Expression of ovarian tumour suppressor OPCML in the female CD-1 mouse reproductive tract

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Abstract

Opioid binding protein/cell adhesion molecule-like gene (OPCML) is frequently inactivated in epithelial ovarian cancer, but the role of this membrane protein in normal reproductive function is unclear. The ovarian surface epithelium (OSE) is thought to be the cell of origin of most epithelial ovarian cancers, some of which arise after transformation of OSE cells lining ovarian inclusion cysts, formed during ovulation. We used immunohistochemistry, immunoblotting and quantitative RT-PCR (qRT-PCR) to investigate OPCML expression in the uteri and ovaries of cycling 3-month CD-1 mice, as well as in ovaries from older mice containing inclusion cysts derived from rete ovarii tubules. Immunoblotting showed OPCML bands in uterine, but not whole ovarian or muscle extracts. Strong OPCML immunoreactivity was observed in oviduct, rete ovarii and uterus, whereas in ovary more immunoreactivity was seen in granulosa cells than OSE. No staining was observed in OSE around ovulation sites, where OSE cells divide to cover the site. OPCML immunoreactivity was also weaker in more dysplastic cells lining large ovarian inclusion cysts, compared with normal rete ovarii. No significant changes in Opcml mRNA expression were observed in whole ovarian and uterine extracts at different stages of the cycle. We conclude that murine OPCML is more consistently expressed in cells lining the uterus, oviduct and rete ovarii than in ovary and is not expressed in OSE associated with ovulation sites. This observation supports the hypothesis that a proportion of epithelial ovarian cancers arise from ductal cells and other epithelia of the secondary Mullerian system, rather than the OSE.

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Introduction

Opioid binding protein/cell adhesion molecule-like gene (OPCML) has been identified as a tumour suppressor protein that is frequently inactivated by allele loss and CpG island promoter methylation in ovarian and lung adenocarcinoma (Sellar et al. 2003, Tsou et al. 2007). OPCML, also called opioid-binding cell adhesion molecule (OBCAM), was originally isolated from brain (Schofield et al. 1989), but has also been shown to be expressed in other tissues, including human stomach (Wang et al. 1989), whole ovary (Ntougkos et al. 2005, Teodoridis et al. 2005, Czekierdowski et al. 2006, Chen et al. 2007) and ovarian surface epithelium (OSE; Sellar et al. 2003, Mei et al. 2006), but not in rat kidney or liver (Hachisuka et al. 1996). OPCML belongs to the IgLON limbic system-associated membrane protein (LSAMP, OPCML/OBCAM, neurotrimin) family of IgG domain-containing glycosylphosphatidylinositol-anchored cell adhesion molecules (Lodge et al. 2000, Miyata et al. 2003a, 2003b, Reed et al. 2004, Ntougkos et al. 2005). In vivo, epithelial ovarian cancers show reduced OPCML expression as the result of epigenetic inactivation or mutation (Sellar et al. 2003, Ntougkos et al. 2005). Conversely, transfection of epithelial ovarian cancer cell lines in vitro with sense OPCML transcripts results in reduced rates of culture growth, relative to the parent cell line (Sellar et al. 2003) or to transfected normal CD-1 mouse ovarian surface epithelial cells (Yao et al. 2006), suggesting OPCML functions to control cell proliferation and tumour size. The rodent Opcml cDNA shows > 90% identity and the protein > 98% identity to the human sequences, implying conservation of function between these species (Shark & Lee 1995). However, the role of this protein in normal ovarian tissue remains to be described.

We have examined Opcml mRNA and protein expression in the mouse ovary and uterus, using quantitative real-time RT PCR (qRT-PCR), immunoblotting and immunohistochemistry, with the aim of
characterising expression of OPCML in the normal female mouse reproductive system. We hypothesised that OPCML expression would be reduced in cells undergoing cell division, for example, in the OSE at the edges of recent ovulation sites (Tan & Fleming 2004). Our previous studies demonstrated that ovaries of CD-1 out-bred mice subjected to incessant ovulation (IO) showed an increased number of surface invaginations, stratification of the OSE, cortical inclusion cyst formation and dilation of the rete ovarii tubules, similar to preneoplastic changes in the human ovary (Fleming et al. 2006, 2007). We have therefore investigated OPCML protein immunoreactivity in the cystic ovaries of CD-1 mice subjected to IO and containing large cystic structures, likely to be dilated rete ovarii tubules (Tan et al. 2005), to determine if OPCML expression changes in these cystic structures. As far as we are aware, this is the first report on the localisation of expression of OPCML in the mammalian reproductive system to date.

Results

OPCML immunoblot
The chicken anti-human OPCML MAB showed OPCML immunoreactive bands on western blots of uterine protein extracts, but not in whole ovarian or muscle extracts. Two bands at approximately 42 and 50 kDa were observed (Fig. 1), similar to results previously reported in rat brain (Miyata et al. 2000, Yamada et al. 2007).

OPCML immunohistochemistry

Strong membrane staining in the luminal epithelium and endometrial glands of mouse uterus was observed by immunohistochemistry with the chicken anti-human OPCML antibody (Fig. 2a). In addition, strong staining was seen in the oviduct epithelium (Fig. 2e and f) and on the luminal surfaces of cells lining the rete ovarii (Figs 2d and 3a). Immunostaining intensity in the different ovarian cellular compartments was graded in 3- or 6-month mice at oestrus. Average ±S.D. staining intensities are shown in Table 1. The mouse OSE showed patchy immunoreactivity, with parts of the OSE positively stained and other parts remaining unstained (Figs 2b, c and 3a). All ovaries examined had areas of positive OPCML immunoreactivity in the OSE. More consistent staining was seen in the cytoplasm of granulosa cells of healthy and atretic antral ovarian follicles (Table 1; Figs 2b, c and 3c), especially in fresh ovulation sites, whereas the OSE associated with fresh ovulation sites was negative (Fig. 2c). The granulosa cells of primordial and primary follicles were rarely immunopositive for OPCML (Table 1). The zona pellucida of oocytes stained in some, but not all small and antral follicles (Table 1; Figs 2b and 3c).

OPCML immunoreactive staining was observed in cells lining normal (Fig. 2d) and dilated rete ovarii in the ovaries of 9- and 12-month-old mice subjected to IO (Fig. 3a, c and e). Flattened or ciliated cells and columnar secretory cells lining cystic rete ovarii tubules...
showed less reactivity compared with signet ring cells, with no clear membrane delineation of the immunoreactivity (Fig. 3c–f). OSE stained noticeably weaker than cyst epithelium in some sections (Fig. 3c). Stronger OPCML staining was seen on the cells lining the one cortical inclusion cyst observed in this study (Fig. 3b), primarily on the basal surface of the luminal cell layer.

Quantitative RT-PCR

No difference in the average yield of total RNA (mean ± s.d.) between ovarian and uterine samples was observed (17 ± 10 μg RNA), and samples had an average OD260:OD280 ratio (mean ± s.d.) of 1.93 ± 0.16. Higher expression of Opcml (relative to β-actin) was measured in mouse uterus than whole ovary, but no significant differences in relative Opcml expression were observed in either tissue across the stages of the oestrous cycle (Table 2). There was considerable inter-animal variation in the relative amounts of Opcml expression measured in whole extracts of both tissues.

### Table 1

<table>
<thead>
<tr>
<th>Cellular compartment</th>
<th>Stain intensity</th>
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<tr>
<td>OSE</td>
<td>1 (0–3)</td>
</tr>
<tr>
<td>Granulosa cells in primordial or primary follicles</td>
<td>0 (0–0.5)</td>
</tr>
<tr>
<td>Oocytes/zona pellucida in primordial or primary follicles</td>
<td>1 (0.5–2)</td>
</tr>
<tr>
<td>Granulosa cells in healthy antral follicles</td>
<td>1 (1–3)</td>
</tr>
<tr>
<td>Oocytes/zona pellucida in healthy antral follicles</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Granulosa cells in atretic antral follicles</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Oocytes/zona pellucida in atretic antral follicles</td>
<td>1.5 (1–2)</td>
</tr>
<tr>
<td>Corpora lutea</td>
<td>1 (0–2)</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Ovary</th>
<th>Opcml/β-actin</th>
<th>n</th>
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<tbody>
<tr>
<td>Proestrus</td>
<td>1.02±0.52</td>
<td>5</td>
</tr>
<tr>
<td>Oestrus</td>
<td>1.37±1.04</td>
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</tr>
<tr>
<td>Met/dioestrus</td>
<td>1.32±0.38</td>
<td>7</td>
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<table>
<thead>
<tr>
<th>Uterus</th>
<th>Opcml/β-actin</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Proestrus</td>
<td>1.50±0.53</td>
<td>5</td>
</tr>
<tr>
<td>Oestrus</td>
<td>1.62±0.44</td>
<td>5</td>
</tr>
<tr>
<td>Met/dioestrus</td>
<td>2.22±0.63</td>
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### Discussion

OPCML is a member of the IgLON family of OBCAM (including LSAMP, neuronal growth regulator 1 (NEGR1/Kilon) and neurotrimin), which form homo- or heterodimeric DigLONs that affect cell adhesion and cell signalling (Reed et al. 2004, 2007). The IgLons are most highly expressed in brain and other neural tissue, largely in dendritic membranes, and may modify dendrite growth (Reed et al. 2007). Loss of OPCML mRNA has been reported in sporadic epithelial ovarian cancer (Sellar et al. 2003, Ntougkos et al. 2005), gastric cancer (Wang et al. 2006), gliomas and other brain tumours (Reed et al. 2007) using either qRT-PCR or microarray. The current study of OPCML expression is the first to detail the localisation of immunoreactivity of this protein in the female mammalian reproductive system.

More OPCML expression was detected in uterus than in ovary using a variety of techniques. Expression of OPCML protein has been observed (GC Sellar, unpublished data), but not reported previously in uterus. We could detect no significant changes in expression via free access
through the oestrous cycle, in extracts of whole uterine horn, despite strong epithelial immunoreactivity. When the SymAtlas database (http://symatlas.gnf.org/SymAtlas/) was used to determine the relative expression of Opcml in a variety of tissues in silico, low average levels of expression were recorded for most tissues, including ovary and uterus, in comparison with neural tissues, for both human and mouse. Given the proliferative changes shown in the uterus throughout the cycle, the lack of variation with cycle is surprising, but may have been a result of once a day sampling of tissues. A more detailed study on the expression of OPCML in uterine epithelia and in endometrial carcinoma is warranted. Although two OPCML splice variants, alpha1 and alpha2, have been identified recently in humans (Reed et al. 2007), the primers used to amplify Opcml in ovary and uterus would not have distinguished between the variants.

Immunoblotting with a chicken anti-human OPCML MAB showed two bands at approximately 42 and 50 kDa, in mouse uterine extracts, but not in ovary or skeletal muscle. The size of the murine OPCML protein has been reported as 58 and 51 kDa in membrane preparations of bovine, rat, mouse, guinea pig and rabbit brains (Hachisuka et al. 1996). Two immunoreactive bands of 46 and 51 kDa have also been reported for rat OPCML protein (Miyata et al. 2000). These are thought to be isoforms that differ in the amount or type of glycosylation (Yamada et al. 2007). The lack of immunoreactivity on immunoblots of whole ovarian extracts suggests low concentrations of OPCML protein are expressed, or that expression of the protein is confined to a small number of specific cells in this tissue. Alternatively, the protein extraction methods may decrease epitope availability for the antibody used.

The immunoblotting results contrast with those obtained by immunohistochemistry, in both mouse and human tissues, with the same antibody (GC Sellar, unpublished data). OPCML immunoreactivity was detected inconsistently in mouse OSE by immunohistochemistry, despite staining in granulosa cells of larger follicles, particularly around ovulation sites. The granulosa cell immunostaining appeared not to be membrane delineated, compared with the strong immunoreactivity in uterus, oviduct and rete ovarii tubules. No OPCML immunoreactivity was observed in the OSE at the edges of fresh ovulation sites (Fig. 2c). This is consistent with the protein having a role in the suppression of cell proliferation (Sellar et al. 2003, Ntougkos et al. 2005, Reed et al. 2007, Cui et al. 2008), since cell division is frequently observed in the OSE at the base of ovulation sites (Tan & Fleming 2004).

Normal extra-ovarian rete ovarii tubules showed strong membrane-limited OPCML immunostaining and this immunoreactivity decreased as the rete ovarii tubules dilated and formed cysts, again consistent with the hypothesis that OPCML expression decreases in dividing cells. The more dysplastic cells in cysts showed the least staining, suggesting a loss of epitope. Whilst this mouse model of ovarian inclusion cyst formation does not produce ovarian tumours (Fleming et al. 2007), the changes in OPCML immunoreactivity observed during cyst formation parallel those observed during human epithelial ovarian carcinogenesis (Sellar et al. 2003). These results contrast with those observed for E-cadherin, where ciliated, cuboidal cyst cells maintained strong immunoreactivity (Fleming et al. 2007).

The epithelia of the fimbria and distal fallopian tube have been implicated recently in serous epithelial ovarian carcinogenesis, particularly in carriers of BRCA1 mutations (Medeiros et al. 2006, Callahan et al. 2007, Crum et al. 2007, Kindelberger et al. 2007, Jarboe et al. 2008). The observation of strong OPCML immunoreactivity in the mouse oviduct, but not the OSE, supports the hypothesis that a proportion of epithelial ovarian cancers arise from ductal cells. We conclude from this initial study that a decrease in OPCML expression is associated with sites of increased cell division in the normal and cystic CD-1 mouse ovary. The results obtained in extracts from whole mouse ovary and uterus and the presence of OPCML immunoreactivity in uterine epithelia and granulosa cells suggest further studies of this important tumour suppressor at a cellular, rather than whole tissue, level are warranted.

Materials and Methods

Animals

The University of Otago Animal Ethics Committee approved all experiments. Female out-bred CD-1 (Swiss Webster) mice were housed post-weaning in a temperature- and light-controlled facility with 12h light:12h darkness. Mice were held in cages divided by a screen, alongside a male, to induce continuous ovulation cycles, as previously described (Clow et al. 2002, Tan & Fleming 2004). Stage of oestrous cycle was determined by vaginal cytology before 1000 h, using the lavage method, until regular cycles were detected (Blaustein 1981, Clow et al. 2002). Animals (3–4 months old) were killed with halothane gas and exsanguination on the days of proestrus (n=5), oestrous (n=5), dioestrous (n=3) or metoestrus (n=4). One ovary and samples of uterus were fixed overnight in 4% paraformaldehyde for immunohistochemistry, as described (Fleming et al. 2007). Ovarian and whole uterine horn samples were also frozen in liquid nitrogen and stored at −80 °C for RNA analysis.

Induction of ovarian inclusion cysts with age and IO

CD-1 mice were housed beside, but not in contact with, a male in a divided cage, to induce IO (IO group) and prevent breeding, as previously described (Fleming et al. 2007), from age of weaning until 6, 9 or 12 months of age. All animals were killed and dissected on the afternoon of oestrus (Fleming et al. 2007).
OPCML expression in mouse ovary and uterus

**Immunoblotting**

A chicken anti-human OPCML MAB was used for immunoblot and immunohistochemical analysis. Samples of mouse whole ovary, uterus and skeletal muscle (negative control) were snap frozen, pulsed in liquid nitrogen and the protein extracted as previously described (Fleming et al. 2007). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein extracts (5 and 20 μg protein) were separated on discontinuous 10% SDS-PAGE gels and electroblotted onto PVDF membranes (PVDF, Roche Pharmaceuticals). A 10–250 kDa pre-stained protein marker was loaded onto all gels. Blots were incubated with 10% skim milk powder in PBS, pH 7.4 (PBS) plus 0.1% Tween 20, for 1 h at room temperature to reduce non-specific binding, then probed for 1 h at room temperature with a 1:4000 dilution of OPCML primary antibody in PBS containing 0.1% (v/v) Tween 20 and 3% (w/v) skim milk powder (PBS-TM). Membranes were washed and incubated with HRP conjugated anti-chicken secondary antibody (Amersham Pharmacia Biotech; diluted 1:2000 in PBS-TM) for 1 h at room temperature, prior to detection of bound antibody with the ECL chemiluminescent detection system (Amersham Pharmacia Biotech) according to the manufacturer’s directions.

**Immunohistochemistry**

Serial sections (4 μm) were cut from each ovary and 10–20 sections cut from uterine samples, dewaxed and rehydrated as described (Fleming et al. 2007). Up to 10 sections were stained per tissue sample. Antigen retrieval was performed by boiling in a 700 watt microwave for 10 min in 0.01 M citrate buffer (pH 6.0). Slides were left to stand for 15 min in hot buffer before being washed in PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 5 min and, after further washes in PBS plus 1% (v/v) Triton X-100, non-specific binding was minimised by blocking with avidin-biotin blocking reagents (Zymed Laboratories Inc., San Francisco, CA, USA) and 20% (v/v) normal goat serum (Sigma–Aldrich Inc., 1:20 in PBS, 30 min). Sections were incubated overnight at 4°C with a 1:30 dilution of primary antibody, followed by incubation in biotinylated goat anti-chicken secondary antibody for 30 min (Amersham Pharmacia Biotech, 1/200) in PBS). Further washing in PBS plus 1% (v/v) Triton X-100 was followed by incubation in streptavidin biotinylated HRP for 30 min (Amersham Pharmacia Biotech, 1/100) in PBS. Antibody complex was detected with diaminobenzidine (Vector Laboratories Inc., Peterborough, UK). Sections were counterstained with Gills haematoxylin.

OPCML immunoreactivity was examined in 70 stained 4 μm sections from 20 CD-1 mice (3 or 6 months, in oestrus). The intensity of the immunoreactive staining in a range of cellular compartments in the stained ovarian sections was graded on a range of 0–3, where 0 was negative, 1 was mild, cytoplasmic staining and 3 indicated strong, membrane-situated OPCML immunoreactivity.

**RNA extraction**

Total RNA was extracted from frozen whole ovaries and uteri (30±6 μg tissue), using the Qiagen RNeasy Mini Kit according to manufacturer’s directions (Qiagen Pty. Ltd). RNA concentration and quality were determined by spectrophotometry in an ND-1000 Nanodrop u.v./Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Samples were diluted 1:10 in RNase-free water for concentration measurements and in 10 mM Tris HCl, pH 7.5, for purity measurements. RNA quality was also assessed by electrophoresis of 1 μg each sample through a 1% non-denaturing agarose gel (data not shown). Samples showing signs of RNA degradation were not assessed for Opcml mRNA expression by qRT-PCR.

**RT and semi-quantitative PCR**

Total RNA (1–2 μg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and 200 ng random primers (Invitrogen) in a total volume of 20 μl, at 50°C for 60 min and at 70°C for 15 min. A stock of reference cDNA for qRT-PCR was obtained by reverse transcribing 5 μg total RNA from mouse uterus using random hexamers. A serial dilution of this stock cDNA was used to estimate relative Opcml and endogenous β-actin mRNA concentrations for both ovarian and uterine samples. Primers and FAM-labelled probes for Taqman qRT-PCR of mouse Opcml and β-actin sequences were obtained from Applied Biosystems Assays-on-Demand (Applied Biosystems, Foster City, CA, USA; Mm00625983 m1 and 4352933E corresponding to NM_177906 and NM_007393.1 respectively). The Opcml probes amplified the sequence between exons 3 and 4 of the mouse Opcml gene (Probe database, http://www.ncbi.nlm.nih.gov/).

Opcml mRNA concentration was then normalised relative to the concentration of endogenous β-actin mRNA. Opcml and β-actin cDNA were amplified in the same Taqman run. The mean (± S.D.) correlation coefficient (R) for standard curves was 0.994±0.005. Samples for qRT-PCR analysis were run in duplicate in 20 μl reaction volumes with 300 nM target-specific primers and 200 nM fluorescent probe, using the Taqman Universal PCR mix in an ABI Prism 7000 quantitative gene amplification system (Applied Biosystems). Controls without RT were included in each run to assess the contribution of genomic DNA in the reactions (not detectable; data not shown).

**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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References


