

Comparison between Embryonic Stem Cell versus Adult Stem Cell to Restore Cartilage Repair: An Experimental Study

Manunta AF¹, Manunta ML², Ortu S^{1*}, Uboldi FM¹

¹Department of Orthopaedic Surgery, University of Sassari, Italy

²Department of Veterinary Medicine, University of Sassari, Italy

*Corresponding author: Ortu Sebastiano, Department of Orthopaedic Surgery, University of Sassari, Italy, Tel: +393396744945; E-mail: sebastiano.ortu10@gmail.com

Abstract

This study aimed at comparing quantitative and qualitative results of experiments based on embryo cells (embryonic stem-like – ESL) relative to medullary stimulation (BMS) for chondral lesions treatment of Medial Femur Condyle (MFC) in sheep. Male ESL cells embedded in fibrin glue were engrafted into chondral defects in the MFC of 4 ewes. An identical defect was created in other 4 sheep and treated with Nanofracture technique for bone marrow stimulation. Sheep were euthanized at 12 months from surgery. The evaluation of regenerated tissue was performed by macroscopic, histological, immunohistochemical (collagen type II) and Fluorescent in situ Hybridization (FISH) assay. When the BMS and ESL scores at 12 months were compared, statistically significant differences in some macroscopic categories scores were found between the two treatments: ESL samples recorded significantly higher matrix staining score ($p = 0.01$), and tidemark score ($p = 0.04$); BMS samples had significantly higher points in subchondral bone category ($p = 0.01$). No significant differences were found in the others categories. At 12 months, collagen type II immunostaining was detected and marked in all ESL samples. In BMS samples the repaired tissue resulted positive for type II collagen especially around the performed perforations. FISH showed positive intranuclear signals only in the ESL cells found in the newly formed tissue. This study demonstrated that ESL cells, engrafted into chondral defects in sheep knee condyles, enhanced the quality of the regeneration of articular hyaline cartilage. In addition, it confirmed that BMS technique can provide for a good subchondral bone remodeling and a fair quantitative filling of chondral defects.

Received date: August 27, 2017

Accepted date: October 20, 2017

Published date: October 27, 2017

Citation: Ortu Sebastiano, et al. Comparison between Embryonic Stem Cell versus Adult Stem Cell to Restore Cartilage Repair: An Experimental Study. (2017) J Nanotechnol Mater Sci 4(2): 87- 91.

DOI: 10.15436/2377-1372.17.1661

Keywords: Stem; Cell; Cartilage; Repair; Medullary stimulation; Arthroscopy



Introduction

Nomenclature	
ESL	embryonic stem like
BMS	bone medullary stimulation
MFL	medial femur condyle
FISH	fluorescent in situ hybridization
ACI	autologous chondrocyte implantation

Background

Osteoarthritis is characterized by degeneration of the

articular cartilage, with loss of matrix, fibrillation, formation of fissures, and ultimately complete loss of the cartilage surface. Cartilage self-renewal potential is limited and articular cartilage defects rarely heal spontaneously. Numerous surgical procedures have been developed to address focal cartilage defects, yet controversy remains in distinguishing a superior technique. Regenerative procedures aiming at recovering the articular cartilage with a high qualitative repair tissue as similar as possible to the hyaline physiological one: good and longer-lasting results have been reported using Autologous Chondrocyte Implantation (ACI) and successively using matrix assisted techniques that have been introduced to improve the intrinsic limits of ACI; in



Copyrights: © 2017 Ortu Sebastiano. This is an Open access article distributed under the terms of Creative Commons Attribution 4.0 International License.

the end, biphasic scaffolds have been introduced to address both cartilage and subchondral bone problems. None of the different methodologies currently available has been proved efficient to regenerate the articular surface both from a biological and a mechanical point of view^[1]. Currently, the surgical technique mostly applied by clinicians is the medullary stimulation through perforations (micro fractures) first developed by Steadman at the beginning of this century^[2]. The biological rationale behind medullary stimulation is ascribed to the role of mesenchymal stem cells recruited by the subchondral bone. Although the advantages of such technique include the fact that is a minimally invasive, simple, fast and cost-saving procedure (favorable cost-effectiveness ratio), it results into a reduced quality of the newly formed tissue; moreover, this procedure often only delays the occurrence of degenerative processes without effectively stopping them^[3]. Among the different methods of BMS, subchondral needling, characterized by smaller-diameter and deeper perforations (nanofracture), seems to offer an advantage over micro fracture because perforation depth and diameter are consistent and reproducible, the smaller diameter causes less trauma to the subchondral bone surface and may provide a greater perforation density, deeper access channels to the marrow stroma produce superior cartilage repair when compared to shallower channels. If medullary stimulations represent the most applied methodology (80.000 procedures performed every year in U.S.^[4]), instead cutting-edge techniques such as stem cells represent the most advanced scientific frontier. Systemic or local stem cells administration might promote cartilage repair or inhibit the progressive loss of cartilage tissue^[5].

This study aimed at comparing quantitative and qualitative results of experiments based on embryo cells (embryonic stem-like – ESL) relative to medullary stimulation (BMS) for chondral lesions treatment of Medial Femur Condyle (MFC) in sheep, to better understand the future care possibilities compared with traditional techniques.

Materials and Methods

Study design

Eight Sardinian ewes, about 5,5 years of age and weighting approximately 45 kg were divided into two groups of four (group-A and group-B). All animals were given a general health examination, routinely dewormed and had their claws inspected prior to the experimental surgery. All animals were found to be in good health. Standardized circular full-thickness chondral defects were created in the MFCs of the sheep in the load bearing area. In group-A, five perforations by using the nanofracture system (Arthrosurface, Franklin, MA, USA) were performed in the defects after removing the entire calcified cartilage. In group-B, surgical defects created received 60 µl of fibrin glue (Tissucol - Baxter International Inc., Deerfield, IL, USA) containing about 500.000 - 700.000 ESL cells engrafted derived from 2 - 3 male ESL colonies. Macroscopic and histological evaluation of cartilage repair was performed after 12 months post-surgery.

Surgical technique

Surgery was performed by use of sterile conditions and with sheep under general anesthesia. All sheep were intubated after the administration of thiopentone (25 mg/kg) and ventilated

with O₂ in N₂O by volume control. Anesthesia was maintained with 1.5 to 2% isoflurane; a bolus dose of 0.1 mg of fentanyl was given before surgery. In all animals a medial parapatellar arthrotomy was performed to expose the MFC of the left knee. An 8 mm diameter (area: 50,3 mm²), full-thickness chondral lesion in the load bearing area of each MFCs was created using an arthroscopic burr (Figure 1). The calcified layer was removed; vertical walls were created at the periphery of the cartilage lesion.

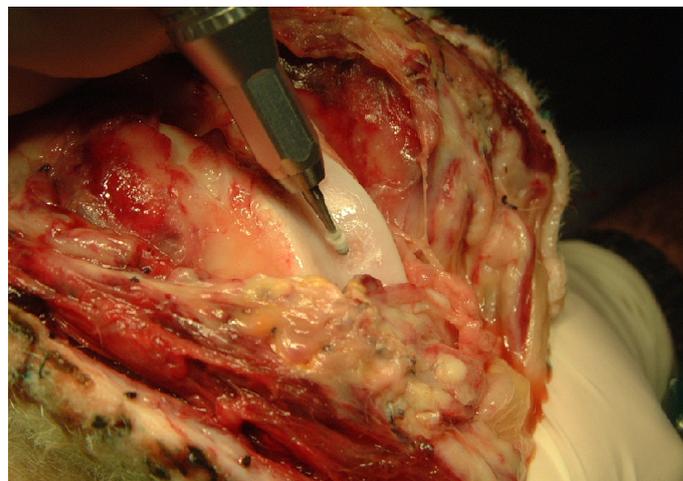


Figure 1: Full thickness defect of medial femoral condyle of right knee obtained with abradar.

In group-A, the defects were then treated with Nanofracture: each cartilage lesion was treated with 5 channels having a 9 mm depth using a cannulated awl and a 1mm thick Nitinol needle with a 3 mm distance between each channel; the surgical technique was previously described^[6]. In group-B, liquid phase duplex Polymerase Chain Reaction (PCR) was performed on approximately 90 *in vitro* produced and vitrified blastocysts at day 6 - 7 of culture according to the technique of Mara *et al.*^[7] as previously described^[8]. About 500.000 - 700.000 cells were embedded in 60 µl of fibrin glue and engrafted into the cartilage defects. Upon completion of the cartilage repair procedures, all incisions were closed in layers according to standard surgical practice, with 5 days of prophylactic antibiotic treatment and an anti inflammatory agent administration (40 mg/kg/day amoxicillin and 2 mg/kg/day ketoprofen). Postoperatively, the animals were kept in stalls with limited movement and weight bearing. After surgery, the animals were left free to roam in their fencings without any immobilization of the operated limb. Full weight-bearing was allowed as tolerated and no specific exercise regimen was adopted. General health and weight-bearing status were monitored by a veterinarian doctor during recovery.

Evaluation of articular cartilage repair

Immediately after euthanasia at 12th month, the defects were photographed to allow assessment by double blind observers. A semi-quantitative scoring system (Table 1) was used to assess the regenerated tissue and obtained values were averaged.

Table 1: Semi-quantitative scoring system for macroscopic evaluation of regenerated cartilage defects.

Categories	Score	Qualifications
• Filling of defect	3	100% normal
• Architecture of surface	2	> 75% normal
• Bone	1	50 - 75% normal
• Tidemark	0	< 50% normal
• Matrix staining	Graded on a scale of 0 to 10, with 0 = no	
• Subchondral bone	repair and 10 = complete regeneration	
• Edges integration	3	100% of perimeter
	2	> 75%
	1	50 - 75%
	0	< 50%

Condylar articular defects containing regenerated tissue, adjacent host cartilage and subchondral bone were harvested using a water-cooled circular saw. The tissue blocks were fixed in 10% neutral-buffered formalin for 4 days and then placed in a decalcification solution for 4 - 10 days. After washing in running tap water for 4 - 8 h to remove all traces of decalcification solution, the osteochondral specimens were paraffin-embedded and stained with safranin orange/fast green (Safranin O) to reveal the presence and distribution of proteoglycans and hematoxylin-eosin to evaluate general tissue morphology as described by Undale *et al.*^[9]. In group-A, immunohistochemical determination of type I and type II collagen contents was conducted on paraffin-embedded sections using a 1/50 dilution of a monoclonal mouse anti-type I or anti-type II collagen IgG and a biotinylated secondary anti-mouse antibody. In group-B, deparaffinization through xylene and hydration through alcohol at different concentrations were followed by antigen unmasking by protease K digestion. After Tissue-Blocker treatments, the slides were incubated overnight at 4°C with a primary antibody (anti-Collagen type II CIICI-DSHB) followed by a biotinylated secondary anti-mouse antibody and then 3% peroxidase solution. Immunoreactivity to type I collagen in the repair tissue was compared with that of the adjacent subchondral bone, serving as positive internal control, with following rating system: 0 = no immunoreactivity; 1 = significantly weaker; 2 = moderately weaker; 3 = similar; 4 = stronger immunoreactivity. To detect ESL cells in the regenerated tissue of the group-B, a Fluorescent *in situ* Hybridization (FISH) assay was performed on one sample according to the method of Sanna *et al.*^[10].

Statistical analysis

Statistical analysis was performed both on macroscopic and histological data. The level of significance was $p < 0.05$.

Result

No problems related to locomotion were noted in any of the animals during the study.

Fluorescent in situ Hybridization (FISH) procedure

Dot-Blot showed positives spots in DNA ESL cells sample and male fibroblasts (positive control), whereas female fibroblasts (negative control) were negative, confirming the specificity of the chosen probe. FISH showed positive intranuclear signals only in ESL cells found in the newly formed tissue.

Histological evaluation

When the BMS and ESL scores at 12 months were compared, statistically significant differences in some macroscopic categories scores were found between the two treatments (Figure 2). ESL samples recorded significantly higher matrix staining score ($p = 0.01$), and tidemark score ($p = 0.04$). BMS samples had significantly higher points in subchondral bone category ($p = 0.01$). No significant differences were found in the others categories (Table 2).

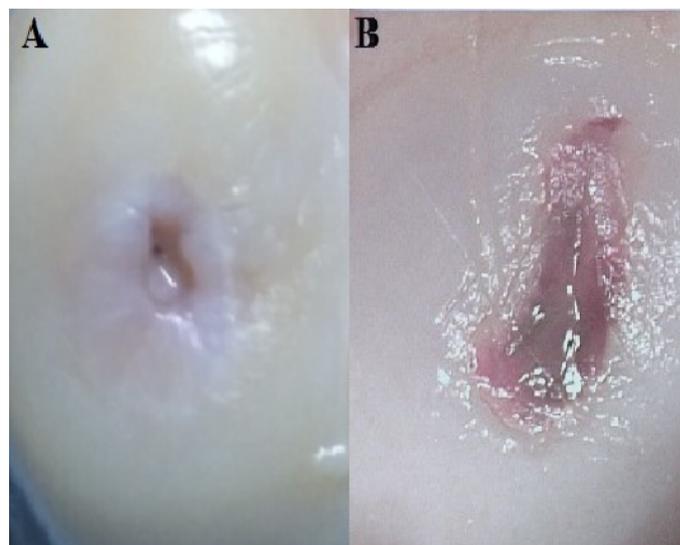


Figure 2: Macroscopic appearance at 12 months. A: ESL sample. B: Nanofracture (BMS) sample.

Table 2: Values are expressed as mean ± SD; values in bold indicate a significant difference between groups ($p < 0.05$).

Categories (12 months)	BMS (group-A)	ESL (group-B)	p value
Defect filling	1.75 ± 0.9	2.5 ± 0.5	0.22
Surface architecture	2.25 ± 0.5	2.75 ± 0.5	0.2
Matrix staining	5.75 ± 0.9	8.25 ± 0.9	0.01
Subchondral bone	9 ± 0.8	7 ± 0.8	0.01
Edges integration	1.75 ± 0.9	2.75 ± 0.5	0.11
Tidemark	1 ± 0.8	2.25 ± 0.5	0.04

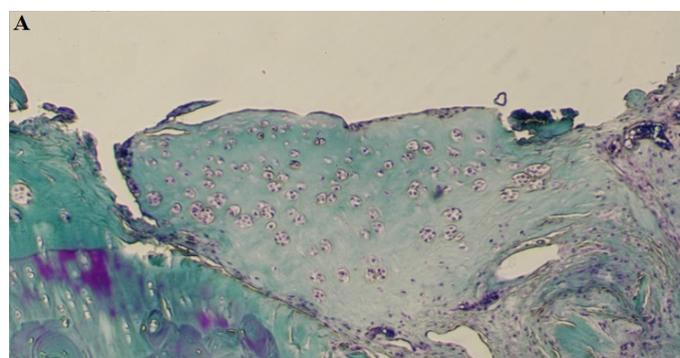


Figure 3A: Immunostaining ESL: in the reparative tissue can be observed numerous clones homogeneously located inside the matrix.

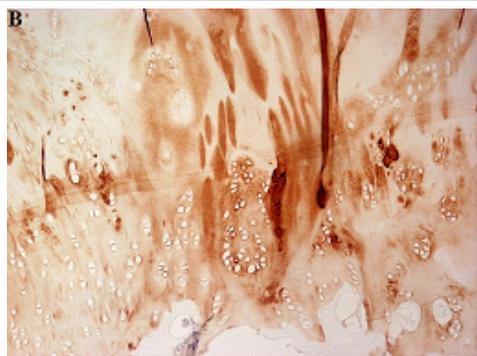


Figure 3B: Positivity for type II collagen after BMS, particularly around the chondrocytes clusters grown up in the deep layers.

Immunohistochemistry

At 12 months collagen type II immunostaining was detected and marked in all ESL samples. In BMS samples the repaired tissue showed many clones and positivity for type II collagen especially around the performed perforations (Figure 3).

Discussion

The most important finding of this study is that both techniques are effective to quantitatively fill a cartilage defect in a preclinical animal model at 12 months postoperatively; besides, the regenerated tissue shows higher quality for what concerns ESL cells compared to BMS cells.

The repair of defects involving articular cartilage through healthy hyaline cartilage continues to be a challenge.

Since the first use of microfracture by Steadman in the 1980's, BMS has become the treatment of choice for the management of chondral lesions^[2]. The technique is based on the direct stimulation of mesenchymal cells of the subchondral bone which have a high potential for differentiation into various connective tissues, including cartilage, bone, tendon and ligaments^[11,12]. The regenerative effects of mesenchymal stem cells achieve their aim in promoting tissue repair and leading to the resolution of inflammation through direct cell-to-cell interaction or by secretion of bioactive components^[13]. Recently, the use of standard micro fracture awls is no longer supported by basic science evidences, while best results are shown by smaller-diameter and deeper perforations. Eldrach et al. and Orth et al. recently published their comparative results treating full thickness defects in animal models reporting significant improvement in overall histological score using small diameter awls versus traditional ones^[14,15]. Our study is in agreement with the latter, showing beneficial effects using small diameter needle perforation.

In our previous study we found that ESL colonies were positive for SSEA mAbs and gene expression for Oct 4, *Nanog*, *Sox 2* and *Stat 3* genes, and negative for all the mAbs indicating the differentiated state, demonstrating that the engrafted cells were real stem cells^[8,16]. The histological results showed better healing in ESL as compared to BMS samples. The more complete filling of the defect in the ESL treatment was probably due to a faster cell replication rate and a higher level of metabolic activity. Microscopic examination showed a more normal histological architecture and a better quality of the matrix in the newly formed cartilage in ESL samples. This healed cartilage

was comparable to the normal hyaline cartilage in one animal: with a superficial zone with proliferating germinal cells and collagen fibers distributed parallel to the articular surface, and a deep zone with calcification and formation of a discontinuous tidemark. The presence of the tidemark is an important finding; this calcification front is in a dynamic equilibrium state, where factors promoting mineralization are probably counterbalanced by substances that inhibit or limit the extent of calcification, and its presence is a joint health signal. In none of the BMS samples tidemark was detected (Figure 4).

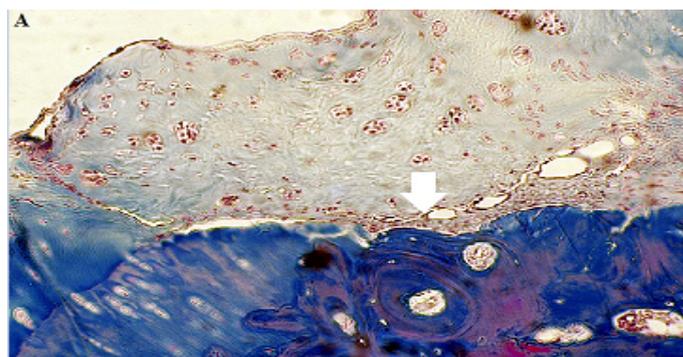


Figure 4A: The aspect of ESL osteochondral junction does not appear as a usual adult hyaline cartilage one, but tundulation and tight contact are prerequisites to tidemark formation.



Figure 4B: in Nanofracture (BMS) sample the tidemark is not detectable.

Immunolocalization of type-II collagen, the main type of hyaline cartilage, was detected in both treatment groups; in ESL was more intense as compared to BMS samples. Similarly, Safranin-O staining showed higher synthesis of proteoglycans in ESL samples. The complex interplay existing between these two components, is reported to be of crucial importance: the collagen fibrils serve to anchor the proteoglycan matrix and contribute to resisting extrinsic forces during loading and intrinsic swelling that occurs metabolically within the proteoglycan domain^[17], while proteoglycan are responsible for the generation of hydrostatic pressure within cartilage matrix which allows it to counteract the loads transmitted to it from the long bones during normal joint movement. In group-A (BMS), the newly formed tissue showed fibro-hyaline repairing features in which clustered and diffusely distributed small round cell elements can be observed. The use of ESL cells engrafted in fibrin glue apparently enhanced a better distribution in filling the defect resulting into a higher quality of regenerated tissue attained compared to mesenchymal cells originated from canals created into the subchondral bone using BMS.

Subchondral bone remodeling was superior in BMS samples, showing natural irregularities of the nanofracture channel walls, with absence of trabecular compaction around the perforation and remarkable communication between pre-existing trabecular canals and the perforation as well as neo-trabecular remodeling inside the channels. None of the nanofracture samples showed subchondral cyst formation. Recent studies support the concept that subchondral bone and articular cartilage should be considered an interdependent functional unit, and a direct stimulation of this layer can promote a better healthy remodeling, that provides the right biomechanical support for hydrostatic compression in the cartilage, which is of critical importance for the differentiation of hyaline-like cartilage^[18]. Further studies are needed to evaluate the possibility of using the two tested techniques together.

Overall, inferences from an animal study are limited because the functional status cannot be assessed at baseline and follow-up evaluations. However, the animal model used in this study is considered suitable for cartilage defect testing. Additionally, limitations due to small cohort size should be reported. The strength of this study was the use of clinically available instrumentation and their application according to previously described methods.

Conclusion

In conclusion, this study demonstrated that ESL cells, engrafted into chondral defects in sheep knee condyles, enhanced the quality of the articular hyaline cartilage regeneration. In addition, it confirmed that BMS technique can provide for a good subchondral bone remodeling and a fair quantitative filling of chondral defects.

Acknowledgement: MAF conceived the study and performed the experiments. MML, OS and UFM helped MAF to realize the study in equal contribution. All authors read and approved the final manuscript.

Funding: There was no funding for the research for this manuscript for any of the authors.

Competing Interests: The authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.

Ethics Approval and Consent to Participate: This study was approved by the University Ethics Committee and all procedures were conducted according to the Institutional Animal Care regulations which conformed to the National Institute of Health Guidelines on the Care of Laboratory Animals.

References

1. Mazar, M., Lespessailles, E., Coursier, R., et al. Mesenchymal stem-cell potential in cartilage repair: an update. (2014) *J Cell Mol Med* 18(12): 2340-2350.
[PubMed](#) | [Crossref](#) | [Others](#)
2. Steadman, J.R., Rodkey, W.G., Rodrigo, J.J. Microfracture: surgical technique and rehabilitation to treat chondral defects. (2001) *Clin Orthop Relat Res* 391: S362-S369.
[PubMed](#) | [Crossref](#) | [Others](#)

3. Gill, T.J., Asnis, P.D., Berkson, E.M. The treatment of articular cartilage using the microfracture technique. (2006) *J Orthop Sports Phys Ther* 36(10): 728-738.
[PubMed](#) | [Crossref](#) | [Others](#)
4. McCormick, F., Harris, J.D., Abrams, G.D., et al. Trends in the surgical treatment of articular cartilage lesions in the United States: an analysis of a large private-payer database over a period of 8 years. (2014) *Arthroscopy* 30(2): 222-226.
[PubMed](#) | [Crossref](#) | [Others](#)
5. Murphy, J.M., Fink, D.J., Hunziker, E.B., et al. Stem cell therapy in a caprine model of osteoarthritis. (2003) *Arthritis Rheum* 48(12): 3464-3474.
[PubMed](#) | [Crossref](#) | [Others](#)
6. Benthien, J.P., Behrens, P. Reviewing subchondral cartilage surgery: considerations for standardised and outcome predictable cartilage remodeling: a technical note. (2013) *Int Orthop* 37(11): 2139-2145.
[PubMed](#) | [Crossref](#) | [Others](#)
7. Mara, L., Pilichi, S., Sanna, A., et al. Sexing of in vitro produced ovine embryos by duplex PCR. (2004) *Mol Reprod Dev* 69(1): 35-42.
[PubMed](#) | [Crossref](#) | [Others](#)
8. Manunta, A.F., Zedde, P., Pilicchi, S., et al. The use of embryonic cells in the treatment of osteochondral defects of the knee: an ovine in vivo study. (2016) *Joints* 4(2): 70-79.
[PubMed](#) | [Crossref](#) | [Others](#)
9. Undale, A.H., Westendorf, J.J., Yaszemski, M.J., et al. Mesenchymal stem cells for bone repair and metabolic bone disease. (2009) *Mayo Clin Proc* 84(10): 893-902.
[PubMed](#) | [Crossref](#) | [Others](#)
10. Sanna, E., Sanna, M.P., Loddo, C., et al. Endogenous jaagsiekte sheep retrovirus RNA is expressed by different cell types in ovine foetus and placenta. (2002) *Eur J Histochem* 46(3): 273-280.
[PubMed](#) | [Crossref](#) | [Others](#)
11. Caplan, A.L., Dennis, J.E. Mesenchymal stem cells as trophic mediators. (2006) *J Cell Biochem* 98(5): 1076-1084.
[PubMed](#) | [Crossref](#) | [Others](#)
12. Lodi, D., Iannitti, T., Palmieri, B. Stem cells in clinical practice: applications and warning. (2011) *J Exp Clin Cancer Res* 30: 9.
[PubMed](#) | [Crossref](#) | [Others](#)
13. Chen, F.H., Tuan, R.S. Mesenchymal stem cells in arthritic disease. (2008) *Arthr Res Ther* 10(5): 223.
[PubMed](#) | [Crossref](#) | [Others](#)
14. Eldracher, M., Orth, P., Cucchiari, M., et al. Small subchondral drill holes improve marrow stimulation of articular cartilage defects. (2014) *Am J Sports Med* 42(11): 2741-2750.
[PubMed](#) | [Crossref](#) | [Others](#)
15. Orth, P., Duffner, J., Zurakowski, D., et al. Small-diameter awls improve articular cartilage repair after microfracture treatment in a translational animal model. (2016) *Am J Sports Med* 44(1): 209-219.
[PubMed](#) | [Crossref](#) | [Others](#)
16. Dattena, M., Chessa, B., Lacerenza, D., et al. Isolation, culture and characterization of embryonic cell lines from vetrified sheep blastocysts. (2006) *Mol Reprod Dev* 73(1): 31-39.
[PubMed](#) | [Crossref](#) | [Others](#)
17. Miosge, N., Hartmann, M., Maelicke, C., et al. Expression of collagen type I and type II in consecutive stages of human osteoarthritis. (2014) *Histochem Cell Biol* 122(3): 229-236.
[PubMed](#) | [Crossref](#) | [Others](#)
18. Martiel-Pelletier, J., Pelletier, J.P. Is osteoarthritis a disease involving only cartilage or other articular tissue? (2010) *Eklek Hastalik Cerrahisi* 21(1): 2-14.
[PubMed](#) | [Crossref](#) | [Others](#)