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# Combinations of antioxidants and/or of epigenetic enzyme inhibitors allow for enhanced collection of mouse bone marrow hematopoietic stem cells in ambient air

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## Abstract

Hematopoietic cell transplantation (HCT) is a treatment for malignant and non-malignant disorders. However, sometimes the numbers of donor hematopoietic stem cells (HSC) are limiting, which can compromise the success of HCT. We recently published that collection and processing of mouse bone marrow (BM) and human cord blood cells in a hypoxic atmosphere of 3% $O_2$ or in ambient air (~21% $O_2$) in the presence of cyclosporine A yields increased numbers of HSC. We now show that collection and processing of mouse BM cells in ambient air in the presence of specific combinations of anti-oxidants and/or inhibitors of epigenetic enzymes can also enhance the collection of HSC, information of potential relevance for enhanced efficacy of HCT.

### Keywords

hematopoietic stem cell; antioxidants; hypoxia; epigenetic enzyme inhibitors

## Introduction

Hematopoietic stem cells (HSCs) reside in a hypoxic microenvironment *in vivo* (~1–5% $O_2$). [1–6] However, upon contact with atmospheric levels of oxygen in ambient air (~21% $O_2$), HSCs rapidly begin the process of differentiation to form hematopoietic progenitor cells (HPC). Most studies and hematopoietic cell transplantation (HCT) are carried out with cells collected in ambient air, but our previous work demonstrated that collection of cells in a hypoxia chamber (at 3% $O_2$), not in ambient air, greatly increased HSC recovery from mouse bone marrow (BM) and human cord blood (CB). [7–8] We termed the phenomenon of ambient oxygen induced loss of HSCs as extraphysiologic oxygen shock/stress (EPHOSS). We could counteract the effects of EPHOSS generated when collecting mouse BM or human CB in ambient air if the collections in air were done in the immediate presence of cyclosporine A. However, use of cyclosporine A is not without technical problems, as the potency of cyclosporine A can vary from batch to batch and the exact amount of cyclosporine A used has to be carefully titrated, plus exposure of cells to cyclosporine A for too long can have toxic effect on the cells. [9]

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In order to delineate alternative means to collect mouse BM cells in ambient air but without loss of HSC, we assessed the collection of mouse BM cells in ambient air, but in the absence and presence of different compounds and their combinations to attempt to mimic the effects of 'hypoxia harvest' on HSCs. We found that combinations of either antioxidants or of epigenetic enzyme inhibitors, but not in the presence of only single agents, could enhance collection of mouse BM HSCs, and of additional interest, could further enhance the collection of HSCs after collection/processing in a 3% $O_2$ hypoxic environment. These studies are of potential interest for clinical translation.

## Methods

### Mice

Female, 8–10 week old C57BL/6, Boy/J and C57BL/6J x Boy/J F1 (herein referred to as F1) mice were obtained from the on-site breeding core facility at Indiana University School of Medicine. All animal procedures were approved by the Indiana University Committee on Use and Care of Animals. Animals were maintained under temperature- and light-controlled conditions (21–24°C, 12 hour light/dark cycle) and were group-housed according to age and sex. Mice were fed *ad libitum*. Where indicated, BM was flushed from the femurs of C57BL/6 mice either at ambient air conditions or in a hypoxia chamber kept at 3% $O_2$ as previously published. [7]

### Inhibitors, antibodies and flow cytometry

All inhibitors used were from Selleck Chemicals (Houstin, TX). All inhibitors were used at the concentrations indicated. Inhibitors were present in all media starting at when the BM was flushed from the mouse femurs. All cells were in the presence of the inhibitor(s) for at least 1 hour prior to use in experiments. The inhibitors were not washed out. However, in the case of the transplantation experiments inhibitors were diluted out while creating the proper cell dose for transplantation. For flow cytometry, HSC were stained at room temperature for 15 minutes with the following antibodies: Lineage cocktail (Lin)-FITC (BioLegend; cat. # 133302), ckit-APC-H7 (BD Bioscience; clone # 2B8), Sca1-PE/Dazzle™594 (BioLegend; clone # D7), Flt3-APC (BioLegend; clone # A2F10) and CD150-BV421 (BD Bioscience; clone # Q38-480). HSC populations are defined as $Lin^{-}Sca1^{+}ckit^{+}$ (LSK) $CD150^{+}$ (see gating strategy provided in Figure 1A). CD3-APC-H7 (BD Bioscience; clone #145-2C11), B220-PE-CF594 (BD Bioscience; clone # RA3-6B2), CD11b-BV421 (BD Bioscience; clone #M1/70), CD45.1-FITC (BD Bioscience; clone # A20), CD45.2-APC (BD Bioscience; clone #104) were used for *in vivo* transplantation to assess donor BM cell engraftment. FACS analysis was performed with a modified BD Bioscience LSRII and FlowJo software (version 7.6.2; TreeStar, WA). The negative portion was determined by using relevant isotype antibody controls.

### *In vitro* colony-forming unit (CFU) assay

Mouse BM cells were seeded in triplicate in 1.0 mL of methylcellulose culture medium (1% methylcellulose) supplement with 30% FBS, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.1 mM hemin (Sigma-Aldrich), 5% vol/vol pokeweed mitogen mouse spleen cell conditioned medium and cytokines: 1 U/mL recombinant human erythropoietin

(Amgen; Thousand Oaks, CA), 50 ng/mL recombinant mouse SCF (R&D Systems). Plates were incubated at 5% $CO_2$ and lowered 5% $O_2$ in a humidified chamber. The number of colonies was scored at day 7 with an inverted microscope.[7,10]

### *In vivo* transplantation

F1 mice ($CD45.1^+CD45.2^+$) were lethally irradiated (550cGy, two doses, 24 hours apart) and transplanted with 50,000 antioxidant or vehicle control treated C57BL/6 ($CD45.1^-CD45.2^+$) BM cells and 100,000 Boy/J ($CD45.1^+CD45.2^-$) BM competitor cells within 24 hours after irradiation. [7,11] Peripheral blood (PB) was collected at various time points from host animals by submandibular vein bleeds. The blood samples were treated with red blood cell lysis buffer and then washed in PBS+0.5% BSA buffer before staining with CD45.1, CD45.2, CD3 (to determine T cells), B220 (to determine B cells) and CD11b (to determine myeloid cells) antibodies as described above. Mice were sacrificed 12 weeks after transplantation then BM cells were stained and analyzed by flow cytometry, but peripheral blood was assessed at 4 and 12 weeks.

### Statistical analysis

Results are expressed as mean values ± standard deviation. *P* value less than 0.05 (two-tailed Student's t-test) was considered as statistically significant.

## Results and Discussion

### Combination antioxidant treatment mimics 'hypoxia harvest'

An environmental difference between 'hypoxia harvest' and 'normoxia (ambient air collected) harvest' is the oxygen level. We hypothesized that antioxidants, which inhibit the oxidation of molecules, may be able to prevent the effect of oxidative stress on HSCs induced by EPHOSS. Mouse BM cells were collected with 1 mM N-Acetyl-Cysteine (NAC), a classic antioxidant which has been proved be useful in many oxidative stress studies [12,13], in the flush media collected in ambient air. Cells were incubated with the inhibitor for 1 hour and remained in all media for the rest of the experiment. In our system, there was no significant change in numbers of collected HSC in ambient air in the presence of 1 mM NAC (Figure 1B–C). A higher dose of NAC (3 mM) was tested, with no increase in numbers of collected HSCs (data not shown). Then we added another antioxidant, ascorbic acid 2-phosphate (AAP) [14], to check whether this anti-oxidant, alone or in combination with NAC, could produce protection and increased numbers of HSCs collected in ambient air under the stress of EPHOSS. Co-treatment with 1 mM NAC and 0.22 mM AAP, but not AAP alone, significantly increased numbers of HSCs from 99 ± 6 per million BM cells to 208 ± 17 per million BM cells ($p<0.001$), which is similar to the number of HSC collected in the hypoxia chamber without these added reagents: 235 ± 25 per million BM cells (Figure 1A–C).

To check whether antioxidants treatment influenced HPC numbers, mouse BM cells treated with either antioxidants or vehicle control were seeded in methylcellulose medium and cultured for 7 days in the presence of cytokines. As shown in Figure 1D–F combination antioxidant treatment resulted in significantly decreased numbers of granulocyte/

macrophage (CFU-GM), erythroid (BFU-E), and granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) progenitors, demonstrating that combination antioxidant treatment produces the same decrease in progenitor cell numbers as does hypoxia collection and processing (Figure 1D–F).

### Antioxidant treatment enhances HSC engraftment

To test the functional significance of antioxidant treatment on HSCs, lethally irradiated F1 mice were transplanted with 50,000 C57BL/6 BM cells treated with antioxidant combination or vehicle control, and 100,000 competitive Boy/J BM cells. Significantly increased engraftment in PB at 1 and 3 months and BM at 3 months post donor cell infusion was observed in the combination antioxidant treated group, compared to the vehicle control group (Figure 2A–B), consistent with our previously published findings of enhanced engraftment when using hypoxia collected and processed mouse BM cells. [7] The increased chimerism of the anti-oxidant treated cells was apparent for B cells, T cells and myeloid cells (Figure 2C–E).

### Epigenetic enzyme inhibitor treatment mimics and expands on hypoxia harvest

We also considered the possibility of chromatin remodeling and/or epigenetic change(s) during EPHOSS, and conducted a small-scale epigenetic inhibitor library screen to test whether selected epigenetic inhibitors could prevent the EPHOSS-induced HSC cell loss. Aurora A Inhibitor I (s1451) is an inhibitor of Aurora kinase. RG108 (s2821), is an inhibitor of DNA methyltransferase. Olaparib (s1060) is an inhibitor of PARP1/2. As shown in Figure 3A, combinatory inhibitor treatment (with inhibitors in all medias from flush through experimental processing) for cell collection in ambient air with 10μM s1451 + 10μM s2821, or 10μM s1451 + 10μM s1060 significantly increased numbers of collected HSCs in air (2.1 to 2.3 fold change; Figure 3A). As shown in Figure 3B–D, the combination of other inhibitors of Aurora kinase (s1103 and/or s1147; 10μM) with other inhibitors of DNA methyltransferase (s1200 and/or s1782; 10μM), or other inhibitors of PARP1/2 (s1004s and/or s2886; 10μM) also significantly increased HSC numbers. Thus, using combinations of anti-oxidants, or an inhibitor of Aurora kinase with either an inhibitor of DNA methyltransferase, or of PARP1/2 enhanced collections of mouse BM HSC in the presence of ambient air resulted in numbers that equated with those cells collected in hypoxia.

Of additional interest, combinatory inhibitor treatment for cells collected in the hypoxia chamber also showed dramatic increases of HSC compared to the numbers of HSCs collected in hypoxia without these reagents (Figure 3E). Thus, we have now identified a number of different means to further enhance collection of HSCs even during hypoxic collection of the BM cells.

## Conclusions

Our previous study showed that brief exposure of mouse BM cells to air limits the efficiency of HSC recovery. [7,8] In this present study, based on the fact that the only difference between ‘normoxia harvest’ and ‘hypoxia harvest’ is the oxygen level, we tested several antioxidants and their combinations and found using *in vitro* and *in vivo* assays that one

antioxidant combination (NAC + AAP) could mimic 'hypoxia harvest'. We also evaluated epigenetic enzymatic regulators by conducting a small-scale epigenetic enzyme inhibitor library screen, and found that some combinations of epigenetic enzyme inhibitors could also mitigate the apparent EPHOSS-induced HSC differentiation in air.

HCT, especially with CB, is limited to a degree by the numbers of HSCs found in single CB collections. [9,15,16] Means to enhance the collection of HSCs could have significant therapeutic effect. We have now identified with mouse BM cells alternative means to enhance the collection of HSC, information that may be of relevance to future efforts to enhance HCT. We consider this a start, not an end, to determining the most efficacious means to enhance the collection of HSC from different tissue sources. It is likely that other means may work to enhance collections of HSC. Whether these combinations of reagents will be useful for collection of human HSCs remains to be determined. Also, it may be possible to enhance the engraftment of these increased numbers of collected HSCs by increasing the homing capacities of these cells. [16–18]

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## Abbreviations

| | |
|---|---|
| **HCT** | hematopoietic cell transplantation |
| **HSC** | hematopoietic stem cell |
| **BM** | bone marrow |
| **HPC** | hematopoietic progenitor cell |
| **CB** | cord blood |
| **EPHOSS** | extraphysiologic oxygen shock/stress |
| **F1** | C57BL/6J x Boy/J |
| **Lin** | lineage |
| **LSK** | $Lin^{-}Sca1^{+}ckit^{+}$ |
| **CFU** | colony-forming unit |
| **PB** | peripheral blood |
| **NAC** | N-Acetyl-Cysteine |
| **AAP** | ascorbic acid 2-phosphate |
| **CFU-GM** | granulocyte/macrophage progenitor |
| **BFU-E** | erythroid progenitor |

**CFU-GEMM** granulocyte/erythrocyte/macrophage/megakaryocyte progenitor

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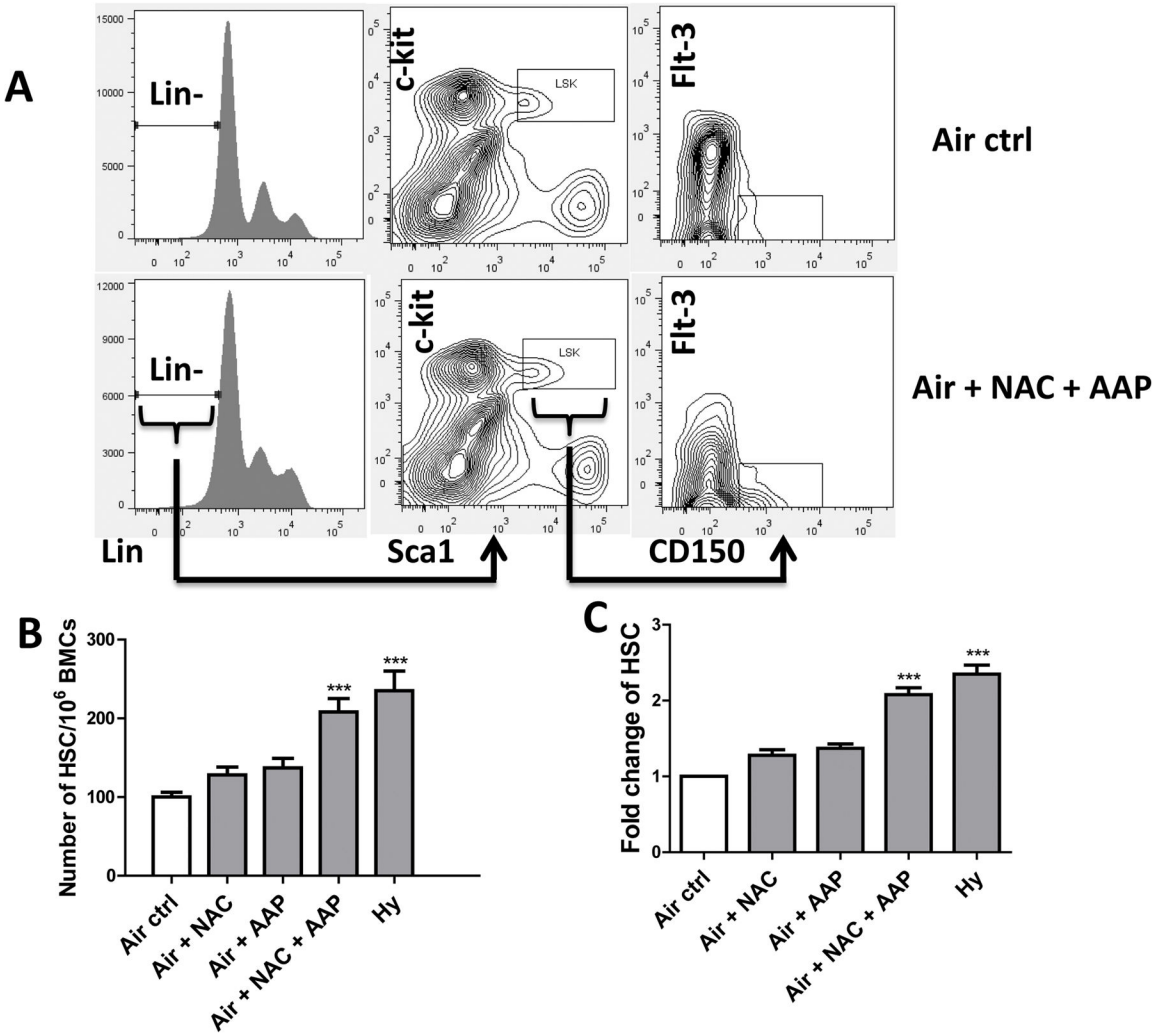

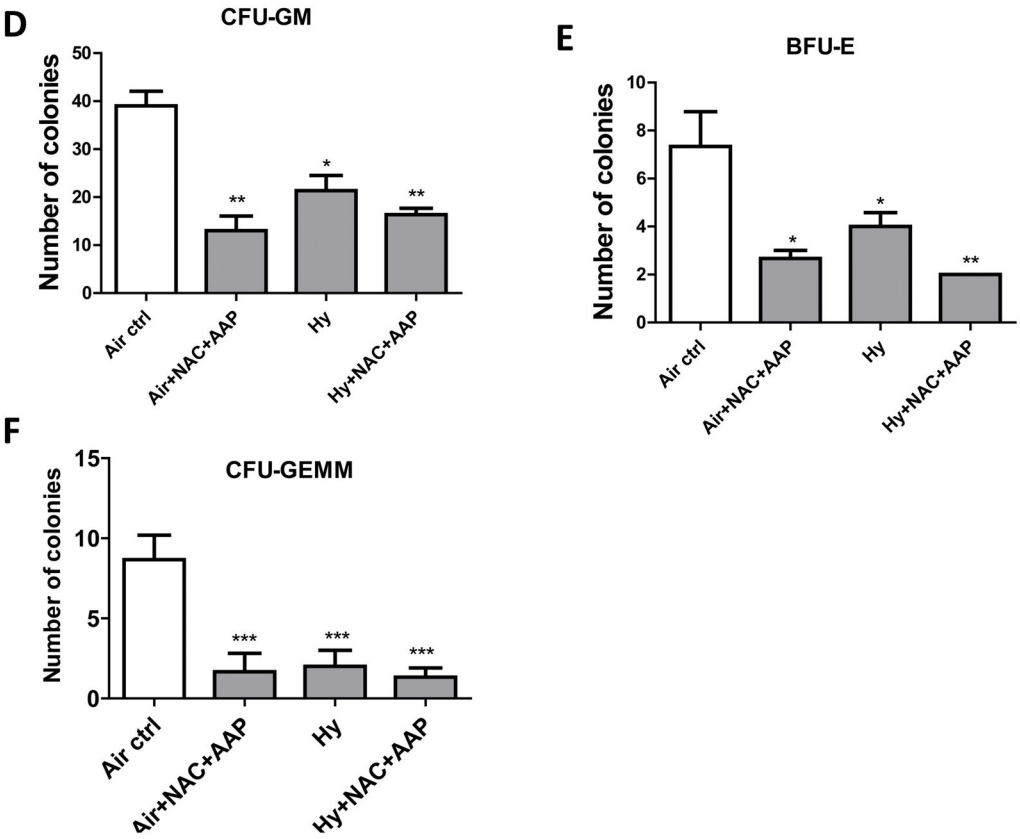


**Figure 1. Antioxidant treatment mimics hypoxia harvest**

Bone marrow cells were harvested and processed in a hypoxic chamber. Groups exposed to air were removed from the chamber and placed in ambient air for 60 minutes. A: Representative contour plots of HSCs treated with antioxidant or vehicle. B: Numbers of HSC in 1 million mouse bone marrow cells (BMCs) treated by 1mM NAC and/or 0.22 mM AAP. C: Fold change of HSCs treat with NAC and AAP. D–F: Colony formation assay (CFU) shows treatment with NAC and AAP, similar to that of hypoxia collection/processing, prevents HSC differentiation, like hypoxia harvest. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

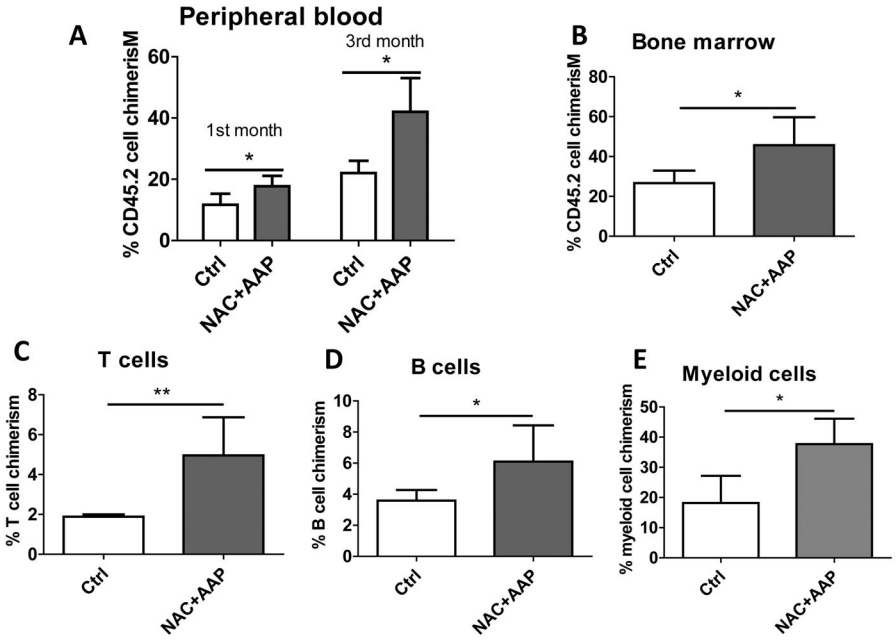


**Figure 2. Effect of antioxidant treatment on competitive mouse HSC engraftment**

50000 antioxidant or vehicle control treated CD45.2$^{+}$ C57BL/6 mouse bone marrow cells and 100000 CD45.1$^{+}$ Boy/J competitive cells were infused into lethally irradiated dual CD45.2$^{+}$/CD45.1$^{+}$ F1 recipients. Data was collected 1 and 3 months after injection. A: Peripheral blood (PB) was collected at 1$^{st}$ month and 3$^{rd}$ month after transplantation and cells were stained for CD45.2 percentage. B: CD45.2 percentage in mouse bone marrow 3 month after transplantation. C. CD45.2$^{+}$ B cell, T cell and myeloid cell percentages in mouse bone marrow 3 month after transplantation. n=5 for mice each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

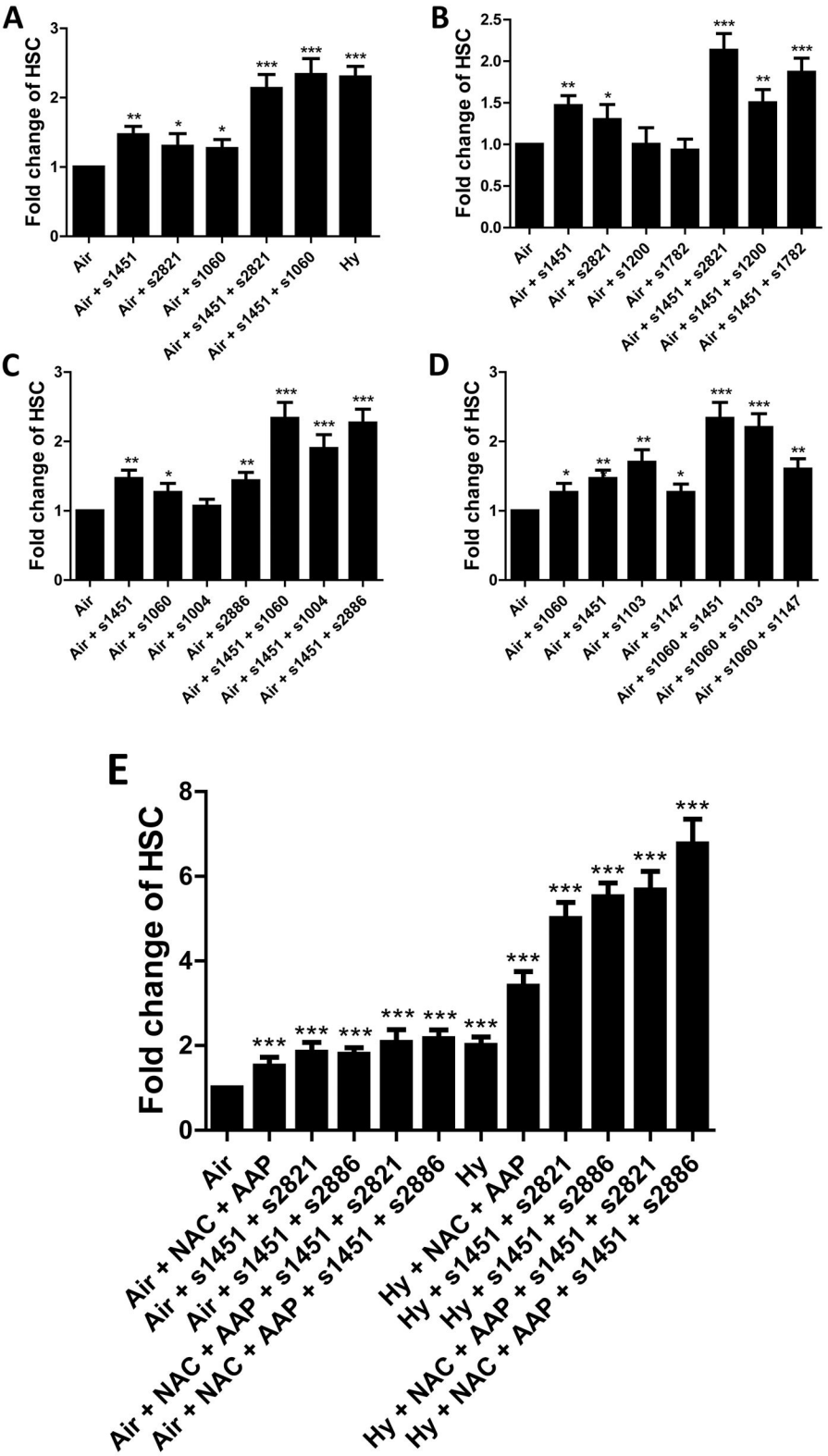


**Figure 3. Epigenetic enzyme inhibitor library screen identified compounds which mimic hypoxia harvest**

A: Fold change of HSCs after treatment with s1451 (Aurora kinase inhibitor), s2821 (DNA methyltransferase inhibitor) and s1060 (PARP1/2 inhibitor) (concentration: 10 μM) for 1 hour. B: Fold change of HSCs after treatment with s1451 and another two DNA methyltransferase inhibitors, s1200 and s1782 (concentration: 10 μM). C: Fold change of HSCs after treatment with s1451 and another two PARP1/2 inhibitors, s1004 and s2886 (concentration: 10 μM). D: Fold change of HSCs after treatment with s1060 (PARP1/2 inhibitor) and another two Aurora kinase inhibitors, s1103 and s1147 (concentration: 10 μM). E: Fold change of HSCs after treatment with antioxidants and inhibitors in air and hypoxia chamber. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n=3, 7 mice.