Your manuscript JoVE52262R1 'Deriving Retinal Pigment Epithelium from Induced-Pluripotent Stem Cells by Different Sizes of Embryoid Bodies' has been peer-reviewed and the following comments need to be addressed.

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors investigated how EB sizes impact their iPSC differentiate into RPE. They concluded the optimal range of the cell number per EB is 500 to 3000.

Major Concerns:

Figures 1-5 lack quantitatively data. Statistical analysis has not mentioned in the text as well as legends. This may become an issue if the comparison of EB sizes is the focus of this manuscript. Statistical analysis and error bar is added for figure 5.

Figure 4 does not have isotype or unstained control. Isotype staining was done during the experiment for negative control. It was not included for publication.

Minor Concerns:

Figure 1 missed A, B, and C. Corrected.

Reviewer #2:

Manuscript Summary:

The manuscript describes methods for the differentiation of retinal pigmented epithelial cells from iPS cells using a variation of the embryoid body technique.

Major Concerns:

No major concerns.

Minor Concerns:

1. Throughout the manuscript, "media" should be "medium" unless the plural is meant (e.g. a mixture of two kinds of media). Proper changes are made.

2. "induced-pluripotent stem" should not be hyphenated. Changes are made.

3. Line 134: which supplement? Which basal medium? Feeder free Stem Cell culture medium comes in two components, basal medium and growth factors supplement, because of their different storage conditions.

4. Line 138: which commercial medium? Aggrewell medium is the EB formation media as described in the reagents table. It was not included in the text because of the editorial policy.

5. Line 138: which ROCK inhibitor? It is rho-associated, coiled-coil containing protein kinase (Rock) inhibitor (Y-27632). It’s added in the text.

6. Line 141: this should be KSR = knockout serum replacement. We have changed it.

7. Line 164: does this really mean "Prepare protein matrix from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells"? It means Matrigel presumably - if so, which type? “Matrigel” was removed as per the request of the editor. It’s mentioned in the reagent’s table.

8. Line 183: "feeder-free stem cell culture media" - which medium is this? It’s mTeSR1 medium and is described in the reagent’s table.

9. Line 265 onward: There seems to be a section missing from the protocol. This section deals with plating the EBs, using "ultra-low adherence" plates - so they don't attach? But the next part of the protocol (line 360) refers to cutting around pigmented colonies. Where do these colonies come from? They are recognized by pigmentation presumably - but how is this recognized in practice - and the photos don't really show it clearly. Step 5.1 is rewritten. After these EBs were plated on a matrigel coated six well culture plate, black pigmented colonies appeared after 4 weeks. These polygonal and pigmented colonies can be recognized easily under the stereomicroscope and can be isolated manually for further culture.

10. Line 375: is this also Matrigel? Protein matrix used in this paper is matrigel. “Matrigel” was not used as it is a commercial name. It’s denoted in the reagent’s table.

Reviewer #3:

Why ultra-low adherent plates in step 5.1. Is it meant to be a suspended differentiation culture. But the images for differentiation culture shown (fig 3, 7) appears to be adherent cultures, probably derived by plating EB and culturing with retinal differentiation medium. And no mention about the culture substrate/matrix used. We have modified the step 5.1.

In 9.1, no mention about the days after differentiation RPE, cell isolation is done for enrichment.

Time point is added in the text.

In 9.4, no mention about the culture matrix used for sub culture. Change has been made.

In results, the authors quote that 200 cell EBs had developed astrocyte and fibroblast morphology (line 386) (Figure 3A). Deffinitely they look fibroblastic but unless it express a specific marker it is not right to comment on astrocytic differentiation. Astrocyte has been removed.

It is better if they show representative FACS profiles for Fig 6A. Mitf data does not have error bars. Also, Pax6 being a common marker for CNS and eye field specification, it is not ideal to use it for

quantifying RPE differentiation efficiency. RPE-specific markers like Bestrophin and RPE 65 will provide better evidence. Same is true for IHC examinations. ZO-1 is expressed by many epithelial cells. MITF staining is weak, though it appears to be nuclear (fig 4). Though PAX6 is a common marker for CNS and eye field specification and Mitf is an early marker for RPE , expression of both PAX6 and Mitf is usually used to confirm the RPE phenotype.

RT-PCR data does not show much of a difference between 200/500/3K/15K cell EBs and it is surprising to find RPE65 expression at d17 itself (Figure: 5). Mitf expression is more prominent in 500 and 3k cell EBs. Though RPE65 expression appears on day 17, it’s more prominent after day 29.

Line: 410. Timeline for subculture, maturation and pigmentation time was not given.

RPE differentiation and enrichment details have to be specific and more clearly elaborated. Technical description on RT-PCR, IHC and FACS can be reduced. RPE colonies were manually isolated and passaged starting from day 29. The timeline for subculture varied based on the confluency of the cells.