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Hematopoietic defects in response to reduced Arhgap21

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ABSTRACT

Arhgap21 is a member of the Rho GTPase activating protein (RhoGAP) family, which function as negative regulators of Rho GTPases. Arhgap21 has been implicated in adhesion and migration of cancer cells. However, the role of Arhgap21 has never been investigated in hematopoietic cells. Herein, we evaluated functional aspects of hematopoietic stem and progenitor cells (HSPC) using a haploinsufficient *(Arhgap21*$^{+/-}$*)* mouse. Our results show that *Arhgap21*$^{+/-}$ mice have an increased frequency of phenotypic HSC, impaired ability to form progenitor colonies *in vitro* and decreased hematopoietic engraftment *in vivo*, along with a decrease in LSK cell frequency during serial bone marrow transplantation. *Arhgap21*$^{+/-}$ hematopoietic progenitor cells have impaired adhesion and enhanced mobilization of immature LSK and myeloid progenitors. *Arhgap21*$^{+/-}$ mice also exhibit reduced erythroid commitment and differentiation, which was recapitulated in human primary cells, in which knockdown of *ARHGAP21* in CMP and MEP resulted in decreased erythroid commitment. Finally, we observed enhanced RhoC activity in the bone marrow cells of *Arhgap21*$^{+/-}$ mice, indicating that Arhgap21 functions in hematopoiesis may be at least partially mediated by RhoC inactivation.

1. Introduction

Hematopoiesis is a complex process initiated by the differentiation of hematopoietic stem cell (HSC) through progressive stages of lineage commitment (Sun et al., 2014; Yu et al., 2016). HSC functions are finely regulated by intrinsic and extrinsic factors, which prevent their exhaustion and allow hematopoiesis to be sustained throughout the life (Baryawno et al., 2017).

Hematopoietic stem and progenitor cells (HSPCs) are constantly undergoing fate-decisions, including quiescence *vs.* proliferation, self-renewal *vs.* differentiation, lineage commitment, and mobilization *vs.* adhesion. These processes are regulated by growth factors, cell-cell interactions, transcriptional networks, and epigenetics, many of which lead to cytoskeletal rearrangements (Nayak et al., 2013; Narla & Mohandas, 2016).

Rho GTPases are central regulators of cytoskeletal dynamics (Ridley, 2015) that cycle between an inactive GDP-bound and an active GTP-bound state. This cycle is tightly controlled by regulatory proteins, such as RhoGEFs and RhoGAPs, which respectively catalyze Rho activation and inactivation (Infante & Ridley, 2013). Despite efforts to understand the participation of Rho GTPases, such as Cdc42 and RhoA, in hematopoiesis, there are few studies regarding the role of RhoC and its regulators (GEFs and GAPs) in this system.

ARHGAP21 is a RhoGAP protein (Basseres et al., 2002) that contains a PDZ and a pleckstrin homology (PH) domain in addition to the RhoGAP domain.(Basseres et al., 2002; Dubois et al., 2005) ARHGAP21 has been shown RhoGAP activity for Cdc42,(Dubois et al., 2005; Bigarella et al., 2009) RhoA and RhoC (Lazarini et al., 2013) and is thought to integrate signals from multiple pathways. Our group has previously identified the participation of ARHGAP21 in cell adhesion and migration of solid tumor cell lines, and described an increase of ARHGAP21 mRNA expression during erythroid differentiation of primary human CD34^{+} cells (Bigarella et al., 2009; Lazarini et al., 2013; Barcellos et al., 2013).

Here we investigate the role of Arhgap21 in hematopoiesis using a heterozygous knockout mouse model. We show that reduction of Arhgap21 levels leads to changes in the relative frequencies of hematopoietic stem and progenitor cell populations, and mobilization of immature progenitor and myeloid cells. Using both murine and human primary cells, we observed that ARHGAP21 is important for erythroid commitment of common myeloid progenitor (CMP) and megakaryocyte-erythroid progenitor (MEP) cells. To provide

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Table 1
Hematologic parameters of WT and *Arhgap21*$^{+/-}$ mice.

Hematologic Parameters	WT	*Arhgap21*$^{+/-}$	p value
WBC ($\times 10^3$ cells/μL)	9.6 ± 1.82	12.4 ± 1.8	0.018
Lymphocytes (cells/μL)	7884 ± 1587	9842 ± 1392	0.09
Neutrophils (cells/μL)	1715 ± 189.7	2265 ± 157.6	0.04
RBC ($\times 10^6$ cells/μL)	10.93 ± 0.34	10.42 ± 0.28	0.015
Hemoglobin (g/dL)	16.27 ± 0.36	15.63 ± 0.35	0.0087
Hematocrit (%)	55.0 ± 1.73	53.5 ± 1.51	≥0.05
MCV (fL)	50.04 ± 0.48	51.05 ± 0.88	0.024
Platelets (10^5 cells/μL)	1.84 ± 0.05	1.74 ± 0.07	0.020
MPV (fL)	7.05 ± 0.12	7.2 ± 0.08	0.042

Blood samples were collected from 9 WT and 8 *Arhgap21*$^{+/-}$ mice (8 wk. old littermates). Counts were performed by automated hematology analyzer (Poch 100iv Diff; Sysmex; Roche). RBC: red blood cell count; MCV: mean cell volume; WBC: White blood cell count; MPV: mean platelet volume.

mechanistic insight, we show that there is increased RhoC activity (but not Cdc42 or RhoA) in the bone marrow, and decreased fibronectin adhesion *in vitro*, both of which likely play a role in causing the hematopoietic defects observed. These results provide strong evidence that Arhgap21 participates in cellular processes related to the maintenance of hematopoiesis, mainly through the regulation of RhoC activity.

2. Methods

2.1. Generation and genotyping of Arhgap21 haploinsufficient mice (*Arhgap21*$^{+/-}$)

An embryonic stem cell line containing vector insertion in the *Arhgap21* gene was obtained from the GeneTrap consortium (Gene Bank Accession number: CG784642) and injected into blastocysts of C57/Bl6 mice. Chimeras were genotyped for genomic insertion of the β-Geo cassette (Fig. S1A) and backcrossed with wild-type C57/Bl6 mice for 10 generations before performing experiments. Arhgap21$^{-/-}$ mice were embryonic lethal at E8. The reasons for embryonc lethality at 8 days post-conception are currently under investigation. Because hematopoietic stem cells emerge in the aorto-gonad-mesonephros region at E10.5, which occurs after Arhgap21$^{-/-}$ embryos have died, we have characterized the hematopoietic compartment of the haplo-insufficient mice.

Arhgap21$^{+/-}$ mice were genotyped by PCR, using DNA extracted from tail and primers targeting the β-Geo cassette (β-Geo forward: GGCGCCTCATGAATATTAACC; β-Geo reverse: CACTCCAACCTCCGCAAA CTC). All procedures were approved by the Ethics Committee for Experimental Research at the University of Campinas.

2.2. Isolation of bone marrow cells

Bone marrow cells were isolated by crushing the femurs, tibias and humerus of 6–10 week old mice. Cells were passed through a 70 μM strainer and red blood cells were lysed with lysis solution (155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA). For histology, femurs were fixed in 10% formalin and embedded in paraffin, sectioned and placed on silanized slides followed by hematoxylin and eosin staining. Five random high-powered fields from stained slides were captured at 10× objective magnification and visualized for manual counting for megakaryocytes, using ImageJ (http://imagej.nih.gov/ij/).

2.3. Real time PCR

RNA was purified with Illustra RNAspin Mini Kit (GE Healthcare Life Sciences, UK) and reverse transcribed with RevertAid H minus First Strand cDNA synthesis Kit (ThermoScientific, Inc., USA). Real time quantitative PCR was carried out as previously described (Xavier-Ferrucio et al., 2015), in an Eppendorf MasterCycler using SYBR green master mix (ThermoScientific, Inc., USA). Gene expression was determined, using specific primers: murine *Arhgap21* (NM_001128084) *Arhgap21* forward: GAGGAAAGCTTCAAGCACCA, Arhgap21 reverse: GATGACAGC AGATCAGGAA; Hprt forward: GGGGGCTATAAGTTCTTTGCT and HPRT reverse: GGCCTGTATCCAACACTTCG; human *ARHGAP21* (NM_020824) ARHGAP21 forward: CAATGGATACCATATTTGTTAAGCAAGTT, ARHGAP21 reverse: CACTTTCTCCATTGACTTTTATAATTCG, HPRT forward: TTGCTTTCCTTGGTCAGGCA and HPRT reverse: TTCGTGGGGTC CTTTTCACC.

2.4. Flow cytometric analysis

Bone marrow, spleen or peripheral blood cells were incubated with specific antibodies for 15 min at room temperature in order to characterize the following hematopoietic populations: HSC: Lineage markers (CD4, CD8, B220, Ter119, Gr1, Mac1) conjugated to FITC, Sca1-PerCP, c-Kit-APC-Cy7, CD150-PE and CD48-PE-Cy7; LSK: Lineage markers conjugated to FITC, Sca1-PE and c-Kit-APC; erythroid committed cells: Ter119-FITC; myeloid cells: Gr1-PE, Mac1-APC; PreMegE, MkP and ErP: Lineage markers conjugated to PeCy7, Sca1-BV421, c-Kit-APC, CD150-PE, FcgR-APCR700, CD41-BV510 and CD105-BB515. Ter119-FITC and CD71-PE staining accessed the erythroid differentiation stages. Gating of murine BM subpopulations was performed as in Pronk et al. (2007).

Expression of CXCR-4, α4β1 and α5β1 integrins in hematopoietic progenitor cells was evaluated by staining total bone marrow cells with APC-conjugated lineage markers (Lineage APC cocktail) and CXCR-4-PE, CD49d/CD29-PE or CD49e/CD29-PE. Unless specified, all antibodies were from BD Biosciences. For intracellular assessment of CXCR-4, cells were fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.2% BSA, 0.1% azide and 0.5% saponin. Fluorescence analysis was performed with a FACSCalibur or FACS Canto (Becton–Dickinson) and analyzed with FlowJo software (TreeStar Inc.)

2.5. Murine colony-formation assay

A total of 5×10^4 murine bone marrow cells were cultured in 1 mL of methylcellulose medium supplemented with growth factors (M3434, StemCell Technologies). After 10 days at 37 °C under 5% CO_2 and high humidity, the numbers of granulocyte–macrophage colony forming units (CFU-GM) and burst forming unit–erythroid (BFU-E) were assessed. Secondary plating was performed to determine sequential clonogenicity and the number of colonies (CFU-GM) was counted after an additional 10 days.

Fig. 1. HSPC and myeloid compartments are altered in *Arhgap21*$^{+/-}$ mice. (A–B) Flow cytometry analysis show increased frequency of Lin$^-$ Sca-1$^+$c-Kit$^+$(LSK) and of Lin$^-$ Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^-$ (LSK CD150$^+$CD48$^-$) cells in bone marrow of *Arhgap21*$^{+/-}$ mice (WT n = 6 and *Arhgap21*$^{+/-}$ n = 4; LSK BM $P = 0.04$; HSC $P = 0.03$). (C–D) Flow cytometry based on Pronk et al. (2007) to quantify MkP (Lin$^-$ FcgRII/III$^-$ cKit$^+$ Sca$^-$ CD150$^+$ CD41$^+$), ErP (Lin$^-$ FcgRII/III$^-$ cKit$^+$ Sca$^-$ CD150$^+$ CD41$^-$ CD105$^+$), PreMegE (Lin$^-$ FcgRII/III$^-$ cKit$^+$ Sca$^-$ CD150$^+$ CD41$^-$ CD105$^-$) and GMP (Lin$^-$ FcgRII/III$^+$ cKit$^+$ Sca$^-$ CD150$^-$ CD41$^-$) shows no change in GMP or PreMegE frequency, an increase of MkP ($P = 0.02$) and a decrease of ErP ($P = 0.02$) (WT n = 5 and *Arhgap21*$^{+/-}$ n = 3). Confirming the megakaryocytic over erythroid change, there was an increase in (E) CD41$^+$ cells by FACS analysis ($P = 0.03$) and in (F) megakaryocyte number assessed by morphology ($P = 0.002$) in 5 high power fields (hpf) of hematoxylin eosin stained bone marrow sections of each mouse (WT n = 8 and *Arhgap21*$^{+/-}$ n = 5). There was a decrease in (G) Ter119+ cells in bone marrow ($P = 0.01$) and spleen ($P = 0.02$). (H–I) Flow cytometric characterization of erythroid differentiation showing a decreased frequency *Arhgap21*$^{+/-}$ splenic proerythroblasts (I, $P = 0.03$), basophilic erythroblasts (II, $P = 0.03$), and late basophilic/polychromatophilic erythroblasts (III, P = 0.01), but no difference in the late subtypes of the erythroid differentiation such as orthochromatophilic erythroblasts (IV, WT n = 6 and *Arhgap21*$^{+/-}$ n = 9). (J) Flow cytometry results showing reduced frequency of Gr1$^+$/Mac-1$^+$ cells in bone marrow ($P = 0.04$) and increased frequency in peripheral blood ($P = 0.0009$) of *Arhgap21*$^{+/-}$ mice (WT n = 6 and *Arhgap21*$^{+/-}$ n = 10).