Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation

C. S. Wright¹, D. L. Becker², J. S. Lin³, A. E. Warner²
and K. Hardy¹

¹Department of Reproductive Science and Medicine, Division of Paediatrics, Obstetrics and Gynaecology, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK; ²Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1 6BT, UK; and ³Department of Anatomy with Radiology, School of Medicine, University of Auckland, New Zealand

Gap junction communication plays an essential role in follicle growth. Immunocytochemistry and confocal microscopy were used to examine the expression of gap junction connexins of the α and β subfamilies in follicles from primordial to preovulatory stages in the ovaries of prepubertal and adult mice. Connexin-specific antibodies detected α_1 , α_4 , α_6 , β_1 , β_2 and β_4 connexins within follicles. In adult ovaries connexin immunolabelling was stronger in larger (more mature) follicles than it was in smaller follicles, with comparatively reduced labelling detected in prepubertal ovaries. In healthy follicles, labelling for α subfamily con-

nexins was detected between granulosa cells, whereas labelling for β subfamily connexins was found in the theca. Labelling for β subfamily connexins and α_4 connexin (preantral stage) was detected on the oocyte surface membrane. In atretic follicles, labelling for β_4 connexin appeared between the granulosa cells. These results demonstrate that α and β connexin subfamilies are segregated to separate cellular compartments in the mouse follicle. The results are discussed in the light of possible roles for differential gap junctional communication in the regulation of folliculogenesis, oocyte maturation and atresia.

Introduction

In the developing ovarian follicle, the oocyte is separated from the local blood supply by an increasing number of granulosa cell layers, the cells of the theca externa being the only follicle cells in direct contact with ovarian capillaries. In this avascular system, intercellular communication between the oocyte and the surrounding somatic cells (the granulosa and theca) is essential for the correct functioning and development of the follicle. An important route for this interaction is provided by gap junctions that occur between follicular cells (Albertini and Anderson, 1974; Anderson and Albertini, 1976).

Gap junctions form channels that directly link the cytoplasm of adjoining cells, and are present in almost all adult tissues (for review, see Kumar and Gilula, 1996). These channels are composed of two opposing hexameric hemi-channels (connexons), consisting of six transmembrane protein subunits known as connexins (Goodenough, 1976). At least seventeen members of the connexin protein family have been sequenced and allocated to subfamilies – α , β , γ and δ – on the basis of sequence homology (Sohl *et al.*, 1998; for review, see Kumar, 1999).

Gap junction communication allows the direct exchange between cells of ions and small molecules such as

cAMP and inositol 1,4,5-trisphosphate (IP₃) (for review, see Kumar and Gilula, 1996). In the ovarian follicle, gap junctions facilitate the uptake by the oocyte of nutrients such as amino acids from somatic cells (Biggers *et al.*, 1967). Gap junctions mediate metabolic co-operation between the oocyte and its somatic companion cells (Heller *et al.*, 1981) and transmit endocrine, paracrine and growth factor effects (for review, see Eppig, 1991). Gap junction communication may also play a role in the co-ordination of follicular growth and steroid hormone production (Grazul-Bilska *et al.*, 1997). Follicular gap junctions maintain the meiotic arrest of the oocyte in the follicle via low tonic amounts of cAMP signalling from the granulosa to the oocyte (Dekel, 1988; Downs *et al.*, 1989; Eppig, 1989). Follicular gap junction communication also plays a role in the final maturation of the oocyte by LH-induced signalling pathways (Dekel, 1988).

Northern blotting, *in situ* hybridization and RT-PCR have detected mRNA for eight connexin types (α_1 (Cx43), α_4 (Cx37), α_5 (Cx40), α_6 (Cx45), α_9 (Cx60), β_1 (Cx32), β_2 (Cx26), β_5 (Cx30.3)) in the ovaries of a number of species (Beyer *et al.*, 1992; Reed *et al.*, 1993; for review, see Grazul-Bilska *et al.*, 1997; Itahana *et al.*, 1998). Immunocytochemistry has confirmed the presence of five of these connexins: α_1 , α_4 , α_6 , β_1 and β_2 (for review, see Grazul-Bilska *et al.*, 1997, 1998; Lenhart *et al.*, 1998; Johnson *et al.*, 1999). In addition, studies have shown that cultured

Email: catherine.wright@ic.ac.uk

ovaries of mouse α_1 -knockouts have very poor follicular development (Juneja *et al.*, 1999), and α_1 -heterozygotes have reduced fertility (C. Lo, personal communication). α_4 -Knockout mice are also infertile (Simon *et al.*, 1997).

Investigations of oocyte and follicle maturation *in vitro* have been undertaken mainly in mice. However, to date, there has been no systematic description of connexin expression in this species. In the present study, the spatio-temporal distribution of connexins forming gap junctions in the mouse ovarian follicle during follicular maturation were studied with the aim of increasing understanding of the role of gap junctions in the follicle.

Materials and Methods

Animals and tissue preparation

F₁ (C57BL/6 $\times$ CBA/Ca; Olac, Bicester) female mice of either 3–6 weeks old (adult) or 12 days old (prepubertal) were used. At least five mice were included in each animal group. Animals were killed by cervical dislocation, and their ovaries were immediately dissected free and collected into filtered PBS ('A' Oxoid, Basingstoke). Ovaries were further cleaned of all fat and other tissue, fixed in 4% paraformaldehyde overnight (Sigma, Poole), and then transferred to 30% (w/v) sucrose (BDH, Poole) in PBS solution overnight. The tissue was then coated in OCT compound (BDH), and frozen by immersion in an isopentane (BDH) slush bath surrounded by liquid nitrogen. Sections (10–20 μ m) were cut on a Bright cryostat, and mounted onto poly-lysine-coated slides (Polysine, BDH). Hearts and livers of 6-week-old mice were dissected at the time of ovary collection and processed as above for use as controls.

Generation and characterization of monoclonal and polyclonal antibodies

Peptides matching sequences from the intracellular loop of connexins α_1 (Cx43) and β_1 (Cx32) were synthesized by Immune Systems, Bristol. The following peptides were used: α_1 : EIKKFKYGIEEH; β_1 : LEGHGDPLHLEE. Peptides were coupled at the C-terminal end to bovine thyroglobulin at a final molar ratio peptide:thyroglobulin of 40–60:1. Coupled peptides were used to generate monoclonal antibodies in mice by the Monoclonal Antibody Unit, University College London. Two injections of peptide were made, 4 weeks apart. One week after the second injection, primary screening was carried out on small samples of serum from each mouse by ELISA with the immunizing peptide and checked by immunolabelling on sections to identify samples that recognized the parent connexin. Mice that were generating promising antibodies were killed 3 weeks later, the spleens were removed and homogenized, and red cells and excess protein were removed. Spleen cells were fused to JK immortal myeloma cells with 50% (v/v) polyethylene glycol (Sigma) to generate antibody secreting hybridoma cells. After fusion, cells

were plated onto a feeder containing 10% (w/v) hybridoma-enhancing substance and surviving hybridomas were cloned. An ELISA screen identified clones secreting antibodies that recognized the immunizing peptide, followed by immunocytochemistry on sections to confirm connexin recognition. Clones generating satisfactory antibodies by both criteria were then expanded and supernatants were taken for further characterization.

Selected antibodies were screened on frozen sections of paraformaldehyde-fixed heart tissue, which expresses predominantly α_1 connexin (Cx43), and liver, which expresses both connexins β_1 (Cx32) and β_2 (Cx26). These screens revealed several monoclonal antibodies for the two connexins and monoclonal antibodies 1A (α_1) and 2A (β_1) were chosen for further analysis. Both antibodies gave excellent, specific labelling of their parent proteins. Immunolabelling was abolished when the peptide used as an antigen was included in the labelling mixture. The connexin specificity of the antibodies was confirmed by assay on pre-implantation mouse embryos, which express predominantly α_1 connexin (Becker *et al.*, 1995) and sections of chick and mouse limb buds, which express both α_1 and β_1 connexins (Makarenkova *et al.*, 1997). The monoclonal antibodies reproduced precisely the labelling patterns published using polyclonal peptide antibodies to the same region of the connexin proteins (Gap 15, Des 1 and Des 5; Becker *et al.*, 1995; Makarenkova *et al.*, 1997). This finding indicates that these monoclonal antibodies have wide species specificity. Given the sequence conservation between the same connexin cloned from a variety of species, this conservation is not unexpected, and indicates that these monoclonal antibodies will be widely useful.

Epitope mapping of monoclonal antibodies 1A and 2A was carried out by 'peptide walking', in which peptides overlapping small regions along the length of the immunizing peptide were tested for the ability to compete with the antibodies for their binding sites and abolish immunolabelling. These experiments showed that the epitope for monoclonal antibody 1A lay within amino acids 136–142 of α_1 connexin (KYGIEEH) and that for 2A lay within amino acids 112–119 of β_1 connexin (GDPLHLEE). These epitopes are close to, but not identical with, the epitopes identified for the peptide polyclonal antibodies Gap 13, Gap 15 and Des 5 (Becker *et al.*, 1995).

β_4 connexin (Cx31.1) antibodies were raised to a 15 amino acid sequence matching hydrophilic residues at the C-terminal end of the β_4 sequence (rat and mouse). The sequence selected is specific to β_4 connexin and has no homology with other known connexin sequences. The peptide was synthesized, conjugated to keyhole limpet haemocyanin (KLH, Sigma) and polyclonal antibodies raised in rabbits by Research Genetics Inc., AL.

Immunocytochemistry and confocal microscopy

Immunolabelling for connexins α_1 (Cx43), α_4 (Cx37), α_5 (Cx40), α_6 (Cx45), β_1 (Cx32), β_2 (Cx26), β_4 (Cx31.1) and β_6

Table 1. Amino acid sequences of peptides used as immunogens to produce primary antibodies used to detect connexins^a

Antibody	Type ^b	Connexin	Amino acid sequence	Location	Reference
R45	RP	α_6 (45)	QYTELSNAKIAYKQN	Carboxyl tail	Coppen <i>et al.</i> , 1998
Gap 15	RP	α_1 (43)	EIKKFKYGIEEH	Cytoplasmic loop 131–142	Becker <i>et al.</i> , 1995
1A	MM	α_1 (43)	EIKKFKYGIEEH	Cytoplasmic loop 131–142	
Anti- α_5	RP	α_5 (40)	AELSCNKEVNG	Cytoplasmic loop 225–270	Coppen <i>et al.</i> , 1998
Gap 23	RP	α_4 (37)	ALLAIEHQMAKIC	Cytoplasmic loop 126–137	Carter <i>et al.</i> , 1996
Anti- α_4 (DP)	RP	α_4 (37)	VHL to QYV	C-terminus 229–333	Goliger and Paul, 1994
Des 5	RP	β_1 (32)	LEGHGDPLHLEEC	Cytoplasmic loop 108–119	Monaghan <i>et al.</i> , 1994
Des 1	RP	β_1 (32)	EKKMLRLEGH-GHLEEVKRHK	Cytoplasmic loop 102–112 + 116–124	Rahman and Evans, 1991
2A	MM	β_1 (32)	LEGHGDPLHLEE	Cytoplasmic loop 108–119	
Anti- β_4	RP	β_4 (31.1)	Proprietary	Proprietary	
Anti- β_6	RP	β_6 (30)	Proprietary	Proprietary	
Des 3	RP	β_2 (26)	FMKGEIKNEFKDIEC	Cytoplasmic loop 106–119	Monaghan <i>et al.</i> , 1994
28H	MM	β_2 (26)	SEFKDIEEIKTQ	Cytoplasmic loop 113–124	Diez <i>et al.</i> , 1999
Gap 10	RP	β sub-family	MNWTGLYTLTSGVNRHSTAIG	Amino terminus of β_1 1–21	Rahman and Evans, 1991

^aAmino acid sequences for β_4 (Cx31.1) and β_6 (Cx30, Zymed) antibodies are not available.

^bType of antibody: RP, rabbit polyclonal; MM, mouse monoclonal.

(Cx30) was performed with primary antibodies that had been raised against short peptide sequences of connexins as described by Becker *et al.* (1995). Antibodies to α_4 , β_4 and α_6 connexins were kind gifts of David L. Paul (Harvard Medical School), Colin Green (University of Auckland) and Rob Gourdie (Medical University of South Carolina), respectively. Details of all primary antibodies are given (Table 1). Secondary fluorescein isothiocyanate (FITC)-conjugated antibodies (Dako, Copenhagen) were either swine anti-rabbit or rabbit anti-mouse, according to the primary antibody used. Most antibodies were diluted in filtered PBS to concentrations of 1:40–50 except Gap 23 and anti- β_4 connexin, which were used at 1:100 and 1:1000, respectively.

In brief, slides were washed with filtered PBS (all PBS was filtered with a 0.22 μ m Millipore filter), incubated with a 0.1 mol lysine l⁻¹ (Sigma) in PBS block containing 0.1% (v/v) Triton-X100 (Sigma) for 30 min at room temperature, incubated with the primary antibody directed against the connexin of interest for 1 h at 37°C, washed three times with PBS and incubated with a secondary antibody for 1 h at 37°C in the dark, washed twice with PBS, then washed with 1 μ g ml⁻¹ propidium iodide (Sigma) in PBS for 2 min, and finally washed twice with PBS. An avidin–biotin (BDS, Cambridge) multiplication step was also used in conjunction with anti- α_4 (DP). Slides were mounted with Citiflour (Chem.lab, Canterbury) and immediately observed on a Leica TCS4D confocal microscope. Confocal images were collected and stored digitally for subsequent analysis of gap junction distribution.

The specificity of connexin labelling was checked by: (1) using multiple antibodies for connexins α_1 (Cx43), β_1 (Cx32) and β_2 (Cx26); (2) parallel staining for connexins in mouse heart, liver and skin sections (positive controls, Fig. 1); (3) omitting the primary antibody for each connexin (negative control); and (4) peptide competition experi-

ments (when peptides were available) for α_1 (using Gap 15 antibody), β_1 (Des 5 antibody) and β_2 (Des 3 antibody) connexins, performed as described by Becker *et al.* (1995). Examples are shown of immunolabelling to check the ability of antibodies 1A (Fig. 1a), 2A (Fig. 1b) and anti- β_4 (Fig. 1c) to recognize their parent proteins in frozen sections. Antibody 1A (Fig. 1a) identified rat heart intercalated discs of the cardiac myocytes, the locations of α_1 connexin (Cx43)-containing gap junctions (Becker *et al.*, 1995). Antibody 2A (Fig. 1b) detected β_1 connexin (Cx32)-containing gap junctions in mouse liver cell plasma membranes (Becker *et al.*, 1995); and anti- β_4 recognized gap junctions in rat epidermis (Fig. 1c). There was no immunolabelling of connexins in negative controls. Peptide competition experiments for α_1 , β_1 and β_2 connexins showed that peptide concentrations of 1:100 reduced labelling markedly, and concentrations of 1:10 and undiluted peptide (10 μ g ml⁻¹) prevented antibody labelling.

Western blotting

Protein was extracted from rat heart and intercalated discs (Colaco and Evans, 1981), mouse liver plasma membranes (Ali *et al.*, 1990) and rat epidermis. Blots were resolved by SDS-PAGE using 10% or 12.5% acrylamide gels. All other antibodies had been characterized previously (Table 1). Western blotting was carried out as described by Burnette (1981) and the location of antigens was determined with an enhanced chemiluminescence kit (ECL+plus; Amersham Pharmacia Biotech, Little Chalfont). It has been reported frequently that good immunocytochemical staining is not necessarily associated with good recognition of the denatured proteins on western blots. In accordance with these observations, the monoclonal antibodies tested here showed the same variability. However, both monoclonal antibodies 1A and 2A and

Fig. 1. Connexin antibodies 1A, 2A and anti- β_4 recognize their parent proteins. (A) Monoclonal antibody 1A identifies the intercalated discs (arrowheads) and punctate gap junctions composed of α_1 connexin (Cx43) that lie in the parallel membranes of myocytes in a frozen section of rat heart. The panel to the right shows the single clear band at 43 kDa identified on a western blot against protein isolated from rat heart. The band is relatively broad and may include the minor bands that indicate the phosphorylated forms of the connexin protein. (B) Frozen section through mouse liver revealing extensive punctate labelling of β_1 (Cx32)-containing gap junctions in the intercellular membranes of liver cells identified by monoclonal antibody 2A. The panel to the right shows a western blot against protein isolated from mouse liver cell membranes with 2A. The band recognized by 2A runs at

anti- β_4 polyclonal identified single bands at positions appropriate for connexins α_1 (Cx43), β_1 (Cx32) and β_4 (Cx31.1), respectively (Fig. 1). The size of the protein identified by each antibody was determined for each gel by plotting the distance travelled by protein markers against their molecular weight, measuring the position of the band identified by the antibody under test, and reading the molecular weight of the stained protein from the graph. The estimated size for the band recognized by each antibody is indicated (Fig. 1). The blots for testing 2A were run on non-reducing 10% acrylamide gels. The β_1 connexin protein is notorious both for its sensitivity to the percentage of acrylamide in the gel and a tendency to form dimers and trimers (Green *et al.*, 1988). In addition, Green *et al.* (1988) demonstrated that when protein loading of a gel is relatively low, the dimeric form of the β_1 protein dominates. Because of this anomalous behaviour, in the illustrated 10% gel, the β_1 dimer was prominent at the predicted size of 46 kDa (Fig. 1). Parallel samples of protein extracted from mouse heart and liver showed that 1A recognized proteins only in the heart while 2A recognized proteins only in the liver, according to the expression patterns of the parent proteins.

Results

Propidium iodide labelling of cell nuclei allowed clear identification of follicles at all developmental stages. The ovaries of 12-day-old prepubertal mice contained primordial, primary and secondary follicles. Ovaries of adult mice contained a range of follicle stages from primordial to antral and preovulatory follicles (see Fig. 2 for follicle staging). Healthy follicles of the same developmental stage in 3–6-week-old (adult) mice showed similar immunolabelling, and observations on adult animals are treated as a single group. Differences in nuclear shape and spatial organization differentiated between stromal, thecal, granulosa and oocyte cells. Connexins in gap junctions were visible as discrete spots or lines between cells. Immunolabelling results are summarized (Tables 2 and 3).

Labelling of connexins in ovarian follicles of prepubertal mice

Gap junctions containing α_1 connexin (Cx43) were detected by Gap 15 between granulosa cells of secondary follicles in the central region of ovaries from 12-day-old mice. Expression was similar to that in secondary follicles of adult animals (Table 2). Primordial and primary follicles in the outer regions of the ovaries did not show any

46 kDa, the predicted position for β_1 protein run at low loading on a 10% acrylamide gel (Green *et al.*, 1988). (C) Frozen section through rat skin labelled with anti- β_4 . Punctate gap junctions are visible in the epidermis (arrowheads). The panel to the right shows a western blot of the single band at 31 kDa recognized by this antibody. Scale bars represent 50 μ m.

Fig. 2. Classification of the stages of mouse ovarian follicular development examined in the present study. Stages are grouped into preantral (primary to large preantral: approximately 50–200 µm), antral (early antral to large antral: approximately 250–500 µm) and preovulatory (approximately 600 µm) stages.

detectable connexin expression (Fig. 3a,b). Gap junction density was greater between mural granulosa cells than between cumulus cells. The β_1 connexin (Cx32) antibodies Des 1, Des 5 and 2A showed weak labelling of gap junctions between theca cells in some central secondary follicles, and also some weak labelling of the oocyte (Table 2). Although β_2 connexin (Cx26) antibodies labelled the ovarian epithelium, no follicular labelling was seen for other antibodies (Table 2).

Labelling of connexins in ovarian follicles of adult mice

In general, the labelling intensity of healthy follicles increased with follicle size (Figs 3c and 4b; Table 3). Primordial and primary follicles had some low-intensity labelling, whereas large antral and Graafian follicles had high-intensity labelling.

α_1 connexin (Cx43) was detected only between follicle granulosa cells (Fig. 3c). Preantral follicles (primordial to late preantral stages) showed less labelling than was seen in larger antral follicles (early antral to preovulatory stages; Table 3). The extent of labelling of secondary follicles for α_1 connexin was similar to that seen in prepubertal animals. An oblique section of a large antral follicle revealed dense immunolabelling of α_1 connexin proteins, which were widely distributed between granulosa cells (Fig. 3d). There was dense expression of α_1 connexin-containing gap junctions between mural cells close to the basal lamina (which appeared linear) and between cells close to the antrum (which appeared more macular), and a lower density in between (Figs 3d and 4e). Both α_1 antibodies – 1A (monoclonal, Fig. 3c) and Gap 15 (polyclonal, Fig. 3d) – gave similar labelling patterns (Table 3).

Immunolabelling with anti- α_4 (DP) for α_4 connexin (Cx37) on the oocyte surface of a preantral follicle is

Table 2. Immunolabelling by antibodies directed against connexins of gap junctions in ovarian follicles of 12-day-old prepubertal mice

Immunolabelling of connexins in gap junctions of prepubertal follicles				
Connexin	Antibody	Theca	Granulosa	Oocyte
α_6 (45)	R45	–	–	–
α_1 (43)	Gap 15	–	++	–
α_1 (43)	1A	–	++	–
α_4 (37)	Gap 23	–	–	–
β_1 (32)	Des 1	+	–	+/-
β_1 (32)	Des 5	+	–	+/-
β_1 (32)	2A	+/-	–	+/-
β_2 (26)	Des 3	–	–	–

Immunolabelling was scored as follows: –, no immunolabelling apparent ('none'); +/-, possible immunolabelling ('very weak'); +, a low density of immunolabelling clearly above background ('weak'); ++, a high density of immunolabelling with plaques visible ('moderate'); +++, very high density immunolabelling with lines of plaques clearly visible ('strong').

shown (Fig. 3e). Expression of α_4 on the oocyte was restricted to preantral stages. α_4 connexins were detected with anti- α_4 (DP) between granulosa cells in antral follicles (Fig. 3e, inset). In secondary, preantral and early antral follicles, there was weak α_4 connexin immunolabelling between granulosa cells, which was generally not evenly distributed but was restricted to patches of the granulosa (Table 3). However, Gap 23 detected α_4 connexin-containing gap junctions widely distributed between granulosa cells in large antral follicles (Fig. 3f), with a gradient of gap junction density from close to the antrum (stronger) to the mural granulosa cells (weaker) (Table 3).

Table 3. Immunolabelling by antibodies directed against connexins of gap junctions in healthy and atretic ovarian follicles of adult mice

Connexin	Antibody	Immunolabelling of connexins in gap junctions of adult mouse follicles											
		Preantral			Antral			Preovulatory			Atretic		
		T	G	O	T	G	O	T	G	O	T	G	O
α_6 (45)	R45	–	–	–	–	–	–	–	+	–	–	–	–
α_1 (43)	Gap 15	–	+	–	–	++	–	–	+++	–	–	+	–
α_1 (43)	1A	–	++	–	–	+++	–	–	+++	–	–	+	–
α_5 (40)	Anti- α_5	–	–	–	–	–	–	–	–	–	–	–	–
α_4 (37)	Gap 23	–	–	–	–	+	–	–	++	–	–	+	–
α_4 (37)	Anti- α_4 (DP)	–	–	+	–	+	–	–	–	–	–	+	–
β_1 (32)	Des 5	+	–	+	+	–	+	++	–	++	+	–	–
β_1 (32)	Des 1	+	–	+	++	–	+	++	–	++	+	–	–
β_1 (32)	2A	+	–	+	+	–	+	+	–	+	–	–	–
β_4 (31.1)	Anti- β_4	–	–	–	–	–	–	–	–	–	–	++	–
β_6 (30)	Anti- β_6	–	–	–	–	–	–	–	–	–	–	–	–
β_2 (26)	Des 3	+	–	+	++	–	+	++	–	+	–	–	–
β_2 (26)	Gap 28H	+	–	+	++	–	+	++	–	+	–	–	–
β subfamily	Gap 10	+	–	+	++	–	+	++	–	+	+	–	–

Follicle compartments are classified into theca (T), granulosa (G) and oocyte (O).

Immunolabelling was scored as follows: –, no immunolabelling apparent ('none'); +/-, possible immunolabelling ('very weak'); +, a low density of immunolabelling clearly above background ('weak'); ++, a high density of immunolabelling with plaques visible ('moderate'); +++, very high density immunolabelling with lines of plaques clearly visible ('strong').

α_6 connexins (Cx45, detected with R45) were restricted to gap junctions between granulosa cells of large antral follicles. α_6 connexin-containing gap junctions were less frequent, and possessed smaller plaques than did α_1 -containing gap junctions (Fig. 4a).

The distribution of gap junctions containing members of the β connexin subfamily in the thecal layer and on the oocyte surface (Fig. 4b) was detected with Gap 10 antibody, which was raised against the amino terminus sequence of β_1 (Cx32) and, therefore, recognizes several members of the β subfamily. Both preantral and antral follicles have immunolabelling between thecal cells, with stronger labelling in the larger follicles (Fig. 4b). Gap 10 detected β subfamily connexins on the oocyte surface, which appeared to be both inside and outside the zona pellucida (Fig. 4b, inset). Specific labelling for β_1 connexins with Des 1 is shown (Fig. 4c) and confirms the presence of β_1 -containing gap junctions between thecal cells and on the oocyte surface. Specific labelling of β_2 con-

nexin (Cx26) with Gap 28H, which matches the distribution of β_1 connexin, was demonstrated between thecal cells (Fig. 4d) and on the oocyte (Table 3). It is concluded that gap junctions expressed between thecal cells and on the oocyte surface membrane contain both β_1 and β_2 connexins.

The connexin expression pattern was modified in atretic follicles, which were identified by the presence of pyknotic nuclei in the granulosa cells and degenerated oocytes. Granulosa cells in an atretic follicle expressed fewer α_1 connexin (Cx43)-containing gap junctions than did granulosa cells in an adjacent healthy follicle (Fig. 4e), indicating that α_1 connexin expression is suppressed. In healthy follicles, members of the β connexin subfamily are not detected between granulosa cells. However, in atretic follicles, granulosa cells express gap junctions containing β_4 connexin (Cx31.1) (Fig. 4f).

Ovarian blood vessel endothelial cells expressed connexins α_1 (Cx43), α_4 (Cx37), α_5 (Cx40) and α_6 (Cx45)

Fig. 3. Distribution of connexin types within gap junctions of adult and prepubertal mouse ovarian follicles. Nuclei are stained with propidium iodide (red) and connexins are stained with fluorescein isothiocyanate (FITC) (green). (A) Immunolabelling of α_1 connexin (Cx43)-containing gap junctions with Gap 15 between granulosa cells of secondary follicles in the central region of a prepubertal ovary. (B) Immunolabelling (arrowhead) of α_1 connexin with Gap 15 showing expression restricted to the granulosa (g) of inner secondary follicles (2°) but not outer primary follicles (1°) in a prepubertal ovary. (C) Immunolabelling of α_1 connexin in an adult ovary with 1A in gap junctions between granulosa cells (g) at several different follicular stages, showing strong expression in large follicles (arrow) and weaker expression in smaller follicles (arrowhead) t: theca. (D) Immunolabelling of α_1 connexin with Gap 15 in a large antral follicle from an adult ovary showing gap junctions between granulosa cells (g) but not thecal cells (t). (E) Immunolabelling of α_4 connexin (Cx37) with anti- α_4 (DP) on the oocyte (o) surface (arrowhead) but not between granulosa cells (g) in an adult preantral follicle, and (inset) between granulosa cells in a larger follicle. (F) Immunolabelling of α_4 connexin with Gap 23 between granulosa cells (g) in a large antral follicle of an adult ovary. Scale bars represent 50 μ m.

Fig. 5. Connexin expression in gap junctions within mouse ovarian follicle blood vessels. (A) Immunolabelling of α_1 connexin (Cx43, arrowhead). (B) Immunolabelling of α_4 connexin (Cx37, arrowhead). (C) Immunolabelling of α_5 connexin (Cx40, arrowhead). (D) Immunolabelling of α_6 connexin (Cx45, arrowheads). Scale bars represent 50 μ m.

(Fig. 5). α_5 connexins were expressed exclusively in blood vessels and were not detected in ovarian tissue or follicles. β_6 connexin (Cx30) was not detectable in ovarian tissue. Connexin β_6 has been found to have sequence homology with β_2 (Cx26); therefore, it is possible that antibodies raised to β_2 recognize β_6 -containing gap junctions. However, when non-communicating cells were transfected with β_2 and β_6 connexins, no crossreactivity was observed (D. L. Becker, unpublished), confirming the specificity of β_2 antibodies.

Discussion

The present study examined the distribution and composition of gap junctions in developing ovarian follicles of the

mouse, by following protein expression for members of the α and β connexin subfamilies with antibodies specific to connexins α_1 (Cx43), α_4 (Cx37), α_5 (Cx40), α_6 (Cx45), β_1 (Cx32), β_2 (Cx26), β_4 (Cx31.1) and β_6 (Cx30). Connexin labelling was not detected in primordial follicles and only seen occasionally in primary follicles. Secondary follicles, whether in prepubertal or adult mice, always expressed gap junctions. Thus, connexin expression only became substantial after initiation of granulosa cell division. Gap junctions between granulosa cells were composed of α connexins (α_1 , α_4 and α_6), whereas those between thecal cells comprised β connexins (β_1 and β_2). This rule was transgressed only in atretic follicles, where α_1 connexin expression between granulosa cells decreased, whereas β_4 connexin could be detected. Gap junctions between the

Fig. 4. Distribution of connexin types within gap junctions of adult mouse ovarian follicles. (A) Immunolabelling of α_6 connexin (Cx45)-containing gap junctions with R45 (arrowhead) between granulosa cells at the edge of an antral follicle (a), but not a preantral follicle (pa). (B) Immunolabelling between thecal cells (t, arrow) and on the oocyte surface (arrowhead) by Gap 10, an antibody that recognizes β subfamily members, and (inset) between the cumulus granulosa and the oocyte (o, arrowhead). (C) Immunolabelling of β_1 connexin (Cx32)-containing gap junctions with Des 1 in the theca layers (t, arrowheads) of a small antral follicle. (D) Immunolabelling of β_2 connexin (Cx26)-containing gap junctions between thecal cells (t) with Gap 28H. (E) Strong immunolabelling of α_1 connexin (Cx43) with gap 15 in a healthy follicle (h) and reduced immunolabelling in an atretic follicle (at) with brightly staining apoptotic nuclei (arrowheads). (F) Immunolabelling of β_4 connexin (Cx31.1)-containing gap junctions between granulosa cells of an atretic follicle (at) with no labelling in a healthy follicle (h). Scale bars represent 50 μ m.

oocyte and granulosa cells were constructed from β subfamily connexins; in preantral follicles α_4 connexin was also detected.

In prepubertal 12-day-old mice, connexin expression was restricted to the central region of the ovary, which contains secondary follicles. These follicles may be hormone responsive, reflecting the influence of hormones on gap junction expression. The expression pattern may also reflect developmental regulation, since growing follicles express gap junctions first. Toshimori and Oura (1982), using freeze–fracture techniques which can detect very small gap junctions, detected gap junctions in 7–10-day-old mice. Low α_1 connexin (Cx43) expression was also detected in 11-day-old rats (Granot and Dekel, 1997).

In the healthy follicles of adult mice, α_1 (Cx43) was the most abundant connexin. Moderate granulosa α_1 expression began at preantral stages, along with weak β_1 (Cx32) and β_2 (Cx26) expression in the theca and β_1 , β_2 and α_4 (Cx37) expression on the oocyte surface. At antral stages, this pattern was maintained with stronger expression of α_1 , β_1 and β_2 and additional expression of α_4 in the granulosa. In preovulatory follicles, α_6 (Cx45) expression was also apparent in the granulosa. Other studies in the mouse have detected α_1 and β_1 connexin mRNA and protein (localized to the cumulus and oocyte surface) in oocyte–cumulus complexes (Valdimarsson *et al.*, 1993) and α_1 connexin protein in the granulosa layer of whole follicles (Koike *et al.*, 1993).

Simon *et al.* (1997) reported α_4 connexin (Cx37) expression in oocyte–granulosa gap junctions. In their study, α_4 -knockout mice were infertile, and had lost granulosa–oocyte dye transfer, indicating a loss of gap junction communication. Follicular development in these mice was arrested, with concurrent ‘premature’ corpus luteum formation, symptoms reminiscent of polycystic ovary syndrome (PCOS) in women. In PCOS, LH concentrations are increased (Gilling-Smith and Franks, 1993), which could cause gap junction breakdown and premature follicular maturation. In the present study, α_4 connexin (detected with both Gap 23 and anti- α_4 (DP)) was found mostly between granulosa cells of antral follicles (in a patchy localization in early antral follicles and more widely distributed in large antral–preovulatory follicles). However, a pattern of oocyte immunolabelling (with anti- α_4 (DP)) in preantral follicles similar to that described by Simon *et al.* (1997) was observed in the present study. The differences in α_4 immunolabelling may be due to the different strains of mice being used in the two studies (the genetic background of α_4 -knockouts is C57 or 129 (D. Paul, personal communication)), or to a greater specificity of α_4 antibody (Gap 23) used in the present study. The phenotype seen in α_4 -knockout mice could also have been produced by loss of communication between granulosa cells.

Connexin expression increased with follicular size. As the number of granulosa cell layers between the oocyte and theca increase during follicle growth, the distance between the oocyte and the blood supply increases,

necessitating highly efficient intercellular communication mechanisms to maintain oocyte health. Gap junctions are known to contribute to the control of cell division (for review, see Bruzzone *et al.*, 1996); indeed, reducing α_1 connexin (Cx43) expression reduces cell proliferation in the developing chick retina (Becker and Mobbs, 1999). α_1 -knockout mouse follicles develop only to the primary stage (Juneja *et al.*, 1999) and, therefore, gap junction communication is required for granulosa proliferation during follicle maturation. Communication via gap junctions may also be important for cellular reorganization, such as in antrum development. Addition of the gap junction inhibitor lindane to granulosa–oocyte cultures inhibits follicle formation (Li and Mather, 1997).

Increased connexin expression in larger follicles may be induced by oestrogen production from granulosa cells, which increases with follicular size. Oestrogen increases α_1 (Cx43) expression in the rat ovary (Schreiber *et al.*, 1993; Wiesen and Midgley, 1993; Risek *et al.*, 1995) and HeLa cells (Yu *et al.*, 1994), and hormone-responsive connexin gene promoters can regulate connexin expression (De Leon *et al.*, 1994; Yu *et al.*, 1994). In the uterus, oestrogen and progesterone are involved in the control of gap junction activation during labour (Tabb *et al.*, 1992; Lefebvre *et al.*, 1995; Risek *et al.*, 1995).

In atretic follicles, connexin expression is reduced. In rats and sheep, α_1 connexin (Cx43) expression was reduced after an atresia-inducing stimulus (Wiesen and Midgley, 1994; Huet *et al.*, 1998). The cellular mechanism of follicular atresia is apoptosis (Tilly, 1996). Gap junctions may transmit survival signals between cells and, thus, reduced intercellular communication may be part of an apoptotic cascade, with cessation of survival factor signalling resulting in global cell death in the follicle. Reduced cell contact is a feature of apoptosis (Wyllie, 1997), so reduced connexin expression may simply reflect cell death. An alternative hypothesis may be that gap junctional communication within the dying follicle may be involved in the co-ordination of atresia, which is an ‘all or none’ event. Expression of β_4 connexin (Cx31.1) has been shown, in the present study, to be restricted to atretic follicles. The gap junctions may transmit apoptosis-regulating signals, co-ordinating apoptosis in all cells of an atretic follicle. Indeed, gap junctions have been shown to pass apoptotic signals between neurones and astrocytes, co-ordinating cell death (Lin *et al.*, 1998).

Compartmentalization of connexin expression appears to occur in the mouse follicle, such that gap junction communication occurs between oocyte and granulosa cells, between granulosa cells and between theca cells but not between granulosa and theca cells. The granulosa and theca are physically separated by a basal lamina, through which growth factors can pass, but gap junctional communication cannot take place (Rodgers *et al.*, 1995). There may be several reasons for this compartmentalization. The permeability and conductance of gap junctions may be influenced by cellular microenvironment, which may

differ in cellular compartments (Bennett *et al.*, 1988). Similar kinases may have different actions on gap junctions formed by different connexin types (Bennett *et al.*, 1991), which may also allow discrimination of second messengers (Kumar and Gilula, 1996). Compartmentalization of gap junctions may be important in the co-ordination of hormone production in cellular compartments, for example, oestrogen from the granulosa and androgens from the theca (Grazul-Bilska *et al.*, 1997).

Differential expression of connexins in follicular compartments may be a mechanism by which global events have cell-specific effects. Oocyte maturation is an example of this, since at the LH surge, the whole ovary is exposed to the marked increase in LH. However, only preovulatory follicles respond to this event by ovulation, and smaller follicles do not, as only preovulatory follicles have sufficient LH receptors to transmit the signal (Hillier, 1994). Although LH-induced cAMP signalling is thought to interrupt granulosa–oocyte gap junction communication (Dekel, 1988; Sherizly *et al.*, 1988; Granot and Dekel, 1994), gap junctions containing β subfamily connexins in the theca may continue to communicate, preserving follicular function. The ovulatory follicle must remain functional through ovulation, as it develops into a corpus luteum to support the potential pregnancy.

In conclusion, this study, carried out with highly specific connexin antibodies, confirms and greatly extends previous observations on the location and changing distribution of gap junctions in mouse ovarian follicles. Thecal and oocyte–granulosa connexins are members of the β connexin subfamily, and granulosa connexins are members of the α subfamily. Atretic follicles exhibit generally reduced gap junction expression, but specific upregulation of β_4 connexin (Cx31.1). The localization of connexins α_6 (Cx45), β_1 (Cx32), β_2 (Cx26) and β_4 in the intact mouse follicle is novel. The distribution and structure of follicular gap junctions reveals potential pathways for cell–cell communication between cell populations in the developing follicle. However, dye transfer experiments will be necessary to discover whether pathways revealed by connexin labelling are mirrored by functional cell–cell communication via gap junctions.

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