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Role of focal adhesion kinase in oocyte-follicle communication

Lynda K. McGinnis¹ and William H. Kinsey^{2,*}

¹ Department of Molecular and Integrative Physiology, University of Kansas Medical Center
Kansas City, KS USA

²Department of Anatomy & Cell Biology, University of Kansas Medical Center Kansas City, KS
USA

Abstract

Germ cells require communication with associated somatic cells for normal gametogenesis as exemplified by the oocyte which interacts with granulosa cells via paracrine factors as well as gap junctions located at sites of contact between these two cell types. The objective of the present study was to define the mechanisms by which cell-cell contact with the oocyte is controlled and determine the extent to which the oocyte actively participates in this association. Focal adhesion kinase (PTK2) was found to be activated at sites of contact between the oocyte and trans-zonal cell processes from the surrounding granulosa cells. In order to determine the functional significance of oocyte-derived PTK2 signaling in oocyte-follicle communication, an oocyte-specific *ptk2* knockout was produced through a breeding strategy pairing a floxed *ptk2*-CAT-*eGFP* mouse with the *Zp3-cre* line. Since *ptk2*-null mice never develop to birth, this represents the first opportunity to define the role of *ptk2* in oocyte-follicle communication. Ablation of *ptk2* within the developing oocyte resulted in lower fertility with reduced numbers of pups, lower rates of blastocyst formation, and reduced cell numbers per blastocyst. Follicles containing *ptk2*-null oocytes exhibited reduced oocyte diameter, reduced numbers of connexin 37 and 43 foci at the oocyte surface, and impaired dye coupling between oocyte and granulosa cells. These findings are consistent with a model in which PTK2 plays a critical role in establishing or maintaining oocyte-granulosa cell contacts that are essential for gap junction - mediated communication between granulosa cells and the oocyte.

Keywords

Focal adhesion kinase; oocyte; granulosa; follicle

Introduction

Development of germ cells into functional gametes involves communication between the germ cell and specialized somatic cells that provide metabolic support and transduce

*To whom correspondence should be addressed: Department of Anatomy & Cell Biology Univ. of Kansas Medical Center 3901 Rainbow Blvd. Kansas City, KS 66160 Tel: 913-588-2721, FAX: 913-588-2710 wkinsey@kumc.edu.

Conflict of Interest

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endocrine or paracrine stimuli into signals that control the progress of the germ cell through meiosis. In the mammalian ovary, for example, the oocyte is surrounded by granulosa cells which respond to gonadotropins to direct growth and maturation of the oocyte. The granulosa cells are coupled by gap junctions and, as the follicle develops an antrum, a subset of granulosa cells (cumulus cells) maintains close association with the oocyte. The innermost tier of cells (corona radiata) maintain gap junction communication with the oocyte via trans-zonal cell processes that traverse the zona pellucida and contact the oocyte plasma membrane. Oocyte – follicle communication through gap junctions and paracrine factors is needed to support the metabolic costs of oocyte growth as well as cytoplasmic and nuclear maturation of the oocyte (Carabatsos *et al.*, 2000; Kidder and Vanderhyden 2010; Conti *et al.*, 2011; Dieci *et al.*, 2013; Wigglesworth *et al.*, 2013). Metabolic coupling between oocyte and the granulosa cell compartment occurs through gap junctions that enable diffusion of pyruvate, amino acids, lipids, etc. in response to the changing requirements of the developing oocyte (Schultz, R.M., 1985; Eppig, J.J., 1991). The junctions between oocyte and granulosa cells initially develop as adherens contacts and mature into adherens junctions (Mora *et al.*, 2012) that participate in follicle organization and likely provide stable cell-cell contacts that promote gap junction formation. Permeability of the gap junctions connecting granulosa cells (including mural granulosa and cumulus cells) appears to be regulated differently than those between the corona radiata cells and the oocyte (Norris *et al.*, 2009; Wigglesworth *et al.*, 2013), especially during the onset of meiotic maturation. While gap junction permeability is a critical element controlling metabolic coupling (Dyce *et al.*, 2012), active regulation of adherens junction stability represents an additional control point that might respond to endocrine or other signals and enable gap junction formation and turnover in the follicle.

Adherens and tight junction formation and turnover requires the activity of Focal Adhesion Kinase (PTK2) which acts as a master integrator responding to extracellular and intrinsic signals. PTK2 functions to promote or reverse assembly of cytoskeletal elements that stabilize nectin and cadherin-based adhesion complexes which can subsequently mature into adherens or tight junctions (Kim *et al.*, 2005; Wheelock *et al.*, 2008). PTK2 plays a prominent role regulating adherens junction assembly (Mitra *et al.*, 2005) and enabling gap junction formation in other cellular systems (Giepmans, 2004; Mitra *et al.*, 2006; Sachdev *et al.*, 2009). Typically, cell-cell contact is initially stabilized by nectin-mediated junctions which promote subsequent recruitment of cadherin dimers (Ogita *et al.*, 2008). These immature adherens junctions are unstable, however junction assembly can be accelerated by PTK2 activation, shifting the balance toward mature adherens junctions stabilized by robust interaction with bundled actin filaments (Kim *et al.*, 2005; Wheelock *et al.*, 2008). Therefore, PTK2 is in a position to act as an important control point integrating multiple endocrine, paracrine, and cell-matrix signals to control oocyte-granulosa cell-cell contacts that enable gap junction formation between these cells.

The *ptk2*-family of protein kinases includes *ptk2* and the Proline-rich Tyrosine Kinase 2 (*ptk2b* or PYK2) which are large (120-125Kda) cytoplasmic protein tyrosine kinases specialized to interact with trans-membrane proteins involved in cell adhesion, such as nectins and cadherins, or with the integrins (Orr and Murphy-Ullrich, 2004). PTK2 and

PTK2b are cytoplasmic protein tyrosine kinases that transduce signals from cell surface receptors to cytoplasmic pathways through their unique capability as scaffolding proteins as well as through their kinase activity. The PTK2 protein consists of multiple protein interaction domains (Lawson and Schlaepfer, 2012; Schlaepfer *et al.*, 1999). An N-terminal Ezrin, Radixin, Moesin homology domain termed FERM plays a key role in regulating PTK2 kinase activity through an autoinhibitory intramolecular interaction (Cooper *et al.*, 2003). The FERM domain also binds to Actin-Related Proteins (ARPs) that promote actin polymerization (Quadri, 2012), growth factor receptors (Chan *et al.*, 2006), as well as P53 and its regulator murine double minute 2 (Mdm2) (Lim *et al.*, 2008). This domain also performs a membrane targeting function via its affinity for PtdIns4,5,P₂-rich membrane domains (Cai *et al.*, 2008). The single catalytic site of PTK2 is followed by two proline-rich domains with high affinity for SH3 domains as well as tyrosine phosphorylation sites thought to interact with SH2 domains of Src-family PTKs. *In vivo*, PTK2 is normally maintained in a 'closed' inactive configuration through intramolecular interaction between the FERM and catalytic domains and is activated in response to stimuli (ex. clustering of integrins, cadherins, etc) that promote trans-phosphorylation of adjacent PTK2 molecules (Toutant *et al.*, 2002) at Y³⁹⁷ within the catalytic domain. Y³⁹⁷ acts as a binding site for Src-family PTKs which stabilize the open configuration of PTK2 and subsequently phosphorylate tyrosines-576, 861, and 925 (Brunton *et al.*, 2005; Hanks *et al.*, 2003). The C-terminal Focal Adhesion Targeting (FAT) domain interacts strongly with paxillin, talin and Grb2 (Schlaepfer *et al.*, 1994; Arold, 2011; Lawson *et al.*, 2012;). These diverse protein interaction domains enable *ptk2* family kinases to integrate a wide variety of extracellular and intracellular signals to regulate Rho activity (Okigaki *et al.*, 2003), implement actin cytoskeleton functions relating to cell-cell junction turnover (Sun *et al.*, 2011), cell process formation (Gil-Henn *et al.*, 2007) and phagocytosis (Owen *et al.*, 2007). PTK2 and PTK2b can also function in more complex events such as mechanical and osmotic stress response (Koh *et al.*, 2001; Takahashi *et al.*, 2003; Klemm *et al.*, 2010), proliferation (Cox *et al.*, 2006), and survival (Hanks *et al.*, 2003).

The potential of PTK2 to regulate adherens junction formation and turnover, and to respond to diverse endocrine and paracrine signaling pathways raised the possibility that this kinase may play an important role in controlling the formation and disassociation of adherens junctions between granulosa cells and the oocyte and thus play a critical role on oocyte-follicle communication. The objective of this study was to test the hypothesis that PTK2 is required for the formation and/or maintenance of normal cell-cell communication between the oocyte and granulosa cells in the mouse ovary. An oocyte-specific *ptk2* -knockout model was developed in order to block *ptk2* expression in oocytes of developing follicles and the impact of *ptk2* ablation on oocyte-cumulus cell communication and oocyte quality was assessed. The results revealed a complex pattern of *ptk2* expression and activation within the follicle and indicate a significant role for oocyte PTK2 signaling in communication between oocyte and follicle.

Results

ptk2 expression and activation in the ovary

ptk2 is normally expressed in all cells at some level and western blot analysis demonstrated that an antibody directed against the C-terminal domain of the PTK2 protein could detect the 125kDa protein even in small numbers of germinal vesicle and metaphase II stage oocytes (**Fig 1A**). A truncated, 55KDa isoform (Nolan et al., 1999) was also detected although at much lower abundance. Confocal immunofluorescence analysis of histological sections labeled with the same antibody (**Fig 1 B, B'**) revealed that PTK2 was expressed in oocytes and at elevated levels in the granulosa cell compartment. Controls incubated with non-immune rabbit IgG exhibited much lower fluorescence (**Fig 1B inset**). The distribution of PTK2 in oocytes was punctuate in nature as described earlier (McGinnis, *et al*, 2013), but appeared more uniformly distributed within the cytoplasm of granulosa cells. In small pre-antral follicles, elevated PTK2 expression was present in those granulosa cells closest to the oocyte (**arrows Fig 1 C, C'**). Secondary follicles with two layers of granulosa cells expressed high levels of PTK2 throughout the cytoplasm of both cell layers (**Panel D, D'**), while in larger follicles, almost all of the granulosa cells exhibited PTK2 immunofluorescence that was significantly higher than in the oocyte (**Fig 1E, E',F,F'**).

The fact that PTK2 activation requires phosphorylation of specific tyrosines in the catalytic or C-terminal domain has provided a means to detect activation of this kinase by antibodies specific for the phosphorylated tyrosines and flanking residues such as PY³⁹⁷ and PY⁸⁶¹ (Nakamura *et al.*, 2001). An affinity purified polyclonal antibody against PTK2 PY⁸⁶¹ was chosen for use for immunofluorescence analysis because this antibody consistently resulted in a stronger signal than antibodies against the other phosphorylation sites. The specificity of this antibody was tested in paraffin sections by competition with the phosphopeptide epitope (**Supplemental Fig 1**) which almost completely eliminated antibody binding to the ovarian tissue. In order to determine the extent to which PTK2 was activated in different compartments of the follicle, ovarian sections were labeled with anti-PTK2 PY⁸⁶¹ under conditions that suppressed phosphatase activity during processing as described in 'Materials and Methods'. Phosphorylated PTK2-PY⁸⁶¹ was detected as a 125KDa band in western blots of WT COCs (**Fig 2C**). Confocal immunofluorescence revealed that PYK2 PY⁸⁶¹ was most abundant in scattered stromal cells and in the granulosa cell compartment while control sections labeled with alexa488 conjugated goat anti-rabbit IgG exhibited no visible labeling (**Fig 2 A inset**). Phosphorylated PTK2-PY⁸⁶¹ was concentrated at the cell borders (**Fig 2 A' large arrows**) suggesting that PTK2 might be actively signaling at these sites of cell-cell or cell-matrix association. Interestingly, the inner layer of granulosa cells exhibited intense PTK2-PY⁸⁶¹ labeling at the cell borders that were in contact with the zona pellucida (**Fig 2B' open arrow**). Discrete foci of intense PTK2-PY⁸⁶¹ labeling were detected within the zona pellucida, possibly associated with trans-zonal processes which traverse this layer to make contact with the oocyte surface (**Fig 2B, B' small arrows**). In an effort to visualize the distribution of activated PTK2 at the oocyte surface without artifacts that are frequently caused by ethanol dehydration and embedding, isolated COC's were labeled in aqueous whole mount preparations (**Fig 3**). In these samples, PTK2-PY⁸⁶¹ was detected in small foci arranged at the oocyte cortex in close approximation to the cortical actin layer (arrows) and

near sites of contact with actin-rich trans-zonal processes from the corona radiata cells (**Fig 3 B,C**). The proximity of activated PTK2 to the sites of contact between the germ cell and the somatic cell compartment raised the possibility that PTK2 signaling played a role in formation, stabilization, or function of junctions between oocyte and the trans-zonal projections from the granulosa cell compartment.

Role of oocyte-derived PTK2 signaling in fertility and oocyte quality

Ptk2-null mice die during early embryonic development while those carrying a single copy of that gene have normal fertility and developmental rates (Ilic *et al.*, 1995). In order to define the role of PTK2 signaling in oocyte quality and the impact on developmental competence, we developed an oocyte-specific *ptk2* knockout (Luo *et al.*, 2014) by crossing a floxed *ptk2*-CAT-*egfp* mouse developed elsewhere (Beggs *et al.*, 2003) with the *zp3-cre* mouse (Kemler *et al.*, 2004). In this model, *zp3* expression in the oocyte drives *cre*-mediated excision of *ptk2* exon 2, together with activation of *egfp* expression. Since *zp3* promoter-driven expression begins in ‘activated’ oocytes of primary follicles (de Vries *et al.*, 2000), this model provides the opportunity to prevent accumulation of PTK2 protein by the oocyte as soon as follicle growth begins in response to local growth factor stimulation. The presence of the CAT-*egfp* gene allowed confirmation of *cre* expression by detection of the eGFP protein by live cell imaging. Oocytes recovered from flox-*ptk2*-CAT-*egfp* homozygous females carrying the *zp3-cre* transgene expressed eGFP protein (**Fig 4A-B**) while control oocytes from littermates that did not contain *cre* showed no evidence of eGFP expression (**Fig 4C-D**). Western blot analysis of these oocytes using an antibody to PTK2 (**Fig 4E**) revealed that PTK2 expression was below the level of detection in oocytes from *ptk2^{flox/flox} :zp3^{cre+}* females and these will therefore be referred to as *ptk2*-null oocytes.

The mature (MII) *ptk2*-null oocytes were nearly normal in appearance with an average diameter of 66.6µm ± 0.7µm (excluding the zona pellucida), a value which was significantly (P=0.008) smaller than oocytes from littermate controls (68.4µm ± 0.4µm). In order to assess the effect of oocyte-targeted *ptk2* ablation on fertility, a breeding study was carried out as described in “Materials and Methods” to compare pup production and survival of control, *ptk2^{flox/flox}* females with that of *ptk2^{flox/flox} :zp3^{cre+}* females. The *ptk2^{flox/flox} :zp3^{cre+}* females exhibited a progressive decline in the number of pups produced per litter, reaching approximately 50% of control by the fourth litter. The number of pups produced during the fourth litter and during the combined litters was significantly lower than that produced by control females (**Table 1**) and a significantly higher percentage of these died prior to weaning (15% ± 6.5) vs (0%) with control females.

To evaluate the developmental competence of embryos derived from *ptk2*-null oocytes without possible secondary effects from the reproductive tract, fertilized eggs were collected from control and *ptk2^{flox/flox} :zp3^{cre+}* females following mating with WT males and placed into culture between 48 and 72hr post-hCG. The number of fertilized oocytes recovered from the *ptk2^{flox/flox} :zp3^{cre+}* females was not significantly different from controls. By 72 hrs post-hCG, the percent of *ptk2^{flox/flox} :zp3^{cre+}* embryos (heterozygous for *ptk2* after fertilization and zygotic genome activation) that developed to the 8-cell stage was slightly (not significantly) lower than WT or littermate controls (**Fig 5**). However, the percent of

embryos derived from *ptk2*-null oocytes that were classified as having undergone compaction by 72 hrs was significantly lower than controls. At 120 hrs post hCG, the percent that developed into expanded blastocysts remained significantly lower than those derived from WT or *ptk2^{flox/flox}* littermate controls. The percent of embryos that developed into expanded blastocysts did not increase by 144 hrs post hCG. In addition, those embryos derived from *ptk2*-null oocytes that succeeded in forming expanded blastocysts were viable but contained significantly fewer inner cell mass, trophoctoderm, and total cells (**Table 2**).

Role of oocyte-specific PTK2 signaling in oocyte-follicle communication

Oocytes communicate with the follicle by a combination of paracrine factors and by direct gap junction-mediated communication which occurs via trans-zonal cell processes that cross the barrier imposed by the zona pellucida. Since stable gap junction communication depends on stable cell-cell contacts that usually involve adherens or tight junctions, it seemed likely that PTK2 would be required to maintain these junctions. In order to assess the effect of oocyte - targeted *ptk2* ablation on the number of trans-zonal processes and gap junctions between oocyte and the granulosa cells, a combination of dark field histology and immunofluorescence techniques were employed. The trans-zonal processes were initially examined in hematoxylin-eosin stained ovarian sections by dark field imaging of follicles where the section passed through the equator of the oocyte. In these images (**Supplemental Fig 2**), the trans-zonal processes appeared as white filamentous structures within the zona pellucida which could be quantified by linescan analysis of the zona-pellucida. Analysis of over twenty five multilayered follicles from *ptk2^{flox/flox}* control ovaries detected an average of 72.8 trans-zonal processes at the oocyte equator which was significantly greater ($P<0.001$) that the 49.2 detected in *ptk2^{flox/flox};zp3^{cre}+* follicles. In order to rule out the possibility that fixation artifacts induced in the zona pellucida proteins might be mistaken for trans-zonal processes, a separate analysis based on the high filamentous actin content of TZPs was performed. Alexa568-conjugated phalloidin provided a more sensitive way to detect these cell processes in the zona pellucida of freshly isolated (dissected under fixative) COCs imaged in wet mount preparations. The confocal images presented in **Fig 6A, B** demonstrate that the density of filamentous actin contained within trans-zonal processes transiting the zona-pellucida of *ptk2*-null oocytes was lower than in littermate control oocytes. Linescan quantitation detected an average of 72.0 trans-zonal processes at the equator of *ptk2*-null oocytes vs 107.8 in the littermate controls, a difference that was statistically significant as determined by one way analysis of variance ($P<0.05$).

In order to determine whether the reduced numbers of trans-zonal processes in contact with *ptk2*-null oocytes correlated with reduced numbers of gap junctions at the oocyte surface, the pattern of connexin foci at the interface between the oocyte and the granulosa derived trans-zonal cell processes was examined. The gap junctions that connect oocytes with the granulosa cells are thought to be homotypic composed of connexin 37, while gap junctions between granulosa cells rely primarily on connexin 43 (Veitch et al., 2004). COCs isolated from control (WT) females and labeled with anti- connexin 43 exhibited numerous connexin foci between granulosa cells as well as numerous small plaques within the zona pellucida (**Fig 6D**). COCs isolated from control (WT) females, then labeled with anti-connexin 37 (**Fig 6F**) exhibited numerous connexin foci in close proximity to the surface of the oocytes.

Both connexin 43 and connexin 37 foci were greatly reduced in number in COCs containing *ptk2*-null oocytes (**Fig 6C, E**). For example, the number of connexin 37 foci at the equator of *ptk2*-null oocytes (3.7 ± 0.7 (n=27)) was significantly ($P=0.034$) lower than in WT oocytes (6.5 ± 1.0 (n=43)). Ablation of *ptk2* in oocytes also resulted in significantly ($P<0.01$) fewer connexin 43 foci (27.8 ± 2.6 (n=24)) presumably formed by granulosa cell processes within the zona pellucida than were observed in COCs from control ovaries (49.2 ± 4.4 (n=23)).

While the above data is consistent with the hypothesis that *ptk2* ablation in the oocyte had a negative impact on cell-cell communication by gap junctions, a more direct method of assessing oocyte-follicle communication was needed to confirm this theory. Dye diffusion across gap junctions connecting the oocyte and cumulus cells has been used extensively to assess the level of communication between these two cell types. This technique was applied to COCs isolated from WT and *ptk2*-null follicles by injecting the cumulus enclosed oocyte with lucifer yellow and monitoring the diffusion of the dye into the associated cumulus cells by confocal fluorescence microscopy. The injected dye was initially confined to the oocyte, but slowly accumulated in the cumulus cells (**Fig 7A**). Dye did not transfer to adjacent COCs that were not injected demonstrating that dye diffusion was limited to intact cell-cell contacts with permeable channels. In order to quantify the rate at which dye accumulated in the cumulus cells, injected oocytes were imaged at 5 second intervals for a period of up to 20 minutes. The resulting micrographs were analyzed with Metamorph 7.1 by creating measurement regions matching cumulus cells in close proximity (innermost cell layer) to the zona pellucida. Since individual cumulus cells were clearly resolved by the confocal technique, eight cells from each COC were routinely measured as representative of the cumulus mass and two additional regions of adjacent culture medium visible in the microscopic field were used to assess background fluorescence. Fluorescence intensity was quantified by measuring integrated pixel intensity within the measurement areas. The frequent exposure of COCs to the beam from a 488nm laser eventually resulted in significant photobleaching, however the accumulation of dye in the cumulus cells consistently remained linear for the first minute of recording and the data presented here is limited to that period. Comparison of the rate at which fluorescence intensity increased in cumulus cells from COCs containing WT or *ptk2*-null oocytes revealed that the cumulus cells enclosing WT oocytes accumulated dye much faster than those surrounding *ptk2*-null oocytes (**Fig 7B**). Analysis of two COCs from each of four WT and four *ptk2*^{flox/flox};*zp3*^{cre+} females (8 COCs from each genotype) produced an average dye coupling rate of 1.45 within COCs enclosing WT oocytes and 0.15 within COCs enclosing *ptk2*-null oocytes, values which were significantly different ($p=0.02$). This result confirms that ablation of *ptk2* in oocytes significantly impaired gap-junction-mediated communication between oocyte and follicle.

Discussion

Oocyte-granulosa cell communication is critical to fertility, playing an essential role in oocyte growth as well as cytoplasmic and nuclear maturation. Paracrine factors perform an essential role in communication between these two cell types (Rodrigues *et al.*, 2008), while gap junction channels provide opportunity for direct cell-cell communication through

diffusion of small molecules as well as some polypeptides (Gittens *et al.*, 2005; Cheng *et al.*, 2013). The mechanism by which gap junction formation and permeability are controlled represents an important aspect of follicle development that is just beginning to be understood (Santiquet *et al.*, 2012). The very presence of functional gap junctions requires that stable cell-cell contact has been achieved, and while trans connexin-connexin interactions provide significant adhesive capability, gap junctions usually occur in combination with adherens junctions stabilized by cadherin and or integrin interactions (Derangeon *et al.*, 2009). The complexity and significance of adherens junction dynamics during folliculogenesis has recently been demonstrated (Mora *et al.*, 2012), raising the obvious question as to how these events are controlled in the follicle. The formation and turnover of adherens and tight junction components is usually under control of the PTK2 kinase which acts to integrate multiple upstream signals and implement stabilization of cell-cell contacts by controlling the extent to which cell adhesion protein complexes are tethered to the cytoskeleton. PTK2 could play an important role during follicle development by transducing endocrine or paracrine signals to control the extent to which oocyte-granulosa cell contacts are stabilized and thereby the extent to which gap junction stabilization could occur. The goal of the present study was to test the hypothesis that PTK2 signaling in the oocyte is required for establishment and or maintenance of oocyte-granulosa cell contacts essential for communication between these two cell types.

The PTK2 protein was more highly expressed in the granulosa cell compartment than in other compartments of the ovary. During the primary follicle stage, PTK2 protein was distributed uniformly within the granulosa cell cytoplasm. As the secondary follicle developed, both layers of granulosa cells exhibited PTK2 accumulation that was significantly more intense than in other cell types within the ovary. Once antral formation began, the level of PTK2 expression among granulosa cells became less uniform, although PTK2 levels appeared to remain higher than in other compartments of the ovary. In contrast to the distribution of PTK2 protein, the accumulation of activated (phosphorylated) PTK2 PY⁸⁶¹ was highest at sites of cell contact between granulosa-granulosa cells, and the interface between the innermost tier of granulosa cells and the zona pellucida. Higher resolution imaging of isolated COCs confirmed that small foci of activated PTK2 were present at sites of contact between oocyte and the trans-zonal processes from the corona radiata cells suggesting that PTK2 may play a role in stabilizing these junctions.

In order to test whether PTK2 was required for formation and/or maintenance of the trans-zonal junctions and for oocyte-follicle gap junction-mediated communication, the floxed *ptk2*-CAT-eGFP mouse line (Beggs *et al.*, 2003) was crossed with the *Zp3-cre* line (Kemler *et al.*, 2004), to target *ptk2* excision to oocytes once zona pellucida formation was underway. The resulting flox-*ptk2*-CAT-eGFP homozygous females heterozygous for *Zp3-cre* produced oocytes in which PTK2 protein levels were much lower than in WT oocytes and below the level of detection by western blot. The array of trans-zonal processes associated with these oocytes was irregular and reduced in density suggesting that oocyte-follicle communication would likely be reduced. This possibility was confirmed by the observation that the number of connexin 37 foci at the surface of *ptk2*-null oocytes was greatly reduced relative to WT oocytes. Interestingly, the number of connexin 43 foci along the granulosa

trans-zona processes was also reduced indicating that absence of PTK2 signaling in the oocyte also had an impact on the granulosa cells in proximity to the zona pellucida.

However, given the possibility that gap junctions at the surface of *ptk2*-null oocytes might be larger with more connexons or otherwise more conductive as a result of phosphorylation state, dye diffusion experiments were undertaken to get a more accurate evaluation of the communication status of the oocyte. Quantification of the dye coupling state confirmed that *ptk2* ablation in the oocyte severely reduced but did not eliminate the ability of the oocyte to communicate with the granulosa cells via gap junctions. When considered with the reduced frequency of connexin 37 foci at the oocyte surface, the reduced dye coupling between oocyte and granulosa cells likely results from a reduced number of gap junctions linking these two cell types. Since the PTK2 protein can function during both assembly and disassembly of adherens junctions, *ptk2* ablation early during the oocyte growth phase might retard the formation of additional junctions between the oocyte and granulosa cells, but also impair disassembly of any junctions that were matured prior to loss of PTK2 protein. This 'freezing' of cell contacts due to *ptk2* ablation has been reported previously (Ilic *et al.*, 1995; Lawson *et al.*, 2012; Mitra *et al.*, 2005) and may explain why some trans-zonal processes persist even in large follicles containing *ptk2*-null oocytes. While gap junction permeability is a critical element controlling metabolic coupling, the above results highlight the importance of active regulation of adherens junction stability by the oocyte.

The impact of *ptk2* ablation on oocyte quality was significant as demonstrated by the reduced fertility of *ptk2^{lox/lox}:zp3^{cre}* female mice demonstrated by lower pup production and survival. While *ptk2^{lox/lox}:zp3^{cre}* females produced normal numbers of oocytes, the oocytes were smaller in diameter. Embryonic development during in vitro culture appeared near normal to the eight cell stage, but a significant number of embryos derived from *ptk2*-null oocytes failed to undergo compaction and form expanded blastocysts. This defect seemed to represent a form of developmental arrest as prolonged culture did not increase the frequency of expanded blastocysts. Finally, those embryos derived from *ptk2*-null oocytes that succeeded in forming blastocysts, exhibited reduced inner cell mass (ICM) and trophoctoderm (TE) cell numbers indicating a lower developmental potential. This result indicates that oocyte PTK2 activity is not required for fertilization and early cleavage, however, the reduced developmental success evident at the compacted morula and blastocyst stage suggests either that oocyte quality was poor or that PTK2 activity was required during the compaction or blastocyst formation stages. Embryos produced by fertilization of *ptk2*-null oocytes by WT sperm would be heterozygous for *ptk2* and could accumulate PTK2 protein after zygotic genome activation was complete. These embryos might be expected to achieve a level of PTK2 expression that was half of normal by the compaction stage where they tended to fail. However, since embryos produced by fertilizing oocytes heterozygous for *ptk2* developed normally (Ilic *et al.*, 1995), the reduced developmental success of embryos derived from *ptk2*-null oocytes likely is a result of poor oocyte quality.

The oocyte-specific *ptk2* knockout model presented here has revealed the significance of PTK2 signal transduction for oocyte-follicle communication. The control mechanisms responsible for the germ cell–somatic cell interactions that are required for successful

gametogenesis are just beginning to be understood (Mora *et al.*, 2012; Siu *et al.*, 2011). The observation that signal transduction within the germ cell (here PTK2 signaling in the oocyte) is required for junction formation with somatic cells has implications for both male and female gametogenesis and particularly for development of in vitro methods of oocyte culture.

Materials and Methods

Animal source and care

Mice were housed in a temperature and light-controlled room on a 14L:10D light cycle and experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences 1996). All experimental procedures were approved by the KUMC IACUC committee. An oocyte-specific *ptk2* knockout mouse line was produced by crossing the *ptk2^{flox/flox}*-CAT-*egfp* line (Beggs *et al.*, 2003) with the C57BL/6-TgN(*zp3-cre*)93Kw (*zp3^{cre}*) line (Kemler *et al.*, 2004) obtained from Jackson laboratories (Bar Harbor, ME). The resulting *ptk2^{flox/wt} zp3^{cre+}* mice were re-crossed to produce *ptk2^{flox/flox} zp3^{cre+}* animals (Luo *et al.*, 2014). For production of *ptk2*-null oocytes for experimental use, *ptk2^{flox/flox} zp3^{cre+}* males were crossed with *ptk2^{flox/flox}* females and the resulting *ptk2^{flox/flox} zp3^{cre+}* females were used for oocyte or embryo collection.

In order to quantify pup production and survival, five breeding pairs were set-up for each female genotype with either *ptk2^{flox/flox}* (control) or *ptk2^{flox/flox} : zp3^{cre+}* (experimental) females, aged 6-8 weeks. The females were paired with *ptk2^{flox/flox} : zp3^{cre+}* males (*zp3* gene is not expressed in males). The pairs remained caged together until the birth and weaning of their fourth litter or until 9 months of age. Breeding cages were checked every morning and the number of pups born or died was recorded.

Genotyping of mice was conducted according to a published protocol (Beggs *et al.*, 2003); Jackson Laboratory protocol: <http://jaxmice.jax.org/strain/003651.html>). Additional strains used included B6/129SF2/J (#101045, Jackson Labs, Bar Harbour, ME, USA) and B6D2F1 males (#B6D2F1/Hsd, Harlan Labs, Indianapolis, IN, USA).

Oocyte collection

Cumulus-oocyte-complexes (COCs) were collected from females stimulated with 5 IU of equine chorionic gonadotropin i.p. (eCG; PG600 Intervet Inc, Millsboro, DE, USA). COCs were dissected from large antral follicles at 42-46 hours (h) post-eCG and released into flushing and handling medium (FHM) supplemented with 0.1 μ M Cilostamide to prevent resumption of meiosis. For experiments in which ovulated oocytes or embryos were used, female mice were stimulated with 5 IU eCG followed 48h later with 5IU human chorionic gonadotropin (hCG; Chorulon, Intervet Inc, Millsboro, DE, USA) to induce ovulation. To generate fertilized embryos, each female was mated overnight with an adult male of appropriate genotype (see results for specific combinations used for each experiment). Mature MII or fertilized oocytes were flushed from oviducts with HEPES-buffered KSOM (FHM, Chemicon-Millipore, Billerica MA) supplemented with 4 mg/ml BSA.

Fixation and immunofluorescence labeling

Methods for fixation and immunofluorescence of COCs were similar to those previously reported (McGinnis, *et al.*, 2007). COCs were fixed for 1h in microtubule stabilization buffer (0.1 M PIPES, pH 6.9, 5 mM MgCl₂·6H₂O, 2.5 mM EGTA) containing 2% formaldehyde, 0.1% Triton X-100, 1 µM Taxol, 10 U/ml aprotinin and 50% deuterium oxide (Messinger and Albertini, 1991), a permeabilizing fixative that enhances antibody penetration through the intact cumulus cell layers to reach the oocyte. Following fixation, COCs were washed 30 min on a rocker (wash solution: PBS containing 2% BSA, 2% powdered milk, 2% normal goat serum, 0.1 M glycine and 0.01% Triton X-100). All fixatives and wash solutions were supplemented with phosphatase inhibitors (40 µM phenylarsine oxide, 100 µM sodium orthovanadate and 1 µM Calyculin-A).

Cumulus-enclosed oocytes were labeled with primary antibodies at 4°C overnight on a rocking plate followed by secondary antibody for 2h at room temperature. After secondary labeling, COCs were transferred to wash solution containing 1 µg/ml Hoechst 33258 and 1:100 Alexa 568-phalloidin (Molecular Probes) for 1h. The COCs were mounted and imaged immediately after labeling (mounting medium consisted of 1:1 glycerol: PBS supplemented with 5 mg/ml sodium azide and 1 µg/ml Hoechst 33258 or SlowFade from Invitrogen). All chemicals, hormones and reagents were purchased from Sigma Chemical Company, (St. Louis, MO) unless otherwise stated.

Ovaries were fixed overnight in 4% PFA in PBS with 0.002% sodium azide supplemented with phosphatase inhibitors as described above. Fixed ovaries were processed for paraffin embedding as previously described (Rodrigues *et al.*, 2008). Deparaffinized sections were incubated in blocking buffer containing PBS and 10% Goat serum for 1h at room temperature, then labeled with primary antibody overnight at 4°C. Sections were then washed 3 times and labeled with Alexa-conjugated secondary for 1.5h at room temperature. Control sections were labeled with secondary antibody and Hoechst only. Lastly, sections were labeled with Hoechst 33258 for 5 min, mounted with Fluoromount-G (Southern Biotech, Birmingham, AL) or SlowFade (Invitrogen) and sealed with clear nail polish.

Antibodies used included anti-PTK2 C20 (#sc-558), anti-connexin-43 (sc-9059; Santa Cruz, Santa Cruz, CA, USA), anti-connexin 37 (Invitrogen, Carlsbad, CA USA) activated PTK2 pY⁸⁶¹ (BioSource #44-652, Invitrogen, Grand Island, NY, USA). Secondary antibodies were Alexa 488 and Alexa 568 conjugated goat anti-mouse or goat anti-rabbit (depending on the source of the primary antibody; Molecular Probes-Invitrogen, Eugene OR).

Embryo Culture

To examine oocyte developmental competence, *ptk2*-null, littermate controls, and B6/129 control (WT) females (aged 42-60 days) were treated with eCG and hCG as described above and mated with WT B6D2F1 or B6/129 males. At 48 to 54h post-hCG, embryos were flushed from oviducts with FHM. Oocytes and embryos were counted and graded according to stage of development, then transferred to 50 µl culture drops of KSOMaa (Millipore, Billerica, MA or Zenith Biotech Co, Guilford, CT, USA) under oil as previously described (Biggers *et al.*, 2004). Embryo development was observed and graded at 72 & 120h post-

hCG (20x objective on a Nikon TE200 with Hoffman differential interference contrast optics). Blastocyst cell counts were performed on embryos fixed and prepared for immunofluorescence as above, then stained with alexa 568 conjugated phalloidin to label filamentous actin as a marker for cell boundaries and Hoechst 33258 as a marker for nuclei. The number of nuclei within the inner cell mass (ICM) and trophectoderm (TE) were then counted by inspection of serial z-plane confocal images.

Dye coupling and fluorescence quantitation

Dye coupling assays were performed in a fully enclosed, temperature controlled live cell imaging system based on a Nikon TE2000-U confocal microscope and images were recorded using a 40X SuperFluor objective lens. Cumulus enclosed oocytes maintained in FHM with 4% BSA and 10uM cilostimide were immobilized with a holder pipet and injected with 5-10pl of 100mM Lucifer yellow (InVitrogen, Carlsbad, CA) using a BioRad celltram-vario injector (BioRad Corp., Hercules, CA). Time lapse imaging with a 488nm laser was begun approximately 15 seconds before injection and continued at 5 second intervals for 10-20 minutes. Images recording dye fluorescence were analyzed with Metamorph 7.2 software to quantify the Lucifer yellow fluorescence in eight different corona radiata cells. Background measurements from the adjacent media were subtracted from each measurement, then the mean fluorescence intensity of the eight cells was normalized to the fluorescence at time= 0 and plotted against time.

Western Blot

All cumulus cells were removed from oocytes at GV and MII stages. Oocytes were then solubilized in 2x SDS-gel sample buffer containing 40 μ M phenylarsine oxide and 100 μ M sodium orthovanadate, and stored at -20°C . Samples of oocytes (20-30 per lane) were resolved on a 10% SDS-polyacrylamide gel with a 4% stacking gel as previously reported (McGinnis *et al.*, 2011). The blots were probed with anti-PTK2 C20 rabbit monoclonal antibody and with anti-GAPDH (ab1881, Abcam, Cambridge, MA, USA). Bound antibody was detected with horseradish peroxidase-coupled goat-anti-rabbit IgG followed by chemiluminescence.

Statistical Analysis

Experimental results were analyzed by Students t-test or ANOVA as defined within each experiment using SigmaStat or SigmaPlot software (Jandell scientific Software, San Jose, CA USA). P values less than or equal to 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSA	bovine serum albumin
EGTA	ethylene glycol-bis(β -aminoethyl ether) N,N,N,N tetraacetic acid
FHM	flushing and handling medium, Chemicon-Millipore Billerica MA
GAPDH	glyceraldehyde phosphate dehydrogenase
GV	germinal vesicle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KSOM	potassium simplex optimization medium
KUMC IACUC	Kansas University Medical Center Institutional Animal Care and Use Committee
MII	metaphase 2
Pipes	piperazine-N,N'-bis(2-ethanesulfonic acid)

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Fig 1.

Distribution of PTK2 in oocytes and granulosa cells. The PTK2 protein was detected with an antibody directed to the C-terminal domain of human PTK2 which could detect PTK2 as a 125KDa band in samples of GV and MII stage oocytes (30 per lane) (A, arrow). A second 55KDa isoform present in most cells was also expressed in oocytes (A, open arrow) although it was not as abundant as the 125KDa form. Blots were re-probed with anti-GAPDH (bottom) to indicate the relative protein loading in each lane. The anti-PTK2 C-20 antibody was also used to label paraffin sections of ovaries and bound antibody was detected with alexa 488-goat anti-rabbit secondary antibody (green) and the DNA dye Hoechst 33258 (blue) (B,-F) as described in 'Materials and Methods'. Panels B and B' show the labeling pattern of a representative region of ovary. The inset to panel B presents an additional section labeled only with control, non-immune rabbit IgG followed by the secondary antibody to rule out non-specific autofluorescence. Specific examples of a primary follicle (C), secondary follicle (D), multilayer follicle (E), and early antral follicle (F) demonstrate the different labeling patterns characteristic of each stage. Arrows in panels C' and D' indicate the higher concentration of PTK2 that occurs in the inner tier of granulosa cells. Arrows in panel E' demonstrate that regions of elevated PTK2 content are no longer limited to the inner tier of granulosa cells in multilayer follicles. Magnification is indicated by the bars which represent 10μm in panels C' through F'.

FIG 2.

PTK2 activation occurs at sites of cell-cell and cell-zona contact.

Activated (phosphorylated PTK2) was localized in paraffin sections with anti-PTK2 PY⁸⁶¹ and bound antibody was detected with alexa 488-goat anti-rabbit secondary antibody (green). Nuclei were localized with Hoechst 3328 (here represented as red). The zona pellucida is indicated by the white bar (ZP), the granulosa cells (GC) are also labeled. The large arrows in panels A' and B' indicate cell borders between granulosa cells. The large open arrow in B' represents the granulosa cell border with the zona pellucida. Small arrows in panels B and B' indicate small foci of phosphorylated PTK2 within the zona pellucida and at the oocyte surface. Western blot analysis (Panel C) was performed with groups of 10 WT COCs and blots were probed with anti-PTK2 C20 (PTK2), anti-PTK2 PY⁸⁶¹, or with rabbit IgG (Con) as a control. PTK2 and PTK2 PY⁸⁶¹ are visible as 125KDa bands.

Fig 3.

PTK2 activation in the oocyte cortex.

Cumulus-enclosed oocyte complexes (COCs) were released from antral follicles directly into a permeabilizing fixative supplemented with phosphatase inhibitors and labeled with anti-PTK2 PY⁸⁶¹ (green) and co-labeled with rhodamine-phalloidin (red) and Hoechst 3328 (blue). Panel A includes all color channels and the DIC image, panel B includes green, red and blue, and C includes only green and blue. Activated PTK2 concentrated in small foci at the oocyte cortex is indicated (panel B, C arrows).

Fig 4.

Specificity and extent of PTK2 ablation in oocytes from *ptk2^{flox/flox}:zp3^{cre+}* females. COCs isolated from *ptk2^{flox/flox}:zp3^{cre+}* females (A,B) or from *ptk2^{flox/flox}* WT females (C,D) were imaged under UV light to detect eGFP fluorescence (green) indicative of *cre* expression. In panel E, western blots of 30 cumulus free WT or *ptk2*-null oocytes were probed with the anti-PTK2 C-20 antibody and bound antibody was detected by chemiluminescence. Panel E demonstrates that the 125 KDa PTK2 protein present in control oocytes (arrow), was not detected in *ptk2*-null oocytes.

Fig 5.

Developmental competence of embryos derived from *ptk2*-null oocytes. Naturally cycling WT (B6/129) females (black bar), littermate control *ptk2*^{fl/fl} females (grey bar), and *ptk2*^{flox/flox} : *zp3*^{cre+} (white bar) females were mated with WT males and the fertilized oocytes were recovered and cultured as described in 'Materials and Methods'. The percent of oocytes that successfully reached the 8-cell cleavage stage or compacted morula stage by 72hrs post hCG, as well as the percent that reached the expanded blastocyst stage by 120hrs post hCG is represented by the bars. Values represent the mean from 7 WT, 15 *ptk2*^{flox/flox} littermate controls, and 14 *ptk2*^{flox/flox}:*zp3*^{cre+} females $\pm$ SEM. (*) indicates that the mean value was significantly different from the littermate controls ($P < 0.05$).

Fig 6.

Effect of *ptk2* ablation on the frequency of transzonal processes and connexin plaques. COCs containing *ptk2*-null (A,C,E) or WT (B,D,F) oocytes were labeled with either rhodamine-phalloidin (A, B) to detect the filamentous actin core (arrows) of transzonal processes (white); with anti-connexin 43 followed by alexa565—goat anti-rabbit (C,D) where connexin 43 foci (arrows) are labeled red ; or with anti- connexin 37 followed by alexa488-goat anti- rabbit (E,F) where connexin 37 foci (arrows) labeled green.

Fig 7.

Effect of *ptk2* ablation on dye coupling between oocyte and cumulus cells. COCs dissected from follicles were injected with Lucifer yellow and imaged via time lapse confocal microscopy at 5 second intervals as described in 'Materials and Methods'. Panel A demonstrates the extent of dye coupling of a single COC containing a WT oocyte over a long (20minute) period of time. The upper panels overlay the green fluorescence image on the differential interference contrast image to show what fraction of the cumulus cells accumulated dye. The lower panels represent dye fluorescence only. Panel B represents the rate of accumulation of dye in cumulus cells during the first minute after injection. Data points represent the mean dye fluorescence in cumulus cells (mean of 8 cumulus cells per COC) recorded from 8 WT (solid circles) and 8 *ptk2*-null (open circles) COCs taken from 4 different WT and 4 *ptk2*^{flox/flox}:*zp3*^{cre+} females.

Table 1

Effect of oocyte-targeted FAK suppression on pup production and survival.

Maternal Genotype		Mean pups / litter +/- SD			
Genotype	litter 1	litter 2	litter 3	Litter 4	all litters
<i>Ptk2^{flox/flox}</i>	9.4 +/- 0.6	10.0 +/- 1.7	10.2 +/- 0.8	8.8 +/- 1.8	9.4 +/- 0.8
<i>Ptk2^{flox/flox} : zp3^{cre+}</i>	8.4 +/- 1.9	8.0 +/- 2.6	7.8 +/- 3.0	4.6 * +/- 3.4	7.2 * +/- 0.9

Breeding pairs were set-up with *ptk2^{flox/flox}* (control) or *ptk2^{flox/flox} : zp3^{cre+}* (experimental) females, aged 6-8 weeks. They were paired with *ptk2^{flox/flox} : zp3^{cre+}* males (*zp3* gene is not expressed in males). The pairs remained caged together until the birth and weaning of their fourth litter or until 9 months of age. Breeding cages were checked every morning and the number of pups born or died was recorded. Values represent the mean +/- SD.

* indicates that the value was significantly different from Control as determined by t test ($P < 0.05$). In total, 126 pups were successfully weaned from *Ptk2^{flox/flox}* females and 109 pups were weaned from *Ptk2^{flox/flox} : zp3^{cre+}* females.

Table 2

Embryos derived from *ptk2*-null oocytes exhibit reduced cell numbers

Maternal Genotype	Total Cells	ICM cells	TE cells
<i>Ptk2^{flox/flox}</i>	92.5 $\pm$ 4.8	25.9 $\pm$ 1.6	66.6 $\pm$ 3.9
<i>Ptk2^{flox/flox}zp3^{cre+}</i>	63.4 $\pm$ 7.7 *	15.5 $\pm$ 2.9 *	47.9 $\pm$ 5.6 *

Embryos recovered from the oviducts of *ptk2^{flox/flox}zp3^{cre+}* females and control *ptk2^{flox/flox}* females following mating with WT males were cultured to the blastocyst stage and cell numbers were determined as described in 'Materials and Methods'. Values represent the mean $\pm$ SD.

* indicates significantly different from control as determined by t test ($P < 0.05$).