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Ablation of Undifferentiated Human Embryonic Stem Cells: Exploiting Innate Immunity Against the Gal α1-3Galβ1-4GlcNAc-R (α-Gal) Epitope

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Key Words. Regenerative medicine • Selective ablation • Human embryonic stem cells • Stem cell therapy Complement-mediated lysis • Gal α1,3-galactosyltransferase

ABSTRACT

Although undifferentiated human embryonic stem cells (hESCs) are tumorigenic, this capacity is lost after differentiation, and hESCs are being widely investigated for applications in regenerative medicine. To engineer protection against the unintentional transplantation of undifferentiated cells, we generated hESCs carrying a construct in which the α1,3-galactosyltransferase (*GalT*) open reading frame was transcribed from the *hTERT* promoter (*pmGT*). Because the endogenous *GalT* gene is inactive, *GalT* expression was limited to undifferentiated cells. A second chimeric construct (*pmfGT*) differed by replacement of the GalT leader sequence for that of the fucosyltransferase gene. Two subclones containing stable integrations of *pmGT* and *pmfGT* (M2 and F11, respectively) were assessed for their response to human serum containing antibodies to the Galα1-3Galβ1-4GlcNAc-R (α-gal) epitope. The low-variegation line, M2, and to a lesser extent the more variegated line F11, were sensitive to human serum when exposed in the undifferentiated state. However, M2 cells were largely insensitive after differentiation and retained both a normal karyotype and the ability to differentiate into derivatives of the three germ layers in severe combined immunodeficient mice. These data exemplify a method of protection against residual, undifferentiated hESCs prior to engraftment and may provide ongoing immune surveillance after engraftment against dedifferentiation or against de novo tumorigenesis involving *hTERT* reactivation. Untransfected H9 cells were not sensitive to the human serum used in this study. Hence, in our system, interactions of hESCs with other circulating antibodies, such as anti-Neu5Gc, were not observed. STEM CELLS *2007;25:10–18*

INTRODUCTION

ESCs have the dual properties of self-renewal and pluripotency. The potential to give rise to diverse cell types raises the prospect of their use in regenerative medicine. However, self-renewal is also associated with tumorigenicity, and undifferentiated mouse ESCs and human ESCs (hESCs) give rise to teratomas when transplanted ectopically in immunocompromised severe combined immunodeficient (SCID) mice. Although benign, teratomas or other forms of cellular overgrowth still constitute a health risk and could contribute to functional failure of the graft. Differentiated ESCs are not known to be tumorigenic, but the formal risk of transplantation of residual populations of undifferentiated ESCs must be addressed. This problem has previously been tackled by the generation of hESCs expressing a suicide gene that renders cells sensitive to administration of ganciclovir [1]. This is a very promising approach that, in addition to tumorigenesis per se, offers the opportunity to remove grafts that simply overproduce factors, a suggested reason for the functional failure of some fetal grafts in patients with Parkinson disease [2]. However, this strategy eliminates all cells of the graft in response to ganciclovir, thus negating any benefit from the procedure and requiring additional intervention. We have developed an alternative strategy that allows for the elimination of undifferentiated cells prior to engraftment and may offer continuing immune surveillance against undifferentiated or dedifferentiated cells within the graft.

The organs of nonprimate mammals transplanted into humans are rejected within a few hours or minutes because of the presence of α-gal epitopes. Human serum normally contains high levels of circulating antibody to these epitopes [3, 4], due to a fixed stop mutation at the human α1,3-galactosyltransferase (GalT) locus (the product of which catalyzes α-gal presentation), rendering us immunoreactive to dietary and gut flora sources of the epitope. We have engineered hESCs that express a functional copy of the *GalT* gene under the transcriptional control of the human telomerase reverse transcriptase *(hTERT*) promoter (Fig. 1A), normally active only in blastomeres, undifferentiated ESCs, and germ cells [5]. We show that exposure of undifferentiated cells to active human serum leads to cell death in vitro. However, when exposure of the same cell line follows a period of differentiation, the majority of hESC-derived cells are resistant. These data demonstrate an efficient method for removal of undifferentiated cells prior to engraftment and suggest that ongoing in vivo surveillance against residual undifferentiated cells may be effective in their continued elimination.

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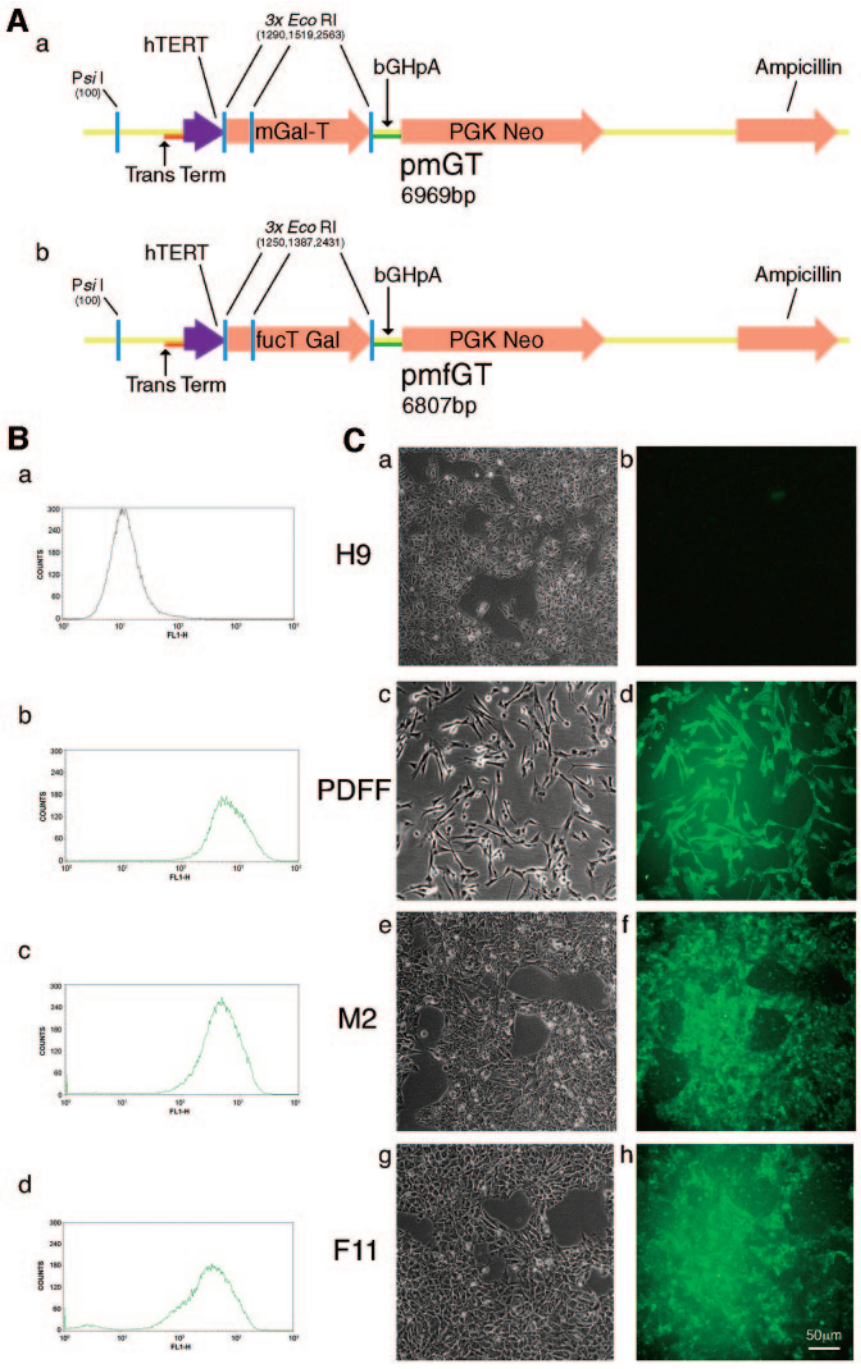

Figure 1. GalT construct design and expression. **(A):** Schematic representation of constructs used to engineer H9 human embryonic stem cells. **(a):** The mouse *GalT* cDNA driven by the hTERT promoter (*pmGT*) and cloned upstream of the bovine growth hormone polyadenylation sequence (bGHpA). **(b):** The same construct containing a fucosyltransferase leader sequence (*pmfGT*). A transcriptional termination sequence (Trans Term) was cloned upstream of the hTERT promoter to prevent any upstream sequences from driving expression of the *GalT* cDNA. **(B):** Flow cytometry for the presence of α-gal using FITC-labeled *BS*-IB_4 isolectin: H9 **(a)**, PDFF **(b)**, M2 **(c)**, and F11 **(d)**. Ninety-five point four percent of the cells in the M2 cell line expressed the α-gal epitope, whereas only 84.74% of the cells expressed in the more variegated F11 population. **(C):** *BS*-IB_4 staining for the presence of α-gal. **(a, c, e, g):** Phase-contrast images taken after staining with the *BS*-IB_4 at 4°C for 90 minutes in a phosphate-buffered saline-based buffer. **(b, d, f, h):** The corresponding FITC-fluorescence images. All images were taken using a ×10 objective. There was no apparent α-gal expression on H9 cells **(a, b)**, strong expression of α-gal on PDFF cells **(c, d)** and M2 cells **(e, f)**, and variegated expression on F11 cells **(g, h)**. Scale bar = 50 μm. Note that cell morphology in the phase-contrast images is affected by incubation with lectin at 4°C for 90 minutes. Abbreviations: *BS*-IB_4, *Bandeiraea simplicifolia* Isolectin B_4; FITC, fluorescein isothiocyanate; GalT, α1,3-galactosyltransferase; hTERT, human telomerase reverse transcriptase.

Materials and Methods

Constructs

Mouse kidneys were used as a source of *GalT* mRNA. Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and was reverse-transcribed using the Superscript system (Invitrogen) with oligo(dT) for first-stand synthesis. The *GalT* coding sequence was amplified using 5′-ggcctgtactacatttgcctgga-3′ and 5′-gaaatagtgtcaagtttccatcacaa-3′ as 5′- and 3′-primers, respectively. The resulting coding sequence was designated mGT. A fucosyltransferase (Fuc T) leader sequence was engineered at the N terminus of mGT prior to the transmembrane domain, following Osman et al. [6], and the resulting coding sequence was designated mfGT. Polymerase chain reaction was performed on the mGT cDNA using the above 3′-primer and 5′-cgatgtggctgcggagccaccggcaggtaatcctgttgatgctgattgtctcaac-3′ as a 5′-primer to produce the mfGT coding sequence. An expression vector (HPV40) was built using a minimal (200 base pair [bp]) *hTERT* promoter and upstream transcription termination signal (kind gift of Geron Corporation, Menlo Park, CA, http://www.geron.com) followed by a multiple cloning site for cDNAs and a bovine growth hormone polyadenylation signal (kind gift of Geron Corporation). To provide selection in eukaryotic cells, a cassette comprising a PGK promoter, neomycin-resistance gene, and polyadenylation signal was incorporated into the expression vector (kind gift of E. Gallagher, Roslin Institute, Roslin, Midlothian, U.K.). Both the mGT and mfGT coding sequences were subcloned into the multiple cloning site of HPV40 to allow their expression under the control of the *hTERT* promoter.

Cell Culture and Transfection

H9 hESCs were a generous gift from Geron Corporation. hESCs were cultured as described previously by Xu et al. [7] on growth factor-reduced Matrigel substrate (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) in mouse embryonic fibroblast-conditioned hESC medium (knockout Dulbecco's modified Eagle's medium [DMEM] and 20% knockout serum replacement supplemented with 2 mM L-glutamine, 1× nonessential amino acids, and 100 μM β-mercaptoethanol; Invitrogen) with 4 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). The cells were passaged using TEG (92.7 mM NaCl, 0.845 mM Na_2HPO_4, 1.58 mM KH_2PO_4, 4.46 mM KCl, 5 mM D-glucose, 22.28 mM Tris-HCl, 0.0009% phenol red, 0.25% trypsin, 1.05 mM EGTA, and 0.000105% polyvinylalcohol).

Exponentially growing cells were used for transfection as previously described [8]. They were rendered into a single-cell suspension by treatment with TEG and swollen in hypoosmolar buffer (Eppendorf, Hamburg, Germany, http://www.eppendorf.com) at room temperature for 20 minutes. One $\times$ 10^6 cells were then electroporated with 50 μg of linearized mGT or mfGT expression vector in a volume of 800 μl of hypoosmolar buffer in a 0.4-cm gap cuvette (Bio-Rad, Hercules, CA, http://www.bio-rad.com) and pulsed at 300 V for 100 μS in a Multiporator (Eppendorf). Cells were left at room temperature for 10 minutes and then plated onto a matrigelled 15-cm plate in conditioned hESC medium. After 48 hours, 100 μg/ml G418 was applied and maintained until colonies appeared after 10 days of selection. Colonies were physically picked up with a micropipetter into 48-well plates and expanded.

Fluorescence Microscopy for the Cell Surface α-Gal Epitope

Unfixed cells in a tissue culture well were stained with fluorescein isothiocyanate (FITC)-conjugated *Bandeiraea simplicifolia* Isolectin B_4 (*BS*-IB_4) (Sigma-Aldrich), which specifically binds the α-gal epitope [9]. Cells were washed with phosphate-buffered saline (PBS) (with Ca^{2+} and Mg^{2+}) and incubated at 4°C for 90 minutes in 5 μg/ml *BS*-IB_4-FITC in PBS (with Ca^{2+} and Mg^{2+}) with 10% newborn calf serum. Cells were then washed twice for 15 minutes with PBS at room temperature and analyzed in PBS in the tissue culture well with a fluorescence microscope.

Karyotyping

Exponentially growing cultures were arrested in metaphase using Karyomax colcemid solution (Invitrogen) at 100 ng/ml for 2 hours at 37°C. Cells were disaggregated by treatment with TEG, pelleted at 200*g*, and resuspended in 0.56% potassium chloride for 10 minutes at room temperature. Cells were pelleted and resuspended in a fixative of three parts methanol, one part acetic acid. Cells were then washed twice with fixative by pelleting and resuspending. Cells in fixative were dropped onto a slide and dried for 3 days. Resulting chromosome spreads were washed in ×2 SSC at 60°C for 2 hours and partially digested with 0.01% Bacto trypsin (Difco Labs, Oxford, U.K., http://www.bdbiosciences.com) before being stained with 5% Gurr's improved Giemsa R66 (BDH, Lutterworth, U.K., http://www.vwr.com) in Gurr's phosphate buffer.

In Vitro Differentiation of hESCs

hESCs were disaggregated with TEG and seeded at high density into a nonadherent bacterial Petri dish in conditioned hESC medium. After 2 days, the medium was changed to differentiation medium (knockout DMEM [Invitrogen], 10% FBS [Globepharm, Surrey, U.K.], 2 mM L-glutamine [Invitrogen], 1× nonessential amino acids [Invitrogen], and 100 μM β-mercaptoethanol [Invitrogen]). After a further 5 days of suspension culture, the resulting aggregates (embryoid bodies) were plated onto gelatin in differentiation medium and allowed to reattach to the culture surface. Differentiation was allowed to proceed for a further 2 weeks. Alternatively, hESCs were spontaneously differentiated as monolayers by removing conditioned hESC medium and bFGF and replacing it with differentiation medium, as described above, for a specified time scale (0–22 days).

Neural Differentiation of hESCs

hESCs were differentiated into neural progenitors in accordance with the protocol of Gerrard et al. [10]. Briefly, hESCs at near confluence were passaged with 0.5 mM EDTA/PBS at a ratio of 1:5 onto tissue culture plastic coated with poly(L-lysine)/laminin (Sigma-Aldrich). These cells were then cultured in N2B27 medium (1:1 mix of DMEM/F-12 supplemented with N2 and Neurobasal medium supplemented with B27 all from Invitrogen) in the presence of 100 ng/ml mouse recombinant noggin (R&D Systems, Inc., Minneapolis, http://www.rndsystems.com) for 3 weeks [10].

Immunofluorescence

In all cases, differentiated cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) at room temperature for 20 minutes. When intracellular staining was performed, cells were washed a further two times with PBS and permeabilized with absolute ethanol at room temperature for 2 minutes, followed by a further two PBS washes. Nonspecific protein binding was blocked with 10% normal goat serum for 1 hour at room temperature. Primary antibodies were bound to their antigens in PBS with 1% normal goat serum plus 0.02% Triton-X for 1–2 hours at room temperature. Antibody dilutions were as follows: monoclonal anti-β-tubulin III (Sigma-Aldrich) at a 1:200 dilution, monoclonal muscle-specific actin (Dako Cytomation, Glostrup, Denmark, http://www.dakocytomation.com) at a 1:50 dilution, monoclonal anti-α-fetoprotein (Sigma-Aldrich) at a 1:500 dilution, monoclonal anti-human specific nestin (Chemicon International, Temecula, CA, http://www.chemicon.com) at a 1:200 dilution, and polyclonal anti-human musashi (Chemicon International) at a 1:200 dilution. Unbound antibody was removed by three 5-minute room temperature washes with PBS. FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich), Alexa Fluor 568-conjugated goat anti-mouse IgG, and Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen) were used as secondary antibodies at dilutions of 1:200 (Sigma-Aldrich) and 1:400 (Invitrogen) in PBS with 1% normal goat serum incubated at room temperature for 30 minutes, and unbound antibody was washed away as before. Coverslips were mounted in Vectashield with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and viewed with a fluorescence microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Formation of Tumors in SCID Mice and Histological Analysis

SCID mice were obtained from Harlan UK Limited (Bicester, Oxon, U.K., http://www.harlanseralab.co.uk) and maintained in a sterile environment. hESCs for injection into SCID mice were disaggregated by treatment with TEG, washed once in PBS, and resuspended in PBS at 1 $\times$ 10^8 cells per milliliter. A 100-μl aliquot was injected into the leg muscle of each of three SCID mice.

Three to five months later, the mice were sacrificed, and the tumors were removed. Tumors were fixed whole or, if very large, cut into segments. Fixation was by treatment with 4% PFA for 20 minutes at room temperature. Tumors were embedded in paraffin wax in an automated system using a Shandon Hypercenter XP processor (Shandon Scientific UK Ltd., http://www.thermo.com). Once embedded, tumors were cut into 10-μm sections using a Microm HM325 rotary microtome (MICROM International GmbH, Walldorf, Germany, http://www.microm-online.com) and stained using standard hematoxylin and eosin protocols. Tumor sections were analyzed and photographed by Dr. David Brownstein (University of Edinburgh, Scotland, U.K.).

Lysis of Cells with Human Serum

Human sera from healthy screened volunteers of blood group A was either purchased from Harlan Sera-Lab Limited (Loughborough, Leicestershire, U.K., http://www.harlanseralab.co.uk) as a pooled sample or collected fresh and pooled from blood group A healthy

volunteers from the Roslin Institute. Blood group B serum was purchased from Harlan Sera-Lab Limited.

Where required, heat inactivation of complement in serum was achieved by heating to 56°C for 30 minutes. Cells were disaggregated with TEG, pelleted (200*g*), resuspended at 1×10^5 cells per 0.1 milliliter per replicate in undiluted human serum, and incubated for 1 hour at 37°C. Experiments were performed in triplicate. Disaggregation with TEG generated robust single-cell suspensions with, on average, 93% viability as determined by flow cytometry for propidium iodide (PI) inclusion (data not shown).

Calcein-Release Assay

A calcein-release assay was used to measure cell lysis, based upon the methods of Spiller [11] and Iwanowicz et al. [12]. Calcein AM (Invitrogen) is weakly fluorescent and nonpolar and is readily taken up into cells where the acetoxymethyl ester group is cleaved, leaving fluorescent, polar calcein trapped in the cell. Cells were incubated with Calcein AM in conditioned hESC medium at 40 μg/ml for 2 hours at 37°C to load the cells. For lysis in suspension, all pelleting of cells was performed at 260*g* to ensure a robust pellet and no carry-over of cells in the supernatant. One $\times 10^5$ cells were loaded with Calcein AM in a volume of 50 μl, and cells were washed with knockout DMEM prior to lysis in 100 μl of active or heat-inactivated serum for 1 hour at 37°C. As controls, cells were also incubated with either 100 μl of conditioned hESC medium as a measure of spontaneous release or 0.1% Triton X-100 as a measure of total release. A 50-μl sample of the supernatant after pelleting of unlysed cells was transferred to a black 96-well assay plate containing 50 μl of 0.2% Triton X-100. Cells were then resuspended in 100 μl of 0.1% Triton X-100 and incubated for 15 minutes at 37°C to lyse intact cells. A 50-μl sample of lysate was transferred to an assay plate as before. Fluorescence was measured using a Victor [2] plate reader (PerkinElmer Wallac, Boston, http://www.perkinelmer.com) with an excitation filter for 485 nm and an emission filter for 535 nm. Calcein release was calculated as the fluorescence in the serum lysate as a percentage of the fluorescence in the sum of the fluorescence in the serum lysate and Triton lysate.

RESULTS

Engineered hESCs Express *GalT*

Two constructs were generated expressing, respectively, the murine *GalT* (*pmGT*) and a chimeric *GalT* in which the leader sequence of *GalT* was exchanged for that of the Fuc T gene (*pmfGT*) (Fig. 1A). The latter construct was developed because the native *GalT* has been reported to compete poorly with Fuc T for location within the Golgi apparatus, thus conferring dominance on the Fuc T [6]. Both coding sequences were subcloned into an expression vector to come under the transcriptional control of the minimal *hTERT* promoter [5] and juxtaposed to a constitutive neomycin-resistance cassette. H9 hESCs [13] were transfected with pmGT and pmfGT yielding, respectively, 558 and 585 colonies, an overall transfection efficiency of 5.7×10^{-4}. Twenty-four colonies from each transfection were picked, expanded, and stained with FITC-conjugated *BS*-IB_4 that specifically binds the α-gal epitope [9]. The epitope was clearly detected on four of the mGT clones and eight of the mfGT clones. However, there was no obvious improvement in expression level associated with the use of the mfGT version of *GalT*, and all mfGT clones analyzed showed variegated patterns of expression.

One mGT clone, M2, was selected for further analysis due to its low level of variegation and was compared with a more variegated mfGT clone, F11. Near ubiquitous *GalT* expression in M2 cells and variegated expression in F11 cells is illustrated by lectin binding in Figure 1. *GalT* expression in M2 cells (Fig. 1Ce, 1Cf) was similar to that observed with a positive control ovine fetal fibroblast cell line (PDFF) (Fig. 1Cc, 1Cd), and consistent with their dysfunctional *GalT* genotype, H9 cells showed no detectable level of expression (Fig. 1Ca, 1Cb). Cytometric analysis of the same populations (Fig. 1B) confirmed that 95.40% of the cells in the M2 cell line expressed the α-gal epitope (mean fluorescence intensity of 478.29 compared with 615.27 for PDFF), whereas only 84.74% of the cells expressed in the more variegated F11 population (mean fluorescence intensity 395.96).

The specificity of isolectin *BS*-IB_4 binding was confirmed by costaining with anti-α-gal antibody. Flow cytometry analysis for costaining confirmed that 93.8% of M2 cells both expressed *GalT* and stained with the isolectin *BS*-IB_4 (supplemental online Fig. 1C). As expected, the variegated clone F11 showed two subpopulations, one costaining with both reagents and the other negative with both reagents. Few cells were stained for only one marker (supplemental online Fig. 1D), further confirming the specificity of the isolectin staining.

GalT-Expressing hESCs, but Not Nonexpressing hESCs, Are Lysed by Exposure to Human Serum

To investigate their sensitivity to human serum, M2, F11, positive control PDFF, and negative control parental hESCs (H9) were exposed to human serum for 1 hour. Cell death was quantified using a calcein-release assay [11, 12]. In initial experiments, we tested the capacity of serum from blood groups A and B to lyse M2 cells as monolayers (data not shown) and found that serum from blood group A led to higher levels of lysis than blood group B. This is consistent with previous reports that AB serum results in lower lysis of *GalT*-expressing cells [14, 15]. This is presumed to arise from cross-reactivity of anti-B and anti-GalT antibodies, due to homology between the B blood group antigen and the Gal epitope. We observed improved lysis when cells were exposed to serum as a suspension. This lysis was specific to active serum and did not result from the method of disaggregation. Flow cytometry for propidium iodide inclusion indicated that TEG generated a robust single-cell suspensions with, on average, 93% viability (data not shown). Therefore, in subsequent experiments, cells were exposed in suspension only to serum from blood group A.

Positive control ovine (PDFF) cells and M2 hESCs were both sensitive to lysis in active human serum, and for both cell types approximately 90% of total calcein was released in response to a 1-hour incubation with active human serum (Fig. 2A). H9 hESCs were insensitive to active human serum, as predicted by their lack of *GalT* activity, and F11 cells were intermediate in their level of lysis, which varied between experiments as indicated by the increase in standard deviation. This is consistent with their variegated pattern of transgene expression. Figure 2B shows H9, PDFF, M2, and F11 cells stained with Giemsa 24 hours after incubation with either active or heat-inactivated human serum. Both PDFF and M2 cultures exposed to active serum for 1 hour contained small residual numbers of surviving cells. However, there was an increase in the number of surviving cells observed by Giemsa staining in the F11 cultures after exposure to serum, confirming the results suggested by the calcein-release assay.

We next asked whether the progeny of surviving cells expressed *GalT*. Cells were exposed to active human serum for 1 hour, cultured for a further 7–10 days, and stained with the FITC-conjugated lectin *BS*-IB_4 (Fig. 3). After in vitro culture for 7–10 days, sporadic single colonies did emerge, suggesting that a single incubation was insufficient to eliminate all hESCs and that even a single cell may recover (Fig. 3Ae). Both PDFF and M2 cultures incubated with active serum contained surviving cells expressing *GalT* (Fig. 3). Hence, a 1-hour incubation was sufficient to kill most, but not all, undifferentiated M2 cells within a large population.

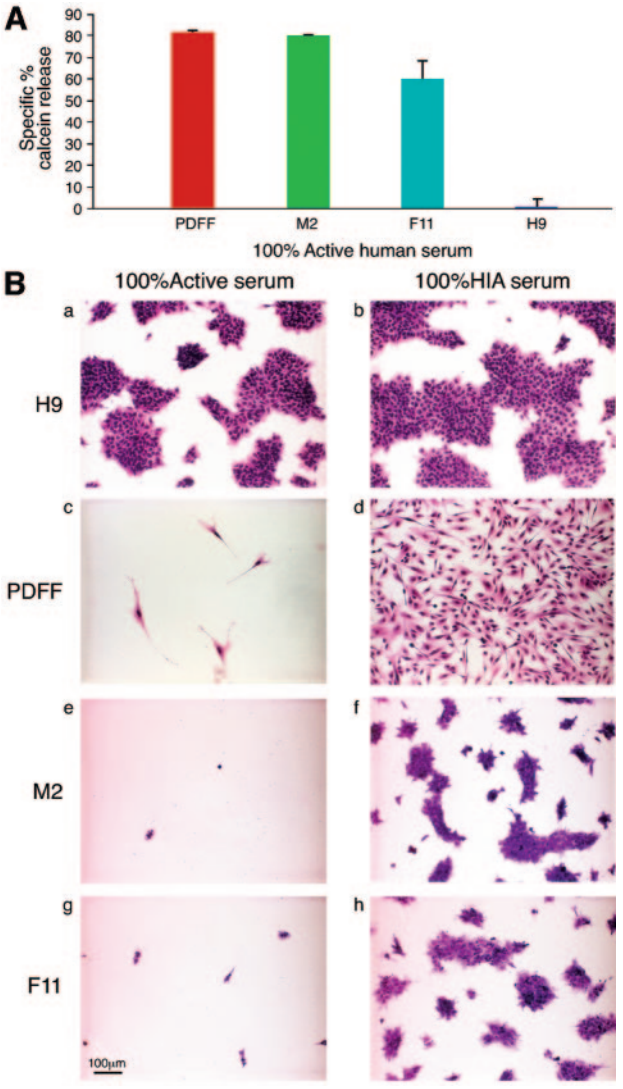

Figure 2. Cell survival following exposure to human serum. **(A):** Average Calcein AM release over three assays showing the cytolytic efficiency of undiluted active and heat-inactivated (HIA) human serum after a 1-hour incubation with PDFF (red), M2 (green), F11 (sky blue), and H9 (navy blue) cells. Cells were loaded with 40 μM Calcein AM for 2 hours at 37°C. Error bars represent the SEM. **(B):** Giemsa staining of cells 24 hours after a 1-hour exposure to active (left panels) and heat-inactivated (right panels) human serum. **(a)** and **(b)** show that H9 human embryonic stem cells (hESCs) are insensitive to both active and inactive human serum. **(c)** and **(d)** confirm that ovine cells (PDFF) are sensitive to active, but not inactivated, human serum. **(e)** and **(g)** demonstrate that hESCs engineered to express *GalT* behave like non-human cells and are sensitive to active human serum but not heat-inactivated serum **(f, h)**.

To determine whether survival of M2 cells occurred due to downregulation of the transgene, we compared the number of live cells after one or two exposures, of 1 hour each, to human serum. A double-exposure to human serum led to a doubling in nonspecific cell death, even in negative control H9 cells (Fig. 3B). However, the number of surviving M2 cells decreased fivefold from 1,500 with a single 1-hour exposure to 300 after a second 1-hour exposure. No such cell loss was observed with negative control H9 cells or M2 cells in heat-inactivated serum. Hence, surviving M2 cells remain sensitive to prolonged or repeated exposure to human serum. We conclude that M2 cells retain expression of the *GalT* transgene.

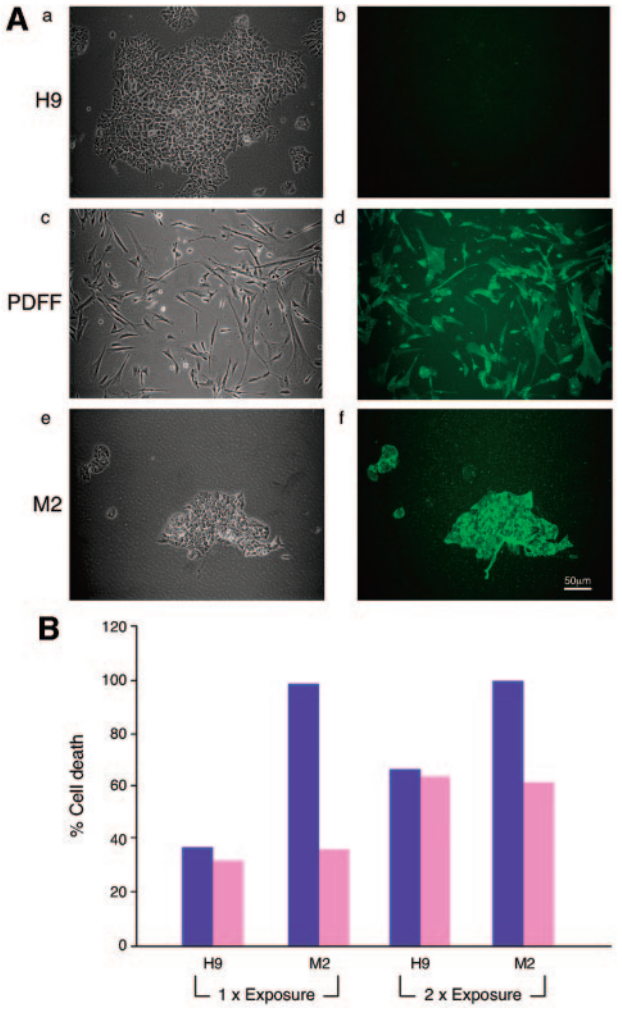

Figure 3. Characterization of surviving cells after 7–10 days in culture. **(A):** The level of cell survival 7–10 days after serum lysis, represented by *BS*-IB$_4$ staining for the presence of α-gal on H9 **(a, b)**, PDFF **(c, d)**, and M2 **(e, f)** cells. **(a)**, **(c)**, and **(e)** are phase-contrast images ($\times$10) indicating the level of cell survival after incubation with undiluted active human serum for 1 hour at 37°C, whereas **(b)**, **(d)**, and **(f)** represent fluorescein isothiocyanate-fluorescence microscopy showing that residual PDFF and M2 cells continue to express α-gal after incubation with active serum. Scale bars = 50 μm. **(B):** Numbers of surviving cells after single and double 1-hour incubations with human serum (mean of two replicates). Data are plotted as percentage of cell death in active (blue bars) and heat-inactivated (pink bars) human serum. Bars on the left are after a single 1-hour exposure, and righthand bars are after two consecutive 1-hour exposures.

Minority Populations of *GalT*-Expressing (M2) Cells Are Efficiently Lysed by Exposure to Human Serum in Mixed Populations

In the context of regenerative medicine, in which differentiated populations are engrafted, residual *GalT*-expressing cells would normally be small in number. To determine whether different proportions of *GalT*-expressing cells could be efficiently killed when cocultured with *GalT*-negative cells, we mixed varying numbers of M2 cells with HEK 293 cells that do not express *GalT*, exposed the mixture to active or inactive human serum, and then selected for surviving M2 cells in G418 for 7–10 days. A single G418-resistant colony was observed among three replicates when the proportion of M2 cells exposed to active human serum was less than 25%, although no colonies were observed

at proportions less than 1.6% (Fig. 4). At higher concentrations of M2 cells, small numbers of colonies were occasionally observed after lysis. It was not possible to count the number of surviving colonies in heat-inactive controls at these concentrations and therefore the frequencies of surviving ESC colonies provided in Figure 4 are overestimations based on a count greater than 500 colonies. These data indicated again that, at high cell concentrations, a single 1-hour incubation killed most, but not all, *GalT*-expressing cells.

GalT-Expressing (M2) Cells Downregulate *GalT* and Become Insensitive to Lysis After Differentiation

hESCs remain undifferentiated when cultured in mouse embryonic fibroblast-conditioned medium supplemented with bFGF. If these conditions are replaced with medium containing FBS and no bFGF, hESCs begin an unregulated process of differentiation. A critical feature of the *GalT* strategy is to achieve downregulation of the α-gal epitope on differentiated derivatives of M2 such that only undifferentiated cells are subject to complement-mediated lysis. To determine whether M2 cells downregulated *GalT* expression when they differentiated, we first cultured cells as monolayers in spontaneous differentiation conditions for 10 days and stained with *BS*-IB_4 (Fig. 5A). After 10 days in these conditions, M2 cells were seen to have begun downregulating *GalT* expression. Continuing expression may reflect the transient persistence of epitope after downregulation of transcription, the persistence of undifferentiated hESCs, or the possible retention of *hTERT* expression in some progenitor populations. There was no evidence of persistent expression on phenotypically differentiated neuronal progenitor cells after a directed differentiation protocol (Fig. 5Bb).

We then exposed M2-derived, differentiating cultures to active human serum at time points between 0 and 21 days after onset of differentiation. The level of specific complement-mediated lysis as measured by percentage of calcein release was significantly reduced over time: 85%, 62%, 25%, and 8% at 0, 7, 14, and 21 days of differentiation, respectively. These data are summarized in Figure 5D and correspond closely with the proportion of cells still expressing α-gal at similar time points, as determined by flow cytometry (Fig. 5C). Hence, at day 21 of differentiation, we observed 8% calcein release (Fig. 5D), and at day 22 of differentiation, 8.8% of cells were expressing α-gal (Fig. 5Cf). The M2 population in Figure 5Ca (green) can be seen as distinct from the parental H9 population (pale green), but with time the two populations gradually merge (Fig. 5Cf), indicating that by 22 days of differentiation, *GalT* expression is minimal in M2 cells. Figure 5C also indicates (red overlays) a similar pattern for the downregulation of an hESC-specific marker, Tra-1-81, with differentiation.

GalT-Engineered hESCs (M2) Are Karyotypically Normal and Have Normal Potentiality

Fifty chromosome spreads of M2 cells were examined at 10 passages after transfection and were shown to have a normal 46XX karyotype (data not shown). Cells at the same passage were injected intramuscularly into SCID mice and gave rise to broadly differentiated tumors (Fig. 6A). M2 cells differentiated in vitro in a similar manner to the parental H9 line and gave rise to cells staining for representative markers of all three germ layers (Fig. 6B). We conclude that there is no overt evidence of abnormality or restriction of potentiality.

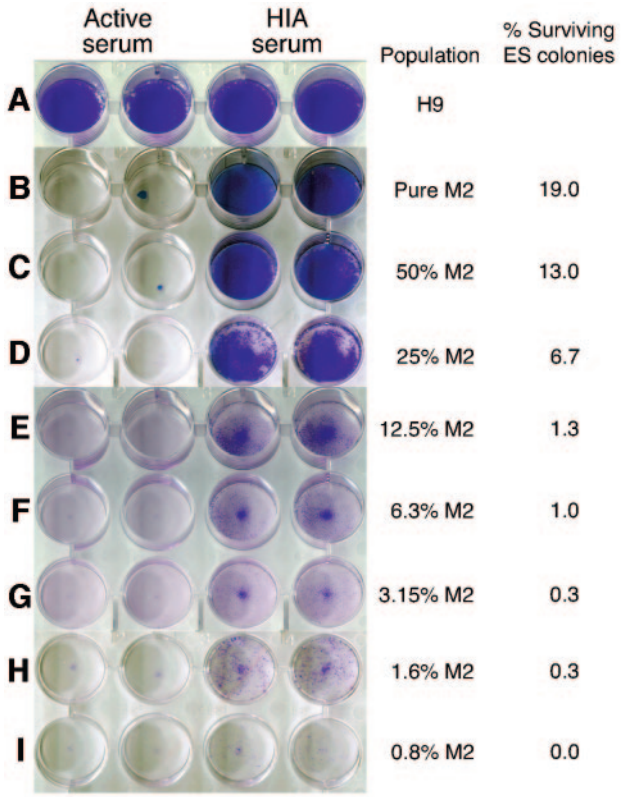

Figure 4. Complement lysis of M2 eliminates minority populations within a mixed culture. M2 cells were suspended along with HEK 293 cells **(a)** such that they comprised 100% **(b)**, 50% **(c)**, 25% **(d)**, 12.5% **(e)**, 6.3% **(f)**, 3.15% **(g)**, 1.6% **(h)**, and 0.8% **(i)** of the total population. Mixed populations were exposed to active and heat-inactivated (HIA) human serum in duplicate. Cells were then replated, and surviving M2 cells were selected in 1 mg/ml G418. Survival was assessed by Giemsa staining 7–10 days after plating, and the frequency of survival was estimated as a percentage of the colonies observed after exposure to HIA human serum.

DISCUSSION

As predicted from the known specificity of the epiblast- and germ line-specific *hTERT* gene [5], the *hTERT*/*GalT* (*pmGT*) transgene is expressed in M2 cells and downregulated after differentiation to mimic expression at the endogenous *hTERT* locus. As a consequence, M2 cells are susceptible to active human serum in vitro when undifferentiated and lose susceptibility in response to differentiation by downregulating the *GalT* transgene. In the context of regenerative medicine, this provides protection against undifferentiated hESCs by exposing differentiated populations to active human serum prior to engraftment.

M2 cells exposed to human serum for 1 hour still contained small numbers of undifferentiated cells as indicated by lectin binding of the colonies arising after 7 days (Fig. 3). This result would be anticipated in a variegated population if a subset of undifferentiated cells, not expressing the transgene at the time of exposure, subsequently reinitiated transcription and formally shows that survival did not arise as a consequence of loss of the transgene. Alternatively, colonies arising after lysis could simply reflect incomplete lysis. In either event, multiple exposures might be expected to eliminate these cells. Minority populations of M2 cells, when mixed with HEK 293 cells, were efficiently

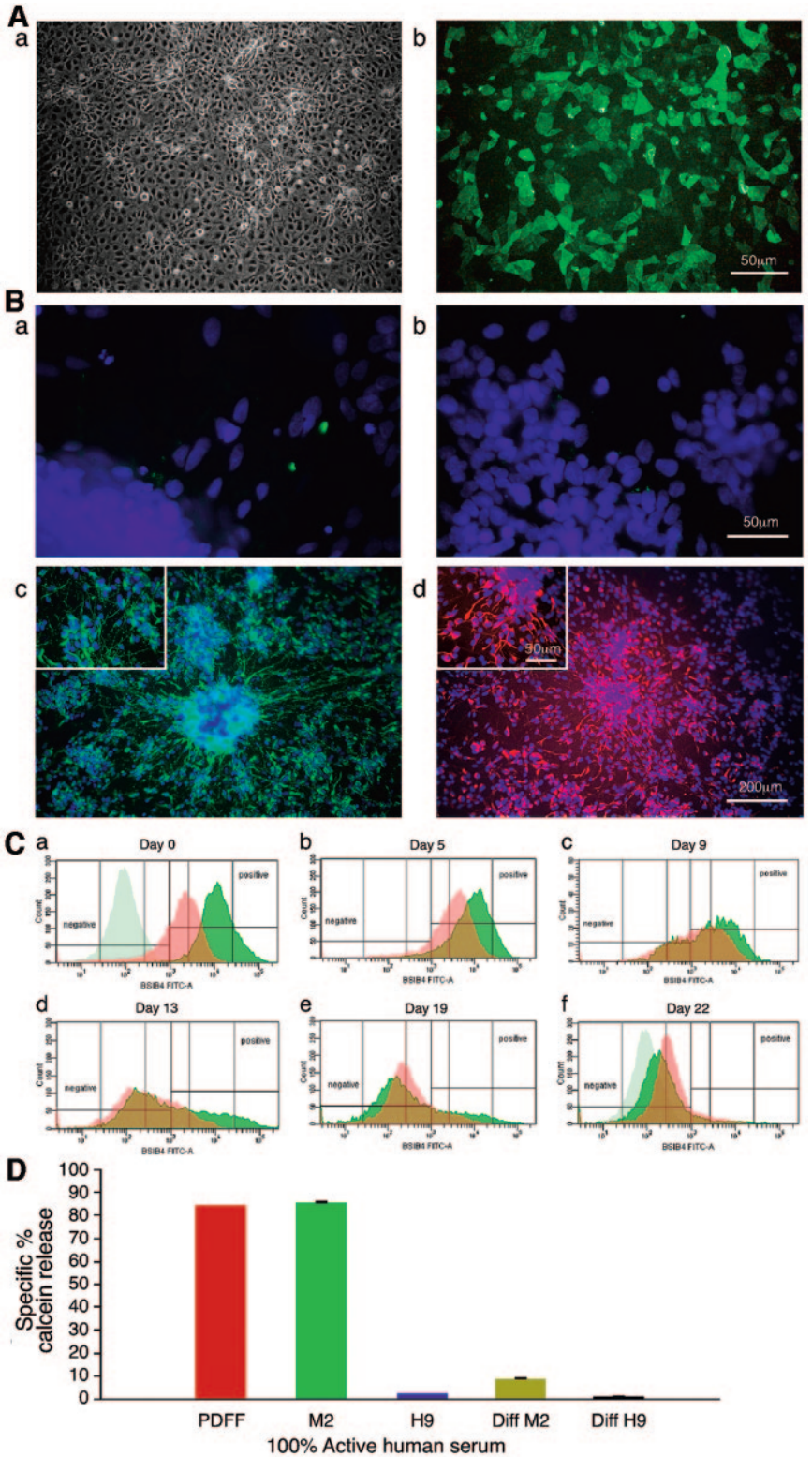

Figure 5. Expression of galT and sensitivity to human serum following differentiation. **(A):** M2 cells were cultured for 10 days and stained with *BS*-IB_4. **(B):** M2 and H9 cells were directed to differentiate into neural progenitors. After 3 weeks, presence of α-gal was assessed via *BS*-IB_4 staining. **(a)** H9 and **(b)** M2 cells were both negative, with only minimal background being observed. The presence of neural progenitors within the differentiated population was determined by staining differentiated M2 cells for the neural specific markers musashi **(c)** and nestin **(d)**. **(C):** M2 cells were spontaneously differentiated for 0, 5, 9, 13, 19, and 22 days, incubated with *BS*-IB_4 or Tra-1-81, and analyzed by flow cytometry to detect expression of *hTERT/GalT* (dark green) or continued expression of the ESC-specific marker Tra-1-81 (red). Staining with undifferentiated H9 hESCs is shown in light green in **(a)** and **(i)** to illustrate that with increasing differentiation, M2 cells acquire an expression pattern similar to the parental H9 population. Loss of Tra-1-81 expression accompanies loss of *BS*-IB_4 staining, consistent with the loss of undifferentiated hESCs among the population. **(D):** PDFF (red), undifferentiated M2 (green), undifferentiated H9 (navy blue), and differentiated M2 (yellow) and H9 (black) cells (21 days of differentiation) showing levels of calcein release after a 1-hour incubation with undiluted active human serum. By contrast to undifferentiated M2 and PDFF cells, differentiated M2 cells are almost wholly resistant to lysis. Error bars represents the SD of the six replicates. Abbreviations: *BS*-IB_4, *Bandeiraea simplicifolia* Isolectin B_4; GalT, α1,3-galactosyltransferase; hESC, human embryonic stem cell; hTERT, human telomerase reverse transcriptase.

killed. When the same mixed populations were exposed to heat-inactivated serum and the cells grown in conditions that allow outgrowth of only hESC colonies, we could detect the number of potentially tumorigenic cells in the mixed population. Hence, at 0.8% hESCs, an average of 308 colonies arose from the mixed population exposed to inactive serum, whereas no colonies survived in a sister culture exposed to active serum (Fig. 4i). Sporadic colonies (a single colony in three replicates) appear when the hESC population increases to 12.5% of the population, although it is not possible at this density to count colonies in the heat-inactivated control (Fig. 4e).

Although the majority of *GalT*-expressing cells in a population of M2 cells are lysed in human serum within 1 hour, the proportion of M2 hESCs that express *GalT* remains less than

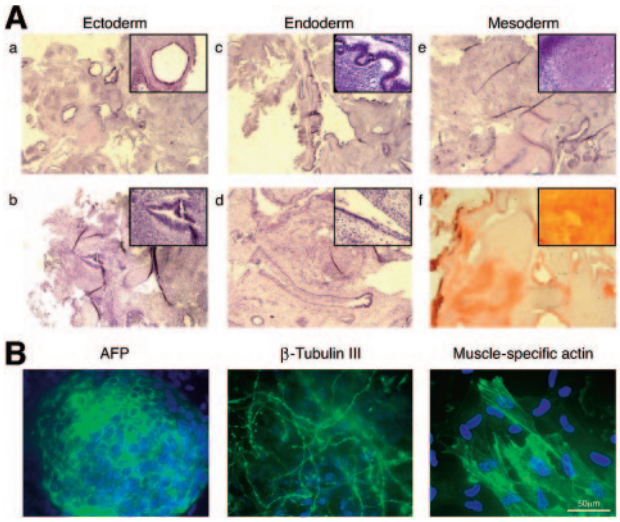

Figure 6. Differentiation of M2 cells. **(A):** M2 cells when injected intramuscularly into SCID mice formed teratomas that contained **(a)** sensory epithelium and **(b)** neural epithelium from the ectoderm lineage, **(c)** secretary epithelium and **(d)** transitional ciliated epithelium from the endoderm lineage, and **(e)** cartilage mesenchyme and **(f)** smooth muscle from the mesoderm lineage. Main images were taken using a ×20 objective; inserts were taken using a ×100 objective, except **(b)**, which used a ×200 objective. **(B):** In vitro differentiation of M2 cells, through embryoid body formation followed by disaggregation and adherent culture on gelatin. Immunohistochemistry showing the expression of markers from each of the three germ layers: **(a)** α-fetoprotein (endoderm), **(b)** β-tubulin III (ectoderm), and **(c)** muscle-specific actin (mesoderm). Scale bar = 50 μm. Images were captured using a ×40 objective. Abbreviation: AFP, α-fetoprotein.

100%. If nonexpressing cells have lost *GalT* expression as a consequence of variegated transgene expression, such cells could present a continuing risk of tumorigenesis. However, we show that increasing the exposure to human serum further reduces the number of surviving cells, suggesting that these cells are sensitive to active serum and continue to express the transgene. In addition, the mixing experiments provide evidence that, in practice, M2s that fail to express *GalT* and hence are insensitive to human serum and appear at very low frequency after incubation. Nevertheless, this theoretical risk could, in principle, be reduced by targeting the transgene (using homologous recombination to acquire the native *hTERT* or another ESC-specific promoter), by targeting an *hTERT*/*GalT* cassette to a "neutral" genomic site unassociated with position effect variegation, or by incorporating insulator elements to confer site-independent expression.

The core *hTERT* promoter sequence used in this study has higher transcriptional activity than the full *hTERT* promoter. When used to drive expression of *neo* in transfected hESCs, it provided higher colony number than *neo* cassettes transcribed from the *PGK*, *SV40*, or *CMV* promoters (data not shown). The *hTERT* gene is expressed in some adult progenitor cells; however, the core promoter is known to be downregulated in at least some of these populations, such as mesenchymal stem cells [16]. Hence, we anticipate that the *hTERT*/*GalT* transgene will be widely useful for application with multiple lineages. However, there may prove to be some lineages for which alternate promoters will be preferable, and it will be useful to generate additional modifications with *GalT* transcribed from promoters such as *Oct4*, *Nanog*, and *Sox2*.

A recent report has demonstrated that hESCs grown in medium containing serum replacement and other components of animal origin express an immunogenic nonhuman sialic acid, Neu5Gc, leading to complement-mediated cell death when exposed to human serum [17]. These authors attribute a major source of Neu5Gc to serum replacement. In contrast to that report, we did not detect cell death when H9 hESCs grown under our culture conditions (also including serum replacement) were incubated with human serum. In the absence of commercial sources of anti-Neu5Gc antiserum, we could not directly determine whether cells in our culture regime also express that epitope. Instead, we cultured H9 cells for 3 days with the addition of up to 3.0 mM Neu5Gc to the normal culture medium. After 3 days of exposure, cells were again incubated with human serum, and we again observed no difference in cell death in active versus heat-inactivated sera (data not shown). This result suggests that the sera used in this study did not contain significant levels of anti-Neu5Gc activity.

A novel feature of the *pmGT* approach is that M2 or similar cells encoding an *hTERT*/*GalT* transgene and engrafted into a patient may experience continuing in vivo immune surveillance [18–20] against residual hESCs or cells that re-express the *GalT* transgene due to dedifferentiation or as a consequence of secondary tumorigenesis. This possibility suggests an important advantage of the use of the *GalT* gene over *TK*-based approaches because in the latter case the tumor must be diagnosed (and hence well developed) before gancyclovir treatment can begin. With ongoing immune activity against *GalT*, it is anticipated that the tumors will simply never become established. In vivo surveillance for undifferentiated *hTERT*/*GalT* expression could be demonstrated in principle, using similarly modified murine *hTERT*/*GalT* ESCs in a syngeneic *GalT* knockout mouse, although this would not directly demonstrate the utility of M2 cells. More powerful still would be to induce tolerance in the *GalT* knockout mouse to M2 cells themselves, possibly by treatment with anti-CD4 antibodies or by inducing hematopoietic chimerism (reviewed in [21]). Such models could then be used to compare the tumorigenic potential of M2 versus parental cells.

These data demonstrate that hESCs can be routinely modified to provide clones with appropriate regulation of a *GalT* transgene and that such clones are sensitive to normal human serum when undifferentiated and insensitive after differentiation.

SUMMARY

We describe a new approach to eliminate undifferentiated hESCs from an hESC-derived population in vitro prior to engraftment. We show that hESCs engineered to express *GalT* under the control of the *hTERT* promoter (M2 cells) are sensitive to complement-mediated lysis when undifferentiated cells are exposed to human serum. By contrast, differentiated derivatives of M2 were resistant to complement-mediated attack, as were unmodified hESCs. To test the efficiency of selective elimination, M2 cells were seeded as minority populations within HEK 293 cultures and exposed to active human serum. When present at less than 1.6% of the total population, all M2 cells were eliminated after a single 1-hour incubation with human serum. In the context of regenerative medicine, the pretreatment of therapeutic populations with human serum prior to engraftment should provide a fail-safe system against the accidental transplantation of potentially tumorigenic hESCs.

In addition, the presence of circulating antibody against the α-gal epitope may provide continuing in vivo immune surveillance against cells expressing the *GalT* transgene. By harnessing the restricted expression of hTERT, nontumorigenic compo-

nents of a graft would be preserved while undifferentiated or dedifferentiated cells would be targeted. This approach could be further improved by directing the transgene to a site free of position effect variegation using homologous recombination and by generating homozygous cells to guard against mutation or chromosome loss.

ACKNOWLEDGMENTS

Z.H. was supported by a CASE studentship from the Biotechnology and Biological Sciences Research Council (BBSRC) in collaboration with Geron Corporation. H.P. was supported by a BBSRC ROPA (Realising Our Potential Award) award. We thank Judy Fletcher for careful analysis of karyotypes, Tim King and Steve Pells for assistance with blood collection, Martin Waterfall for assistance with blood collection and flow cytometry analysis, David Brownstein for providing analysis of tumor sections, Norrie Russell and Elliot Armstrong for photography, and John Ansell and Craig Taylor for valuable discussions. Z.H. and H.P. contributed equally to this work. H.P. is currently affiliated with the Molecular Embryology and Stem Cell Lab, School of Human Development, Division of Obstetrics and Gynaecology, Queens Medical Centre, Nottingham, United Kingdom.

DISCLOSURES

J.M. has consulted for Geron Corporation within the last two years.

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