Isolation, Culture and Characterization of Klinefelter Spermatogonial Stem Cells

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ISOLATION, CULTURE AND CHARACTERIZATION OF KLINEFELTER SPERMATOGONIAL STEM CELLS

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I confirm that Mr. Guillermo Galdon has been my Ph.D. student, and he completed his Doctoral Thesis under my direct mentorship. I authorize him to deposit his thesis book for the final defense.
Please feel free to contact me with any questions!

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Dedicado a todas las personas con la ilusión de crear una familia.

Dedicated to all the people hoping to start a family.

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1. Ethics:

All studies involving human patients and tissues have been conducted according to FDA requirements and following eIRB approved protocols.

All studies involving animal tissue have been conducted according to FDA animal welfare guidelines and following IACUC approved protocols.

However, this study as designed had limited if any contact with live animals or patients and focused on cryopreserved tissue and cells.

2. Glossary:

Ab: Antibody
ART: Advanced reproductive techniques
CN-D: Chromosomal Non-Disjunction
ECM: Extracellular Matrix
FACS: Fluorescence Activated Cell Sorting
FBS: Fetal Bovine Serum
FISH: Fluorescence in situ hybridization
hCG: Human Chorionic Gonadotropin
HRT: Hormonal Replacement therapy
HTO: Human Testicular Organoids
ICSI: Intra Citoplasmatic Sperm Injection.
IVF: In Vitro Fertilization
KS: Klinefelter Syndrome
MACS: Magnetic Activated Cell Sorting
MEM: Minimal Essential Media
micro-TESE: micro Testicular Sperm Extraction
NHP: non-human primates
NOA: non-obstructive azoospermia
PCR: Polymerase Chain Reaction
dPCR: digital PCR
qPCR: quantitative PCR
ROSI: Round Spermatid Injection
SSC: Spermatogonial Stem Cells

3. List of the articles:

This doctoral thesis is articulated in the form of a collection of published articles:

1. **Galdon, G.;** Deebel, N. A.; Zarandi, N. P.; Pettenati, M. J.; Kogan, S.; Wang, C.; Swerdloff, R. S.; Atala, A.; Lue, Y.; Sadri-Ardekani, H., In Vitro Propagation of XXY Undifferentiated Mouse Spermatogonia: Model for Fertility Preservation in Klinefelter Syndrome Patients. Int J Mol Sci 2021, 23, (1).
    - **Impact Factor:** SJR 1.176; JCR (5.542-5.924); H Index [195]; Quartile 1 in Medicine

2. **Galdon, G.;** Deebel, N. A.; Zarandi, N. P.; Teramoto, D.; Lue, Y.; Wang, C.; Swerdloff, R.; Pettenati, M. J.; Howard, S.; Kearns, W. G.; Kogan, S.; Atala, A.; Sadri-Ardekani, H In Vitro propagation of XXY Human Klinefelter Spermatogonial Stem Cells; a step towards new fertility opportunities. Front Endocrinol (Lausanne) 2022 Sep 28;13:1002279 doi: 10.3389/fendo.2022.1002279.eCollection 2022.

    - **Impact Factor:** SJR 1.375; JCR (5.364-5.828); H Index [83]; Quartile 1 in Endocrinology, Diabetes and Metabolism

3. **Galdon, G.;** Zarandi, N. P.; Zhang, S.; Cornett, O.; Pettenati, M. J.; Bishop, C.; Atala, A.; Sadri-Ardekani, H., In vitro Generation of Haploid Germ Cells from Human XXY Testicular Organoids ***(Under preparation)***

4A. Abstract Spanish version

Introducción:

El síndrome de Klinefelter (SK) es una alteración cromosómica que afecta aproximadamente a 1 de cada 500-1000 nacidos varones. Su cariotipo más frecuente es 47XXY y hasta un 10% de ellos presentan mosaicismo significativo en el estudio de sangre periférica. En la mayoría de casos, los pacientes SK permanecen asintomáticos hasta la edad adulta cuando la infertilidad masculina orienta al diagnóstico. Se ha descrito una pérdida progresiva de células germinales que se acelera durante la pubertad hasta establecer una fibrosis testicular extensa dejando a más del 90% de los pacientes azoospérmicos.

Según la literatura actual, aproximadamente 8% de los pacientes SK presentan espermatozoides funcionales en el eyaculado, dando lugar a descendencia euploide y sana mediante concepción natural o terapias de fertilidad avanzada. En los pacientes SK azoospérmicos, se han descrito focos aislados de tejido testicular con espermatogénesis preservada por lo que la biopsia testicular se ha establecido como tratamiento de elección. No obstante, la tasa de éxito para la extracción de espermatozoides en pacientes SK permanece por debajo del 50% y las probabilidades de conseguir un recién nacido sano se encuentra alrededor del 15%.

Incluso en pacientes SK sin espermatozoides viables en biopsia testicular, el análisis anatomopatológico de las muestras ha descrito la presencia de espermatogonias indiferenciadas. Las células madre espermatogoniales (SSC) son una pequeña población de espermatogonias especialmente indiferenciadas presentes en la membrana basal de los túbulos seminíferos. Las SSC tienen tanto la capacidad para la propia autorenovación así como para la diferenciación hacia la espermatogénesis, manteniendo el equilibrio de los túbulos seminíferos. A su vez, la cualidad más característica atribuida a las SSC es la capacidad para migrar hasta la membrana basal de túbulos seminíferos de sujetos azoospérmicos y reestablecer la espermatogénesis.

Se ha postulado que las SSC podrían ser utilizadas en nuevas terapias de fertilidad experimentales para pacientes SK sin espermatozoides disponibles en biopsias testiculares. Dentro de esta nueva generación de técnicas de fertilidad avanzada experimentales se incluyen: trasplante de SSC, espermatogénesis *in vitro*, maduración *ex vivo* de tejido testicular e injerto de tejido testicular. Todas ellas han mostrado resultados prometedores en modelos animales, así como en estudios preclínicos y se encuentran a diferentes niveles de desarrollo hacia la aplicabilidad clínica.

Un aspecto esencial de cualquier tratamiento de fertilidad es el valor del uso de material autólogo del paciente a fin de transmitir su material genético a su descendencia. La mayoría de las terapias experimentales basadas en SSC previamente mencionadas requieren un número significativo de células para ser viables. Algunos investigadores han logrado propagar *in vitro* células testiculares euploides de roedores y humanos manteniendo las características propias de una putativa población de SSC. No obstante, se desconoce si estas técnicas serían reproducibles en sujetos aneuploides como SK.

Hipótesis:

Nuestra hipótesis es que los actuales métodos de aislamiento y propagación *in vitro* de células testiculares podría ser adaptado a las necesidades específicas del tejido testicular de ratones y humanos con SK.

Organoides testiculares formados a partir de células testiculares SK podrían aportar conocimiento sobre la fisiopatología del SK. Los organoides testiculares podrían también suponer un avance hacia nuevas terapias de fertilidad para pacientes SK.

Objetivos:

1. Aislar, propagar y caracterizar células testiculares procedentes de ratones XXY, incluida una población de putativas SSC.
2. Aislar, propagar y caracterizar células testiculares procedentes de humanos XXY, incluida una población de putativas SSC.
3. Formar organoides testiculares en 3D a partir de células testiculares humanas XXY y evaluar su potencial como modelo de SK *in vitro;* y como herramienta para la espermatogénesis *in vitro*.

Métodos:

El método de aislamiento y cultivo de células testiculares descrito previamente para tejido humano adulto y prepuberal fue adaptado para tejido SK peripuberal de ratón y humano hasta conseguir su propagación consistente, como se describe en los artículos correspondientes. A su vez, el método de formación y diferenciación de organoides testiculares previamente descrito por nuestro grupo fue optimizado.

Algunas de las más significativas variaciones del protocolo identificadas como beneficiosas durante el proceso son:

La transición de una digestión enzimática basada en la combinación de hialuronidasa, coleganasa, tripsina y DNAsa a una combinación de colagenasa (SERVA, aprobada por la FDA para ser aplicada en humanos) y DNAsa, manteniendo buena eficacia durante el proceso.

Diferentes medios de cultivo fueron comparados hasta describir una nueva variante de StemPro Complete especialmente enriquecida en FBS a la que hemos dado el nombre de Gonomedia y que en nuestra experiencia estimula la propagación inicial de células testiculares inmaduras.

En cuanto a la formación de organoides testiculares 3D, pasamos de un sistema de cultivo *hanging drop* a placas de cultivo ultrantiadherentes y añadiendo centrifugación tras la siembra para facilitar la formación de organoides. Se estableció 10.000 células testiculares por organoide como una medida adecuada para evitar necrosis del núcleo a lo largo del cultivo manteniendo un alto número de células resultantes.

Resultados:

En el primer artículo se reportan células testiculares del modelo murino SK siendo satisfactoriamente aisladas y propagadas en cultivo hasta incrementar más de 10^9 veces su número. Estos resultados son comparables a los obtenidos en controles XY de la misma camada. La posterior caracterización de las células mediante RT-qPCR y dPCR identificó en cultivo los cuatro principales tipos celulares esperados en el tejido tescticular: espermatogonias, células de Leydig, células de Sertoli y células peritubulares. A su vez, mediante citometría de flujo se caracterizó una población de putativas SSC presente a lo largo de todo el tiempo en cultivo. Se realizó hibridación *in situ* fluorescente (FISH) de las células en cultivo observando mosaicismo presente a lo largo de cultivo e identificándose células XXY, XX y XY tanto estacionarias como en división.

En el segundo artículo se presentan los resultados obtenidos del aislamiento y cultivo de células testiculares humanas de pacientes SK peripuberales. El tejido testicular fue cedido por pacientes incluidos en el proyecto de criobanco experimental de tejido testicular de pacientes en riesgo de infertilidad masculina del Wake Forest Baptist Medical Center. El número de células experimentó un crecimiento exponencial durante 90 días en cultivo hasta alcanzar más de 10^9 veces su número original. La expresión génica de las células en cultivo mostró rasgos característicos propios de espermatogonias indiferenciadas, células de Leydig, células de Sertoli y células peritubulares. Mediante RNAseq para células individuales se pudo identificar un agregado células con un perfil de expresión génica superponible la descripción actual de SSC humana. La presencia de una putativa población de SSC en cultivo fue a su vez identificada mediante citometría de flujo a lo largo del tiempo de cultivo. Cariotipado molecular de las células en cultivo fue realizado mediante secuenciación genómica de nueva generación (NGS) describiendo una población homogénea 47 XXY con un filtro de confianza del 10%. A continuación, se caracterizaron los cromosomas sexuales de las células en cultivo mediante hibridación *in situ* fluorescente (FISH). Los resultados identificaron de nuevo mosaicismo presente a lo largo de cultivo e identificándose células XXY, XX y XY tanto estacionarias como en división. Al enriquecer la población de espermatogonias mediante marcadores magnéticos anti-CD9, la proporción de células euploides se vio incrementada. Al observar estos resultados, se realizó un análisis FISH en las secciones histológicas tejido testicular utilizado en el estudio. Los resultados demostraron la presencia de mosaicismo minoritario intratesticular a pesar de haber sido clínicamente diagnosticados como 47, XXY no mosaicos, mediante cariotipo sobre sangre periférica.

Por último, a partir de las células testiculares de pacientes SK aisladas y propagadas *in vitro* en el apartado anterior se crearon organoides testiculares para su cultivo en medio 3D. Durante 23 días en cultivo (2 días de formación y 21 días de diferenciación) los organoides permanecieron viables, sin signos de necrosis y produciendo ATP. Histológicamente los organoides presentaban un aspecto compacto con multitud de núcleos celulares repartidos tanto en la superficie como en el núcleo. No se apreciaron significativos signos de fibrosis. Para evaluar la capacidad de los organoides de reproducir in vitro la fisiología testicular se evaluaron sus dos principales funciones: la esteroidogénesis y la espermatogénesis semanalmente durante los 21 días de cultivo en media de diferenciación. En cuanto la producción de testosterona, los organoides produjeron cantidades analíticamente relevantes a lo largo de su tiempo en cultivo. No se observó una diferencia significativa en la producción de testosterona en aquellos organoides testiculares SK sometidos a estimulación de pulsos de gonadotropina coriónica humana(hCG), a diferencia de lo observado en organoides testiculares de pacientes adultos o prepuberales euploides. Para valorar la espermatogenesis sustentada *in vitro*, se evaluó la evolución de la expresión génica. Inicialmente, la expresión de marcadores spermatogenesis intermedia (SYCP3) y tardía (Acrosina y PRM1) eran indetectables. Tras 1 semana en cultivo de diferenciación la expresión de SYCP3 se positivizó mostrando un pico significativo que decreció posteriormente. En cambios, marcadores de espermatogénesis tardía como Acrosina y PRM1 aumentaron significativamente su expresión tan solo en la tercera semanas en cultivo. Mediante PCR digital se estimó que el porcentaje de células del organoide expresando Acrosina y PRM1 era del 0.2%.
Finalmente se realizó análisis de la dotación cromosómica de las células conformando los organoides mediante tinción FISH para los cromosomas X, Y y 18. No se apreciaron células haploides al inicio del estudio ni tras 1 o 2 semanas en cultivo. No obstante, tras 3 semanas en cultivo, células haploides tanto 18X como 18Y eran distinguibles en el núcleo de los organoides testiculares. Tras contabilizar estas poblaciones se pudo estimar que el 10.9% de las células era X/18 y el 4.6% Y/18.

Discusión:

Este es el primer registro del que tenemos conocimiento sobre propagación *in vitro* de células testiculares procedentes de sujetos SK. Supone un paso crucial y necesario hacia el desarrollo de nuevas terapias de fertilidad en pacientes azoospermicos, incluidos SK.

En términos cuantitativos los resultados de propagación obtenidos son suficientes para potencialmente permitir su aplicación a la práctica clínica mediante trasplante de SSC o espermatogénesis *in vitro*.

No obstante, la confirmación definitiva del éxito de este sistema de cultivo celular será la eficacia con la que células propagadas *in vitro* son potencialmente capaces de restituir espermatogénesis en túbulos seminíferos inactivos. Para ello, nuevos estudios de seguridad y eficacia clínica son necesarios hasta que suponga razonable su aplicación experimental en pacientes.

El mosaicismo intratesticular observado en pacientes SK sin signos de mosaicismo en sangre periférica confirma la sospecha de mosaicismo tejido-específico postulada en estudios previos. A su vez, podría representar una plausible explicación para los focos de actividad espermatogénica remanentes en adultos SK azoospérmicos.

Por otra parte, la observación de que las células euploides en cultivo son preferentemente espermatogonias concuerda con el hallazgo clínico documentado de gametos intratesticulares SK mayoritariamente euploides. De este modo, se afianza la importancia de desarrollar nuevas terapias de fertilidad para pacientes SK ya que pueden dar lugar a descendencia sana.

En cuanto a los organoides testiculares, el hecho de que reproduzcan adecuadamente la esteroidogenesis y espermatogénesis *in vitro* confirman su valor como herramienta de recreación de la fisiología testicular y su aplicabilidad a estudio de toxicidad o exploración farmacológica.

La falta de respuesta a la estimulación con pulsos de hCG de los organoides SK arrojaría nuevos datos sobre los mecanismos fisiopatológicos del SK y apoyaría las expectativas de su uso como modelo de enfermedad.

En cuanto a la espermatogénesis, el hallazgo de células haploides tras tres semanas de cultivo de diferenciación supone un gran avance hacia la espermatogénesis *in vitro* como terapia experimental de fertilidad. Estudios posteriores deberán evaluar el potencial de fertilización y la viabilidad de los embriones resultantes obtenidos a partir de gámetos desarrollados *in vitro*.

Conclusiones:

a) Por primera vez se ha conseguido aislar y propagar *in vitro* células testiculares de ratón XXY. Las células en cultivo mantuvieron la expresión génica característica de los cuatro principales tipos celulares testiculares: Espermatogonias, células de Sertoli, células peritubulares y células de Leydig. A su vez una población de putativas células madre espermatogónicas, potencialmente adecuada para ser trasplantada, fue identificada.

b) Por primera vez se ha conseguido aislar y propagar *in vitro* células testiculares humanas XXY de pacientes con síndrome de Klienfelter. Las células en cultivo mantuvieron la expresión génica característica de los cuatro principales tipos celulares testiculares: Espermatogonias, células de Sertoli, células peritubulares y células de Leydig. A su vez una población de putativas células madre espermatogónicas, potencialmente adecuada para ser trasplantada, fue identificada.

c) Mosaicismo de cromosomas sexuales fue identificado, caracterizado y cuantificado tanto en células cultivadas *in vitro* como en biopsias testiculares obtenidas *in vivo* de pacientes clínicamente diagnosticados como Klinefelter no-mosaico.

d) Organoides testiculares tridimensionales fueron creados con éxito a partir de células humanas XXY, recreando in vitro características de la fisiología testicular como la producción de testosterona, la producción de ATP y su expresión génica característica.

e) Tras tres semanas en cultivo, los organoides testiculares humanos con síndrome de Klinefelter experimentaron diferenciación espermatogénica resultando en la producción de células haploides.

4B. Abstract Catalan version

Introducció:

La síndrome de Klinefelter (SK) és una alteració cromosòmica que afecta aproximadament 1 de cada 500-1000 infants nascuts. El seu cariotip més freqüent és 47XXY i fins a un 10% presenten mosaïcisme significatiu a l'estudi de sang perifèrica. A la majoria de casos, els pacients SK romanen asimptomàtics fins l'edat adulta quan la infertilitat masculina orienta el diagnòstic. S'ha descrit una pèrdua progressiva de cèl·lules germinals que s'accelera durant la pubertat fins a establir una fibrosi testicular extensa que deixa més del 90% dels pacients azoospèrmics.

Segons la literatura actual, aproximadament el 8% dels pacients SK presenten espermatozous funcionals a l'ejaculat, donant lloc a descendència euploide i sana mitjançant concepció natural o teràpies de fertilitat avançada. Als pacients SK azoospèrmics, s'han descrit focus aïllats de teixit testicular amb espermatogènesi preservada i en conseqüència la biòpsia testicular s'ha establert com a tractament d'elecció. Tot i així, la taxa d'èxit per a l'extracció d'espermatozous en pacients SK roman per sota del 50% i les probabilitats d'aconseguir un nadó sa es troba al voltant del 15%.

Fins i tot en pacients SK sense espermatozous viables a la biòpsia testicular, l'anàlisi anatomopatològica de les mostres ha descrit la presència d'espermatogonies indiferenciades. Les cèl·lules mare espermatogonials (SSC) són una petita població d'espermatogonies especialment indiferenciades presents a la membrana basal dels túbuls seminífers. Les SSC tenen tant la capacitat per a la pròpia autorenovació com per a la diferenciació cap a l'espermatogènesi, mantenint l'equilibri dels túbuls seminífers. Alhora, la qualitat més característica atribuïda a les SSC és la capacitat per migrar fins a la membrana basal de túbuls seminífers de subjectes azoospèrmics i restablir l'espermatogènesi.

S'ha postulat que les SSC podrien ser utilitzades en noves teràpies de fertilitat experimentals per a pacients SK sense espermatozous disponibles a biòpsies testiculars. Dins aquesta nova generació de tècniques de fertilitat avançada experimentals s'inclouen: trasplantament de SSC, espermatogènesi *in vitro*, maduració *ex vivo* de teixit testicular i empelt de teixit testicular. Totes han mostrat resultats prometedors en models animals, així com en estudis preclínics i es troben a diferents nivells de desenvolupament cap a l'aplicabilitat clínica.

Un aspecte essencial a qualsevol tractament de fertilitat és el valor de l'ús de material autòleg del pacient per tal de transmetre el material genètic a la seva descendència. La majoria de les teràpies experimentals basades en SSC prèviament esmentades requereixen un nombre significatiu de cèl·lules per a ser viables. Alguns investigadors han aconseguit propagar *in vitro* cèl·lules testiculars euploides de rosegadors i humans mantenint les característiques pròpies d'una putativa població de SSC. No obstant això, es desconeix si aquestes tècniques serien reproduïbles en subjectes aneuploides com SK.

Hipòtesi:
La nostra hipòtesi és que els mètodes actuals d'aïllament i propagació *in vitro* de cèl·lules testiculars podrien ser adaptat a les necessitats específiques del teixit testicular de ratolins i humans amb SK.

Organoides testiculars formats a partir de cèl·lules testiculars SK podrien aportar coneixement sobre la fisiopatologia del SK. Els organoides testiculars podrien també suposar un avenç cap a noves teràpies de fertilitat per a pacients SK.

Objectius:

1. Aïllar, propagar i caracteritzar cèl·lules testiculars procedents de ratolins XXY, inclosa una població de putatives SSC.

2. Aïllar, propagar i caracteritzar cèl·lules testiculars procedents dels humans XXY, inclosa una població de putatives SSC.

3. Formar organoides testiculars en 3D a partir de cèl·lules testiculars humanes XXY i avaluar-ne el potencial com a model de SK in vitro; i com a eina per a l'espermatogènesi in vitro.

Mètodes:
El mètode d'aïllament i cultiu de cèl·lules testiculars descrit prèviament per a teixit humà adult i prepuberal va haver de ser adaptat a teixit SK peripuberal de ratolí i humà fins a aconseguir-ne la propagació consistent, com es descriu als articles corresponents. Alhora, el mètode de formació i diferenciació d'organoides testiculars prèviament descrit pel nostre grup va ser optimitzat.

Algunes de les més significatives variacions del protocol identificades com a favorables durant el procés van ser:

La transició d'una digestió enzimàtica basada en la combinació de hialuronidasa, col·leganasa, tripsina i DNAsa a una combinació de col·lagenasa (SERVA, aprovada per la FDA per ser aplicada en humans) i DNAsa, mantenint bona eficàcia durant el procés.

Diferents mitjans de cultiu van ser comparats fins a descriure una nova variant de StemPro Complete especialment enriquida amb FBS a la qual hem donat el nom de Gonomedia i que en la nostra experiència estimula la propagació inicial de cèl·lules testiculars immadures.

Pel que fa a la formació d'organoides testiculars 3D, passem d'un sistema de cultiu tipus hanging drop a plaques de cultiu ultrantiadherents i afegint-hi centrifugació després de la sembra. Es va establir 10.000 cèl·lules testiculars per organoide com una mesura adequada per evitar necrosi del nucli al llarg del cultiu mantenint un alt nombre de cèl·lules resultants.

Resultats:
Al primer article es reporten cèl·lules testiculars del model roent de SK sent satisfactòriament aïllades i propagades en cultiu fins a incrementar més de 109 vegades el seu nombre. Aquests resultats són comparables als obtinguts en controls XY de la mateixa ventrada. La posterior caracterització de les cèl·lules mitjançant RT-qPCR i dPCR va identificar en cultiu els quatre principals tipus cel·lulars esperats en el teixit tescticular: espermatogonies, cèl·lules de Leydig, cèl·lules de Sertoli i cèl·lules

peritubulars. Alhora, mitjançant citometria de flux es va caracteritzar una població de putatives SSC present al llarg de tot el temps en cultiu. Es va realitzar hibridació in situ fluorescent (FISH) de les cèl·lules en cultiu observant mosaïcisme present al llarg de cultiu i identificant-se cèl·lules XXY, XX i XY tant estacionàries com en divisió.

Al segon article es presenten els resultats obtinguts de l'aïllament i cultiu de cèl·lules testiculars humanes de pacients SK peripuberals. El teixit testicular va ser cedit per pacients inclosos al projecte de criobanc experimental de teixit testicular per a pacients en risc d'infertilitat masculina del Wake Forest Baptist Medical Center. El nombre de cèl·lules va experimentar un creixement exponencial durant 90 dies en cultiu fins a assolir més de 109 vegades el seu nombre original. L'expressió gènica de les cèl·lules en cultiu va mostrar trets característics propis d'espermatogonies indiferenciades, cèl·lules de Leydig, cèl·lules de Sertoli i cèl·lules peritubulars. Mitjançant RNAseq per a cèl·lules individuals es va poder identificar un agregat cèl·lules amb un perfil dexpressió gènica superponible la descripció actual de SSC humana. La presència d'una putativa població de SSC en cultiu va ser identificada al mateix temps mitjançant citometria de flux al llarg del temps de cultiu. Cariotip molecular de les cèl·lules en cultiu va ser realitzat mitjançant seqüenciació genòmica de nova generació (NGS) descrivint una població homogènia 47 XXY amb un filtre de confiança del 10%. A continuació, es van caracteritzar els cromosomes sexuals de les cèl·lules en cultiu mitjançant hibridació in situ fluorescent (FISH). Els resultats van identificar de nou mosaïcisme minoritari present al llarg de cultiu i identificant-se cèl·lules XXY, XX i XY tant estacionàries com en divisió. En enriquir la població d'espermatogonies mitjançant marcadors magnètics anti-CD9, la proporció de cèl·lules euploides es va veure incrementada. En observar aquests resultats, es va realitzar una anàlisi FISH a les seccions histològiques teixit testicular utilitzat a l'estudi. Els resultats van demostrar la presència de mosaïcisme intratesticular malgrat haver estat clínicament diagnosticats com a 47, XXY no mosaics, mitjançant cariotip sobre sang perifèrica.

Finalment, a partir de les cèl·lules testiculars de pacients SK aïllades i propagades *in vitro* a l'apartat anterior es van crear organoides testiculars per al seu cultiu 3D. Durant 23 dies en cultiu (2 dies de formació i 21 dies de diferenciació) els organoides van romandre viables, sense signes de necrosi i produint ATP. Histològicament els organoides presentaven un aspecte compacte amb multitud de nuclis cel·lulars repartits tant a la superfície com al nucli. No es van apreciar significatius signes de fibrosi. Per avaluar la capacitat dels organoides de reproduir in vitro la fisiologia testicular se'n van avaluar les dues funcions principals: l'esteroidogènesi i l'espermatogènesi setmanalment durant els 21 dies de cultiu en mitjana de diferenciació. Pel que fa a la producció de testosterona, els organoides van produir quantitats analíticament rellevants al llarg del seu temps en cultiu. No es va observar una diferència significativa en la producció de testosterona als organoides testiculars SK sotmesos a estimulació de polsos de gonadotropina coriònica humana (hCG), a diferència del que s'observa en organoides testiculars de pacients adults o prepuberals euploides. Per valorar l'espermatogènesi sustentada in vitro, es va avaluar l'evolució de l'expressió gènica. Inicialment, l'expressió de marcadors spermatogènesi intermèdia (SYCP3) i tardana (Acrosina i PRM1) eren indetectables.

Després d'una setmana en cultiu de diferenciació, l'expressió de SYCP3 es va positivitzar mostrant un pic significatiu que va decréixer posteriorment. En canvi, marcadors d'espermatogènesi tardana com Acrosina i PRM1 van augmentar significativament la seva expressió tan sols a la tercera setmana en cultiu. Mitjançant PCR digital es va estimar que el percentatge de cèl·lules de l'organoide expressant Acrosina i PRM1 era del 0,2%.

Finalment es va realitzar anàlisi de la dotació cromosòmica de les cèl·lules conformant els organoides mitjançant tinció FISH per als cromosomes X, Y i 18. No es van apreciar cèl·lules haploides a l'inici de l'estudi ni després d'1 o 2 setmanes en cultiu. No obstant això, després de 3 setmanes en cultiu, cèl·lules haploides tant 18X com 18Y eren visibles al nucli dels organoides testiculars. Després de comptabilitzar aquestes poblacions es va poder estimar que el 10,9% de les cèl·lules era X/18 i el 4,6% Y/18.

Discussió:

Aquest és el primer registre del que tenim coneixement sobre propagació in vitro de cèl·lules testiculars procedents de subjectes SK. Suposa un pas crucial i necessari cap al desenvolupament de noves teràpies de fertilitat en pacients azoospermics, inclosos SK.

En termes quantitatius, els resultats de propagació obtinguts són suficients per potencialment permetre'n l'aplicació a la pràctica clínica mitjançant trasplantament de SSC o espermatogènesi in vitro.

Tot i així, la confirmació definitiva de l'èxit d'aquest sistema de cultiu cel·lular serà l'eficàcia amb què cèl·lules propagades in vitro seran potencialment capaces de restituir espermatogènesi a túbuls seminífers inactius. Per això, nous estudis de seguretat i eficàcia clínica són necessaris fins que suposi raonable la seva aplicació experimental en pacients.

El mosaïcisme intratesticular observat en pacients SK sense signes de mosaïcisme en sang perifèrica confirma la sospita de mosaïcisme teixit-específic postulada a estudis previs. Alhora, podria representar una plausible explicació per als focus d'activitat espermatogènica romanents en adults SK azoospèrmics.

D'altra banda, l'observació de que les cèl·lules euploides en cultiu són preferentment espermatogonies concorda amb la troballa clínica documentada de gàmetes intratesticulars SK majoritàriament euploides. D'aquesta manera, es consolida la importància de desenvolupar noves teràpies de fertilitat per a pacients SK, ja que poden donar lloc a descendència sana.

Pel que fa als organoides testiculars, el fet que reprodueixin adequadament l'esteroidogènesi i espermatogènesi in vitro confirmen el seu valor com a eina de recreació de la fisiologia testicular i la seva aplicabilitat a l'estudi de toxicitat o exploració farmacològica.

La manca de resposta a l'estimulació amb polsos de hCG dels organoides SK donaria noves dades sobre els mecanismes fisiopatològics del SK i recolzaria les expectatives del seu ús com a model de malaltia.

Pel que fa a l'espermatogènesi, la troballa de cèl·lules haploides després de tres setmanes de cultiu de diferenciació suposa un gran avenç cap a l'espermatogènesi in vitro com a teràpia experimental de fertilitat. Estudis posteriors han d'avaluar el potencial de fertilització i la viabilitat dels embrions resultants obtinguts a partir de gàmets desenvolupats in vitro.

Conclusions:

a) Per primer cop s'ha aconseguit aïllar i propagar *in vitro* cèl·lules testiculars de ratolí XXY. Les cèl·lules en cultiu van mantenir l'expressió gènica característica dels quatre principals tipus cel·lulars testiculars: Espermatogònies, cèl·lules de Sertoli, cèl·lules peritubulars i cèl·lules de Leydig. Tanmateix una població de putatives cèl·lules mare espermatogòniques, potencialment adients per ser trasplantades, fou identificada.

b) Per primer cop s'ha aconseguit aïllar i propagar *in vitro* cèl·lules testiculars humanes XXY de pacients amb síndrome de Klinefelter. Les cèl·lules en cultiu van mantenir l'expressió gènica característica dels quatre principals tipus cel·lulars testiculars: Espermatogònies, cèl·lules de Sertoli, cèl·lules peritubulars i cèl·lules de Leydig. Tanmateix una població de putatives cèl·lules mare espermatogòniques, potencialment adients per ser trasplantades, fou identificada.

c) Mosaicisme de cromosomes sexuals vas ser identificat, caracteritzat y quantificat tant en cèl·lules cultivades *in vitro* com a biòpsies testiculars obtingudes *in vivo* de pacients clínicament diagnosticats com Klinefelter no-mosaic.

d) Organoides testiculars tridimensionals varen ser creats amb èxit a partir de cèl·lules humanas XXY, recreant *in vitro* característiques de la fisiologia testicular como la producció de testosterona, la producció de ATP i la seva expressió gènica característica.

e) Després de tres setmanes en cultiu, els organoides testiculars humans amb síndrome de Klinefelter van experimentar diferenciació espermatogènica resultant en la producció de cèl·lules haploides.

5. Introduction:

Klinefelter Syndrome (KS) is a chromosomal disease first described in 1948 as a group of men presenting with gynecomastia, testicular atrophy, hypogonadism, and infertility (1). The most common genotype is 47 XXY, but many other less common variants have been described (mosaicism, 47 iXqY, 48XXXY, 48 XXYY) (1–3).
It is estimated that KS affects approximately 1 out of every 500-1000 males born (4–8). In most cases, KS remains asymptomatic and undiagnosed until adulthood, when male infertility affecting more than 90% of KS patients leads to the diagnosis (1,4). Therefore KS is considered the most common genetic cause of male infertility and represents up to 10% of all men with non-obstructive azoospermia (9–11).

Meiosis is a key element of sexual reproduction in which through two consecutive cell divisions four haploid cells with recombined genetic material are produced from a single diploid cell. During the first cell division (Meiosis I) homologous chromosomes are segregated into different cells while in the second cell division (Meiosis II) sister chromatids are divided(12).

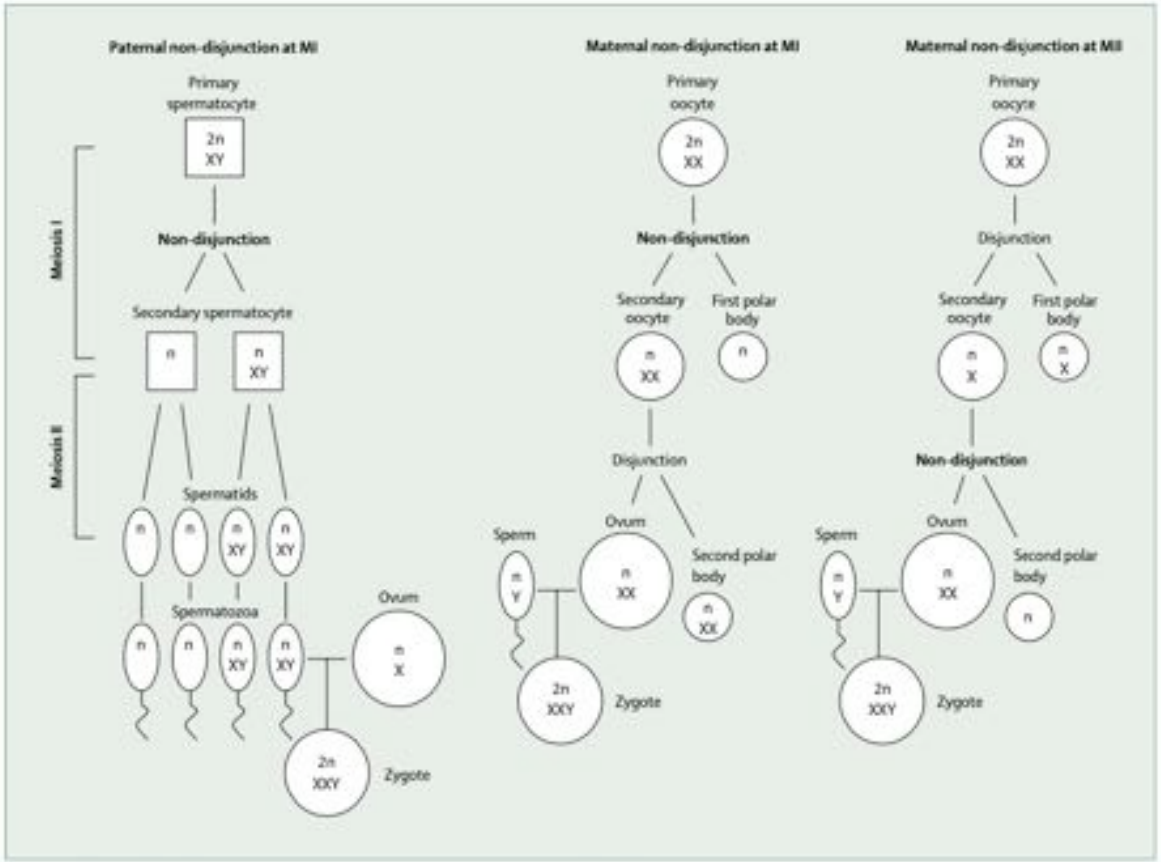


Figure 1: Different forms of non-disjunction leading to the 47,XXY karyotype of Klinefelter's syndrome. *Incorporated from Lanfranco et al Lancet 2004; 364: 273–83*

The most common cause of KS is chromosomal non-disjunction (CN-D) during meiosis producing disomic gametes. In contrast to other chromosomal diseases, KS can originate from either maternal or paternal germlines in a similar proportion (1,13,14) (Figure 1). Maternal CN-D may occur during either meiosis I or II but paternal CN-D can only occur in meiosis I phase as CN-D during paternal meiosis II would result in either 47XXX or 47XYY zygotes (13,15). Some studies have linked increaded risk of KS to advanced maternal age (1,4,13).

Differences during cell division in the early stages of fetal development may lead to mosaicism consisting of cell populations with different chromosomal dotation coexisting in the same organism. Around 10-20% of KS patients present some degree of mosaicism in peripheral blood cells analysis (1,13). However, even in patients clinically diagnosed as non-mosaic 47XXY, tissue-specific hidden mosaicism has been described (16–18).

At a hormonal level, KS patients usually present with hypergonadotropic hypogonadism starting at puberty. High luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels are frequently associated with low or lower limits of normality testosterone (1,3,19). High sex-hormone-binding globulin (SHBG) levels decrease the bioavailable active free testosterone and further exacerbate hypogonadism status (1,3,19). Inhibin B and Anti-Müllerian Hormone (AMH) remain within normal limits in prepubertal boys but decrease during puberty proportionally to the loss of functional seminiferous tubules (20,21). Similarly, GnRH appears to be within normal limits in prepubertal boys but rises after puberty in response to the lack of negative feedback (22). Recent studies have refuted previous beliefs that estradiol was significantly increased in KS patients (23) in favor of a relative hyperestrogenism with a high estradiol/testosterone ratio (1,23).

Previous clinical practices used to initiate hormonal replacement therapy (HRT) with testosterone upon KS diagnosis assuming clinically significant hypogonadism. Current literature suggest that only some patients may benefit from HRT, but the optimal time to start treatment remains unclear (24–31). If KS diagnose comes early, close follow up while transitioning into puberty is recommended to evaluate the adequate indication of HRT in a case-to-case basis (30). Randomized placebo-controlled clinical trials are still needed to clarify the real efficacy of HRT in KS patients. Before starting HRT, patients should be informed about its possible deleterious impact on male fertility in order to better assess the risk-benefit balance (24,26,27,32).

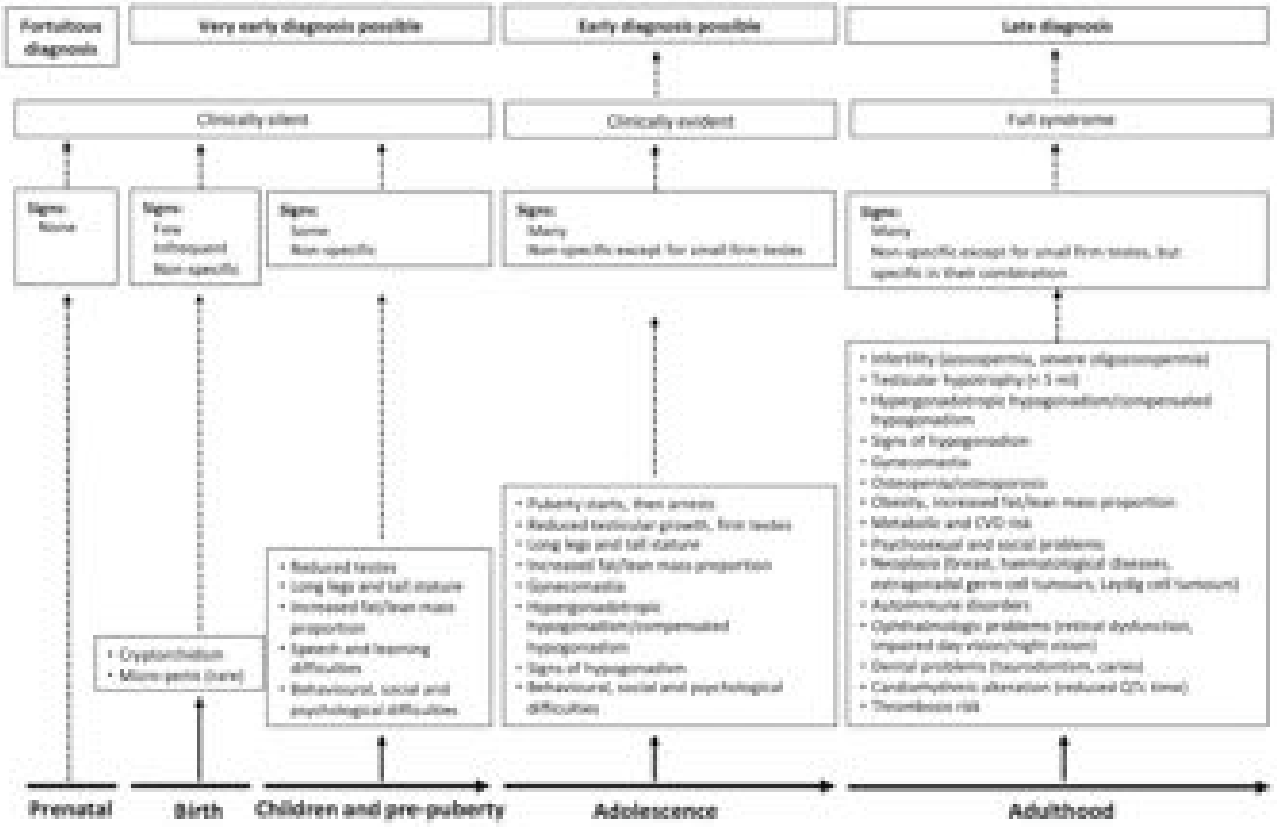
The KS classical phenotype included high stature, feminized fat distribution, hypogonadism, gynecomastia, testicular atrophy, and learning difficulties (14,24). Some of the other signs and symptoms described in KS are cryptorchidia, micropenis, diabete, metabolic syndrome, osteopenia and increased thrombotic risk. (14,24) (Table 1)

Feature	Frequency (%)
Infertility (adults) (8, 57)	91–99[b]
Small testes (bi-testicular size <6 ml) (8)	>95
Increased gonadotropin levels (57)	>95
Azoospermia (adults) (57)	>95
Learning disabilities (children) (74)	>75
Decreased testosterone levels (57)	63–85
Decreased facial hair (adults) (57)	60–80
Decreased pubic hair (adults) (57)	30–60
Gynecomastia (adolescents, adults) (8, 33, 74)	38–75
Delay of speech development (children) (74)	40
Increased height (prepubertal, adults) (74, 123)	30
Abdominal adiposity (adults) (36)	~50
Metabolic syndrome (adults) (36)	46
Osteopenia (adults) (51, 124)	5–40
Type 2 diabetes (adults) (19, 36)	10–39
Cryptorchidism (8, 74)	27–37
Decreased penile size (children) (74)	10–25
Psychiatric disturbances (children) (74)	25
Congenital malformations, cleft palate, inguinal hernia (125)	~18
Osteoporosis (adults) (124)	10
Mitral valve prolapse (adults) (126, 127)	0–55
Breast cancer (adults) (16, 128)	Increased risk (~50 fold)
Mediastinal cancers (children) (22)	increased risk (~500 fold)
Fractures (17, 18)	Increased risk (2–40 fold)

Table 1: Abnormalities associated with KS and their tentative frequencies. *Incorporated from Groth et al J Clin Endocrinol Metab, January 2013, 98(1):20 –30* →

However, only a small portion of patients present as such and most KS patients remain completely asymptomatic until adulthood being infertility the sign most frequently leading to the diagnosis (3,27) (Figure 2).

Figure 2: Signs and symptoms of Klinefelter syndrome (KS) at various stages in life. It is indicated that these symptoms may be seen in some or many patients with KS, depending on their age. *Incorporated from Zitzman et al Andrology. 2021;9:145–167.*



Mosaicism, paternal origin of the extra numeracy chromosome, differences in skewed X chromosome genes expression, genes escaping inactivation, and androgen receptor polymorphism may account for the high variability in KS clinical features (3,33–37).

A high incidence of diabetes, metabolic syndrome, and obesity has been described in KS patients most likely due to multifactorial etiology including hypogonadism, aneuploidy, and androgen or INS-like receptor polymorphism (24,38,39). Only discrete changes in the metabolic profile of KS patients were observed after HRT was started, supporting its multifactorial nature (38).
The previously described endocrinological profile may account for some of KS clinical features. Low circulating testosterone combined with the underlying chromosomal abnormality and INS-like receptor 3 levels may contribute to KS elevated height, osteoporosis, reduced muscle mass, and eunuchoid physical habit (19,24,40–42). Clinically significant hypogonadism usually presents in KS patients as decreased libido and erectile dysfunction from an early age (19) that improves after HRT (43). It may also explain the higher incidence of undescended testis compared to the general population (9,44). Androgen deficiency is also linked to elevated thromboembolic events in KS patients (25,45).
The overall combination of endocrinological misregulation slightly impacts their overall survival and life expectancy of KS patients (46,47). No clear connection between KS and cancer has been established, although currently available data is insufficient to rule out its association (37,47,48).

Testicular tissue has a complex histologic architecture with many cell types distributed between two differentiated compartments separated by the basal membrane: the seminiferous tubules where spermatogenesis takes place and the testicular interstitium where neurovascular structures and androgen producing cells are located (49) (Figure 3)

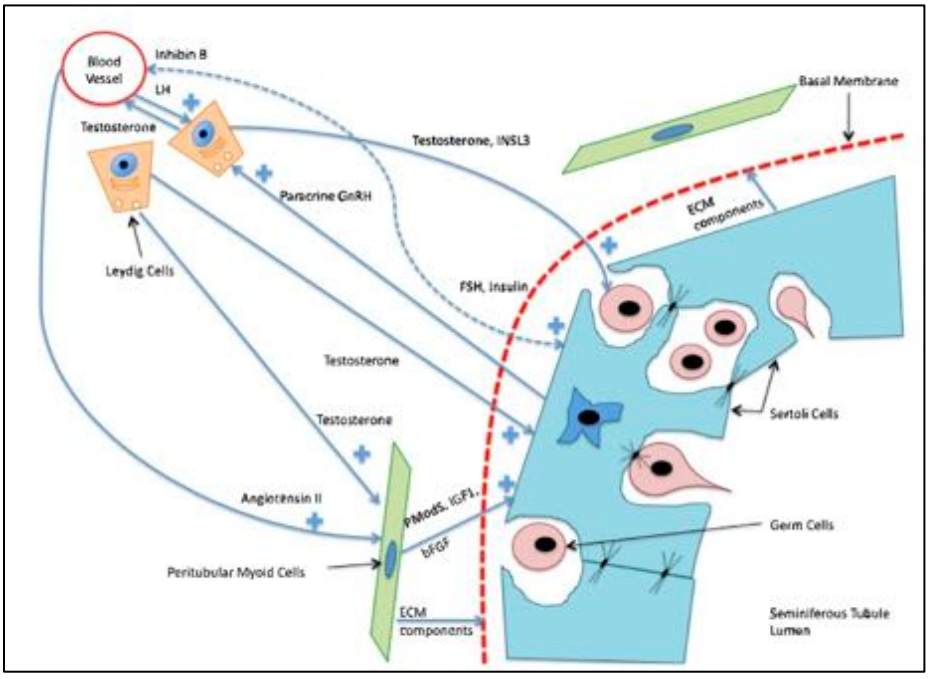


Figure 3: Hormonal and Paracrine control of Spermatogenesis. *Incorporated from Neto et al Seminars in Cell & Developmental Biology 59 (2016) 10–26*

The seminiferous tubules are formed by a tightly sealed layer of Sertoli cells resting on the inner side of the basal membrane (Figure 3). Sertoli cells provide adequate immunologic protection, metabolic environment and paracrine regulation to support spermatogonial lineage thrive (49).
During testicular development, male primordial germ cells migrate from tubules lumen and settle in close contact to Sertoli cell on the basal membrane where they are conditioned to become male immature spermatogonia (49,50).
At the onset of puberty, under the influence of testosterone, retinoic acid, and other co-factors, some immature spermatogonias commit to spermatogenesis and go through a gradual differentiation process to become mature spermatozoa as they advance into the seminiferous tubules' lumen(49–51) (Figure 3 and Figure 4). This process remains active in a wave fashion of 42-75 days per cycle approximately (49). Cell-to-cell interactions are crucial to support and maintain functional spermatogenesis.

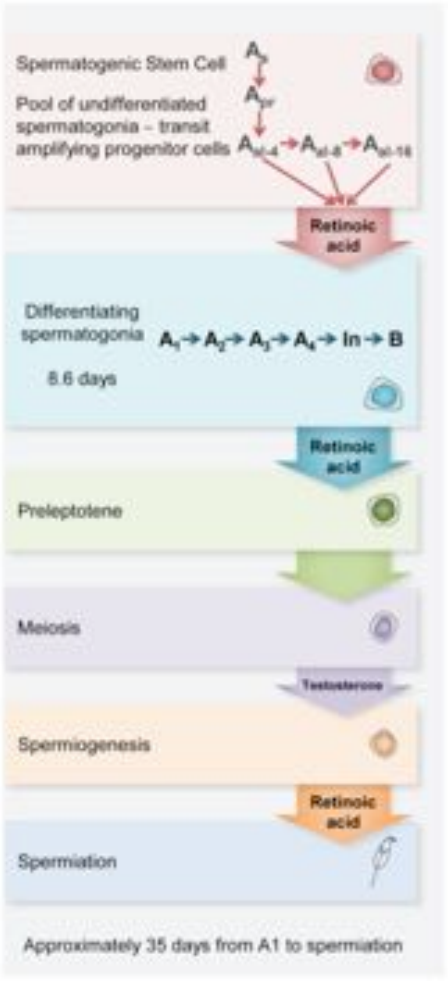


Figure 4: Overview of germ cells differentiation in mice. *Incorporated from Griswold Physiol Rev 96: 1–17, 2016* →

Spermatogonial stem cells (SSC) are a small subpopulation of undifferentiated spermatogonia located in the basal membrane both able to self-renewal and differentiate into spermatogonia lineage, keeping balanced the seminiferous tubules niche (52–54). Moreover, SSCs present the unique capability to migrate to the basal membrane of inactive seminiferous tubules and reestablish spermatogenesis on azoospermia subjects (55).

Many research groups have focused their efforts on the study of SSC population however a single definitive SSC-specific marker has not been identified yet. A combination of undifferentiated spermatogonia markers has proved to enrich SSC population and improve SSC transplantation efficacy (52,56–65). Recent studies using novel single cell RNAseq data analysis (66) have been able to further characterize testicular gene expression (67,68) including SSC population (69–71) even in azoospermia patients (72–74). Therefore SSC enshrine great therapeutic potential for male infertility even in azoospermic patients with no available sperm.

In the testicular interstitium, immediately outside the tubules, layers of peritubular myoid cells continuously repair and maintain the basal membrane and secrete co-factors required for spermatogenesis (Figure 3). Peritubular cells also present contractile functions similar to

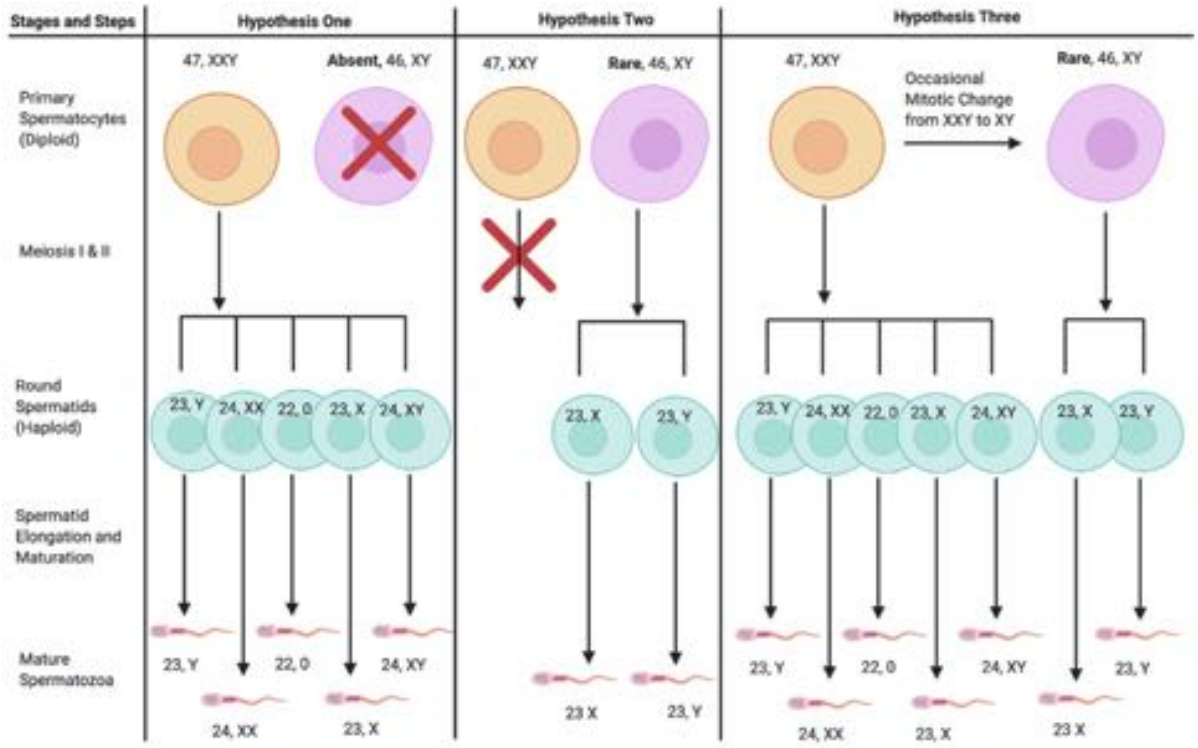
smooth muscles that contribute to propel spermatozoa through the seminiferous tubules (49,50,75). In the testicular interstitium, Leydig cells aggregated in clusters present an intense metabolic activity dedicated to steroid production in response to hormonal and non-hormonal stimulation (49,50) (Figure 3).

KS patients present a progressive loss of spermatogonia (75–80) which has been described to accelerate at the onset of puberty (19,34,80–86). Adult patients usually develop extensive testicular fibrosis and impaired fertility characterized as Sertoli cell-only morphology in most seminiferous tubules (44,77,80,86,87). However, the specific physiopathology mechanism of this process remains unclear. Some reports suggested X chromosome genes overexpression, testicular microenvironment misbalance, or altered meiosis in aneuploid cells as likely causes for germ cell depletion (18,79,82,83,85,88,89). Leydig cell hyperplasia, although not always present, is another common histologic finding in KS testes (24,34,44,77,81).

Animal models mimicking the clinical features observed in KS humans have been established to better understand its pathophysiology (90–99). Lue et al developed an XXY mouse model with testicular fibrosis, germ cell depletion, osteopenia and learning difficulties (90–92,99). Wistuba et al used their XXY mouse model to further describe the pathophysiologic mechanism behind germ cell loss (96,97), Sertoli alteration (95) and Leydig misfunction (94) Hirota *et al* used their XXY mouse model to study the mosaicism dynamics *in vivo* and *in vitro* (98). Joerg *et al* developed a Klinefelter bull reproducing the germ cell loss observed in humans (93).

Current literature estimates that up to 8% of non-mosaic KS patients may have functional sperm in their semen (1,26,100,101). In those cases; healthy euploid offspring has been reported by either natural conception (101–104) or advanced reproductive techniques (ART) (105–109), suggesting that cells that complete meiosis may develop into euploid sperm (18,110,111). The mechanism behind KS euploid gametes production remains unknown but different theories have been postulated (Figure 5) (88).

Figure 5: Competing Spermatogenesis Hypotheses in Klinefelter Syndrome.
(1.) All spermatogenesis derives from 47, XXY Spermatocytes, which results in multiple chromosomal options in produced sperm. No 46, XY spermatocytes are present.
(2.) All successful spermatogenesis derives from rare 46, XY spermatocytes, which may be present even in non-mosaic Klinefelter patients resulting in sperm with only normal 23, X, and 23, Y genotypes.
(3.) Both 47, XXY spermatocytes and rare 46, XY spermatocytes are responsible for undergoing spermatogenesis with an occasional mitotic change between 47, XXY, and 46, XY at the diploid spermatocyte developmental level. *Adapted from our research group work: Deebel NA, Bradshaw AW, Sadri-Ardekani H. Infertility considerations in klinefelter syndrome: From origin to management. Best Pract Res Clin Endocrinol Metab. 2020 Dec;34(6):101480.* (88)



More than 90% of KS adults do not present spermatozoa on their semen, making KS the most common genetic cause of non-obstructive azoospermia (NOA) (4). Even azoospermic KS patients may still have foci of preserved intratesticular spermatogenesis (18,80,86,111). Nowadays, the gold standard of care to treat male infertility in KS patients with azoospermia is TESE (Testicular Sperm Extraction) or micro-TESE (microscopic assisted Testicular Sperm Extraction) combined with advanced reproductive techniques (ART) like ICSI (Intra Cytoplasmic Sperm Injection) in order to achieve pregancy (88). However, the success rate of testicular sperm retrieval remains lower than 50% (1,24,86,112–116). It needs to be combined with subsequent *in vitro* Fertilization (IVF) with a pregnancy rate around 40% for a combined Live Birth Rate of about 15% (24,114,117). It remains unclear if there is a difference between using freshly retrieved sperm compared to frozen-thawed sperm in KS ART success (114,118,119). Recent studies evaluating histologic architecture and tissue integrity showed no significant difference between fresh and thawed testicular tissue (120)

There is a high variability on microTESE/TESE sperm retrieval rates as testicular histology varies from patient to patient (16,77,114), even in monozygotic twins (121). Many studies have analyzed variables like testosterone, estradiol, LH, FSH, Prolactin, Inhibin B levels and testicular volume trying to anticipate sperm retrieval results in KS patients. No factor other than testicular histology has consistently predicted microTESE/TESE success (32,112–115,119,122–130). The latest reports have shown promising results centered around the origin of the extra X Chromosome (131) and AMH levels at the time of the biopsy (132) . The optimal age for sperm retrieval remains controversial (114,118,128,133,134). The chances of

finding sperm in prepubertal KS patients is remote. Some experts advocate for early TESE in pubertal or peripubertal young adults to minimize testicular fibrosis while others prefer adult TESE to add the benefit of avoiding sperm cryopreservation.
Active testosterone treatment could also negatively impact spermatogenesis in KS, although available data is still insufficient (27,44,114). Some authors recommend discontinuing testosterone treatment six months before TESE or microTESE to optimize sperm retrieval rates (1,19).

Intratesticular sperm retrieved from KS patients is euploid in more than 90% of cases (135). However, a higher percentage of aneuploid sperm than healthy controls and euploid azoospermic patients has been reported (89,117,135–137). Consequently, if available, preimplantation genetic diagnosis and genetic counseling are highly recommended (117,135).

KS patients, KS parents and medical providers treating underaged KS boys in risk to develop male infertility have shown significant interest in fertility preservation treatments (138–140). Even in cases where no sperm is found on testicular biopsy, histology reports may show un-differentiated spermatogonia and putative SSC present in the seminiferous tubule's basal membrane(84,86,141). The presence of this population might be affected by age and HRT (84,88).

Unfortunately, most KS patients remain undiagnosed or the diagnose is delayed until adulthood when infertility is already stablished (4,24). Current efforts from clinical community are concentrating on early diagnose and follow up in order to improve clinical management and quality of life in KS patients (26,43,142). Since the introduction of prenatal genetic diagnostic techniques (Amniocentesis, Chorion Villus Sample, Cell-Free-Fetal DNA determination, Quantitative Fluorescence PCR, Multiplex Ligation Dependent Probe Amplification, Preimplantation Genetic Screening, Methylation specific PCR) it is now possible to follow KS patients since the time of birth. Consequently, there is a new window for early medical interventions in KS patients (27,143–146).

As previously mentioned, the current standard of care for patients with NOA relies on TESE followed but ICSI or ROSI using retrieved sperm. However, there is a call for new fertility therapies for patients with no available sperm. That might be the case for prepubertal and peripubertal patients pending to receive gonadotoxic treatment, presenting bilateral cryptorchidia, or KS. Experimental SSC-based fertility treatments under development include SSC transplantation, *in vitro* spermatogenesis, testicular tissue *ex vivo* culture, or testicular tissue grafting (88,147–149). It's been hypothesized that early testicular biopsy and cryopreservation (29,88,140,141,150–155) (Figure 6) could provide a source of autologous SSC required to consider future SSC-based fertility treatments. Therefore some reference fertility-centers are currently offering testicular tissue cryopreservation to their patients. Several studies have optimized testicular tissue cryopreservation methods and process in order to protect and maintain viability of SSC for future ART applications (156–165).

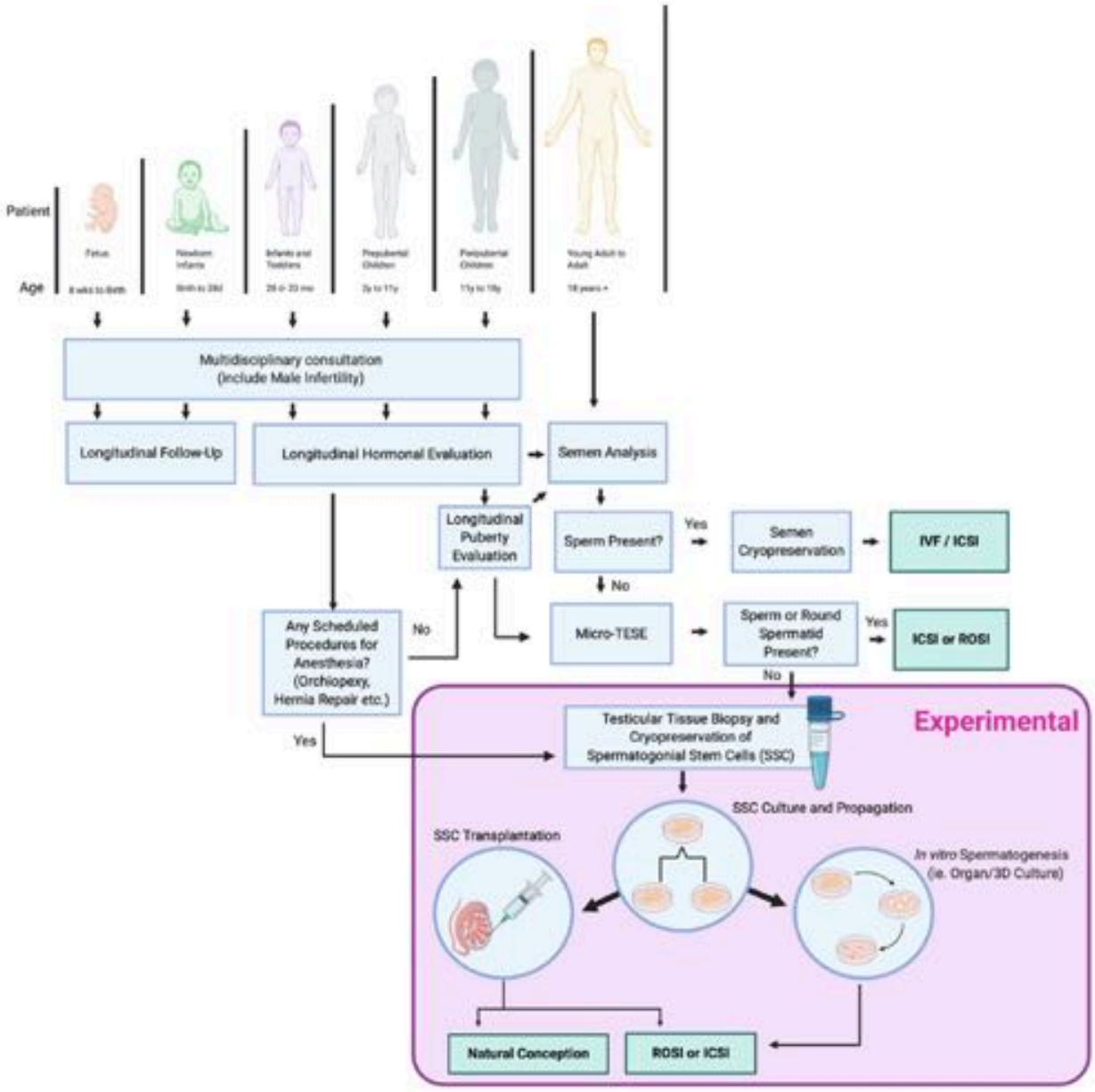


Figure 6. Management of Klinefelter Syndrome for Fertility Preservation. Following Klinefelter syndrome diagnosis, all ages from fetal through peripubertal children are recommended to undergo multidisciplinary consultation, including male infertility specialists. Newborns will be followed longitudinally, as will infants and children with attention paid to regular hormonal evaluations up to, and especially during, puberty. In children, if at any point a surgical procedure requiring anesthesia is scheduled, the authors recommend undergoing experimental testicular tissue biopsy and cryopreservation of SSC at that time. In adults desiring fertility, if sperm is present on semen analysis, this may be preserved and used for IVF or ICSI. If no sperm are found, mTESE is the preferred next step. If sperm or round spermatid is found on mTESE, then it may be preserved for ICSI or ROSI. If no sperm are found, then experimental testicular tissue biopsy and cryopreservation of SSC is suggested. In adults and children who underwent experimental SSC cryopreservation, SSCs can be cultured and

propagated, with the goal of SSC auto-transplantation leading to potentially increasing the number of 46XY SSCs to support in vivo spermatogenesis and subsequent natural conception or ROSI/ICSI. An additional experimental possibility following SSC propagation is in vitro spermatogenesis, which is possible in 3D Organoid culture and which may allow for ROSI or ICSI conception. Abbreviations: SSC, Spermatogonia Stem Cells; TESE, Testicular Sperm Extraction; ROSI, Round Spermatid Injection; ICSI, Intracytoplasmic Sperm Injection. *Adapted from our research group work: Deebel NA, Bradshaw AW, Sadri-Ardekani H. Infertility considerations in Klinefelter syndrome: From origin to management. Best Pract Res Clin Endocrinol Metab. 2020 Dec;34(6):101480.(88)*

SSC transplantation consists of testicular cells suspension infusion into the rete testis so they retrogradely reach the semniferous tubules, colonize the basal membrane and restore spermatogenesis. SSC transplantation has already been successfully used to restore male fertility in animals for more than 25 years (55), and since its methods have been refined (166,167). The technique has been translated into several animal models (93,168–172) including non-human primates (NHP) (173–175) (Figure 7) and xenotransplantation of human testicular cells (176–178). The system is effective in azoospermia subjects after busulfan ablation (55,168), radiation exposure (174,175), or congenital infertility models (92,93,179). In the 41 XXY mouse KS model, seminiferous tubules were successfully repopulated by Lue *et al* using SSC transplantation from 40XY mouse after a fully established testicular fibrosis (92). Unfortunately, spermatogenesis restoration wasn't achieved after SSC transplantation into the Klinefelter bull model (93). SSC transplantation has shown efficiency in restoring spermatogenesis even in non-ablated animals Clonidine 11 KO mice in meiotic arrest (180). Proof of concept has been provided for the autologous SSC transplantation (174,175,179). In a recent blinded preclinical study carried out in mice undergoing SSC transplantation no significant differences in overall health were found in two consecutive offspring generations compared to wild-type controls (181). Successful SSC transplantation has been achieved using cryopreserved testicular tissue (182) even after 14 (162) and 20 years in cryostorage (183). The application of this particular fertility restoration method would represent an important advance as it would potentially enable natural conception of children without the need to go through IVF.

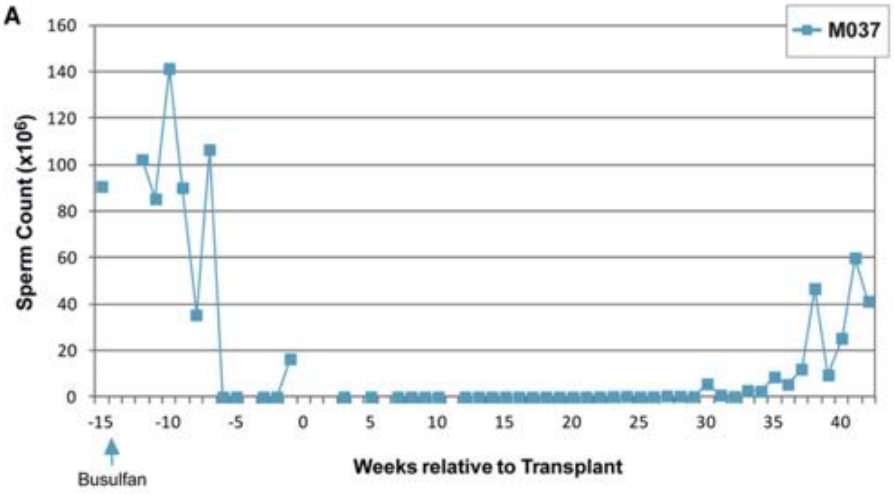


Figure 7: Sperm was detected in the ejaculate from a busulfan ablated NHP 35 weeks after autologous SSC transplantation. *Adapted from Hermann et al Cell Stem Cell 11, 715–726, November 2, 2012*

In vitro spermatogenesis consists of *in vitro* culture systems that recreate testicular physiologic conditions in order to achieve cell differentiation into male gametas. It has been explored as means to produce differentiated haploid germ cells suitable for ART (184). This method might be preferred when concerns are raised regarding reintroducing undifferentiated cells back into the patient, especially in cancer survivors (185–187). Literature on animal models show promising reports of *in vitro* spermatogenesis in 3D culture systems from both testicular cells (188–191) and somatic stem cells (98,192–194).
Significantly, Hirota *et al.* used a 3D culture system to complete *in vitro* spermatogenesis from XXY mouse induced Pluripotent Stem Cells (iPSC), achieving the conception of healthy offspring (98).
In humans, few groups have reported successful haploid germ cells produced *in vitro* from testicular cells culture (195–197). Moreover, human testicular organoids have been able to recreate key features of testicular physiology like testosterone production (196). It has been proposed as a toxicity model for drug screening alone (196) and included in a multiorganoid body-in-chip platform (198–200). 3D testicular organoids are also being explored as an infectious disease pathogenesis model (201).

By testicular tissue culture and *ex vivo* differentiation we refer to the culture of whole tissue pieces, without previous cell isolation, trying maintain the original histologic architecture. This method has shown some success in animal models (202–208). However, recent attempts to translate this system into human tissue are still in the early stages of development (209,210).

Testicular tissue grafting consists on surgically implanting testicular tissue from a donor on to a living host so the graft can support spermatogenesis under the hormonal control from the host. We talk about autologous graft when the tissue is implanted on to the donor, allogenic graft when the tissue is implanted on a host of the same species as the donor and xenograft when the tissue is implanted on a host from a different species. We can also differentiate between orthotopic graft when the testicular tissue is implanted inside the host's scrotum and ectopic graft when it is implanted anywhere else.

Testicular tissue grafting has shown promising results in the *ex vivo* maturation (211,212) enabling conception and healthy offspring in wild-type NHP (Figure 8). In humans, it has been suggested that testicular tissue grafting could play a role in KS disease modeling (213). Nevertheless, no germ cell differentiation was reported after four weeks. The scarcity of germ cells in SK patients may compromise the viability of this experimental fertility option.

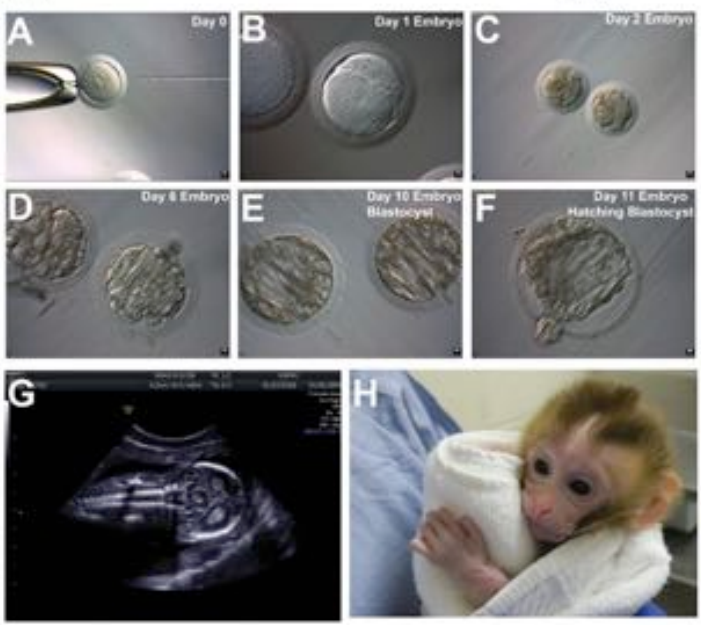


Figure 8: Intracitoplasm Injection of rhesus spermatids retrieved from testicular graft and resultant embryo development until birth. *Incorporated from Fayomi et al Science. 2019 March 22; 363(6433): 1314–1319*

All these different methods are not mutually exclusive, and a holistic approach will help decide what specific strategy or combination best suits a particular patient. Novel approaches in the field combine the previously described methods to optimize differentiation and spermatogenesis (214).

A crucial aspect of any fertility treatment is the value of using autologous material from the patient to pass down its genetic information through his offspring. Most of the previously mentioned experimental fertility therapies require a high number of cells to become feasible. However, testicular tissue from patients at risk of male infertility is usually scarce, and the number of viable cells is reduced.
Few research groups have been able to propagate testicular cells *in vitro* while preserving a putative SSC population from rodents (64,215–221) and humans (64,177,222–231) (Figure 9).

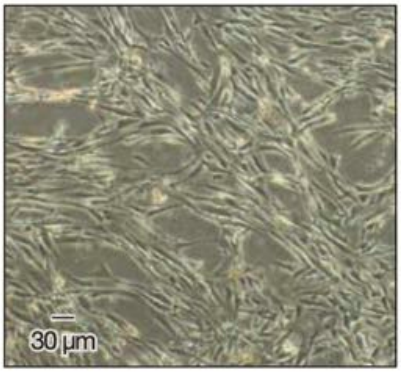


Figure 9: Human testicular cells, including a population of putative SSC, in culture. *Incorporated from Sadri-Ardekani et al JAMA, November 18, 2009—Vol 302, No. 19*

However, whether these systems would be reproducible in KS patients' testicular cells remains unknown.

Abstract:

Klinefelter syndrome is a chromosomal disease characterized by the presence of a masculine phenotype and at least one supernumerary X chromosome (47 XXY most commonly). Frequency has been estimated to be 1/500-1000 born males. The most prominent clinical feature is testicular fibrosis starting at the onset of puberty, leaving more than 95% of them azoospermics[1].

Despite widespread testicular fibrosis, adult Klinefelter patients may still present small focuses of full spermatogenesis or at least some viable surviving spermatogonia[19]. Current fertility therapies available for azoospermic patients include Testicular Sperm Extraction (TESE) combined with *in vitro* fertilization (IVF), although the probability of finding fully differentiated viable sperm is very limited[3].

To improve fertility treatments in azoospermic patients, including Klinefelter, it's been hypothesized that spermatogonia-based methods could be used when sperm-based therapies aren't available.[16,18] Many of these methods including Spermatogonial Stem Cell (SSC) transaplantation[14,15] and *in vitro* Spermatogenesis[17] have shown great progress in the later years, although remain experimental.

However, these procedures require a significant number of testicular cells and, unfortunately, the viable testicular tissue present in a Klinefelter testicular biopsy is very limited. In order to make autologous procedures feasible, it is critically important to increase the number of spermatogonia *in vitro*. There have been reports of *in vitro* propagation of testicular cells in different species including mouse[9,10] and human[6,7], but it remains unknown if these results are reproducible in Klinefelter patients.

This study aims to isolate, propagate and characterize testicular cells, including SSC, from Klinefelter mouse model and Klinefelter human patients. To achieve that goal, current culture systems will have to be adapted. Klinefelter mouse model developed by Dr. Lue et al will be used in the pilot study. Then the optimized method will be applied to human Klinefelter testicular tissue donated by patients enrolled in Wake Forest Baptist testicular bank.

If Klinefelter testicular cells are successfully isolated and propagated in culture a new generation of autologous fertility treatments could be pursued.

Hypothesis

I hypothesize that current methods of isolation and *in vitro* propagation of testicular cells could be optimized to fit the specific needs of KS testicular tissue from both mice and humans.

Testicular organoids formed from KS testicular cells could provide a better understanding of KS physiology. KS testicular organoids could also support spermatogenesis *in vitro* and bring us closer to new therapies for infertile KS patients.

Objectives:

1. Isolate, Propagate and Characterize mouse XXY testicular cells, including Spermatogonial Stem Cells population
2. Isolate, Propagate and Characterize human XXY testicular cells, including Spermatogonial Stem Cells population
3. Form 3D Testicular Organoids from human XXY testicular cells and evaluate its potential as Klinefelter Syndrome in *vitro* disease model and *in vitro* spermatogenesis tool

Materials and methods:

Article 1: " ***In Vitro*** **Propagation of XXY Undifferentiated Mouse Spermatogonia: Model for Fertility Preservation in Klinefelter Syndrome Patients**" addressing the objective: *Isolate, Propagate and Characterize mouse XXY testicular cells, including Spermatogonial Stem Cells population*

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Article

In Vitro Propagation of XXY Undifferentiated Mouse Spermatogonia: Model for Fertility Preservation in Klinefelter Syndrome Patients

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Abstract: Klinefelter syndrome (KS) is characterized by a masculine phenotype, supernumerary sex chromosomes (usually XXY), and spermatogonial stem cell (SSC) loss in their early life. Affecting 1 out of every 650 males born, KS is the most common genetic cause of male infertility, and new fertility preservation strategies are critically important for these patients. In this study, testes from 41, XXY prepubertal (3-day-old) mice were frozen-thawed. Isolated testicular cells were cultured and characterized by qPCR, digital PCR, and flow cytometry analyses. We demonstrated that SSCs survived and were able to be propagated with testicular somatic cells in culture for up to 120 days. DNA fluorescent in situ hybridization (FISH) showed the presence of XXY spermatogonia at the beginning of the culture and a variety of propagated XY, XX, and XXY spermatogonia at the end of the culture. These data provide the first evidence that an extra sex chromosome was lost during innate SSC culture, a crucial finding in treating KS patients for preserving and propagating SSCs for future sperm production, either in vitro or in vivo. This in vitro propagation system can be translated to clinical fertility preservation for KS patients.

Keywords: spermatogonia; spermatogonia stem cells; Klinefelter syndrome; male infertility; fertility preservation

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1. Introduction

Klinefelter syndrome is a chromosomal alteration presenting with a masculine phenotype, with one Y chromosome and at least two X chromosomes (47 XXY being the most common genotype) [1]. It affects around 1 out of 650 newborn males and, despite remaining widely underdiagnosed, represents the leading genetic cause for male infertility [2,3]. The most common pathologic finding in Klinefelter adult patients is small testis due to seminiferous tubule fibrosis [4]. However, little is known about the preceding pathophysiologic process except that germ cell loss starts in the fetus [5–8], accelerates around the onset of puberty [9], and progresses to azoospermia in most adult patients [10,11].

Several authors have studied this process of progressive germ cell loss [9,10,12], resulting often in hyalinized or Sertoli cell only seminiferous tubules. However, it remains unclear if the germinal loss is due to an intrinsic dysfunction of the germ cells [8], a dysfunction of supporting Sertoli cells [13], or defective spermatogonial stem cell (SSC) renewal [14]. Studies analyzing these germ cells entering meiosis have described a meiotic arrest either

at spermatogonia [9] or early spermatocytes [15]. In addition to germ cell loss, Leydig cell hyperplasia is characteristic in KS patients [4,9].

Testosterone production and testicular growth are often within the normal range during early puberty, whereas LH, FSH, and estradiol concentrations are increased. Serum testosterone concentration decreases when mid puberty is reached and remains low or in a lower than normal range in adult KS patients [16]

Despite extensive testicular fibrosis and germ cell depletion in the testis, it has been shown that adult KS patients may have focal areas of conserved spermatogenesis in the testis [1,4,9,10,17]. Moreover, few KS patients may still have some spermatozoa present in the ejaculate [1,18] that could be potentially used for in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [19–21].

Current therapeutic options for adult KS patients presenting with non-obstructive azoospermia in the ejaculate include surgical testicular sperm extraction (TESE), with or without utilization of a surgical microscope, followed by ICSI [14,22,23]. According to recent meta-analyses, the success rate of sperm retrieval using TESE in KS patients is up to 47% regardless of the age of the patient at the time of surgery and should be decided on a patient to patient basis [24,25]. Moreover, the subsequent pregnancy rate using harvested sperm is 43% per ICSI cycle [24,26].

There have also been reports of spermatogonia in the testes of patients without intratesticular spermatazoa on micro-TESE [27,28]. These findings suggest that SSC technology could be an alternative option in the future.

It has been hypothesized that novel cell therapies may allow the use of autologous cryopreserved testicular tissue to treat Klinefelter patients' infertility [29–32]. However, a significant challenge to prove this concept is the lack of acceptance for performing testicular biopsy before puberty when there are still a significantly higher number of undifferentiated spermatogonia in Klinefelter testes as shown based on either H&E or IHC staining [9,12,33,34]. The effectiveness of these therapies might also be influenced by testosterone replacement treatment, making the timing of the testicular biopsy a vital issue for discussion amongst the patient, their family, and their clinician [33]. More consistent data is needed to establish a better standard of care for KS patients [27,28].

Several Klinefelter animal models have been introduced in the past decade to better understand the disease [35–43]. Our group established a colony of the most advanced 41 XXY mouse models sharing many clinical characteristics with human 47XXY [35–37]. Previous studies found that gonocyte numbers were similar in XY and XXY mice at postnatal day 1, followed by an over 60% decrease in the number of gonocytes in the XXY mice on day 3 and further progressive loss of spermatogonia by days 5 and 7. Only a few spermatogonia remained in the XXY mice testis on day 10, mimicking the findings in pubertal boys [36].

One of the main limitations of potential SSC-based fertility treatments [44,45] in KS patients is the scarcity of SSC in small testicular biopsies. To make autologous therapies feasible [44,45], it is critically essential to propagate these cells in culture and increase their number successfully. SSCs' in vitro propagation has been developed in different species in the past two decades, including wild-type mice [46–49]. We also reported the first SSC in vitro propagation system using adult [50] and prepubertal [51] cryopreserved human testis tissue. This study aimed to culture testicular cells from frozen prepubertal XY (as a control) and XXY mice testes to maintain and increase the population of putative SSCs. This technique may overcome the germ cell loss observed at puberty [36].

2. Results

2.1. Histology and Immunohistochemistry (IHC) Comparison of XY and XXY Neonatal Mouse Testes

Histologic analysis of testes from both XY and XXY mice was performed to assess the effect of the extra X chromosome on 3-day-old animals. Hematoxylin and Eosin (H&E) staining (Figure 1A,B) showed XXY mouse testis had a tubular structure that was primarily

conserved. Although, the number of undifferentiated spermatogonia appeared to be lower in the XXY mouse compared to that in XY control mice (Figure 1C,D). Interstitial testicular tissue was more prominent in XXY mouse testes than in XY control mice, with the presence of characteristic Leydig cell hyperplasia as previously described [37].

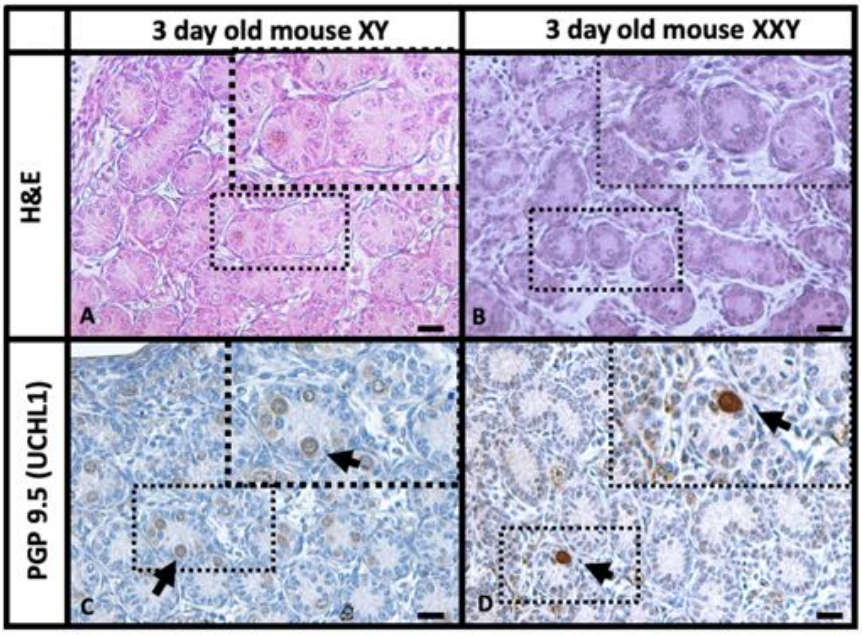

Figure 1. Histology images from a paraffin section of the whole testis tissue with H&E and IHC for PGP9.5 (UCHL1) as an undifferentiated spermatogonia marker. Hematoxylin and Eosin (H&E) staining of 3-day-old XY (**A**) and XXY (**B**) mice testes. UCHL1 staining of 3-day-old XY (**C**) and XXY (**D**) mice testes. Arrows point to cells positive for UCHL1 (DAB, brown color) inside the seminiferous tubules, most likely gonocytes. Scale Bar 20 μm.

The immunohistochemistry for PGP 9.5 (UCHL1) results (Figure 1) were especially useful as this marker is expressed in undifferentiated spermatogonia from seminiferous tubules and Leydig cells in the testicular interstitium tissue [52] but not in other somatic cells within the testis. The staining in 3-day-old XY mouse testis showed bright positive signals on intraluminal gonocytes and undifferentiated spermatogonia migrating to the basal membrane as well as a dimmer signal on interstitial cells. When the same staining was performed on 3-day-old XXY mouse testis, very few positive cells were observed inside the seminiferous tubules or migrating to the basal membrane. Nevertheless, interstitial cells showed prominent signals suggesting the presence of Leydig cell hyperplasia and validating the staining. These findings are consistent with previous characterizations of the XXY mice [35–37].

2.2. Isolation and Culture of XXY Neonatal Mouse Testicular Cells

The testicular cell isolation process was performed based on both mechanical and enzymatic dissociation of testicular tissue as described by Shinohara's group [46–49] and our previous works [50,51]. Supplemented StemPro-34 is a widely used media for testicular cell culture and has already shown promising results in both mice [46–49] and men [50,51]. However, for this experiment, a different enriched version of StemPro media under the name of "Gonomedia" (details in the methods section) was used. Our group proposes that Gonomedia better supports neonatal mice testicular cell expansion.

The frozen testes came from the Klinefelter XXY mice maintained at The Lundquist Institute and Harbor-UCLA Medical Center kept frozen at −80 °C and sent to Wake Forest Institute for Regenerative Medicine (WFIRM) [35–37]. The same isolation and culture method was applied to both the 3-day-old XXY mouse and its XY littermate mice in parallel. For each isolation, 5 frozen testes were pooled for a total weight of 1 mg. After mechanical and chemical tissue digestion, 300,000–350,000 cells were retrieved and seeded in Gonomedia.

In our isolation and culture system of testicular cells [50,51], both somatic and germ cells coexist, supporting and nurturing the expansion of each other. The former population presents an elongated morphology and usually attaches to the culture dish early, creating intercellular connections and forming patches. The latter population presents a small round form and usually attaches later, using the somatic cell patches as a physiologic feeder layer (Figure 2).

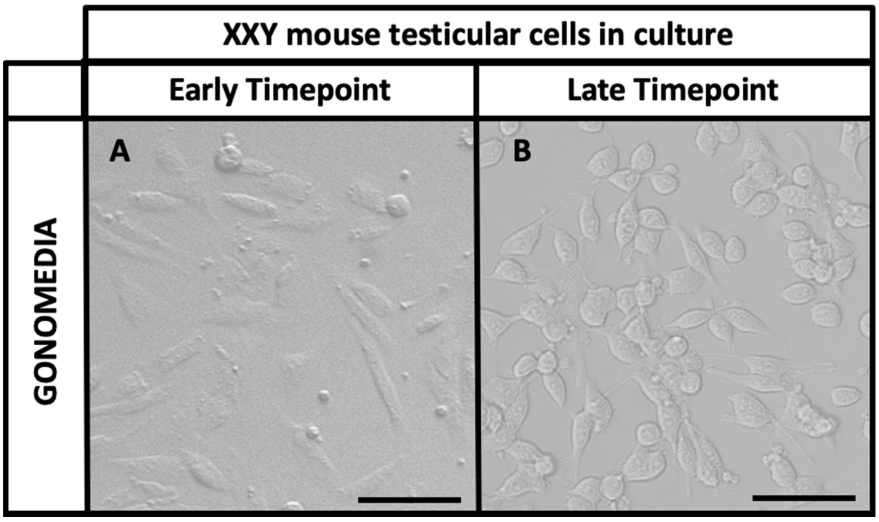

Figure 2. Optic microscopy images of XXY mouse testicular cells in culture: (**A**) Cells re-seeded in Gonomedia after five days in culture (1st passage, Early timepoint). (**B**) Cells re-seeded in Gonomedia after 89 days in culture (10th passage, Late timepoint). Scale Bar 20 µm.

In our laboratory, testicular cells from XXY mice remained viable and stable in culture with a discrete propagation rate, less than a 10-fold increase, during the first 60 days. However, after 65–70 days in culture, cells started to expand exponentially at least until day 110, when the study goal was considered met and the study was terminated. At this point, cells in culture had increased over one million fold its number (Figure 3). To our best knowledge, this is the first report of cells from XXY testes being successfully propagate in long-term cultures, overcoming the previously known period of apoptosis in the XXY mouse model. Testicular cells from XY mice testes, as the control, grew in similar pattern in parallel (Figure 3).

2.3. Gene Expression Analyses of Cultured Cells

Different spermatogonia markers were used to assess the character and differentiation of the cells in culture. This was crucial because only undifferentiated spermatogonia cells containing an SSC subpopulation can repopulate seminiferous tubules after transplantation.

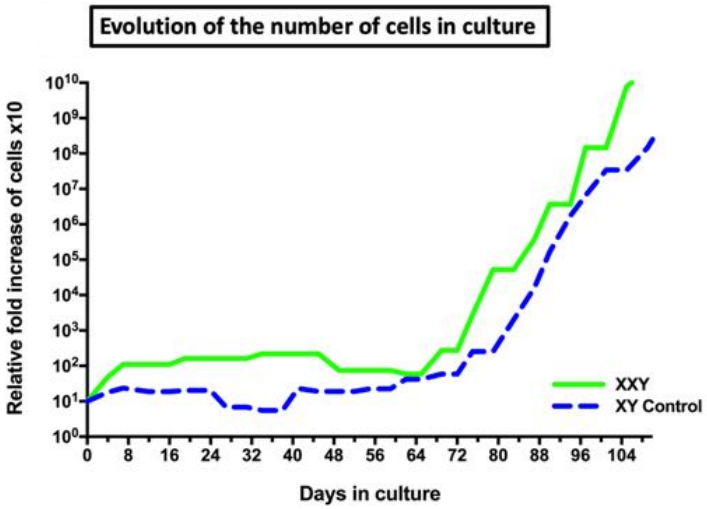

Figure 3. Evolution of the number of cells in culture from XY and XXY mice testes. Both conditions include data from two replicas of cell isolation from 5 testes each (n = 10) seeded in Gonomedia to improve the transition from gonocyte to spermatogonia and potentiate cell propagation.

Characterization of cultured XXY cells confirmed positive expression for markers of all four major cell types expected in the immature mouse testis: undifferentiated spermatogonia (THY1, CD9, ITGA6, ITGB1), Sertoli (GATA4, SOX9, CYP19A1), Leydig (CYP11A1), and peritubular (a heterogeneous population with multiple cell types including peritubular myoid cells and telocytes [53]) (CD34, ACTA2) cells (Figure 4A).

To detect differences in gene expression from XXY cells in culture, qPCR was performed at an early timepoint (Passage 1, 7 days in culture) and late timepoint (Passage 14, 110 days in culture) in the same culture media. Results show a significant increase in the increment in gene expression for markers of non-differentiated spermatogonia (ZBTB16), Sertoli cells (SOX9), and peritubular cells (CD34), while no significant difference was found in the Leydig cell (STAR) marker (Figure 4B).

Undifferentiated spermatogonia markers PGP9.5 (UCHL1) and ZBTB16 were highly expressed in cultured cells from both XXY and XY testes at several checkpoints along its time in culture. When expression levels between XXY and XY cells were compared, PGP9.5 (UCHL1) expression was higher in XXY than XY cells while ZBTB16 expression levels were comparable (Figure 4C). The expression of the differentiating spermatogonia marker STRA8 was undetectable in both cultured cells from XY (data not shown) and XXY (Figure 4A) testes, indicating that cells in culture conserve a non-differentiated status potentially suitable for transplantation or use for in vitro differentiation.

In addition to quantitative gene expression analysis, digital PCR was used to assess the portion of all cells expressing the ZBTB16 undifferentiated spermatogonia marker, compared to a POLR2A housekeeping gene. After seven days in culture, the results showed that 6.4% of cells expressed ZBTB16. Then, after 110 days in culture, digital PCR analysis was repeated, showing 29.07% of ZBTB16-positive cells (Figure 5).

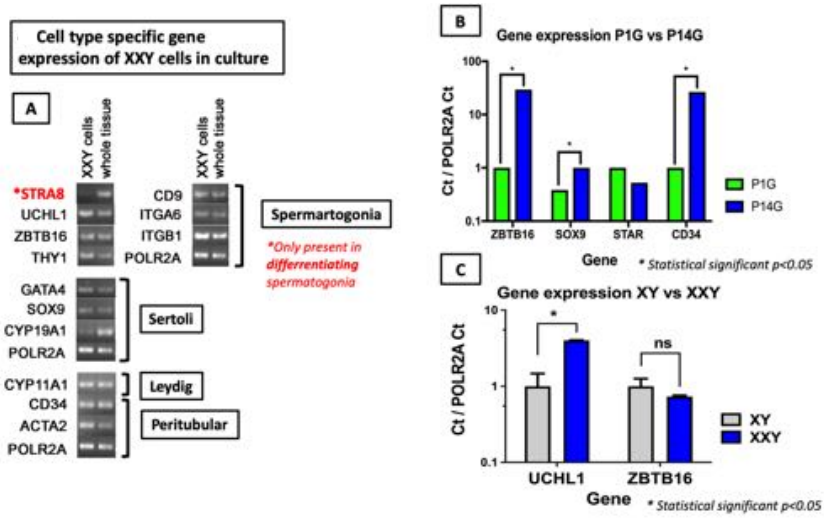

Figure 4. Gene expression analysis of cultured cells from XXY testes. (**A**) qPCR analysis of XXY cells after 31 days in culture showed significant expression of markers from all four major testicular cell types: spermatogonia, Sertoli, Leydig, and peritubular cells. (**B**) Comparative gene expression between XXY testicular cells from early (Passage 1, 7 days in culture) and late time points (Passage 14, 110 days in culture) in Gonomedia using paired t student comparison via Prism9 software; (**C**) Comparative gene expression between XXY and XY testicular cells that were long-termed cultured (Passage 14, 110 days) using a t student comparison via Prism9 software; Results expressed as a Delta Delta Ct value normalized with the housekeeping gene POLR2A. Statistically significant $p < 0.05$ shown as *.

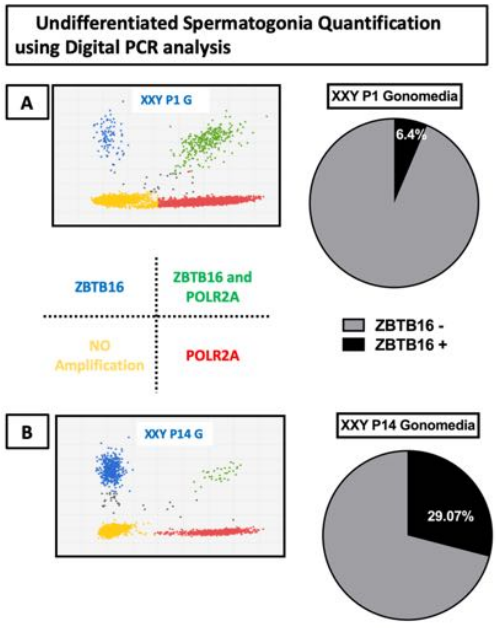

Figure 5. Digital PCR analysis of gene expression for the undifferentiated spermatogonia marker ZBTB 16 (FAM) and housekeeping gene POLR2A (VIC): (**A**) Gene expression from XXY mouse testicular cells after seven days in culture (1st passage) in Gonomedia; (**B**) XXY mouse testicular cells after 110 days in culture (14th passage) in Gonomedia.

2.4. Flow Cytometry Analyses of Cultured Cells from XXY Testes

When enough cells were propagated in culture, flow cytometry was used to identify the characteristic protein expression of the enriched cells that are SSC. To date, there is no single marker to identify the SSC population. On the other hand, the combination of MHC I-/CD9+/CD49f+ is well described in the literature as an enriched SSC population and a good predictor for SSC [54–59] transplantation success. The same analysis was repeated at several time points during our culture process (Figure 6).

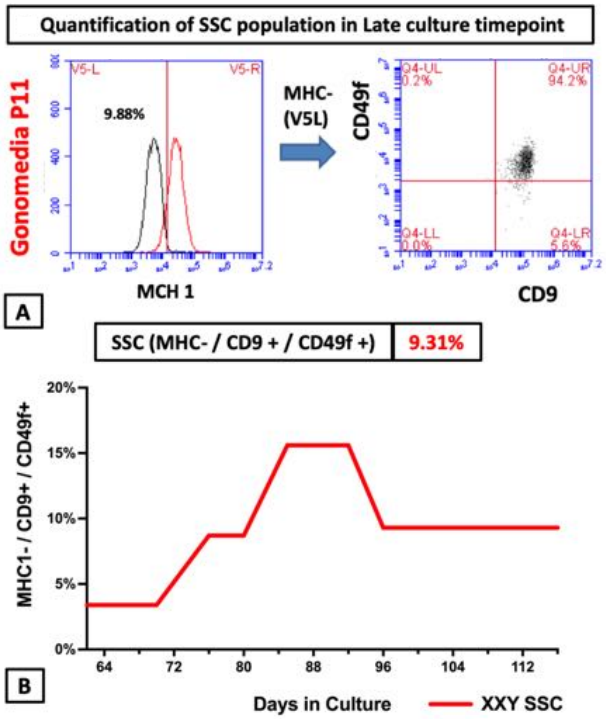

Figure 6. (**A**) Flow cytometry analysis to identify putative spermatogonial stem cells (MHC I- CD9+ CD49f+) XXY mouse testicular cells after 96 days in culture (11th passage) in Gonomedia; (**B**) Evolution of the percentage of putative SSC (MHC I- CD9+ CD49f+) along with culture.

A putative SSC population was identified in all conditions at every checkpoint in the study. The percentage of cells expressing SSC potential markers remained between 3.3 and 18.8% (Figure 6). These values were comparable to analysis of XY mouse controls testicular cells (data not shown). These findings are promising and suggest that in vitro propagated cells using the described culture system could play a role in SSC transplantation.

2.5. DNA FISH Analysis for X and Y Chromosomes

FISH analysis was performed on the samples to characterize X and Y chromosomes. Cells were cytospinned into slides for chromosome hybridization with fluorescent probes showing specific staining for X (orange) and Y (green) chromosomes. Our system correctly identified both X and Y chromosomes in an XY control mouse blood smear and XXY mouse specimen's fibroblasts. Then, the same experiment was reproduced with cultured testicular cells from the XXY mouse testes (Figure 7).

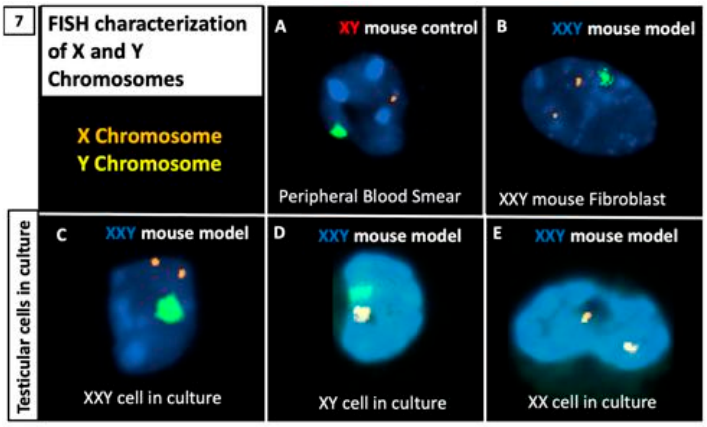

Figure 7. Characterization of Klinefelter (XXY) mouse and isolated testicular cells using FISH: (**A**) FISH staining on peripheral blood smear from control, XY mouse; (**B**) FISH staining on skin fibroblast from the Klinefelter model XXY mouse before sacrifice; (**C**–**E**) FISH staining on isolated and propagated testicular cells from the Klinefelter model (XXY) mouse testes; variations of XXY, XY and XX cells were identified in KS testicular cells in culture.

Systematic analysis of the X and Y chromosomes from cells in culture at different time points was carried out to identify diverse cell populations and quantify possible mosaicism. From each cyto-spun slide, five representative areas were imagined, and every cell was classified depending on its number of X and Y chromosomes. Both stationary and dividing cells were included. The results showed that mosaicism was present at the initiation of the culture. Initially, the most prevalent population was XXY cells accounting for 60% of the cells in culture, followed by XY cells representing 35%, and the final 5% was a mix of XX, XYY, and XXXY cells. For the first eight passages, the percentages of both XXY and XY remained most common although slowly decreased while XX and XXXY populations became more common. After passage 10, the XX population became the most common, representing at least 42% of the cells in culture while XXY and XY cells followed with 28% and 5%, respectively. From then on, the XX population continued to increase up to 80% of the cells in culture at passage 15 (Figure 8A).

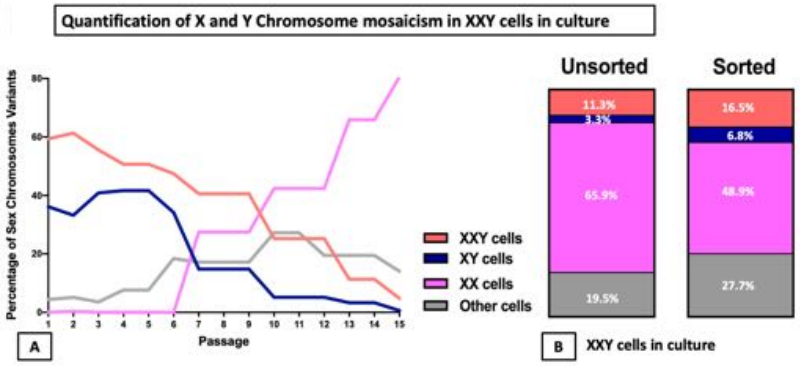

Figure 8. To quantify the presence of X and Y chromosomes in the testicular cell culture from the XXY mice, FISH staining and systematic counting were performed in consecutive passages. (**A**) Evolution of the different mosaic populations in culture. (**B**) Comparison of mosaicism of the cells 96 days in culture (12th passage) between unsorted and FACS sorted for putative SSC (MHC I- CD9+ CD49f+).

In a subsequent study, we assessed if germ or somatic cells preferentially presented a particular karyotype. Parallel FISH analysis was performed in unsorted and FACS-sorted for MHC-/CD9+/CD49f+, as previously described as an enriched SSC population. The results showed a more significant proportion of XXY cells in sorted cells than unsorted (16% vs. 11%) as well as XY cells (6.7% vs. 3.2%), while XX cells were more frequent in the unsorted samples than the sorted (65% vs. 48%) (Figure 8B).

3. Discussion

Previous studies have thoroughly characterized the 41 XXY mice as a model for Klinefelter syndrome and showed that the number of spermatogonia significantly decreased in XXY mice by day three after birth [35–37]. Histological analysis carried out in this study showed alteration of the testis architecture with empty seminiferous tubules and prominent interstitial PGP9.5 (UCHL1) positive cells. These findings support that XXY mice are a reliable model for Klinefelter patients presenting early germ cell loss and Leydig cell hyperplasia as is seen in the human KS patient.

The 3-day-old XXY mice were expected to show some degree of germ cell loss without reaching complete depletion. At this age, the mice would mimic a scenario where peripubertal or adult KS patients with testicular fibrosis but not complete germ cell depletion may undergo testicular biopsy [27,32–34,60]. With the same clinical setting in mind, a decision was made to used frozen mice testes as this would be translatable to pre-pubertal Klinefelter patients that undergo testicular biopsy and cryopreserve testicular tissue for fertility treatments later in their life [28–31].

The culture system used in this study was optimized for the neonatal XY mouse testes before attempting to apply it on isolated cells from the XXY mice testes. This step was necessary because of the well-known challenges with growing 3-day-old C57Bl6 mice testicular cells in vitro [49]. One possible explanation is that after birth, the germ cell population of mice is transitioning from quiescent gonocytes [61] into mitotic spermatogonia [48], which may represent a delay for in vitro propagation. Two different culture surfaces were tested: regular plastic culture plates and laminin-coated culture plates. The rationale to test laminin-coated wells as a culture surface follows some reports that suggested supporting cell attachment and proliferation [46]. A decision was made not to use a commercially available cell-based feeder layer that other authors have reported to improve neonatal mouse SSC attachment [48,62]. The explanation is that these biological products are not approved for clinical application, making the culture system not translatable to clinical settings. We also tested two different media: supplemented StemPro, which has been previously shown to support mouse SSC propagation in vitro [46], as well as our formulation called Gonomedia. This is a variant of supplemented StemPro enriched with 20% FBS, Follistatin, PDGF, and FSH (see the method section for more details). Based on the present literature [63,64], our group hypothesized that Gonomedia could help push quiescent neonatal gonocytes into spermatogonia and favor cell propagation. In our hands, the laminin-coated culture surface did not improve cell attachment nor cell propagation.

In our experience, a higher cell seeding concentration potentiates cell attachment but limits the propagation yield of cells in culture. For this study, seeding concentrations from 3500 to 25,000 cells/cm^2 were tested. In our hands, only Gonomedia combined with the plastic surface and up to 250,000 cells/cm^2 seeding concentration allowed cells to propagate initially while conserving the gene expression and surface marker characteristics of SSC during the first 55–70 days in culture. Nevertheless, once cells started propagating consistently, we reduced the seeding concentration to as low as 3000 cells/cm^2 to optimize the yield of cell propagation with no negative impact on cell behavior.

The cells usually took between 55 and 70 days to start growing steadily, with subsequent exponential propagation of both XY and XXY cells. The initial quiescent stage suggested by these findings is similar to what was previously reported by Kanatsu-Shinohara et al. on neonatal mouse testicular cell culture, although our novel formulated Gonomedia was not used previously.

Another promising finding of this study was the presence of all significant testicular cell types (Spermatogonia, Sertoli, Leydig, and peritubular cells) within the culture. Maintaining the viability of all different cell types is paramount to recreating the in vivo physiology of the testes. However, more studies could be done to assess specific up- and downregulated gene expression pathways of every cell type to identify new treatment targets. Moreover, when we compared the ZBTB16 expression level in cultured testicular cells from XXY and XY mice, both presented a similar expression level while PGP9.5 (UCHL1) was significantly higher in XXY cultured cells (Figure 4C). ZBTB16 is highly specific for undifferentiated spermatogonia while PGP9.5 (UCHL1) is expressed in both spermatogonia and Leydig cells [52]. Therefore, the difference in the UCHL1 expression level in XXY culture is most likely related to Leydig cells' hyperplasia in KS testes. Although a final answer is not provided at this point in our study, these results suggest that some of the differences between in vitro cultured testicular cells from XY and XXY mice are relevant to in vivo environments. Therefore, it can be considered as a tool for future physiologic studies.

Consecutive DNA FISH analysis in cultured cells from KS mouse testes demonstrated sexual chromosome mosaicism from the onset of the culture. The populations of cells in culture varied significantly over time. At first, most cells presented with XXY, although a significant population of XY was present with a scarce population of XX. Later in the culture process, both XXY and XY populations experienced a substantial decline mirrored by a significant increment of the XX population. This suggests instability in the Y and extra X chromosomes in this KS mouse model. A similar phenomenon has been described as trisomy-biased chromosome loss (TCL) in induced pluripotent stem cells (iPSCs) derived from KS mice fibroblasts [38].

In previous studies characterizing XXY mice, karyotype was performed on cultured fibroblasts. However, in this study, we analyzed hundreds of cultured testicular cells using FISH at each timepoint of the culture, detecting the populations that may have remained concealed in the past studies. Another critical point is long-term culture may alter the chromosome stability of cells. However, previous reports have looked deeply into this matter without finding definitive reasons for concern [65,66]. Additionally, the presence of few mosaic testes between the XXY mice pooled before cell isolation may contribute to the mixture population of XY and XXY cells at the beginning of cell culture. Although the percentage of the XY population in propagated KS testicular cells decreased during the culture (Figure 8), the cumulative number of XY cells increased dramatically from around 120,000 (40% of 300,000 cells) to at least 9,000,000,000 (3% of 3×10^{12} cells) (Figures 3 and 8). The finding of XX cells in late passage but not before culture demonstrated that the trisomy-biased chromosome loss (TCL) occurred in XXY mouse testicular cell culture [38]. Importantly, these data indicated that tissue- and organ-specific mosaicism may cause a variety of phenotypes in XXY aneuploidy [67].

We have shown previously that testes from adult XXY mice were successfully repopulated after SSC transplantation from XY littermates [36]. In the current study, testicular cells from XXY mice were isolated and propagated in culture and, using flow cytometry, a putative SSC population (MHC I-/CD9+/CD49f+) was identified. Therefore, we believe that a population of SSC may remain viable after initial germ cell loss. This needs to be proven by transplantation or in vitro differentiation of cultured XXY testicular cells to haploid germ cells in the future.

Taking these findings into account, along with previous reports of preserved foci of spermatogenesis in adult KS [1,4,9,10,17], the data suggest that adult human Klinefelter testes could potentially sustain spermatogenesis using SSC technology. A critical step to bring this therapy to the clinic setting would be to expand cultured human KS SSCs to the point that either autologous SSC transplantation or in vitro differentiation could be possible. We believe this study is the first step in that direction. Moreover, as previously mentioned, the specific conditions for this study were selected to support an efficient technique in a clinical setting. Therefore, we hope these data will help to establish an SSC culture system for KS patients.

4. Materials and Methods

4.1. Animals

In total, 41 XXY mice (n = 5) and 40XY littermates (n = 5) with a C57Bl6 genetic background were generated at the Lundquist Institute lab as described previously [35–37]. Immediately after birth, XXY puppies were identified using DAPI staining on fibroblast spread. The 3-day-old mice were sacrificed, and orchiectomy was performed under a sterile technique. Each testis was cut in half and cryopreserved in 1.5 mL cryovials (VWR, PA, USA) using cryopreservation solution: MEM (Invitrogen, MA, USA), 20% Fetal Bovine Serum (FBS, ThermoFisher Scientific, MA, USA), and 8% dimethyl sulfoxide (DMSO, Mylan, WV, USA). An isopropyl alcohol-based constant slow freezing device (Mr. Frosty, Nalgene, Sigma-Aldrich, MO, USA) was used to freeze the samples −1 °C/min up to −80 °C overnight [50,51,54,66]. The next day, cryovials were transferred into liquid nitrogen tanks for long-term storage. No experiments with living animals were performed. The creation of 3-day-old XXY mice for testicular cell culture was done at THE LUNDQUIST INSTITUTE FOR BIOMEDICAL INNOVATION AT HARBOR-UCLA MEDICAL CENTER under LUNDQUIST INSTITUTE IACUC approval #32014-02 (Ref#052444), last renewal 2/18/2021 through 2/17/2022.

4.2. Histology, Immunohistochemistry, and Viability Assay

Freshly recovered testes from XY and XXY mice were fixed in Bouins for 2 h and then transferred to 70% ethanol. The tissue was washed three times for 5 min in 70% ethanol before processing for paraffin embedding. Samples were then paraffinized using an automated system (Leica ASP300S, Buffalo Grove, IL, USA) following increasing concentrations of isopropyl alcohol followed by xylene and then paraffin on a three-hour program. Paraffinized tissue was then embedded into blocks. Using a Leica RM 2255 manual microtome, 5 µm thick sections were obtained and placed on histology glass slides. Slides were then placed in a 60 °C oven for 30 min to eliminate the excess paraffin.

4.2.1. Hematoxylin and Eosin Staining

Hematoxylin and Eosin (H&E) staining was performed using an automated stainer (Leica autostainer XL, Buffalo Grove, IL, USA) following a house protocol. Finally, slides were covered with MM24 mounting media and plastic cover slides. Microscopic images were taken using a LEICA DM4000B microscope, Olympus camera DP73, and Olympus Cellsens software.

4.2.2. Immunohistochemical Staining

Immunohistochemical staining of PGP9.5 (UCHL1) was performed following the protocol optimized at WFIRM. Slides were deparaffinated and rehydrated using the Leica Autostainer XL protocol. Immunohistochemistry staining was then performed manually using the following steps. Initially, antigen retrieval was achieved using sodium citrate (0.01 M, pH = 6) at 98 °C for 15′ and then allowed to cool down at room temperature. Slides were then incubated in 0.2% Triton X-100 in PBS for 7 min to help permeabilize membranes. For the next step, endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol for 30 min. Non-specific protein binding was prevented by incubating the slides in Serum-Free Protein Block solution (Dako, Santa Clara, CA, USA) for 15 min. Then slides were incubated on primary antibody overnight at 4 °C (Positive Slides: anti-PGP9.5 (UCHL1) Ab Rabbit Ig against human Abd Serotec 7863-0504 1:1000 in Dako Antibody Diluent; Negative controls: Rabbit IgG Sc-2027-1/1000 in Dako Antibody Diluent). To keep the slides moist during the incubation time, they were covered with parafilm and maintained in a humidity chamber (Sigma-Aldrich H6644). The next day, positive and negative slides were washed separately with phosphate buffer saline (PBS) for 5 min three times. Subsequently, incubation on secondary antibody was performed for 1 h at room temperature (goat anti-rabbit biotin IgG-BA1000 VECTOR (1/300) in Dako Antibody Diluent). After washing the remaining unbound antibody with PBS, the slides were

incubated on Avidin-Biotin-Peroxidase solution for 30 min (Ready to Use Elite ABC Reagent by Vector Laboratories). Finally, DAB Chromagen Substrate (Vector Laboratories SK-4100) was prepared as directed by the manufacturer, then added by the drop to both positive slides and negative controls in parallel. Development was monitored under light microscopy. The optimal color was reached after 1 min and 30 s. Development was stopped by dipping the slides in water. All reagents were at room temperature unless mentioned otherwise. At this point, slides were counterstained with Gill's hematoxylin using a Leica Autostainer XL. Finally, slides were over-slipped with MM24 mounting media. Microscopic images were taken using a LEICA DM4000B microscope, Olympus DP73 camera, and Olympus Cell sens software.

4.3. Cell Isolation, Culture, and Cryopreservation

Our testicular cell isolation protocol was on based on Shinohara's group's work [46–49] and previous works from our group [50,51] including both mechanical and enzymatic digestion of testicular tissue. For each experiment, five 3-day-old XY testes and five 3-day-old XXY testes were used, all DMSO frozen previously. Initially, cryovials were quickly thawed using running warm tap water, and testicular tissue was transferred into a petri dish with 1× MEM 8 µg/mL DNAse (Roche). Tunica albuginea was carefully removed using tweezers and a surgical blade before weighing the tissue. The next step was separating single seminiferous tubules of the testis from the surrounding conjunctive tissue with tweezers under a dissecting microscope (Leica S6D) for mechanical digestion. Once single tubules were obtained, the sample was transferred into a 15 mL centrifuge tube, and tubules were allowed to sink by gravity to the bottom. The supernatant was carefully removed, and tubules were then resuspended on enzymatic solution: 1× MEM; 12 µg/mL DNAse (Roche); Collagenase type I (CLS1) 225 U/mL; Hyaluronidase type IV from sheep (Sigma) 450 U/mL; Trypsine TRL3 (Worthington) 250 U/mL. Testicular tissue on the enzymatic solution was placed underwater in a shaking water bath at 120 rpm and 32 °C for a one-hour incubation. The solution was then centrifuged for 5 min at 16× *g* without brake, and supernatant carefully removed to discard early released cells. The remaining testicular tubules were re-suspended on a second enzymatic solution: 1× MEM 12 µg/mL DNAse (Roche); Collagenase type I (CLS1) 225 U/mL; Hyaluronidase type IV from sheep (Sigma) 450 U/mL and placed entirely under water in a shaking water bath at 120 rpm and 32 °C for enzyme incubation for 45 min. At this point, digested tubules were vigorously pipetted to help release remaining cells from the tubules and centrifuged for 5 min at 350× *g* with a brake. The supernatant was removed, and the pellet was resuspended on Trypsin 0.25% EDTA (Invitrogen) and incubated at 37 °C for 25 min to dissociate clumps of cells further. At this point, the sample was centrifuged for 5 min at 350× *g* with brake, and the supernatant was carefully removed. Cells were finally resuspended on Gonomedia (Table 1). Trypan Blue staining (1:1) and a Hematocytometer was used to assess the number of cells and viability. Cells were then seeded at 20,000–25,000 cells/cm^2 on culture cell plastic plates (Falcon) and kept in an incubator at stable conditions of 37 °C 5% CO_2.

Culture media was refreshed every three to four days. When confluency of the attached cells was approached, 80% of cells were passaged and split using Trypsin 0.25% EDTA (Life Technologies). A surplus of cells were cryopreserved in MEM 20% FBS and 8% DMSO and kept overnight in Mr. Frosty at −80 °C. The next day, cryotubes were transferred into liquid nitrogen tanks for long-term storage.

4.4. Quantitative Reverse Transcriptase Polymerase Chain Reaction (q RT-PCR)

RNA was extracted using an RNEasy mini kit (Qiagen, Germantown, MD, USA) from Snap Frozen tissue or cells. The quality and quantity of the resultant product were tested with a spectrophotometer (Nanodrop 2000, ThermoFisher, Waltham, MA, USA). RNA was then converted to cDNA using a Reverse Transcriptase Kit (Life Technologies, Carlsbad, CA, USA) and through the following thermocycler (Simpli amp thermal cycler, life technologies) conditions: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and then

hold on 4 °C. The resulting cDNA samples underwent PCR amplification using Taqman primers (Supplementary Table S1) and an applied Biosystems 7300 Real-Time PCR system. The cycling conditions followed were 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All primers (Supplementary Table S1) were previously tested for not amplifying genomic DNA. POLR2A was selected as a housekeeping gene [68] and the expression of genes was normalized to this gene; relative expression was determined with the Delta CT method.

Table 1. Gonomedia formulation.

Reagent	Company	Catalog #	Final Concentration
Stem Pro-34 SFM	Invitrogen	10639-011	Base Medium
Stem Pro Supplement	Invitrogen	10639-011	26 µL/mL
Bovine Albumin	Roche	10735094001	5 mg/mL
D(+) Glucose	Sigma	G7021	6 mg/mL
Ascorbic acid	Sigma	A4544	1×10^{-4} M
Transferrin	Sigma	T1147	100 µg/mL
Pyruvic acid	Sigma	P2256	30 mg/mL
d-Biotin	Sigma	B4501	10 µg/mL
2-beta Mercatoethanol	Sigma	M7522	5×10^{-5} M
DL-lactic acid	Sigma	L4263	1 µL/mL
MEM-non essential aa	Invitrogen	11140-035	10 µL/mL
Insulin	Sigma	I1882	25 µg/mL
Sodium Selenite	Sigma	S1382	30 nM
Putrescine	Sigma	P7505	60 µM
L-Glutamine	Invitrogen	25030-024	2 mM
MEM Vitamine solution	Invitrogen	11120-037	10 µL/mL
b-Estradiol	Sigma	E2758	30 ng/mL
Progesterone	Sigma	P8783	60 ng/mL
Human EGF	Sigma	E9644	20 ng/mL
Human bFGF	Sigma	F0291	10 ng/mL
Human LIF	Chemicon	LIF1010	10 ng/mL
GDNF	Sigma	G1777	10 ng/mL
FCS	Invitrogen	10106-169	20%
Pen/Strep	Invitrogen	15140122	0.5%
Platelet-Derived Growth Factor (PDGF)	Sigma	SRP3228-10UG	10 ng/mL
Follistatin (Ft)	Sigma	F1175-25UG	100 ng/mL
Follicle Stimulant Hormone (FSH)	Sigma	F8174-1VL	200 ng/mL

Amplified cDNA from RT qPCR was subsequently used for an electrophoresis study on the gel to visualize the specific product bands. A 2% agarose gel was used, given that our target primers were between 50 and 120 base pairs of length (Supplementary Table S1). Ethidium Bromide was included in the gel formulation at a concentration of 5 µL/100 mL. After samples and DNA ladder were loaded, a 140 V Voltage (Enduro power supply, Labnet, Edison, NJ, USA) was applied for 20 min when the DNA dye reached 2/3 of the

total gel size. Images of the gel were taken using a UV light Camera system (Gel logic 200 imaging system).

4.5. Digital Reverse Transcriptase Polymerase Chain Reaction (d RT-PCR)

A QuantStudio 3D Digital PCR system (Life Technologies, Carlsbad, CA, USA) was used to estimate the population of cells expressing specific markers for each cell type expected in culture. Every chip was loaded with 2 Taqman assay primers: one with FAM signal for the specific targeted gene and one with VIC signal for the housekeeping gene POLR2A. The chip load was completed following the manufacturer's manual with commercially available dPCR Master Mix and cDNA from RNA extracted from Snap Frozen cells. The quantity of cDNA loaded in each 20,000 wells chip was 50 ng. Assuming each mammalian cells has around 10–30 pg of total RNA, each well should represent at most one cell with a significant number of empty wells as a safety margin. Cycling conditions were 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.

4.6. Flow Cytometry Analyses

The population of putative spermatogonial stem cells (SSCs) in the cells in culture was estimated as the MHC I/CD9+/CD49f+ population [54–59] using a BD Accuri C6 Flow cytometry system without sorting. BD antibodies were used (Supplementary Table S2) at a concentration of 5 µL of antibody per 50,000 cells in 100 µL. Cells were incubated with the antibody for one hour at room temperature and then washed with FACS Buffer (1% FBS in PBS). The obtained data were analyzed using BD Accuri software. Unstained cells and isotype controls (Supplementary Table S2) were used to optimize channel compensation and as a negative control. In every condition, at least 10,000 events were evaluated.

In separate experiments, the same staining method was used to perform Fluorescence-Activated Cell Sorting (FACS) using BD FACS ARIA. Cells expressing MHC I-/CD9+/CD49f+ were sorted and used for DNA FISH analysis.

4.7. X and Y Chromosome Fluorescent In Situ Hybridization (FISH)

After cells in culture were harvested by trypsin, 50,000 cells were attached to the glass microscopic slides by cytocentrifuge (Cytospin) for 10 min at 1000 RPM on glass poly-l-lysine slides (citopro, ELI Tech Biomedical Systems, Puteaux, France) using the cytopro 7620 cytocentrifuge system (Wescor). The slides were left to dry at room temperature overnight. Then, slides were soaked in 2× saline sodium citrate (made from stock 20xSSC from ABBOTT/VYSIS Company, Chicago, IL, USA) at 37 °C for 35 min. Subsequently, cells were incubated in Pepsin (AVANTOR PERFORMANCE MATERIALS, INC 2629, company) 0.5 mg/mL solution of HCl 0.1 M at 37 °C for 35 min. Slides were then washed at room temperature in 1XPBS for 5 min, and Post-Fixation Solution (0.9% formaldehyde *w/v*; 4.5 mg/mL $MgCl_2$ in PBS) was added for 5 min of incubation at room temperature. Slides were rewashed at room temperature in PBS for 5 min, and the dehydration process was performed by submerging slides in increasing concentrations of ethanol (70%, 80%, 100%) for 1 min each at room temperature. At this point, working solutions of X and Y chromosome probes (EMPIRE GENOMICS Company kit MCEN-Y-10-GR and MCEN-X-10-OR 1:1:4 probe buffer dilution) were added to the sample, and slides were kept at 75 °C for 5 min overnight incubation at 40 °C (16 h minimum). The following day, slides were washed in 0.3% IGEPAL/solution (SCI-GENE, Stanford, CA, USA) on 0.4X saline sodium citrate/for 2 min at 73 °C and 0.1% IGEPAL (SCI-GENE, Stanford, CA, USA) solution on 2X saline sodium citrate for 1 min at room temperature. Slides were finally mounted with ABBOTT/VYSIS DAPI II and coverslips applied. Imaging of the slides was performed using a Zeiss Axiophot microscope and Applied Spectral Imaging Software.

5. Conclusions

To the best of our knowledge, this is the first report of long-term culture and propagation of mouse XXY testicular cells, well beyond the previously noted and expected time of germ cell loss. Moreover, the comparable in vitro growth rates of XXY testicular cells and cultured cells from wild-type mice testes open the door to pursue new cell-based therapies to treat infertility in Klinefelter syndrome patients. The growing mosaicism observed in the cells in culture may lead to a better understanding of the stem cell selection dynamics in the Klinefelter patient's testis. Finally, we hope this body of work may be reproduced in human Klinefelter tissue.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms23010173/s1.

Author Contributions: Conceptualization, G.G., S.K., C.W., R.S.S., Y.L. and H.S.-A.; methodology, G.G., Y.L. and H.S.-A.; software, G.G., Y.L. and H.S.-A.; validation, G.G., M.J.P., Y.L. and H.S.-A.; formal analysis, G.G., N.A.D., Y.L. and H.S.-A.; investigation, G.G., N.A.D., N.P.Z., Y.L. and H.S.-A.; resources, G.G., Y.L. and H.S.-A.; data curation, G.G., Y.L. and H.S.-A.; writing—original draft preparation, G.G., Y.L. and H.S.-A.; writing—review and editing, G.G., N.A.D., N.P.Z., M.J.P., S.K., C.W., R.S.S., A.A., Y.L. and H.S.-A.; visualization, G.G., Y.L. and H.S.-A.; supervision, Y.L. and H.S.-A.; project administration, H.S.-A., funding acquisition, A.A. and H.S.-A. All authors have read and agreed to the published version of the manuscript.

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References

1. Lanfranco, F.; Kamischke, A.; Zitzmann, M.; Nieschlag, E. Klinefelter's Syndrome. *Lancet* **2004**, *364*, 273–283. [CrossRef]
2. Nielsen, J.; Wohlert, M. Sex Chromosome Abnormalities Found among 34,910 Newborn Children: Results from a 13-Year Incidence Study in Arhus, Denmark. *Birth Defects Orig. Artic. Ser.* **1990**, *26*, 209–223.
3. Bojesen, A.; Juul, S.; Gravholt, C.H. Prenatal and Postnatal Prevalence of Klinefelter Syndrome: A National Registry Study. *J Clin. Endocrinol. Metab* **2003**, *88*, 622–626. [CrossRef] [PubMed]
4. Gordon, D.L.; Krmpotic, E.; Thomas, W.; Gandy, H.M.; Paulsen, C.A. Pathologic Testicular Findings in Klinefelter's Syndrome. 47,XXY vs. 46,XY-47,XXY. *Arch. Intern. Med.* **1972**, *130*, 726–729. [CrossRef]
5. Autio-Harmainen, H.; Rapola, J.; Aula, P. Fetal Gonadal Histology in XXXXY, XYY and XXX Syndromes. *Clin. Genet.* **1980**, *18*, 1–5. [CrossRef] [PubMed]
6. Coerdt, W.; Rehder, H.; Gausmann, I.; Johannisson, R.; Gropp, A. Quantitative Histology of Human Fetal Testes in Chromosomal Disease. *Pediatr. Pathol.* **1985**, *3*, 245–259. [CrossRef]
7. Mikamo, K.; Aguercif, M.; Hazeghi, P.; Martin-Du Pan, R. Chromatin-Positive Klinefelter's Syndrome. A Quantitative Analysis of Spermatogonial Deficiency at 3, 4, and 12 Months of Age. *Fertil. Steril.* **1968**, *19*, 731–739. [CrossRef]
8. Winge, S.B.; Dalgaard, M.D.; Jensen, J.M.; Graem, N.; Schierup, M.H.; Juul, A.; Rajpert-De Meyts, E.; Almstrup, K. Transcriptome Profiling of Fetal Klinefelter Testis Tissue Reveals a Possible Involvement of Long Non-Coding RNAs in Gonocyte Maturation. *Hum. Mol. Genet.* **2018**, *27*, 430–439. [CrossRef]
9. Wikstrom, A.M.; Raivio, T.; Hadziselimovic, F.; Wikstrom, S.; Tuuri, T.; Dunkel, L. Klinefelter Syndrome in Adolescence: Onset of Puberty Is Associated with Accelerated Germ Cell Depletion. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 2263–2270. [CrossRef]
10. Aksglaede, L.; Wikstrom, A.M.; Rajpert-De Meyts, E.; Dunkel, L.; Skakkebaek, N.E.; Juul, A. Natural History of Seminiferous Tubule Degeneration in Klinefelter Syndrome. *Hum. Reprod. Update* **2006**, *12*, 39–48. [CrossRef]
11. Groth, K.A.; Skakkebaek, A.; Host, C.; Gravholt, C.H.; Bojesen, A. Clinical Review: Klinefelter Syndrome–a Clinical Update. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 20–30. [CrossRef]

12. Wikstrom, A.M.; Hoei-Hansen, C.E.; Dunkel, L.; Rajpert-De Meyts, E. Immunoexpression of Androgen Receptor and Nine Markers of Maturation in the Testes of Adolescent Boys with Klinefelter Syndrome: Evidence for Degeneration of Germ Cells at the Onset of Meiosis. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 714–719. [CrossRef] [PubMed]
13. Yamamoto, Y.; Sofikitis, N.; Mio, Y.; Loutradis, D.; Kaponis, A.; Miyagawa, I. Morphometric and Cytogenetic Characteristics of Testicular Germ Cells and Sertoli Cell Secretory Function in Men with Non-Mosaic Klinefelter's Syndrome. *Hum. Reprod.* **2002**, *17*, 886–896. [CrossRef]
14. Paduch, D.A.; Bolyakov, A.; Cohen, P.; Travis, A. Reproduction in Men with Klinefelter Syndrome: The Past, the Present, and the Future. *Semin. Reprod. Med.* **2009**, *27*, 137–148. [CrossRef]
15. Foresta, C.; Galeazzi, C.; Bettella, A.; Marin, P.; Rossato, M.; Garolla, A.; Ferlin, A. Analysis of Meiosis in Intratesticular Germ Cells from Subjects Affected by Classic Klinefelter's Syndrome. *J. Clin. Endocrinol. Metab.* **1999**, *84*, 3807–3810. [CrossRef] [PubMed]
16. Salbenblatt, J.A.; Bender, B.G.; Puck, M.H.; Robinson, A.; Faiman, C.; Winter, J.S. Pituitary-Gonadal Function in Klinefelter Syndrome before and during Puberty. *Pediatr. Res.* **1985**, *19*, 82–86. [CrossRef]
17. Wikstrom, A.M.; Dunkel, L. Testicular Function in Klinefelter Syndrome. *Horm. Res.* **2008**, *69*, 317–326. [CrossRef] [PubMed]
18. Aksglaede, L.; Jorgensen, N.; Skakkebaek, N.E.; Juul, A. Low Semen Volume in 47 Adolescents and Adults with 47,XXY Klinefelter or 46,XX Male Syndrome. *Int. J. Androl.* **2009**, *32*, 376–384. [CrossRef]
19. Cruger, D.; Toft, B.; Agerholm, I.; Fedder, J.; Hald, F.; Bruun-Petersen, G. Birth of a Healthy Girl after ICSI with Ejaculated Spermatozoa from a Man with Non-Mosaic Klinefelter's Syndrome. *Hum. Reprod.* **2001**, *16*, 1909–1911. [CrossRef]
20. Kitamura, M.; Matsumiya, K.; Koga, M.; Nishimura, K.; Miura, H.; Tsuji, T.; Matsumoto, M.; Okamoto, Y.; Okuyama, A. Ejaculated Spermatozoa in Patients with Non-Mosaic Klinefelter's Syndrome. *Int. J. Urol.* **2000**, *7*, 88–92; discussion 93–94. [PubMed]
21. Akashi, T.; Fuse, H.; Kojima, Y.; Hayashi, M.; Honda, S. Birth after Intracytoplasmic Sperm Injection of Ejaculated Spermatozoa from a Man with Mosaic Klinefelter's Syndrome. *Asian J. Androl.* **2005**, *7*, 217–220. [CrossRef]
22. De Sanctis, V.; Ciccone, S. Fertility Preservation in Adolescents with Klinefelter's Syndrome. *Pediatr. Endocrinol. Rev.* **2010**, *8* (Suppl. 1), 178–181. [PubMed]
23. Forti, G.; Corona, G.; Vignozzi, L.; Krausz, C.; Maggi, M. Klinefelter's Syndrome: A Clinical and Therapeutical Update. *Sex. Dev.* **2010**, *4*, 249–258. [CrossRef] [PubMed]
24. Corona, G.; Pizzocaro, A.; Lanfranco, F.; Garolla, A.; Pelliccione, F.; Vignozzi, L.; Ferlin, A.; Foresta, C.; Jannini, E.A.; Maggi, M.; et al. Sperm Recovery and ICSI Outcomes in Klinefelter Syndrome: A Systematic Review and Meta-Analysis. *Hum. Reprod. Update* **2017**, *23*, 265–275. [CrossRef] [PubMed]
25. Ly, A.; Sermondade, N.; Brioude, F.; Berthaut, I.; Bachelot, A.; Hamid, R.H.; Khattabi, L.E.; Prades, M.; Lévy, R.; Dupont, C. Fertility Preservation in Young Men with Klinefelter Syndrome: A Systematic Review. *J. Gynecol. Obstet. Hum. Reprod.* **2021**, *50*, 102177. [CrossRef]
26. Vicdan, K.; Akarsu, C.; Sozen, E.; Buluc, B.; Vicdan, A.; Yilmaz, Y.; Biberoglu, K. Outcome of Intracytoplasmic Sperm Injection Using Fresh and Cryopreserved-Thawed Testicular Spermatozoa in 83 Azoospermic Men with Klinefelter Syndrome. *J. Obstet. Gynaecol. Res.* **2016**, *42*, 1558–1566. [CrossRef]
27. Deebel, N.A.; Galdon, G.; Zarandi, N.P.; Stogner-Underwood, K.; Howards, S.; Lovato, J.; Kogan, S.; Atala, A.; Lue, Y.; Sadri-Ardekani, H. Spermatogonia Stem Cell Technology: A New Avenue for All Age Klinefelter Patients. *Hum. Reprod. Update* **2021**, *15*, dmab025. [CrossRef]
28. Van Saen, D.; Gies, I.; De Schepper, J.; Tournaye, H.; Goossens, E. Can Pubertal Boys with Klinefelter Syndrome Benefit from Spermatogonial Stem Cell Banking? *Hum. Reprod.* **2012**, *27*, 323–330. [CrossRef]
29. Damani, M.N.; Mittal, R.; Oates, R.D. Testicular Tissue Extraction in a Young Male with 47,XXY Klinefelter's Syndrome: Potential Strategy for Preservation of Fertility. *Fertil. Steril.* **2001**, *76*, 1054–1056. [CrossRef]
30. Gies, I.; De Schepper, J.; Goossens, E.; Van Saen, D.; Pennings, G.; Tournaye, H. Spermatogonial Stem Cell Preservation in Boys with Klinefelter Syndrome: To Bank or Not to Bank, That's the Question. *Fertil. Steril.* **2012**, *98*, 284–289. [CrossRef]
31. Gies, I.; Oates, R.; De Schepper, J.; Tournaye, H. Testicular Biopsy and Cryopreservation for Fertility Preservation of Prepubertal Boys with Klinefelter Syndrome: A pro/Con Debate. *Fertil. Steril.* **2016**, *105*, 249–255. [CrossRef]
32. Van Saen, D.; Vloeberghs, V.; Gies, I.; Mateizel, I.; Sermon, K.; De Schepper, J.; Tournaye, H.; Goossens, E. When Does Germ Cell Loss and Fibrosis Occur in Patients with Klinefelter Syndrome? *Hum. Reprod.* **2018**, *33*, 1009–1022. [CrossRef] [PubMed]
33. Mehta, A.; Paduch, D.A. Klinefelter Syndrome: An Argument for Early Aggressive Hormonal and Fertility Management. *Fertil. Steril.* **2012**, *98*, 274–283. [CrossRef] [PubMed]
34. Deebel, N.A.; Galdon, G.; Zarandi, N.P.; Stogner-Underwood, K.; Howards, S.; Lovato, J.; Kogan, S.; Atala, A.; Lue, Y.; Sadri-Ardekani, H. Age-Related Presence of Spermatogonia in Patients with Klinefelter Syndrome: A Systematic Review and Meta-Analysis. *Hum. Reprod. Update* **2020**, *26*, 58–72. [CrossRef] [PubMed]
35. Lue, Y.; Jentsch, J.D.; Wang, C.; Rao, P.N.; Hikim, A.P.; Salameh, W.; Swerdloff, R.S. XXY Mice Exhibit Gonadal and Behavioral Phenotypes Similar to Klinefelter Syndrome. *Endocrinology* **2005**, *146*, 4148–4154. [CrossRef] [PubMed]
36. Lue, Y.; Liu, P.Y.; Erkkila, K.; Ma, K.; Schwarcz, M.; Wang, C.; Swerdloff, R.S. Transplanted XY Germ Cells Produce Spermatozoa in Testes of XXY Mice. *Int. J. Androl.* **2010**, *33*, 581–587. [CrossRef] [PubMed]
37. Lue, Y.; Rao, P.N.; Sinha Hikim, A.P.; Im, M.; Salameh, W.A.; Yen, P.H.; Wang, C.; Swerdloff, R.S. XXY Male Mice: An Experimental Model for Klinefelter Syndrome. *Endocrinology* **2001**, *142*, 1461–1470. [CrossRef]

38. Hirota, T.; Ohta, H.; Powell, B.E.; Mahadevaiah, S.K.; Ojarikre, O.A.; Saitou, M.; Turner, J.M.A. Fertile Offspring from Sterile Sex Chromosome Trisomic Mice. *Science* **2017**, *357*, 932–935. [CrossRef]
39. Joerg, H.; Janett, F.; Schlatt, S.; Mueller, S.; Graphodatskaya, D.; Suwattana, D.; Asai, M.; Stranzinger, G. Germ Cell Transplantation in an Azoospermic Klinefelter Bull. *Biol. Reprod.* **2003**, *69*, 1940–1944. [CrossRef]
40. Wistuba, J.; Luetjens, C.M.; Stukenborg, J.B.; Poplinski, A.; Werler, S.; Dittmann, M.; Damm, O.S.; Hamalainen, T.; Simoni, M.; Gromoll, J. Male 41, XXY* Mice as a Model for Klinefelter Syndrome: Hyperactivation of Leydig Cells. *Endocrinology* **2010**, *151*, 2898–2910. [CrossRef]
41. Werler, S.; Demond, H.; Damm, O.S.; Ehmcke, J.; Middendorff, R.; Gromoll, J.; Wistuba, J. Germ Cell Loss Is Associated with Fading Lin28a Expression in a Mouse Model for Klinefelter's Syndrome. *Reproduction* **2014**, *147*, 253–264. [CrossRef]
42. Werler, S.; Poplinski, A.; Gromoll, J.; Wistuba, J. Expression of Selected Genes Escaping from X Inactivation in the 41, XX(Y)* Mouse Model for Klinefelter's Syndrome. *Acta Paediatr.* **2011**, *100*, 885–891. [CrossRef]
43. Hunt, P.A.; Worthman, C.; Levinson, H.; Stallings, J.; LeMaire, R.; Mroz, K.; Park, C.; Handel, M.A. Germ Cell Loss in the XXY Male Mouse: Altered X-Chromosome Dosage Affects Prenatal Development. *Mol. Reprod. Dev.* **1998**, *49*, 101–111. [CrossRef]
44. Sadri-Ardekani, H.; Atala, A. Testicular Tissue Cryopreservation and Spermatogonial Stem Cell Transplantation to Restore Fertility: From Bench to Bedside. *Stem Cell Res. Ther.* **2014**, *5*, 68. [CrossRef]
45. Galdon, G.; Atala, A.; Sadri-Ardekani, H. In Vitro Sperm.matogenesis: How Far from Clinical Application? *Curr. Urol. Rep.* **2016**, *17*, 49. [CrossRef] [PubMed]
46. Kanatsu-Shinohara, M.; Inoue, K.; Ogonuki, N.; Morimoto, H.; Ogura, A.; Shinohara, T. Serum- and Feeder-Free Culture of Mouse Germline Stem Cells. *Biol. Reprod.* **2011**, *84*, 97–105. [CrossRef] [PubMed]
47. Kanatsu-Shinohara, M.; Miki, H.; Inoue, K.; Ogonuki, N.; Toyokuni, S.; Ogura, A.; Shinohara, T. Long-Term Culture of Mouse Male Germline Stem Cells under Serum-or Feeder-Free Conditions. *Biol. Reprod.* **2005**, *72*, 985–991. [CrossRef]
48. Kanatsu-Shinohara, M.; Ogonuki, N.; Inoue, K.; Miki, H.; Ogura, A.; Toyokuni, S.; Shinohara, T. Long-Term Proliferation in Culture and Germline Transmission of Mouse Male Germline Stem Cells. *Biol. Reprod.* **2003**, *69*, 612–616. [CrossRef] [PubMed]
49. Kanatsu-Shinohara, M.; Ogonuki, N.; Matoba, S.; Morimoto, H.; Ogura, A.; Shinohara, T. Improved Serum- and Feeder-Free Culture of Mouse Germline Stem Cells. *Biol. Reprod.* **2014**, *91*, 88. [CrossRef]
50. Sadri-Ardekani, H.; Mizrak, S.C.; van Daalen, S.K.; Korver, C.M.; Roepers-Gajadien, H.L.; Koruji, M.; Hovingh, S.; de Reijke, T.M.; de la Rosette, J.J.; van der Veen, F.; et al. Propagation of Human Spermatogonial Stem Cells in Vitro. *JAMA* **2009**, *302*, 2127–2134. [CrossRef] [PubMed]
51. Sadri-Ardekani, H.; Akhondi, M.A.; van der Veen, F.; Repping, S.; van Pelt, A.M. In Vitro Propagation of Human Prepubertal Spermatogonial Stem Cells. *JAMA* **2011**, *305*, 2416–2418. [CrossRef] [PubMed]
52. Wilson, P.O.; Barber, P.C.; Hamid, Q.A.; Power, B.F.; Dhillon, A.P.; Rode, J.; Day, I.N.; Thompson, R.J.; Polak, J.M. The Immunolocalization of Protein Gene Product 9.5 Using Rabbit Polyclonal and Mouse Monoclonal Antibodies. *Br. J. Exp. Pathol.* **1988**, *69*, 91–104.
53. Marini, M.; Rosa, I.; Guasti, D.; Gacci, M.; Sgambati, E.; Ibba-Manneschi, L.; Manetti, M. Reappraising the Microscopic Anatomy of Human Testis: Identification of Telocyte Networks in the Peritubular and Intertubular Stromal Space. *Sci. Rep.* **2018**, *8*, 14780. [CrossRef] [PubMed]
54. Nickkholgh, B.; Mizrak, S.C.; Korver, C.M.; van Daalen, S.K.; Meissner, A.; Repping, S.; van Pelt, A.M. Enrichment of Spermatogonial Stem Cells from Long-Term Cultured Human Testicular Cells. *Fertil. Steril.* **2014**, *102*, 558–565. [CrossRef] [PubMed]
55. Zohni, K.; Zhang, X.; Tan, S.L.; Chan, P.; Nagano, M. CD9 Is Expressed on Human Male Germ Cells That Have a Long-Term Repopulation Potential after Transplantation into Mouse Testes. *Biol. Reprod.* **2012**, *87*, 27. [CrossRef]
56. Guo, Y.; Hai, Y.; Gong, Y.; Li, Z.; He, Z. Characterization, Isolation, and Culture of Mouse and Human Spermatogonial Stem Cells. *J. Cell Physiol.* **2014**, *229*, 407–413. [CrossRef]
57. Hutter, H.; Dohr, G. HLA Expression on Immature and Mature Human Germ Cells. *J. Reprod. Immunol.* **1998**, *38*, 101–122. [CrossRef]
58. Harichandan, A.; Sivasubramaniyan, K.; Hennenlotter, J.; Poths, S.; Bedke, J.; Kruck, S.; Stenzl, A.; Buhring, H.J. Molecular Signatures of Primary Human Spermatogonial Progenitors and Its Neighboring Peritubular Stromal Compartment. *Stem Cells Dev.* **2017**, *26*, 263–273. [CrossRef]
59. Shinohara, T.; Avarbock, M.R.; Brinster, R.L. Beta1- and Alpha6-Integrin Are Surface Markers on Mouse Spermatogonial Stem Cells. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 5504–5509. [CrossRef]
60. Deebel, N.A.; Bradshaw, A.W.; Sadri-Ardekani, H. Infertility Considerations in Klinefelter Syndrome: From Origin to Management. *Best Pract. Res. Clin. Endocrinol. Metab.* **2020**, *34*, 101480. [CrossRef] [PubMed]
61. Culty, M. Gonocytes, the Forgotten Cells of the Germ Cell Lineage. *Birth Defects Res. Part C Embryo Today Rev.* **2009**, *87*, 1–26. [CrossRef]
62. Kubota, H.; Avarbock, M.R.; Brinster, R.L. Growth Factors Essential for Self-Renewal and Expansion of Mouse Spermatogonial Stem Cells. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16489–16494. [CrossRef]
63. Meehan, T.; Schlatt, S.; O'Bryan, M.K.; de Kretser, D.M.; Loveland, K.L. Regulation of Germ Cell and Sertoli Cell Development by Activin, Follistatin, and FSH. *Dev. Biol.* **2000**, *220*, 225–237. [CrossRef]
64. Hasthorpe, S. Clonogenic Culture of Normal Spermatogonia: In Vitro Regulation of Postnatal Germ Cell Proliferation. *Biol. Reprod.* **2003**, *68*, 1354–1360. [CrossRef]

65. Kanatsu-Shinohara, M.; Ogonuki, N.; Iwano, T.; Lee, J.; Kazuki, Y.; Inoue, K.; Miki, H.; Takehashi, M.; Toyokuni, S.; Shinkai, Y.; et al. Genetic and Epigenetic Properties of Mouse Male Germline Stem Cells during Long-Term Culture. *Dev. Camb. Engl.* **2005**, *132*, 4155–4163. [CrossRef] [PubMed]
66. Baert, Y.; Braye, A.; Struijk, R.B.; van Pelt, A.M.; Goossens, E. Cryopreservation of Testicular Tissue before Long-Term Testicular Cell Culture Does Not Alter in Vitro Cell Dynamics. *Fertil. Steril.* **2015**, *104*, 1244–1252. [CrossRef] [PubMed]
67. Martin, G.V.; Kanaan, S.B.; Hemon, M.F.; Azzouz, D.F.; El Haddad, M.; Balandraud, N.; Mignon-Ravix, C.; Picard, C.; Arnoux, F.; Martin, M.; et al. Mosaicism of XX and XXY Cells Accounts for High Copy Number of Toll like Receptor 7 and 8 Genes in Peripheral Blood of Men with Rheumatoid Arthritis. *Sci. Rep.* **2019**, *9*, 12880. [CrossRef]
68. Radonic, A.; Thulke, S.; Mackay, I.M.; Landt, O.; Siegert, W.; Nitsche, A. Guideline to Reference Gene Selection for Quantitative Real-Time PCR. *Biochem. Biophys. Res. Commun.* **2004**, *313*, 856–862. [CrossRef] [PubMed]

Materials and methods:

Article 2: "***In Vitro*** **Propagation of XXY Human Klinefelter Spermatogonial Stem Cells: a step towards new fertility opportunities**" addressing the objective: *Isolate, Propagate and Characterize human XXY testicular cells, including Spermatogonial Stem Cells population*

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In vitro propagation of XXY human Klinefelter spermatogonial stem cells: A step towards new fertility opportunities

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Klinefelter Syndrome (KS) is characterized by a masculine phenotype, supernumerary sex chromosomes (47, XXY), and impaired fertility due to loss of spermatogonial stem cells (SSCs). Early testicular cryopreservation could be an option for future fertility treatments in these patients, including SSCs transplantation or *in vitro* spermatogenesis. It is critically essential to adapt current *in vitro* SSCs propagation systems as a fertility option for KS patients. KS human testicular samples (13,15- and 17-year-old non-mosaic KS boys) were donated by patients enrolled in an experimental testicular tissue banking program. Testicular cells were isolated from cryopreserved tissue and propagated in long-term culture for 110 days. Cell-specific gene expression confirmed the presence of all four main cell types found in testes: Spermatogonia, Sertoli, Leydig, and Peritubular cells. A population of $ZBTB16^{+}$ undifferentiated spermatogonia was identified throughout the culture using digital PCR. Flow cytometric analysis also detected an HLA^{-}/$CD9^{+}$/$CD49f^{+}$ population, indicating maintenance of a stem cell subpopulation among the spermatogonial cells. FISH staining for chromosomes X and Y showed most cells containing an XXY karyotype with a smaller number containing either XY or XX. Both XY and XX populations were able to be enriched by magnetic sorting for CD9 as a spermatogonia marker. Molecular karyotyping demonstrated genomic stability of the cultured cells, over time. Finally, single-cell RNAseq analysis confirmed transcription of ID4, TCN2, and

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NANOS 3 within a population of putative SSCs population. This is the first study showing successful isolation and long-term *in vitro* propagation of human KS testicular cells. These findings could inform the development of therapeutic fertility options for KS patients, either through *in vitro* spermatogenesis or transplantation of SSC, *in vivo*.

KEYWORDS

spermatogonia, stem cell, germ cell transplantation, fertility preservation, Klinefelter Syndrome, male infertility, cell culture

Introduction

Klinefelter Syndrome (KS) is a classically underdiagnosed cause of male infertility characterized by a male phenotype and altered karyotype, usually presenting as non-mosaic 47 XXY (1). However, variants have been described with additional X chromosomes and different degrees of mosaicism. Recent studies using non-invasive genetic diagnostic technology estimated KS may affect one of every 600-1000 males born (2, 3). Hence, KS is now considered the most common chromosomal cause of male infertility.

The pathophysiological mechanisms underlying male infertility in KS patients are not yet entirely understood. However, several studies have attempted to describe KS testicular morphology from the fetus into adulthood (4–6). Testicular morphology remains largely unaffected in prepubertal patients. During puberty, when testicular fibrosis accelerates, distortion of the tubular architecture was seen (7). By the end of puberty, over 95% of KS patients are azoospermic (8). Leydig cell hyperplasia occurs in the interstitial space in response to elevated LH. Despite Leydig cell hyperplasia, KS patients commonly present with low or low normal range serum testosterone levels. Nevertheless, the degree of testicular fibrosis differs from patient to patient. Foci of active spermatogenesis have been found in KS patients with testicular fibrosis. Current literature shows that only 8% of KS patients have sperm present in the ejaculate (9). These KS patients with adequate sperm in the ejaculate can achieve natural conception with euploid offspring suggesting that cells that complete meiosis may develop into normal gametes (10). The current gold standard of care for treatment of infertility in KS patients is micro-TESE (microscopic testicular sperm extraction) with a reported 44% success rate in testicular sperm retrieval (11, 12), 43% pregnancy rate, and 43% birth rate from *in vitro* fertilization (IVF) or intracytoplasmatic sperm injection (ICSI). No other fertility options for KS patients are currently available.

Since the development of prenatal genetic diagnostic techniques (e.g. Amniocentesis, Chorion Villus Sample, Cell-Free-Fetal DNA determination, Quantitative Fluorescence PCR, Multiplex Ligation Dependent Probe Amplification, Preimplantation Genetic Screening), it is now possible to diagnose and follow KS patients in the prepubertal period, providing social support and enhanced education for preservation of occult sperm that would provide fertility options, should infertility develop. Several reports showed that in patients from whom no spermatozoa could be retrieved by microTESE, viable spermatogonia, including SSCs, may be found in testicular biopsies (13, 14). SSCs are a subpopulation of undifferentiated spermatogonia present in the testis. Their primary function is both continuous self-renewal and differentiation into germ cells committed to undergo spermatogenesis (15). It has been hypothesized that SSCs should be capable of complete restoration of spermatogenesis following transplantation into infertile patients (16). This hypothesis has been supported by several different animal studies (17–22). However, successful SSCs transplantation in humans has yet to be accomplished (23). Experimental SSCs-based therapies may potentially treat KS patients' infertility, even in those patients with unsuccessful TESE. Testicular tissue banking has been implemented in KS patients to provide a source of spermatogonia for new fertility treatments (24, 25). As previously mentioned, germ cell loss in KS accelerates with the onset of puberty. Therefore, retrieval of viable spermatogonia in testicular biopsy is higher in peripubertal than in adult KS patients (8). About 25% of KS patients are diagnosed before puberty (1), and storing testicular tissue from these patients is not a standard practice. Our Wake Forest Baptist School of Medicine group has established an experimental testicular tissue banking for boys and men with a high risk of infertility. KS is one of the approved indications (26). Participating patients are given the option to donate a portion of their testicular samples for research voluntarily.

Our setting provides a unique opportunity to explore the ability to isolate and propagate KS testicular cells from testicular biopsy performed before puberty. Expanding the number of KS

Abbreviations: KS, Klinefelter Syndrome; SSCs, Spermatogonial Stem Cell; SSC, Sodium Chloride-Sodium; dPCR, Digital PCR; MACS, Magnetic Activated Cell Sorting; FISH, Fluorescent *In Situ* Hybridization; PGM, Personal Genome Machine.

testicular cells *in vitro* would be critically important when considering autologous cell-based fertility treatments. It would also provide material for research to preserve and enhance fertility in KS patients, and improve our understanding of why XXY males lose germ cells at puberty. Our previous work tested SSC isolation and culture on 41 XXY KS mice (27–30). Cells were successfully propagated in culture for up to 120 days while expanding 650,000-fold in number. Moreover, characteristic phenotypes of all four common cell types were maintained in culture, including a population of putative SSCs (30). Similar findings are now confirmed using human frozen testicular tissue from KS patients.

Material and methods

Patients

Selected Klinefelter adolescent patients eligible for micro-Testicular Sperm Extraction (mTESE) were offered the opportunity to store their testicular tissue for potential stem cell therapy at Wake Forest Baptist experimental testicular tissue bank under IRB approved protocols at Wake Forest School of Medicine (IRB00021686 and IRB00061265). Surgery was performed by experienced pediatric and adult reproductive urologists (26). Intraoperative testicular tissue examination to evaluate the presence of sperm was performed by the clinical embryologist. The protocol included several rinses, microbiology testing, and histology tissue processing. Testicular samples were dissected into 2-4mm^3 portions and cryopreserved in 1ml cryovials using cryoprotectant solution: Hank Balances Saline Solution 5% Human Serum Albumin (CSL Behring LLC) 5% DMSO (Mylan Institutional). A controlled rate freezing machine was used to freeze the samples following the in house validated protocol (26, 31). Cryovials were transferred into vapor nitrogen tanks at the Manufacturing and Development Center (MDC) of Wake Forest Institute for Regenerative Medicine (WFIRM) for long-term storage. A maximum of 20% of stored tissue was used in this study. The remaining portion (80%) was kept for future clinical use.

A portion of the testicular biopsies was fixed in 4% Formalin, 4% Paraformaldehyde, and Bouin fixatives. Tissue was then processed, paraffinized, and mounted by a clinical pathology lab for staining and histology.

Hematoxylin-Eosin staining and immunohistochemical staining with PGP 9.5 (UCHL1) as a spermatogonia marker were performed on 5 micron thick tissue sections using an automated stainer. Immunostaining was performed using the Leica Microsystems Bond 3 autostainer at the Wake Forest University clinical pathology laboratory. After 20 minutes of antigen retrieval using Bond Epitope Retrieval Solution 2 (ER2), the primary antibody (PA0286; mouse monoclonal PGP9.5 (UCHL1)) was incubated for 15 minutes at room temperature. To detect and visualize the antigen, a Bond Polymer Refine Detection kit (peroxide block, post-primary, polymer reagent, DAB chromogen/Leica, DS9800, followed by hematoxylin counterstain) was used. Counterstaining of hematoxylin identified the nucleus of cells. Isotype control (mouse IgG) was used as a negative control for the primary antibody. Age-matched controls (testes biopsies from 46 XY patients *via* National Disease Research Interchange, NDRI) were used to compare testicular morphology. Microscopic images were acquired using LEICA DM4000B microscope, Olympus camera DP73, and Olympus Cellsens software.

Cell isolation, culture, and cryopreservation

Testicular cells were isolated from cryopreserved testicular tissue. The process was performed under pre-clinical Good Laboratory Practice (GLP)-conditions following a protocol previously described by our group (30, 32, 33) with some modifications for potential clinical application of human SSCs isolation and culture. Selected cryovials were thawed uniformly by immersion in warm water. Immediately after, DMSO was washed out from the tissue using 1x MEM 8μg/ml DNAse (Roche). External connective tissue was removed, and single seminiferous tubules were dissected apart using a dissecting microscope (Leica S6D) and jeweler tweezers.

Tissue was transferred into a 1.5 ml Eppendorf tube due to the small sample size and resuspended in an enzymatic digestion solution made of: 1x MEM; 12μg/ml DNAse (Roche); 0.4 PZU/ml Collagenase NB4 Standard Grade (SERVA); 0.02 DMCU/ml Natural Protease NB (SERVA). Samples were incubated in a shaking water bath at 120 rpm and 32°C. The enzyme mix was exchanged after every hour of incubation. Tubules and cells were pelleted by centrifugation at 16g for 5 min. A sample of the pellet was examined under the microscope, and the process was repeated with fresh enzymatic solution until disassociation of the tubules was confirmed by these examinations.

Once the enzymatic digestion disrupted the seminiferous tubules and many single floating cells were visible floating, the sample was pipetted up and down energetically to help release the remaining cells. The tube was then centrifuged for 5 min at 350g (1500 rpm) with a brake. The resultant supernatant was discarded, and pellets containing released cells were used for culture.

During the isolation process released tubular cells might form tight clumps. In these cases, it was helpful to additionally incubate the sample in 0.25% Trypsin-EDTA (FisherScientific-Gibco) at 37°C for 5-30 minutes, checking every 5 minutes until a single cell suspension was observed again. Then the sample was

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TABLE 1 Stempro Complete culture media composition.

Reagent	Company	Catalog #	Final concentration
Stem Pro-34 SFM	Invitrogren	10639-011	–
Stem Pro Supplement	Invitrogren	10639-011	–
Bovine Albumine	Roche	1.07E+10	5 mg/ml
D(+) Glucose	Sigma	G7021	6 mg/ml
Ascorbic acid	Sigma	A4544	1x 10-4
Transferrin	Sigma	T1147	100 µg/
Pyruvic acid	Sigma	P2256	30 mg/ml
d-Biotin	Sigma	B4501	10 µg/ml
2-beta Mercatoethanol	Sigma	M7522	5x 10-5
DL-lactic acid	Sigma	L4263	1 µl/ml
MEM-non essential	Invitrogen	11140-035	10 µl/ml
Stem Pro Supplement	Invitrogen	10639-011	26 µl/ml
Insulin	Sigma	I1882	25 µg/ml
Sodium Selenite	Sigma	S1382	30 nM
Putrescine	Sigma	P7505	60 µM
L-Glutamine	Invitrogen	25030-024	2 mM
MEM Vitamine	Invitrogen	11120-037	10 µl/ml
b-Estradiol	Sigma	E2758	30 ng/ml
Progesterone	Sigma	P8783	60 ng/ml
Human EGF	Sigma	E9644	20 ng/ml
Human bFGF	Sigma	F0291	10 ng/ml
Human LIF	Chemicon	LIF1010	10 ng/ml
GDNF	Sigma	G1777	10 ng/ml
FCS	Invitrogen	10106-169	1%
Pen/Strep	Invitrogen	15140122	0,5%

centrifuged again for 5 min at 350g (1500 rpm) with brake, trypsin supernatant was removed, and the pellet containing released cells was used for culture.

The final pellet of cells was resuspended on MEM 10% FBS for culture. This culture media was used to improve the initial attachment of cells. Hematocytometer should be used to assess cell numbers in the final sample and all the previously removed supernatants to ensure no cell is wasted. Viable cells were seeded at 10.000 cells/cm^2 on plastic plates (Falcon) and kept in an incubator constant 37°C 5% CO2 in a room with positive air pressure GLP conditions. The next day, culture media was switched from MEM 10% FBS into an enriched StemPro medium, Table 1 (32, 33), previously reported to support testicular cell propagation *in vitro*.

During the culture, samples were checked every other day. The media was refreshed every 2-3 days. Cells were passaged and split when cell confluency approached 80% using 0.25% Trypsin-EDTA (FisherScientific-Gibco).

After every passage, when possible, a portion of cells were cryopreserved for backup in a 2ml cryotube (Sigma Aldrich). Cryopreservation media used was MEM 20% FBS and 8% DMSO, and Mr.Frosty (Nalgene) at -80˚C was used for slow freezing overnight. The next day, cryotubes were transferred into liquid nitrogen tanks for long-term storage.

Quantitative reverse transcriptase polymerase chain reaction

RNA was extracted using RNEasy Minikit (Qiagen) from Snap Frozen tissue or cells. The quality and quantity of extracted RNAs were tested with a spectrophotometer (Nanodrop 2000, ThermoFisher). RNA was then converted to cDNA using Reverse Transcriptase Kit (Life Technologies) and through the following thermocycler (Simpli amp thermal cycler, life technologies) conditions: 25°C for 10min, 37°C for 120min, 85°C for 5 min, and 4°C hold.

The cDNA samples underwent PCR amplification using Taqman primers (Table 2) and applied Biosystems 7300 Real Time PCR system. The cycling conditions followed were 95˚C for 10 minutes, then 40 cycles of 95˚C for 15 seconds, and 60˚C for 1 minute.

All primers were chosen intron spanning and previously tested for not amplifying genomic DNA. A minus RT (water)

TABLE 2 Taqman assay primers used for PCR.

Gene	Catalog #	Primer length
UCHL1	Hs00985157 m1	80
ZBTB16	Hs00957433 m1	65
THY1 (CD90)	Hs00174816 m1	60
PRM1	Hs00358158 g1	99
GATA4	Hs00171403 m1	68
Clusterin	Hs00971656 m1	93
CD34	Hs02576480_m1	63
STAR	Hs00264912 m1	85
TSPO	Hs00559362 m1	57
CYP11A1	Hs00897320 m1	81
POLR2A	Hs00172187 m1	61

control has been used to rule out any contamination in PCR mixtures (not shown in the gel picture, Figure 4). POLR2A was selected as a housekeeping gene, and the expression of genes were normalized to this gene; relative expression was determined with the Delta CT method.

Amplified cDNA from RT qPCR was later used for an Electrophoresis study on a gel to visualize the specific product bands. A 2% Agarose gel was used, given that our target primers were between 50-120 bp in length (Table 2). Ethidium Bromide was included in the gel formulation at a concentration of 5ul/100ml. After the Samples and DNA ladder were loaded, a 140V Voltage (Enduro power supply, Labnet) was applied for 20 mins when the DNA dye reached 2/3 of the total gel size. Images of the gel were taken using a UV light Camera system (Gel logic 200 imaging system).

Digital reverse transcriptase polymerase chain reaction

Digital PCR is a molecular biology tool that evenly distributes sample cDNA along with RT PCR mix into independent microchip wells. Then independent thermocycling reactions are conducted separately in each well prior to fluorescent signal reading. Digital PCR is especially useful for small population assessment or rare events analysis. This system has been validated by both manufacturer and independent researchers (34), even in a clinical setting (35, 36).

To estimate the percentage of undifferentiated spermatogonia during the culture, we used a Digital PCR system (Quant Studio, Life Technologies) and Taqman assays labeled with VIC (for POLR2A as housekeeping gene) and FAM for target gene (ZBTB16). As each mammalian cell has a range of 10–30 pg of total RNA, we used the cDNA made from 50 ng of total RNA in 20 000 wells of a digital PCR chip for each assay.

The chips were loaded following the manufacturer's protocol with commercially available dPCR Master Mix (Life Technologies), water, cDNA, and Taqman primers. cDNA for this project was obtained using retrotranscriptase reaction on RNA extracted from Snap Frozen cells. Cycling conditions were: 95˚C for 10 minutes, then 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute.

Flow cytometry analysis and MACS-sorting

The population of putative spermatogonial stem cells in culture was estimated as HLA-ABC-/CD9+/CD49f+ population (37–41) using BD Accuri C6 Flow cytometry system without sorting. BD antibodies were used (Table 3) at a concentration of 5ul of antibody per 50.000 cells in 100ul Flow Cytometry buffer (PBS 1% FBS). Cells were incubated with the antibody for 30 minutes at room temperature and then washed with flow cytometry buffer. Obtained data were analyzed using BD Accuri software. Unstained cells and cells incubated with isotypes control antibodies (Table 3) were used to optimize channel compensation and negative control. In every condition, 10,000 events were evaluated.

The same staining method was used in separate experiments to perform Fluorescence Activated Cell Sorting using BD FACS ARIA. Cells expressing HLA-ABC-/CD9+/CD49f+ were sorted and used for FISH analysis.

TABLE 3 Antibodies used for Flow cytometry and Magnetic-activated cell sorting (MACS).

Antigen	Reactive species	Raised in	Flourochrome	Company	Catalog #
HLA-ABC	Anti-Human	Mouse	FITC	BD Biosciences	555552
CD9	Anti-Human	Mouse	PE	BD Biosciences	341637
CD49f (Integrin alpha 6)	Anti-Human	Mouse	APC	Thermofisher	17-0495-80
Mouse IgG			FITC	BD Biosciences	340755
Mouse IgG			PE	BD Biosciences	340756
Mouse IgG			APC	BD Biosciences	340754
FcR Blocking Reagent	Anti-Human		MACS Microbeads	Miltenyl Biotec	120-000-442
Anti-PE	Anti-Human		MACS Microbeads	Miltenyl Biotec	120-000-294

Single-cell RNA sequencing

Cultured and cryopreserved testicular cells from XXY and XY adolescent individuals were sent from WFIRM to UCLA. Upon arriving at the Lundquist Institute of Harbor-UCLA Medical Center, testicular cells were revived and cultured in a 25 cm^2 flask with 4 ml of enriched StemPro-34 medium/flask for five days. Cells were harvested, washed, resuspended in 0.04% BSA in PBS, and delivered to UCLA Technology Center for Genomics and Bioinformatics (TCGB). The cell concentration and viability were determined at TCGB using a countess II automated cell count. Per manufacturer recommendations, a single cell suspension (1000 cells/μL) from each sample was loaded onto the 10x Chromium chip and controller. Ten thousand cells were targeted for capture per sample. Cell capture and 10X single cell 3' gene expression sequence library preparation were performed. The resultant library was sequenced using a NovaSeq 6000 SP (100 Cycles). Mapping, cell identification, and clustering analysis were performed using 10x Cell Ranger software at the UCLA-TCGB.

For the data analysis, raw count matrices generated from 10X Genomics and Cell Ranger were imported to the Seurat package in R, where cells were screened based on QC metrics (42). An object of class Seurat 19618 features across 9580 cells of XY testicular sample and 19284 features across 11845 cells of XXY sample were subject to data analysis. Data normalization, scaling, and feature selection was performed as described in the SeuratV3 procedure. These were followed by unsupervised cell clustering and a UMAP analysis (43, 44). Differential gene expression among clusters was determined in Seurat using a Wilcoxon rank sum test, and gene expression probability across clusters was visualized with VlnPot and FeaturePlot.

X and Y chromosome fluorescent *in situ* hybridization

After cells in culture had been trypsinized, fifty thousand cells were cytospin for 10 min at 1000 RPM on glass poly-l-lysine slides (Cytopro, ELI Tech Biomedical Systems) using the cytopro 7620 cytocentrifuge system (Wescor). The slides were left to dry at room temperature overnight. In parallel, testicular tissue samples from the same patients archived in pathology were processed by for the same X and Y hybridization.

Slides were soaked in 2X saline sodium citrate (made from stock 20xSSC from ABBOTT/VYSIS Company) at 37°C for 35 minutes. Subsequently, cells were incubated in Pepsin (AVANTOR PERFORMANCE MATERIALS, INC 2629, company) 0.5mg/ml solution of HCl 0.1M at 37 degrees C for 35 minutes. Slides were then washed at room temperature in 1XPBS for 5 minutes, and Post-Fixation Solution (0.9% formaldehyde W/V; 4.5 mg/ml MgCl2 in PBS) was added for 5 minutes incubation at room temperature. Slides were re-washed at room temperature 1XPBS for 5 minutes, and the dehydration process was performed by submerging slides in increasing concentrations of ethanol (70%, 80%, 100%) for 1 minute each at room temperature.

A working solution of X and Y chromosome probes (EMPIRE GENOMICS Company kit 1:1:4 probe buffer dilution) was added to the sample, and slides were kept at 75°C for 5 minutes, followed by overnight incubation at 40°C (16 Hours minimum). The following day, slides were washed in 0.3% IGEPAL/solution (SCI-GENE) on 0.4X saline sodium citrate/for 2 minutes at 73° C and 0.1% IGEPAL (SCI-GENE) solution on 2X saline sodium citrate for 1 minute at room temperature.

Slides were finally mounted with ABBOTT/VYSIS DAPI II and coverslipped.

Imaging of the slides was performed using a Zeiss Axiophot microscope and Applied Spectral Imaging Software.

Molecular karyotyping with next generation sequencing

The cryopreserved cultured cells were transported from WFIRM to AdvaGenix and Johns Hopkins Medicine lab. After DNA extraction, fifty to 100 ng of amplified DNA underwent library preparation (Thermo Fisher Scientific, Waltham, MA). First, the pooled DNA samples underwent enzymatic shearing to produce fragment sizes of approximately 200 bp. The DNA fragments were purified using an AMPure Bead (Beckman Coulter, Sharon Il) wash, stabilized by the ligation of adapters and barcodes at either end of each fragment, and size selected using a second AMPure Bead wash. Each DNA fragment was bound to one ion sphere particle (ISP) and amplified thousands of times using an emulsion PCR reaction. The positive template ISPs were recovered using Dynabeads MyOne Streptavidin CI bead washes (Invitrogen, Carlsbad, CA). Following recovery, sequencing primers and Ion Hi-Q sequencing polymerase were added to the samples, and the samples were loaded onto a sequencing chip and analyzed at a depth of 1X across the entire genome by a Personal Genome Machine (PGM) or S5 (Thermo Fisher Scientific, Waltham, MA). Sequencing data were processed by a Torrent Browser Server (Thermo Fisher Scientific, Waltham, MA) to provide initial sequencing information and ensure adherence to our required quality assurance metrics. The data was then transferred to an Ion Reporter Server (Thermo Fisher Scientific, Waltham, MA) for comprehensive data analysis and interpretation. The PGM or S5 sequencing provided a minimum of over 3.5 million reads with a median sequencing fragment length of 181 bp.

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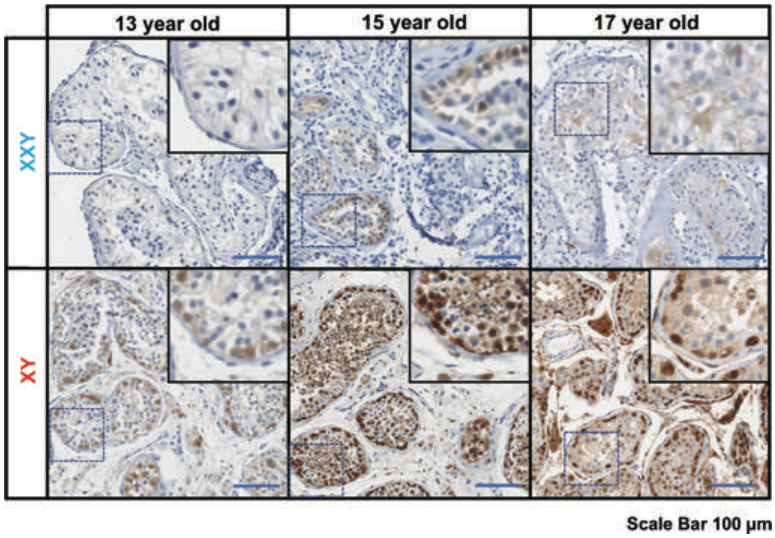

FIGURE 1
Immunostaining for undifferentiated spermatogonia marker UCHL1 (PGP 9.5), subjects' ages: 13, 15 and 17 years old. On the top panel, three Klinefelter patients included in the study. On the bottom panel, three aged-matched controls. Scale bar 100µm.

Results

Presence of undifferentiated spermatogonia in TESE negative KS patients' testes

Standard of care at Wake Forest testicular tissue bank includes histologic analysis and pathology review of all samples. Every KS patient case is discussed and compared with age-matched controls from our archives. Three KS patients enrolled in the testicular tissue bank were selected for this study (13 years old, 15 years old, and 17 years old). Pathology slides from testicular biopsy stained immunohistochemically for PGP 9.5 (UCHL1) and Hematoxylin-Eosin and compared with age-matched controls (Figure 1).

In all KS subjects, most seminiferous tubules were either hyalinized or only contained Sertoli cells. Rarely, some tubules contained the cells expressing the PGP 9.5, a marker for undifferentiated spermatogonia, with no further spermatogenic cells such as spermatocytes and spermatid. Leydig cell hyperplasia was prominent between tubules. No evidence of germ cell neoplasia or carcinoma *in situ* was observed. In contrast, age-matched controls presented a well-organized tubular architecture containing spermatogonia at different stages of differentiation into elongated spermatids.

Given that sperm were not found using micro TESE in any of the three patients (TESE negative), they were considered ideal candidates to benefit from SSCs isolation and culture strategy.

Long term *in vitro* propagation of KS testicular cells

Compared to previous experiences by our group in isolating cells from human testes, KS samples presented as a different consistency. Tubules were thickly packed and more difficult to dissect. In our hands, pieces took between 2-2.5 h of enzymatic digestion to release cells, but it varied from sample to sample. Once the isolation method was tuned and optimized for KS tissue, cell isolation yield was comparable to XY age matched controls. Similarly, the morphology of cells in short- and long-term cultures was similar to previous testicular cells cultured from euploidy patients (Figure 2).

Isolated testicular cells from KS patients remained viable in culture for more than 90 days. For the first eight days in culture, the number of cells did not significantly increase. Cells then started growing exponentially until the end of the study. The number of cells expanded more than 100 million fold within 90 days (Figure 3). Cell viability was always ≥ 95%, as determined by trypan blue staining at each passage. These findings are

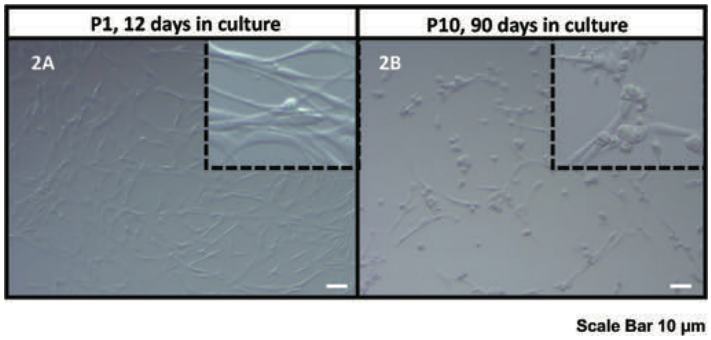

FIGURE 2
Bright field microscope images of the human KS testicular cells in culture at passage 1, 12 days in culture **(A)** and passage 10, 90 days in culture **(B)**. As it was also reported previously (30, 32, 33), the current culture system includes both spermatogonia and testicular somatic cells. Somatic cells usually attach earlier forming an extensive network of plain elongated cells. Then round spermatogonia cells attach forming clusters on top of somatic cell feeder layer (showed in inserts). Scale Bar 10 µm.

comparable to previous data from our group in propagating testicular cells from peripubertal euploid patients (32).

Presence of undifferentiated spermatogonia and somatic cells in culture

After testicular cell isolation, a heterogeneous mixture of cells is expected in this culture system. Somatic cells are expected to quickly attach to the culture surface and form a feeding layer that provides an excellent environment for spermatogonia propagation. It is critically important to confirm the presence of all significant testicular cell types during the culture period, as a misbalance could prevent the other cell types from expanding.

qPCR analysis was used to confirm the presence of characteristic gene expression from the main four testicular cell types expected in culture: undifferentiated spermatogonia (UCHL1, ZBTB16, and THY1), Leydig (TSPO, STAR, CYP11A1), Sertoli (GATA4, Clusterin) and peritubular cells (CD34) (Figure 4). A

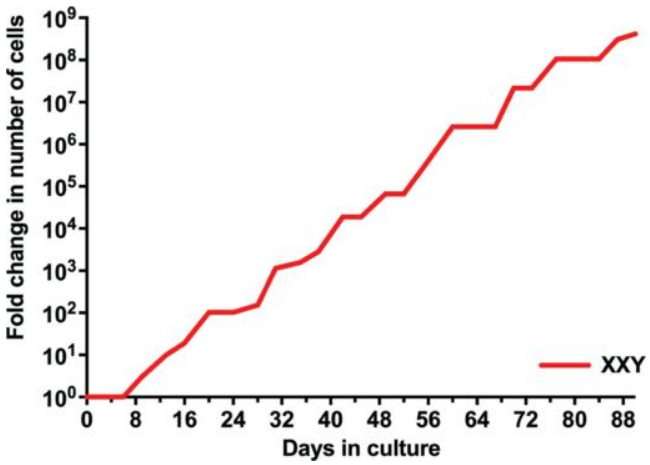

FIGURE 3
Evolution of the number of isolated human KS testicular cells in culture. Results show the average from KS subjects' testicular tissue. The initial number of cells was standardized to 1 to better assess growth along time in the graph and compare subjects with different initial cell numbers.

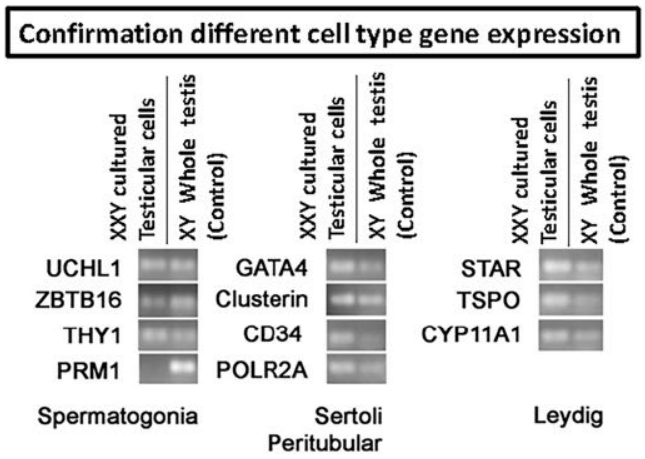

FIGURE 4
RT-PCR to confirm the presence of the four main testicular cells in culture based on cell-specific gene expression: spermatogonia (UCHL1, ZBTB16, THY1, PRM1), Sertoli (Gata4, Clusterin), peritubular (CD34) and Leydig (STAR, TSPO, CYP11A1). POLR2A was used as a housekeeping gene for internal control. Results confirmed the presence of all four common cell types. However, differentiated germ cell marker PRM1 remained negative, indicating the undifferentiated status of the germ cells in culture.

positive signal for all of these was obtained from cultured cells. Conversely, the expression of differentiated germ cell marker PRM1 remained negative (Figure 4). This demonstrated that spermatogonia remained undifferentiated and capable of maintaining a putative SSCs population suitable for transplantation.

After confirming the phenotypes of cells present in the culture, the focus moved to determining the percentage of spermatogonia throughout the culture period. Digital PCR analysis was performed (Figure 5) on isolated testicular cells from frozen tissue with 2.3% of cells shown to be ZTBTB16^{+}. This population grew during the initial stages of culture, climbing from 20- 37% between days 13-66. In the culture's late stages, the percentage of undifferentiated spermatogonia dropped to 14% but remained present throughout the entire culture period (Figure 5). Therefore, our culture system for Klinefelter testicular cells propagated every cell type expected

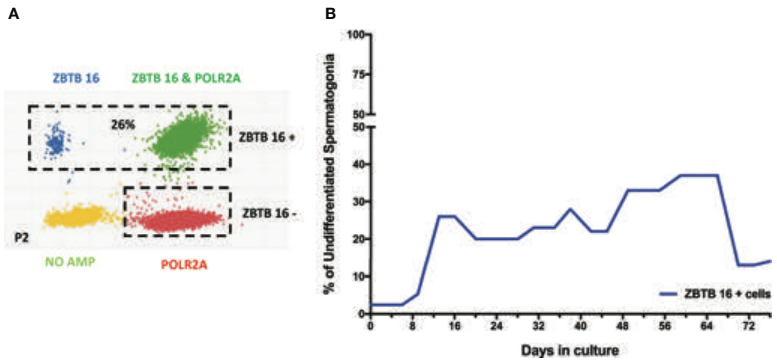

FIGURE 5
Digital PCR analysis was used to assess the population of undifferentiated spermatogonia expressing ZBTB16 (PLZF) marker and housekeeping gene POLR2A in testicular cells in culture: After 2nd passage from 15-year-old KS patient **(A)**. The same analysis was repeated at different time points. The graph shows the average undifferentiated spermatogonia population during the culture of testicular cells from KS patients **(B)**.

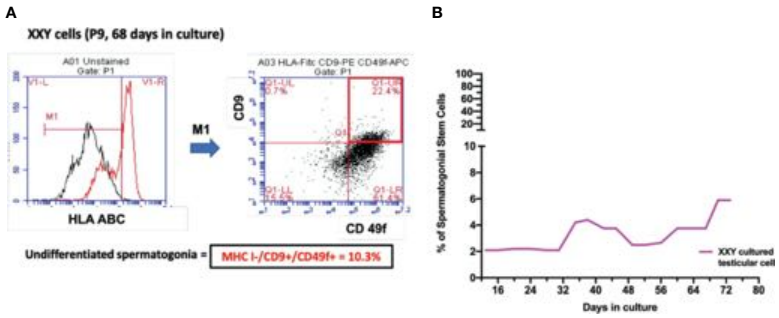

FIGURE 6
(A) Spermatogonial stem cells (SSC) population was estimated by combining HLA-/CD9+/CD49f+ markers on Flow Cytometry analysis. After 68 days in culture and nine passages, the percentage of putative SSC was 10.1% of a 17-year-old KS patient **(B)**. Several time points were analyzed to evaluate the evolution of this population over time in all subject samples in culture. The graph shows the average of the SSCs population during the culture in KS patients **(B)**.

while maintaining a significant population of undifferentiated spermatogonia throughout the 90 days culture.

The constant presence of SSCs during the culture

At the time of transplantation, only SSCs are expected to migrate to the basal membrane of the seminiferous tubules and restore spermatogenesis in azoospermic patients. So far, the scientific community has struggled to identify a single marker that could fully characterize this population of cells. Meanwhile, a combination of HLA-ABC -/CD9 +/CD49f + has been postulated as markers for the enrichment of SSCs and predicting therapeutic success following transplantation (38–41, 45).To assess putative SSCs in culture, flow cytometry analysis was utilized.

Successfully propagated testicular cells from KS patients presented putative SSCs at every analyzed time point (Figure 6). Quantitative analysis suggested a consistent population in culture, representing between 2-10% of cells in the culture. This indicates that our culture system not only promotes cell propagation, but also provides an excellent environment for maintaining SSCs phenotype, *in vitro*. These results align with prior data using dPCR that identified viable undifferentiated spermatogonia within the culture.

ID4 positive cells in culture as an SSCs sub-population

To further characterize the cells in culture, ScRNA Seq analysis was carried out. Cell partitioning *via* UMAP identified 6 clusters in cultured XY and 7 clusters in cultured XXY testicular cells. As shown in Figure 7, the ID4, TCN2, and NANOS 3 positive SSCs (46–48) are predominately located within clusters 0 and 1. The data showed that ID4 positive SSCs were more abundant in the XY testicular cell culture than in the XXY culture. The number of TCN2 and NANOS 3 positive SSCs was similar between XY and XXY testicular cell cultures. This data demonstrated that our human testicular cell culture system could provide a favorable environment for SSCs to grow and support survival, *in vitro*.

The stable genotype of SSCs in culture

NGS technology has been successfully established to assess chromosome copy number and integrity by applying linear regression to the amplification of chromosome-specific sequences for 24-chromosome aneuploidy screening (49). In this study, chromosome copy number analysis was essential to address the concern of genotype instability of cells in culture.

Fluorescence-activated cell sorting (FACS) was used to isolate putative SSCs using the markers HLA$^-$ABC. $^-$/CD9 $_+$/CD49f $^+$ and compared to peripheral blood XX and XY controls. Results showed no significant difference in the number of somatic chromosomes using up to a 10% confidence filter (Figure 8). Sexual chromosome analysis identified an XXY sample compared to XX and XY controls. The sample was finally characterized as non-mosaic 47XXY.

These data suggested that cells in culture remained karyotypically stable. These findings are interpreted as a favorable indication of clinical application potential. The same experiment was conducted on non-sorted cells with equivalent results (data not shown).

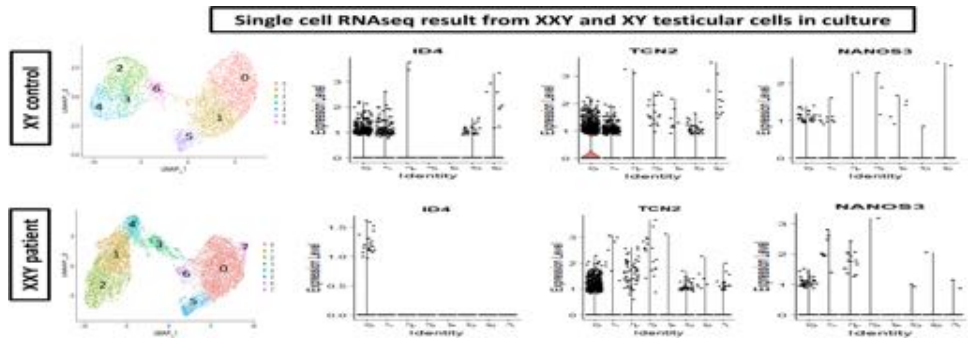

FIGURE 7
Single cell RNAseq analysis of cultured human XY (upper panel) and XXY (lower panel) testicular cells. Cell partitioning *via* UMAP identified 6 clusters in cultured XY and 7 clusters in cultured XXY testicular cells. Expression patterns of ID4, TCN2 and NANOS 3 (violin plot) supported the presence of SSCs during the culture.

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization (FISH) staining for X and Y chromosomes was performed to demonstrate the presence of KS XXY cells in culture. The probes used for this study marked the X chromosome with a red signal (5-ROX) and the Y chromosome with a green signal (5-Fluorescein).

The initial goal of this analysis was to confirm the aneuploidy of the cells in culture. Cytospun slides were systematically examined, XXY cells were identified. Additionally, XY and XX and their dividing counter partners were also seen (Figure 9A).

After these findings were confirmed, we hypothesized that although all of the patients included in this study had been clinically diagnosed as non-mosaic 47XXY Klinefelter, we might have some mosaicism in the testicular cell culture. An experiment was designed to analyze FISH slides in consecutive passages to identify different mosaic populations and describe their evolution in culture over time. Moreover, when the total cell number was sufficient, FISH staining was performed in parallel in CD9+ Magnetic Activated Cell Sorter (MACS) cells and non-sorted controls.

Results showed that even at the earliest time point at four days, there was a small population of both XY (1.4%) and XX

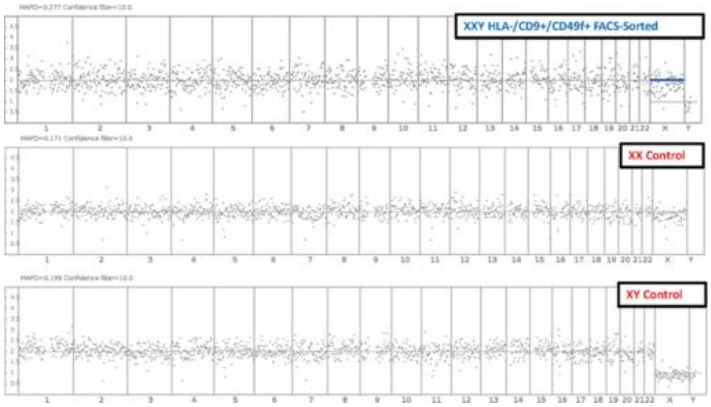

FIGURE 8
Molecular Karyotyping by next-generation sequencing. After 47 days and 6 passages, Klinefelter cells in culture were FAC Sorted for putative SSC markers HLA-/CD9+/CD49f+. Then cells were analyzed with next generation sequencing and compared to XX and XY controls. Results identified cells in culture as 47 XXY with a 10% confidence filter.

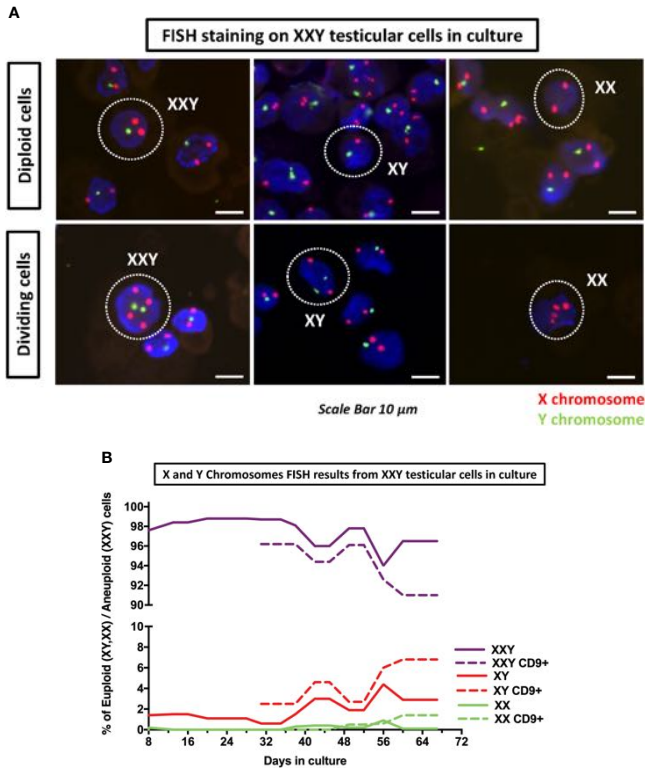

FIGURE 9
DNA FISH analyses of cultured cells was used to assess the number of copies of X (red probe) and Y (green probe) chromosomes on KS testicular cells in culture. **(A)** Detection of XXY, XY and XX in culture as well as their respective dividing counterparts. **(B)** Quantification of XXY, XY and XX populations in consecutive passages during the culture. The results showed a consistent mosaic population (solid lines). Moreover, when cells in culture were enriched for undifferentiated spermatogonia marker CD9+ using MACS, both XY and XX population increased (dot lines) suggesting that higher number of euploid cells in spermatogonia population.

(0.2%) (Figure 9A). As time passed, both populations thrived and came to represent (4.4%) and (0.9%) respectively. Furthermore, when cells were MACS sorted using CD9 to enrich spermatogonia, XY and XX populations were enriched up to 6.8% and 1.4%, respectively (Figure 9B). These findings confirmed that small populations of XY and XX cell mosaicism among XXY cells in culture and suggested XX and XY cells were preferably spermatogonia. This is critical, as euploid spermatogonia may impact the product of spermatogenesis and might, at some level, provide an explanation for the presence of healthy offspring produced from azoospermic KS patients with intratesticular sperm available.

A question was raised about the possibility of KS patients presenting some congenital testicular mosaicism that had remained undiagnosed with standard clinical Karyotype techniques. FISH staining was then performed in testicular tissue histology slides from the same patients. The results showed most cells presenting XXY signal. However, few XX and XY cells were found inside the few preserved seminiferous tubules (Figure 10). In the basal membrane from one of the tubules, few XY cells were found to be dividing. These findings strongly support some mosaicism in the testes of non-Mosaic 47 XXY KS patients diagnosed by karyotyping.

Discussion

Although testicular morphology was severely affected by fibrosis in all KS subjects, PGP 9.5 positive spermatogonia cells were still found inside several seminiferous tubules (Figure 1). Following isolation and culture of testicular cells from all three subjects, undifferentiated spermatogonia cells were positive for PGP 9.5 ZBTB16 and THY1. These findings suggest that even in patients with negative IHC for spermatogonia, viable

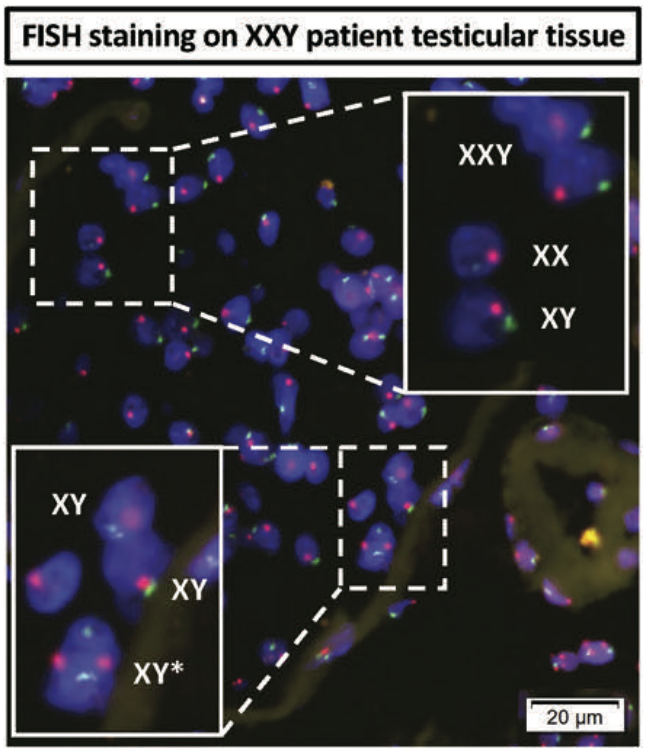

FIGURE 10
DNA FISH analyses of KS testicular tissue was used to assess any mosaicism in KS testes: X (red probe) and Y (green probe) chromosomes. The top magnified region showed all three XXY, XY and XX cells in non-mosaic KS subject. The bottom magnified region shows an XY cell actively dividing in the basal membrane of seminiferous tubules of non-mosaic KS subject.

spermatogonia are likely present in the cryopreserved samples. Cell isolation and culture under optimal conditions may be able to selectively expand these spermatogonia for fertility treatments. The age of the subjects and other developmental factors may be vital in predicting the success of spermatogonia retrieval and optimizing cell culture.

SSC transplantation is the definitive test for SSCs identification. However, regulatory limitations do not allow for human SSC transplantation. The connection between HLA-/CD9+/CD49f+ enriched cells and SSC transplantation success has long been characterized in autologous animal models and xenotransplantation (37–41). The presence of SSC in our culture system has previous already been tested using xenotransplantation into nude mice (32). We feel that SSC characterization using FACS and RNAseq can reasonably assess the SSC population and may predict SSC transplantation efficacy without the economic costs, ethical challenges, and time associated with low efficient xenotransplantation.

By reaching a 20 million-fold increase in the number of cells in culture, the goal of *in vitro* propagation of spermatogonia cells was achieved. The number of cells isolated per biopsy varied between subjects from 275,000 to 400,000, and the propagation culture system could provide around 5.5 and 8 trillion cells. Conservatively estimating the enriched population of SSCs (HLA-ABC-/CD9 +/CD49f+ or ID4 +) as 2% of propagated testicular cells, 110 billion SSCs could be provided after 50 days of culture. Based on previous studies in non-human primates (19, 21, 22), these numbers of cells might potentially enable SSC transplantation. An even lower number of cells might be needed for *in vitro* spermatogenesis (45, 50). Another advantage to this strategy is that only XY SSCs from KS subjects (1% of all propagated SSCs) are likely to go through complete spermatogenesis. Also worth mentioning is that a maximum of 20% of the original testicular biopsy sample was used for this project, as 80% remained untouched for future clinical applications. When the full samples are used, the starting number of cells in culture will increase, and the duration of culture reduced.

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Due to characterization challenges, it has been difficult to quantify the SSC population *in vivo*. However, recent reports by Brinster and Kubota (15) in different mammals estimated this population to be somewhere between 0.01-12.5% of spermatogonia. Using HLA-/CD9+/CD49f+ markers, a population of 2-10% putative SSC was identified in culture. RNAseq data provided confirmation of SSC presence, identifying ID4, TCN2, and NANOS 3 positive clusters of cells. These findings correlate with current literature describing SSC dynamics and their cellular niche, which could indicate an *in vitro* system mimicking *in vivo* physiology (38, 40, 41, 46, 47, 51). Although further research is required on this topic, we expect these data to help optimize the timing between testicular cell culture and SSC transplantation.

Different hypotheses may explain the fluctuation in the number of undifferentiated spermatogonia in culture. It is possible that somatic cells, although able to be maintained in culture long term, do not have the ability to propagate indefinitely. Therefore, somatic testicular cells become less proliferative and efficient in supporting the SSCs population.

FISH and NGS analyses were combined to better characterize the chromosomal stability of cells in culture. Molecular Karyotyping by NGS gave a broad overview of chromosomal integrity across hundreds of thousands of cells using a high number of specific gene loci, very reproducible technology, increased statistical power. The technical limitations of statistical analysis provided a 10% confidence filter, meaning that mosaicisms representing less than 10% of the cells could not be detected.

The limitation of detecting mosaicism in cultured cells was overcome using FISH staining for X and Y chromosomes and manually counting over 5000 cells per subject. Using this method, XY and XX cells were identified within the predominantly XXY population, and the ratio of these karyotypes were characterized during the time in culture. Consistent with NGS data, mosaicism never reached 10%, meaning at least 90% of the cells in culture presented a 47XXY karyotype. Up to 10% might present some mosaicism, including XX and XY karyotypes. Therefore, looking to potential clinical fertility applications, preimplantation genetic screening (PGS) should be recommended for these patients. However, our data support a reasonable level of genetic stability and safety in the cells in culture.

Previous studies have shown sex chromosome mosaicism in cultured iPSCs (52). Similar mosaicism was also described by Hirota et al. (45) using iPSC derived from both XXY mouse and human fibroblasts. Cells in our culture system were not immortalized and achieved long-term culture, up to 100 days. Future studies should compare different cell sources in parallel to verify which options presents the greatest chromosomal stability in culture.

Another interesting finding was that observation of XY and XX cells, even at the earliest timepoint (day four) in our culture system. Similar FISH staining was performed on testis tissue from the same patients before isolation to validate this data. A general review of the slides identifed both XX and XY cells. This fact contradicted previous clinical genetics reports that diagnosed these subjects as non-mosaic Klinefelter 47 XXY. Standard clinical karyotyping usually analyzes no more than 20 cells in peripheral blood smear and cannot rule out organ-specific mosaicism. Although these procedures are beneficial for diagnosing minor defects on the chromosomes constantly present in cells, it lacks the sensitivity to detect small mosaic populations. Further studies are needed to systematically analyze the histology and establish a better quantification method for testicular mosaicism of the KS patient testis, *in vivo*, and its fertility repercussions.

To assess the impact of this finding for future KS fertility treatments, the suitability of KS testis for transplantation should be evaluated. Previous reports by Lue et al. (29) successfully demonstrated SSC transplantation from XY mice into the seminiferous tubules of azoospermic young adult XXY mice. This study suggests that azoospermic KS adult testis, although severely fibrotic, may still retain the basic seminiferous architecture required to perform SSC transplantation. Subsequent studies have shown SSC transplant efficacy in structurally defective seminiferous tubules (53), even without prior germ cell ablation (54) Nevertheless, more studies are needed to confirm that these animal model results are reproducible in humans.

On the other hand, Hirota et al. (45) successfully transplanted primordial iPSC derived germ cell-like cells from XXY mouse fibroblasts into the seminiferous tubules of azoospermic KS mice. Donor XXY SSCs successfully migrated into the basal membrane and restored spermatogenesis. Fertilization and healthy offspring were achieved by ICSI using testicular sperm (45). Our current study suggests that the use of primary human SSCs may provide a better strategy for clinical fertility applications.

Conclusions

To the best of our knowledge, this is the first report of *in vitro* propagation of human SSCs in long-term culture. This study describes the dynamic chromosomal changes in primary testicular cells from KS subjects in culture. This is a critical step forward in utilizing SSC technology to preserve fertility in KS patients.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

This study was reviewed and approved by Wake Forest School of Medicine, IRB00021686 and IRB00061265. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author Contributions

Conceptualization, GG, SK, CW, RS, YL and HS-A. Methodology, GG, YL and HS-A. Software, GG and HS-A. Validation, GG, MP, WK, YL and HS. Formal analysis, GG,ND, WK, YL and HS-A. Investigation, GG,ND, NZ, YL and HS-A. Resources, GG,YL and HS. Data curation, GG,YL and HS-A. Writing—original draft preparation, GG, YL and HS-A. Writing—review and editing, GG, ND, NZ, MP, SK, CW, RS, WK, SH, AA, YL and HS-A. Visualization, GG,YL and HS-A. Supervision, YL and HS-A. Project administration, HS-A. Funding acquisition, AA, YL and HS-A. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Bonomi M, Rochira V, Pasquali D, Balercia G, Jannini EA, Ferlin A, et al. Klinefelter syndrome (KS): Genetics, clinical phenotype and hypogonadism. *J Endocrinol Invest* (2017) 40(2):123–34. doi: 10.1007/s40618-016-0541-6

2. Nielsen J, Wohlert M. Sex chromosome abnormalities found among 34,910 newborn children: results from a 13-year incidence study in arhus, Denmark. *Birth Defects Orig Artic Ser* (1990) 26(4):209–23.

3. Bojesen A, Juul S, Gravholt CH. Prenatal and postnatal prevalence of klinefelter syndrome: A national registry study. *J Clin Endocrinol Metab* (2003) 88(2):622–6. doi: 10.1210/jc.2002-021491

4. Aksglaede L, Wikstrom AM, Rajpert-De Meyts E, Dunkel L, Skakkebaek NE, Juul A. Natural history of seminiferous tubule degeneration in klinefelter syndrome. *Hum Reprod Update* (2006) 12(1):39–48. doi: 10.1093/humupd/dmi039

5. Wikstrom AM, Hoei-Hansen CE, Dunkel L, Rajpert-De Meyts E. Immunoexpression of androgen receptor and nine markers of maturation in the testes of adolescent boys with klinefelter syndrome: evidence for degeneration of germ cells at the onset of meiosis. *J Clin Endocrinol Metab* (2007) 92(2):714–9. doi: 10.1210/jc.2006-1892

6. Shiraishi K, Matsuyama H. Klinefelter syndrome: From pediatrics to geriatrics. *Reprod Med Biol* (2019) 18(2):140–50. doi: 10.1002/rmb2.12261

7. Wikstrom AM, Raivio T, Hadziselimovic F, Wikstrom S, Tuuri T, Dunkel L. Klinefelter syndrome in adolescence: onset of puberty is associated with accelerated germ cell depletion. *J Clin Endocrinol Metab* (2004) 89(5):2263–70. doi: 10.1210/jc.2003-031725

8. Groth KA, Skakkebaek A, Host C, Gravholt CH, Bojesen A. Clinical review: Klinefelter syndrome–a clinical update. *J Clin Endocrinol Metab* (2013) 98(1):20–30. doi: 10.1210/jc.2012-2382

9. Lanfranco F, Kamischke A, Zitzmann M, Nieschlag E. Klinefelter's syndrome. *Lancet* (2004) 364(9430):273–83. doi: 10.1016/S0140-6736(04)16678-6

10. Gonsalves J, Turek PJ, Schlegel PN, Hopps CV, Weier JF, Pera RA. Recombination in men with klinefelter syndrome. *Reproduction* (2005) 130(2):223–9. doi: 10.1530/rep.1.00641

11. Vicdan K, Akarsu C, Sozen E, Buluc B, Vicdan A, Yilmaz Y, et al. Outcome of intracytoplasmic sperm injection using fresh and cryopreserved-thawed testicular spermatozoa in 83 azoospermic men with klinefelter syndrome. *J Obstet Gynaecol Res* (2016) 42(11):1558–66. doi: 10.1111/jog.13090

12. Corona G, Pizzocaro A, Lanfranco F, Garolla A, Pelliccione F, Vignozzi L, et al. Sperm recovery and ICSI outcomes in klinefelter syndrome: A systematic review and meta-analysis. *Hum Reprod Update* (2017) 23(3):265–75. doi: 10.1093/humupd/dmx008

13. Van Saen D, Tournaye H, Goossens E. Presence of spermatogonia in 47, XXY men with no spermatozoa recovered after testicular sperm extraction. *Fertil Steril* (2012) 97(2):319–23. doi: 10.1016/j.fertnstert.2011.11.009

14. Deebel NA, Galdon G, Zarandi NP, Stogner-Underwood K, Howards S, Lovato J, et al. Age-related presence of spermatogonia in patients with klinefelter syndrome: A systematic review and meta-analysis. *Hum Reprod Update* (2020) 26(1):58–72. doi: 10.1093/humupd/dmz038

15. Kubota H, Brinster RL. Spermatogonial stem cells. *Biol Reprod* (2018) 99(1):52–74. doi: 10.1093/biolre/ioy077

16. Sinha N, Whelan EC, Brinster RL. Isolation, cryopreservation, and transplantation of spermatogonial stem cells. *Methods Mol Biol* (2019) 2005:205–20. doi: 10.1007/978-1-4939-9524-0_14

17. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci U S A* (1994) 91(24):11298–302. doi: 10.1073/pnas.91.24.11298

18. Joerg H, Janett F, Schlatt S, Mueller S, Graphodatskaya D, Suwattana D, et al. Germ cell transplantation in an azoospermic klinefelter bull. *Biol Reprod* (2003) 69 (6):1940–4. doi: 10.1095/biolreprod.103.020297

19. Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, et al. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. *Cell Stem Cell* (2012) 11(5):715–26. doi: 10.1016/j.stem.2012.07.017

20. Harkey MA, Asano A, Zoulas ME, Torok-Storb B, Nagashima J, Travis A. Isolation, genetic manipulation, and transplantation of canine spermatogonial stem cells: progress toward transgenesis through the male germ-line. *Reproduction* (2013) 146(1):75–90. doi: 10.1530/REP-13-0086

21. Shetty G, Mitchell JM, Meyer JM, Wu Z, Lam TNA, Phan TT, et al. Restoration of functional sperm production in irradiated pubertal rhesus monkeys by spermatogonial stem cell transplantation. *Andrology* (2020) 8(5):1428–41. doi: 10.1111/andr.12807

22. Shetty G, Mitchell JM, Lam TNA, Phan TT, Zhang J, Tailor RC, et al. Postpubertal spermatogonial stem cell transplantation restores functional sperm production in rhesus monkeys irradiated before and after puberty. *Andrology* (2021) 9(5):1603–16. doi: 10.1111/andr.13033

23. Sadri-Ardekani H, Atala A. Testicular tissue cryopreservation and spermatogonial stem cell transplantation to restore fertility: From bench to bedside. *Stem Cell Res Ther* (2014) 5(3):68. doi: 10.1186/scrt457

24. Gies I, Oates R, De Schepper J, Tournaye H. Testicular biopsy and cryopreservation for fertility preservation of prepubertal boys with klinefelter syndrome: a pro/con debate. *Fertil Steril* (2016) 105(2):249–55. doi: 10.1016/j.fertnstert.2015.12.011

25. Gies I, Tournaye H, De Schepper J. Attitudes of parents of klinefelter boys and pediatricians towards neonatal screening and fertility preservation techniques in klinefelter syndrome. *Eur J Pediatr* (2016) 175(3):399–404. doi: 10.1007/s00431-015-2657-7

26. Sadri-Ardekani H, McLean TW, Kogan S, Sirintrapun J, Crowell K, Yousif MQ, et al. Experimental testicular tissue banking to generate spermatogenesis in the future: A multidisciplinary team approach. *Methods* (2016) 99:120–7. doi: 10.1016/j.ymeth.2016.02.013

27. Lue Y, Rao PN, Sinha Hikim AP, Im M, Salameh WA, Yen PH, et al. XXY male mice: an experimental model for klinefelter syndrome. *Endocrinology* (2001) 142(4):1461–70. doi: 10.1210/endo.142.4.8086

28. Lue Y, Jentsch JD, Wang C, Rao PN, Hikim AP, Salameh W, et al. XXY mice exhibit gonadal and behavioral phenotypes similar to klinefelter syndrome. *Endocrinology* (2005) 146(9):4148–54. doi: 10.1210/en.2005-0278

29. Lue Y, Liu PY, Erkkila K, Ma K, Schwarcz M, Wang C, et al. Transplanted XY germ cells produce spermatozoa in testes of XXY mice. *Int J Androl* (2010) 33(4):581–7. doi: 10.1111/j.1365-2605.2009.00979.x

30. Galdon G, Deebel NA, Zarandi NP, Pettenati MJ, Kogan S, Wang C, et al. *In vitro* propagation of XXY undifferentiated mouse spermatogonia: Model for fertility preservation in klinefelter syndrome patients. *Int J Mol Sci* (2021) 23 (1):173. doi: 10.3390/ijms23010173

31. Zarandi NP, Galdon G, Kogan S, Atala A, Sadri-Ardekani H. Cryostorage of immature and mature human testis tissue to preserve spermatogonial stem cells (SSCs): a systematic review of current experiences toward clinical applications. *Stem Cells Cloning Adv Appl* (2018) 11:23–38. doi: 10.2147/SCCAA.S137873

32. Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt AM. *In vitro* propagation of human prepubertal spermatogonial stem cells. *JAMA* (2011) 305(23):2416–8. doi: 10.1001/jama.2011.791

33. Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, et al. Propagation of human spermatogonial stem cells in vitro. *JAMA* (2009) 302(19):2127–34. doi: 10.1001/jama.2009.1689

34. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The ultimate qPCR experiment: Producing publication quality, reproducible data the first time. *Trends Biotechnol* (2019) 37(7):761–74. doi: 10.1016/j.tibtech.2018.12.002

35. Fehse B, Badbaran A, Berger C, Sonntag T, Riecken K, Geffken M, et al. Digital PCR assays for precise quantification of CD19-CAR-T cells after treatment with axicabtagene ciloleucel. *Mol Ther Methods Clin Dev* (2020) 16:172–8. doi: 10.1095/biolreprod.112.098913

36. Nykel A, Woźniak R, Gach A. Clinical validation of novel chip-based digital PCR platform for fetal aneuploidies screening. *Diagn Basel Switz* (2021) 11(7):1131. doi: 10.3390/diagnostics11071131

37. Nickkholgh B, Mizrak SC, Korver CM, van Daalen SK, Meissner A, Repping S, et al. Enrichment of spermatogonial stem cells from long-term cultured human testicular cells. *Fertil Steril* (2014) 102(2):558–565.e5. doi: 10.1016/j.fertnstert.2014.04.022

38. Zohni K, Zhang X, Tan SL, Chan P, Nagano M. CD9 is expressed on human male germ cells that have a long-term repopulation potential after transplantation into mouse testes. *Biol Reprod* (2012) 87(2):27.

39. Guo Y, Hai Y, Gong Y, Li Z, He Z. Characterization, isolation, and culture of mouse and human spermatogonial stem cells. *J Cell Physiol* (2014) 229(4):407–13. doi: 10.1002/jcp.24471

40. Harichandan A, Sivasubramaniyan K, Hennenlotter J, Poths S, Bedke J, Kruck S, et al. Molecular signatures of primary human spermatogonial progenitors and its neighboring peritubular stromal compartment. *Stem Cells Dev* (2017) 26 (4):263–73. doi: 10.1089/scd.2016.0042

41. Hutter H, Dohr G. HLA expression on immature and mature human germ cells. *J Reprod Immunol* (1998) 38(2):101–22. doi: 10.1016/S0165-0378(98)00032-1

42. Butler A, Hoffman P, Smibert P, Papalexi E, Satija R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* (2018) 36(5):411–20. doi: 10.1038/nbt.4096

43. Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell gene expression data. *Nat Biotechnol* (2015) 33(5):495–502. doi: 10.1038/nbt.3192

44. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, et al. Comprehensive integration of single-cell data. *Cell* (2019) 177(7):1888–902. doi: 10.1016/j.celrep.2018.10.026

45. Hirota T, Ohta H, Powell BE, Mahadevaiah SK, Ojarikre OA, Saitou M, et al. Fertile offspring from sterile sex chromosome trisomic mice. *Science* (2017) 357 (6354):932–5. doi: 10.1126/science.aam9046

46. Sohni A, Tan K, Song HW, Burow D, de Rooij DG, Laurent L, et al. The neonatal and adult human testis defined at the single-cell level. *Cell Rep* (2019) 26 (6):1501–17. doi: 10.1016/j.celrep.2019.01.045

47. Hermann BP, Cheng K, Singh A, Roa-De La Cruz L, Mutoji KN, Chen IC, et al. The mammalian spermatogenesis single-cell transcriptome, from spermatogonial stem cells to spermatids. *Cell Rep* (2018) 25(6):1650–67. doi: 10.1016/j.celrep.2018.10.026

48. Di Persio S, Tekath T, Siebert-Kuss LM, Cremers JF, Wistuba J, Li X, et al. Single-cell RNA-seq unravels alterations of the human spermatogonial stem cell compartment in patients with impaired spermatogenesis. *Cell Rep Med* (2021) 2 (9):100395. doi: 10.1016/j.xcrm.2021.100395

49. Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G, et al. Development and validation of a next-generation sequencing-based protocol for 24-chromosome aneuploidy screening of embryos. *Fertil Steril* (2014) 101(5):1375–82. doi: 10.1016/j.fertnstert.2014.01.051

50. Pendergraft SS, Sadri-Ardekani H, Atala A, Bishop CE. Three-dimensional testicular organoid: A novel tool for the study of human spermatogenesis and gonadotoxicity *in vitro*. *Biol Reprod* (2017) 96(3):720–32. doi: 10.1095/biolreprod.116.143446

51. Guo J, Grow EJ, Mlcochova H, Maher GJ, Lindskog C, Nie X, et al. The adult human testis transcriptional cell atlas. *Cell Res* (2018) 28(12):1141–57. doi: 10.1038/s41422-018-0099-2

52. Robertson EJ, Evans MJ, Kaufman MH. X-Chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. *J Embryol Exp Morphol* (1983) 74:297–309. doi: 10.1242/dev.74.1.297

53. Kanatsu-Shinohara M, Ogonuki N, Matoba S, Ogura A, Shinohara T. Autologous transplantation of spermatogonial stem cells restores fertility in congenitally infertile mice. *Proc Natl Acad Sci U S A* (2020) 117(14):7837–44. doi: 10.1073/pnas.1914963117

54. Morimoto H, Ogonuki N, Kanatsu-Shinohara M, Matoba S, Ogura A, Shinohara T. Spermatogonial stem cell transplantation into nonablated mouse recipient testes. *Stem Cell Rep* (2021) 16(7):1832–44. doi: 10.1016/j.stemcr.2021.05.013

Materials and methods:

Article 3: "***In vitro* Generation of Haploid Germ Cells from Human XXY Testicular Organoid**" addressing the objective: *Form 3D Testicular Organoids from human XXY testicular cells and evaluate its potential as Klinefelter Syndrome in vitro disease model and in vitro spermatogenesis tool*

bioengineering

MDPI

Article

In vitro Generation of Haploid Germ Cells from Human XXY Testicular Organoid

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Abstract: Klinefelter Syndrome (KS) (47 XXY) is the most common genetic cause of male infertility. KS patients present with progressive testicular fibrosis accelerating in puberty leading to azoospermia in adults. The increasing availability of genetic testing has facilitated diagnosis of KS early on in life, opening the door for early fertility interventions. This study was designed to explore new spermatogonia-based fertility therapies to meet the needs of these patients.

Testicular cells were isolated from cryopreserved human testes tissue from XXY peripubertal patients and propagated in 2-dimensional culture. Cells were then incorporated into a 10.000 cells 3D organoid culture system.

During a 3-week culture period in an enriched media for spermatogenesis *in vitro*, organoids maintained their structure, viability, and metabolic activity according to live/dead assay and ATP production. Cell-specific PCR and Flow Cytometry markers identified spermatogonia, Sertoli, Leydig, and peritubular cells along the culture. Testosterone was produced by the organoids with and without hCG stimulation. Upregulation of post-meiotic germ cell markers was detected. Fluorescent in situ hybridization (FISH) of chromosomes X, Y, and 18 identified haploid cells in the organoids after the completion of spermatogenesis *in vitro* maturation.

Thus, 3D organoids were successfully generated from isolated peripubertal human testicular cells from Klinefelter patients. Moreover, our 3D culture system provided support for androgen production and germ cell differentiation *in vitro*.

Keywords: spermatogonia; spermatogonia stem cells; spermatogenesis *in vitro*; 3D culture; organoid; Klinefelter syndrome; male infertility; fertility preservation.

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1. Introduction

The mammalian testis is a complex multicellular organ that can be divided into two main compartments, each with a unique role. In the seminiferous tubules, Sertoli cells and spermatogonia provide the adequate environment to enable germ cell differentiation to haploid gametes [1,2]. On the interstitial tissue surrounding the tubules, Leydig cells produce testosterone through steroidogenesis [3,4]. Maintaining these two functions requires a complex communication network in this multicellular environment [5].

Genetic, epigenetic, and environmental factors can disrupt this network during spermatogenesis, causing infertility which has become a major challenge for the human health

and reproduction [6,7]. Reportedly 8-12% of couples who are trying to conceive, are suffering from this problem; half of them due to male factor [8]. Technical revolutions in the past decades such as microsurgical testicular sperm extraction (m-TESE), intracytoplasmic sperm injection (ICSI), and more recently round spermatid injection (ROSI), all have successfully improved the chances of fathering children for men with infertility [9,10]. Nevertheless, these therapeutic strategies require the presence of fully differentiated haploid cells to be considered. Therefore, patients whose conditions are established before puberty urgently need alternative methods to overcome infertility.

Klinefelter syndrome (KS) is a chromosomal disease characterized by male phenotype and supernumerary X chromosomes being 47XXY its most frequent karyotype [11,12]. Although seriously underdiagnosed KS affects 1 in every 600-1000 born males and it's currently considered the most common genetic cause of male infertility [13–15]. KS's most prominent clinical feature is severe testicular fibrosis that accelerates germ cell loss at the onset of puberty leaving most KS patients completely azoospermic by the time they reach adulthood[16,17]. Due to the lack of available sperm, previously mentioned fertility treatments like mTESE and ICSI have only found partial success in KS patients[9,18].

It's been hypothesized that prepubertal boys at risk of infertility with no sperm available could undergo early testicular tissue cryopreservation to save spermatogonial stem cells (SSC) for future experimental fertility treatments [19–21]. This initiative is currently in place in many different centers worldwide and their main indications are prepubertal boys undergoing gonadotoxic chemotherapy, bilateral un-descendent testis, and KS [22–26]. Many spermatogonia-based fertility therapies are been explored with promising preliminary results including: *in vitro* spermatogenesis[27,28], SSC transplantation [29], testicular tissue grafting [30], and testicular ex vivo maturation [31,32].

In vitro spermatogenesis has long been considered a possible solution for these patients. However, and despite many efforts from different research groups, reliable spermatogenesis *in vitro* system isn't available for humans yet [33].

In recent years, SSCs isolation from cryopreserved testicular tissue and propagation *in vitro* has been established for prepubertal XY boys, XXY mice, and finally, XXY human [34–36] (Galdon *et al* 2022 Front. Endocrinol., *accepted in print*.) paving the way for SSC clinical application in the future.

In previous works, our group successfully created testicular organoids from isolated and propagated testicular cells from the adult testicular tissue [27]. In that study, organoids not only maintained viability and morphology in a 3-week 3D culture period but were also able to produce androgen in response to hormonal stimulation. Moreover, *in vitro* maturation of spermatogonia was observed in terms of gene expression and immunostaining positive for postmeiotic markers. These organoids can also serve as a scalable high-throughput 3D culture system mimicking the native functionality of human testes *in vitro* and suitable for toxicity [27] and drug discovery studies [37,38]. In addition, this model was used to study the pathophysiology of testicular diseases such as chronic infection by Zika virus [39].

In the current study, a similar system was applied using isolated and propagated primary testicular cells from peripubertal KS patients to form 3D human testicular organoids and attempt spermatogenesis *in vitro*.

2. Materials and Methods

2.1 Human testis material.

KS testicular tissue (testicular biopsies) was donated by 15 and 17-year-old KS patients enrolled in Wake Forest Medical Baptist testicular bank under IRB-approved protocols at Wake Forest School of Medicine (IRB00021686 and IRB00061265). Testicular tissue was processed into small fragments (~2-4 mm) and slow freeze rate cryopreservation was performed according to in-house protocol [21,23]. Cryotubes were then moved to liquid nitrogen (-196° C) for long-term storage.

2.2 Testicular Tissue Evaluation

Preliminary analyses were conducted to evaluate the presence of spermatogonia in testicular tissue from KS testicular biopsies before cell isolation and culture. XXY peripubertal patients were expected to mirror a realistic clinical scenario where KS patients undergo micro-TESE and no sperm is found.

Hematoxylin-Eosin staining and immunohistochemical staining with non-differentiated spermatogonia marker PGP 9.5 (UCHL1) (PA0286; mouse monoclonal) were performed on testicular tissue histology slides by clinical pathology lab, as previously described in our work (Galdon *et al* 2022 Front. Endocrinol., *accepted in print*). Negative control was provided by isotype control (mouse IgG) stained slides. Testicular tissue from age-matched 46XY controls obtained via National Disease Research Interchange (NDRI) was used as positive control. Microscopic images were acquired using LEICA DM4000B microscope, Olympus camera DP73, and Olympus Cellsens software

2.3. Cell Isolation, Propagation, Characterization, and Quantification

2.3.1. Isolation and propagation.

Previously cryopreserved testicular tissue pieces of around 1-2 mg were thawed slowly in 37° C running water. Testicular cell isolation, including an enriched spermatogonia stem cells (SSCs) population, was performed using a two-step mechanical and enzymatic digestion system [34,40] (Galdon *et al* 2022 Front. Endocrinol., *accepted in print*). For this study, FDA-approved collagenase (NB 4 Standard Grade SERVA Electrophoresis, Heidelberg, Germany) and protease (Natural Protease NB, SERVA Electrophoresis, Heidelberg, Germany) were used to facilitate the transition of this method from research into the clinical setting.

Isolated cells were cultured on uncoated 6-well plates at a seeding density of 10,000-20,000 cells/cm2 in supplemented 1X MEM (1X MEM with 10% FBS, 1X non-essential amino acids (Invitrogen), 15mM HEPES (Invitrogen), 50 μg/ml gentamicin (Invitrogen), 4mM L-glutamate (Invitrogen) 0.12% sodium bicarbonate, streptomycin (100 μg/ml) – penicillin (100 IU/ml) (sigma)) overnight. The next day, the media was changed to supplemented StemPro-34 (Invitrogen) media (Stempro-34 with recombinant human GDNF (40 ng/mL) (Sigma), recombinant human EGF (20 ng/mL), recombinant human Leukemia inhibitory factor (10 ng/mL), streptomycin (100 μg/mL) - penicillin (100 IU/mL) (Sigma)) (Table 1).

Media was refreshed every 4 days and cells were passaged when 80% confluent (approximately every 7-10 days). Somatic cells from initial isolation were utilized as a feeder layer for proliferating germ cells. Excess cells after each passage were cryopreserved at -196° C using 1X MEM containing 20% FBS and 8% DMSO.

Table 1: Testicular cells culture, testicular organoid formation, and testicular organoid differentiation medias composition

Component	Manufacturer	Catalogue #	Final conc.	StemPro Complete	Formation Media	Differentiating Media
Bovine Serum Albumin	Sigma-Aldrich	A3059	5 mg/ml	✓	✓	✓
D (+) Glucose	Sigma-Aldrich	G7021	6 mg/ml	✓	✓	✓
Ascorbic acid	Sigma-Aldrich	A4544	1×10^{-4} M	✓	✓	✓
Transferrin (Apo)	Sigma-Aldrich	T1147	100 µg/ml	✓	✓	✓
Pyruvic Acid	Sigma-Aldrich	P2256	30 mg/ml	✓	✓	✓
d-Biotin	Sigma-Aldrich	B4501	10 µg/ml	✓	✓	✓
2β-mercaptoethanol	Sigma-Aldrich	M3148	5×10^{-5} M	✓	✓	✓
DL-Lactic Acid	Sigma-Aldrich	L4263	1 µl/ml	✓	✓	✓
MEM NEAA	Invitrogen	11140-050	10 µl/ml	✓	✓	✓
Stem Pro-34 Supplement	Gibco	10641-025	26 µl/ml	✓	✓	✓
Insulin	Cell Applications	128-100	25 µg/ml	✓	✓	✓
Sodium Selenite	Sigma-Aldrich	S1382	30 nM	✓	✓	✓
Putrescine	Sigma-Aldrich	P7505	60 µM	✓	✓	✓
L-Glutamin	Invitrogen	25030-081	2 mM	✓	✓	✓
MEM Vitamin Solution	Invitrogen	11120-052	10 µl/ml	✓	✓	✓
B-Estradiol	Sigma-Aldrich	E2758	30 ng/ml	✓	✓	✓
Progesterone	Sigma-Aldrich	P8783	60 ng/ml	✓	✓	✓
Epidermal Growth Factor (EGF)	Sigma-Aldrich	E9644	20 ng/ml	✓	✓	✓
Human basic Fibroblast Growth Factor (hbFGF)	Sigma-Aldrich	F0291	10 ng/ml	✓	✓	✓
Glial Cell Line-Derived Neurotrophic Factor (GDNF)	Sigma-Aldrich	G1777	10 ng/ml	✓	✓	✓
Leukemia Inhibitor Factor	Chemicon	LIF1010	10 ng/ml	✓	✓	×
Fetal Calf Serum (FCS)	Invitrogen	10437010	1 %	✓	**30%**	✓
Penicilline/Streptomycine (Pen/Strep)	Invitrogen	15140-122	0.5 %	✓	✓	✓
Gentamycin	Gibco	15750078	50 µg/ml	×	✓	✓
Recombinant human stemcell factor (SCF)	Peprotech	300-07	100 ng/ml	×	×	✓
Retinoic Acid	Sigma-Aldrich	R2625	10 µM	×	×	✓
Human chorionic gonadotropin (hCG)	Sigma-Aldrich	C8554	1 mIU/ml	×	×	✓
Follicle Stimulating Hormone (FSH)	Sigma-Aldrich	F4021	2.5×10^{-5} IU/ml	×	×	✓
Human testis Extra-Cellular Matrix (ECM)	n/a	n/a	1 µg/ml	×	✓	✓

2.3.2. Characterization of the cultured cells.

To characterize different cell types of testicular tissue in our two-dimensional (2D) culture, 200K cells were snap-frozen in liquid nitrogen and RNA was isolated later and converted to cDNA. Taqman® gene expression assays include: for undifferentiated spermatogonia: PLZF (ZBTB16), UCHL1(PGP 9.5), THY1 (CD90); for differentiated spermatogonia: PRM1; for Sertoli cells: CLUSTERIN and GATA4; for Leydig cells: STAR, TSPO, CYP11A1; and for peritubular cells: CD34 were used (Table 2). POLR2A was used as an internal control. All reactions were performed using standard Taqman® Universal PCR Master Mix and run on ABI-7300 FAST System. For each reaction, 25-50 ng of cDNA was used. PCR productions were later run on 3% Agarose gel in TAE buffer in 120mA current for 20-30 minutes and pictures of the bands were taken using Kodak Gel Logic 200 Imaging system.

Table 2: Taqman® Gene Expression Assays primers used

Gene ID	Amplicon Length	Catalog #
PRM1	99	4331182 (Hs00358158_g1)
ZBTB16 (PLZF)	65	4331182 (Hs00957433_m1)
UCHL1	80	4331182 (Hs00985157_m1)
THY1 (CD-90)	60	4331182 (Hs009174816_m1)
GATA4	68	4331182 (Hs00171403_m1)
CLUSTERIN	93	4331182 (Hs00971656_m1)
STAR	85	4331182 (Hs00264912_m1)
TSPO	57	4331182 (Hs00559362_m1)
CYP11A1	81	4331182 (Hs00897320_m1)
CD-34	63	4448892 (Hs02576480_m1)
POLR2A	61	4331182 (Hs00172187_m1)

The combination of HLA-/CD9+/CD49f+ is known to enrich the SSC according to current literature [43–48] and was used for Flow Cytometry to assess the putative SSC population in culture. Regular mouse IgG for each condition was used as isotype control (Table 3). Briefly, after trypsinization and counting, an appropriate number of cells (100K for each marker/condition) were washed with flow cytometry buffer (PBS + 0.5% FBS). Cells were pelleted, resuspended in the buffer, and aliquoted in flow cytometry tubes, 100K in 100 µl of buffer in each tube. 10 µl of the respective antibody was introduced to the cell in each condition and kept in darkness for 20 minutes in RT. Then cells were washed, pelleted, and resuspended in the buffer (100 µl per each condition) and then carried on ice to the flow cytometry machine. Flow cytometry was done using a BD Accuri C6 machine. 10,000 events were run per condition. Gating, compensation, and analysis were done using BD Accuri C6 software.

Specificity	Host	Type	Conjugated Fluorochrome	Manufacturer	Catalog #
HLA-ABC	MOUSE	Monoclonal Anti-Human	FITC	BD Pharmigen	555552
CD-49f	MOUSE	Monoclonal Anti-Human/Mouse	APC	eBiosience	17-0495-80
CD-9	MOUSE	Monoclonal Anti-Human	PerCP-Cy5.5	BD Pharmigen	341649
Control	MOUSE	IgG1 Isotype	FITC	BD Pharmigen	340755
Control	MOUSE	IgG1 Isotype	APC	BD Pharmigen	340754
Control	MOUSE	IgG1 Isotype	PerCP-Cy5.5	BD Pharmigen	347212

Table 3: Antibodies used for Flow Cytometry

2.4. Klinefelter Human Testicular Organoid (HTO) Formation and differentiation

Cells were seeded in formation media (Table 1) into 96-well Ultra-Low attachment round-bottom plates using 10000 cells per organoid. The plates were centrifuged at 150g for 30 seconds. Cells were cultured at 37°C and 5% CO2 for 48 hours until organoid formation was completed. Then, HTOs were refreshed with differentiating media (Table 1) every other day and kept for additional 3 weeks in culture at 34°C and 5% CO2.

2.4.3. Diameter and viability assay.

HTOs' diameter and viability were determined on days 2, 9, 16, and 23. For viability, Molecular Probes Live-Dead cell imaging (Invitrogen, L3224) and ATP content were utilized. For the live-dead assay, n=8 HTOs were incubated at 37°C in media with Calcein AM (Green, Live cells) and Ethidium Homodimer (Red, dead cells) for 20 minutes, then were washed in 1X PBS and imaged using Leica TCS-LSI Macro Confocal microscope. HTOs ATP content was evaluated using relative light units (RLUs) from luminescence ATP kit following manufacturer's instructions (CellTiter-Glo Luminescent Cell Viability Assays, Promega). For each time point, n=8 HTOs were harvested for ATP measurement.

2.4.4. Cell count and RNA isolation.

Additionally, at each time point, N=48-96 HTOs were dissociated in Collagenase NB 4 Standard Grade for 2 hours and then mechanically using a pipette. The dissociated cells were counted using a hemocytometer, and the number of counted cells per organoid was calculated. Cells were spun in the centrifuge and the media was removed. 350 µL of RLT/BME was added to the cells in an Eppendorf tube and then were snap frozen in liquid nitrogen to be used for RNA extraction using RNeasy QIAGEN Mini Kit.

2.4.5. *Histology evaluation.*

HTOs were harvested for analysis on days 2, 9, 16, and 23 of culture. At each time point, N=24 HTOs were pooled and fixed in 4% PFA for 30 minutes at room temperature. Fixed HTOs were embedded in HistoGel (Thermofisher, HG-4000-012), paraffin-embedded, and 5µm sections were mounted on a slide. These sections were deparaffinized and Hematoxylin and Eosin staining was performed according to standard in-house protocols

using an autostainer. Organoids were visualized on a Leica DM400 B LED microscope and pictures were captured.

2.4.6. Testosterone production.

HTOs ability to produce androgen was evaluated using a clinical setting for measuring testosterone in the Wake Forest Baptist Health Medical Center laboratory by competitive binding immunoenzymatic assay (Beckman DXI800 analyzer). A total of N=32 organoids per time point were used in this assay, half of them stimulated with 10 mIU of hCG for 3 hours. Then, organoids were harvested and their media was kept in a -80C freezer until measurement. The measured testosterone level in stimulated HTOs was compared to non-stimulated HTOs at each time point.

2.4.7. Gene expression assay.

HTOs were evaluated for relative gene expression changes for both functional somatic cell markers and spermatogenesis markers after 23 days in culture following a spermatogenic cell differentiation protocol. As previously mentioned, total RNA was isolated from cells of the dissociated HTOs (N=48) using QIAGEN RNeasy Mini Kit following manufacturer's instructions. Isolated RNA was converted to cDNA using a high-capacity cDNA Reverse Transcription Kit (Life Technologies). For quantitative reverse transcriptase PCR (qRT-PCR) analysis, Taqman® gene expression assays were used to evaluate testicular cell type-specific gene expression changes over time. Reactions were performed using standard Taqman® Universal PCR Master Mix (96-well plate format) and run on an ABI 7300 FAST system (Life Technologies). Cycling conditions were as follows: 95°C for 10 min, 95°C for 15 seconds (40 cycles), and 60°C for 1 minute. The gene POLR2A was used as an internal normalization control. Expression of all genes was normalized to POLR2A housekeeping gene; relative expression of 23-day-old HTOs compared to day 2 HTOs as controls using the 2-ΔΔCT method. All runs were performed in duplicate. Statistical analyses were determined by student t-test using GraphPad Prism 8 software.

2.4.8. Fluorescent in situ Hybridization (FISH)of the HTOs for haploid cells.

23-day-old HTOs were harvested (n=24) and washed with PBS 1X once. Then they were fixed in 4% PFA for 30 minutes in RT, then placed on Histogel base and embedded in paraffin following in-house protocol. 5-μm consecutive sections were cut and FISH for chromosomes 18, X, and Y using AneuVysion Multicolor DNA Probe Kit (Vysis CEP 18/X/Y - alpha satellite) was performed following manufacturer instructions. Nuclei were counterstained with DAPI. Pictures of the hybridized slides were taken using a filter microscope and software. Due to color similarities of X and 18 probes, the color of chromosome 18 was changed to yellow (pseudo color) digitally. The presence of only one X or one Y accompanied by one 18 chromosome in a nucleus was considered a haploid cell. Cells were counted in consecutive sections, among different HTOs.

2.5 Statistics

Statistical analysis of all quantitative results is presented as mean ± standard deviation (SD). Statistical significance was determined via GraphPad Prism 8 software using Student t-test, with p values < 0.05 considered statistically significant.

3. Results

3.1. Testicular tissue characterization before cell isolation.

Testicular tissue from peripubertal KS patients showed severe alteration of testicular architecture with signs of established testicular fibrosis and no apparent active spermatogenesis present. Conversely, age-matched controls testicular tissue presented prominent seminiferous tubules and signs of active spermatogenesis inside the tubules. (Figure 1)

Immunohistochemistry staining was performed against the non-differentiated spermatogonia marker PGP 9.5. While PGP 9.5 was highly present inside the adult control testicular tissue, only a few positive cells were found in KS testicular tissue (Figure 1).

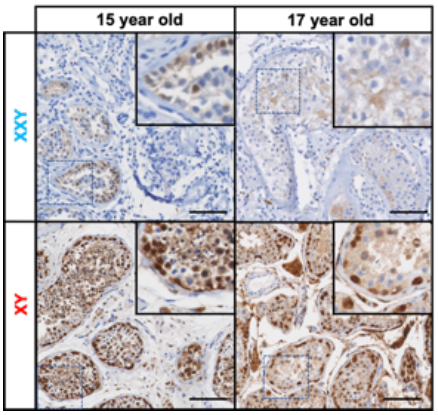

Figure 1. Immunostaining for undifferentiated spermatogonia marker UCHL1 (PGP 9.5). On the top panel, Klinefelter patients aged 15 and 17 years old respectively included in the study. On the bottom panel, age-matched controls. *Scale bar 100µm.* (Adapted from Galdon *et al* 2022 Front. Endocrinol., *accepted in print*.).

3.2. Cell isolation and propagation.

To isolate cells, previously cryopreserved tissue was digested both mechanically and enzymatically according to an established method for SSC isolation and previously mentioned enzyme modifications [34–36] (Galdon *et al* 2022 Front. Endocrinol., *accepted in print*.). Cells were initially cultured in a 2-dimensional (2D) system with supplemented Stempro-34 media (Table 1). Isolated cells from 15- and 17-years old KS peripubertal patients were propagated until P4, 34 days, and P3,31 days respectively. Media was refreshed every 2-3 days and cells were passaged at 80% confluency as previously described (Galdon *et al* 2022 Front. Endocrinol., *accepted in print*.).

3.3. Characterization of the cultured cells.

To identify different cell types present in our culture, qRT-PCR was performed on snap-frozen propagated cells for key markers of each of the testicular tissue cell types. Expression of ZBTB16, UCHL1, and THY1 for Un-differentiated spermatogonia; PRM1 for differentiated spermatogonia; GATA4 and Clusterin (CLU) for Sertoli cells; STAR, TSPO, and CYP11A1 for Leydig cells; and CD34 for peritubular cells, all together proved the presence of all 4 different testicular cell types in culture and no signs of spermatogonia differentiation (Figure 2).

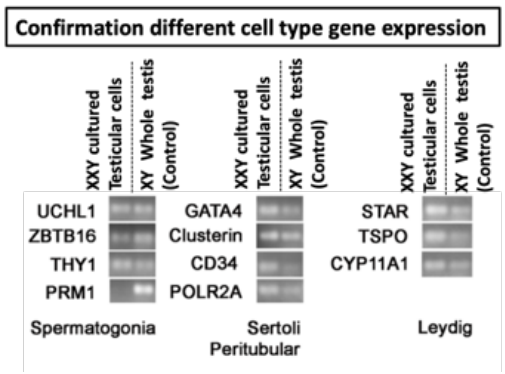

Figure 2. RT-PCR to confirm the presence of the four main testicular cells in culture based on cell-specific gene expression. POLR2A was used as a housekeeping gene for internal control. Results confirmed the presence of all four common cell types. However, differentiated germ cell marker PRM1 remained negative, indicating the undifferentiated status of the germ cells in the culture. Adapted from Galdon *et al* 2022 Front. Endocrinol., *accepted in print*.

Using flow cytometry, the presence of HLA-/CD9+/CD49f+ putative SSC was confirmed (Figure 3). These results indicate cells in culture retain the ability to initiate spermatogenesis.

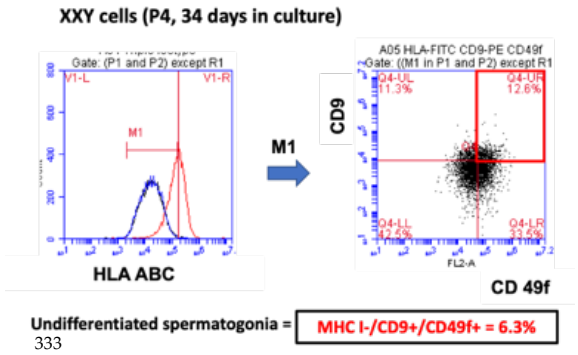

Figure 3: Assessment of SSC in a culture based on Flow Cytometry for a combination on HLA-/CD9+/CD49f+

3.5. Human Testicular Organoids (HTOs) formation and differentiation.

HTOs were successfully formed from 10,000 cells in 96-well Ultra-low attachment round-bottom plates and centrifugation at 150g for 30 seconds from KS peripubertal cells during 48hs in formation media at 37° C and 5% CO2 (Table 1, Figure 4). During the following 3 weeks of 3D culture, HTO were kept in differentiating media (Table 1) at 34°C and 5%CO2.

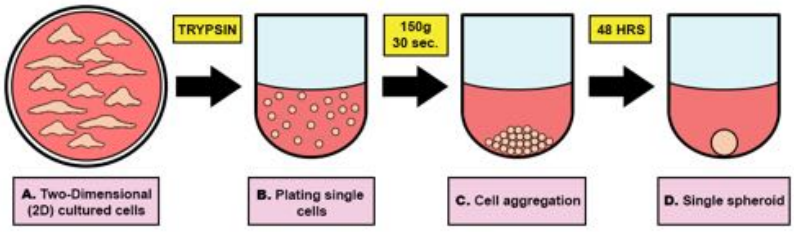

Figure 4: Schematic picture of creating 3-dimensional testicular organoids from cultured testicular cells using Corning Costar® Ultra Low Attachment Round Bottom 96-well plates (Ref. #7007):

A. 2D cultured cells were trypsinized and made single cells in a suspension of formation media.
B. Single cells were plated in 10000 cells/100 µL/well concentration.
C. Plates were centrifuged at 150g for 30 seconds to make cell aggregates.
D. After 48 hours single spheroids were formed.

HTO presented a morphology of cohesively packed cells with a prominent nucleus. (Figure 5). Using LIVE/DEAD staining the organoids showed positive viability along the culture. (Figure 5). Core necrosis was only barely observed in the last timepoint of the study (Figure 5).

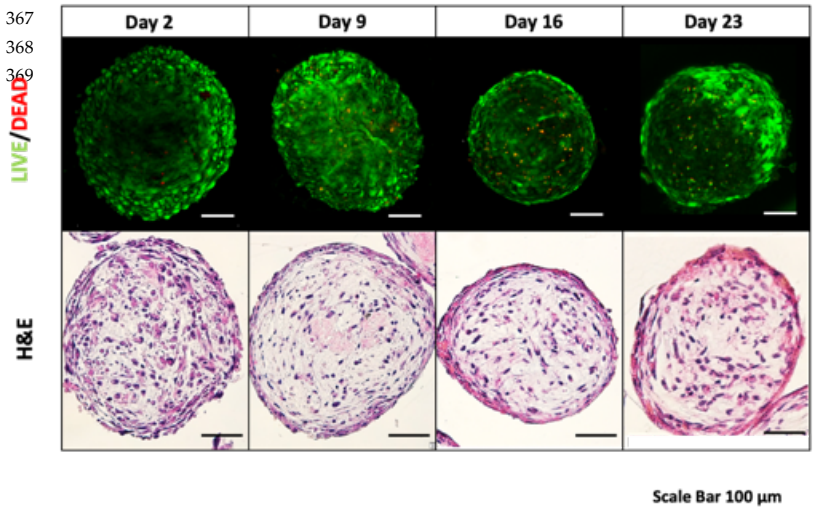

Figure 5: KS organoids morphology and viability along the culture. **Top row:** Organoids were stained using Life Technologies LIVE/DEAD Cell Imaging Kit (live cells = green, dead cells = red) and freshly analyzed with a confocal microscope. **Bottom row:** Organoids were fixed, paraffinized, and processed for Hematoxylin and Eosin staining and analyzed with an optic microscope. *Scale bars 100 µm.*

Both size (Figure 6A) and ATP production (Figure 6B) were monitored weekly and showed a statistically significant drop after the first week of culture, followed by stability during the next two weeks until the completion of the study.

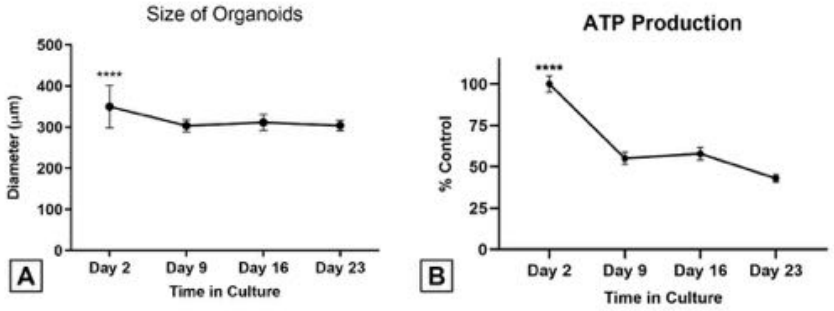

Figure 6: Testicular organoid size and ATP production was monitored during culture. A) Organoid size measured with optic microscopy. B) Organoid ATP production was measured in RLUs using a luminescence kit. *Data presented as mean ± SD. Significance $p < 0.05$*

At each of the weekly time points, testosterone production was detected from both hCG stimulated and not stimulated HTOs, showing that they were able to continuously produce testosterone during culture (Figure 8). The hCG stimulation didn't significantly increase testosterone production in KS organoids (Figure 8) conversely to previous work from our group on euploid adult cells [27].

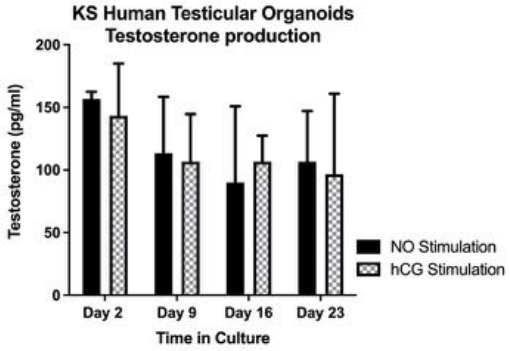

Figure 7: Testicular organoids androgen production during culture. Each time point represents 16 organoids with or without hCG stimulation. *Data presented as mean ± standard deviation.*

Gene expression analysis showed upregulation of meiotic (SYPC3) and postmeiotic (PRM1) markers after 21 days of testicular organoids culture on differentiation media. (Figure 9A). Moreover, using dPCR it was possible to estimate a population of 0.2% expressing PRM1 and 0.2% cells expressing Acrosin (Figure 9B).

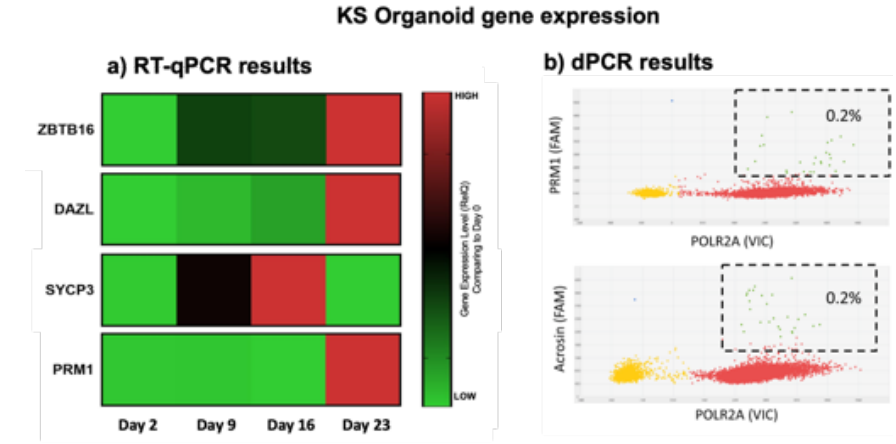

Figure 8: KS organoid gene expression **9A)** RT-qPCR results expressed as a Heat map showing gene expression compatible with active spermatogenesis in KS organoids. **9B)** digital PCR analysis for PRM1 (FAM) and Acrosin (FAM) and housekeeping gene POLR2A (VIC) showing 0.2% of positive cells for both genes.

Finally, Fluorescent in situ Hybridization (FISH) for X, Y, and 18 chromosomes was performed on fixed organoids after three weeks of differentiation. Among mostly diploid cells on the organoid's surface, haploid cells were identified in the core. (Figure 10A) Quantification of haploid cells observed a presence of 10.9% of 18X and 4.6% of 18Y (Figure 10B) in contrast to unpublished data from our group on prepubertal patients showing a 6.42% of 18X cells and a 6.68% of 18Y cells.

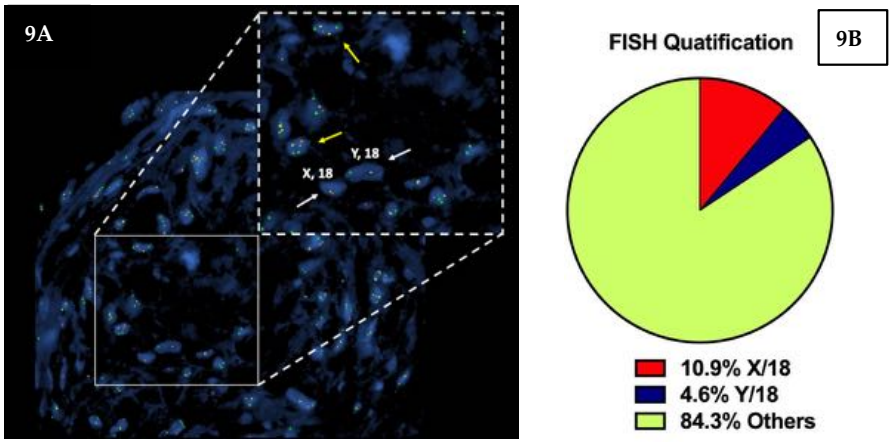

Figure 9: Fluorescent in situ hybridization (FISH) (Cr X green; Cr Y orange; Cr 18 yellow) on KS testicular organoid after 23 days in culture. **9A)** Haploid cells stained for only one chromosome 18 and one sex chromosome either X or Y identified with white arrows. Diploid cells, stained for two chromosomes 18 and both sex chromosomes identified with yellow arrows for comparison. **9B)** Pie chart showing the percentage of the haploid cells (based on their genotype) amongst the total population of the cells in the testicular organoid.

4. Discussion

The first goal of this study was to test the viability of testicular organoids made from Klinefelter tissue. On one hand, LIVE/DEAD staining showed good viability at every weekly timepoint and only mild core necrosis was observed by the end of the study after three weeks (Figure 5). On the other hand, considering ATP production analysis as an indirect method to assess viability, a significant drop was found after the first week followed by stability during the rest of the study.

One possibility is that metabolic transition from a 2D propagation system into a 3D differentiation system modifies the physiologic energy requirements and impacts ATP production without compromising cell viability.

Another feasible is that although cells in the organoid remained viable, the overall number of cells decreased over time. In preliminary studies comparing different organoid seeding concentrations, the results showed a consistent 33% cell into organoid incorporation rate regardless of seeding concentration while maintaining excellent viability (data not shown). The washout of the cells not incorporated into the organoid structure would account for the initial ATP drop.

Perhaps even cells that initially joined the organoid structure die as part of the initial structural remodeling process. The remnants of cellular skeleton and matrix from cells lost during this process may have improved organoid cohesion and helped shape the overall 3D structure. This idea would also be supported by the amount of "conjunctive tissue-like" material found in H&E histology images.

Testes play a critical role in human physiology by both testosterone secretion and male gamete production. Testicular organoids' ultimate goal is to accurately and consistently reproduce testicular physiology *in vitro* for clinical and research applications.

Therefore, a cornerstone of our study was to assess Testosterone production of the 3D organoids in culture with and without previous hCG pulse stimulation. KS organoids

produced clinically detectable amounts of testosterone at every single time point of the study (Figure 7). Let's remark that NO testosterone was added in the media but instead a basal concentration of hCG (Table 1). On the other hand, testosterone production by KS organoids did not significantly respond to hCG pulse stimulation (Figure 7) in contrast to previous reports from our group on adult [27] and prepubertal testicular organoids (Zarandi, Galdon *et al in preparation*). This might be a sign that KS organoids are an accurate disease model; therefore, KS Leydig cells do not respond to hCG pulses in a comparable way to prepubertal and adult controls.

Later reports from Sun et al [28] have presented a testicular organoid model completely spermatogenesis orientated with no Leydig cells. However, our group envisions testicular organoids as similar as possible to human *in vivo* physiology and therefore Leydig cells and Testosterone production are key. Including steroidogenic cells in our 3D culture system could add value to spermatogenesis *in vitro* in more ways than we could anticipate. Moreover, successful recreation of steroidogenic pathways in vitro could open the door to other possible testicular organoid clinical applications including hormone replacement therapy.

Gene expression analyses were conducted to identify signs of *in vitro* spermatogenesis in the cells in the culture. Results accurately showed the transition from undifferentiated spermatogonia to postmeiotic spermatids along the three-week differentiation period mimicking *in vivo* findings (Figure 8).

Initially, cells in the 2D culture system and testicular tissue showed no differentiated spermatogonia expression, and the undifferentiated spermatogonia marker (ZBTB16) was highly expressed (Figure 2). When cells were included in the 3D culture system both undifferentiated spermatogonia (ZBTB16) and differentiating spermatogonia (DAZL) markers increased steadily their level of expression along the culture. Organoids started expressing meiosis marker (SCYP3) after 1 week then the expression level peaked in week 2 and dropped in week 3 (Figure 8). Finally, after 3 weeks in culture positive expression of postmeiotic markers (PRM1 and Acrosin) was detected (Figure 8). These findings fit the spermatogenesis gene expression pattern.

Interestingly, an increment on undifferentiated spermatogonia gene expression markers was observed too. A way to interpret this data is that spermatogonia in our 3D system do not sync for spermatogenesis and while some cells engage in differentiation other spermatogonia are preparing for the next spermatogenesis wave. That would also explain why meiosis marker SCYP3 peaks by week 2 and then negativizes.

Using dPCR postmeiotic cell population was estimated to represent around 0.2% of cells in culture. Using this data, we will be in a position to estimate the number of organoids needed to produce enough haploid cells for clinical applications.

FISH staining in testicular organoids after 3 weeks in culture revealed a population of haploid cells with either X or Y chromosomes. No haploid cells were identified inside testicular organoids in earlier timepoints of the study (Data not shown)

In previous work from our group, evidence of *in vitro* maturation of spermatogonia into post-meiotic stage using 3D culture system [27] using adult testicular cells. Although it represented a good proof of concept for our current work, some questions rose along the way regarding the use of adult testicular tissue. We wondered whether cells included in the 3D system were already committed to spermatogenesis and organoids just supported this process or on the contrary 3D system pushed spermatogonia into differentiation strictly *in vitro*. Similarly, the latest report by Sun et al [28] used their *in vitro* spermatogenesis system cells from a patient diagnosed with Obstructive Azoospermia with full spermatogenesis confirmed by the pathology lab. In this study, by using Klinefelter peripubertal testicular tissue without active spermatogenesis confirmed by pathology the possibility of already having differentiated spermatogonia is negligible. Hence it can be safely concluded that any differentiation observed in this study strictly occurred *in vitro* and therefore could potentially represent a future clinical option for Non-Obstructive Azoospermia KS patients.

This study tried to bring testicular organoids closer to future clinical applications. Consequently immortalized Sertoli and Leydig cells used in our previous work [27] were substituted by primary somatic cells instead. Only FDA-approved digestive enzymes (Collagenase NB 4 Standard Grade and Natural Protease NB SERVA Electrophoresis, Heidelberg, Germany) were used during the cell isolation process. KS testicular tissue came from an experimental testicular tissue bank. As only a small portion of their banked material was used for research those same patients could potentially benefit from the techniques developed in this study and tested in their own tissue for future clinical applications.

Previous studies in mice have used an organotypic 3D culture system for successful *in vitro* maturation leading to fertile offspring [31,47,48]. Lately, this study was reproduced using human cells from Obstructive Azospermic human patients producing haploid cells capable of fertilizing mouse eggs [28]. However, these studies noticeably identify Knock Out Serum Replacement as a key factor in cell differentiation. In our studies, we avoid using KSR due to concerns about their known impact on the chromosome instability [49]. In the same way, Sun et al [28] meticulously selected Spermatogonia and Sertoli cells with a high level of purity to allegedly improve spermatogenesis efficiency. However, in this study, a decision was made to include Sertoli, Leydig, and peritubular cells as they may support spermatogenesis and better recreating the overall testicular physiology.

Although Klinefelter Syndrome is considered the most common genetic cause of male infertility its physiopathological mechanism remains widely unknown. The fact that using our 3D organoid culture system both meiosis and androgen production were replicated suggests its suitability as a Klinefelter Syndrome disease model. Therefore, it could potentially play a key role in better understanding the Klinefelter syndrome and how to clinically address it.

This study is presented as promising evidence of germ cell *in vitro* differentiation. With recent advances in reproductive techniques with intratesticular germ cells (ICSI, ROSI, ELSI) follow-up studies exploring the fertilization potential of *in vitro* produced haploid cells may unveil significant results. Overall, this work hopes to push *in vitro* spermatogenesis a step closer to clinical application for KS patients with no sperm retrieved by TESE/microTESE.

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Institutional Review Board Statement: The KS testicular tissue samples used in this study were obtained in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Wake Forest School of Medicine (IRB00021686 and IRB00061265). For the rest of the study, only biological tissue and cells were involved and no additional ethical review was required by our institution.

Data Availability Statement: Not applicable

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References

1. De Felici, M.; Dolci, S. From Testis to Teratomas: A Brief History of Male Germ Cells in Mammals. *Int J Dev Biol* **2013**, *57*, 115–121, doi:10.1387/ijdb.130069md.
2. Eddy, E.M. Regulation of Gene Expression during Spermatogenesis. *Semin Cell Dev Biol* **1998**, *9*, 451–457, doi:10.1006/scdb.1998.0201.
3. Hanukoglu, I. Steroidogenic Enzymes: Structure, Function, and Role in Regulation of Steroid Hormone Biosynthesis. *J Steroid Biochem Mol Biol* **1992**, *43*, 779–804, doi:10.1016/0960-0760(92)90307-5.
4. Huhtaniemi, I.; Toppari, J. Endocrine, Paracrine and Autocrine Regulation of Testicular Steroidogenesis. *Adv Exp Med Biol* **1995**, *377*, 33–54, doi:10.1007/978-1-4899-0952-7_3.
5. Oatley, J.M.; Brinster, R.L. The Germline Stem Cell Niche Unit in Mammalian Testes. *Physiol Rev* **2012**, *92*, 577–595, doi:10.1152/physrev.00025.2011.
6. De Kretser, D.M.; Baker, H.W. Infertility in Men: Recent Advances and Continuing Controversies. *J Clin Endocrinol Metab* **1999**, *84*, 3443–3450, doi:10.1210/jcem.84.10.6101.
7. Hirsh, A. Male Subfertility. *BMJ* **2003**, *327*, 669–672, doi:10.1136/bmj.327.7416.669.
8. Agarwal, A.; Baskaran, S.; Parekh, N.; Cho, C.-L.; Henkel, R.; Vij, S.; Arafa, M.; Panner Selvam, M.K.; Shah, R. Male Infertility. *Lancet* **2021**, *397*, 319–333, doi:10.1016/S0140-6736(20)32667-2.
9. Okuyama, N.; Obata, R.; Oka, N.; Nakamura, Y.; Hattori, H.; Nakajo, Y.; Aono, N.; Koizumi, M.; Toya, M.; Nagao, K.; et al. Long-Term Clinical Outcomes of Testicular Sperm Extraction and Intracytoplasmic Sperm Injection for Infertile Men. *Reprod Med Biol* **2018**, *17*, 82–88, doi:10.1002/rmb2.12073.
10. Tournaye, H. Update on Surgical Sperm Recovery--the European View. *Hum Fertil (Camb)* **2010**, *13*, 242–246, doi:10.3109/14647273.2010.522677.
11. Lanfranco, F.; Kamischke, A.; Zitzmann, M.; Nieschlag, E. Klinefelter's Syndrome. *Lancet* **2004**, *364*, 273–283, doi:10.1016/S0140-6736(04)16678-6.
12. Bonomi, M.; Rochira, V.; Pasquali, D.; Balercia, G.; Jannini, E.A.; Ferlin, A.; Klinefelter Italia, N.G. Klinefelter Syndrome (KS): Genetics, Clinical Phenotype and Hypogonadism. *J Endocrinol Invest* **2017**, *40*, 123–134, doi:10.1007/s40618-016-0541-6.
13. Bojesen, A.; Juul, S.; Gravholt, C.H. Prenatal and Postnatal Prevalence of Klinefelter Syndrome: A National Registry Study. *J Clin Endocrinol Metab* **2003**, *88*, 622–626, doi:10.1210/jc.2002-021491.
14. Zhao, Y.; Gardner, E.J.; Tuke, M.A.; Zhang, H.; Pietzner, M.; Koprulu, M.; Jia, R.Y.; Ruth, K.S.; Wood, A.R.; Beaumont, R.N.; et al. Detection and Characterization of Male Sex Chromosome Abnormalities in the UK Biobank Study. *Genet Med* **2022**, S1098-3600(22)00777-8, doi:10.1016/j.gim.2022.05.011.
15. Nielsen, J.; Wohlert, M. Sex Chromosome Abnormalities Found among 34,910 Newborn Children: Results from a 13-Year Incidence Study in Arhus, Denmark. *Birth Defects Orig Artic Ser* **1990**, *26*, 209–223.
16. Aksglaede, L.; Wikstrom, A.M.; Rajpert-De Meyts, E.; Dunkel, L.; Skakkebaek, N.E.; Juul, A. Natural History of Seminiferous Tubule Degeneration in Klinefelter Syndrome. *Hum Reprod Update* **2006**, *12*, 39–48, doi:10.1093/humupd/dmi039.
17. Willems, M.; Gies, I.; Van Saen, D. Germ Cell Loss in Klinefelter Syndrome: When and Why? *Am J Med Genet C Semin Med Genet* **2020**, *184*, 356–370, doi:10.1002/ajmg.c.31787.
18. Corona, G.; Pizzocaro, A.; Lanfranco, F.; Garolla, A.; Pelliccione, F.; Vignozzi, L.; Ferlin, A.; Foresta, C.; Jannini, E.A.; Maggi, M.; et al. Sperm Recovery and ICSI Outcomes in Klinefelter Syndrome: A Systematic Review and Meta-Analysis. *Hum Reprod Update* **2017**, *23*, 265–275, doi:10.1093/humupd/dmx008.

19. Wallace, W.H.B. Oncofertility and Preservation of Reproductive Capacity in Children and Young Adults. *Cancer* **2011**, *117*, 2301–2310, doi:10.1002/cncr.26045.
20. Ly, A.; Sermondade, N.; Brioude, F.; Berthaut, I.; Bachelot, A.; Hamid, R.H.; Khattabi, L.E.; Prades, M.; Lévy, R.; Dupont, C. Fertility Preservation in Young Men with Klinefelter Syndrome: A Systematic Review. *J Gynecol Obstet Hum Reprod* **2021**, *50*, 102177, doi:10.1016/j.jogoh.2021.102177.
21. Zarandi, N.P.; Galdon, G.; Kogan, S.; Atala, A.; Sadri-Ardekani, H. Cryostorage of Immature and Mature Human Testis Tissue to Preserve Spermatogonial Stem Cells (SSCs): A Systematic Review of Current Experiences toward Clinical Applications. *Stem Cells Cloning* **2018**, *11*, 23–38, doi:10.2147/SCCAA.S137873.
22. Lakhoo, K.; Davies, J.; Chakraborty, S.; Berg, S.; Tennyson, R.; Fowler, D.; Manek, S.; Verrill, C.; Lane, S. Development of a New Reproductive Tissue Cryopreservation Clinical Service for Children: The Oxford Programme. *Pediatr Surg Int* **2019**, *35*, 1271–1278, doi:10.1007/s00383-019-04503-3.
23. Sadri-Ardekani, H.; Atala, A. Testicular Tissue Cryopreservation and Spermatogonial Stem Cell Transplantation to Restore Fertility: From Bench to Bedside. *Stem Cell Res Ther* **2014**, *5*, 68, doi:10.1186/scrt457.
24. Tournaye, H.; Dohle, G.R.; Barratt, C.L.R. Fertility Preservation in Men with Cancer. *Lancet* **2014**, *384*, 1295–1301, doi:10.1016/S0140-6736(14)60495-5.
25. Picton, H.M.; Wyns, C.; Anderson, R.A.; Goossens, E.; Jahnukainen, K.; Kliesch, S.; Mitchell, R.T.; Pennings, G.; Rives, N.; Tournaye, H.; et al. A European Perspective on Testicular Tissue Cryopreservation for Fertility Preservation in Prepubertal and Adolescent Boys. *Hum Reprod* **2015**, *30*, 2463–2475, doi:10.1093/humrep/dev190.
26. Valli-Pulaski, H.; Peters, K.A.; Gassei, K.; Steimer, S.R.; Sukhwani, M.; Hermann, B.P.; Dwomor, L.; David, S.; Fayomi, A.P.; Munyoki, S.K.; et al. Testicular Tissue Cryopreservation: 8 Years of Experience from a Coordinated Network of Academic Centers. *Hum Reprod* **2019**, *34*, 966–977, doi:10.1093/humrep/dez043.
27. Pendergraft, S.S.; Sadri-Ardekani, H.; Atala, A.; Bishop, C.E. Three-Dimensional Testicular Organoid: A Novel Tool for the Study of Human Spermatogenesis and Gonadotoxicity in Vitro. *Biol Reprod* **2017**, *96*, 720–732, doi:10.1095/biolreprod.116.143446.
28. Sun, M.; Yuan, Q.; Niu, M.; Wang, H.; Wen, L.; Yao, C.; Hou, J.; Chen, Z.; Fu, H.; Zhou, F.; et al. Efficient Generation of Functional Haploid Spermatids from Human Germline Stem Cells by Three-Dimensional-Induced System. *Cell Death Differ* **2018**, *25*, 749–766, doi:10.1038/s41418-017-0015-1.
29. Shetty, G.; Mitchell, J.M.; Lam, T.N.A.; Phan, T.T.; Zhang, J.; Tailor, R.C.; Peters, K.A.; Penedo, M.C.; Hanna, C.B.; Clark, A.T.; et al. Postpubertal Spermatogonial Stem Cell Transplantation Restores Functional Sperm Production in Rhesus Monkeys Irradiated before and after Puberty. *Andrology* **2021**, doi:10.1111/andr.13033.
30. Fayomi, A.P.; Peters, K.; Sukhwani, M.; Valli-Pulaski, H.; Shetty, G.; Meistrich, M.L.; Houser, L.; Robertson, N.; Roberts, V.; Ramsey, C.; et al. Autologous Grafting of Cryopreserved Prepubertal Rhesus Testis Produces Sperm and Offspring. *Science* **2019**, *363*, 1314–1319, doi:10.1126/science.aav2914.
31. Sato, T.; Katagiri, K.; Kojima, K.; Komeya, M.; Yao, M.; Ogawa, T. In Vitro Spermatogenesis in Explanted Adult Mouse Testis Tissues. *PLoS One* **2015**, *10*, e0130171, doi:10.1371/journal.pone.0130171.
32. Wang, D.; Hildorf, S.; Ntemou, E.; Mamsen, L.S.; Dong, L.; Pors, S.E.; Fedder, J.; Clasen-Linde, E.; Cortes, D.; Thorup, J.; et al. Organotypic Culture of Testicular Tissue from Infant Boys with Cryptorchidism. *Int J Mol Sci* **2022**, *23*, 7975, doi:10.3390/ijms23147975.
33. Galdon, G.; Atala, A.; Sadri-Ardekani, H. In Vitro Spermatogenesis: How Far from Clinical Application? *Curr Urol Rep* **2016**, *17*, 49, doi:10.1007/s11934-016-0605-3.
34. Sadri-Ardekani, H.; Akhondi, M.A.; van der Veen, F.; Repping, S.; van Pelt, A.M. In Vitro Propagation of Human Prepubertal Spermatogonial Stem Cells. *JAMA* **2011**, *305*, 2416–2418, doi:10.1001/jama.2011.791.

35. Sadri-Ardekani, H.; Mizrak, S.C.; van Daalen, S.K.; Korver, C.M.; Roepers-Gajadien, H.L.; Koruji, M.; Hovingh, S.; de Reijke, T.M.; de la Rosette, J.J.; van der Veen, F.; et al. Propagation of Human Spermatogonial Stem Cells in Vitro. *JAMA* **2009**, *302*, 2127–2134, doi:10.1001/jama.2009.1689.
36. Galdon, G.; Deebel, N.A.; Zarandi, N.P.; Pettenati, M.J.; Kogan, S.; Wang, C.; Swerdloff, R.S.; Atala, A.; Lue, Y.; Sadri-Ardekani, H. In Vitro Propagation of XXY Undifferentiated Mouse Spermatogonia: Model for Fertility Preservation in Klinefelter Syndrome Patients. *Int J Mol Sci* **2021**, *23*, 173, doi:10.3390/ijms23010173.
37. Rajan, S.A.P.; Aleman, J.; Wan, M.; Pourhabibi Zarandi, N.; Nzou, G.; Murphy, S.; Bishop, C.E.; Sadri-Ardekani, H.; Shupe, T.; Atala, A.; et al. Probing Prodrug Metabolism and Reciprocal Toxicity with an Integrated and Humanized Multi-Tissue Organ-on-a-Chip Platform. *Acta Biomater* **2020**, *106*, 124–135, doi:10.1016/j.actbio.2020.02.015.
38. Skardal, A.; Aleman, J.; Forsythe, S.; Rajan, S.; Murphy, S.; Devarasetty, M.; Pourhabibi Zarandi, N.; Nzou, G.; Wicks, R.; Sadri-Ardekani, H.; et al. Drug Compound Screening in Single and Integrated Multi-Organoid Body-on-a-Chip Systems. *Biofabrication* **2020**, *12*, 025017, doi:10.1088/1758-5090/ab6d36.
39. Strange, D.P.; Zarandi, N.P.; Trivedi, G.; Atala, A.; Bishop, C.E.; Sadri-Ardekani, H.; Verma, S. Human Testicular Organoid System as a Novel Tool to Study Zika Virus Pathogenesis. *Emerg Microbes Infect* **2018**, *7*, 82, doi:10.1038/s41426-018-0080-7.
40. Kanatsu-Shinohara, M.; Inoue, K.; Ogonuki, N.; Morimoto, H.; Ogura, A.; Shinohara, T. Serum- and Feeder-Free Culture of Mouse Germline Stem Cells. *Biol Reprod* **2011**, *84*, 97–105, doi:10.1095/biolreprod.110.086462.
41. Nickkholgh, B.; Mizrak, S.C.; Korver, C.M.; van Daalen, S.K.; Meissner, A.; Repping, S.; van Pelt, A.M. Enrichment of Spermatogonial Stem Cells from Long-Term Cultured Human Testicular Cells. *Fertil Steril* **2014**, *102*, 558-565 e5, doi:10.1016/j.fertnstert.2014.04.022.
42. Nickkholgh, B.; Mizrak, S.C.; van Daalen, S.K.M.; Korver, C.M.; Sadri-Ardekani, H.; Repping, S.; van Pelt, A.M.M. Genetic and Epigenetic Stability of Human Spermatogonial Stem Cells during Long-Term Culture. *Fertil Steril* **2014**, *102*, 1700-1707.e1, doi:10.1016/j.fertnstert.2014.08.022.
43. Zohni, K.; Zhang, X.; Tan, S.L.; Chan, P.; Nagano, M. CD9 Is Expressed on Human Male Germ Cells That Have a Long-Term Repopulation Potential after Transplantation into Mouse Testes. *Biol Reprod* **2012**, *87*, 27, doi:10.1095/biolreprod.112.098913.
44. Hutter, H.; Dohr, G. HLA Expression on Immature and Mature Human Germ Cells. *J Reprod Immunol* **1998**, *38*, 101–122.
45. Griswold, M.D.; Oatley, J.M. Concise Review: Defining Characteristics of Mammalian Spermatogenic Stem Cells. *Stem Cells* **2013**, *31*, 8–11, doi:10.1002/stem.1253.
46. Guo, Y.; Hai, Y.; Gong, Y.; Li, Z.; He, Z. Characterization, Isolation, and Culture of Mouse and Human Spermatogonial Stem Cells. *J Cell Physiol* **2014**, *229*, 407–413, doi:10.1002/jcp.24471.
47. Sato, T.; Katagiri, K.; Gohbara, A.; Inoue, K.; Ogonuki, N.; Ogura, A.; Kubota, Y.; Ogawa, T. In Vitro Production of Functional Sperm in Cultured Neonatal Mouse Testes. *Nature* **2011**, *471*, 504–507, doi:10.1038/nature09850.
48. Yokonishi, T.; Sato, T.; Katagiri, K.; Ogawa, T. In Vitro Spermatogenesis Using an Organ Culture Technique. *Methods Mol Biol* **2013**, *927*, 479–488, doi:10.1007/978-1-62703-038-0_41.
49. Hogg, K.; Western, P.S. Differentiation of Fetal Male Germline and Gonadal Progenitor Cells Is Disrupted in Organ Cultures Containing Knockout Serum Replacement. *Stem Cells Dev* **2015**, *24*, 2899–2911, doi:10.1089/scd.2015.0196.

Discussion

As previously described by our group we envision a clinical scenario where peripubertal, young adults, or even adult KS patients with no sperm detected in semen would undergo TESE. Most likely these patients would already present some degree of testicular fibrosis. Then, if no sperm was to be found during TESE, immature spermatogonia possibly present in the testicular biopsy could be isolated and propagated *in vitro* for SSC-based future fertility treatments with or without previous cryopreservation (88). To mirror this scenario, a decision was made to use 3 day-old XXY Klinefelter mice where testicular fibrosis had been documented but spermatogonia may still be present (91). These samples proved to be adequate as, despite the significant testicular fibrosis observed in histology analysis, spermatogonias were successfully isolated. When the

Only a few research groups have reported successful testicular cell propagation *in vitro*. Even fewer groups have been able to do it with testicular cells obtained from NOA patients' testicular biopsies (225,228) despite being some of the patients who would benefit most from SSC-based fertility treatments. To the best of our knowledge, this is the first-ever report on long-term *in vitro* propagation of testicular cells from KS mouse or KS human subjects. It is crucial, as KS is the most common genetic cause of NOA.

Along the way, our culture system has evolved and improved to suit the specific needs of KS testicular tissue and showed consistency between different KS patients. In our hands, KS testicular cells from both mouse and human initially required more favorable culture conditions to attach and propagate mainly determined by enriched culture media, high seeding concentration and close monitoring. However, once initial stages of the culture were overcome, the propagation rate was comparable to euploid controls. Significantly, our work focused on non-immortalized primary testicular cells to better adapt current health and safety regulations and facilitate its future clinical application. For the same reason a decision was made to avoid culture media co-factors with clinical safety concerns as Knock-out serum replacement and all the digestive enzymes used during cell isolation were FDA-approved for clinical use.

When the different experimental SSC-based ARTs are compared one main difference comes up. While testicular grafting and testicular tissue *ex vivo* culture are tissue-based strategies, SSC transplantation and spermatogenesis *in vitro* are cell-based strategies. Tissue-based strategies are limited by the total amount of testicular tissue available through biopsy. On the other hand, cell-based strategies can be enabled by *in vitro* propagation to overcome testicular tissue scarcity, as shown in this study. The propagation results obtained would theoretically be sufficient to reproduce in humans for SSC transplantation clinical application, as demonstrated in non-human primate recently (174,175). If spermatogenesis *in vitro* is considered the number of cells might be even lower. This factor might be a critical advantage to bring SSC-based ART closer to clinical application.

Moreover, the KS human testicular tissue used in this study belongs to real patients enrolled in a experimental testicular tissue bank. There is a real chance that at some point these patients decide to pursue offspring, present with NOA and no sperm is retrieved by TESE and then experimental SSC-based ARTs is considered. This study will provide invaluable real data on the viability of SSC isolation and propagation from their own cryopreserved testicular tissue.

As previously mentioned, there is currently no consensus on what age should TESE be performed on KS patients. Nos clear clinically significant differences have been reported on sperm retrieval between peripubertal young adults and adults(114,127). However, early testicular biopsy likely increases the chances to find immature spermatogonia and possibly SSC (84). Paving the way for SSC-based male fertility treatment could impact current clinical practice to lean into early-testicular biopsy. Then, if no sperm are found, testicular tissue samples could be cryopreserved to consider SSC-based ART in the future (88,232).

Results from this study reasonably identify a population of SSC present in culture. Previous studies have used SSC xenotransplantation to complete SSC characterization (177). However, it is inefficient, not cost-effective, involves extensive animal work with its own ethical concerns and its findings aren't categorically translatable into humans. We consider that the combination of gene expression, flow cytometry and RNAseq data provides reasonable confirmation of the presence of SSCs in culture at this point. The systematic and consistent manner on how this study has been conducted will also provide valuable information on when to attempt SSC transplantation to optimize SSC presence in culture. Definitive confirmation of the functionality of SSC in culture would require experimental SSC transplantation in patients. More preclinical safety and efficacy studies are needed before it becomes acceptable, but when that time comes this study will have meant a significant step forward.

Gene expression analysis confirmed the presence of all four expected testicular cell types in culture from both mice and human cells. This is a key aspect of our culture system as it aims to faithfully recreate testicular environment where somatic cells likely support spermatogonia in more ways that we can anticipate. While it is definitely important to maintain a certain balance so somatic cells don't overgrow spermatogonia we aren't looking to over-purify the spermatogonia population either.

The intratesticular mosaicism observed in KS patients without signs of mosaicism in peripheral blood supports the hypothesis of tissue-specific mosaicism postulated in previous studies(17). Tissue-specific mosaicism may also influence the clinical variability observed in KS patients. It could also explain the remaining foci of spermatogenic activity in azoospermic KS adults (114). Furthermore, the observation that euploid cells in culture are preferentially spermatogonia is consistent with the documented clinical finding of predominantly euploid intratesticular KS gametes (88) as well as adding valuable insight on the SSC niche dynamics on KS. In this way, the importance of developing new SSC based fertility therapies for KS patients is strengthened since they can give rise to healthy offspring.

After sex chromosomal mosaicisim was observed, a reasonable concern could be risen on wether it reflected chromosomal instability in the cells in culture. However, next-generation sequencing analysis for ploidy did not detected significant presence of somatic chromosomes aneuploidy nor deletions or translocations. These results further support the safety and stability of our culture system as currently described.

Testicular organoid formed with KS testicular cells maintain good viability through 23 days in culture (2 days in formation media followed by 21 days in differentiation media) with no significant core necrosis observed. During the first differentiation week a slight decline in ATP production was observed probably due to the transition from 2D to 3D culture system as well as the loss of cells not incorporated to the 3D organoid structure. Then, ATP production remained stable for the rest of the study. These findings overall suggest that the optimized 3D culture system in terms of culture surface, media, number of cells per organoid, organoid size, and organoid handling technique described in this study was adequate.

Characteristic gene expression from all four main testicular cell-types was present through the study, suggesting that all of them had been incorporated into the organoid structure. Testosterone production was consistent during the culture. Needed is to remark that no testosterone was present in the culture medie but basal hCG instead. On the other hand, the lack of response to hCG pulse stimulation, in contrast to previous findings on euploid testicular organoids, might yield new data on the pathophysiological mechanisms behing KS and support its use as a KS disease model. Successful *in vitro* testosterone production may eventually open the door for autologous hormone replacement therapy.

Moreover, sequential gene expression analysis indicated spermatogonia differentiation process taking place *in vitro.* Finding haploid cells after three weeks of differentiation culture confirmed the completion of meiosis and represent a significant advance for *in vitro* spermatogenesis. Subsequent studies should evaluate the fertilization potential from gametes developed *in vitro* as well as the viability of the resulting embryos.

The overall data reaffirmed the value of 3D testicular organoids as a valuable tool to recreate testicular physiology in toxicity studies, pharmacological exploration and a promising experimental fertility therapy.

I hope the accomplishment of this work will open the door for new autologous cell-based infertility treatments. It may as well improve our understanding of KS and hopefully provide a reliable *in vitro* disease model.

Conclusions:

a) Isolation and *in vitro* propagation of murine XXY testicular cells was achieved for the first time. Cells in culture maintained characteristic gene expression of the main four testicular cell types: Spermatogonia, Sertoli, peritubular and Leydig cells. A population of putative spermatogonial stem cells potentially suitable for transplantation was identified.
b) Isolation and *in vitro* propagation of human XXY testicular cells was achieved for the first time. Cells in culture maintained characteristic gene expression of the main four testicular cell types: Spermatogonia, Sertoli, peritubular and Leydig cells. A population of putative spermatogonial stem cells potentially suitable for transplantation was identified.
c) Sex chromosome mosaicism was identified, characterized and quantified both on *in vitro* cultured testicular cells and *in vivo* testicular biopsies from clinically diagnosed non-mosaic Klinefelter patients.
d) Three-dimensional testicular organoids were successfully created from human XXY testicular cells, recreating *in vitro* characteristics of testicular physiology including testosterone production, ATP production and characteristic gene expression.
e) After three weeks in culture, human Klinefelter syndrome testicular organoids underwent spermatogenesis differentiation resulting in haploid cell production.

References:

1. Lanfranco F, Kamischke A, Zitzmann M, Nieschlag E. Klinefelter's syndrome. Lancet. 2004 Jul 17;364(9430):273–83.

2. Jacobs PA, Strong JA. A case of human intersexuality having a possible XXY sex-determining mechanism. Nature. 1959 Jan 31;183(4657):302–3.

3. Bonomi M, Rochira V, Pasquali D, Balercia G, Jannini EA, Ferlin A, et al. Klinefelter syndrome (KS): genetics, clinical phenotype and hypogonadism. J Endocrinol Invest. 2017 Feb;40(2):123–34.

4. Bojesen A, Juul S, Gravholt CH. Prenatal and postnatal prevalence of Klinefelter syndrome: a national registry study. J Clin Endocrinol Metab. 2003 Feb;88(2):622–6.

5. Coffee B, Keith K, Albizua I, Malone T, Mowrey J, Sherman SL, et al. Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA. Am J Hum Genet. 2009 Oct;85(4):503–14.

6. Herlihy AS, Halliday JL, Cock ML, McLachlan RI. The prevalence and diagnosis rates of Klinefelter syndrome: an Australian comparison. Med J Aust. 2011 Jan 3;194(1):24–8.

7. Berglund A, Stochholm K, Gravholt CH. The epidemiology of sex chromosome abnormalities. Am J Med Genet C Semin Med Genet. 2020 Jun;184(2):202–15.

8. Zhao Y, Gardner EJ, Tuke MA, Zhang H, Pietzner M, Koprulu M, et al. Detection and characterization of male sex chromosome abnormalities in the UK Biobank study. Genet Med. 2022 Jun 9;S1098-3600(22)00777-8.

9. Forti G, Corona G, Vignozzi L, Krausz C, Maggi M. Klinefelter's syndrome: a clinical and therapeutical update. Sex Dev. 2010 Sep;4(4–5):249–58.

10. Vincent MC, Daudin M, De MP, Massat G, Mieusset R, Pontonnier F, et al. Cytogenetic investigations of infertile men with low sperm counts: a 25-year experience. J Androl. 2002 Feb;23(1):18–22; discussion 44-45.

11. Tüttelmann F, Werny F, Cooper TG, Kliesch S, Simoni M, Nieschlag E. Clinical experience with azoospermia: aetiology and chances for spermatozoa detection upon biopsy. Int J Androl. 2011 Aug;34(4):291–8.

12. Ohkura H. Meiosis: an overview of key differences from mitosis. Cold Spring Harb Perspect Biol. 2015 Jan 20;7(5):a015859.

13. Maiburg M, Repping S, Giltay J. The genetic origin of Klinefelter syndrome and its effect on spermatogenesis. Fertil Steril. 2012 Aug;98(2):253–60.

14. Bojesen A, Gravholt CH. Klinefelter syndrome in clinical practice. Nat Clin Pract Urol. 2007 Apr;4(4):192–204.

15. Thomas NS, Hassold TJ. Aberrant recombination and the origin of Klinefelter syndrome. Hum Reprod Update. 2003 Aug;9(4):309–17.

16. Bergère M, Wainer R, Nataf V, Bailly M, Gombault M, Ville Y, et al. Biopsied testis cells of four 47,XXY patients: fluorescence in-situ hybridization and ICSI results. Hum Reprod. 2002 Jan;17(1):32–7.

17. Garcia-Quevedo L, Blanco J, Sarrate Z, Català V, Bassas L, Vidal F. Hidden mosaicism in patients with Klinefelter's syndrome: implications for genetic reproductive counselling. Hum Reprod. 2011 Dec;26(12):3486–93.

18. Blanco J, Egozcue J, Vidal F. Meiotic behaviour of the sex chromosomes in three patients with sex chromosome anomalies (47,XXY, mosaic 46,XY/47,XXY and 47,XYY) assessed by fluorescence in-situ hybridization. Hum Reprod. 2001 May;16(5):887–92.

19. Wikström AM, Dunkel L. Klinefelter syndrome. Best Pract Res Clin Endocrinol Metab. 2011 Apr;25(2):239–50.

20. Christiansen P, Andersson AM, Skakkebaek NE. Longitudinal studies of inhibin B levels in boys and young adults with Klinefelter syndrome. J Clin Endocrinol Metab. 2003 Feb;88(2):888–91.

21. Aksglaede L, Christiansen P, Sørensen K, Boas M, Linneberg A, Main KM, et al. Serum concentrations of Anti-Müllerian Hormone (AMH) in 95 patients with Klinefelter syndrome with or without cryptorchidism. Acta Paediatr. 2011 Jun;100(6):839–45.

22. Salbenblatt JA, Bender BG, Puck MH, Robinson A, Faiman C, Winter JS. Pituitary-gonadal function in Klinefelter syndrome before and during puberty. Pediatr Res. 1985 Jan;19(1):82–6.

23. Santi D, De Vincentis S, Scaltriti S, Rochira V. Relative hyperestrogenism in Klinefelter Syndrome: results from a meta-analysis. Endocrine. 2019 May;64(2):209–19.

24. Groth KA, Skakkebaek A, Host C, Gravholt CH, Bojesen A. Clinical review: Klinefelter syndrome--a clinical update. J Clin Endocrinol Metab. 2013 Jan;98(1):20–30.

25. Chang S, Christiansen CF, Bojesen A, Juul S, Münster AMB, Gravholt CH. Klinefelter syndrome and testosterone treatment: a national cohort study on thrombosis risk. Endocr Connect. 2020 Jan;9(1):34–43.

26. Zhang M, Fan HT, Zheng HS, Zhang QS, Feng SQ, Li RW. Clinical characteristics of men with non-mosaic Klinefelter syndrome in northeastern China: implications for genetic counseling. Genet Mol Res. 2015 Sep 10;14(3):10897–904.

27. Zitzmann M, Aksglaede L, Corona G, Isidori AM, Juul A, T'Sjoen G, et al. European academy of andrology guidelines on Klinefelter Syndrome Endorsing Organization: European Society of Endocrinology. Andrology. 2021 Jan;9(1):145–67.

28. Nielsen J, Pelsen B, Sørensen K. Follow-up of 30 Klinefelter males treated with testosterone. Clin Genet. 1988 Apr;33(4):262–9.

29. Mehta A, Paduch DA. Klinefelter syndrome: an argument for early aggressive hormonal and fertility management. Fertil Steril. 2012 Aug;98(2):274–83.

30. Gies I, Unuane D, Velkeniers B, De Schepper J. Management of Klinefelter syndrome during transition. Eur J Endocrinol. 2014 Aug;171(2):R67-77.

31. Ross JL, Roeltgen DP, Stefanatos G, Benecke R, Zeger MPD, Kushner H, et al. Cognitive and motor development during childhood in boys with Klinefelter syndrome. Am J Med Genet A. 2008 Mar 15;146A(6):708–19.

32. Mehta A, Bolyakov A, Roosma J, Schlegel PN, Paduch DA. Successful testicular sperm retrieval in adolescents with Klinefelter syndrome treated with at least 1 year of topical testosterone and aromatase inhibitor. Fertil Steril. 2013 Oct;100(4):970–4.

33. Zitzmann M, Depenbusch M, Gromoll J, Nieschlag E. X-chromosome inactivation patterns and androgen receptor functionality influence phenotype and social characteristics as well as pharmacogenetics of testosterone therapy in Klinefelter patients. J Clin Endocrinol Metab. 2004 Dec;89(12):6208–17.

34. Aksglaede L, Wikström AM, Rajpert-De Meyts E, Dunkel L, Skakkebaek NE, Juul A. Natural history of seminiferous tubule degeneration in Klinefelter syndrome. Hum Reprod Update. 2006 Feb;12(1):39–48.

35. Wikström AM, Painter JN, Raivio T, Aittomäki K, Dunkel L. Genetic features of the X chromosome affect pubertal development and testicular degeneration in adolescent boys with Klinefelter syndrome. Clin Endocrinol (Oxf). 2006 Jul;65(1):92–7.

36. Navarro-Cobos MJ, Balaton BP, Brown CJ. Genes that escape from X-chromosome inactivation: Potential contributors to Klinefelter syndrome. Am J Med Genet C Semin Med Genet. 2020 Jun;184(2):226–38.

37. Bojesen A, Gravholt CH. Morbidity and mortality in Klinefelter syndrome (47,XXY). Acta Paediatr. 2011 Jun;100(6):807–13.

38. Bojesen A, Kristensen K, Birkebaek NH, Fedder J, Mosekilde L, Bennett P, et al. The metabolic syndrome is frequent in Klinefelter's syndrome and is associated with abdominal obesity and hypogonadism. Diabetes Care. 2006 Jul;29(7):1591–8.

39. Overvad S, Bay K, Bojesen A, Gravholt CH. Low INSL3 in Klinefelter syndrome is related to osteocalcin, testosterone treatment and body composition, as well as measures of the hypothalamic-pituitary-gonadal axis. Andrology. 2014 May;2(3):421–7.

40. Breuil V, Euller-Ziegler L. Gonadal dysgenesis and bone metabolism. Joint Bone Spine. 2001 Feb;68(1):26–33.

41. van den Bergh JP, Hermus AR, Spruyt AI, Sweep CG, Corstens FH, Smals AG. Bone mineral density and quantitative ultrasound parameters in patients with Klinefelter's syndrome after long-term testosterone substitution. Osteoporos Int. 2001;12(1):55–62.

42. Wikström AM, Bay K, Hero M, Andersson AM, Dunkel L. Serum insulin-like factor 3 levels during puberty in healthy boys and boys with Klinefelter syndrome. J Clin Endocrinol Metab. 2006 Nov;91(11):4705–8.

43. Fabrazzo M, Accardo G, Abbondandolo I, Goglia G, Esposito D, Sampogna G, et al. Quality of life in Klinefelter patients on testosterone replacement therapy compared to healthy controls: an observational study on the impact of psychological distress, personality traits, and coping strategies. J Endocrinol Invest. 2021 May;44(5):1053–63.

44. Zitzmann M, Rohayem J. Gonadal dysfunction and beyond: Clinical challenges in children, adolescents, and adults with 47,XXY Klinefelter syndrome. Am J Med Genet C Semin Med Genet. 2020 Jun;184(2):302–12.

45. Winkler UH. Effects of androgens on haemostasis. Maturitas. 1996 Jul;24(3):147–55.

46. Swerdlow AJ, Hermon C, Jacobs PA, Alberman E, Beral V, Daker M, et al. Mortality and cancer incidence in persons with numerical sex chromosome abnormalities: a cohort study. Ann Hum Genet. 2001 Mar;65(Pt 2):177–88.

47. Swerdlow AJ, Higgins CD, Schoemaker MJ, Wright AF, Jacobs PA, United Kingdom Clinical Cytogenetics Group. Mortality in patients with Klinefelter syndrome in Britain: a cohort study. J Clin Endocrinol Metab. 2005 Dec;90(12):6516–22.

48. Swerdlow AJ, Schoemaker MJ, Higgins CD, Wright AF, Jacobs PA, UK Clinical Cytogenetics Group. Cancer incidence and mortality in men with Klinefelter syndrome: a cohort study. J Natl Cancer Inst. 2005 Aug 17;97(16):1204–10.

49. Neto FTL, Bach PV, Najari BB, Li PS, Goldstein M. Spermatogenesis in humans and its affecting factors. Semin Cell Dev Biol. 2016 Nov;59:10–26.

50. O'Shaughnessy PJ, Fowler PA. Development of the human fetal testis. Ann Endocrinol (Paris). 2014 May;75(2):48–53.

51. Griswold MD. Spermatogenesis: The Commitment to Meiosis. Physiol Rev. 2016 Jan;96(1):1–17.

52. Kubota H, Brinster RL. Spermatogonial stem cells. Biol Reprod. 2018 Jul 1;99(1):52–74.

53. Griswold MD, Oatley JM. Concise review: Defining characteristics of mammalian spermatogenic stem cells. Stem Cells. 2013 Jan;31(1):8–11.

54. Yoshida S. Stem cells in mammalian spermatogenesis. Dev Growth Differ. 2010 Apr;52(3):311–7.

55. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A. 1994 Nov 22;91(24):11298–302.

56. Hutter H, Dohr G. HLA expression on immature and mature human germ cells. J Reprod Immunol. 1998 Jul;38(2):101–22.

57. Shinohara T, Avarbock MR, Brinster RL. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5504–9.

58. Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci U S A. 2000 Jul 18;97(15):8346–51.

59. Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A. 2003 May 27;100(11):6487–92.

60. Tokuda M, Kadokawa Y, Kurahashi H, Marunouchi T. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod. 2007 Jan;76(1):130–41.

61. Hermann BP, Sukhwani M, Lin CC, Sheng Y, Tomko J, Rodriguez M, et al. Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. Stem Cells. 2007 Sep;25(9):2330–8.

62. Izadyar F, Wong J, Maki C, Pacchiarotti J, Ramos T, Howerton K, et al. Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod. 2011 Jun;26(6):1296–306.

63. Kanatsu-Shinohara M, Morimoto H, Shinohara T. Enrichment of mouse spermatogonial stem cells by melanoma cell adhesion molecule expression. Biol Reprod. 2012 Jun;87(6):139.

64. Nickkholgh B, Mizrak SC, Korver CM, van Daalen SK, Meissner A, Repping S, et al. Enrichment of spermatogonial stem cells from long-term cultured human testicular cells. Fertil Steril. 2014 Aug;102(2):558-565 e5.

65. Zheng Y, He Y, An J, Qin J, Wang Y, Zhang Y, et al. THY1 is a surface marker of porcine gonocytes. Reprod Fertil Dev. 2014;26(4):533–9.

66. Suzuki S, Diaz VD, Hermann BP. What has single-cell RNA-seq taught us about mammalian spermatogenesis? Biol Reprod. 2019 Sep 1;101(3):617–34.

67. Bellil H, Ghieh F, Hermel E, Mandon-Pepin B, Vialard F. Human testis-expressed (TEX) genes: a review focused on spermatogenesis and male fertility. Basic Clin Androl. 2021 Apr 22;31(1):9.

68. Zhao L, Yao C, Xing X, Jing T, Li P, Zhu Z, et al. Single-cell analysis of developing and azoospermia human testicles reveals central role of Sertoli cells. Nat Commun. 2020 Nov 10;11(1):5683.

69. Hermann BP, Cheng K, Singh A, Roa-De La Cruz L, Mutoji KN, Chen IC, et al. The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. Cell Rep. 2018 Nov 6;25(6):1650-1667.e8.

70. Tan K, Wilkinson MF. A single-cell view of spermatogonial stem cells. Curr Opin Cell Biol. 2020 Dec;67:71–8.

71. Guo J, Grow EJ, Mlcochova H, Maher GJ, Lindskog C, Nie X, et al. The adult human testis transcriptional cell atlas. Cell Res. 2018 Dec;28(12):1141–57.

72. He H, Yu F, Shen W, Chen K, Zhang L, Lou S, et al. The Novel Key Genes of Non-obstructive Azoospermia Affect Spermatogenesis: Transcriptomic Analysis Based on RNA-Seq and scRNA-Seq Data. Front Genet. 2021;12:608629.

73. Di Persio S, Tekath T, Siebert-Kuss LM, Cremers JF, Wistuba J, Li X, et al. Single-cell RNA-seq unravels alterations of the human spermatogonial stem cell compartment in patients with impaired spermatogenesis. Cell Rep Med. 2021 Sep 21;2(9):100395.

74. Winge SB, Dalgaard MD, Jensen JM, Graem N, Schierup MH, Juul A, et al. Transcriptome profiling of fetal Klinefelter testis tissue reveals a possible involvement of long non-coding RNAs in gonocyte maturation. Hum Mol Genet. 2017 Nov 24;

75. Van Saen D, Vloeberghs V, Gies I, De Schepper J, Tournaye H, Goossens E. Characterization of the stem cell niche components within the seminiferous tubules in testicular biopsies of Klinefelter patients. Fertil Steril. 2020 Jun;113(6):1183-1195.e3.

76. Mikamo K, Aguercif M, Hazeghi P, Martin-Du Pan R. Chromatin-positive Klinefelter's syndrome. A quantitative analysis of spermatogonial deficiency at 3, 4, and 12 months of age. Fertil Steril. 1968 Oct;19(5):731–9.

77. Gordon DL, Krmpotic E, Thomas W, Gandy HM, Paulsen CA. Pathologic testicular findings in Klinefelter's syndrome. 47,XXY vs 46,XY-47,XXY. Arch Intern Med. 1972 Nov;130(5):726–9.

78. Autio-Harmainen H, Rapola J, Aula P. Fetal gonadal histology in XXXXY, XYY and XXX syndromes. Clin Genet. 1980 Jul;18(1):1–5.

79. Coerdt W, Rehder H, Gausmann I, Johannisson R, Gropp A. Quantitative histology of human fetal testes in chromosomal disease. Pediatr Pathol. 1985;3(2–4):245–59.

80. Foresta C, Galeazzi C, Bettella A, Marin P, Rossato M, Garolla A, et al. Analysis of meiosis in intratesticular germ cells from subjects affected by classic Klinefelter's syndrome. J Clin Endocrinol Metab. 1999 Oct;84(10):3807–10.

81. Wikstrom AM, Raivio T, Hadziselimovic F, Wikstrom S, Tuuri T, Dunkel L. Klinefelter syndrome in adolescence: onset of puberty is associated with accelerated germ cell depletion. J Clin Endocrinol Metab. 2004 May;89(5):2263–70.

82. Wikström AM, Hoei-Hansen CE, Dunkel L, Rajpert-De Meyts E. Immunoexpression of androgen receptor and nine markers of maturation in the testes of adolescent boys with Klinefelter syndrome: evidence for degeneration of germ cells at the onset of meiosis. J Clin Endocrinol Metab. 2007 Feb;92(2):714–9.

83. Willems M, Gies I, Van Saen D. Germ cell loss in Klinefelter syndrome: When and why? Am J Med Genet C Semin Med Genet. 2020 Jun;184(2):356–70.

84. Deebel NA, Galdon G, Zarandi NP, Stogner-Underwood K, Howards S, Lovato J, et al. Age-related presence of spermatogonia in patients with Klinefelter syndrome: a systematic review and meta-analysis. Hum Reprod Update. 2020 01;26(1):58–72.

85. Müller J, Skakkebaek NE, Ratcliffe SG. Quantified testicular histology in boys with sex chromosome abnormalities. Int J Androl. 1995 Apr;18(2):57–62.

86. Van Saen D, Vloeberghs V, Gies I, Mateizel I, Sermon K, De Schepper J, et al. When does germ cell loss and fibrosis occur in patients with Klinefelter syndrome? Hum Reprod. 2018 Jun 1;33(6):1009–22.

87. Skakkebaek NE. Two types of tubules containing only Sertoli cells in adults with Klinefelter's syndrome. Nature. 1969 Aug 9;223(5206):643–5.

88. Deebel NA, Bradshaw AW, Sadri-Ardekani H. Infertility considerations in klinefelter syndrome: From origin to management. Best Pract Res Clin Endocrinol Metab. 2020 Dec;34(6):101480.

89. Vialard F, Bailly M, Bouazzi H, Albert M, Pont JC, Mendes V, et al. The high frequency of sperm aneuploidy in klinefelter patients and in nonobstructive azoospermia is due to meiotic errors in euploid spermatocytes. J Androl. 2012 Dec;33(6):1352–9.

90. Lue Y, Rao PN, Sinha Hikim AP, Im M, Salameh WA, Yen PH, et al. XXY male mice: an experimental model for Klinefelter syndrome. Endocrinology. 2001 Apr;142(4):1461–70.

91. Lue Y, Jentsch JD, Wang C, Rao PN, Hikim AP, Salameh W, et al. XXY mice exhibit gonadal and behavioral phenotypes similar to Klinefelter syndrome. Endocrinology. 2005 Sep;146(9):4148–54.

92. Lue Y, Liu PY, Erkkila K, Ma K, Schwarcz M, Wang C, et al. Transplanted XY germ cells produce spermatozoa in testes of XXY mice. Int J Androl. 2010 Aug 1;33(4):581–7.

93. Joerg H, Janett F, Schlatt S, Mueller S, Graphodatskaya D, Suwattana D, et al. Germ cell transplantation in an azoospermic Klinefelter bull. Biol Reprod. 2003 Dec;69(6):1940–4.

94. Wistuba J, Luetjens CM, Stukenborg JB, Poplinski A, Werler S, Dittmann M, et al. Male 41, XXY* mice as a model for klinefelter syndrome: hyperactivation of leydig cells. Endocrinology. 2010 Jun;151(6):2898–910.

95. Wistuba J, Beumer C, Brehm R, Gromoll J. 41,XXY * male mice: An animal model for Klinefelter syndrome. Am J Med Genet C Semin Med Genet. 2020 Jun;184(2):267–78.

96. Werler S, Poplinski A, Gromoll J, Wistuba J. Expression of selected genes escaping from X inactivation in the 41, XX(Y)* mouse model for Klinefelter's syndrome. Acta Paediatr. 2011 Jun;100(6):885–91.

97. Werler S, Demond H, Damm OS, Ehmcke J, Middendorff R, Gromoll J, et al. Germ cell loss is associated with fading Lin28a expression in a mouse model for Klinefelter's syndrome. Reproduction. 2014 Mar;147(3):253–64.

98. Hirota T, Ohta H, Powell BE, Mahadevaiah SK, Ojarikre OA, Saitou M, et al. Fertile offspring from sterile sex chromosome trisomic mice. Science. 2017 Sep 1;357(6354):932–5.

99. Swerdloff RS, Lue Y, Liu PY, Erkkilä K, Wang C. Mouse model for men with klinefelter syndrome: a multifaceted fit for a complex disorder. Acta Paediatr. 2011 Jun;100(6):892–9.

100. Kitamura M, Matsumiya K, Koga M, Nishimura K, Miura H, Tsuji T, et al. Ejaculated spermatozoa in patients with non-mosaic Klinefelter's syndrome. Int J Urol. 2000 Mar;7(3):88–92; discussion 93-4.

101. Aksglaede L, Jorgensen N, Skakkebaek NE, Juul A. Low semen volume in 47 adolescents and adults with 47,XXY Klinefelter or 46,XX male syndrome. Int J Androl. 2009 Aug;32(4):376–84.

102. Kaplan H, Aspillaga M, Shelley TF, Gardner LI. Possible fertility in Klinefelter's syndrome. Lancet. 1963 Mar 2;1(7279):506.

103. Laron Z, Dickerman Z, Zamir R, Galatzer A. Paternity in Klinefelter's syndrome--a case report. Arch Androl. 1982 Mar;8(2):149–51.

104. Terzoli G, Lalatta F, Lobbiani A, Simoni G, Colucci G. Fertility in a 47,XXY patient: assessment of biological paternity by deoxyribonucleic acid fingerprinting. Fertil Steril. 1992 Oct;58(4):821–2.

105. Bourne H, Stern K, Clarke G, Pertile M, Speirs A, Baker HW. Delivery of normal twins following the intracytoplasmic injection of spermatozoa from a patient with 47,XXY Klinefelter's syndrome. Hum Reprod. 1997 Nov;12(11):2447–50.

106. Cruger D, Toft B, Agerholm I, Fedder J, Hald F, Bruun-Petersen G. Birth of a healthy girl after ICSI with ejaculated spermatozoa from a man with non-mosaic Klinefelter's syndrome. Hum Reprod. 2001 Sep;16(9):1909–11.

107. Tachdjian G, Frydman N, Morichon-Delvallez N, Dû AL, Fanchin R, Vekemans M, et al. Reproductive genetic counselling in non-mosaic 47,XXY patients: implications for preimplantation or prenatal diagnosis: Case report and review. Hum Reprod. 2003 Feb;18(2):271–5.

108. Komori S, Horiuchi I, Hamada Y, Hasegawa A, Kasumi H, Kondoh N, et al. Birth of healthy neonates after intracytoplasmic injection of ejaculated or testicular spermatozoa from men with nonmosaic Klinefelter's syndrome: a report of 2 cases. J Reprod Med. 2004 Feb;49(2):126–30.

109. Akashi T, Fuse H, Kojima Y, Hayashi M, Honda S. Birth after intracytoplasmic sperm injection of ejaculated spermatozoa from a man with mosaic Klinefelter's syndrome. Asian J Androl. 2005 Jun;7(2):217–20.

110. Gonsalves J, Turek PJ, Schlegel PN, Hopps CV, Weier JF, Pera RA. Recombination in men with Klinefelter syndrome. Reproduction. 2005 Aug;130(2):223–9.

111. Sciurano RB, Luna Hisano CV, Rahn MI, Brugo Olmedo S, Rey Valzacchi G, Coco R, et al. Focal spermatogenesis originates in euploid germ cells in classical Klinefelter patients. Hum Reprod. 2009 Sep;24(9):2353–60.

112. Fullerton G, Hamilton M, Maheshwari A. Should non-mosaic Klinefelter syndrome men be labelled as infertile in 2009? Hum Reprod. 2010 Mar;25(3):588–97.

113. Aksglaede L, Juul A. Testicular function and fertility in men with Klinefelter syndrome: a review. Eur J Endocrinol. 2013 Apr;168(4):R67-76.

114. Corona G, Pizzocaro A, Lanfranco F, Garolla A, Pelliccione F, Vignozzi L, et al. Sperm recovery and ICSI outcomes in Klinefelter syndrome: a systematic review and meta-analysis. Hum Reprod Update. 2017 May 1;23(3):265–75.

115. Boeri L, Palmisano F, Preto M, Sibona M, Capogrosso P, Franceschelli A, et al. Sperm retrieval rates in non-mosaic Klinefelter patients undergoing testicular sperm extraction: What expectations do we have in the real-life setting? Andrology. 2020 May;8(3):680–7.

116. Almardawi AH, Akram R, Kattan Y, Al Suhaibani SS, AlAli H, Kattan S, et al. Micro-dissection testicular sperm extraction in Klinefelter's syndrome patients, King Faisal Specialist Hospital and Research Center, Riyadh experience. Urol Ann. 2022 Mar;14(1):43–7.

117. Staessen C, Tournaye H, Van Assche E, Michiels A, Van Landuyt L, Devroey P, et al. PGD in 47,XXY Klinefelter's syndrome patients. Hum Reprod Update. 2003 Aug;9(4):319–30.

118. Vicdan K, Akarsu C, Sozen E, Buluc B, Vicdan A, Yilmaz Y, et al. Outcome of intracytoplasmic sperm injection using fresh and cryopreserved-thawed testicular spermatozoa in 83 azoospermic men with Klinefelter syndrome. J Obstet Gynaecol Res. 2016 Nov;42(11):1558–66.

119. Okuyama N, Obata R, Oka N, Nakamura Y, Hattori H, Nakajo Y, et al. Long-term clinical outcomes of testicular sperm extraction and intracytoplasmic sperm injection for infertile men. Reprod Med Biol. 2018 Jan;17(1):82–8.

120. Rives-Feraille A, Liard A, Bubenheim M, Barbotin AL, Giscard d'Estaing S, Mirallié S, et al. Assessment of the architecture and integrity of frozen-thawed testicular tissue from (pre)pubertal boys with cancer. Andrology. 2022 Feb;10(2):279–90.

121. Karibe J, Kuroda S, Saito T, Ishibashi Y, Usui K, Takeshima T, et al. Monozygotic adult twins with nonmosaic Klinefelter syndrome with different results of sperm retrieval. Andrologia. 2022 Feb;54(1):e14266.

122. Tournaye H, Verheyen G, Nagy P, Ubaldi F, Goossens A, Silber S, et al. Are there any predictive factors for successful testicular sperm recovery in azoospermic patients? Hum Reprod. 1997 Jan;12(1):80–6.

123. Silber SJ, Nagy Z, Devroey P, Tournaye H, Van Steirteghem AC. Distribution of spermatogenesis in the testicles of azoospermic men: the presence or absence of

spermatids in the testes of men with germinal failure. Hum Reprod. 1997 Nov;12(11):2422–8.

124. Ezeh UI, Taub NA, Moore HD, Cooke ID. Establishment of predictive variables associated with testicular sperm retrieval in men with non-obstructive azoospermia. Hum Reprod. 1999 Apr;14(4):1005–12.

125. Westlander G, Ekerhovd E, Granberg S, Hanson L, Hanson C, Bergh C. Testicular ultrasonography and extended chromosome analysis in men with nonmosaic Klinefelter syndrome: a prospective study of possible predictive factors for successful sperm recovery. Fertil Steril. 2001 Jun;75(6):1102–5.

126. Rohayem J, Fricke R, Czeloth K, Mallidis C, Wistuba J, Krallmann C, et al. Age and markers of Leydig cell function, but not of Sertoli cell function predict the success of sperm retrieval in adolescents and adults with Klinefelter's syndrome. Andrology. 2015 Sep;3(5):868–75.

127. Yücel C, Keskin MZ, Kose C, Kucuk U, Ilbey YO, Kozacioğlu Z. Relationship between testicular histopathology and the success of testicular sperm extraction in patients with non-mosaic Klinefelter syndrome. Rev Int Androl. 2021 Mar;19(1):9–15.

128. Lin YM, Huang WJ, Lin JSN, Kuo PL. Progressive depletion of germ cells in a man with nonmosaic Klinefelter's syndrome: optimal time for sperm recovery. Urology. 2004 Feb;63(2):380–1.

129. Madgar I, Dor J, Weissenberg R, Raviv G, Menashe Y, Levron J. Prognostic value of the clinical and laboratory evaluation in patients with nonmosaic Klinefelter syndrome who are receiving assisted reproductive therapy. Fertil Steril. 2002 Jun;77(6):1167–9.

130. Li H, Chen LP, Yang J, Li MC, Chen RB, Lan RZ, et al. Predictive value of FSH, testicular volume, and histopathological findings for the sperm retrieval rate of microdissection TESE in nonobstructive azoospermia: a meta-analysis. Asian J Androl. 2018 Feb;20(1):30–6.

131. Franik S, Smeets D, van de Zande G, Gomes I, D'Hauwers K, Braat DDM, et al. Klinefelter syndrome and fertility-Impact of X-chromosomal inheritance on spermatogenesis. Andrologia. 2018 Jun;50(5):e13004.

132. Benderradji H, Prasivoravong J, Marcelli F, Barbotin AL, Catteau-Jonard S, Marchetti C, et al. Contribution of serum anti-Müllerian hormone in the management of azoospermia and the prediction of testicular sperm retrieval outcomes: a study of 155 adult men. Basic Clin Androl. 2021 Jun 17;31(1):15.

133. Shepherd S, Oates R. At what age should we attempt to retrieve sperm from males with Klinefelter syndrome. Transl Androl Urol. 2021 Mar;10(3):1432–41.

134. Chehrazi M, Rahimiforoushani A, Sabbaghian M, Nourijelyani K, Sadighi Gilani MA, Hoseini M, et al. Sperm Retrieval in Patients with Klinefelter Syndrome: A Skewed Regression Model Analysis. Int J Fertil Steril. 2017 Sep;11(2):117–22.

135. Levron J, Aviram-Goldring A, Madgar I, Raviv G, Barkai G, Dor J. Sperm chromosome analysis and outcome of IVF in patients with non-mosaic Klinefelter's syndrome. Fertil Steril. 2000 Nov;74(5):925–9.

136. Hennebicq S, Pelletier R, Bergues U, Rousseaux S. Risk of trisomy 21 in offspring of patients with Klinefelter's syndrome. Lancet. 2001 Jun 30;357(9274):2104–5.

137. Morel F, Bernicot I, Herry A, Le Bris MJ, Amice V, De Braekeleer M. An increased incidence of autosomal aneuploidies in spermatozoa from a patient with Klinefelter's syndrome. Fertil Steril. 2003 Jun;79 Suppl 3:1644–6.

138. Sadri-Ardekani H, Akhondi MM, Vossough P, Maleki H, Sedighnejad S, Kamali K, et al. Parental attitudes toward fertility preservation in boys with cancer: context of different risk levels of infertility and success rates of fertility restoration. Fertil Steril. 2013 Mar 1;99(3):796–802.

139. Ginsberg JP, Li Y, Carlson CA, Gracia CR, Hobbie WL, Miller VA, et al. Testicular tissue cryopreservation in prepubertal male children: an analysis of parental decision-making. Pediatr Blood Cancer. 2014 Sep;61(9):1673–8.

140. Gies I, Oates R, De Schepper J, Tournaye H. Testicular biopsy and cryopreservation for fertility preservation of prepubertal boys with Klinefelter syndrome: a pro/con debate. Fertil Steril. 2016 Feb;105(2):249–55.

141. Van Saen D, Gies I, De Schepper J, Tournaye H, Goossens E. Can pubertal boys with Klinefelter syndrome benefit from spermatogonial stem cell banking? Hum Reprod. 2012 Feb;27(2):323–30.

142. Ferlin A. Strategies to improve early diagnosis of Klinefelter syndrome. Expert Rev Endocrinol Metab. 2020 Nov;15(6):375–8.

143. Mehta A, Malek-Jones M, Bolyakov A, Mielnik A, Schlegel PN, Paduch DA. Methylation-specific PCR allows for fast diagnosis of X chromosome disomy and reveals skewed inactivation of the X chromosome in men with Klinefelter syndrome. J Androl. 2012 Oct;33(5):955–62.

144. Dey M, Sharma S, Aggarwal S. Prenatal screening methods for aneuploidies. N Am J Med Sci. 2013 Mar;5(3):182–90.

145. Zheng H, Jin H, Liu L, Liu J, Wang WH. Application of next-generation sequencing for 24-chromosome aneuploidy screening of human preimplantation embryos. Mol Cytogenet. 2015;8:38.

146. Ottesen AM, Garn ID, Aksglaede L, Juul A, Rajpert-De Meyts E. A simple screening method for detection of Klinefelter syndrome and other X-chromosome aneuploidies based on copy number of the androgen receptor gene. Mol Hum Reprod. 2007 Oct;13(10):745–50.

147. Goossens E, Van Saen D, Tournaye H. Spermatogonial stem cell preservation and transplantation: from research to clinic. Hum Reprod. 2013 Apr;28(4):897–907.

148. Sadri-Ardekani H, Atala A. Testicular tissue cryopreservation and spermatogonial stem cell transplantation to restore fertility: from bench to bedside. Stem Cell Res Ther. 2014 May 28;5(3):68.

149. Doungkamchan C, Orwig KE. Recent advances: fertility preservation and fertility restoration options for males and females. Fac Rev. 2021;10:55.

150. Damani MN, Mittal R, Oates RD. Testicular tissue extraction in a young male with 47,XXY Klinefelter's syndrome: potential strategy for preservation of fertility. Fertil Steril. 2001 Nov;76(5):1054–6.

151. Gies I, De Schepper J, Goossens E, Van Saen D, Pennings G, Tournaye H. Spermatogonial stem cell preservation in boys with Klinefelter syndrome: to bank or not to bank, that's the question. Fertil Steril. 2012 Aug;98(2):284–9.

152. Pietzak EJ, Tasian GE, Tasian SK, Brinster RL, Carlson C, Ginsberg JP, et al. Histology of Testicular Biopsies Obtained for Experimental Fertility Preservation Protocol in Boys with Cancer. J Urol. 2015 Nov;194(5):1420–4.

153. Sadri-Ardekani H, McLean TW, Kogan S, Sirintrapun J, Crowell K, Yousif MQ, et al. Experimental testicular tissue banking to generate spermatogenesis in the future: A multidisciplinary team approach. Methods. 2016 Apr 15;99:120–7.

154. Valli-Pulaski H, Peters KA, Gassei K, Steimer SR, Sukhwani M, Hermann BP, et al. Testicular tissue cryopreservation: 8 years of experience from a coordinated network of academic centers. Hum Reprod. 2019 Jun 4;34(6):966–77.

155. Lakhoo K, Davies J, Chakraborty S, Berg S, Tennyson R, Fowler D, et al. Development of a new reproductive tissue cryopreservation clinical service for children: the Oxford programme. Pediatr Surg Int. 2019 Nov;35(11):1271–8.

156. Izadyar F, Matthijs-Rijsenbilt JJ, den Ouden K, Creemers LB, Woelders H, de Rooij DG. Development of a cryopreservation protocol for type A spermatogonia. J Androl. 2002 Aug;23(4):537–45.

157. Frederickx V, Michiels A, Goossens E, De Block G, Van Steirteghem AC, Tournaye H. Recovery, survival and functional evaluation by transplantation of frozen-thawed mouse germ cells. Hum Reprod. 2004 Apr;19(4):948–53.

158. Jahnukainen K, Ehmcke J, Hergenrother SD, Schlatt S. Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts. Hum Reprod. 2007 Apr;22(4):1060–7.

159. Keros V, Hultenby K, Borgström B, Fridström M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod. 2007 May;22(5):1384–95.

160. Goossens E, Frederickx V, Geens M, De Block G, Tournaye H. Cryosurvival and spermatogenesis after allografting prepubertal mouse tissue: comparison of two cryopreservation protocols. Fertil Steril. 2008 Mar;89(3):725–7.

161. Milazzo JP, Vaudreuil L, Cauliez B, Gruel E, Massé L, Mousset-Siméon N, et al. Comparison of conditions for cryopreservation of testicular tissue from immature mice. Hum Reprod. 2008 Jan;23(1):17–28.

162. Wu X, Goodyear SM, Abramowitz LK, Bartolomei MS, Tobias JW, Avarbock MR, et al. Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. Hum Reprod. 2012 May;27(5):1249–59.

163. Wu JJ, Hu TJ, Guo B, Yue ZP, Yang ZT, Zhang XM. Cryopreservation of adult bovine testicular tissue for spermatogonia enrichment. Cryo Letters. 2011 Oct;32(5):402–9.

164. Baert Y, Braye A, Struijk RB, van Pelt AM, Goossens E. Cryopreservation of testicular tissue before long-term testicular cell culture does not alter in vitro cell dynamics. Fertil Steril. 2015 Nov;104(5):1244-52 e1-4.

165. Zarandi NP, Galdon G, Kogan S, Atala A, Sadri-Ardekani H. Cryostorage of immature and mature human testis tissue to preserve spermatogonial stem cells (SSCs): a systematic review of current experiences toward clinical applications. Stem Cells Cloning. 2018;11:23–38.

166. Medrano JV, Martínez-Arroyo AM, Sukhwani M, Noguera I, Quiñonero A, Martínez-Jabaloyas JM, et al. Germ cell transplantation into mouse testes procedure. Fertil Steril. 2014 Oct;102(4):e11-12.

167. Medrano JV, Acimovic I, Navarro-Gomezlechon A, Noguera I, Pellicer A, Andrés MM, et al. Timing of spermatogonial stem cell transplantation affects the spermatogenic recovery outcome in mice. In Vitro Cell Dev Biol Anim. 2021 Jan;57(1):21–9.

168. Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL. Rat spermatogenesis in mouse testis. Nature. 1996 May 30;381(6581):418–21.

169. Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, Nieschlag E. Germ cell transfer into rat, bovine, monkey and human testes. Hum Reprod. 1999 Jan;14(1):144–50.

170. Harkey MA, Asano A, Zoulas ME, Torok-Storb B, Nagashima J, Travis A. Isolation, genetic manipulation, and transplantation of canine spermatogonial stem cells: progress toward transgenesis through the male germ-line. Reproduction. 2013 Jul;146(1):75–90.

171. Pieri NCG, Mançanares ACF, de Souza AF, Fernandes H, Diaza AMG, Bressan FF, et al. Xenotransplantation of canine spermatogonial stem cells (cSSCs) regulated by FSH promotes spermatogenesis in infertile mice. Stem Cell Res Ther. 2019 May 20;10(1):135.

172. Honaramooz A, Behboodi E, Blash S, Megee SO, Dobrinski I. Germ cell transplantation in goats. Mol Reprod Dev. 2003 Apr;64(4):422–8.

173. Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, et al. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell. 2012 Nov 2;11(5):715–26.

174. Shetty G, Mitchell JM, Meyer JM, Wu Z, Lam TNA, Phan TT, et al. Restoration of functional sperm production in irradiated pubertal rhesus monkeys by spermatogonial stem cell transplantation. Andrology. 2020 Sep;8(5):1428–41.

175. Shetty G, Mitchell JM, Lam TNA, Phan TT, Zhang J, Tailor RC, et al. Postpubertal spermatogonial stem cell transplantation restores functional sperm production in rhesus monkeys irradiated before and after puberty. Andrology. 2021 May 6;

176. Brook PF, Radford JA, Shalet SM, Joyce AD, Gosden RG. Isolation of germ cells from human testicular tissue for low temperature storage and autotransplantation. Fertil Steril. 2001 Feb;75(2):269–74.

177. Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt AM. In vitro propagation of human prepubertal spermatogonial stem cells. JAMA. 2011 Jun 15;305(23):2416–8.

178. Wang D, Hildorf S, Ntemou E, Dong L, Pors SE, Mamsen LS, et al. Characterization and Survival of Human Infant Testicular Cells After Direct Xenotransplantation. Front Endocrinol (Lausanne). 2022;13:853482.

179. Kanatsu-Shinohara M, Ogonuki N, Matoba S, Ogura A, Shinohara T. Autologous transplantation of spermatogonial stem cells restores fertility in congenitally infertile mice. Proc Natl Acad Sci U S A. 2020 Apr 7;117(14):7837–44.

180. Morimoto H, Ogonuki N, Kanatsu-Shinohara M, Matoba S, Ogura A, Shinohara T. Spermatogonial stem cell transplantation into nonablated mouse recipient testes. Stem Cell Reports. 2021 Jul 13;16(7):1832–44.

181. Serrano JB, van Eekelen R, de Winter-Korver CM, van Daalen SKM, Tabeling NC, Catsburg LAE, et al. Impact of restoring male fertility with transplantation of in vitro propagated spermatogonial stem cells on the health of their offspring throughout life. Clin Transl Med. 2021 Oct;11(10):e531.

182. Avarbock MR, Brinster CJ, Brinster RL. Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med. 1996 Jun;2(6):693–6.

183. Whelan EC, Yang F, Avarbock MR, Sullivan MC, Beiting DP, Brinster RL. Reestablishment of spermatogenesis after more than 20 years of cryopreservation of rat spermatogonial stem cells reveals an important impact in differentiation capacity. PLoS Biol. 2022 May;20(5):e3001618.

184. Galdon G, Atala A, Sadri-Ardekani H. In Vitro Spermatogenesis: How Far from Clinical Application? Curr Urol Rep. 2016 Jul;17(7):49.

185. Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE. Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. Hum Reprod. 2011 Dec;26(12):3222–31.

186. Dovey SL, Valli H, Hermann BP, Sukhwani M, Donohue J, Castro CA, et al. Eliminating malignant contamination from therapeutic human spermatogonial stem cells. J Clin Invest. 2013 Apr;123(4):1833–43.

187. Sadri-Ardekani H, Homburg CH, van Capel TMM, van den Berg H, van der Veen F, van der Schoot CE, et al. Eliminating acute lymphoblastic leukemia cells from human testicular cell cultures: a pilot study. Fertil Steril. 2014 Apr;101(4):1072-1078.e1.

188. Abu Elhija M, Lunenfeld E, Schlatt S, Huleihel M. Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. Asian J Androl. 2012 Mar;14(2):285–93.

189. Huleihel M, Nourashrafeddin S, Plant TM. Application of three-dimensional culture systems to study mammalian spermatogenesis, with an emphasis on the rhesus monkey (Macaca mulatta). Asian J Androl. 2015 Dec;17(6):972–80.

190. Lei Q, Zhang E, van Pelt AMM, Hamer G. Meiotic Chromosome Synapsis and XY-Body Formation In Vitro. Front Endocrinol (Lausanne). 2021;12:761249.

191. Delessard M, Stalin L, Rives-Feraille A, Moutard L, Saulnier J, Dumont L, et al. Achievement of complete in vitro spermatogenesis in testicular tissues from prepubertal mice exposed to mono- or polychemotherapy. Sci Rep. 2022 May 6;12(1):7407.

192. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007 Jul 19;448(7151):313–7.

193. Kerkis A, Fonseca SAS, Serafim RC, Lavagnolli TMC, Abdelmassih S, Abdelmassih R, et al. In vitro differentiation of male mouse embryonic stem cells into both presumptive sperm cells and oocytes. Cloning Stem Cells. 2007;9(4):535–48.

194. Khampang S, Cho IK, Punyawai K, Gill B, Langmo JN, Nath S, et al. Blastocyst development after fertilization with in vitro spermatids derived from nonhuman primate embryonic stem cells. F S Sci. 2021 Nov;2(4):365–75.

195. Riboldi M, Rubio C, Pellicer A, Gil-Salom M, Simón C. In vitro production of haploid cells after coculture of CD49f+ with Sertoli cells from testicular sperm extraction in nonobstructive azoospermic patients. Fertil Steril. 2012 Sep;98(3):580-590.e4.

196. Pendergraft SS, Sadri-Ardekani H, Atala A, Bishop CE. Three-dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity in vitro. Biol Reprod. 2017 Mar 1;96(3):720–32.

197. Sun M, Yuan Q, Niu M, Wang H, Wen L, Yao C, et al. Efficient generation of functional haploid spermatids from human germline stem cells by three-dimensional-induced system. Cell Death Differ. 2018 Mar;25(4):749–66.

198. Skardal A, Aleman J, Forsythe S, Rajan S, Murphy S, Devarasetty M, et al. Drug compound screening in single and integrated multi-organoid body-on-a-chip systems. Biofabrication. 2020 Feb 26;12(2):025017.

199. Rajan SAP, Aleman J, Wan M, Pourhabibi Zarandi N, Nzou G, Murphy S, et al. Probing prodrug metabolism and reciprocal toxicity with an integrated and humanized multi-tissue organ-on-a-chip platform. Acta Biomater. 2020 Apr 1;106:124–35.

200. Baert Y, Ruetschle I, Cools W, Oehme A, Lorenz A, Marx U, et al. A multi-organ-chip co-culture of liver and testis equivalents: a first step toward a systemic male reprotoxicity model. Hum Reprod. 2020 May 1;35(5):1029–44.

201. Strange DP, Zarandi NP, Trivedi G, Atala A, Bishop CE, Sadri-Ardekani H, et al. Human testicular organoid system as a novel tool to study Zika virus pathogenesis. Emerg Microbes Infect. 2018 May 9;7(1):82.

202. Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, et al. In vitro production of functional sperm in cultured neonatal mouse testes. Nature. 2011 Mar 24;471(7339):504–7.

203. Sato T, Yokonishi T, Komeya M, Katagiri K, Kubota Y, Matoba S, et al. Testis tissue explantation cures spermatogenic failure in c-Kit ligand mutant mice. Proc Natl Acad Sci U S A. 2012 Oct 16;109(42):16934–8.

204. Hogg K, Western PS. Differentiation of Fetal Male Germline and Gonadal Progenitor Cells Is Disrupted in Organ Cultures Containing Knockout Serum Replacement. Stem Cells Dev. 2015 Dec 15;24(24):2899–911.

205. Dumont L, Arkoun B, Jumeau F, Milazzo JP, Bironneau A, Liot D, et al. Assessment of the optimal vitrification protocol for pre-pubertal mice testes leading to successful in vitro production of flagellated spermatozoa. Andrology. 2015 May;3(3):611–25.

206. Reda A, Hou M, Winton TR, Chapin RE, Söder O, Stukenborg JB. In vitro differentiation of rat spermatogonia into round spermatids in tissue culture. Mol Hum Reprod. 2016 Sep;22(9):601–12.

207. Yokonishi T, Sato T, Katagiri K, Ogawa T. In vitro spermatogenesis using an organ culture technique. Methods Mol Biol. 2013;927:479–88.

208. Sato T, Katagiri K, Kojima K, Komeya M, Yao M, Ogawa T. In Vitro Spermatogenesis in Explanted Adult Mouse Testis Tissues. PLoS One. 2015;10(6):e0130171.

209. Medrano JV, Vilanova-Pérez T, Fornés-Ferrer V, Navarro-Gomezlechon A, Martínez-Triguero ML, García S, et al. Influence of temperature, serum, and gonadotropin supplementation in short- and long-term organotypic culture of human immature testicular tissue. Fertil Steril. 2018 Nov;110(6):1045-1057.e3.

210. Wang D, Hildorf S, Ntemou E, Mamsen LS, Dong L, Pors SE, et al. Organotypic Culture of Testicular Tissue from Infant Boys with Cryptorchidism. Int J Mol Sci. 2022 Jul 19;23(14):7975.

211. Fayomi AP, Peters K, Sukhwani M, Valli-Pulaski H, Shetty G, Meistrich ML, et al. Autologous grafting of cryopreserved prepubertal rhesus testis produces sperm and offspring. Science. 2019 Mar 22;363(6433):1314–9.

212. Ntemou E, Kadam P, Van Saen D, Wistuba J, Mitchell RT, Schlatt S, et al. Complete spermatogenesis in intratesticular testis tissue xenotransplants from immature non-human primate. Hum Reprod. 2019 Mar 1;34(3):403–13.

213. Willems M, Seβenhausen P, Gies I, Vloeberghs V, Tournaye H, Van Saen D, et al. Intratesticular xenografting of Klinefelter pre-pubertal testis tissue as potential model to study testicular fibrosis. Reprod Biomed Online. 2022 May;44(5):896–906.

214. Kanatsu-Shinohara M, Ogonuki N, Matoba S, Morimoto H, Shiromoto Y, Ogura A, et al. Regeneration of spermatogenesis by mouse germ cell transplantation into allogeneic and xenogeneic testis primordia or organoids. Stem Cell Reports. 2022 Apr 12;17(4):924–35.

215. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells in vitro. Biol Reprod. 2003 Jun;68(6):2207–14.

216. Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod. 2004 Sep;71(3):722–31.

217. Kanatsu-Shinohara M, Inoue K, Ogonuki N, Morimoto H, Ogura A, Shinohara T. Serum- and feeder-free culture of mouse germline stem cells. Biol Reprod. 2011 Jan;84(1):97–105.

218. Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, et al. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod. 2005 Apr;72(4):985–91.

219. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, et al. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod. 2003 Aug;69(2):612–6.

220. Kanatsu-Shinohara M, Ogonuki N, Iwano T, Lee J, Kazuki Y, Inoue K, et al. Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development. 2005 Sep;132(18):4155–63.

221. Kanatsu-Shinohara M, Ogonuki N, Matoba S, Morimoto H, Ogura A, Shinohara T. Improved serum- and feeder-free culture of mouse germline stem cells. Biol Reprod. 2014 Oct;91(4):88.

222. He Z, Kokkinaki M, Jiang J, Dobrinski I, Dym M. Isolation, characterization, and culture of human spermatogonia. Biol Reprod. 2010 Feb;82(2):363–72.

223. Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, et al. Propagation of human spermatogonial stem cells in vitro. JAMA. 2009 Nov 18;302(19):2127–34.

224. Mizrak SC, Chikhovskaya JV, Sadri-Ardekani H, van Daalen S, Korver CM, Hovingh SE, et al. Embryonic stem cell-like cells derived from adult human testis. Hum Reprod. 2010 Jan;25(1):158–67.

225. Lim JJ, Sung SY, Kim HJ, Song SH, Hong JY, Yoon TK, et al. Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture conditions. Cell Prolif. 2010 Aug;43(4):405–17.

226. Nickkholgh B, Mizrak SC, van Daalen SKM, Korver CM, Sadri-Ardekani H, Repping S, et al. Genetic and epigenetic stability of human spermatogonial stem cells during long-term culture. Fertil Steril. 2014 Dec;102(6):1700-1707.e1.

227. Kokkinaki M, Djourabtchi A, Golestaneh N. Long-term Culture of Human SSEA-4 Positive Spermatogonial Stem Cells (SSCs). J Stem Cell Res Ther. 2011 Nov 11;2(2):2488.

228. Koruji M, Shahverdi A, Janan A, Piryaei A, Lakpour MR, Gilani Sedighi MA. Proliferation of small number of human spermatogonial stem cells obtained from azoospermic patients. J Assist Reprod Genet. 2012 Sep;29(9):957–67.

229. Conrad S, Azizi H, Hatami M, Kubista M, Bonin M, Hennenlotter J, et al. Differential gene expression profiling of enriched human spermatogonia after short- and long-term culture. Biomed Res Int. 2014;2014:138350.

230. Guo Y, Liu L, Sun M, Hai Y, Li Z, He Z. Expansion and long-term culture of human spermatogonial stem cells via the activation of SMAD3 and AKT pathways. Exp Biol Med (Maywood). 2015 Aug;240(8):1112–22.

231. Dong L, Kristensen SG, Hildorf S, Gul M, Clasen-Linde E, Fedder J, et al. Propagation of Spermatogonial Stem Cell-Like Cells From Infant Boys. Front Physiol. 2019;10:1155.

232. Deebel NA, Galdon G, Zarandi NP, Stogner-Underwood K, Howards S, Lovato J, et al. Spermatogonia stem cell technology: a new avenue for all age Klinefelter patients. Hum Reprod Update. 2021 Jul 29;dmab025.

I, Guillermo Galdón López, solemnly declare that the doctoral thesis "ISOLATION, CULTURE, AND CHARACTERIZATION OF KLINEFELTER SPERMATOGONIAL STEM CELLS" is an original work and doesn't constitute plagiarism in any form. I give my consent to verify its originality to the competent authority.

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I confirm that Mr. Guillermo Galdon has been my Ph.D. student, and he completed his Doctoral Thesis following all codes of ethics and good practice. His work is original, and I am not aware of any plagiarism.

Please feel free to contact me with any questions!

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