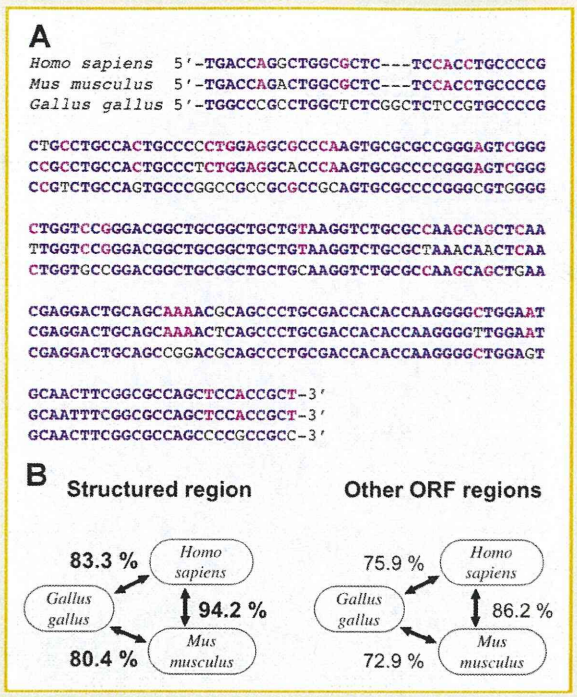

Fig. 7. Evolutionary conservation of the GC-rich region. A: Alignment of the cDNA sequences of the GC-rich regions in the *ccn1* genes from three vertebrates. Nucleotide sequences conserved among the three are shown in blue bold face letters, whereas those conserved between two species are shown in purple. NCBI accession numbers are; NM_001554.4 for *homo sapiens*, NM_010516.2 for *mus musculus*, and NM_001031563.1 for *gallus gallus*, respectively. B: Comparison of maximum matching scores between two species shown in panel A. The matching scores of the GC-rich structured region and those of the other ORF regions are shown at the left and right, respectively.

corresponding cDNA was constructed by assembling four synthetic oligonucleotides into a parental reporter construct at the upstream end of the luciferase gene. Two chimeric constructs containing the subfragments of the structured region were constructed as well (Fig. 8A). The results of DNA transfection and luciferase assay clearly revealed that the minimal element, RPC, was a positive *cis*-regulatory element *per se* (Fig. 8B). Interestingly, the former-half of the element, RPF, containing two upstream loops was found to be as potent as the entire element, whereas the latter-half, RPL showed no activity. These findings suggest the critical requirement of the two loops for the regulatory function.

DISCUSSION

The *ccn1* mRNA is approximately 1.8 kb-long, and its overall GC-content is <50%; whereas that of its 5′ region (1–500 bases from the initiation codon) of the ORF is nearly 70%. We initially found that the PCR products of the corresponding region were distinctly shorter than the expected, by approximately 200 bp (Fig. 1; RP-A). In such a case, involvement of alternative splicing products may be suspected. However, since the PCR product recovered the expected length (Fig. 1; RP-B) in the presence of PCRx Enhancer Solution, a PCR supplement for problematic and GC-rich templates, we concluded that the shortening to RP-A was not the result of an alternative splicing of mRNA, but of miselongation by DNA polymerase. This conclusion was further supported by the reproduction of the same results by the experiments with purified RP-B DNA (data not shown). Nucleotide sequencing of RP-A (Fig. 1A) demonstrated that this shortening arose from the consistent deletion of a central 209-bp segment with 73.5% GC-content. It was previously reported that DNA polymerases, including *Taq* DNA polymerase, skip a large region during the synthesis of template DNA, if the region has a stable secondary structure [Dignam et al., 1983; Hew et al., 1999, 2000; Kontoyiannis et al., 2001]. Hence, the observations above indicate a stable secondary structure in the 5′ region of the ORF of *ccn1* cDNA, over which the elongation of DNA continued, skipping the site of the secondary structure during PCR.

Next, the results of the analytical RNase protection assay of the folded RNA (Fig. 6) revealed that the radio-labeled RNAs corresponding to the 5′-region (RP-B) and the minimal structured region (RPC) were resistant to digestion with RNase, indicating the presence of a stable secondary structure in the GC-rich region on the RNA as well. Digestion of RPC as well as RP-B S with RNase T1 resulted in a residual product of 100-nt in length. These fragments are most likely to have arisen from the digestion of the structured region at the major loops that are assumed to be sensitive to RNase T1. Additionally, our other results showing that RNase T1 was able to completely digest both the sense and anti-sense strands of RP-A and the anti-sense strand of RP-B (Fig. 2C) confirmed the specific formation of a secondary structure in the GC-rich RP-B region of the ORF in the *ccn1* mRNA.

The stable secondary structure of the 5′-region of the ORF of *ccn1* mRNA suggests that the fragment may play a role in the regulation of gene expression as a *cis*-element. Therefore, REMSA (Fig. 3) was performed, and the results clearly demonstrated the specific binding of a cytoplasmic protein or multimolecular complex of proteins from CEF cells to the sense strand of the RP-B region. Since no such binding was observed for RP-A, we concluded that the secondary structure was required for the binding between the *ccn1* mRNA and the cytoplasmic protein(s). Of note, no binding protein was detected in the nuclear fraction, suggesting a cytoplasmic event, in which this mRNA segment might play a significant role (Fig. 3B).

Finally, luciferase reporter gene analysis (Fig. 4) revealed an enhancing effect of the corresponding region (RP-B) on the gene expression. Importantly, the enhancing effect of RP-B′ was much stronger at the 5′-region of the reporter gene than at the 3′-region, indicating a location-dependent enhancing effect. Since RP-B is originally located in the 5′-region of the *ccn1* ORF, this segment ought to contribute to the enhancement of *ccn1* gene expression in the natural context. Indeed, nucleotide sequence alignment of the GC-rich regions from three vertebrate species showed that this region has been highly conserved during evolution (Fig. 7A). Of note, the matching scores representing evolutionary conservation were markedly higher in the GC-rich region than the other regions of the ORF (Fig. 7B). These findings indicate another indispensable function of the GC-rich region than encoding protein sequences. Consistent with this indication, the minimal structured segment was confirmed to be functional *per se*. However, the enhancing effect by

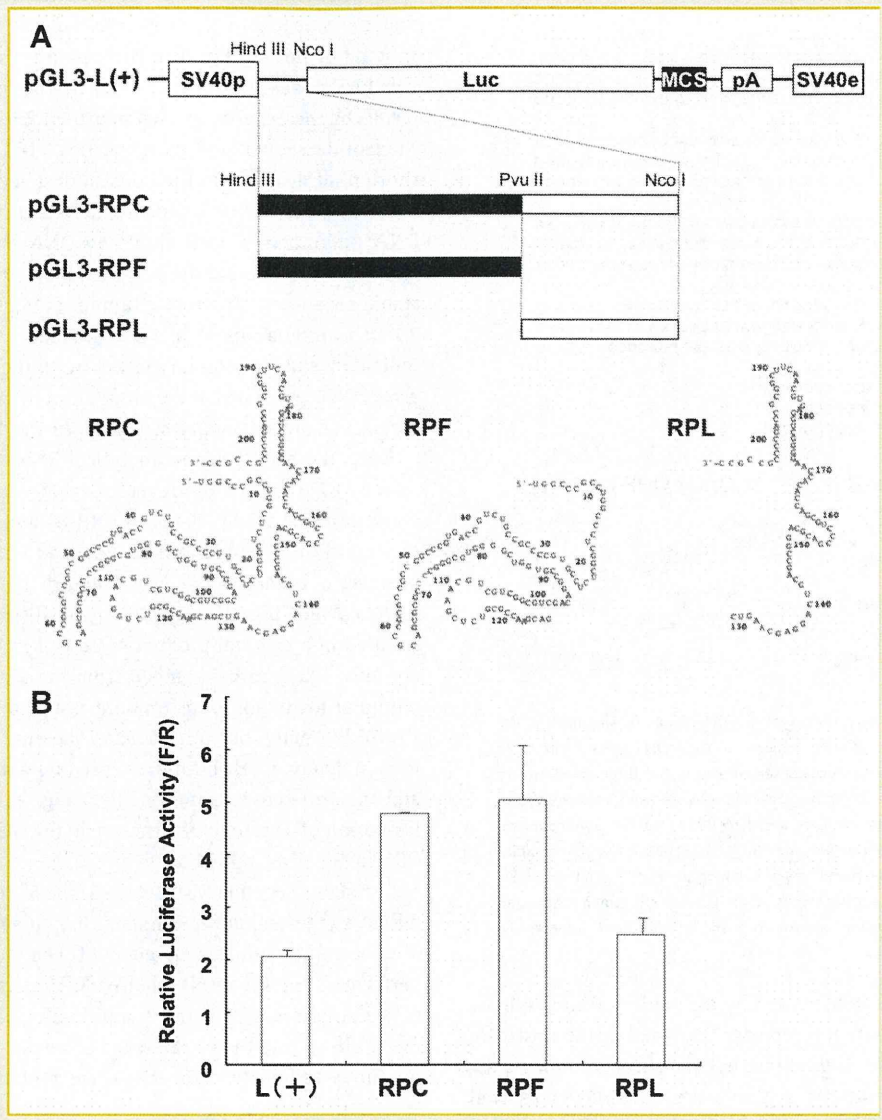

Fig. 8. Functional definition of the GC-rich region as a regulatory element. A: Construction of the plasmids for the evaluation of the GC-rich region and its subfragments. Structures of the parental plasmid and built-in *ccn1* fragments are displayed, together with the names of the resultant reporter plasmids. B: Firefly luciferase activities from the plasmids described in panel A. Experiments and computation of the data obtained were performed essentially as described in the legend to Figure 4. The data are representative of two sets of independent experiments.

this element, RPC, was not so strong as that by RP-B′, as yet suggesting a functional contribution of another segment in RP-B′.

Several reasons can be considered to account for the mechanism of the observed effect of the RP-B′ on gene expression in *cis*. It is widely recognized that a number of mRNA *cis* elements, especially those in the UTRs, possess signal sequences for mRNA export from the nucleus [St Johnston, 1995]. Also, a number of structured RNA elements that affect mRNA translation have been described. For example, in another CCN family member, CCN2, *cis*-acting element of structure-anchored repression was discovered [Kondo et al., 2000; Kubota et al., 2000, 2005]. Furthermore, our group recently clarified that chicken *ccn2* mRNA level is regulated by selective mRNA degradation under the collaboration of a structured mRNA element (5′–100/50) and nucleophosmin/B23 [Mukudai et al., 2008]. Additionally, regulation of *ccn2* gene by miR-18a *via* a target in the 3′-UTR has also been reported [Ohgawara et al., 2009]. In the present study, the RNase protection assay revealed that RP-B markedly increased the amount of the corresponding mRNA in the ribosomal fraction (5.6-fold vs. control: Fig. 5B), whereas it slightly enhanced the level of total mRNA of the reporter gene (1.8-fold vs. control: Fig. 5A). These results suggest that the enhancing effect of RP-B′ mainly resulted from the promotion of transportation and/or entry of mRNA into the ribosome, with modest up-regulation of the steady-state expression level of mRNA. Therefore, the structured segment might possess the ability to transport the mRNA from the nucleus into ribosome through interaction with cytoplasmic protein(s), as observed in the case of internal ribosomal entry site (IRES) of viral mRNAs. As such, regulation of the translation process

is strongly suspected as the major function of RP-B at the original location in the *ccn1* mRNA. Studies are currently ongoing in order to address these issues.

CCN1 plays important physiological roles in cell growth [Kireeva et al., 1996; Babic et al., 1998], migration [Kireeva et al., 1996; Babic et al., 1998], adhesion [Kireeva et al., 1996, 1997, 1998; Jedsadayanmata et al., 1999; Chen et al., 2001] and differentiation [O'Brien and Lau, 1992; Mo et al., 2002] of a variety of cells. Moreover, CCN1 is also involved in tumorigenesis, either positively or negatively [Kireeva et al., 1996; Tsai et al., 2000; Tong et al., 2001; Xie et al., 2001; Juric et al., 2009]. In executing such various functions, CCN1 is thought to be under complex regulation not only at the transcriptional level, but also at the post-transcriptional level. In the present study, we discovered that the RP-B region of the ORF of *ccn1* mRNA was able to regulate gene expression through interaction with as yet some unidentified cytoplasmic protein(s). Moreover, we also demonstrated that this RNA segment enhanced gene expression mainly by altering the transportation and/or entry of the mRNA into the ribosome. It should be noted that RP-B region itself encodes part of the amino acid sequence of CCN1 and thus undergoes translation process. Therefore, identification of this binding protein(s) and characterization of its (their) interaction with eukaryotic initiation and elongation factors will uncover the entire regulatory system mediated by the RP-B segment of *ccn1* mRNA.

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Role of low-density lipoprotein receptor related protein 1 (LRP1) in CCN2/connective tissue growth factor (CTGF) protein transport in chondrocytes

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Summary

LRP1 is known to be a receptor for signal transmission and endocytosis. We formerly reported that LRP1 regulates WNT/β-catenin and protein kinase C signaling in chondrocytes and represses the hypertrophy of chondrocytes during endochondral ossification, and that LRP1 is co-localized with a ligand, CCN2, which conducts endochondral ossification, on chondrocytes. However, the role of LRP1 in endocytotic transport of CCN2 in chondrocytes is not yet understood. In the present study, we investigated the interaction between LRP1 and CCN2 during endocytotic trafficking.

RNAi-mediated knockdown of *LRP1* in chondrocytic HCS-2/8 cells showed that the amount of exogenous CCN2 binding/incorporation was decreased in the *LRP1* down-regulated cells. Importantly, we observed that CCN2 internalization in chondrocytes was dependent on clathrin and internalizated CCN2 was co-localized with an early or recycling endosome marker. Transcytosis of CCN2 through HCS-2/8 cells was confirmed by performing experiments with a trans-well apparatus, and the amount of transcytosed CCN2 was decreased by an LRP1 antagonist. These findings rule out possible leakage and confirm the critical involvement of LRP1 during experimental transcytosis. Moreover, under the hypoxic condition mimicking the cartilaginous microenvironment, the production level of LRP1 and the amount of transcytosed CCN2 were increased, which increases were neutralized by the LRP1 antagonist. The distribution of LRP1 and its antagonist in the growth plate in vivo was consistent with that of CCN2 therein, which was produced by and transported from the chondrocytes in the prehypertrophic layer.

These findings suggest that LRP1 mediates the transcytosis of CCN2, which may be a critical event that determines the distribution of CCN2 in cartilage.

Keywords: low-density lipoprotein receptor-related protein 1 (LRP1), chondrocytes, CCN2/connective tissue growth factor (CTGF), endocytosis, transcytosis

Running title: LRP1 induced CCN2 transcytosis

Introduction

The low-density lipoprotein receptor (LDLR)-related protein-1 (LRP1), is a 600-kDa type I membrane protein and a member of the LDLR family (Herz and Strickland, 2001). By interacting with over 40 distinct ligands, LRP1 is thought to regulate lipid homeostasis, extracellular proteolysis, growth factor/cytokine activity, composition of the extracellular matrix (ECM), and even immune responses (Herz, 2001; Herz and Strickland, 2001; Lillis et al., 2008). A significant part of these LRP1 functions is thought to be related to clathrin-dependent endocytosis (Hussain, 2001) and cellular signal transduction pathways (Herz, 2001; Herz and Strickland, 2001; Lillis et al., 2008), such as protein kinase C (PKC) cascades (Hayashi et al., 2007). Recently, LRP1 was shown to interact with human frizzled-1 to down-regulate the canonical WNT/β-catenin signaling pathway (Zilberberg et al., 2004). Consistent with its functional diversity, LRP1 is essential for embryonic development. It was reported that conventional *Lrp1*-deficient animals failed to develop normally and died during early to mid-gestation (Herz et al., 1992). Of note, the involvement of LRP1 in the prevention of atherosclerosis was also indicated by utilizing conditional gene targeting technology (Boucher et al., 2003). Also, the expression and function of LRP1 in the central nervous system (May and Herz, 2003), vascular smooth muscle cells (Boucher et al., 2003), and macrophages (Gardai et al., 2003) have been relatively well characterized.

We formerly demonstrated the distribution of LRP1 in normal cartilage (Kawata et al., 2006) and reported that LRP1 initiates the hypertrophy of chondrocytes during endochondral ossification through the WNT/β-catenin and PKC signaling (Kawata et al., 2010). Vertebrate cartilage is of two distinct types, permanent cartilage represented by articular cartilage, and temporary cartilage represented by the growth plate cartilage where endochondral ossification occurs. During endochondral ossification, chondrocytes first proliferate, and then become mature cells that produce abundant ECM components such as type II collagen. Thereafter, the cells eventually

differentiate into hypertrophic chondrocytes, which produce alkaline phosphatase and type X collagen. At the terminal stage of endochondral ossification, the cartilage matrix becomes mineralized and is invaded by blood vessels; and then these hypertrophic chondrocytes are thought to undergo apoptosis. Thus, through this process, cartilage is replaced by bone (Nakanishi et al., 1997; Takigawa et al., 2003).

In our series of studies, we have uncovered critical roles of CCN2 (also known as CTGF; connective tissue growth factor) in endochondral ossification (Ivkovic et al., 2003; Kubota and Takigawa, 2011; Nakanishi et al., 1997; Nakanishi et al., 2000; Nishida et al., 2002; Perbal and Takigawa, 2005; Takigawa et al., 2003) and regeneration of articular cartilage (Nishida et al., 2004). Interestingly, in vivo, CCN2 molecules are distributed in a layer different from that containing the *Ccn2* mRNA-positive cells. Namely, whereas the chondrocytes expressing the mRNA of the *Ccn2* gene are detected in the prehypertrophic chondrocytes, the CCN2 protein itself is broadly detected from the prehypertrophic zone to the hypertrophic chondrocyte one (Oka et al., 2007). Here, it should be noted that CCN2 is one of the ligands of LRP1, which mediates intracellular protein transport (Gao and Brigstock, 2003; Perbal, 2004; Segarini et al., 2001; Yang et al., 2004).

We hypothesized that the difference in the distribution of *Ccn2* mRNA expression and CCN2 protein in vivo was caused by the function of LRP1 in CCN2 protein trafficking. In this study, we for the first time show that LRP1 induces CCN2 transcytosis through chondrocytes in vitro.

Results

Effect of *LRP1* knockdown on CCN2 association with chondrocytic cells

Firstly, to examine whether LRP1 participates in associating CCN2 with chondrocytes or not, we performed RNAi experiments to knockdown *LRP1* in HCS-2/8 cells, a human chondrocytic cell line, as previously performed (Kawata et al., 2010). We

confirmed that the production of LRP1 protein was substantially knocked down by si-1163 and si-13157 (Fig. 1). We added a recombinant CCN2 with a Flag tag at the N-terminus to the control/ *LRP1* knockdown HCS-2/8 cells, and then collected the cells after 1 hour and performed immunoblotting by using anti-Flag tag antibody. As a result, the bound/ incorporated amount of recombinant CCN2 was decreased in the *LRP1* knockdown HCS-2/8 cells compared with that in the control cells (Fig. 1). The result of immunoblotting with anti-His tag antibody recognizing a 6xHis tag fused to the CCN2 C-terminus was similar to that obtained by using the anti-Flag tag antibody (Fig. 1). These results indicate that LRP1 participated in associating CCN2 with chondrocytes.

Effect of clathrin inhibition on CCN2 association with chondrocytic cells

Secondly, to examine whether CCN2 association with chondrocytes depend on clathrin or not, we evaluated the effect of endocytosis inhibitors. Namely, we added a recombinant CCN2 with a Flag tag at the N-terminus to the HCS-2/8 cells pretreated either with a clathrin-dependent endocytosis inhibitor, chlorpromazine or a caveolin-dependent endocytosis inhibitor, MβCD and then collected the cells after 1 hour and performed immunoblotting by using the anti-Flag tag antibody. As a result, the bound/ incorporated amount of recombinant CCN2 was decreased in the HCS-2/8 cells pretreated with chlorpromazine compared with that in the control cells with vehicle only (Fig. 2A). The result of immunoblotting with anti-His tag antibody recognizing the C-terminal 6xHis tag was comparable to that obtained with the anti-Flag tag antibody (Fig. 2A). In contrast, in the HCS-2/8 cells pretreated with MβCD, the bound/ incorporated amount of recombinant CCN2 was unchanged compared with that in the control cells (Fig. 2B). These results indicate that association of CCN2 with chondrocytes is dependent on clathrin.

Intracellular destination of CCN2 taken up into HCS-2/8 cells

Subsequently, we added recombinant human CCN2 (rhCCN2) and chased the fate after the application onto HCS-2/8 cells. In fact, exogenously added rhCCN2 and endogenous LRP1 were also partially co-localized inside of the HCS-2/8 cells (Fig. 2C, D). This result again suggests the contribution of LRP1 in the endocytotic incorporation of CCN2. Excluding the signals from nascent CCN2 in exosomes on the way to secretion, we chased the fate of exogenous CCN2 after internalization by anti-Flag antibody. As confirmed by double-staining with the organelle-specific markers, incorporated CCN2 was directed to clathrin (Fig. 2E, F), early (Fig. 2G, H) and recycling endosomes (Fig. 2I, J). Particularly, exogenously added rhCCN2 and, the recycling endosomes marker were predominantly co-localized in HCS-2/8 cells (Fig. 2I, J). Therefore, CCN2, which was internalized in HCS-2/8 cells, was directed to recycle out of the cells toward any direction, following partially the pathway directed by LRP1.

Effect of LRP1 on CCN2 transcytosis in chondrocytes

We considered that the broad localization of CCN2 protein from the prehypertrophic zone to the hypertrophic one of growth plate cartilage is possibly realized by LRP1-mediated transcytosis of this protein from the chondrocytes expressing *ccn2* in the prehypertrophic zone. To test the validity of this hypothesis, we performed a transcytosis assay using CCN2 with/ without an LRP1 antagonist, i.e., LRP-associated protein 1 (LRPAP1)/ receptor-associated protein (RAP). After the addition of exogenous CCN2 having an N-terminal Flag tag to the cells in the upper transcytosis chamber (Fig. 3A), the exogenous full-length CCN2 was detected in cell lysates by using anti-Flag tag antibody (Fig. 3B). Moreover, the result of immunoblotting with anti-His tag antibody that recognizing a 6xHis tag fused to the CCN2 C-terminus was similar to that obtained with by using the anti-Flag tag antibody (Fig. 3B). These signals were decreased by LRPAP1 (Fig. 3B). Results similar to those found with the

cell lysate were obtained with the medium in the lower chamber (Fig. 3C), which indicates the functional involvement of LRP1 and rules out the possibility of major leakage through the uncovered part of the membrane. Since we basically use an Escherichia coli (E. coli) derived CCN2, we repeated the same analysis with another recombinant CCN2 from HeLa cells, in order to rule out the possibility of the contamination of E.coli components therein. As a result, the exogenous biotin-labeled full-length CCN2 was detected in cell lysates by using horse radish peroxidase (HRP) conjugated avidin, which was inhibited by LRPAP1 (Fig. 3D). Results similar to those found with the cell lysate were obtained with the medium in the lower chamber (Fig. 3E). These results clearly indicate that CCN2 is transcytosed, as well as bound/ incorporated in chondrocytes mediated by LRP1.

Effect of hypoxia on the levels of LRP1 mRNA and protein in HCS-2/8 cells

Cartilage is an avascular tissue; therefore, it has been assumed that the low oxygen partial pressure in the chondrocytic growth plate imposes energetic limitations on the cells as they evolve from a proliferative to a terminally differentiated state (Rajpurohit et al., 1996). Suspecting the contribution of the oxygen pressure gradient to the hypertrophic layer-specific localization of CCN2, we investigated transcytosis of CCN2 by LRP1 under hypoxic conditions. Firstly, we examined the levels of *LRP1* mRNA expression and production of LRP1 protein under the hypoxic condition. Exposure to hypoxia resulted in a time-dependent increase in *LRP1* mRNA expression level (Fig. 4A). Moreover, the production level of LRP1 protein was increased under hypoxic condition as well (Fig. 4B). Consistent with previous reports(Semenza and Wang, 1992; Shimo et al., 2001), the production level of CCN2 and HIF1α protein was also increased under hypoxic condition (Fig. 4B). Thus, to determine whether HIF1α mediates the hypoxia-induced LRP1 and CCN2 production, we examined the effect of the antisense HIF1α oligonucleotides on LRP1 and CCN2 production, respectively,

under hypoxic condition. Cultured cells in 5% O_2 with antisense oligonucleotides against HIF1α (but not with sense oligonucleotide-treated) abolished LRP1, CCN2 as well as HIF1α induction (Fig. 4C), but β-Actin was unaffected (Fig. 4C). Decreased CCN2 by HIF1α downregulation agrees with the results of a previous study (Hong et al., 2006). More importantly, these data indicate that HIF1α regulates not only CCN2 but also LRP1 production under hypoxic condition. Additionally, these results agree with the results of a previous study, in which the level of LRP1 was found to be drastically decreased in the hypertrophic zone of the cartilage near the bone marrow (Kawata et al., 2006).

Effect of hypoxia on CCN2 transcytosis in chondrocytes

Secondly, we evaluated the effect of hypoxia on actual CCN2 transport in the same experimental system using the Transwell. As a result of immunoblotting with anti-Flag or His tag antibody, the exogenous CCN2 in the cell lysate was increased under the hypoxic condition compared with the signals under the normoxic condition (Fig. 5A). In the medium in the lower chamber, results similar to those for the cell lysate were obtained (Fig. 5B). In both cases these signals were decreased by LRPAP1, again confirming the functional involvement of LRP1 (Fig. 5). These results indicate that transcytosis of CCN2 in chondrocytes by LRP1 was increased under the hypoxic condition compared with that under the normoxic condition.

Higher expression and production levels of LRPAP1 in the chondrocytic cell line

We formerly reported that the LRP1 levels are higher in HCS-2/8 cells than in other types of cells (Kawata et al., 2006). Based on this finding, we next compared expression and production levels of the LRP1 antagonist LRPAP1 in the chondrocytic HCS-2/8 cells with those in the other cell lines. LRP1 and LRPAP1 were analyzed comparatively in HCS-2/8 cells, breast cancer-derived MDA-MD-231 cells (MDA-231),

and cervical carcinoma-derived HeLa cells by use of real-time RT-PCR and immunoblotting. As a result, the expression level of *LRP1* was certainly higher in HCS-2/8 cells than in HeLa and MDA-231 cells, as previously reported (Kawata et al., 2006). Similarly, the mRNA level of *LRPAP1* was higher in the HCS-2/8 cells than in the other cells (Fig. 6A). Furthermore, although LRPAP1 protein was detected in all of the cell lines tested, it was more abundant in HCS-2/8 cells than in HeLa and MDA-231 cells (Fig. 6B). This result indicates that LRPAP1 was specifically induced in chondrocytic HCS-2/8 cells.

Distribution of LRPAP1 in growth-plate cartilage in vivo

Next, to investigate the production of LRPAP1 in growth-plate cartilage, we performed immunostaining analysis using anti-LRPAP1 antibody and tibial sections prepared from mice. As a result, LRPAP1 was clearly detected, particularly in resting chondrocytes, where CCN2 was absent (Fig. 7A). We formerly reported that LRP1 is present in the growth-plate cartilage, but is drastically decreased along the hypertrophic zone of the cartilage (Kawata et al., 2006). Therefore, LRP1-mediated transcytosis was supposedly suppressed by LRPAP1 in resting chondrocytes.

Changes in the expression of *lrpap1* mRNA in chondrocytes at various differentiation stages in vitro

Following the in vivo analysis, we analyzed the changes in the levels of *lrpap1* mRNA in chondrocytes during differentiation in vitro. To do this, we employed an established differentiation system using primary chicken chondrocytes (Iwamoto et al., 1995). For the evaluation of gene expression, real-time quantitative RT-PCR was performed (Fig. 7B). We initially confirmed that lower sternum (LS), upper sternal peripheral (USP) and upper sternal core (USC) cells represented resting, proliferating, and hypertrophic chondrocytes, respectively. Indeed, the *col2a1* mRNA level was the highest in the LS

cells, whereas the *col10a1* mRNA level was the highest in the USC cells. Moreover, the *alp* mRNA level was lower in the LS cells than in the other cells. Under this condition, the expression of *lrpap1* mRNA was the highest in LS cells, confirming strong distribution of LRPAP1 protein in resting chondrocytes in vivo.

Discussion

CCN2 acts in a harmonized manner on all cells involved in the promotion of endochondral ossification. In the growth-plate in vivo, CCN2 protein is distributed in a pattern different from that of its mRNA. Namely, whereas *Ccn2* mRNA expression is found in the prehypertrophic chondrocytes, CCN2 protein is broadly detected from the prehypertrophic to the hypertrophic zone (Oka et al., 2007). This broad distribution enables CCN2 to contact all the target cells, and thus is of critical importance. Here, we should note that CCN2 is one of the ligands of LRP1 (Gao and Brigstock, 2003; Perbal, 2004; Segarini et al., 2001; Yang et al., 2004). We considered that such a distribution of CCN2 protein was realized by LRP1-mediated transcytosis. To test the validity of this hypothesis, we performed this study and found that not only CCN2 binding/ incorporation but also CCN2 transcytosis was indeed mediated by LRP1 (Figs. 3, 5).

Since the growth-plate is an avascular tissue, it has been assumed that the low oxygen partial pressure in the chondrocytic growth-plate imposes energetic limitations on the cells as they differentiate from a proliferative to a terminally differentiated state (Rajpurohit et al., 1996). Therefore, mimicking such in vivo conditions, we examined the levels of *LRP1* mRNA expression and LRP1 protein production under the hypoxic condition in vitro. As a result, exposure to hypoxia increased both mRNA expression and protein production levels of LRP1 (Fig. 4). These results support a previous finding that the level of LRP1 is drastically decreased along the hypertrophic zone of the cartilage (Kawata et al., 2006). Moreover, in a previous study, increased mRNA

expression of the *lrp1* gene in carcinoma-derived cells under hypoxia was demonstrated as well (Koong et al., 2000). Consistent with these findings, transcytosis of CCN2 by LRP1 was increased under the hypoxic condition compared with that under the normoxic one in chondrocytes (Fig. 5). Thus, we propose that the distribution of CCN2 in growth-plate cartilage is controlled possibly by LRP1 regulated by hypoxia (Fig. 8).

Although cartilage is avascular, the cartilage matrix is invaded by blood vessels at the terminal stage of endochondral ossification (Nakanishi et al., 1997; Takigawa et al., 2003). Therefore, the level of the oxygen tension might be different according to the difference in the quantity of the oxygen supply between the entirely avascular layer of resting chondrocytes and layer of hypertrophic chondrocytes that is invaded by blood vessels. According to such an oxygen tension gradient, LRP1 production would be suspected to be repressed in the late hypertrophic layer, as described previously, which may prevent the flow-through of CCN2 into the bone marrow.

Finally, we should comment on the distribution of LRPAP1, the natural LRP1 antagonist, in the growth plate. We formerly reported that the LRP1 levels were higher in HCS-2/8 cells than in the other cells (Kawata et al., 2006). As was shown here, the mRNA and protein levels of LRPAP1 were also higher in HCS-2/8 cells than in HeLa and MDA-231 cells (Fig. 6). These results indicate that LRPAP1 was specifically induced in chondrocytes, and concomitant expression and production of LRP1 and LRPAP1 suggests a particular role of LRPAP1 in the endocytotic pathway in chondrocytes. Importantly, LRPAP1 was detected particularly in resting chondrocytes, where CCN2 was absent (Fig. 7). Thus, in the growth-plate cartilage, CCN2 produced in the prehypertrophic chondrocyte layer may not be transcytosed to the resting chondrocyte layer, being prevented by the higher level of LRPAP1. Collectively, localization of CCN2 in the growth plate would supposedly be maintained by LRP1 under the interaction with LRPAP1 and the hypoxic gradient therein (Fig. 8).

Materials and Methods

Antibodies and reagents

For immunoblotting and/ or immunofluorescence microscopy, anti-FLAG M2 MONOCLONAL (Sigma Aldrich, St. Louis, MO), anti-6-His (BETHYL, Montgomery, TX), monoclonal 5A6 (Progen, Heidelberg, Germany) recognizing the 85-kDa LRP1 light chain, a rabbit polyclonal H-80 antibody (Santa Cruz Biotech, Santa Cruz, CA), which recognizes amino acids 206–285 of LRP1, anti-β-Actin AC-74 (Sigma Aldrich), anti-Clathrin Heavy Chain P1663 (Cell Signaling Technology, Inc. Danvers, MA), anti-EEA1 (Cell Signaling Technology, Inc.), anti-Rab11 (C-19; Santa Cruz Biotech), anti-CTGF ab6992 (abcam, Cambridge, England), anti-Hypoxia Inducible Factor (HIF) 1 α clone H1α67 (Millipore, Billerica, MA), and anti-LRPAP1 rabbit monoclonal (Epitomics, Inc., Burlingame, CA) antibodies were employed. As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody was purchased from GE Healthcare UK Ltd (Buckinghamshire, England), HRP-conjugated anti-rabbit IgG antibody, from BETHYL; and Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-mouse IgG, Alexa Fluor 488 donkey anti-goat IgG, from Molecular Probes (Eugene, OR). Streptavidin HRP conjugate was purchased from Zymed Laboratories (San Francisco, CA). Chlorpromazine and methyl-β-cyclodextrin (MβCD) were purchased from LKT Laboratories, Inc. (St. Paul, MN) and Sigma Aldrich, respectively.

Cells

HCS-2/8 cells (a human chondrocytic cell line; (Takigawa et al., 1991; Takigawa et al., 1989), HeLa (a human cervical cancer cell line), and MDA-MB-231 (MDA-231; a human breast cancer cell line) were cultured in Dulbecco's modification of minimum essential medium (D-MEM) containing 10% fetal bovine serum (FBS). The cells were

cultured at 37°C in humidified air with 5% CO_2. Hypoxia experiments were performed for the desired times in a humidified triple gas model BL-40M incubator (BIO-LABO, Tokyo, Japan) calibrated to deliver 5% CO_2, 5% O_2, and 90% N_2 at 37°C for 8-48 h. Primary chicken chondrocytes were isolated from the caudal one-third portions (LS) of the sterna, the peripheral regions (USP), and central core regions (USC) of the cephalic portions of the sterna of day-17 chick embryos by using the method described earlier (Iwamoto et al., 1995).

LRP1 siRNA transfection

To knockdown LRP1 protein production, we used RNA interference technology. Two designed pairs of RNA oligoduplexes targeting human *LRP1* (gene accession No. NM_002332) were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The target nucleotide sequences of those oligoduplexes were 5′-UGG ACU AUA UUG AAG UGG UGG ACU AAG-3′ and 5′-CCU GUA CCA UGA ACA GCA AAA UGA UAG-3′. The former was termed LRP-1163; and the latter, LRP-13157. A nonspecific oligoduplex (nonsilencing control, targeting 5′-UUA GGG GAU AAG UAC GGU UGA AUC UAG-3′) was used as a negative control at the same final concentrations as used for the human LRP1-targeting RNA duplexes. Prior to transfection, the cells were transferred to each well in 6-well plates (density: 4×10^5 cells/well). Transient transfection with a 70 nM concentration of siRNA was performed by using siPORT NeoFX™ Transfection Agent (Applied Biosystem, Foster City, CA) according to the manufacturer's protocol. At 24 h after the transfection, the medium was exchanged for fresh medium; and the cells were then cultured for another 48 h.

Expression and purification of full-length recombinant human CCN2 (rhCCN2)

Expression and purification of full-length rhCCN2 protein derived from HeLa cells or

E. coli were carried out, as described previously (Aoyama et al., 2009; Nakanishi et al., 2000).

Association and transcytosis assay of CCN2

The HCS-2/8 cells were washed 3 times on ice with cold phosphate-buffered saline (PBS), and LRPAP1 were added the cells. After 15 minutes, the cells were allowed to association with CCN2 in serum free D-MEM containing 2 μg/ml recombinant CCN2 at 37 °C for 1 h. For inhibitor assays, prior to addition of CCN2, HCS-2/8 cells were preincubated for 5 min at 37°C in medium lacking FBS and with 5 μM chlorpromazine or 3 mM MβCD that specifically inhibited endocytic pathways. Then, the cells were washed 3 times on ice with cold PBS. After that, cell layers (total binding samples) were harvested in 100 μl of lysis buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 5% glycerol, 40 mM ammonium molybdate, and 1 mM phenylmethylsulfonyl fluoride). For the transcytosis assays of CCN2, HCS-2/8 cells were seeded in Transwell chambers with the pore size of 0.4 μM (Millipore, Billerica, MA), which had been inserted into 6-well culture plates (density: 6 × 10^5 cells/well) containing D-MEM supplemented with 10% FBS and incubated at 37°C for 1 week. The cells were washed 3 times on ice with cold PBS and then allowed to associate with CCN2 at 37°C for 1 h in serum-free D-MEM containing 2 μg/ml recombinant CCN2. All cells were subsequently washed 3 times on ice with cold PBS. Cell lysates (total binding samples) on upper chambers were harvested in 100 μl of lysis buffer. The medium in the lower chamber (transcytosis sample) was harvested, and then anti-FLAG® M2 affinity gel freezer-safe (Sigma Aldrich) or Ni-NTA (Ni^{2+}-nitrilotriacetate)−agarose gel (Qiagen, Hilden, Germany) was added to it, which mixture was subsequently incubated for 2 h with gentle rotation to capture the Flag or His-tagged protein in the medium. After the removal of the supernatant, then PBS and 1× SDS sample buffer (50 mM Tris–HCl [pH6.8], 2% SDS, 5% glycerol, 2%

bromphenol blue) with 2-mercaptoethanol was added to the gel to elute the bound proteins.

Immunoblot analysis

HCS-2/8 cells were lysed in the lysis buffer. The lysate diluted in 1× SDS sample buffer with or without 2-mercaptoethanol was boiled for 3 min, and was then subjected to SDS–PAGE in 9% or 12% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes with a blotting apparatus. The membranes were then incubated for 1 h in a blocking buffer (3% dry non-fat milk in PBS) and subsequently incubated overnight with anti-Flag (1:1000), anti-GAPDH (1:100), anti-6-His (1:1000), anti-LRP1 5A6 (1:100), anti-β-Actin (1:5000), anti-CCN2 (1:1000), anti-HIF1α (1:1000), anti-LRPAP1 (1:500) antibody, or Streptavidin HRP conjugate (1:2000) in the blocking buffer. Next, the membrane was washed 5 times in PBS and then incubated for 2 h with HRP-conjugated anti-mouse (1:5000) or anti-rabbit (1:5000) IgG in the blocking buffer. After extensive washes with PBS, immunoreactive proteins were detected by using an ECL Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Immunocytochemistry

HCS-2/8 cells were cultured on glass coverslips, fixed in 4% paraformaldehyde (w/v) in a phosphate buffer (PB) for 15 min and permeabilized with 0.2% Triton X-100 for 15 min. Primary anti-LRP1 H-80 (1:40), anti-Flag (1:200), anti-clathrin (1:300), anti-EEA1 (1:100), and anti-Rab11 C-19 (1:40), antibodies were used for detection. Alexa Fluor-labeled secondary antibodies were also utilized at 1:500.

Confocal laser-scanning microscopy

Confocal laser microscopy was performed using a ZEISS Confocal Laser Scaning