Supplemental Material

Supplemental Methods

TF-1a lymphoblastic leukemia cell line: marking with GFP, phenotyping and sorting

In order to determine if the multi-parameter FACS approach would be successful across different human malignancies, another human leukemic cell line, TF-1a was used in a second spiked sorting experiment. TF-1a, a lymphoblastic cell line derived from a 35year old Japanese male with erythroblastic leukemia was obtained from American Type Culture Collection (ATCC)(1). Previous sensitivity analyses had demonstrated that TF-1a cells do not form solid tumors as consistently as MOLT-4 cells when transplanted into the testes of immune-deficient nude mice. Thus, TF-1a cells were transduced with a lentivirus containing GFP driven by the ubiquitin-C promoter (generously provided by Dr. Carlos Lois, University of Massachusetts (2)) to enable tracking of malignant cells through the multi-parameter FACS experiments. The cell culture was then expanded and cloned by limiting dilution. Cells derived from a single GFP-expressing clone, TF-1a (C2), were used for all experiments in this study. Cultures were established in RPMI-1640 media (GIBCO, Invitrogen) with 10% FBS and supplemented with antibiotic-antimycotic solution containing penicillin, streptomycin, and amphotericin (Anti-Anti, GIBCO Invitrogen Cell Culture). Fresh media was added every 2-3 days and cells were passaged at or before they reached a density of 2×10^6 cells/mL as per manufacturer recommendations.

Initial flow cytometry experiments using the TF-1a-GFP clone demonstrated that over >95% of cells expressed the markers CD45 and CD49e, but not HLA-ABC (as we had observed for the MOLT-4 leukemic cells). Additionally, EpCAM was expressed on <1% of the TF-1a cells. Thus, our multi-parameter sorting approach with TF-1a utilized CD45-PE and CD49e-PE as markers for TF-1a leukemic cells, and EpCAM-APC as a marker of spermatogonia. Spiked

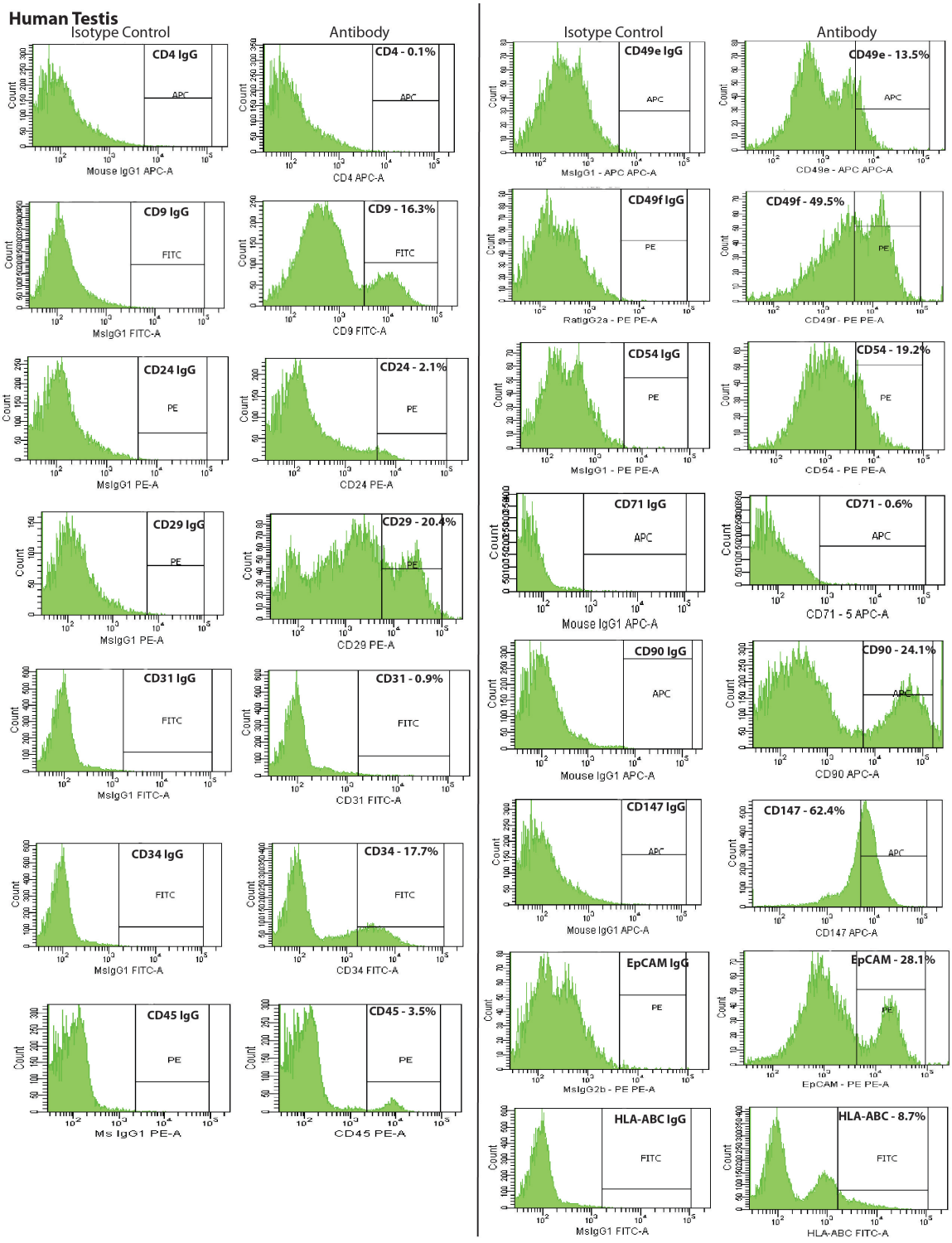
sorting experiments were carried out as described in the main body of the manuscript by adding TF-1a-GFP cells to a suspension of human testicular cells and performing multi-parameter FACS.

Immunohistochemistry of testicular tumors with NuMA

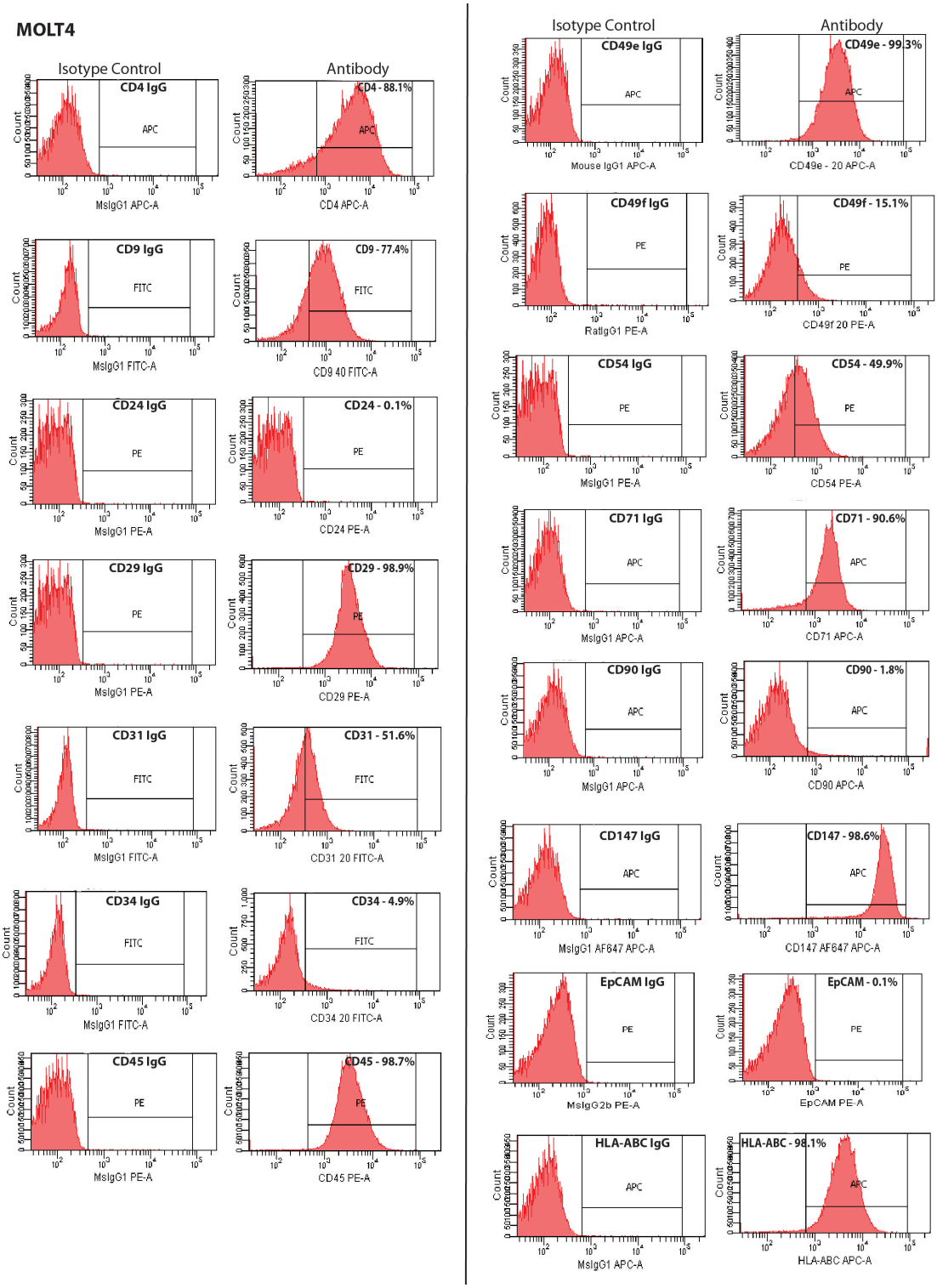
In order to demonstrate that the testicular tumors observed after transplantation of MOLT-4 cells and the $EpCAM^{-}/CD49e^{+}/HLA\text{-}ABC^{+}$ fraction resulted from the MOLT-4 cells injected and are of human origin, immunohistochemistry was performed with a human-specific polyclonal antibody directed against the nuclear mitosis apparatus protein (NuMA), a protein involved in the formation and maintenance of the mitotic spindle. To accomplish this, the tumors were fixed with 4% paraformaldehyde overnight, paraffin-embedded and sectioned (5 μm). The tissue slides were then deparaffinized, rehydrated, and incubated in sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% Tween-20) for antigen retrieval. The tissue was then blocked with a buffer containing 3% bovine serum albumin and 5% normal serum from the host species of the secondary antibody. Subsequently, sections were stained for 90 minutes at room temperature with anti-NuMA antibody (1:100, Abcam, Cambridge, MA). Isotype matched normal IgG was used as negative control. Primary antibody was detected using AlexaFluor-488 conjugated secondary antibody. The slides were mounted with VectaShield mounting medium containing DAPI (Vector Laboratories) for detection of nuclei. Sections were observed with a Nikon Eclipse E600 Fluorescence microscope and images captured with MetaView Digital Imaging software.

Supplemental Table 1: Expression pattern of various cell surface antigens on MOLT-4 lymphoblastic leukemia cells and human testicular cell suspensions (expressed in percentage)

Cell surface antigen	MOLT-4	Human testis
CD4	75.9 ± 13.5	0.14 ± 0.02
CD9	68.9 ± 6.0	16.1 ± 0.4
CD24	0.17 ± 0.07	2.46 ± 0.3
CD29 (β1 integrin)	98.3 ± 0.5	24.4 ± 3.7
CD31 (PECAM-1)	51.8 ± 2.3	1.1 ± 0.3
CD34	4.5 ± 1.7	15.96 ± 1.1
CD45	97.9 ± 0.9	3.56 ± 0.6
CD49e (α5 integrin)	97.6 ± 0.9	23.2 ± 4.2
CD49f (α6 integrin)	12.5 ± 1.7	53.2 ± 8.7
CD54	56.1 ± 7.7	26.1 ± 3.5
CD71 (transferrin receptor)	91.6 ± 1.0	0.46 ± 0.2
CD90 (Thy 1)	2.2 ± 0.5	21.1 ± 1.3
CD147	98.3 ± 0.5	53.1 ± 14.6
CD326 (EpCAM)	0.1 ± 0.0	16.4 ± 3.9
HLA-ABC	96.9 ± 1.2	9.42 ± 0.7



Supplemental Figure 1A: Histograms from flow cytometry demonstrating the percentage of human testicular cells that stained positively for the cell surface antigens analyzed, relative to isotype (IgG) controls. Gating was established to exclude 99.9% of isotype control events.



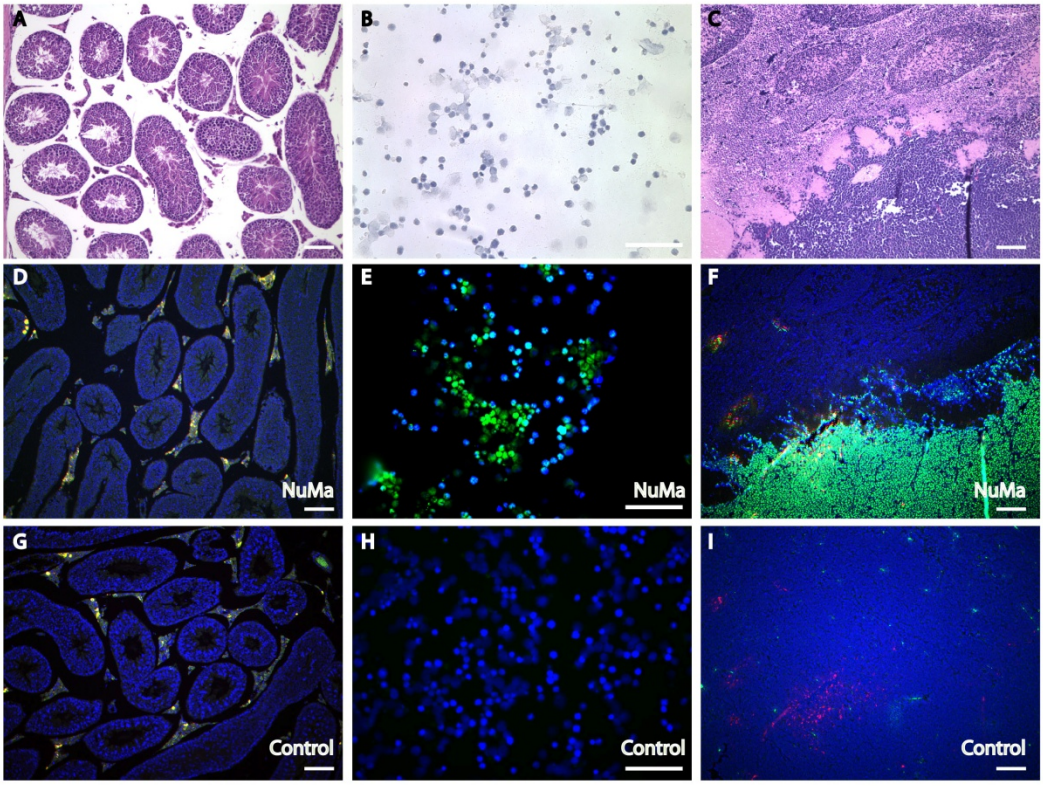
Supplemental Figure 1B: Histograms from flow cytometry demonstrating the percentage of MOLT-4 leukemic cells that stained positively for the cell surface antigens analyzed, relative to isotype (IgG) controls. Gating was established to exclude 99.9% of isotype control events.

Supplemental Table 2: Characteristics of human testis organ donors

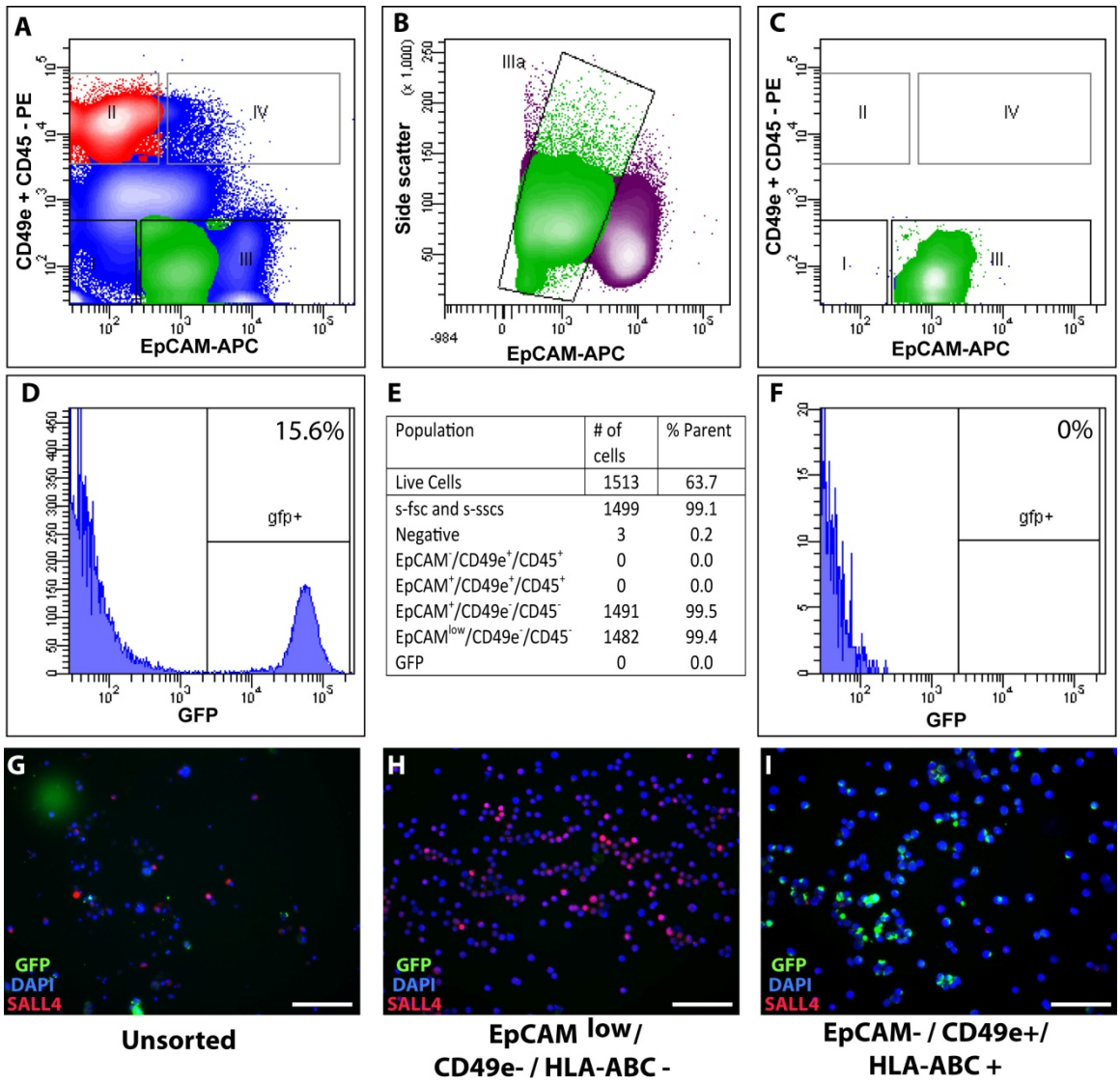
Donor	Age range	Race	Time to tissue processing (hours)	Cell viability prior to cryopreservation
1	10-19	Caucasian	11	93.2%
2	21	Caucasian	7.5	91.9%
3	20-29	Unknown	14	92.6%
4	14	Caucasian	12.5	90.2%
5	16	Caucasian	8	79.1%
6	40-49	Caucasian	20.8	96.3%
7	41	Unknown	9	92.7%

Supplemental Table 3: Manufacturing company, fluorophore, clone, and staining concentration of antibodies used in flow cytometry with human testicular cell suspensions and the MOLT-4 cell line

Cell surface antigen	Antibody-conjugated fluorophore	Company	Clone	Concentration
CD4	APC	BD Pharmingen	L200	20 uL/10^6 cells
CD9	FITC	BD Pharmingen	M-L13	20 uL/10^6 cells
CD24	PE	BD Pharmingen	2H7	10 uL/10^6 cells
CD29 (β1 integrin)	PE	BD Pharmingen	MAR4	40 uL/10^6 cells
CD31 (PECAM-1)	FITC	BD Pharmingen	WM59	20 uL/10^6 cells
CD34	FITC	AbCAM	QBEND/10	20 uL/10^6 cells
CD45	PE	BD Pharmingen	TU116	20 uL/10^6 cells
CD49e (α5 integrin)	APC PE	BioLegend	NK1-SAM-1	20 uL/10^6 cells
CD49f (α6 integrin)	PE	BD Pharmingen	GoH3	20 uL/10^6 cells
CD54	PE	BioLegend	HCD54	20 uL/10^6 cells
CD71 (transferrin receptor)	APC	BD Pharmingen	L01.1	10 uL/10^6 cells
CD90 (Thy 1)	APC	BD Pharmingen	5E10	1 ug/10^6 cells
CD147	AlexaFluor 647	BioLegend	HIM6	5 uL/10^6 cells
CD326 (EpCAM)	PE	BioLegend	9C4	20 uL/10^6 cells
CD326 (EpCAM)	APC	BioLegend	9C4	5 uL/10^6 cells
HLA-ABC	APC	BD Pharmingen	G46-2.6	20 uL/10^6 cells



Supplemental Figure 2: Testicular tumors observed after tranplantation of the EpCAM$^-$/CD49e$^+$/HLA-ABC$^+$ fraction following FACS are of human origin. **A, D and G)** Cross-section from nude mouse testis demonstrating normal architexture of the seminiferous tubules stained with hematoxylin and eosin (H&E, Panel A). Panel D demonstrates negative staining with the human-specific nuclear mitotis apparatus protein (NuMA). Panel G is stained with an IgG isotype control. **B, E and H)** MOLT-4 leukemic cells stained with H&E (panel B), NuMA antibody (panel E), and with an IgG isotype control (panel H). NuMA is expressed by a variety of human malignancies, including MOLT-4 leukemic cells, as demonstrated in panel E. **C, F and I)** Testis from nude mouse demonstrating gross tumor formation following transplantation of EpCAM$^-$/CD49e$^+$/HLA-ABC$^+$ cells. Disruption of the normal architexture of the seminiferous tubules by the MOLT-4 leukemic cells can be visualized in panel C (bottom right), and these cells stain positively for NuMA (panel F). Panel I is MOLT-4-derived tumor stained with an IgG isotype control. Scale bar = 100μm.



Population	# of cells	% Parent
Live Cells	1513	63.7
s-fsc and s-sscs	1499	99.1
Negative	3	0.2
$EpCAM^-/CD49e^+/CD45^+$	0	0.0
$EpCAM^+/CD49e^+/CD45^+$	0	0.0
$EpCAM^+/CD49e^-/CD45^-$	1491	99.5
$EpCAM^{low}/CD49e^-/CD45^-$	1482	99.4
GFP	0	0.0

Supplemental Figure 3: Separation of TF-1a–GFP cells and human spermatogonia from a contaminated human testis cell suspension using a multi-parameter FACS approach. **A and D)** Human testicular cell suspensions were "spiked" with TF-1a-GFP cells (15.6%) and then FACS was performed using EpCAM-APC, CD49e-PE and CD45-PE. **B)** Fraction III in (A) was further analyzed with side scatter, as previously described, to identify the spermatogonial fraction, $EpCAM^{low}$/side scatter ^{high} (green, fraction IIIA). **C and E)** Purity check indicated that the $EpCAM^{low}$/side scatter ^{high} (green, fraction IIIA) fraction was 99.4% pure and contained and contained no GFP positive cells (**E and F)**, representing the TF-1a leukemic cells. **G-I)** Unsorted and sorted cell fractions were evaluated by immunocytochemistry for SALL-4 (human spermatogonia) and GFP (TF-1a-GFP). We focused on fractions II (red) and IIIA (green) because these were expected to contain TF-1a leukemic cells and human spermatogonia, respectively. The $EpCAM^{low}/CD49e^-/CD45^-$ fraction (IIIA) contained SALL-4 positive spermatogonia, but not GFP positive TF-1a cells (panel H). The $EpCAM^-/CD49e^+/CD49^+$ fraction (II) contained GFP positive TF-1a cells, but not SALL4 positive spermatogonia (panel I). Scale bars = 100 µm.

Supplemental References

1. Hu, X., Moscinski, L.C., Hill, B.J., Chen, Q., Wu, J., Fisher, A.B., and Zuckerman, K.S. 1998. Characterization of a unique factor-independent variant derived from human factor-dependent TF-1 cells: a transformed event. *Leukemia research* 22:817-826.
2. Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868-872.