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A knock-out model of paroxysmal nocturnal hemoglobinuria: Pig-a$^-$ hematopoiesis is reconstituted following intercellular transfer of GPI-anchored proteins

(embryonic stem cells/embryoid bodies/developmental biology/bone marrow failure syndromes/paroxysmal nocturnal hemoglobinuria)

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ABSTRACT **We created a "knock-out" embryonic stem cell via targeted disruption of the phosphatidylinositol glycan class A (Pig-a) gene, resulting in loss of expression of cell surface glycosyl phosphatidylinositol-anchored proteins and reproducing the mutant phenotype of the human disease paroxysmal nocturnal hemoglobinuria. Morphogenesis of Pig-a$^-$ embryoid bodies (EB) *in vitro* was grossly aberrant and, unlike EB derived from normal embryonic stem cells, Pig-A$^-$ EB produced no secondary hematopoietic colonies. Chimeric EB composed of control plus Pig-A$^-$ cells, however, appeared normal, and hematopoiesis from knock-out cells was reconstituted. Transfer *in situ* of glycosyl phosphatidylinositol-anchored proteins from normal to knock-out cells was demonstrated by two-color fluorescent analysis, suggesting a possible mechanism for these functional effects. Hematopoietic cells with mutated PIG-A genes in humans with paroxysmal nocturnal hemoglobinuria may be subject to comparable pathophysiologic processes and amenable to similar therapeutic protein transfer.**

Certain cell surface membrane proteins lack a hydrophobic membrane-spanning region and are instead tethered to the cell through a glycosyl phosphatidylinositol (GPI) anchor (1). As a result of the distinctive biophysical properties of the GPI anchor, these proteins may be localized in specific membrane microdomains or cell surface subregions (2–4), but the biologic advantage of this mode of cell surface expression remains poorly understood. The GPI anchor is synthesized through a complex sequence of biochemical reactions (5, 6); the initial step, attachment of *N*-acetylglucosamine to phosphatidylinositol, depends in part on the product of a recently cloned X chromosome gene termed phosphatidylinositol glycan class A (PIG-A in humans, Pig-a in mice) (7, 8). Somatically acquired deletions or mutations in the PIG-A gene of hematopoietic stem cells have been identified in all cases of the human disease paroxysmal nocturnal hemoglobinuria (PNH) studied to date (9, 10). Cells lacking GPI-anchored proteins (GPI-AP) comprise a variable proportion of all hematopoietic lineages in these patients (11). The absence of two GPI-AP, decay-accelerating factor (CD55), and membrane inhibitor of reactive lysis (CD59) results in decreased resistance of erythrocytes to lysis induced by complement *in vitro* and *in vivo* and produces the disease's characteristic intermittent intravascular hemolysis (12–15).

The reason why GPI-AP-deficient clones expand within the hematopoietic system of patients with PNH remains to be elucidated. The clinical observation that PNH frequently evolves from acquired aplastic anemia (16–18), bone marrow failure secondary to T-cell-mediated hematopoietic stem cell destruction, has led to the hypothesis that the absence of certain GPI-AP on the surface of the mutant cells may permit them to evade immune attack, thus conferring a relative growth advantage (19, 20). When hematopoietic progenitors from PNH patients have been evaluated *ex vivo*, no intrinsic growth advantage has been demonstrated (21–24); hematopoietic colony formation from both the GPI-AP$^+$ and GPI-AP$^-$ CD34$^+$ cells was significantly lower than that of CD34$^+$ cells of normal volunteers (J. Maciejewski and N.Y., unpublished data). Interpretation of such experiments, however, is confounded by the use of clinical samples, as colony formation may reflect either the microenvironment from which the cells are derived or the direct consequence of GPI-AP deficiency on cell growth and differentiation. Accordingly, to study the direct effects of mutation of the PIG-A gene in the context of an otherwise normal hematopoietic system, we generated a murine Pig-A knock-out embryonic stem cell (ESC) line and studied the developmental and hematopoietic potential of these cells *in vitro*.

METHODS

ESC Line Culture and Embryoid Bodies (EB) Culture. ESC of the CCE lineage (a kind gift of Ashok Kulkarni, National Institute of Neurological Disorders and Stroke, National Institutes of Health) were maintained as monolayer cultures in 35-mm wells (Costar 3506) coated with 0.1% porcine skin gelatin (United States Biochemical 16045, Bloom 175) and passaged every 3–5 days by brief trypsinization with 0.5 ml of 0.1% trypsin in 0.02% EDTA (Advanced Biotechnologies, Columbia, MD, 01-513-100) at 37°C for 2 min. Enzymatic digestion was quenched with an equal volume of fetal calf serum (FCS), and cells were washed once. The culture medium consisted of high-glucose DMEM (Bio-Fluids 104) plus 15% heat-inactivated FCS (Bio-Fluids 200C), 2 mM L-alanyl-L-glutamine (GIBCO/BRL 35050-012), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (GIBCO/BRL 11140-019), 0.1 mM 2-mercaptoethanol, exogenous nucleosides (Specialty Media, Lavellette, NJ, ES-008), 20 mM Hepes (Bio-Fluids

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Abbreviations: GPI, glycosylphosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria; GPI-AP, GPI-anchored protein; PIG-A or Pig-a, phosphatidylinositol glycan class A; ESC, embryonic stem cells; EB, embryoid bodies; LIF, leukemia inhibitory factor; PE, phycoerythrin; neo^R, neomycin resistance or neomycin-resistant ESC line.

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321), and 1000 units/ml murine leukemia inhibitory factor (LIF) (GIBCO/BRL 13275-029).

EB were generated in ultra-low binding 6-well plates (Costar 3471) to prevent attachment and foster aggregation. Initial cultures were 10,000 cells in 2 ml. The culture medium consisted of Iscove's modified Dulbecco's medium (GIBCO/BRL 31980-022) with 15% FCS (Biofluids 200C), 0.2 mg/ml bovine holo-transferrin (Sigma T-1283), 10 μg/ml bovine insulin (Sigma I-6643), and 0.45 mM monothioglycerol (Sigma M-1753). Hematopoietic growth factors were added on day 6 or 7 of culture to enhance development of colony-forming units and consisted of recombinant murine stem cell factor at 100 ng/ml (R & D Systems 455-MC), recombinant human granulocyte-colony stimulating factor at 100 ng/ml, recombinant human interleukin 6 at 50 ng/ml, and recombinant human erythropoietin at 5 units/ml (kindly provided by Amgen Biologicals). An additional 2 ml of culture medium (without additional hematopoietic growth factors) was added after 1 week to feed cultures. G418 (GIBCO/BRL 11811-031) was added at a final active concentration of 1 mg/ml for the final 2 days of culture, where indicated. G418 at one-tenth of this concentration, 0.1 mg/ml, was found sufficient to kill 100% of untransfected ESC in preliminary experiments.

Construction of Vector with Targeted Disruption of Mouse Pig-A Gene. A 0.7-kb *Eco*RI to *Sma*I fragment encompassing the upstream intronic sequence and the 5′ end of exon 2 was deleted, and the phosphoglycerol kinase (Pgk)-Neo construct was inserted as described (25). The Pgk-Neo construct consisted of a 500-bp *Eco*RI to *Taq*I fragment containing the promoter of the mouse Pgk gene linked to an 832-bp fragment containing the Neo coding region in turn linked to 500 bp of the Pgk 3′-terminal sequence. Before electroporation, the full-length sequence was linearized with *Sal*I.

Generation of Pig-A Knock-Out and Control Neomycin-Resistant (neo^R) ESC Lines. Cells (50–60 × 10^6 CCE ESC with 25–50 μg of the linearized (*Sal*I) disrupted Pig-A/neo^R construct in 0.8 ml of cold PBS) were electroporated with 240 V and 500 μF in a 0.4-cm cuvette. The cells were plated on gelatinized dishes in LIF-containing medium, and G418 (0.4 mg/ml) was added 1 day later. After 1 week, neo^R cells were stained with anti-CD24-PE, and cells staining less intensely than 99% of untransfected cells were sorted directly into microwells at limiting dilution. One of the 26 clones screened was found to be CD24$^-$ and designated C25. Unsorted (i.e., uncloned) neo^R ESC were cultured in parallel and used as controls where indicated. Both the C25 and neo^R lines remained resistant to 1 mg/ml G418 at all times.

Southern Blot Analysis of Insertion of the Neomycin Resistance Gene in ESC Lines. Samples (10 μg) of genomic DNA prepared from 10^7 ESC by the proteinase K method were digested with *Eco*RI or *Sac*I. After electrophoresis on a 0.6% agarose gel, the DNA was transferred to a Hybond nylon membrane and crosslinked by ultraviolet irradiation. Hybridization with a ^{32}P-labeled neo^R probe prepared by the random primer method (GIBCO/BRL 8187SA) was carried out in QuikHyb (Stratagene 201220). Nonspecific ^{32}P-labeled neo^R probe binding was eliminated by several high stringency washes (0.2× standard saline citrate at 50°C). Autoradiograms were developed after overnight exposure.

Cell Surface Antigen Immunofluorescence and Sorting. ESC were stained after brief trypsinization (0.1%, 37°C, 2′), which was found not to remove significant numbers of CD24 epitopes. Cells (10^5 to 10^6) were incubated at 4°C for 30 min with 1 μg of phycoerythrin (PE)-conjugated rat anti-mouse CD24 (M1/69; PharMingen 01579D) or as an isotype control, PE-conjugated rat IgG$_{2b}$, κ antibody (PharMingen 11035A), and then washed twice with cold 10% FCS in PBS. Fluorescently labeled cells were analyzed and sorted by a Coulter Elite, model T07112.

Methylcellulose Culture for Enumeration of Murine Hematopoietic Progenitors. EB were collected after 11–13 days of culture, treated with 2.5 mg/ml collagenase (Sigma C-1639) in 20% FCS/PBS at 37°C for 1 hr, washed once with PBS, and then digested for 10 min with 0.25% trypsin with 1 mM EDTA (GIBCO/BRL 25200-056) at 37°C and vortexed vigorously. Only cells excluding trypan blue were counted. About 200,000 viable cells were plated in 1.1 ml of methylcellulose supplemented with recombinant murine cytokines (Stem Cell Technologies, Vancouver, British Columbia; MethoCult GF M3434). Colonies were enumerated on day 6 or 7 of culture and scored as either erythroid or granulocytic/monocytic based on color and appearance; lineage determinations were periodically confirmed by Giemsa staining of individual colonies. C25- and control-derived hematopoietic colonies were morphologically indistinguishable, both as colonies and upon Giemsa staining. No hematopoietic colonies were observed in methylcellulose cultures in which hematopoietic growth factors were not included. Secondary EB developing in methylcellulose were not counted.

Co-Culture of C25 or neo^R ESC with Normal ESC in EB Differentiation Cultures. To provide an environment of normal GPI-AP in which the knock-out C25 or control neo^R ESC could develop, chimeric EB were generated from mixtures of 80% untransfected plus either 20% C25 or 20% neo^R ESC. The resultant EB yielded numbers of colonies roughly equivalent to those from EB derived entirely from normal ESC (data not shown), from which it was inferred that the presence of the knock-out C25 ESC did not exert a negative effect on the generation of hematopoietic precursors from the chimeric EB. Addition of G418 (1 mg/ml) during the last 2 days of culture was lethal for EB composed of 100% untransfected ESC and produced marked cell loss in the chimeric cultures, as assessed microscopically and by viable cell yield. Cultures from disaggregated untransfected EB (grown without G418) produced no colonies when G418 was included in the 6- to 7-day secondary methylcellulose cultures.

Green Fluorescent Intracellular Labeling of ESC, Co-Culture with Unlabeled ESC, and Analysis of Intercellular GPI-AP Transfer. C25 cells were labeled intracellularly by incubation at 37°C for 30 min with the green fluorescent probe CMFDA (16 μM, Molecular Probes C-2925). After washing, the labeled cells were incubated in fresh medium for an additional 30 min and washed again. Green fluorescence-labeled knock-out cells were incubated with a 4-fold excess of normal, unlabeled ESC for 2–4 days in suspension (i.e., EB) culture. A single-cell suspension of the resultant culture was obtained after gentle trypsinization (0.1%, 37°C, 2 min) and stained with anti-CD24-PE (red fluorescence). Cytofluorometric analysis was performed on a Coulter Elite, model T07112. Two-color histograms of green (abscissa) and red (ordinate) fluorescence vs. cell number were obtained. A progressive increase in both the percentage of C25 cells staining with PE-conjugated anti-CD24 and the level of staining were seen with increased time of coincubation; similar but lower levels of staining of C25 with anti-CD24 were seen with lower ratios of normal cells/knock-out cells (data not shown). Negligible transfer of the green intracellular dye to the unlabeled, CD24^{bright} cells was observed (see Fig. 4).

RESULTS

Generation of a Pig-A Knock-Out ESC Line. To assess more directly the intrinsic hematopoietic potential of GPI-AP-deficient cells, we developed a Pig-A knock-out ESC. First, a plasmid was constructed in which a neomycin resistance cassette was inserted into the second exon of the murine Pig-A gene (Fig. 1). The plasmid was linearized and introduced into ESC by electroporation. After selection of transfectants by culture in G418 for 1 week, cells in which the normal Pig-A

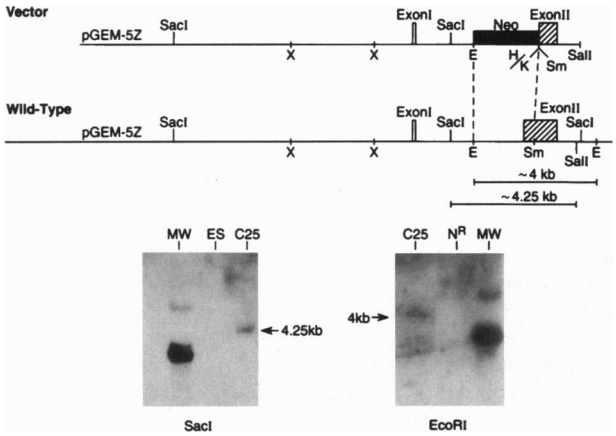

FIG. 1. Targeted mutation in the mouse Pig-A gene. (*Upper*) A 1.8-kb insert consisting of a neomycin resistance cassette and the mouse phosphoglycerol kinase promoter was substituted for a 0.7-kb segment of the mouse Pig-A gene composed of the 5′ end of the second exon and its adjoining upstream intronic sequence. X, *Xba*I; E, *Eco*RI; H, *Hin*dIII; K, Klenow; Sm, *Sma*I. (*Lower*) Hybridization of a ^{32}P-labeled neo^R probe with restriction endonuclease digests of DNA from the three cell lines: ES (normal, i.e., untransfected, ESC; CD24$^+$), C25 (Pig-A knock-out ESC; CD24$^-$), and N^R (neo^R, i.e., control transfected, ESC; Pig-A$^+$, CD24$^+$). As predicted from the restriction map, discrete *Eco*RI and *Sac*I restriction fragments of 4 and 4.25 kb, respectively, were seen only with the C25 line, in which homologous recombination of the disrupted construct had occurred. No discrete band is seen with the control neo^R line for either restriction enzyme as it was not cloned after transfection and G418 selection, and hence the integration sites of the cassette would be expected to be random and manifold. MW, molecular weight markers.

gene (which is located on the X chromosome in mice, as well as humans) had been disrupted by homologous recombination could be identified by loss of expression of the murine GPI-AP CD24, which is normally expressed on ESC (unpublished observations). CD24$^-$ cells were enriched by fluorescence-activated cell sorting and cloned at limiting dilution. DNA analysis by Southern hybridization of one clone, called C25, confirmed insertion of the neomycin resistance cassette into the second exon of the Pig-A gene (Fig. 1, *Lower*). Staining of the C25 ESC with phycoerythrin-conjugated anti-murine CD24 (Fig. 2) confirmed the expected absence of this GPI-AP.

Aberrant Development of Pig-A Knock-Out EB and Its Correction. Presence of the Pig-A knock-out mutation in the mouse germ line may be lethal (6) and congenital absence of GPI-AP in humans has not been reported; these observations suggest an essential role of GPI-AP in embryogenesis (26). We investigated the functional consequences of Pig-A deficiency, in particular as they relate to hematopoiesis. ESC, when grown in suspension culture in the absence of LIF, form pseudoembryos called EB; disaggregated cells from EB can be induced to differentiate along different cell lineage pathways, including hematopoietic (27–32). Consistent with the apparent lethality of germ-line Pig-A mutations, the morphologic development of EB derived from the knock-out C25 ESC line was strikingly abnormal (Fig. 2*D*): Pig-A-deficient EB did not achieve the same dimensions as normal or neo^R control EB, nor did they develop the characteristic cystic structure of a typical EB (Fig. 2*B*). Thus, Pig-A-deficient EB appeared arrested at an early stage of embryonic differentiation and failed to progress beyond the stage of simple spherical aggregates.

When EB derived from either C25 or neo^R control ESC were cultured for 12–13 days, enzymatically disaggregated, and then assayed for hematopoietic progenitors in methylcellulose colony cultures (33, 34), C25-derived colonies were conspicuously absent (Table 1). To determine whether this was due to failure of progenitors to develop in a GPI-AP-deficient environment (at the EB stage) or due to a deleterious effect of the knock-out mutation on maturation of hematopoietic progenitors into recognizable colonies, we performed cell mixing experiments. Pilot experiments revealed that EB morphology was preserved in chimeras with an initial composition of up to 20% knock-out C25 cells and, accordingly, a four ratio of untransfected ESC for every one C25 or neo^R ESC was used in all subsequent experiments (as depicted schematically in Fig. 3.) Chimeric EB composed of 80% normal ESC and 20% of either C25 or neo^R ESC were exposed to G418 for several days before the termination of culture to select for the progeny of C25 or neo^R control cells. The EB were then enzymatically disaggregated and assayed in standard methylcellulose medium (supplemented with G418) for hematopoietic colony formation (Fig. 3). C25-derived hematopoiesis was thereby restored to a level comparable to that seen in control cultures (Table 1). These experiments showed that the hematopoietic potential of Pig-A$^-$ cells was restored when they developed in a GPI-AP$^+$ environment.

Intercellular Transfer of GPI-AP in EB. Exchange of GPI-AP between membranes has been demonstrated in several *in vitro* and *in vivo* experimental systems (15, 35–41). We speculated that transfer of GPI-AP from normal to C25 cells might lead to phenotypic and functional correction of the GPI-AP deficiency. Evidence for protein transfer was sought in several independent experimental systems. First, we coincubated normal and C25 ESC in suspension culture (i.e., EB culture) for 3–5 days and selected against the normal cells by adding G418 to the medium for the final 2 days. About 25% of G418-resistant cells showed expression of CD24, but at levels lower than that seen with normal or control neo^R ESC cells (data not shown). We then separated by fluorescence-activated cell sorting CD24$^+$ and CD24$^-$ G418-resistant cells and analyzed them for the neomycin resistance cassette by gene amplification; these CD24$^+$ cells were strongly positive on PCR analysis (data not shown), consistent with their origin from knockout C25 cells.

In a second series of experiments, C25 cells were labeled in the cytoplasm with the fluorescein derivative CMFDA before mixing with unlabeled normal ESC (CD24$^+$). After coincubation (in the absence of G418) for 2–4 days (again in suspension culture), cells were assayed for green fluorescence, as a marker of origin from C25 cells, and for the presence of CD24 on the cell surface, as a measure of protein transfer from normal ESC (Fig. 4*B*). Most cells derived from the Pig-A-deficient clone became CD24$^+$, and again the levels of surface CD24 antigen were approximately an order of magnitude lower than those of normal or control ESC. Similar results were obtained when normal and C25 cells were coincubated in adherent (i.e., undifferentiated) culture, although slightly lower levels of transfer were observed (data not shown). Transfer of GPI-AP via culture supernatant, however, could not be demonstrated (data not shown), suggesting that close cell–cell apposition may be important. When CD24$^+$ CMFDA$^+$ cells were sorted and cloned at limiting dilution, each subclone (n = 23) was positive for the neomycin resistance gene (by PCR, data not shown), confirming that these cells were indeed of C25 origin. Fluorescence-activated cell sorting analysis of each expanded subclone, moreover, demonstrated that after the multiple rounds of cell division attendant to each expansion, surface expression of the CD24 antigen became undetectable (data not shown). Transfer of GPI-AP between the closely apposed cells of the EB may permit participation of Pig-A$^-$ cells in normal EB development. Once the EB cells are disaggregated and induced to differentiate terminally, the already low concentrations of transferred GPI-AP are probably further reduced by dilution with each cell division, but without apparent adverse functional effect on colony formation.

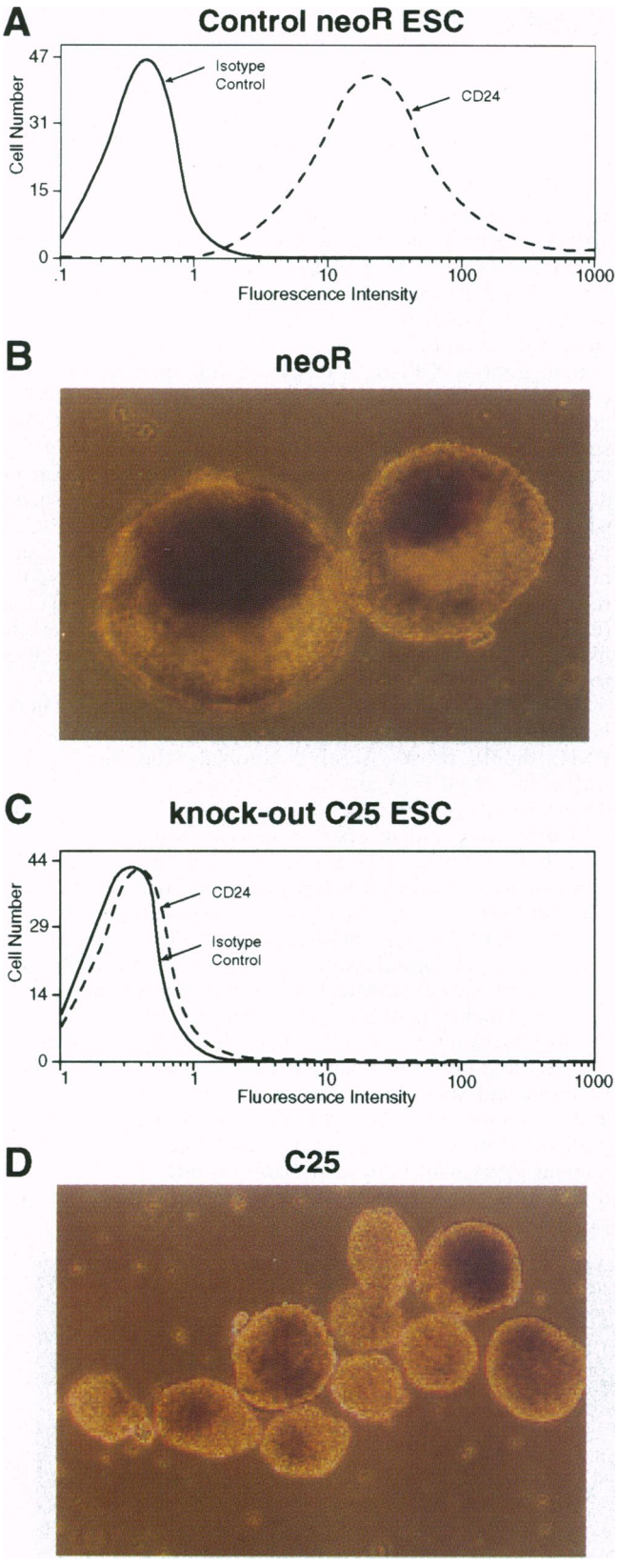

FIG. 2. CD24 expression and morphologic characterization of control (neo^R) and knock-out (C25) ESC lines. (*A* and *C*) Flow cytometric analysis of the two lines with a phycoerythrin-conjugated monoclonal antibody to CD24, a GPI-AP present on normal ESC, and with a phycoerythrin-conjugated isotype control. (*B* and *D*) EB derived from C25 or neo^R ESC were photographed with a phase contrast microscope after 7 days in LIF-free suspension culture. Chimeric EB containing 20% of either C25 or neo^R cells were morphologically indistinguishable from normal EB (*B*).

Table 1. Enumeration of neo^R- and C25-derived hematopoietic progenitors from unmixed and chimeric EB*

		Colonies†	
Experiment	ESC line	Unmixed EB	Chimeric EB
1	neo^R	51/63	16/20
	C25	0/0	12/14
2	neo^R	30/35	7/16
	C25	0/0	15/27
3	neo^R	33/53	12/13
	C25	0/0	11/14

*EB (12–13 days old) derived entirely from neo^R or C25 ESC ("unmixed"), or "chimeric" EB composed of 80% normal (untransfected, G418-sensitive) ESC plus 20% of either neo^R or C25 cells, were grown in suspension culture. EB were enzymatically disaggregated, cultured in standard methylcellulose medium supplemented with colony-stimulating factors for 6–7 days, and scored for erythroid or granulocyte/macrophage colonies. Cultures of chimeric EB included 1 mg/ml G418 over the last 2 days to select for C25- or neo^R-derived cells; methylcellulose colony assays of cells derived from chimeric EB cultures contained 1 mg/ml G418 as further selection as well. The overall lower levels of progenitors derived from the cultures of both C25- and neo^R-chimeric EB cultured with G418 (relative to the unmixed neo^R EB cultures) were likely due to the fact that the C25- or neo^R-derived cells in the chimeras were exposed to cellular debris over the final hours to days of culture and were deprived of the trophic and accessory functions that these cells would have otherwise ordinarily provided. G418 at the same concentration did not impair development of hematopoietic progenitors from EB derived from 100% neo^R control ESC (data not shown) but was lethal (i.e., >99%) to normal (untransfected) ESC and EB after 2 days in culture. (See Fig. 3 for a schematic representation of the design of the chimera experiments.)

†Per 2×10^5 viable cells plated. The first number represents erythroid colonies, and the second number represents granulocyte/monocyte colonies.

DISCUSSION

The PNH Conundrum: Are PIG-A Mutations Deleterious, Neutral, or Advantageous? Lack of success in developing nullizygous knock-out mice deficient in GPI-AP expression (6) is consistent with the central importance of this class of cell surface molecules in ontogeny. Inactivation of the Pig-A gene in a subset of more mature cells, however, can be tolerated in the experimental system of EB described here or when due to a somatic mutation in a human hematopoietic stem cell in patients with PNH. Although inferences from experimental murine *in vitro* systems to human disease must be made with caution, our results indicate that in an otherwise normal environment, the loss of a functional Pig-A gene confers neither a gross intrinsic hematopoietic growth advantage or disadvantage. The paradox of PNH, then, is that of a bone marrow failure syndrome, in which the mutant PIG-A$^-$ hematopoietic stem cells are nevertheless preferentially expanded but in which the mutant phenotype (GPI-AP deficiency) results in a decreased life span of at least one class of mature blood cells—erythrocytes (due to lower than normal resistance to membrane damage by physiologically activated complement).

A mixture of normal and deficient cells must exist in the marrow of patients with evolving PNH at the time the initial PIG-A$^-$ clone expands; without an intrinsic growth advantage, some external selective pressure would therefore appear to favor the expansion of the PIG-A$^-$ clone. Possibly, a GPI-AP is a target for attack by some environmental agent such as a chemical or a microorganism [the GPI-AP CD55, for example, is a receptor for several ECHO- and coxsackie-viruses (42–44)]. Alternatively, as we and others have proposed (19, 45), one or more GPI-AP may serve as critical costimulatory ligands in the activation of the immune system by target

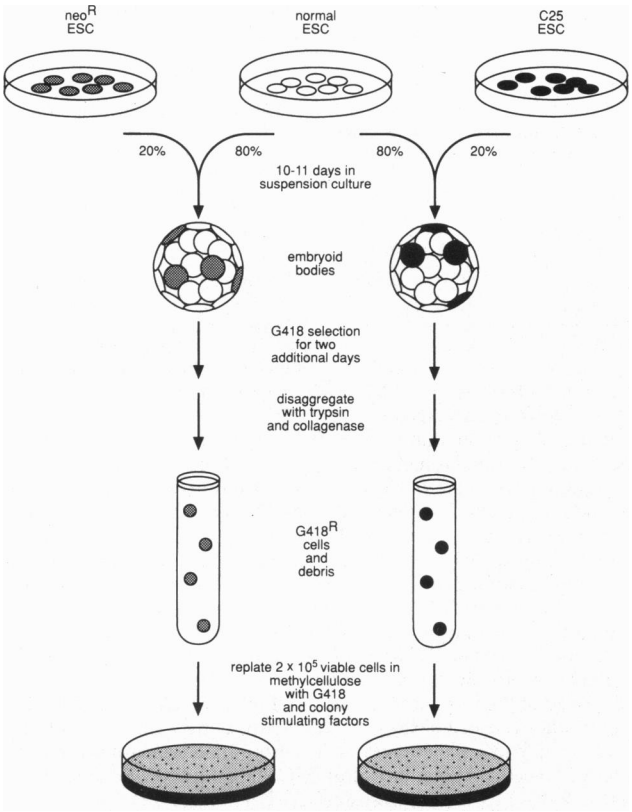

FIG. 3. Experimental design of chimeric EB culture and G418 selection for Table 1.

hematopoietic stem cells. A paradigm of such an interaction is that between CD2 and two of its ligands, CD58 and CD59, both of which exist in GPI-anchored forms (46). A recently described example of iatrogenic immune selection of the GPI-AP$^-$ phenotype is provided in the cases of three lymphoma patients who were treated with a humanized antibody against the GPI-AP CD52; cell surface phenotyping of lymphocytes from patients after multiple rounds of treatment revealed emergence of a substantial GPI-AP$^-$ population. Single-strand conformation polymorphism analysis of two T-cell clones suggested mutations in the PIG-A gene (47). After treatment with the antibody to CD52 was discontinued, cells with the GPI-AP$^-$ phenotype gradually disappeared, a result that argues against any intrinsic proliferative advantage conferred by the PIG-A mutation, at least for lymphocytes. Immune selection for GPI-AP deficiency may also occur in the autoimmune disease psoriasis; GPI-AP expression was lower than normal in nonlesional psoriatic skin and virtually absent in lesional psoriatic skin (48). In PNH, deficiency of GPI-AP on the surface of the affected clone(s) may not become absolute until the progeny leave the close proximity of GPI-AP$^+$ cells present in the bone marrow (49) and enter the circulation, thus separating temporally and physically positive selection for GPI-AP$^-$ cells in the marrow from negative selection against CD55/59$^-$ cells (in particular erythrocytes) in the periphery. Experiments are currently underway to elucidate the mechanism(s) of intercellular GPI-AP transfer and address the clinical observation that most erythrocytes in PNH patients remain entirely GPI-AP$^-$ despite a life span of weeks to months in an environment of unaffected (i.e., GPI-AP$^+$) nonhematopoietic cells (i.e, endothelia) replete with CD59. Erythrocytes with intermediate complement sensitivity and intermediate CD59 expression (type II PNH cells) can be demonstrated in some PNH patients, and it is unresolved whether these cells represent the progeny of a second PIG-A mutation (45) with only partial inactivation of the PIG-A gene product or are the result of GPI-AP exchange. Given the restriction of expression of many GPI-AP to specific lineages (e.g., CD14 on monocytes or CD16 on neutrophils), it seems likely that transfer is not an arbitrary event, but rather a tightly regulated process.

Clinical Implications. The system we have described here, in addition to providing an *in vitro* hematopoietic model for PNH, should prove useful in studying the biology and trafficking of GPI-AP in embryogenesis, as well as in fully differentiated cells of other lineages. Differentiation of ESC into primitive cardiac (29) or neural tissues (50) is well described and other systems are being developed (51, 52). Once biological processes dependent on GPI-AP are identified in these systems, the effects of reconstitution of all or selected GPI-AP via chimerism, genetically engineered transmembrane homologues, or even crude membrane extracts may allow identification of the physiologic role of individual members of this class of cell surface molecules. In the specific example of PNH, it may ultimately be possible to differentiate between those GPI-AP necessary for normal function and survival of mature erythrocytes, leukocytes, and platelets, and the GPI-AP that participate on the pathologic attack on hematopoietic stem cells in the aplastic anemia/PNH syndrome. If the appropriate GPI-AP can be delivered to deficient cells, such therapy could potentially prove beneficial to PNH patients.

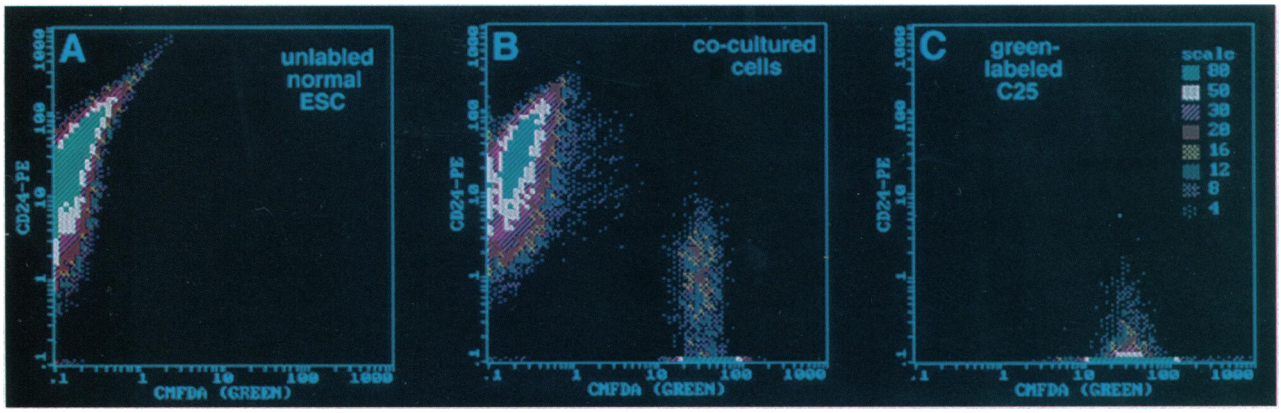

FIG. 4. Transfer of GPI-AP from normal to C25 cells. C25 cells labeled intracellularly with the green fluorescent probe CMFDA were incubated in suspension (EB) culture with a 4:1 excess of normal, unlabeled ESC for 3 days. A single-cell suspension of the resultant culture was obtained after gentle trypsinization (0.1%, 37°C, 2 min) and stained with anti-CD24-PE (red fluorescence). Cytofluorometric analysis was performed on a Coulter Elite, model T07112. Two-color histograms of green (abscissa) and red (ordinate) fluorescence vs. cell number are shown for 3-day cultures of unlabeled normal (*A*), CMFDA (green)-labeled C25 (*C*), and chimeric EB (*B*).

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