E- and N-cadherin expression and distribution during luteinization in the rat ovary

N. H. Machell¹ and R. Farookhi^{2*}

¹Departments of Physiology and ²Obstetrics and Gynecology, McGill University, Montreal, Quebec, Canada H3A 1A1

Cadherins, a family of Ca^{2+} -dependent cell adhesion molecules, play an important role in ovarian tissue remodelling processes. The aim of this study was to examine the expression pattern of E- and N-cadherin in rat preovulatory follicles, luteinizing follicles and corpora lutea. Immature female rats were treated with equine chorionic gonadotrophin (eCG) to promote preovulatory follicle development. At 48 h after eCG treatment, the rats were injected with an ovulatory dose of hCG. Ovaries were analysed by western blot analysis and immunofluorescence for E- and N-cadherin expression at 48 h after eCG injection, and at 24 and 72 h after hCG injection. Ovaries of cyclic adult rats were examined to assess whether the changes in the expression pattern of cadherin were in agreement with those of the gonadotrophin-treated rats. Finally, expression of E-cadherin in luteinizing granulosa cells *in vitro* was assessed by RT-PCR and western blot analysis. Immunofluorescence results indicate that E-cadherin is expressed in the theca-interstitial cells surrounding preovulatory follicles. N-cadherin expression

is prominent in the membrana granulosa of these follicles. The initiation of luteinization with hCG leads to a decreased expression of N-cadherin in the membrana granulosa, whereas expression of E-cadherin starts within the luteinizing follicle. Both cadherins are prominently expressed in the fully formed corpus luteum at 72 h after hCG treatment. Immunofluorescence results revealed that the patterns of E- and N-cadherin expression in the gonadotrophin-treated rats were similar to those of the cyclic adult rats. Western blot analysis reflected similar changes for N-cadherin in the ovaries of both the cyclic adults and gonadotrophin-treated rats; however, they were different in E-cadherin expression. The expression of E-cadherin mRNA and protein was induced *in vitro* in luteinized granulosa cells. These results support the hypothesis that modulation of cadherin expression is an integral component of remodelling processes, including corpus luteum formation, in the ovary. The results also indicate that expression of E- and N-cadherin in granulosa-lutein cells appear to be under hormonal control.

Introduction

Cell interactions and rearrangements are an important aspect of ovarian function and structure (Hirshfield, 1991; Richards *et al.*, 1995). Although the ovarian follicle undergoes extensive remodelling during its growth and development, particularly marked changes in follicular structure and function are seen after ovulation and corpus luteum formation (Murphy, 2000). Over the peri-ovulatory period the basement membrane of the follicle degenerates allowing theca cells and vascular elements, as well as connective tissue cells, to invade the previously avascular membrana granulosa (Van Blerkom and Motta, 1978; Mori *et al.*, 1983; for a review, see Murphy, 2000).

The subsequent consolidation of these cells results in a functional corpus luteum.

The characteristics of both the theca and granulosa cells change markedly during luteinization. The cells increase in size, acquire a polyhedral shape, accumulate lipids and produce increased amounts of progesterone (Van Blerkom and Motta, 1978; Mori *et al.*, 1983). In some species, including humans, the theca- and granulosa-lutein cells are separated in layers and can be distinguished from one another (Greenwald and Rothchild, 1968; Mori *et al.*, 1983). However, in rats the two types of luteal cells become intermingled as early as 36 h after ovulation (Pedersen, 1951; Greenwald and Rothchild, 1968). Clearly, changes in cell adhesion molecules (CAMs) must occur during this remodelling process to permit the migration of theca cells and to facilitate the association of luteinizing theca and granulosa cells into a functional corpus luteum.

The cadherins are a family of calcium-dependent CAMs that have been studied extensively for their role in

*Correspondence address: Royal Victoria Hospital, F3.44 Women's Pavilion, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1
Email: riaz.farookhi@muhc.mcgill.ca

embryogenesis and organogenesis, as well as in cell sorting and migration (Nose *et al.*, 1988; Friedlander *et al.*, 1989; Radice *et al.*, 1997a,b; Simonneau and Thiery, 1998; Vallin *et al.*, 1998). A number of studies have demonstrated that cadherins are important regulators of reproductive tissue structure, function and viability (Peluso, 1997; Makrigiannakis *et al.*, 2000; Peluso, 2000; reviewed by Rowlands *et al.*, 2000). Trollice *et al.* (1997) have shown that N-cadherin is involved in maintaining the viability of granulosa cells in rats. Cell adhesion affects the ability of rat granulosa cells to acquire LH receptors (Farookhi and Desjardins, 1986) and also influences their responsiveness to gonadotrophin signals (Harandian and Farookhi, 1998). Thus, cadherins can affect the survival, differentiation and function of ovarian cells.

Previous studies focused on the developmental expression and distribution of the classic cadherins, E- and N-cadherin, in the prepubertal rat ovary (Machell *et al.*, 2000). More recently, it has been shown that additional cadherins, specifically P-, K- and OB-cadherin, are expressed in the prepubertal rat ovary and they too display distinct developmental localization (Machell *et al.*, 2002). One marked feature of follicular cadherin expression is the absence of E-cadherin from the membrana granulosa of large preantral and antral follicles and the presence of this CAM in theca cells (Machell *et al.*, 2000). In contrast, expression of N-cadherin is maintained in the membrana granulosa at all stages of follicle development, including large antral follicles, and is present in the theca–interstitium (Machell *et al.*, 2002). Sundfeldt *et al.* (2000) reported that there was a decrease in the expression of ovarian E-cadherin after induction of ovulation with hCG in equine chorionic gonadotrophin (eCG)-primed rats. Together, these findings raise the question of what happens to the expression of these CAMs in the periovulatory period and during corpus luteum formation and establishment. In an attempt to address this issue, the expression of E- and N-cadherin was examined in ovaries of gonadotrophin-treated immature rats. Ovaries of adult cyclic rats were examined on each day of the oestrous cycle to assess changes in the expression of cadherin under endogenous hormonal conditions. Finally, changes in cadherin expression were examined in isolated granulosa cells undergoing luteinization *in vitro*.

Materials and Methods

Animals

All animal care and experimental treatments followed the regulations established by the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Royal Victoria Hospital (Montreal, QC). Female Sprague–Dawley rats were purchased from Charles River (St Constant, QC) and housed under a

12 h light:12 h dark photoperiod with food and water available *ad libitum*.

Gonadotrophin-treated rats

In vivo luteinization. Twelve immature rats of 22 days of age were injected s.c. with 10 iu eCG (Equinex, Ayerst Laboratories, Montreal, QC) in PBS containing 0.1% (w/v) gelatin (PBS–GM). An additional four control rats were injected with an equivalent volume (0.1 ml) of PBS–GM. After 48 h, four eCG-treated rats were killed by exposure to CO₂ and their ovaries recovered. One ovary from each animal was frozen on dry ice and stored at –80°C until processed for protein extraction. The other ovary was fixed in cold 100% methanol, post-fixed in 70% ethanol and then embedded in paraffin wax. The remaining eight eCG-treated rats were injected s.c. with 10 iu hCG (APL, Ayerst Laboratories) in PBS–GM. The four control (PBS–GM injected) rats received another injection at the same time with PBS–GM (0.1 ml). At 24 h after administration of hCG, four of the rats treated with eCG and hCG were killed and their ovaries allocated and processed as described above. At 72 h after the administration of hCG, the remaining four rats treated with eCG and hCG were killed. The four control rats (PBS–GM-injected) were killed at the same time. The ovaries from these two groups of animals were allocated and processed as described above.

In vitro luteinization. Sixteen rats of 22 days of age were injected s.c. with 10 iu eCG in PBS–GM. After 48 h, eight animals were killed and granulosa cells were isolated from their ovaries. Cells were obtained by first puncturing the large preovulatory follicles with a 27-gauge syringe needle and then gently pressing on the ovary with a microscalpel to expel the cells. The granulosa cells were collected in PBS and aliquots of the cell suspension were assessed for cell viability by Trypan blue exclusion. Cells with 60–70% viability were routinely recovered. Cells derived from the ovaries of each animal were maintained separately.

The isolated granulosa cells were plated in 35 mm culture dishes in 1 ml of Dulbecco's modified Eagle's medium and Ham's F12 (1:1 v:v) media containing penicillin (100 U ml^{–1}) and streptomycin (100 µg ml^{–1}). Cells were plated at a density of 500 000 cells per dish and cultured either in the absence or presence of hCG (1 iu ml^{–1}). At least two dishes per treatment (± hCG) were established using cells derived from a single animal. Media were removed at 24 h intervals for progesterone analysis and replaced with fresh media. Cell cultures were maintained for 3 days after which cells were washed with fresh media and harvested by scraping with a rubber policeman. The cell suspensions were centrifuged at 800 *g* for 5 min and the resulting cell pellets frozen for either protein or RNA extraction.

Granulosa cells from the remaining eight eCG-treated rats and from eight age-matched rats that received

PBS–GM were collected as controls to compare cadherin expression with that of the cultured granulosa cells. Granulosa cells were collected from the large preovulatory follicles of the ovaries from eCG-treated rats and from the surface follicles of rats that received PBS–GM. As described previously, cells from both ovaries of a single animal were collected and maintained as a separate sample. The residual ovarian tissue, after granulosa cell isolation, was collected from rats that received PBS–GM. The granulosa cells and residual ovarian tissue were washed in PBS and flash frozen for later protein or RNA extraction.

Cyclic adult rats

Adult cyclic female rats were approximately 50 days of age on arrival. Only animals showing regular 4-day cycles, as assessed over a 3 week period, were used. Oestrous cycles were monitored each day by examination of vaginal smears. Groups of rats were killed on the morning of each day of the oestrous cycle. The ovaries were dissected and then either frozen on dry ice and stored at -80°C until processed for protein extraction (four to five rats per group) or fixed for tissue sectioning (three rats per group) as described above.

Protein extraction

Ovaries. Frozen whole ovaries were pulverized on dry ice and processed for protein extraction as crude membrane preparations (Machell *et al.*, 2000). Briefly, cold extraction buffer (10 mmol Tris l^{-1} , pH 7.4, with 1 mmol CaCl_2 l^{-1} and 1 $\mu\text{g ml}^{-1}$ of protease inhibitors: pepstatin A, phenylmethylsulphonyl fluoride, leupeptin, soybean trypsin inhibitor and aprotinin) and solubilization buffer (18.75 μmol ; final concentrations of 62.5 mmol Tris l^{-1} , pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) β -mercaptoethanol) were added to the pulverized ovaries. The extracts were sonicated and centrifuged (10 000 *g* for 10 min) to remove insoluble material. The supernatants were centrifuged at high speed (100 000 *g* for 1 h at 4°C) and the resultant pellets resuspended in solubilization buffer and heated for 5 min at 100°C .

Isolated cells and residual ovarian tissue

Owing to the small amount of material recovered from cells luteinized *in vitro*, the granulosa cells and residual ovarian tissue were prepared as total protein extracts. The cells were extracted as described above except that the centrifugation at 10 000 *g* was eliminated. The protein content of all samples was assayed as described by Machell *et al.* (2000).

SDS-PAGE and western blot analysis

Protein samples were resolved by SDS-PAGE and blotted as described by Machell *et al.* (2000). Four micro-

grams of protein of crude membrane preparations from whole ovaries and 20 μg protein of total extracts from granulosa cells or residual ovarian tissue were loaded on to the gels. An ovary extract from a pro-oestrous rat was included on each gel to allow standardization between gels (Machell *et al.*, 2000). Membranes were immersed in 0.2% (w/v) blocking reagent (Roche Diagnostics, Laval, QC) for 1 h at room temperature (20°C) and incubated overnight at room temperature with mouse monoclonal antibodies against either E-cadherin (clone 36) (Transduction Laboratories; Bio/Can Scientific, Mississauga, ON) or N-cadherin (Zymed Laboratories Inc., San Francisco, CA), diluted 1:125 and 1:150, respectively. Alkaline phosphatase-conjugated second antibodies (rabbit anti-mouse IgG, Bio/Can Scientific) diluted 1:2500 in blocking reagent were incubated with the membranes for 2 h at room temperature. The results were visualized using a chemiluminescent detection system (Bio/Can Scientific Inc., Mississauga, ON).

Immunofluorescent staining

Immunofluorescent staining for E- and N-cadherin was performed as described by Machell *et al.* (2000). Specificity of the antibodies has been demonstrated by Machell *et al.* (2000). Sections incubated with either non-specific mouse IgG antibodies (Cedarlane Laboratories Ltd., Hornby, ON) or with the fluorescent second antibody alone served as negative controls. Images were captured using an image analysis computer system (Applied Imaging, Pittsburgh, PE) attached to an Olympus BX60 fluorescence microscope.

RNA extraction and RT-PCR

RNA was extracted from frozen ovaries, residual ovarian tissue and granulosa cells as described by Machell *et al.* (2000). Reverse transcription and PCR were performed using primers specific for E-cadherin as described by Machell *et al.* (2000). PCR products were resolved on 1.5% (w/v) agarose gels and visualized after ethidium bromide staining.

Progesterone assay

Functional luteinization of granulosa cells was assessed by measurement of the progesterone content of the media collected from the cell cultures. Progesterone was assayed using the progesterone enzymeimmunoassay test kit (ICN Pharmaceuticals, Orangeburg, NY) by following the manufacturer's instructions.

Statistical analysis

Densitometric analysis of scanned films was used to quantify the western blots. Absorbance values of all bands were normalized to the value obtained for the

Fig. 1. Expression of E- and N-cadherin in crude membrane preparations derived from total ovary extracts of equine chorionic gonadotrophin (eCG)–hCG-treated prepubertal rats. The top panels are representative western blots that were probed with either (a) anti-E-cadherin or (b) anti-N-cadherin specific antibodies. The corresponding reactive bands at approximately 120 and 130 kDa for E- and N-cadherin, respectively, are shown. The bottom panels are graphical representations derived from the densitometric analyses of similar western blots from four separate groups of rats treated in a similar way. Densitometric values were first standardized against a pro-oestrus sample that was included on all gels and then normalized against the highest mean value for each data set to eliminate reference to the external standard. Data were analysed by ANOVA using Duncan's multiple-range test. Columns that do not share a common letter are significantly different ($P < 0.05$). The molecular mass ($\times 10^{-3}$) markings corresponding to 120 and 130 kDa are shown to the left of the western blots. PBS–GM refers to control animals injected with PBS and gelatin instead of gonadotrophins that were killed at the same time as P/hCG-72 rats; eCG refers to animals killed at 48 h after eCG injection; P/hCG-24 and P/hCG-72 refer to rats killed at 24 and 72 h, respectively, after hCG injection of the eCG-treated rats. The expected occurrence of ovulation in response to this treatment paradigm is indicated.

standard sample (pro-oestrous extract of rat ovary) that was included on each gel (Machell *et al.*, 2000). This procedure allowed comparison between samples run on different gels and corrected for differences in background levels. Each value was then calculated as a percentage of the mean peak value (the highest mean value for the data set). Data were analysed by ANOVA and multiple comparisons were made using Duncan's multiple-range test with a significance level of $P < 0.05$. The mean daily progesterone production by cultured granulosa cells was compared between the hCG-treated and untreated cell cultures using one-tailed *t* tests and a significance level of $P < 0.05$.

Results

Ovarian expression of E- and N-cadherin in gonadotrophin-treated rats

Ovulation and luteinization were induced in the ovaries of immature rats by eCG and hCG treatments. Representative E- and N-cadherin immunoblots from the ovaries of these rats are shown (Fig. 1). The means ($\pm$ SEM) derived from the densitometric analyses of

four separate immunoblots for each cadherin are shown (Fig. 1).

Treatment with eCG led to a significant, approximately threefold, increase in the expression of ovarian E-cadherin compared with that of control rats that received PBS–GM (Fig. 1a). There was no change in the ovarian content of E-cadherin protein at 24 h after hCG administration in eCG-primed rats, but by 72 h after hCG treatment, E-cadherin decreased to amounts comparable to those seen in the PBS–GM control rats. In contrast, ovarian N-cadherin was unaffected by eCG treatment (Fig. 1b). However, N-cadherin increased significantly at 24 h after hCG administration and this increase was maintained at 72 h after hCG treatment. Examination of ovarian morphology at 48 h after eCG treatment showed the presence of many large antral and preovulatory follicles (Fig. 2a). At 24 h after hCG administration to these rats, many large follicles were observed together with recently ovulated follicles (Fig. 2b). Well-formed corpora lutea were visible at 72 h after hCG treatment (Fig. 2c).

Immunofluorescent staining for E-cadherin in eCG-treated rats revealed expression in thecal–interstitial cells, but staining was limited or in low amounts

Fig. 2. Histological and immunofluorescent staining of E- and N-cadherin in ovaries from (a,d,g) equine chorionic gonadotrophin (eCG)- and (b,c,e,f,h,i) eCG-hCG-treated prepubertal rats. (a–c) Haematoxylin and eosin stained ovary sections visualized at low magnification showing ovarian morphology resulting from the gonadotrophin treatments. (d–f) and (g–i) show E- and N-cadherin immunostaining, respectively, in ovary sections derived from eCG- and eCG-hCG-treated rats. E-cadherin staining in eCG-treated rats is seen predominantly in (d) the theca–interstitium, but appears in (e) the luteinizing granulosa cells after hCG injection. (f) Luteal cells show prominent E-cadherin. N-cadherin staining predominates in the (g) granulosa cells of eCG-treated rats and is expressed in both the (h) theca–interstitium and granulosa cells of hCG-treated rats and in (i) luteal cells. CL: corpus luteum; GR: granulosa cells; LG: luteinizing granulosa cells; PO: preovulatory follicle; SA, small antral follicle; t/i: thecal–interstitial cells. The dashed lines denote the (d,g) actual or (e,h) deduced location of the basement membrane. The arrowheads indicate autofluorescent red blood cells. Scale bars represent (a–c) 200 μ m, (d,e,h,i) 50 μ m and (f,g) 100 μ m.

in granulosa cells of large antral follicles (Fig. 2d). After treatment with hCG, cells in recently ovulated follicles were weakly stained for E-cadherin (Fig. 2e). The breakdown of the basement membrane was apparent as the autofluorescent red blood cells in the luteinizing follicle were visible (Paterakis, 1996). By 72 h after treatment with hCG, the corpora lutea were brightly stained for E-cadherin (Fig. 2f).

N-cadherin immunostaining was present in thecal–interstitial cells and in granulosa cells of follicles of

eCG-treated rats (Fig. 2g). Staining appeared brighter in granulosa cells of preovulatory follicles compared with the smaller antral follicles (Fig. 2g). N-cadherin staining was decreased in the cells of the luteinizing follicles at 24 h after hCG treatment (Fig. 2h), as was the case for E-cadherin, but N-cadherin stained brightly in the corpora lutea (Fig. 2i). Incubation of ovarian sections from gonadotrophin-treated or cyclic rats (see below) with non-specific mouse IgG or with the secondary antibody were not stained (not shown).

Fig. 3. E- and N-cadherin protein expression in crude membrane extracts derived from ovaries of cyclic adult rats at pro-oestrus (PE), oestrus (E), metoestrus (ME) and dioestrus (DE). The top panels represent western blots that were probed with either (a) anti-E-cadherin or (b) anti-N-cadherin specific antibodies. The corresponding reactive bands at approximately 120 and 130 kDa for E- and N-cadherin, respectively, are indicated. The bottom panels are graphical representations derived from the densitometric analyses of similar western blots from four separate groups of rats at similar stages of the ovarian cycle. Densitometric values were first standardized against a pro-oestrus sample that was included on all gels and then normalized against the highest mean value for each data set to eliminate reference to the external standard. The expected occurrence of ovulation, with respect to the oestrous cycle, is shown. Data were analysed by ANOVA using Duncan's multiple-range test and were considered significantly different at $P < 0.05$. Columns with different letters are significantly different.

Ovarian E- and N-cadherin in cyclic rats

Ovaries from adult cyclic rats were examined to assess the effects of the cyclic changes in endogenous hormones on ovarian cadherins. Representative E- and N-cadherin immunoblots derived from ovary extracts of cyclic rats are shown (Fig. 3). The means ($\pm$ SEM) derived from the densitometric analyses of four individual immunoblots for each cadherin are shown (Fig. 3).

The highest expression of E-cadherin was observed at dioestrus. This high expression was reduced by approximately 50% at pro-oestrus and reduced further at oestrus to approximately 15% of expression at dioestrus. Expression of E-cadherin at metoestrus was higher than at oestrus, but was still significantly lower than expression at dioestrus. Only the expression of E-cadherin at oestrus and metoestrus was significantly different from expression at dioestrus (Fig. 3a).

Expression of N-cadherin showed a marked and significant increase between pro-oestrus and oestrus. This increase in expression was followed by a decline at metoestrus and then a slight increase at dioestrus. Note that the expression patterns for N- and E-cadherin during the oestrous cycle are different. E-cadherin shows

a progressive increase starting from a nadir at oestrus (when N-cadherin is maximum) to maximal E-cadherin expression at dioestrus. In contrast, an increase in N-cadherin expression is seen at oestrus and dioestrus.

Evaluation of ovaries from cyclic rats showed staining for E-cadherin in extrafollicular cells including the surface epithelium, theca-interstitial cells and corpora lutea (Fig. 4a,b for metoestrous ovaries; data not shown for other cycle stages). N-cadherin was present in granulosa cells, the surface epithelium, portions of the theca-interstitium (Fig. 4c), as well as in oocytes (not shown). N-cadherin staining was pronounced in the corpus luteum (Fig. 4d). No obvious differences in staining distribution for either E- or N-cadherin were observed between ovaries collected at different stages of the oestrous cycle.

E-cadherin in granulosa cells luteinized in vitro

Progesterone production *in vitro* for granulosa cells derived from eCG-treated rats is shown (Fig. 5). Cells cultured with hCG showed a significant increase in progesterone production compared with cells cultured without hCG. This difference was seen only after 1 day

Fig. 4. Immunofluorescence staining for ovarian (a,b) E- and (c,d) N-cadherin in metoestrous rats. E-cadherin staining is seen predominantly in (a) the theca–interstitial cells (t/i) surrounding follicles and in (b) luteal and ovarian surface epithelial cells. Arrowheads indicate the ovarian surface epithelium. N-cadherin immunostaining is seen in both (c) the theca–interstitium and membrana granulosa as well as (d) the luteal cells. CL: corpus luteum; G: granulosa cells. Scale bars represent (a,c) 100 μm and (b,d) 50 μm .

Fig. 5. Progesterone production *in vitro* by luteinizing granulosa cells. Granulosa cells, isolated from equine chorionic gonadotrophin (eCG)-treated prepubertal rats, were cultured in either the absence (■) or presence (▲) of 1 iu hCG for 3 days. Progesterone production was assessed each day by measurement of the progesterone concentration in the media. The data points illustrate the mean ($\pm$ SEM) progesterone concentration for cultures of granulosa cells derived from eight eCG-treated rats. Each rat provided cells for at least four separate cultures which were assigned, in duplicate, to the two treatment groups. The mean daily progesterone production was compared between the two treatment groups using a one-tailed *t* test. Significant differences between the two treatment groups are indicated by an asterisk ($P < 0.05$).

in culture and persisted over the entire 3 day culture period. After 3 days in culture, granulosa cells treated with hCG acquired an enlarged, flattened appearance with a high cytoplasm to nucleus ratio and the presence of several cell extensions. In contrast, untreated granulosa cells maintained their smaller, rounded appearance (data not shown).

Evaluation of N- and E-cadherin expression in the cultured granulosa cells is shown (Fig. 6). N-cadherin protein was present in residual ovarian tissue from PBS–GM rats as well as in granulosa cells derived from rats that received PBS–GM and eCG-treated rats, and from granulosa cells luteinized in culture (Fig. 6a). RT–PCR analysis, with primers specific for E-cadherin, showed the presence of PCR products in ovarian residual tissue and luteinized granulosa cells, but not in granulosa cells from rats that received PBS–GM or rats treated with eCG (Fig. 6b). Western blot analysis for E-cadherin expression are in agreement with the PCR data (Fig. 6c). The absence of an E-cadherin signal in the western blots for the PBS–GM and eCG derived granulosa cells is not due to the lack of protein since subsequent re-probing for β -catenin revealed similar amounts of this cadherin-associated protein in all of the lanes of the immunoblot (data not shown).

Fig. 6. E-cadherin is expressed *in vitro* by luteinizing rat granulosa cells. (a) Western blot analysis for expression of N-cadherin (approximate molecular mass of 130 kDa) protein in total extracts derived from ovarian residual tissue (RT) and granulosa cells from rats injected with PBS containing 0.1% (w/v) gelatin (GC), granulosa cells from rats treated with equine chorionic gonadotrophin (eCG) (GC-P) and granulosa cells from rats treated with eCG after 3 days in culture with 1 iu hCG ml⁻¹ (GC-L). (b) The results for RT-PCR analysis of RNA derived from ovarian residual tissue and granulosa cells corresponding to the same treatments. PCR was conducted using specific primers for E-cadherin, which yield an expected product of 432 bp. The amplified products were visualized on a 1% (w/v) agarose gel stained with ethidium bromide. (c) Western blot analysis for expression of E-cadherin (approximate molecular mass of 120 kDa) in total extracts of ovarian residual tissue and granulosa cells derived from the same treatment groups.

Discussion

The establishment of cell associations and interactions is an important component of tissue morphogenesis and development. The ovary, perhaps more than other organs, exemplifies such events. Changes in cell associations and segregation of cell populations are characteristics of follicular development, ovulation and formation of the corpus luteum (Hirshfield, 1991; Murphy, 2000). Machell *et al.* (2000, 2002) described classical and atypical cadherin expression in the prepubertal rat ovary. These cadherins are expressed within specific compartments of the ovary and their expression shows changes during the different stages of follicular development. The present study reports expression of E- and N-cadherin in preovulatory follicles and during corpus luteum formation. The results demonstrate that changes in expression of cadherin accompany the formation and consolidation of follicular-lutein cells in the corpus luteum. In addition, the present study demonstrates that functional luteinization of isolated rat granulosa cells is accompanied by repeated expression of E-cadherin in these cells. Together, these observations further support the contention that remodelling and differentiation processes in the ovary involve modulation of cadherin expression.

The results of the present study indicate that enhanced expression of E-cadherin is an important event in the early stages of luteinization. Expression of ovarian E-cadherin increased after eCG treatment and remained high after hCG-induced ovulation. However, on the basis of immunofluorescence evaluation, the types of cell contributing to the enhanced expression of ovarian E-cadherin differ as a consequence of these hormone stimulations. The eCG-mediated increase in expression arises from the theca-interstitial cells surrounding enlarged preovulatory follicles. However, with induction of ovulation it appears that both the luteinizing theca and granulosa cells contribute to ovarian E-cadherin content. The expression of E-cadherin by luteinizing granulosa cells was confirmed *in vitro* when these cells were induced to express this CAM and produce progesterone in response to hCG.

The results from the present study with respect to the expression of E-cadherin in luteinizing follicles are not in agreement with the findings of Sundfeldt *et al.* (2000). Sundfeldt *et al.* (2000) reported an abrupt decrease in ovarian E-cadherin protein within 4 h after hCG treatment of eCG-treated rats. This low expression of E-cadherin was maintained for up to 12 h after the hCG treatment. In contrast, a decrease in E-cadherin occurred only by 72 h after hCG treatment. Immunostaining results of the present study demonstrated the presence of E-cadherin in the luteo-follicular complex 24 h after hCG treatment, indicating that the luteinizing theca and granulosa cells were positive for this cadherin. Differences in tissue fixation procedures and the use

of antigen retrieval may explain the ability to detect E-cadherin more readily in the tissue sections in the present study. There are also differences in the time intervals after hCG that were examined versus those examined by Sundfeldt *et al.* (2000). In addition, a membrane-enriched fraction of the ovarian homogenates was used for immunoblotting purposes, whereas these were not used in other studies. It is possible that there is an acute decrease in E-cadherin expression which is restored by 24 h. This finding raises the possibility that there may be two competing drives for E-cadherin expression in follicular, specifically, luteinizing theca cells. Initially, the downregulation of E-cadherin may be involved in facilitating the migration of theca cells into the ovulated follicle. The absence of E-cadherin has been associated with cell migratory behaviour (Streit *et al.*, 1996). The subsequent increase in E-cadherin could be involved in promoting adhesion between luteal cells leading to the consolidation of the corpus luteum.

Western blot analysis revealed that eCG treatment does not change ovarian N-cadherin content, although immunofluorescence indicated more intense staining of the membrana granulosa in preovulatory follicles. Failure to detect changes in ovarian N-cadherin is a consequence of the more general distribution of N-cadherin in the ovary (Machell *et al.*, 2000). Thus, small increases in expression in a subset of follicles, apparent by immunostaining, may not be detected as a change in total ovarian content. However, ovulation at oestrus or as a result of hCG treatment evokes an increase in N-cadherin indicating that luteinization is accompanied by increases in N-cadherin expression that may be related to luteal cell hypertrophy and hyperplasia. The increase in the expression of N-cadherin is maintained with the formation of the corpus luteum at 72 h after hCG administration.

As expected, western blot analyses reveal differences between gonadotrophin-treated and cyclic animals. The variations are due, most likely, to differences in composition between the ovaries from the two groups (for example, the presence of corpora lutea from previous cycles and degenerating follicles in the adults). One of the advantages of using gonadotrophin-treated immature animals is that degenerating corpora lutea or corpora lutea from previous cycles are not present in the ovary. In addition, events can be followed in a temporal manner on the basis of the time of administration of the gonadotrophins.

The observed changes in the expression of cadherin in the gonadotrophin-treated animals indicate that ovarian E- and N-cadherin are affected in different ways by hormones. Oestrogen can regulate E- and N-cadherin mRNA content in the mouse ovary (MacCalman *et al.*, 1994b, 1995) and N-cadherin in granulosa cells (Farookhi *et al.*, 1997). The presence of E-cadherin mRNA and protein in luteinized granulosa cells indicates

that LH may be involved in transcriptional regulation of this cadherin in granulosa-lutein cells. In human cultured cytotrophoblasts, hCG alters cadherin expression (Shi *et al.*, 1993). Expression of E-cadherin may be dependent on concentrations of progesterone. The experimental design used in the present study does not allow distinction between gonadotrophin or progesterone dependence. E-cadherin is regulated by both oestrogen and progesterone in reproductive tissues (MacCalman *et al.*, 1994a,b; Potter *et al.*, 1996).

The immunostaining results of the present study indicate that similar changes in the expression of E- and N-cadherin occur during corpus luteum formation regardless of whether the luteinization process is evoked by exogenous gonadotrophin treatment or as the consequence of changes in endogenous cyclic hormone. Considering that luteal E- and N-cadherin expression has been demonstrated in other species (that is humans and baboons) (Khan-Dawood *et al.*, 1996a,b; Makrigiannakis *et al.*, 2000), their role in corpus luteum organization may be general across mammalian species.

In summary, the present study has demonstrated that the expression of two classic cadherins (E- and N-cadherin) is modulated during the periovulatory period and formation of the corpus luteum in gonadotrophin-treated immature and adult cyclic rats. In addition, granulosa cells express E-cadherin after *in vitro* luteinization. These results, in association with previous studies, provide compelling evidence that expression of cadherin in the ovary is hormonally regulated and is an important component of ovarian function.

This research was supported by a CIHR grant to R. Farookhi and an MRC Doctoral research award to N. H. Machell.

References

- Farookhi R and Desjardins J (1986) Luteinizing hormone receptor induction in dispersed granulosa cells requires estrogen *Molecular and Cellular Endocrinology* **47** 13–24
- Farookhi R, Geng CS, MacCalman CD and Blaschuk OW (1997) Hormonal regulation of N-cadherin mRNA levels in rat granulosa cells *Annals of the New York Academy of Sciences* **816** 165–172
- Friedlander DR, Mege RM, Cunningham BA and Edelman GM (1989) Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces *Proceedings National Academy of Sciences USA* **86** 7043–7047
- Greenwald GS and Rothchild I (1968) Formation and maintenance of corpora lutea in laboratory animals *Journal of Animal Science* **27** 139–162
- Harandian F and Farookhi R (1998) Contact-dependent cell interactions determine hormone responsiveness and desensitization in rat granulosa cells *Endocrinology* **139** 1700–1707
- Hirshfield AN (1991) Theca cells may be present at the outset of follicular growth *Biology of Reproduction* **44** 1157–1162
- Khan-Dawood FS, Yang J and Dawood MY (1996a) Immunocytochemical localization and expression of E-cadherin and beta-catenin in the human corpus luteum *Molecular Human Reproduction* **2** 753–757
- Khan-Dawood FS, Yang J, Ozigi AA and Dawood MY (1996b) Immunocytochemical localization and expression of E-cadherin, beta-catenin, and plakoglobin in the baboon (*Papio anubis*) corpus luteum *Biology of Reproduction* **55** 246–253

- MacCalman CD, Farookhi R and Blaschuk OW** (1994a) Estradiol and progesterone regulate E-cadherin mRNA levels in the mouse uterus *Endocrine* **2** 485–490
- MacCalman CD, Farookhi R and Blaschuk OW** (1994b) Estradiol regulates E-cadherin mRNA levels in the surface epithelium of the mouse ovary *Clinical and Experimental Metastasis* **12** 276–282
- MacCalman CD, Farookhi R and Blaschuk OW** (1995) Estradiol regulates N-cadherin mRNA levels in the mouse ovary *Developmental Genetics* **16** 20–24
- Machell NH, Blaschuk OW and Farookhi R** (2000) Developmental expression and distribution of N- and E-cadherin in the rat ovary *Biology of Reproduction* **63** 797–804
- Machell NH, Blaschuk OW and Farookhi R** (2002) Expression and localization of P-, K- and OB-cadherin in the prepubertal rat ovary *Molecular Reproduction and Development* **61** 142–154
- Makrigiannakis A, Coukos G, Blaschuk O and Coutifaris C** (2000) Follicular atresia and luteolysis. Evidence of a role for N-cadherin *Annals of the New York Academy of Sciences* **900** 46–55
- Mori T, Nihnobu K, Takeuchi S, Onho Y and Tojo S** (1983) Interrelation between luteal cell types in steroidogenesis *in vitro* of human corpus luteum *Journal of Steroid Biochemistry* **19** 811–815
- Murphy BD** (2000) Models of luteinization *Biology of Reproduction* **63** 2–11
- Nose A, Nagafuchi A and Takeichi M** (1988) Expressed recombinant cadherins mediate cell sorting in model systems *Cell* **54** 993–1001
- Paterakis GS** (1996) Reticulocyte counting in thalassaemia *Clinical and Laboratory Haematology* **18** Supplement **1** 17–28
- Pedersen** (1951) Histogenesis of lutein tissue of the albino rat *American Journal of Anatomy* **88** 397
- Peluso JJ** (1997) Putative mechanism through which N-cadherin-mediated cell contact maintains calcium homeostasis and thereby prevents ovarian cells from undergoing apoptosis *Biochemical Pharmacology* **54** 847–853
- Peluso JJ** (2000) N-cadherin-mediated cell contact regulates ovarian surface epithelial cell survival *Biological Signals and Receptors* **9** 115–121
- Potter SW, Gaza G and Morris JE** (1996) Estradiol induces E-cadherin degradation in mouse uterine epithelium during the estrous cycle and early pregnancy *Journal of Cellular Physiology* **169** 1–14
- Radice GL, Ferreira-Cornwell MC, Robinson SD, Rayburn H, Chodosh LA, Takeichi M and Hynes RO** (1997a) Precocious mammary gland development in P-cadherin-deficient mice *Journal of Cell Biology* **139** 1025–1032
- Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M and Hynes RO** (1997b) Developmental defects in mouse embryos lacking N-cadherin *Developmental Biology* **181** 64–78
- Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Alliston T and Sirois J** (1995) Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes *Recent Progress in Hormone Research* **50** 223–254
- Rowlands TM, Symonds JM, Farookhi R and Blaschuk OW** (2000) Cadherins: crucial regulators of structure and function in reproductive tissues *Reviews of Reproduction* **5** 53–61
- Shi QJ, Lei ZM, Rao CV and Lin J** (1993) Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts *Endocrinology* **132** 1387–1395
- Simonneau L and Thiery JP** (1998) The mesenchymal cadherin-11 is expressed in restricted sites during the ontogeny of the rat brain in modes suggesting novel functions *Cell Adhesion and Communication* **6** 431–450
- Streit M, Schmidt R, Hilgenfeld RU, Thiel E and Kreuser ED** (1996) Adhesion receptors in malignant transformation and dissemination of gastrointestinal tumors *Journal of Molecular Medicine* **74** 253–268
- Sundfeldt K, Piontkewitz Y, Billig H and Hedin L** (2000) E-cadherin–catenin complex in the rat ovary: cell-specific expression during folliculogenesis and luteal formation *Journal of Reproduction and Fertility* **118** 375–385
- Trolice MP, Pappalardo A and Peluso JJ** (1997) Basic fibroblast growth factor and N-cadherin maintain rat granulosa cell and ovarian surface epithelial cell viability by stimulating the tyrosine phosphorylation of the fibroblast growth factor receptors *Endocrinology* **138** 107–113
- Vallin J, Girault JM, Thiery JP and Broders F** (1998) *Xenopus* cadherin-11 is expressed in different populations of migrating neural crest cells *Mechanisms of Development* **75** 171–174
- Van Blerkom J and Motta P** (1978) A scanning electron microscopic study of the luteo-follicular complex. III. Repair of ovulated follicle and the formation of the corpus luteum *Cell and Tissue Research* **189** 131–153

Received 24 May 2002.

First decision 23 July 2002.

Revised manuscript received 27 November 2002.

Accepted 19 February 2003.