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Analysis of the expression of the Pregnancy Zone Protein in the human placenta in disorders of early pregnancy

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Für meine Familie

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1. Introduction

1.1 Disorders in early pregnancy

1.1.1 Miscarriage

Miscarriage is defined as a loss of pregnancy upon fertilization until 24 weeks of gestation or a fetus weight < 500 g by the time of parturition [1]. However, about 80% of miscarriages occur in the first trimester of pregnancy [2]. In the past, low human Choriongonadotropin (hCG) levels in maternal plasma have been associated with early pregnancy loss [3, 4]. Miscarriage is subclassified into recurrent (RM) and spontaneous miscarriage (SM). SM describes the loss of one pregnancy and affects up to 50% of all women of child-bearing age [5]. Chromosomal disorders or structural defects of the embryo are main causes for SM [6]. RM is specified as loss of at least two consecutive pregnancies, which distresses about 5% of conceived females worldwide [7, 8]. The etiology of recurrent abortion is multifactorial. For example, it has been associated with different autoimmune disorders, uterine malformations, thrombophilia or parental chromosomal abnormalities [1, 9]. Even after thorough work-up of the affected patients, in nearly 50% the reason for RM cannot be detected [10]. In these patients, exaggerating maternal immune effector functions are considered as one important mechanism particularly for unexplained recurrent loss of early pregnancy [1].

1.1.2. Hydatidiform mole

Hydatidiform mole (HM) is a pre-malignant disorder with an reported incidence of 1 out of 1000 pregnancies [11]. It is defined either as complete or incomplete mole and derives from trophoblastic tissue of the placenta after abnormal fertilization [12]. Recent studies showed that 80% of the gestational trophoblastic diseases (GTD) are hydatidiform moles, 15% are invasive moles and 5% are chorioncarcinomas [12]. The diagnostic work up of HM includes analysis of hCG levels in maternal plasma as extraordinarily high values are distinctive for HM [13]. Due to routine ultrasonography and hCG determination in maternal plasma in early gestation, mole pregnancy is

usually diagnosed during the first trimester [12]. The initial treatment of hydatidiform mole is suction dilation of the uterus with ultrasound guidance in order to extract all molar tissue [14]. Usually, preservation of fertility is possible [15]. The final diagnosis of hydatidiform mole is made by histopathological and cytogenetic examination of the obtained placental tissue [16, 17]. Postoperative surveillance of the patients includes weekly controls of serum hCG levels until 3 consecutive controls are normal [18]. Patients with complete moles should receive monthly serum hCG controls for one year [18]. Postoperative persistent or rising serum hCG levels indicate persistent intrauterine trophoblastic tissue or the subsequent development of a gestational trophoblastic neoplasia (GTN) like invasive mole or chorioncarcinoma [19]. At this point it is necessary to examine the patients for metastases in brain, lung, liver and the gastro-intestinal tract by cross-sectional imaging [12]. The further therapy and prognosis depend on the staging according to the International Federation of Gynecology and Obstetrics (FIGO 2000) and World Health Organization (WHO) scoring system [20, 21]. The therapy includes single agent or combination chemotherapy and eventually hysterectomy.

1.2 Mechanisms for a successful pregnancy

1.2.1. Establishment of immunotolerance at the feto-maternal interface

Pregnancy is an immunological phenomenon [22]. In contrast to organ transplantation, in which the allograft is rejected by the host`s immune system, the semi-allogenic embryo is not rejected by the maternal immune system [23]. A balanced maternal immune reaction towards the semi-allogenic embryo plays a crucial role in pregnancy maintenance [24]. Consequently, any imbalance might result in a complicated course like intrauterine growth restriction (IUGR) of the fetus, miscarriage or preeclampsia [25]. Up to date, several immunological mechanisms have been described that promote maternal immunotolerance towards the fetus. To begin with, the decidua has an unique resident leukocyte population where decidual natural killer cells (NK) represent the predominant leukocyte subpopulation [26]. These cells are characterized by high cytokine secreting activity and reduced cytotoxicity in early pregnancy [27].

Increased cytotoxic activity of NK cells is associated with recurrent miscarriage and infertility [28].

To continue, a complex cytokine network secreted by maternal T lymphocytes is pivotal for implantation, placentation and fetal survival [1, 29] . Human T lymphocytes are classified into CD8+ T cells (cytotoxic T cells), regulatory T cells (Treg) and CD4+ T cells (T helper cells, Th) [30]. Furthermore, Th cells can be subclassified by their different cytokine profiles into Th1, Th2 and Th17 cells. [29]. Former studies have shown that these cytokines need to be tightly regulated in order to establish maternal immunotolerance towards fetal antigens [28]. Importantly, a switch from a Th1- to a Th2 cytokine profile seems to play a key role for a successful ongoing pregnancy [29, 31]. In this context, it is assumed that elevated levels of Th1 cytokines are associated with a higher risk for miscarriage and that Th2 cytokines play a protective role [24, 31]. Besides a specialized decidual leukocyte population and the Th1 to Th2 cytokine shift, several immunomodulatory proteins like Glycodelin A (GdA) are reported to play an important role for the maintenance of a pregnancy [32, 33]. In gestation, GdA is secreted by the decidua reaching highest concentrations between 6 and 12 weeks of gestation [32, 34]. Several studies investigated the role of GdA in the context of feto-maternal tolerance. For example, monocytes release Th2 cytokines in response to GdA stimulation [35]. Furthermore, Dixit et al. demonstrated that GdA regulates the function of peripheral cytotoxic NK cells in pregnancy [36]. In addition, GdA promotes a tolerogenic differentiation of dendritic cells, which modulate maternal immune reactions [37, 38]. Functional failure of these cells has been associated with pregnancy complications like preeclampsia and IUGR [37, 38]. *In vitro* experiments with primary trophoblast cells have shown that these cells produce hCG upon GdA stimulation [39, 40]. Moreover, GdA has an impact on the extend of trophoblast invasion [27]. An aberrant expression of GdA in the decidua has already been associated with spontaneous miscarriage and mole pregnancy [33].

1.2.2. Alternative Splicing

Alternative splicing of messenger-RNA (mRNA) is a crucial mechanism for the regulation of gene expression and is therefore an important source for protein diversity [41]. It is also suggested that functional complexity of several proteins is accomplished

by alternative splicing of various genes [42]. To begin with, extravillous trophoblasts express the non-classical class I human leukocyte antigen (HLA)-G, which has important immunomodulatory functions to prevent rejection of the semi-allogenic embryo [43]. HLA-G is associated with limited genetic polymorphism, but due to alternative splicing there are seven isoforms of HLA-G described [43]. Aberrant HLA-G expression and its genetic polymorphism in the extravillous trophoblast seems to be associated with pregnancy complications like preeclampsia and recurrent miscarriage [44-46]. To continue, Revil et. al. demonstrated in a mouse model, that alternative splicing is an important regulatory mechanism during embryonic development [47]. However, the exact function of alternative splicing in gestation and in pregnancy disorders is still not clear. The elongation factor Tu GTP binding domain containing 2 (EFTUD2) seems to be essential in the process of alternative splicing [48]. Recently, it was identified to be associated with fetal malformations [49, 50]. Furthermore, EFTUD2 expression was described in the mouse placenta and EFTUD2 mutation in mice embryos resulted in embryogenic death [51]. Hence, EFTUD2 might play an important role during embryogenesis and placentation [51]. Additionally, EFTUD2 affects innate immune responses of macrophages [52]. Macrophages are the second most abundant leukocyte population in the decidua and are reported to be involved in several key events in pregnancy [27]. Stremmel et al. demonstrated in mice embryos that macrophage progenitor cells are crucial for embryogenic development [53]. Furthermore, macrophages with a changed polarization in early pregnancy, especially the loss of the peroxisome proliferator-activated receptor γ (PPARγ), are associated with RM [54].

1.3. Pregnancy Zone Protein

1.3.1. Structure and expression

Pregnancy Zone Protein (PZP) is a protease inhibiting glycoprotein belonging to the α2-Macroglobulin (α2M) family [55, 56]. PZP mRNA can be modulated by alternative splicing, which results in two major splice variants with differences affecting exons 3b,1c and 1a [57]. Native PZP is a disulfide-bridged 360 kDa homodimer with a bait region and a cyclic thiolester bond within each subunit [55, 58, 59]. The bait region,

representing a proteinase-sensitive sequence, is cleaved when PZP interacts with a proteinase or amine molecules [55, 58, 59]. This process leads to exposure of the thiolesters and thereby induces a structural change, which enables PZP to bind to its receptor “low density lipoprotein receptor-related-protein” (LRP) [55, 58, 59]. By binding to the LRP receptor, which is expressed in liver, lung, brain and the placenta, PZP is cleared from circulation [55, 58, 59]. There is striking evidence that most of PZP in the plasma is secreted by the liver [60]. However, PZP expression is also described in immune cells, the brain and placenta [30, 55, 60]. PZP serum levels are highly elevated in pregnant women and decline rapidly after delivery [61]. In addition, high PZP serum levels have also been described in women taking conjugated estrogens and oral contraceptives indicating that PZP expression might be estrogen dependent [62, 63].

1.3.2. Impact of PZP on immune response

Initially, PZP was described as a protease inhibitor with remarkable structural and functional similarity to α2M [64]. But unlike α2M, the protease inhibiting properties of PZP are limited [65]. PZP has been shown to modulate general immune responses of the innate immune system [30]. PZP is reported to facilitate leukocyte migration and inhibit allograft rejection as well as activation-induced T cell proliferation [30, 66, 67]. It has been shown that PZP inhibits Interleukin 2 (IL-2) secretion, which is a classic Th1 cytokine [68]. This function of PZP is discussed to support the Th1 to Th2 cytokine shift. Consequently, suppression of IL-2 leads to a reduced macrophage killer activity and activation of NK cells [69, 70].

1.3.3 Carrier protein

There is striking evidence that PZP functions as a carrier protein. For example, transforming growth factor-β (TGF- β) 1 and 2 are considered important proteins for reproduction as they have a significant impact on embryogenesis, organogenesis and different immune responses [71, 72]. Especially the loss of function of the of TGF-β type 1 receptor in mice led to embryogenic death [72]. It was demonstrated that PZP is able to bind both proteins in a noncovalent manner [73]. Therefore, PZP might be a

carrier protein for TGF-β 1 and 2, but the biological importance of the PZP-TGF- β interaction is not fully understood [73]. Interestingly, a recent study demonstrated that loss of function of PZP may promote breast cancer progression by activating the pro-oncogenic TGF-β/SMAD signaling [74]. Moreover, PZP has been described as a carrier protein for the immunomodulatory GdA [30]. Both PZP and GdA are able to suppress the production of the Th1 cytokine IL-2 and to inhibit T cell proliferation [68, 75]. Skornicka et al. showed, that the inhibitory effect on T cells and IL-2 is even higher, when GdA is bound to PZP [30]. Surprisingly, GdA and PZP do not affect the secretion of Interleukin-4 (IL-4), which is known as a Th2 cytokine. Therefore, it is assumed that the interplay of GdA and PZP is able to promote the Th1 to Th2 cytokine shift [30].

1.3.4 The role of PZP in pregnancy

Up to date, there is limited insight into the role of PZP in gestation and pregnancy complications like miscarriage, hydatidiform mole and preeclampsia. Most of the published studies came to contradictory conclusions. Dember et al. observed in a prospective study low PZP serum levels in women experiencing spontaneous abortion and postulated a high prognostic accuracy for PZP regarding pregnancy outcome in the first trimester [76]. On the other hand, an another study examined blood samples from women with threatened abortion and did not found significant changes in PZP serum levels [77]. Low PZP maternal serum levels were observed in patients suffering from mole pregnancy [77]. Several studies demonstrated an accumulation of the misfolded protein amyloid beta peptide (Aβ) in the placenta of women diagnosed with preeclampsia [78, 79]. Recently, it was described that PZP efficiently suppresses the aggregation of this protein, which might indicate an important function of PZP particularly in late pregnancy [80]. This reported function of PZP is interesting especially in view of the fact that PZP serum levels are peaking in the third trimester [61].

1.4. Aim of the study and study design

PZP is one of the most abundant immunosuppressive proteins in maternal plasma, but its role in pregnancy is still unclear [61, 81]. This thesis investigates the role of PZP in the first trimester from the 6th to the 14th week of gestation. The intention was to analyze the PZP expression in the placenta of healthy human first trimester pregnancy (control) and of early pregnancy disorders like HM, RM and SM.

Study approval was obtained by the ethical committee of the Ludwig-Maximilians-University, Munich (Approval number: 337-06). An informed consent was signed by all participants of the study, which permitted analysis of the obtained tissue and all clinical data.

For the first publication the PZP protein expression in the syncytiotrophoblast and decidual glands of controls (n = 15), SM (n = 15) and HM (n = 14) was examined via immunohistochemistry [82]. As described earlier, EFTUD2 is a protein regulating alternative splicing and PZP is a protein, whose expression is regulated by alternative splicing [48, 57]. Therefore, a potential correlation of protein expression between PZP and EFTUD2 in controls, SM and HM was analyzed in order to get more insight into the role of alternative splicing in early pregnancy [82]. For *in vitro* analysis, the chorioncarcinoma cell line JEG-3 was used to investigate a possible regulation of PZP and EFTUD2 by hCG, a key hormone for pregnancy maintenance in the first trimester [3, 4, 82]. After stimulation of JEG-3 cells with hCG, PZP expression was examined by immunocytochemistry and EFTUD2 expression by RealTime PCR (RT-PCR) [82].

In the second publication the expression of GdA and PZP was investigated in the decidua of healthy pregnancies (n = 15), SM (n = 19) and RM (n = 17) [83]. GdA is the most abundant immunosuppressive glycoprotein in the decidua and low serum levels in maternal plasma are associated with RM [27, 34]. As mentioned before, when PZP carries GdA, their combined immunosuppressive potential is higher than the immunosuppressive property of each protein [30]. Hence, the investigation of GdA and PZP expression in miscarriage as well as their potential co-localization might lead to a better understanding of early pregnancy loss [83]. The expression on protein level was evaluated by immunohistochemical staining [83]. The mRNA expression was investigated by RT-PCR for PZP and by in situ hybridization for GdA [83]. In addition, double-immunofluorescence staining was applied to examine a co-expression of GdA and PZP in the decidua [83].

1.5 Overview on results

In the first publication differences were found in the PZP and EFTUD2 expression pattern in HM and SM placentas [82]. PZP protein level was lower in the syncytiotrophoblast of patients with HM ($p = 0.006$) and SM ($p = 0.028$) compared to healthy controls [82]. There was no significant difference in decidual glands for PZP expression in both HM and SM, but the EFTUD2 protein level was significantly increased ($p = 0.002$ for SM and $p = 0.003$ for HM) [82]. Also, EFTUD2 protein expression was significantly upregulated in HM ($p = 0.003$) and SM ($p= 0.003$) in the syncytiotrophoblast [82]. Correlation analysis revealed a negative correlation between EFTUD2 and PZP protein expression in the trophoblast ($r = -,369$, $p = 0.012$) [82]. *In vitro* cultivation of JEG-3 cells with hCG resulted in an elevated EFTUD2 protein level ($p = 0.027$) [82]. In contrast, after stimulation of JEG-3 cells with hCG there was a significant downregulation of PZP mRNA expression ($p = 0.027$) [82].

In the second publication a divergent expression pattern of GdA and PZP was observed in both compartments of the decidua [83]. The protein expression of PZP was significantly increased in decidual stroma cells of RM and SM in comparison to healthy controls ($p = 0.047$, $p = 0.015$, respectively) [83]. In decidual glands there was a likewise trend ($p = 0.130$ for RM, $p = 0.078$ for SM) [83]. The expression of PZP mRNA was significantly elevated in RM ($p < 0.001$) and SM ($p < 0.001$) patients in comparison to healthy pregnancies whereas a significant decrease of GdA mRNA expression was observed ($p = 0.014$ for SM, $p < 0.001$ for RM) [83]. The GdA protein expression was significantly lower in decidual stroma in SM ($p = 0.011$) and RM ($p = 0.021$) [83]. The protein expression of GdA and PZP showed a significant negative correlation only in decidual stroma cells of RM patients ($r = -0.975$, $p = 0.005$) [83]. In decidual glands of SM there was a significant reduction of GdA protein ($p = 0.012$) and mRNA ($p = 0.007$) expression [83]. In RM specimen, GdA expression in decidual glands was significantly downregulated only on mRNA ($p = 0.012$) level [83]. A co-expression of GdA and PZP in the decidual stroma and decidual glands in both RM and the control group was demonstrated by double-immunofluorescence staining [83].

1.6. Contribution to publications included in this thesis

Sanja Löb (SL) performed all experiments as described in section two (materials and methods) of publication 1 and 2 except the immunohistochemical staining of GdA and the in-situ hybridization of GdA for publication 2. SL completed the statistical analysis of the obtained data and made substantial contribution to data interpretation. SL wrote the manuscripts for both publications and designed the figures.

2. Publications included in this thesis

2.1 Publication 1

Title:

*"*Spliceosome protein EFTUD2 is upregulated in the trophoblast of spontaneous miscarriage and hydatidiform mole*"*

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2.2. Publication 2

Title:

"Pregnancy Zone Protein (PZP) is significantly upregulated in the decidua of recurrent and spontaneous miscarriage and negatively correlated to Glycodelin A (GdA)"

Authors:

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3. Summary

Pregnancy is a unique example for natural immunological tolerance [22, 23]. This immunological phenomenon is the result of various modulations of the maternal immune response especially at the feto-maternal interphase [24, 25]. For example, the overexpression of Th2 cytokines seems to be crucial to secure immunological acceptance of the semi-allogenic fetus [29, 31]. It is also suggested that alternative splicing of mRNA of different receptors and proteins is an important mechanism for the maintenance of a pregnancy [43-47]. PZP for example, is an immunosuppressive protein with two reported isoforms due to alternative splicing [30, 57, 66-68]. In addition, it reaches high serum levels in maternal plasma [61, 81]. The role of PZP in the first trimester, the most vulnerable phase of gestation [2], is still not well understood. Hence, the intention of this thesis was to analyze the expression of PZP in the human placenta of normal and disturbed early pregnancy. Additionally, the expression of two important proteins and their correlation to PZP was analyzed: GdA, a major immunosuppressive glycoprotein in early pregnancy, and EFTUD2, which is an important regulator of alternative splicing [27, 34, 48, 52]. Also, *in vitro* experiments were performed to evaluate a possible regulation of PZP by hCG.

The first publication demonstrates that the PZP expression is significantly downregulated in the syncytiotrophoblast of hydatidiform mole and spontaneous abortion [82]. In contrast, EFTUD2 was shown to be to significantly upregulated [82]. A negative correlation between EFTUD2 and PZP was observed [82]. PZP mRNA expression was reduced following hCG stimulation of JEG-3 cells, whereas EFTUD2 protein expression was upregulated [82]. Therefore, hCG, a key hormone for the maintenance of early pregnancy, seems to be involved in the regulation of the expression of both proteins [82].

Subsequent, in the second publication GdA and PZP expression was examined in the decidua of patients suffering from recurrent and spontaneous abortion [83]. A co-expression of GdA and PZP was demonstrated in both compartments of the decidua via double-immunofluorescence staining [83]. While PZP expression was significantly upregulated on mRNA- and protein level in both miscarriage groups, the GdA expression was downregulated [83]. The overexpression of PZP might be a compensatory mechanism in the placenta of patients diagnosed with miscarriage due to low hCG levels [83]. The correlation analysis of the expression between both

proteins revealed a significant negative correlation only in the decidual stroma in recurrent miscarriage cases [83]. Therefore, an imbalance of GdA and PZP expression could be associated with unexplained recurrent pregnancy loss [83].

In conclusion, the results of this thesis indicate that PZP might be an important factor for a successful ongoing pregnancy. However, further studies need to evaluate the exact pathophysiological mechanisms and the potential role of PZP as a diagnostic marker for recurrent miscarriage.

4. Zusammenfassung

Die Schwangerschaft ist ein einzigartiges Beispiel für immunologische Toleranz [22, 23]. Dieses immunologische Phänomen wird unter anderem durch diverse Veränderungen des mütterlichen Organismus vor allem im Bereich der feto-maternalen Interphase ermöglicht [24, 25]. Hierbei scheint eine veränderte maternale Immunantwort durch zum Beispiel vermehrte Sekretion von Th2 Zytokinen eine Schlüsselposition einzunehmen [29, 31]. Zudem wird angenommen, dass alternatives Spleißen der mRNA verschiedener Rezeptoren und Proteine ein notwendiger Mechanismus für eine erfolgreiche Schwangerschaft darstellt [43-47]. Als Beispiel ist PZP zu nennen, ein immunsuppressives Protein, welches hohe Serumwerte im maternalen Plasma erreicht [30, 61, 66-68, 81]. Als Folge von alternativem Spleißen der PZP mRNA existieren verschiedene Isoformen [57]. Die Bedeutung von PZP vor allem im ersten Trimenon, der vulnerabelsten Phase einer Schwangerschaft [2], ist noch weitgehend unbekannt. Daher wurde in der vorliegenden Dissertation die Expression von PZP in Plazenten von gesunden Schwangerschaften aus dem ersten Trimenon, spontanen und rezidivierenden Aborten sowie Blasenmolen systematisch untersucht. Ergänzend wurde die Expression von zwei weiteren wichtigen Proteinen und deren Korrelation zu PZP untersucht: Glycodelin A, als wichtiges immunsuppressives Protein in der Frühschwangerschaft, und EFTUD2, welches als Schlüsselprotein für die Regulation von alternativem Spleißen fungiert [27, 34, 48, 52]. Ebenfalls wurde eine mögliche Regulation von PZP durch hCG in der Chorionkarzinom Zelllinie JEG-3 *in vitro* untersucht.

In der ersten Publikation wurde demonstriert, dass die Proteinexpression von PZP signifikant inhibiert ist im Synzytiotrophoblasten von Spontanaborten und Blasenmolen im Vergleich zur Kontrollgruppe [82]. Zudem korreliert die PZP Proteinexpression negativ mit EFTUD2 [82]. Die Expression von EFTUD2 wiederrum ist in Spontanaborten und Blasenmolen im Synzytiotrophoblasen sowie dezidualen Drüsen signifikant hochreguliert [82]. Nach der Kultivierung der Chorionkarzinom Zelllinie JEG-3 mit hCG wurde eine signifikante Verminderung der PZP mRNA Expression festgestellt sowie eine signifikante Hochregulation von EFTUD2 [82]. Somit scheint hCG, ein zentrales Hormon für die Erhaltung der Frühschwangerschaft, die Expression von PZP als auch EFTUD2 zu regulieren [82].

Anschließend wurde in der zweiten Publikation die Expression von GdA und seinem Trägerprotein PZP in der Dezidua von Patientinnen mit spontanen und rezidivierenden Aborten sowie gesunden Kontrollen untersucht [83]. Zum ersten Mal konnte eine gemeinsame Expression von PZP und GdA in der Dezidua von Patientinnen mit rezidivierenden Aborten und gesunden Plazenten aus dem ersten Trimenon mittels Doppelimmunfluoreszenz gezeigt werden [83]. Zudem konnte in der Veröffentlichung demonstriert werden, dass die PZP Expression im dezidualen Stroma von Patientinnen mit spontanen und rezidivierenden Aborten auf Protein- und mRNA - Ebene signifikant hochreguliert ist [83]. Im Gegensatz dazu zeigte sich für GdA eine signifikant verminderte Protein- und mRNA-Expression in der Dezidua [83]. Die Hochregulation von PZP scheint ein kompensatorischer Mechanismus in der Dezidua zu sein, welcher durch verminderte hCG Werte vermittelt sein könnte [83]. Interessanterweise wurde nur im Stroma der Dezidua von Patientinnen von rezidivierenden Aborten eine signifikant negative Korrelation von PZP und GdA festgestellt [83]. Eine Imbalance in der Expression dieser beiden Proteine könnte somit im Zusammenhang mit rezidivierenden Fehlgeburten stehen [83].

Die im Rahmen dieser Dissertation erhobenen Ergebnisse deuten darauf hin, dass das PZP eine zentrale Rolle für die Erhaltung einer Schwangerschaft spielen könnte. Weitere Untersuchungen sind jedoch notwendig, um den genauen pathophysiologischen Mechanismus zu klären. Insbesondere die Bedeutung und die Erforschung von PZP als potentieller Marker für ungeklärte rezidivierende Aborte ist von großem klinischem Interesse.

5. Abbreviations

hCG	Human Choriongonadotropin
SM	Spontaneous miscarriage
RM	Recurrent miscarriage
HM	Hydatidiform mole
GTD	Gestational trophoblastic disease
IUGR	Intrauterine growth restriction
NK	Natural killer cells
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
Treg	T regulatory
GdA	Glycodelin A
mRNA	messenger-RNA
HLA-G	non-classical class I human leukocyte antigen
EFTUD2	Elongation factor Tu GTP binding domain containing 2
PPARγ	Peroxisome proliferator-activated receptor γ
PZP	Pregnancy Zone Protein
α2M	α2-Macroglobulin
LRP	Low densitiy lipoprotein receptor-related-protein
IL-2	Interleukin 2
TGF- β 1	Transforming growth factor-β 1
TGF- β 2	Transforming growth factor-β 2
IL-4	Interleukin 4
Aβ	Amyloid beta peptide
RT-PCR	RealTime PCR

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7. EIDESSTATTLICHE VERSICHERUNG

Eidesstattliche Versicherung

Löb, Sanja (geb. Milak)
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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Analysis of the expression of the Pregnancy Zone Protein in the human placenta in disorders of early pregnancy

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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9. Appendix: Publication 1 and 2

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Spliceosome protein EFTUD2 is upregulated in the trophoblast of spontaneous miscarriage and hydatidiform mole

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ABSTRACT

Background: Elongation factor Tu GTP binding domain containing 2 (EFTUD2) is an alternative splicing factor that modulates cell differentiation and activation processes. EFTUD2 is known to modulate immune responses and mutation of the *EFTUD2*-gene lead to fetal malformation. Little is known about its expression and role in normal and disturbed first trimester pregnancy.

Patients and methods: We investigated the expression of EFTUD2 in placental tissue obtained from patients with normal (n = 14), spontaneous miscarriage (n = 15) and molar (n = 14) pregnancy by immunohistochemistry. The expression of EFTUD2 was correlated on the protein level with known immune modulatory proteins like pregnancy zone protein (PZP) and in addition with human chorionic gonadotropin (hCG). Furthermore, we analysed the EFTUD2 and PZP expression *in vitro* after stimulation of the chorioncarcinoma cell line JEG-3 with hCG.

Results: EFTUD2 is significantly upregulated in the syncytiotrophoblast of spontaneous miscarriage ($p = 0.003$) and molar pregnancy ($p = 0.003$) compared to week of gestation-adjusted normal first trimester placentas. PZP is negatively correlated ($p = 0.021$) to EFTUD2 in the syncytiotrophoblast and is therefore significantly downregulated in miscarriage ($p = 0.028$) and mole pregnancy ($p = 0.006$). In addition, hCG is positively correlated to EFTUD2 in mole pregnancy. The addition of hCG to chorioncarcinoma cell lines JEG-3 *in vitro* stimulated EFTUD2 expression in these cells ($p = 0.027$).

Conclusion: Regulation of alternative splicing seems crucial for a successful ongoing pregnancy. The up-regulated elongation factor EFTUD2 may have a critical role in miscarriage.

1. Introduction

Miscarriage, which is defined as either spontaneous or recurrent, is a common disorder in pregnancy (Makrigiannakis et al., 2011). It is affecting 25–50% of all reproductive aged women. Immunologic, endocrine and metabolic mechanisms are involved in the success of human pregnancy and disturbances in any of these processes can lead to fetal loss (Knabl et al., 2016). Developmental defects of the embryo and fetal chromosomal abnormalities are often the cause for spontaneous miscarriage in the first trimester. The most common genetic disorders are trisomy 15, 16, 21 and monosomy X (Philipp et al., 2003). Established risk factors, especially for recurrent pregnancy loss, are the antiphospholipid syndrome, thrombophilia or maternal anatomical malformations (Toth et al., 2010). In nearly 50% of affected patients, however, the cause of miscarriage remains unknown (Arck et al., 2008).

Hydatidiform mole pregnancy is a rare disease which occurs in 1 of 1000 pregnancies (Brown et al., 2017; Khoo, 1982). Determination of hCG-overproduction is the gold standard for the diagnosis of this disease (van Trommel et al., 2005). Already earlier studies revealed that in mole pregnancy the peptide chain of hCG as well as the glycosylation of hCG is disturbed (Elliott et al., 1997). Our former studies showed that the glycosylation of hCG produced in chorion carcinoma cells JEG-3 is altered and carries Sialyl-Lewis structures (Jeschke et al., 2003).

Another target protein for alternative splicing in a developmental disorder is the pregnancy zone protein (PZP) (Cheong et al., 2016). Two major splice variations for PZP are known with variations especially in

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exons 1a, 1c and 3b (Gerhard et al., 2004). PZP is an immunosuppressive protein (Stigbrand et al., 1978; Svendsen et al., 1978) that is produced by the placenta and the endometrium during pregnancy (Lin and Halbert, 1976; Tayade et al., 2005). PZP is the carrier of another major immunosuppressive glycoprotein called glycodelin or PP14 (Skornicka et al., 2004). The expression pattern of glycodelin is dramatically changed in placentas with miscarriage or mole pregnancy (Toth et al., 2008; Wood et al., 1990). In addition, PZP seems to play an important role in the inhibition of *in vitro* aggregation of misfolded proteins, including the amyloid beta peptide that is implicated in pre-eclampsia as well as with Alzheimer's disease (Cater et al., 2019).

In general, the process of alternative splicing and protein misfolding and its role during gestation and placental development is not well understood. A recent study showed, that alternative splicing of Progesterone induced blocking factor (PIBF) is related to pregnancy outcome (Bogdan et al., 2014). A main protein for alternative splicing is the elongation factor Tu GTP binding domain containing 2 (EFTUD2) (Gordon et al., 2012), which was identified only very recently to be responsible for fetal malformation (Mouthon et al., 2019; Silva et al., 2019). In addition, EFTUD2 is an alternative splicing factor for the modulation of the innate immune response in macrophages (Lv et al., 2019). Macrophages in different polarization status are important for a successful ongoing pregnancy and may be inducers for miscarriage in the first trimester placenta (Stremmel et al., 2018; Kolben et al., 2018).

Therefore, the aim of this study was to analyse the combined expression and induction of EFTUD2, PZP and hCG in first trimester normal, miscarriage and hydatidiform mole placental tissue.

2. Materials and methods

2.1. Patient data

The Institutional Review Board of the Ludwig-Maximilian-University, Munich, (Number of approval: 337-06, 29th of December 2006) approved this study. All women signed an informed consent allowing analysis of all clinical and laboratory data mentioned in this study. Placental tissue from spontaneous miscarriages (SM) ($n = 15$) and hydatidiform mole pregnancy (HM) ($n = 14$) at gestational weeks 6–14 was obtained at the Department of Obstetrics and Gynecology, LMU Munich. Placental tissue from legal terminations of healthy pregnancies ($n = 14$) served as control group. The tissue was collected at a private practice clinic in Munich, Germany. The control group specimens were confirmed as healthy by a blinded independent pathologist. All placental material was acquired by dilatation and curettage, without any prior pharmaceutical induction. In cases of SM and HM, the operation was performed within 24 h after diagnosis. Instantly, after the uterine curettage, the obtained tissue was either frozen or formalin fixed for further analysis. All patients included had an inconspicuous family and medical history, which was obtained systematically. Patients with common disorders, autoimmune diseases and microbiological infections (Bacteria and Chlamydia trachomatis) were excluded. Likewise we excluded patients from our study, who suffered from thrombophilia like factor V Leiden, antithrombin-, protein C-, protein S-deficiency and a prothrombin G20210A-mutation or if the patient suffered from a thromboembolic event in the past.

Chromosomal abnormalities were ruled out by karyotype analysis in all samples, as described recently (Ziegelmuller et al., 2015). Table 1 summarizes the number of samples used for immunohistochemical staining for each gestational week. Table 2 shows the demographic and clinical characteristics of the study population.

2.2. Immunohistochemistry

Formalin-fixed tissue slides were embedded in paraffin wax for immunohistochemistry. Samples were deparaffinized in xylol for 20 min and rinsed in 100% ethanol. Methanol/H_2O_2 incubation for 20 min was performed to inhibit endogenous peroxidase reaction. Afterwards, the specimens were rehydrated in deescalating alcohol gradients, starting with 100% ethanol and ending with distilled water. The samples were cooked in a pressure pot, containing a sodium citrate buffer (pH = 6.0), which consisted of 0.1 mM citric acid and 0.1 mM sodium citrate in distilled water. Subsequently, samples were washed in PBS twice and incubated with a blocking solution (Power Block, diluted 1:10 in Aqua dest, Universal Blocking Reagent, BioGenex, San Ramon, USA) for 3 min. Incubation with the primary antibody was performed with each section for 16 h at 4 °C. All antibodies used are listed in Table 3. Following every subsequent step, samples were washed twice in PBS (pH = 7.4). Blocking solutions, containing post block (reagent 2, ZytoChem Plus HRP Polymer System (Goat), Zytomed, Berlin, Germany) for 30 min and HRP-Polymer (reagent 3) for 30 min, were applied. The chromogen-substrate staining was carried out using the Liquid DAB + Substrate Chromogen System (Dako Scientific, Glostrup, Denmark), 2 min for Pregnancy Zone Protein, 2 min for EFTUD 2 and 2 min for hCG. The reaction was stopped by applying distilled water. Finally, tissue samples were counterstained with Hemalaun for 2 min and blued in tap water. Specimens were dehydrated in an ascending alcohol gradient and cover slipped with Eukitt® quick hardening mounting medium (Sigma Aldrich, St. Louis, MO, USA). The intensity and distribution pattern of the immunochemical staining reaction were evaluated by two independent blinded observers. In one case (n = 7.1%), the evaluation of the two observers differed. This case was re-evaluated by both observers together. After the re-evaluation, both observers came to the same result. The concordance before the re-evaluation was 91.9%.

Table 1
Number of sampels used for immunohistochemical staining for each gestational week.

Gestational Age	Normal Pregnancy	Spontaneous Miscarriage	Hydatidiform mole pregnancy
6th week	2	1	1
7th week	1	3	2
8th week	4	3	1
9th week	2	2	3
10th week	2	2	2
11th week	1	2	2
12th week	1	1	1
13th week	0	0	1
14th week	1	1	1
	$n = 14$	$n = 15$	$n = 14$

Positive controls were carried out with kidney tissue for PZP detection and colon tissue for detection of EFTUD 2 following the staining protocol as described above. Negative controls were performed by replacement of the primary antibodies by species specific isotype control antibodies (Dako).

2.3. Cell culture of JEG-3 cells

The choriocarcinoma cell line JEG-3 (ATCC, Washington, DC, USA) was used for the *in vitro* experiments. JEG-3 cells were cultured in dulbeco modified eagle medium (DMEM, 3.7 g/l $NaHCO_3$, 4.5 g/l D-glucose, 1.028 g/l stable glutamine, and Na-Pyruvate; Biochrom, Berlin, Germany), which was supplemented with 10% heat-inactivated FCS and incubated at 37 °C and at an atmospheric CO_2 concentration of 5% without antibiotics and antimycotics.

2.4. RNA extraction from JEG-3 Cells after stimulation with hCG

JEG-3 cells were grown on sterile 12-well Multiwell slides (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 80 000 cells/mL DMEM medium. For the stimulation the cells were incubated with fresh DMEM. After 24 h, the cells were stimulated with 10 , 100 and 1000 IU/

Table 2
Demographic and clinical characteristics of the study population.

Characteristics*	Normal Pregnancy $n = 14$	Spontaneous Miscarriage $n = 15$	Hydatidiform mole $n = 14$	p Value (Kruskal Wallis Test)
Maternal age (years)	31.18 ± 8.06 (18.7 − 43.3)	37.8 ± 4.51 (29.2–43.2)	31.0 ± 5.1 (23.0–40.0)	0.287
Gestational age (weeks)	9.00 ± 1.30 (6–14)	9.56 ± 1.40 (6–14)	9.79 ± 1.41 (6–14)	0.681
Gravidity	2.6 ± 1.5 (1–6)	1.6 ± 0.9 (1–7)	2.0 ± 1.1 (1–4)	0.356
Parity	2 ± 1.1 (0–4)	0.9 ± 0.8 (0–2)	0.4 ± 0.1 (0–1)	0.273

Values are Mean ± S.D. * Mean, standard deviation, range.

Table 3
Antibodies used for immunohistochemical/-cytochemical characterization of placental tissue samples and cell culture slides.

Antibody	Isotype	Clone	Dilution	Source
PZP	IgG	Polyclonal	1:200	Santacruz Biotechnology
EFTUD 2	IgG	Polyclonal	1:300, 1:800*	Lifespan Biosciences
hCG	IgG	Polyclonal	1:1000	DAKO

PZP = pregnancy zone protein, EFTUD2 = Elongation factor Tu GTP binding domain containing 2, hCG = human chorionic gonadotropin, *Dilution 1:300 for immunohistochemistry, 1:800 for immunocytochemistry.

mL hCG for 2, 4 and 8 h. Control cells were incubated without the stimulants. We used the NucleoSpin RNAII Kit (Macherey-Nagel, Düren, Germany) for the investigation of the total RNA according to the manufacturer's protocol. Quantification and evaluation of the purity of the purified RNA was carried out with a NanoPhotometer (Implen, Munich, Germany). Three independent experiments were performed.

2.5. Reverse transcription

According to the protocol reverse transcription (RT) was carried out with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Fisher Scientific Company, Waltham, MA USA) and placed in a mastercycler® gradient (Eppendorf, Hamburg; Germany). RT conditions were as follows: 10 min at 25 °C, 2 h at 37 °C, 5 min at 85 °C and continued by a hold step at − 20 °C.

2.6. Real-time reverse transcription PCR

After conversion of RNA to cDNA, PCR was performed on all samples individually. Real-Time Reverse Transcription PCRs were covered with optical caps in optical 96-well (Applied Biosystems™, Fisher Scientific Company, Waltham, MA USA) reaction microtiter plates. Each reaction was accomplished with a volume of 20 µL, including 1 µL cDNA, 8 µL H_20 (DEPC treated DI water; Sigma, Taufkirchen, Germany) and 10 µL TaqMan® Fast Universal PCR Master Mix 2× (Applied Biosystems, Nr. 4367846; 50 mL). The total contained 1 µL TaqMan® Gene Expression Assay 20× (Hs00161140_m1 for PZP, Applied Biosystems). The temperature protocol was as follows: 20 s at 95 °C, 40 cycles of amplification, denaturation for 3 s at 95 °C and denaturation plus annealing process for 30 s at 60 °C. Processing the PCR assays was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems), and quantification was accomplished by the $2^{-\Delta\Delta Ct}$ method using GAPDH as housekeeping gene (Applied Biosystems, Hs99999905_m1).

2.7. Evaluation of EFTUD-2 with immunocytochemistry

Cell culture of JEG-3 cells was performed as described above (Section 4.3.1). JEG-3 cells were separately grown to near confluency on sterile 8-well chamber slides (Nunc, Rochester, NY, USA) at a density of 50 000 cells/500 µl DMEM medium. Medium change was carried out after 24 h, which was followed by treatment of the cells with 1000 IU/mL hCG (Sigma–Aldrich) for 48 h. Untreated cells served as controls.Afterwards the slides were fixed in 1:1 ethanol/methanol solution for 15 min and between each following step the slides were washed with PBS(Biochrome). At first the slides were incubated with diluted horse serum for 20 min (10 mL PBS and 150 µl horse serum; Vector Laboratories, Burlingame, CA, USA) at room temperature, then an incubation with the EFTUD2 antibody, diluted 1:800 in PBS, was performed for 16 h at 4 °C. Afterwards the slides were incubated with the secondary antibody from the Vectastain Elite ABC Goat IgG Kit (Vector Laboratories) for 30 min. Then, the ABC complex (Vector Laboratories) was applied on the slides and incubation lasted for 30 min. Finally, the slides were stained with 3-amino-9-ethylcarbazole plus (AEC plus; Dako, Glostrup, Denmark) for 12 min, counterstained with hemalaun for 30 s, washed in tap water and cover-slipped. In total, three independent experiments were conducted and evaluated.

All slides were analyzed using the microscope Leitz Wetzlar (Wetzlar, Germany; Type 307-148.001 514686). The immunoreactive score (IRS) was used for evaluation of the intensity and distribution pattern of antigen expression. This semi-quantitative score is calculated as follows: the optical staining intensity (grades: 0 = none, 1 = weak, 2 = moderate, 3 = strong staining) is 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. Analysis of all slides was performed independently by two experienced staff members.

2.8. Statistics

Analysis of the collection and statistical data was processed with the SPSS software version 24 (SPSS; Chicago; IL; USA) and Excel version 12.3.1 (Microsoft Windows 2016; Redmond, WA; USA). The Mann-Whitney U signed-rank test was used for the comparison of two independent and Kruskal-Wallis test for more than two independent groups. p-values < 0.05 were considered to be statistically significant. Correlation analysis was performed using the Spearman correlation coefficient.

3. Results

3.1. EFTUD2-expression in the trophoblast

The expression of EFTUD2 in the syncytiotrophoblast was analyzed in tissue from healthy pregnancies (14 cases), spontaneous miscarriages (SM, 15 cases), and hydatidiform mole pregnancy (HM 14 cases; Fig. 1a–d). The syncytiotrophoblast revealed a significantly higher expression in the group with spontaneous abortions (Fig. 1b) than in the group with healthy placentas (Fig. 1a, IRS 12 *vs.* 6, $p = 0.003$). There was also a significant upregulation of EFTUD2 in the syncytiotrophoblast of hydatidiform mole placentas (Fig. 1c, IRS 9 *vs.* 6, $p = 0.003$) compared to the control group. Briefly, the staining results are shown in the boxplot in Fig. 1d. Controls (colon tissue) are presented in Fig. 1e-f.

3.2. EFTUD2-expression in the decidua

EFTUD2 expression was investigated in the glandular epithelial cells in the decidua of healthy pregnancies (14 cases), SM (15 cases), and HM

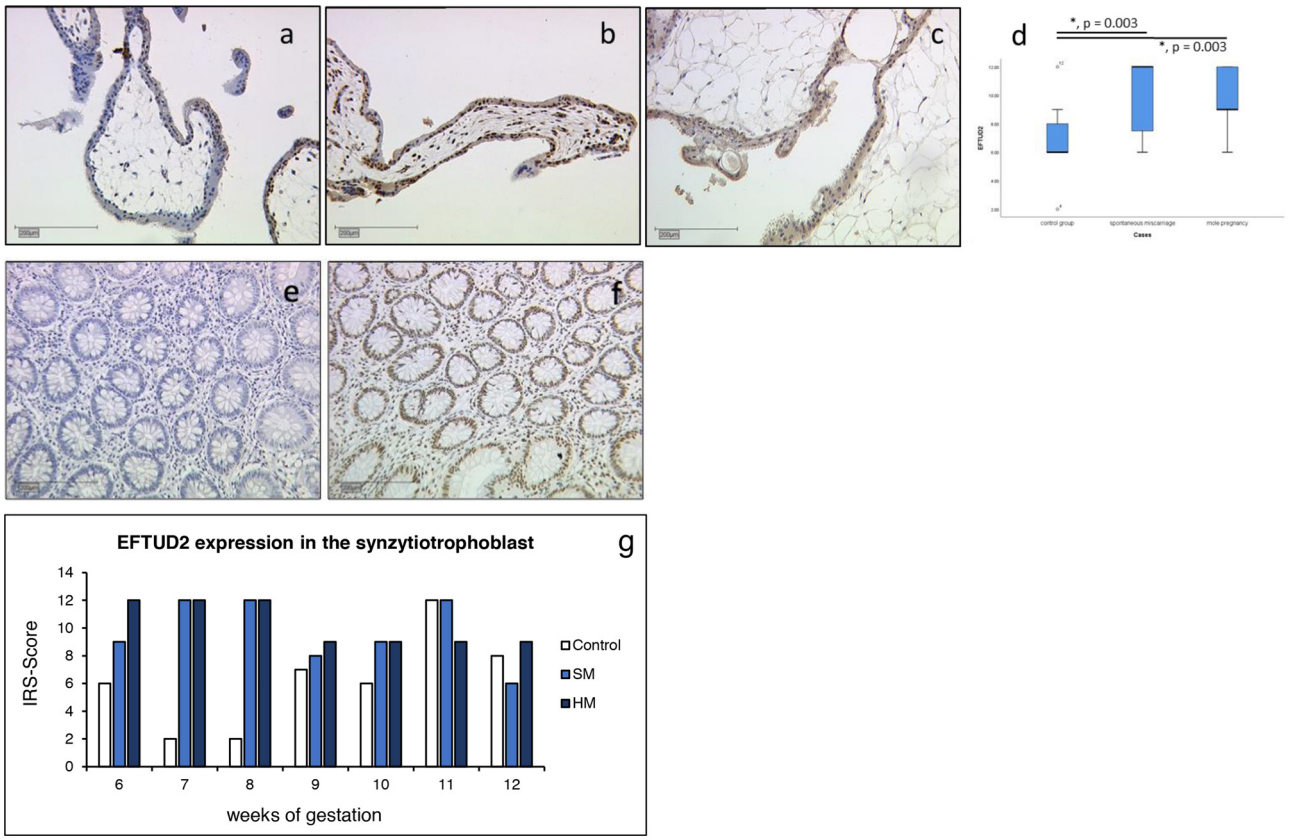

Fig. 1. Immunohistochemical staining of EFTUD2 in the syncytiotrophoblast. EFTUD2 expression is found with moderate distribution and intensity in the syncytiotrophoblast of first-trimester placentas. The control group (14 cases) showed a moderate expression pattern (a). EFTUD2 was significantly upregulated ($p = 0.003$) in the syncytiotrophoblast of spontaneous miscarriage (SM, 15 cases, (b). EFTUD2 expression in the syncytiotrophoblast of hydatidiform mole tissue (HM, 14 cases), (c) was significantly increased compared to the control ($p = 0.003$). The boxplot summarizes the statistical data of the immunohistochemical staining results (d). A negative (e) and a positive (f) control in colon tissue were performed during each staining. Scale is 200 µm. (g) Staining intensity of EFTUD2 in the syncytiotrophoblast of normal, spontaneous miscarriage and hydatidiform mole pregnancy was determined by the semiquantitative immunohistochemical IRS score. Data shown represent the expression of EFTUD2 from the 6th to the 12th week of gestation in all three groups.

(14 cases; Fig. 2a–d). The expression of EFTUD2 was moderate in glandular epithelial cells of the decidua basalis of control specimens (Fig. 2a). The expression of EFTUD2 was significantly increased in decidua basalis SM samples ($p = 0.002$; Fig. 2b). EFTUD2 was also significantly increased in the decidua basalis HM group ($p = 0.003$; Fig. 2c). A summary of the staining results is shown in Fig. 2d.

3.3. PZP-expression in the syncytiotrophoblast

PZP expression was investigated in the syncytiotrophoblast of healthy pregnancies (14 cases), SM (15 cases), and HM (14 cases; Fig. 3a–d). The syncytiotrophoblast showed a significantly reduced expression in the SM-group (Fig. 3b) compared to the group of healthy placentas (Fig. 3a, IRS 3 *vs.* 4, p = 0.028). There was also a significant reduction of PZP in the syncytiotrophoblast of HM-placentas (Fig. 3c, IRS 4 *vs.* 3, p = 0.006) compared to the control group. Briefly, the staining results are shown in the boxplot in Fig. 3d.

3.4. Correlation analyses of EFTUD2, PZP and hCG

There are significant correlations with EFTUD2, PZP and hCG within the trophoblast of the placentas investigated. EFTUD2 expression showed a negative correlation with PZP in the syncytiotrophoblast (correlation coefficient (cc) = −0.369, p = 0.021). PZP showed an additional negative correlation with hCG (cc = −0.380, p = 0.014). In addition, EFTUD2- and PZP-expression was correlated to the week of gestation. Neither EFTUD2 nor PZP showed significant changes in the period investigated (6th-14th week of gestation; p = 0.321 and 0.304, respectively).

3.5. Evaluation of PZP expression in JEG-3 cells after stimulation with hCG with Real-Time RT-PCR (TaqMan)

PZP mRNA (*PZP*) expression was analyzed in JEG-3 cells after stimulation with hCG by quantitative RT-PCR. *PZP* was significantly downregulated at concentration of 100 and 1000 IU/mL (Fig. 4a; p = 0.027, respectively). In addition, we carried out time dependent investigation of PZP inhibition with 1000 IU/mL hCG. Results show significant downregulation of *PZP* after 2, 4 and 8 h respectively (Fig. 4b).

3.6. Upregulation of EFTUD2 in JEG-3 cells in vitro with hCG

Chorion carcinoma cells JEG-3 were stimulated with hCG. Stimulation of JEG3 cells with 1000 IU hCG for 48 h resulted in significant upregulation of EFTUD2 on protein level as investigated with immunocytochemistry (Fig. 4c–e, p = 0.027).

4. Discussion

Within this study we could show that the Elongation factor Tu GTP binding domain containing 2 (EFTUD2) is significantly upregulated in the syncytiotrophoblast of spontaneous miscarriage and hydatidiform mole pregnancy compared to week of gestation-adjusted normal first

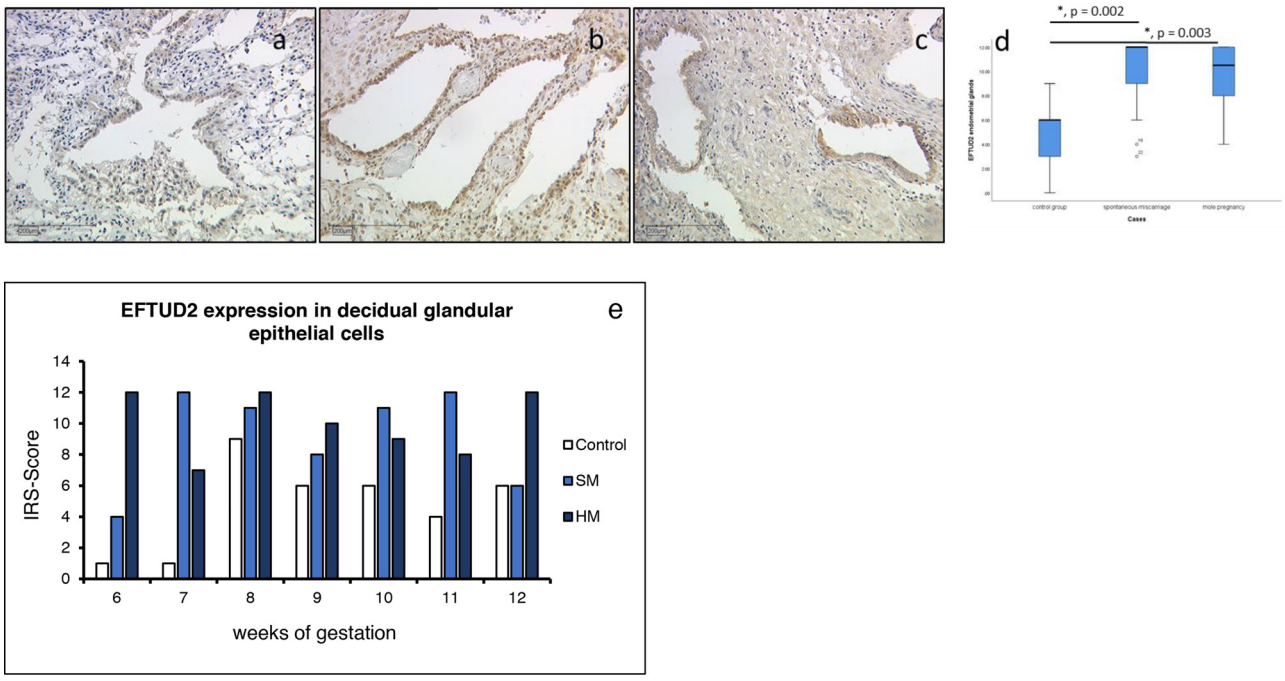

Fig. 2. Immunohistochemical staining of decidual glandular epithelial cells with ani-EFTUD2 antibodies. Decidual EFTUD2 expression was increased in SM (15 cases) and HM samples (14 cases) compared to the control group (14 cases), (a). In SM specimens, EFTUD2 is significantly upregulated ($p = 0.002$) (b). In HM samples, the EFTUD2 was significantly higher compared to the control ($p = 0.003$) (c). Summary of staining results of EFTUD2 (d). Scale is 200 µm. (e) Staining intensity of EFTUD2 in decidual glandular epithelial cells of normal, spontaneous miscarriage and hydatidiform mole pregnancy was determined by the semiquantitative immunohistochemical IRS score. Data shown represent the expression of EFTUD2 from the 6th to the 12th week of gestation in all three groups.

trimester placentas. PZP is negatively correlated to EFTUD2 in the syncytiotrophoblast and therefore downregulated in miscarriage and hydatidiform mole pregnancy. In addition, hCG is positively correlated to EFTUD2 in hydatidiform mole pregnancy. The addition of hCG to cultivated chorion carcinoma cells JEG-3 *in vitro* stimulated EFTUD2 expression in these cells.

It is known already for some years that mutation of EFTUD2 causes mandibulofacial dysostosis with microcephaly (Lines et al., 2012). But recently it was found that EFTUD2 mutation causes a variety of malformations like syndromic oesophageal atresia (Gordon et al., 2012) and Nager syndrome (Czeschik et al., 2013). It was also recently described that oto-facial syndrome, esophageal atresia, intellectual disability and zygomatic anomalies are associated with EFTUD2 mutations (Voigt et al., 2013). EFTUD2 mutations are also found in cases with autosomal dominant retinitis pigmentosa (Benaglio et al., 2014). Cases with EFTUD2 mutations displayed abnormal brain development with evident neuronal apoptosis while the development of other organs appeared less affected in an animal model (Lei et al., 2017).

In addition to this fetal malfunction studies, it was found that loss of function mutation of EFTUD2 leads to pre-implantation arrest in mouse (Beauchamp et al., 2019). Within this study, EFTUD2 was found to be expressed in the mouse placenta and therefore it seems to be evident that EFTUD2 may also be involved in placental development and function (Beauchamp et al., 2019).

In addition, in a comparative genomics RNAi screening approach it was described that one of a novel conserved regulators of innate immunity is the mRNA splicing regulator EFTUD2 (De Arras et al., 2014). This protein seems to control the alternate splicing of the MyD88 innate immunity signaling adaptor and to modulate the extent of the innate immune response (De Arras et al., 2014). A potential mechanism to link innate immune signaling in human macrophages to DNA repair was described by White et al. recently (White et al., 2019). The proposed mechanism is that simultaneous activation of SCN5A and SCN10A increases expression of PPP1R10 protein expression through cleavage of a retained intron in PPP1R10 transcripts (White et al., 2019). This increase in PPP1R10 protein expression is dependent on expression of EFTUD2. Therefore EFTUD2 expression in macrophages might be a protective factor that prevents cytotoxicity during inflammatory responses (White et al., 2019).

In a recent mouse model on cancer development, it was found that knockout of EFTUD2 suppresses inflammation and tumorigenesis, which is associated with decreased production of inflammatory cytokines and tumorigenic factors (Lv et al., 2019). Repression of inflammation and tumour development in EFTUD2-deficient mice was found to be related to impaired activation of NF-κB signaling in macrophages (Lv et al., 2019). Furthermore, the alteration of EFTUD2-mediated alternative splicing involving the components of TLR4-NF-κB cascades underlies the impairment of NF-κB activation (Lv et al., 2019).

Macrophages are also involved in high affinity receptor mediated binding, uptake and degradation of the pregnancy zone protein (PZP) (Jensen et al., 1988), a protein that is elevated in maternal plasma during pregnancy (Yding Andersen et al., 1992). PZP is an immune-modulating factor that binds a variety of immunosuppressive proteins like glycodelin A (also called PP14) (Skornicka et al., 2004; Tayade et al., 2005). In addition, PZP was found to efficiently inhibit the aggregation of misfolded proteins, including the amyloid beta peptide (Aβ) that is implicated in preeclampsia as well as with Alzheimer's disease (Cater et al., 2019). In a recent study of Carter et al. it was proposed that the up-regulation of PZP during pregnancy represents a major maternal adaptation that helps to maintain extracellular proteostasis during gestation in humans (Cater et al., 2019). In a former study we identified an upregulation of macrophages in spontaneous miscarriage placentas (Guenther et al., 2012; Kolben et al., 2018). In a similar set of cases we also identified a diminished expression of glycodelin A in placentas of spontaneous miscarriages (Toth et al., 2008). It has been speculated that there might be a regulative feedback loop between Glycodelin A and hCG. This hypothesis was supported by experiments showing that first trimester trophoblast cells expressed more hCG after incubation with Glycodelin A *in vitro* (Jeschke et al., 2005). Within our study we found a downregulation of PZP in placental tissue

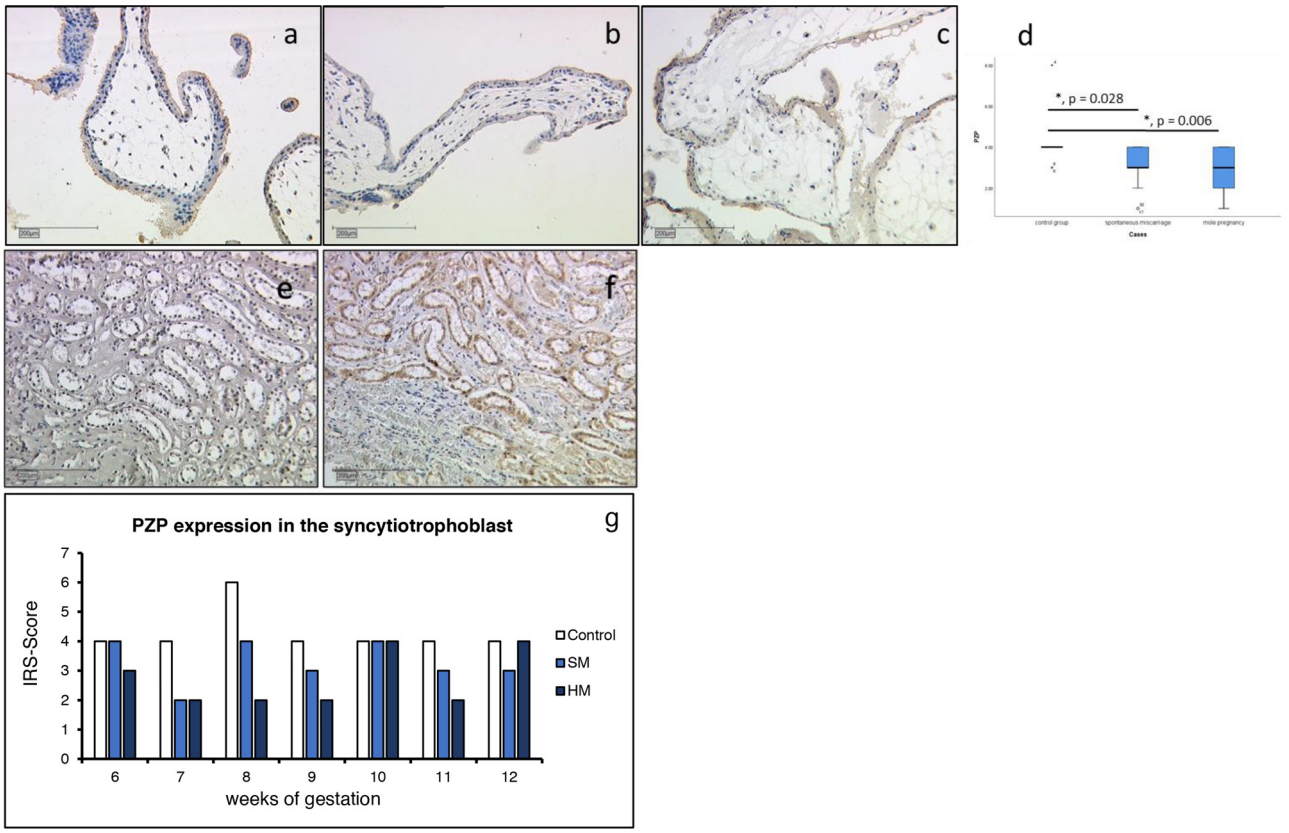

Fig. 3. Immunohistochemical staining of PZP. PZP expression is found with moderate intensity on the apical syncytiotrophoblast of first-trimester placentas. The control group (14 cases) showed a moderate expression pattern (a). PZP was significantly reduced (p = 0.028) on the syncytiotrophoblast of SM, 15 cases, (b). PZP expression in the syncytiotrophoblast of hydatidiform mole tissue (HM, 14 cases), (c) was also significantly reduced compared to the control (p = 0.006). The boxplot summarizes the statistical data of the immunohistochemical staining results (d) and (e) shows a negative and (f) a positive control. Scale is 200 µm.
(g) Staining intensity of PZP in the syncytiotrophoblast of normal, spontaneous miscarriage and hydatidiform mole pregnancy was determined by the semi-quantitative immunohistochemical IRS score. Data shown represent the expression of PZP from the 6th to the 12th week of gestation in all three groups

of spontaneous miscarriage cases. We might speculate that the enhanced number of macrophages in spontaneous miscarriage tissue could be involved in rapid PZP uptake and degradation as proposed by Jensen et al. (Jensen et al., 1988). In addition, we found that PZP was also downregulated in hydatidiform mole pregnancy placenta. This is an agreement with a former study showing that PZP serum level are downregulated in hydatidiform mole pregnancy as well as in chorion carcinoma cases (Teng et al., 1994). These results could be confirmed by our *in vitro* JEG-3 cell culture model by adding excess amounts of hCG.

On the other hand, EFTUD2 was upregulated in spontaneous miscarriage cases and also in hydatidiform mole pregnancy placentas. Because EFTUD2 is a protective factor that prevents cytotoxicity during inflammatory responses (White et al., 2019), we might speculate that the upregulation of this protein is a compensatory effect for the increase of inflammation during miscarriage. EFTUD2 expression was also upregulated in hydatidiform mole pregnancy. The gold standard for the identification of hydatidiform moles are elevated hCG serum level (Kishino et al., 2014; Matsui et al., 2001). Therefore we incubated chorion carcinoma cells JEG-3 *in vitro* with hCG. Interestingly, we could mimic the hydatidiform mole expression pattern of PZP as well as of EFTUD2 in this cell culture model. The addition of hCG leads to a downregulation of PZP and an upregulation of EFTUD2. Therefore we assume that hCG is an inducer of the elongation factor Tu GTP binding domain containing 2 (EFTUD2), which could be an interesting additional function for the major trophoblast protein hCG. Another explanation for the EFTUD2 upregulation after hCG treatment could be a polymorphism in the alternative donor site of the cryptic exon of LHCGR, the receptor of hCG (Liu et al., 2017). A cryptic exon (LHCGR-exon 6A) was found to be derived from alternative splicing in intron 6 of the LHCGR gene, which includes two transcripts, has functional consequences (Liu et al., 2017). Interestingly, there is an association of this alternative splicing event with testosterone levels (Liu et al., 2017). Testosterone is upregulated in hydatidiform mole pregnancy (Chew et al., 1979).

In conclusion, alternative splicing seems to be crucial for a successful ongoing pregnancy. The elongation factor EFTUD2 is an important factor in this process. Upregulation of EFTUD2 in spontaneous miscarriage could be related to elevate inflammatory signaling in which EFTUD2 is involved. EFTUD2 upregulation in hydatidiform mole pregnancy could be due to the fact that elevated testosterone levels, which are accompanied with moles, induce alternative splicing. Therefore hCG could upregulate EFTUD2 expression in chorion carcinoma cells JEG-3 *in vitro*. Chorion carcinomas can derive from hydatidiform mole pregnancies.

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CRediT authorship contribution statement

Sanja Löb: Conceptualization, Investigation. **Aurelia Vattai:** Software, Validation. **Christina Kuhn:** Methodology, Validation, Formal analysis. **Elisa Schmoeckel:** Data curation, Writing - original draft. **Sven Mahner:** Resources, Funding acquisition. **Achim Wöckel:** Resources. **Thomas Kolben:** Project administration. **Julia Szekeres-**

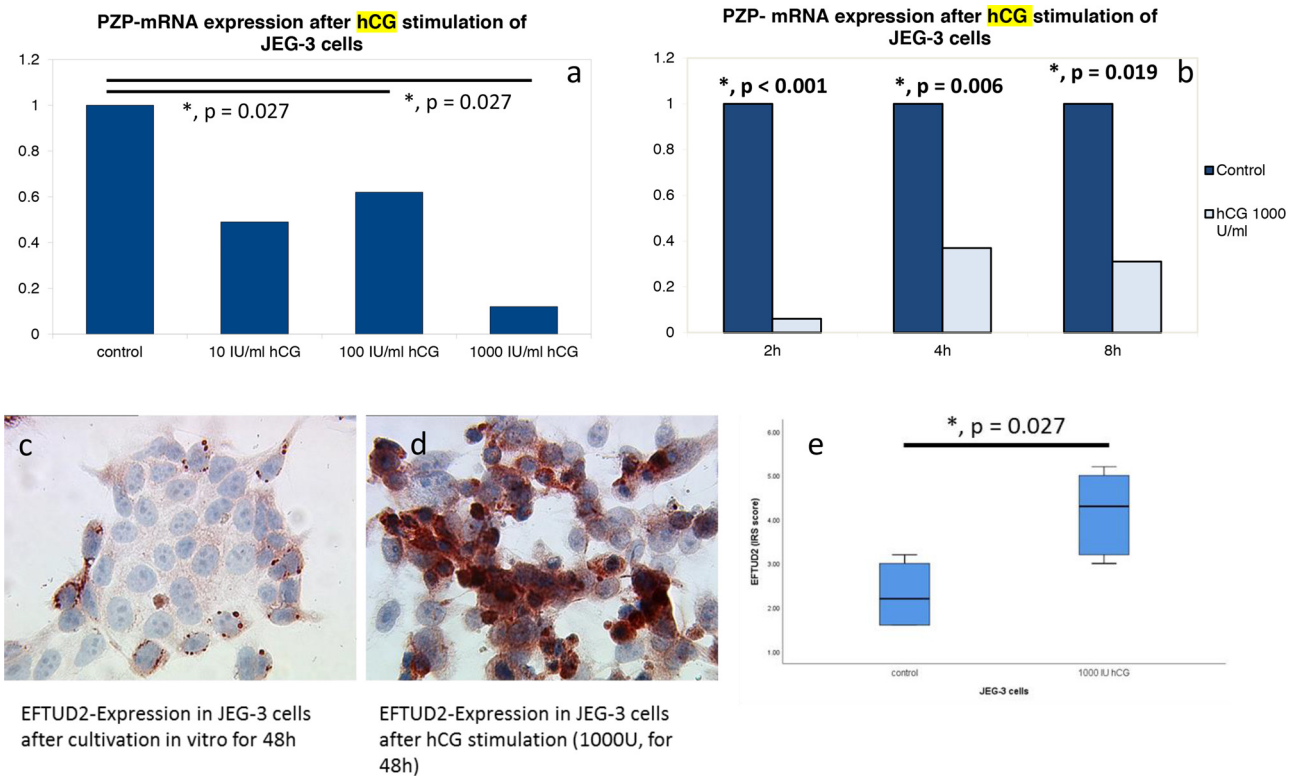

Fig. 4. a) Results of PZP mRNA expression analysis with TaqMan RT-PCR from JEG-3 cells *in vitro*. PZP mRNA expression was significantly downregulated in concentrations of 100 and 1000 IU/mL (p = 0.027, respectively). This bar graph shows the mean of relative *PZP* expression; therefore, the presentation of error bars is not appropriate. b) Results of PZP mRNA expression analysis with TaqMan RT-PCR from JEG-3 cells *in vitro* after incubation with 1000 U/mL hCG for 2, 4 and 8 h. PZP mRNA expression was significantly downregulated (p < 0.001, 0.006 and 0.019, respectively). This bar graph shows the mean of relative *PZP* expression; therefore, the presentation of error bars is not appropriate c) -e. Results of EFTUD2 immunocytochemistry in JEG-3 cells *in vitro*. EFTUD2 is expressed in JEG-3 cells with low to median intensity in unstimulated cells (median IRS score 2) after 48 h of cultivation (c). Addition of hCG (1000 IU) resulted in significant upregulation of EFTUD2 in JEG3 cells after 48 h of cultivation (median IRS score 4; d). A summary of the stainings is presented as Box-Plot in e.

Bartho: Methodology. **Udo Jeschke:** Conceptualization, Validation, Writing - original draft, Supervision, Project administration. **Theresa Vilsmaier:** Visualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Pregnancy Zone Protein (PZP) is significantly upregulated in the decidua of recurrent and spontaneous miscarriage and negatively correlated to Glycodelin A (GdA)

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ABSTRACT

Background: Pregnancy Zone Protein (PZP) is an immunosuppressive protein that is expressed by the placenta and has also been identified in immune cells. When PZP and Glycodelin A (GdA) are combined, they act synergistically to inhibit Th-1 immune response. Little is known about its combined expression and role in normal and disturbed first trimester pregnancy.

Patients and methods: We investigated the expression of PZP and GdA in placental tissue obtained from spontaneous miscarriage (SM) (n = 19) and recurrent miscarriage (RM) (n = 17) at gestational weeks 6–13 by immunohistochemistry and on mRNA-level by either TaqMan PCR or in situ hybridization. Placental tissue from legal terminations of healthy pregnancies (n = 15) served as control group. Immunofluorescence double staining was used to analyse the combined expression of PZP and GdA in decidual tissue.

Results: The protein level of PZP was significantly increased in decidual stroma of SM samples compared to the decidua of control specimens and also significantly upregulated in the decidual stroma cells in the RM group. Concerning GdA, the decidual stroma revealed a significantly decreased protein level in the group with spontaneous abortions than in the group with healthy pregnancies. There was also a significant downregulation of GdA in the decidual stroma of RM samples compared to the control group. We observed a significant negative correlation of PZP and GdA in decidual stromal tissue of recurrent abortion. We could confirm the staining results for PZP as well as for GdA on mRNA level. Both proteins are co-localized in decidual stroma as analysed by immunofluorescence double staining.

Conclusion: A balanced expression of GdA and its carrier protein PZP in the decidua seems crucial for a successful ongoing pregnancy. According to our data, these immunosuppressive proteins are co-localized in the decidual tissue and show a negative correlation only in patients suffering from recurrent abortion.

1. Introduction

Pregnancy is an immunological paradox (Moffett and Loke, 2004). The semi-allogenic fetus is accepted by the mother's adaptive immune system, which contradicts the rules of classic transplantation immunology (Chavan et al., 2017). The establishment of maternal immunotolerance towards the embryo at the fetomaternal interphase is crucial for a successful pregnancy (Toth et al., 2010a). Any disturbance of this complex system can lead to pregnancy disorders like preeclampsia or miscarriage (Negishi et al., 2018). In 25–50 % of all reproductive-aged women, miscarriage occurs at least once (Regan and Rai, 2000). About 5% of conceived females undergo two or more consecutive pregnancy losses, which is defined as recurrent abortion (Practice Committee of American Society for Reproductive, M., 2013; RPL and E. G.G.o., 2018). Spontaneous abortion in the first trimester can be caused by developmental defects of the embryo and fetal chromosomal

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abnormalities like trisomy 15, 16, 21 and monosomy X (Philipp et al., 2003). The antiphospholipid syndrome, thrombophilia or maternal anatomical malformations are established risk factors for recurrent pregnancy loss (Toth et al., 2010b). Although pregnancy loss is associated with embryonic chromosomal rearrangements, endocrine dysfunction, autoimmune disorders, maternal infections and life style factors (Ali et al., 2020), in nearly 50 % of affected patients the cause of miscarriage remains unknown (Arck et al., 2008). For these cases, rejection of the embryo by an exacerbated maternal immune system is discussed as one significant reason for unexplained recurrent miscarriage (Ali et al., 2020).

The immunosuppressive Glycodelin A (GdA), previously known as placental protein 14, may play an important role at the fetomaternal interphase for the maintenance of a pregnancy (Toth et al., 2008). GdA shows high concentrations in the decidua, especially from the 6th to 12th week of gestation, and is not synthesized by the trophoblast (Lee et al., 2011a; Seppala et al., 2002). The immunosuppressive properties of GdA are various. GdA is able to stimulate Th-2-type cytokine production in monocytes (Miller et al., 1998) and thereby promotes the cytokine shift from Th-1 to Th-2 cytokines, which appears crucial for a successful pregnancy (Jeschke et al., 2003a). Also, GdA induces a tolerogenic phenotype in dendritic cells, these modulate immunological tolerance at the feto-maternal interphase and are involved in diseases of insufficient placentation (Scholz et al., 2008a, b). Further, during gestation, GdA modulates the function and the number of natural killer (NK) cells (Dixit and Karande, 2020), these represent the most abundant leukocyte population in the decidua (Moffett-King, 2002). In addition, GdA stimulates hCG secretion in isolated primary trophoblast cells *in vitro* (Jeschke et al., 2005a, b) and functions as a modulator of trophoblast invasion (Lee et al., 2016). It has been known for some years that GdA secretion is pathologically decreased in the decidua of patients suffering from early pregnancy loss (Toth et al., 2008).

A carrier and modulator of GdA is the Pregnancy Zone Protein (PZP). PZP is an immunosuppressive protein (Stigbrand et al., 1978; Svendsen et al., 1978) that is produced by the placenta and has also been identified in immune cells (Stimson, 1977; Stimson and Blackstock, 1975). When PZP and GdA are combined, they act synergistically to inhibit Th-1 cell proliferation and IL-2 production (Skornicka et al., 2004), thereby, protecting the allogenic fetus from the maternal immune system (Wyatt et al., 2016). PZP inhibits allograft rejection (Svendsen et al., 1978), facilitates leukocyte migration (Birkeland et al., 1979) and seems to have an important role in human gestation (Wyatt et al., 2016). It has been reported that PZP inhibits in vitro aggregation of the Alzheimer's disease-associated amyloid beta peptide (Cater et al., 2019), which accumulates in the placenta in patients suffering from preeclampsia.

However, there is still limited knowledge about the role of PZP in miscarriage. Recently we could demonstrate that the protein level of PZP shows a pathological pattern in the syncytiotrophoblast of patients with spontaneous miscarriage and hydatidiform mole pregnancy (Lob et al., 2020). Additionally, we observed a negative correlation of PZP and hCG (Lob et al., 2020). The aim of this study was to systematically analyze the expression of GdA and its carrier protein PZP on protein and mRNA level in the decidua of healthy first trimester pregnancies, spontaneous and recurrent miscarriage. Furthermore, we were interested in the combined localization of GdA and PZP.

2. Material and methods

2.1. Patient data

The Institutional Review Board of the Ludwig-Maximilian-University, Munich, (Number of approval: 337−06, 29th of December 2006) approved this study. All women signed an informed consent allowing analysis of all clinical and laboratory data mentioned in this study. The study was conducted in accordance with the Declaration of Helsinki.

Placental tissue from spontaneous miscarriage (SM) (n = 19) and recurrent miscarriage (RM) (n = 17) at gestational weeks 6–13 was obtained at the Department of Obstetrics and Gynecology, LMU Munich. Recurrent abortion was defined as two or more consecutive failed pregnancies (Practice Committee of American Society for Reproductive, M., 2013; RPL and E.G.G.o., 2018). Placental tissue from legal terminations of healthy pregnancies (n = 15) served as control group and was collected at a private practice clinic in Munich, Germany. The tissue samples were all obtained by dilatation and curettage, without any prior pharmaceutical induction. In cases of SM and RM, the operation was performed within the first 24 h after diagnosis. Instantly, after the uterine curettage, the placental tissue was either frozen or formalin fixed for further analysis. The control group specimens were confirmed as healthy by a blinded independent pathologist. We obtained the patients family and medical history systematically, which was inconspicuous for all patients included in our study. Patients with common disorders and intrauterine infections (Bacteria and Chlamydia trachomatis) were excluded such as patients suffering from thrombophilia and autoimmune diseases, already known as aggravating factors for increased risk for miscarriage. Chromosomal abnormalities were ruled out by karyotype analysis in all samples, as described recently (Ziegelmuller et al., 2015). Table 1 summarizes the number of samples used for immunohistochemical staining for each gestational week. Table 2 shows the demographic and clinical characteristics of the study population.

2.2. Immunohistochemistry

Formalin-fixed paraffin-embedded sections (3 μm) were deparaffinized in xylol for 20 min and rinsed in 100 % ethanol. Methanol/H_2O_2 incubation for 20 min was performed to inhibit endogenous peroxidase reaction. Rehydration of the slides was performed using an alcohol gradient, starting with 100 % ethanol and ending with distilled water. The samples were cooked in a pressure pot, containing a sodium citrate buffer (pH = 6.0), which consisted of 0.1 mM citric acid and 0.1 mM sodium citrate in distilled water. Next, all samples were washed in PBS (pH = 7.4) twice. Afterwards, for the PZP staining, each slide was incubated with Power Block (diluted 1:10 in Aqua dest, Universal Blocking Reagent, BioGenex, San Ramon, USA) for 3 min. Blocking Solution (Reagent 1, ZytoChem Plus HRP Polymer System (Rabbit), Zytomed, Berlin, Germany) was used for each section for the GdA staining for 5 min. Incubation with the primary antibody was performed for 16 h at 4 °C for each slide. All antibodies used are listed in Table 3. Following every subsequent step, samples were washed twice in PBS. Reactivity was detected by the horseradish peroxidase-polymer system (Zytomed Systems; Goat/Rabbit, Berlin, Germany) according to the manufacturer's protocol. Afterwards, the chromogen-substrate staining was carried out using the Liquid DAB + Substrate Chromogen System (Dako Scientific, Glostrup, Denmark) for 2 min. The reaction was stopped by applying distilled water. Finally, tissue samples were counterstained with Hemalaun for 2 min and blued in tap water. Specimens were dehydrated in an ascending alcohol gradient and cover slipped

Table 1
Amount of slides used for immunohistochemical staining for each gestational week.

Gestational Age	Normal Pregnancy	Spontaneous Miscarriage	Recurrent Miscarriage
6th week	2	0	0
7th week	0	1	0
8th week	2	0	5
9th week	3	5	4
10th week	2	4	3
11th week	3	3	1
12th week	1	4	2
13th week	2	2	2
	n = 15	n = 19	n = 17

Table 2
Demographic and clinical characteristics of the study population.

Characteristics	Normal Pregnancy n = 15	Spontaneous Abortion n = 19	Recurrent Abortion n = 17	p Value (Kruskal Wallis Test)
Maternal age (years, median (min-max)))	33.0 (22.0 – 41.0)	31.5 (19.0–43.0)	34.30 (25.0–39.0)	0.813
Gestational age (weeks, median (min-max))	9.7 (6–13)	10.4 (7–13)	9.8 (8–13)	0.370
Gravidity (n, median (min-max))	3.1 (1–7)	2.2 (1–9)	2.9 (1–4)	0.077
Parity (n, median, min-max))	1.2 (0–4)	1.2(0–8)	0.7 (0–2)	0.475

Table 3
Antibodies used for immunohistochemistry and double-immunofluorescence staining.

Antibody	Isotype	Clone	Dilution	Source
PZP	Goat IgG	Polyclonal	1:100, 1:200*	Santacruz Biotechnology
GdA**	Rabbit IgG	Polyclonal	1:50	Zytomed
GdA	Mouse IgG	6F2, Monoclonal	1:50	Zytomed

PZP = Pregnancy Zone Protein, GdA = Glycodelin A.
* Dilution 1:200 for immunohistochemistry, 1:100 for double-immunofluorescence.
** Antibody used for immunohistochemistry.

with Eukitt® quick hardening mounting medium (Sigma Aldrich, St. Louis, MO, USA). All slides were analyzed using the microscope Leitz Wetzlar (Wetzlar, Germany; Type 307-148.001 514686). The intensity and distribution pattern of the immunochemical staining reaction were evaluated by two independent blinded observers using the semi-quantitative immunoreactive score (IRS). The IRS score is calculated by multiplying the percentage of positively stained cells (0, no staining, 1, <10 % of the cells; 2, 11–50 %; 3, 51–80 %, 4, >80 %) with the cells staining intensity (0, none; 1, weak; 2, moderate; 3, strong). Four representative fields were examined at the decidua of every single placenta at a 10x and 40 x objective magnification. In one case (n = 7.1 %), the evaluation of the two observers differed. This case was re-evaluated by both observers together. After the re-evaluation, both observers came to the same result. The concordance before the re-evaluation was 91.9 %.

Positive controls were carried out with kidney tissue for PZP detection and term placenta for GdA following the staining protocol as described above. Negative controls were performed by replacement of the primary antibodies by species specific isotype control antibodies (Dako). Positive and negative control staining are shown in Fig. 1

2.3. Evaluation of PZP mRNA-expression with real time (RT)PCR (TaqMan)

2.3.1. RNA extraction from placental tissue

Total RNA was extracted from placental tissue of 19 women with spontaneous miscarriage, 17 women with recurrent miscarriage and 15 healthy pregnancies. RNA extraction using 10 mg tissue of each sample was performed with RNeasy®Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.3.2. Reverse transcription

Reverse transcription (RT) was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany), according to the protocol using a master cycler® gradient (Eppendorf, Hamburg, Germany). RT conditions were 10 min at 25 °C, 2 h at 37 °C, and 5 min at 85 °C, followed by a hold step at −20 °C.

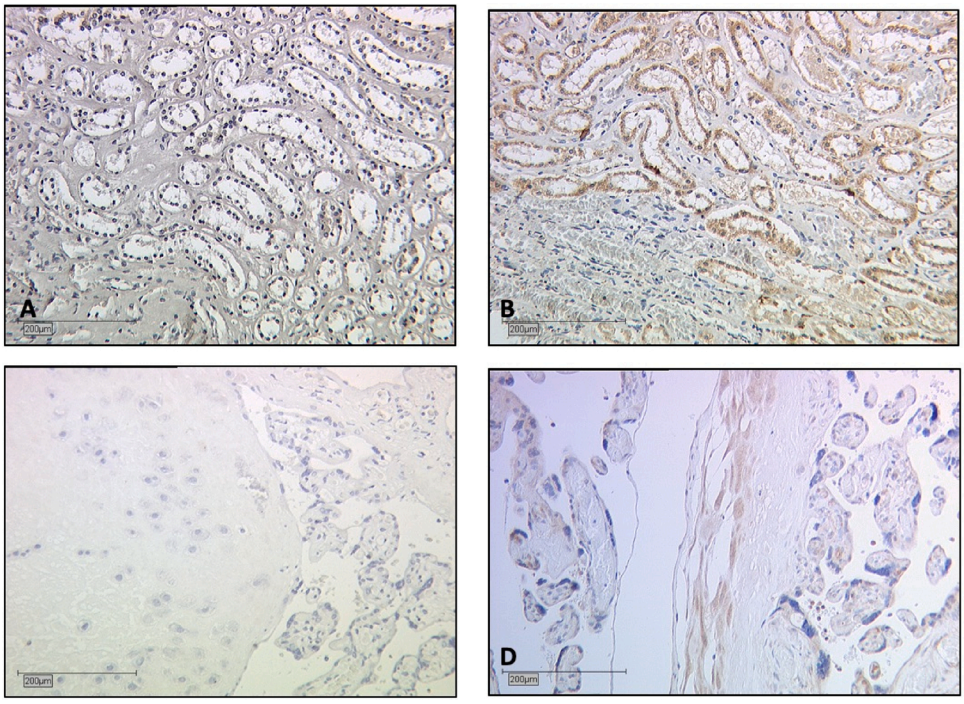

Fig. 1. Positive and negative control staining. Kidney tissue was used for negative (A) and positive (B) control staining of the PZP antibody. Term placental tissue was used for negative (C) and positive (D) control staining of the GdA antibody.

2.3.3. Real-time reverse transcription-PCR

After conversion of RNA to cDNA, RT-PCR was performed on all samples individually. Optical, 96-well microtiter reaction plates (Applied Biosystems™, Fisher Scientific Company, Waltham, MA USA) were covered with optical caps and Real-Time Reverse Transcription PCRs were accomplished in a volume of 20 µL containing 1 µL 20× TaqMan® Gene Expression Assay (Hs00161140_m1 for PZP, Applied Biosystems), 10 µL 2×TaqMan® Universal PCR Master Mix (Applied Biosystems), 8 µL H_2O (DEPC-treated DI water; Sigma-Aldrich, Taufkirchen, Germany) and 1 µL template. Thermal cycling conditions were 95 °C for 20 s, followed by 40 cycles of amplification with at 95 °C for 3 s and 60 °C for 30 s. The ABI PRISM 7500 Fast (Applied Biosystems) was used for PCR assays. Quantification was carried out by the $2^{-\Delta\Delta CT}$method using GAPDH (Hs99999905_m1) as a housekeeping gene.

2.4. Evaluation of mRNA expression of GdA by in-situ hybridization

2.4.1. Preparation of riboprobes

A 227-bp fragment of the Gd cDNA (positions +41 to +268) was cloned into the EcoR1 restriction site of pBluescript SK2 (Stratagene; Amsterdam, The Netherlands) and labeled with digoxigenin (DIG) by *in vitro* transcription using the DIG RNA labeling Kit (SP6/ T7; Roche Biochemicals, Mannheim, Germany) as described previously (Keil et al., 1999). The antisense cRNA was used for the detection of Gd mRNA, whereas the sense cRNA probe served as a negative control.

2.4.2. In situ hybridization of GdA on paraffin sections

In situ hybridization of GdA was performed as described previously (Toth et al., 2008; Jeschke et al., 2005b). Non-radioactive *in situ* hybridization was performed on paraffin sections (4 µm) that had been fixed in 4% paraformaldehyde. Sections were rehydrated and permeabilized by pepsin digestion (750 mg/mL pepsin in 0.2 M HCl, 37C, 30 min). Post fixation (paraformaldehyde 4%, 20 min, 4C) was followed by acetylation using 0.25 % acetic anhydride in triethanolamine (0.1 M, pH 8.0, 15 min). After dehydration in ethanol (70 %, 95 %, and 100 %), the sections were hybridized for 16 h (56 °C) in a solution containing 50 % formamide, 50 % solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0), 0.5 % blocking reagent (Roche Biochemicals), 210 mg/mL t-RNA derived from E. coli MRE 600 (Roche Biochemicals), and 125 ng DIG-labeled cRNA probe. After washing with decreased concentrations of SSC (sodium-sodium-chlorid: 20x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.4), sections were incubated 1 h with blocking reagent (Roche Biochemicals). Bound riboprobe was visualized by incubation with alkaline phosphatase–conjugated anti-DIG antibody (Roche Biochemicals) and subsequent substrate reaction using 5-bromo-4-chloro-3- indolyl phosphate/nitroblue-tetrazolium chloride.

2.4.3. Computerized analysis of GdA mRNA expression

Computerized analysis was performed as described previously (Toth et al., 2008; Jeschke et al., 2005b). The level of GdA mRNA expression was determined in a blinded fashion in one run with identical staff, equipment and chemicals. From each section, five digital pictures were taken at random of different places of the decidual tissue (200-fold magnification; 3CCD color camera, HV-C20 M; Hitachi, Denshi, Japan, and Axiolab; Carl Zeiss, Jena, Germany). For standardization of the measurement in each picture, the optical density of white background color was attuned to 250. For all sections, the mean optical density and the quantity of pixels having a positive reaction for GdA was assessed using KSRun software (imaging system KS400, release 3.0; Zeiss).

2.5. Double-immunofluorescence staining

Identification of PZP localization in the decidua was performed in samples of healthy controls and recurrent miscarriages. Double immunofluorescence was used to prove the localization of PZP in the glandular epithelium and decidual stroma. The used antibodies are filed in Table 3. Glycodelin A (GdA) was used as a specific marker for glandular epithelial and decidual stromal cells.

First, the specimens were deparaffinized in Roticlear (Roth, Germany) for 20 min and rinsed in ethanol 100 %, rehydrated in an alcohol gradient (70 % and 50 %). Then the slides were stationed in a pressure cooker with sodium citrate (pH 6.0) for 5 min and washed in distilled water and PBS. Next, the samples were blocked with Ultra V blocking solution (Lab Vision, Thermo Scientific Inc., Fremont, CA, USA) for 15 min to minimize non-specific background staining. Incubation with monoclonal Anti-GdA mouse IgG and polyclonal goat Anti-PZP IgG (Table 3) was performed for 16 h at 4 °C. Afterwards, the slides were washed in PBS, and the secondary antibodies were applied to the specimen. We used the Cy2-labelled rabbit-anti-mouse IgG (Dianova, Hamburg, Germany), diluted 1:100, and the Cy3-labelled donkey-anti-goat IgG (Dianova, Hamburg, Germany), diluted 1:500, for 30 min at room temperature. Finally, the samples were embedded in Vectashield® mounting medium with DAPI (Vector Laboratories; Burlingame, CA; USA). The specimens were examined with a fluorescent Axioskop photomicroscope (Zeiss, Oberkochen, Germany). Pictures were taken using a digital Axiocam camera system (Zeiss).

2.6. Statistics

Analysis of the collection and statistical data was processed with the SPSS software version 24 (SPSS; Chicago; IL; USA) and Excel version 12.3.1 (Microsoft Windows 2016; Redmond, WA; USA). The Mann-Whitney U signed-rank test was used for the comparison of two independent and Kruskal-Wallis test for more than two independent groups. *p*-values < 0.05 were considered to be statistically significant. Correlation analysis was performed using the Spearman correlation coefficient.

3. Results

3.1. Evaluation of GdA mRNA expression in decidual stroma and glandular epithelial tissue with in situ hybridization

In situ hybridization was applied to investigate the GdA mRNA expression in placental tissue of spontaneous miscarriage, recurrent miscarriage and normal first trimester pregnancies. In glandular epithelial tissue we observed a significantly lower GdA mRNA expression in spontaneous miscarriage ($p = 0.007$, Fig. 2C) and recurrent miscarriage ($p = 0.012$, Fig. 2D) compared to normal pregnancies (Fig. 2A). We identified also a significantly decreased GdA mRNA expression in decidual stroma cells of spontaneous miscarriage ($p = 0.014$, Fig. 3B) and recurrent miscarriage ($p < 0.001$, Fig. 3C) compared to the control group (Fig. 3A). Briefly, the results are summarized in the boxplots in Fig. 2E and 3 D.

3.2. GdA protein level in decidual stroma and glandular epithelial tissue

The protein level of GdA was analyzed in the decidua from healthy pregnancies, spontaneous miscarriage and recurrent miscarriage (Fig. 4A–D). The decidual stroma revealed a significantly decreased protein level in the group with spontaneous abortions (Fig. 4B) than in the group with healthy pregnancies (Fig. 4A, $p = 0.011$). There was also a significant downregulation of GdA in the decidual stroma of RM samples (Fig. 4C $p = 0.021$) compared to the control group. Briefly, the staining results are shown in the boxplot in Fig. 4D.

The GdA protein level is significantly lower in glandular epithelial cells of spontaneous abortions compared to healthy pregnancies (Fig. 5A and B, $p = 0.012$). The GdA protein level was not significantly changed in glandular epithelial cells of RM compared to control specimens (Fig. 5C, $p = 0.172$). A summary of the staining results is shown in Fig. 5D

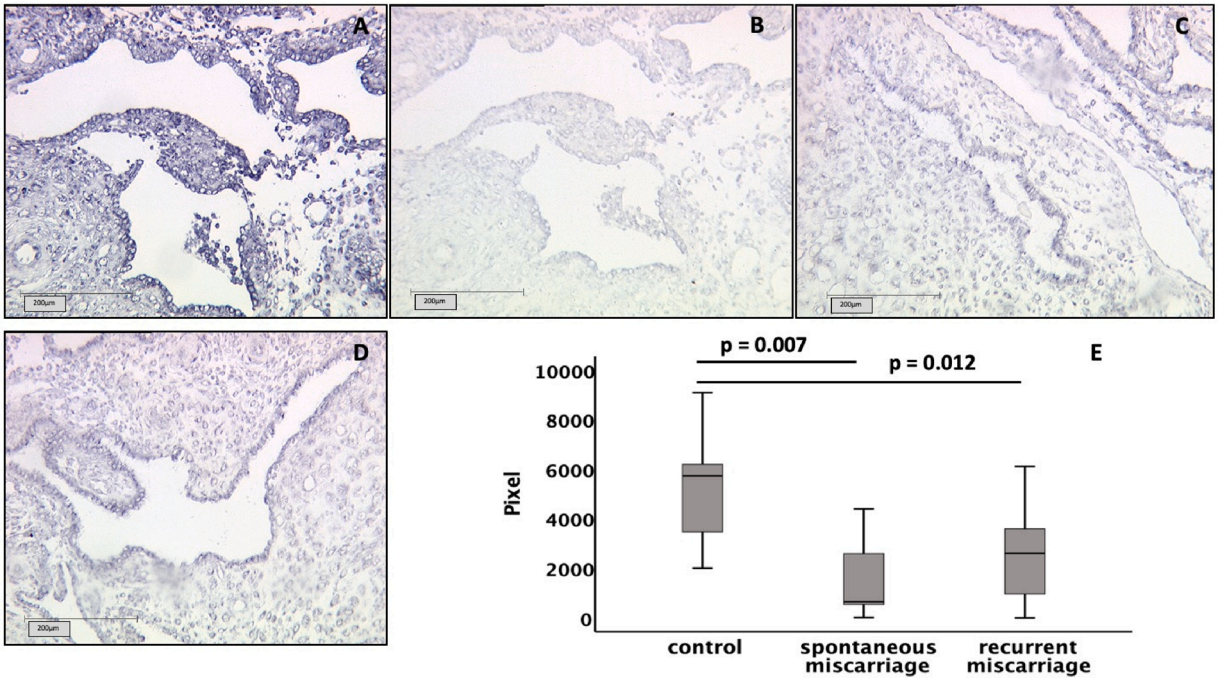

Fig. 2. In situ hybridization of GdA in glandular epithelial tissue. We identified a strong GdA mRNA expression in the glandular epithelial tissue of healthy first trimeter placenta (A, antisense). (B) shows a sense image of a healthy first trimester placenta (negative control). There was a significant lower expression of GdA mRNA in glandular epithelial tissue of spontaneous abortion ($p = 0.007$) (C, antisense) and recurrent miscarriage ($p = 0.012$) (D, antisense) compared to the control group (A, antisense). Summary of staining results of GdA (E). Scale is 200 μm.

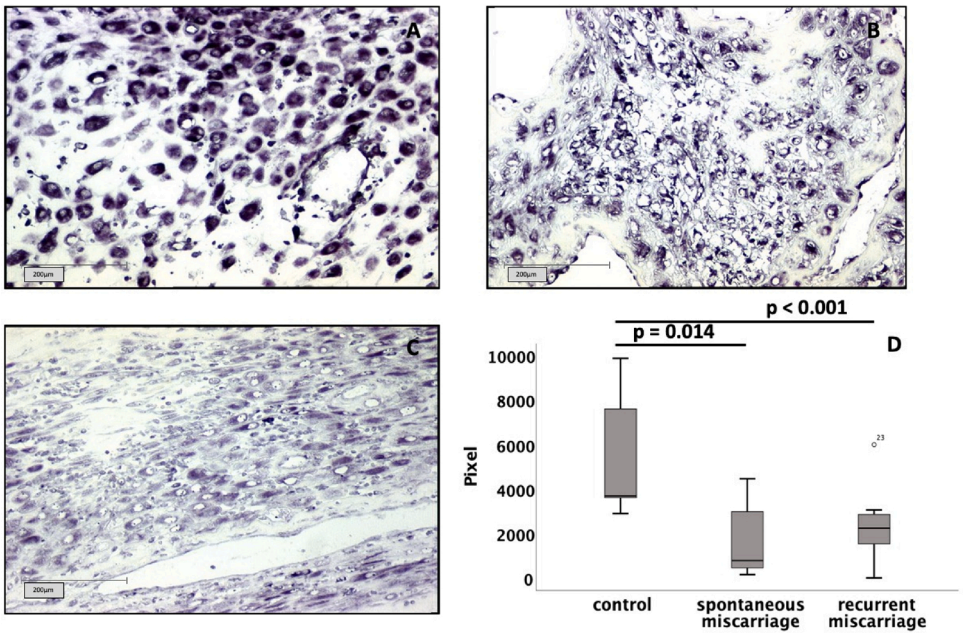

Fig. 3. In situ hybridization of GdA in decidual stomal cells. We identified a strong GdA mRNA expression in the decidual stroma of healthy pregnancies (A, antisense). There expression of GdA mRNA was significantly downregulated in the decidual stroma of spontaneous miscarriage ($p = 0.014$) (B, antisense) and recurrent miscarriage ($p < 0.001$) (C, antisense) compared to the control group (A, antisense). Summary of staining results of GdA (D). Scale is 200 μm.

3.3. Evaluation of PZP mRNA expression in placental tissue with RT-PCR (TaqMan)

The PZP mRNA-expression was examined by real-time-PCR (TaqMan) in placental tissue of healthy first-trimester pregnancies, spontaneous abortion and recurrent abortion. The mRNA expression of PZP was significantly increased 11.26-fold in placental tissue in patients with recurrent abortion ($p < 0.001$, Fig. 6) and 14.75-fold higher in patients with spontaneous abortion ($p < 0.001$, Fig. 6) compared to control participants, confirming the results of immunohistochemistry.

3.4. PZP protein level in decidual stroma and glandular epithelial tissue

PZP protein level was investigated in decidual stromal tissue of

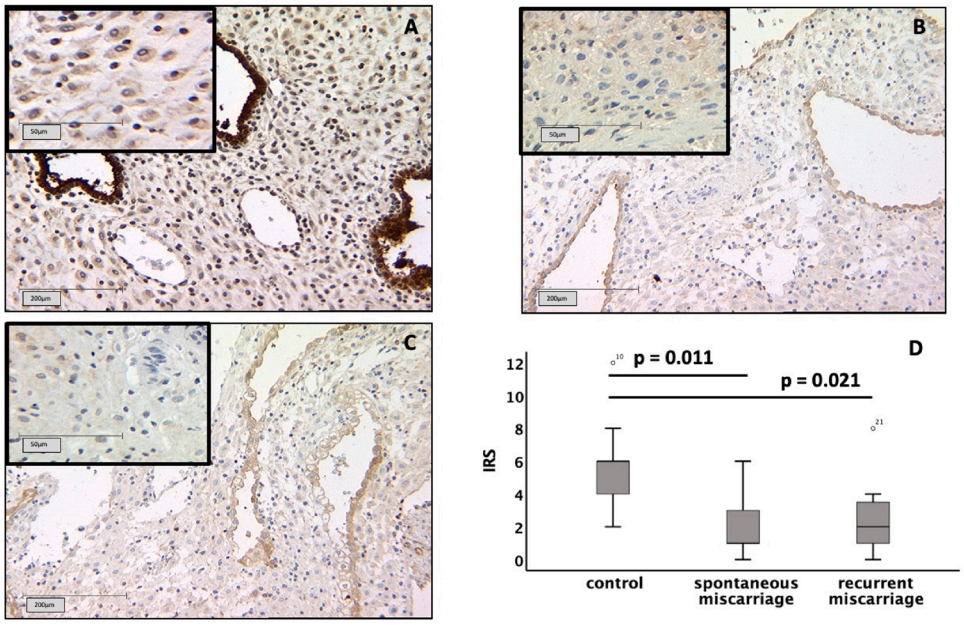

Fig. 4. Immunohistochemical staining of GdA in decidual stomal cells. Decidual GdA was significantly decreased in SM and RM samples compared to the control group (A). In SM specimens, GdA protein level is significantly lower expressed compared to healthy pregnancies ($p = 0.011$) (B). In RM samples, GdA protein level is significantly downregulated compared to the control ($p = 0.021$) (C). Summary of staining results of GdA (D). Scale is 200 μm and 50 μm.

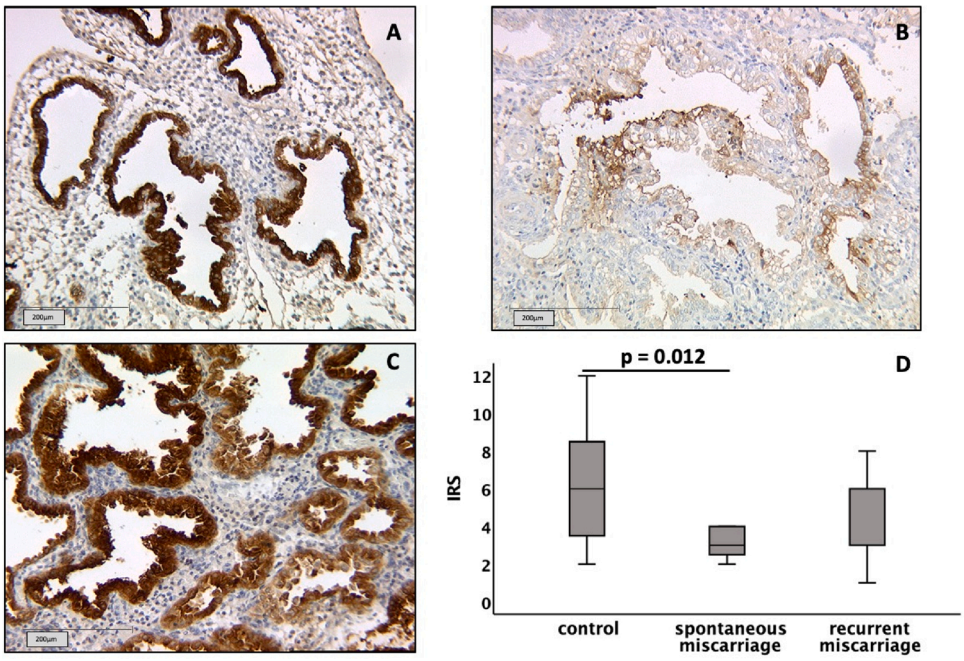

Fig. 5. Immunohistochemical staining of GdA in glandular epithelial cells. GdA protein level was significantly reduced in glandular tissue of SM (B) compared to healthy pregnancies ($p = 0.012$) (A). In RM specimen, GdA protein level showed no significant difference when compared to the control group (p = 0.172) (C). Summary of staining results of GdA (D). Scale is 200 μm.

healthy pregnancies, spontaneous miscarriage (SM) and recurrent miscarriage (RM) (Fig. 7A–D). The protein level of PZP was significantly increased in decidual stroma of SM samples ($p = 0.015$; Fig. 7B) compared to the decidua of control specimens (Fig. 7A). PZP was also significantly upregulated in the decidual stroma cells in the RM group ($p = 0.047$; Fig. 7C). A summary of the staining results is shown in Fig. 7D.

In glandular epithelial cells we observed a higher protein level of PZP in SM and RM samples compared to healthy controls, but the difference was not significant (p = 0.078 for SM, p = 0.130 for RM) (data not shown).

3.5. Correlation analyses between GdA and PZP

We investigated the correlations of GdA and PZP protein level within the different parts of the decidua. We observed a significant negative correlation of PZP and GdA in decidual stromal tissue of recurrent abortion (spearman correlation coefficient

$r = -0.975$, p = 0.005). The PZP and GdA protein level of patiens

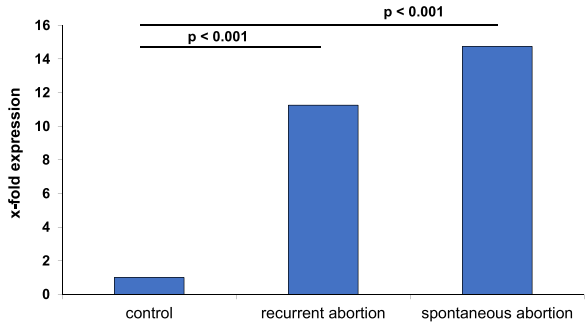

Fig. 6. Quantitative real-time polymerase chain reaction (RT-PCR) showed a significant upregulation of PZP mRNA expression in placental tissue of patients with spontaneous and recurrent abortion compared to normal controls ($p < 0.001$). This bar graph shows the mean of relative PZP expression.

suffering from recurrent miscarriage from the 6th-13th week of gestation is summarized in Fig. 8. In healthy pregnancies and spontaneous abortion we did not detect any correlations. Likewise we did not detect significant correlations in glandular epithelial cells in the control group or spontaneous abortion. In addition, GdA and PZP protein level was correlated to the week of gestation. Neither GdA nor PZP showed significant changes in the period investigated (6th-13th week of gestation; $p = 0.348$ and 0.518, respectively).

3.6. Double-immunofluorescence staining

Double-immunofluorescence staining was performed on placentas of healthy pregnancies and recurrent abortion (Fig. 9). In the decidual tissue GdA producing cells are shown in green and cells producing PZP are shown in red. Cells producing neither PZP nor GdA are stained in blue and cells producing both GdA and PZP are stained in yellow. Double-immunofluorescence staining shows that PZP and GdA are secreted by the same cells (glandular tissue of the decidua and decidual stroma). In addition, a diminished protein level of GdA in the decidua of recurrent miscarriages was found (Fig. 9D), whereas PZP protein level is

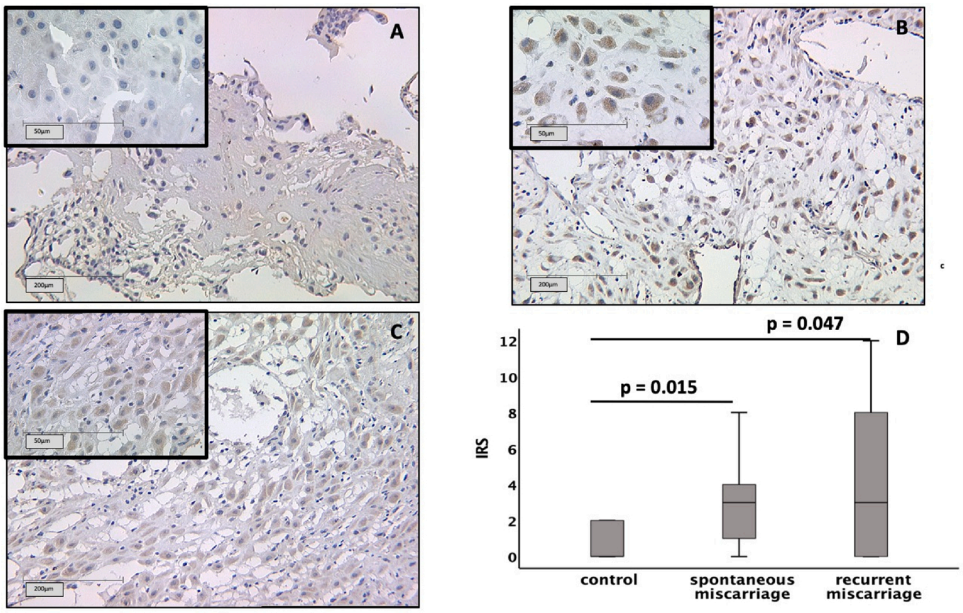

Fig. 7. Immunohistochemical staining of PZP in decidual stomal cells. Decidual PZP protein level was significantly increased in SM and RM samples compared to the control group (A). In SM specimens (B), PZP shows a significantly higher protein level in comparison to healthy pregnancies ($p = 0.015$). In RM samples (C), PZP protein level is significantly upregulated compared to the control ($p = 0.047$). Summary of staining results of PZP (D). Scale is 200 μm and 50 μm.

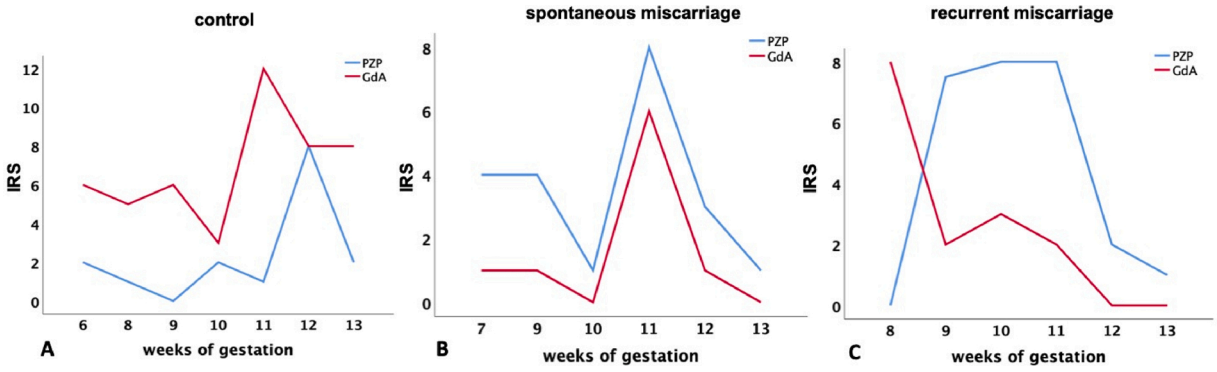

Fig. 8. Protein level of PZP and GdA in the decidual stroma from the 6th-13th week of gestation in controls, spontaneous and recurrent miscarriage. Immunohistochemical staining intensity was determined by the semiquantitative immunoreactive score (IRS). IRS values are presented in mean. In the decidua of healthy pregnancies (A) and spontaneous miscarriage (B) we did not detect a significant correlation of PZP and GdA. We observed a significant negative correlation ($r = -0.975$, $p = 0.005$) of PZP and GdA in the decidua of patients suffering from recurrent abortion (C).

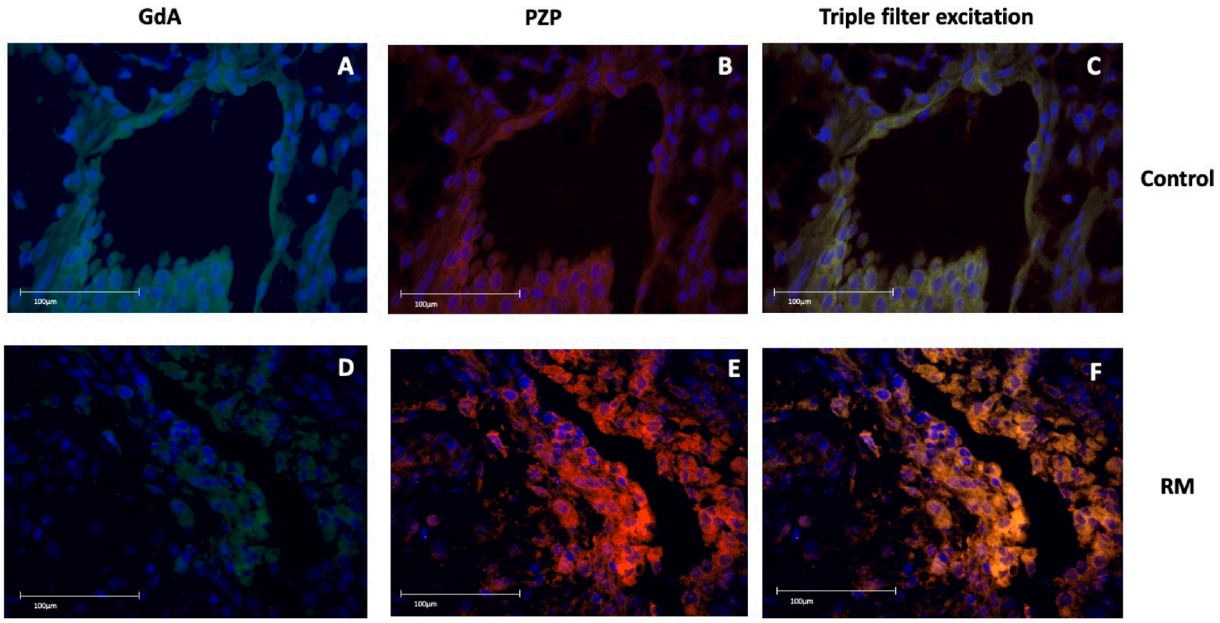

Fig. 9. Double-immunofluorescence staining of GdA and PZP in the decidua of healthy pregnancies and recurrent miscarriage. (A) GdA protein level (Cy2, green) shows a high distribution in normal decidua. (B) PZP protein level in the decidua of healthy pregnancies appears red (Cy3). (C) Triple filter excitation in the control group shows that most decidual cells are expressing both markers. (D) The decidua of recurrent miscarriage shows a decreased protein level of GdA (Cy2, green) in comparison to the GdA protein level in the control group. (E) PZP (Cy3, red) protein level is increased in the decidua of recurrent miscarriage. Triple filter excitation (F) shows decidual cells producing both PZP and GdA. Scale is 100 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

more intense in both decidual stroma and glandular epithelial cells of recurrent miscarriage specimens (Fig. 9E). Triple filter excitation showed a localization of PZP and GdA in the same decidual stroma and glandular epithelial cells, indicated by yellow staining (Fig. 9 C and F). Therefore, we demonstrated a co-localization of PZP and GdA in the decidua of controls and recurrent miscarriage.

4. Discussion

The first trimester appears to be the most vulnerable phase of a pregnancy as 80 % of miscarriages occur during this period (Chen and Creinin, 2007). Upon fertilization, a precise interaction between both maternal and fetal cells is indispensable. Especially the implementation of a tolerogenic environment at the fetal-maternal interface, which allows the maternal immune system to accept the semi-allogenos fetus, is crucial for a successful ongoing pregnancy (Lee et al., 2016).

Within this study we could demonstrate that PZP protein level is significantly upregulated in the decidual stroma of patients suffering from spontaneous and recurrent abortion compared to normal first trimester pregnancy. In glandular epithelial cells we observed a likewise trend. GdA shows a significantly lower protein level in decidual stroma in both miscarriage groups and in recurrent abortion specimens we found a negative correlation to PZP. Furthermore, the results obtained on protein level were confirmed on mRNA level. In glandular epithelial cells of spontaneous abortion we found a decreased protein level of GdA. In cases of recurrent miscarriage we observed a significant down-regulation on mRNA but not on protein level. Furthermore, using double-immunofluorescence, we demonstrated for the first time a co-localization of PZP and GdA in the decidua of healthy pregnancies and recurrent abortion.

PZP is a proteinase inhibiting glycoprotein with reported immunomodulatory properties (Tayade et al., 2005). Serum levels are low in men and non-pregnant women (normally <0.03 mg/mL), but in maternal plasma it reaches high levels (up to ~3 mg/mL) (Ekelund and Laurell, 1994), therefore indicating an important role during pregnancy. PZP is reported to inhibit activation-induced T cell proliferation (Skornicka et al., 2004). There are two subsets of differentiated $CD4^{+}$T-cells: T-helper-1 (Th-1) and Th-2 cells (Saito et al., 2010). In early gestation, Th-1 cytokines are associated with abortion, whereas Th-2 cytokines are thought to protect pregnancy (Wegmann et al., 1993). There is a shift from Th-1 to Th-2 cytokine production at the feto-maternal interphase and a proper cytokine balance seems crucial for the maintenance of a normal pregnancy (Saito et al., 2010). PZP suppresses Interleukin 2 (IL-2) production, a key Th-1-type cytokine, by inhibiting the stimulus conduction system at the T cell surface (Saito et al., 1990). Thereby, PZP could be important in supporting the Th-1 to Th-2 shift in pregnancy. Furthermore, when IL-2 production is inhibited, also activated NK cells (Grimm et al., 1983) and macrophage killer activity are suppressed (Malkovsky et al., 1987). Additionally, it was reported that PZP binds transforming growth factors- β (TGF-) 1 and 2 *in vitro* (Philip et al., 1994). TGF- β are important regulatory proteins in embryogenesis and modulation of the immune system (Sanjabi et al., 2017). It was demonstrated in different mouse models that global inactivation of genes encoding the TGF-β type 1 receptors resulted in embryonic lethality (Monsivais et al., 2017). Philipp et al. suggested, that PZP could function as a carrier protein for TGF- β (Philip et al., 1994). In addition, PZP is a carrier protein and modulator of GdA (Skornicka et al., 2004).

GdA, as one of the most abundant glycoproteins in the decidua, has an effect on different immune cells (Miller et al., 1998; Lee et al., 2016, 2011b). Like PZP, GdA is reported to inhibit the secretion of the Th-1 cytokine IL-2 (Pockley and Bolton, 1989). When PZP non-covalently carries GdA, they synergistically suppress T cell proliferation and IL-2 production in a greater amount than the individual protein (Skornicka et al., 2004). Interestingly, they do not have an inhibitory effect on the Th-2 cytokine IL-4 (Skornicka et al., 2004). Thereby, the interaction of PZP and GdA promotes the Th-1 to Th-2 shift at the feto-maternal interphase.

We assume that the upregulation of the immunosuppressive PZP in the decidua is a compensatory effect due to low hCG levels in miscarriage. hCG is essential for the maintenance of a pregnancy and plays an important role in several key events like embryo implantation,

trophoblast invasion, modulation of maternal immune response towards the fetus etc. (Gridelet et al., 2020) In daily clinical routine, hCG is used as a diagnostic tool especially in early pregnancies with a risk of abortion as low hCG serum levels are indicative for patients suffering from miscarriage. Recently, we were able to demonstrate that the protein level of hCG is negatively correlated to PZP in the syncytiotrophoblast (Lob et al., 2020). Furthermore, our *in vitro* studies showed that after addition of hCG to the chorioncarcinoma cell line JEG-3 the cells produced less PZP in a dose-dependent manner (Lob et al., 2020). On the other hand, evidence suggest that there is a regulative feedback loop between hCG and GdA. After stimulation of the chorioncarcinoma cell lines BeWo and JEG-3 with GdA there is a noteable upregulation of hCG (Bergemann et al., 2003). In addition, when human endometrial cancer cells are incubated with hCG the secretion of GdA is increased (Toth et al., 2008).

The negative correlation of PZP and GdA protein level in cases of recurrent abortion in the decidual stroma is a key finding of our study as we detected an altered expression ratio compared to healthy pregnancies. Different expression ratios of interacting immunological molecules in disturbed pregnancy is a known phenomenon. Freis et al. showed in a prospective study that the ratio of GCSF/ IL-1ra and TGF- beta /MIP1a is significantly increased in blood samples from patients suffering from miscarriage (Freis et al., 2018). Especially, when MIP1a and IL-1ra were put in relation to hCG after four weeks of gestation they were more predictive for pregnancy outcome (Freis et al., 2018). In preeclampsia, circulating maternal serum levels of soluble fms-like tyrosine kinase 1 (sFlt-1) are increased and placental growth factor (PlGF) levels are decreased (Levine et al., 2006; Maynard et al., 2003). The ratio of sFlt-1 to PlGF is elevated and a ratio of 38 or lower can predict the absence of preeclampsia for one week, which is used in clinical practice for women in whom the disorder is suspected (Zeisler et al., 2016). As the significant negative correlation of PZP and GdA is restricted to patients suffering from recurrent abortion, we hypothesize that a disturbed PZP-GdA interaction might be accountable for cases of unexplained recurrent miscarriage.

In our present study we demonstrated a significant upregulation of PZP in the decidual stroma and a likewise trend in glandular epithelial cells whereas our former work showed a decreased protein level of PZP in the trophoblast (Lob et al., 2020). This variying secretion pattern in the placenta is in line with a study about PZP serum levels (Ekelund and Laurell, 1994). Although PZP is associated with high serum levels in pregnancy, protein levels in maternal plasma are extremely variable between individuals and some women have low PZP levels throughout inconspicuous pregnancy (Ekelund and Laurell, 1994). Therefore, we suggest that PZP shows a variable placental secretion pattern due to different functions of PZP in the maternal and fetal part. Skornicka et al. suggested that the anti-proteinase activity of PZP and its role as a carrier protein are independent from each other (Skornicka et al., 2004). Another explanation for the variable secretion of PZP in the placenta might be that PZP is a target protein for alternative splicing (Cheong et al., 2016). Two major splice variations for PZP are known with variations especially in exons 1a, 1c and 3b (Gerhard et al., 2004). In addition, we demonstrated that PZP is associated to Elongation factor Tu GTP binding domain containing 2 (EFTUD2), a protein regulating alternative splicing, in spontaneous miscarriage and hydatidiform mole pregnancy. This could indicate that maternal and fetal cells synthesize different isoforms of PZP in early pregnancy.

The expression of GdA in healthy first trimester and abortive placenta has been characterized before (Toth et al., 2008). Toth et al. demonstrated a significantly decreased synthesis of GdA in decidual tissue in abortive patients in comparison to healthy first trimester pregnancies. However, the authors did not clearly define if the miscarriage group consisted of patients with only one or more pregnancy losses. In the present study we were able to confirm a reduced protein and mRNA level of GdA in the decidua of spontaneous abortion specimen. In addition, we could show that GdA is significantly downregulated on protein and mRNA level in cases of recurrent abortion in the decidual stroma. Interestingly, we did not observe a significant downregulation of GdA in glandular epithelial cells on protein but on mRNA level. Glandular epithelial cells mainly produce GdA (Toth et al., 2008) reaching highest levels from 6^{th} to 12^{th} week of gestation (Lee et al., 2011a; Seppala et al., 2002). This leads us to suggest that in our cases investigated we did not find a significant difference in GdA protein expression due to accumulation of GdA based on incorrect protein degradation in recurrent miscarriage. Another explanation for our observation might be the semiquantitative immunoreactivity score (IRS) we used for the analysis of GdA. The analysis via IRS might have induced a higher degree in uncertainty and might have led to a higher protein level than expected.

We investigated the expression of immunosuppressive proteins in decidual tissue of abortive patients that might have been necrotic for a few days and therefore this could be a limitation of our study. However, our results clearly show in both miscarriage groups that the expression of PZP and GdA is significantly changed independently of the week of gestation and the time of diagnosis and operation. Therefore, we interpret our results as a consequence of disturbed immune responses at the feto-maternal interphase rather immune activation upon apoptotic processes. Another limitation of our study might be that the gestational age of our study population is defined in weeks of gestation and not in days. It is important to distinguish whether a pregnancy lasted for example 6 weeks or a pregnant woman is in 6^{th} week of gestation. Furthermore, the gestational age in days could give a more precise insight into gestational age dependence of protein levels in early pregnancy. However, in literature, GdA and PZP expression in pregnancy has often been described in weeks of gestation, which makes our results comparable to already published data (Toth et al., 2008; Cater et al., 2019; Lob et al., 2020; Jeschke et al., 2005b; Freis et al., 2018). Moreover, in the present study GdA and PZP protein level was correlated to the week of gestation (6^{th}-13^{th} week of gestation) and neither GdA nor PZP showed significant changes.

In conclusion, a balanced expression of GdA and its carrier protein PZP in the decidua seems crucial for a successful ongoing pregnancy. According to our data, these immunosuppressive proteins are co-localized in the decidua and show a negative correlation only in patients suffering from recurrent abortion. Therefore, further studies need to evaluate PZP and GdA serum levels as a biomarker for unexplained recurrent miscarriage.

Declaration of Competing Interest

The authors report no declarations of interest.

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