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学位論文題目	Novel effects of CCN3 that may direct the differentiation of chondrocytes. （軟骨細胞分化に対するCCN3蛋白質の新たな制御効果）
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学位論文内容の要旨

Introduction: Chondrocytes are divided in two groups: transient and permanent ones. The former one goes through the complex process of physiological endochondral ossification; whereas in the later ones, ossification is prevented by unknown molecules. In this context, the mechanism of articular cartilage development within synovial joints is particularly interesting. Articular cartilage is a thin strip of cartilage that persists throughout the entire growth and development process and maintains its integrity. The molecules acting locally to divide the epiphyseal chondrocytes (EC) into two these populations in the epiphysis have not yet been identified.
The CCN family influences cartilage homeostasis. As CCN3 has been shown to repress proliferation and matrix calcification of costal chondrocytes (CC) undergoing endochondral ossification, we hypothesized that CCN3 might be one of the factors involved in driving the articular chondrocyte phenotype, acting by slowing down their proliferation and preventing their maturation towards endochondral ossification. After verifying the production of CCN3 in vivo, we evaluated the effect of exogenous CCN3 mainly on the cells from the distal femoral epiphysis of rats before secondary ossification.

Methods: Immunohistochemistry (IHC) for CCN3 was performed on femurs from 5-day-old Sprague–Dawley rats and immunocytochemistry for tenascin-C was done on cells from distal third of femoral epiphysis and from rib cage of 5-day-old Sprague–Dawley rats. Western blotting (WB) analysis for CCN3 was also performed with tissue lysate from the distal halves of epiphysis.
Cells for *in vitro* analysis were obtained from femoral distal epiphysis and from the ribcage. Cells grown to confluence were treated with one of the following proteins: human recombinant CCN3 fused with GST (GST-NOV) or GST only and glycosaminoglycan synthesis was estimated through [^{35}S] sulfate incorporation. Proteoglycan accumulation in culture was also evaluated by alcian blue staining upon the same treatment described above along 2 weeks
Cells grown to sub-confluence were treated with recombinant CCN3 (rCCN3) and DNA synthesis was estimated through [^{3}H] thymidine incorporation. Viable cell numbers were estimated by WST-1 assay upon the same treatment above to further confirm the results from DNA synthesis estimation.

For matrix calcification assay, cells were treated for two weeks with medium supplemented with ascorbic acid and β-glycerophosphate. Mineralization degree was assayed through alizarin red stain.
The effect of exogenous CCN3 on ECs and CCs, as well as that of CCN3 knockdown on ATDC5 cells, was evaluated by the mRNA level of marker genes by RT-PCR.

Results: CCN3 was found throughout the cartilage template of the distal epiphyseal pole, being mainly observed in the chondrocyte territorial matrix. Significant signals were also present associated with the cells. A distinct signal could also be observed by WB analysis.
Estimated by [^{35}S] sulfate incorporation, enhanced proteoglycan synthesis was observed upon addition of rCCN3 at 2 nM. CCN3 increased the proteoglycan content accumulated during the course of 2 weeks, as revealed by alcian blue staining.
DNA synthesis, estimated by [^{3}H] thymidine incorporation, was decreased by 2 nM rCCN3. The decreased DNA synthesis was accompanied by reduced mitochondrial metabolic activity, as shown by the WST-1 assay.
Phenotype assessment showed that ECs were close to articular chondrocytes. We found that the number of cells producing tenascin-C, a typical marker of articular chondrocytes, was higher in ECs than in CCs. The higher matrix calcification level presented by CCs than by ECs corroborates these phenotypical differences. The expression level of the CCN3 gene was shown to be remarkably higher in ECs. In these cells, the expression of aggrecan core protein was up-regulated at 48 h after the addition of rCCN3. Expression of tenascin-C also displayed up-regulation in those cells, while type X collagen gene expression was repressed after 48 h of rCCN3 treatment. Tenascin-C was, at a marginally significant level, up-regulated also in CCs upon the same treatment.
ATDC5 cells, after infection with a retroviral vector expressing a short hairpin siRNA against CCN3 mRNA, were induced to differentiate by the addition of ITS. The degree of silencing was not striking, but it resulted in significant changes in the gene expression of tenascin-C and type X collagen. These results are consistent with the data with rCCN3 and further indicate the contribution of CCN3 in directing immature chondrocytes towards the pathway of differentiation to articular chondrocytes

Discussion: IHC confirmed that epiphyseal cartilage strongly produced CCN3 denoting critical roles in articular cartilage development. The proliferation-inhibiting effect and the enhanced matrix production, exerted by CCN3 bestowed these cells with low mitotic activity and active matrix production, which are two remarkable characteristics of chondrocytes forming articular cartilage.
CCN3 also remarkably increased tenascin-C expression in ECs, which represent the promotion of differentiation toward articular chondrocytes. Similar effect was observed also on CCs undergoing endochondral ossification. The repression of collagen type X gene expression by CCN3 also indicates that it directs chondrocytes away from the endochondral pathway. This hypothesis was supported by a knockdown strategy on ATDC5 cells. These results together suggest a novel effect of CCN3 that may direct EC towards articular cartilage construction.
In light of the new data presented here, we propose that CCN3 is the molecule behind the non-calcifying phenotype of articular chondrocytes even in the presence of CCN2, which promotes maturation towards endochondral ossification in CCs. We also propose that the down-regulation of CCN3 in articular cartilage may be involved in the genesis of osteoarthritis. External factors, such as mechanical overload to the articular cartilage, could turn CCN3 off, inducing chondrocytes to enter the endochondral ossification pathway to form osteophytes.

学位論文審査結果の要旨

関節の発生過程においては、骨端軟骨原基を形成する均質な軟骨細胞は二つの異なる分化過程を経て関節軟骨と骨組織を形成する。しかしながらこれら軟骨細胞を二つの分化方向に振り分けるメカニズムは明らかではない。本研究ではそこで役割を演ずる局所シグナル分子としてCCNファミリーメンバー3（CCN3）に着目し、その機能検証を行った。

その結果、明らかになったことは下記のとおりである

1） 生後5日目ラットの骨端軟骨においてCCN3タンパク質が局在する。

2） ラット骨端軟骨原基を形成する細胞の大部分は関節軟骨細胞マーカーであるtenascin-C（TNC）陽性である一方、肋軟骨ではTNC陰性細胞が大部分を占めていた。興味深いことにCCN3遺伝子発現も骨端軟骨細胞の方が肋軟骨より高く、TNC発現量と正の相関を見せた。

3） rCCN3添加によって骨端軟骨細胞の増殖は抑制されると同時に軟骨基質産生が増強された。

4） rCCN3はtenascin-C遺伝子発現に有意な増強効果を示す一方typeX collagen遺伝子発現に有意の抑制効果を示すのが観察された。

5） ATDC5細胞を軟骨細胞初期分化に導き、shRNAによってCCN3遺伝子ノックダウンを行ったところ、rCCN3添加実験とほぼ逆に内軟骨性骨化へ向けての軟骨細胞の分化誘導促進現象が観察された。

これらの知見はCCN3が骨端軟骨組織に存在し、関節軟骨細胞への分化に軟骨細胞を誘導していることを強く示唆している。またノックダウン実験の結果からは、CCN3欠乏と変形性関節症との関連も示唆され極めて興味深い。

よって、審査委員会は本論文に博士（歯学）の学位論文としての価値を認める。