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Assessment of folliculogenesis in ovarian tissue from young patients with Turner syndrome using a murine xenograft model

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Objective: To study the impact of aneuploid granulosa and stromal cells on folliculogenesis of small ovarian follicles from patients with mosaic Turner syndrome (TS) using a murine xenograft model.
Design: Laboratory study.
Setting: ■ ■ ■
Patient(s): Ovarian cortical tissue was obtained by laparoscopic surgery from 18 patients with mosaic TS (aged 5–19 years) and 13 controls (aged 5–18 years).
Intervention(s): Part of each tissue fragment was used to karyotype ovarian cells in nongrafted tissue by fluorescence in situ hybridization. The remaining tissue was xenografted to severe combined immunodeficient mice for 5 months. Grafted tissue was analyzed for aneuploidy, and follicle density and morphology were determined. Expressions of proliferating cell nuclear antigen and anti-Müllerian hormone were investigated by immunohistochemistry.
Main Outcome Measure(s): The impact of aneuploid granulosa and stromal cells on folliculogenesis. Fluorescence in situ hybridization of ovarian tissue before grafting was performed to determine the level of aneuploidy in stromal cells and oocytes and granulosa of small follicles. After xenografting, the level of aneuploidy of the newly formed layers of granulosa cells was again determined in secondary and antral follicles.
Result(s): Follicle density in ovarian tissue from patients with TS was significantly lower than in controls before grafting. Fluorescence in situ hybridization analysis confirmed that 101 of 104 oocytes from nongrafted tissue of patients with TS showed normal X chromosome content, whereas granulosa and stromal cells were mainly 45,X. Fragments from 12 patients with TS contained follicles at all stages after xenografting, including secondary and antral follicles. Follicle density in patients with TS and controls decreased significantly after grafting. Moreover, a shift from high to low proportions of 45,X granulosa cells was observed during folliculogenesis. Expression of proliferating cell nuclear antigen in follicles from patients with TS increased significantly during grafting. Secretion of anti-Müllerian hormone was impaired before grafting in peripubertal/postpubertal girls with TS, but recovered after grafting.

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Conclusion(s): Our study showed that small follicles from patients with mosaic TS undergoes folliculogenesis, despite the presence of aneuploid granulosa and stromal cells. Ovarian tissue cryopreservation could therefore be a valid option to preserve fertility in young patients with mosaic TS if sufficient numbers of follicles are present, thus preferably before the age of 12. (Fertil Steril® 2023;■:■–■. ©2023 by American Society for Reproductive Medicine.)

Key Words: Turner syndrome, fertility preservation, xenografting, mosaicism

Turner syndrome (TS), affecting 1 in every 2,500 girls, is one of the most common sex chromosome disorders in females and is associated with congenital malformations of the heart and kidneys, thyroid disease, hearing loss, and overweight (1, 2). In most women with TS, fertility is also severely compromised as a result of accelerated germ cell apoptosis and impaired folliculogenesis in the ovaries during fetal life (3, 4). Postnatal loss of oocytes further depletes the oocyte pool and leads to premature exhaustion of the ovarian reserve by the time these women are ready to conceive (more than 80% of cases) (5). Fertility loss is indeed a major challenge for women with TS, regardless of their age (6, 7). In view of the rapid decline of the ovarian pool in most patients with TS, fertility preservation should be considered at the earliest age possible to preserve as many oocytes as possible (8, 9). Ovarian tissue cryopreservation (OTC) is an effective option for the preservation of fertility in prepubertal girls facing gonadotoxic chemotherapy. Although this technique is already being considered in patients with TS in an experimental setting, there are currently no data on whether the frozen tissue will actually function when reimplanted (10–12).

Reports on levels of aneuploidy in ovarian tissue from patients with TS are limited. In 3 case studies, including five 45,X/46,XX patients in total, 90% of oocytes had normal X chromosomal content, whereas granulosa cells (GCs) and cortical stromal cells were either largely or exclusively 45,X (13–15). The high degree of aneuploidy in GCs may have functional consequences for bidirectional signaling between oocytes and GCs, which is essential for normal folliculogenesis (16, 17). Moreover, abnormal X chromosomal content in ovarian cortical stromal cells could disrupt follicle growth, because they constitute the follicular microenvironment that is crucial to the recruitment of theca cells (17, 18).

The goal of this paper is to assess the capacity of the primordial ovarian follicles from patients with TS to progress to secondary and antral stages after OTC. Follicular density and follicular morphology were determined in a series of 18 young patients with TS with a mosaic karyotype. In addition, the X chromosomal content of ovarian cells and the expression of proliferating cell nuclear antigen (PCNA) and anti-Müllerian hormone (AMH) were analyzed before and after long-term xenografting to immunodeficient mice to explore the changes induced by transplantation of the ovarian cortex of patients with mosaic TS, and to determine whether OTC is a realistic option to preserve fertility in TS.

MATERIALS AND METHODS

Participants

Frozen-thawed ovarian tissue fragments from 18 patients with mosaic TS (6 from UCLouvain, Belgium, and 12 from Radboudumc, The Netherlands) and 13 age-matched controls (from UCLouvain) were used in this study. All patients had undergone cryopreservation of their ovarian tissue at different ages to preserve their fertility. Among patients with TS (aged 5–19 years, mean age: 11.8 ± 3.6 years), 7 were prepubertal and 11 peripubertal or postpubertal at the time of OTC. Levels of 45,X/46,XX mosaicism in lymphocytes varied considerably between patients (range: 11%–100% 45,X cells). Among controls (aged 5–18 years, mean age: 11.4 ± 5.7 years), 6 were prepubertal and 7 peripubertal or postpubertal. The characteristics of participants, including the indication for OTC in the controls, are shown in Supplemental Tables 1 and 2 (available online).

Slow Freezing and Thawing Procedure

Cryopreservation of all ovarian tissues from controls and patients was performed according to clinical standards using the slow freezing protocol (19). After obtaining patient consent, one cortical fragment per patient was thawed, as previously described (19). Part of the thawed ovarian cortex was then subjected to analyses before grafting, whereas the other part was xenografted for 5 months.

Xenotransplantation to Immunodeficient Mice

Thirty-one 6- to 9-week-old female severe combined immunodeficient (SCID) mice (Charles River Laboratories, France) were used for ovarian tissue transplantation (OTT). One frozen-thawed ovarian fragment per patient was grafted to the intraperitoneal lining for 5 months. Appropriate housing and breeding conditions and surgical procedures were applied, as previously reported (20, 21). Ovarian stimulation was performed during the last 2 weeks before graft retrieval using 7.5 IU recombinant follicle-stimulating hormone daily.

Histology

After fixation in 4% formaldehyde, the tissue was embedded in paraffin and serially cut into 4-μm-thick sections. To ascertain the viability and classification of follicles after xenotransplantation, every seventh section was stained with hematoxylin and eosin (Sigma-Aldrich, Machelen, Belgium). On the basis of the hematoxylin and eosin–stained slides, ovarian cortical sections containing follicles were selected for immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) analyses. For histological evaluation, 5 sections per sample (taken at 84-μm intervals) were scrutinized. Stained slides were then scanned with the Pannoramic P250 Digital Slide Scanner (3DHISTECH, Hungary) and analyzed using CaseViewer (3DHISTECH, Hungary). Follicle density was determined by dividing the total number of follicles by the

volume of evaluated sections, as previously detailed (22, 23). Only follicles with a visible oocyte nucleus were counted, ensuring that no preantral follicles were counted twice. Follicles were classified into primordial, intermediate, primary, secondary, antral, and atretic (22, 23). Abnormal follicles were divided into 3 categories, namely vacuolated follicles, di-oocytes or multinucleated follicles, and oocytes invaded by GCs (24).

Immunohistochemistry

Expressions of essential proteins for GC function, PCNA, and AMH were evaluated by IHC, as previously reported (25). In brief, slides were incubated for 1 hour at room temperature with primary antibodies, including monoclonal mouse anti-human AMH (1:100, MCA2246, Bio-Rad, Oxford, United Kingdom) and monoclonal rabbit anti-PCNA (1:600, 13110T, Cell Signaling, MA). For each marker, diaminobenzidine (Vector, CA) was used as a chromogen. Finally, the nuclei were counterstained with hematoxylin. The sections were digitized with the Pannoramic P250 Digital Slide Scanner (3DHISTECH, Hungary) using the following filters: green (SR-FITC-Zero) for Alexa Fluor 488, yellow (SP-CY3-4040C-Zero) for Alexa Fluor 555, and blue (DAPI-5060c-000-Zero) for Hoechst 33342. The slides were then analyzed using CaseViewer software (3DHISTECH, Hungary). Negative controls for IHC and immunofluorescence were incubated with FLEX polyclonal rabbit anti-human CD3 (IS600, Dako). Follicles were considered positive for PCNA if the nucleus of at least one GC expressed PCNA, and follicles were considered positive for AMH if the cytoplasm of at least one GC showed staining for AMH (26, 27).

FISH Analysis of Ovarian Cells in the Nongrafted Ovarian Cortex Tissue

Some of the nongrafted ovarian cortical tissues were digested enzymatically to obtain stromal cells and individual small follicles. Purified follicles were treated with trypsin to separate oocytes and GCs from the same follicle. Next, the X chromosomal content of ovarian cells was determined by FISH using centromere-specific probes for chromosome X (green) and chromosome 18 for control purposes (red). The X chromosomal content of oocytes was analyzed by determining the surface ratio of chromosome X- and chromosome 18-specific signals, as already described (14).

FISH Analysis of GCs in Secondary and Antral Follicles after Xenografting

The X chromosomal content of GCs in secondary and antral follicles was evaluated by FISH on 4-μm histological sections of grafts after long-term xenotransplantation. To accurately ascertain the number of X chromosomes per GC on histological sections of ovarian tissue from patients with TS, it was essential to first establish the detection sensitivity of X chromosome-specific FISH signals and the control chromosome in GCs from normal 46,XX individuals. To this end, 14 secondary and antral follicles from histological sections of the ovarian cortex from seven 46,XX transgender individuals were hybridized using chromosome X- and (control) chromosome 18-specific probes. The ratio of chromosome X-/chromosome 18-specific FISH signals in the GC layers of these normal follicles was 1.01 (95% CI 0.98–1.05), indicating that the detection sensitivity of the 2 probes was very similar (Supplemental Fig. 1, available online). The percentage of FISH signals lost because of sectioning of the tissue was determined by counting hybridization signals per GC in follicles of 46,XX transgender patients, and was found to be 24.0% in secondary and 21.5% in antral follicles. On the basis of these data, the degree of aneuploidy could be assessed in the GC layers of secondary and antral follicles in histological sections of ovarian tissue from patients with TS after grafting.

Ethical Approval

Use of human ovarian tissue was approved by the Ethics Committee of the Cliniques Universitaires Saint-Luc, the Institutional Review Board of the Université Catholique de Louvain (institutional review board references 2012/23MAR/125 and 2020/11MAR/152), and the Dutch Central Committee on Research Involving Human Subjects (CCMO NL57738.000.16). Written informed consent was obtained from all participants and/or their parents. Animal welfare was fully respected and complied with all guidelines endorsed by the Committee on Animal Research of the Université Catholique de Louvain (reference 2014/UCL/MD/007).

Statistical Analysis

Statistical parameters were calculated using GraphPad Prism software, version 8 (GraphPad Software Inc., CA). Student's unpaired *t*-test was conducted to compare results of follicle densities, survival, follicle classification, and expression of different markers between patients with TS and controls, prepubertal and peripubertal/postpubertal tissues from participants, and nongrafted and 5-month-xenografted ovarian tissues. In case of abnormal distribution or small sample size, the Mann-Whitney test was applied. Statistical significance was set at $P \leq .05$.

RESULTS

Viability, Survival, and Classification of Follicles in Ovarian Tissue from Patients with TS before and after Xenotransplantation

Before xenografting. Ovarian follicle density (Fig. 1) was significantly lower in prepubertal patients with TS than in controls (131–2,121 follicles/mm^3 vs. 2,946–7,401 follicles/mm^3, $P=.0012$) and in peripubertal/postpubertal girls with TS compared with controls (50–872 follicles/mm^3 vs. 1,553–2,045 follicles/mm^3, $P<.0001$). Onset of puberty affected follicle densities in both patients with TS and controls, causing loss of more than 50% of the follicle population.

Classification of follicles (Supplemental Fig. 2, available online) showed that quiescent-stage follicles (primordial and intermediate) accounted for the largest proportion of the follicle pool in ovarian tissue from patients with TS (65% in prepubertal and 68.3% in peripubertal/postpubertal girls), which was significantly lower than values in control

patients (87.3% and 86.3%, respectively, $P<.05$). Growing follicles from primary to antral stages only constituted a small fraction of follicles in TS tissues (both prepubertal and peripubertal/postpubertal). In contrast, proportions of abnormal follicles were almost 3 times higher in patients with TS than in controls (31.2% vs. 11.5%, $P=.0055$, in prepubertal girls; and 29.6% vs. 9.8%, $P<.0001$, in peripubertal/postpubertal girls).

After xenografting. Follicle densities in ovarian tissue from both patients with TS and controls decreased significantly after long-term grafting compared with before grafting (considered as 100%; Fig. 1). It is noteworthy that 27% (3 of 11 patients, aged 16, 17, and 19 years) of ovarian samples from peripubertal/postpubertal girls with TS had lost all follicles by 5 months after grafting.

In patients with TS, long-term xenografting revealed a remarkable drop in the proportion of abnormal follicles, from 31.2% to 8.4% in prepubertal girls and 29.6% to 6.2% in peripubertal/postpubertal patients, making them comparable with controls. Xenografted ovarian tissue from peripubertal/postpubertal patients with TS also exhibited a significant decline in proportions of small follicles (68.3% before grafting vs. 40.3% after grafting, $P\leq.05$). This was accompanied by a considerable rise in the proportion of growing follicles (2.1% before grafting vs. 53.5% after grafting, $P=.0052$) compared with control patients (3.5% before grafting vs. 27.9% after grafting, $P<.0001$). Secondary follicles were observed in grafts from 12 patients with TS, 6 of which also showed visible antral follicles (Supplemental Fig. 3, available online). Two mice died before graft retrieval and no follicles were observed in grafts from the 4 remaining patients with TS.

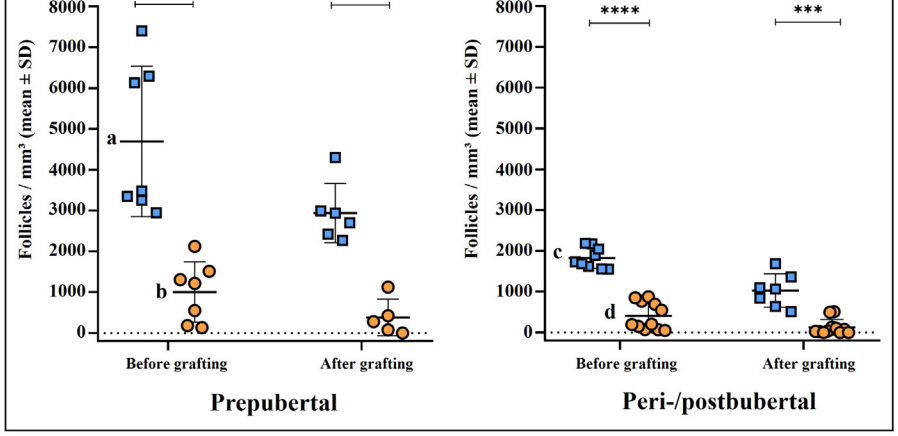
X Chromosome Mosaicism in cells from the Ovarian Cortex before and after Grafting

Before xenografting. Like lymphocytes, the stromal cell compartment also displayed highly variable levels of X chromosome mosaicism, ranging from 0% up to 80% of 45,X cells. Some stromal cells from patient I were 47,XXX, a cell line that was not detected in the analysis of lymphocytes from this patient (Fig. 2).

Counterstaining with DAPI revealed the meiotic DNA of oocytes to be more diffused than that of GCs, allowing GC- and oocyte-specific FISH signals to be easily distinguished (Fig. 3). In this manner, the X chromosomal content of 104 oocytes derived from small follicles from 10 patients with TS could be determined. Of that number, 101 were normal, whereas 3 oocytes (2.9%) from 3 different individuals contained only half of the X chromosome DNA strands (Supplemental Fig. 4, available online).

The X chromosome content of 2,153 GCs from 87 small follicles isolated from 10 patients with TS was also evaluated (Fig. 2). The number of karyotyped GCs ranged from 11 to 58 per follicle. Notably, follicles from most patients with TS

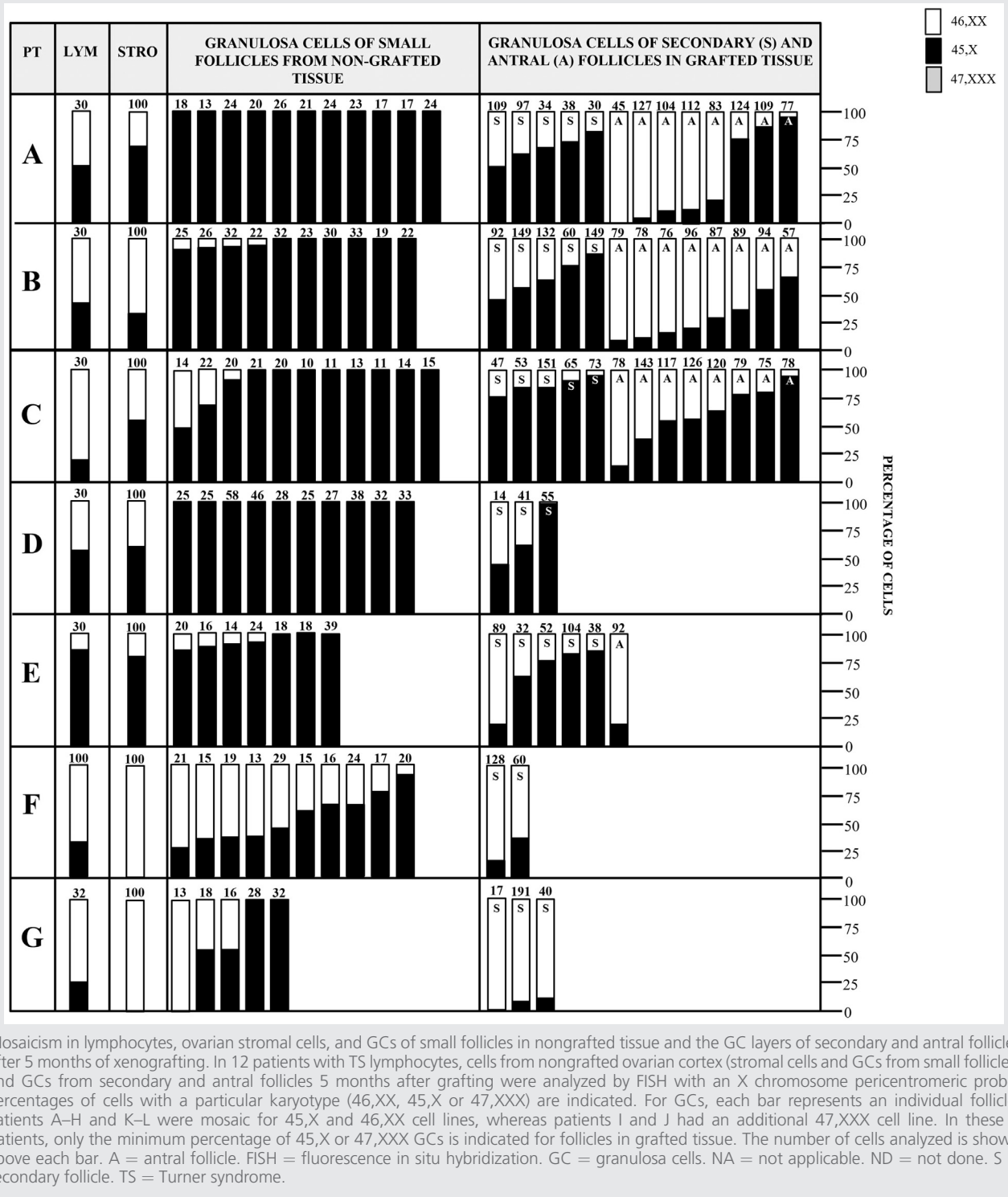
FIGURE 1



Follicle densities 5 months after xenotransplantation of ovarian cortex from patients with TS and control patients. Follicle densities (mean number of follicles/mm^3) in ovarian tissue from patients with TS (orange circle) were significantly lower than in controls (blue square) in both prepubertal (A) and peripubertal/postpubertal (B) groups before and after grafting. Prepubertal girls had a significantly higher follicle reserve compared with peripubertal/postpubertal patients in both TS (b>d, $P<.05$) and control (a>c, $P<.001$) groups. Significant differences among groups are indicated by *$P<.05$, **$P<.01$, ***$P<.001$ and ****$P<.0001$. TS = Turner syndrome.

Peek. Folliculogenesis in Turner syndrome. Fertil Steril 2023.

FIGURE 2



Mosaicism in lymphocytes, ovarian stromal cells, and GCs of small follicles in nongrafted tissue and the GC layers of secondary and antral follicles after 5 months of xenografting. In 12 patients with TS lymphocytes, cells from nongrafted ovarian cortex (stromal cells and GCs from small follicles) and GCs from secondary and antral follicles 5 months after grafting were analyzed by FISH with an X chromosome pericentromeric probe. Percentages of cells with a particular karyotype (46,XX, 45,X or 47,XXX) are indicated. For GCs, each bar represents an individual follicle. Patients A–H and K–L were mosaic for 45,X and 46,XX cell lines, whereas patients I and J had an additional 47,XXX cell line. In these 2 patients, only the minimum percentage of 45,X or 47,XXX GCs is indicated for follicles in grafted tissue. The number of cells analyzed is shown above each bar. A = antral follicle. FISH = fluorescence in situ hybridization. GC = granulosa cells. NA = not applicable. ND = not done. S = secondary follicle. TS = Turner syndrome.

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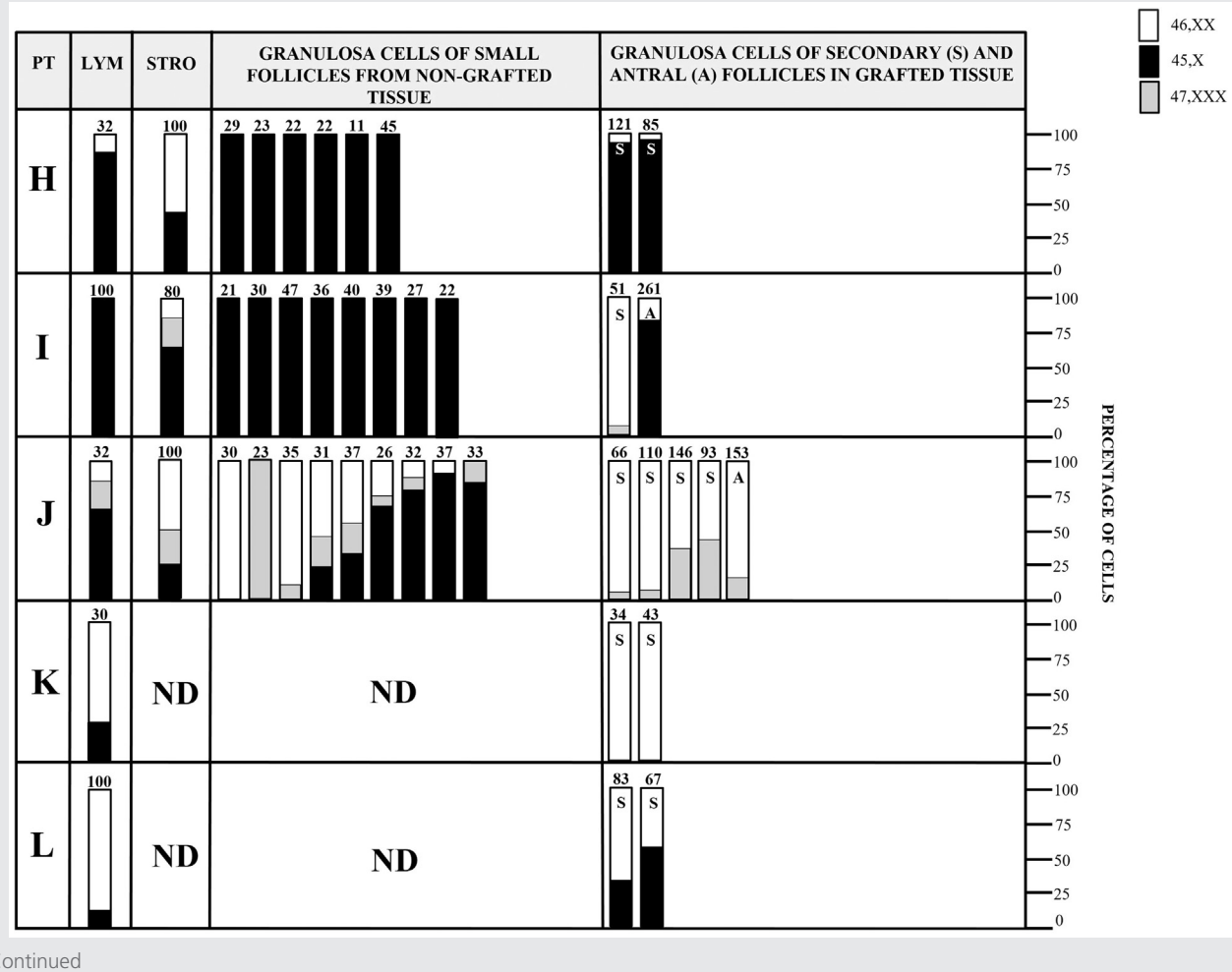
contained only or mainly 45,X GCs. Ratios of 45,X/46,XX or 45,X/46,XX/47,XXX GCs of individual follicles varied considerably, not only between patients, but also between follicles from the same patient.

After xenografting. The X chromosomal content of GC layers was investigated in 39 secondary and 27 antral follicles by FISH (Fig. 2). Percentages of aneuploid GCs from secondary follicles after 5 months of grafting were generally much lower

FIGURE 2



Continued

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than in small follicles in the nongrafted tissue (Fig. 2). In patients I and J, the 47,XXX cell line observed in the stromal cell compartment was also present in GCs from secondary and antral follicles. At more advanced stages of folliculogenesis, GC layer aneuploidy further decreased, but considerable variations were again observed between patients and between antral follicles from the same patient.

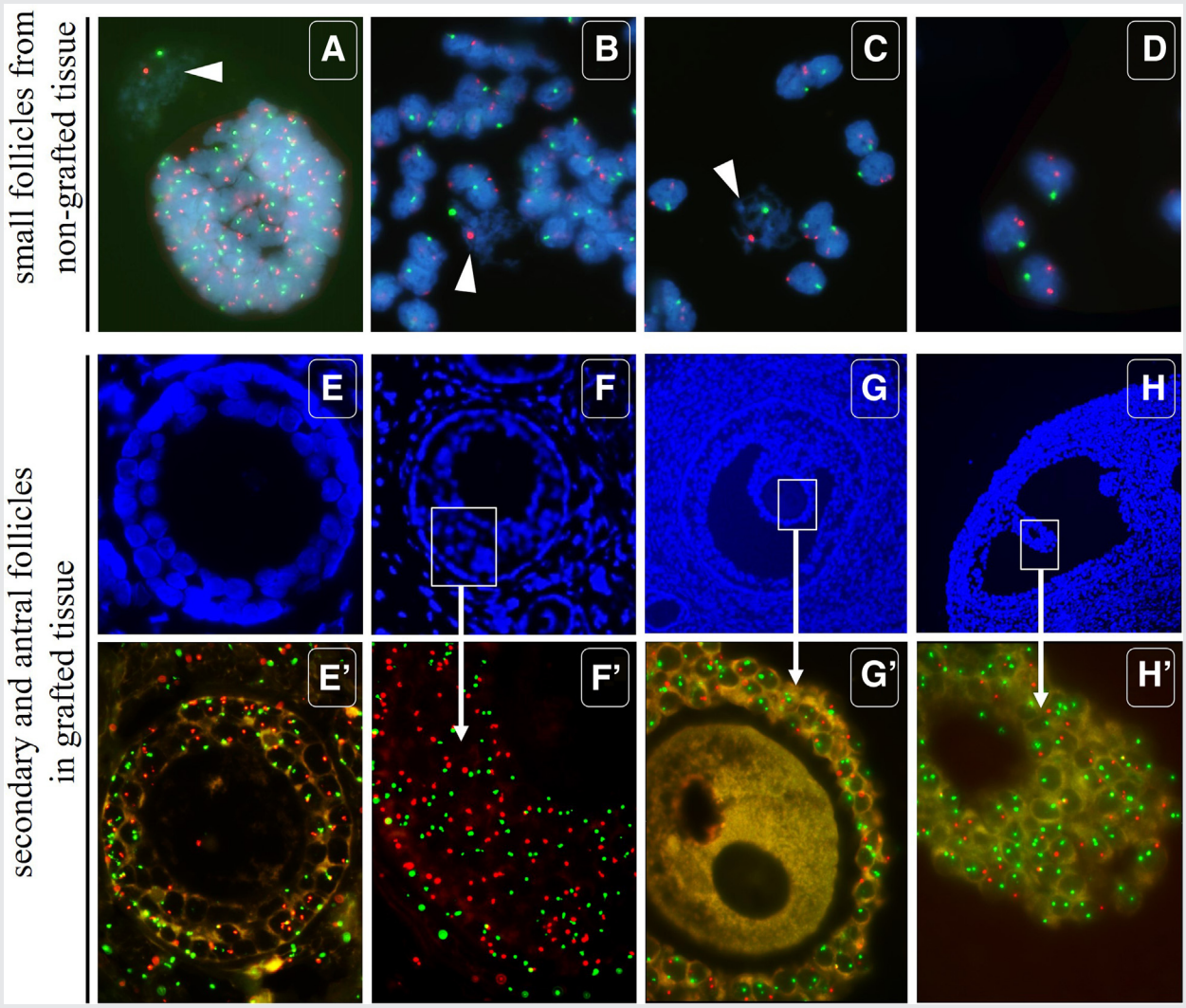
Immunohistochemical Analysis of PCNA and AMH

Proliferating cell nuclear antigen. A total of 4,368 (before grafting) and 1,292 (after grafting) primordial follicles were analyzed: 2,047 and 269 from patients with TS and 2,321 and 1,023 from controls (Fig. 4). Follicles were considered positive when they contained at least one GC with a labeled nucleus. Mean proportions (mean ± SD) of primordial follicles expressing PCNA were, respectively, 35.9% ± 22.7% and 32.2% ± 15.7% in prepubertal and peripubertal/postpubertal girls with TS. In the control group, primordial follicles also showed high levels of PCNA-positive GCs in both prepubertal (51.6% ± 14.8%) and peripubertal/postpubertal (52.9% ± 15.5%) girls.

Five months after grafting, proportions of PCNA-positive primordial follicles in both prepubertal and peripubertal/postpubertal patients with TS had increased considerably and were significantly higher than in controls (99.5% ± 1.1% vs. 56.9% ± 30.4%, $P=.0095$; and 99.3% ± 1.6% vs. 83.7% ± 13.1%, $P=.0331$).

Anti-Müllerian hormone. Follicles expressing AMH were identified by the presence of at least one labeled GC. Immunostaining for AMH was detected in GCs of follicles in ovarian tissue from both patients with TS and controls from the primordial to the antral stage (Fig. 4). The weakest positivity was encountered at the primordial stage, and the strongest at the secondary stage. In prepubertal patients with TS, 7.2% of follicles expressed AMH, which was not statistically different from controls (4.5%) before grafting. During or after puberty, AMH levels in control follicles rose significantly to 22.8%, suggesting that AMH secretion was upregulated in GCs to maintain follicle pool dormancy.

FIGURE 3



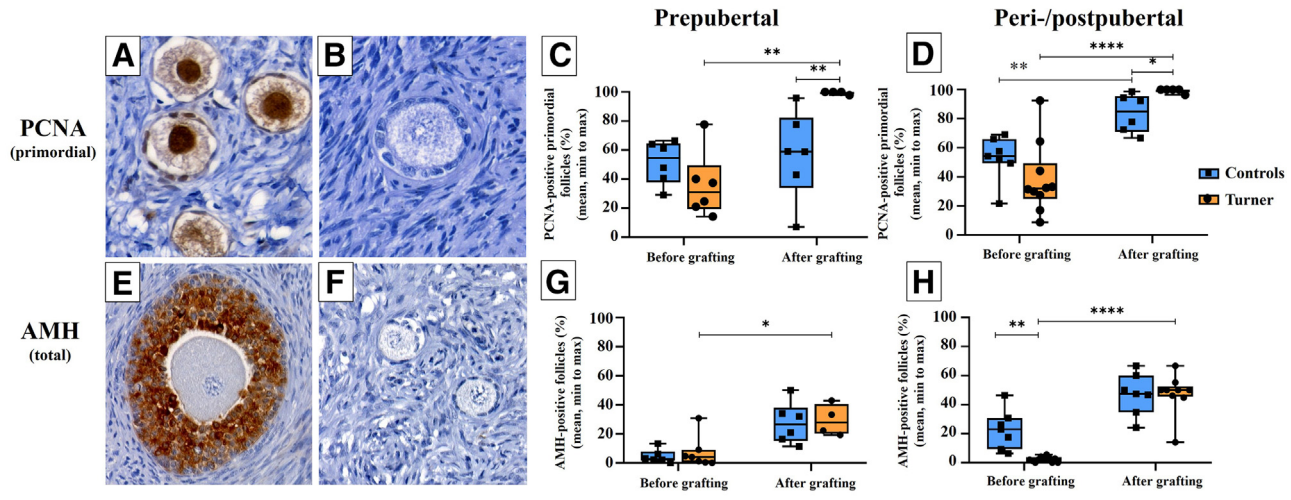
Determining levels of mosaicism in small follicles from nongrafted tissue and in the GC layers of secondary and antral follicles after 5 months of xenografting. Nongrafted ovarian cortex from patients with TS (patients A–J) was subjected to enzymatic digestion to release follicles from stromal cells. In some follicles, the oocyte had already been expelled from the GC mass and could be easily distinguished by diffuse DAPI counterstaining of nuclear material and intense FISH labeling of 4 tightly clustered chromosomes in these tetraploid cells (A). Isolated follicles were subjected to additional digestion with trypsin to further dissolve the GC mass and allow FISH analysis of individual GCs and the oocyte in the same follicle (B and C). FISH of three 45,X stromal cells is also shown (D). Levels of mosaicism in the GC layers of secondary and antral follicles in grafts were determined by FISH analysis of follicles in 4-μm histological sections. GCs from secondary follicles of patient G show a chromosome X (red)/chromosome 18 (green) signal ratio close to 1, indicating a normal 46,XX karyotype for most GCs (E and E'). By contrast, in the GC layers of a secondary follicle from patient J, the number of signals for chromosome X (red) clearly exceeds chromosome 18 (green), indicating that some GCs had a 47,XXX karyotype (F and F'). Similar observations were made of GC layers in antral follicles, whose FISH signal ratio was close to 1 in an antral follicle from patient G (indicative of mainly 46,XX cells; G and G'), but close to 0.5 in an antral follicle from patient C (indicative of mainly 45,X cells; H and H'). Arrowheads point to FISH signals from oocytes. Note that the X chromosome is shown in red in A–D and in green in E'–H'. Not all FISH signals could be captured in a single plane of focus. The original magnification of FISH signals was ×630. DAPI. FISH = fluorescence in situ hybridization. GC = granulosa cells. TS = Turner syndrome.

Peek. Folliculogenesis in Turner syndrome. Fertil Steril 2023.

However, this phenomenon was not seen in peripubertal/postpubertal patients with TS, with only 2.1% of follicles containing AMH-positive GCs. The proportion of AMH-positive cells was significantly lower than in controls (P=.0069), suggesting that the secretion of AMH by GCs was impaired.

After 5 months of grafting, a significant increase was observed in the percentages of AMH-positive follicles in patients with TS (up to 29.5%, P=.0152, in prepubertal girls; and 47.1%, P<.0001, in peripubertal/postpubertal subgroups), reaching levels similar to controls (27.5% in prepubertal and 47.1% in peripubertal/postpubertal patients).

FIGURE 4



Expression of PCNA (A–D) and AMH (E–H). Follicles were considered positive for PCNA (A) when they contained at least one GC with a brown-stained nucleus. A secondary follicle expressing AMH was identified by the presence of at least one labeled GC (E). Negative controls (B and F) failed to show any DAB-positive immunostaining. PCNA-positive primordial follicle proportions before and after grafting in prepubertal (C) or postpubertal (D) patients. AMH-positive follicle proportions (G+H) before and after grafting in prepubertal (G) and postpubertal (H) patients. Values show mean minimum-to-maximum percentages of positive-stained follicles (blue represents controls, and orange patients with TS) analyzed using Student's *t*-test and the Mann-Whitney *U* test. Statistically significant differences between patients with TS and controls before and after grafting are indicated by *$P<.05$, **$P<.01$, and ****$P<.0001$. AMH = anti-Müllerian hormone. PCNA = proliferating cell nuclear antigen. TS = Turner syndrome.

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DISCUSSION

Reviews summarizing the efficacy of OTT of cryopreserved ovarian cortex have indicated that OTC is very successful in oncological patients, and the chances of achieving a live birth after a transplant are as high as 40% (10–12). Although OTC is well documented in women facing gonadotoxic therapy, little is known about its effectiveness in other conditions that cause premature depletion of the resting pool of primordial follicles, such as TS (10, 11). Levels of X chromosome aneuploidy in ovarian cells and the possible impacts of ovarian aneuploidy on folliculogenesis in patients with TS are largely uncharted (13–15, 28).

To investigate these effects, ovarian cortical fragments containing follicles from 18 young patients with mosaic TS with numerical X chromosome aberrations (45,X/46,XX or 45,X/46,XX/47,XXX) were analyzed before and after xenografting to SCID mice. We limited our study to patients with mosaic TS with numerical X chromosome aberrations, as these patients are much more likely to benefit from OTC. The probability of finding ovarian follicles in this subgroup is thought to be markedly higher than in patients with TS with 45,X only, or structural abnormalities of the X chromosome (14, 24, 29).

Mean follicle density in TS tissues before grafting was considerably lower than that in age-matched controls. This is in line with previous reports showing a reduced ovarian reserve in girls with TS (14, 24, 28). Aberrant morphology in up to 30% of follicles, also reported by Mamsen et al. (24), strongly suggests that only part of the already reduced pool of follicles in affected women is potentially capable of recovering fertility after OTT. In ovarian xenografts retrieved after 5 months, the number of follicles was found to have decreased substantially in both the TS group and controls, especially in peripubertal/postpubertal patients. Follicle loss after OTT is widely documented and known to be caused by an ischemic phase before revascularization of the graft is completed (30). In the grafts of 4 patients with TS, no follicles were observed. Possibly, the ovarian tissue fragment may have had very few follicles before transplantation (31). Alternatively, the transplantation procedure could have been suboptimal in these cases, leading to an even more extensive decrease in the number of follicles than normally observed after transplantation.

Interestingly, the high percentage of morphologically abnormal follicles in TS tissues returned to normal after grafting. It is possible that these aberrant follicles are preferentially lost from the tissue during grafting, contributing to massive follicle loss in patients with TS after grafting. After OTT, a significant proportion of the remaining follicle pool consisted of growing follicles in tissues from both patients with TS and controls. Large numbers of secondary and even visible antral follicles were observed. In grafts from peripubertal/postpubertal girls with TS, more than 50% of follicles were classified as growing. This strongly indicates that at least part of the

population of small follicles in TS tissue is capable of reinitiating folliculogenesis and advancing to secondary and antral stages.

In the current study, 101 of 104 (97%) small follicles from 10 patients with TS contained an oocyte with normal X chromosomal content. There are robust selection mechanisms that eliminate oocytes lacking a second X chromosome, thereby disrupting synapsis during the diplonema of meiosis prophase I (32). Impaired transition of primordial germinal cells to oocytes during meiotic prophase I, observed in 45,X fetuses, suggests that these mechanisms are responsible for the absence of primordial follicles in the ovaries of these fetuses (4). In patients with 45,X/46,XX mosaic TS, elimination of the aneuploid portion of the germ cell population during meiosis I might explain the relatively small number of primordial follicles with a normal karyotype in these individuals (14).

Ovarian cortical stromal cells from most patients with TS displayed a high degree of aneuploidy, up to 78% in 45,X/46,XX patients and as high as 86% in 45,X/46,XX/47,XXX patients. Elevated levels of X chromosome aneuploidy in ovarian stromal cells and GCs in the majority of follicles from patients with TS could in turn impair the process of folliculogenesis. Primordial follicle activation requires bidirectional signaling between the oocyte and surrounding somatic cells (17). Aneuploid stromal cells and GCs that are either missing an X chromosome (45,X) or have gained an X chromosome (47,XXX) are likely to show abnormal expression of genes in pseudoautosomal regions that escape silencing during X chromosome inactivation in 46,XX females (33).

We have demonstrated, for the first time, that the level of aneuploidy in the GC layers of secondary and antral follicles in the grafted tissue from patients with TS decreases considerably compared with GCs in small follicles in the same tissue before grafting. This decline may be explained by 2 different mechanisms: preferential recruitment of small follicles with a relatively high proportion of 46,XX GCs; or preferential expansion of 46,XX cells in the mosaic layer of GCs during folliculogenesis. In the course of folliculogenesis, the monolayer of GCs in small follicles undergoes at least 10 mitotic divisions to produce several thousand GCs in antral follicles (34). The increase in the percentage of 46,XX GCs during the process of folliculogenesis in grafts, advancing from primordial to secondary and finally antral stages, is therefore most probably the result of the preferential expansion of 46,XX GCs. This is substantiated by the observation that 45,X cells are more prone to apoptosis and have a longer cell cycle than 46,XX cells (35–37). Taken together, our results indicate that despite the presence of aneuploid stromal cells and GCs, small ovarian follicles from patients with mosaic TS are capable of developing to morphologically normal secondary and antral stages, particularly if their tissue is cryopreserved before puberty.

The percentage of PCNA-positive follicles in TS tissue increased during grafting to almost 100%, significantly exceeding the percentage observed in controls. Remarkably, around 50% of small follicles in the tissue before grafting were also PCNA-positive, suggesting that some follicles had been activated before OTC or during fragmentation of cortical tissue, known to induce follicle activation (38). Expression of AMH was upregulated during grafting, which is in line with its function in maintaining follicle pool dormancy. Although AMH values in the nongrafted tissue from peripubertal/postpubertal patients with TS were lower than in controls, similar levels were reached after grafting. It is possible that follicles from patients with TS with abnormal AMH secretion are lost during long-term grafting, leaving those with normal AMH secretion to survive and grow.

In summary, our results are the first to show that small ovarian follicles from patients with mosaic TS are capable of reinitiating folliculogenesis after xenografting to SCID mice and are able to progress to secondary and antral stages. These findings suggest that folliculogenesis is feasible in patients with TS, despite high proportions of aneuploid ovarian stromal cells and GCs. In addition, freezing ovarian cortical tissue before follicle depletion occurs around the age of puberty enhances the chances of restoring future fertility. Our data therefore support the notion that cryopreservation of ovarian cortical tissue in girls with mosaic TS is a realistic option to preserve their fertility, as long as sufficient numbers of follicles are present.

DATA AVAILABILITY

Pseudonymized patient data, study protocol, analysis plan, or other specific data sets can be made available after receiving a proposal. Proposals should be directed to the corresponding author M.-M.D.. A signed data access agreement is required to gain access to the data.

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