The Ontogeny of Immunoreactive, Endogenous FSH and LH in the Rat Ovary during Early Folliculogenesis[1] (42834)

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Abstract. **Rabbit antisera to rat pituitary follicle-stimulating hormone (FSH) and to rat luteinizing hormone (LH) were used, in an immunocytochemical probe, to determine the ontogeny and distribution of immunoreactive, endogenous, intraovarian FSH and LH in immature rats. Ovaries from rats 4, 8, 12, and 21 days of age were studied. Both gonadotrophins were first immunodetectable on Day 8. In reactive primordial follicles, LH was restricted to the cytoplasm and nuclei of the surrounding follicle cells. In those follicles possessing both squamous and cuboidal follicle cells, i.e., transitional between primordial and primary, LH was found in both the cytoplasm and nuclei of both follicle cell types. In primary follicles, LH was no longer present in granulosa cells but was concentrated in germ cell cytoplasm. In contrast, in primordial follicles, FSH was restricted to the germ cell but was present in both the oocyte cytoplasm and germinal vesicle. In transitional and primary follicles, FSH remained within the oocyte cytoplasm and germinal vesicle but also became detectable within the cytoplasm and nuclei of granulosa cells. These findings raise some important new questions regarding the role(s) of the gonadotrophins in early follicular development.** [P.S.E.B.M. 1989, Vol 190]

In the rat, folliculogenesis begins during fetal life and results, at birth, in the presence of primordial follicles composed of an oocyte approximately 10 μm in diameter that is surrounded by a single layer of squamous follicle cells. These quiescent, primordial follicles number approximately 35,000 (1). From this reserve pool follicles begin to grow and develop into morphologically distinct primary follicles. In the transition from primordial to primary follicle, the oocyte increases in size and the surrounding follicle cells multiply and become cuboidal. Once a follicle has begun to grow, i.e., once it has left the nongrowing reserve pool, it is destined to undergo either atresia or ovulation.

Although the factors which might regulate the loss of follicles from the reserve pool remain unidentified, historically those factors have been thought not to be the classical pituitary gonadotrophins. The evidence upon which this idea is based comes primarily from work in immature rats. However, we have recently reported the accumulation of endogenous, immunoreactive follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by selected primordial follicles and by primary follicles in adult cycling rats (2). This work, therefore, was undertaken to examine both the onset and distribution of immunodetectable FSH and LH in the developing, immature rat ovary.

Materials and Methods

Animals. Sprague-Dawley rats (Charles River Inc., Wilmington, MA) were maintained in accordance with Public Health guidelines under controlled temperature (±22°C) and light (14:10). Daily vaginal smears were taken, and adult male rats were placed overnight in cages with those females in proestrus. Pregnant rats, identified by the presence of a vaginal plug, were kept

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one per cage and provided food and water *ad libitum*. The day of parturition was designated Day 0. Female offspring, 4, 8, 12, and 21 days of age (three per age group), were killed by cervical dislocation, after which their ovaries were collected, fixed in Bouin's fluid, embedded in paraffin, cut into 5-μm serial sections, and mounted four sections per microscope slide in preparation for immunocytochemical staining. Representative slides from each age group were prepared for routine histology with hematoxylin and eosin. Pituitaries collected from adult Sprague-Dawley rats, bilaterally ooph-orectomized 3 months earlier, were prepared as described above for immunocytochemistry and used to validate antisera specificity.

Reagents. Rabbit antisera to rat pituitary FSH (NIH-FSH S-9, S-11) and LH (NIH-S-9, S-10) were diluted in phosphate-buffered saline (PBS, pH 7.0) containing 0.05 *M* EDTA to working concentrations of 1:100 and 1:200, respectively. Highly purified rat FSH (NIH-FSH I-6) and rat LH (NIH-LH I-6) were used to test for antiserum specificity. The purified hormone was reconstituted and diluted with PBS. Both the antisera and purified hormone preparations were a gift from the National Pituitary Agency.

Nonconjugated goat anti-monkey γ-globulin (Antibodies, Inc., Davis, CA) diluted 1/1000 in PBS (pH 7.0) was used to block direct binding of the secondary antiserum to the tissue. Goat anti-rabbit γ-globulin conjugated to peroxidase (Antibodies, Inc.) and diluted 1/1000 with PBS-0.01% gelatin was the secondary antiserum. Diaminobenzidine-H_2O_2 (Sigma Chemical Co., St. Louis, MO) served as the chromogenic substrate.

Immunocytochemistry. The immunocytochemical protocol used has been described in detail (3). Briefly, deparaffinized, rehydrated, PBS-washed tissue sections were exposed to the following incubation sequence: (i) a series of six 15-min incubations with anti-monkey γ-globulin; (ii) one 4-day incubation with anti-LH or anti-FSH; and (iii) a series of up to ten 15-min incubations with anti-rabbit γ-globulin-peroxidase followed by a 2-min diaminobenzidine-H_2O_2 incubation. Each incubation step was preceded by a PBS wash (pH 7.0) and followed by a tap water wash (pH 5.5) and was done at room temperature in a moist chamber.

Antisera Specificity Tests. Absorption tests were done, using 21-day-old ovaries and oophorectomized adult rat pituitaries, with the same concentrations of antisera that were used to detect tissue immunoreactivity. In each test, hormones were added in 2-μl aliquots directly to 8 μl of the antiserum that had just been applied to the tissue section. Ovary sections were incubated in the primary antiserum admixtures for 4 days as described above; pituitary gland tissue sections were incubated for 1 day. Cross-absorption tests were done as additional antisera specificity tests. Purified rat FSH I-6 was crossed with anti-LH and rat LH I-6 was crossed with anti-FSH at hormone concentrations which completely absorbed their respective antisera.

Results

Antisera Specificity Tests. Neither anti-LH nor anti-FSH cross-reacted with any of the other pituitary hormones when tested on pituitary glands from the adult oophorectomized rats. All antisera were immunolocalized within both normal size and enlarged gonadotrophins, characteristic of this cell type following castration (Fig. 1A). Evidence for complete absorbability (20 μg LH/ml anti-LH and 25 μg FSH/ml anti-FSH) in the pituitary is given in Figure 1B.

Anti-FSH activity in the antisera directed against FSH could be completely absorbed with highly purified, iodination grade hormone (24 μg FSH/ml anti-FSH; Fig. 1C cf, Fig. 1D). Anti-FSH potency was not lost when these antisera were premixed with purified LH. Likewise, anti-LH activity in the antisera directed against LH could be abolished with purified LH (200 μg LH/ml anti-LH; Fig. 1E cf, Fig. 1F), but did not lose immunopotency when premixed with purified FSH.

General Histology. The rat ovary at 4 days of age consists largely of undifferentiated stromal cells and primordial follicles averaging 30 μm in diameter (Fig. 2A). By 8 days of age, some of these follicles have entered the growing pool. By then, in addition to the many primordial size follicles, the rat ovary contains many transitional follicles (those follicles having characteristics shared by both primordial and primary follicles, namely, both squamous and cuboidal follicle cells surrounding the same oocyte), and both small (one to two granulosa cell layers) and medium (three to four granulosa cell layers) sized primary follicles which have undergone hypertrophy and hyperplasia and acquired definable theca layers (Fig. 2B). By 12 days of age, larger primary follicles (approximately 100 μm) and small antral follicles are also present (Fig. 2C). The 21-day-old rat ovary contains even larger antral follicles with well-defined theca (Fig. 2D).

Ontogeny of Immunoreactivity. Neither FSH nor LH was detectable at 4 days with any of the four antisera tested but both were detectable in primordial, transitional, and primary follicles at 8, 12, and 21 days of age. The immunodistribution patterns once established did not vary with the age of the animal but were heterogeneous with respect to both hormone and stage of follicle development. The results are summarized in Table I.

Distribution of Immunoreactivity. LH was immunodetectable in both the nuclei and cytoplasm of the squamous follicle cells of primordial follicles (Fig. 3A). In most follicles, LH immunoreactivity was not detectable in either the oocyte nucleus or cytoplasm. When it was, it was restricted to the cytoplasm. Immunostain-

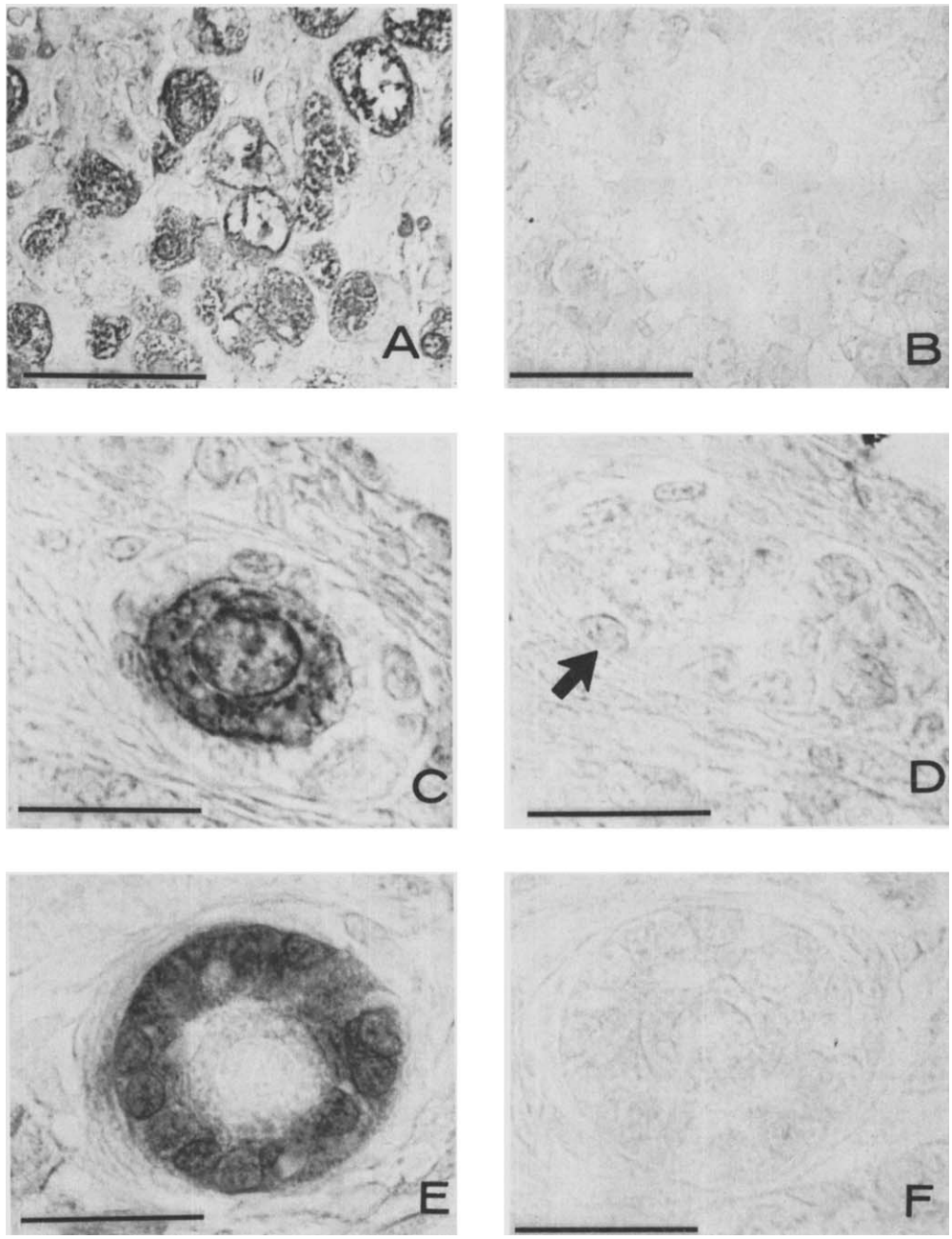

Figure 1. Antisera controls. A, LH-positive pituitary gonadotrophins from bilaterally oophorectomized rats. Bar = 50 μm. B, Pituitary gonadotrophins. Absorbed control (20 μg LH/ml anti-LH). C, Typical FSH-positive early transitional follicle. Bar = 30 μm. D, Transitional follicle on adjacent tissue section. FSH absorption control (24 μg FSH/ml anti-FSH). E, Typical LH-positive transitional follicle. Bar = 30 μm. F, Transitional follicle on adjacent tissue section. LH absorption control (200 μg/ml anti-LH).

able FSH was found in oocyte cytoplasm and occasionally in oocyte nuclei; follicle cells were nonreactive (Fig. 3D).

Although both squamous and cuboidal follicle cells of transitional follicles contained cytoplasmic and nuclear LH immunostaining, oocytes were completely devoid of LH immunoreactivity (Fig. 3B). FSH was found in the cytoplasm of oocytes and occasionally in the oocyte nucleus. Additionally, immunoreactive FSH was homogeneously distributed in both the cytoplasm and nuclei of both squamous and cuboidal follicle cells (Fig. 3E).

In primary follicles, regardless of the number of granulosa cell layers, immunodetectable LH was found in oocyte cytoplasm and was occasionally associated with the germinal vesicle (Fig. 3C). In contrast, FSH was immunodistributed in oocyte cytoplasm and follicle cell nuclei and cytoplasm (Fig. 3F).

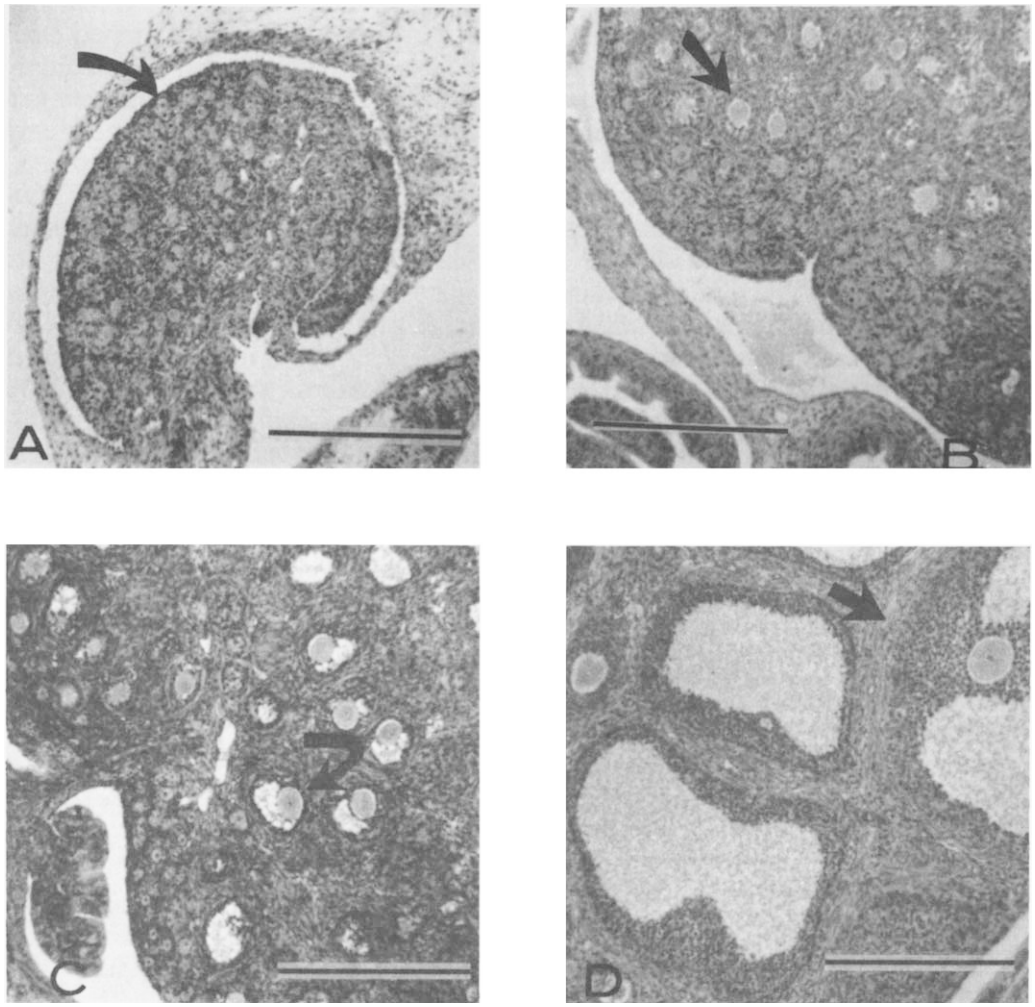

Figure 2. Immature rat ovaries stained with hematoxylin and eosin. Bars = 480 μm. A, 4-day-old ovary. Note abundance of primordial follicles. B, 8-day-old ovary. Note primordial and small primary follicles. C, 12-day-old ovary. Note presence of small antral follicles. D, 21-day-old ovary. Note large antral follicles.

Table I. Summary of Immunocytochemical Distribution Patterns of FSH and LH within Primordial and Primary Follicular Compartments

Results	Primordial		Primary	
	FSH	LH	FSH	LH
Oocyte germinal vesicle	+[a]	−	−	−
Oocyte cytoplasm	+	−	+	+
Granulosa cell nuclei	−	+	+	−
Granulosa cell cytoplasm	−	+	+	−

[a] +, stained; −, no stain.

Discussion

A predominant feature of early postnatal ovarian development is the initiation of nongrowing primordial follicles into the growing pool, a process that continues throughout reproductive life. The evidence that this initiation of primordial follicle growth and the subsequent follicle development up to antrum formation is independent of the pituitary is overwhelming (4–20). *What then is the meaning of our findings?* We believe the half-lives of FSH and LH may be sufficiently long in the maturing ovary to obviate the need for their continuous availability from the anterior pituitary.

Our absorption studies satisfy the criterion of antisera specificity which is most important for validating immunocytochemical data. Antisera were fully absorbable with their respective purified hormones. Thus, the results clearly indicate that the antisera are detecting molecules which have immunoreactive epitopes in common with pituitary gonadotrophins, although this is not to say that the ovarian versions of FSH and LH are precisely identical to the pituitary forms. In addition, cross-absorption could not be obtained by admixture, indicating single hormone specificity. Further evidence that these antisera do not cross-react with each other is provided by the ovary itself, where FSH was distributed differently from LH, sometimes even in the same follicle. These antisera do not cross-react recip-

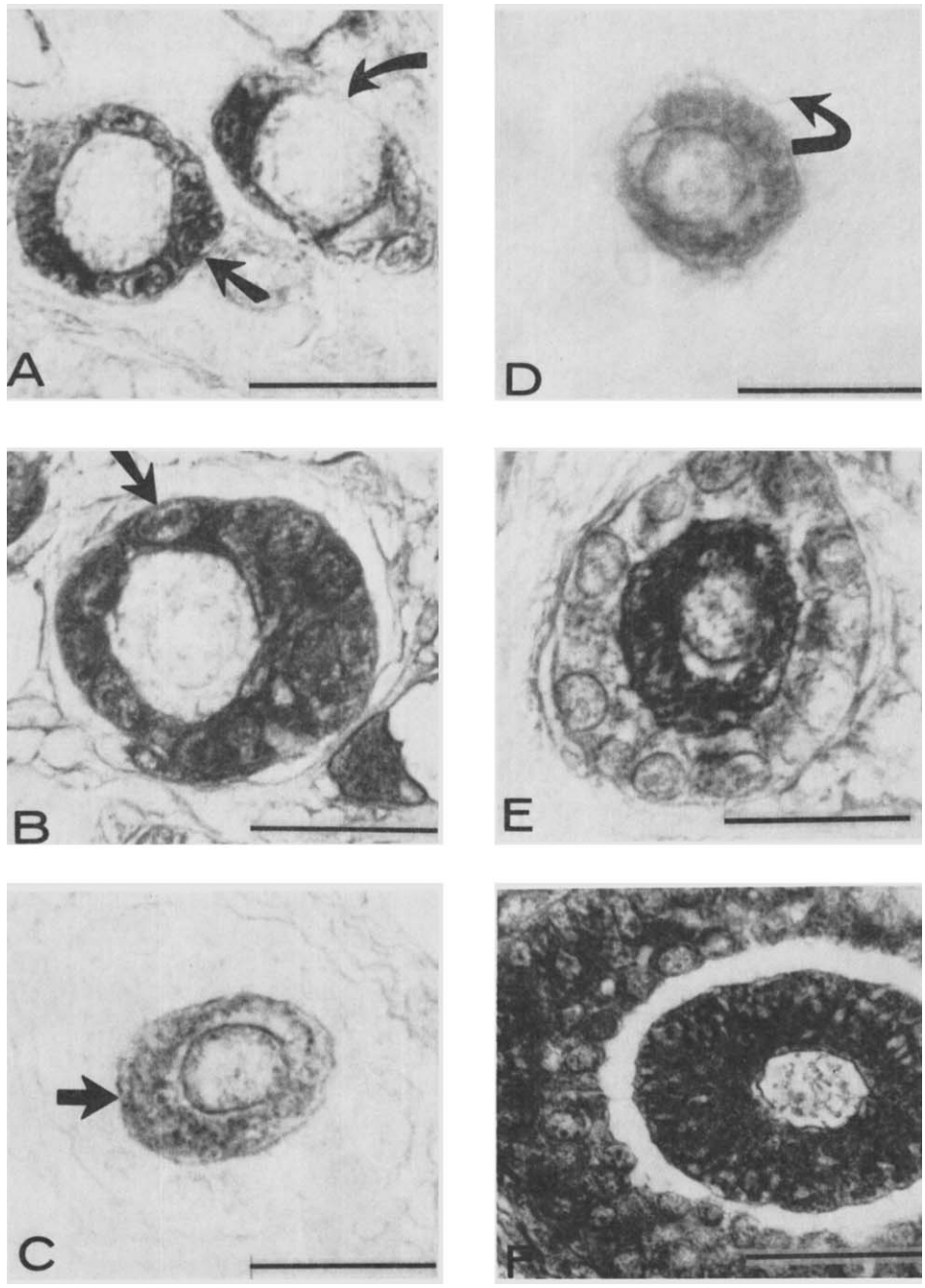

Figure 3. Follicles reacted with anti-LH (1:375) and anti-FSH (1:125) demonstrating the transition from primordial to primary follicle and the associated distributional changes in immunoreactive LH and FSH. Bars = 30 μm. A, Two primordial follicles from a 21-day-old ovary reacted with anti-LH. Note LH-positive cytoplasm and nuclei of follicle cells and LH-negative oocytes. B, An LH-positive transitional follicle from a 12-day-old ovary. Note immunoreactivity is restricted to granulosa cell cytoplasm and nuclei. C, An early primary follicle from a 21-day-old ovary showing LH immunoreactivity exclusively within the oocyte cytoplasm. D, Primordial follicle from an 8-day-old ovary stained with anti-FSH. Note FSH-positive oocyte cytoplasm and germinal vesicle and FSH-negative follicle cell. E, A transitional follicle from a 12-day-old ovary showing FSH immunoreactivity within both cytoplasm and nuclei of both granulosa cells and the oocyte. F, FSH uniformly distributed throughout both oocyte and granulosa cells of a large primary follicle from a 21-day-old ovary.

rocally and they do not cross-react with any of the other anterior pituitary hormones. Their activity is confined to the morphologically identifiable cell population in which such reactivity would be expected (i.e., in the pituitary gonadotrophs), indicating their specificity.

Thus, not only do immunoreactive FSH and LH appear to be present in small "gonadotrophin-independent" follicles in the adult rat, where the number of positive primordial follicles increases at proestrus (2), we have now found similar reactivity in a few, select follicles at the time of the very first recruitment of primordial follicles into the growing pool. Both the work in the adult and in the immature rat represent completely physiologic, unmanipulated circumstances.

Nothing was done to the animals nor did we study unoccupied "receptors" or "binding sites." It should be noted that although we clearly did not study unoccupied receptors, the detection of endogenous hormones includes membrane receptor-bound, intracellular receptor-bound, and free intracellular gonadotrophins.

Both gonadotrophins were first detectable by 8 days of age. This corresponds chronologically with (i) the initial postpartum rise in serum gonadotrophins (21); (ii) the earliest detectability of unoccupied binding sites on both oocytes and granulosa cells (22, 23); and (iii) the ontogeny of functional gonadotrophin receptors in the neonatal ovary (24). It is noteworthy that in our work, only a small percentage of primordial follicles in any given cross-section contained immunoreactive gonadotrophins (data not shown). This suggests a role for FSH and LH in the recruitment of follicles into the growing pool.

Although follicle recruitment per se may represent one of the intracellular functions of intrafollicular gonadotrophins, they may also be involved in the subsequent development of small follicles. A change in the immunolocalizations of both FSH and LH occurred as primordial follicles became primary follicles. FSH, restricted to oocytes in primordial follicles, was detected in both oocyte and granulosa cells of both transitional and primary size follicles. In contrast, LH localization in transitional and primary size follicles resembled that seen in primordial follicles but was quite different in primary follicles where it was restricted to oocytes. The loss of immunodetectable LH from the granulosa cells of primary follicles agrees with the findings of Bortolussi *et al.* (25) who found no human chorionic gonadotrophin binding to primary follicle granulosa cells from immature rats. This very transition in immunolocalizations may be descriptive of follicle functions that are under gonadotrophin control.

Other data tend to support our overall concept. Amsterdam *et al.* (26) discovered that FSH stimulates flattened epithelioid granulosa cells to assume a spherical shape as cytoskeletal components respond *in vitro.* This conforms with our finding FSH only in cuboidal shaped granulosa cells. Perhaps, FSH is essential for early granulosa cell differentiation. Funkenstein *et al.* (27) and, more recently, Carson and Smith (28) have demonstrated that the onset of follicular steroidogenic competence in the rat occurs by the second week; the time at which we first identified intrafollicular, immunodetectable FSH and LH. Both gonadotrophins have been shown to stimulate RNA synthesis in primordial and small primary follicles *in vitro* when given exogenously to immature rats (29). FSH has been shown to increase DNA polymerase α activity in immature rat ovaries (30), which might explain the presence of FSH in granulosa cell nuclei reported here. Furthermore, the presence of gonadotrophin binding sites on intracellular organelles including nuclei, isolated from human and bovine ovaries, has been reported (31–34). Beyond this, in a series of recent articles reporting on studies in adult animals, Roy and Greenwald have recorded (i) binding of ^{125}I-labeled rat FSH to a mixture of isolated oocytes, each from rat, mouse, and hamster and of ^{125}I-labeled human chorionic gonadotrophin to hamster and mouse, but not rat(?) oocytes (35); (ii) an apparent dependence on FSH and LH for DNA synthesis that is dependent on follicle size in enzymatically dissociated preantral follicles from adult hamsters (36); and (iii) *in vitro* steroidogenesis in response to gonadotrophins not only by primary but also by primordial follicles (37). Evidence is thus accumulating now, from a variety of new end points, to support the concept that both recruitment and early follicular development are in fact dependent on the gonadotrophins. *But what about 50 years of evidence to the contrary?*

The answer may be simple. Each experiment which led to the conclusion that early follicular growth is gonadotrophin-independent included one universal assumption, that gonadotrophins were unavailable to the gonad because of hypophysectomy, isolation by tissue culture, or antigonadotrophin treatment. Previous studies did not consider the possibility that the half-lives of FSH and LH in the ovary may be sufficiently long to obviate the need for continuously available extraovarian hormone which we raise here. The work of Kim and Greenwald (38) where they found FSH bound to hamster granulosa cells 4 days posthypophysectomy in the absence of detectable serum FSH levels supports our hypothesis. Clearly, the next step is to examine the half-life of immunodetectable, intracellular gonadotrophins in a posthypophysectomy time course study. We suggest that the gonadotrophins may function as modulators of follicular growth initiation and differentiation and that they form the basis of a working hypothesis for understanding how these processes may be both pituitary gonadotrophin dependent and "independent"

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