Dear editor and reviewers,

Thanks for all the comments and suggestions. The modifications that we made in the manuscript were changed to red color and were listed below:

**Editor’s suggestions**

**1) The protocol is >3 pages and is not highlighted. The authors will need to highlight 2.75 pages of material to be filmed.**

Following the requirement of the journal, we now have highlighted 2.75 pages of material for filming in the revised manuscript.  **2) Figures 1-5 are not Results; these should be changed to Supplemental Figures, and Figs. 6-10 should be renumbered appropriately.**

As suggested, we moved figures 1-5 to supplemental figures and renumbered figures 6-10 accordingly.   
 **3) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.**

Thanks for the suggestion, we have checked and corrected the spelling and grammar errors.   
  
**Reviewer #1’s suggestions**  
**Major Concerns:  
I did not find major flaws in the paper. It should be mentioned that DESeq is not the only application available for differential gene expression analyses. Cuffdiff could be explained. Alternative apps may be explained. The advantages of DEseq could be detailed some more here.**

We appreciate reviewer’s comments on the manuscript. Following the reviewer’s suggestion, we have explained different methods for differential gene expression analyses including DESeq and Cuffdiff in the result part of the revised manuscripts. In addition we also addressed the advantages of DESeq. It is true that DESeq is not the only application available for differential gene calling. We chose DESeq because it is a widely used count based method for DE gene calling from RNA-Seq data. In our experience, cuffdiff gives significantly different numbers of DE genes among different versions of software packages. The count based method DESeq is more stable in this sense.

**Minor Concerns:  
1 The RNA manipulation steps (2.2.2) could be provided in more detail. The make of the machines used for the analyses should be provided.**

Following the reviewer’s advice, the step 2.2.2 is provided in more detail. The machines used for analyses have already been listed out in the material part. It is not allowed to write any commercial names (e.g. NanoVue Plus spectrophotometer) of instruments in the text.

**2 The Lineage antibodies used for negative selection should be listed.**

Lineage antibodies used for negative selection are now listed in the step 1.3.3. The antibodies were contained in the Lineage Cell Depletion Kit and the kit has been mentioned in the material part.

**3 Fig 9 legend, delete FU, it is not applicable to base pair. Editor’s note: Please write the legends in a new sentence.**

Thanks for the suggestion; FU has been deleted and a new legend has been written.

**Reviewer #2’s suggestions**  
  
**Major Concerns:  
1) As is, the portion of the manuscript discussing culture of EML cells is not novel nor particularly useful beyond the study described. A brief discussion of the potential use of EML cells as a model for differentiation or gene function (e.g. shRNA knockdown) coupled with RNA-seq would broaden the utility of this protocol.**

Following the reviewer’s suggestion, we added a discussion of using EML cells as a model for differentiation and gene function study coupled with RNA-sequencing technology in the introduction and the discussion.

**2) As the most useful part of the manuscript is the description of the RNAseq process, there should be more discussion about enrichment of mRNA from total RNA (Ribo minus vs. PolyA enrichment), how to decide what kinds of reads to generate (paired end vs. single end; 50nt vs. 75nt), and different sequencing platforms. In fact, the sequencing platform is not even discussed.**

Thanks for the reviewer’s comments. We described and compared different sequencing platforms in the introduction part. In addition, we added discussions about different methods for enrichment of mRNA and the criteria of choosing different kinds of reads and read lengths in the result session.

**The protocol jumps from the cDNA library to uploading data files. It seems as this protocol is intended for Illumina based sequencing. But the readers should be made aware that other sequencers exist (and may be the only choice for the reader) and that they will require different adaptor kits and possibly different means of data analysis.**

We added the description and comparison of different sequencing platforms in the introduction. In addition we explained in the protocol session that the library preparation part described in the paper is for Illumina sequencing platform.

Minor Concerns:  
**1) In the introduction, CD34+ cells are referred to as self-renewing population. However, in mice, the long-term reconstituting HSCs are Kit+, Sca+, CD150+, and CD34-. Does this population exist in EML cells? Moreover, it can be argued that Sca+, Kit+, CD34+ cells are multipotent progenitors with limited self-renewing potential.**

The reviewer is correct. The closest normal proximity of Lin-CD34+ cells are short-term (ST)-HSC or multipotent progenitors (MPP).

**The mechanisms of self-renewal in MPPs may be different than those of true, quiescent HSCs.**

We agree with the reviewer that the mechanisms of self-renewal in MPPs might be different from that of quiescent HSCs. To address this question, we are in the process of performing experiments to test the functions of candidate key transcription factors in human HSCs.

**The expression of c-Kit should also be briefly mentioned.**

Following reviewer’s suggestion, we added the expression of c-Kit in EML cells in the introduction part of the manuscript.

**2) The citation for the derivation of the EML cell line is not the original paper describing the EML. Please reference Tsai, S., et al (1994) Genes & Development.**

A citation for the original paper has been added according to the reviewer’s suggestion  
  
**3) For culture of EML cells, is there variation between lots of horse serum used to culture the cells in terms of proliferation and differentiation?**

According to our experience, different lots of horse serum had no significant influence on EML cell proliferation and differentiation

**In the discussion, it is mentioned that the cells need to be treated with "gentle operation." This should be stated in the procedure.**

Following the reviewer’s suggestion, we added “gentle operation” into the step 1.2.2.

**The warning that the ratio of CD34+ to CD34- is critical is too vague-- at what point should the user discard the cultures (e.g. >60% CD34-).**

The percentage at which EML cell should be discarded is still debatable. We normally discard the culture after passaging for 10 generations.

**Also, how many cells are required to get RNA in the 0.1-4ug range? Is this before or after mRNA enrichment?**

We usually use 105 cells for extracting total RNA and get about 1-2 ug RNA. The 0.1-4 ug RNA refers to the amount of total RNA before mRNA enrichment. We have already mentioned this information in the step 2.2.1.   
  
**4) A table of the software needed would be useful for someone who has no prior experience with analyzing this kind of data.**

As suggested, we added a table (Table 2) of the software used in this manuscript.   
  
**5) Would be useful if described how to generate a file to use as a custom track on the UCSC Genome Browser.**

Thanks for the reviewer’s suggestion. We have added a description about how to generate .bigwig file in the protocol part. There is excellent information on how to load custom track on UCSC Genome Browser website. We added a reference link to the website, because the detailed information on this would be too long for the manuscript.   
**6) Will the commands in the figures be legible? The font is very small in the proof.**

We have changed the commands to larger font size.  
  
**Additional Comments to Authors:**  
**There are many grammatical errors in the text. Please proofread carefully.**

We are sorry for the grammatical errors. We proofread the manuscript and made corrections.