Glucose transporter expression in eutopic endometrial tissue and ectopic endometriotic lesions

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Abstract

Endometriosis is an extremely prevalent disorder characterized by the growth of endometrial tissue at ectopic locations. Glycolysis is an energy-producing mechanism that occurs in almost all cells and requires an adequate uptake of glucose mediated by glucose transporter (GLUT) proteins. At present, however, very little is known about their expression in either the endometrium or the endometriotic lesions. The objective of this study was to examine the expression of *SLC2A* genes in the endometrium of women with and without endometriosis and in the matching ectopic tissue, and to confirm the presence of the GLUT proteins in ectopic lesions. There was a significantly higher expression of *SLC2A3* and a significantly lower expression of *SLC2A4* in women with endometriosis compared with those without. In women with endometriosis, the ectopic expression of *SLC2A3*, *SLC2A4* and *SLC2A5* was significantly higher than that observed in the matching eutopic tissue. GLUT1 protein expression was present in both epithelial and stromal cells and GLUT3 was confined to CD45-positive leukocytes. GLUT4 expression was strong in both ectopic epithelial and stromal cells and localized to the cellular membrane in epithelial cells. These results show that GLUT expression is altered between eutopic and ectopic tissue and between women with and without endometriosis, and that GLUT4 may represent a significant entry route for glucose into the endometriotic epithelial cells. The inducible nature of GLUT4 and its limited cellular expression may make GLUT4 an attractive target for non-hormone-based treatments of endometriosis.

Key Words

- ▶ endometriosis
- ▶ GLUT4
- ▶ endometrium
- ▶ glucose
- ▶ SLC2A

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Introduction

Endometriosis is an extremely prevalent estrogen-dependent gynecological disorder affecting at least 10% of reproductive-aged women worldwide (Eskenazi & Warner 1997). It is characterized by the growth of endometrial tissue outside the uterine cavity and can result in severe

pelvic pain (Evans *et al.* 2007) and subfertility (D'Hooghe *et al.* 2003). Currently, the most widely accepted theory of endometriosis etiology purports that retrograde menstruation allows the implantation of viable endometrial cells at ectopic sites (Sampson 1927, Halme *et al.* 1984). Ectopic

lesions then secrete chemotactic molecules that recruit immune cells, further stimulating the continued growth of the lesion.

Glycolysis is an energy-producing mechanism that requires an adequate supply of intracellular glucose uptake mediated by the glucose transporter (GLUT) proteins encoded by the *SLC2A* gene family. These proteins transport glucose into the cell via a facilitated diffusion down a concentration gradient (Scheepers *et al.* 2004). Cloning of the first gene to encode a GLUT protein occurred in 1985 (Mueckler *et al.* 1985), after which a further 13 related members of the *SLC2A* gene family were subsequently identified in human (Uldry & Thorens 2004).

Little information is available on GLUT expression in both endometrium and endometriotic lesions. GLUT1 is a ubiquitously expressed protein, responsible for the basal glucose uptake and is the most prominent GLUT in the endometrium (Frolova & Moley 2011b). The high-affinity GLUT3 transporter has also been reported in eutopic endometrial tissue, although its expression is confined to CD45-positive leukocytes (von Wolff *et al.* 2003, Korgun *et al.* 2005). The insulin-sensitive GLUT4 has also been identified in endometrial tissue, but with contradictory results. No RNA expression was detected in endometrial biopsies (von Wolff *et al.* 2003) or in isolated endometrial stromal cells (Frolova & Moley 2011a), although it has been detected in epithelial endometrial cells (Mioni *et al.* 2004, Mozzanega *et al.* 2004). The mRNA expression of the *GLUT6*, *GLUT8*, *GLUT10* and *GLUT12* genes has also been identified in a single study (Frolova & Moley 2011b), although their role in the endometrium has not been examined.

The objective of this study was therefore to examine both the eutopic endometrial tissue of women with and without endometriosis, as well as matching ectopic endometriotic lesions to determine the expression of the *SLC2A* genes and their cellular localization.

Subjects and methods

Sample collection and patient data

Women with idiopathic infertility or pelvic pain were recruited for the study, and prior to laparoscopic surgery, informed consent, patient details and medication history were collected. All laparoscopies were performed in the proliferative phase of the menstrual cycle, and during surgery any endometriotic lesions identified were removed and their location noted. In a subset of patients,

endometrial biopsies were also collected using a soft curette (Pipelle-de-Cornier, Laboratoire CCD, Paris, France) and were split into two groups based on the presence or absence of histologically-confirmed endometriosis. No endometrial biopsies were included if the patients reported hormonal medication use in the 3 months prior to surgery. All tissue collected was stored in RNAlater at -80°C until further use.

In cases where an ectopic endometrial lesion was identified, irrespective of whether a matching biopsy was collected, all ectopic tissue removed during surgery and collected and stored in RNAlater (Invitrogen Life Technologies) at -80°C for subsequent analysis, as was the peritoneal fluid. For subsequent analysis of ectopic tissue, all samples collected were first split into groups based on hormonal treatment including no hormonal medication, estrogen- or progesterone-based hormonal contraceptives (HCs), or gonadotropin-releasing hormone analogs (GnRHa). In addition, within the no hormonal treatment cohort, an additional three groups based on lesion location were established (peritoneal, ovarian and recto-vaginal septum (RVS)). As only two lesions with no hormonal treatment were available from the RVS group, this region was excluded from the statistical analysis.

The presence of endometriosis was confirmed by histological examination, and the menstrual stage of women not using hormonal medication was confirmed by measuring progesterone in the peritoneal fluid with a RIA (Coata-count, DPC; Buhlmann Laboratories, Allschwil, Switzerland) (Supplementary Figure 1, see section on supplementary data given at the end of this article). The cutoff value was 27 nmol/l. Institutional Review Board approval was obtained from the ethical committee prior to the commencement of the study.

Analysis of *SLC2A* gene expression in endometrial and endometriotic tissue

For both the eutopic endometrial biopsies and ectopic lesions, ~ 30 mg tissue was excised and homogenized in the FastPrep 120 tissue homogenizer (30 s at 4.0 m/s) in cell lysis buffer (Qiagen). The remaining RNA isolation was performed with the RNeasy Mini Kit (Qiagen) and TurboDNase (Ambion, Life Technologies, Zug, Switzerland) for genomic DNase digestion. One microgram of total RNA was reverse transcribed in a 25 μl reaction with the Moloney murine leukemia virus reverse transcriptase (Promega) and random primers. The resulting cDNA was diluted 1:20 and the absence of genomic DNA confirmed with a reverse transcriptase-negative control.

Table 1 Primer sequences used for real-time PCR

Gene	GenBank no.	Forward	Reverse	Product size (bp)
<i>GAPDH</i>	NM_002046	TGCACCACCACTGCTTAGC	GGCATGGACTGTGGTCATGAG	86
<i>ACTB</i>	NM_001101	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	96
<i>YWHAZ</i>	NM_003406	ACTTTTGGTACATTGTGGCTTCAA	CGCCAGGACAAACCAGTAT	92
<i>RPL13A</i>	NM_012423	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTGTCAA	125
<i>SLC2A1</i>	NM_006516	AAGGTGATCGAGGAGTTCTACA	ATGCCCCCAACAGAAAAGATG	119
<i>SLC2A2</i>	NM_000340	TTGCTGGAAGAAGCATATCAGG	TGACTAATAAGAATGCCGTGAC	148
<i>SLC2A3</i>	NM_006931	GTTCCCTCACTGGATGAAA	TATTTGGATGGCTCTCCAC	95
<i>SLC2A4</i>	NM_001042.2	CGTCGGGCTTCCAACAGATA	CGCAGAGAACACAGCAAGGA	89
<i>SLC2A5</i>	NM_003039	CAAGAAAGCCCTACAGACGC	AACAGCTTCAGCACGGAGAT	117
<i>SLC2A7</i>	NM_207420	TGTGCAGGCATCTCCTACAG	CGAAAACCTCGGTCATTGTT	97
<i>SLC2A8</i>	NM_014580.3	GTCCTCACCAACTGGCTCAT	CAAGCCAGAAGGCTCCATAG	91

The quantitative real-time qPCR was performed with the Rotor-gene SYBR Green PCR Kit (Qiagen) in a Rotor-gene RG 2000 (Corbett Research, Sydney, NSW, Australia) under the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. Primers were purchased from Microsynth (Balgach, Switzerland), and the sequences are defined in Table 1. Specificity of amplification was confirmed by melt curve analysis.

The four most stable reference genes among all samples were selected via the geNORM Software program (Zwijnaarde, Ghent, Belgium) and the geometric mean of all four reference genes was used to normalize the expression of the genes of interest (Vandesompele *et al.* 2002). The reaction efficiency of each assay was determined via linear regression (Ruijter *et al.* 2009) and the relative mRNA expression calculated with the qBASE-plus Software (Biogazelle, Zwijnaarde, Belgium).

The comparison between *SLC2A* gene expression in eutopic endometrium from women with and without endometriosis and the comparison between eutopic expression and ectopic expression in matching samples from women with endometriosis were both performed using the non-parametric Mann–Whitney *U* test, as was the mRNA comparison between peritoneal and ovarian lesions. The comparison between hormonal treatments was performed with a non-parametric one-way ANOVA test with a *post-hoc* Dunn's multiple comparison tests for each group.

Immunodetection in endometriotic lesions and surrounding tissue

After RNA had been harvested from the ectopic tissue, any remaining parts of the samples were fixed in formalin and embedded into paraffin (FFPE) blocks. Previous studies have confirmed the effectiveness of using tissue stored in RNAlater for immunohistochemistry (Florell *et al.* 2001). For imaging studies, serial sections of 4 µm thickness were

cut from each FFPE block and mounted onto glass slides (SuperFrost, Braunschweig, Germany). Prior to analysis, the sections were dewaxed in xylene and rehydrated through a series of ethanol. Epitope retrieval for all antibodies was performed via heating slides in 10 mM citrate buffer at pH 5.5 for 5 min in a 450 W microwave oven. Endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide (H₂O₂) for 10 min and non-specific binding blocked by incubation with 3% BSA in Tris-buffered saline (TBS; Tris 100 mM, NaCl 0.15 M; pH 7.4) for 30 min.

Primary antibody incubations were performed overnight in TBS containing 3% BSA with the following dilutions: 1:50 for rabbit anti-human GLUT1 and rabbit anti-human GLUT4, 1:25 for rabbit anti-human GLUT3, and 1:75 for rabbit anti-human CD45 (all from Abcam, Cambridge, UK). After incubation, slides were washed with TBS containing 0.1% Tween 20 (TBST) and subsequently incubated with affinity purified, biotin-conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark) for 90 min at room temperature. After washing, the sections were incubated with avidin–biotin–HRP complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 45 min. Detection of bound antibody was via 1–5 min incubation at room temperature with 3,3'-diaminobenzidine substrate. Slides were counterstained with hematoxylin and mounted in Aquatex (Merck). Negative controls were performed in the absence of primary antibody. Immunostained slides were photographed with a Nikon Eclipse E800 microscope (Nikon, Japan) at a magnification of 20× and 60×, and the endometriotic lesions, large nerve fiber trunks and blood vessels were identified via the hematoxylin staining. Individual leukocytes were identified via CD45 reactivity.

For immunofluorescence, the sample was prepared exactly as described for the immunohistochemistry and co-staining was performed by incubating the sections

with both the rabbit anti-human GLUT4 (1:25) and the goat anti-human cytokeratin 19 (CK19; 1:500 dilution, Santa Cruz Biotechnology) antibody in 3% BSA solution of TBS. Secondary antibodies were anti-goat IgG Cy5 (Millipore, Zug, Switzerland) and anti-rabbit IgG Alexa-Fluor 488 (Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and slides mounted with Prolong Gold Antifade (Invitrogen). Images were photographed with the Zeiss LSM 710 Confocal Microscope. Negative controls were performed in the absence of the primary antibodies.

Results

Sample collection

Endometrial biopsies were collected from 13 women who were confirmed to have no visible endometriotic lesions and 15 women with endometriosis. There was no significant difference in either the age or the BMI between the two groups (Table 2). We also collected a total of 18 ectopic lesions from the 15 endometriotic women, as multiple ectopic samples were collected from two women. One woman had three lesions, two of which were peritoneal and one in the RVS; whereas the second woman had two lesions, one each from the peritoneal cavity and the ovary.

Additional ectopic tissue without matching eutopic biopsy was collected; 48 samples in total. Of these samples, 24 were from women with no hormonal treatment and 12 of these lesions were located in the peritoneal region, ten on the ovaries, and only two in the RVS. To determine the effect of hormonal treatment on *SLC2A* gene expression, a further 14 samples were available from

women taking estrogen- or progesterone-based HC and ten women using GnRHa.

For the immunoanalysis of ectopic lesions, sufficient remaining tissue was available from 19 patients. These samples included eight from women who were not using hormonal treatments, six using HC, and five using GnRHa. The tissue collected for this study is listed in Table 2.

SLC2A gene expression in eutopic and ectopic endometrial tissue

There was a significantly increased expression of *SLC2A3* in the endometrium of women with endometriosis compared with those without. By contrast, there was a significantly decreased expression of *SLC2A4* in the endometrial tissue of women with endometriosis compared with women without endometriosis (Fig. 1A). No significant difference was observed between the two groups for any of the other genes examined. No expression of *SLC2A2* was observed in either group.

In women with endometriosis, there was a significant increase in *SLC2A3*, *SLC2A4* and *SLC2A5* observed in the ectopic lesions compared with their matching eutopic endometrium (Fig. 1B). No significant variation was observed in *SLC2A1*, *SLC2A7* and *SLC2A8*. No expression was observed for *SLC2A2* in any of the ectopic or eutopic samples.

SLC2A gene expression in endometriotic tissue from different locations and under hormonal treatments

The comparison of mRNA expression of the *SLC2A* family in different regions was only performed in samples

Table 2 Clinical data and sample parameters

	Eutopic tissue			Ectopic tissue			
	<i>n</i>	Age	BMI	Total	P	O	RVS
RNA analysis							
Healthy eutopic endometrium	13	30.2 ± 1.7	24.4 ± 1.7				
Matching eutopic and ectopic tissue	15	33.3 ± 1.8	23.7 ± 1.3	18 ^a	9	7	2
<i>P</i> value		0.2339	0.7522				
Ectopic samples	–			48	15	24	9
No hormonal treatment	–			24	12	10	2
Estrogen/progesterone	–			14	1	9	4
GnRHa	–			10	2	5	3
Protein analysis							
Ectopic samples	–			19	4	9	6
No hormonal treatment	–			8	3	3	2
Estrogen/progesterone	–			6	1	3	2
GnRHa	–			5	0	3	2

P, peritoneal; O, ovarian; RVS, recto-vaginal septum.

^aSome eutopic samples have multiple matching ectopic samples.

Figure 1

SLC2A gene expression in the eutopic and ectopic endometrial tissue of women with and without endometriosis. (A) A significantly higher expression of *SLC2A3* was observed in women with endometriosis (0.38 ± 0.11 , $n=15$) compared with those without endometriosis (0.13 ± 0.04 , $n=13$, $P=0.0448$). A significantly lower expression of *SLC2A4* was observed in women with endometriosis (0.13 ± 0.02 , $n=15$) compared with those without endometriosis (53.87 ± 21.07 , $n=13$, $P<0.0001$). No significant difference for *SLC2A1*, *SLC2A5*, *SLC2A7* or *SLC2A8* was observed between women with or without endometriosis. No expression in either group was observed in *SLC2A2*. (B) The eutopic expression of *SLC2A3* ($Eu=0.38 \pm 0.11$, $n=15$ vs $Ec=1.98 \pm 0.51$, $n=18$; $P<0.0001$), *SLC2A4* ($Eu=0.13 \pm 0.02$, $n=15$ vs $Ec=2.88 \pm 1.03$, $n=18$; $P<0.0001$), and *SLC2A5* ($Eu=0.15 \pm 0.07$, $n=15$ vs $Ec=0.27 \pm 0.10$, $n=18$; $P=0.0464$) was significantly lower than the paired ectopic expression. No significant change was observed in *SLC2A1*, *SLC2A7* and *SLC2A8*. All values are expressed as mean $\pm$ s.e.m. * $P<0.05$ and **** $P<0.0001$.

from women who had not taken hormonal medication. Results show a significantly higher expression of both *SLC2A1* and *SLC2A8* in the peritoneal lesions compared with ovarian lesions (Fig. 2A). No significant difference was observed between any of the other genes examined.

An analysis on the effect of hormonal treatment on *SLC2A* gene expression found no significant variation between any of the genes examined (Fig. 2B).

GLUT1, GLUT3 and GLUT4 protein expression and their localization in endometriotic lesions and surrounding tissue

In the endometriotic lesions, there was strong immunoreactivity of GLUT1 in both the epithelial and the stromal

cells (Fig. 3A). In the stromal cells, immunoreactivity was predominantly nuclear, whereas epithelial cells showed both nuclear and membranous localization (Fig. 3B). GLUT3 showed only sparse immunoreactivity in scattered cells throughout the endometriotic lesions (Fig. 3C and D). The GLUT3 immunoreactivity coincided with the immunoreactivity observed for CD45 (Fig. 3E and F, black arrows). GLUT4 showed strong immunoreactivity in both the epithelial and the stromal cells of the endometriotic lesions (Fig. 3G). In the stromal cells this immunoreactivity appeared predominantly nuclear whereas the epithelial immunoreactivity was both nuclear, and membranous (Fig. 3H). No background signal was observed in the negative controls (Fig. 3I and J).

As dissected, endometriotic lesions will include structures other than endometrial tissue. Non-endometrial tissue, including blood vessels (Fig. 4) and large nerve fiber trunks (Fig. 5) were also examined in these samples. Strong GLUT1 immunoreactivity was observed in the endothelial cells lining the blood vessels surrounding the endometriotic lesions (Fig. 4A). This immunoreactivity appeared to be predominantly nuclear, with some membranous localization (Fig. 4B). GLUT3 showed very limited immunoreactivity in the cells surrounding the blood vessels (Fig. 4C and D). GLUT4 immunoreactivity was

Figure 2

SLC2A gene expression in endometriotic lesions from different locations and under hormonal treatment. (A) A significantly stronger expression of *SLC2A1* (peritoneal = 2.93 ± 0.69 , $n=12$ vs ovarian = 1.23 ± 0.49 , $n=10$; $P<0.011$) and *SLC2A8* (peritoneal = 4.71 ± 2.03 , $n=12$ vs ovarian = 1.71 ± 1.37 , $n=10$; $P<0.019$) was observed in peritoneal lesions compared with ovarian lesions. No significant variation was observed in *SLC2A3*, *SLC2A4*, *SLC2A5* and *SLC2A7*. (B) A comparison of *SLC2A* gene expression in the ectopic tissue of women using hormonal treatment found no significant variation in all genes examined. All values represent mean $\pm$ s.e.m. * $P<0.05$.

Figure 3

GLUT protein localization in endometriotic lesions. (A) GLUT1 immunoreactivity was detected in endometriotic lesions. (B) A higher magnification showed that moderate GLUT1 expression was present in both epithelial (black arrow) and stromal cells (red arrow). (C) Sparse GLUT3 immunoreactivity was observed in endometriotic lesions. (D) A higher magnification identified cells with GLUT3 immunoreactivity (black arrows). (E) CD45 immunoreactivity was observed within endometriotic lesions. (F) A higher magnification identified immunoreactivity of CD45 in cells

similar to that of GLUT3 (black arrows). (G) Strong GLUT4 immunoreactivity was observed in endometriotic lesions. (H) A higher magnification shows immunoreactivity is predominantly nuclear in the stromal cells (red arrow) and both nuclear and membranous in the epithelial cells (black arrow). (I and J) Negative control showed limited background staining in either epithelial or stromal cells. (A, C, E, G and I) Scale bars represent 50 μ M and the areas outlined in black represent the region of higher magnification. (B, D, F, H and J) Scale bars represent 10 μ M.

also observed in the endothelial cells lining the blood vessels (Fig. 4E and F). The negative control showed no background signal in the endothelial cells (Fig. 4G and H).

The large nerve fiber trunks showed a positive immunoreactivity to the GLUT1 antibody (Fig. 5A), which appeared to be predominantly nuclear, although with some immunoreactivity also in the cytosol and the membrane (Fig. 5B). GLUT3 showed some weak to moderate immunoreactivity in these large nerve fiber trunks (Fig. 5C), which was predominantly nuclear (Fig. 5D). GLUT4 immunoreactivity was also moderate to strong in the large nerve fiber trunks (Fig. 5E), although this immunoreactivity appeared to be almost exclusively nuclear (Fig. 5F). Negative controls showed no background signal (Fig. 5G and H).

Localization of GLUT4 transporter protein to the membrane of endometriotic epithelial cells

The nuclei of all cells in both the positive (Fig. 6A) and the negative control (Fig. 6B) were identified with DAPI. Immunofluorescence of GLUT4 (Fig. 6C), above the background and autofluorescence observed in the negative control (Fig. 6D), was identified in the epithelial cell membranes. Immunofluorescence of CK19 (Fig. 6E), above the background and autofluorescence observed in the negative control (Fig. 6F), was also observed in the epithelial cell membranes. An overlay of the images showed a significant co-localization (arrow) of the CK19 (red staining) and the GLUT4 (green staining) immunofluorescence in the epithelial membranes (Fig. 6G).

Figure 4

GLUT expression in surrounding blood vessels. (A) Strong GLUT1 immunoreactivity was observed in the endothelial cells surrounding endometriotic lesions. (B) A higher magnification showed that immunoreactivity was nuclear with some membranous staining (black arrows). (C and D) Very little GLUT3 expression was observed in endothelial cells. (E) Moderate GLUT4 immunoreactivity was observed in endothelial cells surrounding the

blood vessel. (F) A higher magnification showed a predominantly nuclear but with some membrane localization (black arrows). (G and H) Negative controls showed limited background immunoreactivity in either epithelial or stromal cells. (A, C, E and G) Scale bars represent 50 μ m and the areas outlined in black represent the region of higher magnification. (B, D, F and H) Scale bars represent 10 μ m.

The absence of significant immunofluorescence in the negative control sample in the same regions confirmed the specificity of both the GLUT4 and CK19 immunofluorescence in the endometriotic epithelial cell membranes (Fig. 6H).

Discussion

GLUT proteins are the principle route for glucose entry into the cells. Very little is known, however, about the expression of these transporters in endometrial tissue and even less in endometriotic lesions. The results of this study show a significant variation in the expression of both the *SLC2A3* and *SLC2A4* genes in the eutopic endometrial tissue of women with and without endometriosis. It also reports an upregulation of *SLC2A3*, *SLC2A4* and *SLC2A5* in the ectopic tissue compared with the matching eutopic tissue. Further analysis of the GLUT1, GLUT3 and GLUT4 protein expression indicates that GLUT1 expression was present in endometriotic lesions, GLUT3 expression was restricted mainly to CD45-positive leukocytes and large nerve fiber trunks, and GLUT4 was expressed extensively in the ectopic endometrial epithelial and stromal cells with a strong expression in the membrane of the epithelial cells. The increased expression of *SLC2A4* and the localization of GLUT4 to the epithelial cell membranes

could allow GLUT4 to provide a significant route of glucose entry into endometriotic epithelial cells.

In eutopic endometrial tissue, we found a significant variation in the gene expression of *SLC2A3* and *SLC2A4* between women with and without endometriosis. *SLC2A3*, which was found to be significantly increased in women with endometriosis, is expressed in endometrial macrophages (von Wolff *et al.* 2003) and it is possible that the increase in *GLUT3* gene expression in the endometrium of women with endometriosis observed in this study was due to the increased number of macrophages found in the endometrium of these women (Ota *et al.* 1996). By contrast, GLUT4 was significantly reduced. The variation in *SLC2A4* expression between women with and without endometriosis is difficult to interpret in the context of older studies as it has not previously been examined in women with endometriosis, but in women without endometriosis, both the presence (Mioni *et al.* 2004, Mozzanega *et al.* 2004) and absence (von Wolff *et al.* 2003) of GLUT4 has been reported. It is also possible that these large variations and contradicting results for GLUT4 expression may be due to factors not controlled in this or other studies, such as serum insulin or glucose levels. The large difference observed, however, represents an interesting finding and a further investigation of the difference in *SLC2A4* expression between women with and

Figure 5

GLUT expression in surrounding nerves. (A) Moderate GLUT1 immunoreactivity was observed in large nerve fiber trunks surrounding endometriotic lesions. (B) A higher magnification showed that immunoreactivity was both nuclear and membranous (black arrows). (C) Weak to moderate GLUT3 immunoreactivity was observed in large nerve fiber trunks. (D) A higher magnification showed that immunoreactivity was predominantly nuclear (black arrows). (E) Moderate to strong GLUT4

immunoreactivity was observed in the large nerve fiber trunks. (F) A higher magnification showed almost exclusive nuclear localization (black arrows). (G and H) Negative control showed limited background staining in either epithelial or stromal cells. (A, C, E and G) Scale bars represent 50 μ M and the areas outlined in black represent the region of higher magnification. (B, D, F and H) Scale bars represent 10 μ M.

without endometriosis, taking into account factors such as the insulin and glucose serum concentrations, should be performed on a larger sample size.

An analysis of gene expression in ectopic tissue found a significantly higher expression of *SLC2A1* and *SLC2A8* in the peritoneal lesions compared with the ovarian lesions. The difference supports the notion that endometriotic lesions may be a collection of related but individual diseases (Nisolle & Donnez 1997) and that ovarian and peritoneal lesions may acquire different amounts of glucose or may even utilize different mechanisms to acquire sufficient glucose. In addition, as previous studies have shown that both ectopic lesions and the peritoneal microenvironment can be significantly affected by hormonal treatments (Nirgianakis *et al.* 2013), we also examined whether these had an effect on *SLC2A* expression. No significant variation, however, was observed in the expression of any genes after either HC treatment or GnRHa use, indicating that the effectiveness of these drugs is not involved with a modulation of the metabolic capabilities of the lesions.

In a comparison between the *SLC2A* gene expression in eutopic and matching ectopic tissue, we found a significant upregulation of expression in *SLC2A3*, *SLC2A4* and *SLC2A5*. Of these three genes, there was a large

expression of both *SLC2A3* and *SLC2A4* in the ectopic tissue. *SLC2A5*, although significantly higher in ectopic tissue, still showed a very low expression and thus was not considered for further investigation. By contrast, *SLC2A1* surprisingly showed no significant change in expression event although it is significantly increased by the hypoxic environment created during malignant lesion growth (Goldman *et al.* 2006, Szablewski 2013). We therefore chose to further analyze the presence of *GLUT1*, *GLUT3* and *GLUT4* at the protein level in the ectopic tissue.

SLC2A1 gene expression in endometriotic lesions has not previously been reported; however, the GLUT1 protein expression in benign endometriotic lesions was found in 16–19% of ovarian endometrioma, which increased to 95% in endometriosis-associated clear cell carcinoma (Kato *et al.* 2012). In the ectopic tissue, we found no significant variation in the *SLC2A1* gene expression compared with the matching eutopic tissue, but did find GLUT1 expression in most of the cell types examined with immunohistochemistry. These results in combination suggest that GLUT1 is present in ectopic endometriotic lesions but it is not upregulated, at least at the genetic level, by the change to benign but pathological ectopic endometriotic lesions.

Figure 6

GLUT4 membrane localization in endometriotic epithelial cells. (A, B, E and G) Endometriotic lesions were incubated with both GLUT4 and CK19 primary antibodies and fluorescent-conjugated secondary antibodies. (B, D, F and H) Negative controls were performed in the absence of primary antibodies. (A and B) Nuclei in both the positive and the negative controls were visualized with DAPI. (C) Strong GLUT4 immunofluorescence (green) above the background and autofluorescence observed in (D) the negative control was observed predominantly in apical and basal regions of the

glandular structures of the endometriotic lesions (white arrows). (E) CK19 immunofluorescence above the background and autofluorescence observed in (F) the negative control was also observed in the epithelial cell membranes (white arrows). (G) An overlay of the GLUT4 and CK19 immunofluorescence showed a significantly stronger signal of both GLUT4 and CK19 in the epithelial cell membranes above the background and autofluorescence observed in (H) the negative control. Scale bars represent 50 μ M.

GLUT3 is a high-affinity transporter and is usually expressed in tissue with high metabolic requirements, such as the brain (Shepherd *et al.* 1992). It has also been identified in normal endometrium; however, in these studies it was confined to the CD45-positive cells (von Wolff *et al.* 2003). The immunohistochemistry results of this study indicate that GLUT3 expression was also primarily in the CD45-positive leukocytes that infiltrate the endometriotic lesions, as well as the surrounding large nerve fiber trunks in the ectopic tissue. Increased numbers of immune cells have been detected in endometriotic lesions (Halme *et al.* 1983), and nerve fibers are found proximal to lesions, particularly in the peritoneal cavity and the RVS (Mechsner *et al.* 2007, McKinnon *et al.* 2012). It is hence possible that the increase in *SLC2A3* expression observed in this study is derived from the increased infiltration of leukocytes and nerve fibers and therefore that GLUT3 may not contribute substantially to the glucose requirements of the ectopic tissue.

GLUT4 is an inducible, insulin-sensitive transporter predominantly expressed in skeletal muscle and

adipose tissue. In response to insulin, GLUT4 translocates from intracellular compartments to the plasma membrane, resulting in a rapid increase in glucose uptake (Bryant *et al.* 2002). Once at the membrane, GLUT4 can increase glucose uptake into the cell by 10- to 40-fold (Shepherd & Kahn 1999). The immunohistochemical and immunofluorescence images in this study confirm that the GLUT4 protein is found in significant concentrations in the endometriotic epithelial and stromal cells, and in particular at the epithelial plasma membrane. This expression and membrane localization indicates that GLUT4 has the potential to provide glucose to the lesions and thus may represent an important transporter for glucose entry into ectopic tissue.

In summary, the results of this study show that there is an altered expression of the *SLC2A* genes between women with and without endometriosis, as well as between the eutopic endometrial tissue of women with endometriosis and their matching ectopic lesions. Moreover, we were able to show that there was a significant expression of the GLUT4 transporter at the membrane of

endometriotic epithelial cells. The inducible nature of GLUT4 and its limited cellular expression may make GLUT4 an attractive target for non-hormone-based pharmaceuticals.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0194>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

B M conceived the experimental design, performed experiments and prepared the manuscript. C W and D B contributed to experimental procedures. N A B contributed to the collection of tissue and manuscript editing. J E contributed to tissue collection. M D M supervised the project and edited the manuscript.

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