Response to Reviewer 1 Comments

Point 1: This is an original article by Penolazzi and colleagues that seeks to further characterize the role of TRPS1 and miR-221 in IVD homeostasis and degeneration. The study is based on previous published data by the group showing that miR-221 promotes IVD degeneration in part through suppression of TRPS1 and FOXO3. However, the data provided in the present follow up study is not entirely novel and just incremental over what the group has already published. Most of the claims in this manuscript are not sufficiently supported by data.

Response 1: We thank the Reviewer who through his/her comments gave us the opportunity to clarify some points. The anatomy of the spinal column clearly illustrates that the vertebrae in each region (cervical, thoracic, lumbar, sacrum, coccyx) have unique features that help them perform their main functions. Therefore, it is reasonable that intervertebral disc cells may have specific molecular characteristics as affected by different biophysical microenvironments: this is what emerges from our work which, due to the complexity and diversity of the mechanisms involved, led to the realization of two manuscripts. In our previous published paper (Penolazzi, L. et al. MicroRNA-221 silencing attenuates the degenerated phenotype of intervertebral disc cells. Aging (Albany NY) 2018, 10, 2001-2015.) we focused on degenerated cervical IVD and on the miR-221 – FOXO3 axis (not TRPS1: it was only marginally analyzed) demonstrating the efficacy of miR-221 silencing in reverting the degenerated phenotype of these cells that are characterized by a significant increase of miR-221 during the de-differentiation process. Continuing the research in this field, on the contrary, we found the cells from lumbar IVD were characterized by comparable miR-221 expression levels regardless of the degree of degeneration, but a strong decrease of TRPS1 expression: here, in the Cells paper, we describe a new molecular mechanism supporting the degeneration of lumbar IVD microenvironment that may be counteract by TRPS1 overexpression approach. Therefore, the current study certainly represents a continuation of the previous work as it tries to answer pending questions and it is within a research project that seeks to shed light on the mechanisms that support IVD degeneration, but let me say that it adds completely new data.

Point 2: Sample collection is a major concern. It is not clearly indicated whether the isolated cells are just NP or a mix of NP+AF cells. This is a major limitation that can greatly confound the results.

Response 2: We thank the Reviewer for her/his observation which gives us the opportunity to better specify experimental model we used. In the Material an Methods section we stated “Disc sampling was obtained from the central core of the disc, in order to avoid anterior and posterior longitudinal ligament, anulus and calcified portions of the disc.” therefore the isolated cells are mainly NP cells. It is well known that discs from donors undergoing surgery for IDD exhibit a general disorganization with loss of NP/AF demarcation, and loss of AF cellularity. As previously reported by us (reference 18): in the degenerated IVD “the nucleus infiltrates the annulus and the cellular components mix together. Consequently, a variety of cells coexist in the degenerated microenvironment such as neurons, chondrocytes, and osteoblasts which come from both surrounding spinal tissue or differentiation of progenitor cells resident in the disc […]. Therefore, when investigating IDD local microenvironment it must take into account the difficulties of both acquiring a uniform IVD tissue or obtaining homogeneous cell sub-populations. However, in a scenario like this it is not always necessary/convenient to sort single cell populations, but rather to try to preserve in vitro the properties of the endogenous microenvironment to obtain informative results. Therefore, the idea of not selecting the different types of cells, but of using the whole cell population with a part of resident extracellular matrix (ECM), is becoming increasingly
convincing.”
We referred to our previous paper (reference 18, I apologize because, erroneously, I reported the number 16 instead of 18 in the previous version) regarding the method of isolation of human IVD cells (Row 138). However, in order to meet the Reviewer’s request we defined again the method we used, and added an explanatory sentence in the Results (335-338).

Point 3: Correlation analysis of TRPS1 expression with different sample parameters (grade, age, region of the spine) is indicated in the text but no data is shown.

Response 3: We agree with the Reviewer, we improperly used the term “correlation”. The description of the results have been modified in the revised version (305-311).

Point 4: Although SOD2 expression correlates with TRPS1 expression, SOD2 is regulated by several transcription factors (such as FOXOs, NRF2, etc…).

Response 4: We agree with the Reviewer, SOD2 is regulated by many signaling in different context. In order to improve the explanation of the meaning of our data, we added the following statement: SOD2 has been recently demonstrated to be an important effector of FOXO signaling in disc degeneration, and its reduction is correlated with decrease in the expression of FOXO transcription factors [25]. Therefore, TRPS1 decrease by us observed can be considered part of biomolecular damage accumulation. (Row 315-318)

Point 5: Despite authors claim that TRPS1 and miR-221 have antagonistic expression, levels of miR-221 in normal and degenerated IVD samples are not statistically different.

Response 5: We don’t think we fully understand the Reviewer's request. We don't seem to have said that TRPS1 and miR-221 have antagonistic expression. Moreover, we compare IVD with different grade of degeneration (not “normal” versus “degenerated”). We state that when TRPS1 is overexpressed, miR-221 dramatically decreased, indicating that the cells receive a benefit from high levels of TRPS1 that are able to suppress an antichondrogenic factor such as miR-221. This does not conflict with the fact that lumbar IVD cells express miR-221 at comparable levels regardless of the grade of degeneration. Most likely in the degenerated microenvironment there are factors that may counteract the TRPS1 action supporting the expression of miR-221.

Point 6: In Figure 2, a small legend indicating the color code for the bar graphs would improve clarity.

Response 6: A small legend has been included.

Point 7: The method to analyze protein expression in Figure 2A is rather unusual. Why more traditional and better quantitative methods such as PCR or western blot were not used?

Response 7: We are sorry, but we don't completely agree with the Reviewer. The immunocytochemistry together with quantitative image analysis as a method to evaluate protein expression is not unusual and we used it many other times (Lolli et al., Stem Cell Rev Rep. 2014,10, 841-855; Lolli et al., Stem Cells 2016, 34, 1801-1811; Penolazzi et al., Aging 2018, 10, 2001-2015). In literature there are many examples in this regard, above all when, as in our case, a really limited number of cells is available. From a human disc surgical sample the number of cells obtainable is low. Therefore, in order to perform all the evaluations that are reported in the
manuscript in the same sample, the only possibility is represented by immunocytochemical analysis.

One possibility to overcome this limitation would be to use cadaveric samples, but unfortunately the Italian legislation does not allow it. For this reason, we are looking for collaborations with foreign research centers interested in our research and able to recruit cadaver samples through organ donation program.

For what concerns RT-PCR analysis, we have chosen to insert the figure only for the revision process (see below), but not in the text of the manuscript in order not to burden the Results for the following reasons:

- we have adequate experience regarding the evaluation of gene expression by qRT-PCR that in many cases has led us to show that there is no correlation between RNA and protein levels. In particular, this happens with soluble factors and matrix proteins. In many cases (Collagen 1, Collagen 2, Collagen 15) we found an appreciable amount of protein also in samples with very low or scarcely measurable levels of corresponding mRNA (in agreement with literature data)

- in accordance with what has just been said, and as the Reviewer can see in the Figure below, the TRPS1 overexpression increased TRPS1 and SOX9 mRNA levels, but mRNA levels of COL2, ACAN, and COL1 remained substantially unchanged between the three groups (CTR, hTRPS1 and EMPTY).

Point 8: SOX2 expression levels alone are not a good indication of cell stemness.

Response 8: The observation is correct: the comment on the result was appropriately “mitigated” and paragraph changed (Row 359-362).
Point 9: Only chondrogenic genes were analyzed upon TRPS1 overexpression, but not specific genes more relevant to IVD biology. The role of TRPS1 in the maintenance of the IVD phenotype remains largely unexplored in this study.

Response 9: We agree with the Reviewer, there is still much to investigate on the role of TRPS1 in the maintenance of the IVD phenotype. It is known that “Degeneration is characterized by the loss of chondrocyte-like phenotype by the cells”, and we added this statement in Introduction (Row 51-52) together with the sentence in the Results (Row 342-343): “Although disc cell phenotypes still remain to be defined in detail, it is accepted that IVD cells exhibit a chondrocyte-like phenotype.” in order to strengthen the aim of our work. Therefore, in our opinion one of the first things to ascertain to demonstrate the efficacy of an in vitro treatment like ours is represented by increase of chondrogenic markers.

We know and appreciate many works in the literature that show data regarding a progress in the understanding of the IVD phenotype: they represent for us an inspiration to continue our work and to better investigate the role of TRPS1 in the maintenance of the IVD phenotype. But once again, we need much more biological material (which, maintaining the human model, may be obtained only from cadaver - see the previous reply) to perform experiments such as RNA Seq, Microarrays, Functional and pathway enrichment analysis together with biological network analysis, various immunohistochemical detections or Western blot.