12

# Physical Limitations to Tissue Engineering of Intervertabral Disc Cells

Shigeru Kobayashi,[1,2] Hisatoshi Baba,[1,2] Kenichi Takeno,[1]
Tsuyoshi Miyazaki,[1] Adam Meir[3] and Jill Urban[3]
[1]*Department of Orthopaedics and Rehabilitation Medicine, Faculty of Medical Sciences, The University of Fukui, Fukui, Japan.*
[2] *Research and Education Program for Life Science.The University of Fukui, Fukui Japan.*
[3] *Dept of Physiology, Anatomy and Genetics, Oxford University, Parks Road, Oxford, UK*

## 1. Introduction

In humans, the intervertebral disc becomes avascular by the fourth year of life. It has been the major target of basic and clinical research on low back pain. Disc degeneration is a common cause of chronic low back pain and neurological dysfunction. So far, there have been numerous advances in our basic knowledge of the anatomy, biochemistry, physiology, and biomechanical features of the disc cells. However, disc cell research and fundamental knowledge are certainly lacking in comparison to research on cells of other organs.

It is generally considered that the notochord gives rise to the nucleus pulposus during development of the intervertebral discs. However, it remains unclear why the cells composing the nucleus pulposus differ among animal species and stages of maturation and what role notochordal cells play in the growth process. Development of the human spinal column begins in about embryonic week of 3 (Keys & Compere, 1932). In about week 8 of gestation, the cartilaginous vertebral bodies are completed, and the notochord is gradually pushed into the gaps between the vertebral bodies, where it later becomes the intervertebral discs. In week of 10, the notochord disappears from the vertebral bodies and the nucleus pulposus is formed. Embryonic notochordal cells remain active producers of matrix components until the end of the first decade of life, at which time a ''notochordal'' nucleus pulposus can no longer be defined because of its increased collagen content and the loss or metaplasia of notochordal cells (Keys & Compere, 1932, Smith, 1930, Coventry, et al., 1945a, Peacock, 1952, Taylor & Twomey, 1988). Thus, the notochordal cells disappear from the nucleus pulposus during adolescence and are replaced by nonnotochordal (chondrocyte-like) cells. Although the role of notochordal cells in the nucleus pulposus is unknown, it is interesting to note that the loss of these cells from the nucleus pulposus occurs before early signs of degeneration appear, and that the combination of notochordal cells and cartilage-like cells actually determines the properties of this tissue. During weeks 12 to 14 of gestation, blood vessels invade the cartilaginous vertebral bodies and primary ossification centers begin to appear. Primary cartilaginous ossification remains active until about puberty, and a cartilaginous region including the growth plate is observed at the vertebral

epiphysis. From the early stage of puberty, small secondary ossification centers appear and gradually extend around the periphery of the vertebral body to form a ring apophysis. At this stage, the cartilage canal plays an important role in nourishment and growth of the intervertebral disc and osteogenesis at the epiphyseal nucleus. The ring apophysis fuses completely with the vertebral body to form the vertebral bone at 18 to 25 years of age, if growth is normal, and the cartilage canal becomes buried in the bone marrow of vertebral body during the process of ossification (Peacock, 1951, Walmsley, 1953, Verbout, 1985).

The largely avascular intervertebral disc relies for nutrition on the outer one-third of the annulus fibrosus, which contains clearly defined blood vessels, and vascular channels in the cartilaginous end plate (Crock & Yoshizawa, 1977). In maintaining the nucleus pulposus, the central region of the end plate is probably more important than the outer region since it has a relatively higher density of vascular channels. Nutrient supply to the disc is affected by the architecture of the vascular buds and the porosity of the subchondral plate. nucleus pulposus cells may be up to 8 mm away from the closest blood supply in the adult lumbar disc (Bibby, et al., 2001). Steep gradients in metabolites develop with oxygen and glucose concentrations low in the disc center and lactic acid concentrations high. The pH in the disc center is thus acidic. The endplate does not normally provide a barrier to diffusion. In aging and degeneration, however, this end plate tends to calcify by unknown mechanisms and the apparent permeability of this plate decreases with age, as the end plate becomes more sclelotic (Robert et al., 1993). The consequent fall in supply of nutrients to the disc cells inhibits their ability to synthesise and maintain the matrix and even leads to cell death. Thus, it is noteworthy that the first signs of the disc degeneration are seen in the disc center.

There is now an increasing interest in developing biological repair methods for the treatment of disc degeneration that achieves the correct biomechanical properties for a successful outcome. Many researchers have performed basic studies on regeneration and transplantation of intervertebral discs that have applied tissue engineering using cell culture methods (Alini et al., 2002, Ganey &b Meisel, 2002, Masuda et al., 2004, Shimer et al., 2004, Brisby et al., 2004, Richardson et al. 2007, O'Halloran & Pandit, 2007, Sebastine & Williams, 2007, Kalson et al., 2008). So far, rat (Walsh et al., 2002, Yoon et al., 2003a, Risbud et al., 2003), rabbit (Nishida et al., 1999, Okuma et al., 2000, Kroeber et al., 2002, Yoon et al., 2003b, Masuda et al., 2003, An H, et al., 2005, Sakai et al., 2006, Iwashina et al., 2006)., porcine (Baer et al., 2001, Chen et al., 2004), canine (Thompson et al., 1991, Ganey et al., 2003, Hohaus et al., 2008), ovine (Sun et al., 2001, Mizuno et al., 2004), and bovine discs (Osada et al., 1996, Matsumoto et al., 2001, Alini et al., 2003, Hamilton et al., 2005) have been used in previous studies as tissue sources. However, the effects of aging and degeneration on the metabolism of human discs have been less frequently studied because suitable human discs for in vitro experiments are difficult to obtain (Alini et al.,2008, Hunter et al., 2003b). Therefore, comparison of metabolic activity between notochordal and nonnotochordal cells is considered to be important for determining the type of cell to use for transplantation when attempting to regenerate intervertebral discs. And also, tissue engineering and biological approach for disc degeneration is a potential approach for disc repair. However, these attempts to develop tissue with properties similar to those of native disc have been a challenge largely because of the difficulty of providing optimum physiological environments for the cells and lack of good control of extracellular conditions. The balance cellular demand and transport limits the number of cells that can be supported in any tissue

and governs the inverse relationship between thickness and cell density. Such limitations apply to all avascular tissues, including tissue-engineered constructs.

## 2. Nutritional supply to the disc by cartilage canal

It is well known that a cartilage canal is present in the epiphyseal cartilage of the long bones and spine from the fetal to juvenile stages, and this canal plays an important role in osteogenesis at the epiphysis as well as in nourishment and growth of the epiphyseal cartilage (Strayer, 1943, Brookes, 1971, Gardner & Gray, 1970). It has been variously reported that the cartilage canal was derived from differentiation during angiogenesis (Haines, 1933, Hurrell, 1934), progression of angiogenesis (Hurrell, 1934, Anderson & Matthiessen, 1966), transformation of mesenchymal cells to chondrocytes (Levene, 1964), and extension of the perichondrium (Moss-Salentijn, 1975, Wilsman, 1970), but no definitive conclusion has been reached. Even at the early stage of vertebral formation, a cartilage canal nourishing the unossified cartilaginous part of the vertebrae and intervertebral discs is present in the unossified cartilaginous region, and is subsequently buried within the vertebral marrow during the progression of ossification (Fig.1A) (Crock & Yoshizawa, 1977, Kobayashi et al., 2008). During the formation of vertebrae, the cartilage canal enlarges and undergoes anastomosis to form the bone marrow of the secondary epiphyseal center (Fig.2A,B). It also persists in the unossified cartilaginous region at a suitable location to provide nourishment for the proliferating cartilage until the completion of ossification (Haines, 1933, Yoshizawa et al., 1986). The cartilage canal made up of arteries, veins, capillaries and mesenchymal elements surrounded by chondral tissue (Fig.2C). The blood vessels in the cartilage canal have an important role in providing nutrition for intervertebral discs and enchondral ossification. True capillaries are classified into 3 types, which are discontinuous, fenestrated, and continuous: the capillary permeability increases in this order and the morphological features of the endothelium indicate organ specificity (Bennett et al. 1959). Fenestrated capillaries are observed in metabolically active tissues, such as endocrine glands, the dorsal root ganglion, the kidney, and the intestinal mucosa (Kobayashi et al., 1993, Kobayashi & Yoshizawa, 2002). In the cartilage canal, fenestrated capillaries were observed. This suggests that there was enhanced permeability and active metabolism in the canal, with the capillaries providing nutritional support for immature cells surrounding the vessels and chondrocytes outside the cartilage canal.

A perichondrium exists between the cartilage canal and surrounding cartilage matrix, and this is composed of fibroblast-like cells (Fig.2C). Electron microscopy suggested that immature chondrocytes observed near the cartilage canal might have originated from the perichondrium. A large number of fibroblastic-like cells as well as blood vessels were observed in the cartilage canal, and these cells were connected by processes to form an intercellular network in the cartilage canal (Fig.2D). Therefore, these cells were assumed to be the source of fibroblasts forming the perichondrium and chondrocytes outside the cartilage canal (Fig.3). This network was also considered to be involved in intercellular signaling that controlled the direction of cell differentiation. Chondroclasts also appeared in the cartilage canal to make the bone marrow of the secondary epiphysial center (Fig.2E). Concerning the origin of macrophages and chondroclasts, some hold that they arise from osteoprogenitors (Crelin & Koch, 1967), others that they are derived from monocytes (Fischman & Hay, 1962).

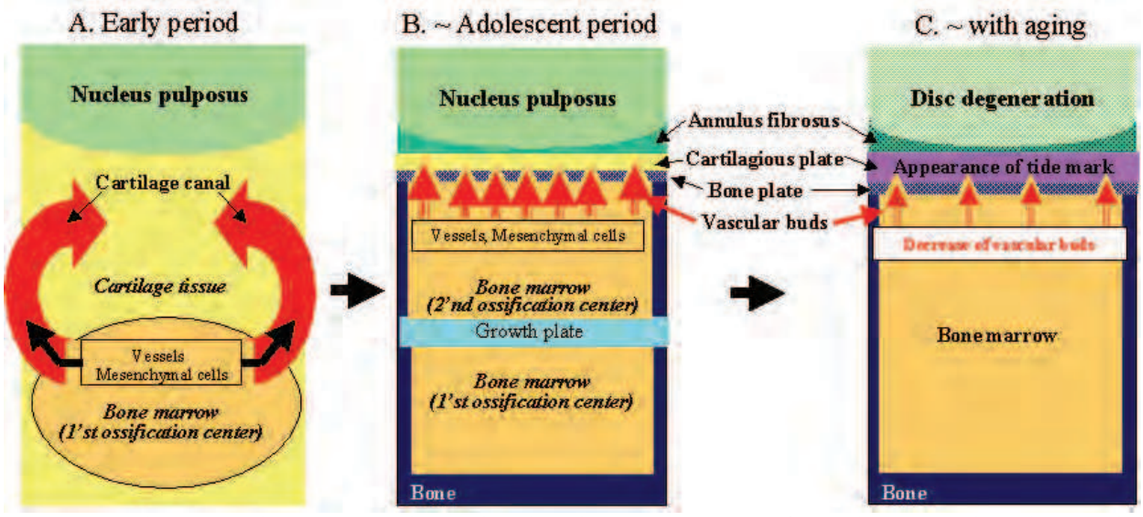


Fig. 1. A schematic drawing to show the developing process of cartilage canal and vascular bud in the vertebral end-plate. (Reproduced with permission from Kobayashi S, et al: Fine structure of cartilage canal and vascular buds in the vertebral end-plate in rabbit. J Neurosurg Spine 2008; 9: 96-103.)

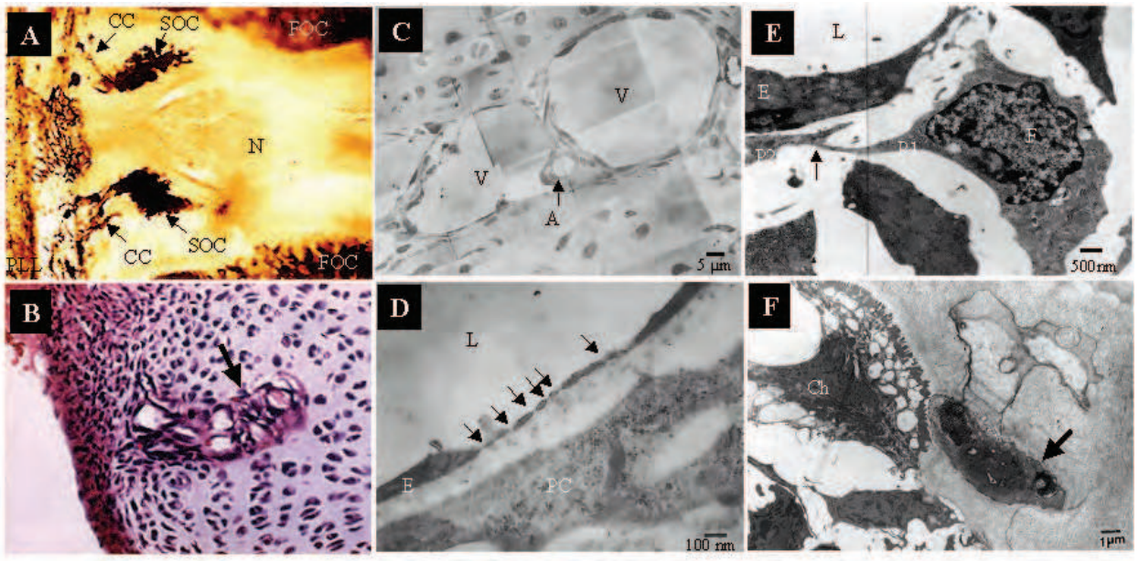


Fig. 2. The cartilage canal in the chondral cap of 5th lumbar vertebral body after its birth.

(A) A transparent specimen showing cartilage canal of a thin median sagittal section at 1 week after its birth. This specimen showing cartilage canal and second ossification center. Cartilage canals and second ossification center stained with India ink. CC: cartilage canal, FOC: first ossification center, N: nucleus pulposus, PLL: posterior longitudinal ligament, SOC: secound (epiphysial) ossification center.

(B) A hematoxylin and eosin stained medial saggital section showing immature chondrocytes around the cartilage canal (arrow).

(C) A transverse electron photograph of the cartilage canal. The cartilage canal is made

up of vessels of various size and fibroblastic-like cells around vessels. A: arteriole, V: venule.

(D) A high magnification of endothelium. This capillary has many fenestrations with a diaphragm in an endothelial cell (arrows). E: vascular endothelium, L: vascular lumen, PC: perichondral cell (fibroblast-like cell).

(E) A fibroblastic-like cell in the cartilage canal. These cells were connected each other by gap junction (arrow) and thus forming an intercellular network. Ch: chondroclast, E: vascular endothelium, L: vascular lumen, M: macrophage, P1 and P2: process of fibroblastic-like cells.

(F) A chondroclast with ruffled border was presented adjacent to the uncalcified matrix in canal. Chondrocytes undergoing apoptosis were seen around cartilage canal, the cells and nuclei were reduced in size and chromatin condensation was visible in the nuclei (arrow). Ch: chondroclast. (Reproduced with permission from Kobayashi S, et al: Fine structure of cartilage canal and vascular buds in the vertebral end-plate in rabbit. J Neurosurg Spine 2008; 9: 96-103.)

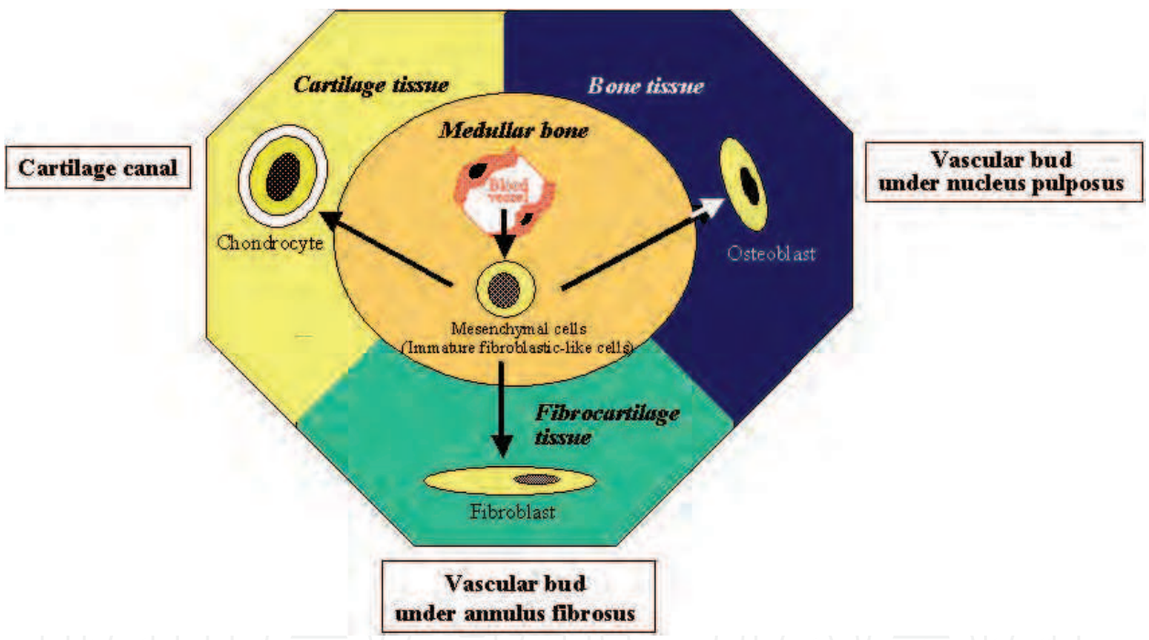


Fig. 3. A schematic drawing to show the developing process of mesenchymal cells in cartilage canal and vascular bud. The mesenchymal cells seen surrounding the cartilage canal and vascular bud represent a common precursor for the three main types connective tissue cells seen during early vertebral development. (Reproduced with permission from Kobayashi S, et al: Fine structure of cartilage canal and vascular buds in the vertebral end-plate in rabbit. J Neurosurg Spine 2008;9:96-103.)

## 3. Nutritional supply to the disc by vascular buds and disc degeneration

Previous histological studies of the bone-disc junction have pointed out that the bone-cartilage interface is perforated by small holes corresponding to the bone marrow the vascular terminations projecting into the cartilage, which are called as capillary beds or

vascular buds (Crock & Yoshizawa, 1977, Nachemson et al., 1970, Ogata & Whiteside, 1981, Crock et al., 1973, Maroudas et al., 1975). These vascular buds in the bone-disc junction area are structurally very similar to the cartilage canal made up of vessels and mesenchymal elements (Crock & Goldwasser,1984, Yoshizawa et al., 1986). A segmental arrangement of small vessels communicating with the bone marrow cavity is known to exist in the adult human vertebral body end-plate, and Crock et al. found a large number of small blood vessels that were systematically arranged around the end-plate in clear specimens of adult human or canine vertebral bodies, which they termed capillary buds (Crock & Yoshizawa, 1977, Crock & Goldwasser, 1984). Ogata et al. assigned the term vascular buds to these small blood vessels (Ogata & Whiteside, 1981). Thus, capillary buds are identical to vascular buds, and these are suggested to be an important nutritional pathway for the intervertebral disc. When ossification of vertebral body has progressed and the vertebral end-plate has formed, vascular buds appear in small foramina. These vascular buds consist of capillaries and mesenchymal cells, with a structural resemblance to the cartilage canal, although smaller in size, and their tips are in contact with the intervertebral disc. That is, special tissue consisting of blood vessels and mesenchymal cells is present in the undifferentiated and unossified part of the vertebra, while the cartilage canal persists in the bone marrow after it is buried during the process of ossification of the vertebral body. It then reappears in the form of vascular buds occupying numerous small foramina at the vertebral end-plate in association with formation of the end-plate, and plays an important role in supplying nutrition to the intervertebral discs. These vascular buds are morphologically similar among humans, monkeys, and rabbits, and numerous buds are observed in the central part of the end-plate corresponding to the nucleus pulposus of the intervertebral disc (Yoshizawa et al., 1986). This gives us the impression that these vascular buds are important structure for the nutrition of the intervertebral disc, especially nucleus pulposus.

In the case of rabbits, this vascular bud starts to project into the bone cartilage interface through the bone plate from the bone marrow about 4 weeks after its birth (Fig. 4A). This corresponds to the adolescent period of the human being (Yoshizawa et al., 1986, Kobayashi et al., 2008). Mature vascular buds are present in the bony end-plate and their ends are in contact with the cartilage of the intervertebral disc, so nutrients are considered to be transferred across this junction and distributed to the deeper areas of the intervertebral discs by diffusion (Fig.1B, 4B). In maintaining the nucleus pulposus, the central region of the end plate is probably more important than the outer region since it has a relatively higher density of vascular channels. There are reports that these channels disappear with increasing age, and eventually become occluded by calcification (tide mark) (Fig.1C, 4C). Schmorl and Junghanns noted “ossification gaps” representing degenerative foci in the mature cartilage end plate (Schmorl & Junghanns, 1971). These were seen to appear at the same time as the blood vessels disappeared and with the concomitant degeneration of the disc, it was assumed that the vessels had a major role in disc nutrition. Histological studies of intervertebral disc degeneration conducted by Conventry et al. (Coventry, 1945b, Coventry et al., 1945c) and Roberts et al. (Robert et al., 1993). Roberts et al., 1996) have demonstrated that degeneration begins near the vertebral end-plate with aging, while a study by Ogata et al. demonstrated that blockade of the vertebral route interfered greatly with intervertebral disc nutrition (Ogata & Whiteside, 1981). Thus, the tide mark which is the certified zone in the cartilage, covers the bone-disc junction by the end of growing period and looks to close the nutritional route through these vascular buds. On the other

hand, the tide mark may need to tolerance the body weight with age. These is still controversy concerning the role of the vascular buds after the appearance of this tide mark, and further studies may be required.

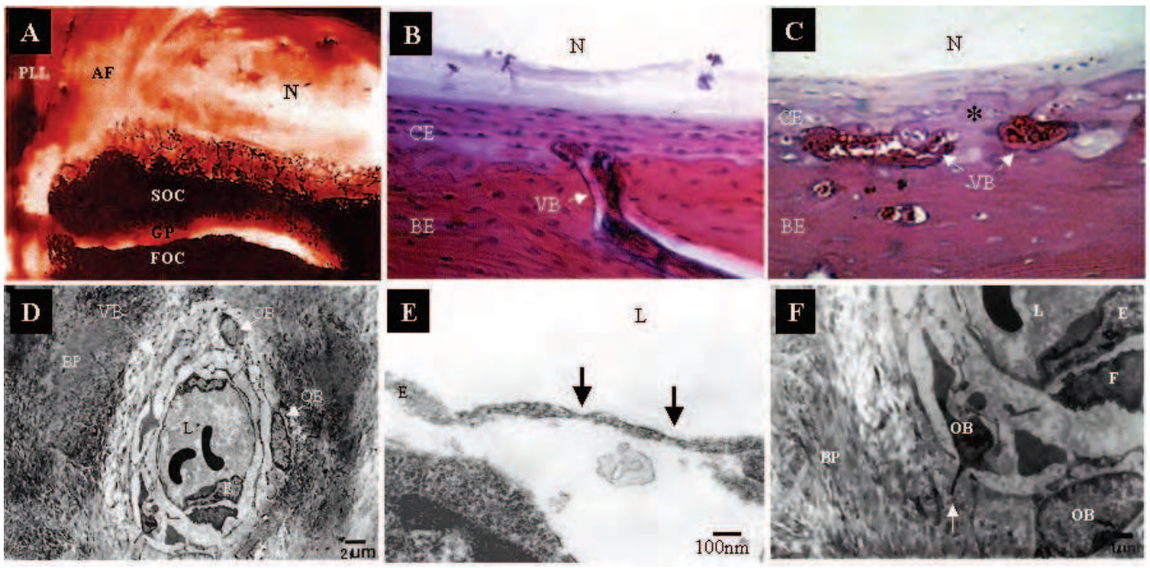


Fig. 4. The vascular buds in the vertebral end-plate of adult rabbit.

(A) A transparent specimen showing vascular buds at 6 month after its birth. The vascular buds were numerous in number in the central potion of the end-plate.

(B) A vascular bud in the central portion under the light microscope at 6 month after its birth. A vascular bud is contacting directly to the cartilage end-plate (Hematoxylin and eosin–stained section).

(C) A vascular bud in the central portion under the light microscope at 2 year after its birth. The tide mark (*) which is the calcified zone in the cartilage plate, covers the bone-disc junction by the end of growing period and looks to close the nutritional route through the vascular buds.

(D-F) Transverse sections of transmission electron microscope at 6 month after its birth.

(D) A photograph of the vascular bud in the center portion. A diameter of a capillary is around 10 μm and some fibloblastic-like cells surround it. These cells surrounding the capillary were connected each other by gap junction and thus forming an intercellular network.

(E) High magnification shows fenestrae in the endothelium (arrows). The capillary is fenestrated type.

(F) A osteoblastic-like cell that had differentiated from fibroblastic-like cells were observed and this cell were bound to the surrounding bone through processes (arrow). AF: annulus fibrosus, BP: bone plate, CP: cartilaginous end-plate, E: vascular endothelium, F: fibroblastic-like cell, FOC: first ossification center, GP: growth plate, L: vascular lumen, N: nucleus pulposus, OB: osteoblastic like cell, PLL: posterior longitudinal ligament, SOC: secound (epiphysial) ossification center, VB: vascular bud. (Reproduced with permission from Kobayashi S, et al: Fine structure of

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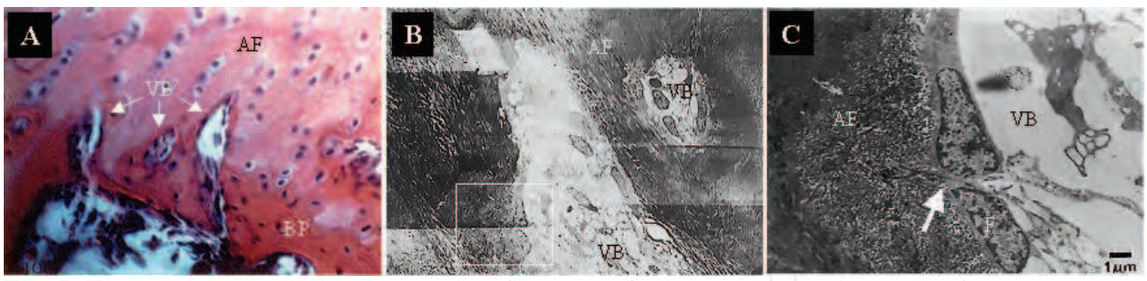


Fig. 5. The vascular buds in the annulus fibrosus of adult rabbit (at 6 month of age).

(A) A sagittal section showed some vascular buds in the annulus fibrosis (Hematoxylin and eosin–stained section).
(B) A sagittal constructed photograph of the vascular buds. The vascular buds are made up of vessels of various size and fibroblastic cells around vessels.
(C) Some fibroblastic cells were observed in the wall of vascular buds. Fibroblastic cells producing collagen fibers were observed (arrow). AF: annulus fibrosus, BP: bone plate, FB: fibroblastic like cell, N: nucleus pulposus, VB: vascular bud. (Reproduced with permission from Kobayashi S, et al: Fine structure of cartilage canal and vascular buds in the vertebral end-plate in rabbit. J Neurosurg Spine 2008; 9: 96-103.)

Vascular buds are resemble glomerulus-like capillary vessels (Oki et al., 1996). These are fenestrated capillaries and show increased vascular permeability like blood vessels in the cartilage canal, suggesting active metabolism of tissues around the vertebral end-plate (Fig.4D,E). A large number of fibroblastic-like cells were observed around capillaries in the vascular buds, and these cells was connected with each other by gap junctions to form an intercellular network as seen the cartilage canal. Osteoblast-like cells that had probably differentiated from fibroblastic-like cells were also observed, and some of the processes of these cells were buried in the bone tissue (Fig.3, 4F). In the vascular buds of the annulus fibrosus (Fig.5A,B), fibroblast-like cells produced collagen fibers in the region adjacent to the fibrocartilage, a finding which suggests that vascular buds in the annulus fibrosus have a marked influence on the composition of the fibrocartilagious matrix (Fig.3, 5C). Bessett et al. cultured mesenchymal cells under various conditions, and reported that bone tissue was generated when compression was applied under aerobic conditions and cartilaginous tissue was generated when compression was applied under anaerobic conditions (Bassett, 1962). When tension was applied under aerobic conditions, connective tissue was formed. Although cell differentiation is not only determined by the local oxygen tension and mechanical force, this research is interesting because blood vessels are not present in the cartilage although abundant vessels are observed in the bone.

## 4. A phenotypic comparison of morphological features of nucleus pulposus cells

It is generally considered that the notochord gives rise to the nucleus pulposus during development of the intervertebral discs. However, it remains unclear why the cells composing the nucleus pulposus differ among animal species and stages of maturation and what role notochordal cells play in the growth process. Morphological changes with aging have been actively studied since a report by Luschka in 1858 (von Luschka, 1858), and comparative studies of humans and animals have also been performed (Hansen, 1959, Bulter, 1988). As a result, it has been demonstrated that the nucleus pulposus of cattle (Hansen, 1959), horses (Bulter, 1988), sheep (Carlier, 1890), and chondrodystrophoid dogs (e.g., beagles) (Hansen, 1959, Bulter, 1988, Hansen,1952, Braund et al.,1975) shows similar changes to that of humans, while the nucleus pulposus of mice (Berry, 1961), rats (Williams,1908), rabbits (Souter & Taylar,1970, Scott et al.,1980), pigs (Hansen, 1959, Williams,1908), cats (Hansen, 1959, Bulter, 1988, Butler & Smith, 1967) and nonchondrodystrophoid dogs (e.g., mongrels) (Hansen, 1959, Bulter, 1988, Hansen,1952, Hunter et al.,2003a) retains notochordal cells. These studies have also demonstrated that notochordal and cartilaginous nucleus pulposus cells display considerable morphological differences. Although the role of notochordal cells in the nucleus pulposus is unknown, it is interesting to note that the loss of these cells from the nucleus pulposus occurs before early signs of degeneration appear, and that the combination of notochordal cells and cartilage-like cells actually determines the properties of this tissue.

Here, comparison of morphological features between notochordal and nonnotochordal cells is considered to be important for determining the type of cell to use for transplantation when attempting to regenerate intervertebral discs. In this study, we used notochordal cells from the nucleus pulposus of rats (Fig.6A) and rabbits (Fig.6B), as well as chondrocyte-like bovine nucleus pulposus cells (Fig.6C). At first, we determined the age of the animals based on their skeletal maturity so that nucleus pulposus tissues from different species could be compared under similar conditions. The age when skeletal maturity is reached is 2, 4-6, and 24-30 months after birth for rat (Adler et al., 1983, Moskowitz et al.,1990), rabbits (Kobayashi et al., 2008, Scott et al.,1980, Smith & Serafini-Fracassini,1968), and cattle (Lawrence et al.,2001), respectively. Loss of notochordal cells occurs at 12 and 6 months after birth in rats (Adler et al., 1983, Moskowitz et al.,1990) and rabbits (Scott et al.,1980, Smith & Serafini-Fracassini,1968), respectively, while notochordal cells are completely replaced by cartilage-like nucleus pulposus at birth in cattle (Hansen,1956). Accordingly, the discs from 2-month-old rats, 6-month-old rabbits, and 18 to 24-month-old cattle were considered to correspond approximately to the age of adolescence in humans. The cell density of the nucleus pulposus was noticeably higher in notochordal discs and was low (4,264 cells/$mm^3$ on average) in bovine discs (Fig.6G), which was close to the value (about 6,000 cells/$mm^3$) reported for the human nucleus pulposus by Maroudas et al (Maroudas et al., 1975). Butler and Smith et al. classified the nucleus pulposus cells into 3 stages: in stage 1, the NP consists entirely of notochordal cells; in stage 2, notochordal cells form clusters and the extracellular matrix increases; and in stage 3, notochordal cells show a marked decrease and extracellular matrix and cartilage-like cells become predominant (Butler & Smith, 1967). According to this classification, humans are already in stage 2 at birth and progress to stage 3 in their teens.

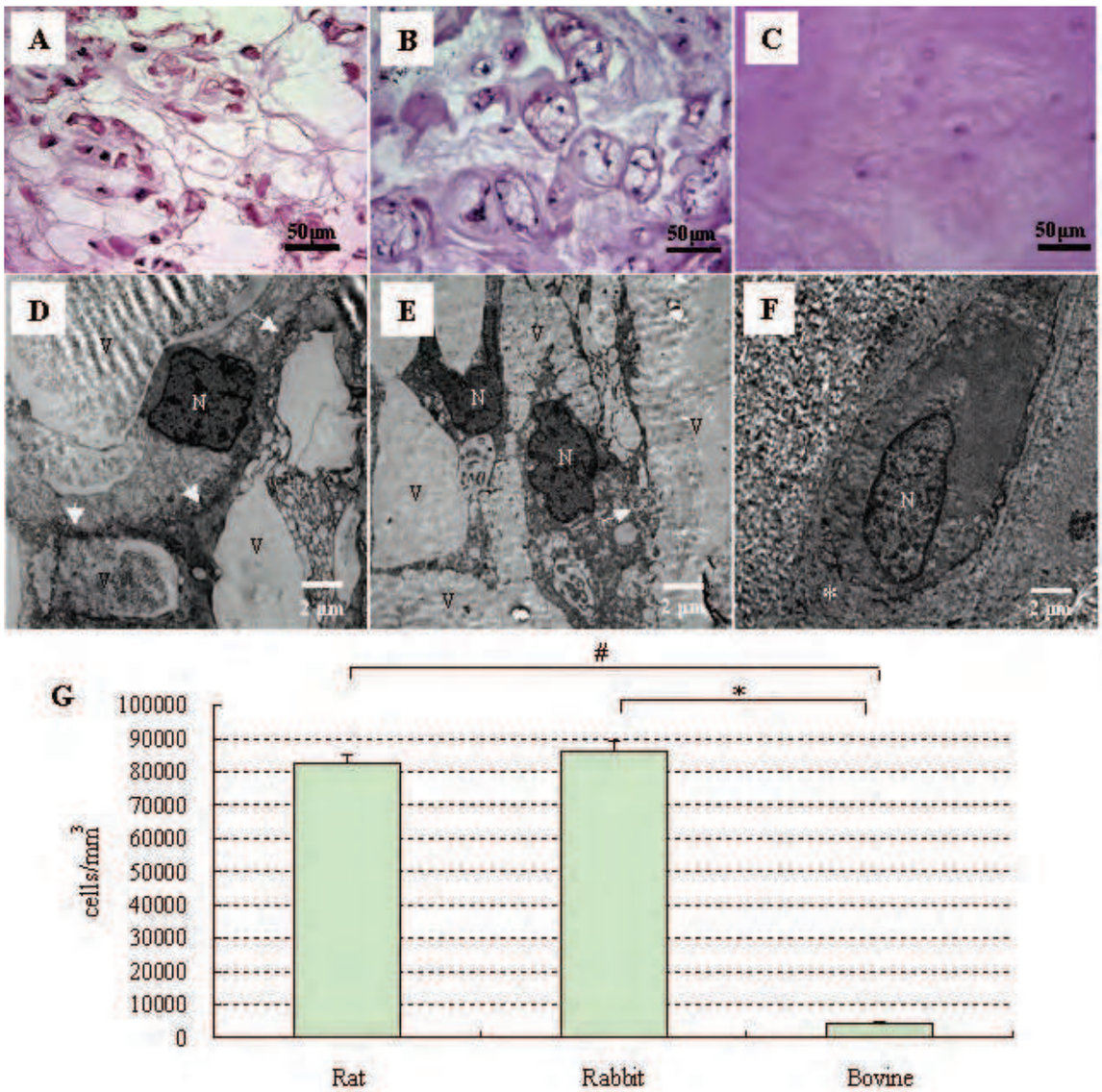


Fig. 6. Photomicrographs (A–C) and electron micrographs (D-F) of transverse sections of rat, rabbit, and bovine lumbar nucleus pulposus.

(A–C) Photomicrographs: Rat and rabbit discs have a gelatinous and well-hydrated nucleus pulposus. In contrast, the bovine nucleus pulposus is much more fibrous and cartilage like. The rat nucleus pulposus only contains notochordal cells with a number of vacuoles (A). In the rabbit nucleus pulposus, a few notochordal cells form clusters surrounded by mucoid material (B). In the bovine nucleus pulposus, no notochordal cells are observed, and chondrocyte-like cells with lacunae are scattered in a hyaline-like matrix (C) (X40, scale bar.50 mm, hematoxylin and eosin–stained sections).
(D-F) Transmission electron micrographs of the rat nucleus pulposus (D), rabbit nucleus pulposus (E), and bovine nucleus pulposus (F). In the rat nucleus pulposus, a number of vacuoles of various sizes, which are empty or contain an amorphous substance, can be

observed around the cells. Cells are connected to adjacent cells by tight junctions (white arrow) (D). In the rabbit nucleus pulposus , as in rats, notochordal cells with a number of vacuoles of varying sizes are observed. The cells are connected with adjacent cells to form an intercellular network as in rats (E). Bovine nucleus pulposus cells have larger nuclei than notochordal cells. The cytoplasm does not contain vacuoles and the cells are found individually in the matrix. Lacunae (*) are observed around the cells (F). The cells are completely different from notochordal cells, closely resembling chondrocytes from articular cartilage. N: nucleus; V: vacuoles.
(G) The number of nucleus pulposus cells per unit volume was counted in 10 discs from each animal, and the mean values were compared. As a result, the number of cells in the bovine nucleus pulposus (chondrocyte-like cells) was significantly lower than that of notochordal cells in the nucleus pulposus of rats and rabbits (*, #: $p<0.05$). (Reproduced with permission from Miyazaki T, Kobayashi S, et al. A phenotypic comparison of proteoglycan production of intervertebral disc cells isolated from rats, rabbits, and bovine tails; Which animal model is most suitable to study tissue engineering and biological repair of human disc disorders? Tissue Eng Part A. 2009 Aug 15. [Epub ahead of print] PMID: 19681728 [PubMed - as supplied by publisher].)

The cells of the adult nucleus pulposus more closely resemble articular chondrocytes. The rat, rabbit, and bovine nucleus pulposus used in this study were considered to correspond to stages 1, 2, and human stage 3, respectively. Overall, the structure of bovine and human discs is similar, but some differences exist with regard to the diameter, height, and thickness of the wall of the annulus (Race et al., 2000). Although bovine tails support a lower load than human lumbar discs, the swelling pressure of bovine coccygeal discs has been shown to be similar to that of the discs in a person resting in the prone position (Ohshima et al., 1993, Ishihara H, et al. 1996). Biochemically, similar rates of *in vitro* proteoglycan synthesis (Ohshima et al., 1993). and matrix synthesis in response to hydrostatic pressure (Ishihara H, et al. 1996, Ohshima et al., 1995) have also been found in human and bovine coccygeal discs. Both bovine and human discs are also similar with regard to the types and distribution of aggrecan and collagen (Ohshima et al., 1993, Roberts et al., 1991). The bovine disc closely resembles the human disc because adult human and adult bovine nucleus pulposus have almost no notochordal cells and contain chondrocyte-like nucleus pulposus cells that produce a hyaline cartilage-like matrix. It has been demonstrated that notochordal cells persist throughout life in rats and rabbits, but decrease with growth, and are replaced by hyaline cartilage-like matrix and chondrocyte-like nucleus pulposus cells infiltrating from the annulus fibrosus (Keys, & Compere, 1932, Coventry, et al., 1945, Peacock, 1951, Kim et al., 2003). It is unknown, however whether this decrease is caused by differentiation of notochordal cells into non-notochordal cells, apoptosis, or lack of nutrients diffusing from the end plates. Thus, in each animal species, notochordal cells decrease and chondrocyte-like cells become predominant in the matrix of the nucleus pulposus with aging, but the reason for the decrease of notochordal cells and the origin of the chondrocyte-like nucleus pulposus cells remain to be elucidated. Therefore, investigation of the relationship between the decrease of matrix formation by notochordal cells and the onset of intervertebral disc degeneration is an important subject for the future.
When compared with a report by Trout et al., who observed the human nucleus pulposus at various ages from fetal to 91 years by electron microscopy (Trout et al., 1982a, Trout et al.,

1982b), the morphological characteristics of notochordal cells observed in rats and rabbits in this study were similar to those of nucleus pulposus cells observed in human discs up to adolescence, while the features of cartilage-like cells observed in bovine discs were similar to human nucleus pulposus cells from adolescence onward. That is, the notochordal discs contained physaliphorous cells aggregated in close contact with each other (Fig.6D,E), while non-notochordal discs contained cartilage-like cells scattered through the matrix and characteristically surrounded by lacunae (Fig.6F), which were not observed around notochordal cells. The mechanism leading to formation of lacunae and their significance remains unclear, but lacunae may reflect the influence of the cell over its immediate pericellular matrix, either through accumulation of excreted products or some other process. The formation of lacunae may also be related to changes of the nucleus pulposus that occur with age, including the apparent increase of some nutrients and substrates, making the extracellular matrix less accessible to cell products (Happy & Bradford,1964, Happy, et al. 1969). Changes in the ability of substances to diffuse in or out of the cell and a reduction in the number of viable cells might gradually alter the extracellular matrix and thereby lead to age-related changes in the mechanical properties of the disc.

## 5. Metabolic activities of notochordal cells and non-notochordal cells

Proteoglycans, particularly the large proteoglycan known as aggrecan, play a major role in load-bearing by the intervertebral disc. Because of the high osmotic pressure due to the sulphated glycosaminoglycans in aggrecan, it absorbs water that expands the collagen network and maintains tissue turgor (Grodzinsky, 1983, Maroudas & Bannon, 1981). The stiffness of intervertebral disc tissues is thus strongly dependent on the aggrecan content (Kempson et al., 1970, Treppo et al., 2000). Thus, it is necessary to have an adequate content of proteoglycans (glycosaminoglycans) when attempting to produce intervertebral disc tissue with the mechanical strength to withstand stress of about 2.8-13.0 kN by tissue engineering (Adams & Hutton, 1982). The structure and composition of the intervertebral discs are intimately related to their function. By studying species differences and similarities, many researchers have attempted to define the functions of the discs. However, caution is necessary when interpreting and extrapolating to humans from such data, because even though all vertebrates have discs, not all discs are the same. For example, comparison of proteoglycan synthesis in the discs of chondrodystrophoid dogs (beagles) and nonchondrodystrophoid dogs (greyhounds) has revealed significantly lower proteoglycan synthesis in chondrodystrophoid dogs (Cole et al., 1985). The relative risk of disc herniation is about 10 to 12 times higher in nonchondrodystrophoid dogs than in chondrodystrophoid dogs (Gage, 1975, Priester, 1976). Autoradiography with $^{35}$S-sulfate, a radio label that is largely incorporated by the glycosaminoglycan chains of Proteoglycans, has established that notochordal cells also synthesize proteoglycans (Souter & Taylar, 1970). A similar finding has been reported for human fetal and pediatric discs in which notochordal cells are more abundant compared with mature discs (Bayliss, et al., 1988, Johnstone & Bayliss, 1995). Aguiar et al. have suggested that the notochordal cells found in the human nucleus pulposus up to approximately 10 years of age play an active role in nucleus pulposus development and in the maintenance of disc integrity through the production of soluble factors that induce nucleus pulposus cells to increase proteoglycan synthesis (Aguiar et al., 1999). Interestingly, it has been found that co-culture of immature

cells such as mesenchymal stem cells with notochordal cells and adult nucleus pulposus cells (chondrocyte-like cells) stimulates proteoglycan synthesis more rapidly, and this method has been extensively used in studies on disc regeneration (Sakai et al., 2006, Iwashina et al., 2006, Cappello et al., 2006, Erwin & Inman, 2006). However, previous studies on proteoglycan production by notochordal cells and chondrocyte-like nucleus pulposus cells have only compared proteoglycan synthesis on the basis of sulphate synthesis, and no studies have compared glycosaminoglycan accumulated by these cells.

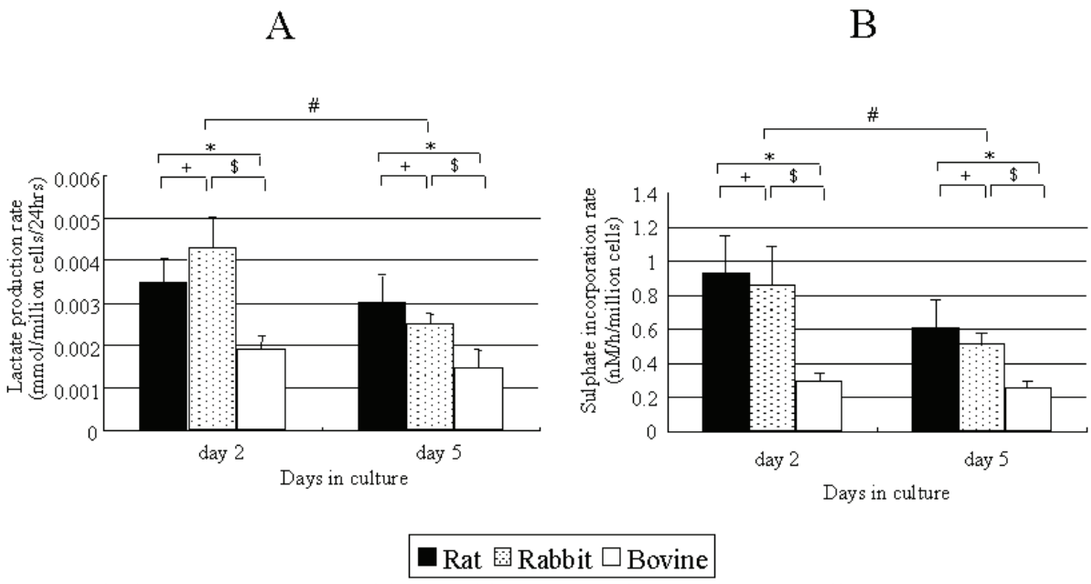


Fig. 7. Metabolic activity of rat, rabbit, and bovine nucleus pulposus cells.

(A) Lactate production rate. Cells were cultured under standard conditions in beads containing 4 million cells/ml (1.0ml medium and five beads per well) for up to 5 days, with a complete medium change everyday. Representative beads were dissociated for cell counting, and the viable cell density per bead was determined. Lactate in the medium was measured after each 24-h culture period and production per million cells/24 hours is shown. Notochodal nucleus pulposus cells from rats and rabbits produced about twice as much lactate as bovine nucleus pulposus cells (*: $p<0.05$ by the paired t-test for rat vs. bovine cells; $: $p<0.05$ by the paired t-test for rabbit vs. bovine cells; +: p=n.s., by the paired t-test for rat vs. rabbit cells.) Lactate production decreased over time during culture (#: $p<0.05$ by two-way ANOVA with repeated measures for comparison among days 2 and 5).

(B) Sulfate incorporation rate. On days 2 and 5, radiolabeled sulfate was added to fresh medium in three wells, and the beads were cultured for 4 hours. Then the beads were dissociated and cell density and sulfate incorporation were measured. Results are given as the mean_standard error of six independent experiments. The sulfate incorporation rate decreased over time (#: $p<0.05$ by two-way ANOVA with repeated measures for 2 vs. 5 days). The rate was highest for rat cells and lowest for bovine cells up to 5 days of culture (*: $p<0.05$ by the paired t-test for rat vs. bovine cells; $: $p<0.05$ by the paired t-test for rabbit vs. bovine cells; +: p=n.s., by the paired t-test for rat vs. rabbit cells.). ANOVA, analysis of variance. (Reproduced with permission from Miyazaki T,

Kobayashi S, et al. A phenotypic comparison of proteoglycan production of intervertebral disc cells isolated from rats, rabbits, and bovine tails; Which animal model is most suitable to study tissue engineering and biological repair of human disc disorders? Tissue Eng Part A. 2009 Aug 15. [Epub ahead of print] PMID: 19681728 [PubMed - as supplied by publisher].)

We examined that a 3-dementional culture system was employed to compare the metabolism of notochordal cells from rat and rabbit nucleus pulposus, as well as chondrocyte-like cells from the bovine nucleus pulposus (Miyazaki et al., 2009). As a result, notochordal nucleus pulposus cells showed levels of lactate production (Fig.7A) and proteoglycan synthesis (Fig.7B) about twice as high as those of chondrocyte-like nucleus pulposus cells, indicating the high metabolic activity of notochordal cells.

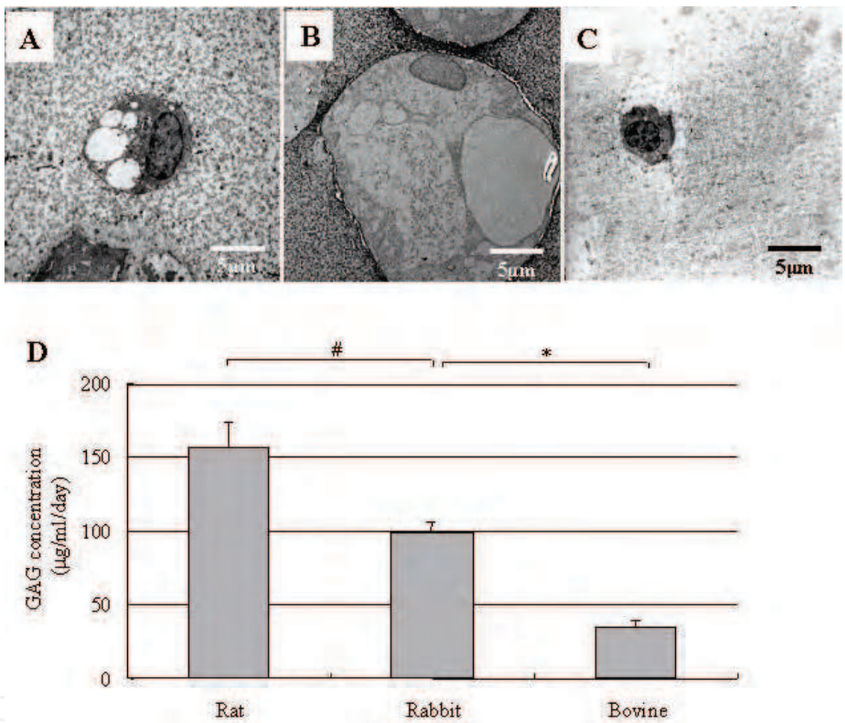


Fig. 8. Morphological changes and glycosaminoglycan production after three-dimensional culture. Cells were encapsulated in alginate beads and cultured in Dulbecco's modified Eagle's medium with 6% serum under air for 5 days.

(A-C) Electron micrographs of rat (A), rabbit (B), and bovine (C) nucleus pulposus cells cultured in alginate beads for 5 days. Notochordal (A,B) and non-notochordal (cartilaginous)(C) nucleus pulposus cells showed essentially similar findings to those observed *in vivo*. (D) Glycosaminoglycan accumulated/day in rat, rabbit, and bovine nucleus pulposus cells. The GAG concentration was measured after 5 days. Pooled data from six separate experiments are shown. After culture for 5 days, accumulative glycosaminoglycan production was higher in notochordal nucleus pulposus cells than in cartilaginous nucleus pulposus cells, being about 156.5, 99.5, and 35.4 μg/ml/day for rat, rabbit, and bovine nucleus pulposus cells, respectively. The glycosaminoglycan

accumulated/day was lowest in the bovine cells. Values are the mean ± standard error. ( *, #: $p<0.05$). (Reproduced with permission from Miyazaki T, Kobayashi S, et al. A phenotypic comparison of proteoglycan production of intervertebral disc cells isolated from rats, rabbits, and bovine tails; Which animal model is most suitable to study tissue engineering and biological repair of human disc disorders? Tissue Eng Part A. 2009 Aug 15. [Epub ahead of print] PMID: 19681728)

When nucleus pulposus cells were encapsulated in alginate beads, cultured for 5 days, and examined under a electron microscope, notochordal nucleus pulposus cells of rats (Fig.8A) and rabbits (Fig.8B) were larger than chondrocyte-like bovine nucleus pulposus cells (Fig.8C), and both cell types were surrounded by gaps. However, electron microscopy only revealed lacunae around the bovine nucleus pulposus cells (Fig.8C) and not around rat or rabbit nucleus pulposus cells, which contained a number of vacuoles. Both types of cell showed essentially similar findings to those observed in vivo and contained few intracellular organelles. After culture for 5 days, accumulative glycosaminoglycan production was higher in notochordal nucleus pulposus cells than in cartilaginous nucleus pulposus cells, being about 156.5, 99.5, and 35.4 μg/ml/day for rat, rabbit, and bovine nucleus pulposus cells, respectively (Fig.8D). Thus, the notocordal cells were more active metabolically than were chondrocyte-like nucleus pulposus cells. One index that in used for disc repair and regeneration is the glycosaminoglycan content of the disc. It has been reported that a 7-10 % glycosaminoglycan content is required to obtain disc tissue with enough strength for clinical application (Urban & Roberts, 1995). Based on the results of the present study, it was calculated that it would respectively take at least 1 year, 2 years, and 5 years for rat, rabbit, and bovine nucleus pulposus cells to produce a 7%-glycosaminoglycan content (i.e., 70 mg/ml). This provides important information about prospective autologous transplantation of cells with a high proteoglycan production capacity such as notochordal cells and mesenchymal stem cells.

## 6. Which animal model is most suitable to study tissue engineering and biological repair of human disc disorders?

Accordingly, glycosaminoglycan accumulation by chondrocyte-like bovine and adult human nucleus pulposus cells is extremely slow compared with that by notochordal cells, and in vitro production of a construct with a 7%-glycosaminoglycan content by using chondrocyte-like nucleus pulposus cells alone would be assumed to take an extremely long time and thus would be impractical. In fact, transformation of the matrix by cells in human discs is very slow, and studies have shown that proteoglycan turnover takes about 20 years (Roughley, 2004) and collagen turnover takes more than 100 years (Verzijl et al., 2000). However, autologous transplantation of cells with a high proteoglycan production capacity such as notochordal cells and mesenchymal stem cells may lead to successful repair of degenerated discs (Sakai et al., 2006, Iwashina et al., 2006, Aguiar et al.,1999, Cappello et al., 2006, Erwin & Inman, 2006). Even if the transplantation of cells with a high proteoglycan production capacity into the nucleus pulposus is feasible, it is also essential for preventing the progression of disc degeneration that the transplanted cells survive in the degenerated disc and continue to produce appropriate macromolecules for maintenance of disc mechanical strength throughout life.

When cells with a high metabolic activity such as notochordal cells are implanted at such a location, it is unclear that the cells will be able to obtain enough nutrients to achieve adequate regeneration of the extracellular matrix. In fact, notochordal nucleus pulposus cells have a higher metabolic activity than chondrocyte-like nucleus pulposus cells, and thus should require a larger amount of nutrients to maintain their metabolism than the chondrocyte-like cells. It can be assumed from the results of this study that the glycosaminoglycan content of the nucleus pulposus decreases when chondrocyte-like cells take over the major role of extracellular matrix production from notochordal cells during the growth process in humans. The immature disc shows a much higher cell density in vivo than the mature nucleus pulposus with chondrocyte-like cells, and lactate and glycosaminoglycan production were elevated when culture was done at the same cell density ($4 \times 10^6$ cells/mL). This indicates that notochordal nucleus pulposus cells have a higher metabolic activity than chondrocyte-like nucleus pulposus cells and require more nutrients. Based on these results, it was considered preferable to study disc regeneration using discs from animals with chondrocyte-like cells resembling those in the adult human disc.

One of the initial signs of disc degeneration is said to be a decrease of proteoglycan, particularly glycosaminoglycans, in the nucleus pulposus, and one possible cause may be a difference of metabolic activity between nucleus pulposus cells with different phenotypes. Although such changes are often considered to be signs of degeneration, these changes may merely be stages in the normal evolution of discs subjected to mechanical stress. Also, given that many degenerated discs are asymptomatic in humans (Boden et al., 1990, Jensen et al., 1994) identification of the features that distinguish physiological from pathological degeneration is critical. That is, the decrease of the proteoglycan content in the nucleus pulposus that is observed in human discs during the growth process does not necessarily indicate pathological degeneration. Its major cause is considered to be a decline in the function of chondrocyte-like nucleus pulposus cells or the death of cells due to impairment of nutrient supply from the end plates, which occurs after notochordal cells are completely replaced by chondrocytelike cells in the nucleus pulposus from adolescence onward.

## 7. Changes of physico-chemical environments of the disc cells with aging.

Nutritional supply to the disc is affected by the architecture of the vascular buds and the porosity of the end plate (Fig.9A). Nutrients move from the vascular buds that supply the disc, through the end plates and the dense matrix of the disc, to the cells. Its limits transport of large molecules into and out of the disc (Roberts et al., 1996). For small solutes such as glucose, lactate acid, and oxygen, both experimental and modeling studies have shown that solute transport is accomplished mainly by diffusion (Urban et al.,1982, Katz et al., 1986, Ferguson et al., 2004, Holm et al.,1981), hence, the movement of fluid in and out of the disc as a result of the diurnal loading pattern has little direct influence on transport. Gradients in the concentration arise depending on the balance between the rate of supply of glucose or oxygen from the blood supply to the cells and the rate of cellular consumption (Holm et al.,1981, Sélard, et al., 2003). The intervertebral disc is avascular, and the metabolic activity of its cells is regulated by various factors in the extracellular matrix, such as oxygen (Holm et al.,1981, Bibby et al., 2005), osmolality (Urban & Maroudas, 1979, Maroudas, 1981, Ishihara et al, 1997, Takeno et al., 2007, Negoro et al., 2008), and pH (Ohshima & Urban,

1992, Razaq et al., 2004). The cell density of the normal human nucleus pulposus is 2– 4 x $10^6$ cells/mL, and the extracellular environment differs markedly from that of other tissues, with an oxygen saturation of 1–5%, pH of 7.2–7.4, and extracellular osmolality of 370–400mOsm. Disc cells activity is regulated by extracellular oxygen concentrations and extracellular pH. Synthesis rates appear to be highest at around 5% oxygen, where they are greater than the rates found in air (Ishihara & Urban, 1999). However, once oxygen tension falls below 5%, synthesis is inhibited appreciably in an oxygen-tension dependent manner. Extracellular pH also has a marked effect on the synthesis rates of matrix components. The synthesis rates are 40% higher at pH 7.0 than pH 7.4 but fall steeply once the environment become acidic (Ohshima & Urban, 1992). Extracellular matrix degradation, however, appears less sensitive to pH. In fact, the production of active matrix metalloproteinases is similar at pH 7.0 and pH 6.4. Thus, acidic pH levels, by inhibiting synthesis but not degradation, may increase the rate of matrix breakdown.

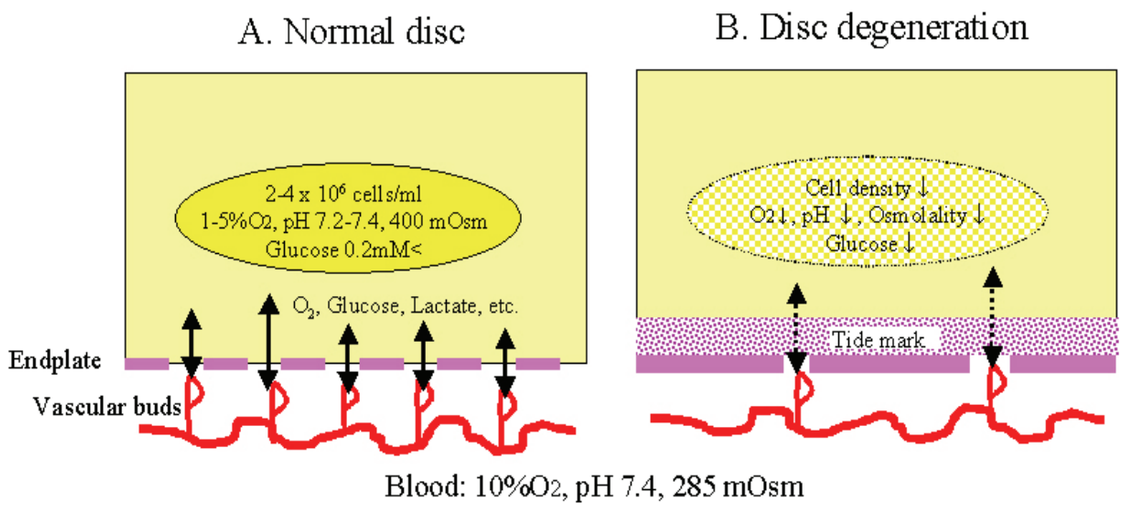


Fig. 9. Changes of nutrition and the extracellular environment in nucleus pulposus with aging. (A) Normal disc, (B) Degenerated disc.

The number of disc cells decreases with aging, but it is unknown whether this decline is caused by differentiation of notochordal cells into nonnotochordal cells, apoptosis, or insufficient supply of nutrients from the end plate. Ossification of the end plate (tide mark) occurs with aging and is one of the major causes of disc degeneration. This leads to deterioration of the extracellular environment in the nucleus pulposus and causes cellular impairment that is followed by a decline of matrix metabolism, resulting in progression to disc degeneration. The nutrient supply for cells and the extracellular environment of the disc have a considerable influence on the outcome of treating disc degeneration by bioengineering techniques. (Reproduced with permission from Miyazaki T, Kobayashi S, et al. A phenotypic comparison of proteoglycan production of intervertebral disc cells isolated from rats, rabbits, and bovine tails; Which animal model is most suitable to study tissue engineering and biological repair of human disc disorders? Tissue Eng Part A. 2009 Aug 15. [Epub ahead of print] PMID: 19681728)

In aging, the end plate tends to calcify by unknown mechanisms (Fig.9B). This tide mark (calcification) acts as a barrier to nutrients transport and is thought to be a major factor in the development of disc degeneration (Urban & Roberts, 1995, Nachemson et al., 1970, Rajasekaran et al., 2004). Cellular parameters are very important in regulating nutrient levels, with levels of oxygen or pH falling with increases in rates of cell metabolism or cell density (Sélard, et al., 2003, Mokhbi-Soukane et al., 2005). For the disc cells to remain viable, the levels of extracellular nutrients and pH must remain above critical values.

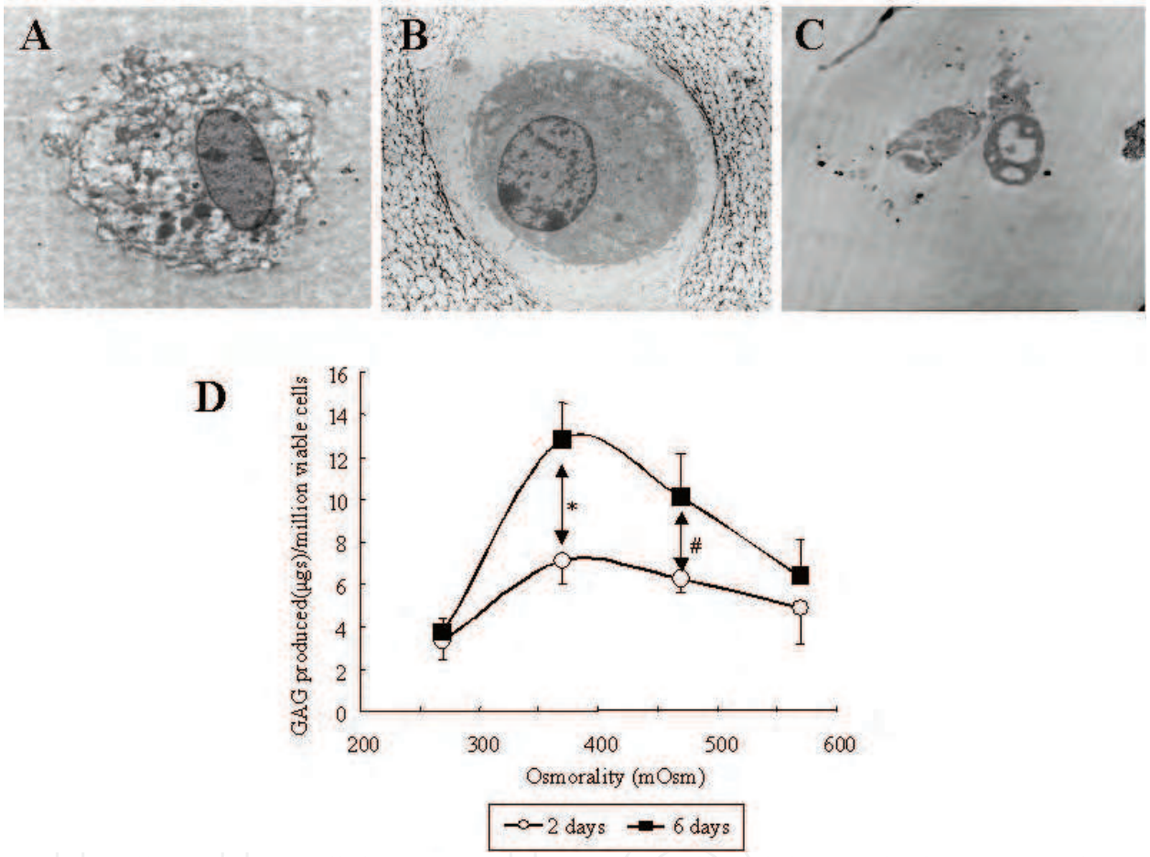


Fig. 10. Effect of extracelluler osmotic change on cell viability and glycosaminoglycan production. Cells were isolated from the nucleus pulposus of 18-24 month bovine caudal discs. They were cultured for 6 days in alginate beads at 4 million cells/ml in Dulbecco's modified Eagle's medium containing 6% serum under 21% $O_2$, Medium osmolality was altered by NaCl addition over the range 270-570 mOsm and monitored using a freezing point osmometer.

(A-C) Electron micrograms of nucleus pulposus cells in the centre of the beads. At 270mOsm, the cell was swelling with numerous vacuoles and cytoplasmic organelles destroyed were visible (A). This cell undergoing oncosis were seen. At 370 mOsm, all cells appeared viable (B). At 570mOsm, the cells were reduced in size and budding was visible pinching off from the cytoplasm (C). (D) Effect of extracelluler osmotic change on glycosaminoglycan produced per million cells. Total glycosaminoglycans produced per million cells was the highest in the 370 mOsm group and the lowest in the hypo-osmolality

(270 mOsm) group. Glycosaminoglycan produced per million cells was significantly increased at 370 and 470 mOsm with time in culture (Paired t test between day 2 and day 6 [*,#: $P<0.05$]. However, there was no significant values of glycosaminoglycan produced per million cells at 270 and 570 mOsm with time in culture. Values are mean ± standard error. (Reproduced with permission from Takeno K, Kobayashi S, et al.: Physical limitations to tissue engineering of intervertabral disc cells. Effect of extracellular osmotic change on glycosaminoglycan production and cell metabolism. J Neurosurg Spine 7: 637-644, 2007).

Because disc cells obtain ATP primarily by glycolysis, glucose is a critical nutrient. The cells start to die within twenty-four hours if glucose concentration falls below 0.2 mM and the efficiency of glucose transport into the cell is likely reduced at this glucose concentration (Windhaber et al., 2003). The rate of cell death increases when pH levels are acidic. The cell viability is reduced even with adequate glucose at pH 6.0. The osmotic environment of nucleus pulposus cells in the discs changes with loading and pathologic states. The osmolality of the extracellular matrix is regulated by negatively charging the glycosaminoglycan chains of proteoglycans which adjust ionic composition. Particularly, extracellular osmolarity is controlled by negatively charged proteoglycans. It is now evident that an increase in the concentration of proteoglycans which control ionic composition causes an increase in the osmolality, and conversely, a decrease in proteoglycans reduces osmolality (Ishihara et al., 1997, Maroudas, 1981). Urban and Maroudas et al. assessed the osmotic pressure across the sagittal section of the discs and noted that the osmolality in the nucleus pulposus was about 370-400 mOsm (Maroudas, 1975, Urban & Maroudas, 1979), and the osmolality was decreased in degenerated disc. glycosaminoglycan production was largest in the 370mOsm, and the capacity for glycosaminoglycan production and cell metabolism (lactate production) was low under hypo-osmolality and hyper-osmolality, and cell deaths were observed on electron microscopy (Fig. 10) (Takeno et al., 2007). Thus, it may be said that osmotic pressure gradient disturbance associated with reduced proteoglycans is an important factor contributing to the development of disc degeneration. The results also suggest that standard culture mediums do not provide an appropriate ionic and osmotic environment for nucleus pulposus cells.

The physico-chemical environment created and maintained by disc cells in turn has a powerful effect on disc cell metabolism. However, the supply of nutrients from vascular buds at the end plates to the nucleus pulposus of a degenerative disc is likely to be affected, causing the extracellular environment to deteriorate (Urban & Roberts, 1995, Nachemson et al.,1970, Rajasekaran et al., 2004). Roberts et al. demonstrated that the cells in degenerate discs are senescent (Roberts et al., 2006). This environment is often neglected by it can strongly influence matrix turnover or the responses of disc cells to growth factors or other external stimuli. Such limitations apply to all avascular tissues including tissue engineered constructs.

## 8. Effect of cell density on the rate of glycosaminoglycan accumulation by nucleus pulposus cells

Glycosaminoglycan accumulation in constructs is dependent on the rate of glycosaminoglycan production per cell and on the cell density. It seems intuitive, therefore, that increasing cell density should increase rate of glycosaminoglycan deposition, as indeed

has been shown in several studies (Almarza & Athanasiou, 2005, Mauck et al., 2002, Mauck et al., 2003, Saini & Wick, 2003, Williams et al., 2005, Kobayashi et al., 2008). However, it is apparent from these studies that glycosaminoglycan accumulation in the construct does not increase in proportion to cell density and, indeed, glycosaminoglycan production per cell appears to fall at high cell densities. When bovine nucleus pulposus cells were cultured in three-dimensional constructs such as alginate beads, the amount of glycosaminoglycan accumulated increased with time in culture and also with increase in cell density as expected (Fig. 11A) (Kobayashi et al., 2008). However, the rise in cell density did not lead to a proportional rise in the amount of glycosaminoglycan accumulated; rather the amount of glycosaminoglycan produced per live cell fell with cell density and was significantly lower in beads cultured at a density of 22.7 million cells per ml than in those cultured at 2.02 million cells per ml for nucleus pulposus cells (Fig. 11B). This difference arose at least in part from a fall in metabolic activity of the cells rather than because of increased loss of glycosaminoglycan; the rate of energy production (Fig.12A) and of sulfated glycosaminoglycan production/viable cell (Fig.12B) was lower in cells cultured at high cell densities.

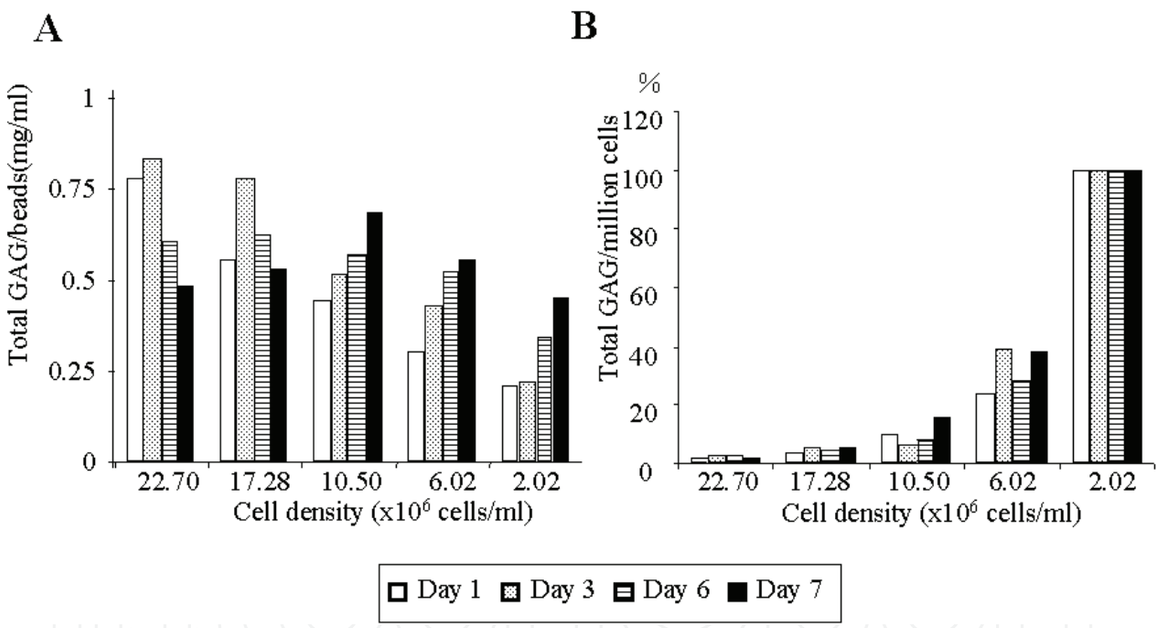


Fig. 11. Effect of cell density on glycosaminoglycan production.

Typical results showing effect of cell density on glycosaminoglycan deposition (A) and on glycosaminoglycan accumulation per million cells (B) by nucleus pulposus cells. Cells were isolated, encapsulated in alginate beads at cell densities ranging from 2.02 to 22.7 million cells/ml. Beads were cultured for 7 days at 5 wells/bead in 2 ml medium, 2 wells for each cell density and cultured for 7 days in Dulbecco's modified Eagle's medium containing 6% serum. Beads were then dissociated for cell counting and assay of total glycosaminoglycans. (A). Effect of cell density on glycosaminoglycan accumulation per beads (mg/ml). More glycosaminoglycan at high cell density than low cell density. Amount of

glycosaminoglycan/bead increased with time in culture at low cell density, but not at high cell density.
(B). Effect of cell density in beads on glycosaminoglycan production per million cells. glycosaminoglycan per cell higher at low cell density. Data normalized to results at 4 million cells/ml.

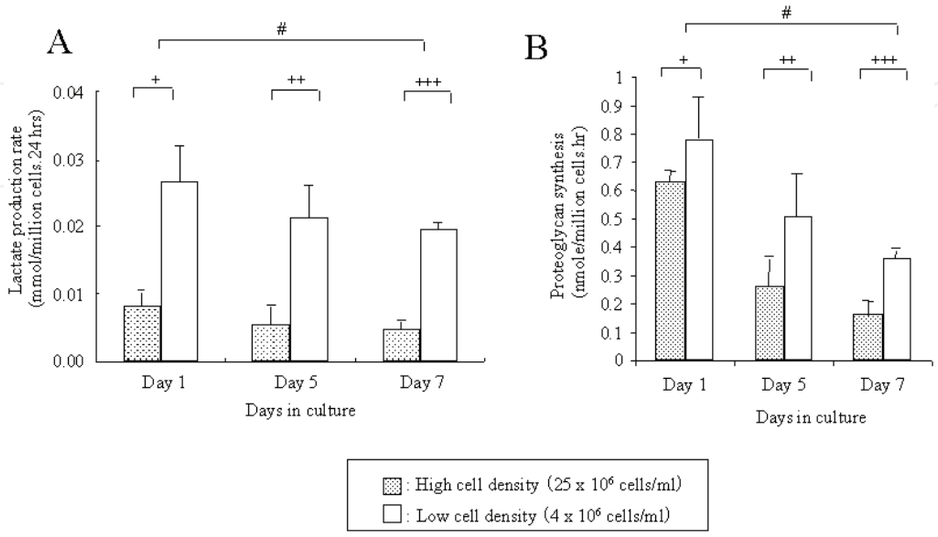


Fig. 12. Effect of cell density on lactate production rate (A) and $^{35}$S-sulfate incorporation rate (B). (A) Cells were cultured under standard conditions in beads containing 4 and 25 million cells/ ml (1.0ml medium, 5 beads/well) for up to 7 days, with complete medium change daily. Representative beads were dissociated for cell counting and viable cell density/bead recorded. Lactate in the medium was measured at days 1,5 and 7, after 24 hours culture and rates per million cells/24 hrs reported. High cell density lead to a fall in cellular metabolism (+,++,+++: $P<0.05$, Paired t test between high [25 million cells/ml] and low cell density [4 million cells/ml]). Lactate production rate fall with time in culture (#: $P<0.05$, 2 way ANOVA with repeated measures among 1, 5 and 7 days). (B) At days 1,5 and 7, tracer sulphate was added to the fresh medium of 3 wells, the beads were cultured in the radioactive solution for 4 hours, the beads dissociated and cell density and sulphate incorporation measured (Fig 3B). Results are given as means standard error of 3 independent experiments. Sulfate incorporation rates fall with increase in cell density (+,++,+++: $P<0.05$, Paired t test between high [25 million cells/ml] and low cell density [4 million cells/ml]) and with time in culture (#:$P<0.05$, 2 way ANOVA with repeated measures among 1, 5 and 7 days). (Reproduced with permission from Kobayashi S, Meir A, Urban J. Effect of cell density on the rate of glycosaminoglycan accumulation by disc and cartilage cells in vitro. J Orthop Res 26:493-503,2008.)

The change in percentage of live and dead cells with time in culture at the periphery and centre of beads is shown in Fig.13 for cells cultured at low (4 million cells/ml) and high cell densities (25 million cells/ml), respectively. For cells cultured at 4 million cells/ml, 100% of the cells were viable at the both the periphery (Fig 13A) and in the centre (Fig 13B). It can be seen that by day 2 of culture at high cell densities, while almost all the cells at the periphery were alive (Fig.13C,E), 30 percent of the cells in the bead centre were dead (Fig.13D,E).

Similar percentages were dead at day 5 of culture, suggesting the profile of viable cells across the bead was established early in culture (Fig.13E). At low cell density, transmission electron micrographs indicated that all cells appeared viable and active.

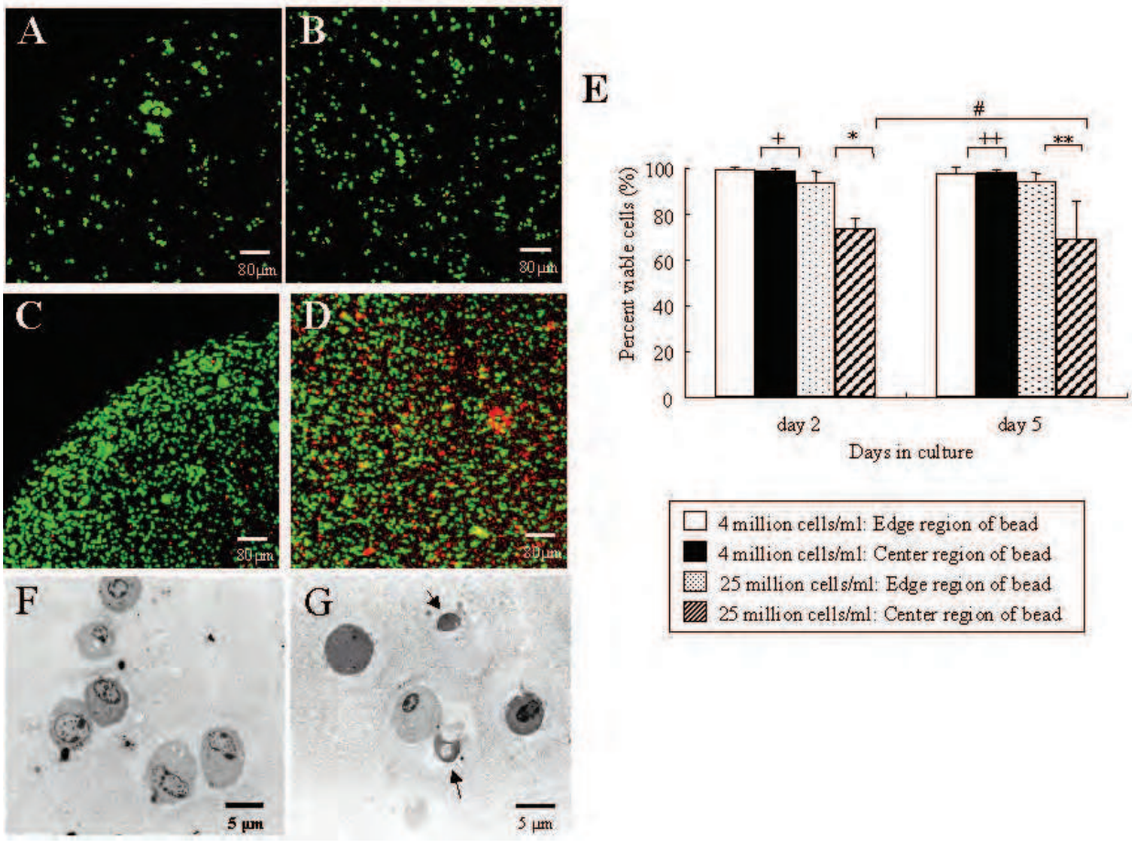


Fig. 13. Effect of cell density on cell viability under conforcal (A-E) and electron (F,G) microscope.

(A-E) A and B shows the periphery and central region respectively of a typical bead cultured at 4 million cells/ml after 5 days. C and D shows the periphery and central region of a bead cultured at 25 million cells/ml after 5 days. Cell viability was determined using a live/dead assay kit; live cells (green) and dead cells (red) were counted manually. E shows the variation of cell viability at the edge and centre of beads with time and cell density. Results are means and s.e.ms of percentage of viable cell from 4 representative beads. At high cell density (25 million cells/ml), cell viability is lower in the centre than at the edge (+: $P=0.961$, ++: $P=0.932$, *, **: $P<0.05$, 2 way ANOVA with repeated measures between edge and centre).
(F,G) Electron micrographs of peripheral (F) and central (G) nucleus pulposus cells (A-D) cultured at high cell density. The figures show representative cells from the central and peripheral regions of beads cultured at high (25 million cells/ml) for 5 days. In the bead periphery, some cells appear normal. However, the central region showing cells undergoing apoptosis (arrows). The cells and nuclei were reduced in size and chromatin condensation was seen in the nuclei in comparison of the cells in the periphery. (Reproduced with permission from Kobayashi S, Meir A, Urban J. Effect of cell density on

the rate of glycosaminoglycan accumulation by disc and cartilage cells in vitro. J Orthop Res 26:493-503,2008.)

Nucleus pulposus cells cultured at high cell density appeared viable at the bead periphery (Fig.6F). However cells undergoing apoptosis were seen in the centre; the cells and nuclei were reduced in size and chromatin condensation was visible in the nuclei (Fig.6G). These results are in agreement with those of others who have found regions of cell death in the center of constructs or even of microsphere aggregates (Martin et al., 1999, Mercier et al., 2004, Obradovic et al., 1999), and that glycosaminoglycan accumulation may highest at the construct peripheries. In addition, others have also found that increasing cell density or cell number does not necessarily increase matrix accumulation (Mercier et al., 2004).

These avascular constructs, unless experimentally perfused, rely on diffusion for supply of nutrients to the cells (Grunhagen et al., 2006, Obradovic et al., 2000) simulating the condition seen in intervertebral disc. In avascular tissues and in constructs, there are steep gradients of oxygen and other nutrients between the surface and center of the tissue or constructs (Kellner et al., 2002, Malda et al., 2004). The steepness of these gradients, and hence the nutrient concentrations in the center of the construct, depend not only on the geometry and properties of the tissue or construct but also on the cell density and the cellular activity. (Haselgrove et al., 1993, Zhou et al., 2004, Soukane et al., 2005). Thus, in any particular construct or tissue, an increase in cell density will lead to a corresponding fall in the concentration of nutrients such as oxygen and glucose, and an increase of metabolic by-products such as lactic acid (Zhou et al., 2004), leading, once cell density has risen sufficiently, to a fall in rates of cell metabolism and glycosaminoglycan synthesis (Gray et al., 1988, Ysart & Mason, 1994). If cell density is sufficiently great, oxygen and glucose concentrations and pH levels can fall to levels which can no longer sustain viable cells (Horner & Urban, 2001) leading to the necrotic region in the construct center. Diffusional nutrient transport is thus a limitation on the number of viable and active cells which can be maintained in any construct or tissue; indeed, viable cell density is inversely related to diffusion distance both in disc and in constructs (Horner & Urban, 2001, Stockwell, 1971).

## 9. Physical limitations to biological repair and tissue engineering

The interrelationships between cell density, cell viability and activity, and diffusion distance resulting from nutrient supply constraints, limit the rate at which glycosaminoglycan can be accumulated in three-dimensional constructs. Glycosaminoglycan accumulation depends on glycosaminoglycan production per cell and on cell density. At low cell densities, cells may be functioning optimally but the low cell density limits the rate of glycosaminoglycan accumulation. At high cell densities, more glycosaminoglycan is deposited at least initially, but nutrient gradients particularly in the center of constructs, reduce the rate of glycosaminoglycan deposition per cell and may even lead to a fall in cell number if cells die. Glycosaminoglycan accumulation thus appears necessarily slow, and the general finding that cultures of >7 months are required to achieve concentrations of glycosaminoglycan similar to those seen in vivo may not be easily overcome (Kellner et al., 2002, Roughley, 2004). The different maneuvers which have been tried to increase glycosaminoglycan production all have limitations. An increase in glycosaminoglycan production rate per cell can be induced by addition of growth factors, by providing mechanical or ultrasound stimulation or through alterations to scaffold properties (Blunk et al., 2002, Richmon et al.,

2005, van der Kraan et al., 2002, Kuo & Lin, 2006), but the relative increase which can be achieved is limited (usually two–threefold under optimal conditions) and the consequent increase in metabolic demand can lead to a fall in pH in the construct center (Razaq et al., 2004) and thus severely limit growth factor efficacy. Indeed, addition of growth factors to constructs was found to have little effect on the concentration of accumulated glycosaminoglycan although it increased construct size (Walsh et al., 2002, Yoon et al., 2003a, Yoon et al., 2003b, Masuda et al., 2003, An et al., 2005, Malda et al., 2004). In addition, glycosaminoglycan production rates appear to fall with time in culture in many different systems also limiting glycosaminoglycan accumulation (Mercier et al., 2004). Increasing cell density potentially should increase glycosaminoglycan deposition, but leads to a lower activity per cell, and also, in general, has not been found to increase glycosaminoglycan deposition rates (Panossian et al., 2001). It should also be noted that tissue *in vivo* cannot support too high a cell density, so *in vitro* culture of constructs at high cell density could lead to cell death after implantation.

Culture conditions such as stirring or perfusion (Freyria et al., 2000, Seidel et al., 2004) appear able to overcome diffusive transport initially, but as glycosaminoglycan concentrations rise and the hydraulic permeability of the construct falls, convective transport also is reduced and rates of glycosaminoglycan deposition slow. Glycosaminoglycan concentrations were reported to reach 5%by wet weight within 2 months but took a further 5 months to increase to 7% glycosaminoglycan. In view of the long culture times which appear necessary to achieve the required glycosaminoglycan composition in vitro, achievement of in vivo concentration before implantation of a construct may be an unrealistic and possibly unnecessary goal for tissue engineered disc.

Cellular repair using autologous chondrocyte transplantation appears successful even though chondrocytes are implanted with no matrix at all. Under these conditions, remodeling in vivo appears to produce a cartilage-type matrix under some conditions. Tissue engineered composites implanted with low glycosaminoglycan appeared to accumulate glycosaminoglycan in vivo, withstand physiological loading, and remodel towards a hyaline-type matrix. Perhaps optimization of such processes is a more useful goal.

## 10. Conclusions

There is increasing interest in the using biological methods to repair degenerate discs. Biological repair depends on the disc maintaining a population of viable and active cells. Adequate nutrition of the disc influences the outcome of such therapies and, hence, must be considered to be a crucial parameter. Therefore, it is very important to maintain an appropriate physicochemical environment to achieve successful disc repair by biological methods and tissue engineering procedures.

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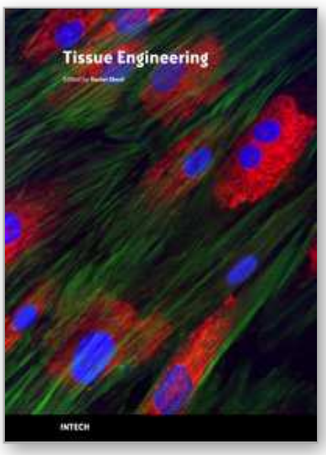


**Tissue Engineering**

Edited by Daniel Eberli

ISBN 978-953-307-079-7
Hard cover, 524 pages
**Publisher** InTech
**Published online** 01, March, 2010
**Published in print edition** March, 2010

The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

**How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Shigeru Kobayashi, Hisatoshi Baba, Kenichi Takeno, Tsuyoshi Miyazaki, Adam Meir and Jill Urban (2010). Physical Limitations to Tissue Engineering of Intervertabral Disc Cells, Tissue Engineering, Daniel Eberli (Ed.), ISBN: 978-953-307-079-7, InTech, Available from: http://www.intechopen.com/books/tissue-engineering/physical-limitations-to-tissue-engineering-of-intervertabral-disc-cells

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