Clonal reversal of ageing-associated stem cell lineage bias via a pluripotent intermediate

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Ageing associates with significant alterations in somatic/adult stem cells and therapies to counteract these might have profound benefits for health. In the blood, haematopoietic stem cell (HSC) ageing is linked to several functional shortcomings. However, besides the recent realization that individual HSCs might be preset differentially already from young age, HSCs might also age asynchronously. Evaluating the prospects for HSC rejuvenation therefore ultimately requires approaching those HSCs that are functionally affected by age. Here we combine genetic barcoding of aged murine HSCs with the generation of induced pluripotent stem (iPS) cells. This allows us to specifically focus on aged HSCs presenting with a pronounced lineage skewing, a hallmark of HSC ageing. Functional and molecular evaluations reveal haematopoiesis from these iPS clones to be indistinguishable from that associating with young mice. Our data thereby provide direct support to the notion that several key functional attributes of HSC ageing can be reversed.

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Aging associates with a profound predisposition for an array of diseases, which in the blood includes a higher prevalence for anaemia, leukaemia and compromised immunity. While age-related diseases evidently can arise due to changes that compromise or alter the function of mature effector cells, this is harder to reconcile with organs such as the blood, that rely on inherently short-lived effector cells in need of continuous replenishment. Rather, accumulating data have suggested that the de novo production of subclasses of haematopoietic cells shifts in an age-dependent manner, akin to that seen during more narrow time windows in early development. These findings have to a large extent also challenged the classically defining criteria of haematopoietic stem cells (HSCs) as a homogenous population of cells with differentiation capacity for all haematopoietic lineages. Rather, the differentiation capacity of HSCs might be more appropriately defined by a continuous multilineage haematopoietic output, but which might not necessarily include the production of all types of blood cells at all points in time.

While many of the changes in the ageing adult are underwritten by alterations in HSC function, the individual constituents of the HSC pool can display a significant variation in function. Apart from individual HSCs being preset differentially, which could gradually alter the composition of the HSC pool with age, other mechanisms leading to segmental changes within the HSC pool, including environmental influences, uneven proliferative rates and acquisition of DNA mutations in individual cells, are also possible. Hence, by merely evaluating chronologically aged cell populations, the heterogeneity of individual cells is not accounted for.

The mechanisms that drive ageing at both the organisinal and cellular level have attracted significant attention as they represent prime targets for intervention. For instance, prolonged health span and lifespan has been reported in a variety of model organisms by caloric restriction and/or by manipulating the IGF1 and mTOR axes. Moreover, an increased function of aged cells by caloric restriction and/or by manipulating the IGF1 and mTOR axes may be more appropriately defined by a continuous multilineage haematopoietic output, but which might not necessarily include the production of all types of blood cells at all points in time.

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a very low abundance of T cells and an increased frequency of myeloid cells (Fig. 2a). To investigate the clonal repopulation dynamics in detail, we next analysed the barcodes retrieved from peripheral B-, T- and granulocyte/myeloid cells, as well as from bone marrow (BM) erythroid progenitors (Fig. 2b–e; Supplementary Fig. 1). In agreement with a previous barcoding study of aged HSCs, both young and aged HSC clones contributed actively to haematopoiesis (Supplementary Fig. 1a). Of these clones, the proportion that contributed to B cells and erythropoiesis was similar regardless of age. A higher frequency of aged clones contributed to myeloid cell outputs (38% of young, 60% of aged clones). However, when investigating the output of these multipotent clones, they were responsible for a vast majority of the T-cell output observed in aged mice (Supplementary Fig. 1c).

As a second part of our analysis, we intersected the barcode data from all lineages to infer the clonal combinatorial contributions from young and aged HSCs (Fig. 2d–e; Supplementary Fig. 1b,c). While HSCs contributed to a spectrum of different lineage outputs regardless of age, myeloid-restricted clones and bipotent clones with combined myeloid and erythroid potential increased in frequency with age (6 ± 7% (mean ± s.d.) versus 13 ± 3% and 10 ± 7% versus 19 ± 6% for young and aged HSCs respectively), while all combinations that involved T cells were reduced (Fig. 2d,e). In addition, our analyses revealed that HSC ageing associated with a pronounced decrease in the ability of candidate HSCs to contribute to all of the four lineages evaluated (13 ± 9% (mean ± s.d.) of young and 1 ± 1% of aged clones). However, when investigating the output of these multipotent clones, they were responsible for the vast majority of the T-cell output observed in aged mice (Supplementary Fig. 1c).
Altogether, these data demonstrated that the altered output of mature effector cells with age is underwritten by distinct shifts in the abundance of different HSC clone types, but also in the magnitude, whereby individual HSC clones contribute to haematopoiesis. At the same time, these data established that ageing does not affect all HSCs in a similar manner.

**Functional reversal of aged HSC clones.** We next challenged the heritability of the ageing phenotype by generating iPS cells from a mix of aged barcoded progenitors from one of the original recipients that completely lacked donor-derived barcoded T cells (Figs 2a and 3a, aged HSC mouse #1). These parental cells represent the immediate progeny of the barcoded HSCs (Fig. 1) and were chosen over HSCs, as we have been unable to generate iPS cells from highly purified HSCs, likely owing to the high level of dormancy of stringently purified HSCs.

This approach led to the generation of 20 barcoded iPS lines. Following retrospective analysis of the retrieved barcodes to their haematopoietic lineage affiliations, we identified three unique barcodes originating from HSCs with a combined myeloid and erythroid, but no B- or T-cell potential (M/E clones 1–3) and two additional myeloid-biased barcodes with myeloid/erythroid, B- but no T-cell potential (M/B/E clones 1–2; Table 1).

To assess the haematopoietic repopulation potential of the iPS-derived cells, we next transplanted BM cells from the blastocyst/morula chimeras into lethally irradiated hosts (Fig. 1, 2°/C176 transplant). Because of the mixture of iPS-derived and endogenous in primary chimeras, these experiments are by nature competitive. This showed that both the quantitative...
levels of donor-derived cells) and qualitative (contribution to each assessed lineage) properties from each of the iPSc-derived HSCs (iPS-HSCs) were similar to that of the endogenous (young) control HSCs (Table 1). Given the complete lack of T-cell lineage contribution of the parental HSCs (Figs 2f and 3a), we complemented our investigations by more detailed analysis of T-cell development in the thymi of both iPScs and 2nd transplants (Fig. 3). This revealed comparable frequencies of CD3+/CD4−/CD8−, CD3+/CD4+CD8−, CD3+/CD4+CD8+ and CD3+/CD4−CD8+ cells to the endogenous (young) control cells (Fig. 3b) and verified persistent re-establishment of T-cell competence from T-cell incompetent parental donor cells.

Having established that the haematopoiesis derived from iPScs was functionally equivalent to young haematopoiesis in terms of differentiation and regenerative potential following transplantation, iPSc-HSCs were next interrogated more directly. Apart from alterations in the lineage output of HSCs, ageing coincides with a numerical expansion of the HSC pool17,10,16. We therefore first investigated the frequency of HSCs in iPSc chimeras (Fig. 4a). This revealed that the iPSc-HSC frequency was equivalent to that of the young controls (Fig. 4b). In addition, the iPSc-HSC pool was not, as opposed to the aged HSCs originally barcoded, dominated by cells that express high levels of CD150 and instead expressed levels of this marker comparable to young controls (Supplementary Fig. 2a,b). We thereafter applied a multiplexed single-cell quantitative PCR with reverse transcription approach on sorted iPSc-HSCs for a panel of genes that are differentially expressed with HSC ageing (Fig. 4c; Supplementary Table 1). Principal component analyses were used to evaluate their relationships to normal young (endogenous control cells from each iPSc chimera) or aged HSCs. We here also included HSCs isolated from normal mid-aged (11 months old) mice, reasoning that this potentially could unveil a phenotype intermediate to that of young and aged HSCs. This revealed two distinct patterns; most middle-aged and aged HSCs clustered together and away from iPSc-HSCs and the young HSCs, the latter two cell types clustering tightly together in all instances (Fig. 4d). Finally, although rather few cells were analysed for mid-aged and aged HSCs, more mid-aged HSCs overlapped with young and iPSc-HSCs, suggesting that the composition of the HSC compartment changes gradually with age. Collectively, these data indicate that rejuvenation appears complete with the measured signatures of chronological age being erased.

Discussion
Most organs contain adult/somatic stem cell populations that function to maintain homeostasis throughout life. Given the growing evidence that adult stem cells are not spared from ageing, it appears reasonable that the widespread decline in tissue function with age is influenced by an altered function of their associated stem cells. At the same time, any pool of ageing stem cells (blastocyst/morula-derived; % ± s.d.)

| Aged parental clone | Origin | Overall haematopoietic chimerism (% ± s.d.) | Overall relative chimerism (% ± s.d.) | Lineage distribution of test cells in 1st transplants, iPSc chimera and 2nd transplants (% ± s.d.) | Lineage distribution of young cells (blastocyst/morula-derived; % ± s.d.) |
|---------------------|--------|-----------------------------------|-----------------------------------|----------------------------------|------------------------------------------------|
| M/E clone 1         | Transplant | NA                               | NA                               | 0 0 100                          | NA NA NA                                               |
| iPS chimera         |        | 26.9 ± 6.3                       | 100                              | 11.2 60.6 28.2                   | 45.5 32.3 22.2                                        |
| 2nd Transplant, iPS origin (n = 4) |        | 25.8 ± 3.6                       | 113.9 ± 45.4                    | 8.7 ± 4.9 75.4 ± 10.9 15.9 ± 8.9 | 22.7 ± 9.5 62 ± 6.2 15.3 ± 3.5 |
| M/E clone 2         | Transplant | NA                               | NA                               | 0 0 100                          | NA NA NA                                               |
| iPS chimera         |        | 25.5 ± 1.3                       | 100                              | 20.1 60.9 19                     | 58.5 26 ± 4.4 26.1 12.5 ± 4.2 |
| 2nd Transplant, iPS origin (n = 4) |        | 18.6 ± 3.3                       | 73.1 ± 12.8                     | 10.1 ± 1.7 76.9 ± 6.1 13 ± 6.8 | 26 ± 4.4 61.5 ± 3.6 12.5 ± 4.2 |
| M/E clone 3         | Transplant | NA                               | NA                               | 0 0 100                          | NA NA NA                                               |
| iPS chimera         |        | 55.4 ± 10.4                      | 109.9 ± 18.7                    | 32.9 36.8 30.3                   | 55.8 42.7 ± 5.2 27.2 ± 17 16.3 ± 6.3 |
| 2nd Transplant, iPS origin (n = 4) |        | 60.9 ± 10.4                      | 109.9 ± 18.7                    | 22.3 ± 6 60.9 ± 1.1 16.8 ± 6.4 | 42.7 ± 5.2 41 ± 7.2 16.3 ± 6.3 |
| M/B/E clone 1       | Transplant | NA                               | NA                               | 0 16.5 83.5                      | NA NA NA                                               |
| iPS chimera         |        | 33.7 ± 9.7                       | 100                              | 11.5 69.2 19.3                   | 36.9 51.8 ± 11.3                                     |
| 2nd Transplant, iPS origin (n = 5) |        | 27.6 ± 9.7                       | 79.7 ± 32.8                     | 22.4 ± 6.2 64.7 ± 10.1 13 ± 5.9 | 43.7 ± 6.1 40.7 ± 7.8 15.6 ± 2.3 |
| M/B/E clone 2       | Transplant | NA                               | NA                               | 0 24.5 75.5                      | NA NA NA                                               |
| iPS chimera         |        | 22.4 ± 4.1                       | 71.9 ± 31.5                     | 29.5 31.7 38.8                   | 62.3 22.3 ± 4.4 15.4 ± 6.3 |
| 2nd Transplant, iPS origin (n = 5) |        | 21.4 ± 4.1                       | 71.9 ± 31.5                     | 12.8 ± 3.7 78.6 ± 3.3 8.3 ± 2.6 | 29.8 ± 5.3 54.3 ± 17.5 15.9 ± 6.3 |

BM, bone marrow; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; iPSc, induced pluripotent stem; NA, not applicable; PB, peripheral blood.

The PB lineage distribution of the parental HSCs used for somatic cell reprogramming was determined by the barcode read distribution in the different lineages obtained from barcode sequencing of the 1st transplanted mouse, and in iPSc chimera and 2nd transplants by FACS analysis (Figures determined among CD45.2+CD45.1− cells). GFP was not used in analysis of iPSc-derived haematopoiesis due to a large degree of viral silencing coinciding with the iPSc-cell generation. The relative overall PB iPSc chimerism maintained following transfer of iPSc BM in 2nd transplants is also provided. Shown values are expressed as average values ± s.d. The data are from one experiment for each iPSc line for iPSc chimeras and 2nd transplants. Bold entries denote the complete absence of detectable donor-derived B/T or T cells from evaluated clones in primary transplants.
compared to middle-aged (92 cells) and aged HSCs (47, 47, 47, 91, 45 cells with a retained aged transcriptome15. Therefore, a rather complete epigenomic ‘reset’, like that shown here, might be needed to normalize the alterations that arise and govern cellular ageing. While it remains to be determined how this could be achieved in a more tractable manner, we believe our results should be encouraging for the prospects of such efforts, which in the long term would pave the way for the development of therapeutic modalities aimed at achieving overall healthier late stages of life.

Methods

Mice. Rosa26ERTA2;Col1a14F2A mice (4F2A mice) were kindly provided by Dr Rudolf Jaenisch and acquired via the Jackson laboratory (stock number 011004). Cells from these mice express Doxycycline inducible Cre recombine (Dox) in c-Kit+ bone marrow cells in iPS (n = 1 chimera per iPS clone) and 2 iPS transplants (n = 4, 4, 2, 5 mice for M/E clone 1, M/E clone 2, M/E clone 3, M/B/E clone 1 and M/B/E clone 2, respectively). (a) FACS gating strategy to distinguish iPS-HSCs from young endogenous HSCs in iPS chimeras (pregated on single, viable and lineage-negative cells.). (b) The frequencies of HSCs were determined among iPS and young Lin− c-Kit+ bone marrow cells in iPS (n = 1 chimera per iPS clone) and 2 iPS transplants (n = 4, 4, 2, 5 mice for M/E clone 1, M/E clone 2, M/E clone 3, M/B/E clone 1 and M/B/E clone 2, respectively). (c) Heatmap showing the relative expression of the 45 genes selected for single-cell PCR with reverse transcription (RT–PCR) in previously published data sets of young and aged HSCs. (d) Single iPS-HSCs and chronologically young endogenous HSCs were selected from each iPS chimera and subjected to multiplexed quantitative RT–PCR. Principal component analyses for each analysed iPS-derived HSCs and young control HSCs isolated from the same iPS chimera (n = 90, 90, 89, 67, 88 cells and 47, 47, 47, 91, 90 cells for the iPS-HSC and young HSCs isolated from M/E clone 1, M/E clone 2, M/E clone 3, M/B/E clone 1 and M/B/E clone 2, respectively) as compared to middle-aged (n = 92 cells) and aged HSCs (n = 90 cells). Data are from one experiment for each iPS line and endogenous blastocyst control cells, and from one experiment for middle-aged and aged HSCs.
cells were stained with fluorescently labelled antibodies directed against CD3, CD4 and CD8. For blood analysis and sorting of mature effector cells, PB was collected from the tail vein and red blood cells were sedimented with 1% Dextran T-200 (Sigma). Remaining red blood cells were lysed with ammonium chloride before staining with antibodies (CD3, CD11b and CD19). When preparing the BM, thymus and PB samples, CD45.1 and CD45.2 antibodies were included to distinguish iPS-derived cells from endogenous blood cells, and propidium iodide (used according to the manufacturer's instructions, Thermofisher Scientific) to exclude dead cells. Cells were sorted on a FACS Aria III or cell sorter and analysed on an LSRII or an LSR Fortessa (Becton Dickinson) made available at the FACS core at Lund Stem Cell Center. Representative fluorescence-activated cell sorting (FACS) profiles and gating strategies are shown in Supplementary Fig. 3 and the antibodies used are listed in Supplementary Table 2.

Investigations into the clonality of HSC ageing. 1 Transpl. Twenty-three-month-old 4P2A and 8- to 10-week-old C57BL/6 HSCs (CD45.2+ or CD45.1+, respectively) were isolated and transduced overnight with a lentiviral vector barcode library2, resulting in 29% and 31% transduction, respectively (as determined by a GFP reporter gene). Sixteen hours later, 6,900 aged or 1,000 young cells were PCR reactions designed to amplify a 238 bp amplicon containing the barcode sequence, sample multiplex information and IonTorrent sequencing adaptors. In cases where an excess of 25,000 cells were obtained, PCR products were pooled following the PCRs. The reactions were amplified using Q5 High-Fidelity DNA Polymerase (NEB) with cycling conditions: 30 s at 98 °C, 32 cycles of 10 s at 96 °C, 10 s at 55 °C, and 30 s at 72 °C followed by 10 min at 72 °C. The genomic DNA (gDNA) in the lysates was next purified using Ampure XP beads (Beckman Coulter), and 5,000–25,000 cell equivalents of gDNA were used as templates for PCR amplification using barcoded, random, single positive cells in the PB. At 11–14 weeks of age, clonotypic mice were killed, and their RBMs, PBs and thymus were collected for FACS analysis and sorting.

2 Transplants. To investigate the competitive ability of iPS-derived HSCs, four to five lethally irradiated (950 cGy) 8- to 10-week-old CD45.1+ or CD45.1+/2+ Fl mice were transplanted with 2 × 106 unfractonated BM cells from each iPS clone. At the time of transplantation, aged cells were assessed that aged candidate HSCs would be a prerequisite for a correct clonality analysis. To this end, clonal analysis of GFP+ donor hematopoietic cells were performed periodically in PB. Long term after transplantation (20–24 weeks), donor-derived hematopoietic cells were pooled following the PCRs. The reactions were amplified using Q5 High-Fidelity DNA Polymerase (NEB) with cycling conditions: 30 s at 98 °C, 32 cycles of 10 s at 96 °C, 10 s at 55 °C, and 30 s at 72 °C followed by 10 min at 72 °C. The genomic DNA (gDNA) in the lysates was next purified using Ampure XP beads (Beckman Coulter), and 5,000–25,000 cell equivalents of gDNA were used as templates for PCR amplification using barcoded, random, single positive cells in the PB. At 11–14 weeks of age, clonotypic mice were killed, and their RBMs, PBs and thymus were collected for FACS analysis and sorting.

Generation and characterization of clone-specific chimeric mice. iPS chimeras. The iPS clones of choice (originally CD45.2+/2+) were injected into morulas and blastocysts from CD45.1+/2+ mice at the Core Facility for Transgenic Mice (Copenhagen University, Denmark). Resulting chimeras were investigated for agouti fur colour, by PCR against the specific barcode and by the presence of CD45.2+ single-positive cells in the PB. At 11–14 weeks of age, clonotypic mice were killed, and their RBMs, PBs and thymus were collected for FACS analysis and sorting.

To investigate the molecular resemblance of the iPS-derived HSCs to HSCs of different chronological ages, we first selected 3 highly expressed HSC-related genes, 3 reference genes (Actb, Hprt and Gapdh) and 42 genes differentially expressed in HSCs as a consequence of age. The latter category was obtained by analysis of the GEO data of young and aged HSCs (six arrays per age). Data were preprocessed by extracting probe level expression values using RMA28 and further analysed using dChip29 by filtering out probes with a lower expression than 50 in all subsets to eliminate noise and with a differential expression lower than 1.5-fold. Next, up- and downregulated genes were identified to select gene sets for a gene set enrichment analysis30 of the steady-state young and aged HSC arrays and 42 genes were selected from the leading edge gene lists of these analyses.

The age-associated differential expression of the selected genes was confirmed in independently generated microarray RNA sequence (RNA-seq) dataset. In this experiment, we included 3 independently obtained datasets. Below and the analysis of the RNA-seq data set was performed using provided fragments per kilobase of exon per million fragments mapped (FPKM) values. The heatmap in Fig. 3c was generated in dChip. Next, iPS-HSCs and endogenous young HSCs isolated from the same iPS chimeras (11–14 weeks of age), middle-aged (11 months of age) and aged HSCs (22 months of age) were used for multiplex quantitative PCR with reverse transcription analyses using the Fluidigm Biomark platform as previously described31. In brief, single HSCs were sorted into 5 μl lysis buffer (10 mM TRIS-HCl pH 8.0, 0.1 mM EDTA, 0.1 μM l-1SUPRease-In (Clontech) and 0.5% NP40 (Igepal-CA630 Sigma Aldrich, St Louis, MO)). Sorted plates were preamplified in multiplex with target specific primers (Supplementary Table 1) using TATAA PreAmp GrandMaster Mix (TATAA Biocenter, Gothenburg, Sweden) for 22 cycles of 96°C for 15 s and 60°C for 6 min. After amplification, the PCR products were treated with Exonuclease I (NEB, Ipswich, MA) to remove unused primers. Gene expression analysis was performed using the 48.48 Dynamic array Integrated Fluidic Circuits (48 IFC) on the Biomark HD platform (Fluidigm, San Francisco, CA). For each gene assay, 2.5 μl 2× Assay loading reagent (Fluidigm, San Francisco, CA) was mixed with 2.5 μl of diluted assays from Fluidigm and in-house designed assays (forward and reverse primers mixed at a final concentration of 5 μM). Samples were diluted 5× in low EDTA TE buffer (10 mM TRIS-HCl pH 8.0 and 0.1 mM EDTA) before loading the 48 IFC. A premix of 2.7 μl diluted sample and 3.3 μl of 2× TaqFast EvaGreen Supermix with low ROX (Biorad, Hercules, CA) and 20× DNA-binding Dye (Fluidigm, San Francisco, CA) was applied. A premix of 4 μl of each sample and assay were loaded into individual sample and assay inlets on the 48 IFC. Using the IFC controller MX (Fluidigm), the samples and assays were loaded into the reaction chambers and the 48 IFC was then transferred to the BioMark HD unit for quantitative PCR. The Fast PCR program included an initial hot start of 1 min at 95°C and then 30 cycles of 96°C for 5 s and 60°C for 20 s. Upon completion of the PCR, a melting curve analysis was performed with a ramp from 60 to 95°C at 1°C/30 s. Data were analysed using the Fluidigm Real-time PCR Analysis software using the Linear (Derivative) Baseline Correction method and the Auto (Global) Ct Threshold Method. The Ct values determined were exported to SGEA32 and Excel for further processing.

Statistics. Data were analysed using Microsoft Excel (Microsoft, http://www.microsoft.com) and Prism (GraphPad Software). All FACS data were analysed using the Flowjo software (TreeStar, Ashland, OR, http://www.flowjo.com). Venn diagrams were generated using Venny30. Significance values were calculated
by Student’s two-tailed t-test and a P value of < 0.05, indicated as **, was used to determine significance. No statistical method was used to predetermine sample size and experiments were not randomized. No data were excluded from analysis. The investigators were not blinded to allocation during experiments or outcome assessment.

Data availability. The microarray data sets used to determine genes deregulated with age are found in Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo) under accession numbers GSE27686 and GSE44923. The microarray and RNA-seq data sets used to independently validate the ageing gene lists can also be found in GEO under accession numbers GSE55525, GSE43729 and GSE47817.

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Author contributions
M.W. and D.B. designed the study. M.W. and E.E. performed experiments and J.M.G. and C.B. executed and supervised blastocyst/morula injections. T.K., R.L. and J.Y. were involved in the design and interpretation of the barcoding experiments and provided critical reagents. D.B. conceived and supervised the study, and wrote the paper together with M.W.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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