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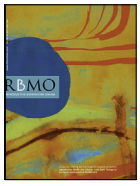

ARTICLE

Adenomyotic glands are highly related to endometrial glands

BIOGRAPHY

Dr Lutz Konrad has a PhD in genetics and is currently a laboratory manager at the Institute of Gynecology and Obstetrics. Current research interests include endometriosis, male fertility and TGF-betas. In addition to basic research, the Institute has been conducting qualitative research through questionnaires for endometriosis patients.

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KEY MESSAGE

The protein expression pattern of calcyphosine, and msh homeobox 1, two highly specific and abundant endometrial proteins, in adenomyosis is nearly identical to those of the endometrium with and without endometriosis externa, thus suggesting endometrial glands as the main source for adenomyotic glands.

ABSTRACT

Research question: How closely related are adenomyotic and endometrial glands?

Design: In this study, the mRNA and protein database www.proteinatlas.org was searched for proteins expressed predominantly in the endometrial glands. Specificity was tested with tissue microarrays. Biopsy specimens of endometrial, adenomyotic tissue, or both, were collected after surgery from 21 women without endometriosis, 20 women with endometriosis, 18 women with adenomyosis together with endometriosis and 12 women with adenomyosis alone. Tissue expression was analysed by immunohistochemistry.

Results: Two proteins were identified: calcyphosine (CAPS), and msh homeobox 1 (MSX1). A high abundance and good specificity in endometrial glands were found. Both proteins, CAPS and MSX1, showed a high specificity for endometrium and are both localized in the luminal cells and epithelial cells of the glandular and adenomyotic glands. No significant differences were found between CAPS- and MSX1-positive endometrial glands between cases with and without endometriosis. Also, no cycle-specific different expression was found. Furthermore, a close relationship between the adenomyotic glands and the endometrial glands for CAPS (range 63.0–98.3%) and for MSX1 (range 87.1–99.3%) could be demonstrated. Only 11.2% and 6.8% negative glands for CAPS and MSX1 were identified in all tissues from all patients, respectively; none were negative for both proteins.

Conclusions: Taken together, our results show that the protein expression pattern of adenomyosis is nearly identical to those of the endometrium with and without endometriosis, thus suggesting endometrial glands as the main source for adenomyotic glands.

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KEYWORDS
Adenomyosis
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Endometrium
Epithelial cell marker
Msh homeobox 1
Pathogenesis

INTRODUCTION

Adenomyosis is a benign uterine disease affecting 24.4% of women of reproductive age and is associated with endometriosis in 35% of cases as found in a hospital centre (*Puente et al., 2016*). In the general population, it might be lower and was estimated to be around 2% (*Morassutto et al., 2016*). Possible risk factors are early menarche, short menstrual cycles, increased body mass index, surgical tissue damage (*García-Solares et al., 2018*), history of depression (*Taran et al., 2010*) and multiparity (*Ferraz et al., 2017*).

Adenomyosis is histologically characterized by the presence of endometrial tissue within the myometrium, producing an enlarged uterus with endometrial glands and stroma most often surrounded by hypertrophic and hyperplastic myometrium (*Siegler and Camilien, 1994*). Adenomyosis is a commonly diagnosed oestrogen-dependent gynaecological disorder that causes pelvic pain, abnormal uterine bleeding and infertility (*Abbott, 2017*) but is asymptomatic in about one-third of cases (*Graziano et al., 2015*). Its diagnosis may be improved by high-quality imaging, such as transvaginal ultrasonography and magnetic resonance imaging (*Agostinho et al., 2017*). The gold standard for the diagnosis of adenomyosis, however, is still histological examination after surgery (*Gordts et al., 2018*), although histological differences in pathological diagnosis vary from centre to centre and even between pathologists (*Seidman and Kjerulff, 1996*).

Several molecular pathways involved in adenomyosis have been investigated, such as focal adhesion kinase (*Mu et al., 2015*; *Zheng et al., 2018*), matrix metalloproteinases (MMP2, 7, 9) (*Herndon et al., 2016*; *García-Solares et al., 2018*), apoptosis, extracellular matrix remodelling or steroid hormone responsiveness (*Herndon et al., 2016*). Recently, it has been suggested that invasiveness of adenomyosis is not driven by a single mechanism of migration but by a time-dependent combination of at least two processes, namely epithelial–mesenchymal transition (EMT) in the early phase and collective cell migration in the later phase (*García-Solares et al., 2018*).

Despite the prevalence and severity of symptoms, the pathogenesis of adenomyosis has not yet been elucidated (*Vannuccini and Petraglia, 2019*). Two main theories have been proposed to explain the origin. One suggests the involvement of tissue injury and repair mechanism in the endometrium leading to stromal invagination into the inner layer of the myometrium with gland invasion (*Leyendecker et al., 2009*), in conjunction with microenvironmental factors to stimulate smooth muscle cell growth (*Koike et al., 2013*). An alternative hypothesis proposes that adenomyosis may develop *de novo* from metaplasia of displaced embryonic pluripotent Müllerian remnants (*García-Solares et al., 2018*). Other hypotheses, such as differentiation or activation of adult stem cells after tissue injury (*Gargett, 2007*; *Vannuccini et al., 2017*), pale cells (*Ibrahim et al., 2015*) or EMT (*García-Solares et al., 2018*) should not be dismissed lightly.

We have developed a hypothesis similar to the analysis of unknown primary tumours in cases of metastases (*Conner and Hornick, 2015*), in which immunohistochemistry can illucidate the tumour of origin. The higher the identity of the immunostaining profile, the more likely the metastases are derived from it. Similarly, endometrial and adenomyotic glands in the endometrium with two different but highly abundant endometrial epithelial tissue biomarkers were examined and the identify quantified. A similar analysis was conducted with annexin A (*Liu et al., 2019*) and KISS-1 (*Kolioulis et al., 2017*), and showed a significant increase of both proteins in adenomyosis. In both studies, however, only the HScore was used, and no information was provided on the number of positive endometrial and adenomyotic glands as in our study. Furthermore, because adenomyosis and endometriosis may be linked to changes that occur in the inner portion of the myometrium or the junctional zone and to molecular abnormalities in the eutopic endometrium (*Benagiano et al., 2014*), eutopic endometrium of women with endometriosis, in addition to women with adenomyosis, was included in our study.

MATERIALS AND METHODS

Patients and ethical approval

This study was approved by the Ethics Committee of the Medical Faculty of the Justus-Liebig-University, Giessen, Germany, on 2 July 2009 (registry number 95/09) and all participants gave written informed consent. All specimens were obtained by hysterectomy from patients mainly suffering from pain (TABLE 1) or in cases of endometriosis by laparoscopy (*Konrad et al., 2018*). In addition, two healthy Fallopian tubes were obtained, which were used as positive controls. In particular, the tissues from four patient groups (TABLE 1) were analysed: healthy endometrium (controls), endometrium with endometriosis only (EM-En), endometrium with endometriosis and adenomyosis (EM-En-Ad) and adenomyotic tissues (Ad).

The intraoperative findings were classified by the pathologist, who also diagnosed adenomyosis. Dating of the endometrial tissue was based on the dates of the last menstrual period and histological evaluation by the pathologist. Specimens were fixed in Bouin's solution (and partly in formaldehyde for the histological evaluation by the pathologist) and embedded in paraffin. After staining 5-µm sections with haematoxylin and eosin, the histological evaluation was carried out.

TABLE 1 OVERVIEW OF THE TISSUE SAMPLES

Tissues	(ctrl) EM, healthy	EM-En	EM-En-Ad	Ad[a]
All samples, *n*	21	20	18	30
Median age	43 ± 7.5	41 ± 7.5	44 ± 6.1	44 ± 6.0
Proliferative, *n*	10	9	11	17
Median age	45 ± 10.0	39 ± 8.6	45 ± 4.8	45 ± 4.6
Secretory, *n*	11	11	7	13
Median age	42 ± 6.7	42.5 ± 6.2	45 ± 4.6	42 ± 7.3

[a] Includes adenomyotic tissue from 18 patients with endometriosis and adenomyosis and from 12 with adenomyosis only. The median age is given with the SD.

Ad, adenomyosis; Ctrl, control group; EM, endometrium; EM-En, EM-endometriosis only; EM-En-Ad, EM-endometriosis-adenomyosis.

TABLE 2 ANTIBODIES USED FOR IMMUNOHISTOCHEMICAL CHARACTERIZATION OF ADENOMYOSIS

Protein	Source	Catalogue number	Species	Clonality	Dilution
Calcyphosine	Sigma	HPA043520	Rabbit	Polyclonal	1:5000
MSX1	Sigma	HPA073604	Rabbit	Polyclonal	1:100
Anti-rabbit HRP	DAKO	K4002	Goat	IgG	Ready
IgG isotype	Invitrogen	02-6102	Rabbit	IgG	1:2500

MSX1, msh homeobox 1; Cat-No, catalogue number; HRP, horse radish peroxidase; IgG, immunoglobulin G.

Screening of the database

The database www.proteinatlas.org was searched for differently expressed genes with the keyword tissue specificity_ rna:any; tissue enriched AND sort by: tissue specific score. In a second step, the restricted protein presence especially in the female reproductive tract was searched for.

Immunohistochemical analysis and quantification

Serial sections of 5 µm were used to ensure that the same lesions could be examined. Immunohistochemistry of bouin-fixed specimens was carried out as published previously (*Konrad et al., 2018*). The EnVision Plus System (DAKO, Hamburg, Germany) was used according to the manufacturer`s instructions. Briefly, a citrate buffer (pH 6) (DAKO, Hamburg, Germany) was used for antigen retrieval, and jars with the slides were then placed into a steamer (Braun, Multi Gourmet) at 100°C for 20 min and then cooled for 20 min. Primary antibodies (TABLE 2) were added and the slides incubated in a humidified chamber overnight at 4°C. After washing with phosphate buffered saline, incubation with the appropriate secondary antibody (TABLE 2) was carried out for 30 min at room temperature. Staining was visualized with diaminobenzidine (Liquid DAB K3467, DAKO). Mayer's haematoxylin (Waldeck, Germany) was used for counterstaining. After dehydration in ethanol, slides were mounted with Eukitt. Negative controls for immunohistochemistry were prepared by omission of the primary antibody and by using Rabbit immunoglobulin G Isotype Control (Cat-No. 02-6102, Invitrogen/ThermoFisher) instead of the primary antibody (Supplementary Figure 1). The same concentration as for msh homeobox 1 (MSX1) (2.0 ng/ml) was used.

Leica DM 2000/Leica MC170/Leica application suite LAS 4.9.0 (Leica, Wetzlar, Germany) was used to obtain digital images. They were then processed with Adobe Photoshop CS6 (Adobe Systems Software, Dublin, Ireland). Additionally, the human normal tissue array II (Cat-No. 4011120; Provitro, Berlin, Germany) was used to analyse tissue expression of calcyphosine (CAPS) and MSX1 as described above. The tissue array includes tissues from the following organs: brain, colon, heart, kidney, liver, lung, muscle, spleen, testis, ovary, uterus, pancreas, small intestine, rectum and skin.

Conventional HScore was not used for quantification of the endometrial and adenomyotic glands; instead, all stained and unstained endometrial and adenomyotic glands in all samples were counted; therefore, the whole slide was evaluated for every patient. Because of the scarcity of the material, only one slide per patient per antibody could be used. The evaluation of 11,126 glands for CAPS and 11,326 glands for MSX1 in all patients was carried out by two people at a magnification of 200x.

Statistical analysis

All values are presented as means ± SE of the mean or median ± SD. Differences between groups were analysed using one-way analysis of variance. Then, comparison between the groups was conducted with the non-parametric multiple test of Kruskal–Wallis, to compare all groups with each other. $P \leq 0.05$ was considered to be significant. GraphPad Prism 6.01 (www.graphpad.com, San Diego, USA) was used for statistical analyses.

RESULTS

The database www.proteinatlas.org was searched for differently expressed genes, with a restricted protein presence in a few organs but with a strong expression in the endometrial glands. Two proteins, CAPS and MSX1, were identified, which were expressed mainly in the female reproductive duct and in only a few other organs. Next, the protein expression with tissue arrays was validated, and an abundance of strong CAPS was found in the endometrium (FIGURE 1A) as well as in the Fallopian tubes from our biobank (FIGURE 1B). A faint staining could be also observed in the kidney and pancreas (FIGURE 1C and FIGURE 1D).

Msh homeobox 1 was strongly expressed in the glandular epithelial cells of the endometrium, in the Langerhans cells of the pancreas and nerve cells of the brain (FIGURE 2). In the Fallopian tube, only a few cells stained positive for MSX1 (data not shown). After determination of the tissue specificity of the two proteins, they were analysed immunohistochemically in tissues from the control group compared with EM-En, EM-En-Ad and Ad (TABLE 1). In the last group (Ad), patients with endometriosis and adenomyosis (n = 18) and 12 cases with adenomyosis only were grouped together (TABLE 1), because analysis of adenomyotic glands with MSX1 (97.1% 2.4 versus 100% 0.0) and CAPS (93.7% 3.5 versus 98.4% 0.9) revealed no significant differences between both groups.

Localization of CAPS was found in most but not all endometrial glandular epithelial cells of the proliferative and secretory phases (FIGURE 3A and FIGURE 3B) as well as in the luminal cells (FIGURE 3C and FIGURE 3D) in the control group. Similarly, CAPS was also localized in the glandular cells (FIGURE 3E and FIGURE 3F) and luminal cells of EM-En (data not shown). Quantification of the CAPS-positive glands showed no difference between control versus EM-En secretory phases, as well as between control versus EM-En proliferative phases (TABLE 3). Comparison of the localization of CAPS in EM-En-Ad and Ad similarly demonstrated positive glands (FIGURE 4), but the numbers of positive adenomyotic glands was significantly higher in the Ad group compared with the control group, in the proliferative phase ($P < 0.01$) and secretory phase ($P \leq 0.05$) (TABLE 3).

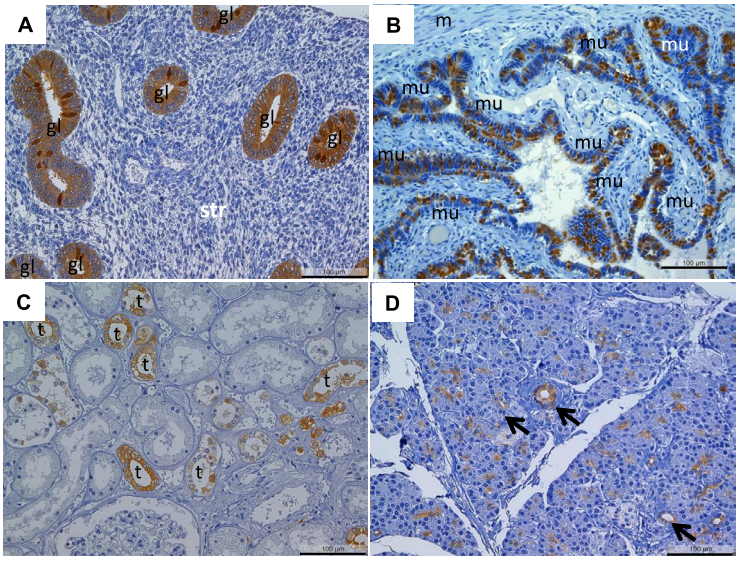

FIGURE 1 Immunohistochemical detection of calcyphosine in human tissues. Calcyphosine was found in (A) the glands of the endometrium tubules; (B) in the epithelial cells of the mucosa of the Fallopian tubes; (C) in the tubules of the kidney; and (D) and in the ducts (arrows) of the pancreas. Haematoxylin was used for counterstaining. gl, glands; m, muscles; mu, mucosa; str, stroma; t, tubules. Magnification A–D 20x; scale bars 100 μm

Msh homeobox 1 was highly abundant in nearly all endometrial glandular epithelial cells of the proliferative and secretory phases (FIGURE 5A and FIGURE 5B) as well as in the luminal cells (FIGURE 5C and FIGURE 5D) in nearly all glands in the control group (TABLE 3). Similarly, MSX1 was also present in the glandular cells (FIGURE 5E and FIGURE 5F) and in the luminal cells in the EM-En group (data not shown).

The localization of MSX1 in the Ad group (FIGURE 6) demonstrated a similar percentage of positive glands compared with all other groups, except for a statistically significant difference between secretory endometrium of the controls and proliferative phase of the Ad group ($P < 0.01$). These results suggest a high similarity between endometrial and adenomyotic glands (TABLE 3).

Although some negative glands for CAPS (11.2%) and MSX1 (6.8%) were identified in all tissues from all patients, we never observed double negative adenomyotic glands, which is exemplarily shown for the adenomyotic glands of one patient (FIGURE 7A and FIGURE 7B). Additionally, the strong staining of nerve fibres in the myometrium by MSX1 is demonstrated (FIGURE 7C).

In one patient of the Ad group without endometriosis, the beginning invasion of the myometrium by CAPS- and MSX1-positive endometrial glands could be identified (FIGURE 8). Remarkably, the invading glands were positive for both biomarkers tested.

DISCUSSION

Adenomyosis shares many similarities with endometriosis but resides inside the uterine wall; however, in both cases, the pathogenesis is still poorly understood. Also, besides hysterectomy, few medical options are currently available (*Groothuis and Guo, 2018*). Because many investigators regard adenomyosis as a condition starting with the deposition of endometrial stroma and epithelium within the myometrium (*García-Solares et al., 2018*), we hypothesized that the analysis of the similarities or differences with specific tissue biomarkers with a high abundance in the endometrium as well as in adenomyosis will provide us with a fair estimation about how many adenomyotic glands might be derived from endometrial glands.

Our approach started with the identification of two highly expressed endometrial proteins in the www.proteinatlas.org database. We identified CAPS and MSX1 to be specific for endometrial epithelial cells, which we further substantiated by analysing the protein presence in human tissues. Calcyphosine was found with a high protein presence in the epithelial cells in the endometrium and Fallopian tubes. A fainter protein expression was also evident in kidney and pancreas. Msh homeobox 1 was highly specific for the

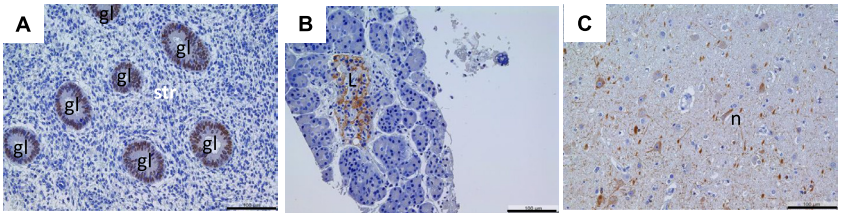

FIGURE 2 Immunohistochemical detection of msh homeobox 1 in human tissues. Localization of msh homeobox 1 was found in (A) the glandular epithelial cells of the endometrium; (B) in the Langerhans cells of the pancreas; and (C) in the nerve cells of the frontal cortex. Haematoxylin was used for counterstaining. Magnification A–C 20x; scale bars 100 μm; gl, glands; L, Langerhans cells; n, nerve cells; str, endometrial stroma.

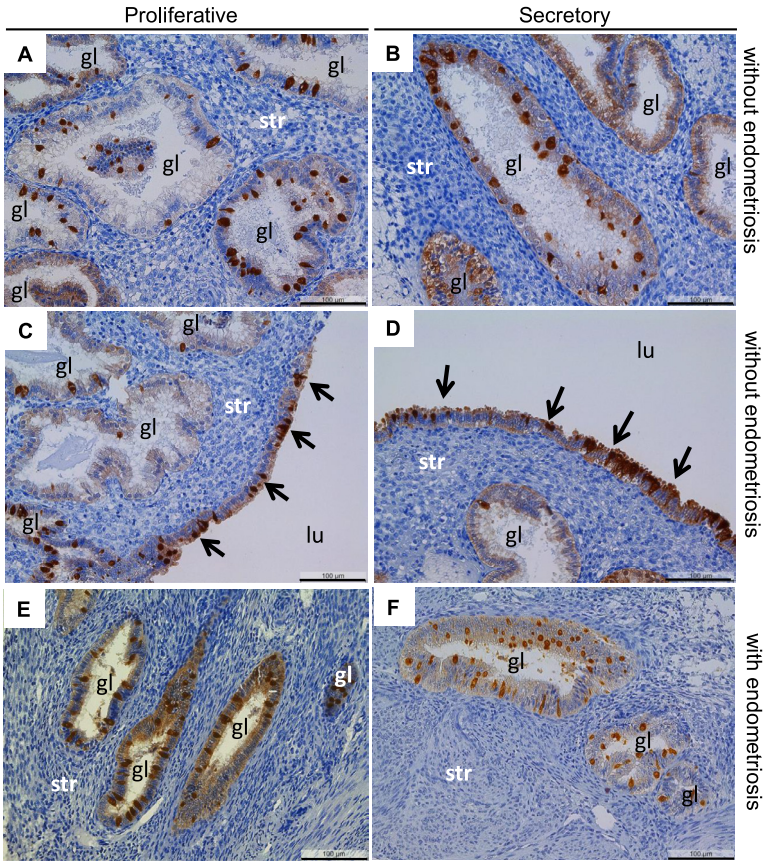

FIGURE 3 Immunohistochemical detection of calcyphosine in endometrial tissue from healthy and women with endometriosis only. Calcyphosine was found in proliferative (A, C, E) and secretory (B, D, F) endometrium without endometriosis (A–D) or with endometriosis (E, F). The luminal samples (C, D, arrows) correspond to the endometrial samples (A with C, B with D). Haematoxylin was used for counterstaining. Magnification A–F 20x, scale bars 100 µm; gl, gland; lu, lumen; str, endometrial stroma.

TABLE 3 PERCENTAGE OF ENDOMETRIAL AND ADENOMYOTIC GLANDS SHOWING EXPRESSION OF CALCYPHOSINE AND, MSH HOMEOBOX 1

	(ctrl) EM, healthy		EM-En		EM-En-Ad		Ad	
CAPS	**Proliferative (a)**	**Secretory (b)**	**Proliferative (c)**	**Secretory (d)**	**Proliferative (e)**	**Secretory (f)**	**Proliferative (g)**	**Secretory (h)**
Mean (%)	72.6	93.1	63	96.8	89.1	93.2	98.3	94.0
SEM	8.2	2.9	4.8	3.5	4.3	5.1	0.8	3.5
P-value	NS	NS	NS	NS	NS	NS	$<0.01^{a,g}$	$\leq 0.05^{a,h}$
n	10	11	9	10	11	7	17	12
Age	45 ± 10	43 ± 1.3	33 ± 8.8	42 ± 6.7	44 ± 4.6	45 ± 4.6	45 ± 4.6	39.5 ± 7.4
MSX1	Proliferative (a)	Secretory (b)	Proliferative (c)	Secretory (d)	Proliferative (e)	Secretory (f)	Proliferative (g)	Secretory (h)
Mean (%)	94.6	87.1	98.9	95.3	98.1	99.3	98.2	99.2
SEM	3.3	8.2	0.8	2.7	1.3	0.4	1.9	0.6
P-value	NS	NS	NS	NS	NS	NS	$<0.01^{b,g}$	NS
n	10	10	9	11	9	7	16	13
Age	45 ± 9.1	44 ± 3.9	33 ± 8.8	42 ± 6.4	45 ± 1.5	45 ± 1.5	45 ± 1.2	42 ± 7.3

All groups have been compared with each other.

Superscript letters indicate groups with statistically significant differences.

Age is given as the median ± SD.

[a] Includes adenomyotic tissue from 18 patients with endometriosis and adenomyosis and from 12 with adenomyosis only.

Ad, adenomyosis; CAPS, calcyphosine; Ctrl, control group, EM, endometrium; EM-En, EM-endometriosis; EM-En-Ad, EM-endometriosis-adenomyosis; MSX1, msh homeobox 1; NS, not significant compared with any other group; SEM, standard error of the mean.

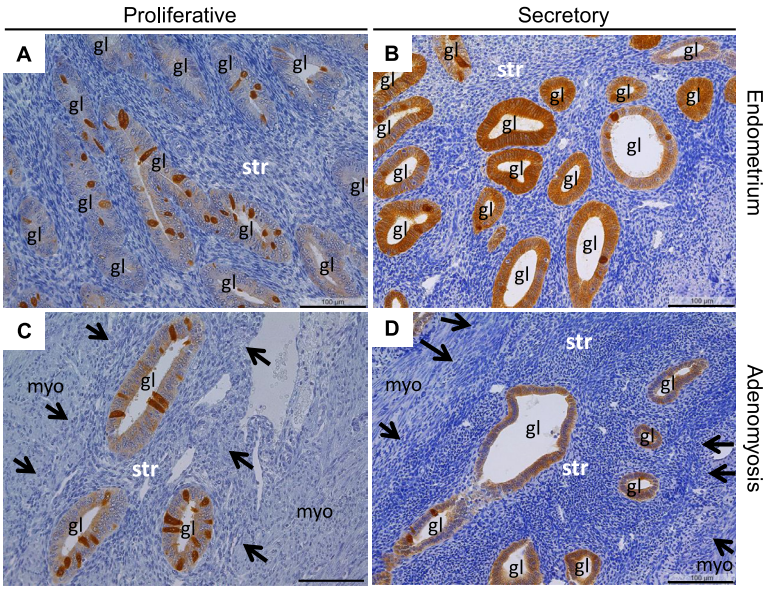

FIGURE 4 Immunohistochemical detection of calcyphosine in endometrial and adenomyosis tissue. Calcyphosine is localized in proliferative (A, C) and secretory (B, D) endometrium with endometriosis and adenomyosis (A, B) and adenomyosis only tissue (C, D). The adenomyotic stains (C, D) correspond to the endometrial stains from the same patient (A with C, B with D). Haematoxylin was used for counterstaining. Magnification A–F 20x, scale bars 100 μm; gl, gland; myo, myometrium; str, endometrial stroma.

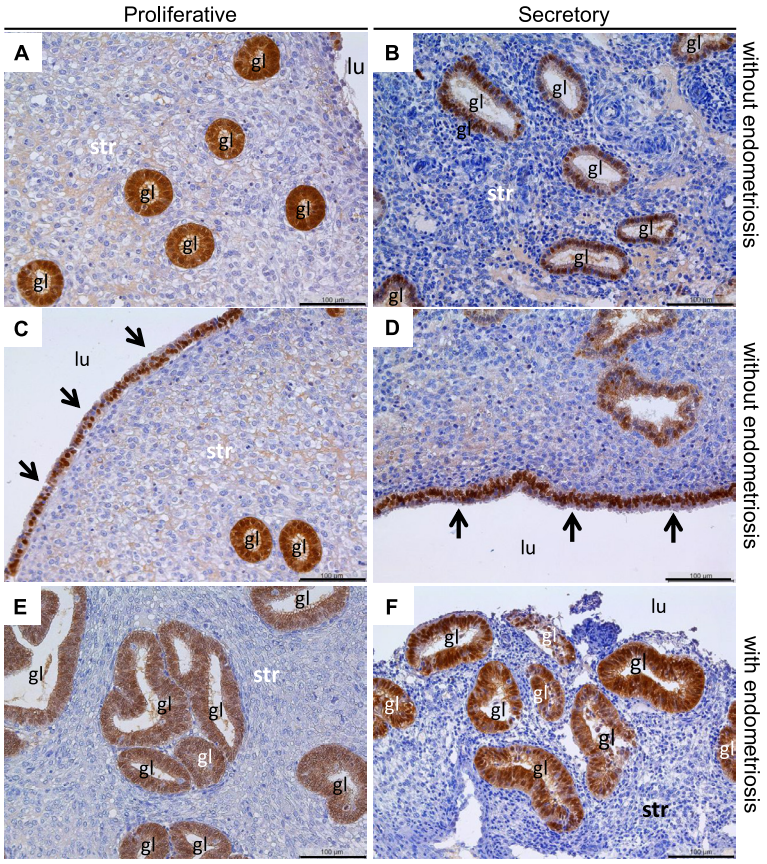

FIGURE 5 Immunohistochemical detection of msh homeobox 1 in the endometrial tissue from healthy and women with endometriosis only. Msh homeobox 1 was found in proliferative (A, C, E) and secretory (B, D, F) endometrium without endometriosis (A–D) or with endometriosis (E, F). The luminal stains (C, D, arrows) correspond to the endometrial stains (A with C, B with D). Haematoxylin was used for counterstaining. Magnification AF 20x, scale bars 100 μm; gl, gland; lu, lumen; str, endometrial stroma.

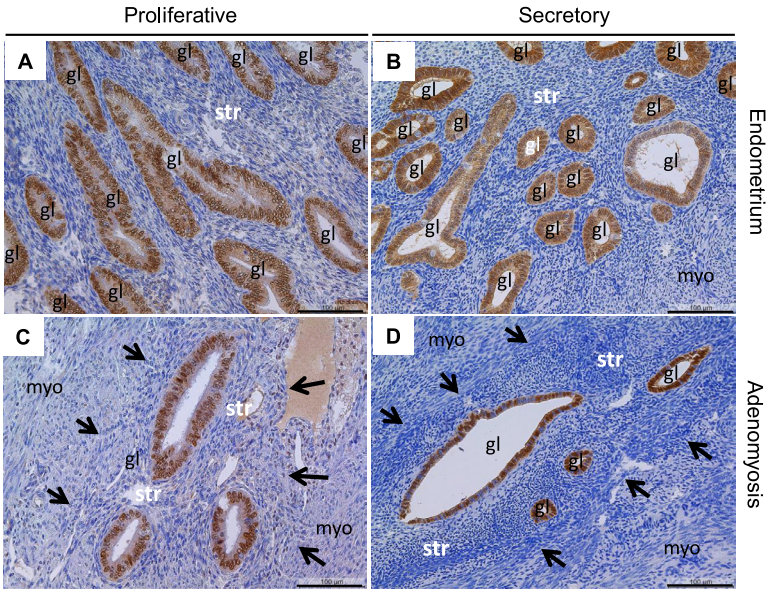

FIGURE 6 Immunohistochemical detection of msh homeobox 1 in endometrial and adenomyosis tissue. Msh homeobox 1 was localized in proliferative (A, C) and secretory (B, D) endometrium with endometriosis and adenomyosis (A, B) and adenomyosis only tissue (C, D). The adenomyotic stains (C, D) correspond to the endometrial stains (A with C, B with D). Hematoxylin was used for counterstaining. Magnification A–D 20x, scale bars 100 µm; gl, gland; myo, myometrium; str, endometrial stroma.

human endometrium and additionally showed only positivity in Langerhans cells and nerve cells in the brain.

The calcyphosine gene encodes a Ca^{2+}-binding protein and the crystal structure analysis revealed a new EF-hand-containing family involved in both Ca^{2+}-phosphatidylinositol and cyclic AMP signalling (*Dong et al., 2008*) as well as in other cellular functions like proliferation, colony formation, migration and invasion (*Shao et al., 2016*). Increased protein abundance might be correlated with endometrial carcinoma (*Li et al., 2008*), colorectal cancer (*Shao et al., 2016*) and oesophageal squamous cell carcinoma (*Li et al., 2017*). In contrast, a decrease in the presence of CAPS protein was suggested to be associated with metastatic lung adenocarcinoma (*Yang et al., 2018*). Recently, CAPS mutations have been shown to be involved in unexplained recurrent pregnancy loss (*Pan et al., 2019*).

Msh homeobox 1 is part of a highly conserved family of homeobox-containing genes encoding transcription repressors (*Ramos and Robert, 2005*). During embryogenesis, MSX1 is expressed mainly at sites of epithelial–mesenchymal interactions, is involved in neural development (*Ramos and Robert, 2005*), odontogenesis, palatogenesis

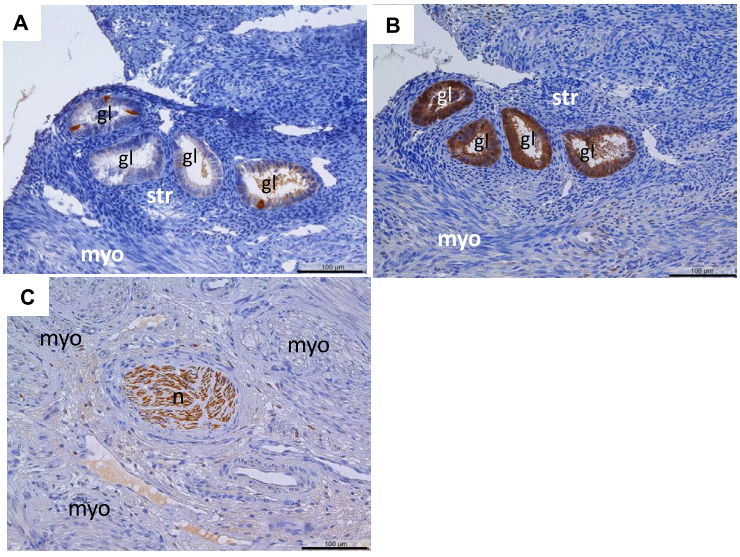

FIGURE 7 Immunohistochemical localization of calcyphosine and msh homeobox 1 (MSX1) in adenomyotic glands. Calcyphosine (A) and MSX1 (B, C) can be found in adenomyotic glands from a woman without endometriosis in the proliferative phase (A, B). A strong staining of a nerve fibre in the myometrium by MSX1 is shown (C). Haematoxylin was used for counterstaining. Magnification A–C 20x, scale bars 100 µm; gl, gland; myo, myometrium; n, nerve fibre; str, stroma.

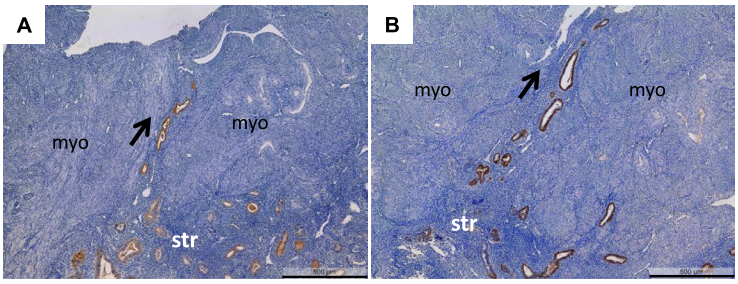

FIGURE 8 Immunohistochemical localization of calcyphosine and msh homeobox 1 in the endometrium and myometrium. (A) Calcyphosine and (B) msh homeobox 1 in the endometrial proliferative glands are beginning to invade the myometrium as denoted by arrows. The patient showed adenomyosis but no endometriosis. Haematoxylin was used for counterstaining. Magnification A, B 5x, scale bars 500 µm. myo, myometrium; str, endometrial stroma.

(*Liang et al., 2016*) and primordial germ cell migration in the mouse (*Sun et al., 2016*). Remarkably, conditional ablation of MSX1 in the mouse uterus leads to female infertility owing to a failure in implantation because of constant proliferation of the uterine epithelium and failure to attach to the embryo (*Nallasamy et al., 2012*). Similarly, reduced expression of MSX1 in infertile patients in the secretory phase was observed (*Bolnick et al., 2016*). In accordance with our results, MSX1 was also described as a highly specific endometrial gene (*Xu et al., 2016*).

Analysis of the tissue specificity of CAPS and MSX1 showed a high endometrial specificity, as can be seen in the www.proteinatlas.org database and in our analysis of the tissue array. The detection of CAPS and MSX1 in the Fallopian tube is not in conflict with our results, because endosalpingiosis in the myometrium is extremely rare (*Yiğıt et al., 2014*) and can be distinguished by CD10, which is not expressed in the Fallopian tube but in adenomyosis (data not shown).

We found a high abundance of CAPS (range 63.0–98.3%) and MSX1 (range 87.1–99.3%) in glands of healthy endometrium, in endometrium with endometriosis and in adenomyosis. Of note, we could not identify significant differences between healthy endometrium and endometrium with endometriosis in the expression of the two proteins. This finding is interesting and is in accordance with genome-wide studies such as mRNA/cDNA (*Fassbender et al., 2012*), methylation patterns (*Wang et al., 2019*), subtractive hybridization (*Meola et al., 2010*) and miRNA (*Braza-Boïls et al., 2014*), who also found a high identity of healthy endometrium compared with endometrium with endometriosis. Furthermore, *Dior et al. (2019)* also found no differences in gene expression between endometrium from women with and without adenomyosis and after adjusting for cycle phase and the presence or absence of endometriosis. In contrast, the healthy endometrium and endometrium with endometriosis have often been reported as different (*Benagiano et al., 2014*).

To date, no medical therapy is available to treat the symptoms of adenomyosis, which still allows patients the chance to conceive (*Pontis et al., 2016*). The failure to find an appropriate medical treatment was recently attributed to the lack of understanding of the natural history of adenomyosis, and it was suggested that fibrogenesis and EMT to myofibroblasts are a possible cause for the pathogenesis (*Guo and Groothuis, 2018*). Although this is an important aspect, we believe that this represents the endpoint of the disease, and other causes such as invasion should be further elucidated. Recently, it was shown by microarray analysis that the eutopic endometrium in patients with adenomyosis has abnormalities such as apoptosis, steroid hormone responsiveness and genes involved in extracellular matrix remodelling that may predispose to invasion and survival beyond the myometrial interface (*Herndon et al., 2016*). Therefore, we suggest that medical treatments in the future should concentrate on inhibition or attenuation of myometrial infiltration as shown recently, e.g. for resveratrol in a mouse model of adenomyosis (*Zhu et al., 2015*).

Although the invagination or implantation hypothesis is preferred by most investigators (*García-Solares et al., 2018*), alternative hypotheses such as metaplasia (*García-Solares et al., 2018*), stem cells (*Gargett, 2007*), pale cells (*Ibrahim et al., 2015*) or EMT have been suggested (*García-Solares et al., 2018*).

Processes like metaplasia, EMT or stem cells and pale cells, however, require the differentiation into two distinct and opposite cellular phenotypes, epithelium and stroma. Furthermore, it remains unclear whether this process starts from one cell type, e.g. stem cells or pale cells, or rather from two cell types, which then undergo differentiation or metaplasia into two distinct cell types (stromal and epithelial). Additionally, our study showed that adenomyotic and endometrial glands share a high abundance of CAPS and MSX1. Consequently, stem cells, pale cells or myometrial cells undergoing differentiation must then also acquire protein expression of MSX1 and CAPS. To summarize, the hypotheses of stem cells and pale cells, metaplasia and EMT must explain how two distinct and clearly different cell types (epithelial and stromal) arise from precursor(s), and how these cells and glands in most people acquire a similar protein expression of CAPS and MSX1 as shown in this study. To the best of our knowledge, no hypothesis of how this might be achieved has been presented. In our opinion, these scenarios are highly unlikely compared with the implantation hypothesis, in which epithelial and stromal cells co-migrate and co-invade the myometrium.

On the basis of our results of a high similarity of adenomyotic and endometrial glands as shown by CAPS and MSX1, we propose that, in most cases of adenomyosis, the endometrium is the most important source of adenomyotic glands. Therefore, the invasion hypothesis (of endometrial glands and stroma) seems to be the best hypothesis to satisfactorily explain our results.

In conclusion, although we cannot exclude alternative hypotheses, such as metaplasia or stem cells, including pale cells, our results suggest that the invasion hypothesis explains the overwhelming amount of cases of adenomyosis. Our findings pave the way to focus on adenomyosis as an invasive disease comparable to cancer and thus prevention of invasion of endometrial glands into the myometrium might be feasible.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2019.11.007.

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