Response to Reviewer 4 Comments

**Point 1:** I like the idea of the current study very much. Gene expression control happens at multiple levels and TRPS1-miR axis may be very important.

**Response 1:** We thank the Reviewer for her/his positive comment.

**Point 2:** However, the authors should describe more specifically about the significance of their study and why TRPS1-miR-221 axis is so critical? Is there any intervertebral disc phenotype in TRPS1 knockout mice or is there any spine intervertebral disc phenotype because of TRPS1 mutation in human?

**Response 2:** In order to satisfy the Reviewer request and better specify the significance of our study, we added some sentences in the Introduction (Row 51; Row 73-75; Row 79; Row 86-87). As far as we know, to date there is no direct evidence on IVD phenotype in TRPS1 knockout mice or spine intervertebral disc phenotype because of TRPS1 mutation in human, probably due to the difficulty of investigating this tissue. However, as we reported in Introduction and in Discussion there is strong evidence on the pro-chondrogenic role of TRPS1.

**Point 3:** In method section 2.2, I am not sure about the Streptomycin concentration (100mg/mL or 100ug/mL??).

**Response 3:** We are sorry for the mistake, the right Streptomycin concentration is 100 μg/mL (we corrected in the text).

**Point 4:** In method section 2.4, please mention the species you cloned the 739 bp UTR region of TRPS1 mRNA, also with the positions of the nucleotides. Is this region containing the SEED seq for miR-221-3p or 5p please indicate their positions? ([http://www.targetscan.org/cgi-bin/targetscan/vert_72/view_gene.cgi?rs=ENST00000395715.3&taxid=9606&members=&showcnc=0&shownc=0&showncf1=&showncf2=&subset=1#miR-221-3p/222-3p)?

**Response 4:** We added the required information in the Material and Methods section (Row 162-163).

**Point 5:** In method section 2.8, I am curious whether miR-221/222 is inter or intragenic? There are three species of non-coding RNA transcribed from opposite strand. Did authors have any idea what happens to miR-222 and IncRNA miR222HG expression in IVD cells? ([https://useast.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000207870;r=X:45746157-45746266;t=ENST00000385135].

**Response 5:** The miR-221/222 cluster is intergenic and positioned in the chromosome X (spanning from 45746157 to 45746266). We added this information as required (Row 251). We agree with the Reviewer: the scenario regarding regulatory elements is broad. Without entering into a too speculative discussion or listing the numerous data present in the literature for different experimental models, we added new References with a general sentence in Discussion: “In recent years, research on gene expression modulation during both physiological and disease processes of IVD is focusing on attributing critical roles to transcription factors and non-
coding RNAs (microRNAs and long non-coding RNAs), not only for what concerns their impact in diverse target genes, but also their interplay” (Row 461-465).

There is no evidence in literature on a specific role of lnc miR22HG in IVD cells. As regards miR-222, some data are present in literature as reported in reference n. 20 (mentioned in the first version of the paper) and in the new references added in the revised version (41-43). At the moment, the stored material from the samples used in the current study is very little and insufficient for further analysis: we should wait a few months to get a useful number of samples to perform other investigations. However, data from literature demonstrated that miR-222 is upregulated in IDD tissues. Therefore, in order to respond to the Reviewer curiosity, we added our hypothesis in Discussion taking into account evidence from literature (Row 508-514).

**Point 6:** Figure 1, Please replace Fig.1 (A & B) with higher quality.

**Response 6:** The images we uploaded in the document appear to us to be of good quality, perhaps in the Document for peer review quality was lost.

**Point 7:** Figure 2, western blot needed for TRPS1 in panel A and a known target for miR-221 in Panel B.

**Response 7:** From a human disc surgical sample the number of cells obtainable is very 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. Therefore, at this moment we are unable to satisfy the Reviewer request because the material is insufficient to perform the Western blot analysis. We are trying to overcome this limitation by using 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 target for miR-221, the experiments reported in Figure 2 are designed to demonstrate effectiveness of TRPS1 overexpression, not the action of miR-221 silencing. However, in order to satisfy Reviewer request we included in the Reviewer response the Figure below showing the increase of FOXO3 expression, a validated miR-221 target gene, in TRPS1 overexpressing IVD cells.
Point 8: Figure 3, fold enrichment over IgG does not specify percent binding over input. IgG nonspecific binding may be varied depending on the regions. I want to see the % binding over input using $=100 \times 2^{\text{adjusted input CT-IP CT}}$. Do authors have data on promoter activity (reporter assay) using promoter region 3 or region 1 including WT and mutation in the TRPS1 binding site?

Response 8: We agree with the Reviewer: we know that ChIP-qPCR data need to be normalized for sources of variability, including amount of chromatin, efficiency of immunoprecipitation, and DNA recovery. For this reason all qPCR results reported in Figure 3, were analyzed by the $2^{-\Delta\Delta Ct}$ method, normalized for Input signal (dCT) and presented as fold increase (mean ± SD) relative to the background signal (IgG). Accordingly, all data collected for all samples and regions, were corrected both for input content and background. This was specified with a new sentence we added in the legend of Figure 3.

In order to satisfy the Reviewer request, we included below a graphic representation showing the % binding over input using $=100 \times 2^{\text{adjusted input CT-IP CT}}$. As shown in the Figure, using % input, the binding profile of the analyzed samples evaluated for the different regions of miR-222/221 promoter, does not change. Consequently, our considerations reported in paragraph 3.2 are confirmed.
In this moment we have no data on promoter activity using promoter region 3 or region 1 including WT and mutation in the TRPS1 binding site.

**Point 9:** I also want to see ChIP assay for the level of H3K4me3 or H3K27ac modifications in region 3 and H3K36me3 modification on region 1 to support miR-221 transcription repression and reduced transcription elongation.

**Response 9:** Epigenetic chemical modification involved in the regulation of gene expression is a very interesting issue that certainly may strengthen the data we obtained. However, in order to perform chromatin immunoprecipitation assay with many antibodies high cells number is mandatory: from a human disc surgical sample the number of cells obtainable is very low and this preclude to satisfy the Reviewer request. 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 specific organ donation program.

**Point 10:** Figure 4D, I am confused about how anti-miR-221 degrading endogenous miR-221-3p? I thought anti-miR blocks the loading of the corresponding miR on to the RISC complex resulting inhibition. Do you know the mechanism of miR-221 degradation because of miR-221 antagomir treatment?

**Response 10:** The Reviewer considerations are corrects. The anti-miR oligonucleotides sequester the mature miRNA on the RISC complex, leading to functional inhibition of the miRNA and derepression of the direct targets. RT-qPCR is the gold standard technique for the evaluation of the efficacy of anti-miR treatment, as the generation of anti-miR/miR duplex can both lead to RNase-
H–mediated cleavage of the target miRNA or interfere with the reverse transcription and amplification process, with the resulting decrease in miR expression level.

**Point 11:** The same binding site of TRPS1 mRNA is targeted by miR-222-3p and TRPS1 transcriptionally controls both of them, did you check level of miR-222-3p. Finally, TRPS1 expression and control of miR-221 transcriptional fate and miR-221 expression and TRPS1 post transcriptional fate are highly and tightly regulated by tissue specific fashion. Any idea, how this fine balance is maintained? How you study is going to decipher that tissue specific balance?

**Response 11:** The questions asked by the Reviewer concern a very fascinating area. We are planning to continue the research by performing RNA Seq and Microarrays experiments in TRPS1 overexpressing cells and antagomiR-221 treated cells by using cadaveric IVD tissues.