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13. ABSTRACT ***(Maximum 200 Words)***

We implemented an approach toward investigating the development and progression of ovarian cancer that involved "species hopping". This approach made use of strong genotype-phenotype correlations in *Drosophila melanogaster* to identify a fly tumor suppressor gene, *discs large 1* (*dlg1*), whose mouse homologs could serve as candidates for study into the etiology of mammalian ovarian cancer. The proposed study was designed (1) to identify *dlg1* homologs whose gene products were localized to the cells of the ovarian surface epithelium (OSE), (2) to generate mice bearing a systemic deletion of a *dlg1* homolog normally expressed in the OSE, and (3) to characterize the ovarian phenotype resulting from such systemic deletion of the selected homolog. As a result of effort toward accomplishing the first goal, protein products of the mouse genes SAP102 and ZO-1 were identified by immunodetection in granulosa cells, but not in OSE. ZO-3 protein was identified by immunodetection in oviduct lumen epithelium, but not in OSE. Both ZO-2 and CASK proteins were immunodetected in OSE, but were also present in other ovarian cell types. Efforts at localizing the Dlgh1, Dlgh2, Dlgh3, p55 and PSD95 proteins are ongoing and may yet provide a candidate for further study by systemic deletion.

14. SUBJECT TERMS
discs large 1 (*dlg 1*), tumor suppressor genes, epithelial cell junctions, immunohistochemistry, mammalian homologs of a *Drosophila* gene, generation of knockout mice, cDNA macroarray

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INTRODUCTION

Cancerous growth of the germinal epithelium of the human ovary gives rise to the most common and virulent form of human ovarian cancer. Since conventional approaches to investigating the biology of the ovarian surface epithelium have yielded frustratingly slow progress toward understanding the development and progression of ovarian cancer, we have implemented an approach involving "species hopping". The fundamentals of cellular biology and pathology are highly conserved through much of the evolutionary tree. Therefore, we have made use of strong genotype-phenotype correlations in the fruit fly, *Drosophila melanogaster*, to identify a gene whose mouse homologs could serve as candidates for study into the etiology of mammalian ovarian cancer. Homologs of the fruit fly tumor suppressor gene *discs large 1* (*dlg 1*) were identified as such candidates. The present study was designed to determine the ovarian expression of ten mouse *dlg 1* homologs, to identify one or more homologs whose products were localized to the cells of the ovarian surface epithelial layer, to generate mice bearing a systemic deletion of a *dlg 1* homolog normally expressed in the germinal epithelium, and to characterize the ovarian phenotype resulting from such systemic deletion of the select homolog.

BODY

Following submission of the original proposal, two additional mouse homologs of *discs large 1* (*dlg 1*) were identified through computer-based BLAST analysis (1). The 10 genes ultimately identified included those encoding three discs large homologs (Dlgh1, Dlgh2, Dlgh3), a calcium/calmodulin-dependent serine kinase (CASK), an erythrocyte membrane protein (p55), post-synaptic density protein 95 (PSD95), synapse-associated protein 102 (SAP102), and three zona occludens proteins (ZO-1, ZO-2, ZO-3). The expression of these ten genes in mouse ovary homogenate preparations was verified by using RT-PCR analysis.

Task 1. *To determine which of the known mouse* dlg 1 *homologs are expressed in the germinal epithelium of the mouse ovary.*

We have attempted to characterize the protein localization patterns for each *dlg 1* homolog in sections of the mouse ovary by immunohistochemical and immunofluorescent detection methods. Commercially available antibodies were used whenever they were available, and polyclonal antibodies were preferentially chosen for these studies in order to avoid high levels of background and false positive immunolabeling associated with the use of mouse monoclonal antibodies on mouse tissues. The samples probed with the antibodies included paraformaldehyde-fixed ovary cryosections, paraformaldehyde-fixed paraffin-embedded ovary sections, and ovary sections that were processed by a "hydrated heat" antigen retrieval method in which slide-mounted sections were heated in 80°C sodium citrate buffer (pH 6.0) for 30 minutes prior to processing for immunodetection. All referenced figures are located in the appendix.

Dlgh1. A rabbit polyclonal anti-Dlgh1 primary antibody (Affinity BioReagents; Golden, CO) was used in conjunction with a fluorophore-conjugated goat anti-rabbit secondary antibody (Molecular Probes; Eugene, OR) to assess the potential ovarian localization of the Dlgh1 protein. Both cryosections and paraffin-embedded sections of mouse ovary were used as substrates for immunolocalization studies, but neither preparation yielded positive immunoreactivity for Dlgh1. Subsequent antigen retrieval failed to expose any

additional epitopes specific for the Dlgh1 protein. A mouse monoclonal anti-Dlgh1 primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA) and a Mouse-On-Mouse Immunodetection Kit (Vector Laboratories; Burlingame, CA) were also used in a further attempt to identify possible ovarian localization of the protein. This combination also detected no immunoreactive Dlgh1 in the mouse ovary.

Dlgh2. Since no antibodies were available against this protein, Bethyl Laboratories (Montgomery, TX) was contracted to produce a goat polyclonal anti-Dlgh2 primary antibody against a peptide sequence unique to the Dlgh2 protein. This antibody, along with a fluorophore-conjugated donkey anti-goat secondary antibody (Molecular Probes), was used to probe for the Dlgh2 protein in mouse ovary cryosections, paraffin-embedded sections, and paraffin-embedded sections following antigen retrieval. No positive immunoreactivity against Dlgh2 was detected in any of the processed mouse ovary samples.

Dlgh3. Since no antibodies were available against this protein, Bethyl Laboratories was contracted to produce a goat polyclonal anti-Dlgh3 antibody against a peptide sequence unique to the Dlgh3 protein. This antibody, along with a fluorophore-conjugated donkey anti-goat secondary antibody (Molecular Probes), was used to probe for the Dlgh3 protein in mouse ovary cryosections, paraffin-embedded sections, and paraffin-embedded sections following antigen retrieval. No positive immunoreactivity against Dlgh3 was detected in any of the processed mouse ovary samples.

CASK. A mouse monoclonal anti-CASK primary antibody (Chemicon International, Inc.; Temecula, CA), used with the Mouse-On-Mouse Immunodetection Kit (Vector Laboratories), detected no immunoreactive CASK in cryosections of mouse ovary. In contrast, a combination of rabbit polyclonal anti-CASK primary antibody (Zymed Laboratories, Inc.; San Francisco, CA) and fluorophore-conjugated goat anti-rabbit secondary antibody (Molecular Probes) did result in detectable fluorescence throughout ovary cryosections that was somewhat brighter in the cells of the surface epithelial layer. Subsequent antigen retrieval resulted in immunofluorescence associated more strongly with cell membranes, especially noticeable in cells of the surface epithelial layer (Figure 1).

p55. Since no antibodies were available against this protein, Bethyl Laboratories was contracted to produce a goat polyclonal anti-p55 antibody against a peptide sequence unique to the p55 protein. This antibody, along with a fluorophore-conjugated donkey anti-goat secondary antibody (Molecular Probes), was used to probe for the p55 protein in mouse ovary cryosections, paraffin-embedded sections, and paraffin-embedded sections following antigen retrieval. No positive immunoreactivity against p55 was detected in any of the processed mouse ovary samples, including those retaining erythrocytes following fixation.

PSD95. A goat polyclonal anti-PSD95 primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA) was used in conjunction with a fluorophore-conjugated donkey anti-goat secondary antibody (Molecular Probes) to assess the potential ovarian localization of the PSD95 protein. Both cryosections and paraffin-embedded sections of mouse ovary were used as substrates for immunolocalization studies, but neither preparation yielded positive immunoreactivity for PSD95. Antigen retrieval treatment of these sections failed to expose any additional epitopes specific for the PSD95 protein. Brain cryosections, used as positive controls, unfortunately also lacked PSD95 immunoreactivity. A different method

of antigen retrieval, consisting of a pepsin treatment (2), has been used successfully by others to expose PSD95 epitopes in adult mouse brain sections but has not yet been attempted in our preparations.

SAP102. A goat polyclonal anti-SAP102 primary antibody (Santa Cruz Biotechnology) was used in conjunction with a fluorophore-conjugated donkey anti-goat secondary antibody (Molecular Probes) to assess the potential ovarian localization of the SAP102 protein. Immunoreactivity was detected specifically in granulosa cells (Figure 2) and could be blocked by pretreatment of the primary antibody with the immunizing peptide. This immunoreactivity appeared to co-localize at small ring-shaped structures with the gap junction protein connexin43 (Figure 3).

ZO-1. Two goat polyclonal anti-ZO-1 primary antibodies (Santa Cruz Biotechnology) were used in conjunction with a fluorophore-conjugated donkey anti-goat secondary antibody (Molecular Probes) to assess the potential ovarian localization of the ZO-1 protein. One primary antibody was specific for an N-terminal region of the protein while the other was specific for a C-terminal region of the protein. The N-terminal antibody detected no immunoreactive ZO-1 in ovary sections. The C-terminal antibody did localize to small punctate structures in the granulosa cells (Figure 4), similar to the localization of anti-SAP102 antibodies, but this immunoreactivity could not be completely blocked by pretreatment of the primary antibody with the immunizing peptide.

ZO-2. A goat polyclonal anti-ZO-2 primary antibody (Santa Cruz Biotechnology) was used in conjunction with a fluorophore-conjugated donkey anti-goat secondary antibody (Molecular Probes) to assess the potential ovarian localization of the ZO-2 protein. Immunoreactivity was detected in multiple cell types in the mouse ovary sections (e.g. granulosa cells, theca cells, and oviduct lumen epithelial cells), and was strongly and clearly localized to lateral cell membranes of the surface epithelial cells (Figure 5). Immunoreactivity could be blocked by pretreatment of the primary antibody with the immunizing peptide.

ZO-3. Two rabbit polyclonal anti-ZO-3 primary antibodies (Chemicon International, Inc.) were used in conjunction with a fluorophore-conjugated goat anti-rabbit secondary antibody (Molecular Probes) to assess the potential ovarian localization of the ZO-3 protein. One primary antibody was specific for an N-terminal region of the protein while the other was specific for a C-terminal region of the protein. The N-terminal antibody detected no ZO-3 in sections of ovary or attached oviduct. The C-terminal antibody also detected no ZO-3 in ovary sections, but instead was strongly reactive at epithelial cell membranes in the lumen of the oviduct (Figure 6).

Task 2. *To generate mice bearing a systemic deletion of a* dlg 1 *homolog expressed in the germinal epithelium of the mouse ovary.*

Since the two genes expressed in the ovarian surface epithelium (CASK and ZO-2) are also expressed in other cell types in the ovary and in the oviduct, systemic deletion may result in ovarian phenotypes that are unrelated to alterations in the cells of the germinal epithelium. Therefore, these genes are not good candidates for systemic deletion. We continue our investigation for a candidate gene among the five remaining homologs to identify a protein that is surface epithelium-specific or surface epithelium-selective.

Task 3. *To characterize the phenotype of the ovarian germinal epithelium of mice bearing a systemic deletion of a* dlg 1 *homolog.*

As no knockout mice have been generated through this project, this task has not been started.

KEY RESEARCH ACCOMPLISHMENTS

1. The genes CASK and ZO-2 are expressed in the cells of the mouse ovarian surface epithelium. They are also expressed in other cell types within the ovary.

2. The genes SAP102 and ZO-1 are not expressed cells of the mouse ovarian surface epithelium, but are expressed in granulosa cells.

3. The gene ZO-3 is not expressed in cells of the mouse ovarian surface epithelium, but is expressed in epithelial cells lining the lumen of the oviduct.

REPORTABLE OUTCOMES

Presentation (poster). Carrie A. Stoltzman and Eli Y. Adashi. Program #2391, "Ovarian expression of mouse orthologues of the *Drosophila* tumor suppressor, *discs large 1* (*dlg 1*)". American Society for Cell Biology 40th Annual Meeting, December 2000, San Francisco, CA.

Presentation (oral). Carrie Stoltzman. "Identification of *discs large 1* (*dlg 1*) homologs in the mouse ovary". University of Utah Department of Obstetrics and Gynecology Academic Faculty Meeting, March 2001.

Presentation (poster). Carrie A. Stoltzman and Eli Y. Adashi. Program #435, "Association of the MAGUK SAP102 with granulosa cell gap junctions in the mouse ovary". American Society for Cell Biology 41st Annual Meeting, December 2001, Washington, DC.

CONCLUSIONS

Of the genes investigated in this study as candidates for systemic deletion, thus far only CASK and ZO-2 were expressed in the ovarian surface epithelium. Unfortunately, these two genes were also expressed in other cell types in the ovary and in the oviduct. Systemic deletion of either gene potentially may result in ovarian phenotypes completely unrelated to alterations in the cells of the germinal epithelium. Furthermore, the expression of these genes in other organs of the mouse could produce possibly lethal consequences in mice carrying such a systemic deletion.

While we find that the length of time needed to determine which mouse *dlg 1* homologs are expressed in the ovarian surface epithelium has exceeded that predicted in the original proposal, we feel that even the negative results obtained through this study are useful. For example, we were able to exclude SAP102, ZO-1, and ZO-3 as candidates for further investigation in this project based upon the absence of their expression in cells of the surface epithelium. However, the presence of both the neuronal "synaptic protein" SAP102 and the epithelial "tight junction protein" ZO-1 at what appear to be granulosa cell gap junctions may

provide valuable insight into possible tissue-specific variability in the roles of the MAGUK family proteins. And the presence of the ZO-3 protein in oviduct lumen epithelium, but not in ovarian surface epithelium, demonstrates that members of this protein family can be expressed in a very tissue-specific and organ-specific manner.

We continue our investigation for a candidate gene for systemic deletion among the five remaining homologs to be characterized in the ovary. Further work needs to be performed toward determining whether these yet undetected proteins are present in the ovary and in which cell type(s). This work will include the use of additional antigen retrieval methods on the ovary sections, the use of additional organ or tissue sections as possible positive controls, and the search for additional antibodies against the Dlgh1, Dlgh2, Dlgh3, p55, and PSD95 proteins from other laboratories or commercial sources.

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2. Fukaya, M. & Watanabe, M. (2000) *J Comp Neurol* **426,** 572-86.

APPENDIX

Figure 1 through Figure 6 are located on pages 9 through 12.

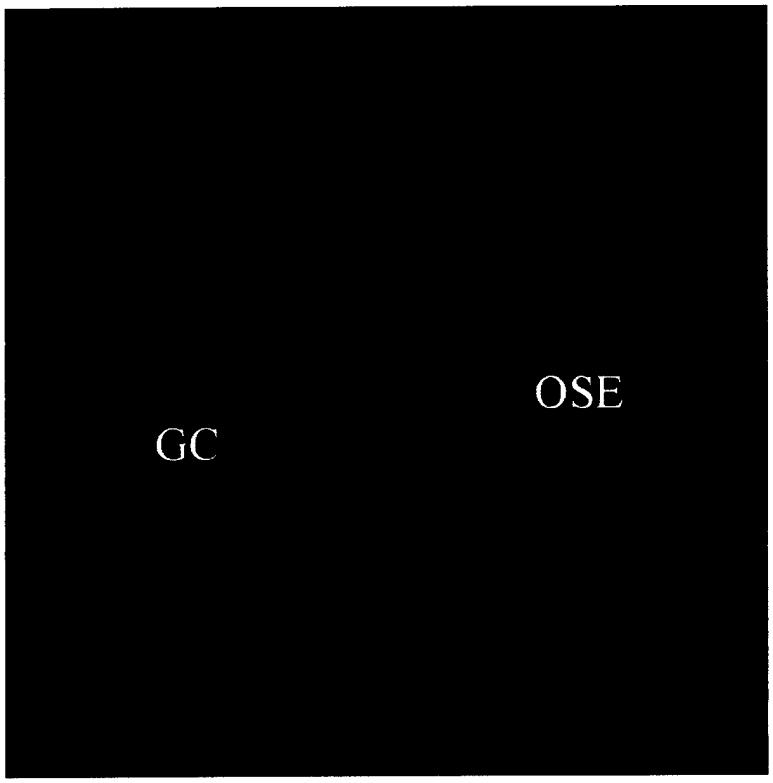

Figure 1. Immunodetection of mouse CASK protein in mouse ovary sections following antigen retrieval (70X magnification). OSE=ovarian surface epithelium, GC=granulosa cells

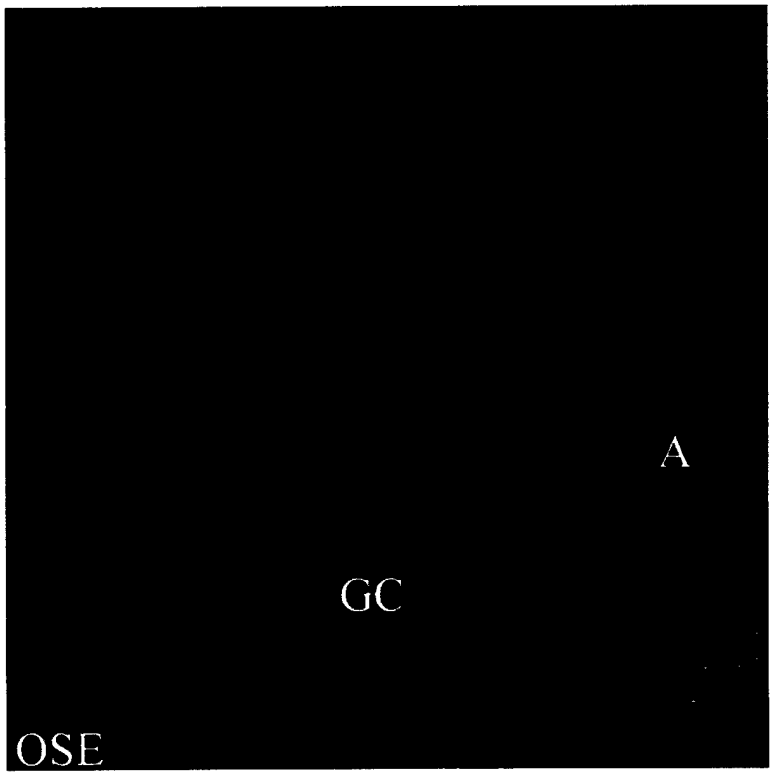

Figure 2. Immunodetection of mouse SAP102 protein in mouse ovary section (14X magnification). OSE=ovarian surface epithelium, GC=granulosa cells, A=antrum of follicle

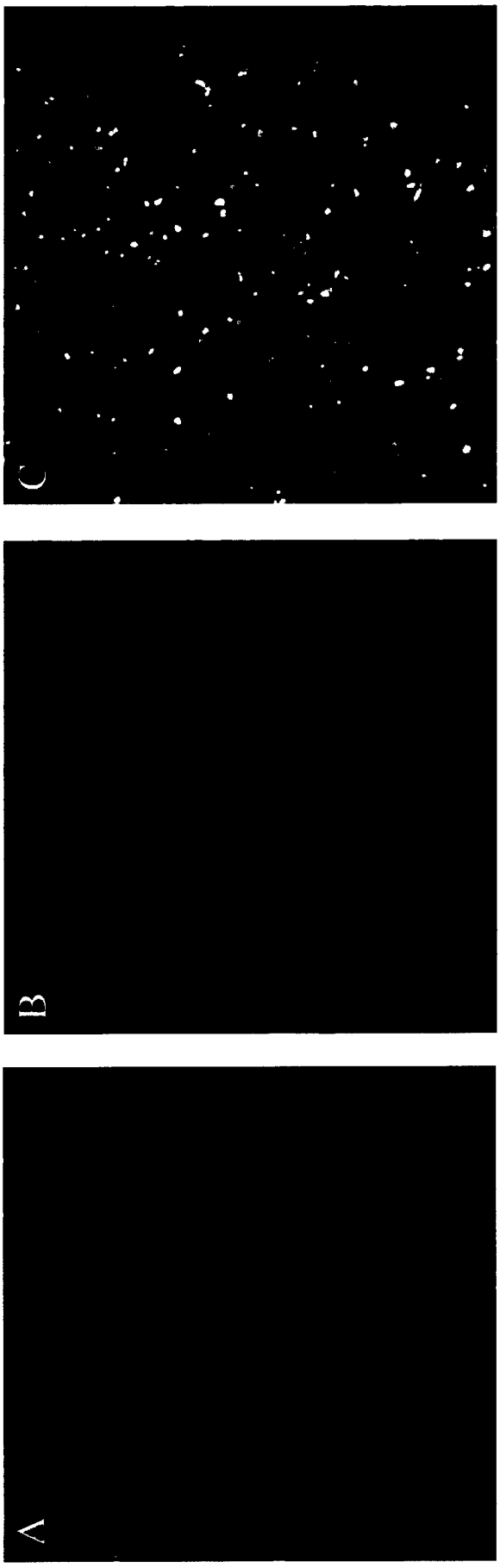

Figure 3. Immunodetection of mouse SAP102 (A, green) and the gap junction protein connexin43 (B, red) in granulosa cells (100X magnification). The merged images (C) suggest points of colocalization (yellow).

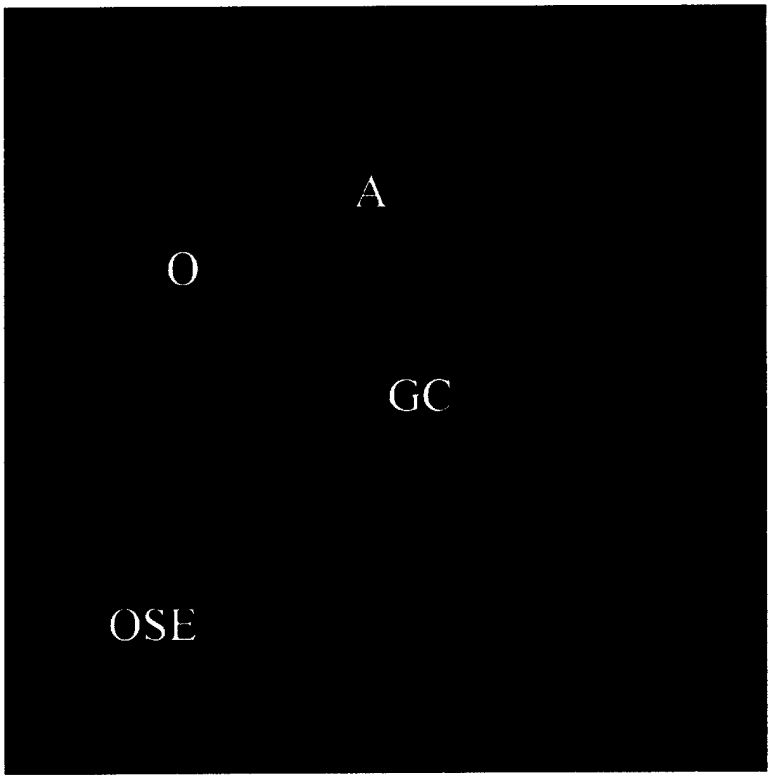

Figure 4. Immunodetection of mouse ZO-1 in mouse ovary sections (20X magnification). OSE=ovarian surface epithelium, GC= granulosa cells, A=antrum of follicle, O=oocyte

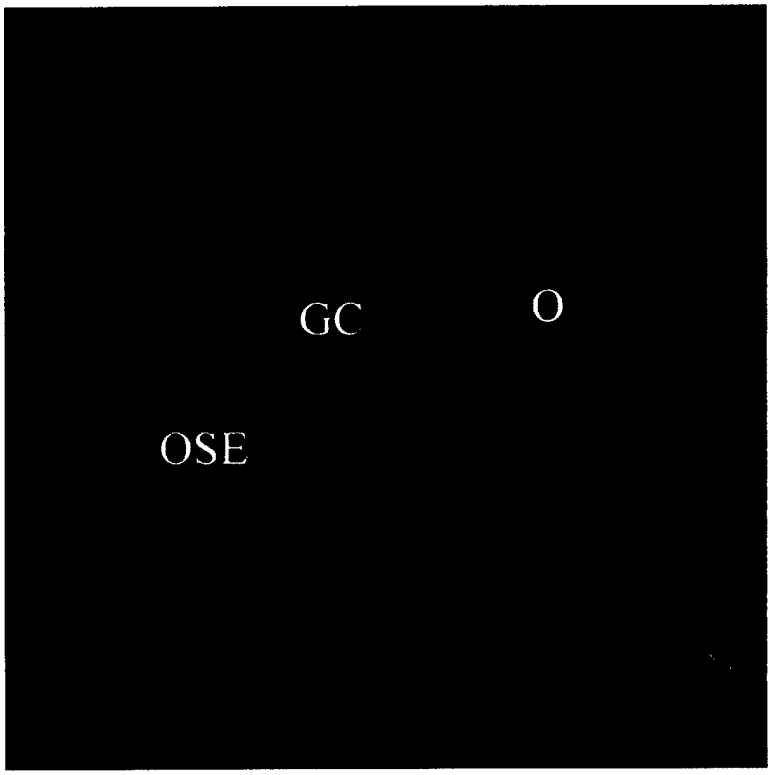

Figure 5. Immunodetection of mouse ZO-2 in mouse ovary sections (28X magnification). OSE=ovarian surface epithelium, GC=granulosa cells, O=oocyte

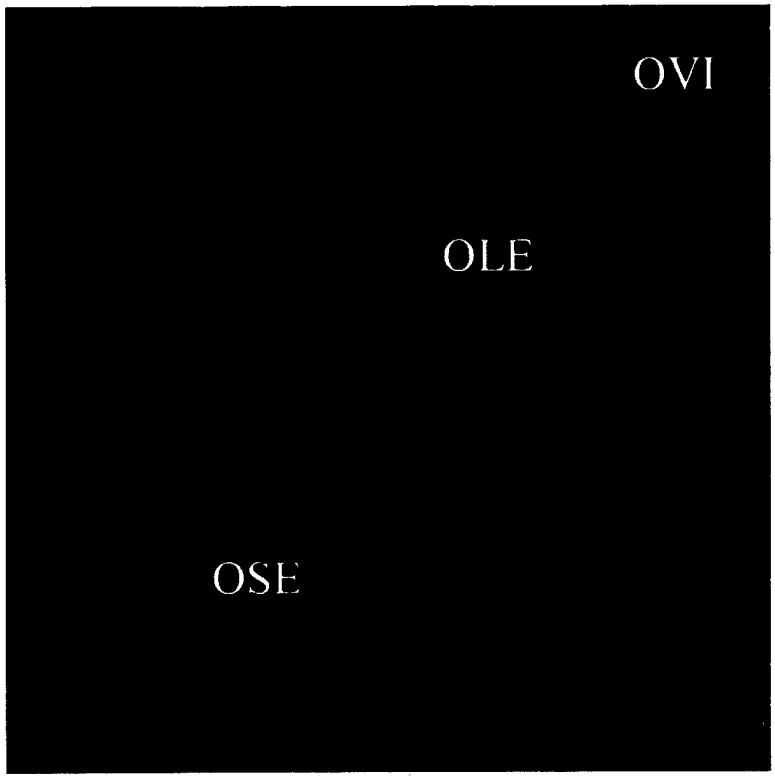

Figure 6. Immunodetection of mouse ZO-3 in mouse ovary and oviduct section (28X magnification). OSE=ovarian surface epithelium, OVI=oviduct, OLE=oviduct lumen epithelium