

The embryotrophic activity of oviductal cell derived complement C3b and iC3b – a novel function of complement protein in reproduction

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Running title: C3 on mouse preimplantation embryo development

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Summary

Oviduct derived embryotrophic factor, ETF-3, enhances the development of trophectoderm 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 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 blastocyst with larger size and higher hatching rate, consistent with previous reported embryotrophic activity of ETF-3. Embryo treated with iC3b contained iC3b immunoreactivity. The oviductal epithelium produced C3 as evidenced by the presence of C3 immunoreactivity and mRNA in human fallopian tube and cultured oviductal cells. Cyclical change in the expression of C3 immunoreactivity and mRNA was also found in mouse oviduct with 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, CR3). In conclusion, the present data showed that the oviduct produced C3/C3b which was converted to iC3b to stimulate embryo development.

Introduction

Since the birth of the first in vitro fertilization-embryo transfer (IVF/ET) baby, researches have been ongoing to optimize the human embryo culture condition. A large proportion of time when embryos are cultured in vitro is when they should be developing in the oviduct in vivo. It is generally accepted that the oviductal microenvironment provides the best support to early embryo development. Oviductal cell coculture, the culture of embryo with oviductal cells, had been shown to improve the success rate in prospective randomized control clinical trials (Yeung et al. 1996b;Yeung et al. 2002). Our in vitro experiments and others show that human oviductal cell coculture enhances the hatching rate and reduces the fragmentation rate of human embryos (Bongso et al. 1989;Yeung et al. 1992), increases the blastulation rate and total cell count per blastocyst of mouse embryos (Liu et al. 1995).

Despite the success of coculture, it is not commonly used in IVF/ET program because of the complexity in its implementation as a routine service. Therefore, sequential culture, the use of different media for culturing embryos at different stages of development, is currently the method of choice for improving the outcome of human IVF (Gardner et al. 1998). However, the culture condition in sequential culture systems is still suboptimal as the development of human embryo in these systems can be further improved by supplementation of granulocyte-macrophage colony-stimulating factor (Sjoblom et al. 1999), a cytokine with peak expression during the preimplantation period in human fallopian tube (Zhao and Chegini

1994). Our unpublished data (Xu JS and Yeung WSB) also show that mouse blastocyst after human oviductal cell coculture in G1.2/G2.2, the most commonly used sequential culture system, have better trophectoderm development and therefore, hatch more often than those cultured in sequential media 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 cell improves the development of mouse embryo *in vitro* by the production of growth factors, cytokines (e.g. (Yeung et al. 1996a) and other factors with unknown identities. We have purified 3 embryotrophic fractions termed ETF-1, ETF-2 and ETF-3 from human oviductal cell conditioned medium by various liquid chromatographies (Liu et al. 1995;Liu et al. 1998). While ETF-1 and ETF-2 preferentially stimulate the development of inner cell mass, ETF-3 enhances the development of trophectoderm cells, which leads to increase in blastocyst size, hatching and attachment of the hatched blastocyst (Xu et al. 2001). ETF-3 is most abundant among the three human oviductal cell derived fractions (Liu et al. 1998).

In this article, we report the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Henzel et al. 1993;O'Connell and Stults 1997;Scheler et al. 1998) 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 mouse, complement receptor-1-related gene/protein (Crry) served the function of MCP and Decay Accelerating Factor (DAF) (Molina 2002). In this article, we demonstrate the presence of these molecules in the oviduct/embryo, suggesting that C3b is converted to iC3b, which is the biologically active embryotrophic molecule of ETF-3.

Material and Methods

Production of Anti-ETF-3 Antibody

Anti-ETF-3 monoclonal antibodies were generated by immunizing Balb/cByJ mice subcutaneously with 100 µg affinity-purified ETF proteins isolated from oviductal cell conditioned medium (Liu et al. 1998) in 200 µl of emulsion containing equal volume of PBS and complete Freund adjuvant (Sigma) (Day 0). Booster doses were similarly given in Freund's incomplete adjuvant on day 28 and 42. Three days prior to fusion, a final injection of ETF-3 in sterile PBS was given through tail vein. Fusion of spleen cells and mouse plasmacytoma Sp2/0 cells was carried out as described previously (Luk et al. 1990), using polyethyleneglycol PEG4000 (Sigma Co., St. Louis, MO, USA) as fusion agent. Hybridomas were cultivated and selected in RPMI 1640 (Gibco BRL, Paisley, Scotland) standard medium, containing 10% heat-inactivated fetal bovine serum, HAT (0.1 mM hypoxanthine, 0.016 mM thymidine and 0.4 µM aminopterin), 1 mM sodium pyruvate, 100 U penicillin-streptomycin and 5 mM L-glutamine. Screening was performed by enzyme immunoassay 10-12 days after fusion against purified ETF-3. Positive [hybridomas](#) were cloned by limiting dilution and subsequently mAbs were produced in serum-free medium or ascites fluids.

[Antibodies clone 14 \(IgG....\)](#) were purified by affinity chromatography using HiTrap Protein G Sepharose (Amersham Pharmacia Biotech) and concentrated by the Centricon-30 (Amicon, Inc., Beverly, CA). The immunoreactivity of the purified monoclonal antibody was

confirmed by western blot analysis of three different batches of ETF-3.

Protein-G purification of ETF-3

The conditioned media derived from immortalized oviductal cells, OE-E6/E7, were fractionated as previously described (Lee et al. 2001). Briefly, the cells were grown in Dulbecco modified Eagle medium/Ham F12 (DMEM/F12) supplemented with 0.3% (w/v) BSA (Sigma, St. Louis, MO). Fifty milliliters of oviductal cell conditioned medium were passed through a concanavalin-A affinity column using a fast-performance liquid chromatographic system (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was washed with a start buffer (20 mM Tris, 0.5 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM MnCl₂, pH 7.4) at a flow rate of 0.3 ml/min for 30 min to remove unbound molecules. The bound glycoproteins were eluted with the same buffer containing 0.3 M α -D-methylglucoside at a flow rate of 0.3 ml/min. The eluate was dialyzed against PBS in porous tubing with molecular size cutoff of 12-14 kDa (Spectrum Laboratories, Inc. Rancho Dominguez, CA). Two hundred microgram of purified monoclonal antibody (clone 14) was added to the dialysed concanavalin-A eluate and incubated overnight at 4°C on a rocking platform. The mixture was allowed to pass through a 5-ml HiTrap Protein G column (Amersham Pharmacia Biotech) and washed with 20 mM sodium phosphate buffer, pH 7.0. The bound antibody-antigen complex was eluted with 0.1 M glycine-HCl buffer, pH 2.7 and collected in

neutralizing 1 M Tris-HCl buffer, pH 9.0. The purified fraction was concentrated by the Centricon-30 (Amicon, Inc., Beverly, CA). To confirm that the purity of protein-G purified ETF-3, Western blot analysis of the purified ETF-3 was performed using purified clone 14 antibody (1:100) and anti-mouse IgG antiserum conjugated with horseradish peroxidase (1:5000). The signal was visualized by enhanced chemiluminescence (ECL) according to the manufacturer's recommendations (Santa Cruz, CA, USA).

Two-dimensional gel electrophoresis and silver staining

About 10 µg of affinity purified ETF-3 was reconstituted in 250 µl of rehydration solution (8 M urea, 4% w/v CHAPS, 2% v/v pharmalyte 3-10, 0.002% bromophenol blue), and loaded onto a 13 cm IPG strip in Ettan IPGphor Strip Holder (Amersham Pharmacia Biotech, Uppsala, Sweden) for overnight in-gel rehydration. The first dimension was run on an Ettan IPGphor isoelectric Focusing System (Amersham Pharmacia Biotech) for a total of 16000 kVh at 20°C. The IPG strip was then placed in the equilibration buffer (2% SDS, 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 0.002% bromophenol blue) containing 10 mg/ml DTT for 15 min, followed by another 15 min in equilibration buffer containing 25 mg/ml of iodoacetamide. A 10% slab gel (10% acrylamide, 0.27% N,N'-methylenebisacrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate, 0.005% TEMED) was casted using Hoefer SE 600 system. The IPG strip was then

sealed onto the SDS slab gel with 0.5% agarose in SDS electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% w/v SDS). The second dimension was performed in the electrophoresis buffer at 15 mA/gel for 15 min and followed by 5 hr at 30 mA/gel.

After overnight fixation in 40% ethanol/10% acetic acid, sensitization of the gel was performed using 0.2% w/v sodium thiosulfate and 30% ethanol for 1 hr followed by five 8 min washes in double distilled water. The gel was then incubated in 0.25% silver nitrate for 1 hr, briefly washed in double distilled water and finally developed with a solution containing 2.5% sodium carbonate and 0.015% formaldehyde (37%) until a clear image was seen. Staining was stopped by rinsing the gel with 5% acetic acid.

MALDI-TOF peptide mass fingerprint analysis

The 115-kDa protein band from one-dimensional SDS-PAGE was used for mass spectrometry analysis. The excised gel was sliced into 1x1 mm pieces and equilibrated with 0.5 ml of 50 mM NH_4HCO_3 for 10 min with 700 rpm agitation at room temperature. The gel was destained by washing twice with 500 μl of 50% acetonitrile in 50 mM NH_4HCO_3 for 30 min and with 100% acetonitrile for 30 min. It was then dried in a centrifugal vacuum concentrator. In gel digestion was performed using 15 μl of trypsin solution (10 $\mu\text{g/ml}$ trypsin in 25 mM NH_4HCO_3 , pH 8.0) overnight at 37°C.

About 10% of the digest was analyzed in a Voyager-DE STR MALDI-TOF mass

spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Briefly, 2 μ l of the digested sample was applied onto the sample plate and allowed to dry to 1 μ l before adding with 1 μ l of matrix solution (4 mg/ml α -cyano-4-hydroxycinnamic acid in 35% acetonitrile and 1% trifluoroacetic acid). The spot was dried at room temperature and subjected to mass analysis. The mass spectrum was obtained in delayed extraction mode using an accelerating voltage of 25 kV and 175 nsec delay. Trypsin peptides with mass-to-charge ratio of 906.5049, 1153.5741 and 2163.0570 were used for internal calibration. Database searching was performed with ProteinProspector MS-Fit (<http://prospector.ucsf.edu>).

Western Blot Analysis using anti-C3 and purified clone-14 antibody

To confirm that ETF-3 secreted from OE-E6/E7 cells was C3 and that the monoclonal antibody raised against ETF-3 also recognized C3, different C3 fragments (C3, C3b and iC3b) purified from human serum (Calbiochem, CA, USA), concanavalin-A eluted fraction and ETF-3 derived from OE-E6/E7 after Centricon-100 centrifugation were separated by 8% SDS-PAGE. The proteins were then transferred to PVDF membrane, on which Western blot were performed using [either](#) goat anti-human C3 antibody (1:20,000 dilution) [or](#) [clone-14 \(1:100 dilution\)](#) in blocking solution containing 5% skim milk in PBS with 0.05% Tween-20 (PBST) overnight at 4°C. The membrane was then successively washed 5 times with PBST for 5 min at room temperature, incubated with anti-goat IgG conjugated [or](#) [anti-mouse IgG](#)

conjugated with horseradish peroxidase (1:5000) for 1 hr, washed 5 times with PBST and visualized by ECL technique (Santa Cruz, CA, USA). The protein bands in all the samples were observed with Coomassie Blue staining.

Embryotrophic activity of C3 fragments

Mature MF1 female mice (age, 6–8 wk) were superovulated with 5 IU of PMSG (Sigma), followed by an injection of 5 IU of hCG (Sigma) 46 hours later before allowing to mate with BALB/c males of proven-fertility. The zygotes were recovered 24 hours post-hCG from the oviductal ampullae into HEPES-buffered CZB (CZB/HEPES) (Chatot et al. 1989) containing 0.8 mg/ml of hyaluronidase (Sigma) to remove the cumulus mass. They were washed three times in 250 μ l of CZB/HEPES, followed by one wash in CZB, before being pooled and allocated randomly in groups of 20–30 for culturing in CZB alone, CZB supplemented with 10 μ g/ml of C3, C3b and iC3b (Calbiochem) for the first 48 hours. They were then transferred to CZB containing 5 mM glucose (CZB+G) and appropriate supplementation of ETF-3, C3 or its fragments.

The percentages of embryos reaching fully expanded blastocyst and hatching blastocyst were recorded at approximately 120 and 144 hours post-hCG, respectively. The image of each expanded blastocyst was captured with a phase-contrast inverted microscope. The area of each expanded blastocyst was determined using the MetaMorph imaging system (version 3.51;

Universal Imaging Corp., West Chester, PA) and compared among different C3 fragments' treatment and medium-alone culture groups. The data obtained from four batches of mouse embryo were combined and analyzed by Chi-square test or Student *t*-test where appropriate.

Immunohistochemical staining

Human fallopian tube tissue was obtained from patients admitted for tubal ligation or hysterectomy due to uterine fibromyoma. The Ethics Committee of the University approved the study protocol. Formalin-fixed and paraffin-embedded human oviduct tissue sections were dewaxed, permeabilized using microwave at high power for 3 min and low power for 17 min in Target Retrieval Solution (Dako, CA), and blocked in 10% normal rabbit serum in PBS for 2-4 hours at room temperature. The section was then incubated with the anti-C3 antibody (1:2000; Calbiochem, Germany) in blocking solution at 4°C overnight in a humidified chamber. The second antibody used was fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat IgG antibody (1:100; v/v). The section was counterstained with propidium iodide. The negative control sections were incubated in parallel with omission of the first antibody or with OE-E6/E7 derived ETF-3 or iC3b preabsorbed anti-C3 antibody. The sections were observed with a confocal microscope (MRC-600, BioRad, CA, USA). The expression of C3 protein in OE-E6/E7 cells was also studied with the cells cultured in chamber slide (Nunc, Inc., Naperville, IL), fixed with 4% (v/v) paraformaldehyde in PBS (pH 7.35) and permeated

with 0.1% (v/v) Triton X-100/PBS on ice. The protocol for immunostaining of the wax section was then applied. The expression of factor I protein in the human oviduct section was also studied with the same protocol using [goat anti-factor I \(1:2000; Calbiochem, Germany\)](#) [with or without Factor I pre-absorption](#).

Mouse preimplantation embryos at different stages of development cultured with or without iC3b treatment were collected, washed with PBS containing 0.3% PVP (PVP/PBS) and had zona pellucida removed with acid Tyrode (Hogan et al. 1994). They were fixed in 4% formaldehyde in PBS (pH 7.35) for at least 30 minutes. Prior to staining, the embryos were washed 6 times in PBS containing 0.1% Tween-20 (PBST), permeated in 0.1% Triton X-100 on ice for 1 min. Blocking was performed by incubating with 10% goat serum in PBST for 1 hour. The embryos were successively incubated with purified clone 14 antibody (1:100 v/v) in blocking solution overnight at 4°C, washed 6 times in PBST, incubated with FITC-labeled goat anti-mouse IgG antibody (Sigma) (1:500 in blocking solution) for 1 hour and were washed 6 times in PBST before examination under a confocal microscope.

RT-PCR for human C3 and factor I expression

Total RNAs from two primary oviductal cell samples (OE₈₉ and OE₁₀₉), OE-E6/E7 cells at passages 14, and 25, human oviductal epithelium tissue, SKOV-3, CHO-K1 cell lines and human liver tissue were isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) according to

the manufacturer's protocol. The quantity and quality of total RNA samples were analyzed by UV spectrophotometry. One hundred nanogram of total RNA was subjected to RT-PCR using the Access RT-PCR System (Promega, Madison, WI). In brief, the samples were incubated at 48°C for 45 minutes for first strand cDNA synthesis. PCR amplification was carried out for 40 cycles at 94°C for 30 seconds, 60°C for 1 minutes and 68°C for 2 minutes using human gene-specific primers (Table 1). The PCR products were analyzed in a 2% agarose gel (Gibco GRL).

C3 protein and mRNA expression in mouse oviducts in estrous cycle

Sexually mature (6- to 8- week old) MF1 female mice were used. The estrous cycle was determined by vaginal smear. The protein expression of C3 in MF1 mouse oviducts at different estrus cycles, pro-estrus (P), estrus (E), met-estrus (M) and di-estrus (D) was studied by immunohistochemical staining described above using polyclonal antibody against the β -chain of C3 (sc-14612, Santa Cruz, CA). The antibody was omitted in the negative control. Semi-quantitative RT-PCR was used to determine the relative changes of C3 mRNA transcripts in mouse oviducts in the estrus cycles. Mouse C3 specific primers (Table 1) were used to generate a 480 bp DNA fragment. Total RNAs of pooled oviducts from 4-5 individual mice at each estrous stage were extracted as mentioned above. Reverse transcription using the First strand cDNA synthesis kit (Amersham Biosciences, Uppsala, Sweden) was performed to

generate the single strand cDNA and followed by PCR amplifications with conditions: 94°C for 30 seconds, 60°C for 1 minutes, and 68°C for 2 minutes for 26–38 cycles. The relative amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of mRNA loading. A 2 % NuSieve 3:1 (w/w) agarose (FMC BioProducts, Rockland, MN) was used to resolve the PCR products. The images were captured and the relative amount of amplified products quantified by Labwork Image Acquisition and Analysis Software (UVP, Inc., Upland, CA). A minimum of four replicates of semiquantitative RT-PCR was performed.

Messenger RNA expression of complement binding proteins in mouse embryos

The expression of cofactors/receptor possibly involved in the conversion of C3b to iC3b was studied. In vivo developed mouse embryos at the 1-cell, 2-cell, 4-cell, 8-cell/morula, and blastocyst stages were flushed from either oviducts or uteri at 22–24, 42–46, 67–71, 88–92, and 100–104 h post-hCG, respectively. Two-step RT-PCR was used to determine the complement receptor-1-related gene/protein (Crry), CD11b, CD18, decay accelerating factor (DAF; CD55) mRNA transcripts in these embryos. Gene-specific primers were designed and listed in Table 1. Briefly, mRNA of embryos was isolated using Dynabeads mRNA Direct Kit (DynaL AS) as described (Lee et al. 2003) and subjected to two step RT-PCR analysis as described above.

Results

Identification of ETF-3

In order to purify ETF-3 to homogeneity for identification, monoclonal antibodies against ETF-3 was raised. The antiserum of the mouse from which the hybridoma clones were raised nullified the activity of ETF-3 on blastulation and hatching of mouse embryos (Lee et al., 2003). The antiserum also bound to the epithelial cells of human fallopian tube and to the blastomeres of ETF-3 treated mouse embryos (Lee et al., 2003). Monoclonal antibody from hybridoma (clone-14) abolished the embryotrophic effect of ETF-3 (Fig. 1).

Western blot analysis showed the presence of an extra 115 kDa protein (PG-115, lane A, Fig. 2) in ETF-3 immunocomplex when compared with protein-G purified anti-ETF-3 antibody alone (lane B, Fig. 2). In two-dimensional gel electrophoresis, the protein appeared as dots with same size but with slightly different pI ranging from 6-7 (data not shown). This was consistent with our previous conclusion that ETF-3 was glycoprotein in nature (Liu et al. 1998). Database search showed that the peptide mass fingerprint from MALDI-TOF mass spectrum of PG-115 after in-gel tryptic digestion was complement C3 precursor (Table 2).

Fig. 3A shows the protein patterns of commercially available C3 and its fragments, C3b and iC3b. Polyclonal antibody against C3 recognized all the subunits in C3 and its fragments (Fig. 3C). Anti-ETF-3 antibody (clone-14) reacted with the 115 kDa α -chain of C3 (α -115) and the 106 kDa α' -chain of C3b (α' -106), and the 40 kDa α -chain of iC3b (α -40) (Fig. 3B),

indicating that the binding epitope of the antibody was in the α -40 of C3 fragments.

Anti-C3 polyclonal antibody detected the presence of C3 fragments in purified and partially purified ETF-3 (concanavalin-A bound fraction of OE-E6/E7 conditioned medium) with sizes of 115, 106, 75 and 40 kDa (Fig. 3F). Mass spectrometry analysis of the 115, 106 and 75 kDa bands confirmed their identities as C3 precursor (Data not shown). A band of about 190 kDa was also found in purified ETF-3 and partially purified samples. This possibly represented C3 precursor in the lysate of OE-E6/E7 that had been concentrated after purification. C3 precursor is a single chain molecule with size about 180 kDa. It is cleaved into the α and β chains of C3 during post-translational modification. Anti-ETF-3 antibody recognized the 115, 106, and 40 kDa bands of purified and partially purified ETF-3 (Fig. 3E).

Embryotrophic activity of C3 and its fragments

The development of mouse embryo in medium alone and medium supplemented with different C3 fragments is shown in Table 3. The rate of embryo development was based on the number of 2-cell embryos after 24 hours of culture. The embryos incubated with iC3b for 4 days had significantly more expanded blastocysts and 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 blastocyst was also significantly larger than the other groups ($p < 0.05$). Both

C3b and iC3b stimulated hatching of the treated embryo. Result of immunostaining of iC3b treated embryos at different stages of development using purified clone-14 antibody are shown in Fig. 4. iC3b bound to the mouse embryos starting from 2-cell stage. The immunoreactivities persist at all developmental stages, from 2-cell stage to blastocyst stage. Medium alone cultured embryo did not possess the immunoreactivity.

Protein and mRNA expression of oviduct and oviductal cells

C3 immunoreactivity was localized to the epithelial lining of human fallopian tube obtained from patients admitted for tubal ligation or hysterectomy due to uterine fibromyoma (Fig. 5). After pre-absorption of the anti-C3 antibody with ETF-3 or iC3b, no signal was found in the sections. Immortalized human oviductal cells, OE-E6/E7 possessed C3 immunoreactive signal, which was absent when anti-C3 antibody pre-absorbed with ETF-3 was used (Fig. 6). Factor I was localized to the epithelial lining of the human oviduct and no signal was obtained in the section when anti-factor I antibody was preabsorbed with factor I (Fig. 7).

The expression of human C3 mRNA (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 2 patients, SKOV-3 and human liver tissue (Fig. 8). No such mRNA expression was found in CHO-K1 cell lines.

Positive staining of C3 was found in the epithelial lining of mouse oviduct at different stages (Fig. 9 A-D). The C3 immunoreactivity was strong during estrus and metestrus when compared to that diestrus and proestrus. The change in the intensity of immunoreactivity was positively correlated with the mRNA expression levels (Fig. 10). C3 mRNA expression was highest during estrus and lowest during diestrus. Statistical significant difference ($p < 0.05$) was found between these two stages.

Crry, CR3 and DAF expression in mouse preimplantation embryo

The expression of mouse Crry, CD11b (α_M) and CD18 (β_2) of CR3 and DAF mRNA was determined in the preimplantation mouse embryo at different developmental stages (Fig. 11). Crry (520 bp) and DAF (563 bp) were constitutively expressed throughout preimplantation development. The α_M (498 bp) and β_2 (282 bp) of CR3 were expressed in mouse embryo, but the expression of α_M was absent at the 3-4 cell and morula stages while that of β_2 was present from 2-cell onwards. All the transcripts were present in mouse liver (positive control).

Discussion

We describe here the identification of ETF-3 as complement C3 and that its derivatives are embryotrophic. Our previous data showed that the ETF-3 enhanced the development of trophectoderm and hatching of the treated embryos (Xu et al. 2001). The beneficial effect of ETF-3 on mouse embryo development is also seen when it is supplemented to G1.2/G2.2 sequential embryo culture system (Xu J.S. and Yeung W.S.B. unpublished observation). Monoclonal antibody that abolishes the embryotrophic effect of ETF-3 recognizes a 115 kDa protein with identity confirmed by MALDI-TOF mass spectrometer to be complement C3. This is verified by the cross-reactivities between anti-C3 antibody with ETF-3 and anti-ETF-3 antibody with C3 and its derivatives.

Previous study demonstrated the presence of C3 protein or its derivatives in human oviduct (Tauber et al. 1985). 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. 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. Preliminary data also demonstrated that C3 is secreted from the human oviduct as immunoassay showed the presence of C3 in hydrosalpinx fluid (data not shown). In addition, the presence of C3 mRNA indicates that the protein is synthesized *de novo* in the oviduct. The conclusion is further supported by the expression of C3 mRNA and

protein in immortalized human oviductal cells, OE-E6/E7. The latter observation is consistent with our previous finding regarding the localization of ETF-3 immunoreactivity in OE-E6/E7 cells (Lee et al. 2003).

C3 immunoreactivity is also present in the luminal epithelium of mouse oviduct. Complement C3b was among one of the four proteins synthesized and secreted by porcine oviducts (Buhi et al. 2000). Its expression has recently been localized mainly to the luminal epithelium at the apical border of the oviduct (Buhi and Alvarez 2003). C3-like molecule in oviduct of an amphibian, *B. arenarum* (Llanos et al. 2000) has also been reported. The presence of this complement protein in widely different animal species suggests that the molecule may have important physiological function in the oviduct.

Our previous finding demonstrated that ETF-3 had a temporal effect on trophectoderm development with maximal efficiency between 3-4 cells and blastocyst stage, leading to higher hatching rate (Xu et al. 2001). Preimplantation embryos up to the morula stage are developed within the oviduct (Hogan et al. 1994). The cyclical changes of C3 expression in mouse oviduct with higher immunoreactivity at estrus and metestrus and mRNA expression at estrus in comparison to that at diestrus are in line with the embryotrophic activity of ETF-3. The expression of C3b in the oviductal ampulla of pig is under hormonal regulation (Buhi et al. 1992). Its concentration in the luminal fluid of pig is high at estrus and during early embryo cleavage stage of pregnancy (Buhi and Alvarez 2003). C3 immunoreactivity is also

detected in human oviduct at 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 human (Tao et al. 1997), mouse (Li et al. 2002) and rat (Puy et al. 1993) and the higher biosynthetic activity of C3 in human endometrium in the luteal phase than that in the proliferative phase (Hasty et al. 1994). Therefore, it is likely that the observed embryotrophic phenomenon of ETF-3 is of physiological importance (or function). We hypothesize that the oviduct of human and mouse secretes C3 and its derivatives to enhance the development of early embryo.

C3 plays important role in the complement system (Morgan and Holmes 2000). Recent data suggest that complement proteins may exert novel functions other than those in the immune system, possibly by modulating cellular responses and cell-cell interactions that are important to early development and cell differentiation (Mastellos and Lambris 2002). C3 has been implicated in the development of bone and cartilage (Sato et al. 1993), B-cell proliferation (Servis and Lambris 1989) and urodele regeneration (Rio-Tsonis et al. 1998).

Apart from involving in antipathogenic effect and removal of dead or dysfunctional spermatozoa from the female reproductive tract, there are two possible functions of C3 in reproduction. First, C3 may facilitate the fertilization process by enhancing sperm-egg membrane apposition (Anderson et al. 1993). The supporting evidence include (1) human sperm acrosomal protease released during acrosome reaction cleaves C3 to C3b and facilitates

the binding of C3b to MCP; (2) presence of CR1 and CR3 on the surface of human oocyte; (3) C3b stimulates while anti-MCP and anti-C3 antibodies inhibit the fusion of human spermatozoa with hamster oocytes. A similar role for C3 was also suggested to occur in amphibian (Llanos et al. 2000).

The second function is related to the survival of the embryo/fetus, a foreign body, in the maternal body. In human, normal host cells are protected against complement attack by cell surface complement regulatory protein like DAF and MCP (Caucheteux et al. 2003). In human, DAF inhibits C3 cleavage by accelerating the decay of C3 convertases (Lublin and Atkinson 1989; Nicholson-Weller et al. 1982) and MCP serves as a cofactor of factor I for cleavage of C3b and C4b (Seya et al. 1986; Seya and Atkinson 1989). In contrast to DAF, MCP has high affinity towards C3b, which is abrogated after cleavage of C3b to iC3b by factor I (Morgan and Harris 1999b). These complement regulatory proteins are present in the embryo (Fenichel et al. 1995; Taylor and Johnson 1996) and reproductive tract of human (Jensen et al. 1995). In mice, the rodent-restricted Crry regulates the deposition of activated C3 on the surface of autologous cells in vitro by exhibiting MCP- and DAF-like activities (Kim et al. 1995) (Molina 2002) (Morgan and Harris 1999a). Crry-deficient embryos die in the early post-implantation period and C3-deficient mother can rescue the Crry-deficient conceptus (Xu et al. 2000) indicating that mouse Crry is important in the protection of maternal-mediated fetal loss. Apart from these two functions, the embryotrophic activity of

C3b and iC3b described in this report here is thus a novel function of the molecules.

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 during the formation of C3b (Morley and Walport 2000a). ETF-3 contains both C3 and C3b as the main components. The identities of both chains were confirmed by mass spectrometry (data not shown). In the formation of iC3b, the α' chain is cleaved into 3 fragments of sizes 63-kDa (α' -63), 40-kDa (α -40) and 75-kDa (β -75) after removal of 3-kDa (C3f) (Morley and Walport 2000a). A faint band with molecular size about 40-kDa was detected in the Western blot analysis of ETF-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 by mass spectrometry. 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 cases, iC3b cannot be a major component in the ETF-3 preparation. ETF-3 improves mouse embryo development in terms of size, blastulation and hatching rate (Xu et al. 2001), which is similar to iC3b. The reason that C3b alone only increase the hatching rate could possibly be due to high concentration of C3b used in the present experiment. Previous study has also demonstrated that saturating doses ($>10\mu\text{M}$) of dimeric C3b can inhibit the sperm-oocyte interaction (Anderson et al. 1993). Dose responses of C3 and its fragments on the development of mouse embryo are being studied in this laboratory.

Although C3 and C3b are the major constituents of ETF-3, iC3b is more potent than C3b in stimulating mouse 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 into iC3b and C3f. Factor I mRNA has been detected in rat uterus (Schlaf et al. 1999). In the present study, we demonstrated the de novo synthesis of factor I in human oviduct and cultured oviductal cells, suggesting that the oviduct produces factor I, which together with membrane bound cofactors on mouse embryo, e.g. Crry, cleave C3b into iC3b. The observation that exogenous iC3b alone can stimulate embryo development to the greatest extent indicates that the molecule is the actual active form in stimulating embryo development. The presence of iC3b immunoreactivity in the embryo after treatment supports this hypothesis. The lack of factor I in embryo culture medium may explain the low embryotrophic activity of exogenous C3b when being used alone in culturing embryo.

To test this hypothesis, expression of membrane bound cofactors and iC3b binding protein on mouse embryo were studied. Our data demonstrated that mouse Crry transcript were present in embryos from 1-cell to blastocyst stage. In mouse, Crry serves as a cofactor for factor I-mediated cleavage of C3b (Kim et al. 1995). Complement receptor 3 (CR3; $\alpha_M\beta_2$; CD11b/CD18; Mac-1) is a member of the β_2 integrin family with iC3b binding activity (Morley and Walport 2000b). Although β_2 transcript is present from the 2 cell to blastocyst

stages, the α_M transcripts were present only from 1-cell to 3-4 cell stages, indicating that CR3 may be functional only in early cleavage stages. This may account for some embryotrophic activity of ETF-3 observed at these stages (Xu et al. 2001). However, the maximal efficiency of ETF-3 treatment is between 3-4 cells and blastocyst stage (Xu et al. 2001). Therefore, it is unclear whether the embryotrophic effect of iC3b acts via interaction with β_2 integrins at later stages.

The I domain that appears in the α_M subunit but not in other integrins is the ligand recognition site for iC3b (Diamond et al. 1993; Shimaoka et al. 2002). The I domain like region of the β_2 chain is also involved in binding to iC3b (Goodman and Bajt 1996). Complement receptor 3 binds to the α -40 chain (aa 1383-1403) of iC3b (Morley and Walport 2000a). Interestingly, the binding epitope of the monoclonal antibody used in the present study 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 at later stages of preimplantation embryo development cannot be excluded.

Integrins involve in cell-cell and cell-matrix interactions, cell signaling and survival (Bloor et al. 2002). Previous studies demonstrated that β_1 and β_3 subunits were constitutively expressed throughout preimplantation development in mouse (Sutherland et al. 1993) and human with expression localized to trophectoderm cells (Bloor et al. 2002). Although β_2 integrins leukocyte restricted (Kolanus et al. 1996), the presence of its member, CR3, in

oocyte is controversial; CR3 was reported to be present in human oocyte in one study (Anderson et al. 1993) but could not be substantiated in another study (Taylor and Johnson 1996).

A recent study on the role of complement in systemic tolerance after injection of antigen into the eye demonstrated that the binding of iC3b to CR3 on antigen-presenting cells induced the production of transforming growth factor- β 2 (TGF β 2) (Sohn et al. 2003). The presence of TGF β and its receptors in preimplantation embryo and oviduct of mouse (Chow et al. 2001) supports a role of TGF β during preimplantation embryo development. This is consistent with the observation that injection of anti-TGF β 2 antibody into the blastocoel markedly reduced the implantation rate of mouse embryo (Slager et al. 1993). ETF-3 increases the implantation rate of mouse embryo (Liu et al. 1998). The relationship between iC3b, CR3 and TGF β in mouse preimplantation embryo is being investigated in this laboratory.

In conclusion, we have demonstrated a novel function of C3 in reproduction. The oviduct produces C3/C3b, which is converted to iC3b. iC3b stimulates the development of mouse blastocyst in terms of size and hatching. This is consistent with our previous observations of increased hatching rate of human embryos after oviductal cell coculture (Yeung et al. 1992) and improved trophectoderm development of mouse embryos after ETF-3 treatment (Xu et al. 2001). The detailed regulation of C3 production and the mechanism of action of the iC3b on embryo development await further investigation.

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Legends

Fig. 1: Effects of monoclonal anti-ETF-3 antibody on the embryotrophic activity of ETF-3.

Monoclonal antibody from hybridoma (clone-14) abolishes the embryotrophic effect of ETF-3. ** represent percentage significantly different from other groups ($p < 0.05$).

Fig. 2: SDS-PAGE and western blot analysis of protein-G purified ETF-3. Immunoblot using

purified clone 14 antibody showed a band of 115 kDa (P-G-115) in immunocomplex (lane A)

whereas no such band was found in purified antibody alone (B).

Fig. 3: SDS-PAGE and western blot analysis of complement proteins C3 (lane 1), C3b (lane

2), iC3b (lane 3), purified (lane a) and partial purified ETF-3 (lane b) using monoclonal

anti-ETF-3 antibody (B and E) and anti-C3 polyclonal antiserum (C and F). Immunoblot

using anti-C3 showed bands of α -115 and β -75 in C3 (lane C1), α' -106 and β -75 in C3b (lane

C2) and β -75, α' -63 and α -40 in iC3b (lane C3) whereas only α -115, α' -106 and α -40 were

found using anti-ETF-3 antibody in C3 (lane B1), C3b (lane B2) and iC3b (lane B3)

respectively. Bands of 190, 115, 106, 75 and 40 were obtained in both purified (lane Fa) and

partial purified ETF-3 (lane Fb) using anti-C3 whereas only bands of 115, 106 and 40 were

obtained using purified clone 14 antibody (E). A and D were the Coomassie blue staining of

the two sets of protein respectively.

Fig. 4: Immunostaining of iC3b treated embryos using purified clone 14 antibody under confocal microscope (A-R, 200x). A-E and K-N are bright field images; F-J and O-R are the corresponding fluorescent images localizing clone 14 antibody with FITC (green).

Immunoreactivities were found in iC3b treated embryo at 2-cell (G), 4-cell (H), morula (I) and blastocyst (J). No staining was found in medium alone cultured embryos at different developmental stages (O-R) as well as the 1-cell embryo (A). Bar: 20 μ m

Fig. 5: Immunohistochemical staining of complement C3 in paraffin section of human oviduct under confocal microscope (A-I, 630x) using anti-C3 polyclonal antibody. A, D, G are bright field images; B, E, H are fluorescent images with C3 stained with FITC (green); C, F, I,

fluorescent images showing nuclei stained with propidium iodide (red). C3

immunoreactivity is localized to the epithelial lining of the human oviduct (B). The signal for C3 immunoreactivity is absent after the antibody is preabsorbed with ETF-3 (E) and iC3b (H).

Bar: 20 μ m

Fig. 6: Immunohistochemical staining of complement C3 in immortalized human oviductal cells, OE-E6/E7 using anti-C3 antibody. The cells are stained positively with anti-C3 antibody (B). The signal is absent after preabsorption with ETF-3 (D). A and C are phase

contrast images of cultured OE-E6/E7.

Fig. 7: Immunohistochemical staining of human factor I in paraffin section of human oviduct under fluorescent microscope (A-D) using anti-factor I polyclonal antibody. A and C are the fluorescent images with factor I stained with FITC (green); B and D are fluorescent images showing nuclei stained with propidium iodide (red). Factor I immunoreactivity is localized to the epithelial lining of the human oviduct (A). The signal for factor I immunoreactivity is absent after the antibody is preabsorbed with factor I (C).

Fig. 8: Detection of human C3 and factor I gene in different cells and tissues. Products of size 972 bp and 722 bp respectively were obtained in cultured primary OE cell from patient number 109 (lane 1), 89 (lane 2), immortalized OE-E6/E7 at passages 14 (lane 3), 25 (lane 4), human oviductal epithelium tissue from 2 different patients (lane 5 and 6), SKOV-3 cell (lane 7) and human liver tissue (lane 9). No C3 and factor I was found in CHO-K1 cell (lane 8) and dH₂O control (lane 10). M: One-kb DNA marker.

Fig. 9: Immunohistochemical staining of complement C3 in paraffin section of mouse oviduct at different estrous cycles (P, Proestrus; E, estrus; M, metestrus; D, diestrus) (A-P, 200x). A-D are fluorescent images with C3 stained with FITC (green); C3 immunoreactivity is localized

to the epithelial lining of the mouse oviduct with stronger signal observed in estrous and metestrus (B and C). No signal was obtained when anti-C3 antibody was omitted (I-L). E-H and M-P are fluorescent images showing nuclei stained with propidium iodide of A-D and I-L respectively (red).

Fig. 10: Relative expression of mouse C3 in mouse oviduct with different estrous cycles (P, Proestrus; E, estrus; M, metestrus; D, diestrus) after being normalized with mouse GAPDH. Transcript levels of mouse oviduct at proestrus have an arbitrary value of 1. Bars with * are statistically significant ($P < 0.05$, Student-Newman-Keuls test). Representative diagram of mRNA transcripts in mouse oviduct was shown (A: mouse C3, 480bp; B: mouse GAPDH, 452bp).

Fig. 11: Fluorograph of the RT-PCR products of mRNA samples of mouse preimplantation embryo at 1-cell (1), 2-cell (2), 3-4 cell (3), morula (4), blastocyst (5), mouse liver (6) and dH₂O control (7). Ethidium bromide-stained PCR products of Crry (520 bp), DAF (563bp) and GAPDH (452 bp) were present in all stages of embryos. CD11b (498bp) was absent in 1-cell embryos whereas CD18 (282bp) was only present in 1-cell to 3-4 cell embryos.

Table 1: Details of human and mouse primers for PCR

Gene	Primers (F: forward; R: reverse)	Size (bp)	Accession no.
Human C3	F: 5'-GGTCAAGCAGGACTCCTTGT-3' R: 5'-CCCTTGTTTCATGATGAGGTAG-3'	973	NM_000064
Human factor I	F: 5'-GTCTTCTGCCAGCCATGGCAGAGA-3' R: 5'-GTAATGCAGTCCACCTCACCATT-3'	722	NM_000204
Mouse C3	F: 5'-AGAAGACTGCCTGACCTTCA-3' R: 5'-TTTTCTCCCCAGAGGTCAGA-3'	480	NM_009778
Mouse Crry	F: 5'-CATCACAGCTTCCTTCTGCCA-3' R: 5'-ATCGTTGCTGGTACAGTATA-3'	520	NM_013499
Mouse CD11b	F: 5'-CAGATCAACAATGTGACCGTATGG-3' R: 5'-CATCATGTCCTTGTACTGCCGC-3'	498	NM_008401
Mouse CD18	F: 5'-CATATGTGACGAAGGCTACCA-3' R: 5'-TCTAGACTGTCCTCCACATG-3'	282	NM_008404
Mouse DAF	F: 5'-CAATGGGAATAATGCGAGGGGA-3' R: 5'-TATTGTATCCATTCTTCTTG-3'	563	NM_010016
Human/mouse GAPDH	F: 5'-ACCACAGTCCATGCCATCAC-3' R: 5'-TCCACCACCCTGTTGCTGTA-3'	452	BT006893 NM_008084

Table 2: Identification of protein-G purified 115 kDa protein by MALDI-TOF MS

Fractions	Mass submitted	Mass matched	Start	End	Peptide sequence
1	833.4410	833.4885	835	841	LPYSVVR
2	845.4060	845.4270	1255	1260	WLNEQR
3	887.3990	887.4587	842	848	NEQVEIR
4	898.4470	898.4787	849	855	AVLYNYR
5	1083.6350	1083.5587	1052	1060	GYTQQLAFR
6	1190.6690	1190.6210	1245	1254	DFDFVPPVVR
7	1211.7220	1211.6537	1311	1320	IHWESASLLR
8	1421.6860	1421.6371	1451	1462	VSHSEDDCLAFK
9	1471.7380	1471.7446	914	926	AAVYHHFISDGVR
10	1657.7390	1657.7644	1172	1185	AGDFLEANYMNLQR
11	1687.8700	1687.8478	1186	1201	SYTVAIAGYALAQMGR
12	1841.9770	1841.9914	1463	1478	VHQYFNVELIQPGAVK
13	2146.9330	2147.0733	1285	1303	DAPDHQELNLDVSLQLPSR

Protein sequence of human complement component 3 precursor as retrieved from

ProteinProspector MS-Fit software search result (<http://prospector.ucsf.edu>) (accession no.

NM_000064, NCBI Entrez).

Table 3: Effects of C3 and its fragments on mouse embryo development in vitro

	No. of	Expanded blastocyst		hatching
	2-cell Embryo	No. (%)	area (μm^2)	Blastocyst, n (%)
CZB	230	128 (56) ^a	9265 \pm 170 ^a	51 (22) ^e
C3	129	69 (53) ^a	8961 \pm 208 ^a	35 (27) ^g
C3b	140	85 (61) ^c	9514 \pm 223 ^a	49 (35) ^f
iC3b	131	88 (67) ^b	11171 \pm 245 ^b	58 (44) ^h

a-b, b-d, c-d, e-f, e-h, g-h $p < 0.05$ for values within the same column

Fig. 1

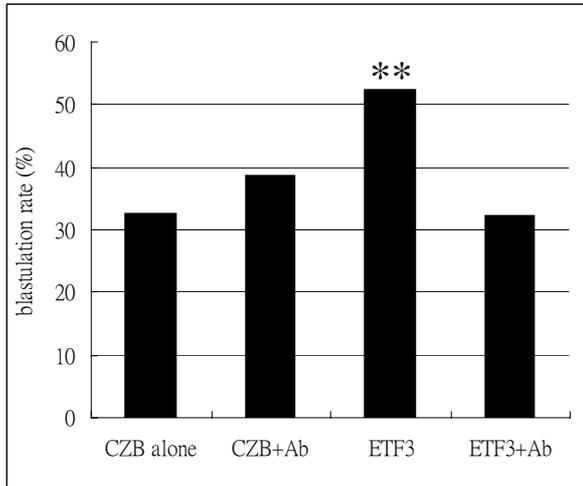


Fig. 2

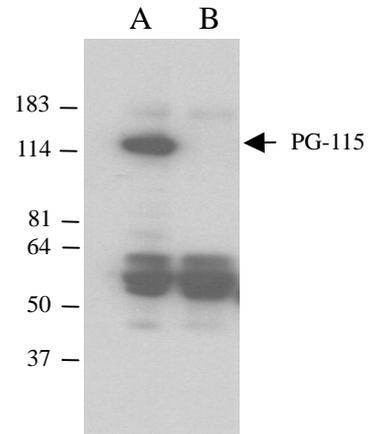


Fig. 3

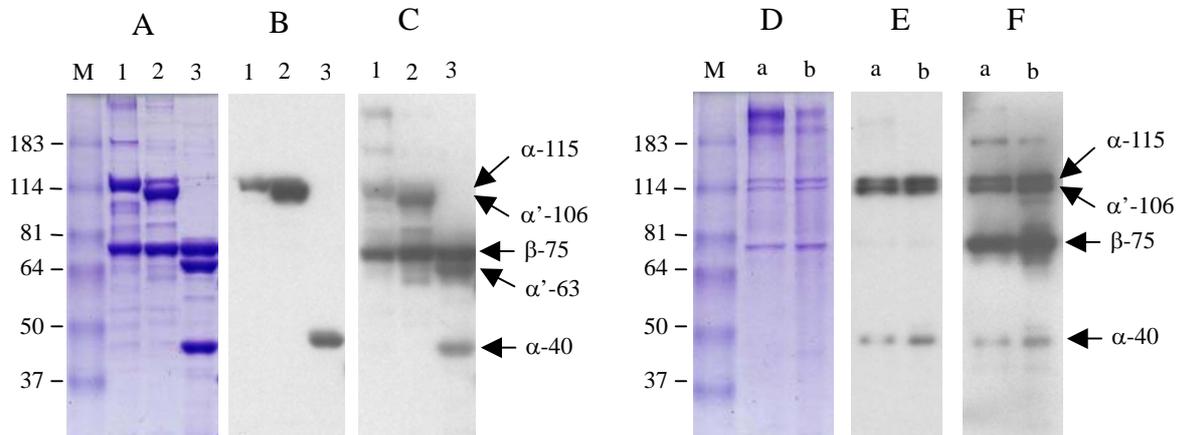


Fig. 4

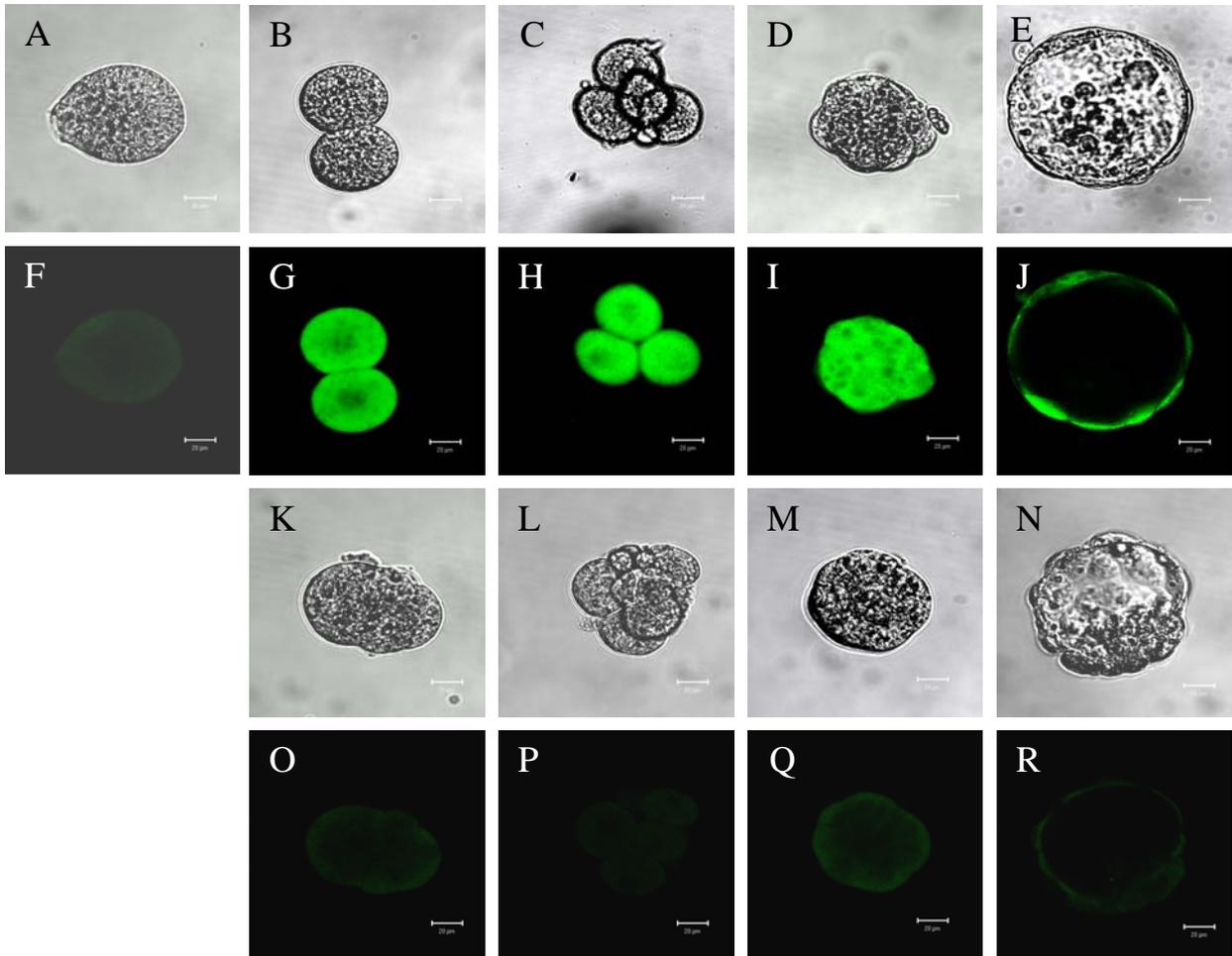


Fig. 5

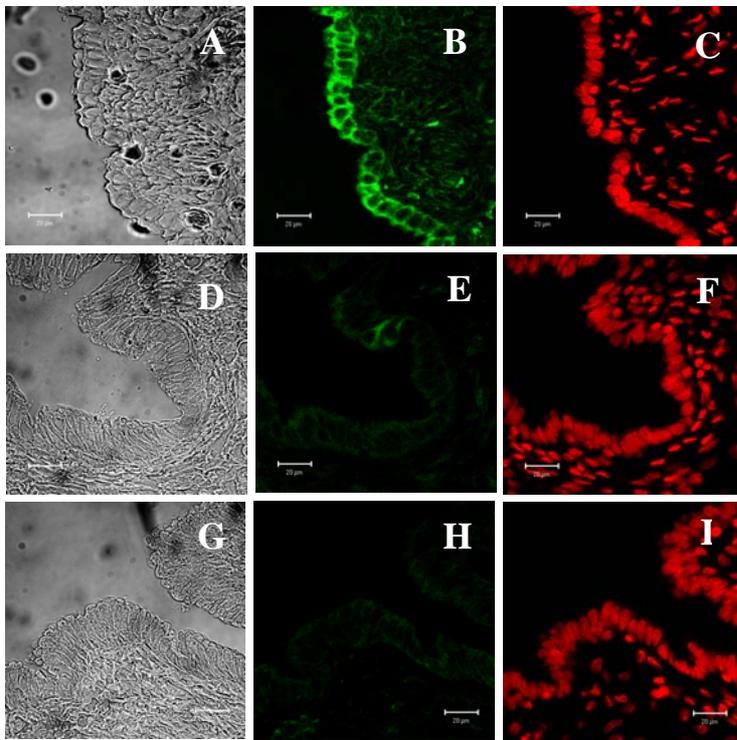


Fig. 6

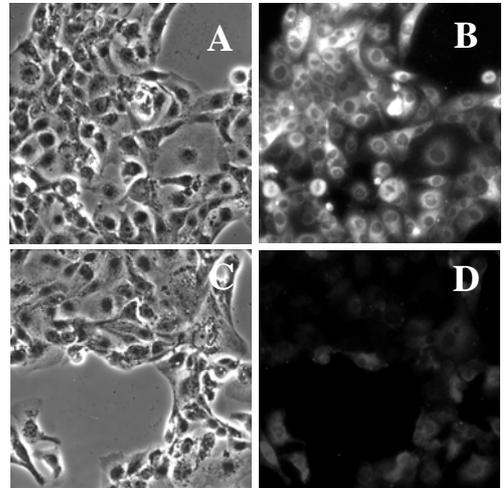


Fig. 7

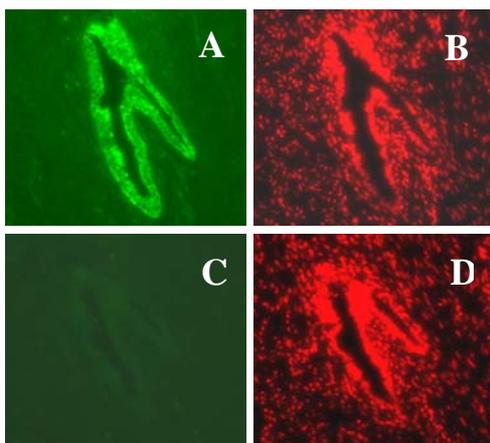


Fig. 8

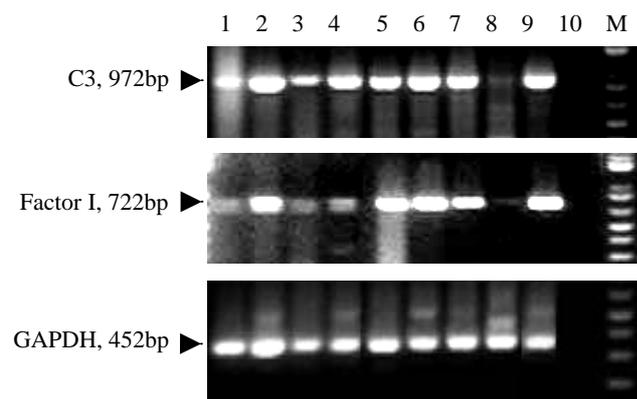


Fig. 9

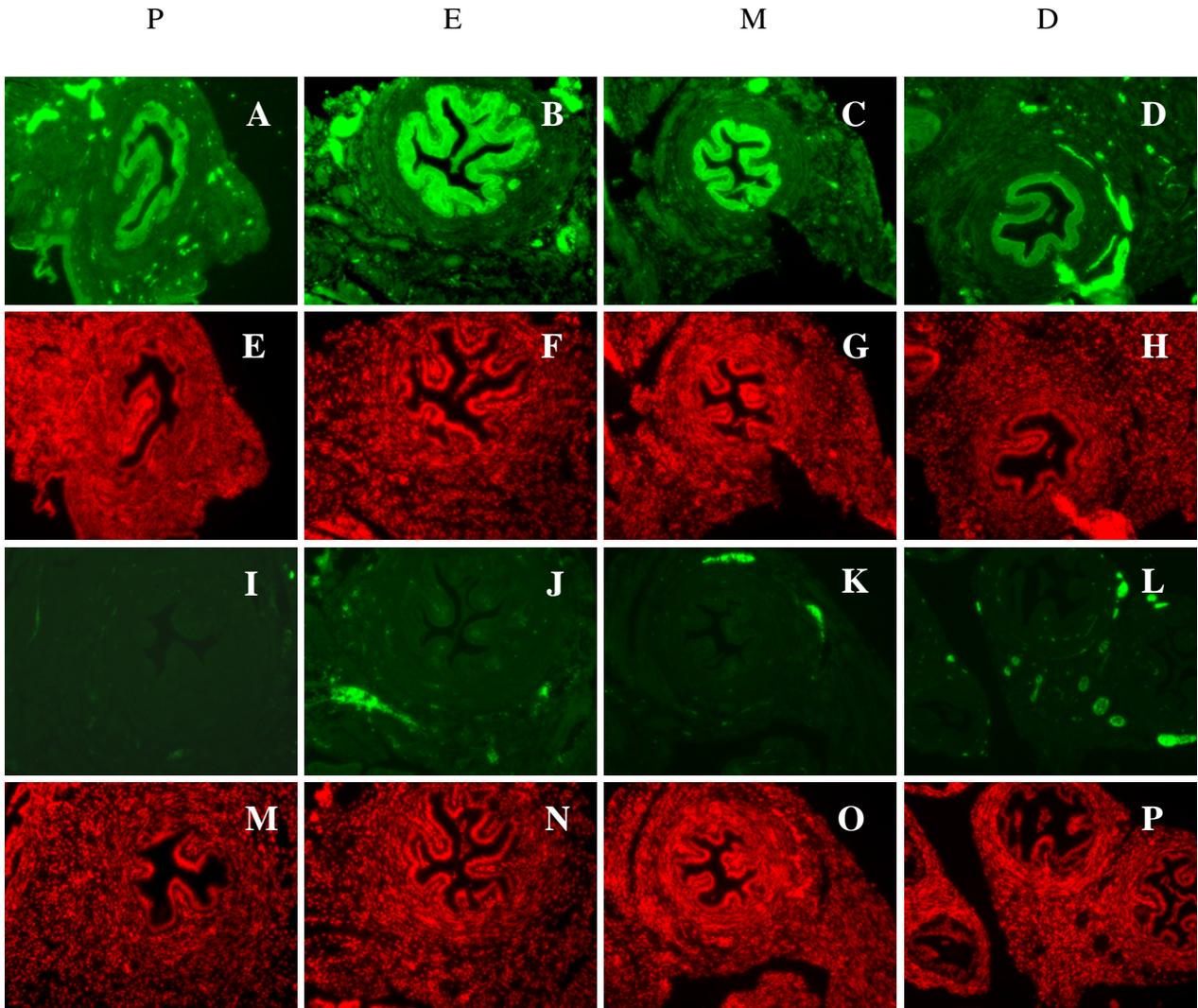


Fig. 10

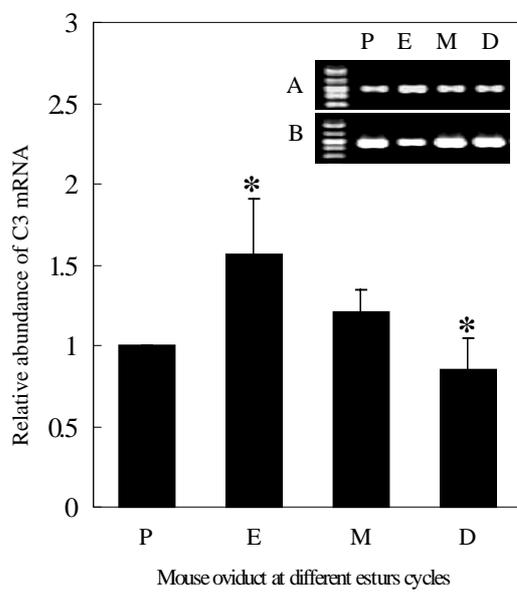


Fig. 11

