

Basic Science

Animal Models of Disc Degeneration and Major Genetic Strategies

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The establishment of a reliable animal model of lumbar disc degeneration (AMDD) is important for studying pathogenesis and evaluating treatment effectiveness. However, an ideal AMDD for use in laboratory studies has not yet been produced. This retrospective study reviews and compares several common AMDD and discusses their strengths and weaknesses. We also suggest a new method for establishing future AMDD. The identified genes associated with disc degeneration are susceptibility genes, which elevate risk but do not necessarily lead to disease occurrence. We propose to identify families with hereditary disc degeneration, find major casual genes with exome sequencing, and establish transgenic animal models. This approach may help us to build an improved AMDD.

Key words: Disc degeneration, animal model, major gene strategy, exome sequencing

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Normal discs are located between adjacent vertebrae and consist of an endplate, annulus fibrosus, and nucleus pulposus. Fibrocartilagenous endplates are found at the top and bottom of vertebral bodies. Its average thickness is approximately one mm, which helps vertebrae to withstand pressure. The annulus fibrosus is divided into 2 tiers: the outer layer made of collagen fibers and the inner layer made of fibrous cartilage. The annulus fibrosus is arranged in concentric circles and is closely attached to the cartilage endplate to maintain spinal stability. The nucleus pulposus is a fibrocartilagenous tissue with a high water content. An intervertebral disc resembles a water bag with constant volume, and the nucleus pulposus resembles a ball wrapped with a fibrous ring that evenly distributes pressure (1). At the molecular and cellular levels, collagen type II, proteoglycans, and non-collagenous proteins synthesized by the cartilage cells form the nucleus

pulposus matrix and spinal cartilage endplate. Types I and II collagen fibers constitute the fibrous ring.

Disc degeneration is a class of diseases that can lead to bulging, endplate calcification, osteophyte formation, and spinal stenosis (2). It is a common orthopedic disease that often causes low back pain and seriously hinders patient quality of life. The mechanisms underlying disc degeneration are not fully elucidated. Traditional risk factors include age, gender, smoking history, and biomechanical factors. However, recent studies suggest that genetic factors might play an important role (3). Since 1998, a number of genes that can increase the risk of disc degeneration have been identified (3), and these discoveries have increased our understanding of the biological mechanisms of disc degeneration. Now, many researchers agree that similar to other complex diseases, disc degeneration is affected by both environmental and genetic factors (4-5).

This review will discuss the factors believed to underlie disc degeneration, explain the animal models that are routinely employed to mimic these pathological events, and describe a potential new strategy to generate new and improved animal models of disc degeneration (AMDDs).

FACTORS THAT CAUSE DISC DEGENERATION

Age

Studies have shown that discs gradually deteriorate over time. Disc matrix synthesis capacity decreases; the number of cells in the intervertebral disc is reduced; the nucleus pulposus loses water and elasticity; the annulus fibrosus around the nucleus pulposus becomes thick, which leads to further wear and tear; and the cartilage endplate thins and become uneven and calcified (6-7).

Genetics

In recent years, genetic factors underlying disc degeneration have attracted significant attention. Battié et al (8) studied 147 monozygotic and 153 dizygotic male twin pairs and found that lumbar disc degeneration showed a high degree of consistency with regard to signal intensity, disc stenosis, disc herniation, and endplate changes. The study also showed that family members of patients with intervertebral disc had higher incidences of disc herniation compared to the general population. Varlotta et al (9) assessed 63 young patients who had undergone disc surgery and the same number of normal controls and found that 32% of patients had a positive family history of disc disease compared with 7% of controls. Similarly, Simmons et al (10) studied 65 patients undergoing disc surgery and found that 44.6% had a family history of low-back pain compared with 25.4% of controls.

Biomechanical Factors

Biomechanical factors play an important role in disc degeneration. Abnormal physical stress can directly damage the disc structure and ultimately result in disc degeneration. Mechanical factors can also affect the biological characteristics of the disc tissue, resulting in intervertebral disc cell synthesis and abnormal inflammatory factor secretion (11-12). Epidemiological studies have shown that long-term heavy physical labor, bending over, and mechanical vibration exposure history are closely associated with disc degeneration. Animal experiments confirmed that long-term intervertebral disc compression or decompression lead to disc degen-

eration. Goff and Landmesser (13) amputated the upper limbs of mice to generate a bipedal animal model and found that mouse lumbar discs underwent changes similar to those observed in human intervertebral disc degeneration.

Trauma

Animal experiments and clinical observations show that traumatic factors clearly influence disc degeneration, and most existing AMDDs employ this mechanism. Trauma increases type I collagen fiber synthesis in the nucleus pulposus and simultaneously decreases type II collagen fiber synthesis, which leads to nucleus pulposus fibrosis and loss of elasticity. Trauma caused by vascular invasion, granulation tissue formation, and vasogenic cells destroys notochord cells and degrades the extracellular to facilitate disc degeneration. Trauma may directly lead to the annulus fibrosus tearing, which is also an important underlying cause of disc degeneration (14).

Other

Intervertebral discs are the body's largest enclosed structure that lacks a separate blood supply. During endplate injury, antigen components of the nucleus pulposus enter the circulatory system and elicit an immune response. Limiting expression of Fas ligand in disc tissue is important for maintaining disc immunological properties. Animal experiments confirmed that if the intervertebral disc physiological barrier is destroyed, Fas ligand can cause disc cell apoptosis (15-16). Other long-term, low-toxicity forms of damage, such as smoking, can also cause disc degeneration. Battié and colleagues (17) found that the average score of disc degeneration severity in smokers is 18% higher than that in non-smokers. Kim et al (18) reported that nicotine could lead to disc degeneration by inhibiting nucleus pulposus matrix proteoglycan synthesis and type II collagen expression.

Disc degeneration is a common cause of discogenic low-back pain. Mechanical compression causes nerve root congestion, edema, and inflammation, which are associated with pain. Furthermore the nucleus pulposus leaks due to disc degeneration and annular disruption can stimulate nerve roots and cause lower back pain. Lastly, during inflammation, a variety of chemical mediators increase blood vessel protein permeability and histamine release, this stimulates nerves and causes pain.

At present, the AMDD used in laboratories are mainly established based on the pathological mechanisms described above and in Fig. 1. A good animal

model is important for studying disease mechanisms and evaluating treatment effectiveness (19). At present, we lack a good animal model of disc degeneration; establishing a reliable, reproducible AMDD will increase our ability to study disease pathogenesis, progression, and provide an effective way to test new treatments (20).

ANIMAL MODELS OF DISC DEGENERATION

Many species, including mice, rats, sheep, dogs, and others, have been used to study disc degeneration. In general, AMDDs can be divided into 2 general categories: experimentally induced and spontaneous.

Experimentally Induced Models

This type can be divided into mechanical and

structural models in which degeneration is induced by experimental methods. The principle is to cause disc degeneration by directly damaging the intervertebral disc structure.

Mechanical Type

In this type of model, disc degeneration is induced by mechanical interventions, including load increasing/decreasing, exercise, or physical injury. In 1957 Lindblom (19) built a rat model of fixed tail in the U-bend position, and observed the degeneration of the concave side of the annulus fibrosus. Maclean JJ et al (21) fixed the rats' tails of the study group and gave daily 2-hour dynamic pressure for controls (Fig. 2). They observed overall downregulation of collagen types I and II and upregulation of aggrecan, collagenase, and

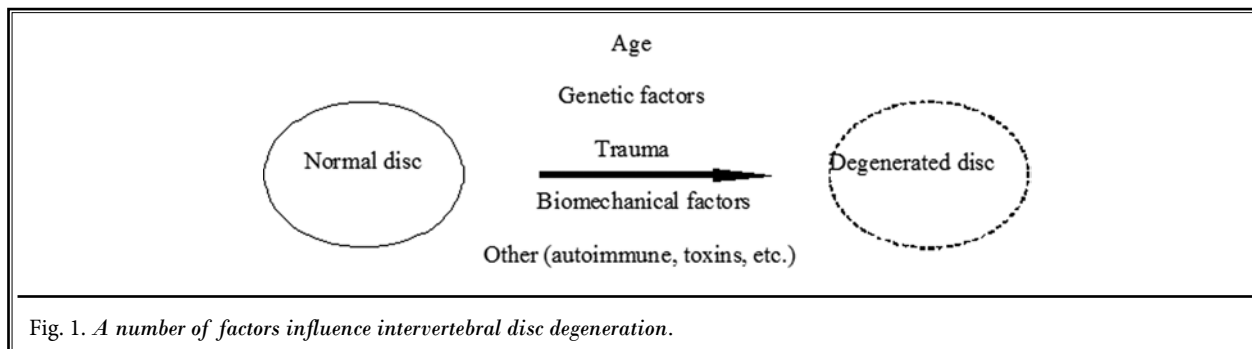


Fig. 1. A number of factors influence intervertebral disc degeneration.

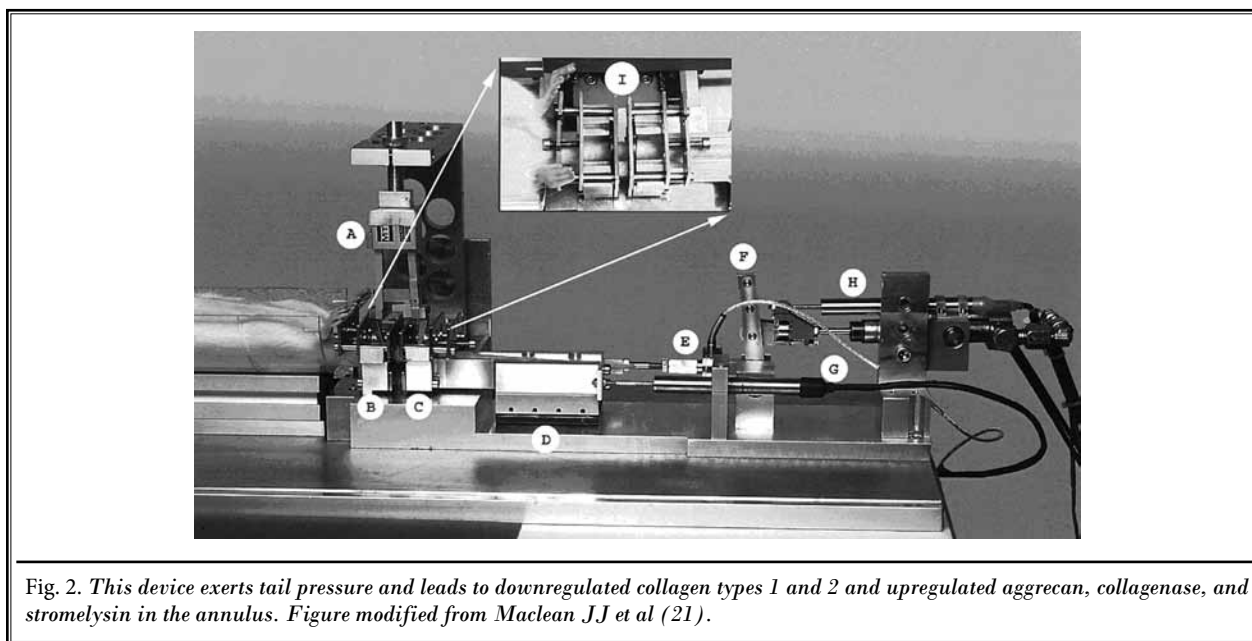


Fig. 2. This device exerts tail pressure and leads to downregulated collagen types 1 and 2 and upregulated aggrecan, collagenase, and stromelysin in the annulus. Figure modified from Maclean JJ et al (21).

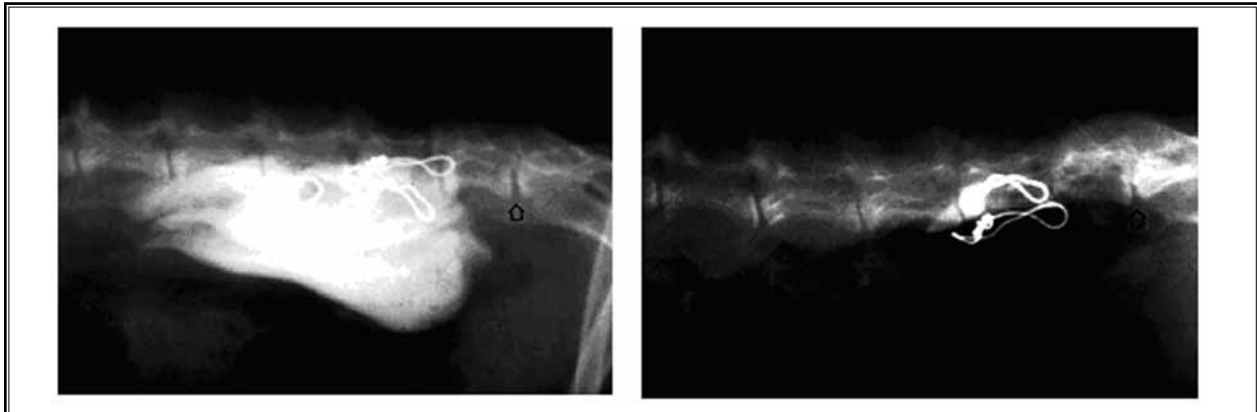


Fig. 3. Phillips et al (21) established a rabbit model of disc degeneration by fusing of lumbar 4, 5 to narrow the disc space and observed endplate sclerosis and osteophyte formation at adjacent disc spaces.

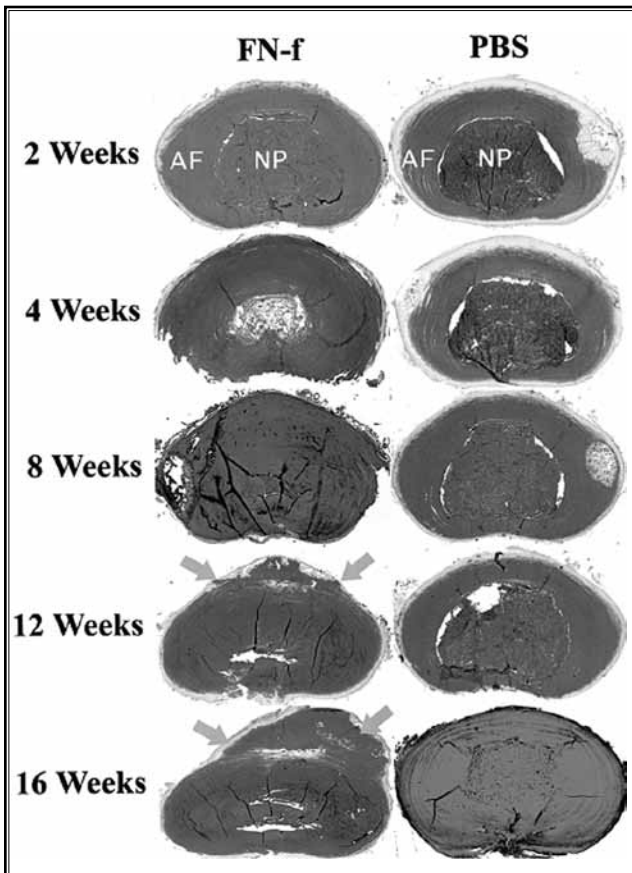


Fig. 4. Anderson et al (25) used a fine needle to inject a 30-kDa N-terminal fibronectin fragment (Fn-f-injected) into the center region of separate rabbit lumbar discs and compared the results with that of animals that received PBS injections. Over time, they observed disc degeneration similar to what occurs in human patients in the FN-f-treated animals. AF, annulus fibrosus; NP, nucleus pulposus.

stromelysin in the annulus in both the study and control groups. Phillips and colleagues (22) established a rabbit model of disc degeneration to mimic what is observed in humans at levels proximal (L4-L5) and caudal (L7-S1) using simulated lumbar fusion. The experimental animals were studied for up to 9 months after arthrodesis. By month 3, they observed collagen bundled within the annular lamellae and a loss of normal parallel arrangement. There was further disorganization at month 6, and at month 9, the structure of the disc had been replaced by disorganized fibrous tissue (Fig. 3). This type of animal model relies on biomechanical factors. Abnormal stress directly damages intervertebral disc structure and results in disc degeneration. Although this model is popular, it has its limitations. Human disc degeneration is a complex disease caused by multiple factors, and is not fully replicated by introducing a single mechanical factor in an animal model.

Structural Type

In structural models, degeneration is induced by injuring intervertebral disc structure. Osti et al (23) established a disc degeneration model by surgically injuring the front edge of the fibrous ring in sheep. They observed postoperative changes in intervertebral discs: The annulus fibrosus formed granulation tissue in the outer third, the inner annulus was broken, and the nucleus pulposus-like tissue was mislocalized to the incision site within 180 days. Fazzalari and colleagues (24) punctured sheep anterior left annulus fibrosus and observed mechanical changes in annular lamellar thickness and vertebral body bone volume fraction. Figure 4 shows an example of such lamellar thickening. Anderson et al (25) injected a

30-kDa N-terminal fibronectin fragment (Fn-f-injected) or phosphate-buffered saline (PBS) into rabbit intervertebral discs. The animals were sacrificed 2, 4, 8, or 12 weeks after the procedure, and histological assessments revealed a progressive loss of the normal architecture of the nucleus pulposus and annulus fibrosus over the 16-week study period (Fig. 5). This type of animal model is the most commonly used method that directly damages the intervertebral disc structure to achieve induction of disc degeneration. This low-cost approach for generating animal models takes a relatively short time and is highly reproducible. However, its limitations include the use of a single pathogenic factor to mimic a complex pathological process and the need for surgical intervention, which may result in infection.

Spontaneous Type

In this type of animal model, disc degeneration is a spontaneous natural process, and thus is the closest to human intervertebral disc degeneration among existing AMDDs. The sand rat is the most commonly used spontaneous degeneration model. Silberberg and co-workers (26) first described changes in intervertebral discs of the sand rat in 1979. They observed notochord cells apoptosis, endplate sclerosis, annular disruption, and peripheral osteophyte formation in the majority of 18 - 30-month-old sand rats. Gruber et al (27) studied intervertebral disc imaging features of 158 sand rats in a cross-sectional study and 22 in a longitudinal study. They noted bulging discs at weeks 6 - 12 follow-up and lumbar endplate calcification in 12-month-old animals. They found intervertebral disc narrowing and bulging in the majority of the rats. Although this model most closely resembles what occurs in humans in that it is spontaneous, it has its own limitation, including limited choice of animal species, unclear degeneration mechanism, and difficulties in predicting degeneration incidence. For these reasons, the applicability of these animal models is limited.

Susceptibility Gene Selection Strategy

Although there are many different AMDDs, we are still in great need of an appropriate, reliable, and reproducible model, which would help us to understand the underlying mechanism of disc degeneration in humans and validate treatment feasibility and efficacy. It is well known that genetic factors play crucial roles in the development of disc degeneration. Battié and Videman (28) estimated the proportion of disc degeneration attributable to genetic factors to be as high as

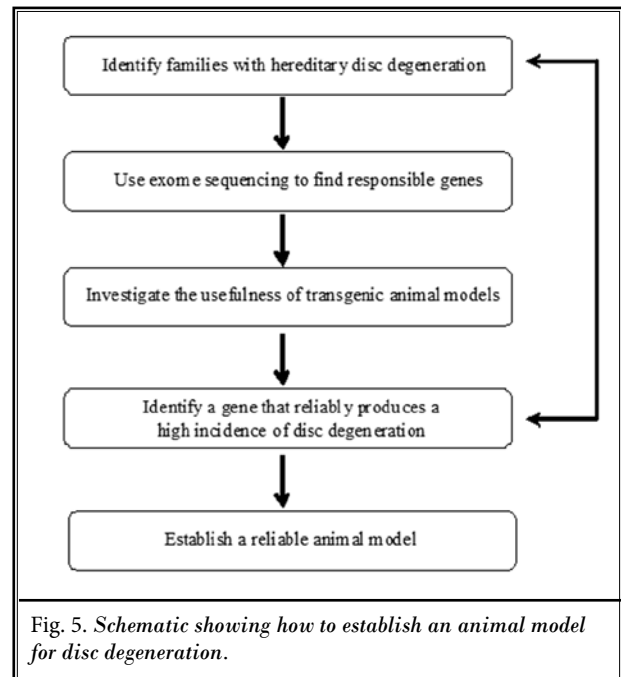


Fig. 5. Schematic showing how to establish an animal model for disc degeneration.

74%, and Videman et al (29) posited that 47 - 66% of disc degeneration may be influenced by genetic factors. Therefore, we propose a new method of establishing AMDDs by exploiting recent breakthroughs in genetic sequencing and molecular biology techniques. In our research, we have found significant familial aggregation characteristics in disc disease, which provides an excellent starting point for exploring disc disease gene susceptibility (30). We propose identifying major genes of intervertebral disc degeneration and using these to create new transgenic animal models. At present, some researchers have already started down this road. Sahlman et al (31) conducted a study on the role of the COL2a1 gene in growth, development, and degeneration in rats and found that limb, skull, and spine shortening; fibrous ring changes; and vertebral endplate thickening and glycosaminoglycan reduction is associated with COL2a1 knockout. Hamrick (32) studied rats lacking the GD8 gene and reported similar findings. However, there are still some inherent problems in this type of animal model: some transgenic animals have short survival times, it is difficult to predict the incidence of disc degeneration, and the cultivation cycle is relatively long. We believe that effective screening for major genes within families with hereditary disc degeneration could increase the incidence of degeneration in model systems and shorten the onset time.

In recent years, researchers have employed both

whole-genome scanning and candidate gene approaches to identify the genetic factors of complex diseases. Often times, one may compare the potential susceptible genes between cases and controls to determine whether this target gene is associated with the phenotype of interest (33). We propose to use the classification strategy developed by Zhang et al (3) to identify candidate genes in the first step. Disc degeneration-related genes are divided into 4 categories. The first includes intervertebral disc structure-related genes, such as the collagen I and IX genes and the aggrecan gene. The second category includes genes associated with intervertebral disc matrix-degrading enzymes, e.g., the matrix metalloproteinase (MMP)-3 and -9 genes. The third category consists of genes associated with bone structure, and the last category includes all other genes.

Type IX Collagen

Collagen 9 encoded by the type IX collagen gene is the collagen component of most hyaline cartilage. This gene is divided into COL9A1, COL9A2, and COL9A3, which encode the 3 chains of type IX collagen fiber. Mutations in these genes may induce disc degeneration, and type IX collagen allele is a suitable candidate gene. Specifically, COL9A2 and COL9A3 are current study foci.

COL9A2

COL9A2 gene encodes the A2 chain of type IX collagen fibers. The coding of Glu326 or Arg326 is substituted by Trp326 (Trp2), which can cause structural abnormalities of intervertebral disc collagen that renders discs prone to degeneration. Jim et al (34) conducted a large case-control study in southern China and found that people who carried the Trp2 polymorphism had an increased risk of disc degeneration with an odds ratio (OR) of 4 in the 30 - 39 age group and an OR of 2.4 in the 40 - 49 age group. Higashino and colleagues (35) studied 84 patients who had undergone discectomy and found that 21.4% of patients had the Trp2 allele. It has been found that patients younger than 40 with the Trp2 polymorphism are prone to severe disc degeneration. However, this effect appears to be age-dependent; the phenomenon was not observed in patients over 40 years old. Wrocklage et al (36) employed restriction endonuclease technology to analyze disc tissue samples from 250 patients and discovered that Trp2 can affect disc tissue stability by reducing the number of collagen crosslinks. Seki and colleagues (37) studied the COL9A2 gene polymorphism in 470 patients with lumbar disc disease and 658 normal controls and found that Trp2 was common in the Japanese population. They

identified a COL9A2-specific haplotype related to severe disc degeneration, which suggests that disc degeneration susceptibility genes might vary in different ethnic groups.

COL9A3

Arginine103 is replaced by tryptophan103 (Trp3) in a known COL9A3 gene mutation. In a case-control study of 171 patients with disc degeneration and 321 normal controls, Paasilta et al (38) found that 12.2% of patients had Trp3 genotypes compared to only 4.7% in the control group and concluded that the Trp3 genotype was associated with disc degeneration susceptibility. By studying serum samples of 135 middle-aged men with occupation predisposing factors, Solovieva and colleagues (39) found that in the absence of a specific interleukin-1 (IL-1) genotype, Trp3 genotype increases the risk of disc degeneration by 6 fold. Their findings suggest that the effect of Trp3 gene on disc degeneration risk can be greatly attenuated by IL-1 genotype. That same group further investigated the interplay between Trp3 gene and obesity, which is also a risk factor, and found that 45 - 71% of disc degeneration could be attributed to the synergistic effect between the 2 factors (40). Kales et al (41) studied 105 patients who underwent surgery for disc disease and 102 normal controls and found that the frequencies of COL9A3 gene mutation were 12.3% and 4.9%, respectively.

Sox9

Berta and co-workers (42) cloned testis-determining factor (SPY gene) in humans and mice and found that the SPY gene product contained a section similar to the binding sequence of chromosomal proteins high mobility group (HMG)-1 and -2. An HMG box is a DNA binding motif of about 79 amino acids. Sox transcription factors belong to the superfamily of genes that contain an HMG box. Paul et al (43) hypothesized that sox9 could increase the amount of type 2 collagen fibers in disc tissue, which was then tested by transfecting rabbits with sox9 adenovirus. Greater intervertebral content of type 2 collagen fibers reduces the incidence of disc degeneration. Gruber et al (44) measured sox9 in disc tissue from 12 controls and 25 disc degeneration patients and found that sox9 expression was inversely related with age. They speculated that intervertebral disc degeneration is associated with sox9 expressing deletion.

AGC-1

Aggrecan (AGC), which is found in the nucleus pulposus, is an important intervertebral disc proteo-

glycan and is associated with pressure load. Individuals with low levels of aggrecan may be prone to disc degeneration. Videman et al (5) studied 25 disc degeneration-related genes in 588 men and concluded that AGC-1 was related to disc signal and height reduction on magnetic resonance imaging. Solovieva et al (39) had studied 132 cases of middle-aged men in Finland and found that AGC gene increased the occurrence of bulging disc and disc height reduction.

MMP-3 and -9

MMPs are a family of enzymes that can degrade the extracellular matrix. MMP3 can degrade proteoglycans, laminin, and fibronectin (45). Because the intervertebral matrix is mainly comprised of collagen, proteoglycan, water, and elastin, it is logical that MMPs are associated with disc degeneration. Yuan et al (46) studied MMP-3 alleles in 178 lumbar disc degeneration cases and 284 controls and found that the risk of disc degeneration is positively associated with genotype 6A5A or 5A5A. Sun et al (47) investigated the MMP-9 gene in 408 lumbar disc degeneration patients and 451 normal controls in northern China and found that cytosine1562 can be replaced by thymine1562, which increases the risk of disc degeneration with an OR of 2.14. This finding demonstrates that the MMP9 gene 1562 C/T polymorphism is related to disc degeneration in the Chinese population.

IL-1, IL-6

Solovieva et al (39) confirmed that replacing thymine3954 with cytosine in the IL-1 gene also increases the risk of disc degeneration. By studying 588 male patients with disc degeneration, Videman and colleagues (5) also demonstrated that IL-1 is related to disc signal reduction. In a case-control study, Noponen-Hietala et al (48) reported that the replacement of thymine15 with adenine in the fifth exon of the IL-6 gene also increases the risk of disc degeneration.

Vitamin D Receptor

Vitamin D receptor (VDR) is closely related to bone and cartilage metabolism. Vitamin D is a necessary factor for maintaining normal bone metabolism; vitamin D deficiency may lead to rickets in children and osteoporosis in adults. A study conducted by Eser and colleagues (49) in Turkey demonstrated that the tt/Tt genotype confers increased risk for disc degeneration. Furthermore, patients with the tt genotype tend to have more severe disc degeneration than those with Tt.

Currently, the disc disease-related COL9a2 gene

attracts more attention than other candidates. The underlying mechanisms related with these genes are far from clear and further research is required (50).

Major Gene Strategy

Disc degeneration disease is a complex multifactorial disease. In addition to trauma and biomechanical issues, it is also influenced by multiple genes. The genes mentioned above are susceptibility genes for disc degeneration rather than major genes, which would lead to the inevitable occurrence of disc degeneration. Therefore, they cannot be used to generate ideal transgenic animal models.

We usually employ linkage analysis to study genetic diseases. However, such a method requires a large pedigree, which would be difficult for a study of disc degeneration. Unlike linkage analysis, exome sequencing is an efficient strategy to selectively sequence the coding regions of the genome to identify novel genes associated with both rare and common disorders. Exon sequencing has been widely used in the study of Mendelian disease because of its economy and effectiveness. For example, Ng et al (51) successfully used this technique to determine that mutations in the MYH3 gene were the cause of Freeman-Sheldon syndrome in 8 unrelated individuals. They subsequently screened DHODH mutations in 4 patients with Miller syndrome (52). Although it is less frequently used for studying complex disease, exon sequencing offers a promising approach in this research area.

We hope to be able to identify a single gene capable of causing disc degeneration and use it to create a viable animal model. The first step is to find a significant disc degeneration pedigree, then use exome sequencing to identify a major gene, and mutate this gene to produce a transgenic AMDD. We often examine families with hereditary disc degeneration in China, which laid the foundation for the establishment of the disc degeneration gene bank. It is our hope that identifying major genes associated with disc disease to establish transgenic animal models will become an important future direction for disc disease research.

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