The Embryotrophic Activity of Oviductal Cell-derived Complement C3b and iC3b, a Novel Function of Complement Protein in Reproduction*

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The oviduct-derived embryotrophic factor, ETF-3, enhances the development of trophoectoderm and the hatching process of treated embryos. Monoclonal anti-ETF-3 antibody that abolishes the embryotrophic activity of ETF-3 recognized a 115-kDa protein from the conditioned medium of immortalized human oviductal cells. Mass spectrometry analysis showed that the protein was complement C3. Western blot analysis using an antibody against C3 confirmed the cross-reactivities between anti-C3 antibody with ETF-3 and anti-ETF-3 antibody with C3 and its derivatives, C3b and iC3b. Both derivatives, but not C3, were embryotrophic. iC3b was most efficient in enhancing the development of blastocysts with larger size and higher hatching rate, consistent with the previous reported embryotrophic activity of ETF-3. Embryos treated with iC3b contained iC3b immunoreactivity. The oviductal epithelium produced C3 as evidenced by the presence of C3 immunoreactivity and mRNA in the human oviduct and cultured oviductal cells. Cyclical changes in the expression of C3 immunoreactivity and mRNA were also found in the mouse oviduct with the highest expression at the estrus stage. Molecules involving in the conversion of C3b to iC3b and binding of iC3b were present in the human oviduct (factor I) and mouse preimplantation embryo (Crry and CR3), respectively. In conclusion, the present data showed that the oviduct produced C3/C3b, which was converted to iC3b to stimulate embryo development.

Research is ongoing to optimize the embryo culture condition in human in vitro fertilization and embryo transfer programs. A large proportion of time when embryos are cultured in vitro is when they should be developing in the oviduct in vivo. Oviductal microenvironment is generally accepted to provide the best support to early embryo development. Oviductal cell coculture, the culture of embryo with oviductal cells, has been shown to improve the success rate in prospective randomized fertility and embryo transfer programs (5). However, the culture condition in sequential culture systems is still suboptimal as the development of human embryos in these systems can be further improved by supplementation of granulocyte-macrophage colony-stimulating factor (6), a cytokine with peak expression during the preimplantation period in the human fallopian tube (7). Our unpublished data also show that mouse blastocysts after human oviductal cell coculture in G1/2/G2.2, the most commonly used sequential culture system, have better trophoectoderm development and therefore hatch more often than those cultured in sequential medium alone. Thus, the beneficial effect of coculture and sequential culture on embryo development can be merged if the embryotrophic factors from the cocultured cells are known and are supplemented to the sequential culture system. Human oviductal cells improve the development of mouse embryos in vitro by the production of factors like cytokines and other factors with unknown identities (1). We have purified three embryotrophic fractions termed ETF-1, ETF-2, and ETF-3 from human oviductal cell conditioned medium by various liquid chromatographies (4, 8). Whereas ETF-1 and ETF-2 preferentially stimulate the development of inner cell mass, ETF-3 enhances the development of trophoectoderm, which leads to an increase in the blastocyst size, hatching, and attachment of the hatched blastocyst (9). ETF-3 is most abundant among the three ETFs (8).

In this paper, we report the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (10) and Western blot to identify ETF-3 as a mixture containing complement protein 3 (C3) and its derivative, C3b. We demonstrate that the oviductal epithelium produces the complement protein in a cyclical manner. In the classical immune cascade, C3 is cleaved to C3b and C3a when being activated. C3b is further cleaved to iC3b during inactivation by factor I in the presence of cofactors, such as membrane cofactor protein (MCP; CD46). In mice, the rodent-re-

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stricted transmembrane C3 convertase inhibitor, Crry, served the function of MCP and DAF (11). In this paper, we report the presence of these molecules in the oviduct/embryo, suggesting that C3b is converted to iC3b, which is the biologically active embryonic protein of ETF-3.

EXPERIMENTAL PROCEDURES

Production of Anti-ETF-3 Antibody—Purified ETF-3 isolated from oviductal cell conditioned medium was used to generate anti-ETF-3 monoclonal antibodies. Screening was performed by enzyme immunoassay 10–12 days after fusion (12). Positive hybridomas were cloned and used to produce antibodies in serum-free medium. Anti-ETF-3 monoclonal antibodies clone-14 (ETF3-C14) was purified by affinity chromatography using HiTrap Protein G-Sepharose (Amersham Biosciences) and concentrated by the Centricon-30 concentrator (Amicon, Beverly, CA). The immunoreactivity of the antibody was confirmed by Western blot analysis of three different batches of ETF-3.

Protein G Purification of ETF-3—The conditioned medium derived from the immortalized oviductal cells, OE-E6/E7 (13), was passed through a concanavalin A affinity column using a fast performance liquid chromatographic system (Amersham Biosciences). Two hundred micrograms of ETF3-C14 was incubated overnight with the dialyzed concanavalin A eluate. The mixture was then passed through a 5-ml HiTrap Protein G column (Amersham Biosciences) and concentrated by ProteinProspector MS-Fit (available on the World Wide Web at prospector.ucsf.edu) (14).

MALDI-TOF Peptide Mass Fingerprint Analysis—The 115-kDa protein band from one-dimensional SDS-PAGE (Coomassie Blue-stained) was used for mass spectrometry analysis. In-gel digestion was performed as described elsewhere (14), and the digest was analyzed in a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA). The mass spectrum was obtained in the delayed extraction mode using an accelerating voltage of 20 kV and a 175-ns delay. Trypsin autolytic fragment peaks of 906.5049, 1153.5741, and 2163.0570 were used for internal calibration. Data base searching was performed with ProteinProspector MS-Fit (available on the World Wide Web at prospector.ucsf.edu) (14).

RT-PCR for Human C3 and Factor I Expression—Total RNAs from two oviductal cell samples (OE& and OE&c), OE-E6/E7 cells at passages 14, and 25, human oviductal epithelial tissue, SKOV-3, CHO-K1 cell line, and human liver tissue were isolated using TriReagent (Invitrogen) according to the manufacturer’s protocol. The quantity and quality of total RNA samples were analyzed by UV spectrophotometry. One hundred nanograms of total RNA was subjected to RT-PCR using the Access RT-PCR system (Promega, Madison, WI) according to the manufacturer’s protocol using specific primers for human C3 (5′-GCTCAAGGACCGACCTCTTTG-3′ and 5′-CCCTTTCTCATGATGAGGTAG3′) and human Factor I (5′-GTCTTTCTGCGACCCTATGGAGGA-3′ and 5′-GTAATGCTGACACCTTTTC-3′). The amount of glyceraldehyde-3-phosphate dehydrogenase (5′-ACACAGTCCATGATGCGTCA-3′ and 5′-TCCACACCCCTGTTGTCGTA-3′) was used for normalization of the mRNA loading. The relative amount of amplified products was quantified by Labwork Image Acquisition and Analysis Software (UVp, Inc., El Segundo, CA). A minimum of four replicates of semiquantitative RT-PCR were performed.

Messerger RNA Expression of Complement-binding Proteins in Mouse Embryos—In vivo developed mouse embryos at different stages of development were flushed from oviducts or uteri. mRNA of the embryos was isolated using the Dynabeads mRNA Direct kit (Dynal AS) as described (15). Two-step RT-PCR was used to determine the relative changes of C3 mRNA in mouse oviducts in the estrus cycle using mouse C3-specific primers (5′-AGAGACTGCGCTGACCTCT-3′ and 5′-TTCCTCCCAGAGGTGCAAA-3′). The relative amount of gliceraldehyde-3-phosphate dehydrogenase (5′-ACACAGTCCAGGATGCGTCA-3′ and 5′-TCCACACCCCTGTTGTCGTA-3′) was used for normalization of the mRNA loading. The relative amount of amplified products was quantified by Labwork Image Acquisition and Analysis Software (UVp, Inc., El Segundo, CA).

RESULTS

Identification of ETF-3—In order to purify ETF-3 to homogeneity for identification, monoclonal antibodies against ETF-3 were raised. The antiserum of the mouse from which ETF3-C14 was raised nullified the activity of ETF-3 on blastulation and hatching of mouse embryos (15). The antiserum also bound to the epithelial cells of the human oviduct and to the blastomeres of ETF-3-treated mouse embryos (15). ETF3-C14 abolished the embryotrophic effect of ETF-3 (Fig. 1).

Western blot analysis showed the presence of an additional 115-kDa protein (PG-115; Fig. 2, lane A) in ETF3-C14 affinity-purified ETF-3 immunocomplex when compared with ETF3-C14 alone (Fig. 2, lane B). In two-dimensional gel electrophoresis, the protein appeared as dots with the same size but with slightly different pI ranging from 6 to 7 (data not shown), observed with a confocal microscope (MRC-600; BioRad). The expression of C3 protein in OE-E6/E7 cells was also studied with the cells cultured in chamber slide (Nunc, Inc., Naperville, IL). The expression of factor I protein in the human oviduct section was studied with the same protocol using goat anti-factor I (1:2000; Calbiochem) with or without factor I preabsorption. Immunostaining of mouse blastocyst cultured with or without iC3b treatment were preformed as described (15) using anti-ETF-3 antibody (1:2000) and fluorescein isothiocyanate-labeled rabbit anti-goat IgG antibody (1:100). The stained blastocysts were examined under a confocal microscope.

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consistent with our previous conclusion that ETF-3 was glycoprotein in nature (8). A data base search showed that the peptide mass fingerprint from MALDI-TOF mass spectrum of PG-115 was complement C3 precursor (Table I).

When running alongside commercially available C3 and its derivatives, C3b and iC3b (Fig. 3, lanes 1, 2, and 3, respectively), polyclonal anti-C3 antibody recognized all of the subunits in C3 and its fragments. ETF3-C14 reacted with the 115-kDa α-chain of C3 (α-115), the 106-kDa α′-chain of C3b (α′-106), and the 40-kDa α-chain of iC3b (α-40), indicating that the binding epitope of the antibody was in the α-40 fragment of C3.

Antic3 polyclonal antibody detected the presence of C3 fragments in purified and partially purified ETF-3 (concana 
avin A-bound fraction of OE-E6/E7 conditioned medium) with sizes of 115, 106, 75, and 40 kDa (Fig. 3, lanes 4 and 5, respectively). Mass spectrometry analysis of the 115, 106, and 75-kDa bands confirmed their identities as C3 precursor (data not shown). ETF3-C14 recognized the 115-, 106-, and 40-kDa bands of purified and partially purified ETF-3.

Embryotrophic Activity of C3 and Its Fragments—The development of mouse embryos with or without C3 derivatives treatment is shown in Table II. The rate of embryo development was based on the number of two-cell embryos after 24 h of culture. The embryos incubated with iC3b for 4 days had significantly more expanded blastocysts and a higher hatching rate (p < 0.05) when compared with those cultured in medium alone and media supplemented with other C3 fragments. The size of the expanded blastocysts in the iC3b group as determined by the area of the expanded blastocysts was also significantly larger than the other groups (p < 0.05). Both C3b and iC3b stimulated hatching of the treated embryos. iC3b immunoreactivity was found in the trophectoderm of iC3b treated mouse blastocysts but not in medium alone-cultured blastocysts (Fig. 4).

Protein and mRNA Expression of Oviduct and Oviductal Cells—C3 immunoreactivity was localized to the epithelial lining of human oviduct (Fig. 5A, a). After preabsorption of the anti-C3 antibody with ETF-3 or iC3b, no signal was found in the sections (b and c). Immortalized human oviductal cells, OE-E6/E7, possessed C3 immunoreactivity, which was absent when anti-C3 antibody preabsorbed with ETF-3 was used (Fig. 5A, d and e, respectively). Factor I was localized to the epithelial lining of the human oviduct, and no signal was obtained when anti-factor I antibody was preabsorbed with factor I (Fig. 5A, f and g, respectively).

The mRNA expression of human C3 (972 bp) and human factor I (722 bp) were detected in two primary oviductal cell samples, OE-E6/E7 cells at passages 14 and 25, oviductal epithelium tissue from two patients, SKOV-3 cells, and human liver tissue (Fig. 5B, lanes 1–7 and 9). No such mRNA expression was found in the CHO-K1 cells (lane 8).

Positive staining of C3 was found in the epithelial lining of the mouse oviduct at different stages (Fig. 6A). The C3 immunoreactivity was stronger at estrus and metestrus than at other stages. This change in immunoreactivity was positively correlated with the mRNA expression levels (Fig. 6B), highest at estrus and lowest at diestrus. A statistically significant difference (p < 0.05) in the mRNA expression level was found between these two stages.

Crry, CR3, and DAF Expression in Mouse Preimplantation Embryo—The expression of mouse Crry, CD11b (αM), and CD18 (β2) of CR3 and DAF mRNA was determined in the mouse embryos at different developmental stages (Fig. 7). Crry (520 bp) and DAF (563 bp) transcripts were constitutively expressed throughout the preimplantation development. Mouse embryos expressed αM (498 bp) and β2 (282 bp) mRNA of CR3. The expression of αM was absent at the three- to four-cell and morula stages, whereas that of β2 was present from the two-cell stage onwards. All of the transcripts were present in mouse liver (positive control).

DISCUSSION

We describe here the identification of ETF-3 as complement C3 and its derivatives are embryotrophic. Our previous data showed that ETF-3 enhanced the development of trophectoderm and the hatching of the treated embryos (9). The beneficial effect of ETF-3 on mouse embryo development is also seen when it is supplemented to a C12G22.2 sequential embryoblast culture system.2 The ETF3-C14 antibody abolishes the embryotrophic effect of ETF-3 and recognizes a 115-kDa protein in ETF-3 identified by mass spectrometry to be complement C3. This is verified by the cross-reactivities between anti-C3 antibody with ETF-3 and ETF3-C14 with C3 and its derivatives.

A previous study has reported the presence of C3 immunoreactivity in human oviduct (17). However, the cellular location of the protein has not been studied. The present study localized C3 immunoreactivity to the cytoplasm of the epithelial lining of human oviduct (Fig. 5). The lack of staining on oviduct sections using anti-C3 antibody preabsorbed with ETF-3 and iC3b confirms that the immunoreactivity is due to at least a C3-like molecule, if not C3. The presence of C3 mRNA in the oviduct indicates that the protein is synthesized de novo. The conclusion is supported by the expression of C3 mRNA and protein in immortalized human oviductal cells, consistent with our previous finding on the localization of ETF-3 immunoreactivity in OE-E6/E7 cells (15). In addition, our preliminary data showing the presence of C3 immunoreactivity in hydrosalpinx fluid lends further support to the secretion of C3 by the human oviduct.3

C3 immunoreactivity is also present in the luminal epithelium of the mouse oviduct. Complement C3b was synthesized and secreted by the luminal epithelium at the apical border of the porcine oviducts (18, 19). C3-like molecule in the oviduct of an amphibian, B. arenarum (20), has also been reported. The

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3 Y. L. Lee and W. S. B. Yeung, unpublished observations.
presence of C3 or its derivatives in widely different species suggests that the molecule may have important physiological function in the oviduct.

Our previous report demonstrated that ETF-3 had a temporal effect on trophectoderm development with maximal efficiency between the three- to four-cell and blastocyst stages, leading to a higher hatching rate (9). Preimplantation embryos up to the morula stage are developed within the oviduct (21). The cyclical changes of C3 expression in mouse oviduct with the embryotrophic activity of ETF-3 in the oviduct. The expression of C3b in the oviductual ampulla of pig is under hormonal regulation (22). Its concentration in the luminal fluid of pig is high at estrus and during the early embryo cleavage stage of pregnancy (19). C3 immunoreactivity is detected in human oviduct at the luteal phase in this study, and its expression at other phases of the menstrual cycle is being investigated in this laboratory. These are in line with the detection of C3 immunoreactivity in the endometrium of humans (23), mice (24), and rats (25) and the higher biosynthetic activity of C3 in human endometrium at the luteal phase than that at the proliferative phase (26). Therefore, it is likely that the observed embryotrophic activity of ETF-3 is physiologically relevant. We propose that the oviduct of human and mouse secretes C3 and its derivatives to enhance the development of early embryos.

The contribution of C3 to fertility in vivo is not known. Although C3-deficient mice are fertile and can produce offsprings with normal appearance (27), the fecundity of normal and C3-deficient mice has not been compared. It is likely that the role of C3 on embryo development is facilitatory and not obligatory. This is supported by the observation that pregnancy can be obtained by transferring in vitro cultured embryos to the uterus without the need to expose the embryos to an oviductal environment. We hypothesize that the fecundity of the C3-deficient mice is inferior to that of the wild type mice. This is being tested in our laboratory.

Whereas C3 is well known for its role in immunity, it has been implicated in the development of bone and cartilage (28), B-cell proliferation (29), and urodele regeneration (30). In reproduction, it has only one proposed function (i.e. facilitation of fertilization by enhancing spermatozoa-egg membrane apposition) (31). This is supported by the following: 1) human sperm acrosomal protease cleaves C3 to C3b and facilitates the binding of C3b to C3 regulatory protein, MCP; 2) presence of complement receptor 1 (CR1; CD35) and CR3 (CD11b/CD18) on human oocyte; and 3) C3b stimulates whereas anti-MCP and
anti-C3 antibodies inhibit the fusion of human sperm with hamster oocyte. C3 may have a similar role in amphibian (20). Our identification of the embryotrophic activity of C3 derivatives is thus a novel function of the molecule in reproduction.

C3 regulatory proteins (e.g. DAF and MCP) (32) regulate the activity of the complement protein. In humans, DAF inhibits C3 cleavage by accelerating the decay of C3 convertases (33), and MCP serves as a cofactor of factor I for cleavage of C3b (34). These complement regulatory proteins are present in the embryo (35) and the reproductive tract of human (36). In mouse, the rodent-restricted transmembrane C3 convertase inhibitor, Crry, regulates the deposition of activated C3 on the surface of autologous cells in vitro by having MCP- and DAF-like activities (11, 37).

C3 contains a 115-kDa α-chain and a 75-kDa β-chain. The α-chain is cleaved to become a 106-kDa α’-chain in the formation of C3b (38). ETF-3 contains both C3 and C3b as the main components. The α’-chain is cleaved into three fragments of sizes 63 kDa (α’-63), 40 kDa (α-40), and 75 kDa (β-75) after removal of the 3-kDa C3f to form the iC3b fragment (38). A faint band with molecular size about 40 kDa was detected in the Western blot analysis of ETF-3 (Fig. 3). This could be the α-40-chain of iC3b or a degradation product of C3/C3b during purification. However, the amount of this band obtained was insufficient for characterization. No α’-63 band was found, which could be due to its genuine absence or presence at concentration below the sensitivity of detection. In any case, iC3b cannot be a major component in the ETF-3 preparation. Similar to ETF-3 (9), iC3b improves mouse embryo development in terms of blastocyst size, blastulation, and hatching rate.

Although C3 and C3b are the major components of ETF-3, iC3b is more potent than C3b in stimulating embryo development, and C3 is not embryotrophic. In the activation of complement cascade, factor I in the presence of cofactors (MCP, factor H, CR1) cleaves C3b to iC3b and C3f. Factor I mRNA is present in the rat uterus (39). In the present study, we reported the mRNA expression of factor I in the human oviduct and

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**Fig. 5.** A, immunohistochemical staining of complement C3 and human factor I. Paraffin section of human oviduct at luteal phase (α-c) × 630) and OE-E6/E7 (d and e) using anti-C3 polyclonal antibody. C3 immunoreactivity was localized to the cytoplasm of the epithelial lining of the human oviduct (a). The signal was absent after anti-C3 was preabsorbed with ETF-3 (b) and iC3b (c). OE-E6/E7 cells were also stained positively with anti-C3 antibody (d). The signal was absent after preabsorption with ETF-3 (e). Human factor I immunoreactivity was localized to the epithelial lining of the human oviduct (f). The signal was absent after the antibody was preabsorbed with factor I (g). Bar, 20 μm. B, transcripts of human C3 (972 bp) and factor I (722 bp) were obtained in cultured primary oviductal cells from patient 109 (lane 1), 89 (lane 2), OE-E6/E7 at passages 14 (lane 3), 25 (lane 4), human oviductal epithelium tissue from two patients (lanes 5 and 6), SKOV-3 cell (lane 7), and human liver tissue (lane 8). No C3 or factor I was found in CHO-K1 cell (lane 9) and distilled H2O control (lane 10). M, 1-kb plus DNA marker.

**Fig. 6.** Cyclical changes of C3 expression in mouse oviduct. A, C3 immunoreactivity was localized to the epithelial lining of mouse oviduct with stronger signal observed in estrous (E) and metestrus (M) when compared with proestrus (P) and diestrus (D). No signal was obtained when anti-C3 antibody was omitted (negative) control. Magnification was ×200. B, RT-PCR analysis of mouse C3 mRNA in mouse oviduct with different estrous cycles after being normalized with mouse glyceraldehyde-3-phosphate dehydrogenase. The transcript level of mouse oviduct at proestrus was given an arbitrary value of 1. The bars with asterisks were statistically significant (p< 0.05, Student-Newman-Keuls test).

**Fig. 7.** Fluorograph of the RT-PCR products of mRNA samples of mouse preimplantation embryo at one-cell (lane 1), two-cell (lane 2), three- to four-cell (lane 3), morula (lane 4), blastocyst (lane 5), mouse liver (lane 6), and distilled H2O control (lane 7). Ethidium bromide-stained PCR products of Crry (520 bp), DAF (563 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (452 bp) were present at all developmental stages. CD18 (282 bp) was absent in one-cell embryos, whereas CD11b (498 bp) was only present in one-cell to three- to four-cell embryos.
cultured oviductal cells, suggesting that the oviduct synthesizes factor I, which together with membrane-bound cofactors on mouse embryo, e.g. Crry, could cleave C3b into iC3b. The exact reasons for the observed variation in the expression levels of C3 and factor I mRNA among primary and immortalized oviductal cell samples (Fig. 5B) are unknown. In the primary oviductal cells, the variation could be a reflection of possible regional or cyclic changes of these transcripts in the parental oviductal tissue as has been shown in the mouse oviduct. Quantitative analyses on the expression of these genes using real time polymerase chain reaction in different oviductal regions and at different stages of the menstrual cycle are being performed in this laboratory. The variation in the gene expression of the immortalized oviductal cells could be associated with increasing number of cell passages. In this connection, the telomerase activity of the immortalized cells increases with increasing number of cell passages. In this connection, the telomerase activity of the immortalized cells increases to iC3b (42). CR3 binds to the α2β1-integrin family with iC3b binding activity (40). Although the αβ2 integrin transcript is present from the two-cell stage onward, the αβ2 integrins are believed to be leukocyte-restricted at later stages of preimplantation embryo development cannot be excluded. The I-domain region of the αβ2-chain is also involved in the binding to iC3b (42). CR3 binds to the α-40-chain (amino acids 1383–1403) of iC3b (38). Interestingly, the binding epitope of ETF3-C14 antibody with neutralization effect on ETF-3 embryotrophic activity also lies in the α-40-chain. The possible presence of isoforms of CR3 to mediate the embryotrophic action of iC3b was repeated in a subsequent study (35).

In conclusion, we have reported a novel function of C3 in mouse embryo development (3). The complement C3 derivatives play an active role in mouse embryo development (3). The Complement FactsBook (34). Morley, B. J., and Walport, M. J. (2000) J. Immunol., 165(10), 5255–5260. He, Q. Y., Lau, G. K., Zhou, Y., Yuen, S. T., Lin, M. C., Kung, H. F., and Chiu, J. F. (2003) Mol. Hum. Reprod. 9, 165–172. Tauber, P. F., Wettich, W., Nohlen, M., and Zaneveld, L. J. (1985) J. Immunol. 135, 2557–2564. Lee, Y. L., Lee, K. F., Xu, J. S., Wang, Y. L., Tsao, S. W., and Yeung, W. S. (2001) Mol. Reprod. Dev. 59, 400–409. Lee, Q. Y., Lau, G. K., Zhou, Y., Yuen, S. T., Lin, M. C., Kung, H. F., and Chiu, J. F. (2006) Proteomics 6, 2063–2074. Lee, Y. L., Lee, K. F., Xu, J. S., Wang, Y. L., Tsao, S. W., and Yeung, W. S. (2001) Mol. Reprod. Dev. 59, 400–409. Lee, Q. Y., Lau, G. K., Zhou, Y., Yuen, S. T., Lin, M. C., Kung, H. F., and Chiu, J. F. (2003) Proteomics 6, 2063–2074. Lee, Y. L., Lee, K. F., Xu, J. S., Wang, Y. L., Tsao, S. W., and Yeung, W. S. (2001) Mol. Reprod. Dev. 59, 400–409. Lee, Q. Y., Lau, G. K., Zhou, Y., Yuen, S. T., Lin, M. C., Kung, H. F., and Chiu, J. F. (2003) Proteomics 6, 2063–2074.