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# Clusterin, a novel modulator of TGF-β signaling, is involved in Smad2/3 stability

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**Abstract**

Clusterin (CLU) is known as a multifunctional protein involved in a variety of physiological processes including lipid transport, epithelial cell differentiation, tumorigenesis, and apoptosis. It is known that CLU interacts with TGF-β type ll receptor (TβRll). However, the relationship of CLU and TGF-β signaling is unclear. Here we present that CLU is a novel modulator of TGF-β signaling by regulating Smad2/3 proteins. Overexpression of CLU enhanced TGF-β-induced transcriptional activity and increased the amount of Smad2/3 proteins, while CLU siRNA repressed TGF-β-induced transcriptional activity and decreased the amount of Smad2/3 proteins in Hep3B cells. We also found that CLU was involved in Smad2/3 stability at the protein level. These findings suggest that CLU regulates TGF-β signaling pathway by modulating the stability of Smad2/3 proteins.


*Keywords:* Clusterin; TGF-β; Smad2; Smad3

Clusterin (CLU) is a ubiquitously expressed glycoprotein that has been implicated in a variety of physiological processes, including cell–cell interaction, lipid transport, tissue remodeling, chaperone activity, and apoptosis [1,2]. It was first described as a protein isolated from ram testis fluid with the ability to cluster sertoli cells [3]. Human CLU is the 60 kDa translation product of 449 amino acids, which is cleaved into the α and β subunits [4]. It has been shown that the gene is upregulated in response to a variety of stress- and/or apoptosis-inducing agents. For example, tumor necrosis factor α (TNF-α), transforming growth factor-β (TGF-β), heat shock, UV exposure, and oxidative stresses induce the expression of CLU [5–8]. CLU was known as a secreted protein in mammalian cells. Truncated CLU resulting from alternative mRNA splicing was also reported by several groups [8,9]. Truncated CLU binds to the DNA binding protein ku 70 in cell lysates [10].

TGF-β superfamily includes the TGF-β, bone morphogenic proteins (BMPs), and activins. TGF-β plays multifunctional roles in regulating the cell cycle, apoptosis, differentiation, and extracellular matrix remodeling [11]. In TGF-β signaling, the activated type I receptor phosphorylates Smad2/3 to transduce the signal [12].

The relationship between CLU and TGF-β signaling has been reported: The striking accordances in the expression pattern of CLU and TGF-β have been demonstrated during mouse embryogenesis [13], cardiac valve morphogenesis [14], and various patho-physiological conditions such as sclerosis and Alzheimer's disease [15,16]. In yeast two hybrid analyses and in cell lysates, CLU is capable of binding with TGF-β receptors [17]. In the epithelial cell

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lines (HepG2 and CCL64), TGF-β treatment makes CLU translocate from cytoplasm into the nucleus [9]. To further determine the relationship between CLU and TGF-β signaling, we performed the reporter assay and found that CLU modulated the TGF-β signaling by the Smad2/3 stabilization.

## Experimental procedures

*Plasmids.* Human CLU ORF (open reading frame) constructs were cloned from the human brain cDNA library (Clontech) and were sequenced. The truncated CLU was generated from these constructs and was subcloned into the HA-tagged mammalian expression vector. A Flag-tagged-Smad2 and -Smad3 in pCMV5 and $SBE_4$-Luc reporter constructs were used. pNull (Promega) was used as the internal control for the reportor assay.

*Reagents and antibodies.* Recombinant human TGF-β1 was purchased from R&D systems (Minneapolis, MN) and reconstituted as 1 μg/mL stock solution in 4 mM HCl with 1 mg/mL bovine serum albumin. Z-Leu-Leu-al (MG132) was purchased from Sigma (St. Louis, MO) and was made as 10 mM stock solution in DMSO. Antibodies against Smad2, P-Smad2, P-Smad3 (Cell Signaling), and Smad3 (Zymed) were used.

*Cell culture.* Hep3B and PLC/PRF5 liver cancer cell lines were grown in RPMI 1640 and EMEM, respectively, with 10% fetal bovine serum at 37 °C in a humidified incubator with 5% $CO_2$. 293T and Mv1Lu cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

*Luciferase assays.* Hep3B, PLC/PRF5, and Mv1Lu cells were seeded on 12-well plates at the density of $2 \times 10^5$/well. Transfection was performed 24 h later with the indicated plasmid DNAs or siRNA oligomers along with pRL-null vector (10 ng/well) as a control for transfection efficiency. After 12 h of transfection, 2 ng/mL TGF-β1 was added and the cells were incubated for the additional 12 h. The luciferase and Renilla activity were measured with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Each experiment was repeated at least three times and the data were presented as the mean (±S.E.).

*Transient transfection and immunoblotting.* pCMV/HA-CLU and CLU siRNA (SAMCHOLLY Pharm. Co., Ltd., Korea) were prepared for overexpression and knockdown. Hep3B cells were cultured in 60 mm plates at the density of $1 \times 10^6$ cells for 24 h before transfection. The transfection was performed with Lipofectamine & Plus reagent (Invitrogen). After 24 h, the transfected cells were in the reduced serum condition (0.5% FBS) for 6 h and then treated with TGF-β1 (2 ng/mL). These cells were lysed with the extraction buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 40 mM NaF, and protease inhibitor cocktail (Roche). Western blot analyses were performed with anti-phospho-Smad2, anti-phospho-Smad3, anti-Smad2, and anti-Smad3 antibodies. 293T cells were transfected with pCMV/Flag-Smad2 and pCMV/Flag-Smad3, respectively, on the different concentration of pCMV/HA-CLU. Twenty-four hours later, the intensity of Flag-Samd2 and Flag-Smad3 was confirmed by the Western blot analysis. The transfection efficiency was normalized by the mammalian GST expression vector.

## Results and discussion

### *Effect of CLU on TGF-β1 signaling*

There are two main CLU isoforms: the conventional glycosylated secretory form and the truncated intracellular form. In comparison with the secreted form, the truncated form has no signal peptide targeted to the endoplasmic reticulum (ER) and therefore resides in the cytoplasm [18]. In our overexpression system, the truncated constructs were used. It has been reported that CLU translocates from the cytoplasm to the nucleus by various stimuli including TGF-β [9,18]. CLU also interacts with the intracellular domain of TβRII [17]. To examine the effects of CLU on TGF-β signaling, we carried out luciferase assays with the SBE reporter, containing four tandem repeats of the Smad-binding element [19], in Hep3B, PLC/PRF5, and MvlLu cells. These cell lines are responsive to TGF-β. Overexpression of CLU significantly increased luciferase activity in all three TGF-β-treated cell lines in a dose-dependent manner, though the magnitude of the activity was different. In Hep3B cells, CLU increased TGF-β-induced transcription up to 2.8-fold compared with the pCMV/Mock transfected cells, while CLU increased the transcription by 2.1- and 2.0-fold in PLC/PRF5 and Mv1Lu cells, respectively (Fig. 1A). We next determined the effect of CLU knockdown on TGF-β signaling in Hep3B and PLC/PRF5 cell lines. The addition of CLU siRNA decreased the activity around 50% compared with the treatment of GFP siRNA in Hep3B and PLC/PRF5 cell lines (Fig. 1B). These results indicate that CLU enhances the TGF-β-induced transcriptional activity. In the subsequent experiment, we performed the confocal microscopic analysis to test whether these actions of CLU on TGF-β signaling were related to translocation of CLU into the nucleus. However, most CLU was on the cytoplasm in the TGF-β treated Hep3B cells (data not shown). It suggests that CLU may function on the cytoplasm to activate TGF-β signaling.

### *Activation of Smad2/3 proteins by CLU*

TGF-β signal is transmitted into the nucleus via the Smad proteins (Smad2, Smad3, and Smad4) and especially Smad2/3 phosphorylation plays a key role in this transduction [12]. To examine whether TGF-β-mediated phosphorylation of Smad2/3 is regulated by CLU, we tested the phosphorylated Smad2/3 (P-Smad2/3) in Hep3B cells under the conditions of CLU overexpression and CLU downregulation. In CLU overexpressed Hep3B cells, P-Smad2/3 distinctly increased with a concomitant increase of the amount of their wild types compared with the mock-transfected cells. The levels of phosphorylated Smad2 peaked about 30 min after TGF-β treatment and gradually declined. Smad3 was also phosphorylated by TGF-β with similar kinetics of Smad2 phosphorylation (Fig. 2). Treatment of CLU siRNA decreased wild Smad2/3 and P-Smad2/3 compared with Smad proteins in GFP siRNA-treated cells (Fig. 3). From these data, we reasoned that the increased intensity of P-Smad2/3 was facilitated by the increase of wild Smad2/3 proteins by CLU. However, we cannot completely exclude the possibility of the regulation in the phosphorylation level. Though TGF-β/Smad signaling is regulated by multiple and complex mechanism, there are two major ways to modulate the actions of Smad2/3 proteins. One is the phosphoryla-