Peer Review File

Manuscript Title: Foetal hepatocytes protect the HSPC genome via fetuin-A

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In this manuscript by Guo et al., the authors explore the genomic integrity of fetal liver HPCs over time and evaluate the role of fetuin A, produced by fetal liver hepatocytes, in regulating HPC genomic integrity. Several of the findings are interesting, but I have some concerns with the data analyses and strength of the conclusions.

Major Comments
Figure 1b-e: The data convincingly show the differences in tail moments in fetal liver ckit+sca1+lin- cells at different time points in development. This population is mostly HPCs, not hematopoietic stem cells (HSCs). SLAM markers have been shown to enrich for HSCs in fetal liver as well as adult tissues (Kim et al. Blood 2006;108:737). In addition to the analyses shown in Figures 1b-d, authors should compare SLAM marked HSCs at same time points in fetal liver to demonstrate the sensitivities of more purified HSCs.

Figure 1e: The order of x axis labeling of E12.5 and E13.5 appears out of order; show the time points in order to be consistent with other panels.

In the Results text associated with Figure 1d, the authors speculate that the difference in gamma-H2AX staining in fetal liver HPCs compared to placental HPCs reflects the “microenvironment” in the fetal liver not providing suitable “protection”. There is no evidence to support that conclusion at this point in the manuscript; this statement should be removed and the data should be presented in order, leaving discussion/interpretation to the Discussion section.

Extended Data Figure 3: Ext Data Fig 3B does not show representative chromosome damage in the E12.5 vs E16.5 cell populations following etoposide treatment; please show the representative chromosomal damage in both groups of mice so that the reader can better understand the total results shown in Ext Data Fig 3c.

Figure 1g: The data shown indicate that there is no difference or increase in BM lin- cells in the ETOP-treated E12.5 hematopoietic system compared to the ETOP-untreated or E16.5 time point; these results appear at odds or non contributory to the argument that the E12.5 HPCs are more susceptible to leukemic transformation than other developmental time points. Data that demonstrates leukemic

transformation directly in these different populations would be more supportive of the authors' argument (e.g. clonality or direct evidence of leukemic cells in hematopoietic or other tissues).

Figure 1h: What were the criteria for "disease free" or having disease in the mice shown? As noted above, authors need to show more clear, direct measurements of leukemia in these mice groups to be convincing and these data should be shown in the main figures.

Figure 2b and 2c: The representative cell images shown in 2b suggest a very modest difference in gamma-H2AX levels between the populations from the 2 groups of mice. Perhaps additional representative samples can be shown to be more convincing. The bar graphs shown in 2c suggest that there is not an overall difference in gamma H2AX + cell percentages between the 2 groups, but rather a difference in "severity". Authors need to better explain/justify what the significance of the latter analysis is or rather simply show the gamma H2AX percentages as shown in Figure 1.

Also, since the maximal genomic sensitivity of embryonic HPCs appears to be at E12.5 – E15.5 as shown in Figure 1, why don't the authors compare the gamma-H2AX percentages in HPCs at E12.5 – E15.5 in Figure 2b and 2c in order to better assess the role of intact hepatocytes on the genomic integrity of the developing fetal liver HPCs. Analysis at E16.5 appears to be a flawed strategy based on the data shown in Figure 1.

Figure 3a: Did the authors test the effect of contact and non-contact (transwell) co cultures of E12.5-E16.5 HPCs with fetal liver hepatocytes? This should be done to show fundamentally whether contact interactions are important or not for HPC genomic stability at this point in fetal development.

These in vitro culture studies with fetal liver conditioned media and non contact cultures with fetal liver cells should be performed with purified fetal liver HSCs as well as HPCs to elucidate whether the observed effects are direct on HSCs or indirect via action on HPC cells.

Figure 3e: The resolution of the higher powered microscopic images shown here are insufficient to be convincing of the conclusion drawn by the authors. Please show additional higher magnification microscopic views and consider adding cell surface markers for HSPCs beyond ckit to more convincingly show association of the fetuin protein with the cell surface of HSPCs in situ.

Figure 3f: Please show representative cell preps supporting the bar graph analyses shown in 3f.
As a general concern with the analysis, why is % gamma H2AX shown in some panels, e.g. Figure 3h, whereas in other panels, fluorescence density classification of gamma H2AX is shown, e.g. Figure 3f. The data would be more convincing throughout if consistent measurements of gamma H2AX percentages were shown. The selective application of the different measurements related to gamma H2AX raises concern about the magnitude of the effects.

Figure 3g and 3j: The experiments performed here and the results presented are more convincing of the role of Fetuin A in regulating HPC genomic integrity during development.

Figure 4g-4i: Which BLM inhibitor was utilized in these studies? Please specify in legend. Also, please comment on the specificity of the BLM inhibitor utilized for the target versus non specific effects expected.

Figure 5a and 5c: The panels show a clear difference in G0 and G2/S/M phase percentages between the E12.5 and E16.5 HPCs. However the percentages in panel 5c suggest these populations are similar in cell cycle stage and panel 5e suggests that ENU incorporation is comparable between E12.5 and E16.5 HPCs. Please explain these nuances or represent the data in such a way to be less confusing.

Also, representative percentage numbers should be added to the flow figure panels in 5a and 5c.

Would treatment with the BLM inhibitor correct the cell cycle progression that occurs upon transit of HPCs to E12.5 in fetal liver? Can this experiment be performed to mechanistically link the role of BLM DNA repair with the cell cycle effects?
Does Fetu A treatment or expression correct these cell cycle differences in E12.5 vs. PL12.5 HPCs vs. E16.5 HPCs? It seems imperative to connect the mechanistic effects of Fetu A and BLM regulation with the HPC cell cycle effects shown in this Figure.

Figure 6. Did the presence or absence of Fetuin A affect the correlation between R loop and mutation frequencies shown in panel 6e? Was expression of fetuin A associated significantly with less mutations in leukemogenic genes in this analysis. The demonstration that fetuin A expression was associated with a longer latency to leukemia presentation is interesting. It would be helpful to clarify what effect fetuin A expression had on mutations in leukemia-associated genes.

Referee #2 (Remarks to the Author):

In this manuscript the authors study the role of the fetal liver as a HSPCs niche. They show that HSPCs from the early fetal liver at the E12.5 are more susceptible to genotoxic stress than HSPCs from the placenta at E12.5 or HSPCs from the late fetal liver at E16.5. They show this unique feature from E12.5FL HSPCs both ex vivo and in vivo. They also identify an intrinsic higher burden of R-loops in replicating HSPCs at the fetal liver stages and link this increased R-loop formation to an increased mutation risk at developmental genes. The authors propose that the fetal liver microenvironment provides a genome protective environment but that the early fetal liver lack this genome protection ability. They identify the paracrine secretion from hepatocytes of FetuA and the implication of the FetuA-Tlr4-MyD88-bZip-Blm axis as the key mechanism providing HSPCs genome protection by inducing the transcription of the Blm helicase who help process R-loops, thereby reducing their toxicity. Lastly, they show that this mechanism identified in mice embryonic development is preserved in human development. Altogether, the mechanism identified in this manuscript provides new important information on hematopoietic embryonic development and in leukemia development. I think that this manuscript meets the

requirement for publication in Nature but I have several questions that should be addressed.

Major points:

1. The authors conclude that the microenvironment of the fetal liver provides the protective advantage, but this initial conclusion stems from ex vivo experiments where HSPCs were harvested and isolated in single-cell suspensions and at line 106 the authors conclude that "[…] the microenvironment within the early fetal liver does not provide newly colonized HSPCs with sufficient protection." These cells are separated from their microenvironment so this conclusion is difficult to make from these initial experiments. It is conceivable considering the later findings of the manuscript on the signaling pathway and transcriptional regulation of Blm and the other in vivo data presented, but the manuscript would gain quality from preserving a better linearity with conclusions that are not affected from later results.

2. The protective role of secreted FetuA raises several unanswered questions. If HSPCs are more prone to genotoxic agents and need FetuA from the hepatocytes to protect them, this would mean that once they migrate to the bone marrow, they still need the FetuA protection. Does the perinatal BM HPSCs also depend on FetuA for its genome protection? Do the fetal liver stop to produce FetuA explaining the need to migrate again? Why do HSPCs migrate in early fetal liver if the early fetal liver does not protect them?

3. Fig2b-c. These results support the importance of hepatocytes for protecting HSPCs from genotoxic agents as in iDTA +/- mice, E16.5 HSPCs have a sensitivity to Etoposide similar to ex vivo/in vivo E12.5 HSPCs. When comparing E16.5 HSPCs in Fig 1e and Fig 2c (both in vivo quantification of Eto-induced y-H2A.X), the percentage of y-H2A.X negative cells is similar but when compared to E16.5 HSPCs in Extended data Fig 2b (ex vivo quantification of Eto-induced y-H2A.X), there is a 1.5x diminution in y-H2A.X positive cells. Does this suggest that ex vivo HSPCs are less susceptible to genotoxic stress than in situ HSPCs?

4. Fig3. In Fig3b, Co-SHI media from E12.5 and E16.5 hepatocytes were used and tested but in the following panels and text, it is not specified if the protein enriched in Co-SHI comes from E12.5 or E16.5 Co-SHI or both. I would expect it to be E16.5 and these are protein found to be lacking from E12.5 FL but I think it should be specified as depending on the source of the analyzed Co-SHI would alter the conclusions.

5. Fig3b. As E12.5 Co-SHI decrease the percentages of y-H2A.X cells compared to SHI, why did the authors decide to look at secreted proteins that are lacking at E12.5? Most of the changes displayed in Fig3b are between SHI and Co-SHI, not between E12.5 and E16.5 Co-SHI.

6. The authors use S9.6 staining for R-loop quantification by immunofluorescence. As the increase in R-loops is an important result in the paper and specificity of the S9.6 antibody has often been debated in the field, I would like to see the same results with a complementary method that is not relying on S9.6 antibody (e.g. GFP-dRNH1, https://doi.org/10.1083/jcb.202101092).

7. Fig 4g and Extended data Fig8: In Extended data Fig 8 it is hard to see if the S9.6 signal is nuclear or outside of the nucleus, especially in E12.5 PL HPSCs. Can the author maybe use the same representation as in Fig4g, representing the nuclei by showing the DAPI signal outline instead of the DAPI signal itself. Still, from Fig 4g, a major part of the signal seems to be outside of the DAPI border, is it due to non-specific nuclear membrane binding of the antibody or is it still inside the nuclei? In Fig4h, does the quantification shown represent S9.6 signal inside the DAPI region only or take in this perinuclear region also?

8. Fig5e,f: I don't think it is possible to conclude on accelerated gene transcription based on those EU data. The data shown only display % of EU+ cells which display a reduction in the transcriptionally silent cell population. The author could quantify the intensity of EU incorporation and conclude if there is an increase in transcription output, but I would still refrain using the terms "accelerated gene-transcription" as it sounds like the authors are concluding on the speed of RNA polymerases. The level of EU incorporation may also be heavily influenced by RNA Polymerase I transcription which accounts for a large proportion of newly synthesized RNA per cell.

9. Extended data Fig8. Considering that 40% of E12.5 PL HSPCs are not incorporation EU (Fig5e,f), it is difficult to reconcile the image shown and the quantification shown here. Is this a representative image of E12.5 PL HPSCs? Shouldn't we see only 60% of E12.5 PL HPSCs showing S9.6 signal? Also, does the quantification in b have excluded s9.6 negative cells? I do not think so because it seems that there is a decent amount of datapoints in the different samples showing absence of staining, but if negative cells have not been removed, it doesn't seem to show that 40% of the cells are negative based on this graph.

10. Fig6: the R-loop quantification suggests a small decrease in R-loops when comparing E12.5FL and E16.5FL while in extended data fig8, the quantification of R-loops show a level of R-loops in E16.5FL even lower than E12.5PL. Why is there such a difference in the levels of R-loops at E16.5 as evaluated by these two approaches?

11. Fig6: Has the R-loop quantification by cut-and-tag of E12.5PL been normalized in any way to address the fact that there exists a large amount of transcriptionally silent cells at this stage? Can the lower R-loop levels at that stage be explained by a lower population of transcribing cells (instead of transcribing cells show less R-loops)?

Minor points:
Intro: basic description of fetuin-A would be nice.
Fig1: the abbreviation AGM is not explained
Fig 2a. Are the fluorescence histogram values standardized between the panels? It seems that between the c-kit/albumin panels and the merge panels that the values are not the same (see intensity of c-kit in E16.5 FL for example)

Referee #3 (Remarks to the Author):

In this work, the authors investigated the genomic integrity of fetal HSPCs and the genome-protective mechanism from hepatocytes. Firstly, by assessing the fetal HSPC sensitivity to genotoxic agents, they found that early fetal liver (E12.5-E14.5) HSPCs exhibited more severe DNA damage and shorter latent period of induced leukemia than late fetal liver (E15.5-E18.5) HSPCs. Then, to detect the genome protection effect of HSPC from microenvironment hepatocytes, they analyzed the developmental dynamics between hepatocytes and HSPCs using Alb-cre+/−Rosa-iTomato+/− line, and hematopoietic phenotype after hepatocyte depletion using Alb-cre+/−Rosa-iDTA+/− line, respectively. As a result, the development of hepatocytes lags than that of HSPCs. Besides, the cell numbers and genome integrity of HSPCs were reduced after hepatocyte depletion. Mechanistically, by performing proteomic assay and ex vivo culture assay, they found that FetuA secreted by hepatocytes can protect the genomic stability of HSPCs from genotoxic agents. Moreover, HSPCs in E16.5 FetuA-/- fetal liver exhibited higher sensitivity to genotoxins than that in sibling mice. Finally, by integrating RNA-seq and ATAC-seq analysis, their results showed that FetuA-Tlr4-Myd88-bZIP pathway is involved in genomic protection of HSPCs by interfering R-loop formation. Overall, this work detected the genome integrity of HSPCs during embryonic development and revealed the genome protective mechanism, i.e. FetuA secreted by hepatocyte protected HSPC by preventing R-loop accumulation.

Comments

(1) To detect DNA damage by immunofluorescence staining, the authors used the sorted fetal liver HSPCs by FACS and co-stained with γ-H2A.X. However, flowcytometry would cause some injury to cells. the immunofluorescence staining of whole-mount tissues should be provided, which would be better to reflect DNA sensitivity of HSPC in native niche.

(2) To test the regulatory role of hepatocyte to HSPCs, the authors manipulated hepatocytes by using Alb-cre+/−; Rosa-iDTA+/− mice. However, hepatocyte is an important cell type in fetal liver and the depletion of hepatocyte may cause a severe developmental disability to fetal liver. How to exclude the regulatory role of other niche components for HSPC development in Alb-cre+/–Rosa-iDTA–/– mice, in the absence of hepatocytes? In addition, the DTA approach is very likely toxic which would cause a systemic effect to the whole environment, rather than just affecting hepatocytes alone, therefore the functional specificity of the DTA approach as well as of the hepatocytes should be considered.

(3) In this study, the authors only used c-Kit labeling HSPCs on fetal liver sections. The authors need to use more HSPC markers to confirm this result.

(4) By performing proteomic assay and co-culture, the authors found that hepatocytes secrete Dfactors FetuA to protect HSPC genome integrity. Can FetuA rescue the HSPC phenotype via intra-placental injection of recombinant FetuA in Alb-cre+/–Rosa-iDTA–/– pregnant mice?

(5) In terms of the mechanism, the authors proposed that FetuA–Tlr4–Myd88–bZip–Blm axis signaling protected HSPC by preventing R-loop amassment. However, the functional validation for the signaling

relationship is weak, especially biochemical experiments. Besides, it is unclear how these signaling coordinately regulate HSPC genomic stability.

(6) Genotoxic agents caused DNA damage for fetal liver HSPCs, how about the cell fate of HSPC after Eto treatment? Do cell death, cell cycle arrest or others occur? More phenotypical validation should be provided.

Author Rebuttals to Initial Comments:

The point-by-point response

We greatly appreciate the reviewers' encouraging comments and helpful comments on the manuscript. We have substantially addressed most of the issues that the reviewers raised. The point-by-point responses are as follows.

Referee #1 (Remarks to the Author):

In this manuscript by Guo et al., the authors explore the genomic integrity of fetal liver HPCs over time and evaluate the role of fetuin A, produced by fetal liver hepatocytes, in regulating HPC genomic integrity. Several of the findings are interesting, but I have some concerns with the data analyses and strength of the conclusions.

Response: We are very grateful for the reviewer's encouraging comments. We have carefully addressed all concerns to validate our findings and strengthen our conclusions.

Major Comments

(1) Figure 1b-e: The data convincingly show the differences in tail moments in fetal liver ckit+sca1+lin- cells at different time points in development. This population is mostly HPCs, not hematopoietic stem cells (HSCs). SLAM markers have been shown to enrich for HSCs in fetal liver as well as adult tissues (Kim et al. Blood 2006;108:737). In addition to the analyses shown in Figures 1b-d, authors should compare SLAM marked HSCs at same time points in fetal liver to demonstrate the sensitivities of more purified HSCs.

Response: We agree with this comment. We have performed the suggested experiments. LSK cells were divided into three populations based on SLAM marker expression: long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs). Eto treatment consistently induced DNA damage across these three fractions of foetal haematopoietic cells, as measured by a DNA comet assay. The data are presented in the new Extended Data Fig. 2.

(2) Figure 1e: The order of x axis labeling of E12.5 and E13.5 appears out of order; show the time points in order to be consistent with other panels.

Response: The order has been corrected.

(3) In the Results text associated with Figure 1d, the authors speculate that the difference in gamma-H2AX staining in fetal liver HPCs compared to placental HPCs reflects the "microenvironment" in the fetal liver not providing suitable "protection". There is no evidence to support that conclusion at this point in the manuscript; this statement should be removed and the data should be presented in order, leaving discussion/interpretation to the Discussion section.

Response: The statement has been removed, and the data are presented in order.

(4) Extended Data Figure 3: Ext Data Fig 3B does not show representative chromosome damage in the E12.5 vs E16.5 cell populations following etoposide treatment; please show the representative chromosomal damage in both groups of mice so that the reader can better understand the total results shown in Ext Data Fig 3c.

Response: Representative images of chromosomal damage in both groups of mice are shown in the new Extended Data Fig. 3b.

(5) Figure 1g: The data shown indicate that there is no difference or increase in BM lin- cells in the ETOP-treated E12.5 hematopoietic system compared to the ETOP-untreated or E16.5 time point; these results appear at odds or non contributory to the argument that the E12.5 HPCs are more susceptible to leukemic transformation than other developmental time points. Data that demonstrates leukemic transformation directly in these different populations would be more supportive of the authors' argument (e.g. clonality or direct evidence of leukemic cells in hematopoietic or other tissues).

Response: To address this issue, we treated pregnant mice at E12.5 or E16.5 with Eto and kept them until delivery to determine whether the treatment resulted in phenotypical, functional, chromosomal, and leukaemia-vulnerable alterations in HSPCs that were detectable postnatally after they migrated into the bone marrow (Fig. 1f). The proportion of 3-week-old BM LSK populations was greater in Eto-treated mice at E12.5 than in Eto-treated mice at E16.5 and in untreated mice (Fig. 1g, h). These cells exhibited stronger colony formation ability, as assessed by a colony-forming cell (CFC) assay (Fig. 1i, j). Whole-genome sequencing revealed that 3-week-old BM LSK cells from Eto-treated mice at E12.5 carried more mutations than those from Eto-treated mice at E16.5 (Fig. 1k). Additionally, mice that were Eto-treated at E12.5 were more vulnerable to leukaemia induction than were those that were Eto-treated at E16.5, with leukaemic cell infiltration in the BM and spleen convincingly shown (Fig. 1l-o and Extended Data Fig. 4). Please see the details in the revised manuscript.

(6) Figure 1h: What were the criteria for "disease free" or having disease in the mice shown? As noted above, authors need to show more clear, direct measurements of leukemia in these mice groups to be convincing and these data should be shown in the main figures.

Response: The occurrence of leukaemia was monitored by daily observation of leukaemic symptoms and signs including white toes, swollen lymph nodes, and weight loss, along with weekly measurements of leukaemia-like cells by Giemsa staining of peripheral blood smears. Once these symptoms appeared (Fig. 1l and Extended Data Fig. 4b, c), the animals were sacrificed, and leukaemia was confirmed by the presence of enlarged spleens and lymph nodes (Extended Data Fig. 4d), increased proportions of immature Lin^- cells in the bone marrow (Extended Data Fig. 4e), and definite leukaemic cell infiltration in the bone marrow (Fig. 1m, n) and spleen (Fig. 1n). The latency period of induced leukaemia was significantly shorter in Eto-treated mice at E12.5 than in Eto-treated mice at E16.5 or in untreated mice (Fig. 1o). This information has been added to the revised manuscript, and the major data are presented in Fig. 1l-o.

(7) Figure 2b and 2c: The representative cell images shown in 2b suggest a very modest difference in gamma-H2AX levels between the populations from the 2 groups of mice. Perhaps additional representative samples can be shown to be more convincing. The bar graphs shown in 2c suggest that there is not an overall difference in gamma H2AX+ cell percentages between the 2 groups but rather a difference in "severity". Authors need to better explain/justify what the significance of the latter analysis is or rather simply show the gamma H2AX percentages as shown

in Figure 1.

Response: Better representative and more convincing images are shown (new Fig. 2i), and the gamma H2AX percentages are simply shown (new Fig. 2j).

(8) Also, since the maximal genomic sensitivity of embryonic HPCs appears to be at E12.5 – E15.5 as shown in Figure 1, why don't the authors compare the gamma-H2AX percentages in HPCs at E12.5 – E15.5 in Figure 2b and 2c in order to better assess the role of intact hepatocytes on the genomic integrity of the developing fetal liver HPCs. Analysis at E16.5 appears to be a flawed strategy based on the data shown in Figure 1.

Response: Thank you for the insightful comment. The data were possibly presented improperly in the previous version of the manuscript, which might have led to misunderstanding. To clarify this, we have moved the panels from the previous Extended Data Fig. 5 to the main Fig. 2 in the revised manuscript and explain as follows: We first showed that embryonic HSPCs were more sensitive to genotoxins in the early foetal liver at E12.5–E14.5 than in the later foetal liver (Fig. 1). Additionally, hepatocyte development lagged behind HSPC development, resulting in relatively low hepatocyte numbers in the early foetal liver, as demonstrated in hepatocyte-tracing (Alb-tomato) mice (new Fig. 2c). We thus speculated that hepatocytes are essential for maintaining the genomic stability of HSPCs in the foetal liver. To test this hypothesis, we used a hepatocyte depletion tool, mice (Alb-cre$^{+/-}$Rosa-iDTA$^{+/-}$), to interfere with hepatocyte development and observe the effect on the genomic sensitivity of embryonic HSPCs (new Fig. 2d-j). Analysis at E16.5 was chosen because the HSPC genome was well protected in wild-type and noninterfered control mice, whereas hepatocyte development was substantially disrupted (50% reduction in number) in Alb-Cre$^{+/-}$Rosa-iDTA$^{+/-}$ mice (new Fig. 2e, f). Eto treatment induced more severe DNA damage in the HSPCs of Alb-Cre$^{+/-}$Rosa-iDTA$^{+/-}$ mice than in those of control mice (new Fig. 2i, j). These results thus proved that hepatocytes play an important role in protecting HSPCs from genotoxic insults, and this protection is lacking in the early foetal liver.

(9) Figure 3a: Did the authors test the effect of contact and non-contact (transwell) co cultures of E12.5-E16.5 HPCs with fetal liver hepatocytes? This should be done to show fundamentally whether contact interactions are important or not for HPC genomic stability at this point in fetal development.

Response: These experiments were performed. Eto treatment induced markedly less DNA damage in the HSPCs in the cocultures, either in contact or in transwells, than in the control cultures without hepatocytes (new Fig. 3a-c).

(10) These in vitro culture studies with fetal liver conditioned media and non contact cultures with fetal liver cells should be performed with purified fetal liver HSCs as well as HPCs to elucidate whether the observed effects are direct on HSCs or indirect via action on HPC cells.

Response: Cocultures in transwell and genome stability tests were also performed on LT-HSCs, ST-HSCs, and MPPs. Consistent results were obtained for all three fractions of cells (Extended Data Fig. 5a, b).

(11) Figure 3e: The resolution of the higher powered microscopic images shown here are insufficient to be convincing of the conclusion drawn by the authors. Please show additional higher

magnification microscopic views and consider adding cell surface markers for HSPCs beyond ckit to more convincingly show association of the fetuin protein with the cell surface of HSPCs in situ.

Response: Higher-magnification microscopy images showing the interaction between FetuA and HSPCs with additional markers ($CD150^{+}Lin^{-}CD41^{-}CD48^{-}$) *in situ* were obtained and are shown in the new Extended Fig. 5g. Please also refer to Fig. 4a, b for the interaction.

(12) Figure 3f: Please show representative cell preps supporting the bar graph analyses shown in 3f.
As a general concern with the analysis, why is % gamma H2AX shown in some panels, e.g. Figure 3h, whereas in other panels, fluorescence density classification of gamma H2AX is shown, e.g. Figure 3f. The data would be more convincing throughout if consistent measurements of gamma H2AX percentages were shown. The selective application of the different measurements related to gamma H2AX raises concerns about the magnitude of the effects.

Response: Representative IF images are now shown together with the statistical bar graph (new Fig. 3f, g). All related panels show the percentages of gamma-H2AX foci.

(13) Figure 3g and 3j: The experiments performed here and the results presented are more convincing of the role of Fetuin A in regulating HPC genomic integrity during development.

Response: Many thanks for the positive comment.

(14) Figure 4g-4i: Which BLM inhibitor was utilized in these studies? Please specify in legend. Also, please comment on the specificity of the BLM inhibitor utilized for the target versus non specific effects expected.

Response: A highly specific inhibitor of BLM, ML216, was utilized in our studies, which has been specified in the legend and the text of the revised manuscript. ML216 was shown to be a potent inhibitor of the DNA unwinding activity of BLM and to exert antiproliferative effects on BLM-expressing cells in cell line cultures[1]. In our studies, we demonstrated that short-term treatment (2 h) had potent R-loop unwinding activity in foetal liver HSPCs (new Fig. 4h, i) but had no significant effect on their cell cycle progression (new Extended Data Fig. 8c, d).

(15) Figure 5a and 5c: The panels show a clear difference in G0 and G2/S/M phase percentages between the E12.5 and E16.5 HPCs. However, the percentages in panel 5c suggest these populations are similar in cell cycle stage and panel 5e suggests that ENU incorporation is comparable between E12.5 and E16.5 HPCs. Please explain these nuances or represent the data in such a way to be less confusing.
Also, representative percentage numbers should be added to the flow figure panels in 5a and 5c.

Response: Consistent data are presented in the new Fig. 5, and representative numbers have been added.

(16) Would treatment with the BLM inhibitor correct the cell cycle progression that occurs upon transit of HPCs to E12.5 in fetal liver? Can this experiment be performed to mechanistically link the role of BLM DNA repair with the cell cycle effects?
Does Fetu A treatment or expression correct these cell cycle differences in E12.5 vs. PL12.5 HPCs vs. E16.5 HPCs? It seems imperative to connect the mechanistic effects of Fetu A and BLM regulation with the HPC cell cycle effects shown in this Figure.

Response: In the experimental setting, cells were treated with FetuA and a BLM inhibitor for two hours, and no significant effect on the cell cycle of E12.5 foetal liver HSPCs was detected (Extended Data Fig. 8c, d), indicating that FetuA did not provide HSPCs with genome protection through a direct effect on their cell cycle.

(17) Figure 6. Did the presence or absence of Fetuin A affect the correlation between R loop and mutation frequencies shown in panel 6e? Was expression of fetuin A associated significantly with less mutations in leukemogenic genes in this analysis. The demonstration that fetuin A expression was associated with a longer latency to leukaemia presentation is interesting. It would be helpful to clarify what effect fetuin A expression had on mutations in leukaemia-associated genes.

Response: To address this question, genome-wide R-loop sequencing and whole-genome sequencing were performed on E16.5 foetal liver HSPCs from FetuA knockout FetuA$^{-/-}$ mice and control wild-type mice. As expected, R-loop accumulation (Fig. 6b, c) and nutation numbers (Fig. 6d) were much greater in the absence of FetuA in FetuA$^{-/-}$ mice than in the presence of FetuA in control mice. The Circos plot shows the genome-wide distribution of the identified R-loop events and mutations (Fig. 6e). The R-loop distribution was positively correlated with mutations both in the presence and absence of FetuA (Fig. 6f). We also observed that numerous important haematopoietic regulatory genes were located in these R-loop-enriched and highly mutated regions (Fig. 6g). These results thus indicate that the lack of FetuA protection may result in vulnerability to leukaemogenesis. This passage has been added to the revised manuscript, and the data are presented in the new Fig. 6.

Referee #2 (Remarks to the Author):

In this manuscript the authors study the role of the fetal liver as a HSPCs niche. They show that HSPCs from the early fetal liver at the E12.5 are more susceptible to genotoxic stress than HSPCs from the placenta at E12.5 or HSPCs from the late fetal liver at E16.5. They show this unique feature from E12.5FL HSPCs both ex vivo and in vivo. They also identify an intrinsic higher burden of R-loops in replicating HSPCs at the fetal liver stages and link this increased R-loop formation to an increased mutation risk at developmental genes. The authors propose that the fetal liver microenvironment provides a genome protective environment but that the early fetal liver lack this genome protection ability. They identify the paracrine secretion from hepatocytes of FetuA and the implication of the FetuA-Tlr4-MyD88-bZip-Blm axis as the key mechanism providing HSPCs genome protection by inducing the transcription of the Blm helicase who help process R-loops, thereby reducing their toxicity. Lastly, they show that this mechanism identified in mice embryonic development is preserved in human development. Altogether, the mechanism identified in this manuscript provides new important information on hematopoietic embryonic development and in leukemia development. I think that this manuscript meets the requirement for publication in Nature but I have several questions that should be addressed.

Response: Many thanks to the referee for the encouraging comment. All concerns have been seriously addressed, as detailed below.

Major points

(1) The authors conclude that the microenvironment of the fetal liver provides the protective advantage, but this initial conclusion stems from ex vivo experiments where HSPCs were harvested and isolated in single-cell suspensions and at line 106 the authors conclude that "[…] the microenvironment within the early fetal liver does not provide newly colonized HSPCs with sufficient protection." These cells are separated from their microenvironment so this conclusion is difficult to make from these initial experiments. It is conceivable considering the later findings of the manuscript on the signaling pathway and transcriptional regulation of Blm and the other in vivo data presented, but the manuscript would gain quality from preserving a better linearity with conclusions that are not affected from later results.

Response: The manuscript has been edited accordingly.

(2) The protective role of secreted FetuA raises several unanswered questions. If HSPCs are more prone to genotoxic agents and need FetuA from the hepatocytes to protect them, this would mean that once they migrate to the bone marrow, they still need the FetuA protection. Does the perinatal BM HPSCs also depend on FetuA for its genome protection? Do the fetal liver stop to produce FetuA explaining the need to migrate again? Why do HSPCs migrate in early fetal liver if the early fetal liver does not protect them?

Response: We agree that these questions are interesting but, in our opinion, beyond the scope of this paper. To address these questions, we measured the FetuA concentration and its genome-protective role in perinatal BM. Indeed, perinatal BM HPSCs also depend on FetuA for genome protection, and these data have been added to the manuscript (newly extended data Fig. 5j-l). HSPCs begin to migrate from the foetal liver at E16.5, but FetuA remains at a high concentration (newly extended data Fig. 5f). Thus, neither FetuA expression nor its genome-protective role could be a driver of HSPC migration. The exact drivers should be identified, but this is beyond the scope of this paper.

(3) Fig2b-c. These results support the importance of hepatocytes for protecting HSPCs from genotoxic agents as in iDTA+/- mice, E16.5 HSPCs have a sensitivity to Etoposide similar to ex vivo/in vivo E12.5 HSPCs. When comparing E16.5 HSPCs in Fig 1e and Fig 2c (both in vivo quantification of Eto-induced y-H2A.X), the percentage of y-H2A.X negative cells is similar but when compared to E16.5 HSPCs in Extended data Fig 2b (ex vivo quantification of Eto-induced y-H2A.X), there is a 1.5x diminution in y-H2A.X positive cells. Does this suggest that ex vivo HSPCs are less susceptible to genotoxic stress than in situ HSPCs?

Response: Thank you for reviewing the paper so carefully. Different dosages of Eto were utilized in the *ex vivo* and *in vivo experiments*. It is unsurprising that the severity of DNA damage varies between these settings. This discrepancy does not suggest that HSPCs are less susceptible to genotoxic stress *ex vivo* than *in vivo,* in our opinion.

(4) Fig3. In Fig3b, Co-SHI media from E12.5 and E16.5 hepatocytes were used and tested but in the following panels and text, it is not specified if the protein enriched in Co-SHI comes from E12.5 or E16.5 Co-SHI or both. I would expect it to be E16.5 and these are protein found to be lacking from E12.5 FL but I think it should be specified as depending on the source of the analyzed

Co-SHI would alter the conclusions.

Response: Given that Co-SHI media conditioned from either E12.5 or E16.5 hepatocytes conferred similar genome protection on HSPCs (newly extended data Fig. 5c, d), Co-SHI media conditioned from E16.5 hepatocytes were used for mass spectrometry analysis because E16.5 hepatocytes were easier to obtain. This selection does not alter the conclusion, which has been clarified in the revised manuscript (Lines 192-197). The lack of factors in E12.5 FL was due to the relatively low number of hepatocytes.

(5) Fig3b. As E12.5 Co-SHI decrease the percentages of γ-H2A.X cells compared to SHI, why did the authors decide to look at secreted proteins that are lacking at E12.5? Most of the changes displayed in Fig3b are between SHI and Co-SHI, not between E12.5 and E16.5 Co-SHI.

Response: Hepatocytes isolated from either E12.5 or E16.5 foetal livers secreted proteins, including FetuA. However, the number of hepatocytes is relatively low in the E12.5 foetal liver, and the concentration of secreted proteins is thus too low to provide genome protection to HSPCs at early stages. Please also refer to our response to question 4.

(6) The authors use S9.6 staining for R-loop quantification by immunofluorescence. As the increase in R-loops is an important result in the paper and specificity of the S9.6 antibody has often been debated in the field, I would like to see the same results with a complementary method that is not relying on S9.6 antibody (e.g. GFP-dRNH1, https://doi.org/10.1083/jcb.202101092).

Response: Thank you for the comment and the recommendation. We utilized two specific antibodies, the recommended dRNH1[2] and 2 x HBD[3], for these experiments and obtained consistent results (new Fig. 4h, i, and extended data Fig. 7). The experiments were also performed on more enriched long-term hematopoietic stem cells (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs) (extended data Fig. 7e, h).

(7) Fig 4g and Extended data Fig8: In Extended data Fig 8 it is hard to see if the S9.6 signal is nuclear or outside of the nucleus, especially in E12.5 PL HPSCs. Can the author maybe use the same representation as in Fig4g, representing the nuclei by showing the DAPI signal outline instead of the DAPI signal itself. Still, from Fig 4g, a major part of the signal seems to be outside of the DAPI border, is it due to non-specific nuclear membrane binding of the antibody or is it still inside the nuclei? In Fig4h, does the quantification shown represent S9.6 signal inside the DAPI region only or take in this perinuclear region also?

Response: These experiments were performed using the recommended specific dRNH1 antibody. The same representation was used for the two panels in the new Fig. 4h and the new Extended Data Fig. 7a-d. The DAPI borders are marked. The signals outside of the DAPI border are not due to nonspecific binding of the antibody; they are in the plasma[4]. The amount of IF signal inside the nuclei was quantified and is presented (new Fig. 4i; Extended Data Fig. 7b, d, f, h).

(8) Fig5e,f: I don't think it is possible to conclude on accelerated gene transcription based on those EU data. The data shown only display % of EU+ cells which display a reduction in the transcriptionally silent cell population. The author could quantify the intensity of EU incorporation and conclude if there is an increase in transcription output, but I would still refrain using the terms "accelerated gene-transcription" as it sounds like the authors are concluding on the speed of RNA polymerases. The level of EU incorporation may also be heavily influenced by

RNA Polymerase I transcription which accounts for a large proportion of newly synthesized RNA per cell.

Response: We agree with this comment and have edited the manuscript accordingly.

(9) Extended data Fig8. Considering that 40% of E12.5 PL HSPCs are not incorporation EU (Fig5e, f), it is difficult to reconcile the image shown and the quantification shown here. Is this a representative image of E12.5 PL HPSCs? Shouldn't we see only 60% of E12.5 PL HPSCs showing S9.6 signal? Also, does the quantification in b have excluded s9.6 negative cells? I do not think so because it seems that there is a decent amount of datapoints in the different samples showing absence of staining, but if negative cells have not been removed, it doesn't seem to show that 40% of the cells are negative based on this graph.

Response: As explained in the responses to questions 6 and 7, using the specific dRNH1 antibody for the IF assay resulted in consistent data and figure panels. Representative images are shown. Approximately 60% of E12.5 PL HPSCs exhibited a positive dRNH1 signal in the nucleus (newly extended data Fig. 7a, b). Indeed, dRNH1 signal-negative cells in the nucleus were included in the quantification shown in the statistical graph (new Extended Data Fig. 7b). However, the signal outside of the nucleus was not quantified because it is beyond the scope of this paper[4]. Please also refer to our response to question 11.

(10) Fig6: the R-loop quantification suggests a small decrease in R-loops when comparing E12.5FL and E16.5FL while in extended data fig8, the quantification of R-loops show a level of R-loops in E16.5FL even lower than E12.5PL. Why is there such a difference in the levels of R-loops at E16.5 as evaluated by these two approaches?

Response: When specific dRNH1 and 2xHBD antibodies were used, the IF data were consistent with the cut-tag sequencing data. These data are presented in the new Extended Data Figures 7a-d.

(11) Fig6: Has the R-loop quantification by cut-and-tag of E12.5PL been normalized in any way to address the fact that there exists a large amount of transcriptionally silent cells at this stage? Can the lower R-loop levels at that stage be explained by a lower population of transcribing cells (instead of transcribing cells showing fewer R-loops)?

Response: The cut-and-tag assay was performed on bulk cells, and the data were not normalized to distinguish between transcriptionally silent and active cells. Certainly, a lower population of transcribing cells contributed to the lower R-loop levels in E12.5PL cells. Nevertheless, this does not compromise the major point of the paper that foetal hepatocytes secrete FetuA to protect the HSPC genome by preventing pathogenic R-loop accumulation in the foetal liver, and this protection is lacking in the early developmental stage of the foetal liver.

Minor points:

Intro: basic description of fetuin-A would be nice.

Fig1: the abbreviation AGM is not explained

Fig 2a. Are the fluorescence histogram values standardized between the panels? It seems that between the c-kit/albumin panels and the merge panels that the values are not the same (see intensity of c-kit in E16.5 FL for example)

Reponses: These minor points have been addressed.

Referee #3 (Remarks to the Author):
In this work, the authors investigated the genomic integrity of fetal HSPCs and the genome-protective mechanism from hepatocytes. Firstly, by assessing the fetal HSPC sensitivity to genotoxic agents, they found that early fetal liver (E12.5-E14.5) HSPCs exhibited more severe DNA damage and shorter latent period of induced leukemia than late fetal liver (E15.5-E18.5) HSPCs. Then, to detect the genome protection effect of HSPC from microenvironment hepatocytes, they analyzed the developmental dynamics between hepatocytes and HSPCs using Alb-cre$^{+/-}$Rosa-iTomato$^{+/-}$ line, and hematopoietic phenotype after hepatocyte depletion using Alb-cre$^{+/-}$Rosa-iDTA$^{+/-}$ line, respectively. As a result, the development of hepatocytes lags than that of HSPCs. Besides, the cell numbers and genome integrity of HSPCs were reduced after hepatocyte depletion. Mechanistically, by performing proteomic assay and ex vivo culture assay, they found that FetuA secreted by hepatocytes can protect the genomic stability of HSPCs from genotoxic agents. Moreover, HSPCs in E16.5 FetuA-/- fetal liver exhibited higher sensitivity to genotoxins than that in sibling mice. Finally, by integrating RNA-seq and ATAC-seq analysis, their results showed that FetuA-Tlr4-Myd88-bZIP pathway is involved in genomic protection of HSPCs by interfering R-loop formation. Overall, this work detected the genome integrity of HSPCs during embryonic development and revealed the genome protective mechanism, i.e. FetuA secreted by hepatocyte protected HSPC by preventing R-loop accumulation.

Response: We are very grateful to the reviewer for reading the manuscript and summarizing the story. We have substantially addressed his/her questions.

Comments

(1) To detect DNA damage by immunofluorescence staining, the authors used the sorted fetal liver HSPCs by FACS and co-stained with γ-H2A.X. However, flowcytometry would cause some injury to cells. the immunofluorescence staining of whole-mount tissues should be provided, which would be better to reflect DNA sensitivity of HSPC in native niche.

Response: Immunofluorescence staining of whole-mount tissues was indeed performed. The data are presented in Fig. 1d, e; Fig. 2i, j; Fig. 3i, j, l, m; Extended Data Fig. 2d-e; Extended Data Fig. 5k, l, n, o, q, r in the revised manuscript.

(2) To test the regulatory role of hepatocyte to HSPCs, the authors manipulated hepatocytes by using Alb-cre$^{+/-}$; Rosa-iDTA$^{+/-}$ mice. However, hepatocyte is an important cell type in fetal liver and the depletion of hepatocyte may cause a severe developmental disability to fetal liver. How to exclude the regulatory role of other niche components for HSPC development in Alb-cre$^{+/-}$ Rosa-iDTA$^{-/-}$ mice, in the absence of hepatocytes? In addition, the DTA approach is very likely

toxic which would cause a systemic effect to the whole environment, rather than just affecting hepatocytes alone, therefore the functional specificity of the DTA approach as well as of the hepatocytes should be considered.

Response: In the foetal liver, hepatocytes are essential for HSPC development. Recently, vascular vessel endothelial cells ($CD144^+$) and perivascular cells ($Nestin^+$) were proven to be important niche cells for HSPC development in the foetal liver[5]. In this paper, we showed that the number of hepatocytes decreased by approximately 50% in Alb-$cre^{+/-}$Rosa-$iDTA^{+/-}$ mice at E16.5 compared with control Alb-$cre^{+/-}$Rosa-$iDTA^{-/-}$ mice (new Fig. 2e, f). To answer the reviewer's questions, we measured the possible alterations in vascular vessel endothelial cells ($CD144^+$) and perivascular cells ($Nestin^+$). No visible difference was observed in these niche components between Alb-$cre^{+/-}$Rosa-$iDTA^{+/-}$ mice and control Alb-$cre^{+/-}$Rosa-$iDTA^{-/-}$ mice (new Fig. 2g, h), indicating that the regulatory effect of hepatocytes on HSPCs is unlikely to occur through these niche components despite the lack of a functional assay. Nevertheless, the data shown in Fig. 3 provide compelling evidence that hepatocytes confer genome protection to HSPCs via the paracrine factor FetuA. Cre-inducible diphtheria toxin fragment A (DTA) mice are broadly used to specifically deplete Cre-expressing cells and thus study their functions. Once the DTA-expressing cells die, the released DTA is unable to enter other cells lacking its partner fragment B[6,7]. Therefore, the functional specificity of the Alb-cre-DTA approach is assured, and the functional specificity of hepatocytes has been proven by the data presented in Fig. 3.

(3) In this study, the authors only used c-Kit labeling HSPCs on fetal liver sections. The authors need to use more HSPC markers to confirm this result.

Response: HSPCs were identified with additional markers ($CD150^+Lin^-CD41^-CD48^-$) *in situ*. The data are presented in the new Extended Data Fig. 2d, e.

(4) By performing proteomic assay and co-culture, the authors found that hepatocytes secrete D factors FetuA to protect HSPC genome integrity. Can FetuA rescue the HSPC phenotype via intra-placental injection of recombinant FetuA in Alb-$cre^{+/-}$Rosa-$iDTA^{-/-}$ pregnant mice?

Response: Indeed, intraplacental injection of recombinant FetuA can rescue the HSPC phenotype in Alb-$Cre^{+/-}$Rosa-$iDTA^{-/-}$ pregnant mice. These findings are presented in the new Extended Data Fig. 5p-r.

(5) In terms of the mechanism, the authors proposed that FetuA–Tlr4–Myd88–bZip–Blm axis signaling protected HSPC by preventing R-loop amassment. However, the functional validation for the signaling relationship is weak, especially biochemical experiments. Besides, it is unclear how these signaling coordinately regulate HSPC genomic stability.

Response: In our previous submission, we demonstrated that FetuA binds to its receptor Tlr4, inducing the formation and activation of the Tlr4-Myd88 complex. This activation leads to an increase in the expression and phosphorylation of the transcription factor bZip, which in turn upregulates the transcription of the helicase Blm, reducing R-loop accumulation and consequent

DNA damage in HSPCs. To further validate this signalling pathway, we conducted additional experiments. Western blot assays confirmed that FetuA treatment enhances Blm expression, an effect that was nullified by using the bZip inhibitor SR11032 (Fig. 4c, g and Extended Data Fig. 6g, h). Cut and tag-seq assays confirmed that bZip binds directly to the Blm gene promoter (Extended Data Fig. 6d, e). Collectively, these results strongly demonstrate that FetuA protects the HSPC genome by activating the Tlr4–bZip–Blm cascade, thereby preventing R-loop accumulation (Fig. 4l).

(6) Genotoxic agents caused DNA damage for fetal liver HSPCs, how about the cell fate of HSPC after Eto treatment? Do cell death, cell cycle arrest or others occur? More phenotypical validation should be provided.

Response: To address this issue, pregnant mice at E12.5 or E16.5 were treated with Eto and monitored until delivery to their offspring to evaluate whether the treatment led to phenotypical, functional, chromosomal, and leukaemia-vulnerable alterations in HSPCs detectable postnatally after their migration into the bone marrow (Fig. 1f). Initially, the proportion of 3-week-old BM LSK populations was greater in mice treated with Eto at E12.5 than in those treated at E16.5 or in untreated controls (Fig. 1g, h). These cells exhibited enhanced clonality, as determined by colony-forming cell (CFC) assays (Fig. 1i, j). Whole-genome sequencing revealed that 3-week-old BM HSPCs from mice treated at E12.5 harbored more mutations than those from mice treated at E16.5 (Fig. 1k). Furthermore, mice treated at E12.5 exhibited greater susceptibility to leukaemia induction than did those treated at E16.5 (Figs. 1l-o). The detailed results are presented in the revised manuscript.

RERFERENCE

1. Nguyen, Giang H., Dexheimer, Thomas S., Rosenthal, Andrew S., Chu, Wai K., Singh, Dharmendra K. *et al.* A Small Molecule Inhibitor of the BLM Helicase Modulates Chromosome Stability in Human Cells. *Chemistry & Biology* **20**, 55-62, (2013).
2. Crossley, M. P., Brickner, J. R., Song, C., Zar, S. M. T., Maw, S. S. *et al.* Catalytically inactive, purified RNase H1: A specific and sensitive probe for RNA–DNA hybrid imaging. *Journal of Cell Biology* **220**, e202101092, (2021).
3. Kang Wang, H. W., Conghui Li, Zhinang Yin, Ruijing Xiao, Qiuzi Li,Ying Xiang, Wen Wang, Jian Huang, Liang Chen, Pingping Fang, Kaiwei Liang,. Genomic profiling of native R loops with a DNA-RNA hybrid recognition sensor. *Sci Adv* **7**, eabe3516, (2021).
4. Crossley, M. P., Song, C., Bocek, M. J., Choi, J.-H., Kousouros, J. N. *et al.* R-loop-derived cytoplasmic RNA–DNA hybrids activate an immune response. *Nature* **613**, 187-194, (2022).
5. Khan, J. A., Mendelson, A., Kunisaki, Y., Birbrair, A., Kou, Y. *et al.* Fetal liver hematopoietic stem cell niches associate with portal vessels. *Science* **351**, 176-180, (2016).
6. Collier, R. J. Diphtheria toxin: mode of action and structure. *Bacteriol Rev* **39**, 54–85, (1975).
7. Jr, A. M. P. Diphtheria toxin. *Annu Rev Biochem* **46**, 69-94, (1977).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have satisfactorily addressed all of my concerns.

Referee #2 (Remarks to the Author):

In this revised manuscript, the authors provide an extensive amount of additional experiments that convincingly address the majority of my initial concerns and criticism. Overall, the revised manuscript makes a strong case that HSPCs from the early fetal liver at the E12.5 are more susceptible to genotoxic stress that is derived from the lack of FetuA levels that reduce genotoxic R-loop accumulation via a characterized signaling pathway involving the BLM helicase. The statistical tests and quantifications have been unified (e.g yH2AX quantifications) and appear appropriate to this reviewer. Importantly, the authors have improved their R-loop detection method by using dRNH1 and 2xHBD probes and show consistent results with these complementary approaches. Overall, I'm fully supportive of publication if one minor issue can be resolved:

line 277-278: The statement 'Considering that pathogenetic R-loops are primarily formed by collisions between DNA replication and gene transcription' needs to be revised as this is not established in the field. There are many R-loops formed outside of S-phase/DNA Replication that also show pathogenic potential.

Referee #3 (Remarks to the Author):

Although the authors addressed several of my previous concerns, I found that some important issues still remained in the revision, thus being problematic for its consideration at this stage.

1. For the major comment on the immunofluorescence staining of whole-mount tissues, the authors have examined the DNA damage with CD150+Lin−CD41−CD48− labeling HSPCs and observed similar trend (ETO treatment induced DNA damage more easily in HSPCs in the early fetal liver stage). However, the markers used for labeling HSPCs in this text are very different, such as, ckit, LSK and CD150+Lin−CD41−CD48−. It is desirable to use well established HSPC markers in the field throughout the paper either in mice or in humans, otherwise it seems that the authors used a cherry-picking approach to support their claim of the genome-protective role of hepatocytes on HSPCs. Moreover, the treatment of ETO is not specific for HSCs and may cause a severe DNA damage for whole fetal liver cells. It is difficult to exclude its non-direct effect on HSPCs.

2. For the major comment on the regulatory role of hepatocyte to HSPCs by using Alb-cre+/−; Rosa-iDTA+/− mice, the authors have detected the vascular cells and perivascular cells and found no visible difference in these niche components. However, the authors only detected vascular area and perivascular area. It remains unclear whether fetal liver showed impaired development and these vascular niche components exhibit normal biological function upon treatment.

3. More importantly, the authors employed Alb-Cre; ROSA26-LSL-DTA mice to show that hepatocytes are required for preventing DNA damage in the HSPCs. Given that the genome-protective role of hepatocytes is developmental stage-dependent, the temporally-specific depletion of Alb+ hepatocytes (by using Alb-CreERT2; ROSA26-LSL-DTA mice) is a more suitable strategy.

4. What are the expression and protein levels of FetuA in different FL cell types? If the authors aim to highlight the genome-protective role of hepatocyte-derived FetuA, the data on hepatocyte-specific FetuA deficiency is more convincing.

Author Rebuttals to First Revision:

The point-by-point response

Referee #3 (Remarks to the Author):

A*lthough the authors addressed several of my previous concerns, I found that some important issues still remained in the revision, thus being problematic for its consideration at this stage.*
Response: Thank you very much for your encouraging comment and remaining concerns for the paper revision. Following the editor's instruction we humbly provide the responses below to address your concerns and thus revise the manuscript. The changes in the revised manuscript are marked in red

1. For the major comment on the immunofluorescence staining of whole-mount tissues, the authors have examined the DNA damage with CD150+Lin−CD41−CD48− labeling HSPCs and observed similar trend (ETO treatment induced DNA damage more easily in HSPCs in the early foetal liver stage). However, the markers used for labeling HSPCs in this text are very different, such as, ckit, LSK and CD150+Lin−CD41−CD48−. It is desirable to use well established HSPC markers in the field throughout the paper either in mice or in humans, otherwise it seems that the authors used a cherry-picking approach to support their claim of the genome-protective role of hepatocytes on HSPCs. Moreover, the treatment of ETO is not specific for HSCs and may cause a severe DNA damage for whole foetal liver cells. It is difficult to exclude its non-direct effect on HSPCs.

Response: We agree with you on that it is desirable to use well established HSPC markers in the field throughout the paper. We indeed made great efforts to do so. In the first version, we used LSK for flow-sorting (Extended Data Fig. 1a), flow-cytometric analysis (Fig. 5a-f) and c-kit for tissue-staining *in situ* throughout the paper (Fig. 1d, e, Fig. 2c, l, m, Fig. 3i, j, l, m, Extended Data Fig. 5o, p); in the last revision, according to the reviewers' suggestion, we utilised slam gene expression (CD150 and CD48) to distinguish HSCs from progenitors in flow-sorting (Extended data Fig. 2a), flow-cytometric analysis (Extended Data Fig. 8a, b) and tissue staining *in situ* (Extended data Fig. 2e, f,). Consistent results were obtained in these experimental settings (Fig. 1b-e and Extended Data Fig. 2b, c, e, f; Fig. 4h, i and Extended Data Fig. 7g, h; Extended Data Fig. 7a, b and Extended Data Fig. 7e, f; Fig. 5a-d and Extended Data Fig. 8a, b), and HSPCs are thus referred to as LSK enrichment unless further specified in the paper. These have been clarified in the revised manuscript (Lines 126-128).

Indeed, Eto-treatment caused a DNA damage for whole foetal liver cells including HSPCs and non-HSPCs (Extended Data Fig. 2d), and it is difficult to fully exclude the non-direct effect

on HSPCs. Nevertheless the results of our functional experiments *ex vivo* could convince the direct effect of Eto on HSPCs (Fig. 1b, c, Extended Data Fig. 1b, c, Extended Data Fig. 2b, c). These have been clarified in the revised manuscript (Lines 116-118, 124-125).

2. For the major comment on the regulatory role of hepatocyte to HSPCs by using Alb-cre+/−; Rosa-iDTA+/− mice, the authors have detected the vascular cells and perivascular cells and found no visible difference in these niche components. However, the authors only detected vascular area and perivascular area. It remains unclear whether foetal liver showed impaired development and these vascular niche components exhibit normal biological function upon treatment.

Response*:* Additional immunofluorescence assay on Sca-1 was performed, no obvious difference on arterioles was observed in between Alb-cre$^{+/-}$; Rosa-iDTA$^{+/-}$ mice and the control mice, the same as seen in the assays on CD144 and Nestin (Fig. 2f, i). Even so, we have to acknowledge that more subtle modifications of the niche, and so indirect effects on HSPCs via their niche, cannot be fully ruled out. Nevertheless, the results of our functional assays *ex vivo* could demonstrated that hepatocyte provide genome protection for HSPCs in a paracrine manner (Fig. 3b, c and Extended Data Fig. 5a-d). These have been clarified in the revised manuscript (Lines 171-173, 175-178).

3. *More importantly, the authors employed Alb-Cre; ROSA26-LSL-DTA mice to show that hepatocytes are required for preventing DNA damage in the HSPCs. Given that the genome-protective role of hepatocytes is developmental stage-dependent, the temporally-specific depletion of Alb+ hepatocytes (by using Alb-CreERT2; ROSA26-LSL-DTA mice) is a more suitable strategy.*

Response: We agree that the temporally-specific depletion of Alb^{+} hepatocytes (by using Alb-CreERT2; ROSA26-LSL-DTA mice) would be a more suitable strategy; the limitations of our current depletion strategy (i.e. possible effects earlier in development) should be considered. Nevertheless, the results of our comparative functional assays across developmental stages (Fig. 1b-e, Extended Data Fig. 1b-e, Extended Data Fig. 2b, c, e, f and Extended Data Fig. 5c, d) and lineage tracing observation (Fig. 2a-c) could demonstrate that hepatocytes provides genome-protection for HSPCs in a paracrine manner, and the protection is lacking in the early foetal liver due to the low number of hepatocytes. These have been clarified in the revised manuscript (Lines 178-182).

4. What are the expression and protein levels of FetuA in different FL cell types? If the authors aim to highlight the genome-protective role of hepatocyte-derived FetuA, the data on hepatocyte-specific FetuA deficiency is more convincing.

Response: Additional double immunofluorescence stainings were performed. The results showed the presence of FetuA inside hepatocytes (E-cadherin^{+}) and absence inside haematopoietic (CD45/Ter119^{+}), endothelial (Scal-1/CD144^{+}) and mesenchymal (Nestin^{+}) cells (Extended Data Fig. 5g). Hence, FetuA is primarily expressed by hepatocytes and so considered as a hepatokine, consistent with the published findings[1,2]. Even so, we have to agree that a contribution of FetuA from cells other than hepatocytes cannot be fully ruled out in the absence of tissue-specific deletion of FetuA. Nevertheless, the results of our functional assays *ex vivo* (Fig. 3b-g) and rescue experiments *in vivo* (Fig. 3k-m and Extended Data Fig. 5n-s) demonstrated that hepatocyte-secreted FetuA provide genome protection for HSPCs. These have been clarified in the revised manuscript (Lines 211-215, 233-236).

1 Jirak, P. *et al.* Clinical implications of fetuin-A. *Advances in Clinical Chemistry* **89**, 79-130, (2019).

2 Icer, M. A. & Yıldıran, H. Effects of nutritional status on serum fetuin-A level. *Critical Reviews in Food Science and Nutrition* **60**, 1938-1946, (2019).