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Apoptosis of Granulosa Cells and Female Infertility in Achondroplastic Mice Expressing Mutant Fibroblast Growth Factor Receptor 3^{G374R}

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Fibroblast growth factors play an important role in the control of ovarian folliculogenesis, but the complete repertoire of ovarian receptors which can transduce the fibroblast growth factor signals and their precise localization in the ovary have not yet been characterized. The most common form of inherited human dwarfism results from a point mutation in the transmembrane region of fibroblast growth factor receptor 3. A mouse model for achondroplasia was generated by introducing the human mutation (glycine 380-arginine) into the mouse fibroblast growth factor receptor 3 (G374R) by a "knock-in" approach using gene targeting leading to a constitutively active receptor. This resulted in the development of dwarf mice that share many features with human achondroplasia. Here we report that female (fibroblast growth factor receptor 3 G374R) dwarf mice become infertile. While no significant changes were observed in the anatomical and histological appearance of ovaries of 3-wk-old dwarf mice, a dramatic difference was observed in ovaries of 3-month-old mice. The normal ovary consists mainly of healthy corpora lutea and follicles at different stages of development, whereas the ovaries of the dwarf mice remain small and contain mainly follicles with a progressive apoptosis in the granulosa cells, and no corpora lutea could be observed. The levels of LH, FSH, and progesterone were lower by 72.3%, 38.0%, and 40.0%, respectively, in the blood of the dwarf mice compared with normal mice, and the total bioactivity of pituitary FSH and LH was lower by 65.6% and 79.6%, respectively, in the dwarf mice compared with normal mice. However treatment with PMSG and human CG of the dwarf mice led to rapid follicular development and formation of corpora lutea. Interestingly, the expression of the tumor suppressor gene p53 was increased dramatically in ovaries of the dwarf mice. The presence of the fibroblast growth factor receptor 3 cellular receptors in both normal and dwarf animals was demonstrated by Western blot and immunostaining. However, the distribution of the fibroblast growth factor receptors in the two strains shows significant differences. In the normal ovaries fibroblast growth factor receptor 3 was homogeneously distributed on the cell membrane of the granulosa cells and was absent in theca as well as corpora lutea cells, whereas in dwarf mice ovaries it was highly clustered on granulosa cells and very often appears in endocytic vesicles. Aged oocytes were more frequently observed in preantral follicles of ovaries of the dwarf mice. Nevertheless, oocytes isolated from antral follicles resume their meiotic division at a high percentage, similar to oocytes obtained from normal ovaries. The results imply fibroblast growth factor receptor 3 involvement in the control of follicular development through regulation of granulosa cell growth and differentiation, and that unovulation in the dwarf mice could be overcome in part by administration of exogenous gonadotropins. Moreover, it is suggested that the infertile phenotype is partially due to defects in the pituitary-gonadal axis. (*Molecular Endocrinology* 15: 1610–1623, 2001)

FIBROBLAST GROWTH FACTORS (FGFs) comprise at least 19 mitogenic ligands (1, 2) which bind to four high-affinity FGF receptors (FGFR1–4) and require heparan sulfate proteoglycans for signaling through these receptors (3, 4). FGFs are implicated in diverse biological processes such as cell growth, motility, and differentiation, angiogenesis, and wound healing (5). Their role was also demonstrated in limb, skeletal, lung, and brain development (6, 7).

FGFs also play an important role in follicular development and ovarian function. FGF was found to be mitogenic in cultured bovine, porcine, rabbit, guinea pig, and human granulosa cells and to delay their differentiation (8–10). In porcine granulosa cells, basic FGF (bFGF) suppresses induction of LH/CG receptors and progesterone secretion. It suppresses FSH induction of LH receptor also in rat granulosa cells in culture as well as aromatose activity (11–14) but enhances progesterone synthesis in preovulatory rat granulosa cells alone and synergistically with FSH and LH stim-

Abbreviations: bFGF, Basic fibroblast growth factor; DAPI, 4′6′-diamido-2-phenylindole hydrochloride; FGF, fibroblast growth factor; FGFR, FGF receptor; mAb, monoclonal antibody; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

ulation (15, 16). It was recently demonstrated that the FGF effect on enhanced progesterone production is due to up-regulation of the expression of the steroidogenic acute regulatory protein, which facilitates the transfer of cholesterol into mitochondria where conversion of cholesterol to pregnenolone take place (16). Furthermore, bFGF stimulates tissue-type plasminogen activation expression, PGE synthesis, and resumption of oocyte maturation in rat follicles *in vitro* (17). Thus, although FGF seems to delay the earlier steps of granulosa cell differentiation stimulated by FSH, it seems to mimic some of the ovulatory actions of LH (18).

Recently, more than 75 germ-line mutations at various domains of FGFR1–3 were found to be responsible for various forms of skeletal dysplasias, resulting in at least seven syndromes of craniofacial and limb anomalies such as craniosynostosis and achondroplasia (reviewed in Refs. 19–21). Various studies indicated that the common cause of these phenotypes was the gain-of-function due to ligand-independent activation of the mutant receptors (22, 23). The severity and location of the skeletal dysplasia was correlated with the site of receptor expression and the extent of activation of the mutant receptor (19, 21).

A notable example is the G380R mutation in the transmembrane region of FGFR3, which is responsible for 98% of all cases of human achondroplasia (24). To study this phenotype we generated a mouse model for achondroplasia using gene targeting (knock-in) to generate the mouse with mutant $FGFR3^{G374R}$ (25). This mutant mouse strain was shown to share many features with human achondroplasia. The achondroplastic phenotypes develop after birth and begin to differ from their normal littermates 1 wk or 10 d after birth (25). Our analysis also showed that some of the histological features in the growth plate of these mice were more severe than those reported for human achondroplasia. Surprisingly, the murine females were infertile whereas no female infertility was reported in human achondroplasia.

In this paper we studied the changes in the ovary of the achondroplastic female mice. We found that FGFR3 is expressed on the membrane of the granulosa cells but not in the oocyte. The granulosa cells of the mutant mice showed aggregation and endocytosis of the mutant receptor accompanied by high incidence of apoptosis. These results implicate FGFR3 in the regulation of granulosa cell differentiation. The cause of infertility was in the failure of follicular maturation, ovulation, and corpora lutea formation, due to apoptosis of the granulosa cells, which could be overcome, at least in part, by exogenous administration of gonadotropins to the dwarf mice.

RESULTS

To investigate the differences between dwarf and normal mice, ovaries from 3-wk- and 3-month-old mice were inspected. No significant differences were found in the ovaries of 3-wk-old mice, which contained primordial primary, preantral, and antral follicles (Fig. 1). In contrast, striking differences were observed in ovaries of 3-month-old female mice. While the mean diameter of the 3-month ovary was 2.4 ± 0.35 mm, the mean diameter of the dwarf mice ovaries was significantly smaller; 1.2 ± 0.31 mm ($n = 5$) $P < 0.01$ (Figs. 2 and 3).

The basal level of progesterone in the sera of 3-month-old normal and dwarf animals was 30.15 ± 0.58 ng/ml ($n = 6$) and 18.16 ± 0.60 ng/ml, respectively (40% reduction). The level of LH in the normal mice was 1.80 ± 0.09 ng/ml while in dwarf mice it was 0.50 ± 0.10 ng/ml (73% reduction). The level of FSH in the normal mice was 7.05 ± 0.40 ng/ml compared with 4.37 ± 0.30 ng/ml in the dwarf mice (38% reduction) (Fig. 4A). To examine whether the reduction of LH and FSH blood concentration is due, at least in part, to the biosynthesis of these hormone we checked the total bioactivity of these hormones in pituitary homogenates prepared from 3-month-old female mice. While the bioactivity of LH and FSH was $2{,}275 \pm 543$ mIU (mean $\pm$ SD, $n = 6$) and 607 ± 142 mIU (mean $\pm$ SD, $n = 6$) per normal mouse pituitary (equivalent to 159 ± 28 ng and 42 ± 9 ng of pure active LH and FSH, respectively) the bioactivity of these hormones was 466 ± 148 mIU for LH and 209 ± 92 mIU for FSH (mean $\pm$ SD, $n = 6$) per dwarf mouse pituitary (equivalent to 33 ± 4 ng and 15 ± 3 ng of pure active LH and FSH, respectively) (Fig. 5). It should be noted that while the total weight of 3-month dwarf mice decreased by 44.8% (27 ± 0.5 g *vs.* 14.9 ± 0.6 g), the weight of the pituitary decreased by 59.3% (4.9 ± 0.3 mg *vs.* 1.9 ± 0.2 mg) with no change in the weight of the hypothalamus (53 ± 3 mg *vs.* 56 ± 3 mg, $n = 6$) (Fig. 5).

While most of the ovarian tissue in the normal animals contained corpora lutea and follicles at different stages of development, ovaries of 3-month-old dwarf mice remained smaller in size, similar to ovaries obtained from 3-wk-old animals. However, numerous follicles were atretic with pyknotic nuclei (Fig. 3).

The incidence of apoptosis was analyzed in ovarian sections of 3-month-old dwarf and normal mice by 4′6′diamido-2-phenylindole hydrochloride (DAPI) and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) techniques (Figs. 4, 6, and 7). In the normal ovary of 3-month-old mice some apoptosis could be detected, but most of the follicular tissue did not show signs of apoptosis. In contrast, in ovaries of 3-month-old dwarf animals, condensed nuclei and large DNA aggregates were evident mainly in antral follicles where most of the apoptotic nuclei were confined to the inner follicular layers of the membrana granulosa (Fig. 7). Tissue sections stained for TUNEL showed occasionally intensive labeling of fragmented DNA at the antrum of the large follicles (data not shown), which suggests that the apoptotic cells released their content into the follicular antrum. The incidence of apoptosis was extremely low (<2%) in

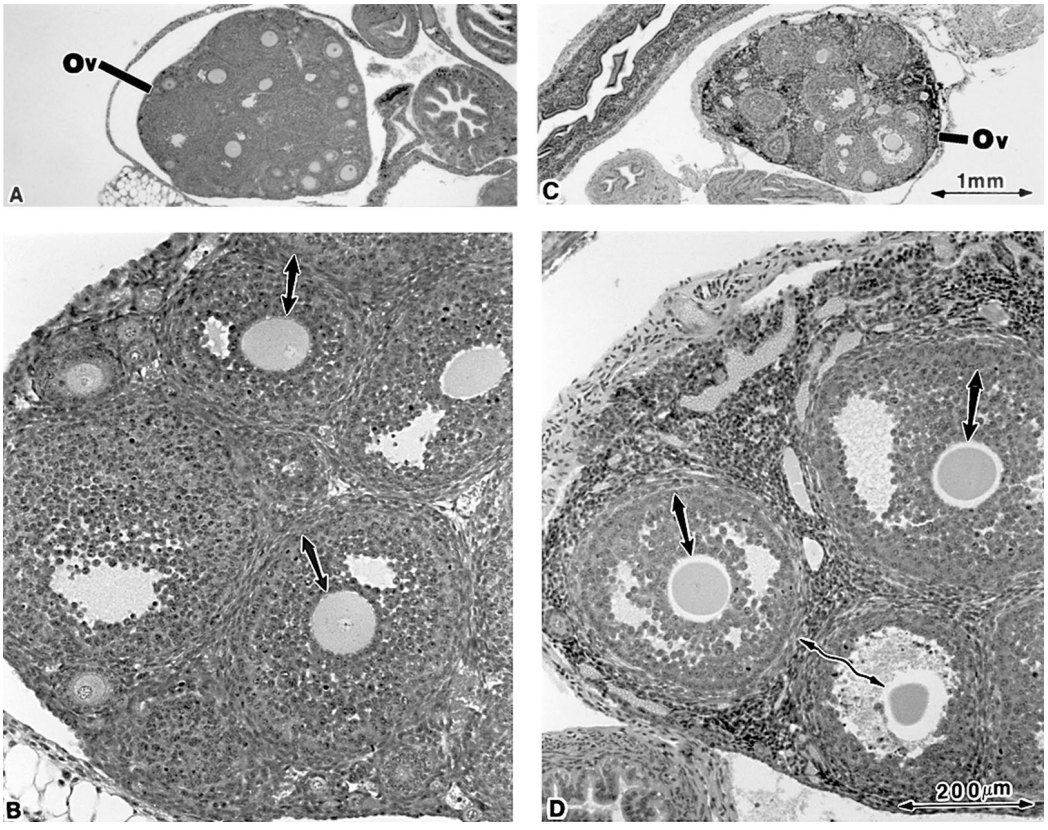

Fig. 1. Sections of Ovaries Obtained from 3-wk-Old Normal and Dwarf Female Mice

A, Normal ovary (Ov) is loaded with preantral and small antral follicles. B, Higher magnification of part of the ovary showing developed follicles and normal oocyte (*arrows*). C, Ovary (Ov) of dwarf mice essentially in a similar size. D, Higher magnification of part of the ovary showing atretic follicle with condensed nuclei at the inner layers of the granulosa cells and in the follicular antrum (*bipolar twisted arrow*). Two other antral follicles appear normal (*bipolar arrows*).

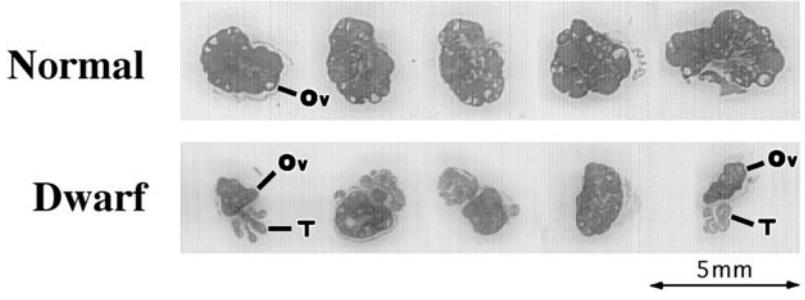

Fig. 2. Gallery of Ovaries (Ov) of Normal and Dwarf Mice Obtained from 3-Month-Old Female Sectioned Through the Center of the Glands

Ovaries of dwarf mice were often removed with the fallopian tubes (T) because of their smaller size. Their average diameter was 50% of the normal ovary.

primordial and primary follicles of 3-wk-old ovaries, which show normal oocytes with no difference between normal and dwarf mice. The incidence of apoptosis among large preantral and antral follicles of 3-wk-old normal ovaries was 31 ± 0.7%, while that of dwarf mice of the same age was 33 ± 1.4% with no significant difference between these two strains of mice (Fig. 4B). In contrast, the incidence of apoptosis in 3-month-old normal ovaries was 32 ± 5.9%, while in the dwarf mice it was 66 ± 1.7% (increase of 2.06-fold).

Ovarian tissue sections were also stained with anti-FGFR3 antibodies (Figs. 6 and 7). In ovaries of normal 3-month-old mice, positive staining was evident on the circumference of granulosa cells. The staining was found to be homogeneously distributed at the cell circumference, but oocytes were devoid of specific staining. No staining was detected in theca and cor-

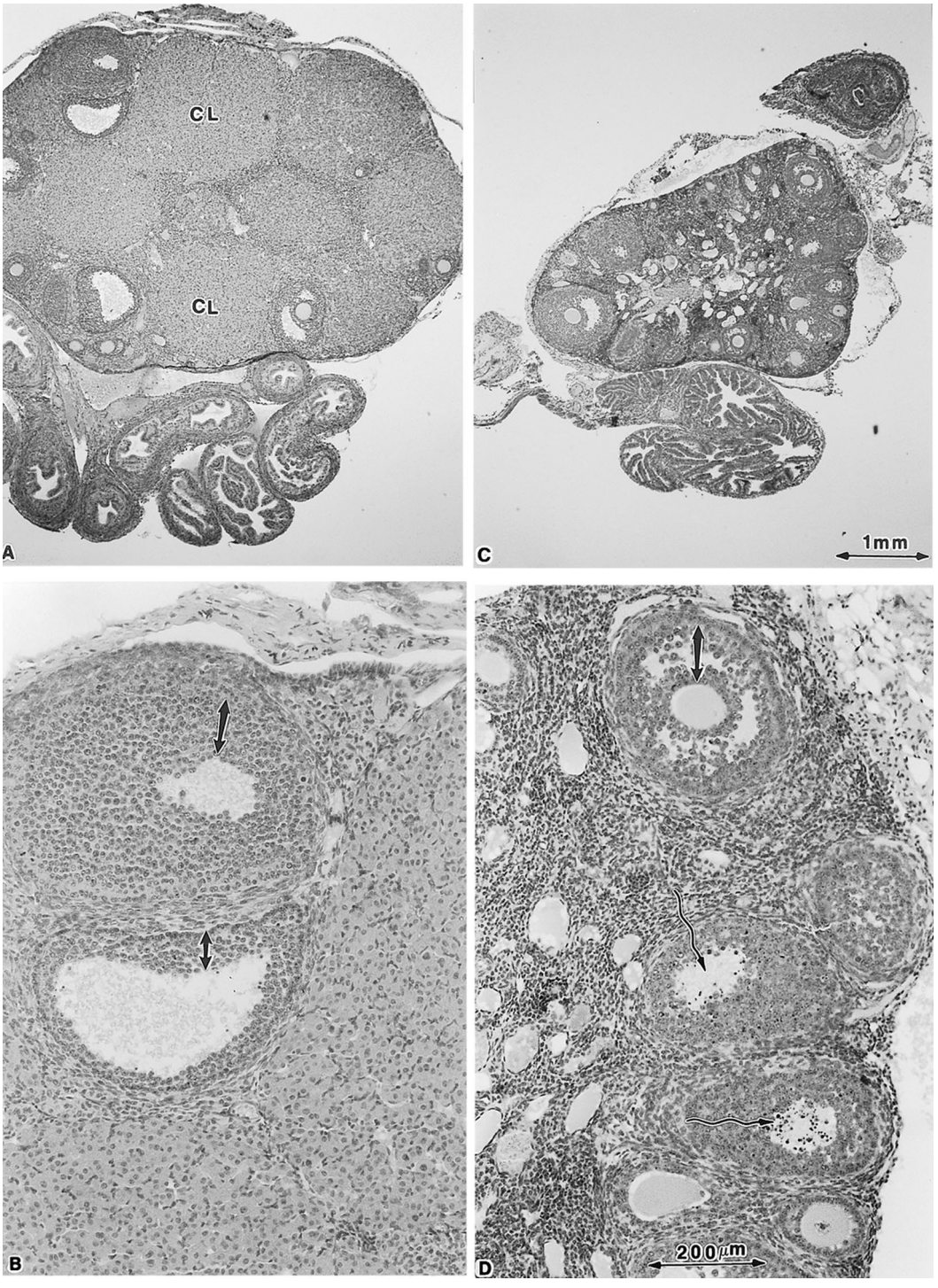

Fig. 3. Sections of Ovaries Obtained from 3-Month-Old Normal and Dwarf Female Mice

A, Normal ovary loaded with corpora lutea (CL) and follicles at different stages of development. B, Higher magnification of part of the normal ovary showing two antral follicles (*bipolar arrow*) and luteal tissue. C, Ovary of dwarf mouse demonstrating small antral follicles at the periphery of the ovary. D, Higher magnification of a part of the ovary with atretic follicles showing condensed nuclei at the inner layers of granulosa cells of small antral follicles (*twisted arrows*).

pora lutea cells. In dwarf mice the labeling was also confined to the granulosa cells. However, staining with anti-FGFR3 of the cell membrane was not homogenous but showed aggregates and patches. In atretic follicles internalization of the receptors in endocytic vesicles was clearly evident. Immunoblots of ovarian homogenates clearly show the expression of FGFR3 both in normal and dwarf mice (Fig. 8A). The receptor

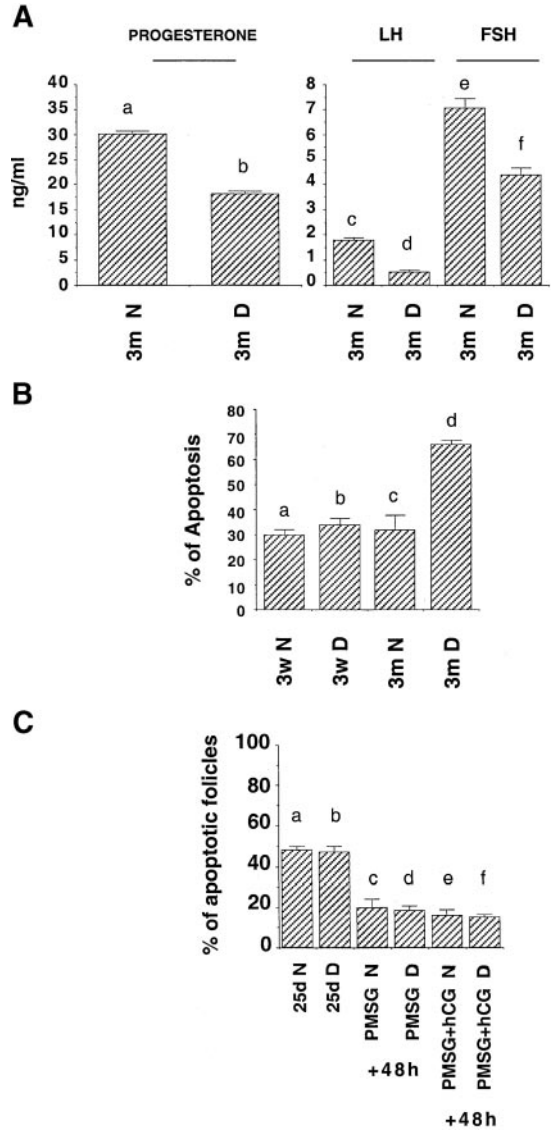

Fig. 4. Hormone Concentrations in Sera and Incidence of Apoptosis in Ovaries of Normal *vs*. Dwarf Female Mice

A, Hormones in sera of 3-month-old normal and dwarf female mice. Basal levels of progesterone, LH, and FSH were measured at the day of diestrus in normal mice and at exactly the same day of age in dwarf mice. Progesterone was assayed by RIA. LH and FSH were assayed for their bioactivity using highly purified rat LH and FSH (iodination grade) as standard and rLHR-15 and rFSH-17 responsive cell lines (see *Materials and Methods*). Data are mean ± SD (n = 6), a>b; c>d, and e>f, $P < 0.01$. B, Incidence of apoptosis in preantral and antral follicles of 3-wk- (3w) and 3-month (3 m)-old ovaries of dwarf and normal mice. Data are mean ± SD of scoring of apoptosis in sections of six ovaries doubly stained for DAPI and TUNEL. c<d, $P < 0.01$. C, Apoptosis in follicles of normal and dwarf mice stimulated with PMSG/hCG. Female mice (25 d old) were injected with 15 IU PMSG, 48 h later with 15 IU/animal of hCG. Animals were killed at 0 min time of injections (three animals of each group) 48 h after PMSG injection and 48 h after hCG injection. Data are mean ± SD obtained by scoring of at least four sections of each gland doubly stained for DAPI (DNA) and TUNEL (apoptosis). a>c and b>d, $P < 0.01$. m, Month; w, wk; D, dwarf; N, normal; hCG, human CG.

also shows an intensive band in brain homogenate, which serves as a positive control, and also in liver and spleen, while only weak staining was found in the adrenal (not shown). Intensity of staining of the FGFR3 in Western blot was essentially the same in normal and dwarf mice. We also examined the expression of oncogenes and tumor suppressor genes by gel electrophoresis followed by Western blots. While there were no differences in MDM2 expression, there was a dramatic elevation of p53 expression in ovary, lung, and spleen of dwarf mice (Fig. 8, B and C), which may suggest that modulation of p53 expression may play an important role in the high apoptotic incidence in the ovaries.

We intended to examine whether infertility and the absence of ovulation in dwarf mice was due to the inability of follicles to reach the Graafian follicle stage or to the inability of the oocytes to resume their meiotic division. Ovaries of 3-month-old mice were punctured with a syringe needle, and oocytes from both dwarf and normal ovaries were released (six ovaries for each group) and incubated in isotonic solutions at 37 C for 12–24 h. The vast majority of oocytes from both normal (87 ± 2.8%) and dwarf mice (85 ± 3.1%) resumed their meiosis at the same rate as observed by the extrusion of the first polar body (Fig. 9, A and B), indicating that the infertility of the dwarf mice is due to failure in follicular maturation rather than to a defect in oocyte maturation.

To examine whether the failure in follicular development and enhanced apoptosis in dwarf mice are due to deficiencies in circulating gonadotropins, we injected 25-d-old female of both dwarf and normal mice with 15 IU of PMSG. Forty-eight hours later 15 IU of hCG were injected. The ovaries of both strains were examined by histology and TUNEL method at 0 time after injection of PMSG and at 0 and 48 h after injection of hCG. This hormonal treatment was reported to accelerate follicular development and sexual maturation leading to massive ovulation (26, 27). The 25-d-old ovaries of the dwarf mice were smaller (72% in diameter) than those of the normal mice and consisted mainly of large preantral follicles and a few antral follicles (Fig. 10, A and B). The interstitial tissue in the normal ovaries was more developed compared with the dwarf mice ovaries. Forty-eight hours after PMSG injection, there was a dramatic increase in the development of the normal mice ovaries as expected, the diameter of which increased by 54%. The ovaries contained many large preovulatory follicles (Fig. 10C). Similarly, there was also a dramatic development of the dwarf mice ovaries (61% enlargement in diameter), and the ovaries consisted mainly of large preantral follicles and some antral follicles (Fig. 10D). The incidence of apoptosis was reduced significantly [from 48 ± 2% and 47 ± 2.6% to 20 ± 4.3% and 18 ± 2.4%, respectively ($P < 0.01$)] for normal and dwarf ovaries (Fig. 4C). After 48 h of hCG injection the normal ovaries contained about six corpora lutea per ovary with no further significant increase in size (Fig. 10E). On the

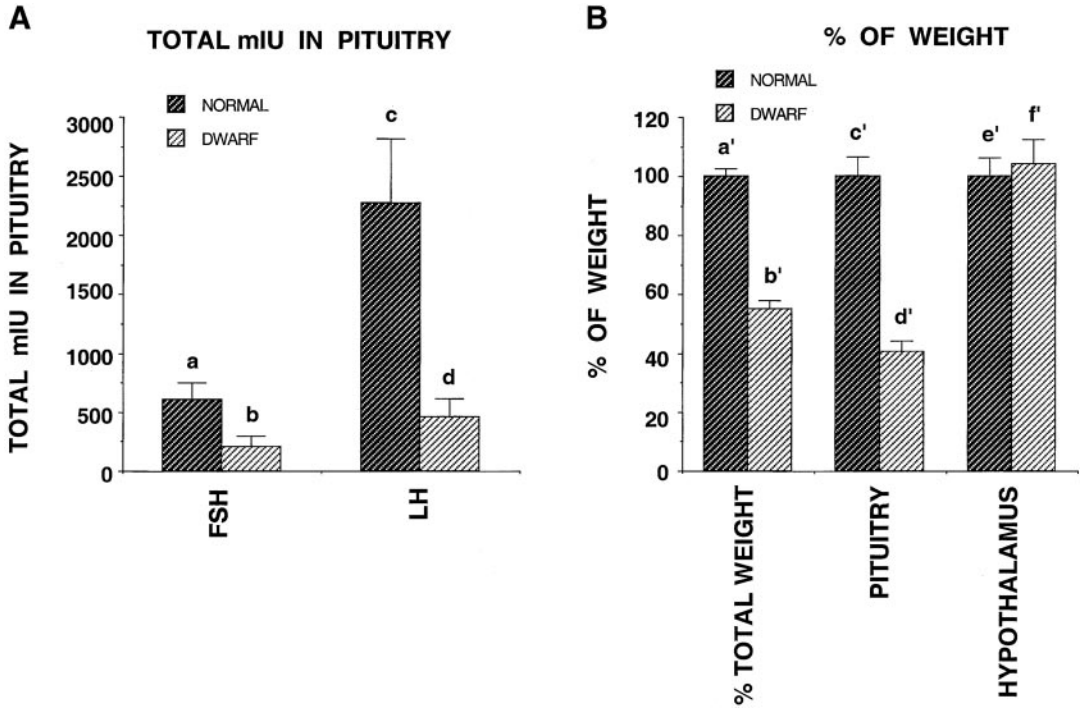

Fig. 5. Pituitary Gonadotropin Bioactivity and Body, Pituitary, and Hypothalamic Weights of Normal *vs*. Dwarf Female Mice

A, Bioactivity of LH and FSH and pituitary weight in normal and dwarf 3-month-old mice. LH and FSH bioactivity was monitored as specified in legend to Fig. 3 and in *Materiasl and Methods*. Data are calculated as mean ± SD per whole pituitaries (a>b, $P <$ 0.001; c>d, $P < 0.001$). B, Relative weight of animals, pituitaries, and hypothalami. Original data are expressed in grams or milligrams in *Results*. Data are mean ± SD (n = 6). a′>b′, $P < 0.001$; c′>d′, $P < 0.001$.

other side, dwarf ovaries continued to develop (15.5% increase in the mean diameter, $P < 0.01$) containing a large number of antral follicles, and one to two corpora lutea (Fig. 10F). The incidence of apoptosis remained low in both strains after PMSG and hCG treatment (Fig. 4C). Therefore, it seems that gonadotropin treatment of the dwarf mice can repair, at least in part, the development of the ovary by reducing the apoptotic incidence and accelerating follicular maturation and ovulation.

DISCUSSION

Previous studies demonstrated the expression of FGFR1, FGFR2, and FGFR4 in the ovary (28, 29). Here, we have shown that in mouse ovary FGFR3 is expressed in the granulosa cells but not in the theca cells or the oocyte. Using immunostaining with antibodies to FGFR3, the receptor was found to be localized to the membrane of the granulosa cells. On the other hand, in granulosa cells of dwarf mice containing $FGFR3^{G374R}$, aggregation and internalization of the receptor were observed.

Activation of the receptors by FGF leads to clustering and internalization followed by receptor degradation or recirculation. Therefore, it is possible that constant activation of the mutant receptors even without ligand may lead to intensive internalization. The role of FGFR3 in ovarian development is still obscure, but our results suggest that it plays a role in the maturation of the follicle via the function of the granulosa cells. This conclusion can be drawn since granulosa cells expressing $FGFR3^{G374R}$ undergo apoptosis, and at 3 months of age the ovary of the dwarf mice showed many immature follicles and lack of Graafian follicles. On the other hand, the oocytes in the ovaries of the mutant mice did not show any defect and when released from the immature follicles could complete the meiotic cycle similar to oocytes from wild-type mice. An alternative possibility, which should be taken in consideration, is that the entire phenotype of the ovary described in the present work may be indirect and result from changes in pituitary function, namely chronic low levels of FSH and LH that cannot support complete follicular development or luteinization. Pituitary insufficiency clearly could lead to apoptosis. Moreover, as demonstrated in this study, the failure in follicular growth and luteinization can be recovered to a large extent upon PMSG/hCG treatment. These observations would strengthen the notion of an endocrine effect. An additional possibility is that the ovarian phenotype leading to infertility in achondroplastic mice expressing mutant $FGFR3^{G374R}$ may be due to the small size of the mice.

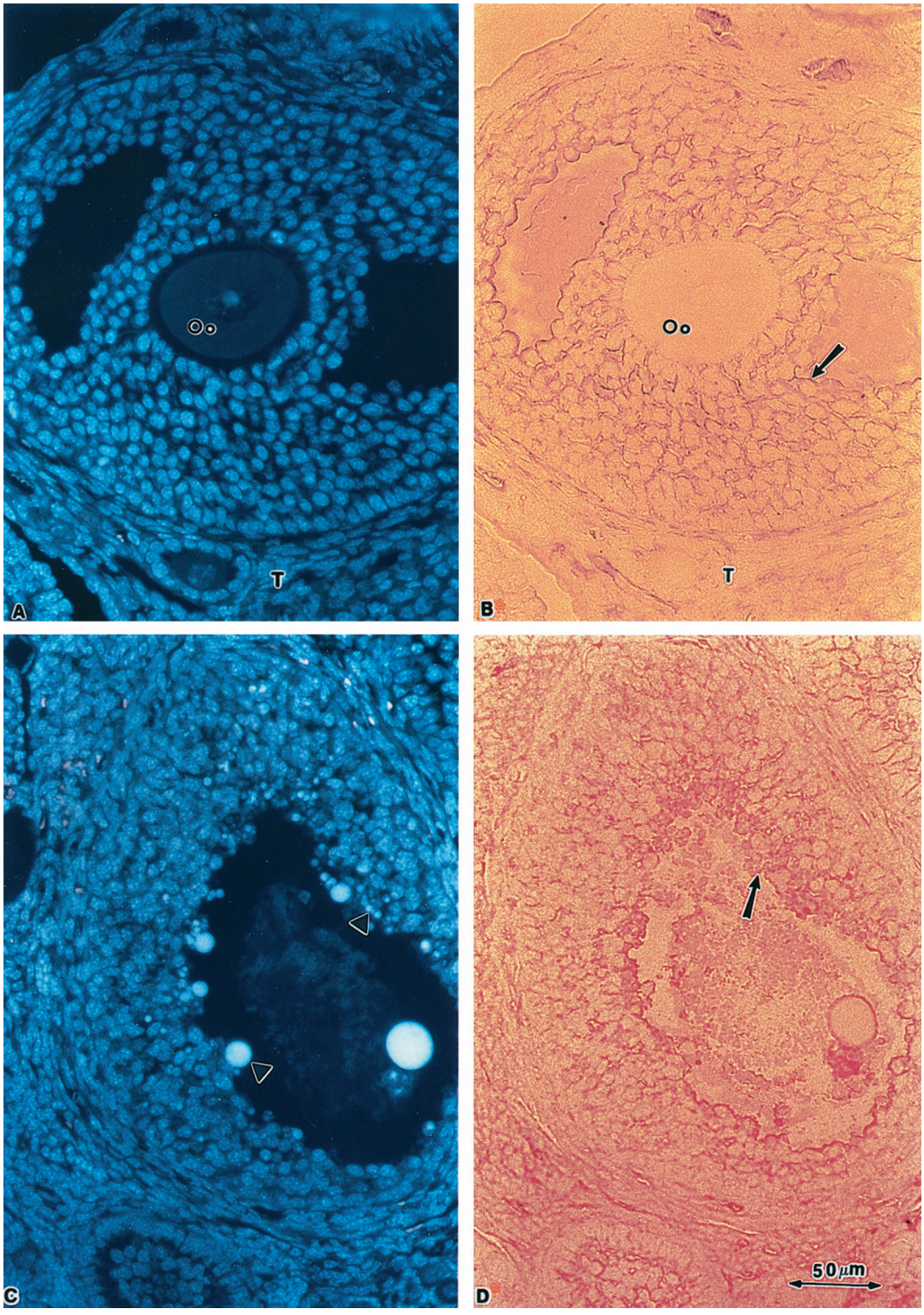

Fig. 6. Localization of FGFR3 in Normal and Dwarf Mice

A, DAPI staining and FGFR3 staining by immunocytochemistry of normal follicle from ovary of 3-month-old female mouse. Nonapoptotic nuclei delineating the organization of the follicular tissues. B, Staining of FGFR3 in the some follicle with specific antibodies delineating the surface of granulosa cells (*arrow*) with no labeling within the oocyte (Oo) or theca cells (T). C, Apoptotic follicle stained with DAPI. Note the fragmented DNA in nuclei of the inner layers of granulosa cells and large spheres of DNA within the antrum indicating intensive apoptosis (*arrowheads*). D, The same follicle subsequent to staining with FGFR3 antibodies. Receptors are located at the circumference of granulosa cells showing nonhomogenous fragmented distribution of the receptor (*arrow*).

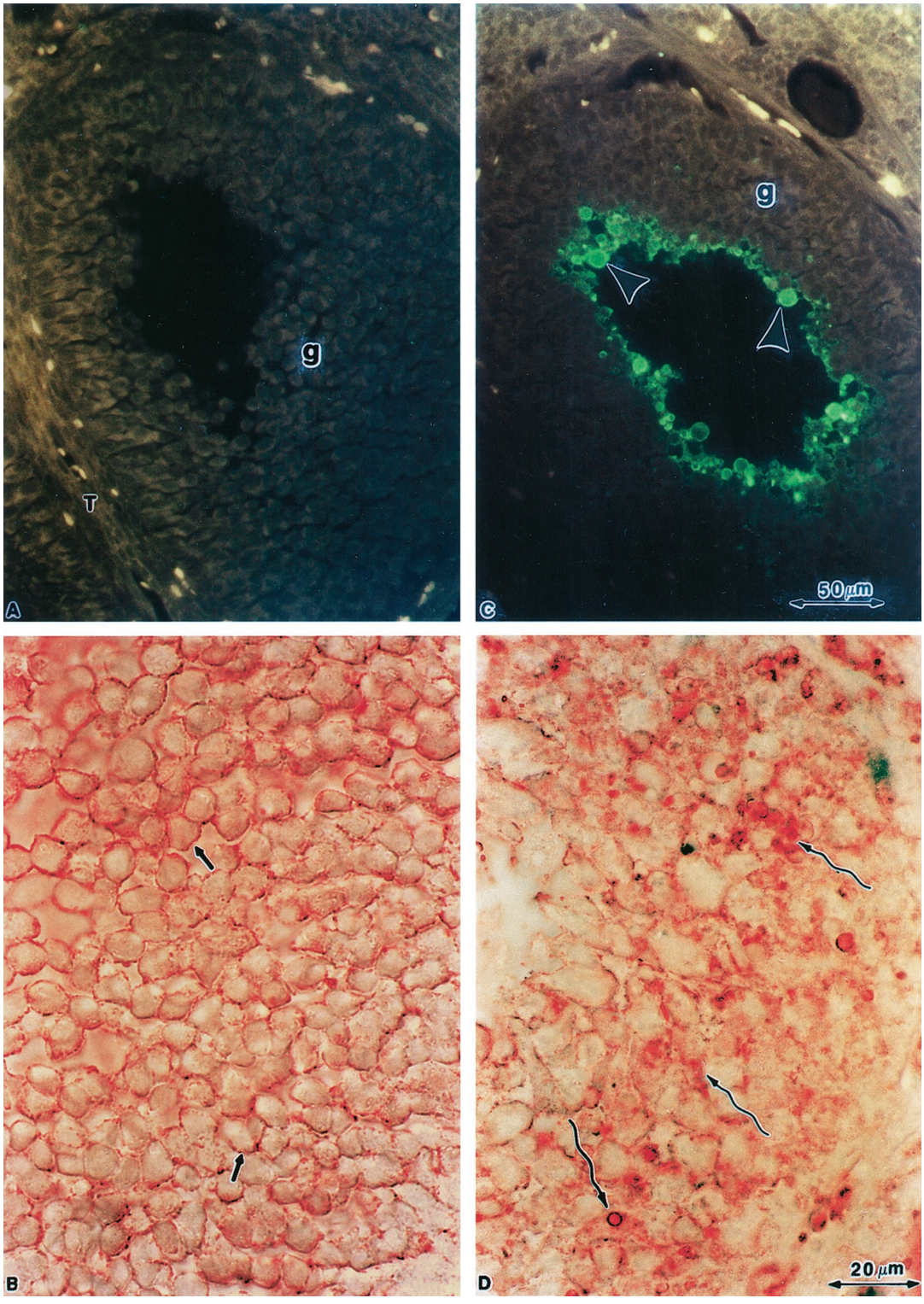

Fig. 7. TUNEL Staining and FGFR3 Localization in Ovaries Obtained from 3-Month-Old Normal or Dwarf Mice

A, Note the absence of apoptotic granulosa cells (g) in the antral follicle of a normal mouse. The labeling of the blood capillaries within the theca (T) is not specific. B, Localization of FGFR3 at the circumference of granulosa cells (*arrows*). C, TUNEL labeling of an antral follicle of a dwarf mouse. Note the labeling at the inner layers of granulosa cells (*arrowheads*). D, Fragmented labeling of FGFR3 on granulosa cell surface and in endocytic vesicles (*twisted arrows*).

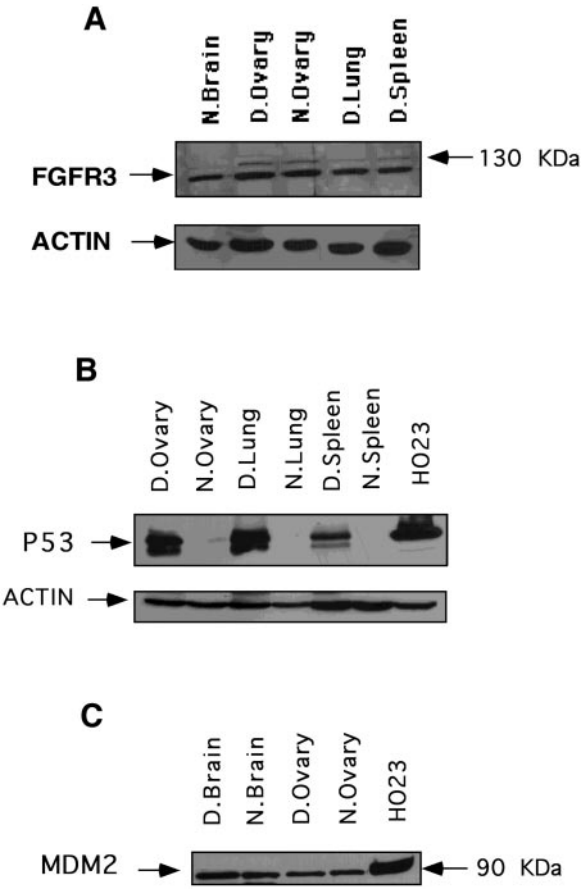

Fig. 8. Western Blot of FGFR3, p53, and MDM2

A, The receptor is expressed in normal and dwarf mice (3-month-old) in the brain, ovary, lung, and spleen. B, High expression of p53 in dwarf ovary, lung, spleen, and immortalized granulosa cell line (HO23) expressing the temperature-sensitive mutant of p53 (Val 135 p53), which serves as a positive control (37). C, No difference in expression of Mdm2 in normal and dwarf mice.

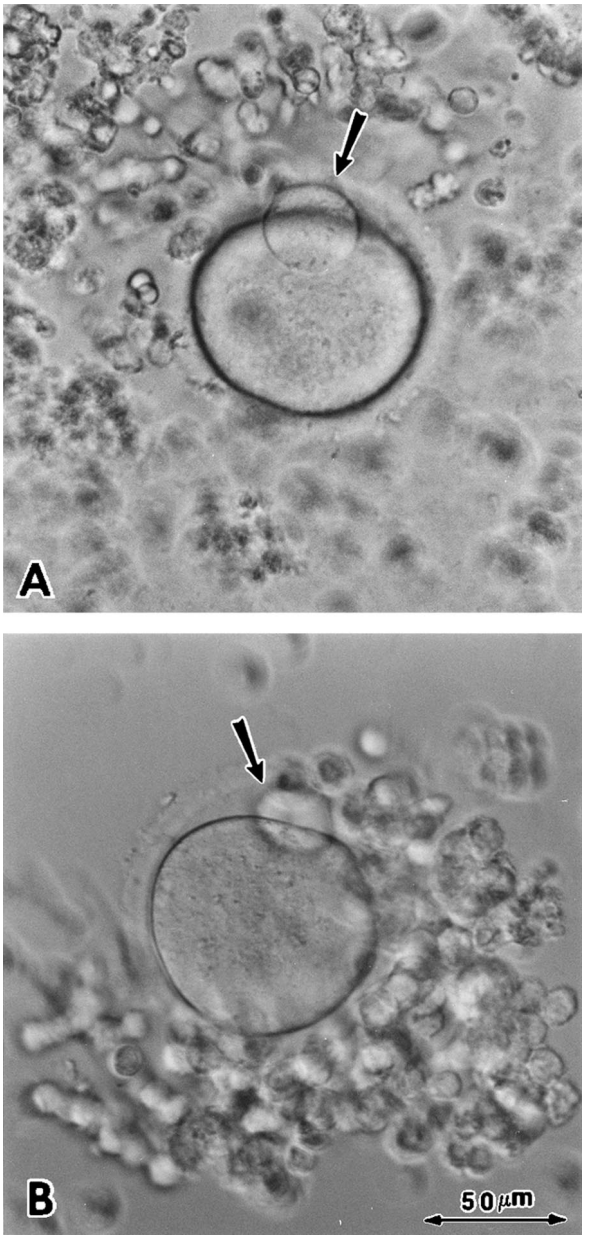

Fig. 9. Oocytes Removed from Antral Follicles of 3-Month-Old Normal Female Mouse (A) and 3-Month-Old Dwarf Mouse (B)

Note extrusion of the first polar bodies after 24 h of incubation of the oocytes *in vitro*. Cumulus cells are still surrounding the oocyte.

The effect of the mutant FGFR3 on granulosa cells should be viewed in light of recent studies on the signaling pathways of FGFR3 in various cell types. These studies demonstrated that FGFR3 signaling could follow three different pathways, depending on cell type and stage, as described in the following examples. First, in fibroblasts the signaling by FGFR3 leads to cell division, and this may be considered as the proliferative or mitogenic function of receptor activation which operates through the FRS, ras, and MAPK pathway (30). Second, in chondrocytes, signaling by FGFR3 probably functions through the signal transducer and activator of transcription (STAT) pathway and transcription activation of cyclin-dependent kinase (cdk) inhibitors (*e.g.* $p21^{waf}$ or p16), which lead to cell growth arrest (31, 32). Indeed, FGFR3-deficient mice showed bone overgrowth (33, 34), and $FGFR3^{G374R}$ heterozygotes showed achondroplasia and inhibition of bone growth (25, 35). Thus, FGFR3 was defined in this cell type as a negative growth regulator. Recent work demonstrated a third pathway as shown in osteoblast cell lines at certain stages of cell differentiation, where the signaling through FGFR3 caused apoptosis (36). Also, in human chondrocytes expressing the mutant $FGFR3^{G380R}$, apoptosis was observed (37). It is therefore possible that in granulosa cells also, the activation of FGFR3 may lead to apoptosis and inhibit granulosa function and follicular development. These signaling pathways can be activated either by overstimulation with FGF, or by the FGFR3 mutation, which

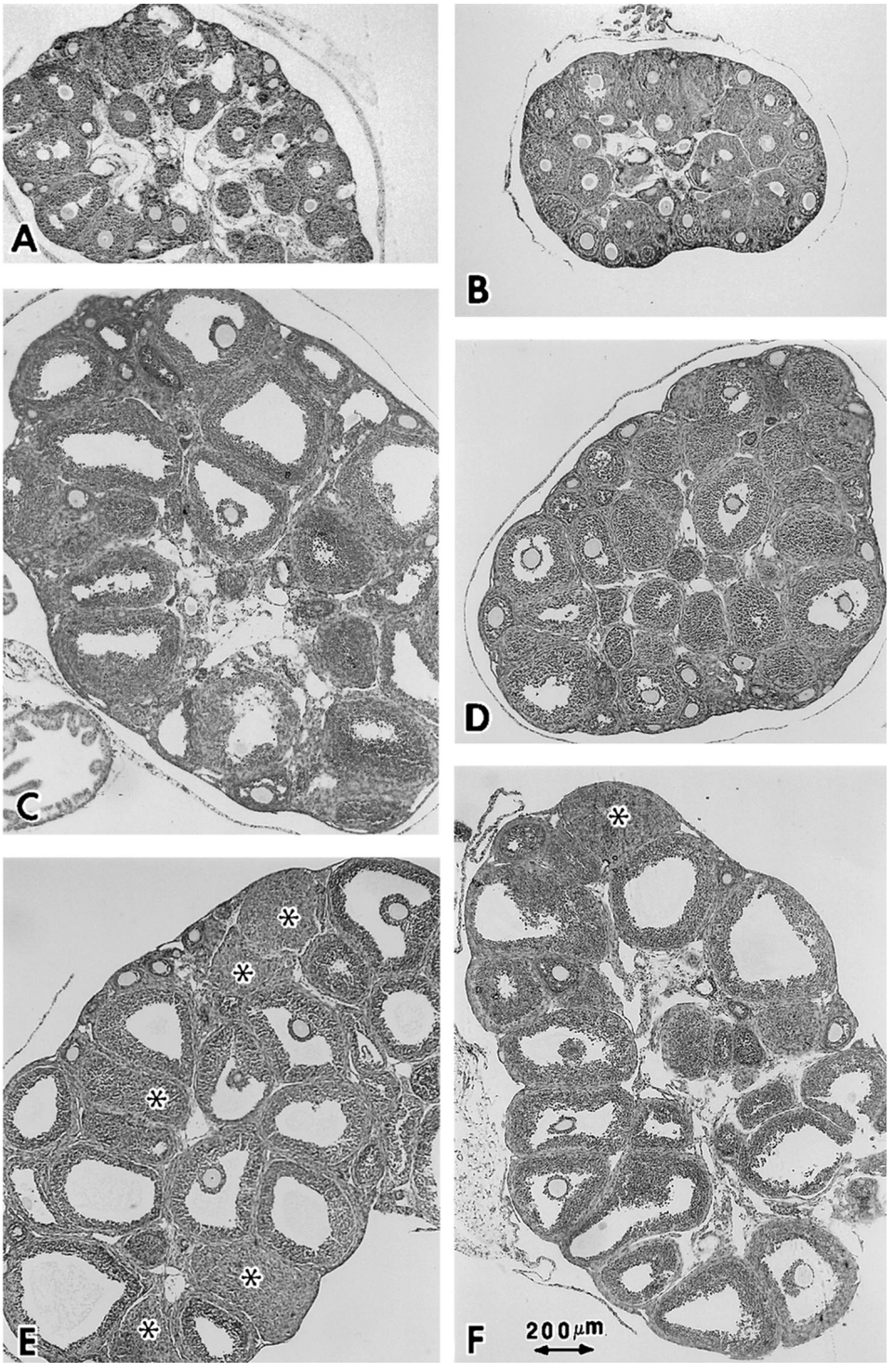

Fig. 10. Stimulation of 25-d-Old Female Normal and Dwarf Mice with PMSG and hCG

Females (25 d old) were injected with 15 IU of PMSG for 48 h followed by 15 IU of hCG, and animals were killed 48 h after hCG injection and ovaries were processed for histology. A, Section of ovary of 25-d-old normal mouse. Note preantral and small antral follicles, which occupy most of the peripheral ovarian tissue of normal ovary. B, Section of ovary of 25-d-old dwarf mouse. Note the smaller size of the ovary (70% to that of the control in diameter), which mainly consists of preantral and some small antral follicles. C, Development of the normal ovary after PMSG injection (15 IU PMSG for 48 h), which contains mainly large antral and preovulatory follicles. D, Development of the dwarf mouse ovary 48 h after PMSG injection, which contains mainly large preantral and medium sized antral follicles. E, Normal ovary showing massive ovulation and formation of corpora lutea (*) after 48 h of PMSG (15 IU) injection and 48 h after hCG injection. F, Pronounced increase in the size of the antral follicle in dwarf mice ovary after the same hormonal treatment. One corpus luteum is visible in the ovarian section (*).

may activate the receptor in a ligand-independent manner. However, one cannot exclude the possibility that the chronic low levels of FSH and LH play a major role in the induction of granulosa cell apoptosis in the $FGFR3^{G374R}$ achondroplastic mice.

It was recently demonstrated that bFGF is a survival factor in granulosa cells (15, 16, 38). In contrast, in the present study we show that the mutant FGFR3 exerts apoptosis. This may reflect the different pathway of FGFR signaling at different stages of cell differentiation. For example, a recent analysis of the mutation K644E in the kinase region of FGFR3 was shown to cause chondrocyte proliferation on days 14 and 15 of embryonal differentiation and inhibition of proliferation at a later stage of development on days 18 and 19 (39). Since the demonstration that FGF is a survival factor in granulosa cells was done in cultured cells and the apoptosis of granulosa cells shown here was during the postnatal development of the mutant mice, this result is in agreement with the various effects of FGFR signaling during different cell stages (36, 37). Alternatively, the degree of activation of the FGFR3 and its duration may determine whether it will transduce survival or apoptotic signals (36, 37).

Significantly lower levels of FSH, LH, and progesterone in the blood of dwarf mice, compared with normal mice (40), suggest that the dwarf mice may have reduced amounts or lower ability to release LH and FSH compared with normal animals. Our observation of a pronounced reduction in bioactivity of both LH and FSH in pituitaries of dwarf mice compared with normal animals strongly support this notion. Alternatively, they might have a defect in releasing GnRH, which is known to stimulate the release of LH and FSH from the pituitary to the circulation (for review see Refs. 41 and 42). This view is strongly supported by the pronounced effect exerted by exogenous PMSG, which exhibits both LH/CG and FSH activity (43), followed by the effect of hCG on rapid ovarian development in dwarf mice, which was associated with reduction of apoptosis and reaching sexual maturation, as was demonstrated in the present study. This would support the notion that concerted action of growth factors and gonadotropins is essential for ovarian development and maturation (44, 45).

Our results on the elevated expression of p53 in the mutant ovary suggest a possible correlation between granulosa apoptosis and p53 expression (46). Activation of p53 was previously demonstrated to induce granulosa cell apoptosis both *in vitro* and *in vivo* (reviewed in Ref. 47). Nevertheless, the mechanism by which granulosa cells undergo apoptosis in mutant mice is still obscure and requires further investigation. It is of interest that although mutations in FGFR3 were reported mainly to be associated with skeletal dysplasia, the receptor is expressed also in many other tissues such as brain, lung, bladder, and ovary. Nevertheless, no pathological effect of receptor activation was reported in these tissues. The study reported here provides an example of female infertility due to impairment of follicular development in the ovary, resulting probably from activation of FGFR3 by the achondroplastic mutation, and shows that a major effect can emerge from a pituitary defect in the $FGFR3^{G374R}$ achondroplastic mice in failing to provide optimal survival signals of gonadotropins essential for follicular development. This effect of the mutation was not reported for achondroplasia in humans. It is therefore possible that it is specific to mice carrying this mutation. Nevertheless, our data demonstrate the importance of FGFR3 in normal development of ovarian follicles, since the mutated receptor causes a severe defect in follicular maturation, which leads to female infertility. Interestingly, it was recently reported that a $Ser^{365} \rightarrow Cys$ mutation of FGFR3 in mice led to achondroplasia and reduced fertility of the mutant mice in which no abnormalities were apparent in their testis and ovaries (56). These authors suggest that the infertility may be due to the small size of the mice. Taken together, our data and the data of Chen *et al.* (56) would suggest that different mutations in FGFR3 may lead to infertility not necessarily by the same mechanism.

MATERIALS AND METHODS

Specific Reagents

Polyclonal antibodies to FGFR3 (C-15) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were used both for Western blot and immunohistochemistry. Polyclonal antibodies to mouse FGFR3 were also kindly provided by Dr. D. Ornitz (Washington University, St. Louis, MO). Reagents for TUNEL staining were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Monoclonal mouse Mdm2 antibodies were from Santa Cruz Biotechnology, Inc. p53-specific monoclonal antibody, mAb421 (directed against human and mouse p53 antigen) and mAb240 (directed again mouse p53 antigen) were kindly provided by Dr. M. Oren (Weizmann Institute of Science, Rehovot, Israel). Both goat antimouse or antirabbit IgG coupled to horseradish peroxidase were obtained from Sigma (Nes Ziona, Israel). A kit (Auto Prob III) for histochemical staining of FGFR3 by the immunoperoxidase method was obtained from Biomeda Corp. (Foster City, CA). ^3H progesterone was obtained from Sigma, Rehovot. Highly purified rat LH, FSH [iodination grade (rat FSH-I-8, IOD; rat LH-I-9, IOD)] were obtained from the NIH and Dr. Parlow. PMSG was obtained from N.V. Organon (Oss, The Netherlands).

Animals

At selected time points, 3 wk- or 3-month-old normal or dwarf mice (25) were killed by ip injection of 0.2 ml thiopentone sodium (50 mg/ml) (Abbott S.A., Campovera, Italy), and the ovaries, kidneys, brains, lungs, and liver were removed. One ovary of each animal was fixed in 4% paraformaldehyde, and the other one was snap frozen in liquid nitrogen and kept at −80 C. Liver and other organs were also snap frozen in liquid nitrogen and kept at −80 C. The experiments were repeated three times with at least three animals per each time point. In some experiments, 25-d-old normal and dwarf mice were injected (nine animals of each group) with 15 IU per animal of PMSG followed by 15 IU hCG 48 h later. Ovaries were re-

moved and fixed with 4% paraformaldehyde immediately after each injection as well as 48 h after the hCG treatment.

Histology

After fixing with 4% paraformaldehyde for 24 h at 24 C, the ovaries were embedded in 70% ethanol and subsequently immersed in paraffin, and 6- to 10-μm sections were cut with a microtome and stained with hematoxylin and eosin. Sections were inspected using a Carl Zeiss light microscope (Axioscope -Carl Zeiss, Oberkochen, Germany). For examinations of apoptosis, unstained sections were deparaffinized by washing three times with xylene for 5 min, followed by ethanol 100%, ethanol 95%, ethanol 70%, and H_2O for 3 min each. Sections were doubly stained for terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) (48) and DAPI (15, 16) as follows: 1) TUNEL method was performed by labeling DNA strand breaks with the *In-Situ* Cell Death Detection Kit (Roche Molecular Biochemicals) using fluorescein-deoxyuridine triphosphate (dUTP). 2) After the TUNEL staining the sections were incubated with 100 μl of 125 ng/ml of DAPI (Sigma Ltd.) for 30 min at 24 C. Microscopic examination of the specimens was carried out using a Carl Zeiss Axioskop microscope in a bright field or in fluorescent mode.

Collection of Blood and Hormonal Assays

Aliquots of blood were collected from 3-month-old normal and dwarf mice (six animals from each group). Sera were prepared at 4 C and kept at −80 C until hormonal determinations. Pituitaries were dissected immediately after the animals were killed and were homogenized in 50 μl of PBS containing 1 mM of phenylmethylsulfonyl fluoride to avoid protein degradation at 4 C. Homogenates were kept at −80 C until hormonal determinations. Progesterone in serum was determined by RIA as described previously (49, 50). FSH and LH bioactivity in sera and pituitary homogenates was determined by FSH-responsive or LH/CG-responsive cell lines [rat FSHR-17 (see Ref. 51) and rat LHR-15 cell lines (see Ref. 52)] as described earlier (53). In brief, the amount of progesterone secreted into the medium in response of the appropriate cell lines to aliquots of sera (5–50 μl) or 1–3 μl pituitary homogenates were measured, and the bioactivities of the hormones were calculated according to calibration curves run in parallel containing the appropriate cell culture incubated with increasing hormone concentrations of highly purified (iodination grade) rat LH (rat LH-I-9, IOD) or rat FSH (rat FSH-I-8, IOD). Samples of serum and pituitary homogenates were assayed in triplicate experiments.

Immunohistochemical Staining for FGFR3

Sections were deparaffinized, as described above, and incubated overnight at 4 C with FGFR3 polyclonal antibodies followed by anti-IgG coupled to peroxidase. After completing the peroxidase reaction on the slides, the tissue was either stained or left without staining with diluted hematoxylin solution. For nonspecific staining, sections of microscopic slides were stained with nonimmune rabbit sera. Sections were visualized with the Carl Zeiss microscope.

Protein Analysis and Western Blot

Frozen tissues were homogenized in lysis buffer containing: 20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 20 μM leupeptin, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, and 5 μg/ml aprotinin. Proteins were quantified by the Bradford method (54). Lysates containing 50 μg proteins were boiled in sample buffer for 10 min and separated by 12% SDS-PAGE, transferred onto nitrocellulose membranes. The blots were then blocked using 5% milk powder in PBS plus 0.05% Tween 20 and incubated overnight at 4 C with the corresponding first antibodies followed by 1 h incubation at 24 C with goat antirabbit or goat antimouse IgG conjugated to horseradish peroxidase. For the detection of p53, the first antibody solution consisted of a 1:1 mixture of mAb421 and mAb240 antibodies, which recognize different epitopes of the mouse p53 and significantly increase the sensitivity of detection of p53 compared with the use of each antibody alone. The detection of the second antibody was carried out by the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech Co, Buckinghamshire, UK). Western blot analysis was carried out in three separate experiments.

Morphometric Analysis

Fixed ovaries embedded in paraffin were sectioned at the center of the gland and the mean diameter was calculated to determine the size of the ovary. Measurements were taken from six different glands that received the same treatment. For scoring the apoptosis, at least four sections of each ovary were used. Follicles were scored as apoptotic if they demonstrated at least five apoptotic cells (positively stained by the TUNEL method) in a given section. The number of corpora lutea per gland was scored on the intact gland and confirmed by scoring the corpora lutea in serial sections.

Oocyte Recovery and First Polar Body Extrusion

Oocytes were recovered by puncturing the antral follicles of six ovaries of 3-month-old normal or dwarf mice and cultured for 24 h as described recently (55). Each group (normal and dwarf mice) contained 30–40 oocytes. At the end of incubation the oocytes were analyzed for maturation by differential interference contrast microscopy, and the percent oocytes demonstrating extrusion of the first polar body were scored.

Statistical Analysis

Mean values of ovarian diameter, and other morphological measurements, and hormone values were compared using ANOVA followed by Fishers' least significance difference test. Differences between treatment groups were considered statistically significant at $P < 0.05$.

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