Supplementary materials

Successful Bone Marrow Stroma Transplantation is enabled by Preliminary Recipient's Stromal Compartment Injury

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CFU-F seeding and harvest

Thirty days after irradiation and BM injection, recipient mice were sacrificed by cervical dislocation. Under sterile conditions, the body cavity was opened, the diaphysis of the femur was isolated, and the medullary cylinder was washed into a 1.5 ml polypropylene tube using a syringe jet filled with pure αMEM medium. The BM fragment was converted into a single cell suspension by repeated passage through a syringe with a G23 needle. The concentration of cells was measured in a light microscope using a Goryaev chamber after staining the cells with gentian violet. 3 x 10^6 cells were placed in T25 flasks containing 5 ml of complete nutrient medium consisting of αMEM medium (Merck, Germany), 20% fetal calf serum (FBS Xtra, Fetal Bovine Serum collected in South America, Capricorn Scientific, Germany), 5 ng/ml FGF2 (SciStore, Russia), 2 mM L-glutamine (Macklin, China) and antibiotics 100 μg/ml streptomycin and 100 units/ml penicillin (PanEco, Russia). From each mouse, 2-3 flasks for CFU-Fs were seeded. Cells were cultured for 3 weeks, with weekly monitoring of cell colonies under a light microscope. After 2 weeks of culture, the medium was completely changed to fresh medium. After a further week, one of the flasks was stained with crystal violet to count CFU-F colonies. The cells in the remaining flasks were used for stromal cell sorting. The medium was removed and each flask was washed twice with 5 ml Versen solution (Dulbecco's PBS + 0.5 mM EDTA). 700 μl of 0.25% trypsin solution was added and the cells were incubated for 5-10 min until they detached. Complete medium with 20% FBS was then added to inhibit the action of trypsin. Cells were counted, the cell suspension was washed twice with 1 ml PBS, each time gently mixing the cells by pipetting, precipitating the cells by centrifugation at 300g at room T and removing the supernatant. Cells were resuspended in 50 μl PBS, stained with a monoclonal antibody to CD45-APC (clone 30-F11, Biolegend)

and 7-AAD (Sigma-Aldrich), adding 1 µl of antibody and 1 µl of 7-AAD to each sample. Cells were incubated with the antibody in the dark at +4°C for 30 min, washed twice with PBS, and resuspended in 300 µl PBS for sorting.

Assessment of the specificity of primers and probes used to determine donor chimerism

Primers and probes to murine genes *Gapdh* and *Prssly* were used (Table 1). The specificity of primers and probe on *Gapdh* was confirmed by the results of BLAST tool (blastn suite, database: mouse genomic + transcript, expect threshold = 0.5, word size = 7, match/mismatch stores = 2/-3, gap costs = existence: 5, extension: 2; filters for low complexity regions and species-specific repeats for Rodentia (Rodents) (Fig. S1).

Table 1. Primers and probes used in the study

Gapdh_mm_DNA_F	GTAAGTGGCCGGAAAGCTGAA
Gapdh_mm_DNA_R	AGTGGGCCTGTTTAAGGGAAAG
Gapdh_mm_DNA_probe	R6G-CCTTGATATGGTGCAACCTGAAAACCA-BHQ2
Prssly_DNA_mm_F	AGTGGGGCTGGTTGATCTTC
Prssly_DNA_mm_R	AGGGGCTCCTTCAGGAAGAT
Prssly_DNA_mm_probe	FAM-AAGGTAAAATCGTAGGTATCCACCGT-RTQ1

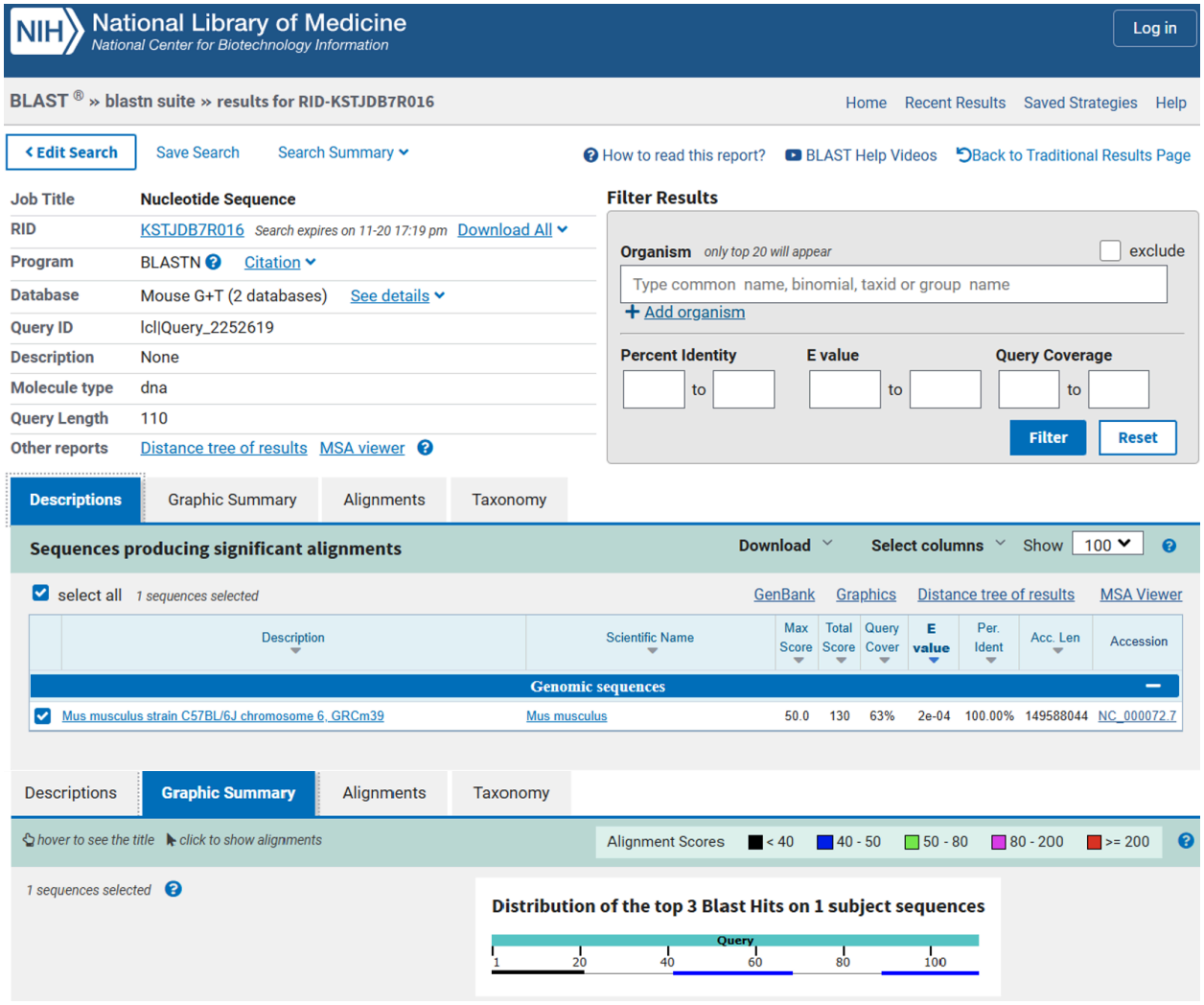


Fig S1. Results of primer specificity analysis on the murine *Gapdh* gene using the BLAST tool.

To further confirm the specificity of the primers used, real-time PCR was performed using the intercalating dye EvaGreen, followed by melting curve analysis. The latter showed the presence of only one amplification product (Fig. S2).

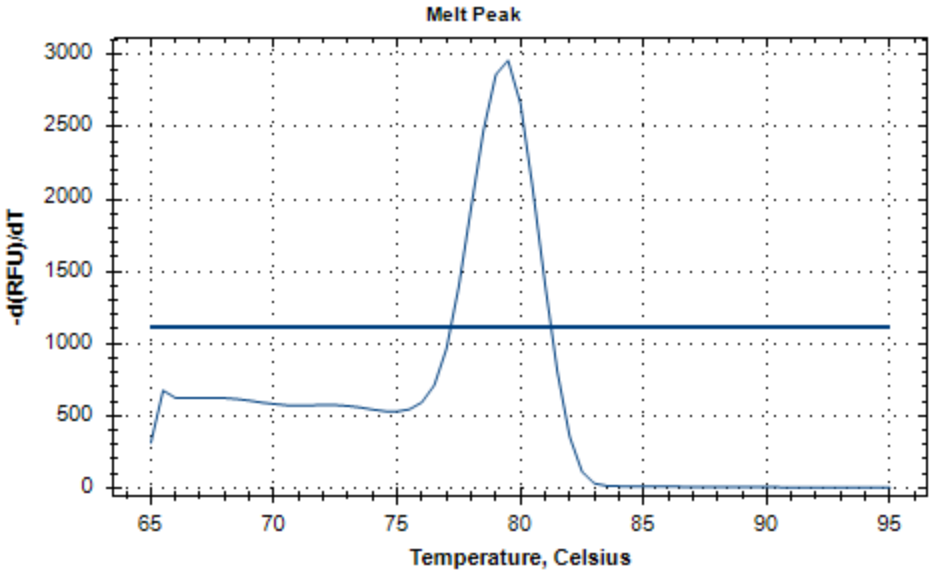


Figure S2. Result of primer specificity analysis for the mouse *Gapdh* gene using the melting curve of the PCR product.

The specificity analysis was also carried out for primers and probe on the mouse *Prssly* gene located on the Y chromosome, with some modifications. Application of the BLAST tool to the mouse genome, using the same parameters as for the *Gapdh* gene analysis, did not reveal any non-specific primer and probe landing sites on the mouse autosomes (no significant similarity found). Since the distal region of the mouse Y chromosome containing the *Prssly* primary sequence and its pseudogenes is not covered by the commonly used mouse reference genome (GRCm39), for additional specificity analysis we used the sequence of the Y chromosome-derived bacterial artificial chromosome BAC CH29-45G4 (AC279166. 1) and a mixed unplaced genomic scaffold (NW_001034423.1) from the whole genome shotgun sequence (GCF_000002165.2) [1]. Using the Find Motifs tool built into the Vector NTI Advance v 11.5 software package, we identified two highly homologous primer and probe landing sites in a configuration capable of amplification and free fluorophore accumulation in the Taqman RQ-PCR reaction (Figure S3).

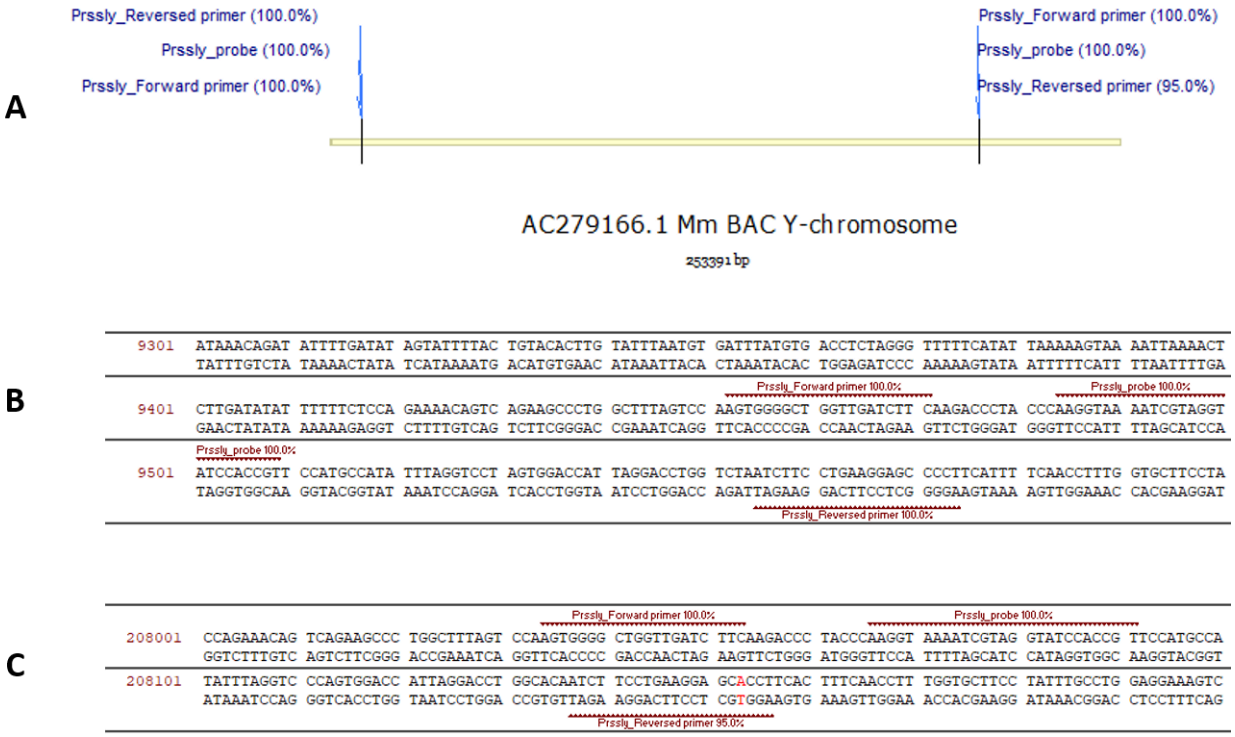


Figure S3. Result of primer specificity analysis on the mouse *Prssly* gene using Vector NTI Advance. A. General view of artificial chromosome AC279166 with marked primers and probe landing sites (homology threshold > 80%). B. First (left from A) primer and probe landing site: 100% homology for both primers and probe. C. Second (right from A) primer and probe landing site: 100% homology for forward primer and probe and 95% homology for reverse primer (mismatch highlighted in red).

The first landing site corresponded to coordinates 9452-9574 bp (PCR product length 123 bp) and belonged directly to the *Prssly* gene. The second landing site corresponded to coordinates 208034-208156 bp (PCR product length also 123 bp) and belonged to one of the *Prssly* pseudogenes. To confirm the presence of two PCR products, real-time PCR was performed using the intercalating dye EvaGreen, followed by melting curve analysis. DNA isolated from male mice was used as a matrix. The presence of two PCR products with close melting temperatures was confirmed. (Figure S4). The difference in melting temperature may be explained by differences in the GC composition of the amplicons.

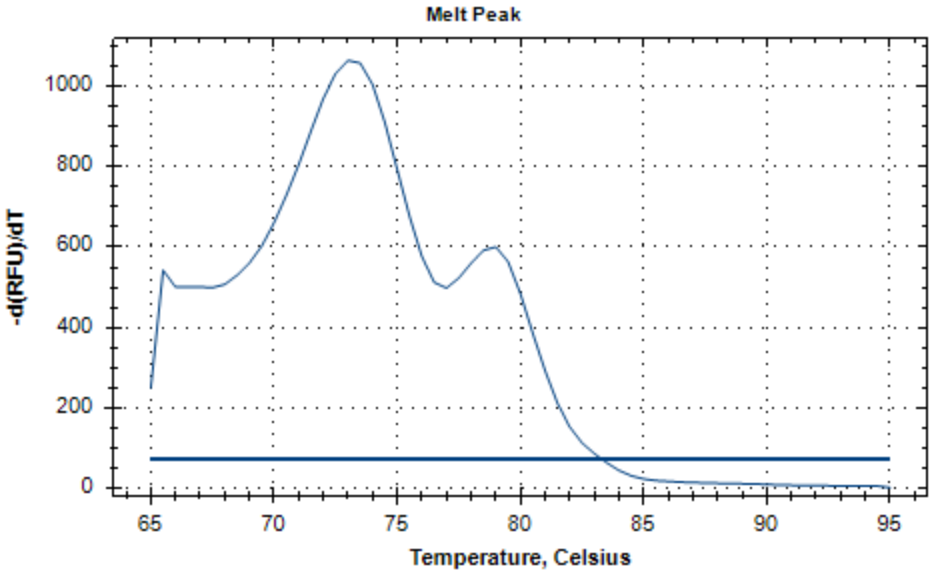


Figure S4. Result of primer specificity analysis on the mouse *Prssly* gene using the melting curve of the PCR product.

The PCR products were purified by agarose gel electrophoresis, the PCR product of estimated length 123 bp was excised from the gel, DNA was isolated and sequenced by Sanger method. Sequencing confirmed the presence of two sequences corresponding to the above primer landing sites (Fig. S5).

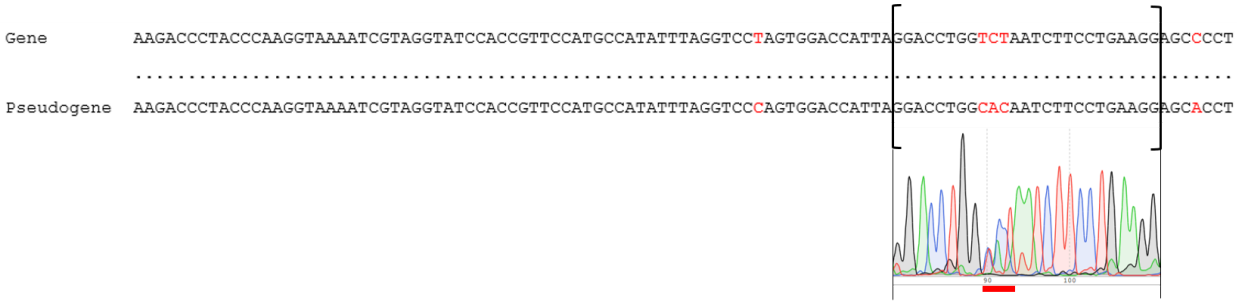
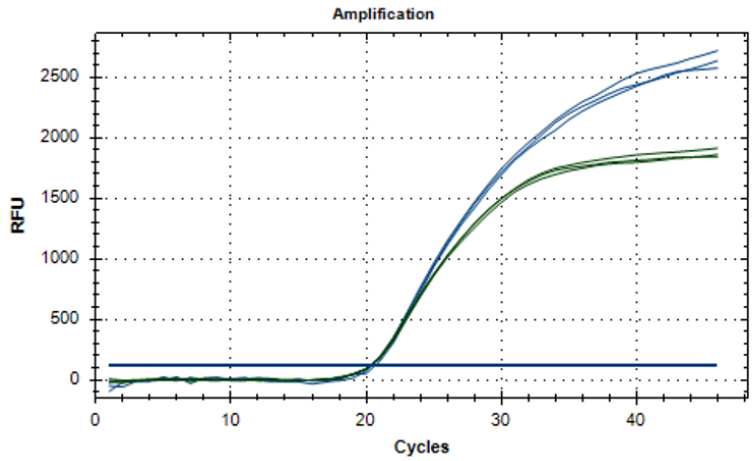


Figure S5. Nucleotide sequence analysis of PCR products obtained using primers for the *Prssly* gene. Reference sequences of the gene fragments and the pseudogene are shown above. Distinct nucleotides are highlighted in red. The sequence fragment of the PCR product is shown below; the red rectangle indicates differentiating nucleotides, confirming the presence of two sequences in the PCR product.

Thus, each male mouse genome contained two amplifiable targets for primers and probe on *Gapdh* (one on each sister chromosome 6) and two amplifiable targets for primers and probe on *Prssly* (the first directly on the Prssly gene, near locus #1 according to Holmlund et al [1]; the second on its pseudogene on the same Y chromosome, near locus #3 according to Holmlund et al [1]). This is confirmed by the fact that in multiplex PCR for *Gapdh* and *Prssly*, the threshold cycles of both PCR products were identical (Fig. S6).



Well	Fluor	Target	Sample	Cq	Cq Mean
A01	FAM	Prssly	XY DNA	20,32	20,46
A02	FAM	Prssly	XY DNA	20,64	
A03	FAM	Prssly	XY DNA	20,41	
A01	HEX	Gapdh	XY DNA	20,35	20,34
A02	HEX	Gapdh	XY DNA	20,33	
A03	HEX	Gapdh	XY DNA	20,33	

Figure S6. Results of multiplex PCR for *Gapdh* and *Prssly* on DNA matrix isolated from male mice. Amplification plots in three technical replicates are shown on the left. Green curves are *Prssly* (FAM dye), blue curves are *Gapdh* (ROX dye). On the right are individual and average Cq threshold cycle values for *Prssly* and *Gapdh*, respectively.

Calibration curve construction for the determination of donor chimerism by real-time PCR

The following sequence of steps was used to construct the calibration curve. DNA isolated from the BM of one male (XY) and one female (XX) was used. The DNA concentration was measured on a Qubit 3.0 fluorometer using the dsDNA Quantitation, High Sensitivity kit (ThermoFisher Scientific, USA). The concentration of both DNA solutions was equalised to 48.8 ng/µL. The male DNA was then serially diluted with the female DNA in steps of 2. In this way, calibration standards were obtained in which the proportion of male DNA was 100%, 50%, 25% and so on down to 0.0003%. The calibration standards were used to perform a

multiplexed real-time PCR in a BioRad CFX Connect amplifier, in which the signals from the *Prssly* and *Gapdh* targets were detected simultaneously in each sample. Primer and probe sequences are summarised in Table 1. Each standard was measured in three technical replicates. The resulting threshold cycle values were averaged over the technical replicates. The average threshold cycle values of each standard Cq(*Prssly*) and Cq(*Gapdh*) were used to construct a calibration curve (Table 2). The difference in threshold cycles ΔCq = Cq(*Prssly*) - Cq(*Gapdh*) and the value of the parameter $2^{-\Delta Cq}$ were calculated (Table 2). A graph was plotted in the coordinates "male DNA fraction" vs "$2^{-\Delta Cq}$" (Figure S7). A trend line was drawn through the points obtained using the method of least squares. The equation for the trend line was $y = 87{,}291x^3 - 47{,}851x^2 + 61{,}802x + 0{,}5931$, where y is the proportion of male DNA ("donor chimerism") and x is $2^{-\Delta Cq}$. The goodness of fit index of the experimental points R^2 was 0.9999. This equation was used as the calibration curve. To calculate donor chimerism in experimental DNA samples isolated from the bones of recipient mice, multiplex PCR was performed under the same conditions (5 mM $MgCl_2$, T_m = 60 ^{0}C, Table 3) and with the same primers and probes on the *Prssly* and *Gapdh* genes, determining Cq (also in three technical replicates for each gene), ΔCq and $2^{-\Delta Cq}$. We substituted the value of the latter parameter into the trend equation and calculated the donor chimerism in the sample studied.

Table 2. Data for calibration curve construction

% XY	Cq mean (Prssly)	Cq mean (Gapdh)	ΔCq	$2^{-\Delta Cq}$
100,0	21,62	21,61	0,01	0,99182
50,0	22,57	22,05	0,52	0,69764
25,0	23,36	22,13	1,23	0,42583

12,5	24,63	22,36	2,27	0,20779
6,25	25,63	22,42	3,20	0,10851
3,13	27,10	22,47	4,64	0,04024
1,56	28,63	22,41	6,22	0,01343
0,78	30,87	22,50	8,37	0,00302
0,39	34,74	22,82	11,92	0,00026
0,20	38,53	22,54	16,00	0,00002
0,10	37,29	22,48	14,81	0,00003
0,05	37,67	22,46	15,21	0,00003
0,024	N/A	22,36	N/A	N/A
0,012	N/A	22,41	N/A	N/A
0,006	N/A	22,56	N/A	N/A
0,003	N/A	22,56	N/A	N/A

0	N/A	22,58	N/A	N/A

Table 3. RQ-PCR amplification parameters

Temperature	Time	Cycles
95 °C	5 min	1x
95 °C	15 sec	50x
60 °C	30 sec	

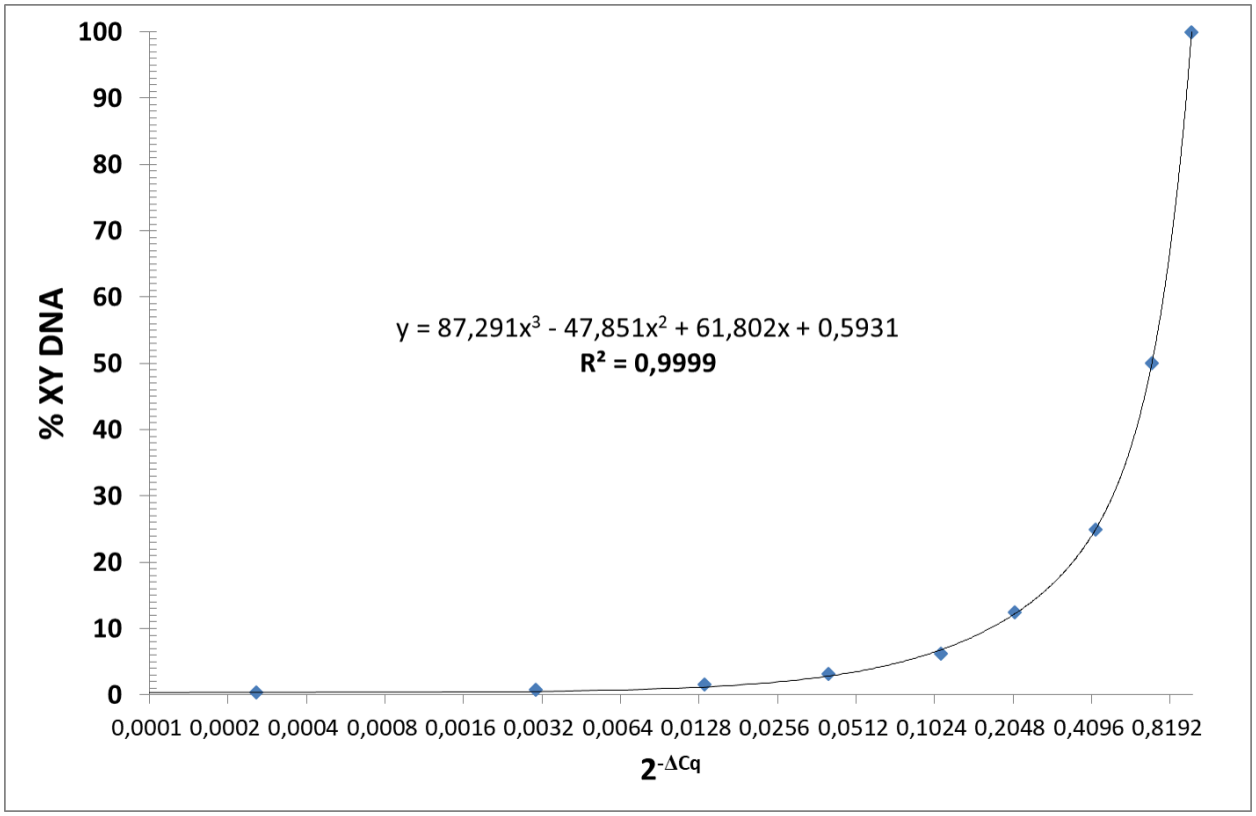


Figure S7. Calibration curve for calculating donor chimerism using RQ-PCR.

Calculation of donor chimerism error from the calibration curve

To determine the value of donor chimerism, a calibration curve has previously been constructed. The calibration curve is an approximation curve constructed from the points of the value $x = 2^{-d}$ determined for a set of standard DNA samples with known specified values of donor chimerism (in the range of 100% to 0.003%). The value of *d* is defined as the difference between the values of the C_q threshold cycles determined in each standard DNA sample for targets located on chromosome Y (*Prssly* gene) and chromosome 6 (*Gapdh* gene): $d = C_{q(Prssly)} - C_{q(Gapdh)}$. The value of *x* depends on the threshold cycle C_q. So we are looking for a functional dependence $x = f(C_q)$.

Each value of $C_{q(Prssly)}$ and $C_{q(Gapdh)}$ is the arithmetic mean of three experimentally measured values of $C^{i}_{q(Prssly)}$ and $C^{i}_{q(Prssly)}$, i = 1,2,3.

To estimate the error of $C_{q(Prssly)}$ and $C_{q(Gapdh)}$, the RMS error ΔC of the arithmetic mean was used:

$$\Delta C = \sqrt{\frac{\sum_{i=1}^{n} (C^{i} - \overline{C})^{2}}{n(n-1)}}, \qquad n = 3$$

The error of d can be estimated as $\Delta d = \Delta C_{q(Prssly)} + \Delta C_{q(Gapdh)}$, where $\Delta C_{q(Prssly)}$ and $\Delta C_{q(Gapdh)}$ are the RMS errors of each of the quantities. Then the error of x for each d can be estimated as

$$\Delta x = \left|\frac{dx}{dd}\right| \Delta d = 2^{-d} \cdot ln2 \cdot \Delta d$$

and the relative error of x as $\varepsilon = \frac{\Delta x}{x}$

The results of the error estimation are given in Table 4.

Table 4. Parameters for calculation of donor chimerism value error by calibration curve

Donor chimerism in standards, D_{ch}^{std}	$C_{q(Prssly)}$	$\Delta C_{q(Prssly)}$	$C_{q(Gapdh)}$	$\Delta C_{q(Gapdh)}$	d	Δd	x	Δx	Relative error, ε
100	21,62	0,07	21,61	0,05	0,01	0,12	0,992	0,079	0,140
50	22,57	0,07	22,05	0,05	0,52	0,12	0,698	0,060	0,154
25	23,36	0,07	22,13	0,02	1,23	0,10	0,426	0,028	0,098

12.5	24,63	0,02	22,36	0,03	2,27	0,06	0,208	0,008	0,033
6.25	25,63	0,23	22,42	0,07	3,20	0,30	0,109	0,023	0,127
3.13	27,10	0,04	22,47	0,07	4,64	0,11	0,040	0,003	0,023
1.56	28,63	0,27	22,41	0,07	6,22	0,34	0,013	0,003	0,040
0.78	30,87	0,38	22,50	0,22	8,37	0,60	0,003	0,001	0,029

The table shows that the relative error ε is between 2% and 15%. The points of dependence $D_{ch}^{std} = g(x) = f^{-1}(x)$ are approximated by the polynomial $D_{ch}^{std} = 87.921x^3 - 47.851x^2 + 61.802x + 0.5931$ with the degree of approximation reliability $R^2 = 0.9999$.

Then the relative error of the determination of donor chimerism in the tested experimental DNA samples D_{ch}^{exp} at the experimentally determined value of x also does not exceed 15%.

Survival analysis

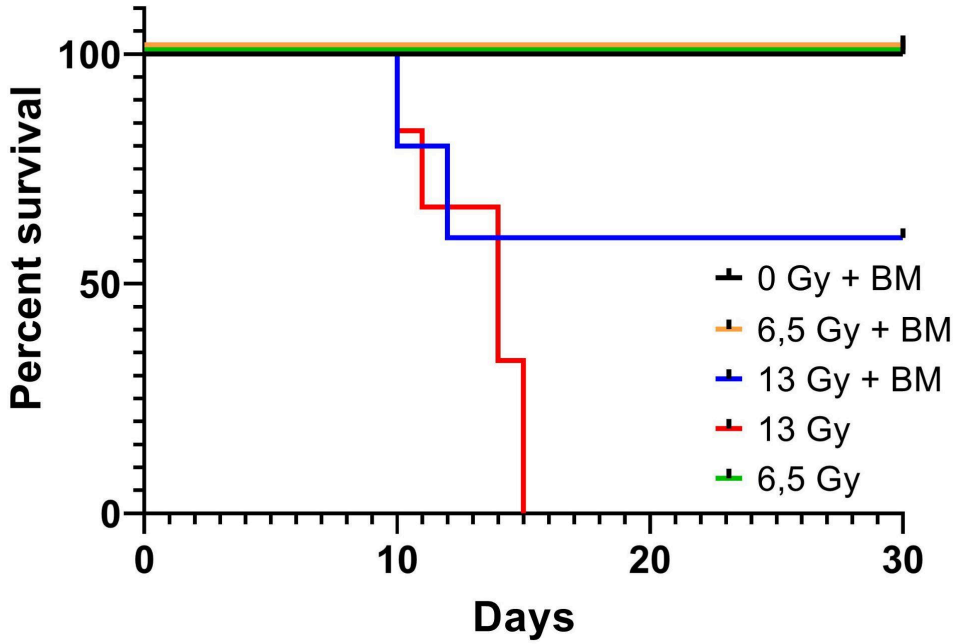


Figure S8. Kaplan-Meier curves of animal survival. “0 Gy + BM”, “6.5 Gy + BM” and “13 Gy + BM” are the following experimental groups: unirradiated, irradiated with 6.5 Gy and 13 Gy, respectively, injected with BM from a male donor. “13 Gy” and “6.5 Gy” - control groups of irradiated mice not injected with donor BM.

Comparison of CFU-F concentration in experimental groups

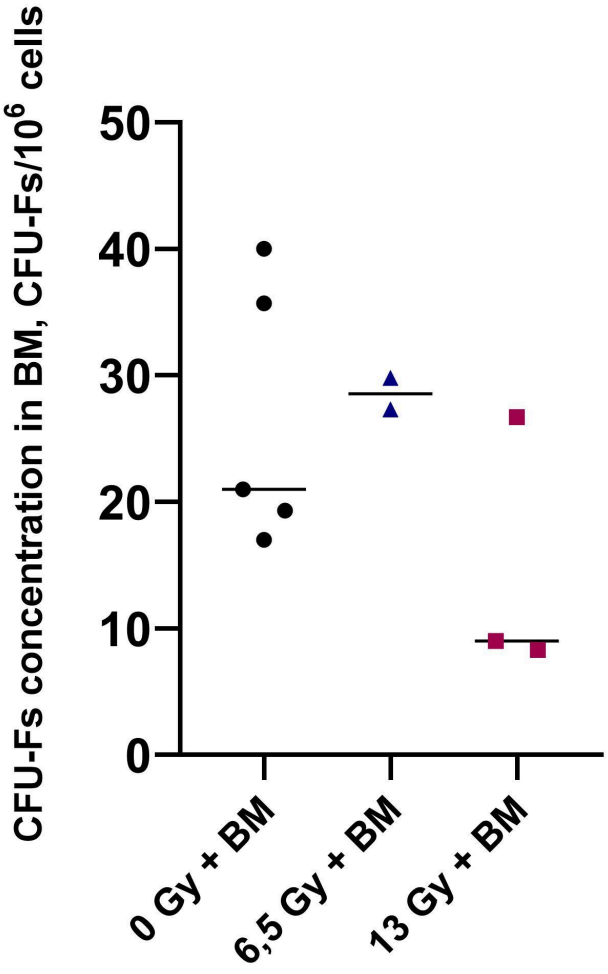


Figure S9. Concentration of CFU-F in the experimental groups. Horizontal bars indicate medians.

Sorting of CD45$^-$ cells from vials containing CFU-F

The first step in isolating the population of interest for sorting was to exclude cell debris in the forward scatter (FSC) vs. side scatter (SSC) plot (Figure S10), then to exclude cell doublets in the plot showing FSC in area format (FSC-A) and peak height format (FSC-H). The next step was to exclude non-viable cells in the side light scatter plot against 7-AAD (7-AMINO-ACTINOMYCIN D). In the final dot plot (SSC vs. CD45), the central region of the CD45-negative cell population was highlighted to exclude CD45$^+$ cells from the target region.

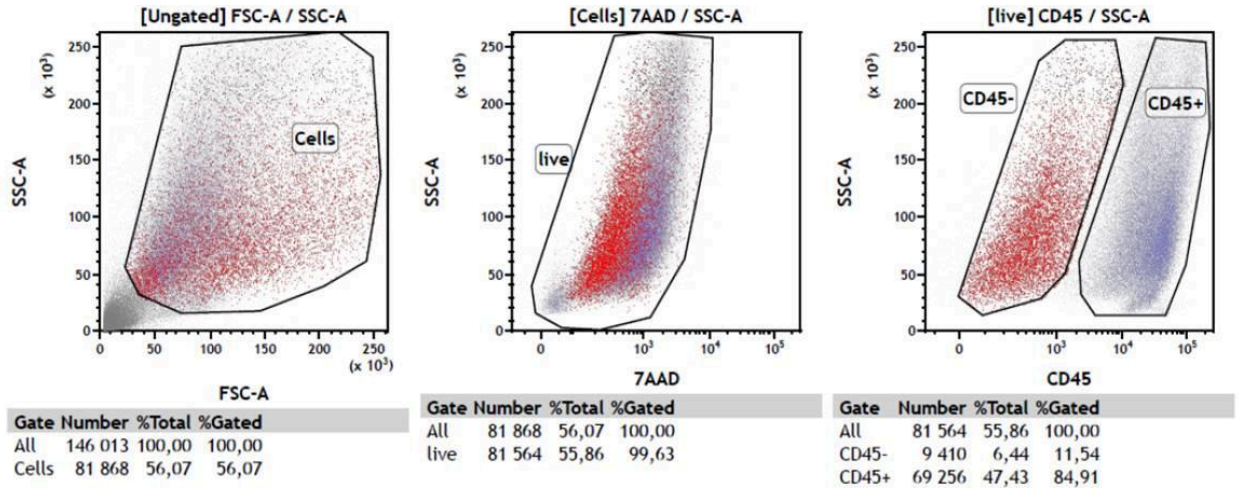


Figure S10. Typical example of a cell sample from a vial of CFUf on a cell sorter.

Sorting resulted in the fraction of CD45 cells from the total population of live nuclear cells in each vial (Table 5).

Table 5. Proportion of CD45⁻ cells in flasks with CFU-Fs

Mouse ID	Group	% CD45⁻ cells in flasks with CFU-F
1	0 Gy	5,2
2	0 Gy	4,9
3	0 Gy	11,8
4	0 Gy	5,0

5	0 Gy	13,9
6	6,5 Gy	11,1
7	6,5 Gy	19,7
8	6,5 Gy	12,2
9	6,5 Gy	13,3
10	6,5 Gy	4,1
11	13 Gy	3,3
12	13 Gy	3,5
13	13 Gy	6,7
Mean		8,8

References

1. Holmlund H, Yamauchi Y, Durango G, Fujii W, Ward MA. Two acquired mouse Y chromosome-linked genes, Prssly and Teyorf1, are dispensable for male fertility‡. *Biol Reprod*. 2022;107(3):752–764.