41,XXY* male mice: An animal model for Klinefelter syndrome

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Abstract

Klinefelter syndrome (KS, 47,XXY) is the most frequent male chromosomal aneuploidy resulting in a highly heterogeneous clinical phenotype associated with hormonal dysbalance, increased rate of co-morbidities, and reduced lifespan. Two hallmarks of KS-affecting testicular functions are consistently observed: Hypergonadotropic hypogonadism and germ cell (GC) loss resulting in infertility. Although KS is being studied for decades, the underlying mechanisms for the observed pathophysiology are still unclear. Due to ethical restrictions, studies in humans are limited, and consequently, suitable animal models are needed to address the consequences of a supernumerary X chromosome. Mouse strains with comparable aneuploidies have been generated and yielded highly relevant insights into KS. We briefly describe the establishment of the KS mouse models, summarize the knowledge gained by their use, compare findings from the mouse models to those obtained in clinical studies, and also reflect on limitations of the currently used models derived from the B6Ei.Lt-Y* mouse strain, in which the Y chromosome is altered and its centromere position changed into a more distal location provoking meiotic non-disjunction. Breeding such as XY* males to XX females, the target 41,XXY*, and 41,XXY males are generated. Here, we summarize features of both models but report in particular findings from our 41,XXY* mice including some novel data on Sertoli cell characteristics.

KEYWORDS

41,XXY* mouse, chromosomal imbalance, germ cell loss, Klinefelter syndrome, Sertoli cell

1 | INTRODUCTION

Men carrying one or more supernumerary X chromosomes are defined as being Klinefelter patients (Lanfranco, Kamischke, Zitzmann, & Nieschlag, 2004). The syndrome is the most frequent male chromosomal disorder with an incidence of approximately 0.2% in the population. The clinical phenotype covers a wide variety of features associated with this genetic condition, that is, changed body proportions, gynecomastia, cognitive impairment, changed retina composition, disturbed bone metabolism, increased cardiovascular risks, and other metabolic disorders as well as altered socioeconomic traits. This plethora of factors results in increased mortality and morbidity, reducing life expectancy for up to 2 years (Bojesen & Gravholt, 2011, Gravholt, Jensen, Høst, & Bojesen, 2011, Foresta et al., 2012, Skakkebæk, Wallentin, & Gravholt, 2015, Zitzmann et al., 2015, Jørgensen, Skakkebaek, Anders, Pedersen, et al., 2015, Brand et al., 2017, Gravholt et al., 2018). However, the clinical phenotype is extremely heterogeneous, ranging from mild to severe presentation of one or more symptoms (Nieschlag et al., 2016). There are only two features found consistently in all Klinefelter men: hypergonadotropic hypogonadism and elevated gonadotropin but lowered testoster-

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axis, and the production of sperm are affected most by the syndrome and reflected in a massively disturbed architecture of the gonadal tissues. The underlying mechanisms are almost impossible to analyze in depth in patients for ethical reasons as especially developmental studies, experimental manipulations, and access to various tissues are either excluded or limited. Up to now only few approaches were conducted so far in vitro, as human cell lines with an XXY karyotype are rare (Bortolai & Melaragno, 2001, Panula, Kurek, Kumar, Albulfushi, et al., 2019), and cell culture experiment suffer from the missing systemic interplay of organs and endocrine milieu characteristic for the complex phenotype of KS (Lanfranco et al., 2004).

Thus, there is a strong need for the use of animal models to experimentally address the consequences of a supernumerary X chromosome on male physiology. However, because the most prominent consequence of KS is male infertility—this causes a dilemma considering that an animal model has to be bred in sufficient numbers to be utilized in experimental settings. Nevertheless, for KS, several mouse strains have been successfully established in the past and produced highly relevant insights into the mechanisms along which the supernumerary sex chromosome acts (Table 1). In this review, we will briefly describe the history of the KS mouse models, summarize the knowledge gained by their use, and report on some novel findings but also reflect on limitations thereof. Furthermore, we will in particular report findings from our 41,XXY* mice including some novel data on Sertoli cell (SC) characteristics.

2 | GENERATION AND CHARACTERIZATION OF MOUSE MODELS

As mentioned above, an animal model is crucial for analyzing any disorder that provokes systemic effects, especially when those are additionally of developmental plasticity. A bundle of genetically or hormonally caused comorbidities and increased mortality occurs. Only an experimental animal model can mimic and integrate such a number of crosslinked effects (Wistuba, 2010; Wistuba, Werler, Lewejohann, Brand, & Damm, 2017).

Males with a supernumerary X chromosome are observed in a broad range of mammalian species, including primates, rodents, ruminants, canids, and felids (Wistuba, 2010, Wistuba, Werler, et al., 2017). However, common to all of these males from different species is that they are occurring sporadically—found as single cases by chance—, because the supernumerary X chromosome derives from a chromosomal disjunction during meiosis (Tüttelmann et al., 2014), and that they are naturally infertile. Therefore, these naturally occurring cases are not suited to generate an animal model system as this would require regular breeding to provide sufficient numbers of animals for subsequent experimental use (Wistuba, 2010). A breed that regularly produces males with a supernumerary X chromosome with a reasonable frequency is needed to generate an animal model for Klinefelter syndrome.

There had been previous attempts to generate chimeric models by injecting embryonic stem cells with an extra X chromosome and those had been in use to produce a “KS-like” male phenotype but those males were difficult to obtain and only small numbers could be produced (Lue, Rao, Sinha Hikim, Im, & Swerdloff, 2001; Wistuba, Werler, et al., 2017).

Fortunately, a mouse strain was discovered approximately three decades ago by Eicher et al. (1991), when a mutant mouse line, the B6Ei.Lt-Y* mouse, was described in which the Y chromosome was altered. In this chromosome—designated Y*—, the centromere position changed into a more distal location and by that, the rate of meiotic non-disjunction I significantly increased (Wistuba, 2010). Upon breeding, the XY* males to XX females numerous aberrant karyotypes occur, among those also 41,XXY* (in the first generation) and 41,XXY males (after four generations of recurrent breeding with mice of various karyotypes Lue et al., 2005, Wistuba, 2010). Both models have been intensively characterized for their value to serve as an animal model for Klinefelter syndrome (Table 1, Wistuba, 2010, Wistuba, Werler, et al., 2017).

However, it should be noted that there is one major difference on the genetic level between the 41,XXY and the 41,XXY* mouse model for Klinefelter syndrome. Although the 41,XXY mice possess the full content of all three sex chromosomes, in the 41,XXY* males, one X and the Y chromosome fused end-to-end at their PAR, with an aberrant pseudoautosomal region (PAR) that lacks the Sats gene present in wild type PAR (Burgoyne & Arnold, 2016); however, the Sats gene is generally lacking in the mutant XY* males used for the four-generation breeding scheme. This difference is reflected in the generally accepted nomenclature by using a superscript Y when describing the 41,XXY* males to point this difference in chromosomal content out (Wistuba, 2010). Although these differences are present—physiologically and phenotypically—, there is almost no difference between the 41,XXY* and the 41,XXY male mice as the comparison of similar studies using either the one or the other model showed (Table 1, Wistuba, 2010, Wistuba, Werler, et al., 2017); both appear useful to experimentally study effects of a supernumerary X chromosome on the male physiology. Characteristic for male mice with a supernumerary X chromosome is the presence of small firm testes due to germ cell (GC) loss during the postnatal phase, altered body proportions, a hypergonadotropic hypogonadal endocrine state (elevated LH and FSH levels accompanied by lowered testosterone), behavioral and cognitive problems an altered vascularization (at least at the testicular level), as well as disturbed bone metabolism (Chen et al., 2013; Lue et al., 2009; Liu, Erkkila, et al., 2010; Lue et al., 2005; Lue et al., 2010; Werler et al., 2014; Wistuba et al., 2010; Wistuba, Brand, et al., 2017). In summary, the mouse models resemble many features of the human disorder and thus can serve as an animal model overcoming the limits of clinical studies to explore and manipulate the basic molecular mechanisms of the chromosomal imbalance. Many of the adverse features of Klinefelter syndrome have been thought to be related to a disturbed testicular function and—as a consequence of that—the altered endocrine milieu. However, in elegant experimental settings manipulating the endocrine regulation of mice, it was found that beneath the immediate hormone effects, also several genetic factors provoke the phenotype directly, for example, for bone metabolism (Liu, Kalak, et al., 2010).
| Feature                          | Mouse model                  | Human                                                                 | References                                                                                                                                                                                                                                                                                                                                 |
|---------------------------------|------------------------------|----------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Testis**                      |                              |                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                  |
| Appearance                      | Small and firm               | Small and firm                                                       | Lanfranco et al., 2004, Lewejohann, Damm, Luetjens, Hämäläinen, et al., 2009                                                                                                                                                                                                                                                  |
| Germ cell loss                  | Present from day 1 pp onwards, completed by day 10 | Prepubertal onset likely, apart from focal spermatogenesis in a certain proportion of patients complete after puberty | Werler, Demond, Ehmcke, Damm, et al., 2014, Rohayem, Bongers, Czeiło, Malzidis, et al., 2015, Corona et al., 2017, Zitzmann & Rohayem, 2020                                                                                                                                                                                             |
| Focal spermatogenesis           | Rarely present, often arrested | Present in approximately 40% of patients, spermatozoa formed        | Werler et al., 2014, Corona et al., 2017                                                                                                                                                                                                                                                                                                   |
| Testicular vascularization      | Altered, impaired blood supply | No data available                                                    | Wistuba et al. submitted                                                                                                                                                                                                                                                                                                                |
| **SC and SC function**          |                              |                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                  |
| BTB                             | Ocludin more diffusely distributed or even missing, Cx43 showed irregular diffuse expression | Cx43 and claudin-11 significantly reduced with a disorganized pattern | Figures 2 and 3. Giudice, Vermeulen, & Wyns, 2019                                                                                                                                                                                                                                                                                       |
| Maturation                      | Delayed, AMH expression extended | No data available                                             | Werler et al., 2014                                                                                                                                                                                                                                                                                                                   |
| Germ cell support               | Working in focal spermatogenesis | Working in focal spermatogenesis                                   | Scirano et al., 2009, Lue et al., 2010                                                                                                                                                                                                                                                                                               |
| Numbers                         | Altered                      | Normal until puberty, for adults no data available                 | Werler et al., 2014, Wikström & Dunkel, 2011                                                                                                                                                                                                                                                                                           |
| **LC and LC function**          |                              |                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                  |
| LC hyperplasia                  | Present                      | Present                                                             | Wistuba et al., 2010, Wikström & Dunkel, 2011                                                                                                                                                                                                                                                                                       |
| LC marker gene expression       | Overexpression in vitro      | No data available                                                  | Wistuba et al., 2010                                                                                                                                                                                                                                                                                                                  |
| **Endocrine function**          |                              |                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                  |
| Androgenisation                 | Normal until puberty onset   | Almost normal until puberty onset, whether testosterone is slightly reduced during early development is under debate | Wistuba et al., 2010, Lue et al., 2005, Fennoy, 2011                                                                                                                                                                                                                                                                                 |
| Serum testosterone/gonadotropins| Lowered testosterone/elevated FSH and LH (hypergonadotropism) | Lowered testosterone/elevated FSH and LH (hypergonadotropism) | Lue et al., 2005, Lewejohann et al., 2009, Wistuba, 2010, Lanfranco et al., 2004                                                                                                                                                                                                                                                   |
| Intratesticular testosterone    | Comparable to control values per organ | Comparable to control values per organ                             | Tüttelmann et al., 2014                                                                                                                                                                                                                                                                                                               |
| Steroidogenesis                 | Hyperactivated in vitro      | No data available                                                  | Wistuba et al., 2010                                                                                                                                                                                                                                                                                                                  |
| **X chromosome**                |                              |                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                  |
| X chromosomal escapee genes     | 13 (of which only 4 are in common with human) | Approximately one third of the x-chromosomal genes (~270 of 800 coding X-linked genes) with some tissue specific plasticity | Werler, Poplinski, Gromoll, & Wistuba, 2011, Carrel & Willard, 2005, Tukiainen, Villani, Yen, Rivas, & MacArthur, D.G., 2017                                                                                                                                                                                                 |
| X inactivation                  | Comparable to controls       | Comparable to controls                                             | Werler et al., 2011, Poplinski, Wieacker, Kiesch, & Gromoll, 2010                                                                                                                                                                                                                                                                     |
| Body proportions                | Unaffected                   | Altered, tall stature with longer legs and arms                    | Lanfranco et al., 2004, Lewejohann et al., 2009                                                                                                                                                                                                                                                                                     |
| Bone metabolism                 | Increased risk of osteoporosis and increased mortality due to femoral fractures | Testosterone-replaced XXY mice show reduced bone volume despite similar blood testosterone levels pointing to genetic causes | Liu et al., 2010, Bojesen, Juul, Birkebæk, & Gravholt, 2006, Swerdlow, Higgins, Schoemaker, Wright, & Jacobs, 2005                                                                                                                                                                                                                  |

(Continues)
In placental mammals, inactivation of the second X chromosome (XCI) by X-inactive specific transcript (XIST) expression from the inactivated X chromosome (Xi) compensates for sex-specific gene dosages (Brown & Willard, 1994; Csankovszki, Panning, Bates, Pehrson, & Jaenisch, 1999; Lyon, 1961). XCI is mainly regulated by DNA-methylation in which a highly methylated promoter region represses Xist expression on the activate X chromosome, whereas the lack of DNA methylation of XIST leads to Xist transcription on the inactivated X chromosome (Xi) (Chow et al., 2010).

However, due to the lack of detailed molecular analysis, we have no clear picture whether the processes of X inactivation in the KS mouse models are identical between the models which exhibit different chromosomal content, nor do we currently know whether the inactivation of the second X chromosome is identical with the inactivation in females. The only data available reported that Xist methylation in 41,XXY* males was comparable to their female 40,XX littermates (Werler et al., 2011). A similar methylation pattern was observed in a study in humans (Poplinski et al., 2010). This assumption is further supported by the expression of X-linked genes which was mainly observed for the escapee but no other X chromosomal genes neither in mice with a supernumerary X chromosome nor in KS men (Werler et al., 2011; Wistuba, Brand, et al., 2017; Zitzmann et al., 2015).

In the case of euploid men (46, XY), the Xist promoter region is fully methylated giving rise to an active X chromosome. In patients with KS having two X chromosome, Xist methylation and expression patterns are highly similar to women indicating a normally inactivated X chromosome (Poplinski et al., 2010). Although most genes on the Xi are silenced, some remain active and "escape" inactivation ("escapee genes"); in human about 30% of the gene content of the X chromosome, in the mouse only 13 genes (Carrel & Willard, 2005; Tukiainen et al., 2017; Werler et al., 2011; Wistuba, Werler, et al., 2017, Lewejohann et al., 2009, Lüer et al., 2010, Liu et al., 2005, Chen, Williams-Barris, McClusky, Njam, & Jordan, 2013; Niu, Bishop, Brookman-Byrne, Garton, Gay, et al., 2019).

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Genes are necessarily differently expressed between XY and XXY males. It is obvious that the escapee genes (genes that escape from silencing of the X chromosome [see below]) are the most prominent contributors to the altered gene dosage as they are not silenced in XXY and thus higher expressed per se than from the one active X in XY. However, there are other potential mechanisms which also influence expression differences between the karyotypes, for example, mosaic expression of X alleles and parental imprints in XXY but not
XY, hemizygous exposure of X alleles in XY but not XXY, the presence of paternal imprint of X-linked genes in XXY but not XY (Golden, Itoh, Itoh, Iyengar, et al., 2019, humans and outbred animals) and finally epigenetic effects of an inactive X chromosome in XXY but not XY (Wijchers & Festenstein, 2011).

The syndrome affects various organs—also during development—likely not only due to the endocrine changes which it provokes but also by the impact of the altered gene expression likely mainly provoked by a mixture of the above mentioned mechanisms of which the expression of escapee genes is the most obvious (Disteche et al., 2002; Werler et al., 2011; Wistuba, Brand, et al., 2017; Zitzmann et al., 2015). In case of the presence of one (or more) supernumerary X chromosome, those effects are the result of a combined impact of chromosomal imbalance leading to altered escapee gene activity in the male and subsequent features as a disturbed endocrine feedback along the hypothalamic-pituitary-gonadal (HPG) axis leading to hypogonadotropic hypogonadism, almost complete GC loss and infertility.

3 | LESSONS FROM THE MOUSE MODEL

3.1 | Cardiovascular effects and vascularization

There has been growing evidence that the circulatory system, including vascularization and angiogenesis, is substantially affected by the presence of a supernumerary X chromosome and that patients with KS seem to be generally at a higher risk for cardiovascular disease. Patients with KS have reduced artery diameters when compared to healthy men, but more similar to women ("feminized" phenotype) also an increased risk of deep venous thrombosis or pulmonary embolism was found in patients with KS (Foresta et al., 2012; Salzano et al., 2016). In addition, a cardiovascular phenotype was observed in men with KS in two independent studies in which a substantially shorter QTc interval while recording ECGs close to a pathological range was noted. It was speculated that the chromosomal imbalance could impact the regulation of the QTc interval and, in addition, that treatment of patients with KS may influence this mechanism which leads to possibly increased risk of cardiac infarction (Jørgensen et al., 2015; Zitzmann et al., 2015). In sum, the results point to a possibly increased risk of cardiac infarction due to shortened QTc times. For all of these observations in the circulatory system, it was suggested that they might be mainly due to genetic reasons resulting from the chromosomal imbalance rather than to the endocrine situation. Recently it was shown that also the vascularization of the eyes in men with KS is different from healthy controls (Brand et al., 2017).

Although the testis is the organ affected most by the disorder and a significant link between a functioning germ line and testicular blood vessels as well as between gonadal endocrine function and circulation is obvious (Wistuba, Werler, et al., 2017; Yoshida, Sukeno, & Nabeshima, 2007), studies dealing with the testicular angiogenesis and vascularization in males with a supernumerary X chromosome have not been addressed in detail. Consequently, we decided to use our 41,XXY* mouse model to analyze testicular vascularization and blood flow (Wistuba et al. submitted) by a morphometrical and functional approach. We found both, altered vascularization during development and a significant loss of blood supply in the testes of 41,XXY* males as we did find a different vessels distribution in 41,XXY* testes. In addition, using contrast enhanced ultrasound, we also observed significantly longer times needed to flood the testis with contrast agent. In conclusion, we suggested that the altered testicular vascularization might be a so far overlooked contributor to the endocrine phenotype as the hormone transport via the blood stream into and/or from the testis in the periphery might be hampered (Wistuba et al. submitted; Tüttelmann et al., 2014).

Although plausible, it remains to be elucidated whether the testicular situation with regard to vascularization and blood supply as observed in our mouse model holds true for patients with KS. To our knowledge, there are no data available yet from human testes yet, mainly for reasons of invasiveness of the functional tests and that the entire organ has to be available for the morphometrical evaluation.

3.2 | Hypergonadotropic hypogonadism

In line with the vascularization data described above, we also had obtained data on Leydig cell (LC) function and intratesticular testosterone in a previous study which appear to support the suggestion that hampered circulation might be involved into the endocrine phenotype. Surprisingly, these data had disproved the assumption that the hypergonadotropic hypogonadism, that is, the lower serum testosterone values could be due to impaired LC function. In contrast, LC appeared not only to be normally functioning when challenged in an in vitro approach by chorionic gonadotropin stimulation (used as a surrogate of luteinizing hormone [LH]) but even to be hyperactivated, that is, that their LH receptor was normal, their transcription level was generally elevated, and that they produced more testosterone than wildtype LC isolated from their littermates (Wistuba et al., 2010). In line with this finding, we also found intratesticular testosterone to be not different per testis neither between males of the 41,XXY* and the 40,XY* karyotype nor between patients with KS and healthy controls (Tüttelmann et al., 2014). Preliminary evaluation of the mouse testes pointed to a reduction of blood vessels in the testicular tissues (Tüttelmann et al., 2014). Consequently, we hypothesized that the hypergonadotropic hypogonadal endocrine milieu seems to be encountered by compensatory mechanisms (LC hyperactivation) but that these might be insufficient to rescue the endocrine phenotype due to hampered testicular blood supply.

3.3 | GC loss

Concerning the infertility aspect, we could also demonstrate 41,XXY* male mice to be an adequate model for KS (Wistuba, 2010; Wistuba,
Brand, et al., 2017; Wistuba, Werler, et al., 2017). In these mice, the number of GC is already reduced at birth and expression of spermatogonial markers such as Lin28 fading neonatally (Werler et al., 2014). In 41,XXY* male mice as well as in patients with KS frequently focal spermatogenesis is observed underlining the validity of the mouse model for the human. We hypothesize that at spermatogonia stage or even earlier, the karyotype is randomly corrected due to loss of the supernumerary X which allows spermatogonia eventually to survive and progress into differentiation (Werler et al. 2014). This assumption was first raised by Sciurano et al. (2009) by demonstrating the presence of only one X chromosome in spermatogonia of KS patients by using FISH. Interestingly, in foci of spermatogenesis, GC marker expression appeared fully correct (Werler et al., 2014).

3.4 | Testicular consequences

Concerning the testicular phenotype most attention was paid so far to the GC loss and the altered testicular architecture, for example, the degeneration of the tubular structures and the vascular situation. However, analyses of the testicular somatic components such as peritubular cells (PTC), LCs, and SC under the genetic condition are relatively scarce and remarkably underrepresented. In the case of SCs, this might be due to the fact that also SC with the aberrant XXY karyotype appear to support full germ line differentiation because such differentiation occurs when undifferentiated XY GCs were transplanted into a GC depleted murine XXY testes (Lue et al., 2010). Studying SC in the 41,XXY* mouse testes, we however found notable differences between male mice with a supernumerary X chromosome and control mice. First, when stereologically analyzed, SC numbers were found to be altered as although they were increased per volume unit their total number was significantly reduced per testis in adulthood due to the much lower testis volume of 41;XXY* males (Werler et al., 2014). But not only the abundance of SC was changed, also expression of SC markers was found to be different in 41,XXY* mice. It was previously assumed that the Doublesex and Mab-3 Related Transcription factor 1 (Dmr1), essential for the maintenance of the SC identity could be affected due to the sex chromosomal imbalance. However, immunohistochemical analyses in our mouse model showed that its expression remains unaffected from the postnatal phase to adulthood (Figure 1).

However, when Anti-Muellerian hormone (AMH), a marker for SC maturation was studied we noticed an extended expression window during the postnatal phase. Already gone by day 10 post partum (dpp) in controls, AMH expression was maintained in 41,XXY* males beyond 14 dpp and only no longer expressed in adult animals. Interestingly, this expression pattern was not found in tubuli with remaining focal spermatogenesis, indicating that the GC–SC communication might play a role for normal expression (Werler et al., 2014). We also analyzed the expression of the androgen receptor (Ar) in SCs to check the maturation state at the age of 3 weeks and in adulthood and found regular expression in both karyotypes. Taken together our findings that although AMH expression is extended in SCs of 41,XXY* male mice, the overall differentiation as revealed by missing AMH and detection of Ar appears to be correct latest at 3 weeks of age (Figure 2).
Apart from their nutritive and regulative function for the germ line, SC have crucial tasks to fulfill which is such as establishing and maintaining the blood–testis barrier (BTB) and by this forming the immunopriviliged inner space of the seminiferous tubules. For the BTB gap and tight junctions at the cells’ basal side enable complete closure to the basal lamina (Wistuba, Stukenborg, & Luetjens, 2007). It is well known that failure of the BTB closure can result in degenerative processes damaging the testicular structure as well as affecting

**FIGURE 2**  Immunohistochemical detection of androgen receptor (AR), occludin, and ZO-1, expression in testicular cross sections from male mice: (a), (c), (e) 40,XY* control, (b), (d), (f) 41,XXY* tissue; (a), (b), (e), (f) 21 days post partum; (c), (d) adult. Briefly, staining was performed as follows: After deparaffinization and inhibition of the endogenous peroxidase activity with 3% H$_2$O$_2$ in 80% ethanol for 30 min, sections for IHC were microwave pretreated with sodium citrate buffer (pH 6.0) for 3× 5 min at 800 W. Sections were then cooled down for 30 min at room temperature, blocked with 20% normal goat serum for 20 min, and incubated with the respective primary antibody (anti-Occludin, Invitrogen, Catalog-No.: 71-500, dilution 1:300; anti-ZO-1, Invitrogen, Catalog-No.: 61-7300, dilution 1:50; anti-AR, Santa Cruz Biotechnology INC., Catalog-No.: AR [N-20] sc-816, dilution 1:250) over night at 4°C. With the exception of ZO-1, sections were then exposed for 30 min at room temperature with the EnVision™ +Kit HRP Rabbit DAB+ (Dako, Hamburg, Germany, Catalog-No.: K4011). For the detection of ZO-1, sections were incubated with a secondary antibody (biotin-labeled goat anti-rabbit, Vector, Catalog-No.: BA-1000, dilution 1:200) for 60 min and, after rinsing, 30 min with the ABC-System (Vector, Catalog-No.: PK-6100). After visualization with DAB, sections were counterstained with hematoxylin for 2 sec. Omission and isotype controls with IgG served as negative controls. (a, b) Expression of the AR at the age of 3 weeks was found in peritubular cells (ptC) and Sertoli cells (SC) in both karyotypes indicating the SC differentiation to be correct. Note that germ cell differentiation in the wild type has progressed from spermatogonia (spg) to pachytene spermatocytes (P), whereas all germ cells are lost in 41,XXY* tissue at this time point forming the typical aspect of SC only. Proteins of the blood–testis barrier (BTB), that is, occludin (c, d) and the BTB-associated Zona occludens-1 (ZO-1, e, f); ZO-1 expression was present and correctly localized and appeared only a little weaker in 41,XXY* cells, and some unspecific staining was found in Leydig cells (LC) of both karyotypes; occludin, however, was detectable at the basis of 41,XXY* SCs but more diffusely distributed or even missing (arrows) when compared to wild type.
germ line differentiation. Also for patients with KS such degenerative processes have been described (Giudice et al., 2019). We therefore used our mouse model and examined SC-specific proteins which are involved into or associated with the formation of the BTB, that is, occludin, Zona occludens-1 (ZO-1), and connexin (Cx) 43 (Figures 2 and 3) as well as vimentin (Vim, data not shown). Although Vim was normally expressed in both karyotypes and age groups, and ZO-1 expression was present and correctly localized and appeared only a little weaker in 41,XXY* cells, occludin was detectable at the basis of 41,XXY* SC but more diffusely distributed or even missing when compared to wild type. The most prominent changed expression pattern were observed for the gap junction protein Cx43, 41,XXY* testes exhibited an altered pattern with an irregular diffuse expression likely indicating an impaired formation of the gap junctions when compared to littermate 40,XY* control tissues, which showed distinct signals marking exclusively the basal parts of the SC in a structurally regular way (Figure 3). Interestingly, very recently Giudice et al. (2019) also reported the expression of BTB proteins Cx43 and claudin-11 to be significantly reduced with a disorganized pattern in KS patients concluding a contribution to the degeneration of seminiferous tubules likely.

In conclusion, the presence of a supernumerary X-chromosome provokes a dramatically altered testicular phenotype in both organ architecture and cellular composition, and these features start to develop likely already in utero before they become manifest after puberty (Werler et al., 2014).

4 | KS MOUSE VERSUS HUMAN KS COMPARISON

The studies summarized above characterized male mice with a supernumerary X chromosome to mimic several features of the human KS. Not surprising, not all of the changes provoked by the presence of the chromosomal imbalance are identical in both species; however, in particular, the most important ones—GC loss and endocrine situation—are of high conformity as are the presence of focal spermatogenesis. At the genetic level, X chromosomal genes which escape from silencing are detected and are shared between the two species. Of advantage might be the fact that in mice, only 13 genes belong to these group (Werler et al., 2011; Yang et al., 2010) compared to almost approximately one third of the at least 800 protein coding X-chromosomal genes (with some tissue specific plasticity) escapee that escape silencing on the human X chromosome (Tukiainen et al., 2017). Noteworthy is the fact that only very few genes of the mouse X chromosome provoke an almost identical phenotype making it relatively easier to address genotype phenotype correlations. In Table 1, we have summarized similarities and discrepancies between the mouse model and patients with KS. In addition also illustrated open questions, some not yet analyzed in the mouse model or vice versa not yet observed in humans. It will be of importance to close these gaps in the future to understand the full spectrum of KS (Table 1).

The importance of this and the reciprocal benefits are shown by studies on cognition and behavior. Previously, it has been reported from both, mouse models as well as KS men that social behavior, learning ability, and language skills are effects by the presence of a supernumerary X chromosome. In mouse models, for Klinefelter Syndrome, also social interaction, motor performance, and pavlovian learning were demonstrated to be altered in male mice carrying a supernumerary X chromosome (Table 1; van Rijn et al., 2006, van Rijn, 2015, Ross et al., 2017, Wistuba, Werler, et al., 2017, Liu, Erkkila, et al., 2010, Lue et al., 2005, Chen et al., 2013). In addition, we analyzed a basic behavioral skill in male 41,XXY* mice which is requirement of non-pavlovian learning behavior, that is, memory recognition.
For this, we used the novel object task, a test integrating both, explorative skills, as well as the ability to learn and remember objects. A significant worse memory recognition in 41,XXY* males when compared to their 40,XY* littermates was noticed (Lewejoehann et al., 2009). Interestingly, when the experimental setting was adapted and used for human probands, Bruining et al. (2011) could show that comparable mechanisms also impair the recognition ability in patients with KS. This example underlines the usefulness and the necessity of mouse models which can add important knowledge for the further exploration of the human disorder.

5 | LIMITATIONS OF KS MICE AS A DISEASE MODEL

Mouse model for KS were proven in the past as the best animal model available for KS. However, they are exactly that—a model—and therefore, mouse studies have also limitations which should be mentioned.

The most important difference is that, compared to the human phenotype which is phenotypically very heterogeneous with a wide range of severity of the various symptoms present, male mice with a supernumerary X chromosome are phenotypically by far more homogeneous as they are bred from a mutated inbred strain (Eicher et al., 1991; Wistuba, 2010). Furthermore, mouse and human physiology are substantially different. For example, the differences found in the ECGs of KS men were not confirmed in 41,XXY* mice which exhibited perfectly normal patterns likely due to the peculiar murine heart physiology (Wistuba et al. submitted).

Concerning the GC loss in KS, the 41,XXY* mouse model indicates a very early GC loss might be true also in the human (Werler et al., 2014). However, there are only very few data available yet from KS fetuses or young boys (Heckmann, Langenstroth-Röwer, Pock, Wistuba, et al., 2018), and although those seem to provide some confirmatory evidence for the early loss of the germ line (Davis, Lahlou, Bardsley, Temple, et al., 2016), it has to be proven that the fading of the germ line is of a comparable sequence in the patients.

As the spermatogonial stem cell systems of rodents and primates (including the human) are significantly different (Sharma, Wistuba, Pock, Schlatt, & Neuhaus, 2019), the mechanism behind the GC loss might also differ to some extent and only result in similar testicular damage. For example, focal spermatogenesis is seen in approximately 40% of KS men (Corona et al., 2017), but only very rarely in the KS mice, a fact which seems to suggest a careful interpretation of these data when it comes to clinical consequences.

Moreover, on the chromosomal level, in humans, there is evidence that the X inactivation occurs elsewhere, that is, either the paternal or the maternal X is inactivated (Tüttelmann et al., 2014); however, the inactivation is possibly skewed with regard to the CAG repeats preferring the shorter repeat length (Zitzmann, Depenbusch, Gromoll, & Nieschlag, 2004). Other than in human, no data are available from the mouse models whether the inactivated X chromosome is always maternal, paternal, or silenced randomly. As long as this has not been addressed in the models, it might be also seen as another limitation of the model as it might for example make a difference if 41,XXY* mice have only the maternal X inactivated, whereas the maternal X is active in wild type XY or 41,XY* males.

6 | CONCLUDING REMARKS

As the discovery of the mutant B6Elite* mouse models for KS in the 90’s these animals served to explore general molecular and cellular mechanisms that underlie the male phenotype provoked by this chromosomal condition. As X-chromosomal inactivation was reported is seemingly normal in mice and men, it was concluded that the X-linked genes escaping from silencing are of prime importance for the phenotypical features as they are normally not expressed in a normal male chromosomal environment with one X chromosome only. These genes were ought to build the basis of all downstream events that interfere with the male metabolism resulting finally in GC loss, disturbed endocrinology, impaired recognition, cardiovascular alterations, and all other comorbidities of the syndrome (Wistuba, Werler, et al., 2017). Interestingly, there are only very few of such genes in mice but wide parts of the human phenotype—actually the most important ones—are resembled in male mice with a supernumerary X-chromosome suggesting them as a perfect model to analyze genotype—phenotype correlations. When comparing data published on mouse models with those from clinical studies some findings from animal studies are obviously in good consistency with results from patients like behavioral aspects, bone metabolism, infertility, and testicular degeneration as well as hypogonadism, whereas others are not like the cardiovascular phenotype and other morbidities. As also pointed out above, there is a bundle of findings from both species which have not yet been addressed either in patients or in the models and should be examined in further studies. In particular, developmental aspects for which only very limited options for exploration are present in clinical settings, and it can be expected that studies in the mouse models will offer deeper and important insights even when used for follow up analyses of aspect that have been examined before as our new results on SC presented here illustrate.

In brief, future studies using mouse models could contribute to a number of open questions concerning the genetic conditions, for example, the altered endocrine phenotype. So far, all studies dealing with males carrying a supernumerary X chromosome consistently reported lowered peripheral testosterone values but elevated numbers of Leydig cells. To date, the mechanisms underlying this phenomenon remain unsolved. What exactly provokes the Leydig cell proliferation under the genetic condition should be a target of future studies. As it is likely that developmental processes are involved, the mouse model will serve as the logical experimental tool to elucidate this, as access to human material from boys and adolescents is rather limited.

Furthermore, as intratesticular testosterone values are apparently normal but peripheral values lowered, the transport between testis tissues and circulation seem impaired by a so far unknown
mechanism. Although we could provide some evidence that the testicular blood supply might be involved (Wistuba et al. submitted), it is not yet clear, whether the export of testosterone, the import of gonadotropins, or both are the underlying reason. This has to be clarified in follow up studies using in vivo and in vitro approaches.

As work in animal models always should aim at providing evidence for therapeutic progress, the mouse models should be used to test potential treatments interfering into these mechanisms. For example, with regard to the endocrine/circulatory problems mentioned above, it would be possible to test treatments with substances supporting the testicular blood stream or to administer agonistic compound enhancing LH action via the LH receptor.

On the molecular level, as the number of X-linked genes escaping silencing is small in mice, the models will help to identify genotype-phenotype correlations which should be easier to follow than in humans and—more important—can allow also to understand how expression of genes not directly linked to the X chromosome but also involved into the syndrome are regulated. Combining such findings with transgenic mouse models, this would open a route for a far more exact assessment of gene dosage effects than it would be possible in humans.

Taken together, the mouse models are the best available model for KS—although there are also limitations (as in every model)—as male KS mice are the only tool to study the genetic condition in its complexity in vivo. In addition, their availability will allow developing and testing any novel and alternative treatments derived from both, clinical, or experimental approaches and by that stressing their importance for the field of KS research.

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CONFLICT OF INTEREST
The authors declare to have no conflict of interest.

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