N-cadherin mediated cell contact inhibits germinal vesicle breakdown in mouse oocytes maintained *in vitro*

J J Peluso

Departments of Cell Biology and Obstetrics and Gynecology, University of Connecticut Health Center, Farmington, Connecticut 06030

Correspondence should be addressed to J J Peluso; Email: peluso@nso2.uchc.edu

Abstract

The effect of granulosa cell contact on the ability of zona-free oocytes to undergo germinal vesicle breakdown (GVBD) was assessed using a granulosa cell co-culture system. Oocytes contacted granulosa cells in a site-specific manner such that their GV was away from the granulosa cells. Also contact with granulosa cells reduced the percentage of oocytes undergoing GVBD from about 40% to 15%. GVBD was inhibited by contact with granulosa cells but not a granulosa cell-secreted product, since oocytes cultured in the same culture, that were adjacent to the granulosa cell monolayer underwent GVBD at the same rate as controls. Similarly, media collected from granulosa cell cultures did not attenuate the rate of GVBD. The ability of granulosa cell contact to inhibit GVBD was equal to that of db-cAMP. Moreover, the ability of granulosa cells to inhibit GVBD was not mimicked by spontaneously immortalized granulosa cells. This cell specificity appeared to be related to N-cadherin, since granulosa cells and oocytes express N-cadherin and a N-cadherin antibody attenuates the effect of granulosa cell contact. The mechanism through which N-cadherin mediated cell contact maintains meiotic arrest is unknown. It is possible that homophilic N-cadherin binding between the granulosa cells and oocyte acts through a junxtacrine mechanism, which in part may lead in the activation fibroblast growth factor (FGF) receptors that are expressed by the oocyte. The involvement of FGF receptors is supported by the observations that FGF and a N-cadherin peptide known to activate FGF receptors inhibit GVBD.

Reproduction (2006) **131** 429–437

Introduction

Of the many primordial germ cells (PGCs) that enter the developing ovary, only those PGCs that establish contact with presumptive granulosa cells and form primordial follicles survive. The remaining germ cells undergo apoptosis (De Felici 2000, 2001). Once associated with granulosa cells, the PGCs exit the cell cycle and enter into prophase I of meiosis (De Felici 2000, 2001). The PGCs, now referred to as oocytes remain in meiotic arrest throughout the course of follicular development (De Felici 2000, 2001). It is important to appreciate that in non-atretic follicles at all stages of development the oocyte maintains contact with the granulosa cells (Albertini & Barrett 2003). It is only in mature females at the time of the ovulatory gonadotropin surge, that granulosa cell-oocyte contact is disrupted and oocytes within preovulatory follicles undergo germinal vesicle breakdown (GVBD) and resume meiosis (Hyttel *et al.* 1986, Albertini & Barrett 2003). Contact between granulosa cells and the oocyte is also lost during follicular atresia at which time the nuclear membrane of the oocyte degenerates (Peluso *et al.* 1979, Hyttel

et al. 1986). Thus, these observations suggest that somehow granulosa cell contact is essential for both oocyte viability and the maintenance of meiotic arrest.

The capacity to undergo germinal vesicle breakdown and resume meiosis is developmentally regulated (Eppig *et al.* 2004). This occurs when the oocyte has grown to a diameter of 60 and 65 μm (Eppig *et al.* 2004). Interestingly, both oocyte growth and the development of meiotic competence are dependent in part on the formation of gap junctions between the oocyte and the surrounding granulosa (cumulus) cells (Salustri *et al.* 2004). This concept is supported by the observation that the ovulatory gonadotropin surge decreases the frequency of gap junctions between the granulosa cells and the oocytes and induces GVBD (Salustri *et al.* 2004). Moreover, pharmacological inhibitors of gap junction communication stimulate the hypoxanthine-treated cumulus cell-enclosed oocytes to undergo GVBD *in vitro* (Downs 2001).

It is well known that agents that elevate intracellular cAMP inhibit GVBD *in vitro* (Voronina & Wessel 2003, Eppig *et al.* 2004). One mechanism that increases

intra-oocyte cAMP levels is the gap junction-mediated transfer of cAMP, which is synthesized in the granulosa cells, to the oocyte (Webb *et al.* 2002). However, rodent oocytes possess an adenyl cyclase that is likely to be involved in generating intra-oocyte cAMP (Horner *et al.* 2003). Moreover, recent studies indicate that oocytes possess a G-protein coupled receptor that may ultimately stimulate cAMP production within the oocyte (Mehlmann *et al.* 2002, 2004). Although the ligand for this G-protein coupled receptor is not known, these studies suggest that cAMP derived from the oocyte as well as the surrounding granulosa cells act to maintain oocyte meiotic arrest.

In addition to gap junctions, oocytes interact with granulosa cells through adhesion junctions that are formed through homophilic binding of cadherins (Rufas *et al.* 2000, Machell & Farookhi 2003). In a primordial follicle, the oocyte maintains contact with the granulosa cells through adhesion junctions composed of both E-cadherin and N-cadherin (Machell & Farookhi 2003). Although E-cadherin expression decreases once follicular growth is initiated, N-cadherin levels are maintained throughout the course of follicle development (Machell & Farookhi 2003).

Previous studies of homophilic cadherin binding have historically focused on their role in mediating specific cell–cell associations. However, more recent studies have shown that cadherin binding can activate various signal transduction kinases including the FGF receptor (Peluso 2000, Williams *et al.* 2001, Williams *et al.* 2002) and PI3 kinase/AKT kinase (Peluso *et al.* 2001, Rieger-Christ *et al.* 2004, Reddy *et al.* 2005). Since these kinases are expressed in mammalian oocytes (Haffner-Krausz *et al.* 1999, Voronina & Wessel 2003), it is possible that N-cadherin mediated cell contact could activate a signaling cascade that maintains the oocyte in meiotic arrest. To test this hypothesis, a series of *in vitro* experiments were designed to determine whether: 1) granulosa cell contact per se inhibits GVBD; 2) N-cadherin mediated cell contact attenuates GVBD and 3) a soluble N-cadherin peptide mimetic prevents GVBD.

Materials and Methods

Chemicals and reagents

The following reagents were used in these studies. The db-cAMP (Sigma Chemical Co., St Louis, MO, USA) was used at a final concentration of 250 μ M. IgG and anti-N-cadherin (Clone GC-4) were purchased from Sigma and used at a final concentration of 1 mg/ml. Human recombinant basic fibroblast growth factor (bFGF, R & D Inc, Minneapolis, MN, USA) was used at a final concentration of 5 ng/ml. The peptides were synthesized by Aves labs (Tigard, OR, USA) and were purified by reverse-phase HPLC to a purity of >98%. The dimeric peptide, N-Ac-CHAVDINGHAVDIC-NH₂, was made cyclic by making a disulfide bond between the cysteine residues. A linear

dimeric peptide sequence (N-Ac-HAVDINGHAVDI-NH₂) was used as a control. Both peptides were diluted in DMSO and used at a final concentration of 20 μ g/ml (Williams *et al.* 2002).

Animals and granulosa cell cultures

All protocols involving mice were conducted with the approval of the Animal Care Committee of the University of Connecticut Health Center. Immature female CF-1 mice (21 days of age) were obtained from Charles River Laboratory (Wilmington, MA, USA) and housed under controlled conditions of temperature, humidity and photoperiod (12 h light:12 h darkness; lights on at 07:00 h). For most experiments, granulosa cells were obtained from two immature animals that were 23 or 25 days of age as previously described (Lederer *et al.* 1995). Briefly, ovaries were sequentially placed in EGTA- and EGTA/sucrose-supplemented DMEM/F-12 medium. The ovaries were then pressed to release the granulosa cells. The granulosa cells were washed in 5% serum-supplemented DMEM/F12 medium and plated in a 20 μ l drop of serum-supplemented media, which was in a 35 mm culture dish covered with sterile light mineral oil. After 24 h of culture at 37°C in 5% CO₂, these cells formed a monolayer.

Spontaneously immortalized granulosa cells (SIGCs) were generously provided by Dr Robert Burghardt of Texas A & M University (College Station, TX, USA). SIGCs were also cultured in a 20 μ l microdrop culture as described above.

Oocyte-granulosa cell co-cultures

To isolate zona-free oocytes, the residual ovaries from two immature mice, obtained after the granulosa cells were isolated, were incubated in DMEM/F12 supplemented with 0.01 g/ml of Collagenase ($\approx$ 5000 units), 0.08 mg/ml of DNAase ($\approx$ 2000 units) and 1% BSA for 35 min in a shaking water bath at 37°C (Roy & Greenwald 1996). The residual ovaries were passed through a Pasteur pipette then filtered through sequential stainless steel grids with pore sizes of 230, 140 and 73 μ m, respectively. The last filtrate contained single cells and zona-intact and zona-free oocytes. The zona-free oocytes with intact nuclear membranes were isolated with a micropipette, washed twice in serum-supplemented media and then 15 to 25 oocytes were placed into 20 μ l microdrop of culture media according to the specific experimental design. After culture, the presence or absence of the oocyte GV was assessed under phase optics. In some experiments, the granulosa cell-oocyte co-cultures were fixed in 3% paraformaldehyde for 10 min, permeabilized with 0.1% Triton-X for 5 min, stained with propidium iodide (0.5 μ g/ μ l) for 40 min, washed twice in PBS and then observed under fluorescent optics with the TRITC filter set.

Immunochemical localization of N-cadherin and fibroblast growth factor receptor

For immunohistochemical assessments, mouse ovaries from immature 23 day old mice were removed, fixed in formalin, embedded in paraffin, sectioned at 5 μ m and mounted on glass slides. Representative slides from each ovary were stained as outlined in the following protocol. Endogenous peroxidase activity was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Slides were incubated overnight at 4°C with N-cadherin (1:100 dilution) or with FGF receptor antibody (1:200 dilution). Slides were then incubated with either biotinylated goat-anti-mouse IgG or biotinylated goat-anti-rabbit IgG, for 30 min at room temperature. The slides were washed in PBS and incubated with Avidin/Biotinylated Complex (ABC) reagent for 30 min at room temperature. The slides were then developed using a 3,3'-diaminobenzidine (DAB)-peroxidase substrate for 5 min. Finally, the slides were counter stained with methyl green for 10 s, rinsed in distilled water, dehydrated, cleared and mounted. As a negative control, an IgG replaced the primary antibody in this immunohistochemical protocol. The presence of N-cadherin or FGF receptor was revealed by the presence of a reddish-brown precipitate.

Statistical analysis

All experiments were repeated three to five times. For each experiment, the data from each replicate were pooled and analyzed using a chi-square test. *P* values of less than 0.05 were considered to be significant.

Results

Collagenase/DNAase treatment of immature mouse ovaries resulted in the isolation of numerous oocytes that possessed intact germinal vesicles and their zonae were either completely or partially depleted. Their average diameter was $66 \pm 2 \mu$ m. When placed in serum-supplemented medium, these zona-free oocytes underwent nuclear breakdown with approximately 40% undergoing

Figure 1 The morphology (A) and capacity of zona-free oocytes isolated from immature mouse ovaries to undergo germinal vesicle breakdown (GVBD) *in vitro* (B). In this study a total of 36 zona-free oocytes were sequentially observed under phase optics at 0, 1, 2, 3 and 4 h. Scale bar 20 μ m.

GVBD by 4 h (Fig. 1). In contrast, zona-free oocytes plated onto a monolayer of granulosa cells established contact almost immediately with most oocytes remaining in the GV stage. The granulosa cells did not engulf these oocytes and only a small segment of the oolemma interacted with the granulosa cell monolayer. In this co-culture system the oocytes were not tightly bound to the granulosa cells but rather loosely tethered to the granulosa cell monolayer. This cellular association did not change even after 24 h of culture. In addition the oocyte orientated itself such that the GV was always away from the granulosa cell monolayer. This was evidenced by fact that the focal plane of the oocyte GV was always well above that of the granulosa cells (Fig. 2).

The effectiveness of granulosa cell contact in preventing GVBD was then compared with that of 250 μ M db-cAMP. As seen in Fig. 3, only about 5% of the oocytes that were exposed to db-cAMP underwent GVBD compared with 43% for the serum controls (*P* < 0.05). Likewise, granulosa cell contact suppressed GVBD to 15% (*P* < 0.05 compared with serum controls). This percentage was not significantly different from the db-cAMP treatment (*P* > 0.05).

As with any co-culture study, there is concern that a secreted factor could account for the effect of granulosa cells on GVBD. Two different treatments were designed to assess this possibility. First, a microdrop culture was set up in which two 10 μ l drops of media were placed within a few mm of each other in the same culture dish. Granulosa cells were plated in one drop. After 24 h the media from both drops were removed and replaced with fresh media. Then oocytes were added to both drops and the 'oil bridge' between the two drops broken thereby combining the two cultures. As shown in Fig. 3 those oocytes within the same microdrop that did not establish granulosa cell contact underwent GVBD at a rate comparable to that of the serum controls, while GVBD was inhibited in those oocytes that interacted with the granulosa cells. Similarly, media taken from granulosa cells after 24 h of culture (i.e. spent media) did not inhibit GVBD (Fig. 3).

To address the issue of cell specificity, the ability of SIGCs to inhibit GVBD was compared with that of granulosa cells. This study revealed that granulosa cells but not SIGCs suppressed the rate of GVBD (Fig. 4). SIGCs differ from granulosa cells in that they express E-cadherin (Peluso *et al.* 2001). Immunohistochemical analysis confirmed that N-cadherin was present at the plasma membrane of granulosa cells and oocytes. Moreover, N-cadherin was also detected in the transzonal projections (Fig. 5 A,B). To determine if disrupting N-cadherin homophilic binding influences the rate of GVBD, oocytes were incubated for 5 min with either an N-cadherin antibody or IgG and then plated on a granulosa cell monolayer. This study revealed that the N-cadherin antibody significantly increased the percentage of oocytes that underwent GVBD compared with the IgG controls (Fig. 5 C; *P* < 0.05). Interestingly, the N-cadherin antibody did not prevent the oocytes from tethering to the granulosa cell layer.

Figure 2 Morphology of oocytes maintained in co-cultured on a monolayer of granulosa cells. Images in panels A, B and C, D are taken from the same co-cultures, respectively. Images in panels A and C focused on the granulosa cell monolayer, while images in B and D focused on the germinal vesicle. Panels A and B were taken under phase optics. The granulosa cell-oocyte culture shown in C and D was stained with propidium iodide. Panel D shows the heterochromatin associated with the germinal vesicle and the nucleolus (arrowhead). Note that the fluorescence associated with the granulosa cell nuclei that are beneath the oocyte is not detectable in panel D.

Figure 3 The effect of db-cAMP, cell contact and spent media collected from granulosa cell cultures on the percentage of zona-free oocytes undergoing GVBD after 4 h of culture. Values at the base of each bar represent the number of oocytes examined. * indicates a value that is significantly different from the serum only control group. All treatments were not conducted each time the experiment was run. However as a positive control, some oocytes were treated with the serum only on each day the experiments were conducted and all studies were part of the same experimental series.

These observations suggested that N-cadherin might be stimulating a signal transduction pathway as well as being involved in cell-cell adhesion. To test this possibility a cyclic dimeric peptide mimetic of N-cadherin (i.e. HAVDI) that is known to bind and activate the FGF receptor (Williams *et al.* 2002), was assessed for its ability to inhibit GVBD. A linear dimeric peptide and bFGF were used as negative and positive controls, respectively. After 4 and 20 h of culture both the cyclic HAVDI peptide and bFGF suppressed rate of GVBD compared with DMSO control (Fig. 6; $P < 0.05$). At 20 h, cyclic HAVDI peptide and bFGF suppressed rate of GVBD compared with DMSO and the linear peptide control (Fig. 6; $P < 0.05$). Immunohistochemical analysis confirmed that both the oocyte and granulosa cells express FGF receptors (Fig. 7) with the FGF receptors being localized in the transzonal projections.

Discussion

Oocytes, isolated from immature mouse ovaries, are capable of maturing with approximately 40% undergoing GVBD within 4 h of culture. This rate is consistent with previous reports (Donahue 1968, Sorensen & Wassarman 1976), but it is somewhat lower than the rate of maturation for oocytes isolated from preovulatory follicles (Sorensen & Wassarman 1976). Oocytes develop the capacity to undergo GVBD *in vitro* when they are approximately 60 to 65 μm in diameter (Sorensen & Wassarman 1976). In the present study, the oocytes averaged 66 ± 2 μm in diameter indicating that most oocytes were of sufficient size to undergo GVBD. However, some of the oocytes were smaller than 60 μm, which would account for the somewhat lower GVBD rate compared with oocytes of preovulatory follicles.

Figure 4 The effect of granulosa cell (GC) and Spontaneously Immortalized Granulosa Cell (SIGC) contact on the rate of germinal vesicle breakdown. Zona-free oocytes were plated on a monolayer of either granulosa cells or SIGCs. The oocytes were scored for nuclear breakdown after 4 h of culture. The number of oocytes examined in each treatment is shown at the base of each bar. * indicates a value that is significantly different from oocytes cultured without a cell monolayer (i.e. no cell contact) or in the presence of a SIGC monolayer.

Figure 5 The immunohistochemical localization (A, B) and effect of N-cadherin on the rate of germinal vesicle breakdown (C). Panel A is a negative control. An oocyte that can only be seen under phase optics is in the center of this panel. Panel B shows an oocyte within very early stage antral follicle that was stained for N-cadherin. N-cadherin is revealed by a reddish-brown stain. N-cadherin is expressed by both granulosa cells and oocyte. Further, N-cadherin is also detected in the transzonal projections. In panel C the effect of either IgG or N-cadherin antibody on the ability of granulosa cell contact to maintain the germinal vesicle is shown. The number of oocytes examined in each treatment is shown at the base of each bar. * indicates a value that is significantly different from oocytes exposed to IgG.

In addition to their capacity to undergo GVBD, many of the oocytes isolated from immature mouse ovary after the collagenase/DNAase protocol were either zona-free or had a very diminished zona. This allowed these oocytes to directly interact with a granulosa cell monolayer. The contact established between oocyte and granulosa cells under these culture conditions are not tight or strong. Rather the oocytes appear tethered to the granulosa cells. This attachment is strong enough to withstand the mechanical forces generated by shaking the culture dish, but it is easily disrupted by aspirating the oocyte into a micropipette.

Figure 6 The effect of linear HAVDI, cyclic HAVDI and bFGF on the rate of germinal vesicle breakdown after 4 and 20 h of culture. The number of oocytes examined in each treatment is shown at the base of each bar. * indicates a value that is significantly different from oocytes exposed to DMSO, which was used as the peptide solvent. ** indicates a value is different from both the DMSO control and the linear peptide.

The use of this culture system revealed three interesting aspects of oocyte–granulosa cell communication. First, the oocyte binds to the granulosa cells in a site-specific manner such that it is the oolemma, that is opposite the GV, binds to the granulosa cells. This implies that there exists a polarity within the oolemma. Whether this polarity was pre-existing or occurred as a result of the cell contact being established *in vitro* cannot be determined from the present experiments. However *in vivo*, oocyte polarity exists as demonstrated by the eccentric localization of the GV (Albertini & Barrett 2004), the increased number of transzonal projections opposite the GV (Albertini & Barrett 2004) and the distribution pattern of the leptin receptor and Stat3 within the oolemma (Antczak & Van Blerkom 1997). Specifically, both the leptin receptor and Stat3 localize to an area of the oolemma that is opposite from the GV. While this establishes polarity within the oolemma with regards to leptin receptor and Stat3, whether these signal transduction components play a role in regulating the location and/or maintenance of the GV remains to be determined. However in normal breast and tumor cells, cell contact can activate Stat3, which results in mitotic arrest (Vultur *et al.* 2004). It is possible that a similar relationship between cell contact, Stat3 activation and meiotic arrest exists *in vivo*.

The second aspect is that granulosa cell contact inhibits GVBD and not a factor that is secreted into the culture media by granulosa cells. This is supported by the following observations. First, oocytes cultured adjacent to granulosa cells undergo GVBD at a rate similar to that of oocytes cultured in the absence of granulosa cells. Secondly, oocytes cultured in medium collected from granulosa cell cultures, undergo GVBD at the same rate as those cultured with fresh media. It has been known for a long time that the presence of granulosa cells can inhibit GVBD. It was

Figure 7 The immunohistochemical localization of FGF receptor in a preantral follicle within the ovary of a 23 day old immature mouse. The FGF receptor is detected by a reddish-brown stain. Panel B reveals that FGF receptor is present in granulosa cells, oocyte and the transzonal projections. Panel A is a negative control.

assumed that the granulosa cells did so by secreting an oocyte maturation inhibitory peptide into the follicular fluid (Eppig *et al.* 2004). Although many attempts have been made to identify this inhibitor, they have been unsuccessful (Eppig *et al.* 2004). While granulosa cell secreted products may influence the GVBD, our data suggest that the major effect of granulosa cells in maintaining meiotic arrest is mediated by cell contact per se. This is consistent with the concept put forth by Racowsky and Baldwin for hamster oocytes (Racowsky & Baldwin 1989).

The third aspect is that cell contact is as effective as db-cAMP in preventing GVBD. Moreover, only a small segment of the oolemma, which is located opposite the GV, is required to be in contact with the granulosa cell membrane in order to attenuate the rate of GVBD. This is consistent with the finding that transzonal projections are more frequently observed opposite the GV (Albertini & Barrett 2003). These observations imply that the signal cascade that maintains meiotic arrest is initiated from the segment of oolemma that is in contact with the granulosa cell monolayer.

In vivo, oocyte–granulosa cell contact is mediated through transzonal projections that connect the oocyte and cumulus cells by both gap junctions and cadherin-mediated adhesion junctions. These junctions remain intact until either the gonadotropin surge induces GVBD (Hyttel *et al.* 1986, Albertini & Barrett 2003) or the follicle begins to undergo atresia (Hyttel *et al.* 1986). *In vitro* studies has shown that when cumulus cell–oocyte complexes are placed in culture in the presence or absence of hypoxanthine, FSH induces GVBD (Downs 2001, Albertini & Barrett 2003, Eppig *et al.* 2004). Under these conditions, FSH also induces the withdrawal of the transzonal projects that connect the oocyte and cumulus cells.

Moreover, the withdrawal of the transzonal projections is necessary for maturation to progress to completion (Albertini & Barrett 2003).

The present study confirms other immunohistochemical studies that demonstrate that N-cadherin is expressed by granulosa cells and oocytes at all stages of development (Machell & Farookhi 2003). It is important to appreciate that although *in vivo* the granulosa cells and oolemma are separated by the zona pellucida, N-cadherin localizes to the transzonal projects, which likely results in the establishment of homophilic N-cadherin binding between the granulosa cells and the oocyte *in vivo*.

To assess N-cadherin's role in mediating the cell-contact inhibition of GVBD, zona-free oocytes were used to allow for the immediate establishment of homophilic binding of N-cadherin molecules between the granulosa cell and oolemma *in vitro*. Using this co-culture system, an antibody to the extracellular domain of N-cadherin was shown to inhibit the ability of cell contact to prevent GVBD. This indicates that homophilic N-cadherin binding is an important component of the mechanism through which cell contact attenuates GVBD. This concept is also supported by the observation that SIGCs, which express E-cadherin (Peluso *et al.* 2001) do not inhibit GVBD.

How then might N-cadherin mediated cell contact prevent GVBD? There are at least three possible junctacrine mechanisms. First, it is well known cadherins lead to the formation of gap junctions (Rowlands *et al.* 2000). Gap junctions are thought to function as a conduit for transporting cAMP from the cumulus cells to the oocyte, thereby preventing GVBD (Webb *et al.* 2002). Thus, by facilitating the formation of gap junctions, homophilic

N-cadherin binding could play an essential role in maintaining meiotic arrest.

A second junxtacrine mechanism could involve an interaction with the recently identified G_s -linked receptor, GPR3 (Mehlmann *et al.* 2004). This receptor is expressed by mouse oocytes and appears to be responsible for the intra-oocyte production of cAMP and ultimately meiotic arrest (Mehlmann *et al.* 2004). Granulosa cells could secrete a putative GPR3 ligand that could remain associated with the granulosa cells and therefore would not be present throughout the culture media. Alternatively, this GPR3 ligand may not be secreted but rather localized to the extracellular surface of the granulosa cells. In either case, homophilic N-cadherin binding could bring the GPR3 receptor into close proximity to the putative GPR3 ligand thereby stimulating intra-oocyte cAMP production and meiotic arrest.

A third junxtacrine mechanism could be related to N-cadherin's ability to directly activate various signal transduction pathways. Insight into this aspect of N-cadherin's mechanism of action is provided by studies involving peptide mimetics of N-cadherin. Previous studies have shown that the HAVDI sequence that is present within the extracellular domain of N-cadherin binds to and inhibits the activation (i.e. tyrosine phosphorylation) of the FGF receptor (Williams *et al.* 2001, Williams *et al.* 2002). Therefore, peptides containing the HAVDI sequence inhibit the interaction between N-cadherin and the FGF receptors, in a manner like FGF receptor antagonists. This is likely because the linear sequence binds a single FGF receptor molecule and prevents FGF receptor dimerization and activation (Williams *et al.* 2001, Williams *et al.* 2002). However, if dimeric HAVDI sequences are made cyclic by forming disulfide bonds between cysteine residues, then this cyclic HAVDI can bind to two FGF receptors promoting dimerization and activation of the FGF receptor (Williams *et al.* 2002). As a result, the cyclic dimeric peptide functions as an FGF receptor agonist (Williams *et al.* 2002). The present studies revealed that the cyclic dimeric HAVDI peptide but not the linear dimeric HAVDI peptide inhibited GVBD. Previous studies have shown that bFGF had a slight stimulatory effect on GVBD in cumulus-enclosed hypoxanthine-blocked oocytes (Downs 1989). In contrast, the present data showed that bFGF blocks GVBD. The discrepancies in bFGF action may be due to the use of hypoxanthine and the presence of cumulus cells (Downs 1989). Since granulosa cells express FGF receptors (Asakai *et al.* 1993, 1994, Amsterdam *et al.* 2001), it is likely that the granulosa (cumulus) cells were the major site of FGF action (Downs 1989). In the present studies, the oocytes are the sole site of action for both bFGF and the cyclic HAVDI peptide. Thus under the present culture conditions, the ability of the cyclic HAVDI peptide and bFGF, to attenuate the rate of GVBD is consistent with the activation the FGF receptors that are present in the oocyte (Haffner-Krausz *et al.* 1999, present study). Interestingly,

RT-PCR studies reveal that mouse oocytes express the type IIIc isoform of FGF receptor 2 (Haffner-Krausz *et al.* 1999), making it likely that this isoform of the FGF receptor mediates the action of bFGF and N-cadherin. However, the putative activation of the oocyte FGF receptor cannot account for all of the effect of cell contact since cyclic peptide and bFGF do not appear to be as effective as cell contact in preventing GVBD. Given that these three proposed junxtacrine mechanisms are not mutually exclusive, it is possible that all three are involved in promoting meiotic arrest.

Although agents that increase cAMP within the oocyte inhibit GVBD (Eppig *et al.* 2004) an increase in cAMP may not be the sole intracellular messenger that blocks meiosis. Activation of FGF receptor does not appear to increase cAMP but can stimulate several different signal transduction cascades that increase protein kinase C (PKC) activity (Szebenyi & Fallon 1999, Klint & Clasesson-Welsh 2000). Recent studies indicate that activation of PKC can inhibit both the initiation of GVBD or meiotic progression of metaphase I oocytes (Eppig *et al.* 2004, Viveiros *et al.* 2004). Moreover, studies in epithelial cells demonstrate N-cadherin binding to the FGF receptor can stabilize the FGF receptor at the cell surface thus prolonging FGF signaling (Wheelock & Johnson 2003). *In vivo*, this may be an important mechanism since oocytes can synthesize FGF (Knee *et al.* 1994, Nilsson & Skinner 2004), which can be secreted and then bind to the zona where it, together with homophilic N-cadherin binding, could continuously activate the FGF receptor. This autocrine/junxtacrine feedback system would be an effective component of the mechanism that maintains meiotic arrest *in vivo*.

In summary, the present studies show that contact between a small segment of the oolemma and granulosa cells is as effective as db-cAMP in preventing GVBD. The mechanism through which granulosa cell contact maintains meiotic arrest is unknown but is likely to involve homophilic N-cadherin binding between the granulosa cells and oocyte, and possibly the activation of the FGF receptors that are expressed by the oocyte. Since the present studies were conducted with zona-free oocytes, future works must determine whether N-cadherin mediated cell contact maintains meiotic arrest in zona-enclosed oocytes at different stages of follicular development.

Acknowledgements

The author would like to thank Dr Robert Burghardt of Texas A&M University for providing the SIGC cells and Nancy Ryan of the Histology and Histochemistry Core of the University of Connecticut Health Center for conducting the immunohistochemical staining. The author would also like to thank Dr Lawrence Engmann for his careful review of this manuscript.

Funding

This work was supported by NIH grant RO3 HD044428. The author declares that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Albertini DF & Barrett SL 2003 Oocyte-somatic cell communication. *Reproduction Supplement* **61** 49–54.
- Albertini DF & Barrett SL 2004 The developmental origins of mammalian oocyte polarity. *Seminars in Cell Development and Biology* **15** 599–606.
- Amsterdam A, Kannan K, Givol D, Yoshida Y, Tajima K & Dantes A 2001 Apoptosis of granulosa cells and female infertility in achondroplastic mice expressing mutant fibroblast growth factor receptor 3G374R. *Molecular Endocrinology* **15** 1610–1623.
- Antczak M & Van Blerkom J 1997 Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Molecular and Human Reproduction* **3** 1067–1086.
- Asakai R, Tamura K, Eishi Y, Iwamoto M, Kato Y & Okamoto R 1993 Basic fibroblast growth factor (bFGF) receptors decrease with luteal age in rat ovarian luteal cells: colocalization of bFGF receptors and bFGF in luteal cells. *Endocrinology* **133** 1074–1084.
- Asakai R, Song SY, Itoh N, Yamakuni T, Tamura K & Okamoto R 1994 Differential gene expression of fibroblast growth factor receptor isoforms in rat ovary. *Molecular and Cellular Endocrinology* **104** 75–80.
- De Felici M 2000 Regulation of primordial germ cell development in the mouse. *International Journal of Developmental Biology* **44** 575–580.
- De Felici M 2001 Twenty years of research on primordial germ cells. *International Journal of Developmental Biology* **45** 519–522.
- Donahue RP 1968 Maturation of the mouse oocyte in vitro I. Sequence and timing of nuclear progression. *Journal of Experimental Zoology* **169** 237–249.
- Downs SM 1989 Specificity of epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus in vitro. *Biology of Reproduction* **41** 371–379.
- Downs SM 2001 A gap-junction-mediated signal, rather than an external paracrine factor, predominates during meiotic induction in isolated mouse oocytes. *Zygote* **9** 71–82.
- Eppig JJ, Viveiros MM, Bivens CM & De La Fuente R 2004 Regulation of mammalian oocyte maturation. In *The Ovary*, pp 113–129. Eds PCK Leung & EY Adashi. Amsterdam: Elsevier Academic Press.
- Haffner-Krausz R, Gorivodsky M, Chen Y & Lonai P 1999 Expression of Fgfr2 in the early mouse embryo indicates its involvement in preimplantation development. *Mechanisms of Development* **85** 167–172.
- Horner K, Livera G, Hinckley M, Trinh K, Storm D & Conti M 2003 Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Developmental Biology* **258** 385–396.
- Hyttel P, Westergaard L & Byskov AG 1986 Ultrastructure of human cumulus-oocyte complexes from healthy and atretic follicles. *Human Reproduction* **1** 153–157.
- Klint P & Claesson-Welsh L 2000 Signal transduction by fibroblast growth factor receptors. *Frontiers in Bioscience* **4** d165–d177.
- Knee RS, Pitcher SE & Murphy PR 1994 Basic fibroblast growth factor sense (FGF) and antisense (gfg) RNA transcripts are expressed in unfertilized human oocytes and in differentiated adult tissues. *Biochemical and Biophysical Research Communications* **205** 577–583.
- Lederer KJ, Luciano AM, Pappalardo A & Peluso JJ 1995 Proliferative and steroidogenic capabilities of rat granulosa cells of different sizes. *Journal of Reproduction and Fertility* **103** 47–54.
- Machell NH & Farookhi R 2003 E- and N-cadherin expression and distribution during luteinization in the rat ovary. *Reproduction* **125** 791–800.
- Mehlmann LM, Jones TL & Jaffe LA 2002 Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. *Science* **297** 1343–1345.
- Mehlmann LM, Saeki Y, Tanaka S, Brennan TJ, Evsikov AV, Pendola FL, Knowles BB, Eppig JJ & Jaffe LA 2004 The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science* **306** 1947–1950.
- Nilsson EE & Skinner MK 2004 Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Molecular and Cellular Endocrinology* **214** 19–25.
- Peluso JJ 2000 N-cadherin-mediated cell contact regulates ovarian surface epithelial cell survival. *Biology of Signals Receptors* **9** 115–121.
- Peluso JJ, Bolender DL & Perri A 1979 Temporal changes associated with the degeneration of the rat oocyte. *Biology of Reproduction* **20** 423–430.
- Peluso JJ, Pappalardo A & Fernandez G 2001 E-cadherin-mediated cell contact prevents apoptosis of spontaneously immortalized granulosa cells by regulating Akt kinase activity. *Biology of Reproduction* **64** 1183–1190.
- Racowsky C & Baldwin KV 1989 *In vitro* and *in vivo* studies reveal that hamster oocyte meiotic arrest is maintained only transiently by follicular fluid, but persistently by membrane/cumulus granulosa cell contact. *Developmental Biology* **134** 297–306.
- Reddy P, Liu L, Ren C, Lindgren P, Boman K, Shen Y, Lundin E, Ottander U, Rytinky M & Liu K 2005 Formation of E-cadherin mediated cell–cell adhesion activates Akt and mitogen activated protein kinase (MAPK) via phosphatidylinositol 3 kinase and ligand-independent activation of epidermal growth factor (EGF) receptor in ovarian cancer cells. *Molecular Endocrinology* **19** 2564–2578.
- Rieger-Christ KM, Lee P, Zagha R, Kosakowski M, Moizadeh A, Stofel J, Ben-Ze'ev A, Libertino JA & Summerhayes IC 2004 Novel expression of N-cadherin elicits *in vitro* bladder cell invasion via the Akt signaling pathway. *Oncogene* **23** 4745–4753.
- Rowlands TM, Symonds JM, Farookhi R & Blaschuk OW 2000 Cadherins: crucial regulators of structure and function in reproductive tissues. *Reviews of Reproduction* **5** 53–61.
- Roy SK & Greenwald GS 1996 Methods of separation and *in-vitro* culture of pre-antral follicles from mammalian ovaries. *Human Reproduction Update* **2** 236–245.
- Rufas O, Fisch B, Ziv S & Shalgi R 2000 Expression of cadherin adhesion molecules on human gametes. *Molecular and Human Reproduction* **6** 163–169.
- Salustri A, Fulop C, Camaioni A & Hascall VC 2004 Oocyte-granulosa cell interactions. In *The Ovary*, pp 131–143. Eds PCK Leung & EY Adashi. Amsterdam: Elsevier Academic Press.
- Sorensen RA & Wassarman PM 1976 Relationship between growth and meiotic maturation of the mouse oocyte. *Developmental Biology* **50** 531–536.
- Szebenyi G & Fallon J 1999 Fibroblast growth factors as multifunctional signaling factors. *International Reviews in Cytology* **185** 45–106.
- Viveiros MM, O'Brien M & Eppig JJ 2004 Protein kinase C activity regulates the onset of anaphase I in mouse oocytes. *Biology of Reproduction* **71** 1525–1532.
- Voronina E & Wessel GM 2003 The regulation of oocyte maturation. *Current Topics in Developmental Biology* **58** 53–110.
- Vultur A, Cao J, Arulanandam R, Turkson J, Jove R, Greer P, Craig A, Elliott B & Raptis L 2004 Cell-to-cell adhesion modulates Stat3 activity in normal and breast carcinoma cells. *Oncogene* **23** 2600–2616.

- Webb RJ, Marshall F, Swann K & Carroll J** 2002 Follicle-stimulating hormone induces a gap junction-dependent dynamic change in [cAMP] and protein kinase a in mammalian oocytes. *Developmental Biology* **246** 441–454.
- Wheelock MJ & Johnson KR** 2003 Cadherin-mediated cellular signaling. *Current Opinion in Cell Biology* **15** 509–514.
- Williams EJ, Williams G, Howell FV, Skaper SD, Walsh FS & Doherty P** 2001 Identification of an N-cadherin motif that can interact with the fibroblast growth factor receptor and is required for axonal growth. *Journal of Biological Chemistry* **276** 43879–43886.

- Williams G, Williams EJ & Doherty P** 2002 Dimeric versions of two short N-cadherin binding motifs (HAVDI and INPISG) function as N-cadherin agonists. *Journal of Biological Chemistry* **277** 4361–4367.

Received 30 June 2005

First decision 25 August 2005

Revised manuscript received 6 September 2005

Accepted 14 September 2005