From:em.cell-reports.0.611b1e.82764994@editorialmanager.com <em.cell-reports.0.611b1e.82764994@editorialmanager.com>
Date:2019-02-07 11:04:58
Recipient: Qizhou Lian <qzlian@aliyun.com>
Subject:CELL-REPORTS-D-18-04341 - Editorial Decision

Dr. Qizhou Lian
University of Hong Kong
Department of Medicine, Li Ka Shing Faculty of Medicine,
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Hong Kong Nil
HONG KONG

Feb 06, 2019

RE: CELL-REPORTS-D-18-04341
"Notochordal differentiation and integrative transcriptomic analysis using human pluripotent stem cells"

Dear Dr. Lian,

Thank you for submission of your paper to Cell Reports. I have included the comments that reviewers made on your paper, which I hope you will find useful and constructive. As you will see, the overall view is that the study is potentially interesting, but the recommendation is against publication of the paper in Cell Reports and we have therefore closed the submission.

That said, the reviewers do make suggestions for further experiments and analyses that might guide the paper toward being a stronger candidate for the journal. If you are interested in pursuing further consideration of this work at Cell Reports, you would first need to submit a point-by-point response that outlines how you might be able to address the reviewer concerns, and outlines how any corresponding results and/or edits would be incorporated into a revised manuscript. We would then assess the plan for revision, possibly in consultation with the reviewers. Based on these considerations, we would either reactivate the manuscript file to enable resubmission, or reiterate our decision to reject the paper. Please note that we take into account the published literature up until the date of our final editorial decision.

I know that this outcome is disappointing, given the hard work that you and your colleagues have put into the paper. I hope that the reviewers' comments will be useful to you as you consider next steps, and I hope that you will consider Cell Reports for future submissions as your interesting work progresses.

Sincerely,

Quan Wang, Ph.D.
Scientific Editor, Cell Reports

Reviewers' comments: (25 major questions + 15 minor questions)

Reviewer #1:

The manuscript describes the differentiation of human ESCs and iPSC into notochord cells, and further into their descendants; the nucleus pulposus cells, with the hope to transplant these cells in degenerated intervertebral discs for treatment of the disc disease and the neurological symptoms like back pain that is often associated with disc degeneration. The differentiation of cells is characterized by using a few notochord markers, and RNAseq. The RNAseq data was further compared with previously published microarray data from non-degenerated nucleus pulposus cells. The differentiated cells were transplanted in a rat disc injury model to test their potential for disc regeneration. Overall, this is an interesting study, however, requires further experiments for validation of the cells at each stage of differentiation, profiling and transplantation studies. There are conceptual and technical flaws in some of the experiments like using Noto-GFP reporter to identify nucleus pulposus cells. Also, details are missing in the methods, figure legends, and results. In addition, several references are incorrectly cited, and most of the statements in introduction and discussion are not supported by references (over 20 such statements). The flow of manuscript is currently raw, the results sections have information that is best suited for introduction, discussion, or methods section. The manuscript requires editing for scientific as well as the English language. Correct gene annotation should be followed throughout the manuscript, and abbreviations should be limited to standard and described when first used.

Major comments:

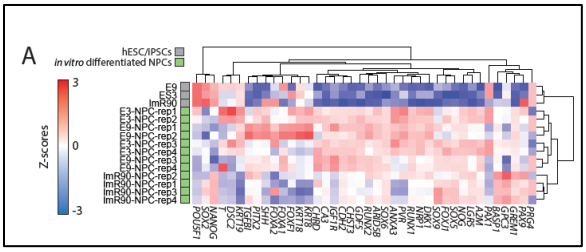
R1-1. It is well established that Noto is expressed transiently in the primitive streak and caudal notochord, but disappears soon after the notochord is elongated and formed. Hence, the Noto-eGFP knockin cells should not express eGFP once they are thought to be differentiated past notochord stage and into nucleus pulposus cells. A better explanation for eGFP expression under Noto in the differentiated NP cells is required.

> **Answer**: Thanks for comments. We are sorry for the misunderstanding caused about using the Noto-GFP reporter in this study, if we didn't explain clearly in the manuscript.
>
> Indeed our aim is not using Noto-GFP reporter to identify nucleus pulposus cells because NOTO is not expressed in nucleus pulposus after the differentiation pasted through notochord stage into nucleus pulposus cells. Instead, the reason that we established Noto-GFP reporter is to monitor notochord differentiation of human pluripotent stem cells (hPSC) , but not to use it to identify nucleus pulposus cells. Lineage tracing studies have indicated that Nucleus pulposus cells were generated from notochord and NOTO is transiently expressed during notochord formation. Therefore, we here use NOTO-GFP reporter to monitor differentiating human PSCs toward notochord stage for further nucleus pulposus cells genereation. After we confirm NOTO-GFP detectable next we can further induce these cells toward NP cells in vitro.
> We have add data of NOTO expression at notochord stage and NP cells (see fig xxx) . More explanation for Noto expression in notochord stage and the differentiated NP cells is included at discussion (paged xxx .

R1-2. In addition to analyzing expression of Noto, Foxa2, and Brachyury, validation of ESCs/ iPSCs into

notochord should be using all the known markers: Shh, Noggin, Chordin, and Foxj1. Also, all markers should be analyzed at mRNA and protein level.

Answer: [by Peikai: fixed, see heatmap in new Figure 3A and barcharts in new suppl. Fig S1-3.]



← **Figure 3A**

R1-3. Expression of Shh at day 5 should be validated at mRNA level. Considering that DMEM only media also stimulated the Light2 cells, ELISA should be performed on the culture media collected from each cohort to quantify the amount of Shh produced.

Answer:

R1-4. In addition to Oct4, the absence of stemness should be validated by analyzing the expression of Nanog and Sox2. All markers should be analyzed at mRNA and protein level.

Answer: [by Peikai: fixed, see new Figure 3A and barcharts in new suppl. Fig S1-3.]

R1-5. Tie2 is an endothelial cell marker. The Sakai et al., 2012 study showed that Tie2 and GD2 are expressed by a subset of NP cells, and not all. This should be correctly reflected.

Answer:

R1-6. Type II Collagen and Aggrecan are not specific markers of notochord or nucleus pulposus cells. These ECM proteins are expressed by several cells including chondrocytes, tenocytes, fibroblasts, cardiocytes, etc. Hence, their expression cannot be concluded to demonstrate the differentiation of cells into notochord or nucleus pulposus cells.

Answer: need comments on more molecular signatures to define NP

[by Peikai: fixed, see below table of NP marker lists from literature. I have added them to new Figure 3A.]
[by Peikai: literature also added to new texts]:

Genome-wide transcriptomic analysis reveals differentiated cells have strong characteristics of the nucleus pulposus

To assess the genome-wide behaviors of the differentiated products, we performed bulk RNA sequencing on the before- (n=3) and after-differentiation samples (n=12) (Methods). Many important genes previously reported to be defining young adult NP [PMID: 25411088] or involved in NP development [PMID: 30902259] show strong up-regulation patterns in the differentiations (Figure 3A; Figures S1-3). Hierarchical

Table 2. Proposed Secondary Markers[a] of Young Healthy NP and NP Progenitor Cells and Their Relevance to NP Cell Physiology

Identified NP phenotypic marker	Relevance to NP physiology	Species	Method
Integrins α3, α6, β4	Cell-matrix adhesion	Human, porcine[55,56]	Flow cytometry, IHC
Microarray and proteomics studies			
Annexin A3	Unknown	Rat[46]	Microarray
Glypican 3		Rat[46]	Microarray
α2-macroglobulin		Human, dog[47,64]	Microarray
Desmocollin-2		Dog[64]	Microarray, IHC
CD56		Dog, mouse[64,77]	Microarray, flow cytometry, IHC
SNAP25		Bovine, human[39]	Microarray, qPCR
CDH2		Bovine, human[39]	Microarray, qPCR
BASP1		Bovine, rat[39,83]	Microarray
SOSTDC1		Bovine[39]	Microarray
PAX1		Human[67,71]	Microarray, qPCR
FOXF1		Human[67,71]	Microarray, qPCR
Hemoglobin β-chain		Human[67]	Microarray
Ovostatin		Human[67]	Microarray
Neurochondrin		Rat[79]	Microarray, qPCR, IHC
Neuropilin-1		Rat[79]	Microarray, IHC
CD155		Rat[79]	Microarray
CD221		Rat[79]	Microarray, IHC
Lubricin (PRG4)		Human[73,74]	IHC, LC-MS/MS
Progenitor markers			
Tie2	Receptor for angiopoietin-1; drives proliferation of progenitor cells	Human, mouse[77]	IF, flow cytometry

Table 1. Proposed Primary Markers[a] of Young Healthy NP Cells and Their Relevance to NP Cell Physiology

Identified NP phenotypic marker	Relevance to NP physiology	Species	Method
Stabilized HIF-1/2α	Transactivate many pro-survival genes in NP; absolutely necessary for post-natal NP cell survival	Human, mouse rat, sheep[21,22,27]	Western blot, IF, IHC, qPCR
GLUT-1	Glucose transporter expressed in hypoxic tissues; expression controlled by HIF-1	Human, rat[28,31]	Western blot, qPCR, IHC
Shh	Signaling ligand necessary for post-natal function of NP cells	Human, mouse[36–38]	Western blot, IF, *in situ* hybridization
Brachyury (*T*)	Transcription factor necessary for notochordal morphogenesis and patterning	Human, mouse, dog, bovine[16,39–41]	Western blot, IHC, *in situ* hybridization, microarray, qPCR, flow cytometry
Aggrecan /collagen II ratio >20	High PG content maintains hydration to resist loads	Human, many others[53,54]	DMMB/DMAB, qPCR, Western blot, IHC
Carbonic anhydrase 3/12	Acid-base balance	Human, mouse[67,68,70]	Microarray, qPCR, IHC, Western blot
Microarray studies			
CD24	Unknown	Human, rat, mouse[63,77,78]	Microarray, flow cytometry, IHC, qPCR
Cytokeratins 8, 18, and 19	Cellular structural integrity and possibly signaling	Human, rat, bovine, dog[39,46,64]	Microarray, IHC

[a]Primary markers were chosen based on criteria including: (i) specific expression in young healthy NP cells, (ii) requirement for proper NP cell function and relevance to NP cell physiology, and (iii) mRNA and protein expression validated across different species.

Table 5 Genes and proteins involved in nucleus pulposus development.

Brachyury	T	Transcription factor	N/A
Collagen II Type 1A	Col2A|I	Protein	Diastrophic dysplasia, OMIM#222600
CYR61, CTGF, and NOV	CCN	Protein	Childhood Progressive Pseudorheumatoid Arthropathy, OMIM#208230
Forkhead Box A	FOXA	Transcription factor	N/A
Forkhead Box O	FOXO	Transcription factor	N/A
Hypoxia Inducible Factor 1 Subunit Alpha	HIF-1α	Transcription factor	N/A
Sickle Tail Gene	Skt	Protein	Lumbar Disc Disease, OMIM#617367
Smoothened	Smo	G protein-coupled receptor	Curry-Jones syndrome, OMIM#601707
Sonic Hedgehog	Shh	Secreted ligand	Brachydactyly Type A1, OMIM#112500
Sox 5	Sox 5	Transcription factor	Multiple synostoses syndrome, OMIM#186500

R1-7. Provide complete details for the methods employed to obtain the results described in the manuscript. This includes details on the correct product name, catalog number, vendor, for all reagents including antibodies. Dilution of antibodies and protocol for the immunostainings. Doses of the pharmacological molecules. Also, provide a rationale for the dose used for each molecule in the current study.

Answer:

R1-8. Provide detail on the age, gender, and strain of the rats used for the transplantation study. Were these immunocompromised rats? As the human stem cells were differentiated and transplanted. Provide details on post-surgery and post-transplantation care for the rats.

Answer:

R1-9. In Figure 4, provide histological data from the disc of rats 2 weeks post-injury and before transplantation.

Answer:

R1-10. In Figure 4, provide GFP+DAPI images for the entire disc and for all the four levels to distinguish between the native and transplanted cells.

Answer:

R1-11. Figure 5Ai, provide counterstain data for nuclei and cell membrane. The data provided does not show cell survival as concluded in the manuscript. The only conclusion that can be drawn from the data provided is that there are fewer GFP+ve cells 8 weeks post-transplantation.

Answer:

R1-12. Figure 5Aii, the data should be compared with the naive controls.

Answer:

R1-13. Figure 7A, the contrast for the three images should be similar.

Answer:

R1-14. Figure 7B, several GFP+ve cells are negative for Noto. The results should correctly reflect this. Data should be quantified on the percentages of cells that are positive cells.

Answer:

R1-15. From the schema provided in the Figure. xx it is unclear where the T2A and eGFP are inserted in the Noto gene.

Answer: zhang zhao pls fill in more details

R1-16. The information on replicates is unclear. For example, the figure legend for Figure 1 says "n=3". However, it is unclear whether these are technical replicates, or the study was repeated three times. Also, how many technical replicates were used in each experiment, and which data was used for statistical analysis? Such details are also missing in the methods section. This information should be provided throughout the manuscript.

Answer:

R1-17. To confirm the structure observed in the dark-field images are vacuoles, electron microscopy should be performed.

Answer: who can help perform electron microscopy?

Figure 6 may be combined with Figure 5, or moved to supplemental figures.

R1-18. Several parts of the manuscript describe that the notochord is "thought" to give rise to nucleus pulposus. However, this was genetically proved a decade ago and should be discussed accordingly.

Answer: what notochord genetically proved a decade ago?

R1-19. Several parts of the manuscript suggest that disc degeneration leads to back pain. However, it is well established that not all degenerated discs cause back pain, and back pain may not always be due to degenerated discs, and it is only one of the several causes. This information should be correctly presented.

Answer:

R1-20. When discussing current literature, please identify the model organism and use the correct gene annotation specific for that organism.

Answer:

R1-21. All the schemas presented in the current figures are not well illustrated and the experimental plan is not correctly reflected. Either they should be removed or modified.

Answer:

R1-22. The manuscript needs editing for flow, scientific and English language.

Answer: will send to English editing again

R1-23. Incorrect references:

a). Introduction, first para, Kennon et al. 2018, replace with the reference for Lancet GBD 2018 study, and with updated demographics.

Answer:

b). Introduction, third para, the McCann and Sequin 2016 is incorrect for the statement "We and other ..... progenitors of NP cells". This will be Choi et al., 2008. Also, none the authors of the current manuscript were on that article.

Answer:

c). Results, Early notochord....., "The expression of key..... determine notochord differentiation" the reference of Risbud 2010, and Sivakamasundari 2012 is incorrect for the information. Please cite the original research articles for each of the genes.

Answer:

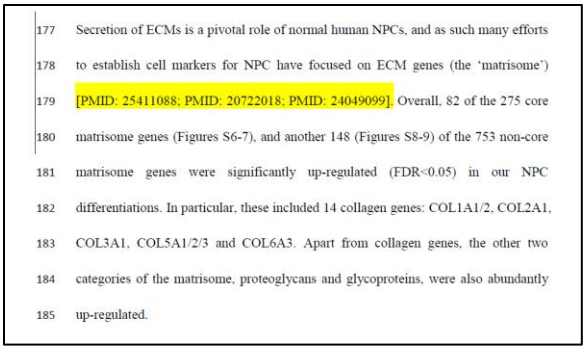
d). Results, Differentiation of NP-like...., Choi et al 2012 did not show release or expression of Shh by the NP cells. This was first demonstrated by DiPaola et al., 2005.

Answer:

e). Results, Genome-wide transcriptomic ....., Hynes and Naba 2012 did not analyze the martisome of nucleus pulposus.

Answer:

[By Peikai: Fixed, see below(pls replace the PMIDs with ref. as I don't have the endnote lib for this doc.)]



177 Secretion of ECMs is a pivotal role of normal human NPCs, and as such many efforts
178 to establish cell markers for NPC have focused on ECM genes (the 'matrisome')
179 [PMID: 25411088; PMID: 20722018; PMID: 24049099]. Overall, 82 of the 275 core
180 matrisome genes (Figures S6-7), and another 148 (Figures S8-9) of the 753 non-core
181 matrisome genes were significantly up-regulated (FDR<0.05) in our NPC
182 differentiations. In particular, these included 14 collagen genes: COL1A1/2, COL2A1,
183 COL3A1, COL5A1/2/3 and COL6A3. Apart from collagen genes, the other two
184 categories of the matrisome, proteoglycans and glycoproteins, were also abundantly
185 up-regulated.

Please check all references.

R1-24. Missing references:

a). Introduction, first para, "Surgical intervention.....metallic compounds"

Answer:

b). Introduction, second para, "The NP, which is ..... notochord", refer Choi et al., 2008 here.

Answer:

c). Introduction, third para, "A notochord ..... notochordal development".

Answer:

d). Introduction, third para, "Human pluripotent ..... regenerative medicine". Please cite examples and provide a reference for each.
This list is enormous, and hence the suggestion is to refer the original research article for each statement from previously known literature.

Answer:

R1-25. Minor comments:
1. Gene annotations should be used for each species discussed per current guidelines.

Answer:

2. Nucleus pulposus (NP) cells should be written as NP cells, and not "NPCs".

Answer:

3. Notochord cells should be written as such and not further abbreviated as "NCCs".

Answer:

4. Correct "FASC" to "FACS" (Fluorescence-Assisted Cell Sorting) throughout the manuscript.

Answer:

[By Peikai: fixed. See below:]

824	C. The GD2/Tie2 double positive cells were sorted by FACS (i).

93	enhanced green fluorescent protein (eGFP) to the loci of the Noto gene in a human

5. Correct "enhance fluorescent protein (GFP)" to "enhance green fluorescent protein (eGFP)" throughout the manuscript.

Answer:

6. The heading for each result sections showed be changed to describe the major findings.

Answer:

7. Instead of using "up-DEGs", use "upregulated genes".

Answer:

[By Peikai: fixed.]

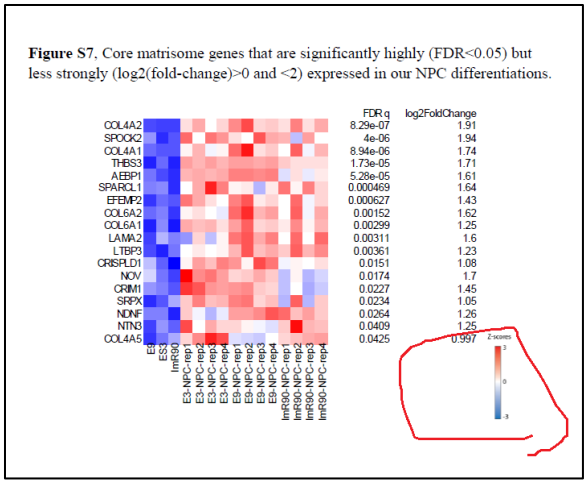
8. In what plane were the discs sectioned?

Answer:

9. Provide scale for all heat-maps in supplemental data.

Answer:

[By Peikai: fixed, see below, and new supplementary figures]



10. Figure legend: Fig 1Biii, are the iPSCs IMR-90-iPScs?

Answer:

11. Figure legend for 5A, is the data for immunostaining for GFP protein, or detecting natural fluorescence using epifluorescence? Please correct accordingly.

Answer:

12. The methods describe that only ESC9 was used for creating the Noto-eGFP knock-in allele. However, the method on "Rat model of IDD.... " describes "ESC/ iPSC-NP like cells". Please clarify which cells were used for transplantation experiments. Also, were the "GFP-labeled" cells the "Noto-GFP cells"?

Answer:

13. What time point post-transplantation is shown in Figure 5B?

Answer:

14. In Results, Early notochord..... section, change "blockage" to "blockade".

Answer:

15. In Results, Early notochord..... section, please provide model organism for "Xnot".

Answer:

Reviewer #2: Cell Reports (13 major questions)

The study carries out an important and timely set of experiments aimed at generating notochord and nucleus pulposus cells from human pluripotent cells. The paper presents a large amount of data with strong overall experimental strategy. The "cell product" is robustly characterized and an in depth comparison to endogenous human tissues is presented. **Bioinformatic analysis here is a strength**.

1. In general the manuscript would benefit from editing for grammar and language. Paragraph breaks are missing throughout the Results and Discussion. Language is not always clear.
Figure legends should be extensively edited to ensure all data is presented is clearly described.

Answer: we will revise and send for English editing

2. Throughout the paper (i.e. Introduction as well as the background rationale provided in the Results), citations need to be revisited. In many places review papers are cited in lieu of primary research papers - this is highly inappropriate and needs to be revisited.

Answer: need correction

3. There are also many places throughout the manuscript (especially Discussion) where citations are completely missing and should be included for original work.

Answer:

Specific comments:

R2-1. First paragraph of Results - This text presents the rationale and background on notochord patterning. This content may be better suited for the Introduction. Within this text, the context of many of the findings are not adequately presented - some citations are EB models of hESC differentiation, some are Xenopus gastrulae… The developmental rationale provided needs to be more precise in explaining in what model system these pathways have been implicated.

Answer: need write clearly

R2-2. Fig.1 T staining is oversaturated - are the authors convinced that all cells gain T expression at day 5 (as suggested by the IHC images). Quantification of percentage of positive cells for these markers would be informative. Particularly the double-positive cells given that T and Foxa2 are upstream of Noto.
In the mouse, noto expression is only detected for a very brief window of notochord differentiation. How does this relate to the stage of cells generated here.

Answer: Quantification of percentage of positive cells

R2-3. Fig 2- The labeling on the data presented in this figure is not clear. Panel B is labeled for cells from days

14-19. Is that days post NC differentiation? Or total days in culture?

Why are vacuolated cells only detected following full NP differentiation? This is not in keeping with the phenotype of cells in vivo - the hallmark of notochord cells in the embryo is the presence of large intracellular vacuoles. These are no longer detected in "differentiated" NP cells.

Answer: Notocordal cells vacuoles. NP cells no vacuoles?

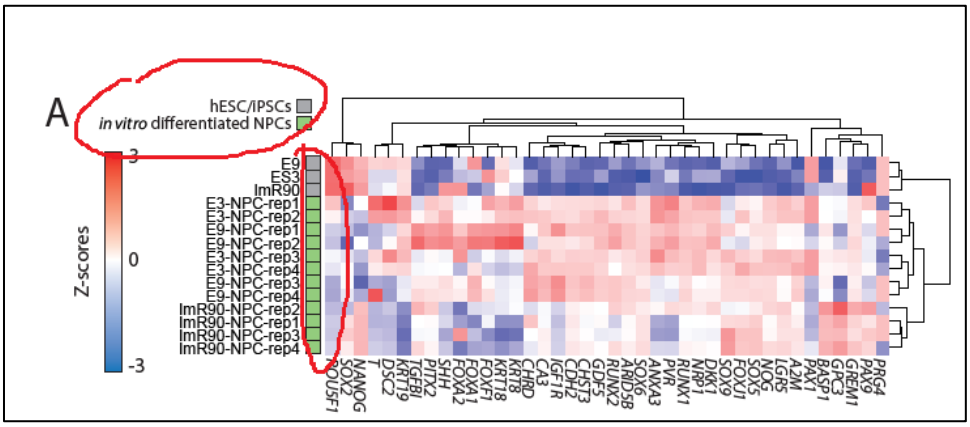
R2-4. FACS data presented in panel C is not convincing of a distinct population of Tie2 /GD2 positive cells. Gate is seems randomly drawn with no separation from the bulk population of cells.

Answer:

R2-5. The transition to transcriptome analysis is not clear in terms of the work flow of the paper. Which "differentiated" cells were analyzed? Fig 2 and Results outline many populations - bulk differentiated cells, FACS sorted Tie2 /GD2 positive cells, clonogenic cells. Which of these was used for subsequent characterization?

Answer: Peikai , need make it easier to catch up

[By Peikai; explained; see new Figure 3A]

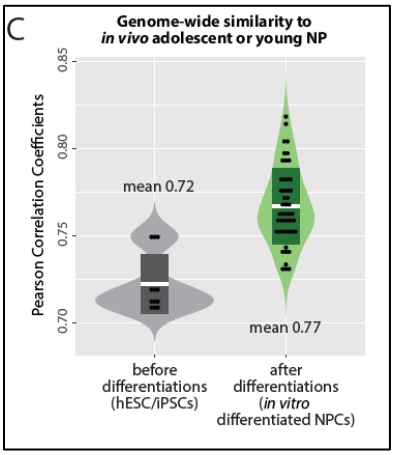


← new Fig 3A

R2-6. Fig 3 - Appropriate labeling and clarity lacking for all this data. Figures labeled with investigator name is not appropriate - should be labeled by sample type. What does the label "our NPC and Chan NPCs" mean? Was there a comparison between different differentiation protocols?

Answer:

[By Peikai: fixed, see new Figure 3C.]



← new Figure 3C.

R2-7. In Results section detailing the cross comparison of data - what is the difference between samples indicated as healthy human NPC and non-degenerated NPC - these descriptors seem to mean the same thing. Better description of the "purified gene sets" used for analysis is required. Does this just mean candidate genes? Selected using what criteria? In large omic data sets it is often challenging to distinguish possible "other cell types present" from relevant data. Moreover, the differentiated cells being characterized are inherently heterogenous based on the characterization shown - this is not addressed.

Answer: Peikai , correct

[By Peikai:]

- I cannot find the term ‘non-degenerated NPC’ in main text or supplementary.
- ‘healthy human NPC’ all changed to ‘human *in vivo* NP control’.
- "purified gene sets" not mentioned any more
- ‘*Moreover, the differentiated cells being characterized are inherently heterogenous based on the characterization shown - this is not addressed.*’:

408 to 77%, suggesting sizeable heterogeneity in both *in vivo* and *in vitro* samples.
409 Another reason we are not seeing higher similarities might be that the reference *in*
410 *vivo* NP was taken from individuals with scoliosis conditions or burst fracture, whose
411 transcriptomes by themselves may have deviated from the healthy state to a certain
412 degree. Nonetheless the proportion of our cells with strong NP characteristics is
413 projected to have been reasonably high in order to bring out those DEGs and increases
414 in similarities to *in vivo* NP. An ideal and popular tactic may be to perform single-cell
415 transcriptomics of our cells to further delineate their heterogeneity and assess the

← see my discussion.

R2-8. Fig 4/5 IVD puncture - Methods / results / figures need to be revised to clearly indicate these as caudal IVDs. Why are they labeled as L4-5, L6-7, L7-8?
The model needs to be better explained.

Answer: Yuelin and Victor ,revise pls

R2-9. Fig 4 needs better presentation of the histological appearance of discs post transplantation - at all time points shown in fig 5. Histological sections should be presented to better show whole IVD, as well as high magnification view of NP cells.
Were any implanted cells detected elsewhere? In the AF? Outside the IVD? No indication that other areas of ectopic cell differentiation were detected, but this is not specifically addressed.

Answer: Yuelin and Victor ,revise pls

R2-10. Fig 5 - Data presented here would benefit from additional low magnification images to better present the IVD as a whole - were any cells ever detected in the AF? What does rest of disc look like? Is the localization of cells within the NP homogeneous? Are there any non-GFP cells?

Answer: Yuelin and Victor ,revise pls

R2-11. Did the group test the effects of injection of notochord cells, in addition to NP cells?

Answer：

R2-12. Fig 6 & 7 - this data does not seem to fit with the narrative of the study. Use of these cells seems preliminary and not well characterized. Many references to the poor viability of these cells. The authors should consider removing this data.

Answer：

R2-13. Details regarding the location of GFP insertion in the Noto locus are not provided.
While it is interesting that a noto-reporter line was generated, it was not applied to refine the differentiation protocol. Need to see this reporter line put through and characterized at all steps of differentiation to NP - not just NC. It would be interesting to use the line carrying the Puro selection cassette to test the effect of purifying the Noto+ cell subpopulation (after day 5) prior to subsequent NP differentiation. The effect of heterogeneity within the "post-differentiation" cell population is not adequately addressed or characterized.

Answer: zhang zhao –if don't remove noto-GFP , need explain more details GFP insert at noto locus and why cant be used with Puro selection