GENERAL GYNECOLOGY

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Arrangement of myofibroblastic and smooth muscle-like cells in superficial peritoneal endometriosis and a possible role of transforming growth factor beta 1 (TGFβ1) in myofibroblastic metaplasia

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Abstract

Purpose Superficial peritoneal endometriotic (pEM) lesions are composed of endometrial glands and stroma, in addition to a third component—myofibroblasts and smooth muscles (SM)-like cells. The latter develops secondary to a metaplasia. In this study, we characterised the third component cells in pEM according to differentiation markers in different micro-compartments. Furthermore, a possible effect of TGFβ1 on myofibroblastic metaplasia in endometriotic epithelial cells was studied.

Methods Seventy-six premenopausal patients were included. Peritoneal biopsies were excised from EM patients ($n = 23$), unaffected peritoneum (peritoneum from EM patients but without EM components, $n = 5/23$) and non-EM patients ($n = 10$). All peritoneal biopsies were immunolabeled for ASMA, calponin, collagen I, desmin, TGFß receptor 1 (R1), R2 and R3 in addition to ultrastructure examination by transmission electron microscopy (TEM) ($n = 1$). TGFß1 level was measured in peritoneal fluid (PF) (EM, $n = 19$ and non-EM, $n = 13$) collected during laparoscopy. Furthermore, TGFß1 effect on myofibroblastic metaplasia was studied in vitro.

Results At the centre of pEM lesions, calponin immunolabeling outweighs the collagen I while in the periphery the reverse occurs. SM-like cells expressing desmin predominate at the periphery, while ASMA immunolabeling was detectable in all micro-compartments. Both indicate an abundance of myofibroblasts at the centre of pEM lesions and SM-like cells in the periphery. Although activated TGFß1 in PF did not differ between EM and non-EM, it inhibited the cell proliferation of the endometriotic epithelial cells and induced an upregulation in ASMA and collagen IA2 expression as well.

Conclusion The abundance of the myofibroblasts and SM-like cells points to a myofibroblastic metaplasia in pEM. Both cells are differentially arranged in the different micro-compartments of pEM lesions, with increasing cell maturity towards the periphery of the lesion. Furthermore, TGFß1 may play a role in the myofibroblastic metaplasia of the endometriotic epithelial cells. These findings provide a better insight in the micro-milieu in EM lesions, where most of the disease dynamics occur.

**Keywords** Peritoneal endometriosis · Myofibroblastic metaplasia · TGFβ1 · Smooth muscle-like cells

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Introduction

Endometriosis (EM) is an enigmatic disease affecting 10–15% of women of reproductive age [1, 2]. The ectopic presence of endometrial glands and/or stroma marks the histological diagnosis of the disease [3]. Nevertheless, a third component, smooth muscle-like cells, is frequently seen in all different forms of EM lesions [4–10]. Although their origin is still unknown, it is presumed that they develop from endometriotic stromal cells by the process of metaplasia. This is supported by the expression of uterine markers;

ASMA immunolabeling in pEM lesion

Desmin immunolabeling in pEM lesion
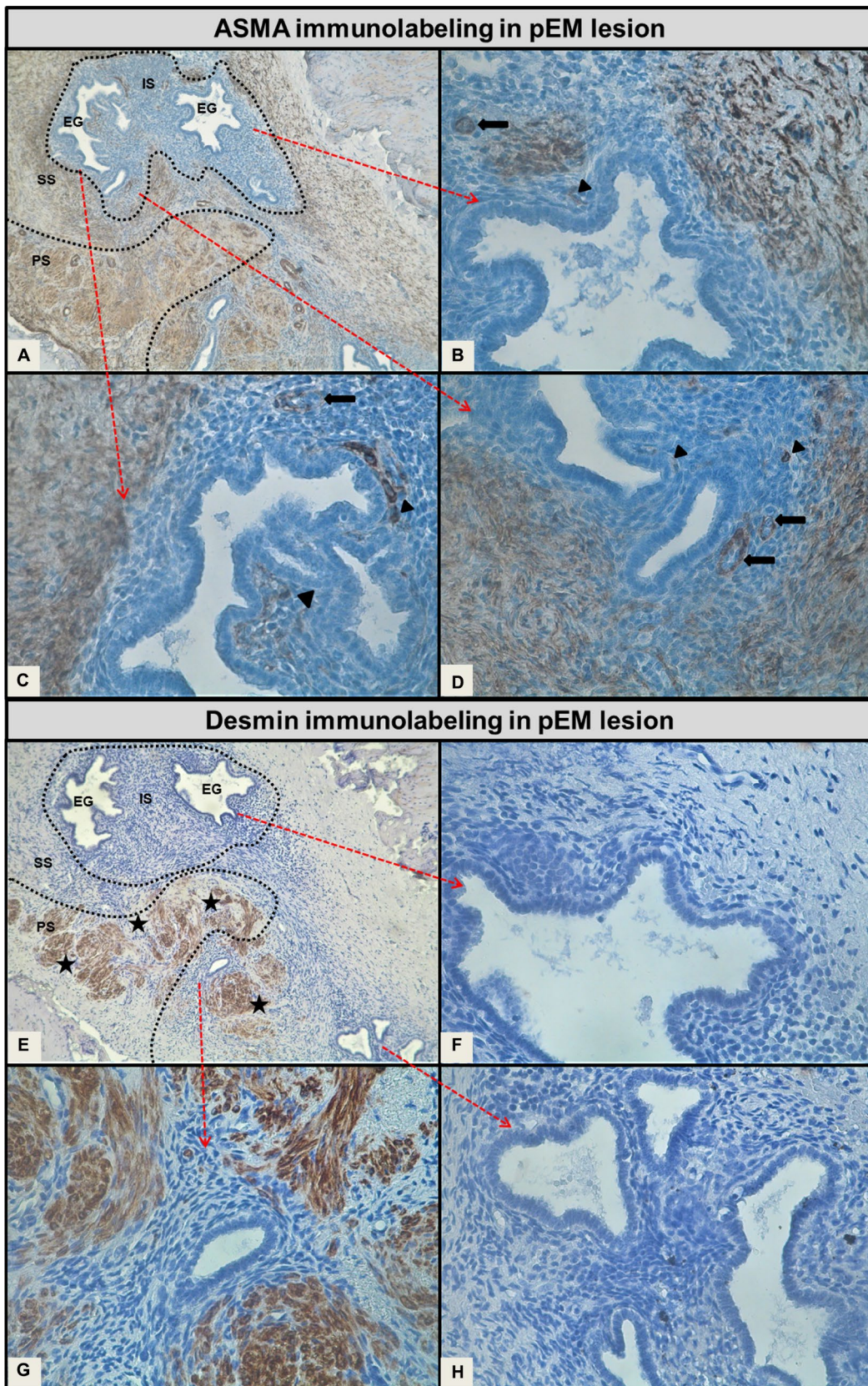
◂**Fig. 1** A representative microscopic photo of ASMA and desmin immunolabeling (brown) in peritoneal endometriosis in a serial section. **a** Stromal cells and mesenchymal cells in IS (arrowheads, **b**–**d**), SS and PS compartments express ASMA, ×200. Small blood vessels express ASMA (black arrows, **b**–**d**). Magnification (×400) of the different sites of the lesions is shown (**b-d**). **e** Furthermore, only stromal and mesenchymal cells in PS compartment (asterisk) express desmin. Both IS and SS compartment show almost no desmin expression, ×200. Magnification (×400) of the different sites of the lesions is shown (**f**–**h**). *EG* endometrial gland, *IS* intra-stroma compartment, *SS* surrounding stroma compartment, *PS* peripheral stroma compartment

namely oxytocin and vasopressin receptors as well as oestrogen and progesterone receptors [11]. Another theory postulates that peritoneal mesothelial cells undergo epithelial–mesenchymal transition (EMT), and develop into myofibroblasts and smooth muscle cells; the same is seen in peritoneal dialysis. This can be secondary to a tissue reaction to the retrograde shed endometrium [12, 13].

The arrangement of these smooth muscle-like cells exhibits a unique pattern in EM lesions. They are arranged into three micro-compartments (Fig. 1): intra-stromal (IS), surrounding stroma (SS) and peripheral stroma (PS) compartments [11]. Here the smooth muscle cells are in different stages of differentiation. In the IS compartment, the cells express only alpha smooth muscle actin (ASMA) (immature cells), while in the PS compartment they express desmin and caldesmon in addition to ASMA (the earlier are of mature smooth muscle cells) [11]. Also oxytocin and vasopressin receptor expression is higher in the periphery than when it is closer to the lesion. This has led to the assumption that EM lesions are actually mini-uteri, composed of endometrial glands and stroma (mimicking the endometrium) surrounded by smooth muscle-like cells (mimicking the myometrium) [5, 11].

Metaplasia seems to be one main pathway for the development of EM, as the lesions show a characteristic profile of decreased epithelial markers (E-cadherin and cytokeratin) and increased mesenchymal markers (N-cadherin and vimentin) [14, 15]. Transforming growth factor beta 1 (TGFβ1) mediates metaplasia through its three different TGFß receptors 1, 2 and 3 [7]. TGFß1 is usually secreted by endometrial epithelial and stromal cells, as well as immune cells (infiltrating into endometrium) especially macrophages [16]. Decidualised stromal cells [17] and peritoneal mesothelial cells [16] can also secrete TGFß1. TGFß1 is one of many growth factors that can be detected in the peritoneal fluid (PF) of women with and without EM [18]. Tissue damage is a known stimulus for TGFß1 secretion and the latter helps tissue repair, the regulation of cell proliferation, differentiation, extracellular matrix (ECM) synthesis, fibrosis and angiogenesis [17].

Myofibroblast formation is the result of metaplasia. These cells play a pivotal role during tissue injury and are usually recruited to the site of tissue injury by TGFß1 [19]. They retain their fibroblastic ability to produce collagen I to replace the lost ECM. Moreover, they acquire contractile properties, as ASMA is integrated in the contractile apparatus, to ensure their mobility in the tissue.

In our study, we attempted to further characterise the ASMA-expressing cells (especially at the centre of EM lesions) by studying their immunohistochemical expression of calponin, desmin, and intracellular collagen I. Calponin, a calcium-binding protein in smooth muscle cells, is a component of the contractile apparatus of both myofibroblasts and smooth muscle cells [20–23]. Desmin is a recognised marker for differentiated smooth muscle cells [11], whereas collagen I is usually expressed by fibroblasts. Furthermore, the Van Gieson stain was used to study the extracellular collagen I (Table 1). Myofibroblasts in peritoneal EM lesions were characterised ultrastructurally by transmission electron microscopy as well. Additionally, TGFß1 level in the PF of EM and non-EM patients was measured and a possible in vitro effect on cell proliferation and myofibroblastic metaplasia was studied.

Materials and methods

Specimen collection

All patients included in this study were operated on via laparoscopy at Charité University of Medicine and gave written informed consent. The study was approved by the local research and ethics committee at the Charité University of Medicine, Berlin-Germany (EA4/071/07).

Premenopausal patients with EM ($n = 23$) were biopsied at the pelvic peritoneum. Biopsies were taken from macroscopically affected peritoneum ($n = 23/23$) as well as from macroscopically unaffected areas (away from the endometriotic lesions and looking normal intra-operatively, $n = 5/23$). All peritoneal biopsies were excised from the lateral pelvic wall and lesions of deep infiltrating endometriosis were excluded. The average age of the patients was 35 years (range 24–43 years old). All peritoneal lesions were later histopathologically proven to have the two basic components of EM: endometriotic glands and stroma. There were two patients in the menstrual phase, eight in the proliferative phase, and nine in the secretory phase and in four patients the cycle phase was unknown. They were staged according to rASRM as follows: stage 1 ($n = 7$), stage 2 ($n = 3$), stage 3 ($n = 4$), stage 4 ($n = 8$) and unknown in a single patient. All patients did not take pre-operative hormonal treatment in the last 3 months.

Patients in the non-EM group ($n = 10$) were operated on for benign gynaecological indications other than EM and were included as a control group. Healthy-looking (no white,

Table 1 Expression of different markers in myofibroblasts, smooth muscle-like cells, fibroblasts and stromal cells

Cell type	ASMA	Calponin	Desmin	Collagen I
Myofibroblasts	+	+	−	+
Smooth muscle-like cells	+	+	+	−
Fibroblasts	−	−	−	+
Stromal cells	−	−	−	+

(+) expressing, (−) not expressing, *ASMA* alpha smooth muscle actin [4, 5, 11, 19]

red or black lesions and appears glistening) peritoneum was excised and collected in the non-EM group; therefore endometriosis was excluded by laparoscopy and histopathology. These patients were not on hormonal contraceptives except in two, who were on intrauterine devices, and in three patients the status was unknown. All peritoneal samples were rapidly fixed in paraformaldehyde 4% and embedded after 24 h in paraffin.

PF ($n = 42$) was collected undiluted during laparoscopy (32 for the ELISA experiment and 10 for the in vitro studies). It yielded variable volumes (range 2–10 ml, blood-stained samples were excluded) (supplementary Fig. 1).

All samples used in this study were collected according to the standardised EPHect guidelines for tissue and fluid biospecimen collection [24, 25].

Immunolabeling of ASMA, calponin, collagen 1, Desmin, TGFßR-1, -2 and -3

The paraffin blocks were cut to 4-µm slices, deparaffinised in xylene and finally rehydrated in a series of decreasing ethanol concentrations as previously described [5]. The antigens of interest were retrieved by being cooked in a steamer (Multi Gourmet, type 3216, Braun, Germany) in the appropriate buffer solution for 20 min, then cooled down in a Tris buffer for another 20 min. Blocking with 10% foetal calf serum (FCS) for 30 min, then by avidin–biotin blocking agents (Avidin/Biotin blocking kit, SP-2001, Vector Laboratories, Canada) for 10 min. The slides were incubated with the primary antibody (supplementary Table 1) for 60 min followed by the appropriate secondary antibody (rabbit anti-mouse IgG (H + L) and mouse anti-rabbit IgG (H + L) Biotin-SP-conjugated AffiniPure, 1:400, Jackson ImmunoResearch) for 40 min at room temperature (RT). Streptavidin-AP was incubated for 40 min at RT, followed by 20 min incubation with 2-solution DAB kit (Invitrogen) or Fast Red (Fast Red chromogen system, Covance, USA) to develop the final colour. Finally, counterstaining with Mayer`s hemalum solution (Merck KG a.A., Germany) for 35 s followed, and the slides were covered with Eukitt quick-hardening mounting medium (Sigma-Aldrich) and cover slips.

Immunoreactive scores

A light microscope (Carl Zeiss Axiophot Microscope, Göttingen, Germany) with a × 400 power of magnification was used for evaluation by two independent observers without bias. The intensity of the staining was classified as follows: 0 for no staining, 1 for mild, 2 for moderate and 3 for strong (intense) staining, with regard to a positive control. The percentage of stained cells among the total cells was then multiplied by the intensity and the immunoreactive score (IRS) was then calculated with the highest value at 300 as previously described [5, 26]. In the manuscript the terms immunolabeling and IRS are interrelated. The images were edited using the Adobe Photoshop Program (Adobe Systems, Unterschleissheim, Germany). No nonlinear adjustments were performed.

Specimen preparation for electron microscopy examination

The peritoneal endometriotic lesion (from the lateral pelvic wall) from a single patient was fixed in Karnovsky solution (7.5% glutaraldehyde and 3% paraformaldehyde in phosphate buffered saline), washed in 0.1 M cacodylate buffer (cacodylic acid sodium salt trihydrate; Roth, Karlsruhe, Germany), incubated in 1% osmium tetroxide (Chempur, Karlsruhe, Germany) for 120 min, dehydrated in a descending series of ethanol and washed in the intermedium propylene oxide (1,2 Epoxypropan; VWR, Germany). Specimen was subsequently embedded in a mixture of agar 100 (epoxy resin), DDSA (softener), MNA (hardener) and DMP 30 (catalyst) (all: Agar Scientific; Stansted, UK). Polymerisation was done at 45 °C and 55 °C each for 24 h.

Semi- and ultrathin sections were cut using an ultramicrotome Reichert Ultracut S (Leica, Wetzlar, Germany). Semi-thin sections (0.5 µm) were stained with modified Richardson solution [27]. Embedding was done in epoxy resins for ultrathin sectioning in electron microscopy for 45 s on an electric hot plate adjusted to 80 °C. Sections were checked under a light microscope Olympus CX 21 (Olympus, Stuttgart, Germany) to ensure the presence of endometriotic glands and stroma. Ultrathin (80 nm) sections were mounted on nickel grids (Agar Scientific, Stansted, UK) and examined with a transmission electron microscope (Zeiss EM 900, Oberkochen, Germany). Photos were taken and edited by an Adobe Photoshop Program (Adobe Systems; Unterschleissheim, Germany).

Van Gieson's stain

This stain was used to evaluate the extracellular collagen (as a main component of ECM) in the peritoneum of non-EM patients as well as around the peritoneal endometriotic

glands in EM patients. Collagen fibres stain red, while the cytoplasm of all cells stains brown. Paraffin-embedded slides were deparaffinised and rehydrated as previously described [26] and then transferred to an autostainer (Leica XL). Slides were stained with resorcinol for 12 min and then immersed in Weigert's hematoxylin (Sigma-Aldrich) for 13 min, rinsed with distilled water, and finally stained with van Gieson´s solution for 8 min.

ELISA for the TGFß1 level in the peritoneal fluid

PF ($n = 42$) was collected during laparoscopy (32 for the ELISA experiment and 10 for the in vitro studies). PF was aspirated from the pouch of Douglas, from EM ($n = 19/32$) and non-EM ($n = 13/32$) patients. The PF was centrifuged (within 15 min after its collection) at $300g$ at 4 °C for 5 min. The supernatant was then collected and stored at -80 °C for further use, while the sediment was discarded. No hormonal contraception was recorded for the 7 patients in the EM group and the 10 in the non-EM group. The thawing of the PF at room temperature was followed by the activation of the latent TGFß1 in the PF according to the manufacture's manual (Human TGF-ß1 ELISA KIT, catalog number OKAA00026_96W, Aviva systems biology, USA). The samples were added to the primary antibody-coated wells for 90 min at 37 °C, then washed and lastly incubated with biotin-conjugated secondary antibody for another 60 min at 37 °C. After washing, streptavidin-HRP was added for 30 min at 37 °C. For the production of the final colour, the substrate was incubated for 20 min away from light at 37 °C. Reading at 450 nm within 30 min was done with the spectrophotometer (Multiskan FC, type 357, Thermo scientific, China) and the accompanying software (Skanit software 3.0, Thermo scientific, China) showed the curves.

In vitro effect of 10% PF and TGFß1 on the cell proliferation

We used two different cell lines in our in vitro studies because EM is a disease characterised by extensive fibrosis; the mouse fibroblast cell line L-929, a representative of the collagen-producing cells, and the immortalised endometriotic epithelial cell line 12Z [28], a representative of endometriotic epithelial cells. The culture medium for 12Z cell line was composed of: Dulbecco's modified Eagle's medium (DMEM—low glucose and without phenol red/L-glutamine, BioConcept, Switzerland), 10% foetal calf serum (FCS) (Biochrom AG, Germany), 1% glutamine, 1% ampicillin (Roche, Germany), 1% streptomycin and 1% amphotericin B. The culture medium for the L-929 cell line was composed of: Roswell Park Memorial Institute medium (RPMI 1640—without phenol red/L-glutamine, PAA laboratories, Austria), 10% FCS (Biochrom AG, Germany), 1% glutamine,

1% ampicillin (Roche, Germany), 1% streptomycin and 1% amphotericin B.

The primary outcome was to compare the cell proliferation of two cell lines; L-929 and 12Z cell lines incubated with either 10% PF (peritoneal fluid diluted 1:10) from women with ($n = 5$) and without ($n = 5$) EM, or TGFß1 (hBA-1, a biologically active GST-TGFß1 fusion protein, sc-4561, Santa Cruz) in a concentration series (0.1–0.5–1.0–5.0 and 10 ng/ml) up to 72 h. An automated cell counter (Countess, Invitrogen, Korea) was used to count the total and living cells after staining with trypan blue.

In vitro effect of TGFß1 on myofibroblastic metaplasia

To study if myofibroblastic metaplasia in peritoneal endometriotic lesions is TGFß1-mediated, an in vitro study was carried out as follows: group I, the 12Z cell line was incubated with TGFß1 (conc. 5 ng/ml) for 72 h. Group II, the TGFß1-post-receptor signalling pathway was blocked by adding TGFβ1 activin receptor-like kinase inhibitor (ALK-I) (conc. 10 uM, SB 431542, Sigma-Aldrich) together with addition of TGFß1 (conc. 5 ng/ml) in the 12Z cell line for 72 h. Group III (control), the 12Z cell line was cultured untreated for 72 h. ASMA and collagen I A2 expression was studied before and after treatment in all groups. All experiments were done in triplicate.

Quantitative real-time PCR

RNA isolation and reverse transcription

Isolation of RNA was performed using the innuPREP RNA Mini Kit (Biometra) according to the manufacturer's instructions. Quality of RNA was controlled photometrically at 260 nm/280 nm. Reverse transcription of mRNA was performed using the First Strand cDNA Synthesis Kit (Fermentas), using random hexamer primers and M-MuLV reverse transcriptase.

Polymerase chain reaction

Polymerase chain reaction (PCR) analysis complementary DNA corresponding to 0.5 ng of total RNA was used as a template in the PCR analysis. The reaction mix consisted of SYBR Select Master Mix (Applied Biosystems) and the following primer sequences, normalising ASMA and Col I A2 expression to the housekeeping gene GAPDH:

ASMA (length: 111 bp; [29])

Forward: 5′-ACTGAGCGTGGCTATTCCTTCGTT-3′

(bp 756-779 of ASMA, Gen Bank accession number NM_001613.3)

Reverse: 5′-GCAGTGGCCATCTCATTTTCA-3′
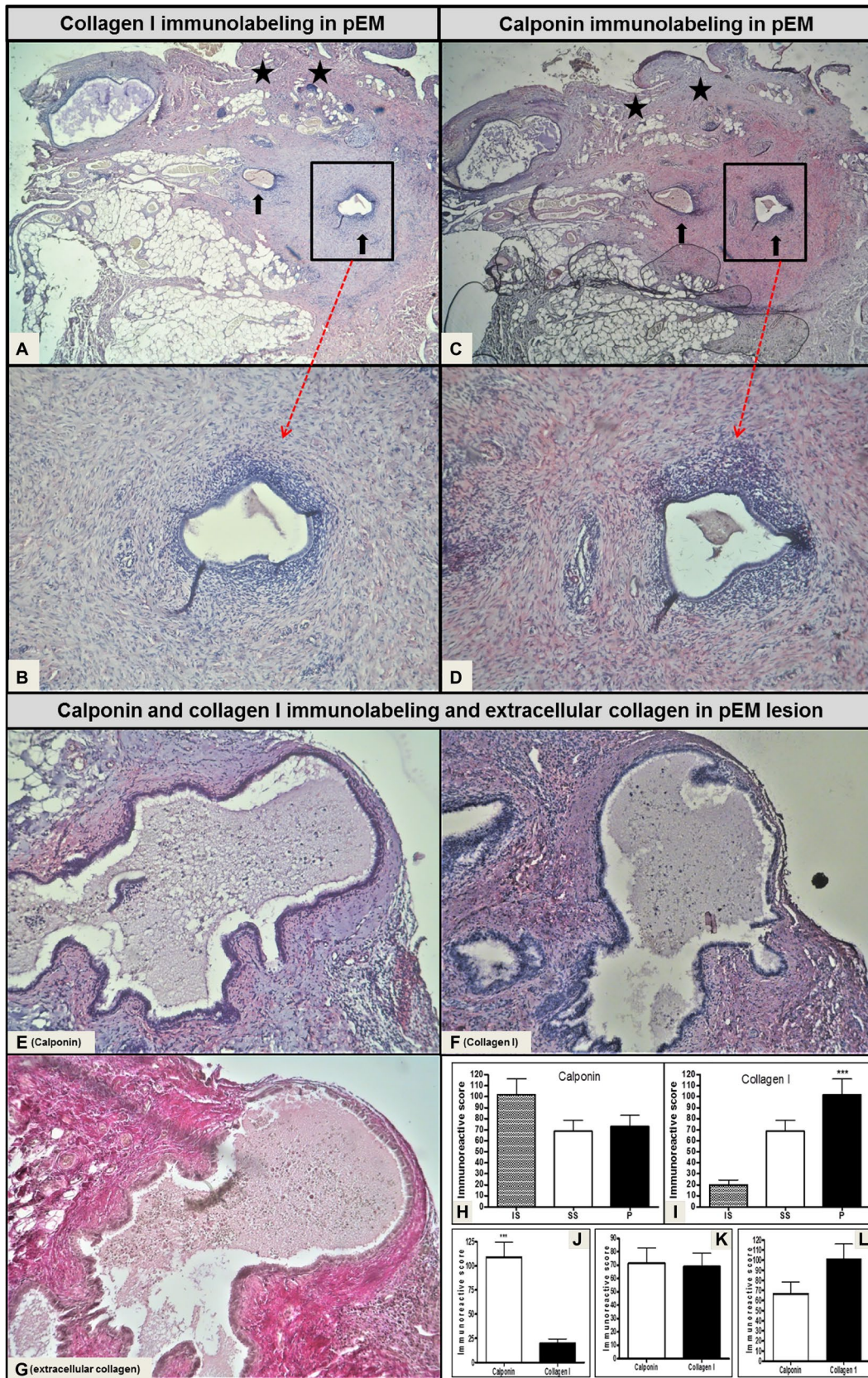
◄**Fig. 2** A representative microscopic photo of collagen I and calponin immunolabeling and extracellular collagen (red) in peritoneal endometriosis in a serial section. Stromal cells and mesenchymal cells in PS compartment preferentially express collagen I (asterisk) than in IS compartment (arrows) (**a**, **b**), while the pattern of calponin immunolabeling (**c**, **d**) shows the reverse (**a**, **c**: ×100, **b**, **d**: ×400). Furthermore, while the stromal and mesenchymal cells in PS express lower calponin (**e**) and higher intracellular collagen I (F) than in IS compartment, the extracellular collagen (stained red by van Gieson stain, G) is tightly packed in PS compartment, ×400. Calponin (**h**) and collagen I (**i**) immunolabeling in the different microcompartments of endometriotic lesion shows an interesting pattern. While calponin immunolabeling significantly outweighs the collagen I immunolabeling in IS compartment (**j**), the reverse tends to occur in PS (**l**), though statistically insignificant. In SS compartment the immunolabeling of both is almost equal (**k**). *IS* intra-stroma compartment, *SS* surrounding stroma compartment, *PS* peripheral stroma compartment

(bp 845-865 of ASMA, Gen Bank accession number NM_001613.3)
Collagen I alpha 2 (length: 161 bp, BioMol VHPS-2104)
Forward: 5′-AACCAAGGATGCACTATGAG-3′
(bp 4018-4037 of Col1A2, Gen Bank accession number NM_000089.3)
Reverse: 5′-GCTGCCAGCATTGATAGTTT-3′
(bp 4179-4160 of Col1A2, Gen Bank accession number NM_000089.3)
GAPDH (length: 74 bp)
Forward: 5′-GCACCGTCAAGGCTGAGAAC-3′
(bp 249-268 of GAPDH, Gen Bank accession number NM_002046.6)
Reverse: 5′-AGGGATCTCGCTCCTGGAA-3′
(bp 323-305 of GAPDH, Gen Bank accession number NM_002046.6)"

Quantitative PCR (qPCR) was performed in a 96-well plate format using the 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)]. The comparative cycle threshold method was used for relative quantification.

Statistical analysis

All statistical analyses were performed using Graph Pad prism 4, a Student's *t* test and ANOVA—when applicable—was used. All statistical tests were two-sided and with a 95% confidence interval width ($p < 0.05$).

Results

Differential expression of myofibroblastic and SM-like cells markers in the different pEM micro-compartments

The immunolabeling of ASMA, desmin (Fig. 1), calponin, collagen I, extracellular collagen (Fig. 2), TGFßR-1, -2 and -3 (supplementary Fig. 2) was studied in the previously described compartments of the endometriotic lesions [11]; intra-stroma compartment (IS), surrounding stroma compartment (SS) and peripheral stroma compartment (PS).

In the IS compartment, calponin immunolabeling is significantly higher than collagen I ($p < 0.0001$), and its immunolabeling tends to decrease towards the PS compartment, though insignificant ($p = 0.2456$). No desmin immunolabeling is observed. TGFßR-1 and -R3 immunolabeling is significantly higher than in SS and PS compartments ($p = 0.0081$ and 0.0166, respectively). Extracellular collagen fibres are loosely packed as shown by van Gieson stain. Myofibroblasts are ultrastructurally seen with TEM. Their cytoplasm is rich in organelles (ribosomes and mitochondria). Dense bodies are localised at the cytoplasmic membrane where the cellular cytoskeleton (stress fibres) attach (supplementary Fig. 2).

In the PS compartment, collagen immunolabeling tends to be higher than calponin, though insignificant ($p = 0.1532$), however its immunolabeling significantly decreases towards the IS compartment ($p < 0.0001$). Desmin immunolabeling is massively detected. Extracellular collagen fibres are tightly packed by van Gieson stain.

In all compartments, ASMA immunolabeling is detected. TGFßR-2 immunolabeling is consistent and higher than TGFßR-1 and -3 immunolabeling in IS and PS compartments, though insignificant in the former ($p = 0.0593$ and $p = 0.001$, respectively).

Unaffected peritoneum from EM patients and healthy peritoneum from non-EM patients are both phenotypically similar

Both groups show a similar immunolabeling pattern of calponin, collagen I, TGFßR-1, -2 and -3. Of note, calponin and collagen I immunolabeling is lower than that in EM patients ($p = 0.0002$ and $p = 0.0341$, respectively), while the immunolabeling of TGFßR-1, -2 and -3 is higher ($p < 0.0001$, $p = 0.0014$ and $p = 0.0536$, respectively) (supplementary Fig. 3).

TGFß1 level in PF

There was no significant difference in TGFß1 level between endometriosis peritoneal fluid (EMPF) and

non-endometriosis peritoneal fluid (non-EMPF) ($p = 0.4130$). The measured level varies between 2 and 5 ng/ml (supplementary Fig. 2).

TGFß1 inhibits cell proliferation and induces a myofibroblastic metaplasia in the 12Z cell line

TGFß1 (0.5, 1.0, 5.0 and 10 ng/ml, but not 0.1 ng/ml) inhibits the cell proliferation significantly ($p < 0.05$, $p < 0.01$, $p < 0.05$, $p < 0.01$ and $p > 0.05$, respectively). Interestingly, EMPF shows a reverse effect, stimulating the cell proliferation of the fibroblast cell line ($p < 0.05$) but not that of 12Z ($p > 0.05$) (supplementary Fig. 4). Furthermore, TGFß1 induces a significant upregulation in both ASMA, ($p = 0.005$), and collagen IA2 expression ($p = 0.01$) in 12Z cell line after 72 h incubation. Blocking the TGFß1 post-receptor signalling pathway downregulates both ASMA ($p = 0.005$), as well as collagen IA2 expression ($p = 0.007$) (supplementary Fig. 5).

Discussion

Arrangement of myofibroblastic and smooth muscle-like cells in peritoneal endometriosis

Endometrial glands and stroma cells are two well-known basic components of EM lesions. However, many authors describe a third component: namely smooth muscle-like cells in different EM lesions [4–11].

In our study, all three micro-compartments exhibited an abundance of ASMA-expressing stromal and mesenchymal cells, while the desmin-expressing one was only seen in the PS compartment. This points to a special arrangement of the smooth muscle-like (SM) cells in the endometriotic lesions, being immature at the centre and getting matured towards the periphery. Nevertheless, those ASMA$^+$/Desmin$^-$ cells are unique myofibroblasts, and are mostly localised in the IS- and SS-compartments. The myofibroblasts combine the acquisition of ASMA-expression in addition to the intracellular expression of collagen I. Ultrastructurally and as a representative method of the myofibroblasts, the latter exhibited a cytoplasm rich in both cellular organelles and well-developed contractile apparatus. They were seen surrounding the endometriotic glands.

These myofibroblasts play a pivotal role during tissue injury and usually exhibit two phenotypes, a contractile and a secretory one [20, 22]. The overexpression of calponin in IS compartment may highlight the predominance of the contractile phenotype, whereas the overexpression of collagen I in PS compartment may hallmark the secretory phenotype. However, a further functional in vitro experiment is recommended to prove this theory. Extracellular collagen

was also seen tightly packed in the PS compartment, but less packed in the IS compartment. This special arrangement of the myofibroblasts/SM-like cells together with peripheral deposition of collagen I can favour the implantation of the retrograde shed endometrium, favouring the building of new lesions [30]. As no correlation exists between the menstrual phase and the immunolabeling of the different myofibroblastic/smooth muscle metaplasia markers in endometriotic lesions [4, 5, 11], no corresponding correlation was investigated in our study.

The myofibroblastic metaplasia in PS compartment may point to a remote effect exerted by the endometrial glands and stroma on the cells, as they do not have a direct link to EM glands and stroma [31, 32]. Nevertheless, this effect seems to be a restricted one, as both the unaffected peritoneum of EM patients as well as the healthy peritoneum from non-EM patients did not show any significant changes in the myofibroblastic markers. This observation might be related to excess collagen I seen at the periphery of the EM lesions [33, 34], restricting this remote effect to only few millimetres away from EM glands.

Tissue microtrauma is a well-known stimulus for the myofibroblastic metaplasia, and induces TGFß1 secretion, which in turn mediates this myofibroblastic transformation [20]. Moreover, tissue microtrauma is evident ultramicroscopically in endometriotic lesions, and the myofibroblasts share in exerting mechanical tension on the ECM, causing tissue micro-disruption [35]. The latter favours further endometriotic tissue implantation through the exposure of the underlying ECM [36, 37]. The same is seen in adenomyosis uteri [19].

TGFß1-mediated myofibroblastic metaplasia in endometriotic epithelial cells

In our study, TGFß1 induced an upregulation of both ASMA and collagen I A2 expression in the endometriotic epithelial cells. This assumes that the endometriotic epithelial cells seem to acquire mesenchymal characters upon exposure to TGFß1, while slowing down its proliferation rate. Furthermore, the process is probably TGFß1-dependent, as blockage of the post-receptor signalling of TGFß1 down-regulated the expression of both markers. This may highlight a possible involvement of TGFß1 in the myofibroblastic metaplasia in EM lesions.

Although the total TGFß1 (both active and latent forms) level was significantly higher in EMPF than in non-EMPF in other studies [16, 38], in our study the measured activated TGFß1 did not differ between both groups. Its level ranged between 2 and 5 ng/ml.

We propose the following explanations for our finding: (i) TGFß1 increases COX-2 production [39] with a subsequently higher level of PGF$_2\alpha$, which in turn

down-regulates TGFß1 in the decidual cells [32, 40]. (ii) The extensive fibrosis, which is usually associated with EM, might prevent the free release of TGFß1 into the PF. The same is seen in the chocolate fluid of endometriomas with higher TGFß1 than that of the PF of the same patients [41]. (iii) Metaplasia might be mediated by growth factors other than TGFß1, namely the nerve growth factor (NGF). (iv) The desquamated endometrium may behave differently than the eutopic endometrium with reduced TGFß1 in its cytoplasm together with an increased expression of oxidative stress markers [42].

Although our study included a relatively low number of patients for an immunohistochemical/electron microscopy study, the inclusion criteria were strict to superficial peritoneal endometriosis as a single independent entity. Nevertheless, in ELISA of TGFß1 level in PF the number of included patients is higher than that in previous studies [16, 38].

Conclusion

The abundance of the myofibroblasts and SM-like cells points to a myofibroblastic and smooth muscle metaplasia in pEM. Both cells are differentially arranged in the different micro-compartments of pEM lesions, with increasing cell maturity towards the periphery of the lesions. Furthermore, TGFß1 may play—independently—a role in the myofibroblastic metaplasia of the endometriotic epithelial cells. These findings provide a better insight in the micro-milieu in EM lesions, where most of the disease dynamics occur. This argues further basic and clinical research of the endometriotic micro-milieu being a potential therapeutic target of the disease.

Compliance with ethical standards

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