Brief report

Fanconi anemia type C–deficient hematopoietic stem/progenitor cells exhibit aberrant cell cycle control

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The pathogenesis of bone marrow failure in Fanconi anemia is poorly understood. Suggested mechanisms include enhanced apoptosis secondary to DNA damage and altered inhibitory cytokine signaling. Recent data determined that disrupted cell cycle control of hematopoietic stem and/or progenitor cells disrupts normal hematopoiesis with increased hematopoietic stem cell cycling resulting in diminished function and increased sensitivity to cell cycle–specific apoptotic stimuli. Here, we used Fanconi anemia complementation type C–deficient (Fancc−/−) mice to demonstrate that Fancc−/− phenotypically defined cell populations enriched for hematopoietic stem and progenitor cells exhibit increased cycling. In addition, we established that the defect in cell cycle regulation is not a compensatory mechanism from enhanced apoptosis occurring in vivo. Collectively, these data provide a previously unrecognized phenotype in Fancc−/− hematopoietic stem/progenitor cells, which may contribute to the progressive bone marrow failure in Fanconi anemia. (Blood. 2003;102:2081-2084)

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Study design

Mice

Six- to 10-week-old WT, Fancc−/−, and p21WAF1−/− mice (C57Bl/6 strain) were used as previously described. The Indiana University laboratory animal committee approved all studies.

BrdU assays

WT and Fancc−/− mice were injected intraperitoneally with 100 mg/kg of bromodeoxyuridine (BrdU; Sigma, St Louis, MO) and killed 90 minutes later. For in vitro studies, WT and Fancc−/− BM cells were cultured in Iscove modified Dulbecco medium (IMDM; GIBCO BRL, Gaithersburg, MD), 20% fetal calf serum (FCS; Biowhittaker, Walkersville, MD), and 10 μM BrdU for 45 minutes. BrdU-pulsed cells were stained with anti-CD3–fluorescein isothiocyanate (FITC), anti-B220–FITC, anti-Gr1–FITC, anti-ckit–allophycocyanin (APC; BD Biosciences), fixed in 1% formaldehyde, and 10 μM BrdU for 45 minutes. BrdU-pulsed cells were stained with anti-CD3–fluorescein isothiocyanate (FITC), anti-B220–FITC, anti-Gr1–FITC, anti-Mac1–JTITC, and anti-Ter119–FITC (BD Biosciences, San Diego, CA) and enriched for lineage negative (lin−) cells using a FACStar fluorescence cytometer. Lin− cells were stained with anti-Scal–phycoerythrin (PE) and anti-ckit–allophycocyanin (APC; BD Biosciences), fixed in 1% formaldehyde overnight, and permeabilized with 0.1% saponin phosphate-buffered saline (PBS) plus 2% formaldehyde for 10 minutes. Cells were then analyzed by flow cytometry.

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permeabilized with 0.2% Tween 20 for 10 minutes and treated with 100 to 300 Kunitz Units of DNase I (Sigma) in Hanks Balanced salt solution (Biowhittaker) for 60 minutes before incubating with anti-BrdU–FITC (BD Biosciences). Cells were then analyzed by fluorescence cytometry for simultaneous detection of Sca1, ckit, and BrdU as previously described.16

3H-thymidine incorporation/PKH26 studies

BM-derived mast cells (BMMCs) were derived as previously described.17,18

G0/G1 analysis

BM cells or BMMCs were stained with Hoechst 33342 (Molecular Probes, Eugene, OR) and Pyronin Y (Sigma) and analyzed as previously described. Fluorescence cytometer settings were determined using WT and p21<sup>cip1/waf1</sup>−/− samples as controls.

Apoptosis

WT and Fancc<sup>−/−</sup> SCL cells were evaluated by the TUNEL (TdT-mediated dUTP nick end labeling) assay similar to previously described methods except fluorescence cytometry was used for analyses.

Western blotting

BMMCs were lysed and protein lysates were quantitated, separated on a PAGE gel, and transferred to nitrocellulose as previously described.17 Membranes were incubated for 1 hour with rabbit antiretinoblastoma (Rb) antibody (1 ug/mL; BD Biosciences) followed by a 1-hour incubation with antirabbit–horseradish peroxidase antibody (1:1000; Amersham, Piscataway, NJ) before visualizing by chemiluminescence (Amersham). Equal loading was documented with β-actin (Sigma).

Results and discussion

To evaluate cycling of primitive phenotypically defined cells enriched for hematopoietic stem/progenitors, we compared BrdU incorporation of Fancc<sup>−/−</sup> and WT Sca1<sup>+</sup> ckit<sup>+</sup>lin<sup>−</sup> (SCL) cells after pulsing with BrdU. We observed a 2- to 3-fold increase in BrdU incorporation in Fancc<sup>−/−</sup> SCL cells compared with WT controls (Figure 1A-B), analogous to clonogenic progenitors.15 Similar data were obtained using a 3H-thymidine incorporation assay (data not shown). We next examined whether Fancc<sup>−/−</sup> cells were less quiescent in vivo by comparing the percent of Fancc<sup>−/−</sup> and WT lin<sup>−</sup> cells in G0 using our previously described method of Hoechst 33342 and Pyronin Y staining.19 WT and p21<sup>cip1/waf1</sup>−/− samples were used as controls to validate fluorescence cytometer settings. Consistent with increased BrdU incorporation, we detected fewer Fancc<sup>−/−</sup> lin<sup>−</sup> cells in G0 compared with WT controls (Figure 1C-D). Since primitive Fancc<sup>−/−</sup> hematopoietic cells display a proapoptotic phenotype15,20 and differentiated cell numbers in Fancc<sup>−/−</sup> mice are similar to WT (Haneline et al.,15 Chen et al.,21 and data not shown), an increase in stem/progenitor cell cycling may be a compensatory response to maintain normal numbers of cells in vivo. However, at time points that cell cycle alterations were observed in Fancc<sup>−/−</sup> SCL cells, apoptosis was not different from WT controls (fresh SCL cells < 2% TUNEL<sup>+</sup> in both genotypes and 24 hours cultured SCL cells 6% ± 2% versus 9% ± 3% TUNEL<sup>+</sup> for WT and Fancc<sup>−/−</sup> SCL cells, respectively, n = 4). Collectively, these data argue that Fancc<sup>−/−</sup> stem/progenitor cells exhibit increased cycling and are less quiescent compared with WT controls.

To further examine whether Fancc<sup>−/−</sup> hematopoietic cells have a cell autonomous cell cycle control defect, we established BMMCs and conducted in vitro experiments, since cultured cells do not require maintenance of cell numbers in vitro. BMMCs were selected as the cellular model system because these cells are a ckit<sup>+</sup> myeloid progenitor population that retains a high clonogenic capacity analogous to other myeloid progenitors.17,22 To assess whether Fancc<sup>−/−</sup> BMMCs exhibit altered cell cycle regulation, we compared %G0 cells (Figure 2A) and proliferation (Figure 2B) of WT and Fancc<sup>−/−</sup> BMMCs after 24 hours of serum/cytokine starvation. Similar to phenotypically defined Fancc<sup>−/−</sup> stem/progenitor cells (Figure 1C-D), fewer Fancc<sup>−/−</sup> BMMCs were in G0 compared with WT cells (Figure 2A). Additionally, 3H-thymidine incorporation was detected earlier (12 hours) and was

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Figure 1. Fancc<sup>−/−</sup> hematopoietic stem/progenitor cells are less quiescent than WT control cells. (A) A representative experiment demonstrating the gating method used for in vivo and in vitro BrdU pulse assays of SCL cells. Either mice or lin<sup>−</sup> cells harvested from Fancc<sup>−/−</sup> and WT mice were pulsed with BrdU and analyzed for simultaneous detection of Sca1, ckit, and BrdU incorporation as described in “Study design.” (B) Mean BrdU incorporation in SCL cells. Data shown are the mean of 5 experiments for both in vitro and in vivo pulsing methods. *P < .05. (C) Cell cycle analysis of a representative experiment. Lin<sup>−</sup> cells from Fancc<sup>−/−</sup>, WT, and p21<sup>cip1/waf1</sup>−/− mice were stained with Hoechst 33342 and Pyronin Y. WT and p21<sup>cip1/waf1</sup>−/− samples were used as controls to set fluorescent cytometer parameters. Data shown are one of 5 representative experiments with similar results. (D) Mean %G0 lin<sup>−</sup> cells. The mean of 5 independent experiments is shown. **P < .002. A Student t distribution and P values were determined using GraphPad Prism 3.0a software (San Diego, CA).
significantly higher in Fancc<sup>−/−</sup> BMMCs at all time points evaluated compared with WT BMMCs (Figure 2B). Similar data were obtained using PKH26, a membrane dye used to track divisional history<sup>18</sup> (Figure 2C), verifying that the observed increase in H-thymidine incorporation in Fancc<sup>−/−</sup> BMMC was from increased cycling and not secondary to unscheduled DNA synthesis from increased damage/repair. To confirm these data with a biochemical marker, Rb phosphorylation was examined. Unphosphorylated Rb is a transcriptional corepressor that inhibits E2F family members and Rb hyperphosphorylation results in subsequent E2F-mediated transcription with S-phase initiation. Consistent with cell biology data (Figure 2A-C), significantly higher phosphorylated Rb was detected earlier in Fancc<sup>−/−</sup> BMMCs compared with WT controls (Figure 2D), providing a biochemical correlate for cell cycle data. Using BMMCs as a model system, together these data argue that the alteration in hematopoietic cell cycle regulation observed in vitro and in vivo is, at least in part, cell autonomous.

Cumulatively, these data demonstrate a previously unrecognized phenotype in Fancc<sup>−/−</sup> hematopoietic stem/progenitor cells. Furthermore, these data suggest that increased Fancc<sup>−/−</sup> stem/progenitor cell cycling may contribute to the apoptotic phenotype. Previously identified mechanisms accounting for enhanced apoptosis in Fancc<sup>−/−</sup> cells include perturbed double-stranded RNA-dependent protein kinase–mediated apoptotic signaling<sup>23,24</sup> and increased DNA damage secondary to either aberrant cell cycle checkpoint control and/or DNA repair.<sup>1,2</sup> Our data implicate an additional mechanism since increased HSC cycling predisposes to stimuli that predominantly damage cycling cells.<sup>10-12</sup> Furthermore, if the DNA damage was insufficient to signal apoptosis and was not adequately repaired, an accumulation of mutations in a single HSC could result over time, increasing the risk of clonal evolution. Alternatively, altered cytokine signaling in Fancc<sup>−/−</sup> stem/progenitor cells may affect their cycling activity, arguing an indirect role of increased cycling on enhanced apoptosis in Fancc<sup>−/−</sup> stem/progenitor cells. The pathogenesis of BM failure and leukemogenesis in FA is likely multifaceted including defects in cytokine signaling, DNA repair, and cell cycle control, emphasizing the complexity of this disease and the need to carefully dissect the function(s) of individual FA proteins in order to improve current treatment strategies.

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**References**


