Cellular basis for paracrine regulation of ovarian follicle development*

David F. Albertini, Catherine M. H. Combelles,
Elizabeth Benecchi and Mary Jo Carabatsos

Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA 02111, USA

Paracrine factors secreted by oocytes and somatic cells regulate many important aspects of early ovarian follicle development in mammals. From activation of dormant primordial follicles to selection of secondary follicles, locally acting factors have been identified that appear to exert important effects on the growth and differentiation of oocytes and granulosa cells. This article summarizes evidence to support a model for bi-directional paracrine communication that is based on developmental regulation of the delivery and reception of paracrine factors at the oocyte–granulosa cell interface. Transzonal projections that originate from granulosa cells and terminate at the oocyte plasma membrane provide a polarized means to orient the secretory organelles of somatic cells. Characterization of transzonal projections in follicles from normal and genetically modified mice reveals dynamic changes in the density and stability of transzonal projections. On the basis of new data analysing the orientation and cytoskeletal content of transzonal projections in mammalian oocytes, a model is proposed for regulation of paracrine growth factor secretion by follicle-stimulating hormone. These findings have immediate implications for ovarian hyperstimulation protocols and follicle culture models as related to the production of mammalian embryos by assisted reproductive technologies.

Although the physiological, molecular and genetic determinants that initiate and sustain follicular growth and maturation have received much attention (see reviews by McNatty *et al.*, 1999; McGee and Hsueh, 2000), comparatively little information has been forthcoming with regard to the changes in cellular behaviour that attend the process of folliculogenesis. When viewed in the context of other developmental systems, in which paracrine control of cell differentiation is considered implicitly relative to cell signalling, cell–cell adhesion and cell–extracellular matrix (ECM) interactions, our knowledge of cellular behaviours in the ovarian follicle is lacking, despite the wealth of information available on the role of growth factors and hormones in ovarian follicle development (for a review see Elvin and Matzuk, 1998). Recently, Rodgers *et al.* (1999, 2000) have drawn attention to the role of the ECM during bovine follicle growth as related to changes in the follicular basal lamina that may influence the growth and differentiation of both granulosa and theca cells. Given the

general importance of the ECM in regulating diverse cell behaviours including migration, division, adhesion and death, this direction of study will add measurably to our understanding of the cellular forces that shape remodelling of the follicle during normal growth, differentiation and atresia.

In the context of paracrine factor access and presentation to target cells, it is helpful to consider the disposition of the ECM relative to the three basic types of cell present in preantral follicles. Although much of the growth of preantral follicles is characterized by gradual expansion of the follicular basal lamina as oocyte size and the number of granulosa cells increase, thecal cells also associate with the follicle and begin their course of differentiation. Given that growth factors such as growth differentiation factor 9 (GDF-9) and kit ligand (KL) have been implicated in the paracrine control of early follicle development (Yoshida *et al.*, 1997; Elvin and Matzuk, 1998; Elvin *et al.*, 1999a; Galloway *et al.*, 2000), it is essential to consider the cellular origins and targets of signalling molecules in relation to the cell–ECM interfaces present at these early stages. The follicular basal lamina encapsulates granulosa cells and separates these cells from the surrounding theca cells, which are encased by basal lamina-like material (see Rodgers *et al.*, 2000). The

Email: david.albertini@tufts.edu

'personalized' ECM of the oocyte, the zona pellucida, also becomes highly organized and serves to limit contact between the oocyte and surrounding granulosa cells. If oocytes, granulosa and theca cells act as cellular sources or receivers of paracrine growth factors like GDF-9 or KL, there must be mechanisms for the bi-directional exchange of such factors through these intervening matrices. Given this problem of the spatial partitioning of discrete types of cell by the ECM, and the well-known role of the ECM as a selective filtration or barrier device, this article reviews what is known about the oocyte–granulosa cell interface with respect to cellular mechanisms that could facilitate and regulate bi-directional exchange of paracrine signals (Buccione *et al.*, 1990).

Transzonal projections at the oocyte–granulosa cell boundary

Transzonal projections (TZPs), follicle cell extensions that traverse the zona pellucida and terminate on the oocyte cell surface, have been well characterized in many mammals by electron microscopy (Hertig and Adams, 1967; Anderson and Albertini, 1976; reviewed in Motta *et al.*, 1994). The studies of Motta *et al.* (1994) on human ovarian follicles were the first to demonstrate that dynamic alterations in the number and form of TZPs occur at specific stages of follicle development and might serve to co-ordinate or modulate information exchange between the oocyte and follicle cells. This work showed that, in preantral follicles, TZPs were most numerous forming both adhesive and gap junctional contacts at the oolemma, and further demonstrated that during peak periods of oocyte growth, TZPs extended as deep invaginations that impinged upon the germinal vesicle. After further antral follicle development, TZPs retracted and maintained fewer terminal connections with the oocyte than in preantral follicles. During ovulation, active retraction of TZPs was noted (Motta *et al.*, 1994).

These seminal findings merit reinterpretation given recent observations on the composition and structure of TZPs and assembly of the zona pellucida. The continued synthesis and secretion of zona pellucida proteins throughout ovulation indicates that remodelling of the zona pellucida is an ongoing process during folliculogenesis (Wassarman and Albertini, 1994). Since there is abundant evidence consistent with the contention that cumulus cell uncoupling from the oocyte involves TZP retraction and remodelling during maturation of cumulus–oocyte complexes *in vivo* and *in vitro* (Allworth and Albertini, 1993; Albertini and Rider, 1994; Suzuki *et al.*, 2000), the continued synthesis and secretion of zona proteins may allow for closure of channels left after cellular retraction. Thus, marked changes in granulosa–oocyte and granulosa–granulosa cell adhesion, induced by the LH surge at ovulation, co-ordinate changes in the shape and secretory activity of cumulus cells with the final stages of oocyte and follicle maturation. But what about earlier transitions in follicle development?

Differences in gonadotropin-dependent events in antral follicle development, during what McGee and Hsueh (2000) refer to as cyclic recruitment, probably involve very different signalling requirements compared with the initial recruitment of primordial follicles into the growing pool of preantral follicles. Plasticity and remodelling of the zona pellucida may similarly allow for regulated changes in the establishment and maintenance of functional TZPs during these different critical transitions of follicle development.

The observations of Motta *et al.* (1994) on preantral follicles should be considered in light of the predominant, but not exclusive, role of known paracrine factors during these early developmental stages. TZPs were reported to be most abundant in preantral follicles. Studies in our laboratory have defined functional properties of TZPs in preantral follicles that would support their role in the localized secretion or uptake of factors at the oocyte–granulosa cell interface (Can *et al.*, 1997). Numerous microtubule-containing TZPs are present within the zona pellucida in preantral follicles as evidenced by optical sectioning of intact follicles by confocal microscopy (Fig. 1a). Moreover, mitochondrial vital staining of intact cultured mouse follicles with MitoTracker™ shows active bi-directional movements of these organelles within TZPs when monitored by time-lapse confocal microscopy (Fig. 1b). Similarly, loading follicles with non-metabolizable and neutral charge endocytic tracers like fluorescent dextrans shows active translocations of endosomes and lysosomes across the zona pellucida due to organelle movement within TZPs (Fig. 1c). These findings extend ultrastructural observations on the organellar contents of TZPs and document their capacity to support polarized organelle movement, an observation consistent with the demonstration of microtubules within TZPs and the role of microtubules in directed organelle movement (Allworth and Albertini, 1993; Can *et al.*, 1997; Carabatsos *et al.*, 1998). If these structures mediate directed transport, secretion, or selective uptake of factors secreted by the oocyte (for example GDF-9), it would be expected that granulosa cells apposed to the zona pellucida would be anchored and oriented to support localized macromolecular exchange. This contention is supported by electron microscopy of normal follicles and follicles from mutant lines of mice blocked at various stages of preantral development.

In control mice, TZPs observed in preantral follicles are anchored by F-actin bearing follicle cell extensions at the periphery of the zona pellucida, but send solitary and usually corkscrew-shaped projections through the zona pellucida (Fig. 2a). Follicles from GDF-9 null (–/–) animals lack anchoring TZPs but show prominent organelle-rich TZPs that envelope the oocyte surface, indicating that, in this situation, an unregulated exploration of the oocyte is allowed to occur (Carabatsos *et al.*, 1998; Fig. 2b). In marked contrast, animals null for the FSH-β gene, in which follicle development is arrested at the late secondary preantral stage (Kumar *et al.*, 1997), show prominent organelle-rich and anchoring TZPs (Fig. 2c). Furthermore,
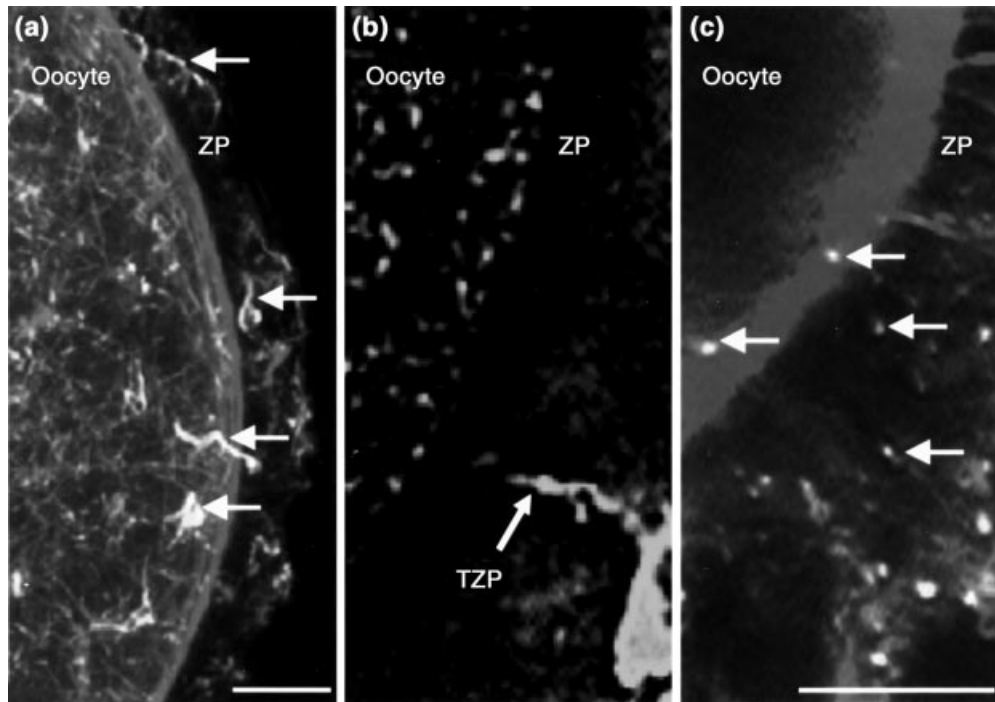
Fig. 1. Confocal microscope images of intact preantral mouse ovarian follicles. (a) Dense network of microtubule-containing transzonal projections (TZPs) derived from follicle cells (arrows). (b) A single confocal image of vitally stained mitochondria within a follicle cell TZP (arrow) obtained from a living cultured follicle labelled with MitoTracker™. (c) Fluorescent dextran-labelled endosomes (arrows) within follicle cell TZPs from a follicle cultured in the presence of endocytic marker before fixation and analysis by confocal microscopy. ZP: zona pellucida. Scale bars represent 5 μm.

the centrosome–Golgi complex is oriented towards the oocyte in nearly all follicle cells apposed to the outer surface of the zona pellucida in this FSH-β-deficient animal model (Fig. 2c). Thus, in the absence of FSH, follicle arrest at the secondary stage is associated with a striking degree of granulosa cell polarity that spatially approximates the secretory machinery with an increased density of TZPs. The implications of this finding are discussed below with respect to processing, secretion and transcytotic transport of factors between oocyte, granulosa and theca. In addition, a central question that emerges from observations of enhanced input in the absence of FSH-β is: what regulates the orientation and stability of TZPs?

Factors regulating TZP stability

As noted earlier, changes in cell–cell (junctional) and cell–zona adhesion underscore both the retraction of TZPs and the migration of cumulus cells during hormone-induced expansion (Albertini and Rider, 1994), and, in some systems, selective loss of anchoring of granulosa cells to the zona pellucida is associated with invasion of microtubule TZPs during hormone-induced oocyte maturation (Allworth and Albertini, 1993). As in axons, microtubules form the cytoskeletal core of granulosa cell TZPs and would provide tracks for the polarized translocation of secretory pathway

organelles. Thus, it is important to consider factors intrinsic to granulosa cells that influence the stability of TZPs, since, as indicated earlier, developmental changes in their stability would have direct consequences on the processing and action of paracrine factors originating from the oocyte or somatic follicular cells.

One mechanism for stabilizing microtubules involves post-translational acetylation of the α-tubulin subunit that occurs subsequent to microtubule polymerization (Can and Albertini, 1997). Cultured human ovarian granulosa cells show a subpopulation of drug-resistant acetylated microtubules (Can and Albertini, 1997) and these are present in human cumulus cells. Typically, all TZPs present in the zona pellucida of human oocytes retrieved for *in vitro* fertilization show acetylated microtubules (Fig. 3). In addition to the staining observed within branching TZPs, staining is found within circular hoop-like structures that represent terminal portions of TZPs which associate with the oocyte surface (Fig. 3b, arrows). Thus, the prevalence of acetylated microtubules within TZPs of human oocytes indicates that this mechanism for maintaining microtubule stability may persist through the latest stages of follicle development to maintain oocyte–granulosa cell connections during cumulus expansion. This mechanism for TZP stabilization has been evaluated in follicles at earlier developmental stages.
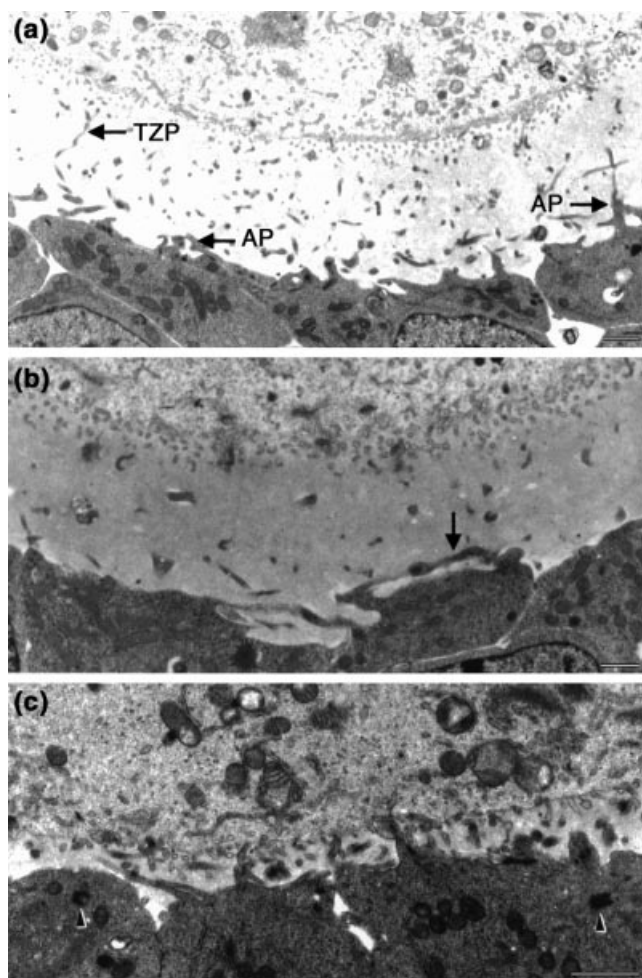
Fig. 2. Electron micrographs of the oocyte–granulosa cell interface from control (a), growth differentiation factor 9 (GDF-9) null (b) or FSH-β null (c) mouse preantral follicles. In control follicles, anchoring projections (AP) and zona-traversing projections (TZP) are evident (a). Prominent organelle-rich TZPs (arrow) are typical in GDF-9 null follicles (b). Follicles from FSH-β null animals show a striking degree of granulosa cell orientation towards the oocyte as evidenced by the apposition of the centrosome–centriole complex (c, arrowheads). Scale bars represent 1 μm.

Fig. 3. Comparison of total tubulin (a) and acetylated tubulin (b) staining patterns within zona-enclosed human oocytes retrieved for *in vitro* fertilization. Note the abundance of oocyte microtubules (a) that lack acetylated α-tubulin epitope after immunostaining compared with those within TZPs with acetylated α-tubulin epitope (b). TZPs containing acetylated microtubules often terminate as hoops (arrows) that are located at the oocyte surface. Scale bar represents 20 μm.

A similar approach was used to analyse TZPs in preantral mouse follicles with respect to the possible consequences of protein kinase A activation in granulosa cells, one of the most important signalling pathways invoked to regulate granulosa cell differentiation (McGee and Hsueh, 2000). Untreated oocytes isolated from preantral follicles of prepubertal mice show a high density of TZPs that are enriched in the acetylation epitope (Fig. 4a,b). Brief treatments with the phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX), an agent commonly used to increase intracellular cyclic monophosphate concentrations in granulosa cells, causes both a reduction in TZP density and a decrease in immunodetectable acetylated tubulin (Fig. 4c,d). Together, these observations imply that
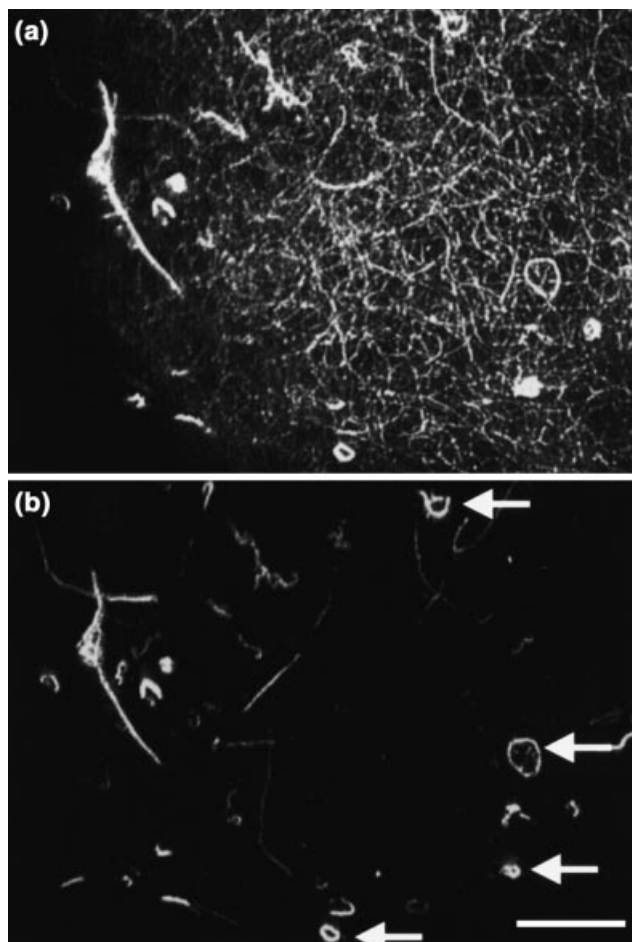
TZPs are dynamic granulosa cell structures, the stability and function of which may be subject to both developmental and hormonal regulation. As specialized conduits for the transport, processing and reception of paracrine factors at the oocyte–granulosa cell interface, TZPs may represent a novel mechanism for the regulation of paracrine signalling in the developing follicle. A model for this hypothesis is presented below.

Regulation of paracrine signalling

Given the importance of modulating local forms of cell communication within the developing ovarian follicle, especially with respect to the changing demands for oocyte–granulosa cell cross talk at different developmental stages, this review of cellular behaviours has focused on
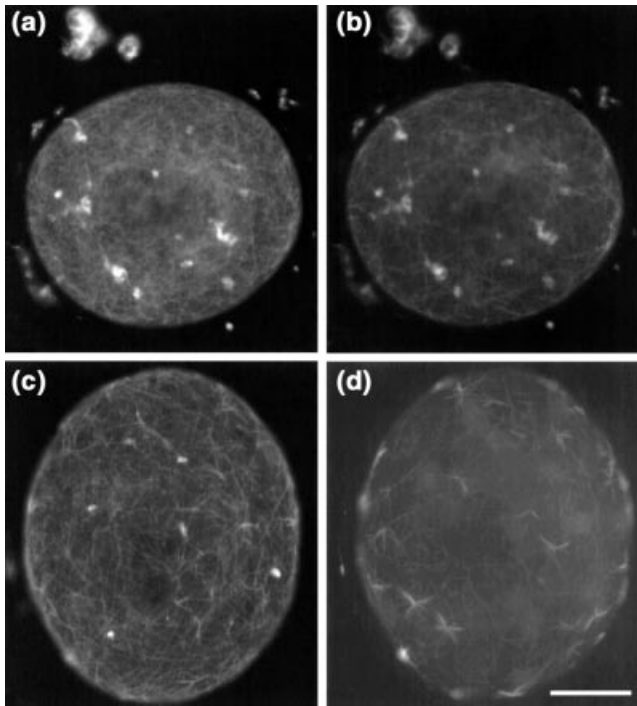
Fig. 4. Effects of isobutylmethylxanthine (IBMX) on the density and tubulin acetylation of TZPs in mouse oocytes isolated from preantral follicles. Total tubulin (a) and acetylated tubulin (b) in control oocytes cultured for 2 h before fixation and processing. (c,d) Respective tubulin patterns after exposure to 100 μmol IBMX l^{-1} for 2 h; note the reduction in TZP density and expression of acetylation epitope. Scale bar represents 20 μm.

two important points. First, modifications in cell–cell and cell–matrix interactions are likely to influence both the availability and actions of growth factors derived from each of the cellular compartments of the follicle (oocyte, granulosa and theca). Thus, the architectural remodelling associated with oocyte growth, epithelial expansion, antrum formation and differentiation of a vascularized theca represents significant consequences of differential gene expression that both reinforce functional distinctions in the germ and somatic cell lineages of the follicle and co-ordinate the processes of oogenesis and folliculogenesis. Second, since oocyte development is a critical aspect of follicle development and oocytes exert profound effects on granulosa cells, the interface between oocyte and granulosa cell emerges as a major control site for the co-ordination of follicle development. Historically, the properties of this interface have been viewed as fundamental to the regulation of oocyte growth and maturation and follicular luteinization (Elvin *et al.*, 1999a,b, 2000; Mermillod *et al.*, 1999; Nagyova *et al.*, 2000). Here, we provide a novel framework for considering aspects of paracrine signalling that would depend directly on dynamic changes in the connections between oocyte and granulosa cells.

On the basis of work summarized earlier in this review,

the model proposed (see Fig. 5) has been developed to explain how the interplay between growth factors and hormones may mediate the selection, support and demise (atresia) of the follicle. Implicit in this model is the notion that TZPs serve as specialized devices for mediating information exchange between oocyte and granulosa cells. This exchange includes, but is not limited to, the exchange of small molecules via gap junctions that support metabolite exchange. Thus, in preantral follicles, in which oocyte growth is supported and follicle growth is protracted, bi-directional exchange of cytoplasmic signals, whether nutrients or second messengers, co-ordinates directly certain aspects of metabolism shared by oocytes and granulosa cells (Buccione *et al.*, 1990). Possibly just as important is the need to establish and maintain terminal TZP connections that would mediate both the local delivery of growth factors and possibly facilitate receptor occupation and activation by ligands like KL, a granulosa cell product (Yoshida *et al.*, 1997; Elvin *et al.*, 1999a). Delivery of follicle cell-derived leptin and STAT3 to the oocyte by TZPs has been reported (Antczak and Van Blerkom, 1997). TZPs would also support uptake of oocyte products, like oocyte-specific members of the transforming growth factor β superfamily (GDF-9), which could be transported and possibly processed during transcytosis through granulosa cells for presentation to more distal granulosa cells and even surrounding theca (Dube *et al.*, 1998; Solovyeva *et al.*, 2000). Central to this model are key properties of TZPs: (i) that they possess the appropriate subcellular machinery for orienting the trafficking of paracrine factors; and (ii) that their dynamics are controlled by other factors (for example FSH) such that localized delivery and uptake is regulated. It appears that criteria to support the idea of polarized secretion and endocytosis have been met, at least on a preliminary level and, as the model proposes, FSH may be one factor causing TZP retraction given the data mentioned above.

In many ways, TZPs resemble axons, as has been previously alluded to (Allworth and Albertini, 1993), and recent data indicate additional similarities to synapse-like modes of signal transmission (Grosse *et al.*, 2000). The cytoskeletal composition, organellar contents and overall disposition of TZPs at the oocyte–granulosa cell interface (Motta *et al.*, 1994) support a view consistent with TZPs functioning in the directed delivery and uptake of factors within the perivitelline space. Although future work will be needed to define the factors involved in the formation and function of TZPs, especially with respect to the actions of GDF-9, KL and related signalling molecules, there is already some evidence to support their role in the acquisition of developmental competence by mammalian oocytes (Liu *et al.*, 1997; Carabatsos *et al.*, 1998; Mermillod *et al.*, 1999). As mentioned, Antczak and Van Blerkom (1997) have shown that leptin and STAT3, factors that may influence embryo metabolism, assume a polarized distribution in oocytes and embryos. On the basis of confocal microscopy studies in intact follicles, it was proposed that leptin and STAT3 are maternally derived from a subpopulation of
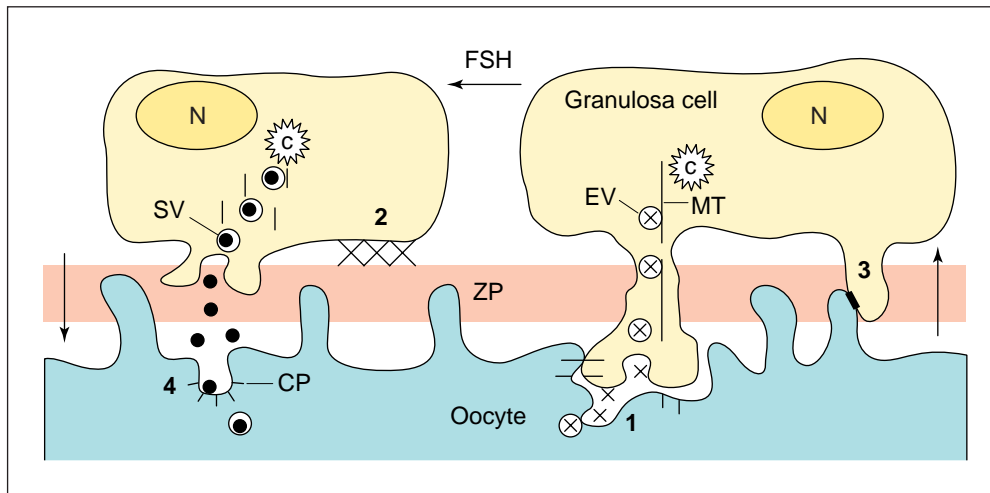
Fig. 5. Model proposing regulated delivery of paracrine factors at the oocyte–granulosa cell interface. Four (1–4) communication modalities are described. (1) Localized uptake of oocyte factors ($\times$), like GDF-9, by endocytosis at stabilized attachment sites of transzonal projections (TZPs) at the oolemma; vectorial transport of endocytic vesicles (EV) to granulosa cell body occurs along microtubules (MT) in preparation for intracellular processing and release of factors after transcytosis. (2) Granulosa–zona pellucida anchoring required for TZP orientation. Contact sites may play a signalling role for oocytes and granulosa cells, and changes in adhesion would occur in response to changes in composition of the zona pellucida. (3) Gap junctions that allow for direct intercellular communication between oocyte microvilli and granulosa cell TZPs. (4) Pathway for delivery of granulosa cell-derived factors ($\bullet$) packaged in secretory vesicles (SV) that are subsequently endocytosed by receptor-mediated endocytosis at the oocyte surface through coated pits (CP). Note that remodelling of the microtubule cytoskeleton (MT) in response to granulosa cell stimulation by FSH would lead to TZP retraction and thus modulating either the delivery of granulosa cell factors ($\bullet$) to the oocyte (arrow, left) or uptake of oocyte secreted factors ($\times$) by granulosa cells (arrow, right). c: centrosome; FSH: follicle-stimulating hormone; N: nucleus; MT: microtubules; ZP: zona pellucida; $\bullet$: granulosa cell factors; $\times$: oocyte-secreted factors.

follicle cells and that TZPs deliver these products into the perivitelline space for endocytosis by the oocyte.

The extent to which these and other protein factors secreted by granulosa cells gain access to the oocyte needs to be investigated more thoroughly given current ART protocols that involve removal of somatic cells. This commonly practised manipulation may compromise efficient delivery of factors to the oocyte at crucial stages of development that normally would have been subserved by the function of TZPs proposed here. In light of the data presented herein, the success of hormone-induced follicle development *in vivo* or *in vitro* may depend on experimental protocols that maintain the structural integrity of the granulosa–oocyte cell interface (Hayashi *et al.*, 1999).

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