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Female Sex Bias in Human Embryonic Stem Cell Lines

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The factors limiting the rather inefficient derivation of human embryonic stem cells (HESCs) are not fully understood. The aim of this study was to analyze the sex ratio in our 42 preimplantation genetic diagnosis (PGD)-HESC lines, in an attempt to verify its affect on the establishment of HESC lines. The ratio between male and female PGD-derived cell lines was compared. We found a significant increase in female cell lines (76%). This finding was further confirmed by a meta-analysis for combining the results of all PGD-derived HESC lines published to date (148) and all normal karyotyped HESC lines derived from spare in vitro fertilization embryos worldwide (397). Further, gender determination of embryos demonstrated that this difference originates from the actual derivation process rather than from unequal representation of male and female embryos. It can therefore be concluded that the clear-cut tendency for female preponderance is attributed to suboptimal culture conditions rather than from a true gender imbalance in embryos used for derivation of HESC lines. We propose a mechanism in which aberrant X chromosome inactivation and/or overexpression of critical metabolic X-linked genes might explain this sex dimorphism.

Introduction

Disease-associated human pluripotent stem cell lines provide a powerful tool for studying a wide range of pathological conditions in humans, specifically those for which no good animal or cellular model systems are available [1]. In particular, these human embryonic stem cell (HESC) lines can be utilized to gain new insights on aberrant events that take place during early human embryo development, which are inaccessible for research. HESCs can also be used to explore new therapeutic protocols, including gene therapy–based treatments and disease-oriented drug screening and discovery. For these reasons, much effort is invested in the establishment of new pluripotent HESC lines that carry mutations for a myriad of genetic conditions and serve as cell-based systems for basic and applied research.

One approach to obtain diseased pluripotent SC lines is to derive them directly from preimplantation diseased embryos [2,3] that are frequently obtained from preimplantation genetic diagnosis (PGD) programs. PGD is performed on carrier couples who are at high risk of transmitting a genetic defect to their offspring and wish the birth of an unaffected baby. As PGD requires in vitro fertilization (IVF), the embryos are available for biopsy and genetic analysis at a very early stage, prior to implantation [4–7]. Using various single-cell molecular diagnostic techniques, the embryos are tested for the genetic defect for which PGD is performed, and only disease-free embryos are transferred into the uterus for implantation. Affected embryos are usually discarded. They can, however, serve as a valuable source for the derivation of HESC lines carrying the naturally inherited mutations associated with particular disorders. The great advantage of this approach for establishing mutant HESC lines is that it is based on natural transmission of inherited disorders that have been characterized with an identified phenotype [1,8,9]. To date, derivation of HESC lines from genetically abnormal embryos has been reported by a number of groups, including ours, for the generation of cellular models for a variety of heritable conditions [2,3,10–12].

An alternative approach for establishing mutant pluripotent SC lines is to generate patient-specific induced pluripotent stem (iPS) cells [13–17]. Although iPS cells are easier to obtain and may even complement HESCs under specific conditions, they still need to be compared with HESCs, which are still considered the gold standard by which all other pluripotent SCs are judged [18–20]. There are, however, factors that affect the establishment and maintenance of HESC lines that are not fully understood. Embryo gender was suggested as being one

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of them. The aims of this study were to analyze the sex ratio in our disease-bearing HESC lines and to attempt to identify the origins of sex discrepancy.

Materials and Methods

Donors

Couples undergoing PGD were asked to donate their affected embryos for SC derivation rather than have them discarded. The use of preimplantation genetically diagnosed affected embryos for HESC derivation was performed in compliance with protocols approved by the National Ethics Committee (7/04-043; 87/07) and the acceptance of a written informed consent. The couples' participation in the study was voluntary and there was no monetary compensation for their embryo donation.

Preimplantation genetic diagnosis

PGD was performed as previously described [4,7]. Single-cell biopsies were molecularly analyzed by PCR or FISH analysis for the particular mutation carried by the parent(s). Based on the results of the genetic analysis, embryos unsuitable for reproductive needs were donated for HESC derivation and further cultured to the blastocyst stage.

HESC derivation protocol

Derivation was carried out using established protocols [21]. In short, the inner cell masses (ICMs) were isolated either by immunosurgery or mechanically, by laser-assisted micromanipulation (Fig. 1), or by manual cutting with an ultrasharp splitting blade (Bioniche). The intact ICM clumps were placed on a feeder cell layer of mitomycin C–inactivated treated mouse embryonic fibroblasts and cultured in HESC media (knockout DMEM supplemented with 20% KO-serum replacement, 1% nonessential amino acids, 1 mM L-glutamine, 0.5% insulin–transferrin–selenium, 50 U/mL penicillin, 50 mg/mL streptomycin, 0.1 mM beta-mercaptoethanol, and 30 ng/mL bFGF). Outgrowths of proliferating HESCs were manually propagated using the cut-and-paste method. Following 5–7 passages, the newly established cell lines were

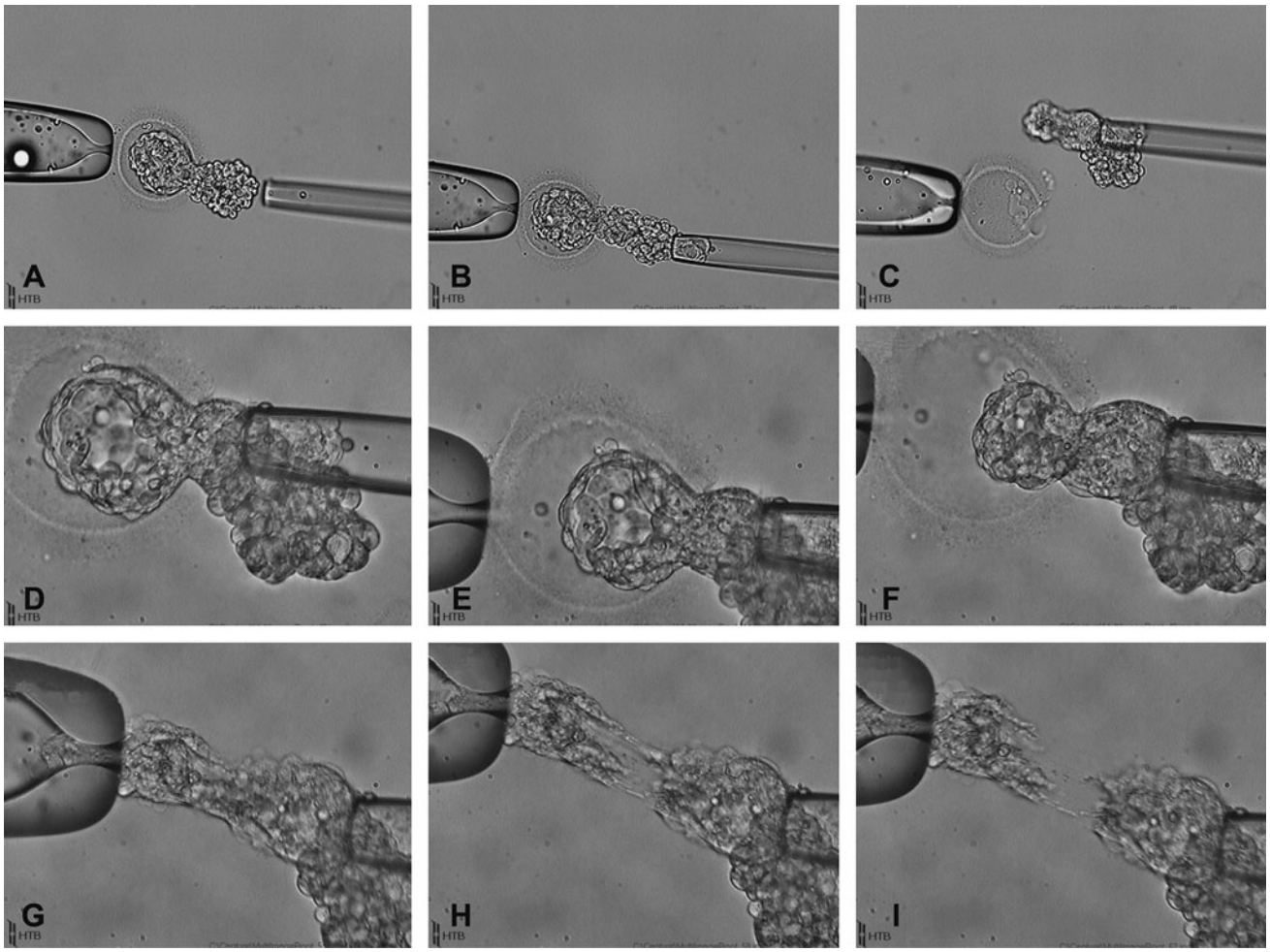

FIG. 1. Laser micromanipulation to isolate the inner cell mass (ICM) during derivation of human embryonic stem cells. A day-7 blastocyst diagnosed by preimplantation genetics as being affected is removed from the zona pellucida (ZP) using laser-assisted micromanipulation technology. The biopsy pipette is used to pull the blastocyst through the hole that was made in the ZP during embryo biopsy at day 3 postfertilization **(A–F)**. A few laser shots of 980 μs (300 mW) were used to separate the ICM from the trophoblast cells at the opposite side of the blastocyst **(G–I)**.

further propagated by collagenase type IV and then frozen for future use.

Characterization of HESC lines

The newly established cell lines were characterized for self-renewal ability, expression of undifferentiated cell specific markers (Oct4, Rex1, Nanog, SOX2, alkaline phosphatase, Tra-1-60, and SSEA3), karyotype, and pluripotent potential by forming embryoid bodies in vitro or by teratoma induction in vivo. Established cell lines were then molecularly tested for the genetic defect that is carried by the couple to confirm their genotype using the same protocols applied for the one-cell PGD tests.

Gender determination

The gender of the preimplantation embryo was determined by molecular testing for the presence of the Y-linked Sry gene, in addition to the characterization of Amelogenein, Zfx, and several other X-linked polymorphic markers. The gender of the derived ES cell line was determined by analyzing genomic DNA extracted from the HESCs using the same PCR primers and reactions used for the PGD analysis.

Statistical analysis

The one-sample binomial test was used to test whether the proportion of females significantly deviated from the 50% expected. This test was performed for a sample size of >6. Two-sample proportion tests were used to compare blastocyst development of female versus male embryos.

Results

In this report, we describe a list of disease-bearing HESC lines, which we established in the Shaare Zedek and Tel Aviv Sourasky Medical Centers. These HESC lines were all exclusively established from genetically affected embryos that were donated by couples undergoing PGD treatment (Table 1). Our large collection includes 42 mutant HESCs lines with dominant (17 lines) and recessive (3 lines) conditions as well as X-linked disorders (20 lines) and chromosomal rearrangements (2 lines). Among those, to the best of our knowledge, is the largest collection of HESC lines associated with trinucleotide repeats expansion disorders, counting fragile X syndrome (9 lines), myotonic dystrophy type 1 (7 lines), and spinal and bulbar muscular atrophy X-linked (1 line). In addition, we have several lines that carry cancer predisposition mutations, such as retinoblastoma, familial adenomatous polyposis, and susceptibility to breast and ovarian cancer (BRCA1). Moreover, as we perform diagnosis of all possible conditions regardless of their prevalence in the general population, we have established cell lines for a wide range of diseases, some of which are extremely rare in the general population and are considered as private mutations, such as hypohidrotic ectodermal dysplasia, congenital insensitivity to pain with anhidrosis, branchiootorenal syndrome, as well as HESC lines that carry unbalanced translocations that are naturally embryonic lethal (t(12;11) and t(1;12)). We are currently employing these HESC lines for studying different aspects of human genetics, embryogenesis, and cell therapy [2].

Examination of the sex distribution among our mutant HESC lines revealed that 76% of them were females (29 female vs. 9 male HESC lines; Table 1). This sex ratio is statistically different from the 50% ($P<0.01$) that would be expected. This value is even more notable when taking into account the fact that more male ICMs were plated for HESC derivation than female ones (53% of all the blastocysts; data not shown).

After having obtained such a high ratio of females among our PGD-HESC lines, we performed a meta-analysis to integrate the results on gender distribution in all PGD-HESC lines published to date in scientific journals and publications from international HESC banks. The gender of all 148 PGD-derived HESC lines was reported and the findings are summarized in Table 2. Altogether, we found that 67% of PGD-derived HESC lines are female, significantly different from the 50% expected distribution ($P=0.0001$) and confirming our data. As PGD-related cell lines are genetically abnormal, it is theoretically possible that male embryos are preferentially less viable because of distinct mutations, particularly in X-linked conditions. This is very unlikely, however, because the increase in the sex ratio of females observed among the mutant HESC lines persisted after excluding the cases of X-linked conditions, reaching a bias of up to 80% females (data not shown). In addition, the bias toward female gender was also evident among cell lines derived from embryos that had undergone biopsy for preimplantation genetic screening for chromosomal aberrations but were eventually found to be karyotypically normal [10].

In contrast to PGD for sex selection in which only embryos with the desired gender are transferred, PGD for X-linked diseases enables determination of the gender of an embryo from the very early cleavage stage through the blastocyst stage and until birth. Therefore, we attempted to determine the origin of the sex bias toward females in our HESC lines by examining embryo gender prior to and following embryo biopsy (days 3 and 5 postfertilization, respectively). These data were obtained from PGD cycles for X-linked diseases (Table 3). There was no bias toward females in cleavage-stage embryos (day 3 postfertilization), and the percentage of females was even lower than the 50% expected (45.9% females, 629/1370, $P<0.01$). Moreover, the developmental potential toward the blastocyst stage was not statistically different between males and females (21.6% and 23.1%, respectively; $P>0.05$, 2-sample proportion test). In addition, only 48.0% (96/200) of the babies born following PGD were females, which is not significantly different from the 51.3% females born following IVF-intracytoplasmic sperm injection (ICSI) in both medical centers ($P>0.05$). Overall, we showed equal representation of males and females among embryos, both prior to and following embryo biopsy. This is in accordance with our PGD policy for X-linked diseases, in which both female carriers as well as affected males are considered unsuitable for transfer and therefore are equally available for HESC line derivation.

In light of the above findings, we hypothesized that the significant increase in XX female HESC lines is most likely attributed to culture conditions upon derivation rather than an a priori overrepresentation of female embryos available for manipulation. To test this hypothesis, we analyzed the sex ratio of all, to the best of our knowledge, wild-type HESC lines with normal karyotype thus far established from surplus IVF embryos. Such embryos are donated by infertile

Table 1. Gender Distribution Among Preimplantation Genetic Diagnosis-Derived Human Embryonic Stem Cell Lines

	Indication for PGD			*Molecular analysis of the HESC line*	
HESC line ID[a]	*Disease*	*Inheritance*	*Familial mutation*	*Genotype*[b]	*Sex*
SZ-Rb2	Retinoblastoma	Autosomal dominant	C1572insAA	Affected	XX
SZ-Rb26	Retinoblastoma	Autosomal dominant	C1572insAA	Affected	XX
SZ-Rb18	Retinoblastoma	Autosomal dominant	C1572insAA	Affected	ND
SZ-DM1	Myotonic dystrophy	Autosomal dominant	Expansion in paternal DMPK allele	Affected	**XY**
SZ-DM2	Myotonic dystrophy	Autosomal dominant	Expansion in paternal DMPK allele	Affected	XX
SZ-DM4	Myotonic dystrophy	Autosomal dominant	Expansion of the paternal DMPK allele	Affected	XX
SZ-DM7	Myotonic dystrophy	Autosomal dominant	Expansion in paternal DMPK allele	Affected	XX
SZ-DM5	Myotonic dystrophy	Autosomal dominant	Expansion in paternal DMPK allele	Affected	**XY**
SZ-FX1	Fragile X	X-linked	75 CGG repeats in the FMR1 gene	Affected/premutation carrier	XX
SZ-FX3	Fragile X	X-linked	100–300 CGG repeats in the FMR1 gene	Affected	XX
SZ-FX6	Fragile X	X-linked	170 CGG repeats in the FMR1 gene	Affected	**XY**
SZ-FX2	Fragile X	X-linked	70 CGG repeats in the FMR1 gene	Premutation carrier	**XY**
SZ-FX5	Fragile X	X-linked	75 CGG repeats in the FMR1 gene	Affected/premutation carrier	XX
SZ-FX10	Fragile X	X-linked	75 CGG repeats in the FMR1 gene	Affected/premutation carrier	XX
SZ-HED7	Hypohydrotic ectodermal dysplasia	Autosomal dominant	Mutation D50N in the GJB2 gene	Affected	**XY**
SZ-CIPA1	Congenital insensitivity to pain with anhidrosis (CIPA)	Autosomal recessive	1926 insT in the TrkA gene	Affected	XX
SZ-BOR1	Branchio-oto-renal syndrome	Autosomal dominant	Mutation 433del G in exon 5 of the EYA1 gene	Affected	XX
SZ-Hun4	Hunter	X-linked	Carrier of exon 4–7 del in the IDS	Carrier	XX
SZ-BRCA1	BRCA1 breast cancer predisposition	Autosomal dominant	Carrier of 5382 insC in BRCA1 gene	Affected (predisposition)	XX
SZ-SBMA	Kennedey disease (SBMA)	X-linked	CAG expansion in the androgen receptor	Carrier	XX
Lis01_HEFX[c,d]	Fragile X	X-linked	180 CGG repeats in the FMR1 gene	Affected	**XY**
Lis02_FXS2[d]	Fragile X	X-linked	300 CGG repeats in the FMR1 gene	Affected	XX
Lis03_FXS4[d]	Fragile X	X-linked	250 CGG repeats in the FMR1 gene	Affected	XX

(*continued*)

Table 1. (Continued)

	Indication for PGD			Molecular analysis of the HESC line	
HESC line ID[a]	*Disease*	*Inheritance*	*Familial mutation*	*Genotype*[b]	*Sex*
Lis04_Twist1[d]	Saethre-Chotzen Syndrome	Autosomal dominant	Ala 129pro (twist)	Affected	XX
Lis05_t(11,22)[d]	Translocation (11; 22)	Balanced translocation inheritance	Translocation (11;22)	Affected	**XY**
Lis06_Gaucher1[d]	Gaucher	Autosomal recessive	N370S	Affected	XX
Lis07_AIS1[d]	Androgen insensitivity syndrome	X-linked	AR gene. Small deletion in intron 5	Carrier	XX
Lis08_AIS2[d]	Androgen insensitivity syndrome	X-linked	AR gene. Small deletion in intron 5	Carrier	XX
Lis09_DYS1[d]	Dystonia	Autosomal dominant	GAG deletion	Affected	XX
Lis10_DMD1[d]	Duchenne muscular dystrophy	X-linked	Deletion exons 44–47	Carrier	XX
Lis11_DMD2[d]	Duchenne muscular dystrophy	X-linked	Deletion exons 44–47	Affected	**XY**
Lis12_DM1[d]	Myotonic dystrophy	Autosomal dominant	Maternal CAG expansion (>2 kb)	Affected	XX
Lis13_Alport2[d]	Alport syndrome	X-linked Paternal	Sex selection for male, unknown mutation	Affected	XX
Lis14_Alport3[d]	Alport syndrome	X-linked Paternal	Sex selection for male, unknown mutation	Affected	XX
Lis15_t(l;12) 1	Translocation (1;12)	Balanced translocation inheritance	Translocation (1;12)	Affected	ND
Lis18_Connexin2	Nonsyndromic deafness	Autosomal recessive	167delT/35del G	Affected	XX
Lis19_DM2	Myotonic dystrophy	Autosomal dominant	Maternal CAG expansion (>1.5 kb)	Affected	ND
Lis20_DMD3	Duchenne muscular dystrophy	X-linked	R2870×point mutation	Carrier	XX
Lis21_noonan	Noonan	Autosomal dominant	922A to G	Affected	ND
Lis22_DMD4	Duchenne muscular dystrophy	X-linked	R2870X point mutation	Carrier	XX
Lis23_DMD5	Duchenne muscular dystrophy	X-linked	R2870X point mutation	Affected	**XY**
Lis25_FAP	Familial adenomatous polyposis (FAP)	Autosomal dominant	R332X point mutation	Affected	XX

[a]SZ, Shaare Zedek Medical Center, Jerusalem, Israel; Lis, Lis Maternity Hospital, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel.
[b]In Fragile X, all HESC lines inherited the expanded allele; however, only following Southern blot analysis full mutation (>200 CGGs) can be confirmed.
[c]Ref. [2].
[d]Ref. [21].
HESC, human embryonic stem cell; PGD, preimplantation genetic diagnosis.

Table 2. Human Embryonic Stem Cell Lines Derived Following Preimplantation Analysis

Stem cell Center	*XX*	*XY*	*Sample size*	*Female proportion*	P *value*[a]
Tel Aviv Sourasky Medical Center (Lis in Table 1)	15	4	19	78.9%	0.022
Shaare Zedek Medical Center (SZ in Table 1)	14	5	19	73.7%	0.067
Cedar-Sinai Medical Center, Los Angeles (Narwani et al. [22])	9	3	12	75.0%	0.149
VUB, Brussel, Belgium (Mateizel et al. [23])	15	0	15	100.0%	0.000
Reproductive Genetics Institute, Chicago, Illinois NIH Human Embryonic Stem Cell Registry; http://stemcells.nih.gov/research/registry/	10	16	26	38.5%	0.327
Hadassah HESC Research Center, Jerusalem, Israel (Turetsky et al. [24])	3	1	4	75.0%	–
Hadassah HESC Research Center, Jerusalem, Israel (Steiner et al. [25])	1	2	3	33.3%	–
INSERN, France; European HESC Registry; www.hescreg.eu/	6	6	12	50.0%	0.773
King's Colledge, UK; European HESC Registry; www.hescreg.eu/	0	2	2	0.0%	–
Royan Institute for Stem Cell Biology and Technology, Tehran, Iran (Taei et al. [26])	2	0	2	100.0%	–
IGBMC, Illkirch, France (Tropel et al. [27])	5	4	9	55.6%	1.000
Istanbul Memorial Hospital, Turkey (Candan and Kahraman [28])	8	4	12	66.7%	0.387
Sydney IVF Stem Cells, Australia (Peura et al. [29] and Bradley et al. [30])	11	2	13	84.6%	0.027
Total	**99**	**49**	**148**	**66.9%**	**0.0001**

[a]One-sample proportion test was performed only when sample size was >6. Only karyotypic normal lines (46XX, 46XY) were included.

couples undergoing IVF treatment, which resulted in the cryopreservation of their excess embryos. Gender type of all these 397 cell lines, derived in 35 different SC centers worldwide, was examined. Although when examining the sex ratio within each SC center separately, differences are not always apparent; a significant bias favoring female over male HESC lines is observed when accumulating all data (55.4%, $P < 0.05$; Table 4).

Discussion

In this study, we describe a unique set of disease-bearing PGD-derived HESC lines that can serve as new and improved models for studying various human pathologies. Our findings demonstrate a clear-cut propensity toward higher success in the derivation of female cell lines over male ones. In contrast to PGD for sex selection in which only embryos with the desired gender are transferred, PGD for X-linked diseases enables embryo gender determination from the very early cleavage stage through the blastocyst stage and prior to their manipulation for SC derivation. It therefore makes it possible to study the origin of this sex bias in the established HESC lines. Our results demonstrate that the source of the gender predisposition is the derivation process itself, rather than from unequal representation between male and female embryos at any stage during development. We support our findings by integrating them with the results of all SC centers worldwide who are engaged in the derivation of HESC lines following PGD analysis. To rule out any contribution of embryo biopsy as a cause for the sex bias among the established HESC lines, we performed a meta-analysis of the sex ratio of all wild-type

Table 3. XX/XY Ratio During Development of Preimplantation Genetically Diagnosed Embryos

	XX	*XY*	*Sample size*	*% XX*	P *value*
Day 3 embryos[a]					
SZ	93	108	201	46.27%	0.32
LIS	536	633	1169	45.85%	0.005
Total (SZ + LIS)	629	741	1370	45.91%	0.003
Blastocyst development on Day 5[a,b]					
SZ[c]	ND	ND			
LIS	21.6% (88/408)	23.1% (110/475)			0.629
Babies born following PGD[d]					
SZ	65	57	122	53.28%	0.526
LIS	31	47	78	39.74%	0.089
Total (SZ + LIS)	96	104	200	48.00%	0.621
Babies born following IVF[e]					
Babies born	1436	1363	2799	51.30%	0.174

[a]Data collected based on PGD for X-linked diseases.
[b]Including early, full, and hatched blastocysts.
[c]PB biopsy was applied; therefore, no data on embryo sexing were available.
[d]Babies born following PGD for monogenic disorders or chromosomal translocations.
[e]Babies born following IVF/ICSI in Lis Maternity Hospital, Tel Aviv Medical Center.
IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

Table 4. Gender Distribution Among Human Embryonic Stem Cell Lines Derived from Surplus In Vitro Fertilization Embryos

Country	*Stem cell center*		*XX*	*XY*	*Sample size*	*Female proportion*	P *value*[a]
Australia	Sydney IVF	www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx	25	14	39	64.1%	0.109
China	Central South University	www.hescreg.eu/	0	3	3	0.0%	
China	Peking University Third Hospital	www.hescreg.eu/	1	2	3	33.3%	
China	Shanghei Second Medical University	www.hescreg.eu/	5	3	8	62.5%	0.724
Czech Republic	Masaryk University	www.hescreg.eu/	3	4	7	42.9%	1
Denmark	Aalborg University	www.hescreg.eu/	1	3	4	25.0%	
Denmark	University of Copenhagen	www.hescreg.eu/	3	2	5	60.0%	
Denmark	University of South Denmark	www.hescreg.eu/	3	1	4	75.0%	
Finland	University of Helsinki	www.hescreg.eu/	1	5	6	16.7%	
Finland	University of Tampere	www.hescreg.eu/	5	3	8	62.5%	0.724
Iran	Royan Institute	www.hescreg.eu/	2	1	3	66.7%	
Israel	Technion Israeli Institute of Technology	www.hescreg.eu/	2	1	3	66.7%	
Japan	Kyoto University	www.hescreg.eu/	2	1	3	66.7%	
Korea	Maria Infertility Hospital	www.hescreg.eu/	4	5	9	44.4%	1
Korea	Mizmedi Hospital Korea	www.hescreg.eu/	9	5	14	64.3%	0.423
Korea	Seoul National University	www.hescreg.eu/	1	5	6	16.7%	
Netherlands	Leiden University Medical Center	www.hescreg.eu/	3	1	4	75.0%	
Singapore	ES Cell Internation Pte. Ltd.	www.hescreg.eu/	3	2	5	60.0%	
Singapore	Singapore Stem Cell Consortium	www.hescreg.eu/	5	1	6	83.3%	
Spain	Center of Regenerative Medicine in Barcelona	www.hescreg.eu/	2	8	10	20.0%	0.114
Spain	Principe Felipe Centro de Investigecion	www.hescreg.eu/	3	3	6	50.0%	
Sweden	Cellartis AB Sweden	www.hescreg.eu/	6	11	17	35.3%	0.332
Sweden	Karolinska Institutet	Strom et al. [31]	10	14	24	41.7%	0.540
Taiwan	Kaohsiung Medical University	www.hescreg.eu/	2	2	4	50.0%	
Turkey	Istanbul Memorial Hospital	Candan and Kahraman [28]	5	1	6	83.3%	
United Kingdom	Axordia Ltd.	www.hescreg.eu/	3	2	5	60.0%	
United Kingdom	King's College	www.hescreg.eu/	0	3	3	0.0%	
United Kingdom	National Institute for Biological Standards and Control	www.hescreg.eu/	11	9	20	55.0%	0.823
United Kingdom	Roslin Cells Ltd.	www.hescreg.eu/	5	4	9	55.6%	1
United Kingdom	University of Edinburgh	www.hescreg.eu/	4	1	5	80.0%	
United Kingdom	University of Newcastle	www.hescreg.eu/	5	4	9	55.6%	1
United States	Reproductive Genetics Institute	www.hescreg.eu/; www.stemride.com/	45	21	66	68.2%	0.005
United States	WiCell Research Institute	www.hescreg.eu/; Thompson et al. [32]	2	3	5	40.0%	
United States	Children's Hospital Boston; Harvard Stem Cell Institute	Lerou et al. [33]	7	4	11	63.6%	0.547
United States	Harvard University, Harvard Stem Cell Institute	Cowan et al. [34]; Chen et al. [35]	32	25	57	56.1%	0.427
		Total	**220**	**177**	**397**	**55.4%**	**0.035**

[a]One-sample proportion test was performed only when sample size was >6. Only karyotypic normal lines (46XX, 46XY) were included.

HESC lines with published karyotypes that had been established worldwide from surplus IVF embryos. When accumulating all data in this large group of WT HESC lines, a significant propensity toward females is observed, although to a lower extent. This further supports the results obtained with PGD-derived lines. Interestingly, these results accord with the findings related to the derivation of embryonic stem cells in mouse, demonstrating female sex bias following plating of intact ICMs or epiblasts [36].

Assisted reproduction technologies have been proposed to have a slight effect on the sex ratio of new born babies (1.5%–2.5%; reviewed in [37]). It was suggested that there are more male blastocysts selected for embryo transfer, because they grow faster, but this cannot be proved when sex determination is retrospectively made following delivery. Our results determining the embryo gender prior to implantation in X-linked PGD cycles unequivocally show that no such sex bias exists among new born babies following assisted reproduction technologies.

In the clinical setting, there is a well-documented disproportionate loss of males following natural conception and at birth, reaching gender equilibrium by the third or fourth decade of life and followed by a further decline into old age. Previous studies on singleton pregnancies have found male fetuses (compared with females) to be an independent risk factor for adverse pregnancy outcome [38]. These results are in agreement with our findings on female HESC lines being more adaptive to the suboptimal culture conditions.

The question arises as to which mechanism might explain the more efficient derivation of female HESCs over males. It would appear that pluripotent XY cells are more vulnerable than pluripotent XX cells. As the major difference between the two genders is the presence of an additional X chromosome, it is tempting to suggest that a difference in the epigenetic state and activity of this extra chromosome may account for the preference of female ICM survival in vitro. The state of X chromosome inactivation has been shown to considerably vary among different WT-HESC lines [39,40] because of suboptimal culture conditions, such as exposure to oxygen stress. This possibility is supported by the recent publication of Guenther et al., who concluded that uncontrolled laboratory-specific conditions may contribute to differential gene expression between pluripotent stem cell lines [41]. It is interesting to note that, in contrast to humans, the maintenance of stable XX ESC lines from mouse blastocysts is relatively difficult because of the frequent loss of one of the two active X chromosomes [42]. It has been also shown that DNA methylation is globally reduced in XX mouse ESC lines and that this may provide the basis for X-chromosome instability [42]. Using conventional methods for HESC derivation may not only induce precocious X inactivation, but also impose cellular stress, leading to proliferation of specific cells that are best adapted to the suboptimal growth conditions available. We herein suggest a possible mechanism by which aberrant X chromosome inactivation in female undifferentiated cells eventually favors XX HESC outgrowth and proliferation over their XY counterparts. A complementary explanation may be overexpression of genes critical for metabolism and survival under stress conditions, which are located on the X chromosome (eg, G6PD and HPRT) and are known to control key metabolic functions [43]. This may lead to the favoring of XX cell growth in suboptimal culture conditions, which do not exactly mimic the natural embryo environment and can expose cells to stressful conditions. Although we are unable to pinpoint the culture-associated factors that lead to this female propensity because of the lack of information regarding the methodologies and conditions that were used by the different centers at the time of derivation, our results highlight the importance of optimizing culture conditions at the time of derivation as well as during culture of HESCs. This is further supported by the recent publications related to epigenetic errors and genomic instabilities acquired during long-term culture of HESCs and derivation of iPS cells [44,45]. Further work is warranted to reveal this yet unknown mechanism.

Conclusions

We here describe a gender dimorphism in the establishment and maintenance of HESC lines, resulting in the availability of more XX lines than XY lines. This may be due to the conventional culture conditions that provide a growth advantage to cells in which either two X chromosomes are expressed or, alternatively, demonstrate precocious X inactivation. Future investigations into the factors controlling derivation and proliferation of HESCs are warranted to elucidate the mechanism responsible for this phenomenon.

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Author Disclosure Statement

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