



Patient-Specific Embryonic Stem Cells Derived from Human SCNT Blastocysts

Woo Suk Hwang, *et al.*
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ally intense ecologically, even if their macro-evolutionary consequences are unlikely to have been significant.

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- Our data provide multiple estimates for the mid-to-late Paleozoic increase in drilling frequencies. The evidence for late Mesozoic increase is more circumstantial, however, being based on the late Cenozoic (Holocene) estimates only. However, the Paleozoic-to-Holocene increase is consistent with the Mesozoic Marine Revolution hypothesis. Moreover, recent literature compilations (4, 17) do not indicate any significant increase in drilling between Paleozoic and Mesozoic brachiopods (i.e., the post-Paleozoic increase must have happened either in the latest Mesozoic or in the Cenozoic). Also, recent case studies indicate that exceptionally high drilling frequencies are occasionally observed in Cenozoic brachiopods, yielding HDLT estimates as high as 40% (19): a value higher than any of the 34 Paleozoic estimates reported here (Fig. 2).
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~3% and represents a slow increase over a period of ~500 million years.

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Supporting Online Material

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Patient-Specific Embryonic Stem Cells Derived from Human SCNT Blastocysts

Woo Suk Hwang,^{1,2*} Sung Il Roh,³ Byeong Chun Lee,¹
Sung Keun Kang,¹ Dae Kee Kwon,¹ Sue Kim,¹ Sun Jong Kim,³
Sun Woo Park,¹ Hee Sun Kwon,¹ Chang Kyu Lee,² Jung Bok Lee,³
Jin Mee Kim,³ Curie Ahn,⁴ Sun Ha Paek,⁴ Sang Sik Chang,⁵
Jung Jin Koo,⁵ Hyun Soo Yoon,⁶ Jung Hye Hwang,⁶
Youn Young Hwang,⁶ Ye Soo Park,⁶ Sun Kyung Oh,⁴ Hee Sun Kim,⁴
Jong Hyuk Park,⁷ Shin Yong Moon,⁴ Gerald Schatten^{7*}

Patient-specific, immune-matched human embryonic stem cells (hESCs) are anticipated to be of great biomedical importance for studies of disease and development and to advance clinical deliberations regarding stem cell transplantation. Eleven hESC lines were established by somatic cell nuclear transfer (SCNT) of skin cells from patients with disease or injury into donated oocytes. These lines, nuclear transfer (NT)-hESCs, grown on human feeders from the same NT donor or from genetically unrelated individuals, were established at high rates, regardless of NT donor sex or age. NT-hESCs were pluripotent, chromosomally normal, and matched the NT patient's DNA. The major histocompatibility complex identity of each NT-hESC when compared to the patient's own showed immunological compatibility, which is important for eventual transplantation. With the generation of these NT-hESCs, evaluations of genetic and epigenetic stability can be made. Additional work remains to be done regarding the development of reliable directed differentiation and the elimination of remaining animal components. Before clinical use of these cells can occur, preclinical evidence is required to prove that transplantation of differentiated NT-hESCs can be safe, effective, and tolerated.

Many human injuries and diseases result from defects in a single cell type. If defective cells could be replaced with appropriate stem cells, progenitor cells, or cells differentiated in vitro, and if immune rejection of transplanted cells could be avoided, it might be possible to treat disease and injury at the cellular level in the clinic (1). By generating hESCs from human NT blastocysts, in which the somatic cell nucleus comes from the individual patient—a situation where the nuclear [though not mitochondrial DNA (mtDNA)] genome is identical to that of the NT donor—the possibility of immune rejection might be eliminated if these cells were to be used for human treatment (2, 3).

Recently, mouse models of severe combined immunodeficiency (SCID) (4) and Parkinson's disease (PD) (5) have been successfully treated through the transplantation of autologous differentiated mouse embryonic stem cells (mESCs) derived from NT blastocysts, a process also referred to as therapeutic cloning (6). In 2004, evidence was presented that a human NT-hESC line (NT-hESC-1) was derived by transferring the donor's cumulus cell nucleus into her own enucleated oocyte (6); however, questions remained as to whether the cell line had a parthenogenetic origin. In addition, it was not known whether NT-hESCs could be generated from NT procedures using nuclei from males, prepubescent girls, or postmenopausal

Table 1. Establishment of patient-specific NT-hESCs.

(A) Cell line	(B) Cell donors			(C) No. injected oocytes (no. of donors)	(D) No. fused oocytes (%)	(E) NT blastocysts		(F) NT-hESC lines established			
	Age (years)	Sex	Status			No.	% per fused oocyte	No.	% NT-hESCs per blastocyst	Mean no. oocytes injected per line	Mean no. fused oocytes per line
NT-hESC-2	10	M	SCI	8 (1)	5 (62.5)	1	20.0	1	100.0	8.0	5
NT-hESC-3	6	F	JD	18 (2; 1 > 30 years old)	6 (33.3)	2	33.3	1	50.0	18.0	6.0
NT-hESC-4 and -5	36	M	SCI	22 (1)	21 (95.5)	7	33.3	2	28.6	11.0	10.5
NT-hESC-6 and -7	24	F	SCI	23 (1)	18 (78.3)	6	33.3	2	33.3	11.5	9.0
NT-hESC-8*	33	F	SCI	5 (1)	4 (80.0)	2	50.0	1	50.0	5.0	4.0
NT-hESC-9	2	M	CGH	9 (1)	7 (77.8)	3	42.9	1	33.3	9.0	7.0
NT-hESC-10	56	M	SCI	12 (1)	7 (58.3)	1	14.3	1	100.0	12.0	7.0
NT-hESC-11	30	M	SCI	22 (2; 2 > 30 years old)	15 (68.2)	3	20.0	1	33.3	22.0	15.0
NT-hESC-12	35	M	SCI	48 (5; 4 > 30 years old)	34 (70.8)	6	17.6	1	16.6	48	34
Attempt I†	23	M	SCI	10 (2; 1 > 30 years old)	8 (80.0)	0	-	-	-	-	-
Attempt II†	20	M	SCI	8 (1)	4 (50.0)	0	-	-	-	-	-
Compilations				Σ Oocytes = 185, 125‡ Σ/Cycle = 10.2 oocytes; 12.5 oocytes‡	Σ = 129 (69.7)	Σ = 31	Mean = 24.0%	Σ = 11	Mean = 35.4% 40.9%‡	16.8 13.8‡	11.7 10.0‡

*Autologous SCNT was performed; that is, the donor's own fibroblast nuclei were transferred back into her own enucleated oocytes. †Successful NT development without blastocyst formation. ‡Rates with oocytes donated by women <30 years old (table S3).

women, or when using cell components from unrelated women. Finally, because NT-hESC-1 was grown on mouse feeders with likely xeno-graft contamination (7), the utility of those cells is largely preclinical. In our study, patient-specific NT-hESCs were established reliably and efficiently (~1 NT-hESC line per oocyte donation cycle) on human feeder cells and regardless of somatic cell donor sex or age. In addition, some lines were derived without the animal products used during immunosurgery. Immunosurgery exposes the ESC precursors, the inner cell mass cells (ICMs), through lysis of the blastocyst's outer trophoctoderm cells by sequential exposure to antibodies followed by complement; however, residual animal byproducts, including calf serum and enzymes, still remain. Furthermore, 10 of the 11 new cell lines were generated from NT procedures using oocyte and somatic cell donors that were obtained from biologically unrelated individuals.

Oocyte donations by healthy women (with cell line -8 as the only exception) and somatic

cell donations by patients conformed to the regulations and law in the Republic of Korea, in accordance with responsible institutional review board (IRB) review and oversight (8). Donors were fully aware of the scope of this study and each signed an informed consent form. Both of the parents of children under 18 years old donating somatic cells were similarly counseled, and each signed informed consent forms on behalf of their child. Patients voluntarily donated oocytes and somatic cells for therapeutic cloning research and relevant applications but not for reproductive cloning. Although expenses for public transportation and injections administered by medical personnel could have been provided, none of the donors requested this, and therefore no financial reimbursement in any form was paid (9).

Recruited patients had a genetic immunodeficiency disease [congenital hypogammaglobulinemia (CGH)], disorders caused by injury [spinal cord injury (SCI)], or another condition caused by complex autoimmune mechanisms [juvenile diabetes (JD)]. These three diseases are proposed to be treatable by single-cell-type transplantation with hematopoietic stem cells (of mesodermal origin), motor neurons or neuroprogenitors (ectodermal origin), or β-islet cells (endodermal origin), respectively. Patient-specific stem cells derived in this study are now expected to provide cells in a disease state that can be used to understand disease progression and assist in drug development. Because the stem cells generated with the use of patient cells are still likely to be defective, they probably cannot be directly used in cell transplantation to patients. In addition, before the cells can be used in the clinic, the biological properties of

the patient-specific NT-hESCs must be defined, reliable differentiation procedures must be established, and the cells must be free of contaminating undifferentiated cells and potential pathogens.

Donor patients' fibroblasts were grown from skin biopsies (9). Individual cells were retrieved from the monolayer by trypsinization (9). Heterologous NTs were performed, in which donor somatic cell nuclei were transferred individually into enucleated oocytes from a biologically unrelated individual. However, for one cell line, NT-hESC-8, autologous NT was performed, in which the donor's own fibroblast nuclei were transferred into her own enucleated oocytes. Nine of the generated lines (and one of the unsuccessful attempts) used donated oocytes from unrelated individuals (biological or otherwise), whereas another successful line and one unsuccessful attempt used oocytes from a biologically unrelated family member. Details on oocyte and somatic cell donations and ovarian stimulation protocols are described, and the Korean version and translations by the Korean team into English of the informed consent forms are appended in (9). Enucleation, confirmation of the oocyte's DNA removal, NT, fusion, and activation were performed as described (6).

Eleven NT-hESC lines were derived using somatic cells from patients with SCI, JD, and CHG of both sexes and ranging from 2 to 56 years old (Table 1). Figure 1 shows results from male NT-hESC-2 (left columns) and female NT-hESC-3 (right columns), in which stem cell colonies display characteristic cobblestone-like appearances with circumscribed borders (Fig. 1, A and B) and express hESC pluripotency markers, including alkaline

¹College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea. ²School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea. ³Medical Research Center, MizMedi Hospital, Seoul 135-280, Korea. ⁴College of Medicine, Seoul National University, Seoul 110-744, Korea. ⁵Hanna Women's Clinic, Seoul 137-872, Korea. ⁶School of Medicine, Hanyang University, Seoul 471-701, Korea. ⁷Pittsburgh Development Center, Magee-Womens Research Institute, Department of Obstetrics, Gynecology, and Reproductive Sciences and Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA.

*To whom correspondence should be addressed. E-mail: hwangws@snu.ac.kr (W.S.H.); gschatten@pdc.magee.edu (G.S.)

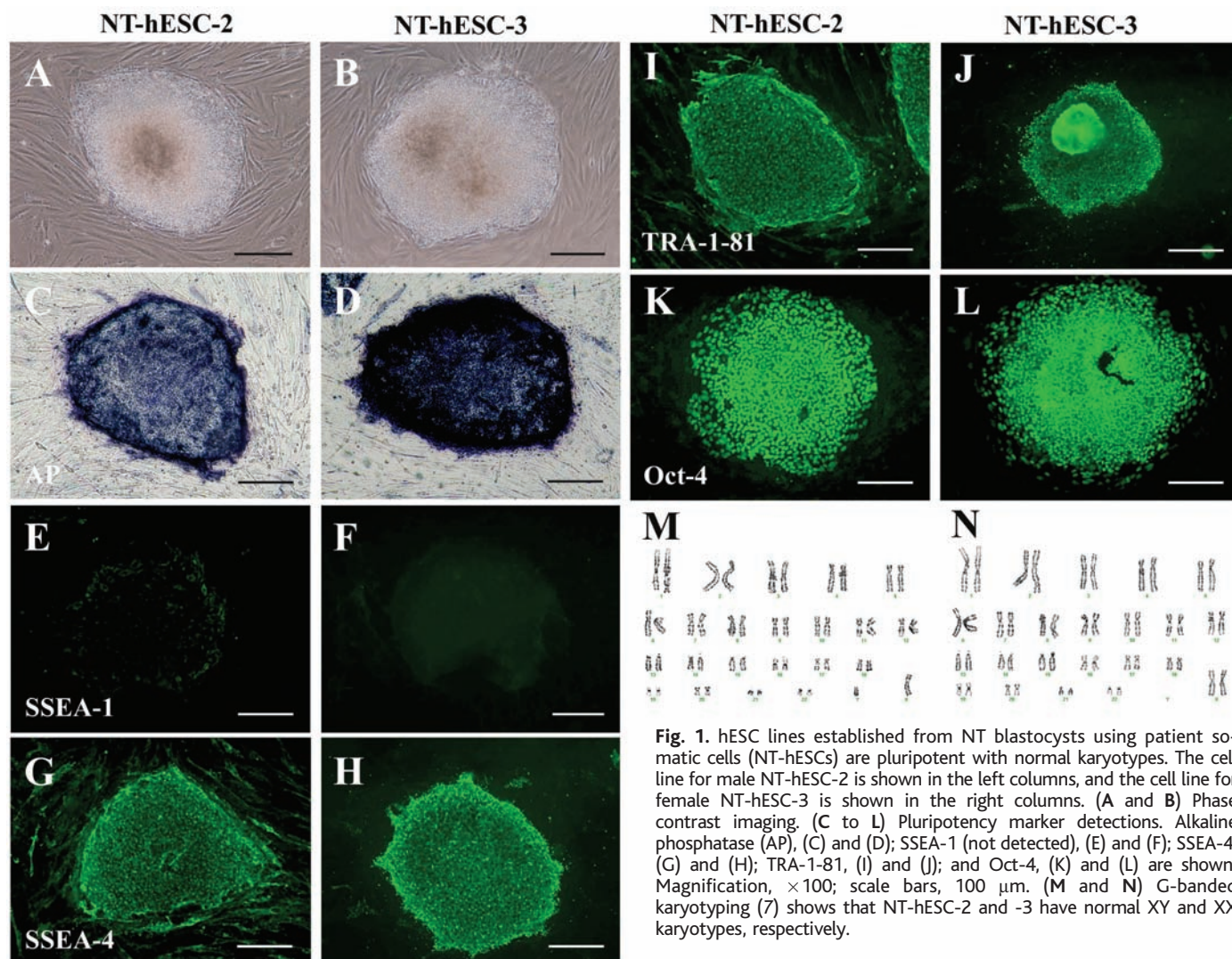


Fig. 1. hESC lines established from NT blastocysts using patient somatic cells (NT-hESCs) are pluripotent with normal karyotypes. The cell line for male NT-hESC-2 is shown in the left columns, and the cell line for female NT-hESC-3 is shown in the right columns. (A and B) Phase contrast imaging. (C to L) Pluripotency marker detections. Alkaline phosphatase (AP), (C) and (D); SSEA-1 (not detected), (E) and (F); SSEA-4, (G) and (H); TRA-1-81, (I) and (J); and Oct-4, (K) and (L) are shown. Magnification, $\times 100$; scale bars, 100 μm . (M and N) G-banded karyotyping (7) shows that NT-hESC-2 and -3 have normal XY and XX karyotypes, respectively.

phosphatase (AP) (Fig. 1, C and D), stage-specific embryonic antigen 4 (SSEA-4) (Fig. 1, G and H), SSEA-3 (fig. S1A), tumor rejection antigen 1-81 (Tra-1-81) (Fig. 1, I and J), Tra-1-60 (fig. S1A), and octamer-4 (Oct-4) (Fig. 1, K and L), but not SSEA-1 (negative control; Fig. 1, E and F). Normal male (Fig. 1M) and female (Fig. 1N) karyotypes are shown for NT-hESC-2 and NT-hESC-3, respectively. For complete data on NT-hESC-4 through -11, see fig. S1, B and C, and Table 2.

DNA fingerprinting with human short tandem-repeat probes (Fig. 2 and fig. S2, A to D) shows with high certainty that every NT-hESC line derived here originated from the respective patient donor and that these lines were not the result of enucleation failures and subsequent parthenogenetic activation. In Fig. 2 (red), isogenic analysis in the loci amelogenin, D5S818, and the fibrinogen alpha chain gene (FGA) shows that the male lines -2 and -4 each precisely match the respective DNA fingerprints of the male NT donors, just as the female line -3 is an identical match with

the female NT donor. The other male lines -5 and -9 to -12 also each match the respective male NT donors, just as the other female lines -6 to -8 each match the other female NT donors (fig. S2A; red are isogenic analyses in the amelogenin, D5S818, and FGA loci comparing NT-hESC-5 through NT-hESC-12, with their respective patient donor's DNA). DNA fingerprinting of donor oocytes was not performed because of their limited numbers and their central importance for the NT procedure. Data shown in fig. S2B [black; isogenic analysis in loci D19S433, von Willebrand factor gene (vWA), thyroid peroxidase gene (TPOX), and D18S51], fig. S2C [green; isogenic analysis in loci D3S1358, tyrosine hydroxylase gene 1 (THO1), D13S317, D16S539, and D2S1338], and fig. S2D [blue; isogenic analysis in loci D8S1179, D21S11, D7S820, and c-fms proto-oncogene for CSF-1 receptor gene (CSF1PO)] further confirmed the identical matches of NT-hESCs with each respective donor. The statistical probability that these lines may have been derived from another person is $<4.1 \times 10^{-16}$.

NT-hESCs have been efficiently established from a diverse group of patients. In Table 1, the somatic cell donor's sex, age, and disorder or disease (Table 1B) are shown in the left columns for each of the 11 new NT-hESC lines (NT-hESC-2 to -12; Table 1A). Eighteen women donated 185 oocytes for these studies (Table 1C), of which 125 oocytes were donated by 10 women under 30 years old (Table 1C, bottom row). On average, 10.2 oocytes were donated during each assisted reproductive technology (ART) stimulation cycle, and an average of 12.5 oocytes were donated per cycle by women under 30 (Table 1C, bottom row). Success varied, with nine lines derived from single cycles (Table 1C). NT-hESC-2 was established with five oocytes from a single cycle, but the derivation of NT-hESC-12 required 48 oocytes from five cycles (however, four of these donations were by women over 30; Table 1C). Lines NT-hESC-4 and -5 were established from a single cycle, as were NT-hESC-6 and -7 (Table 1C). The only line in which the oocytes and somatic cell were biologically related was NT-hESC-8, in which

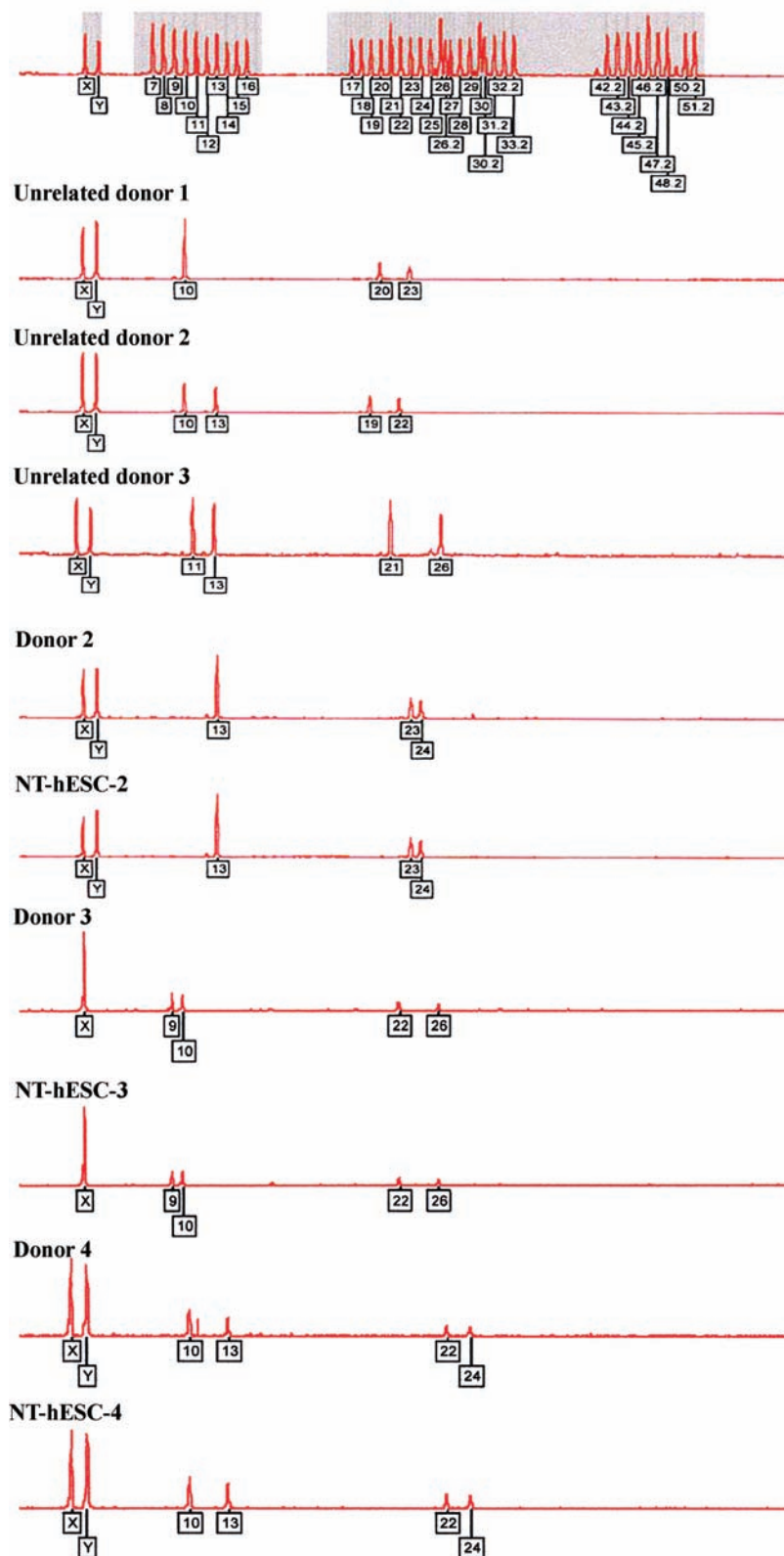


Fig. 2. DNA fingerprinting analysis of 3 of 11 NT-hESCs cell lines (-2 to -4) demonstrates genetic identities with donor patient nuclei (DNA fingerprinting of NT-hESC-5 through -12 are shown in fig. S2A). Isogenic analysis in loci amelogenin (chromosome location: X, p22.1 to 22.3; Y, p11.2); D5S818 (chromosome location 5p22 to 31); and FGA (chromosome location 4q28) is shown. The boxed numbers and corresponding peaks represent locations of polymorphisms for each short tandem-repeat marker at loci amelogenin (peak: X, Y); D5S818 (peak no. 9 to 13); and FGA (peak no. 18 to 26). Figure S2 provides additional DNA fingerprinting evidence.

the donor's own somatic cell was transferred into her own oocytes. Two cycles each were required for NT-hESC-3 and -11 (Table 1C). Only two NT-hESC attempts [NT-hESC attempt I, with oocytes from two donors (one donation from a woman older than 30), and attempt II, with a single donor's oocytes] were unsuccessful; i.e., NT constructs did not develop into blastocysts (Table 1A, bottom rows). Blastocyst development after NT occurred at high rates, with 129 fused NT constructs recovered from the original 185 injected oocytes (69.7%; range from 33.3 to 95.5%; Table 1D). Thirty-one NT blastocysts (24.0% of the fused NT oocytes; Table 1E) were generated. This rate was less than half the ~60% in vitro fertilization (IVF) blastocyst development rate with gametes from infertility patients reported by ART clinics (10).

Eleven NT-hESC lines were established from these 31 NT blastocysts (Table 1F; 35.4% average; range, 16.6 to 100%). 16.8 injected oocytes or 11.7 NT-fused oocytes were required for each established NT-hESC line (range, 5 to 48 injected or 4 to 34 fused NT oocytes, respectively). NT-hESC lines were established from 40.9% of NT blastocysts generated from oocytes donated by women under 30 (Table 1F, bottom). For each NT-hESC line when the oocytes were donated by women under 30, 13.8 injected oocytes or 10.0 NT-fused oocytes were needed (Table 1F, bottom). Derivation rates for NT-hESC lines from NT blastocysts were similar to those with fertilized blastocysts (11, 12).

Neither nuclear donor sex (table S1, 33.3% NT-hESC lines per male blastocyst versus 40.0% NT-hESC lines per female blastocyst) nor age (table S2) influenced cell-line establishment with statistical significance. Oocyte donor age appears to be negatively correlated with success [oocytes donated by women older than 30 years were less successful (table S3), with 40.9% NT-hESC lines per NT blastocyst for oocytes donated by women younger than 30 years versus 22.2% for oocyte donors older than 30 years]. Whereas the limited sample size precludes findings of statistical significance, only two NT-hESC lines were established from the 60 oocytes donated by women older than 30 (3.3% NT-hESCs per oocyte), and the other nine lines were derived from the 125 oocytes donated by younger women (7.2%). It is premature to conclude whether technical confounders, the natural variability of oocytes and donor nuclei, or individual patient characteristics account for either very favorable outcomes (when some lines were established with a few oocytes) or unsuccessful outcomes [when NT-hESCs were not generated (Table 1, attempts I and II)].

Stem cells are defined (13) by their ability to self-renew as well as differentiate into somatic cells from all three embryonic germ layers: ectoderm, mesoderm, and endoderm. Differentiation of 7 of the 11 new NT-hESC lines (NT-hESC-2 through -11) (Table 2) was

analyzed both in teratomas (Fig. 3, A to X) and into embryoid bodies (EBs) (Fig. 3, Y to e, and fig. S3), and the remaining four cell lines investigated so far differentiated only in embryoid bodies (Table 2). Each NT-hESC line differentiated into all germ layers. The convoluted surfaces characteristic of ectodermal lineages, including skin epithelium, and retinal and primitive neuroepithelium, are shown in Fig. 3, A, B, I, J, Q, and R. Muscle cell bundles, distinctive cartilage nodules, renal tubules, and bone matrix, all derived from mesoderm, are shown in Fig. 3, C, D, G, K, L, O, Q, S, T, W, and X. Endoderm derivatives, including gastrointestinal and respiratory epithelia, are shown in Fig. 3, E to H, M, N, P, U, V, and X. Figure 3, Y to e, demonstrates in embryoid bodies that each line differentiates into cells labeled by mono-specific lineage probes; for example, ectodermal cells [Fig. 3, Y to a; Y, NT-hESC-5; Z, NT-hESC-6; and a, NT-hESC-7; paired box gene (*Pax 3/7*), microtubule-associated protein 2 (*MAP-2*), and glial fibrillary acidic protein (*GFAP*)], mesodermal cells [Fig. 3, b to d; b, NT-hESC-8; c, NT-hESC-9; d, NT-hESC-10; atrial natriuretic peptide (*ANP*), *CD34*, and *desmin*, respectively], and endodermal cells [Fig. 3e, NT-hESC-11, myosin heavy chain (*MHC*)]. Figure S3 shows panels of immunohistochemical markers specific for each lineage in every line analyzed to date (Table 2).

The discoveries of Wilmut *et al.* (14) in sheep cloning, together with those of Thomson *et al.* (1) in deriving hESCs, have generated considerable enthusiasm for regenerative cell transplantation based on the establishment of patient-specific hESCs derived from NT blastocysts generated from a patient's own nuclei. This strategy, aimed at avoiding immune rejection through autologous transplantation, is perhaps the strongest clinical rationale for therapeutic cloning. By the same token, derivations of complex disease-specific NT-hESCs may accelerate discoveries of disease mechanisms. For cell transplantations, innovative treatments of murine SCID and PD models with the individual mouse's own NT-mESCs are encouraging (4, 5). Human populations, unlike those of inbred mice, are genetically very diverse; consequently, conclusive proof of immune matching between the specific patient and her or his individualized NT-hESC line demands rigorous evidence. Toward this immune matching, MHC human leukocyte antigen (MHC HLA) isotypes of our stem cell lines are identical to those of the somatic cells from the corresponding patient donors (Table 3 for lines NT-hESC-2 through -4; table S4 for lines -5 through -12). MHC-HLA matching is crucial for immunological tolerance during organ donations, and in the absence of MHC-HLA matching, immunosuppressive medicines are required. This MHC-HLA matching provides additional evidence that parthenogenetic errors had not occurred. Each NT-hESC line matches the respective donor's for

all HLA isotypes shown in Table 3 and table S4, suggesting that transplanted NT-hESCs will be tolerated. Yet caution is warranted in extrapolating from these *in vitro* data. Some histocompatibility antigens traffic through mitochondria (15), and mitochondria in these NT-hESCs are likely to be of either oocyte or heteroplasmic origin (except for autologous NT-hESC-8 and the original NT-hESC-1); thus, meticulous preclinical tolerance investigations in relevant preclinical animal models are a prerequisite for any consideration of clinical experimentation.

NT-hESCs were derived from 35.4% of the NT blastocysts (11 NT-hESC lines/31 NT blastocysts). This rate is more than 10 times the 3.3% reported earlier (6). In contrast, the rate of blastocyst development remains at ~24%. Direct derivations from either zona-free or zona-enclosed intact blastocysts were superior to derivations by immunosurgery (Table 2). Also, immunosurgery involves antibodies and com-

plement, so the elimination of this method avoids animal contaminants, although alternatives for the animal enzymes and serum used in the fibroblast dissociation must now be perfected.

This 10-fold increase in NT-hESC derivation resulted from five protocol improvements discussed here, combined with 10 that were previously reported (6). The five improvements that we developed are as follows: (i) Human feeder cells, rather than murine ones, were established from the skin biopsy of donor 2, obtained under local anesthesia, and grown in 10% fetal bovine serum, 1% nonessential amino acids, and 10 μ g of penicillin-streptomycin per milliliter of culture medium at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. (ii) Donor nuclei were retrieved with 0.25% trypsin-EDTA for 30 s at 37°C and monitored carefully to avoid damage. (iii) Cumulus cell removal from the recipient oocytes demanded limited hyaluronidase exposure. (iv) Direct ES derivations from NT

Table 2. Summary of patient-specific NT-hESC lines. ZF-blast, zona-free blastocyst; ImmS, immunosurgery; Plurip, pluripotent. The check marks denote pluripotency demonstrated by both EBs and teratomas. Normal karyotypes have been shown for each line. Lines derived from male patients are shown in blue; lines derived from female patients are in pink.

NT-hESC	Isolation	Plurip	Differ	Pass no.	DNA	HLA
-2	ZF-blast	✓	✓	P40	Identical	Match
-3	Blast	✓	✓	P35	Identical	Match
-4, -5	ImmS	✓	✓	P26	Identical	Match
-6, -7	ImmS	✓	✓	P25	Identical	Match
-8	Blast	✓	✓	P21	Identical	Match
-9	ZF-blast	✓	EB	P20	Identical	Match
-10	ImmS	✓	EB	P19	Identical	Match
-11	ZF-blast	✓	EB	P19	Identical	Match
-12	ZF-blast	✓	EB	P7	Identical	Match

Table 3. Identical matches of MHC-HLA isotypes among NT-hESC lines -2, -3, and -4 with donors. A, B, C, DRB, and DQB represent gene loci. M, male; F, female. Asterisks indicate genotyping analysis. See table S4 for MHC-HLA matches with NT-hESC-5 through -12.

	MHC-I			MHC-II	
	HLA-A	HLA-B	HLA-C	HLA-DRB	HLA-DQB
Donor 2 (M)	A*01	B*37	Cw*06	DRB1*10	DQB1*0501
	A*31	B*51	Cw*14	DRB1*14	DQB1*0502
NT-hESC-2	A*01	B*37	Cw*06	DRB1*10	DQB1*0501
	A*31	B*51	Cw*14	DRB1*14	DQB1*0502
Donor 3 (F)	A*24	B*13	Cw*06	DRB1*04	DQB1*03
	A*24	B*44	Cw*14	DRB1*11	DQB1*03
NT-hESC-3	A*24	B*13	Cw*06	DRB1*04	DQB1*03
	A*24	B*44	Cw*14	DRB1*11	DQB1*03
Donor 4 (M)	A*02	B*60	Cw*03	DRB1*09	DQB1*0303
	A*11	B*62	Cw*08	DRB1*15	DQB1*0602
NT-hESC-4	A*02	B*60	Cw*03	DRB1*09	DQB1*0303
	A*11	B*62	Cw*08	DRB1*15	DQB1*0602

blastocysts were performed, rather than immunosurgery (Table 2). (v) Scientist-specific micromanipulation improvements were made during the most exacting steps of the oocyte's enucleation and during NT injection and fusion. The previous 10 important steps for NT blastocyst development are presented in (6, 9). Neither autologous cytoplasmic matches nor fructose now appears essential. These combined 15 steps result in an NT-hESC line established with 16.8 injected or 11.7 NT-fused oocytes [fewer if donated by women younger than 30 (13.8 injected or 10.0 fused oocytes)] (Table 1F), which compares favorably with 10.5 oocytes donated each cycle (12.5 oocytes per cycle from women under 30 (Table 1C).

Our NT-hESC derivation rate is in line with some of the highest rates from IVF blastocysts (12). However, direct comparisons need to be tempered by the recognition that most IVF-hESC lines have been derived from clinically discarded, frozen embryos from infertility patients (11, 12), whereas our studies relied on only prime fresh oocytes donated by fertile women expressly for this research.

Here we have described the establishment of patient-specific NT-hESCs with high success rates (Table 2): Average rates indicating that each oocyte donation cycle leads to the establishment of one patient-specific NT-hESC line. Furthermore, discoveries of the mechanisms of complex and multifactorial diseases,

also called Research Cloning, are enabled because NT-hESC-9 is derived from a patient with CGH, and NT-hESC-3 is derived from a patient with JD. By extending NT-hESC procedures from previously autologous (6) to now heterologous NT, regardless of donor patient age (2 to 56 years old) or sex, these NT-hESCs can be evaluated in vitro and after transplantation into appropriate animal models for tolerance, efficacy, and safety.

NT-hESC derivation rates from NT-blastocysts increased more than 10-fold over our previous results (6). Although the cell lines in this study were derived on human feeder cells and were found to be free of known contaminants, the method for dissociating the patient's skin biopsy and primary fibroblast culture included fetal calf serum, trypsin, and collagenase (animal products). If preclinical results are encouraging, long before hESC lines could be used in the clinic, greater stringency is mandated, including methods to avoid xenotransplants. Furthermore, exacting documentation of the meticulous derivation, maintenance, and differentiation procedures would all need to be performed within current good manufacturer practice facilities, so that regulatory authorities such as the Korean and/or U.S. Food and Drug Administration could evaluate investigational new drug applications. However, these rates of NT-hESC establishment, combined with a time

frame of less than 1 year from skin biopsy and oocyte donation to NT-hESC establishment, might be clinically relevant if therapeutic cloning were shown to be of medical value.

Molecular deviations between animals developing after fertilization versus those developing after reproductive cloning have been noted. In particular, epigenetic aberrations have been discovered in the genomic imprints of both cloned fetuses and offspring, as well as their placentas (2). Notwithstanding therapeutic interest, learning whether the erasure, reestablishment, and stability of genomic imprints in these NT-hESCs compare with those of IVF-derived hESCs (16) is essential. Other extranuclear or epigenetic influences include mitochondrial inheritance patterns in hESCs, let alone those in NT-hESCs, which are not understood. mtDNA heteroplasmy (17) could influence hESC stability or differentiation, as might homoplasmic oocyte mtDNA incompatibilities with the donor nucleus. Furthermore, the cells' mitotic spindle poles, or centrosomes, which are contributed by the sperm during human fertilization (18) and which, if imbalanced, have been shown to cause cancers (19, 20), might replicate or divide inaccurately, leading to aneuploidies. Consequently, learning whether somatic centrosome transfer occurs during human NT, as it does during bovine NT (21), is important. Inactivation of the X

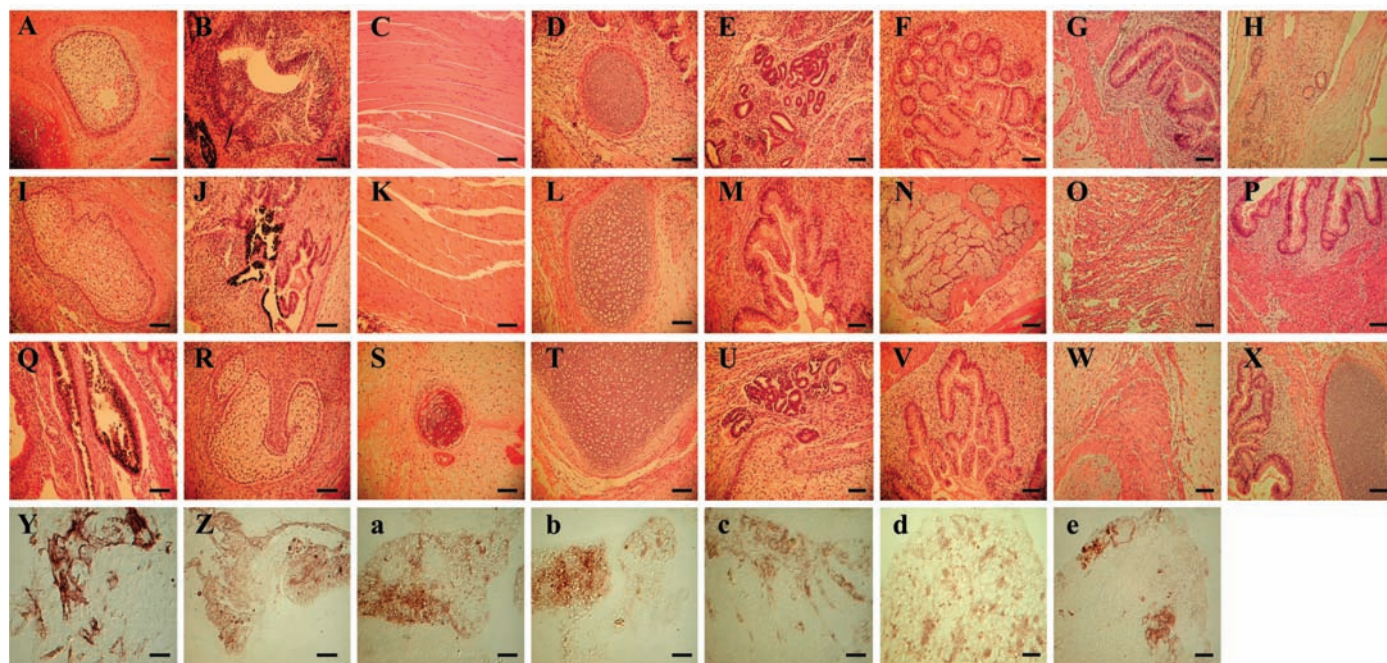


Fig. 3. Patient-specific human NT-hESCs differentiate into tissues from all three germ cell layers in vivo in teratomas (first three rows) and in vitro in EBs (bottom row). NT-hESC-2 (A to H), -3 (I to P), and -4 (Q to X) differentiated into all of the following somatic tissue types: skin [(A), (I), and (R)]; primitive neuroepithelium (B); striated muscle [(C) and (K)]; cartilage [(D), (L), and (T)]; renal tissues [(E) and (U)]; gastrointestinal epithelium [(F), (M), and (V)]; retina and primitive neuroepithelium [(J) and (Q)]; smooth muscle and respiratory epithelium [(G), (P), and (X)];

colon epithelium (H); mucosa gland (N); smooth muscle [(O) and (W)]; and bone (S). Immunohistochemical staining for EBs was performed for Pax3/7 (Y), MAP-2 (Z), GFAP (a), ANP (b), CD34 (c), desmin (d), and MHC (e). EBs also differentiated into all three germ layers expressing ectodermal [(Y), (Z), and (a)], mesodermal [(b) to (d)], and endodermal (e) marker genes. Figure S3 shows differentiation details for all NT-hESC lines. Magnification: $\times 100$, (A) to (R); $\times 200$, (S) to (Y). Scale bars, 100 μm .

chromosome, which is typically random in female mammals, is skewed in cloned female mice (22, 23), causes recurrent spontaneous abortions in some pregnant women (24), and could result in misexpressions in NT-hESCs derived from women. Finally, the genomic stability of NT-hESCs, as well as their differentiation fidelity, including aging and telomerase/telomere behavior, also require rigorous investigations.

The somatic cell's adaptation to in vitro conditions may predispose human NT embryos to cell culture proliferation, with negligible potentials for implantation and none for normal development. Neither NT embryonic development nor NT-hESC establishment rates provide any encouragement for dangerous human reproductive cloning attempts. Cloned animals have adverse pregnancy outcomes, so regardless of cruel hoaxes (25), scientific evidence should further discourage reckless notions regarding human reproductive cloning. Human SCNT was optimized from porcine SCNT procedures in which ~150 NT embryos were transferred for pregnancy establishment (26–28). Furthermore, in rhesus monkeys, 135 cloned embryos transferred into 25 surrogates using some of these improved SCNT techniques (29) did not result in any pregnancies, although rhesus NT blastocysts developed and NT-ICMs were isolated.

Our work described here shows that stem cell lines can be generated using somatic cells from patients with disease and injury. It may also be possible to generate NT-hESC lines from patients with diseases and disorders of unknown causes. For example, NT-hESCs derived from early-onset Alzheimer's disease or autism patients might prove invaluable for mechanistic studies in vitro after differentiation into neuroprogenitors (30, 31). In addition, biological insight gained through studying hESCs might find application to ART and assist in understanding genomic imprinting. The derivations of patient-specific NT-hESCs grown without animal cell co-culture may advance cell transplantation therapies as well as aid in the discovery of human developmental processes and the causes of many complex diseases.

References and Notes

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8. Before beginning any experiments, we obtained approval for this study from the IRB for Human Subjects Research and Ethics Committee at Hanyang University Hospital, Seoul, Korea, which was required by existing regulations that were in place up to 31 December 2004. IRB approvals are included in (9). Oocyte and/or somatic cell donors were counseled by two IRB mem-

bers to ensure that they were fully aware of the scope of the investigation, and each donor signed informed consent forms. Both of the parents of children under 18 years old donating somatic cells were similarly counseled, and each signed informed consent forms on behalf of their child. On 1 January 2005, the Republic of Korea's new regulation entitled Bioethics and Biosafety Act—Act No. 7150, requiring governmental licensing of SCNT using human oocytes and subsequent derivation of NT-hESCs (Therapeutic Cloning), became effective. On 12 January 2005, we received governmental approval in accordance with this new stem cell law. This law also required IRB approval from the College of Veterinary Medicine, Seoul National University, which was granted on 25 January 2005. When our previous report on NT-hESCs appeared online on 12 February 2004 (6), we imposed a voluntary moratorium on new NT-hESC derivations. In September 2004, we announced that we were again performing SCNT and deriving NT-hESCs, under the auspices and oversight of the Hanyang University IRB for Human Subjects Research and Ethics Committee. All IRB documents are included in (9).

9. Materials, methods, and IRB documents are available as supporting material on Science Online.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/1112286/DC1
Materials and Methods
SOM Text
Figs. S1 to S3
Tables S1 to S4
References

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Cladosporium Avr2 Inhibits Tomato Rcr3 Protease Required for Cf-2–Dependent Disease Resistance

Henrietta C. E. Rooney,^{1*} John W. van 't Klooster,^{2*}
Renier A. L. van der Hoorn,² Matthieu H. A. J. Joosten,²
Jonathan D. G. Jones,^{1†} Pierre J. G. M. de Wit^{2‡}

How plants recognize pathogens and activate defense is still mysterious. Direct interaction between pathogen avirulence (Avr) proteins and plant disease resistance proteins is the exception rather than the rule. During infection, *Cladosporium fulvum* secretes Avr2 protein into the apoplast of tomato leaves and, in the presence of the extracellular leucine-rich repeat receptor-like Cf-2 protein, triggers a hypersensitive response (HR) that also requires the extracellular tomato cysteine protease Rcr3. We show here that Avr2 binds and inhibits Rcr3 and propose that the Rcr3-Avr2 complex enables the Cf-2 protein to activate an HR.

Plant disease resistance (*R*) genes mediate race-specific recognition of pathogens via perception of avirulence (*Avr*) gene products (*I*).

Tomato (*Lycopersicon esculentum*) *Cf* genes confer resistance to leaf mold caused by *Cladosporium fulvum* and encode trans-

ERRATUM

post date 16 December 2005

Reports: "Patient-specific embryonic stem cells derived from human SCNT blastocysts" by W. S. Hwang *et al.* (17 June, p. 1777). There were errors in Table 2. The corrected table appears here.

NT-hESC	Isolation	Plurip	Differ	Pass #	DNA	HLA
-2	ZF - Blast	✓	✓	P40	Identical	Match
-3	Blast	✓	✓	P35	Identical	Match
-4, -5	ImmS	✓	✓, EB	P26	Identical	Match
-6, -7	ImmS	✓	EB	P25	Identical	Match
-8	Blast	✓	EB	P21	Identical	Match
-9	ZF - Blast	✓	EB	P20	Identical	Match
-10	ImmS	✓	EB	P19	Identical	Match
-11	ZF - Blast	✓	EB	P19	Identical	Match
-12	ZF - Blast	TBD	TBD	P7	Identical	Match

Table 2. Summary of patient-specific human NT-ESC lines. ZF-blast, zona-free blastocyst; ImmS, immunosurgery; Plurip, Pluripotent; TBD, to be determined; EB, embryoid body; ✓, pluripotency demonstrated by both EBs and teratomas. Normal karyotypes have been shown for each line (female, pink; male, blue).