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Preventing mucopolysaccharidosis type II (Hunter syndrome): PGD and establishing a Hunter (46, XX) stem cell line

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Objectives Preimplantation genetic diagnosis (PGD) enables the identification of affected embryos prior to implantation. We present for the first time three families in which either the oocytes or embryos obtained from female carriers of mutations in the iduronate-2-sulfatase (*IDS*) gene underwent PGD for mucopolysaccharidosis type II (Hunter syndrome). Furthermore, we report the first ever derivation of a Hunter's syndrome (46, XX) human stem cell line from embryos (HESC) carrying the IDS and oculocutaneus albinism type 2 mutations.

Methods Combined polar body (PB) 1 and 2 or a single cell of a six- to eight-cell embryo (blastomere) was used for genetic analysis by multiplex polymerase chain reaction assay using six microsatellite polymorphic markers flanking the gene and mutation.

Results One couple underwent four PB-PGD cycles, with birth of a healthy girl; the second couple with one PB-PGD cycle had healthy twins; the third couple underwent seven cycles of double PGD for Hunter and Albinism syndrome with birth of healthy twins. One novel Hunter 46, XX HESC line was established displaying typical characteristics of HESC cells.

Conclusions PGD is a reliable method to prevent pregnancy of children affected with Hunter syndrome. In addition, derived HESC can be further utilized for drug testing and better understanding of the pathogenesis of this syndrome. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS: mucopolysaccharidosis type II; Hunter syndrome; preimplantation genetic diagnosis; single cell; polar body; multiplex PCR; stem cell

INTRODUCTION

Mucopolysaccharidosis type II (MPS II) or Hunter syndrome is a lysosomal storage disease caused by a deficiency of the enzyme, iduronate-2-sulfatase (IDS) (Wraith *et al*., 2008b). It is an X-linked disorder manifesting in males and rarely in females (Sohn *et al*., 2010). There are two different types of Hunter/MPS II, classified by age of onset and the severity of the symptoms: the early-onset type is characterized by more severe symptoms and the late-onset form is characterized by less severe symptoms. Although the phenotype, age of onset, disease manifestations, and rate of progression may vary, the severe form is characterized by central nervous system involvement including cognitive deterioration, progressive airway disease, and cardiac disease which usually result in death in the first or second decade of life (Yong and Harper, 1983). Furthermore, genotype–phenotype correlations for point mutations, most of which are private, are not reliable for diagnosis of MPS II (Vafiadaki *et al*., 1998; Moreira da Silva *et al*., 2001). Symptoms of both forms include coarse facial features, a large head, stiff joints, hearing loss, increased hair production, enlarged liver and spleen, and carpel tunnel syndrome (Young *et al*., 1982; Martin *et al*., 2008; Wraith *et al*., 2008a). Hunter/MPS II is estimated to affect one in 1 00 000 to 1 50 000 births worldwide (Young and Harper, 1982).

The early-onset form of Hunter/MPS II manifests shortly after 2 years of age. Symptoms include those that are common to both forms as well as mental deterioration leading to mental retardation, aggression, and hyperactivity (Young and Harper, 1983) Although the phenotype is variable and the rate of symptom progression varies widely, Hunter syndrome is usually progressive and life-limiting (Hunter, 1917).

Enzyme replacement therapy (ERT) is available today for the treatment of Hunter syndrome but the improvement in symptoms is only modest and, as in other ERTs for other lysosomal disorders, does not impact the neurological features in a clinically meaningful manner (Davison *et al*., 2010).

Preimplantation genetic diagnosis (PGD) was developed two decades ago for couples at genetic risk for having pregnancies with potentially affected embryos. PGD is performed by blastomere (one cell of a six- to eight-cell embryo) or PB biopsy (extruded by the

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Table 1—Demographic data of the three families

Family	Disease	Gene	Mutation	Age proband (years)
Family 1[a]	Hunter	*IDS*	L410P	34
Family 2[a]	Hunter	*IDS*	L410P	25
Family 3	Hunter and albinism	*IDS* and tyrosinase	Del exons 4–7 G47D and R402Q	24

IDS, iduronate-2-sulfatase.
[a] Mothers in Families 1 and 2 are sisters.

oocyte), or more recently by blastocyst biopsy (Zhang *et al*., 2009), for Mendelian and chromosomal disorders (Handyside *et al*., 1990). Combined analysis of PB1 from the first meiosis and PB2 from the second meiosis can be used for the assessment of maternal autosomal dominant and X-linked disorders (Rechitsky *et al*., 2004). In these cases, only the by-product of the oocytes is analyzed without the need for embryo biopsy, thereby preserving the integrity of the embryo.

Although in theory, PGD could be accomplished using mutation analysis alone, because of the phenomenon of allele dropout (ADO), this would be accompanied by an unacceptably high error rate, potentially as high as 20% (Thornhill *et al*., 2001). Therefore, PGD protocols include several linked polymorphic microsatellite markers flanking the disease gene to minimize misdiagnosis due to ADO (Verlinsky *et al*., 2004; Sanchez-Garcia *et al*., 2005).

We present three families (of two sisters and one unrelated family) in which the females were carriers of mutations in the *IDS* gene and underwent PGD. One healthy girl and one set of healthy twins were born, respectively, to the two sisters. In the third couple healthy twins were born after undergoing combined PGD for Hunter and oculocutaneus albinism (OCA) type IA (of which both parents were carriers). The mutant embryos donated by the third family were used to develop Hunter embryonic stem cells.

MATERIALS AND METHODS

Patients

In the first two couples, the females were sisters aged 34 and 25 years and were carriers of a missense L410P mutation in the *IDS*. They had a brother who had died in childhood of Hunter syndrome. Both sisters had delivered healthy children after chorionic villous sampling testing to rule out an affected fetus. In the third couple, the female was a carrier of a deletion of exons 4–7; she had two brothers who had died of Hunter syndrome. This couple had one pregnancy termination because of the identification of an affected male fetus. This couple was also found to be carriers of the G47D and R402Q in the *OCA2* gene (based on a relative who had suffered from OCA and subsequent genetic testing of both members of the couple). For this couple, a double PGD for Hunter syndrome and OCA was performed (Table 1).

Ovarian stimulation and eggs retrieval

In vitro fertilization (IVF) treatment was performed using the long downregulation protocol as previously described (Altarescu *et al*., 2007)

PB biopsy, intracytoplasmic sperm injection, and embryo cultures

PB biopsy and intracytoplasmic sperm injection (ICSI) were performed as previously described (Altarescu *et al*., 2008).

Molecular analysis

Genetic testing for the missense L410P mutation and the deletion of exons 4–7 in the *IDS* gene, in conjunction with microsatellite markers analysis, was performed with DNA extracted from peripheral blood cells using high salt precipitation (Miller *et al*., 1988).

Biopsied PB1 and PB2 were transferred (individually) to a tube containing 5 μL of proteinase K lysis buffer (Thornhill *et al*., 2001) and incubated at 45 °C for 15 min, followed by inactivation at 94 °C for 15 min. Ten microsatellite informative polymorphic markers were identified flanking the *IDS* gene [DXS6687, DXS8091, DXS2496, DXS1193, DXS457, DXS1123, Hunter-AC1 (chromosome X: 148 404 995), DXS8377, DXS8069, DXS7423 (UCSC genomic browser March 2006)] (Karolchik *et al*., 2003), which were used for linkage (Figure 1) and haplotype building. A multiplex polymerase chain reaction (PCR) was prepared as previously described (Altarescu *et al*., 2009): 1.5 μL from each reaction was used as a template with a hemi-nested primer 5′ fluorescently labeled with 6-FAM, HEX, or TAMRA (Metabion, Germany) with one outside primer for an additional 35 cycles for each of the six individual PCRs. Reaction products were diluted and run on an ABI Prism 3100xl Avant automated sequencer and analyzed using Genemaper software, ABI.

HESC derivation and maintenance

Derivation and maintenance of undifferentiated Shaare Zedek (SZ) Hunter cells were carried out according to routinely applied protocols (Eiges *et al*., 2007; Epsztejn-Litman *et al*., 2010).

The embryos were given by the couple following a written informed consent, under the approvals of

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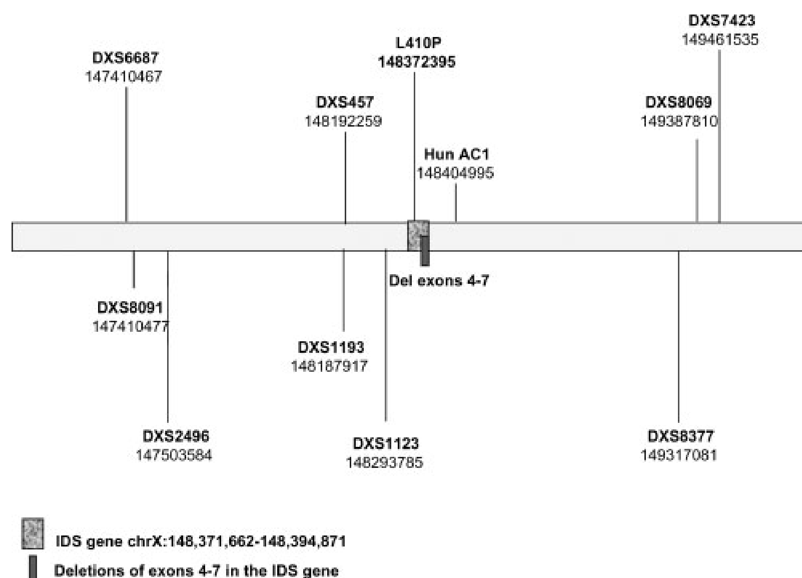

Figure 1—Schematic representation of microsatellite polymorphic markers flanking the iduronate-2-sulfatase (*IDS*) gene together with the two familial mutations (L410P and del exons 4–7) in the *IDS* gene. Numbers represent the locations of the markers on chromosome X according to the genome browser USCS March 2006 assembly

the National Ethics Committee for Genetic and Reproductive Research and our institution's IRB Committee (87/07). The donated embryos remained in culture until they developed into fully grown hatched blastocysts (6 days post-fertilization). They were then manually manipulated to remove the extraembryonic material (trophoblast cells) with an Ultra-sharp Splitting Blade (Bioniche). Intact inner cell mass cell clumps were then placed on a feeder cell layer of inactivated mouse embryonic fibroblasts (MEFs) and cultured in HESC media. Following several days in culture (5–10 days) outgrowths of proliferating cells appeared in one of the cell clumps. The newly proliferating cells were manually propagated using the cut-and-paste method (five to seven passages) until a stable cell line was established. The undifferentiated cells were derived and propagated on a supporting feeder layer of mitomycin-C-inactivated MEFs, in 85% knockout DMEM medium (Gibco-BRL), supplemented with 15% knockout serum replacement (Gibco-BRL), 1 mM glutamine, 0.1 mM B-mercaptoethanol, 1% non-essential amino acids stock, penicillin (50 units/mL), streptomycin (50 ug/mL), and 8 ng/mL basic fibroblast (Peprotech) in the presence of 0.4% insulin–transferin–selenium supplement (Gibco-BRL). Cell cultures were split by incubation with 2 mg/mL collagenase type IV (Gibco-BRL) for approximately 1 h at 37 °C.

Spontaneous differentiation in vitro

Undifferentiated cells were propagated by collagenase type IV treatment and induced to form embryoid bodies (EBs) by allowing them to aggregate in suspension culture through growth in non-adherent plastic petri bacterial dishes (Itskovitz-Eldor *et al.*, 2000; Schuldiner *et al.*, 2001) in the absence of basic FGF. The EBs were collected for karyotype, morphology, proliferation, and expression analysis following 30 days of cell aggregation in culture to confirm the status of the HES derivation.

DNA extraction and genotyping

Total DNA was extracted using FlexiGene DNA Kit (Qiagen). Haplotype analysis for genotype confirmation was performed as described above.

RNA extractions and marker expression

Total RNA was extracted using TRI-reagent (Sigma) and 1 µg of RNA was reverse transcribed by random hexamer priming using MultiScribe™ reverse transcriptase (Applied Biosystem) (Table 2). Amplification was performed on cDNA using Supertherm Taq DNA polymerase (Roche), in the presence of 1× Taq Buffer, 200 µM dNTPs each, and 0.4 µM of each primer. Final products were assessed by gel electrophoresis on 2% agarose ethidium-bromide stained gels.

Chromosome analysis and biochemical analysis

Chromosome biochemical analyses were performed as previously described (Stock AD *et al.*, 1972) and Giemsa banding of meiotic chromosomes with the description of a procedure for cytological preparations from solid tissues (Neufeld *et al.*, 1976).

RESULTS

PGD analysis and results

In each couple, informative markers were identified using genomic DNA prior to the PGD cycle. For the two

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Table 2—Primers for marker expression of stem cell line

Target	Primer forward	Primer reverse
Oct3/4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
Nanog	CAGCCCCGATTCTTCCACCAGTCCC	CGGAAGATTCCCAGTCGGGTTCACC
REX1	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA

related families six microsatellite markers (DXS2496, DXS1193, DXS457, Hunter AC1, DXS8377, and DXS8069) in conjunction with the familial mutation L410P were used for analysis. For the third family, another combination of six microsatellite markers (DXS6687, DXS8091, DXS1123, Hunter AC1, DXS8069, and DXS7432) was used in conjunction with microsatellite markers surrounding the *OCA2* gene for diagnosis of OCA (Figure 2). The results of the PGD cycles for Hunter syndrome in the three families are presented in Table 3.

The first couple (the 34-year-old sister) underwent four PGD cycles, all performed by PB analysis (Table 3). In one cycle, of four retrieved and three fertilized oocytes, no embryos were transferable, but in the remaining three cycles 9–13 oocytes were retrieved and two to four wild-type embryos were available for transfer. Two embryos were transferred in each of these three cycles, the last transfer resulting in the birth of a healthy girl at the 40th week of gestation, birth weight 3665 g. Postnatal analysis of the L410P mutation, confirmed the wild-type allele. The mean number of oocytes retrieved per cycle was ten oocytes, fertilization rate was 79%, and diagnostic rate was 92%. The non-diagnosed embryos were frozen for further blastomere biopsy.

In the second couple (the 25-year-old younger sister of the above woman), one cycle resulted in retrieval of 16 oocytes that extruded PB1. Of these, 11 fertilized and extruded PB2. Both PB1s and PB2 were analyzed resulting in four wild-type embryos, five mutant embryos, and two in which PB2 did not amplify (giving no result). Two embryos were transferred resulting in the birth of healthy twins (boy and girl) at 35th week of gestation, birth weights 2300 and 2280 g. Postnatal analysis of the L410P mutation showed that both children received the wild-type maternal allele.

In the third couple, the 24-year-old female was a carrier of a deletion of exons 4–7 in the *IDS* gene. She had two brothers who had died of Hunter syndrome and a pregnancy termination because of an affected male fetus. Both members of the couple were carriers of different mutations in the *OCA2* gene requiring simultaneous PGD for both Hunter syndrome and OCA (recessive disorder). Since in cases of autosomal recessive disorders the information of both maternal and paternal contribution is required for complete diagnosis, blastomere biopsy of a six- to eight-cell stage embryo was used for PGD analysis. This couple underwent seven PGD cycles, resulting in birth of healthy twins. One of the seven cycles combined fresh and frozen blastomere analysis. Embryos that were found to bear the mutant allele of the *IDS* gene or mutant for either or both alleles of the *OCA2* gene were not transferred. The mean number of oocytes retrieved per cycle was 14, fertilization rate was 83%, and diagnostic rate was 94%. Two of the seven cycles resulted in no transfer because of there were no wild-type embryos for Hunter syndrome and/or no wild-type or carrier embryos for OCA. In the remaining five cycles, one to three embryos were available for transfer.

Hunter HESC line derivation and characterization

Three embryos, which were shown to carry the deleted allele at the *IDS* gene according to PGD analysis, were donated for HESC line derivation. Of those, one HESC line was successfully established. This new cell line, termed SZ-Hunter, displays all the characteristics typical of HESCs. SZ-Hunter cells express a panel of undifferentiated markers, including NANOG, OCT4, SOX2, and REX (Figure 3A). Chromosomal analysis by Giemsa staining carried out on metaphase spreads at passage 8 showed a normal 46, XX human karyotype (Figure 3B). The mutant undifferentiated cells, termed SZ-Hunter cells, were propagated for a long period in culture (currently at passage 30) and have a cell morphology that is typical of HESC (Figure 3C). Moreover, SZ-Hunter cells were induced to spontaneously differentiate in suspension culture, generating mature EBs that are composed of various cell types (Figure 3D). The mutant genotype of this cell line was verified by PCR. Biochemical analysis for the enzymatic activity of IDS protein in these cells revealed the levels that are comparable with wild-type XX HESC counterparts, in accordance with most carrier females being asymptomatic.

DISCUSSION

We present the development of a reliable method for preventing birth of affected fetuses with Hunter syndrome and its application in three families. Two families have been reported in which one brother has the severe phenotype whereas the other brother shows an attenuated phenotype (Yatziv *et al*., 1977). Moreover, there is a serious ethical dilemma in differentiating between the 'expected' course in males versus that expected in females. In boys with gene deletions or complex gene rearrangements, severe central nervous system presentation of the disease is inevitable. However, although most carrier females are asymptomatic, albeit at risk of passing the mutation to the next generation, several cases with a severe phenotype have been described in females as well (Manara *et al*., 2010). Thus, although termination

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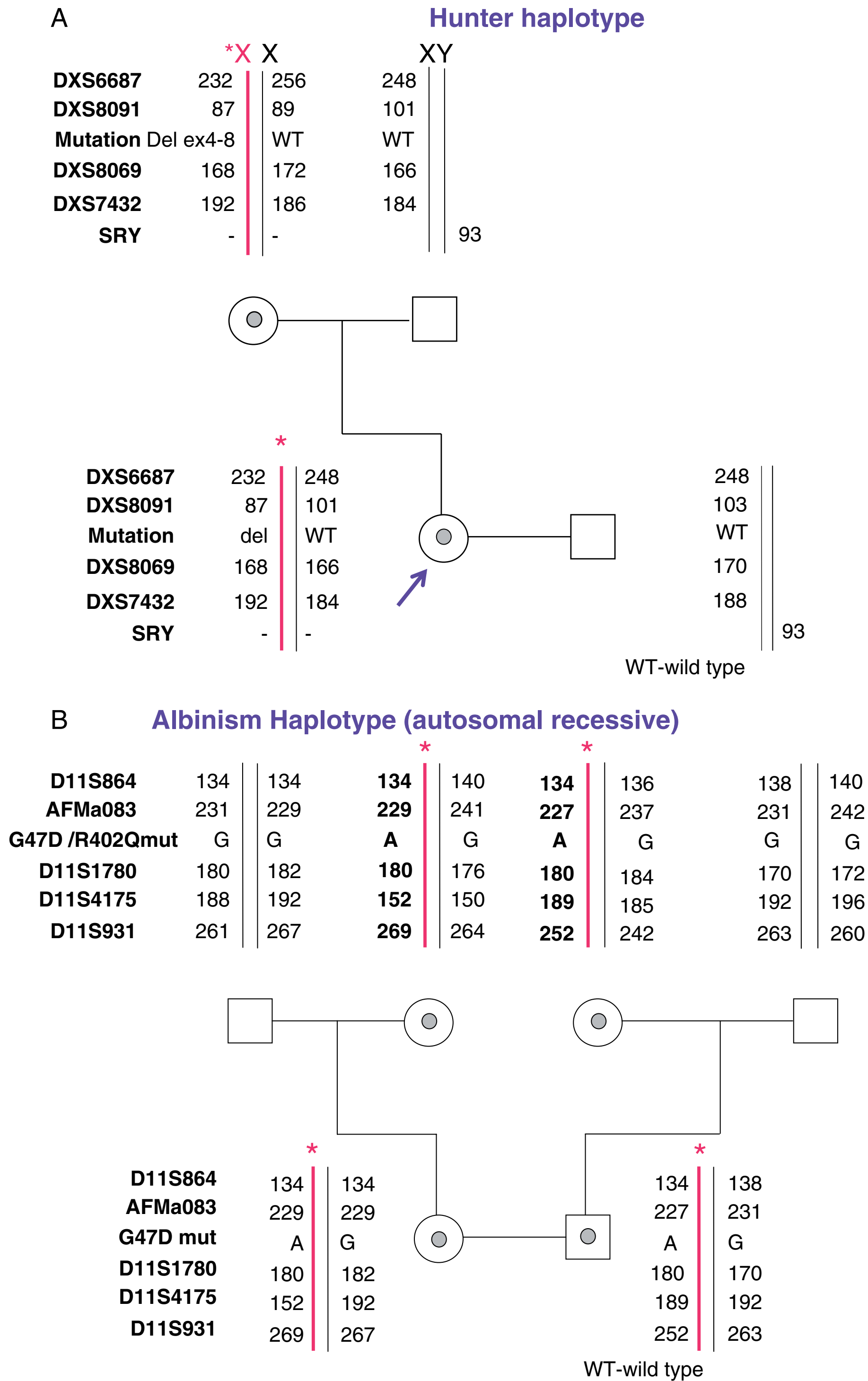

Figure 2—Family 3 haplotype map based on five polymorphic microsatellite markers for Hunter syndrome (SRY marker for sex identification) and five polymorphic microsatellite markers for oculocutaneus albinism syndrome in conjunction with the genes mutations. Asterisks (*) indicate the affected allele. Allele length in base pairs is indicated next to every marker for each family member

of pregnancy of an affected male embryo may be justified, abortion of a carrier female is a more problematic ethical decision.

PGD is a technique that precludes the need for pregnancy termination in cases of an affected fetus by virtue of analysis of the six- to eight-cell stage embryos (obtained by IVF) and transfer of only unaffected embryos. For maternal autosomal dominant and X-linked disorders, diagnosis at an even earlier stage can be performed by analyzing the PBs that are extruded by oocytes. Genetic diagnosis using PBs for PGD is an indirect analysis of the oocyte genotype based on both

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Table 3—Results of the preimplantation genetic diagnosis cycles performed in the three families

Family	Cycle number	Oocyte/MII	Fertilized	Oocyte/polar body results	Embryos biopsied	Transferable[a] embryos	Embryos transferred	Pregnancy or babies
1	1	9	6	Four mutants, two wild type	—	—	2	0
	2	6	4	Three mutants, one inconclusive	—	—	0	0
	3	12	5	Two mutant, three wild type	—	—	2	0
	4	13	12	Eight mutant, four wild type	—	—	2	1 (girl)
2	1	16	13	Seven mutant, four wild type	—	—	2	Twins
3	1	34	20	—	8[b]	0[c]	0	0
	2	21	13	—	12	2[c]	2	Chemical pregnancy
	3	34	27	—	17[d]	3[c]	3	0
		Frozen/thawed cycle		—	14	0[c]	0	—
	4	23	13	—	12	2[c]	2	0
	5	16	13	—	13	1[c]	2	Abortion
	6	12	9	—	9	3[c]	3	Twins

[a] As per text.
[b] Fourteen embryos cryopreserved without biopsy.
[c] Wild type for Hunter syndrome and wild type or carriers for oculocutaneus albinism.
[d] Seven embryos cryopreserved without biopsy.

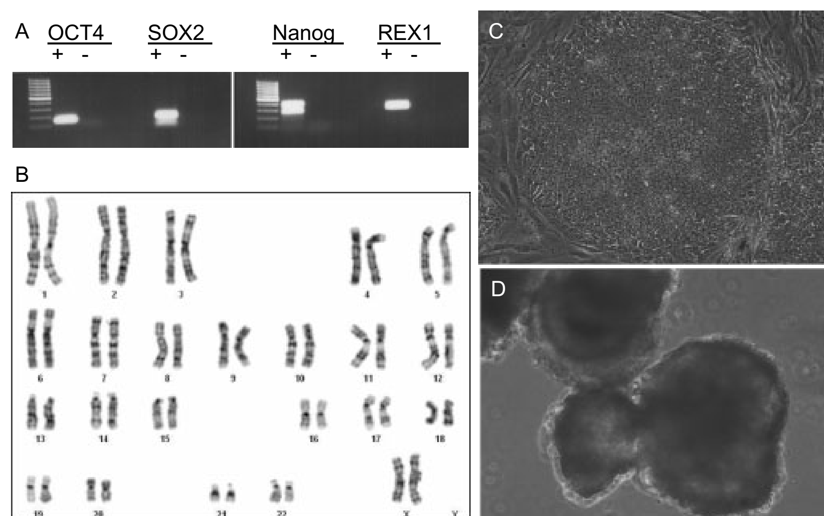

Figure 3—Characterization of Shaare Zedek (SZ)-Hunter HESCs for the expression of undifferentiated cell-specific markers, karyotype and pluripotent potential. (A) RT-PCR products for the undifferentiated gene-specific markers OCT4, SOX2, NANOG, and REX-1, using cDNA-specific primers, in undifferentiated SZ-Hunter HESCs at passage P17. (B) Karyotype analysis for SZ-Hunter cells by Giemsa staining. (C) A typical morphology of an undifferentiated SZ-Hunter HESC colony. (D) A cystic embryoid body established from SZ-Hunter HESCs, grown for 20 days in suspension culture

PB1 and PB2, requiring two biopsies and two analyses for each oocyte, including those which will not develop to the six- to eight-cell stage embryo. The use of PB1 and PB2 analysis in PGD was pioneered by Verlinsky (Verlinsky *et al*., 1997).

This method has several advantages: (1) PB biopsy allows embryo analysis without the removal of any embryonal cells; (2) if the PB analysis is inconclusive, a second analysis using a single blastomere can be performed at the six- to eight-cell stage; (3) ADO rates have been shown to be significantly less in PB than in blastomeres (Verlinsky *et al*., 2004); and (4) informative markers are easier to identify as only the maternal alleles are present. Therefore, we elected to perform PGD using PBs in most cycles of the two sisters. In the third couple, because PGD for an X-linked syndrome (MPS II) was combined with analysis for a recessive disorder (OCA), blastomere analysis was performed as mutant oocytes require analysis of the paternal allele to differentiate between carrier and affected embryos. It is to be noted that there is a significant diminution in the number of oocytes retrieved relative to the number of embryos that

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can be transferred to the woman. This decrease includes the commonly accepted rate of 80% fertilization of oocytes so that only about 75% of zygotes reach the six- to eight-cell stage on the third day; the rate of 5–10% of unclear results of the embryo biopsied; and the fact that sometimes embryos stop developing and therefore are not transferable even if they are genetically normal. In cases in which two diseases are analyzed (one X-linked and one recessive) the chances that the embryo will be wild type for the X-linked disease and simultaneously wild type or carrier for the recessive disorder are three of eight. This complexity is exemplified by the third couple: although in each of seven cycles 10–17 oocytes were retrieved, two cycles ended in no transfer because of the absence of wild-type IDS embryos who were also wild-type/carrier embryos of the tyrosinase gene.

Although more recent protocols for IVF do not encourage multiple pregnancies by transferring more than one embryo, at the time of these pregnancies, the protocol in our laboratory was to transfer two embryos. The overall pregnancy rate during that year was 35% per embryo transfer and the percentage of twins was 10%. Although recent data show that transfer of one selected embryo of high quality is satisfactory to achieve good pregnancy rates, when the available embryos are of lesser quality, the decision (together with the couple) is to transfer two embryos (Guerif *et al*., 2011) rather than await another cycle in the hopes of a single high-quality embryo. Generally, the incidence of twin pregnancies because of transfer of two embryos is higher than in single embryo transfer.

Finally, we show that diseased embryos obtained through PGD provide a novel cell source for the derivation of mutant HESC lines. Such cell lines can be exceptionally useful for basic research studies as well as for drug screening and development. Much effort is invested in the development of new drugs by trying to understand the pathology of the disease and thereby impact it with disease-specific treatment. This disease-oriented approach to drug screening requires model systems that are based on human cells rather than animals, which are frequently inadequate. Obviously, the best model system to study Hunter syndrome would be specific human cell types that manifest the clinical phenotype. However, the currently available cellular models for the disease in human are limited in their potential because of the lack of relevant and validated cell types. Mutant Hunter HESCs, however, may be uniquely useful for drug screening and development, as they can potentially differentiate into all cell types and consequently can be used for large-scale drug screening. Therefore, the derivation of this SZ-Hunter HESC line provides proof of principle how mutant PGD-derived embryos can be utilized for the derivation of novel cells that may, in the future, lead to the identification of new drug targets and to the improvement of currently available treatments.

In conclusion, we developed an efficient and safe protocol for preventing birth of affected children with Hunter syndrome by preimplantation genetic analysis, comparable to its use in other lysosomal diseases. This method is efficient, highly accurate, and can be extended to other mucopolysacharidoses. In addition, we here report for the first time the derivation of a Hunter 46, XX HESC line.

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