Response to Reviewer 2 Comments

**Point 1:** The IVD is a complex joint consisting of NP, AF, and cartilaginous endplates. In this study, the authors used the mixture cells. Is it possible to investigate possible mechanisms in single type of cells?

**Response 1:** We thank the Reviewer for her/his observation which gives us the opportunity to better specify experimental model we used. We believe in the added value of the protocol we use: it is a previously established quick protocol to minimize artifacts from extended in vitro culture, without selecting the different types of cells from intervertebral disc (IVD) or completely disrupting extracellular matrix (ECM), but by using the whole cell population with a part of resident ECM. In the Material and 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 we used are mainly NP cells. However, 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 by us reported (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.” In addition, 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 139). However, in order to meet the Reviewer’s request we defined again the method we used, and added an explanatory sentence in the Results section (Row 335-338)

**Point 2:** Further studies are needed to investigate possible relationships of these molecules in disc degeneration animal model.

**Response 2:** In many cases animal models are very useful and in the literature many interesting evidences on IDD come just from disc degeneration animal models. However, IDD is a complex and multi-factorial process that is influenced by genetic predispositions, aging, biomechanical loading and physical activities, lifestyle and other health-associated factors: all these factors play a critical role in modulating cell behavior. In particular, the human posture can hardly be mimicked in an animal model and this is an important issue, in my opinion, that can risk producing errors in the interpretation of the data and in the proposal of therapeutic approaches. For this reason, with all the limitations that even human cellular experimental models can have, we would like to try to continue along this way, and possibly setting up 3D cell/scaffold based construct to obtain as much information as possible, before moving on to the animal model.
**Point 3:** Further studies are needed to investigate possible relationships of these molecules in different regions of the degenerated spine, ex: cervical and lumbar IVDs.

**Response 3:** We agree with the Reviewer. We believe that different spinal column regions may have molecular specific characteristics. We are very interested in this aspect that we are pursuing (obviously with the times dependent on the recruitment of biological samples from patients who are undergoing surgery). In fact, in the text we have mentioned this issue referring also to data previously published by us (reference 18 in the paper):

Row 306-311 “Highly degenerated discs, classified according to Pfirrmann grading system, expressed low levels of TRPS1, on the contrary high-level expression of TRPS1 was significantly associated with lower pathological stage. It is noteworthy that we not observed this association in the IVD from cervical spine which expressed TRPS1 at comparable levels regardless of the degree of degeneration [18].”

Row 410-412 “Unlike what was by us observed in freshly isolated (passage zero, P0) cells from cervical IVD samples [18], cells from lumbar IVD expressed substantial miR-221 at comparable levels regardless of the grade of degeneration (Figure 4A).”

Row 530-542 “A last aspect that will be worthy of further investigation regards the possibility that aberrant gene regulation occurs differently in diseased degenerative discs and normal aged discs, and in different regions of the degenerated spine [50-55]. This hypothesis is supported by the evidence we found examining cervical and lumbar IVDs. TRPS1 was expressed by cervical discs at comparable levels regardless of the degree of degeneration [18], whereas highly degenerated lumbar discs (Pfirrmann grades IV-V) expressed low levels of TRPS1 respect to discs with lower pathological stage. On the contrary, cells from lumbar disc expressed miR-221 at comparable levels regardless of the degree of degeneration, whereas high expression levels of miR-221 were found in highly degenerated IVDs from cervical spine. Although this observation deserves to be studied in depth, however the hypothesis that IVD from different spine regions may have molecular specific characteristics is to be kept in mind, especially in relation to the use of these data for the development of targeted therapies for diseases affecting neck and low back [3-5,56].”