MET is crucial for embryonic and placental development (Bladt et al., 1995). The function of c-MET in the equine trophectoderm remains unknown (Stewart, 1996).

IGF-1 and their receptors participate in mammalian embryonic and fetal development (Klein et al., 2010). The blastocoel cavity is an important source of IGF-1 (Walters et al., 2001). Both the embryo and uterine endometrium express IGF-1 in an estrogen-independent manner, unlike swine (Walters et al., 2001). IGFBP1 is highly expressed in the pregnant mare's uterus (Klein et al., 2010). Researchers suggested than IGFBP1 may regulate the transportation of IGFs between the uterine lumen and endometrium of the pregnant mare (Klein et al., 2010). EGF from

the maternal endometrial gland, is in charge of originating and maintaining the tissues growing

and remodeling the feto-maternal interface in both sides in the horse (Allen and Stewart, 2002).

Equine iTr cells express FGF2 receptors 1,3 and 4 but not the corresponding ligand,

indicating the absence of and autocrine loop (Reinholt et al., 2017). Interestingly, equine iTr cells

expressed FGF7 ligand but not its receptor FGFR2b, however its role has not yet been described

in the horse. In humans, FGF7 promotes cytotrophoblasts differentiation towards

synctiotrophoblasts (Massabbal et al., 2005). In pigs, during early pregnancy, FGF7 receptor and

ligand are expressed in the endometrial epithelia. The receptor is also expressed by the TE,

suggesting a possible paracrine interaction between the embryo and the endometrium (Ka et al.,

2000). A study done in horses showed that FGF2 was present in the equine embryo and its

mRNA expression decreased throughout the embryo development (Klein and Troedsson, 2011b).

In bovine embryos, close to the elongation, FGF2 increases in expression (Cooke et al., 2009).

Although FGF2 was not present in the equine iTrs' transcriptome, expression of the

corresponding receptors (FGFR1,3,4) was, suggesting that in this species, the endometrium may

be the source of the ligand (Reinholt et al., 2017).

Steroid and prostaglandin hormones

In several species, hormones like prostaglandin, progestin and estrogens play a central

role in the establishment of pregnancy (DeFranco, 2002).

Estrogen and its receptors play an important role in luteolysis in ruminants, upregulating

OXT receptors in the endometrium. Oxytocin binds to its receptors and initiates PGF2α pulsatile

secretion (Bazer et al., 1986). In the early gestational ewe, ESR1 is inhibited by IFNT which

blocks OXT receptors inhibiting luteolysis (Spencer and Bazer, 1995). In cyclic mares, OXT

plays a main role in luteolysis, increasing the endometrial secretion of PGF2α. ESR1 might be

involved in the induction of luteolysis in a similar way than in ruminants however, the mechanism of action has not been documented (Klein et al., 2010). Variations in ESR1 expression during the estrous cycle have been shown in different species. For example, in pregnant ewes, ESR1 is lower during early pregnancy (day 11 to 14) increasing between day 15 to 25. Non-pregnant ewe express high levels of ESR1 as early as at day 1 and between 11-15, and it is lower between day 1-6 when progesterone is higher (Spencer and Bazer, 1995). In cycling mares, expression ESR1 and progesterone receptors is higher during estrus and lower during mid/late diestrus (between days 11-14) while in pregnant mares it is lower during early pregnancy (up to day 20 PO) (Hartt et al., 2005). A study performed by Klein et al also identified that ESR1 expression was lower at day 13.5 PO in pregnant mares than in non-pregnant mares. Therefore, the low expression of ESR1 in pregnant mares could be related to low OXT, no PGF2α secretion resulting in inhibition of luteolysis (Klein et al., 2010).

Equine embryos also express enzymes involved in steroid hormones synthesis (Fig 1-2). Steroidogenic acute regulatory protein (STAR), is a transport protein which regulates the cholesterol transport in the mitochondria, a rate limiting step for the production of steroid hormones. In the sow, STAR is the main regulator of steroidogenesis (Blomberg and Zuelke, 2005). This enzyme is highly expressed in equine embryos day 12-14 PO compared to day 8 embryos (Klein and Troedsson, 2011b), but was not present in the iTr transcriptome.

The cholesterol side-chain cleavage enzyme (CYP11A1), is the rate limiting enzyme involved in steroidogenesis and converts cholesterol into pregnenolone (Tu et al., 2014). This enzyme is expressed by the horse embryo (Klein, 2015) and the iTr cells (Reinholt et al., 2017). CYP17A1 and CYP19A1, a cytochrome P450 enzyme and an aromatase respectively, start increasing their expression in the equine embryo at day 12 PO and both are present in iTr cells.

3Beta Hydroxysteroid dehydrogenase (HSD3), is also present in equine embryos and iTr cells. This enzyme is very important in the production of hormonal steroids of all classes. Its expression increases in embryos by day 12-14 PO (Klein and Troedsson, 2011b). CYP11A1, CYP19A1 and HSD3B1 haven't been found to be expressed in endometrial samples (Klein, 2015). Suggesting that the embryo is involved in steroidogenesis around the time range of MRP.

Rate limiting enzymes of the PLA2 family, such as SPLA2 (also known as PLA2G2A) and PLA2G4A (cPLA2) are required for prostaglandin synthesis (Fig 1-3) (Murakami et al., 1998). The arachidonic acid coming from the phospholipid bilayer is cleaved by SPLA2 and mobilized by PLA2G4A (Klohonatz et al., 2015).

PLA2G4A regulates the secretion of PGF2α and its expression increase in the mare endometrium close to luteolysis. PLA2G4A expression is higher during estrus and lower but maintained steady around day 8 PO. There is no difference between pregnant and non-pregnant mares with high P4 at day 15, but is lower than non-pregnant mare with low P4 (Ababneh et al., 2011). SPLA2 has been involved in degradation of the embryo's capsule. During early pregnancy, increase in SPLA2 after PGF2α administration blocks embryo fixation with resulting in early loss of the pregnancy (Hayes et al., 2008). SPLA2 is not expressed in the iTr transcriptome, but PTGS2 and PLA2G4A are. The latest is also present in the equine embryo (Simpson et al., 1999).

Apolipoproteins are proteins which bind to lipids to form lipoproteins for transport. APOB, one of the 5 apolipoproteins, plays a main role in the mouse embryos growth. Via the yolk sac, APOB transports lipid nutrients to the mouse embryo, regulating the synthesis of lipoproteins. A study of horse embryos showed that apolipoproteins are up-regulated. These are involved in cholesterol transport and are present in the equine embryo. The authors suggested

that apolipoproteins could have a similar function in the horse embryo than in mice, by providing

nutrients for the embryo development (Klein and Troedsson, 2011a). Klohonatz et al compared

the increase in apolipoproteins in embryos (day 8, 10, 12 and 14 PO) with a decreased in SPLA2

in endometrial samples from pregnant mares (day 12, 14, 16 and 18 PO) around the same time of

embryo development, suggesting that the embryo could be controlling its own cholesterol

transport (Klohonatz et al., 2015).

Experimental objectives and hypothesis

       Given the absence of a defined MRP for the horse, the objective of the study was to

examine the role of EGF, FGF2, HGF and IGF-1 on equine iTr cells. Our hypothesis was that

iTr, similar to TE, demonstrate altered transcription of steroidogenic genes and growth kinetics

in response to growth factors. Understanding the impact of growth factors on equine TE may

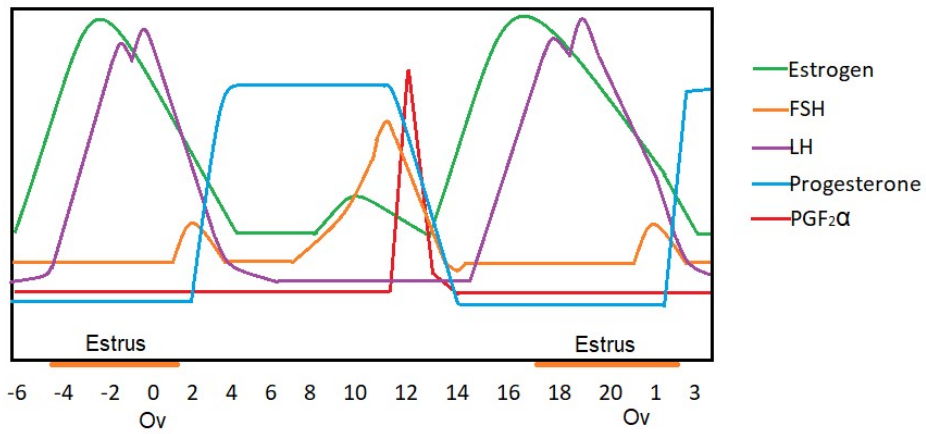lead to further insight into MRP in the horse.

Figure 1-1. The estrous cycle of the mare (Adapted from (Brinsko et al., 2010).
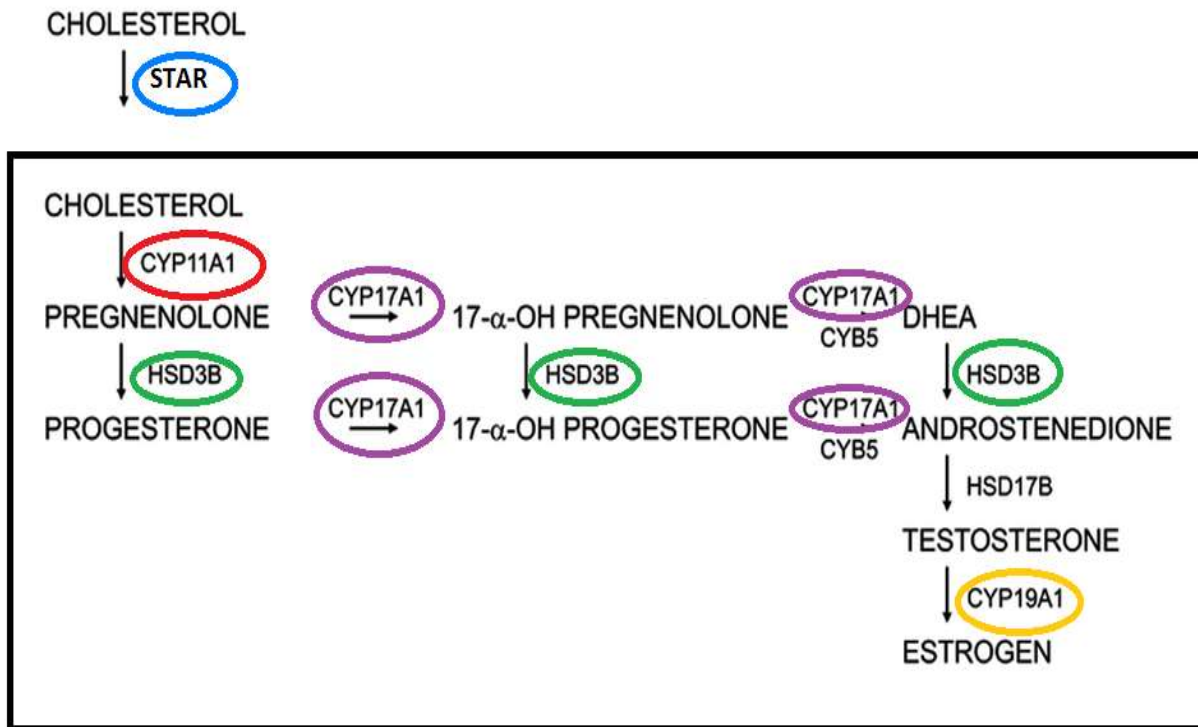
Figure 1-2. Simplified scheme of the classic steroid biosynthetic pathway (Adapted from (Hofland et al., 2010)).
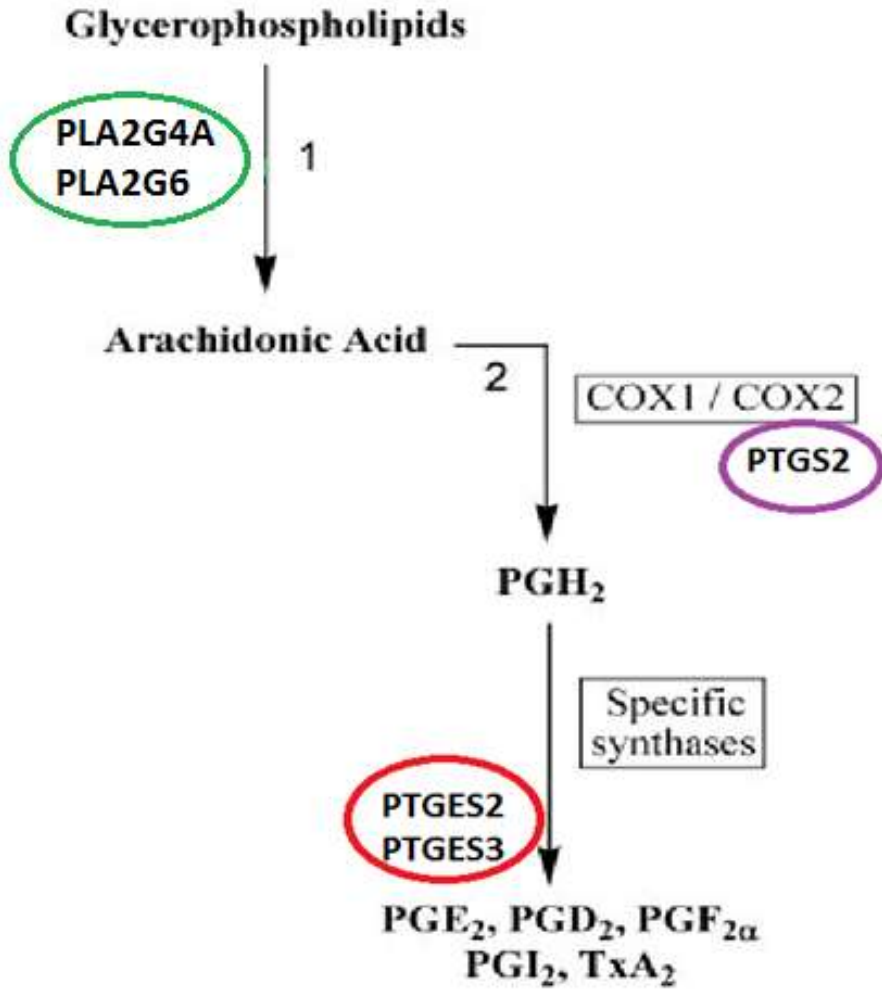
Figure 1-3. Cellular pathways of prostaglandin (PG) metabolism (Adapted from (Phillips et al., 2014)).

CHAPTER II

Growth factor modulation of equine trophoblast mitosis and prostaglandin gene expression.

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Running title: Growth factors and trophoblast proliferation

ABSTRACT

To provide insight into maternal recognition of pregnancy control in equids, the mitogenic and developmental effects of endometrium-expressed growth factors (epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF2) and hepatocyte growth factor (HGF), were examined in equine iTr cells, an equine trophectoderm cell line. Initial Western blots revealed that HGF and IGF-1 stimulate phosphorylation of AKT serine/threonine kinase 1 (AKT1) and EGF, FGF2 or HGF resulted in phosphorylation of both extracellular signal-regulated kinase 1 (ERK1) and ERK2. Mitotic activity was stimulated ($P < 0.05$) by EGF, FGF2 and HGF. Chemical disruption of mitogen activated protein kinase 1 and 2 (MEK1/2) phosphorylation suppresses ($P < 0.05$) proliferation in control and growth factor treated cells demonstrating a dependence on ERK1/2 for mitotic activity. Treatment of iTr cells with EGF or HGF in the presence of an AKT1 inhibitor prohibits ($P < 0.05$) growth factor stimulated proliferation. The effect of EGF, FGF2, HGF and IGF-1 on steroid biosynthetic enzyme gene expression, including *prostaglandin-endoperoxide synthase 2* (*PTGS2*), was determined by real-time PCR. Neither EGF, FGF2 nor IGF-1 affected *PTGS2* expression while HGF caused a two-fold increase ($P < 0.05$) in expression. Co-supplementation with HGF and an AKT1 inhibitor did not block *PTGS2* expression whereas providing a MEK1/2 inhibitor prevented ($P < 0.05$) the HGF-mediated increase in *PTGS2*. These results provide novel evidence of a role for HGF in equine trophectoderm proliferation and prostaglandin biosynthesis.

KEY WORDS: hepatocyte growth factor, proliferation, prostaglandin, trophectoderm

INTRODUCTION

Early embryogenesis in the horse is unique amongst domestic animals. Following fertilization of the ovum, the embryo travels through the oviduct and enters the uterus at 6 days post-ovulation whereas non-fertilized ova are not expelled into the uterus (Betteridge et al., 1982). Soon thereafter the blastocyst is surrounded by a thickened glycocalyx capsule that protects the embryo as it moves throughout the uterus because of intrauterine contractions that are mediated by prostaglandins E and F, a requirement for establishment of pregnancy (McDowell et al., 1988; Stout and Allen, 2001). By day 10 of development, an embryonic disc is present, and gastrulation is complete by day 14 to 16, when fixation within an uterine horn occurs (Gaivão et al., 2014; Klein, 2015).

Both capsule formation and uterine mobility are hallmarks of equid pregnancy establishment, but the underlying mechanism(s) of equine maternal recognition of pregnancy (MRP) remain largely unresolved. What is known is that manipulation of prostaglandin F2α (PGF2α) secretion is the apparent key to MRP in mares, and that the MRP signal(s) should exist by 14 post-ovulation to prevent endometrial PGF2α from lysing the corpus luteum (Boerboom et al., 2004). Uterine fluids at day 14-16 post-ovulation contain less PGF2α than cycling mares (Berglund et al., 1982). *In vitro* exposure of endometrial explants to embryo secretions from this time period decreased *prostaglandin synthetase 2* (*PTGS2*) (Ealy et al., 2010). At day 18 post-ovulation, uterine fluids contain PGF2α concentrations that are similar to cyclic mares undergoing luteolysis, suggesting that the equine embryo doesn't prevent PGF2α secretion during pregnancy, but delays it (Stout and Allen, 2002). However, little is known about the actual MRP signal or group of signals. Dialysis of conditioned media from equine embryos at day 14 blastocysts and co-cultured with equine endometrial explants determined that a protein of

1 to 6 kDa will inhibit PGF2α synthesis (Sharp et al., 1989). Others report that the anti-luteolytic activity released by embryos was refractile to proteinase treatment but was removed by charcoal treatment, indicating that the proposed MRP was a lipophilic, non-polar compound (Ababneh et al., 2000). One cannot discount, however, the possibility that two or more MRP signals exist in the mare. The trophectoderm (i.e. outermost layer of embryonic cells) synthesizes and releases both cytokines and hormones during the MRP timeframe, and apparently several of these secreted factors are capable of suppressing PGF2α secretion. The question that remains unanswered is to decipher which signal or set of signals are essential for the establishment of pregnancy.

Cytokines and growth factors within the uterine microenvironment support the growth and development of the conceptus prior to implantation. In other species, many of these same signaling molecules also facilitate MRP either directly by affecting the expression of the MRP signal or indirectly by promoting embryogenesis, and specifically early placental development (Spencer et al., 2004). For example, culture of bovine blastocysts with fibroblast growth factor 2 (FGF2) causes an increase in the production and release of interferon tau (IFNT), the MRP factor for ruminants (Michael et al., 2006; Cooke et al., 2009). Other endometrial factors, such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF1) stimulate bovine trophoblast cell proliferation and increase bovine blastocyst size (Dilly et al., 2010; Xie et al., 2017; Mesalam et al., 2018). Also, treatment of early stage embryos with IGF-1 initiates intracellular signals that protect the developing blastocyst from an environmental heat insult (Bonilla et al., 2011). The mare's endometrium produces FGF2 (de Ruijter-Villani et al., 2013), VEGF (Silva et al., 2011), EGF (Allen et al., 2017), IGF-1 and IGF-2 (Sessions-Bresnahan et al., 2018) as well as the cytokines LIF (de Ruijter-Villani et al., 2015), IL6, TNFα and IL1β

(Sessions-Bresnahan et al., 2018). Their participation and requirement in early embryogenesis in the horse remain undefined.

We propose that describing how various endometrial-derived growth factors influence trophoblast cell proliferation and prostaglandin gene expression may provide new clues into the embryo-derived mechanisms that control MRP in horses. Our hypothesis was tested using iTr cells, an equine trophoblast cell line created through a partial cellular reprogramming event, that is similar in morphology to trophoblast cells and share a common genetic signature with native trophectoderm (TE) (Reinholt et al., 2017).

MATERIALS AND METHODS

Animals and biopsy

All animal protocols were reviewed and approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee. Mares (n=3) were evaluated daily by transrectal ultrasonography to map follicular development and ovulation. Mares were sedated (Xylazine, MWI Animal Health, Boise, ID) prior to biopsy collection. Endometrial biopsies were recovered at day 7 post-ovulation by insertion of a sterile alligator-type biopsy device through the cervix into the uterus and physical resection of tissue (~75 mg). Tissue was briefly rinsed with ice-cold sterile phosphate buffered saline (PBS) and snap frozen in liquid nitrogen for RNA isolation.

Cell Culture

Induced trophoblasts (iTr) were cultured on tissueware coated with extracellular matrix (Matrigel, Corning, Corning, NY) in high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10 mM non-essential amino acids, 15% fetal bovine serum (FBS), 2 mm glutamine, 1% penicillin-streptomycin, 0.5% gentamicin and 55 μM β-mercaptoethanol. All media and supplements were purchased from Invitrogen (Thermofisher, Carlsbad, CA). For the

measurement of proliferation, cells were serum starved overnight followed by treatment with 10 ng/mL bovine FGF2, human EGF, human HGF or human IGF-1 for 24 hr. Growth factors were purchased from R&D Systems (Minneapolis, MN). During the final 2 h of culture, 10 µM 5-ethynyl-2′-deoxyuridine (EdU) was included for the detection of DNA synthesis. Chemical inhibition of AKT serine/threonine kinase 1 (AKT1) or mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) was accomplished by supplementation of the culture media with 1 nM MK2206 and 1 nM PD0325901, respectively. Cells were fixed with 4% paraformaldehyde in PBS and EdU was detected by click chemistry (Click-iT EdU AlexaFluor 488, Invitrogen). Nuclei were detected following incubation with 10 µg/mL Hoechst 33245 (Invitrogen). Total nuclei and EdU-positive nuclei were visualized with epifluorescence (Eclipse TS100, Nikon, Melville, NY) and representative images captured with a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ) with shutter speed controlled by NIS Elements software (Nikon). A mitotic index was calculated as EdU(+)/Hoechst(+) X 100.

RNA isolation and polymerase chain reaction (PCR*)*

Total RNA was extracted and purified from iTr cells and endometrial tissue using TRIzol (Ambion, Austin, TX). Contaminating DNA was removed with DNase I (Ambion). A High Capacity cDNA Reverse Transcription Kit (ThermoFisher) was used to convert RNA into cDNA, according to the manufacturer's instructions. Real-time PCR reactions were performed with Power SYBR® Green PCR Master Mix (ThermoFisher) and gene specific primers (Table 1) using an Eppendorf Realplex thermocycler (Eppendorf, Hamburg, Germany). The fold change for all the samples was calculated using the $2^{-\triangle\triangle Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control; GAPDH Ct values were unaffected by treatment. Verification that a single amplicon was produced was completed by electrophoresis through 2% agarose gels impregnated with SYBR Safe DNA gel stain (Thermofisher) in 40 mM

Tris-acetate buffer (pH 8.3) containing 1 mM ethylenediaminetetraacetic acid. Amplicons were visualized at 280 nm on a BioDoc-It (UVP).

Western Blot

Cells were washed with ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA; Thermofisher) buffer containing protease and phosphatase inhibitors (Halt; Thermofisher). Insoluble material was removed by centrifugation at 10,000 X g for 5 minutes at 4 C. Protein concentration was measured colorimetrically (BCA Assay, Thermofisher). Proteins were diluted in loading buffer containing SDS (LDS, Thermofisher) and heated at 95 ˚C for 5 min. Equivalent amounts of protein were electrophoretically separated through denaturing 10% polyacrylamide gels (NuPAGE, Thermofisher). Proteins were transferred to nitrocellulose (iBlot2 Dry Transfer, Thermofisher). Membranes were incubated with 10 mM TRIS, pH 8.0, 150 mM NaCl (TBS) containing 2% bovine serum albumin (BSA) and 0.1% Tween20 (TBST) for 30 min at room temperature to block nonspecific antigen sites. Subsequently, membranes were incubated at 4 ℃ overnight with anti-AKT1 (pan 11E7; Cell Signaling Technologies (CST), Danvers, MD), anti-phosphoAKT1^{S473} (D9E; CST), anti-ERK1/2 (137F5; CST), anti-phosphoERK1/2 (197G2; CST) or anti-α/β-tubulin (2148; CST) diluted 1:2,000 in TBS containing 2% BSA. Blots were washed extensively with TBST followed by incubation with donkey anti-rabbit IgG-peroxidase (Invitrogen) diluted 1:2,000 in TBS containing 2% BSA for 45 min at room temperature. After washing with PBS, immunocomplexes were visualized by chemiluminescence (ECL, Thermofisher) using a BioDoc-It (UVP, Upland, CA).

Statistics

Data were analyzed by one- or two-way ANOVA using Prism 7 (Graphpad Software, La Jolla, CA). Technical replicates were averaged for each biological replicate prior to calculation of means and SEM. Post-test comparisons were performed using Tukey's adjustment to analyze

pre-planned comparisons between groups. A minimum of 3 replicates of each experiment was performed. Significance was set at $P < 0.05$.

RESULTS

Equine iTr cells express receptors for EGF, FGF, HGF and IGF-1 (Reinholt et al., 2017). Expression of the corresponding ligands was examined by RT-PCR using total RNA isolated from the uterine endometrium, archived adult liver tissue and iTr cells. Expression of *EGF*, *FGF2*, *HGF* and *IGF-1* was detected in endometrium and liver (positive control) RNA isolates (Fig 2-1). Expression of *EGF*, *FGF2* and *IGF-1* was detected in iTr cells whereas HGF was not detected.

Each growth factor docks with its cognate receptor tyrosine kinase (RTK) and initiates several intracellular signals that modify cell behavior. As a first step to ensure that the ligands can affect iTr biology, each growth factor was examined for its ability to phosphorylate AKT1 and ERK1/2, two prominent intracellular signaling effectors of RTKs. Serum-starved equine iTr cells were treated with recombinant EGF, FGF2, HGF or IGF-1 and analyzed with total and phospho-specific antibodies to AKT1 and ERK1/2. Weak phosphorylation of AKT1 was observed in response to EGF, FGF2, HGF or IGF-1 exposure. By contrast, substantial amounts of phosphorylated ERK1/2 were detected following treatment with EGF, FGF2 and HGF (Fig 2-2). Direct phosphorylation of ERK1/2 in response to IGF-I was no different than control. No differences in the relative amounts of total AKT, ERK1/2 or α/β tubulin proteins were noted.

Initial experiments examined the role of AKT1 as a mediator of iTr proliferation. In brief, equine iTr cells were treated with each growth factor in the presence or absence of the AKT inhibitor, MK2206. Weak phosphorylation of AKT1^{S473} occurred in response to EGF, FGF2, HGF and IGF-1 (Fig 2-3A). Phosphorylation was inhibited completely by inclusion of 1 nM MK2206 in the culture media. The next experiment examined whether these growth factors

influence iTr proliferation. Results revealed that EGF, FGF2, HGF and IGF-1 increased ($P < 0.05$) the percentage of cells incorporating EdU (Fig 2-3B). Supplementation of the culture media with the individual growth factors and 10 nM MK2206 suppressed ($P < 0.05$) EGF, HGF and IGF-1-directed proliferation. The AKT inhibitor affected neither control nor FGF2-mediated cell division.

The importance of ERK1/2 signals as mediators of mitogenic effects was evaluated. Phosphorylation of ERK1/2 occurred in response to EGF, FGF2, HGF and IGF-1, although to varying intensity levels. Supplementation with 1 nM PD0325901 suppressed ($P < 0.05$) both basal and growth factor-initiated phosphorylation of ERK1/2 (Fig 2-4A) as well as proliferation (Fig 2-4B).

A final set of studies examined whether the growth factors under investigation affect gene expression of enzymes controlling prostaglandin E2 and F2α production. Treatment of iTr with either EGF, FGF2, HGF or IGF-1 failed to alter basal transcript amounts for *cytochrome P450 family 11 subfamily A member 1* (*CYP11A*), *prostaglandin E synthase 2* (*PTGES2*) and *PTGES3* (Fig 2-5 A, C, D, respectively). Treatment of iTr cells with HGF caused a 10-fold increase ($P < 0.05$) in *prostaglandin synthase 2 (PTGS2)* mRNA abundance. No other growth factor elicited an effect on *PTGS2* (Fig 2-5B). Media supplementation with 1 nM MK2206 (AKT inhibitor) did not affect either basal transcription of *PTGS2* or the HGF-directed response (Fig 2-6A). By contrast, iTr cells incubated with HGF and PD0325901 (MEK1/2 inhibitor) resulted in a reduction ($P < 0.05$) in *PTGS2* mRNA content to levels comparable to controls (Fig 2-6B).

DISCUSSION

The incidence of pregnancy loss in the mare is 7.9%, and the majority of these losses occur within the first 42 days post-ovulation (Rose et al., 2018). The underlying causes of these losses remain largely unknown due to our limited understanding of embryology and MRP in the

horse. Communication between the uterine endometrium and the TE of the developing

blastocyst is a critical determinant in establishing pregnancy. Transcriptome analysis of the early

TE reveals the presence of conventional RTK signaling systems as well as chemokine and

interferon cell signaling genes (Iqbal et al., 2014; Reinholt et al., 2017).

This work determined that iTr cells can respond to FGF2. *Fibroblast growth factor receptors 1,*

2 and *3* are expressed in TE with a temporal increase over the initial 21 days of the pregnancy

that coincide with an increase in *FGF2* expression within the endometrium (de Ruijter-Villani et

al., 2013). Treatment of iTr cells with FGF2 caused an increase in cell proliferation, similar to

that observed in bovine trophoblast cells and blastocysts (Xie et al., 2017). The ligand initiated

weak phosphorylation of AKT1, and AKT1 was not involved in either mitotic responses or

steroidogenesis enzyme expression in equine iTr cells. Thus, the intracellular signaling pathway

used by FGF2 for equine trophoblast proliferation remains unknown. It is possible that FGF2

utilizes ERK1/2-dependent pathways, but we were unable to tease apart mitosis in the absence of

ERK1/2 activity, which is an absolute requirement for iTr cell division.

Insulin-like growth factor-1 contributes to multiple events in early embryogenesis and

trophoblast biology specifically. Treatment of primary TE cells isolated from day 12 porcine

embryos with an equivalent amount of IGF (20 ng/mL) does not affect proliferation but does

promote migration (Jeong et al., 2014). Proliferation was increased by 60% over controls,

however, by treatment of porcine trophoblasts with 100 ng/mL. The IGF-1 mediated effect on

mitosis was abolished by chemical inhibition of PI3K, mammalian target of rapamycin (mTOR),

MEK1/2 and p38 directed signaling events. This suggests that a complex kinase network

regulates porcine trophoblast proliferation. Because we examined the effects of a single IGF-1

concentration, it is possible that mitogenic effects could occur at greater concentrations as seen

with porcine trophoblasts. Indeed, 50 ng/mL of IGF-1 is required to elicit a mitogenic effect on bovine CT1 trophoblast cells (Xie et al., 2017). Future efforts should include examination of greater concentrations of IGF-1 on iTr biology as well as measurement of IGF-1 content in the uterine milieu.

Ligand-initiated receptor recruitment of PI3Ks to the plasma membrane followed by AKT phosphorylation and formation of the PI3K/AKT/mTOR signalosome underlies cell proliferation, metabolism and growth (Laplante and Sabatini, 2012). Ablation of individual components (PI3K p110α, PI3K p110β, mTORC) and downstream effectors (Sin1) of the signaling module is embryonic lethal in mice (Jason and Cui, 2016). Activation of the module by EGF and HGF triggers equine iTr proliferation and offers a potential pathway for early embryo development in the horse. In support of this premise, EGF improves bovine embryo quality in a serum-free media *in vitro* (Mesalam et al., 2018). Culture of bovine blastocysts with EGF affected neither total blastomere number nor CDX2(+) trophoblast numbers but worked synergistically with FGF2 and IGF-I to increase both parameters (Xie et al., 2017). Proliferation of trophoblast cells derived from bovine placentomes is increased by EGF treatment through a pathway that involves both ERK1/2 and PI3K activity (Dilly et al., 2010). The discrepant results likely reflect differences in developmental age of the cell types.

The most striking result of the experiments was the observation that HGF initiated ERK1/2 signals and this resulted in a 10-fold increase in *PTGS2* expression. The enzymatic actions of PTGS2 convert arachidonic acid to PGH2, a precursor to PGE2 and PGF2α. Equine embryos secrete and release PGs into the uterine milieu, and this contributes to smooth muscle contraction within the uterus and subsequent embryo mobility (Watson and Sertich, 1989; Stout and Allen, 2002). Inhibition of PTGS2 does not affect uterine tone but substantially reduces

embryo motility within the uterus in mares (Stout and Allen, 2001). Our results fill a void through identification of HGF, a growth factor secreted by the uterine endometrium, as a modulator of trophoblast *PTGS2* expression and subsequent PG production during early pregnancy. These actions are dependent upon the MEK/ERK signaling module. By contrast, HGF signals through the PI3K/AKT module are critical for trophoblast proliferation. The capacity to use divergent signaling networks to control distinct cellular functions makes HGF an intriguing possible modulator of MRP. During the preimplantation stage of gestation, the embryokine may support trophectoderm expansion while ensuring continued mobility prior to fixation.

Maternal recognition of pregnancy in the mare remains a mystery but likely involves endometrial derived growth factor communication with the outer trophectoderm layer of the embryo. Results presented herein provide evidence that EGF, FGF2, HGF and IGF-1 may support trophoblast proliferation during early equine embryogenesis. More importantly, HGF mediated signals uniquely affect both trophoblast proliferation and steroidogenesis gene transcription supporting a role for this endometrial-derived growth factor as a novel facilitator of MRP in the horse.
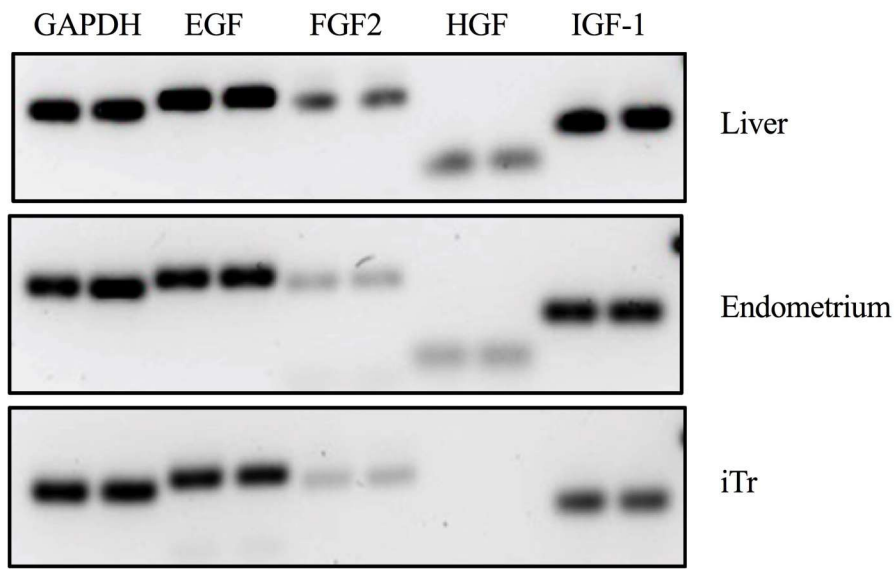
Figure 2-1. Expression of potential embryokines in equine liver, endometrium and iTr cells.
Total RNA was isolated from adult mare liver and day 7 post-ovulation endometrium, and
equine iTr cells, reverse transcribed and amplified with gene-specific primers for EGF, FGF2,
HGF, IGF-1 and GAPDH. Amplicons were separated through Syber-safe agarose gels and
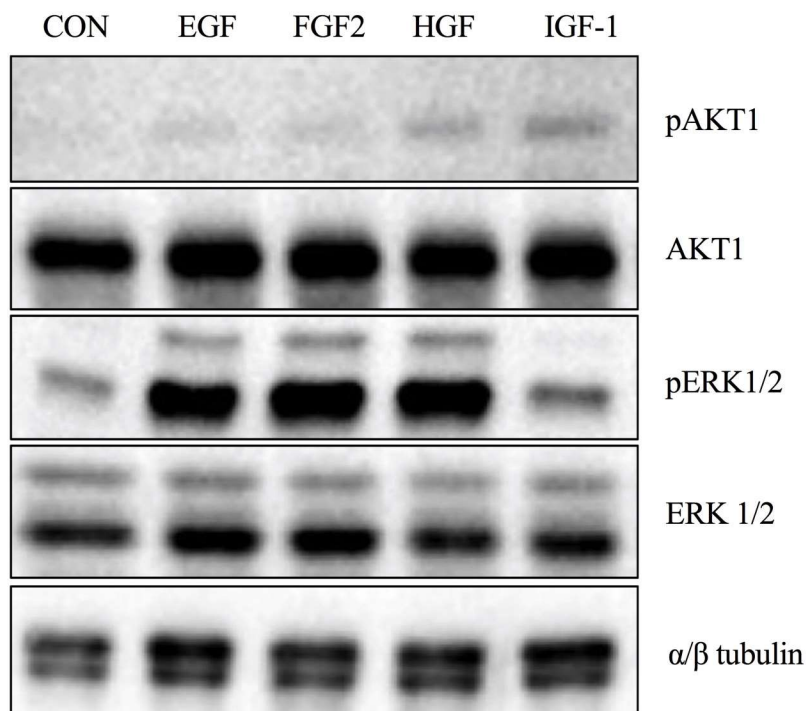imaged. Gel is representative of three independent mares.

Figure 2-2. Phosphorylation of AKT1 and ERK1/2 in response to selected growth factors.
Equine iTr cells were serum starved and treated with 10 ng/mL of EGF, FGF2, HGF or IGF-1
for 20 minutes prior to lysis. Proteins were separated through SDS-PAGE, transferred to
nitrocellulose and analyzed by Western blot for total and phosphorylated version of AKT and
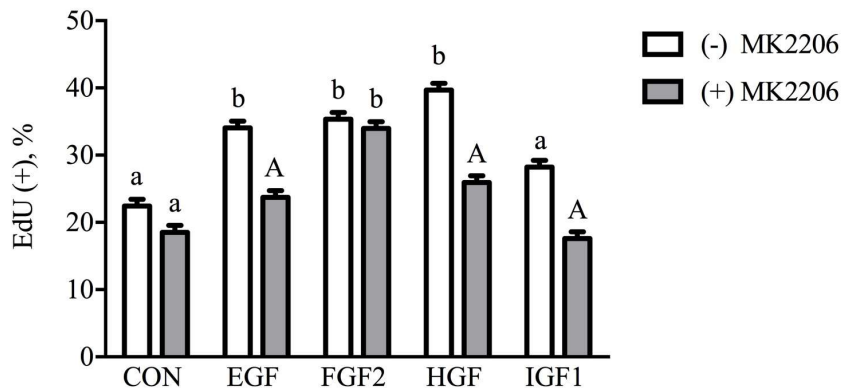ERK1/2. Tubulin expression was monitored as a loading control.

A.
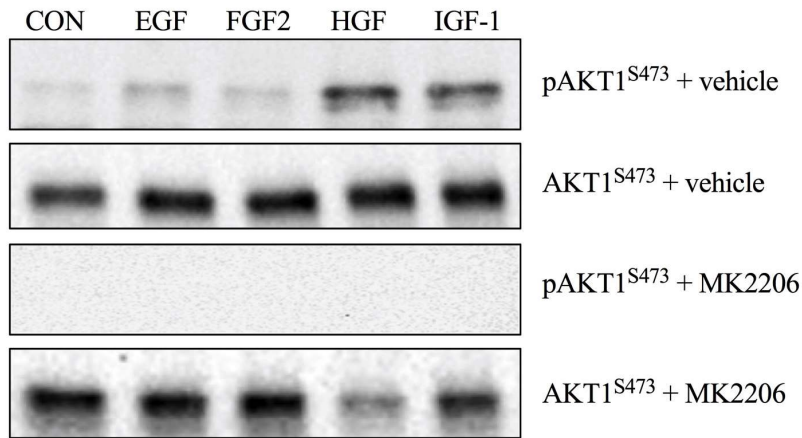
B.

Figure 2-3. Equine iTr cells proliferate in response to EGF, HGF and IGF-1 in an AKT1-dependent manner. Equine iTr cells were treated with the respective growth factors in the presence or absence of 1 nM MK2206, an AKT inhibitor. Lysates were analyzed by Western blot for total and phosphorylated AKT1 (**A**). Parallel plates of cells were treated in a similar manner with EdU included 2-h prior to fixation. Total and EdU (+) cells were enumerated (**B**). Means and SEMs of 3 replicate experiments shown. Different letters represent significance at P < 0.05. Capital letters indicate significance within treatment at P < 0.05.

A.
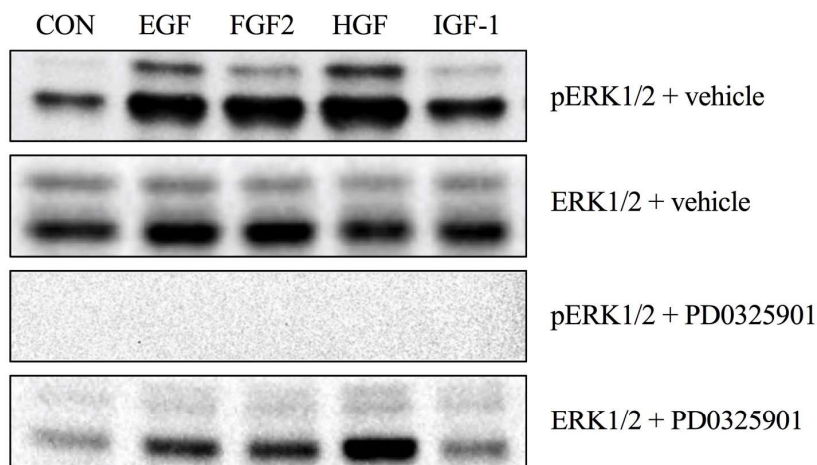
B.
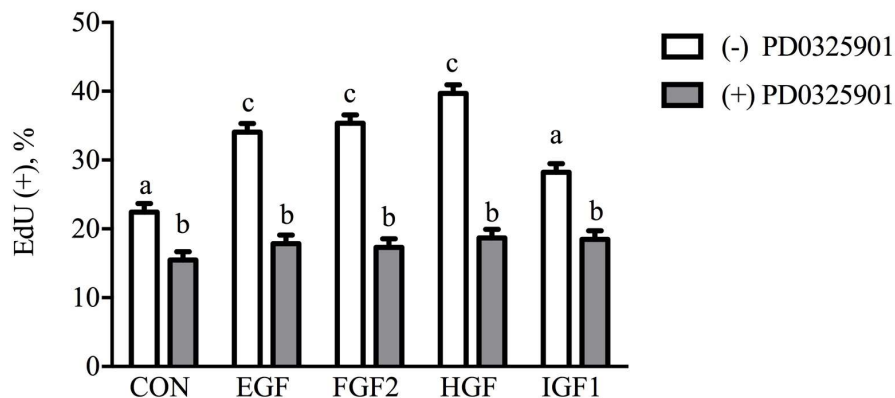
Figure 2-4. Equine iTr cells require MEK/ERK signals for proliferation. Equine iTr cells were treated with the respective growth factors in the presence or absence of 1 nM PD0325901, a MEK inhibitor. Lysates were analyzed by Western blot for total and phosphorylated ERK1/2 (**A**). Parallel plates of cells were treated in a similar manner with EdU included 2-h prior to fixation. Total and EdU (+) cells were enumerated (**B**). Means and SEMs of 3 replicate experiments shown. Different letters represent significance at P < 0.05.
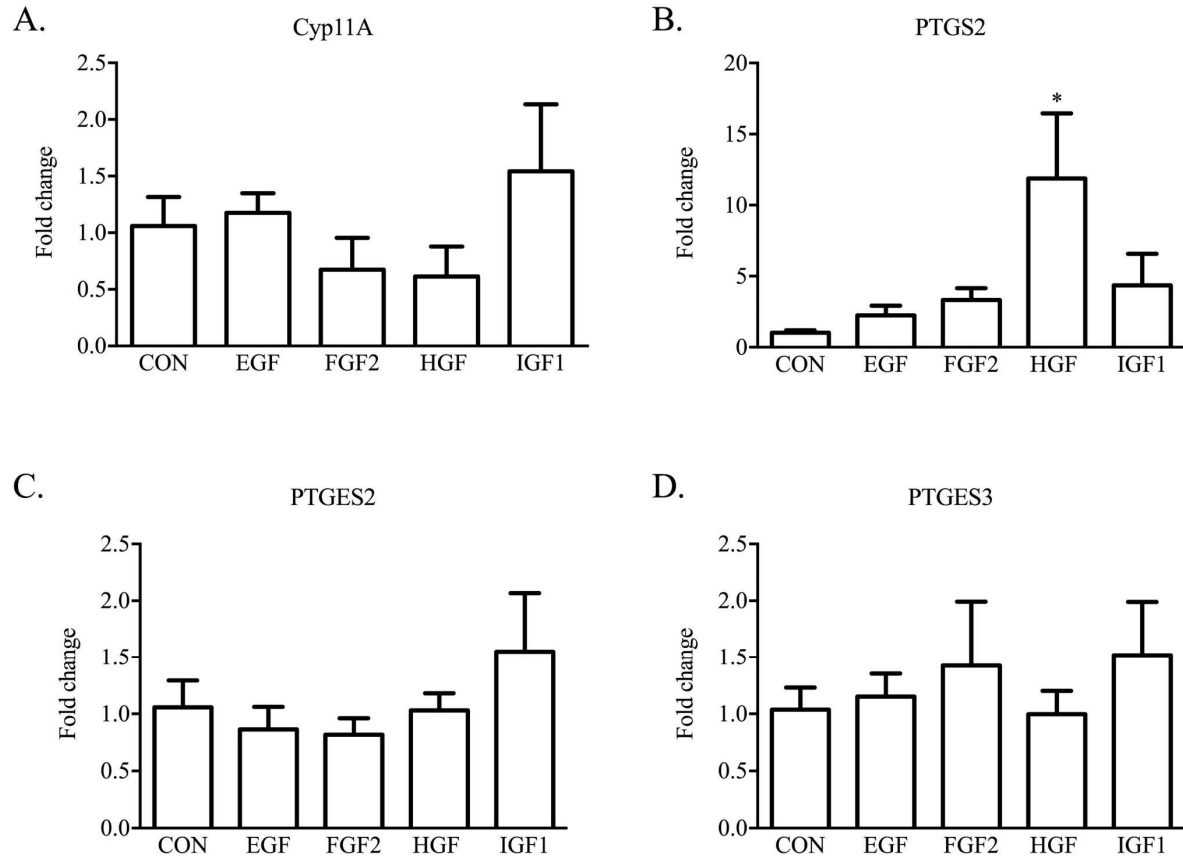
Figure 2-5. Growth factor treatment of equine iTr cells differentially affects steroidogenic gene expression. Equine iTr cells were cultured for 36 h with 10 ng/mL of EGF, FGF2, HGF or IGF-1. Total RNA was isolated, reverse transcribed and analyzed by real time PCR for Cyp11A, PTGS2, PTGES2 and PTGES3 mRNA abundance. Fold change calculated relative to CON cells treated with vehicle-only. Mean and SEM of 3 replicate experiments shown. * denotes significance at $P < 0.05$.
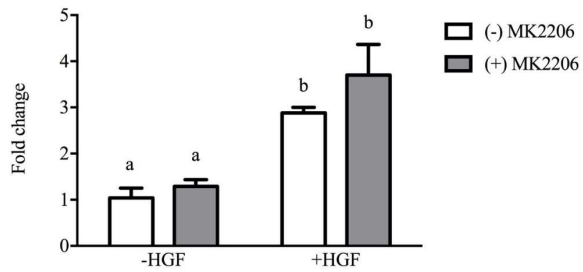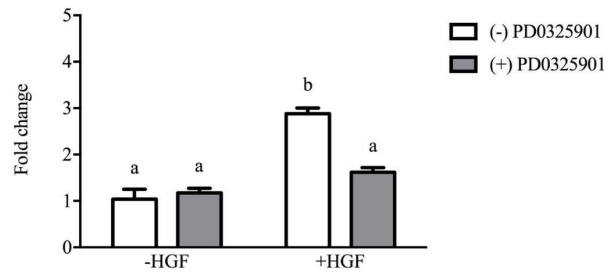
Figure 2-6. HGF stimulates PTGS2 expression through a MEK-dependent signaling pathway. Equine iTr cells were cultured for 36 h with 10 ng/mL of HGF in the presence or absence of 1 nM MK2206 (**A**) or 1 nM PD0325901 (**B**). Total RNA was isolated, reverse transcribed and analyzed by real time PCR for PTGES2 mRNA abundance. Fold change calculated relative to CON cells treated with vehicle-only. Mean and SEM of 3 replicate experiments shown. * denotes significance at $P < 0.05$.

Table 2-1. List of equine primers

Gene	Amplification product (bp)	Primer sequence
F-GAPDH	150	CCACCCCTAACGTGTCAGTC
R-GAPDH		AATCGCAGGAGACAACCTGG
F-CYP11A1	159	AACGTTACCGAGATGCTGGC
R-CYP11A1		CACCATCGTGCTCGTGTCTC
F-CYP17A1	108	CTCTGTGGTGAAGTGGATTG
R-CYP17A1		AGTGTTGGTGTGCGGCTGAA
F-CYP19A1	100	AGTGCTCCTCCCATCCCATT
R-CYP19A1		ATCGGCCTGGTCTTTCTAAT
F-STAR	186	GAAGCTCCCCTTGAGAGTAGGA
R-STAR		CAGTTCAGCTCCTGGCCAATG
F-PTGS2	188	GCTTGTTCCAGACGAGCAGG
R-PTGS2		GGGATGCCAGTGATAGAGCG
F-PTGES2	164	TGATCTGGCTGTGTATGGCG
R-PTGES2		ATCTTCCGTTGCCTCTCGTC
F-PTGES3	196	CACGTTCATTCTCCGTCCTCG
R-PTGES3		GCAGTCGACTCTTCTCCGTTG
F-EGF	126	TTCTGGACTGATATGGGGATT
R-EGF		GGCATCGCACCAATACAACTT
F-FGF2	132	TGCTATGAAGGAAGATGGAAG
R-FGF2		TTATACTGCCCCGTTCGTTTC
F-HGF	70	CGCTACGAAGTCTGTGACATTCC
R-HGF		TTCCCCATTGCAGGTCATG
F-IGF1	89	GAAGCAATGGGAAAAATCAGC
R-IGF1		AGGTAGAAGAGATGTGAGGAG

CHAPTER III

Implications and future directions

In several species prostaglandins and growth factors play a central role in the establishment of pregnancy (DeFranco, 2002; Spencer and Bazer, 2004). In horses, embryonic migration to the uterus and mobility within it, are stimulated by prostaglandins E and F and required for successful establishment of pregnancy (McDowell et al., 1988; Stout and Allen, 2002). However, the role of embryo derived prostaglandins in the MRP are still not completely characterized in the horse.

Our study revealed that HGF induced an increase in iTr proliferation and simultaneous PTGS2 gene expression. In conjunction to findings from Stout & Allen, 2001, results of this study suggest that HGF signaling from the endometrium participates in the embryo derived PGE and PGF2a secretion, required to suppress the production of the endometrium derived PGF2a responsible for luteolysis.

Different types of growth factors such as IGF-1 and FGF-2 are involved in signaling pathways that participate in mammalian embryonic and fetal development (Klein et al., 2010). Also, EGF from the maternal endometrial gland is responsible for originating and maintaining the growth and remodeling of feto-maternal interface tissues (Allen and Stewart, 2002).

This study identified that supplementation with growth factors like EGF, HGF, FGF2 and IGF1 to the iTr cells, stimulates their proliferation. Our findings also suggest that HGF and IGF-1 may play an important role in cell survival, signaling and embryonic growth, by increasing AKT1 phosphorylation, reinforcing previously reported findings in humans (Vincent et al., 2011). Similar findings were observed following EGF, FGF2, HGF and IGF-1 supplementation and consecutive ERK1/2 activation.

The aforementioned improve our understanding of the role of such GFs in equine trophectoderm expansion during embryonic development. Findings of some roles these GFs may play in embryonic development and prostaglandin expression during MRP may provide ways to improve *in vitro* studies of early embryonic development. These observations can be useful in designing new approaches aimed at improving successful embryo development during *in vitro* fertilization and cloning. Nonetheless, such findings are not enough to propose therapeutic measures to approach mares facing issues to become pregnant or those that lose pregnancy in the first weeks of gestation.

Co-cultures of endometrial explants with equine embryos or iTr spheres, and comparison of their protein and gene expression, could allow identification of the roles of TE in MRP and more strongly validate the use of iTr as an in vitro model to study MRP. In addition, comparing effect of iTr spheroids on CL persistency as a consequence of iTr derived MRP signal mimicking pregnancy could provide information of TE/iTr derived signaling. Moreover, transcriptomic and proteomic analysis of day 8-15 embryos compared with day 8-15 endometrium from pregnant and non-pregnant mares would provide a more complementary set of information regarding MRP, and seem the most logical step in future studies targeting characterization of the equine MRP.

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