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Research Article

Generation of Induced Pluripotent Stem Cells from a Female Patient with a Xq27.3-q28 Deletion to Establish Disease Models and Identify Therapies

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Abstract

Since it is extremely difficult to establish an animal model for human chromosomal abnormalities, induced pluripotent stem cells (iPSCs) provide a powerful alternative to study underlying mechanisms of these disorders and identify potential therapeutic interventions. In this study we established iPSCs from a young girl with a hemizygous deletion of Xq27.3-q28 who exhibited global developmental delay and intellectual disability from early in infancy. The deletion site on the X chromosome includes Fragile X Mental Retardation 1 (FMR1), the gene responsible for fragile X syndrome, which likely contributes to the patient's neurodevelopmental abnormalities. The FMR1 gene was expressed in approximately half of the iPSC clones we generated while it was absent in the other half due to the random inactivation of normal and abnormal X chromosomes. The normal or absent expression pattern of the FMR1 gene was not altered when the iPSCs were differentiated into neural progenitor cells (NPCs). Moreover, chromosome reactivating reagents such as 5-aza-2-deoxycytidine, trichostatin A, and UNC0638, were tested in an attempt to reactivate the suppressed FMR1 gene in affected iPSC-NPCs. The affected and control isogenic iPSCs developed in this study are ideal models with which to identify downstream consequences caused by the Xq27.3-q28 deletion and also to provide tools for high-throughput screening to identify compounds potentially improving the well-being of this patient population.

Keywords: induced pluripotent stem cells, X-linked disorders, partial X chromosome deletion, intellectual disability, chromosomal reactivation

Introduction

Global developmental delay and intellectual disability (GDD/ID) are observed among 1%–3% of all children (Michelson et al., 2011; Shevell et al., 2003). The etiology for GDD/ID is highly variable in published reports due to multiple influences, including specific disorder criteria, extent of diagnostic investigations, and technological advances over time. However, X-linked intellectual disability (XLID) consistently accounts for a relatively high proportion of these with estimates ranging from 10% of all ID cases (Michelson et al., 2011) to 5%–10% of ID cases in males (Lubs et al., 2012). The most common XLID is fragile X syndrome (FXS), which is caused by abnormalities in the FMR1 (Fragile X Mental Retardation (1) gene located at Xq27.3.

Large deletions of the Xq27-q28 region have been previously reported in more than a dozen patients with various mental and physical developmental delays. Autistic traits are often observed in these patients, which may be related to loss of the FMR1 gene included within the deleted region. Male cases with large deletions of the region, including FMR1 and AFF2 (AF4/FMR2 family member (2) genes show a severe phenotype (Birot et al., 1996; Brusius-Facchin et al., 2012; Cavani et al., 2011; Fengler et al., 2002; Moore et al., 1999; Wolff et al., 1997). On the other hand, female cases tend to show milder phenotypes in general. Some individuals are asymptomatic and are later determined to be carriers following diagnosis of affected offspring.

Others show mild-to-moderate developmental delays (Marshall et al., 2013; Probst et al., 2007; Wolff et al., 1997), while a few female individuals show severe phenotypes

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similar to male patients (Clarke et al., 1992; Dahl et al., 1995). All the female patients reported to date are heterozygous for the mutation, as the deletion is present in one X chromosome while the other chromosome is unaffected. Due to X chromosome inactivation (XCI) in females, only one X chromosome is expressed while the other is inactivated within individual cells. The XCI process occurs randomly during early embryogenesis; thus, Xq27-q28 patients are expected to express the deleted X chromosome in approximately a half of their somatic cells unless cells expressing a deleted X chromosome are selected for in a negative or positive manner during development.

As it is extremely difficult to establish an animal model for human chromosomal abnormalities such as Xq27-q28 deletions, induced pluripotent stem cells (iPSCs) provide a powerful alternative to study underlying mechanisms of these disorders and identify potential therapeutic interventions. iPSCs can be easily generated from somatic cells such as skin fibroblasts or peripheral blood mononuclear cells (PBMCs), and subsequently differentiated into disease-relevant cell types such as neurons and myocytes *in vitro*. Based upon these attributes, iPSCs and their descendent differentiated cell types have been widely used for disease pathobiology studies and/or drug discoveries for many genetic disorders. High-throughput drug screening using such cells has demonstrated, for instance, that statins and rapamycin can ameliorate disease conditions of chondrodysplasia and fibrodysplasia ossificans progressiva, respectively (Hino et al., 2017; Yamashita et al., 2014).

In this report, we generated iPSCs from a young girl showing GDD/ID with a 7.3 Mb deletion at Xq27.3-q28 (chrX: 142, 271, 441–149, 561, 309). The deleted region includes well-characterized disease-associated genes, such as FMR1, AFF2, and IDS (iduronate 2-sulfatase), which are associated with FXS, fragile XE syndrome, and Hunter syndrome, respectively (Bondeson et al., 1995; Mulley et al., 1995; Verkerk et al., 1991). This is the first report of iPSC generation from a female with a Xq27-q28 deletion syndrome. iPSC clones from this patient were established in equal numbers from PBMCs expressing either the affected or unaffected X chromosome.

We further differentiated these isogenic iPSCs into neural progenitor cells (NPCs), and performed studies to determine whether chromatin-reactivating drugs can derepress the FMR1 gene from the inactivated (unaffected) X chromosome when delivered to affected cells to explore potential therapeutic interventions for Xq27-q28 deletion syndromes.

Materials and Methods

Clinical summary

The patient is a 3-year-old girl who presented at age 14 months to the University of Florida Pediatric Specialties with global developmental delays of unknown cause. At full-term gestation, she was born to a 21-year-old mother by normal spontaneous vaginal delivery with no complications and discharged within 48 hours. Her birth weight was eight pounds and three ounces (70th percentile) and length 20.5 inches (80th percentile). The parents report the earliest observations of delays in development around age 5 months, when solid foods were introduced. Severe gagging and vomiting, along with excessive drooling, tongue protrusion, and tactile defensiveness prompted early intervention at 10 months. She started occupational therapy and physical therapy for hypotonia. She began to crawl at 13 months and walk at 16 months.

Other observed behaviors included: hyperactive deep tendon reflexes, wide gait, forward lunging, arm flapping, high excitability, sleep trouble, and partial complex seizures. Chronic sinus and ear infections improved somewhat after tonsil and adenoid removal and two tympanostomies, but persistent infections have also prompted immunological blood work. These tests did not show any immunoglobulin deficiency but did reveal a poor immune response to vaccinations.

Initial biochemical laboratory studies were normal for quantitative serum lactate, pyruvate, ammonia, and creatine kinase. Spinal X-rays showed minimal dextrocurvature of the thoracolumbar spine and the remaining osseous structures were within normal limits. Her abdomen was sonographically normal with no gross organomegaly. She received a diagnosis of autism, ID, developmental delay, and speech/language delay, and ranked in the first percentile for speech and language as evaluated by the Receptive-Expressive Emergent Language Test-third edition (REEL-3). She had learned to point and use some sign language.

Comparative genomic hybridization (CGH array; hg19) studies performed by Quest Diagnostics revealed a 7.3 megabase deletion at Xq27.3q28 (142, 271, 441–149, 561, 309 bp). This deletion resulted in hemizygosity for 76 RefSeq genes, including Online Mendelian Inheritance in Man (OMIM) disease-associated genes like FMR1, AFF2, and IDS genes, which are associated with FXS, FRAXE, and Hunter syndrome, respectively. Androgen Receptor differential methylation, examined by Greenwood Genetic Center, demonstrated a random pattern of X-inactivation at 51:49 in a blood sample.

Ethics

This study was approved by the University of Florida Institutional Review Board (IRB201602056).

Establishment of patient-derived iPSCs

Approximately 5 mL of the patient's peripheral blood was collected into an EDTA tube. PBMCs were isolated using SepMate tubes and Lymphoprep (StemCell Technologies, Vancouver, Canada) following the manufacturer's instructions. Around 4×10^5 cells of PBMCs were suspended in blood growth medium (RPMI 1640, 10% fetal bovine serum, and 1% penicillin/streptomycin solution) and infected with Sendai viral vector SeVdp (KOSM) 302L encoding four reprogramming factors (Oct4, Sox2, Klf4, and c-Myc) (Nishimura et al., 2011) with a multiplicity of infection of 2 for 2 hours at 37°C.

The cells were then seeded into six-well plates coated with Corning Matrigel hESC-qualified Matrix (Corning, Corning, NY) at 2×10^5 cells per well. They were cultured in blood growth medium for the first 3 days and then in ReproTeSR medium (StemCell Technologies) until iPSC colonies were ready for isolation. Individual iPSC colonies were manually isolated $\sim$3 weeks after the viral infection and transferred onto Matrigel-coated dishes in mTeSR1 medium (StemCell Technologies) supplemented with 10 μM Y-27632 dihydrochloride (Tocris Bioscience, Bristol, UK). They were maintained in mTeSR1 medium thereafter with daily medium changes and passaged approximately every 6 days using ReLeSR (StemCell Technologies).

Trilineage (mesodermal and endodermal) differentiation culture

To validate the ability of established iPSCs to differentiate into the three germ layers, we used the STEMdiff Trilineage Differentiation Kit (StemCell Technologies). We omitted the ectodermal differentiation because iPSCs were separated differentiated into NPCs with the method described below. Mesoderm and endoderm differentiation were performed following the manufacturer's manual.

In brief, iPSCs were dissociated using StemPro Accutase (Gibco; Thermo Fisher Scientific, Waltham, MA). After centrifugation, the cells were resuspended with mTeSR1 medium supplemented with 10 μM Y-27632. They were then seeded onto Matrigel-coated 24-well plates at 1×10^5 cells per well for mesodermal differentiation and 4×10^5 cells per well for endodermal differentiation, respectively. On the following day, the medium was switched to either STEMdiff Trilineage Mesoderm Medium or STEMdiff Trilineage Endoderm Medium. Daily medium changes were performed for 5 days until differentiation was assessed.

Differentiation of iPSCs to NPCs

Selected patient-derived iPSC clones were used for differentiation at approximately passage 7. The STEMdiff Monolayer Culture Protocol was utilized from NPC differentiation and expansion following the manufacturer's instructions (Stemcell Technologies).

In brief, confluent cultured iPSCs were dissociated using StemPro Accutase. After centrifugation, they were resuspended with STEMdiff Neural Induction Medium supplemented with SMADi (StemCell Technologies) and 10 μM Y-27632. Cells were plated onto Matrigel-coated six-well plates at 2×10^6 cells per well and maintained by daily medium changes with STEMdiff Neural Induction Medium supplemented with SMADi. Cells were passaged approximately every 7 days using StemPro Accutase. Cells were expanded until passage 2 and cryopreserved using Neural Progenitor Freezing Medium (StemCell Technologies) and stored in liquid nitrogen for later use. Thawed NPCs were subsequently maintained in STEMdiff Neural Progenitor Medium (StemCell Technologies).

Gene expression analysis

RNA extraction and cDNA synthesis were performed according to the manufacturer's protocol and as we described previously (Biel et al., 2015). Real-time polymerase chain reaction (PCR) was performed using the TaqMan Fast Advanced Master Mix (Applied Biosystems; Thermo Fisher Scientific) and TaqMan Assays listed below mixed with 10 ng of cDNA following the manufacturer's protocol and the StepOnePlus Real-Time PCR system (Applied Biosystems). The following TaqMan Assays were used: FMR1 (Hs00924547_m1, FAM-MGB); MECP2 (Hs04187588_m1, FAM-MGB); SLC25A5 (ANT2) (Hs00854499_g1, FAM-MGB); and GAPDH (Hs02786624_g1, VIC-MGB). Each well was a duplex reaction with GAPDH assay mixed or FMR1, MECP2, or SLC25A5 to normalize the expression on a per well basis. Relative quantitation of target genes was compared using the $\Delta\Delta$CT calculation.

Flow cytometry cell analysis

Cultured cells were detached using StemPro Accutase. After fixation in 4% paraformaldehyde for 15 minutes, cells were washed and incubated with antibodies for 30 minutes on ice in the dark. In the case of intracellular markers, cells were treated with Intracellular Staining Permeabilization Wash Buffer (BioLegend, San Diego, CA) before staining. The cells were then analyzed with a BD Accuri C6 Plus (BD Biosciences, San Jose, CA). The following conjugated antibodies were used: PE anti-Oct4 (BioLegend); APC anti-SSEA4 (BioLegend); AF647 anti-Pax6 (BD Biosciences); PE anti-Sox1 (BD Biosciences); APC anti-Brachyury (R&D Systems, Minneapolis, MN); PE anti-FoxA2 (BD Biosciences); and AF647 anti-Sox17 (BD Biosciences).

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde for 20 minutes and washed, then blocked in 4% bovine serum albumin with PBS for 30 minutes. For Oct4 and Sox2 staining, permeabilization was performed with 0.1% Triton X-100 with PBS for 10 minutes before blocking. Cells were incubated in primary antibodies at 4°C overnight. Following 3× wash in PBS, secondary antibodies were added to the cells and incubated for 1 hour in the dark. The 4′,6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. Fluorescence images were visualized using IX70 inverted microscope and CellSens software (Olympus Tokyo Japan).

The following primary antibodies were used: anti-Oct4 (1:50; MilliporeSigma, Burlington, MA); anti-Sox2 (1:50; Santa Cruz Biotechnology, Dallas, TX); anti-SSEA4 (1:50; MilliporeSigma), and anti-Nestin (1:50; StemCell Technologies). As secondary antibodies, rabbit anti-mouse IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 488 (1: 200, Invitrogen; Thermo Fisher Scientific) or donkey anti-goat IgG (H+L) cross-adsorbed secondary antibody Alexa Fluor 568 (1:200, Invitrogen; Thermo Fisher Scientific) were used.

CDr3 stain

Cultured iPSC-derived NPCs were incubated in 2 μM of NeuroFlour CDr3 (StemCell Technologies) diluted with growth medium for 1 hour at 37°C. After replacement with fresh medium, cells were maintained in incubator for $\sim$20 hours. The NucBlue Live ReadyProbes Reagent (Invitrogen; Thermo Fisher Scientific) was added (two drops per mL) as a nuclear counterstain. After 15 minutes, cells were imaged by fluorescence microscopy.

NPC drug treatment

Two NPC clones expressing the deleted X chromosome as well as two control clones were seeded onto Matrigel-coated 24-well plates at a density of 5×10^6 cells per well and cultured in STEMdiff Neural Progenitor Medium (StemCell Technologies). Following a 24-hour incubation, 5-Aza-2′-deoxycytidine (5-Aza-dC; Sigma #A3656), trichostatin A (TSA; Sigma), and/or UNC0638 (Abcam, Cambridge, UK) were added into the culture medium at the indicated concentrations. After 72 hours, RNA was isolated from drug-treated NPCs using the RNAqueous Total RNA Isolation Kit (Invitrogen; Thermo Fisher Scientific) following the manufacturer's protocol. Gene expression of

FMR1 as well as the control ANT2 was analyzed by quantitative PCR as described above.

Results

Selection of iPSC clones expressing normal or deleted X chromosome

PBMCs from the patient were reprogrammed using previously described Sendai virus methodology (Biel et al., 2015; Santostefano et al., 2015). Individual iPSC colonies were isolated ~3 weeks after virus infection and cultured for additional 2–3 passages. Eleven clones were then analyzed by quantitative RT-PCR (Reverse Transcription-Polymerase Chain Reaction) to determine which expressed the X chromosome containing the deleted region or unaffected X chromosome. Each of the clones were evaluated in their ability to express the following genes: FMR1, MECP2, and SLC25A5 (ANT2), which are located on Xq27.3, Xq28, and Xq24, respectively (Fig. 1A). As a control, RNA isolated from a healthy female donor-derived iPSC line was also used. Using this method, clones were screened based upon their ability to express the FMR1 gene located in the deleted region.

Five clones showed negligible expression of FMR1 (clones #1, 4, 6, 7, and 9), while the remaining six clones showed FMR1 expression levels that were equal to that of the healthy control (clone #2, 3, 5, 10, 11, and 13) (Fig. 1B). In contrast, all the iPSC clones showed MECP2 and ANT2 gene expression levels that were equal to that of healthy controls (Fig. 1B). This was as expected as these genes lie outside the deleted region (Xq27.3-q28) and therefore were not predicted to be impacted by the presence of the deletion. Four iPSC clones, including two disease phenotypes (clone #4 and #6) and two healthy phenotypes (clone #5 and #11) were then used for subsequent analyses.

Characterization of iPSCs

The four selected iPSC clones were next examined for their pluripotency marker expression and trilineage differentiation ability as we described elsewhere (Santostefano et al., 2015; Taylor et al., 2018). All four iPSC clones expressed the pluripotency markers, including Oct4 and SSEA4, by immunocytochemistry (Fig. 2A). Expression of Oct4 and SSEA4 in over 90% of the cells was also confirmed by flow cytometry analysis (data not shown).

The four clones also demonstrated ability to differentiate to both mesodermal and endodermal lineages in culture assays. In flow cytometry analyses, the mesodermal marker, Brachyury, and the endodermal markers, FoxA2 and Sox17, revealed positive expression in the majority of cells, respectively (over 90%, data not shown). As all four iPSC clones were differentiated into NPCs as described below, these selected clones were all functionally validated in their abilities to differentiate to all three germ layers.

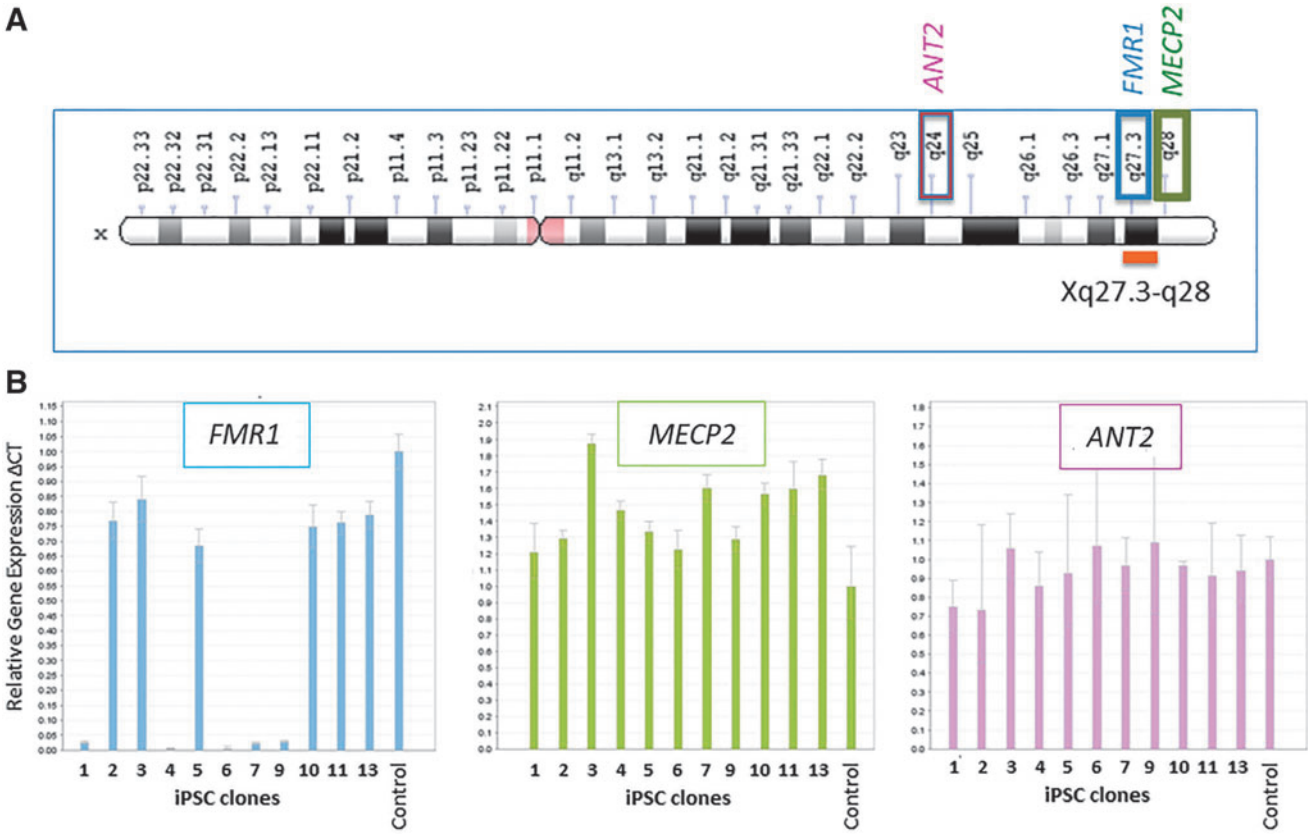

FIG. 1. Gene expression of FMR1, MECP2, and ANT2 genes in iPSCs derived from Xq27.3-q28 patient. **(A)** The deletion site of X chromosome (Xq27.3-q28) and the location of ANT2 (Xq24), FMR1 (Xq27.3), and MECP2 (Xq28) genes are illustrated. **(B)** Eleven iPSC clones were established from peripheral blood of a female patient with Xq27.3-q28 deletion. RNA expression of FMR1, MECP2, and ANT2 was examined by qRT-PCR. Relative expression to GAPDH is shown as ΔΔCT. FMR1, Fragile X Mental Retardation 1; iPSC, induced pluripotent stem cell; qRT-PCR, quantitative RT-PCR.

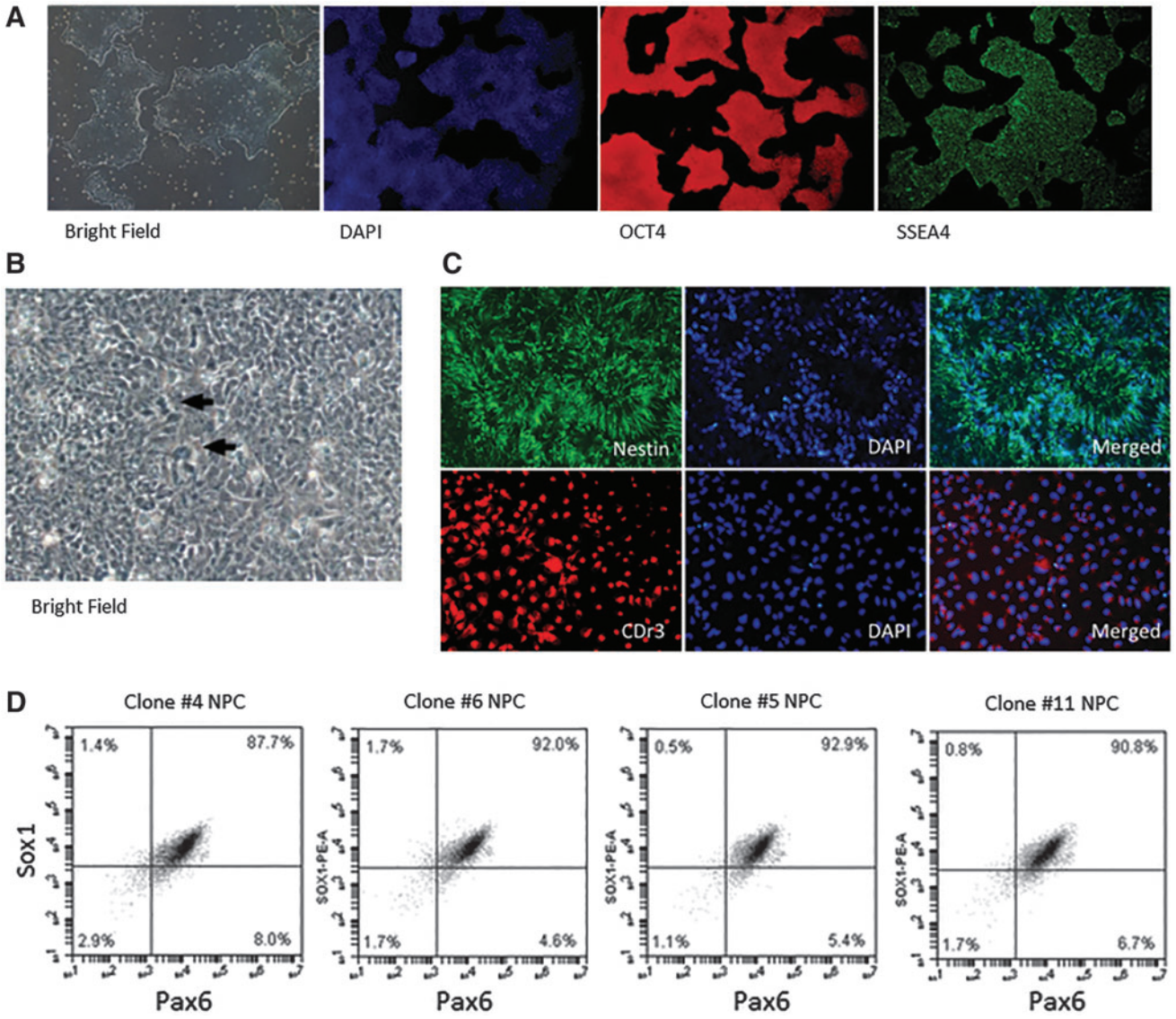

FIG. 2. Characterization of iPSCs and NPCs derived from Xq27.3-q28 patient. **(A)** Morphology (bright field), DAPI, OCT4, and SSEA4 staining of a representative iPSC clone in the study. **(B)** Rosette formation of NPCs (indicated by *arrows*) of representative NPCs in the study. **(C)** NPCs differentiated from iPSC clones (#4, 5, 6, 11) showed a similar Sox1 and Pax6 expression profile. **(D)** Nestin, CDr3, and DAPI staining of representative NPCs in the study showing rosette formation. DAPI, 4′,6-diamidino-2-phenylindole; NPC, neural progenitor cell.

iPSC differentiation into NPCs

The four selected iPSC clones (#4, 5, 6, 11) were induced to become NPCs at approximately passage 7. Throughout this induction, the morphology of iPSCs gradually changed as they became larger cells arranged in a cobblestone pattern accompanied with scattered cells projecting dendrite-like fibrillary processes (Fig. 2B). The majority of cells (over 90%) expressed neural markers for both PAX6 and Sox1 by flow cytometry analysis in all the four clones tested, regardless of X chromosome status (Fig. 2C). They were also positive for Nestin by immunocytochemistry and the fluorescent CDr3 stain, which detects living neural stem cells (Yun et al., 2012), while simultaneously showing rosette-like formation (Fig. 2D).

Drug treatment tests to derepress FMR1 in affected NPCs

NPCs derived from iPSC clones expressing the deleted X chromosome (#4, 6) as well as those expressing the normal X chromosome (#5, 11) were exposed to chemical agents known to have the capacity to potentially reactivate repressed genes. These include a chemical analog of cytidine, 5-Aza-dC, which induces DNA hypomethylation; TSA, an inhibitor of class I and II histone deacetylase; and UNC0638, an inhibitor of histone methyltransferase G9a (Schmidt et al., 1990; Vedadi et al., 2011; Yoshida et al., 1990). NPCs were incubated with 5-Aza-dC, TSA, UNC0638, and combinations of two compounds (5-Aza-dC+TSA, 5Aza-dC+UNC0638) at various dosages and

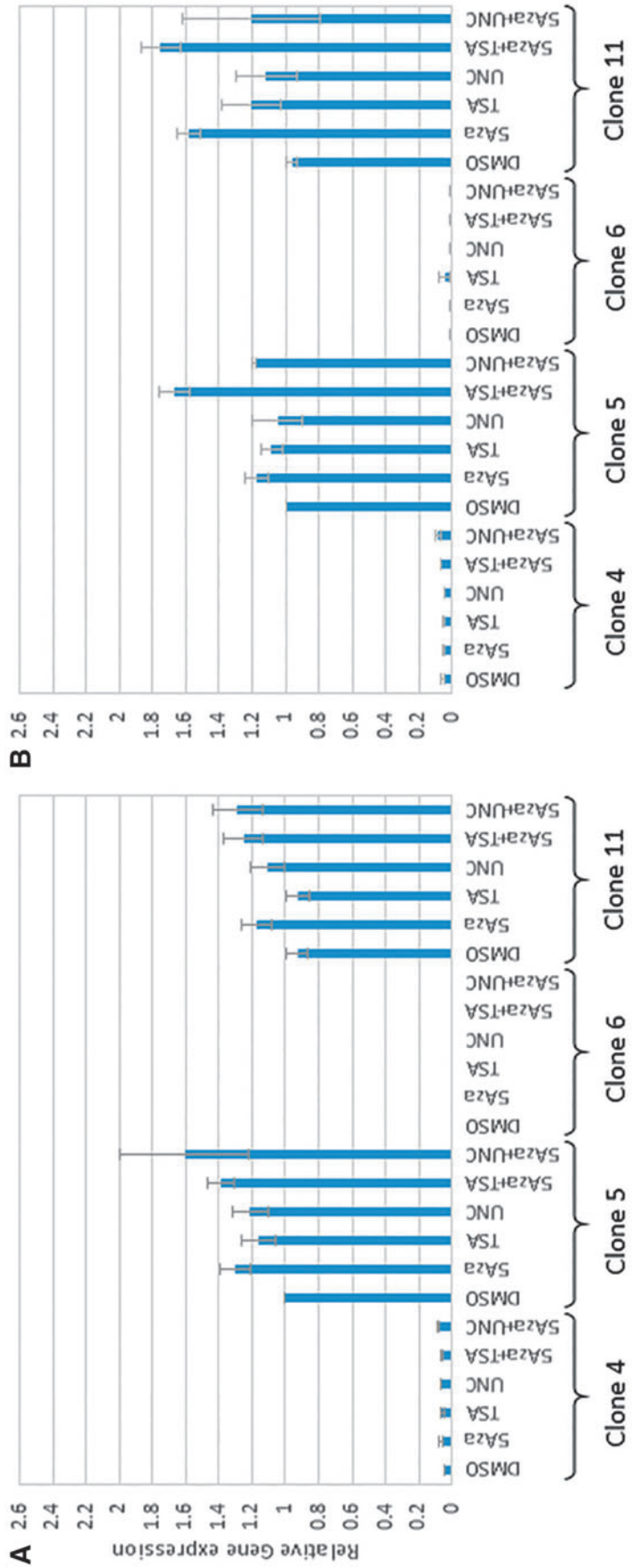

FIG. 3. Effects of chromatin reactivation agents on NPCs from Xq27.3-q28 patient. **(A)** NPCs differentiated from iPSC clones (#4, 5, 6, 11) were treated with either vehicle (DMSO), 5-aza-2′-deoxycytidine (5Aza), trichostatin A (TSA), UNC0638 (UNC), or in combination for 72 hours with (A) a low dose (5 μM 5Aza, 50 nM TSA, 8 μM UNC) or **(B)** a high dose (7.5 μM 5Aza, 75 nM TSA, 12 μM UNC). FMR1 gene expression was examined by qRT-PCR. Relative expression to GAPDH is shown as $\Delta\Delta$CT. Clones #4 and #6 express the deleted X chromosome, whereas clones #5 and #11 express the normal X chromosome.

durations according to previously published literature (Brower et al., 2009; Kim et al., 2017; Ng et al., 2016; Rodić et al., 2005).

No morphological alterations were observed under microscopic visualization during drug exposures, suggesting the drugs were not toxic to the cells. To test for how successful these were at derepression of the region of interest in the inactivated X-chromosome in affected lines, FMR1 gene expression assays were performed. Despite multiple trials testing a wide variety of conditions, these drugs failed to increase FMR1 RNA expression in NPCs expressing the deleted X chromosome (Fig. 3A, B).

Discussion

To the best of our knowledge, this is the first report of iPSC generation from a female with a Xq27-q28 deletion syndrome. Large chromosomal deletions can be generated with CRISPR technologies in mouse (Boroviak et al., 2016; Korablev et al., 2017); however, since chromosomal synteny is not conserved well between humans and rodents across large chromosomal regions, it is extremely difficult to generate rodent models that accurately recapitulate human chromosomal abnormalities of this nature. However, as iPSCs can differentiate into a wide variety of cell types and three-dimensional tissue organoids, the present system provides a powerful alternative for elucidating disease mechanisms and exploring potential therapeutic interventions to improve the well-being of this patient population.

As shown in Table 1, more than a dozen female patients have been reported to date with Xq27-q28 deletion. One genetically interesting observation from the information in this table is the differences in XCI patterns between patients which vary between random and skewed inactivation profiles. Relationships between these XCI patterns and the varied impact they ultimately have upon patient phenotypes represent one aspect of these disorders that may be more fully explored through the continued generation of isogenic iPSC models such as those described in this study. While the patient in our study exhibited the expected random XCI pattern in both blood samples and subsequently in reprogrammed iPSCs, it is unknown whether this is the same pattern that is present in the patient's brain or neural cells which are among the cell types most impacted by the deletion (Bittel et al., 2008).

It should also be noted that the XCI status in human female iPSCs has been controversial (Barakat et al., 2015; Briggs et al., 2015; Geens and Chuva De Sousa Lopes, 2017). Although studies have demonstrated that the majority of human female iPSCs retain the XCI pattern of the original somatic cells after reprogramming, it has also been shown that some iPSCs display a reactivation of the inactive X chromosome. The reason for this discrepancy has yet to be fully elucidated. Potential factors that may play a role in this include variations in culture conditions, reprogramming methods, techniques implemented to evaluate XCI status, and instability of the inactivated X in human iPSCs (Cantone and Fisher, 2017).

An additional caveat is that XCI erosion may occur during the culture and maintenance of iPSCs (Mekhoubad et al., 2012). In iPSCs generated from a female Lesch–Nyhan carrier initially showed XCI, which then eroded and became reactivated following repeated passages in culture. Therefore, whenever using iPSCs for modeling X-linked disorders, it is highly critical to reevaluate the XCI status following reprogramming, repeated passages, and/or post-differentiation.

The retention of XCI following iPSC reprogramming has been demonstrated in the vast majority of reports describing cells representing various X-linked disorders, including the present case (Table 2). This is a substantial advantage for the use of iPSCs when modeling X-linked disorders. When using patient iPSCs for disease mechanism studies and/or drug discoveries, it is critical to have control iPSCs, ideally

Table 1. Previous Reports of Females with Large X Chromosome Deletion Involving Xq27-q28

Author	*Deleted site*	*Phenotype*	*XCI pattern (inactivated allele)*
Schmidt et al. (1990)	Xq27.1-q27.3	Moderate MR	Skewed (normal)
Clarke et al. (1992)	Xq27-q28	Hunter disease with severe developmental delay	Skewed (normal)
Gedeon et al. (1992)	Xq27.3	Mild ID	Random
Dahl et al. (1995)	Xq27.3-q28	Myotubular myopathy with MR	Skewed ($\sim$80%) (normal)
Birot et al. (1996)	Xq27.2-q28 (two cases)	Mild MR and severe MR, respectively	Skewed (90%) (deleted) and random, respectively
Wolff et al. (1997)	Xq26.3-q27.3 (three cases)	Normal, ID, and moderate-to-severe MR, respectively	Skewed (>95%) (deleted), skewed (80%–85%) (deleted), and random, respectively
Probst et al. (2007)	Xq27.3-q28	GDD/ID	Random
Yachelevich et al. (2011)	Xq27.3-q28 (one case)	POI (one case)	Skewed (deleted) (all cases)
	Xq27.3 to terminus (three cases)	Normal (three cases)	
Marshall et al. (2013)	Xq27.3-q28	GDD/ID	Skewed (deleted)
Our case	Xq27.3-q28	GDD/ID	Random

GDD, global developmental delay; ID, intellectual disability; MR, mental retardation; POI, premature ovarian insufficiency; XCI, X chromosome inactivation.

Table 2. Previous Studies Reporting Post-X Chromosome Inactivation Status of Induced Pluripotent Stem Cells Generated from X-Linked Disease Female Patients or Carriers

Author	*Disease*	*Somatic cell origin for iPSC generation*
Tchieu et al. (2010)	Duchenne muscular dystrophy	Fibroblast
Cheung et al. (2011)	Rett syndrome	Fibroblast
Amenduni et al. (2011)	Rett syndrome	Fibroblast
Kim et al. (2011)[a]	Rett syndrome	Fibroblast
Pomp et al. (2011)	Rett syndrome	Fibroblast
Ananiev et al. (2011)	Rett syndrome	Fibroblast
Mekhoubad et al. (2012)[b]	Lesch-Nyhan syndrome	Fibroblast
Reboun et al. (2016)[c]	Hunter syndrome	PBMCs
Ng et al. (2016)	Danon disease	Fibroblast
Yoshida et al. (2018)	Danon disease	Peripheral blood T cells
Present case	Xq27.3-q28 deletion	PBMCs

[a]They observed both pre-XCI and post-XCI status, and isolated both types of iPSC clones, respectively.
[b]They observed XCI status was retained in early passage, which was subjected to ''erosion'' in extended passage.
[c]They added cultural condition which led to naive state besides normal cultural condition, which ended up to partial XCI reset in Fabry disease iPSC clones but not in Hunter syndrome iPSC clones.
iPSC, induced pluripotent stem cell; PBMC, peripheral blood mononuclear cell.

having the same genetic background. In general, such control iPSCs must be created through genome editing-based methodologies to either correct a particular mutation in patient-derived iPSCs or recapitulate a mutation in healthy iPSCs using CRISPR/Cas9.

The ability to generate both affected and unaffected isogenic control iPSCs through the same one reprogramming scheme by using X-linked carrier cells is a powerful alternative. This is not only due to the less-laborious nature of direct reprogramming but also because genome editing approaches are not currently capable of repairing/mimicking enormous chromosomal deletions, unless we use a chromosomal transplantation approach (Paulis et al., 2020). In some cases, it can be difficult to obtain the disease-relevant iPSCs when the XCI status of the founder cells are highly skewed. In one report, selection procedures were implemented to divide host fibroblasts expressing either the intact or the mutant allele before reprogramming. This then enabled generation of isogenic pairs of iPSCs derived from a Danon disease patient (Ng et al., 2016).

Following confirmation of the post-XCI in our study, we tested several known chromosomal reactivation agents in an attempt to reactivate the genes found to be deficient in the affected NPCs. As gene expression is null from the deleted loci (as demonstrated with FMR1 expression the cells expressing the abnormal X), even a partial derepression of the missing genes may have a significant impact upon patient health. In the X-linked Danon syndrome study described above, administration of a DNA demethylating agent (5-aza-dC) into iPSCs as well as iPSC-derived cardiomyocytes did indeed achieve partial reactivation of the silent LAMP2 gene and ameliorated the autophagy failure phenotype observed in untreated cells (Ng et al., 2016). In addition to 5-aza-dC, we also tested two other agents. One HDAC inhibitor (TSA) and a HMT inhibitor (UNC0638). Each of these drugs have previously demonstrated the potential to reactivate epigenetically suppressed genes.

Unfortunately, in the present study, none of the agents, alone or in combination, was able to derepress the FMR1 gene in iPSCs expressing the affected X-chromosome. Although we were not able to identify any effective chemical agents within the present study, the models we have developed provide tools for high-throughput screening to identify such compounds in the future. In addition, the affected and control iPSCs developed in this study are ideal models with which to identify downstream consequences caused by the Xq27.3-q28 deletion in a variety of disease-relevant cells or organoid systems.

Author Disclosure Statement

The authors declare they have no conflicting financial interests.

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References

Amenduni, M., De Filippis, R., Cheung, A.Y., Disciglio, V., Epistolato, M.C., Ariani, F., Mari, F., Mencarelli, M.A., Hayek, Y., Renieri, A., Ellis, J., and Meloni, I. (2011). iPS cells to model CDKL5-related disorders. Eur. J. Hum. Genet. 19, 1246–1255.

Ananiev, G., Williams, E.C., Li, H., and Chang, Q. (2011). Isogenic pairs of wild type and mutant induced pluripotent stem cell (iPSC) lines from Rett syndrome patients as in vitro disease model. PLoS One 6, e25255.

Barakat, T.S., Ghazvini, M., de Hoon, B., Li, T., Eussen, B., Douben, H., van der Linden, R., van der Stap, N., Boter, M., Laven, J.S., Galjaard, R.-J., Grootegoed, J.A., de Klein, A., and Gribnau, J. 2015. Stable X chromosome reactivation in female human induced pluripotent stem cells. Stem Cell, Rep. 4, 199–208.

Biel, N.M., Santostefano, K.E., DiVita, B.B., El Rouby, N., Carrasquilla, S.D., Simmons, C., Nakanishi, M., Cooper-DeHoff, R.M., Johnson, J.A., and Terada, N. (2015). Vascular smooth muscle cells from hypertensive patient-derived induced pluripotent stem cells to advance hypertension pharmacogenomics. Stem Cells Transl. Med. 4, 1380–1390.

Birot, A.M., Delobel, B., Gronnier, P., Bonnet, V., Maire, I., and Bozon, D. 1996. A 5-megabase familial deletion removes the IDS and FMR-1 genes in a male Hunter patient. Hum. Mutat. 7, 266–268.

Bittel, D.C., Theodoro, M.F., Kibiryeva, N., Fischer, W., Talebizadeh, Z., and Butler, M.G. 2008. Comparison of X-chromosome inactivation patterns in multiple tissues from human females, J. Med. Genet. 45, 309–313.

Bondeson, M.L., Dahl, N., Malmgren, H., Kleijer, W.J., Tonnesen, T., Carlberg, B.M., and Pettersson, U. (1995). Inversion of the IDS gene resulting from recombination with IDS-related sequences is a common cause of the Hunter syndrome. Hum. Mol. Genet. 4, 615–621.

Boroviak, K., Doe, B., Banerjee, R., Yang, F., and Bradley, A. (2016). Chromosome engineering in zygotes with CRISPR/-Cas9. Genesis 54, 78–85.

Briggs, S.F., Dominguez, A.A., Chavez, S.L., Reijo and Pera, R.A. (2015). Single-cell XIST expression in human preimplantation embryos and newly reprogrammed female induced pluripotent stem cells. Stem Cells 33, 1771–1781.

Brower, J. V, Lim, C.H., Han, C., Hankowski, K.E., Hamazaki, T., and Terada, N. (2009). Differential CpG island methylation of murine adenine nucleotide translocase genes. Biochim. Biophys. Acta. 1789, 198–203.

Brusius-Facchin, A., CF, D. sausa, Schwartz, I., Riegel, M., Melaragno, M., Correia, P., Moraes, L., Lierena, J.J., Giugliani, R., and Leistner-Segal, S. (2012). Severe phenotype in MPS II patients associated with a large deletion including contiguous genes. Am. J. Med. Genet. A. 158A, 1055–1059.

Cantone, I., and Fisher, A.G. (2017). Human X chromosome inactivation and reactivation: Implications for cell reprogramming and disease. Philos. Trans. R. Soc. L. B. Biol. Sci. 372, 20160358.

Cavani, S., Prontera, P., Grasso, M., Ardisia, C., Malacarme, M., Gradassi, C., Cecconi, M., Mencarelli, A., Donti, E., and Pierluigi, M. (2011). FMR1, FMR2, and SLITRK2 deletion inside a paracentric inversion involving bands Xq27.3-q28 in a male and his mother. Am. J. Med. Genet. A. 155, 221–4.

Cheung, A.Y., Horvath, L.M., Grafodatskaya, D., Pasceri, P., Weksberg, R., Hotta, A., Carrel, L., and Ellis, J. (2011). Isolation of MECP2-null Rett Syndrome patient hiPS cells and isogenic controls through X-chromosome inactivation. Hum. Mol. Genet. 20, 2103–2115.

Clarke, J.T., Wilson, P.J., Morris, C.P., Hopwood, J.J., Richards, R.I., Sutherland, G.R., and Ray, P.N. (1992). Characterization of a deletion at Xq27-q28 associated with unbalanced inactivation of the nonmutant X chromosome. Am. J. Hum. Genet. 51, 316–322.

Dahl, N., Hu, L.J., Chery, M., Fardeau, M., Gilgenkrantz, S., Nivelon-Chevallier, A., Sidaner-Noisette, I., Mugneret, F., Gouyon, J.B., and Gal, A., Kioschis, P., d'Urso, M., and Mandel, J.-L. (1995). Myotubular myopathy in a girl with a deletion at Xq27-q28 and unbalanced X inactivation assigns the MTM1 gene to a 600-kb region. Am. J. Hum. Genet. 56, 1108–1115.

Fengler, S., Fhchs, S., Konig, R., and Arnemann, J. (2002). Mosaicism for FMR1 and FMR2 deletion: A new case. J. Med. Genet. 39, 200–201.

Gedeon, A.K., Baker, E., Robinson, H., Partington, M.W., Gross, B., Manca, A., Korn, B., Poustka, A., Yu, S., and Sutherland, G.R., and Mulley, J.C. (1992). Fragile X syndrome without CCG amplification has an FMR1 deletion. Nat. Genet. 1, 341–344.

Geens, M., and Chuva De Sousa Lopes, S.M. (2017). X chromosome inactivation in human pluripotent stem cells as a model for human development: Back to the drawing board? Hum. Reprod. Update 23, 520–532.

Hino, K., Horigome, K., Nishio, M., Komura, S., Nagata, S., Zhao, C., Jin, Y., Kawakami, K., Yamada, Y., Ohta, A., Toguchida, J., and Ikeya, M. (2017). Activin-A enhances mTOR signaling to promote aberrant chondrogenesis in fibrodysplasia ossificans progressiva. J. Clin. Invest. 127, 3339–3352.

Kim, K.Y., Hysolli, E., and Park, I.H. (2011). Neuronal maturation defect in induced pluripotent stem cells from patients with Rett syndrome. Proc. Natl. Acad. Sci. U. S. A. 108, 14169–14174.

Kim, Y., Lee, H.-M., Xiong, Y., Sciaky, N., Hulbert, S.W., Cao, X., Everitt, J.I., Jin, J., Roth, B.L., and Jiang, Y. (2017). Targeting the histone methyltransferase G9a activates imprinted genes and improves survival of a mouse model of Prader–Willi syndrome. Nat. Med. 23, 213–222.

Korablev, A.N., Serova, I.A., and Serov, O.L. (2017). Generation of megabase-scale deletions, inversions and duplications involving the Contactin-6 gene in mice by CRISPR/Cas9 technology. BMC Genet. 18, 112.

Lubs, H.A., Stevenson, R.E., and Schwartz, C.E. (2012). Fragile X and X-linked intellectual disability: Four decades of discovery. Am. J. Hum. Genet. 90, 579–590.

Marshall, L.S., Simon, J., Wood, T., Peng, M., Owen, R., Feldman, G.S., and Zaragoza, M.V. (2013). Deletion Xq27.3q28 in female patient with global developmental delays and skewed X-inactivation. BMC Med. Genet. 14, 49.

Mekhoubad, S., Bock, C., de Boer, A.S., Kiskinis, E., Meissner, A., and Eggan, K. (2012). Erosion of dosage compensation impacts human iPSC disease modeling. Cell. Stem. Cell. 10, 595–609.

Michelson, D.J., Shevell, M.I., Sherr, E.H., Moeschler, J.B., Gropman, A.L., and Ashwal, S. (2011). Evidence report: Genetic and metabolic testing on children with global developmental delay: Report of the Quality Standards Subcommittee of the American Academy of Neurology and the Practice Committee of the Child Neurology Society. Neurology 77, 1629–1635.

Moore, S.J., Strain, L., Cole, G.F., Miedzybrodzka, Z., Kelly, K.F., and Dean, J.C.S. (1999). Fragile X syndrome with FMR1 and FMR2 deletion. J. Med. Genet. 36, 565–566.

Mulley, J.C., Yu, S., Loesch, D.Z., Hay, D.A., Donnelly, A., Gedeon, A.K., Carbonell, P., Lopez, I., Glover, G., and Gabarron, I., Yu, P.W.L., Baker, E., Haan, E.A., Hockey, A., Knight, S.J.L., Davies, K.E., Richards, R.I., and Sutherland, G.R.. (1995). FRAXE and mental retardation. J. Med. Genet. 32, 162–169.

Ng, K.M., Mok, P.Y., Butler, A.W., Ho, J.C., Choi, S.W., Lee, Y.K., Lai, W.H., Au, K.W., Lau, Y.M., Wong, L.Y., Esteban, M.A., Siu, C.W., Sham, P.C., Colman, A., and Tse, H.F. (2016). Amelioration of X-linked related autophagy failure in danon disease with DNA methylation inhibitor. Circulation 134, 1373–1389.

Nishimura, K., Sano, M., Ohtaka, M., Furuta, B., Umemura, Y., Nakajima, Y., Ikehara, Y., Kobayashi, T., Segawa, H., Takayasu, S., Sato, H., Motomura, K., Uchida, E., Kanayasu-Toyoda, T., Asashima, M., Nakauchi, H., Yamaguchi, T., and Nakanishia, M. (2011). Development of defective and persistent Sendai virus vector: A unique gene delivery/expression system ideal for cell reprogramming. J. Biol. Chem. 286, 4760–4771.

Paulis, M., Susani, L., Castelli, A., Suzuki, T., Hara, T., Straniero, L., Duga, S., Strina, D., Mantero, S., Caldana, E., Sergi,

L.S., Villa, A., and Vezzoni, P. (2020). Chromosome transplantation: A possible approach to treat human X-linked disorders. Mol. Ther. Methods Clin. Dev. 17, 369–377.

Pomp, O., Dreesen, O., Leong, D.F., Meller-Pomp, O., Tan, T.T., Zhou, F., and Colman, A. (2011). Unexpected X chromosome skewing during culture and reprogramming of human somatic cells can be alleviated by exogenous telomerase. Cell. Stem. Cell. 9, 156–165.

Probst, F.J., Roeder, E.R., Enciso, V.B., Ou, Z., Cooper, M.L., Eng, P., Li, J., Gu, Y., Stratton, R.F., Chinault, A.C., Shaw, C.A., Sutton, V.R., Cheung, S.W., and Nelson, D.L. (2007). Chromosomal microarray analysis (CMA) detects a large X chromosome deletion including FMR1, FMR2, and IDS in a female patient with mental retardation. Am. J. Med. Genet. A. 143a, 1358–1365.

Reboun, M., Rybova, J., Dobrovolny, R., Vcelak, J., Veselkova, T., Storkanova, G., Musalkova, D., Hrebicek, M., Ledvinova, J., Magner, M., Zeman, J., Peskova, K., and Dvorakova, L. (2016). X-chromosome inactivation analysis in different cell types and induced pluripotent stem cells elucidates the disease mechanism in a rare case of mucopolysaccharidosis type II in a female. Folia. Biol. 62, 82–89.

Rodić, N., Oka, M., Hamazaki, T., Murawski, M.R., Jorgensen, M., Maatouk, D.M., Resnick, J.L., Li, E., and Terada, N. (2005). DNA methylation is required for silencing of Ant4, an adenine nucleotide translocase selectively expressed in mouse embryonic stem cells and germ cells. Stem Cells 23, 1314–1323.

Santostefano, K.E., Hamazaki, T., Biel, N.M., Jin, S., Umezawa, A., and Terada, N. (2015). A practical guide to induced pluripotent stem cell research using patient samples. Lab. Invest. 95, 4–13.

Schmidt, M., Certoma, A., Du Sart, D., Kalitsis, P., Leversha, M., Fowler, K., Sheffield, L., Jack, I., and Danks, D.M. (1990). Unusual X chromosome inactivation in a mentally retarded girl with an interstitial deletion Xq27: Implications for the fragile X syndrome. Hum. Genet. 84, 347–352.

Shevell, M., Ashwal, S., Donley, D., Flint, J., Gingold, M., Hirtz, D., Majnemer, A., Noetzel, M., and Sheth, R.D. (2003). Practice parameter: Evaluation of the child with global developmental delay: Report of the Quality Standards Subcommittee of the American Academy of Neurology and The Practice Committee of the Child Neurology Society. Neurology 60, 367–380.

Taylor, J.P., Cash, M.N., Santostefano, K.E., Nakanishi, M., Terada, N., and Wallet, M.A. (2018). CRISPR/Cas9 knockout of USP18 enhances type I IFN responsiveness and restricts HIV-1 infection in macrophages. J. Leukoc. Biol. 103, 1225–1240.

Tchieu, J., Kuoy, E., Chin, M.H., Trinh, H., Patterson, M., Sherman, S.P., Aimiuwu, O., Lindgren, A., Hakimian, S., Zack, J.A., Clark, A.T., Pyle, A.D., Lowry, W.E., and Plath, K. (2010). Female human iPSCs retain an inactive X chromosome. Cell Stem Cell 7, 329–342.

Vedadi, M., Barsyte-Lovejoy, D., Liu, F., Rival-Gervier, S., Allali-Hassani, A., Labrie, V., Wigle, T.J., Dimaggio, P.A., Wasney, G.A., Siarheyeva, A., Dong, A., Tempel, W., Wang, S.-C., Chen, X., Chau, I., Mangano, T.J., Huang, X.-P., Simpson, C.D., Pattenden, S.G., Norris, J.L., Kireev, D.B., Tripathy, A., Edwards, A., Roth, B.L., Janzen, W.P., Garcia, B.A., Petronis, A., Ellis, J., Brown, P.J., Frye, S. V, Arrowsmith, C.H., and Jin, J. (2011). A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. Nat. Chem. Biol. 7, 566–574.

Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., and Zhang, F.P. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65, 905–914.

Wolff, D.J., Gustashaw, K.M., Zurcher, V., Ko, L., White, W., Weiss, L., Van Dyke, D.L., Schwartz, S., and Willard, H.F. (1997). Deletions in Xq26.3-q27.3 including FMR1 result in a severe phenotype in a male and variable phenotypes in females depending upon the X inactivation pattern. Hum. Genet. 100, 256–261.

Yachelevich, N., Gittler, J.K., Klugman, S., Feldman, B., Martin, J., Brooks, S.S., Dobkin, C., and Nolin, S.L. (2011). Terminal deletions of the long arm of chromosome X that include the FMR1 gene in female patients: A case series. Am J Med Genet A 155a, 870–874.

Yamashita, A., Morioka, M., Kishi, H., Kimura, T., Yahara, Y., Okada, M., Fujita, K., Sawai, H., Ikegawa, S., and Tsumaki, N. (2014). Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. Nature 513, 507–511.

Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J. Biol. Chem. 265, 17174–17179.

Yoshida, S., Nakanishi, C., Okada, H., Mori, M., Yokawa, J., Yoshimuta, T., Ohta, K., Konno, T., Fujino, N., Kawashiri, M.A., Yachie, A., Yamagishi, M., and Hayashi, K. (2018). Characteristics of induced pluripotent stem cells from clinically divergent female monozygotic twins with Danon disease. J. Mol. Cell. Cardiol. 114, 234–242.

Yun, S.W., Leong, C., Zhai, D., Tan, Y.L., Lim, L., Bi, X., Lee, J.J., Kim, H.J., Kang, N.Y., Ng, S.H., Stanton, L.W., and Chang, Y.T. (2012). Neural stem cell specific fluorescent chemical probe binding to FABP7. Proc. Natl. Acad. Sci. U. S. A. 109, 10214–10217.

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