TGF superfamily and *MMP$_2$, MMP$_9$, TIMP$_1$* genes expression in the endometrium of women with impaired reproduction

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Abstract: During the putative "implantation window", a period of maximal endometrial receptivity that spans 7-9 days after ovulation, a series of changes on the structural and molecular level occur that render the endometrium susceptible to implantation for the human embryo. Many members of the TGFβ$_s$ are expressed by human endometrium at different stages of menstrual cycle. Also studies regarding the MMP$_2$ gene expression and activity of MMP$_2$ in the implantation window have shown a higher expression and activity of MMP2 in women with impaired fertility. We have examined by RT-PCR the expression of TGFβ$_2$ and MMP$_2$, MMP$_9$ and TIMP$_1$ in 28 patients with idiopathic infertility, 16 patients with unexplained recurrent miscarriage and 16 control women were enrolled in this study. Seven to nine days after ovulation endometrial biopsy by Pipelle or hysteroscopy was performed to assess the expression of TGFβ$_2$, MMP$_2$, MMP$_9$ and TIMP$_1$. We found that in endometria from women with idiopathic infertility TGFβ$_2$ expression was 2.8 fold higher than in endometria from control group and 2.1 fold higher in endometrial samples from women with unexplained recurrent miscarriage compared to the control group. The MMP$_2$, MMP$_9$ and TIMP$_1$ expression in endometrial samples revealed no significant differences between the study groups and control group. There was a statistically significant negative correlation between TGFβ$_2$ and MMP$_9$ expression in endometria from women in control group. The present investigations suggest that dysregulated TGFβ$_2$, MMP$_2$, MMP$_9$ and TIMP$_1$ expression are associated with infertility and early pregnancy loss. However the exact mechanism of how overexpression of endometrial TGFβ$_s$ and MMP$_s$ interferes with implantation may be more complex.

Key words: Imaired reproduction - Endometrium - TGFβ$_2$ - MMP$_2$ - MMP$_9$ - TIMP$_1$

Introduction

During the putative "implantation window", a period of maximal endometrial receptivity that spans 7-9 days after ovulation, a series of changes on the structural and molecular level occur that render the endometrium susceptible to implantation for the human embryo [1].

There are number of cytokines, adhesion molecules and receptors that possibly might play a role in increasing the receptivity of the endometrium, however the exact "composition" needed for implantation to occur is not known [1-3].

The TGFβ superfamily comprises at least 42 distinct mammalian dimeric proteins, that share a similar structure [4].

These are divided into two subfamilies, the TGFβ (activin) nodal subfamily and second subfamily including bone morphogenetic protein, müllerian inhibitory substance, and growth and differentiation factor [2].

The TGFβ [1-3] are each synthesized as a large precursor molecule from which a propeptid must be cleaved. After secretion, most TGFβ is stored bound to extracellular matrix components as a complex of

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TGFβ, propeptide and a peptide called latent TGFβ - binding protein. Release of active TGFβ from the complex is a critical regulatory step [5]. Many members of the TGFβ$_s$ are expressed by human endometrium at different stages of menstrual cycle. The three TGFβ isoforms have been localized to both epithelial and stromal cells [6] with TGFβ$_2$ staining being more intense in the stroma while TGFβ - 1 and - 3 is of equal staining intensity in stromal and epithelial cells [7].

Cyclical changes in expression level are not evident for TGFβ$_1$ and TGFβ$_2$, only TGFβ$_3$ varies across the cycle, with higher expression level during the late secretory phase [2].

The production and secretion of TGFβ by epithelial cells in the secretory phase suggests a role in the preparation of the endometrium for implantation. TGFβ$_s$ may play role in human implantation via their stimulation of fibronectin or vascular endothelial growth factor production [8] or by promotion of adhesion of trophoblast cells to the [9].

More recently it has been shown, that both TGFβ1 and activin A enhance the production of the pro-implantatory cytokine, leukemia inhibitory factor in endometrial cells [10] and have profound effects on ECM production and degradation of enzymes [11]. In addition retroviral overexpression of TGFβ antagonist in the mouse uterus in the preimplantation phase reduces the number of implantation sites [12]. Also its abnormally elevated expression was observed in human endometrium during the receptive phase in women suffering from infertility [12].

In the recent years much attention was given to extracellular matrix. It is known, that improper turnover of endometrial extracellular matrix is associated with disturbances of implantation and functional bleeding. Studies regarding the *MMP$_2$* gene expression and activity of *MMP$_2$* in the implantation window have shown a higher expression and activity of MMP$_2$ in women with impaired fertility [13,14].

Our current study is based on working model hypothesis that disturbances in endometrial extracellular matrix and dysregulated expression of TGFβ$_s$ genes during the implantation window might be a cause of impaired reproduction. To further test this hypothesis, we examined the expression of TGFβ$_2$ and *MMP$_2$*, *MMP$_9$* and TIMP$_1$ in normal human endometria and in the endometria of women with idiopathic infertility and unexplained recurrent fetal loss.

Materials and methods

Patients. Endometria have been harvested from 60 women in reproductive age. Twenty-eight patients with idiopathic infertility, sixteen patients with unexplained recurrent miscarriage and sixteen control women were enrolled in this study. Infertile women had undergone a standard panel of tests that included hysterosalpingogography, hormonal profiles and semen analysis. Women with recurrent miscarriage were evaluated for the presence of antiphos-

Table 1. Sequences of the primers that have been used in the evaluation of expression.

Gene	Tm	Length (nn)	Sequence
GAPDH			
FF	60.02	20	ACAGTCAGCCGCATCTTCTT
REV	59.97	20	ACGACCAAATCCGTTGACTC
MMP2			
FF	59.84	20	AAGTATGGCTTCTGCCCTGA
REV	59.97	20	ATTTGTTGCCCAGGAAAGTG
MMP9			
FF	59.91	20	TCTTCCCTGGAGACCTGAGA
REV	60.32	20	ATTTCGACTCTCCACGCATC
TIMP1			
FF	60.11	20	TGACATCCGGTTCGTCTACA
REV	59.99	20	TGCAGTTTTCCAGCAATGAG
TGF2 BETA			
FF	60.1	20	CGCCAAGGAGGTTTACAAAA
REV	59.98	20	CTCCATTGCTGAGACGTCAA

pholipid antibodies, karyotype anomalies and uterine malformation. The control group comprised of fertile women (at least one live birth, no miscarriages, with regular menses) who were admitted for non-endometrial associated diseases (*e.g.* benign ovarian cysts or myoma). Exclusion criteria included patients receiving hormonal replacement therapy, patients using intrauterine contraceptive devices or oral contraception within last six months and patients with any major diseases (requiring use of any drugs).

The mean age of infertile women was 29,9 years (±2.6), 31.6 (±4.6) years of age for women with recurrent miscarriages and 41.8 (±5.9) years in control group. Mean number of miscarriages in the recurrent miscarriage group was 2.7 (range: 2-4) and the mean duration of infertility was 5.6 years (range: 1-17 years).

Tissue samples. All women underwent serial ultrasound assessments to track follicular growth and the formation of corpus luteum. During the same cycle, 7-9 days after ovulation (the putative implantation window) endometrial biopsy by Pipelle or hysteroscopy was performed to assess the expression of TGFβ$_2$, *MMP$_2$*, *MMP$_9$* and *TIMP$_1$*. Part of the material was assigned to histological assessment according to Noyes and Hertig criteria. The rest of the material was transferred to RNAlater solution (Qiagen, Australia) and frozen in -80°C till the isolation of total RNA.

Isolation of the RNA from biopsies was conducted according to manufacturers instructions, with the Rnaesy Protect Mini Kti (Qiagen, Australia).

The evaluation of expression of studied genes was done with the RT-qPCR in two phases:

1. Reverse transcription with QuantiTect Reverse Transcription Kit (Qiagen, Australia). From each sample, a solution containing no more than 1 μg of isolated RNA had undergone the reverse transcription reaction according to manufacturers instructions. The procedure included the elimination of potential DNA presence. The end result of this phase was complementary DNA resulting from conversion of mRNA.
2. The real-time PCR: 2 μl of matrix, containing no more than 200 ng of cDNA from the first phase was used to conduct quantitative analysis of the expression of studied genes with the use of DyNAmo HS SYBR Green qPCR Kit (Finnzymes,
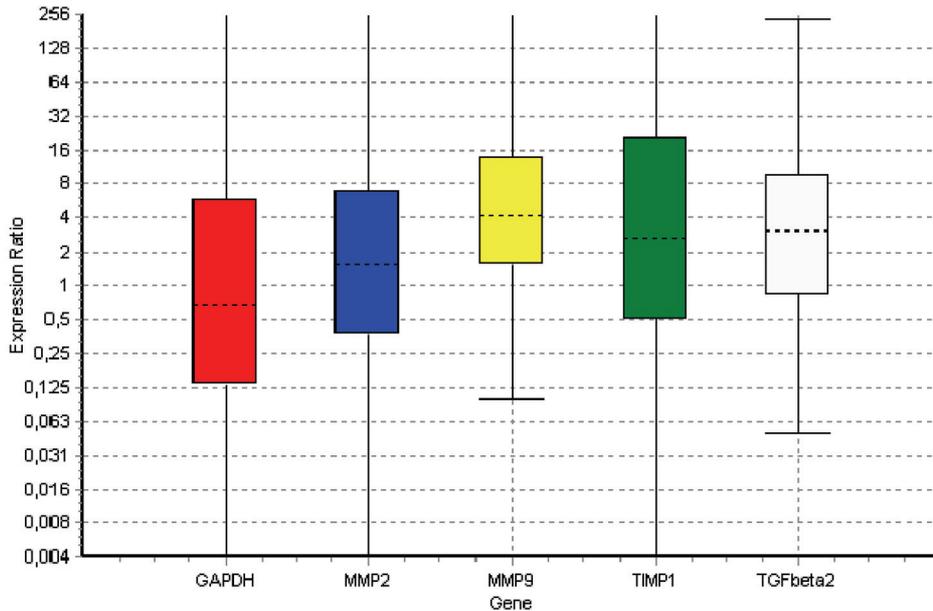
Fig. 1. The relative TGFβ₂ expression in idiopathic infertility group. Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.
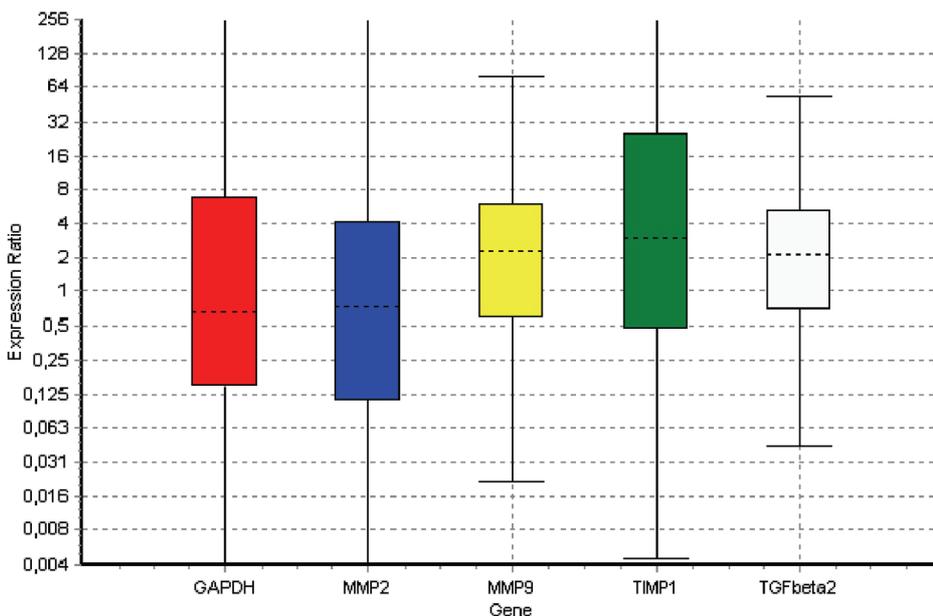
Fig. 2. The relative TGFβ₂ expression in unexplained miscarriage group. Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

Finland). The reaction profile and the mix content was done according to manufacturers instructions. Primers for the qPCR reaction were designed with Primer3 software.

The specificity of oligonucleotides was confirmed using NCBI BLAST. To assess the efficacy of the qPCR reaction 6 subsequent 10 times dilutions of DNA was used, that was a specific reaction from the PCR of each studied transcript.

The qPCR was done on Rotor-Gene3000 thermocycler (CorbettResearch, Australia). During the qPCR, after each cycle the increase in amount of generated product was assessed, and at the end of a reaction the thermocycler program assigned the fluorescence level (Ct) at which the pace of increase of generated product was logarithmic.

To assess the expression levels and statistical analysis following programs were used:
- REST2005 (http://www.gene-quantification.de/download.html - rest-2005)
- MedCalc (http://www.medcalc.be/) [15].

Results

TGFβ₂ expression in human endometrium during the implantation window

The RT-PCR used to analyse TGFβ₂ expression in endometrial RNA samples revealed that in endometria from women with idiopathic infertility TGFβ₂ expression was 2.8 fold higher than in endometria from control group (Fig. 1).

Similarly TGFβ₂ expression was 2.1 fold higher in endometrial samples from women with unexplained recurrent miscarriage compared to the control group (Fig. 2). Overexpression of TGFβ₂ was found in 59% of endometria from women with infertility and in 64% of endometria from women with recurrent pregnancy

loss compared to the results achieved in control group. Unfortunately there was no statistically significant differences.

MMP$_2$, MMP$_9$, TIMP$_1$ expression in human endometrium during the implantation window

The RT-PCR studies of MMP$_2$, MMP$_9$ and TIMP$_1$ expression in endometrial samples revealed no significant differences in their expression between the study groups and control group (Fig. 1 and 2).

However MMP$_2$, MMP$_9$ and TIMP$_1$ expression in endometria harvested from women suffering from idiopathic infertility compared to endometria from women without reproductive disorders was 1.3, 5.2 and 3.9 fold higher, respectively. Higher MMP$_2$, MMP$_9$, and TIMP$_1$ expression than in control group was observed in endometria harvested from women with infertility (58%, 75% and 92% higher, respectively). Also the expression of MMP$_9$ and TIMP$_1$ in endometrial samples collected from women with unexplained recurrent miscarriage compared to endometrial samples in control group was 2.4 and 3.8 higher, respectively. We found overexpression of MMP$_9$ and TIMP$_1$ in 63% and 88%, respectively, in endometrial samples from women with recurrent pregnancy loss compared to control group.

Although MMP$_2$ expression in endometrial samples didn't differ between unexplained miscarriage and control group, there was a higher MMP$_2$ expression in 43% of endometrial samples from unexplained miscarriage group.

The correlation between TGFβ$_2$, MMP2, MMP9 and TIMP1 expression

There was a statistically significant negative correlation between TGFβ$_2$ and MMP$_9$ expression in endometria from women in control group (Fig. 3), which means, that a higher TGFβ$_2$ expression was accompanied by lower MMP$_9$ expression. In both studied groups we have not been able to show any correlation between expression of TGFβ$_2$ and MMP$_2$, nor between MMP$_9$ and TIMP$_1$.

Discussion

While many techniques have been developed that are used to diagnose different causes of impaired fertility still in the etiology of about 50% of recurrent miscarriages and 10% of infertility remains unexplained. Many authors tried to explain the etiologies of idiopathic infertility and recurrent miscarriage by endometrial disturbances both at the tissue and also molecular level [16-18].

It is becoming increasingly clear, that transforming growth factor beta (TGFβ is involved in cellular pro-
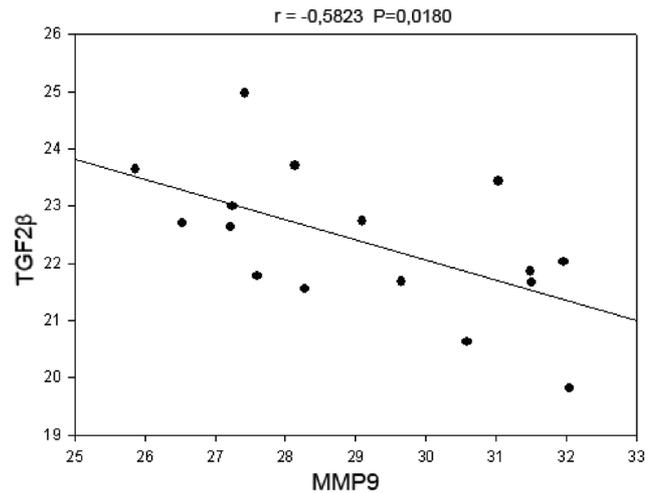
Fig. 3. Correlation between MMP$_9$ and TGFβ$_2$ expression in control group.

liferation and differentiation, extracellular matrix modification, tissue remodeling, angiogenesis and decidualization of uterine endometrium during the implantation [19].

All three isoforms of TGFβ have been localized in rodent and human endometrial epithelium and stroma. It has been shown, that TGFβ$_s$ modulate maternal immunotolerance during implantation and regulate *in vitro* several molecules related to implantation as VEGF, IGFBP$_1$, LIF and MMP$_9$ [2,20]. A role for TGFβ in the modulation of endometrial receptivity in monkey has also been reported [21].

Our RT-PCR investigation showed trend for higher expression of TGFβ$_2$ in endometrial samples from women with idiopathic infertility and unexplained recurrent miscarriages compared to endometria from control group, but we didn't found any statistically significant differences. Kothapalli *et al.* [22] identified ebaf/lefty - a novel human gene of the TGFβ superfamily during a search for genes highly expressed during the non-receptive phase in human endometrium. Lefty proteins were present in low amounts during the receptive phase in the endometria and sera of normal fertile women, but were abundant in the endometria of a number of infertile patients subjects during the receptive phase of the cycle [23]. The authors speculated, that successful implantation occurs in the presence of a low level of ebaf protein in human endometrium, and that a high level of ebaf would be associated with infertility. In their study in over 50% of endometria from infertile patients, a mRNA that hybridized with full-length ebaf cDNA was up-regulated during the endometrial receptivity period. The infertility in these women was associated with endometriosis, polycystic ovary syndrome, bilateral tube occlusion, anovulatory cycle, luteal phase defect,

premature ovarian failure and habitual abortion. In some women, the underlying reason of infertility remained unknown.

In our study only patients with unexplained infertility were included. In 59% of their endometria we have observed higher $TGF\beta_2$ expression than in control group. Also in 64% of endometria from women with unexplained recurrent miscarriages we have noticed a higher $TGF\beta_2$ expression than in endometria from women in control group.

It has been known, that nearly 50% of early pregnancy losses occur when implantation occurs after postovulatory day 10, when the ebaf protein is relatively abundant in endometrium [24]. These data and our observation can suggest, that dysregulated $TGF\beta_s$ gene expression might be a contributing factor leading to the impairment of implantation.

Lately much attention was paid to the role of MMPs for endometrial receptivity and the implantation process. Expression of genes taking part in the production and degradation of endometrial extracellular matrix in women with unexplained infertility and recurrent miscarriages was the subject of studies from Jokimaa *et al*. [14]. These author have observed higher mRNA level for collagen type 1, MMP_2 and catepsin H in studied patients compared to control group. Compared to normal endometria, from control group, in our own RT-PCR study we have observed higher MMP_2 expression in 58% endometria from women with idiopathic infertility and only in 43% endometria from women with unexplained recurrent miscarriages. These data can lead to the conclusion, that the extracellular matrix participate in the endometrial receptivity and that the imbalances in the extracellular matrix might offer explanation for same case of unexplained infertility and recurrent miscarriages.

Our results regarding MMP_9 and TIMP1 mRNA expression in endometrium during the mid-luteal phase in patients with idiopathic infertility differ from results in Wu and Zhon study [25]. While these authors have notice significantly lower expression of MMP_9 and $TIMP_1$ mRNA in endometrium from women with idiopathic infertility, we have observed a trend for higher expression of these molecules, however without statistical significance. We have also found in endometria from women with unexplained recurrent miscarriage a higher expression of endometrial MMP_9 and $TIMP_1$ (63% and 88%, respectively) compared to control group. Bany *et al*. [26] have shown significantly higher content of MMP_9 mRNA in stimulated decidualization compared with non-stimulated one in the horn of mouse uterus. In our previous study [27] we have observed a lower MMP_9 and $TIMP_1$ concentration in uterine fluid collected from women with idiopathic infertility and women with unexplained cause for recurrent miscarriage than in controls. These discordance can be explained by hypothetic disturbances between modules expression and their secretion and transport.

Fact, that decasualization also occurs in the presence of inhibitors, points to a conclusion, that MMP_s are not indispensable able for endometrial transformation. It has already been mentioned above, that $TGF\beta_1$ has profound effect on ECM production and degradation of enzymes [11]. To support this hypothesis we have found statistically negative correlation between the $TGF\beta_2$ and MMP_9 expression but only in endometria from control group.

Also Tabibzadeh *et al*. [23] have found, that ebaf up-regulates the expression of matrix metalloproteinase -3 and -7, therefore they my be involved in tissue shedding.

The present investigations suggest that dysregulated $TGF\beta_2$, MMP_2, MMP_9 and $TIMP_1$ expression are associated with infertility and early pregnancy loss. However the exact mechanism of how overexpression of endometrial $TGF\beta_s$ and MMPs interferes with implantation may be more complex.

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