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Human embryonic stem cells express an immunogenic nonhuman sialic acid

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Human embryonic stem cells (HESC) can potentially generate every body cell type, making them excellent candidates for cell- and tissue-replacement therapies. HESC are typically cultured with animal-derived 'serum replacements' on mouse feeder layers. Both of these are sources of the nonhuman sialic acid Neu5Gc, against which many humans have circulating antibodies. Both HESC and derived embryoid bodies metabolically incorporate substantial amounts of Neu5Gc under standard conditions. Exposure to human sera with antibodies specific for Neu5Gc resulted in binding of immunoglobulin and deposition of complement, which would lead to cell killing *in vivo*. Levels of Neu5Gc on HESC and embryoid bodies dropped after culture in heat-inactivated anti-Neu5Gc antibody–negative human serum, reducing binding of antibodies and complement from high-titer sera, while allowing maintenance of the undifferentiated state. Complete elimination of Neu5Gc would be likely to require using human serum with human feeder layers, ideally starting with fresh HESC that have never been exposed to animal products.

The pluripotent abilities of human embryonic stem cells (HESC)[1,2] have potential for treating many diseases by transplantation of HESC-derived tissues[3]. Although safety is a major issue regarding infection or tumorigenicity, the possibility of rejection is also of concern[1]. Current culture methods use animal products, carrying the risk of infection by nonhuman pathogens. HESC lines are traditionally cultured on mitotically inactivated mouse embryonic fibroblasts (so-called 'feeder layers'), and in media containing fetal calf serum[4]. To avoid animal serum, certain proprietary serum replacements are in use, but these also contain animal products[5–7]. When HESC are removed from the feeder layer and grown in suspension, they differentiate into aggregates called embryoid bodies[8]. Embryoid bodies are formed by precursors of several cell lineages and can be induced to differentiate into many cell types[9,10]. Although the feeder layer is no longer necessary, embryoid bodies must still be maintained in 'serum replacement' medium.

Sialic acids are a family of acidic sugars displayed on the surfaces of all cell types, and on many secreted proteins. The two most common mammalian sialic acids are *N*-glycolylneuraminic acid (Neu5Gc) and *N*-acetylneuraminic acid (Neu5Ac), with Neu5Ac being the metabolic precursor of Neu5Gc. Humans are genetically unable to produce Neu5Gc from Neu5Ac[11], because of a mutation that occurred after our common ancestor with great apes[12]. Thus, although human cells have no overall loss of sialic acids, they express primarily Neu5Ac. But they can potentially take Neu5Gc up from media containing animal products, activate it into CMP-Neu5Gc, and metabolically incorporate it using the same Golgi transporter and sialyltransferases as CMP-Neu5Ac[12,13]. The mechanism by which Neu5Gc enters cells has been defined[14]. Most normal healthy humans have circulating antibodies specific for Neu5Gc[13]. Thus, xenogenic culture methodology could compromise transplantation success, resulting from uptake and expression of Neu5Gc on the surface of any tissue developed from HESC. Such incorporation could induce an immune response upon transplantation.

RESULTS

Presence of Neu5Gc on HESC grown under standard conditions

We detected Neu5Gc on HESC using an affinity-purified chicken polyclonal monospecific antibody against Neu5Gc[13]. HESC stably expressing enhanced green fluorescent protein (EGFP) were gated for EGFP positivity to separate them from contaminating feeder layer fibroblasts. The antibody stained HESC growing in standard conditions, and binding was partially blocked by Neu5Gc-containing glycoproteins from chimpanzee serum (**Fig. 1a**). Blocking was incomplete, probably because not all possible epitopes recognized by the polyclonal antibody are present in chimpanzee serum[13].

To chemically analyze the sialic acid content of HESC, we separated the cells from the feeder layer fibroblasts by fluorescence-activated cell sorting (FACS) using their EGFP signal. Feeder layer–free embryoid bodies derived from HESC were also examined without sorting. Both the membrane and cytosolic fractions from HESC and embryoid bodies had a peak corresponding to Neu5Gc (**Fig. 1b**), whose identity was confirmed by electrospray mass spectrometry (data not shown)[13]. HESC membranes contained 17.88 ± 1.47 pmol sialic acid/μg protein with 9.31 ± 3.70 pmol sialic acid/μg protein in the cytosolic fraction. The percentage of total sialic acids present as Neu5Gc varied from 6–10.5% in the membranes and from 2.5–9% in the cytosolic fraction. Embryoid body membranes had 16.59 ± 3.88 pmol sialic acid/μg protein with 9.13 ± 0.10 pmol sialic acid/μg protein in the cytosolic fraction. The percentage

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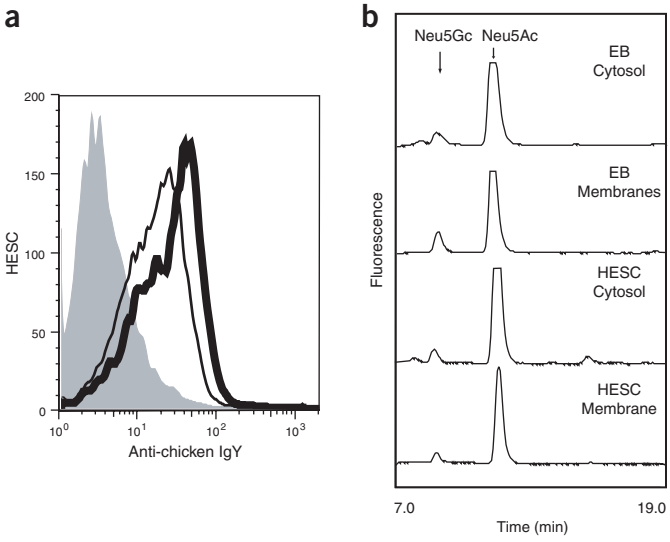

Figure 1 Detection of Neu5Gc on HESC cultured under conventional conditions. EGFP-transfected HESC were grown under conventional conditions using a mouse feeder layer and medium containing 20% KnockOut serum replacement. (**a**) HESC were released with 2mM EDTA and studied by flow cytometry using a previously described affinity-purified polyclonal monospecific antibody against Neu5Gc followed by a secondary Cy5-conjugated anti-chicken IgY antibody. Gray shaded plot: secondary antibody only; thick line: primary and secondary antibody; thin line: cells incubated with chicken anti-Neu5Gc in the presence of 1% chimpanzee serum that contains Neu5Gc. (**b**) HESC were isolated by FACS sorting using the intrinsic EGFP fluorescence. Embryoid bodies were derived by removing the feeder layer and growing the HESC in reduced serum medium for 5 d. Both types of cells were fractionated into membrane and cytosolic components. Sialic acids were released and analyzed by DMB derivatization and high-performance liquid chromatography. A peak corresponding to Neu5Gc is seen in all fractions.

of total sialic acids present as Neu5Gc in embryoid bodies varied from 5–17% for the membranes and 6.5–11% for the cytosolic fraction.

Identifying potential sources of Neu5Gc in HESC

Because human cells are unable to synthesize Neu5Gc[12], the detected Neu5Gc probably originated elsewhere, eventually being metabolically incorporated by the HESC. As expected for other mammals, Neu5Gc represented 20% of total sialic acid in the mouse feeder layer (0.92 ± 0.13 nmol/10^6 cells). But uptake from feeder cells cannot explain all the Neu5Gc found in HESC, because removal of the layer to obtain embryoid bodies did not eliminate it. Our ongoing studies have shown that human cells can take up Neu5Gc from the medium and metabolically incorporate it into membrane glycoconjugates. The serum replacement–containing medium used to support HESC growth was found to contain 35.93 nmol Neu5Gc/ml, representing 54% of total sialic acids. The commercial KnockOut serum replacement, used for preparing this medium, is the major source of Neu5Gc because it contains 129 nmol/ml. In contrast, medium without any additives contains little Neu5Gc (0.008 nmol/ml).

Reduction of Neu5Gc content of HESC

Culture in heat-inactivated pooled normal human serum could markedly reduce Neu5Gc in human colon carcinoma cells[13], apparently as the result of metabolic replacement by Neu5Ac in the human serum. We therefore incubated HESC in medium containing heat-inactivated human serum instead of the standard serum replacement (an approach already suggested by others for different reasons)[15,16]. We first screened and defined a lot of pooled human serum in which natural antibodies specific for Neu5Gc were very low (normal human serum; NHS). In case any residual antibodies were active, we used heat inactivation to eliminate complement. HESC incubated in such NHS remained undifferentiated on the feeder layer, expressing typical levels of markers of nondifferentiation (alkaline phosphatase, Oct-4, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81) and lacking all differentiation markers tested (**Fig. 2a**). After feeder layer removal, these HESC were able to develop into normal embryoid bodies.

Neu5Gc incorporation into HESC membranes dropped after 3 d (from ~4 pmol/μg protein to 0.34 ± 0.06 pmol/μg protein) and down to 0.13 ± 0.01 pmol/μg protein after 1 week (~1% of total sialic acid as Neu5Gc; **Fig. 3**). The required presence of the mouse feeder layer apparently prevented complete elimination of Neu5Gc from HESC. After growing for 3 d in human serum, some HESC were differentiated into embryoid bodies either in 10% commercial serum replacement, or in 10% NHS, without a feeder layer. After 1 week in serum replacement,

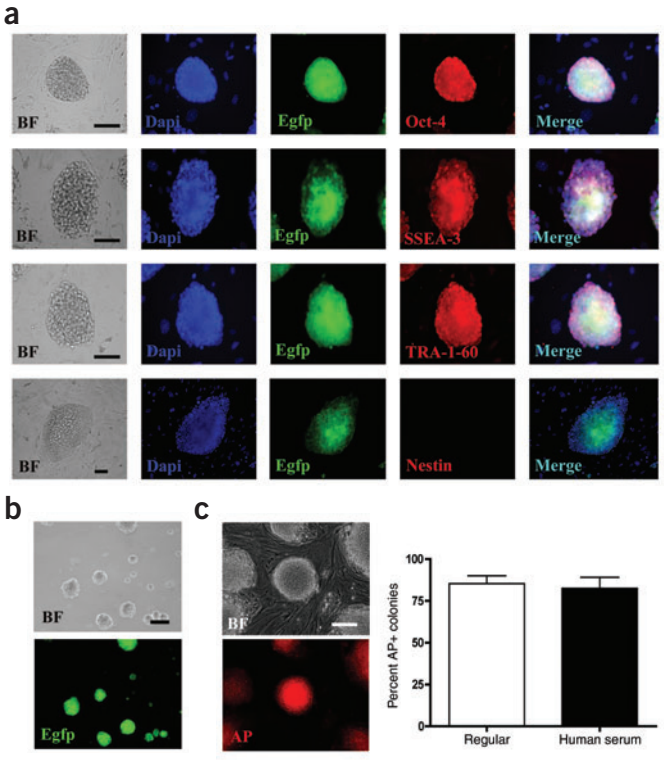

Figure 2 HESC stably expressing EGFP can remain undifferentiated when NHS is substituted for animal-derived culture medium components. (**a**) Bright-field images (BF) of undifferentiated HESC, scale bars correspond to 100 μm. HESC are positive for EGFP and for several nondifferentiation markers, such as Oct-4, SSEA-3 and TRA-1-60. Staining for nestin, a neural progenitor marker, was negative, indicating that the HESC are not differentiated. (**b**) Bright field image of embryoid body derived from EGFP-expressing HESC. They maintain the EGFP expression after differentiation. (**c**) HESC colonies were positive for alkaline phosphatase (AP) activity, as shown in the fluorescent image, both with the standard serum replacement (regular) and in human serum. Cells were also negative for SSEA-1 and for neural markers such as nestin, Tuj-1, Map2(a + b), NeuN, astrocyte markers GFAP and S100-β; and oligodendrocyte markers O4, GSTπ and RIP (data not shown).

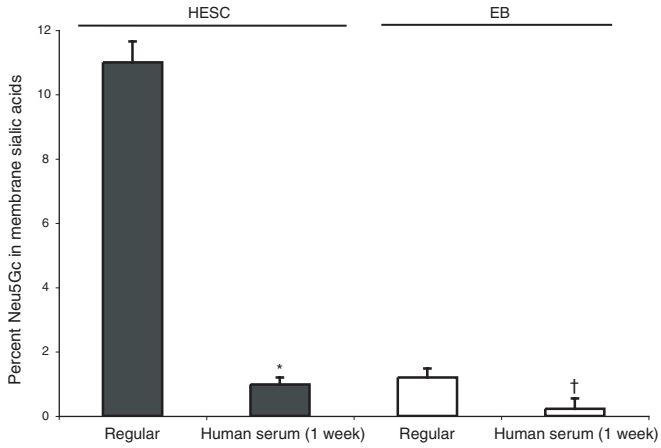

Figure 3 Effect of growth in NHS on Neu5Gc content of HESC and embryoid bodies. HESC or embryoid bodies were grown in NHS instead of the standard serum replacement. Membrane-bound sialic acids were studied for percentage of Neu5Gc as described in the **Fig. 1b** legend. Data represent the mean of two different experiments (mean ± s.d.). $*P < 0.005$, $\dagger P < 0.01$.

the amount of Neu5Gc on the embryoid body membranes increased (from 0.34 ± 0.06 to 0.40 ± 0.10 pmol/μg protein). In contrast, continued incubation in NHS further reduced Neu5Gc levels to 0.047 ± 0.06 pmol/μg protein.

Natural human anti-Neu5Gc antibodies bind to HESC

Healthy humans have variable levels of 'natural' circulating antibodies to Neu5Gc[13]. We asked whether such antibodies could recognize Neu5Gc-containing epitopes on HESC grown under standard conditions. Cells exposed to a high-level anti-Neu5Gc antibody–containing human serum (Hi-GcAbHS) showed human immunoglobulin G (IgG) binding (**Fig. 4**). In contrast, staining of cells exposed to a low-level anti-Neu5Gc antibody–containing human serum (Lo-GcAbHS) was almost similar to that of nonexposed controls. Antibody deposition was related to the amount of Neu5Gc on the HESC, because cells growing in NHS did not show any IgG binding when exposed to the same Hi-GcAbHS (**Fig. 4**).

Cell-surface antibody deposition can activate the classical complement pathway, eventually leading to killing or phagocytosis. We asked whether complement C3b deposition occurred after exposure to Hi-GcAbHS. As before, we show the data gated for EGFP + HESC. When HESC were grown under the standard conditions, 37% were positive for C3b (**Fig. 5c**; compare to 0% background in **Fig. 5a**). Only 22% of the cells were positive after exposure to Lo-GcAbHS, with actual levels on individual cells being much lower (**Fig. 5b**). When HESC were grown for 5 d in medium containing NHS, C3b positivity after exposure to Hi-GcAbHS dropped to 13% (**Fig. 5d**). These data are consistent with deposition of anti-human IgG under the same conditions (**Fig. 4**) and also with the substantial reduction in Neu5Gc on the HESC after incubation in medium containing human serum (**Fig. 3**).

Such binding of antibody and complement to HESC would target them for death *in vivo*, through recognition by macrophages and natural killer cells. Regardless, attempts were made to directly determine antibody:complement-mediated cytolysis on HESC *in vitro*. The standard single-cell suspension required for such analyses caused extensive cell death even under control conditions (without serum). Exposure to Hi-GcAbHS caused increased death above background levels seen with NHS, from 40% to 60–70%. In contrast, the percentage of dead HESC after exposure to Lo-GcAbHS was similar to that of the control. When the assay was performed directly on the culture dish for shorter time, more HESC remained alive. Cell death with Hi-GcAbHS was higher than that of the control (14% versus 10%), whereas the death rate after exposure to Lo-GcAbHS remained unchanged.

DISCUSSION

HESC and embryoid bodies can incorporate the nonhuman sialic acid Neu5Gc from the mouse feeder layer and/or the medium, leading to an immune response mediated by natural antibodies to Neu5Gc present in most humans. In effect, HESC seem like animal cells to the human immune system. Pooled, heat-inactivated human serum selected for low titers of antibodies specific for Neu5Gc could be substituted for the traditional animal serum or serum replacement, supporting the undifferentiated growth of HESC. This approach markedly reduced the immune response, by reducing the Neu5Gc content on the HESC.

Most existing HESC lines (including all those that are currently approved for study under federal funding in the United States) have been grown on or derived from mouse feeder layer[10]. Standard culture conditions also include animal serum, or a serum replacement. We show here that the commercial serum replacement is also a rich source of Neu5Gc, and both HESC and embryoid bodies are able to incorporate it. The composition of this serum replacement is under an international patent (WO 98/30679), and the formulation includes proteins like transferrin, which are likely to be from animal sources and therefore, would carry Neu5Gc. Human orthologs or recombinant proteins synthesized in bacteria could be used instead.

Many efforts have been recently made to eliminate these animal-derived components[17]. The use of a feeder-free system, such as Matrigel or other components of the extracellular matrices, have been

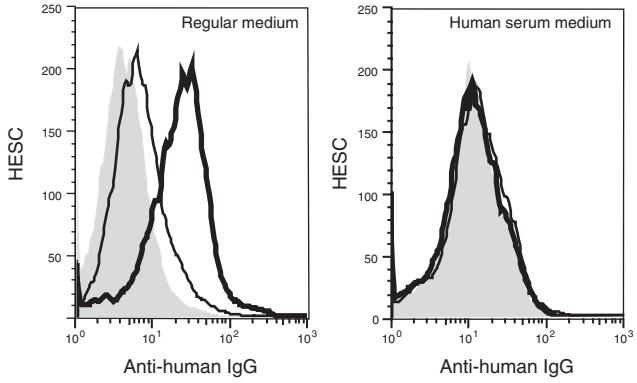

Figure 4 Binding of 'natural' antibodies from sera of normal human donors to HESC. HESC were grown in regular medium or in NHS as in **Fig. 3** for 5 d. The cells were released with 2mM EDTA and then exposed to a normal serum from a human with a high level of anti-Neu5Gc antibodies (thick line) or from another individual with a low level of such antibodies (thin line) for 55 min, then stained with a secondary goat anti-human IgG conjugated to Alexa 594, and studied by flow cytometry, with gating on the EGFP-positive HESC. The gray shaded plot shows the result with secondary antibody alone. Immunoglobulin deposition was markedly reduced when cells were first grown in NHS-containing medium for 5 d (although nonspecific background levels were increased, see lower panel). The somewhat higher background seen when HESC were grown in NHS-containing medium could be due to a nonspecific IgG absorption, but it had no major consequences, such as complement deposition (see **Fig. 5**).

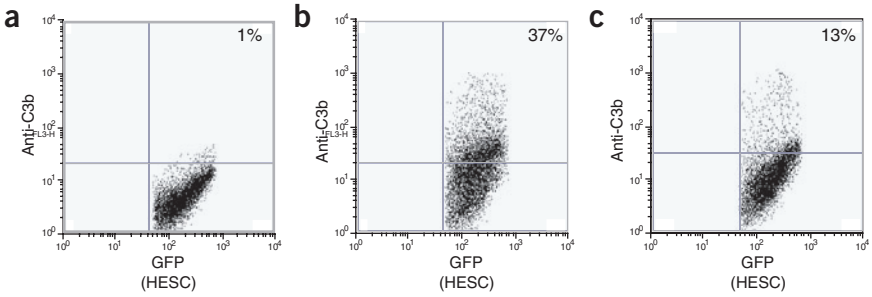

Figure 5 Binding of complement C3b from human sera to HESC. HESC were grown in regular medium or in NHS-containing medium for 5 d and harvested with 2 mM EDTA as in Figure 3. The cells were then exposed to a normal human serum from an individual with a high level of anti-Neu5Gc antibodies for 15 min at 37 °C, and also to the serum from an individual with a low level of anti-Neu5Gc antibodies. Deposited C3b was detected using a goat anti-human C3b, then stained with a goat anti-human C3b conjugated to Alexa 594 and studied by flow cytometry. (**a**) Control cells that were not exposed to the any human sera showed no positive staining for C3b. In the case of the cells that were exposed to the serum with a low level of anti-Neu5Gc antibodies, only 22% of the EGFP-positive cells were positive for human C3b (data not shown). Note that the levels appear much lower because the *y* axis is on a log scale. After exposure to the high level of anti-Neu5Gc antibodies serum, the double fluorescence plot shows that 37% of the EGFP-positive HESC grown in regular medium showed positive staining for human C3b (**b**), whereas only 13% of the EGFP-positive cells grown in medium containing human serum were positive for C3b (**c**).

explored[6,18,19]. But feeder-free conditions seem to facilitate *in vitro* evolution of HESC, selecting for aneuploid cells[20]. Moreover, many of the medium and matrix components are still from animal sources and contamination with Neu5Gc can be expected.

Human feeders of different origins have also been tried[21–23]. Successful derivation and culture of some HESC lines in the complete absence of nonhuman components, using feeder layers from human tissues with human serum and supplements, further showing the ability to develop teratomas (*i.e.*, confirming the maintenance of pluripotentiality)[15,16] was recently reported. Although it was not the primary aim of our work, we also noted that human serum did not cause any change in the undifferentiated state of the HESC. Others have also tried similar xeno-free techniques on hematopoietic stem cells by growing them on human stromal cells and using medium containing human blood type AB serum[24]. Of course, the use of an 'all-human' environment carries a different set of risks (unexpected contamination with novel or newly emerging pathogens).

There are also potential implications for the incorporation of Neu5Gc with regard to general HESC biology. Many characteristic markers of HESC (SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81)[4] are glycolipids or glycoproteins, many of which can carry sialic acids. SSEA-4, which is highly expressed even in long-term cultures of HESC[9], is the sialylated form of the globo-series glycolipid SSEA-3 (Gb5). TRA-1-60 is a sialylated keratan sulfate protein and the TRA-1-81 epitope became accessible only after sialidase treatment[25]. Neural lineage cells derived from embryoid bodies express the polysialylated form of NCAM[26], as well as the antigen A2B5 (ref. 27), which corresponds to polysialylated gangliosides[28]. Because sialic acids are involved in self-recognition events, the presence of Neu5Gc instead of Neu5Ac could lead to unexpected impairments of cell function and tissue development[12].

Another possible solution is growth in heat-inactivated serum from the actual patient who is going to receive the therapy. Similar alternatives have been suggested for hematopoietic stem cells[24]. Even if the patient serum contains anti-Neu5Gc antibodies, heat inactivation could prevent complement activation, until such time as the preexisting Neu5Gc in the HESC is metabolically eliminated by the Neu5Ac in the serum. An added advantage to this approach is that it would screen for allogeneic cytotoxic antibodies in the recipient's serum.

None of these approaches guarantees the complete elimination of Neu5Gc (or any other unknown animal antigen or pathogen) from existing cultures. Therefore it would seem safest to start over again with newly derived HESC that have never been exposed to any animal products containing Neu5Gc (and ideally, only ever exposed to serum from the intended transplant recipient). The current regulatory climate in the United States precludes this type of approach, when using federal grant dollars[29,30].

METHODS

Culture of HESC and embryoid bodies. The H1 embryonic stem cell line (WiCell Research Institute, Inc.) cells were cultured on mitotically inactivated (mitomycin C–treated) mouse embryonic fibroblasts (MEF, Specialty media) in DMEM/F12 Glutamax (Gibco), 20% KnockOut serum replacement (Gibco) or pooled human blood type AB serum (Pel-Freeze), 0.1 mM nonessential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Gibco), and 4 ng/ml βFGF-2 (R&D Systems). For embryoid body culture, H1 embryonic stem cells were grown in suspension for 7–10 d, using the same medium without FGF-2 and 10% serum. Cells were moved to a new dish every day to eliminate eventual fibroblast contamination.

HESC transfection. H1 HESC were stably transfected to express green fluorescent protein (GFP) by CAG-EGFP self-inactivating lentivirus infection. The self-inactivating lentiviral vector expressing EGFP under control of the CAG promoter was derived from a multiply attenuated HIV vector system, but included a U3 deletion and introduction of a cPPT element. Vectors were produced by triple transfection of 293 cells followed by ultracentrifugation and titration as previously described[31]. Undifferentiated cells were exposed to the virus at a titer of 0.5×10^{10} gene transfer unit (gtu)/ml for 1 h followed by a 2-d recovery period. EGFP was detected by native fluorescence at day 3 after transduction. Cells expressing EGFP were FACS sorted for uniform EGFP expression. We observed no loss in EGFP expression during propagation or embryoid body differentiation and up to 10 months after transduction. The EGFP-positive cells derived from these colonies are thus polyclonal in origin. The GFP-positive embryonic stem cells maintain a similar phenotype to the wild-type cells (SSEA-4, SSEA-3 and Oct4-positive).

Oct-4 antibody (1:500) was from Santa Cruz, the other marker antibodies (SSEA-3, TRA-1-60, alkaline phosphatase and nestin) were from Chemicon (dilution, 1:100), and the secondary Cy3 antibody from Sigma (dilution, 1:250). We measured alkaline phosphatase activity using the Vector Red Alkaline Phosphatase Substrate Kit I from Vector Laboratories.

Human sera. We obtained sera from several healthy human donors after written consent and Institutional Review Board approval, and anonymously numbered them before further use. We determined levels of antibody specific for Neu5Gc in several serum samples as described elsewhere[13]. Two specific sera, corresponding to the lowest and highest extremes of the range, were selected for the experiments. Another serum with a high level of antibodies specific for Neu5Gc was also tried with identical results to those presented in the figures.

Determination of Neu5Gc content. Sialic acids from HESC, feeder layer cells, embryoid bodies or culture medium were released by mild acid, derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB) and analyzed by high-performance liquid chromatography to determine the percentage of Neu5Gc in total sialic acids[13].

Flow cytometry. Cells were harvested into 2mM EDTA in phosphate-buffered saline (PBS) and washed with PBS. We incubated 1×10^5 cells with a chicken anti-Neu5Gc (1.5μg/100 μl) and stained them with a donkey anti-chicken IgY conjugated to Cy5 (Jackson; dilution, 1:100 in PBS). Neu5Gc-specific antibody binding was partially blocked by coincubation with 1% chimpanzee serum, which (unlike human serum) is rich in Neu5Gc.

For human serum antibody deposition studies, we harvested HESC and exposed them to individual human sera. Human IgGs deposited on the cells were stained with an anti-human IgG conjugated to Alexa 594 (Molecular Probes; dilution, 1:100 in PBS). For C3b deposition, we exposed HESC to human serum, then incubated them with a goat anti-human C3b (Fitzgerald; dilution, 1:100 in PBS) and finally stained them with an anti-goat IgG conjugated to Alexa 594 as above.

Cytotoxicity assays. A standard procedure for testing antibody:complement-mediated cytotoxicity after exposure to human sera was followed. We harvested HESC as described and resuspended them in GVB^{2+} buffer (Sigma) alone (control) or in GVB^{2+} containing 25% human serum. We incubated cells for 2 h at 37 °C and gently shook them. Dead cells were stained with propidium iodide (5 μg/ml) and analyzed by FACS. For cytotoxicity assays on the plate, cells were exposed to serum-free HESC culture medium containing 25% of the test human sera. After 30 min at 37 °C, we harvested and stained them with propidium iodide.

Statistical analysis. Sialic acid content from at least two experiments run in duplicate was analyzed using the T test in Microsoft Excel. Data are expressed as mean ± s.d.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. D'Amour, K. & Gage, F. H. New tools for human developmental biology. *Nat. Biotechnol.* **18**, 381–382 (2000).
2. Keller, G. & Snodgrass, H. R. Human embryonic stem cells: the future is now. *Nat. Med.* **5**, 151–152 (1999).
3. Bradley, J. A., Bolton, E. M. & Pedersen, R. A. Stem cell medicine encounters the immune system. *Nat. Rev. Immunol.* **2**, 859–871 (2002).
4. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
5. Amit, M. *et al.* Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* **227**, 271–278 (2000).
6. Draper, J. S., Moore, H. D., Ruban, L. N., Gokhale, P. J. & Andrews, P. W. Culture and characterization of human embryonic stem cells. *Stem Cells Dev.* **13**, 325–336 (2004).
7. Koivisto, H. et al. Cultures of human embryonic stem cells: serum replacement medium or serum-containing media and the effect of basic fibroblast growth factor. *Reprod Biomed Online* **9**, 330-337 (2004).
8. Itskovitz-Eldor, J. *et al.* Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol. Med.* **6**, 88–95 (2000).
9. Carpenter, M. K., Rosler, E. & Rao, M. S. Characterization and differentiation of human embryonic stem cells. *Cloning Stem Cells* **5**, 79–88 (2003).
10. Conley, B. J., Young, J. C., Trounson, A. O. & Mollard, R. Derivation, propagation and differentiation of human embryonic stem cells. *Int. J. Biochem. Cell Biol.* **36**, 555–567 (2004).
11. Chou, H. H. *et al.* A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence. *Proc. Natl. Acad. Sci. USA* **95**, 11751–11756 (1998).
12. Varki, A. Loss of N-glycolylneuraminic acid in humans: mechanisms, consequences and implications for hominid evolution. *Yearb. Phys. Anthropol.* **44**, 54–69 (2002).
13. Tangvoranuntakul, P. *et al.* Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc. Natl. Acad. Sci. USA* **100**, 12045–12050 (2003).
14. Bardor, M., Nguyen, D.H., Diaz, S. & Varki, A. Mechanism of uptake and incorporation of the non-human sialic acid N-glycolneuraminic acid into human cells. *J. Biol. Chem.* published online 29 November 2004 <http://www.jbc.org/cgi/reprint/M412040200v2>
15. Richards, M., Fong, C. Y., Chan, W. K., Wong, P. C. & Bongso, A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat. Biotechnol.* **20**, 933–936 (2002).
16. Richards, M. *et al.* Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells* **21**, 546–556 (2003).
17. Nakashima, K., Colamarino, S. & Gage, F. H. Embryonic stem cells: staying plastic on plastic. *Nat. Med.* **10**, 23–24 (2004).
18. Rosler, E. S. *et al.* Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev. Dyn.* **229**, 259–274 (2004).
19. Amit, M., Shariki, C., Margulets, V. & Itskovitz-Eldor, J. Feeder layer- and serum-free culture of human embryonic stem cells. *Biol. Reprod.* **70**, 837–845 (2004).
20. Draper, J. S. *et al.* Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat. Biotechnol.* **22**, 53–54 (2004).
21. Cheng, L., Hammond, H., Ye, Z., Zhan, X. & Dravid, G. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cells* **21**, 131–142 (2003).
22. Hovatta, O. *et al.* A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Hum. Reprod.* **18**, 1404–1409 (2003).
23. Miyamoto, K. *et al.* Human placenta feeder layers support undifferentiated growth of primate embryonic stem cells. *Stem Cells* **22**, 433–440 (2004).
24. Yamaguchi, M. *et al.* Bone marrow stromal cells prepared using AB serum and bFGF for hematopoietic stem cells expansion. *Transfusion* **42**, 921–927 (2002).
25. Badcock, G., Pigott, C., Goepel, J. & Andrews, P. W. The human embryonal carcinoma marker antigen TRA-1-60 is a sialylated keratan sulfate proteoglycan. *Cancer Res.* **59**, 4715–4719 (1999).
26. Zhang, S. C., Wernig, M., Duncan, I. D., Brustle, O. & Thomson, J. A. *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* **19**, 1129–133 (2001).
27. Reubinoff, B. E. *et al.* Neural progenitors from human embryonic stem cells. *Nat. Biotechnol.* **19**, 1134–1140 (2001).
28. Dubois, C., Manuguerra, J.-C., Hauttecoeur, B. & Maze, J. Monoclonal antibody A2B5, which detects cell surface antigens, binds to ganglioside GT3 (II3 (NeuAc)3LacCer) and to its 9-O-acetylated derivative. *J. Biol. Chem.* **265**, 2797–2803 (1990).
29. Cowan, C. A. *et al.* Derivation of embryonic stem-cell lines from human blastocysts. *N. Engl. J. Med.* **350**, 1353–1356 (2004).
30. Gearhart, J. New human embryonic stem-cell lines—more is better. *N. Engl. J. Med.* **350**, 1275–1276 (2004).
31. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L. & Trono, D. Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nat. Biotechnol.* **15**, 871–875 (1997).