THESIS

TRANSPOSABLE ELEMENTS AND EARLY PREGNANCY IN THE HORSE

Submitted by
Sierra L. Southerland
Department of Animal Sciences

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Master's Committee:

Advisor: Stephen Coleman

Jason Bruemmer
Carleigh Fedorka
Pablo Pinedo
Adam Chicco

ABSTRACT

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Understanding the molecular events and physiological dynamics in the endometrium during early pregnancy is crucial for improving our understanding of reproductive outcomes in horses. Much of the work investigating early pregnancy in the horse has focused on maternal recognition of pregnancy (MRP). This critical signal, which initiates the pathways and regulatory changes to help make the endometrium receptive to pregnancy, remains elusive in the horse. Transposable elements (TEs), once overlooked components of the genome, have been implicated in regulating gene expression during critical stages of embryo and placental development. The current study investigated the expression of TEs during the early phase of pregnancy post-ovulation. The hypothesis tested was that pregnancy status would impact the expression patterns of TE-derived transcripts in the endometrium around the time of maternal recognition. It was anticipated that the TE transcripts would increase in the endometrium of pregnant mares either in response to or as a part of the maternal recognition signal. RNA sequencing data was generated from endometrial biopsies at days 9, 11, and 13 in pregnant and non-pregnant mares. Bioinformatics analyses identified distinct patterns of TE expression across the duration of early pregnancy. Contrary to expectations, these findings did not reveal the accumulation of TE transcripts in the pregnant endometrium compared to the non-pregnant endometrium during early pregnancy. Instead, there was a significant decrease in the number of differentially expressed transcripts over the time points studied. These results challenge the initial assumption and suggest that the regulation of TE transcriptional activity during early pregnancy may be more

complex than previously thought, highlighting the need for further research into the roles of TEs in equine reproductive physiology. An alternative explanation for the results of this analysis is that maternal recognition of pregnancy may involve the downregulation of transcripts expressed from TEs, potentially serving as a switch to prevent luteolysis and maintain pregnancy. This study underscores TEs as potential biomarkers and regulators in equine reproduction, providing insights into endometrial processes and offering avenues for improving fertility outcomes and breeding efficiency in horses.

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CHAPTER 1

REVIEW OF LITERATURE

Summary

This review delves into the intricate relationship between transposable elements and pregnancy physiology, shedding light on the pivotal role of these repetitive elements in the genomic landscape and their influence on early pregnancy processes. By exploring the significance of TEs in genome evolution, organization, and function, alongside their specific involvement in pregnancy-related mechanisms, this review aims to elucidate the complex interplay between genetic elements and reproductive biology. Through an examination of current research, the review seeks to provide insights into the molecular underpinnings of early pregnancy, highlighting the potential diagnostic and therapeutic implications of TE dysregulation in pregnancy disorders. By synthesizing existing knowledge and identifying future research directions, this review endeavors to advance our understanding of TE biology in the context of pregnancy, offering new avenues for exploration in reproductive health and fertility studies.

Introduction

Understanding the dynamic interplay between genomic elements and pregnancy physiology is crucial for understanding the physiology of reproduction. At the heart of this intricate relationship lie transposable elements (TEs), once dismissed as “junk DNA’ but now recognized as pivotal players in genome evolution and function. Comprising a significant portion, approximately half, of the mammalian genome, TEs and other repetitive elements (REs),

like tandem and interspersed repeats, exert profound effects on genomic organization and regulation [Medstrand et al., 2005; Buckley and Adelson, 2014].

During pregnancy, the role of REs, particularly TEs, gains prominence due to their potential involvement in pregnancy-related processes, such as embryonic development, endometrial vascularization, and maternal-fetal communication [Keighley et al., 2023; Friedli and Trono, 2015; Senft and Macfarlan, 2021]. In early pregnancy, a critical phase marked by complex and cellular events, understanding the dynamics of TEs becomes paramount for deciphering the mechanism underlying successful reproduction. This is particularly evident in the mare, where early pregnancy entails intricate interactions between maternal tissues and the developing embryo [Ginther, 1998; Swegen, 2021].

Despite the recognized importance of TEs in genomic regulation and pregnancy physiology, gaps persist in our understanding of their specific roles in early pregnancy. Clarifying these roles is essential for elucidating the underlying mechanisms and potential implications for reproductive health. Hence, this review provides an overview of the involvement of TEs and, by extension, REs in early pregnancy.

This review begins by stating the significance of TEs in genome evolution and function, establishing their relevance to pregnancy biology. It then delves into the definition and importance of TEs in genomes, emphasizing their role in pregnancy-related processes. Subsequently, the review explores the specific early pregnancy stages in the mare, highlighting the physiological and anatomical aspects relevant to TE activity.

Throughout the review, a synthesis of existing knowledge and recent findings sheds light on the intricate interplay between genomic regulation and reproductive biology during early

pregnancy. By delving into existing knowledge and highlighting recent findings, this review provides insights into the potential implications of TE dynamics in early pregnancy physiology and pathology.

Early Pregnancy Physiology and Anatomy in the Mare

Successful pregnancy establishment in the mare involves a series of finely coordinated events following ovulation and fertilization. Understanding the physiological changes that occur during the early phase of pregnancy physiology and interactions with the mare's anatomy is crucial for elucidating the molecular mechanisms underlying conceptus development and maternal-fetal interactions. This section provides a detailed overview of the key stages and physiological changes occurring during early pregnancy in mares.

In mares, the estrous cycle is a recurring reproductive cycle characterized by distinct phases, including proestrus, estrus, diestrus, and anestrus. Proestrus marks the transition from sexual quiescence to sexual receptivity, driven by increasing estrogen levels. Estrus, commonly known as “heat,” is the period of sexual receptivity and ovulation. During this stage, maximal estrogen secretion from ovarian follicles promotes behavioral signs such as frequent urination, tail raising, and lordosis [Vandeplassche et al., 1981; Pryor and Tibary, 2005; Crowell-Davis, 2007; Satue et al., 2013; Raz and Aharonson-Raz, 2012]. Ovulation marks the cessation of estrous, triggered by a surge in luteinizing hormone (LH) secretion from the anterior pituitary gland [Ginther et al., 2007; Alexander and Irvine, 1987]. Essential details and events associated with early pregnancy in the mare are discussed in the following sections.

Ovulation and Fertilization

The ovulation process involves the rupture of the mature preovulatory follicle, releasing the oocyte into the oviduct [Hamilton and Day, 1945; Aurich, 2011]. Before ovulation, the dominant follicle undergoes rapid growth and maturation under the influence of follicle-stimulating hormone (FSH), inhibin, and LH. Following ovulation, the released oocyte enters the oviduct, which undergoes final maturation processes to become competent for fertilization [Leemans et al., 2016; Hunter, 1991]. Following ovulation, spermatozoa within the oviduct are stimulated to undergo capacitation, rendering them capable of fertilizing the oocyte. Capacitated spermatozoa migrate through the reproductive tract toward the site of fertilization in the oviduct. Fertilization occurs within the ampullary region of the oviduct, where the oocyte and capacitated spermatozoa meet. The fusion of spermatozoon and oocyte membranes results in the formation of the zygote, marking the initiation of early embryonic development.

Conceptus Development and Mobility

Following fertilization, the zygote, a diploid cell resulting from the fusion of two haploid gametes, undergoes a series of developmental stages within the mare's reproductive tract. Around day five post-ovulation, the zygote develops into a multicellular embryo called the morula. By day six post-ovulation, the morula progresses into a blastocyst as it enters the uterus, characterized by an outer trophoblast layer and an inner cell mass (ICM) [Betteridge et al., 1982]. Unlike the embryos of other species, equine embryos do not undergo elongation within the uterus due to a glycoprotein capsule surrounding the blastocyst, which maintains embryo mobility until the later stages of pregnancy [Betteridge, 1989].

The embryo, propelled by uterine contractions, reaches peak mobility around day 12 post-ovulation, allowing for free movement and distribution within the uterine horns and body [Betteridge et al., 1982; Oriol et al., 1993; Ginther, 1983]. By approximately day 16 post-ovulation, fixation occurs, marking the cessation of embryo mobility as the embryo becomes lodged in the caudal uterine horn. Fixation is facilitated by factors such as the diameter, longitudinal folds, and size of the endometrial crypts, influenced by the increased contact between the embryo and the endometrial epithelium during mobility [Ginther, 1983; Aurich and Budik, 2015].

Maternal recognition of pregnancy (MRP), a critical event in early pregnancy establishment, must occur to ensure the maintenance of the CL and progesterone secretion to support embryonic development [Maria and Stout, 2007]. In mares, MRP research has focused on lipid-based signals [Vanderwall et al., 1994; Wilsher and Allen, 2011; Diel de Amorium et al., 2016], protein-based interactions [Smits, 2018], focal adhesions [Klohonatz et al., 2016, Klohonatz et al., 2019a], as well as the role of embryo contact with the endometrium through mechanical and chemical signals [Klohonatz et al., 2019; McDowell et al., 1988; Wilsher et al., 2010; Sharp et al., 1984; Boerboom et al., 2004; Ealy et al., 2010]. Recent studies have also focused on investigating gene expression changes in the endometrium, focusing on both protein-coding [Klohonatz et al., 2019b; Gebhardt et al., 2012; Klein et al., 2010; Klein and Troedsson, 2011; Klein, 2015] and non-coding [Klohonatz et al., 2019c] expression. Studies have also found various signaling molecules that mediate MRP across different species, such as human chorionic gonadotropin (hCG) in humans, interferon-tau (INFT) in ruminants [Ross, 1979; Fishel et al., 1984; Moor and Rowson, 1988; Betteridge et al., 1980; Northey and French, 1980], and estradiol-17b in pigs.

Endocrinology of Early Pregnancy

Progesterone secreted by the corpus luteum (CL), a temporary endocrine structure remaining in the ovary from the ovulated follicle, plays a central role in maintaining the receptive state of the endometrium and preventing luteolysis [McDowell et al., 1988]. Progesterone levels rise following ovulation. The surge of progesterone promotes endometrial gland development and secretion factors essential for embryonic development [Silva et al., 2019].

One of the primary roles of progesterone is to sustain a receptive state, ensuring optimal conditions for embryo development, fixation, and even implantation later in pregnancy. This is achieved by stimulating the endometrial glands to produce and secrete various proteins, glycoproteins, and other molecules. These secretions provide essential nutrients, growth factors, and immunomodulatory substances necessary for embryo nourishment, growth, and immune tolerance [Jones et al., 2020; Hayes et al., 2018; Stewart et al., 1995]. Progesterone also enhances endometrial receptivity by orchestrating biochemical changes within endometrial cells, including alterations in gene expression and protein synthesis [Wong et al, 2023; De Castro et al., 2024]. These changes help facilitate attachment and subsequent fixation and implantation. Progesterone exerts immunomodulatory effects within the endometrium, suppressing inflammatory responses and promoting immune tolerance towards the semi-allogeneic embryo, allowing for its survival and development [Antczak, 2020; Figarska et al., 2022]. One of the most important roles of progesterone is to inhibit the contractility of the uterine smooth muscle by modulating the activity of ion channels and neurotransmitters involved in muscle contractions [Hirsbrunner et al., 2006; Gastal et al., 1998]. The suppression of uterine contractions creates a stable and

nurturing environment that is conducive to embryo development and prevents the premature expulsion of the embryo from the uterus [Kastelic et al., 1987].

Simultaneously, estrogen, predominantly secreted by the developing conceptus, further modulates endometrial receptivity during early pregnancy. Increasing estrogen levels further enhances endometrial gland secretion and promotes uterine vascularization, both essential for providing vital nourishment and support to the developing embryo in its early stages [Allen, 2000; Klein, 2016; Pinto, 2020]. Estrogen achieves this by instigating the proliferation and expansion of endometrial glands, thereby enhancing their capacity to generate imperative secretions crucial for embryo nourishment [Wilsher, 2019; Pinto, 2020]. Additionally, estrogen fosters the differentiation of endometrial glandular cells, bolstering their biochemical machinery to facilitate secretions of substances such as mucus, proteins, and glycoproteins indispensable for nurturing the embryo's microenvironment [Geisert and Malayer, 2013]. Estrogen orchestrates the augmentation of uterine vascularization by promoting the formation of new blood vessels within the endometrium, thereby increasing blood flow and supply [Haneda et al., 2021; Ferreira-Dias and Serrao, 2001; Silva et al., 2005]. By initiating these processes, estrogen primes the endometrium for embryo fixation and facilitates vascular remodeling, thus preparing the uterine environment for successful pregnancy establishment.

The balance between progesterone and estrogen signaling is crucial for maintaining embryo receptivity and preventing luteolysis during early pregnancy [Aurich and Budik, 2015; Knowles et al., 1993; Shikichi et al., 2017]. As long as the corpus luteum continues to secrete progesterone, progesterone production will support embryonic development. With suppression of luteolysis and the prolongation of CL function, maternal recognition can occur. [Maria and Stout, 2007; Klein and Troedsson, 2011; Newcombe et al., 2023].

Endometrial Changes during Early Pregnancy

The endometrium undergoes dynamic changes, such as vascularization, during early pregnancy to facilitate embryo development and MRP [Klein, 2016; Allen, 2000; Griffin and Ginther, 1991; Hayes et al., 2018]. Hormonal cues and molecular interactions between the embryo and the maternal endometrium cause these modifications. These uterine adaptations are crucial to ensure endometrial receptivity [Stout, 2020; Schjenken and Robertson, 2014].

Repetitive Elements: Structure and Classification

Repetitive elements, sequence features characterized by repeated DNA motifs, constitute a significant portion, about half, of the mammalian genome [de Koning et al., 2011; Jurka et al., 2007; Shapiro and von Sternberg, 2005]. These repetitive elements are characterized as either tandem repeats or interspersed repeats. The family and classes of repetitive elements can be seen in Figure 1.1.

Tandem Repeats

Tandem Repeats, also known as microsatellites or short tandem repeats (STRs), are intricate patterns of DNA sequences recurring in tandem arrays [Gymrek, 2017; Rao et al., 2010]. These repeats, where the same sequence motif is repeated consecutively multiple times, typically range from one to six base pairs in length and play essential roles in genomic stability [Weber and Wong, 1993]. Tandem; Fungtammasan et al., 2015]. Repeats can be found throughout the genome, occurring both within coding and non-coding regions, and are known for their high degree of polymorphism, or variation, within populations [Tomilin, 2008; Zhou et al.,

2022]. The structure of tandem repeats allows for variation in the number of repeating units, resulting in allelic diversity among individuals. This polymorphic nature makes tandem repeats valuable genetic markers for various applications, including forensic identification, genetic linkage analysis, and population genetics studies [Madhumati, 2014; Buowle et al., 1990].

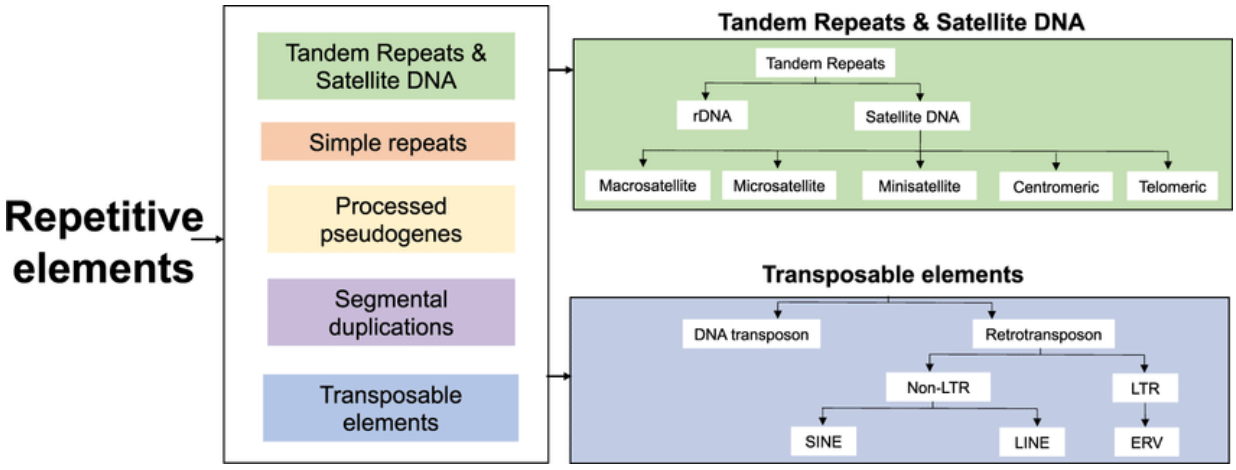

Figure 1.1 - Repetitive Element Classes in the Mammalian Genome. Adapted from Billingsley et al., 2019.

The centromere and telomere structures of chromosomes are composed of tandem repeats that influence their function and maintenance. Centromeres are crucial structures of chromosomes and are responsible for ensuring accurate chromosome segregation during cell division [Verdaasdonk and Bloom, 2011]. Tandem repeats, particularly those within the centromeric region, help establish and maintain centromere function. These repetitive sequences provide a platform for the assembly of protein complexes, known as kinetochores, which attach to microtubules and facilitate the movement of chromosomes during cell division [Fukagawa,

2004]. The repetitive nature of tandem repeats in centromeres allows for the formation of specialized chromatin structures necessary for proper chromosomal segregation.

Telomeres at the ends of linear chromosomes protect chromosome ends from degradation and prevent fusion with neighboring chromosomes [Grandin and Charbonneau, 2008, O'sullivan and Karlseder, 2010]. Tandem repeats, specifically the TTAGGG sequence in vertebrates, comprise the bulk of telomeric DNA. These repeats are binding sites for telomere-binding protein, which helps maintain telomere length and integrity. Telomerase, an enzyme complex, adds repetitive TTAGGG sequences to telomeres, counteracting the gradual shortening that occurs with each round of cell division [Bolzán, 2017]. Thus, tandem repeats in telomeres play a crucial role in preserving genome integrity by ensuring the stability and functionality of chromosome ends.

Tandem repeats' adaptability, highlighted by dynamic changes in repeat lengths, contributes to genetic variability, allowing organisms to navigate evolutionary challenges. However, this variability comes with a caveat, as abnormal expansions of specific tandem repeats are associated with genetic disorders such as Huntington's disease, Fragile X syndrome, and myotonic dystrophy [Sutherland and Richards, 1995; Paulson, 2018; Siwach and Ganesh, 2008]. This shows the intricate balance between these repetitive elements' adaptability and genomic stability.

Interspersed Repeats

Interspersed repeats, predominantly comprising transposable elements (TEs), intricately weave throughout the genome, substantially influencing its size and complexity [Smit, 1996]. These elements actively contribute to shaping genomic structure. TEs are hotspots for

chromosomal rearrangements, mutations, and crossing over during recombination [Kidwell, 2005; Kent et al., 2017; Underwood and Choi, 2019]. These genetic alterations can be implicated in developing diseases that propel evolutionary changes.

TEs can undergo amplification within the genome through processes such as transposition and retrotransposition. This amplification can expand certain genomic regions, resulting in increased genome size [Zhou et al., 2020]. Conversely, the deletion or inactivation of TEs can lead to genomic contraction, influencing genome size dynamics [Gregory, 2004]. The cumulative effect of TE amplification and deletion events can shape the overall structure and size of the genome.

TEs are hotspots for chromosomal rearrangements, including insertions, deletions, inversions, and translocations. These rearrangements can alter the order and orientation of genomic segments, leading to structural variation within chromosomes [Kidwell, 2005]. For example, inserting a TE into a coding region can disrupt gene functions. At the same time, the deletion of a TE can result in the loss of genetic material [Bennetzen and Wang, 2014]. Chromosomal rearrangements mediated by TEs contribute to genomic diversity and can drive evolutionary changes.

TEs can induce genomic instability through various mechanisms, including ectopic recombination, DNA double-strand breaks, and insertional mutagenesis [Hedges and Deininger, 2007]. Ectopic recombination between repetitive sequences can result in chromosomal rearrangements, such as duplications or deletions. Additionally, TEs can cause DNA double-strand breaks during their mobilization, leading to genomic instability. Insertional mutagenesis, where TEs are inserted into coding or regulatory regions of genes, can disrupt gene function and contribute to the development of genetic disorders.

TEs can also serve as sources of regulatory elements that influence gene expression and genomic function. For example, TEs may contain enhancers, promoters, or transcription factor binding sites that modulate the expression of nearby genes. The insertion of TEs near genes can alter their expression patterns, leading to changes in cellular processes and phenotypic traits [Fueyo et al., 2022]. Additionally, TEs may contribute to the evolution of gene regulatory networks by providing raw material for developing novel regulatory elements.

Interspersed repeats play a multifaceted role in shaping genomic structure. The ability to undergo amplification, induce chromosomal rearrangements, cause genomic instability, and contribute to regulatory element evolution profoundly influences genome architecture and function. The structure, classification, and role in genome dynamics of interspersed repeats are essential for comprehensively understanding the impact on genome evolution and function.

Classification of Transposable Elements

TEs can be broadly classified into two main classes based on their transposition mechanisms: Class I TEs, known as retrotransposons, and Class II TEs, known as DNA transposons.

Class I TEs

Class I TEs replicate via an RNA intermediate and utilize a “copy-and-paste” mechanism for transposition [Wag and Kunze, 2015; Wicker et al., 2007]. Retrotransposons are divided into two main subclasses based on the presence or absence of long terminal repeats (LTRs). These subclasses are LTR and non-LTR retrotransposons [Wicker et al., 2007].

LTR Retrotransposons

LTR retrotransposons are characterized by the presence of LTRs at their ends, which contain sequences necessary for transcriptional regulation and reverse transcription [Galindo-Gonzalez et al., 2017; McCarthy et al., 2002]. LTR retrotransposons replicate via an RNA intermediate, which is reverse transcribed into DNA by the enzyme reverse transcriptase [Wilhelm and Wilhelm, 2001]. The resulting DNA copy is then integrated into a new genomic location, often generating target site duplications upon insertion. Endogenous retroviruses (ERVs) are a well-known group of LTR retrotransposons derived from ancient retroviral infections and have played significant roles in shaping genomic structure and function [Grandi and Tramontano, 2018].

ERVs constitute a substantial portion of the mammalian genome, ranging approximately 8-10%, with some species harboring thousands of ERV copies [Cordaux and Baxter, 2009]. Integrating ERVs into the host genome can lead to genomic expansion, contributing to the overall size and complexity of the genome. ERVs at specific genomic loci can also influence chromosomal structure and organization, affecting genetic density and distribution [Jern and Coffin, 2008].

ERVs contain regulatory elements, such as promoters and enhancers, with LTRs. These regulatory elements can influence the expression of nearby genes by acting as alternative promoters and enhancers [Fu et al., 2019; Enriquez-Gasca et al., 2020]. ERV-derived regulatory sequences may modulate the transcriptional activity of adjacent genes, leading to changes in gene expression patterns and cellular phenotypes. Additionally, ERVs can provide binding sites for transcription factors, further impacting gene regulation and cellular processes [Geis and Goff, 2020].

ERVs contribute to genomic innovation by serving as a source of genetic material for the evolution of novel genes and regulatory elements [Popov et al., 2018]. Integrating ERVs into the genome can introduce new coding sequences or regulatory motifs, which may undergo subsequent evolutionary changes and adaptations. ERV-derived sequences have been co-opted for various biological functions, including placental development, immune response, and antiviral defense mechanisms [Frank and Feschotte, 2017; Buttler and Chuong, 2022].

Non-LTR Retrotransposons

Non-LTR retrotransposons lack LTRs and are further classified based on their length into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). LINEs are autonomous retrotransposons capable of transposing independently, while SINEs are non-autonomous elements that rely on the enzymatic machinery provided by LINEs for their mobility. LINEs and SINEs contribute significantly to genomic diversity and evolution and constitute a substantial portion of mammalian genomes [Böhne et al., 2008]. LINEs and SINEs contribute to genomic evolution by inserting themselves into new genomic locations, creating genetic diversity and structural variation [Warren et al., 2015; Jurka et al., 2007]. They can influence gene regulation by altering gene expression patterns and can drive functional innovation by providing new regulatory elements or coding sequences.

LINEs and SINEs undergo amplification within the genome through retrotransposition. This results in the generation of additional copies of these elements, contributing to increased repetitive DNA content and overall genome size. This genomic expansion can lead to the

duplication of coding and non-coding sequences, potentially generating raw material for evolutionary innovation [Chuong et al., 2017].

Non-LTR retrotransposons contain regulatory elements, such as promoters and enhancers, within their sequences. These regulatory elements can influence the expression of nearby genes by acting as alternative promoters and enhancers [Galindo-Gonzalez et al., 2017]. The insertion of LINEs and SINEs near genes can modulate expression patterns, leading to gene regulation and cellular phenotype changes.

LINEs and SINEs play diverse and dynamic roles in genomic evolution. They contribute to genomic diversity, structural variation, gene regulation, and functional innovation. The widespread distribution and abundance of these elements in mammalian genomes underscore the significance of shaping the genome across species.

Class II TEs

Class II TEs transpose directly as DNA molecules employ a “cut-and-paste” mechanism for transposition, mediated by a transposase enzyme [Kidwell, 2005]. DNA transposons are characterized by terminal inverted repeats (TIRs) flanking the transposon sequence, which are recognized by the transposase enzyme [Muñoz-López and García-Pérez, 2010]. The transposase catalyzes the excision of the transposon from its original genomic location and its integration into a new site in the genome, often generating target site duplications (TSDs) upon insertion. DNA transposons are diverse and include families such as hAT (hobo, Activator, and Tam3) transposons and Mariner elements, which have played significant roles in genome evolution and adaptation across diverse organisms [Robertson, 2007; Muñoz-López and García-Pérez, 2010]. They have contributed to genome evolution by facilitating horizontal gene transfer and

promoting genetic diversity. For instance, they have been implicated in the development of insecticide resistance in insects and the evolution of new genes and regulatory networks in plants [Rostant et al., 2012; Qui and Köhler, 2020].

DNA transposons have the potential to mediate chromosomal rearrangements that contribute to genomic diversity and evolution. Through recombination, DNA transposons can induce structural variations in the genome [Fambrini et al., 2020; Krasileva, 2019]. Unequal crossing over between DNA transposon copies located on different chromosomes or at distant genomic loci can lead to chromosomal duplications, deletions, inversions, or translocations. These rearrangements alter the organization of the genome, influencing gene distribution, regulatory elements, and genome stability [Federoff, 2012].

The transposition activity of DNA transposons plays a significant role in genome evolution by contributing to genomic plasticity and structural variations [Pimpinelli and Piacentini, 2020; Schrader and Schmitz, 2019]. The insertion of DNA transposons into new genomic locations generates genetic diversity and can lead to the emergence of novel genomic architectures. These structural variations influence gene distribution, regulatory elements, and genome stability, shaping the evolution of organisms [Feschotte and Pritham, 2007].

Host organisms have evolved mechanisms to regulate the activity of DNA transposons to maintain genome stability and integrity. Transcriptional and post-transcriptional regulatory mechanisms, including DNA methylation, histone modifications, and small RNA-mediated silencing pathways, control the expression and mobility of DNA transposons within the genome. These regulatory mechanisms ensure that DNA transposons are tightly regulated to prevent genomic instability and disruptions [Slotkin and Martienssen, 2007].

Examples of Transposable Elements in the Horse Genome

Like many mammalian genomes, the equine genome harbors a diverse array of TEs that significantly contribute to its structure and function. These elements exhibit characteristics in terms of abundance, distribution, and impact on genomic dynamics. In horses, the major families of TEs encompass a spectrum of repetitive sequences, including Equine SINEs (EqSINEs), Equine LINEs (EqLINEs), and Equine endogenous retroviruses (ERVs) [Batmagnai et al., 2018; Enkhbaatar, 2012]. These families showcase a remarkable diversity in structural features and insertion patterns within the genome. Notably, Equus caballus clade-specific LINE 1 (L1) repetitive sequences have undergone recent rapid expansion, which is evidenced by an increased number of insertions across the genome [Zhao et al., 2023]. This expansion highlights their dynamic nature, as they actively contribute to genomic variability and evolution by creating new insertion sites that can affect gene function and regulation. Additionally, ERVs constitute a significant portion of TE insertions in coding sequences, with implications for gene regulation and disease susceptibility [Fueyo et al., 2022].

Distribution and Abundance of TEs in the Horse Genome

Transposable elements pervade the horse genome, contributing substantially to its repetitive DNA content. Through high-throughput sequencing and computational analyses, researchers have elucidated the distribution patterns and abundance of TEs across equine chromosomes [Liu et al., 2023; Khalkhali-Evrigh et al., 2019]. Certain TE families exhibit preferential insertion into specific chromosomal locations, reflecting underlying genomic dynamics and evolutionary processes [Ivancevic et al., 2013]. The equine genome features

diverse hybrid repetitive sequences alongside typical eutherian mammal repeats, underscoring the interplay between ancient and lineage-specific TE elements [Ivancevic et al., 2013].

Regulation of Transposable Elements

TEs are tightly regulated within genomes to maintain genomic stability and integrity. At the transcriptional level, TEs are subject to intricate regulatory mechanisms that govern their expression dynamics. Several key factors and processes, like epigenetic modifications, transcription factor binding, RNA-mediated silencing pathways, and post-transcriptional regulatory mechanisms, contribute to TE transcriptional control, ensuring tight regulation across diverse cellular contexts [Slotkin and Martienssen, 2007].

Mechanisms of Transposable Element Transcriptional Control

TE Transcriptional regulation encompasses a repertoire of mechanisms aimed at suppressing or activating TE expression in a spatially and temporally controlled manner. One prominent mechanism involved epigenetic modifications, such as DNA methylation and histone modifications, which regulate TE accessibility to the transcriptional machinery [Slotkin and Matienssen, 2007]. DNA methylation, mediated by DNA methyltransferases, is a potent silencing mechanism that inhibits TE promoter activity and recruits repressive chromatin remodeling complexes [Roberson, 2019]. Histone modifications, including methylation, acetylation, and phosphorylation, modulate chromatin structure and accessibility, thereby influencing TE transcriptional activity.

Transcription factors and RNA-binding proteins play crucial roles in TE expression. Transcription factors bind TE regulatory elements, such as promoters and enhancers, to either activate or repress TE transcription. Conversely, RNA-binding proteins regulate TE expression post-transcriptionally by modulating TE mRNA stability, splicing, and translation efficiency [Sassone-Corsi and Borrelli, 1986; Hermant et al., 2020; Fueyo et al., 2022].

Small RNA-mediated silencing pathways, including RNA interference (RNAi) and the PIWI-interacting RNA (piRNA) pathway, exert robust control over TE expression [Siomi et al., 2011; Gebert and Rosenkranz, 2015]. These pathways involve the generation of small non-coding RNAs that target complementary TE sequences for degradation or translational inhibition. This effectively silences TE activity at the post-translational level.

Factors Influencing TE Expression

TE expression is influenced by a myriad of intrinsic and extrinsic factors, reflecting the dynamic interplay between TE sequences and the cellular environment. Intrinsic factors, like TE sequence composition, genomic location, and epigenetic status, dictate TE transcriptional activity within specific genomic contexts. For instance, TE sequences harboring active promoters or enhancers are more prone to transcriptional activation, whereas TE sequences embedded within heterochromatic regions are often transcriptionally silenced [Ali et al., 2021].

Extrinsic factors, including developmental stage, environmental cues, and cellular stress responses, modulate TE expression in a context-dependent manner. Developmental transitions and cellular differentiation programs often entail dynamic changes in TE expression patterns, reflecting the regulatory plasticity of TEs during ontogeny [Del Toro-De Leon et al., 2014; Guo et al., 2018]. Environmental stimuli, like nutrient availability, temperature fluctuations, and

exposure to pathogens, can elicit rapid changes in TE expression as part of the host defense response or adaptive cellular stress responses [Bloomfield et al., 2014; Federoff, 2012; Burns, 2020; Ravel-Godreuil et al., 202].

Post-Transcriptional Regulation

Post-transcriptional regulation plays a critical role in controlling the activity of TEs within genomes. RNAi pathways, mediated by small RNA molecules, are crucial for regulating TE activity post-transcriptionally. These pathways involve the generation of small non-coding RNAs that guide sequence-specific recognition and degradation of TE transcripts, thereby suppressing TE mobilization and maintaining genomic stability [McCue, 2015].

RNA Interference Pathways

RNA interference pathways encompass several distinct mechanisms, including microRNA (miRNA), short interfering RNA (siRNA), and piwi-interacting RNA (piRNA) pathways, each of which contributes to TE regulation. In the miRNA pathway, precursor miRNAs are processed into mature miRNAs, which then guide the RNA-induced silencing complex (RISC) to target TE transcripts for degradation of translational repression [MacFarlane and Murphy, 2010]. Similarly, siRNAs derived from long double-stranded RNA molecules trigger RNAi-mediated degradation of TE transcripts by guiding the RISC to complementary sequences within TE transcripts. In the piRNA pathway, PIWI proteins interact with piRNAs to form effector complexes that recognize and silence TE transcripts, particularly in germ cells, thereby safeguarding germ cell genomes against TE mobilization [Siomi et al., 2011; Tóth et al., 2016].

Impact of Small RNA Molecules on TE Activity

Small RNA molecules, including miRNAs, siRNAs, and piRNAs, exert profound effects on TE activity by targeting TE transcripts for degradation or translational repression. These small RNAs recognize complementary sequences within TE transcripts and guide the RNAi machinery to degrade TE transcripts, preventing their expression and subsequent mobilization. The specificity of small RNA-mediated TE regulation ensures precise control over TE activity, thereby ensuring genome integrity and stability.

Integration of RNA Interference Pathways

The coordinated action of RNA interference pathways ensures robust and effective regulation of TE activity post-transcriptionally. miRNAs, siRNAs, and piRNAs target different classes of TEs and are expressed in a cell- and tissue-specific manner, allowing for fine-tuning of TE regulation in response to developmental and environmental cues [Carotti et al., 2023]. Dysregulation of RNA interference pathways can lead to aberrant TE activation and genomic instability, highlighting the importance of maintaining proper small RNA-mediated TE regulation.

Epigenetic Regulation

Epigenetic mechanisms are pivotal in regulating TEs' transcriptional activity and genomic mobility. DNA methylation and histone modifications represent two fundamental epigenetic mechanisms contributing to TE silencing and regulation across different cellular contexts [Kim et al., 2009; Choi and Lee, 2020].

DNA Methylation and TE Silencing

DNA methylation, the addition of methyl groups to cytosine residues in DNA, is a well-established mechanism for transcriptional silencing of TEs [Gibnet and Nolan, 2010]. In many eukaryotic organisms, including mammals, plants, and fungi, DNA methylation occurs predominantly at CpG dinucleotides within TE sequences, leading to stable transcriptional repression and genomic stability [Wolfee and Matzke, 1999]. Maintenance of DNA methyltransferases, such as DNMT1, ensures faithful propagation of DNA methylation patterns during DNA replication, thereby perpetuating TE silencing across cell divisions [Hsieh, 2016]. De novo DNA methyltransferases, such as DNMT3A and DNMT3B, establish DNA methylation patterns during development and in response to environmental cues, providing dynamic regulation of TE activity [Dean et al., 2005].

Histone Modifications and TE Regulation

Histone modifications, including acetylation, methylation, phosphorylation, and ubiquitination, also play critical roles in TE regulation by modulating chromatin structure and accessibility to transcriptional machinery. Histone methyltransferases and demethylases catalyze the addition and removal of methyl groups from histone tails, thereby regulating TE transcriptional activity. Histone acetyltransferases and deacetylases control the acetylation status of histone proteins, influencing TE expression and chromatin accessibility [Sundar and Rahman, 2016]. Histone modifications associated with active transcription, such as H3K4me3 and H3K9ac, are enriched at transcriptionally active TE loci. In contrast, repressive histone marks, such as H3K9me3 and H3K27me3, are associated with transcriptionally silent TE loci, contributing to TE silencing and genomic stability [Rugg-Gunn et al., 2010].

Integration of DNA Methylation and Histone Modifications

DNA methylation and histone modifications are interconnected epigenetic marks that collaborate to regulate TE activity and maintain genomic integrity. Crosstalk between DNA methylation and histone modifications, such as H3K9me3 and DNA methylation, reinforces transcriptional silencing of TEs by stabilizing repressive chromatin states [Di Stefano, 2022]. Conversely, active histone modifications, such as H3K4me3, can antagonize DNA methylation and promote transcriptional activation of TEs, leading to their expression under specific developmental or environmental conditions. The dynamic interplay between DNA methylation and histone modifications provides a flexible and adaptable mechanism for regulating TE activity in response to cellular cues.

Transposable Elements in Early Pregnancy

Expression Patterns of TEs in Early Pregnancy

Research has increasingly highlighted the involvement of TEs in early pregnancy, shedding light on their dynamic expression patterns and functional roles during implantation and early embryonic development. Transcriptomic analyses utilizing next-generation sequencing techniques have identified distinct TE expression signatures in the maternal-fetal interface, including the uterus, placenta, and developing embryo. These analyses have uncovered a complex relationship between TEs and host gene expression networks, suggesting their involvement in crucial biological processes underlying pregnancy establishment and maintenance.

Research on TEs in early pregnancy spans various mammalian species, providing insights into their diverse roles in reproductive biology. In humans, research has investigated TE expression dynamics in the endometrium, placenta, and early embryos, elucidating their involvement in implantation, trophoblast differentiation, and immune modulation during pregnancy [Lynch et al., 2015; Senft and Macfarlan, 2021; Moon et al., 2019]. Mice models have been instrumental in deciphering the functional significance of TEs in early pregnancy, with research focusing on TE-mediated regulation of placental development, fetal growth, and maternal-fetal immune interactions [Lynch et al., 2015; Emera and Wagner, 2015; Todd et al., 2019]. Research in cattle has explored TE expression patterns in the developing conceptus and uterine tissues, revealing their potential roles in conceptus elongation, implantation, and placental angiogenesis [Alessio et al., 2016; Russel et al., 2017; Wenwren et al., 2014; Böhne et al., 2008]. Studies in pigs have investigated TE dynamics in the maternal-fetal interface, highlighting their contributions to placental morphogenesis, nutrient transport, and fetal development during gestation [Hwang et al., 2017; Zang et al., 2021; Wang et al., 2023; Jiang et al., 2024]. In sheep, research has focused on TE-mediated regulation of trophoblast invasion, placental hormone production, and maternal immune tolerance [Emera and Wagner, 2012; Spencer and Palmarini, 2012]. Investigation in horses has examined TE expression profiles in the developing embryo and endometrium, implicating their roles in early pregnancy events, such as embryo development and placental function [Dini et al., 2021; Imakawa and Nakaggawa, 2017; Goszcynski et al., 2022]. Studies in rats have explored TE involvement in uterine receptivity, decidualization, and spiral artery remodeling during early pregnancy [Huang et al., 2009; Dong et al., 2017; Finley, 2018]. These offer unique advantages for studying TE dynamics and their relevance in pregnancy [Mika and Lynch, 2022].

Dynamic Changes in TE Expression During Implantation and Early Embryonic Development

During implantation and early embryonic development, TE expression undergoes dynamic regulation to facilitate tissue remodeling, cell differentiation, and establishment of maternal-fetal interactions [Huang et al., 2023]. TE families, such as ERVs, LINEs, and SINEs, exhibit temporally and spatially specific expression patterns within the developing embryo and surrounding maternal tissues. Differential expression of TEs during critical developmental stages implicated their roles in modulating gene regulatory networks, chromatin remodeling, and epigenetic reprogramming, which is essential for successful pregnancy outcomes.

In most mammalian species, TEs have been found to display specific expression patterns during embryonic development and in various maternal tissues, contributing to the intricate regulatory networks in pregnancy establishment and maintenance [Lynch et al., 2015; Senft and Macfarlan, 2021]. Several TE families undergo transcriptional activation during early embryogenesis, contributing to the regulatory landscape of gene expression critical for embryo development [Fueyp et al., 2022; Oron and Ivanova, 2012]. L1 is transcriptionally active during embryogenesis [Jachowicz et al., 2017]. SINE families, such as Alu elements in humans and B1 elements in mice, are also activated during early embryonic development, contributing to the generation of noncoding RNAs [Ichiyanagi, 2013; Ge, 2017]. LTRs have been seen to contribute to establishing cell lineage-specific gene expression programs.

In the endometrium, TE expression is regulated during the peri-implantation period, contributing to uterine receptivity and establishing an environment for embryo implantation [Stout, 2015]. TE expression patterns also undergo changes during decidualization, the process by which uterine stromal cells differentiate into decidual cells to support embryo implantation and development. TE-derived regulatory elements are involved in placental development and

function, with specific TE families being expressed in trophoblast cells and contributing to placental morphogenesis, vascularization, and nutrient exchange.

Functional Roles of TEs in Pregnancy

Impact on Placental Development

TEs play diverse functional roles in early pregnancy, with emerging evidence implicating their involvement in placental development, immune modulation, and maternal-fetal tolerance. TEs contribute to placental development by influencing trophoblast differentiation, syncytialization, and angiogenesis, crucial processes for establishing and maintaining proper placental function [Malhotra et al., 2016; Zhou et al., 2023]. They do this by integrating into the genome and providing retrotransposon-derived regulatory elements, such as promoters, enhancers, and non-coding RNAs, which modulate the expression of genes involved in placental morphogenesis and vascularization [Emera and Wagner, 2012; Medstrand et al., 2005]. For instance, TEs can insert regulatory sequences that activate gene expression in trophoblast cells, promoting their differentiation into specialized cells necessary for placenta formation. Additionally, TEs can enhance the fusion of these cells into syncytia, a process essential for nutrient and gas exchange between mother and fetus [Emera and Wagner, 2012; Senft and Macfarlan 2021]. By driving these regulatory changes, TEs highlight their significant role in shaping placental architecture and function [Etchegaray et al., 2021].

Potential Involvement of TEs in Immune Modulation during Pregnancy

TEs have been implicated in immune modulation during pregnancy, which may influence maternal-fetal tolerance and immune homeostasis at the maternal-fetal interface. ERVs encode viral-like proteins with immunomodulatory properties, capable of eliciting innate immune responses and regulating the activity of immune cells within the placenta [Rosenkrantz et al., 2021]. Additionally, TE-derived small RNAs, such as piRNAs and siRNAs, may regulate the expression of immune-related genes and suppress inflammatory responses, contributing to immune tolerance and protection of the developing embryo from maternal immune attack [Rosenkrantz et al., 2021].

Regulation of Transposable Elements in Pregnancy

Influence of Reproductive Hormones on TE Activity

Reproductive hormones, such as estrogen, progesterone, and hCG, have been shown to influence TE activity during pregnancy [Spencer and Bazer, 1995]. Estrogen and progesterone, for example, exhibit dynamic changes in concentration throughout gestation, coinciding with alterations in TE expression patterns [Lynch et al., 2015; Emera, 2012]. Estrogen can activate certain TE families by binding to estrogen receptors, which then interact with estrogen response elements with the transposable elements, leading to their transcriptional activation. For instance, estrogen has been shown to activate LINE-1 retrotransposons and ERV families through these mechanisms, influencing genomic regulation and contributing to processes like placental development [Chenais, 2022; Galantonu, 2017]. Studies have demonstrated that estrogen can activate certain TE families, while progesterone may have suppressive effects on TE activity.

Progesterone may have suppressive effects by binding to progesterone receptors, which recruit transcriptional repressors and promote the formation of repressive chromatin structures, thereby reducing TE transcription [Leonhardt and Boonyaratanakornkit, 2002]. Progesterone can enhance DNA methylation and histone modifications that further silence TEs. Human chorionic gonadotropin (hCG), secreted by the placenta, regulates TE expression in maternal and fetal tissues [Burton et al., 2020; Etchegaray et al., 2021]. Human chorionic gonadotropin does this by binding to the luteinizing hormone/chorionic gonadotropin receptor and activating downstream signaling pathways [Lazzaretti et al., 2020; Choi and Smitz, 2014; Casarini et al., 2012]. By altering the epigenetic landscape, hCG can either enhance or suppress the activity of certain TEs, thus influencing their expression in both maternal and fetal tissues.

Hormonal Control of TE Silencing Mechanisms

Hormonal signaling pathways are intricately involved in regulating TE silencing mechanisms, particularly those mediated by epigenetic modifications. DNA methylations, histone modifications, and small RNA pathways are vital mechanisms through which TEs are silenced in the genome. Reproductive hormones can influence these silencing mechanisms by modulating the activity of enzymes involved in epigenetic modifications or by directly affecting the expression of small RNA molecules involved in TE regulation [Sabry et al., 2019; Sibuh et al., 2023].

Reproductive hormones can influence DNA methylation patterns, which play a crucial role in silencing TEs. Estrogen and progesterone, for example, have been shown to affect DNA methyltransferase activity [Yamagata et al., 2009]. This results in the modulation of the methylation status of TE-associated genomic regions. Hormonal signaling pathways can also

impact histone modifications, such as methylation, acetylation, and phosphorylation, which regulate TE expression [Suganauma and Workman, 2011]. Changes in hormone levels during pregnancy may influence the recruitment of histone-modifying enzymes to TE loci, leading to alterations in chromatin structure and TE silencing. Reproductive hormones can affect the expression of small RNA molecules. Hormonally regulated miRNAs and piRNAs can target TE transcripts for degradation or translational repression, thereby suppressing TE activity in maternal and fetal tissues [Sabry et al., 2019].

Tissue-Specific Expression Patterns of TEs During Early Pregnancy

Transcriptomic analyses have revealed tissue-specific expression patterns of TEs during early pregnancy, highlighting the differential regulation of TE activity in various maternal and fetal tissues [Chuong et al., 2017; Emera and Wagner, 2012; Lee et al., 2022]. For example, the placenta, uterus, ovaries, and developing embryo exhibit distinct TE expression profiles, reflecting tissue-specific transcriptional programs and developing processes. TEs may be dynamically activated or silenced in response to tissue-specific cues and signaling pathways, contributing to the unique regulatory landscape of each tissue during gestation.

Tissue-specific factors, including transcription factors, chromatin modifiers, and non-coding RNAs, are critical in regulating TE activity context-dependently [Navarro-Martin et al., 2020]. These factors interact with TE-derived regulatory elements and genomic loci to modulate TE expression dynamics and integrate TE activity into tissue-specific gene regulatory networks [Hutchins and Pei, 2015]. For example, tissue-specific transcription factors may bind to TE-associated enhancers or promoters, influencing nearby gene expression patterns and contributing to tissue-specific phenotypes during pregnancy [Jiang et al., 2024].

TEs may contribute to placental nutrient transport, hormone production, and immune regulation, by modulating the expression of genes involved in trophoblast differentiation, angiogenesis, and immunomodulation [Maltepe and Fisher, 2015; Yu et al., 2022]. The uterus undergoes dynamic changes in gene expression and tissue remodeling during early pregnancy to support embryo implantation and fetal development [Cha et al., 2012]. TEs may be involved in uterine receptivity [and decidualization processes by regulating the expression of genes critical for embryo attachment, endometrial remodeling, and immune tolerance. Ovarian tissues are essential in ovulation, follicular development, and hormone production during early pregnancy [Nagashima et al., 2011]. TEs may influence ovarian function by modulating gene expression in folliculogenesis, steroidogenesis, and oocyte maturation, impacting reproductive success and embryo implantation. The developing embryo undergoes dynamic transcriptional changes during early pregnancy, orchestrating cell fate specification, morphogenesis, and organogenesis [Shahbazi, 2020]. TEs may contribute to embryonic development by regulating the expression of genes essential for cell proliferation, differentiation, and patterning, thereby influencing embryo viability and developmental potential.

Implications of TE Dysregulation

Dysregulated transposable elements (TEs) during pregnancy can significantly impact maternal health and fetal development, leading to complications such as recurrent miscarriage, pre-eclampsia, gestational diabetes mellitus (GDM), fetal growth restriction (FGR), and preterm birth [Lou and Qiang, 2020; Sharif, 2019; Keighley et al., 2023; Sun et al., 2016]. Aberrant TE activity can disrupt placental development, impair maternal-fetal immune tolerance, and alter maternal metabolic homeostasis. TEs influence gene expression and epigenetic landscapes

critical for placental function, and their dysregulation may interfere with trophoblast differentiation and angiogenesis, resulting in placental insufficiency and adverse pregnancy outcomes [Senft and Macfarlan, 2021; Gebriel, 2023; Keighley et al., 2023].

TEs also modulate maternal-fetal immune interactions, where their dysregulation can trigger immune activation and inflammation, contributing to conditions like pre-eclampsia [Ravel-Godreuil et al., 2021; Fueyo et al., 2022; Jönsson et al., 2020]. Additionally, TEs affect metabolic pathways, and their aberrant expression is linked to GDM and other metabolic disorders [Du et al., 2016; Osipovich et al., 2023]. Studies have shown elevated retrotransposon expression in pre-eclampsia and altered DNA methylation in GDM, underscoring the role of TEs in these disorders [Keighley et al., 2023; Lokossou et al., 2014; Anwar, 2021]. Animal models further support the association between TE dysregulation and pregnancy complications, emphasizing the importance of TEs in reproductive health [Hemberger et al., 2020; Swanson and David, 2015; Zhang et al., 2023].

Clinical Relevance and Future Perspectives

Diagnostic and Therapeutic Implications of TEs in Pregnancy

Dysregulated TE activity has emerged as a promising biomarker for diagnosing and predicting pregnancy complications [Hromadnikova et al., 2022; Tang et al., 2017; Forest et al., 2012]. By analyzing TE expression patterns or epigenetic modifications in maternal blood or placenta tissues, clinicians can gain insights into the underlying pathophysiology of pregnancy disorders, such as preeclampsia, fetal growth restriction, and preterm birth [Gheorghe et al., 2010; Apicella et al., 2019]. Furthermore, TE profiles may serve as noninvasive indicators of

fetal health, offering valuable prognostic information for identifying high-risk pregnancies and guiding personalized management strategies.

In addition to diagnostic applications, targeting dysregulated TE activity holds therapeutic potential for mitigating pregnancy complications and outcomes. Emerging evidence suggests that modulating TE expression or activity through epigenetic editing, small molecule inhibitors, or RNA-based therapeutics could restore placental function, enhance maternal-fetal immune tolerance, and improve pregnancy outcomes [Chen and Zhang, 2020; Thompson, 2012]. By developing innovative interventions that specifically target TE-mediated pathophysiology, researchers aim to revolutionize prenatal care and reduce the burden of adverse pregnancy outcomes on maternal and fetal health.

Moreover, understanding TE activity in early pregnancy holds particular significance in equine reproduction, where maternal recognition of pregnancy plays a vital role in pregnancy outcomes. While the mechanisms underlying maternal recognition in horses remain elusive, TE expression dynamics may relate to this process. Investigating the transcriptional profiles during the pre-fixation period in equine embryos and maternal endometrial tissues could uncover potential TE-mediated signaling pathways in maternal-fetal communication and pregnancy establishment.

Future Directions for Research in TE Biology and Pregnancy

Research in TE biology and its implications for pregnancy physiology will continue to expand, driven by advances in genomics, transcriptomics, and epigenetics. Future studies will focus on elucidating the molecular mechanisms underlying TE dysregulation in pregnancy

disorders, unraveling the interplay between TEs and hose genes in placental development, and characterizing the role of TE-derived small RNAs in maternal-fetal communication. There is a growing need to explore the long-term consequences of dysregulated TE activity on offspring health and development. By integrating multi-omic approaches, longitudinal cohort studies, and functional genomic assays, researchers can unravel the complex relationship between TEs, environmental factors, and maternal health on offspring phenotype and disease susceptibility.

The development of innovative technologies, such as single-cell sequencing, spatial transcriptomics, and CRISPR-based genome editing, will facilitate in-depth investigations into the dynamic regulation of TEs during early pregnancy and their impact on maternal-fetal health. By leveraging these cutting-edge tools and interdisciplinary collaborations, researchers can unravel the complexities of TE biology in pregnancy and pave the way for precision medicine and therapeutic approaches to improve maternal and fetal outcomes.

TE activity holds significant diagnostic and therapeutic implications for pregnancy complications, offering insights into the underlying pathophysiology and guiding personalized management strategies. Future research directions can focus on unraveling the molecular mechanisms of TE dysregulation, exploring its long-term effects on offspring health, and harnessing innovative technologies to advance our understanding of TE biology in pregnancy. By addressing these critical knowledge gaps, researchers aim to revolutionize prenatal care and improve outcomes for mothers and their offspring.

Concluding Remarks

In conclusion, this review has provided a comprehensive overview of the intricate interplay between TEs and pregnancy physiology. Several key findings have emerged by exploring TEs' significance in genome evolution, organization, and function, alongside their specific roles in pregnancy-related processes. TEs have been shown to exert profound influences on early pregnancy stages, impacting placental development, maternal-fetal immune tolerance, and metabolic homeostasis. Dysregulated TE activity has been implicated in various pregnancy complications, underscoring the importance of understanding TE biology in reproductive health.

This review has also highlighted the diagnostic and therapeutic implications of TE dysregulation in pregnancy disorders, emphasizing the need for further research to elucidate the underlying mechanisms and identify potential therapeutic targets. However, it is important to acknowledge certain limitations inherent in the current literature, including the complexity of TE regulation and the need for more comprehensive animal-based studies, specifically in horses.

Future research directions should focus on unraveling the molecular mechanisms governing TE activity in pregnancy, leveraging advanced genomic technologies and animal models to dissect the intricate regulatory networks. Additionally, longitudinal studies examining TE dynamics throughout gestation and across diverse populations are warranted to elucidate temporal and population-specific variations in TE expression patterns.

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CHAPTER 2

TRANSPOSABLE ELEMENTS AND EARLY PREGNANCY IN THE HORSE

Summary

The equine endometrium undergoes critical physiological and molecular changes during early pregnancy to ensure the successful development of the embryo. Embryo development and pregnancy recognition are vital processes during the early pregnancy phase. Many aspects of gene expression during this phase have been studied in an effort to better understand these crucial events. One aspect that has received limited study is the expression of repetitive elements, specifically transposable elements (TEs). We hypothesized that pregnancy status would impact the expression of TE transcripts in the endometrium around the time of maternal recognition. This study tested the hypothesis by assessing the differential expression of TEs in the endometrium during early pregnancy, specifically on days 9, 11, and 13 post-ovulation, to determine the influence of pregnancy status. TEs constitute a significant portion of the mammalian genome and have demonstrated an impact on chromosome structure, genome stability, and gene expression. This investigation relied on next-generation sequence data from endometrial biopsies and bioinformatics analysis to examine TE expression from pregnant and non-pregnant mares. A total of 873 TEs showed expression across the three time points analyzed. On day 9, 381 TEs exhibited significant differential expression between pregnant and non-pregnant mares. Somewhat surprisingly, these differentially expressed TEs predominately showed a decrease in their abundance in the pregnant samples. Differentially expressed TEs decreased to 5 on day 11 and 4 on day 13. This trend contrasts with the protein-coding gene

expression results from the same sample set, where the number of differentially expressed transcripts increased from day 9 to day 11 in pregnant versus non-pregnant mares. This reduction from day 9 to day 11 suggests a transition from heightened TE activity during the early phase of endometrial remodeling, preparing for a possible pregnancy, to a more targeted regulation as pregnancy is established and progresses. The observed decline in TE activity aligns with the progression of embryo development and successful pregnancy recognition, indicating a shift in the endometrial molecular landscape to support embryo survival. Contrary to the initial expectation that TE expression would increase in pregnant mares relative to non-pregnant, our findings did not show such an increase. This study enhances our understanding of the role of TEs in early pregnancy in the mare. It suggests that TE regulation may play a role in genomic stability and endometrial receptivity during this period. These insights could inform future research on the physiology of early pregnancy and be used to support reproductive success and potential therapeutic interventions in equine pregnancy.

Introduction

Pregnancy in the mare is a multifaceted and intricate process involving a series of critical physiological and molecular events that ensure the successful development and maintenance of the developing fetus. The gestation period in horses is approximately 340 days, divided into three main phases: early pregnancy, mid-pregnancy, and late pregnancy [Jeffcott and Rossdale, 1977; Rezac et al., 2013]. Distinct developmental milestones, endocrinology, and physiological changes within the reproductive tract characterize each phase.

Early pregnancy in the mare encompasses embryo development and pregnancy establishment, beginning with ovulation [Stout, 2009; Allen and Wilsher, 2009; Ginther, 1998]. The mature preovulatory follicle ruptures, releasing an oocyte into the oviduct, where fertilization occurs [Allen,1970; Betteridge et al., 1982; Hunter, 1991; Yoon, 2012]. By day six post-ovulation, the embryo enters the uterus, propelled by oviductal contractions [Griffin and Ginther, 1993; Ginther, 1998; Pinto, 2020; Benammar et al., 2021; Jones et al., 2021; Gaivão and Stout, 2012]. The embryo exhibits peak mobility around day 12, freely moving and distributing itself within the uterine horns and body [Betteridge et al., 1982; Oriol et al., 1993; Ginther, 1983]. By day 16 post-ovulation, the embryo lodges into the caudal uterine horn, ceasing mobility [Ginther, 1983; Aurich and Budik, 2015].

Maternal recognition of pregnancy (MRP) ensures continued progesterone production, preventing luteolysis [Short, 1969]. Progesterone, secreted by the corpus luteum, maintains the receptive state of the endometrium, while estrogen from the conceptus enhances endometrial receptivity [McDowell et al., 1988; Walters et al., 2001].

This study focuses on early pregnancy, specifically days 9, 11, and 13 post-ovulation, a critical period for pregnancy success. Maternal recognition of pregnancy must occur before day 15 to prevent the release of prostaglandin F2α (PGF2α) , which would regress the corpus luteum [Klein, 2016; Klohonatz, 2013; Stout, 2020; Daels, et al., 1989; Jimoh et al., 2022; Sauberli, 2014; Maria and Stout, 2007; Newcombe et al., 2023]. Substantial research has been conducted on MRP, yet the exact signaling mechanisms remain unclear, suggesting it may involve a series of regulatory events rather than a single signal [Klein, 2016; Klohonatz, 2013; Stout; 2020]

To gain more insights into MRP and early pregnancy, our research investigates the non-coding portions of the genome – sequences that regulate gene expression without encoding

proteins [Santosh and Varshney, 2015; Shanmugam and Nagarajan, 2017]. This study leverages next-generation sequencing data and bioinformatic analyses to examine the temporal dynamics of transposable element (TE) expression in the equine endometrium. Transposable elements, which make up a significant portion, approximately half, of the mammalian genome, can influence gene expression, chromosome structure, and genome stability [Platt et al., 2018; Jern and Coffin, 2008; Fambrini et al., 2020; Chenais et al., 2012].

The hypothesis of this study is that pregnancy status would impact transposable element expression in the endometrium around the time of maternal recognition. It was anticipated that these transcripts would accumulate in pregnant mares compared to non-pregnant mares as pregnancy progresses. This hypothesis is based on previous research using the same data set to investigate protein-coding gene expression as a signal for MRP. The data from our study came from research evaluating endometrial gene expression changes during MRP via RNA sequencing, which revealed significant transcript changes on days 9, 11, and 13 post-ovulation [Klohonatz et al., 2019]. Results from that study showed an increase in gene expression from day 9 to day 11 in pregnant mares, and we assume that TE expression will follow a similar pattern.

Recent studies highlight TEs' involvement in early pregnancy, showing dynamic expression patterns at the maternal-fetal interface, including the uterus, placenta, and developing embryo [Huang et al., 2023; Lynch et al., 2015; Emera and Wagner, 2012]. By examining TE regulation in the equine endometrium, we aim to uncover new insights into early pregnancy mechanisms and identify potential biomarkers for reproductive success. Understanding TE dynamics during early pregnancy could enhance reproductive outcomes in horses and provide avenues for therapeutic intervention.

Materials and Methods

Experimental Design, Sample Collection, and RNA-seq

RNA sequence data were generated from endometrial biopsy samples collected during early pregnancy as previously described by Klohonatz et al., 2019b. In brief, three mares were randomly assigned to each of three collection days (days 9, 11, and 13 post-ovulation) in a cross-over design where each horse served as a non-mated control for their pregnant sample (Figure 2.1). Animal use approval was obtained from the Colorado State University Institutional Animal Care and Use Committee. Mares (n = 9) were housed at the Colorado State University Bud and Jo Adams Equine Reproduction Laboratory (Fort Collins, CO, USA) and maintained on a dry lot, fed a grass-alfalfa hay mix with free-choice mineral and salt supplement.

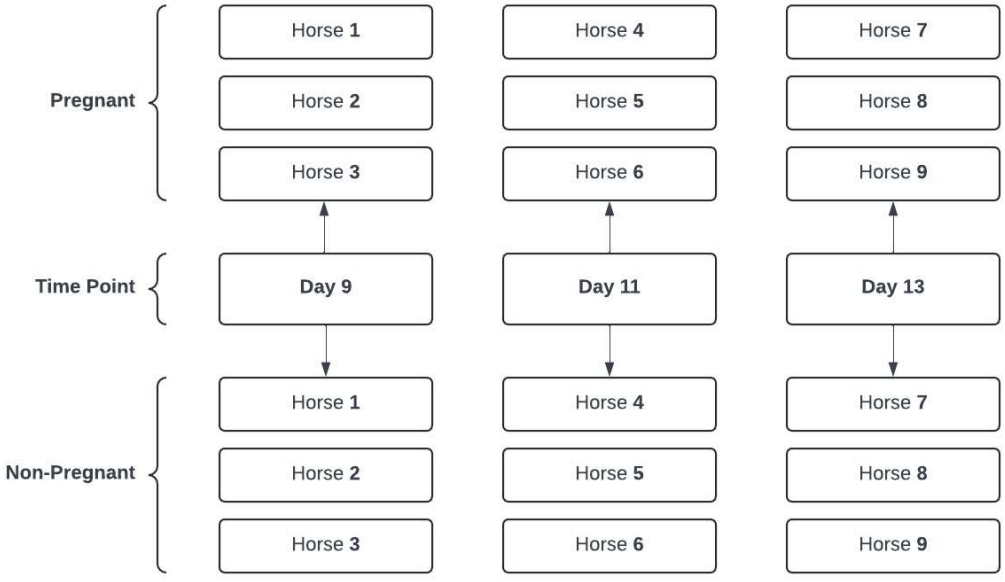

Figure 2.1 - Experimental design [Klohonatz et al., 2019] showing the random assignment of mares 1-9 to each of the three collection days (days 9, 11, and 13 post-ovulation).

Follicular development was monitored via transrectal palpation and ultrasonography every other day. Pregnant mares were inseminated with at least 500 x 10^6 progressively motile sperm when the follicle reached a diameter of 35mm or greater. Transrectal ultrasonography was conducted daily, with insemination occurring every other day until ovulation (day zero). Non-mated cycles followed the same monitoring procedure except for insemination. Pregnancy status was diagnosed via transrectal ultrasonography and confirmed by uterine lavage on the assigned collection day. Endometrial samples were obtained non-surgically via a trans-cervical biopsy punch, rinsed in DPBS/Modified 1x (Hyclone Laboratories, Logan, UT, USA) and stored at -80°C immediately. A luteolytic dose of PGF2α was administered after embryo and biopsy collection. Subsequent estrous cycles were utilized for non-pregnant control cycles.

Total RNA was isolated from approximately 30 mg of frozen tissue using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) for lysis and extraction. This was followed by purification with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with RNase-Free DNase (Qiagen, Valencia, CA, USA) treatment to remove DNA contamination. RNA purity and quantification were assessed using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE, USA).

Following the manufacturer's protocol, library prep was performed on 1 ug of total RNA for each sample using the Illumina TruSeq Sample Preparation Kit v2 (Illumina, San Diego, CA, USA). Sequencing generated 100 bp single-end reads and was performed on Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). The raw RNA-seq data from the experiment was retrieved from BioProject PRJNA545717 in the NCBI Sequence Read Archive.

Transposable Element Analysis

Identifying and quantifying transposable elements (TEs) in the RNA-seq data involved several steps performed on the Alpine high-performance computing resource (University of Colorado, Boulder, CO, USA). The workflow is presented in Figure 2.2. The Equus Caballus genome assembly (equCab3.fa.gz) and corresponding annotation file (equCab3.ncbiRefSeq.gtf.gz) were retrieved from the UCSC Genome Browser (genome.ucsc.edu). Additionally, sequence repeat information, including transposable element annotation (equCab3_rmsk_TE.gtf.gz), was obtained from RepeatMasker (https://www.repeatmasker.org). These files serve as the reference for mapping sequencing reads and associating them with known genomic features.

Raw RNA-seq data in FASTQ format were obtained from the NCBI Sequence Read Archive (SRA) using the 'fastq-dump' utility. Each sample was processed individually to ensure accurate downstream analysis.

Sequencing reads were aligned to the Equus caballus genome using the STAR aligner [Dobin et al., 2013]. A genome index was constructed using the STAR aligner to facilitate efficient read alignment. The 'genomeGenerate' command of STAR was employed with the following parameters: the genome directory ('--genomeDir'), the GTF annotation file ('--sjdbGTFfile'), an overhang parameter ('--sjdbOverhang') of 99 to accommodate sequencing reads flanking splice junctions, and the genome fasta file ('--genomeFastaFiles'). This step utilized 24 computational threads ('-- runThreadsN') for parallel processing to expedite the index generation process. The 'alignReads' mode of STAR was utilized with parameters specifying the genome index directory, GTF annotation file, and read files. Additionally, filtering and mapping quality parameters were set to ensure robust alignment. Raw count data for annotated features

was determined using HTSeq [Anders et al., 2015] TE expression levels were quantified using the TEtranscript tool [Jin et al., 2015] after alignment. The 'TEcount' command was executed with parameters specifying the aligned BAM file ('-b'), the GTF annotation file ('--GTF'), and the TE annotation file ('--TE'). Analysis was performed in multi-mapping mode to account for reads mapping to multiple genomic locations.

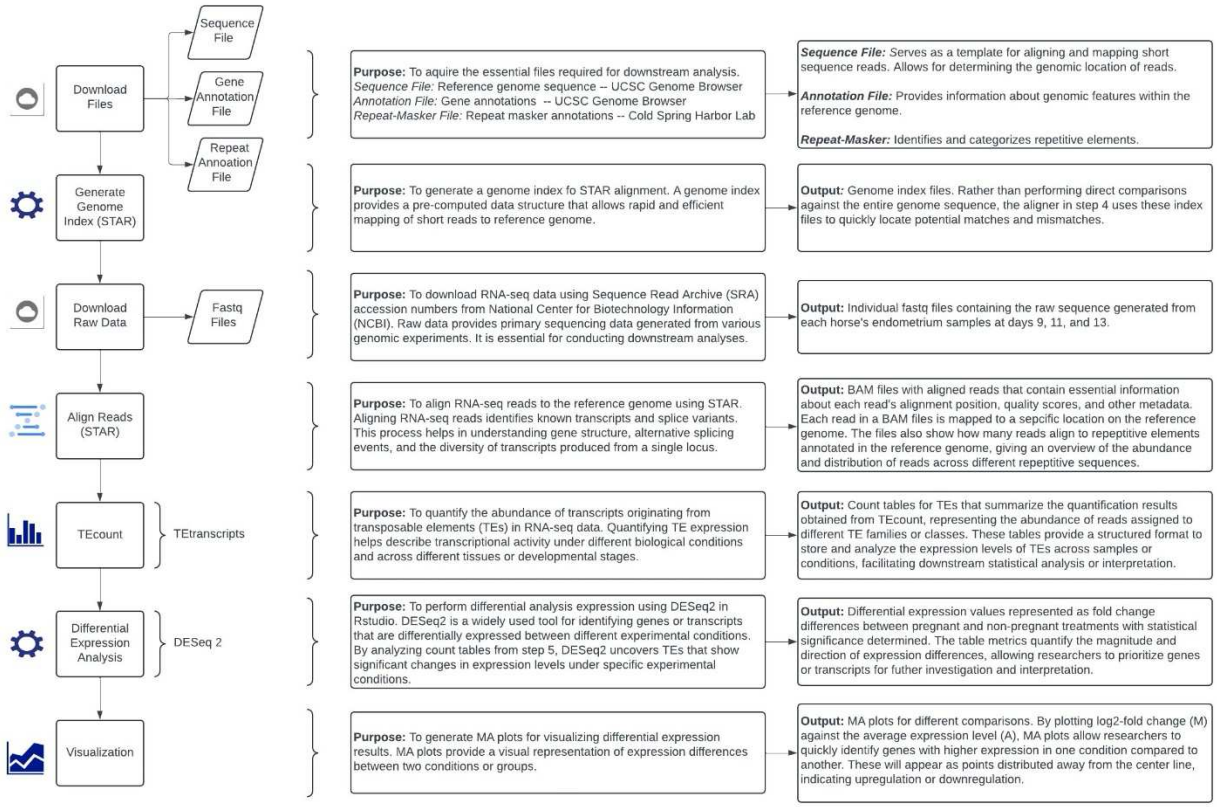

Figure 2.2 - Workflow for Transposable Element and Differential Expression Analyses. Describes the order of analyses with the intended purpose and expected outcomes for each step.

Differential Expression Analysis

Differential expression analysis was conducted in R Studio utilizing the DESeq2 package [Figure 2; Love et al., 2014]. DESeq2 provides robust statistical methods for identifying

differentially expressed genes between experimental conditions while accounting for variability inherent in RNA-seq data. To begin the analysis, the DESeq2 library was loaded into the R environment. Subsequently, the raw count data obtained from HTSeq counts were used to create a DESeqDataSet object, a crucial step in preparing the data for differential expression analysis. This object incorporates information about sample conditions, allowing for proper statistical modeling of gene expression differences. The condition column in the sample metadata table was converted into a factor with levels representing the experimental conditions of interest. Specifically, samples were categorized into pregnant and non-pregnant groups based on the conditions under study. This conversion ensures that the analysis model appropriately represents the experimental conditions. Differential expression was determined by implementing a negative binomial generalized linear model (GLM) to test for differential expression between groups while accounting for sample-specific variation and sequencing depth. Non-parametric statistical tests were used to identify genes that exhibit significantly different read alignment distributions indicating expression differences associated with the experimental conditions.

Visualization of Differential Expression

MA plots were generated to visualize the differential expression analysis results. These plots provide a graphical representation of the log-fold change (M) versus each gene's mean average expression (A). These plots facilitate the identification of genes that are significantly upregulated or downregulated between pregnant and non-pregnant conditions. Additionally, MA plots can reveal expression changes over different time points within each experimental group, providing insights into the temporal dynamics of gene regulation.

Results

Sequencing Results

The sequencing analysis of endometrial samples from pregnant and non-pregnant horses across distinct time points within the estrous cycle, including days 9, 11, and 13, revealed consistent patterns indicative of high-quality sequencing data. Over 93% of the input reads were uniquely mapped to the reference genome, with an average mapped length exceeding 98 base pairs (Table 2.1). Analysis of splicing events identified many annotated splices at each time point, with a minimal mismatch rate per base and absence of chimeric reads. These findings underscore the reliability and robustness of the sequencing data obtained from horses at specific time points within the estrous cycle, providing a solid foundation for further investigation into gene expression dynamics during pregnancy and the luteal phase in mares.

Table 2.1 - Mapping Summary

Run ID	Horse #	P/NP	DPF	Total Reads	Uniquely Maping Reads	Multiple Mapping Reads	Too Many Mapping Reads	Unmapped Reads - Too Short	Unmapped Reads - Other
SRR9678907	2250	NP	D9	39,730,545	37,006,560	1,483,029	4,028	1,224,430	12,498
SRR9678908	2250	P	D9	41,974,017	39,122,478	1,633,528	4,085	1,202,334	11,592
SRR9678909	312	NP	D11	42,475,854	39,676,992	1,647,899	3,697	1,136,722	10,544
SRR9678910	312	P	D11	38,213,837	35,606,002	1,463,202	3,005	1,132,758	8,870
SRR9678911	2212	NP	D13	37,161,016	34,597,673	1,541,941	2,339	1,013,053	6,010
SRR9678912	2212	P	D13	37,605,997	34,976,151	1,590,207	2,312	1,031,235	6,092
SRR9678913	1436	NP	D9	41,871,538	39,411,093	1,583,290	3,813	863,044	10,298
SRR9678914	1436	P	D9	39,047,228	36,660,543	1,563,393	3,215	811,707	8,370
SRR9678915	198	NP	D11	31,623,491	29,561,570	1,369,283	2,717	685,458	4,463
SRR9678916	198	P	D11	34,831,992	32,737,059	1,355,080	2,024	732,653	5,176
SRR9678917	231	NP	D9	37,268,781	35,031,738	1,363,124	3,472	861,533	8,914
SRR9678918	231	P	D9	33,584,539	31,416,488	1,530,793	2,518	628,146	6,594
SRR9678919	1289	NP	D13	33,525,004	31,499,622	1,287,355	2,246	730,512	5,269
SRR9678920	1289	P	D13	33,232,969	31,143,255	1,345,807	2,501	735,203	6,203
SRR9678921	785	NP	D13	34,447,102	32,129,881	1,604,937	3,817	700,944	7,523
SRR9678922	785	P	D13	35,108,370	32,673,928	1,771,042	3,151	652,026	8,223
SRR9678923	346	NP	D11	35,026,795	32,896,724	1,372,788	2,242	749,756	5,285
SRR9678924	346	P	D11	34,159,497	32,094,360	1,472,066	2,932	583,909	6,230
			MEAN	*36,716,032*	*34,346,784*	*1,498,820*	*3,006*	*859,746*	*7,675*
			MAX	*42,475,854*	*39,676,992*	*1,771,042*	*4,085*	*1,224,430*	*12,498*
			MIN	*31,623,491*	*29,561,570*	*1,287,355*	*2,024*	*583,909*	*4,463*
			SD	*3,291,595*	*3,059,619*	*130,694*	*682*	*209,450*	*2,379*

Run ID: Unique identifier for the sequencing run. Horse #: Identifier for the individual horse sample. P/NP: Indicates whether the sample was collected from a pregnant or non-pregnant mare. DPF: Days post-fertilization. Total Reads: Total number of sequencing reads generated for each sample. Uniquely Mapping Reads: Number of reads uniquely mapped to the reference genome. Multiple Mapping Reads: Number of reads mapped to multiple locations in the reference genome. Unmapped Reads – Too Short: Reads that did not map to the reference

genome due to being too short. Unmapped Reads – Other: Reads that did not map to the reference genome for reasons other than length.

TE Expression and Quantification

Our analysis identified 873 transposable elements (TEs) expressed in the endometrial samples from horses across different time points. These TEs were classified into various families, including DNA transposons, Long Interspersed Nuclear Elements (LINEs), Short Interspersed Nuclear Elements (SINEs), and Long Terminal Repeat (LTR) retrotransposons (Figure 2.3).

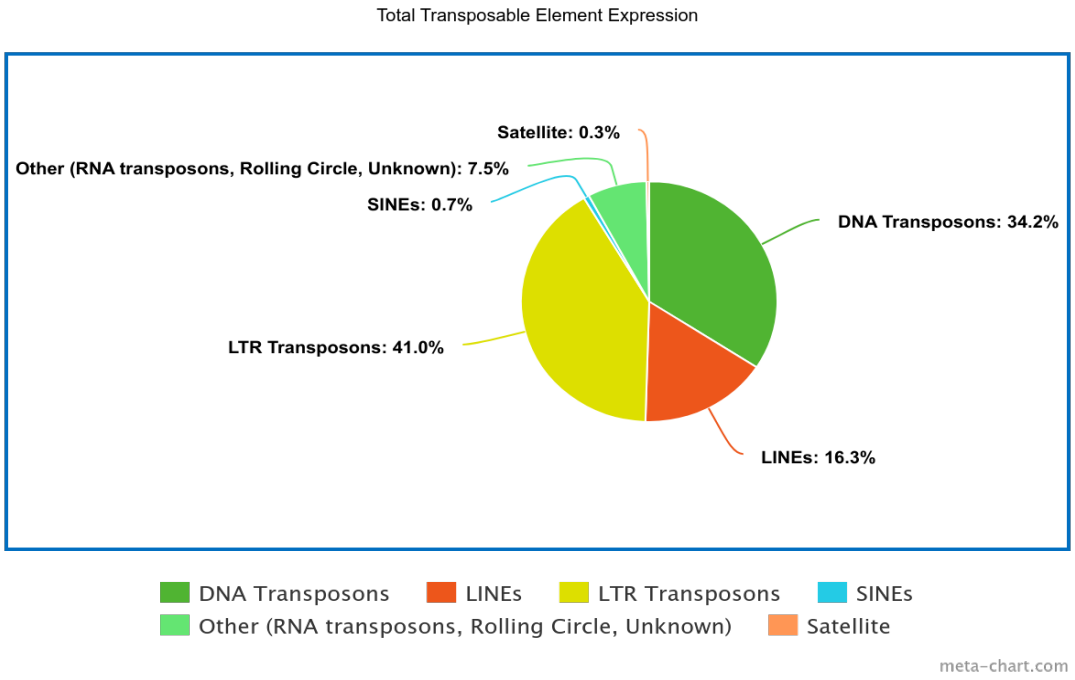

Figure 2.3 - Total Transposable Element Expression. A total of 873 TEs exhibited expression between the two groups.

Day 9 Horses

On day 9 of the estrous cycle, comparing endometrial samples from pregnant and non-pregnant horses revealed notable differences in TE expression profiles. A total of 381 TEs exhibited significant differential expression between the two groups (pregnant and non-pregnant), meeting the predefined threshold of statistical significance (p-value <0.1) [Table 2]. The distribution of these differentially expressed TEs across various TE classes is shown in Figure 2.4.

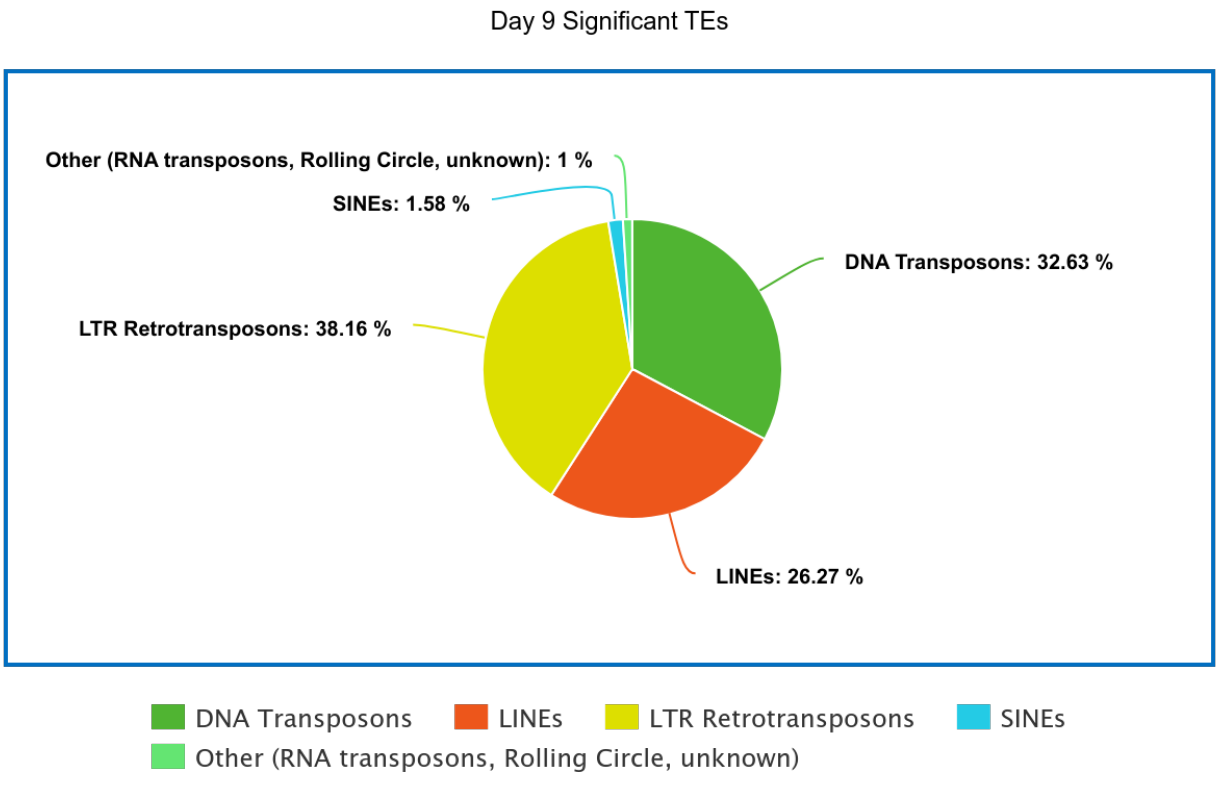

Figure 2.4 - Differentially expressed TEs by type day 9 post-ovulation. A total of 381 TEs exhibited significance between the two groups.

Significant Transposable Element Transcripts Day 9

The comparison of endometrial samples from pregnant and non-pregnant mares on day 9 revealed significant differential expression of TEs (Figure 2.5). Notably, the majority of these significant TEs exhibited negative log2FoldChange values. The downregulated TEs included various classes of TEs, suggesting a broad suppression of TE activity in the endometrial environment during early pregnancy. The top 10 significant TEs and their statistical information are presented in Table 2.2.

Day 11 Horses

On day 11 of the estrous cycle, comparing endometrial samples from pregnant and non-pregnant horses revealed notable differences in TE expression profiles. A total of 5 TEs exhibited significant differential expression between the two groups (pregnant and non-pregnant), meeting the predefined threshold of statistical significance (p-value <0.1) [Table 3]. The distribution of these differentially expressed TEs across various TE classes is shown in Figure 2.6.

Table 2.2 - Top 10 Significant Transposable Elements at Day 9

TE Name	TE Family	TE Type	Base Mean	log2FoldChange	lfcSE	stat	p-value	adjusted p-value
7SK RNA	RNA	RNA	669.48	0.43	0.24	1.81	0.0708	0.860
L1ME3E	L1	LINE	8620.74	-0.60	0.13	-4.56	5.09E-06	0.00765
LTR13B_EC	ERV1	LTR	1667.74	-0.90	0.21	-4.35	1.36E-05	0.0156
L1-5B_EC	L1	LINE	10918.41	-0.44	0.11	-4.12	3.85E-05	0.0339
MER45B	hAT-Tip100	DNA	1154.96	-0.65	0.16	-4.07	4.64E-05	0.0375
X24_LINE	L2	LINE	257.54	-0.65	0.17	-3.94	8.12E-05	0.0505
L1ME3A	L1	LINE	19482.62	-0.29	0.08	-3.81	0.000138	0.0736
MER21-int	ERVL	LTR	1030.62	-0.50	0.13	-3.79	0.000149	0.0770
L1-5A2_EC	L1	LINE	34299.93	-0.30	0.08	-3.75	0.000179	0.0868
Charlie20a	hAT-Charlie	DNA	987.40	-0.37	0.10	-3.69	0.000222	0.0942

Table includes the TE name, family, type, baseMean, log2FoldChange, IfcSE, stat, p-value, and adjusted p-value (padj) for each significant TE.

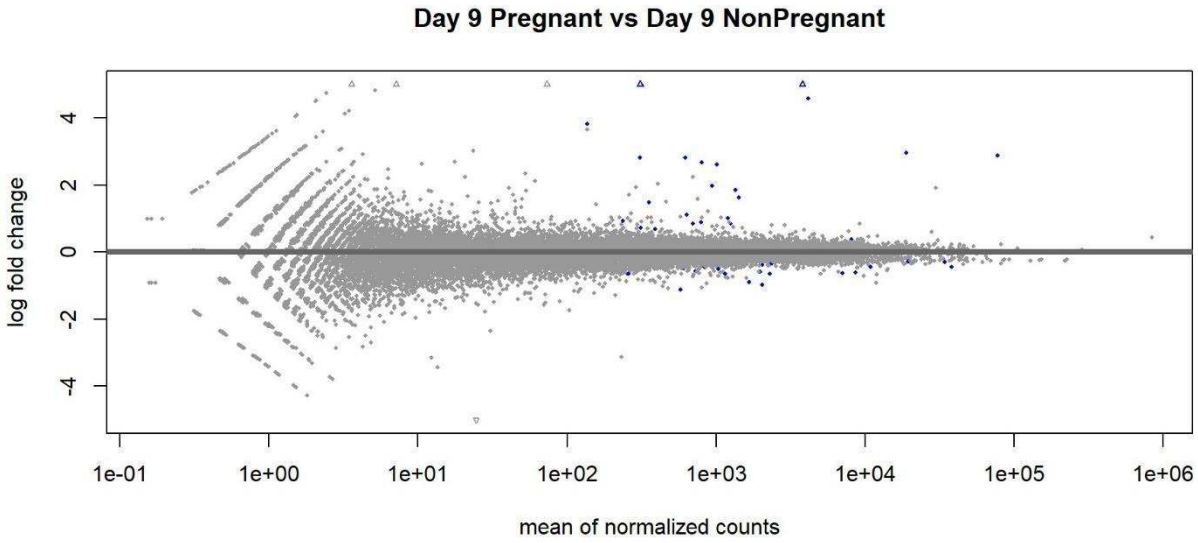

Figure 2.5 - MA plot comparing the differential expression profiles between pregnant and non-pregnant mares on day 9. Differential expression is represented by points that deviate from the horizontal line at M=0, indicating genes that are upregulated (above the line) or downregulated (below the line) in one condition compared to the other.

Significant Transposable Element Transcripts Day 11

The detailed statistical analysis of these TEs showed varying degrees of differential expression (Figure 2.7 and Table 2.3]. RNA transposon 7SK RNA exhibited a positive log2FoldChange value, indicating upregulation in pregnant mares. This suggests a possible role in facilitating the changes necessary for sustaining early pregnancy. DNA transposons MER123 and MER53 displayed negative log2FoldChange values, indicating downregulation in pregnant mares, suggesting that suppressing these TEs might be part of a broader strategy to maintain genomic stability and integrity of the developing embryo. LTR retrotransposons/ERV1, LTR12_Ec, and MLT2E showed negative log2FoldChange values, indicating downregulation. This suggests a potential mechanism to prevent genomic instability that could interfere with pregnancy establishment.

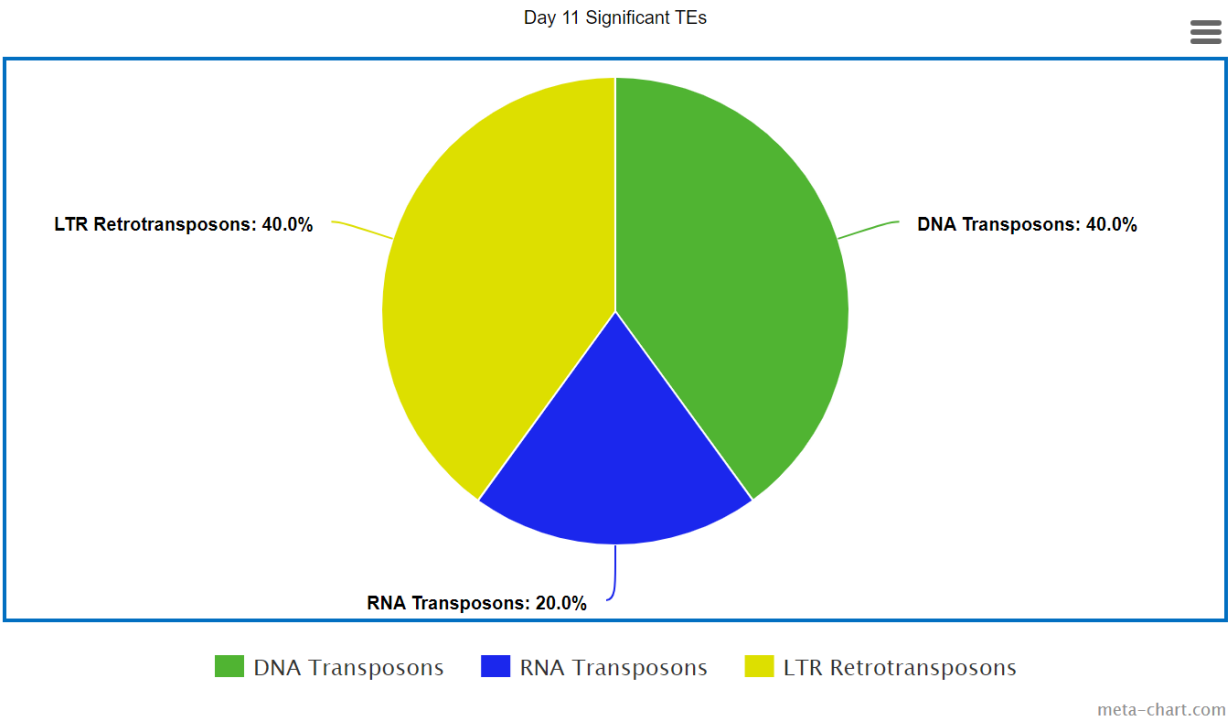

Figure 2.6 - Differentially expressed TEs by type on day 11 post-ovulation. A total of 5 TEs exhibited significance between the two groups.

Day 13 Horses

On day 13 of the estrous cycle, comparing endometrial samples from pregnant and non-pregnant horses revealed notable differences in TE expression profiles. A total of 4 TEs exhibited significant differential expression between the two groups (pregnant and non-pregnant), meeting the predefined threshold of statistical significance (p-value <0.1) (Table 2.4). The distribution of these differentially expressed TEs across various TE classes is shown in Figure 2.8.

Table 2.3 - Significant Transposable Elements at Day 11

TE	Class	Subclass	BaseMean	log2FoldChange	lfcSE	stat	pvalue	padj
7SK	RNA	RNA	548.87	0.92	0.35	2.61	0.009	0.9997
MER124	DNA	DNA?	749.77	-0.53	0.25	-2.16	0.031	0.9997
LTR12_EC	ERV1	LTR	48.83	-0.76	0.40	-1.90	0.057	0.9997
MLT2E	ERVL	LTR	237.47	-0.87	0.46	-1.88	0.060	0.9997
MER53	hAT	DNA	32.23	-0.84	0.50	-1.66	0.096	0.9997

Table includes the TE name, family, type, baseMean, log2FoldChange, IfcSE, stat, p-value, and adjusted p-value (padj) for each significant TE.

Significant Transposable Element Transcripts Day 13

The detailed statistical analysis of these TEs showed varying degrees of differential expression (Figure 2.9 and Table 2.4). LTR transposons LTR53 and MER70-int both showed a negative log2FoldChange value, suggesting down-regulation. This pattern aligns with the trend of reducing LTR retrotransposon activity to protect genomic integrity. DNA transposon MER97b, along with UCON60, also displayed a negative log2FoldChange value, indicating down-regulation.

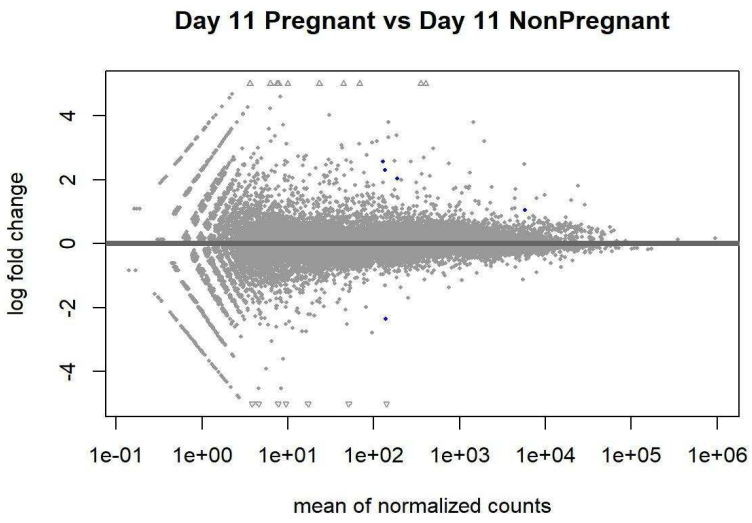

Figure 2.7 - MA plot comparing the differential expression profiles between pregnant and non-pregnant mares on day 11. Differential expression is represented by points that deviate from the horizontal line at M=0, indicating genes that are upregulated (above the line) or downregulated (below the line) in one condition compared to the other.

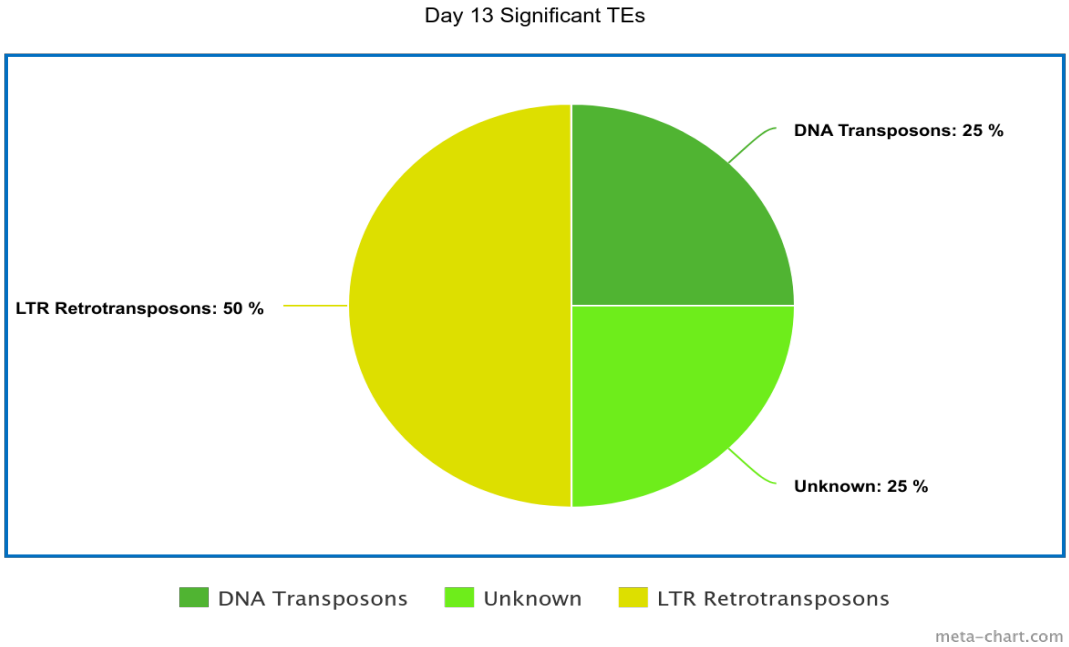

Figure 2.8 - Differentially expressed TEs by type on day 13 post-ovulation. A total of 4 TEs exhibited significance between the two groups.

Table 2.4 - Significant Transposable Elements at Day 13

TE	Class	Type	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
UCON60	Unknown	Unknown	4.21	-2.43	1.28	-1.89	0.059	0.99997
MER97b	hAT-Tip100	DNA	392.74	-0.81	0.43	-1.87	0.062	0.99997
LTR52	ERVL	LTR	502.54	-0.48	0.28	-1.73	0.084	0.99997
MER70-int	ERVL	LTR	171.77	-0.60	0.35	-1.68	0.092	0.99997

Table includes the TE name, family, type, baseMean, log2FoldChange, IfcSE, stat, p-value, and adjusted p-value (padj) for each significant TE

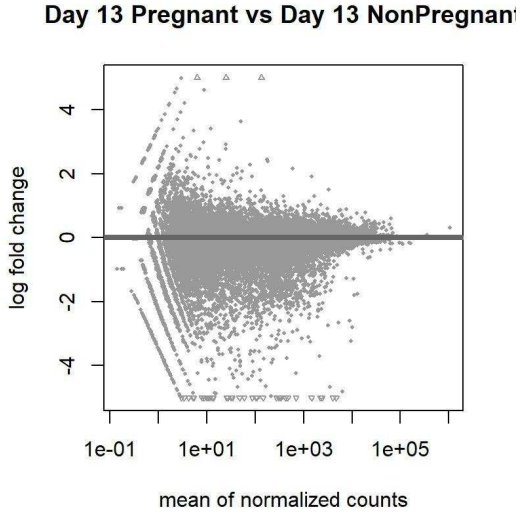

Figure 2.9 - MA plot comparing the differential expression profiles between pregnant and non-pregnant mares on day 13. Differential expression is represented by points that deviate from the horizontal line at M=0, indicating genes that are upregulated (above the line) or downregulated (below the line) in one condition compared to the other.

Discussion

Interpretation of Results and Decline in TE Accumulation

A striking observation in our study is the significant reduction in differentially expressed transposable elements (TEs) from day 9 to day 11 of the estrous cycle. This reduction may indicate the transition from heightened TE activity and endometrial remodeling during the early stages of receptivity (day 9) to a more defined and selective regulation of TE expression as pregnancy progresses. The decline in TE activity coincides with the progression of embryo development and early pregnancy processes, suggesting a potential shift in the molecular landscape of the endometrium to support embryo survival and recognition of pregnancy.

The data analyzed in this study were derived from the same dataset used by Klohonatz et al., [2019b] to examine protein-coding gene expression during early pregnancy. Their research identified significant increases in protein-coding gene expression in pregnant mares, with the number of differentially expressed genes (DEGs) peaking on day 11 [Klohonatz et al., 2019b]. This robust transcriptional response is likely associated with the endometrium's preparation for embryo implantation and successful pregnancy maintenance.

Given the observed trends in protein-coding gene expression, my initial expectation was that TE transcripts would exhibit a similar trend, increasing in pregnant mares as early as pregnancy progresses. This was based on the premise that the molecular mechanisms driving increased gene expression during early pregnancy would similarly affect TE expression, reflecting a coordinated genomic response.

Contrary to the trends observed for protein-coding genes, TE expression differences significantly decreased in pregnant mares from day 9 to day 11. On day 9, we observed 381

differentially expressed TEs, primarily downregulated in pregnant mares. By day 11, only 5 TEs showed significant differential expression, and this trend continued on day 13 with just 4 TEs significantly differentially expressed. This suggests a suppression of TE activity as pregnancy progresses, possibly to maintain genomic stability and protect the developing embryo from potential TE-induced disruptions.

Estrogen and Transposable Elements

The relationship between estrogen and TE expression further informed our hypothesis. Estrogen is known as an activator of TEs, and levels of this hormone differ between pregnant and non-pregnant mares [Sato et al., 1977; Daels et al., 1991; Dini et al., 2021]. Given estrogen's role, we expected to see an increase in TE expression in pregnant mares, correlating with elevated estrogen levels. However, the observed decline in TE expression suggests that other regulatory mechanisms may be at play.

During early pregnancy, the endometrium undergoes significant changes to accommodate and support the developing embryo [Achache and Revel, 2006; Allen and Wilsher, 2009; Morresy, 2011]. While estrogen may activate TEs under certain conditions, the hormonal milieu of early pregnancy, including the interplay with progesterone and other factors, likely exerts a more complex regulatory influence on TEs. This complexity may result in the observed suppression of TE activity, which could be a protective mechanism to ensure genomic integrity during a critical period of embryo development.

Conclusion

In summary, this study revealed a decreasing pattern of differentially expressed transposable elements during early pregnancy in mares, contrasting with the trends observed in protein-coding gene expression. These results may indicate the action of TE-specific regulatory mechanisms. The interplay between estrogen and TE expression, along with the implications of these findings, provides a deeper understanding of the molecular events during early pregnancy. Further research is warranted to explore the regulatory networks involving TEs and their potential roles in reproductive success.

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CHAPTER 3

REFLECTION

Introduction

This has been quite the process. I initially started my research by investigating repetitive elements (REs) and a possible threshold level as the mechanism for maternal recognition of pregnancy (MRP). However, my research evolved, leading me to focus specifically on the dynamic changes around the timing of MRP. To better understand MRP, we need to do more research on what is happening in the endometrium of the mare during these critical processes throughout early pregnancy. This redirection allowed me to explore the nuanced regulatory mechanisms, providing a foundation for proposing an alternative hypothesis.

For my redirected research, I hypothesized that TEs would show distinct temporal patterns of expression during early pregnancy, reflecting their roles in embryo development, pregnancy detection, and placental function. This hypothesis was grounded in the need to elucidate the molecular mechanisms underlying early pregnancy establishment and maternal-fetal communication in mares. By leveraging next-generation sequencing techniques and bioinformatic analyses, I aimed to delineate the temporal dynamics of TE expression and their potential roles in early pregnancy.

Research Limitations

It is essential to recognize the research limitations I underwent and noticed that impacted both my initial and refined hypotheses. One major limitation was the sample size, which included nine horses, with three horses assigned to each time point (days 9, 11, and 13). This limited sample size restricts the ability to make clear, definitive conclusions when comparing different samples at the time points. Additionally, each mare's physiological and anatomical uniqueness, including factors such as timing of pregnancy recognition and parity, adds another layer of variability that may influence the results.

The study design captured TE expression at specific time points but may have missed critical windows of TE activity. Because the data was collected with MRP in mind, the time points were focused on the timing of that signal. My initial assumption was that the accumulation of TE transcripts in the endometrium might act like a threshold triggering maternal recognition of pregnancy signaling, but the observed dynamic nature of repetitive element expression challenges this assumption.

Practically, the study was constrained by the availability of horses and resources, limiting the scope of experimental approaches. While I am forever grateful for the opportunity to use already collected data, both studies could have benefitted from a fresh and more current set of data. Moreover, the complexity of TE regulation and its interplay with other genomic elements requires more sophisticated techniques to understand their functional roles fully.

Each of these limitations has influenced the validity and reliability of my findings. The limited sample size and variability among the mares may have introduced noise into the data, affecting the ability to detect subtle differences in TE expression. The focus on specific time

points may have overlooked critical temporal dynamics, while the lack of functional studies limits the interpretation of the observed expression patterns.

Alternative Hypothesis/Study

Given these limitations, I propose an alternative hypothesis regarding MRP. Instead of a threshold-based model where increased RE expression initiates MRP signaling, it is plausible that MRP signaling involves the downregulation of RE expression, potentially as a switch for luteolysis prevention and pregnancy maintenance.

The estrous cycle, considered 'normalcy' in my opinion, typically spans 21-22 days, involving ovulation, hormone production, secretion, and corpus luteum regression. Pregnancy, viewed as a "foreign object", disrupts this normal cycle. REs are known to have regulatory roles in the genome, potentially maintaining this "normalcy". They might have a close relationship with prostaglandin F2 alpha, a hormone involved in luteolysis.

During regular estrous cycles, REs might regulate processes necessary for maintaining the cycle. When pregnancy changes the "normal, stable cycle", RE expression may decrease, disrupting their regulatory role and preventing luteolysis. This hypothesis can be visualized as a dimming light switch.

- Light On: Active regulatory mechanisms by REs maintaining the estrous cycle.
- Light Off: Inhibition of regulatory mechanisms by REs, preventing Prostaglandin F2 alpha production and thereby luteolysis.

The well-established role of TEs in genome regulation and epigenetic modifications supports this alternative hypothesis. TEs can influence gene expression by introducing regulatory elements, modifying chromatin structure, and providing alternative promoters and enhancers. Moreover, they are subject to epigenetic regulation through DNA methylation and histone modifications, leading to transcriptional silencing of TEs and nearby genes.

The observed fluctuations in TE activity across different time points may reflect the dynamic interplay between RE expression and MRP signaling pathways. The downregulation of RE expression, rather than its upregulation, may serve as a crucial molecular event triggering MRP, thereby preventing luteolysis and supporting pregnancy maintenance. This alternative interpretation aligns with the intricate regulatory mechanism governing TE activity in equine reproductive physiology.

Supporting Evidence

The evidence supporting this alternative hypothesis includes the observed fluctuations in TE activity across the different time points, which may reflect the dynamic interplay between RE expression and MRP signaling pathways. For instance, the significant reduction in TE expression from day 9 to days 11 and 13 likely represents a protective mechanism to ensure genomic integrity during early pregnancy, preventing TE-induced mutations that could affect embryo development and pregnancy establishment.

Furthermore, the continued suppression of LTR retrotransposons on days 11 and 13 underscores the importance of genomic stability during this period. These findings align with the well-established role of TEs in genome regulation and epigenetic modifications, where TEs can

influence gene expression through various mechanisms, including introducing regulatory elements, modifying chromatin structure, and providing alternative promoters and enhancers.

Implications and Goals

The implications of this research are far-reaching. Understanding the mechanisms of MRP has the potential to impact reproductive management, fertility issues, and therapeutic interventions in horses. The ultimate goal of this research is to provide insights that could lead to practical applications in equine reproductive health.

I was drawn to this research due to my interest in overpopulation control in wild horse populations. My final project in my undergraduate equine reproduction course involved developing a reproductive technique to address this issue. By solving MRP or at least better understanding the changes in the endometrium and the mechanisms involved in pregnancy detection, it is possible that we could intervene and use this research as a preventative intervention.

Furthermore, the findings from this research can be applied to reproductive management and therapeutic interventions to help mares with pregnancy success. Understanding the dynamic regulation of TEs and their role in MRP could lead to developing novel strategies to enhance fertility, prevent pregnancy loss, and improve overall reproductive health in horses.

Personal Reflections

Reflecting on this research project, I feel a sense of accomplishment in contributing to understanding TEs in equine reproductive physiology. The process has taught me the importance of considering dynamic regulatory mechanisms and the need for comprehensive experimental approaches to uncover complex biological phenomena. Although the initial hypothesis could not be supported, revamping the study to focus on the specific roles of TEs in early pregnancy provided insights and a valuable foundation for future MRP research.