Microscope Model LSM510 (Carl Zeiss, Oberkochen, Germany) belonging to Central Research Laboratory, Okayama University Medical School.

Biotin labeled CCN2 protein derived from HeLa cells

CCN2 protein derived from HeLa cells was biotin-labeled by a commercially available kit, following the manufacturer's instructions (Biotin Labeling Kit-NH_2; Dojindo Molecular Technologies, Inc, Kamimashiki-Gun, Japan).

RNA extraction and cDNA synthesis

Cells were collected, and total RNA was extracted by following the manufacturer's instructions (RNeasy kit, Qiagen). Total RNA (500 ng) was reverse-transcribed by AMV Reverse Transcriptase (Takara, Ohtsu, Japan) at 42°C for 30 min, according to the manufacturer's protocol.

Real-time PCR

Real-time PCR was performed by using TOYOBO SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan) in a LightCycler™ system (Roche, Basel, Switzerland). Reactions were performed in a 10-μl reaction mixture containing 1 μl of cDNA, 0.4 μl of each primer (5 μM), and 5 μl of 1× SYBR Green master mix. Primer sets and optimized conditions for the PCR of each target are listed in Table 1. Absence of non-specific PCR products was checked by melting curve and electrophoresis analyses. Relative copy numbers were computed based on data obtained with a serial dilution of a representative sample for each target gene.

Antisense oligonucleotides

To inhibit the expression of HIF1α, we prepared an antisense phosphorothioate oligonucleotide (AS-HIF) and a sense oligonucleotide (S-HIF: control) according to the

nucleotide sequence of the human HIF1α gene (Caniggia et al., 2000). The nucleotide sequences of the AS-HIF and S-HIF were 5′-GCCGGCGCCCTCCAT-3′ and 5′-ATGGAGGGCGCCGGC-3′, respectively. These oligonucleotides were added directly to medium in HCS-2/8 cells culture at a concentration of 10 μM.

Animals and preparation of tissue

After Balbc/j mice (2 weeks of age) had been anesthetized with sodium pentobarbital (Nembutal, Abbott laboratories, North Chicago, IL; 25 mg/kg), proximal tibiae were harvested and immersed in 4% paraformaldehyde (w/v) in phosphate buffer (PB: 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4; pH 7.4) at 4°C overnight. After having been rinsed in PBS, the tibiae were decalcified in 0.5 M EDTA, pH 7.4, at 4°C and then embedded in paraffin wax. The sections were prepared at a thickness of 7 μm and mounted on silane-coated slides. The Animal Committee of Okayama University approved all of the procedures.

Immunohistochemistry

Tibial sections were dewaxed in xylene and rehydrated through a graded series of ethanol to water, blocked in a blocking buffer (5% dry non-fat milk in Tris-buffered saline), and incubated overnight at 4°C with the primary anti-LRPAP1 antibody (1:100) and subsequently with an HRP-conjugated anti-rabbit IgG (1:1000) for 1 h at room temperature. Color development was performed by using 3, 3′-diaminobenzidine tetrachloride (Dojindo, Tokyo, Japan). The sections were also counterstained with hematoxylin and mounted. Control samples were processed with the omission of the primary antibody.

Statistics

Data were presented as means ± standard deviations, and the statistical significance

of differences in mean values was assessed by performing Student's unpaired *t*-test. Differences among the mean values were considered significant at a *P* value of <0.05.

All experiments were repeated at least twice, and similar results were obtained.

Acknowledgements

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Figure Legends

Fig. 1. Effect of *LRP1* knockdown on CCN2 association with chondrocytic cells. Recombinant CCN2 was designed to possess a Flag tag, which was captured or internalized by LRP1 (left panel). Dual-tagged recombinant CCN2 was added to control or *LRP1* knockdown HCS-2/8 cells, the medium was removed after 1 h, and the cellular protein was collected. Immunoblotting was performed by using anti-Flag or His tag antibody. Positions of molecular weight markers (35, 75 kDa) are shown at the right of the images (right panel). NC, non-silencing siRNA as a negative control; si-1163, LRP1 siRNA (target sequence position 1163); si-13157, LRP1 siRNA (target sequence position 13157).

Fig. 2. Effect of chlorpromazine on the CCN2 association with chondrocytic cells. Dual-tagged CCN2 was added to control or chlorpromazine (A) / MβCD (B) -treated HCS-2/8 cells and the cellular protein was collected after 1 h. Immunoblotting was performed by using anti-Flag or anti-His tag antibody. As a result, the bound/incorporated CCN2 was decreased in the chlorpromazine-treated HCS-2/8 cells (A), while it was not in MβCD-treated ones (B). Positions of molecular weight markers (35, 75 kDa) are shown at the right of the images. (C, D) Internalization of the exogenously added rhCCN2 into HCS-2/8 cells and intracellular co-localization with endogenous LRP1. The Flag-tagged CCN2 was added and analyzed by laser-scanning confocal microscopy after 15 minutes. The distribution of LRP1 in HCS-2/8 cells was visualized with an antibody for LRP1 (H-80 for α-subunit). The intracellular CCN2 uptake and co-localization with LRP1 was evident. (E-L) Intracellular delivery of exogenously added rhCCN2 into certain organelle of HCS-2/8 cells. Staining of clathrin (E, F), EEA1 (G, H; a marker of early endosomes) or Rab11 (I, J; a marker of recycling endosomes) is shown. The squares in panels C, E, G and I indicate the areas enlarged in the panels D, F, H and J, respectively. Incorporated rhCCN2 was partially

targeted to clathrin (C, D) and early endosomes (E, F). Interestingly, exogenously added rhCCN2 and, particularly, the recycling endosome marker were predominantly co-localized in HCS-2/8 cells (I, J). Merge: merged images of rhCCN2 with LRP1 (C, D), clathrin (E, F), EEA1 (G, H), or Rab11 (I, J) staining. Scale bars, 5 μm.

Fig. 3. Effect of LRPAP1 on CCN2 transcytosis in chondrocytes. (A) Schemaic representation of the sampling strategy is shown. E. coli-derived dual-tagged CCN2 (B, C) was added to control or LRPAP1-treated HCS-2/8 cells in the upper chamber of a Transwell, the medium in the upper chamber was removed after 1 h; and the cellular protein (**B**) and the medium in the lower chamber (**C**) were collected as illustrated. Immunoblotting was performed by using anti-Flag or His tag antibody. As a result, the bound/ incorporated (**B**) and transcytosed (**C**) amount of CCN2 was decreased in the LRPAP1-treated HCS-2/8 cells. Comparable results were obtained with HeLa cell-derived biotinylated recombinant CCN2 detected by the Streptavidin conjugate (D, E). Positions of molecular weight markers (35, 75 kDa) are shown at the right of the images. NC, the mixture of anti-FLAG® M2 affinity gel or Ni-NTA−agarose gel and serum-free D-MEM without Flag or His-fusion protein as a negative control.

Fig. 4. Effect of hypoxia on levels of LRP1 mRNA and protein in HCS-2/8 cells. (**A**) The *LRP1* mRNA level in HCS-2/8 cells under hypoxia. The level of mRNA was standardized to that of *18s* mRNA. Exposure to hypoxia resulted in a time-dependent increase in the *LRP1* mRNA level. The values represent the means ± SD. $^*P < 0.05$. (**B**) LRP1, CCN2 and HIF1α protein level in HCS-2/8 cells under hypoxia for 48 h. Immunoblotting was carried out with anti-LRP1, CCN2 and HIF1α antibody. Stronger signals for the LRP1 subunit, CCN2 and HIF1α were detected under hypoxia. (**C**) Effect of antisense HIF1α oligonucleotides on HIF1α, LRP1 and CCN2 production under hypoxic condition for 48 h. Treating the cells in 5% O_2 with antisense

oligonucleotides to HIF1α (AS-HIF) for 48 h abolished LRP1, CCN2 and HIF1α production. S-HIF: control experiments with the sense oligonucleotide. The position of the molecular weight marker used (35 and 75 kDa) is shown at the left of the images.

Fig. 5. Effect of hypoxia on CCN2 transcytosis in chondrocytes. Transcytosis assay was performed as described in the legend of Figure 2. As a result, the bound/ incorporated (**A**) and transcytosed (**B**) amount of CCN2 increased in the HCS-2/8 cells under hypoxia, and the increase was suppressed by LRPAP1. NC, the mixture anti-FLAG® M2 affinity gel or Ni-NTA−agarose gel and serum free D-MEM without Flag or His-fusion protein as a negative control.

Fig. 6. LRPAP1 in HeLa, MDA-231, and HCS-2/8 cells. (**A**) The mRNA level of *LRP1* and *LRPAP1* in HeLa, MDA-231, and HCS-2/8 cells. The level of each mRNA was standardized to that of *GAPDH* mRNA. *LRP1* and *LRPAP1* mRNAs were highly expressed in chondrocytic HCS-2/8 cells. The values represent the means ± SD. $*P < 0.05$. (**B**) The protein level of LRPAP1 in HeLa, MDA-231, and HCS-2/8 cells. Immunoblotting was carried out with anti-LRPAP1 antibody. The strongest signal for the LRPAP1 was detected in the HCS-2/8 cells. Positions of molecular weight markers (35kDa) are shown at the right of the images.

Fig. 7. Difference in expression and distribution of LRPAP1 among chondrocytes of various differentiation stages. (**A**) Immunohistochemical analysis of LRPAP1 in the growth plate. Tibial sections from mice were stained with anti-LRPAP1 antibody. The ECM in the entire growth-plate cartilage was immunopositive for LRPAP1, with the strongest signal in the resting zone. The dark gray circles in the bottom panel represent the cells that express *Ccn2* gene. The hatched circles therein represent the cells that accumulated CCN2 protein. Scale bars, 2 mm. (**B**) Change in the

expression levels of *lrpap1* mRNA and other mRNAs in chicken sternum chondrocytes of various differentiation stages. LC, USP, and USC represent resting, proliferating, and hypertrophic chondrocytes in the growth plate, respectively. The expression of *lrpap1* mRNA was the highest in the LC cells. Thus these results support the data obtained in vivo (**A**). The values represent the means ± SD. $*P < 0.05$.

Fig. 8. Schematic representation of the molecular mechanism determining the polarity of CCN2 distribution. CCN2 protein is transported from prehypertrophic chondrocytes, where it is produced, through transcytosis mediated by LRP1 to the hypertrophic chondrocytes. The high levels of LRPAP1 in the resting zone interfere with this transportation, whereas this interference is presumably attenuated by down-regulation of LRPAP1 in the hypertrophic layer and down-regulation of LRP1 by oxygen in the late hypertrophic layer. As a result of the down-regulation of LRP1 by oxygen in the late hypertrophic layer, CCN2 is accumulated in the hypertrophic layer.

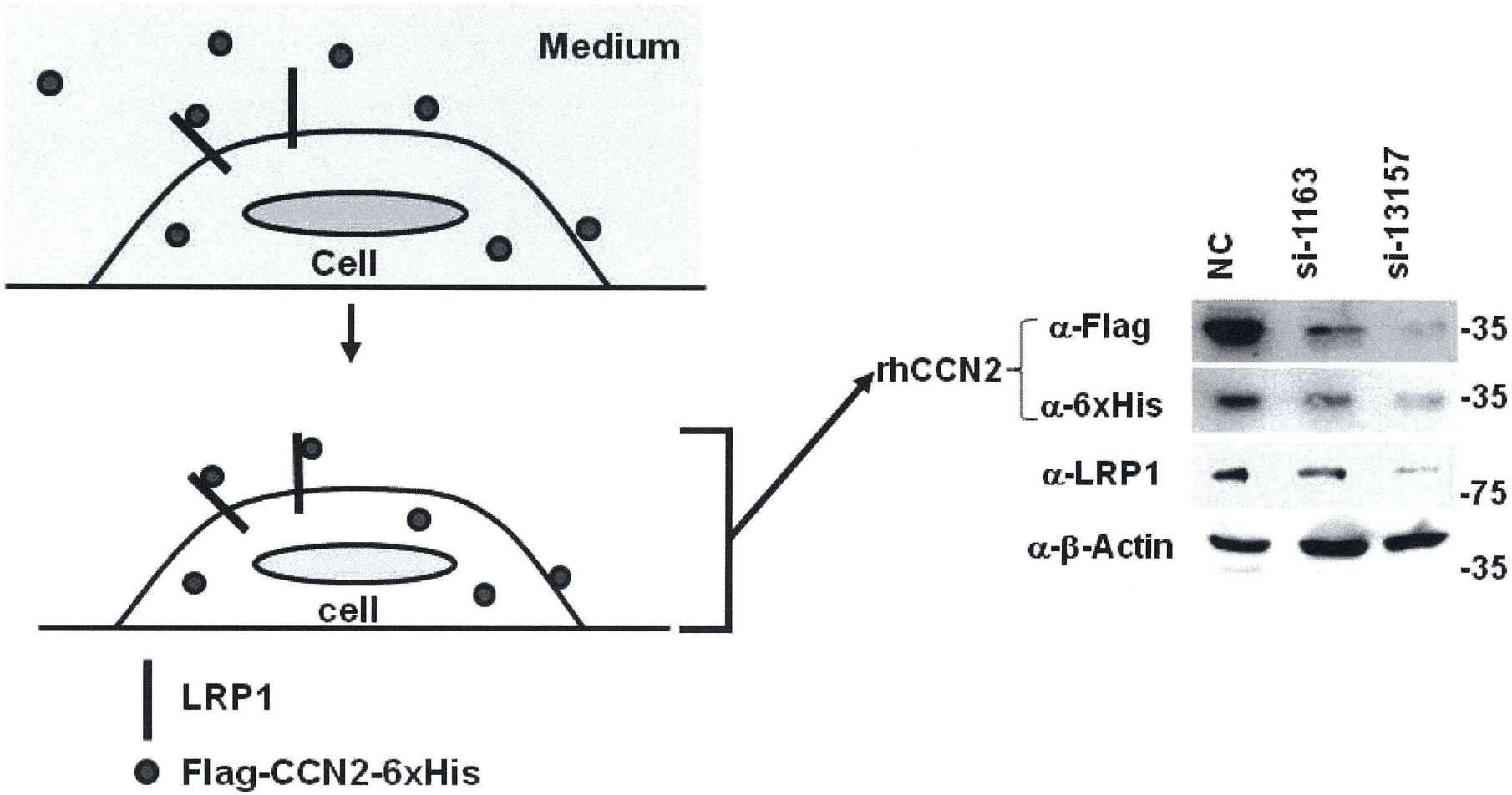


Fig. 1

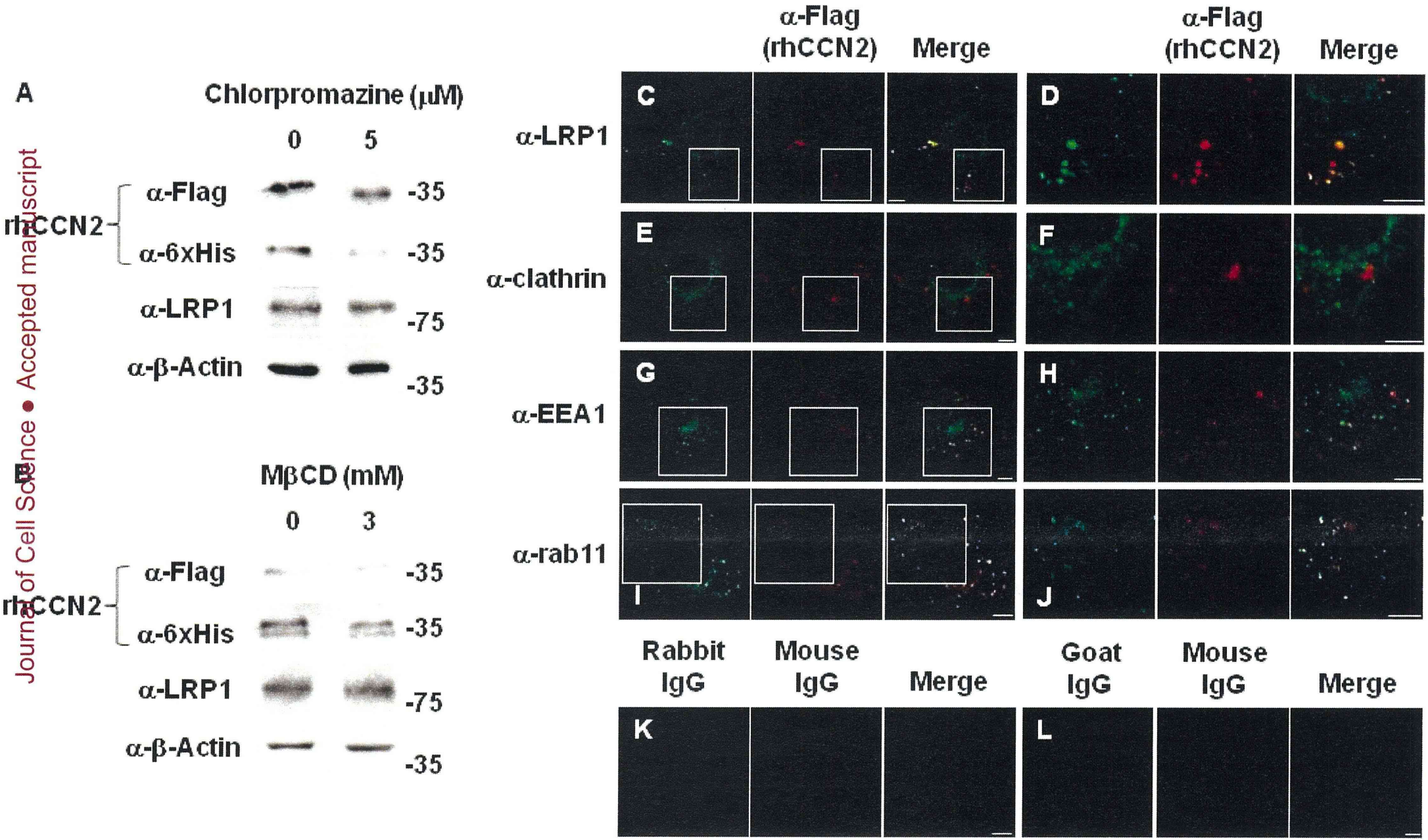
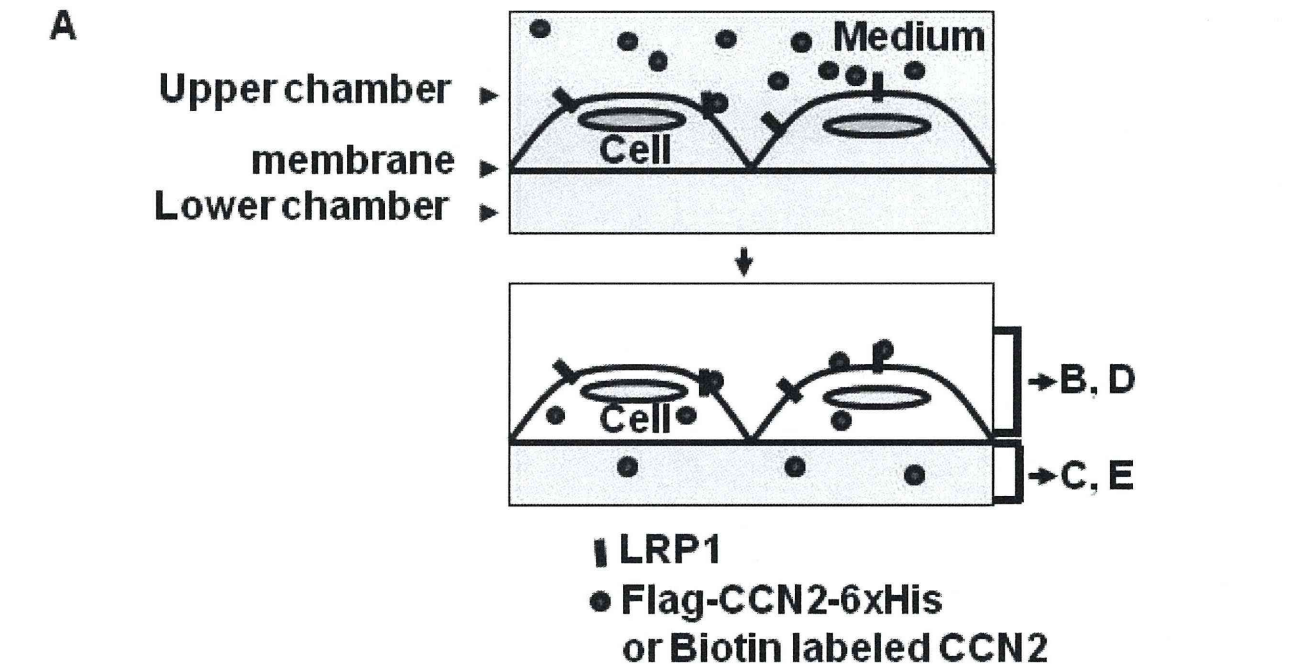
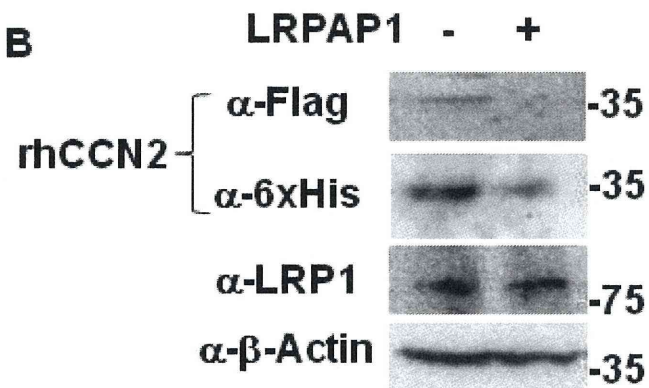
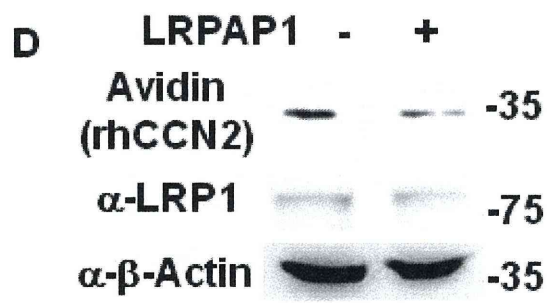
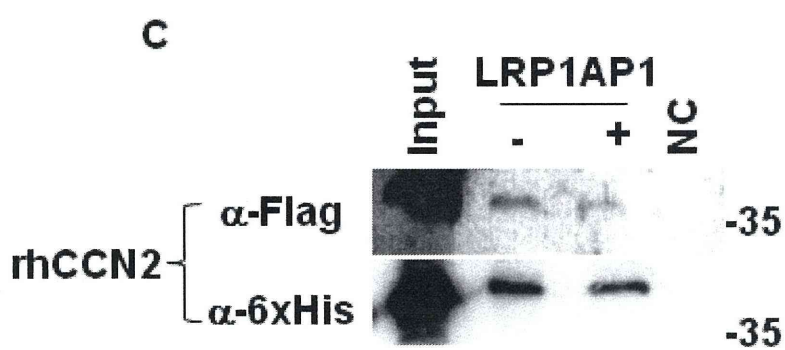


Fig. 2









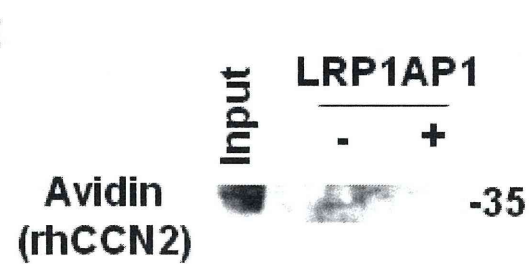


Fig. 3

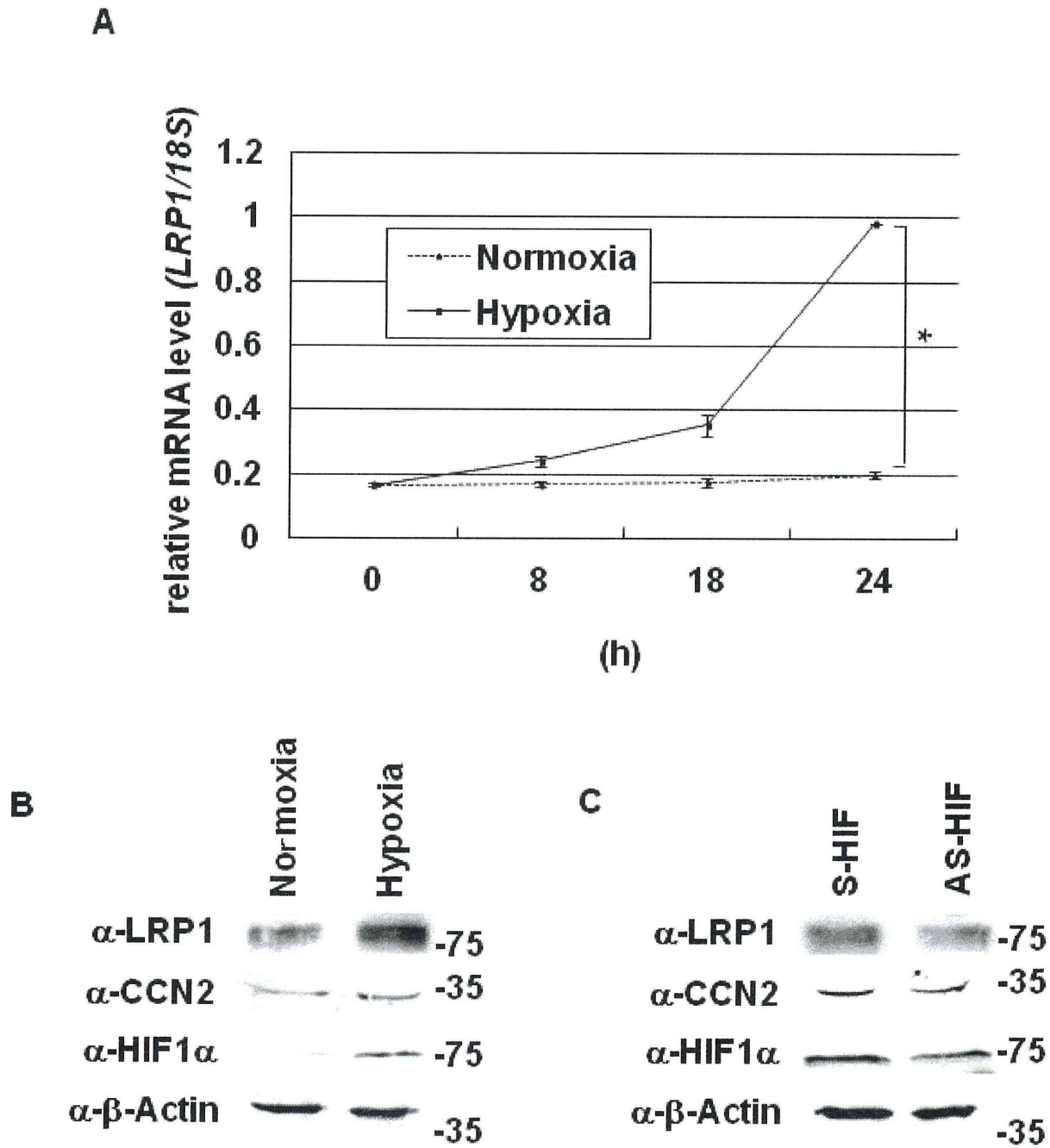


Fig. 4

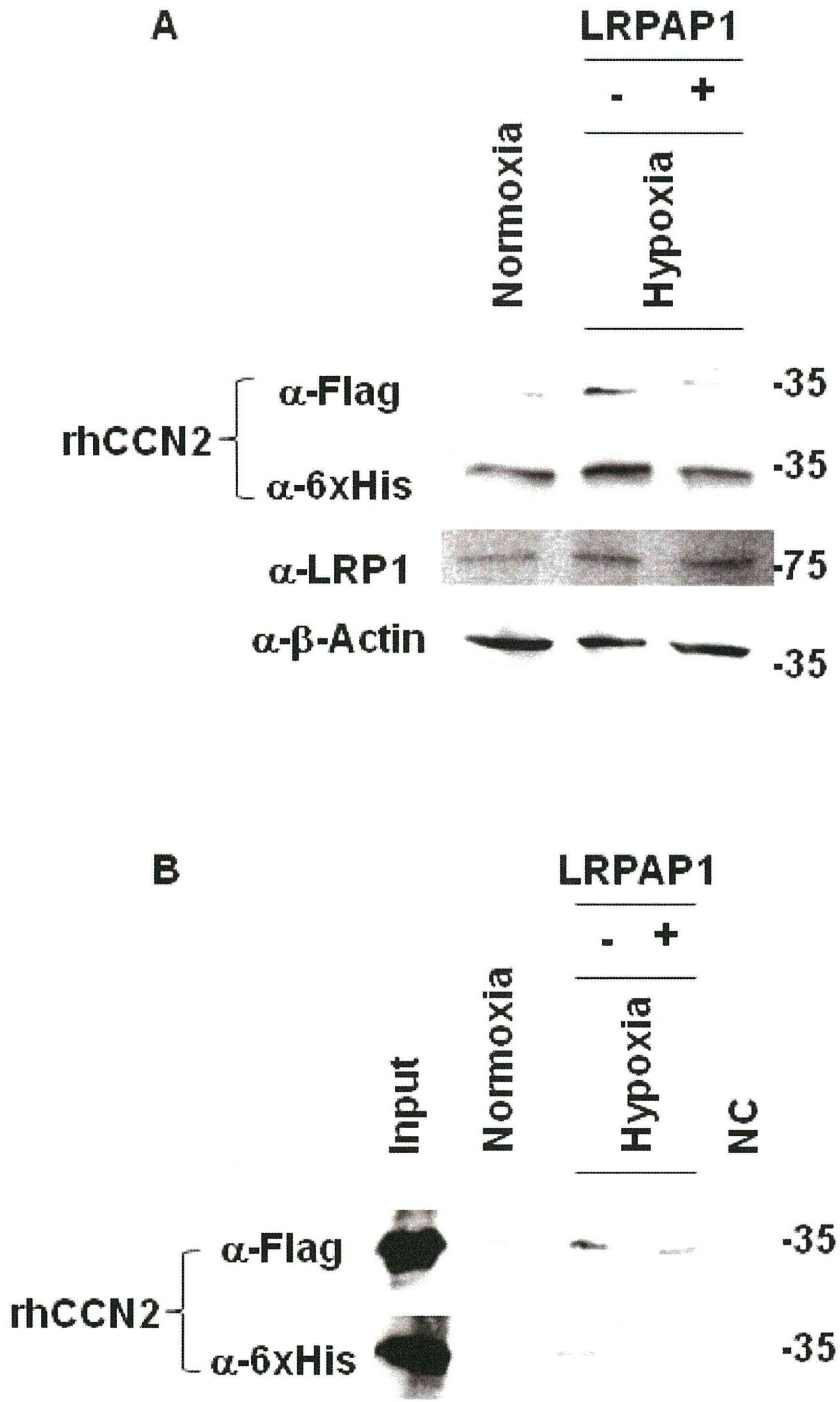
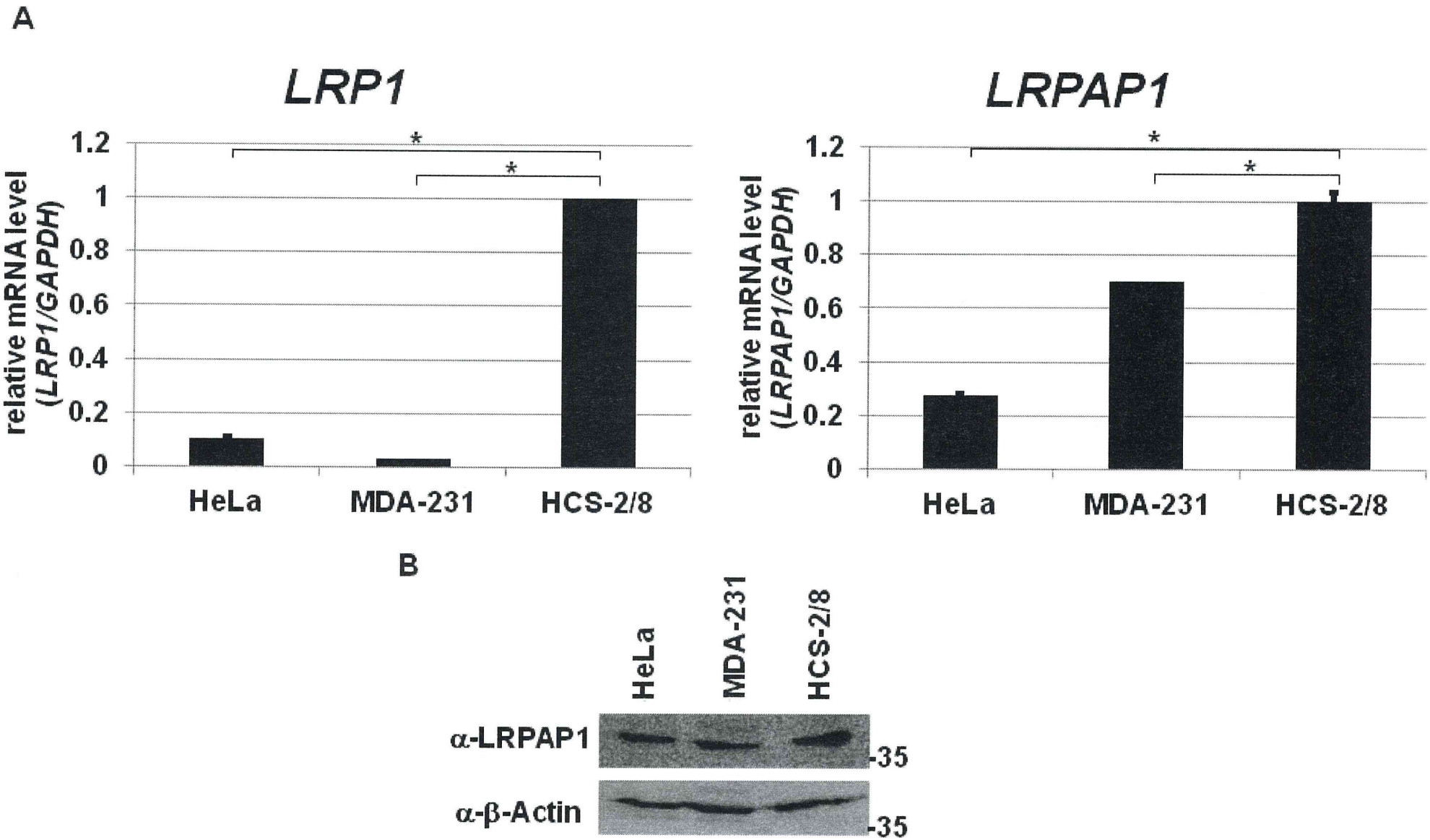


Fig. 5



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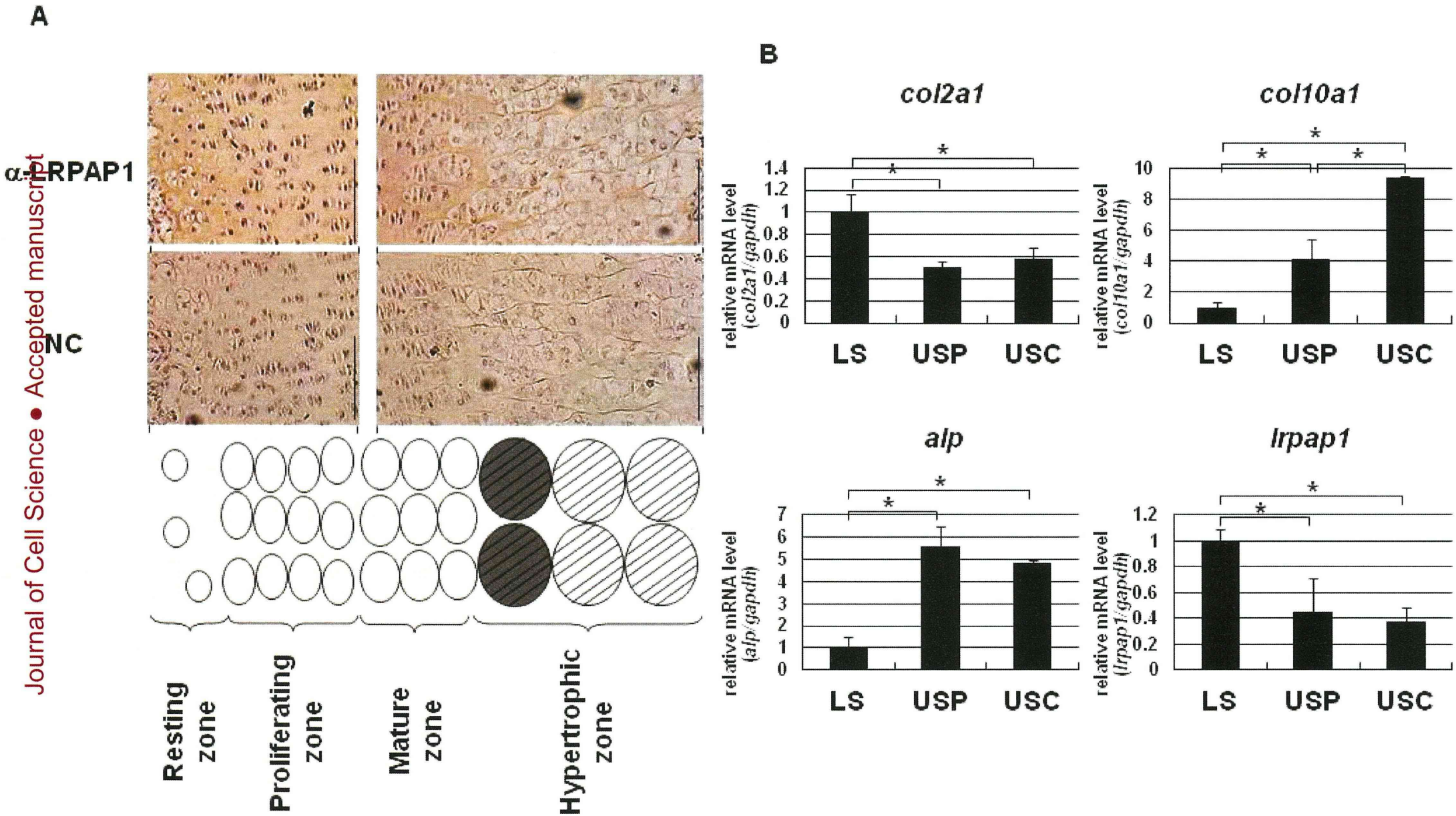

Fig. 7

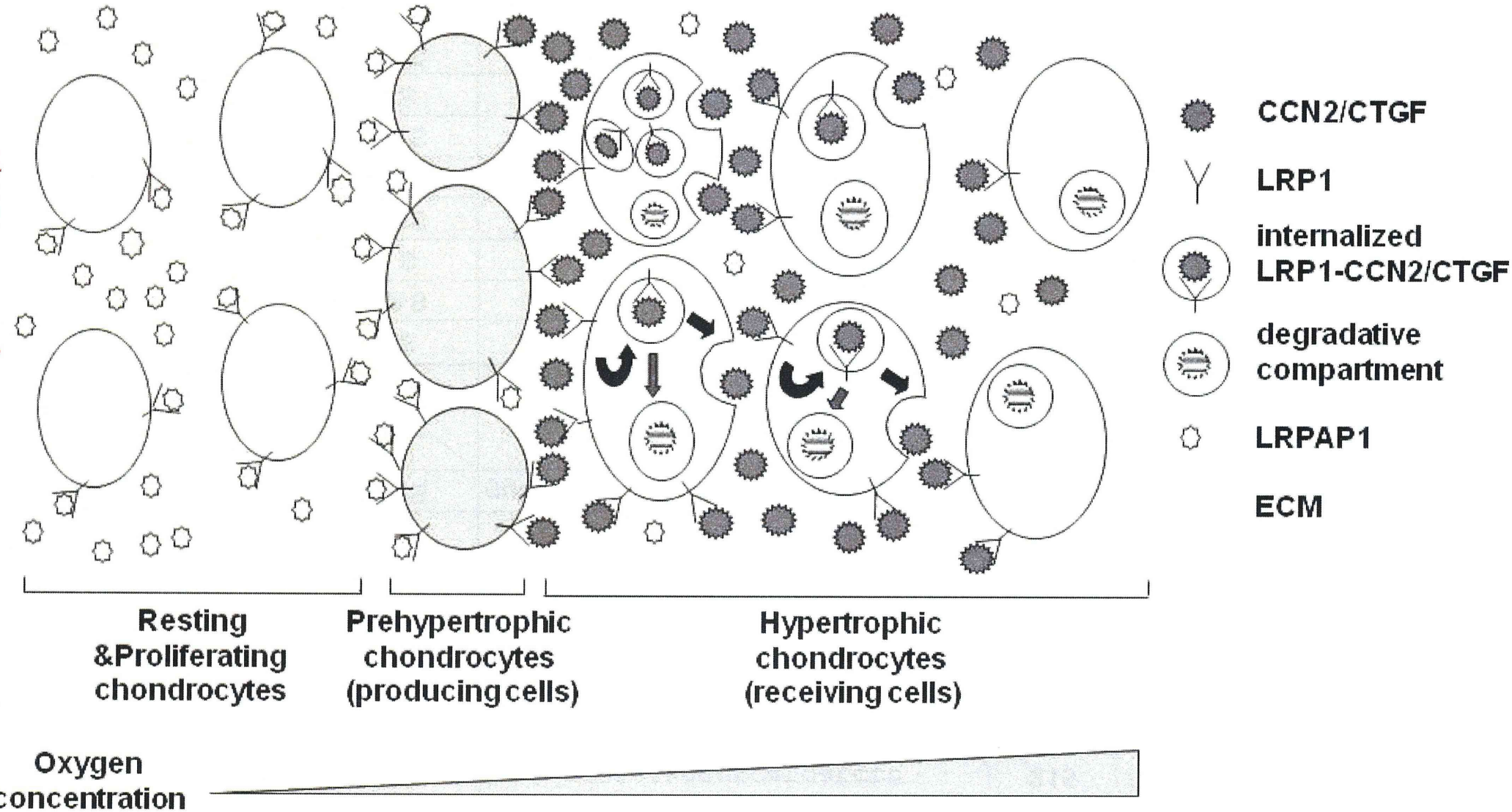

Fig. 8

Table 1.

Primers and experimental conditions for real-time PCR

target gene (human)	Primer direction	Sequence(5'→3')	Length of PCR product	Annealing temperature (℃)
GAPDH	S	gccaaaagggtcatcatctc	215	65
	AS	gtcttctgggtggcagtgat		
LRP1	S	acatatagcctccatcctaatc	152	65
	AS	ttccaatctccacgttcat		
LRPAP1	S	ctgaggctgagttcgaggag	150	65
	AS	gctgcttctggtagtggttg		
18S	S	gcgaattcctgccagtagcatatgcttg	140	60
	AS	ggaagcttagaggagcgagcgaccaaagg		
Target gene (chicken)				
gapdh	S	aggctgtggggaaagtca	202	65
	AS	gacaacctggtcctctgtgtat		
col2a1	S	agaaaggaatccagcccaat	236	65
	AS	acacctgccagattgattcc		
col10a1	S	acatgcatttacaaatatcgttac	160	60
	AS	aaaatagtagacgttaccttgactc		
alp	S	aacggccctggctataagat	186	60
	AS	tgggggatgtagttctgctc		
lrpap1	S	acccggtgaaagaggaagtc	164	65
	AS	tgccatgtcccacaaatc		

S, sense; AS, anti-sense.