Germ cell loss is associated with fading *Lin28a* expression in a mouse model for Klinefelter's syndrome

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Abstract

Klinefelter's syndrome is a male sex-chromosomal disorder (47,XXY), causing hypogonadism, cognitive and metabolic deficits. The majority of patients are infertile due to complete germ cell loss after puberty. As the depletion occurs during development, the possibilities to study the underlying causes in humans are limited. In this study, we used the 41,XX^{Y*} mouse model to characterise the germ line postnatally. We examined marker expression of testicular cells focusing on the spermatogonial stem cells (SSCs) and found that the number of germ cells was approximately reduced fivefold at day 1pp in the 41,XX^{Y*} mice, indicating the loss to start prenatally. Concurrently, immunohistochemical SSC markers LIN28A and PGP9.5 also showed decreased expression on day 1pp in the 41,XX^{Y*} mice (48.5 and 38.9% of all germ cells were positive), which dropped to 7.8 and 7.3% on 3dpp, and were no longer detectable on days 5 and 10pp respectively. The differences in PCNA-positive proliferating cells in XY* and XX^{Y*} mice dramatically increased towards day 10pp. The mRNA expression of the germ cell markers *Lin28a* (*Lin28*), *Pou5f1* (*Oct4*), *Utf1*, *Ddx4* (*Vasa*), *Dazl*, and *Fapb1* (*Sycp3*) was reduced and the *Lin28a* regulating miRNAs were deregulated in the 41,XX^{Y*} mice. We suggest a model for the course of germ cell loss starting during the intrauterine period. Neonatally, SSC marker expression by the already lowered number of spermatogonia is reduced and continues fading during the first postnatal week, indicating the surviving cells of the SSC population to be disturbed in their stem cell characteristics. Subsequently, the entire germ line is then generally lost when entering meiosis.

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Introduction

Klinefelter's syndrome (karyotype 47,XXY; KS) is the most frequent sex-chromosomal disorder in men. Although the syndrome presents heterogenous manifestations, the vast majority of patients suffer from hypergonadotropic hypogonadism, reflected by high levels of luteinising hormone (LH) and follicle-stimulating hormone (FSH), together with low levels of testosterone (Lanfranco *et al.* 2004). Most patients are infertile due to germ cell loss, which is thought to start during childhood and progresses with the onset of puberty. Therefore, the most prominent features of KS are disturbed endocrinology and a dramatically altered testicular architecture (i.e. Sertoli cell-only syndrome (SCO) and Leydig cell hyperplasia).

Certain key issues remain unresolved, namely the reason for the germ cell loss and the exact time-point when germ cells begin to fade. Studies have reported that reduced germ cell numbers are already present before the pubertal period (Ferguson-Smith 1959,

Coerdts *et al.* 1985). One study reported reduced germ cell numbers in testicular biopsies from 47,XXY foetuses at gestational age of 18–22 weeks, although the density and number of testicular tubules and mesenchymal structures appeared to be normal (Coerdts *et al.* 1985). This finding is in contrast to other studies that report normal testicular histology and germ cell numbers during the foetal period of KS patients (Gustavson & Kjessler 1978, Flannery *et al.* 1984). Other studies analysing testis material from the neonatal and prepubertal period have found reduced numbers of germ cells (Ferguson-Smith 1959, Mikamo *et al.* 1968, Edlow *et al.* 1969, Muller *et al.* 1995, Wikstrom *et al.* 2004). With such disparate data, it is difficult to derive any meaningful conclusions, a situation further complicated by the fact that the KS boys were partially cryptorchid, a factor known to cause a marked decrease in germ cell number independent of karyotype (Hadziselimovic & Herzog 2001). Wikstrom *et al.* (2004) showed that the depletion of germ cells accelerated with increasing testosterone levels at the

onset of puberty, culminating in the complete loss of germ cells. A finding that led to the proposition that meiotic failure occurring during this phase (i.e. when germ cells start to enter meiotic differentiation) could be the driving force behind the germ cell loss (Lanfranco *et al.* 2004, Tuttelmann & Gromoll 2010).

Against this background of contradictory data, it is obvious that studies are needed which systematically analyse the entire time course of germ cell development and loss during postnatal life, a task which cannot be sufficiently addressed in patients as access to testis material from KS children is extremely limited. As a consequence for the analyses to be conducted, the use of an animal model is essential. For KS, two mouse models exist (Lue *et al.* 2005, 2010, Liu *et al.* 2010a, 2010b, Wistuba 2010, Swerdloff *et al.* 2011), of which the 41,XX^{Y*} mouse (Lewejohann *et al.* 2009, Wistuba 2010, Wistuba *et al.* 2010, Werler *et al.* 2011) has been shown to closely resemble the sequelae of the human syndrome, namely small testes in adulthood due to complete germ cell loss, hypergonadotropic hypogonadism and cognitive deficits (Wistuba 2010).

As for the reasons for the germ cell loss in males with a supernumerary X-chromosome, it has been suggested that it results from a combination of meiotic and mitotic failure due to the germ cells' inability to deal with the aberrant number of chromosomes during division. Such a mechanism, however, would leave the spermatogonial stem cells (SSCs) behind and a seminiferous epithelium consisting of components reminiscent of spermatogonial arrest, a situation not seen in KS. We hypothesise that germ cell loss is not solely due to failure of division but also linked to a disturbed SSC population which is predisposed to fade during development.

Using the 41,XX^{Y*} model, we tested our proposition by examining the expression of LIN28A and its associated microRNAs, as LIN28A has been shown not only to be essential for successful development of primordial germ cells (PGCs) but also marks undifferentiated spermatogonia in mice throughout their lifetime (West *et al.* 2009, Zheng *et al.* 2009). LIN28A is an RNA-binding protein that is highly expressed in pluripotent mouse embryonic stem cells (Moss & Tang 2003), is discussed as a marker for SSCs in primates (Aeckerle *et al.* 2012), and is specifically expressed in undifferentiated spermatogonia in the mouse. Epigenetically, LIN28A expression is regulated by the microRNAs 125a and 125b. Two conserved miRNA-responsive elements are located in the UTR of *Lin28a*, which can mediate the repression by miR-125a and miR-125b so that an increase in miR-125b results in the downregulation of *Lin28a* mRNA transcripts. This downregulation involves the reduction of both translational efficiency and mRNA abundance (Wu & Belasco 2005). LIN28A in turn regulates other microRNAs from the let-7 family. The biosynthesis of the microRNAs from the let-7 family is inhibited by LIN28A due to blocking of the procession of

pri-miRNAs into mature miRNAs, and this feedback loop between LIN28A and the let-7 family is highly important during embryonic development. Induction of let-7 by declining levels of LIN28A inhibits the self-renewal of undifferentiated cells and promotes differentiation (Melton *et al.* 2010, Thornton & Gregory 2012).

To characterise the transcriptional regulatory mechanisms underlying germ cell loss, we assessed the mRNA expression of SSC markers (*Pou5f1* (*Oct4*) – expressed in PGCs, postproliferative prospermatogonia and A spermatogonia (Nichols *et al.* 1998, Pesce *et al.* 1998); *Utf1* – expressed in embryonic stem cells and in gonocytes and A spermatogonia of germ line tissue (Okuda *et al.* 1998, van Bragt *et al.* 2008); *Dazl* – a marker of differentiating germ cells with the main expression in pachytene spermatocytes (Nicholas *et al.* 2009); *Fabp1* (*Sycp3*) – synaptonemal complex protein-3 – a meiotic marker (Yuan *et al.* 2000) and *Ddx4* (*Vasa*) – a general germ cell marker that is expressed at stages from spermatogonium to round spermatids with the strongest expression at the early spermatocyte stage (Toyooka *et al.* 2000)). Furthermore, the expression of somatic cell marker anti-Muellerian Hormone (AMH) was assessed (Lee & Donahoe 1993). In addition, we characterised the protein expression of another marker for early spermatogonia, the protein PGP9.5 (Kon *et al.* 1999), and examined general proliferative as well as apoptotic events during postnatal spermatogenesis. By performing these various approaches, we aimed to obtain a detailed characterisation of the complete germ cell loss observed in males with a supernumerary X-chromosome.

Materials and methods

Animals

In total 26 41,XX^{Y*} and 24 40,XY* littermate control mice were obtained from our colony by breeding males with a mutated Y-chromosome (Y*) from the strain B6Ei.Lt-Y* with WT females. The karyotype of immature mice was determined by Xist-PCR, whilst adult mice were karyotyped using fluorescence *in situ* hybridisation (FISH) as described previously (Lewejohann *et al.* 2009, Wistuba *et al.* 2010, Werler *et al.* 2011). All animals were kept under standardised conditions: at 24 °C, 12 h light:12 h darkness cycle, and food and tap water were available *ad libitum*. All procedures and protocols were in accordance with the national and European (86/609/EEC) legislation for animal care and experiments (animal licences No. A87/05 and 8.87–50.10.46.09.016; RP Muenster and LANUV Northrhine-Westfalia).

Tissue and organ collection

The animals were killed by inhalation of CO₂ followed by decapitation and their testes were removed and weighed. One testis was fixed in Bouin's solution for 24 h, the other was snap-frozen in liquid nitrogen and stored at –80 °C until analysis.

RNA-isolation and quantitative real-time PCR

Twenty nanograms of luciferase was added to the testis tissue in lysis solution (QIAzol Lysis Reagent; QIAGEN) before the extraction of mRNA and miRNA using the miRNeasy Kit (QIAGEN). In total, 250 ng of mRNA were transcribed into cDNA using iScript (Bio-Rad). Primer sequences for the genes *Lin28a*, *Pou5f1*, *Utf1*, *Fapb1*, *Dazl*, *Ddx4*, and *Amh* are listed in Table 1. Quantitative real-time PCR analyses were conducted using SYBR Green Technology (Applied Biosystems). All samples were measured in duplicate and amplification was performed with the StepOnePlus PCR system (Applied Biosystems). As a standard amount of luciferase was added to each testis, the Ct value of luciferase was measured and the Ct value of the sample was normalised using the $\Delta\Delta C_t$ -method (Livak & Schmittgen 2001). To normalise the different cell compositions of the testes from different developmental stages and karyotypes, the $2^{(-\Delta\Delta C_t)}$ value was related with testis weight. The microRNAs were reverse transcribed with the TaqMAN microRNA reverse transcription kit (Applied Biosystems). Quantification of the microRNAs let-7-g and miR125-b was performed using TaqMANMicroRNA assays (Applied Biosystems) on the StepOnePlus PCR system (Applied Biosystems) and normalised with the U6 snRNA.

Histology and immunohistochemical staining

The fixed testes were transferred to and stored in 70% ethanol until they were embedded in paraffin, using routine methods. The tissue sections (5 μ m) were cut using a Leica SM2000R microtome (Leica Microsystems GmbH, Wetzlar, Germany). Primary antibodies for LIN28A (rabbit-anti-LIN28A (A177) Cell Signaling Technology, Inc., Danvers, MA, USA; dilution: 1:50) and PGP9.5 (rabbit-anti-PGP9.5 (Z5116) DakoCytomation, Copenhagen, Denmark A/S 1:500) were used for subsequent analyses with IgG (rabbit-IgG) and the omission of the primary antibodies serves as negative controls.

The sections were deparaffinised in paraclear, rehydrated in graded alcohol and rinsed in tap water. Antigen retrieval was conducted by incubation in 10 mM citrate buffer (pH 6.0) at 90 °C for 10 min before sections were washed in Tris-buffered saline (TBS; 0.05 mM Tris and 0.85% (w/v) NaCl, pH 7.6) then treated in 3% H₂O₂. The H₂O₂ reaction was stopped by rinsing the slides in distilled water and washing in TBS for 3 $\times$ 5 min. Blocking was performed to suppress non-specific antibody binding by incubating with 25% chicken serum in 0.5% BSA in TBS for 30 min at room temperature. After blocking, the first antibody (diluted in the blocking solution) was directly applied to the sections overnight at 4 °C, in a dark humid chamber.

Afterwards, sections were washed in TBS and incubated with the corresponding secondary antibody (anti-rabbit) for 1 h at room temperature in a dark humid chamber. After washing in TBS, the streptavidin-HRP reagent was applied and the sections were incubated at room temperature for 30 min, before being washed and stained with diaminobenzidine (DAB, Sigma Fast 3,3'-diaminobenzidine tablets; Sigma-Aldrich) for 2–20 min. After DAB staining, the sections were counterstained with haematoxylin for 10–15 s, rinsed in tap water, dehydrated in ethanol, paraclear and mounted (Merckoglas, Merck).

For PCNA immunostaining, deparaffinised sections (see above) were incubated overnight at 4 °C with the primary antibody (monoclonal rabbit-anti-PCNA EPR 3821, Epitomics, Burlingame, CA, USA, 1:500 and 1:750) diluted in PBS with 0.2% BSA and 0.1% sodium azide. Immunoreactivity was visualised using the EnVision kit (DAKO, Hamburg, Germany) followed by the nickel-glucose oxidase method (Davidoff *et al.* 1995). For negative controls, primary antibodies were omitted.

Determination of germ cell numbers and labelling index

In testicular cross-section of 40,XY* and 41,XX^{Y*} animals (1dpp, 3dpp, 5dpp, 10dpp) the number of germ cells was assessed. For each animal, the number of germ cells in 100 tubular cross-sections was counted.

Immunohistochemical stainings were semi-quantified by the determination of the labelling index (LI). In 100 tubular cross-section of each animal, the number of positively stained germ cells was put in relation to the total number of germ cells.

Determination of absolute Sertoli cell number via stereology

The animals were anaesthetised with CO₂ gas and killed by cervical dislocation. Their testis was obtained by dissection and fixed in Bouin's solution for up to 24 h. Then the testis tissues were washed three times in 70% ethanol to remove the fixative; then imbedded in Technovit (Heraeus Kulzer GmbH, Wehrheim, Germany), an artificial resin based on 2-hydroxyethylmethacrylate; and dehydrated in 3 $\times$ 100% ethanol each time for 1 h and two times in pure isobutanol for 1 and 3 h respectively. The tissues were converted into an infiltration solution, consisting of 1 g hardener 1 in 100 ml Technovit 7100 solution and incubated in the dark for at least 20 h at room temperature on a shaker. Then they were embedded in 1 ml Technovit 7100 hardener 2 mixed with 15 ml infiltration solution (for ten tissue samples). Polymerisation proceeded overnight at room temperature. The tissues in polymerised technovit were placed onto blocks and adhered with

Table 1 Primer sequences used for mRNA analysis.

Gene	Forward primer sequence	Reverse primer sequence
<i>Ddx4</i>	5'-AAAGCAGTGATAGTCAAGGTCCAA-3'	5'-GCAAAGATGGAGTCCTCATCCT-3'
<i>Dazl</i>	5'-CCTCCTTATCCAAGTTCACCAAGT-3'	5'-ACTGCGGTGGCATCTGGTA-3'
<i>Fapb1</i>	5'-AGAGGAAATACAGAAGCTTAACAATGAA-3'	5'-CCTTGTCCTCAAATTTCTGTATATCC-3'
<i>Utf1</i>	5'-GCACCGGTGGAGCAAGAG-3'	5'-TTCGTCGTGGAAGAAGTGAATCT-3'
<i>Lin28a</i>	5'-GGTGGTGTCTCTGTATTGGGA-3'	5'-AGTTGTAGCACCTGTCTCCCTTTG-3'
<i>Luciferase</i>	5'-GCACATATCGAGGTGAACATCAC-3'	5'-GCCAACCGAACGGACATTT-3'
<i>Pou5f1</i>	5'-CTACCCCTGGGCGTTCTCT-3'	5'-AGGCCTGGAAGCGACAGAT-3'

Technovit 3040. The blocks were cut into 20- μ m cross-sections using microtome (HM 360, Microm International GmbH, Walldorf, Germany).

Absolute Sertoli cell numbers were counted in the testis of adult 41,XX^{Y*} mice ($n=4$) and 40,XY* ($n=4$) littermate controls using the optical dissector technique (according to Wistuba *et al.* (2003)). The average age of all adult animals analysed was 30 (range 21–38) weeks. Two slides were scored for each testis and the Sertoli cell number was calculated per organ and volume unit.

Detection of apoptotic cells

Apoptosis was histochemically analysed in the testis sections of 40,XY* mice aged 1 ($n=3$), 3 ($n=3$), 5 ($n=4$) and 10dpp ($n=3$) as well as in 41,XX^Y mice aged 1 ($n=3$), 3 ($n=2$), 5 ($n=3$) and 10dpp ($n=2$). In addition, a 41,XX^{Y*} male aged 21dpp with focal spermatogenesis was analysed and testis tissue from adult 40 XY* mice served as controls. The DeadEnd colorimetric TUNEL system (Promega) was carried out according to the manufacturer's protocol. The number of apoptotic germ cells was determined by counting 100 tubular cross-sections of each animal.

Statistical analysis

Analysis of data was performed with two-way ANOVA followed by Bonferroni post-test, using the statistical software package GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). The values were considered as statistically significantly different if $P<0.05$.

Results

Dynamics of the germ cell population in the 41,XX^{Y*} mice

In order to address systematically the developmental time-course of the germ cell loss in the first 10 days *post partum*, we counted the total number of germ cells in the 41,XX^{Y*} mice in comparison with their littermate controls on day 1, 3, 5 and 10pp. In each animal, the number of the germ cells in 100 tubular cross-sections was counted. A significant difference between 41,XX^{Y*} and their littermate controls was detectable on day 1pp. At each timepoint (1dpp–5dpp), ~100 germ cells were found in 100 tubular cross-sections of the 41,XX^{Y*} mice, whereas in 40,XY* littermate controls the number of germ cells was always five times greater. On 10dpp, this difference increased markedly as the germ cells entered mitosis and started to differentiate (Fig. 1).

Apoptotic cells within the 41,XX^{Y*} seminiferous tubules

From the findings, we examined whether the cause of decrease in germ cell numbers in the 41,XX^{Y*} testis was cell death resulting from the apoptotic pathway. In 1dpp XY* mice, a few apoptotic germ cells/100 seminiferous

Figure 1 Total germ cell number counted in 100 tubular cross-sections of 41,XX^{Y*} and 40,XY* mice during testis development at 1dpp ($n=3$), 3dpp ($n=2$), 5dpp ($n=3$) and 10dpp ($n=3$). Data are shown as mean $\pm$ S.E.M.; *** $P<0.001$.

tubules (4.3 ± 2.36 ; mean $\pm$ S.D.; Fig. 2B) were detected, whereas in the XX^{Y*} mice no apoptotic germ cells were found (Fig. 2A). In both XY* and XX^{Y*} mice at 3dpp and 5dpp, few apoptotic germ cells per 100 seminiferous tubules (1 ± 0.5 – 1.5 ± 0.82 respectively) were present (Fig. 2C, D, E, and F). On day 10pp, in the XY* controls a mean of $47.67 (\pm 14.08)$ apoptotic cells was detected compared with $11 (\pm 2)$ in the 41,XX^{Y*} mice (Fig. 2G and H and Table 2). On day 10pp, a higher number of apoptotic germ cells was seen in 40,XY* mice due to the presence of differentiating germ cells, which were not detected in the 41,XX^{Y*} mice.

Then, we tested whether differences in postnatal cell proliferation could be responsible for reduced germ cell number in 41,XX^{Y*} mice. For this, PCNA immunostaining was performed (Fig. 3). In both 40,XY* and 41,XX^{Y*} mice at days 1 and 3pp, PCNA immunoreactivity was predominantly found in peritubular and Sertoli cells, whereas PCNA-positive germ cells were barely detectable. At day 5 and especially at day 10pp, however, a high number of proliferating germ cells was detectable in 40,XY* mice, whereas PCNA-immunostained germ cells nearly completely failed in seminiferous tubules from 41,XX^{Y*} mice.

Characterisation of the germ cell population in the 41,XX^{Y*} mice

In 40,XY* controls, protein expression of the SSC marker LIN28A was found in gonocytes and spermatogonia at all developmental time-points. In contrast, in 41,XX^{Y*} mice LIN28A was only expressed in a few gonocytes at day 1pp and in some spermatogonia up till day 3pp, and from day 5pp onwards no expression could be observed (Fig. 4). Semi-quantification of the results showed a LI (defined as the number of positive germ cells related to all germ cells in 100 tubules per animal) of $48.5\% \pm 10.7$ (mean $\pm$ S.E.M.) in the 41,XX^{Y*} mice on day 1pp that decreased to $6.7 \pm 4.7\%$ on day 3pp (Fig. 5A) and was

Figure 2 Apoptotic cells detected with TUNEL staining in the testis of 41,XX^{Y*} male mice (left column) and 40,XY* littermate controls (right column). Few apoptotic cells were found in the testis of 1dpp (B), 3dpp (C and D), 5dpp (E and F) and 10dpp (G and H) old mice. In 1dpp old XX^{Y*} mice (A) no apoptotic cell was detected. Scale bar: 100 μ m.

no longer detectable at day 5pp and 10pp. The LI in control testes was significantly higher and remained stable from day 1pp to day 5pp ($\sim 80\%$). On day 10pp, a decrease of $59 \pm 1.6\%$ was determined in LI (Fig. 5A), findings consistent with the increased presence of differentiating germ cells due to the meiotic entry. These differentiating cells are negative for LIN28A expression.

Immunohistochemical detection of another marker of undifferentiated germ cells, PGP9.5, showed a pattern similar to LIN28A, of which the LI on day 1pp was

significantly lower ($38.9\% \pm 4.5$) in 41,XX^{Y*} compared with controls ($83.3\% \pm 0.3$); it further decreased to $7.8\% \pm 1.7$ on day 3pp, $7.3\% \pm 3.4$ on day 5pp and on day 10pp its expression was no longer detected (Fig. 5B). In the control mice, the LI from day 1pp to day 10pp did not change, remaining at $\sim 80\%$ (Fig. 5B).

Characteristics of germ cells in spermatogenic foci of 41,XX^{Y*} mice

As we observed spermatogenic foci in the 41,XX^{Y*} mice, which were also detected in another unpublished study, we investigated whether the SSC markers LIN28A and PGP9.5 were expressed in the seminiferous tubules. In the two 41,XX^{Y*} animals aged 14 and 21dpp, we detected tubules displaying ongoing spermatogenesis, surrounded by tubules lacking any kind of germ cells, and we observed LIN28A and PGP9.5 protein expression in the spermatogenic foci, whereas the surrounding tubules did not show any expression. The tubules with focal spermatogenesis showed the expression of LIN28A and PGP9.5 in the spermatogonia (Fig. 6) and had normal SSC marker expression that was comparable with the littermate controls.

Marker mRNA expression of testicular cells

The mRNA expression of the germ cell markers, *Dazl* and *Ddx4*, as well as spermatogonia-specific markers *Pou5f1*, *Lin28a*, *Utl-1* and a marker for meiotic cells *Fapb1* were analysed over the developmental time-course (1, 3, 5, 10, 14dpp, adult). In controls, both *Ddx4* and *Dazl* were expressed in all germ cells and their expression increased with the onset of propagation and of germ cell differentiation from day 5pp onwards (Fig. 7). In contrast, in the testicular tissue of the 41,XX^{Y*} mice, the expression level of *Dazl* relative to adult XY* mice was very weak ranging from 0.017 arbitrary units (a.U.) (1dpp), 0.004 a.U. (3dpp), 0.004 a.U. (5dpp), 0.003 a.U. (10dpp), 0.007 a.U. (14dpp) to no expression of *Dazl* in adult 41,XX^{Y*} mice. The expression of *Ddx4* in the 41,XX^{Y*} mice was also weak compared with adult controls, ranging from 0.001 a.U. to 0.003 a.U. with the exception of a higher expression (0.033 a.U.) on day 14pp (Fig. 7).

Whilst the mRNA expression of SSC markers *Lin28a*, *Pou5f1* and *Utl-1* was detected in the 40,XY* littermates throughout the entire postnatal period, in the 41,XX^{Y*} mice the expression of *Lin28a* mRNA was detected from day 1 until day 10pp at levels 14 to 600 times lower than

Table 2 Number of TUNEL-positive cells in 100 seminiferous tubules counted (mean $\pm$ s.d.).

Karyotype/age	1dpp	3dpp	5dpp	10dpp
40,XY*	4.33 ± 2.36	1 ± 0.82	1 ± 0.71	47.67 ± 14.08
41,XX ^{Y*}	0 ± 0	1.5 ± 0.5	1 ± 0.82	11 ± 2

Figure 3 Proliferating cells labelled by PCNA immunostaining in the testis of 41,XXY* male mice (left column) and 40,XY* littermate controls (right column) at days 1pp (A and B), 3pp (C and D), 5pp (E and F) and 10pp (G and H). Besides proliferating peritubular (arrows) and Sertoli (arrowheads) cells, proliferating germ cells (asterisks) were clearly detectable only at days 5 (F) and 10 (H) of 40,XY* mice. Scale bar: 50 μm.

the corresponding controls and not at all in the adult 41,XXY* mice. Similarly *Pou5f1* mRNA was not present in adult XXY* mice and its expression during the early postnatal development was drastically decreased compared with littermate controls (Fig. 7).

Utf1 expression was detected in the germ cells of 41,XXY* mice, but to a lesser extent (0.21 a.U. compared with 0.75 a.U. on day 1pp, 0.084 a.U. compared with 1 a.U. in adult mice) than in littermate controls. In the 41,XXY* mice, the meiotic marker *Fapb1* was expressed very weakly, whilst it was highly expressed in controls from day 10pp onwards (Fig. 7).

Amh expression and Sertoli cell number

Despite being statistically not significant, during the postnatal development a higher expression of *Amh* in 41,XXY* mice was observed (Fig. 8A). This indicated the presence of more Sertoli cells in the 41,XXY* mouse testes. Therefore, we analysed the number of Sertoli cells

in the adult testis. In 41,XXY* mice, Sertoli cell number per ml (863.5 ± 182.6) was significantly increased compared with littermate controls (*t*-test; $P < 0.05$; Fig. 8B). In contrast, Sertoli cell number per testis showed a different pattern than per volume unit in 30-week-old mice, because in each testis it was significantly decreased in 41,XXY* mice compared with controls ($12\,340 \pm 6319$, $n = 4$, $P < 0.01$; Fig. 8C).

Expression of microRNAs regulating *Lin28a* expression

The microRNA 125-b has a regulatory function by inhibiting the expression of *Lin28a*. Expression of *Lin28a* was present from day 1pp onwards in littermate controls and increased during postnatal development up to day 14pp, whilst it was found to be reduced afterwards in adulthood (Fig. 9C), a change which can be explained by the rising numbers of differentiating germ cells diluting the signal. In line with this, related to the *Lin28a* expression, an elevated expression of miR-125 on day 1pp was observed, starting to decrease on day 3pp and was further lowered from day 5pp onwards (Fig. 9A). In addition, microRNAs from the let-7 family, e.g. let-7g, associated with the expression of *Lin28a*, were examined. In controls, a decrease in let-7g expression was found to accompany rising levels of *Lin28a* expression (Fig. 9E). In 41,XXY* mice, however, only very weak mRNA expression of *Lin28a* (0.007–0.07 a.U.) was observed from day 1 to 14dpp and complete absence of expression was observed in adult animals (Fig. 9D). Consequently, the regulatory microRNAs miR-125b and let-7g were found to be completely deregulated in the XXY* testis, although *Lin28a* expression is almost lacking from day 1 to 10pp, as these microRNAs are not upregulated as expected, but from day 14pp onwards, and in adulthood the expression of both microRNAs increased (Fig. 9B and F).

Discussion

Despite being one of the most prominent features of the KS, the molecular mechanisms as well as the timecourse and the processes associated with the germ cell loss remain poorly understood. In this regard, the availability of mouse models resembling the features of the syndrome has already contributed substantially to reveal the testicular phenotype of males with a supernumerary X-chromosome, e.g. in demonstrating that Leydig cells are not immature nor functionality impaired but are hyperactivated in what is obviously a compensatory manner (for review see Wistuba (2010)). The similarities extend to the progressive loss of germ cells of KS, making our 41,XXY* mice an invaluable means to analyse the characteristics of SSCs, the postnatal developmental timecourse of germ line fading and decipher parts of the regulatory mechanisms behind this phenomenon.

Figure 4 Immunohistochemical analysis of *Lin28a* expression in the testis of 41,XX^{Y*} male mice (bottom row) and 40,XY* mice (top row) for four ontogenic stages ((A and E) 1dpp; (B and F) 3dpp; (C and G) 5dpp; (D and H) 10dpp). The scale bar applies to the entire figure. Arrowheads indicate LIN28A negative germ cells. The figure visualises that LIN28A is expressed in spermatogonia of littermate controls throughout life. In 41,XX^{Y*} mice LIN28A expression can only be found in a few spermatogonia in newborn mice (1dpp, 3dpp) and is lost from the age of 5dpp, even though spermatogonia are still present at that age. Insert at day 1pp (40,XY*) shows a LIN28A-positive germ cell, insert at day 1pp (41,XX^{Y*}) shows LIN28A-negative germ cells. The insert at day 3pp (40,XY*) shows a negative IgG-control.

Based on our experimental findings, we propose a new model for the course of the germ cell loss, hypothesising that it comprises a three-step sequence of events, culminating in a germ cell depleted testis (Fig. 10).

In our previous study, we did not find a significant difference in the number of tubules containing germ cells in 41,XX^{Y*} and 40,XY* mice from day 1pp to 10pp (Wistuba *et al.* 2010) and no obvious difference was detected in germ cell appearance. However, in contrast to the previous analysis in the recent study, we have decided to reanalyse the germ cell number in more detail, by scoring the actual germ cell numbers in the tubules. Interestingly, we observed changes in the composition of testicular cell in 41,XX^{Y*} mice already from day 1pp onwards, i.e. the number of gonocytes was already significantly reduced at this early postnatal timepoint, in contrast to our previous assumption that the germ cell loss becomes manifested at the onset of puberty. Thus, the reanalysis of germ cell number pointed to a phenomenon of germ cell reduction (i.e. of the SSC population) much earlier in life than thought so far. In addition, this finding is supported by Hunt *et al.* (1998) who reported the loss of germ cells occurring in XXY mice during the early postnatal timeperiod and demonstrated that it was due to disturbed prenatal mitotic proliferation of these germ cells. Similar to female germ cells in the differentiating ovary, the male XXY PGCs undergo X-reactivation when they enter the genital ridge (Mroz *et al.* 1999a, 1999b). In a healthy

Figure 5 Labelling index (defined as the number of positive germ cells related to all germ cells) of the SSC marker (A) LIN28A and (B) PGP9.5 in 41,XX^{Y*} mice and 40,XY* littermate controls during testis development in the first-postnatal week of 1dpp ($n=3$), 3dpp ($n=2$), 5dpp ($n=3$) and 10dpp ($n=3$) old animals. Data are shown as mean $\pm$ S.E.M.; *** $P<0.001$.

Figure 6 Immunohistochemical analysis of LIN28A, PGP9.5 expression in the testis of pubertal 41,XXY* mice with focal spermatogenesis. (A) Testis of a 21dpp old 41,XXY* mouse stained for Lin28A. The entire testis is depleted of germ cells and no LIN28A expression can be observed, except for four tubules in the centre. (B) In these tubules, LIN28A-positive spermatogonia are present and proliferating. (C) PGP9.5 immunoexpression of a 41,XXY* mouse at day 14pp, showing positive spermatogonia in tubules sections containing focal spermatogenesis.

testis, the PGCs of the foetus differentiate into gonocytes and proliferate, then after a certain period of mitotic arrest, the proliferation is resumed several days after birth (Zhao & Garbers 2002). In our study, the latter point is reflected by the dramatic increase in PCNA immunopositive germ cells from day 5pp to day 10pp in the 40,XY* mice. Mroz *et al.* (1999a, 1999b) speculate that the demise of testicular XXY germ cells during the quiescence period is due to the inability of germ cells with three sex chromosomes to survive.

After we detected that the germ cell number was already remarkably reduced on day 1pp in the 41,XXY* mice, we examined the expression of SSC markers of the remaining germ cells. SSC markers are only expressed by those testicular gonocytes that can differentiate to some extent and enter the early steps of the spermatogenic pathway (Dym *et al.* 2009). The current approach was to first aim at the analysis of the expression patterns of specific SSC markers in mice with a supernumerary X chromosome. A loss of expression of both LIN28A and PGP9.5 proteins was observed in the

early developmental period before germ cells enter the mitotic propagation and the meiotic pathway, and this may be indicative of an early loss of the full stem cell potential of the SSC population, i.e. of a proportion of the SSCs that would not be able to fulfil the maintenance of the population by providing daughter cells entering the differentiation path and would remain as fully active SSCs in 41,XXY* mice, although we cannot directly prove for stem cell self-renewal. This proposition is corroborated by the mRNA expression profile of the SSC markers which were found to be almost absent in the testis of XXY* mice. This loss of the expression after birth points to an inability of the remaining germ cells to undergo proliferation, visible as the absence of PCNA-positive germ cells in the XXY* mice and thus the mitotic propagation of germ cells. However, it is also likely that the process of self-renewal would be affected by this fact as self-renewing divisions share mechanisms that occur in mitotic proliferation. Thus the lack of proliferatory germ cells very likely is closely associated with the fact that the spermatogonia are already lost at this timepoint

Figure 7 Developmental timecourse of germ cell marker expression in 41,XXY* and 40,XY* mouse testes. qPCR results are shown for the genes *Lin28a*, *Pou5f1*, *Ddx4*, *Dazl*, *Utl1* and *Fapb1*. $n=3$ per group (data are shown as mean $\pm$ s.e.m.). * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Figure 8 (A) Expression of *Amh* during the developmental time-course from day 1pp up to the adult stage ($n=3$) per group and timepoint in $41,XX^{Y*}$ mice and $40,XY^*$ mice. (B) Mean Sertoli cell numbers ($\pm$ S.E.M.) per volume unit in the testis of $41,XX^{Y*}$ mice and $40,XY^*$ littermate controls during adulthood (30 weeks). (C) Mean Sertoli cell numbers ($\pm$ S.E.M.) per testis of $41,XX^{Y*}$ mice and $40,XY^*$ littermate controls during adulthood (30 weeks; * $P<0.05$ and ** $P<0.01$).

as we no longer found any LIN28A- and PGP9.5-positive cells. The proliferation of the SSCs is a highly regulated mechanism that is, amongst other factors, controlled by the Sertoli cells which are involved in the niche formation. Thus the fading of the SSC markers in the prenatal XX^{Y*} testis suggests that Sertoli cells may possibly not be functioning normally under the prevailing conditions. As a first step towards the characterisation of the Sertoli cell population of the $41,XX^{Y*}$ mice, we therefore determined the absolute Sertoli cell number in

testes of adult $41,XX^{Y*}$ mice and found this cell type to be more frequent in XX^{Y*} testes when expressed per volume unit, but when normalised for much smaller testes, the numbers were significantly lower. The latter result is likely to be caused by the enormous shrinkage as a result of the complete germ cell loss. In contrast, the higher numbers per volume unit suggests that the Sertoli cell density per tubule is altered in $41,XX^{Y*}$ mice and might, by this, also influence the integrity of the niches and subsequently contribute to the germ cell loss and the fading expression of SSC markers. Alternatively, the Sertoli cell proliferation might be affected during the perinatal period by endocrine effects (i.e. FSH changes) but this has to remain speculative as no data are available yet.

This is of interest, as it is known that Sertoli cells with aberrant function can support full germ cell differentiation in males with a supernumerary X-chromosome as seen in those cases of focal spermatogenesis (Sciurano *et al.* 2009, Lue *et al.* 2010, for review see Wistuba (2010)). In our study, LIN28A and PGP9.5 were normally expressed in seminiferous tubules with focal spermatogenesis, whereas in the surrounding tubules that completely lacked germ cells, no LIN28A expression could be observed. Thus, the expression of the marker proteins associated with normal SSC function was found to be perfectly normal in tubules containing clonal and spermatogenic expansion and was apparently unaffected. In Klinefelter's patients undergoing testicular sperm extraction, the

Figure 9 Expression of the microRNA miR125b and miRlet-7g in relation to *Lin28a* expression in testicular tissue at different developmental time-points. Quantitative real-time PCR results for (A) microRNA125b, (C) *Lin28a*, (E) miRlet-7g in $40,XY^*$ littermate controls and (B) miR125b, (D) *Lin28a*, (F) miRlet-7g in $41,XX^{Y*}$ mice (data are shown as mean $\pm$ S.E.M.).

Figure 10 Schematic model of the germ cell loss in the 41,XXY* mouse. A reduced number of gonocytes is proposed already during the intrauterine period. Consequently, at birth, a reduced number of germ cells expressing SSC markers is present and the stem cell potential is lost during the mitotic spread several days after birth. When the germ cells afterwards enter meiosis, they are finally depleted in the 41,XXY* testis whereas in normal 40,XY* control mice differentiation into spermatogenic progress starts.

karyotype of meiotic spermatocytes in the spermatogenic foci was found to be euploid (46,XY), whereas the surrounding Sertoli cells still possessed a supernumerary X-chromosome (47,XXY) (Sciurano *et al.* 2009). The correct expression of the SSC markers also suggests that the germ cells found in such tubules discard the supernumerary X-chromosome. This was also shown in a study analysing spermatozoa of KS patients, demonstrating that meiosis occurred only in euploid spermatocytes and was never complete in aneuploid spermatocytes (Vialard *et al.* 2012). As XXY germ cells are unable to undergo meiosis (Mroz *et al.* 1999a, 1999b), it is likely that these germ cells are of a 'corrected 46,XY karyotype', a hypothesis that could be tested using either FISH on mouse testicular sections or PCR investigations of laser-microdissected germ cell-bearing tubules, although these approaches are challenging due to technical limitations.

To further gauge the regulatory mechanisms behind the fading expression of the SSC markers, we investigated the post-transcriptional control of *Lin28a*, as *Lin28a* expression is strictly regulated by the miRNAs 125a and 125b, which in turn regulates miRNAs of the let-7 family; an important feedback loop for the differentiation process (Wu & Belasco 2005, Thornton & Gregory 2012). Consistent with findings of Zheng *et al.* (2009) and Gaytan *et al.* (2013), we detected the highest *Lin28a* mRNA expression in the controls on day 14pp, whereas in the adult state, expression was slightly declined. In our control group, we observed a high expression of miRNA 125b accompanied with lower levels of *Lin28a* and *vice versa*. However, in the 41,XXY*

mice, this regulatory mechanism was disturbed from day 1pp to day 10pp, in that low expression of the miRNA and low expression of *Lin28a* were found. In addition, similar misregulation was found for miRNA let-7g, a downstream target of LIN28A, during the early development. Although the low level of *Lin28a* in the prenatal 41,XXY* mice failed to provoke an upregulation of miRNA let-7g, the very low expression of mature let-7g miRNA in XY* mice on days 1pp–3pp could possibly be involved in the inhibition of the differentiation processes. Although the particular cell type in which the miRNAs are expressed cannot be directly determined, our data strongly propose that miRNAs are expressed in germ cells as well as in somatic cells: because the levels of the miRNAs and *Lin28a*, being expressed in undifferentiated spermatogonia, are correlated in XY* littermate controls, as described by Gaytan *et al.* (2013), it is likely that the miRNAs are expressed in germ cells. Furthermore, PGCs were shown to exhibit a high expression of let-7g and the miRNA-125a, which also regulate *Lin28a* (Hayashi *et al.* 2008). Nevertheless, the expression of miR-125b and miR-let7g being highest in adult 41,XXY* mice points to the fact that these miRNAs are also expressed in somatic cells (although in a deregulated way in a disturbed testicular environment) as adult XY* mice lack any germ cells. The molecular reasons for the loss of stem cell characteristics and post-transcriptional deregulation, however, remain to be resolved. Genes escaping X-inactivation, that are directly linked to the supernumerary X-chromosome, were found to be expressed at higher levels in various tissues of the 41,XXY* mouse (Werler *et al.* 2011).

Thus, an elevated expression of these genes might also be likely to occur in the testis. Escaping from X-inactivation, *Kdm6a*, which codes a H3K27 demethylase, is essential for the induction of pluripotency and is associated with *Pou5f1*, *Klf4*, *Sox* and *Nanog*, known factors for pluripotency maintenance and the correct epigenetic reprogramming of germ cells (Mansour *et al.* 2012, Wang *et al.* 2012). The elevated expression of such an escapee gene in the testis and the regulatory consequences of the altered expression could be possibly linked to the observed SSC loss.

In the developmental period from days 1pp until 10pp, the number of germ cells remaining in the KS mouse testis is stable. Although these spermatogonial germ cells do not proliferate and thus lose their stem cell potential, they do not become apoptotic. On day 10pp, the starting point of the early wave of apoptosis, more apoptotic germ cells are detected compared with the earlier stages; however, even at this stage not all germ cells became apoptotic. As we did not observe any germ cells in the 41,XX^{Y*} mice on day 14pp (data not shown), it is likely that they die for other reasons, e.g. necrosis. Other than apoptosis, necrosis is marked by cytoplasmic swelling, release of cytoplasmic enzymes and breakdown of organelles (Galluzzi *et al.* 2007). As the number of germ cells in the 1dpp-old-KS mouse testis is already dramatically decreased, it may be indicative that apoptosis occurs before birth.

In conclusion, our current findings suggest a hypothetical model, in which the presence of a supernumerary X-chromosome in males results in a three-step germ cell loss: it disturbs the correct development of the gonocytes during the intrauterine period, which results not only in a lower number of germ cells at birth but also in destabilisation of their stem cell characteristics, reflected by the loss of SSC marker expression. The germ cells that manage to survive are finally lost when meiotic differentiation processes drive them into a default pathway (see Fig. 10).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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