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Long-Term Bovine Hematopoietic Engraftment with Clone-Derived Stem Cells

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ABSTRACT

Therapeutic cloning by somatic cell nuclear transfer offers potential for treatment of a wide range of degenerative disease. Nuclear transplantation with *neo*^{r}-marked somatic nuclei from 10–13-year-old cows was used to generate cloned bovine fetuses. Clone fetal liver (FL) hematopoietic stem cells (HSC) were transplanted into two busulfan-treated and one untreated nuclear donor cows. Hematopoiesis was monitored over 13–16 months by *in vitro* progenitor and HSC assays. Chimerism was demonstrated by PCR in blood, marrow, lymph nodes, and endothelium, peaking at levels of 9–17% in blood granulocytes but at lower levels in lymphocyte subsets (0.1–0.01%). Circulating progenitors showed high levels of chimerism (up to 60% *neo*^{r}+) with persisting fetal features. At sacrifice, the animal that had no pre-transplant myelosupression showed persisting donor cells in blood and lymph nodes, and in marrow 0.25% of progenitor cells and a detectable fraction of stem cells were *neo*^{r}+. The fetal HSC showed a 10-fold competition advantage over adult HSC. Cloning generated histocompatible HSC capable of long-term multilineage engraftment in a large animal model.

INTRODUCTION

THERAPEUTIC CLONING by somatic cell nuclear transfer offers the potential for treatment of a wide range of degenerative disease. We have used nuclear transplantation in a bovine model to create bioengineered tissues from cardiac and skeletal muscle and renal cells obtained from cloned bovine fetuses, and have shown successful engraftment and functioning of these tissues in nuclear donor animals (Lanza et al., 2002a). Despite expression of a different mitochondrial DNA haplotype, there was no evidence of a rejection response to cloned cells. Cloning strategies also offer the potential of rejuvenation of senescent somatic cells, since successful cloning of a number of calves was achieved using senescent fibroblasts with short telomeres as nuclear donors (Lanza et al., 2002b).

Stem cell transplantation can be curative in marrow failure disorders, various genetic diseases, and hematopoietic malignancies. The success of

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such a strategy is primarily determined by the closeness of the donor-recipient genetic match. Embryonic stem (ES) cell lines derived by nuclear transfer are attractive as an infinitely expandable source of histocompatible, multipotent HSCs amenable to genetic manipulation. Human ES cells have been used to generate hematopoietic cells of erythroid, granulocytic, and megakaryocytic lineage (Kaufman et al., 2001; Chadwick et al., 2003).

In the present study, we have utilized nuclear transfer from somatic cells of old cows together with a *neo*^{r} gene marker to produce a number of cloned bovine fetuses. Fetal liver (FL) from these clones was then used to engraft three nuclear donor cows, with or without busulfan conditioning. The FL serves as a source of lympho-myeloid HSC, endothelial stem/progenitor cells, and mesenchymal stem cells. There is no convincing evidence that FL could be a source of more totipotential stem cells with properties comparable to ES cells. Hematopoietic and lymphoid chimerism was observed in peripheral blood (PB) leukocytes, and circulating progenitors cells, lymph nodes, and bone marrow, and endothelial chimerism in the vasculature of the non-myeloablated cow at 414 days post-transplant.

MATERIALS AND METHODS

Fibroblast isolation and transfection

Dermal fibroblasts were isolated from three 10–13-year-old cows (#31, #33, and #35). Skin fibroblasts were obtained as described (Lanza et al., 2002a) and transfected with a marker phosphoglycerine kinase (PGK)–neomycin resistance cassette (*neo*^{r}). Cells were selected with G418 for >2 weeks, and neomycin-resistant colonies were isolated for nuclear transfer. Experimental protocols were approved by the University of Pennsylvania (Philadelphia, PA) and Advanced Cell Technology (Worcester, MA) Institution Animal Care and Use Committees.

Nuclear transfer and embryo culture

Bovine (*Bos taurus*) oocytes were obtained from abattoir-derived ovaries as previously described (Lanza et al., 2002a). Oocytes were mechanically enucleated at 18–22 h post-maturation, and complete enucleation of the metaphase plate was confirmed with *bis*Benzimide (Hoechst 33342) dye under fluorescence microscopy. A suspension of the transgenic cells was prepared immediately prior to nuclear transfer. The cell suspension was centrifuged at $800 \times g$ and 5 μL of the resulting cell pellet was used for the donor cells. A single cell was selected and transferred into the perivitelline space of the enucleated oocyte. Fusion of the cell–oocyte complexes was accomplished by applying a single pulse of 2.4 kV/cm for 15 μsec. Nuclear transfer embryos were activated as previously described (Presicce and Yang, 1994) with slight modifications (Lanza et al., 2002a). Cleavage rates were recorded, and development to blastocyst stage was assessed on days 7 and 8 of culture. Resulting blastocysts were non-surgically transferred into synchronized heifer recipients.

Busulfan treatment

Nuclear donor cow #33 was administered busulfan (1.0 mg/kg lean weight) via an indwelling catheter (Busulfex™, Orphan Medical, Minnetonka, MN) once daily for 4 days (AUCest of 508 μMol-min, myelosuppressive exposure). Cow #35 was administered 2.0 mg/kg busulfan for 4 days (AUCest of 1326 μMol-min, myeloablative exposure), and cow #31 was untreated. After the final 2-h intravenous infusion, there was a drug washout period of 48 h (cow #35) and 72 h (cow #33) prior to infusion of the FL cells. Blood plasma samples were collected for pharmacokinetic (PK) parameter data analysis at pre- and 0, 15, 30, 60, and 120 min post-busulfan infusion.

Animal #35, which received complete myeloablation, was sacrificed on study day 20 despite a blood transfusion and a course of human recombinant granulocyte colony stimulating factor (Neupogen 1 mcg/kg s.q., MSKCC Pharmacy, New York, NY). Clinical signs included fever, hypoactivity, and decreased appetite. Pathologic examination revealed internal hemorrhages secondary to thrombocytopenia. Animals #31 and #33 survived to scheduled necropsies on study days 414 and 488, respectively.

Fetal liver cell preparation

One control and five cloned fetuses were removed at 100–120 days gestation (crown-rump length, 21–28 cm), and FL cells were dissected out. After mincing with scalpel and scissors, a single cell suspension was prepared by passing the tissue through a metal sieve, followed by Ficoll-Hypaque centrifugation, and resuspension in Iscove's Modified Dulbeccos Medium (IMDM,

GIBCO-BRL, Life Technologies, Grand Island, NY), with 10% FBS. Viable nucleated cell hemocytometer counts were undertaken on trypan blue–stained cell suspensions. Aliquots of <5% of each FL were retained for PCR, and progenitor assay and the remainder were infused into the nuclear donor cows.

Fetal liver transplantation and post-transplant monitoring

Cow #35 received a pool of three FLs from clones #404, #408, and #410, equivalent to 7.0×10^9 ficoll-separated cells; cow #33 received one FL from clone #396, equivalent to 5.4×10^9 cells; and cow #31 received one FL from clone #405, equivalent to 9.3×0^9 cells. Cells were suspended in 1 L, of tissue culture media, infused over 30–60 min. Post-treatment monitoring included daily physical examination; collection of blood (3 mL) for CBC daily for 14 days, then weekly for 3 months; and collection of blood (5 mL) for chemistry screen weekly for 1 month, then monthly for 3 months. Larger volumes of blood were drawn prior to cell infusion, and at intervals thereafter, for flow cytometry, and PCR testing for the *neo*^{r} marker.

To stimulate granulocyte production and mobilize progenitors, cow #31 was treated with rhG-CSF, 1 μg/kg daily for 7 days, beginning at day 183 post-engraftment, and PB samples obtained at baseline and days 3–7.

Femoral and pelvic marrow aspirates were undertaken at intervals. At necropsy, bone marrow was removed from the ribs, and samples of various tissues were isolated for PCR analysis (spleen, liver, intestine, brain, muscle, multiple lymph nodes, arteries, veins). In some cases, viable cells were isolated from minced and sieved tissues, and used for hematopoietic colony assay (marrow, spleen), immunophenotyping (lymph nodes, spleen), and endothelial cell isolation (arteries, veins, marrow, and spleen; and in cow #33, subcutaneous sites of Matrigel injection and cutaneous wound healing sites).

Hematopoietic progenitor cell cultures. Ficoll-Hypaque separated FL cells (1×10^5/mL), and adult PB mononuclear cells or bone marrow cells (2×10^5/mL) were plated in triplicate in 35-mm tissue culture dishes containing 1 mL of assay medium consisting of IMDM, 1.2% methylcellulose (Dow Chemical, Midland, MI), 30% FBS, 5×10^{-5} M-mercaptoethanol, 2 mM L-glutamine (GIBCO), 0.05 mM hemin (Sigma, St Louis, MO), supplemented with recombinant human (rh) cKit Ligand (rhKL 20 ng/mL), rhIL-3 (20 ng/mL), rhG-CSF (100 ng/mL), and rhEpo (6 U/mL). After 14 days of incubation at 37°C in 5% CO_2, granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and multilineage (CFU-GEMM) progenitors were scored.

Long-term culture-initiating cell (LTC-IC) and cobblestone-area forming cell (CAFC) assays. CAFC assays were performed as previously described (Jo et al., 2000). Briefly, appropriate numbers of FL or adult marrow cells were seeded onto confluent MS-5 stroma in 12.5-cm^2 flasks in α-MEM medium plus 12.5% FBS, 12.5% horse serum and hydrocortisone (10^{-6} M), incubated at 37°C in 5% CO_2 in air and demi-depopulated weekly. Cobblestone areas were scored at 2 and 5 weeks using an inverted phase microscope to identify phase-dark hematopoietic areas of at least five cells beneath the stromal layer. The LTC-IC content was determined by assaying for secondary CFC in methylcellulose culture after 5 weeks of stromal co-culture.

Endothelial cell culture. Cells from various vascular sites were dissociated by trypsinization and plated onto petri dishes pre-coated with 0.1% gelatin, in endothelial growth medium consisting of DMEM with low glucose (GIBCO, Grand Island, NY), 20% FBS, L-glutamine 2 mM, bFGF 2 ng/mL, VEGF 10 ng/mL, and heparin 1 μg/mL. Individual endothelial colonies were isolated and expanded. Adherent cells were incubated with 2.4 μg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (Dil-Ac-LDL) for 1 h to confirm endothelial differentiation. DNA extracted from multiple clones was subjected to *neo*^{r} PCR analysis.

DNA extraction. Genomic DNA from cells and tissues was extracted by the Nucleon BACC2 DNA extraction kit (RPN 8502, Amersham Life Science, Buckinghamshire, U.K.) according to the manufacturer's instruction. Tissue specimens were homogenized prior to DNA extraction.

Primer synthesis. Primers were synthesized by Sigma-Genosys (The Woodlands, TX), or the Molecular Biology Core (Mayo Clinic, Rochester, MN) with retention of the 5′-protecting group dimethoxytrityl ^{a}PE (Applied Biosystems, Foster City, CA).

PCR and nested PCR analysis. Polymerase chain reaction (PCR) was performed using a 5′ primer,

Table 1. Total Cell and Hematopoietic Precursor Content of Control and Clone Fetal Livers

		Fetus I.D. (cow I.D.)				
	Control	*#404 (#35)*	*#408 (#35)*	*#410 (#35)*	*#396 (#33)*	*#405 (#31)*
Liver weight (g)	62.0	14.5	13.2	12.3	20.1	29.0
Total cells $\times 10^9$	5.96	3.15	2.47	1.66	5.40	9.30
Total week 5 CAFC $\times 10^5$	9.24	3.00	2.53	2.16	3.00	6.50
Total BFU-E $\times 10^5$	9.8	7.6	16.3	12.0	35.3	47.8
Total CFU-M/CFU-GM $\times 10^5$	3.8	24.2	22.0	24.1	63.2	84.9

CTTGGGTGGAGAGGCTATTC, and a 3′ primer, AGGTGAGATGACAGGAGATC, with 200 ng of genomic DNA template. Thirteen cycles at 94°C for 20 sec, 64°C for 30 sec, and 72°C for 35 sec, 26 cycles of 94°C for 20 sec, 58°C for 30 sec, and 72°C for 35 sec were performed. A 280 bp PCR product was visualized by 2% agarose gel. Nested PCR analysis was carried out using the following primers: first round 5′ primer, CAGCGGCCAATAGCAGCTTTGCTCC; 3′ primer, CCCAGTCATAGC CGAATAGCCTCTCC; second round 5′ primer, CTCCTTCGCTTTCTGGGCTC; 3′ primer, CCCAAGCGGCCGGAGAACC. Cycling parameters were as described above. Twenty nanograms of genomic DNA was used as template and native Pfu DNA polymerase (Stratagene, La Jolla, CA) was used for PCR reaction. Cycling was performed in a T3 Thermocycler (Biometra, Gottingen, Germany), and the 242-bp PCR product was visualized by 2% agarose gel. Semi-quantification of PCR product was established by serial dilution of donor fetal liver genomic DNA in which 100% of FL cells were assumed to be *neo*^{r} positive. The data was expressed as percentage of *neo*^{r} signal in cells versus the equivalent number of control FL cells.

Mitochondrial DNA analyses. Mitochondrial DNA products included all protein-coding sequences, and those encoding tRNAs, ranging in size from 3 to 3.8 kb, were amplified by PCR using Advantage-GC Genomic Polymerase (Clontech) and total genomic DNA templates from clones and nuclear donors sequenced by the Molecular Biology Core (Mayo Clinic, Rochester, MN) as previously described (Lanza et al., 2002a).

RESULTS

Fetal liver

FL weight and total viable nucleated cell recovery are shown in Table 1. Morphologically, 96% of FL cells were erythroblasts at all stages of differentiation with a small percentage of macrophages, and early myeloid and undifferentiated blast cells (Fig. 1A). In the progenitor assay, CFU-GM and CFU-M frequencies were comparable to adult marrow, while BFU-E frequency exceeded adult values (Table 1). Very large "giant" colonies of macrophage and mixed morphology were

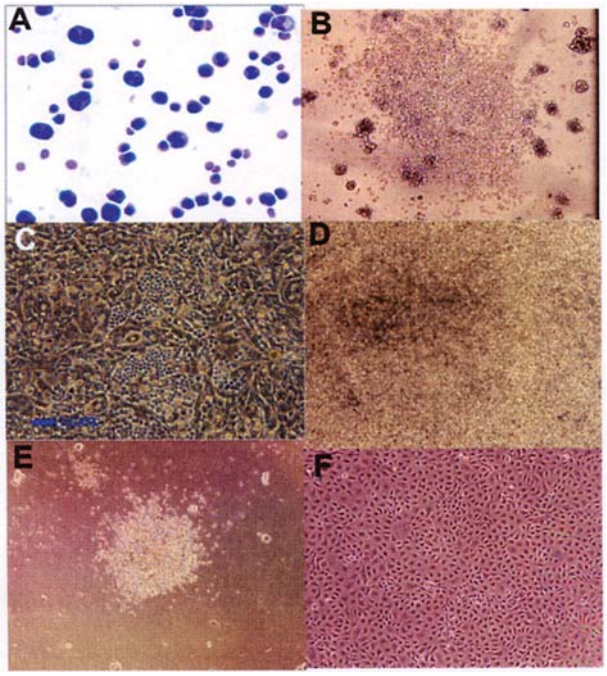

FIG. 1. (**A**) Cytospin preparation of dissociated cloned fetal liver (cloned fetus #405). Note predominance of erythroid lineage. Hematoxylin and eosin stained. ×400. (**B**) Colony types, "giant" HPP-colony in day 14 methylcellulose culture of cloned fetal liver. ×10. (**C**) Cobblestone area formation at week 5 in co-culture of clone fetal liver cell on MS5 stroma. ×100. (**D**) "Giant" HPP-colony in the peripheral blood of cow #31 at 12 weeks after clone fetal liver transplantation. ×10. (**E**) GM-colony in the marrow of cow #31 at necropsy. ×40. (**F**) A cloned population of endothelial cells established from aortic endothelium of cow #31 at the time of sacrifice 414 days post-transplantation. ×100.

noted exclusively in fetal cultures, reminiscent of high proliferative potential (HPP)–CFC (Fig. 1B). In stromal co-culture, cobblestone area formation was observed at 2 and 5 weeks (Fig. 1C), and the latter were scored as a measure of stem cell content, as were the numbers of secondary colonies developing after replating suspension cells and dissociated adherent cells at week 5 (Table 1). By PCR, a strong *neo*^{r} signal was detected in all five cloned livers and in individual hematopoietic colonies (Fig. 2). By progressive dilution of FL cells, a lower limit of detection of the signal by the more sensitive nested PCR technique was established with detection at the 0.001–0.01% level with FL #405, #406, and #408, at the 0.01% level with FL #410, and at the 0.1% level with FL #369 (Fig. 2A–D).

Post-transplant

Hematologic recovery. Cow #35 received 1×10^7 nucleated cells/kg, 15,100 CFC/kg, and 1,099 CAFC/kg, administered 48 h post-busulfan. Cow #33 received 0.77×10^7 nucleated cells/kg, 11,000 CFC/kg, and 429 CAFC/kg administered 72 h post-busulfan. Cow #31 received 1.33×10^7 nucleated cells/kg, 19,000 CFC/kg, and 929 CAFC/kg with no busulfan pretreatment. Cow #35 had the lowest baseline WBC (Fig. 3) and was highly sensitive to busulfan, with PMN falling below 500/μL by day 7 and being almost absent between day 10 and 16, with platelets at 10% of baseline by day 15. Administration of G-CSF failed to stimulate neutrophil recovery, and the animal was euthenized. Cow #33 received half the busulfan dose and a longer washout period before cell infusion. PMN reached a nadir at day 13, with a platelet nadir at day 20 and recovery to near baseline by 23–28 days.

PCR determination of PB engraftment. PCR evaluation of *neo*^{r} expression in nucleated blood cells was undertaken at regular intervals in cows #33 and #31. At the level of sensitivity (0.01%), engraftment was detected at day 42 in cow #31 (Figs.

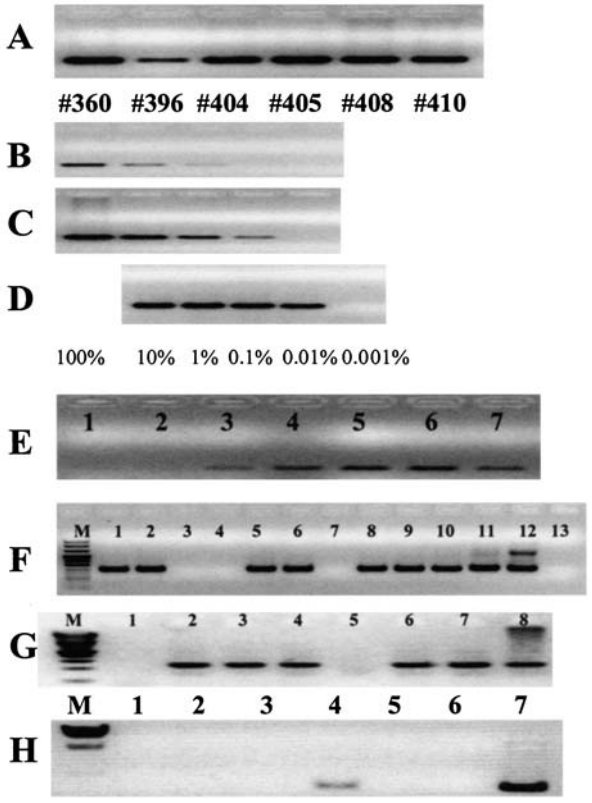

FIG. 2. PCR analysis of *neo*^{r} expression in clone fetal liver, in PB leukocytes and CFC from cows #31 and #33 at intervals post-transplantation, and in various tissues at autopsy. (**A**) Comparison of PCR product from six cloned cow FLs in which 0.2 μg of genomic DNA was used for one round of PCR amplification. (**B**) PCR product of DNA titration from cow FL #396. One round PCR amplification indicated a threshold of detection of 1%. (**C**) PCR product of DNA titration from cow FL#405 indicated a threshold of detection of 0.1%. (**D**) Nested PCR product of DNA titration from cow fetal liver #405 indicating a threshold of detection of 0.01%. (**E**) *Neo*^{r} expression with one round of PCR amplification of 0.2 μg of genomic DNA from PB leukocytes of cow #31 at different time intervals post-transplantation of FL from clone #405; lane 1, d0; lane 2, d9; lane 3, d44 (0.2% *neo*$^{r+}$); lane 4, d86 (1.0% *neo*$^{r+}$); lane 5, d122 (1.85% *neo*$^{r+}$); lane 6, d155 (1.14% *neo*$^{r+}$); lane 7, d177 (1.2% *neo*$^{r+}$). (**F**) Nested PCR analysis of *neo*^{r} expression in various tissues from cow #31 at sacrifice. M, 100-bp DNA ladder. Lane 1, pooled hematopoietic colonies from bone marrow; lane 2, marrow mononuclear cells; lane 3, spleen cells; lane 4, peripheral blood mononuclear cells; lane 5, peripheral blood granulocytes; lane 6, leukocytes generated at week 5 of co-culture of marrow on MS5 stroma; lane 7, leukocytes at week 5 of co-culture of spleen cells on MS5 storma; lanes 8–11, leukocytes at week 5 of co-culture of cow marrow on cow marrow stroma; lane 12, *neo*^{r} plasmid positive control; lane 13, PCR negative control. (**G**) Nested PCR analysis of 0.1 μg of genomic DNA from lymphoid tissue of cow #31 at sacrifice. M, 100-bp ladder. Lane 1, negative control; lanes 2–4, pre-scapular, prefemoral, and supra-mammary lymph nodes; lane 5, spleen; lanes 6–8, jejunal, inguinal, and ileocecal lymph nodes, respectively. (**H**) PCR analysis of *neo*^{r} expression in cloned endothelial populations established from vascular tissue of cow #31. M, Hind III marker. Lane 1, negative control; lanes 2 and 3, endothelial clones from the aorta; lane 4, *neo*$^{r+}$ aortic endothelial clone illustrated in Figure 1F; lanes 5 and 6, endothelial clones from vena cava; lane 7, *neo*^{r} plasmid positive control.

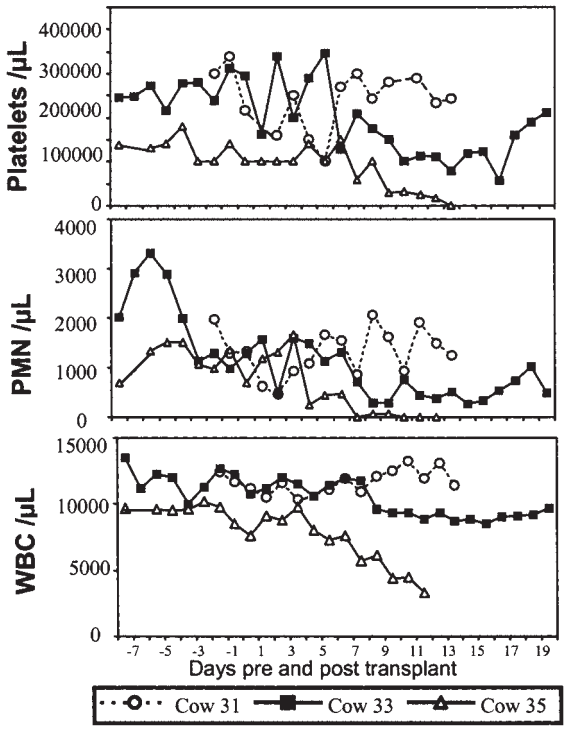

FIG. 3. Peripheral blood parameters (platelet, WBC, and PMN/μL) at intervals from pre-treatment baseline (cows #31, #33, #35) through busulfan treatment (cows #33, #35), and for 2–3 weeks after (day 0) transplantation of clone FL cells. Cow #35 was sacrificed at 12 days post-transplant.

2E and 4), peaking at day 86 (1.85%) and remaining elevated until day 178, when a *neo*^{r} signal was detected in the PMN fraction (0.96%) but not in the mononuclear fraction. Since the *neo*^{r} signal was expressed at levels of 0.01–0.1% in purified lymphocytes obtained between weeks 12 and 24, the majority of observed chimerism was confined to the PMN and monocyte population, comprising 10–17% of the PB leukocytes analyzed over this period. The calculated fetal donor contribution to the myeloid compartment during this period was 7–19%. The loss of a *neo*^{r} PCR signal at day 184 prompted us to administer G-CSF (1 μg/kg) daily for 7 days to stimulate PMN production and mobilize CFC. PMN rose sevenfold (to 15,252/μL). *Neo*$^{r+}$ cells were first detected in PMN fraction after 6 days of G-CSF, and 2.16% of PMN were *neo*$^{r+}$ at day 7 (Figs. 2E and 4). At necropsy on day 414, *neo*$^{r+}$ cells were still present in the PMN fraction at a low level (0.1%), but were not detected in the mononuclear cell population. In cow #33, *neo*$^{r+}$ cells were present in the PB leukocyte population at 12 and 18 weeks at levels corresponding to 7–10% of the granulocyte/monocyte population, but fell below detectable levels at later stages (Fig. 4).

Progenitor assay of PB mononuclear cells revealed absence or low numbers (<1 per 2×10^5), of circulating CFC in all three old cows prior to treatment. In cow #31 post-treatment, circulating CFU-GM and BFU-E/CFU-E were present between day 8 and 177, but not thereafter (Figs. 1, 4, and 5). PCR analysis was possible with individual colonies of >200 cells and showed no *neo*$^{r+}$ colonies at day 22 (0/17), 10% *neo*$^{r+}$ at day 43 (2/20), 44% at day 87 (8/18), 25% at day 123 (4/16), and 60% at day 178 (12/20) (Fig. 5). Coinciding with the presence of FL-derived progenitors in circulation was the appearance of "giant" colonies or HPP-CFC, and these comprised the majority of progenitors at day 177 (Fig. 1D). In cow #33, elevated numbers of circulating CFC were detected from days 14 through 128 (Fig. 5). The initial wave of circulating progenitors coincided with the time of recovery from busulfan-induced myelosuppression, and at this stage, the colonies were almost exclusively CFU-GM and *neo*^{r} negative. *Neo*$^{r+}$ CFC comprised 5.6% and 27% of total CFC at day 56 and 86, respectively, with HPP-CFC comprising 50% of CFC at day 86 (Fig. 5). The almost 10-fold lower level of *neo*^{r} expression in the cloned FL used to engraft cow #33 (Fig. 2B) resulted in a higher threshold (0.1%) for detection of *neo*$^{r+}$ cells. This could account for detection of *neo*$^{r+}$ circulating CFC at day 56, with almost no detectable *neo*^{r} signal in PB leukocytes.

Bone marrow hematopoiesis and detection of neo^{r+} *cells.* Attempts to obtain bone marrow by aspiration of long bones or pelvis were unsuccessful due to replacement of red marrow by yellow adipose tissue in these sites, which is normal in cows of this age. At necropsy, red marrow was found in ribs and was assayed for CFC and CAFC. The marrow of cow #33 had lower frequency ($30 \pm 8/10^5$) of CFC (Fig. 1E) than in clone FLs ($160 \pm 20/10^5$, $n = 5$), while cow #31 had a higher frequency ($208 \pm 36/10^5$). The CAFC frequency in the marrow of cow #33 ($0.62 \pm 0.20/10^5$) and cow #31 ($1.8 \pm 0.40/10^5$) was significantly lower than that in clone FLs ($9.1 \pm 1.3/10^5$, $n = 5$). The number of secondary CFC per CAFC was 3.3 and 3.7 in cow #33 and #31 respectively, similar to the 2.7 obtained with FL CAFC.

PCR analysis was undertaken on primary and

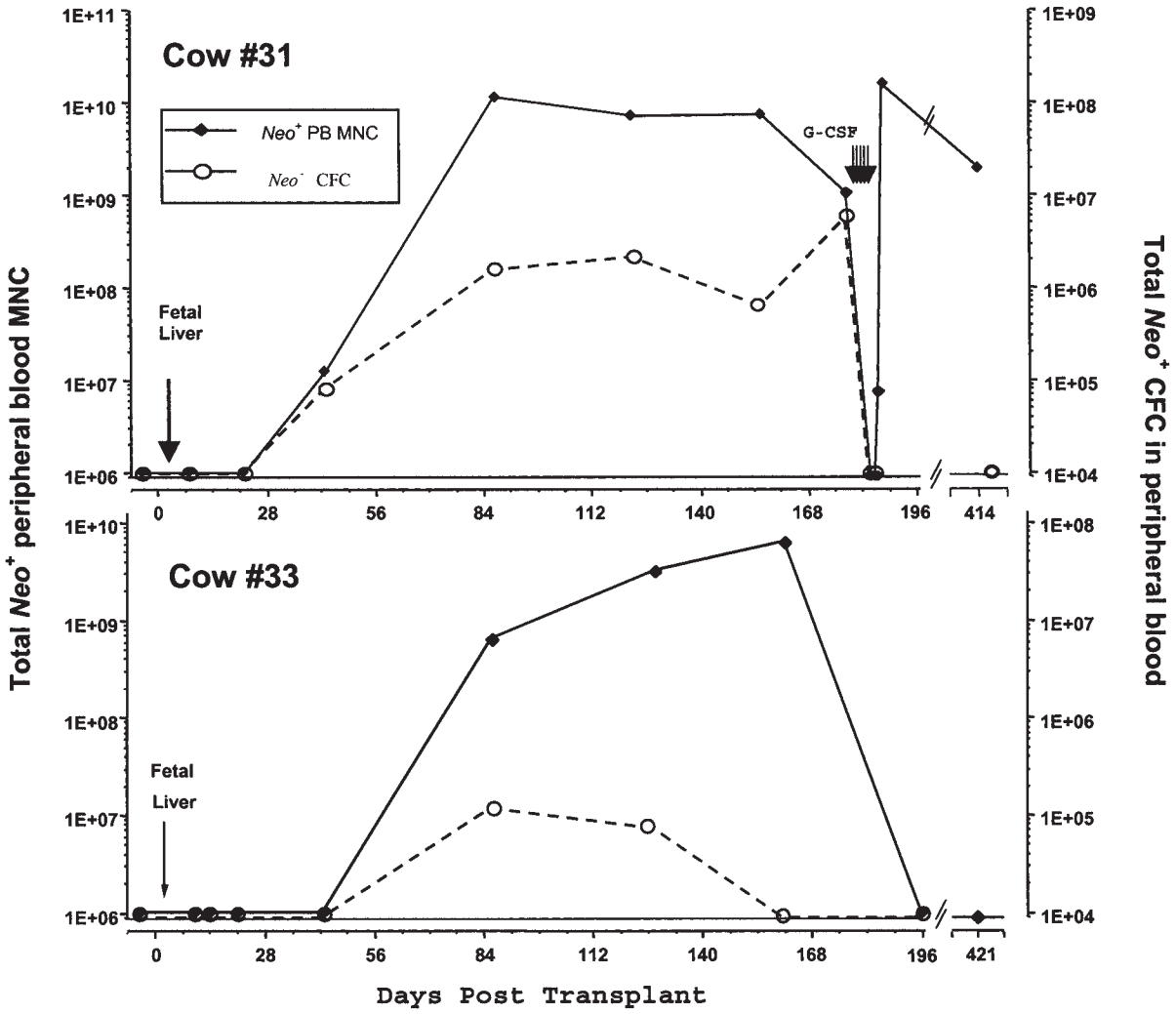

FIG. 4. Graphs showing the absolute numbers of *neo*$^{r+}$ leukocytes (solid diamonds), and *neo*$^{r+}$ hematopoietic colonies (open circles) in the peripheral blood of cows #31 and #33 at intervals following transplantation of clone FL cells. Date based on the *neo*$^{r+}$ frequency determined by PCR of leukocyte DNA, or on the frequency of *neo*$^{r+}$ hematopoietic colonies that simultaneously were positive for β-actin. Total numbers calculated on observed WBC counts and an estimated blood volume of 50 L. On day 181 post-transplant, cow #31 was treated with 1 μg of recombinant human G-CSF (Neupogen) per kg daily for 7 days.

secondary hematopoietic colonies (Fig. 2F). Colonies were pooled (100 per pool), and 5/20 pools from cow #31 were *neo*$^{r+}$, indicating that ~0.25% of the marrow progenitor pool was derived from cloned FL at day 414. Analysis of secondary colonies from week 5 stromal co-culture of bone marrow of cow #31 showed no *neo*$^{r+}$ in a total of 110 colonies analyzed (Fig. 2F). However, a *neo*^{r} signal was obtained from suspension cells generated at week 5 from three separate marrow-stromal co-cultures, indicating that up to 1% of cells generated long-term in these cultures was of FL origin (Fig. 2E). In cultures of cow #33 marrow, nested PCR analysis of 400 primary colonies and 40 secondary colonies, as well as suspension cells at week 5, failed to detect a *neo*^{r} signal.

Detection of the neo^{r} *transgene in lymphoid tissue*

Genomic DNAs from PBLs of cow #33 (2, 12, and 24 weeks) and cow #31 (2, 3, and 24 weeks) were amplified in *neo*^{r}-specific, nested PCRs. All genomic DNA samples, with exception of the 2-week sample from cow #31, yielded relatively faint *neo*^{r}-specific products that were considerably lower than the amounts obtained with ter-

minal lymphoid tissues from cow #31. Lymphocytes were harvested at time of sacrifice from seven lymph node sites in cow #31 and six lymph node sites in cow #33. Genomic DNAs from these lymphocyte samples, without cell-type–specific separations, were used as templates in nested PCRs to assay for the presence of the *neo*^{r} transgene. Although no *neo*^{r}-specific amplification was observed with lymphoid samples from cow #33, the *neo*^{r} transgene was amplified from all lymph node samples harvested from cow #31, but not from spleen (Fig. 2G). The need for nested PCRs to generate PCR products was an indicator that the population of lymphocytes derived from cloned cells was relatively small at the time of sacrifice, probably at the level of 0.1–0.01%.

The persistence of cloned cells in lymphoid tissues of cow #31 prompted us to return to the analysis of PBL samples from cow #31 and #33 at sequential timepoints following transplantation. As a group, these data suggest that transplanted, cloned FL stem cells engrafted in lymphoid tissue and, in cow #31, persisted to time of sacrifice. However, their contribution to circulating lymphoid cell populations was limited.

Detection of neo^r *in vascular endothelium*

At the time of autopsy of cow #31, endothelium was isolated from a number of vascular sites (aorta, vena cava, major and minor arteries and veins), dissociated, and cultured under endothelial growth conditions. Colonies of Dil-Ac-LDL+ endothelial cells were individually isolated and clonally propagated in culture prior to DNA extraction and evaluation of *neo*^{r} expression (Fig. 1F). Of 20 clones an-

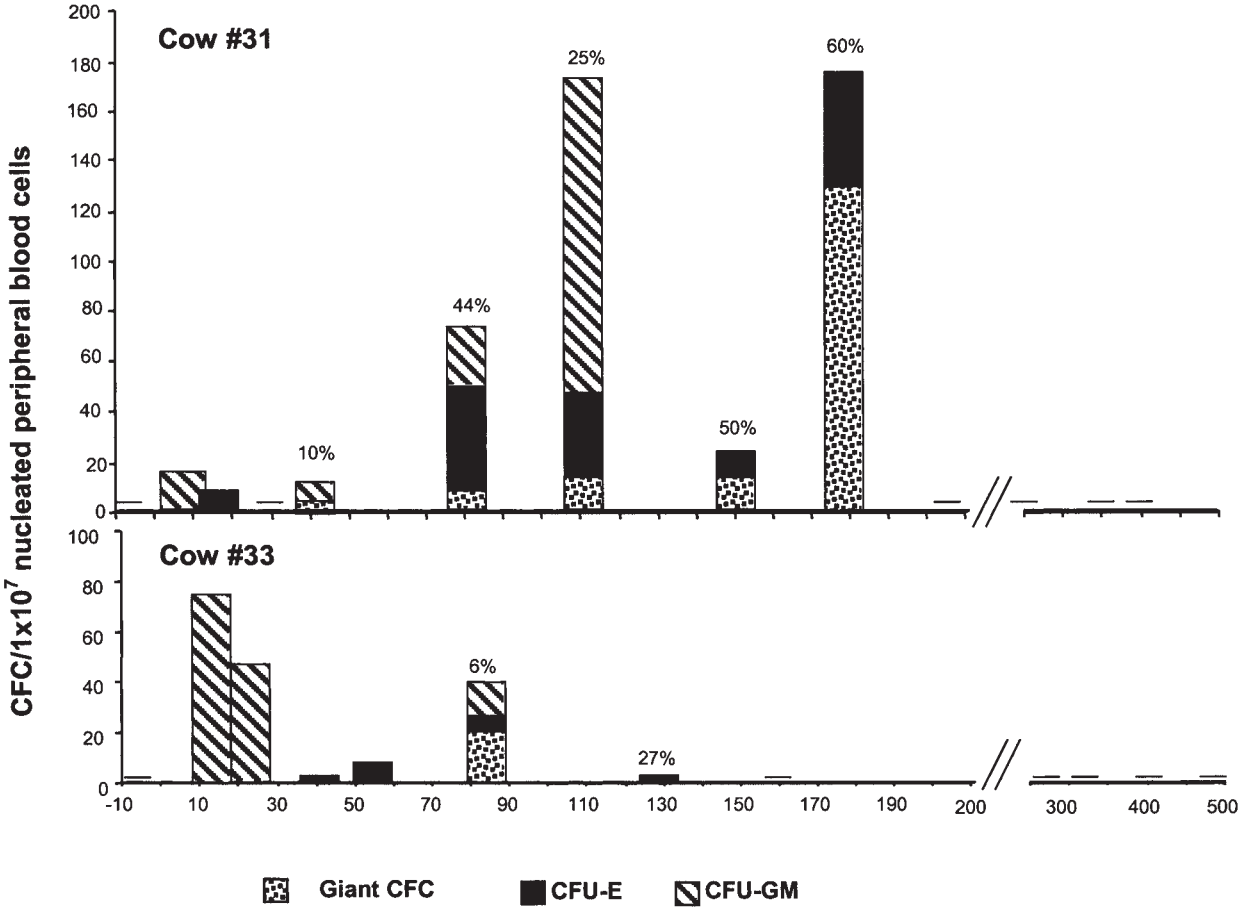

FIG. 5. Hematopoietic colony numbers in Ficoll-separated peripheral blood obtained at various intervals post-transplantation in cows #31 and #33. Colonies were typed as "giant" high proliferative potential, erythroid, predominantly forming single erythroid colonies (solid squares CFU-E) rather than multifocal BFU-E, and granulocyte-macrophage (CFU-GM). Based on analysis of 150 colonies, the percentages of colonies that were positive for *neo*^{r} and β-actin are shown. Colonies appearing in the circulation in the first 2 weeks were predominantly small GM type, and were either negative for *neo*^{r} or were excluded due to lack of a β-actin signal.

alyzed, one, of aortic origin, was *neo*$^{r+}$ (Fig. 2H). Prior to sacrifice, cow #33 was implanted with Matrigel subcutaneously, and small incisions were established to stimulate wound healing. At autopsy, endothelial cells were obtained from these sites as well as from various blood vessels. Endothelial cells were successfully propagated from all these sites, but none of the 27 analyzed were found to be *neo*$^{r+}$.

PCR analysis of tissues from other sites

Tissue samples obtained from brain, liver, kidney, pancreas, heart, intestine, skeletal muscle, and skin of cow #31 and #33 gave no detectable *neo*^{r} signal.

*Mitochondrial DNA (*mt*DNA) analysis*

Nucleotide sequencing revealed seven nucleotide substitutions for the FL#405/cow#31 combination, with three of these resulting in an amino acid interchange (I>T, V>I, T>A) (Table 2). In the FL#396/cow #33 combination, there were eight nucleotide substitutions and one amino acid interchange (A>V).

DISCUSSION

This study is based on analysis of five cloned fetal calves and engraftment into three adult animals. Each engrafted animal was treated as an individual subject, allowing us to provide a case report on individual survival and engraftment status. While small animal transplant studies usually present pooled data on groups of animals, this was not logistically feasible in the large animal experiments that we have undertaken. In one animal (cow #31), with no myelosuppression, hematopoietic chimerism with *neo*^{r} marked circulating leukocytes was detected from 6 weeks post-transplantation up to time of sacrifice at 59 weeks post-transplant. The level of chimerism was 1–2% for 12–27 weeks, falling to 0.1–0.2% over the subsequent 32 weeks. PCR analysis of PB lymphocytes indicated a level of chimerism of 0.01–0.1%. Consequently, the majority of circulating *neo*$^{r+}$ cells were granulocytes and monocytes. By adjusting for the frequency of these in the PB for 12–27 weeks (10–17%), it can be estimated that 7–19% of circulating myeloid lineage cells were derived from FL during this period, with production of $\sim 3.4 \times 10^{12}$ PMN over the 59 weeks of engraftment. The extent of PB chimerism in the busulfan treated cow #33 was less than in cow #31, being observed only between 12 and 23 weeks post-transplantation. The inability to detect *neo*$^{r+}$ cells at earlier or later time-points, including all tissues analyzed at autopsy at 69 weeks, probably reflects the 10-fold lower sensitivity of PCR detection attributed to the lower *neo*^{r} copy number in the nuclei used to generate clone #396 versus clone #405. The

Table 2. Mitochondrial Nucleotide and Amino Acid Substitutions that Distinguish the Clones from Fibroblast Nuclear Donors

Clone nucleotide	*Donor nucleotide*	*Position*[a]	*Gene*	*Amino acid substitution*
Clone 31 vs. fetal liver 405				
G	A	69	ND1	—
T	C	1,599	ND2	I>T
A	T	1,684	ND2	—
G	A	7,984	ND4	V>I
G	A	9,425	ND5	—
A	G	10,167	ND5	T>A
C	T	10,439	ND5	—
Clone 33 vs. fetal liver 396				
T	C	1,312	ND2	—
C	T	2,162	ND2	A>V
C	T	4,751	CO II	—
T	C	5,246	ATPase6	—
A	G	8,376	ND4	—
G	A	9,605	ND5	—
C	T	10,864	ND6	—
T	C	12,329	Cyt B	—

[a]Relative to start of protein-encoding sequence.

dose of busulfan and the initial 48-h washout period used to treat cow #35 was based upon human pharmacokinetic data, and subsequent irreversible myeloablation was not reversed by FL grafting. It should be noted that this cow had baseline PMN and platelet levels a third and a half, respectively, of the values in cow #31 and #33, and may thus have been more susceptible to busulfan, resulting in myeloablation rather than the projected myelosuppression. The number of FL cells engrafted in this animal was 10.4×10^6/kg, and the number of CFC was 15,000/kg, which may have been insufficient to achieve adequate platelet and neutrophil reconstitution within a time frame compatible with survival. It should be noted that, in human transplantation with umbilical cord blood, a minimum number of 15×10^6 nucleated cells/kg or >50,000 CFC/kg is considered necessary to achieve optimal engraftment, and avoid delayed platelet and neutrophil recovery with associated morbidity and mortality (Grewal et al., 2003; Migliaccio et al., 2000). Cow #33 received a lower dose of busulfan and had a 72-h washout period prior to engraftment. Busulfan blood plasma measurements showed that we achieved an Area Under the Curve of 508 μMol-min—a range considered nonmyeloablative in humans. While this resulted in modest myelosuppression with return of PMN and platelets to baseline levels within 3–4 weeks, it did not appear to provide a selective advantage for engrafted cells as reflected in the levels of PB chimerism.

Circulating progenitors were rare in normal cow blood and in pre-transplant blood in the three old cows; however, a wave of progenitor cells appeared in cow #33 blood 2–3 weeks after busulfan treatment, coinciding with recovery from myelosuppression. These progenitors were not derived from the graft and were exclusively CFU-GM. A second wave of progenitors appeared in the blood of cows #31 and #33 between 6 and 18–25 weeks. A significant percentage of these progenitors were of FL origin (up to 60%), and a substantial number formed giant colonies containing ~100,000 cells. Colonies of this type were found in FL cultures, but not in adult marrow at autopsy. In addition, substantial numbers of erythroid colonies of a CFU-E rather than BFU-E type were present, and these also were not seen in significant numbers in normal circulation. Based upon the level of granulocyte chimerism, it is likely that the contribution of FL to the progenitor population of the marrow at this time was in the range of 1–5%; thus, the FL-derived progenitors must have had a propensity to mobilize relative to their adult counterparts.

Assuming a 24-h circulating lifespan of progenitors, cow #31 generated 2.56×10^9 circulating neo^{r+} CFC during this mobilization period. It is possible that FL-derived stem/progenitor cells mobilize into circulation as a consequence of ontogenetic programming, recapitulating the egress of such cells from the liver to colonize developing bone marrow. At time of sacrifice at week 59, cow 31# was still chimeric but at a lower level than seen between 12 and 27 weeks. The 0.1% neo^{r+} cells in marrow represents 1.5×10^{10} cells of clone FL origin. Since 0.25% of the marrow CFC were also neo^{r+} and knowing the frequency of CFC in the marrow and estimating total marrow volume to be 1.5×10^{13} cells, there were 7.7×10^7 CFC of fetal origin in the marrow and approximately 6.7×10^5 stem cells as defined by CAFC assay. Murine studies have shown that FL was fivefold more effective than adult marrow in providing long-term repopulation (Harrison et al., 1997). This may be attributed to a sevenfold higher concentration of HSC in FL (Morrison et al., 1995) and threefold greater clonal expansion of fetal HSC relative to adult (Silvassy et al., 2003). Studies in non-ablated mice have challenged the assumption that myeloablation was necessary to create "niches" for colonization by engrafted stem cells (Ramshaw et al., 1995). The extent of chimerism obtained in non-myeloablated mice reflects the number of HSCs injected relative to total HSC pool of the host. We can estimate that the fetal liver graft in cow #33 was 0.03% and in cow #31 0.06% of total adult marrow population of 1.5×10^{13} nucleated cells. At the peak of engraftment, the FL-derived cells comprised 0.5–2% of PB leukocytes and up to 19% of the circulating myeloid cells, while at sacrifice over a year post-transplant, the total number of engrafted cells were close to the number transplanted. The greater expansion of donor cells at 4–6 months relative to >12 months could be explained by a higher frequency of short-term repopulating HSC, relative to rarer long-term repopulating HSC in the FL inoculum. There is no data in human or large animal systems defining the minimal duration of engraftment that would be necessary to satisfy the criteria of long-term repopulation, but we believe that it is a reasonable assumption that hematopoietic engraftment beyond 12 months would require HSC that fit this criteria. The bovine FL obtained at 100–120 days

of gestation was approximately mid-way through the phase of hepatic hematopoiesis (Moore and Williams, 1973) and by analogy with murine development, extensive further stem cell expansion would be anticipated in the liver over a further 80–100 days. Based on total cell and stem cells infused, transplantation of whole liver from older cloned fetuses into unconditioned cows would be expected to give significantly higher levels of long-term chimerism.

Participation of transplanted cells in angiogenesis/vasculogenesis was evaluated by PCR analysis of endothelial cells grown from different sites in both cows at necropsy. Only one endothelial clone, derived from the aorta of cow #31, was neo^{r+} so it is not possible to determine with any accuracy the degree of participation donor cells in host endothelium. Nevertheless, the data support the concept that endothelial progenitors within adult marrow (Lyden et al., 2001) and FL (Shi et al., 1998) participate in neovascularization.

In our previous bovine study (Lanza et al., 2002a), where there was a two–amino acid substitution distinguishing donor from host mitochondrial proteins, there was no evidence of rejection of cloned tissue. Furthermore, there was no evidence of anti-mitochondrial protein immunity through both delayed type hypersensitivity testing *in vivo* and Elispot analysis of IFNγ-secreting T cells *in vitro*. The presence of substantial numbers (1×10^{10}) of hematopoietic cells and progenitors at 414 days post-engraftment in cow #31 with three amino acid substitutions distinguishing donor from host further attests to a lack of detectable rejection response specific for mitochondrial-encoded proteins expressed by cloned FL-derived cells.

This study has extended the use of clone-derived populations that can be transplanted without rejection into adult nuclear donors to the lymphohematopoietic and endothelial lineages. It has also shown that clone-derived HSCs present in FL have a competitive advantage over the recipient HSC population and can engraft in an unmanipulated adult animal. The low degree of lymphoid engraftment relative to myeloid suggests that, in old cows, aging-associated defects in the environment of primary and secondary lymphoid organs impair lymphoid reconstitution. Improvement in engraftment may be anticipated if the number of stem cells transplanted were to be increased, either by utilizing older fetuses or pooling FL. Alternatively, more effective myeloablation strategies could be employed.

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REFERENCES

Chadwick, K., Wang, L., Li, L., et al. (2003). Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood 102, 906–915.

Grewal, S.S., Barker, J.N., Davies, S.M., et al. (2003). Unrelated donor hematopoietic cell transplantation: marrow or umbilical cord blood? Blood 101, 4233–4244.

Harrison, D.E., Zhong, R.K., Jordan, C.T., et al. (1997). Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term. Exp. Hematol. 25, 293–297.

Jo, D.Y., Rafii, S., Hamada, T., et al. (2000). Chemotaxis of primitive hematopoietic cells in response to stromal cell–derived factor–1. J. Clin. Invest. 105, 101–111.

Kaufman, D.S., Hanson, E.T., Lewis, R.L., et al. (2001). Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. USA 98, 10716–10721.

Lanza, R.P., Chung, H.Y., Yoo, J.J., et al. (2002). Generation of histocompatible tissues using nuclear transplantation. Nat. Biotechnol. 20, 689–696.

Lanza, R.P., Cibelli, J.B., Blackwell, C., et al. (2002). Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. Science 288, 665–669.

Lyden, D., Hattori, K., Dias, S., et al. (2001). Impaired recruitment of bone-marrow–derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat. Med. 7, 1194–1201.

Migliaccio, A.R., Adamson, J.W., Stevens, C.E., et al. (2000). Cell dose and speed of engraftment in placental/umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. Blood 96, 2717–2722.

Moore, M.A., and Williams, N. (1973). Analysis of proliferation and differentiation of foetal granulocyte-macrophage progenitor cells in haemopoietic tissue. Cell Tissue Kinet. 6, 461–476.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M., et al. (1995). The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. USA 92, 10302–10306.

Presicce, G.A., and Yang, X. (1994). Parthenogenetic development of bovine oocytes matured *in vitro* for 24 hr and activated by ethanol and cycloheximide. Mol. Reprod. Dev. 38, 380–385.

Ramshaw, H.S., Crittenden, R.B., Dooner, M., et al. (1995). High levels of engraftment with a single infusion of bone marrow cells into normal unprepared mice. Biol. Blood Marrow Transplant. 1, 74–80.

Shi, Q., Rafii, S., Wu, M.H., et al. (1998). Evidence for circulating bone marrow–derived endothelial cells. Blood 92, 362–367.

Szilvassy, S.J., Ragland, P.L., Miller, C.L., et al. (2003). The marrow homing efficiency of murine hematopoietic stem cells remains constant during ontogeny. Exp. Hematol. 31, 331–338.

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