Dissertation Summary

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学 位 論 文 題 目

Title of dissertation

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）

The demand for caviar in the global market far exceeds the capacity of the sturgeon industry, leading to its high prices (Bronzi et al., 2019). Given the vertically integrated farming model mandated for sturgeon aquaculture (spanning hatchery operations to caviar production), the sustainable utilization of by-products has become particularly valuable for small-to-medium scale farmers seeking additional revenue streams. To our knowledge, by-products from sturgeon aquaculture have been explored and utilized across multiple disciplines, including artistic painting, the food industry, wound healing, and tissue engineering applications, particularly for bone and cartilage regeneration. Thus, to promote a healthy and sustainable aquaculture operation and ensure favorable market prices, the industry's interest in extending its merit is high. Therefore, in this study, I set the objective to establish a novel linkage between sturgeon-derived by-products that contain type II collagen and cartilage regeneration research, aiming to (i) unlock new valorization pathways for the aquaculture industry, while (ii) pioneering diversified biomaterial strategies for cartilage regeneration.

The risk of zoonotic diseases and religious restrictions limit terrestrial collagens' applications to various fields, including wound healing, drug delivery, therapeutic intakes, and tissue engineering research for bone, cartilage, dental, and vascular tissues. Instead, collagens derived from marine resources have become a major topic as a substitute, attributed to the absence

of reports associated with transmissible diseases and their universal acceptance across various religious practices. To date, type II collagen from marine resources primarily comes from sharks and other cartilaginous fish, jellyfish, and sturgeon. Currently, research on scaffold development using marine-derived type II collagen for cartilage tissue engineering (CTE) is severely lacking. Type II collagen constructs the intricate network within articular cartilage (AC) tissue and significantly influences cellular behavior. Despite its promising applications in CTE, the utilization of type II collagen is hampered by its high viscosity as a solution and the instability of its traditional shark sources. Thus, type II collagen derived from sturgeons has begun to gain attention in CTE due to its stable supply, relatively simple extraction, and high purity. This scarcity of knowledge has motivated me to explore this field and contribute to its development. 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.

Type II collagen forms the fibril network responsible for the overall shape of the AC tissue, and the fibrillar morphology plays a significant role in controlling cellular behavior. *In vivo*, type II collagen fibrils serve as the habitat for chondrocytes; thus, theoretically, the type II collagen fibril-based biomimetic material has a positive effect on chondrogenesis. Initially, I pioneered an *in vitro* fibrogenesis model for NC-II via a self-assembly approach under optimized physicochemical conditions, utilizing the first-of-its-kind "Flip-Contact Method" to achieve spatially controlled fibril formation. The notochord material underwent sequential processing comprising alkaline pretreatment, pH neutralization, enzymatic hydrolysis, salt precipitation, dialysis, and freeze-drying, with all operations being performed at 4 °C or below to preserve the structural integrity of the collagen triple-helical conformation. SDS-PAGE analysis with CBB staining demonstrated successful extraction of NC-II, as evidenced by intense band enrichment at approximately 135 kDa. While conventional fibril coating methods (Moroi et al., 2019; originally developed for type I collagen fibrillogenesis) could initiate NC-II fibrillogenesis on culture surfaces, systematic screening across four NC-II concentrations (4-10 mg/mL) and six phosphate buffer gradients (10-120 mM, pH 7.6) via electron microscopy revealed that NC-II's inherent high viscosity coupled

with its unique sensitivity to ionic environment fundamentally limited uniform fibril deposition. This critical limitation was overcome by our innovative "Flip-Contact Method", which uniquely enables homogeneous fibril surface formation, representing a significant advance in NC-II matrix engineering. In the novel method, the following critical parameters for reproducible NC-II fibrillogenesis were employed: 8 mg/mL collagen concentration (35 μL/coverslip) in 30 mM phosphate buffer (pH 7.6), with a 48 h fibrillogenesis period followed by 48 h crosslinking at 12°C using PET substrates. Quantitative analysis via ImageJ revealed that the resulting fibrils exhibited a diameter range of 50–500 nm (mean: 140.02 ± 74.62 nm), demonstrating the method's precision in generating nanostructured matrices that closely mimic native collagen architecture. In the screening process, the present study showed that the fibril formation of type II collagen is influenced by time, temperature, collagen concentration, ion concentration, and the material of the contact surface. Despite sharing fundamental collagenous characteristics, types I and II collagens demonstrate strikingly divergent fibrillogenic behaviors under identical assembly conditions. The self-assembly process of collagen may be affected by the naturally different components of the α chains. Type I collagen consists of $\alpha1(I)_2\alpha2(I)$, while type II collagen consists of $\alpha1(II)_3$. Not only do they differ in their heteropolymer and homopolymer of α chains, but their proα amino acid residue compositions also exhibit distinctive features. As in prior studies (Zhang et al., 2016, 2019), distinct collagen types exhibit variations in: (i) amino acid profiles, (ii) proα-chain composition, and (iii) glycosides amount. These intrinsic molecular differences likely influence type-specific fibrillogenesis behaviors. While the precise mechanistic determinants of NC-II assembly remain elusive, from this study, I surmise that the self-assembly process of NC-II collagen might be significantly influenced by electrostatic forces, such as amino acid sequences and glycosides in α chains, along with the ionic species and their concentrations in the fibrillogenesis solution. Therefore, if molecular physics studies are employed, designed fibrillogenesis, such as adjustable diameter, may be possible under guided electrostatic conditions, which will beneficially regulate cellular behaviors grown on the material in the future. Through the innovative "Flip-Contact Method", I successfully achieved the first-reported *in vitro* self-assembly of marine-derived type II collagen (NC-II) into nanofibrillar coatings on cell-culture coverslips. This breakthrough establishes a novel biomimetic platform for NC-II matrix engineering. The innovation bridges a critical technological gap between marine

collagen utilization and CTE applications, opening new avenues for biomaterial development in regenerative medicine.

Cell-laden scaffolds support cell proliferation and differentiation to achieve lesion repair after transplantation. Several studies have used type I collagen, the proportion of which is high in bone but low in cartilage, as a scaffold material for CTE (Irawan et al., 2018; Yang et al., 2020). However, clinical trials have also reported disadvantages of its use, such as incomplete cartilage repair (Christensen et al., 2016). In contrast, in a rabbit model with full-thickness articular cartilage defects, implants made of bovine type II collagen scaffolds repaired the cartilage better than those made of type I collagen (Buma et al., 2003). Therefore, theoretically, using type II collagen to construct CTE scaffolds has become reasonable because type II collagen fibrils are a major component of cartilage ECM *in vivo* (Wu et al., 2021). Owing to terrestrial animal-to-human disease transmission concerns, low-risk aquatic sources are considered suitable for biomaterial development (Laasri et al., 2023). Therefore, the present study aimed to test the chondroinductive ability of this new material in search of a new CTE scaffold. In this section, the pre-chondrogenic ATDC5 cells experimental design incorporated comprehensive morphological and proliferative analyses across critical timepoints during the chondrogenic differentiation on the fibrils, extending up to day 28. Time-lapse imaging demonstrated robust cellular adhesion to the fibril matrix without observable detachment events. Phase-contrast microscopy revealed morphological adaptations as early as 24 h post-seeding. Unlike the flattened, spindle-shaped morphology observed on the control group (type II collagen molecule-coated surface), ATDC5 cells on fibrils adopted a relatively rounded shape with pronounced cellular filopodia anchoring to the fibril matrix. The cells on the fibril-coated surface had shorter filopodia than those on the control surface on day 0 (24 h in the growth medium; hereafter, the counting of culture days begins after replacing the growth medium with the differentiation medium). Subsequently, the filopodia did not increase in number but extended over the culture. On day 14, the cells approached monolayer confluence, and filopodia connected the cells. The CCK-8 assay revealed that ATDC5 cells grew rapidly on the control surface. In contrast, they proliferated slowly on the fibril-coated surface. Slower proliferation on the fibril-coated surface was also confirmed in time-lapse monitoring. Phase-contrast microscopy revealed that the cells on the control surface reached almost 100% confluency around day 3, but on

the fibril-coated surface, the cells did not reach confluence until day 14. Alcian blue (AB) staining was performed to confirm the extracellular matrix (ECM) secretion of the ATDC5 cells. The control surface without cells was negative for AB staining. When ATDC5 cells were cultured, the weakly AB-positive area first appeared around the cells on day 14 in both the control and the fibril-coated groups, suggesting that ECM secretion had started. In addition to the weakly AB-positive area, the intensely AB-positive area existed only in the fibril-coated group, and the area became larger through the culture, confirming the continuous differentiation of cells and ECM-secretory activity in the fibril-coated group. In contrast, the intensely stained area never appeared in the control group, despite the increased number of AB-positive cells. These findings suggest qualitative differences in AB-positive cells, resulting in a qualitative difference in the secreted ECMs between the groups. The hallmark chondrogenic-related genes were also quantitatively analyzed by the real-time quantitative polymerase chain reaction (qPCR). Gene expression revealed significantly enhanced chondrogenic differentiation in the fibril-coated group: *Sox9* peaked specifically on day 21 (5.21-fold increase vs controls, 95% CI = 4.12–6.3, $p < 0.001$). Sustained elevation of *Acan* expression (2.5-19.5-fold across timepoints) and *Col2a1* (5.5-fold on day 21, 95% CI = 2.33–8.67, $p = 0.0049$) confirmed robust matrix production, demonstrating the fibrillar matrix's superior ability to promote chondrogenesis. For CTE, the designed scaffold materials refer to the cartilage ECM, which is expected to facilitate efficient cell landing, chondrogenic differentiation, and chondrogenesis. In this study, both the time-lapse observations and CCK-8 measurements consistently showed low proliferation and metabolic rates in the fibril-coated group, directly highlighting the unique characteristics of type II fibrils to alter the energy of the mitotic cycle during chondrogenesis. The combined evidence from ECM secretion analysis by AB staining and qPCR confirms that the NC-II fibrils trigger the chondroinductive signaling pathway. Therefore, I propose that the NC-II fibril material activates partial signal pathways related to chondrogenesis in cells through integrin subunits. Through advantageous signal transduction, ATDC5 cells respond to the fibril material and are functionalized as chondrocytes. Thus, the *in vitro* model presented herein may contribute to a better understanding of the molecular signaling pathways of chondrogenesis and might lead to further pharmacokinetic studies and/or CTE scaffold exploration in the future.

Extending the biomimetic concept, we further implemented a novel temperature adjustment to challenge more possibilities of the cell culture system. The core body temperature of terrestrial mammals is conventionally regarded as 37 °C. In general, most cellular and related research on mammals is conducted at 37°C. Inspired by sports medicine observations revealing that normal knee joint temperatures (via Infrared thermal imaging) remain below 32 °C, and considering both the denaturation threshold of NC-II and its post-fibrillogenesis mechanical reinforcement properties, I pioneered a crosslinker-free, 30 °C-culture experiments that simultaneously achieves (i) enhanced physiological relevance by mimicking articular joint temperature, and (ii) avoidance of exogenous chemical modifications that could confound biological responses. Most studies on CTE scaffolds primarily focus on assessing their ability to induce chondrogenic-related differentiation markers. Research on the differentiation process for chondrocytes in relation to the pericellular matrix (PCM) and extracellular matrix (ECM) production is limited and lacks depth, though both PCM and ECM are synthesized and maintained by chondrocytes. In this section, the growth, differentiation, and morphology of ATDC5 were also analyzed; beyond the commonly known genes related to chondrogenesis, I initially explored the presence of minor collagens in this culture system. 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 that 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 the use of crosslinkers. 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.

The study concludes that **sturgeon-derived notochord type II collagen (NC-II)** is a highly promising alternative for CTE, offering a sustainable and effective solution for scaffold development. Key findings and their implications include:

1. **Successful Scaffold Fabrication**: Using an innovative "Flip-Contact Method," NC-II was self-assembled into thin fibril layers with a mean diameter of 140.02 ± 74.62 nm, closely resembling those of mature human cartilage. This biomimetic structure provides an ideal environment for chondrogenic differentiation, marking the first successful fabrication of marine-derived type II collagen fibrils for CTE applications.
2. **Enhanced Chondrogenic Differentiation**: The NC-II fibril scaffolds demonstrated remarkable potential in promoting chondrogenesis. ATDC5 cells cultured on these scaffolds exhibited active ECM secretion and significant upregulation of chondrogenesis-related genes (*Sox9*, *Col10a1*, *Acan*, *Col2a1*) by day 21. These results indicate that the scaffolds not only support cell adhesion and proliferation but also actively drive chondrogenic differentiation, making them highly suitable for cartilage regeneration.
3. **Low-Temperature Culture Advantage**: By shifting the cell culture conditions to 30°C, mimicking the acceptable temperature of the knee joint (26.05–31.13°C), the study revealed that NC-II fibrils remain stable without the need for crosslinking. This low-temperature environment induced cell aggregation and early ECM secretion (before day 14), suggesting a synergistic effect between uncrosslinked fibrils and reduced temperature. This finding opens new avenues for studying chondrogenesis under physiologically relevant conditions.
4. **Crosslinker-Free Approach**: The study demonstrated that uncrosslinked NC-II fibrils can effectively induce chondrogenesis, eliminating the need for chemical crosslinkers. This not

only simplifies the scaffold fabrication process but also reduces potential cytotoxicity, offering a safer and more efficient strategy for CTE research.

5. **Marine Resource Utilization**: This work highlights the potential of sturgeon-derived NC-II as a sustainable marine resource for CTE. Unlike traditional sources, sturgeon collagen benefits from a reliable aquaculture supply and ease of extraction, making it an environmentally friendly and scalable option for future applications.
6. **Gene Expression Insights**: The study provided valuable insights into the expression patterns of chondrogenesis-related and ECM collagen genes. While early differentiation markers (*Sox9*, *Icam1*) and major ECM proteins (*Acan*, *Cspg4*, *Col2a1*) showed significant upregulation, the low expression of specific genes (*Col12a1*, *Col16a1*) at key time points might suggest a state conducive to mature chondrocyte formation. However, the absence of time-dependent upregulation in some mechanical performance-related genes (*Col14a1*, *Col22a1*) indicated that further optimization is needed to achieve complete chondrogenesis *in vitro*.
7. **Novel *In Vitro* Model**: The study established a new model for studying chondrogenesis *in vitro*, combining marine-derived NC-II fibrils with low-temperature culture conditions. This model not only accelerates chondrogenic differentiation but also provides a platform for exploring the mechanisms of cartilage formation and ECM deposition.

In summary, this research advances the field of cartilage tissue engineering by introducing **sturgeon-derived NC-II as a sustainable and effective biomaterial** for scaffold development. The findings underscore the potential of marine resources in CTE, offer a crosslinker-free approach to scaffold fabrication, and provide a novel *in vitro* model for studying chondrogenesis. These contributions pave the way for future research into optimizing expressions of mechanical performance-related genes and achieving complete chondrogenesis *in vitro*, ultimately enhancing the development of functional cartilage tissue for regenerative medicine.

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