Identification of Pathways and Genes Associated With Meniscus Degeneration Using Bioinformatics Analyses

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Abstract

There are few studies on the genetic changes of meniscus degeneration. We used anterior cruciate ligament resection of Wuzhishan pig to prepare a meniscus degeneration model, and applied gene chip technology to detect differentially expressed genes in degenerative meniscus tissue. Then we applied GO analysis, Pathway analysis, Core gene network analysis and Relevant miRNAs analysis to discover relevant regulatory networks of meniscus degeneration. As a result, we detected 893 differentially expressed genes, mainly involving hormone, apoptosis, inflammation and other mechanisms, and obtained MUC13, Inflammatory mediator regulation of TRP channels, MDFI, mir-335-5p and so on that may play a key role. In summary, we have established a reliable animal model of meniscus degeneration and found that meniscus degeneration involves several possible molecular mechanisms, which will provide molecular targets for further research of the disease in the future.

Introduction

The meniscus has the functions of buffering load, absorbing impact, decompressing and improving joint stability. And the blood circulation of the meniscus is poor, the healing ability is limited, and it is easy to degenerate. The degenerative meniscus not only cannot fully protect the knee joint, but also tends to tear itself, leading to knee cartilage damage, pain, and restricted knee movement\(^1\text{,}\,^2\). In addition, Meniscus degeneration can lead to osteoarthritis\(^3\). Therefore, how to protect the meniscus and delay the degeneration of the meniscus has important scientific significance for preventing meniscus injury in young and middle-aged patients and alleviating the symptoms of elderly patients with knee arthritis.

Meniscus and articular cartilage are very similar in tissue composition, many researchers speculate that meniscus degeneration may be similar to cartilage degeneration, and the imbalance between anabolism and catabolism leads to degeneration\(^4\text{,}\,^5\). But the specific mechanism is not clear. Trauma, chronic inflammation, apoptosis and so on may be involved unilaterally or in multiple ways, and there are countless kinds of genes that may play a role, which brings great trouble to the research of meniscus degeneration mechanism\(^6\text{,}\,^7\). With the advantages of large amount of information, reliable operation and repeatability, gene chip technology has been successfully used in gene expression detection, DNA sequencing, search for new genes, diagnosis of diseases and other research fields\(^8\). This study used gene chip technology to screen differentially expressed genes (DEGs) between normal meniscus and degenerated meniscus, and tried to analyze the mechanism of meniscal degeneration to provide theoretical basis for the diagnosis, prevention and treatment of meniscal degeneration.

Results

Gross morphological observation

In the normal meniscus group, the meniscus was smooth and complete, with a white and shiny surface, without any signs of degeneration. In the degenerative meniscus group, the surface of meniscus was rough, the color was dim, the elasticity was poor, and there were some small defects and erosion (Fig. 1).

Figure 1. (a) In the normal meniscus group, the meniscus was crescent shaped, with complete and smooth surface, no tear, white color and good elasticity. And the medial part of the meniscus was thin and the lateral part was thick. (b) In the degenerative meniscus group, the color of the meniscus was light yellow, the elasticity was worse than that of the normal meniscus, the surrounding synovial membrane was congested and edema, the inner part was thinner with uneven wear, and the free edge was incomplete and cracked.

Histological examination
In the normal meniscus group, HE staining of the meniscus showed normal meniscus tissue. In the degenerative meniscus group, HE staining of the meniscus showed disorder, uneven staining and sparse arrangement of collagen fibers, disorder, reduction and swelling of chondrocytes, reduction or disappearance of cartilage lacunae, increased local fibers and hyaline changes, which were consistent with meniscal degeneration (Fig. 2).

Figure 2. (a-b) The HE staining of normal meniscus tissue. The chondrocyte nucleus was large and round, the cell distribution was regular, the collagen fibers were abundant, and the fiber bundles were thick and neat. (c-f) The HE staining of degenerative meniscus. The arrangement of collagen fibers was disordered, the staining was uneven, and the arrangement was sparse; the chondrocytes were arranged disorderly and reduced, and the visible cells were swollen, the cartilage lacuna was reduced or disappeared, and the local area was fibrotic and hyalinized. a: x40, b: x100, c: x40, d: x100, e: x200, f: x400.

**Quality control**

For RNA quantification and quality assurance performed by NanoDrop ND-1000, the ratio of A260/A280 for all samples was close to 2.0, and the ratio of O.D. A260/A230 was greater than 1.8. The result of Agilent 2100 Bioanalyzer showed that all RINs ≥ 6.3, indicating that the integrity of total RNA was good and there was no obvious degradation, so follow-up experiments could be carried out (Table 1).

Table 1. RNA quality control result

| Sample ID | OD260/280 Ratio | OD260/230 Ratio | Conc. (ng/µl) | RIN  | Pass or Fail |
|-----------|----------------|----------------|--------------|------|--------------|
| NM1       | 2.00           | 1.90           | 319.41       | 6.9  | Pass         |
| NM2       | 1.88           | 1.96           | 195.83       | 6.5  | Pass         |
| NM3       | 2.00           | 1.99           | 314.36       | 6.5  | Pass         |
| NM4       | 2.02           | 1.91           | 299.40       | 6.4  | Pass         |
| NM5       | 2.01           | 1.97           | 335.84       | 6.3  | Pass         |
| NM6       | 1.94           | 1.85           | 164.94       | 7.2  | Pass         |
| NM7       | 2.00           | 1.94           | 316.15       | 6.3  | Pass         |
| DM1       | 1.97           | 1.92           | 358.41       | 6.3  | Pass         |
| DM2       | 2.02           | 1.97           | 721.61       | 7.5  | Pass         |
| DM3       | 2.03           | 1.90           | 400.45       | 6.3  | Pass         |
| DM4       | 1.83           | 1.96           | 112.25       | 7.2  | Pass         |
| DM5       | 1.98           | 1.89           | 267.03       | 6.5  | Pass         |
| DM6       | 1.86           | 1.90           | 251.31       | 6.4  | Pass         |
| DM7       | 2.01           | 2.07           | 345.64       | 6.7  | Pass         |

NM: normal meniscus; DM: degenerative meniscus
**DEGs**

There were 893 DEGs in the two groups, including 537 up-regulated genes and 356 down-regulated genes. The result was showed in the volcano plot and the dendrogram (Fig. 3). The top 10 most significant genes were cyp2c33, gcnt7, ncdn, exd3, MUC13, ppp1r3d, nphp3, upb1, CD81 and prph (Table 2).

Figure 3. (a) The volcano plot. Red and green represent DEGs; the downregulated DEGs are green, and the upregulated DEGs are red. (b) The dendrogram. The ordinate represents the grouping information of the sample. Red indicates high relative expression and green indicates low relative expression. NM: normal meniscus; DM: degenerative meniscus.

Table 2. The top 10 most significant genes.

| ProbeName     | P-value         | FDR            | Fold Change | Regulation | GeneSymbol | Rank |
|---------------|-----------------|----------------|-------------|------------|------------|------|
| A_72_P526917  | 1.2593E-07      | 0.000537464    | 2.0593695   | up         | CYP2C33    | 1    |
| A_72_P362758  | 1.38326E-07     | 0.000537464    | 3.2506906   | up         | GCNT7      | 2    |
| A_72_P050406  | 6.10533E-07     | 0.001031403    | 2.5418055   | up         | NCDN       | 3    |
| A_72_P258107  | 8.6355E-07      | 0.001198329    | 2.5599119   | up         | EXD3       | 4    |
| A_72_P165116  | 9.46523E-07     | 0.001225904    | 2.046065    | up         | MUC13      | 5    |
| A_72_P442485  | 1.08108E-06     | 0.001272895    | 2.2891991   | up         | PPP1R3D    | 6    |
| A_72_P222892  | 1.13988E-06     | 0.001302652    | 2.0178437   | up         | NPHP3      | 7    |
| A_72_P234107  | 1.23101E-06     | 0.001328635    | 2.5152315   | up         | UPB1       | 8    |
| A_72_P496803  | 1.4119E-06      | 0.001443671    | 3.0181273   | up         | CD81       | 9    |
| A_72_P352838  | 1.81842E-06     | 0.001613149    | 2.4487246   | up         | PRPH       | 10   |

**GO analysis**

A total of 55 biological processes enriched by the DEGs were obtained. The top 10 biological processes were cell response to hormone stimulus, response to hormone, sex determination, muscle fiber development, mehcheme morphogenesis, cell response to endogenous stimulus, C21 steroid hormone metallic process, neuropeptide signaling pathway, negative regulation, respectively of reactive oxygen species metallic process and regulation of nitric oxide biosynthetic process (Table 3).

Table 3. The enriched GO terms for the DEGs.
| Term                                                | Count | P-value          | Regulation | Rank |
|-----------------------------------------------------|-------|-----------------|------------|------|
| cellular response to hormone stimulus               | 7     | 0.000311929     | Up         | 1    |
| response to hormone                                 | 7     | 0.001152029     | Up         | 2    |
| sex determination                                   | 2     | 0.001281445     | Down       | 3    |
| muscle fiber development                            | 2     | 0.002753211     | Down       | 4    |
| mesenchyme morphogenesis                            | 2     | 0.002753211     | Down       | 5    |
| cellular response to endogenous stimulus            | 8     | 0.003566943     | Up         | 6    |
| C21-steroid hormone metabolic process               | 2     | 0.003588081     | Up         | 7    |
| neuropeptide signaling pathway                      | 3     | 0.003940886     | Up         | 8    |
| negative regulation of reactive oxygen species metabolic process | 2 | 0.004359628 | Up | 9 |
| regulation of nitric oxide biosynthetic process     | 2     | 0.007086781     | Up         | 10   |
| response to endogenous stimulus                     | 8     | 0.007145167     | Up         | 11   |
| negative regulation of reproductive process         | 2     | 0.008950734     | Down       | 12   |
| nitric oxide biosynthetic process                   | 2     | 0.009235862     | Up         | 13   |
| nitric oxide metabolic process                      | 2     | 0.009235862     | Up         | 14   |
| reactive nitrogen species metabolic process         | 2     | 0.009235862     | Up         | 15   |
| gland development                                   | 4     | 0.010284414     | Up         | 16   |
| hormone metabolic process                           | 3     | 0.011308564     | Up         | 17   |
| male gonad development                              | 2     | 0.011498663     | Down       | 18   |
| development of primary male sexual characteristics  | 2     | 0.011498663     | Down       | 19   |
| regulation of reactive oxygen species biosynthetic process | 2 | 0.011638114 | Up | 20 |
| body fluid secretion                                | 2     | 0.012931151     | Up         | 21   |
| striated muscle cell development                    | 2     | 0.013594657     | Down       | 22   |
| hormone biosynthetic process                        | 2     | 0.015695135     | Up         | 23   |
| male sex differentiation                            | 2     | 0.016626965     | Down       | 24   |
| muscle cell development                             | 2     | 0.016626965     | Down       | 25   |
| reproductive structure development                  | 3     | 0.017568602     | Down       | 26   |
| reproductive system development                     | 3     | 0.017988302     | Down       | 27   |
| cellular hormone metabolic process                  | 2     | 0.018688631     | Up         | 28   |
| regulation of hormone levels                        | 3     | 0.018844593     | Down       | 29   |
| reactive oxygen species biosynthetic process        | 2     | 0.020268615     | Up         | 30   |
| epithelial cell differentiation                     | 3     | 0.020624915     | Down       | 31   |
| neurotransmitter biosynthetic process               | 2     | 0.021902613     | Up         | 32   |
| feeding behavior                                    | 2     | 0.023589533     | Up         | 33   |
| Pathway                                             | P-values | q-values | Adjusted P-value | Adjusted q-value |
|----------------------------------------------------|----------|----------|------------------|------------------|
| response to chemical                               | 0.023617031 | Up      | 34               |                  |
| cellular response to chemical stimulus             | 0.023892904 | Up      | 35               |                  |
| hormone metabolic process                          | 0.026281932 | Down    | 36               |                  |
| bone remodeling                                    | 0.027117856 | Up      | 37               |                  |
| striated muscle cell differentiation               | 0.033355701 | Down    | 38               |                  |
| tissue development                                 | 0.035003106 | Down    | 39               |                  |
| gonad development                                  | 0.035500212 | Down    | 40               |                  |
| development of primary sexual characteristics      | 0.035500212 | Down    | 41               |                  |
| mammary gland development                          | 0.036791312 | Up      | 42               |                  |
| mesenchyme development                             | 0.038815337 | Down    | 43               |                  |
| regulation of reproductive process                 | 0.038815337 | Down    | 44               |                  |
| muscle organ development                            | 0.041089286 | Down    | 45               |                  |
| response to oxygen-containing compound             | 0.041364725 | Up      | 46               |                  |
| hormone-mediated signaling pathway                 | 0.043140041 | Up      | 47               |                  |
| neurotransmitter metabolic process                 | 0.043140041 | Up      | 48               |                  |
| regulation of reactive oxygen species metabolic process | 0.043140041 | Up      | 49               |                  |
| response to peptide                                | 0.048362819 | Up      | 50               |                  |
| body morphogenesis                                 | 0.048805944 | Down    | 51               |                  |
| forebrain neuron differentiation                   | 0.048805944 | Down    | 52               |                  |
| regulation of interleukin-4 production             | 0.048805944 | Down    | 53               |                  |
| positive regulation of mitotic nuclear division    | 0.048805944 | Down    | 54               |                  |
| labyrinthine layer morphogenesis                   | 0.048805944 | Down    | 55               |                  |

Pathway analysis

A total of 36 pathways were affected by the alteration of the gene expression. The top 10 pathways are Type II diabetes mellitus, Thyroid hormone synthesis, Taste transduction, Prolactin signaling pathway, Longevity regulating pathway, Ovarian steroidogenesis, Neuroactive ligand-receptor interaction, Inflammatory mediator regulation of TRP channels, Pantothenate and CoA biosynthesis and Cocaine addiction (Table 4).

Table 4. The enriched pathways for the DEGs.
| Definition                                                                 | Fisher P-value | regulation | Rank |
|---------------------------------------------------------------------------|---------------|------------|------|
| Type II diabetes mellitus                                               | 0.000759595   | Up         | 1    |
| Thyroid hormone synthesis                                               | 0.002669959   | Down       | 2    |
| Taste transduction                                                       | 0.002858715   | Up         | 3    |
| Prolactin signaling pathway                                              | 0.003352503   | Up         | 4    |
| Longevity regulating pathway                                             | 0.007875137   | Up         | 5    |
| Ovarian steroidogenesis                                                  | 0.009215694   | Up         | 6    |
| Neuroactive ligand-receptor interaction                                  | 0.01021098    | Up         | 7    |
| Inflammatory mediator regulation of TRP channels                         | 0.01098087    | Up         | 8    |
| Pantothenate and CoA biosynthesis                                        | 0.01205545    | Up         | 9    |
| Cocaine addiction                                                       | 0.01468413    | Down       | 10   |
| Metabolism of xenobiotics by cytochrome P450                             | 0.01528523    | Down       | 11   |
| Legionellosis                                                            | 0.01844662    | Down       | 12   |
| AMPK signaling pathway                                                   | 0.02220957    | Up         | 13   |
| Bile secretion                                                           | 0.02225764    | Up         | 14   |
| Glycerolipid metabolism                                                 | 0.02257206    | Down       | 15   |
| Chemical carcinogenesis                                                  | 0.02329364    | Down       | 16   |
| Adipocytokine signaling pathway                                          | 0.0249136     | Up         | 17   |
| Amphetamine addiction                                                    | 0.02627419    | Down       | 18   |
| Acute myeloid leukemia                                                   | 0.02627419    | Down       | 19   |
| Adherens junction                                                        | 0.03020581    | Down       | 20   |
| Drug metabolism - other enzymes                                          | 0.03062382    | Up         | 21   |
| Insulin signaling pathway                                               | 0.0323184     | Up         | 22   |
| African trypanosomiasis                                                  | 0.03438215    | Up         | 23   |
| Dilated cardiomyopathy (DCM)                                             | 0.0360517     | Up         | 24   |
| Primary immunodeficiency                                                | 0.03632543    | Up         | 25   |
| Insulin secretion                                                        | 0.03717241    | Up         | 26   |
| GABAergic synapse                                                        | 0.03831094    | Up         | 27   |
| Phospholipase D signaling pathway                                        | 0.0386556     | Up         | 28   |
| Salmonella infection                                                     | 0.03961531    | Down       | 29   |
| Protein digestion and absorption                                        | 0.04064128    | Up         | 30   |
| GnRH signaling pathway                                                   | 0.04304219    | Up         | 31   |
| Progesterone-mediated oocyte maturation                                  | 0.04304219    | Up         | 32   |
| Protein digestion and absorption                                        | 0.04516019    | Down       | 33   |
Morphine addiction 0.04551314 Up 34
Endocrine resistance 0.0467747 Up 35
Hematopoietic cell lineage 0.04706952 Down 36

**Core gene network and Relevant miRNA analysis**

Core network analysis yielded 40 core genes, of which the gene MDFI had the largest number of connections, indicating that it was in the most central position (Fig. 4, Table 5). Relevant miRNA analysis yielded 101 related miRNAs, among which miR-335-5p was the most connected related miRNA (Fig. 5, Table 6).

Table 5. The Correspondence and data source of the Core genes.
| Gene 1 | Protein 1 | Gene 2 | Protein 2 | Relationship | Experiment | Source |
|--------|-----------|--------|-----------|--------------|------------|--------|
| MDFI   | Q99750    | GREM1  | Q60565    | protein interaction | two hybrid array | PMID:19060904 |
| MDFI   | Q99750    | ZNF408 | Q9H9D4    | protein interaction | two hybrid array | PMID:19060904 |
| MDFI   | Q99750    | ZNF408 | Q9H9D4    | protein interaction | two hybrid array | PMID:unassigned1304 |
| MDFI   | Q99750    | ZNF408 | Q9H9D4    | protein interaction | two hybrid pooling | PMID:16189514 |
| MDFI   | Q99750    | RIPPLY1| Q0D2K3    | protein interaction | two hybrid array | PMID:unassigned1304 |
| MDFI   | Q99750    | ADRA2C | P18825    | protein interaction | two hybrid array | PMID:unassigned1304 |
| SSX2IP | Q9Y2D8    | ZNF408 | Q9H9D4    | protein interaction | two hybrid array | PMID:unassigned1304 |
| IL2RG  | PRL       | activation |          |              |            | pathway_id:hsa04630 |
| PRL    | IL2RG     | activation |          |              |            | pathway_id:hsa04630 |
| LEF1   | CTNND2    | inhibition |         |              |            | pathway_id:hsa04310 |
| CTNND2 | Q9UQB3    | TTR    | P02766    | protein interaction | two hybrid | PMID:21900206 |
| ZNF408 | Q9H9D4    | MDFI   | Q99750    | protein interaction | two hybrid array | PMID:19060904 |
| ZNF408 | Q9H9D4    | MDFI   | Q99750    | protein interaction | two hybrid array | PMID:unassigned1304 |
| ZNF408 | Q9H9D4    | MDFI   | Q99750    | protein interaction | two hybrid pooling | PMID:16189514 |
| ZNF408 | Q9H9D4    | SSX2IP | Q9Y2D8    | protein interaction | two hybrid array | PMID:unassigned1304 |
| RIPPLY1| Q0D2K3    | MDFI   | Q99750    | protein interaction | two hybrid array | PMID:unassigned1304 |
| ADRA2C | P18825    | MDFI   | Q99750    | protein interaction | two hybrid array | PMID:unassigned1304 |
| PDYN   | ATF4      | activation |         |              |            | pathway_id:hsa05030 |
| PDYN   | ATF4      | activation |         |              |            | pathway_id:hsa05031 |
| RAB33A | Q14088    | RTN4   | Q9NQC3    | protein interaction | two hybrid pooling | PMID:16189514 |
| RTN4   | Q9NQC3    | RAB33A | Q14088    | protein interaction | two hybrid pooling | PMID:16189514 |
| CFAP58 | Q5T655    | RIPPLY1| Q0D2K3    | protein interaction | two hybrid array | PMID:unassigned1304 |
| Protein1  | Protein2  | Protein3  | Interaction Type       | Method                        | PMID          |
|-----------|-----------|-----------|------------------------|-------------------------------|---------------|
| RSPH14    | Q9UHP6    | TRIM69    | protein interaction    | two hybrid array              | PMID:unassigned1304 |
| TRIM69    | Q86WT6    | RSPH14    | protein interaction    | two hybrid array              | PMID:unassigned1304 |
| HTR2C     | P28335    | RTN4      | protein interaction    | ubiquitin reconstruction      | PMID:28298427  |
| SLC2A4    | ADIPOQ    |           | indirect relationship  |                               |               |
| TG        | ATF4      |           | activation expression  |                               | pathway_id:hsa04918  |
| ADIPOQ    | Q15848    | FASN      | protein interaction    | two hybrid array              | PMID:unassigned1304 |
| AFDN      | SSX2IP    |           | binding/protein interaction |                           | pathway_id:hsa04520  |
| SSX2IP    | AFDN      |           | binding/protein interaction |                           | pathway_id:hsa04520  |
| SSX2IP    | Q9Y2D8    | ZNF408    | protein interaction    | two hybrid array              | PMID:unassigned1304 |
| ARID2     | Q68CP9    | CD14      | protein interaction    | anti tag coimmunoprecipitation | PMID:26496610  |
| CD14      | P08571    | ARID2     | protein interaction    | anti tag coimmunoprecipitation | PMID:26496610  |
| CD14      | P08571    | CD81      | protein interaction    | fluorescent resonance energy transfer | PMID:11745332  |
| ATF4      | P18848    | CEP83     | protein interaction    | two hybrid array              | PMID:unassigned1304 |
| ATF4      | P18848    | TTR       | protein interaction    | two hybrid pooling approach   | PMID:16189514  |
| TTR       | P02766    | ATF4      | protein interaction    | two hybrid pooling approach   | PMID:16189514  |
| CEP83     | Q9Y592    | ATF4      | protein interaction    | two hybrid array              | PMID:unassigned1304 |
| ATXN2     | Q99700    | PABPC1    | direct interaction     | x-ray crystallography         | PMID:20181956  |
| ATXN2     | Q99700    | PABPC1    | protein interaction    | surface plasmon resonance     | PMID:20181956  |
| PABPC1    | P11940    | ATXN2     | direct interaction     | x-ray crystallography         | PMID:20181956  |
| PABPC1    | P11940    | ATXN2     | protein interaction    | surface plasmon resonance     | PMID:20181956  |
| CCR10     | P46092    | S100A10   | protein interaction    | pull down                      | PMID:26941067  |
| S100A10   | P60903    | CCR10     | protein interaction    | proximity ligation assay       | PMID:26941067  |
| CD81      | P60033    | CD14      | protein interaction    | fluorescent resonance energy transfer | PMID:11745332  |
| COBLL1 | Q53SF7 | DLG3 | Q92796 | protein interaction | anti tag coimmunoprecipitation | PMID:26496610 |
|--------|--------|------|--------|---------------------|-------------------------------|----------------|
| DLG3   | Q92796 | COBLL1 | Q53SF7 | protein interaction | anti tag coimmunoprecipitation | PMID:26496610 |

| CYP17A1 | ATF4 | activation expression | pathway_id:hsa04927 |
|---------|------|------------------------|---------------------|
| CYP17A1 | ATF4 | activation expression | pathway_id:hsa04934 |
| CYP17A1 | CYP2E1 | compound | pathway_id:hsa00140 |
| CYP17A1 | NR5A1 | activation expression | pathway_id:hsa04927 |
| CYP17A1 | NR5A1 | activation expression | pathway_id:hsa04934 |
| CYP2E1 | CYP17A1 | compound | pathway_id:hsa00140 |
| CYP2E1 | EPHX1 | compound | pathway_id:hsa00980 |
| NR5A1  | CYP17A1 | Activation expression | pathway_id:hsa04927 |
| NR5A1  | CYP17A1 | activation expression | pathway_id:hsa04934 |
| CES1   | CYP2E1 | compound | pathway_id:hsa00983 |
| EPHX1  | CYP2E1 | compound | pathway_id:hsa00980 |
| GREM1  | O60565 | MDFI | Q99750 | protein interaction | two hybrid array | PMID:unassigned1304 |

Table 6. The Correspondence and data source of miR-335-5p
| MiRNA name | Target gene name | Experimental verification method | PMID    |
|------------|------------------|---------------------------------|---------|
| miR-335-5p | ABCC8            | Microarray                      | 18185580|
| miR-335-5p | ACE              | Microarray                      | 18185580|
| miR-335-5p | ADCY6            | Microarray                      | 18185580|
| miR-335-5p | ADGRE3           | Microarray                      | 18185580|
| miR-335-5p | AFDN             | Microarray                      | 18185580|
| miR-335-5p | CD14             | Microarray                      | 18185580|
| miR-335-5p | CFAP58           | Microarray                      | 18185580|
| miR-335-5p | CPEB4            | Microarray                      | 18185580|
| miR-335-5p | CYP2E1           | Microarray                      | 18185580|
| miR-335-5p | DLG3             | Microarray                      | 18185580|
| miR-335-5p | ESRP1            | Microarray                      | 18185580|
| miR-335-5p | EXD3             | Microarray                      | 18185580|
| miR-335-5p | GABBR1           | Microarray                      | 18185580|
| miR-335-5p | MACF1            | Microarray                      | 18185580|
| miR-335-5p | MDFI             | Microarray                      | 18185580|
| miR-335-5p | MLN              | Microarray                      | 18185580|
| miR-335-5p | MYOD1            | Microarray                      | 18185580|
| miR-335-5p | MYOT             | Microarray                      | 18185580|
| miR-335-5p | NCDN             | HITS-CLIP                       | 27418678|
| miR-335-5p | PNLIP            | Microarray                      | 18185580|
| miR-335-5p | PPP1R3D          | Microarray                      | 18185580|
| miR-335-5p | PTK2B            | Microarray                      | 18185580|
| miR-335-5p | SLC2A4           | Microarray                      | 18185580|
| miR-335-5p | SLC35A5          | HITS-CLIP                       | 23313552|
| miR-335-5p | SLC7A9           | Microarray                      | 18185580|
| miR-335-5p | SPTB             | Microarray                      | 18185580|
| miR-335-5p | TRIM40           | Microarray                      | 18185580|
| miR-335-5p | TRIM69           | Microarray                      | 18185580|
| miR-335-5p | TSPAN15          | Microarray                      | 18185580|
| miR-335-5p | ZNF609           | Microarray                      | 18185580|

*Real-time RT-PCR*
In order to verify the reliability of the microarray results, the expression levels of the seven mRNAs (GCNT7, NCDN, EXD3, MUC13, PPP1R3D, NPHP3 and CD81) detected by RT-PCR were equivalent to the microarray data. And the difference of mRNA expression between the degenerative meniscus group and the normal meniscus group was statistically significant (P < 0.05) (Table 7).

Table 7. Primer and differential expression of mRNA

| Gene      | Primer(5’ - 3’)                        | Sham (x±s,n=7) | OA (x±s,n=7) | P-value |
|-----------|----------------------------------------|----------------|--------------|---------|
| GAPDH     | F:5’ TCTCTGCTCCTCCCCGT 3’             |                |              |         |
|           | R:5’ CGGCCAAATCGTTCACT 3’             |                |              |         |
| GCNT7     | F:5’ GGCTTACACTGCTTCTAGGAG 3’         | 2.41±0.80      | 5.92±1.44    | 0.000   |
|           | R:5’ AGTTGGCTTGTCTTGGATTTA 3’         |                |              |         |
| NCDN      | F:5’ AGACCTGCTGTCACTCTTCC 3’          | 0.47±0.33      | 3.41±1.44    | 0.000   |
|           | R:5’ AGCGACGCCATCAGAGTGT 3’           |                |              |         |
| EXD3      | F:5’ GCATTTCACGTGCAGTCA 3’            | 0.26±0.17      | 1.33±0.41    | 0.000   |
|           | R:5’ CGAGCTGCTTCATCATGCTCTT 3’        |                |              |         |
| MUC13     | F:5’ ATGTCCAAGCTGTCCATT 3’            | 0.02±0.02      | 0.14±0.11    | 0.014   |
|           | R:5’ TGCAATCACCAGGGTCGAG 3’           |                |              |         |
| PPP1R3D   | F:5’ GTCAAGGTGTCAAGCAGCTG 3’          | 3.33±2.39      | 14.75±3.87   | 0.000   |
|           | R:5’ GCCGACCGCAGTGTGAG 3’             |                |              |         |
| NPHP3     | F:5’ TGAACGCTGCTCTACTA 3’             | 1.97±0.94      | 5.04±1.72    | 0.001   |
|           | R:5’ TGCTCGTCTCAGAAGTCTAA 3’          |                |              |         |
| CD81      | F:5’ ACTGACGGGCTCTCCTACGT 3’          | 47.33±53.47    | 225.55±99.88 | 0.001   |
|           | R:5’ ACAGGCAAAGGAGATCACGA 3’          |                |              |         |

F: forward primer; R: reverse primer

**Discussion**

In this study, an Agilent-026440 Sus scrofa (Pig) Oligo Microarray v2 (Probe Name version) chip was used to detect the DEG of degenerative meniscus tissue. The results showed that there were 893 DEGs between the meniscus degeneration group and the normal group, of which 537 genes were up-regulated, 356 genes were down-regulated. We found 10 genes with the most significant expression. They are CYP2C33, GCNT7, NCDN, EXD3, MUC13, PPP1R3D, NPHP3, UPB1, CD81 and PRPH. Among them, we are interested in MUC13. The transmembrane mucin encoded by MUC13 has a variety of physiological functions, mainly to lubricate and protect the mucosal epithelium. Under pathological conditions, MUC13 expression is abnormal and participates in the occurrence and development of inflammation and tumors. Our study found that MUC13 is highly expressed in degenerative meniscus tissues, suggesting that MUC13 may play an important role in it, presumably through the anti-inflammatory effect of MUC13. IL-8 is an important inflammatory factor. MUC13
promotes NF-κB activity through NF-κB-dependent pathways and increases IL-8 production. OA synovial fluid contains high levels of IL-6 and IL-8. The increased expression of IL-8 has a chemotactic effect on inflammatory cells and stimulates the secretion of IL-6, thereby promoting joint inflammation\(^\text{11}\). Therefore, MUC13 may play a role in degenerative meniscus tissue through the anti-inflammatory effect of MUC13.

According to the GO analysis results, 55 BPs were significantly expressed. After comprehensive analysis, DEGs participated in cellular response to hormone stimulus, response to hormone, sex determination, cellular response to endogenous stimulus, C21-steroid hormone metabolic process, neuropeptide signaling pathway, negative regulation of reactive oxygen species metabolic process, regulation of nitric oxide biosynthetic process, response to endogenous stimulus, negative regulation of reproductive process, nitric oxide biosynthetic process, nitric oxide metabolic process, reactive nitrogen species metabolic process, regulation of hormone levels, regulation of reactive oxygen species metabolic process, and regulation of interleukin-4 production. It is suggested that hormones, apoptosis, oxidation and other mechanisms are involved in the meniscus degeneration process. Studies\(^\text{12-14}\) have reported that the inhibitory effect of sex hormones seems to be related to cystic degeneration of meniscus tissue, and growth hormone and parathyroid hormone have effects on meniscus and chondrocytes.

NO is the main cause of human articular chondrocyte apoptosis\(^\text{15-18}\). These studies are consistent with our finding. The regulatory biosynthesis process of nitric oxide is one of the most enriched biological processes in GO analysis. Studies have shown that NO induces chondrocyte apoptosis, and NO is the main cause of human articular chondrocyte apoptosis\(^\text{15,19-23}\). Our study shows that the biological process involved in meniscus degeneration involves NO, suggesting that NO-induced apoptosis also occurs in meniscus cells, which could eventually lead to the degeneration of meniscus tissue. The consistency between our finding and previous findings provides strong support for our hypothesis that degenerate meniscus cells are different from normal meniscus cells and may play an active role in the development of OA, in order to verify this finding, further research is still needed.

Inflammatory mediator regulation of TRP channels is one of the 10 lowest p-values in Pathway analysis. As a variety of cellular signal receptors, it plays a very important role in the inflammatory response\(^\text{24,25}\). This study found that inflammatory mediator regulation of TRP channels were upregulated in the meniscus of swine OA, suggesting that degeneration of the meniscus involves TRP channels and is associated with inflammatory responses.

Our research found MDFI and miR-335-5p, both of which regulate the WNT signaling pathway\(^\text{26,27}\), which is the core signal pathway of OA synovitis\(^\text{28-30}\). This shows that synovitis and meniscal degeneration may have similar mechanisms of action. Synovial and meniscal tissues are also involved in the pathogenesis of OA. Pilar Tornero-Esteban's\(^\text{31}\) study shows correlation between miR-335-5p expression and OA. Our study found a strong correlation between miR-335-5p and meniscus degeneration. MiR-335-5p could play an important role in meniscus degeneration, indicating that miR-335-5p may become a new target for clinical prevention and treatment of meniscus degeneration. The specific mechanism and how to mediate meniscus degeneration are the next research the key of.

This study has shortcomings. The experimental study uses a Wuzhishan pig meniscus degeneration model. The knee joint is different from humans in biomechanics, so the animal model research cannot be completely equivalent to the study of meniscus degeneration's patients. In addition, this model is more similar to traumatic meniscus degeneration, such as human meniscus and cruciate ligament injury, rather than primary meniscus degeneration. However, compared with many shortcomings such as the ethical issues involved in the study of human specimens, the lack of sample size, the obvious heterogeneity of specimens, and the difficulty of obtaining specimens in special parts, this animal model is an ideal research tool.

In summary, it is necessary to better understand the pathogenesis of meniscus degeneration, identify potential drug targets, and regulate gene expression of meniscus degeneration. This study is the first time that the whole gene spectrum
analysis of the meniscus tissue has been performed in the Wuzhishan pig animal. Our findings expand current understanding of the genetic mechanisms of meniscus degeneration. Therefore, these data provide a basis for future studies on the function of a large number of newly discovered genetic materials in the pathogenesis of meniscal degeneration and the suitability of the target as a drug target for meniscal degeneration.

Materials And Methods

Animals

A total of 14 healthy adult male Hainan Wuzhishan pigs (Institute of Animal Husbandry and Veterinary Medicine, Hainan Academy of Agricultural Sciences, China) were randomly divided into normal meniscus group and meniscus degeneration group. Anterior cruciate ligament resection was done in the meniscus degeneration group, and only the skin was cut in the normal meniscus group. The weight of each pig was 15-18kg, the age was 6-7 months, and normal illumination and diet and non-single cage feeding were given. The breeding environment temperature was 20-28 degrees centigrade. All pigs were sacrificed 24 weeks after the operation. All operations were performed under pentobarbital sodium anesthesia. The ethics committee had approved the experiment (Ethics Committee of Hainan Provincial People's Hospital of China, Ethical approval No: Med-Eth-Re [2018] 01). All methods were performed in accordance with the relevant guidelines and regulations of the ethics committee.

Gross morphological observation and HE staining

The medial meniscus was taken out and the smoothness, gloss and color of the surface to be observed. After a little cleaning, it was fixed with 10% formalin. After 72 hours, it was decalcified, dehydrated, and embedded in paraffin, and then sectioned. After HE staining, the morphological and structural changes of the tissue were observed under a microscope.

RNA extraction

According to the manufacturer’s requirements, TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from meniscal tissue. Quality control was performed by NanoDropND-1000 and Agilent 2100 Bioanalyzer.

Microarray

The Pig Gene Expression 4x44K Microarray V2 (Agilent Technologies, Santa Clara, CA, USA) were used to compare mRNA expression profiles, respectively, in normal and degenerative meniscus tissue. Microarray analysis was performed by GCBI analysis platform (Shanghai, China, https://www.gcbi.com.cn). The data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE156132.

Strategy

The flow chart of the analysis was shown in the Fig. 6. First, the differentially expressed gene analysis used the two samples Welch's t-test (unequal variances) to identify significantly different mRNAs which were screened by p < 0.05, fold change > 1.5 and FDR < 0.05 and sorted by p value. Second, the gene ontology system is used to classify these differentially expressed genes according to their biological functions. Similarly, pathway analysis was used to identify affected KEGG pathways. Third, differential expression genes were further analyzed by core gene network analysis and related miRNAs analysis. The core gene network analysis displays the interaction relationship between the input genes, making it easier to understand the core genes of the input genes. The core network is based on the relationship between genes and genes that have database basis and literature basis in Pubmed, Mesh, KEGG and other databases. Related miRNAs analysis show at least two miRNAs that interact with the input gene. Related miRNAs are based on the
relationship between genes and miRNAs that are documented in Pubmed, miRBase and other databases, so as to construct a network of related miRNAs. All of the data mentioned above were analyzed by GCBI analysis platform.

**Real-time RT-PCR**

Changes in the expression of selected genes (GCNT7, NCDN, EXD3, MUC13, PPP1R3D, NPHP3 and CD81) were confirmed by RT-PCR. The 7 genes were chosen because they had the lowest p-value. TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from meniscus tissue, and then 500ng RNA was taken to synthesize cDNA. PCR was performed on a ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using a 2X PCR master mix (Arraystar, Rockvile, MD, USA). The primers were chemically synthesized by Kangcheng, Shanghai, China and are listed in Table 7. GAPDH was used as internal controls to determine the relative expression of target mRNA. All reactions were repeated three times.

**Statistical Analysis**

The data of PT-PCR was analyzed using SPSS 19.0 software, and the counting data were expressed as (x ± s). One-way ANOVA was used for comparison between two groups, and P < 0.05 indicated statistical difference.

**Declarations**

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**Author contributions statement**: H.H. conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper. J.Z. and H.T. conceived and designed the experiments, analyzed the data, prepared figures and/or tables, reviewed drafts of the paper. G.W. and W.W. analyzed the data, wrote the paper, reviewed drafts of the paper. H.H. and Y.F. performed the experiments, reviewed drafts of the paper. J.L. and M.D. conceived and designed the experiments, wrote the paper, reviewed drafts of the paper.

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