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## The RNA binding protein *Ars2* supports hematopoiesis at multiple levels

Seerat Elahi<sup>a,b</sup>, Shawn M. Egan<sup>b</sup>, G. Aaron Holling<sup>b</sup>, Rachel L. Kandefer<sup>b</sup>, Michael J. Nemeth<sup>b,c</sup>, and Scott H. Olejniczak<sup>a,b</sup>

<sup>a</sup>Department of Pathology and Anatomical Sciences, State University of New York at Buffalo, Buffalo, NY, USA

<sup>b</sup>Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA

<sup>c</sup>Department of Medicine, Roswell Park Cancer Institute, Buffalo, NY, USA

### Abstract

Recent biochemical characterization of Arsenic resistance protein 2 (*Ars2*) has established it as central to determining the fate of nascent RNA polymerase II (RNAPII) transcripts. Through interactions with the nuclear 5'-7-methylguanosine (7mG) cap binding complex (CBC), *Ars2* promotes co-transcriptional processing coupled with nuclear export or degradation of several classes of RNAPII transcripts, allowing for gene expression programs that facilitate rapid and sustained proliferation of immortalized cells in culture. However, rapidly dividing cells in culture do not represent the physiological condition of the vast majority of cells in an adult mammal. To examine functions of *Ars2* in a physiological setting we generated inducible *Ars2* knockout mice and found that deletion of *Ars2* from adult mice resulted in defective hematopoiesis in bone marrow and thymus. Importantly, only some of this defect could be explained by the requirement of *Ars2* for rapid proliferation, which we found to be cell-type specific *in vivo*. Rather *Ars2* was required for survival of developing thymocytes and for limiting differentiation of bone marrow resident long-term hematopoietic stem cells (LT-HSCs). As a result, *Ars2* knockout led to rapid thymic involution and loss of the ability of mice to regenerate peripheral blood following myeloablation. These *in vivo* data demonstrate that *Ars2* expression is important at several steps of hematopoiesis, likely because *Ars2* acts on gene expression programs underlying essential cell fate decisions such as the decision to die, to proliferate, or to differentiate.

### Introduction

Arsenic resistance protein 2 (*Ars2*) is an essential, highly conserved protein with mammals sharing more than 98% amino acid identity [1]. *Ars2* was initially described as a modulator

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**Corresponding author:** Dr. Scott H. Olejniczak, PhD., Department of Immunology, Roswell Park Comprehensive Cancer Center, L5-306 Center for Genetics and Pharmacology, Elm and Carlton Streets, Buffalo, NY 14263, +1(716)845-8538, Scott.olejniczak@roswellpark.org.

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of arsenic sensitivity in a cDNA screen [2]; subsequent analysis determined that the ability of Ars2 to modulate arsenic sensitivity likely resulted from a dominant-negative effect of the partial cDNA sequence used in the screen [3]. Full length Ars2 did not impart arsenic resistance, but rather was found to be a component of the 5'-7-methylguanosine (7mG) cap binding complex (CBC) that coordinated RNA polymerase II (RNAPII) transcription with nuclear RNA processing and export [3–9]. Ars2 was required for rapid proliferation of cells in culture [2, 3] and, unlike other protein components of the CBC, Ars2 could be induced by mitogenic signals [3, 10]. The intimate relationship between Ars2 and cellular proliferation likely stems from its role in 3' end processing of replication-dependent histone (RDH) mRNAs [4, 11, 12]. In addition to RDH mRNAs, Ars2 was shown to be involved in maturation and/or nuclear export of microRNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and select mRNAs [10, 11, 13–15]. As a balance to its anabolic effects on RNAPII transcripts, Ars2 supports transcription termination-coupled degradation of superfluous transcripts such as promoter upstream transcripts (PROMPTs) and enhancer RNAs (eRNAs) [5, 16, 17]. Overall, data indicate that the net effect of Ars2 is increased efficiency of productive RNAPII transcription.

*In vivo* characterization of Ars2 in mammals, while limited, demonstrated roles for Ars2 that do not stem from its requirement for rapid cellular proliferation. Wilson *et al.* demonstrated that embryos at the blastocyst stage rely on Ars2 to promote cell survival [1], while Gruber *et al.* found similar viability-promoting properties of Ars2 in adult peripheral immune organs [3]. Finally, Andreu-Agullo *et al.* demonstrated that Ars2 is essential for maintenance of neural stem cells in the absence of effects on their proliferation or viability [18]. Rather, Ars2 activated transcription of *Sox2*, a transcription factor that suppresses differentiation of neural stem cells.

In the current study we set out to identify additional physiologic roles for Ars2 in adult mammals. To this end, we generated inducible whole-body Ars2 knockout (Ars2 iKO) mice by crossing Ars2-floxed mice [3] with mice expressing the Cre-ERT2 transgene [19]. Initial characterization of Ars2 iKO mice following induced whole-body Ars2 deletion confirmed that developing blood cells are sensitive to Ars2 deletion, as originally observed by Gruber *et al.* following MxCre-mediated Ars2 deletion [3]. Adult mammals continually regenerate blood cells in a well characterized, step-wise process termed hematopoiesis. Hematopoiesis begins in the bone marrow with multipotent long-term hematopoietic stem cells (LT-HSC) that are capable of indefinite self-renewal and differentiation to generate all blood cells throughout the life of an organism [20, 21]. In adult mammals, the majority of LT-HSCs are quiescent to prevent exhaustion and malignant transformation [22, 23]. Upon activation by peripheral signaling, LT-HSCs enter cell cycle and undergo asymmetrical division to self-renew and differentiate into downstream lineage committed stem and progenitor cells that ultimately produce every mature blood cell [23–25].

While many blood cells mature prior to leaving the bone marrow, most T-cells require additional, antigen-dependent maturation that takes place in the thymus. Bone marrow derived T-cell progenitors enter the thymus and progress through a series of maturation and selection processes termed thymopoiesis that ultimately yields mature CD4<sup>+</sup> helper T-cells and CD8<sup>+</sup> cytotoxic T-cells [26–28]. Early thymic maturation during the CD4/CD8 double-

negative (DN) stage of thymopoiesis is driven by recombination of T-cell receptor (TCR) genes to produce TCR diversity [29]. Following TCR recombination, thymopoiesis proceeds to the CD4/CD8 double-positive (DP) stage during which positive and negative selection eliminate non-MHC-restricted and autoreactive T-cell progenitors. The small fraction of T-cell progenitors that survive positive and negative selection down-regulate either CD4 or CD8 to become mature CD8<sup>+</sup> or CD4<sup>+</sup> T-cells, respectively [30].

The current study extends cursory observations regarding roles for *Ars2* in normal mammalian hematopoiesis made by Gruber *et al* [3]. Specifically, we show that thymopoiesis is critically dependent on expression of *Ars2*, both in developing thymocytes and in thymic stroma. Additionally we report that hematopoiesis within the bone marrow of *Ars2* deficient mice is compromised as the result of defective proliferation of progenitor cells combined with loss of self-renewal capacity of LT-HSCs. Taken together, data indicate that *Ars2* is essential for multiple processes involved in production of mature blood cells and that *Ars2* influences these processes through intrinsic effects on developing blood cells along with effects on stromal cells that support hematopoiesis.

## Results

### Deletion of *Ars2* is lethal to adult mice

While growing evidence has established *Ars2* as a vital factor for transcription regulation and nuclear RNA processing, little is known about the relevance of *Ars2* to normal mammalian physiology. To begin to explore physiological relevance of *Ars2* in mammals, we generated inducible *Ars2* knockout (iKO) mice by cross breeding *Ars2*<sup>fl/fl</sup> mice [3] with mice expressing the tamoxifen-inducible Cre recombinase Cre-ERT2 [19]. In these mice exons 2–20 of the *Srrt* gene, which codes for *Ars2*, are deleted by daily intraperitoneal injection of tamoxifen for five consecutive days (Fig. 1A). Deletion of *Srrt* following tamoxifen injection was confirmed by endpoint PCR in multiple organs (Fig. S1A) and changes in *Ars2* mRNA expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Consistent with previous observations [1, 3], *Ars2* mRNA expression was high in hematopoietic tissues of control mice. Reduced expression of *Ars2* mRNA was observed in most organs eight days following first tamoxifen injection of *Ars2* iKO mice (Fig. 1B). Several organs from *Ars2* iKO mice failed to demonstrate reduction in *Ars2* mRNA expression despite clear evidence of *Srrt* deletion (Fig. S1A), suggesting extended half-life of *Ars2* mRNA in these organs. Importantly, tamoxifen treatment of *Ars2* iKO mice led to rapid weight loss (Fig. 1C) and death (Fig. 1D) within two weeks, with no differences observed between male and female mice (Fig. S1B). In contrast, *Ars2*<sup>fl/fl</sup> mice injected with tamoxifen survived for the duration of each experiment (20 to 60 days) and showed no signs of distress (Fig. 1C, D). *Ars2* iKO mice injected with vehicle control corn oil and *Ars2*<sup>fl/WT</sup> mice injected with tamoxifen also demonstrated normal survival (data not shown).

### *Ars2* is required for normal thymopoiesis

Necropsy of tamoxifen-treated *Ars2* iKO mice revealed no obvious cause of death, but a marked reduction in the size of *Ars2* deleted thymi when compared to littermate control

mice (Fig. 2A). Reduction in size was reflected in significant reduction in cellularity and mass (Fig. 2B) of thymi from tamoxifen-treated *Ars2* iKO mice when compared to littermate controls. In contrast, other parenchymal and immune organs such as kidney, liver and spleen appeared normal on gross examination (Fig. S2A, B).

To assess whether thymocyte populations were altered by *Ars2* depletion, multicolor flow cytometry was performed [31]. Significant decrease in the percent of CD4/CD8 double positive (DP) thymocytes ( $p = 0.007$ ) with concomitant increase in the percent of CD8 single positive (CD8SP,  $p = 0.04$ ), CD4 single positive (CD4SP,  $p = 0.003$ ), and CD4/CD8 double negative (DN,  $p = 0.002$ ) thymocytes was observed in thymi of *Ars2* iKO mice one week following initial tamoxifen injection (Fig. 2C). These changes likely reflect rapid loss of immature *Ars2* knockout thymocytes, as the number of mature thymocytes remained relatively stable (Fig. 2D, S2C).

To further explore effects of *Ars2* deletion on thymic T-cell development, *Ars2*<sup>fl/fl</sup> mice were crossed with mice expressing Cre recombinase driven by the *Lck* promoter to induce deletion of *Ars2* in thymocytes [32]. *Lck-cre* expressing *Ars2*<sup>fl/fl</sup> mice were viable (Fig. S3A) yet exhibited significant reduction in thymic cellularity and mass (Fig. S3B), potentially as a result of increased thymocyte apoptosis (Fig. S3C). Comparison of immature thymocyte populations from mice in which *Ars2* deletion was driven by Cre-ERT2 versus *Lck-cre* demonstrated similar decrease in the number of DP thymocytes in both models, suggesting that thymocyte-intrinsic *Ars2* is required for maturation beyond the DN stage (Fig. S3D). In contrast, the number of DN thymocytes decreased when *Ars2* deletion was driven by Cre-ERT2 but not when *Ars2* deletion was driven by *Lck-cre* (Fig. S3D).

Expression of Cre recombinase under the control of the *Lck* promoter was found to increase basal apoptosis of immature thymocytes [33], making interpretation of increased apoptosis observed when *Ars2* deletion was driven by *Lck-Cre* difficult. To circumvent this difficulty and to determine if effects of *Ars2* on thymocytes apoptosis are cell-intrinsic, we generated mixed chimeras in which *Ars2* iKO thymocytes could be distinguished from Cre negative *Ars2*-floxed thymocytes by expression of fluorescent reporter proteins. In this model, 1:1 ratio of bone marrow from Cre-ERT2-positive (*Ars2* iKO) and Cre-ERT2-negative (control) *Ars2*<sup>fl/fl</sup> mice with transgenic expression of the mT/mG Cre reporter, which expresses membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision [34], was transplanted into lethally irradiated C57BL/6J mice (Fig. 2E). Decreased expression of *Ars2* in mG<sup>+</sup> thymocytes was confirmed by qRT-PCR (Fig. S3E). After two months of reconstitution, the gene coding *Ars2* was deleted by five daily tamoxifen injections and apoptosis of thymocytes populations was analyzed by Annexin V staining. High rates of apoptosis were found in mG<sup>+</sup>, *Ars2* iKO thymocytes within the two most immature thymocytes populations analyzed, CD4/CD8 double negative (DN) and CD8 immature single positive (CD8 ISP) (Fig. 2F,  $P = 0.01$  and  $<0.0008$ , respectively). These data conclusively demonstrate a cell-intrinsic role for *Ars2* in limiting apoptosis of immature thymocytes.

### Ars2 is required for maintenance of long-term hematopoietic stem cells (LT-HSCs)

During characterization of mice in which *Ars2* was deleted by *Mx-Cre*, Gruber *et al.* found decreased bone marrow cellularity and attributed this finding to the role of *Ars2* in promoting cellular proliferation [3]. To begin to assess bone marrow function in our *Ars2* iKO mice, we performed serial complete blood counts (CBCs) prior to and following tamoxifen injection. *Ars2* iKO mice showed progressive reduction in peripheral white blood cells (WBC) and red blood cells (RBC) following tamoxifen injection, indicating underlying bone marrow dysfunction (Fig. 3A, S4A, B). Consistent with this observation, bone marrow cellularity was significantly reduced in tamoxifen-treated *Ars2* iKO mice compared to littermate controls (Fig. 3B,  $p = 0.002$ ).

To explore the underlying cause of bone marrow dysfunction in *Ars2* iKO mice, cell cycle and apoptosis of mature bone marrow resident cells ( $lin^+$ ), immature bone marrow resident cells ( $lin^-$ ), and several  $lin^-$  sub-populations (Fig. 3C) were measured by flow cytometry three, five and eight days after initiation of tamoxifen injections. In contrast to thymocytes, *Ars2* knockout bone marrow cells exhibited very little apoptosis ( $<10\%$ ) as assessed by flow cytometry (Fig. 3D, S5A, B) and immunofluorescence staining for active caspase-3 (Fig. 3D). Furthermore, when comparing *Ars2* knockout to littermate control mice no significant changes in apoptosis were observed in bone marrow sub-populations examined (Fig. 3D, S5C). These data indicate that reduced bone marrow cellularity in *Ars2* iKO mice did not result from increased apoptosis of bone marrow resident cells following *Ars2* deletion.

When cell cycle of bone marrow sub-populations was examined, no change in bulk  $lin^+$  or  $lin^-$  populations was observed in tamoxifen-treated *Ars2* iKO mice compared to littermate controls (Fig. 3E). In contrast, hematopoietic progenitor sub-populations from tamoxifen-treated *Ars2* iKO mice, including  $Lin^-$ ,  $Sca^+$ ,  $c-Kit^+$  (LSK) [35], multipotent progenitors (MPP:  $lin^-$ ,  $Sca^+$ ,  $c-Kit^+$ ,  $CD34^+$ ,  $Il7ra^-$ ,  $Flt3^+$ ) and short-term hematopoietic stem cells (ST-HSC:  $lin^-$ ,  $Sca^+$ ,  $c-Kit^+$ ,  $CD34^+$ ,  $Il7ra^-$ ,  $Flt3^-$ ) [36], exhibited decreased frequency of cells in S and G2/M phase of the cell cycle (Fig. 3E). These cell types normally express high levels of *Ars2* (Fig. S6A) and like immortalized cell lines in culture [3] appear to rely on *Ars2* to maintain continued proliferation.

Despite observed reductions in proliferating cells within the LSK, MPP and ST-HSC bone marrow populations, the frequency of these cells within bone marrow of tamoxifen-treated *Ars2* iKO mice was similar to littermate controls one week after initiation of tamoxifen treatment (Fig. 4A, B). Frequencies of additional downstream bone marrow progenitor populations, including common lymphoid progenitors (CLPs:  $lin^-$ ,  $Sca^{lo}$ ,  $c-Kit^{lo}$ ,  $Il7ra^+$ ), common myeloid progenitors (CMPs:  $lin^-$ ,  $Sca^-$ ,  $c-Kit^+$ ,  $Il7ra^-$ ,  $CD34^+$ ,  $CD16/32^{lo}$ ), granulocyte-monocyte progenitors (GMPs:  $lin^-$ ,  $Sca^-$ ,  $c-Kit^+$ ,  $Il7ra^-$ ,  $CD34^+$ ,  $CD16/32^{hi}$ ), and megakaryocyte-erythrocyte progenitors (MEPs:  $lin^-$ ,  $Sca^-$ ,  $c-Kit^+$ ,  $Il7ra^-$ ,  $CD34^-$ ,  $CD16/32^-$ ) were also similar between tamoxifen-treated *Ars2* iKO mice and littermate controls (Fig. S6B, C). Strikingly, only long-term hematopoietic stem cells (LT-HSCs:  $lin^-$ ,  $Sca^+$ ,  $c-Kit^+$ ,  $CD34^-$ ,  $CD48^-$ ,  $CD150^+$ ) [36] were significantly reduced in tamoxifen-treated *Ars2* iKO mice when compared to littermate controls (Fig. 4A,B,  $p = 0.008$ ). This reduction was rapid, as 3 of 5 *Ars2* iKO mice examined displayed reduced LT-HSC frequency relative to littermate control mice five days following initiation of tamoxifen injections (Fig. S5D).

Addition of Flt3 to our multicolor flow cytometry panel [23] had no effect on observed loss of LT-HSC from bone marrow of *Ars2* deficient mice (Fig. S7A), while elimination of CD34 as a marker of HSC [36] resulted in loss of the phenotype (Fig. S7B). Since CD34 staining was essential to discriminate the LT-HSC population sensitive to *Ars2* deletion, we used a second CD34 antibody to stain *Ars2* iKO and littermate control bone marrow and confirmed that  $lin^{-}$ ,  $Sca^{+}$ ,  $c-Kit^{+}$ ,  $CD34^{-}$ ,  $CD48^{-}$ ,  $CD150^{+}$  LT-HSCs were reduced from *Ars2* iKO bone marrow (Fig. S7C, D).

### Effects of *Ars2* deletion on bone marrow are stroma-independent

Bone marrow stroma has myriad effects on hematopoietic progenitor cells, including LT-HSCs [37]. To determine if *Ars2* deletion from stromal cells was the cause of phenotypes observed in tamoxifen-treated *Ars2* iKO mice, lethally irradiated wild-type C57BL/6 mice were reconstituted with bone marrow from either *Ars2* iKO mice or *Ars2*<sup>fl/fl</sup> mice as controls (Fig. 4C). Similar to induced whole-body *Ars2* deletion, thymic involution occurred rapidly (data not shown) and examination of *Ars2*-deleted bone marrow from chimeric animals a week to ten days after initial tamoxifen injection (a similar timeframe as whole-body knockouts) revealed significant reduction in bone marrow cellularity (Fig. 4D,  $p = 0.01$ ) and the LT-HSC population (Fig. 4E,  $p = 0.04$ ) relative to control chimeras. However, unlike whole-body *Ars2* knockout, induced deletion of *Ars2* from bone marrow (and bone marrow derived cells) did not result in death and weight loss of mice (Fig. 4F).

### *Ars2* is required for hematopoiesis within bone marrow following myeloablation

Maintenance of LT-HSC population within the bone marrow relies on a balance between differentiation, self-renewal, and cell death, all of which can be affected by signals from bone marrow stroma cells [25]. Since data indicate that deletion of *Ars2* does not alter viability of LT-HSCs and that wild-type stroma cannot rescue reduced numbers of *Ars2* knockout LT-HSCs (Fig. 3D, 4E), we examined the self-renewal capacity of *Ars2* deficient LT-HSCs by performing stressed hematopoiesis experiments.

We generated mT/mG chimeric mice by transplanting bone marrow from Cre-ERT2-positive (*Ars2* iKO) or Cre-ERT2-negative (control) *Ars2*<sup>fl/fl</sup> mice expressing mT/mG Cre reporter into lethally irradiated C57BL/6J mice. After a two month reconstitution period, mT/mG chimeras were injected with tamoxifen to induce *Ars2* deletion from bone marrow and bone marrow-derived cells, followed by weekly injections of a sub-lethal dose of 5-fluorouracil (5-FU, 120 mg/kg IP, Fig. 5A). Depletion of *Ars2* from mG<sup>+</sup> cells was confirmed by qRT-PCR in bone marrow and peripheral immune organs (Fig. S3E). Tamoxifen-treated *Ars2* iKO chimeric mice lacked the ability to reconstitute peripheral white and red blood cells (WBC, RBC) following initial 5-FU injection (Fig. S8A). Additionally, rapid weight loss (Fig. 5B) and death (Fig. 5C) of tamoxifen-treated *Ars2* iKO chimeric mice occurred following a second ( $n = 7$ ) or third ( $n = 3$ ) weekly 5-FU injections. In contrast, control chimeras demonstrated more robust reconstitution of peripheral WBC following each of three weekly 5-FU injections (Fig. S8A), stabilized their weight over the course of the experiment (Fig. 5B), and did not die until after third ( $n=1$ ) or fourth ( $n=5$ ) weekly 5-FU injections (Fig. 5C).

Upon necropsy, tamoxifen-treated *Ars2* iKO chimeras had significantly reduced bone marrow cellularity relative to control chimeras (Fig. 5D,  $p=0.03$ ). Flow cytometry revealed that repeated exposure of control chimeras to 5-FU (four weekly doses) resulted in approximately 70% reduction in  $mT^+$ , *Ars2*-expressing LT-HSCs relative to control chimeras that never received 5-FU. Strikingly,  $mG^+$ , *Ars2* knockout LT-HSCs were reduced to an undetectable level in all mice examined following 2 to 3 weekly doses of 5-FU (Fig. 5E,  $p=0.003$ ).

Self-renewal of LT-HSCs is accomplished by asymmetric division to produce one daughter cell identical to its parent and one daughter cell that can expand and differentiate into all blood lineage cells [25]. Complete lack of identifiable LT-HSCs in tamoxifen-treated *Ars2* iKO chimeras following multiple doses of 5-FU precluded analysis of their proliferation and/or differentiation in response to hematopoietic stress. Additionally, severe defects in peripheral blood reconstitution in 5-FU treated *Ars2* knockout chimeras could multiply hematopoietic stress signals, potentially exacerbating depletion of LT-HSCs. To mitigate these possibilities we generated mixed chimeras by transplanting a 1:1 mix of bone marrow cells from  $mT/mG$ -expressing *Ars2* iKO and control mice into lethally irradiated C57BL/6J mice. Following reconstitution, mixed  $mT/mG$  chimeras were given five daily injections of tamoxifen (75 mg/kg IP) followed by a single dose of 5-FU (150, mg/kg IP) (Fig. 5F).

As expected 5-FU treatment strongly induced proliferation of wild-type,  $mT^+$  LSK cells measured by Ki-67 staining ( $57.7\% \pm 6.1\%$  Ki-67<sup>+</sup>). In contrast, proliferation was only moderately induced in *Ars2* knockout  $mG^+$  LSK cells ( $23.3\% \pm 7.7\%$  Ki-67<sup>+</sup>, Fig. 5G, S8B,  $p=0.02$ ). Delineation of LSK sub-populations using CD34, a marker whose expression is unchanged by 5-FU treatment [38], revealed that the proliferation defect observed in *Ars2* knockout  $mG^+$  cells was limited to CD34<sup>+</sup> cells (Fig. 5H,  $p=0.02$ ), while CD34<sup>-</sup>,  $mT^+$  wild-type and CD34<sup>-</sup>,  $mG^+$  *Ars2* knockout LSK cells demonstrated similar Ki-67 positivity (Fig. 5H,  $p=0.3$ ). When CD34<sup>-</sup> LSK cells were further subdivided using markers of LT-HSC (CD48<sup>-</sup>, CD150<sup>+</sup>), the vast majority of wild-type  $mT^+$  LT-HSCs were found to be Ki-67<sup>+</sup> whereas *Ars2* knockout  $mG^+$  LT-HSCs were not detected in Ki-67 stained bone marrow ( $p=0.02$ , Fig. 5G,I). In fact,  $mG^+$  *Ars2* knockout LT-HSCs were detected in only 2 of 7 mixed chimeras exposed to a single dose of 5-FU while *Ars2* wild-type  $mT^+$  LT-HSCs were observed in 6 of 7 (Table 1).

### Self-renewal defect in *Ars2* knockout LT-HSCs is cell-intrinsic

Near complete loss of *Ars2*-deficient LT-HSCs, but not *Ars2*-expressing LT-HSCs, from 5-FU treated mixed chimeras strongly suggests that *Ars2* is necessary for self-renewal of LT-HSCs following *in vivo* stress. Alternatively, rapid loss of LT-HSCs following 5-FU exposure could have resulted from increased sensitivity of *Ars2* knockout LT-HSCs to death induced by 5-FU. Examination of tamoxifen-treated mixed chimera mice in the absence of 5-FU treatment (Fig. 6A) revealed a profound decrease in  $mG^+$ , *Ars2* knockout LT-HSCs relative to  $mT^+$ , wild-type LT-HSCs one week following initiation of tamoxifen treatment ( $p=0.002$ , Fig. 6B,C). Importantly, mixed chimera mice displayed no signs of bone marrow failure and had normal bone marrow cellularity upon necropsy ( $86.6 \times 10^6 \pm 15.2 \times 10^6$  bone marrow cells). Consistent with lack of self-renewal potential of *Ars2* knockout LT-HSCs,

singly sorted LT-HSCs from *Ars2* knockout chimeras failed to produce large, differentiated colonies in single cell colony formation assays (Fig. 6D,E) [39]. As expected control LT-HSCs formed large colonies consisting mainly of morphologically mature blood cells. In contrast, LT-HSCs from tamoxifen-treated *Ars2* iKO chimeras produced few small colonies consisting of uniform, morphologically undifferentiated cells ( $p = 0.007$ , Fig. 6F).

## Discussion

Several recent studies have established *Ars2* as a critical mediator of transcription and co-transcriptional RNA processing [4, 6, 11, 12]. However, little is known about physiological roles for *Ars2* in mammals, as its germline deletion led to early embryonic lethality [1]. Here we describe generation and characterization of whole-body inducible *Ars2* knockout mice. Using these mice we provide mechanistic insight into the requirement for *Ars2* in hematopoiesis, both in the bone marrow and during thymic T-cell maturation.

Our data indicate that the *in vivo* ability of long-term hematopoietic stem cells (LT-HSCs) to self-renew is critically dependent on expression of *Ars2*. Additionally, proliferation of several bone marrow progenitor populations decreased upon *Ars2* deletion. These data likely explain reduced bone marrow cellularity initially observed when *Ars2* was inducibly deleted from bone marrow cells using MxCre [3] and again when inducibly deleted from the whole body (Fig. 3,4) or from bone marrow chimeras (Fig. 4) using Cre-ERT2. In addition to confirming and extending the bone marrow phenotype of *Ars2* knockout mice reported by Gruber *et al.* [3], we found that *Ars2* supports T-cell development in the thymus (Fig. 2). Interestingly, reported phenotypes required deletion of both alleles of *Ars2*, as heterozygous mice or cells from heterozygous mice displayed no phenotype (data not shown).

Self-renewal of stem cells is accomplished through cell division in the absence of differentiation of one daughter cell [21, 22]. Determining whether *Ars2* influences self-renewal of LT-HSCs through effects on proliferation versus differentiation has proven difficult, although data point to a role for *Ars2* in limiting LT-HSC differentiation. Depletion of the *Ars2*<sup>fl/fl</sup> Cre-reporter (mG<sup>+</sup>) LT-HSC pool from mixed chimera mice within one week of tamoxifen injection (Fig. 6) provides the best evidence for spontaneous differentiation of LT-HSCs following *Ars2* knockout. In these mice downstream bone marrow and peripheral blood populations were maintained by wild-type progenitors co-occupying the bone marrow, thus limiting signals that activate LT-HSCs to enter cell cycle. Moreover, LT-HSCs from tamoxifen-treated *Ars2* iKO mice displayed a cell cycle profile containing predominantly 1N, non-proliferating cells (Fig. 3E) suggesting that *Ars2* knockout alone is insufficient to stimulate LT-HSCs to proliferate. Rather, data point to a scenario in which LT-HSC differentiation is suppressed by a gene expression program that relies on continued expression of *Ars2*. Mechanistic studies similar to those performed by Andreu-Agullo *et al.* in neural stem cells [18] are required to conclusively establish that *Ars2* suppresses differentiation of LT-HSCs. However, these studies are beyond the scope of the current manuscript.

Previous studies focusing on cellular context and consequences of *Ars2* expression have demonstrated that high levels are necessary for cells to rapidly proliferate in culture [3, 10].



In contrast, *Ars2* has been found to maintain viability [1, 3] or cell identity [18] when examined in the context of normal mammalian physiology. Studies of *Ars2* expression in blood cancers further suggest that *Ars2* may be less important for *in vivo* proliferation, as low expression of *Ars2* was found to correlate with aggressive pediatric acute lymphocytic leukemia [40] and the region of human chromosome 7 containing *SRRT* is commonly deleted in acute myeloid leukemia [41]. Our whole-body inducible *Ars2* knockout mice reveal that *in vivo* effects of *Ars2* are context-dependent, with thymocytes relying on *Ars2* for survival (Fig. 2) [3], bone marrow progenitor cells relying on *Ars2* for cell cycle progression (Fig. 3), and long-term hematopoietic stem cells relying on *Ars2* to limit differentiation (Figs. 4–6). These findings are not surprising in light of the molecular role of *Ars2* in coordinating transcriptional and co-transcriptional events underlying gene expression programs. Whether context-dependent effects of *Ars2* reflect cell type-specific molecular functions of *Ars2* (transcriptional regulation of *Sox2* to maintain neural stem cells [18] vs. 3' end processing during replication-dependent histone mRNA biogenesis to support proliferation of cultured cells [4, 11]) or universal molecular functions of *Ars2* resulting in distinct phenotypic outcomes due to underlying context-dependent gene expression programs remains an open question.

A striking consequence of whole-body *Ars2* deletion was rapid demise of 100% of *Ars2* deficient animals accompanied by clear signs of bone marrow failure (rapid weight loss, decreased peripheral blood and bone marrow cellularity). Surprisingly, deletion of *Ars2* from bone marrow derived cells using two distinct models (inducible knockout in chimeras in this study and *MxCre* driven knockout in the Gruber et al. study [4]) did not kill mice even though reduced bone marrow cellularity was observed. This raises several possibilities, 1) *Ars2* deletion from LT-HSCs was incomplete, 2) *Ars2* expressed in bone marrow stroma cells is needed to support hematopoiesis downstream of LT-HSCs, or 3) death following *Ars2* deletion did not result from bone marrow failure. An organ site whose failure following *Ars2* deletion could explain rapid weight loss and death is the intestine. Microscopic examination of intestines from *Ars2* knockout mice showed marked reduction in crypt depth and organization (data not shown). Similar to bone marrow, cells of the intestine are continuously replenished through differentiation of tissue-specific stem cells [42]. If, as data presented here and in Andreu-Agullo *et al.* study [18] suggest, *Ars2* is broadly required for maintenance of stem cell populations, its deletion from intestinal stem cells could lead to rapid loss of intestinal epithelium and result in malabsorption, weight loss and death of *Ars2* knockout animals.

Although further efforts are required to fully appreciate the importance of *Ars2* in normal mammalian physiology, this study provides insight into roles for *Ars2* in hematopoiesis. Data demonstrate that *Ars2* is required for adult mice to maintain a pool of undifferentiated hematopoietic stem cells, likely by suppressing their differentiation, and that *Ars2* is essential for survival of thymocytes during T cell maturation. While molecular studies will provide mechanistic details of how *Ars2* affects LT-HSCs and thymocytes, the current study identifies a second type of stem cell that requires *Ars2* to maintain its population *in vivo*, suggesting that *Ars2* may play an especially important role in the biology of stem cells.

## Material and methods

### Mice

Mice were maintained in Laboratory Animal Shared Resource (LASR) at Roswell Park Cancer Institute and all procedures were approved by the institutional animal care (IACUC). Four different strains of mice were used in this study: C57BL/6J (B6), Stock No: 000664, B6.Cg-Tg(UBC-cre/ERT2)1Ejb/1J (Cre-ERT2), Stock No: 007001 and Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (mT/mG), Stock No:007576 were purchased from Jackson Laboratory. *Ars2* floxed (*Ars2*<sup>fl/fl</sup>) mice were obtained from Thompson Lab[3]. Cre-ERT2 *Ars2*<sup>fl/fl</sup>, mT/mG *Ars2*<sup>fl/fl</sup> and mT/mG Cre-ERT2-*Ars2*<sup>fl/fl</sup> were generated by crossbreeding of Cre-ERT2 and mT/mG with *Ars2*<sup>fl/fl</sup> mice. To delete *Ars2* gene from its floxed sites, Cre-ERT2 was activated by intra-peritoneal injection of 75µg Tamoxifen (Sigma, 10540-29-1) per gram of 6-8 weeks old mouse for 5 days. Chimera mice were generated by tail vein injection of 2.5-5 million bone marrow cells from *Ars2*<sup>fl/fl</sup> or Cre-ERT2 *Ars2*<sup>fl/fl</sup> or mT/mG *Ars2*<sup>fl/fl</sup> or mT/mG Cre-ERT2-*Ars2*<sup>fl/fl</sup> into lethally irradiated (600 Rad) B6 mice. Mixed chimeras were generated by tail vein injection of 1:1 ratio of bone marrow cells from both *Ars2*<sup>fl/fl</sup> and Cre-ERT2-*Ars2*<sup>fl/fl</sup> or mT/mG *Ars2*<sup>fl/fl</sup> and mT/mG Cre-ERT2-*Ars2*<sup>fl/fl</sup> into lethally irradiated C57BL/6J mice.

### Flow cytometry

To identify the stem/progenitor cell populations, bone marrow was harvested by flushing the hind limb bones using FACS buffer (1% bovine serum albumin (BSA) and 0.5% sodium azide in 1X PBS) and filtered through 40µm cell strainer. Red blood cells were lysed using ACK lysing buffer (ThermoFisher scientific). Cells were stained in a two-step manner with CD34 as the first step and the rest of the antibodies as the second step. Data was collected and analyzed by BD LSR II A flow cytometer and flowjo V10 software, respectively. For cell cycle analysis, cells were stained with surface markers, fixed, permeabilized (Intrasure Kit) and DAPI stained. To analyze T cells, harvested thymus was meshed through a 40µm cell strainer and stained for flow cytometry. Markers used in this study are listed in supplementary Table-1.

### Immunohistochemistry

Femur bones were formalin fixed, decalcified and 10µm sectioned by pathology core facility of Roswell Park Cancer Institute. Sections were deparaffinized by xylene and ethanol method [43], antigen retrieved (heat induced antigen retrieval using Reveal Decloaker, Biocare), permeabilized by Triton 0.5% and blocked by 5% normal goat serum in PBS. Slides were incubated with primary antibody (anti caspase 3 (Rb), 1:200) overnight. Next day, slides were washed (1XPBS) and incubated with secondary antibody (Goat anti rabbit alexa fluor 488, 1:2000) for 2 hours. Lastly, slides were washed (1XPBS) and DAPI mounted. Images were taken by fluorescence microscope. Antibodies are listed in supplementary Table-1.

### Self-renewal (5-FU) assays

At day 7 post tamoxifen injection, 5-fluorouracil (5-FU) was administered intraperitoneally at either four weekly 120mg/kg doses or single 150mg/kg dose. Blood was drawn from sub-mandibular vein and collected in 50  $\mu$ M EDTA tubes. CBC was performed using HemaTrue. Moribund mice (identified by ruffled fur, hunched back, more than 20% reduction in weight) were euthanized according to IACUC protocol and bone marrow was harvested for flow cytometry.

### Colony formation assay (CFA)

Single cell colony formation assay was performed according to Ema et al. protocol [39] from single sorted Lin<sup>-</sup>Sca<sup>+</sup>kit<sup>+</sup>CD34<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup> LT-HSCs. After 14 days of culture, colonies were imaged and analyzed by ImageJ. Colonies were cytopined and stained for hemacolor according to its protocol (EMD Millipore).

### Quantitative Real Time PCR (qRT-PCR)

RNA from harvested organs was extracted using trizol and miRNeasy kit (Qiagen). cDNA was generated using super script IV (Thermofisher Scientific) and qPCR with SYBR green master mix (Thermofisher Scientific) was performed using Quant studio 6 flex device. Primers are listed in supplementary Table-2. Expression of *Ars2* was normalized by TBP using  $^{-CT}$  method. (Expression level =  $2^{(CT_{TBP} - CT_{Ars2})}$ ).

### Statistical analysis

All values are expressed in mean + SD/SEM. Unpaired student t-test was used to compare two experimental groups using GraphPad Prism 7 software. P value less than 0.05 was considered significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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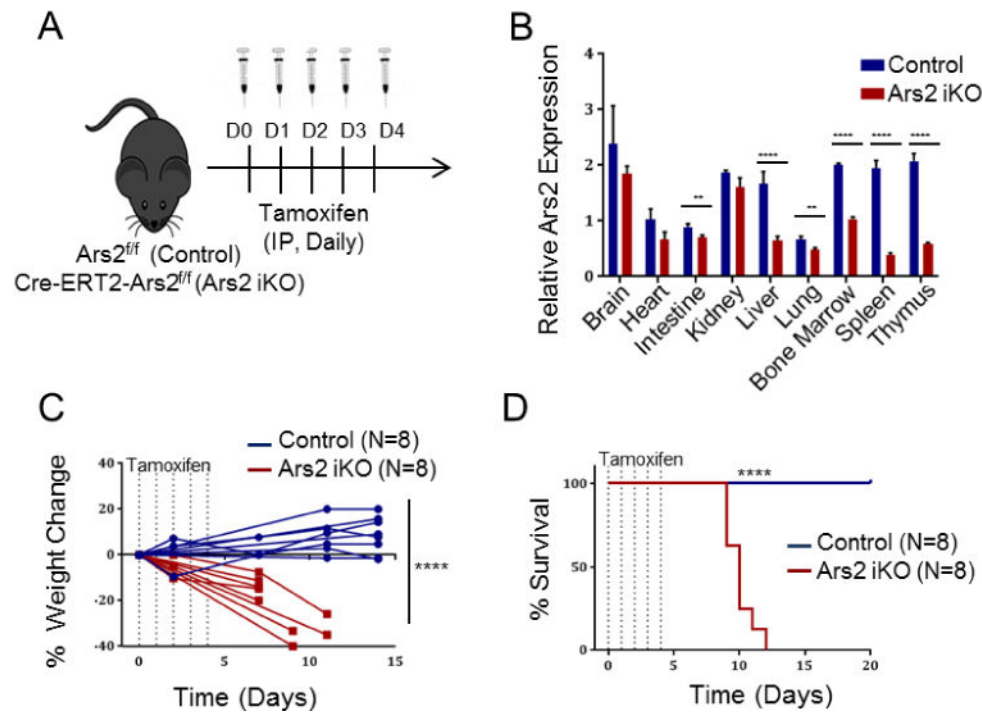
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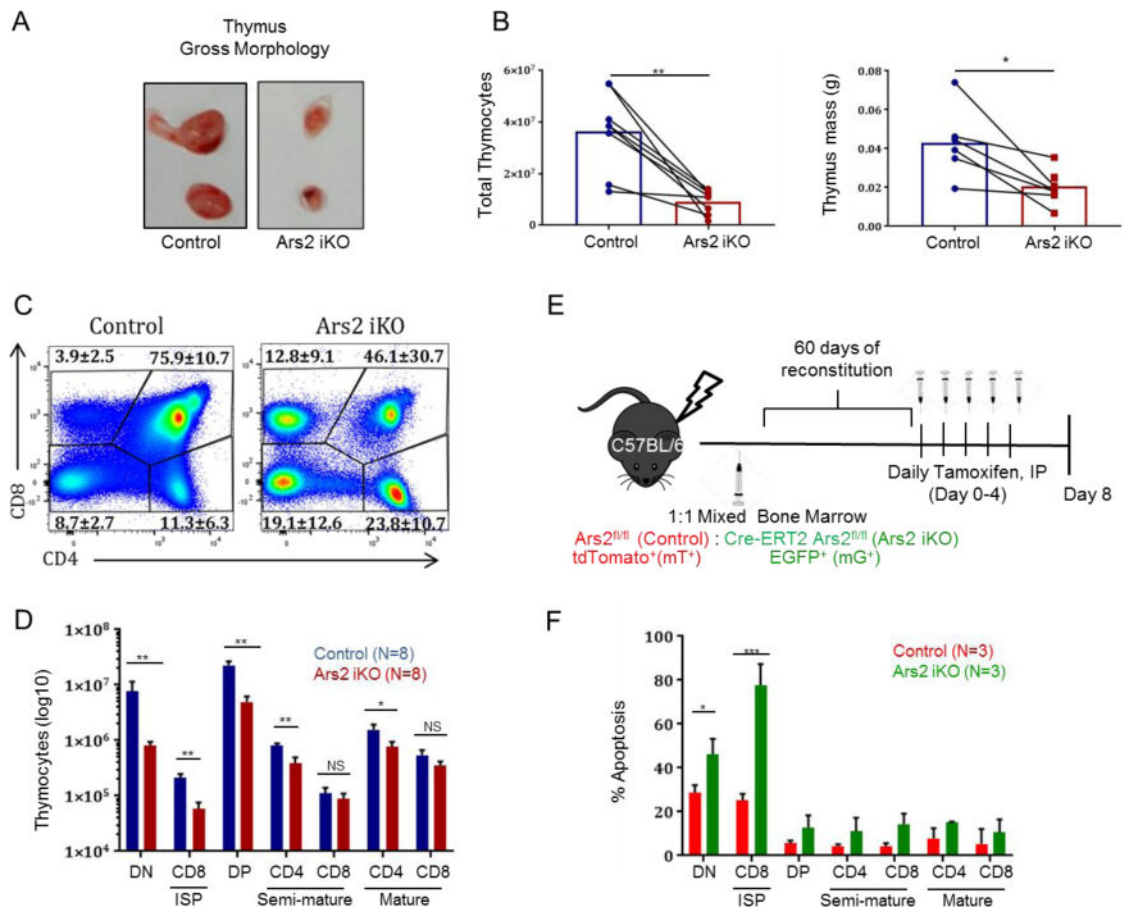
**Highlights**

- *In vivo* maintenance of LT-HSCs pool relies on RNA-binding protein Ars2.
- Ars2 supports the proliferation of early hematopoietic progenitors.
- Deletion of Ars2 results in thymocyte apoptosis.
- Physiological effects of Ars2 are context-dependent.



**Figure 1. Induced whole-body *Ars2* knockout leads to rapid death of adult mice**

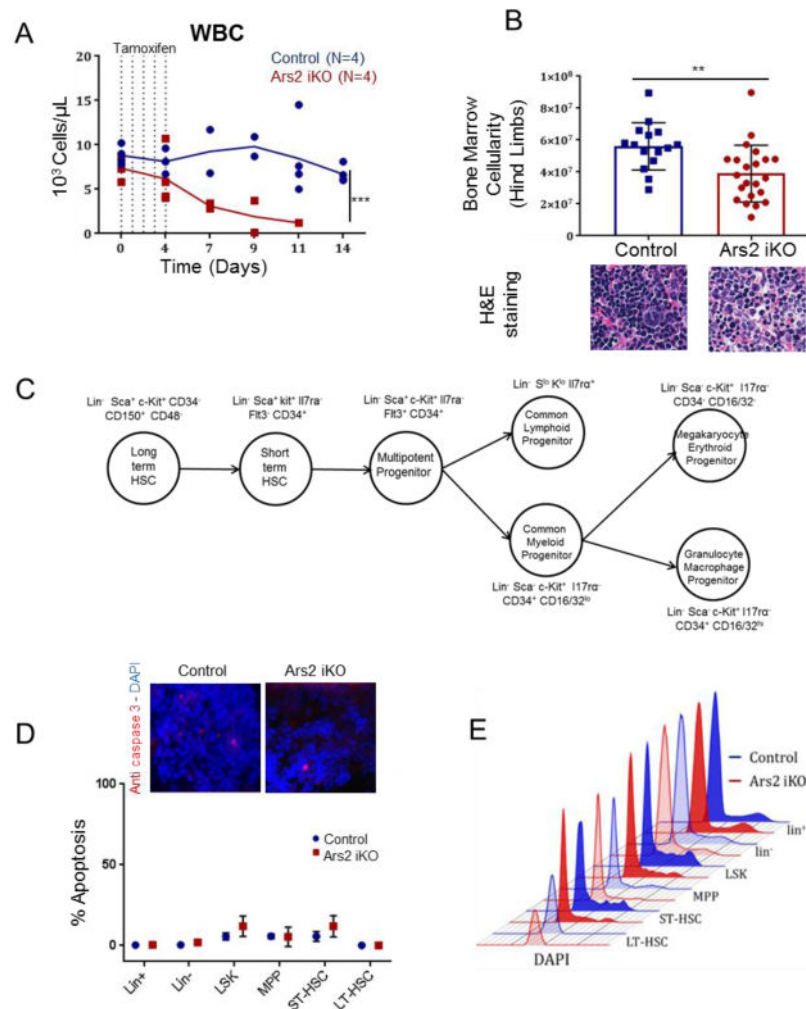
A) Schematic of whole-body inducible *Ars2* knockout model. Littermate mice with either *Ars2<sup>fl/fl</sup>* (Control) or *Cre-ERT2<sup>+</sup>, Ars2<sup>fl/fl</sup>* (*Ars2* iKO) genotype were injected intraperitoneally with tamoxifen (75 mg/kg) for five consecutive days and then monitored for up to 60 days. B) qRT-PCR was performed on RNA isolated from several organs removed from *Ars2* iKO or control mice eight days following initial tamoxifen injection. Relative *Ars2* expression was calculated using the  $\Delta\Delta C_T$  method with TBP as endogenous control. Bars represent mean  $\pm$  SD of technical quadruplicates. C) Rapid weight loss of *Ars2* iKO following *Ars2* deletion. Lines represent change in weight of individual mice relative to initiation of tamoxifen injection. D) Survival of *Ars2* iKO mice following tamoxifen injection (red line) was reduced relative to littermate controls (blue line). \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ .



### Figure 2. Ars2 supports survival of immature thymocytes

A) Gross anatomy of thymi showing smaller size following tamoxifen-induced Ars2 depletion. B) (left) Cellularity and (right) mass of thymi from 6 to 8 weeks old Ars2 iKO (n=8) mice was reduced compared to littermate controls (n=8) seven to ten days following initial Tamoxifen injection (\*\*p = 0.001 and \*p=0.01, respectively). Dots represent individual mice with lines connecting littermates. C) Representative dot plots showing distribution of lineage-negative (B220<sup>-</sup>, CD11b<sup>-</sup>, Ly-6G<sup>-</sup>, NK1.1<sup>-</sup>) thymocytes between four major developmental stages based on CD4 and CD8 staining. Numbers represent mean  $\pm$  standard error for each of four thymocyte populations, CD4/CD8 double-negative (DN, p = 0.002), CD4/CD8 double-positive (DP, p = 0.007), CD4 single-positive (ISP, p = 0.003) and CD8 single-positive (Mature, p = 0.04) comparing Ars2 iKO (n=8) versus control (n=8) mice 7 to 8 days following initial tamoxifen injection. D) Average number  $\pm$  standard deviation of DN, immature single positive CD8 (CD8ISP, TCR $\beta$ <sup>-</sup>, CD24<sup>+</sup>), DP, semi-mature (TCR $\beta$ <sup>+</sup>, CD24<sup>+</sup>) CD4 and CD8 single positive and mature (TCR $\beta$ <sup>+</sup>, CD24<sup>-</sup>) CD4 and CD8 single-positive thymocytes isolated from Ars2 iKO (n=8) versus control (n=8) mice 7 to 8 days following initial tamoxifen injection (NS: P > 0.05, \*: P = 0.05, \*\*: P = 0.01). E) Schematics of mixed mT/mG chimera model. F) Percent apoptosis (Annexin V+)  $\pm$  standard deviation of immature, semi-mature and mature thymocytes from mixed mT/mG chimeric mice (n=4, \*P = 0.01, \*\*\*p < 0.0008).





**Figure 3. Ars2 sustains proliferation of bone marrow progenitor cells**

A) Serial counting of white blood cells (WBC) from Ars2 iKO and control mice prior to and following five daily tamoxifen injections. Solid lines represent mean values. Dotted lines represent tamoxifen injections. Dots represent individual mice (\*\**P* = 0.001). B) (Top) Total number of bone marrow cells obtained from hind limbs of Ars2 iKO ( $39.01 \times 10^6 \pm 17.8 \times 10^6$ ) or littermate control ( $56.09 \times 10^6 \pm 14.7 \times 10^6$ ) mice 7-10 days following initial tamoxifen injection (\*\**p* = 0.002). Dots represent individual mice. (Bottom) H&E staining of paraffin-embedded femurs from control and Ars2 iKO mice 10 days following initial tamoxifen injection. C) Hematopoiesis hierarchy demonstrating various populations and their corresponding surface markers used in this study. D) (Top) Immunohistochemistry staining for anti-caspase 3 marker on bone marrow of Ars2 iKO and control mice 8 days post first tamoxifen injection. (Bottom) Percent apoptosis (Annexin V<sup>+</sup>, DAPI<sup>+</sup>) mean  $\pm$  standard deviation among bone marrow mature and immature populations in control (*n*=2) and Ars2 iKO (*n*=2) mice 8 days post first tamoxifen injection. E) Bone marrow cells were stained for surface markers and then fixed and processed according to DAPI staining protocol for cell cycle analysis. Representative cell cycle histograms are

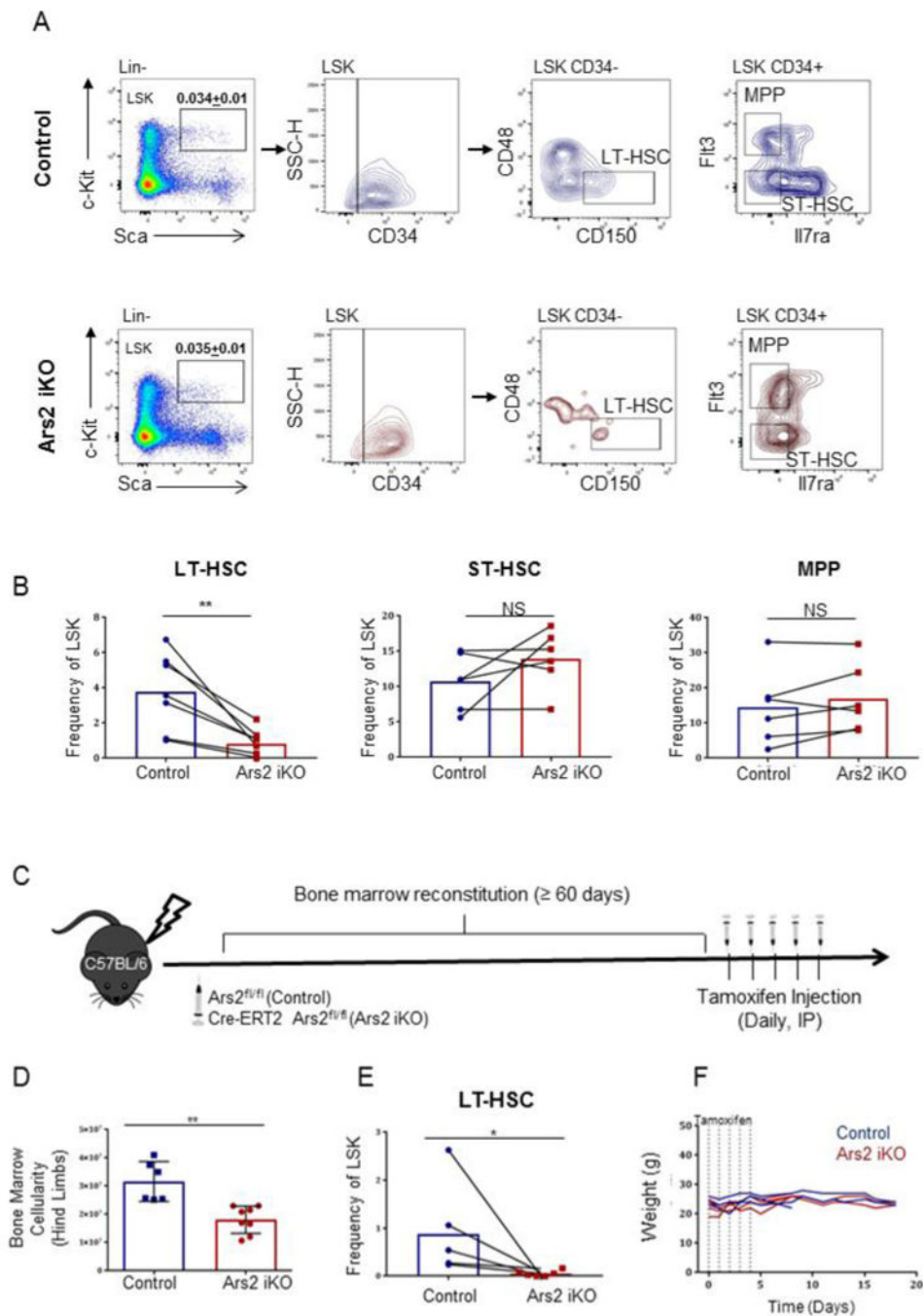
presented for each population from control (blue) and *Ars2* iKO (red) mice one week following initial tamoxifen injection.

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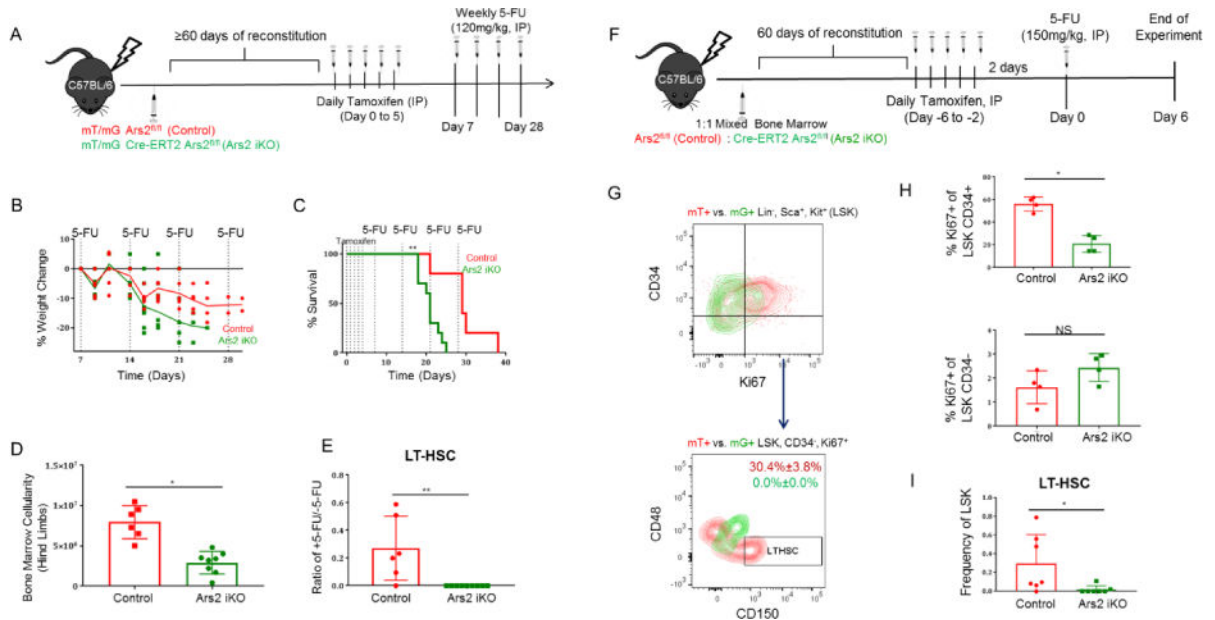
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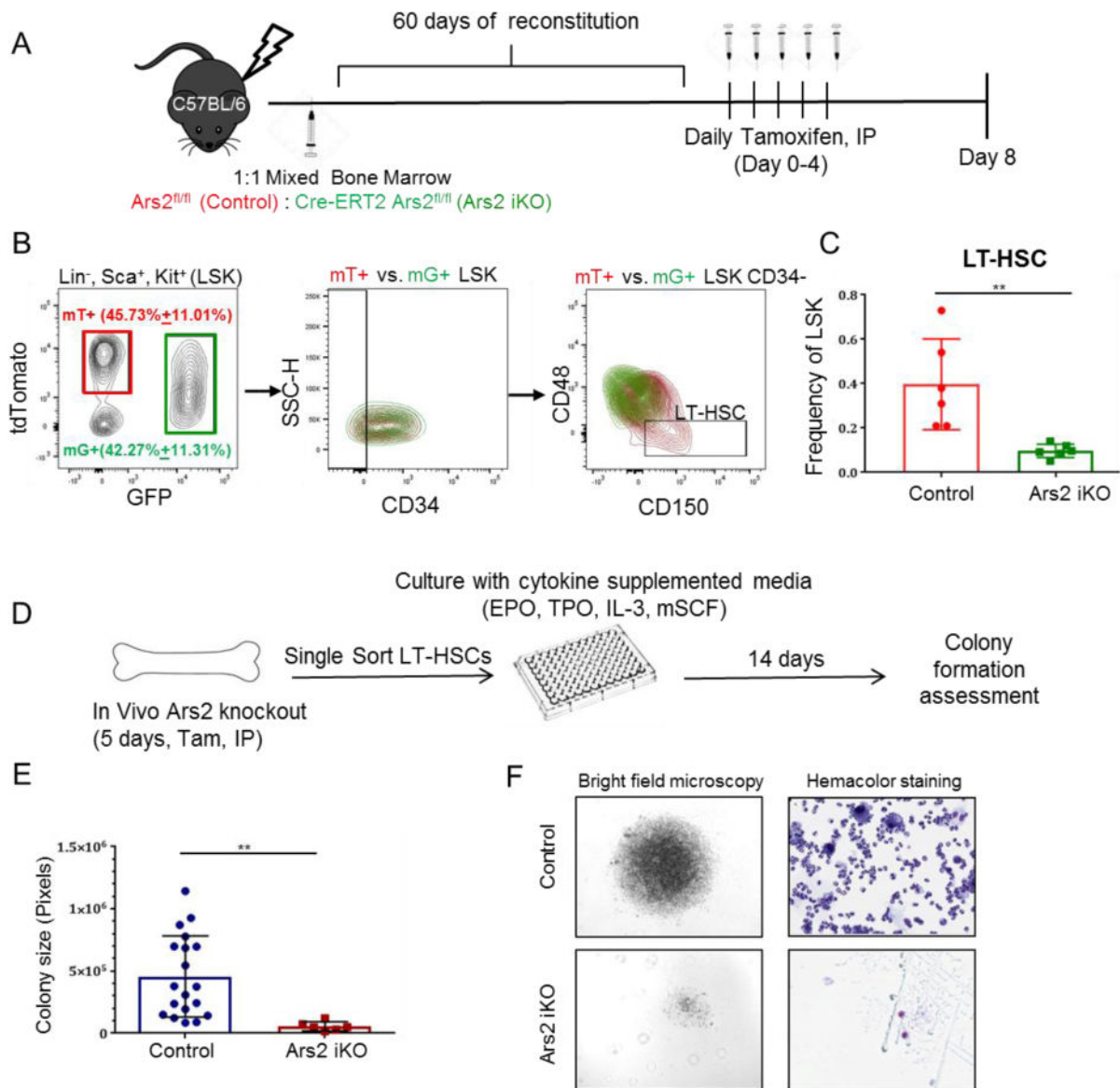
**Figure 4. Arс2 is required for maintenance of long-term hematopoietic stem cells (LT-HSCs)**  
 A) Representative flow cytometry plots and gating strategy for lin<sup>-</sup> (CD3<sup>-</sup>, CD11b<sup>-</sup>, B220<sup>-</sup>, Ly6-G<sup>-</sup>, TER119<sup>-</sup>) Sca<sup>+</sup>, c-Kit<sup>+</sup> (LSK), long-term hematopoietic stem cell (LT-HSC), short-term hematopoietic stem cell (ST-HSC) and multipotent progenitor (MPPs) populations. B) Frequency of LT-HSCs, ST-HSCs and MPPs within the LSK population comparing Arс2 iKO (n=6) mice to littermate controls (n=6) 7-8 days following initial tamoxifen injection. Dots represent individual mice with lines connecting littermates. \*\*p = 0.008, NS: p > 0.05. C) Schematic depicting experiments performed using bone marrow chimera mice. Donor

bone marrow obtained from either  $Ars2^{fl/fl}$  (control) or Cre-ERT2- $Ars2^{fl/fl}$  ( $Ars2$  iKO) mice was transplanted into lethally irradiated wild-type C57BL/6J mice. After 2 months of bone marrow reconstitution, mice were injected with five daily doses of tamoxifen (75 mg/kg IP) and sacrificed 8 days after first tamoxifen injection or monitored for survival. D) Bone marrow cellularity was significantly reduced in  $Ars2$  iKO bone marrow chimeras ( $18.54 \times 10^6 \pm 4.98 \times 10^6$ ) compared to control chimeras ( $28.47 \times 10^6 \pm 6.02 \times 10^6$ , \*\* $p = 0.01$ ). E) LT-HSC frequency of LSK was significantly reduced in  $Ars2$  iKO chimeras ( $0.05 \pm 0.02$ ) compared to littermate control chimeras ( $0.88 \pm 0.36$ , \* $p = 0.04$ ). Dots represent individual mice with lines connecting littermates. E) Bone marrow chimeras were able to survive after  $Ars2$  depletion with no significant changes in weight ( $Ars2^{fl/fl}$  donor into wildtype host, N=3, Cre-ERT2- $Ars2^{fl/fl}$  donor into wildtype host, N=4).



**Figure 5. *Ars2* is required for hematopoiesis within bone marrow following myeloablation**

A) Schematic of stress hematopoiesis experiments performed using Cre-reporter (mT/mG) bone marrow chimeric mice. B) Change in weight of control (n=6, red line) and *Ars2* iKO (n=10, green line) bone marrow chimera mice following *Ars2* deletion and subsequent weekly 5-FU injections (120 mg/kg IP). Solid lines represent mean values. Each dot represents a mouse. C) Survival of control (n=6, red line) and *Ars2* iKO (n=10, green line) bone marrow chimera mice prior to and following *Ars2* deletion and subsequent weekly 5-FU injections (120 mg/kg IP). \*\*p= 0.001. D) Total number of bone marrow cells obtained from hind limbs of control ( $7.95 \times 10^6 \pm 0.83 \times 10^6$ , red bar) and *Ars2* iKO ( $2.94 \times 10^6 \pm 0.438 \times 10^6$ , green bar) chimeras following repeated 5-FU exposure. \*p = 0.03. E) Change in frequency of LT-HSCs obtained from bone marrow of control (red bar) and *Ars2* iKO (green bar) chimeras following repeated 5-FU exposure. \*\* p=0.003. F) Schematic depicting generation and use of mixed chimera mice. G) Proliferation of mT<sup>+</sup> LSK cells overlaid with mG<sup>+</sup> LSK cells and subdivided by CD34 and Ki67 markers from mixed chimera mice six days following injection of 5-FU (150 mg/kg IP). Proliferating (Ki67<sup>+</sup>) CD34<sup>-</sup> LSK cells were further examined for expression of LT-HSC markers (CD48<sup>-</sup>, CD150<sup>+</sup>). Quiescent (Ki67<sup>-</sup>) CD34<sup>-</sup> LSK cells showed a similar lack of mG<sup>+</sup>, CD48<sup>-</sup>, CD150<sup>+</sup> LT-HSCs (data not shown). H) (Top) Percent of Ki67<sup>+</sup> cells among mG<sup>+</sup> CD34<sup>+</sup> LSK cells was significantly reduced (\*p= 0.02) compared to mT<sup>+</sup> CD34<sup>+</sup> LSK cells while (bottom) Ki67 positivity was unaffected in CD34<sup>-</sup> compartment of LSK cells (n=4). I) *Ars2* knockout, mG<sup>+</sup> LT-HSC frequency was significantly reduced compared to wild-type, mT<sup>+</sup> LT-HSC frequency in bone marrow from mixed chimeras exposed to single dose of 5-FU (n=7, \*p=0.03). (See Table 1). (D, E, H, I) Each dot represents a mouse.



**Figure 6. Defect in *Ars2* knockout LT-HSCs is cell-intrinsic**

A) Schematic of mixed mT/mG chimera generation. Two months after reconstitution with 1:1 bone marrow cells from both Cre+ mT/mG and Cre- mT/mG mice, mice were injected with daily tamoxifen and bone marrow was analyzed at day 8. B) Representative density plots with mT<sup>+</sup>, wild-type (red) and mG<sup>+</sup>, *Ars2* knockout (green) cells isolated from a single mixed chimera overlaid to demonstrate gating strategy and intrinsic differences between cell populations. C) Frequency of *Ars2* knockout, mG<sup>+</sup> LT-HSCs (green bar) was reduced compared to wild-type, mT<sup>+</sup> LT-HSCs (red bar) in mixed chimeras eight days following initial tamoxifen injection. n=6, \*\*p=0.002. D) Schematic of single cell colony formation assay. Single LT-HSCs from tamoxifen-treated control or *Ars2* iKO chimeras were FACS sorted into 96 well plates and cultured for 14 days in cytokine supplemented media. E) Size of colonies formed from *Ars2* iKO LT-HSCs was significantly less than size of colonies formed from control LT-HSCs (\*\*p = 0.007). Each dot represents a colony. F) Comparison

of representative colony size (left) and differentiation (right) obtained from a singly sorted LT-HSC from a tamoxifen-treated control chimera (top) versus a tamoxifen-treated *Ars2* iKO chimera (bottom).

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**Table 1**  
Mixed mT/mG chimera bone marrow analysis, single dose of 5-FU (150mg/kg, IP).

Frequency of LSK						
Mouse	LT-HSC		ST-HSC		MPP	
	mT+	mG+	mT+	mG+	mT+	mG+
1	0.082	0	0.71	0.066	5.9	3.75
2	0.48	0.11	0.032	0	8.6	3.82
3	0.063	0.021	0.079	0.021	6.6	3.1
4	0.096	0	N/A	N/A	N/A	N/A
5	0.56	0	N/A	N/A	N/A	N/A
6	0.79	0	N/A	N/A	N/A	N/A
7	0	0	N/A	N/A	N/A	N/A
Average	0.29	0.018	0.27	0.029	7.03	3.55