Human Reproduction vol.13 no.5 pp.1133–1138, 1998

OUTSTANDING CONTRIBUTION

Development of human primordial follicles to antral stages in *SCID/hpg* mice stimulated with follicle stimulating hormone

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In contrast to the many detailed studies of Graafian follicles, the biology of small follicles in the human ovary is poorly understood and the trigger for follicular growth initiation remains unknown. No practical model exists to study preantral follicle growth in the human because of their slow growth rate and lack of an effective culture system. We therefore tested ovarian xenografts as a new strategy to study the early stages of ovarian follicular growth *in vivo*. Mice homozygous for severe combined immunodeficiency (*SCID*) and hypogonadism (*hpg*) received human ovarian xenografts under their kidney capsules. Follicle growth was assessed by morphology and proliferating cell nuclear antigen (PCNA) immunostaining. The grafts were recovered after 11 (short-term) and 17 weeks (long-term), and serially sectioned. During the last 6 weeks of long-term grafting, mice were randomized to receive either placebo or 1 IU of purified follicle stimulating hormone (FSH) s.c. on alternating days. After 11 weeks of grafting, the most advanced follicles had a maximum of two granulosa cell layers. In the absence of FSH administration, follicles did not progress beyond the two-layer stage even after 17 weeks of grafting, and the oestradiol levels remained undetectable. In the FSH-treated long-term grafts, follicles had grown to antral stages and resulted in oestradiol levels as high as 2070 pmol/l. Growth initiation indices did not differ between control and FSH-treated grafts. This study demonstrates that follicles can survive and grow in human ovarian tissue grafted under the renal capsules of immunodeficient mice for at least 17 weeks, and indicate that xenograft models are potentially useful for studying human follicle development. Using this physiological model, we showed that FSH is required for follicle growth beyond the two-layer stage, although growth initiation is independent of gonadotrophin stimulation.

Key words: follicle growth/ovary/PCNA/SCID mouse/xenograft

Introduction

Despite advances in our knowledge of the cell biology of granulosa cells and of the character of the pituitary gonadotrophins and their receptors, the signals that trigger follicle growth and control the proportion of follicles in the growing population are poorly understood. For practical reasons, it has been difficult to monitor the early stages of follicle development in human ovaries or to investigate the ontogeny of FSH and LH responsiveness. The formation of pre-ovulatory follicles in patients with clinical hypogonadotrophism 2 weeks after injecting exogenous gonadotrophins indicates either that undetectable hormone concentrations in these women are biologically significant or that preantral development is gonadotrophin independent (Santen and Paulson, 1973), as is indicated in studies of the hypogonadal mouse (Halpin *et al.*, 1986). The answer to this question has a bearing on whether elevated FSH is responsible for accelerated follicle disappearance during the decade before the menopause (Faddy *et al.*, 1992) or if repeated stimulation during IVF treatment advances the time when the follicle store in the ovary is exhausted.

Considering the practical difficulties of studying small follicles *in vivo*, the most attractive strategy is follicle culture. While it is possible to recover viable primordial and preantral follicles in moderate numbers from ovarian biopsies (Oktay *et al.*, 1997a), culture technology is still in its infancy, at least for human tissue (Gosden and Oktay *et al.*, 1996). A more tractable approach at present is to grow follicles in cortical slices, as follicles survive and the early stages of development can be observed in human (Baker and Neal, 1974; Hovatta *et al.*, 1997), in baboon (Wandji *et al.*, 1997) and in bovine species (Wandji *et al.*, 1996). Even so, these methods are scarcely adequate because it is not possible to control the numbers of follicles at different stages or to monitor growth continuously. Moreover, it has not been possible to culture ovarian tissue for longer than a few weeks (Oktay *et al.*, 1998). Because it takes >3 months for primordial follicles to grow (Gougeon,1986), it is unlikely that any morphological evidence of follicle growth can be seen after only a few weeks in culture.

Against this background, we have developed xenografting in immunodeficient animals as a model for investigating early stages of follicle growth and for verifying follicle viability after cryopreservation (Gosden *et al.*, 1994; Newton *et al.*, 1996). The mouse carrying the *SCID* mutation has been chosen as the preferred model because it is immunologically anergic but has a healthy lifespan, and tolerates surgery well in a

sterile environment (Bosma *et al.*, 1983). These advantages do not overcome all the drawbacks of follicle culture, but it is possible to maintain ovarian xenografts indefinitely compared with the short range studies only possible *in vitro*. Moreover, combining this mutation with that for hypogonadism (*hpg)* carries a double benefit because the animals are rendered profoundly deficient in pituitary gonadotrophins at the same time (Mason *et al.*, 1986). Thus, *SCID*/*hpg* animals can be used to study the influence of gonadotrophins *in vivo* on ovarian tissue from virtually any species.

We here describe the dynamics of human follicular growth and test the hypothesis that the early stages of development are independent of follicle stimulating hormone (FSH) stimulation. Because human follicles grow slowly and assessment of growth initiation in primordial follicles and incipient atresia in larger ones is difficult, it is necessary to use a more sensitive indicator of growth than morphology alone. Proliferating cell nuclear antigen (PCNA), a 35 kDa nuclear protein, is a valuable growth marker because it is universally expressed throughout the G_1/S-phase interface and reaches a plateau during G_2 (Xiong *et al.*, 1992). PCNA is expressed from the earliest stages of follicular growth in the rat but is undetectable by immunochemistry in either granulosa cells in atretic follicles or pregranulosa cells of primordial follicles as expected (Oktay *et al.*, 1995). We have therefore used PCNA to label the developing follicles in the ovarian xenografts.

Materials and methods

Human ovarian tissue was obtained with informed consent under a protocol approved by the Research Ethics Committee of the United Leeds Teaching Hospitals NHS Trust. A 17 year old patient undergoing gynaecological surgery provided strips of healthy ovarian cortical tissue ~1 mm thick from a uniformly smooth area devoid of antral follicles or remains of luteal tissue. The tissue was cut into cubes ~1 mm^3.

Mice homozygous for severe combined immunodeficiency (*SCID*) and hypogonadism (*hpg*) were shipped from the Jackson Laboratories (Bar Harbor, ME, USA) for a study approved under a Home Office licence. They were maintained in groups of up to three per cage in positive pressure isolators with sterile food and water. Verification of homozygosity for *hpg* was necessary because the hypogonadal phenotype of the reproductive tract was obscured in animals treated with gonadotrophins. This was performed by screening the tail tips of the animals using the polymerase chain reaction, as described previously (Lang, 1991). The risk of leakage of the *SCID* mutation was monitored in the animal colony by measuring serum immunoglobulins to ensure they did not exceed 20 μg/ml. When approximately 6 months old, the mice were anaesthetized with a halothane–oxygen mixture and their right kidneys were exteriorized via a mid-line skin incision using a strictly aseptic technique. All but three cubes of ovarian tissue were inserted under the kidney capsule, one or two grafts in each animal (Oktay, 1998*)*. Three cubes were fixed in Bouin's fluid to serve as controls.

The grafts were recovered after either 11 weeks (n = 3) or 17 weeks (n = 13). Animals in the long-term group were randomized to receive either sterile saline (n = 7) or 1 IU of FSH s.c. (n = 6) (Metrodin HP, Serono Laboratories, Welwyn Garden City, Herts, UK) on alternate days for 6 weeks prior to autopsy. This dose was adjusted from an earlier study (Oktay *et al.*, 1997b) to produce a mean peak level of 25–30 mIU/ml plasma in mice, and to avoid any risk of down-regulating FSH receptors by overstimulation (Lapolt *et al.*, 1992). The animals were killed using carbon dioxide gas, and a terminal blood sample was collected by cardiac puncture. Total serum oestradiol was measured by radioimmunoassay after ether extraction in a single assay with a sensitivity of 30 pmol/l (Atkinson *et al.*, 1996). The uterine horns were removed, trimmed and weighed after blotting to remove surface moisture.

Ovarian grafts were fixed in Bouin's fluid (3 h) and sectioned serially at 5 μm thickness. The majority of slides were stained with haematoxylin and eosin, but every fifth slide was stained with antibodies for PCNA (Novocastra Laboratories, Newcastle-upon-Tyne, UK) as described previously (Oktay *et al.*, 1995). Briefly, the sections were deparaffinized, rehydrated and quenched in 3% H_2O_2 for 30 min to block endogenous peroxidase activity. After washing, normal horse serum was added for 10 min at room temperature to inhibit non-specific binding. The first antibody was incubated at a dilution of 1:150 for 18 h at room temperature followed by washing and incubation in equine anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) at 1:250 dilution for 30 min. Finally, avidin–biotin complex was added for 30 min at 37°C, followed by diaminobenzidine (DAB) for a further 3 min. After completing the reactions, the sections were counterstained with haematoxylin.

The slides were number-coded to avoid observer bias. Follicles were counted and classified according to their morphological stage of development and number of granulosa cell layers (Table I). Those with cuboidal granulosa cells or more than one layer of cells were regarded as growing. Follicles were counted only when the nucleolus was present.

Statistical analysis

Statistical comparisons of mean follicle densities in each group were made using one way analysis of variance. Follicle growth rates and initiation indices were compared using the χ^2 test. To increase the study power and enable χ^2 comparisons, follicles from each treatment arm (baseline, 11 weeks; short term, 17 weeks; long term with or without FSH administration) were grouped together after a heterogeneity test confirmed that no difference existed between individual grafts in terms of growth rates and initiation indices. A P value of $\leqslant 0.05$ was considered statistically significant.

Results

Ovarian xenografts were easily identified at autopsy, except in two cases. A graft was missing in a saline-treated and an FSH-treated animal, probably due to dislodgement. Number of sections varied between 40 and 95 per graft (mean ± SE, 62.3 ± 4.0). Histological examination revealed that all grafts were well vascularized and free of necrotic tissue. The interstitial tissue was unremarkable (Figures 1–5). Primordial follicles

Table I. Follicle classification[a]

Stage	Description
Primordial	Oocyte partially or completely encapsulated by squamous pregranulosa cells
Primary	Single layer of cuboidal granulosa cells
Preantral	Enlarged oocyte encapsulated by >1 granulosa cell layers without an antrum
Antral	Enlarged oocyte with multiple granulosa cell layers and antrum formation

[a]Modified from Gougeon (1986).

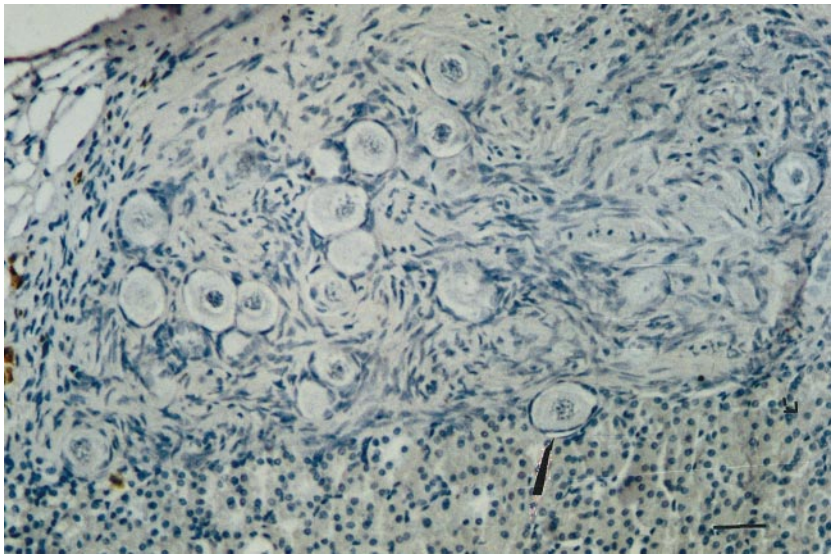

Figure 1. Primordial follicles in an 11 week old ovarian graft under the renal capsule of a *SCID*/*hpg* mouse, demonstrating that primordial follicles were negative for proliferating cell nuclear antigen (PCNA) staining. Note the primordial follicle bulging into renal tissue (arrow). Counterstained with haematoxylin (original magnification ×200). Bar = 50 μm.

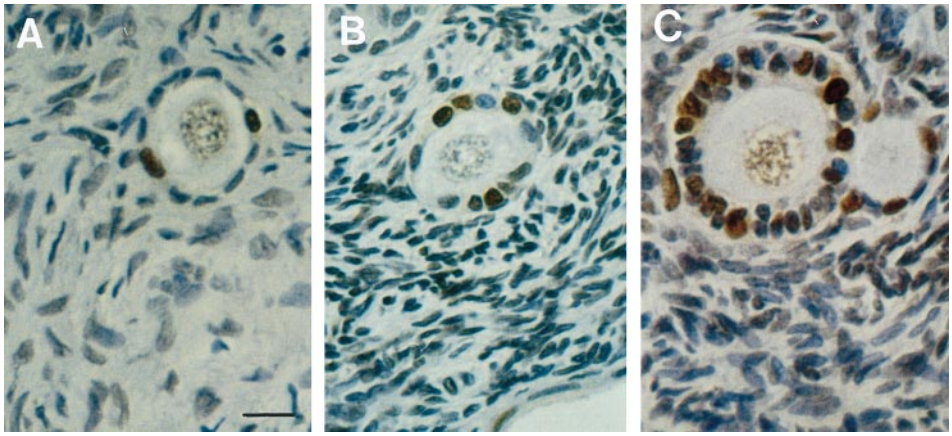

Figure 2. Follicles in early stages of growth, which stained for proliferating cell nuclear antigen (PCNA), were present in hypogonadotrophic ovarian xenografts after 17 weeks. (**A**) and (**B**) show primary follicles with one or more cells staining for PCNA, whereas (**C**) shows a PCNA-positive follicle in transition between the primary and two-layer stage. Counterstained with haematoxylin (original magnification ×400). Bar = 25 μm.

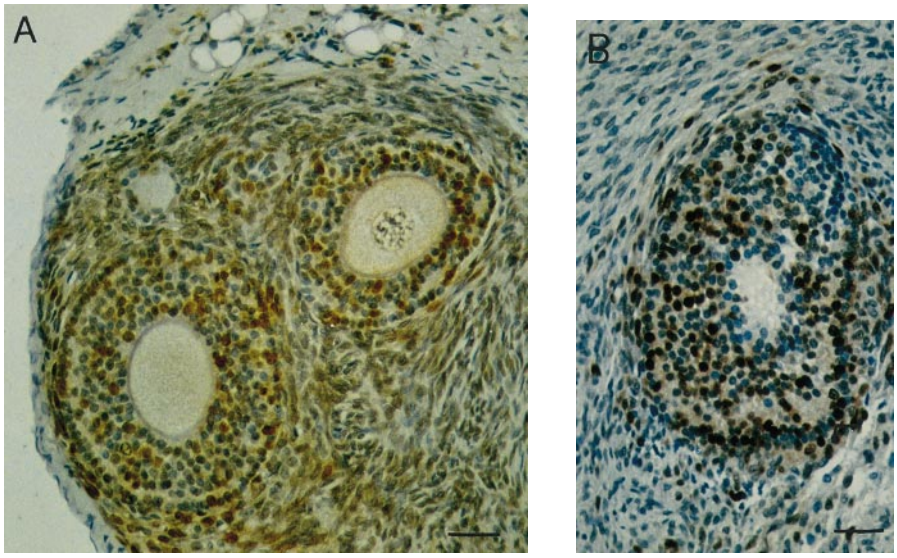

Figure 3. (**A**) Multilaminar, proliferating cell nuclear antigen (PCNA)-positive follicles in an ovarian graft stimulated with follicle stimulating hormone (FSH) for the last 6 weeks of 17 weeks. (**B**) A PCNA-positive follicle from the normal ovary of a 32 year old woman. Counterstained with haematoxylin (original magnification ×200). Bar = 50 μm.

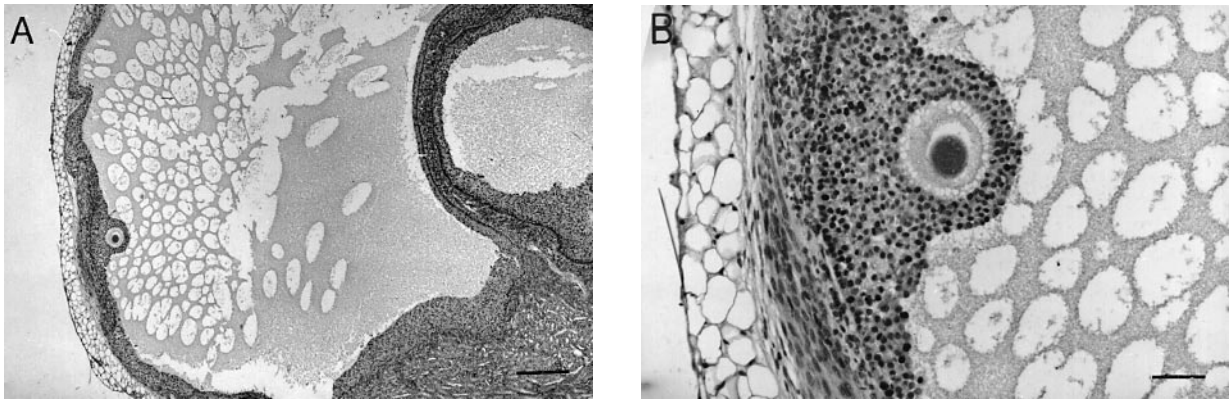

Figure 4. (**A**) Low magnification of a Graafian follicle (3 mm diameter) in human ovarian tissue xenografted 17 weeks previously and stimulated with follicle stimulating hormone (FSH) for the last 6 weeks (original magnification ×20). Bar = 50 μm. (**B**) Higher magnification of the same follicle showing a germinal vesicle oocyte surrounded by cumulus cells (original magnification ×200). Bar = 50 μm. Haematoxylin and eosin.

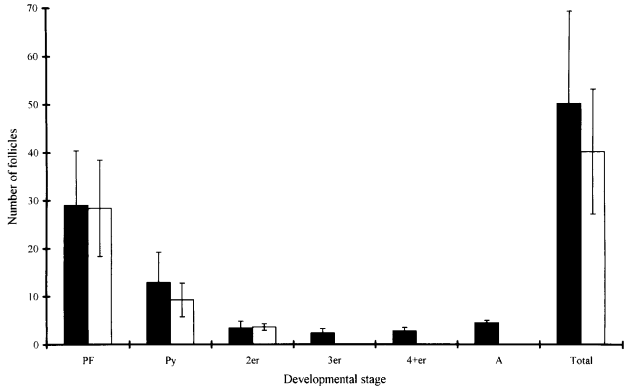

Figure 5. Relationship between the mean number of follicles ±SEM per graft and the stage of development in long-term ovarian xenografts. Grafts stimulated with follicle stimulating hormone (FSH) (black bars) contained a continuum of growing follicles up to the antral stage, while in the control grafts (white bars), follicles did not advance beyond the two-layer stage. Primordial (PF), primary (PY), two-layer (2er), three-layer (3er), more than three-layer (4+er) and antral (A) follicles.

were present in all grafted and ungrafted specimens and were similar in both appearance and size (30–50 μm diameter) (Figure 1). When expressed as the total number per number of sections, follicle density showed a decline in parallel to the length of duration of grafting. However, this difference did not reach statistical significance between control tissue and short and long term grafts respectively (0.62 ± 0.04, 0.57 ± 0.08 and 0.53 ± 0.06). Growing follicles, defined by PCNA staining and the morphology of the granulosa and oocyte size, were more frequent in the FSH treated grafts than saline treated ones (42 versus 31%, $P = 0.009$). The growth initiation indices, as expressed by the total number of primary follicles per primordial follicles, were not significantly different in the treated and untreated groups at 0.38 (76/202) and 0.44 (77/174) respectively.

In the short term grafts, after 11 weeks of grafting, no follicles exceeded the two layer stage. In addition, in animals which received saline, the follicles still did not grow beyond two-layer stage after 17 weeks of grafting (Figure 2). Those treated with FSH produced a spectrum of follicles ranging from primary to large preantral and antral stages (Figures 3A, 4 and 5). Preantral follicles had as many as 10–12 granulosa cell layers. Antral follicles were as large as 5 mm in diameter (Table II), and the histological appearance of the oocytes, granulosa and theca cells was indistinguishable from normal (Figure 4).

PCNA staining suggested that follicles were still growing and had not halted in development (Figures 2, 3A and 4) with primordial follicles unstained in all of the grafts (Figure 1). Granulosa cells in follicles as early as the primary stage were labelled, as were the nuclei of their oocytes (Figures 2–4). The staining characteristics of large preantral follicles were similar to those of a normal ovary from a 32 year old patient (Figures 3A, B).

Where large follicles were present, the reproductive tract of the host animal revealed that they were secretory (Table II). The median wet weight of the uterus of adult hypogonadal mice was 17 mg and the vaginas were closed. The median weight of uteri of animals treated with FSH was 128.5 mg and the vaginas were patent and cornified. Serum concentrations of oestradiol in mice with no xenografts and in animals with unstimulated xenografts were undetectable (<30 pmol/l), but those with xenografts stimulated by FSH had risen to supra-physiological concentrations (Table II).

Discussion

This study demonstrates for the first time, and extends an earlier study of non-primate xenografts in SCID mice (Gosden *et al.*, 1994), that human ovarian tissue not only survives in the *SCID* mouse, but that follicles can grow to antral–secretory stages. It also provides information about the effects of the gonadotrophins in early stages of follicle growth by utilizing a sensitive cell-proliferation marker, PCNA, together with the *SCID*/*hpg* model.

By eliminating the possible effects of endogenous murine gonadotrophins, this study indicates that follicle growth initiation in humans proceeds in the absence of gonadotrophins. This conclusion is strengthened by several indications that follicles at the primary stage or later had started growing after

Table II. Assessment of oestrogen secretion by ovarian xenografts in *SCID/hpg* mice stimulated with follicle stimulating hormone (FSH)

Graft	No. of antral follicles (mm diameter)	Serum oestradiol (pmol/l)	Uterine weight (mg)	Vaginal introitus
1	1 (5)	2070	212 (ballooned)	Patent
2	2 (3,4)	780	131	Patent
3	0	35	123	Patent
4	1 (2.5, haemorrhagic)	126	126	Patent

grafting and were advancing in development rather than remaining static since the time of surgery. Few, if any, growing follicles survive the transplantation of an ischaemic graft (Gosden, 1992); thus the growing follicles that were found in the xenografts could be considered to have initiated growth after grafting. The evidence of PCNA staining provides even more conclusive proof that the follicles had initiated growth, and that their granulosa cells were proliferating at the time of tissue harvesting.

The halting of follicle development at the two-layer stage indicates that this is a critical period and that further growth depends on gonadotrophic stimulation, probably mainly FSH. These results are in agreement with earlier animal studies in which relatively more advanced, albeit still preantral, stages are found in hypogonadal mice (Halpin *et al.*, 1986; Wang and Greenwald, 1993) as well as in bovine cortical tissue incubated in defined medium (Wandji *et al.*, 1996). What is more, the conclusion that the initiation of growth is independent of gonadotrophins is consistent with evidence that FSH receptor expression commences at the primary stage in humans (Oktay *et al.*, 1997c) and at a correspondingly early stage in animal species (Tisdall *et al.*, 1995; Zheng *et al.*, 1996). As in sheep, FSH binding was shown in one- to three-layer hamster follicles (Roy *et al.*, 1987). It has also been reported that FSH supplementation enhances the in-vitro growth of both mouse (Corvrindt and Van Steirteghem, 1997) and human preantral follicles (Roy and Treacy, 1993; Wandji *et al.*, 1997). Follicle development beyond the two-layer stage has been demonstrated in histological sections of ovaries from women with Kallman's syndrome, who are relatively gonadotrophin deficient (Goldenberg *et al.*, 1976). However, the sensitivity of gonadotrophin assays was low, and in some cases gonadotrophin concentrations were not reported.

Though overall growth rates were higher in the FSH-treated group, compared with the saline-treated one, the growth initiation indices were similar. This could be explained by the fact that once the follicles initiate growth, they are more likely to survive and continue growth in the presence of FSH. Only when exogenous FSH was given did follicles develop further, and the growth appeared to be normal on morphological and immunochemical grounds. In addition, aromatase was evidently expressed in antral follicles since the serum oestradiol levels were high and exceeded those of in the normal oestrous cycle of the host species (Nelson *et al.*, 1981). Feline and ovine xenografts also produced high concentrations of oestradiol in SCID mice, although in those studies, exogenous FSH was not given and the only source of stimulation was endogenous (Gosden, 1992; Gosden *et al.*, 1994, Nugent *et al.*, 1997).

According to the data of Gougeon (1986), 17 weeks should be sufficient for follicles to reach pre-ovulatory stages but the most advanced follicles were still some 2 weeks short of ovulatory size in the xenografts. Whether they could grow larger than the maximum recorded of 5 mm with continued stimulation is not clear, especially given the fact that larger follicles were never found in ovine xenografts (Gosden *et al.*, 1994). Indeed, it would not be surprising if this size was the upper limit, as it is doubtful that a pre-ovulatory follicle could be adequately accommodated at the renal site in this species. Transplantation to another site in a larger species might permit full development as long as immunological tolerance could be assured. It is also possible that luteinizing hormone (LH) may be necessary for further follicle growth, and the absence of LH administration in our experiment may explain the lack of progress beyond 5 mm.

Previous studies suggested that the time course for follicular growth in xenografts is largely independent of the host, since the full growth span of follicles is <4 weeks in mice (Hirshfield, 1991), whereas in the present study, the time span for follicles to grow to antral stages was consistent with the >120 day estimate by Gougeon (1986). Thus the time course for follicular development in human xenografts is also mainly determined by the graft rather than the host.

In conclusion, this study demonstrates that a model utilizing ovarian xenografts in *SCID/hpg* mice and PCNA immunostaining can be useful to study the dynamics of follicular growth *in vivo*. In this physiological model, human primordial follicles can initiate growth in the absence of gonadotrophins and FSH promotes growth at an earlier stage than had been suspected hitherto. These data, taken together with the evidence that the FSH receptor is expressed in one- to two-layer follicles (Oktay *et al.*, 1997c), may change our current understanding of the role of gonadotrophins in regulation of early follicle growth as well as pharmacological ovarian stimulation.

Acknowledgements

This work was supported by an RCOG/ACOG Exchange Fellowship from WellBeing (London). We thank Dr Wes Beamer (Jackson Laboratory, USA) for the gift of *SCID/hpg* mice and the Centre for Steroid Hormones at Leeds University for assistance.

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Received on October 13, 1997; accepted on February 2, 1998