**

Aug 30th, 2017

Dear Dr. DSouza:

We would like to thank the reviewers for their thorough review of our manuscript entitled “Evaluation of stem cell therapies in a bilateral patellar tendon injury model in rats” (JoVE56810). Their comments were well-taken and constructive.

In the revised manuscript, we incorporated the reviewer’s comments. A detailed response to the reviewer’s comments is provided below.

We believe that the revised manuscript is much improved and will be of great interest to the readers of Journal of Visualized Experiments and the wider scientific community. We are looking forward to hearing from you soon.

Best regards,

Dominique Griffon, DVM, PhD.

1. Please include at least six keywords/phrases.

*Sixth keyword are added.*

1. 2.10 Should be made into a note.

*It was made into a note part of 2.9.*

1. 1.1: Mention the animal type/strain used.

*Pregnant mare. No particular strain noted.*

1. 1.4: Do you use a microscope.

*No, anatomical feature of each component was identified by gross appearance.*

1. 3.1.1.1: Is this done on the plates from 2.9?

*No, as indicated earlier in the text, it is explanation on how to treat cells in standard plates. Anyone who has done cell culture should be able to understand what this sentence is talking about. If needed, we can add “From step 1.14”.*

1. 3.1.2.1: How do you collect/handle the spheroids? Mention pipette tip size if relevant.

*The sentences were changed and more details were added as shown below:*

*“3.1.2.1. Collect all spheroids by aspirating medium using pipette with 1000 µL tip. Transfer collected medium into 15 mL conical tube. After collecting medium, wash well by adding 1 ml of PBS, and transfer washed PBS into same 15 ml conical tube.“*

1. 3.1.2.3: Mention pipette tip size if relevant.

*The sentences were changed and more details were added as shown below:*

*“Add 500 µL of the cell dissociation reagent and incubate for 5-10 min at room temperature. Mix by pipetting using 1 ml tip until spheroids dissociate and are no longer visible.”*

1. 3.3.3: What are the final antibody concentrations?

*The antibody concentration was not provided for the ones we purchased. Instead, the recommendation from manufacture was to add 10 micro litter per 1 million cells, as described in manuscript.*

1. 3.3.8: Describe the gating scheme.

*The sentences were changed and more details were added as shown below:*

*“3.3.8. Analyze stained cell samples by flow cytometer. Exclude debris by their smaller SSC and FSC, and identify viable cells with lower uptake of 7-AAD. Plot FL1 and FL2 on y- and x- axis, respectively. Use isotype control to create a gate above diagonal line. Measure percentage of positively stained cells in the area. Count at least 20000 events/sample.”*

1. Section 4: Please add a step to mention and describe anesthesia (with drug dosage etc).

*Our anesthesia protocol is disclosed:*

*4.2. Anesthetize the rat with 8 % sevoflurane in 2 L 100 % oxygen delivered via mask, until disappearance of pinch-toe reflex in the induction chamber.*

*4.3. Inject Meloxicam (1mg/kg) subcutaneously as preemptive analgesia.*

*4.4. Place the rat between 2 0.5L water bottles filled with warm water and covered with a cloth to maintain body temperature and position, while preventing skin injury. Tape each extremity to the table with tape. To reduce risk of hypothermia, cover the body with bubble wrap, (Figure 1).*

*4.5. Maintain anesthesia with continuous flow of 5 % sevoflurane in 1 L 100 % oxygen mixture via nose cone with the animal on dorsal recumbency on water heating pad.*

1. 4.1: Mention animal strain, age, sex, weight.

*There is description on animal strain, age, sex, weight:*

*“Collect 5 mL blood by cardiac puncture from the anesthetized Sprague-Dawley rat (adult male, 4-5 months old, body weight 350-375 g), in a 5 mL syringe with 20 gauge needle containing 1 mL of acid-citrate-dextrose (5:1 v/v). “*

1. 4.5: “A clot will form, retaining cells at the surgical site.” This is confusing, isn’t this in the suspension? Where does the surgical site feature here?

*Cell suspension will be gel-like clot due to mixture with 100 micro litter of ACP. The surgical site is the central portion of patella removed.*

1. 4.5: Please mention the dissection steps, how is the tendon exposed? Mention surgical tools used.

*The tendon is immediately visible once the skin is incised.*

*Surgical approach and tools used are described in section 5. 7-8:*

*“4.8. Incise the skin with a #15 scalpel blade in a proximal to distal direction, on the craniomedial aspect of the stifle. The incision starts about 1 cm proximal to the level of the patella and extends approximately 5 mm distal to the tibial tubercle.*

*4.9. Reflect the skin to expose the patellar tendon by freeing the underlying subcutaneous tissue with a #15 scalpel blade.”*

1. Section 4 should like appear after Section 5 for clarity.

*Order of section 4 and 5 were switched.*

1. 5.3: What is the purpose of the bubble wrap?

*It was used to prevent hypothermia. Amended*

1. 5.4: With scalpel?
2. 5.10: When is the clot (4.5) placed in the defect? How many days after the surgery in section 5?

*Clot was placed after creating window defect in patellar tendon. The sentence was changed accordingly:*

*“5.7. Mix cell suspension (20 µL) and ACP (100 µL) to form a clot (Figure 6). Place the clot within the patellar tendon defect created before closing the fascia of the stifle.”*

1. 5.12 should likely appear before 5.11.

*The order of 5.11 and 5.12 was switched.*

1. 7.1: Mention anesthesia dosage.

*Sentence was changed accordingly:*

“*Euthanize rats at 7 days (to evaluate inflammation) or 28 days (to evaluate tissue healing) post treatment by intracardial injection of pentobarbital (100 mg/kg) under anesthesia with 8 % sevoflurane and 2 L 100 % oxygen delivered via mask.”*

1. 1.7,1.8,1.9 should be highlighted for continuity.

*These steps have been highlighted.*

1. Section 3: Antibody incubation steps should be highlighted.

*Section 3. 3. 3 (antibody incubation step) was highlighted.*

1. Please specify the format of quantitative results presented, for example, was mean ± standard deviation used? Mention sample sizes. Please mention the statistical tests performed.

*The sentences were changed and more details were added as shown below:*

*“In the current study, results are presented as mean ± SD (standard deviation). Cells were isolated from umbilical cord of 6 mares and percentage of isolated cell lines expressing each cell surface marker under standard or chitosan conditioning were compared with a Friedman test, as non-parametric analysis of variance with repeated measures. For tendon defect model creation, 8 rats were used for 7 days post-surgery assessment and 12 rats were used for 28 days assessment. Results of functional outcome are presented as mean ± SEM (standard error of the mean) and compared using t-test. UCM was selected as cellular source due to its”*

1. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Accutase.

*We replaced ‘Accutase’ with cell dissociation reagent (Accutase).*

1. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

*Figure 9 was adopted from previous publication. However, we have not obtained re-using permission. Additionally, the result of surface marker expression is not essential in the article, as our focus is surgical procedure to create patellar tendon defect in rat. Therefore, figure 9 was removed, but results were kept in manuscript with proper citation.*

Reviewer 1.

1. The authors used special staining techniques that did not allow them to evaluate Tendon repair. While Collagen is an important element of tendon structure but as stated by the authors Elastin fibers are the essential ECM component in the Tendon. Also, authors could use formation of Tenocytes as another indicator. Using markers for Elastin and tenocytes after cell therapy could be a useful tool to determine the degree of tendon repair. Adding this information will substantially increase the value of the paper.

*Thank you for these constructive suggestions on. The use of Masson’s Trichrome and evaluation of Collagen was chosen because Collagen grade of repaired tissue was one of the criteria used in the histology scoring system used in our study. The system was adopted from a previously validated histological scoring for tendon repair, which had been shown to correlate with tensile strength1. Further studies with Elastin staining or other tendon markers such as Scx, Tnmd would certainly be beneficial in evaluating tendon healing and repair for future studies, listed at the end of our manuscript:*

*“In the future, the ability of our model to discriminate treatments based on histological characteristics may be improved by integrating additional criteria such as presence of tenocytes, extracellular matrix organization, proteoglycan content, and distribution of elastin fibers52,59.”*

1. Authors used equine umbilical cord derived MSCs in an immune competent rat model. While xenogeneic transplantation of MSCs has been reported but the authors need to elaborate on this aspect. The inflammation described by the authors at day 7 post transplantation may be related to the immune response to the xenogeneic cells. Also, any information about the immune modulatory property of the equine umbilical cord derived MSC could be beneficial to the reader to understand justification of a xenogeneic test model. It is also important to know if this material is safe for clinical application. Are there adventitious agent concerns or allergic reaction considerations?

*Equine cells were selected in this project in view of clinical application in horses with tendon disease. This study was a pre-clinical trial. We believe equine MSCs did not elicit immune reaction in immune competent rat. There were inflammation in tendons at 7 days post-surgery in treated as well as empty defects, as described in the sentence “Upon histological examination, inflammation was observed in all of the tendons tissue sections examined at 7 days after surgery, regardless of the presence of treatment.” And the inflammation was not noted at 28 day post-surgery, when inflammation phase had passed and repair was transitioning from proliferative to remodeling phase. Report on in vivo implantation of equine UCM-MSCs is limited and there has been only one report using equine UCM-MSCs implanted in healthy horse muscle (allogenic)2. And our study was the first to report equine UCM-MSCs implanted in rat model. Regarding xenotransplantation, one review had reported 93.6% of studies using MSCs xenotransplantation had evidence of MSCs engrafted and functioned, and only 6.4% had evidence of failure to function3. So that safety of allogenic or even xenogenic transplantation have been extensively studied and established in MSCs treatment.*

*However, the scope of this manuscript is not to discuss the treatment or cells, but instead, to focus on the creation of a bilateral tendon injury model in rats, without significant morbidity. This model may be applied to any stem cell. Therefore, a discussion on immune privilege and immunomodulatory properties of MSCs was not included in this manuscript.*

1. What is the conclusion with this study. Did the stem cell therapy assist with tendon healing? And was there a difference between the chitosan cultured cells and non-chitosan cultured cells? The authors describe disorganization of collagen fibers, angiogenesis and chondrocyte formation after cell therapy. These histological observations do not match the clinical improvement. Any explanation?

*The conclusion of this study has been clarified:*

*“This bilateral patella tendon defect model controls inter-individual variation via creation of an internal control in each rat, was associated with acceptable morbidity and allowed detection of differences between untreated tendons and treatments.”*

*The effects of treatment are presented and discussed in another publication.4*

*Regarding contradicting result of clinical improvement and increasing histological score, we only observed an increase in cartilage formation between day 7 and 28. Cartilage formation and fibrous scar tissue formation is one of the main problems why damaged tendon tend to re-injure at the site of cartilage or fibrous scar. We were able to observe functional improvement of animals and biomechanical properties of harvested tendon. However, treatment did not prevent cartilage formation, which is a natural response to tendon injury. We invite the reviewer to access the reference (open access) for further details.*

1. The paper did not have a page number or line number so it makes it difficult to pin point to special section of the manuscript.

*Line numbering was added.*

1. In second page, first paragraph of the introduction, the authors stated that they have previously observed the formation of spheroids and gave some references (11-16). Almost all these references point to promoted chondrogenic potential under this condition. Seeing chondrocyte and moderate cartilage formation in the tendon might also reflect that perhaps this system of culture is more suitable for cartilage repair than tendon repair. MSCs are multipotent and are known to differentiate into at least three cell types (chondrocyte, osteocyte and adipocyte). One of the big challenges in cell therapy is to hope the stem cells differentiate into the cell type that is surrounded. Fig.11, C and F contains some adipocytes. It is difficult to say if these are originated from the equine cells or are rat adipocytes. These points need to be stated and discussed in the revised manuscript.

*It is true that many spheroid formation of MSCs have been found to correlate with better chondrogenesis as stated in references. In addition, hypoxia conditioning is known to enhance chondrogenesis of MSCs5,6. Adipose tissue found in Fig. 11 C and F might be equine MSCs origin or rat native tissue. Interestingly, there appeared to be more adipocyte in tendon of conditioned cell treatment group. Therefore, it can be interpreted implanted MSCs differentiated to chondrocyte or adipocyte due to their multi-lineage differentiation capacity. However, all these evidences on differentiation capacity are in vitro phenomenon and there has been no answer to the question whether implanted MSCs indeed differentiated to desired lineage or not. MSCs might have functioned to improve healing by trophic factor release or immunomodulatory properties under acute inflammatory environment. It is very important and interesting to know what properties of MSCs contributed clinical improvement or biomechanical strength of tendon. However, the scope of this study was to investigate the effect of preconditioning on clinical outcome or biomechanical properties of repaired tendon, which has been discussed in previous publication4. Although further investigation on MSCs behavior after implantation is warranted, this issue was beyond our scope and therefore not included in the manuscript.*

1. Protocol, 1.3. Trice is not a common English word. Please replace that with three times. 1.6. How much of the tissue is digested after 3-4h and on average how many cells per gram of the umbilical cord tissue is expected to obtain?

*The term thrice was replaced with three times throughout the manuscript. The number of isolated cells were not counted as we did not perform red blood lysis to minimize negative effect on MSCs. MSCs were selectively isolated using their plastic adherent property following 12 - 24 h incubation by medium change. However, we had used 12 – 15 g of tissue and SVF was seeded onto T25 flask, which then lead to 80 – 90 % confluence by day 7. As a reference, isolation of 10 – 50 \* 10^3 cells/cm of umbilical cord from human is reported7.*

1. Results, second page, second paragraph, the authors stated that rats recover after 28 days of transplantation. This is very fast as compared to tendon repair in the horse that takes months to occur. May be the size of the injury and the weight of animal or species differences are the reason for the fast recovery in rat as compared to slow recovery in horse. Using some references discussing the time require for full tendon recovery in horse could help the reader to understand the difference.

*We appreciate the suggestion to discuss on function recovery differences between species. It is very important to appreciate rat model we had created differ from horse model or patient in terms of weight or biomechanics. Indeed, functional recovery of rat model was relatively faster than horse patients and this might be due to light weight and lower load on injured tendon. Although not included in this manuscript, even functionally normal rats had lower biomechanical properties of tendon compared to normal rats’ tendon. This suggest full recovery of tendon had not been achieved by day 28.*

*The scope of this study was comparison of different treatments using small rodent model as proof-of-concept for the horse application. The results obtained by using this rat model may not be directly extrapolated to horse patients, but it is a robust model to test a new treatment before trial in larger animals, because small size and fast life cycle reduce the cost and time needed for study. Also we believe fast functional recovery is advantageous to observe difference in treatments’ efficacy in short term. Therefore, species difference of tendon recovery was beyond our scope and not discussed in this manuscript.*

1. Discussion, first page last three lines and the first line in the second page of discussion, the authors compare the spheroid with bioreactor and conclude that spheroid is a more cost effective for scale up manufacturing of MSCs. It is important for the reader to know how many cells can be grown on the spheroids as compared to bioreactor. This is debatable, as for scale-up patient therapy it becomes very demanding and labor intensive to handle so many culture dishes at a time.

*Bioreactor, ultra-low attachment culture plate, hanging drop method, chitosan film coated plate all forms spheroids. And spheroid size, number, and cellular number growing within spheroids vary depending on initial cell number, medium properties, etc., but it is consistently observed cells proliferate much slower within spheroid independent of which method used. Therefore, spheroid formation itself is not suited for scale-up therapy of MSCs. Instead, as several study reported previously, spheroid formation has been found to improve properties of MSCs4,8.*

*The advantage of chitosan film is low cost of the material itself due to the fact that chitosan is by-product of seafood industry. As discussed in the manuscript, other methods require either equipment to create specific condition (e.g. rotating reactor) or expensive coating of culture flask. On the other hand, chitosan film coating is easy and can coat any size of surface area, so that it is relatively easier when scale-up of spheroid formation is necessary. To make easier for readers to understand, the sentences were changed and more details were added as shown below:*

*“Among various 3D culture techniques such as spinner flasks, rotating cell bioreactors, non-adherent plate, natural and synthetic matrices, chitosan film seems appealing because there is no necessity for expensive equipment, it is cost effective compared to non-adherent plate with synthetic matrices due to the fact that chitosan is by-product of seafood industry, and it is easy to change medium, which makes this technique suitable for scale-up patient therapy.”*

1. Figure legends. Fig 11. The arrows describing the blood vessels are in Fig 10. The statement describing the blood vessel belongs to Fig 10. It seems that Fig legends for Fig. 10 and Fig 11 need to be replaced.

*Figure legends 10 and 11 were switched and changes were made also in manuscript accordingly.*

1. Could NSAID administration falsely improved ambulation and reduced pain post surgery? Also, could it have reduced the effectiveness of the MSCs?

*NSAIDs were administered to all rats in the study as a therapeutic dose for pain control. There is rather weak evidence to absolute contraindicate the use of NSAIDs in subjects that have had post-operative surgery. And NSAIDs definitely improved and reduced post-surgical pain, which then lead to no difference in functional score between standard and conditioned cell treatment group4. However, functional score was measured not to evaluate the difference of treatment group, but to prove creation of bilateral tendon injury does not cause significant morbidity by NSAIDs administration. Although there have been studies that NSAIDs may interfere with stem cells, such as effect on hematopoietic stem cells (HSCs) migration pattern9, the exact mechanism behind the relationship of NSAIDs and MSCs is yet to be found.*

1. The expression of some surface markers changed in the presence of Chitosan. What could be the meaning of these changes? Are the cells differentiating in culture? Although small and arguably negligible MHC-II increase, could this increase raise concern for an immune response with allogenic application in the clinic? Could this increase have been caused by the use of Chitosan from shellfish shells?

*It will be very interesting to investigate phenotypical change along with surface marker expression change. It is possible that those cells with different surface markers are differentiating within spheroids. There are reports on relationship with reduced CD90 and CD105 expression and enhance osteogenesis or adipogenesis10,11. However, this issue was also beyond our scope and we put emphasis on enhancement of stemness genes (Oct4, Sox2, Nanog) and biomechanical strength improvement4. Further investigation on surface marker expression change and differentiation caused by culturing on chitosan is warranted.*

*As suggested by reviewer 1, slight increase of MHC II raise a concern to some extent. There is a report on mismatched haplotype of allogenic MSCs induced immune reaction, whereas MHC II negative MSCs suppressed immune reaction12. In the literature, MSCs which caused immune reaction expressed mostly above 50%, so that slight increase observed in our study may not elicit immune reaction upon allogenic application. However, it’ll be beneficial for future allogenic application to investigate on threshold to cause immune reaction.*

Reviewer 2.

1. The MSCs used for wounded rat tendon healing were isolated from mare placenta. Were any immunoreactions found in the rats caused by the mare MSCs? Because this is a kind of xenotransplantation. The healed tissue should be tested for anti-mare-MSCs antibodies, such as anti-rat collagen type I and anti-horse collagen type I antibodies. To demonstrate the healed tissue was from implantation of mare MSCs, not from rat self.

*There were inflammation from all injured tendon with or without MSCs treatment at day 7, which is not observed at day 28. And immune reaction associated with MSCs application was not observed in any tissue collected. Although collected tissue was not tested with α-horse or α-rat collagen type I Abs, the tracking of MSCs were performed by GFP expression of MSCs4. And presence of implanted MSCs were confirmed up to 28 days post-surgery. It’ll be difficult to determine which MSCs from horse or rat deposited collagen type I and contributed to tendon healing. Yet, we were able to conclude that tendon with MSCs treatment had better biomechanical properties compared to tendon without treatment. Therefore, MSCs from horse contributed to tendon healing either by differentiation, trophic factor, or immunomodulation. We appreciate very constructive suggestion on collagen staining as it will partly answer the question about what function of MSCs lead to improvement of biomechanical strength. This assay should be included in future study.*

1. Fig. 2 shows that a water bottle was used for temperature keeping. I suggest using an electric blanket to instead of bottle (better control the temperature and convenient for operation).

*Given the short anesthetic surgical procedure, warm water bottles were used. We can absolutely substitute for an electric blanket, or a circulating warm water heating pad.*

1. The picture in Fig. 3 is too small and it looks like the wire just past through the paratenon, not tendon. Please tell the thickness of the tissue above the wire (or provide a high-quality picture to see more details).

*Replaced - Figure 3 is a Tiff file at 1,600kb, which should suffice for online publication.*

*The wire is placed on the center of tendon. The tendon measures about 3mm in width but the thickness estimated at 1-2mm. Details should be easier to see online as readers will be able to magnify images. In addition, a video will be provided. We are willing to let the photographer take additional pictures during the session, if the Editor finds it necessary.*

1. The picture in Fig. 4 is not clear. It is difficult to find the wounds (too much blood). Please provide a larger picture shown the wound area.

*Replaced - Figure 4 is a Tiff file at 790kb. The central portion is clearly identified as the band of tissue positioned over the forceps and identified with an arrow.*

*Details should be easier to see online as readers will be able to magnify images. In addition, a video will be provided. We are willing to let the photographer take additional pictures during the session, if the Editor finds it necessary.*

1. Where is the defect in Figure 5? Please provide a high-quality picture and add an arrow to tell the wound area.

*Replaced- The Tiff file is 765kb*

*Details should be easier to see online as readers will be able to magnify images. In addition, a video will be provided. We are willing to let the photographer take additional pictures during the session, if the Editor finds it necessary.*

1. Why are right tendons bigger than left tendons shown in Figure 7?

*The changes are consistent with postoperative inflammation, but we do not have evidence to explain this inconsistent finding. In Figure 7, two tendons from right hind limbs showed thickening. However, thickening of tendon was observed in both right and left limbs. Also there was no relationship between tendon thickening and presence of treatment. This was discussed in result section as shown below:*

*“About 37% of tendons collected at 28 days appeared markedly thickened (Figure 7). This thickening was equally distributed between empty and treated defects.”*

1. All the pictures should be larger so that the wound and treatment can be seen more clearly.

*Addressed above*

1. The staining results on stem cell markers should be added in Figure 8.

*Due to the fact that we did not obtain permission to adopt Figure 9 (staining of stem cell markers) from previous publication and limited time frame, we removed Figure 9. However, the same result was left in result section with proper citation.*

1. How were the results shown in Figure 9 obtained? By Flow cytometry? Or immunostaining? If the results were obtained by flow cytometry, please provide flow cytometry graph (or staining picture).

*Result of Figure 9 was obtained by flow cytometry. Scattered plot of flow cytometry is previously published and was excluded from this manuscript, because the focus of this manuscript was methodological explanation of surface marker expression from MSCs within spheroids and brief presentation of result. However, citation of our previous publication for surface marker expression level will aid in obtaining detailed information of flow cytometry data13.*

1. Figure 10: were these tissue sections from patellar tendons? Why is the organization of the collagen fibers so poor? The normal tendon sections with the same staining should be provided.

*These sections were from patellar tendons. It is unclear what caused the disorganization of collagen after surgery. The reason of disorganized collagen fibers is possibly due to change in biomechanical properties after surgery, such as dislocation of well aligned collagen toward central defect or change in direction of tensile stress moves through tendon. For comparison, the normal tendon histology was added to the figure 10,11, and 12 for comparison.*

1. Figure 11: please indicate the wound area and the healed tendon area in each picture.

*In all the figure of histology including Figure 11, the images represented as empty or treated (A – L) shows only wound area because each images were approximately 800 x 600 μm and were not large enough to include both normal and wound area of tendon. Instead, images of normal tendon were added (M, N).*

**References**

1 Rosenbaum, A. J. *et al.* Histologic stages of healing correlate with restoration of tensile strength in a model of experimental tendon repair. *HSS J.* **6** (2), 164-170, doi:10.1007/s11420-009-9152-5, (2010).

2 Dias, M. C. *et al.* Intramuscular Transplantation of Allogeneic Mesenchymal Stromal Cells Derived from Equine Umbilical Cord. *International Journal of Stem Cells.* **9** (2), 239-249, doi:10.15283/ijsc16011, (2016).

3 Li, J., Ezzelarab, M. B. & Cooper, D. K. C. DO MESENCHYMAL STEM CELLS FUNCTION ACROSS SPECIES BARRIERS? RELEVANCE FOR XENOTRANSPLANTATION. *Xenotransplantation.* **19** (5), 273-285, doi:10.1111/xen.12000, (2012).

4 Griffon, D. J. *et al.* Effects of Hypoxia and Chitosan on Equine Umbilical Cord-Derived Mesenchymal Stem Cells. *Stem Cells Int.* **2016** 2987140, doi:10.1155/2016/2987140, (2016).

5 Adesida, A. B., Mulet-Sierra, A. & Jomha, N. M. Hypoxia mediated isolation and expansion enhances the chondrogenic capacity of bone marrow mesenchymal stromal cells. *Stem Cell Res Ther.* **3** (2), 9, doi:10.1186/scrt100, (2012).

6 Buravkova, L. B., Andreeva, E. R., Gogvadze, V. & Zhivotovsky, B. Mesenchymal stem cells and hypoxia: where are we? *Mitochondrion.* **19 Pt A** 105-112, doi:10.1016/j.mito.2014.07.005, (2014).

7 Weiss, M. L. *et al.* Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells.* **24** (3), 781-792, doi:10.1634/stemcells.2005-0330, (2006).

8 Hsu, S. H., Huang, G. S. & Feng, F. Isolation of the multipotent MSC subpopulation from human gingival fibroblasts by culturing on chitosan membranes. *Biomaterials.* **33** (9), 2642-2655, doi:10.1016/j.biomaterials.2011.12.032, (2012).

9 Hoggatt, J. *et al.* Differential Stem and Progenitor Cell Trafficking by Prostaglandin E(2). *Nature.* **495** (7441), 365-369, doi:10.1038/nature11929, (2013).

10 Moraes, D. A. *et al.* A reduction in CD90 (THY-1) expression results in increased differentiation of mesenchymal stromal cells. *Stem Cell Research & Therapy.* **7** (1), 97, doi:10.1186/s13287-016-0359-3, (2016).

11 Anderson, P., Carrillo-Gálvez, A. B., García-Pérez, A., Cobo, M. & Martín, F. CD105 (Endoglin)-Negative Murine Mesenchymal Stromal Cells Define a New Multipotent Subpopulation with Distinct Differentiation and Immunomodulatory Capacities. *PLoS ONE.* **8** (10), e76979, doi:10.1371/journal.pone.0076979, (2013).

12 Schnabel, L. V., Pezzanite, L. M., Antczak, D. F., Felippe, M. J. B. & Fortier, L. A. Equine bone marrow-derived mesenchymal stromal cells are heterogeneous in MHC class II expression and capable of inciting an immune response in vitro. *Stem Cell Research & Therapy.* **5** (1), 13-13, doi:10.1186/scrt402, (2014).

13 Taguchi, T. *et al.* Influence of hypoxia on the stemness of umbilical cord matrix-derived mesenchymal stem cells cultured on chitosan films. *Journal of Biomedical Materials Research Part B: Applied Biomaterials.* n/a-n/a, doi:10.1002/jbm.b.33864, (2017).