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Proliferating Cell Nuclear Antigen Marks the Initiation of Follicular Growth in the Rat[1]

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ABSTRACT

Despite expanding knowledge on the kinetic aspects of folliculogenesis, the question of what initiates follicle growth remains unanswered. Efforts to solve this problem have been thwarted by the absence of sensitive markers to identify the onset of follicular growth. In this study, we determined whether increased proliferating cell nuclear antigen (PCNA) correlates with initiation of follicle growth and might therefore be useful for studying early events in this process. Paraffin sections of ovaries from cycling adult rats, prepubertal eCG + hCG-primed rats, and prepubertal control rats were processed for immunocytochemistry by use of a PCNA primary antibody. In primordial follicles, neither granulosa cells nor oocytes stained for PCNA. PCNA immunoreactivity coincided with the earliest sign of follicle growth, appearing in pregranulosa cells of early primary follicles just beginning to grow. In all primary follicles, some granulosa cells were PCNA-positive. PCNA immunoreactivity in oocytes first appeared in primary follicles, preceding oocyte enlargement. In preantral and antral follicles, granulosa cell PCNA staining was uniform throughout the granulosa cell layers. Oocytes were positive for PCNA in both preantral and antral follicles. PCNA expression diminished in atretic follicles. In CL, granulosa cell PCNA expression was also decreased. Theca cell PCNA expression was first evident in the transitional follicle (1–2-layer granulosa cells) and was present in all stages thereafter. The pattern of PCNA expression did not differ among adult cycling, prepubertal eCG + hCG-stimulated, and control rat ovaries. These results suggest that the expression of PCNA, an essential regulator of the cell cycle, marks the initiation of follicle growth, coinciding with and in some cases preceding the first sign of granulosa cell growth and preceding the oocyte growth.

INTRODUCTION

In mammals, the pool of primordial follicles is established in fetal or neonatal life depending on the species [1–4]. Once established, the reserve of primordial follicles begins to diminish as follicles initiate growth or die in situ [3]. Because the reserve of primordial follicles is never replenished, it ultimately becomes exhausted, usually after midlife [5].

Despite growing understanding of the kinetic aspects of folliculogenesis, what initiates follicle growth remains unanswered. Approaches to solve this problem have been thwarted by the absence of sensitive markers to identify the onset of follicular growth [3]. Morphological techniques can be used to distinguish the small growing follicles from quiescent ones, but morphological changes are probably consequences of earlier biochemical changes. [^{3}H]Thymidine labeling has also been used to identify growing follicles [6]. However, the mitotic rate of follicle cells is very low during the early phase of follicular growth. Even the premitotic transition from a primordial follicle to one with a single layer of columnar granulosa cells may take several weeks [6]. Consequently, not enough thymidine may be incorporated by these slowly dividing cells to reliably detect them through pulse-labeling. Hirshfield attempted to circumvent this problem by continuous infusion of [^{3}H]thymidine for up to 7 days [6] and noted that uptake of [^{3}H]thymidine by very small follicles increased in parallel to duration of administration. It was estimated that over 3 wk would be required for all the "very small" follicles initiating growth to incorporate thymidine. Such prolonged infusion studies are not compatible with studies involving short-term effects of hormones or other potential initiators of follicle growth. Also, because the method involves radioactive labeling and relatively long incubation time, it is not suitable for human studies [7].

We sought to identify a more sensitive and broadly applicable marker of the earliest events in follicle growth. An ideal marker would be present throughout the cell cycle once G_1 is entered and would be applicable to human studies. The candidates we considered were Ki-67 and Proliferating Cell Nuclear Antigen (PCNA). Ki-67, though not well defined, is a cell cycle-regulated antigen that is present throughout the cycle and can be detected by immunohistochemical techniques. However, Ki-67 can only be detected in frozen sections because it may deteriorate above −20°C, a limitation for optimal preservation of ovarian morphology.

By contrast, PCNA has been used successfully on formalin-fixed tissues as a proliferation marker for nearly a decade [7]. PCNA is a 35-kDa nuclear protein that has an essential role in cell cycle regulation [8]. During S phase, PCNA pairs with cyclin D [9], another important regulator of cell proliferation. This complex is modulated by various growth factors and other growth stimuli [8]. If appropriate stimuli are received during G_1, cells become committed to S phase, and PCNA expression increases through G_1/S-

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TABLE 1. Follicle classification*.

Stage	Name	Description
0	Primordial	Oocyte partially or completely encapsulated by squamous pregranulosa cells
1	Early primary	At least one of the pregranulosa cells became columnar (enlarged)
2	Primary	All granulosa cells show enlargement; single layer of granulosa cells
3	Transitional	1–2 layer of columnar granulosa cells; zona pellucida distinguishable
4	Preantral	Oocyte encapsulated by more than 2 layers of granulosa cells; no antrum formation
5	Antral follicle	Oocyte encapsulated by more than 2 layers of granulosa cells with antrum formation

*Modified after Peters et al. [11] and Hirshfield [12].

phase interface, reaching a plateau during G_2 [10]. PCNA expression sharply declines in M phase and in quiescence. These characteristics make PCNA a useful marker for proliferating cells.

The objective of this study was to determine whether increased PCNA expression correlates with initiation of follicle growth and whether it might therefore be a useful tool to study early events in follicular growth. We studied PCNA expression in ovaries from adult cycling rats, prepubertal eCG + hCG-stimulated rats, and prepubertal untreated control rats to assess whether PCNA expression varied across age and gonadotropin exposure.

MATERIALS AND METHODS

Animals and Tissue Handling

Three 60-day-old cycling and six 30-day-old immature inbred AXC/SSh rats from the colony maintained at the University were housed three per cage and fed ad libitum. Lights were on from 0600 h to 2000 h. Smears were taken from adult rats daily to assure cyclicity, and these rats were killed in the afternoon of estrus. Half of the immature rats received 20 IU of eCG s.c. followed by 10 IU hCG i.p. 60 h later. The remaining immature rats received injections of vehicle, and all immature rats were killed 16 h after the last injection. Ovaries were fixed in Bouin's solution for 6 h and transferred into 70% alcohol. After 24 h, they were embedded in paraffin and serially sectioned at 5 μm. Sections were mounted on coated slides (Vectabond; Vector Laboratories, Burlingame, CA).

Immunostaining Technique

Thirty-six sections were randomly selected from each ovary and stained for PCNA immunoperoxidase (Novocastra Laboratories, New Castle upon Tyne, UK). Sections were deparaffinized with 3% xylene for 3 min, rehydrated in ethanol, and quenched in 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. After washes with water and PBS, normal horse serum was added for 10 min at 37°C to prevent nonspecific binding. Then the first antibody was added (1/150), and sections were incubated 18 h at room temperature. After a wash in PBS, the second antibody (1/250) was added, and specimens were incubated for 15 min at 37°C. Avidin-biotin complex was added at 37°C for 20 min followed by diaminobenzadine (DAB) for 3 min. Sections were counterstained with hematoxylin.

Morphometry

All sections were examined at 200× to 1000× magnification under light microscopy to determine the follicle stage and PCNA staining characteristics. Follicles were classified (Table 1) according to a modification of the classifications of Peters et al. [11] and Hirshfield and Midgley [12]. Because our focus was on earlier stages of follicle development, when follicle growth progresses at a very slow rate, the classification is weighted towards earlier stages of follicle growth. We therefore differentiated between primary follicles with one or few enlarged (i.e., columnar no longer squamous) granulosa cells (early primary) and follicles with a single layer of uniformly enlarged granulosa cells (primary) in an effort to better distinguish the early changes during presumptive follicle growth in relation to PCNA. Rat ovaries were examined on the day after presumptive ovulation in cases of cycling and eCG-stimulated ovaries. Previous studies have shown that primordial follicles enter the pool of growing follicles regardless of the day of the estrous cycle [13].

Seven sections from each cycling (n = 3), eCG + hCG-stimulated (n = 3), and control (n = 3) rat were randomly selected for oocyte and follicle measurements. All follicles that contained an oocyte with a nucleus were examined in each section. Oocyte and follicle diameters were measured with a computer-assisted image analyzer (Perceptics, Knoxville, TN), under 400× magnification. Objects displayed on a monitor were outlined by a mouse-driven pointer. The average diameter of the object (oocyte or follicle) was computed in pixels and then converted to microns. Oocyte diameter was measured only when the oocyte nucleus was visible. The total number of oocytes measured was 106, 56, and 53 in cycling, prepubertal control, and prepubertal eCG + hCG-stimulated rats, respectively. For cycling rats, primordial (n = 23) and early primary (n = 12) follicle diameters were also measured on 10 randomly selected sections.

Statistical Analysis

Data are reported as mean ± SD and were analyzed by analysis of variance. When more than two groups were compared, differences between subgroups were tested by Student Newman-Keuls test. Probability of alpha error was set at 0.05.

RESULTS

Unless stated otherwise, descriptions are applicable to all three groups of rats studied.

Granulosa Cell PCNA Expression

Granulosa cells of primordial follicles did not exhibit PCNA immunoreactivity (Fig. 1A). When at least one enlarged granulosa cell was present in a follicle cross-section (early primary follicle), PCNA staining was present in at least one of the granulosa cells (Fig. 1B). However, the PCNA-positive granulosa cells of early primary follicles were not always enlarged. Mean diameter of early primary follicles was greater than that of primordial follicles (21.0 ± 2.1 vs. 18.2 ± 2.0 μm, $p = 0.001$). Hence, PCNA immunoreactivity was detectable at the earliest sign of follicular enlargement, and it was sometimes present in granulosa cells that had not begun to enlarge.

In primary follicles, in which all granulosa cells were enlarged, PCNA immunoreactivity was detectable in many of the granulosa cells (Fig. 1C). In transitional follicles (Fig. 1D) and preantral follicles (Fig. 1E), most granulosa cells displayed PCNA expression. Likewise, in antral follicles, granulosa cell PCNA expression was uniform (Fig. 2A). Atretic follicles varied in their PCNA expression. Follicles with fewer pyknotic granulosa cells, possibly in early atresia, retained their PCNA positivity in the cumulus and oocyte. In follicles demonstrating extensive pyknotic changes, presumably in advanced atresia, PCNA expression was either confined to a few granulosa cells, mainly in the cumulus (Fig. 2B), or was absent. In the fresh CL, PCNA immunostaining of cells was present but sparse (Fig. 2C), while no PCNA staining was seen in the older CL.

Theca Cell PCNA Expression

Theca cell PCNA immunoreactivity was first evident in transitional follicles. These follicles had well-defined theca layers that stained for PCNA (Fig. 1D). Theca cell PCNA expression persisted through the later stages of follicle growth (Figs. 1E and 2A) and in the CL (Fig. 2C). PCNA expression progressively diminished in theca cells parallel to the morphological evidence of atresia. Stromal staining was minimal and did not interfere with interpretation of theca cell staining (Fig. 2D).

Oocyte PCNA Expression

Neither oocytes from primordial nor early primary follicles expressed PCNA. Oocyte PCNA immunoreactivity was first evident in oocytes of primary follicles (Fig. 1C). Oocytes of follicles in later stages of growth were also PCNA-positive (Fig. 1, D and E, and Fig. 2A). In atretic follicles, oocyte PCNA immunoreactivity varied in parallel to the extent of atresia. In follicles displaying signs of early atresia, most oocytes were PCNA-positive. In follicles with more advanced atresia, PCNA was absent in oocytes (Fig. 2B).

Figure 3 summarizes the relation among follicle stage, oocyte diameter, and PCNA immunoreactivity in granulosa cells and oocytes of adult cycling, prepubertal eCG + hCG-stimulated, and control rats. In all groups, enlargement and PCNA immunoreactivity of granulosa cells preceded enlargement and PCNA immunoreactivity of oocytes. In oocytes, PCNA immunoreactivity preceded enlargement in all treatment groups. PCNA was first detected in oocytes of primary follicles; however, oocyte enlargement was not statistically significant until the transitional stage.

DISCUSSION

This study indicates that PCNA is a sensitive marker of early events in follicular growth. Increased PCNA expression correlates with the earliest signs of granulosa cell growth. In addition, oocytes begin to express PCNA early in follicular growth, before beginning to enlarge. PCNA immunoreactivity remains prevalent in granulosa and theca cells of follicles in subsequent stages of follicle growth and progressively diminishes with advancing atresia.

PCNA immunoreactivity did not precede the onset of morphologically detectable follicle growth, as demonstrated by absence of PCNA in cells from primordial follicles. However, in the earliest stage of follicle growth, PCNA began to appear in granulosa cells, even in many with no evidence of enlargement. This result indicates that PCNA immunoreactivity precedes cell enlargement in at least some granulosa cells. Some granulosa cells of follicles in the earliest stage of growth were PCNA-negative despite showing enlargement. Absence of PCNA in these enlarged granulosa cells may be because they were in M phase when PCNA expression is sharply reduced. This could also reflect an incomplete concordance between PCNA expression and the onset of granulosa cell proliferation. For example, some granulosa cells may vary in morphology despite not having initiated growth. Hirshfield also reported that [^{3}H]thymidine labeling patterns did not correlate with granulosa cell morphology in "very small" follicles [6].

Although we defined an "early primary follicle" stage on the basis of morphological changes in one or more granulosa cells and presumed that follicles so identified were initiating growth, some of these follicles may have been quiescent or growth-arrested. In subsequent stages of follicle growth, the patterns of PCNA expression in both granulosa and theca cells were similar to the those previously observed with [^{3}H]thymidine [3]. In a previous study, [^{3}H]thymidine incorporation by granulosa cells was uniform in the antral follicles of prepubertal rats and of cycling rats in estrus, whereas in rats in diestrus or proestrus, labeling was heavier in granulosa cells bordering antrum [14]. We studied only rats in estrus and, likewise, found no difference in PCNA immunostaining patterns of preantral and antral follicles between cycling rats in estrus and prepubertal rats. In general, the results indicate that PCNA immunoreactivity is an early marker of the onset of follicular growth. Whether

FIG. 1. Immunochemistry for PCNA; counterstained with hematoxylin (×1000). **A)** PCNA-negative primordial follicles (arrows). **B)** Early primary follicles with single granulosa cell PCNA staining (arrows). **C)** Primary follicles; both granulosa cells and oocyte stained for PCNA. **D)** Transitional follicle; granulosa cells, oocyte and theca cells PCNA-positive. **E)** Preantral follicle with 3–4 granulosa cell layers.

FIG. 2. PCNA immunochemistry. **A)** Antral follicle (×200). **B)** Atretic follicle; only a few cells in cumulus express PCNA (×200). **C)** Fresh CL showing sparse PCNA staining of cells (×200). **D)** Section of ovary showing PCNA-positive follicles of various stages (×4).

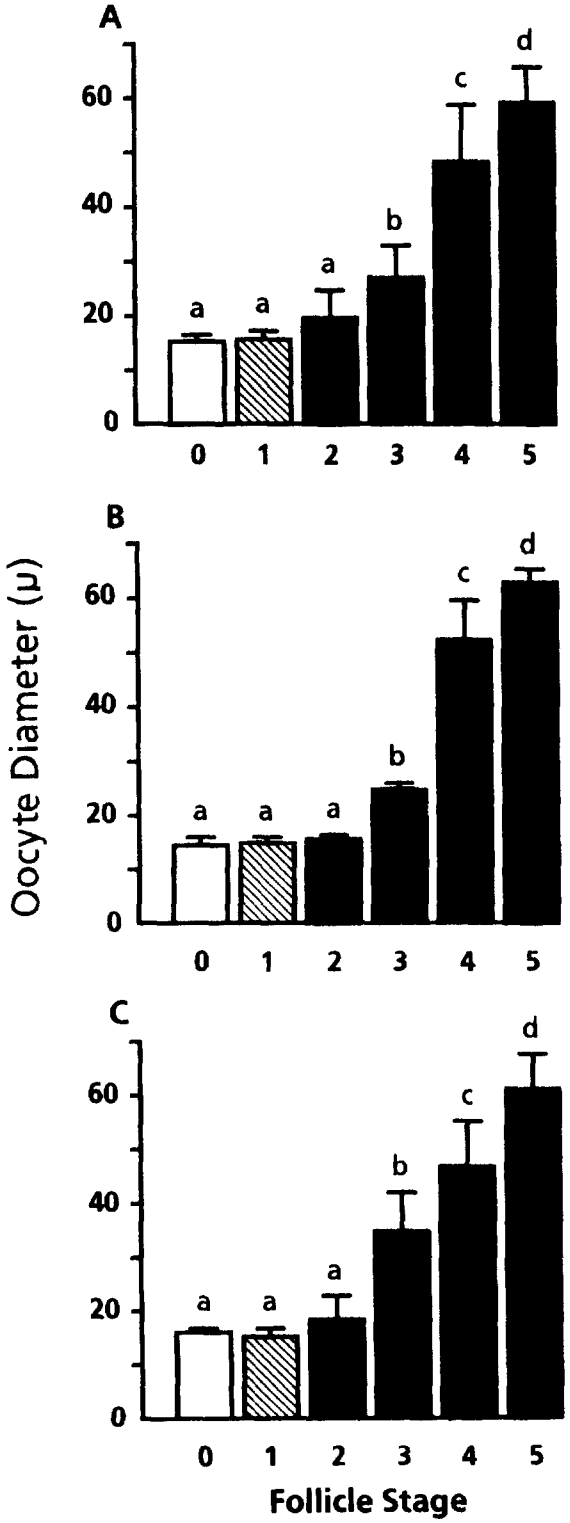

FIG. 3. Relation among follicular stage, oocyte diameters (mean ± SD in microns), and PCNA expression in (**A**) cycling, (**B**) control, and (**C**) eCG + hCG-stimulated prepubertal rats. Open bars, absence of PCNA immunoreactivity in granulosa cells and oocytes; hatched bars, PCNA immunoreactivity only in granulosa cells but not in oocytes; black bars, PCNA immunoreactivity in both oocyte and granulosa cells (see text for explanation). Columns with different letters indicate follicle stages with oocyte diameters that are statistically different ($p < 0.05$). For follicle stage explanation see Table 1.

there is complete correspondence between the appearance of PCNA immunoreactivity and DNA synthesis cannot be determined from these data, and this may be difficult to establish given the technical limitations of the [^{3}H]thymidine studies [6, 14].

There is ample evidence that the PCNA is a reliable indicator of cell proliferation [10, 15, 16]. However, PCNA expression can occur in nonproliferating cells adjacent to tumor cells [16]. Although such deregulation has not been demonstrated in nonneoplastic tissue, PCNA expression can be up-regulated in some quiescent cells by growth factors [17]. Also, the duration of the stages of the cell cycle may fluctuate in the absence of a change in proliferative rate. Thus PCNA expression may change under some conditions without any accompanying change in mitotic activity. We therefore cannot infer from the present results that PCNA expression always indicates that ovarian cells have begun to grow, although its correlation with other measures of follicular growth is consistent with this view.

In contrast to previous studies using [^{3}H]thymidine, in our study we found that PCNA immunoreactivity was a marker of oocyte growth. Regardless of the age or treatment of the animal, PCNA staining was positive in the nucleus of oocytes prior to oocyte enlargement. The increased PCNA expression in growing oocytes cannot be ascribed to cell division, since the oocyte is arrested meiotically. By the time the fetus is born, all oocytes in humans and 96% of oocytes in rodents are arrested in the diplotene stage (G_2) of the first meiotic division [3]. The significance of the presumptive increase in PCNA in the growing oocyte is unknown. Though arrested meiotically, the mammalian oocyte is not quiescent. It begins synthesizing nuclear RNA as it moves from the primordial to the growing phase [18]. Besides being involved in cell cycle regulation, PCNA also serves as an auxiliary protein to DNA polymerase delta, which is involved in DNA synthesis and repair [10]. Although no new DNA synthesis takes place in the growing oocyte, it is possible that DNA polymerase delta might be activated to repair possible damage to the genetic material in the oocytes selected to grow. It has been shown that mitochondrial DNA deletions are increased in the human ovary with age [19]; these deletions may occur in granulosa cells and oocytes. While DNA damage and repair can occur in any cell at any time, long-lived cells such as oocytes may be more prone to DNA deletions compared to rapidly proliferating granulosa cells. Increased PCNA expression in the oocyte nucleus could also reflect increased expression of growth factors in the oocyte since PCNA expression can be stimulated by various growth factors even in quiescent cells [17].

Delineating the sequence of events in follicular growth should increase understanding of the mechanisms that initiate follicle growth. This study demonstrates that granulosa cell enlargement precedes oocyte enlargement by both morphometric and immunochemical criteria, confirming a

previous morphometric study in mice [20]. In that study, although increased size and number of granulosa cells were the first events in follicular growth, mitotic granulosa cells were not seen until the oocyte began to enlarge. The authors suggested that the initial increase in granulosa cells of the growing follicles arose from recruitment of neighboring stromal cells. However, both the study using [^{3}H]thymidine infusion [6] and our findings indicate that granulosa cells begin to proliferate at the earliest morphological sign of growth. These results suggest that the initial increase in granulosa cell numbers arises from mitosis of existing cells rather then recruitment. Furthermore, the findings of the present and previous studies that all measures of growth initiation in granulosa cells—PCNA expression, enlargement, and thymidine incorporation—precede onset of oocyte growth focuses attention on the granulosa cell as the target of growth-initiating signals. Identification of those signals and their targets is an important step toward understanding the mechanism of follicular growth initiation.

In conclusion, the expression of PCNA, an essential regulator of the cell cycle, appears to mark the initiation of follicle growth, coinciding with and in some cases preceding the first sign of granulosa cell enlargement and preceding oocyte enlargement. An examination of its regulation and the basis for the induction of its expression in the granulosa cells and oocyte at the onset of follicular growth may increase understanding of the mechanisms underlying this process.

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