論文内容の要旨

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Study of Novel Chondrocytogenic Regulators
(新規軟骨細胞分化制御因子群に関する研究)

1. Introduction:

Osteoarthritis (OA) is characterized by a progressive degradation of articular cartilage leading to loss of joint function. The molecular mechanisms regulating pathogenesis and progression of OA are poorly understood. Remarkably, chondrocyte differentiation process, addressed as chondrocytogenesis, during skeletal development by endochondral ossification is involved in the progression of OA. Articular chondrocytes in degradated cartilage exhibit progressively differentiation. Thus, regulation factors engaged in chondrocyte differentiation in both growth cartilage and permanent articular cartilage implicated in OA may be possible targets for novel disease-modifying therapies for OA.

Chondrocytogenesis is the process by which cartilage is formed from condensed mesenchymal cells at growth plate, the ends of long bone (epiphyseal plate) and distal end of pubis (apophyseal plate), where longitudinal bone growth occurs. Therefore, this process can determine the length and shape of bone morphology. The process starts the commitment from mesenchymal stem cells (MSCs) to subsequent proliferation of chondrocytes, and then differentiates into hypertrophic chondrocytes (1). It has been documented that various signaling pathways, growth factors, transcription factors (i.e.Sox9) and hormones (i.e. sex steroids) are involved in the regulation of chondrocytogenesis (2).

During puberty, it is well known that the sex differences in bone length as well as bone shape are apparently remarkable. From these physiological evidences, it can be hypothesized that sex steroids, especially androgens, might play a role in chondrocytogenesis. Most of androgens exert their

functions as ligands specifically through the androgen receptor (AR), a member of nuclear receptor superfamily. AR is expressed in chondrocytes of rodents and humans. Hence, AR may perform important function on determination of bone morphology via direct effect on growth plate. Though, no significant difference in longitudinal growth of long bone between the conventional AR knock-out (ARKO) mice and WT littermates is observed, the ARKO mice suffer from endocrine disturbance, inferring that the phenotype on long bone may be not specifically according to androgen-AR signaling (3, 4). Thus, the chondrocyte-specific ARKO mice are required to determine whether AR takes the direct biological function in chondrocytes.

Meanwhile, so far, the researches of chondrocytogenesis-related factors have merely had access to the tip of an iceberg, which unable to clarify the chondrocyte differentiation- involved pathological mechanisms even the regulation mechanisms thoroughly. Lots of novel factors, especially transcriptional and epigenetic regulators should be considered particularly noteworthy.

Therefore, the purpose of this study is clarification of AR function together with identification of the novel transcription and/or epigenetic factors in chondrocytogenesis and determination their underlying molecular mechanisms in chondrocytes *in vitro* and *in vivo*.

2. Results:

1) Determination of the function of androgen receptor in chondrocytogenesis

By employing the Cre/loxP system, the male proliferative chondrocyte-specific ARKO mice (Col2a1-ARKO) and hypertrophic chondrocyte-specific ARKO mice (Col10a1-ARKO) were generated to uncover the biological function of AR in chondrocytogenesis. Unexpectedly, not only in cartilage but actually in testis, the deletion of AR gene was partially observed in both mice lines. According with the analyses associated with testis, the partial AR gene ablation in testis was unlikely to reflect on endocrine system. Thus, the generated chondrocyte specific ARKO mice were tolerating models for morphological analysis of skeletal tissues. In both chondrocyte-specific ARKO mice, there was no significant difference in long bone length and bone mass. However, the expanded proliferating zone with consequence of markedly increased pubis length and pelvic size were exhibited in Col2a1-ARKO, not in Col10a1-ARKO mice. This result suggested that AR may suppress the proliferation of chondrocytes in pubic bone not in long bone.

To determine the molecular mechanism of AR in chondrocytogenesis, chondrocyte differentiation model *in vitro* should be established. In micro mass culture system using C3H10T1/2 (10T1/2) cell line treated with BMP2, the mass cultured 10T1/2 cells differentiated into proliferative chondrocytes at day 5 followed by the differentiation into hypertrophic chondrocytes at day 9. In the chondrogenic 10T1/2 cells, AR was highly expressed in proliferative chondrocytes on both mRNA and protein levels. Moreover, the mRNA expression of proliferative chondrocyte-related genes was down-regulated by an AR ligand, dihydrotestosterone (DHT). These results suggested that liganded AR might be served as a novel negative regulator in early stage of chondrocytogenesis. According to

the distribution and the function of AR in chondrocytogenesis, the early stage chondrogenic ATDC5 cell line was utilized to determine the mechanism by which AR negatively regulated early chondrocytogenesis. Interestingly, the result of luciferase assay with *Col2a1* luciferase reporter containing Sox9 binding site, revealed that AR repressed transcriptional activity of Sox9 in a ligand-dependent manner. Furthermore, AR recruitment was detected in Sox9 binding motif in *Col2a1* enhancer region under the DHT treatment. Meanwhile, acetylation on histone H3 and methylation on H3K4 residues were decreased at the same region in the presence of DHT via the analysis of ChIP-qPCR.

Together, these results suggested that liganded AR may suppress the transcriptional activity of Sox9, subsequently, down-regulate the expression of *Col2a1* via alteration of histone modifications to repress the early chondrocytogenesis. This androgen-AR dependent mechanism might play a role in the determination of pelvic bone morphology.

2) <u>Identification of novel transcriptional and/or epigenetic regulators in chondrocytogenesis</u>

To identify novel transcriptional and/or epigenetic regulators in chondrocytogenesis, differentiation stage dependent gene expression profiles of the mass cultured 10T1/2 cells were analyzed by gene expression microarray. From the results of the differentially expressed candidates among differentiation stages followed by validation through qRT-PCR, autoimmune regulator (Aire), a transcription factor, was targeted, which mutations cause the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) accompanying with reversible metaphyseal dysplasia.

As a result of qRT-PCR, mRNA expression levels of chondrocyte-related genes were decreased in Aire stable knockdown mass cultured 10T1/2 cells and *Aire*$^{-/-}$ primary cultured chondrocytes. These data suggested that Aire might work as a novel positive regulator in chondrocytogenesis. To further clarify the function of Aire, bone morphogenetic protein 2 (BMP2) was focused, which is a member of the transforming growth factor β superfamily and can facilitate chondrocytogenesis via BMP2/Smads signaling pathway, as a possible target gene of Aire, because the expression of *BMP2* is down-regulated in *Aire*$^{-/-}$ thymic epithelial cells (TECs). Interestingly, luciferase assay with *BMP2* promoter suggested that Aire can directly up-regulated *BMP2* expression in ATDC5 cells. Also, the early stage chondrocytogenesis in mass cultured 10T1/2 cells was induced by Aire overexpression even without BMP2 treatment. Furthermore, ChIP-qPCR assay was performed to define the molecular mechanism of Aire on *BMP2* expression. As a result, Aire was recruited to Aire binding motif (T box, TTATTA) in *BMP2* promoter in early stage of chondrocytogenesis, with increased H3K4me2, an active histone marker for gene expression. This modification was partially reduced with the inactivation of Aire. These data indicated that Aire may be a novel transcription factor in chondrocytogenesis via up-regulating *BMP2* expression through the alteration of histone modification to accelerate the chondrocyte differentiation in early stage.

3. **<u>Conclusion:</u>**

In this study, we determined the physiological function of AR via chondrocyte-specific androgen receptor knock-out mice model and the molecular mechanism of AR in differentiated mass cultured 10T1/2 cell line or early chondrogenic ATDC5 cell line. Meanwhile, based on the analysis of microarray of gene expression during the chondrocytogenesis, we also identified Aire, which may be a novel transcription factor in chondrocytogenesis.

From the results of chapter 2 and 3, it had been observed that the increased chondrocyte proliferation in Col2a1-ARKO mice *in vivo* as well as the decreased expression of proliferative chondrocyte-related genes in DHT-dependent manner *in vitro.* It indicated that liganded AR may be regarded as a suppressor of early chondrocytogenesis, which was consistence with the smaller pelvis size in male than in female, however, contrary to that long bone is longer in male comparing with in female. It may be contributed to the different construction of two growth plates resulting in the increased expression of aromatase in long bone not in pubic bone. Hence, in male mice, most of androgens may be aromatized into estrogens, which stimulate the growth spurt to cover the negative regulation of AR via less of androgens on long bone growth.

Meanwhile, results from chapter 4 indicated that Aire prompted the early stage of chondrocyte differentiation by up-regulating *BMP2*. Aire may be recruited to Aire binding motif on *BMP2* promoter via the recognition of hypomethylated H3K4 by PHD1 domain of Aire and associate with histone methyltransferases for methylation on H3K4 or co-regulators to activate the expression of *BMP2*. However, it has been published that the chondrocyte-specific BMP2 knock-out mice exhibit a severe chondrodysplasia phenotype, whereas, no significant phenotype is observed in bone in the reported conventional Aire knock-out mice. The possible reasons of this inconsistency are considered as follows: 1) conventional Aire knock-out mice suffering from immune deficiency may exhibit a growth factors-related disorder, such as IGF-1. 2) Aire may be engaged in transcriptional regulation of *BMP2* as one of the mechanisms, which can be compensated by other factors.

Therefore, considering the temporal and spatial-dependent function of AR, other stages of chondrocyte-specific ARKO mice should be required to elucidate the AR biological function in long bone growth. Furthermore, *in vitro*, the specific AR-associated complexes involving HDACs/HDMs should be identified to further understand the mechanism of AR in early stage of chondrocytogenesis. Besides, generation of the chondrocyte-specific Aire knock-out mice and analyzed in early stage should be required to clarify the physiological function of Aire in bone growth *in vivo*. Further, to deep draw out the molecular mechanism of Aire, the PHD1 domain and Aire-involved complexes in early chondrocytogenesis should be a key point to be clarified.

Together, the activity of proliferative chondrocytes was suppressed by AR and activated by Aire. By the understanding of physiological and molecular function of them, it can bring us incredible wide insight in the fields of chondrocytogenesis regulation as well as the treatment of OA in the future.