A critical review of the recent concept of regulatory performance of DNA Methylations, and DNA methyltransferase enzymes alongside the induction of immune microenvironment elements in recurrent pregnancy loss

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ABSTRACT

Recurrent pregnancy Loss (RPL)is a frequent and upsetting condition. Besides the prevalent cause of RPL including chromosomal defects in the embryo,the effect of translational elements like alterations of epigenetics are of great importance. The emergence of epigenetics has offered a fresh outlook on the causes and treatment of RPL by focusing on the examination of DNA methylation. RPL may arise as a result of aberrant DNA methylation of imprinted genes, placenta-specific genes, immune-related genes, and sperm DNA, which may have a direct or indirect impact on embryo implantation, growth, and development. Moreover, the distinct immunological tolerogenic milieu established at the interface between the mother and fetus plays a crucial role in sustaining pregnancy. Given this, there has been a great deal of interest in the regulation of DNA methylation and alterations in the cellular components of the maternal-fetal immunological milieu. The research on DNA methylation's role in RPL incidence and the control of the mother-fetal immunological milieu is summed up in this review.

1. Introduction

RPL is conventionally characterized as the occurrence of three or more clinical pregnancies that terminate before the 20th week of gestation in human females. Based on the provided definition, it may be inferred that the incidence of RPL is around 1 in 100 pregnancies globally [1]. Nevertheless, the incidence of this frequency rises to 5% when medical professionals establish RPL as the occurrence of two or more pregnancy losses [2]. Furthermore, epidemiological studies have provided evidence indicating that the incidence of recurrent pregnancy loss is around 24% following two pregnancy losses, 30% following three losses, and 40% following four consecutive pregnancy losses [3]. Although the precise reason for many incidents remains unknown, several explanations have been proposed. These consist of genetic

variables, exposure to environmental factors, stress factors, endometrial infections, endocrine abnormalities, antiphospholipid syndrome, hereditary thrombophilias, chromosomal and uterine morphological abnormalities, and alloimmune causes [4]. Although a range of interventions, such as immunological and hormonal therapy, have been used to treat women with RPL [5]. The adequacy of the examination conducted to determine the underlying causes of unsuccessful pregnancies remains a subject of controversy. Hence, there is a strong emphasis on the necessity of conducting thorough examinations and implementing standardized protocols for patients undergoing RPL. This is crucial to accurately identify potential hazards and choose suitable treatment interventions [6]. To get a deeper understanding of the etiology of RPL and establish effective treatment strategies, it is imperative to conduct further extensive investigations at the molecular level. In

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recent times, the fields of genomics, transcriptomics, and proteomics have been employed to explore potential genes or their corresponding products that may be linked to the development of RPL. An examination of the genetic material in chorionic villi from individuals experiencing RPL, whose chromosomal profiles are normal, has identified a correlation between the levels of expression of five specific categories of genes (related to immunosuppression, embryo attachment, angiogenesis, apoptosis, and other causes) and RPL [7]. Furthermore, a recent proteomic investigation has demonstrated that RPL is also linked to the abnormal production of thrombophilic factors, including fibrinogen-γ and antithrombin [7]. Conducting a comprehensive functional investigation of these genes or their gene products in the context of typical pregnancy holds the potential to facilitate the identification of pregnancies that exhibit an elevated risk of RPL. In this analysis, we examine a number of primary variables that have been linked to RPL. Specifically, we focus on immunological and thrombophilic components, which have been identified as crucial elements for successful pregnancy.

Furthermore, we will explore potential therapy approaches for RPL. In this review article, the regulatory performance of DNA Methylations, and DNA methyltransferase enzymes alongside the induction of Immune Microenvironment elements in spontaneous abortion will be discussed.

1.1. Epigenetic mechanisms and infertility

The human body has over 200 distinct cell types, each of which possesses an identical copy of the genome. Nevertheless, it is crucial to acknowledge that various types of cells give rise to varied gene sequences, which are contingent upon the process of epigenetic control. The field of epigenetics investigates the heritable alterations in gene expression that occur during mitosis and/or meiosis, without any accompanying modifications to the DNA sequence. It is commonly accepted that the epigenome functions as an extra layer of information on top of the DNA sequence and that it is essential for maintaining gene expression patterns unique to particular cell types [8]. Gene expression
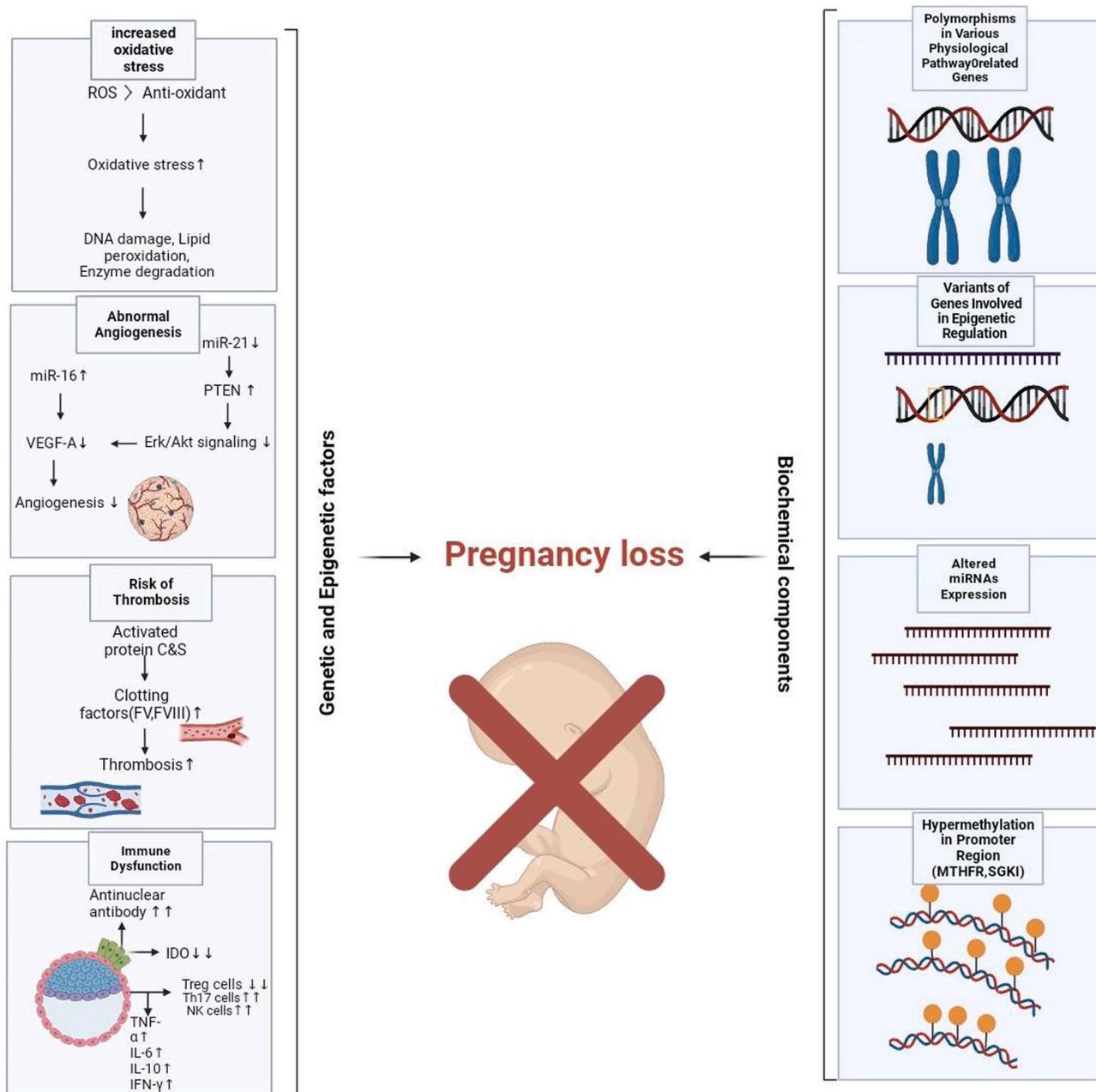

Fig. 1. Factors that cause abortion include immune dysfunction, thrombosis, abnormal angiogenesis, and increased oxidative stress.

is dynamically and reversibly controlled by epigenetic changes because a cell's epigenome is highly malleable and reprogrammable. Cell destiny is changed by epigenetic reprogramming during development and maturity. It is significant to emphasize that environmental conditions are crucial for the establishment and preservation of epigenetic markers (Fig. 1) [9].

1.2. DNA methylation in infertility

The process of embryogenesis is significantly influenced by DNA methylation, which serves to bestow pluripotent capabilities upon embryonic cells, hinder the transfer of acquired epimutations, and facilitate the proper development of germ cells through successive cycles of methylation and demethylation [10]. Epigenetic modifications occurring in primordial germ cells (PGCs) encompass the reprogramming of DNA methylation, resulting in the development of germ cells distinct to each sex and the facilitation of appropriate spermatogenesis [11]. This underscores the need to effectively control epigenetic mechanisms to ensure accurate embryonic development and optimal sperm functionality. During the initial stages of embryo development, a limited number of cells are identified as primordial germ cells (PGCs), which subsequently differentiate into germ cells (sperm or oocytes) for the purpose of reproduction [12]. The aforementioned mechanism plays a pivotal role in the transfer of genetic information to subsequent generations. To produce germ cell-specific epigenomes, it is necessary to eliminate somatic lineage epigenetic markers, such as DNA methylation, from germline germ cells (PGCs) during the process of epigenetic reprogramming in the germline [13]. The process of reprogramming plays a crucial role in ensuring the appropriate growth and operation of germ cells. There are notable disparities in the DNA methylation patterns seen between oocytes and sperm. The variation in methylation patterns plays a pivotal role in ensuring the appropriate operation of male and female germ cells throughout the processes of gametogenesis and conception [14]. The process of oocyte development and the establishment of methylation. Germ cells in female embryos undergo arrest during prophase 1 and remain in a state of inactivity until the moment of birth. Following the process of birth, oocytes undergo a series of developmental stages, including the primary follicle stage and the secondary follicle stage [15]. Methylation at germline differentially methylated regions (gDMRs) is created during this developmental phase, and it plays a crucial role in the formation and function of oocytes. On the other hand, it is noteworthy that male germ cells experience methylation of gDMRs before the initiation of meiosis in prospermatogonia [16]. This process starts in the fetal testis and culminates at birth. The methylation process in male germ cells is facilitated by many cycles of cell division, which enable the alteration of pre-existing methylation patterns through maintenance [17]. Nevertheless, there is an elevated probability of methylation mistakes accumulating over some time. Abnormal DNA methylation, which affects gene expression in spermatogenesis, is associated with male infertility, which affects 30–50% of cases. The DNA fragmentation index (DFI) has been identified as a potential indicator of male infertility, as imbalances in DNA methylation have been linked to atypical sperm characteristics [18]. Abnormalities in the Methylenetetrahydrofolate reductase (MTHFR) gene, such as hypermethylation, hurt the integrity of sperm DNA and can lead to male infertility [19]. DNA methylation and spermatogenesis are significantly influenced by DNMT genes, TET enzymes, and imprinted genes such as MEST and H19. Male infertility and aberrant sperm parameters might result from abnormal methylation of imprinted genes, and smoking is associated with H19 methylation [20]. Aberrant methylation at the IGF2-H19 CTCF region can impact the development of embryos and the outcome of pregnancy, as observed in offspring conceived via assisted reproductive technologies [21]. Understanding the impact of male infertility and DNA methylation on gene expression, sperm quality, and fertility outcomes is of paramount importance in the field of research. The process of oogenesis is significantly influenced by DNA methylation, wherein the genes DNMT1 and DNMT3A/B/L are pivotal in determining the methylation patterns that occur during the development of oocytes [22]. The preferential methylation of imprinting control regions (ICRs) and certain non-imprinted gene regulatory DNA modification regions (gDMRs) has a significant impact on genomic imprinting and embryo development. Developmental issues such as maternal-to-zygotic transcriptional transition or ovulation failure can arise as a result of disruptions in the oocyte methylome [23]. Oocyte activation deficiency (OAD) can occur due to atypical fluctuations in calcium ion levels after fertilization, which can impact the expression of genes and the process of development. OAD may be caused by abnormalities in DNA methylation, which can affect gene expression and the development of offspring after fertilization. There exists a correlation between DNA methylation modifications and female comorbidities that impact reproductive capacity, such as endometriosis, polycystic ovary syndrome (PCOS), and obesity [24]. Differential methylation profiles in endometrial tissue have been observed in individuals with endometriosis, affecting genes associated with steroid hormone signaling and DNA methylation [25]. Patients with polycystic ovary syndrome (PCOS) exhibit genome-wide hypomethylation, which impacts genes such as FKBP5, YAP1, CYP19A1, and LHCGR. The coexistence of obesity and female infertility is associated with abnormal methylation patterns in different organs, which in turn affect genes associated with energy balance and metabolic syndrome [26]. There exists a correlation between parental age at conception, particularly in industrialized countries, and diminished reproductive capacity, infertility, as well as increased health hazards for the developing baby. To accurately predict biological age and disease risks, epigenetic clocks such as Horvath, Hannum, PhenoAge, and GrimAge are employed to measure DNA methylation [23]. Additionally, methylation-based aging markers like DunedinPOAM and DunedinPACE are utilized to investigate the decline in fertility associated with aging in both males and females [27]. The occurrence of male infertility as paternal age increases is linked to changes in DNA methylation patterns in sperm, which impact genes important in embryonic development [28]. Age-related female infertility is caused by changes in DNA methylation, which impact the expression of genes in ovarian cells and can result in early ovarian insufficiency [29]. Epigenetic changes like DNA methylation play a vital role in defining the initial pool of primordial follicles and regulating ovarian function [30]. Turner syndrome, a hereditary condition causing early ovarian insufficiency, is connected to DNA hypomethylation and variable gene expression impacting ovarian function. Epigenetic age acceleration (EAA) assessed by clocks like Horvath is connected with poor ovarian responsiveness to stimulation and numerous infertility problems in women [31]. Pre-eclamptic pregnancies are connected to EAA and accelerated cellular senescence, providing health hazards to both mother and baby. Developing tissue-specific clocks to monitor DNAm Age in the ovary might assist knowledge of age-related reproductive reduction [30]. that DNA methylation variations in sperm as a cause of infertility could have been overstated, and that the functional impact of differential methylation due of ART on fetal health and development is not substantial [32]. Despite extensive research on sperm methylome, only a limited number of genes have received significant attention [33]. Additionally, the list of modifiable risk factors that effect fertility through DNA methylation in both males and females is restricted to smoking and developing research on obesity [34]. It is important to consider the influence of age on epigenetic modifications, such as DNA methylation, when conducting stratified comparisons of the epigenome between persons who are infertile and those who are fertile, based on age [35]. The development of an ovary-specific epigenetic clock, similar to the approach used for sperm, offers valuable insights on the epigenomic changes that occur in ovaries as individuals age, as well as the influence of external variables such as physical fitness, smoking, obesity, and comorbidities. Furthermore, the examination of the influence of DNA methylation on aberrant Ca2+ oscillations during oocyte activation is also ongoing [36].

1.3. Histone modification in infertility

The significance of histone alterations in male and female infertility is of considerable importance [37]. Asthenozoospermia, a condition affecting sperm motility and male fertility, is linked to aberrant S-sulfhydration of histones H3 and H3.3 in men [38]. Histone changes during oogenesis in females can result in genetic abnormalities that cause implantation failure and infertility [39,40]. In addition, the occurrence of male infertility with azoospermia or oligozoospermia can be attributed to deficits in the histone-to-protamine transition that takes place during sperm formation [41]. Epigenetic alterations, such as modifications to histones, have been identified as important elements in the context of male infertility, exerting an impact on the processes of spermogenesis, sperm quality, and embryo development. The regulation of the CYP11A1 gene, which is essential for the generation of steroid hormones and female fertility, is influenced by histone modifications, including H3K4me3.

1.4. Histone phosphorylation in infertility

The post-translational modification known as histone phosphorylation plays a critical role in chromatin dynamics and gene regulation [42]. It has gained prominence as a fundamental regulatory mechanism in the field of reproductive biology, specifically in infertility [43]. The modulation of chromatin structure, accessibility, and transcriptional activity is facilitated by a range of kinases and phosphatases [44]. This process has significant implications for important reproductive processes, including gametogenesis, fertilization, and early embryonic development [45].The process of gametogenesis, which involves the differentiation of germ cells into mature gametes, is significantly influenced by histone phosphorylation [46]. Dynamic alterations in histone phosphorylation patterns play a crucial role in coordinating gene expression programs that are vital for the maturation and functioning of germ cells during the processes of spermatogenesis and oogenesis. Impaired germ cell growth and infertility in both males and females have been linked to dysregulation of histone phosphorylation in gametogenesis [47]. The process of histone phosphorylation plays a crucial role in the epigenetic reprogramming that occurs during germline development. This includes the deletion of DNA methylation and the formation of chromatin states that are exclusive to germ cells [48]. The occurrence of epigenetic aberrations, which contribute to the observed infertility phenotypes in patients, can be attributed to disruptions in histone phosphorylation dynamics during these crucial periods of epigenetic remodeling [49]. Abnormal histone phosphorylation patterns in sperm chromatin have been associated with impaired sperm quality and reproductive outcomes in cases of male infertility [50]. Impaired fertilization and embryo development can occur as a result of dysregulated histone phosphorylation, which can have detrimental effects on chromatin compaction, DNA integrity, and sperm function [48,51]. Histone phosphorylation is essential for controlling the movement of chromatin during the maturation of oocytes, which in turn affects the process of meiosis and the maturation of the cytoplasm [52].The association between dysregulation of histone phosphorylation in oocytes and reduced fertilization rates, suboptimal embryo quality, and female infertility has been shown [53]. The risk of infertility can be influenced by several environmental and lifestyle variables, including exposure to endocrine-disrupting chemicals, oxidative stress, and an unhealthy diet. These factors have the potential to disrupt the patterns of histone phosphorylation in germ cells [54]. A comprehensive comprehension of the intricate relationship between histone phosphorylation and environmental variables is necessary to gain insight into the underlying causes of infertility and to devise precise therapies. Artificial Reproductive Technologies (ART) treatments, such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have the potential to unintentionally disrupt the dynamics of histone phosphorylation in embryos [55]. This raises concerns over the potential long-term health

consequences for babies conceived through ART. Additional investigation is required to assess the effects of epigenetic alterations associated with assisted reproductive technology on fertility outcomes and the health of kids [56]. The therapeutic promise for treating infertility lies in targeting particular kinases and phosphatases involved in histone phosphorylation, which plays a crucial role in reproductive processes. Modulating histone phosphorylation patterns in germ cells and embryos by pharmacological treatments might provide innovative approaches to enhance reproductive results in individuals undergoing assisted reproduction. Non-coding RNAs, such as microRNAs and long non-coding RNAs, control the process of histone phosphorylation by influencing the activity of enzymes that alter histones [57]. The dysregulation of non-coding RNAs has been connected with anomalies in histone phosphorylation in cases of infertility, therefore emphasizing their potential as biomarkers for diagnosis and targets for therapeutic interventions. The interaction between genetics and epigenetics: Genetic variations that impact genes responsible for encoding enzymes that alter histones can make individuals more likely to experience infertility by disturbing the dynamics of histone phosphorylation and the structure of chromatin. Comprehending the intricate relationship between genetics and epigenetics in the development of infertility is crucial in order to develop tailored strategies for the diagnosis and treatment of this condition [58]. Despite notable advancements in comprehending the involvement of histone phosphorylation in infertility, numerous obstacles persist. These challenges encompass the necessity for thorough mechanistic investigations, the creation of non-invasive diagnostic instruments, and the effective application of research outcomes in clinical settings. The integration of genetics, epigenetics, and reproductive biology through collaborative multidisciplinary endeavors is necessary in order to effectively tackle these issues and enhance our comprehension of the causes behind infertility [59]. histone phosphorylation plays a crucial role in regulating infertility, and it has significant implications for gametogenesis, epigenetic reprogramming, outcomes of assisted reproductive technology (ART), and therapeutic interventions. Ongoing research endeavors focused on elucidating the complex molecular pathways that govern the dynamics of histone phosphorylation are imperative in enhancing fertility diagnoses, therapies, and results in persons affected by this condition [60].

1.5. Histone acetylation in infertility

Histone acetylation, a crucial epigenetic alteration, has a significant impact on how genes are expressed and the structure of chromatin [61]. As a result, it affects several elements of reproductive biology, such as gametogenesis, fertilization, and early embryonic development. Recent research indicates that the disruption of histone acetylation dynamics has a role in the development of infertility symptoms in both males and females [62]. The process of histone acetylation is closely associated with the control of gene transcription in gametogenesis [63]. The activation or suppression of gene expression programs necessary for germ cell maturation and function is regulated by dynamic changes in histone acetylation patterns during spermatogenesis and oogenesis [37]. Defective gametogenesis and infertility have been linked to changes in histone acetylation levels. The process of histone acetylation is of utmost importance in the epigenetic reprogramming that occurs during germline development [64]. This process involves the removal of parental histone modifications and the formation of chromatin states that are particular to germ cells [65]. Disruptions in the dynamics of histone acetylation during these crucial periods of epigenetic remodeling can result in epigenetic aberrations, hence playing a role in the development of infertility. Male infertility has been associated with abnormal histone acetylation patterns in sperm chromatin [37]. Imbalanced histone acetylation can impact the compression of chromatin, the integrity of DNA, and the functioning of sperm, eventually hindering the process of conception and the development of embryos [66]. Gaining insight into the function of histone acetylation in the restructuring of sperm

chromatin is crucial for enhancing diagnostic and therapeutic approaches for male fertility. The process of chromatin dynamics during oocyte maturation is regulated by histone acetylation, which has a significant impact on both meiotic development and cytoplasmic maturation [67]. Decreased fertilization rates, poor embryo quality, and female infertility have been linked to dysregulation of histone acetylation in oocytes. Focusing on histone acetylation pathways might provide innovative treatment approaches to enhance female reproductive results [68]. Various environmental and lifestyle variables, including the presence of endocrine-disrupting substances, oxidative stress, and dietary choices, have the potential to impact the levels of histone acetylation in germ cells, hence playing a role in the risk of infertility. The comprehension of the relationship between histone acetylation and environmental variables is crucial in order to gain insight into the causes of infertility and to devise tailored therapies. Assisted reproductive technology treatments, such as in vitro fertilization and intracytoplasmic sperm injection (ICSI), have the potential to disrupt the dynamics of histone acetylation in embryos [69]. This raises concerns over the potential long-term health consequences for babies achieved by ART. Additional investigation is required to evaluate the influence of ART-related epigenetic alterations on reproductive results and the well-being of kids [70]. Targeting particular histone acetyltransferases (HATs) and histone deacetylases (HDACs) has potential for treating infertility, considering the crucial role of histone acetylation in reproductive processes. Modulating histone acetylation patterns in germ cells and embryos by pharmacological treatments might provide innovative approaches to enhance reproductive results in individuals undergoing assisted reproduction. Non-coding RNAs, including microRNAs and long non-coding RNAs, control the process of histone acetylation by influencing the expression of histone acetyltransferases (HATs) and histone deacetylases (HDACs). The aberrant production of non-coding RNAs has been linked to changes in histone acetylation associated with infertility [71]. This emphasizes their potential as biomarkers for diagnosis and targets for treatment. Genetic variations impacting genes responsible for HATs and HDACs can make individuals more likely to experience infertility by changing the dynamics of histone acetylation and the structure of chromatin [72]. Comprehending the intricate relationship between genetics and epigenetics in the development of infertility is crucial in order to devise individualized strategies for the diagnosis and treatment of this condition. Although there has been notable advancement in comprehending the function of histone acetylation in infertility, several obstacles persist. These include the requirement for thorough mechanistic investigations, the creation of non-intrusive diagnostic instruments, and the use of study outcomes in clinical settings. The integration of genetics, epigenetics, and reproductive biology through collaborative multidisciplinary endeavors is necessary in order to effectively tackle these issues and enhance our comprehension of the causes behind infertility [73].

1.6. Male infertility

Epigenetic modifications affect both male fertilization potential and sperm function. The appropriate functioning of epigenetic mechanisms, such as chromatin remodeling, histone tail modifications, and ncRNA DNA methylation during gonadal development, is necessary for normal sperm generation and function [9]. When it comes to clinical settings, semen analysis is frequently employed to identify the trend of male-related infertility; this is because microscopic inspection and DNA fragmentation analysis are insufficient to account for every occurrence. The diagnosis of unexplained male infertility (UMI) is sometimes hampered by the inability to identify spermiograms from those of fertile persons in infertile patients. However, current developments in sequencing technology hold promise for elucidating the reasons behind the idiopathic infertility experienced by certain couples [74]. The major epigenetic changes that take place at the sperm RNAs are reflected in the remodeling of chromatin, residual histone modifications, and DNA methylation. The amount of sperm also appears to be relevant [75]. Sperm motility, morphology, and evolution are influenced by the activity of the enzyme Methylenetetrahydrofolate reductase (MTHFR) and its byproduct, S-adenosylmethionine (SAM). It has been demonstrated that the MTHFR enzyme participates in the metabolism of folic acid and that its activity may have a major effect on spermatogenesis [13]. According to a study, the MTHFR hypermethylation gene promoter is frequently found in sperm taken from infertile people. It is also comparatively more common in men who have previously had spontaneous abortions than in men who have never had one, and it frequently affects the entire sperm population. This finding suggests a novel male characteristic associated with infertility resulting from spontaneous abortion. Because of this, the MTHFR hypermethylation gene promoter seems to be a novel potential risk factor in the etiology of spontaneous abortion [76]. In another study, it was found that patients with impaired sperm DNA integrity exhibited abnormal methylation in the gene promoters associated with imprinting, spermatogenesis, and antioxidant defense [15].

1.7. Female infertility

A study's findings indicate that illnesses and gene dysregulation are caused by epigenetic deviation, a significant biological element. It has been demonstrated that the pathophysiology of endometriosis is effectively influenced by aberrant expression of the HomeoboxA10 (HOXA10) gene and epigenetic constructs [77]. Several studies, like those conducted by Taylor and Gui, have demonstrated that females with endometriosis exhibit anomalies in the endometrium that are associated with the HOXA10 gene, a regulator of bodily growth. Situated on chromosome 7p15.2, this master regulatory gene belongs to a broader group of transcription parameters that bind DNA and share a 183-nucleotide sequence that encodes a homeodomain consisting of 61 amino acids. The HOXA10 homeobox gene controls the formation of the uterus throughout early development and the establishment of a functional endometrium in maturity [17]. The expression of the HOXA10 gene in fertile women who have no health issues is contingent upon the menstrual cycle. HOXA10 messenger RNA (mRNA) expression significantly rises during the mid-secretory phase, coinciding with embryo implantation. This stage is marked by increased histological differentiation and elevated systemic levels of estrogen and progesterone [18]. The increased expression of HOXA10 in the endometrium is vital for promoting the conversion of endometrial stromal cells into decidual cells, a process necessary for the proper attachment of the embryo. A dysregulation in the expression of HOXA10 and its associated regulatory mechanisms leads to recurrent miscarriages and infertility, resulting in compromised implantation and decidualization processes [78]. PCOS, endometriosis, and infertility of unexplained persons account for the majority of infertility cases in women [79]. One of the primary causes of infertility among women globally is PCOS. We also know that developmental programming and epigenetic modifications, in addition to genetic variants, can pass on a vulnerability to PCOS [80]. PCOS is a lifelong environmental factor that can lead to (epi)genetic predisposition to the development of hormonal and metabolic abnormalities. Since hormonal and environmental changes have a direct impact on PCOS development and clinical symptoms, epigenetic modifications may potentially have an impact on PCOS outcomes [9]. RPL is the term used to describe three or more spontaneous abortions with the same partner that occur before 28 weeks of gestation. Approximately 5% of women worldwide who are of reproductive age suffer from this issue [81]. The American Reproductive Medicine recently revealed epidemiological statistics that indicated a 15–20% prevalence of spontaneous abortions, with the frequency of RPL accounting for 2–5% of all pregnancies [82]. The etiology of RPL encompasses a multifaceted mechanism including aberrations in chromosomal structure and function, immunological dysregulation, endocrine disturbances, aberrant reproductive anatomy, maternal factors, prethrombotic conditions, and environmental

influences, among other contributing variables (Fig. 2) [83,84]. Despite extensive study, the underlying causes and mechanisms of around 50% of RPL instances remain poorly understood. These instances are commonly known as unexplained recurrent pregnancy loss (UPRL). Investigating the underlying mechanisms of Unexplained Recurrent Pregnancy Loss (URPL) and implementing timely intervention strategies are of utmost importance in order to enhance the likelihood of successful live births following RPL and to improve overall pregnancy outcomes.

Epigenetics has introduced a fresh viewpoint on the origins of RPL. Epigenetics encompasses the mechanisms by which cells can maintain or modify gene expression in a stable and heritable manner, without altering the DNA sequence. These mechanisms include DNA or RNA methylation, post-translational modifications of histones or chromatin, and regulation by non-coding RNA. Epigenetics significantly influences the onset and progression of RPL by controlling the activity of crucial genes that govern cell specialization, growth, and programmed cell death. These epigenetic modifications can be inherited across generations and impact the health of future offspring. These genes are anticipated to serve as novel targets for RPL treatment as well as prospective biomarkers for diagnosis. RPL has been linked to abnormalities in DNA methylation, methylation modification, and other related epigenetic changes, according to several epigenetic studies. The processes of embryo implantation, growth, and development can be impacted by imprinting barriers, gene expression disorders, spermatozoa defects, and immunological imbalances brought on by aberrant DNA methylation, all of which can eventually result in RPL. This review elaborates on the significance of DNA methylation in the etiology of RPL and its regulatory pathways, offering a novel approach to RPL diagnosis and treatment.
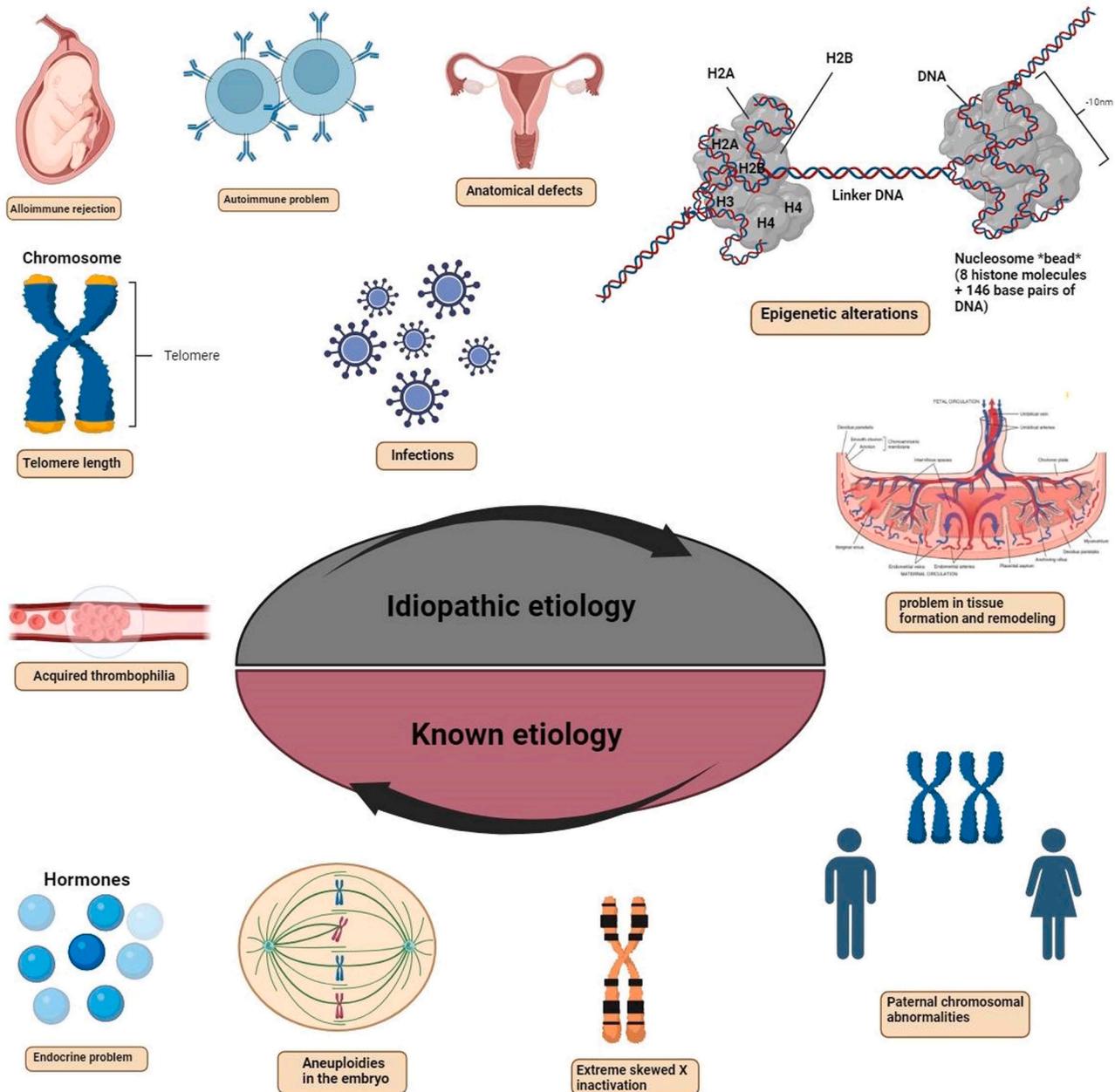

Fig. 2. High risk of RPL [21]. By asking detailed questions about the gestational age and the characteristics of the miscarriage, the etiology of RPL can be diagnosed. Prethrombotic state (PTS), immunological dysfunction, endocrine disorders, aberrant reproductive systems, poor environments, and lifestyle choices may all be associated with early RPL. The function of the uterine cervical, the development of amniotic fluid, and the umbilical cord may also be linked to late RPL. This review specifically examines the atypical immune function of RPL and analyses the significant significance of DNA methylation.

2. DNA methylation and related enzymes

DNA methylation is a well-established and thoroughly researched epigenetic alteration, with a particular focus on CpG islands as a key component. DNA methylation serves as a regulatory mechanism that hinders the binding of transcription factors (TFs) to DNA or facilitates the recruitment of proteins containing methyl-binding domains, hence promoting chromatin remodeling. The process of gene expression silencing is achieved via the generation of 5-methylcytosine (m5C) [85]. De novo methylation (using DNMT3a and DNMT3b) and demethylation (using TET1, TET2, and TET3) are the mechanisms by which protein systems inside biological contexts produce methylation patterns on DNA. Furthermore, these systems duplicate DNMT1 and UHRF1, two components that preserve methylation patterns during DNA replication, accurately [86]. Mammalian development and illnesses are significantly influenced by the formation, maintenance, and clearance functions of DNA methylation, a significant epigenetic change [85]. Numerous biological activities are regulated by DNA methylation, according to prior research. These encompass a variety of processes, including X chromosome inactivation, chromatin structural remodeling, gene transcription, chromosomal stability, and genome imprinting. In the early stages of human embryo development, pregnancy events such as fertilization, embryo implantation, and placental development are also closely associated with DNA methylation [87,88]. Abnormal immune tolerance, improper decidua vascular remodeling during placental development, and unsuccessful cell invasion in the early stages of pregnancy are all potential causes of URPL. These factors are all governed by a complex web of genetic and epigenetic alterations [87]. DNA methyltransferases (DNMTs) play a major role in DNA methylation by facilitating the transfer of a methyl group from S-adenyl methionine (SAM) to a cytosine residue's fifth carbon, which becomes 5-methylcytosine (5McDNMT1, DNMT3a, DNMT3b, and DNMT3L are among the enzymes [31]. The maintenance of the DNA methylation pattern during the S phase of cellular division is the responsibility of DNMT1 [32]. Both the induction of de novo methylation in vivo and the creation of novel DNA methylation patterns throughout embryonic development require DNMT3a and DNMT3b [31]. Despite lacking the conserved catalytic domain commonly seen in DNA methyltransferases, DNMT3L serves as a crucial auxiliary protein for DNMT3a-induced de novo methylation in the germline [33]. DNMT3c, a replicated form of DNMT3b, specifically appears in rodent species and functions to facilitate DNA methylation in repetitive sequences inside the male germline [34,35]. All the DNA methyltransferases (DNMTs) play a significant role in the embryonic development process. The deposition of methylation marks is dependent on the catalytic activity of DNMTs, whereas the active removal is dependent on the activity of ten-eleven translocation enzymes (TET) and thymine DNA glycosylase (TDG) [36]. DNA demethylation has been shown to play a role in various biological processes, including the preimplantation of the embryo and the growth, maintenance, and differentiation of embryonic stem cells (ESCs), as well as neurological function and cancer [37]. Research has demonstrated that the main techniques employed to accomplish global DNA demethylation of the preimplantation embryo genome on a global scale are TET3-mediated oxidation and passive dilution [38]. Female mice lacking TET1 exhibit disruptions in the process of primordial germ cell (PGC) meiosis. This condition is linked to insufficient demethylation, which leads to the failure to activate genes involved in meiosis [39]. Conversely, the absence of TET1 leads to abnormal methylation patterns detected in imprinted genes inside primordial germ cells (PGCs) and sperm cells in male mice [40]. Moreover, it is worth mentioning that the presence of both TET1 and TET2 has been observed in mouse embryonic stem cells (mESC) [41]. The deletion of TET1 can be used to manipulate embryonic stem cells (ESCs) and guide them toward specific lineages. On the other hand, the deletion of TET2 has the potential to limit enhancer activity and cause a delay in transcriptional alterations throughout the process of differentiation [89]. Triple knock-out (TKO) of ESCs with

TET1/TET2/TET3 has [43]. They exhibit pluripotency markers and have essentially normal morphology, but they have compromised developmental potential and differentiation. To sum up, aberrant DNA methylation can alter the development of embryos by obstructing their implantation and placenta formation, potentially resulting in unfavorable pregnancy outcomes.

3. DNA methylation dynamics in early embryo development

One important epigenetic regulation mechanism in animals during embryonic development is DNA methylation. Mammals go through a full genome reprogramming process of demethylation-remethylation throughout gametogenesis and early embryo development phases, realizing the continuation of the germline and the transfer of genetic information [90]. Two demethylation-remethylation waves are thought to have occurred during development since 1987. The early waves of remethylation and demethylation occur during the migration of proliferating primordial germ cells and before mature gametes emerge, respectively. The methylation marks on the imprinted genes are erased from the primordial germ cells during the fetal period. DNA methyltransferases reliably maintain these marks throughout cell divisions and reinstate them during spermatogenesis until the primary spermatocyte stage. During the second wave of demethylation that occurs after fertilization, the epigenetic memory of the gametes is largely eliminated. As a result, the blastocyst exhibits the lowest amount of DNA methylation, which is measured at 45. Following the process of embryo implantation, progressive remethylation takes place, ultimately leading to a restoration of the initial high level [91]. To elucidate, the methylation profile of the genome in the early stages of human embryonic development can be described as follows: before fertilization, the methylation level is notably elevated; thereafter, demethylation events transpire throughout the cleavage stage; ultimately, the methylation level reaches its lowest point upon the formation of the blastocyst. A novel methylation mechanism has been identified to interact with the uterus subsequent to embryo implantation, with the aim of reinstating the initial high level of methylation. The maintenance of pluripotency in the developing embryo relies on the periodic and systematic alteration of DNA methylation [92]. Subsequently, it is mirrored during the ovulation phase, reaching the minimum methylation level created by the blastocyst, and ultimately, the mother-to-child interface's new methylation state. In their examination of the reprogramming process of DNA methylation modification during preimplantation development of mouse-cloned embryos, Gao Shaorong et al. discovered that aberrant DNA remethylation is the main obstacle leading to abnormal development of cloned embryos after implantation [93]. From this work and the literature, it can be deduced that the orderly and periodic changes in DNA methylation that take place in the early phases of embryonic development represent a novel research focus for RPL.

4. RPL and abnormal or defective DNA methylation

DNA methylation takes place during the whole reproductive process, encompassing gametogenesis, embryonic development, and the formation of the maternal-fetal interface. Abnormal DNA methylation might be a potential cause of early pregnancy loss, which refers to miscarriage or abortion occurring within the sixth week of conception. An intricate network of genetic and epigenetic alterations regulates the initial development of the embryo, invasion of trophoblast cells, maintenance of the immune tolerance environment in the uterus, and modification of the uterine spiral artery. These modifications promote the incidence of URPL. Aberrations in DNA methylation have been linked to around 4% of spontaneous abortions globally [50]. Consequently, DNA methylation is essential for both embryo fertilization and the proper development of the fetus. Vasconcelos et al. first discovered the malfunction of imprinted genes and epigenetic modifiers in the placenta and fetal tissues in idiopathic spontaneous abortion. The author noted that methylation of

MEST, KvDMR1, and H19 DMR increased during the trimester, while TET2/3, IGF2, and CDKN1C were up-regulated [51]. The process of DNA methylation might hinder the expression of specific crucial genes in the embryo, impeding its appropriate growth and development and elevating the likelihood of RPL. The process of DNA methylation involves the targeted insertion of DNMTs and the removal of TETs. Patients diagnosed with URPL had a decrease in methylation levels in both chorionic villi and decidual tissues. This decrease in methylation was found to be associated with a decrease in the expression of DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) and an increase in the expression of DNA demethylase (TET1/2/3) [52]. During the receptive phase of the mouse endometrium, there was a significant reduction in the expression of DNMT1 and DNMT3a. Empirical studies have demonstrated that the DNMT3b gene polymorphism (rs1569686) and the DNMT3a gene polymorphism (448 A > G) could potentially serve as genetic indicators for increased vulnerability to RPL [54]. In a typical early pregnancy, the chorionic villus and decidua contain both DNMT1 and DNMT3a. DNMT1 inhibitor interference may result in a reduction in DNA methylation, hinder embryonic development, and prevent embryos from adhering to endometrial cells [55]. In a related study, it was discovered that the expression levels of DNMT1 and DNMT3a in the endometrial tissue dropped in pregnant mice given the DNA methylase inhibitor 5-Aza-CdR. Furthermore, there was a notable reduction in the rate of embryo implantation and inhibition of trophoblast cell growth [56]. Tanaka S. et al. discovered that TET1 and TET2 control cyclin B1 expression using protein-protein interactions. The enzyme also controls the intracellular replication cycle and the integrity of trophoblast cells, both of which have an impact on the fetus's development [94]. Additionally, by interfering with gamete meiosis, early embryo totipotency, and the differentiation of certain lineages, the demethylases TET1/2/3 may contribute to the development of URPL [58]. By using inhibitors of methylation methyltransferase, it is expected that more studies will uncover new strategies for the treatment and prevention of URSA. DNA methylation is intimately related to the metabolism of folic acid, which is essential for the synthesis of methyl donors such as S-adenosylmethionine (SAM). Since the gene 5,10-methylenetetrahydrofolate reductase (MTHFR) is situated in the confluence of pathways that provide methyl groups for DNA methylation, DNA repair, and DNA synthesis, its methylation is a thrombophilic indicator [59]. MTHFR (methylenetetrahydrofolate reductase) gene mutation is much more common in RPL (recurrent pregnancy loss) instances (p=0.002). This discovery emphasizes the critical relationship between MTHFR methylation and the transformation process [60]. The clinical data indicate that the presence of the MTHFR (C677T/A1298C) polymorphism in RPL's spouse may potentially impact sperm concentration and the proportion of forward motility sperm (PR) through its influence on DNA methylation. Therefore, this genetic variant may increase the likelihood of experiencing early spontaneous miscarriage [61]. Furthermore, the presence of the MTHFR (C677T) polymorphism results in diminished enzymatic activity, perturbs the methylation cycle, and decreases the abundance of free methyl groups. The occurrence of preeclampsia (PE) and pregnancy loss (PL) may be associated with low global DNA methylation [95]. Rotondo and colleagues (year) discovered a significant level of methylation in the promoter region of MTHFR in the sperm of the RPL partner. Based on the aforementioned studies, it is postulated that aberrant methylation of the MTHFR gene may exert an influence on typical embryonic growth and development, hence contributing to the manifestation of RPL [63]. The effectiveness of folic acid therapy in treating methylation anomalies in individuals with RPL associated to MTHFR is under doubt, with the goal of improving pregnancy outcomes. Further investigation into the complexities of folate metabolism and potential compensatory mechanisms in patients with RPL is required to assess the validity of this parameter.

5. DNA methylation of imprinted genes and RPL

Genomic imprinting refers to the phenomenon wherein parental alleles undergo modifications during transmission to offspring via gametes. Alleles that undergo parental imprinting exhibit distinct expression properties. The tissue and stage-specific methylation and expression patterns of imprinted genes undergo dynamic changes during the process of development [96]. The placenta expresses almost 50% of known imprinted genes, which are essential for cellular differentiation and embryonic development [97]. Both aberrant production of inactive alleles and aberrant silencing of active alleles can result from aberrant DNA methylation in the imprint control region (ICR) of imprinted genes. This results in metabolic problems, fetal neurological abnormalities, and imprinting disorders or deletions, all of which have an impact on embryo development and lead to unfavorable pregnancy outcomes. Imprinted gene abnormalities have been linked in the past to early pregnancy loss, fetal mortality, protracted delivery, and the growth of embryo cancers [66]. According to Liang et al., down-regulating the imprinted gene PEGIO can result in irreparable pregnancy loss, so maintaining this gene's expression is crucial for early embryo development [67]. Zhang et al. established a correlation between the developmental defects and early pregnancy loss observed in cloned pigs and the changes in the expression of imprinted genes and the hypermethylation profile of the repetitive sections (PRE-1 and satellite DNA) [68]. Empirical investigations have substantiated that the maternal gene PHLDA2 is increased in expression in the placental tissues of RPL cases. Additionally, the deactivation of the Mash2/Peg10 gene can lead to abnormal placental development, potentially leading to the demise of an early pregnancy. Flisikowski et al. discovered that a small deletion in the PEG3 gene, which is inherited from the mother and is present in cattle, leads to the suppression of the paternal gene MIMT1. This deletion is associated with late pregnancy loss and stillbirth [69]. Moreover, during early to mid-pregnancy abortions, there was an upregulation of IGF2, PHLDA2, PEG10, and CDKN1C gene expression. Conversely, in late-pregnancy abortions or stillbirths, there was a downregulation of PEG10 gene expression [70]. The hypothesis that "aberrant methylation and expression patterns of imprinted genes may contribute to RPL " has also garnered support from several clinical investigations [71]. Liu et colleagues. found that both the maternal genetic imprinting gene GRB10 and the paternally inherited genes (IGF2 and PEG3) displayed significantly high levels of DNA methylation in their analysis of the villus samples of RPL [72]. PEG3 is essential for both the formation of progeny and p53-mediated cell death. Mutations and deletions of PEG3 may result in inadequate placental nutrition transfer and growth retardation in offspring, which may cause the death of the offspring [73]. IGF2 influences fetal growth and placental metabolism in addition to contributing to the synthesis of glycogen. Placenta-specific IGF2-knockout mice have much lower-quality placentas and fetuses [74]. GRB10 is situated on chromosome 11, and transgenic mice harboring this duplication showed pre- and postnatal growth retardation [75]. In summary, aberrant DNA methylation-related imprinting gene abnormalities can exacerbate fetal neurological, developmental, metabolic, and embryonic developmental diseases. The placenta is a crucial organ for the increased manifestation of imprinted genes. In the placental villi of RPL, the imprinted gene (H19/PEG1/LIT1/SNRPN) exhibited a significantly higher level of methylation compared to the standard level [76]. The methylation alterations occurring at specific locations in the placental villi may have a direct association with the risk of RPL, as suggested by the abnormal elevation of H19/ICR1 methylation in the villi [52]. During embryonic development, DNMT3L functions as a crucial structural protein that orchestrates DNMT3A/DNMT3b-mediated DNA methylation. Due to the DNMT3L-knockout oocytes' inability to form maternal-specific methylation imprints, the embryonic neural tube development was aberrant, which showed up as SA during the second trimester. Furthermore, it has been demonstrated that the secret to genomic imprinted markers is DNA methylation [98]. Sheng and colleagues obtained placentas from pregnant C57/BL mice at three different stages of development (E7.5, E13.5, and E19.5). They investigated the LncRNA that is influenced by DNA methylation during the

process of placental development. The findings demonstrated that lncRNA MEG8 (RIAN) was aberrantly up-regulated in trophoblast cells, which hindered cell invasion and proliferation, and that the MEG8 promoter was more methylated in RPL villi. The observation was connected by the author to the incidence of URPL [78]. Genetic imprinting issues or deletions linked to abnormal DNA methylation have been found to play a role in RPL by disrupting various aspects of embryo development, including neural-tube development, metabolism, placental nutrition transport, and trophoblast proliferation and invasion [79]. It is posited that the identification of novel prognostic markers, particularly in cases of RPL, particularly those of unexplained etiology, will significantly benefit obstetricians in terms of timely identification and treatment of RPL patients.

6. Sperm abnormal DNA methylation and RPL

Additionally, sperm DNA damage, aberrant semen parameters, and age are linked to RPL in males. The idea that normal embryo development depends on the intact paternal epigenome is becoming more and more supported by data [80]. DNA methylation is a prominent epigenetic change that plays a crucial role in ensuring the proper growth of sperm. It effectively condenses the chromatin structure within the sperm head and permanently suppresses the activity of gene promoters associated with gene imprinting [39]. Several investigations have indicated that the RPL group exhibits deficiencies in the integrity and compactness of sperm chromatin [81]. The presence of atypical DNA methylation in sperm has a disruptive effect on the process of spermatogenesis and the subsequent development of embryos, resulting in inadequate implantation and recurrent pregnancy loss [82]. The research study observed a notable decrease in global methylation levels of sperm in the group with RPL [83]. The methylation levels of promoters associated with development-related genes in mature sperm are frequently seen to be low. The involvement of sperm DNA methylation in the developmental processes of mammalian embryos has been elucidated by multiple researchers. To elucidate the underlying process by which DNA methylation influences sperm function and embryo development, several investigations were conducted utilizing animal models. Previous research conducted on rodents has shown that a reduction in overall methylation levels in sperm cells is linked to the loss of embryos after implantation [83]. The family of proteins known as DNA methyl-transferases facilitates methylation and keeps it stable [84]. Mutations in these proteins have been linked to chromosomal deactivation, disruption of embryonic growth, and subsequent demise, according to studies conducted on knockout mice [85]. Furthermore, research employing strong DNA methylation inhibitors in mice revealed a higher probability of embryo death [86]. It should be mentioned that rather than directly resulting from the suppression of DNA methylation, embryo loss may occur as a consequence of these inhibitors' harmful consequences [87]. In a recent study conducted by Rogenhofer et al., it was found that there were significant differences in the levels of P1 and P2 mRNA, as well as the ratio of these two protamines, between male partners of infertile couples undergoing in vitro fertilization/intracytoplasmic sperm injection and healthy control men [88]. Based on the existing data, it is plausible to hypothesize that alterations in sperm epigenetics have an impact on embryo development and are associated with miscarriage. We expect further investigation on this subject. Ankolkar et al. discovered that there was no discernible modification in the methylation of PEG1/DLK1/GTL2/PLAGL1. Nevertheless, a notable reduction in the methylation of the imprinted gene H19 was noted in the semen of the URPL spouse, in contrast to the typical levels. In their work, Khambata et al. (year) discovered several imprinted genes that displayed aberrant DNA methylation patterns in the sperm of people experiencing unexplained recurrent pregnancy loss (URPL). These genes, including IGF2-H19 DMR, intergenic differentially methylated region (IG-DMR), mesoderm specific transcript (MEST), zinc finger protein regulating apoptosis and cell cycle arrest (ZAC, also known as

PLAGL1), DMR KCNQ1 intron 10 gene (KVDMR), paternally expressed gene 3 (PEG3), and paternally expressed gene 10 (PEG10), were found to be associated with RPL. Additionally, the researchers observed a decrease in global 5-methylcytosine (5mC) levels in the sperm, further implicating these genes in the pathogenesis of RPL. The IGF2-H19 DMR has been identified as a potent mitogen that plays a crucial role in regulating the growth of embryos and placenta, as supported by previous research [89]. The primary symptoms of DLK1 loss are abnormalities in placenta formation and adipocyte differentiation, not embryonic mortality [99]. MEST promotes angiogenesis during trophoblast invasion and is expressed in human villi and invasive trophoblasts in the early stages of pregnancy [100]. PLAGL1 is involved in the control of the cell cycle, apoptosis, and embryonic development; its absence can result in the restriction of embryonic growth and eventual death [91]. Sperm concentration and motility are impacted by the hypo- and demethylation of H19, according to a meta-analysis [92]. Sperm's global 5mc level may be used as a diagnostic marker for RPL to identify "epigenetically abnormal" sperm; the best candidate genes will be those that are imprinted, like IGF-H19. Furthermore, less sperm is linked to MTHFR polymorphism variations [93], which can result in male oligospermia, infertility, and RA. According to Rotondo et al., aberrant embryo development and trophoblast proliferation, apoptosis, and differentiation may result in RA due to hypermethylation of the MTHFR promoter in the sperm of the URPL's spouse [94]. Ultimately, the paternal epigenome influences the quality of sperm, the early stages of embryogenesis, and the potential somatic health of future progeny [82]. More transgenerational research is required, with an emphasis on environmental exposures in the F0 population, impacts on the epigenetics of sperm, and any indications of a possible link to somatic health outcomes in progeny. In the end, this will offer a route to therapy, diagnosis, and prevention actions.

7. DNA methylation of placenta/decidual and RPL

The first organ to develop during pregnancy is the placenta, which is crucial to the control of embryonic growth inside the uterus. During fetal development, the placenta performs a variety of roles, including the exchange of nutrients, waste, and gas, as well as different metabolic and endocrine processes and maternal-fetal immunological tolerance [95]. Intrauterine growth restriction (IUGR) and preeclampsia (PE) can result from placental insufficiency. Placental-related preterm birth (PTBs) and chorioamnionitis (CA) can result from inflammation or infection in the placenta. Hydatidiform mole (HM) or choriocarcinoma (CA) are other terms for tumor formation in the placenta [64]. It is thought that epigenetic alteration is essential to placental growth and function [96]. The human placenta shares several phenotypic traits and epigenetic patterns with malignancies during the early stages of pregnancy, including widespread hypomethylation of the whole genome and localized hypermethylation of CpG islands [97]. Beginning in the pre-implantation phase and continuing during the entire pregnancy, the placenta's epigenetic control over imprinted and non-imprinted genes is significant [64]. Furthermore, because the placenta serves as a facilitator of communication between the mother and fetus and must be sensitive to a range of signals, DNA methylation may be more varied by nature. For this reason, the placenta serves as the primary organ for the expression of genes with imprints. Lim et al. analyzed the gene expression and DNA methylation of human placental tissue using RNA-seq and RRBS (bisulfite sequencing). The author proposed that immune response and cell cycle-related pathways were enriched in significantly changed genes [98]. It is noteworthy that human chorionic gonadotropin (HCG) is a crucial hormone for the development of embryos and the maintenance of pregnancy. Its coding genes, CGB5 and CGB8, carry imprinting sites of placental expression. HCG secretion is decreased and the abortion rate is markedly elevated in RPL when the CGB5 promoter is hemi-methylated [39]. Hannah et al. found a strong correlation between RPL and the DNA methylation of particular placental locations (such as

the raised AXL and decreased defensin β1) in a study that used BSP to detect the methylation of the villi in RPL [99]. In another study, Du et al. examined and contrasted the placental villi's genome-wide DNA methylation in RPL. According to the authors, trophoblast cell migration and death are stimulated, the GATA2-FOXA1 complex is recruited, and RPL is caused by the hypomethylation of the PRDM1 promoter [77]. Primordial germ cells and intestinal cells must be reprogrammed using PRDM1, a transcriptional regulator of embryo cell fate [100]. According to Wu et al.'s microarray analysis of the lncRNA expression profile, aberrant IGF2AS methylation in the placental villi may have an impact on the stability of an early pregnancy [101]. Prior research has indicated that the extracellular matrix's release of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) may be in balance throughout the formation and localization of the placenta. This indicates that the imbalance of MMPs/TIMPs and the emergence of URPL are caused by the expression of MMPs/TIMPs and modifications in the methylation of the promoter [102]. According to a clinical investigation, alterations in the placental DNA methylation of genes related to immune response, environmental adaption, and imprinted genes could potentially have a role in the genesis of RM [86]. Nevertheless, discerning whether these alterations are causative or a result of placental adaptation to an impaired embryo poses a challenge. Furthermore, the presence of various cell types, each of which has a distinct methylome, complicates research on DNA methylation in the placenta. The ensuing studies need to bolster the necessity of concentrating DNA methylation sequencing investigations on specific cell populations as opposed to complete tissue extracts. To sustain a pregnancy, decidua, the maternal portion of the placenta, maintains a precarious balance between immunological tolerance and defense [103]. Proliferation, migration, and invasion of trophoblast cells, as well as endometrial decidualization, occur simultaneously with the creation and maintenance of a normal pregnancy. Abnormalities in trophoblast cell invasion, excessive invasion, or improper decidualization of the endometrium can all lead to RPL. The reprogramming of endometrial stromal cells, the synthesis of different mediators like cytokines and chemokines, and the targeted recruitment of immune cells are all part of the endometrial decidualization process. Decidua is a vital component of the maternal-fetal interaction and is essential to the initiation and continuation of pregnancy. To protect the mother's uterus, it first restricts the overwhelming invasion of trophoblast cells [104]. Secondly, growth hormones, glycogen, and lipid droplets secreted by deciduals supply nourishment for the development of embryos [105]. Third, to sustain a healthy pregnancy, the decidual improves the development of the mother-fetal immunological tolerance milieu [106]. Unusual DNA methylation disrupts decidual-related genes' expression, which lowers immunological tolerance, prevents cell invasion, and results in inadequate remodeling of the uterine spiral artery. Inflammation, poor placental development, and incorrect implantation may result from these [107]. A DNA methylation analysis of the decidual in RPL revealed that overexpression of SGK3, a serine-threonine protein kinase regulated by glucocorticoids involved in epithelial ion transport and cell survival, and CREB5, a cAMP responsive element binding protein 5, can interfere with trophoblast migration, apoptosis, and dysfunction, thereby promoting the occurrence of RPL [108]. According to the GO analysis, the hypo-DMR close to CREB5 may be able to bind P53 and SP1, upregulating the expression of the CREB5 gene. This would increase cell migration and death while inhibiting the cell cycle [109]. Furthermore, CREB5 has been linked to several aspects of the immune response, such as regulating T/B cells, promoting the transcription of immune-related genes (including IL-10), and promoting macrophage survival [109]. That suggests that via controlling the immunological system, CREB5 hypomethylation may encourage the development of RPL. Using a miRNA-mRNA network in decidual, Chen H. et al. discovered several small molecules that are important in the onset and progression of URPL, including FCGR1A/3 A (important receptors for innate and adaptive immune responses), CXCL8 (also known as IL-8, an important

inflammatory factor), HCK [expressed in bone marrow cells, B lymphocytes, and various cancer cells to enhance the expression of myelin growth factor and pro-inflammatory cytokines], PLEK (a crucial substrate of protein kinase C, phosphorylation of PLEK can promote the secretion of pro-inflammatory cytokines in phagocytes), IL10 (low IL-10 are associated with pregnancy complications, such as RA, premature delivery, PE, IUGR], and has-miR-498 and has-miR-4530 [110–112]. In this retrospective observational case-control study, it was found that there was an increase in succinate dehydrogenase complex iron-sulfur subunit (SDHB) DNA methylation and SDHB expression, whereas succinate levels dropped in the decidua of individuals with RPL. Insufficient buildup of chorionic succinic acid hinders the invasion and proliferation of extravillous trophoblast cells via the PHD2-VHL-HIF-1α pathway, hence impacting the process of embryo implantation [113]. Xie et al. discovered that the estrogen receptor (ER), which mediates the transcription of lncHZ08, was upregulated and the DNA methylation of the lncHZ08 promoter was reduced in the decidua of RPL. By down-regulating PI3K, the up-regulated lncHZ08 suppresses the PI3K/p-AKT/p-P21/CDK2 pathway, consequently decreasing trophoblast cell proliferation, migration, and invasion and further inducing abortion [114]. In their study, Fatima et al. [115] employed structural equation modeling to examine the causal association between methyltransferase, methylation, and cell apoptosis in the context of abortion. This study presents novel findings on the association between the methylation of the p53 pathway (BAX/P53/CASPASE-6/BCL-2) mediated by methyltransferase (G9aMT/DNMT1) and the outcome (continuation or cessation) of early pregnancy [116]. It is noteworthy to mention that the atypical reaction of human endometrial stromal cells (HESCs) to decidual signals is strongly associated with RPL. The analysis of MEDIP-Seq data on human embryonic stem cells (HESCs) indicates a substantial correlation between the decrease in methylation levels of CA-rich sequences and the presence of RPL. These CA-rich sequences are known to be extensively distributed across the genome and are particularly abundant in regions around telomeres. This study posited that the aberrant DNA methylation of human embryonic stem cells (HESCs) within the uterine environment can lead to the loss of their epigenetic stemness. Consequently, this can facilitate the senescence of stromal cells, restrict the flexibility of the endometrium, impede the formation of decidua, and ultimately contribute to recurrent pregnancy loss [117]. Studies using HESCs in vitro have demonstrated that the expression of decidual-specific IGFBP-1 can be downregulated in DSC due to DNMT3b inactivation. This suggests that decidualization can be eliminated before or upon implantation by blocking DNA methylation, which can result in pregnancy loss [118]. Down-regulating DNMT1 and DNMT3a in the endometrium of mice injected with DNMT inhibitors (5-Aza-CR) at varying stages of pregnancy led to defects in stromal cell proliferation and endometrial decidualization, which may potentially lower the rate of embryo implantation [112].

7.1. The regulation of the maternal-fetal immune tolerance microenvironment is influenced by DNA methylation, which also affects RPL

A common view is that the endometrium is an allograft that the embryo comes into intimate contact with. The endometrium's main function is to create and maintain the optimal immunological, molecular, and endocrine/paracrine environment necessary for the embryo's proper attachment, implantation, invasion, and growth. The endometrial stromal cells go through a significant morphological and functional reprogramming process known as decidualization during the gestational period. Numerous elements are included in this reprogramming, including tissue remodeling, changes in gene expression, post-translational regulation, and adjustments to cell signaling pathways. Significant alterations in the activity of immune cells also occur within the uterine environment. The maternal-fetal interface refers to a specialized microenvironment characterized by immunological

tolerance. It consists of the maternal decidua and fetal placenta, both of which play crucial roles in facilitating the successful progression of pregnancyThe user's text is empty. This section presents a detailed summary of the composition of immune cells and the corresponding changes in DNA methylation at the interface between the mother and fetus in cases of RPL. This study aims to examine the influence of DNA methylation on the immune environment of the uterus and its connection to RPL. The interplay between the maternal and fetal immune systems is a crucial factor in the processes of embryo implantation and the maintenance of pregnancy. Several academics have postulated that URPL (unexplained recurrent pregnancy loss) can be classified as an allogeneic immunological disorder associated with the breakdown of maternal-fetal immune tolerance. This implies that the outcome of a pregnancy is contingent upon the immune status of the pregnant individual and the embryo's capacity for immune regulation [101]. In their study, Du et al. conducted a comprehensive investigation of DNA methylation across the entire genome in placental villi affected by RPL. Their findings revealed the presence of numerous differentially methylated regions (DMRs) near genes that exhibited dysregulation, including PRDM1, in the context of RPL. The enrichment of differentially expressed genes in the immune response pathway suggests a potential link between aberrant immune regulation and the development of RPL [77]. The disparities in the immunological milieu at the interface between the mother and fetus are often manifested in two dimensions [120]. The absence of negative signal activation for maternal-fetal tolerance can have a detrimental effect on the process of embryo

implantation or formation. Conversely, an excessive amount of immune system activation could cause increased inflammation in the uterus or damage to trophoblast cells. Chuang and colleagues performed a study using a technique called single-cell high-throughput sequencing to thoroughly analyze the cellular lineage of the decidual immune milieu in cases of recurrent pregnancy loss RPL. The researchers documented a variety of cell types, including natural killer cells, macrophages, dendritic cells, T cells, and Invitro decondensation of sperm chromatin in conjunction with modification-dependent retention of sperm histone (Fig. 3)[121].

7.2. Chromatin reorganization in RPL

Spermatozoa efficiently compact a significant quantity of DNA within a compact nucleus by the process of rearranging sperm chromatin. Small proteins known as protonucleins are specific to spermatozoa. Sperm DNA takes up less room in the nucleus because of the protamine-related passage that histones contain. Consequently, this process leads to the formation of a relevant condensation in the sperm nuclei, characterized by a denser structure that facilitates the enhancement of sperm motility. Furthermore, protamination serves the purpose of safeguarding the integrity of the sperm genome by preventing its destruction, oxidation, and exposure to hazardous chemicals present inside the female reproductive system [9]. In the last stages of male gametogenesis, known as spermiogenesis, haploid spherical spermatids give rise to motile spermatozoa. A significant remodeling of
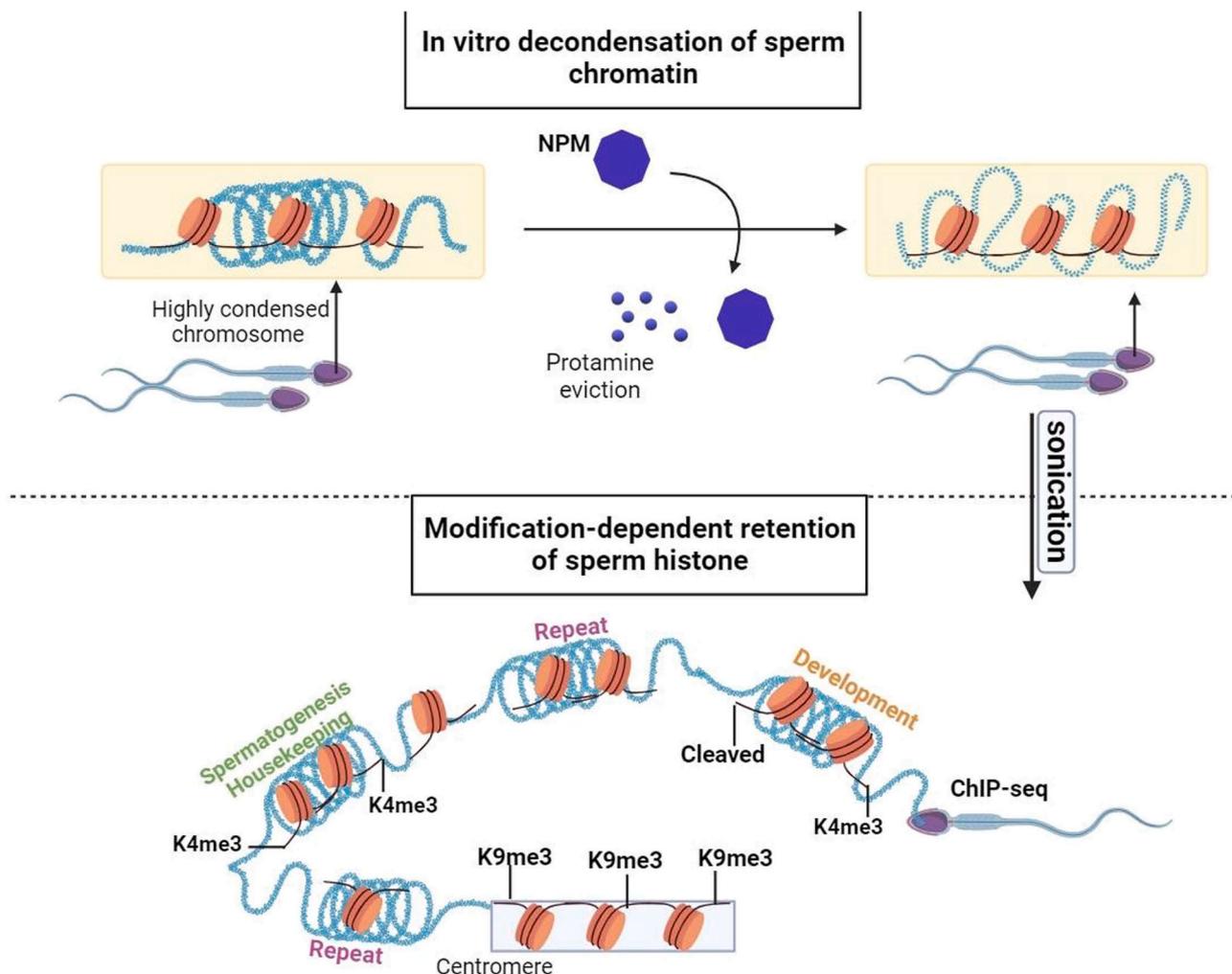

Fig. 3. Invitro decondensation of sperm chromatin in conjunction with modification-dependent retention of sperm histone.

chromatin occurs, with transitional proteins initially replacing nearly all (90–95%) of the nucleosomal core histones H2A, H2B, H3, and H4 (nucleosomal core histones). Protamines then take their place [74]. Spermatozoa are widely recognized as highly specialized cells. During spermatogenesis, approximately 90–95% of chromatin histones are replaced by protamines, which are nuclear proteins that are tiny and rich in arginine. The process described above, which is expected to occur in sperm cells, leads to substantial condensation of DNA, reduced vulnerability to external factors, and functions as a mechanism for gene suppression. Histone acetylation first increases during the early phases of protamination, promoting DNA topoisomerase activity. Next, transition proteins (TP1 and TP2) take the place of histones. Protamine 1 (P1) and protamine 2 (P2), which are both expressed at the same levels, replace histones in a process that is initiated by the previously indicated DNA-binding proteins. After the process of sperm chromatin protamination, three levels of DNA organization may be observed: [1] toroidal structures formed by protamines, which account for 90–95% of the DNA, [2] nucleosomes, which make up 5–10% of the DNA and play a significant role in the early stages of embryo development, and [3] matrix binding sites, also known as non-nucleosomal DNA regions. DNA segments lack toroidal structures and nucleosomes. Matrix Attachment Regions (MARs) have a crucial role in providing structural support to chromatin and functioning as promoters during the creation of the paternal pronucleus following fertilization. Additionally, MARs contribute significantly to the proper development of the embryo. As a result, empirical research has demonstrated that deviations in protamine levels can influence the transmission of epigenetic information carried by paternal DNA. Consequently, the outcome of assisted reproductive technology (ART) is influenced by the status of sperm protamination [12].

7.3. Histone alterations in RPL

Alterations to histone proteins can have either a negative or positive impact on the interaction between regulatory factors and DNA. This, in turn, can lead to a decrease or increase in the activity that is relevant to gene regulation and expression. Testicular tissue has been shown to have enhanced gene expression by a variety of modifications, such as acetylation of histones H3 and H4, methylation of histone H3 at lysine 4 (H3K4), and ubiquitination of histone H2B. In contrast, the process of methylation involving H3K27, H3K9, and ubiquitination involving H2A leads to the suppression of gene expression. Previous studies have proposed that the methylation of H3K4 and H3K27 plays a role in both the activation and inactivation of gene expression [122]. The presence of histones in gene clusters of imprinted nature is observable, indicating that protamination and alterations in residual histones may be a shared factor contributing to paternal infertility. Correspondingly, a study was undertaken on a total of 291 assisted reproductive technology cycles to investigate the role of histone-protamine ratio (HPR) in embryonic development and ART results. The rate of blastocyst formation for HPR, which varied from 6% to 26%, exhibited a significantly higher value (87.8%) compared to the rates reached for HPR greater than 6% (74.6%) or less than 6% (71.2%). Subsequently, it may be observed that HPR exerts an influence on the development of embryos. According to the available information, it is necessary to utilize protamine-free sperm, in addition to addressing any remaining histone abnormalities, in assisted reproductive technology programs [12].

7.4. RNA molecules from sperm that are both coding and non-coding

Research efforts have increasingly focused on identifying the roles of these ribonucleic acids (RNAs). Although sperm are transcriptionally inactive, they have been shown to contain both non-coding (ncRNA) and coding (mRNA) molecules. RNA molecules have been recognized as essential for the transfer of epigenetic information in various biological processes, such as early development and spermatogenesis. Sperm cells contain a diverse array of RNA molecules, including both non-coding RNAs (ncRNAs) such as antisense RNA, tiny interfering RNA (siRNA), micro-RNA (miRNA), long non-coding RNA (lncRNA), and piwi-interacting RNA (piRNA), as well as coding RNAs (mRNAs). These RNA molecules play a role in regulating gene expression by interfering with the translation of mRNA molecules through various mechanisms [123]. The examination of transcriptomic data related to sperm samples obtained from males with idiopathic infertility (specifically normozoospermic), asthenozoospermia (characterized by reduced motility), and known fertility revealed distinct RNA profiles across the different patient groups. These findings underscore the potential significance of these molecules in the context of paternal fertility [124]. There is evidence indicating that environmental toxicants can impact the development of testicular illness and the epigenetic transgenerational transmission of male infertility. This process encompasses modifications in the germline, specifically in sperm cells, which subsequently impact the epigenome and transcriptome of early embryonic stem cells. Testicular illness may play a significant role in the etiology of male infertility by enhancing epigenetic transgenerational inheritance, as evidenced by the sharp rise in infertility and decline in sperm count observed in the male population (125). In a different investigation, significant alterations at 6609 CpG sites connected to long-term infertility ($\geq$60 months) were discovered using methyloma analysis of individual blastocysts in comparison with fertile controls. When compared to assisted reproductive technologies alone, long-term infertility is linked to a changed methylome in euploid blastocysts with a particular focus on regulation relevant to genomic imprinting (Reference Denomme, Haywood, and McCallie 37). Moreover, H19 encodes non-coding RNA (126).

7.5. microRNAs in RPL

RPL is a multifaceted illness that can be influenced by several causes. MicroRNAs (miRNAs) play a crucial role in the development of RPL [102]. Small, non-coding RNA molecules known as microRNAs are of significant importance in a range of biological processes, including embryo implantation, placental development, and interactions between the maternal and fetal immune systems. Research findings indicate that the regulation of certain microRNAs (miRNAs) is disrupted in women diagnosed with RPL in comparison to those with normal pregnancies [103]. The variations in miRNA have the potential to impact many cellular pathways that are involved in trophoblast invasion, angiogenesis, apoptosis, and immunological regulation. These pathways play a crucial role in ensuring the successful progression of pregnancy [104]. An instance of reduced expression of miR-23a and miR-29b has been linked to compromised trophoblast invasion and heightened apoptosis, hence playing a role in the pathogenesis of retinal pigment epithelium RPL [105]. In contrast, the increase in miR-155 levels has been associated with heightened immunological reactions in the mother, resulting in the loss of pregnancy [105]. Moreover, microRNAs can modulate the expression of genes associated with the synthesis of inflammatory cytokines, chemokines, and other molecules that have the potential to influence placental development and function [106]. This plays a significant role in the etiology of RPL. the disruption of certain miRNAs is a vital factor in the occurrence of repeated pregnancy loss by impacting diverse cellular and molecular mechanisms that are necessary for a healthy pregnancy [107].

7.6. Exosomes in RPL

Exosomes are a specific category of extracellular vesicles (EVs) that have a size ranging from 40 to 120 nm. They are produced by cells through the endosomal pathway. Exosomes may be released into the extracellular environment by most cells, including malignant cells [108]. Exosomes host a diverse array of biomolecules, including proteins, nucleic acids (such as miRNAs), and lipids, which facilitate

intercellular communication by enabling their transport across cells [109]. The cargo can impact the receiving cells, such as via facilitating tumor advancement, metastasis, resistance to chemotherapy, and the formation of new blood vessels in the setting of cancer. Exosomes have garnered significant attention as potential diagnostic and therapeutic instruments owing to their distinctive characteristics [110]. The inclusion of these substances in bodily fluids renders them appealing as biomarkers for liquid biopsy in the context of early cancer diagnosis and prognosis. In addition, modified exosomes can serve as carriers for medication delivery, capitalizing on their exceptional biocompatibility and little toxicity [111]. Nevertheless, some obstacles require attention, including the establishment of standardized criteria for the characterization, isolation, and purification of exosomes [112]. Additional investigation is necessary to comprehensively clarify the processes that underlie the anticancer properties of exosomes and to guarantee the safety of their utilization in clinical settings [113]. In brief, exosomes are extracellular vesicles that serve crucial functions in intercellular communication and possess considerable promise as diagnostic and therapeutic instruments, particularly within the realm of cancer and other pathological conditions [114]. The complex involvement of exosomal miRNAs in the development of RPL is highlighted by the finding of maternal circulating exosomal miR-185–5p levels as a possible predictive biomarker [115]. The results of this study indicate that changes in the expression of certain exosomal microRNAs, such as miR-185–5p, might potentially play a role in the fundamental processes that drive retinal pigment epithelium RPL. Furthermore, recent studies have demonstrated that placenta-derived exosomes (PdEs) have heightened concentrations in women diagnosed with gestational diabetes mellitus (GDM) in comparison to those with pregnancies that are considered normal [116]. Considering the acknowledged association between GDM and RPL, it is pertinent to examine the altered levels and biological effects of PdEs within the framework of RPL pathophysiology [117]. Exosomes, which have a size range of 40–120 nm and come from the endosomal pathway, are important carriers of communication between cells and have crucial functions in controlling several cellular processes such as angiogenesis, proliferation, and metabolism. The possible contribution of dysregulation in exosome-mediated processes to the etiology of RPL lies in its impact on placental development and function [118]. The research article published in Pubmed1 emphasizes the potential of maternal circulating exosomal miR-185–5p levels as a prognostic biomarker for RPL. The researchers noted a notable increase in exosomal miR-185–5p levels among women who underwent RPL in comparison to those who had pregnancies without any abnormalities. These findings indicate that examining particular exosomal miRNAs, such miR-185–5p, might be used as a diagnostic method to identify individuals who are at risk of RPL. The elevated concentrations of these exosomal microRNAs may indicate underlying pathogenic alterations in the placenta or the maternal-fetal interface that contribute to the onset of RPL [119]. Despite the encouraging nature of this observation, the existing body of research does not provide any more evidence about the use of exosomes as diagnostic tools for RPL. Additional investigation is necessary to confirm the dependability and effectiveness of exosomal miRNA profiling as a diagnostic method for reproductive pelvic lymphoma RPL. Furthermore, doing further research on the wider scope of exosomal miRNA expression patterns and their correlation with RPL disease will yield significant knowledge on the prospective diagnostic and therapeutic uses of exosomes in this particular domain [120].

8. Endometrial stromal cells regulate local immune function

Proper decidualization of endometrial stromal cells is essential for early pregnancy implantation and maintenance, according to earlier investigations. This development has been linked to the substantial morphological and functional reprogramming of endometrial stromal cells, which differentiate into highly specialized cells with secretory capacities. Overall, it is becoming clear that the process of decidualization of the endometrium entails extensive cell reprogramming, tissue remodeling, modifications to gene expression and post-translation regulation, as well as adjustments to cell signaling pathways (125). According to this research, the endometrium is a highly accurate biosensor of the quality of embryos that have been implanted (126). Following the process of decidualization, normal endometrial cells in females acquire sensitivity to signals sent by embryos. Consequently, low-quality embryos can impede the secretion of substances that are advantageous for successful embryo implantation. In contrast, embryos with robust developmental potential are known to generate signals that facilitate the process of implantation (129). Poor pregnancy consequences result from the RPL's insensitivity to this signal, which permits the implantation of inferior embryos (Fig. 4)[120].

9. Macrophages

Studies on the role of macrophages in the development of RPL are scarce. Only during the luteal phase does the number of macrophages in the non-pregnant state dramatically increase. Near trophoblast cells that are invading the uterus and the modified uterine spiral artery are macrophages, which have grown quickly during pregnancy [117]. They take part in the implantation of the embryo, the invasion of trophoblasts, the remodeling of the uterine spiral artery, the elimination of dead cells and cell debris, and the defense of the developing embryo against infections or microbes (127). The following was reported from a clinical investigation that sought to identify the DNA methylation group of macrophages on the maternal-fetal interface: First, macrophages generated from maternal or fetal cells differ significantly in their DNA methylation patterns (128). Second, fetal-derived macrophages have significant levels of differential methylation in genes linked to immunological response. Abortion is associated with decidua M1/M2 macrophage imbalance (129). M2 macrophages enhance immunological tolerance by generating anti-inflammatory cytokines like TGF-β and IL-10, while M1 macrophages enhance the inflammatory advantage by releasing inflammatory cytokines including TNF-α, IL-β, IL-6, and IL-12 (130). In contrast to a typical pregnancy, there is no discernible decrease in M1 decidual macrophages in URPL (129). Even though there is currently no information on the uterine microenvironment of RPL macrophage DNA methylation. On the other hand, a MeDIP-seq of a preterm placenta revealed that aberrant DNA methylation was enriched in Fcy receptor-mediated macrophage phagocytosis (131), indicating that worse pregnancy outcomes may be associated with aberrant DNA methylation in macrophages.

10. Natural killer cells (NK Cells): are NK cells the "BAD BOYS" that reject embryos?

NK cells were initially identified as a subset of the innate immune system that possessed the ability to spontaneously kill both autologous and allogeneic target cells through cytotoxic action. NK cells can be classified into several subsets according to their phenotype and function, including innate lymphoid cells-1 (ILC1s), tissue-resident NK (trNK) cells, uterine NK (uNK), and peripheral NK (pNK) cells. This review concentrates on uNK cells, primarily CD56bright cells, which have limited cytotoxicity but can generate copious amounts of cytokines to safeguard alloantigens and control the pregnancy process (132). Early in pregnancy, the uNK cells surge and are seen next to the trophoblast cells that are invading the body (133). According to studies, activated uNK can release a wide range of cytokines, including GM-CSF, CSF-1, TNF-α, IFN-γ, TGF-β, LIF, IL2, CXCL10, and CXL12, as well as angiogenic factors including VEGF and ANG2 (134). During pregnancy, uterine natural killer (uNK) cells perform a distinct function in the regulation of trophoblast cell invasion and the remodeling of the uterine spiral artery, which is an essential process for the establishment of placenta formation (135). Furthermore, the uNK functions as an immune cell at the interface between the mother and the fetus, simultaneously taking on the roles of
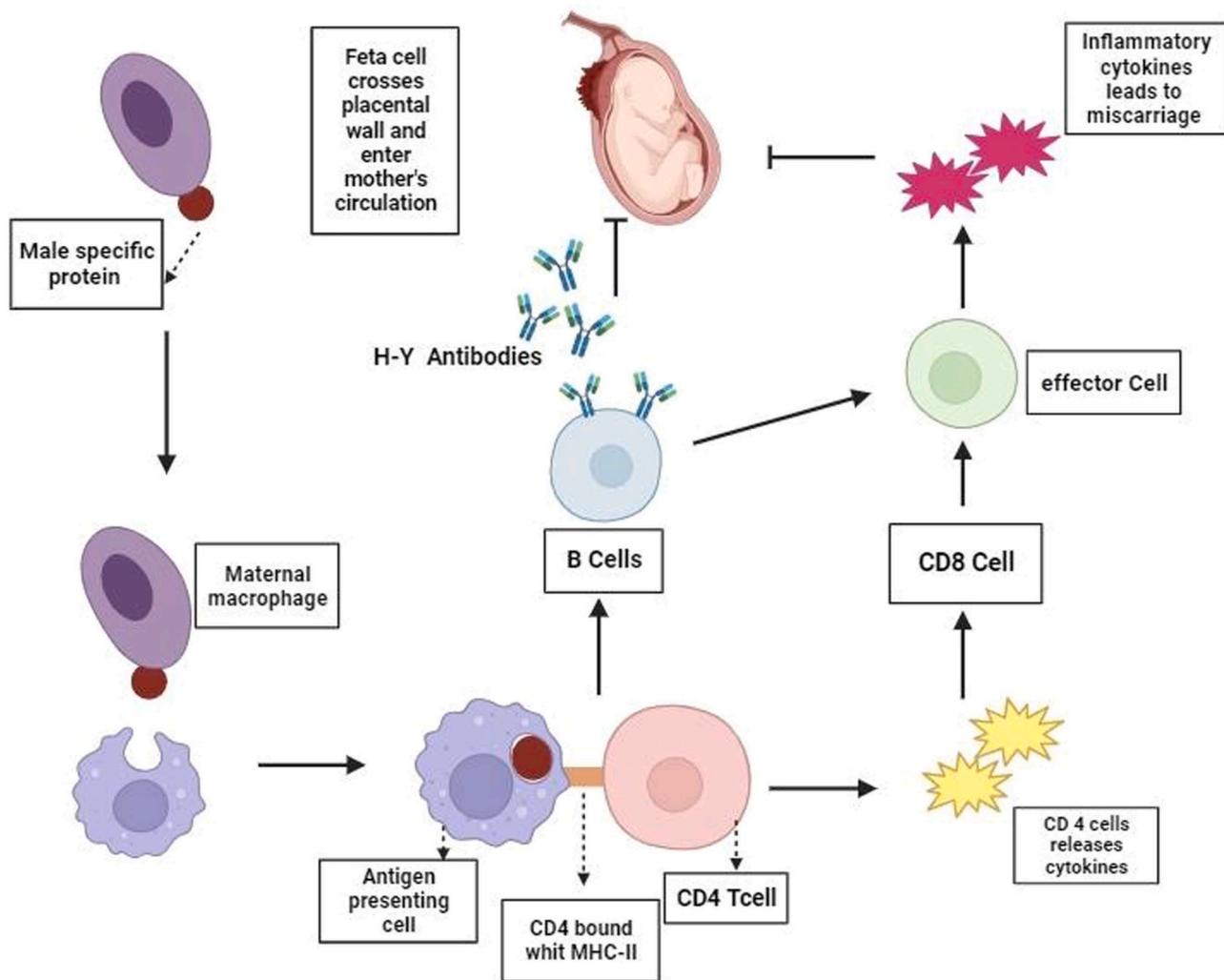
Fig. 4. Poor pregnancy results result from the RPL's insensitivity to this signal, which permits the implantation of inferior embryos.

immune tolerance (like a healthy pregnancy) and immune killing (like eliminating germs to prevent different types of inflammation) (136). Numerous clinical studies have shown that the quantity, caliber, and cytotoxicity of uNk cells in URPL are different from those in normal pregnant women (137). Pregnancy harm could result from the deregulation of uNK cells in RPL in five different ways: [1] The ability of NK cells to cause harm is preserved, albeit with impairments; [2] NK cells have trouble correctly interacting with the particular HLA expressed by trophoblasts; [3] NK cells have trouble effectively taking part in the entire remodeling of the uterine spiral artery; [4] T cell cytotoxicity is hindered; and [5] NK cells obstruct the process of cytokine production (138,140). Although the relationship between NK cells and RPL is currently widely established, the underlying epigenetic regulatory mechanism is yet unknown. It is yet unknown if the number, methylation state, and functional state of NK cells are connected to RPL.

11. The balance of Th1/Th2 or Th17/Treg and RPL

The Th1/Th2/Th17 and Treg cell paradigms, according to Saito et al. (2010), are critical for maternal immunological tolerance (141). Th1 cells are responsible for generating IL2 and INF-α, which are believed to be the starting points for allograft rejection and help support cellular immunity (142). Conversely, Th2 cells contribute to humoral immunity and release IL4, IL5, and IL13, which are essential for establishing and preserving allograft tolerance (143). Th1 cells contribute to immunological surveillance, produce cytokines (IL2/TNF-/IFN-), and provide

defense against excessive trophoblast invasion (144). During embryo implantation, tissue remodeling and angiogenesis are facilitated by a pro-inflammatory Th1 immune response (145). In contrast to typical pregnant women, pregnant women with RPL had increased Th1 levels in their peripheral blood (146). Large amounts of TNF-α are secreted by Th1 and NK cells, which ultimately results in pregnancy loss by causing apoptosis, inhibiting trophoblast development, and preventing the uterine epithelium from secreting granulocyte-macrophage colony-stimulating factor (147). The preponderance of Th1 cells in the immune response eventually transitions to Th2 cells during the implantation period. This transition aids in maintaining the mother's tolerance to fetal antigens until the time of birth (148). By producing IL-4 and IL-13, respectively, Th2 immunity suppresses the growth of Th1 and Th17 immunities and encourages allograft tolerance (149). In the meantime, it has been shown that the Th2 cytokine IL-4 stimulates autoreactive B cell activation, which in turn enhances autoimmunity (150). The heightened Th2 immune response observed during pregnancy has the potential to trigger autoimmune illnesses such as systemic lupus erythematosus. Conversely, the presence of a tolerogenic immunological environment may lead to uncontrolled viral infections, such as the ZIKA virus (151). The timely and sufficient development of Th2 immunity plays a crucial role in ensuring immunotolerance and safeguarding the fetus against infections. Hence, the prompt and appropriate migration and equilibrium of Th1 and Th2 cells during gestation appear to be imperative for the achievement of a viable pregnancy. On the other hand, pregnancy problems such as RPL and PE are linked to an

inappropriate balance throughout pregnancy. In patients with RPL, reversing the Th1/Th2 imbalance and compensating for a shortage of associated cytokines can bring new ideas for the focused therapy of RPL. In a mouse model of LPS-induced miscarriage, the administration of etanercept, which blocks TNF- receptors, and IL-10, which has immunoregulatory properties, decreased LPS-induced pregnancy losses (152). When the Th1 immunity was controlled using intravenous immunoglobulin G (IVIg), TNF blockers, or T cell activation inhibitors such as etanercept, adalimumab, or tacrolimus, the pregnancy result was significantly improved in women with RPL (153). Inducing inflammation and immunological rejection are crucial roles that are played by Th17 cells, which are responsible for the production of the effective proinflammatory IL17 (154). The pathogenicity of autoimmune illnesses, allergic reactions, immunological rejection, and adverse pregnancy outcomes have all been associated with the interaction between Th17 and Th1 (155). Furthermore, Th17 can stimulate dormant natural killer (dNK) cells and reduce the uterine artery's vascular reactivity, both of which can aid in the resorption of embryos [76]. Both the decidua and the peripheral blood of women who had an unavoidable abortion contained an increased number of Th17 cells (156). The process of mediating maternal-fetal tolerance, which is essential for embryo implantation and the initial phases of pregnancy, involves Treg cells (157). Treg proliferation is linked to a healthy pregnancy, while a decrease in Tregs can result in the pregnancy ending prematurely by encouraging the immune system to reject the embryos (157). In animal studies, the ingestion of Tregs results in a statistically significant increase in the incidence of abortion (158). On the other hand, the transfer of Tregs can prevent the loss of pregnancy in mice that are prone to miscarriage (158). In a similar vein, clinical trials have shown that there are fewer Tregs in the peripheral blood (159), endometrium, and decidua of RPL patients as compared to normal pregnant women. This is followed by an increase in the number of Th17 cells (160). FOXP3, an essential transcription factor, supports the hypothesis that regulatory T cells, often known as Tregs, may play a role in RPL. Wang and colleagues [108] discovered that hypermethylation of the Foxp3 promoter leads to a decrease in the expression of FOXP3, resulting in a reduction in the number of regulatory T cells (Tregs). This imbalance between Tregs and Th17 at the interface of the maternal and fetal immune systems promotes the development of RPL. Additionally, Treg cells can take part in the down-regulation of maternal-fetal excessive inflammatory response and embryo implantation (161), both of which might be factors in the development of RPL if they are out of balance or not functioning properly. In conclusion, the extremely plastic Tregs have the potential to develop into effector T cells under specific conditions. Tregs that are out of balance may play a direct role in the rejection of fetal tissue (162). Despite the absence of definitive evidence, researchers continue to speculate that aberrant DNA methylation of Th cells and Treg cells may play a significant role in RPL. Existing research indicates that abnormal DNA methylation may play a role in the development of RPL by influencing the growth, specialization, and cellular functions of both Th cells and Treg cells. Determining the ratio of Th cells at the interface between the maternal and fetal compartments and intervening in the activation of Th cells is beneficial for preventing and treating patients with RPL.

12. Cytokine-chemokine network unbalance in RPL

Numerous cell types within the endometrium, including trophoblast cells, stromal cells, decidual cells, immune cells engaged in innate and adaptive immunity, and endometrial glandular epithelial cells, can synthesize cytokines, chemokines, and their related receptors. A healthy pregnancy, effective embryo implantation, and control over trophoblast migration all depend on the creation of a local immunological balancing milieu. In individuals with RPL or animal models, immune and non-immune cells inside the endometrium and decidua exhibit dysregulation of cytokines associated with embryo implantation and early pregnancy. Therefore, the existence of an unfavorable cytokine environment

will impede the ability of the maternal immune system to develop tolerance towards the trophoblast, ultimately leading to immunological rejection. The occurrence of RPL is linked to a decrease in the secretion of TGF-β by decidual dendritic cells (163). The elevated levels of interferon-gamma (IFN-γ) in the decidua of RPL can initiate apoptosis and embryotoxic effects, thereby facilitating the development of RPL through the stimulation of an exaggerated inflammatory response in the decidua. Similarly, interleukin-10 (IL-10), along with transforming growth factor-beta (TGF-β) and indoleamine 2,3-dioxygenase (IDO), can induce differentiation of dendritic cells (DCs) into a tolerant phenotype and modulate the expansion of regulatory T cells (Tregs). The decrease in interleukin-10 (IL-10) levels inside the decidua of RPL patients is expected to impede the immune defense mechanism (164, 165). Given the intricate nature, delicate responsiveness, and adaptable characteristics of the endometrial cytokine-chemokine network, the comprehensive identification of all implicated cytokines and their cellular origins in RPL remains a challenging endeavor. Further exploration is required to elucidate the mechanisms and particular signaling pathways behind the imbalance in the cytokine-chemokine network generated by aberrant DNA methylation at the maternal-fetal interface, which contributes to the promotion of RPL. The comprehensive investigation of epigenetic mechanisms in immune regulation and associated signaling pathways of RPL is anticipated to offer prospects for individualized diagnosis and therapeutic interventions for RPL. Understanding the mechanisms generating and maintaining the immunological tolerance milieu at the maternal-fetal interface has advanced significantly during the last three decades. The maternal immune system has a role not only in maintaining proper immunological communication at the interface between the mother and fetus, but also in processes such as endometrial decidualization, vascular remodeling, and placentation. The involvement of the maternal-fetal immune tolerance milieu in the various stages of a normal pregnancy, including establishment, maintenance, development, and termination, is readily apparent. The prevention of pregnancy loss resulting from maternal immune dysfunction can be achieved through the induction of comprehensive immunological tolerance at the maternal-fetal interface, while simultaneously ensuring the preservation of complete immunological reactivity against all other foreign antigens. Indeed, the now accessible immunological therapies for RPL are very restricted, primarily based on empirical approaches, and exhibit little effectiveness. Based on the extant body of research, prospects in the field of immunological treatment for RPL may involve targeting the correction of aberrant decidualization and addressing dysfunctions within the maternal-fetal immune tolerance milieu. Despite the current dearth of direct evidence and understanding of the processes by which DNA methylation influences the immunological microenvironment between mother and fetus. Given the same characteristics between pregnancy and malignancies, it is reasonable to anticipate that the emerging class of anti-tumor therapies known as "epi-drugs" may also demonstrate efficacy in the context of RPL immunotherapy.

13. Conclusion

RPL is a challenging and vexing domain within the field of maternal-fetal medicine. The rates of RPL have exhibited a consistent upward trend over the past few decades; yet, the fundamental mechanisms responsible for this phenomenon remain inadequately comprehended. One area of growing attention pertains to the mediation of critical gene expression by epigenetic alteration during the early stages of pregnancy. Epigenetic intergenerational or cross-generational inheritance provides evidence in support of the "Fetal Origins of Adult Disease (FOAD)" hypothesis. When examining the situation from this perspective, one may argue that there is a fortuitous aspect to it, as the atypical burden of epigenetic factors originating from the maternal lineage is eliminated and does not propagate to subsequent generations. The quantity of research about DNA methylation has exhibited a consistent linear growth pattern after its initial identification in the 1980 s, while some

aspects of this phenomenon continue to elude comprehensive understanding. One notable inquiry pertains to the underlying reasons for the premature demise of embryos exhibiting aberrant DNA methylation during the initial stages of development. Although it may appear that all difficulties have been resolved, it is important to acknowledge that challenges continue to persist. The etiological variables contributing to RPL are multifaceted and heterogeneous. DNA methylation is a critical factor in the initial stages of embryonic development, the invasion of trophoblast cells, the remodeling of uterine spiral arteries, and the maintenance of the maternal-fetal interface. It achieves this by effectively controlling the transmission and activation of genes. It elucidates four specific features, namely imprinted genes, placental/decidual genes, sperm DNA, and immune-related genes, with a detailed analysis. Correspondingly, we tried to show attention to the cellular makeup of the immune system and the associated alterations in DNA methylation patterns observed at the interface between the maternal and fetal tissues in cases of RPL. Relatively, the impact of DNA methylation on the immunological milieu of the uterus and its association with RPL in different signaling pathways are different. It also here addresses a notable research gap in the examination of the significant involvement of epigenetic regulatory networks in both embryo development and the maternal-fetal interface. In this section, it is imperative to do additional fundamental studies to provide a comprehensive investigation into the molecular mechanism and associated signaling pathways involved in the epigenetic control of RPL. It is imperative to thoroughly investigate the intricate network of associations between various regulatory approaches and their respective mechanisms in the context of early embryo development, embryo implantation, and the maternal-fetal interface. Our study presents novel opportunities for preconception screening of women at risk of miscarriage and highlights the possibility of epigenetics-based immunity treatments in the prevention of RPL. Ultimately, besides the importance above factors of epigenetics in RPL, the association of different malignancies and viruses [121,122] can be investigated in RPL, and CAR_T cell therapy can be recommended in this way [123,124].

Author contributions

KB and **SEN** wrote the manuscript and were involved in all parts of the project. **MA**, **EM**, **HM**, **SHS,** and contributed to some parts of the paper, and **AAS** designed and supervised this study, and edited and revised the whole manuscript comprehensively.

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CRediT authorship contribution statement

Seyedeh Elham Norollahi: Formal analysis, Investigation, Methodology, Resources, Software. **Ali Akbar Samadani:** Conceptualization, Data curation, Supervision, Validation, Writing – original draft, Writing – review & editing. **Kosar Babaei:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mohsen Aziminezhad:** Validation, Methodology, Investigation. **Ebrahim Mirzajani:** Methodology, Investigation, Formal analysis, Data curation. **Hossein Mozdarani:** Methodology, Investigation, Formal analysis, Data curation. **Seyedeh Hajar Sharami:** Methodology, Investigation, Formal analysis, Data curation. **Seyedeh Elham Norollahi:** Writing – original draft, Validation, Methodology, Investigation. **Ali Akbar Samadani:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Seyedeh Hajar Sharami:** Investigation, Methodology, Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. all authors declare that there is no conflict of interest and also all the ethical standard considered carefully. Remarkably, all the authors studied and confirmed the final edited version of this manuscript.

Data availability

The data that has been used is confidential.

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