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STEM CELL APPLICATIONS IN INTERVERTEBRAL DISC REPAIR

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Abstract – There is increasing rise of interest in stem cell therapy, as it provides new options for treating a broad range of diseases. Several experimental methods are being explored for the use of stem cells in delaying or reversing the degenerative process of the intervertebral disc, a major cause of low back pain. In this article, we review the current strategies for stem cell applications in intervertebral disc repair and present three novel approaches. These are, first, the activation of nucleus pulposus cells by co-culture with mesenchymal stem cells for autologous disc cell reinsertion; second, the in vitro induction of nucleus pulposus-like or annulus fibrosus-like cells from mesenchymal stem cells; and third, the *in vivo* induction study by direct transplantation of mesenchymal stem cells to the intervertebral disc induced to degenerate experimentally. Although still untested, stem cell therapy may become a major option in the treatment of intervertebral disc degeneration.

Key words: Intervertebral Disc Cell, Mesenchymal Stem Cell, Cell Therapy, Nucleus Pulposus, Tissue engineering, Regeneration

INTRODUCTION

Intervertebral disc disorders form one of the most frequent causes of low back pain, which is one of the most common health problems in humans (10, 84, 93, 94). About 80% of the population will experience low back pain at some time during their lifetime (93, 94). Intervertebral disc disorders account for 80% of all elective surgeries on the spine, with an annual cost to the US health care industry of about $33 billion in direct costs and a total annual social cost exceeding $100 billion (54).

The normal intervertebral disc is sandwiched between upper and lower osteochondral endplates and is composed with two distinct components: the nucleus pulposus (NP) and the annulus fibrosus (AF) (19,31,33). The normal NP has a higher content of hydrated aggrecan,the main proteoglycan component, whereas in the normal AF there is a higher content of collagen (5,31,42,83). Degeneration of the intervertebral disc is influenced by multiple factors, including occupation, activity, genetic predisposition, and ageing (25,52,59). Histopathologically, disc degeneration leads to dramatic changes in the cellular and matrix components. Consequently, there are morphological changes and alterations in biomechanical properties (7,32,73,90). The diseased intervertebral disc shows a decrease in water content associated with reduced proteoglycan content of the NP (5,11,62).

Another problem along with degeneration and ageing is a decrease in absolute cell number (72,73). There is no effective therapy to treat and restore the degenerated intervertebral disc (41). Recent experimental studies have explored various biological strategies to address disc. These include strategies involving the induction of cytokines and growth factors, gene therapy, tissue engineering and cell transplantation therapy (1,3,4,8,9,16,24,26,43 44, 45,50,51,53, 63,78,87,88,97).

Among candidates for donor cells in cell transplantation therapy, there is an increasing interest in the use of stem cells, as these promise new options for treating a broad range of diseases

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(12, 58). Several experimental methods are being explored for the use of stem cells in delaying or reversing the degenerative process of the intervertebral disc (17, 76). In this article, we review the current concepts for stem cell applications in intervertebral disc repair and present three novel approaches. First, activation of nucleus pulposus cells by co-culture with mesenchymal stem cells (MSCs) for autologous disc cell reinsertion: second, second the ***in vitro*** induction of nucleus pulposus-like or annulus fibrosus-like cells from mesencymal stem cells, and third the ***in vivo*** induction study by direct transplan-ation of MSCs to the intervertebral disc induced to degenerate experimentally.

ACTIVATION OF NUCLEUS PULPOSUS CELLS BY CO-CULTURE WITH MESENCHYMAL STEM CELLS FOR AUTOLOGOUS DISC CELL REINSERTION

Loss of matrix secreting NP cells has been correlated with disc degeneration. In 1996, Mochida et al. showed the importance of preserving the NP for preventing an acceleration of disc degeneration after discectomy (48). This clinical study led to an experimental approach by Nishimura and Mochida, who showed that reinsertion of autologous fresh or cryopreserved NP cells slows the degenerative process in a rat model (55).

However, the preparation of NP cells for reinsertion is problematic because autologous transplantation requires more cells than can be harvested from a single disc. To obtain more NP cells, Okuma et al. found that NP cell viability could be improved by co-culture with annulus fibrosus cells (60). With low cellular yields and low proliferative activity of NP cells in the early phases of primary culture, further enhancement of the biological and metabolic viability of NP cells was desired. A novel method to obtain further activation of NP cells was reported by Yamamoto et al. with the use of the direct cell-to-cell contact co-culture system with mesenchymal stem cells (MSCs) (85,86) (Fig.1). Besides differentiating into multiple cell types of mesenchymal origin, MSCs serve as feeder or nursing cells for other cells. Such as hematopoietic progenitor cells, plasma cells, and hepatocytes.

Kawada et al. compared multiple co-culture systems in expanding human umbilical cord hematopoietic progenitor cells and found that direct cell-to-cell contact between MSCs and hematopoietic progenitor cells achieved significantly faster cell proliferation, than conventional noncontact co-culture (39). They concluded that direct cellular contact enhances cell signaling pathways and the expression of specific adhesion molecules controlling proliferation, differentiation, and phenotypic expression of cells.

The ability of MSCs to enhance the biological and metabolic viability of NP cells was evaluated using rabbit cell cultures. Results showed significantly better nucleus pulposus cell proliferation, DNA synthesis, proteoglycan synthesis and cytokine/growth factor production in a co-culture system with direct cell-to-cell contact with MSCs than in a conventional co-culture system or using monolayer cultures of NP cells. Furthermore, concentrations of transforming growth factor (TGF) -beta, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF) and platelet derived growth factor (PDGF) were significantly increased in the direct cell-to-cell contact co-culture group, which presumably lead to enhanced NP cell growth (83).

With the positive result of this co-culture system, pre-clinical studies to test its effects on human cells are ongoing (36,49,56,98).

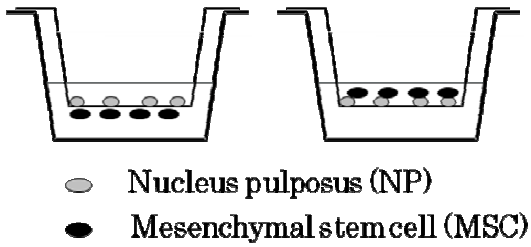

Figure 1. Coculture system having direct cell-to-cell contact demonstrated significant positive effect in activating the viability of NP cells.

IN VITRO INDUCTION OF NUCLEUS PULPOSUS OR ANNULUS FIBROSUS LIKE CELLS FROM MESENCHYMAL STEM CELLS

As mentioned above, MSCs are considered as a useful cell source for regenerative medicine in a variety of organs because of their multipotency (6,15,89,95,96). MSCs are stem cells found in small numbers in the bone marrow stroma. Only about 0.125% of cells in the bone marrow are mesenchymal stem cells (MSCs) (99). They express CD44, CD71, CD90, CD105, CD120a and CD124. But are

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negative for many hematopoietic lineage markers (99). MSCs secrete a distinctive pattern of cytokines different from hematopoietic cells (46). There have been many reports on successful osteogenic, adipogenic and chondrogenic induction of MSCs in vitro; moreover, MSCs can differentiate depending on their environment (12,18,65,66).

One factor compromising ***in vitro*** induction is that there are no specific markers for the phenotypes of NP and AF cells. However, they share similar cell characters with chondrocytes and, because a chondrocyte phenotype can be induced in MSCs, it is believed that chondrocyte-like cells among NP and AF cells may be derived using MSCs. (Fig.2)

The intervertebral disc is the largest avascular organ in the body. Risbud et al. have found that hypoxia is necessary to main the disc cell phenotype in vitro (67,70,71). They found that nucleus pulposus cells produce high levels of hypoxia inducible factor-1 (HIF-1), matrix metalloproteinase-2 (MMP-2) and glucose transporter-1 (GLUT-1) (67). They hypothesized that hypoxia serves to drive differentiation of MSCs towards nucleus pulposus-like cells (69). With the supplementation of TGF**,** MSCs can show differentiation towards NP cells with increased levels of HIF-1, MMP-2 and GLUT-1 along with aggrecan and type II collagen; classical phenotypic markers of the nucleus pulposus phenotype.

The use of three-dimensional culture systems is another key factor in the induction of an intervertebral disc cell phenotype, because NP and AF cells dedifferentiate in monolayer culture (27,30). Steck et al. found that, when cultured in a pellet in the presence of TGF-β3, dexamethasone, and ascorbate, MSCs showed chondrogenic differentiation closer to an intervertebral disc cell phenotype than to that of articular chondrocytes (82).

Sakai et al. used another method for induction with combinig co-culture and three-dimentional culture system. Flow cytometric analysis of human disc cells and articular chondrocytes (AC), showed characteristic tendencies in cell size, internal composition and the expression of cell-associated matrix markers (75).

They applied this profile to distinguish the differentiation status of MSCs after inducing them in a mixed three dimentional co-culture system (41). The concept here is that MSCs are co-cultured in a three-dimensional environment along with the very cell type into which one desires the cells to differentiate. In this situation, MSCs undergo differentiation stimulated by the surrounding environment modified by the co-cultured cell type. MSCs recovered by fluorescent activated cell sorting after having cocultured with NP cells showed similar cell sizes with large cells predominantly staining for keratan sulfate, accompanied with turnover of type I and type II collagen expression. These results were similar with the results of culturing NP cells alone. In MSCs co-cultured with AF cells, MSCs remained small and relatively weak in staining intensity, with no dominance in the expression of proteoglycan epitopes they resembled an AF cell phenotype (74).

Richardson et al. conducted moreover a co-culture study to determine whether human NP cells can initiate the differentiation of human mesenchymal stem cells (68). They used the same co-culture system as Yamamoto mentioned above (85,86). They evaluated the differentiation in MSCs and compared the results with or without cell-to-cell contact using real-time polymerase chain reaction (PCR)amplification of DNA. Human mesenchymal stem cells induced in a co-culture system with cell to cell contact showed more similar cell phenotype with human NP cells, compared with co-culture without cell to cell contact.

Although several studies have now been reported. Much more work is needed to characterize nucleus pulposus and annulus fibrosus cells fully and to obtain complete differentiation from MSCs. Further research in this field to be encouraged.

IN VIVO INDUCTION BY DIRECT TRANSPLANTATION OF MESENCHYMAL STEM CELLS TO THE EXPERIMENTALLY DEGENERATED INTERVERTEBRAL DISC

The final strategy on the use of MSCs in intervertebral disc regeneration involves cell transplantation therapy. The aim is the restoration of such cells that they can produce a proteoglycan rich extra cellular matrix. Cell therapy for treating intervertebral discs has been suggested as a possible method in several experimental studies using animal models (2,3,13,14,22,23,29,34,35,37,38,45,47,53,55, 57,61,63,64,80,81).

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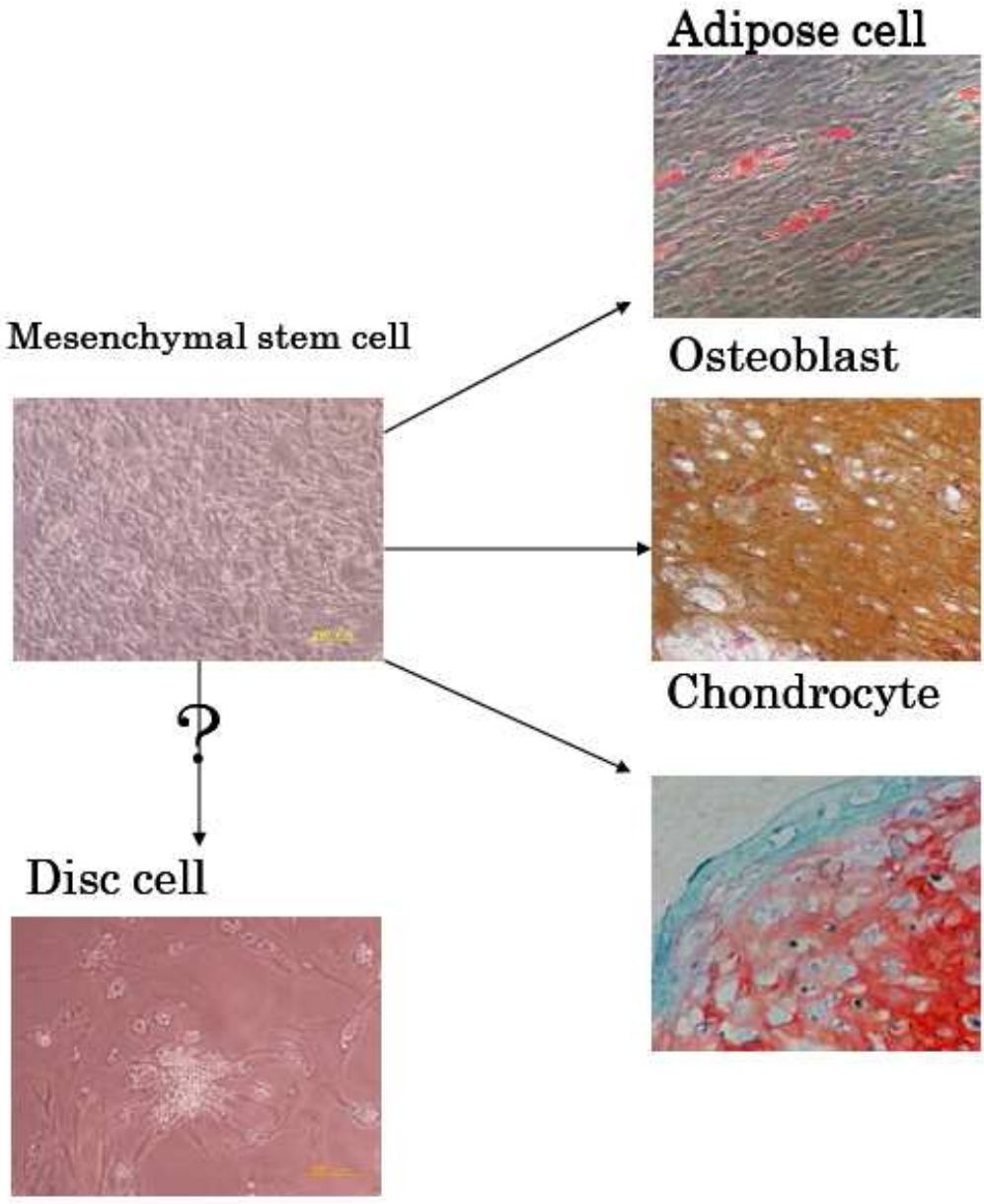

Figure 2. Mesenchymal stem cells(MSCs) have ability to differentiate along multiple connective tissue cell lineages. However, we don't know that MSCs may be induced into disc cell lineage.

Okuma et al. transplanted notochordal NP cells activated by co-culture with AF cells, using a rabbit disc degeneration model, and found that the structural appearance of the nucleus pulposus was maintained histologically (60).

Gruber et al. implanted autologous disc cells in the sand rat (29), an animal that undergoes spontaneous disc degeneration (28,29,79). The cells were harvested from intervertebral discs, expanded in monolayer culture, labelded and then implanted into different disc sites of the donor animals. The cells were either placed on a bioresorbable scaffold or injected directly into the recipient disc. Labeled cells were seen very clearly in the disc of animals as late as eight months after the transplantation. Therefore autologous disc cell implantation can be successful for cell survival and integration into the disc.

Ganey et al. studied a canine model in which disc degeneration was induced by injury (21). Six million autologous disc cells expanded from

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disc aspiration were transplanted to the nucleus region of injured discs without a carrier matrix. The discs in the dogs receiving transplants were significantly better maintained in terms of disc height and structure than in controls. The effects lasted for about 12 months after transplantation.

Although experimental studies confirm that cell transplantation can help to prevent disc degeneration in animal models, in a human clinical setting, it is almost impossible to obtain donor disc cells from healthy discs. From the perspective of regenerative medicine, it seems likely that restoration of a tissue or of an organ will be most efficiently pursued by using cells originating from that particular tissue. However, if a suitable cell source is unavailable, the next obvious candidates are progenitor cells or stem cells.

Sakai et al. studied the potential of mesenchymal stem cells as an alternative cell source (74,77). They transplanted autologous MSCs, tagged with the gene for green fluorescent protein (GFP), to rabbit disc degeneration models and followed them for 48 weeks. Tracking the effects using magnetic resonance imaging and radiography (Fig.3, Fig.4, Fig.5).

They also used immunohistochemistry for C-S, K-S, type I, II, and IV collagen, HIF-1 α and β, HIF-2 α and β, GLUT-1 and GLUT-3, and MMP-2, and applied RT-PCR to for expression levels of the genes for aggrecan, versican, type I, II collagen, IL-1 β, IL-6, TNF-α, MMP-9 and MMP-13. MRI and radiographic results confirmed the regenerative effects of the procedure. GFP-positive cells were detected in the nucleus throughout all periods at proportions rising from 21±6% in 2 weeks to 55 ± 8% in 48 weeks, which proved survival and proliferation of MSCs.

Immunohistochemistry showed positive staining of all proteoglycan epitopes and type II collagen in some of the GFP-positive cells. MSCs produced HIF-1α, MMP2 and GLUT-3 with phenotypic activity compartible with nucleus pulposus cells. The results obtained from RT-PCR demonstrated significant restoration of aggrecan, versican and type II collagen gene expression and significant suppression of TNF-α and IL-1β genes in the transplantation group. Thus, MSCs transplan- ted to degenerating discs ***in vivo*** can survive, proliferate and differentiate into cells expressing the phenotype of nucleus pulposus cells with suppression of inflammatory genes.

Since the first report of mesenchymal stem cell transplantation, several studies have reconfirmed the effectiveness of the procedure. Crevensten et al. demonstrated that injected MSCs in a rat disc using hyaluronan gel as a scaffold maintained viability and proliferated (17). Using cell labeling, viable cells were detected over the 28 days study period, maintaining the disc heighth at normal levels.

Zhang et al. implanted allogeneic MSCs containing the marker gene ***LacZ*** from young rabbits into the rabbit intervertebral disc to determine the potential of this cell-based approach (91,92). They reported that transplanted allogeneic mesenchymal stem cells could survive and increase the proteoglycan content within the disc, supporting its use as a potential treatment for intervertebral disc degeneration.

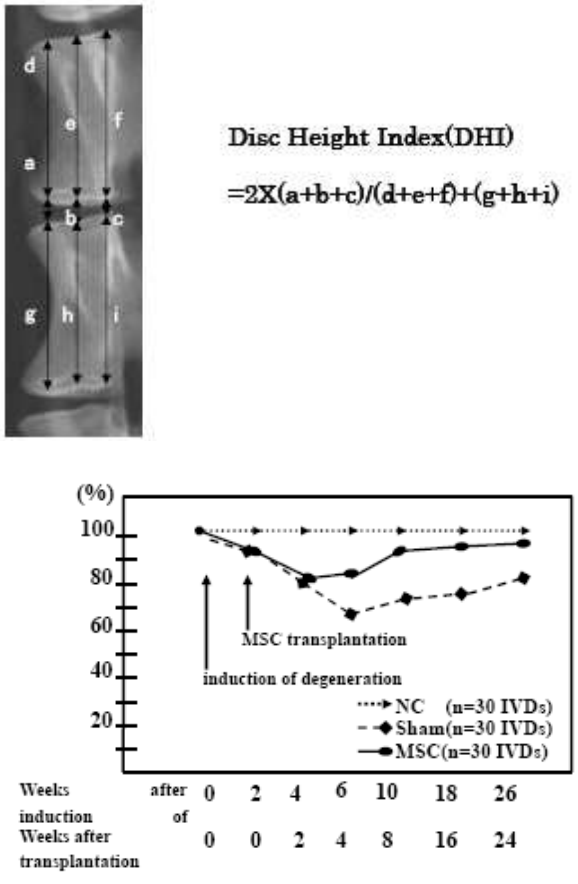

Figure 3. A lateral radiograph image of each group disc and measurement of disc height(DHI) using of Masuda et al.methods. Restoration of %DHI began 4weeks after MSC transplantation while sham animal discs showed constant decrease.

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Figure 4. Macroscopic view of each group disc harvested at period equivalent to 24 weeks after MSC transplantation in the MSC transplantation group. The MSC transplanted group discs demonstrated reappearance of the NP with restoration of disc space narrowing.

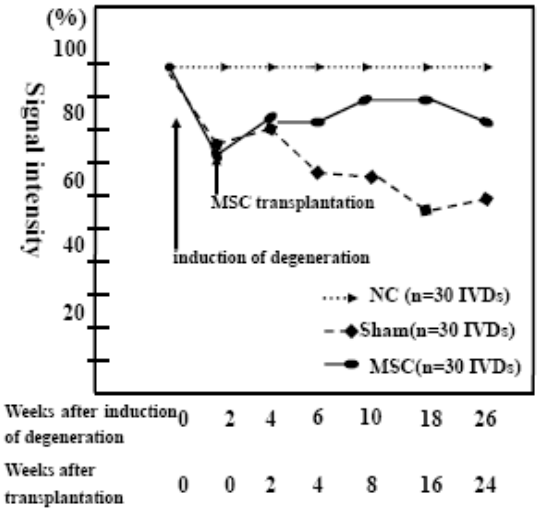

Figure 5. MRI image of each group rabbit until 26weeks post-induction of degeneration. The MRI of the NP area in the MSC transplantation group showed stronger signal intensity than in the sham group.

CONCLUSION

Advances in stem cell research have offered new strategies in treating the irreversible process of intervertebral disc degeneration. It is clear from the reports from multiple research centers that mesenchymal stem cells can play an important role in experimental research aimed at intervertebral disc regeneration. To progress this step forward, an important factor to overcome is to characterize intervertebral disc cells more thoroughly. There may well exist differences between the different species.

Furthermore, lack of a defined cell marker for these cell types is a major obstacle to our ability to detect differentiating or differentiated stem cells. Recently some new insights have been reported regarding cell marker for nucleus pulposus cell phenotype. Fujita et al. performed a microarray screening and cluster analysis to identify cell factors that were expressed specifically in the nucleus pulposus tissue from Wistar rats. They reported that CD24 expression was upregulated in NP cells in a tissue specific manner (20). In a different group, Lee et al. also performed a microarray analysis and compared annulus fibrosus and articular chondrocytes against nucleus pulposus cells, in the rat intervertebral disc. The results showed that GP3 (Glypican-3) and K19 (keratin-19) appear to be promising candidates as markers to distinguish NP cells from AC cells (40). Also in addition, Mwale et al. reported another study that used human lumbar spine specimens from fresh cadavers, and compared the proteoglycan to collagen synthesis ratio of nucleus pulposus cells of normal disc to that of the hyaline cartilage of the endplate. This study demonstrated that the production of an extracellular matrix with a high proteoglycan to collagen ratio of the nucleus pulposus cells can be used in vivo to distinguish nucleus pulposus cells from chondrocytes (50,51).

Although further research is clearly needed on intervertebral disc cell phenotype and function, the use of stem cells in regenerative strategies to treat intervertebral disc degeneration seems promising.

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