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PACAP function during rat folliculogenesis

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INTRODUCTION

The ovaries are amongst the most dynamic and plastic tissues in the body, with the monthly cycle of follicular maturation, ovulation and (in absence of pregnancy) reabsorption of the corpus luteum occurring 300–400 times throughout the average woman's reproductive lifespan. The endocrine control of this process by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) rests on a network of extracellular and intracellular molecular interactions, the complexity of which reflects the unique histogenetic origins and specialized functions of the cell types that constitute the ovaries.

Origin of the Germ Line

The primordial germ cells (PGCs) are derived from the epiblast (Falconer & Avery 1978; Lawson & Hage 1994). There is no evidence for a segregated germ line in the inner cell mass of blastocysts up to 4.5 dpc because single epiblast cells at this stage can give rise to both somatic tissue and gamete. PGCs are first detected during gastrulation at 7.0 dpc as distinct cell population located in the extraembrionic mesoderm of the posterior amniotic fold (Ginsburg *et al.* 1990). Subsequently, they came to underlie the posterior part of streak and became incorporated in the base of allantois, forming a coherent cluster of about 75 cells at the presomite stage. The PGCs leave the gut endoderm and traverse the dorsal mesentery toward the coelom, the first cell reaching the genital ridges by 10.5-11.5 dpc. By 13 dpc, about 25000 PGCs have colonized each gonadal primordium. The genital ridges arise from intermediate mesoderm adjacent to mesonephros and are first visible as a dinstinct urogenital ridge at 10.0 dpc. However, the first sign of overt sexual dimorphism in the gonad primordia are not apparent until 12.5 dpc. Their movements from gut to the genital ridges involves active migratory movements, probably dependent on a suitable substratum and possibly involving chemotactic signals to ensure that the majority of cells converge to the gonadal ridges. Female germ cells that migrate into prospective ovaries cease proliferation and enter meiosis, becaming arrested at prophase of the first meiotic division. In contrast, male germ cells that enter the prospective testes continue to proliferate until meiotic arrest occurs at about 14.0 dpc.

The local gonad environment has a profound influence on germ cell differentiation, because, in general the genetic sex of the gonad determines the type of gamete produced (Swain & Lovell-Badge 1999).

Spermatogenesis

Spermatogenesis is one of the most exquisite examples of continuously synchronized and spatially organized sequence of differentiation. Since the time taken by spermatozoa to differentiate from stem cell is more or less constant (about 5 weeks in a mouse), any transverse section of a seminiferus tubule will contain a standard array of cells from the basement lamina to the luminal surface. This image reflects the succeeding waves of spermatogenesis passing along the tubule. Spermatogenesis relies on a population of true stem cells, which are capable of self-renewal as well as producing progeny for differentiation into spermatozoa (Brinster 2002).

Oogenesis and Ovulation

In rodents, by 5 days after birth, all the oocytes are in the diplotene stage of the prophase of the first meiotic division. They are therefore diploid but contain four times the haploid amounts of DNA. During the prolonged resting of dictyate stage, the paired homologues chromosome are fully extended, and transcription of oocyte (maternal) mRNA takes place. In PGCs and 11.5 dpc oogonia, only one X chromosome is active, but by 12.5 dpc, both chromosomes became active (McLaren & Burgoyne 1983).

Each oocyte is contained within a follicle composed of multiple layers of granulosa cells, which are of the same embryonic origin of Sertoli cells of the testis and have various roles in oocyte growth and differentiation (Matzuk *et al.* 2002; Richards *et al.* 1987). The follicle cells immediately surrounding the oocyte have numerous projections that form specialized junctions with the oocyte. These junction complexes involve gap junctions and allow metabolite transfer. They are maintained even when the follicle cells and the oocyte are gradually separated by the deposition of the zona pellucida, a layer of extracellular material synthesized and deposited by the growing oocyte (Bleil & Wassarman 1980a; Bleil & Wassarman 1980c; Canipari *et al.* 1988; Greve & Wassarman 1985).

As the oocyte increase in size, it gradually acquires the competence to enter the final stages of meiosis in response to either the hormonal stimulus (*in vivo*) or release from the follicle (*in vitro*). Ovulation requires the coordinate response of both the follicle cells and the oocyte. In any natural cycle only a few follicles can respond to the increasing levels of follicle stimulating hormone (FSH), product by the pituitary. The follicle accumulate fluid, swells and move towards the periphery of the ovary, ready for the final steps of maturation and release of oocyte. The mature fluid-filled follicle units are known as antral or graafian follicles, since the scientist Regnier Graaf described them in 1672 (Hsueh *et al.* 1984a; Richards *et al.* 1987).

Ovulation occurs in response to a surge in the level of leutenizing hormone (LH), also produced by the pituitary. After LH stimulation, the oocyte undergoes nuclear maturation. The nucleus (also known as the germinal vesicle, GV) loses its membrane (a process known as germinal vesicle breakdown, GVBD), and the chromosomes assemble on the spindle and move toward the periphery of the cell, where the first meiotic division takes place. One set of homologous chromosome, surrounded by a small amount of cytoplasm, is extruded as the first polar body, whereas the other set remains in metaphase II. It is in this state of arrest, after the first meiotic division, that the oocyte is finally released from the follicle, and meiosis does not resume until after fertilization.

Each ovulated oocyte is surrounded by its zona and a mass of follicle cells (cumulus cells) with their associated protoglycans. The oocytes are swept into the open end, or infundibulum, of the oviduct by the action of numerous cilia, on the surface of the oviduct epithelium. Other cells in the epithelium are secretory, and, at the time of ovulation, the section of the tube adjacent to the infundibulum becomes engorged and enlarged to form an ampulla where the fertilization takes place. After ovulation, the follicle cells remaining in the ovary will differentiate in steroid-secreting cells (luteinized granulosa cells), which help to maintain pregnancy.

Ovarian structure and folliculogenesis

In Mammals the ovaries are the female gonads responsible for the differentiation and the release of mature; ovaries have also endocrine function, in fact they produce steroidal hormones that allow the development of secondary sexual characteristics in women and the maintenance of pregnancy. The ovaries are equal organs covered by a single layer of cuboidal cells that is called ovarian epithelium, just below there is a compact layer of epithelium. The ovary is made up of a

cortical peripheral area, and of an internal medullar part where are located nerves, lymphatic and blood vessels. In the connective cortical area are immersed follicles in various stages of development: primordial, primary, secondary, mature and atretic. Most ovarian follicles (about 90%) is composed of primordial follicles (unilaminar follicles), the remaining is represented by growing and antral follicles (Fig. 1).

During the period that extends from fetal life to the end of reproductive age, the mammalian ovary is in an excellent dynamic balance. This balance enables to maintain in continuous development small follicles from the pool of primordial follicles and, simultaneously, the selection of some of them to complete their development. The dynamics of these events is governed by many factors which make sure that less than 1% of follicles reach ovulation, while the majority become atresic, probably through a mechanism of apoptosis (Tilly *et al.* 1992b).

Folliculogenesis is a process with a precise timeline of morphological and functional changes depending from germinal and somatic cells.

The female germ cells, at birth, are surrounded by a single layer of small, flattened and non proliferating follicular cells (primordial follicle, stage I of folliculogenesis according to Pedersen and Peters, 1968 (Fig. 2). At this stage the oocyte is a cell with a large vesicular nucleus at the stage of diplotene.

The age at which the first primordial follicles leave their quiescent state and begin growth is closely dependent from species. In most mammals, shortly after birth begin to appear the first growing follicles.

In the early stages of development, the follicular cells acquire a different shape, from flattened they became cuboidal and immediately enters a period of intense proliferative activity (primary follicle, stage II and III) (Fig. 3B), in consequence of the numeric increase of somatic cells, the follicles become multi-stratified (secondary or preantral follicle, stage IV - V) (Fig. 3C), while from the stroma new cells that surround the follicle start to differentiate (Theca cells).

At the same time the oocyte increases and deposits a translucent membrane composed of carbohydrates and proteins called zona pellucida (Bleil & Wassarman 1980b).

This first phase of follicular growth appears to be independent from gonadotropins. In fact, preantral follicles grow in hypophysectomized animals, in gonadotrophin-deficient mutants (Halpin *et al.* 1986) or when the circulating gonadotrophins are immunologically neutralized (Eskol A. *et al.* 1970). In the woman, similarly, the process of formation of primary follicles also occurs in cases of congenital deficiency of gonadotropins (GAUTHIER G. 1960), as well as in prepubertal age,

during pregnancy and during the use of steroidal contraceptives (Peters *et al.* 1976). However, in mice it has been shown that the gonadotrophin induction of DNA synthesis indicates competence of follicles to respond to gonadotrophins themselves from the earliest stages of development (Wang *et al.* 1991). This may lead to believe that an optimal development of preantral follicles might require the presence of these hormones (Cortvrindt *et al.* 1997; Fortune & Eppig 1979).

The second interval of growth (from preantral to antral preovulatory follicle) is strictly dependent by the presence of the follicle stimulating hormone (FSH), which induces an intense proliferation of follicular cells, the appearance of the antrum, the increase in the number of its receptors and the appearance on the granulosa cells of receptors for luteinizing hormone (LH) (Eppig 1980).

The mature ovarian follicle (tertiary or early antral follicle,stages VI to VII) (Fig. 3D) is ball-shaped, almost completely embedded within the matrix of the ovarian connective tissue, but visible on the ovarian surface like a translucent vesicle. When the antrum begins to form, the follicle continues to increase, while the oocyte has reached its final size and assumes an eccentric position. The walls of the preantral follicle are composed of three layers:

1. *The external Theca*: made up of 7-11 concentric bands of fibroblasts, connected by collagen fibrils immersed in an array of mucopolysaccharides substances. This composition gives to the wall of the follicle a remarkable elasticity (Fig. 4).

2. *The inner Theca*: containing cells with an abundant smooth endoplasmic reticulum. In the mature follicle, this layer is the main site for steroid secretion (Schaar 1976). An important feature of this cell population is the extensive development of capillaries and lymphatic vessels, distributed on the inside (Fig.4).

3. *The granulosa cells*: form the multi-stratified epithelial wall that demarcates the cavity of the antral follicle and are separate from the theca cells from a basal membrane(Fig. 4).

While in preantral follicle granulosa cells are a relatively homogeneous population, the progress of follicular development and the antrum formation, determined by FSH and assisted by other factors including regulatory factors produced by the oocyte (Eppig & Wigglesworth 2000) (Salustri 2000), divides the population into two subpopulation: the cumulus cells and the mural cells. These cells show a marked biochemical and morphological heterogeneity, suggesting a gradient of differentiation within the follicle (Amsterdam *et al.* 1992). The cells that are in close

contact with the oocyte, also known as corona radiata, communicate with the oocyte itself through cytoplasmic extensions crossing the zona pellucida. The cells that constitute the corona radiata connect the oocyte with the other cumulus cells through gap junctions, thus forming an electrophysiological sincitium (de Loos *et al.* 1991).

The mural granulosa cells, which remain adherent to the wall of the follicle, increase considerably the expression of receptors for LH and for hormones and steroids and express other factors as cKit ligand (Manova *et al.* 1993). While cumulus cells express low levels of LH-R (Channing *et al.* 1981; Ishimura *et al.* 1989) and show higher mRNA levels of some growth factors such as insulin growth factor-like (IGF) (Oliver *et al.* 1989), the vascular endothelial growth factor (vEGF) (Dissen *et al.* 1994) and the βA-inhibin (Braw-Tal *et al.* 1994).

Following the endogenous stimulus of FSH and LH, or the administration of ovulatory doses of hCG, the mural granulosa cells through their production of proteolytic enzymes, promote selective degradation of the perifollicular matrix determining the rupture of the follicle wall. The cumulus cells, instead, synthesize a mucoelastic matrix that promotes the separation of cumulus-oocyte complex by the follicular wall. At the same time, in mice, the cumulus cells decrease the production of a Serin-protease (urochinasic plasminogen activator - UPA) involved in tissue remodeling and in the degradation of the mucoelastic matrix (Canipari *et al.* 1995).

The two follicular sub-populations have a different role in the oocyte development. While the granulosa cells have a steroidogenic function, the cumulus cells create favourable environment to the oocyte development.

Despite the structural and morphological differentiation, all follicular cells function as a coordinated unit, thanks to the presence of gap junctions that permit the passage of small metabolites and cellular mediators, such as cAMP (the second messenger of gonadotropins).

Inside the follicle we find the oocyte that before the peak of LH is still blocked at the profase I of the first meiotic division (dictiato). In antral or preovulatory follicles (stage VIII) the antral cavity expands gradually and is filled by follicular fluid. It was found that in rat the follicular fluid prevented the resumption of meiosis in oocytes and this capacity is attributed to the presence of anti-Müllerian Hormone (AMH), produced by the granulosa cells (Takahashi et al., 1986). The AMH inhibitory effect is dose dependent, reversible and independent of cAMP.

After the LH peak, the oocyte resumes maturation, complete the first meiotic division by ejecting the first polar body and stops at the stage of metaphase II.

Finally, as the last stage of the differentiation process (Fig. 5), the mural cells form the corpus luteum, producing progesterone, necessary for the correct embryo implantation and for the maintenance of pregnancy.

Summarising, the major function of the female gonad is the differentiation and release of the mature oocyte for fertilization and successful propagation of the species. Additionally, the ovary produces steroids that allow the development of female secondary sexual characteristics and support pregnancy. In mammalian ovaries the individual follicle consists of an innermost oocyte, surrounding granulosa cells, and outer layers of theca cells. The fate of each follicle is controlled by endocrine as well as paracrine factors (Gougeon 1996c; Hirshfield 1991; Roy & Greenwald 1989). The follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, whereas only a few of them, under the cyclic gonadotropin stimulation that occurs after puberty, reach the preovulatory stage (Gougeon 1996b; Hirshfield 1991). These preovulatory follicles are the major source of the cyclic secretion of ovarian estrogens in women in the reproductive age. In response to preovulatory gonadotrophin surges during each reproductive cycle, the dominant preovulatory follicle ovulates and releases the mature oocyte for fertilization, whereas the remaining theca and granulosa cells undergo transformation to become the corpus luteum (Fig 5.) (Fauser & Van Heusden 1997b). The pool of oocytes in the mammalian ovary becomes fixed early in life; thus, ovarian senescence is linked to the dwindling supply and eventual exhaustion of the pool of primordial follicles (Fig. 6). This basic biological doctrine cited in the 1921 by Pearl and Schoppe was solidified as a dogma in 1951 by Zuckerman (S.H.Green 1951).

For more than half a century, biologists have claimed the theory that in most mammalian species, oocytes are formed before or shortly after birth, but never in adulthood. However, exceptions to this have previously been found in mammals; in some species of prosimian primates (Loris tardigradus lydekkerianus and Nycticebus coucang), the most ancient of primate families, mitotically active germ cells have been found in adult ovaries (David GF 1974). These studies found that germ cells clustered in nests within the ovarian cortex incorporated tritiated thymidine. The question of whether any of these proliferating germ cells pass through follicular growth and ovulation remains unanswered and the original studies provided no evidence to suggest that they did.

Two recent papers published by the group headed by Jonathan Tilly opened a new discussion : the first paper asserts that mouse germline stem cells (GSCs) replace ovarian follicles that have

been rapidly lost through follicle death (Johnson *et al.* 2004a) and the second paper, recently published in Cell, proposes continuous immigration into mouse ovaries of GSCs derived from bone marrow (Johnson *et al.* 2005). The mouse study (Johnson *et al.* 2004b) may suggest that the prosimian primates are not the only mammals to exhibit this phenomenon but the presence of such cells in other mammalian species remains to be proven. As others authors suggest if the dogma is to be debunked and a new one accepted, at least in the mouse, it is necessary to be challenged and tested.

In the second Johnson paper the authors shown that transplantation of bone marrow from normal donor mice appears to regenerate follicles in the ovaries of young mice sterilized by chemotherapy and in those of the congenitally sterile Atm mutant mouse. With regard to this paper many authors (among which Telfer, Gosden, Albertini, Tzafriri) emphasized the importance of verifying the fertility and the origin of this "new" germ cells: the results published to date do not even show that these new "oocytes" have entered meiosis, far less that they can complete it and undergo fertilization.

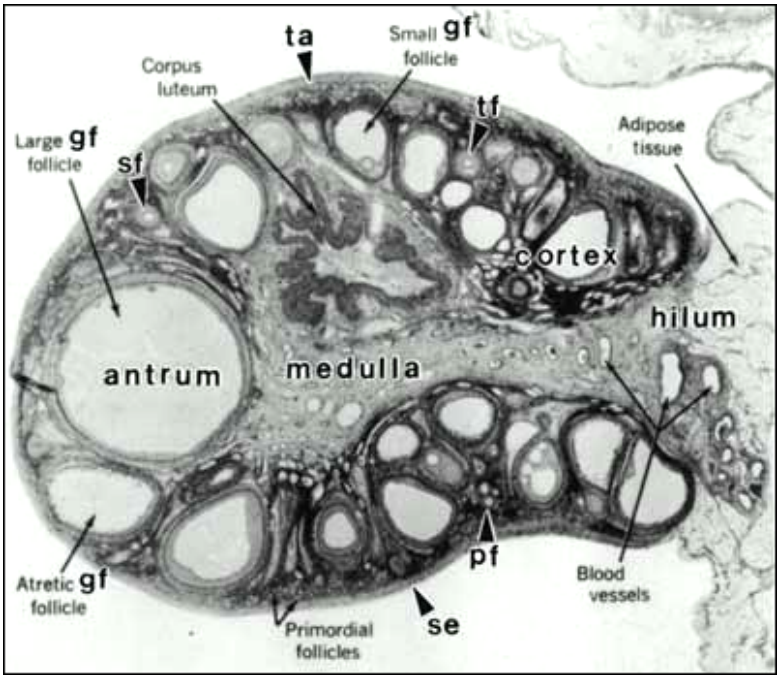


Fig.1:

Adult ovary can be subdivided into three regions: the cortex, medulla, and hilum regions. The cortex consists of the surface epithelium (**se**), tunica albuginea (**ta**), ovarian follicles (primordial, primary (**pf**), secondary (**sf**), small, medium, large Graafian follicle (**gf**)) and corpora lutea (**cl**). The medulla consists of large blood vessels and nerves. The hilum contains large spiral arteries.

Micrograph of Macacus Rhesus ovarian section (Bloom W, Fawcett DW: A Textbook of Histology. Philadelphia, WB Saunders Company, 1975).

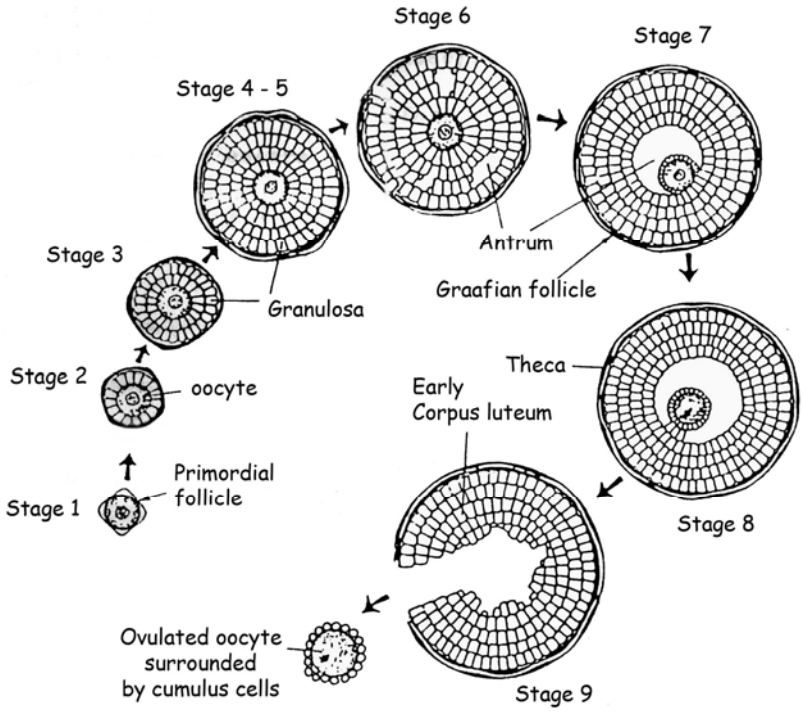


Fig. 2:

Schematic representation of the follicle development in mammals according to Pedersen and Peters (1968).

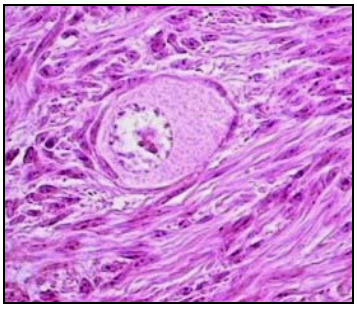


Fig. 3A Primordial follicle

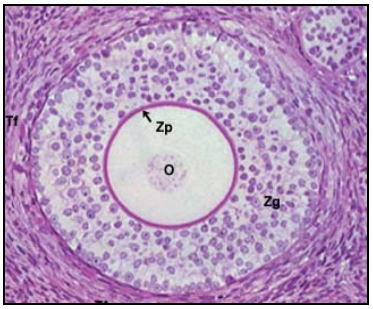


Fig. 3C Secondary follicle

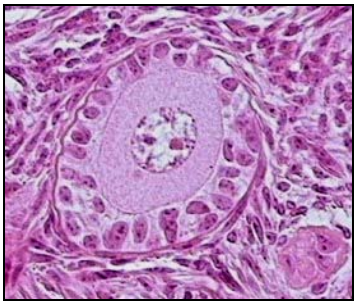


Fig. 3B Primary follicle

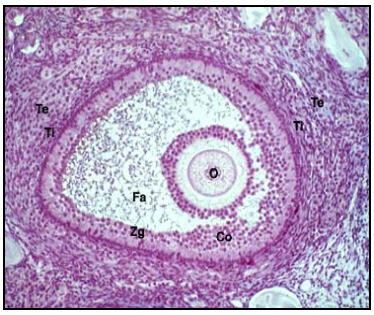


Fig. 3D Antral follicle

Fig. 3:

Fig. 3A – Primordial or unilaminar follicle: The oocyte is surrounded by a single layer of flattened and small follicular cells.

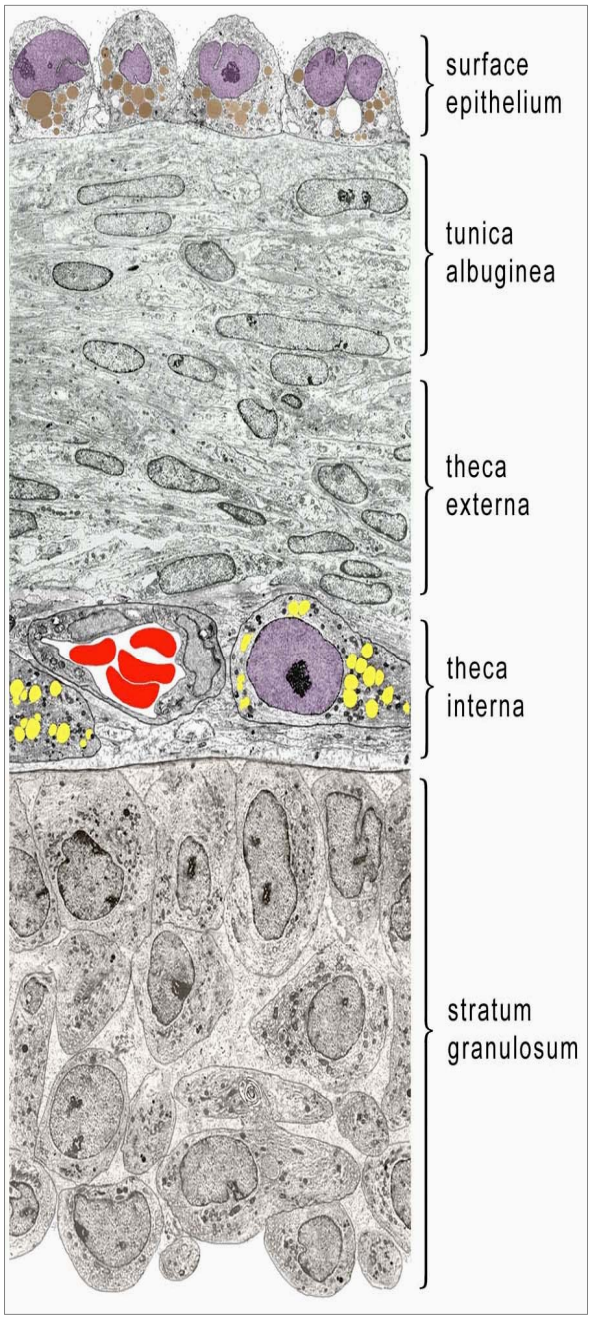
Fig. 3B – Primary follicle: follicular cells become cuboidal and are arranged on a single layer.

Fig. 3C – Secondary follicle: two or more layer of follicular cells surround the oocyte.

Fig. 3D – Antral or preovulatory follicle: The antrum is almost completely formed. The follicular cells are differentiated in the inner and outer Theca cells (Ti and Te), mural granulosa cells and cells of cumulus oophorus.

Co Cumulus oophorus; Fa Antral fluid; O oocyte; Te Outer theca cells; Ti Inner theca cells; Tf Follicular theca cells; ZG Granulose cells; ZP Zona pellucida.

Fig. 4:



Surface Epithelium: In mature ovarian follicles, the surface epithelium is a single layer of cuboidal cells that are loosely attached to a thin basal lamina at the surface of the connective tissue (the tunica albuginea) surrounding the ovary. The most conspicuous feature of these cells is the dense cytoplasmic spheres that are common on the basal side of the cells. The composition of these dense granules is unknown, but it is unlikely that they are involved in the ovulatory process, because the surface epithelium can be gently scraped from the surface of a mature follicle, yet the follicle will still ovulate in response to adequate stimulation by gonadotropin(s). The other interesting feature of the surface epithelium is that the cells contain polymorphous nuclei that somewhat resemble the nuclei of polymorphonuclear leukocytes. It is possible that the surface epithelium functions as a first line of defense to protect the vital procreative elements of the ovary.

Tunica Albuginea: Beneath the ovarian surface epithelium is the layer of dense collagenous connective tissue known as the tunica albuginea. This layer, which surrounds the entire ovary, consists almost entirely of fibroblasts, along with extracellular collagen and related ground substance. The fibroblasts in this thecal tissue give the appearance of spindle-shaped smooth muscle cells if the follicle is sectioned on a plane perpendicular to the apical follicle wall. However, if one cuts thin sections of a follicle on a plane that is tangential to the surface of the ovary, then the cells in this layer appear round, or ovoid, and it is quite obvious that they are platter-shaped fibroblasts that produce substantial amounts of collagen.

Theca Externa: The follicle itself is surrounded by its own layer of collagenous connective tissue called the theca externa. This tissue is quite similar to the tunica albuginea, and these two layers of thecal tissue are so contiguous at the apex of a follicle that it is difficult to distinguish them from one another. The theca externa usually contains a few more fibroblasts than the tunica albuginea, but the outer tunic of connective tissue contains more collagenous extracellular material. There is not a conspicuous difference in the cellular composition of the theca externa at the apex of a follicle (where rupture will occur) versus the base of the follicle (which is surrounded by ovarian stromal tissue).

Theca Interna: This highly differentiated thecal tissue is a thin layer of steroidogenically active cells that are supplied by a number of large capillaries which collectively comprise the bulk of the ovarian circulation. Fibroblasts and collagen are sparse in this thin layer. The secretory cells of the inner theca are characterized by large oval nuclei with prominent nucleoli; and, like most steroid-secreting cells, their cytoplasm is dominated by lipid droplets, numerous mitochondria, and Golgi networks that are distributed throughout their smooth endoplasmic reticulum. These cells are sometimes referred to as "interstitial cells" in the current literature. The interior border of the theca interna is clearly delineated by a thin, but conspicuous, basal lamina called the membrana propria. This basal lamina has been erroneously referred to as a double membrane because of its close association to the plasma membranes of the granulosa cells that adhere to its inner border.

Stratum Granulosum: The granulosa layer at the inner most surface of the follicle wall arises from a single layer of epithelial cells which surround the oocytes of primordial follicles. The granulosa cells that are attached to the membrana propria extend in a columnar pattern from this basement membrane. The remaining cells toward the follicular antrum are more cuboidal and are distributed inward for a total depth of 3-10 cells, depending on the species of animal. The cells of the granulosa layer are metabolically integrated by an extensive labyrinth of gap junctions that couple this layer into a syncytium with the cumulus oophorus. In the vicinity of the tight junctions between granulosa cells it is common to observe invaginations from one cell to the other that become pinched-off and form phagocytic-like vesicles within one or the other of the abutting cells. These vesicles constitute the transfer of cytoplasm from one granulosa cell to another, and their contents can include mitochondria, lipid droplets, or other large areas of cytoplasm. The cumulus mass, which includes the oocyte, consists of granulosa-like cells that protrude inward from any portion of the stratum granulosum, i.e., from either the apical or the basal region of this innermost layer of the follicle. This random morphological arrangement positions the oocyte toward the center of the follicular antrum and probably facilitates its dislodgement and expulsion from the follicle at the time of ovulation. Also, this central location of the fragile germ cell may serve to protect it from the degradative events that occur within the thecal layers of the follicle wall during the ovulatory process.

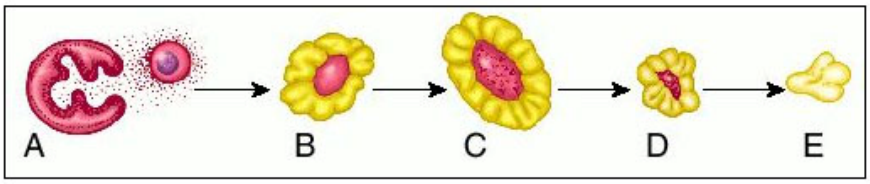


Fig. 5:

Luteinization, beginning after rupture of the ovarian follicle in ovulation (**A**) and progressing trough vascularization and hypertrophy of the maturing corpus luteum (**B**, **C**); it is followed by regression (**D**) to the corpus albicans (**E**).

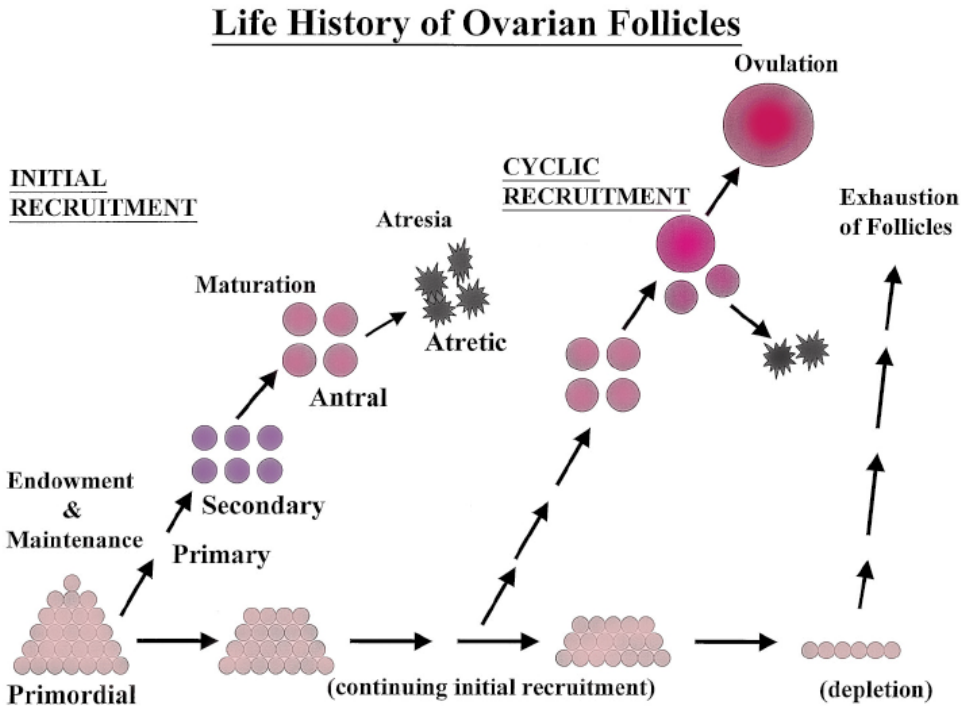


Fig. 6:

Life history of ovarian follicles: a fixed number of primordial follicles are endowed during early life, and most of them are maintained in a resting state. Growth of some of these dormant follicles is initiated before and throughout reproductive life (**Initial recruitment**). Follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage most follicles undergo atresia; however, under optimal gonadotropin stimulation that occurs after puberty, a few of them are rescued (**Cyclic recruitment**) to reach the preovulatory stage. Eventually, depletion of the pool of resting follicles leads to ovarian follicle exhaustion and senescence.

Follicle growth: Initial vs. cyclic recruitment

Folluculogenesis is the result of a sequence of processes, that is:

— The *Recruitment*

— The *Selection*

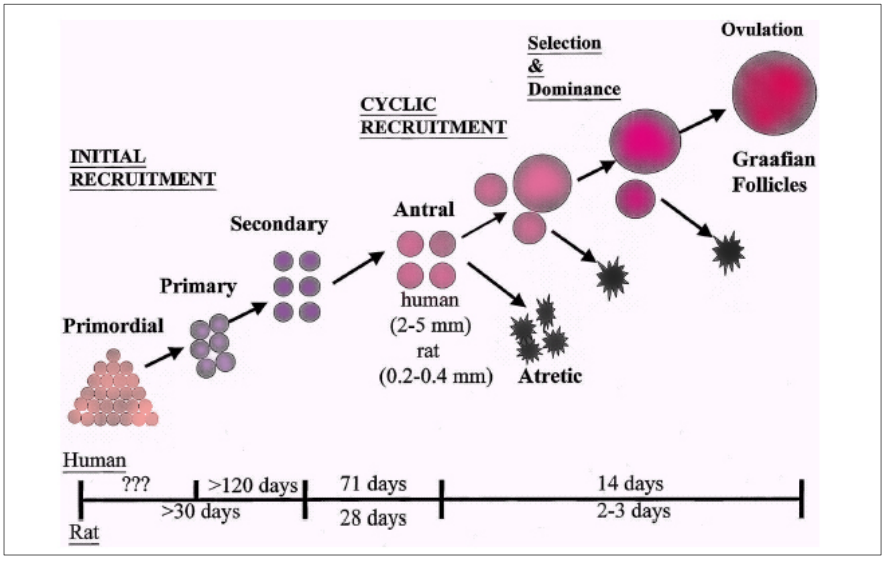
— The *Dominance*

The term recruitment has been used frequently by different investigators to describe two important but distinct decision points during follicle development (Gosden *et al.* 1983; Gougeon & Testart 1990a; Meijs-Roelofs *et al.* 1990). The dormant primordial follicles are *recruited* into the growing follicle pool in a continuous manner, whereas increases in circulating FSH during each reproductive cycle *recruit* a cohort of antral follicles. To avoid confusion, it was proposed designating these branching points as *initial* recruitment and *cyclic* recruitment and have summarized major differences between the two processes in Table 1. During initial recruitment, intraovarian and/or other unknown factors stimulate some primordial follicles to initiate growth, whereas the rest of the follicles remain quiescent for months or years. Alternately, initial recruitment may be due to a release from inhibitory stimuli that maintain the resting follicles in stasis. Initial recruitment is believed to be a continuous process that starts just after follicle formation, long before pubertal onset. After initial recruitment, oocyte growth is a prominent feature of the growing follicles, but these oocytes remain arrested in the prophase of meiosis. For those follicles not recruited, the default pathway is to remain dormant. In contrast, cyclic recruitment starts after pubertal onset and is the result of the increase in circulating FSH during each reproductive cycle that rescues a cohort of antral follicles from atresia. In rodents, the recruitable early antral follicles are 0.2– 0.4mm in diameter, whereas human follicles at the comparable stage are larger (2–5 mm in diameter) and have already acquired antrum. During cyclic recruitment, only a limited number of follicles survive, and the default pathway is to undergo atresia. Oocytes in these follicles have already completed their growth, acquired a zona pellucida, and are competent to resume meiosis (Trounson *et al.* 1998).

	Initial recruitment (initiation of growth)	***Cyclic recruitment*** (escape from atresia)
Stages	Primordial	Antral (human: 2–5 mm in diameter; rodents: 0.2–0.4 mm in diameter)
Hormones involved	Not determined	FSH
Default pathway	Remain dormant	Apoptosis
Timing	Continuous throughout life, begins after follicle formation	Cyclic (human: 28 days, rodents: 4–5 days), starts after puberty onset
Oocyte status	Starting to grow, not capable of undergoing germinalvesicle breakdown	Completed growth, competent to undergo germinal vesicle breakdown

Table 1.
Main differences between initial and cyclic recruitment of ovarian follicles (adapted from McGee 2000).

Fig. 7:



Duration of follicle recruitment and selection in human and rat ovaries. Primordial follicles undergo initial recruitment to enter the growing pool of primary follicles. Due to its protracted nature, the duration required for this step is unknown.

In the human ovary, greater than 120 days are required for the primary follicles to reach the secondary follicle stage, whereas 71 days are needed to grow from the secondary to the early antral stage. During cyclic recruitment, increases in circulating FSH allow a cohort of antral follicles (2–5mmin diameter) to escape apoptotic demise. Among this cohort, a leading follicle emerges as dominant by secreting high levels of estrogens and inhibins to suppress pituitary FSH release. The result is a negative selection of the remaining cohort, leading to its ultimate demise. Concomitantly, increases in local growth factors and vasculature allow a positive selection of the dominant follicle, thus ensuring its final growth and eventual ovulation. After cyclic recruitment, it takes only 2 weeks for an antral follicle to become a dominant Graafian follicle.

In the rat, the duration of follicle development is much shorter than that needed for human follicles. The time required between the initial recruitment of a primordial follicle and its growth to the secondary stage is more than 30 days, whereas the time for a secondary follicle to reach the early antral stage is about 28 days. Once reaching the early antral stage (0.2– 0.4 in diameter), the follicles are subjected to cyclic recruitment, and only 2–3 days are needed for them to grow into preovulatory follicles.

Negative and positive selection leading to dominance

Similar to the confusion associated with two distinct stages of follicle recruitment, the FSH-initiated cyclic recruitment step is sometimes described interchangeably with the process of follicle selection (Gougeon & Testart 1990b; Rombauts *et al.* 1998; Scheele & Schoemaker 1996). Cyclic recruitment and selection of follicles represent a continuous process, eventually leading to the emergence of the preovulatory follicle(s). Cyclic recruitment and final follicle selection are most clearly illustrated during the human menstrual cycle (Fig. 7). After increases in circulating FSH during the perimenstrual period, a cohort of antral follicles escapes apoptosis due to the survival action of FSH. Among this group of antral follicles, one of the leading follicles grows faster than the rest of the cohort and produces higher levels of estrogens and inhibins. Although the exact reasons why one follicle emerges as dominant are unclear, this follicle is likely to be more sensitive to FSH (Fauser & Van Heusden 1997c), perhaps because of enhanced FSH and/or LH receptor expression or increases in local growth factors that augment FSH responsiveness as suggested by bovine studies (Bao *et al.* 1997; Evans & Fortune 1997; Xu *et al.* 1995). Estrogens and inhibins produced by the largest follicle suppress pituitary FSH release during the midfollicular phase. As a result, the remaining growing antral follicles are deprived of adequate FSH stimulation required for survival. In monkeys, it has been elegantly demonstrated that immunoneutralization of the actions of circulating estrogens during the midfollicular phase leads to sustained elevation of circulating FSH, thus allowing the development of multiple preovulatory follicles (Yonkeura *et al.* 1987). Furthermore, administration of exogenous estrogens suppresses follicle development in women (Tsai & Yen 1971), whereas treatment with high levels of exogenous gonadotropins during ovulation induction in women is widely known to stimulate the growth of multiple preovulatory follicles (Fauser & Van Heusden 1997a). Negative selection against subordinate follicles is therefore a result of estrogen and inhibin produced by the dominant follicle exerting negative feedback upon gonadotrophin release. Additionally, this rapidly growing follicle also produces higher levels of autocrine and paracrine growth factors that stimulate increases in vasculature and FSH responsiveness, thus constituting a local positive selection mechanism. Multiple studies have demonstrated the importance of insulin-like growth factors (IGFs) and other local factors in the amplification of FSH action (Giudice 1992). Although remaining to be characterized, atretogenic factors produced by the dominant follicle have been postulated to account for the lack of development of subordinate follicles after exogenous gonadotropin administration (Gougeon &

Testart 1990c). Furthermore, the increased responsiveness of dominant follicle to FSH stimulates the expression of both FSH and LH receptors in the granulosa cells of this follicle (Harlow *et al.* 1988), thus providing a fail-safe mechanism to ensure the eventual ovulation of the selected follicle. Computer modeling of ultrasound images in patients also suggested a suppressive effect of the dominant follicle on its neighbouring subordinate ones (Gore *et al.* 1997). Cyclic recruitment of early antral follicles and selection of dominant follicles in rodents is similar to that of the primates with the major exception that multiple follicles become dominant during each estrous cycle. Monoovulatory and polyovulatory species likely differ in the threshold (or set point) for negative feedback signals, presumably a genetically determined trait (McNatty *et al.* 1986; Spearow 1986). An important role for AMH in cyclic recruitment is supported by the differential pattern of expression of AMH observed at oestrus in non-atretic large preantral and small antral follicles (Baarends *et al.* 1995). Low AMH expression would correlate with increased sensitivity to FSH, allowing these follicles to be selected for continued growth and ovulation in the next oestrous cycle. Indeed, in a preliminary study, using 5-bromodeoxyuridine (BrdU) as a cell proliferation marker, in rats, more proliferating granulosa cells were found in follicles that expressed less AMH, and vice versa (Durlinger *et al.* 2002c). The law of follicular constancy proposed by Lipschutz (Lipschutz A. 1928) emphasizes that the ovulatory number remains constant in a given species even when a single ovary or a large portion of the remaining ovary is removed. Thus, findings of compensatory ovulation (Baker *et al.* 1980) underline the importance of the putative central set point within a given species.

Early follicle development in humans and in rodents

In humans, primordial germ cells arrive in the gonadal ridge from the yolk sac endoderm by the seventh week of gestation to become oogonia, which proliferate by mitosis before differentiating into primary oocytes. Some oogonia begin transformation into primary oocytes and enter the first stages of meiosis at around 11–12 weeks of gestation. The total germ cell number peaks at 20 weeks. After this time, the rate of oogonial division declines. Primordial follicle formation begins around midgestation when a single layer of pregranulosa cells surround each oocyte and continues until just after birth. After oocytes are within the primordial follicles, they remain arrested in the dictyate stage of meiosis I. From a peak of 6 to 7 million at 20 weeks of gestation, the oocyte number falls dramatically so that at birth, there are only 300,000 to 400,000

(Forabosco et al. 1991). Meanwhile, some primordial follicles leave the resting pool by initiating growth (Fig. 7). Once entering the growing pool, most growing follicles progress to the antral stage, at which point they inevitably undergo atresia. After pubertal onset, a small number of the antral follicles can be rescued by gonadotrophins to continue growth (Fig. 7), and normally one preovulatory follicle is formed each month in preparation for ovulation. At the time of puberty there are an average of 200,000 follicles remaining in the ovary. During reproductive life, continuing growth of primordial and primary follicles into secondary and larger follicles leads to a gradual decrease in the original follicle pool. In addition, the primordial follicle pool could also be decreased due to apoptosis of resting follicles. As the result of ovarian follicle exhaustion, menopause occurs at about 51 year of age, a time point that has been constant for centuries. With modern increases in longevity, a significant portion (one-third) of a woman's life is now spent after menopause.

Important landmarks of ovarian development in rats are similar to those in the human; however, the timing is greatly compressed. Primordial germ cells migrate to the gonadal ridge late in embryonic development to become oogonia. At birth, the rat ovary consists of cords and oogonia. Primordial follicles are formed by day 3 of age, and the first wave of follicles develops into antral follicles over the next 3 weeks (Gelety & Magoffin 1997; Hirshfield 1989b; Malamed *et al.* 1992; McGee *et al.* 1997; Rajah *et al.* 1992). Puberty or first estrus occurs around day 34. Noteworthy, the rate of development of the first wave of follicles in juvenile rats (Fig. 5) is more rapid than that in adult cycling animals (Hirshfield & DeSanti 1995).

Role of gonadotrohpins, intraovarian factors, and of the oocyte in primary follicle growth

Mechanisms controlling the initiation of follicle growth have been difficult to investigate because initial follicle recruitment represents a protracted process characterized by the slow growth of a substantial number of small follicles over a prolonged period of time (Hirshfield 1989a). Most investigators have monitored changes in the number of primordial and/or primary follicles that remain in the ovary at any given time. Because of the difficulties involved in distinguishing between nongrowing and growing follicles, primordial and primary follicles have often been considered a contiguous group (Gougeon 1996a; Pedersen & Peters 1968; van Wezel & Rodgers 1996), although primary follicles have been shown to be growing (Hirshfield 1989c).

Resting follicles are likely to be under constant inhibitory influences of systemic and/or local origins to remain dormant (Wandji *et al.* 1996). A decrease of inhibitory influences and/or an increase of stimulatory factors allow the initiation of follicle growth. In hypophysectomized rodents, decreased initial recruitment of follicles is evidenced by a larger resting pool as compared with non operated controls (Wang & Greenwald 1993). Elevated serum FSH levels are associated with accelerated initial recruitment found in both the early and late stages of reproductive life. High tonic LH/human CG (hCG) levels may also reduce the number of resting follicles. In transgenic mice overexpressing a long-acting LH, primordial follicles are lost from the resting pool more rapidly than controls (Flaws *et al.* 1997). However, FSH and LH are unlikely to exert direct actions on primordial follicles because functional gonadotropin receptors have not yet developed in them (Dunkel *et al.* 1994; O'Shaughnessy *et al.* 1997; Oktay *et al.* 1997). Although follicles do not have functional FSH receptors until the secondary stage, pregranulosa cells and primordial follicles respond to activators of the cAMP pathways (e.g., forskolin and cAMP analogs) with increased expression of aromatase and FSH receptors (Ahmed *et al.* 1986b). It has been proposed that endogenous activators of cAMP may play a role in the differentiation of follicles after their initial recruitment (Mayerhofer *et al.* 1997c). Treatment of ovarian explants from neonatal rats with vasoactive intestinal peptide or norepinephrine increases cAMP production and accelerates early follicle development. Because the first follicles that grow in the rat ovary are in the highly innervated corticomedullary junction, the first wave of follicle growth may be facilitated by these local neurotransmitters.

The **AMH** (Anti-Müllerian Hormone), also known as MIS (Müllerian inhibiting Substance), is a peptide identified in 1947 as a factor responsible for the regression of testicular Müllerian duct (Jost 1947). This factor and its type II receptor, were subsequently identified in the postnatal ovaries.

The AMH is not expressed in females of Rodents before birth (Hirobe *et al.* 1992; Taketo *et al.* 1993), ensuring the proper differentiation of female reproductive structures. However, the AMH can be found in the ovaries of postnatal mice in the granulosa cells of early primary follicles. Experiments did by the team of Themmen shown that 2 days old mouse ovaries grown in vitro in the presence of AMH contains about 40% less growing follicles if compared to control (Durlinger *et al.* 2002a). It was also noted a significant increase in the initial recruitment of primordial follicles in AMH KO mice (Durlinger *et al.* 1999).

AMH in the ovary seems to functions as an inhibitory growth factor during the early stages of folliculogenesis. However, another work (McGee *et al.* 2001) conducted on preantral rat follicles describes a stimulating action of AMH on follicular growth induced by FSH. Overall, these data even if contrasting suggest the AMH plays an important role in the regulation of follicular development.

The role of the oocyte in the initial recruitment of follicles has been considered. Because an increase in oocyte size is not evident until formation of the primary follicle, a passive role of the oocyte in initial recruitment has been suggested. The Steel factor or **kit ligand** is expressed by granulosa cells of growing follicles whereas c-kit, a tyrosine kinase receptor of the platelet-derived growth factor receptor family, is located on oocytes and theca cells. Mutations in mice that prevent the production of the soluble form of the kit ligand lead to failure of follicular growth beyond the primary stage (Huang *et al.* 1993; Kuroda *et al.* 1988) (Table 2). Of interest, treatment of neonatal mice with a neutralizing antibody against the c-kit receptor caused apparent disturbances in initial follicle recruitment, primary follicle growth, and antrum formation in larger follicles (Yoshida *et al.* 1997). Mutations affecting the function of c-kit in humans, however, do not seem to affect female fertility (Ezoe *et al.* 1995).

Another interesting factor observed in rat and in cattle ovary (Koos & Olson 1989; Neufeld *et al.* 1987), and in particular localized in oocytes of primordial and primary follicles in various species (van Wezel *et al.* 1995; Yamamoto *et al.* 1997) is the Basic Fibroblast Growth Factor (**bFGF**). The bFGF is a factor with mitogen activity in tissues derived from mesoderm and endoderm, and also has a differentiation activity respect of different cell types, including the ovarian ones. The action of this factor in the ovary has recently been investigated: 4 day old rat ovaries cultured in vitro for 14 days in the presence of bFGF show a significant decrease in the number of primordial follicles and an increase in those that developed as primary and preantral (Nilsson *et al.* 2001). In conclusion, bFGF seems to be able to promote the development of primordial follicles and encourage them to start follicologenesis.

Further evidence of the potential role of the oocyte in early follicle development is provided by studies of growth differentiation factor-9 (**GDF-9**), a homodimeric protein of the transforming growth factor-b (TGFb)/activin family that presumably signals via serine-threonine kinase receptors. GDF-9 is produced by growing mouse, rat, and human oocytes (Aaltonen *et al.* 1999;

Bodensteiner *et al.* 1999; Dong *et al.* 1996; Elvin *et al.* 1999c; Elvin *et al.* 1999a; Hayashi *et al.* 1999; McGrath *et al.* 1995). The deletion of the GDF9 gene determines the failure of theca cells joining, an abnormal oocyte growth and the decrease in the granulosa cells proliferation (Table 2). All this leads to lock follicologenesis at the primary stage of follicle growth (Dong et al., 1996), and the total absence of ovulation. Were carried out further studies in vitro to investigate what was the role of GDF9 during the early stages of follicular development. It has been shown that GDF9, in rat granulosa cells, stimulates the proliferation and inhibits FSH induced differentiation (Vitt *et al.* 2000).

Gene	*Ovarian expression pattern*	*Phenotype of mutant mice*
GDF-9	Growing oocyte	No normal follicle growth beyond the primary stage.
Kit ligand	Granulosa cells	Soluble form necessary for follicle growth beyond primary stage.
Connexin 37	Oocyte-granulosa gap junction	Defective oocyte/granulosa interaction. Small oocytes not meiotically competent. Antral follicles formed but are small in size.
Cyclin D2	Granulosa cells	Reduced number of granulosa cells by secondary stage, small antral follicles.

Table 2.
Mutant mouse models with alterations in preantral follicle development.

Because **kit ligand** and GDF-9 are highly expressed in secondary follicles, they are likely to play important roles in preantral follicle development. Based on its sequence homology to GDF-9 and other TGF-b family proteins, a novel gene, GDF-9B (Laitinen *et al.* 1998), also named as **BMP-15** (Dube *et al.* 1998), has recently been identified. Of interest, the expression of the GDF-9B/BMP-15 transcript, as that for GDF-9, is restricted to the oocyte. It is possible that multiple paracrine factors are involved in the communication between oocyte and somatic cells during early follicle development. Although these studies provide insight into the growth process of very early follicles, the exact mechanisms propelling the primordial follicles to leave the resting pool remain elusive. Of importance, both kit ligand and GDF-9 are first found in primary follicles and their role in primordial follicle activation remains to be established. Further studies are needed to reveal

potential inhibitory factors and/or intraovarian stimulating factors that are involved in the initial stage of follicle.

Another family of growth factors, known as neurotrophin (**NTs**), contributes to the development of non-neural tissues such as pancreas, thymus, heart, adenohypophysis (Tessarollo 1998) and ovary (Dissen *et al.* 1995a; Dissen *et al.* 2001a; Ojeda *et al.* 2000). The ovary contains not only four of the five known NTs (NGF, BDNF, NT-3 and NT-4/5) (Berkemeier *et al.* 1991b; Berkemeier *et al.* 1991a; Ernfors *et al.* 1990; Hallbook *et al.* 1991) but it also expresses their respective receptors. Studies in rats have shown that genes coding for all these neurotrophins and their receptors are expressed in the ovary already before the beginning of follicologenesis (Dissen *et al.* 1995b).

The functions that the NTs have in the ovary have yet to be properly clarified, although it is known that in addition to recruit the ovarian innervation, they also play a direct role in the regulation of two very important stages: the initiation of follicular development and the ovulation. Experiments using mice lacking the NGF (nerve growth factor, the most important member of the family of neurotrophin)gene, have shown that it is required for the initial follicular development (Dissen *et al.* 2001b). Indeed it was noticed that in NGF knock out mice, sacrificed at the end of the first week of life, was evident a great delay in follicular development, characterized by a decrease in the number of primary and secondary follicles (Dissen *et al.* 2001c). The fact that these animals exhibit no significant decrease in the number of primordial follicles, suggests that NGF absence does not damage the formation of follicles, but rather disturbs their subsequent development.

Two neurotransmitters **VIP** (Vasointestinal Polipeptide) and **NE** (norepinephrine), are present in the ovary just before it acquires FSH responsiveness, and act through receptors that are located on the granulosa cells. These receptors trigger the route of of cAMP signaling, therefore is possible that their ligands are involved in the changes that affect the beginning of follicular development. It has been demonstrated in 2 day old rat ovaries grown in vitro in the presence of VIP the increase in the production of mRNA coding for cytochrome P-450 aromatase (P450arom) and for FSH receptor (Mayerhofer *et al.* 1997b). These results suggest that these neurotransmitters contribute to the process of differentiation through which the newly formed primary follicles acquire the ability to respond to FSH.

Recent establishment of a serum free culture of baboon primordial follicles capable of initiating growth in vitro (Wandji *et al.* 1997) could provide further insight into the mechanisms of initial follicle recruitment.

Preantral follicle growth and differentiation

Compared with the initial recruitment process, substantially more is known about the regulation of subsequent follicle differentiation and growth. After initial recruitment, granulosa cells in primary follicles undergo profound changes, progressively acquiring the differentiated characteristics of epithelial cells found in secondary follicles. The oocyte continues to grow, the zona pellucida is formed, theca condenses around the preantral follicle, and the vascular supply develops. In vitro studies have shown that granulosa-oocyte communication is essential for normal oocyte growth in early follicles. Immature oocytes separated from granulosa cells do not grow, but oocytes allowed to maintain gap junctions with granulosa cells grow at a near-normal rate (Tsafriri *et al.* 1987). In mice, a gap junction protein, connexin 37, is expressed at the oocyte-granulosa cell junction by the secondary stage follicles. Whereas follicles of mice that lack connexin 37 do not progress normally (Simon *et al.* 1997) (Table 2). in fact in this animals the oocytes do not reach full size and are not competent to undergo meiosis. Several studies have further demonstrated that oocytes secrete factors that regulate granulosa cell functions (Eppig *et al.* 1997), LH receptor formation (Eppig *et al.* 1998), and steroidogenesis (Nekola & Nalbandov 1971; Vanderhyden & Macdonald 1998) as well as cumulus cell expansion (Elvin *et al.* 1999b). These studies underscore the concept that granulosa - oocyte communication is important for normal preantral follicle development.

Also granulosa-theca cell interactions may have a role in the development of early follicles. Cocultures of theca and granulosa cells enhance proliferation and steroidogenesis of both cell types (Kotsuji & Tominaga 1994). The observed interactions between granulosa and theca cells are probably mediated by paracrine growth factors. Recent studies indicated that keratinocyte growth factor (KGF), or fibroblast growth factor-7, a paracrine hormone secreted by theca cells enhances the growth of preantral rat follicles in culture. In mice, cultured preantral follicles secrete activin, and treatment with recombinant activin enhances FSH-stimulated inhibin and estrogen production (Smitz *et al.* 1998). Furthermore, studies using cocultures of mouse follicles at different stages of

development suggested that activin secreted from secondary follicles causes small preantral follicles to remain dormant (Liu *et al.* 1998; Mizunuma *et al.* 1999).

A body of data exists on the effects of growth factors on monolayer cultures of granulosa and theca-interstitial cells as well as on cultures of antral and preovulatory follicles (Kol & Adashi 1995). It is clear that paracrine growth factors are also involved in preantral follicle development. With recent advances in transgenic technology, more than 30 mouse models with ovarian defects at different stages of follicle development have been described (Elvin & Matzuk 1998). Derivation of additional mutant mice with ovarian phenotypes will further enhance our understanding of early follicle development.

Cyclic Recruitment of Follicles

Before the onset of puberty, the normal fate of growing follicles is atretic demise. After puberty, cyclic stimulation by gonadotropins allows the survival and continued growth of only a limited number of antral follicles that will reach the preovulatory stage. Morphological and biochemical studies have demonstrated that the demise of both somatic and germ cells in the ovary is mediated by apoptosis (Hsueh *et al.* 1994a; Morita & Tilly 1999). Although apoptosis can occur at all stages of follicle development, in rodents, the preantral to early antral transition is most susceptible to atresia (Hirshfield 1991). FSH and LH are important trophic factors for the proliferation and survival of follicular somatic cells and for the cyclic recruitment of antral follicles. Suppression of serum gonadotropins after hypophysectomy leads to atresia and apoptosis of developing follicles (Nahum *et al.* 1996), whereas FSH treatment of cultured early antral follicles prevents the spontaneous onset of follicular apoptosis (Chun *et al.* 1996b). However, LH/hCG treatment alone is ineffective, suggesting that FSH is the predominant survival factor at this stage of follicle development (Chun *et al.* 1996a).

In rats, estrogens are potent antiapoptotic hormones in early antral follicles (Billig *et al.* 1993), although the role of estrogen in human follicles is still unclear. Follicular estrogen production is dependent upon both FSH stimulation of aromatase in the granulosa cells and LH stimulation of androstenedione production by the theca (Hsueh *et al.* 1984b). Therefore, both gonadotrophins play a role in the continued survival of growing follicles, but the cellular mechanism by which FSH or estrogens ensures the survival of early antral follicles is unknown.

It is becoming evident that survival factors are needed to sustain folliculogenesis during the progression of follicle development. In preovulatory follicles, numerous factors promote follicle cell survival (Chun *et al.* 1994c; Chun *et al.* 1995; Eisenhauer *et al.* 1995b; Hsueh *et al.* 1994b), indicating that overlapping hormonal cascades are involved in maintaining follicles that develop to this stage. The differential responsiveness of follicles to hormonal signals at different developmental stages may ensure a staggered supply of maturing follicles during reproductive life. An elaborate intrafollicular control mechanism ensures the survival of preovulatory follicles. The onset of apoptosis in preovulatory follicles in a serum-free culture is prevented by treatment with FSH and LH (Chun *et al.* 1994b). In addition, treatment with GH (Eisenhauer *et al.* 1995a) or local factors including IGF-I, epidermal growth factor, TGFα, and fibroblast growth factor-2, likewise suppresses follicle cell apoptosis (Chun *et al.* 1994a; Tilly *et al.* 1992a). Although gonadotropins are the most important survival factors for preovulatory follicles, this array of extracellular signals acting through endocrine, paracrine, autocrine, or juxtacrine mechanisms, ensures their survival for ovulation.

Control of the cell cycle in the ovary

In all tissues the regulation of cell cycle is a complex phenomenon that involves the proper balance of regulatory molecules, which can be altered by signals that act at different stages of the cycle. The cell cycle progression and proliferation are controlled by a proper balance between positive and negative regulators that converges in the regulation of specific kinases cascades (Sherr 1996).

Molecules have with regulatory role in proliferation and differentiation of the granulosa cells been identified during follicular development: among then there are cyclin D2 (Sicinski *et al.* 1996a) and p27 Kip1 (Fero *et al.* 1996b). The role of these molecules was elucidated after the observation of ovarian altered phenotypes in mice missing cyclin D2 and p27 Kip1. In mice lacking cyclin D2 the proliferation of granulosa cells is decreased, the follicles remain small and lack ovulation (Sicinski *et al.* 1996b). As regards mice lacking p27 Kip1, it was noted that although the follicular growth is not compromised, the granulosa cells do not luteinize properly (Fero *et al.* 1996a). The cyclin D2 (Inaba *et al.* 1992) acts as a positive regulator of the progression through the cell cycle with its ability to tie the ciclin-dependent kinase (cdks) 4 or 6, thus activating the cascade

of events that allows progression through the G1 phase of the cell cycle. Also Ciclin E acts as a positive regulator in the cell cycle progression (Koff *et al.* 1991).

In contrast, p27 Kip1 (one of several inhibitors of CDK), inactivating these cascades of CDKs, stops the cell cycle and the cells remain blocked in G1 phase (Polyak *et al.* 1994).

The fact that the cyclin D2 and p27 Kip1 are expressed and regulated in the ovary in a selective manner indicates that these two molecules have a central role in the growth and follicular differentiation.

PACAP

When in the 1982, the primary structure of GRF (Growth hormone-Releasing Factor) had been determined, it was commonly thought that all major hypophysiotropic neurohormones had been identified. However, the subsequent characterization of other neuropeptides capable of regulating the activity of anterior pituitary cells, such as PACAP (pituitary adenylate cyclise activating polypeptide) (Miyata *et al.* 1989) and PRL (Prolactin Releasing Peptide) has shown that this view was incorrect.

PACAP has been originally isolated from an extract of ovine hypothalamus on the basis of its ability to stimulate cAMP formation in rat pituitary cells (Miyata *et al.* 1989). Like other hypophysiotropic neurohormones, PACAP is contained in extra-hypothalamic neurons as well as in numerous peripheral tissues. Consistent with its widespread distribution, PACAP has been found to exert pleiotropic effects including modulation of neurotransmitter release, vasodilation, bronchodilation, activation of intestinal motility, increase of insulin and histamine secretion, as well as stimulation of cell multiplication and/or differentiation.

Discovery of PACAP

To isolate novel hypophysiotropic neuropeptides, the group of Arimura has screened fractions from ovine hypothalamus extract, by monitoring their stimulatory effect on adenylyl cyclase activity in cultured rat anterior pituitary cells. Using this approach, they have isolated in pure form a peptide, found to markedly increase cAMP formation, that they termed pituitary adenylate cyclase-activating polypeptide. Characterization of the peptide revealed that it comprises 38 amino acid residues and is C-terminally α-amidated (Miyata *et al.* 1989). Two years later, the primary structure of this 38-amino acid form of PACAP (PACAP38) was determined in the European green frog Rana ridibunda. The sequence of PACAP38 comprises an internal cleavage-amidation site (Gly28-Lys29-Arg30), suggesting that the PACAP precursor can generate a 27-residue α-amidated polypeptide (PACAP27). The structure of the biologically active region of PACAP, corresponding to the PACAP27 sequence, has been totally preserved during evolution, from amphibians to mammals (Fig. 8). The sequence of PACAP27 shows 68% identity with

vasoactive intestinal polypeptide (VIP), identifying PACAP as a member of the VIP-glucagon-GRF-secretin superfamily of structurally related peptides (Campbell & Scanes 1992)(Fig 9).

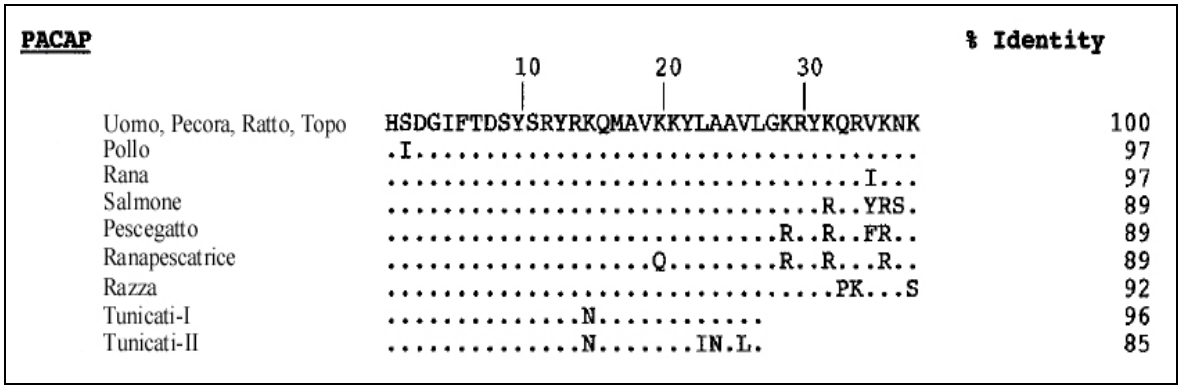


Fig. 8:
Diagram of the amino acid sequence of PACAP38 in various vertebrates and urochordates species: there is clearly a strong conservation of peptide in zoological scale.

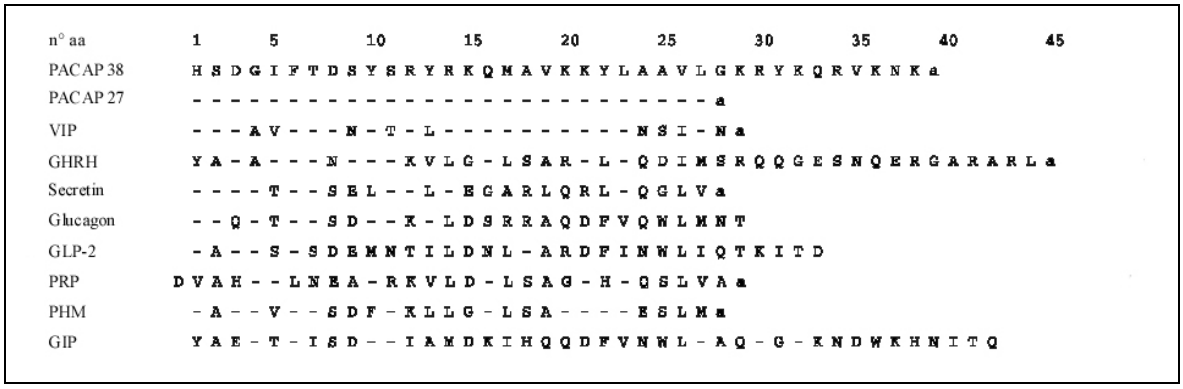


Fig. 9:
Amino acid sequences of the different members of the PACAPVIP-GRF-glucagon superfamily in human.
–: amino acids identical with those of PACAP38.
(a): amydated end.
Adapted from Kieffer and Habener, 1999.

The cDNA encoding the PACAP precursor has been characterized in several vertebrate species (Arimura & Shioda 1995; Kimura *et al.* 1990; Ohkubo *et al.* 1992; Okazaki *et al.* 1995) and in a protochordate, the ascidian Chelyosoma productum (McRory & Sherwood 1997). In humans, the cDNA encodes a 176-amino acid prepro-protein, which comprises a 24-amino acid signal peptide

(Hosoya *et al.* 1992). In all mammalian species studied so far, the sequence of PACAP38 is located in the C-terminal domain of the precursor. In mammals, the primary structure of the PACAP precursor reveals the existence of seven mono or dibasic residues that can be cleaved by various prohormone convertases (PCs) including PC1, PC2, PC4, PC5, PC7, furine, and PACE4 (Seidah *et al.* 1996). In the testis, where PACAP is particularly abundant, PC4 can process the PACAP precursor to generate both PACAP38 and PACAP27 (Li *et al.* 1998). In the 2000 the team of Arimura shown that PC4 is the only prohormon convertase for PACAP, in ovary and testis, where neither PC1 nor PC2 are expressed (Li *et al.* 2000).

In rat, RIA measurements have revealed that the highest concentrations of PACAP occur in the hypothalamic area (Arimura *et al.* 1991a; Ghatei *et al.* 1993b). Reversed-phase HPLC analysis showed that PACAP38 is by far the predominant form, PACAP27 representing less than 10% of the total peptide content in brain tissue (Arimura *et al.* 1991a; Ghatei *et al.* 1993a; Hannibal *et al.* 1995; Masuo *et al.* 1993b). The distributions of PACAP and VIP in the CNS are substantially different (Masuo *et al.* 1993a).

The secretion of gonadotropins by the gonadotrope cells is mainly regulated by GnRH (Conn *et al.* 1981; Waters & Conn 1991). However, there is now evidence that the PACAP act, both alone and in synergy with GnRH, to stimulate the expression of FSH and LH mRNAs (Tsujii & Winters 1995) and their secretion (Culler & Paschall 1991; Ortmann *et al.* 1999b; Tsujii *et al.* 1994). In addition, PACAP stimulates GH, ACTH and PRL release by the hypophysis somatotroph cells, (Goth *et al.* 1992; Jarry *et al.* 1992; Ortmann *et al.* 1999a). The potency of the effects evoked by PACAP on pituitary cells, however, is small if compared to that of classic hypothalamic releasing factors. Therefore, the attention is turned to identify extrahypotalamic roles and effects of this peptide. PACAP can act as paracrine regulator even in body districts where is not produced, since it is able to cross the blood-brain barrier (Banks *et al.* 1993) and is also produced in many tissues (Table 3).PACAP has been identified also in whole ovary (Arimura *et al.* 1991a), although in much lower concentrations than in the testis. In particular in rat ovary it was found an intense expression of PACAP in the granulosa cells of preovulatory follicles after to stimulation with LH (Ko & Park-Sarge 2000c) or hCG (Gras *et al.* 1996g).

Tissue	PACAP 38	PACAP 27
Hypotalamus	2,25	1,42
Cerebral cortex	1,00	0,09
Anterior pituitary gland	0,15	0,07
Posterior pituitary gland	1,68	0,14
Lung	0,09	0,02
Spleen	0,02	0,01
Duodenum	0,39	0,06
Adrenal Medulla	0,61	0,02
Testis	2,29	0,01
Ovary	2,00	0,03

Tab. 3:
Concentration of PACAP38 and PACAP27 assessed as immunoreactivity in various rat tissues (ng / dry weight) (Arimura, 1992).

Pharmacological characteristics and transduction mechanisms associated with PACAP receptors

Type of Binding Sites	Binding Affinity	Receptor Subtypes	Splice Variants	Transduction Mechanisms		
				Adenylyl Cyclase	PLC	Calcium
Type I	K_d	PAC1	S	Stimulates cAMP production	Stimulates IP turnover	Stimulates calcium mobilization
	P38 ≈ P27 ≈ 0.5 nM		Hop1 Hop2			
	VIP > 500 nM		Hip-Hop		P38 > P27 >> VIP	
			Hip	P38 ≈ P27 >> VIP	–	
			Vs		Stimulates IP turnover P38 ≈ P27 >> VIP	
			TM4	–	–	L-type channel
Type II	P38 ≈ 27 ≈ VIP ≈ 1 nM > secretin	VPAC1	?	Stimulates cAMP production P38 > P27 > VIP	+?	Stimulates calcium mobilization
	helodermin > P38 ≈ P27 ≈ VIP ≈ 1 nM	VPAC2		Stimulates cAMP turnover P38 ≈ P27 ≈ VIP	–	Stimulates calcium mobilization

Tab. 4:
Pharmacological charateristics and trasduction mechanisms associated with PACAP receptors.

The PACAP Receptors

Two classes of PACAP binding sites have been characterized on the basis of their relative affinities for PACAP and VIP (Table 4). Type I binding sites exhibit high affinity for PACAP38 and PACAP27 (Kd ≈ 0.5 nM) and much lower affinity for VIP (Kd ≈ 500 nM) (Christophe *et al.* 1989; Gottschall *et al.* 1990; Hosoya *et al.* 1993). Type II binding sites, which are abundant in various peripheral organs including the lung, duodenum, and thymus, possess similar affinity for PACAP and VIP (Kd ≈ 1 nM) (Gottschall *et al.* 1990).

Three PACAP receptors have been cloned so far and termed PAC_1-R, $VPAC_1$-R, and $VPAC_2$-R (Table 4) by the International Union of Pharmacology according to their relative affinity for PACAP and VIP (Sheward *et al.* 1998). The PACAP-specific receptor (PAC_1-R) cDNA sequence encodes a 495-amino acid protein with seven putative membrane-spanning domains and exhibits a high degree of sequence identity with the glucagon, secretin, and calcitonin receptor cDNAs. Five variants resulting from alternative splicing in the third intracellular loop region have been identified in rat (Spengler *et al.* 1993a). The splice variants are characterized by the absence (short variant) or presence of either one or two cassettes of 28 (hip or hop1 variant) or 27 (hop2 variant) amino acids (Journot *et al.* 1994) (Fig. 10). The presence of the hip cassette impairs adenylyl cyclase stimulation and abolishes phospholipase C (PLC) activation, suggesting that the various cassettes are involved in second messenger coupling (Table 4).

In the brain and pituitary, the short variant is the most abundant form, whereas the hop variant predominates in the testes and adrenal gland (Spengler *et al.* 1993b). A very short splice variant of PAC_1-R, characterized by a 21-amino acid deletion in the N-terminal extracellular domain, has also been characterized (Dautzenberg *et al.* 1999; Pantaloni *et al.* 1996a). The existence of this 21-amino acid sequence influences the receptor selectivity for the PACAP38 and PACAP27 isoforms and determines the relative potencies of the two peptides in stimulating PLC. Another PACAP receptor variant termed PAC_1-R transmembrane domain (TM) 4 has been cloned in the rat cerebellum (Chatterjee *et al.* 1996). This latter receptor differs from the short variant of the PAC_1-R by discrete sequence substitutions located in TMs II and IV. Surprisingly, activation of PAC_1-R TM4 has no effect on adenylyl cyclase or PLC activity, but causes calcium influx through L-type voltage-sensitive calcium channels. The mouse PAC_1-R gene spans more than 50 kb and is divided into 18 exons (Aino *et al.* 1995). The proximal promoter region has no apparent TATA box but contains a CCAAT box and two potential specific protein 1-binding sites that act as

transcriptional activators (Dynan & Tjian 1983; Skak & Michelsen 1999). The rat PAC_1-R gene has been localized on chromosome 4 (Cain *et al.* 1995a) and spans 40 kb with 15 exons (Chatterjee *et al.* 1997). The human PAC_1-R gene is located in region p15 of chromosome 7 (Pantaloni *et al.* 1996b).

The rat $VPAC_1$-R cDNA encodes a 459-amino acid protein (Cain *et al.* 1995b) and exhibits 50% amino acid sequence identity with the rat PAC_1-R (Pisegna & Wank 1993). The human $VPAC_1$-R comprises 457 amino acids and possesses 84% sequence identity with the rat $VPAC_1$-R (Sreedharan *et al.* 1993). The promoter region encompasses several potential binding sites for nuclear factors, including specific protein 1, activator protein-2, or autotumorolytic fraction. The $VPAC_1$-R has an affinity slightly more for PACAP-38 compared to PACAP-27. This receptor is able to bind, even if with lower affinity, the elodermin and secretin.

The rat and human $VPAC_2$-R proteins exhibit 87% amino acid identity (Adamou *et al.* 1995; Svoboda *et al.* 1993). The $VPAC_2$-R has an amino acid sequence of 459 amino acids. This receptor is coupled to the activation of the AC. Like the $VPAC_1$-R, $VPAC_2$-R binds with equal affinity PACAP and VIP, showing to preferentially bind elodermin rather than secretin.

The localization of PACAP binding sites and PACAP receptor mRNAs has been investigated thoroughly in the rat brain (Hashimoto *et al.* 1993; Shioda *et al.* 1994). The distribution and relative density of type I (PACAP-specific) and type II (PACAP/VIP) binding sites are compared in Table 4. The major splice variants of PAC1-R in the rat brain is the short isoform that does not contain any hip or hop cassettes (Spengler *et al.* 1993c); Zhou et al., 2000). Anatomical mapping of the VPAC2-R mRNA demonstrates a completely different and, apparently, complementary distribution from that of the VPAC1-R mRNA (Ishihara *et al.* 1992b).

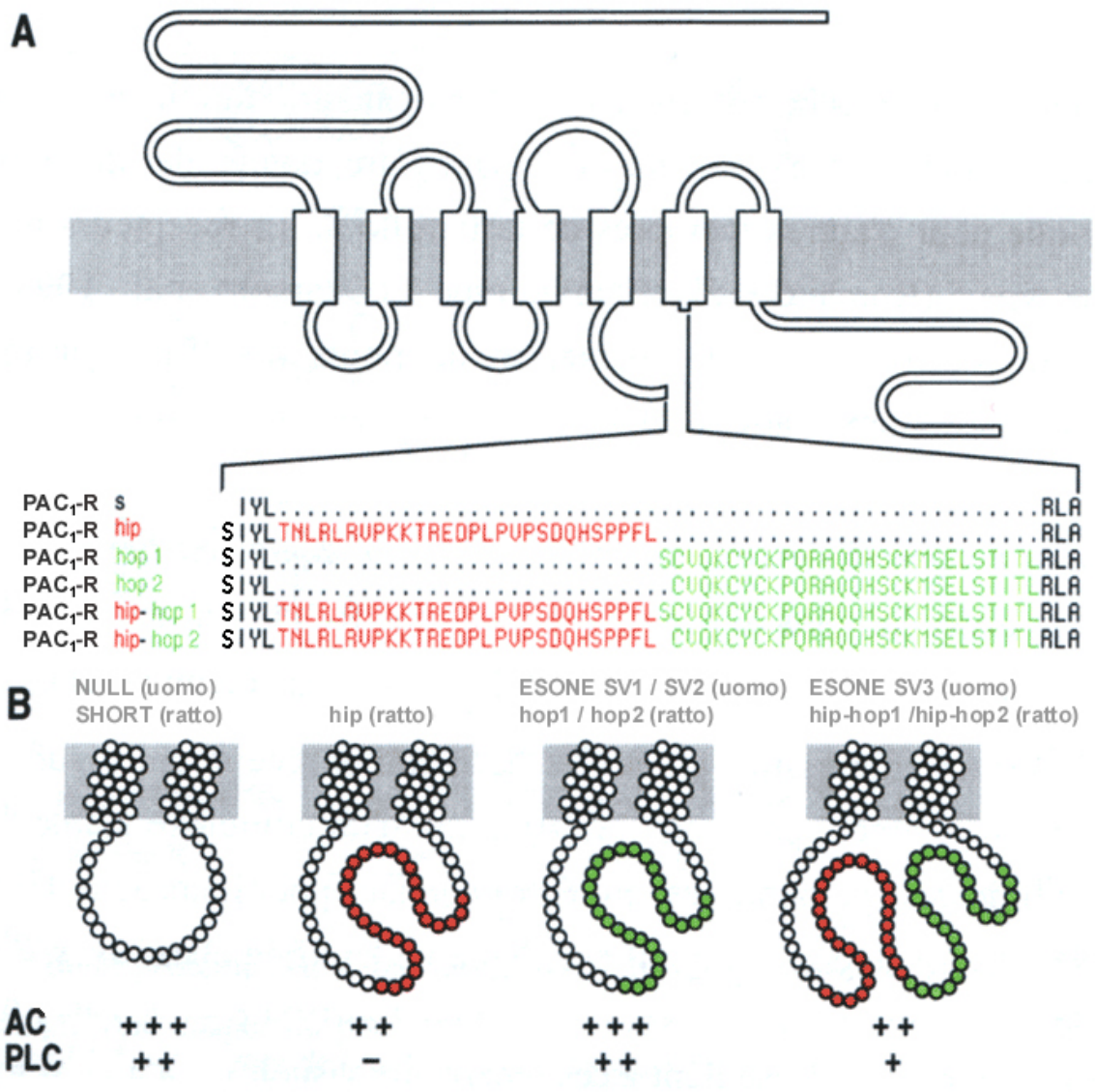


Fig. 10:
Schematic diagram of the PAC_1-R receptor structure and of the six splicing variants identified so far in rats.

PACAP and its receptors in Gonads

PACAP binding sites and receptor mRNAs have been identified in most endocrine glands (Table 3). The presence of PACAP and its receptors in the testis and ovary provides evidence that the peptide may operate as a local regulator of gonadal activity. In the rat testis, the concentration of PACAP is significantly reduced after hypophysectomy and is restored by FSH administration, indicating that the expression of PACAP is under the control of pituitary gonadotrophins (Shuto *et al.* 1995). In vitro, PACAP induces a dose dependent stimulation of testosterone secretion from isolated rat Leydig cells (Romanelli *et al.* 1997b; Rossato *et al.* 1997b) and activates or inhibits protein synthesis in spermatocytes or spermatids, respectively (West *et al.* 1995a). In Leydig cells, PACAP activates both adenylyl cyclase and PLC through an interaction with PAC_1-R (Romanelli *et al.* 1997a). The effect of PACAP on Leydig cells may also be mediated via a novel receptor subtype coupled to a sodium channel through a pertussis toxin-sensitive G protein (Rossato *et al.* 1997a). The effects of PACAP on protein synthesis in spermatocytes and spermatids are both mimicked by dbcAMP (West *et al.* 1995b). In the epididymal epithelium, PACAP stimulates chloride secretion, which is important for sperm activation and storage (Zhou *et al.* 1997a). The occurrence of PACAP-immunoreactive material in epididymal tubules indicates that PACAP is locally synthesized and thus may act as a paracrine regulator of sperm maturation (Zhou *et al.* 1997b).

In the rat ovary, the presence of PAC_1-R and $VPAC_2$-R mRNAs has been reported (Kotani *et al.* 1998b; Scaldaferri *et al.* 1996b; Usdin *et al.* 1994a). Granulosa cells of the developing follicle express the $VPAC_2$-R mRNA (Usdin *et al.* 1994b) whereas the corpus luteum contains the PAC_1-R mRNA (Kotani *et al.* 1998a). In the placenta, Northern blot analysis revealed the presence of both $VPAC_1$-R and $VPAC_2$-R mRNA (Adamou *et al.* 1995; Sreedharan *et al.* 1995). From literature we know that PAC1R mRNA was mainly detected in large preantral follicles of 21 day-old rat ovaries (Park *et al.* 2000b), and recently we have demonstrated the presence of the three PACAP's receptors mRNA in the rat whole ovary at different ages during the first wave of folliculogenesis (Vaccari *et al.* 2006e).

The presence of PACAP in the ovary was first described by Arimura and collaborators in 1991 (Arimura *et al.* 1991b). Later on, PACAP has been identified by immunohistochemistry techniques in mammalian ovary and, especially, into the nerve fibers innervating blood vessels of human ovaries (Steenstrup *et al.* 1995). Following PACAP gene expression was reported in rat ovary (Scaldaferri *et al.* 1996a). The PACAP mRNA has been detected in antral and preovulatory follicles,

especially in the mural granulosa cells and in the cumulus cells, with a maximum of expression between 6 and 18 hours after hCG treatment (Gras *et al.* 1996f).

Human chorionic gonadotropin (hCG) stimulates the expression of both PACAP and progesterone receptor mRNAs (Ko & Park-Sarge 2000b). The peak of expression of progesterone receptor mRNA occurs 3 h after hCG treatment and the peak of PACAP mRNA only after 6 h, suggesting that progesterone receptor activation is required for PACAP gene expression (Ko & Park-Sarge 2000a). In support to this hypothesis, it has been shown that blockage of the progesterone receptor with the progesterone receptor antagonist ZK98299 abrogates the effect of hCG on PACAP gene expression (Ko *et al.* 1999). Exposure of cultured granulosa cells to PACAP causes a dose-dependent increase in progesterone production (Apa *et al.* 1997a; Apa *et al.* 1997d; Gras *et al.* 1999b; Zhong & Kasson 1994). Reciprocally, immunoneutralization of endogenous PACAP reduces progesterone formation and impairs subsequent luteinization, suggesting that PACAP plays an important role in LH-induced progesterone production during the periovulatory period (Gras *et al.* 1999a). Incubation of immature rat preovulatory follicles with PACAP or VIP induces a dose-dependent inhibition of follicle apoptosis (Flaws *et al.* 1995; Lee *et al.* 1999a; Martin *et al.* 1981). In luteinized granulosa cells, PACAP appears to be more potent than LH in stimulating cAMP accumulation (Heindel *et al.* 1996). Stimulation of follicle-enclosed and cumulus-enclosed rat oocytes with PACAP, demonstrate that the peptide is able to affect the meiotic maturation of oocyte in a dose and time dependent manner (Apa *et al.* 1997c). PACAP is also able to directly affect the meiotic maturation in denuded oocyte (Apa *et al.* 1997b). PACAP is able to increase tPA stimulation and inhibits uPA production, in a dose-dependent way (Apa *et al.* 2002). In in vitro preantral follicle cultures, PACAP is able to inhibit several parameter correlated with normal follicle development, such as growth, antrum formation and estradiol production. (Cecconi *et al.* 2004b).

AIM OF THE THESIS

About twenty years after its discovery, PACAP certainly appears as one of the most fascinating neuropeptides ever identified: it belongs to the largest family of regulatory peptides, which encompasses several other prominent members including secretin, glucagon, GRF, and VIP. The primary structure of PACAP has been extremely well conserved from the sea squirt (a protochordate) to humans, indicating that this peptide must be involved in vital functions throughout the animal kingdom. PACAP has been shown to modulate cell proliferation in different systems. This polypeptide not only stimulates primordial germ cell proliferation (Pesce *et al.* 1996) but also inhibits cell proliferation in the development of mouse neural tube, in which it is modulated by the presence of fibroblast growth factors and other growth factors (Lelievre *et al.* 2002). Moreover, PACAP and VIP both inhibit cell proliferation in a murine cell line of neuroblastoma (Lelievre *et al.* 1998). In many systems the action of PACAP is mediated by an increase in cAMP production. Moreover, perturbing cAMP signals by altering its intensity and duration can interfere with the differentiative program of granulosa cells (Park *et al.* 2003) as well as in a prostate cancer cell line (LNCaP) (Farini *et al.* 2003). PACAP is cyclically expressed in granulosa cell of preovulatory follicles in response to the LH surge but is further present in the interstitial space around primordial and primary follicles (Gras *et al.* 1996e).

The spatial and temporal expression of PACAP and its receptors, together with its well documented tasks in the ovary, let us to speculate that its expression is relate with a functional role of the peptide on the follicle development.

As was mentioned before, the beginning of follicle growth is independent from gonadotropins: in fact at this stage follicles do not have FSH receptors and follicular growth can reach the secondary follicle stage even in mice lacking FSH-R (Dierich *et al.* 1998). Beginning from secondary follicles stage, granulosa cells acquire FSH receptors and are therefore FSH responsive. The factors that govern the initiation of follicular growth are still largely unknown. Numerous studies have been interested in the research of the physiological stimulus that triggers the growth of the follicle and the acquisition of receptors for FSH. Factors acting via cAMP play an important role in FSH-R induction. Therefore cAMP may regulate the follicle development inducing the synthesis of gonadotropins receptors, stimulating the synthesis of steroidogenesis key enzymes and increasing the synthesis of molecules involved in the communication between oocyte and somatic cells. In rats neurotransmitters such as VIP and NE produced in the ovarian nerve fibers contribute to the

differentiation process in primary follicles by stimulating the acquisition of FSH receptors (Mayerhofer *et al.* 1997a).

In this thesis the attention is focused on a peptide that belongs to the family of peptides VIP / secretin / glucagon: the PACAP (pituitary adenylate cyclase-activating polypeptide).

PACAP is produced abundantly by the granulosa cells of large preovulatory follicles after LH stimulation (Lee *et al.* 1999b). PACAP production has been studied also in ovaries of adult cycling animals: PACAP production is cyclical and is found only at the stage of estrus. At that stage, in addition to the granulosa cells of preovulatory follicles, PACAP is produced in rats also in theca/interstitial cells near the primordial follicles, the growing follicles and the early preantral follicles (Gras *et al.* 1996d). This suggests that the endogenous cyclical PACAP production could also have an action on follicles during the early growth stages.

The action of PACAP is mediated by three receptors: the type I (PAC_1-R) that specifically binds PACAP and with very low affinity for VIP, and the type II receptors ($VPAC_1$-R and $VPAC_2$-R) that bind with equal efficiency PACAP and VIP (Inagaki *et al.* 1994; Ishihara *et al.* 1992a; Spengler *et al.* 1993d).

In a recent paper we showed the expression of all three receptors in whole ovaries obtained from juvenile and gonadotropin-treated immature rats. A more detailed analysis on cells from preovulatory follicles showed that PAC_1-R and $VPAC_2$-R were expressed in granulosa cells, whereas only VIP receptors were expressed in theca/interstitial (TI) cells and fully grown oocytes presented only PAC_1-R (Vaccari *et al.* 2006d) .

The goal of this study (performed on rats) is to better characterize the expression of PACAP and of its receptors in the rat neonatal ovary. This study also investigated the effect of PACAP on transition from primordial to preantral follicle in vitro, and its ability to influence the gene expression of regulatory proteins of the cell cycle (Cyclin-D2) and of other important genes involved in the differentiation of ovarian somatic (CYP19, inhibin-alpha, FSH-R) and germ cells (GDF9).

MATERIALS AND METHODS

Materials

The materials used in the present study were obtained from the following sources: pregnant mare gonadotropin serum (PMSG) and human chorionic gonadotropin (hCG) from Intervet (Livorno, Italy); PACAP and Bisindolylmaleimide-I from Calbiochem (San Diego, CA); Cell Proliferation KIT RPN20 from Amersham; RNeasy Mini KIT and RNeasy Micro KIT from QIAGEN S.p.A. ; M-MLV Reverse Trascriptase and TOTO-3 from Invitrogen S.R.L (San Giuliano Milanese, Milan , IT) ; Hot Master TAQ from Eppendorf S.R.L. (Milan, IT); The antibody to PAC1-R H-55 (sc-30018), to total ERK (sc-154) and to phospo-ERK (sc-7383) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA); The antibody alpha tubulin mouse (T5168) from Sigma Aldrich (Milan, IT) ; Dulbecco's modified Eagle medium:F12 (DMEM:F12), alpha-MEM and Hank's balanced salt solution from Gibco (Grand Island, NY, USA); the MEK inhibitors H89 and U0126 from TOCRIS biosciences (Bristol, UK); Highly purified ovine follicle-stimulating hormone (NIDDKo- FSH-19-SIAFP, BIO) was kindly provided by Dr Parlow (National Hormone and Pituitary Program of the NIH).

Animals

Immature female Wistar rats were purchased from Charles River (Como, Italy). They were housed in groups, maintained in controlled temperature (25° C) and light (12 h light/day) conditions, and were given free access to food and water. Animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory animals. Experimental protocols were approved by the University "La Sapienza" Committee for Animal Care and Use. Animals aged from 2 to 25 days were killed by cervical dislocation and the ovaries were collected for RNA analysis or processed for each group of experiments as described below. Twenty-four-days-old rats were injected with 10 IU of PMSG. After 48 h the animals were injected with 10 IU of hCG (hCG-rats) and sacrificed after 6 hrs by cervical dislocation.

RNA extraction and RT-PCR

Total RNA from whole ovaries or from preantral follicle was isolated by RNeasy Mini KIT and RNeasy Micro KIT from QIAGEN, according on the amount of the starting material (up to 100µg or to 45 µg respectively). The purity and integrity of the RNA was checked spectroscopically and by gel electrophoresis. Then the total RNA (1–2 mg) was reverse transcribed in a final volume of 20 µl, with M-MLV Reverse Trascriptase from Invitrogen, according to the manufacturer's instructions. The PCRs were carried out using Hot Master Taq DNA polymerase (Eppendorf) according to the manufacturer's instructions. For each primer set, the number of cycles for the PCR was chosen in the exponential phase of amplification, using the annealing temperature provided. For each set of primer used (see Table 5), were done preliminary PCRs experiments at different cycles of amplification, to set-up the range of linearity for each pair of primers (data not shown). For each sample, 10 µl PCR product was submitted to electrophoresis on agarose gel (2%) and stained with ethidium bromide. Amplified products were analyzed using AIDA software (Advanced Image Data Analyzer, 2.11) and mRNA levels normalized against the expression of the chaperon protein Cycliphilin A(CYC) mRNA.

RNA extraction and RT-PCR on single oocytes

Messenger RNA from rat oocytes was amplified by PCR according to the method of Fiorenza et al. (Fiorenza & Mangia 1998), with slight modifications. Briefly, about 80 oocytes, obtained by 15 days-old rats were mechanically isolated from the surrounding granulosa cells and immediately lysed for RNA extraction. For this purpose the oocytes were frozen and thawed twice in order to break their membrane, and then each sample was processed for total RNA extraction by a silica gel-based membrane spin column (RNeasy Micro Kit), according to the manufacturer's instructions. Methods used for the following RT-PCR are the same explained in the previous paragraph.

Organ culture

After the 2-day-old rats had been killed, ovaries were removed aseptically from the abdomen, then were placed in 35mm dish containing Hank's solution 1x. With the help of two syringe 25-gauge needles, the tissue surrounding the ovaries, including the capsule in which the ovary is

enclosed (periovarian sac), was removed. Ovaries were immediately placed in culture upon a particular wire grid in an Organ Culture Dish (Falcon–BD) in DMEM:F12 supplemented with 2mM L-glutamine and antibiotics (100mM penicillin, 100μg/ml streptomycin). The ovaries were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 3, 5 or 6 days. Every 2 days of culture, the medium was removed and fresh medium was added. At the end of the culture period, the ovaries were either fixed for 48 h in Bouin's fixative or snap frozen in liquid nitrogen and stored at -80°C until employment.

Gene	*Primers*	*Product length (bp)*	*Gene bank access number*
PAC1-R	Fw 5'-GGACCCTTCTCAGCCTGT-3' Rw 5'-CACATGATCCGGTCTTGA-3'	343	NM133511
VPAC1-R	Fw 5'-TCCGAGCGGAAGTACTTCTG-3' Rw 5'-ACCTGGGCCTTGAAGTTGTC-3'	359	NM012685
VPAC2-R	Fw 5'-CACTAGTGATGGGTGGTCGG-3' Rw 5'-GCCAGTAGAAGTTCGCCATG-3'	399	NM017238
FSH-R	Fw 5'-CTTGTCGACCTGGATTTGGAGACCTG-3' Rw 5'-CTTGAGCTCCTTAATGCCTGTGTTGG-3'	209	NM199237
Cyclophilin A	Fw 5'-GGCAAGTCCATCTACGGA-3' Rw 5'-ACATGCTTGCCATCCAGC-3'	180	NM017101
CYP19a	Fw 5'-GCACGAGAATGGCATCAT-3' Rw 5'-GTTAGAAGTGTCCAGCATG-3'	219	NP058781
Inhibin α	Fw 5'-GCTGCCTCGAAGACATGC-3' Rw 5'-GTCCCAAGGACACAGGCA-3'	320	NM012590.2
Cyclin D2	Fw 5'-CCTTCATCGCTCTGTGTGCTACCG-3' Rw 5'-GCTCCGTCAGGGCATCGCAC-3'	144	NM022267
LH-R	Fw 5'-GTGGCCTTCGTCGTCATCTGTG-3' Rw 5'-GCGTTTACAGCAGCCGAATCG-3'	330	NM012978
PCNA	Fw 5'-AACCTACAGAGCATGGATTCG-3' Rw 5'-CACAGGAGATCACCACAGCA-3'	382	NM022381.3
GDF9	Fw 5'-CCTAAACCCAGCAGAAGTCAC-3' Rw 5'-GTCACATCCATCTCAATCCAC-3'	323	NM021672
CYP17	Fw 5'-TCCGAGAAGTGCTGCGTATC-3' Rw 5'-TGCGTGGGTGTAATGAGATG-3'	220	NM012753

Fw, forward primer; Rw, reverse primer

Tab. 5:
List of primers used to perform RT-PCR.

Ovarian histology and follicle counting

To investigate the development of ovarian follicles in the cultured ovaries and to compare it with follicle development in vivo, fresh and cultured ovaries were fixed for 48 h in Bouin's fixative, embedded in paraffin, and 7 μm serial sections were mounted on slides and stained with hematoxylin and eosin. Primordial follicles are non-growing follicles and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Early primary follicles have initiated development and contain at least one cuboidal (enlarged) layer of granulosa cells. The number of follicles at each developmental stage (primordial, primary, secondary) was counted in two sections from the largest cross-section of each ovary. The data obtained from this analysis of two middiameter crossections have been shown to provide similar data as analysis of compiled data from all serial sections as shown by Parrott & Skinner (Parrott & Skinner 1999).

Preantral follicle isolation and culture

Individual preantral follicles were mechanically dissected, together with a small clump of thecal stromal tissue attached, from the ovaries of 15 day-old rats. Follicles were measured with a precalibrated ocular micrometer at magnification X40 under stereomicroscope; those measuring 130 ± 10 μm of diameter with a centrally placed spherical oocyte (mean diameter: 65 ± 10 μm) and no signs of somatic cell degeneration were chosen for further culture. Follicles were individually incubated in 96-U-well -plates in 40μl αMEM supplemented with 1% ITS (insulin 5 μg/ml; transferrin 5 μg/ml; sodium selenite 5 ng/ml), antibiotics (penicillin, 100 U/ml; streptomycin, 100 mg/ml), and 5% fetal calf serum (FCS). The medium was overlaid with 70 μl sterile mineral oil (embryo tested, d = 0.84 g/ml). The follicles were cultured in the presence of PACAP 10^{-7} M, FSH (100 ng/ml) or PACAP and FSH (100 ng/ml) for 4 and 8 days at 37 °C in a 5% CO_2 atmosphere. Culture medium and stimulations were changed every 48 hrs. Follicle diameter was monitored daily using a calibrated micrometer. All cultured follicles were carefully opened; the granulosa cells that were released, after being mechanically isolated from the cumulus cells and oocytes, were collected and stored at -80 °C until assayed for gene expression by RT-PCR.

Immunofluorescence

Freshly isolated ovaries from 2, and 15 days rats, were collected, embedded in optimal Tissue Freezing Medium, snap frozen in liquid nitrogen and stored at - 80°C until sectioned. Sections (7 μm) were fixed in 4% paraformaldehyde. Sections were blocked in 10% (v/v) normal goat serum 5% (w/v) BSA to minimize non-specific binding, and incubated for 36h at 4°C with 1:100 rabbit polyclonal anti-PACAP receptor-1 (Santa-Cruz). After repeated washes in PBS, sections were incubated for 1 h at RT with 1:1000 diluted Alexa Fluor 488- conjugated mouse anti-rabbit secondary antibody (Molecular Probes, Invitrogen, Milan, Italy). Nuclei were stained with 1:5000 TOTO-3 (Molecular Probes T- 3604, Invitrogen). Images were acquired with Leica Confocal Software. In control samples, primary antibody was omitted.

In situ hybridization

Freshly-isolated ovaries from 15 days and adult 6h hCG-stimulated rats were fixed in Bouin's fixative solution for 48 h at room temperature, paraffin embedded and then sectioned at 7μm. Sections were mounted on microscope polylysine coated slides, deparaffined and rehydrated. Slides were then post-fixed in 4% paraformaldehyde for 10 min at room temperature, and treated with 10 μg/ml proteinase K. Hybridization was carried out overnight at 55°C in a humidified chamber in a mixture containing 200 mg/ml salmon sperm DNA and 300 ng/ml digoxigenin labelled-DNA PACAP receptor-1 probe. The probe was labelled by direct incorporation of DIG-dUTP during PCR amplification performed using the following primers: upper 5´-GCCTCTCTGGTTGTGATTCC-3´ and lower 5´-TCGGCTGGGTAGTAAAGGG-3´(with an amplicon of 318bp). Sections were then incubated in blocking solution (100mM Tris-HCl, pH 7.5, 100mM NaCl, 2mM MgCl2, 1% (w/v) BSA) and in 1:300 alkaline phosphatase conjugated anti-digoxigenin antibody (Roche Diagnostics) diluted in blocking solution and in 1:300 alkaline phosphatase-conjugated anti-digoxigenin antibody diluted in blocking solution. Colorimetric detection was developed by adding chromogen substrate (NBT, BCIP). Sections were observed by light microscopy and not counterstained. A digoxigenin labelled β-actin DNA probe was used as a positive control, whereas an unlabelled-PACAP DNA probe, as well as competing hybridizations, with different mixtures (1:5 and 1:10) of digoxigenin labelled-/unlabelled-PACAP receptor-1 DNA probes, were used as negative and specificity control reactions.

Proliferation assay

The Amersham Cell Proliferation Kit used consist of Immunocytochemical system for monitoring cell proliferation using BrdU (5-bromo-2'-deoxyuridine), a thymidine analogue, incorporated into replicating DNA and subsequently localized using a specific monoclonal antibody.

The assay was performed on newborn ovaries of 3day-old rats cultured in DMEM:F12 at 37°C in a humidified atmosphere containing 5% CO2 for 3days. At the end of the colture period the ovaries were rinsed in DMEM supplemented with 10% FCS to remove F12, according to the manufacturer's instructions. Then the ovaries were incubated in 1 ml of labeling medium (containing BrdU) at 37°C in a humidified atmosphere containing 5% CO2 for 4 hours. Then the ovaries were fixed for 48 h in Bouin's fixative, embedded in paraffin, and 5 µm serial sections were mounted on slides and processed. Nuclease digestion of DNA is performed to allow antibody access. Detection of bound primary antibody is achieved using peroxidase conjugated antibody to mouse immunoglobulin, polymerizing diaminobenzidine (DAB). Counterstain was not performed.

Tunel assay

The apoptotic assay was performed on 3 day old rat ovaries cultured for 3 days with and without PACAP stimulation as previously described. At the end of colture the ovaries was fixed for 48 h in Bouin's fixative, embedded in paraffin, and 5 µm serial sections were mounted on slides. After the sections were rehydrated the apoptotic cells were detected by the TUNEL method using the APOPTAG peroxidase in situ apoptosis detection kit (Q-BIOgene, Irvine, California) according to the manufacturer's instructions. As positive controls, sections of cultured ovaries were treated with DNase I. Negative controls were carried out omitting terminal deoxynucleotidyl transferase enzyme in the reaction mixture. The sections was not counterstained and analyzed using a Zeiss Axioscope microscope.

Statistical analysis

Data are expressed as the mean ± SE from at least three independent experiments. Statistical analysis was performed using ANOVA followed by the Tukey-Kramer test for

comparisons of multiple groups or by paired Student's t-test for comparison of data derived from two groups. Values with $P<0.05$ were considered statistically significant.

RESULTS

Expression of PACAP receptors in the rat newborn ovary

PACAP/VIP receptors are expressed in the rat ovary during the first wave of folliculogenesis (Vaccari *et al.* 2006c). In order to investigate the relative abundance of the three receptors at the beginning of folliculogenesis, the expression of PACAP/VIP receptors in whole ovaries of 3 days old rats was investigated by semi quantitative RT-PCR. As shown in Figure 11 all three receptors were present in the whole ovary at this age. As already shown in older animals (Vaccari *et al.* 2006b), PAC_1- R was the main receptor, followed by $VPAC_2$-R and $VPAC_1$-R.

Localization of PAC_1-R

To determine the cell type that expresses PAC_1-R mRNA, immunofluorescence analysis was performed on ovarian sections obtained from 3- and 15-day-old animals. PAC_1-R immunoreactivity was barely detectable in 3-day-old animals, whereas a strong signal was found in the granulosa cells of almost all the growing preantral follicles (Fig. 12A and 12B). A strong signal was present also in the oocytes, however very low levels of mRNA for PAC_1-R were detected by RT-PCR in isolated oocytes at this age compared to granulosa cells. (Fig. 12C.).

Expression of PACAP during the first wave of folliculogenesis

PACAP expression in whole ovaries at various ages was examined by RT-PCR. PACAP was already present in 3-day-old ovaries and its expression remain almost unchanged till 19 day-old ovaries (Fig. 13). In vivo stimulation with eCG-hCG significantly increased PACAP expression. In order to identify the cells producing this peptide, ovaries from 15 day-old-rats and from eCG-hCG primed 22-day-old rats were analysed by in situ hybridisation. As already shown by Gras (Gras *et al.* 1996c) a very strong signal was localized in granulosa cells of large preovulatory follicles and in interstitial glandular cells (IGCs) of eCG-hCG treated animals. The IGCs show a strong signal also in ovaries from immature animals (Fig. 14).

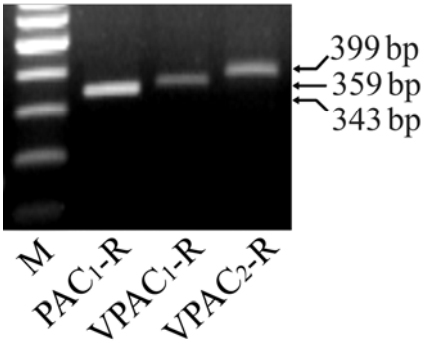


Fig. 11:

Expression of PACAP/VIP receptors in whole ovaries of 3-day-old rats detected by RT-PCR. PAC_1-R is highly expressed, followed by $VPAC_2$-R and $VPAC_1$-R. The figure is representative of three independent experiments.

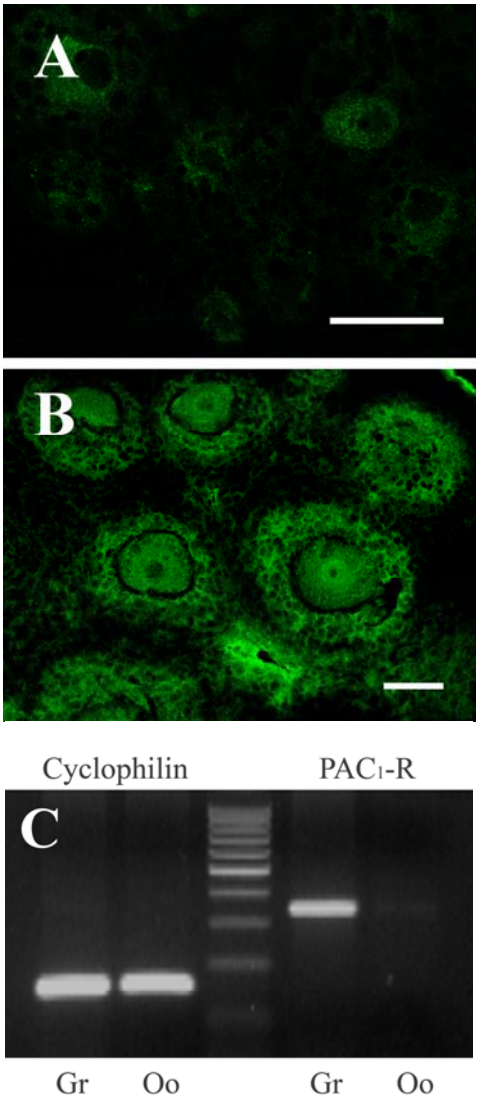


Fig. 12:

To determine the cell type that expresses PAC_1-R, immunofluorescence analysis was performed on ovarian sections obtained from 3-day-old (**A**) and 15-day-old animals (**B**). PAC_1-R immunoreactivity was barely detectable in 3-day-old animals, whereas a strong signal was found in the granulosa cells of almost all the growing preantral follicles. A strong signal was present also in the oocytes (**B**), however no mRNA for PAC_1-R was detected by RT-PCR in isolated oocytes at this age (**C**). The RNA was obtained from 15-day-old animals, respectively from granulosa cells (Gr) and from isolated oocytes (Oo),and was amplified for the same number of cycles. The figure is representative of three independent experiments.

Scale bar = 50µm.

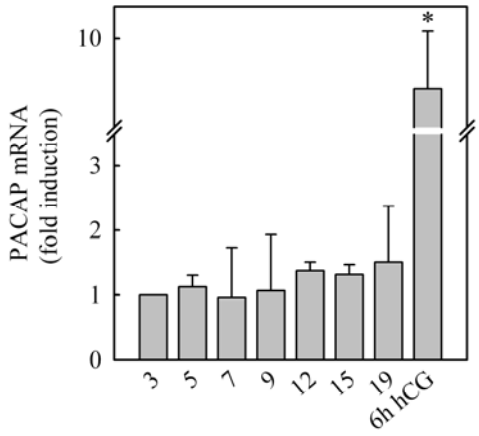


Fig. 13:

PACAP expression in whole ovaries at various ages detected by semiquantitative RT-PCR. The figure is representative of three independent experiments; the values represent the mean ± SEM of them. Result is expressed relative to 3day set equal to 1.

*: P<0,001 vs. 3days

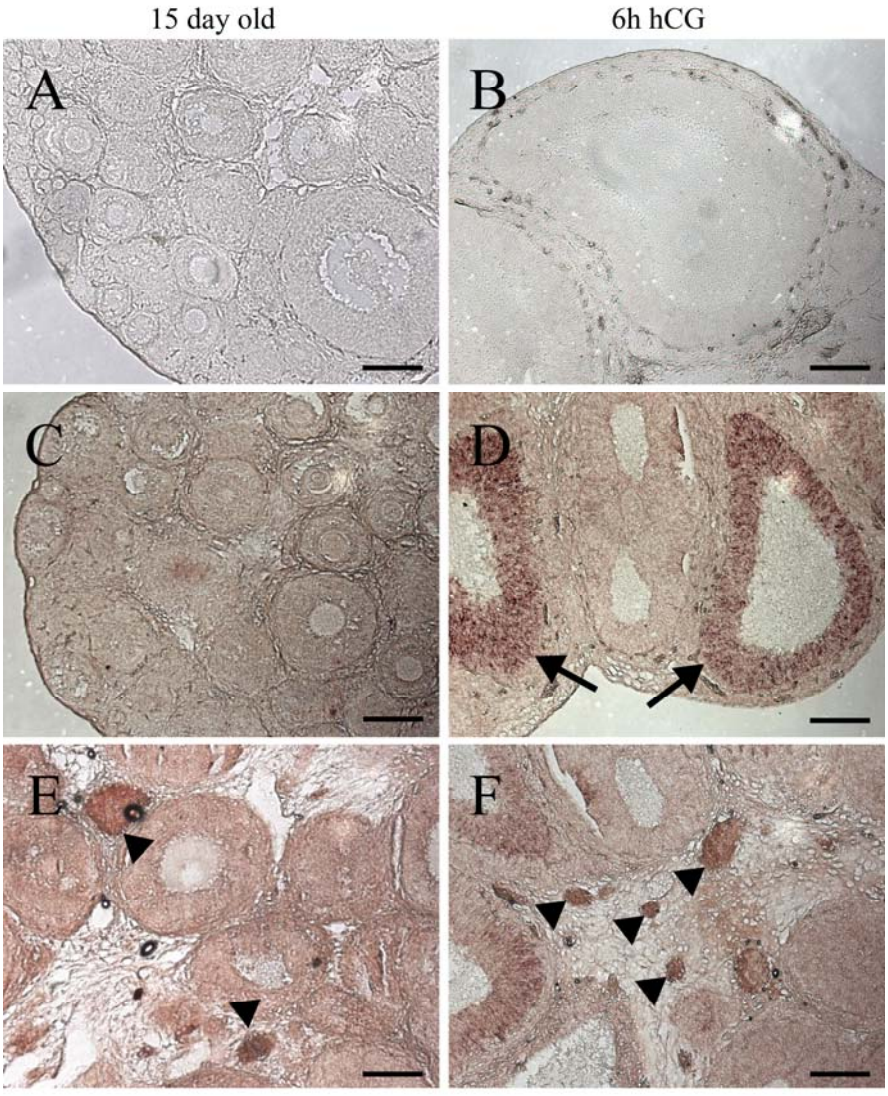


Fig. 14:

In situ hybridisation analysis of PACAP mRNA expression in rat ovary. Ovarian sections were hybridized with DIG-dUTPlabelled cDNA probes generated by PCR, as described in Methods.

PACAP expression was clearly detected 6 h after hCG treatment in granulosa cells of large preovulatory follicles (**D**, black arrows) and in interstitial glandular cells (**F**, black arrowhead). In 15 day-old ovaries PACAP mRNA signal was present in interstitial glandular cells (**E**, black arrowhead) and no expression is detectable in granulosa cells (**C** and **E**).

As a negative control, samples from 15 day-old ovaries (**A**) and 6 h after hCG injection (**B**) were hybridized in the presence of a 1:10 digoxigenin-labelled/unlabelled PACAP DNA probe.

Representative micrographs of three independent experiments.

Scale bar = 100 µm

Effect of PACAP on follicle growth in ovary organ culture

The effect of PACAP on follicle growth was evaluated during a 5-day culture period. To this end, 2-day-old ovaries were incubated in medium alone (C) or in the presence of 10^{-7}M PACAP. As shown in Fig. 15, 2-day-old ovaries contained predominantly primordial follicles and no growing follicles, while in the ovaries of 7-day-old rats it is possible to observe growing secondary and tertiary follicles. Histological examination of the ovaries after 5 days in culture indicated that the culture conditions had no detrimental effect on the ovarian tissue, almost no necrotic or apoptotic cells were present in all conditions. However we noticed, in ovaries obtained from 6 day-old animals, a greater number of growing follicles in the innermost portion of the ovary in comparison to the periphery (Fig. 16). While in our cultures follicles growth is more abundant near the borders where there are better conditions of oxygenation. Moreover when we compared ovaries cultured for 5 days with the morphology of 7-day-old rat ovaries we observed an higher number of secondary follicles in 7-day-old ovaries compared to those cultured for 5 days (Fig. 15B and 15C). After 5 days in culture many oocytes had grown and are enclosed by 1-3 layers of granulosa cells however, as already described in mice the primitive theca cells appeared less defined.

The addition of PACAP significantly reduced the number of growing follicle (Fig.15D).

To evaluate the presence of theca cells we analysed by semiquantitative RT-PCR, the expression of genes typically expressed by theca cells at this age, such as LH-R and 17-alfa-hydroxilase (CYP17). Both genes *in vivo* increased markedly their expression with age, from 2 to 7 day-old animals (Fig. 17). However, no increase was observed in culture.

Proliferation assay

The diminished number of growing follicles was accompanied by a decrease in the proliferation of granulosa cells evaluated as BrdU incorporation (Fig. 18). PACAP treated ovaries showed 59% proliferating cells compared to control ovaries set equal to 100%. This decrease was not dependent on increased apoptosis (data not shown).

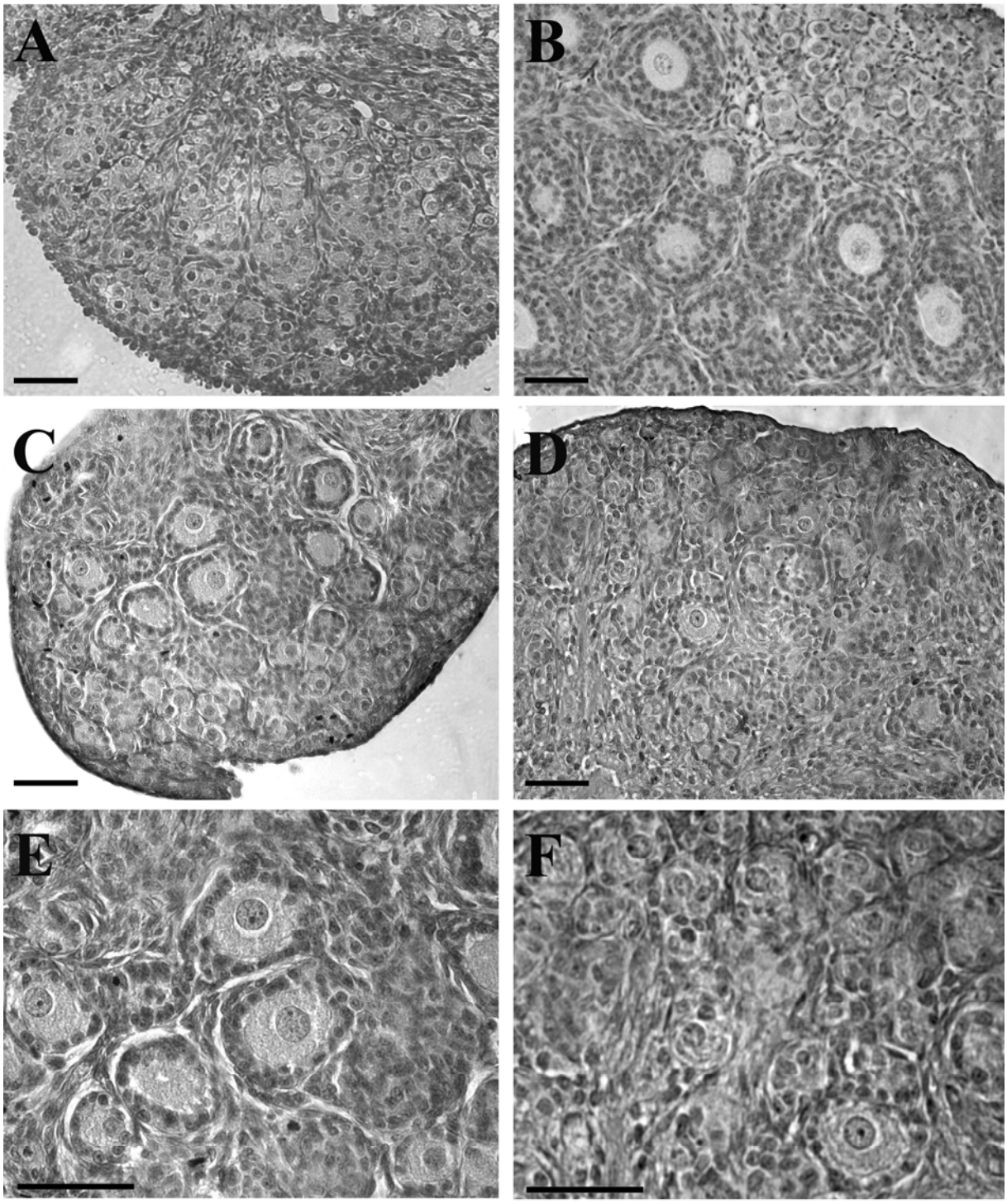


Fig. 15:

Photomicrographs of cultured and noncultured neonatal rat ovaries.

A: Section of an ovary of a 2-day-old rat. Many primordial follicles and some “naked” oocytes are present.

B: Section of a 7-day old rat ovary. Many primordial and some primary and secondary follicles are found. Most primordial follicles are located in the cortex of the ovary.

C and **E**: Sections of an ovary of a 2-day-old rat cultured for 5 days in DMEM:F12 at different magnifications. Many primordial and some growing (almost only primary) follicles are found.

D and **F**: Section of a 2-day-old rat ovary cultured for 5 days in presence of PACAP 10^{-7}M. After 5 days of culture the ovaries contained mainly primordial follicles and only few primary follicles compared to C and E.

Data are representative of at least 3 independent experiments.

Scale bar = 50 µm

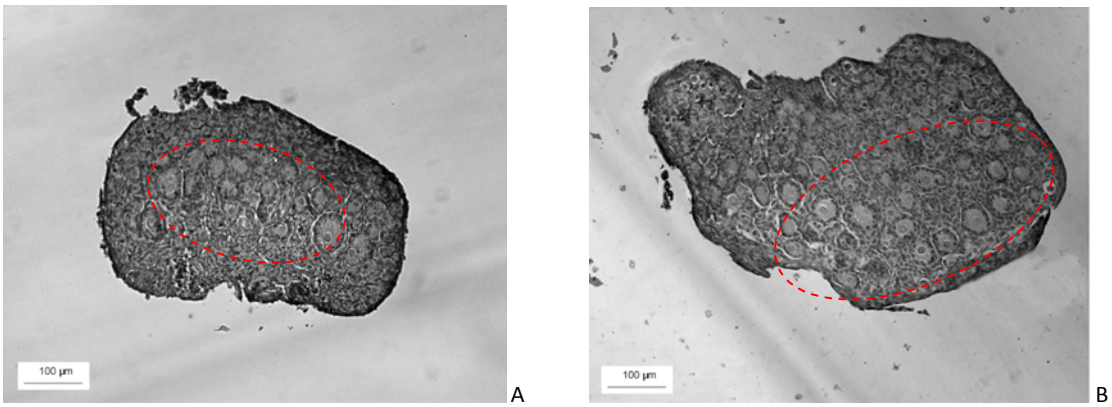


Fig. 16:

Micrograph of a 6 day-old rat untreated ovary A), and of a 2 day-old ovary cultured in vitro for 5 days B). The different distribution of follicles in growth is stressed by the red dotted line.

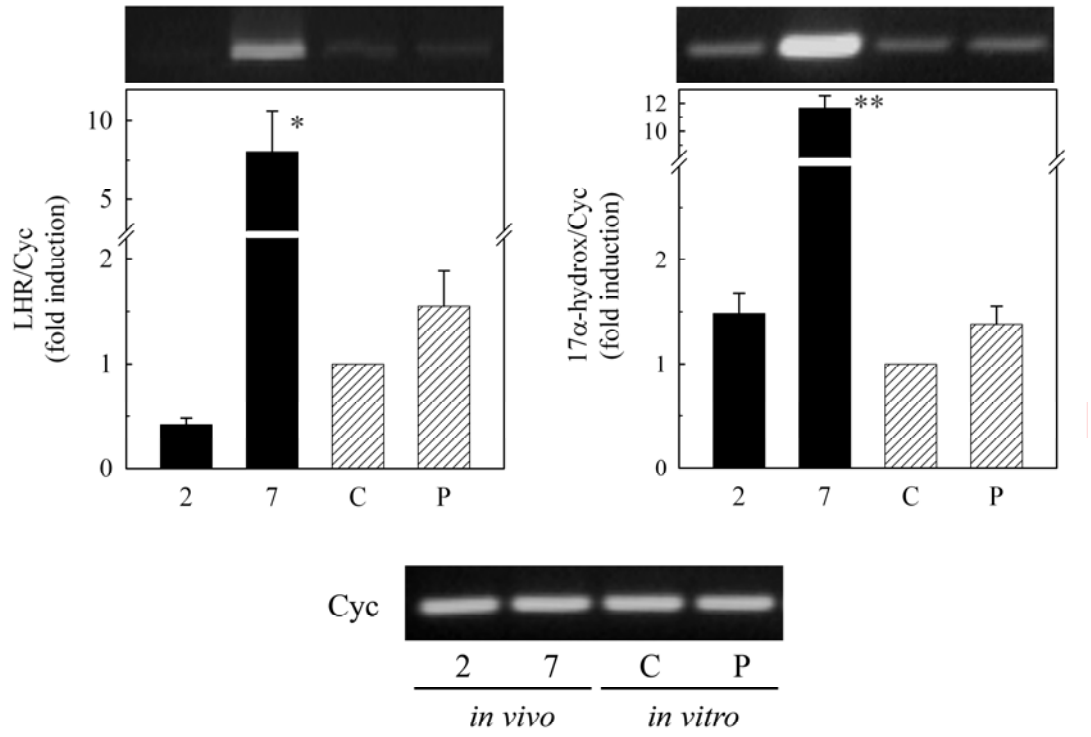


Fig. 17:

Semiquantitative RT-PCR of theca cells genes: LHR and 17α idroxilase in whole ovaries from 2 and 7 day-old rats, and in ovaries of 2-days cultured for 5 days with (P) and without PACAP 10^{-7}M(C).

This figure is representative of three independent experiments.

Optical density values of mRNA in each lane were normalized by respective optical density values of CyclophilinA (Cyc) signals and represent the mean ± SEM of them. Results are expressed relative to C set equal to 1.

*: P < 0,01; **: P < 0,001 vs. respective C.

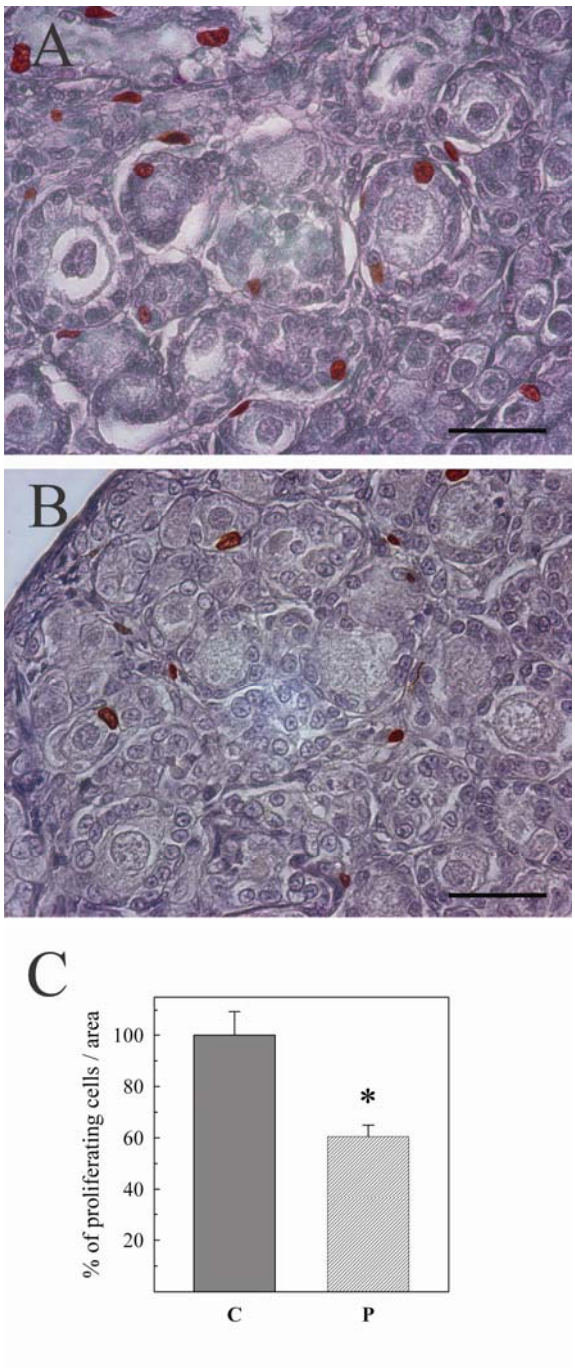


Fig. 18:

Micrographs of the proliferation assay, performed on newborn rat ovaries cultured for 3 days with (B) or without (A) PACAP 10^{-7}M stimulation.

The graph (C) shows the percentage of proliferating cells per area, in ovaries of 3 day old rats cultured for 3 days in absence (c) or presence (p) of 10^{-7}M PACAP, after BrdU incorporation. In PACAP treated ovaries proliferating cells were 59% of those in control ovaries. Values represent the mean ± SEM of four independent experiments.

*P<0.004 vs. C

Effect of PACAP on FSH-induced follicle growth in new born ovaries

Mouse preantral follicles cultured for 5 days in the presence of FSH increase in diameter and form an antrum (Cecconi *et al.* 2004a). Moreover, full-length mRNA for FSH-R appears in rat ovary at day 3 post partum (Rannikki *et al.* 1995). To understand whether PACAP may influence FSH-induced follicle growth, ovaries from 2-day-old animals were cultured for 5 days with 10^{-7}M PACAP, 100µM FSH, PACAP and FSH (administered simultaneously), and PACAP for 2 days followed by FSH for the last 3 days. The number of growing or resting follicles was counted in two sections from the largest cross-section through the centre of the ovary and averaged. Only the follicles in which the nucleus of the oocyte was clearly visible were counted. A significant reduction in growing follicles was evident in the samples stimulated with PACAP compared to control ovaries. FSH treated ovaries presented more growing follicles compared with the control sample. The addition of PACAP together with FSH inhibited FSH induced growth and a greater reduction was observed in the samples cultured with PACAP for 2 days and then with FSH (Fig,. 19).

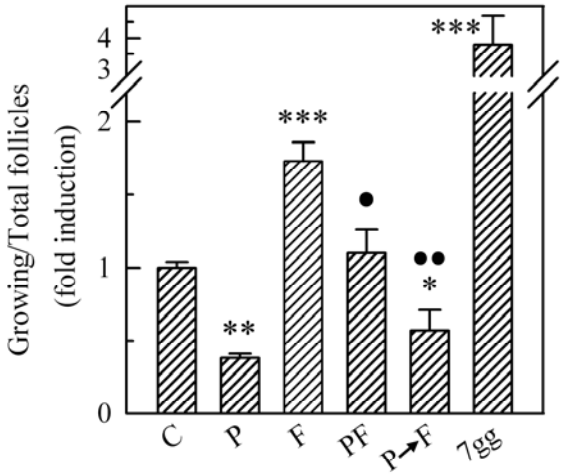


Fig. 19:

As previously shown PACAP treated ovaries presented less growing follicles respect to the Control (C).

FSH treated ovaries presented a significant increase in growing follicles compared with the Control sample. The addition of PACAP together with FSH inhibited FSH induced growth and a greater reduction was observed in the samples cultured with PACAP for 2 days and then with FSH.

Data are the mean ± SEM of four independent experiments with three or four replicates per experiment.

* : P<0,05; ** : P<0,01; *** : P<0,001 vs.C.

●: P<0,01; ●●: P<0,001 vs.F.

Regulation of gene expression of cultured whole ovaries mediated by PACAP

To understand the effect of PACAP on the expression of genes typical of granulosa cells (such as inhibin α, FSH-R, aromatase) and of germinal cells (GDF9) were carried out RT-PCR using cultured whole newborn rat ovaries.

Densitometric analysis of the RT-PCR products showed that genes expressed in granulosa cells increased with age but remained at the same levels in control cultures. FSH increased their expression. PACAP alone did not have any effect whereas it significantly inhibited the FSH-stimulated mRNAs (Fig. 20).

P450 aromatase strongly decreased in control cultures and PACAP induced its expression as already shown by Gras (Gras *et al.* 2005a). However, when added together with FSH, PACAP significantly inhibited FSH-stimulated P450 arom mRNA.

The oocyte specific marker GDF9 was evaluated in the same samples. As already shown (Durlinger *et al.* 2002b) GDF9 mRNA expression levels increased in freshly isolated ovaries from 2 to 7 days. In culture its expression increased in all samples and no variations were found in ovaries cultured with PACAP or FSH either alone or with PACAP.

Effect of PACAP on FSH-induced follicle growth on isolated preantral follicle

The effects exerted by PACAP on follicle development were evaluated also on isolated preantral follicles. Preantral follicles were individually cultured in medium alone or stimulated with 10^{-7} M PACAP, 100 μM FSH or with PACAP and FSH together. As shown in Figure 21 follicles cultured in control medium or with PACAP alone did not growth significantly in diameter. It is important to notice that our culture conditions are not optimal because the growth in vitro is lower than in vivo. After 8 days of culture in vitro, follicles reach a diameter of about 200 μm while in vivo follicles may exceed 400 μm.

As shown in previous studies (Rao *et al.* 1978), even in our cultures FSH causes growth of follicles from preantral (approximately 130 μm) to follicles of about 200 μm in diameter. Although the PACAP alone did not influence follicular growth, it has an inhibitory effect on growth of preantral follicles stimulated by FSH. Indeed at the end of the 8 days of culture, the diameter of follicles grown in the presence of FSH and PACAP together was approximately 170μm against 200 μm achieved with only FSH.

The rate of proliferation was evaluated by measuring the levels of expression of PCNA and Cyclin D2 in the different experimental conditions. At the end of the 5 days of culture, follicles were isolated and PCNA and Cyclin D2 mRNA levels were assessed by means of semiquantitative RT-PCR. Densitometric analysis of the amplified product showed that FSH increased the levels of PCNA and Cyclin D2 mRNA. The addition of PACAP decreased both basal and FSH stimulated levels of PCNA and Cyclin D2 mRNAs (Fig. 22).

Regulation of gene expression mediated by PACAP in cultured isolated preantral follicles.

In order to investigate whether the effect of PACAP on gene expression was due to the decrease of growing follicles or a real decrease of gene expression in the different cell populations the expression of CYP19 and α-Inhibin were analysed by RT-PCR in freshly isolated whole preantral follicles or after 8 days in culture in the presence of 10^{-7}M PACAP, or with 100 ng/ml FSH alone or together with 10^{-7}M PACAP. CYP19 mRNA was expressed at high levels in freshly isolated follicles and decreased in culture (Fig. 23). The addition of PACAP or FSH increased aromatase mRNA levels and the two together further increased its expression. This suggests that the lower levels of mRNAs observed in whole ovary culture in the presence of PACAP is consistent with the observed lower presence of growing follicles in PACAP treated ovaries.

In all conditions examined GDF9 expression did not change significantly.

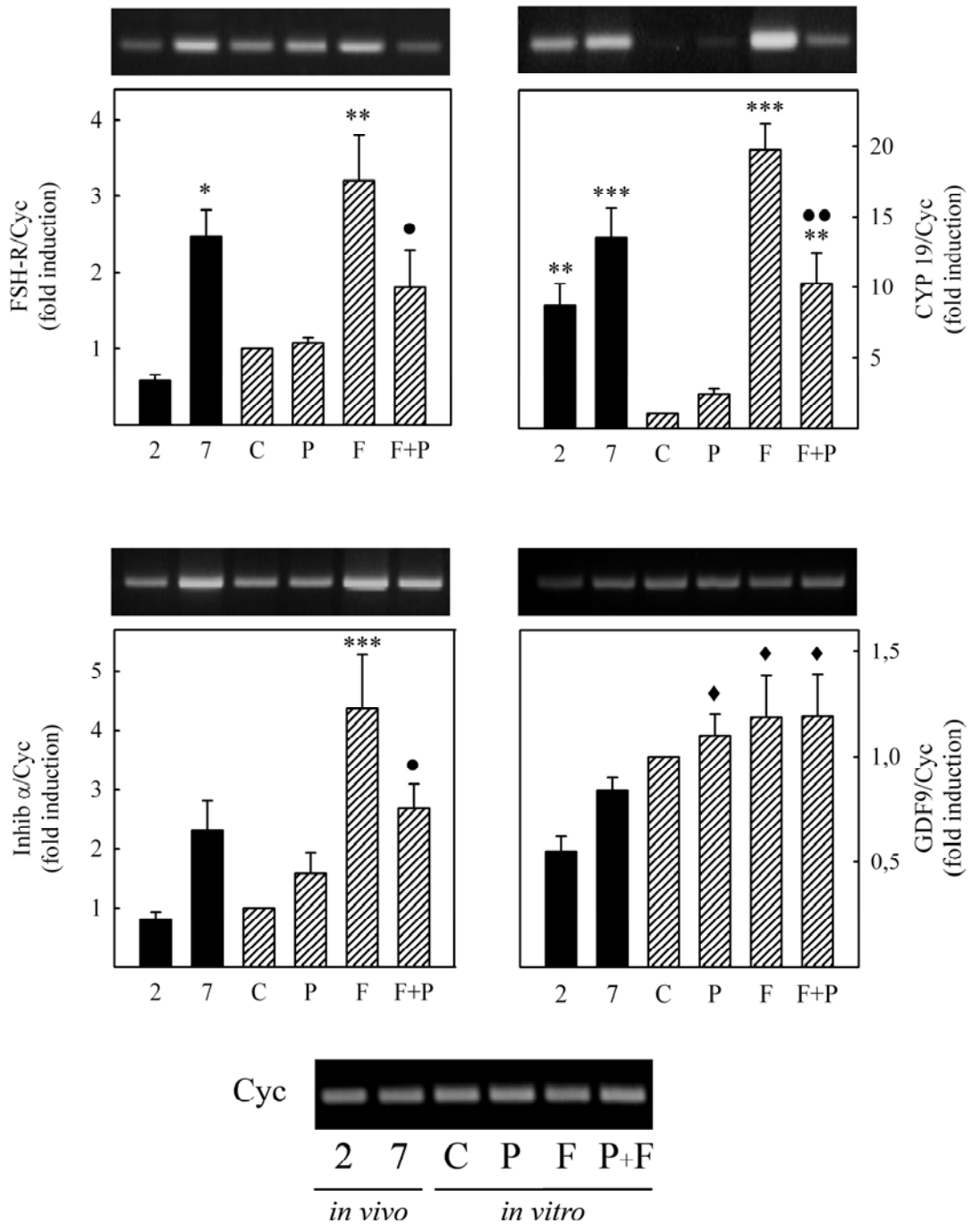


Fig. 20:

Semiquantitative RT-PCR of granulosa cells genes(FSH-R, CYP19, Inhibin α) and of germinal cells gene (GDF9) in whole ovaries from 2 and 7 day-old ovaries, and in 2-day-old ovaries cultured for 5 days with (P) and without PACAP (C), with FSH (F) and with FSH and PACAP administrated simultaneously (F+P).

This figure is representative of three independent experiments.

Optical density values of mRNA in each lane were normalized by respective optical density values of CyclophilinA (Cyc) signals and represent the mean ± SEM of them. Results are expressed relative to C set equal to 1.

*: P <0,05; **: P < 0,01; ***: P <0,001 vs. respective C.

●: P < 0,05; ●●: P < 0,01 vs. respective F.

♦: P < 0,05 vs. 2.

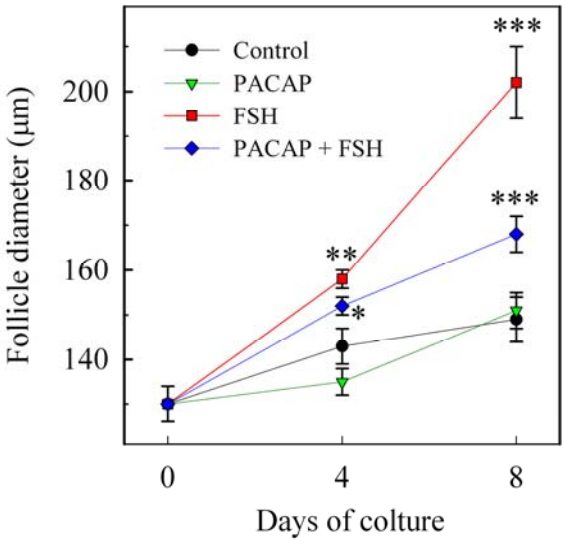


Fig. 21:

Preantral follicles were individually cultured for 4 or 8 days in medium alone (Control), or supplemented with FSH (100ng/ml), PACAP (10-7M), FSH+PACAP. Follicular diameter was monitored using a calibrated micrometer at the fourth day and at the end of the culture.

Values in the graph represent the mean ± SEM of three to five independent experiments. The total number of follicles examined was between 200 and 400 for each point.

*: P< 0,05 ; **: P<0,01; vs.C (day 4)

***: P<0,001 vs. C (day 8)

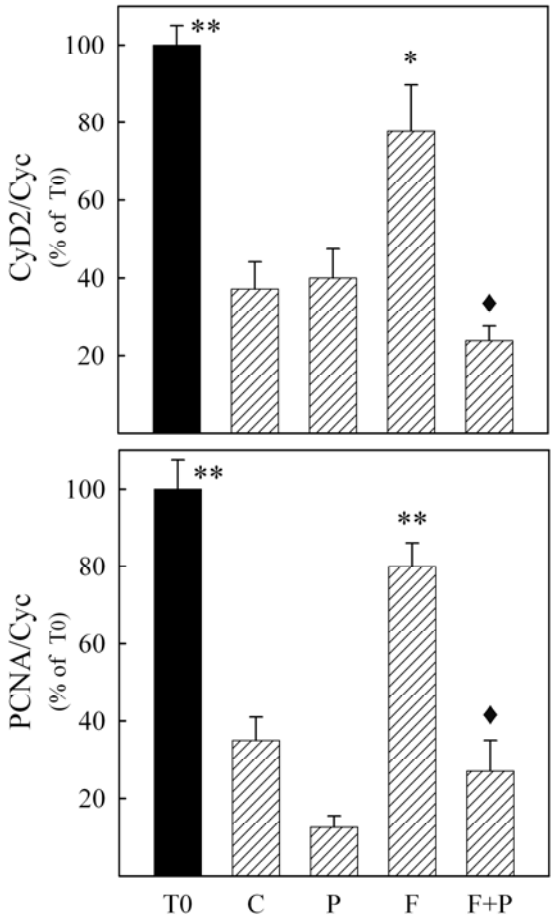


Fig. 22:

Effect of PACAP on the expression of cyclin-D2 and PCNA in isolated preantral follicles. Preantral follicles were cultured for 8 days with medium alone (C), 100ng/ml FSH with or without 10^{-7}M PACAP. The levels of mRNA expression was evaluated by RT-PCR.

Optical density values of mRNA in each lane were normalized by respective optical density values of CyclophilinA (Cyc) signals and represent the mean ± SEM of them. Results are expressed relative to To set equal to 100.

*: P< 0,01; **: P< 0,001 vs. C

♦: P<0,001 vs. F

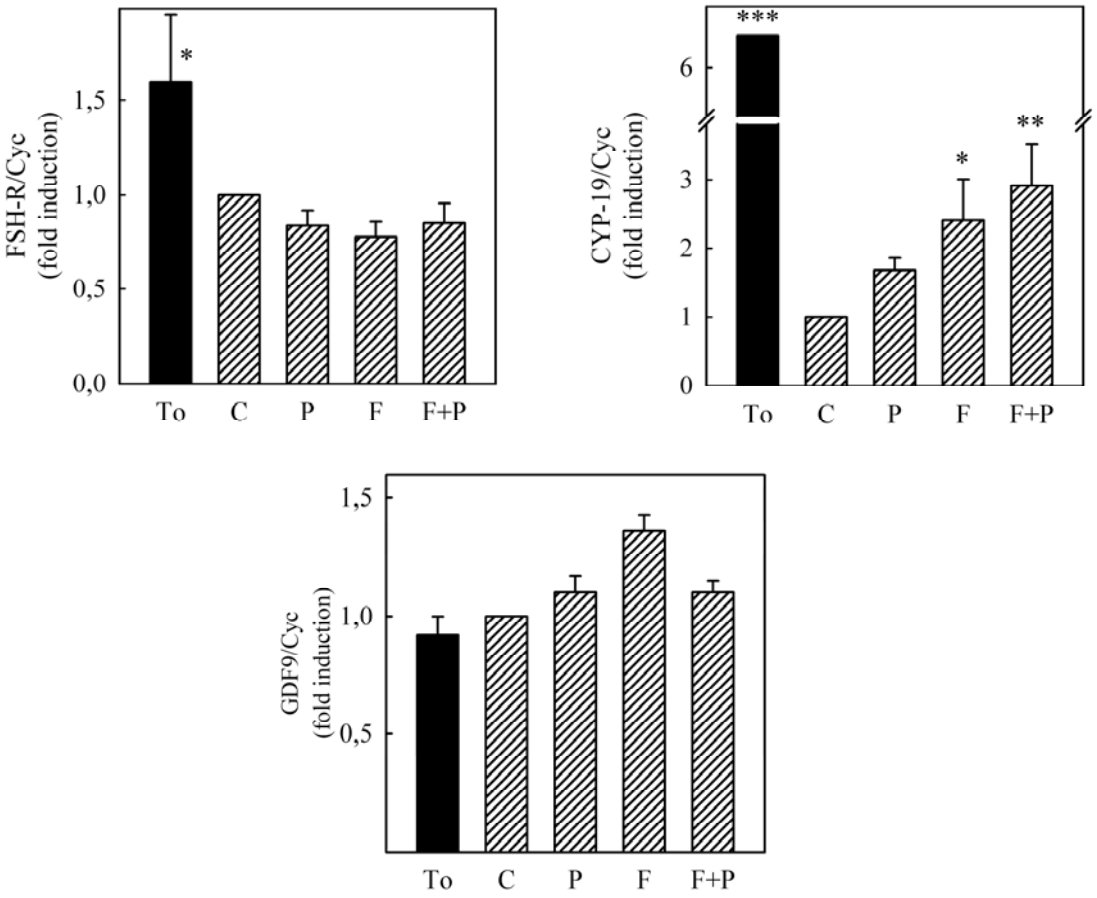


Fig. 23:

Effect of PACAP on the expression of FSH-R, CYP19, and GDF9 in isolated preantral follicles. Follicles were cultured for 8 days with medium alone (C), PACAP 10^{-7} M, 100ng/ml FSH with or without PACAP.

The levels of mRNA expression was evaluated by RT-PCR.

Optical density values of mRNA in each lane were normalized by respective optical density values of CyclophilinA (Cyc) signals and represent the mean ± SEM of them. Results are expressed relative to C set equal to 1.

*: P< 0,05; **: P<0,01; ***: P<0,001 vs. respective C.

DISCUSSION

The exact mechanisms controlling the initiation of the growth of small ovarian follicles are largely unknown. In humans and experimental animals, the removal of tissue from the ovary into culture induces the activation and growth of follicles (Hovatta *et al.* 1999). This suggests the removal of unknown inhibitory factors that were present in situ to maintain the follicles in a dormant state. It is accepted that many local factors produced in the ovary function to control early follicle development.

The presence of PACAP in the proximity of primordial and primary follicles, in hCG stimulated ovaries, (Gras *et al.* 1996b) suggests that PACAP, in addition to its role on preovulatory follicles, may also act by modulating early folliculogenesis.

In a previous paper Park et al. (Park *et al.* 2000a) showed by in situ hybridisation the expression of PAC_1-R mRNA in some small preantral follicles. In this thesis we show by RT-PCR the presence of the PACAP and VIP receptors in juvenile rat ovaries with PAC_1-R being the main receptor form. PAC_1-R was localised by immunohistochemistry in granulosa cells of primary follicles. A strong signal was observed also in the oocytes. However, even thought we previously showed the presence of mRNA for PAC_1-R in fully grown oocytes (Vaccari *et al.* 2006a), here, in experiments of RT-PCR, oocytes showed only a faint band when compared to granulosa cells suggesting very low levels of mRNA for this peptide. We show also by RT-PCR the presence of mRNA for PACAP. However, we were not able to demonstrate by in situ the presence of PACAP mRNA in granulosa cells from juvenile animals except in some interstitial glandular cells. Since it has previously been observed that in rat interstitial glandular cells PACAP is constantly expressed throughout the ovarian cycle (Gras *et al.* 1996a; Koh *et al.* 2000) it is possible that PACAP mRNA found in juvenile ovaries depends on the presence of PACAP in those cells.

In the present paper we investigated the effect of PACAP on initiation of follicle growth culturing ovaries from 2-day-old rats. The ovaries of 2-day-old rats are an useful model to study initiation of follicle growth because there are mainly primordial follicles, some primary follicles and no developing follicles (Fig. 15A). After 5 days of organ culture some primordial follicles start to grow also in the absence of stimulation in control medium, however follicles do not form the multilaminar structure observed in 7-day-old rat ovaries. Theca cells appear more disorganized and genes markers of theca cell differentiation do not increase in culture (Fig. 17). As already

shown in mice (Eppig & O'Brien 1996) even though follicles do not grow at the same rate as in vivo, oocytes are growing reaching a diameter similar to that obtained in older animals

The addition of PACAP to organ culture resulted in a smaller number of growing follicles compared to control cultures. The increase in follicle size depends on a balance between an increase in the number of granulosa cells and the levels of apoptosis. In our cultures the effect of PACAP was not on granulosa cell apoptosis but affected granulosa cell proliferation as evidenced by the decrease of BrdU incorporation in granulosa cells.

Gonadotropins (i.e. FSH and LH) have been shown not to influence the initiation of primordial follicle development, however FSH-Rs are already present at early preantral stage. Even though, in vivo, follicles grow in the absence of FSH, in culture, FSH is necessary to induce follicle growth.

In our organ cultures follicle growth was stimulated by FSH and this effect was significantly inhibited by addition of PACAP.

In order to understand the effect of PACAP on follicle growth and differentiation we studied the expression of genes related to follicle differentiation. In fact, as soon as granulosa cells initiate their differentiation, genes involved in this process start to be expressed. In control cultures we observed a significant decrease in the expression of aromatase and a slight increase in FSH-Rs but not of inhibin-α. PACAP did not affect FSH-R and inhibin-α expression and sligtly stimulated aromatase mRNA levels. FSH was able to stimulate all genes examined and PACAP significantly inhibited this expression. No differences were found for an oocyte specific marker GDF-9 in all culture conditions examined.

It has been previously suggested by Gras et al (Gras *et al.* 2005b) that immature rat follicles stimulated with PACAP may have an increased growing potential since ovaries primed with PACAP have an higher production of estradiol in response to FSH. Moreover, in the same paper they were not able to demonstrate an increase in FSH-Rs in response to PACAP. Our data are partially in accordance to Gras et al: we found stimulation of aromatase expression and no stimulation of FSH-Rs in ovaries treated with PACAP. However, contrasting with their hypotesis we demonstrated that PACAP had a negative effect on follicle growth.

When we incubated isolated preantral follicles, as already shown for mouse follicles, they did not grow unless FSH was present in culture. PACAP alone did not affect follicle growth but significantly inhibited FSH dependent follicle growth (Fig. 21) and inhibited the expression of cyclin D2 and PCNA in isolated follicle cultures.

When we evaluated the effect of PACAP on gene expression, we did not observe inhibition by PACAP on FSH induced expression of FSH-R and aromatase that we found in organ cultures. Actually, we found that PACAP had a sligthly additive effect on aromatase expression. A possible explanation of this apparent discrepancy is that in whole ovary cultures the decreased proliferation of granulosa cells modify the ratio between granulosa cells versus theca interstitial cells in favour of the latters while in follicle cultures the presence of granulosa cells is predominant.

Even if we could not find endogenous PACAP in immature ovaries, it may have an extrinsic origin. In fact, it may be carried in loco by extrinsic innervations, as yet documented for VIP in immature rat ovaries (Ahmed *et al.* 1986a) or simply, PACAP may not have a function during the first wave of folliculogenesis and may act only in cycling ovaries after the LH surge, when PACAP is produced by preovulatory follicles and may modulate the growth of follicles in close proximity.

In conclusion, our results suggest that PACAP and VIP are deeply involved in the ovarian physiology and that they can, at least in the adult ovary after the first LH surge, modulate FSH-dependent growth of developing follicles while acting synergistically with LH on the differentiation of preovulatory follicles.

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