博士論文（要約)

Osteochondral Tissue Engineering using Functionalized Hydrogels and Mechanical Stimulation

(物理刺激と機能性ヒドロゲルによる骨軟骨再生に関する研究)

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The synovial joints represent the hinges of the musculoskeletal system that allow us to perform various motions in daily life from as simple as standing to as complex as playing sports. Despite its simplicity in macro-level appearance, the biochemical composition on the micro-level is astonishingly complex and is what gives the articular joint its superior mechanical functionalities. However at the same time, the complexity also renders its full regeneration to be very difficult. Hydrostatic pressure is one of the major modes of mechanical stress that exists in the native joint environment and is essential for the maintenance of healthy osteochondral tissues. Hence, the application of hydrostatic pressure may have stimulatory effects for tissue-engineering cartilage and bone. In this dissertation, we attempted to elucidate the effects of dynamic hydrostatic pressure on the joint maturation process, as well as its effects on our hydrogel-based tissue engineered models for both cartilage and bone.

In the first study, we used the rat model to first examine the post-natal development of the synovial joint and the role of hydrostatic pressure in the process. By tracking juvenile rats and examining their knee joints at 1 to 7 weeks, we observed the timing of the secondary endochondral ossification and thinning of the cartilage during the tissue maturation progress after birth. The maturation stages also corresponded with the mobility of the animals, suggesting the requirement for tissue function prior to any mechanical stressful movements. When we isolated the epiphyses from the femoral distal end, we were able to maintain the tissue *ex vivo* and even demonstrated growth after one week of culture. However, we observed that the maturation process (specifically the onset of ossification) was halted in *ex vivo* culture, especially under static culture conditions. With the application of dynamic hydrostatic pressure stimulation on the other hand, we were able to initiate the maturation process resulting in changes in the gene expressions of cells and also matrix biochemical composition. Our analysis was performed using the whole epiphysis though, which results in low resolution in regards to which part of the joint the change is really occurring at. Since both bone formation and cartilage maturation progress simultaneously, it may be more meaningful to perform regional analysis for future work.

The second study involved using a photo-crosslinkable HA hydrogel for cartilage tissue engineering. By methacrylating the HA molecules, polymerization between the monomers was easily initiated by UV exposure. By embedding cells in the hydrogel, we were able to create 3D tissue-engineered cartilage constructs. Using this scaffolding material, we examined the effects of mechanical stimulation, namely dynamic compression and hydrostatic pressure, on de-differentiated chondrocytes and hMSCs in 3D culture. From the experimentation, we demonstrated the ability of our hydrogel to re-differentiate the expanded chondrocytes where cell regained their spherical morphology and started producing aggrecan and type II collagen. The effect was further enhanced with the dynamic compression loading and even resulted in constructs with higher elastic and aggregate moduli. A peculiar trend observed, however, was the inconsistency between the mechanical properties and

biochemical composition of the constructs. Intuitively the relation between the amount of matrix proteins and construct strength should be proportional, but the protein quantity was lesser in the dynamically cultured constructs than the static controls. The proposed hypothesis to explain this phenomenon was the concept of collagen fiber orientation, where chondrocytes within the matrix can actively re-orientate their surrounding fibers to better withstand the mechanical stress. Similarly, hydrostatic pressure was applied to tissue-engineered constructs embedded with hMSCs to better promote chondrogenesis. While the results show enhanced induction with the mechanical stimulation, the change was not as significant. Differential control of stem cells is still a major challenge in tissue engineering, where the optimization of numerous parameters is needed to achieve desired outcomes. Nevertheless, both dynamic compression and hydrostatic pressure were shown to be effective in stimulating construct maturation and can potentially be applied in other approaches for cartilage tissue engineering.

For the third study, we developed a bioactive HA hydrogel bone tissue engineering by taking advantage of the osteoconductive potentials of inorganic polyphosphate. In previous works, we have modified HA molecules by attaching adipic dihydrazide and aldehyde groups to form HA-ADH and HA-CHO respectively, which can then be crosslinked to form HAX hydrogels. HA-ADH was further modified to covalently bind PolyP chains to synthesize scaffolds with immobilized PolyP. The functionalized scaffolds were first tested with MC3T3-E1 pre-osteoblasts to examine the potential for osteoconduction. From our experiments, we demonstrated that the embedded cells were indeed stimulated by the immobilized factors and that this stimulation is constantly present throughout the culture period. Up-regulation of bone marker genes, as well as the increase in osteogenic activities and matrix mineralization all verifies the osteoconductive effects of PolyP. In comparison, constructs that were initially loaded with the factors directly or in the culture medium did not exhibit such increase in osteoconduction, denoting the limited effects of the factors in free-floating form. Embedded cells within scaffolds tend to have limited motility, hence immobilization of factors can allow for easier and constant access to such chemical stimulation. These experiments demonstrated the potency of conjugating factors onto scaffolds as a method for efficient factor delivery.

We then used the HAX-PolyP model with hMSCs and attempted to further compliment the osteoconductive effects of immobilized PolyP with dynamic hydrostatic pressure stimulation. The outcome was synergetic, where mechanical loading enhanced osteogenic differentiation of the embedded stem cells. Although further experimentation is required to fully demonstrate osteogenesis and bone formation with hMSCs in the HAX-PolyP hydrogel, we have preliminarily illustrated the potential use of hydrostatic for this purpose. Mechanical loading used in bone tissue engineering are mainly flow-induced shear stresses with little attention to hydrostatic pressure. However, the existence of hydrostatic pressure is significant in the native tissue where it facilitates the bone maintenance. As such, this study presented their role in *in vitro* bone conduction as a start to deeper investigation.

In the last study, we combined the know-hows from the second and third study to develop a tissue-engineered osteochondral model using composition HA hydrogel scaffolds. Although it may be more common to use stiffer materials for the bone layer, problems including low cell inoculation rates and poor interface integration still persist. We have demonstrated that ease of using HA hydrogels for constructing osteochondral tissues with strong interface integrity by layering MeHA with HAX-PolyP and embedding hMSCs. We exploited the osteoconductive capabilities of immobilized PolyP, which are localized only to the HAX-PolyP layer. As such, when we cultured the constructs in chondrogenic medium, hMSCs did not undergo chondrogenesis and were committed to osteogenesis. Two distinct types of matrices were also developed along with the construct maturation as shown by the difference in histology, biochemical composition and mechanical properties. From the results obtained in the previous studies, dynamic hydrostatic pressure loading can also be used to stimulate and promote the maturation of the tissue-engineered osteochondral construct. Since both of these materials are injectable hydrogels and can conform to any shape and size, this approach for treating osteochondral defects is clinically useful. For example, the subchondral defect can first be filled with HAX-PolyP containing hMSCs then topped with MeHA for the cartilage region. Further animal experimentation would be necessary to fully examine how this model perform *in vivo*.

In summary, this dissertation have met the objective set in Chapter 1 by elucidating the effects of dynamic hydrostatic pressure in the post-natal developing joint and also in tissue-engineered cartilage and bone constructs. Several new models for tissue-engineered cartilage, bone and osteochondral constructs have also developed and validated using functionalized hyaluronic acid hydrogels. The data combined gave new insights and a better understanding to the involvement of mechanical stresses in the regulation of the osteochondral tissue. These newly obtained knowledge have also gave us hints and a more definitive direction as to what to further research for achieving the ultimate goal of creating the ideal tissue-engineered osteochondral construct for the treatment of degenerative joint diseases. Hopefully perhaps within the next one or two decades as the demands become even larger with the aging society, clinical application of osteochondral tissue engineering can be commonly implemented worldwide.