学位論文内容の要旨

博士の専攻分野の名称：博士（水産科学）　　氏名：石　林艷（しー　りんいえん）

学 位 論 文 題 目

Studies on the potential utilization of notochord type II collagen derived from bester sturgeon *Huso huso* × *Acipenser ruthenus* in cartilage tissue engineering

（ベステルチョウザメ脊索由来 II 型コラーゲンの軟骨組織工学への応用に関する研究）

Type II collagen constructs the intricate network within articular cartilage (AC) tissue and significantly influences cellular behavior. Despite its promising applications in cartilage tissue engineering (CTE), the utilization of type II collagen is hampered by the instability of its traditional shark sources and high viscosity nature. An aquatic sturgeon, rich in notochord-derived type II collagen (NC-II), offers a promising new source for CTE scaffold development. Its reliable aquaculture supply and ease of extraction further highlight its potential. The goal of this study is to fabricate a fibril-based, thin-layered scaffold using sturgeon NC-II and assess its potential for promoting chondrogenesis in pre-chondrogenic ATDC5 cell culture.

In the fibril fabrication experiments, the NC-II was successfully self-assembled into a thin layer of fibrils *in vitro* using the newly developed “flip-contact method” and coated on a cell-culture coverslip. Specifically, 8 mg/mL NC-II solution (pH 3.0 HCl) was applied using the "flip-contact method" to initiate fibrillogenesis in the presence of 30 mM phosphate buffer, with 48 h fibril-formation time followed by 48 h genipin crosslinking at 12 ℃. Scanning electron microscopy (SEM) analysis revealed that the fibril diameter ranged ＜50–150 nm, with a mean diameter of 140.02 ± 74.62 nm. This marks the first instance of NC fibril coating based on type II collagen sourced from marine life, and the diameter closely resembles that of fibrils found in mature human cartilage. Morphologically, the fibril fabrication effectively mimicked human cartilage.

On the NC-II crosslinked fibril-coated surface, ATDC5 cells were cultured for 28 days at 37 ℃. Time-lapse observations revealed the cell adhesion process. The Cell Counting Kit-8 (CCK-8) assay, light microscopy, and scanning electron microscopy were performed to detect proliferation and filopodium morphology. Alcian blue staining was used to show the deposition of extracellular

secretions, and qRT-PCR was performed to reveal the expression levels of chondrogenesis-related genes. The cell adhesion speed was similar in both fibril-coated and control molecule-coated groups, but the cellular morphology, proliferation, and chondrogenesis activity differed. On fibrils, more elongated finer filopodia showed active inter-cell communications, whereas the slower proliferation suggested an altered cell cycle. The extracellular matrix (ECM) production occurred before day 14 and continued until day 28 on fibrils. The highly significant upregulated expressions of the chondrogenesis-related genes *Sox9*, *Col10a1*, and *Acan* and significant upregulated *Col2a1* were detected in the cells on fibrils at day 21. Thus, fibrils showed rapid cellular affinity and induced active chondrogenesis with extracellular secretions. Those results presented a new model for studying chondrogenesis *in vitro* and a potential alternative material for cell-laden CTE research at a 37 ℃ culture system.

Low-temperature culture on the reaction of ATDC5 was further investigated on NC-II fibrils. The cell culture was conducted at 30°C, a temperature that mimics the temperature of the human knee surface. Due to the stability of NC-II fibrils, the crosslinker was removed. CCK8 assays, phase-contrast microscopy, and SEM results show that cells cultured on fibrils quickly enter the proliferation cycle but have prolonged mitotic cycles and relatively low metabolism. Despite a decrease in temperature to 30 ℃, ECM secretion on fibrils occurs before day 14, with cells displaying an aggregated morphology. Since the cells reached confluency after day 21, this result suggests that uncrosslinked NC fibrils possess a specific capability to induce localized cell aggregation, facilitating chondrogenic differentiation and ECM generation. The uncrosslinked NC-II fibrils also rapidly induce cell proliferation, alter cell morphology, and prompt ECM secretion before day 14, positively impacting chondrogenic differentiation. These effects are likely due to synergistic interactions between the uncrosslinked NC fibrils and cultivation at 30 ℃. Future research on chondrogenesis at 30 ℃ may be up-and-coming. qRT-PCR was conducted to analyze the expression of chondrogenesis-related and ECM-related genes, focusing on minor collagens. Early chondro-induction markers (*Sox9* and *Icam1*) and major ECM proteins (*Acan*, *Cspg4*, and *Col2a1*) in the fibril group exhibited significant upregulation compared with the non-coated or molecule groups. Most of these genes are also upregulated time-dependently during 35 days of culture. Those results validate the tendency of the fibrils group towards chondro-induction.

Combined with ECM secretion, the cells on the fibrils have already shown characteristics of partially mature chondrocytes. Network-forming collagens (*Col4a1* and *Col10a1*), microfibrillar collagen *Col6a1*, and fibril-forming collagens (*Col11a2* and *Col27a1*), except for *Col27a1*, exhibited time-dependent significant upregulations from day 21 to day 35 on the fibrils, and all showed significant intra-group differences on day 35. This highlights the NC-II fibrils directly influence the genetic expressions of extracellular matrix components. In the fibril-associated collagens with interrupted triple helices (FACIT collagens, *Col9a1*, *Col12a1*, *Col14a1*, *Col16a1*, and *Col22a1*), *Col9a1*, *Col12a1*, and *Col16a1* also exhibited time-dependent significant upregulations from day 21 to day 35, along with some intra-group differences. In contrast, *Col14a1* and *Col22a1* expressions did not exhibit time-dependent upregulations on NC-II fibrils, suggesting the absence of positive feedback from the present *in vitro* environment on expressions of these genes that closely relate to the mechanical strength of the ECM. This further indicates the unsuccessful or incomplete attainment of true chondrogenesis *in vitro* in this experiment. Consequently, this study provides valuable insights into collagen research without crosslinker usages. Regarding gene expressions, understanding the expression patterns of all relevant proteins in the ECM remains incomplete. Nonetheless, the data obtained thus will serve as the foundation for more comprehensive investigations in the future.

This study has practical implications for tissue engineering and collagen research. By successfully self-assembling aquatic-derived type II collagen into fibrils *in vitro* and demonstrating its excellent chondro-induction capabilities in the culture of pre-chondrocyte ATDC5 cells, this study has opened up new research directions for CTE. This expands the possibilities for CTE research but also paves the way for the potential expansion and diversification of value chains within the sturgeon industry, in which only caviar and meat make a profit presently.