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Expression of Carbohydrate Lewis Antigens in the Placenta of Patients with Miscarriages

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1. Abbreviations

ALDH	aldehyde dehydrogenase
Ang	angiopoietin
APC	antigen-presenting cell
bFGF	basic fibroblast growth factor
CA-125	carbohydrate antigen 125
CCL	C-C motif ligand
CDC	complement-dependent cytotoxicity
CEA	carcinoembryonic antigen
CSC	cancer stem cell
CXCL	C-X-C motif ligand
DC	dendritic cell
EBP	egg-binding protein
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	Epithelial-mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
ESC	endometrial stromal cell
EVT	extravillous trophoblast
FOXP3	Forkhead box P3
Fuc	Fucose
FUT	fucosyltransferase
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GBM	glioblastoma multiforme
G-CSF	granulocyte colony stimulating factor
GDP	guanosine-diphosphate
Glc	glucose
GlcA	glucuronic acid
GlcNAc	*N*-acetylglucosamine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSEA	gene set enrichment analysis
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HUVEC	human umbilical vein endothelial cell
IdoA	iduronic acid
IF	immunofluorescent
IHC	immunohistochemical
IL-	interleukin

KEGG	Kyoto Encyclopedia of Genes and Genomes
KIR	killer cell immunoglobulin-like receptor
KLH	keyhole limpet hemocyanin
LeA	Lewis A
LeB	Lewis B
LeX	Lewis X
LeY	Lewis Y
LIF	leukemia inhibitory factor
mAbs	monoclonal antibodies
Man	mannose
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MFR	monthly fecundity rate
MIF	migration inhibitory factor
MMP	matrix metalloproteinase
MSK	mitogen- and stress-activated kinase
MUC1	mucin 1
NEU	neuraminidase
Neu5Ac	sialic acid
PADRE	pan-HLA-DR-binding epitope
PAMM	placenta associated maternal macrophage
pNK	peripheral natural killer
POFUT	O-fucosylation
PRL	prolactin
PROK1	prokineticin 1
sLeA	sialyl Lewis A
sLeX	sialyl Lewis X
SSEA-1	Stage-specific embryonic antigen-1
ST	sialyltransferase
TGF-β	transforming growth factor-beta
Th	T helper
TIMP	tissue inhibitors of metalloproteinase
TNF-α	tumor necrosis factor-alpha
Treg	T regulatory
TTP	time-to-pregnancy
uNK	uterine natural killer
uRM	unexplained recurrent miscarriage
VEGF	vascular endothelial growth factor
Xyl	xylose
ZP	zona pellucida

2. Publication list

2.1 Expression of the Carbohydrate Lewis Antigen, Sialyl Lewis A, Sialyl Lewis X, Lewis X, and Lewis Y in the Placental Villi of Patients with Unexplained Miscarriages

Zhi Ma, Huixia Yang, Lin Peng, Christina Kuhn, Anca Chelariu-Raicu, Sven Mahner, Udo Jeschke, and Viktoria von Schönfeldt

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2.2 Targeting Aberrantly Elevated Sialyl Lewis A as a Potential Therapy for Impaired Endometrial Selection Ability in Unexplained Recurrent Miscarriage

Zhi Ma, Huixia Yang, Mirjana Kessler, Markus Sperandio, Sven Mahner, Udo Jeschke, and Viktoria von Schönfeldt

Front Immunol. 2022, 13:919193. DOI: 10.3389/fimmu.2022.919193

3. Contribution to the publications

3.1 Contribution to Paper I

This study was designed and coordinated by Udo Jeschke and Viktoria von Schönfeldt. As the sole first author of this paper, I performed all the experiments and statistical analysis. Huixia Yang, Lin Peng, and Christina Kuhn assisted the immunohistochemistry. Meanwhile, I wrote the manuscript, Anca Chelariu-Raicu and Sven Mahner made significant intellectual content revision.

3.2 Contribution to Paper II

This study was designed and coordinated by Udo Jeschke and Viktoria von Schönfeldt. As the sole first author of this paper, I performed all the experiments, especially optimized the in vitro implantation model. Huixia Yang and Mirjana Kessler assisted the immunohistochemistry, cytokine treatment, and qPCR assay. Statistical analysis and manuscript drafting were also accomplished by me. Mirjana Kessler, Markus Sperandio, and Sven Mahner made significant intellectual content revision.

4. Introduction

4.1 Pregnancy and Miscarriages

4.1.1 Pregnancy related immune cells and cytokines

An inflammatory response involves the upregulation of chemokines and cytokines as well as subsequent recruitment of immune cells from the bloodstream to the injured or infected site, which is a fundamental protection of our human body from invading organisms [1]. Emerging evidence indicates that an inflammatory microenvironment is also required for successful implantation and maintenance of pregnancy [2].

The population of immune cells in human decidua and endometrium has been extensively studied, of which uterine natural killer (uNK) cells (comprising 65-70% of the leukocytes in first trimester), antigen-presenting cells (APCs, make up for about 10-20%) like macrophages and dendritic cells (DCs), and T cells (around 10%) are the three main cell types [1, 3]. Unlike the counterpart cytotoxic peripheral NK (pNK) cells, uNK cells do not appear to be involved in cytotoxic responses to embryos, they mostly express CD56 that differs from the classical markers CD3/4/8/16/57 carried by the major population of pNK cells [4, 5]. Moreover, uNK cells have killer cell immunoglobulin-like receptors (KIRs), which bind to human leukocyte antigen (HLA)-C expressed by the extravillous trophoblasts (EVTs), favoring greater reproductive success [6]. The exact origin of uNK cells remains an enigma, many studies have revealed that they could differentiate from recruited peripheral blood precursors as well as directly proliferate from the local CD34+ precursor cells in decidual tissues driven by factors like interleukin 11 (IL-11) and IL-15 secreted by DCs and endometrial cells, or from recruited pNK cells which is facilitated by transforming growth factor-beta (TGF-β) secreted by macrophages [7-9]. Known cytokines and growth factors produced by uNK cells are tumor necrosis factor-alpha (TNF-α), leukemia inhibitory factor (LIF), interferon-gamma (IFN-γ), IL family members (IL-1β, IL-6, and IL-8), vascular endothelial growth factors (VEGFs), angiopoietin 1 (Ang1), Ang2, TGF-β1,

macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), which have been attributed with crucial roles in promoting spiral artery remodeling and regulating trophoblast invasion [10, 11].

Unlike uNK cells' significantly reduced number in the third trimester, macrophages and DCs remain present at high levels at the implantation site throughout pregnancy [12, 13]. Placenta associated maternal macrophages (PAMM) originate from maternal blood monocytes which are found in the intervillous space [14]. Several lines of evidence suggest that placental villi as well as IL-1β stimulated decidual cells secrete an array of cytokines including macrophage migration inhibitory factor (MIF), C-X-C motif ligand 2 (CXCL2), CXCL3, CXCL8, C-C motif ligand 2 (CCL2), and CCL5, which attract monocytes migration and macrophages recruitment [15-17]. PAMM and DCs themselves also produce a diverse range of enzymes and cytokines like IL4, IL10, and IL13 that control the angiogenesis and tissue remodeling [1, 18]. Besides, macrophages derived LIF and IL-1β contribute to the embryo attachment by regulating α1,2 fucosyltransferases in endometrial cells [19, 20]. In parallel, macrophages have been found to drive trophoblast invasion and clean up debris of apoptotic trophoblast preventing the initiation of semi-allogenic immune response [21, 22]. Accumulation of DCs surrounding the implanted embryo has been suggested to be involved in inducing immunologic tolerance and regulation of T cell-mediated immunity [23]. Further, it has been evident that cross-talks between DCs and uNK cells stimulate the reciprocal maturation and activation [24].

T cells especially T helper (Th1, Th2, and Th17) and T regulatory (Treg) cells are also key players in shaping maternal immune tolerance towards semi-allogenic fetus during pregnancy [22]. These T cell subsets are differentiated from the naïve CD3+CD4+ T cells upon the recognition of active signals including conceptus antigens, a comprehensive bias to stimulate generation of CD4+ Treg cells and suppress Th1 and Th17 cells has been widely acknowledge as the crucial feature in this adaptive immune

process [25]. Th1 cells produce mainly pro-inflammatory cytokines like TNF-α, IFN-γ, IL-1, IL-2, IL-12, IL-15, and IL-18, while Th2 cells exert anti-inflammatory action by secreting IL-4, IL-5, IL-6, IL-10, and IL-13 [26]. Pro-inflammatory Th1 response is required particularly in the peri-implantation period to favor trophoblast invasion and promote tissue remodeling and angiogenesis, while after the accomplishment of embryo implantation, Th1 immune predominance will be gradually taken over by the Th2 anti-inflammatory response [27]. Treg cells recruitment in the uterine starts from the proliferative phase, the number peaks in the mid-secretory phase driven by estrogen and remains elevated throughout the pregnancy [28]. Compelling evidence suggests that engagement of Treg cells with a range of immune cells during implantation lays the foundation for establishing a propitious decidual environment [25, 29]. Through cooperating with progesterone and specific trophoblast derived signals, Treg cells confer macrophages and DCs with anti-inflammatory (M2 subset) and tolerogenic phenotypes, respectively, through TGF-β, IL-10, and CTLA4-mediated mechanisms [29]. In turn, tolerogenic DCs are capable of expanding the Tregs population, while a decline in the number of IL-12-producing decidual DCs contributes to the differentiation and maintenance of Th2 cells in the decidua [27, 30, 31].

In summary, immune cells and cytokine signaling pathways participate as critical mediators of the coordinated interactions between maternal endometrium and fetal blastocyst in maintaining healthy pregnancy. Revealing the functional significance of these mediators at the fetomaternal interface provides important insights into the pathogenesis underlying abnormal pregnancies and sheds light on possible therapeutic strategies.

4.1.2 Endometrial immune perturbations in miscarriages

The term miscarriage refers to the loss of an intrauterine pregnancy visualized by histology or ultrasonography up to 20-24 weeks of gestation [32]. Given that endometrium is the first place where maternal immune system comes into direct contact with fetal cells, endometrial immune perturbation has been proposed as a detrimental

factor in the onset of miscarriage, especially the recurrent miscarriage (RM) [33]. RM, denotes the loss of two or more pregnancies, affects nearly 1–2% of couples attempting to conceive, is one of the most frustrating and challenging areas in reproductive medicine [34]. Current evidence-based diagnostic and treatment strategies for RM are limited since more than half of the cases remain unexplained [32].

A recent meta-analysis shows that CD56+ uNK level is significantly higher in the endometrium of women suffering unexplained RM (uRM) compared with controls, while there is no significant difference regarding the pregnancy outcome in uRM patients stratified by uNK level [35]. uNK cells isolated from uRM patients are found to produce more angiogenic factors including VEGF-A and angiogenin, and have a predisposition to secrete pro-inflammatory Th1-type cytokines (like IFN-γ and TNF-α) while dampening anti-inflammatory Th2-type cytokines (like IL-4 and IL-10) which are crucial in maintaining healthy pregnancy [36, 37]. Hypoxic environment is favorable for the trophoblasts invasion, therefore, excessive angiogenic activity induced by the high level of uNK cells in uRM might induce premature blood flow at the implantation site, which incurs oxidative stress and subsequent cellular injury to the fetal trophoblasts [38, 39]. On the other hand, prominently lower levels of inhibitory receptors such as KIR2DL1/4 and NKG2A are noted in uRM, which may contribute to the overactivation of uNK cells [40-42].

It has been reported that percentages of M1 macrophages are significantly higher in both follicular and luteal phases than in pregnancy, and conversely, much higher M2 macrophages is observed in pregnancy than in menstrual cycles, suggesting that progesterone may drive the M2 macrophages polarization in maintaining healthy pregnancy [43]. Excessive decidual M1 macrophages found in the early pregnancy (prior to 10 weeks) has been related to the occurrence of uRM [43]. Also, the expression of IL-10 is significantly decreased while costimulatory molecules CD80 and CD86 are increased in decidual macrophages from uRM, suggesting the aberrant macrophage regulation capacity [44, 45]. As discussed above, DCs play key roles in inducing

maternal tolerance as well as decidualization and angiogenesis, depletion of DCs interferes implantation and leads to embryo resorption in a transgenic mouse model [46]. In uRM patients, tolerogenic DCs are found to be significantly reduced in the mid-luteal phase endometrium [47].

Both predominant Th1 and shifted Th2 immunity have been documented to be associated with uRM, suggesting that the conventional hypothesis of Th1/Th2 balance is insufficient to explain immune mechanisms underlying pregnancy [48-50]. Later investigation of the Treg and Th17 cell subsets expands our knowledge of the immune enigma at the fetomaternal interface [27]. Women with uRM demonstrate higher ratio of Th17/CD4+ Treg cell and a greater proportion of Th17 cells and associated IL-17A in the peripheral blood during proliferative and secretory phases than those fertile controls, on the other hand, Treg cells and related cytokines IL-10 and TGF-β1 are significantly reduced [51, 52]. In mice, depletion of CD4+CD25+ Treg cells leads to miscarriages and the adoptive transfer of Treg cells from pregnant mice prevent miscarriage in abortion-prone mice of the same strain [53, 54]. Therefore, the imbalance in Th17/Treg cell immunity may deteriorate ongoing pregnancy. The transcription factor forkhead box P3 (FOXP3) is crucial for Tregs development, accumulating evidence shows that there is reduced FOXP3 expression in peripheral blood as well as decidua and increased occurrence of FOXP3 single nucleotide polymorphisms in uRM patients compared with control women, which may hamper the Tregs function and lead to subsequent uRM [55-57].

Collectively, these data indicate the pivotal role of impaired endometrial immune function in the pathogenesis of uRM. While considering the complex interactions among these immune cells and cytokines in the endometrial immune milieu, it is currently not easy for us to fully understand the roles of any single cell or molecule in early pregnancy. Further investigations by using novel research techniques like single cell RNA sequencing might be helpful in deciphering their specific role in physiological and pathological pregnancies.

4.1.3 Hyper-receptive endometrium in recurrent miscarriage

Early embryonic development and gamete formation in humans are marked by poor chromosome segregation [58]. It is estimated that around 50% of human embryos are perished during pregnancy, especially prior to the blastocyst stage, which is largely ascribed to a high prevalence of embryo genetic instability [59, 60]. The biological significance and evolutionary advantages of these common aneuploidy and mosaicism in human embryos are still elusive. Nevertheless, many studies propose that ingenious maternal strategies have evolved to cope discerningly with genomically unstable blastocysts limiting inappropriate investment in invasive, but low competent embryos [60-62]. An emerging concept posits that the properly decidualized endometrium plays a key role in embryo biosensing and selection, which process might be disrupted in uRM [63-65].

The first experimental study indicating the biosensor function of decidualized endometrium at implantation comes from Teklenburg and colleagues, demonstrating that coculture with an arresting human embryo triggered a strong inhibition on the production of key implantation mediators in human endometrial stromal cells (ESCs), including IL-1β, IL-6, IL-10, and heparin-binding epidermal growth factor-like growth factor (HB-EGF) [61]. They also deciphered that an endoplasmic reticulum (ER) stress is induced in human decidual cells by developmentally compromised embryos in contrast to favorable metabolic enzymes and implantation factors activated by competent embryos [62]. Collectively, these distinct positive and negative mechanisms render active embryos selection ability to the decidual endometrium at implantation.

The hypothesis that endometrial selectivity to embryo quality may be impaired in uRM is supported by several robust in vitro evidence [60, 65, 66], and in agreement with clinical observation that many women with uRM report exceptionally high pregnancy rates [64, 67, 68]. In other words, rather than reject viable embryos, uRM patients are more fertile or hyper-receptive toward the implanting embryos that are destined to fail than healthy women. Under in vitro decidualization, ESCs from uRM

patients manifest reduced level of decidual marker prolactin (PRL) and prolonged production of prokineticin 1 (PROK1), a pro-implantation cytokine, which might lead to the enhanced uterine receptivity, but attenuated embryo selection underpins uRM [64, 66]. Furthermore, the ESCs from uRM have a higher migratory potential in response to trophoblast spheroids and are unable to discriminate between high- and low-quality embryos [65]. In parallel, clinical evidence arise from the observation of the higher monthly fecundity rate (MFR) and shorter time-to-pregnancy (TTP) in uRM women than the controls [66, 68]. MFR refers to the probability of conception during a menstrual cycle, the mean MFR in humans ranges between 20%–30%, which is not impressive compared with other mammalian species [60, 64]. Salker et al. reported that 40% uRM women are superfertile with MFR >60% [66]. Moreover, despite the higher maternal age of uRM women, TTP is significantly shorter in uRM than in normal women [68].

Earlier study also found that the expression of anti-adhesion molecule mucin 1 (MUC1), which contributes to the barrier function of uterine epithelium, is lower in the endometrium of uRM women than controls [69]. Selective remove of MUC1 facilitates the implantation in the immediate vicinity of an attaching embryo, thus reduced MUC1 impedes the continuous surface mucin barrier, which might contribute to increased receptivity in uRM [63, 69]. However, there are multiple studies challenge this hypothesis by showing no significant difference of MUC1 expression in the endometrium between uRM and normal controls [70, 71]. Since MUC1 also works as a carrier protein of many adhesion molecules (like sialyl Lewis antigens that will be discussed in next chapter) capable of mediating cell-cell interaction [72], and regulates chemokine secretion [73], a comprehensive understanding of MUC1 function during embryo implantation will have to await more investigations.

4.2 Carbohydrate Lewis antigens

Carbohydrates are remarkably diverse glycans that locate on the surface of every cell

and most secreted macromolecules in the human body [74]. Glycosylation refers to the dynamic process that glycoconjugates are formed though adding sugars to proteins and lipids, conferring the structural and functional complexity to these glycoproteins and glycolipids [75]. Fucose (Fuc), galactose (Gal), N-acetylgalactosamine (GalNAc), glucose (Glc), glucuronic acid (GlcA), N-acetylglucosamine (GlcNAc), iduronic acid (IdoA), mannose (Man), sialic acid (Neu5Ac) and xylose (Xyl) are the ten basic monosaccharides that responsible for the construction of glycans in humans, which are assembled in a stepwise manner in ER and Golgi apparatus under the catalytic effect of specific enzymes (glycosyltransferases and glycosidases) [74].

4.2.1 Classification and synthesis

Lewis antigens are terminal fucosylated carbohydrates that belong to the human Lewis histo-blood group family [76], they are generally divided into two types based on different glycosidic bonds: type I (Galβ1,3GlcNAc) includes sialyl Lewis A (sLeA), Lewis A/B (LeA/B), and H1 antigen; type II (Galβ1,4GlcNAc) includes sialyl Lewis X (sLeX), Lewis X/Y (LeX/Y), and H2 antigen, as shown in Figure 1. The synthesis of Lewis antigens relies on the sequential addition of fucose and sialic acid catalyzed by fucosyltransferases (FUTs) and α2,3 sialyltransferases (STs), respectively, while neuraminidases (NEUs) -namely sialidases- facilitate the removal of sialic acids from sLeA/X [76-80] generating LeA/X (Figure 1).

Fucose, a natural deoxyhexose sugar and the common component of many O- and N-linked glycans, is widely present in various organisms [81]. Fucosylation refers to the incorporation of a fucose residue from donor guanosine-diphosphate (GDP)-fucose into glycan chains on cell surface glycoproteins and glycolipids [82, 83]. To date, 13 FUTs have been well characterized in humans and defined into 4 families based on the site of fucose addition: α1,2 (FUT1/2), α1,3/4 (FUTs3-7 and 9-11), α1,6 (core fucosylation, FUT8), and O-fucosylation (POFUT1/2) [84-89]. The former 3 subfamilies of FUTs are transmembrane Golgi-anchored proteins while O-fucosylation FUTs are soluble proteins locate in the ER [90-93]. α1,2 and α1,3/4 FUTs are involved

in the last steps of synthesis of carbohydrate Lewis antigens in humans (Figure 1). FUTs3-7 and 9-11 all possess the α1,3 activity, but FUT3 and FUT5 also have α1,4 activity conferred by the extended stem region, also hypervariable region, of the membrane proteins [94-96].

Sialic acids are the most important sugar besides glucose in the human body and are frequently found on the non-reducing distal end of glycan chains [97]. The metabolism of sialic acid requires enzymes that catalyze the addition (by STs) as well as removal and degradation (by NEUs) of Neu5Ac to/from a glycoconjugate [98]. In humans, there are 20 STs which can be classified into four families according to the different linkage type and preferential sialylation activity: beta-galactoside alpha-2,3-sialyltransferases (ST3GAL1-6, transferring Neu5Ac to the 3-OH of Gal residues), beta-galactoside alpha-2,6-sialyltransferases (ST6GAL1/2, adding Neu5Ac to the 6-OH of Gal residues), GalNAc alpha-2,6-sialyltransferases (ST6GALNAC1-6, transferring Neu5Ac to the 6-OH of GalNAc residues), and alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferases (ST8SIA1-6, adding Neu5Ac to the 8-OH of another Neu5Ac residue) [99-101]. Each ST catalyzes the sialylation of specific substrate structures, for example, ST3GAL3, ST3GAL4 and ST3GAL6 are potentially involved in the synthesis of sLeA and sLeX on glycoproteins and glycolipids [78, 102, 103], while ST6GAL1 contributes to the sialylation of β1 integrin [104].

So far, 4 neuraminidases have been described in different cellular localizations in mammals: NEU1 in the lysosome, NEU2 in the cytosol, NEU3 in the plasma membrane, and NEU4 in the mitochondrial membrane or lysosome [105-108]. Researchers found that the sialic acids released from sialylated glycoconjugates could be reused in the biosynthesis process [109]. The balance between sialic acid addition and removal plays a crucial role in many physio- and pathophysiological events [110, 111].

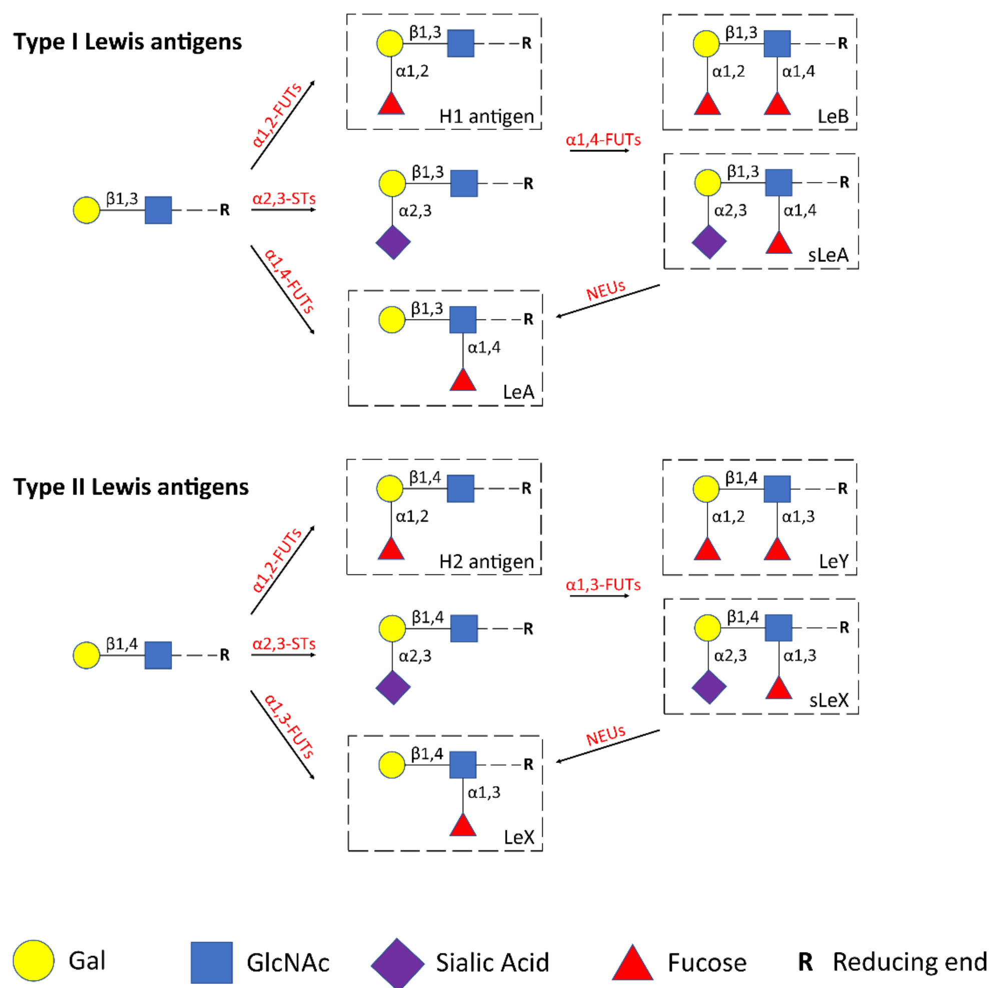

Figure 1. Biosynthesis pathway of Lewis antigens. FUTs, fucosyltransferases. STs, sialyltransferases. NEUs, neuraminidases. Gal, galactose. GlcNAc, N-acetylglucosamine.

4.2.2 Physiological functions

A. Distribution

LeX is widely distributed in many tissues including leukocytes, brain, and gastrointestinal tract [112-116]. The expression of LeY, also known as CD174, in normal tissues is relatively low, it has been detected in blood (erythrocytes and granulocytes), on epithelial cells from the esophagus and stomach, salivary glands and the Paneth cells in duodenum, and scattered cells from the pancreas, tonsils, and testes [117]. In parallel, substantial amounts of LeX and LeY antigens are also found in human seminal plasma and on the surface of sperm [118, 119]. sLeA, also termed CA19-9 antigen, is predominantly expressed on normal fibroblasts, luminal epithelial cells, and

some parenchymatous cells [120, 121], while monocytes and neutrophils usually carry sLeX on their surfaces, which cause them to extravasate to the inflammation sites [122, 123]. In the cycling endometrium, staining of Lewis epitopes are found on the apical membranes of epithelial cells, highest levels of LeX appear in the early- to mid-proliferative phases, while LeY, sLeA, and sLeX are strongly upregulated in the secretory phase [72, 124]. Normal individuals may have high levels of CA19-9 with tea consumption [125, 126].

B. Sperm-egg binding and embryo implantation

During mammalian sperm-egg binding, egg-binding protein (EBP) expressed on the sperm plasma membrane interacts with carbohydrate sequences carried by glycoproteins of the egg's zona pellucida (ZP) [127, 128]. In murine fertilization, LeX was found to be potentially involved in regulating sperm to ZP binding [129]. sLeX is the dominant antenna sequence on the N- and O-glycans attached to human ZP, it's density is more than two orders of magnitude higher than that on somatic cells [130]. Early studies reported the reaction of antibodies against sLeA, sLeX, and LeB epitopes with human ZP [131, 132], Pang et al. further deciphered that only sLeX is expressed at physico-chemically confirmable levels on ZP, the sLeX sequence is the major carbohydrate ligand mediates the sperm-ZP binding [133]. During the window of receptivity, the interaction between strongly upregulated sLeX on the human endometrial epithelial cells and the L-selectin expressed on the trophoblast is critical for the embryo implantation [134].

C. Embryogenesis

LeX, also known as the stage specific embryonic antigen-1 (SSEA-1), was primarily found as a marker of embryonic stem cells in mouse and primordial germ cells in human [135, 136]. In the early stage of mouse embryogenesis, soluble molecules carrying LeX facilitate the decompaction of morula between the end of cleavage and formation of blastocyst [137, 138]. In early human embryogenesis, LeX is preferentially expressed in organ buds progressing in mesenchyme due to the mainly detected α1,3 FUTs gene

transcripts FUT4 and FUT9, which might stimulate the cell-cell interactions allowing the organ buds to grow and branch [139, 140].

D. Nervous system

LeX is temporally and region-specifically expressed in the central nervous system of humans, which participate in the neurite outgrowth, cell–cell interaction, and neuronal migration during development [141, 142]. In mice brain, LeX synthesis is dominantly catalyzed by FUT9, while fully devoid expression of LeX by knocking out Fut9 gene resulted in no gross phenotypic abnormalities except increased emotional anxiety-like behaviors [143]. Also, LeX has been used as a surface marker to select and analyze neural stem cells [144].

4.2.3 In cancer progression

Aberrant glycosylation characterized with overexpressed Lewis antigens along with pertinent FUTs and STs is a hallmark of many types of cancers like lung, breast, hepatocellular, gastrointestinal, renal, and bladder carcinomas, which is frequently associated with cancer progression and poor prognosis [76, 145, 146]. Interestingly, the expression levels of individual STs prominently vary between different types of cancers and even within tumors of the same origin [147, 148].

A. Metastasis and angiogenesis

Tumor metastasis and angiogenesis are two cardinal features during cancer progression. Hematogenous metastasis of cancer is facilitated by the interactions between cancer cells and endothelial cells in distant tissues, in which Lewis antigens expressed on cancer cells are playing a dominant role [146]. Selectins, including E-, P-, and L-selectin, are C-type lectins that favour leukocytes rolling on the vascular endothelium during acute inflammation [149]. Cancer-related epitopes sLeA and sLeX are major selectin ligands, interactions between selectins and sLeA/X promote the formation of circulating emboli containing tumor cells, leukocytes, and platelets, which further extravasate through trans-endothelial migration, which is crucial during metastasis [150].

Numerous studies have reported that downregulation of sLeA/X expression by key glycosyltransferases knockdown in different cancer cell lines resulted in reduced selectin binding and metastatic ability, and vice versa [78, 103, 151-153]. In vivo experiment showed that the formation of pulmonary metastases could be inhibited by peptides mimicking sLeA and was completely abolished in E-selectin knockout mice [154]. Besides, LeX expressed on non-small cell lung cancer cells can also interact with TNF-α induced E-selectin on brain endothelial cells, promoting the attachment and interplay between these two types of cells and facilitating cerebral metastasis, which is in the same manner as sLeX [155, 156].

Although less appreciated, compared with the well-established roles in cancer metastasis, compelling evidence indicates that Lewis antigens are integral to the process of angiogenesis. An example illustrating this concept is that anti-sLeA/X antibodies could markedly inhibit the in vitro tube formation of murine endothelial cells (F-2) induced by the co-culture with human epidermoid cancer cells (A431), and reduce the in vivo A431 tumor size in nude rats [157]. Mathieu et al. found that FUT1-transduced hepatocarcinoma cells HepG2, which sLeX synthesis is strongly inhibited, showed remarkable defect in tumor vascularization [158]. On the other hand, transfection colon cancer cell line HCT-15 with β3Gal-T5 cDNA, generating sLeA, resulted in enhanced angiogenesis and tumor growth [159]. Furthermore, TNF-α induced FUT1 and consequently enhanced LeY expression in endothelial cells are involved in early events during tumor angiogenesis, which may explain the upregulation of cell surface LeY displayed in the tumor-infiltrated capillaries [160]. And using LeY saccharide mimetic inhibited the endothelial functions as well as in vitro angiogenesis [161]. Altogether, these results suggest that multiple Lewis antigens facilitate the tumor angiogenesis, regardless of the underlying carbohydrate structure.

B. Epithelial-mesenchymal transition (EMT)

The EMT is a biological process that allows polarized epithelial cells acquire the mesenchymal phenotypes, which has been widely acknowledged as a critical feature of

cancer malignancy associated with enhanced invasiveness and migratory capacity [162]. During this process, a number of distinct molecular events are engaged, including activation of transcriptional factors like ZEB1/2, SNAIL1/2, and TWIST; expression of specific mesenchymal cell surface proteins like Fibronectin, N-cadherin, and Vimentin; and production of extracellular matrix (ECM)-degrading enzymes like matrix metalloproteinases MMP-2, MMP-3, and MMP-9 [162]. The association between Lewis antigens and EMT has been addressed in many studies [77, 163-165]. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are known EMT inducers, colon cancer cells DLD-1 and HT-29 treated with EGF and/or bFGF exhibited significantly elevated transcript levels of ST3GAL1/3/4 and FUT3 along with increased cell surface sLeA/X expression resulting in enhanced E-selectin binding activity [77]. Similarly, FUT6 was found to be involved in the TGF-β induced colorectal cancer EMT through fucosylation of TGF-β receptors and upregulation of sLeA/X expression facilitating the tumor cells invasion and migration [163]. Also, FUT4 promotes the EMT of breast cancer cells by activating the PI3K/Akt and NF-κB signaling systems, in which key mediators SNAIL and MMP-9 are induced [164]. Studies with ovarian cancer cell line RMG-1 found that LeY promotes the cell metastasis by upregulating MMP-2/9 and downregulating tissue inhibitors of metalloproteinases 1/2 (TIMP1/2) [165].

C. Cancer stemness

Cancer stem cells (CSCs), also referred to as tumor-initiating cells, are a subpopulation of cells within the tumor endowed with increased self-renewal capacity and enhanced ability to generate heterogeneous lineage of cells, CSC enrichment is directly linked to tumor metastasis, recurrence, and treatment failure [166]. Elevated fucosylation has been found as a common glycan change in pancreatic and head and neck cancer CSC-like cells, indicating its potential role as a therapeutic target [167, 168]. In head and neck squamous cell carcinoma (HNSCC), expression of sLeX in membrane-bounded and secreted soluble forms are predominantly detected in carcinomas originating from

cases with developed metastasis and recurrence, the co-expression analysis of sLeX and CSC markers CD44/ALDH (aldehyde dehydrogenase) shows strong overlap in floating orospheres [169]. CSCs derived from human glioblastoma multiforme (GBM) were reported to be enriched for LeX, LeX+ cells are highly tumorigenic in vivo and a secondary tumor with the same characteristics as its primary counterpart could be successively generated [170]. Interestingly, LeX was also proposed to be an important marker for CSCs in medulloblastoma [171]. Two fucosylated antigens LeY and H2 antigen have been reported to be carried by CD44 molecule, a known CSC marker, in three breast cancer cell lines, suggesting their potential role as markers of early progenitors of breast carcinomas [172]. Given the wide distribution of overexpressed Lewis epitopes in solid and blood malignancies, their functional roles related to cancer stemness merit further investigations.

4.2.4 As cancer biomarkers

As discussed above, upregulated carbohydrate Lewis antigens have been detected in various types of cancers, they are currently being extensively investigated as potential tumor biomarkers for cancer detection and evaluation of treatment efficacy. sLeA, more well-known as CA19-9, is the best validated and most routinely used serum biomarker for pancreatic cancer [173], the median sensitivity and specificity in diagnosis is 79% and 82%, respectively [174]. In addition, combination of sLeX and sLeA could provide a higher accuracy for detecting pancreatic carcinoma [175]. Low level of postoperative sLeA is associated with an improved prognosis for patients with resected pancreatic cancer and elevation of sLeA during surveillance is an early and strong predictor for the recurrence [176-179]. Carcinoembryonic antigen (CEA) and carbohydrate antigen 125 (CA-125) are also well established cancer biomarkers [180, 181]. CEA is a glycoprotein expressed during embryonal development, it has been utilized to monitor the tumor progression and predict the recurrence [180]. Huang et al. found tumor CEA proteins contain high levels of sLeX, which is in agreement with previous study that sLeX was identified as a cancer marker in colon cancer [182, 183]. CA-125 has been

serving as the biomarker in ovarian cancer screening for decades, enhanced level of CA-125 carried LeY epitopes after surgery or chemotherapy is associated with poor prognosis and risk of recurrence [181, 184].

4.2.5 As targets of cancer vaccines and therapeutics

Given that aberrant glycans modification is one of the typical hallmarks of several malignancies, and recent successes in cancer vaccines as well as in monoclonal antibody cancer immunotherapy, the development of anticancer preventive and therapeutic treatments targeting cancer-associated Lewis antigens is of great promise and potential [145].

Several studies have reported the potential of LeX as a therapeutic target in different ways. Two monoclonal antibodies (mAbs) FG88.2 and FG88.7, produced by immunizing BALB/c mice with plasma membrane lipid extracts of colorectal cancer COLO205 cells, targeting coexisted LeA-LeX and di-LeA antigens on cancer cells demonstrated excellent in vitro pan-tumor cytotoxicity and effective in vivo antitumor efficacy [185]. A chimeric mouse/human IgG1 mAb counterpart CH88.2 with refined LeA and LeX trisaccharide was further investigated and showed great potential in gastrointestinal tumor imaging [186, 187].

Initial attempt concerning LeY antigen was made for the treatment of ovarian cancer patients using LeY-keyhole limpet hemocyanin (KLH) conjugated vaccine along with the QS-21 immunological adjuvant [188]. The vaccine was well tolerated with no adverse effects related to autoimmunity observed; sera assessment of vaccinated patients showed that the majority produced anti-LeY antibodies with strong anti-tumor cell reactivity and complement-dependent cytotoxicity (CDC), while no clinical response was indicated. Another approach by Westwood and colleagues demonstrated that genetically redirected T cells, which normally do not identify LeY antigen, could cause cytokine secretion and cytolysis in response to high LeY+ ovarian tumor OVCAR3 cells [189]. More recently, peptide-based vaccine P10s, a pan-HLA-DR-binding epitope (PADRE) fused peptide that mimics LeY, has shown enhanced

immunogenicity and possible clinical benefits for stage IV breast cancer subjects in an early clinical study [190].

Ragupathi et al. firstly designed the sLeA-KLH conjugate plus GPI-0100 vaccine, which could induce mice generate high titer IgM and IgG antibodies that mediate potent CDC against sLeA+ human SW626 (adenocarcinoma cell line) and DMS79 (small cell lung cancer cell line) cells [120]. Later on, they generated two fully human mAbs (7E3 and 5B1) with high affinity for sLeA from the blood lymphocytes of patients immunized with the sLeA-KLH vaccine [191]. 7E3 showed high level of CDC against DMS79 cells, while 5B1 displayed strong antibody-dependent cell-mediated cytotoxicity activity on DMS79 cells and is currently under clinical trials evaluation [192, 193]. More recently, Weitzenfeld et al. reported that re-engineering the Fc portion of antibodies derived from patients immunized with an sLeA/KLH vaccines could broadly increase their affinity for activating Fcγ receptors, leading to enhanced therapeutic effects [194].

4.3 Modulation of cytokines on the expression of carbohydrate Lewis antigens

It is now becoming clear that the cytokine-mediated inflammatory microenvironment is an indispensable factor contributing to the neoplastic process in many diseases, altered glycosylation is also found to be modulated by multiple cytokines through specific glycosyltransferases that responsible for the cell surface Lewis antigens biosynthesis [195]. TNF-α is one of the mostly investigated cytokines, it has been found to prominently increase the synthesis of sLeA and/or sLeX in colon (COLO205), lung (QG-95), gastric (AGS), and pancreatic (MDPanc-3) carcinoma cells by inducing the enzyme activities of several α2,3 STs and α1,3/4 FUTs, induced LeY expression is also noticed in MDPanc-3 cells [196-198]. Likewise, IL-1β treatment significantly increases sLeX expression in both human hepatoma-derived HuH-7 cells and pancreatic ductal adenocarcinoma cell line MDAPanc-28, and mildly enhance sLeA and LeX in gastric cancer KATOIII cells but does not reach significance [198-200].

Early study also reported that IL-8 could induce sLeA expression in human liver (HuCC-T1) and pancreatic cancer cells (HuP-T4) and promoted these cells binding to the human umbilical vein endothelial cells (HUVECs), while the underlying mechanisms were not fully deciphered [201]. Groux-Degroote and co-workers have carried out a set of experiments concerning cytokines effect on Lewis epitopes expression in human bronchial mucosa, demonstrating that TNF-α could drive sLeX synthesis through enhancing the transferase activities and transcript levels of FUT3/4 and ST3GAL3/4, IL-6 and IL-8 increased sLeX expression by upregulating FUT3 and ST3GAL6 [202, 203]. Later, they found that MSK1/2 (mitogen- and stress-activated kinase 1/2) mediated the TNF-α induced upregulation of ST3GAL4 BX transcript isoform through the ERK (extracellular-signal-regulated kinase) and p38 MAPK (mitogen-activated protein kinase) pathways [204, 205]. More recently, IL-22 was found to modulate the expression of B3GNT7, a β1,3-N-acetylglucosaminyltransferase, which alone is sufficient to promote LeX expression in human intestinal epithelium [206].

Though most researchers reported stimulated effect of cytokines on Lewis antigens expression, studies in QG-95 and KATOIII cells found no modulatory impact of IL-1β on sLeX expression [197, 198], MKN45 gastric cancer cells even exhibited a decreased expression level of sLeX after IL-1β or IL-6 stimulation [207]. These conflicting results suggest that the modulatory impacts of cytokines on Lewis antigens expression may considerably differ among diverse cell types.

Till now, whether and how pro-inflammatory cytokines influence the Lewis antigens expression during embryo implantation and in the pathogenesis of miscarriages have not been fully elucidated.

4.4 Aim of the study

Aberrant glycosylation frequently contributes to the progression of tumor metastasis, the crucial roles of Lewis antigens have been extensively investigated in various cancers.

It is evident that regulated glycosylation is essential for human gamete binding and embryo implantation, while its involvement in the pathogenesis of miscarriages is still elusive. Our studies were carried out to decipher the expression pattern and functional significances of carbohydrate Lewis antigens in the placenta of women suffering unexplained miscarriages. Specifically, Paper I aimed to reveal the dysregulated 1) glycosylation and inflammatory response at the fetal side-placental villi from uRM patients through Gene set enrichment analysis (GSEA), and 2) the Lewis antigens expression by immunohistochemical (IHC) and immunofluorescent (IF) staining, to determine whether glycosylation alterations and aberrant inflammatory response are involved in the pathogenesis of unexplained miscarriages; Paper II focused on the maternal side-decidua aimed to demonstrate the aberrant expression of Lewis antigens and pertinent glycosyltransferases (FUTs, α2,3 STs, and NEU1) using the same IHC and IF techniques, and elucidate the underlying mechanisms of hyper-receptive endometrium in uRM through an in vitro implantation model.

5. Publication I

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Expression of the Carbohydrate Lewis Antigen, Sialyl Lewis A, Sialyl Lewis X, Lewis X, and Lewis Y in the Placental Villi of Patients With Unexplained Miscarriages

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Background: Lewis antigens such as Sialyl Lewis A (sLeA), Sialyl Lewis X (sLeX), Lewis X (LeX), and Lewis Y (LeY) are a class of carbohydrate molecules that are known to mediate adhesion between tumor cells and endothelium by interacting with its selectin ligands. However, their potential role in miscarriage remains enigmatic. This study aims to analyze the expression pattern of sLeA, sLeX, LeX, and LeY in the placental villi tissue of patients with a medical history of unexplained miscarriages.

Methods: Paraffin-embedded slides originating from placental tissue were collected from patients experiencing a miscarriage early in their pregnancy (6–13 weeks). Tissues collected from spontaneous (n = 20) and recurrent (n = 15) miscarriages were analyzed using immunohistochemical and immunofluorescent staining. Specimens obtained from legally terminated normal pregnancies were considered as control group (n = 18). Assessment of villous vessel density was performed in another cohort (n = 10 each group) of gestation ages-paired placenta tissue. Protein expression was evaluated with Immunoreactive Score (IRS). Statistical analysis was performed by using Graphpad Prism 8.

Results: Expression of sLeA, sLeX, LeX, and LeY in the syncytiotrophoblast was significantly upregulated in the control group compared with spontaneous and recurrent miscarriage groups. However, no prominent differences between spontaneous and recurrent miscarriage groups were identified. Potential key modulators ST3GAL6 and NEU1 were found to be significantly downregulated in the recurrent miscarriage group and upregulated in the spontaneous group, respectively. Interestingly, LeX and LeY expression was also detected in the endothelial cells of villous vessels in the control group but no significant expression in miscarriage groups. Furthermore, assessment of villous vessel density using CD31 found significantly diminished vessels in all size groups of villi (small villi <200 μm, P = 0.0371; middle villi between 200 and 400 μm, P = 0.0010 and large villi >400 μm, P = 0.0003). Immunofluorescent double staining also indicated the co-localization of LeX/Y and CD31.

Conclusions: The expression of four mentioned carbohydrate Lewis antigens and their potential modulators, ST3GAL6 and NEU1, in the placenta of patients with miscarriages was significantly different from the normal pregnancy. For the first time, their expression pattern in the placenta was illustrated, which might shed light on a novel understanding of Lewis antigens' role in the pathogenesis of miscarriages.

Keywords: Lewis antigen, ST3GAL6, NEU1, villous vessel, unexplained miscarriage

INTRODUCTION

Miscarriage is the most common complication of pregnancy, which affects around 9–20% of clinically confirmed pregnancies. In addition, if biochemical loss due to implantation failure is considered, this rate can reach up to 50% (1). The establishment of a healthy pregnancy implies interaction between the embryonal structure and endometrium. Any alterations within this process may trigger miscarriages: chromosomal errors, anatomical uterine defects, autoimmune dysregulations, and endometrial abnormality. Prior studies have shown that more than 50% of cases of miscarriages are associated with the above-mentioned alterations, however the remaining causes are unexplained (2). According to the guidelines of the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), recurrent miscarriage (RM) is defined as the failure of two or more clinically confirmed pregnancies, excluding ectopic and molar pregnancies (3, 4). The average prevalence of recurrent miscarriage is estimated to be between 1 and 4% (1). Most miscarriages, including spontaneous or recurrent events, occur in the first trimester of pregnancy (5).

Lewis antigens are series of carbohydrate epitopes with terminal fucosylation (6). According to different glycosidic bonds, Lewis antigens are classified as type I [H1 antigen, Lewis A (LeA), Lewis B (LeB) and Sialyl Lewis A (sLeA)] and type II [H2 antigen, Lewis X (LeX), Lewis Y (LeY) and Sialyl Lewis X (sLeX)], as shown in **Figure 1**. Lewis antigens are known to mediate adhesion between tumor cells and endothelium by interacting with their selectin ligands. Furthermore, upregulated expression of Lewis antigens has been reported in many types of cancers (7). More recently, it was showed that Lewis epitopes are also involved in early embryogenesis and later development of the pregnancy. Specifically, sLeX participates in the process of sperm–zona pellucida binding (8). During the menstrual cycle, the expression of sLeX in the human endometrium is temporally regulated, reaching the highest level during embryo implantation (9). LeY was also shown to be involved in a cellular model of trophoblast attachment to the epithelium (10). Given the involvement of Lewis antigens and their selectin ligands in the initial steps in implantation (11), we speculate that Lewis antigens may also have potential significance in the pathogenesis of miscarriages.

METHODS

Patient

Placenta samples of recurrent miscarriages (RM, n = 15), spontaneous miscarriages (SM, n = 20), and normal pregnancies (NC, n = 18) were chosen from the tissue bank of the Department of Obstetrics and Gynecology of LMU Munich. The details including both patients' inclusion and exclusion criteria and the process of samples acquisition have been described in our previous work (12). **Supplementary Figure 1** reflects the gestational ages distribution in different groups of samples. **Table 1** summarizes the demographic and clinical characteristics of the study population.

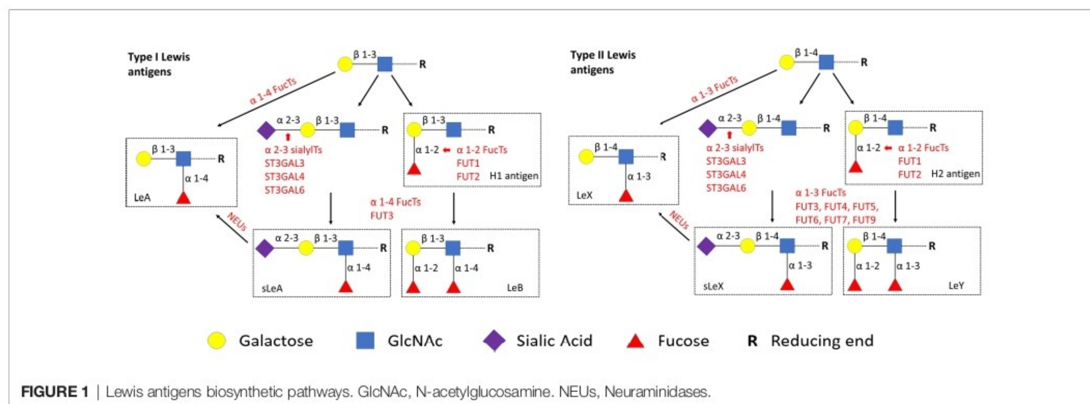

FIGURE 1 | Lewis antigens biosynthetic pathways. GlcNAc, N-acetylglucosamine. NEUs, Neuraminidases.

TABLE 1 | Demographic and clinical characteristics of the study population.

Characteristics	Normal Control n = 18	Recurrent Miscarriage n = 15	Spontaneous Miscarriage n = 20	*P* value
Maternal age (years)	31.28 ± 6.03 (21–43)	34.60 ± 5.96 (22–42)	34.35 ± 4.72 (26–42)	0.14
Gestational age (weeks)	9.04 ± 1.90 (6–13)	9.65 ± 1.66 (7–12)	9.45 ± 1.54 (6–12)	0.57
Gravidity	3.28 ± 1.67 (1–6)	3.27 ± 1.34 (2–6)	1.60 ± 0.82 (1–4)	0.0002
Parity	1.15 ± 1.10 (0–4)	0.80 ± 0.94 (0–3)	0.45 ± 0.76 (0–3)	0.0035

Values are presented as Mean ± S.D.; the range is shown in parentheses.

Gene Expression and Network Analysis

Enrichment of glycosylation and inflammatory response related signatures in RM patients were demonstrated by Gene set enrichment analysis (GSEA) (13) using two gene sets (GO_GLYCOSYLATION, GO:0070085 and HALLMARK_INFLAMMATORY_RESPONSE, M5932) and GSE76862 (14), The functional enrichment was carried out by using the GSEA method based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses with the clusterProfiler package of R (P <0.05 and FDR <0.25). To define the possible crosstalk of glycosylation with immune and adhesion process, differentially regulated GO_GLYCOSYLATION genes in RM were extracted and processed with a web-based tool of Metascape (https://metascape.org) (15) to get significantly enriched Gene Ontology (GO) biological processes and pathways network visualization.

Immunohistochemistry

Paraffin-embedded slides of placenta tissue were dewaxed in xylol for 20 min and rinsed with 100% ethanol for the staining process. For inhibition of the endogen peroxidase reaction, slides were incubated in methanol with 3% H_2O_2 for 20 min followed by rehydration in descending ethanol gradients. Slides were then heated in a pressure pot containing a sodium citrate buffer (pH = 6.0), which consisted of 0.1 mM sodium citrate and 0.1 mM citric acid in distilled water. After cooling in distilled water and rinsing twice in PBS, all slides were incubated with a blocking solution (Reagent 1, ZytoChem Plus HRP Polymer System (mouse/rabbit), Zytomed Berlin, Germany) for 5 min to avoid non-specific binding.

Primary antibody incubation of every slide was performed for 16 h at 4°C, details are shown in **Table 2**. PBS (pH = 7.4) washing was applied between each step. Subsequently, 20 min incubation with post block (Reagent 2) and 30 min with HRP polymer (Reagent 3) were done according to the manufacturer's protocol. Immunostaining was visualized with the DAB chromogen-substrate staining system (Dako, Denmark), the reaction was stopped with distilled water. Slides were counterstained with Hemalaun for 2 min, blued in tap water for 5 min, and rehydrated in an ascending ethanol gradient. Finally, all tissue slides were covered with Eukitt® quick hardening mounting medium (Sigma Aldrich, USA). Negative controls were performed by replacing the primary antibodies with certain species-specific isotype control antibodies (Dako).

The staining results were analyzed under the microscope Leitz (Wetzlar, Germany; Type 307-148.001 514686) by two independent observers. Intensity and distribution patterns of antigens' expression were evaluated with immunoreactive score (IRS), the semi-quantitative score is calculated as the optical staining intensity (grades: 0 = none, 1 = weak, 2 = moderate, 3 = strong staining) multiplied by the total percentage of positively stained cells (0 = none, 1 ≤10%, 2 = 11–50%, 3 = 51–80% and 4 ≥ 81% of the cells). This multiplication has a minimum of 0 and a maximum of 12.

Immunofluorescence

To identify the expression pattern of LeX and LeY in the endothelial cells of villous vessels, double immunofluorescence staining was performed in the specimens of healthy controls. All used antibodies are listed in **Table 2**. CD31 was used as a specific marker for endothelial cells. The same experimental steps were carried out as for immunohistochemistry until the step of blocking: slides were blocked with Ultra V Block solution (Lab Vision, USA) for 15 min and then incubated with specific

TABLE 2 | Antibodies used in this study.

Antibody	Isotype	Clone	Dilution	DAB time	Source
sLeA	Mouse IgG	Monoclonal	1:80 in PBS	30 s	Calbiochem
sLeX	Mouse IgM	Monoclonal	1:200 in PBS	5 min	BD Pharmingen
LeX	Mouse IgM	Monoclonal	1:200 in PBS	5 min	Novocastra
LeY	Mouse IgM	Monoclonal	1:50 in PBS	6 min	LSBio
CD31	Rabbit IgG	Polyclonal	1:50 in PBS	1 min	Abcam
Cy2	Goat IgG anti Mouse	Polyclonal	1:100 in Dako Antibody Diluent	–	Dianova
Cy3	Goat IgG anti Rabbit	Polyclonal	1:500 in Dako Antibody Diluent	–	Dianova
FUT4	Rabbit IgG	Polyclonal	1:100 in PBS	30 s	Prosci
ST3GAL3	Rabbit IgG	Polyclonal	1:500 in PBS	2 min	Invitrogen
ST3GAL4	Rabbit IgG	Polyclonal	1:100 in PBS	2 min	Sino Biological
ST3GAL6	Rabbit IgG	Polyclonal	1:100 in PBS	30 s	Novus Biologicals
NEU1	Rabbit*	Polyclonal	1:50 in PBS	3 min	Sigma-Aldrich

**Isotype is not mentioned in the datasheet.*

primary antibodies for 16 h at 4°C. After washing twice in PBS, slides were incubated with Cy2-/Cy3-labeled fluorescent secondary antibodies (Dianova, Germany) for 30 min at room temperature in darkness to avoid fluorescence quenching. Finally, slides were embedded in Vectashield® mounting medium with DAPI (Vector Laboratories, USA) for blue staining of the nucleus after washing and drying. A Fluorescent Axioskop photomicroscope (Zeiss, Germany) was used to examine all slides. Pictures were taken using a digital Axiocam camera system (Zeiss, Germany).

Evaluation of Villous Vessels in Miscarriage and Control Groups

Another cohort (n = 10 each group) of gestation ages-paired placenta tissues was selected to perform CD31 staining, which visualized the villous vessels. Slides were evaluated by two independent observers using the microscope (Leica, Germany) at a magnification of ×100. Five fields were randomly captured for every slide. Any brown-staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent vessels was considered as a single, countable vessel. The visualization of the vessel lumens was not a prerequisite for a structure to be identified as a vessel (16). To balance the significantly different vessel numbers between large and small villi, all villi were divided into three groups according to their diameter: small villi (<200 μm), middle villi (200–400 μm), and large villi (>400 μm). The final "number of vessels per villous" was calculated (number of vessels/number of villi of every sample) and statistically analyzed in different size groups, respectively.

Statistical Analysis

Statistical differences between experimental groups were analyzed using Graphpad Prism 8 (Graphpad Software Inc., USA). Data in this study were represented as the mean ± SD for quantitative variables. The Gaussian distribution of the continuous variables was tested by the Kolmogorov–Smirnov statistic. Two-tailed Student's paired *t*-test or Wilcoxon matched-pairs signed rank test was performed for the paired cohort. One way ANOVA or Kruskal–Wallis test was used for more than two comparison groups, and Dunnett's test for multiple comparisons. Correlation analysis was performed using Spearman's rank correlation coefficient. A P value <0.05 was considered to be statistically significant.

RESULTS

Aberrant Glycosylation and Inflammatory Response in RM

Our GSEA analysis indicated that glycosylation and inflammatory response were significantly changed in RM (**Figure 2A**). More specifically, glycosylation was downregulated (NES = −1.7706, FDR = 0.0024) and inflammatory response was upregulated (NES = 2.0732, FDR <0.0001). We next performed Metascape analysis including the significantly downregulated GO_GLYCOSYLATION genes in RM. Here, we identified distinctive GO biological processes "Immune system process and Biological adhesion". These results might indicate their significant interaction with the downregulation of glycosylation status in RM (**Figure 2B**). Besides, KEGG pathway and GO enrichment analysis (**Supplementary Figure 2**) also revealed distinctive pathways and cellular components "Focal adhesion and Cell adhesion molecules", with which Lewis antigens have been tightly associated (7). Furthermore, pathways network visualization (**Figure 2C**) indicated that leukocyte adhesion, migration, and developmental process might occur due to changes in glycoprotein metabolic process in RM. Notably, VEGFA-VEGFR2 signaling pathway, which plays the pivotal role in the angiogenesis in ovarian function and embryonic development (17), was also involved in the network.

Downregulated Lewis Antigens in the Miscarriage Groups

Positive expression of sLeA, sLeX, LeX, and LeY was detected in the syncytiotrophoblast across all groups included in this study (**Figure 3**). More specifically, sLeA was prominently lower in both RM and SM group than NC group (P <0.05 and P <0.001, respectively); sLeX expression was also prominently lower in both RM and SM group compared with NC group (P <0.001 and P <0.05, respectively) and sLeX was even lower in RM than SM group (P <0.05); LeX expression was significantly downregulated in both RM (P <0.05) and SM (P <0.001) groups comparing with NC group; LeY showed similar expression pattern to LeX in both RM (P <0.01) and SM (P <0.01) groups. Strikingly, LeX and LeY staining were also clearly identified inside the villi of the normal control group, probably the endothelial cells of villous vessels, but not significant in miscarriage groups (**Figure 4**).

To identify potential key enzymes related to Lewis antigens in miscarriage, we evaluated the expression patterns of three α-2,3 sialyltransferases, ST3GAL3, -4, and -6, which act on the N-Acetyllactosamine structure (Galβ1,3/4GlcNAc) to create sLeA/X and related sialofucosylated glycans. Synthesis of Lewis antigens also requires the synergic action of variable fucosyltransferases (**Figure 1**), here α-1,3 fucosyltransferase FUT4 was investigated. Pretest staining of FUT3 and FUT6 (n = 5 each group) showed their strong expression in the syncytiotrophoblast of every sample but no significant differences between groups (**Supplementary Figure 3**). Meanwhile, neuraminidase 1 (NEU1), a member of the human sialidases (neuraminidases) family that controls cellular sialic acid contents in collaboration with sialyltransferases by catalyzing the removal of sialic acid moieties from glycoproteins and glycolipids, was also investigated. Among three sialyltransferases, ST3GAL6 showed the strongest expression, it was prominently higher in the NC group than the RM group (P <0.05), but not the SM group (**Figure 5**). Expression of ST3GAL3, ST3GAL4, and FUT4 showed no significant differences among groups (**Figure 5**). NEU1 in the SM group but not the NC group was prominently upregulated than the RM group (P <0.05, **Figure 5**). Negative controls are shown in **Supplementary Figure 4**.

Positive Correlation of ST3GAL6 with sLeX and LeY

Significant correlations were found in the expression of ST3GAL6 with sLeX and LeY (**Table 3** and **Figure 6**). In detail, both sLeX and LeY were positively correlated with ST3GAL6 in the syncytiotrophoblast (r = 0.4277, P = 0.0130; r = 0.6377, P <0.0001 respectively). In contrast, there were no prominent correlations of NEU1 with any of the four Lewis antigens (**Table 3**).

Diminished Villous Vessels in the Miscarriage Group

CD31 staining of gestation ages-paired placenta tissues revealed significantly diminished vessels in the villi of the miscarriage group, which was consistent across all types of villi (**Figure 7**). In the miscarriage group, the mean number of vessels per small villi was 0.62 ± 0.59 (*vs* 0.98 ± 0.63, P = 0.0371), per middle villi (1.95 ± 1.32 *vs* 3.88 ± 1.90, P = 0.0010), and per large villi (2.95 ± 2.01 *vs* 9.92 ± 3.50, P = 0.0003).

Existence of LeX/Y in the Endothelial Cells of Villous Vessels

LeX/Y positive cells were stained in green, CD31 positive endothelial cells were red (**Figure 8**). LeX/Y + CD31 double immunofluorescent staining was used to further prove that immunohistochemical staining of LeX/Y inside the villi (**Figure 4**) was exact the endothelial cells of villous vessels. The

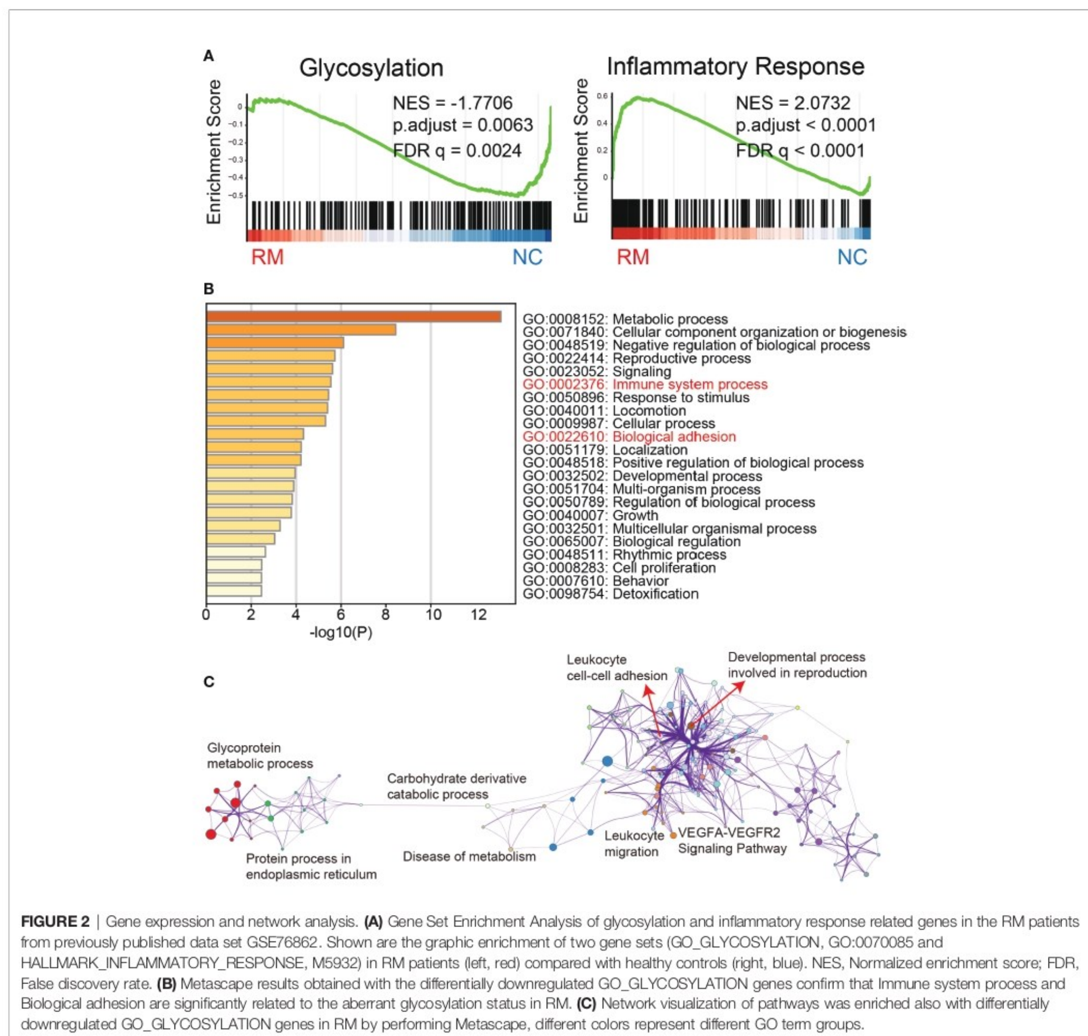

FIGURE 2 | Gene expression and network analysis. **(A)** Gene Set Enrichment Analysis of glycosylation and inflammatory response related genes in the RM patients from previously published data set GSE76862. Shown are the graphic enrichment of two gene sets (GO_GLYCOSYLATION, GO:0070085 and HALLMARK_INFLAMMATORY_RESPONSE, M5932) in RM patients (left, red) compared with healthy controls (right, blue). NES, Normalized enrichment score; FDR, False discovery rate. **(B)** Metascape results obtained with the differentially downregulated GO_GLYCOSYLATION genes confirm that Immune system process and Biological adhesion are significantly related to the aberrant glycosylation status in RM. **(C)** Network visualization of pathways was enriched also with differentially downregulated GO_GLYCOSYLATION genes in RM by performing Metascape, different colors represent different GO term groups.

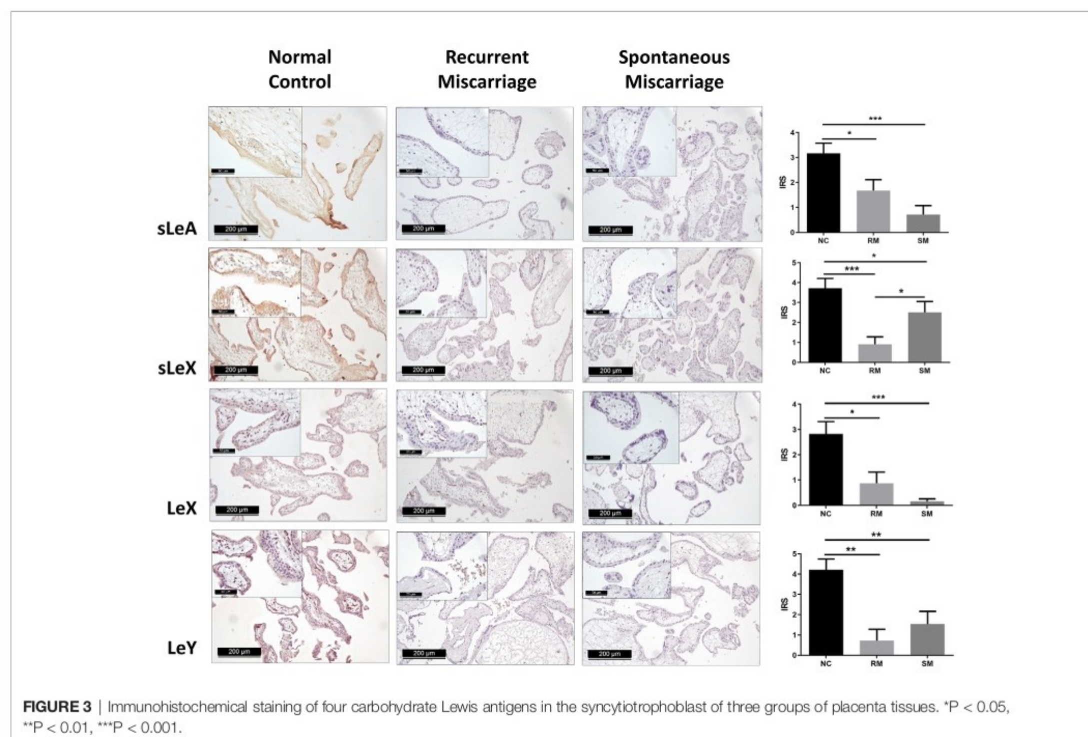

FIGURE 3 | Immunohistochemical staining of four carbohydrate Lewis antigens in the syncytiotrophoblast of three groups of placenta tissues. *P < 0.05, **P < 0.01, ***P < 0.001.

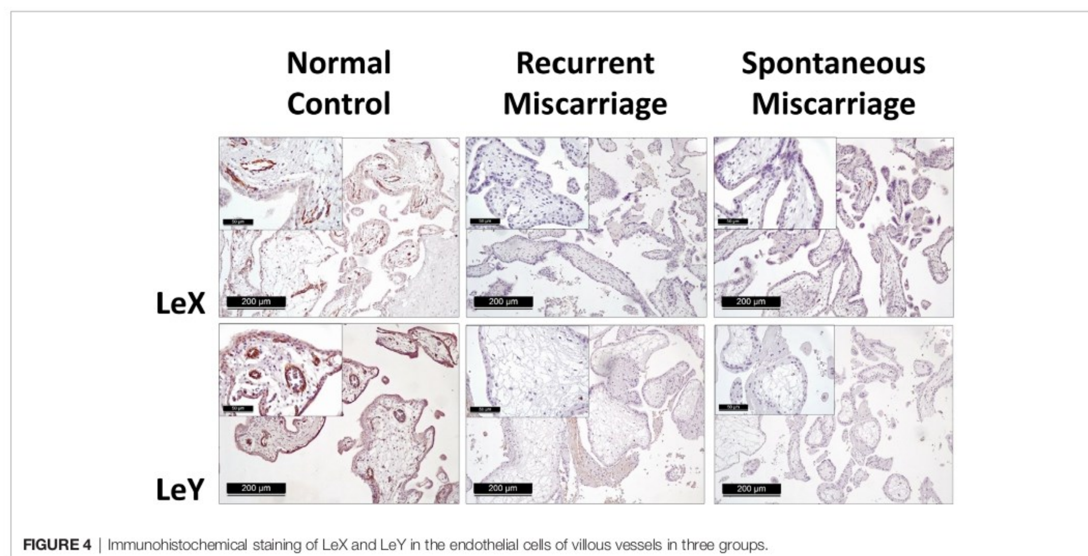

FIGURE 4 | Immunohistochemical staining of LeX and LeY in the endothelial cells of villous vessels in three groups.

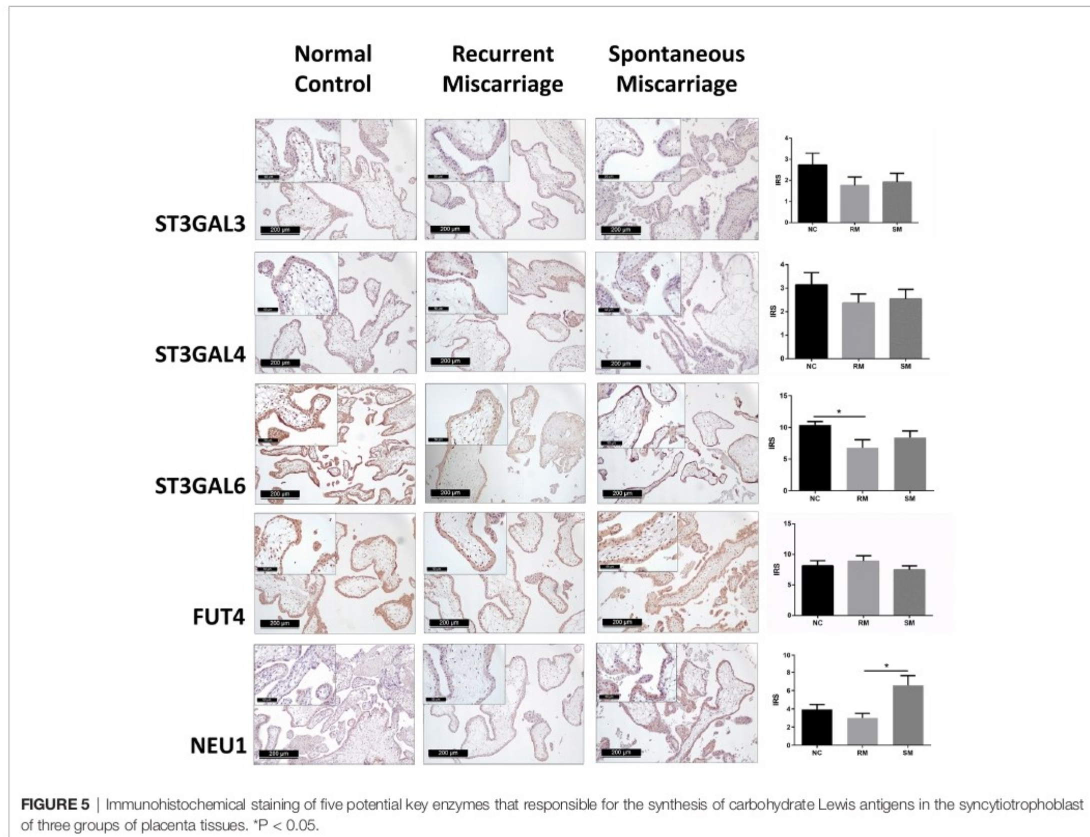

FIGURE 5 | Immunohistochemical staining of five potential key enzymes that responsible for the synthesis of carbohydrate Lewis antigens in the syncytiotrophoblast of three groups of placenta tissues. *P < 0.05.

TABLE 3 | Spearman's rank correlation coefficients of the expression of ST3GAL6 and sLeA, sLeX, LeX, and LeY.

	ST3GAL6			NEU1		
	r	*P* value	95% CI	*r*	*P* value	95% CI
sLeA	0.2336	0.1837	−0.1238 to 0.5373	−0.1606	0.3719	−0.4857 to 0.2035
sLeX	0.4277	**0.0130**	0.08843 to 0.6780	0.1083	0.5486	−0.2540 to 0.4439
LeX	0.2234	0.2190	−0.1464 to 0.5385	−0.07437	0.6858	−0.4213 to 0.2915
LeY	0.6377	**<0.0001**	0.3678 to 0.8085	0.05042	0.7841	−0.3133 to 0.4013

R, correlation coefficient; CI, confidence interval. P < 0.05 is in bold and considered to be statistically significant.

merged pictures in **Figure 8** confirm the co-localization of LeX/Y and CD31.

DISCUSSION

Aberrant glycosylation often indicates the tissue inflammation and neoplasia. Particularly, increased sialylation actuates the key processes of tumor progression and metastasis like cell adhesion, invasion, and immune escape (18–22). Meanwhile, there is increasing evidence that altered glycosylation is important in human gamete binding and embryo implantation (8, 11, 23–26). Establishment of balanced maternal immune tolerance towards the semi-allogenic fetus is a crucial step in maintaining a healthy pregnancy. In particular, an inflammatory microenvironment is required for early implantation (27). However, an excessive inflammatory response would lead to RM and other pregnancy complications like pre-eclampsia and premature labor (28). Whether glycosylation alterations interact with inflammatory response and contribute to the pathogenesis of miscarriage is largely unknown. In this study, we report significant downexpression of glycosylation-related signatures and

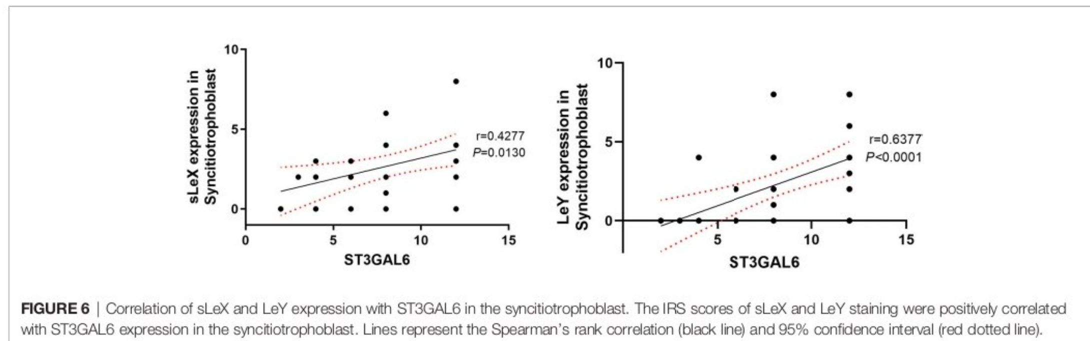

FIGURE 6 | Correlation of sLeX and LeY expression with ST3GAL6 in the syncitiotrophoblast. The IRS scores of sLeX and LeY staining were positively correlated with ST3GAL6 expression in the syncitiotrophoblast. Lines represent the Spearman's rank correlation (black line) and 95% confidence interval (red dotted line).

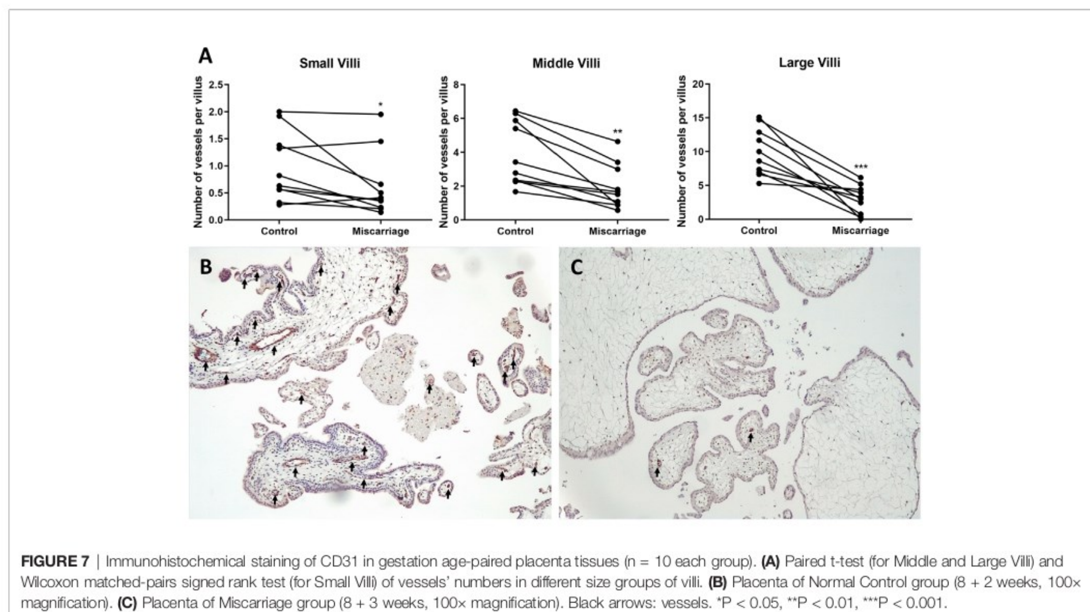

FIGURE 7 | Immunohistochemical staining of CD31 in gestation age-paired placenta tissues (n = 10 each group). **(A)** Paired t-test (for Middle and Large Villi) and Wilcoxon matched-pairs signed rank test (for Small Villi) of vessels' numbers in different size groups of villi. **(B)** Placenta of Normal Control group (8 + 2 weeks, 100× magnification). **(C)** Placenta of Miscarriage group (8 + 3 weeks, 100× magnification). Black arrows: vessels. *P < 0.05, **P < 0.01, ***P < 0.001.

upregulated inflammatory response, which is in line with previous studies (29, 30), in RM through GSEA. Gene enrichment and network analysis performed with downregulated GO_GLYCOSYLATION genes in RM deciphered the significant relationships of aberrant glycosylation with immune system process and biological adhesion, the possible interactions were also visualized (**Figure 2**). Immunohistochemical staining further indicated that Lewis antigens were downregulated in miscarriage groups and key modulators including, ST3GAL6 and NEU1, might be responsible for these alterations.

SLeA, also known as carbohydrate antigen 19-9 (CA19-9), and sLeX are the most common sialylated cell surface glycoconjugates, which mediate the adhesion of leukocytes to endothelial cells and platelets (23). They have been also widely investigated in many types of cancers, and sLeA and sLeX were reported to promote metastasis and malignant transformation (7). SLeA and sLeX were both found to be abundant at the human endometrium during the implantation stage (23). Meanwhile, sLeX was also identified at the level of human zona pellucida, indicating its pivotal role in sperm–egg binding and subsequent adhesion of the embryo to the endometrium (8, 11, 25). Upregulation of sLeX *via* FUT7 transfection promoted the embryo adhesion and implantation were shown in both *in vitro* and *in vivo* model (31, 32). The significantly downregulated sLeA and sLeX in the miscarriage groups revealed by our study may partly explain disturbed trophoblast adhesion and invasion ability during early placentation that leads to subsequent early pregnancy loss.

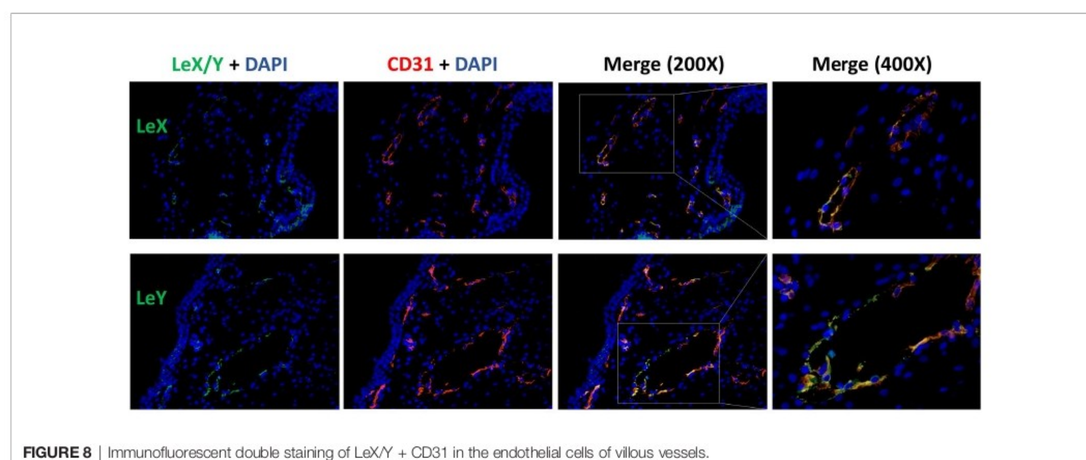

FIGURE 8 | Immunofluorescent double staining of LeX/Y + CD31 in the endothelial cells of villous vessels.

LeX, also Stage-specific embryonic antigen-1 (SSEA-1), is playing a gradually role in human embryogenesis, especially in cell-cell recognition and adhesion processes which is critical on the surface of embryonic ectodermal cells (33). In the brain, LeX predominantly facilitates the cell-cell interactions involved in neuronal development (34). Moreover, LeX is necessary for neutrophil transepithelial migration (35). On the other hand, overexpression of LeX is usually associated with decreased survival, metastasis, and malignant transformation in many types of cancers (36–40). In accordance with a previous study (41), we also found a weak expression manner of LeX in the syncytiotrophoblast in the NC group, but almost no expression in the miscarriage groups. Given the important role of LeX in the processes of adhesion and metastasis, we speculate that downregulated LeX in the syncytiotrophoblast may contribute to miscarriage for insufficient trophoblast function.

As previously described, LeY has been found in human uterine epithelial tissues and its expression was significantly upregulated during the secretory stages of the menstrual cycle in humans (42). Blocking LeY reduced the adhesion of the human trophoblast cell line (JAR) to the uterine epithelial cell line (RL95-2) in an *in vitro* implantation model (10). Here, we report that both LeY and LeX are expressed in the chorionic villi, and their expression is significantly downregulated in the syncytiotrophoblast of miscarriage groups comparing with the NC group. In addition, LeX and LeY expression was also identified inside the villi in the NC group, but not prominent in the miscarriage group. We further showed that LeX and LeY specifically localized in the endothelial cells of the villous vessel. The villous vessels in the miscarriage group were significantly diminished in all sizes of villi compared with the control group. Candelier et al. and Ziganshina et al. reported similar findings in their work on hydatidiform moles and fetal growth restriction (41, 43). Moreover, the role of LeY in endothelial tube formation and angiogenesis has been previously demonstrated in human rheumatoid arthritis and rat cornea (44, 45). Deficient vascularization has been reported in miscarriages and hydatidiform mole, especially the empty sac miscarriages (46, 47). While Reus et al. showed that there was no prominent difference between empty sac or yolk sac miscarriages and embryonic miscarriages concerning the chorionic villous vascularization (48). In our network analysis, VEGFA-VEGFR2 signaling pathway, which plays a prominent role in the angiogenesis, also interacts with aberrant glycosylation. Thus, the downregulated LeY expression may account for the insufficient trophoblast function as well as the defective vascularization in miscarriages.

Leukocyte infiltration in the decidua is a cardinal feature during first trimester pregnancy, which comprises mainly the natural killer cells, macrophages and, comparatively a few T cells (49). The crosstalk between trophoblast and decidual leukocytes *via* complicated cytokine network allows these two parts to attract each other (49). Alterations in leukocytes recruitment and activation have been extensively related to miscarriage (50). Similarly, our results obtained through network analysis indicated that the aberrant glycosylation might be involved in leukocyte adhesion and migration in RM. Moreover, five key modulators in the synthetic pathways of Lewis antigens were investigated, among which ST3GAL6 and NEU1 showed dysregulated expression. To our knowledge, this is the first description of α-2,3 sialyltransferases, neuraminidase, and fucosyltransferase expression patterns in the chorionic villi of patients with unexplained miscarriages.

ST3GAL3, ST3GAL4, and ST3GAL6 all belong to the α-2,3 sialyltransferases family. While having different substrate specificity, they all catalyze the transfer of sialic acid residues to galactopyranosyl residue (51). *In vitro* study suggested that ST3GAL3 preferentially acts on type I disaccharides (Galβ1,3GlcNAc, the backbone of sLeA), but also mildly catalyze the sialylation of type II disaccharides (Galβ1,4GlcNAc, the backbone of sLeX) (52). Sasaki et al. reported that ST3GAL4 recombinant protein extracted from a melanoma library showed enzymatic activity towards both type I and type II disaccharides in *in vitro* assays whereas preferentially catalyzed type II substrates in

in vivo condition (53). While most studies found ST3GAL4 acted on type II disaccharides despite *in vitro* or *in vivo* status (54–57). Similarly, ST3GAL6 mainly catalyzes the transfer of sialic acid residues onto type II disaccharides found on glycoproteins and glycolipids (58, 59). We demonstrated a significant correlation of ST3GAL6 expression with sLeX, which is consistent with previously described exclusive ST3GAL6 effect on type II disaccharides. This is especially important, considering that downregulated ST3GAL6 may play a role in the downregulation of sLeX in the villi of recurrent miscarriages. Notably, we also found a strong positive correlation between ST3GAL6 and LeY expression, which cannot be simply explained by the enzymatic specificity of ST3GAL6 since the synthesis of LeY requires the sequential effect of α-1,2 and α-1,3 fucosyltransferases rather than sialyltransferases. Inhibited sialylation potentially allows greater fucosylation due to competition between fucosyltransferase and sialyltransferase, for the same acceptor substrates (57, 60, 61), downregulated ST3GAL6 should have yielded higher expression of LeX and LeY, which was not found in our study. A possible explanation might be the existence of predominantly impaired fucosyltransferases in the miscarriage villi has not been identified yet, on the other hand, the glycosylation network is far more complex than we have known.

FUT4 was reported in many studies to be vital in controlling the synthesis of LeX and LeY (10, 61, 62), but demonstrated no significant difference among the groups included in our analysis. Gadhoum and Sackstein found that NEU1 predominantly increased LeX expression through desialylation sLeX in the process of human myeloid differentiation (63). NEU4, which is another member of the human neuraminidase family, shows a broader substrate specificity compared with NEU1. Previous study indicates that NEU4 desialylates cell surface sLeA/X to LeA/X without affecting the expression of ST3GAL3, ST3GAL4, FUT3, and FUT7 in colon cancer cells (64). Here, we found significantly higher expression of NEU1 in the SM group comparing with the RM group, but no correlations were identified with Lewis antigens, which may due to its limited sialidase capacity on sLeA/sLeX (64).

To conclude, we report significant downregulation of glycosylation-related signatures and adhesion molecules, as well as upregulated inflammatory response in the chorionic villi of RM through GSEA and KEGG pathway analysis. Accordingly, downregulation of sLeA, sLeX, LeX, and LeY were further identified. Key modulators, such as ST3GAL6 and NEU1 may be involved and account for these alterations. Interestingly, significantly diminished villous vessels were also identified in the miscarriage group. Taken together, our data might indicate that altered expression of Lewis antigens may actuate miscarriages by affecting 1) trophoblast function 2) its interaction with decidual leukocytes and 3) the vascularization of the villi. A better understanding of the vital role that Lewis antigens are playing in pathology of miscarriages will yield the development of novel therapeutic approaches, which still requires more future work.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the LMU Munich. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

UJ and VS conceived of the study and participated in its design and coordination. ZM performed the experiments and statistical analysis and wrote the manuscript. HY, LP, and CK performed technical assistance in immunohistochemistry assays. HY, LP, CK, AC-R, SM, UJ, and VS revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.679424/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors (UJ).

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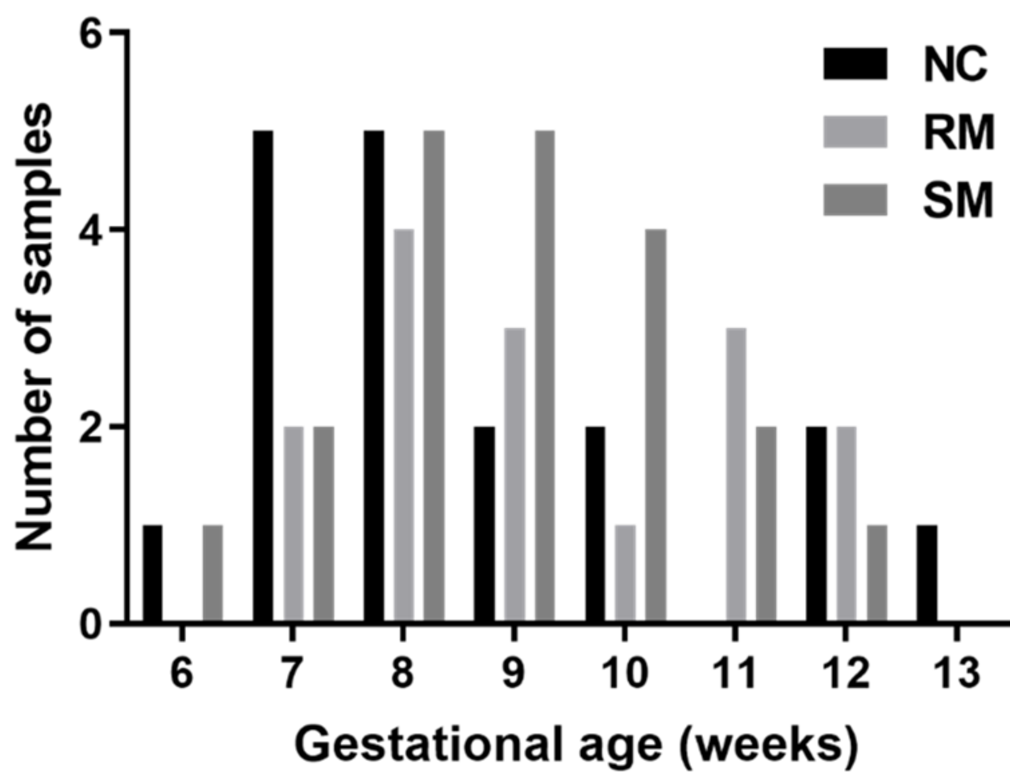
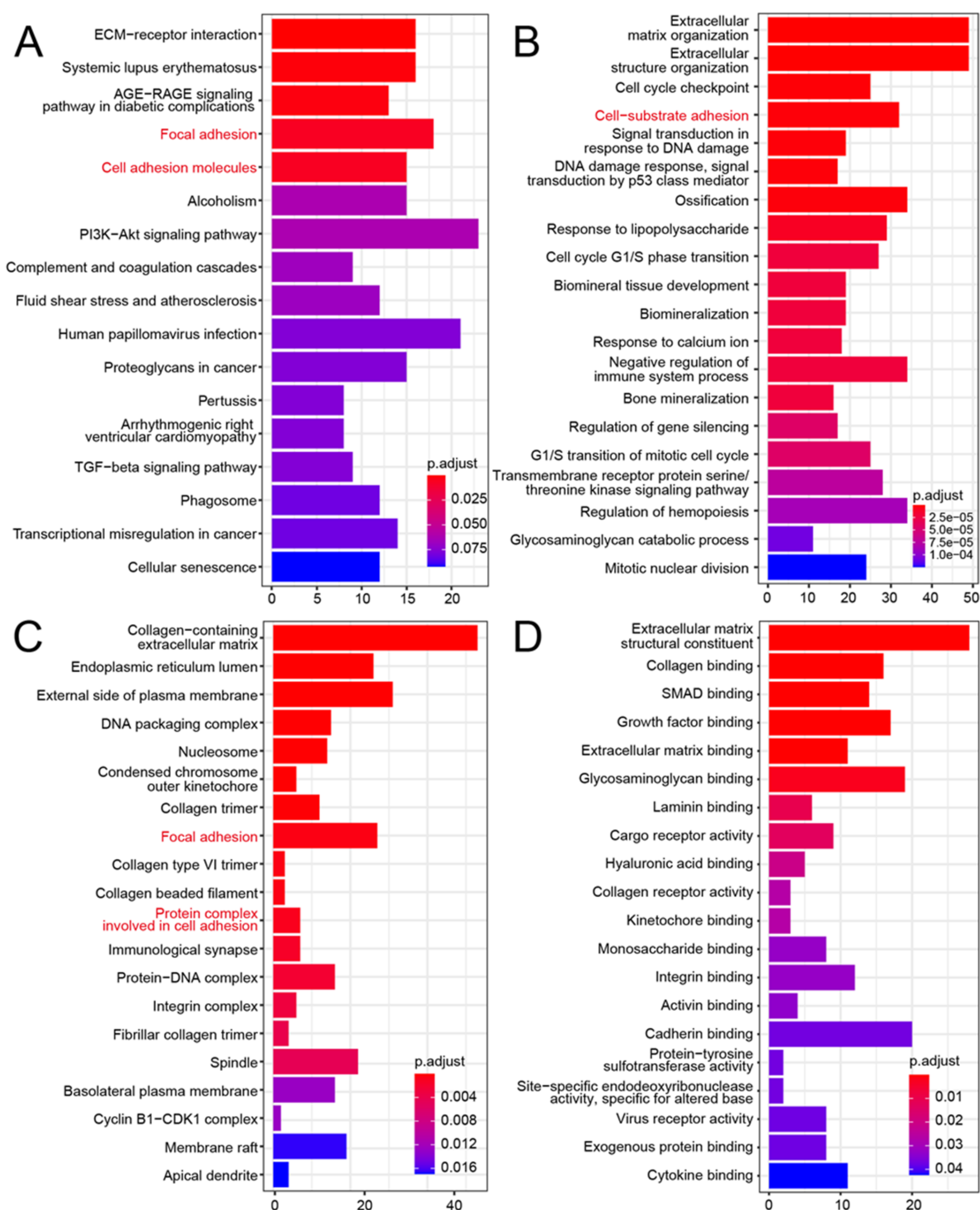
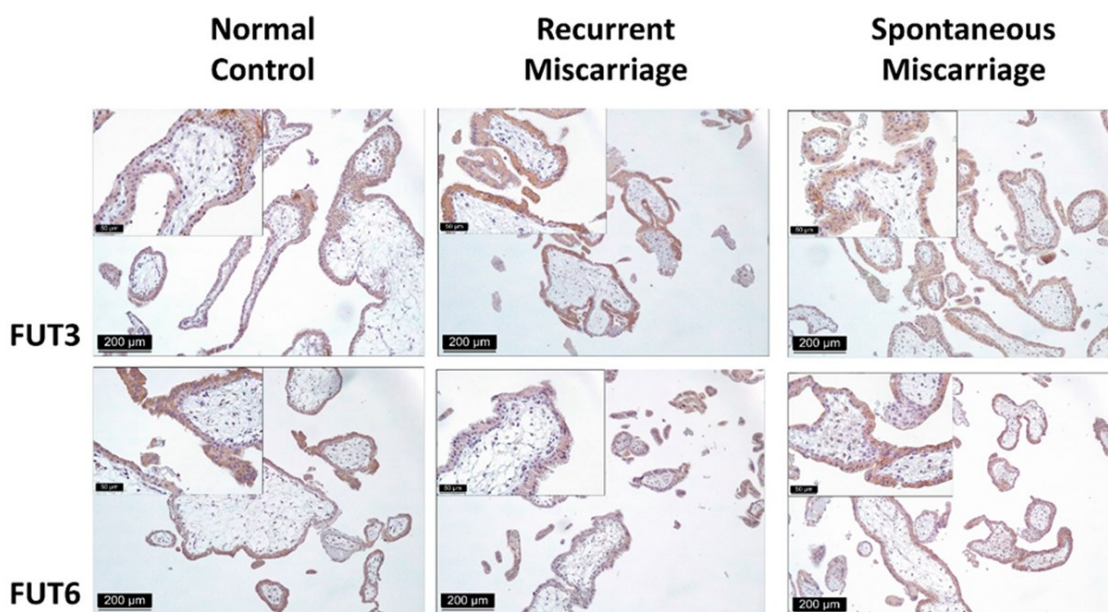
SUPPLEMENTARY MATERIAL



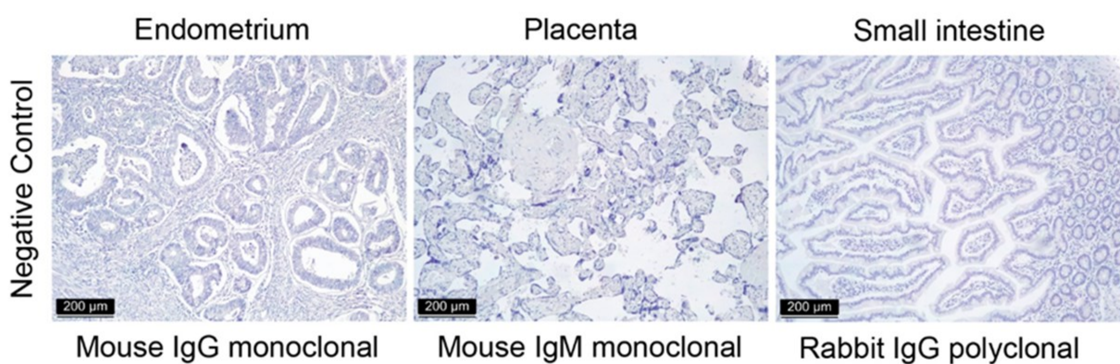
Supplementary Figure 1. Gestational ages distribution in different groups of samples.



Supplementary Figure 2. Gene expression analysis. A) Distinctive pathways obtained from KEGG pathway enrichment analysis, notably "Focal adhesion" and "Cell adhesion molecules" were enriched. Terms in B) Biological Process (BP), C) Cellular Component (CC), and D) Molecular Function (MF) obtained from GO enrichment analysis, notably "Cell-substrate adhesion", "Focal adhesion", and "Protein complex involved in cell adhesion" were also enriched in BP and CC.



Supplementary Figure 3. Immunohistochemical staining of FUT3 and FUT6 in the syncytiotrophoblast of three groups of placenta tissues. Dilution: FUT3 1:200, FUT3 1:400.



Supplementary Figure 4. Immunohistochemical staining of different isotypes of negative controls.

6. Publication II

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ORIGINAL RESEARCH
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Targeting Aberrantly Elevated Sialyl Lewis A as a Potential Therapy for Impaired Endometrial Selection Ability in Unexplained Recurrent Miscarriage

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Background: Carbohydrate Lewis antigens including sialyl Lewis A (sLeA), sialyl Lewis X (sLeX), Lewis X (LeX), and Lewis Y (LeY) are the commonest cell surface glycoconjugates that play pivotal roles in multiple biological processes, including cell adhesion and cell communication events during embryogenesis. SLeX, LeY, and associated glycosyltransferases ST3GAL3 and FUT4 have been reported to be involved in human embryo implantation. While the expression pattern of Lewis antigens in the decidua of unexplained recurrent miscarriage (uRM) patients remains unclear.

Methods: Paraffin-embedded placental tissue slides collected from patients experiencing early miscarriages (6–12 weeks) were analyzed using immunohistochemical (IHC) and immunofluorescent (IF) staining. An *in vitro* assay was developed using endometrial cell line RL95-2 and trophoblast cell line HTR-8/SVneo. Modulatory effect of potential glycosyltransferase on Lewis antigens expression was investigated by target-specific small interfering RNA (siRNA) knockdown in RL95-2 cells. HTR-8/SVneo cells spheroids adhesion assay was applied to investigate the intrinsic role of Lewis antigens in the abnormal implantation process of uRM. The expression of Lewis antigens in RL95-2 cells in response to the treatment with pro-implantation cytokine IL-1β was further measured by flow cytometry and immunocytochemical (ICC) staining.

Results: IHC staining revealed that Lewis antigens are mainly expressed in the luminal and glandular epithelium, IF staining further indicated the cellular localization at the apical membrane of the epithelial cells. FUTs, ST3GALs, and NEU1 located in both stromal and epithelial cells. We have found that the expression of sLeA, LeX, FUT3/4, and ST3GAL3/4 are significantly upregulated in the RM group, while FUT1 is downregulated. SLeX, LeY, ST3GAL6, and NEU1 showed no significant differences between groups. FUT3 knockdown in RL95-2 cells significantly decreased the expression of sLeA and the

spheroids adhesion to endometrial monolayer. Anti-sLeA antibody can remarkably suppress both the basal and IL-1β induced adhesion of HTR-8/SVneo spheroids to RL95-2 cells monolayer. While further flow cytometry and ICC detection indicated that the treatment of RL95-2 cells with IL-1β significantly increases the surface expression of LeX, but not sLeA.

Conclusions: SLeA, LeX, and pertinent glycosyltransferase genes FUT1/3/4 and ST3GAL3/4 are notably dysregulated in the decidua of uRM patients. FUT3 accounts for the synthesis of sLeA in RL95-2 cells and affects the endometrial receptivity. Targeting aberrantly elevated sLeA may be a potential therapy for the inappropriate implantation in uRM.

Keywords: Lewis antigens, sLeA, LeX, FUT3, recurrent miscarriage

INTRODUCTION

Miscarriage is the most common and frustrating disorder of early pregnancy. An estimated 9-20% of clinically recognized pregnancies end in spontaneous loss, and up to 50% of all conceptions are lost at preclinical stages as biochemical loss or implantation failure (1). Recurrent miscarriage (RM), the failure of two or more pregnancies prior to 20 weeks, affects 1-4% of all couples trying to conceive (1, 2). Known risk factors associated with RM are maternal age, previous pregnancy losses, genetic abnormalities, uterine anomalies, and autoimmune disorders. Even after a thorough evaluation, no causes can be identified in more than 50% of RM couples (3), which are defined as unexplained RM (uRM).

Accumulating studies indicate that uRM is associated with the impaired selection ability of decidual cells, allowing embryos of poor viability to implant inappropriately (4–8). Decidualized endometrial stromal cells (ESCs) were reported to be able to sense an arresting embryo, and response with shutting down the production of pro-implantation modulator IL-1β, preventing the implantation of incompetent embryos (9). While ESCs of uRM patients cannot discriminate between high- and low-quality embryos (10).

Sialyl Lewis A (sLeA) and sialyl Lewis X (sLeX) are commonly found on the surface of many types of cancer cells, facilitating the hematogenous metastasis of these cells through interaction with endothelial cells (11). Studies also reveal that sLeA/X are abundant at the endometrium during implantation stage (12). SLeX is the major terminal carbohydrate sequence on zona pellucida, mediating human sperm-egg binding (13). Specific fucosyltransferases (FUTs), sialyltransferases (STs), and neuraminidases (NEUs) are involved in the synthesis of sLeA/X and related epitopes (11, 14–17). Despite the significant roles of sLeX and LeY in normal blastocyst implantation have been investigated in several *in vitro* and animal models (18–20), their expression pattern together with other Lewis antigens in the decidua and association with inappropriate implantation in uRM remains unknown. To clarify this issue, our study aimed to assess whether these Lewis antigens and pertinent glycosyltransferases are altered in the decidua of uRM patients, and elucidate their potential link with the impaired selection ability of ESCs in uRM.

METHODS

Patient

Placental samples of RM (n = 15) and legally terminated normal pregnancies (NC, n = 10) were chosen from the tissue bank of the Department of Obstetrics and Gynecology of LMU Munich as previously described (21). Details of the study population are summarized in **Table 1**.

Immunohistochemistry

Paraffin-embedded placental tissue slides were dewaxed in xylol and rehydrated through descending ethanol. Endogenous peroxidase activity was inhibited in 3% H_2O_2 for 20 min followed by antigen retrieval in a pressure pot with 0.1mM sodium citrate and 0.1mM citric acid buffer. The slides were blocked with Reagent 1 (ZytoChem Plus HRP Polymer System (mouse/rabbit), Zytomed Berlin, Germany) for 5 min before application of the primary antibodies (details are shown in **Table 2**) for 16 h at 4°C. PBS (pH = 7.4) washing was performed between each step. Subsequent incubation with post block (Reagent 2) for 20 min and with HRP polymer (Reagent 3)

TABLE 1 | Demographic and clinical characteristics of the study population.

Characteristics	Normal Control (n=10)	Recurrent Miscarriage (n=15)	*P* value
Maternal age (years)[a]	31.20 ± 2.86	33.50 ± 3.45	0.09
Gestation age (weeks)[a]	9.63 ± 2.40	9.51 ± 1.24	0.87
Gravidity[b]	2 (1-3)	3 (2-6)	0.02
Parity[b]	2 (0-4)	0 (0-2)	0.25

[a]*Data represent mean ± S.D.* [b]*Data presented as median (range).*

TABLE 2 | Details of primary antibodies used in this study.

Antibody	Isotype	Clone	Source	Dilution	Application
E-Selectin	Mouse IgG	Monoclonal	Calbiochem	1:50	IF
L-Selectin	Goat IgG	Polyclonal	R&D Systems	1:20	IF
P-Selectin	Rabbit IgG	Polyclonal	Sigma-Aldrich	1:50	IF
sLeA	Mouse IgG	Monoclonal	Calbiochem	1:80/50/50/50	IHC/IF/FCM/ICC
sLeX	Mouse IgM	Monoclonal	BD Pharmingen	1:200	IHC
LeX	Mouse IgM	Monoclonal	Novocastra	1:200	IHC
			Calbiochem	1:100/100	FCM/ICC
LeY	Mouse IgM	Monoclonal	LSBio	1:50	IHC
EpCAM	Goat IgG	Polyclonal	R&D Systems	1:100	IF
FUT1	Rabbit IgG	Polyclonal	Invitrogen	1:400	IHC
FUT3	Rabbit IgG	Polyclonal	Abcam	1:200	IHC
FUT4	Rabbit IgG	Polyclonal	Prosci	1:100	IHC
ST3GAL3	Rabbit IgG	Polyclonal	Invitrogen	1:500	IHC
ST3GAL4	Rabbit IgG	Polyclonal	Sino Biological	1:100	IHC
ST3GAL6	Rabbit IgG	Polyclonal	Novus Biologicals	1:100	IHC
NEU1	Rabbit*	Polyclonal	Sigma-Aldrich	1:50	IHC

Isotype is not mentioned in the datasheet. IF, immunofluorescence; IHC, immunohistochemistry; FCM, flow cytometry; ICC, immunocytochemistry; EpCAM, Epithelial cell adhesion molecule.

for 30 min was applied following the manufacturer's instructions. The specific antibody binding was visualized with 3,3'-diamionbenzidine substrate-chromogen system (Dako, Denmark) and counterstained with Hemalaun. Certain species-specific isotype control antibodies (Dako) were used for negative control staining. The staining results were analyzed using semi-quantitative immunoreactive score (IRS) as previously described (22).

Immunofluorescence

Placenta tissue slide was processed in the same experimental steps as for immunohistochemistry until the step of blocking: Ultra V Block solution (Lab Vision, USA) was applied for 5 min and then incubated with specific primary antibodies for 16 h at 4° C. After washing twice in PBS, slides were incubated with certain fluorescent secondary antibodies (**Table 3**) according to the host species of different primary antibodies for 30 min at room temperature in darkness. Finally, slides were embedded in Vectashield® mounting medium with DAPI (Vector Laboratories, USA) for nucleus staining after washing and drying. Cell smears were prepared on slides in quadriPERM culture dish (SARSTEDT, Germany). Cells on the slides were fixed with ethanol/methanol solution for 15 min followed by 5 min incubation with Ultra V Block solution, subsequent steps are the same as tissue slide. Double immunofluorescent staining of placenta tissue was examined using Leica SP8 confocal system (Leica Microsystems, Germany) and processed with LAS X software (Leica Microsystems, Germany). Immunofluorescent staining of cell smears was examined by Axioskop photomicroscope (Carl Zeiss Microscopy GmbH, Germany) and processed with ZEN Blue software (Carl Zeiss Microscopy GmbH, Germany).

Cell Lines and Culture

Endometrial cell line RL95-2, general model for receptive epithelial cells (23), and trophoblast cell line HTR-8/SVneo, derived from first trimester villous trophoblast with characteristics of villous cytotrophoblast and extravillous trophoblast (23), were obtained from American Type Culture Collection (ATCC), and were maintained in RPMI 1640 medium + GlutaMAX™ (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) in a 5% CO_2 incubator at 37°C. Medium was refreshed every second day.

Cell Transfection

RL95-2 cells were cultured in 6-well plates (10^6 cells per well, Corning Inc., USA) until 70% confluency and transiently transfected with Silencer Select Pre-designed FUT3 siRNA (12 pmol per well, Thermo Fisher Scientific, USA) with Lipofectamine RNAiMAX (Thermo Fisher Scientific, USA) in Opti-MEM (Gibco, USA) according to the manufacturer's instructions. In the meantime, AllStars Negative Control siRNA (Qiagen, Germany) transfected cells were used as the control. Cells were harvested after 48 h culture for RT-qPCR and

TABLE 3 | Details of fluorescent secondary antibodies used in this study.

Antibody	Source	Dilution	For primary antibody
Goat anti-mouse, Alexa Fluor 488	Dianova	1:100	E-Selectin
Donkey anti-goat, Alexa Fluor Plus 488	Invitrogen	1:300	L-Selectin
Donkey anti-rabbit, DyLight 488	Invitrogen	1:100	P-Selectin
Donkey anti-mouse, Alexa Fluor 488	Invitrogen	1:200	sLeA
Donkey anti-goat, Cy2	Dianova	1:100	EpCAM

72 h culture for flow cytometry detection, respectively. For adhesion assay, after 24 h siRNA transfection, cells were replated into 8-well chamber slides (Millipore, Germany) at 2×10^5 per well and cultured for another 48 h until spheroids adhesion.

RNA Extraction, Reverse Transcription, and RT-qPCR

Total cellular RNA was extracted with NucleoSpin® RNA Mini kit (MACHEREY-NAGEL, Germany) according to manufacturer's instructions. First strand cDNA was prepared from the total RNA (1 µg) using Oligo dT primers and Reverse Transcriptase (Biozym, Germany). For RT-qPCR, cDNA samples were mixed with FAM-labeled TaqMan probes (β-actin: Hs99999903_m1; FUT3: Hs01868572_s1) from Thermo Fisher Scientific (USA) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems, USA), followed by amplification with 7500 Fast Real-Time PCR system (Applied Biosystems, USA) according to manufacturer's protocol. Results were calculated by the comparative CT method, with relative transcript levels determined as $2^{-\Delta\Delta CT}$.

Flow Cytometry Analysis

Detection of cell surface Lewis epitopes was performed by indirect fluorescence. Briefly, RL95-2 cells were detached from the 6-well plate with the Accutase solution (STEMCELL Technologies, USA) digestion, 10^6 cells were stained with respective primary antibodies (**Table 2**) diluted in DPBS-0.5% Bovine Serum Albumin (BSA). After washing twice, cells were incubated with 1:300 diluted fluorochrome-conjugated secondary antibody Alexa Fluor 488 goat anti-mouse IgG (Dianova, Germany). Three independent assays for each sample were analyzed with a BD Accuri™ C6 Cytometer (BD Biosciences, USA).

Pro-Implantation Cytokine IL-1β Treatment

To form a complete endometrial monolayer, RL95-2 cells were firstly cultured in T25 flask with or without 20 ng/ml recombinant human IL-1β (R&D Systems, USA) for 48h to yield enough number of cells. These treated and untreated cells were then subcultured into 6-well plates at a density of 10^6 per well for flow cytometry detection after 48 h, or into 8-well chamber slides for immunocytochemistry (10^5 per well) and *in vitro* implantation assay (2×10^5 per well) after 48h with or without IL-1β treatment. Thus, RL95-2 cells were continuously treated with IL-1β for 4 days in total.

Immunocytochemistry (ICC)

The IL-1β treated RL95-2 cells for immunostaining were prepared in 8-well chamber slides as above explained. Cells on the slides were fixed with ethanol/methanol solution for 15 min. Following steps using ZytoChem Plus HRP Polymer System reagents were the same as immunohistochemical staining except in the last step, slides were not rehydrated in ascending ethanol gradients and were covered with Aquatex® mounting medium (Merck, Germany).

In Vitro Implantation Assay

The RL95-2 cell monolayers were prepared in 8-well chamber slides as above explained. HTR-8/SVneo cells spheroids were created with modified hanging drops method (24). Briefly, 2×10^4 cells per 30 µL drop supplemented with regular culture medium were plated onto the lid of Petri dishes (40 drops/Petri lid). The lid was inverted over the bottom of Petri dish filled with 5ml DPBS. Hanging drops were cultured for 20 h until the spheroids were properly formed, not over aggregated. At the end of spheroids preparation, they were gently collected from the Petri lid to a 50ml Falcon tube and rinsed with complete culture medium. Spheroids suspension was then passed through 200 µm and 100 µm sieve size cell strainer (pluriSelect, Germany) to select spheroids of size between 100 and 200 µm which is similar to human implantation blastocysts.

Selected spheroids were gently transferred onto each well of confluent RL95-2 cells in 8-well chamber slides, and co-cultured for 2 h for adhesion. For the blockade experiment, RL95-2 monolayer cells were pretreated with different concentrations of anti-sLeA/LeX antibody or negative control IgG/M antibody (Dako, Denmark) for 1 h before co-culture. A total number of transferred spheroids in each well were firstly counted, then nonadherent spheroids were aspirated off, washed with DPBS twice and the number of attached spheroids was counted under microscope (Leica Microsystems, Germany). The results were presented as percentage of adhesion spheroids. All experiments were performed in three replicates on three separate days.

Statistical Analysis

Graphpad Prism 8 (Graphpad Software Inc., USA) was used to analyze statistical differences between groups. The normality of data was tested with Shapiro-Wilk's test. Two-tailed Student's *t*-test (for normally distributed data) or Mann-Whitney U test (for non-normally distributed data) was performed for two groups comparison. $P < 0.05$ was determined to be significant.

RESULTS

Upregulated Expression of Lewis Antigens and Associated Key Modulators in uRM

Lewis antigens expression localized mainly in the decidual luminal and glandular epithelium, sparsely in the stromal cells (**Figure 1**). To display the cellular localization of Lewis antigens in the epithelial cells more precisely, double immunofluorescent staining of sLeA and epithelial marker EpCAM (Epithelial cell adhesion molecule) was performed. As the confocal images indicate, sLeA localizes at the apical membrane of epithelial cells (**Figure 2**). The expression of sLeA and LeX both were prominently higher in the RM group than the NC group ($P < 0.05$ and $P < 0.01$, respectively). While sLeX and LeY showed no significant differences between groups.

Cellular glycan maintenance is a complex process, in which FUTs and STs are responsible for the synthesis of fucose residues and sialic acid, while NEUs catalyze the removal of sialic acid moieties (**Figure 3**). To identify the molecular mechanism

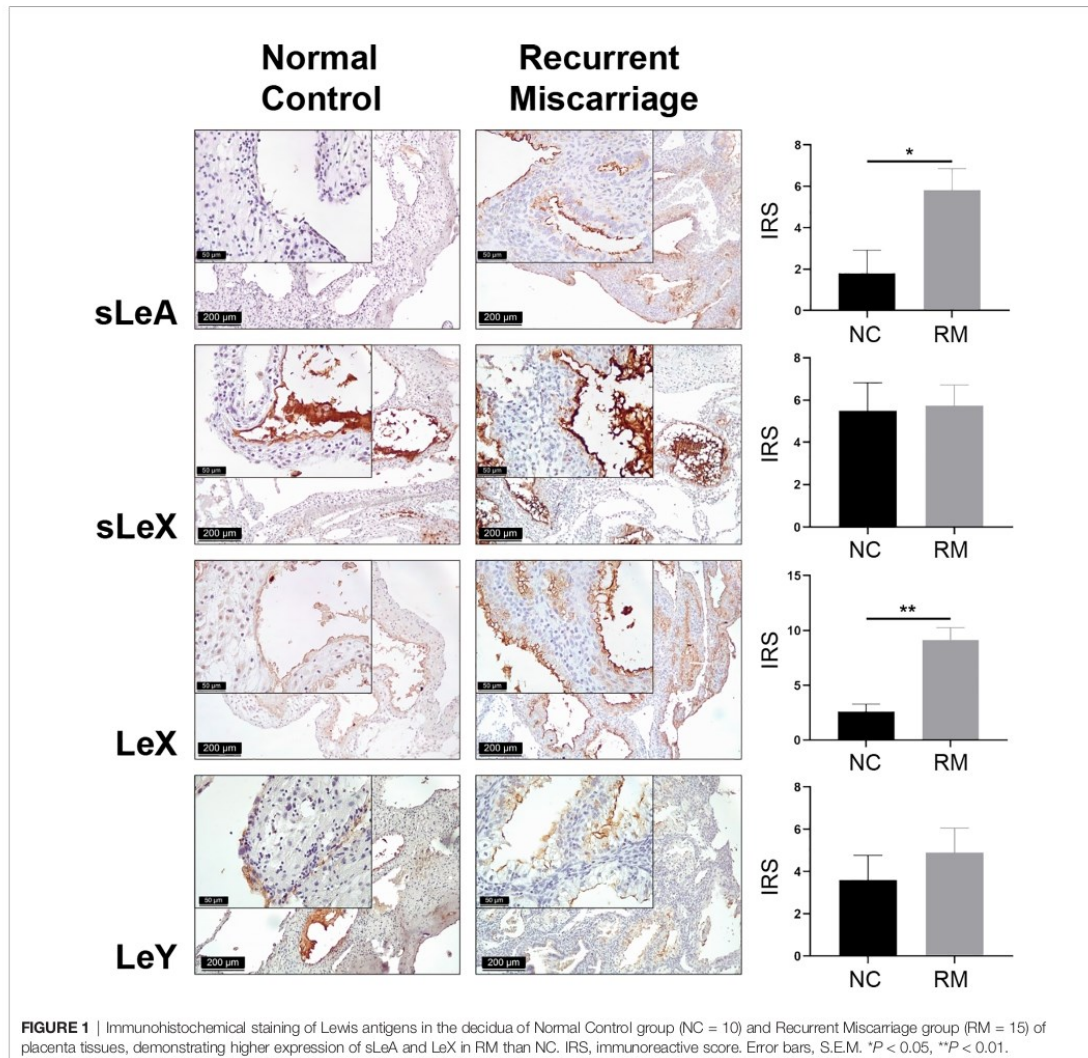

FIGURE 1 | Immunohistochemical staining of Lewis antigens in the decidua of Normal Control group (NC = 10) and Recurrent Miscarriage group (RM = 15) of placenta tissues, demonstrating higher expression of sLeA and LeX in RM than NC. IRS, immunoreactive score. Error bars, S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$.

underlying dysregulated Lewis antigens in uRM, we also evaluated the expression of pertinent glycosyltransferases FUT1/3/4, ST3GAL3/4/6, and NEU1. Here, we found these glycosyltransferases were expressed in both stromal and epithelial cells, and generally higher in the RM group except FUT1 (**Figure 4**). FUT3 and FUT4 were significantly higher in the RM group than the NC group (both $P < 0.05$). Similarly, ST3GAL3 and ST3GAL4 in the RM group were prominently higher than the NC group ($P < 0.01$ and $P < 0.05$, respectively). Notably, FUT1 expression was significantly higher in the NC group than the RM group ($P < 0.05$). ST3GAL6 and NEU1 were slightly higher in the RM group, but the differences were not prominent ($P = 0.083$ and $P = 0.084$, respectively). Negative controls staining is shown in **Supplementary Figure 1**.

Spheroids Formation, Adhesion and Selectins Expression in HTR-8/SVneo Cells

Plenty of spheroids were formed after 20 h culture with modified hanging drops method (**Figure 5A**). After co-cultured for 2 h for adhesion, cell strainer selected spheroids (100 - 200 μm) attached to the confluent RL95-2 cells (**Figure 5B**). Lewis antigens putative ligands E, L, and P selectins could be detected in HTR-8/SVneo cells by immunofluorescent staining (**Figure 5C**).

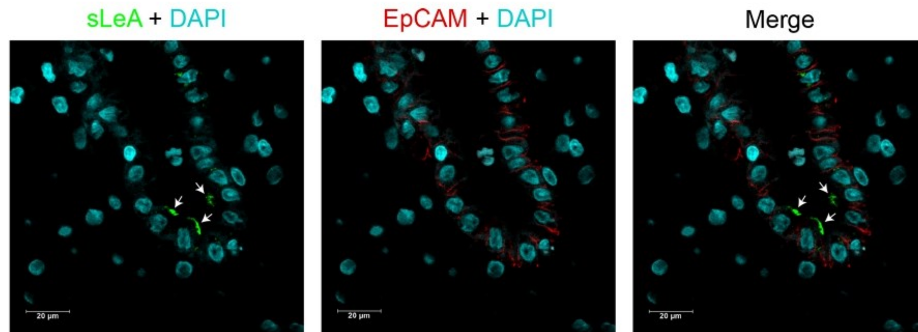

FIGURE 2 | Cellular localization of sLeA in epithelial cells of placenta tissue. sLeA (green) and epithelial cell marker EpCAM (red) were double stained, the confocal images show that sLeA localizes at the apical membrane of epithelial cells as white arrows indicate.

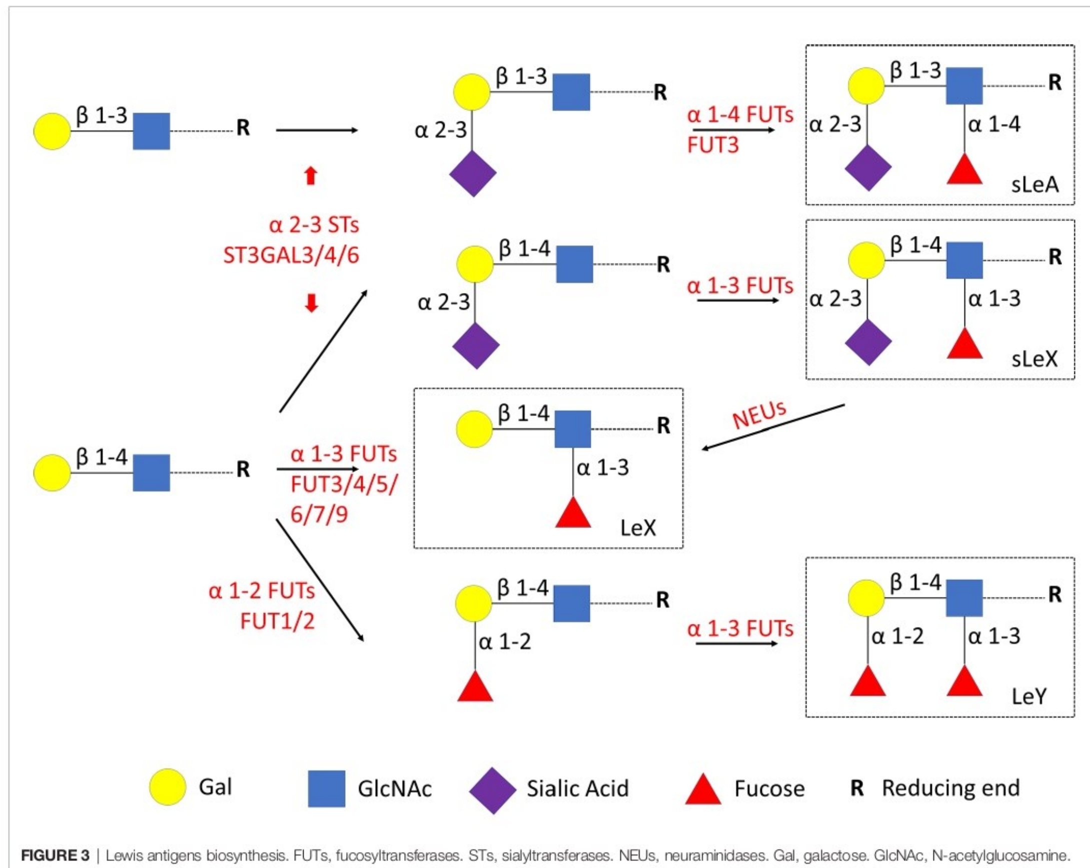

FIGURE 3 | Lewis antigens biosynthesis. FUTs, fucosyltransferases. STs, sialyltransferases. NEUs, neuraminidases. Gal, galactose. GlcNAc, N-acetylglucosamine.

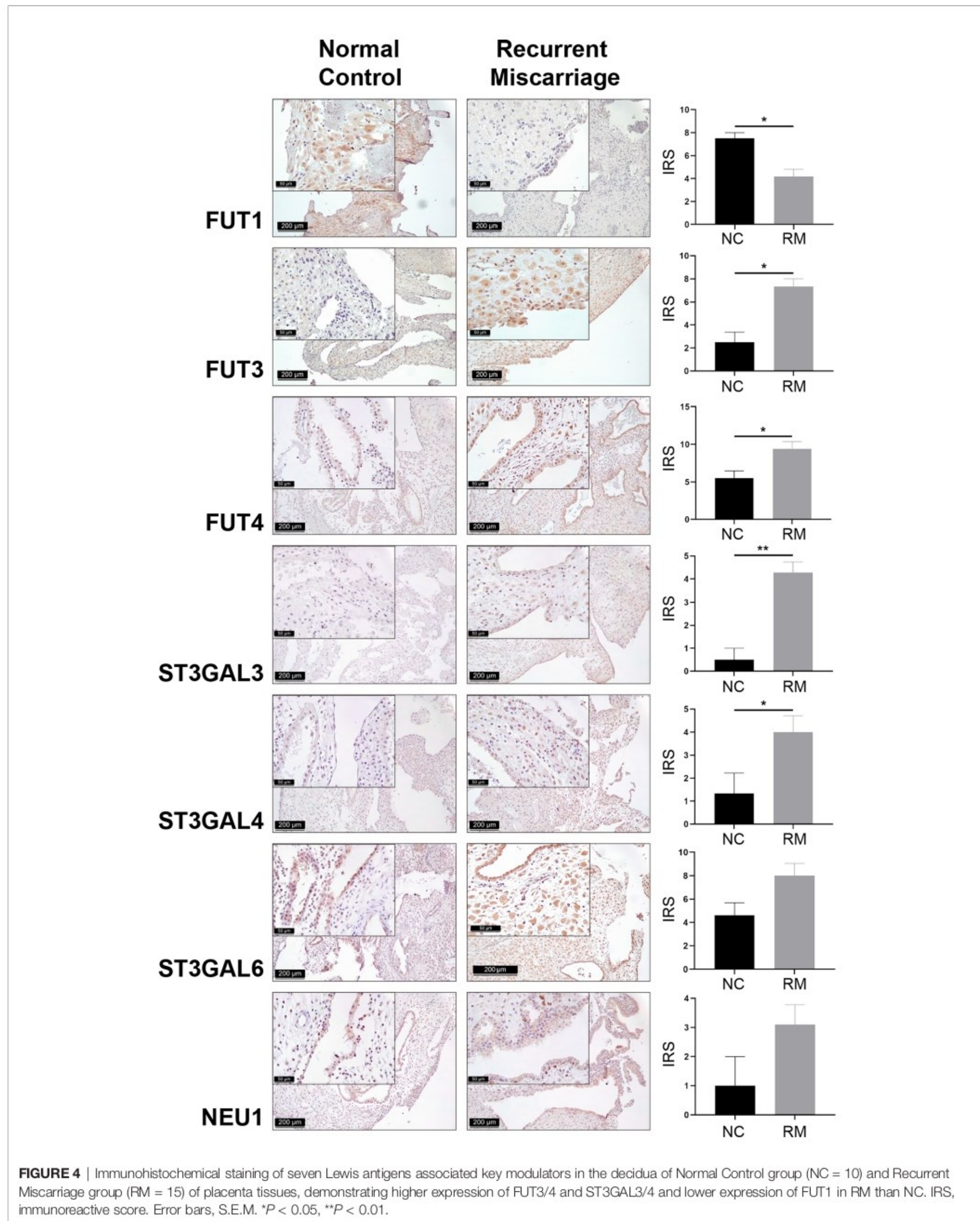

FIGURE 4 | Immunohistochemical staining of seven Lewis antigens associated key modulators in the decidua of Normal Control group (NC = 10) and Recurrent Miscarriage group (RM = 15) of placenta tissues, demonstrating higher expression of FUT3/4 and ST3GAL3/4 and lower expression of FUT1 in RM than NC. IRS, immunoreactive score. Error bars, S.E.M. *P < 0.05, **P < 0.01.

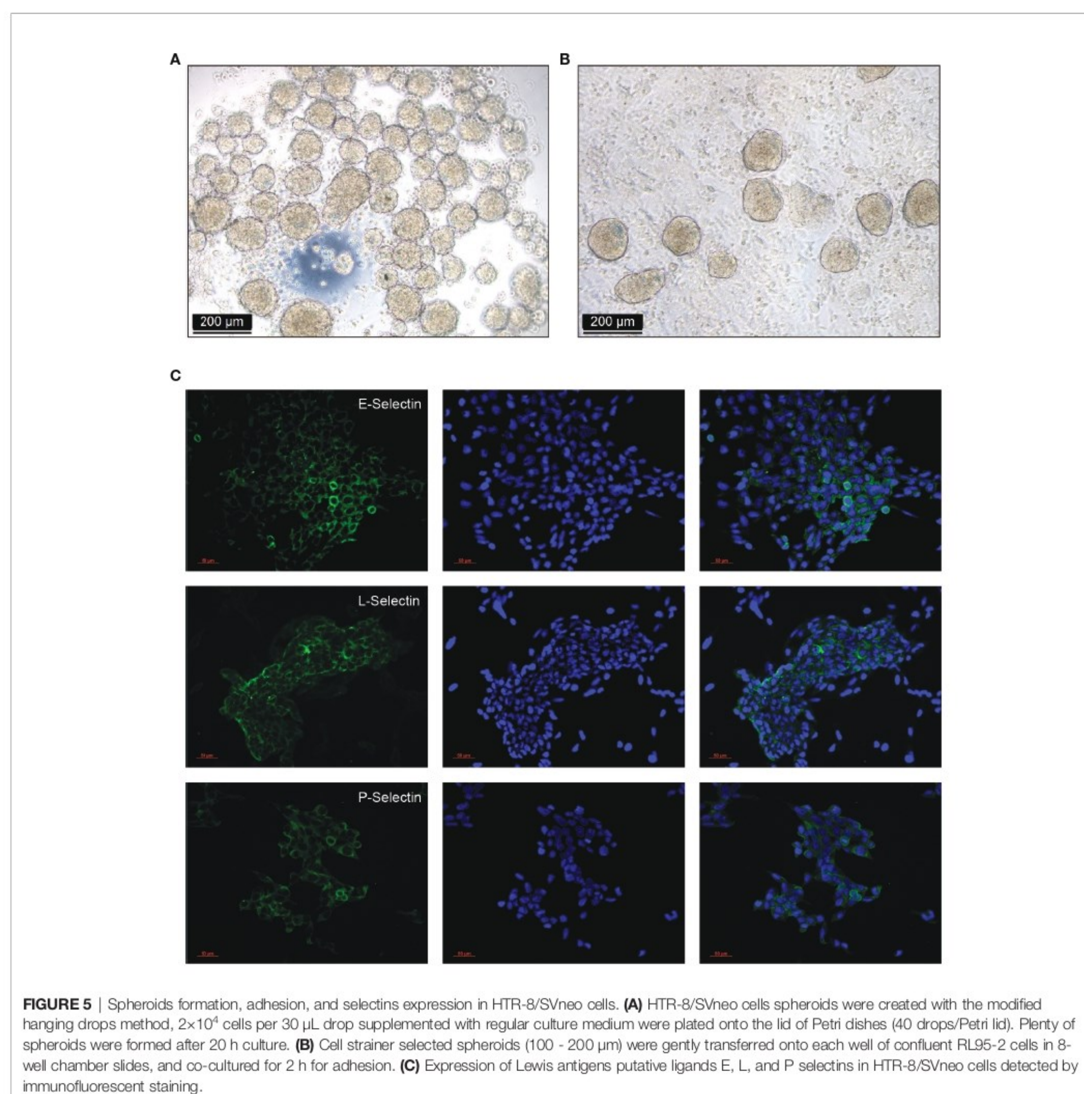

FIGURE 5 | Spheroids formation, adhesion, and selectins expression in HTR-8/SVneo cells. **(A)** HTR-8/SVneo cells spheroids were created with the modified hanging drops method, 2×10^4 cells per 30 µL drop supplemented with regular culture medium were plated onto the lid of Petri dishes (40 drops/Petri lid). Plenty of spheroids were formed after 20 h culture. **(B)** Cell strainer selected spheroids (100 - 200 µm) were gently transferred onto each well of confluent RL95-2 cells in 8-well chamber slides, and co-cultured for 2 h for adhesion. **(C)** Expression of Lewis antigens putative ligands E, L, and P selectins in HTR-8/SVneo cells detected by immunofluorescent staining.

FUT3 Knockdown Decreased sLeA Expression and Spheroids Attachment in RL95-2 Cells

Modulatory effect of FUT3 on the expression of Lewis antigens in RL95-2 cells was confirmed by siRNA knockdown experiments (**Figure 6**). Expression of cell surface sLeA was decreased after FUT3 knockdown in RL95-2 cells ($P<0.05$, **Figure 6B**), while the expression level of LeX remained unchanged (**Figure 6C**). The percentage of attachment of HTR-8/SVneo spheroids to endometrial monolayers was also significantly suppressed by FUT3 knockdown ($P<0.01$, **Figure 6D**).

Anti-sLeA Suppressed IL-1β Induced Trophoblast Spheroid Attachment to Endometrial Cells

To clarify the involvement of sLeA and LeX in the process of blastocyst adhesion, specific antibody blockade was applied in the *in vitro* implantation assay. Blocking sLeA with anti-sLeA antibody significantly suppressed the attachment of spheroids to endometrial monolayers, while the blockade efficacy showed no differences between 20 and 40 µg/ml antibody concentration (**Figure 7A**). Anti-LeX antibody slightly suppressed spheroid attachment but the difference was found to be not significant

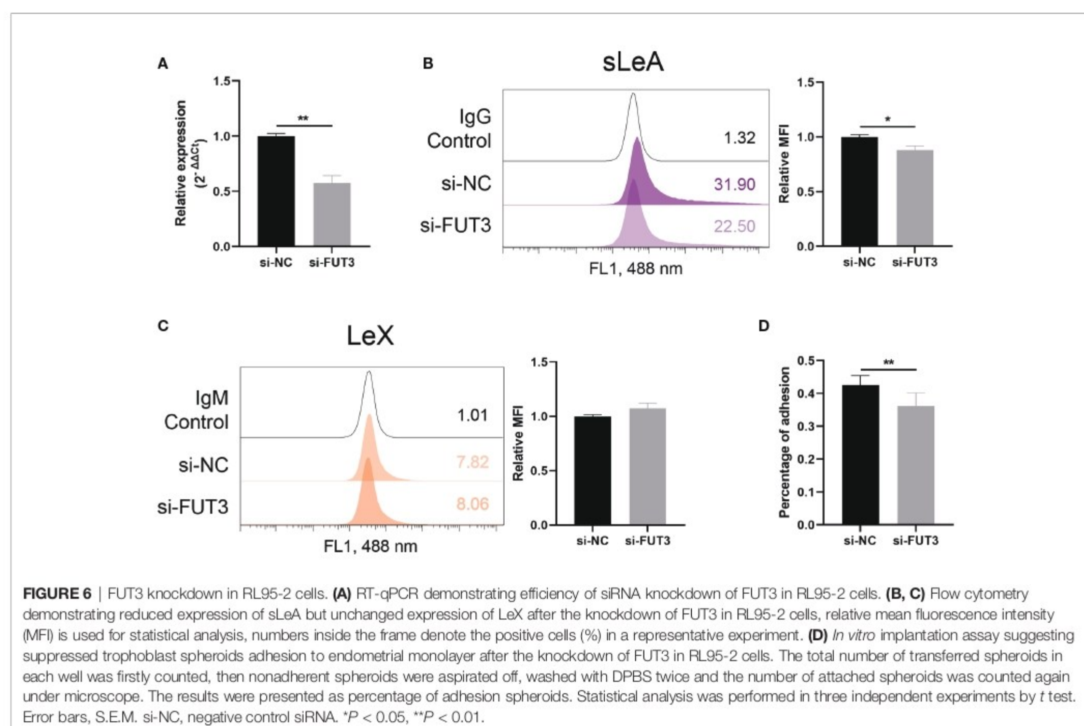

FIGURE 6 | FUT3 knockdown in RL95-2 cells. **(A)** RT-qPCR demonstrating efficiency of siRNA knockdown of FUT3 in RL95-2 cells. **(B, C)** Flow cytometry demonstrating reduced expression of sLeA but unchanged expression of LeX after the knockdown of FUT3 in RL95-2 cells, relative mean fluorescence intensity (MFI) is used for statistical analysis, numbers inside the frame denote the positive cells (%) in a representative experiment. **(D)** *In vitro* implantation assay suggesting suppressed trophoblast spheroids adhesion to endometrial monolayer after the knockdown of FUT3 in RL95-2 cells. The total number of transferred spheroids in each well was firstly counted, then nonadherent spheroids were aspirated off, washed with DPBS twice and the number of attached spheroids was counted again under microscope. The results were presented as percentage of adhesion spheroids. Statistical analysis was performed in three independent experiments by *t* test. Error bars, S.E.M. si-NC, negative control siRNA. $^{*}P < 0.05$, $^{**}P < 0.01$.

(**Figure 7A**). Here, we also found that IL-1β treatment markedly increased the trophoblast spheroids attachment to endometrial cells monolayers (P <0.0001, **Figure 7B**), confirming its function as an implantation promoting factor in the *in vitro* model. Interestingly, blocking sLeA could still suppress the IL-1β induced attachment and the blockade efficacy also did not differ between 20 and 40 μg/ml antibody concentration.

Induction of LeX Expression in Endometrial Cells by IL-1β

Influence of IL-1β treatment on the expression of sLeA and LeX in endometrial cells was investigated by flow cytometry. We found significantly enhanced expression of LeX following stimulation with IL-1β (P <0.0001), with a 1.74-fold increase in mean fluorescence intensity (MFI) values compared to untreated cells (**Figure 8A**). ICC staining also confirmed the induction of LeX expression in RL95-2 cells treated with IL-1β (**Figure 8B**) while changes in the expression level of cell surface sLeA were not detected (**Figures 8C, D**).

DISCUSSION

Pregnancy is characterized by the intimate interactions between a competent embryo and a receptive endometrium. Appropriate blastocyst implantation is widely considered as a crucial factor for achieving successful pregnancy.

Monthly fecundity rates (MFRs) refer to the time taken to achieve pregnancy, which is used to measure the probability of conceiving within one menstrual cycle (25). The average MFR in humans is relatively low at around 20%, while studies have shown that 32-40% of women experiencing RM have exceptionally high pregnancy rates with MFR > 60% (8). However, increased pregnancy rates do not result in increased live birth rates but repeated miscarriages, this paradox leads to the hypothesis of impaired natural embryo selection in RM (4). Biopsies analysis of mid-secretory endometrium from RM revealed decreased expression of mucin 1, an anti-adhesion molecule that maintains the barrier function of endometrial surface (26). *In vitro* decidualization of ESCs from RM manifested by attenuated production of prolactin and enhanced expression of prokinectin-1, a cytokine that facilitates implantation (27). Moreover, ESCs from RM cannot distinguish trophoblast signals between high- and low-quality embryos, resulting in increased migration behavior than normal fertile women (10). Thus, impaired endometrial barrier, aberrant decidualization associated prolonged “window” of receptivity, and less selective decidual phenotype in RM together allow the implantation of low viable embryos which are destined to fail.

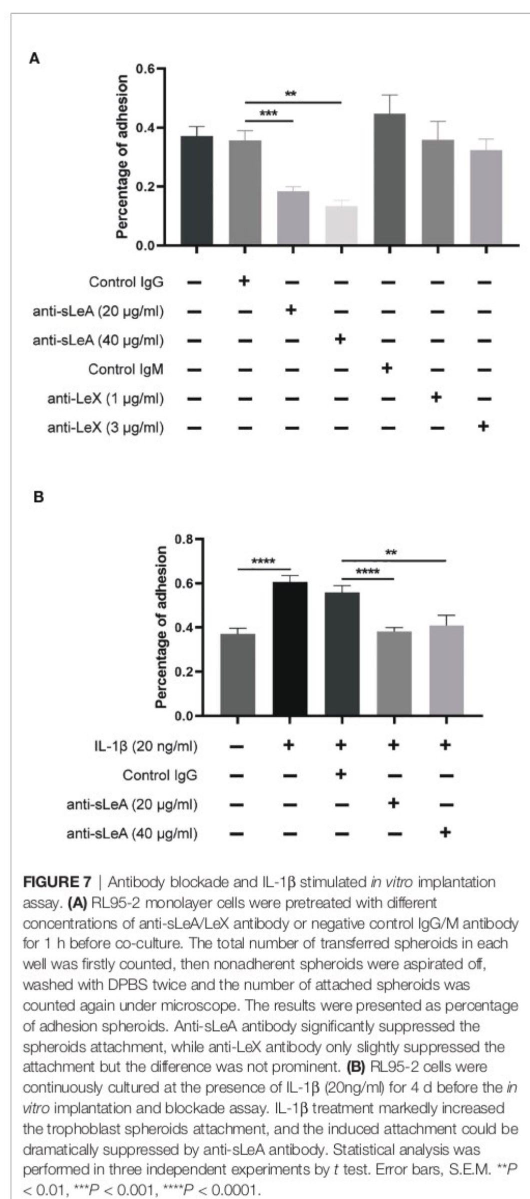

FIGURE 7 | Antibody blockade and IL-1β stimulated *in vitro* implantation assay. **(A)** RL95-2 monolayer cells were pretreated with different concentrations of anti-sLeA/LeX antibody or negative control IgG/M antibody for 1 h before co-culture. The total number of transferred spheroids in each well was firstly counted, then nonadherent spheroids were aspirated off, washed with DPBS twice and the number of attached spheroids was counted again under microscope. The results were presented as percentage of adhesion spheroids. Anti-sLeA antibody significantly suppressed the spheroids attachment, while anti-LeX antibody only slightly suppressed the attachment but the difference was not prominent. **(B)** RL95-2 cells were continuously cultured at the presence of IL-1β (20ng/ml) for 4 d before the *in vitro* implantation and blockade assay. IL-1β treatment markedly increased the trophoblast spheroids attachment, and the induced attachment could be dramatically suppressed by anti-sLeA antibody. Statistical analysis was performed in three independent experiments by *t* test. Error bars, S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Glycoconjugates at the fetomaternal interface play a vital role in mediating the adhesion of blastocyst to the endometrium. SLeA and sLeX are common selectin oligosaccharide ligands, their expression in the endometrium reaches the highest level during the window of implantation (12). Similarly, LeY was highest expressed in the mid-secretory phase during menstrual cycle, while expression of LeX peaked at early- to mid-proliferative phases (28). Biosynthesis of Lewis antigens requires the sequential addition of sialic acid and fucose by α 2-3 STs and FUTs, respectively (22). Genbacev et al. firstly clarified the role of L-selectin/sLeX adhesion system in trophoblast implantation (18). Later functional studies showed that both ST3GAL3 and FUT7 transfection could facilitate the embryo adhesion through upregulating sLeX (19, 29, 30). Integrin αvβ3, biomarker of endometrial receptivity, was reported to be the carrier of LeY, and knocking down FUT4 significantly decreased the adhesion of trophoblast JAR cells to RL95-2 cells by reducing the expression of LeY (20).

Despite the fact that sLeX and LeY play important roles in blastocysts adhesion, our study found no alterations of their expression in the decidua of RM patients, but sLeA and LeX showed dramatic upregulation. SLeA has been intensively studied in various cancers, it promotes the metastasis and malignant transformation by interacting with selectins (14). Similar to sLeX, highest level of sLeA in the endometrium through menstrual cycle is also noticed during the implantation stage, while its significance in the process of human implantation is not fully understood. Our *in vitro* implantation assay showed that anti-sLeA can prominently inhibit the adhesion of trophoblast spheroids to endometrial monolayers, indicating that sLeA also mediates the blastocyst implantation process. Thus, aberrantly elevated sLeA may be a novel marker for the hyper-receptive/less selective endometrium in RM. LeX is a carbohydrate cell adhesion molecule, it plays a crucial role in human embryogenesis and neutrophil transepithelial migration (31, 32). Upregulated LeX usually leads to metastasis and decreased survival in a variety of cancers (33–35). While the absence of LeX on human glioma cells is thought to be the reason that extraneural metastasis of brain tumors is rare (36). LeX expression was also identified in the normal endometrium and can be upregulated by progesterone (37). To test whether LeX is involved in trophoblast adhesion, anti-LeX antibody was used in the *in vitro* implantation assay, while no significant decrease was shown in the spheroids adhesion. This might explain why the highest expression of LeX in the endometrium does not occur at the implantation stage during menstrual cycle. Meanwhile, we also noticed that the basal expression of LeX in RL95-2 cells is around 10% (LeX positive cells) as flow cytometry detected (**Figure 8A**), thus model with RL95-2 cells may not adequately represent LeX expression usually found in receptive endometrium thereby confounding the results of the spheroids adhesion assay. The potential significance of LeX in blastocyst implantation should not be completely excluded, further investigations are merited.

The expression of potential glycosyltransferases FUTs, α2-3 STs, and NEUs were also validated in the decidua of RM. FUT3/4 and ST3GAL3/4 were significantly upregulated in the RM group, while FUT1 was downregulated. ST3GAL6 and NEU1 did not prominently differ between groups. FUT3 is the only fucosyltransferase that generates sLeA through an α1-4 linkage addition of fucose moieties (38). Hepatitis B virus X protein

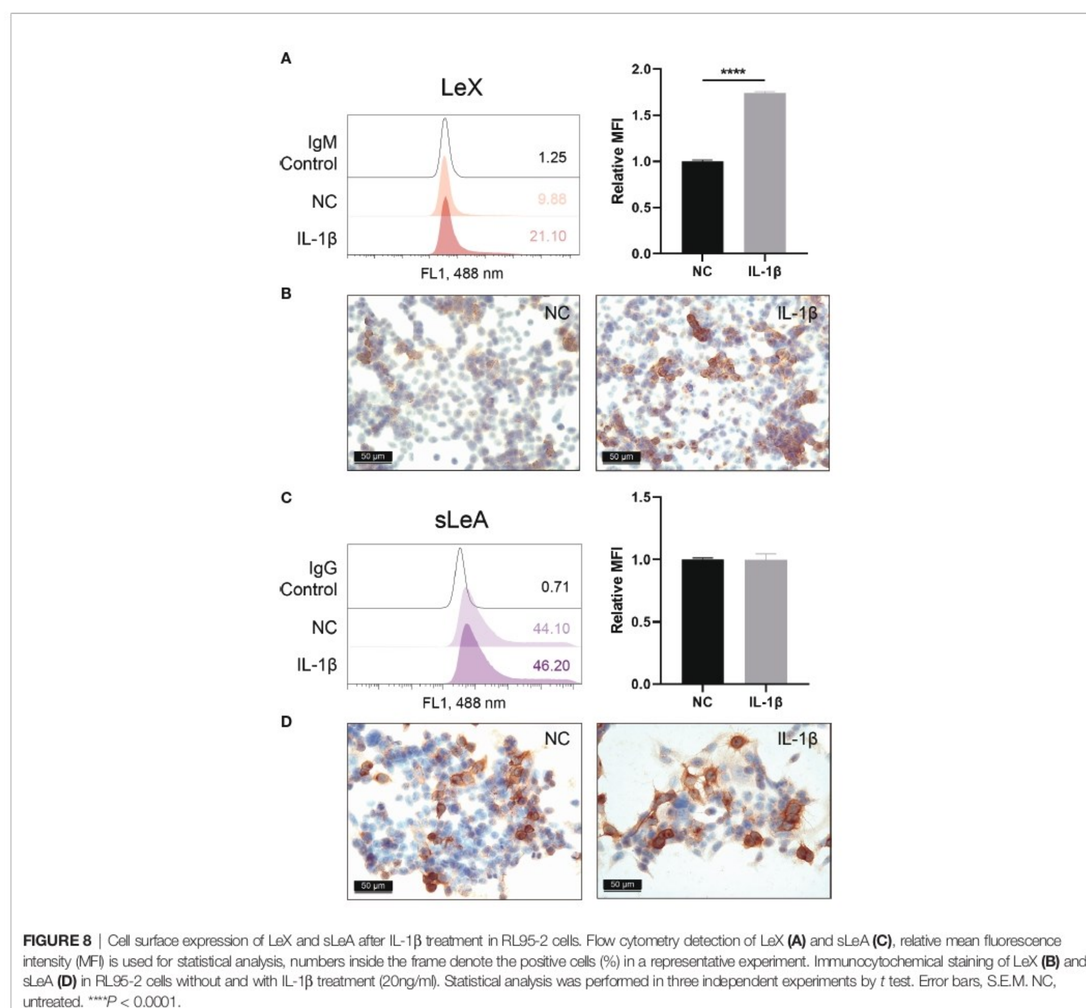

FIGURE 8 | Cell surface expression of LeX and sLeA after IL-1β treatment in RL95-2 cells. Flow cytometry detection of LeX **(A)** and sLeA **(C)**, relative mean fluorescence intensity (MFI) is used for statistical analysis, numbers inside the frame denote the positive cells (%) in a representative experiment. Immunocytochemical staining of LeX **(B)** and sLeA **(D)** in RL95-2 cells without and with IL-1β treatment (20ng/ml). Statistical analysis was performed in three independent experiments by *t* test. Error bars, S.E.M. NC, untreated. ****$P < 0.0001$.

targets FUT3 for the production of sLeA (39). FUT3 was identified as the key enzyme for sLeA synthesis in human intestinal epithelial cells (IECs), its transfection of sLeA-deficient IECs resulted in robust expression of sLeA (40). Here we also found significantly upregulated FUT3 in the decidua of RM and FUT3 knockdown in RL95-2 cells decreased the cell surface expression of sLeA and suppressed the spheroids adhesion, which indicate that FUT3 may account for the aberrantly elevated sLeA and endometrial hyper-receptivity in RM. FUT3 also exhibits α1-3 fucosyltransferase activity that contributes to sLeX expression (11, 41). While upregulated FUT3 in the decidua of RM does not result in higher expression of sLeX as displayed in our study, this might be explained by different catalytic preferences of α1-3 FUTs in different diseases (14). Other α1-3 FUTs like FUT5/6/7/9, which also contribute to the synthesis of sLeX, were not extensively investigated in this study but might be the main modulators for sLeX at the fetomaternal interface. As a member of the human neuraminidases family, NEU1 catalyzes the removal of sialic acid moieties from glycoproteins and glycolipids, it dramatically enhances LeX production *via* sLeX desialylation during human myeloid differentiation (17). Expression of NEU1 in the RM group was mildly elevated but did not significantly differ from the NC group ($P = 0.084$), which is probably due to the relatively small sample sizes. Whether NEU1 is really upregulated in RM and collaborates with FUT3 results in balanced sLeX and enhanced LeX expression needs to be revealed in larger studies. Besides, α1-3 FUTs including FUT3 and FUT4 can directly catalyze the

synthesis of LeX by adding fucose to the N-acetylglucosamine (GlcNAc) residue of glycans through an α1-3 linkage (38, 42). FUT4 expression in the endometrium is dynamically changing during menstrual cycle and reaches the highest level during implantation stage, progesterone shows dramatical induction of FUT4 expression (43). While FUT3 knockdown did not affect the expression level of LeX in RL95-2 cells, other α1-3 FUTs, like elevated FUT4 may explain the higher expression of LeX and contribute to the hyper-receptive endometrium in RM. In the synthesis of LeY, FUT1 mediates the first fucose addition to galactose (Gal) followed by second fucose addition to GlcNAc catalyzed by FUT3/4 (**Figure 3**), upregulated FUT3/4 and downregulated FUT1 in the decidua may result in an unchanged level of LeY in RM.

ST3GAL3/4/6 belongs to the α2-3 STs family that catalyzes the addition of sialic acid to the Gal residue of glycans, yielding sLeA/X (11, 15, 44–46). In humans, ST3GAL3 exhibits a preferential effect on type I disaccharides for the synthesis of sLeA (47). ST3GAL3 expression in the endometrium at secretory phase is significantly higher than the proliferative phase, its downregulation decreases the adhesion of trophoblast cells to endometrial cells (30). ST3GAL4 has been reported to be the major STs regulating sLeX in human myeloid leukocytes (48). This study found prominently higher expression of ST3GAL3/4, but not ST3GAL6, in RM than the control group, which might be also responsible for elevated sLeA expression. Notably, FUTs and STs compete for the same substrate, selective inhibition of fucose addition facilitates greater sialylation (11, 48, 49). Therefore, downregulation of FUT1 may not only maintains the stable level of LeY, but could potentially also allow for more substrates to be modified into LeX and sLeA by FUT3/4 and ST3GAL3/4, respectively.

IL-1β was reported to play pivotal roles in human embryo implantation and has been widely acknowledged as a pro-implantation cytokine (50), it induces endometrial expression of adhesion molecule integrin β3 and promotes the extravillous motility (51, 52). Since anti-sLeA blocked the adhesion of trophoblast to endometrial monolayers at a basal level, we further investigated if anti-sLeA could also exert anti adhesion effect at the presence of IL-1β stimulation. Importantly, while IL-1β treatment of RL95-2 cells, in line with previous studies, significantly enhanced the spheroids adhesion rate, anti-sLeA did partially block the IL-1β induced adhesion. Pro-inflammatory cytokines induced glycosylation alterations have been reported in many studies, IL-8 induces sLeA expression in human pancreatic cancer cells (53), TNF-α stimulation prominently increases sLeA reactivity in gastric adenocarcinoma cells while IL-1β results in mild enhancement of sLeA and LeX but does not reach significance (54). In this study, IL-1β treatment of RL95-2 cells showed no alterations in sLeA while significant increase in LeX expression. Hence, sLeA expression is independent of IL-1β signaling but anti-sLeA still exerts sufficient blockade effect on IL-1β induced trophoblast adhesion. This further elucidated the crucial role of sLeA in blastocyst adhesion and anti-sLeA in attenuating the hyper-receptive endometrium in RM. IL-1β induced LeX expression may suggest that previously reported elevated IL-1β in both endometrium and decidua of RM could be a trigger of the upregulated LeX observed in this study (55, 56).

In summary, this study demonstrates a remarkably elevated glycosylation status in the decidua of uRM patients manifested by prominently upregulated sLeA, LeX, FUT3/4, and ST3GAL3/4, which might contribute to the hyper-receptive/less selective endometrium in RM. Involvement of sLeA in the process of blastocyst adhesion is also deciphered with *in vitro* implantation model. Targeting aberrantly elevated sLeA may be applied as a potential strategy to restore the inappropriate implantation in uRM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of LMU Munich. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

UJ and VS conceived of the study and participated in its design and coordination. ZM performed the experiments and statistical analysis and wrote the manuscript. HY and MK performed technical assistance in immunohistochemistry and cell culture experiments. MK, MS, SM, and UJ revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.919193/full#supplementary-material

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7. Summary

In Paper I, by using GSEA and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, we demonstrate markedly downregulated glycosylation-related signatures and adhesion molecules, but enhanced inflammatory response in the placental villi of uRM. Accordingly, attenuated expression of sLeA, sLeX, LeX, and LeY in the villous syncytiotrophoblast of patients with unexplained miscarriages are further identified through IHC staining. Involvement of key enzymes like ST3GAL6 and NEU1 may contribute to these alterations. Furthermore, pathways network visualization by Metascape analysis indicates that immune system process like leukocyte adhesion and migration are significantly related to the altered glycoprotein metabolic process in uRM. Interestingly, prominently diminished villous vessels have been also observed in the miscarriage patients. Collectively, these results suggest that aberrant Lewis antigens expression may actuate miscarriages by affecting both trophoblast and leukocytes functions as well as the vascularization of the villi. Nevertheless, the exact underlying mechanisms merit further investigations.

While Paper II indicates that the expression pattern of Lewis antigens in uRM decidua is significantly different from that in the placental villi: sLeA, LeX, FUT3/4, and ST3GAL3/4 are upregulated in the uRM compared with the control group, while FUT1 is downregulated. IHC and IF staining reveal that Lewis antigens mainly locate at the apical membrane of luminal and glandular epithelial cells, while key enzymes FUTs, ST3GALs, and NEU1 are expressed in both epithelial and stromal cells. The in vitro human embryo implantation model using endometrial RL95-2 cells formed monolayer and trophoblastic HTR-8/SVneo cells spheroids has been well established. FUT3 knockdown in RL95-2 cells significantly suppressed the cell surface sLeA expression and the spheroids attachment to endometrial monolayer, while surface LeX expression is not influenced. Anti-sLeA antibody can remarkably inhibit both the basal and IL-1β induced spheroids attachment to the endometrial monolayer. In addition, flow cytometry detection demonstrated that cell surface LeX, but not sLeA, is significantly

induced in IL-1β treated RL95-2 cells. In conclusion, upregulated sLeA, LeX, FUT3/4 and ST3GAL3/4 might be related to the hyper-receptive endometrium of uRM patients. FUT3 contributes to the biosynthesis of sLeA in RL95-2 cells and affects the endometrial receptivity. Pro-inflammatory cytokine IL-1β stimulate the expression of LeX in endometrial cells, though sLeA is not influenced, targeting aberrantly elevated sLeA might be a starting point to address inappropriate implantation in uRM.

8. Zusammenfassung

In der Veröffentlichung I zeigen wir unter Verwendung von GSEA und der Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway-Analyse deutlich herunterregulierte glykosylierungsbezogene Signaturen und Adhäsionsmoleküle, aber eine verstärkte Entzündungsreaktion in den Zotten von Plazenten nach einem rezidivierenden Abort (uRM). Dementsprechend wird die abgeschwächte Expression von sLeA, sLeX, LeX und LeY im Syncytiotrophoblast von Patienten mit uRM weiter durch immunhistochemische (IHC) Färbung identifiziert. Die Beteiligung von Schlüsselenzymen wie ST3GAL6 und NEU1 kann zu diesen mit Hilfe der IHC gefundenen Veränderungen beitragen. Darüber hinaus zeigt die Visualisierung des Netzwerks von Signalwegen durch Metascape-Analyse, dass Prozesse des Immunsystems wie Leukozytenadhäsion und -migration signifikant mit dem veränderten Glykoprotein-Stoffwechselprozess in uRM zusammenhängen. Interessanterweise wurden auch bei Patientinnen mit ungeklärten Fehlgeburten deutlich verminderte Zottengefäße beobachtet. Zusammengenommen legen diese Ergebnisse nahe, dass eine abweichende Expression von Lewis-Antigenen das Auftreten von Fehlgeburten auslösen könnte, indem sowohl die Trophoblasten- und Leukozytenfunktionen als auch die Vaskularisierung der Zotten eingeschränkt werden. Dennoch verdienen die genauen zugrunde liegenden Mechanismen weitere Untersuchungen.

In der Veröffentlichung II weisen wir darauf hin, dass sich das Expressionsmuster der Lewis-Antigene in uRM decidua signifikant von dem in den Plazentazotten unterscheidet: sLeA, LeX, FUT3/4 und ST3GAL3/4 sind im uRM im Vergleich zur Kontrollgruppe hochreguliert, während FUT1 herunterreguliert ist. IHC- und IF-Färbung zeigen, dass sich Lewis-Antigene hauptsächlich an der apikalen Membran von luminalen und Drüsenepithelzellen befinden, während die Schlüsselenzyme FUTs, ST3GALs und NEU1 sowohl in Epithel- als auch in Stromazellen exprimiert werden. Das *in-vitro*-Implantationsmodell für menschliche Embryonen unter Verwendung von

endometrialen RL95-2-Zellen, die eine Monoschicht bildeten, und trophoblastischen HTR-8/SVneo-Zellen-Sphäroiden, ist gut etabliert. Der FUT3-Knockdown in RL95-2-Zellen unterdrückte deutlich die Zelloberflächen-sLeA-Expression und die Sphäroidanhaftung an der Endometrium-Monoschicht, während die Oberflächen-LeX-Expression nicht beeinflusst wird. Anti-sLeA-Antikörper können sowohl die basale als auch die IL-1β-induzierte Sphäroid-Anhaftung an die Endometrium-Monoschicht signifikant hemmen. Zusätzlich zeigte der Durchflusszytometrie-Nachweis, dass Zelloberflächen-LeX, aber nicht sLeA, signifikant in mit IL-1β behandelten RL95-2-Zellen induziert wird. Zusammenfassend lässt sich sagen, dass hochreguliertes sLeA, LeX, FUT3/4 und ST3GAL3/4 mit dem hyperrezeptiven Endometrium von uRM-Patienten zusammenhängen könnten. FUT3 trägt zur Biosynthese von sLeA in RL95-2-Zellen bei und beeinflusst die Empfänglichkeit des Endometriums. Das entzündungsfördernde Zytokin IL-1β stimuliert die LeX-Oberflächenexpression in Endometriumszellen. Obwohl sLeA nicht beeinflusst wird, könnte die Ausrichtung auf anormal erhöhte sLeA Expression als Ausgangspunkt dienen, um eine fehlerhafte Implantation in uRM anzuzeigen.

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List of all scientific publications

As first author:

1, Sialyl Lewis X mediates interleukin-1 beta induced trophoblast adhesion to endometrial cells during human embryo implantation. ***Biol Reprod***. 2023; Jan 16:ioad007. DOI: 10.1093/biolre/ioad007.

2, Targeting aberrantly elevated Sialyl Lewis A as a potential therapy for impaired endometrial selection ability in unexplained recurrent miscarriage. ***Front Immunol***. 2022; 13:919193. DOI: 10.3389/fimmu.2022.919193.

3, Expression of the carbohydrate Lewis antigen, Sialyl Lewis A, Sialyl Lewis X, Lewis X, and Lewis Y in the placental villi of patients with unexplained miscarriages. ***Front Immunol***. 2021; 12:679424. DOI: 10.3389/fimmu.2021.679424.

As co-author:

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2, 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. ***Int J Mol Sci***. 2021; 22:9134. DOI: 10.3390/ijms22179134.

3, Comparison of Histone H3K4me3 between IVF and ICSI technologies and between boy and girl offspring. ***Int J Mol Sci***. 2021; 22:8574. DOI: 10.3390/ijms22168574.

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