Intra-hematopoietic cell fusion as a source of somatic variation in the hematopoietic system

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Summary

Cell fusion plays a well-recognized, physiological role during development. Bone-marrow-derived hematopoietic cells have been shown to fuse with non-hematopoietic cells in a wide variety of tissues. Some organs appear to resolve the changes in ploidy status, generating functional and mitotically-competent events. However, cell fusion exclusively involving hematopoietic cells has not been reported. Indeed, genomic copy number variation in highly replicative hematopoietic cells is widely considered a hallmark of malignant transformation. Here we show that cell fusion occurs between cells of the hematopoietic system under injury as well as non-injury conditions. Experiments reveal the acquisition of genetic markers in fusion products, their tractable maintenance during hematopoietic differentiation and long-term persistence after serial transplantation. Fusion events were identified in clonogenic progenitors as well as differentiated myeloid and lymphoid cells. These observations provide a new experimental model for the study of non-pathogenic somatic diversity in the hematopoietic system.

Key words: Copy number variation, Intra-hematopoietic cell fusion, Somatic diversity

Introduction

Non-pathogenic genetic variation and genome-wide copy number variation (CNV) have been demonstrated in somatic tissues of several organisms, including humans, fruit flies and yeast (Conrad et al., 2010; Hastings et al., 2009; Torres et al., 2010). Mechanisms proposed for the generation of CNV implicate cell-intrinsic means of DNA recombination (Zhang et al., 2009). Polyploid progeny of murine hepatocytes undergo ploidy reduction, propagate CNV, and generate genetically diverse events (Duncan et al., 2010). However, a recent study also demonstrates cell fusion between bone-marrow-derived cells (BMDCs) and hepatocytes, suggesting that CNV is generated by merging DNA from two separate cells rather than from recombination events within a single cell (Duncan et al., 2009).

Bone-marrow-derived cells can fuse with hepatocytes, neurons or epithelial cells of the intestine, respectively, in a process that is seemingly amplified by acute tissue damage or inflammation (Bailey et al., 2006; Davies et al., 2009; Johansson et al., 2008; Nygren et al., 2008; Rizvi et al., 2006; Wang et al., 2003; Willenbring et al., 2004). Such products of ‘heterotypic’ cell fusion have been found to acquire functional characteristics of the host tissue and are considered evidence for a physiological regenerative mechanism (Palermo et al., 2009). Other than in the liver, direct tracking of genetic markers in fusion events and serial evaluation of mitotic competence and cell fate have not been extensively performed. This probably reflects the experimental focus of previous studies, technical limitations or the post-mitotic nature of specific fusion partner cell types (e.g. neurons). Aside from rare reports of incidentally detected somatic mosaicism, changes in genomic copy number the hematopoietic system are generally associated with malignant transformation (Piotrowski et al., 2008).

Congenics mice harboring polymorphisms at the Ly5 locus expressing distinct (CD45.1, CD45.2) cell surface markers are frequently used to dissect donor–host contributions for the study of hematopoietic stem cell (HSC) function and cell–cell fusion (McCulloch and Till, 1960; Zebedee et al., 1991). Co-expression of both CD45 donor and host isotype cell surface markers after ablative transplantation is widely attributed to experimental artifact, or considered evidence of membrane protein transfer between hematopoietic cells (Cho and Hill, 2008; Yamanaka et al., 2009). Here, we carefully dissect events with parental marker co-expression and present evidence of hematopoietic ‘homotypic’ cell fusion (i.e. fusion between cells arising in the same tissue) and marker CNV by interphase FISH and SNP-PCR. We observe homotypic hematopoietic fusion at comparable rates under non-injury conditions in a parabiosis model and show that intra-hematopoietic cell fusion produces mitotically competent, clonogenic progenitors that are genotypically diverse for unique informative markers without evidence of malignant transformation.

Results and Discussion

Intra-hematopoietic cell fusion events isolated from irradiated transplant animals

We hypothesized that cell fusion could be a potential mechanism for generating genetic diversity within hematopoietic tissues and sought to identify intra-hematopoietic cell fusion progeny (Anderson et al., 2011; Chandhok and Pellman, 2009). In independent experiments, sublethally irradiated male recipients (CD45.1) received either congenic, CD45 (Ly5)-mismatched c-kit+, sca-1−, Lin− (KSL) cells, or unFractionated bone marrow cells from female donors (CD45.2) transgenic (hemizygous) for human CD46 (Fig. 1A) (Yannoutsos et al., 1996). In an
additional model, sublethally irradiated CD45.1 GFP+ males received whole bone marrow from CD45.2 females transgenic for human CD46 (Fig. 1A). Following hematopoietic reconstitution of recipients with multi-lineage donor chimerism (40–90%) in the peripheral blood, the hematopoietic tissues were harvested for analysis at time points between 1 and 12 months after transplantation. Cells co-expressing human CD46 (donor) and either CD45.1 cell surface antigen or GFP (both host) were serially sorted for improved stringency (Fig. 1B). A ‘doublet discriminator’ was used to exclude isolation of ‘doublets’ (i.e. two attached cells) (Hughes et al., 2009; Wersto et al., 2001). We observed cells co-expressing parental markers in all donor–host combinations and following different FACS sorting strategies (Fig. 1B). Shared marker expression in individual, sorted cells was confirmed by immunofluorescent (IF) deconvolution microscopy and z-stack analysis (Fig. 1C,D), distinguishing cells co-expressing donor–host markers from doublets. To exclude ambiguity from surface antigen or membrane transfer between donor and host hematopoietic cells (Yamanaka et al., 2009), DNA evidence of fusion was demonstrated by single nucleotide polymorphism (SNP) typing (D1Mit421.1) for CD45.1 and CD45.2 alleles. Genomic DNA from flow-cytometrically isolated single CD45.1+ CD46+ cells revealed amplification of both donor and host SNPs (Fig. 1E), whereas cells sorted from control animals demonstrated only their predicted unique donor or host CD45 SNP signature (supplementary material Fig. S1). Validation of cell fusion within the sorted population was further corroborated by interphase fluorescence in situ hybridization (FISH) analysis (Fig. 1F) showing synkarya containing genetic markers of both donor (human CD46) and host (mouse Y chromosome) origin. These observations suggest the acquisition and expression of genetic markers through fusion between hematopoietic cells and illustrate their long-term persistence.

Determination of hematopoietic lineages participating in fusion events
To ascertain the potential lineage restriction of exclusively hematopoietic fusion events generated after transplantation (Fig. 1A), we isolated hematopoietic cells by flow cytometry for co-expression of donor and host immunophenotype and observed expression of T cell (CD3, CD4, CD8), B cell (B220) or macrophage (Mac1) markers (Fig. 2A). Deconvolution fluorescent microscopy further confirmed co-expression of donor, host and select lineage markers in individual cells (Fig. 2B–D), with nuclei containing both donor and host DNA markers identified by interphase FISH at frequencies ranging from 1% to 3% (Fig. 2E–H). Control samples of cells sorted concurrently from the same animals for expression of either donor or host markers did not exhibit evidence of genetic marker blending. To determine the presence of hematopoietic progenitors among fused cells and test their capacity to undergo myeloid differentiation, we plated unfractionated bone marrow of GFP+ hosts (CD45.2 donor) in cytokine-supplemented methylcellulose culture to generate clonogenic colonies (CFU-C). Among GFP-expressing colonies, 9% contained SNP-PCR signatures of both donor and host alleles (Fig. 2H). Because our calculated fusion event frequency reflects only those loci represented by FISH or SNP markers, it is probably an underestimate due to anticipated instances of marker loss.

Fig. 1. Hematopoietic cell fusion following host radiation injury. (A) Transplantation strategy. (B) FACS plots of cells isolated from transplant recipients following serial sort for co-expression of CD45.1+ (host) and human CD46+ (donor). Animal 3* received KSL, all other animals in the table received whole bone marrow. The frequency of co-expressing events recovered following serial sorting was adjusted for sort purity and is recorded in the table. (C) Images of one z-plane of a human CD46+ CD45.1+ sorted whole bone marrow cell or (D) sorted CD45.2+ GFP+ spleen cell. Cytospun cells were stained with antibodies against donor (human CD46–PE, red), and host markers (C, CD45.1–APC, green, or D, anti-GFP Alexa Fluor 488, green) and DAPI (blue), and visualized with deconvolution fluorescent microscopy. Scale bars: 2 μm. (E) Single-cell SNP-PCR on a fused CD45.2+ GFP+ sorted thymus cell of a CD45.1–GFP transplant recipient, shown with controls for each allele. A Beizer correction was applied to Fig. 1E in its entirety to reduce appearance of background smearing; linear adjustments to intensity were applied to all subsequent gel images. L, ladder. (F) Interphase FISH analysis of a fused cell that contains mouse Y (red) and human CD46 (green). Scale bar: 5 μm.
Tissue-specific injury is not required to induce intra-hematopoietic cell fusion events

Experimental models of heterotypic cell fusion use acute tissue damage or inflammation by irradiation or physical injury to initiate cell fusion (Davies et al., 2009; Nygren et al., 2008). To test the requirement for injury induction in intra-hematopoietic fusion we used a parabiosis model (Wright et al., 2001). Congenic, CD45 isotype-mismatched mice were surgically attached to establish cross circulation without injury, resulting in reciprocal parabiont partner bone marrow chimeraism between 6% and 27% (Fig. 3A). At time points ranging from 3 to 9 months after separation, cells co-expressing donor and host markers were serially sorted from hematopoietic tissues of individual parabiosis partners (Fig. 3B). The frequency of co-expressing events was comparable to that observed in the transplantation model (Fig. 3B, supplementary material Table S1). For validation in gender-mismatched parabionts, we examined interphase FISH sex chromosome markers in cells isolated from the bone marrow and spleen of animals at several time points after separation. In serially sorted GFP+ (female) CD45.2 populations, we confirmed a subset of cells with evidence of donor and host genetic marker mixing by FISH (Fig. 3C). Analysis of individual CFU-C derived from unfractionated bone marrow from parabiosis pairs involving one GFP+ transgenenic partner revealed that approximately 25% of the GFP-expressing colonies possessed both donor and host SNPs (Fig. 3D–F). Clearly, these intra-hematopoietic events were non-abortive and mitotically competent. Thus, independent informative SNP and FISH markers in separate parabiosis models reveal that hematopoietic cells can undergo homotypic fusion in the absence of acute irradiation injury.

Frequency of hematopoietic cell fusion events

We used two different methods of screening and enrichment before validation of fusion events by genetic analysis: FACS and GFP+ CFU colony isolation. The frequency of cells co-expressing both parental markers was highest when protein-based assays such as FACS or microscopy were used for detection (supplementary material Fig. S2). This is probably due to cell membrane sharing (trogocytosis) between cells (Yamanaka et al., 2009). As evident from the decreased frequency of fusion events subsequently validated by genetic assays, FACS is suitable for the prospective recovery, but suffers from lower specificity. Conversely, GFP expression-based isolation of CFU-Cs proved a more efficient method. As a host parental marker, GFP represented the minority of plated CFUs. Because CFUs arise from clonal progenitor expansion over 7–10 days in culture, marker expression profiles reflect genomic contributions, rather than residual protein, and provide sensitive and specific validation. As a caveat, the frequency of fusion events in CFUs is biased toward myeloid progenitors; however, it was necessary to use FACS enrichment for all other (i.e. non-clonal) hematopoietic cell types.

Fused hematopoietic cells do not undergo malignant clonal expansion

We investigated whether hematopoietic multipotent progenitor cells (MPPs) participate in fusion events and found that the bone marrow c-kit+sca-1+ lineage (KSL) subset in primary transplant recipients contained up to 0.5% of cells co-expressing donor and host markers (not shown). To investigate the functional...
competence of MPPs, we flow-cytometrically sorted and transplanted whole bone marrow cells co-expressing primary CD45.2 human CD46 donor and GFP+ host markers at up to 1 year after primary transplantation into lethally irradiated CD45.1 GFP+ secondary hosts (Fig. 4A,B). When tested by interphase FISH for genetic evidence of marker mixing, both mouse Y and human CD46 were detected in myeloid and lymphoid lineages cells of secondary recipients (Fig. 4C–E). This observation further supports the notion that long-lived MPPs participate in fusion events and these events contribute to hematopoietic repopulation following serial transplantation. Unlike observations of aneuploidy and genetic instability seen after malignant transformation (Chandhok and Pellman, 2009), our data suggest that hematopoietic cells can fuse without apparent myeloid or lymphoid bias among progeny and that genetic markers from both fusion partners are maintained throughout in vitro progenitor differentiation and in vivo repopulation.

The fusion events discussed here are uniformly hematopoietic in phenotype, with prominent CD45 expression. However, that does not preclude their origin from fusion between a hematopoietic cell and a heterologous cell type with subsequent acquisition of a purely hematopoietic fate (Palermo et al., 2009). Indeed, this would mimic proposed mechanisms for the acquisition of metastatic disease by solid tumors. However, given that no such non-malignant fusion events have been described to date and none of the fusion events described here show malignant evolution, we favor the interpretation of homotypic cell fusion.

Interestingly, examination of donor and host autosomal reporter genes (GFP and CD46) by PCR of CFU-Cs from primary transplant animals revealed independent segregation of alleles in more than half of fusion products. Whereas the donor SNP allele should segregate with the donor reporter allele and the host SNP allele with the host reporter allele, we show several instances (n=4) in which a donor SNP was found to segregate with an autosomal host reporter, and vice versa (Fig. 4F). We observed additional instances (n=4) of parental marker loss. These results were corroborated in immunofluorescent deconvolution microscopy studies, in which hybrid cells with loss of a parental marker were observed (Fig. 4G). Despite these genetic changes, we did not detect evidence of malignant hematopoietic transformation in animals at time points up to 17 months after transplantation or parabiosis separation. Each animal was subjected to gross necropsy, and we analyzed peripheral blood and differential leukocyte cell counts, which showed values within normal limits for strain and gender (supplementary material Table S2). Lineage analysis and CFU-C frequencies obtained from primary and secondary animals showed no abnormalities. Thus, although we observed evidence of marker gain by SNP and FISH analysis in lymphoid and myeloid fusion progeny following transplantation and parabiosis separation, none of the animals demonstrated overt hematopoietic malignancy, lineage restriction or an increasing frequency of homotypic cell fusion over time. Rather, purely hematopoietic fusion events propagate genetically acquired markers for an extended period of time in vivo, without transformation and throughout cytokine-driven clonogenic differentiation in vitro. On the basis of the aggregate SNP and FISH data, we propose the following events to explain how hematopoietic cell–cell fusion can contribute to somatic CNV (Fig. 4H). Subsequent to fusion of the cellular membranes, an intermediate binuclear, tetraploid heterokaryon is formed. Most of
the fusion events observed contained a single nucleus with DNA markers from both parental cells, suggesting nuclear reduction and completion of cell division (Duncan et al., 2009; Duncan et al., 2010).

Our findings suggest that long-lived hematopoietic cells might be more tolerant of limited chromosomal sequence gains than anticipated. Somatic adaptation is thought to contribute to clonal variegation in cancer, presumably owing to increased genetic instability of chromosomally imbalanced cells (Anderson et al., 2011). However, CNV following intra-hematopoietic cell fusion could provide a source of non-pathogenic adaptive diversity (Duelli et al., 2005; Muotri et al., 2010; Piotrowski et al., 2008) and might explain reports of unexpected lymphoid and myeloid lineage marker co-expression in hematopoietic progenitors (Balciunaite et al., 2005; Quesenberry and Aliotta, 2008).

In conclusion, this is the first demonstration of hematopoietic cell fusion resulting in functionally competent, non-pathogenic cells from multiple lineages arising under injury or non-injury conditions. We propose intra-hematopoietic cell fusion as a novel platform to investigate somatic variation in the hematopoietic system.

Materials and Methods
Mice
Mice were maintained in a breeding colony in the animal care facility at OHSU. All procedures were approved by the OHSU Institutional Animal Care and Use Committee. C57BL/6 background mouse strains used in these studies (from the Jackson Laboratory unless indicated) included: C57BL/6 (CD45.2), C57BL/6Ka-Thyl.1-Ly5.1 (CD45.1), B6.FVB-Tg(CD46)2Gsv/J and C57BL/6-TgN(ACTB-EGFP)Osb-YO-1 (referred to as GFP; from Masaru Okabe, Osaka University, Osaka, Japan).

Transplantation studies
Donors and hosts were between 8 and 12 weeks of age at the time of transplant. Female CD46 transgenic donor bone marrow (1 \times 10^6 unfractionated cells or 1000 Lineage^- c-kit^+ Sca-1^+ cells sorted as described (Goldman et al., 2009) was transplanted into CD45.1 or CD45.1 GFP male recipients following 750 cGy gamma irradiation. For secondary transplant studies, 1 \times 10^6 unfractionated bone marrow cells from primary hosts were transplanted into lethally irradiated (1150 cGy), 8- to 12-week-old hosts. Donor engraftment was confirmed 4 weeks after transplant by peripheral blood analysis.

Parabiosis
Parabiotic pairs of 6- to 12-week-old age- and weight-matched mice were generated with CD45 congenic GFP^+ or GFP^- C57Bl/6 mice as previously described (Bailey et al., 2008).

Fig. 4. Fused cells contribute to long-term hematopoiesis in secondary recipients. (A) Secondary transplantation scheme. (B) Cells co-expressing CD45.2^- GFP^- were FACs sorted from spleens collected from secondary hosts. (C) Interphase FISH analysis of a fused lymphoid (CD4, CD8, B220) and (D) myeloid (Mac1, Gr1) cell isolated from spleen. Cells were probed for mouse Y (red) and human CD46 (green). (E) Hematopoietic cells co-expressing donor and host markers isolated from secondary transplant recipients analyzed by interphase FISH. (F) CFU-Cs were isolated from transplant recipients. SNP-PCR and PCR for autosomal reporter genes (CD46 or GFP) was performed on each colony. (G) Whole bone marrow (WBM) was harvested from a primary transplant recipient, FACs sorted for co-expression of parental markers and cytospun onto slides. Cells were stained with antibodies against host (CD45.1, FITC), donor (CD46, PE) and donor (CD45.2, APC). Although all CD46^- cells should be CD45.2^+, there were a few instances in which a host marker was acquired and one of the two donor markers was lost. (H) Model of cell fusion. The genetic markers used in our studies are shown for each cell type. Fusion of the cellular membrane results in a binuclear cell containing a nucleus from each parental cell type. Following mitosis and cytokinesis, a daughter cell will be tetraploid or will undergo ploidy reduction to revert to diploid (or near-diploid) state with concurrent gain or loss of excess chromosomal material. In these cases, the daughter cell is mononuclear.
Fluorescence-activated cell sorting

Cells were prepared from long bones, spleen and thymus of experimental mice. These antibodies (from eBioscience unless indicated) were used in cell sorting: CD46-phycocerythrin (PE), CD45.1-Fluorescein isothiocyanate (FITC; BD Pharmingen), CD45.1-PE (BD Pharmingen), CD45.1-PE-Cy7, CD45.1-APC-efluor 780, CD45.2-APC, CD45.2-PE (BD Pharmingen), F4/80-FITC (Serotec), Macl-Alpha Fluor-488, B220-FITC (BD Pharmingen), CD11 (e-kit)-APC–Alpha Fluor-750, LyAE (Scal)-PE-Cy7 (BD Pharmingen) and an APC-conjugated lineage mixture (B220, Ter119, CD3, CD4, CD5, CD8, Mac1, Gr1; BD Pharmingen). Cells were serially sorted using a BD Influx fluorescence-activated cell sorter. Dead cells were excluded by a combination of scatter gates and propidium iodine, and doublets were eliminated using the pulse-width parameter.

SNP-genotyping PCR

The single nucleotide polymorphism (SNP) at D1Mit421.1 (rs3022832) was used to distinguish CD45.2 cells (C allele) and CD45.1 cells (G allele). Nested SNP-PCR was performed on single sorted cells [collected as previously described (Duncan et al., 2009), and individual CFU-C colonies. Two rounds of PCR (40 cycles each) were performed with Platinum Taq polymerase (Invitrogen) using the outside primers: 5'-TGT TTT TCG AGG GCA TTT GCA CAG CAG-3' and 5'-TGG CAG AGT GTG TGT GAG TCT GTG-3' and the internal primers: 5'-GGG TCT GCC TGT CTT TGT CTT TGA-3' and 5'-GGT TGT GGT TGT GGT TGT GTG TGT-3'. Amplicons were digested overnight with 3 units of NcoI (New England Biolabs) and resolved on a 0.8% agarose gel.

Immunofluorescence and deconvolution microscopy

Hematopoietic cells were prepared as previously described (Skinner et al., 2009). Deconvolution microscopy was performed at the OHUU Advanced Light Microscopy Core with an Olympus IX71 wide field microscope, a Nikon Coolpix HQ Camera, and DeltaVision SoftWoRx software. Deconvolution and color assignments were performed with SoftWoRx software (Applied Precision). Images were acquired using the 60× 1.4 NA oil lens. Z-stacks were acquired at 0.5 μm for the complete depth of the cells and were deconvolved for nine iterations with appropriate point spread function.

Fluorescent in situ hybridization

An Enzo-Green labeled probe for human CD46 (human BAC RP11-454L1; from Empereon Genomics), mouse Y point probe (ID Laboratories) and incubated at 37 ˚C. After 7–12 days of culture, individual, non-crossed colonies were harvested.

Acknowledgements

We gratefully acknowledge Andrea McBeth, Pamela Canaday and Devonish Goldman for experimental assistance and expertise in SNP-PCR, cell sorting and interphase FISH, respectively.

Funding

The project was supported by the National Heart, Lung, and Blood Institute (NHLBI) [grant number HL90765 to P.K. and HL095351 to A.M.S.]; and the National Institutes of Health [grant number HL103109 to William H. Fleming]. The content does not necessarily represent the official views of the NHLBI or the NIH. Deposited in PMC for release after 12 months.

Supplementary material available online at http://www.jcs.biologists.orglookup/suppl doi: 10.1242/jcs.100123/DC1

References


**Fig. S1.** SNP PCR Strategy. (A) Representative images of CFU-Cs under brightfield, magnified 1X under a dissecting microscope. CFUs were plated in 3 ml cytokine-containing methylcellulose on 60 mm plates. All CFUs characterized in our studies were picked under 4x or 10x magnification to ensure single colonies were isolated. Top panel: image of 2 colonies prior to isolation. Bottom panel: image of colony remnants (left) and untouched colony (right) following isolation. (B) SNP-PCR digest of control CD45.1 and CD45.2 CFUs (top) and sequence histograms (bottom) shown alongside a fused CFU. (C) Control experiment in which WBM was isolated and plated in cytokine-containing methylcellulose from a CD45.1 (1) animal, CD45.2 (2) animal, or WBM was mixed (M) at a ratio or 1:2 prior to plating in methylcellulose. Upon colony isolation, all samples were blinded. The genotypes of each colony were correctly determined following SNP-PCR digest. Each colony isolated from the “mixed” plate digested as a single genotype. There were no false positive fusion events.
Fig. S2. Frequency of WBM cells co-expressing both parental markers determined by multiple methods. The average number of WBM cells co-expressing parental markers observed following transplantation is shown (left panel). Error bars represent standard deviation between mice. Enriched co-expressing cells were validated as fusion products using immunofluorescent microscopy with complementary antibodies (i.e. not used in FACS), as well as genetic screens: SNP-PCR or FISH. The number of cells analyzed as well as the number of observed fusion events is shown in each column (right panel). The overall frequency of intra-hematopoietic cell fusion in WBM of transplant animals was calculated by multiplying the average frequency determined by FACS by the frequency of validation by secondary assay (e.g. IF, SNP-PCR, FISH) and is shown below each method.
Supplemental Table 1

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<td><strong>2 (11%)</strong></td>
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Table S1. Single cell events analyzed by SNP-PCR.
Supplemental Table 2: Complete Blood Counts on mice examined in this study

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<th>Hb (g/dL)</th>
<th>platelet (x10&lt;sup&gt;3&lt;/sup&gt;/µL)</th>
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<th>Ly (%)</th>
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<sup>a</sup>: Indicates the number of months elapsed between time of transplant and CBC.

* Data from Mouse Phenome Database (http://phenome.jax.org), 24-month animals for age comparison.

Table S2. Complete Blood Counts on mice examined in this study.