Clarification of the mechanisms underlying vascular endothelial proteoglycan synthesis based on novel research strategies

新しい研究戦略に基づく
血管内皮細胞のプロテオグリカン合成機構解明に関する研究

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Abbreviation

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AhR	Aryl hydrocarbon receptor
ALK	Activin receptor-like kinase
Apo	Apolipoprotein
Ask	Apoptosis signal-regulating kinase
B2M	β2-microgloblin
BCA	Bicinchoninic acid
CBB	Coomassie brilliant blue
Cu-DMP	Dichloro(2,9-dimethyl-1,10-phenanthroline)copper(II)
Cu-Phen	Dichloro(1,10-phenanthroline)copper(II)
DEAE	Diethylaminoethyl
DMP	2,9-Dimethyl-1,10-phenanthroline
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
IdoA	*L*-Iduronic acid
GalNAc	*D*-*N*-Acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlcA	*D*-Glucuronic acid
GlcNAc	*D*-*N*-Acetylglucosamine
HIF	Hypoxia inducible factor
HRP	Horseradish peroxidase
ICP-MS	Inductively coupled plasma mass spectrometry

JNK	c-jun N-terminal kinase
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated Protein Kinase
MEK	MAPK/ERK kinase
ODD	Oxygen-dependent degradation domain
o-Phen	1,10-Phenanthroline
PHD	Prolyl hydroxylase domain-containing protein
RT-PCR	Reverse transcription-polymerase chain reaction
Rh-DMP	Potassium tetrachloro(2,9-dimethyl-1,10-phenanthroline)rhodate(II)
Rh-Phen	Potassium tetrachloro(1,10-phenanthroline)rhodate(III)
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TGF	Transforming growth factor
TIE	TGF-β inhibitory element
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	Polyethylene Glycol Mono-*p*-isooctylphenyl Ether
Tween20	Polyoxyethylene sorbitan monolaurate
VEGF	Vascular endothelial growth factor
Xyl	*D*-Xylose
Zn-DMP	Dichloro(2,9-dimethyl-1,10-phenanthroline)zinc
Zn-Phen	Dichloro(1,10-phenanthroline)zinc

Abstract

Proteoglycans (PGs) are macromolecules that exist in the extracellular matrix and on the cell surface. They are classified based on the types of glycosaminoglycans bound to the core protein. PGs modulate the biological activities of growth factors/cytokines by binding them to the core proteins or glycosaminoglycan side chains. Vascular endothelial cells are aligned on the luminal surface of blood vessels in a monolayer. They synthesize two types of PGs, heparan sulfate PGs (HSPGs) and dermatan sulfate PGs (DSPGs), which contribute to the anticoagulant and fibrinolytic properties of vascular endothelial cells. Although the regulation of endothelial PG expression by growth factors/cytokines expressed in the atherosclerotic vascular wall has been studied, it is still incompletely understood. The purpose of this study is to identify new mechanisms and alternative pathways in the regulation of endothelial PG synthesis by using two novel strategies: one is to use the expression of specific PG (cross-talk strategy) and the other is to use organic-inorganic hybrid molecules (bio-organometallics strategy) to obtain new findings about the regulation of endothelial PG synthesis.

First, a decrease in HSPGs together with an increase in DSPGs, especially biglycan, is observed in the atherosclerotic vascular wall. The cross-talk strategy, which is inspired by this histopathological finding, revealed that the physiological role of endothelial biglycan is to serve as a co-receptor for TGF-β_1–ALK5 system and downregulate the expression of syndecan-4, a HSPG. This downregulation is mediated by the Smad2/3 pathway, whereas the Smad3–p38 MAPK pathway mediates the upregulation of syndecan-4 before the downregulation occurs. The cross-talk strategy effectively clarified the molecular mechanisms underlying the histopathological observation of atherosclerotic intima.

Second, bio-organometallics, the biology of organic-inorganic hybrid molecules, was applied to the biology of endothelial PGs. Hybrid molecules consist of ligand(s) and metal(s) and have contributed to the development of organometallic chemistry as synthetic reagents. However, the molecules have not been commonly used in biological studies. The experiments using zinc complexes revealed for the first time that (3) syndecan-4 is upregulated via the hypoxia inducible factor-1α/1β pathway in vascular endothelial cells and (4) the biological activity of hybrid molecules can be modulated by selection of either organic structure or metal.

Thus, the present study identified additional mechanisms underlying vascular endothelial PG synthesis based on two novel research strategies—the cross-talk strategy and the bio-organometallics strategy.

General introduction

Proteoglycans that are macromolecules that consist of a core protein and sulfated glycosaminoglycan side chain(s), which exist in the cell surface and extracellular matrix (Ruoslahti, 1988). Glycosaminoglycans are attached to serine residues in the core protein, forming a tetrasaccharide linker region (4-β-GlcA-(1-3)-β-Gal-(1-3)-β-Gal-(1-4)-β-Xyl-(1-O)-Ser) and following repeats of disaccharide structures without brunches (Baker et al., 1972; Gallagher et al., 1986; Grebner et al., 1966; Robinson et al., 1966). The types of glycosaminoglycan are classified by the component of disaccharides; chondroitin sulfate chains are composed of the disaccharide unit of 4-α-GlcA-(1-3)-β-GalNAc-1 on the other hand, heparan sulfate chains are composed of the disaccharide unit of 4-α-GlcA-(1-4)-α-GlcNAc-1. GlcA in the chondroitin sulfate chains is enzymatically epimerized to IdoA and called dermatan sulfate. Proteoglycans serve as signal mediators by binding to various kinds of biological factors such as growth factors, cytokines, and lipids via the core protein or glycosaminoglycan chains, and to modulate the biological activities of cytokines/growth factors. The expression levels of core proteins and modification of glycosaminoglycans are independently regulated depending on the cell type.

Vascular endothelial cells are aligned on the luminal surface of blood vessels in a monolayer. The cells synthesize two types of proteoglycans—heparan sulfate proteoglycans and dermatan sulfate proteoglycans—. The former includes a large heparan sulfate proteoglycan, perlecan, that exist extracellular matrix (Saku and Furthmayr, 1989), members of the syndecan family of transmembrane proteoglycans (Kojima et al., 1992), and cell surface-associated proteoglycans, the glypican family (Mertens et al., 1992). On the other hand, the latter includes small leucine-rich proteoglycans, biglycan (Yamamoto et al., 2005) and decorin (Schönherr et al., 1999). These proteoglycans not only modulate the function of endothelial cells such as the regulation of the blood coagulation-fibrinolytic system (Merle et al., 1999; Whinna et al., 1993), cell proliferation and migration (Imamura and Mitsui, 1987; Kinsella et al., 1997; Kinsella and Wight, 1986) but also promote the progression of atherosclerosis (Nakashima et al., 2008). The expressions of proteoglycans in vascular endothelial cells have been studied under the treatment with growth factors/cytokines whose expression is observed in atherosclerotic vascular wall.

The signals generated from the growth factors/cytokines are generally transmitted downstream molecules via highly specific receptors and pathways. However, there may be alternative signal pathways that regulate proteoglycan synthesis independent of growth factors/cytokines-driving pathways. New research strategies will be required for finding such pathways. Herein, we provide two novel research strategies as described below.

The first research strategy is based on the crosstalk of the expression among types of proteoglycans. The progression of atherosclerosis includes several stages such as injuring endothelium, diffuse intimal thickening, lipid accumulation in intima, monocyte infiltration, migration of smooth muscle cells, and lipid intake and plaque formation by macrophage and smooth muscle cells. During the progression of these stages, the amount of proteoglycans are varied species to species in atherosclerotic region (Evanko et al., 1998). Exactly, an increase in chondroitin sulfate chains of versican, a large chondroitin sulfate proteoglycan capable of aggregate formation with hyarulonan, synthesized by vascular smooth muscle cells and dermatan sulfate chains of biglycan, a small leucine-rich proteoglycan, together with a decrease in heparan sulfate chains are observed in atherosclerotic intima (Stevens et al., 1976). There may be an interrelationship among the expression of types of proteoglycans. In addition, little is known about the physiological functions of biglycan. We therefore hypothesized that biglycan may regulate the expression of other types of proteoglycans in vascular endothelial cells.

The second research strategy is based on bio-organometallics. Organic-inorganic hybrid molecules that are composed of an organic molecular structure ligand and metal(s), which have been developed as synthetic reagents like Grignard reagent (Grignard, 1900). Although organic-inorganic hybrid molecules have been markedly contributed to development of organic element chemistry today, the application of the molecules to life science has not achieved yet. Under such circumstances, the laboratory to which the author belongs has been reported that several organic-inorganic hybrid molecules exhibit unique biological useful to analyze novel biological function (Fujie et al., 2016a; Fujie et al., 2016b; Fujie et al., 2016c; Fujie et al., 2016d; Kimura et al., 2012; Kohri et al., 2015; Murakami et al., 2015). In the present study, we searched organic-inorganic hybrid molecules that can modulate the expression of proteoglycans and

investigated the alternative signal pathways for proteoglycan synthesis in vascular endothelial cells, using the hybrid molecules.

The purpose of this study is to clarify the mechanisms underlying vascular endothelial proteoglycan synthesis based on above-mentioned novel research strategies.

Chapter 1. Strategy based on crosstalk among proteoglycans

Section 1. Biglycan intensifies ALK5-Smad2/3 signaling by TGF-β_1 and downregulates syndecan-4 in cultured vascular endothelial cells

1. 1. 1. Abstract

Proteoglycans are macromolecules that consist of a core protein and one or more glycosaminoglycan side chains. A small leucine-rich dermatan sulfate proteoglycan, biglycan, is one of the predominant types of proteoglycans synthesized by vascular endothelial cells; however, the physiological functions of biglycan are not completely understood. In the present study, bovine aortic endothelial cells in culture were transfected with small interfering RNAs for biglycan, and the expression of other proteoglycans was examined. Transforming growth factor-β_1 signaling was also investigated, because the interaction of biglycan with cytokines has been reported. Biglycan was found to form a complex with either transforming growth factor-β_1 or the transforming growth factor-β type I receptor, ALK5, and to intensify the phosphorylation of Smad2/3, resulting in a lower expression of the transmembrane heparan sulfate proteoglycan, syndecan-4. This is the first report to clarify the function of biglycan as a regulatory molecule of the ALK5–Smad2/3 TGF-β_1 signaling pathway that mediates the suppression of syndecan-4 expression in vascular endothelial cells.

1. 1. 2. Introduction

Vascular endothelial cells cover the inner surface of blood vessels and are involved in the regulation of the blood coagulation-fibrinolytic system by synthesizing and secreting not only procoagulants, including tissue factor (Maynard et al., 1977) and plasminogen activator inhibitor type 1 (van Mourik et al., 1984), but also anticoagulants, including prostacyclin (Weksler et al., 1977), thrombomodulin (Esmon and Owen, 1981), and tissue plasminogen activator (Levin and Loskutoff, 1982). The cells also synthesize and secrete anticoagulant proteoglycans, macromolecules that consist of a core protein and one or more glycosaminoglycan side chains (Ruoslahti, 1988). Vascular endothelial cells express two types of proteoglycans. One type is the heparan sulfate proteoglycans, including a large heparan sulfate proteoglycan, perlecan (Saku and Furthmayr, 1989); members of the syndecan family of transmembrane proteoglycans, such as syndecan-1 and syndecan-4 (Kojima et al., 1992); and the cell-associated proteoglycan, glypican (Mertens et al., 1992). The other type is the dermatan sulfate proteoglycans, such as small leucine-rich dermatan sulfate proteoglycans, biglycan (Yamamoto et al., 2005) and decorin (Schönherr et al., 1999).

Vascular endothelial proteoglycans have various physiological functions, such as permeability, lipid metabolism, hemostasis, thrombosis, and extracellular assembly (Berenson et al., 1984; Camejo, 1981). Proteoglycans are also involved in regulating the activity of growth factors and cytokines, such as fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGF-β), to which some proteoglycans bind. When a monolayer of vascular endothelial cells is injured, FGF-2 leaks from the damaged cells and stimulates the migration and proliferation of cells near the damaged site to repair the monolayer (Rifkin and Moscatelli, 1989). During repairment of the damaged endothelial monolayer, perlecan promotes the binding of FGF-2 to its receptor (Aviezer et al., 1994). In addition, the heparan sulfate chains of endothelial heparan sulfate proteoglycans exhibit heparin-like activity and contribute to the anticoagulant properties of vascular endothelial cell monolayers (Mertens et al., 1992). On the other hand, biglycan and decorin activate heparin cofactor II via the dermatan sulfate chains to inhibit a coagulation factor, thrombin (Whinna et al., 1993), and decorin is bound to TGF-β via the core proteins to inactivate this cytokine in vitro (Hildebrand et al., 1994). However, the

physiological functions of dermatan sulfate proteoglycans, especially biglycan, are not fully understood.

TGF-β is a multifunctional cytokine involved in various vascular events and diseases (Ruiz-Ortega et al., 2007). The activity of TGF-β_1 is mediated by type I transmembrane serine/threonine kinase receptors that are activated by type II receptors bound to TGF-β_1 (Wrana et al., 1994). There are two kinds of type I TGF-β receptors: one is activin receptor-like kinase 5 (ALK5), which is expressed in most cell types, and the other is activin receptor-like kinase 1 (ALK1), which is specifically expressed in vascular endothelial cells (Goumans et al., 2002). In vascular endothelial cells, ALK5 and ALK1 transduce different signals from TGF-β_1. Cell migration and proliferation are inhibited by the pathway mediated by transcriptional factors Smad2 and Smad3, which are phosphorylated by ALK5, whereas they are stimulated by the pathway mediated by Smad1, Smad5, and Smad8, which are phosphorylated by ALK1 (Derynck et al., 1998; Goumans et al., 2003).

The synthesis of endothelial proteoglycans is regulated by growth factors and cytokines, such as VEGF165 (Kaji et al., 2006) and connective tissue growth factor (Kaji et al., 2004). TGF-β_1 also regulates endothelial perlecan and biglycan synthesis in a cell density-dependent manner (Kaji et al., 2000). We hypothesized that biglycan may be involved in regulating the expression of other types of proteoglycans by TGF-β_1 in vascular endothelial cells. These data indicated that biglycan intensifies ALK5-Smad2/3 signaling with TGF-β_1 as a co-receptor and then downregulates the expression of syndecan-4 in cultured vascular endothelial cells.

1. 1. 3. Results

Biglycan suppresses syndecan-4 expression

To examine the effects of biglycan knockdown on the expression of messenger RNAs (mRNAs) coding for other types of proteoglycans, either a negative control small interfering RNA (siRNA) (siCont) or bovine biglycan siRNA-1 (siBGN-1) was transfected into vascular endothelial cells, and then the mRNA expression levels were analyzed (Fig. 1-1-1). Suppression of biglycan mRNA expression resulted in the induction of mRNAs for decorin (1.50-fold), syndecan-1 (1.65-fold), and syndecan-4 (1.90-fold). An siRNA for bovine biglycan, siBGN-2, also induced the expression decorin and syndecan-4 mRNAs, but failed to induce syndecan-1 mRNA expression (data not shown). We have investigated the relationship between the expression of biglycan and that of syndecan-4 in vascular smooth muscle cells and found that siRNA-mediated knockdown of biglycan expression results in a higher expression of syndecan-4 mRNA (Sup. Fig. 1-1-1). This suggests that biglycan suppression of syndecan-4 expression occurs in not only vascular endothelial cells but also vascular smooth muscle cells. Since the expression level of syndecan-4 in vascular smooth muscle cells was much lower than that in vascular endothelial cells, we could not show the expression of syndecan-4 core protein.

When the cells were transfected with siBGN-1, the level of biglycan mRNA consistently decreased during a 24 h incubation (Fig. 1-1-2A, upper panel), while the syndecan-4 mRNA level increased during a 6 h incubation (Fig. 1-1-2A, lower panel). Expression of syndecan-4 increased with suppression of biglycan expression during a 24 h incubation (Fig. 1-1-2B). These results suggest that biglycan suppresses the expression of syndecan-4 in vascular endothelial cells.

TGF-β_1 suppresses the expression of syndecan-4

We next examined the effects of exogenous TGF-β_1 on the expression of syndecan-4 in vascular endothelial cells (Fig. 1-1-3), because an interaction between biglycan and TGF-β_1 has been reported (Hildebrand et al., 1994). TGF-β_1 elevated the expression levels of syndecan-4 mRNA after a 3 h treatment at 1 and 5 ng/mL or after a 6 h treatment at 5 ng/mL (Fig. 1-1-3A). The expression of syndecan-4 increased after a 6 h incubation (Fig. 1-1-3B) and then decreased

after a 24 h incubation with TGF-β_1 at 5 ng/mL (Fig. 1-1-3C). A nonspecific band observed at the top of the images in Fig. 1-1-3B did not increase and that in Fig. 1-1-3C did not decrease by TGF-β_1, suggesting that the cytokine specifically modulates the expression of syndecan-4.

To determine the effects of endogenous TGF-β_1 on endothelial syndecan-4 expression, we assessed the expression of syndecan-4 in vascular endothelial cells in which the expression of TGF-β_1 was suppressed by bovine TGF-β_1 siRNA (siTGFB1). As shown in Fig. 1-1-4, both siBGN-1 and siBGN-2 suppressed the expression of biglycan mRNA in the presence or absence of siTGFB1. Similarly, siTGFB1 suppressed the expression of TGF-β_1 in the presence or absence of siBGN-1/siBGN-2 (Fig. 1-1-4A). The expression of syndecan-4 mRNA was elevated by siBGN-1, siBGN-2, and siTGFB1; such an elevation was also observed at the protein level (Fig. 1-1-4B), suggesting that endogenous biglycan and TGF-β_1 each suppress syndecan-4 expression. The effect of siBGN-1/siBGN-2 and siTGFB1 on sydecan-4 expression was additive, indicating that not only endogenous biglycan but also endogenous TGF-β_1 suppresses syndecan-4 expression in vascular endothelial cells.

TGF-β_1 receptor type-I, ALK5, inhibits the expression of syndecan-4

To identify the TGF-β receptor involved in the suppression of syndecan-4, siALK1 and siALK5 were transfected into vascular endothelial cells and the expression of syndecan-4 was evaluated (Fig. 1-1-5). The expression of ALK1 and ALK5 was suppressed by siALK1 and siALK5, respectively (Figs. 1-1-5A and 1-1-5B, left and middle panels), but was not influenced by siALK5 and siALK1 (data not shown). Under such conditions, the expression of syndecan-4 mRNA was elevated by either siALK1 or siALK5 (Fig. 1-1-5A, right panel); however, the expression of syndecan-4 core protein was increased by siALK5 but not by siALK1 (Fig. 1-1-5B), suggesting that ALK5 is involved predominantly in negative regulation of syndecan-4 expression by endogenous TGF-β_1. In fact, a selective inhibitor of ALK5, LY364947, increased the expression of syndecan-4 at both the mRNA and protein levels (Figs. 1-1-5C and 1-1-5D).

Biglycan core protein is bound to either TGF-β_1 or ALK5

Since the data suggested that TGF-β_1–ALK5 signaling negatively regulates the expression

of syndecan-4 in vascular endothelial cells, we analyzed the interaction of biglycan with either TGF-β_1 or ALK5. As shown in Fig. 1-1-6A, biglycan core protein appeared after digestion with chondroitinase ABC, suggesting that the cells synthesized biglycan as a proteoglycan molecule that bears chondroitin/dermatan sulfate chains. The band of TGF-β_1 that had been pulled down by the His-tagged biglycan core protein was detected when recombinant human TGF-β_1 reacted with biglycan core protein after digestion with chondroitinase ABC, suggesting that TGF-β_1 is bound to biglycan core protein, as previously reported by Hildebrand et al. (1994). Fig. 1-1-6B shows the interaction of biglycan core protein with ALK5. The ALK5 band was identified by western blot analysis as an immunoreactive band that was decreased by siRNA-mediated knockdown. ALK5 was increased only when the receptor reacted with the His-tagged biglycan core protein obtained from the conditioned medium of the cells and prepared by digestion with chondroitinase ABC. This suggests that ALK5 is also bound to biglycan core protein. Taken together, these results suggest that the biglycan core protein potentiates TGF-β_1–ALK5 signaling by binding to both TGF-β_1 and ALK5.

Biglycan enhances phosphorylation of Smad2/3

TGF-β_1 is bound to ALK5 and phosphorylates the Smad2/3 C-terminus, which transduces the TGF-β_1 signal to the nucleus. The effect of siBGN-1/2 on the phosphorylation of Smad2/3 was examined to clarify the significance of the formation of a complex of biglycan with TGF-β_1 and ALK5. Although Smad2/3 phosphorylation was observed when the cells were treated with TGF-β_1, phosphorylation was not detected in either siCont- or siBGN-1/2-transfected cells in the absence of TGF-β_1. When the cells were stimulated with TGF-β_1 at 1 ng/mL for 30 min, Smad2/3 phosphorylation was detected. This phosphorylation decreased with siBGN-1/2 (Fig. 1-1-7), indicating that biglycan potentiates TGF-β_1–ALK5 signal transduction by activating Smad2/3 phosphorylation.

1. 1. 4. Discussion

The physiological functions of biglycan in vascular endothelial cells are not fully understood. The present data, however, indicates that (1) the expression of endothelial biglycan induces lower levels of syndecan-4 expression, (2) both exogenous and endogenous TGF-β_1 downregulate syndecan-4 expression, (3) syndecan-4 downregulation is mediated by TGF-β_1-ALK5 signaling, (4) biglycan is bound to both TGF-β_1 and ALK5, and (5) biglycan potentiates the phosphorylation of Smad2/3, which is induced by TGF-β_1. Taken together, these results indicate that biglycan acts as a co-receptor in the TGF-β_1–ALK5–Smad2/3 system and is involved in the downregulation of syndecan-4 expression in vascular endothelial cells. Biglycan has been reported to bind not only TGF-β_1 (Hildebrand et al., 1994) but also other cytokines such as tumor necrosis factor-α (Tufvesson and Westergren-Thorsson, 2002) and receptors (Schaefer et al., 2005). The present study, for the first time, revealed that biglycan serves as a co-receptor in TGF-β signaling and this signaling downregulates the expression of syndecan-4 in vascular endothelial cells.

Biglycan and TGF-β_1 were shown to suppress the expression of syndecan-4 via the TGF-β_1-ALK5 signaling pathway. Activation of the TGF-β_1-ALK5 pathway induces phosphorylation of not only Smad2/3, part of the canonical Smad pathway, but also ERK1/2, JNK1/2/3, and p38 MAPK in a non-Smad pathway. Although the involvement of the non-Smad pathway in the downregulation of endothelial syndecan-4 expression cannot be excluded, the present results indicate that the Smad pathway is certainly involved in this downregulation. There are previous examples of phosphorylated Smads inhibiting gene expression through the formation of complexes with transcription factors E2F4 and DP1 to stabilize the TGF-β inhibitory element (TIE) (Chen et al., 2002; Suzuki et al., 2004), which includes a TTGG sequence (Chen et al., 2001; Kerr et al., 1990). There are consensus TTGG sequences about −1.5 kb from the promoter regions of the syndecan-4 gene in humans, mice, and bovines, according to NCBI data. These TTGG sequences may serve as TIE elements, and some of them are involved in the downregulation of endothelial syndecan-4 expression.

The syndecan family is a group of transmembrane-type heparan sulfate proteoglycans. In this family, syndecan-4 is a type of syndecan that is essential for focal adhesion; the core protein

of syndecan-4 forms focal adhesions and stress fibers in fibronectin substrates (Woods and Couchman, 2001). In fact, degradation of cell surface heparan sulfate chains with heparinase weakens cell-fibronectin adhesion and reduces focal adhesions in vascular endothelial cells (Moon et al., 2005). Heparan sulfate chains of syndecan-4 promote the construction of larger focal adhesions (Gopal et al., 2010). Syndecan-4 is required for the alignment of vascular endothelial cells along the luminal surface of normal blood vessels, and a syndecan-4 deficiency results in the activation of atherosclerotic plaques in ldlr-/- and ApoB100/100 mice fed high-fat diets (Baeyens et al., 2014). In addition, an increase in dermatan sulfate chains together with a decrease in heparan sulfate chains is observed in atherosclerotic intima (Stevens et al., 1976). Excess biglycan activates TGF-β_1-ALK5 signaling and reduces syndecan-4, leading to a disturbance in the normal structure of the extracellular matrix in a vascular endothelial monolayer, which may weaken the barrier function of these cells. Therefore, it is suggested that increased biglycan downregulates endothelial syndecan-4 expression, and consequently affects the adhesion of vascular endothelial cells to the extracellular matrix in atherosclerotic vascular tissue, promoting lesions. Since TGF-β_1 induces the synthesis of biglycan with elongated dermatan sulfate chains in vascular endothelial cells (Kaji et al., 2000), and since TGF-β_1-induced biglycan synthesis in aortic cells is involved in the progression of atherosclerosis (Tang et al., 2013) via lipid accumulation in the vascular wall (Hayashi et al., 2012), an assumption can be made that TGF-β_1 and biglycan participate in a positive feedback loop to enhance endothelial biglycan expression and contribute to the accumulation of lipids in the atherosclerotic vascular wall.

Neovascularization and atherosclerosis progression are interrelated. Plaque neovascularization mainly occurs in the ruptured site of atherosclerotic lesions (Moreno et al., 2004), and the inhibition of neovascularization reduces the progression of advanced atherosclerosis (Moulton et al., 2003). In addition, cultured human umbilical vein endothelial cells highly express ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs 4), an extracellular matrix metalloproteinase that digests biglycan during tube formation in the collagen gel (Melching et al., 2006). At that time, the expression of biglycan is reduced (Obika et al., 2014), suggesting that ADAMTS-4 and its substrate biglycan are involved in

angiogenesis by vascular endothelial cells. On the other hand, it has been reported that TGF-β can suppress the expression of ADAMTS-4 (Ashlin et al., 2013; Salter et al., 2011; Wang et al., 2013). Furthermore, the present data showed that decreased biglycan expression inhibits the TGF-β signaling pathway, which can suppress ADAMTS-4 expression. Therefore, it is postulated that the TGF-β signaling pathway, which was reduced by lower expression of biglycan, mediates higher expression of ADAMTS-4 in vascular endothelial cells during angiogenesis. This positive feedback loop by biglycan, the TGF-β signaling, and ADAMTS-4 may be involved in angiogenesis by vascular endothelial cells in atherosclerotic lesions. The involvement of syndecan-4 in angiogenesis, which is increased by the inhibition of the TGF-β signaling pathway, remains to be elucidated.

Cellular proliferation is promoted by decreasing syndecan-4 in vascular smooth muscle cells in atherosclerosis-susceptible pigeons (Bortoff and Wagner, 2005). On the other hand, the effect of biglycan on proliferation depends on the cell type. For example, biglycan promotes the proliferation of vascular smooth muscle cells but not of vascular endothelial cells (Shimizu-Hirota et al., 2004). Biglycan activates TGF-β_1-ALK5 signaling as a co-receptor as shown in this study. Since ALK5 is ubiquitously expressed in most cell types, including vascular endothelial cells, we postulate that biglycan potentiates TGF-β_1 signaling in vascular endothelial cells as well as other cell types, although the regulation that results may depend on the cell type. Interrelated regulatory mechanisms in the synthesis of different types of proteoglycans has been suggested (Tang et al., 2014), however, little was known about these mechanisms. The present report is the first to reveal the molecular mechanism by which the expression of one proteoglycan type influences that of another proteoglycan type. The present study provides a partial molecular explanation for the histopathological studies on atherosclerosis that show the abundance of proteoglycans varies depending on the progression of atherosclerosis. In addition, the present data suggest the significance of an excess accumulation of biglycan in atherosclerotic vascular walls. Specifically, biglycan contributes not only to the change in proteoglycan types expressed during the progression of atherosclerosis, but also to the accumulation of low density lipoprotein that increases with biglycan (Evanko et al., 1998; O'Brien et al., 1998). There may be crosstalk among different types of proteoglycans

in vascular endothelial and smooth muscle cells. Clarification of this system is essential to understanding the complex histopathological changes associated with variation in the proteoglycan types in atherosclerotic vascular walls.

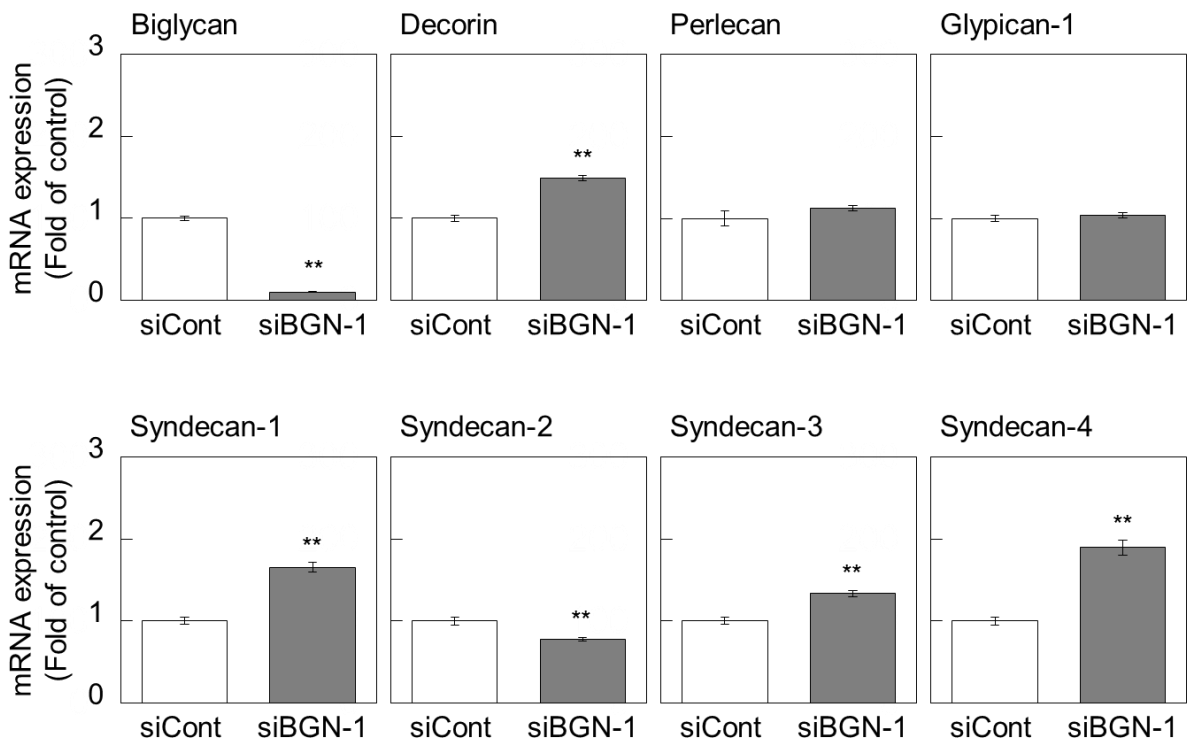


Fig. 1-1-1. Effects of siRNA-mediated knockdown of biglycan on the expression of proteoglycan mRNAs in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siCont or siBGN-1 for 24 h. Values are the mean ± S.E. of three samples. Significantly different from the corresponding siCont, $^{**}P < 0.01$.

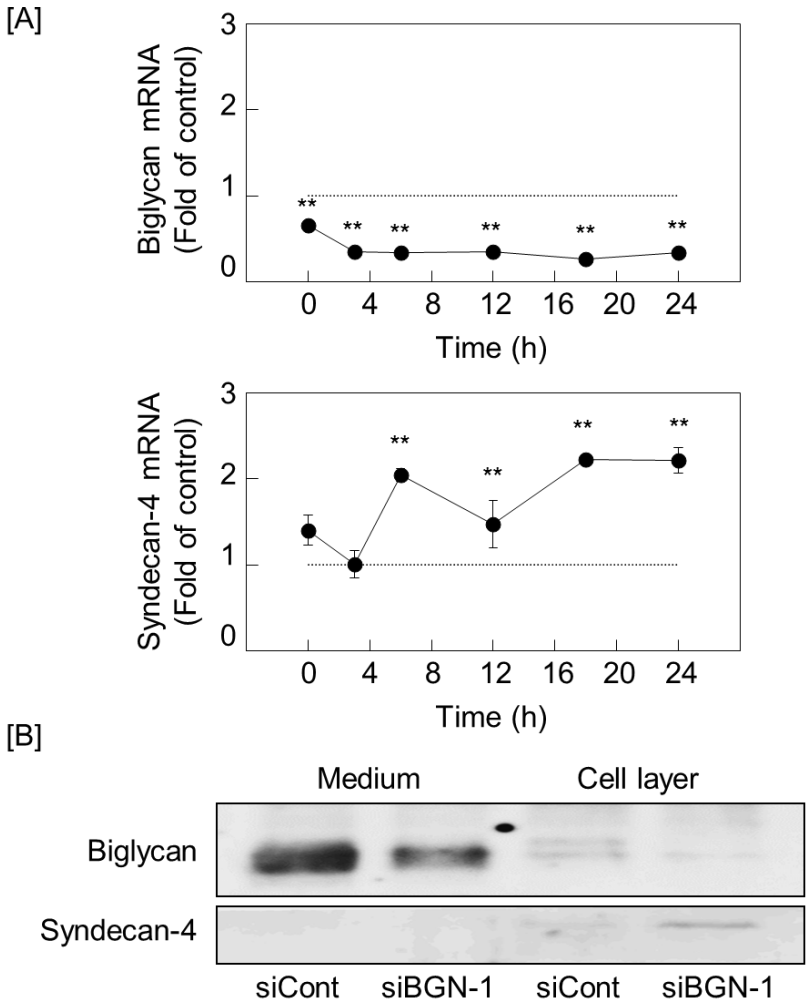


Fig. 1-1-2. Effects of siRNA-mediated knockdown of biglycan on the expression of syndecan-4 in vascular endothelial cells. [A] Expression of biglycan (upper panel) and syndecan-4 (lower panel) mRNAs. Bovine aortic endothelial cells were transfected with siCont or siBGN-1 at 37°C for 3, 6, 12, 18, and 24 h. The time in Fig 2A indicates the incubation time after transfection. Values are the mean ± S.E. of three samples. Significantly different from the corresponding siCont, $^{**}P < 0.01$. [B] Accumulation of biglycan and syndecan-4 core proteins in the conditioned medium and the cell layer transfected with siCont or siBGN-1 for 24 h.

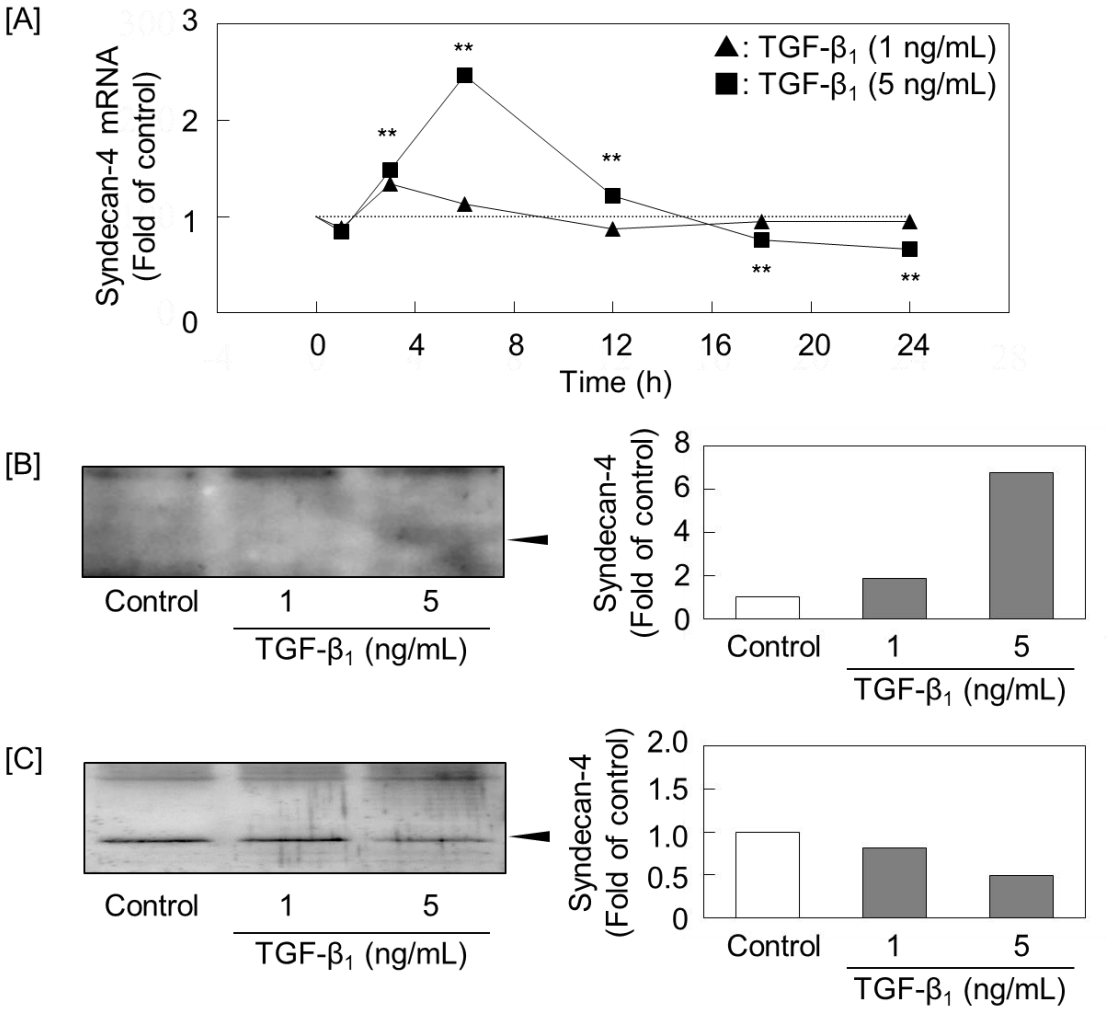


Fig. 1-1-3. Effects of TGF-β1 on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were treated with TGF-β1 at 1 (▲) and 5 (■) ng/mL at 37°C for 1, 3, 6, 12, 18, or 24 h. [A] Expression of syndecan-4 mRNA. Values are the mean ± S.E. of three samples. Significantly different from the corresponding siCont, $^{**}P < 0.01$. Accumulation of syndecan-4 core protein in the cell layer treated with TGF-β1 at 1 or 5 ng/mL for [B] 6 or [C] 24 h. Western blot analysis (left panel) and its quantitative analysis (right panel). Arrow head in the left panel indicates the position of sydecan-4.

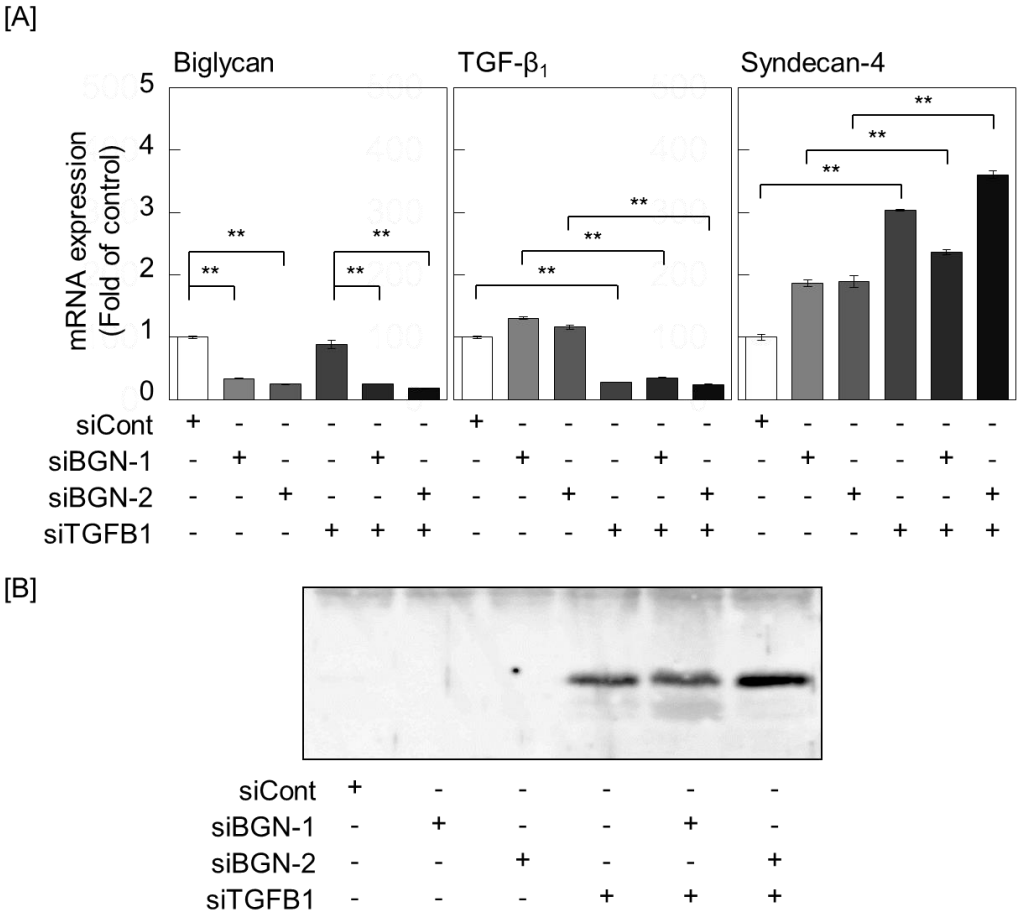


Fig. 1-1-4. Effects of siRNA-mediated knockdown of biglycan or TGF-β$_1$ or both on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siCont, siBGN-1, or siBGN-2 combined with or without siTGFB1 at 37°C for 24 h. [A] Expression of biglycan (left panel), TGF-β$_1$ (middle panel), and syndecan-4 (right panel) mRNAs. Values are the mean ± S.E. of three samples. Significantly different from the corresponding siCont or siTGFB1, $^{**}P < 0.01$. [B] Accumulation of syndecan-4 core protein in the cell layer transfected with siCont, siBGN-1, and siBGN-2 combined with or without siTGFB1 for 24 h.

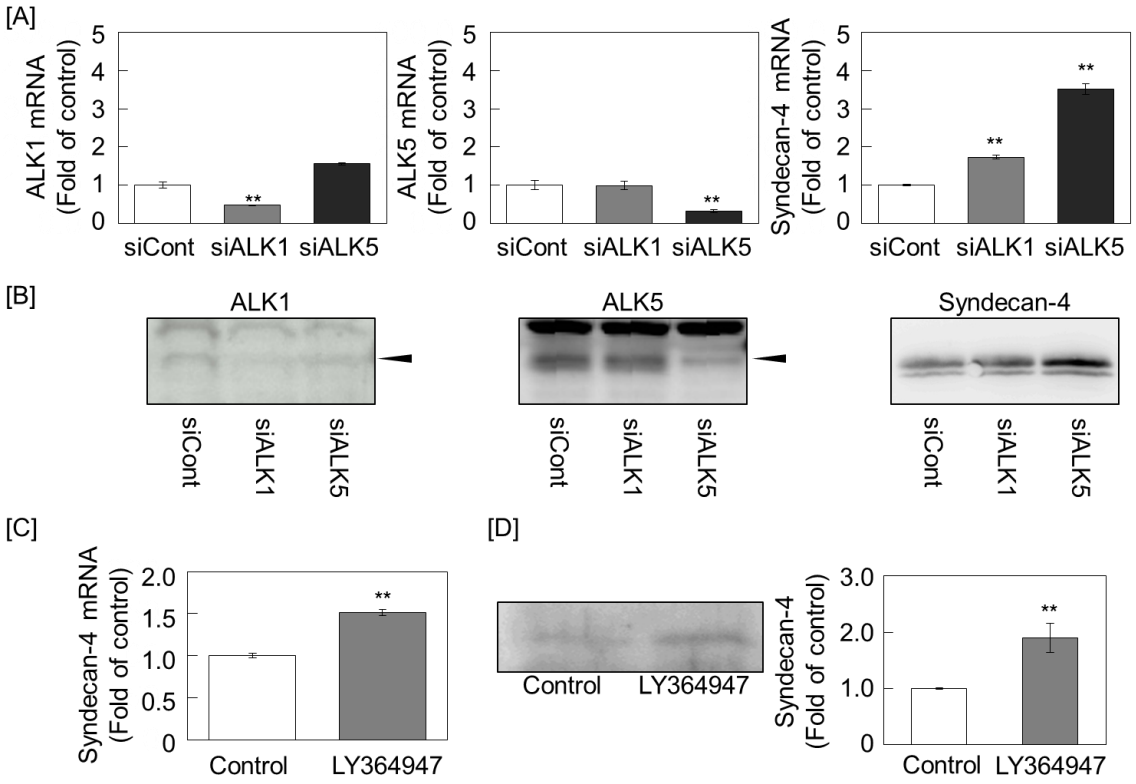


Fig. 1-1-5. Effects of siRNA-mediated knockdown of ALK1 and ALK5 and an ALK5 inhibitor, LY364947, on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siCont, siALK1, or siALK5 at 37°C for 24 h (ALK1 and ALK5) or 36 h (syndecan-4). [A] Expression of ALK1 (right panel), ALK5 (middle panel), and syndecan-4 (right panel) mRNAs. Values are the mean ± S.E. of three samples. Significantly different from the corresponding siCont, $^{**}P < 0.01$. [B] Expression of ALK1 (right panel), ALK5 (middle panel), and syndecan-4 (right panel) proteins. Arrow heads indicate the position of ALK1 (left panel) or ALK5 (center panel). Separately, the cells were treated with an ALK5 inhibitor LY364947 at 1 μM at 37°C for 24 h. [C] Expression of syndecan-4 mRNA. Values are the mean ± S.E. of three samples. Significantly different from the control, $^{**}P < 0.01$. [D] Expression of syndecan-4 core protein in the cell layer. Western blot analysis (left panel) and its quantitative analysis (right panel). Values are means ± S.E. of three experiments performed in duplicate (right panel). Significantly different from the control, $^{\#}P < 0.05$.

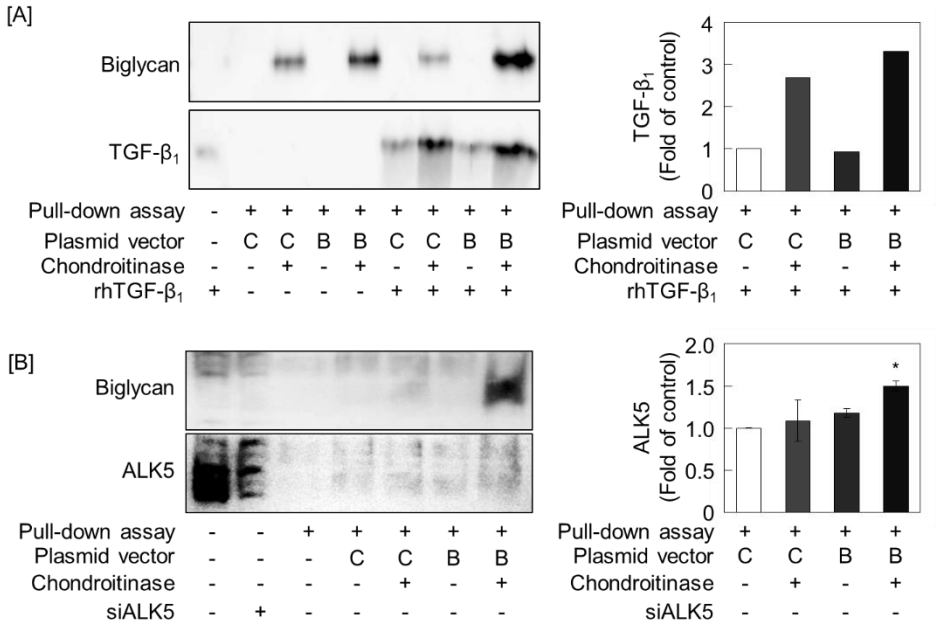


Fig. 1-1-6. Binding of biglycan core protein to TGF-β$_1$ and ALK5. [A] Binding of endothelial biglycan core protein to TGF-β$_1$. Bovine aortic endothelial cells transfected with the pDEST26 (C) or pDEST26-BGN (B) plasmid vector were incubated at 37°C for 24 h to obtain 6 × His-tagged biglycan. The conditioned medium was concentrated and dermatan sulfate chains of biglycan were digested with chondroitinase ABC (+, digested; -, not digested). The biglycan core proteins were subjected to a Ni^{2+} pull-down assay (+, performed; -, not performed) with (+) or without (-) recombinant human TGF-β$_1$ (rhTGF-β$_1$). Western blot analysis (left panel) and its quantitative analysis (right panel). [B] Binding of endothelial biglycan core protein to ALK5. Bovine aortic endothelial cells were transfected with siCont at 37°C for 24 h, and the membrane proteins were extracted. Separately, the cells were transfected with the pDEST26 (C) or pDEST26-BGN (B) plasmid vector to obtain 6 × His-tagged biglycan. The conditioned medium was concentrated and dermatan sulfate chains of biglycan were digested with chondroitinase ABC (+, digested; -, not digested). The membrane proteins and the biglycan core proteins were subjected to a Ni^{2+} pull-down assay (+, performed; -, not performed). The position of ALK5 was confirmed by siRNA-mediated knockdown of ALK5 (+, siALK5; -, siCont). Western blot analysis (left panels) and its quantitative analysis (right panels). Values are means ± S.E. of three experiments performed in duplicate. Significantly different from without chondroitinase ABC, $^{\#}P < 0.05$.

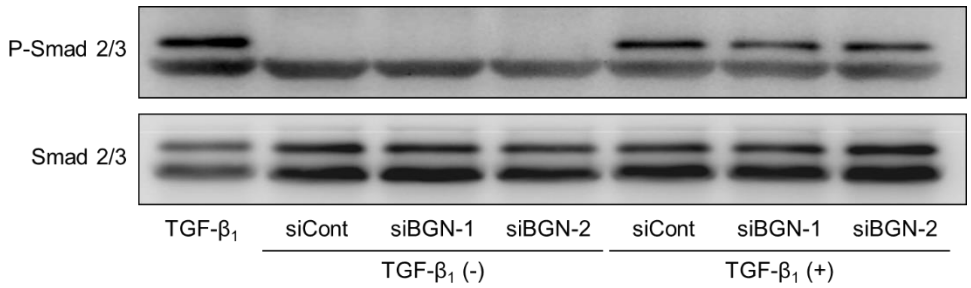
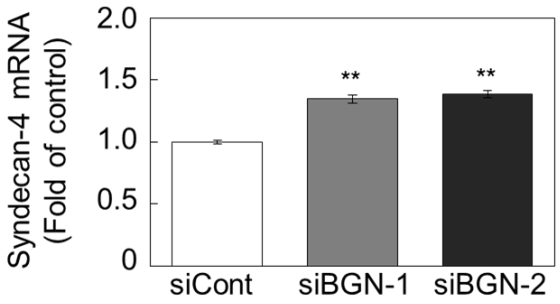


Fig. 1-1-7. Effects of siRNA-mediated knockdown of biglycan on the phosphorylation of Smad2/3 by TGF-β1 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with or without siCont, siBGN-1, or siBGN-2 at 37°C for 24 h and then incubated with or without TGF-β_1 at 1 ng/mL for 30 min.



Supplemental Figure 1-1-1. Effect of siRNA-mediated knockdown of biglycan on the expression of syndecan-4 mRNA in vascular smooth muscle cells. Bovine aortic smooth muscle cells were transfected with siCont, siBGN-1, or siBGN-2 at 37°C for 24 h. Values are the mean ± S.E. of three samples. Significantly different from the corresponding siCont, $^{**}P < 0.01$.

Section 2. Transforming growth factor-β_1 modulates the expression of syndecan-4 in cultured vascular endothelial cells in a biphasic manner

1. 2. 1. Abstract

Proteoglycans are macromolecules that consist of a core protein and one or more glycosaminoglycan side chains. Previously, we reported that transforming growth factor-β_1 (TGF-β_1) regulates the synthesis of a large heparan sulfate proteoglycan, perlecan, and a small leucine-rich dermatan sulfate proteoglycan, biglycan, in vascular endothelial cells depending on cell density. Recently, we found that TGF-β_1 first upregulates and then downregulates the expression of syndecan-4, a transmembrane heparan sulfate proteoglycan, via the TGF-β receptor ALK5 in the cells. In order to identify the intracellular signal transduction pathway that mediates this modulation, bovine aortic endothelial cells were cultured and treated with TGF-β_1. Involvement of the downstream signaling pathways of ALK5—the Smad and MAPK pathways—in syndecan-4 expression was examined using specific siRNAs and inhibitors. The data indicate that the Smad3–p38 MAPK pathway mediates the early upregulation of syndecan-4 by TGF-β_1, whereas the late downregulation is mediated by the Smad2/3 pathway. Multiple modulations of proteoglycan synthesis may be involved in the regulation of vascular endothelial cell functions by TGF-β_1.

1. 2. 2. Introduction

Vascular endothelial cells cover the luminal surface of blood vessels in a monolayer and are thus a unique cell type because they have direct contact with blood. The cells regulate the blood coagulation-fibrinolytic system by synthesizing and secreting several physiological substances, including von Willebrand factor (Jaffe et al., 1974), which facilitates blood coagulation; prostacyclin (Revtyak et al., 1987), which inhibits platelet aggregation; and plasminogen activator (Levin and Loskutoff, 1982), which converts plasminogen to plasmin, which then degrades the fibrin clot. Proteoglycans are macromolecules that consist of a core protein and glycosaminoglycan side chain(s) as a common feature and are a component of the extracellular matrix or are found on the cell surface. Although proteoglycans exhibit multiple functions such as extracellular matrix assembly, lipid metabolism, permeability and thrombosis (Berenson et al., 1984; Camejo, 1981) in vascular tissue, one of the most important functions appears to be the anticoagulant activity of glycosaminoglycan chains such as heparan sulfate and dermatan sulfate. The heparan sulfate and dermatan sulfate chains activate antithrombin III (Mertens et al., 1992) and heparin cofactor II (Tollefsen et al., 1983), respectively, and contribute to the anticoagulant property of vascular endothelium.

The major types of heparan sulfate proteoglycans synthesized by vascular endothelial cells are perlecan, which is the major extracellular matrix proteoglycan (Saku and Furthmayr, 1989); members of the syndecan family of transmembrane proteoglycans such as syndecan-1 and syndecan-4 (Kojima et al., 1992); and the cell surface-associated proteoglycans such as glypican-1 (Mertens et al., 1992). The cells also synthesize small leucine-rich dermatan sulfate proteoglycans such as biglycan and decorin (Järveläinen et al., 1991; Yamamoto et al., 2005). It has been revealed that some cytokines and growth factors regulate proteoglycan synthesis in vascular endothelial cells. For example, fibroblast growth factor-2 and vascular endothelial growth factor-165 enhance the expression of biglycan and perlecan, respectively (Kaji et al., 2006; Kinsella et al., 1997). Transforming growth factor-β_1 (TGF-β_1) induces the expression of biglycan and perlecan in a cell density-dependent manner (Kaji et al., 2000). In addition, connective tissue growth factor suppresses the synthesis of biglycan but newly induces the synthesis of decorin in the cells when cell density is low (Kaji et al., 2004).

Recently, we found that TGF-β_1 regulates the expression of syndecan-4 in vascular endothelial cells in a biphasic manner (Hara et al., 2016b). The cytokine first upregulates and then downregulates endothelial syndecan-4 expression. Two receptors for TGF-β_1 are expressed in vascular endothelial cells: activin receptor-like kinase (ALK) 1 and ALK5 (Goumans et al., 2002; Shi and Massagué, 2003). ALK1 activates its downstream transcriptional factor Smad1/5/8 (Goumans et al., 2003), whereas ALK5 can activate both the canonical Smad2/3 pathway and the non-Smad pathway composed of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase1/2 (ERK1/2), c-jun N-terminal kinase (JNK), and p38 MAPK (Derynck and Zhang, 2003; Macias et al., 2015; Moustakas and Heldin, 2005). It is possible that the biphasic regulation of endothelial syndecan-4 expression by TGF-β_1 is mediated by the Smad pathway, non-Smad pathway, or both.

The present study used a culture system of bovine aortic endothelial cells to clarify the intracellular signal transduction pathways that mediates the early upregulation and the late downregulation of endothelial syndecan-4 expression by TGF-β_1.

1. 2. 3. Results

TGF-β$_1$ activates p38 MAPK and Smad2/3 in vascular endothelial cells

Figure 1-2-1 shows the expression of syndecan-4 mRNA in vascular endothelial cells treated with TGF-β$_1$. The expression of syndecan-4 mRNA was elevated at 6 h and reduced at 24 h by the cytokine at 1 and 5 ng/mL. This result is consistent with our recent study (Hara et al., 2016b), showing that TGF-β$_1$ modulates endothelial syndecan-4 expression in a biphasic manner.

Figure 1-1-2 shows the phosphorylation of MAPKs (ERK1/2, JNK, and p38 MAPK) and Smad2/3, which may be involved in the modulation of syndecan-4 expression by TGF-β$_1$ as the downstream signaling pathways of the cytokine. For the MAPKs, the phosphorylation of only p38 MAPK was elevated by 1 and 5 ng/mL TGF-β$_1$ after 1 h and longer. On the other hand, 1 and 5 ng/mL TGF-β$_1$ increased the phosphorylation of Smad2/3 after 1 h and after 1 h and longer, respectively.

The p38 MAPK pathway mediates the early upregulation of endothelial syndecan-4 expression by TGF-β$_1$

To examine the involvement of MAPKs in the modulation of syndecan-4 mRNA expression by TGF-β$_1$, vascular endothelial cells were pretreated with the MEK1 inhibitor PD98059, JNK inhibitor SP600125, or p38 MAPK inhibitor SB203580 and then stimulated with TGF-β$_1$ (Fig. 1-2-3). Syndecan-4 mRNA expression was upregulated after 6 h and downregulated after 24 h by TGF-β$_1$. PD98059 (Fig. 1-2-3A) and SP600125 (Fig. 1-2-3B) did not influence the early upregulation or the late downregulation. In contrast, SB203580 suppressed the early upregulation of syndecan-4 mRNA expression by TGF-β$_1$, although the late downregulation was unaffected by the inhibitor (Fig. 1-2-3C). In addition, TGF-β$_1$ increased syndecan-4 core protein expression in the cell layer, and this increase was completely diminished by SB203580 (Fig. 1-2-3D); the core protein was not detected in the conditioned medium. These results suggest that TGF-β$_1$ activates p38 MAPK, which mediates the early upregulation of syndecan-4 expression by the cytokine in vascular endothelial cells.

Smad2/3 pathways mediate the late downregulation of syndecan-4 expression by TGF-β$_1$

To examine the involvement of Smad2 and Smad3 in the modulation of syndecan-4 expression by TGF-β_1, vascular endothelial cells were transfected with siSmad2 or siSmad3 and then treated with 5 ng/mL TGF-β_1. siRNA-mediated knockdown alone of either Smad2 (Fig. 1-2-4A) or Smad3 (Fig. 1-2-4B) significantly increased the expression of endothelial syndecan-4 mRNA without treatment with TGF-β_1, suggesting that both Smad2 and Smad3 are involved in the reduction of the basal expression of endothelial syndecan-4. The early upregulation and late downregulation of syndecan-4 mRNA expression by TGF-β_1 were both not affected by siRNA-mediated knockdown of Smad2 (Fig. 1-2-4A). On the other hand, siRNA-mediated knockdown of Smad3 diminished the upregulation of syndecan-4 mRNA expression by TGF-β_1 (Fig. 1-2-4B), suggesting that Smad3 is a member of the signaling pathway that mediates the TGF-β_1-induced upregulation of syndecan-4 expression. The late downregulation of syndecan-4 core protein expression by TGF-β_1 was observed in cells treated with siRNA for Smad2 (Fig. 1-2-3C), but this downregulation was reduced by siRNA for Smad3 (Fig. 1-2-4C). This result indicates that Smad3 is involved in the late downregulation of syndecan-4 core protein expression by TGF-β_1. These results indicate that the early upregulation of syndecan-4 mRNA expression by TGF-β_1 is mediated by Smad3, whereas Smad2 or Smad3 alone is not involved in the late downregulation of syndecan-4 mRNA expression by TGF-β_1; the late downregulation of syndecan-4 core protein expression by TGF-β_1 is mediated by Smad3.

To clarify the role of Smad2/3 in the late downregulation, the effects of the knockdown of both Smad2 and Smad3 on the expression of syndecan-4 mRNA and the core protein were investigated. The results indicated that the siRNA-mediated knockdown of both Smad2 and Smad3 diminished the late downregulation of syndecan-4 mRNA expression by TGF-β_1, although the knockdown of just one could not diminish the downregulation (Sup. Fig. 1-2-1, upper panel). In addition, the results again indicated that siSmad3 with or without siSmad2 diminished the decrease in syndecan-4 core protein expression by TGF-β_1 after a 24 h treatment (Sup. Fig. 1-2-1, lower panel). These results suggest that the late downregulation of syndecan-4 expression by TGF-β_1 is mediated by Smad2/3 at the mRNA level and by Smad3 at the protein level.

Activation of p38 MAPK is mediated by Smad3

In order to examine whether the phosphorylation of p38 MAPK by TGF-β_1 is induced by Smad2 or Smad3, vascular endothelial cells transfected with siSmad2 or siSmad3 were stimulated with TGF-β_1, and the phosphorylation of p38 MAPK was investigated. As shown in Fig. 1-2-5, TGF-β_1 increased the phosphorylation of p38 MAPK after 4 h and longer; the increase was markedly suppressed by siSmad3 but not siSmad2, suggesting that the activation of p38 MAPK is mediated by Smad3.

1. 2. 4. Discussion

Recently, we reported that biglycan intensifies the ALK5-Smad2/3 signaling induced by TGF-β_1 and downregulates syndecan-4 in cultured vascular endothelial cells (Hara et al., 2016b). In this study, we observed that TGF-β_1 first upregulates and then downregulates endothelial syndecan-4 expression. In the present study, we investigated the signaling pathways that mediate the biphasic regulation of endothelial syndecan-4 expression, and the following results were obtained: (i) The biphasic regulation by TGF-β_1 was confirmed. (ii) TGF-β_1 activated MAPKs, including ERK1/2, JNK, and p38 MAPK, as well as Smad2/3. (iii) The early upregulation of syndecan-4 by TGF-β_1 was diminished by either an inhibitor of p38 MAPK or siRNA for Smad3 at both the mRNA and syndecan-4 core protein expression levels. (iv) The late downregulation of syndecan-4 mRNA expression was reduced when cells were treated with siRNAs for both Smad2 and Smad3, whereas the late downregulation of syndecan-4 core protein expression was reduced only by siRNA for Smad3. (v) The activation of p38 MAPK was reduced by siRNA for Smad3 but not by that for Smad2. Together, these results suggest that the early upregulation of endothelial syndecan-4 expression by TGF-β_1 is mediated by the ALK5–Smad3–p38 MAPK pathway, whereas the late downregulation is mediated by the ALK5–Smad2/3 pathway. Specifically, the downregulation of syndecan-4 expression by TGF-β_1 is mediated by the interaction of Smad3 with Smad2 at the mRNA expression level, whereas only Smad3 is crucial at the core protein expression level. The present data showed for the first time that TGF-β_1 and p38 MAPK serve as regulatory molecules for endothelial syndecan-4 expression.

The TGF-β_1 that vascular endothelial cells are exposed to is mainly released from aggregated platelets. When vascular endothelial cells are injured, platelets aggregate at the damaged site, and TGF-β_1 is released from α-granules in the platelets (Assoian and Sporn, 1986). At that time, TGF-β_1 activates p38 MAPK and upregulates plasminogen activator inhibitor type 1 expression to maintain the fibrin clot that prevents bleeding (Woodward et al., 2006). TGF-β_1-activated p38 MAPK increases permeability (Goldberg et al., 2002) and induces the apoptosis of vascular endothelial cells (Hyman et al., 2002). In addition to these inflammatory responses, the TGF-β_1–p38 MAPK pathway also increases the synthesis and activity of focal adhesion kinase,

which is crucial for cell survival, motility, and proliferation (Walsh et al., 2008). Syndecan-4 molecules have three heparan sulfate chains on the extracellular domain of their core proteins that are used for focal adhesion; the heparan sulfate chains play a critical role in stress fiber formation (Gopal et al., 2010). It has also been reported that syndecan-4 is an anti-inflammatory molecule. Specifically, a large number of neutrophils, increased expression of neutrophil chemotactic factors, and higher mortality were reported in syndecan-4 knockout mice injected with lipopolysaccharide (Ishiguro et al., 2001; Tanino et al., 2012). These results and our data, which showed that the induction of endothelial syndecan-4 expression occurs in the early stage of the exposure to TGF-β_1, suggest that the upregulation of endothelial syndecan-4 expression by TGF-β_1 may be a defense mechanism against an acute injury to vascular endothelial cells.

It has been reported that the atherosclerotic vascular wall has fewer heparan sulfate proteoglycans and more dermatan sulfate proteoglycans compared to the normal vascular wall (Stevens et al., 1976). In addition, the lack of syndecan-4 disrupts the alignment of vascular endothelial cells along the direction of blood flow, resulting in the formation of a wide range atherosclerotic lesions, including near vascular branching points where jet laminar flow is impeded to create disturbed flow (Baeyens et al., 2014). The disturbed flow increases thrombogenicity and triggers chronic inflammation (Brooks et al., 2002). Additionally, vascular endothelial cells exposed to disturbed flow exhibit a lower expression of focal adhesion molecules such as VE-cadherin and β-catenin than cells exposed to pulsatile flow (Miao et al., 2005). The functional damage of vascular endothelial cells caused by the disturbed flow induces chronic TGF-β_1 accumulation in the vascular wall, and the accumulated TGF-β_1 suppresses the expression of syndecan-4 by activation of Smad2/3, which contributes to the progression of atherosclerosis (Nesbitt et al., 2009; Popovic et al., 2009; Tull et al., 2006). Together with the results from our previous study, it is suggested that biglycan, a proteoglycan whose synthesis is induced by TGF-β_1 (Kaji et al., 2000), intensifies the TGF-β_1–ALK5 signaling via the core protein and that activated Smad2/3 in the signaling pathway suppresses the expression of syndecan-4 at the late stage of TGF-β_1 exposure in vascular endothelial cells. In other words, TGF-β_1 induces endothelial biglycan synthesis; as a result, syndecan-4 expression is suppressed by enhanced TGF-β_1 signaling. This regulation of endothelial proteoglycan synthesis by TGF-

β_1 may be a component of the mechanisms underlying the histopathological changes in the types of proteoglycans—fewer heparan sulfate proteoglycans and more dermatan sulfate proteoglycans (Stevens et al., 1976)—seen in atherosclerotic vascular walls.

The data from the present study revealed the signaling pathways that mediate the regulation of syndecan-4 expression by TGF-β_1 in vascular endothelial cells. They indicated that TGF-β_1 upregulates syndecan-4 expression via the ALK5–Smad3–p38 MAPK pathway at the early stage, whereas downregulation of the expression occurs via the ALK5–Smad2/3 pathway. The mechanism responsible for switching the signaling pathway from the Smad3–p38 MAPK pathway to the Smad2/3 pathway for syndecan-4 expression appears to be important. It was reported that the signal transduction of Ask1, which is a MAP kinase kinase kinase that regulates the stress responses, is regulated in a biphasic manner by ubiquitination. Ubiquitinated Ask1 is transiently activated and transduces a proliferation signal (Maruyama et al., 2014); however, when Ask1 is not ubiquitinated, the molecule is activated continuously and induces an apoptotic signal (Ichijo et al., 1997). Because Smad3 is also ubiquitinated (Fukuchi et al., 2001; Moren et al., 2003), it may be possible that one of the mechanisms that regulates the signaling pathway for endothelial syndecan-4 is the ubiquitination of Smad3. Further studies are required to clarify the detailed mechanisms by which the signaling pathway for endothelial syndecan-4 expression is switched from the Smad3–p38 MAPK pathway for upregulation at the early stage to the Smad2/3 pathway for downregulation at the late stage.

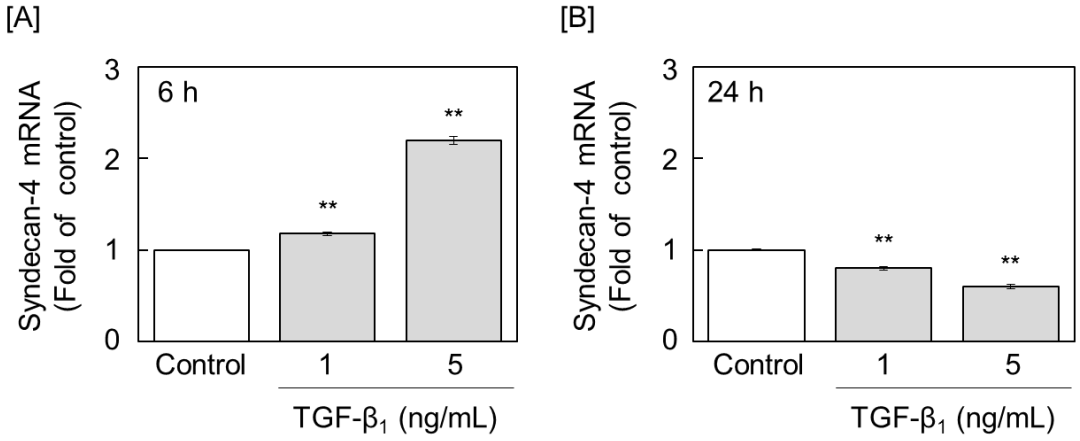


Fig. 1-2-1. Effects of TGF-β1 on the expression of syndecan-4 mRNA in vascular endothelial cells. Bovine aortic endothelial cells were treated with 1 and 5 ng/mL TGF-β_1 at 37°C for 6 or 24 h ([A] and [B], respectively). Values are the mean ± S.E. of four samples. Significantly different from the corresponding control, $^{**}P < 0.01$.

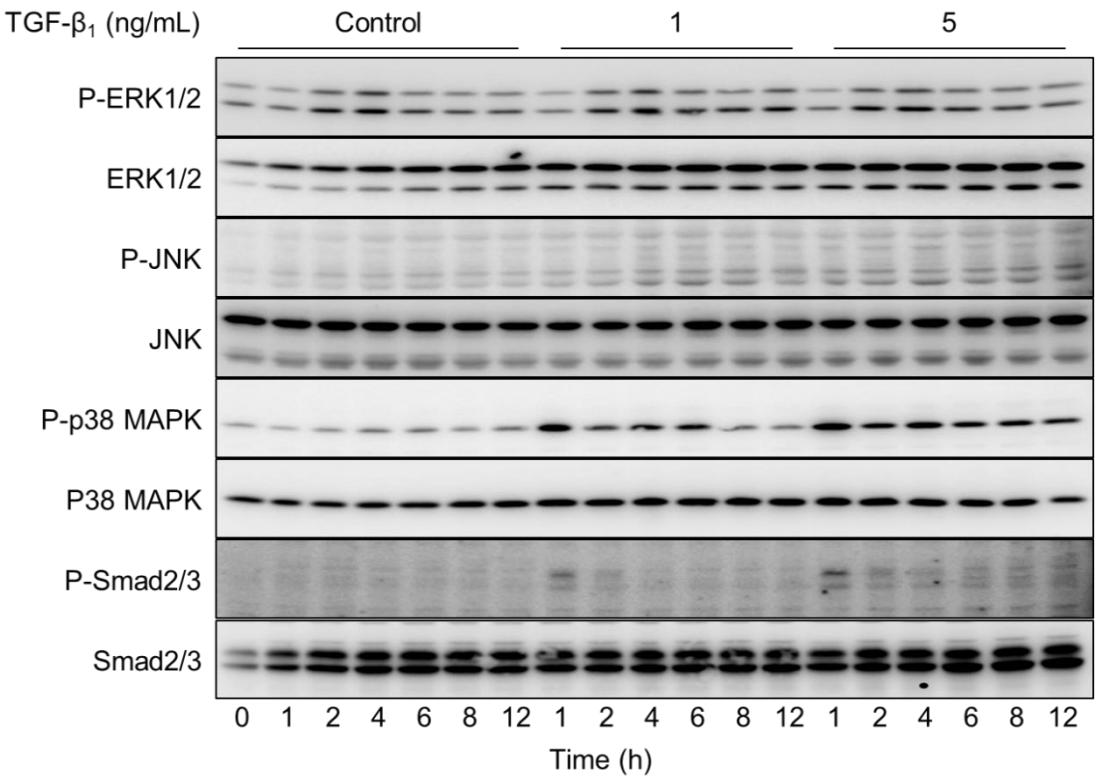


Fig. 1-2-2. Effects of TGF-β1 on the activation of ERK1/2, JNK, p38 MAPK, and Smad2/3 in vascular endothelial cells. Bovine aortic endothelial cells were treated with 1 and 5 ng/mL TGF-β_1 at 37°C for 1, 2, 4, 6, 8, or 12 h.

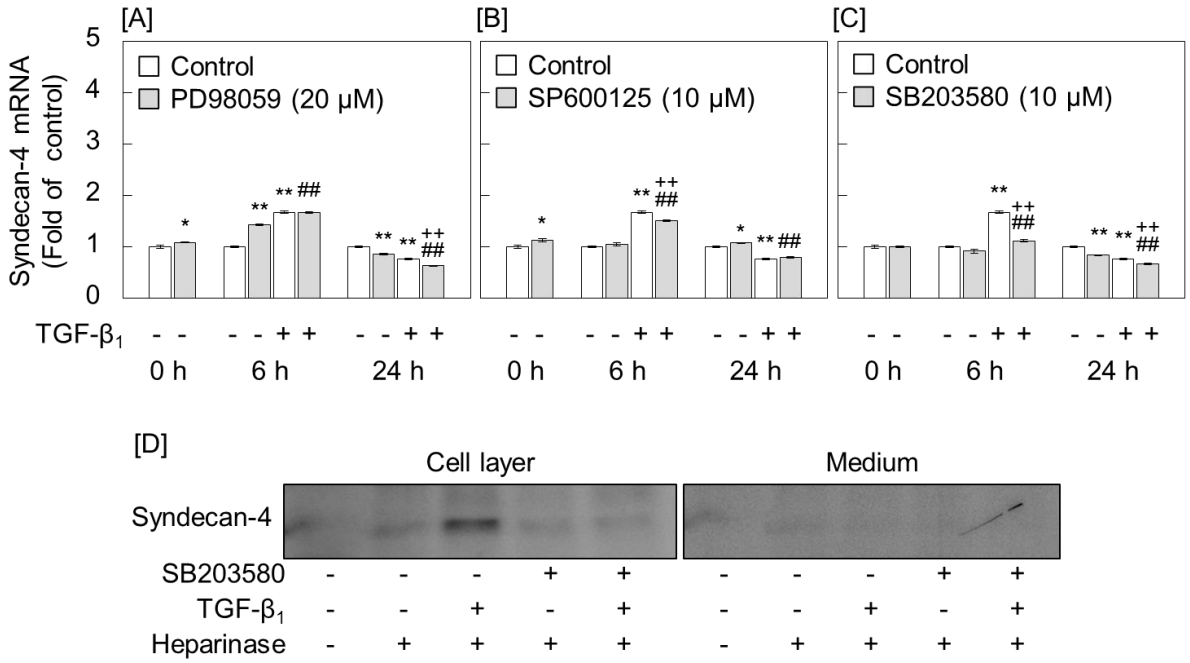


Fig. 1-2-3. Effects of the MAPK pathway inhibitors PD98059, LY364947, and SB203580 on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were pretreated with [A] the MEK1 inhibitor PD98059 at 20 μM, [B] JNK inhibitor SP600125 at 10 μM, or [C] p38 MAPK inhibitor SB203580 at 10 μM at 37°C for 1 h and then treated with 5 ng/mL TGF-β_1 for 6 or 24 h. Values are the mean ± S.E. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs MAPK inhibitor without TGF-β_1; $^{++}P < 0.01$ vs TGF-β_1. [D] The expression of syndecan-4 core protein. Bovine aortic endothelial cells were pretreated with 10 μM SB203580 at 37°C for 1 h and then treated with 5 ng/mL TGF-β_1 for 6 h.

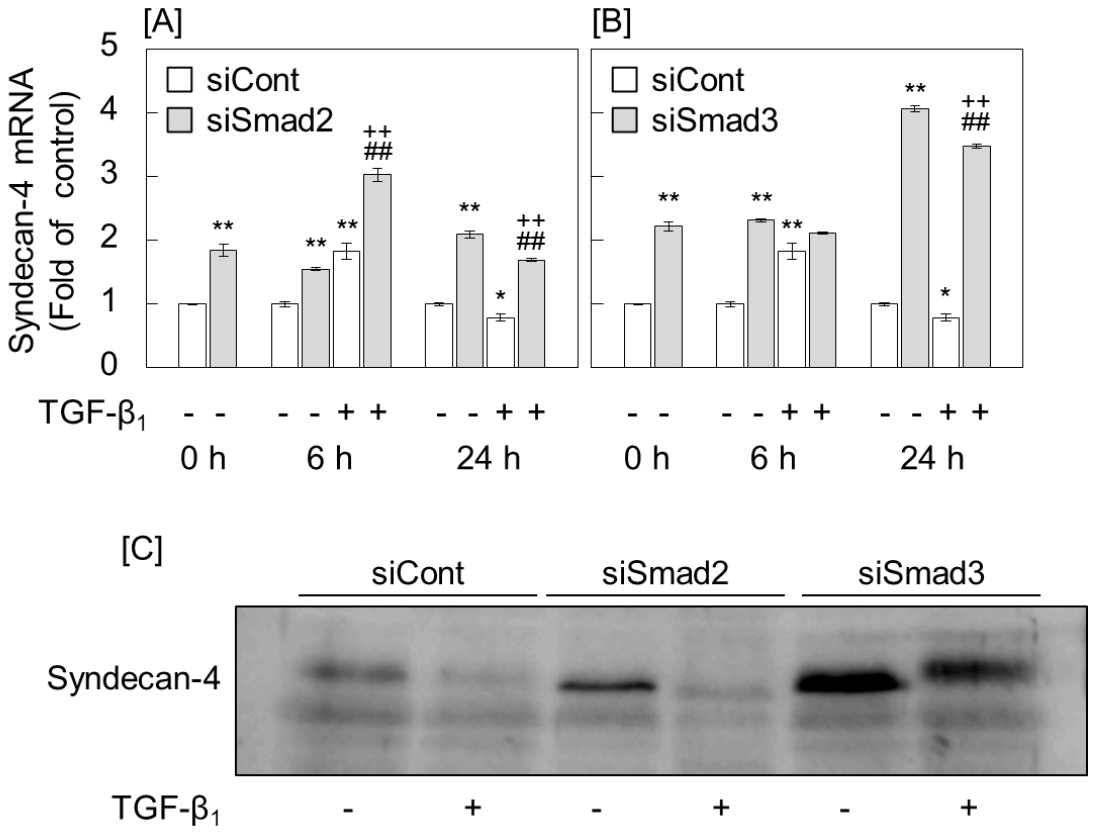


Fig. 1-2-4. Effects of siRNA-mediated knockdown of Smad2 or Smad3 on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with [A] siSmad2 or [B] siSmad3 at 37°C for 24 h and then treated with 5 ng/mL TGF-β_1 for 6 or 24 h. Values are the mean ± S.E. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ vs siCont; $^{\#\#}P < 0.01$ vs siSmad2 or siSmad3 without TGF-β_1; $^{++}P < 0.01$ vs TGF-β_1. [C] The expression of syndecan-4 core protein. Bovine aortic endothelial cells were transfected with siCont, siSmad2, or siSmad3 at 37°C for 24 h and then treated with 5 ng/mL TGF-β_1 for 24 h.

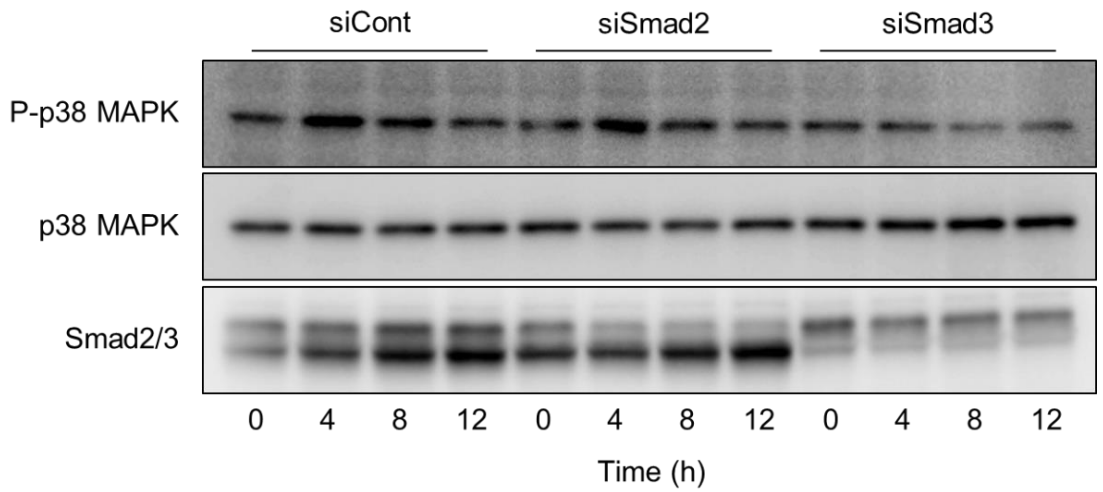
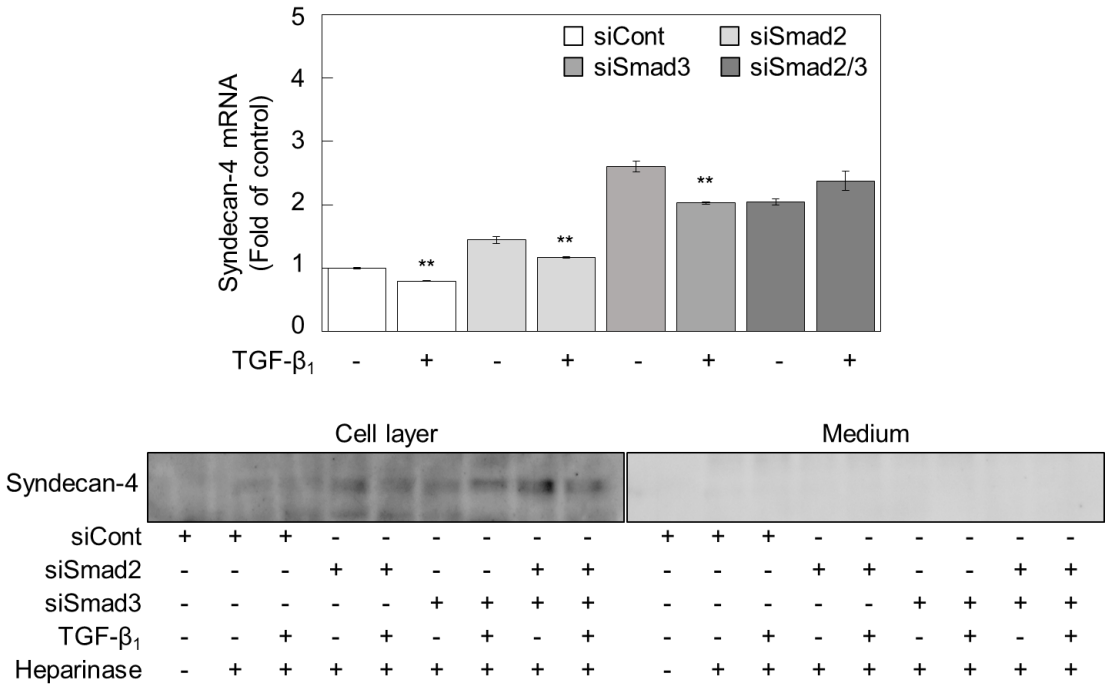
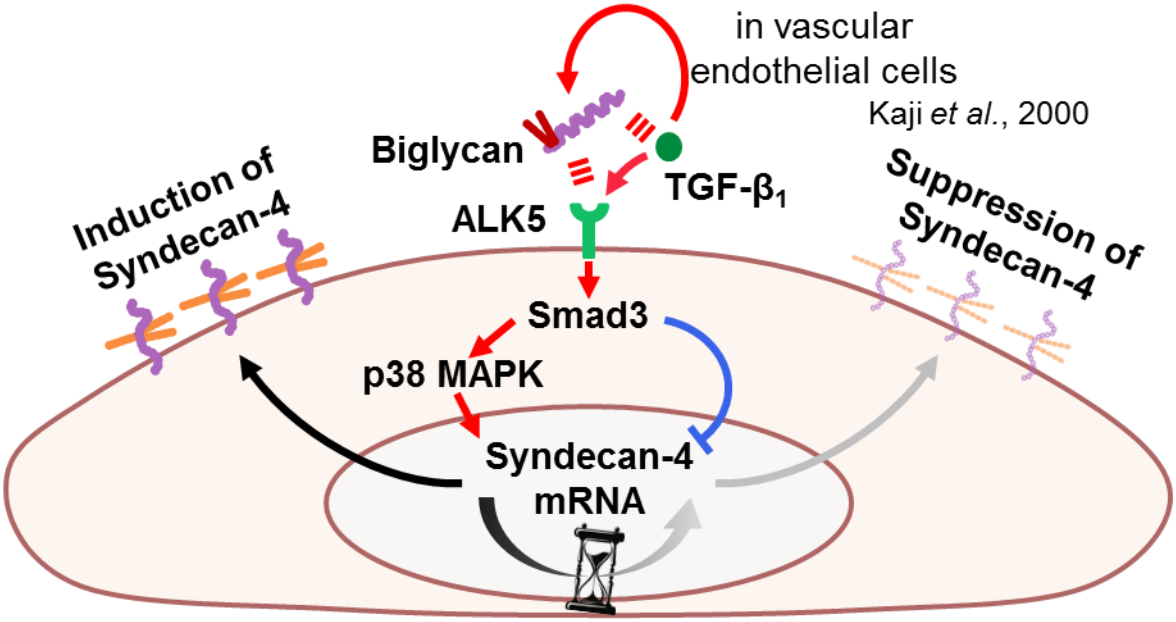


Fig. 1-2-5. Effects of siRNA-mediated knockdown of Smad2 or Smad3 on the activation of p38 MAPK in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siSmad2 or siSmad3 at 37°C for 24 h and then treated with 5 ng/mL TGF-β_1 for 4, 8, or 12 h.



Supplemental Figure 1-2-1. Effects of siRNA-mediated knockdown of Smad2, Smad3, or both on the expression of syndecan-4 in vascular endothelial cells. The expression of syndecan-4 mRNA (upper panel) and core protein (lower panel). Bovine aortic endothelial cells were transfected with siCont, siSmad2, siSmad3, or both at 37°C for 24 h and then treated with 5 ng/mL TGF-β_1 for 24 h. Values are the mean ± S.E. of four samples. $^{**}P < 0.01$ vs without TGF-β_1.



Scheme 1. Figure summary of Chapter 1

Chapter 2. Strategy based on bio-organometallics

Section 1. Selection of zinc compound that modulates proteoglycan expression in vascular endothelial cells.

2. 1. 1. Introduction

Since dysfunction of endothelium can initiate primary cardiovascular diseases such as atherosclerosis (Shimokawa, 1999), rapid repair of the damaged site of endothelium should be required to prevent the progression of the diseases. It is reported that heparan sulfate proteoglycans, especially perlecan and syndecan-4, contribute to the proliferation and defense response, respectively, of vascular endothelial cells (Sakai et al., 2010; Vuong et al., 2015); however, the mechanisms underlying the regulation of the synthesis of these proteoglycans are not fully understood. Zinc, one of essential trace element, stimulates vascular endothelial cell proliferation (Kaji et al., 1994) and enhances the repairmen of damaged endothelial monolayer (Kaji et al., 1995).

Recently, organic-inorganic hybrid molecules—organometallic compounds and metal complexes— are used as chemical reagent in field of organic chemistry because of their unique reactivity. However, organic-inorganic hybrid molecules have not been applied to life sciences yet. As an example of usefulness of the hybrid molecules as a tool to analyze biological systems, it was reported that bis(*L*-cysteinato)zincate(II), a zinc complex, selectively activates a transcriptional factor MTF-1 as a donor of zinc ion (Kimura et al., 2012). This result led us to an idea that there may be metal complexes useful for analysis the expression of proteoglycans such as perlecan and syndecan-4 in vascular endothelial cells.

2. 1. 2. Results and Discussion

To obtain desired molecules for analysis of endothelial proteoglycan synthesis, we used a library of zinc complexes (Table 1 and Fig. 2-1-1). Since perlecan is the predominant type of proteoglycans synthesized and secreted by vascular endothelial cells, we first investigated whether these zinc complexes can modulate the expression of perlecan core protein in cultured bovine aortic endothelial cells (Fig. 2-1-2). Although Zn-06 was cytotoxic, the other compounds were nontoxic in the cells at 10 μM for 24 h. Although perlecan core protein accumulated in cell layer and conditioned medium are increased when the cells treated with Zn-25, while Zn-06, Zn-13, and Zn-33 decreased the accumulation.

Next, we analyzed the expression of syndecan-4 mRNA in vascular endothelial cells treated with these zinc complexes (Fig. 2-1-3). Syndecan-4 mRNA markedly increased in the cell layer by Zn-06, Zn-10, Zn-13, Zn-14, Zn-15, Zn-19, and Zn-33. Although most of these compounds have 1,10-phenanthroline structure as a common ligand, Zn-12, Zn-17, and Zn-21failed to increase the expression of syndecan-4 mRNA. We chose Zn-33—dichroro-1,10-phenanthroline-zinc— as a compound that downregulates perlecan expression and upregulates syndecan-4 expression in vascular endothelial cells.

Table 1

Zinc complexes used in this study

Complex	IUPAC	References
Zn-01	Disodium Bis(L-cysteinato)zincate(II) hexahydrate	(Viladkar et al., 1993)
Zn-02	Bis(L-histidinato)zinc(II) dihydrate	(Viladkar et al., 1993)
Zn-03	Disodium (ehtylenediaminetetraacetato)zincate(II) hemihydrate	(Kołodyńska and Hubicki, 2008)
Zn-05	Bis(maltolato)zinc(II) monohydrate	(Yoshikawa et al., 2000)
Zn-06	Bis(1,4-dihydro-2-methyl-1-phenyl-4-thioxo-3-pyridinolato)zinc(II) monohydrate	(Katoh et al., 2002)
Zn-07	[Tris(2-pyridylmethyl)amine]zinc(II) perchlorate monohydrate	(Anderegg et al., 1977)
Zn-08	Dichloro[bis(2-pyridylmethyl)amine]zinc(II)	(Fernandes et al., 2014)
Zn-10	Dichloro(2,2’:6’,2”-terpyridine)zinc(II)	(Postmus et al., 1967)
Zn-12	Dichloro(2,9-dimetyl-1,10-phenanthroline)zinc(II)	(Dehghani et al., 2012)
Zn-13	Dichloro(4-metyl-1,10-phenanthroline)zinc(II)	
Zn-14	Dichloro(3,4,7,8-tetrametyl-1,10-phenanthroline)zinc(II)	
Zn-15	Dichloro(5-chloro-1,10-phenanthroline)zinc(II)	
Zn-16	Bis(benzenethiolato)(2,2'-bipyridine)zinc(II)	(Anjali et al., 1999)
Zn-17	Bis(benzenethiolato)(1,10-phenanthroline)zinc(II)	(Anjali et al., 1999)
Zn-18	[6,6’-((1*E*,1’*E*)-(1,2-phenylenebis(azanylylidene))bis(methanylylidene))bis(2,4-di-*tert*-butylphenolato)]zinc(II) monohydrate	(Belmonte et al., 2010)
Zn-19	Dichloro(4,7-dimetyl-1,10-phenanthroline)zinc(II)	
Zn-21	Dichloro(2-chloro-1,10-phenanthroline)zinc(II)	
Zn-22	[*N*,*N*,*N*',*N*'-Tetrakis(2-pyridylmethyl)-1,2-ethylendiamine]zinc(II) perchlorate sesquihydrate	(Mikata et al., 2005)
Zn-23	Bis(2-picolinamide)bis(thiocyanato)zinc(II) 1/4hydrate	(Đaković et al., 2008)
Zn-25	Bis[2-(2-benzoxazolyl)phenolato]zinc (II)	TCI, Recrystallization
Zn-26	Bis[2-(2-benzothiazolyl)phenolato]zinc(II)	TCI, Recrystallization
Zn-27	Bis(acetylacetonato)zinc(II)	TCI, Recrystallization
Zn-28	(Toluene-3,4-dithiolato)zinc(II) hemihydrate	Sigma-Aldrich, Recrystallization
Zn-30	Tris(ethylenediamine)zinc(II) dichloride	(Krishnan and Plane, 1966)
Zn-31	Dichloro(*N*,*N*,*N'*,*N'*-tetramethylethylenediamine)zinc(II)	TCI, Recrystallization
Zn-32	Bis(8-quinolinolato)zinc(II) monohydrate	TCI, Recrystallization
Zn-33	Dichloro(1,10-phenanthroline)zinc(II)	(Hara et al., 2016a)
Zn-34	(2,2'-Bipyridine)dichlorozinc(II)	(Postmus et al., 1967)
Zn-35	Dichlorobis(pyridine)zinc(II)	(Anjali et al., 1999)
Zn-36	(1,4,8,11-Tetraazacyclotetradecane)zinc(II) perchlorate	(Kato and Ito, 1985)
Zn-37	Bis(glycinato)zinc(II)	(Viladkar et al., 1993)
Zn-38	Bis(1,4-dihydro-2-methyl-1-phenyl-4-oxo-3-pyridinolato)zinc(II) monohydrate	(Katoh et al., 2002)

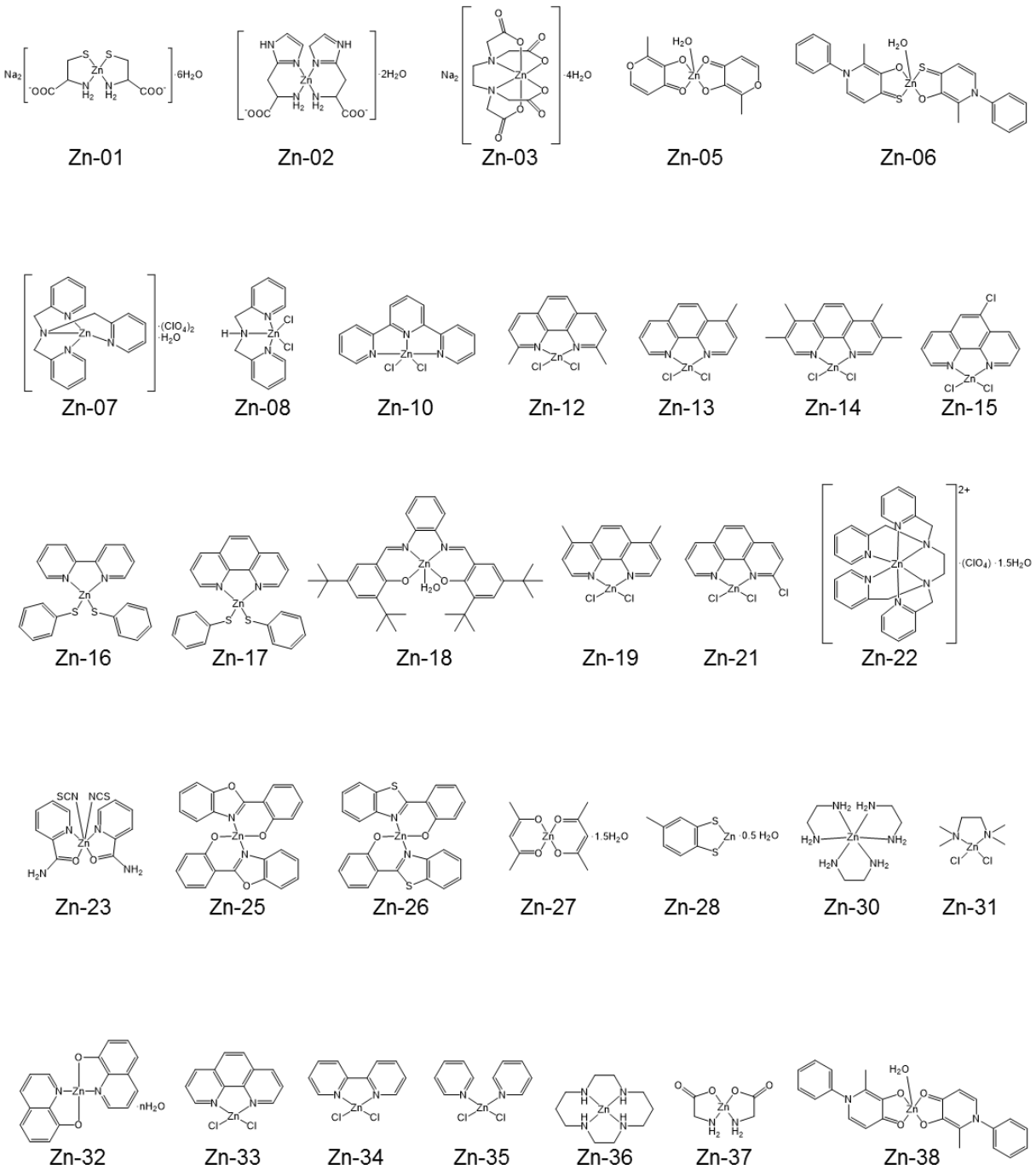


Fig. 2-1-1. Molecular structure of zinc complexes used in this study.

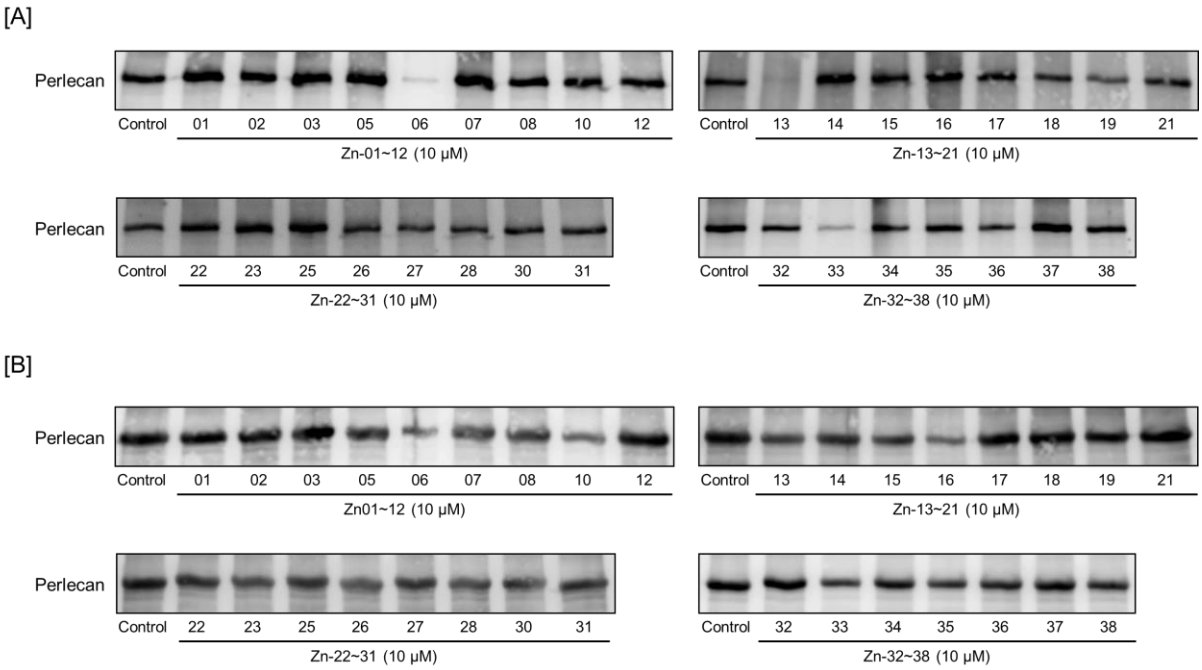


Fig. 2-1-2. Effects of zinc-complexes on the expression of perlecan core protein in vascular endothelial cells. The accumulation of perlecan core protein accumulated in the cell layer [A] and conditioned medium [B] of the cells. Bovine aortic endothelial cells were treated with zinc complexes at 10 μM each at 37°C for 24 h.

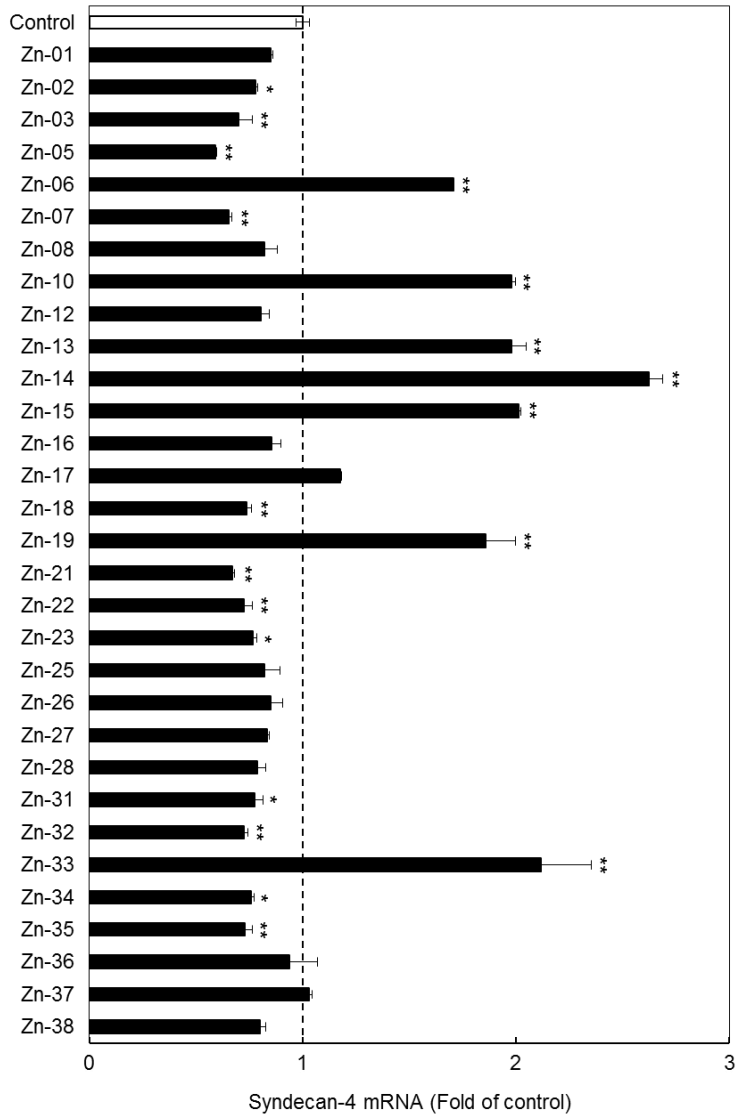


Fig. 2-1-3. Effects of zinc-complexes on the expression of syndecan-4 mRNA in vascular endothelial cells. Bovine aortic endothelial cells were treated with zinc complexes at 10 μM each at 37°C for 12 h. Values are the mean ± S.E. of three samples. $^{**}P < 0.01$ vs control.

Section 2. Cytotoxicity of zinc, copper and rhodium complexes with 1,10-phenanthroline or 2,9-dimethyl-1,10-phenanthroline in cultured vascular endothelial cells

2. 2. 1. Abstract

Organic-inorganic hybrid molecules, which have organic structure and metal atoms, can exhibit various biological activities that are distinctly different from their components. Consequently, organic-inorganic hybrid molecules are considered an effective tool to analyze biological systems. Herein, we investigated the cytotoxicity of zinc, copper, and rhodium complexes, with either 1,10-phenanthroline or 2,9-dimethyl-1,10-phenanthroline as a common ligand, in cultured vascular endothelial cells. The copper complexes, that is, dichloro(1,10-phenanthroline) copper and dichloro(2,9-dimethyl-1,10-phenanthroline) copper, exhibited high cytotoxicity accompanied by considerable accumulation inside the cells. Potassium tetrachloro(1,10-phenanthroline) rhodate also exhibited cytotoxicity and considerable accumulation. Thus, it was found that the cytotoxicity of organic-inorganic hybrid molecules to vascular endothelial cells depends on the interaction between the intramolecular metal and ligand, which facilitates their uptake by the cells.

2. 2. 2. Introduction

Organic-inorganic hybrid molecules, which have an organic structure and metal atoms, are widely used as synthetic reagents in chemical reactions. Little is known about the biological activities of these compounds. Therefore, it is not clear how the intramolecular metal modifies the biological activities of organic-inorganic hybrid molecules. Recently, we reported that the cell's biological response to organic-inorganic hybrid molecules is different from that toward each of the organic or inorganic components separately; this suggests that a distinct biology for organic-inorganic hybrid molecules should be established (Fujie et al., 2016a; Fujie et al., 2016c; Fujie et al., 2016d; Kohri et al., 2015; Murakami et al., 2015).

The intensity of binding between the intramolecular metal and the organic structure appears to be a defining factor for the biological activities of organic-inorganic hybrid molecules. This binding intensity seems to depend on the type of intramolecular metal or on the polarity or configuration of the organic structure (Kimura et al., 2012).

1,10-Phenanthroline (*o*-Phen) is a well-known divalent metal chelating agent, selective for Fe(II) but not Fe(III). Based on this characteristic, it has been used as an assay reagent of Fe(II) in environmental water (Hoshi et al., 1989). In the present study, we investigated the cytotoxicity of o-Phen and 2,9-dimethyl-1,10-phenanthroline (DMP) complexes with zinc (Zn-Phen and Zn-DMP, respectively), copper (Cu-Phen and Cu-DMP, respectively), or rhodium (Rh-Phen and Rh-DMP, respectively) in vascular endothelial cells.

2. 2. 3. Results and Discussion

The organic-inorganic hybrid molecules used in this study are shown in Fig. 2-2-1. Figure 2-2-2 shows the morphological changes and Lactate dehydrogenase (LDH) leakage in vascular endothelial cells treated with *o*-Phen, $ZnCl_2$, Zn-Phen, Zn-DMP, $CuCl_2$, Cu-Phen, Cu-DMP, $RhCl_3$, Rh-Phen, or Rh-DMP for 24 h. Morphologically, Cu-Phen at ≥ 5 μM, Cu-DMP at ≥ 10 μM, and Rh-Phen at ≥ 10 μM exhibited a significant dose-dependent cytotoxicity. This morphological observation was accompanied with considerable leakage of LDH into the medium. However, no cytotoxicity was observed after exposing the cells to o-Phen, $ZnCl_2$, Zn-Phen, Zn-DMP, $CuCl_2$, $RhCl_3$, or Rh-DMP, either morphologically or with regard to LDH leakage. Taking into account the collective results for *o*-Phen and its metal complexes, it was found that (1) *o*-Phen, $ZnCl_2$, and Zn-Phen are all nontoxic; (2) *o*-Phen and $CuCl_2$ are nontoxic, but Cu-Phen is toxic; and (3) *o*-Phen and $RhCl_3$ are nontoxic, but Rh-Phen is toxic. This indicates that the cytotoxicity of *o*-Phen-related metal complexes to vascular endothelial cells was caused by the interaction of *o*-Phen with the introduced metal. In other words, the cytotoxicity of *o*-Phen-related metal complexes is distinctive from that of their structural components. Furthermore, the cytotoxicity of Cu-DMP and Rh-DMP was significantly lower than that of Cu-Phen and Rh-Phen, respectively, suggesting that methylation of *o*-Phen reduces the interaction of Cu/Rh with the ligand. This interaction is critical for exhibiting cytotoxicity to vascular endothelial cells. Similarly, it has been reported that intramolecular metal can modify the cytotoxicity of organopnictogen compounds (Kohri et al., 2015; Murakami et al., 2015).

The intracellular accumulation of organic-inorganic hybrid molecules in vascular endothelial cells was used as an indicator of the relationship between their cytotoxicity and the degree of accumulation. As shown in Fig. 2-2-3, toxic compounds (Cu-Phen, Cu-DMP, and Rh-Phen) significantly accumulated in a dose-dependent manner. On the other hand, nontoxic compounds ($ZnCl_2$, Zn-Phen, Zn-DMP, $CuCl_2$, $RhCl_3$, and Rh-DMP) did not show any significant accumulation. Specifically, the accumulation of ZnCl2, Zn-Phen, Zn-DMP, $CuCl_2$, and $RhCl_3$ was almost equal to that in cells treated with *o*-Phen. However, the intracellular accumulation of Rh-DMP was slightly higher than that of the other compounds in this group, but was

substantially lower than that of Rh-Phen. Thus, it is hypothesized that the cytotoxicity of the compounds tested in this study relied on the level of intracellular accumulation. This is consistent with our previous study regarding the impact of intracellular accumulation on the modification of the cytotoxicity of organopnictogen compounds (Kohri et al., 2015; Murakami et al., 2015).

In conclusion, it is suggested that cytotoxicity of organic-inorganic hybrid molecules to vascular endothelial cells may result from the interaction of intramolecular metal with the ligand. This interaction can lead to higher accumulation within the cells. Therefore, it is difficult to determine the cytotoxicity of organic-inorganic hybrid molecules based on the cytotoxicity of their structural components individually. Further studies are required to clarify the (1) changes in the characteristics of organic-inorganic hybrid molecules, caused by the metal-ligand interaction, including the electron state; and (2) the mechanisms by which these changes facilitate the entry of the molecules into the cells.

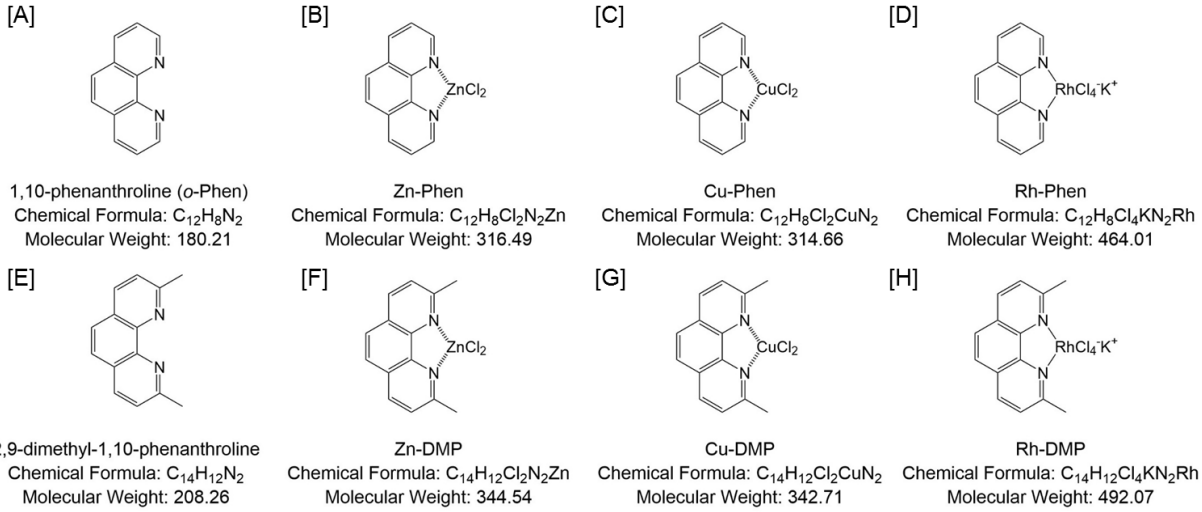


Fig. 2-2-1. Structures of organic-inorganic hybrid molecules used in this study. [A] 1,10-Phenanthroline (*o*-Phen); [B] dichloro(1,10-phenanthroline)zinc (Zn-Phen); [C] dichloro(1,10-phenanthroline)copper (Cu-Phen); [D] potassium tetrachloro(1,10-phenanthroline)rhodate (Rh-Phen); [E] 2,9-dimethyl-1,10-phenanthroline (DMP); [F] dichloro(2,9-dimethyl-1,10-phenanthroline)zinc (Zn-DMP); [G] dichloro(2,9-dimethyl-1,10-phenanthroline)copper (Cu-DMP); [H] potassium tetrachloro(2,9-dimethyl-1,10-phenanthroline)rhodate (Rh-DMP).

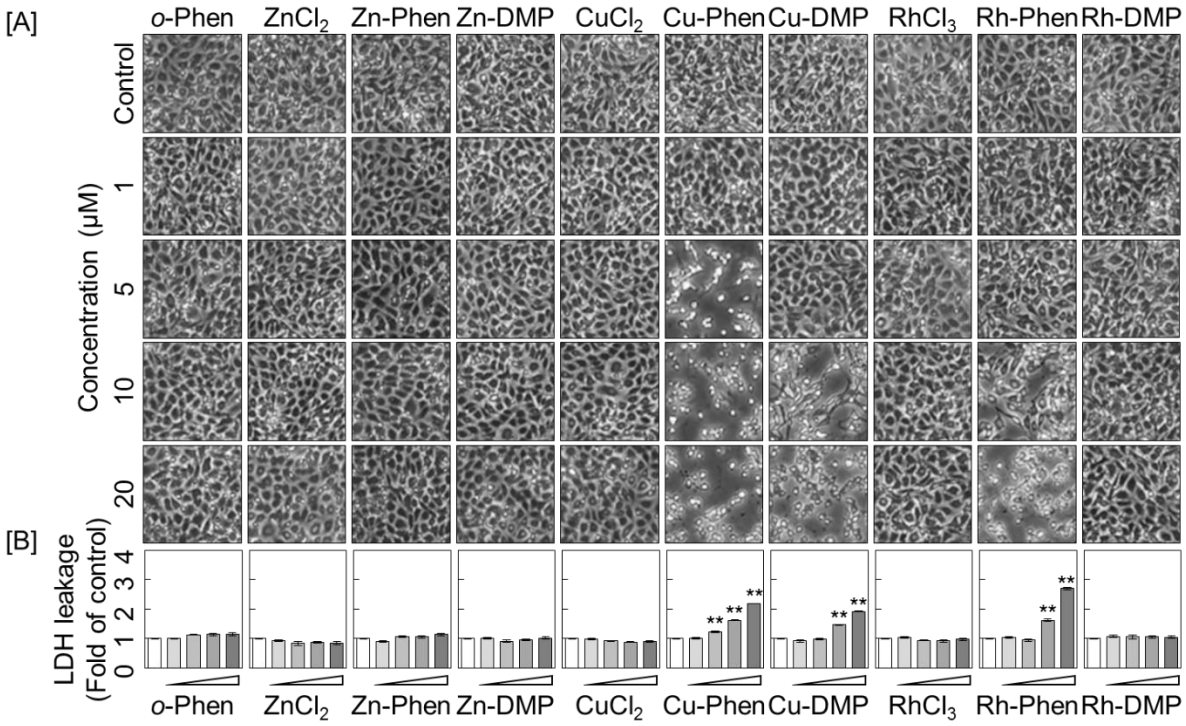


Fig. 2-2-2. Cytotoxicity of *o*-Phen, $ZnCl_2$, Zn-Phen, Zn-DMP, $CuCl_2$, Cu-Phen, Cu-DMP, $RhCl_3$, Rh-Phen, and Rh-DMP. Confluent cultures of bovine aortic endothelial cells were treated with *o*-Phen, $ZnCl_2$, Zn-Phen, Zn-DMP, $CuCl_2$, Cu-Phen, Cu-DMP, $RhCl_3$, Rh-Phen, or Rh-DMP (1, 5, 10, or 20 μM each) for 24 h. [A] Morphological appearance of vascular endothelial cells. [B] The leakage of LDH from the cells into the medium. Values are mean ± S.E. of four samples. Significantly different from the corresponding control, $^{**}P < 0.01$.

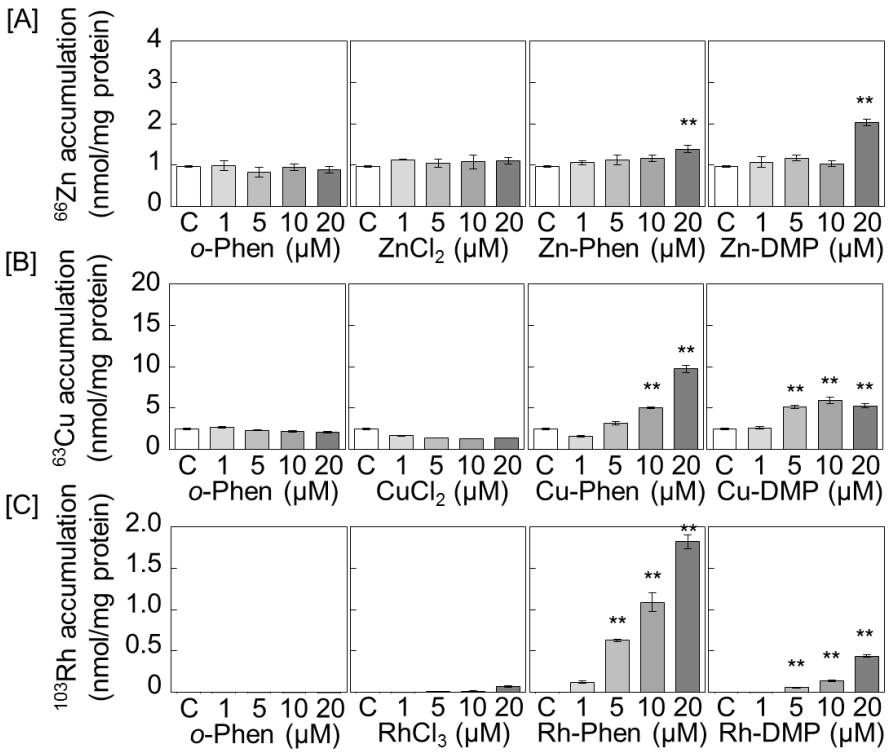


Fig. 2-2-3. Intracellular accumulation of zinc, copper, and rhodiumin vascular endothelial cells. Confluent cultures of bovine aortic endothelial cells were treated with *o*-Phen, $ZnCl_2$, Zn-Phen, Zn- DMP, $CuCl_2$, Cu-Phen, Cu-DMP, $RhCl_3$, Rh-Phen, or Rh-DMP (1, 5, 10, or 20 μM each) for 8 h and then, intracellular [A] ^{66}Zn, [B] ^{63}Cu, and [C] ^{103}Rh were analyzed using ICP-MS. Values are mean ± S.E. of three samples. Significantly different from the corresponding control, $^{**}P < 0.01$.

Section 3. Induction of syndecan-4 by organic-inorganic hybrid molecules with a 1,10-phenanthroline structure in vascular endothelial cells

2. 3. 1. Abstract

Organic-inorganic hybrid molecules are expected as an analytical tool for biological systems. Vascular endothelial cells synthesize and secrete proteoglycans, a macromplecules consist a core protein and glycosaminoglycan side chains. Although it has been reported that the expression of endothelial proteoglycans are regulated by several cytokines/growth factors, there may be alternative pathways for proteoglycan synthesis other than the downstream pathways activated by the cytokines/growth factors. Herein, we searched organic-inorganic hybrid molecules for a tool to analyze the expression of syndecan-4, a transmembrane type heparan sulfate proteoglycan, and found 1,10-phenanthroline (*o*-Phen) with or without zinc (Zn-Phen) or rhodium (Rh-Phen). Bovine aortic endothelial cells in culture were treated with the compounds and the expression of syndecan-4 mRNA and core protein was determined by real-time RT-PCR and western blot analysis, respectively. The experiments indicate that *o*-Phen and Zn-Phen specifically and strongly induced the expression of syndecan-4 in cultured vascular endothelial cells through activation of the hypoxia inducible factor-1α/β pathway by inhibition of prolyl hydroxylase 2. The present study demonstrates an alternative pathway that mediate the induction of endothelial syndecan-4 expression and showed organic-inorganic hybrid molecules as an excellent tool to analyze biological systems.

2. 3. 2. Introduction

Vascular endothelial cells are located in the luminal surface in a monolayer and play a crucial role in regulating in the blood coagulation-fibrinolytic system by synthesizing and secreting several physiological factors. For example, von Willebrand factor and tissue factor derived from the cells promote blood coagulation (Jaffe et al., 1974; Maynard et al., 1977). On the other hand, prostacyclin suppresses platelets aggregation (Revtyak et al., 1987); in addition, tissue plasminogen activator converts plasminogen to plasmin (Levin and Loskutoff, 1982) that degrades fibrin. Proteoglycans that consist of core protein and one or more glycosaminoglycan side chain(s) exist in cell surface and extracellular matrix (Ruoslahti, 1988). Vascular endothelial cells synthesis and secrete two types of proteoglycans —heparan sulfate proteoglycans and dermatan sulfate proteoglycans—. Perlecan is a large heparan sulfate proteoglycan which is a component of basement membrane (Saku and Furthmayr, 1989); the syndecan family is transmembrane type heparan sulfate proteoglycans (Kojima et al., 1992); and the glypican family is glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans (Kojima et al., 1992; Mertens et al., 1992; Saku and Furthmayr, 1989). Vascular endothelial cells synthesize and secrete small leucine-rich proteoglycans —biglycan and decorin— (Schönherr et al., 1999; Yamamoto et al., 2005). Heparan sulfate chains and dermatan sulfate chains activate antithrombin III and heparin cofactor II, respectively, and contribute to the anticoagulant property of vascular endothelium (Mertens et al., 1992; Tollefsen et al., 1983).

Clarification of the mechanisms for regulation of proteoglycan expression is important for understanding the regulation of vascular endothelial cell functions. It has been reported that various cytokines and growth factors regulate the expression of proteoglycans in vascular endothelial cells. For example, vascular endothelial growth factor-165 and fibroblast growth factor-2 induce the expression of perlecan and biglycan, respectively (Kaji et al., 2006; Kinsella et al., 1997). When cell density is low, connective tissue growth factor upregulates decorin but downregulates biglycan (Kaji et al., 2004). In addition, transforming growth factor-β_1 enhances the expression of perlecan and biglycan in a cell density-dependent manner (Kaji et al., 2000). Recently, we have found that transforming growth factor-β_1 first upregulates and then downregulates the expression of syndecan-4 in vascular endothelial cells (Hara et al., 2016b).

However, since it is likely that regulation of endothelial proteoglycan synthesis by growth factors/cytokines is mediated by the downstream signal pathway(s) of the corresponding receptor, it may be difficult to find alternative pathways that regulate endothelial proteoglycan synthesis in studies using growth factors/cytokines as an analytical tool.

Organic-inorganic hybrid molecules are compounds that consist of ligand(s) and metal(s). The molecules have been utilized as synthetic reagents for chemical reactions and have markedly contributed to the development of organic synthetic chemistry. On the other hand, organic-inorganic hybrid molecules have not been generally used in biological studies. Recently, we reported that several organic-inorganic hybrid molecules show distinctive biological activities in cellular responses; the activities including cytotoxicity were not equal to either the organic structure or inorganic metal that formed the hybrid molecules (Kohri et al., 2015; Murakami et al., 2015; Hara et al., 2016a). This suggests that organic-inorganic hybrid molecules may be a good tool to analyze biological systems in certain cases. In fact, we successfully analyzed the intracellular signal pathways that mediate endothelial metallothionein induction using copper/zinc complexes (Fujie et al., 2016b; Fujie et al., 2016d).

In the present study, we attempted to analyze the intracellular signal pathways that mediate syndecan-4 expression in vascular endothelial cells using 1,10-phenanthroline (*o*-Phen) with or without zinc (Zn-Phen) or rhodium (Rh-Phen). It was found that *o*-Phen, Zn-Phen, and Rh-Phen upregulated the expression of syndecan-4 in vascular endothelial cells; the strength of the upregulation was *o*-Phen > Zn-Phen > Rh-Phen. We investigated the intracellular signal pathways that mediate the upragulation of endothelial syndecan-4 expression using these compounds.

2. 3. 3. Results

o-Phen, Zn-Phen, and Rh-Phen induces syndecan-4 in vascular endothelial cells

Since *o*-Phen, Zn-Phen, and Rh-Phen at 5 μM do not exhibit cytotoxicity to vascular endothelial cells (Hara et al., 2016a), the effects of these compounds on the expression of proteoglycans in vascular endothelial cells was investigated at this concentration (Fig. 2-3-1). After a 24-h incubation, syndecan-4 mRNA was significantly increased by *o*-Phen (5.83-fold), Zn-Phen (3.17-fold), and Rh-Phen (1.71-fold).

Figure 2-3-2 shows the expression of syndecan-4 core protein and mRNA in vascular endothelial cells after treatment with Zn-Phen, Rh-Phen, *o*-Phen, $ZnCl_2$, and $RhCl_3$. *o*-Phen (Fig. 2-3-2A and 2-3-2C), Zn-Phen (Fig. 2-3-2A), and Rh-Phen (Fig. 2-3-2C) increased the syndecan-4 core protein expression in the cell layer; the core protein was not detected in the conditioned medium. However, both $ZnCl_2$ (Fig. 2-3-2A) and $RhCl_3$ (Fig. 2-3-2C) failed to increase the endothelial syndecan-4 expression. The expression of syndecan-4 mRNA was significantly increased by *o*-Phen (Fig. 2-3-2B and 2-3-2D), Zn-Phen (Fig. 2-3-2B), and Rh-Phen (Fig. 2-3-2C) in a time-dependent manner; zinc chloride (Figure 2-3-2B) and rhodium chloride (Fig. 2-3-2D) did not exhibit such an activity. The activity of *o*-Phen was marked, that of Zn-Phen was moderate, and that of Rh-Phen was weak (Fig. 2-3-2B and 2-3-2D). The expression of endothelial syndecan-4 was induced by Zn-Phen and Rh-Phen in a dose-dependent manner at 2 μM and more (Sup. Fig. 2-3-1). These results indicate that *o*-Phen has an ability to induce endothelial syndecan-4 expression and zinc and rhodium reduce the ability moderately and markedly, respectively, by binding to the *o*-Phen structure.

The HIF-1α/β pathway specifically mediates upregulation of syndecan-4 by *o*-Phen and Zn-Phen

Since it has been reported that *o*-Phen induces hypoxia inducible factor (HIF) (Cho et al., 2013), we investigated whether Zn-Phen and Rh-Phen also induce the expression of HIF-1α in vascular endothelial cells (Fig. 2-3-3A). *o*-Phen and Zn-Phen significantly increased the expression of HIF-1α protein. However, Rh-Phen, $ZnCl_2$, and $RhCl_3$ failed to induce the expression of HIF-1α protein.

To examine the involvement of HIF-1α in the syndecan-4 induction, vascular endothelial cells were transfected with siHIF-1α and then treated with *o*-Phen, Zn-Phen, or Rh-Phen, and the expression of syndecan-4 mRNA expression was determined. The induction of syndecan-4 mRNA by *o*-Phen and Zn-Phen was significantly suppressed by HIF-1α siRNA transfection (Fig. 2-3-3B). Although siRNA-mediated knockdown of HIF-2α, an isoform of the HIF-α subunit, did not influence the expression of syndecan-4 mRNA (Fig. 2-3-3C), that of HIF-1β, a cofactor of HIF-α, significantly reduced the induction of endothelial syndecan-4 mRNA by *o*-Phen and Zn-Phen (Fig. 2-3-3D). siRNA-mediated knockdown of aryl hydrocarbon receptor (AhR), which forms a complex with HIF-1β in the nuclear, failed to reduce the induction of endothelial syndecan-4 expression by *o*-Phen and Zn-Phen (Sup. Fig. 2-3-2), suggesting that AhR is not involved in the induction. It is suggested that induction of endothelial syndecan-4 expression by *o*-Phen and Zn-Phen is mediated by the HIF-1α/β pathway without involvement of HIF-2α and AhR.

_o_-Phen and Zn-Phen inhibit prolyl hydroxylase domain containing protein 2 (PHD2) activity

The expression level of HIF-1α is regulated by degradation by the ubiquitin-proteasome system after hydroxylation of proline residues in the molecule. PHD2 hydroxylates Pro-564 in the oxygen-dependent degradation domain (ODD) of HIF-1α, resulting in promotion of the HIF-1α degradation by the ubiquitin-proteasome system (Ivan et al., 2001). We constructed pcDNA-ODD-Luc vector and transfected it into vascular endothelial cells (Fig. 2-3-4A). The cells were treated with *o*-Phen, Zn-Phen, and Rh-Phen and the PHD2 activity was measured (Fig. 2-3-4B). The activity was significantly reduced by *o*-Phen and Zn-Phen but not by Rh-Phen. At that time, the amount of ubiquitinated proteins was not changed by treatment with *o*-Phen or Zn-Phen (Fig. 2-3-4C), indicating that the induction of HIF-1α by *o*-Phen and Zn-Phen is not due to a stimulation of the ubiquitin-proteasome system.

2. 3. 4. Discussion

Previously, we have demonstrated that the cytotoxicity of organic-inorganic hybrid molecules does not depend on the hydrophobicity of the molecules and the cytotoxicity of intramolecular metal in the inorganic form (Kohri et al., 2015; Murakami et al., 2015). Specifically, the cytotoxicity of hybrid molecules with a certain molecular structure is caused via an interaction between the molecular structure and the metal atom. It is likely that not only cytotoxicity but also nontoxic biological activities depend on the interaction between the molecular structure and the metal atom. In the present study, we compared the effects of *o*-Phen, Zn-Phen, and Rh-Phen on the synthesis of proteoglycans in vascular endothelial cells, and found that these hybrid molecules induce the expression of syndecan-4 expression in order of *o*-Phen, Zn-Phen, and Rh-Phen. In addition, it was suggested that the mechanism of syndecan-4 induction is mediated by the HIF-1α/β pathway activated by inhibition of PHD2 activity. Little is known about the regulation of syndecan-4 synthesis. The expression of syndecan-4 is increased in vascular smooth muscle cells (Cizmeci-Smith et al., 1997) and osteoblasts (Song et al., 2007) but decreased in satellite cells (Velleman et al., 2008) after exposure to fibroblast growth factor-2. In vascular endothelial cells, tumor necrosis factor-α (Okuyama et al., 2013; Zhang et al., 1999), lipopolysaccharide, and interleukin-1β (Vuong et al., 2015) are known as syndecan-4 inducers. However, the signal pathways that mediate syndecan-4 expression has been unclear. Recently, it was reported that the HIF-1-PHD2 axis upregulates the syndecan-4 expression in cultured nucleus pulposus cells (Fujita et al., 2014). In fact, there are consensus HRE sequence (ACGTG) at -571 bp from the promoter region of the syndecan-4 gene in bovine cells (at approximately -1.6 kbp in human cells), according to NCBI data. The new finding of the present study is to reveal that the HIF-1α/β pathway mediates the upregulation of syndecan-4 expression in vascular endothelial cells, using organic-inorganic hybrid molecules—Zn-Phen and Rh-Phen— and their ligand, *o*-Phen, suggesting that organic-inorganic hybrid molecules are a good tool to analyze biological systems.

Activation of the HIF-1α/β pathway by *o*-Phen and Zn-Phen is postulated to be due to a lower activity of PHD2. *o*-Phen has been used as a reagent to measure Fe(II) in environmental water by its chelating effect of divalent metal (Hoshi et al., 1989). Since PHD2 has Fe(II) in the

active center, it is postulated that *o*-Phen pulls out the Fe(II) from the PHD2 molecules and reduces the activity. The induction of endothelial HIF-1α expression by Zn-Phen was weaker than that by *o*-Phen, and Rh-Phen did not induce the HIF-1α expression. There are coordinate bonds between zinc atom and the ligand in the Zn-Phen molecules, and Rh-Phen molecules has covalent bonds between rhodium atom and the ligand. It is likely that chelating activity of *o*-Phen for Fe(II) of PHD2 is reduced by zinc atom and rhodium atom moderately and strongly, respectively, resulting in a moderate and strong reduction by Zn-Phen and Rh-Phen, respectively, of the HIF-1α induction by *o*-Phen. However, the PHD2 activity was inhibited by *o*-Phen and Zn-Phen in a similar degree, the detailed mechanisms underlying the effects of *o*-Phen, Zn-Phen, and Rh-Phen on endothelial syndecan-4 expression remains to be elucidated. However, the different activity of *o*-Phen, Zn-Phen, and Rh-Phen on the syndecan-4 expression may imply some important mechanisms that regulate the synthesis of syndecan-4 in vascular endothelial cells.

Either HIF-1α or HIF-2α forms a heterodimer with HIF-1β, and both HIF-1α/ HIF-1β and HIF-2α/HIF-1β heterodimers recognize the same consensus sequence, hypoxia response element (HRE), in the promoter regions of hypoxia inducible genes. It was shown that HIF-1α but not HIF-2α is involved in the induction of endothelial syndecan-4 by *o*-Phen and Zn-Phen. Several reports showed that transcriptional factors bound to HRE in the promoter regions of hypoxia inducible genes are highly selective (Mole et al., 2009; Schodel et al., 2011). On the other hand, recently, it was shown that the intranuclear localization of HIF-1α is different from that of HIF-2α, and the half-life of HIF-1α is much shorter than that of HIF-2α (Taylor et al., 2016). As these reports stated, it is possible that there may be epigenetic mechanisms or specific cofactors that interact to HIF-α, which are involved in the modulation of the HIF-α subunit specific genes expression. Therefore, it is likely that the syndecan-4 induction by *o*-Phen and Zn-Phen is mediated by HIF-1α but not HIF-2α in vascular endothelial cells.

Syndecan-4 was originally isolated from rat microvascular endothelial cells as a molecule that binds antithrombin III (Kojima et al., 1992; Shworak et al., 1994). It is reported that the amount of heparan sulfate chains of vascular endothelial cell surface is decreased under hypoxic conditions, whereas the relative synthesis of heparan sulfate chains that bind antithrombin III

is increased under such conditions (Karlinsky et al., 1992). In the present study, it was shown that the syndecan-4 expression in vascular endothelial cells is elevated by the HIF-1α/β pathway that is activated by a lower PHD2 activity, suggesting that hypoxic conditions cause a higher expression of endothelial syndecan-4. It is postulated that syndecan-4 is increased but other types of heparan sulfate proteoglycans such as perlecan may be decreased in vascular endothelial cells under hypoxic conditions. In fact, a lower expression of perlecan mRNA was observed in vascular endothelial cells after treatment with *o*-Phen, Zn-Phen, or Rh-Phen in the present study. Vascular endothelial cells under hypoxic conditions, for example in the unstable angina, the cells would promote the syndecan-4 synthesis and prevent the vascular occlusion through reinforcing the anticoagulant property, which is partly due to the activation of antithrombin III by heparan sulfate chains of syndecan-4, of vascular endothelium. Bio-organometallics— a biology of organic-inorganic hybrid molecules— may be an effective strategy to analyze the regulation of vascular endothelial cells.

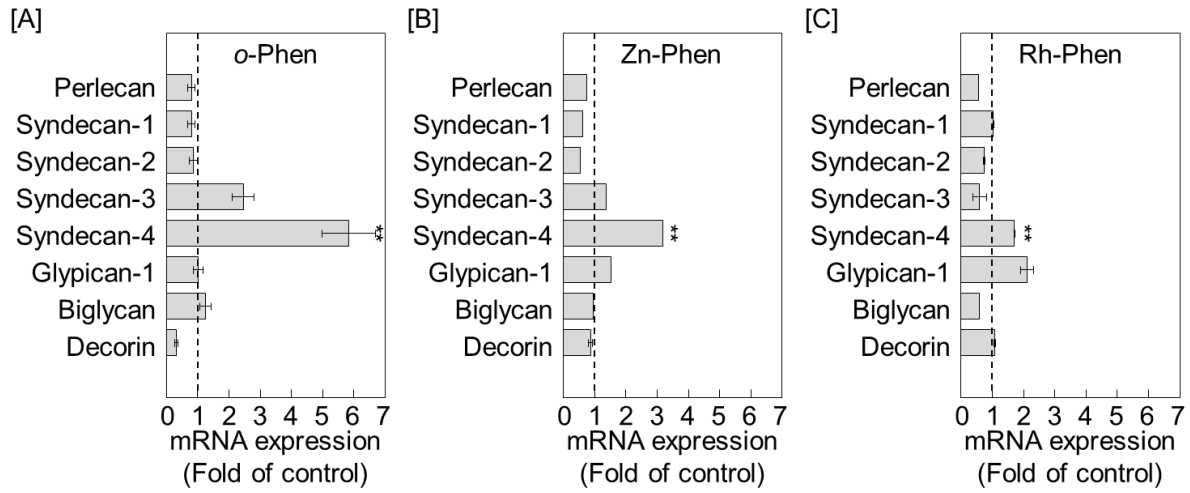


Fig. 2-3-1. Effects of *o*-Phen, Zn-Phen, and Rh-Phen on the expression of proteoglycans in vascular endothelial cells. Bovine aortic endothelial cells were treated with [A] *o*-Phen, [B] Zn-Phen, or [C] Rh-Phen at 5 μM at 37°C for 24 h. Values are means ± S.E. of four samples. $^{**}P < 0.01$ vs the corresponding control.

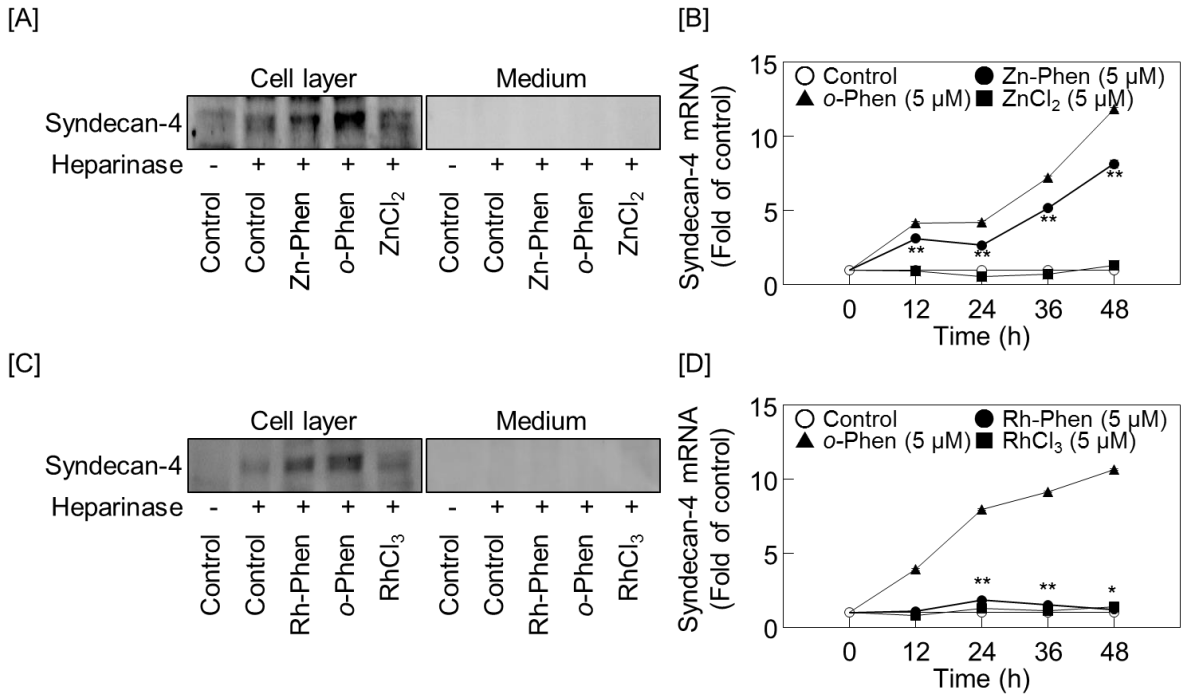


Fig. 2-3-2. Effects of *o*-Phen, Zn-Phen, Rh-Phen, $ZnCl_2$, and $RhCl_3$ on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were treated with [A and B] Zn-Phen *o*-Phen, or $ZnCl_2$ and [C and D] Rh-Phen, *o*-Phen, or $RhCl_3$ at 5 μM each at 37°C for 24 h [A and C], or for or for 12, 24, 36, and 48 h [B and D]. Values are means ± S.E. of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control

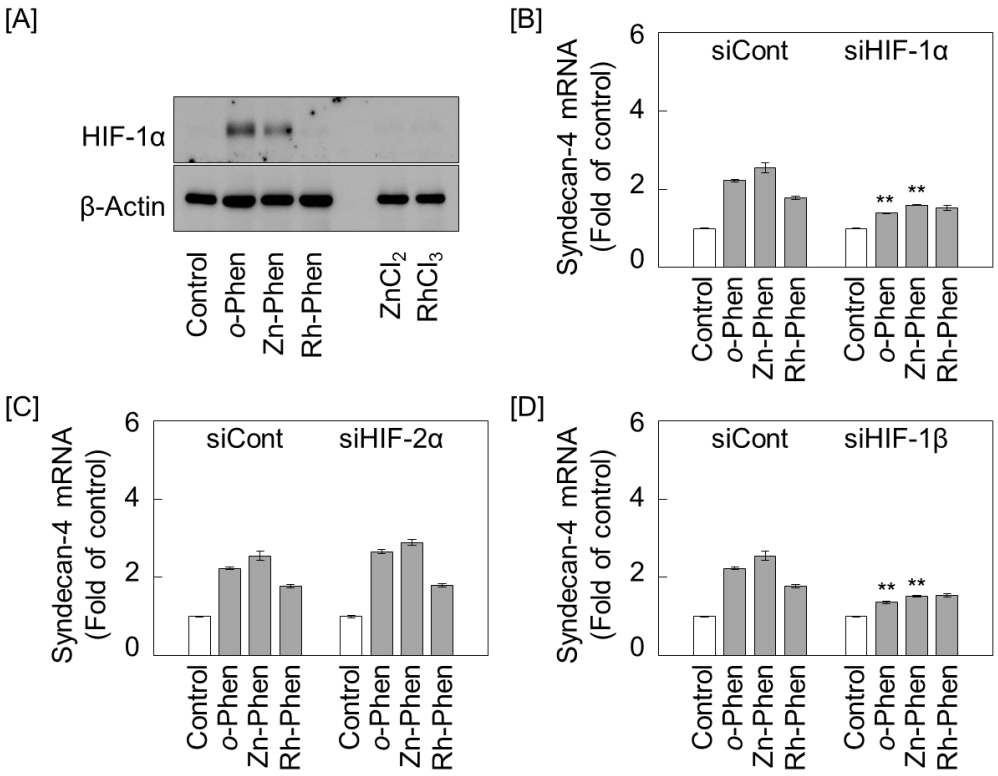


Fig. 2-3-3. Involvement of HIF-1*α*, HIF-2*α*, and HIF-1β in the induction of syndecan-4 by of *o*-Phen, Zn-Phen, and Rh-Phen in vascular endothelial cells. [A] The expression of HIF-1α. Bovine aortic endothelial cells were treated with *o*-Phen, Zn-Phen, Rh-Phen, $ZnCl_2$ or $RhCl_3$ at 5 μM each at 37°C for 1 h. [B] Effects of siRNA-mediated knockdown of HIF-1α on the expression of syndecan-4 mRNA. Bovine aortic endothelial cells were transfected with siHIF-1α at 37°C for 12 h and treated with *o*-Phen, Zn-Phen, or Rh-Phen at 5 μM each at 37°C for 8 h. [C] Effects of siRNA-mediated knockdown of HIF-2α on the expression of syndecan-4 mRNA. Bovine aortic endothelial cells were transfected with siHIF-2α at 37°C for 12 h and treated with *o*-Phen, Zn-Phen, or Rh-Phen at 5 μM each at 37°C for 8 h. [D] Effects of siRNA-mediated knockdown of HIF-1β on the expression of syndecan-4 mRNA. Bovine aortic endothelial cells were transfected with siHIF-1β at 37°C for 12 h and treated with *o*-Phen, Zn-Phen, or Rh-Phen at 5 μM each at 37°C for 8 h. Values are means ± S.E. of four samples. $^{**}P < 0.01$ vs the corresponding siCont.

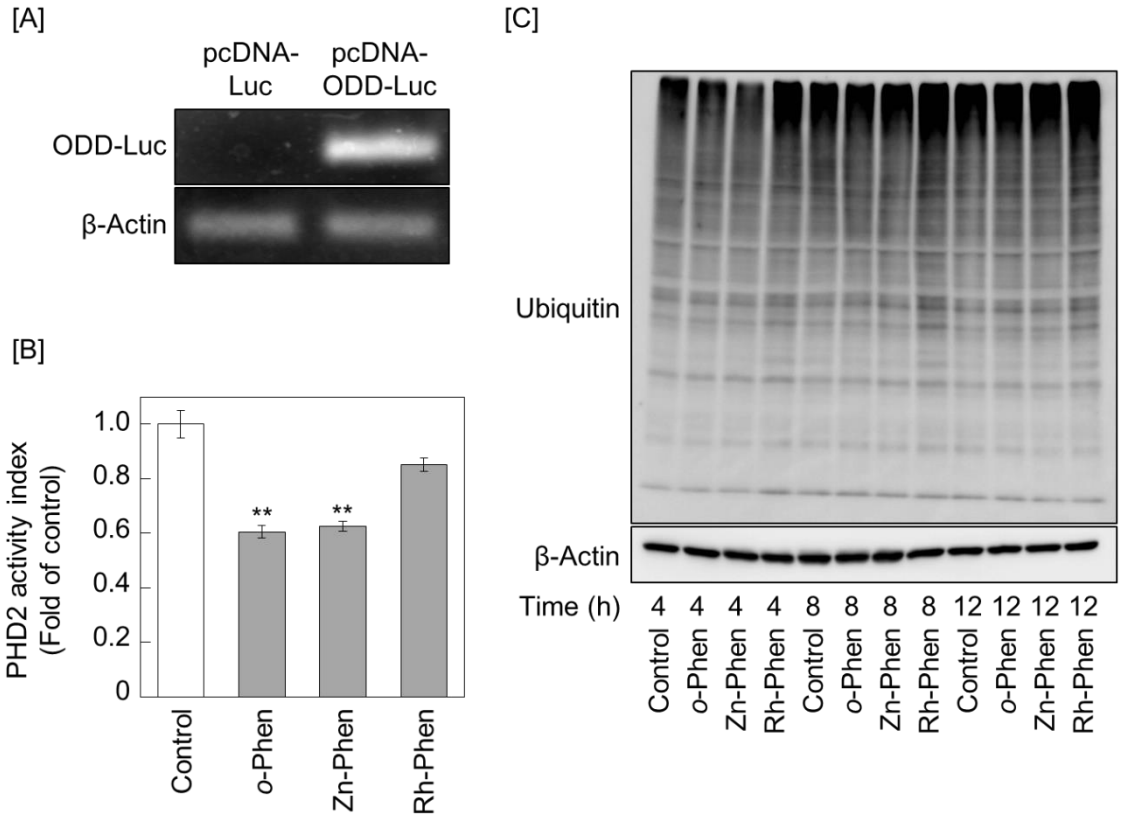
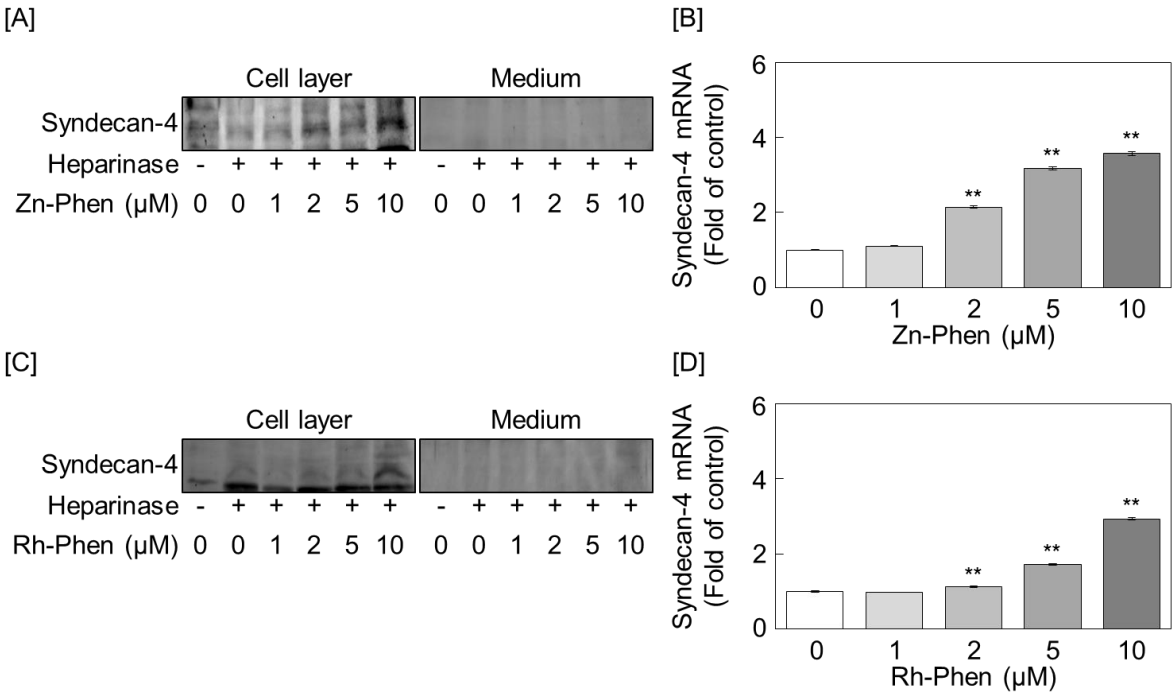
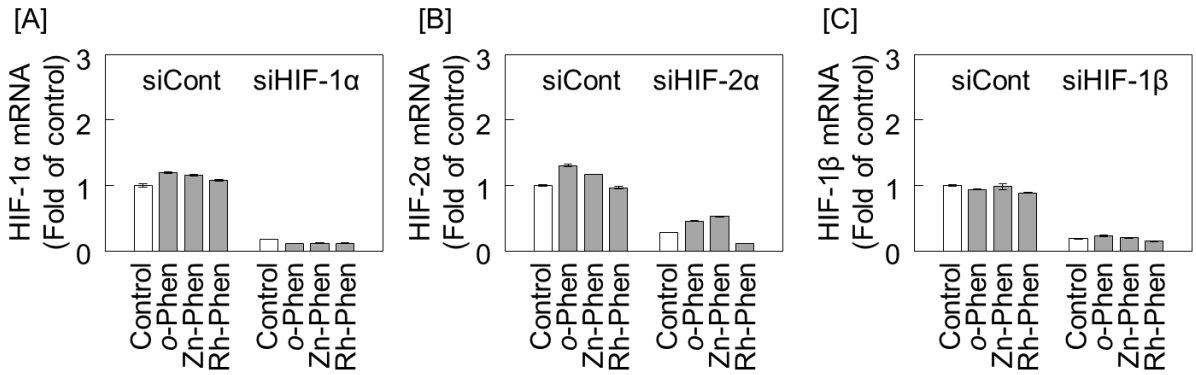


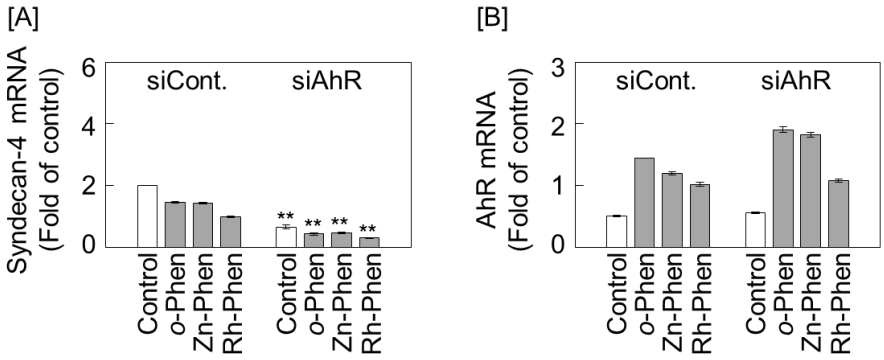
Fig. 2-3-4. Effects of *o*-Phen, Zn-Phen, and Rh-Phen on PHD2 activity in vascular endothelial cells. [A] Amplified DNA products. Bovine aortic endothelial cells were transfected with pcDNA-Luc or pcDNA-ODD-Luc at 37°C for 24 h. [B] PHD2 activity. Bovine aortic endothelial cells were transfected with pcDNA-ODD-Luc at 37°C for 12 h and then treated with *o*-Phen, Zn-Phen, or Rh-Phen at 5 μM each at 37°C for 24 h. Values are means ± S.E. of five samples. $^{**}P < 0.01$ vs the control. [C] The expression of ubiquitinated proteins. Bovine aortic endothelial cells were treated with *o*-Phen, Zn-Phen, or Rh-Phen at 5 μM each at 37°C for 4, 8 and 12 h.



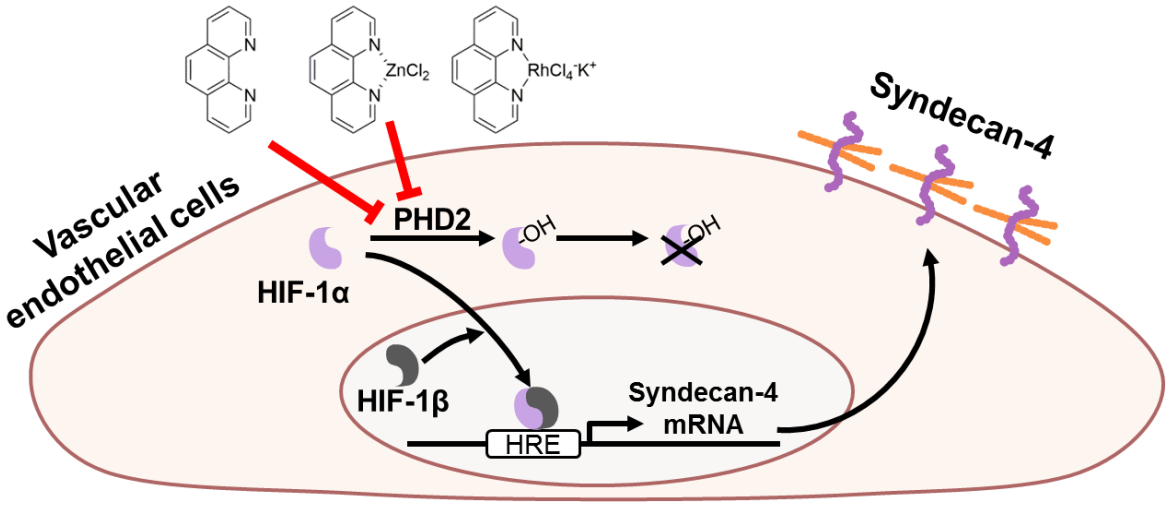
Supplemental Figure 2-3-1. Effects of the concentration of Zn-Phen and Rh-Phen on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were treated with [A and B] Zn-Phen or [C and D] Rh-Phen at 1, 2, 5, or 10 μM at 37°C for 24 h. Values are means ± S.E. of four samples. $^{**}P < 0.01$ vs corresponding control.



Supplemental Figure 2-3-2. siRNA-mediated knockdown of HIF-1α, HIF-2α, and HIF-1β in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siHIF-1α, siHIF-2α, or HIF-1β and the mRNA of the corresponding HIF was determined. Values are means ± S.E. of four samples. $^{**}P < 0.01$ vs the corresponding siCont.



Supplemental Figure 2-3-3. Involvement of AhR in the induction of syndecan-4 mRNA by *o*-Phen, Zn-Phen, and Rh-Phen in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siAhR at 37°C for 12 h and treated with *o*-Phen, Zn-Phen, or Rh-Phen at 5 μM each at 37°C for 8 h. [A] The expression of AhR mRNA. [B] The expression of syndecan-4 mRNA. Values are means ± S.E. of four samples. $^{**}P < 0.01$ vs the corresponding siCont.



Scheme 2. Figure summary of Chapter 2

General conclusion

Proteoglycans regulate vascular endothelial cell functions via interaction with various kinds of biological factors. Although the effects of growth factors/cytokines on the proteoglycans synthesized of vascular endothelial cells have been investigated, imbalance among types of proteoglycans observed in atherosclerotic vascular wall have been incompletely explained. In addition, there are few reports about the tools for analysis of mechanisms underlying proteoglycan expressions. In this study, two novel research strategies—crosstalk among proteoglycans and bio-organometallics— were applied to this clarification and the following results were obtained.

Chapter 1. Strategy based on crosstalk among proteoglycan

Histopathological observations have shown that proteoglycans in atherosclerotic regions are varied types to types. Specifically, chondroitin and dermatan sulfate proteoglycans are increased, while heparan sulfate proteoglycans are decreased in the intima. Although biglycan, a small leucine-rich dermatan sulfate proteoglycan, highly accumulates in the intima from the early to the late stages of atherosclerosis, the physiological roles of biglycan have been unclear. The expression of endothelial biglycan lowered the expression of syndecan-4, which was due to the TGF-β_1-ALK5-Smad2/3 signaling intensified by biglycan; biglycan served as a co-receptor of TGF-β_1 and ALK5. In the process of the study above, it was observed that the expression of syndecan-4 was first upregulated and then downregulated by TGF-β_1 in vascular endothelial cells. This biphasic modulation of endothelial syndecan-4 synthesis by TGF-β_1 is the second point of this chapter. The early upregulation of syndecan-4 by TGF-β_1 is induced via the Smad3-p38 MAPK pathway. On the other hand, the late downregulation of syndecan-4 synthesis was mainly mediated by Smad3. Since TGF-β_1 is a cytokine highly expressed in atherosclerotic vascular wall, the biphasic regulation of endothelial syndecan-4 by the cytokine may contribute to the imbalance between chondroitin/dermatan sulfate proteoglycans and heparan sulfate proteoglycans during the progression of atherosclerosis.

Chapter 2. Strategy based on bio-organometallics

Since organic-inorganic hybrid molecules are expected as useful tools for analyzing

biological functions, we applied these molecules to exploring the systems that modulate proteoglycans independently of the downstream signal transduction of growth factors/cytokines. We chose a non-toxic compound Zn-Phen as a tool for its modulating effects on the expression of endothelial perlecan and syndecan-4, and compared with *o*-Phen, Cu-Phen, and Rh-Phen. Morphological observation and LDH leakage indicated that Cu-Phen has a high toxicity to vascular endothelial cells whereas *o*-Phen, Zn-Phen, and Rh-Phen did not exhibit such a toxicity. Either *o*-Phen, Zn-Phen, or Rh-Phen upregulated the expression syndecan-4; the induction by *o*-Phen and Zn-Phen was stronger than that of Rh-Phen. In addition, *o*-Phen and Zn-Phen increased the expression of HIF-1α and the induction of syndecan-4 expression by *o*-Phen and Zn-Phen was diminished by silencing either HIF-1α or HIF-1β but not HIF-2α. *o*-Phen and Zn-Phen inhibited the activity of PHD2, suggesting that the HIF-1α/1β pathway activated by inhibition of PHD2 mediates endothelial syndecan-4 synthesis.

The first strategy based on the histopathological observation revealed that (1) there are crosstalk mechanisms in the expression among proteoglycan types, which may contribute to the change of proteoglycan expression in atherosclerotic intima; (2) one of the physiological roles of biglycan is to regulate syndecan-4 expression in vascular endothelial cells; and (3) biglycan serves as co-receptor for ALK5 and TGF-β_1. On the other hand, the second strategy based on bio-organometallics, it was revealed for the first time that (4) the biological activity of organic-inorganic hybrid molecules can be modulate by selection of either organic structure or metal; and (5) there is an alternative pathway— the HIF-1α/1β pathway—that upregulates the expression of syndecan-4 independently of the downstream signaling of growth factors/cytokines in vascular endothelial cells. The present study clarified a part of the mechanisms underlying vascular endothelial proteoglycan synthesis based on novel research strategies.

Materials and Methods

Materials

Bovine aortic endothelial cells and bovine aortic smooth muscle cells were obtained from Cell Applications (San Diego, CA, USA). Dulbecco's modified Eagle medium and Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline were purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum was obtained from HyClone Laboratories (Waltham, MA, USA). Tissue culture dishes and plates were purchased from Iwaki (Chiba, Japan). Cytotox 96® Non-Radioactive Cytotoxicity Assay, Firefly luciferase reporter plasmid pGL4.12, *Renilla* luciferase expression plasmid pRL-SV40, and Dual-Luciferase Reporter Assay System were obtained from Promega (Madison, WI, USA). BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 1,10-phenanthroline monohydrate (*o*-Phen), 2,9-dimethyl-1,10-phenanthroline hemihydrate (DMP), 3,4,7,8-teramethyl-1,10-phenanthroline, 5-chloro-1,10-phenanthroline, 4,7-dimethyl-1,10-phenanthroline, 2-chloro-1,10-phenanthroline, bis[2-(2-benzothiazoly)phenolato]zinc(II) (Zn-26), bis(2,4-pentanedionato)zinc (Zn-27), Dichloro(N,N,N',N'-tetramethylethylenediamine)zinc (Zn-31), and bis(8-quinolinolato)zinc (Zn-32) were sourced from Tokyo Chemical Industry (Tokyo, Japan). Copper(II) chloride dihydrate ($CuCl_2$) and a CBB protein assay kit were obtained from Nacalai Tesque, (Kyoto, Japan). TaKaRa Ex Taq, restriction enzymes EcoRI, HindIII, and XbaI were purchased from Takara bio (Shiga, Japan). NEBuilder HiFi DNA Assembly Cloning Kit was obtained from New England Biolabs (Ipswich, MA, USA). Chondroitinase ABC (EC 4.2.2.4, derived from *Proteus vulgaris*), heparinase II (derived from *Flavobacterium heparinum*), heparinase III (EC 4.2.2.8, derived from *Flavobacterium heparinum*), and Diethylaminoethyl–Sephacel (DEAE-Sephacel), 4-methyl-1,10-phenanthroline, dichloro(1,10-phenanthroline)copper(II) (Cu-Phen), and [3,4-toluenedithiolato(2-)]zinc hydrate (Zn-28) were purchased from Sigma-Aldrich (St Louis, MO, USA). PD98059, SP600125, and SB203580 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Recombinant human TGF-β_1 and LY364947, zinc chloride ($ZnCl_2$), rhodium (III) chloride trihydrate ($RhCl_3$), and anti- mouse monoclonal antibody against β-actin were purchased from Wako Purechemical Industries (Osaka, Japan). Rabbit polyclonal antibody against ubiquitin (UG9510) was obtained from Biomol (Hamburg,

Germany). Mouse monoclonal antibody against HIF-1α (610958) was purchased from BD biosciences (Franklin Lakes, NJ, USA). Rabbit polyclonal antibodies against perlecan (H-300), TGF-β_1 (V) or ALK5 (V-22), and goat polyclonal antibodies against biglycan (L-15), syndecan-4 (N-19), or ALK1 (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Smad2/3 (#8685), anti-phospho-Smad2/3 (#8828), anti-ERK1/2 (#9102), anti-phospho-ERK1/2 (#9101), anti-JNK (#9252), anti-phospho-JNK (#9255), anti-p38 MAPK (#9212), anti-phospho-p38 MAPK (#9211), horseradish peroxidase (HRP)-conjugated anti-rabbit (#7074), and HRP-conjugated anti-mouse (#7076) IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated anti-goat IgG antibody (ab6885) was obtained from Abcam (Bristol, UK). pcDNA 3.1(+), Lipofectamine RNAiMAX, Lipofectamine LTX, Opti-MEM, and Mammalian Expression System with Gateway Technology were purchased from Invitrogen (Carlsbad, CA, USA). Polyvinyl difluoride membrane Immobilon-P was obtained from Millipore (Billerica, MA, USA). His Mag Sepharose Ni was purchased from GE Healthcare Bio-Sciences AB (Björkgatan, Sweden). QIAzol lysis reagent was obtained from Qiagen (Hilden, Germany). High-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Waltham, MA, USA). GeneAce SYBR qPCR Mix α (Nippon Gene, Tokyo, Japan). Synthetic siRNAs were obtained from FASMAC (Kanagawa, Japan). Synthetic oligonucleotide primers for real time reverse transcription-polymerase chain reaction (RT-PCR) were purchased from Eurofin Genomics (Tokyo, Japan). Other reagents, which were of the highest grade, were obtained from Nacalai Tesque (Kyoto, Japan).

Synthesis of organo-zinc, copper and rhodium compounds

$ZnCl_2$ was dissolved with *o*-Phen, DMP, 4-methyl-*o*-Phen, 3,4,7,8,-tetramethyl-*o*-Phen, 5-chloro-*o*-Phen, 4,7-dimethyl-*o*-Phen, or 2-chloro-*o*-Phen in ethanol, stirred for 1 h at room temperature, and filtered to obtain a white precipitate of Zn-Phen, Zn-DMP, Zn-13, Zn-14, Zn-15, Zn-19, or Zn-21, respectively. These precipitates were recrystallized from acetonitrile to obtain purified zinc complexes. Other zinc complexes are synthesized according to the references listed in Table 1. A reported procedure was generally followed to synthesize Rh-

Phen and Rh-DMP (Lee et al., 2003). Cu-DMP was synthesized by the reaction of DMP with $CuCl_2 \cdot 2H_2O$ in tetrahydrofuran at room temperature (Wang and Zhong, 2009).

Cell culture and treatment

Bovine aortic endothelial cells and bovine aortic smooth muscle cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum until confluence or subconfluence.

In Chapter 1 - Section 1, the medium was discarded; then vascular endothelial cells were transfected with siRNAs for knockdown of biglycan, TGF-β_1, ALK1, ALK5, or both biglycan and TGF-β_1. In another experiment, cultures of confluent cells were treated with LY364947 or TGF-β_1. The expression levels of proteoglycans and Smad2/3 were determined by real-time RT-PCR or western blot analysis.

In Chapter 1 - Section 2, the medium was discarded; then vascular endothelial cells were transfected with siRNAs for knockdown of Smad2, Smad3, or both and then stimulated with TGF-β_1. In another experiment, confluent cultures of the cells were treated with PD98059, SP600125, or SB203580 and then stimulated with TGF-β_1. The expression levels of syndecan-4, ERK1/2, JNK, p38 MAPK, and Smad2/3 were determined by real-time RT-PCR or western blot analysis.

In Chapter 2 - Section 1, the medium was discarded; then vascular endothelial cells were washed twice with Dulbecco's modified Eagle medium and treated with zinc complexes. The expression levels of perlecan and syndecan-4 were determined by real-time RT-PCR and western blot analysis, respectively.

In Chapter 2 - Section 2, the medium was discarded; then vascular endothelial cells were washed twice with Dulbecco's modified Eagle medium and treated with *o*-Phen, $ZnCl_2$, Zn-Phen, Zn-DMP, $CuCl_2$, Cu-Phen, Cu-DMP, $RhCl_3$, Rh-Phen or Rh-DMP. The cytotoxicity of these compounds was assessed by morphological observation and the leakage of LDH from vascular endothelial cells. The intracellular accumulation of metals was analyzed using inductively coupled plasma mass spectrometry (ICP-MS).

In Chapter 2 - Section 3, the medium was discarded; then vascular endothelial cells were

transfected with pcDNA-Luc or pcDNA-ODD-Luc and then the cells were treated with *o*-Phen, $ZnCl_2$, Zn-Phen, $RhCl_3$, or Rh-Phen after washing twice Dulbecco's modified Eagle medium. In another expresiments, the cells were transfected with siRNAs for HIF-1α, HIF-2α, or HIF-1β and then treated with *o*-Phen, Zn-Phen, or Rh-Phen after washing twice Dulbecco's modified Eagle medium. The expression levels of proteoglycans, HIFs, and ubiquitinated proteins were determined by real-time RT-PCR or western blot analysis, and the PHD2 activity index was analyzed by luciferase assay.

siRNA transfection

Transfection of siRNAs was performed using Lipofectamine RNAiMAX, according to the manufacturer's protocol. Briefly, annealed siRNA duplex and Lipofectamine RNAiMAX were dissolved in Opti-MEM in separate tubes and incubated for 5 min at room temperature. They were then mixed and incubated for 20 min at room temperature. Vascular endothelial cells were grown to about 80% confluence in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and then incubated for 4 h (Chapter 1 - Section 1.), 12 h (Chapter 2 - Section 2.), or 24 h (Chapter 1 - Section 2.) at 37°C in fresh Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and the siRNA/Lipofectamine RNAiMAX mixture. The final concentration of siRNA was 40 nM (Chapter 1 - Section 1. and Chapter 1 - Section 2.) or 18 nM (Chapter 2 - Section 2.), and the final concentration of Lipofectamine RNAiMAX was 0.2% (Chapter 1 - Section 1. and Chapter 1 - Section 2.) or 0.09 % (Chapter 2 - Section 2.). After that, the medium was changed to Dulbecco's modified Eagle medium supplemented with or without 10% fetal bovine serum alone and incubated further. The sequences of sense and antisense siRNAs were shown in Table 2.

Real-time RT-PCR

A monolayer of vascular endothelial cells was washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and lysed with QIAzol lysis reagent. A quarter volume of chloroform was mixed with the lysate, and the mixture was centrifuged. The supernatant was collected, 70% ethanol was added to a concentration of 52.5%, the suspension was centrifuged at 20,000

× *g*, and the supernatant was discarded. The precipitate was suspended again in 70% ethanol and centrifuged at 20,000 × *g*, and the obtained precipitate containing the total RNA was dried. Complementary DNA was synthesized from the mRNA using a high-capacity cDNA reverse transcription kit. Real-time PCR was performed using GeneAce SYBR qPCR Mix α with 1 ng/μl cDNA and 0.1 μM primers (Table 3) on a StepOnePlus Real-Time PCR System (Applied Biosystems). The levels of biglycan, decorin, perlecan, syndecan-1, syndecan-2, syndecan-3, syndecan-4, glypican-1, TGF-β_1, ALK1, ALK5, Smad2, Smad3, HIF-1α, HIF-2α, HIF-1β, AhR, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β2-microgloblin (B2M), and β-actin mRNA were quantified by the relative standard curve method. The fold change of the intensity value of syndecan-4 was normalized by that of GAPDH. In Chapter 1, the fold changes for each gene were normalized by the intensity value of GAPDH. In Chapter 2, the experimental group including Rh-Phen or siRNA transfection, fold change of the intensity value of target gene was normalized by that of B2M, and the other cases, fold change of the intensity value of target gene was normalized by that of β-actin.

Proteoglycan core protein extraction and western blot analysis

Proteoglycans that accumulated in the cell layer and conditioned medium of vascular endothelial cells were extracted under dissociative conditions. Specifically, the conditioned medium was harvested and solid urea was added at a concentration of 8 M. The cell layer was washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and lysed with 8 M urea cell extract solution (pH 7.5) containing 120 mM 6-aminohexanoic acid, 12 mM benzamidine, 10 mM N-ethylmaleimide, 2 mM eEthylenediaminetetraacetic acid (EDTA), 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100. The extracts were applied to DEAE-Sephacel (0.3 mL of resin) columns and washed four times with 8 M urea buffer (pH 7.5) containing 0.25 M NaCl, 2 mM EDTA, 0.5% Triton X-100, and 50 mM Tris base. Proteoglycans were eluted with 0.9 mL 3 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.5% Triton X-100, and 50 mM Tris base, and precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol for 2 h at -20°C; this precipitation step was repeated three times. Precipitated proteoglycans were digested with heparinase II/III in 100 mM Tris-

HCl buffer (pH 7.0) containing 10 mM calcium acetate and 18 mM sodium acetate or with chondroitinase ABC in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mg/mL bovine serum albumin and 3 mM sodium acetate for 3 h at 37°C to determine core proteins of heparan sulfate and dermatan sulfate proteoglycans, respectively. Separately, total proteins from vascular endothelial cells were prepared by lysis in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl buffer solution containing 2% SDS and 10% glycerol, pH 6.8) followed by incubation at 95°C for 10 min. The protein concentration was determined using a BCA Protein Assay Kit before addition of 2-mercaptoethanol and bromophenol blue to the samples. The proteoglycans or cellular proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% or 4-16% polyacrylamide gel and then transferred onto a polyvinyl difluoride membrane at 2 mA/cm^2 for 1 h following the method of Kyhse-Andersen (Kyhse-Andersen, 1984). Membranes were blocked with 2% bovine serum albumin solution or 5% skim milk in 20 mM Tris-HCl buffer solution (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20, and then incubated overnight with a primary antibody against biglycan, perlecan, syndecan-4, ALK1, ALK5, Smad2/3, phosphorylated Smad2/3, ERK1/2, phosphorylated ERK1/2, JNK, phosphorylated JNK, p38 MAPK, phosphorylated p38 MAPK, HIF-1α, or β-actin at 4°C. The membranes were washed and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence western blotting detection reagents (Chemi-Lumi One L, Nacalai Tesque, Kyoto, Japan) and scanned with a LAS 3000 Imager (Fujifilm, Tokyo, Japan).

Ni^{2+} pull-down assay

To prepare 6× His-tagged biglycan, the pDEST26-BGN (NM_001711.3) plasmid vector was constructed using a Mammalian Expression System with Gateway Technology, and the vector was transfected into vascular endothelial cells with Lipofectamine LTX and PLUS reagent. One μg/μL of plasmid vector and PLUS reagent in Opti-MEM and Lipofectamine LTX in Opti-MEM were prepared in separate tubes and mixed; the mixture was then incubated for 5 min at room temperature. Vascular endothelial cells at about 80% confluence were incubated in the mixture for 1 h at 37ºC; the final concentration of the vector, PLUS reagent, and Lipofectamine

RNAiMAX were 1.7 μg/mL, 0.17%, and 0.35%, respectively. The pDEST26 vector was used as a control. After incubation, the medium was changed to Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and the cells were further incubated for 24 h. After incubation, proteoglycans that had accumulated in the conditioned medium were extracted, concentrated, and digested with chondroitinase ABC as described above in "Proteoglycan core protein extraction and western blot analysis." The binding of biglycan to either TGF-β_1 or ALK5 was analyzed by a Ni^{2+} pull-down assay as follows: 40 μL of His Mag Sepharose Ni was equilibrated with a binding/wash buffer, 25 mM Tris-HCl containing 150 mM NaCl and 5 mM imidazole (pH 7.5). Chondroitinase ABC-digested proteoglycans were mixed with either 500 ng recombinant human TGF-β_1 or 50 μg of membrane proteins extracted from endothelial cells using the ProteoExtract Native Membrane Protein Extraction Kit (Merck KGaA, Darmstadt, Germany) and incubated with the beads for 30 min. After incubation, the beads were washed four times with binding/wash buffer and boiled with elution buffer, 50 mM Tris-HCl containing 8% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.005% bromophenol blue, and 500 mM imidazole (pH 6.8) at 95°C for 3 min. The supernatant was collected and used to determine complexes of biglycan with TGF-β_1 or ALK5 by western blot analysis.

Plasmid construction

Firefly luciferase reporter plasmid pGL4.12 was treated with restriction enzymes, HindIII and XbaI. The fragments containing luciferase was inserted into pcDNA3.1(+) cleaved with these two restriction enzymes to make a luciferase-containing pcDNA (pcDNA-Luc). Bovine HIF-1α ODD cDNA and stop codon insertion (STOP) cDNA were cloned from RT-PCR products of bovine vascular endothelial cells using TaKaRa Ex Taq and primers. ODD primers were designed using NCBI database (Accession No. NM_174339_3) and NEBuilder Assembly Tool. The sequence of forward and reverse primers were as follows: ODD, 5′-CTGGCTAGCGTTTAAACTTAATGTTCAAGTTGGAATTGG-3' and 5′-CCAACAGTACCGGATTGCCAAGGAGGTTCTTTAGGTACG-3', respectively; STOP, 5′-GAGAATTCATAACACCCTCAAGATTGTCAGCAA-3' and 5′-CACTCTAGAACAGTCTTCTGGGTGGCAGTGA-3', respectively. The PCR product of

ODD was assembled with pcDNA-Luc cleaved with HindIII, using NEBuilder HiFi DNA Assembly Cloning Kit. After that, the PCR products of STOP treated with both EcoRI and XbaI were inserted into assembled plasmid cleaved with these two restriction enzymes to make pcDNA-ODD-Luc. The DNA sequence of pcDNA-ODD-Luc was analyzed by Eurofin Genomics (Tokyo, Japan). The mRNA expression of ODD-Luc was checked by Real-time RT-PCR. The sequence of forward and reverse primers for ODD-Luc was 5′-CTGGCTAACTAGAGAACCCACTG-3’ and 5′-AGGGGCAAACAACAGATGG-3’, respectively. The amplicon was electrophoresed in 1% agarose gel, stained with ethidium bromide, and visualized using LAS 3000 Imager.

Luciferase assay

pcDNA-ODD-Luc and *Renilla* luciferase expression plasmid pRL-SV40 vector (Promega, Fitchburg, WI, USA) were transfected into vascular endothelial cells with Lipofectamine LTX and PLUS reagent according to the manufacture’s instruction. Confluent cultures of endothelial cells were prepared in 24-well culture plates. Plasmid vectors and PLUS reagent in Opti-MEM and Lipofectamine LTX in Opti-MEM were prepared in separate tubes and mixed; the mixture was then incubated for 5 min at room temperature. The mixture was added to the fresh Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and the cells were incubated at 37°C. The final concentrations of pcDNA-ODD-Luc, pRL-SV40, Lipofentamine LTX, and PLUS reagent were 230 ng/mL, 0.46 ng/mL, 0.09%, and 0.04% respectively. After 12 h, the medium was discarded and the cells were incubated with 5 μM of *o*-Phen, Zn-Phen or Rh-Phen. Twenty-four hours later, luciferase activity of the cells was measured using Dual-Luciferase Reporter Assay System and GloMax 20/20n luminometer (Promega). The luminescence intensity value of Firefly luciferase was normalized by that of *Renilla* luciferase. The PHD2 activity index was calculated from the reciprocal of relative luciferase activity.

Cytotoxicity assay

Confluent cultures of vascular endothelial cells were incubated with *o*-Phen, $ZnCl_2$, Zn-Phen,

Zn-DMP, $CuCl_2$, Cu-Phen, Cu-DMP, $RhCl_3$, Rh-Phen, or Rh-DMP for 24 h. After incubation, the treated medium was collected and used for the determination of LDH activity, as a marker of non-specific cytotoxicity, according to the manufacturer's protocol.

Intracellular accumulation of zinc, copper, and rhodium

Confluent cultures of vascular endothelial cells were incubated with *o*-Phen, $ZnCl_2$, Zn-Phen, Zn-DMP, $CuCl_2$, Cu-Phen, Cu-DMP, $RhCl_3$, Rh-Phen, or Rh-DMP (1, 5, 10, and 20 μM each) for 8 h. After incubation, the medium was discarded and the cell layer was washed and harvested with icecold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. The cell suspension was centrifuged at 20,000 × *g* for 2 min and the pellet was re-suspended in 500 μL of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and sonicated. Cell debris was precipitated by centrifugation at 1,000 × *g* for 2 min and the supernatant (100 μL) was added to 4.9 mL of 0.1 M nitric acid; the mixture was used for the detection of intracellular zinc, copper, and rhodium by ICP-MS (Nexion 300S, PerkinElmer, Waltham, MA, USA) as an indicator of intracellular accumulation of tested compounds. A portion of the supernatant was analyzed for protein concentration using a CBB protein assay kit according to the manufacturer's protocol. The metal content was expressed as nmol/mg protein.

Statistical analysis

Data were tested for statistical significance by analysis of variance and Student's *t*-test, Tukey's or Dunnett's method, as appropriate. *P* values of less than 0.05 or 0.01 were considered statistically significant differences.

Table 2

Bovine gene-specific siRNAs

Gene		sequence (5′→3′)
Biglycan-1 (siBGN-1)	Sense	CCAUCCAGUUUGGCAACUAdTdT
	Antisense	UAGUUGCCAAACUGGAUGGCC
Biglycan-2 (siBGN-2)	Sense	GCUCCGACCUGGGUCUGAAdTdT
	Antisense	UUCAGACCCAGGUCGGAGCAC
TGF-β_1 (siTGFB1)	Sense	GCGUGCUAAUGGUGGAAUAdTdT
	Antisense	UAUUCCACCAUUAGCACGCGG
ALK1 (siALK1)	Sense	CCAGCUUUGAGGACAUGAAdTdT
	Antisense	UUCAUGUCCUCAAAGCUGGGG
ALK5 (siALK5)	Sense	CCAUCGAGUGCCAAAUGAAdTdT
	Antisense	UUCAUUUGGCACUCGAUGGUG
Smad2 (siSmad2)	Sense	UUCAAAACCCUGAUUAACGdTdT
	Antisense	CGUUAAUCAGGGUUUUGAAdTdT
Smad3 (siSmad3)	Sense	UGUUUUCGGGGAUGGAAUGdTdT
	Antisense	CAUUCCAUCCCCGAAAACAdTdT
HIF-1α (siHIF1A)	Sense	GGGAUUAACUCAGUUUGAACUdTdT
	Antisense	UUCAAACUGAGUUAAUCCCAUdTdT
HIF-2α (siHIF2A)	Sense	AUUUUUGAGGCUCAAGUUCUC
	Antisense	GAACUUGAGCCUCAAAAAUGG
HIF-1β (siHIF1B)	Sense	GAACUCUUAGGAAAGAAUAUUdTdT
	Antisense	UAUUCUUUCCUAAGAGUUCCUdTdT
AhR (siAhR)	Sense	AUUAAGUCGGUCUCUAUGCCU
	Antisense	GCAUAGAGACCGACUUAAUAC
Negative control (siCont)	Sense	UUCUCCGAACGUGUCACGUdTdT
	Antisense	ACGUGACACGUUCGGAGAAdTdT

Table 3

Bovine gene-specific primers for real-time polymerase chain reaction

Gene		sequence (5′→3′)
Biglycan	Forward	GCTGCCACTGCCATCTGAG
	Reverse	CGAGGACCAAGGCGTAG
Decorin	Forward	CTGCGGTTGACAATGGC
	Reverse	CTCACTCCTGAATAAGAAGCC
Perlecan	Forward	ATGGCAGCGATGAAGCGGAC
	Reverse	TTGTGGACACGCAGCGGAAC
Syndecan-1	Forward	CAGTCAGGAGACAGCATCAG
	Reverse	CCGACAGACATTCCATACC
Syndecan-2	Forward	CCAGATGAAGAGGACACAAACG
	Reverse	CCAATAACTCCGCCAGCAA
Syndecan-3	Forward	CAAGCAGGCGAGCGTC
	Reverse	GGTGGCAGAGATGAAGTGG
Syndecan-4	Forward	TTGCCGTCTTCCTCGTGC
	Reverse	AGGCGTAGAACTCATTGGTGG
Glypican-1	Forward	GAAGGTCGGCAGGAAGAG
	Reverse	CCAGGAGCAGCAGAGGA
TGF-β_1	Forward	TGGAGTTGTGCGGCAGTGGC
	Reverse	CCGTGAATGGTGGCGAGGTC
ALK1	Forward	CAACCACTACTGCTGCTACA
	Reverse	CCATCTCCTTGAGGCTGC
ALK5	Forward	GTCTGCTTTGTCTGTATCTCACTCA
	Reverse	TCCTCTTCATTTGGCACTCG
Smad2	Forward	CAGAATACCGAAGGCAGACG
	Reverse	TGAGCAACGCACTGAAGG
Smad3	Forward	ACTACAGCCATTCCATCC
	Reverse	ATCTGGTGGTCACTGGTCTC
HIF-1α	Forward	GCTTGCTCATCAGTTGCCAC
	Reverse	GCATCCAGAAGTTTCCTCACAC
HIF-2α	Forward	CAGTGGCAAGGTGGCTGTGTC
	Reverse	GGTCCCGAAATCCAGAGAAATGA
HIF-1β	Forward	TAAGGAGCGGTTTGCCAGGTC
	Reverse	TTCTGTTATGTAGGCTGTCATCTTGTTC

Table 3 (continued)

Bovine gene-specific primers for real-time polymerase chain reaction

Gene		sequence (5′→3′)
AhR	Forward	GTGTCAGTTATCTCAGAGCCAAG
	Reverse	AAAGCCATTTAGTGCCTGTAGTA
GAPDH	Forward	AACACCCTCAAGATTGTCAGCAA
	Reverse	ACAGTCTTCTGGGTGGCAGTGA
B2M	Forward	CCATCCAGCGTCCTCCAAAGA
	Reverse	TTCAATCTGGGGTGGATGGAA
β-actin	Forward	CCTCCCTGGAGAAGAGCTACGA
	Reverse	GGAATTGAAGGTAGTTTCGTGAATG

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