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]. 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
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’ 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) for 20 minutes in a microwave oven. Slides were blocked and examined under light microscopy to select slides for fluorescent imaging of sections using the Leica LSM-2 confocal microscope (Leica Microsystems, Wetzlar, Germany). 4 × 40 images (two images per testis) were taken from each

Table 1

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

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

Table 1 Antibody details.

The number of G/T and SSC/T were then plotted against biopsy age on a scatter graph using SPSS (version 17.0, 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 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...
transformed into SSC at 3.5 weeks in CAIS compared to PAIS patients, although there were insufficient specimens to confirm this statistically. Germ cell numbers in all groups of patients studied also gradually decreased with age. Having some androgen responsiveness (PAIS and 17BHSDD), though, correlates with maintenance of more germ cells compared to CAIS.

These results concur with previous studies which suggest that transformation of gonocytes to SSC is androgen independent. In one study, 95% of testes from CAIS patients contained germ cells. Germ cells were most abundant in the first year of life. Most of these were spermatogonia with an occasional meiotic spermatocyte but with no evidence of further spermatogenesis [11]. In addition, our results suggest that this transformation occurs prior to ‘minipuberty’ and identified germ cells clearly through MVH-staining.

In contrast, Hadziselimovic et al. found that patients with CAIS had defective transformation of AD-S. In 10 out of 12 cases in a biopsy series, abnormal testicular development was obvious as early as 1 month of age, and transformation of gonocytes into AD-S was reported as abnormal even though total germ cell count was within normal limits [14]. The difference between these studies is uncertain but may be due to how the stage of germ cell development was assessed.

We hypothesise that gonocytes transform into SSC earlier than expected in patients with undervirilisation syndromes because a lack of androgenic negative feedback on pituitary gonadotrophins may stimulate non-androgenic regulators to trigger transformation (Fig. 3). This would also explain why at 3.5 weeks, more SSC had transformed in the CAIS testes as compared to the PAIS testes. PAIS testes would presumably have had some androgenic response and this could have fed back on pituitary gonadotrophins to inhibit pro-transformational signalling at this early stage.

Various non-androgenic regulators of gonocyte transformation could be possible. A study by Meehan et al. suggests that a combination of follistatin and FSH may be necessary [15]; follistatin as an antagonist to activin-mediated gonocyte proliferation, and FSH as a stimulus for stem cell factor (a c-Kit ligand) to promote germ cell maturation and migration [15,16]. FSH alone, though, does not appear to affect the maturation of gonocytes to SSC, as no changes in apoptotic and proliferation rates have been observed after acute FSH suppression [17].

**Fig. 1.** A) Gonocytes per tubule (G/T) versus age. B) Spermatogonial stem cells per tubule (SSC/T) versus age. C) G/T versus common log of age. G/T decreases 0.24 for every 10-fold increase in age ($r = -0.52$, p-value = 0.046, n = 15). D) SSC/T versus common log of age. SSC/T decreases 1.4 for every 10-fold increase in age ($r = -0.74$, p-value < 0.0005, n = 30) (CAIS: ○; PAIS: □; 17BHSDD: ×).
Another non-androgenic regulator could be anti-Mullerian hormone (AMH). AMH secretion from Sertoli cells is at its highest level during the first year of life peaking at 4–12 months [18], coinciding with when SSC appear. One study on AMH in cryptorchid patients found that the normal surge in the first year was absent. Mean AMH levels were also lower in cryptorchid compared to control patients and lower in bilateral compared to unilateral cryptorchid patients [18]. This may be due to secondary postnatal testicular degeneration leading to lowered AMH levels and also corresponds with the timing of decreased AD-S production in cryptorchid boys, implying a need for earlier operative intervention [4,18]. TGF-beta 1 and 2, members of the same TGF-beta superfamily as AMH, have also been found to induce gonocyte apoptosis [19], further suggestive of its involvement in physiological germ cell development.

Our results also show overall germ cell numbers decreasing with age, and the cause is probably two-fold. Firstly, it is well established that androgen is necessary for ongoing spermatogenesis. This is evident in our study as the PAIS and 17BHSD testes, which respond partially to androgen, contained some germ cells well into the patients’ teenage years whilst CAIS testes contained no germ cells at the same age. Secondly, most testes in our patient groups were undescended at either abdominal or inguinal position. The undescended testis is retained deep to the inguinal fat pad, which is an effective insulator, keeping the testis at 34–37 °C rather than the normal postnatal temperature of 33 °C. The net effect of undescended testes is germ cell loss, leading to infertility [20,21].

Limitations of this study include low group numbers, although this was sufficient for some critical changes to be elucidated. In particular, more samples during the perinatal period when most of the changes in germ cells seemed to occur would be preferable. However, now that orchidectomy is being delayed until after puberty in many CAIS patients, larger numbers in early infancy may be difficult to obtain. There was also no ethical method of obtaining testicular biopsies from boys with normal descended testes to compare our results to.

In conclusion, not only is early germ cell development androgen-independent, but gonocytes may transform into SSC earlier than expected in patients with undervirilisation syndromes. Perhaps a lack of androgenic action stimulates other non-androgenic regulators to trigger transformation. The subsequent age-associated germ cell reduction may reflect abnormal hormones in undervirilisation syndromes or increased temperature in their undescended testes. Understanding the non-androgenic regulators of gonocyte transformation may enable treatment to optimise spermatogonial development after orchidopexy and thus preserve future fertility.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jpedsurg.2013.11.047.

References


