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Andrology

Long-term culture of human Sertoli cells from adult Klinefelter patients as a first step to develop new tools for unravelling the testicular physiopathology

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ABSTRACT

STUDY QUESTION: Are Sertoli cells (SCs) from adult Klinefelter men (47,XXY) capable of proliferating *in vitro* and maintaining their main phenotypical and functional characteristics as do SCs from adult 46,XY patients?

SUMMARY ANSWER: Isolated SCs from patients with Klinefelter syndrome (KS) can be expanded *in vitro* while maintaining their characteristics and a stable karyotype, similar to SCs from 46,XY patients.

WHAT IS KNOWN ALREADY: The mechanism leading to testicular tissue degeneration in KS is still unknown. A few recent studies highlight the main role played by SCs in the physiopathology of the disease, but new study models based on co-culture or testicular organoids are needed to further understand the SC's involvement in the mechanism of testicular degeneration and fibrosis, and to find therapeutical targets. KS SC expansion could be the first step towards developing such *in vitro* study models. SCs have been isolated from 46,XY men and expanded *in vitro* while maintaining the expression of phenotypical and functional markers, but propagation of SCs from KS men has not been achieved yet.

STUDY DESIGN, SIZE, DURATION: Testicular tissue was obtained during a testicular sperm extraction procedure for infertility treatment between 2019 and 2021 from three azoospermic adult KS (47,XXY) men (33±3.6 years old) and from three control patients (46, XY) (36±2 years old) presenting with obstructive azoospermia. SCs isolated from frozen-thawed tissue of KS and 46,XY patients were cultured for 60 days and compared. All patients signed an informed consent according to the ethical board approval of the study protocol.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Testicular biopsies obtained from KS (n = 3) and 46,XY (n = 3) adult patients were slow-frozen. After tissue thawing SCs were isolated using a double-step enzymatic digestion and differential plating, and cultured for 60 days in DMEM medium containing FBS. Analyses were performed at different culture times (passages 5 (P5) and 10 (P10)). Quantification of cells using immunofluorescence (IF) for cell type-specific markers (Sox9, GATA4, ACTA2, INSL3, MAGEA4), SCs characterization using both IF and quantitative real-time PCR for GDNF, BMP4, AR and CLDN11 and cells karyotyping were performed.

MAIN RESULTS AND THE ROLE OF CHANCE: We demonstrate for the first time that a small population of human SCs isolated from frozen-thawed testis of adult KS patients can be expanded *in vitro* while retaining expression of characteristic markers of SCs and the 47,XXY karyotype, and exhibiting cell-specific functional proteins and gene expression (GDNF, BMP4, AR, and CLDN11) after 60 days in culture. At P10, 83.39 ± 4.2% of cultured cells from KS men and 85.34 ± 4.1% from 46,XY men expressed Sox9, and 88.8 ± 3.9% of KS cells versus 82.9 ± 3.2% of the control cells were positive for GATA4 without any differences between two groups; both Sox9 and GATA4 are typical SC markers. No differences were found between KS and 46,XY SCs *in vitro* in terms of cells expansion (exponential growth between P1 and P10 with an average cell count of $2.8\pm1.5\times10^7$ versus $3.8\pm1.2\times10^7$ respectively for the KS and control groups at P10). There was no significant statistical difference for functional proteins and genes expressions (GDNF, BMP4, AR, and CLDN11) neither between KS SCs and control SCs nor between P5 and P10.

LIMITATIONS, REASONS FOR CAUTION: The small number of donor samples is a limitation but it is due to limited availability of tissue for research in KS populations. Although no differences were observed in SCs function in the culture of isolated SCs after 60 days, the possibility of a SCs dysfunction needs to be investigated in more complex 3-dimensional models allowing the establishment of a proper cell organization and further analyses of cell functions and interactions during longer culture periods.

WIDER IMPLICATIONS OF THE FINDINGS: The demonstration of the possibility to propagate KS SCs *in vitro* could be useful to build new *in vitro* models for deciphering testicular cell interactions, determining deficient signalling pathways involved in impaired spermatogenesis, and identifying targets for infertility treatment in KS. As the cell numbers achieved in this study are higher than cell numbers used to develop testicular organoids, we may expect to be able to understand the behaviour and physiopathology of SCs in

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the disease during the long-term culture of these organoids. Such models could be further applied to understand other causes of deficiencies in seminiferous tubules.

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Introduction

Klinefelter syndrome (KS) is characterized by the presence of an extra X chromosome with the non-mosaic 47,XXY genotype being the predominant form occurring in 80–90% of the patients (Lanfranco *et al.*, 2004). It is the main genetic cause of azoospermia in men with an estimated frequency of 1:600–1:1000 in newborn boys (Bojesen *et al.*, 2003; Bonomi *et al.*, 2017; Berglund *et al.*, 2019). Azoospermia in KS is the consequence of testicular tissue degeneration starting already *in utero* and intensifying during puberty (Aksglaede *et al.*, 2006) leaving mainly hyalinized tubules with reduced numbers of germ cells and Sertoli cells (SCs), along with fibrosis and Leydig cell (LCs) hyperplasia (Wikstrom *et al.*, 2004; Aksglaede *et al.*, 2006). Infertility care commonly requires surgical sperm retrieval and testicular sperm extraction (TESE) with success rates between 40% and 60% (Sa *et al.*, 2023). Using ICSI, these men can achieve fatherhood with a live birth rate of 43% per ICSI cycle (Corona *et al.*, 2017).

The mechanism leading to testicular degeneration in KS patients is poorly understood. Hypotheses concerning an altered somatic compartment in KS have been recently formulated based on transcriptome and histological studies (for review see (Bradshaw *et al.*, 2023)). SCs, which play a key role in spermatogenesis providing an adequate ionic and metabolic environment for germ cell development and assuring the survival of spermatogonial stem cells (SSCs) (Sharpe *et al.*, 2003; Alves *et al.*, 2013; Crisostomo *et al.*, 2018), seem to be the most affected cell type in KS based on transcriptome analyses (D'Aurora *et al.*, 2015), single-cell RNA (scRNA) sequencing (Winge *et al.*, 2018, 2020; Zhao *et al.*, 2020; Mahyari *et al.*, 2021), and histology (Giudice *et al.*, 2019; Van Saen *et al.*, 2020). Upregulation of X-chromosomal transcripts related to markers of immature SCs was found using RNA extracted from fixed paraffin-embedded testicular tissue samples from three adult KS compared to samples showing Sertoli cells only (SCO) tubules, or complete spermatogenesis in 46,XY men or three cellularity-matched controls (Winge *et al.*, 2018). Comparative analyses between scRNA sequencing of one KS patient (Laurentino *et al.*, 2019) and control RNA datasets (Soraggi *et al.*, 2021) also indicate that the most affected genes were related to pathways linked to SCs function (Winge *et al.*, 2020). Furthermore, in comparative scRNA sequencing, using testicular tissue from two KS, one idiopathic non-obstructive azoospermia (iNOA) patient, one obstructive azoospermia (OA) patient and three controls and combining these with published data from adult individuals and two juveniles with normal spermatogenesis, it was found that loss of XIST (X Inactive Specific Transcript) expression only leads to an increase of 11–14% (not 100%) in expression output from the X chromosome in KS SCs, highlighting the upregulated cell signalling pathways (e.g. ligand factors secretion) in KS SCs compared to other testicular cell types (Mahyari *et al.*, 2021). Interestingly, a transcriptome study using microarray analyses showed dysregulated transcripts expressed by SCs in genes involved in the cellular structure of the blood–testis barrier (BTB), the immune response, and inflammation (D'Aurora *et al.*, 2015). In addition, an abnormal immune response of KS SCs presenting a mature pattern was also found when comparing scRNA sequencing results from KS patients to those from iNOA patients (Zhao *et al.*, 2020).

With regards to observations based on histology, immature SC markers appeared to remain in adult KS compared to controls in age-matched stratified cohorts from foetal to adult age (Van Saen *et al.*, 2020). BTB dysregulation was also demonstrated using testis tissue samples of a large adult population of 35 non-mosaic KS where reduced expression of BTB proteins (CLDN11 and CNX43) and androgen receptor (AR) in SCs were observed compared to patients presenting SCO syndrome and OA (Giudice *et al.*, 2019).

While SC's impairment seems to be involved in KS, there are many unknowns with regards to tissue degeneration and germ cell loss. Hence, developing *in vitro* models able to unravel pathological mechanisms in testicular cell interactions and their degenerative process is of paramount importance to identify potential therapeutical targets. However, there are, to the best of our knowledge, no reports on culture of KS SCs.

Our study aims at achieving KS SCs isolation and propagation is the first step in obtaining sufficient cell numbers to generate *in vitro* study tools. Second, to exclude the possibility of culture-dependent effects on KS SCs and thus to point to disease-specific differences, a comparison of *in vitro* cultured KS SCs and 46,XY SCs is required. Comparing SC culture outcomes from patients presenting with normal (OA) and abnormal spermatogenesis (NOA) has already been used to investigate infertility pathogenesis. The authors found differences in SC morphology and reduced expression of SCF, BMP4, and GDNF transcripts and proteins in SCs from NOA compared to OA (Ma *et al.*, 2013) men. Because no studies have reported the culture of KS SCs, we aimed to explore whether isolation and expansion of SCs from adult KS patients would allow the maintenance of their 47,XXY karyotype and original phenotype, including some features related to their function, over a long culture period as a first step to develop more complex *in vitro* models able to unravel the physiopathology of testicular impairment in KS patients.

Materials and methods

Source of testicular tissue

Testicular tissue biopsies taken as part of a TESE or micro-TESE (microdissection TESE) procedure for infertility care were obtained from six adult azoospermic men at the Cliniques Universitaires Saint-Luc, Brussels, Belgium. All participants signed an informed consent after being fully informed of the goal and protocol of our study. Only patients for whom spermatozoa were found during the TESE procedure and could be cryopreserved for future oocyte intracytoplasmic injection were allowed to participate in the study. The study was approved by the Ethics Review Board of the Cliniques Universitaires Saint-Luc (2018/27AVR/196) which implies that it has to be performed according to the Declaration of Helsinki for medical research involving

human subjects, and in accordance with the Guidelines of Local, National and European Ethical Committees.

Three patients with OA, mean age 36 ± 2 years with a normal karyotype (46,XY), in whom percutaneous epididymal sperm aspiration failed and testicular histology showed normal spermatogenesis, and three non-mosaic KS 47,XXY patients presenting NOA and a typical KS testis histology (LC hyperplasia and seminiferous tubule hyalinization), mean age 33 ± 3.6 years, were recruited.

Testicular samples of ± 2 mm^3 donated for research were cryopreserved in 1 ml cryovials using minimum essential media (M4655, Sigma-Aldrich, Belgium) supplemented with 20% human serum albumin (HSA) (CUSL, Brussels, Belgium) and 8% dimethyl sulfoxide (cryosure DMSO) (482, Life science, Belgium). The tissue was frozen using Nalgene® Cryo 1°C 'Mr Frosty' (Thermo Fisher Scientific, Belgium) at −80°C overnight and cryotubes were transferred to −196°C (liquid N2) the following day (Baert et al., 2013; Zarandi et al., 2018).

Multiplex immunofluorescence on tissue sections

After thawing and before digestion, part of the testicular fragment was cut, fixed, and embedded in paraffin. Multiplex immunofluorescence (IF) was performed to characterize the tissue before digestion. Table 1 shows the target proteins for the different testicular cell types and antibodies dilutions (Sox9, MAGEA4, INSL3, and ACTA2). Antigens expression was assessed following an established protocol for evidencing several proteins simultaneously (Huyghe et al., 2023). Histological sections of 5 µm were deparaffinized and rehydrated through a series of toluene and isopropanol baths. Endogenous peroxidase activity was inhibited by incubating the sections with 3% H_2O_2 in isopropanol for 20 min at room temperature (RT). After washing in deionized water, sections were subjected to the first antigen retrieval in citrate buffer pH 5.7. Sections were then washed in Tris-buffered saline (T5912, Sigma-Aldrich, Belgium) and Tween (P1379, Sigma-Aldrich, Belgium) and incubated at RT with bovine serum albumin (BSA) (A7030, Sigma-Aldrich, Belgium,) to block non-specific binding sites for 30 min. The first primary antibody was added to the sections and incubated for 90 min at RT. After three washing steps, sections were incubated with a peroxidase-labelled secondary antibody (Agilent, Belgium) for 40 min at RT. The signal for the first antibody was revealed by applying tyramide signal amplification conjugated with different fluorochromes (Thermo Fisher Scientific, Belgium). The same sequence was repeated three times with three different primary antibodies. Nuclei were stained with Hoescht (62249, Thermo Fisher Scientific, Belgium). Slides were mounted with fluorescence mounting medium (S3023, Agilent, Belgium). For positive tissue controls, we used tissue sections with normal human spermatogenesis. For negative controls, the primary antibody was omitted.

All slides were scanned using the Zeiss Axio Scan Z1 (Carl Zeiss, Göttingen, Germany).

Isolation and culture of cells

After thawing, testes tissue was washed in Dulbecco modified Eagle medium (DMEM) F12 (11330032, Thermo Fisher Scientific, Belgium) containing HSA (CUSL, Brussels, Belgium), penicillin, and streptomycin (15140122, Thermo Fisher Scientific, Belgium) and cut in small pieces. SCs were isolated from testes tissue by a two-step enzymatic digestion using, first, 2 mg/ml Collagenase IV (17104019, Thermo Fisher Scientific, Belgium) and 1 µg/µl DNase I (11284932001, Sigma-Aldrich, Belgium) in a water bath at 37°C for 15 min and, second, 4 mg/ml Collagenase IV, 2.5 mg/ml Hyaluronidase (H1115000, Sigma-Aldrich, Belgium), 2 mg/ml Trypsine (T4549, Sigma-Aldrich, Belgium) and 1 µg/µl DNase I in a 37°C water bath for 10 min, followed by differential plating. For differential plating, cell suspensions were seeded (10 000 cells/cm^2) into uncoated 48-24-12 or 6 wells dishes (VWR, Belgium) containing DMEM-F12 supplemented with 10% foetal bovine serum (FBS) (F7524, Sigma-Aldrich, Belgium) and 100 U/ml penicillin streptomycin (15140122, Thermo Fisher Scientific, Belgium) at 34°C in 5% CO^2 overnight (Ma et al., 2013; Guo et al., 2015). After the overnight incubation, cells in suspension were removed and cells attached to the culture plates were cultured in DMEM F-12 (11330032, Thermo Fisher Scientific, Belgium) supplemented with 10% FBS (FT524, Sigma-Aldrich, Belgium) and 100 U/ml penicillin streptomycin (15140122, Thermo Fisher Scientific, Belgium) at 34°C in 5% CO_2. Cells were passaged when 80% confluence was reached for a total of 10 passages for 60 days. Culture medium was changed every 2–3 days.

Cell viability and quantification

Cell viability was assessed at each passage by exclusion of trypan blue staining and viable cells were counted using a Bürker hemocytometer chamber (VWR, Belgium).

Immunocytochemistry

To analyse the cell population in culture, IF staining was performed at two different culture times: passage 5 (P5) and passage

Table 1. Antibodies references, target cells, and dilutions.

Target protein	Producer—reference	Target	Dilutions/technique	Clone
Sox9	Abcam—AB185966	Sertoli cells marker	1:500 IF 1:300 Multiplex	Rabbit Monoclonal
GATA4	Abcam—AB84593	Sertoli cells marker	1:500 IF	Rabbit Polyclonal
BMP4	Santa Cruz—sc-12721	Sertoli cells function	1:50 IF	Mouse Monoclonal
GDNF	Sigma-Aldrich—SAB1405863	Sertoli cells function	1:150 IF	Mouse Polyclonal
Androgen Receptor (AR)	DAKO—AR441	Sertoli cells function	1:100 IF	Mouse Monoclonal
CLDN11	Santa Cruz—sc-25711	Sertoli cells function	1:300 IF	Rabbit Polyclonal
INSL3	Sigma-Aldrich—HPA028615	Leydig cells marker	1:1000 IF 1:500 Multiplex	Rabbit Polyclonal
ACTA2	Sigma-Aldrich—A2547	Peritubular myoid cells marker	1:1000 IF 1:2000 Multiplex	Mouse Monoclonal
MAGEA-4	Kindly provided by Giulio Spagnoli MD, University of Basel, Switzerland	Spermatogonial cells marker	1:50 IF 1:20 Multiplex	Mouse Monoclonal

IF, immunofluorescence.

10 (P10). Specific markers for testicular cells and dilutions are presented in Table 1. For assessing the culture purity two SC markers, GATA4 as a key transcriptional regulator of SCs (He *et al.*, 2007; Kyronlahti *et al.*, 2011) and Sox9 as a nuclear transcription factor (Hemendinger *et al.*, 2002) were used. For SCs maturation and function, we performed IF staining for AR, GDNF, BMP4, and CLDN11.

Cultured cells were fixed with 4% paraformaldehyde (PFA) (17A184141, VWR Chemicals, Belgium) washed with phosphatase buffer saline (PBS) (RB01, R&D system, Belgium), and permeabilized with Triton-X 0.1% (93443, Sigma-Aldrich, Belgium) for 10 min. Cells were blocked in BSA 5% (A7030, Sigma-Aldrich, Belgium) for 1 h, followed by an overnight incubation at 4°C with primary antibodies (Table 1). For negative controls, the primary antibody was omitted. After extensive washes with PBS, cells were incubated with the secondary antibody Alexa (Thermo Fisher Scientific, Belgium). Nuclei were stained with ProLong Glass Antifade Mountant (P36985, Thermo Fisher Scientific, Belgium).

Images were acquired on an LSM800 fluorescent microscope (LSM800, Zeiss, Zaventem, Belgium) and analysed using QuPath 0.3.0 software (Bankhead *et al.*, 2017).

Quantitative real-time PCR

Total RNA was extracted from cultured cells at two different passages P5 and P10 using the PureLink™ RNA mini kit (12183025, Thermo Fisher Scientific, Belgium), followed by reverse transcription using qScript cDNA (Quanta Biosciences, Gaithersburg, MA, USA). Quantitative real-time PCR (qPCR) was performed with 5 ng of cDNA by using TaqMan fast MasterMix (4444557, Thermo Fisher Scientific, Belgium) and specific primers (Table 2) according to the manufacturer's protocol on a StepOne™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Belgium). All qPCR experiments were performed in duplicates. Relative quantitation was calculated with the Delta-Delta CT (ΔΔCT) method using GAPDH as the reference gene.

Chromosomal analysis

Karyotypes were performed at passage 3 (P3) and P10. SCs were seeded and cultured in a chamber slide system (Nunc Lab-Tek II, Thermo Fisher Scientific, Belgium) for cytogenetic investigation. Cells were incubated with 60 µl of Colcemid (KaryoMAX Colcemid Solution, Thermo Fisher Scientific, Belgium) at 37°C in 5% CO^2 and 15% O^2 for 4 h to arrest cell division. Then 2 ml of pre-heated 0.065 M KCl (Sigma-Aldrich, Belgium) was carefully added for 30 min. Cells were fixed by using 3:1 ratio of methanol (6009, Sigma-Aldrich, Belgium) and acetic acid (695092, Sigma-Aldrich, Belgium,) (Carnoy's fixative) for 60 min. Slides were dried on a heated plate between 70°C and 80°C for 60 min. Metaphase spreads were prepared and studied by GTG banding (G-banding with Trypsin-Giemsa) according to standard techniques. An automated karyotyping system (MetaSystems, GmbH, Altlussheim, Germany) was used for analysis and results were reported according to the International System for Human Cytogenomic Nomenclature (version: 2020) (https://iscn.karger.com/).

Table 2. TaqMan gene primers used for qPCR.

Gene	Reference	FAM-VIC	Chromosome location
BMP4	Hs00370078_m1	FAM	14
GDNF	Hs01931883_s1	FAM	5
AR	Hs00171172_m1	FAM	X
CLDN11	Hs00194440_m1	FAM	3
GAPDH	Hs02786624_g1	VIC	12

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Statistical differences between KS and controls were determined using two-way ANOVA test. Results were expressed as mean ± SEM. *P*-values of <0.05 were considered significant.

Results

Testicular tissue structure and cell populations identified by multiplex IF before digestion

Tissue from OA patients showed seminiferous tubules with a tubular wall underlined by ACTA2-positive peritubular myoid cells (PTMC) and intratubular spermatogonia (MAGEA-4) (Fig. 1A and B). Tissue from the three KS subjects presented typical seminiferous tubules containing, in these patients, only Sertoli cells (Sox9) and Leydig cell (INSL3) hyperplasia in the interstitium (Fig. 1C and D).

Expansion of SCs and characterization of cell populations in culture

SCs culture is shown in Fig. 2A and B. SCs grew exponentially (with 1000-fold increases) during the whole culture duration up to P10 in both groups of patients without significant differences between groups. After digestion, the initial cell numbers varied between 0.026×10^6 and 0.3×10^6. An average cell count of $2.8\pm1.5\times10^7$ versus $3.8\pm1.2\times10^7$ respectively for KS and controls was calculated at P10 without any significant difference between the two patients' groups (Fig. 2C and D). At each passage, cell viability was >96%.

A mean of 83.39 ± 4.2% of cultured cells from KS and 85.34 ± 4.1% from 46,XY men expressed Sox9 and a mean of 88.8 ± 3.9% of KS cells versus 82.9 ± 3.2% of control patients was positive for GATA4. The percentage of positive cells for both markers were not significantly ($P>0.05$) different neither between P5 and P10 nor between the two groups (Fig. 3). A mean of 15.96 ± 1.4% versus 24.03 ± 4.0% of the cultured cells were ACTA2 positive PTMC, respectively for the KS versus control group. No differences ($P>0.05$) were found between the two groups and between P5 and P10 (Fig. 4). No staining was observed for INSL3 and MAGEA4 (data not shown).

Protein expression in SCs from KS and 46,XY OA patients

AR was expressed only in the cytoplasm of cultured SCs with a mean of 31.7 ± 9.3% of cells in KS and 53.0 ± 10.5% in controls. There was no difference ($P>0.05$) in its expression between KS and controls, and between P5 and P10 within the same group (Fig. 5). A mean of 48.1 ± 7.9% versus 64.9 ± 9.2% cells were positive for GDNF and a mean of 42.0 ± 3.9% versus 49.6 ± 6.3% were positive for BMP4, respectively in KS and controls. For both proteins, no statistical differences were found ($P>0.05$) between the two groups neither during culture between P5 and P10 (Fig. 5B).

The BTB protein CLDN11 was present in the cytoplasm (Fig. 6A) of a small percentage of SCs in both groups KLIN (17.18 ± 28.56%) and CTRL (16.56 ± 17.15%), without any difference between the two groups or the culture time (Fig. 6B).

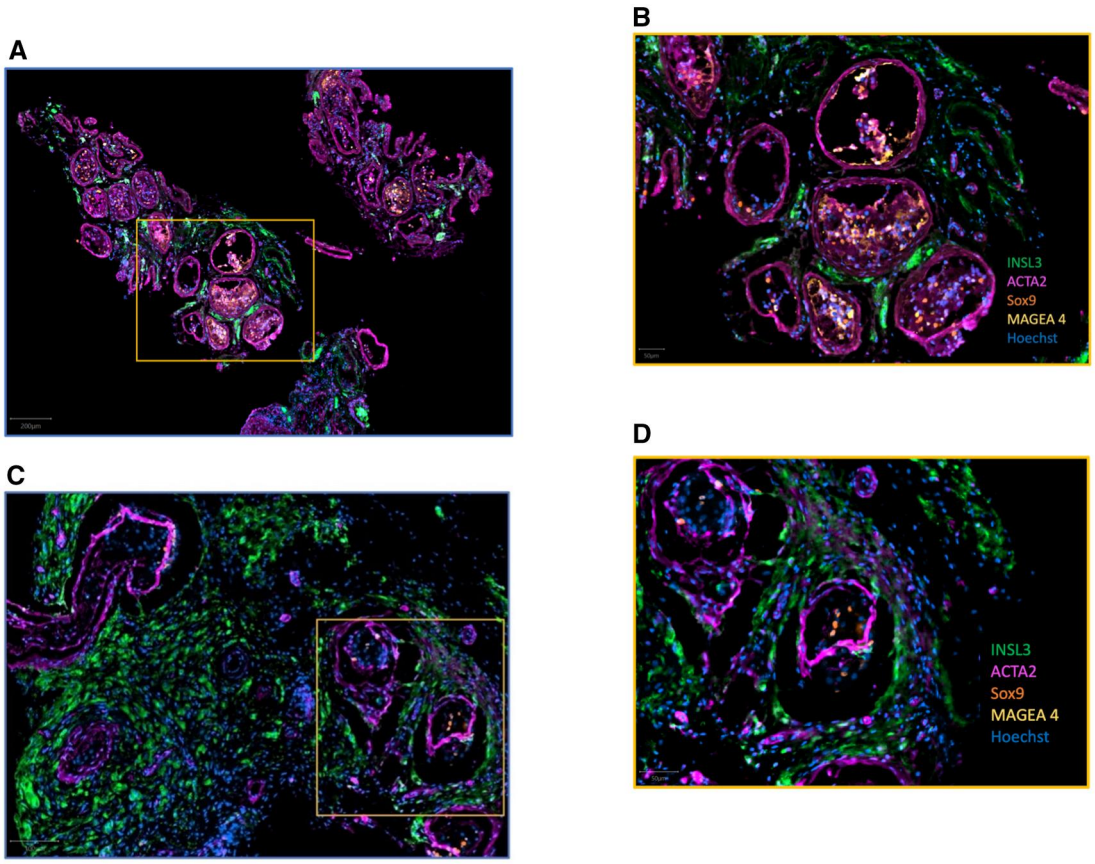

Figure 1. Multiplex assessment of testicular biopsies before digestion. Multiplex immunostaining (Multiplex) of testis tissues of a control patient (**A**, **B**) and a KS patient (**C, D**). Seminiferous tubules (ST) are surrounded by peritubular myoid cells (ACTA2) and contain spermatogonia (MAGEA4) in control but not in KS tissues (magnification scale bar 50 μm in B–D). ST showed reduced numbers of Sertoli cells (SCs) (Sox9) as well as Leydig cell (INSL3) hyperplasia in KS tissues (C). Scale bars=100 μm in A and C. KS, Klinefelter syndrome.

Gene expression in SCs from KS and 46, XY patients

GDNF, BMP4, AR, and CLD11 gene expression is shown in Figs 5C and 6C. There was no significant statistical difference between their expression in KS SCs compared to SCs from 46,XY patients at P5 or P10. GDNF, BMP4, AR, and CLDN11 genes expression was maintained until the end of the culture in both groups and no statistical differences ($P > 0.05$) were found between P5 and P10 within each group (Figs 5C and 6C).

Karyotype analysis during SC culture

Table 3 shows the number of mitoses analysed and the cell's karyotypes at passages 3 and 10. An average of 33 ± 24 mitoses for each donor sample at each passage was analysed. While karyotypes were maintained over the culture period for all 46,XY samples, one of the 47,XXY samples showed a mosaicism in P3 (47/65 mitoses analysed were 47,XXY and 18/65 mitoses analysed were 46,XY) but this mosaicism was not found at the end of the culture (60/60 mitoses were 47,XXY).

Discussion

The purpose of this study was to provide the first evidence that a small population of human SCs isolated from adult KS patients can be expanded *in vitro* while retaining expression of characteristic SC markers and a stable karyotype, and while exhibiting cell-specific functional proteins and gene expression after 60 days.

47,XXY testicular cell expansion is the first step towards developing new *in vitro* models of the disease such as cell co-culture or organoids, to study testicular cell interactions and development *in vitro*. A better understanding of the physiopathology of KS infertility is essential to identify therapeutical targets to improve the reproductive potential of these patients.

Attempts to elucidate the testicular impairment in KS have been made using induced pluripotent stem cells (iPSCs) as a disease model (Panula *et al.*, 2019; Botman *et al.*, 2020) or animal models (Wistuba *et al.*, 2020).

47,XXY-iPSCs derived from a KS patient were used to analyse the differentiation into the germ cell lineage, testing the impact of different combinations of factors, including BMP4 and GDNF. 47,XXY-iPSCs showed a reduced ability to differentiate in germ cells when compared to 46,XY-iPSCs (Botman *et al.*, 2020). The decrease in MAGEA4-positive cells appeared to be more important in BMP4-treated conditions though did not reach statistical significance compared to controls and did not appear to be linked to proliferation failure but rather to cell apoptosis (Botman *et al.*, 2020). Another study compared KS-derived iPSCs to female iPSCs and showed a similar X chromosome inactivation behaviour in culture although the biological relevance of 47,XXY iPSCs for KS genotype-related clinical features, that could be associated with gonadal function and infertility, still needs to be confirmed (Panula *et al.*, 2019). Moreover, using iPSCs as a disease-model for recapitulating the KS testicular environment and functioning is restricted to the germ line behaviour without the possibility of mimicking the testicular niche microenvironment.

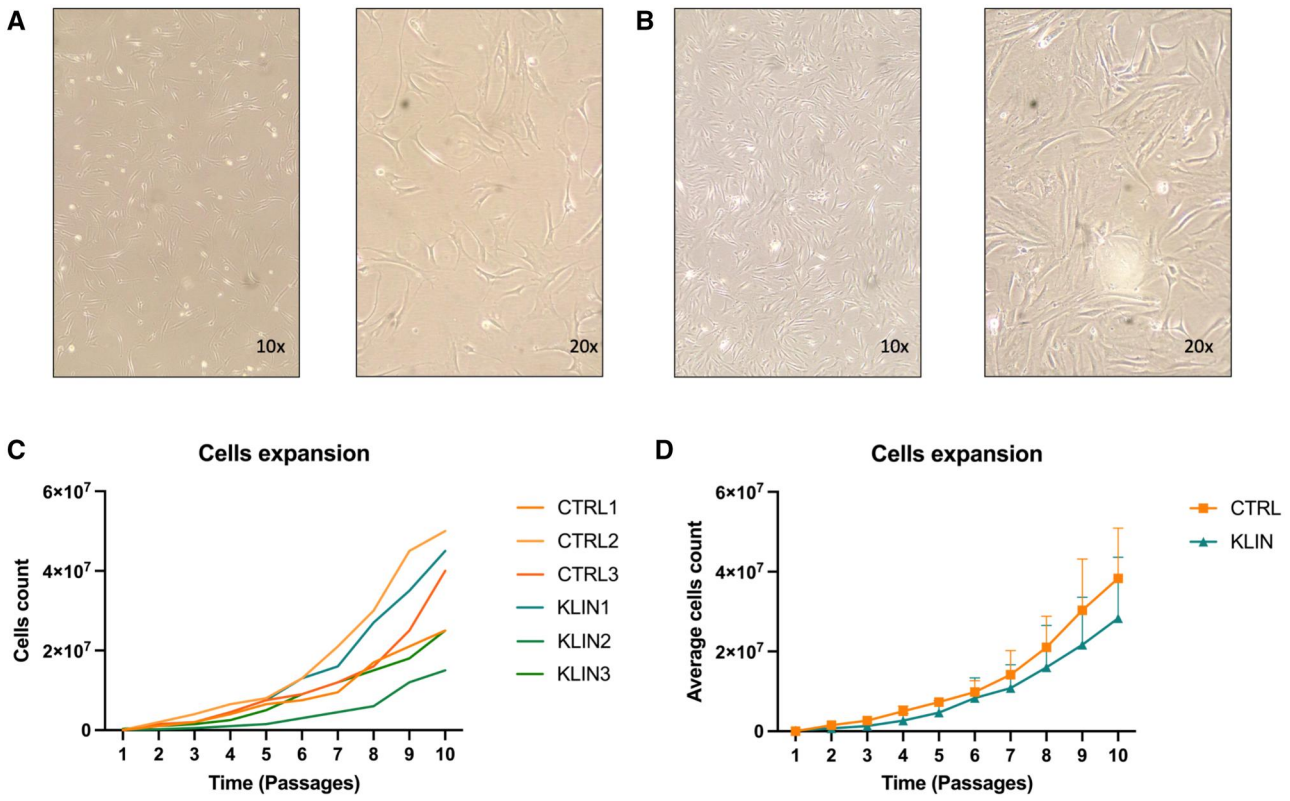

Figure 2. Sertoli cell expansion in culture. Sertoli cell (SC) morphology in phase contrast microscope during *in vitro* culture at different magnifications (10 and 20×) from a KS patient (**A**, **B**). SC proliferation and increased to confluence (80%) before passaging (B). SC expansion during 10 passages (60 days) for the six patients (**C**) with no differences between the two patient groups (Klinefelter versus control) (**D**). KS, Klinefelter syndrome.

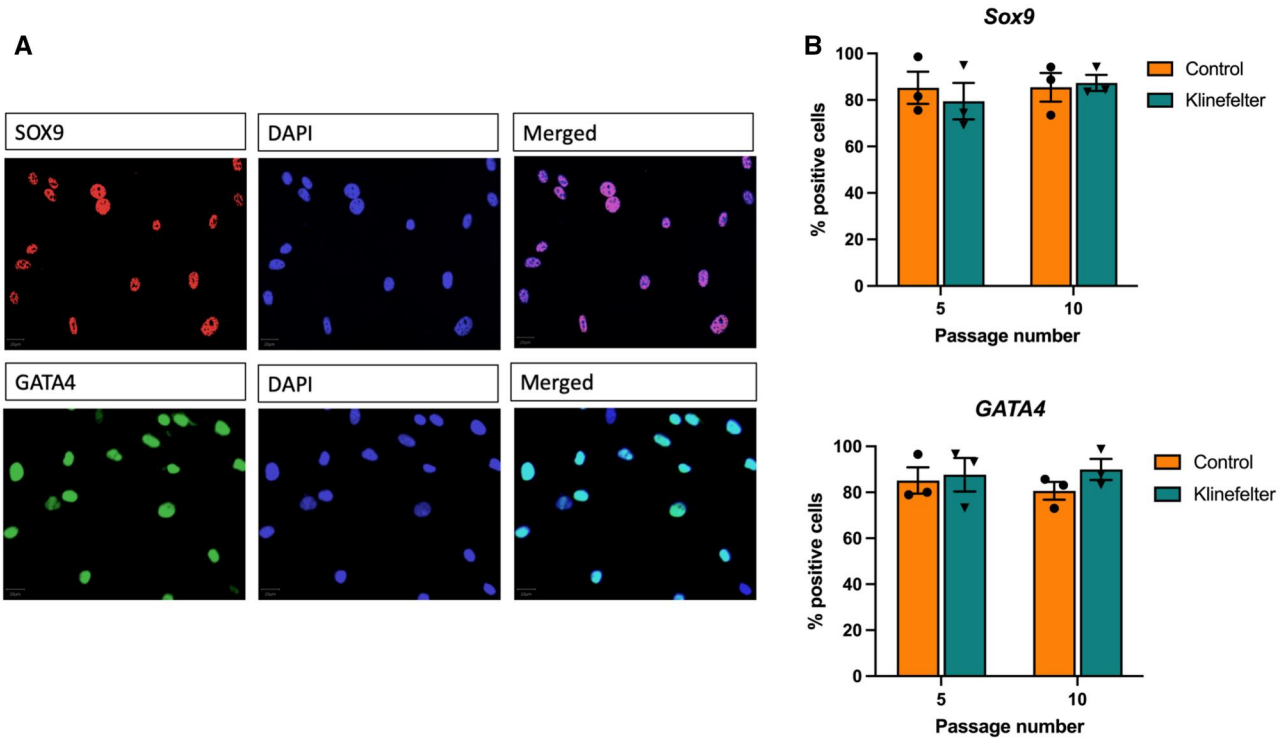

Figure 3. Sertoli cell immunofluorescence characterization in culture. Sox9 positive and GATA4 positive Sertoli cells (SCs) from a control patient at passage (P)10. Scale bars=20 μm (**A**). There was no statistical difference in the proportion of positive cells between the KS and control groups, nor between P5 and P10 (**B**). KS, Klinefelter syndrome.

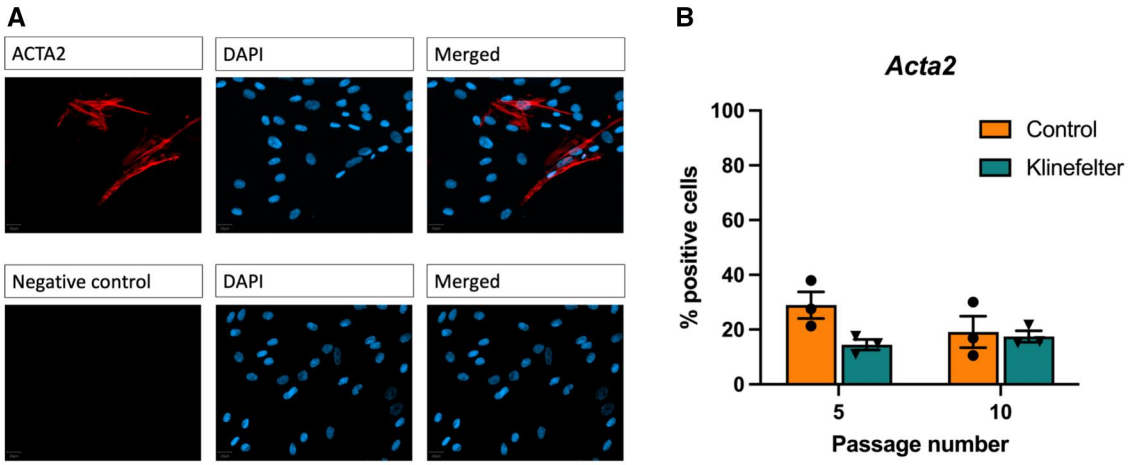

Figure 4. Peritubular myoid cells in culture. ACTA 2 positive peritubular myoid cells and a negative immunofluorescence control from a control patient at passage (P)10. Scale bars=20 μm (**A**). There was no statistical difference in the proportion of ACTA2 positive cells between the KS and control groups, nor between P5 and P10 (**B**). KS, Klinefelter syndrome.

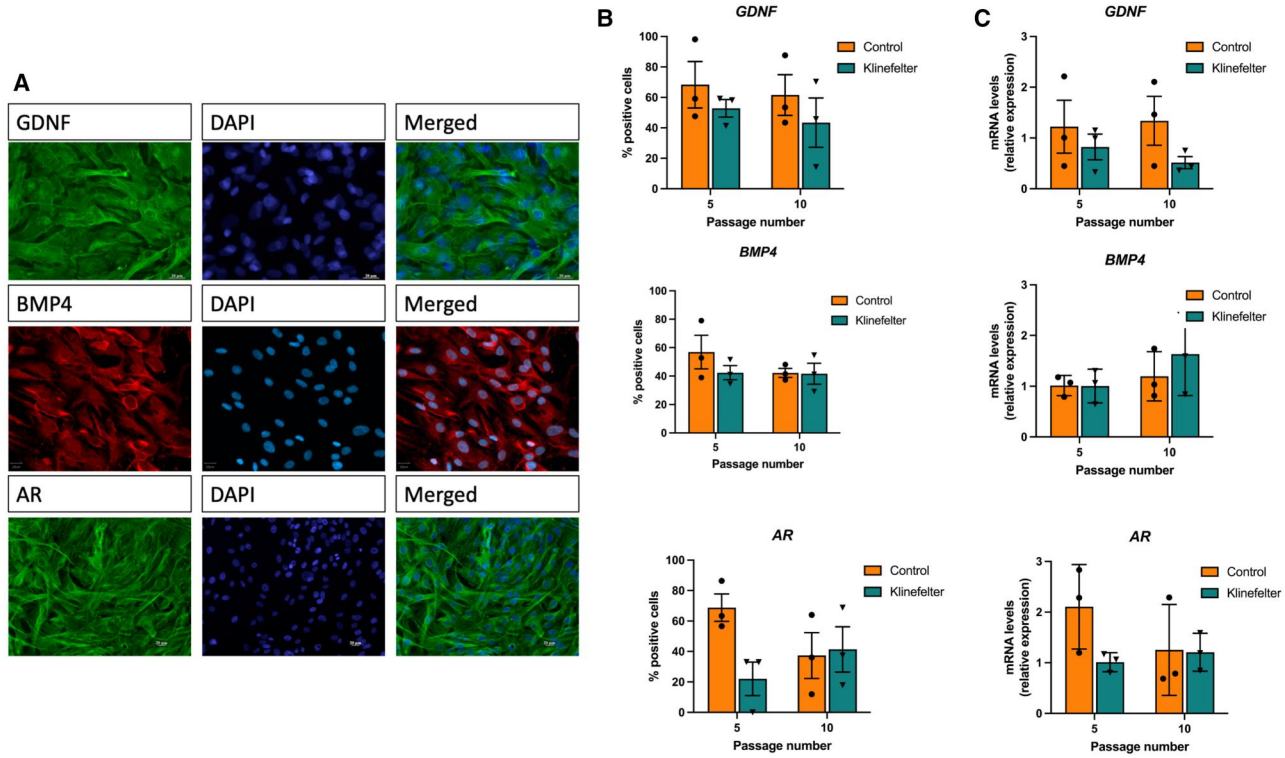

Figure 5. Sertoli cell protein and gene expression in culture. Expression of GDNF, BMP4, and AR in Sertoli cells from a KS patient at P10. Scale bars=20 μm (**A**). There were no significant differences in the protein expression between the KS and control groups, nor between passage (P)5 and P10 (**B**). qPCR results showed no significant differences for GDNF, BMP4, and AR gene expression between the KS and control groups, nor between P5 and P10 (**C**). KS, Klinefelter syndrome.

With regards to animal models for KS, two mouse models exist: 41, XXY and 41, XXY* (the latter resulting from one X and the Y chromosome fused end-to-end) (for review see (Wistuba *et al.*, 2020)). Both models appear useful to experimentally study effects of the supernumerary X chromosome on male physiology, although the 41, XXY* mouse has been shown to more closely resemble the human KS (Wistuba, 2010). However, in mice, there are only 13 X chromosomal genes which escape from silencing (Werler *et al.*, 2011) compared to almost 300 protein-coding X-chromosomal genes escaping silencing in human (Tukiainen *et al.*, 2017), which represents a serious limitation of the rodent models.

For long time, it has been thought that mammalian SCs are terminally differentiated and non-dividing in post-puberty (Sharpe *et al.*, 2003), but several studies showed the capacity of SCs from adult 46,XY men to proliferate and be expanded *in vitro* using standard culture condition adding FBS without any hormones or gonadotropins (Ahmed *et al.*, 2009; Oliveira *et al.*, 2009; Hayrabedyan *et al.*, 2012). While the reason for this remains unclear, some hypotheses may be raised based on observations in literature. Compared to other testicular quiescent cells, adult SCs were found to have a differential expression of cell cycle genes (decreased cell cycle inhibitor and increased cell proliferation inducer) (Chaudhary *et al.*, 2001, 2005). Hence, they would

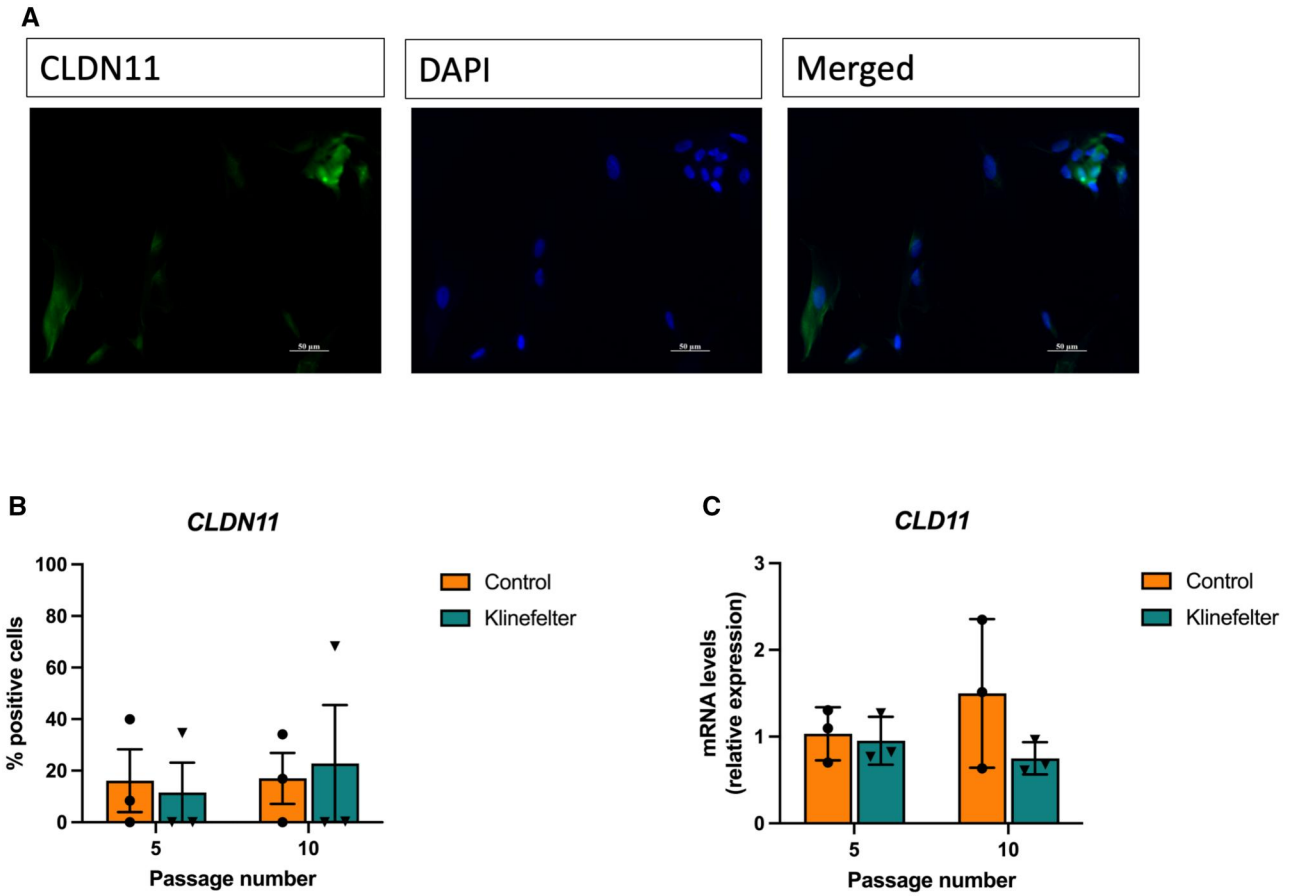

Figure 6. CLD11 protein and gene expression in SCs in culture. Expression of CLDN11 in Sertoli cells from a KS patient at P10. Scale bars=50 μm (**A**). There were no differences in CLDN11 gene expression between the KS and control groups, nor between passage (P)5 and P10 (**B**). KS, Klinefelter syndrome.

Table 3. Cells karyotypes and number of mitoses analysed.

	Passage 3	Passage 10	No. of mitoses analysed at P3	No. of mitoses analysed at P10
CTRL1	46,XY	46,XY	45	20
CTRL2	46,XY	46,XY	87	16
CTRL3	46,XY	46,XY	20	15
KLIN1	mosaic 47, XXY[47]/46, XY[18]	47,XXY	65	60
KLIN2	47,XXY	47,XXY	15	20
KLIN3	47,XXY	47,XXY	13	20

CTRL, control patient; KLIN, Klinefelter patient.

resemble more arrested proliferating cells rather than the classical post-mitotic and terminally differentiated somatic cells that they have always been assumed to be, at least *in vitro* (for review see (Hayrabedyan *et al.*, 2012)).

SC culture was already used in a variety of applications, e.g. to study the impact of metabolic diseases on their metabolism and on spermatogenesis (Alves *et al.*, 2013; Rato *et al.*, 2016) or to disclose the deleterious effects of chemicals on male reproductive health (Reis *et al.*, 2015), as SCs are considered a central target of environmental toxicants responsible of male reproductive defects (Crisostomo *et al.*, 2018). Human adult SCs were found to maintain their typical features *in vitro* based on protein and gene expressions, and ultrastructure and polarized junction morphology after 20 days of culture (Chui *et al.*, 2011). Propagation of differentiated SCs from pooled testicular biopsies of 50 infertile adult men (with OA) up to an almost 60 000-fold expansion after 60 days was also obtained in a 2-dimensional culture system (Guo *et al.*, 2015).

Co-culture of different human testicular cell populations combining 46,XY and 47,XXY cells could be a preferred model to study cell interactions and thus cellular and molecular mechanisms of testicular dysfunction in KS. Recently, co-culture of mixed testicular cells isolated from frozen testis tissue of an adolescent with KS was reported. The authors aimed at expanding SSCs and an increase of ZBTB16+ cells (undifferentiated spermatogonia) identified throughout the culture using digital PCR, scRNA sequencing, and cell sorting could be achieved. However, while the maintenance of all testicular cell types over the culture period was confirmed using PCR, the study did not further analyse the somatic compartment and its interactions with the undifferentiated spermatogonia (Galdon *et al.*, 2022).

With the final objective of understanding the cellular mechanisms involved in testis dysfunction, we performed for the first time a KS SCs culture achieving a cell purity of over 80% for Sox9+ and GATA4+ cells after 60 days of culture.

To be able to detect disease-specific differences, thus culture-dependent effects on SCs, we compared KS SCs to 46,XY SCs *in vitro*. We did not find statistical differences in culture purity between KS and controls. Cells could be expanded *in vitro* in both groups by reaching a 1000-fold increase in the number of cells at P10. Considering cell numbers used in experiments with testicular organoids (Baert *et al.*, 2017; Pendergraft *et al.*, 2017;

Nikmahzar et al., 2023), our culture outcomes of $2.8 \pm 1.5 \times 10^7$ KS SCs represent the first step to allow us to build a more complex *in vitro* system such as spheroids or organoids using 47,XXY SCs with other testicular cells type (either from 46,XY fertile patients or from 47,XXY samples).

We did not find differences in the expression of various proteins involved in SC function such as GDNF and BMP4, both playing an important role in spermatogenesis regulation. GDNF produced by SCs is of particular relevance as it has a critical role in the stimulation of SSC self-renewal, proliferation of progenitors, and maintenance of the undifferentiated state, ensuring germ cell homeostasis (Meng et al., 2000; Naughton et al., 2006). We did not find any difference in SCs GDNF protein and gene expression (IF and qPCR) between KS and OA men, showing that adult KS SCs, even *in vitro*, continue to produce GDNF, further confirming histological data on *in vivo* KS samples (Giudice et al., 2019). BMP4 which is responsible for inducing the differentiation of SSCs (Kee et al., 2006; West et al., 2010) was also equally expressed in both groups as shown by IF and qPCR. The absence of modifications between P5 and P10 of both factors supports that longer-term culture will likely not affect the GDNF and BMP4 gene expression needed for normal spermatogenesis.

While we mainly focused on SC function by exploring the presence of several molecules that are secreted by SCs as a sign of their function, mature SCs after puberty are also involved in the formation and function of the BTB, a dynamic structure that facilitates germ cells movements across the seminiferous tubules (Luaces et al., 2023). We know from a previous study that SCs from six cadaveric men (from 12 to 36 years old) in a 2-dimensional culture with fibronectin could organize tight junctions (Chui et al., 2011). Although we did not use fibronectin in our culture system, we observed the presence (using IF and qPCR) of CLDN11, an essential BTB tight junction protein (Gow et al., 1999). However, the protein was only expressed in the cytoplasm of <20% of SCs without any difference between the 47,XXY and control groups. The lack of an organized pattern of CLDN11 as present *in vivo* in seminiferous tubules (Giudice et al., 2019) where multiple cell types interact synchronously may be explained by our 2-dimensional culture system which is most likely insufficient to reproduce such a complex organization (Luaces et al., 2023). Indeed, the presence of main human BTB components and their organization were not explored in SCs in 2-dimensional culture experiments (Chui et al., 2011; Guo et al., 2015). In addition, in prior studies with rodent samples, only 3-dimensional systems or organotypic testicular culture allowed assessment of the formation and maintenance of tight junctions ((Legendre et al., 2010; Zhang et al., 2014; Rondanino et al., 2017) for review see (Luaces et al., 2023)). Another challenge to explore SC function in a system only aimed at SC expansion is linked to the assumption that SCs will only function properly when terminally differentiated while this would hamper their expansion *in vitro* in a system in which SCs proliferate. Furthermore, the BTB formation in SC culture may be limited by some missing factors such as androgens (Chakraborty et al., 2014) that were not added to our culture media.

We did not observe differences between KS and 46,XY AR gene expression along the culture, in agreement with the observations of Guo *et al.* when culturing 46,XY SCs (Guo et al., 2015). However, IF showed the expression to be only present in the cytoplasm of SCs which can be attributed to the absence of testosterone in the culture medium. Indeed, AR normally appears in SCs nuclei just before the onset of puberty with rising concentrations of testosterone (Gasc and Baulieu, 1986). In a previous study on testicular biopsy specimen of young KS boys, AR expression was mainly localized in the cytoplasm of SCs and only in a smaller proportion of SCs nuclei which was assumed to be related to the lower testosterone levels in these patients (Wikstrom et al., 2007). While medium supplementation proved not to be needed for SCs expansion, this should be considered in the next step when developing more complex models able to further explore SCs function.

All KS patients participating in this study presented a non-mosaic Karyotype 47,XXY performed on leukocytes from peripheral blood before recruitment. We performed SCs Karyotype in the beginning (P3) and at the end of the culture (P10). One KS patient (KLIN1) presented a mosaicism at P3. This was also reported in previous studies reporting the possibility of sex chromosomal loss *in vitro* (Hirota et al., 2017), but when we performed the second analysis at the end of the culture period (P10), all three KS SCs presented with a 47,XXY karyotype.

The small number of donor samples represents the major limitation of our study. Culture outcomes should further be confirmed on samples of KS patients of different ages, including those of pre- and peri-pubertal boys, as seminiferous tubule degeneration accelerates at the onset of puberty (Wikstrom et al., 2004; Aksglaede et al., 2006). Hence, the identification of abnormal cell pathways in the paediatric population is highly needed to find potential therapeutic targets and apply them as early as possible in the evolution of the disease.

SCs kept their main characteristics after long-term culture although this was without any interaction with other testicular cells. Longer culture times could help to further explore KS SC function although this should be considered in more complex models involving interactions with other testicular cell types, especially because of the possibility that KS SCs present a dysfunction in the presence of germ cells.

Conclusions

To our knowledge, this is the first study assessing the capacity of KS SCs to be expanded *in vitro* for 60 days and maintain their main characteristics. This ability to propagate KS SCs represents the first step to build new *in vitro* models for studying testicular cell interactions, determining deficient signalling pathways involved in KS testicular dysfunction, and identifying target molecules for the treatment of infertility in KS patients.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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Authors' roles

M.G.G. designed the study, collected the data, performed the experiments, analysed the results, and wrote the manuscript. M. K. participated in the data collection and revised the manuscript.

J.P. provided technical support for handling patient materials. A. D. supervised the karyotype analyses. C.W. was responsible for the project concept, supervision of the experimental steps, and interpretation of data, as well as critical review and approval of the manuscript.

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Conflict of interest

None declared.

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