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GENERAL GYNECOLOGY

Seminal plasma (SP) induces a rapid transforming growth factor beta 1 (TGFβ1)—independent up-regulation of epithelial–mesenchymal transdifferentiation (EMT) and myofibroblastic metaplasia-markers in endometriotic (EM) and endometrial cells

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Abstract

Purpose To study if short-term exposure (2 h and 6 h) of endometrial/endometriotic tissues and cells to 10% seminal plasma (SP) can induce EMT/metaplasia.

Methods Basic research experimental study was carried out in a University hospital-based fertility center. Semen samples, peritoneal fluid (PF) from endometriosis patients, endometrial biopsy from premenopausal women, immortalized endometriotic epithelial cell line (12Z), and immortalized endometrial stromal cell line (St-T1b) were studied. Rapid stain identification test (RSID), TGFβ1 immunofluorescence of washed sperms, TGFβ1-ELISA of SP and PF, in vitro study (2 h and 6 h incubation) and real-time PCR of endometrial tissue and cell lines to analyze gene expression of EMT/metaplasia markers and mediators were done.

Results SP is still detectable in washed semen. TGFβ1 was expressed on the plasma membrane of the sperms and was significantly more concentrated in SP (88.17 ng/ml) than PF. 10% SP induced an up-regulation of alpha smooth muscle actin expression in endometrial tissue ($p = 0.008$) and in 12Z cells ($p = 0.05$), mostly TGFβ1-independent. TWIST expression was persistently significantly down-regulated while Snail1 and 2 were up-regulated, though insignificant.

Conclusion Our results provide novel evidence to support that even in semen washed twice, SP is still detectable. The changes in EMT/metaplasia markers and mediators give a new insight into a possible effect of SP on the pathogenesis of endometriosis.

Keywords Seminal plasma · Endometriosis · EMT · Metaplasia · TGFβ1

Introduction

Endometriosis is a puzzling disease, affecting up to 10–15% of women in their reproductive age [1, 2]. Notably, the prevalence of endometriosis is higher, reaching up to 40–50% in women suffering from chronic lower abdominal pain [3], causing a severe compromise in both physical and social lives of the patients.

The pathogenesis of the disease remains, however, unclear and at present several theories are being discussed. One of them is the metaplasia theory, stating that the coelomic epithelium of the peritoneal cavity undergoes a metaplasia and develops into the two basic components of endometriosis: endometrial glands and stroma [4, 5]. The loss of the epithelial markers (e.g., E-cadherin) together with the acquisition of mesenchymal markers (e.g., N-cadherin and vimentin) hallmarks an epithelial–mesenchymal transdifferentiation (EMT) in endometriosis [6–8]. Although EMT is a physiological process during embryogenesis [9], it occurs also in pathological conditions; e.g., during fibrosis (e.g.,

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peritoneal dialysis-induced fibrosis) [4, 5] and tumor metastasis [10].

In fibrosis, myofibroblasts predominate. They are transformed fibroblasts which develop secondarily to tissue injury [11–13]. Myofibroblasts produce collagen I and fibronectin replacing lost extracellular matrix (ECM). Furthermore, they acquire cytoplasmic stress fibers to migrate to the injured tissue. Alpha smooth muscle actin (ASMA) is an integral part of these stress fibers [9, 14]. Those ASMA-expressing myofibroblasts are observed abundantly in endometriotic lesions of different locations. They point to a chronic tissue injury in EM lesions [15–21].

This myofibroblastic metaplasia in EM is largely mediated by Transforming Growth Factor beta (TGFβ1) [12]. TGFβ1 is secreted abundantly in the endometrium, specifically in endometrial glands, stroma, and macrophages invading the endometrium [3, 22]. Its expression in the endometrium is menstrual cycle phase-dependent, being higher around the time of menstruation to contribute to tissue repair following endometrial shedding [3].

However, in endometriosis patients, the concentration of TGFβ1 is consistently high in the peritoneal fluid (PF) [22–24]. This may be caused by the abundance of platelets in the endometriotic lesions, secondary to repetitive intralesional bleeding [25, 26] and/or by the mesothelial cells, secreting excess TGFβ1 [22]. TGFβ1 is likewise highly concentrated in the follicular fluid of ovaries affected by endometriotic cysts [27]. The same is observed in the uterine lavage of those patients [28]. The above findings emphasize the abundance of TGFβ1 in endometriosis patients.

Interestingly, TGFβ1 is a growth factor present in seminal plasma (SP), and notably its concentration significantly exceeds all other biological fluids [29, 30]. TGFβ1 in SP plays a pivotal role in immuno-modulation. On one hand, it safeguards a maternal immune tolerance to the paternal antigens contained in the embryo. On the other hand, it acts as a pro-inflammatory factor, recruiting immune cells of the vaginal epithelium to contribute to a protection against infections with sexually transmitted diseases [29]. SP also acts as a vehicle for spermatozoa. The latter expresses TGFβ1 on their cytoplasmic membrane of the post-acrosomal area of their head, on the neck, and on the middle piece of their tail too. This TGFβ1 expression is enhanced by the acidic milieu of the vaginal secretion [31]. It is supposed that TGFβ1-rich SP can reach the uterine cavity directly after coitus [32], on the cytoplasmic membrane of the sperms [31] or through a haematogenous route [33]. Nevertheless, in humans it remains unclear if the SP can reach the uterine cavity.

In this study, we tried to answer the following questions: (1) whether SP and/or its TGFβ1 can be detected in washed semen (2) how much TGFβ1 is contained in SP as well as in PF of endometriosis patients, and (3) if SP as well as its TGFβ1 can induce EMT/metaplasia in endometrial and/or endometriotic tissue. As we thought that SP is rapidly washed out of the genital tract after intercourse, we limited our incubation time in all our in vitro studies to maximum 6 h.

Materials and methods

Detection of seminal plasma

To answer the question if SP can be detected in washed semen, a forensically validated test procedure (Rapid stain identification “RSID” semen, Galantos, Mainz, Germany [34]) was used for highly sensitive detection of semenogelin, the predominant protein in seminal plasma responsible for the formation of the gel matrix that encapsulates spermatozoa after ejaculation [35].

Semen samples were obtained (after at least 48 h abstinence) from men presenting for infertility work-up in the andrology department. Normozoospermia ($n = 9$), teratozoospermia ($n = 1$), oligoteratozoospermia ($n = 1$), and azoospermia ($n = 1$) were all assessed according to WHO criteria 2010 [36], and within 60 min after ejaculation. Clinical characteristics of the patients are listed in Supplementary Table 1. All liquefied semen samples (except the single sample with azoospermia) were then well mixed with 2 ml sperm preparation medium (SPM, Origio), then centrifuged at 2000 rpm for 10 min. The supernatant was then carefully aspirated and discarded. One milliliter of sperm preparation media (SPM) was cautiously layered over the pellet and incubated at 37 °C for 1 h (swim-up technique). In three samples we repeated this washing step twice. Afterwards, 500 µLs from the upper layer of the SPM was aspirated and examined using the RSID. As a negative control ($n = 2$), 200 µL of the SPM was provided. To show the presence of semenogelin in seminal plasma in the absence of spermatozoa, a sample from an azoospermic man (without undergoing a swim-up technique) was transferred onto the fabric and allowed to air-dry. A small cutting of fabric (~2 mm^2) was used for testing.

In the semen sample (azoospermia), washed semen samples (once or twice) and SPM (as a negative control) a sterile cotton swab was dipped into 200–500 µL of the whole sample and then allowed to air-dry. The cotton batting was removed using laboratory clean technique, placed in a 1.5 ml microcentrifuge tube and extracted in 120 µL of RSID™ universal buffer. Samples were incubated in extraction buffer for 2 h at room temperature before applying 80–100 µL buffer into the sample window of the test cassette. Results are read after 10 min incubation time. Extraction negative controls were produced by extracting a clean and unused sterile swab directly alongside the body fluid swab samples.

The rapid stain identification (RSID™) semen kits are lateral flow immunochromatographic strip tests designed for identifying semen from biological residue on forensic evidence. They are qualitative and results are recorded as either positive or negative based on the presence or absence of a visible single red line at the "Test" position by visual inspection of the strip test.

TGFβ1 immunofluorescence staining of washed sperms

To answer the question if TGFβ1 in SP can be detected in washed semen, an immunofluorescence staining was carried out as follows:

Semen samples were obtained (after at least 48 h abstinence) from normozoospermic men ($n=2$) and assessed within 60 min according to WHO criteria 2010 [36]. After liquefaction, samples were centrifuged at 2000 rpm for 10 min, the supernatant (rich in seminal plasma) was discarded, and 1 ml Sperm Preparation Medium (Origio, Denmark) was layered carefully over the sperm pellet to carry out a swim-up preparation. After 1 h incubation at 37 °C in the incubator, the supernatant (rich in motile sperms) was pipetted out.

Sperms were then let to dry overnight on fibronectin-covered glass slides and then fixed with methanol for 10 min at − 20 °C. After washing with PBS, sperms were incubated at room temperature (RT) with PBS/Aurion BSAc for 30 min. Slides were then incubated with the primary antibody (human anti-TGFβ1 antibody, 8 µg/ml, MAB240, R&D, USA) for 1 h. After washing, the secondary antibody (Alexa488-coupled anti-mouse IgG, 1:500, Thermo Fischer Scientific, USA) was added for 30 min at RT in the dark. After washing, the nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI) (1: 10,000, Sigma-Aldrich Co. LLC, USA) for 1 min, afterwards washed with PBS and then mounted with Vectashield (Vector Laboratories, USA). Slides were then left in dark for 2–3 h till hardening and were stored for short-term and long-term usage at 4 °C and − 20 °C, respectively. Sperm samples incubated with PBS without the primary antibodies were used as negative controls. Protein localization was examined at a magnification of ×400 with a fluorescence microscope (Carl Zeiss Inc., USA).

Measurement of TGFβ1 concentration in SP and PF by Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the TGFβ1 level in SP in comparison to PF from endometriosis patients, an ELISA was carried out as following:

- Semen sample collection

Semen samples were obtained (after at least 48h abstinence) from normozoospermic men ($n=23$) and assessed within 60 min according to WHO criteria 2010 [36]. After liquefaction, samples were centrifuged at 2000 rpm for 10 min, and the supernatant was stored at − 80 °C for further use. After thawing at room temperature (RT), SP was pooled and filtered in 0.2 µm mesh and used for in vitro experiments in a 1:10 (10%) dilution.

- Peritoneal fluid sample collection

PF ($n=12$) was collected intra-operatively during laparoscopy (aspirated from the pouch of Douglas) from endometriosis. PF was centrifuged (within 30 min after its collection) at 3000 RPM at 4 °C for 10 min. The supernatant was then collected, aliquoted, and stored at − 80 °C for further use, while the sediment was discarded. All patients were premenopausal [median age 32.4 (range 21–41 years old)] in the proliferative phase of the cycle. The patients had stage I ($n=5$), stage II ($n=5$), and stage III ($n=2$) endometriosis according to the revised classification of the American Society of Reproductive Medicine (rASRM) (Supplementary Table 2).

- Enzyme-linked immunosorbent assay (ELISA)

SP and PF were thawed at RT. TGFβ1 was measured in each single semen sample ($n=23$) and in the pooled semen sample. The latter was further used for all other in vitro studies. The ELISA plate was coated overnight with capture antibody, then washed and subsequently blocked with ELISA diluent according to manufacturer's manual (Human/mouse TGFβ1 ELISA Ready-SET-Go!, 2nd Generation, catalog number 88–8350, affymetrix, eBioscience, Germany). SP and PF were diluted 1:5 and then added to the plate wells and incubated for 2 h at RT. After repeated washing, the detection antibody was added to the plate wells and incubated for another 1 h at RT. Again after repeated washing, avidin–HRP was added and incubated for another 30 min. Finally, plate wells were incubated with TMB solution then with stop solution and the plate was read at 450 nm with a spectrophotometer (Mutliskan FC, type 357, Thermo Fischer scientific, USA). Data were analyzed and plotted using the accompanying software (Skanit software 3.0, Thermo Fischer Scientific, USA).

In vitro studies of effect of SP on EMT/metaplasia markers and mediators

To study a possible time-dependent EMT effect of SP on the endometrial/endometriotic tissues/cells, both endometrial biopsies as well as cell lines were incubated with SP 1:10 (10%) for 2 h and 6 h at 37 °C. It is noticeable that the

undiluted SP is highly toxic to the endometrial cells in vitro [37]. The same was seen in our study, even with SP diluted down to 20% (data not shown), and so that 10% SP was used for the whole in vitro studies.

Gene expression of different EMT markers (Alpha Smooth Muscle Actin “ASMA”, vimentin, E-cadherin “CDH1”, N-cadherin “CDH2”, and fibronectin “FN1”) and EMT mediators (Snail1, Snail2, ZEB2, and TWIST) were studied by quantitative real-time PCR.

- Endometrial biopsy collection

An outpatient transcervical endometrial biopsy using a Probet catheter (Gynemed, Lensahn, Germany) on day 10–12 (proliferative phase, $n=2$) and on day 22–24 (secretory phase, $n=2$) of the menstrual cycle was carried out in premenopausal women presenting in the infertility clinic during routine work-up diagnostics as previously described [38] (Supplementary Table 3). The endometrial tissue was rapidly transferred into HEPES-buffered Dulbecco’s modified essential medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA) to the lab for further in vitro studies.

- Cell lines

Two different cell lines were used, 12Z (immortalized endometriotic epithelial cell) [39] and St-T1b (immortalized endometrial stromal cell line) [40]. Both were cultured in vitro at 37 °C till confluence in Dulbecco’s modified Eagle medium containing 10% FCS, 1% glutamine, insulin (5µg/ml), and 1% penicillin/streptomycin in a humidified atmosphere of 7.5% CO_2.

- Immunofluorescence characterization of both cell lines

To characterize both cell lines phenotypically, an immunofluroscence study was carried out as follows: confluent cells on glass coverslips were washed once with PBS, fixed for 5 min with 3.7% PBS-buffered formaldehyde, followed by permeabilisation with PBS 0.1% Triton X-100. The samples were subsequently washed twice with PBS and were blocked with PBS and Aurion BSAc (1:10, Aurion, Netherlands) for 20 min, and then incubated with monoclonal mouse anti-human alpha smooth muscle actin (ASMA) (1:100, clone 1A4, DAKO, USA), polyclonal rabbit anti-human cytokeratin (1:100, wide-spectrum, abcam 9377, USA), and polyclonal rabbit anti-human vimentin (1:100, Santa Cruz, USA) for 60 min at RT. After repeated washing, the cells were incubated with the suitable secondary antibody anti-mouse IgG (1:500, Alexa Fluor 546, Thermo Fischer Scientific, USA) and anti-rabbit IgG (1:500, Alexa Fluor 488, Thermo Fischer Scientific, USA), respectively, in darkness for 1 h. The slides were then covered with DAPI Fluoromount-G (Southern Biotech, USA), mounted with glycerol/glycerin and then examined under the microscope (Zeiss Axiophot, USA).

- RNA isolation and reverse transcription

Isolation of RNA from cultured cells and endometrial biopsies was performed using the innuPREP RNA Mini Kit (Biometra, Germany) according to the manufacturer’s instructions. RNA quality was controlled photometrically at 260 nm/280 nm. Reverse transcription of mRNA was performed using the High capacity cDNA reverse transcription Kit (Applied Biosystems, Thermo Fischer Scientific, USA), using random hexamer primers and M-MuLV reverse transcriptase according to the manufacturer’s instructions.

- Quantitative real-time polymerase chain reaction

Complementary DNA corresponding to 0.5 ng of total RNA was used as a template in a total reaction volume of 20 µl with Power SYBR Green PCR mix (Invitrogen, Thermo Fischer Scientific, USA) on an ABI PRISM 7300 Sequence Detection System using the default thermal cycling conditions (denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation [95 °C, 15 s] and combined annealing and extension [60 °C, 60 s]). Primers were added at 0.375 µM each. Calculations were based on the $2^{-\Delta\Delta Ct}$ method using beta actin as a housekeeping gene for normalization. Specificity of product formation was controlled by melting curve analysis. Primer sequences are listed in Supplementary Table 4.

In vitro studies of effect of TGFβ1-neutralization in the seminal plasma on EMT/metaplasia markers and mediators

To examine if the reported metaplastic effect of SP is mainly TGFβ1-mediated, an in vitro study was carried out but with neutralization of TGFβ1 in SP. Both cell lines were grown (as above) up to confluence, and then incubated with 10% SP together with anti-TGFβ1 antibody (monoclonal mouse IgG1, clone 9016, R&D Systems, USA) at a concentration of 9 µg/ml for 2 h and 6 h. Another group received only anti-TGFβ1 antibody, while the control group was incubated in the ordinary culture media. RNA isolation, cDNA transcription, and PCR were done as above for both EMT/metaplasia markers and mediators.

Statistics

Statistical analysis was performed using SigmaStat 3.1 and SPSS 15 software (SPSS, Chicago, IL, USA), using the Mann–Whitney rank sum test for immunohistochemical

staining analysis and Student's paired *t* test for qPCR results. A *p* value of < 0.05 was considered statistically significant.

Results

Seminal plasma is detectable in washed semen

To determine if the seminal plasma is detectable upon swim-up preparation of semen samples, we employed an assay for detection of semenogelin. Sperm Preparation Media (SPM) and the negative control reacted negative, as expected. All semen samples collected after swim-up reacted positive indicating the presence of semenogelin. The azoospermic sample yielded a positive test reaction indicating the presence of semenogelin in seminal plasma even though no spermatozoa were present.

TGFβ1 is highly enriched in seminal plasma and expressed on the plasma membrane of sperms

To analyze the presence of the EMT-regulating cytokine TGFβ1 in sperm and seminal plasma, we analyzed its presence by immunofluorescence microscopy and ELISA, respectively. Immunofluorescence microscopy revealed that sperms expressed TGFβ1 on their plasma membrane of the head, neck, and tail regions. The negative control did not show positive signals (Fig. 1).

By ELISA, we could demonstrate that the SP contained a high amount of TGFβ1, with a mean concentration of 92.88 ng/ml, which was not statistically different from the SP pool used later in the in vitro studies (88.17 ng/ml). TGFβ1 concentrations were almost 13,000-fold higher in SP in comparison to PF from endometriosis patients (Supplementary Table 5).

10% SP induces up-regulation of myofibroblastic metaplasia markers in the endometrial tissue

To determine the impact of 10% SP incubation on the expression of EMT markers and mediators in endometrial tissue, we employed a quantitative RT-PCR analysis. Regarding EMT/metaplasia markers, ASMA and fibronectin were significantly higher expressed after 2 h incubation in comparison to the control group ($p = 0.008$ and $p = 0.003$, respectively). Neither vimentin, E-cadherin nor N-cadherin showed any significant changes. After 6 h, ASMA was persistently higher expressed; however, as data were more variable, only a statistical trend was seen ($p = 0.08$). The same was observed for N-cadherin. Other markers remained unchanged.

Regarding EMT/metaplasia mediators, only ZEB2 expression was significantly down-regulated after 2 h incubation ($p = 0.0004$). After 6 h, ZEB2 was significantly higher expressed ($p = 0.004$). The other mediators showed a tendency for higher expression, although not significant (Fig. 2).

10% SP induces EMT/metaplasia markers in the cell lines

Prior to studying the effect of 10% SP incubation on endometriotic and endometrial cell lines, we performed a phenotypic characterization of their marker expression by immunofluorescence microscopy. Vimentin was expressed in the cytoplasm both in 12Z and St-T1b cells, while cytokeratin was only expressed by 12Z cells, as expected for an epithelial cell type. Very few single cells expressed cytoplasmic ASMA in the St-T1b cell line, while 12Z cells did not express ASMA at all (Supplementary Figs. 1, 2).

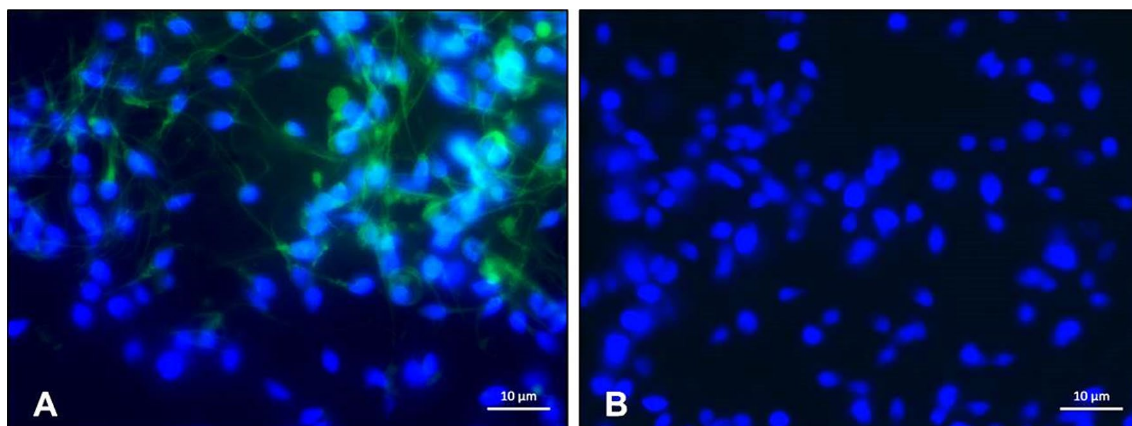

Fig. 1 TGFβ1 Immunofluorescence staining of washed sperms. **a** TGFβ1 is expressed on the plasma membrane of the head, neck, and tail regions of the sperms (green), while (**b**) a negative control shows only the DAPI-nuclear staining (blue)

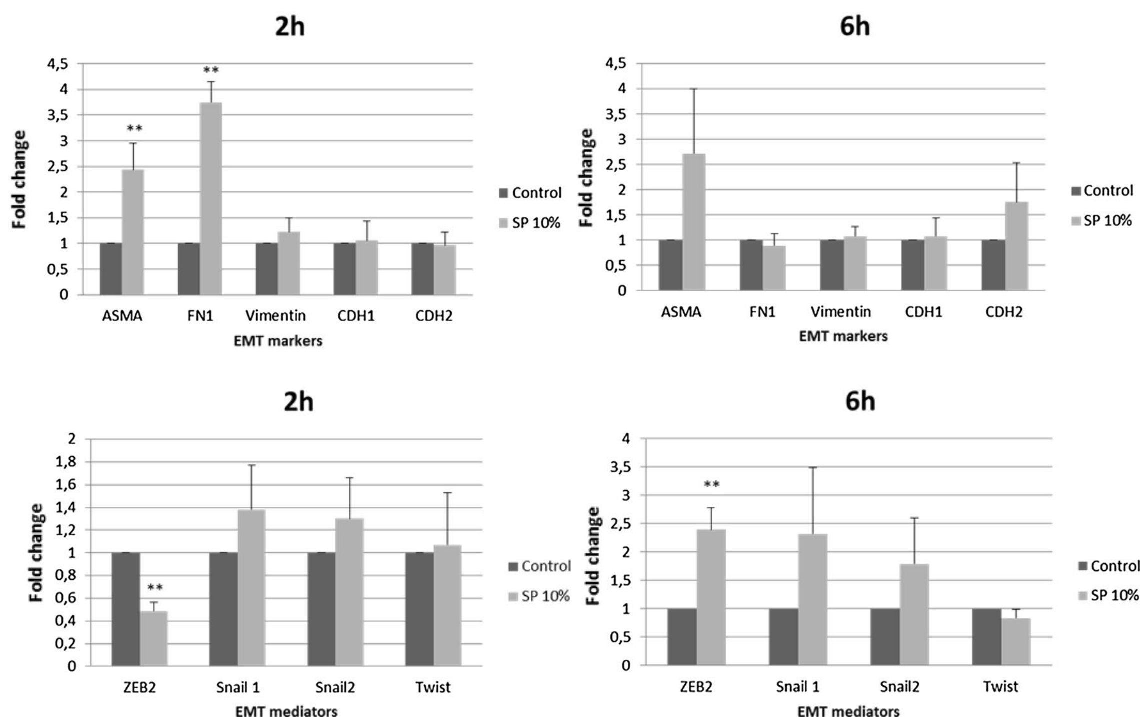

Fig. 2 Seminal plasma induces an up-regulation of EMT/metaplasia markers in endometrial tissue. A 2 h effect was evident in both ASMA and fibronectin gene expressions after incubation with 10% SP, along with down-regulation of ZEB2 gene expression. A 6 h effect showed an up-regulation of ASMA, yet insignificant. Moreover, ZEB2 gene expression was significantly higher expressed. Snail 1 and 2 showed a tendency for up-regulation, but no noticeable changes in TWIST expression. Bars of standard deviation are shown here. *ASMA* alpha smooth muscle actin, *FN1* fibronectin, *CDH1* E-cadherin, *CDH2* N-cadherin, *SP* seminal plasma

Incubation with 10% SP induced a high expression of ASMA in both 12Z and St-T1b cell lines after 2 h incubation; however, surprisingly, data variability was higher compared to the impact on endometrial tissue ($p = 0.05$ and $p = 0.3$, respectively). E-cadherin expression was, significantly up-regulated in 12Z as well as in St-T1b cell lines ($p = 0.04$ and 0.01, respectively). Moreover, N-cadherin was statistically significantly down-regulated in the 12Z cell line ($p = 0.04$).

Extending the incubation time to 6 h kept mean levels of ASMA high compared to controls, in both cell lines; however, due to high variability, data were not statistically significant. However, N-cadherin was significantly down-regulated in St-T1b cell line ($p = 0.04$). Except for a significant down-regulation of E-cadherin expression in the 12Z cell line ($p = 0.03$), all other markers did not show any remarkable changes in both cell lines.

Regarding EMT/metaplasia mediators, TWIST expression was persistently down-regulated in the 12Z and St-T1b cell lines after 2 h and 6 h incubation with 10% SP ($p = 0.007$, 0.02, 0.01, and 0.0001, respectively). The other mediators (ZEB2, SNAIL1, and SNAIL2) showed a similar gene expression pattern in both cell lines. While their mean expression values were up-regulated compared to controls, the data were statistically insignificant due to high variability (Figs. 3, 4).

TGFβ1 neutralization in seminal plasma did not reverse the SP-induced up-regulation of EMT/metaplasia markers

To determine if the effects of 10% SP on the expression of EMT markers and mediators were due to the presence of high TGFβ1 levels, we performed antibody neutralization experiments. Neutralization of TGFβ1 in 10% SP does not seem to affect the SP-induced EMT/metaplasia changes. In the 12Z cell line, adding an anti-TGFβ1 antibody to 10% SP did not affect the down-regulation of N-cadherin expression ($p = 0.04$), allowing the cells to maintain expression of their epithelial markers (Fig. 5a). In the St-T1b cell line, E-cadherin expression showed a persistent significant up-regulation even after adding anti-TGFβ1 antibody ($p = 0.002$). While ASMA was down-regulated, the data did not reach statistical significance (Fig. 5b).

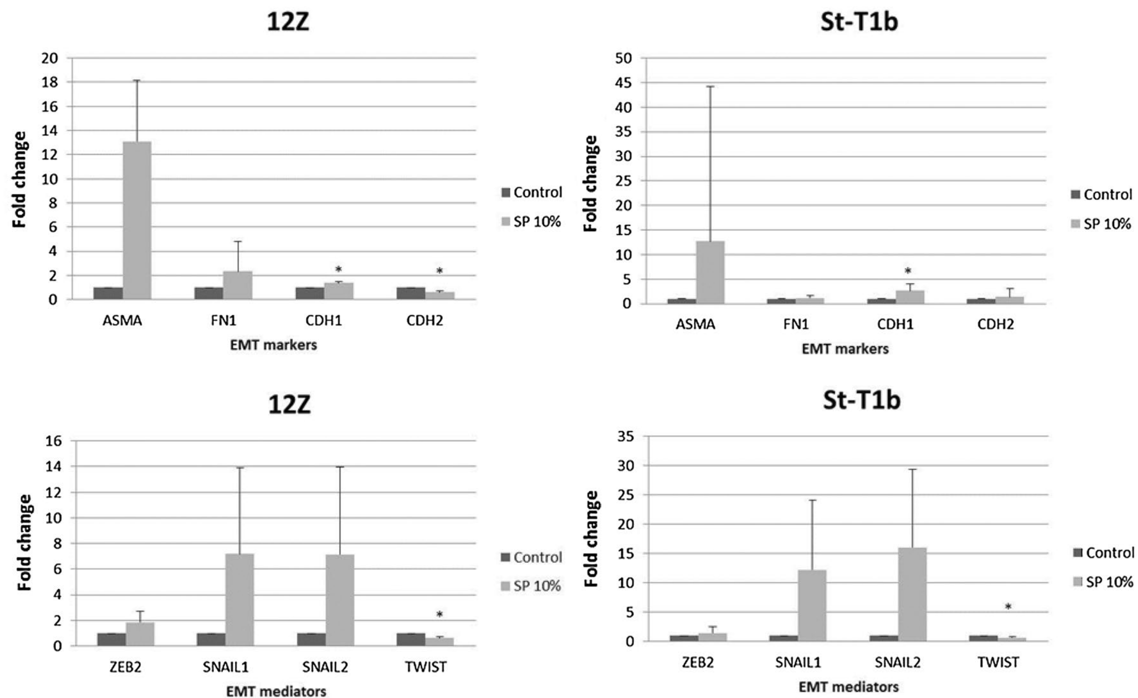

Fig. 3 Seminal plasma induces EMT/metaplasia markers in the cell lines after 2 h incubation. 10% SP induced an up-regulation of ASMA gene expression after 2 h incubation in 12Z and St-T1b cell lines, though statistically insignificant. 12Z cells kept their epithelial markers as E-cadherin was up-regulated and N-cadherin was down-regulated. A mesenchymal epithelial transdifferentiation (MET) was yet evident in St T1b cell line, as E-cadherin was significantly up-regulated (p: 0.0118). TWIST was significantly down-regulated in both cell lines, while other mediators showed up-regulation, though insignificant. Bars of standard deviation are shown here. *ASMA* alpha smooth muscle actin, *FN1* fibronectin, *CDH1* E-cadherin, *CDH2* N-cadherin, *SP* seminal plasma

Discussion

In our study, we provide novel evidence to support that even in semen washed twice, SP is still detectable. This may support the ascension theory of seminal plasma into the uterine cavity. Moreover, TGFβ1 either expressed on the plasma membrane of the sperms or being highly concentrated in SP is believed to get in direct contact with the endometrium by sperms reaching the uterine cavity. Furthermore, 10% SP induced a time-dependent, mostly TGFβ1-independent up-regulation of some EMT/metaplasia markers in both endometrial tissues as well as in endometriotic epithelial cells.

There is still much debate, whether or not SP reaches the uterine cavity in human. Previously, authors proposed different routes by which SP might get in contact with the endometrium. In a study on mice, an ascension/diffusion of SP through the cervical canal following mating was proposed as a possible route [32]. In our study, we used semen samples prepared by the swim-up technique to test this hypothesis. Swim-up technique is a common practice prior to intrauterine insemination (IUI) or in vitro fertilization technique to treat infertility in human. The procedure mimics the function of the cervical canal and ensures not only the extraction of motile sperms but also a washing out of the seminal plasma. Surprisingly, in our study, SP was still detectable in washed semen after swim-up using the RSID semen procedure. RSID is a sensitive and established forensic tool for detecting traces of SP in cases of alleged sexual assault, which can also be applied in the case of azoospermia.

Furthermore, another group described a hematogenous route through the uterine–ovarian counter-current system [33]. The latter supposed absorption of the different growth factors of SP into the blood vessels supplying the endometrium. A third group showed sperms expressing TGFβ1 on their plasma membrane [31], thereby acting possibly like a vehicle for the different growth factors in SP. The latter was reproduced in our study as well. All findings support the hypothesis that the SP can come in contact with the endometrium in human.

How much SP can reach the endometrial cavity remains unclear. A concentration gradient of SP along the female genital tract was supposed. This was tested using TGFβ1 as a main growth factor in the SP. Interestingly, TGFβ1 increases by more than threefold in the uterine fluid following mating in mice to reach 6 ng/ml, while being 30 ng/ml in the ejaculate and 70 ng/ml initially in the seminal vesicles [33].

It is noticeable that the undiluted SP is highly toxic to the endometrial cells in vitro [37]. The same was seen in our

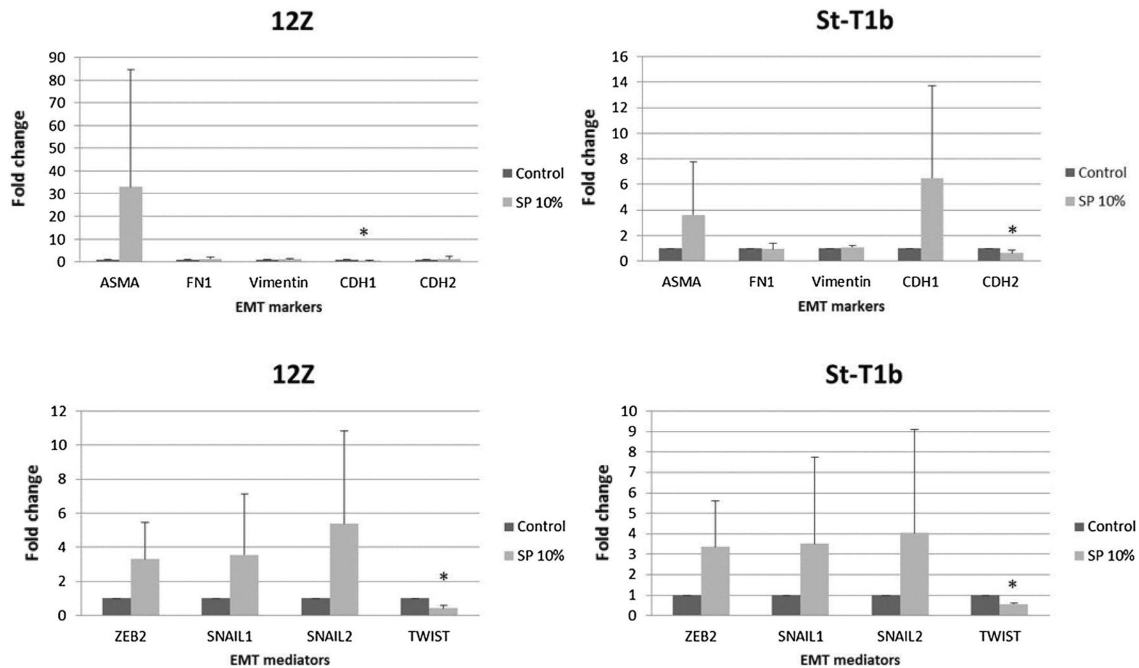

Fig. 4 Seminal plasma induces EMT/metaplasia markers in the cell lines after 6 h incubation. 10% SP induced an up-regulation of ASMA gene expression after 6 h incubation in 12Z and St-T1b cell lines, though statistically insignificant. In the St-T1b cell line N-cadherin was persistently significantly down-regulated, along with an up-regulation of E-cadherin expression. The latter may point to a mesenchymal epithelial transdifferentiation. TWIST was significantly down-regulated in both cell lines, while other mediators showed up-regulation, though insignificant. Bars of standard deviation are shown here. *ASMA* alpha smooth muscle actin, *FN1* fibronectin, *CDH1* E-cadherin, *CDH2* N-cadherin, *SP* seminal plasma

study, even with SP diluted down to 20% (data not shown). This underscores the necessity of a concentration gradient of SP in the female genital tract, being most concentrated in the vagina and probably least concentrated in the endometrial cavity, and hence more tolerable by the endometrial cells.

In our study, it is evident that the TGFβ1 is highly enriched in SP. This was previously reported by other groups [29]. Although in endometriosis, many studies reported an increased TGFβ1 concentration not only in PF [22, 24, 41] but also in the uterine lavage [28]. According to our study, TGFβ1 in SP is concentrated still almost 13,000-fold higher.

Being a key player in endometriosis pathogenesis, TGFβ1 has been the main focus of research in the last few years. It affects major biological steps in the disease pathogenesis. One of them is mediating EMT/metaplasia in endometriotic tissue [3]. TGFβ1 mediates a myofibroblastic metaplasia of the surrounding stromal cells and the fibroblasts. They are involved in tissue repair following tissue injury, and characterized by abundance in ASMA expression to gain a free motility in the injured area [3].

In our study, 10% SP induced a myofibroblastic metaplasia of the endometrial tissue, characterized by up-regulation of ASMA expression. Surprisingly, this effect was evident just after 2 h incubation and persisted until 6 h, although at later time points a higher data variability resulted in lack of significance. For fibronectin, we observed a strong, but transient induction after 2 h, which decreased after 6 h. Indeed, transient induction of fibronectin by TGFβ1 has been previously described in fibrosarcoma cells, and has been attributed to activation of the JNK pathway [42]. Similar mechanisms may play a role in our experimental system. To our knowledge it is the first time to show an early effect of SP causing endometrial metaplasia. The same effect was observed in the endometriotic epithelial cell line.

Furthermore, an early up-regulation of fibronectin in the endometrium was evident, which is a typical feature of metaplasia, supporting cell migration and invasion [7, 43]. Nevertheless, a late effect was lacking.

The endometriotic epithelial cells showed a trend for undergoing an early myofibroblastic metaplasia, hallmarked by up-regulation of the ASMA expression. However, they retained their epithelial markers, where E-cadherin expression was up-regulated and N-cadherin was down-regulated. The lack of significance for several marker changes in 12Z cells is in contrast to the data obtained in endometrial tissues, and may indicate that the effect of SP relies on an interplay between different cell types from the epithelial and stromal compartment, respectively.

Interestingly, the stromal cell line acquired an epithelial marker, as E-cadherin expression was significantly

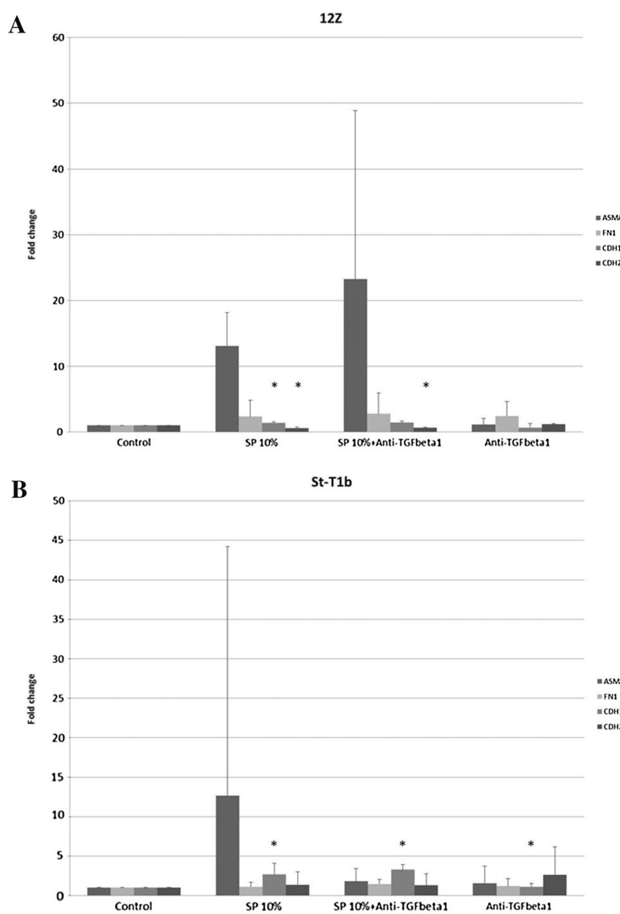

Fig. 5 Neutralization of TGFβ1 in seminal plasma in cell lines. **a** In the 12Z cell line, adding anti-TGFβ1 antibody to 10% SP kept a persistent significant down-regulation of N-cadherin expression, allowing the cells to keep their epithelial phenotype. **b** In the St-T1b cell line, E-cadherin expression showed a persistent significant up-regulation even with adding anti-TGFβ1 antibody. Bars of standard deviation are shown here. *ASMA* alpha smooth muscle actin, *FN1* fibronectin, *CDH1* E-cadherin, *CDH2* N-cadherin, *SP* seminal plasma

up-regulated. The latter may refer to a unique mesenchymal epithelial transdifferentiation (MET), the reverse process of an EMT.

Although the origin of the myofibroblasts is still debatable [7], in our study they can be developed from the endometriotic epithelial and endometrial stromal cells. While the detection of ASMA in the stress fibers would confirm and extend our results, in our study with the necessary short incubation times no changes on the cellular protein level is to be expected. Therefore, our study relied on testing the gene expression which mirrors the very early stage of EMT/metaplasia-associated cellular changes.

Different EMT/metaplasia mediators were analyzed in our study. They showed a similar pattern of gene expression in both 2 h and 6 h incubation groups. We observed a significant down-regulation of TWIST, whereas the other markers show an up-regulation, yet statistically insignificant. This observation may be linked to the E-cadherin up-regulation in stromal cell lines acquiring an epithelial marker.

In our study, neutralization of TGFβ1 in 10% SP could not reverse the SP-induced EMT/metaplastic changes. This may be explained, as SP contains other hormones (e.g., estrogen), growth factors (PDGF, HGF), and prostaglandins which can also mediate a metaplasia [44], which is an aspect worth exploring in future studies.

Conclusion

The detection of seminal plasma in the washed semen may support the ascension theory of seminal plasma into the uterine cavity in human. As TGFβ1 is not only abundantly expressed on the plasma membrane of the sperms, but also highly concentrated in seminal plasma, a contact of this growth factor with the endometrium following insemination is very likely. Expression of selected EMT/metaplasia markers in endometrial tissues, endometriotic and endometrial cells can be rapidly induced upon exposure to 10% SP and this effect is mostly TGFβ1-independent.

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Authors' contributions MGI study design, data analysis, manuscript drafting, and critical discussion. EA statistical analysis and critical discussion. SS, SK and LK manuscript editing and critical discussion. MV RSID test execution, interpretation, and manuscript critical discussion. MGI, SS and SK, ANS samples collection. MG and ANS shared in the study design, manuscript editing and critical discussion.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent All patients included in this study gave written informed consent. The study was approved by the local research and ethics committee of the medical faculty and the regional medical board of Westfalen, Münster (1 IX Greb).

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