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Dynamic Expression Patterns of PRAP1 During Murine Gestation

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Dynamic Expression Patterns of PRAP1 During Murine Gestation

By:

Taylor M. Smith, B.S., Tuskegee University, 2016

Advisor: Andrew S. Neish, M.D.

An abstract of
A thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science
in Biomedical Science
2020

Abstract

Dynamic Expression Patterns of PRAP1 During Murine Gestation

By: Taylor M. Smith

The development of Assisted Reproductive Technologies, such as in vitro fertilization (IVF), has allowed many couples struggling with fertility to conceive. However, IVF has a 75% failure rate. The leading cause of IVF failure is understood to be unsuccessful implantation. Improper or defective implantation leads to improper placentation which can affect fetal growth and development. While it is known that implantation is a complex reproductive process dependent on both competent embryos and a receptive uterus, many aspects of implantation remain unclear. Proline Rich Acidic Protein 1 (PRAP1), a 17 kDa protein, is expressed and secreted by the uterine endometrium. In the absence of PRAP1, female mice show defective implantation suggesting that PRAP1 has a role in this process. Nonetheless, PRAP1 remains largely uncharacterized. The objectives of this study are to characterize the role of PRAP1 during gestation and implantation, specifically. We hypothesize PRAP1 plays a role in implantation/placentation and overall pregnancy maintenance. Using timed pregnancies of wildtype C47BL/6J mice, uterine tissue was collected for each day of gestation and four days post-partum, then analyzed by RT-qPCR, immunofluorescence with α-PRAP1 antibodies, and H&E staining. We found that *Prap1* transcript is highly expressed during gestation compared to non-ovulating females. Additionally, PRAP1 is downregulated during the window of implantation beginning at day 3.5 and increases on day 5.5. This increase continues and peaks just prior to parturition, and PRAP1 expression decreases 1000x within 4 days post-partum. Immunofluorescence analysis demonstrated PRAP1 is localized to the endometrial epithelia during early gestation but localized specifically to the anti-mesometrial epithelia that interfaces with the amnion during mid- and late-gestation. We have also found that our PRAP1$_{-/-}$ female mice have a slightly reduced fecundability (the probability of successful gestation after mating) likely attributable to a reduced rate of implantation. We conclude PRAP1 plays a role in implantation through anti-apoptotic properties.

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Introduction

Reproductive processes and the successful transfer of genetic material are necessary for the survival of a species, yet the exact mechanisms that lead to a successful gestation and parturition (delivery) are not fully known. In mammals, each estrous (menstrual) cycle, a female ovulates, and healthy women have approximately a 30% chance of conceiving (1). According to the CDC, roughly 12% of women of reproductive age have fertility problems (2**)** This may be due to a number of contributory influences that cause fertility problems. In order for a woman to have a successful pregnancy, not only does her reproductive organs have to function properly (ovaries, fallopian tubes, uterus, cervix, etc.) but she must have proper hormonal regulation. Additionally, many non-reproductive factors contribute to a woman's fertility such as environmental exposures, stresses, and substance abuse. Since 1978, assisted reproductive technologies (ART) such as *in* vitro fertilization has allowed women to conceive, who otherwise could not (3). Roughly 2% of all live births in the US are due to ART (2). Unfortunately, ART success rates remain low. Each cycle has a 75% failure rate and most couples must undergo multiple cycles before they are successful. The major factor for failed ART is a failure for the embryo to implant. Implantation failure accounts for more than 70% of all failures (4-5). Implantation, the process by which the blastocyst attaches to and invades the endometrium (6), occurs 6-10 days after ovulation (4-5 days in mice) and requires both a competent blastocyst(s) and a receptive uterus (7-9). Enders, *et al* found implantation in rhesus monkeys consists of three steps: apposition (orienting of the blastocyst), adhesion to the endometrium, and invasion to the stroma. Many factors of this complex process

remain unknown and it is imperative to understand implantation in order to improve IVF rates.

Cycling, conception, and implantation are associated with vast and dynamic physiological and anatomical changes of the female reproductive tract (FRT) in response to steroid hormone transcriptional activation (10-11), particularly estrogens (E) and progesterone (P4), through binding to the estrogen and progesterone nuclear receptors (12-13) directly and indirectly (reviewed by 14). Estrogen is mainly produced by the ovaries and placenta, while progesterone is produced by the corpus luteum. Estrogen signaling induces ovulation (15) as well as many changes in the uterus such as angiogenesis and vasodilation. During estrus and gestation (16), estrogen induces proliferation in the endometrium to prepare for conception (17) and increases receptivity in the uterus (18). Progesterone, commonly referred to as the pregnancy hormone (19-20), inhibits estrogen induced proliferation and allows for the stromal epithelia to differentiate into specialized epithelial cells called decidual cells important for proper implantation. Progesterone receptor knockout mice have non-receptive uteri and therefore, implantation cannot occur (21). Therefore, proper levels of estrogen and progesterone are critical for successful implantation and gestation.

Many hormonally regulated proteins involved in reproductive processes are secreted by the uterus. One example is Lactoferrin, an estrogen regulated iron-binding glycoprotein (22) however, many remain uncharacterized. To better understand the mechanisms that lead to successful gestation, there is a critical need to characterize secreted proteins essential for reproduction and elucidate how these proteins are regulated. Proline-Rich Acidic Protein 1 (PRAP1), a protein of interest in our lab for its possible role in intestinal homeostasis, is also highly secreted by the uterine epithelium (23-24). Although the

function of PRAP1 in the uterus is unknown, PRAP1 has been used as a marker of implantation (25) and has been found to have anti-apoptotic properties in the gut and liver (27-28). *Prap1* is only expressed in placental mammals and is the most abundant transcript in the uterus shortly after mating (unpublished) but is constitutively expressed in the gut of both sexes. Limited data suggest *Prap1* is hormonally regulated in the uterus (25). Previous studies have found *Prap1* to be induced in the uterus of ovariectomized mice by E and P4 injection (24). Additionally, our lab has found PRAP1 to be expressed during estrus (ovulation) (unpublished). PRAP1 expression during gestation has yet to be fully described. We hypothesize that PRAP1 is a hormonally regulated protein and plays a critical role in reproduction. In this study, we aimed to define the spatiotemporal expression of uterine-derived PRAP1 and its requirement in murine gestation. By harvesting the murine uterus before, during and after pregnancy, we observed dramatic changes in gross appearance (Figure 1). Quantitative RT-PCR analysis on the uterine tissue revealed PRAP1 is a very abundant protein in the uterus and expression levels change during gestation, decreasing to undetectable levels immediately after delivery. Furthermore, PRAP1 is exclusively expressed by the anti-mesometrium during

Figure 1. Murine uterus undergoes significant physiological changes during gestation. Representative images of wildtype gravid mouse uteri depicting the physiological changes the uterus undergoes during gestation as well as fetal placement. Dpc, days post-coitus. Dpp, days post-partum

mid- and late gestation. The long-term goal is to define the role of PRAP1 during pregnancy and broaden our understanding of uterine secreted proteins required for reproduction.

Methods

Timed Pregnancy

Wildtype C57BL/6 female mice (Jackson Laboratories, Bar Harbor, ME) where housed with wildtype males. Two females ages 7-10 weeks were housed with one male of similar age and each morning were inspected for a copulatory plug. Presence of a plug was marked as 0.5 dpc (day post coitum). After plugging, females were assigned a day (1.5-23.5 dpc) to be sacrificed. On the day of sacrifice, the uterine horns were dissected, and one fetal unit was fixed in Carnoy's solution (26) for embedding. Uterine tissue was frozen for RT-qPCR.

RT-qPCR

Frozen gravid murine uterine tissue in TRIzol (Invitrogen, Carlsbad, CA) was homogenized in a Magnalyser at 6500rpm for 30 seconds. RNA was extracted using the phenol-chloroform extraction method. Isolated RNA was suspended in 50uL of diH2O at stored at -80°C. cDNA was made using the iScript Reverse Transcription Supermix (Biorad, Hercules, CA) and stored at -20°C. RT-qPCR was performed using the iQ SYBR Green Supermix on a *MyiQ*™ Real time PCR system (Biorad¸ Hercules CA). *β-Actin* as the reference gene. RT-PCR data represented as relative abundance to *β-Actin* using the delta-delta Ct method (ΔΔCT). The primer sequences for mouse *Prap1* detection were 5'-ATCTACAGCTTCGCCATTCG-3' and 5'-GTTTGCCTTTGGTCTTGACAG-3'

Immunofluorescence

Gravid murine uterine tissue was collected and fixed in Carnoy's Solution (26). Tissues were paraffin embedded and sectioned at 5 microns. Sections were rehydrated in xylene and decreasing concentrations of ethanol baths, ending with a tap water and PBS baths. Sections were then circled in wax then blocked in 5% bovine serum albumin (BSA) for 1 hour. After blocking, slides were washed in PBS and the primary rabbit-PRAP1 antibody diluted in 5% BSA was added. The primary antibody remained on the slides overnight at 4°C. After PBS washing, the secondary antibody conjugated to an Alexa Fluor was diluted 1:1000 in 5% BSA and added to the sections for at least 1 hour at room temperature. Sections were then washed again in PBS and DAPI, diluted 1:1000 in 5% BSA, was added as a DNA stain for 5 minutes. Sections were mounted with Prolong Antifade Mountant (Invitrogen, Carlsbad, CA) and stored -80°C. Images were captured on Eclipse 80i digital microscope (Nikon, Tokyo, Japan).

Long-term Reproductive Outcomes

Wildtype (f)-wildtype (m) and *Prap1*$^{-/-}$ (f) – *wildtype (m)* breeding pairs were set up and allowed to mate. *Prap1* whole body knockout mice (Strain: B6;129S5-Prap1^{tm1Lex}/Mmucd, 032532-UCD) were backcrossed with wildtype C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) in our lab (27). Breeders were monitored every day for the presence of a copulatory plug, number of pups, and general well-being. Pups were weighed everyday beginning on day 2 and developmental milestones such as detachment of ears and opening of eyes were recorded.

Results

Prap1 transcript expression during murine gestation

To understand the role of PRAP1 during gestation and immediately post-partum, gravid uterine tissue was collected for each day of gestation and three days post-partum. Diao,

et al has shown *Prap1* to be expressed post-fertilization (0.5 days post coitus, dpc) and during implantation, but drastically decreased immediately post-partum (25). Our RT-PCR data showed high *Prap1* expression for the entirety of gestation with a drastic reduction beginning at 19dpc, the day of delivery. *Prap1* expression was almost undetectable by three days postpartum, as previously shown (Figure 2). We also found that there is a decrease in expression during the implantation window, from 3.5dpc to 6.5dpc. *Prap1* expression then increases beginning 7.5dpc and changes very little mid-gestation until parturition. To summarize, Prap1 is expressed during estrus and throughout gestation, briefly decreasing during the window of implantation, and decreasing post-partum.

PRAP1 localization during gestation

Previous studies have found PRAP1 to be expressed by the uterine epithelia using in situ hybridization (26). Additionally, Zhao et al performed RNA-seq on the mesometrial and the anti-mesometrial epithelia during murine pregnancy and found PRAP1 to be specifically expressed by the anti-mesometrium (29). To confirm these findings, we used immunofluorescence staining to visualize PRAP1 in the uterus throughout gestation. As expected, immunofluorescence staining shows PRAP1 is expressed only by the anti-mesometrium (Figure 3 and 4). Interestingly, during the implantation window and early

Figure 2: *Prap1 is expressed during estrus and throughout gestation decreasing post-partum.* RT-qPCR data of Prap1 transcript during the estrous cycle, gestation, and post-partum. *Prap1* is highly expressed at estrus (ovulation), throughout gestation, decreasing during the implantation window (3.5 dpc-6.5 dpc), and decreasing again post-partum to levels comparable to a non-cycling female.

gestation, before the development and maturation of the placenta, PRAP1 is expressed by the uterine epithelia with no distinction between the mesometrium and the anti-mesometrium.

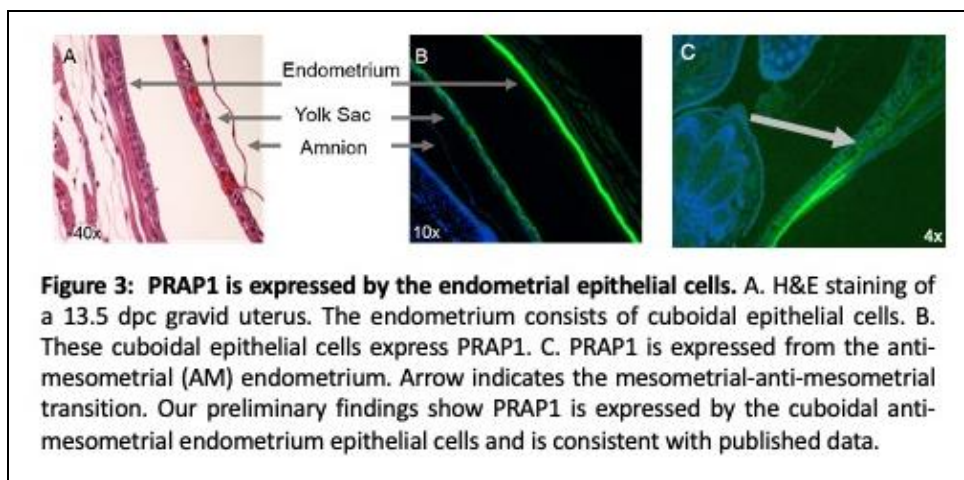

Figure 3: PRAP1 is expressed by the endometrial epithelial cells. A. H&E staining of a 13.5 dpc gravid uterus. The endometrium consists of cuboidal epithelial cells. B. These cuboidal epithelial cells express PRAP1. C. PRAP1 is expressed from the anti-mesometrial (AM) endometrium. Arrow indicates the mesometrial-anti-mesometrial transition. Our preliminary findings show PRAP1 is expressed by the cuboidal anti-mesometrial endometrium epithelial cells and is consistent with published data.

In conclusion, PRAP1 is expressed by the luminal epithelial cells and localizes to the anti-mesometrial (AM) endometrium mid-gestation.

Reproductive Outcomes of *Prap1*$^{-/-}$ females

Although previous studies have implicated PRAP1 during implantation (25), there is currently no reproductive phenotype associated with *Prap1*$^{-/-}$ females. We sought to investigate any possible phenotypes associated with *Prap1*$^{-/-}$ females. We found litters of *Prap1*$^{-/-}$ females are comparable in size and meet developmental milestones at the proper time (ear detachment day 5, eye opening day 14). Interestingly, we found *Prap1*$^{-/-}$ females trend towards having a reduced fecundability, the probability of successful gestation after mating (Table 1). Copulatory plugs from *Prap1*$^{-/-}$ females led to a successful gestation 54% of the time compared to 70% in wildtype mating pairs (OR 1.67). This difference is not statistically significant which may be attributed to low power.

Table 1: Pregnancy Outcomes

	Pregnant	Odds Ratio	p-value
Wildtype females	7/10 (70%)	1.67	0.57
Prap1$^{-/-}$ females	7/13 (53.8%)	0.6	0.57

Discussion

Our lab has previously reported PRAP1 to be an intrinsically disorder protein (IDP),

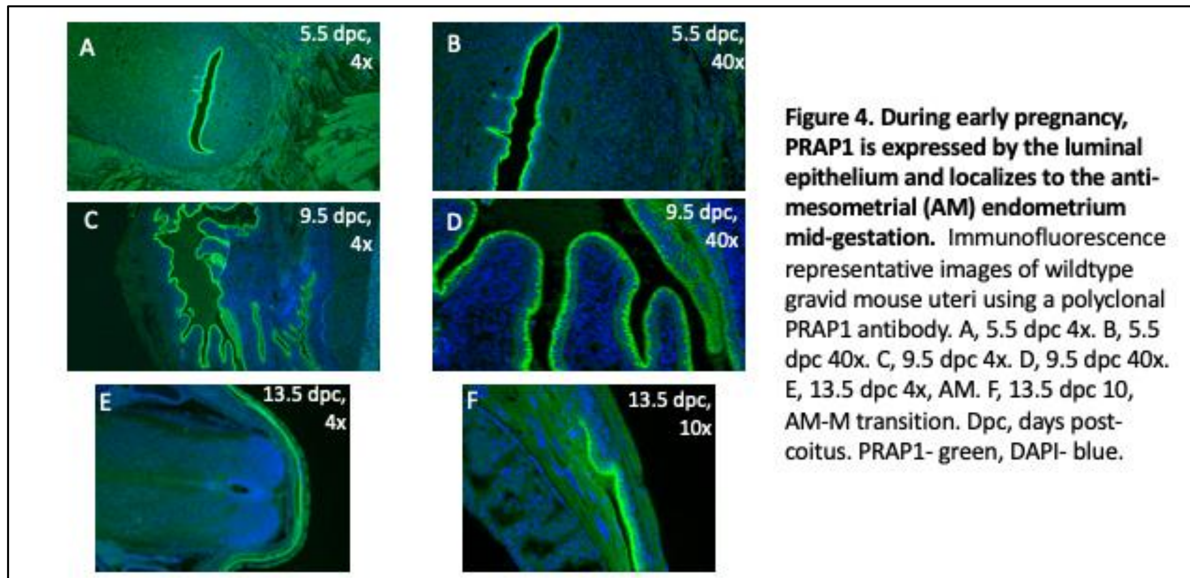

Figure 4. During early pregnancy, PRAP1 is expressed by the luminal epithelium and localizes to the anti-mesometrial (AM) endometrium mid-gestation. Immunofluorescence representative images of wildtype gravid mouse uteri using a polyclonal PRAP1 antibody. A, 5.5 dpc 4x. B, 5.5 dpc 40x. C, 9.5 dpc 4x. D, 9.5 dpc 40x. E, 13.5 dpc 4x, AM. F, 13.5 dpc 10, AM-M transition. Dpc, days post-coitus. PRAP1- green, DAPI- blue.

without any discernable secondary structure (27). Many IDP's remain uncharacterized due to a lack of 3D globular structure, although they may acquire 3D structure upon target binding (30-31). IDPs that have been more fully characterized, such as p21, have been assigned various functions that include transcription, translation regulation and regulation of multiprotein complex assembly (32).

Work by others suggests PRAP1 has a role in implantation. Xiong, et al found when female mice are injected with $\propto$-PRAP1 antibodies, they have implantation defects (33). Uterine fluid is produced in response to mating (34) and plays a critical role in blastocyst attachment to the uterine wall during implantation (35). Alterations in the uterine fluid consistency or contents can cause defective implantation (36). Uterine specific extracellular vesicles (EV) or uterosomes found in uterine fluid contain macromolecules including proteins important for reproductive processes such as implantation. (37-38).

PRAP1 is detected by western blot in uterine fluid (data not published) and it is possible PRAP1 is located within these EVs.

It is also possible PRAP1 has an antimicrobial function. In 1900, Henry Tissier coined the term “sterile womb” (39) and, since then, it has been widely assumed that the uterus is a sterile environment. It is well known that the uterus secretes antimicrobial peptides and these effectors likely maintain the sterility of the uterus or keep the abundance of bacteria very low (40). During the receptive phase of the estrous cycle of mammals (comparable to the proliferative phase of the menstrual cycle of a woman) when ovulation occurs, the cervix is more open which allows not only spermatozoa, but microbes to pass through easily as well. The cervix, characterized by an abundance of endocervical glands comprised of highly secreting epithelial cells, produces large amounts of mucins rich in anti-microbial substances (41). We have shown that shortly after mating, bacteria can be detected in the murine uterus in addition to the expected spermatozoa (data not shown). PRAP1 is highly expressed during this time and may be assisting in the clearance of microbes.

Finally, PRAP1 could function to protect the epithelium from oxidative stress. PRAP1 expression was found to be induced in the colon four hours after an oral gavage of *Lactobacillus rhamnosus* GG (LGG), a probiotic bacterial species, in a screen for cytoprotective genes (42). Recently published data from our lab shows that PRAP1 expression promotes intestinal cell survival after cytotoxic stress (27). When mice are challenged with a lethal dose of irradiation, wildtype mice have reduced apoptosis in the gut and survive longer compared to *Prap1*-/- mice. Furthermore, when exposed to irradiation, *Prap1*-/- enteroids underwent significantly more apoptosis. There was no

significant difference in proliferating cells between wildtype and *Prap1*$^{-/-}$ mice**.** Wolfarth, et al also found p21, a cell cycle regulator, to be increased following irradiation in *Prap1*$^{-/-}$ cells (27) concluding the PRAP1 influences p21 expression. Studies show increased p21 expression correlates with increased apoptosis. Huang, et al also reported PRAP1 may protect cells from apoptosis upon p53 induction (28). Taken together, PRAP1's reported cytoprotective function may explain the implantation phenotype found in *Prap1*$^{-/-}$ mice. Leading up to and during implantation, the uterus undergoes remodeling, the stromal epithelia are proliferating and differentiating into the highly specialized decidua and angiogenesis occurs. During the implantation window, PRAP1 may be suppressing excessive apoptosis and the *Prap1*$^{-/-}$ mice may have increased apoptosis not conducive to successful implantation.

Future Directions

What we hoped to determine is at what day PRAP1 localizes to the anti-mesometrial (AM) epithelia and if that coincides with the maturation of the placenta. Due to the covid-19 pandemic, I was unable to image early gestation uterine tissue. Using CLARITY (43), a whole tissue immunofluorescence staining technique, early gestational uterine tissue samples were collected from wildtype mice, stained, and fixed and some preliminary imaging was done prior to the ramp down of research. In the mouse, the blastocyst attaches to the AM side of the endometrium and the placenta develops on the mesometrial side (44). This orientation is not seen in humans and factors responsible for orienting the blastocyst are unclear. The placenta is the link between the developing fetus and the mother. It provides nutrients to the unborn offspring, removes waste, and offers protection from most bacteria. Srivastava, *et* al found the anti-mesometrial region has endocrine properties and secretes various proteins such as growth factors (45), but many

characteristics of this region remains unknown. Also, though our polyclonal PRAP1 antibody has been extensively validated, prior to covid-19, I set up heterozygous mating pairs in order to obtain null mice to use as negative controls for my IF staining.

The next step in this study is to investigate the possible function of PRAP1 in the female mammalian reproductive tract. Data from the intestinal epithelium suggests PRAP1 has a role in cytoprotection and epithelial survival. PRAP1 could have similar function in the uterine epithelium. I also hypothesize PRAP1 functions in the regulation of proliferation and/or apoptosis leading to the *Prap1*-/- females being less likely to conceive. To test this hypothesis, Ki67 and caspase 3 staining could be compared between wildtype and *Prap1*-/- females during the implantation window under normal conditions or under a challenge such as irradiation similar to Wolfarth, *et al (27)*. Further characterizing the localization and function of PRAP1 in the uterus during implantation and gestation will further our understanding of these critical processes and improve our capacity to advance current fertility therapies.

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