Reviewer’s report

Title: Activation of Intervertebral Disc Cells by Co-culture with Notochordal Cells, Conditioned Medium and Hypoxia

Version: 1 Date: 1 August 2014

Reviewer: Esther Potier

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Major compulsory revisions

MCR1. Introduction. The authors should explain why it is important/interesting to optimize the NC culture conditions to maintain NC in long term culture when NC conditioned medium, produced with only 4 days of culture, has already proven to be efficient in different studies when the NC co-culture did not.

MCR2. Introduction. P.4 L.21-23. The authors explained that culturing the NC under hypoxia should promote “a more authentic phenotype” of these cells. They then present the aggrecan/collagen type 2 expression ratio as a parameter to determine this phenotype, rather than using cytokeratins, brachyury or galectin 3, that have been used in different studies to characterize NCs over NPCs. Can the authors motivate their choice or rephrase the goal? Moreover the targeted ratio of aggrecan/collagen type 2 of 20:1 is “authentic” to NPCs, but not to NCs. Mwale et al. (ref. 30) used human tissue samples in their study, so samples mostly devoided of NCs. Additionally the authors should check their references as the ref.22 is not evaluating the ratio of aggrecan/collagen type 2 but only citing another study (the ref.30). Moreover, in ref. 30 a ratio of 25-27:1 is given for the NP, not 20:1; and Mwale et al. evaluated the glycosaminoglycan/collagen content of the matrix, not the gene expression levels of aggrecan and collagen type 2. Clouet et al. 2009. Rheumatology, however, evaluated the expression of these two genes in a rabbit model.

MRC3. Methods/results. Why did the authors chose to normalize all their data to “pure” cell population and day 0? It makes really difficult to compare their data to other studies where such normalization is not done, except for gene expression levels. Moreover such approach may show increases that are not really here, particularly for the GAG/DNA content. The day 0 is expected to be 0 or very near 0 as no ECM is secreted yet; an increase of 1 µg GAG will therefore represent an important fold increase relative to Day 0, but actually represents almost no ECM being produced. The Fig.5 should therefore be represented as values of GAG/DNA that are not normalized to any D0 or pure population values and the results/discussion modified accordingly to the new results. As the authors only obtained a two fold increase compared to day 0, the GAG production is most likely very limited and should be discussed. As an example, Abbott et al. obtained 800 µg GAG/µg DNA with human NPC after 2 weeks of culture. As day 0 values are always very low, a normalization to day 0 values will most likely
gives fold increase much higher than 800. If the day 0 values obtained by the authors are high, they should make sure that these values are not coming from the alginate, known to interact with the DMMB. Even though a low pH of the DMMB solution will limit the alginate’s effect, a quantity of alginate similar to the one present in the samples should be added in the standard serie to account for its potential effect, as done by Enobakhare et al. Maybe the authors already used this approach, but it is not specified in the methods and they also cited a reference not using alginate in the DMMB assay.

MRC4. Results. The results presented in Fig.3A are a little bit surprising for the first portion of the cells: it seems that the cells initially sorted as NPCs (< 8um) are becoming as large as NCs (SSC of 700) after one passage. The authors may comment that result.

MRC5. Results. P.9 L.30. Claiming that there is an increase, even a trend of an increase, in the GAG/DNA ratio from 80+/− 38.3% to 140.1 +/- 71.5% or 190.62 +/- 112.77 is really a speculation due to the huge SEM obtained. Moreover, the “increase” observed with the NC conditioned medium is clearly neither substantial nor significant, contrary to previous reports using such conditioned medium on NPC. This absence of effect of the conditioned medium may be discussed.

MRC6. Figure. Fig.6. There are more groups detailed below the figures (FCS/CoC +/-) than bars presented in the graphs. It is therefore impossible to know what the bars correspond to. A mistake that should have been corrected before submission. It is therefore impossible to review results and discussion on this part of the study.

MCR7. Results/Figure. Why are the authors indicating a threshold value of 1 for the ACAN/COL2 ratio between an articular cartilage or a disc phenotype? They indicated in the introduction a value of 20:1 (that should be 27:1).

MRC8. Results. In Fig.9, a day 0/1 control may be added for the IHC of T in order to provide a baseline of T expression, so that the reader can evaluate if the expression after 14 days is high or only a small portion of what it is in a fresh NC.

MRC9. Results. For the MS analysis of the conditioned medium, why did the authors analyze the medium after 7 days of culture with NPCs and not directly after the production of the conditioned medium? The protein potentially involved in the NC effects might have been hydrolyzed or consumed by the NPCs by then.

MRC10. Discussion. P.11 L.20. “NC cells seem to be outcompeted by the small chondrocyte-like NPC” : how the authors can be sure that the NC are outcompeted rather than changing phenotype during culture and acquiring a NPC-like phenotype as shown by Kim et al. 2009. Spine (34)?

MRC11. Discussion. P.12 L.28. “we found that GAG/DNA ratio could be increased indeed under hypoxic rather than normoxic conditions on the gene expression level but also at the level of GAG/DNA ratio (Fig. 5)” First, the GAG/DNA ratio can not be determined at the gene level. Second, this statement
may have to be revised after proper presentation of the GAG/DNA data. The same stands for the second point of the conclusions: from the results presented it is impossible to state an increase in GAG/DNA.

MRC12. Discussion. The results of the proteomics analysis of the CM are discussed nowhere in the discussion, when it may be the most interesting outcome of this paper.

Minor essential revisions.

MER0. There are many, many mistakes that should have been corrected in the manuscript before submission. The reviewer is aware that there are always some typing mistakes in a submitted manuscript, but not that much. Many of those could have been spotted with a careful reading of the manuscript.

There are a lot of typing mistake around the references, sometimes the dot is before, sometimes after, sometimes double brackets are used.

Throughout the manuscript the authors should decide for notochordal cells, NC or NC cell as all three terms appear.

P2 L.11 “To test stimulating effects of NC co-cultures of porcine and bovine derived coccygeal IVDs was conducted” is no a correct sentence structure

P.2 L.19 “porcine (p) coccygeal derived pNC” porcine (p)/pNC is redundant.

P.2 L.31 “in equal activating effects as adding solely culturing in NCCM” please rewrite.

P.3 L.12-16 “In dog breeds both IVD types are present, in the so-called non-chondrystrophic dogs (e.g. larger dogs such as greyhound) a high ratio of NC is present and low back pain is less common. In chondrystrophic dog breeder lines, and chondrystrophic (e.g. Beagle, Dachshund). In the non-chondrystrophic dogs which frequently suffer from disc degeneration and low back pain”. The sentence’s structure is not correct.

P.4 L.12-14 “Furthermore, we hypothesized that because NC are more sensitive to culture conditions [28]. These cells can be stimulated by simple exposure to fetal calf serum (FCS), which will then have a stronger activating effect reflected in an increased glycosaminoglycan (GAG) […]”.The sentence’s structure is not correct.

P.4. L.29 “Tails with a high percentage (~80%) were selected and a high percentage of NCs in porcine NP tissue was confirmed by size and presence of vacuoles and the haemocytometer using brightfield microscopy”. With a high percentage of what? By “size and presence of vacuoles and the hematocytometer”: do the authors mean that a haemocytometer was used to assess the cell size and vacuole presence?

P5.L.2 “Both cells were separated from native extracellular matrix (ECM) by 0.19% pronase digestion (Roche, Basel, Switzerland) for 1h and subsequent collagenase type 2 (Worthington, London, UK) digestion 1 overnight (~14h) and primary culture” what is the concentration of collagenase. “and primary culture” what does that mean?
P.5 L.14. “compared to the cell size of NC containing rabbit IVD and porcine IVD” Should not it be “isolated/harvested from” rather than containing?

P.5. L.18 contrary to the other chemical the supplier of the FCS is not indicated.

P.6 L.31 “Relative gene expression at major anabolic genes” of?

P.6 L.32 “ribosomal 18S as a reference gene” used as?

P.9 L.12 “this was due to the fast proliferation of the fast NPC-like cells”. Redundancy.

P. 10 L.3-5. “after 14 days if we added NC conditioned medium or co-culture of NC, which were exposed to 10% FCS during pre-culture, the ratio tended to be increased (140.1 ± 71.5%) or by addition of NCCM (190.62 ± 112.77%, Fig. 5D)” please rewrite the last part.

P.10 L.12 “Fig. 6 lower left” or C? “Fig. 6 upper left” or A?

P.10 L.23 “However, if the ratio of ACAN/col2 was considered the most IVD-like gene expression pattern among all analysed groups was found in hypoxia and by co-culture of NPC with previously activated NC with FCS or by addition of NCCM (Fig. 8)” Please rewrite, not understandable.

P.11 L.8 “A full table of the Swiss Prot search of identified proteins listed per can be obtained as additional supplemental Table S1” something is missing.

P.11. L.22. “Ragosti” should be Rastogi according to the reference list.

P.11 L.32 “It has been demonstrated that these two cell populations differ in cell size, nutrition,[28] surface markers[46] and mechanosensitivity [29]” what are the cell populations the authors are talking about? They just talked about NCs in the sentence before without any indication what the second cell type is.

p.13 L.24 “NC cells responded highly sensitive to the addition of FCS” please rewrite.

p.24 L.2 “identified in free medium (SFM)” serum is missing.

P.21-22. Figure legends. Please use the same way to refer to sub-figures: a) b); A) B); or A) B). the sub-figures A to F are not described in the legend of Fig.4.

P21-22. Similar comment for the coculture, sometimes it is CC, sometimes CoC, sometimes Cocul. With only one abbreviation used the results are easier to follow.

Fig.5. Day 7 and 14 are not indicated in the figure. The graph legend is not complete: the red and green colors are not explained and the reader has to refer to the Fig.4.

Fig.6D. It could be that the SEM are very small, but it looks like they are missing for the normoxia groups.

Fig.6 and 7. The legends read: “respectively after day 7 and 14 days of culture” 1. Redundancy, 2. Only d14 values are displayed.

Fig.7. The caption indicates that Col2A1 was measured but in Table 1 it is indicated that primers for Col2A2 were used for bovine cells. Moreover, this specification is not added in the fig.6 for porcine NCs.
Fig.8. For an easier reading the order of FCS/CoC/CM classification on the side of the graph should be in the same order than for the other graphs. The abbreviation given in the legend, cc, NC, FCS are not those added in the graph.

MER1. Abstract. The abstract is, in general, not of a very good quality as several precisions are needed. The authors should clarify the abstract when keeping the word limit of 350 words.
L.7 “to determine NC conditions” do the authors mean isolation or culture conditions?
L.13: “with no-cell-contact” I guess the authors mean cell-cell rather than cell-matrix contact?
L.14 what are NPC and AFC?
L.16 “Mass spectrometry of the NCCM was conducted” mass spectrometry analysis should be used.
L.18-19 “We provide evidence by flow cytometry that monolayer culture is not favorable for NC culture” for what measured outcome: cell activity, viability, growth?
L.20 “nucleus pulposus cells (NPC)” and L.23 “annulus fibrosus cells” this should be indicated the first time NPC/AFC appears in the abstract.

MER2. Introduction. P.3 L.23-25 “The open question remains about the mesodermal or ectodermal origin of these cells [12-14]. However, recent tracking of the gene “notochord” by a novel notochord specific Cre mouse clearly demonstrated that the notochord indeed gives rise […]” Can the authors explain how the noto gene tracking helps to answer the question raised about the meso/ectodermal origin of the NC?

MER3. Introduction. P.3 L.30-32. It is not clear why the authors chose to highlight the study showing a protective effect of NC against apoptosis and not the effect on matrix synthesis (GAG production or collagen aggrecan gene expression), which may be more pertinent for the work presented.

MER4. Methods. P.7 l.2 “the markers that have been described as notochord specific the cell markers brachyury (T) and CD24” those markers are not specific to NC; Brachyury is also expressed by NPC, only at much lower levels. CD24 is specific to the NP tissue but the study cited does not allow knowing if this marker is solely expressed by NCs.

MER5. Results. P.10 L.13 “we found that the disc specific markers ACAN and col2”, there is no marker specific to the disc and ACAN and COL2 are also largely expressed by articular cartilage.

MRC6. Results. For the MS analysis of the NCCM, the authors should indicate better that all media analyzed are, in fact, conditioned by 7 days of cell culture: NPCs (?) in SFM or NCCM, or NPC:NC in SFM. If that is not the case then they should rewrite more clearly in the methods what were the media analyzed, as it is now difficult to see how the media were produced.
MER7. Table. In Table 1 please provide an accession number of the gene analyzed, such as the GenBank accession number.

MER8. Figures. In Fig.1. some captions are too small and will not be readable when the figure is downscaled. Moreover the first question asked by the authors (2D or 3D culture of NCs) is not represented. Same problem of small caption for hypoxia/normoxia in fig.4,5.

Discretionary Revisions

DR1. Introduction. The different goals of the study are not so easy to understand at the end of the introduction. The last paragraph could be rewritten to clarify these different goals. A main goal, such as “to optimize the culture conditions of NCs to maximize their effects on disc cells” could be introduced and the authors can then describe the different parameters they tested: 2D/3D, with or without FCS, hypoxia or normoxia.

DR2. Methods. P.5 for a better understanding, the authors may specify what the FACS analysis was used for.

DR3. Results. Relative gene expression. The authors may emphasize better in the text the separation between the expression of NC and NPC. As it is now it is not possible to know to what type of cells they are referring to without looking at the figures. For example, “Collagen type 2 expression was up-regulated much stronger in all groups cultured in normoxia but not so much in hypoxia”, we do not know if it refers to NC or NPC. Moreover the authors may discuss more on the NPC a little bit less on NC.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Not suitable for publication unless extensively edited

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests.