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Gonocyte transformation to spermatogonial stem cells occurs earlier in patients with undervirilisation syndromes

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ABSTRACT

Aim: Fertility post-orchidopexy is dependent on transformation of neonatal gonocytes (G) into adult dark spermatogonia at about 3 months, the same time as gonadotrophins stimulate androgen secretion. We examined how androgen blockade affects transformation of gonocytes to spermatogonial stem cells (SSC) during this period in patients with undervirilisation syndromes.
Methods: Patients with undervirilisation syndromes (n = 30, 1.5 weeks–16 years) underwent review of medical records, pathology reports, and H&E slides of testes (ethics HREC32164). Fluorescent immunohistochemistry against anti-Mullerian hormone (AMH, Sertoli cells), mouse VASA homologue (MVH, germ cells) and DAPI (nuclei) allowed the number of MVH-positive gonocytes/spermatogonial stem cells per seminiferous tubular cross-section (G/T or SSC/T) to be counted.
Results: Gonocytes (MVH-positive cells in the tubular lumen) were present in 15/16 patients under 2 years old. SSC (MVH-positive cells on the tubule basement membrane) were present in 25/30 patients. With increasing age, the mean number of SSC/T decreased from ~4 to 0, and G/T decreased from ~1.5 to 0. SSC were present in CAIS and PAIS patients at 1.5 and 3.5 weeks old, respectively.
Conclusions: Gonocytes transform into SSC earlier than expected in patients with undervirilisation syndromes. Lack of androgens may stimulate non-androgenic regulators to trigger transformation. Understanding how gonocytes transform may enable optimization of spermatogonial development to preserve fertility post-orchidopexy.

Infertility is a major consequence of dysfunctional germ cell development in boys. Current evidence suggests that the transformation of gonocytes to adult dark-spermatogonia (AD-S) is crucial for adult fertility [1]. This can be disrupted in cryptorchidism, which if left untreated leads to adult paternity rates of about 66% in unilateral cryptorchidism and less than 33% after bilateral cryptorchidism [2,3]. Although some studies have found that the younger the patients were at orchidopexy, the higher their sperm count, further studies have found that boys lacking AD-S at the time of orchidopexy will nonetheless develop infertility, in particular azoospermia [1,4].

The way in which gonocytes transform into AD-S is still very much a 'missing link' in understanding germ cell development. Primordial germ cells migrate from extra-embryonic ectoderm to the forming gonads on the genital ridge at 4–5 weeks' gestation to mature into gonocytes [5]. A proportion of these gonocytes then transform into spermatogonial stem cells (SSC) 3–12 months after birth and unsuccessful gonocytes undergo apoptosis. These SSC consist of Type A adult dark spermatogonia which become adult pale spermatogonia and then Type B spermatogonia [6].

At the same time, a sudden surge in androgen production stimulated by pituitary gonadotrophins, known as 'minipuberty' occurs [7] and some studies suggest that this is necessary for the transformation of the gonocytes into AD-S [8]. On the other hand, other studies of patients with androgen insensitivity syndromes as well as androgen receptor-knockout mouse models have found that the transformation from gonocytes to various SSC is androgen independent [9–12].

We aimed to determine whether androgen was necessary for the transformation of gonocytes to SSC by studying germ cell development during 'minipuberty' in patients with undervirilisation syndromes.

1. Materials and methods

Testicular biopsies from thirty phenotypically female patients with disorders of sex development (complete androgen insensitivity syndrome (CAIS) n = 17, partial androgen insensitivity syndrome (PAIS) n = 9, 17-beta-hydroxysteroid dehydrogenase deficiency (17BHSDD) n = 4), were selected from the Anatomical Pathology

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Database at the Royal Children's Hospital Melbourne. The human ethics committee approved the proposed use of testicular biopsies and gonadectomies which had previously been obtained for research purposes with the patients' consent. No additional contact with patients was required (ethics number HREC32164). Selection was based on a documented diagnosis of the above conditions in either the patients' pathology reports and/or medical records. Patients diagnosed with 'testicular feminization syndrome' were included as having androgen insensitivity. Patients were classified as 'PAIS' if there were external virilising features such as clitoromegaly or fused labia. All available biopsies from 1961 onwards were selected. Additionally, information was collected on date of birth, age at biopsy, location of gonads at time of biopsy, comorbidities, and the presence of germ cells as outlined on the pathology report. Patients ranged in age from 1.5 weeks to 16 years old.

The biopsies had been fixed in formalin and embedded in paraffin by using conventional methods. The most recent protocol involves the use of a Peloris Rapid Tissue Processor (Leica Microsystems, Wetzlar, Germany) to pass the tissue through a graded series of formalin, ethanol, xylene and wax. The paraffin blocks were retrieved from the Anatomical Pathology archives at the Royal Children's Hospital Melbourne and new sections of the testicular tissue were cut.

Ten micron-thick sections of areas containing testicular tissue were stained with haematoxylin and eosin using a standard protocol and examined under light microscopy to select slides for fluorescent immunohistochemistry (IHC). For IHC, sections were cleared in xylene and rehydrated through graded alcohols. Antigen retrieval was performed by heating slides in 0.2 M borate buffer (pH7.0 in PBS) for 20 minutes in a microwave oven (240 W). Slides were blocked using 10% horse serum (vol/vol) and 5% bovine serum albumin (wt/vol) in 0.1% Triton X/PBS. Primary antibodies against anti-Mullerian hormone (AMH) (also known as Mullerian inhibiting substance) and mouse VASA homologue (MVH) were diluted in the blocking serum which itself had been diluted by one-fifth (Table 1). AMH antibody identified the Sertoli cells, and MVH antibody identified germ cells [13]. Secondary antibodies conjugated to fluorophores, and 4′, 6-diamidino-2-phenylindole, dilactate (DAPI, labels nuclei) (Table 1) were then applied (diluted in one-fifth of the blocking serum) to facilitate fluorescent imaging of sections using the Leica LSM-2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Four × 40 images (two images per testis) were taken from each patient sample and germ cells were counted. MVH-positive cells on the basement membrane of seminiferous tubules were considered spermatogonial stem cells (SSC) and MVH-positive cells in the tubule lumen were considered gonocytes (G) if patients were less than 2 years of age (spermatocytes differentiate from SCC and move into the tubule lumen at 3–4 years and also stain MVH-positive [12,13]). Fifteen patients were under 2 years old. The number of seminiferous tubule cross-sections per image was also counted such that the number of MVH-positive cells per seminiferous tubular cross-section (G/T or SSC/T) could be calculated (30–100 tubules/patient).

The number of G/T and SSC/T were then plotted against biopsy age on a scatter graph using SPSS (version 17.0.0, Polar Engineering and Consulting). G/T and SSC/T both appeared to decline exponentially with age. Thus, a common logarithmic transformation was performed on the independent variable (i.e. biopsy age). This was used to calculate the Pearson correlation coefficients for SSC/T versus the common log of age, and G/T versus the common log of age. The p-values for these two linear regressions were subsequently also found using SPSS.

2. Results

Gonocytes (MVH-positive cells in the tubular lumen) were present in 15/16 patients <2 years old; SSC (MVH-positive cells on the tubule basement membrane) were present in 25/30 patients.

Overall, the mean number of SSC/T decreased from ~4 to 0, and G/T decreased from ~1.5 to 0 with increasing age. This decline appeared to be exponential in nature with SSC/T decreasing 1.4 for every 10-fold increase in age ($r = -0.74$, $P < 0.0005$, $n = 30$), and G/T decreasing 0.24 for every 10-fold increase in age ($r = -0.52$, $P = 0.046$, $n = 15$). (Fig. 1).

On H&E staining, germ cells were large and round with more cytoplasm compared to Sertoli cells within seminiferous tubules. SSC were present and distinct from gonocytes or spermatocytes by their position on the basement membrane. SSC were obviously present even at 1.5 weeks of age, the earliest biopsy available. With increasing age, both gonocytes and SSC appeared to decrease in all patient groups. Additionally, patients with 17BHSDD had testes with increased interstitial fibrosis and thickened basement membranes as age increased.

The amount of cytoplasmic MVH staining in germ cells varied even within each seminiferous tubule. Those germ cells with less MVH staining tended to have a larger volume of cytoplasm and their nuclei were generally less defined and sometimes pale i.e. they appeared necrotic. These germ cells tended to occur more frequently with increased age.

With CAIS patients, SSC were present as early as 1.5 weeks of age but all germ cells had disappeared by 11.1 years. In contrast, whilst SSC were also present in PAIS patients at 3.5 weeks-old, germ cells were still obviously present at 14.2 years despite having decreased in number with age. Also, at 3.5 weeks of age, less gonocytes had transformed into SSC in PAIS (1.25 G/T and 1.65 SSC/T) compared to CAIS patients (0.24 G/T and 2.54 SSC/T). Patients with 17BHSDD also still had some SSC and gonocytes remaining at 16.4 years of age (Fig. 2).

3. Discussion

These results have shown that in children with undervirilisation syndromes, not only do gonocytes transform into SSC but this occurs before minipuberty (3–6 months). In fact, more gonocytes may have

Table 1
Antibody details.

Primary antibody	Company and catalogue number	Host	Neat concentration	Working concentration (vol/vol)
Anti-Mullerian Hormone (AMH)	sc-6886, Santa Cruz Biotechnology, Dallas, TX	Goat	200 μg/ml	1 in 400 Dilute in 1/5 blocking serum
Anti-Mouse Vasa Homologue (MVH)	ab13840, Abcam, Cambridge, MA	Rabbit	0.5–1 mg/ml depending on batch	1 in 10000 Diluted in 1/5 blocking serum
Secondary antibody and fluorophore	Catalogue number*	Host	Absorption wavelength (nm) and emission color	Neat and working concentration (vol/vol)
Anti-goat Alexa 488	A11055	Donkey	494 Green	2 mg/mL 1 in 1000
Anti-rabbit Alexa 594	A21207	Donkey	590 Red	2 mg/mL 1 in 1000
DAPI dye	D3571	N/A	345 Blue	4.000 1 in 1000

* All from Molecular Probes, Invitrogen Australia Pty Ltd, Mulgrave, VIC, Australia.