Journal of Clinical Laboratory Analysis

WILEY

RESEARCH ARTICLE OPEN ACCESS

Integrated Analysis of WES and scRNA-Seq Data Reveals the Genetic Basis of Immune Dysregulation in Unexplained Recurrent Pregnancy Loss

Zhao-Jing Lin[1,2] | Lei Zhu[3] | Yi Dong[1] | Jiao Yun[3] | Ya-Nan Zhi[4] | Wei Zhang[3] | Yan-Mei Sun[4] | Yu-Jie Jiang[5] | Shu Liu[6] | Liang-Liang Fan[1] | Ya-Li Li[4] | Shuai Guo[1]

[1]Department of Cell Biology, Hunan Key Laboratory of Medical Genetics, School of Life Sciences, Central South University, Changsha, China | [2]Department of Anesthesiology, The Second Xiangya Hospital of Central South University, Changsha, China | [3]Department of Obstetrics and Gynecology, Ordos Central Hospital, Ordos, China | [4]Department of Reproductive Genetics, HeBei General Hospital, ShiJiaZhuang, China | [5]Department of Computer Science, Wake Forest University, Winston-Salem, North Carolina, USA | [6]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China

Correspondence: Ya-Li Li (lyl8703@sina.com) | Shuai Guo (ashuai.guo.asg@gmail.com)

Received: 23 December 2023 | **Revised:** 15 May 2024 | **Accepted:** 19 February 2025

Funding: This research is funded by the Natural Science Foundation of Hunan Province (2025JJ60703), the Inner Mongolia Science and Technology Innovation Guidance Project (NM-KJCXYD-018), the Natural Science Foundation of Hebei Province (C2024307001). Ordos key research and development plan project (YF20240053), Inner Mongolia Autonomous Region public hospital research joint fund science and technology project (2024GLLH1285), and Natural Science Foundation of Inner Mongolia Autonomous Region (2024MS08052).

Keywords: immune dysregulation | lipid metabolism | MUC family | single-cell RNA (scRNA) sequencing | unexplained recurrent pregnancy loss (uRPL) | whole-exome sequencing (WES)

ABSTRACT

Objective: This study aimed to identify genetic variants and their functional consequences underlying Unexplained Recurrent Pregnancy Loss (uRPL) through comprehensive genomic and transcriptomic analyses.

Methods: We recruited 13 Chinese uRPL patients and performed Whole Exome Sequencing (WES) on chorionic villi samples from miscarriage tissues. Additionally, we conducted an integrative analysis using single-cell RNA sequencing data from decidual immune cells to examine expression patterns.

Results: WES analysis pinpointed variants in the four *MUC* genes (*MUC4*, *MUC6*, *MUC16*, and *MUC17*), six lipid metabolism genes in immune cells (*ABCA4*, *ABCA7*, *ABCB5*, *ABCC8*, *ADGRV1*, and *ANK3*), and two structural genes (*PIEZO1* and *PKD1*), whose variants impair mucosal barriers and lipid homeostasis, thereby leading to immune dysregulation and contributing to uRPL. To delve deeper into the effects of these genetic variants on cellular expression patterns, we undertook an integrative analysis using a single-cell dataset from decidual immune cells in uRPL cases. We observed significant dysregulation of lipid metabolism within immune cells, reduced heat shock protein expression, and enhanced chemokine signaling in uRPL samples, indicating a pro-inflammatory state.

Conclusions: In summary, our study reveals a complex interplay between genetic variants and immune cell dysfunctions in uRPL, emphasizing the role of identified genetic variants in driving pro-inflammatory states. These findings provide a comprehensive view of the molecular mechanisms underlying uRPL, opening paths for novel therapeutic interventions and improved clinical management.

Zhao-Jing Lin and Lei Zhu contributed equally to this work.

1 | Introduction

Recurrent pregnancy loss (RPL), affecting 1%–5% of couples globally, is defined by two or more consecutive pregnancy losses [1]. RPL can result from multiple causes, including embryo aneuploidy, uterine abnormalities, autoimmune disorders, endocrine disorders, and infections [2]. However, about 50% of these cases remain unexplained recurrent pregnancy loss (uRPL). Currently, clinicians utilize preimplantation genetic testing for aneuploidy (PGT-A) as a therapeutic intervention [3, 4], which screens for chromosomal abnormalities in embryos. However, some patients continue to experience miscarriages [4], indicating unidentified lethal genetic factors. Thus, further investigation of additional molecular factors is urgently needed.

Whole-exome sequencing (WES) offers a promising avenue for uncovering pathogenic variants. A series of recent studies spotlight the genetic underpinnings of uRPL. Tsurusaki et al. [5] highlight the pathogenic role of mutated *IFT122*, a crucial cilia gene integral to embryogenesis [6]. Filges et al. [7] pinpoint rare mutations in *PLCD4* and *OSBPL5*, two genes pivotal for oocyte maturation and fertilization. Qiao et al. [8] discover mutations within *DYNC2H1*, related to cilia formation, and *ALOX15*, vital for placental growth. Xiang et al. [9] map out four significant genes—*FOXA2*, *FGA*, *F13A1*, and *KHDC3L*. Besides, genes critical for embryo sustenance are identified as novel causative factors [10, 11], including *KIF14*, *CEP55*, *STIL*, *FOXP3*, *GLE*, *RYR1*, and *POMT1*. Another recent study [12] reports 18 parental mutations spanning a spectrum from genes linked with cell adhesion and structure (*ANXA5*, *FN1*, *THBS1*, *KDR*, *ITGB1*, *COL4A2*, *LAMA2*, and *LAMA4*) to those central to DNA replication and repair (*APP*, *DNMT1*, *MSH2*, *POLR2B*, *PLK1*, *BUB1B*, *BPTF*, and *MECP2*), and two genes associated with cell development and differentiation (*MMP2* and *SOX21*). Wang et al. [4] unveil six noteworthy genes: *ATP2A2* and *RYR2*, which modulate ion homeostasis, alongside *NAP1L1*, *NRK*, *PLXNB2*, and *SSPO*, which regulate cell growth and differentiation.

While most variants identified by genetic screens enrich in embryo development and cell differentiation, transcriptomic studies spotlight immune dysregulations as critical in uRPL [13–15]. A recent single-cell study further revealed marked immune heterogeneity in the decidua during uRPL pathogenesis [16]. This disparity between genetic and transcriptomic discoveries calls for an integrative study. Drawing from the successes in complex diseases like idiopathic pulmonary fibrosis [17] and inflammatory bowel disease [18], co-analyzing WES and single-cell RNA (scRNA)-seq data allows us to link genetic variants directly to cellular functions and interactions. This detailed insight, capturing aspects like cell type–specific expression, leads us to posit that a multi-omics approach can unravel the multifaceted mechanisms of uRPL.

Building on this premise, we hypothesize that genetic variants identified through WES may contribute to immune dysregulation in uRPL. To test this, we first investigated WES results of 13 uRPL patients to identify potential risk genes. We then map our findings at the single-cell level to reveal the alterations at the expression level. Our study aimed to provide a comprehensive view of the molecular mechanisms underlying uRPL, opening paths for novel therapeutic interventions and improved clinical management.

2 | Materials and Methods

2.1 | Patient Selection Criteria

2.1.1 | Inclusion Criteria

Eligible patients were those with regular menstrual cycles and a pregnancy duration of no more than 14 weeks. Additionally, patients should have experienced at least two previous instances of fetal arrest. The diagnosis of uRPL was primarily based on ultrasound examinations. Detailed criteria for embryonic arrest are outlined in the Diagnostic Examinations section.

2.1.2 | Exclusion Criteria

We excluded patients under several circumstances. Multiple pregnancies during the study period were excluded. Patients with a history of exposure to harmful drugs, radiation, or other adverse environments during the current pregnancy were also not considered. Anatomical issues such as reproductive tract malformations, diseases of the uterus or cervix, endocrine diseases like hyperthyroidism and diabetes, and occurrences of acute or chronic infections during the current pregnancy were grounds for exclusion. Furthermore, patients with a history of autoimmune diseases; familial genetic diseases; or a history of smoking, excessive alcohol consumption, and drug abuse were not included in the study.

2.2 | Diagnostic Examinations

A diagnosis of embryonic arrest was confirmed if one of the following ultrasound criteria was met: (1) a crown-rump length of ≥ 7 mm without observable fetal cardiac activity; (2) a mean gestational sac diameter of ≥ 25 mm without a detectable embryo; (3) absence of a yolk sac, and, even after 2 weeks, there remains no visible embryo or fetal cardiac activity; and (4) presence of a yolk sac, but even after 11 days, there is no detectable fetal cardiac activity.

2.3 | Subjects

This study, including 13 uRPL patients, was conducted at Hebei General Hospital (Tables 1 and S1). All diagnoses were made according to the guidelines from the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), and through multidisciplinary discussion among respiratory specialists, radiologists, and rheumatologists. All the subjects were Han Chinese and were enrolled consecutively. Our study targeted Chinese population to obtain a more precise genetic profile.

2.4 | DNA Extraction and WES

Genomic DNA was extracted by DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The main part of WES was performed in the Beijing Genomics Institute (Beijing, China). The

TABLE 1 | Clinical features of 13 RPL patients.

Characteristics	RPL cases ($n=13$)
Age (years)	33.00 ± 5.20
Height (cm)	160.01 ± 4.48
Weight (kg)	59.00 ± 8.80
BMI	22.96 ± 3.38
Gravidity	3.62 ± 1.39
Parity	4 (30.76%)
History of previous miscarriages	3.23 ± 1.01
Gestational age (days)	65.54 ± 14.19

exomes were captured by Agilent Sure Select Human All Exon V6 kits and high-throughput sequencing was performed in an Illumina HiSeq2000 platform.

2.5 | Filtration Workflow of WES Data

A total of 1,078,586 variants were identified (Figure S1A), consisting of 936,754 single nucleotide polymorphisms (SNPs) and 141,832 insertions and deletions (Indels). The variants were filtered through a multistep preprocessing workflow to prioritize potentially pathogenic variants for further analysis. (1) Variants impacting exonic regions were selected, resulting in 274,674 variants (Figure S1B). (2) As our focus is on the protein structure, we only kept the nonsynonymous SNVs and frameshift indels, resulting in 126,885 variants. (3) Variants with a population frequency of less than 1% were selected to filter out polymorphisms among the population. This step resulted in 8,343 rare exonic variants. (4) These variants were then filtered based on their predicted functional impact, retaining only those classified as pathogenic (P), likely pathogenic (LP), or variants of uncertain significance (VUS).

This filtration workflow yielded 6,260 high-confidence rare deleterious exonic variants for downstream analysis (Figure S1C). The median count of identified variants per sample is 417 (Figure S1D), with most samples demonstrating a consistent variant count. The only outlier is sample MA-12, featuring 1180 variants.

2.6 | Collection and Preprocessing of scRNA-Seq Data

The scRNA-seq data were obtained from the GEO database, GSE164449 [16]. The dataset consists of CD45+ immune cells from six decidua samples—three from normal pregnancies and three from uRPL cases. For the scRNA-seq data analysis, the R package *Seurat* (Version 4.0.2) was employed. We performed quality control procedures as previously described [19]. Briefly, we retained only cells with several detected genes (nFeature_RNA) between 200 and 2500, a total number of RNA counts (nCount_RNA) less than 10,000, and a percentage of mitochondrial gene expression (percent.mt) below 15%.

We obtained 13,350 single cells, comprising 5,455 cells from normal pregnancies and 7,895 from uRPL pregnancies (Figures S3A). Using the graphical-based clustering method within the *Seurat* package, we identified 16 distinct cell clusters. We then annotated these clusters based on known marker genes: dNK cells, T cells, B cells, myeloid cells (including macrophages and monocytes), dendritic cells, and mast cells (Figure S3B; Table S5). Cluster 9 was labeled "undefined" due to the absence of strong expression for any known marker genes. All six cell types included cells from both normal and uRPL samples (Figure S3C). We next subset the cells annotated as dNK cells, T cells, and myeloid cells (monocytes + macrophages) for downstream functional analysis.

2.7 | Detecting Metabolomic Changes in scRNA-Seq Data

We used *scMetabolism* (v 1.0.0) [20] to analyze differences in metabolic pathway activity in the scRNA-seq dataset. *scMetabolism* is an R package that quantifies metabolic activity at the single-cell level based on different databases. In our analysis, we employed the KEGG_metabolism and REACTOME_metabolism datasets to score each cell. We applied this method to the scRNA-seq data from decidual immune cells to compare the metabolic activity between normal and uRPL samples.

2.8 | Differential Expression Genes and Pathway Enrichment Analysis

We used the *FindAllMarkers* function in the *Seurat* package to perform DE analysis on the scRNA-seq data. The statistical significance of the *p*-value was set at 0.05. Log2FC cutoff values were established at 0.75, 0.5, and 0.5 for myeloid, dNK, and T cells, respectively. This analysis led to the discovery of differential expression (DE) in 28 genes in myeloid cells (15 normal, 13 uRPL), 38 genes in dNK cells (23 normal, 15 uRPL), and 32 genes in T cells (25 normal, 7 uRPL). The R package *clusterProfiler* (Version 3.18.1) was used for both GO and KEGG pathway enrichment analyses to explore and visualize the biological functions and pathways.

3 | Results

3.1 | Patient Characteristics and WES Data

In this study, we enrolled 13 unrelated Chinese subjects with a clinical history of RPL in nonconsanguineous marriages (Table 1). Routine examinations, including peripheral blood chromosome karyotypes, revealed no abnormal findings across all subjects, and common causes of RPL were excluded (see the Materials and Methods section 2) [21]. All the 13 subjects included were characterized as uRPL patients. This uRPL cohort consisted of female participants aged between 25 and 41 years, with an average age of 33 years. They had experienced gravidity ranging from two to seven instances and a history of miscarriages spanning two to five instances. Four participants were able to sustain a pregnancy to full term. A detailed summary of the clinical information is delineated in Table S1.

To further unravel the potential molecular etiology of uRPL, we employed WES on chorionic villi sampled from miscarriage products. We achieved a median sequencing depth of 110× for targeted exonic regions and a minimum coverage of 20× for 99% of the targeted sequences. This dataset provides a high level of confidence for the identification of variants with potential functional significance, setting the stage for a comprehensive analysis of the potential genetic architecture underlying uRPL in this cohort.

3.2 | The Landscaped Deleterious Alterations in uRPL Cohort

Our WES data revealed a unique mutational landscape within the uRPL cohort, including 6,260 rare deleterious SNVs and in-frame Indels within our uRPL cohort (see the Materials and Methods section 2). Among these deleterious variants, the majority were missense variants (nonsynonymous variants, $n = 5{,}828$; 93.1%), followed by a smaller proportion of nonsense variants (stop gain variants, $n = 125$; 2.0%), frameshift deletions ($n = 124$; 2.0%), splicing region variants ($n = 93$; 1.5%), frameshift insertions ($n = 66$; 1.1%), start loss ($n = 17$; 0.3%), and stop loss ($n = 6$; 0.1%) (Figure 1A). We explored the chromosomal distribution of these variants and found an above average number of mutational hits on Chromosomes 16, 17, and 19, implying an increased susceptibility to mutation in these genomic regions [22] (Figure 1B). After adjusting for chromosome length, the average variant counts per chromosome are 67. We observed no association between total mutation count, age, or the number of miscarriages (Table 1).

3.3 | Recurrent Deleterious Genetic Variants Impair Embryonic Microenvironment Homeostasis

To elucidate any recurrent genetic etiologies associated with uRPL, we thus confined our analysis to SNPs and Indels frequently presented ($n \geq 4$) across our cohort (Figure 2A). This strategy yielded 74 candidate genes for further investigation (Table S2). We then performed gene ontology (GO) pathway analysis on these recurrent mutations to explore their functional implications [23] (Figure 2B). Our analysis revealed a significant enrichment of mutations within two pivotal functional domains: regulation of tissue homeostasis (implicating genes such as *ABCA4*, *ABCC8*, *ADGRV1*, *FLG*, *MUC4*, *MUC6*, *MUC16*, *MUC17*, *PLEC*, *RP1L1*, *USH2A*, *VPS13B*, and *ZNF516*) and transmembrane transport activity (involving genes like *HNAK*, *AKAP9*, *ANK3*, *NOS1*, *RELN*, and *WNK1*). A group of mutations was found in genes associated with cell adhesion and response to mechanical stimuli (*ADGRV1*, *PIEZO1*, and *PKD1*) across the cohort. A detailed result of the GO analysis for these recurrently mutated genes can be found in Table S3.

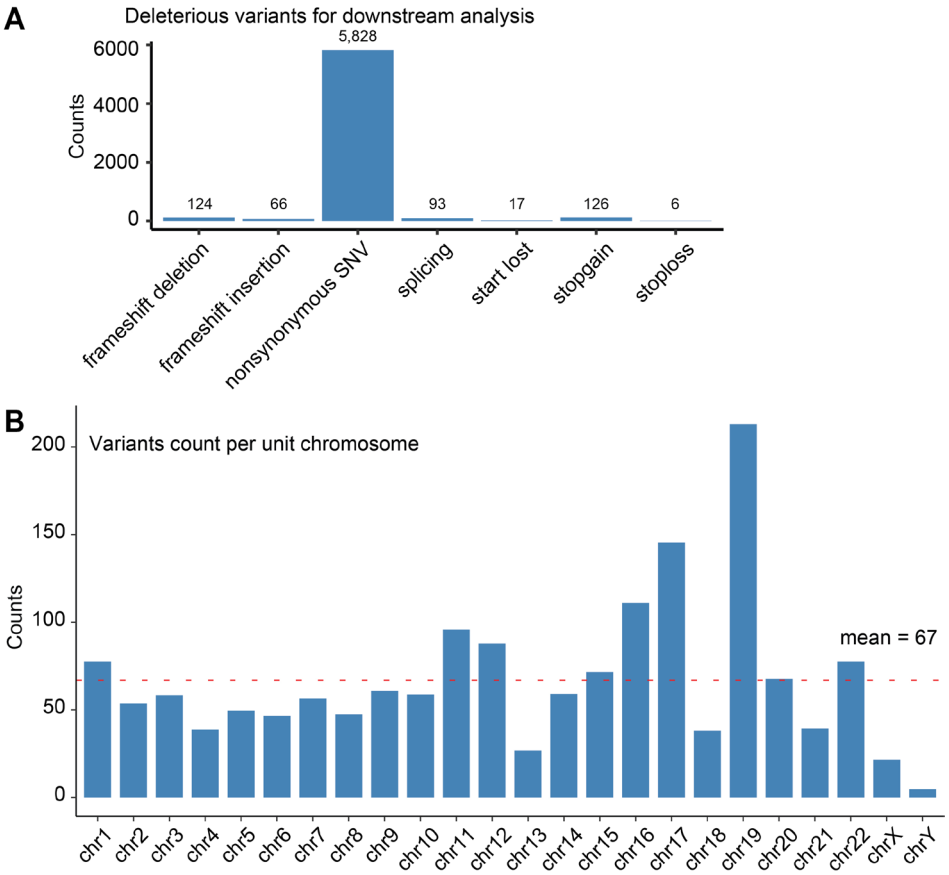

FIGURE 1 | The mutational landscape of 13 RPL samples. (A) A total of 6,260 filtered variants were classified into five groups according to their sample groups and then displayed on the upper part of the chromosome map. Mutation types include missense variants, nonsense variants, splicing region variants, start/stop loss, and frameshift Indels. (B) The bar plot shows variants per unit chromosome.

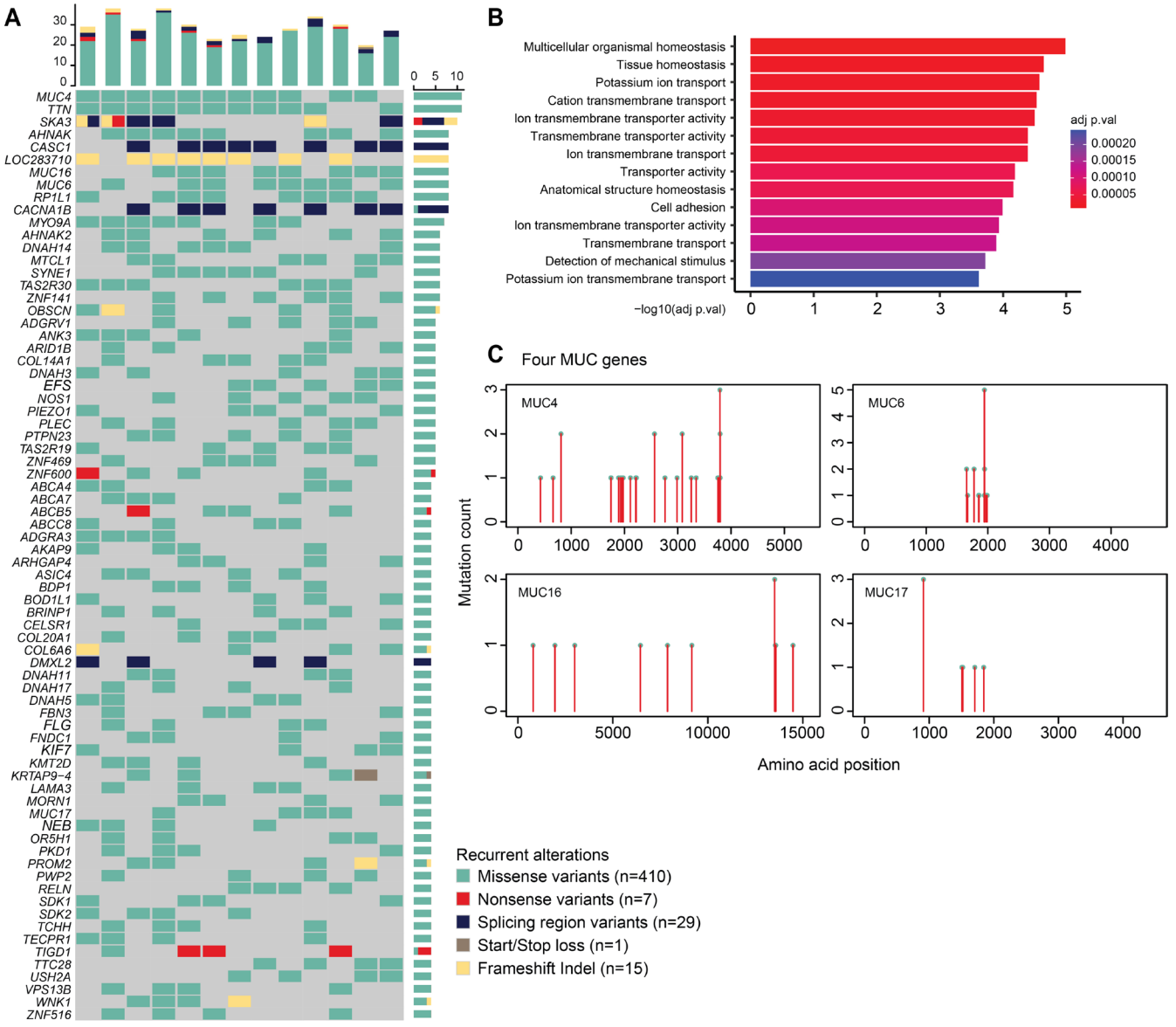

FIGURE 2 | Recurrent deleterious variants in 13 RPL samples. (A) Mutation print plot showed the landscape of 74 recurrent variants. (B) GO enrichment analysis of 74 recurrently mutated genes. (C) The distribution of SNPs along the amino acid sequence for four *MUC* genes. The *X*-axis is the position on the amino acid sequence, and the *Y*-axis is the counts of SNPs hitting the same site.

Among the 74 recurrently mutated candidate genes, four genes (*MUC4*, *MUC6*, *MUC16*, and *MUC17*) belong to the mucin (MUC) family (Figure 2C). MUCs, a protein family of high-molecular-weight glycoproteins, play critical roles in lubricating reproductive tract epithelial surfaces [24, 25]. Produced by epithelial cells in reproductive tissues, MUCs contribute to the formation of cervical and endometrial mucus, thereby playing instrumental roles in reproductive physiology [25]. Beyond acting as mediators of inflammatory and immune responses, MUCs also serve as regulatory agents at transcriptional and posttranscriptional levels [26]. Within the scope of uRPL, altered mucus secretion, a sign of MUC dysregulation, is recognized as a critical pathogenic factor [27–29]. The identified recurrently mutated MUCs are categorized into secreted MUCs (*MUC6*) and transmembrane MUCs (*MUC4*, *MUC16*, and *MUC17*) based on their function and location [24].

Our analysis started with *MUC4*, the most frequently mutated gene in our dataset (Table S4). MUC4 protein, a primary MUC expressed in the endometrial epithelium, has been found to interact with MUC16 [30]. Additionally, MUC4 influences cell growth signaling through interacting with the human epidermal growth factor receptor 2 (HER2) [27, 28]. All *MUC4* variants identified in our study are in the central domain (tandem repeat region, encoded by Exon2). This domain governs MUC4 protein function via glycosylation, modulating the immune response and mediating protein–protein interactions [28, 30]. Therefore, mutations within this crucial region could potentially impair MUC4's immunoregulatory functions and protein interactions, thereby contributing to uRPL [26, 30]. Variants in *MUC6* [31] (Exon31) and *MUC17* [32] (Exon3) are within the glycosylated tandem repeats domain (Table S4). While MUC6 stabilizes the mucus barrier via interacting with lectins [33], MUC17 acts as an immune suppressor, limiting

inflammatory responses in gastric cancer. Consequently, mutations in these genes could disrupt mucus integrity and immune balance, leading to uRPL. The mutational profile of MUC16 is inconclusive; we speculated that its structural changes might affect its interaction with MUC4.

Next, we explored six protein-coding genes renowned for their significant roles in lipid metabolism within immune cells and the regulation of immune homeostasis [34, 35], including *ABCA4*, *ABCA7*, *ABCB5*, *ABCC8*, *ADGRV1*, and *ANK3* (Figure S2). Particularly noteworthy is the *ABC* gene family, which plays a critical role in cholesterol and lipid transport and is crucial for immune regulation. Among these, both *ABCA4* and *ABCB5* have been associated with uRPL [36, 37]. *ABCA7*, expressed across various immune organs, regulates cell cholesterol metabolism via the *SREBP2* pathway and is implicated in phagocytosis. *ABCC8* participates in the inflammatory processes of autoimmune encephalomyelitis. *ADGRV1* interacts with *VLGR1* to form mitochondria-associated ER membranes, an inter-organelle structure that is vital for intracellular processes, including lipid homeostasis, immune response, and autophagy. *ANK3* regulates lipid metabolism through PPAR signaling pathways and modulates immune infiltration processes by linking membrane proteins to the spectrin–actin cytoskeleton.

Additionally, we identified structural genes *PIEZO1* and *PKD1* (Figure S2). PIEZO1, a mechanosensitive channel, responds to mechanical cues and alters its conformation in the presence of specific lipids, thereby modulating signal transduction. PKD1 is important for MyD88-dependent pro-inflammatory responses, affecting signaling pathways and pro-inflammatory gene expression in response to toll-like receptor (TLR) ligands. This connection between structural protein variants and uRPL aligns with prior findings [4, 12]. Detailed mutational information is provided in Table S4.

3.4 | Analysis of scRNA-Seq Data Revealed Overlapping Molecular Pathways

More than membrane formation and energy storage [38], lipid metabolism shapes the plasticity of immune cells, especially myeloid and T cells. Variants in the aforementioned genes can impair lipid balance and disrupt the function of immune cells, potentially contributing to uRPL. To further corroborate our findings, we incorporated publicly available data from GSE164449 [16] (see the Materials and Methods section2, Figures 3A, and S3).

Based on our WES analysis, which hinted at immune cell metabolic dysregulation in uRPL immune cells, we applied the *scMetabolism* package [20] to estimate KEGG and REACTOME metabolism scores. This revealed significant metabolic alterations within three main cell types—natural killer (dNK) cells, T cells, and myeloid cells, further supporting our WES investigations (Figure 3B,C). Our results highlighted an elevated inflammatory score and downregulated fatty acid metabolic activity within uRPL immune cells, especially within myeloid cells (Figure 3D). Detailed examination of myeloid cells (Figure 3E,F) has shown an increased immune response activity in uRPL cases, with pronounced activation of TNFα, INFα/γ, IL6/JAK/STAT3, and ROS pathways. In contrast, metabolic and development pathways, such as fatty acid metabolism, NOTCH, and MTORC1, were inhibited. Interestingly, an upregulation of the cholesterol homeostasis pathway was observed, aligning with recent discoveries linking increased cholesterol metabolism to myeloid cell hyperactivation and subsequent inflammatory response initiation [39]. Similarly, KEGG hallmark pathway analysis within uRPL dNK and T cells echoed patterns observed in myeloid cells, with activated inflammatory pathways and suppressed metabolic ones (Figure 3G,H).

3.5 | Recurrent Genetic Variations Cause Transcriptional Change Leading to uRPL

To elucidate how genetic variations manifested in immune cell signaling pathways in uRPL patients, we performed a DE analysis among three major cell populations: myeloid cells, T cells, and dNK cells (see the Materials and Methods section 2). In uRPL myeloid cells, we observed elevated expression of cell surface proteins like *HLA-DQA2*, *SPP1*, and *IGKC* (Figure S4B). Concurrently, these cells exhibited an upregulation of pro-inflammatory chemokines [40, 41], including four *CCL* genes and *CXCL8*. T cells from uRPL patients displayed enhanced expression of chemokines [42], including *IGKC* and *CXCR4*, with a notable presence of the cytolytic T lymphocyte marker *GZMB* (Figure S4C). Lastly, dNK cells in uRPL demonstrated increased expression of genes [43] such as *IGKC* and *IGFBP2* (Figure S4D). Across these cells, a marked downregulation of HSP family proteins (e.g., *HSPA6*) was evident in uRPL patients compared to the normal controls [44] (Figure S4).

Several key proteins stood out in our findings. *SPP1* exhibited a broad spectrum of roles within the immune landscape [40, 41], including influencing inflammation, immune reactions, and certain immune-related pathologies. Furthermore, it engages in lipid metabolism as a sphingosine 1-phosphate (S1P) phosphatase. CXCR4 [42], a prominent chemokine receptor, modulates immune and inflammatory responses, whose actions are shaped by lipid signals. *IGFBP2* [43] acts as an immunomodulator by promoting PD-L1 expression in M2-like macrophages via *STAT3* activation, leading to immune suppression, and impacting regulators of fatty acid synthesis and triglyceride production. The HSP family [44], essential for cellular resilience under stress, not only directs immune functions but also steers lipid metabolism processes, underpinning immune-metabolic crosstalk. Specifically, *HSPA6* is a key immune regulator in responding to oxidized LDL (oxLDL), activating macrophages and mediating anti-inflammatory signals (e.g., IL10).

Our GO analysis for these DE gene sets revealed similar patterns (Figure 4). Specifically, uRPL immune cells showed increased susceptibility to UPS, evidenced by diminished activity in unfolded protein response (UPR) mechanisms [45], and significantly heightened chemokine-mediated signaling pathways, suggesting an activated pro-inflammatory state [40, 44, 45]. In culmination, our co-analysis of the uRPL WES and scRNA-seq datasets identified genes intricately linked to lipid metabolism

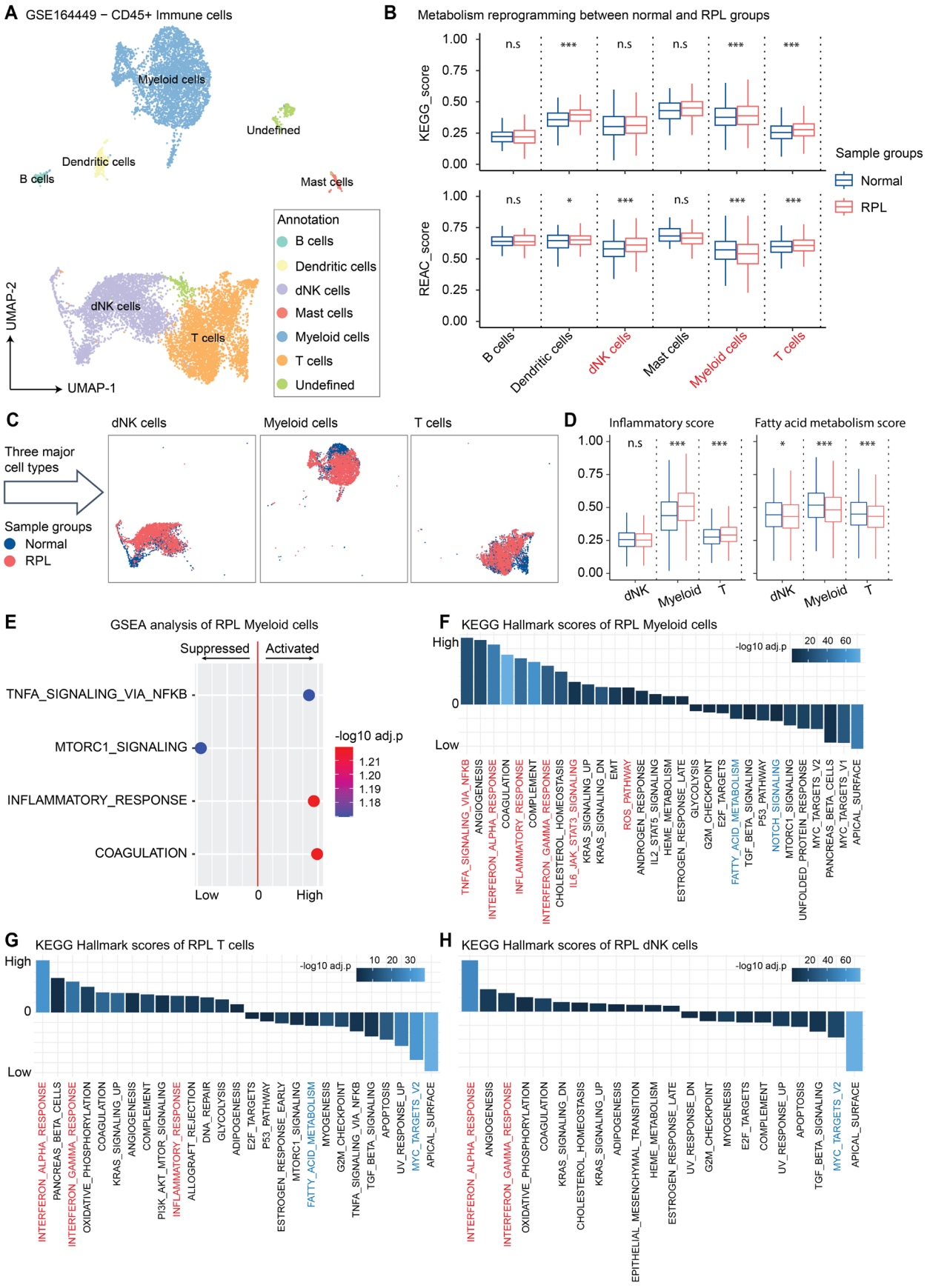

FIGURE 3 | Legend on next page.

FIGURE 3 | Immune cells from RPL samples present metabolism alterations. (A) UMAP presents the annotated scRNA-seq data of immune cells from the dataset GSE164449. (B) Boxplot shows the metabolism score across six annotated immune cell types. The upper panel depicts the KEGG metabolism score; the bottom panel depicts the REACTOM metabolism score. (C) UMAP presents the subset of three major immune cell types, including dNK cells (left), myeloid cells (middle), and T cells (right). (D) The inflammatory score (left) and fatty acid metabolism score (right) in three major immune cell types. (E) Dot plot shows the GESA pathway score of myeloid cells. The bar plot shows KEGG hallmark pathway scores in (F) myeloid cells, (G) T cells, and (H) dNK cells. All the comparisons were performed between normal (blue) and RPL (red) immune cells. Statistical analyses were conducted using Student's *t*-test, with significance determined as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Instances where the statistical difference is not significant are marked as *n.s.*

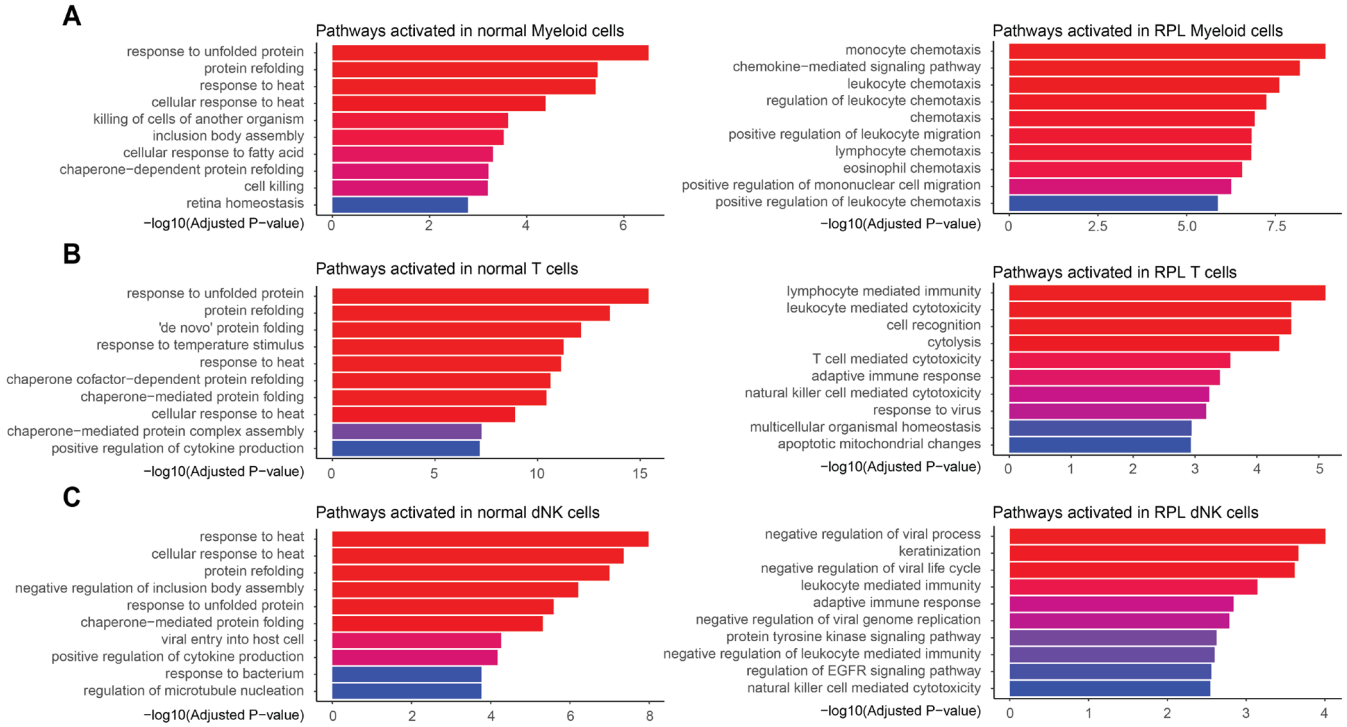

FIGURE 4 | Signature pathways between normal and RPL immune cells. The enriched signaling pathways of differentially expressed genes between normal (left) and RPL (right) immune cells in (A) myeloid cells, (B) dNK cells, and (C) T cells.

and immune processes. This underscores the hypothesis of lipid metabolic anomalies interplaying with immune dysregulation in uRPL.

4 | Discussion

To our knowledge, this study is the first approach to bridging the gap between upstream genetic factors and downstream transcriptomic alterations among uRPL patients. The study identifies specific genetic variations that contribute to the complexity of uRPL. First, we identify variants within the MUC family, which may disrupt mucosal barriers and immune homeostasis. Next, our exploration reveals rare deleterious variants in genes regulating lipid metabolism within immune cells, illuminating the pivotal role of lipid–immune crosstalk in uRPL pathogenesis. These findings are resonated in our scRNA-seq analysis, where altered expression profiles in uRPL immune cells mirrored the importance of lipid metabolism and its consequential effects on immune cell activities. Therefore, our research suggests that uRPL may be affected by a combination of rare SNVs and Indels, especially those that impact lipid–immune interactions.

Our study offers two main advantages in understanding uRPL etiology. First, our multi-omics approach provides a foundation for future comprehensive investigations into the genetic and transcriptomic landscapes of uRPL. Second, we take a step toward bridging the gap between genetic variations and their functional consequences. We identified variations in genes related to lipid metabolism and immune regulation and observed corresponding transcriptional changes in immune cells. These insights highlight the potential interplay between genetic and functional factors in uRPL, guiding future research efforts to elucidate the complex mechanisms underlying the pro-inflammatory environment in uRPL pathology.

There are limitations to be noted in our study. Matched WES and scRNA-seq data would greatly improve our integrative analysis, providing detailed information on the relationship between genetic aberrations and transcriptomic changes [46]. Such precision could reveal more intricate molecular mechanisms and improve the therapeutic approach for uRPL. Additionally, while our findings are compelling, functional studies are needed to validate the roles of the genes and pathways we have identified in uRPL pathogenesis, as well as their potential as therapeutic targets. These areas will be the focus of our future research.

In conclusion, our research provides a comprehensive mutational landscape of uRPL, offering new insights into the possible genetic factors. Our analysis also provides meaningful genetic–functional linkages, revealing a correlation between genetic alterations and immune cell dysregulation in uRPL. This new understanding of uRPL etiology creates opportunities for exploring new therapies and improving the clinical management of this condition.

Author Contributions

Z.-J.L.; investigation, methodology, and writing – original draft. Y.D.; investigation, formal analysis, and methodology. J.Y.; investigation, formal analysis, and methodology. Y.-N.Z.; investigation. W.Z.; methodology. L.Z.; interpretation of biological results and provided expertise in reproductive health and pregnancy complications. Y.-M.S.; supervision and data curation. Y.-J.J.; data visualization. S.L.; data visualization. L.-L.F.; data curation. S.G. and Y.-L.L.; supervision, conceptualization, writing – review and editing, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors thank the patients and their families for participating in this study. They also thank the Beijing Genomics Institute (Beijing, China) for providing WES technical assistance.

Ethics Statement

The study is conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of HeBei General Hospital (protocol code 2022-045 and 3-4-2022).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The scRNA-seq data analyzed in this study were obtained from the GSE164449 dataset. To facilitate data sharing, we have made all relevant data available, including the raw WES data along with the deidentified clinical information, the raw scRNA-seq matrices, and the scripts used for data preprocessing and analysis. These resources can be accessed via our GitHub repository at https://github.com/alfredsguo/rpl_wes_analysis.

References

1. The ESHRE Guideline Group on RPL, R. Bender Atik, O. B. Christiansen, et al., "ESHRE Guideline: Recurrent Pregnancy Loss," *Human Reproduction Open* 2018 (2018): 1–12, https://doi.org/10.1093/HROPEN/HOY004.

2. Practice Committee of the American Society for Reproductive Medicine, "Evaluation and Treatment of Recurrent Pregnancy Loss: A Committee Opinion," *Fertility and Sterility* 98 (2012): 1103–1111, https://doi.org/10.1016/J.FERTNSTERT.2012.06.048.

3. S. Cornelisse, M. Zagers, E. Kostova, K. Fleischer, M. van Wely, and S. Mastenbroek, "Preimplantation Genetic Testing for Aneuploidies (Abnormal Number of Chromosomes) in In Vitro Fertilisation," *Cochrane Database of Systematic Reviews* 9, no. 9 (2020): CD005291, https://doi.org/10.1002/14651858.CD005291.PUB3.

4. X. Wang, W. Shi, S. Zhao, et al., "Whole Exome Sequencing in Unexplained Recurrent Miscarriage Families Identified Novel Pathogenic Genetic Causes of Euploid Miscarriage," *Human Reproduction* 38 (2023): 1003–1018, https://doi.org/10.1093/humrep/dead039.

5. Y. Tsurusaki, R. Yonezawa, M. Furuya, et al., "Whole Exome Sequencing Revealed Biallelic IFT122 Mutations in a Family With CED1 and Recurrent Pregnancy Loss," *Clinical Genetics* 85 (2014): 592–594, https://doi.org/10.1111/CGE.12215.

6. J. Walczak-Sztulpa, J. Eggenschwiler, D. Osborn, et al., "Cranioectodermal Dysplasia, Sensenbrenner Syndrome, Is a Ciliopathy Caused by Mutations in the IFT122 Gene," *American Journal of Human Genetics* 86 (2010): 949–956, https://doi.org/10.1016/j.ajhg.2010.04.012.

7. I. Filges, I. Manokhina, M. S. Peñaherrera, et al., "Recurrent Triploidy due to a Failure to Complete Maternal Meiosis II: Whole-Exome Sequencing Reveals Candidate Variants," *Molecular Human Reproduction* 21 (2015): 339–346, https://doi.org/10.1093/MOLEHR/GAU112.

8. Y. Qiao, J. Wen, F. Tang, et al., "Whole Exome Sequencing in Recurrent Early Pregnancy Loss," *Molecular Human Reproduction* 22 (2016): 364–372, https://doi.org/10.1093/MOLEHR/GAW008.

9. H. Xiang, C. Wang, H. Pan, et al., "Exome-Sequencing Identifies Novel Genes Associated With Recurrent Pregnancy Loss in a Chinese Cohort," *Frontiers in Genetics* 12 (2021): 746082, https://doi.org/10.3389/FGENE.2021.746082/PDF.

10. P. Quintero-Ronderos, E. Mercier, M. Fukuda, et al., "Novel Genes and Mutations in Patients Affected by Recurrent Pregnancy Loss," *PLoS One* 12 (2017): e0186149, https://doi.org/10.1371/JOURNAL.PONE.0186149.

11. E. Rajcan-Separovic, "Next Generation Sequencing in Recurrent Pregnancy Loss-Approaches and Outcomes," *European Journal of Medical Genetics* 63 (2020): 103644, https://doi.org/10.1016/J.EJMG.2019.04.001.

12. J.-T. Mou, S.-X. Huang, L.-L. Yu, et al., "Identification of Genetic Polymorphisms in Unexplained Recurrent Spontaneous Abortion Based on Whole Exome Sequencing," *Annals of Translational Medicine* 10 (2022): 603, https://doi.org/10.21037/ATM-22-2179.

13. O. B. Christiansen, H. S. Nielsen, and B. Pedersen, "Active or Passive Immunization in Unexplained Recurrent Miscarriage," *Journal of Reproductive Immunology* 62 (2004): 41–52, https://doi.org/10.1016/J.JRI.2003.09.003.

14. L. Peng, A. Chelariu-raicu, Y. Ye, et al., "Prostaglandin E2 Receptor 4 (EP4) Affects Trophoblast Functions via Activating the cAMP-PKA-pCREB Signaling Pathway at the Maternal-Fetal Interface in Unexplained Recurrent Miscarriage," *International Journal of Molecular Sciences* 22 (2021): 9134, https://doi.org/10.3390/IJMS22179134.

15. W. J. Wang, C. F. Hao, Q. L. Qu, X. Wang, L. H. Qiu, and Q. D. Lin, "The Deregulation of Regulatory T Cells on Interleukin-17-Producing T Helper Cells in Patients With Unexplained Early Recurrent Miscarriage," *Human Reproduction* 25 (2010): 2591–2596, https://doi.org/10.1093/HUMREP/DEQ198.

16. P. Chen, L. Zhou, J. Chen, et al., "The Immune Atlas of Human Deciduas With Unexplained Recurrent Pregnancy Loss," *Frontiers in Immunology* 12 (2021): 689019, https://doi.org/10.3389/FIMMU.2021.689019.

17. S. Guo, Y. Dong, C. Wang, et al., "Integrative Analysis Reveals the Recurrent Genetic Etiologies in Idiopathic Pulmonary Fibrosis," *QJM: An International Journal of Medicine* 116, no. 12 (2023): 983–992, https://doi.org/10.1093/QJMED/HCAD206.

18. C. S. Smillie, M. Biton, J. Ordovas-Montanes, et al., "Intra- and Inter-Cellular Rewiring of the Human Colon During Ulcerative Colitis," *Cell* 178 (2019): 714–730, https://doi.org/10.1016/J.CELL.2019.06.029.

19. H. Huang, S. Guo, Y. Chen, et al., "Increased RTN3 Phenocopies Nonalcoholic Fatty Liver Disease by Inhibiting the AMPK–IDH2 Pathway," *MedComm* 14 (2023): e226, https://doi.org/10.1002/MCO2.226.

20. Y. Wu, S. Yang, J. Ma, et al., "Spatiotemporal Immune Landscape of Colorectal Cancer Liver Metastasis at Single-Cell Level," *Cancer Discovery* 12 (2022): 134–153, https://doi.org/10.1158/2159-8290.CD-21-0316.

21. K. J. Hyde and D. J. Schust, "Genetic Considerations in Recurrent Pregnancy Loss," *Cold Spring Harbor Perspectives in Medicine* 5, no. 3 (2015): a023119, https://doi.org/10.1101/CSHPERSPECT.A023119.

22. E. Dimitriadis, E. Menkhorst, S. Saito, W. H. Kutteh, and J. J. Brosens, "Recurrent Pregnancy Loss," *Nature Reviews. Disease Primers* 6 (2020): 98, https://doi.org/10.1038/s41572-020-00228-z.

23. T. Wu, E. Hu, S. Xu, et al., "Clusterprofiler 4.0: A Universal Enrichment Tool for Interpreting Omics Data," *Innovations* 2, no. 3 (2021): 100141, https://doi.org/10.1016/j.xinn.2021.100141.

24. J. Ringel and M. Löhr, "The MUC Gene Family: Their Role in Diagnosis and Early Detection of Pancreatic Cancer," *Molecular Cancer* 2 (2003): 9, https://doi.org/10.1186/1476-4598-2-9.

25. C. L. Russo, S. Spurr-Michaud, A. Tisdale, J. Pudney, D. Anderson, and I. K. Gipson, "Mucin Gene Expression in Human Male Urogenital Tract Epithelia," *Human Reproduction* 21 (2006): 2783–2793, https://doi.org/10.1093/HUMREP/DEL164.

26. R. Bhatia, S. K. Gautam, A. Cannon, et al., "Cancer-Associated Mucins: Role in Immune Modulation and Metastasis," *Cancer Metastasis Reviews* 38 (2019): 223–236, https://doi.org/10.1007/S10555-018-09775-0.

27. J. H. Kim, T. H. Kim, Y. S. Kim, et al., "Mucin Gene Polymorphisms Are Associated With Endometriosis in Korean Women," *Archives of Gynecology and Obstetrics* 301 (2020): 801–807, https://doi.org/10.1007/s00404-019-05409-0.

28. J. H. Kim, H. S. Park, J. Y. Lee, et al., "Association Study Between Mucin 4 (MUC4) Polymorphisms and Idiopathic Recurrent Pregnancy Loss in a Korean Population," *Genes* 13, no. 6 (2022): 937, https://doi.org/10.3390/GENES13060937.

29. G. Teklenburg, M. Salker, C. Heijnen, N. S. Macklon, and J. J. Brosens, "The Molecular Basis of Recurrent Pregnancy Loss: Impaired Natural Embryo Selection," *Molecular Human Reproduction* 16 (2010): 886–895, https://doi.org/10.1093/MOLEHR/GAQ079.

30. N. Dharmaraj, P. J. Chapela, M. Morgado, et al., "Expression of the Transmembrane Mucins, MUC1, MUC4 and MUC16, in Normal Endometrium and in Endometriosis," *Human Reproduction* 29 (2014): 1730–1738, https://doi.org/10.1093/HUMREP/DEU146.

31. B. H. Liu, G. B. Liu, B. B. Zhang, et al., "Tumor Suppressive Role of MUC6 in Wilms Tumor via Autophagy-Dependent β-Catenin Degradation," *Frontiers in Oncology* 12 (2022): 756117, https://doi.org/10.3389/FONC.2022.756117.

32. B. Yang, A. Wu, Y. Hu, et al., "Mucin 17 Inhibits the Progression of Human Gastric Cancer by Limiting Inflammatory Responses Through a MYH9-p53-RhoA Regulatory Feedback Loop," *Journal of Experimental & Clinical Cancer Research* 38 (2019): 1–13, https://doi.org/10.1186/S13046-019-1279-8/FIGURES/7.

33. W. Hoffmann, "TFF2, a MUC6-Binding Lectin Stabilizing the Gastric Mucus Barrier and More," *International Journal of Oncology* 47 (2015): 806–816, https://doi.org/10.3892/IJO.2015.3090.

34. T. Aikawa, Y. Ren, Y. Yamazaki, et al., "ABCA7 Haplodeficiency Disturbs Microglial Immune Responses in the Mouse Brain," *Proceedings of the National Academy of Sciences of the United States of America* 116 (2019): 23790–23796, https://doi.org/10.1073/PNAS.1908529116.

35. T. Schatton, J. Yang, S. Kleffel, et al., "ABCB5 Identifies Immunoregulatory Dermal Cells," *Cell Reports* 12, no. 10 (2015): 1564–1574, https://doi.org/10.1016/j.celrep.2015.08.010.

36. H. Lee, E. Ahn, J. Kim, et al., "Association Study of Frameshift and Splice Variant Polymorphisms With Risk of Idiopathic Recurrent Pregnancy Loss," *Molecular Medicine Reports* 18 (2018): 2417–2426, https://doi.org/10.3892/mmr.2018.9202.

37. D. Tavian, D. Degiorgio, N. Roncaglia, et al., "A New Splicing Site Mutation of the ABCB4 Gene in Intrahepatic Cholestasis of Pregnancy With Raised Serum γ-GT," *Digestive and Liver Disease* 41 (2009): 671–675, https://doi.org/10.1016/j.dld.2008.12.101.

38. M. J. Hubler and A. J. Kennedy, "Role of Lipids in the Metabolism and Activation of Immune Cells," *Journal of Nutritional Biochemistry* 34 (2016): 1–7, https://doi.org/10.1016/J.JNUTBIO.2015.11.002.

39. K. Roh, J. Noh, Y. Kim, et al., "Lysosomal Control of Senescence and Inflammation Through Cholesterol Partitioning," *Nature Metabolism* 5, no. 3 (2023): 398–413, https://doi.org/10.1038/s42255-023-00747-5.

40. G. Mor, I. Cardenas, V. Abrahams, and S. Guller, "Inflammation and Pregnancy: The Role of the Immune System at the Implantation Site," *Annals of the New York Academy of Sciences* 1221 (2011): 80–87, https://doi.org/10.1111/j.1749-6632.2010.05938.x.

41. M. Namlı Kalem, N. Akgun, Z. Kalem, B. Bakirarar, and T. Celik, "Chemokine (C-C Motif) Ligand-2 (CCL2) and Oxidative Stress Markers in Recurrent Pregnancy Loss and Repeated Implantation Failure," *Journal of Assisted Reproduction and Genetics* 34 (2017): 1501–1506, https://doi.org/10.1007/S10815-017-0992-5/TABLES/1.

42. S. K. Lee, J. Y. Kim, M. Lee, A. Gilman-Sachs, and J. Kwak-Kim, "Th17 and Regulatory T Cells in Women With Recurrent Pregnancy Loss," *American Journal of Reproductive Immunology* 67 (2012): 311–318, https://doi.org/10.1111/J.1600-0897.2012.01116.X.

43. S. Quenby, H. Nik, B. Innes, et al., "Uterine Natural Killer Cells and Angiogenesis in Recurrent Reproductive Failure," *Human Reproduction* 24 (2009): 45–54, https://doi.org/10.1093/humrep/den348.

44. B. Jee, R. Dhar, S. Singh, and S. Karmakar, "Heat Shock Proteins and Their Role in Pregnancy: Redefining the Function of 'Old Rum in a New Bottle'," *Frontiers in Cell and Development Biology* 9 (2021): 648463, https://doi.org/10.3389/FCELL.2021.648463.

45. J. Grootjans, A. Kaser, R. J. Kaufman, and R. S. Blumberg, "The Unfolded Protein Response in Immunity and Inflammation," *Nature Reviews. Immunology* 16 (2016): 469–484, https://doi.org/10.1038/NRI.2016.62.

46. Y. X. Liu, Y. Q. Hu, S. Y. Zhang, et al., "Identification of arrhythmia-associated gene mutations in chinese patients with primary electrical disorders or sudden cardiac death," *Cardiovascular Innovations And Applications* 9, no. 1 (2024), https://doi.org/10.15212/CVIA.2024.0018.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.